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IONIC BASIS AND MECHANISMS OF ADRENERGIC STIMULATION OF THE ARRHYTHMOGENIC TRANSIENT INWARD CURRENT IN CARDIAC PURKINJE FIBRES

by

Xinqiang Han, M.D.

Submitted in partial fulfilment of the requirements

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ABSTRACT

The mechanisms of adrenergic stimulation of oscillatory afterpotentials (OAP) and transient inward current (TI) and the ionic basis of TI were studied in isolated rabbit cardiac Purkinje fibres with standard intracellular microelectrode recording and two-electrode voltage clamp techniques. Under non voltage clamp, specific α_1-adrenergic stimulation increased the amplitude of OAP and induced triggered activity when sub-threshold OAP were induced by high Ca^{2+}. In contrast, specific α_1-adrenergic stimulation decreased the amplitude of OAP and suppressed triggered activity induced by digitalis. α_2-adrenergic stimulation had no effect on OAP and triggered activity. Thus, specific α_1-adrenergic stimulation may exert either proarrhythmic or antiarrhythmic effects on OAP and triggered activity depending on the method of induction of OAP. Under voltage clamp, TI could be induced routinely by elevation of $[Ca^{2+}]_o$ in the absence of $[Na^+]_o$. High $[Ca^{2+}]_o$ induced TI had a distinct reversal potential (E_{REV}) either in the absence or in the presence of $[Na^+]_o$. This suggests that TI is conducted through TI channels, not Na^+-Ca^{2+} exchange. Since both inward and outward TI were simultaneously abolished by exposure to ryanodine, Ca^{2+} release from the sarcoplasmic reticulum is essential in generation of TI. Elevation of $[Ca^{2+}]_o$ shifted the E_{REV} to more negative potentials if $[Cl^-]_o$ was increased. When $[Ca^{2+}]_o$ was fixed, changing $[Cl^-]_o$ shifted E_{REV}. The magnitude and direction of the shift could be predicted by Nernst equation. Outward TI was greatly suppressed when $[Cl^-]_o$ was decreased. Agents known to block Cl^- conductance in cardiac tissues selectively suppressed outward TI. Ni^{2+} greatly decreased inward TI but increased outward TI. Cd^{2+} strongly suppressed both inward and outward TI at the same time. Mn^{2+} abolished inward TI at a time when outward TI just showed a slight decrease. All these agents had no effect on E_{REV}. These results suggest that Cl^- contributes to generation of TI. Therefore, TI channel conductances are both cationic and anionic, and can be differentially modulated. β-adrenoceptor stimulation itself failed to induce the TI, but significantly increased peak magnitude of spontaneously occurring TI or TI induced by digitalis. The enhancement of TI seemed to be independent of the calcium current (I_{Ca}) because agents known to block I_{Ca} failed to block this stimulation. When TI was induced by elevation of $[Ca^{2+}]_o$, β-adrenergic stimulation had no effect on both inward and outward TI in the absence of $[Na^+]_o$, but significantly increased both inward and outward TI in the presence of $[Na^+]_o$. This suggest that $[Na^+]_o$ is essential in mediating β-adrenergic stimulation of TI. The stimulatory effects were abolished if preparations were previously treated with β-adrenoceptor blockers. However, once the enhancement of TI became apparent, β-adrenoceptor blocker only incompletely reversed the stimulation. Forskolin, a direct adenylate cyclase activator, strongly increased both inward and outward TI. However, these effects were absent in 0 $[Na^+]_o$. β-adrenergic stimulation of either digitalis or high Ca^{2+} induced TI could be blocked by putative Na^+-Ca^{2+} exchanger blockers. Thus, β-adrenergic stimulation of TI seems to be mediated by cAMP-dependent phosphorylation of the Na^+-Ca^{2+} exchanger protein which increases Ca^{2+} loading of the cells.

LIST OF ABBREVIATIONS

APD:	action potential duration
AS:	acetylstrophanthidin
ATP:	adenosine triphosphate
BCL:	basic cycle length (time between stimuli)
$[Ca^{2+}]_i$:	either intracellular Ca^{2+} or free intracellular Ca^{2+} concentration
$[Ca^{2+}]_o$:	either extracellular Ca^{2+} or extracellular Ca^{2+} concentration
CholineCl:	choline chloride
DAD:	delayed afterdepolarization
Driving rate:	stimulation frequency
dV/dt:	maximum upstroke velocity of action potential
hr:	hour
I_{Ca}:	Ca^{2+} current
I_{Na}:	Na^+ current
I_{si}:	refer to L-type calcium current, $I_{Ca,L}$
ISO:	isoproterenol or isoprenaline
I_{ti}:	transient inward current
I-V relation:	current-voltage relation
MDP:	maximum diastolic potential
mg:	milligram
min:	minute
ml:	millilitre
mm:	millimetre
mM:	millimolar
μM:	micromolar

ms:	millisecond
mV:	millivolt
MΩ:	megaohm
nA:	nanoamp
$[Na^+]_i$:	either intracellular Na^+ or free intracellular Na^+ concentration
$[Na^+]_o$:	either extracellular Na^+ or extracellular Na^+ concentration
Na^+-Ca^{2+} exchange:	sodium-calcium exchange
Na^+-K^+ pump:	sodium-potassium ATPase
NMG:	N-methyl-D-glucamine
OAP:	oscillatory afterpotential
PE:	phenylephrine
Prop:	propranolol
PZ:	prazosin
sec:	second
SR:	sarcoplasmic reticulum
TA:	triggered activity
TI:	transient inward current

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INTRODUCTION

I. GENERAL CONSIDERATIONS

The cellular mechanisms that cause cardiac arrhythmias are of great importance and are the subject of intense investigation. One of the most common mechanisms known to cause cardiac arrhythmias is the occurrence of oscillatory afterpotentials (OAP, Ferrier, 1977; 1991) or delayed afterdepolarizations (DAD, Cranefield, 1977).

OAP are oscillations in transmembrane potentials that occur after repolarization of an action potential and that are caused by that action potential. In other words, OAP are "coupled" to the preceding action potentials (Ferrier, 1991). Figure 1 illustrates some of the most commonly used terminology throughout this review. An afterpotential is referred to as an early afterdepolarization (EAD) when it occurs before full repolarization of the cell (Figure 1A). When the membrane potential hyperpolarizes with respect to the resting potential, the voltage excursion is called an early afterhyperpolarization (EAH, Figure 1B). Depolarization occurring after the resting membrane potential has been attained or exceeded is referred to as a delayed afterdepolarization (DAD). The total excursion (EAH + DAD) is termed an oscillatory afterpotential (OAP).

OAP are generated under a variety of clinically associated conditions such as digitalis toxicity (Ferrier, 1977; 1991), ischemia/reperfusion (Ferrier & Guyette, 1991), and adrenergic stimulation with either α- (Kimura et al, 1984; 1987) or β-adrenergic agonists (Wit & Cranefield, 1977; Priori & Corr, 1990). Such conditions may affect each other and worsen cardiac arrhythmias initiated by OAP. For instance, a digitalis glycoside ouabain has been shown to increase both spontaneous (Gillis & Quest, 1980) and stimulation-induced noradrenaline release from the heart (Kranzhofer et al, 1991). Release of endogenous noradrenaline into coronary blood is

known to occur during ischemia (Hirche et al, 1980; Godin et al, 1985) and particularly upon reperfusion of previously ischemic tissues (Godin et al, 1985). Increased α-adrenergic responsiveness has been shown to be associated with cardiac ischemia (Sheridan et al, 1980). More importantly, adrenergic stimulation can not only generate OAP, but also augment OAP induced by digitalis (Hewett & Rosen, 1984) and facilitate the induction of OAP under the conditions of ischemia/reperfusion (Kimura et al, 1987; Lukas & Ferrier, 1989).

An oscillatory membrane current termed transient inward current (TI) which is activated by Ca^{2+} overload of cardiac cells (January & Fozzard, 1988) has been demonstrated to be the current underlying OAP (Lederer & Tsien, 1976; Kass et al, 1978a; 1978b). Despite numerous efforts over more than 15 years of investigation, the ionic mechanism underlying the generation of TI still is a subject of much debate (Giles & Shimoni, 1989a; Wit & Rosen, 1991). As a result, the mechanism(s) by which adrenergic stimulation induces OAP and enhances TI has largely remained elusive. These considerations form the foundation for my Ph.D research work. Therefore, in the following literature review, I will examine some earlier experimental findings which led to the discovery of OAP and triggered arrhythmias, summarize recent experimental evidence pertinent to the mechanisms responsible for OAP and TI, explore the possible charge-carrying systems for TI, and discuss the clinical relevance under a number of pathophysiological conditions including adrenergic arrhythmogenicity.

Figure 1. Schematic illustration of nomenclature of afterpotentials. Panel A: Early afterdepolarization (EAD). Panel B: Early afterhyperpolarization (EAH), delayed afterdepolarization (DAD), and oscillatory afterpotential (OAP). The resting membrane potential is indicated by RMP, and the amplitude of OAP is indicated by AMP.

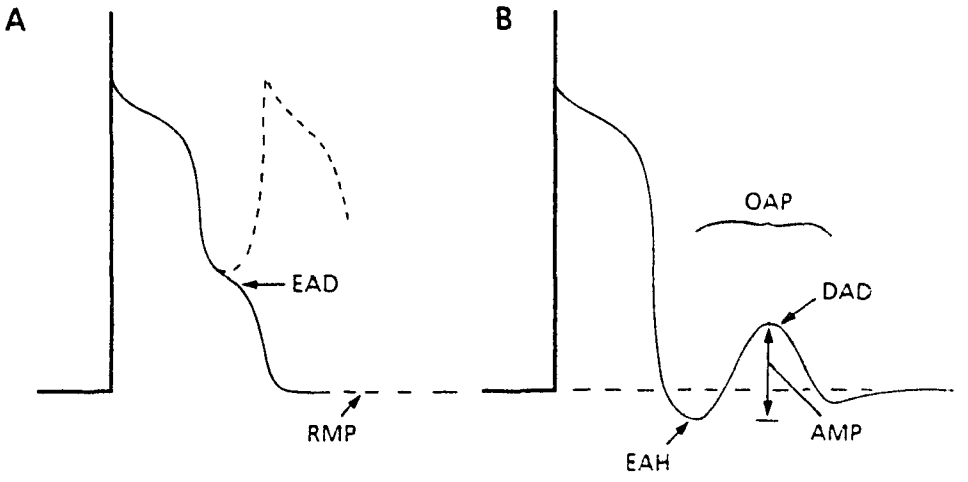


Figure 1.

II. AFTERPOTENTIALS AND TRIGGERED ARRHYTHMIAS

1. HISTORY OF OAP

The rhythmic beating of the heart depends upon oscillatory membrane potential changes in individual cardiac cells. The underlying mechanisms of such activity are important to understanding normal pacemaker function as well as abnormal cardiac rhythms. Two fundamentally different kinds of oscillatory mechanisms which co-exist in various cardiac preparations, and which come into play under various conditions, can be distinguished. The first and most familiar type of oscillation, which is governed by interactions between transmembrane potential and transmembrane ion permeability, is referred to normal pacemaker activity (Noble, 1984). The second kind of oscillation involves a subcellular rhythm generator which drives transmembrane ionic permeability and/or transport and transmembrane potential from within the cell. Unlike the normal pacemaker activity which occurs in natural pacemaker tissues (sinus node, atrial-ventricular node, or Purkinje fibre), the latter type of oscillatory activity which is mostly observed under pathological conditions and can occur in all parts of the heart is regarded as an abnormal activity.

The concept of oscillatory activity as a cause of arrhythmias is not new. In 1926 when Scherf induced arrhythmias in the canine heart, he proposed that the beat that preceded the arrhythmia actually precipitated it (for a review, see Scherf & Schott, 1973). Direct experimental evidence that oscillatory changes in membrane potentials might cause arrhythmias has been observed for nearly half a century (Bozler, 1943a; b). When working with turtle ventricular muscle exposed to calcium-rich, sodium-poor solutions, Bozler (1943a) found small, non-conducted potential changes of oscillatory nature, accompanied by small variations in isometric force which he called "tonus". As he stated in the paper (1943a), "The tonus changes and the local potentials are probably manifestations of a more fundamental process, a fluctuation in

resting metabolism. The mechanical changes are weak and hardly play any role as such. Their chief interest lies in their relation to the automaticity and rhythmicity of the muscle. It may be assumed that an increase in metabolism causes a rise in tonus and a decreased surface polarization. The decrease in polarization in turn may be considered as the last link in the processes leading to the discharge of an impulse".

The terminology "oscillatory afterpotentials" first appeared in another paper by Bozler (1943b). In that study, Bozler commented that ".....oscillatory afterpotentials, for example, provide a simple explanation for coupled extrasystoles and paroxysmal tachycardia. Unfortunately, the study of these weak and localized potentials in the heart in situ presents great technical difficulties".

Although the idea that "decrease in polarization leads to discharge of an impulse" was clearly expressed in the forgoing studies, the concept of oscillatory activity as a cause of arrhythmias did not gain wide acceptance. That oscillations in membrane potential did occur was understood, but a relationship to arrhythmias was either not generally recognized or judged as lacking in experimental proof. For thirty years after Bozler's reports, little concern was expressed about OAP as a cause of arrhythmias. This could not be totally blamed on the "technical difficulties" mentioned above by Bozler. When intracellular microelectrode recording techniques were available in isolated small cardiac tissues, reexamination of the early studies which were performed with extracellular recordings of monophasic action potentials was suggested seriously by Dawes (1952). On the other hand, mechanisms such as conduction disturbances which dominated the focus of research at that time attracted much attention from investigators and left little room for the examination of OAP as a cause of arrhythmias (Rosen & Wit, 1986).

It was probably the studies on the electrophysiological effects of digitalis in the early 1970s which eventually revived interest in reevaluation of oscillatory activity.

This oscillatory activity had been observed frequently both clinically and experimentally for a long time, as a potential cause of arrhythmias. This effort resulted in a sudden burst of publications (Ferrier & Mendez, 1972; Ferrier et al, 1973; Ferrier & Moe, 1973; Hashimoto & Moe, 1973; Saunders et al, 1973; Rosen et al, 1973a; 1973b; Davis, 1973; Hogan et al, 1973) describing the oscillatory nature of membrane electrical activity, under digitalis intoxication. Since then, the cellular and subcellular ionic mechanisms of the membrane oscillations as well as their clinical relevance attracted tremendous attention from both experimental and clinical cardiologists. Soon after, two specific names were given to refer to the membrane oscillatory activity: delayed afterdepolarizations (DAD) and oscillatory afterpotentials (OAP) (Cranefield, 1977; January & Fozzard, 1988; Ferrier, 1991). These two terms remain in use at present, but OAP will be used in this review. Other names such as "transient depolarizations" (Ferrier et al, 1973; Ferrier & Moe; 1973), "low amplitude potentials" (Rosen et al, 1973a; 1973b), and "enhanced diastolic depolarization" (Davis, 1973) will only be mentioned when referring to the original articles.

2. INDUCTION OF OAP

OAP are considered to be a type of triggered activity. Triggering depends on two factors. First, the generation of OAP requires an initiating event such as spontaneously occurring or electrically driven action potentials. OAP or triggering seldom appear in otherwise unstimulated preparations from the heart. Second, while the amplitudes of OAP can be subthreshold, they can also reach threshold voltages for the activation of other membrane ionic currents (i.e. I_{Na}, I_{Ca}) and thereby initiate single or multiple action potentials. Therefore, abnormal impulses can also be triggered by OAP. "The tendency to give afterdischarge is greatest under conditions where the afterpotentials are largest" (Bozler, 1943a).

Although a variety of approaches have been used to induce OAP (Tsien et al,

1979), the prototypical experimental method is to expose cardiac tissues to high concentrations of cardiotonic steroids including ouabain, acetylstrophanthidin (AS), and strophanthidin. The ability to generate OAP seems to be a general property of most cardiac glycosides (Karagueuzian & Katzung, 1981). In fact, most of the important features of OAP were elucidated by using digitalis.

A. Digitalis

Effects of Digitalis on Action Potential: Studies in which both action potentials and contractile tension were measured have reported that digitalis induces a positive inotropic effect well before any changes in the action potential can be discerned. In cat papillary muscles (Dudel & Trautwein, 1958) action potential duration (APD) increases slightly after exposure to low concentrations of strophanthidin (0.66×10^{-8} to 3.3×10^{-7} M), but eventually shortens during exposure to higher concentrations of strophanthidin (3.3×10^{-6} M). In contrast to the biphasic effect on APD, the force of contraction increases monotonically with time during exposure to the drug at all concentrations. Developed tension decreases only when contracture is seens. Vassalle et al (1962) compared the toxic effects of ouabain on canine cardiac Purkinje fibres and on ventricular muscle fibres driven at 60/min. After treatment with ouabain for 30-60 min, no significant changes in the Purkinje fibre action potentials were observed. However, 70 min exposure to ouabain resulted in decreased duration, amplitude, and rate of rise of the upstroke of the action potentials. The maximum diastolic potential also became less negative. After exposure to ouabain for 100 min, these changes became more marked and the Purkinje fibres eventually became quiescent and inexcitable at levels of membrane potential from -40 to -57 mV. Similar changes in the action potential and resting potential of ventricular muscle were observed, but these took much longer (220-400 min) to develop. The resting potential was also more negative (-60 to -83 mV) when the preparations were inexcitable.

Development of the toxic effects depended strongly on the rate of stimulation. Doubling the rate of stimulation (120/min) greatly shortened the time to development of inexcitability of muscle (135-195 min).

The correlation between the effects of digitalis on the electrocardiogram (ECG) and action potentials recorded from isolated dog cardiac Purkinje fibres was studied by Rosen et al (1973a). These studies were done by perfusing isolated Purkinje tissue with blood from an intact "donor" dog that was given ouabain intravenously. The preparation was stimulated at a rate close to that of the spontaneous rate of the donor dog prior to the development of arrhythmias (75-120/min). These investigators found that increased APD in the tissue bath corresponded to ST-T wave changes in the surface ECG before the development of ouabain toxicity. When junctional or ventricular extrasystoles, or supraventricular tachycardia appeared in the ECG, the amplitude, duration, and upstroke velocity of the action potential all decreased. Even more prominent decreases in these parameters were seen with the development of ventricular tachycardia.

Simultaneous measurements of action potential, force of contraction, and intracellular free Ca^{2+} transients ($[Ca^{2+}]_i$) in canine Purkinje tissue treated with ouabain and strophanthin were performed by Wier and Hess (1984). $[Ca^{2+}]_i$ transients were measured by aequorin luminescence signal (Weir & Isenberg, 1982). Exposure to strophanthin (10^{-7} M) for 20 min did not cause significant changes in the action potential but did produce a parallel and progressive increase in both the force of contraction and $[Ca^{2+}]_i$ as reflected by the aequorin signal. After exposure to ouabain for 35 min or more, decrease in the duration and amplitude of the action potential and loss of resting potential were observed. These changes were associated with an increased diastolic level of $[Ca^{2+}]_i$. These studies confirmed that both the inotropic and the toxic effects of digitalis are accompanied by an increase in $[Ca^{2+}]_i$.

Effects of Digitalis on Diastolic Electrical Activity: Induction of OAP: An increase in the slope of spontaneous diastolic depolarization induced by digitalis was initially observed in cardiac Purkinje tissues perfused with Tyrode's solution containing low $[K^+]_o$ (2.7 mM)(Dudel & Trautwein, 1958; Vassalle et al, 1962; Davis, 1973; Rosen et al, 1973b; Hogan et al, 1973). Interestingly, spontaneous diastolic depolarization was never recorded in ventricular muscle intoxicated with digitalis, even when the preparation had developed extrasystoles (Vassalle et al, 1962). On the other hand, elevation of $[K^+]_o$ (5.9 mM) seemed to inhibit digitalis induced enhancement of diastolic depolarization (Kassebaum, 1963). Of the aforementioned studies, clear OAP were also seen in the papers of Rosen et al (1973a; 1973b), Davis (1973), and Hogan et al (1973). However, the OAP in Rosen's studies never reached threshold nor evoked non-driven action potentials. Davis (1973) suggested that "enhancement of diastolic depolarization (i.e. OAP) of Purkinje fibres to the point of spontaneous discharge of action potentials may be a common means by which digitalis produces a variety of ventricular arrhythmias observed in intact animals". However, he did not describe induction of OAP by digitalis, i.e. was this "enhanced diastolic depolarization" simply an ability of digitalis to increase the normal spontaneous diastolic depolarization which can be regularly seen in Purkinje tissue without drug exposure, or an action of digitalis to generate a totally different type of automatic activity which should be considered "abnormal". This was an especially important question for his study because the experiments were performed with only 2.7 mM $[K^+]_o$ which by itself might induce phase 4 depolarization in Purkinje fibres. While Hogan et al (1973) clearly observed a very important phenomenon, "post-overdrive acceleration" in ouabain-treated Purkinje fibres attached to the papillary muscles, they failed to suggest that digitalis-induced changes in diastolic electrical activity might depend on a totally different mechanism from that underlying normal pacemaking activity.

More detailed description of the characteristics of digitalis induced changes in diastolic electrical activity was given in an important study by Ferrier et al (1973) who strived "to define the conditions under which postpacing depression (as a characteristic of normal pacemaker activity) is replaced by postpacing acceleration of spontaneous activity, and to determine the rules which distinguish drug-induced automatic responses from physiologic pacemaker behaviour". These authors found that during regular drive, exposure to acetylstrophanthidin (AS) increased the slope of diastolic depolarization (initial toxicity). Interruption of stimulation revealed one or more subthreshold oscillations in diastolic membrane potential which they referred to as "transient depolarization" (TD, i.e. OAP). OAP appeared in all 40 Purkinje fibres but were absent in all 15 muscle preparations. When driven at shorter cycle length (400-700 msec), OAP could become large enough to trigger one or more nondriven action potentials. Following the last nondriven action potential there was always a subthreshold OAP. No sign of spontaneous diastolic depolarization could be seen following this terminal OAP (advanced toxicity).

As commented by Cranefield & Aronson (1988), "This important study by Ferrier et al offered persuasive evidence that the diastolic electrical activity induced by digitalis differs from spontaneous phase 4 diastolic depolarization observed in Purkinje fibres". This is because 1) normal pacemaker activity exhibits postpacing depression (Lange, 1965) whereas the coupling interval of OAP (measured as the interval from the upstroke of the last action potential to the peak of the OAP) was shortened as the pacing frequency was increased. 2) AS suppressed spontaneous activity dependent on normal diastolic depolarization at a time when frequency-dependent OAP were present. Following this influential study, digitalis-induced OAP in a variety of preparations and their modulation by various agents and approaches were reported in numerous publications. A partial list is given here for reference:

(Ferrier & Moe, 1973; Saunders et al, 1973; Hashimoto & Moe, 1973; Aronson & Cranefield, 1974; Gelles et al, 1975; Vassalle & Musso, 1976; Ferrier, 1976; Lederer & Tsien, 1976; Rosen & Danilo, 1980; Ferrier, 1980; Wasserstrom & Ferrier, 1981; Karaqueuzian & Katzung, 1981; 1982; Binah et al, 1983; Hewett & Rosen, 1984; Lukas & Ferrier, 1986; Aronson & Nordin, 1988; Han & Ferrier, 1990).

B. Ions

Low K^+ Solutions: In addition to digitalis, lowering K^+ concentration in the extracellular solution (to < 0.5 mM) has been shown to be an useful approach to induce OAP. First, lowering $[K^+]_o$ decreases membrane K^+ conductance and therefore, the outward currents carried by K^+. As a result, a small inward current will generate a large depolarization. Second, decreased $[K^+]_o$ inhibits the Na^+-K^+ pump much like digitalis. In fact, phase 4 depolarization can be evoked in working myocardium from calf or sheep by perfusion with K^+-free, Ca^{2+}-free solution (Muller, 1965). Clear OAP and OAP-triggered nondriven action potentials were induced in canine ventricular muscle fibers by exposure to K^+-free solution containing 3.5 to 7.2 mM $[Ca^{2+}]_o$ (Hiraoka et al, 1979; Hiraoka & Okamoto, 1981; Hiraoka et al, 1981). Hiraoka et al (1981) also found that exposure to K^+-free, Ca^{2+}-free solution facilitated the induction of OAP upon return to Ca^{2+}-containing solution. In the presence of normal $[Ca^{2+}]_o$, OAP were also induced in sheep Purkinje fibres and guinea pig papillary muscles in K^+-free solution (Eisner & Lederer, 1979a; 1979b). The effects of K^+-free solution could be divided into two phases. The early phase, developing over tens of seconds included a more negative resting potential and a lengthening of the APD in both Purkinje fibres and papillary muscles. In Purkinje fibres, the principal early effect was a decrease of slope conductance in the steady-state I-V relation. The decrease was more apparent at most negative potentials. This effect tended to depolarize the cells. The late phase developed over minutes. In

ventricular muscle such effects included a shortening of APD, an increase in twitch and tonic tension, and the development of OAP and aftercontractions. The late effects in Purkinje fibres were an increase in twitch tension and voltage dependent tonic tension, the development of OAP and an underlying oscillatory transient inward current. These authors further concluded that these effects were totally due to inhibition of the Na^+-K^+ pump by zero $[K^+]_o$. That inhibition of Na^+-K^+ pump played a major role in the effects of K^+-poor solution was also suggested in a later study by Hiraoka and Hirano (1986) who used low $[K^+]_o$ (1 mM) and high $[Ca^{2+}]_o$ to investigate the passive electrical properties of guinea pig ventricular muscle.

Elevated $[Ca^{2+}]_o$: Increasing $[Ca^{2+}]_o$ without changing concentrations of other ions in Tyrode's solution can induce OAP. This manoeuvre was first used by Ferrier & Moe in 1973. They found that OAP were induced in canine Purkinje fibres by merely increasing $[Ca^{2+}]_o$ from 2.5 to 10 mM, with basic cycle length (BCL) shorter than 400 msec. The Ca^{2+}-induced OAP resembled those induced by digitalis in that they showed a rate-dependent increase in the amplitude and depolarization rate. Nevertheless, the Ca^{2+}-induced OAP seemed to have a slower rising phase and time course than digitalis-induced OAP. They also found that while digitalis-induced OAP were greatly enhanced by elevation of $[Ca^{2+}]_o$, they were abolished after perfusion with Ca^{2+}-free solution or by the Ca^{2+} channel blocker, Mn^{2+}. This study firmly established the crucial role of Ca^{2+} in the generation of OAP. With moderately elevated $[Ca^{2+}]_o$ (8 mM), prominent OAP could be induced at BCL longer than 500 msec if $[K^+]_o$ was decreased to 2 mM, however, 2 mM $[K^+]_o$ itself failed to induce OAP in rabbit Purkinje fibres (Han & Ferrier, 1990).

Na^+-Free, Ca^{2+}-Rich Perfusate: Bozler (1943a; 1943b) was the first to used Na^+-poor, Ca^{2+}-rich solution to induce OAP in turtle ventricular muscle. Bozler found from these studies that triggered action potentials could occur if OAP reached

threshold. Another detailed study on triggered activity in canine Purkinje fibres exposed to Ca^{2+}-rich, Na^{+}-free solution was performed by Cranefield and Aronson (1974). These authors found that three forms of action potential could be recorded by such treatment. In one, no afterpotential was seen following repolarization. In another, early afterhyperpolarization was observed. In the last, OAP which could reach threshold and trigger nondriven action potentials were found following the decay of the early afterhyperpolarization. In some quiescent fibres, a single driven action potential could trigger a burst of up to 20 action potentials.

C. Catecholamines

β-Adrenergic Stimulation: Noradrenaline, adrenaline, and isoproterenol increase the amplitude of OAP induced by a variety of approaches (Bozler, 1943a; Wit & Cranefield, 1977; Karagueuzian & Katzung, 1981; Belardinelli & Isenberg, 1983; Hewett & Rosen, 1984; Johnson et al, 1986; Pogwizd et al, 1986; Lukas & Ferrier, 1989). This potentiating effect will be reviewed later. Stimulation with β-adrenergic agonists, however, can also induce OAP in otherwise untreated normal cardiac tissues, both multicellular preparations and single cells. Wit and Cranefield (1976), when studying triggered activity in fibres of the simian mitral valve, observed that addition of noradrenaline to the superfusates caused the appearance of OAP and triggered activity which never occurred in the absence of the catecholamine. John et al (1986) found that the concentrations of adrenaline needed to induce OAP with an amplitude of 10 mV in canine coronary sinus ranged from 5×10^{-8} to 2×10^{-6} M. In isolated single ventricular myocytes from the guinea pig, Brum et al (1983) demonstrated OAP could be induced by intracellular injection of a catalytic subunit of cAMP-dependent protein kinase. Their results indicated that β-adrenergic stimulation with adrenaline might promote induction of OAP by phosphorylation of same membrane proteins. Priori & Corr (1990) found that isoproterenol elicited single or

multiple (2-5) OAP at concentrations from 10^{-8} to 10^{-6} M, with a corresponding increase in the amplitude of the OAP and decrease in the coupling interval as cells were paced at increasing rates. Priori and colleagues (1988) even found that intense sympathetic stimulation could induce OAP in vivo. While OAP are not readily induced by β-adrenergic stimulation per se in Purkinje fibres (Cranefield & Aronson, 1988), a few studies do suggest that triggered activity can be obtained with β-adrenoceptor agonists (Kassebaum, 1964; Wald & Waxman, 1981; Valenzuela & Vassalle, 1983).

α-Adrenergic Stimulation: Although several studies suggest that under pathological conditions α-adrenergic influence may promote cardiac arrhythmias, possibly by increasing the amplitude of OAP and enhancing triggered activity (Sheridan et al, 1980; Sheridan, 1986), attempts to verify this hypothesis experimentally have generated conflicting observations. In canine Purkinje fibres in which OAP were induced by ouabain, α-adrenergic agonists did not significantly affect OAP (Hewett & Rosen, 1984). Kimura et al (1984) found that when feline cardiac Purkinje fibres were exposed to elevated $[Ca^{2+}]_o$ (8.1 mM) in the presence of propranolol (5×10^{-7} M), no OAP were induced following a series of driven action potentials. However, when 10^{-5} M phenylephrine was added, OAP were seen in 8 of 10 preparations and bursts of triggered activity occurred in three preparations. Another α-adrenergic agonist methoxamine also evoked OAP in the presence of 8.1 mM $[Ca^{2+}]_o$. These effects were suppressed by prazosin, an α_1-adrenergic blocker. Kimura et al (1987) also demonstrated that OAP and triggered activity also could be induced by α-adrenergic stimulation in subendocardial Purkinje fibres after healing of myocardial infarction. Mugelli et al (1986) reported that enhancement of automaticity by noradrenaline in sheep Purkinje fibres exposed to hypoxic glucose-free Tyrode's solution appeared to be mediated by OAP induced by α_2-adrenoceptor stimulation. In

isolated single myocytes from adult canine ventricular muscles, Priori and Corr (1990) found that α_1-adrenergic stimulation with phenylephrine only resulted in a dose-dependent (10^{-8} to 10^{-6} M) prolongation of APD, but failed to induce OAP. These discrepancies seem to depend on use of different methods, which may affect various links leading to the generation of OAP. The discrepancies also may be related to the involvement of different α_1-adrenergic receptor subtypes (Minneman, 1988). At least two distinct α_1-adrenergic receptor subtypes have been demonstrated in adult cardiac tissue (del Balzo et al, 1990). One subtype, which can be selectively antagonized by chloroethylclonidine (CEC), is linked to a decrease in automaticity via a pertussis toxin-sensitive substrate (del Balzo et al, 1990). Activation of this subtype of α_1-adrenoceptor also stimulates the Na^+-K^+ pump current (Shah et al, 1988; Zaza et al, 1990) which may oppose the effect of digitalis and thereby decrease the amplitude of digitalis-induced OAP. Another subtype, which can be selectively blocked by WB 4101, is linked to the stimulation of inositol phosphate accumulation (del Balzo et al, 1990; Otani et al, 1988). Mobilization of $[Ca^{2+}]_i$ by inositol trisphosphate (IP_3) may be an important mechanism underlying the stimulation of OAP induced by high Ca^{2+}.

D. Ischemia and Reperfusion

Convincing evidence exists supporting the hypothesis that ischemia, hypoxia, and acidosis suppress OAP while reoxygenation or reperfusion of previously ischemic tissues (both Purkinje fibres and ventricular muscles) potentiates OAP induced by other means such as digitalis intoxication and β-adrenergic stimulation (Ferrier et al, 1985; Lukas & Ferrier, 1986; Hayashi et al, 1987; Coetzee & Opie, 1987). Recently, it has been found that OAP also could be generated in both isolated canine Purkinje fibre-papillary muscle (Lukas & Ferrier, 1989) and guinea pig ventricular muscles (Ferrier & Guyette, 1991; Li & Ferrier, 1991) upon reperfusion of the

tissues previously exposed to simulated ischemic solutions.

E. Drugs and Toxins

Histamine: Cardiac arrhythmias are frequently found during type IV hypersensitivity reactions. Massive release of histamine from antigen-sensitized heart may be an important factor leading to cardiac dysfunction (Levi, 1986; Han & Vohra, 1991). Levi and Zavecz (1979) studied the effects of histamine on excised guinea pig hearts under Langendorff perfusion; total AV block was created by ligature of the bundle of His. They found that the increases in heart rate were mediated by two separate effects of histamine, one that caused an acceleration of the original rhythm and another that resulted in a new focus. The authors suggested that the new focus might be a site of triggered activity. Later on, histamine was indeed found by Levi et al (1981) to enhance automatic activity in human atrial fibres. OAP also could be induced by histamine during the diastolic interval in cells that showed automatic activity. Increasing the concentration of histamine from 10^{-5} to 5 x 10^{-5} M caused marked increase in the amplitude of OAP and evoked a burst of triggered activity. In guinea pig papillary muscle superfused with Tyrode's solution containing normal $[K^+]_o$ (5.4 mM), histamine failed to induce OAP (Tajima & Dohi, 1985). However, these authors found that when $[K^+]_o$ was reduced to 0.54 mM, no OAP were induced during the first 15 min exposure to K^+-poor solution. Marked OAP could be induced if the K^+-poor solution contained 10^{-5} M histamine.

Caffeine: Caffeine is thought to release Ca^{2+} from and prevent the subsequent uptake of Ca^{2+} by the sarcoplasmic reticulum (Hess & Wier, 1984; Clusin, 1985). OAP were induced in the presence of lowered $[K^+]_o$ in canine Purkinje fibres exposed to 0.5 to 3 mM caffeine while higher concentrations (9-11 mM) of caffeine suppressed OAP (Paspa & Vassalle, 1984). In isolated canine coronary sinus, Aronson

et al (1985) found that OAP could also be induced by 0.5 mM caffeine in the presence of 4 mM $[K^+]_o$. Elevation of caffeine to 5 mM caused bursts of triggered activity within the first few minutes of drug exposure. However, once a steady state was reached, the preparation could not be triggered.

Neuraminidase: Sialic acid is a major component of glycocalyx which covers the external surface of cardiac cells. Langer and co-workers (Langer et al, 1976; 1979; Frank et al, 1977) observed that Ca^{2+} permeability of the membrane was enhanced when sialic acid was removed from the glycocalyx of the cardiac cells by neuraminidase, an enzyme which cleaves sialic acid from the oligosaccharide chains. Therefore, Kimura et al (1984b) undertook to determine whether neuraminidase could facilitate induction of OAP and initiation of triggered activity in canine Purkinje fibers exposed to elevated $[Ca^{2+}]_o$ or digitalis. They found that OAP could be induced in canine Purkinje fibres pretreated with neuraminidase at normal $[Ca^{2+}]_o$ (2.7 mM). Nondriven action potentials could be triggered by elevating $[Ca^{2+}]_o$ to 8.1 mM. Furthermore, ouabain-induced OAP had a larger amplitude in neuraminidase-treated preparations.

Grayanotoxin-III: The grayanotoxins are a class of lipid-soluble toxins from rhododendrons and other Ericaceae (Hille, 1992). Their actions are thought to affect excitable Na^+ channels by causing long-lasting opening of the Na^+ channels and thereby elevating $[Na^+]_i$ (Hille, 1992). Increase in $[Na^+]_i$ may result in elevation of $[Ca^{2+}]_i$ via Na^+-Ca^{2+} exchange. After it had been shown that grayanotoxin I and α-dihydrograyanotoxin II cause cardiac arrhythmias that can be abolished by tetrodotoxin (TTX) (Ku et al, 1977), Brown et al (1981) investigated the possibility that those arrhythmias might be dependent on OAP mechanism. They indeed found that typical OAP and bursts of triggered activity could be induced in feline cardiac Purkinje fibres exposed to grayanotoxin-III for 15 min. A slight depolarization of the

maximum diastolic potential, a shift of the plateau to a more negative level, and a prolongation of APD were also found with the toxin exposure.

Palytoxin: Palytoxin was obtained from certain coelenterates. At a concentration of 4 x 10^{-9} M this toxin decreased the resting potential, overshoot, duration, and the maximum upstroke velocity of the action potential and increased the threshold for excitation of rabbit papillary muscle (Weidmann, 1977). Ito et al (1979) found that triggered activity could be generated by palytoxin at a concentration of 3 x 10^{-8} M. Subthreshold OAP were often seen following termination of triggered activity. These authors suggested that palytoxin might open a class of Na^+ channels which was insensitive to tetrodotoxin (TTX) and increase $[Na^+]_i$, thereby promoting the induction of OAP by elevation of $[Ca^{2+}]_i$ via Na^+-Ca^{2+} exchange.

F. Preparations from Diseased Hearts

Triggered activity and OAP were observed by Gilmour et al (1983) in isolated ventricular preparations from patients with ischemic heart disease. Other abnormalities such as membrane depolarization, slowed upstroke, and abnormal conduction were also present in most of these preparations. Diseased human atrial fibres exposed to adrenaline also developed OAP and bursts of triggered activity (Mary-Rabine et al, 1980). In preparations excised from the canine heart one day after myocardial infarction induced by one-stage ligation of the left anterior descending coronary artery, El-Sherif et al (1983) demonstrated that bursts of triggered activity could be evoked by single or multiple drive and typical OAP occurred following termination of the bursts. Demonstrations of OAP and triggered activity <u>in vivo</u> in dogs surviving 1-day-old myocardial infarction were later reported by the same lab (Hariman et al, 1984). In isolated canine Purkinje fibres, Pogwizd et al (1986) found that certain lysophosphoglycerides like lysophosphatidyl choline (LPC) and lysophosphatidyl ethanolamine which accumulated in ischemic myocardium could cause decrease in

resting potential, amplitude, and upstroke velocity of the action potential. OAP and bursts of triggered activity were seen when moderate loss in resting potential was induced by LPC. These authors also suggested that arrhythmias during early myocardial infarction might result from LPC-induced OAP. It is not clear whether this actually can occur, because ischemic conditions have been shown to inhibit OAP in other studies (Lukas & Ferrier, 1986; Coetzee & Opie, 1987). Other animal models including hypertrophied rat myocardium (Heller, 1979; Heller & Stauffer, 1981; Aronson, 1980; 1981) and streptozotocin-induced diabetic rat hearts (Nordin et al, 1985) were prone to induction of OAP and triggered activity.

3. INITIATION OF TRIGGERED ARRHYTHMIAS

A. Definition of Triggered Activity:

Triggered activity was defined by Cranefield (1975; 1977) as activity in which action potentials arose from afterpotentials that followed and were caused by the previous action potential. According to this definition, both early afterdepolarizations (EAD) and OAP can cause triggered activity (see figure 1). Only triggered activity initiated by OAP will be reviewed. Triggered activity is an important mechanism underlying some clinically observed arrhythmias including single extrasystoles, runs of extrasystoles, junctional tachycardia, and certain exercise- or adrenergic-induced ventricular tachycardia. Triggered activity can occur in virtually any part of the heart.

B. Differentiation from Normal Pacemaker Activity:

Certain cardiac tissues (for instance SA node, AV node, and Purkinje fibres) can spontaneously generate action potentials. These tissues therefore have automatic activity, and sometimes are called automatic tissues. Under a variety of pathological conditions, triggered activity also can occur in these tissues. It is therefore necessary to distinguish between these two phenomena because the underlying mechanisms are

totally different. A number of criteria have been used to differentiate between them (Ferrier, 1977; Cranefield, 1977; Tsien et al, 1979; Cranefield & Aronson, 1988). 1) Automatic tissues, if they become quiescent, usually return to rhythmic activity without being driven, whereas triggerable tissues (such as atria and ventricles), remain quiescent unless driven. 2) Elevation of $[Ca^{2+}]_o$ suppresses normal pacemaker activity but enhances triggered activity. 3) A short period of rapid overdrive causes slowing of normal automatic activity, whereas triggered activity may be initiated or enhanced by such overdrive (overdrive suppression vs acceleration). 4) The ionic currents underlying normal pacemaker activity are I_f and T-type I_{Ca} (Noma et al, 1983; DiFrancesco, 1985; Pelzer & Trautwein, 1987) whereas the current responsible for OAP-initiated triggered activity is the transient inward current (TI, Lederer & Tsien, 1976; Kass et al, 1978a; 1978b; January & Fozzard, 1988; Ferrier, 1991). Both the kinetics and the charge-carrying systems for TI, I_f, and T-type I_{Ca} are believed to be different.

C. OAP-Initiated Triggered Arrhythmias in Animal Models and Human Subjects

The best examples of OAP initiated triggered arrhythmias are digitalis-induced arrhythmias. Scherf (1944) found that when ouabain, lanatoside-C, and digitoxin were injected into the subepicardial surface of the auricle or ventricle of open-chested dogs, all types of extrasystolic arrhythmias seen in patients could be observed in the dogs. Those arrhythmias included ventricular or atrial bigeminal and trigeminal rhythms or tachycardias. Scherf also found that when complete cardiac standstill was produced by vagal stimulation, the digitalis-induced extrasystoles disappeared, and this led him to conclude that each extrasystole was induced by the preceding normal beat. Similar observations also were reported by Vassalle et al (1963) in a more detailed study. When the correlation between the effects of ouabain on the intact canine ECG and action potentials of isolated Purkinje fibre bundles was studied by

Rosen et al (1973a), arrhythmic activities including ST-T changes, junctional or ventricular extrasystoles or supraventricular tachycardia were found in ECG recordings while OAP appeared simultaneously in the isolated Purkinje fibres. Digitalis-induced triggered arrhythmias were further characterized by other investigators (Zipes et al, 1974; Gorgels et al, 1983). Triggered arrhythias attributable to OAP also were seen in dogs with one-day-old myocardial infarction (El-Sherif et al, 1983).

Definite identification of arrhythmias in humans triggered by OAP has not as yet been achieved (January & Fozzard, 1988; Wit & Rosen, 1991). However, some individual arrhythmias tend to occur more readily as heart rate increases and the coupling interval (time between the dysrhythmic beat and its preceeding normal beat) of the first dysrhythmic beat shortens with increasing heart rate. There are essential characteristics of OAP-initiated arrhythmias in animal models. If these features are employed to identify OAP-initiated triggered arrhythmias by statistical analysis, certain arrhythmias including accelerated atrioventricular junction rhythms (Rosen et al, 1980), catecholamine-sensitive ventricular tachycardias (Coumel et al, 1980), and some ischemia and reperfusion induced arrhythmias (Brugada & Wellens, 1984) may be triggered by OAP. It is certainly likely that a broad spectrum of digitalis tachycardias is initiated by OAP.

III. IONIC CURRENT UNDERLYING OAP

1. Transient Inward Current (TI)

A. Initial Demonstration of TI

The ionic current underlying OAP can only be studied by application of voltage clamp techniques. In voltage clamped cardiac tissues of both multicellular preparations (Lederer & Tsien, 1976; Kass et al, 1978a; Karagueusian & Katzung, 1982) and single cells (Mehdi & Sachs, 1978; Matsuda et al, 1982), a transient inward current has been associated with OAP. Lederer and Tsien (1976) were the first to demonstrate that an inward current which they termed "transient inward current (TI)" generated OAP. When calf Purkinje fibres exposed to toxic concentrations of strophanthidin were voltage-clamped at the maximum diastolic potential after the OAP had developed, these authors found a clear inward current deflection (TI) which peaked at a moment corresponding to the rising phase of OAP. They also demonstrated that the TI current was different from that underlying normal pacemaking activity. In sheep cardiac Purkinje fibres treated with ouabain, Aronson and Gelles (1977) also found an oscillatory inward current, which they termed i_{os}, which occurred upon repolarization from a long-lasting previous depolarization pulse of 5 to 12 sec. In many aspects, i_{os} was similar or identical to TI reported earlier by Lederer and Tsien (1976). The TI has been the subject of extensive studies over the past 15 years, yet its ionic mechanisms have still not been fully determined.

B. Evidence Suggesting That TI Generates OAP

In addition to the temporal similarity between TI and OAP, there are several other lines of evidence that suggest that TI is the current underlying OAP. These include: 1) Most agents and conditions used to induce OAP can also generate TI; all agents known to suppress OAP also inhibit TI. 2) The development of OAP, TI, and aftercontractions all appear at similar concentrations of digitalis. 3) Aftercontractions

associated with OAP or TI are very similar (Kass et al, 1978a). 4) The dependence of OAP and TI on frequency of stimulation is similar (Lederer & Tsien, 1976; Vassalle & Mugelli, 1981). 5) Ca^{2+} overload is the prerequisite (as will be discussed later) for the generation of both OAP and TI and various approaches and agents which can modify $[Ca^{2+}]_i$ have identical effects on OAP, TI, and aftercontractions (Kass & Tsien, 1982; Matsuda et al, 1982; Sutko & Kenyon, 1983; Valdeomillos & Eisner, 1985; Marban et al, 1986).

2. Role of Ca^{2+} Overload in the Generation of TI

A. Techniques Used to Measure $[Ca^{2+}]_i$ Transients

Information about Ca^{2+} movements in cells can be obtained by many ways. Some techniques, such as those involving radioisotopes, histochemical methods, or the electron microprobe, cannot distinguish ionized from nonionized Ca^{2+} . Information derived from these techniques may be useful in the interpretation of results obtained with intracellular Ca^{2+} indicators. Techniques using intracellular Ca^{2+} indicators are capable of giving direct indications of ionized calcium concentrations, and can be used to monitor such concentrations over periods of time in living cells.

There are now five general types of methods available for measuring $[Ca^{2+}]_i$ in living cells (Blinks, 1991). They involve the use of 1) Ca^{2+}-selective microelectrodes, 2) Ca^{2+}-regulated photoproteins, 3) fluorescent indicators, 4) metallochromic dyes, and 5) nuclear magnetic resonance (NMR) of ^{19}F-labeled chelators. Until recently, only the first two approaches had been used extensively in cardiac tissues, primarily in isolated multicellular preparations. Now, the fluorescent indicators are being more widely used, especially in the ratio imaging mode, for the study of $[Ca^{2+}]_i$ in isolated cardiac myocytes. The NMR method is being applied to whole hearts. The metallochromic dyes have been used very little in cardiac muscle. The following discussion will address two of the methods mentioned above: a Ca^{2+}-regulated photoprotein,

aequorin, which is isolated from jellyfish of the genus Aequorea, and the fluorescent Ca^{2+} indicators, fura-2 and indo-1. Detailed descriptions of these methods have been previously published (Tsien, 1988, 1989).

B. Pathways for Ca^{2+} Movement across the Sarcolemma

Ca^{2+} overload can be achieved by either increased influx of Ca^{2+} into the cells, or decreased efflux of Ca^{2+} out of the cells, or both. These processes are determined by a number of membrane structures, some of which are less well studied.

Sarcolemmal (SL) Ca^{2+} ATPase: Numerous experimental observations, both physiological and biochemical (Carafoli, 1988; Carafoli & Longoni, 1987), indicate that sarcolemmal Ca^{2+} ATPase plays a relatively minor role in removal of Ca^{2+} from the cardiac cells. In guinea pig ventricular cells, direct determination of the contribution of SL Ca^{2+} ATPase to Ca^{2+} removal was recently performed by Sipido and Wier (1989) who imposed a Ca^{2+} load by activating Ca^{2+} current and observed the decline of that load in the absence of Ca^{2+} current, Na^{+}-Ca^{2+} exchange, and SR function. Under these conditions, the half-time of the fall in $[Ca^{2+}]_i$ from 0.7 μM back to the resting value of 0.3 μM was 8 sec. This extremely slow process of Ca^{2+} removal led the authors to conclude that SL Ca^{2+} ATPase contributed little to Ca^{2+} efflux on the time scale of the physiological $[Ca^{2+}]_i$ transient.

Ca^{2+} Leak: Due to the existence of a large transmembrane concentration gradient for Ca^{2+}, it is possible that there is a leak of extracellular Ca^{2+} into the cell. Although the physical pathway for Ca^{2+} leak is unknown and direct information is unavailable on this issue (Wier, 1990), Rosenberg et al (1988) have demonstrated a B-type Ca^{2+} channel which may mediate Ca^{2+} leak. B-type channels have a small conductance (7 pS) and do not discriminate between Ba^{2+} and Ca^{2+}. Activation of B-type channels does not require depolarization (e.g. channel current can be recorded at potentials negative to -60 mV). B-type channels do not run down and Bay K 8644

has no obvious effect on this type of Ca^{2+} channel. Since B-type channels could not be identified in intack cells (Rosenberg et al, 1988), the contribution of B-type channels to Ca^{2+} leak remains to be determined. On the assumption that the Ca^{2+} leak is ohmic, Wier (1990) suggested that a Ca^{2+} leak is not an important factor on the time scale of the physiological $[Ca^{2+}]_i$ transient because $[Ca^{2+}]_i$ did not change for many seconds when membrane potential was changed by 100 mV.

Ca^{2+} Channels: Ca^{2+} entering the cytoplasm via both L- and T-type Ca^{2+} channels in cardiac tissues makes a direct contribution to Ca^{2+} load. L-type Ca^{2+} channels are more important in causing Ca^{2+} load. Since Ca^{2+} channels are modulated biochemically (Reuter et al, 1988) and are time- and voltage-dependent, Ca^{2+} influx through them will vary with cellular conditions. In the absence of Ca^{2+} entry via Na^+-Ca^{2+} exchange and Ca^{2+} release from SR, the running integral for 100 ms of L-type Ca^{2+} current in rat ventricular cells was almost superimposable with the running intergral of the fura-2 fluorescence change (Wier, 1990). This suggests that the $[Ca^{2+}]_i$ transient is caused mainly by Ca^{2+} entry via L-type Ca^{2+} channel. However, in the absence of SR Ca^{2+} release blocker, it was calculated that Ca^{2+} entering through the channels could have increased total $[Ca^{2+}]_i$ by 5-10 μM and that fura-2 alone bound about 100 μM of Ca^{2+} in the first 25 ms after depolarization (Callewaert et al, 1988). In guinea pig and rat cardiomyocytes, the amount of Ca^{2+} entering via the channels is about 10% or less of the total $[Ca^{2+}]_i$ transient seen upon activation (Wier, 1990). These results indicate that the major part of $[Ca^{2+}]_i$ transients may reflect SR release.

Na^+-Ca^{2+} Exchange: Mullins (1979) first pointed out that an electrogenic Na^+-Ca^{2+} exchange could load the cell with Ca^{2+} during the action potential and also extrude Ca^{2+} from the cell when $[Ca^{2+}]_i$ was high and during diastole. $[Na^+]_i$ is crucial in determining Na^+-Ca^{2+} exchange activity because 3 Na^+ must bind to the

exchanger protein. Half-maximal activation in heart requires 20 mM $[Na^+]_i$ (Kimura et al, 1987). There is a steep dependence of the reversal potential of the exchanger on $[Na^+]_i$ within the physiological range (Blaustein, 1988). Physiological $[Na^+]_i$ is about 10 mM at 37°C or 16 mM at room temperature (Blatter et al, 1986). It seems that Na^+-Ca^{2+} exchange does not contribute significantly to Ca^{2+} loading if the $[Na^+]_i$ is substantially lower (3.75-4.5 mM) than required for physiological conditions (Cannell et al, 1987; Callewaert et al, 1988). However, with measurements of fura-2 or indo-1 fluorescence transients, direct loading of Ca^{2+} through Na^+-Ca^{2+} exchange has been clearly demonstrated in the presence of physiological $[Na^+]_i$ (Barcenas-Ruiz & Wier, 1987; Isenberg et al, 1988). A large body of indirect evidence also supports the idea that Ca^{2+} entry can be achieved through Na^+-Ca^{2+} exchange during depolarization (Bielefeld et al, 1986; Brill et al, 1987; Sheu et al, 1986; Terrar & White, 1989). Ca^{2+} loading via Na^+-Ca^{2+} exchange in the presence of elevated $[Na^+]_i$ is generally believed to occur either by increasing the activity of reverse exchange, or by slowing extrusion of Ca^{2+} in the forward mode.

C. Evidence for Ca^{2+} Overload in the Generation of TI

Involvement of Ca^{2+} in the generation of TI was suggested by a number of earlier studies on OAP. Ferrier and Moe (1973) showed that OAP were enhanced in Ca^{2+}-rich solutions, and reversibly abolished in Ca^{2+}-free solution. OAP were suppressed by agents which were known to inhibit transmembrane Ca^{2+} fluxes, such as Mn^{2+} (Ferrier & Moe, 1973), Mg^{2+} (Ferrier & Saunders, 1972), and verapamil (Rosen et al, 1973b). However, Kass et al (1978a) were the first to demonstrate the crucial importance of Ca^{2+} in the generation of TI. In isolated calf cardiac Purkinje fibres exposed to strophanthidin, Kass et al (1978a) clearly showed that 1) the amplitude of TI varied directly with the extracellular Ca^{2+} concentration and Mg^{2+} had an antagonistic effect; 2) TI thus induced could be abolished by both D600 and Mn^{2+}; 3)

TI and aftercontraction could be evoked by elevating $[Ca^{2+}]_o$ in the absence of digitalis. It was proposed that toxic concentrations of digitalis caused net gain of intracellular Na^+ by inhibiting Na^+-K^+ pump, and the decrease in Na^+ gradient resulted in a net gain of intracellular Ca^{2+} by Na^+-Ca^{2+} exchange. Eventually the cells reached a state of "Ca^{2+} overload" which was accompanied by transient or oscillatory release of Ca^{2+} from intracellular stores, a key step leading to the generation of TI and aftercontraction.

Following this important study, evidence presented by a number of labs indeed suggested that "Ca^{2+} overload" was present under conditions known to generate TI or OAP. With the application of Na^+- and Ca^{2+}-sensitive microelectrodes Lee & Dagostino (1982) found in canine Purkinje fibres that both $[Na^+]_i$ and $[Ca^{2+}]_i$ were increased by toxic concentrations of strophanthidin. By monitoring aequorin fluorescence as a measure of the $[Ca^{2+}]_i$ transient, Allen et al (1984a; 1984b) showed that $[Ca^{2+}]_i$ increased in response to increasing $[Ca^{2+}]_o$ and to exposure to strophanthidin in ferret papillary muscles. In isolated single guinea pig ventricular cells, Matsuda et al (1982) found that intracellular injection of a Ca^{2+} chelator, K-EGTA, abolished both TI and OAP induced by ouabain. The most direct evidence supporting Ca^{2+} overload in the generation of TI comes from a study by Berlin et al (1989). They examined the cellular basis for the rise in $[Ca^{2+}]_i$ that activated TI and aftercontractions in single rat ventricular cells. In their study, $[Ca^{2+}]_i$ was measured both indirectly by cell contraction and directly with fura-2. They found that TI occurred at the time when $[Ca^{2+}]_i$ spontaneously increased and preceded the aftercontraction by 60-90 msec. Examination of cell contraction and digital imaging of fura-2 fluorescence showed that TI was often associated with propagating regions of increased $[Ca^{2+}]_i$, which arose from discrete sites of origin within the cell.

3. Oscillatory Release of Ca^{2+} from Sarcoplasmic Reticulum (SR)

A. SR Ca^{2+} Release and the Generation of TI

Cardiac SR is the major storage site for Ca^{2+} that is released to produce contraction (Katz et al, 1986). Cardiac action potentials activate both T- and L-type Ca^{2+} currents which then may trigger Ca^{2+} release from the SR (Fabiato, 1983; 1985c; Sham et al, 1992; Ferrier & Howlett, 1992). By binding to troponin C, Ca^{2+} initiates cell shortening through interaction of actin and myosin filaments. The released Ca^{2+} is pumped back into the SR by an ATP-dependent Ca^{2+} pump and stored there for the next contraction.

The importance of the SR in the generation of TI (and OAP) has been established primarily by several pharmacological approaches. It is well known that depolarization into the plateau range of the action potential (+10 mV) elicits a large, rapidly rising $[Ca^{2+}]_i$ transient which has been attributed to Ca^{2+} release from the SR. Ryanodine is now known to lock the SR Ca^{2+} release channel in a subconducting state. This in turn results in rapid loss of Ca^{2+} from the SR (Hansford & Lakatta, 1987; Silverman et al, 1988) and interferes with the normal release mechanism (Marban & Wier, 1985). Ryanodine, which reduces this $[Ca^{2+}]_i$ transient by 90% (Barcenas-Ruiz & Wier, 1987; Beuckelmann & Wier, 1988; Callewaert et al, 1988), also suppresses or abolishes TI and aftercontraction associated with this transient (Marban et al, 1986; Sutko & Kenyon, 1983; Valdeomillos & Eisner, 1985; Han & Ferrier, 1992a). Caffeine, another agent known to interfere with the SR Ca^{2+} release mechanism, also abolishes TI and OAP at relatively high concentrations (Kass & Tsien, 1982; Matsuda et al, 1983). TI and OAP also are abolished by intracellular application of Ca^{2+} buffering agents (Marban et al, 1986; Kass & Tsien, 1982) such as ethyleneglycol-bis(β-aminoethylether)-N,N'-tetraacitic acid (EGTA) and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacitic acid (BAPTA). Indeed, TI and aftercontraction cannot be induced in animal species (i.e. frogs) with a small volume of SR (e.g. a smaller

amount of releasable Ca^{2+}, Hume & Uehara, 1986a; 1986b), which further stresses the importance of SR in the induction of TI.

B. Voltage Dependence of SR Ca^{2+} Release

A number of studies have shown that the peak of $[Ca^{2+}]_i$ transient activated upon depolarization has a bell-shaped dependence on membrane potential (Barcenas-Ruiz & wier, 1987; Beuckelmann & Wier, 1988; Callewaert et al, 1988; Cannell et al, 1987). This is interpreted to mean that release of Ca^{2+} from cardiac SR decreases at positive potentials, a feature that is distinctly different from that in skeletal muscle (Rios & Brum, 1987). As mentioned above, this depolarization-induced $[Ca^{2+}]_i$ transient is ryanodine sensitive but cannot be totally abolished by ryanodine. This observation suggests other sources of Ca^{2+} such as those coming through Ca^{2+} channels or Na^+-Ca^{2+} exchange also contribute to the total transient. A $[Ca^{2+}]_i$ transient also has been observed in cardiac cells from guinea pig and rat upon repolarization (Barcenas-Ruiz & wier, 1987; Beuckelmann & Wier, 1988; Callewaert et al, 1988; Cannell et al, 1987). This repolarization generated $[Ca^{2+}]_i$ transient can be totally abolished by ryanodine, which indicates that the Ca^{2+} mostly originates from the SR. The mechanism of Ca^{2+} release from the cardiac SR has been discussed extensively by Fabiato (1982; 1983; 1985a; 1985b; 1985c) and termed Ca^{2+}-induced Ca^{2+} release. Release also can be inactivated by Ca^{2+} (Beuckelmann & Wier, 1988; Fabiato, 1985b).

C. SR Ca^{2+} Pump

Purification of the Ca^{2+}-dependent ATPase from SR was reported in 1970 (MacLennan, 1970). Soon thereafter reconstitution experiments demonstrated that this molecule could pump Ca^{2+} against a concentration gradient at the expense of ATP hydrolysis (Racker, 1972). Both biochemical (Carafoli, 1988) and physiological evidence (Tada & Katz, 1982) supports the idea that an ATP-dependent Ca^{2+} pump in the SR (Inesi, 1985) plays an important role in determining the decline of the $[Ca^{2+}]_i$

transient under physiological conditions. The Ca^{2+} ATPase has a high affinity for Ca^{2+} (Km = 0.5 μM) and a high V_{max} (maximum rate of pumping of Ca^{2+}) relative to the sarcolemmal Ca^{2+} ATPase. β-adrenergic stimulation speeds the decline of $[Ca^{2+}]_i$ transient, most likely via the cAMP-dependent phosphorylation of phospholamban (Tada & Katz, 1982), a regulatory protein which is thought to interact with the Ca^{2+} pump, which has a similar distribution to the Ca^{2+} pump in the SR (Hilgemann & Noble, 1987).

IV. THE CHARGE-CARRYING MECHANISMS OF TI

The cellular basis of the TI has remained controversial (January & Fozzard, 1988; Ferrier, 1991) and forms an important part of my PhD project. Two different charge-carrying mechanisms have been proposed. One hypothesis (Kass et al, 1978a; 1978b; Kass & Tsien, 1982) argues for a nonselective cation membrane channel with its conductance regulated by intracellular free Ca^{2+}. The other mechanism suggested for TI is the electrogenic Na^{+}-Ca^{2+} exchange driven by the transmembrane electrochemical gradients for Na^{+} and Ca^{2+} (Kass et al, 1978b; Noble, 1984; Arlock & Katzung, 1985). Although both mechanisms initially proposed by Kass and his colleagues (1978b) are based on experiments performed on Purkinje fibres, experimental evidence in favour of the electrogenic Na^{+}-Ca^{2+} exchange theory comes mostly from studies in cardiac tissues other than Purkinje fibres. The following literature review will concentrate on summarizing some of the most important studies in both Purkinje and other tissues of the heart.

1. Studies on Purkinje Fibres: Presence of E_{REV}

Shortly after the initial demonstration of the TI current, Kass et al (1978a) studied the role of Ca^{2+} in the development of TI that underlies digitalis-induced OAP. Voltage-clamped calf Purkinje fibres were exposed to strophanthidin during regular electrical stimulation and were perfused with modified Tyrode's solution containing elevated Ca^{2+} (5.4 mM). Tension was simultaneously measured as an indirect measure of $[Ca^{2+}]_i$. These investigators found that: 1) when the membrane potential of a digitalis-intoxicated Purkinje fibre was clamped at the maximum diastolic potential, TI appeared and was accompanied by an aftercontraction; 2) decreasing $[Ca^{2+}]_o$ from 10.8 to 5.4 to 3 mM caused a graded and reversible decrease in the amplitude of TI; 3) TI and aftercontraction could be evoked in the absence of digitalis by large elevation in $[Ca^{2+}]_o$ (18 mM); 4) Ca^{2+} channel blockers Mn^{2+} and D600

inhibited TI but the development and removal of the inhibition lagged far behind the effects on Ca^{2+} current; 5) there was a reversal potential (E_{REV}, about -5 mV) at which no TI could be seen but aftercontractions were still present.

The dissociation between TI and aftercontraction and the temporal dissociation between the inhibitory effects of Ca^{2+} channel blockers on TI and I_{Ca} are consistent with the idea that an oscillatory release of Ca^{2+} from an intracellular store (i.e. SR), rather than Ca^{2+} influx through the sarcolemma, is the primary event underlying both the aftercontraction and the conductance change which generates TI. Furthermore, this important study in which a reversal potential was first demonstrated forms the foundation supporting the hypothesis that a channel might be the charge-carrying system for TI. Since the E_{REV} is about- 5 mV which does not coincide with E_{REV} for Na^+, Ca^{2+}, K^+, or Cl^-, the channel is most likely "non-selective".

In a subsequent study, Kass et al (1978b) used the E_{REV} of TI as a basis for studying the ionic dependence of the current. They found that when $[Na^+]_o$ was decreased from 150 to 75 mM, the state-state effect on TI was to decrease its amplitude and shift E_{REV} from -5 mV to more negative potentials (-18 to -26 mV). Further shift of E_{REV} in the negative direction was found with reduction of $[Na^+]_o$ to 37.5 and 0 mM. There was no obvious difference whether Na^+ was replaced by choline, Tris, or sucrose. When $[Na^+]_o$ was reduced to 0 mM, TI increased briefly before it eventually declined. These observations were interpreted by the authors to indicate that Na^+ participates in carrying TI. However, because of the apparent difference between E_{REV} (-5 mV) and E_{Na} (about +70 mV) Na^+ could not by the sole ion involved in the generation of TI. Since varying $[Ca^{2+}]_o$ and $[Cl^-]_o$ failed to shift E_{REV}, no conclusive role was assigned for these ions in carrying TI. Based on the results reported in these two papers, the investigators suggested that the ionic pathway carrying TI could be either a "leak" conductance which was cationic but non-selective

or an electrogenic inward movement of Na^+ via the Na^+-Ca^{2+} exchange system. However, as the authors pointed out, although Na^+-Ca^{2+} exchange can account for the inward TI recorded at potentials negative to the E_{REV}, it does not readily explain the TI in the outward direction, because, if the stoichiometry of the Na^+-Ca^{2+} exchange is independent of membrane potential, phasic increases of $[Ca^{2+}]_o$ can only promote TI in the inward direction. Thus, "reversal of TI can be accounted for, but only by invoking another mechanism in parallel with the Na^+-Ca^{2+} exchange".

The initial characterization of TI by Kass et al (1978a; 1978b) showed a clear-cut reversal potential at about -5 mV (in Tyrode's solution) and led to the suggestion that TI arises from a Ca^{2+} activated non-selective channel. However, other workers failed to observe an E_{REV} in a variety of cardiac tissues including Purkinje fibres (Vassalle & Mugelli, 1981; Mugelli, 1982; Lin et al, 1986), ventricular muscles or single cells (Karaguazian & Katzung, 1982; Arlock & Katzung, 1985; Fedida et al, 1987a; 1987b), sino-atrial node (Brown et al, 1986); and coronary sinus (Tseng & Wit, 1987a; 1987b). TI induced in these studies seemed to be more easily explained by electrogenic Na^+-Ca^{2+} exchange. Indeed, certain mathematical models also appear to favour electrogenic Na^+-Ca^{2+} exchange as the mechanism for TI (Noble, 1984).

In an attempt to identify the mechanism of the TI, Cannell & Lederer (1986) sought to rule out the Na^+-Ca^{2+} exchange system as the mechanism for TI by replacing $[Na^+]_o$ with $[Ca^{2+}]_o$. When voltage-clamped sheep Purkinje fibres were exposed to zero K^+ solution containing isotonic Ca^{2+} (105 mM) in the absence of $[Na^+]_o$, TI and accompanying aftercontractions which lagged about 80 ms behind TI were both induced following repolarization from a previous brief depolarizing pulse. A distinct E_{REV} occurred at about -37 mV. When TI reversed its direction (outward), the aftercontraction still remained. This TI cannot be explained by the Na^+-Ca^{2+} exchange mechanism because: 1) Influx of Na^+ through the exchanger is disabled by

the absence of $[Na^+]_o$; 2) Appearance of an E_{REV} is not predicted by at least two models of Na^+-Ca^{2+} exchange (Mullins, 1979; 1981; Eisner & Lederer, 1985). On the other hand, these observations strongly argue for a Ca^{2+} activated non-selective cation channel as the mechanism of TI generation.

2. Studies on Cardiac Tissues Other Than Purkinje Fibres: Absence of E_{REV}

A. Ventricular Muscle

Karagueuzian and Katzung (1982) were the first to induce TI in ventricular muscles by digitalis intoxication. Right ventricular papillary muscles from ferret hearts were voltage-clamped with a single sucrose-gap technique and tension was measured simultaneously. Under voltage clamp, TI accompanied by an aftercontraction was recorded on repolarization to the holding potential of -50 mV after a depolarizing clamp to +20 mV for 1 sec. The amplitude of TI thus induced became zero at a potential of -9 mV but did not reverse its direction even when Na^+ was replaced by sucrose or choline chloride. However, replacement of Na^+ by sucrose or choline chloride could reversibly abolish the inward TI. From this early study, Karagueuzian and Katzung (1982) concluded that while the current induced in their experiments was the same novel inward current (TI) seen in Purkinje fibres (Lederer & Tsien, 1976; Aronson & Gelles, 1977; Kass et al, 1978a; 1978b), there was a major apparent difference between the TI induced in Purkinje fibre and ventricular muscle: a detectable reversal, i.e. an outward TI, could not be found in the latter.

Arlock and Katzung (1985) conducted another study designed to investigate the nature of the pathway carrying TI in voltage-clamped guinea pig papillary muscles from the right ventricle. They found that 1) In the presence of normal $[Na^+]_o$, the amplitude of TI induced by digitalis intoxication became progressively smaller at less negative voltages, but never reversed its polarity at voltages up to +30 mV; 2) TI was abolished after perfusion for 10 min with a solution in which 75% of the Na^+

was replaced by Li^+ and no outward TI was observed in these experiments; 3) The I-V relation of TI was remarkably similar to the I-V relationship for the Na^+-Ca^{2+} exchange calculated according to the model of Noble (1984). Therefore, these authors concluded that digitalis-induced TI in ventricular muscles is dependent on $[Na^+]_o$ and that the TI is carried by electrogenic Na^+-Ca^{2+} exchange rather than a passive cation channel.

B. Coronary Sinus

Tseng and Wit (1987a) investigated the characteristics of TI in atrial cells of the canine coronary sinus. Under voltage clamp (two-electrode technique), perfusion of the small preparation with noradrenaline regularly induced TI upon repolarization from a previous depolarizing step (+40 mV). TI thus induced showed biphasic changes in amplitude between the test potentials of -98 and +31 mV: gradual increase in TI amplitude was seen when the test potentials were made positive between -98 to +4 mV, the amplitude of TI then decreased when the test potentials were made further positive between +4 and +31 mV. However, TI never reversed its direction. The time to peak TI decreased nonlinearly from 900 ms at -90 mV to 150 ms at -6 mV and remained nearly constant at test potentials up to +30 mV. The lack of reversal potential in these experiment was taken as evidence for electrogenic Na^+-Ca^{2+} exchange as the charge-carrying mechanism of TI.

C. Single Ventricular Cells

Inhibition of Na^+-K^+ Pump: Fedida et al (1987a) studied TI induced by either digitalis or lowering $[K^+]_o$ to 0.5 mM or less in voltage-clamped single ventricular cells from guinea pig hearts. TI thus induced had some characteristics similar to those reported in multicellular preparations. For instance, longer pre-pulses to more positive potentials increased the size and reduced the latency of TI; TI was maximal at -50 mV but diminishing in magnitude at more negative and positive potentials.

However, TI remained inward at potentials up to +47 mV. Although the I-V relation varied even in the same cell, an inward tail current seen upon repolarization from a previous depolarization pulse progressively increased at negative potentials. Furthermore, replacement of Na^+ by Li^+ initially increased the magnitude of TI, but longer exposure abolished TI although aftercontractions continued to increase. Li^+ is known to pass freely through non-selective cationic channels but cannot substitute for Na^+ in the Na^+-Ca^{2+} exchange. Therefore, the absence of E_{REV} even at very positive potentials and the disappearance of TI when Na^+ was substituted with Li^+ led the authors to favour electrogenic Na^+-Ca^{2+} exchange current as a major component of TI in guinea pig ventricular myocytes.

Reoxygenation: The kinetic properties of TI induced by reoxygenation of the isolated single ventricular cells from the guinea pig hearts were recently investigated by Benndorf et al (1991). These authors also found that the amplitude of inward TI thus induced decreased at more positive potentials, vanished completely between +20 to +60 mV but never reversed. A simple noise analysis of the current fluctuation of TI indicated that the ion channels were not the major system which conducted TI, because their unitary conductance could not exceed 0.36 pS. Therefore, the results were explained by assuming that an increase in cytosolic Ca^{2+} shifted the I-V relation of the Na^+-Ca^{2+} exchanger transiently, and it was the Na^+-Ca^{2+} exchange which mediated the TI.

Oxidant Stress: In another recent report, Matsuura and Shattock (1991) studied the membrane potential fluctuations and TI induced by reactive oxygen intermediates in isolated rabbit ventricular cells under whole-cell voltage and current clamp. They found that while the amplitude of TI was dependent on the magnitude and duration of the preceding voltage step, it remained inward even at test potential up to +30 mV. Replacement of Na^+ by Li^+ decreased TI to approximately 10% of its original value.

Thus, the authors concluded that the major component of oxidant-stress induced TI appeared to be electrogenic Na^+-Ca^{2+} exchange.

D. Sino-Atrial Node

Brown et al (1986) investigated the characteristics of TI in small preparations from rabbit sinus node. Under voltage clamp (two-electrode technique), TI was induced by lowering $[K^+]_o$ to 0.3 mM. The Na^+-K^+ pump is known to be inhibited at this concentration of $[K^+]_o$. These authors found that oscillations of TI became smaller near 0 mV, but there was no potential at which the current records were completely flat. Therefore, no simple reversal potential was recorded. TI was also greatly reduced by 75% substitution of Na^+ by Li^+. A computer model of the sino-atrial node that simulated the experimental conditions (Noble & Noble, 1984) could reproduce the characteristics of the TI as variations in the Na^+-Ca^{2+} exchange current but not the non-specific cation current, even though both of them were dependent on $[Ca^{2+}]_i$. From both the experimental observations and the computer simulations Brown et al (1986) concluded that a large fraction of TI was generated by the Na^+-Ca^{2+} exchange and that only a relatively small portion of TI might be generated by non-specific channels.

E. Cultured Single Atrial Cells

A spontaneous transient inward current (TI) was found by Lipp & Pott (1988; also Mechmann & Pott, 1986) in enzymatically isolated, cultured atrial myocytes from adult guinea pig hearts under voltage clamped conditions with a whole-cell patch-clamp technique. A similar membrane current also could be evoked by superfusion of the cells with caffeine (5-10 mM). This current, while having certain similarities to TI found in other settings, did not require Ca^{2+} overload and occurred spontaneously without triggering by action potentials or depolarizing pulses. This TI remained inward at membrane potentials between -90 and +75 mV and thus, a

reversal of TI was never found. Both the instantaneous I-V relation and voltage dependence of peak TI showed distinct outward rectification which could be described by a formulism suggested for a membrane current caused by electrogenic Na^+-Ca^{2+} exchange with a 3:1 stoichiometry (DiFrancesco & Noble, 1985). The authors concluded from these observations that the electrogenic Na^+-Ca^{2+} exchange was very likely the charge-carrying system for the TI under their experimental conditions.

3. Other Studies

The above discussion suggests that E_{REV} is generally absent in preparations other than Purkinje fibres. However, contradictory evidence also exists. In a recent study, Dinda and Houser (1990) found that in voltage-clamped single feline ventricular myocytes TI had a clear E_{REV} near +40 mV in normal Tyrode's solution (150 mM Na^+). When $[Na^+]_o$ was decreased to 15 mM, the amplitude of TI was reduced and the E_{REV} was shifted to 0 mV. Peak $[Ca^{2+}]_i$ transient, as reflected by changes of indo-1 fluorescence signals, showed no apparent voltage dependence during the spontaneous release elicited by prolonged depolarization. These results were interpreted to support the idea that spontaneous SR Ca^{2+} release activates an ion channel and that Na^+ may be the primary charge carrier. On the other hand, not all studies of TI elicited in Purkinje fibres have a clear E_{REV}. Indeed, failure of TI to reverse its polarity was reported by Lin et al (1986) who studied the mechanism underlying TI in voltage-clamped sheep cardiac Purkinje fibres. The TI was induced by either elevating $[Ca^{2+}]_o$, decreasing $[Na^+]_o$, or adding strophanthidin. Unfortunately, the range of test voltages in their study was limited to negative potentials.

An interesting study that compares the characteristics of Na^+-Ca^{2+} exchanger current and TI in single cells from rabbit ventricle was reported by Giles and Shimoni (1989a; 1989b; also see Shimoni & Giles, 1987). These investigators found that partial inhibition of the Na^+-K^+ pump by either 0.54 mM $[K^+]_o$ or digitalis induced

both TI and the exchanger current termed I_{EX}. TI and I_{EX} could be separated by a number of manoeuvres including varying $[K^+]_o$ and $[Ca^{2+}]_o$, decreasing $[Na^+]_o$ to 37.5 mM (replaced by LiCl), changing patterns of voltage-clamp depolarization, replacing $[Ca^{2+}]_o$ with Ba^{2+} or Sr^{2+}, exposing to Mn^{2+}, and applying caffeine and ryanodine. Since these treatments clearly dissociated TI from the exchanger current, these authors suggested that even though both currents are triggered by increases in $[Ca^{2+}]_i$, they are distinct. While I_{EX} is generated by electrogenic Na^+-Ca^{2+} exchange, TI may be due to Ca^{2+}-activated cation-selective channels in the sarcolemma.

4. Non-Selective Cation Channel Identified at the Single Channel Level

Single channel recordings in cardiac cells of a non-selective cation channel activated by intracellular Ca^{2+} have been obtained by Colquhoun et al (1981) in membrane patches from cultured rat neonatal myocytes. The opening and closing of those channels depended far more on $[Ca^{2+}]_i$ than on the membrane potential: the open-close equilibrium changed less than e-fold for 200 mV. The channels tended to open when $[Ca^{2+}]_i$ was 1.5-6 μM and tended to be closed when $[Ca^{2+}]_i$ was zero. The conductance of the activated channel (30-40 pS at 25-27°C) was ohmic and the permeability to Na^+ and K^+ appeared to be similar. The permeability to Li^+ was not reported. When a salt gradient across the membrane was applied by diluting the pipette saline to 1/5 with distilled water, single channel current recorded reversed polarity at -40 mV as expected for cation selectivity. When Cl^- was replaced by aspartate, E_{REV} did not change, indicating negligible anion permeability of the ion channels.

After this initial report, Ca^{2+}-activated non-selective cation channels were also found in various mammalian non-cardiac cells (Yellen, 1982; Maruyama & Petersen, 1982; Maruyama et al, 1985; von Tscharner et al, 1986; Siemen & Reuhl, 1987). It seems likely that this species of channel is widely distributed. Various membrane

conductances observed in response to Ca^{2+}-overload are explained by assuming activation of this kind of channel.

In contrast to studies in non-cardiac tissues, documentation of single Ca^{2+}-activated non-selective channels in adult cardiac cells is scarce. Reuter (1983) reported a class of Ca^{2+}-activated channels in a brief report with no detailed characteristics of the channel being given. In 1988, a class of Ca^{2+}-activated non-selective cation channels was identified by Ehara et al (1988) in enzymatically isolated ventricular myocytes from adult guinea pig hearts. Kinetics of this channel were not affected by varying the membrane potential. The slope conductance of the channel was about 15 pS at room temperature. The E_{REV} determined in the inside-out patch was at about 0 mV irrespective of the Na^+-, K^+-, Li^+-, or Cs^+-rich solutions on either side of the membrane, thereby indicating that the channel was equally permeable to these cations. The $[Ca^{2+}]_i$ threshold for channel activation was 0.3 μM, and the open probability was half maximal at 1.2 μM $[Ca^{2+}]_i$.

With the techniques of planar bilayer reconstitution, Hill et al (1988) found a class of non-selective cation channels in sarcolemmal vesicles prepared from adult canine ventricular muscles. This type of channel responded to increased $[Ca^{2+}]_i$ with increasing probability of opening. The single channel conductance was about 120 pS. Ionic selectivity experiments indicated that the channels were cation selective but unable to discriminate between Na^+ and K^+. However, channel gating exhibited marked voltage dependence, which was quite different from the channel gating reported by Colquhoun et al (1981) and Ehara et al (1988). Nevertheless, the functional significance of this class of channels with regard to the generation of TI, OAP, and triggered arrhythmias was discussed in all three studies cited.

5. Na^+-Ca^{2+} Exchange Current in Single Cardiac Cells

It is surprising that direct measurement of a current attributable to Na^+-Ca^{2+}

exchange was not reported prior to 1985 (Eisner & Lederer, 1985). Currents attributed to the electrogenic Na^+-Ca^{2+} exchange were first identified in cardiac preparations by several groups of investigators in 1986 (Kimura et al, 1986; Mechmann & Pott; 1986; Bielefeld et al, 1986). With the intracellular perfusion technique combined with the whole-cell voltage clamp, Kimura et al (1986) were able to load Na^+ inside and control Ca^{2+} outside single ventricular cells from guinea pig and succeeded in recording an outward Na^+-Ca^{2+} exchange current while blocking most other membrane currents. The current, with a slope conductance of about 23 pS, was voltage dependent, blocked by La^{3+} and was absent in zero $[Ca^{2+}]_i$. The E_{REV} of the current also was as expected for the Na^+-Ca^{2+} exchange with a stoichiometry of 3 to 1. In cultured adult guinea pig atrial cells Mechmann and Pott (1986) demonstrated that a transient rise in $[Ca^{2+}]_i$ caused by release of Ca^{2+} from the SR generated a membrane current. This current was dependent on both the transmembrane Na^+ and Ca^{2+} gradients and the membrane potential and met the criteria for a current produced by electrogenic Na^+-Ca^{2+} exchange. In voltage clamped single atrial cells from the frog hearts, Bielefeld et al (1986) found a "creep current" when the cells were exposed to a Na^+ ionophore, monensin. Since three agents, dodecylamine, quinacrine, and 3',4'-dichlorobenzamil which were known to block Na^+-dependent Ca^{2+} uptake in sarcolemmal vesicles, blocked the creep current at very low concentrations, the authors suggested that the creep current might represent the activity of a Na^+-Ca^{2+} exchanger.

Since the appearance of the above three reports, detailed studies of the kinetic properties and functional significance of electrogenic Na^+-Ca^{2+} exchange current at the single cell level have been performed by many investigators. Kimura et al (1987) extended their previous report by observing that the exchange current was blocked by heavy metal cations like La^{3+}, Cd^{2+}, Mn^{2+}, Ni^{2+} and partially blocked by amiloride

and D600. The outward current showed a sigmoidal dependence on the $[Ca^{2+}]_o$ and the inward current component showed a sigmoidal dependence on $[Na^+]_o$. While Sr^{2+} could replace Ca^{2+} to generate this current, Mg^{2+} and Ba^{2+} could not. In a subsequent study, the same group (Ehara et al, 1989) measured the E_{REV} of Na^+-Ca^{2+} exchange current and studied its I-V relations under a variety of experimental conditions. When $[Na^+]_i$ and $[Ca^{2+}]_i$ were properly controlled (such as under their experimental conditions), a reversal potential of the Na^+-Ca^{2+} exchange current could be recorded. The E_{REV} was determined from the intersection of the I-V curves obtained in the absence and presence of the Na^+-Ca^{2+} exchange blocker, Ni^{2+} (2 mM). These authors found that the Ni^{2+} sensitive component of the Ca^{2+}- or Na^+-induced current showed changes in E_{REV} which were in agreement with theoretical equilibrium potentials, $E_{Na,Ca}=3E_{Na}-2E_{Ca}$ (Mullins, 1981), under the ionic conditions of 0.2-20 mM $[Ca^{2+}]_o$ and 30-140 mM $[Na^+]_o$. The conclusion from this study was that the $[Ca^{2+}]_o$ or $[Na^+]_o$ induced current was generated by the Na^+-Ca^{2+} exchange system and the stoichiometry was $3Na^+:1Ca^{2+}$. Lipp and Pott (1988) studied the voltage-dependence of the Na^+-Ca^{2+} exchange current which they called spontaneous TI. They used 3',4'-dichlorobenzamil as an inhibitor to identify the Na^+-Ca^{2+} exchange current. It was found that the transient rise in $[Ca^{2+}]_i$ following Ca^{2+} release from the SR could shift the E_{REV} in the positive direction for 85 mV. This suggested that control of the ion concentrations within the cell was indeed prerequisite for measuring the E_{REV} of the Na^+-Ca^{2+} exchange, as noticed by Ehara et al (1989).

A word of caution must be mentioned here. Since there is no specific blocker for the Na^+-Ca^{2+} exchange at present, any study using blockers as the main method of identification must be interpreted with great caution. If the blockers (i.e. Ni^{2+} or 3',4'-dichlorobenzamil) also affect other membrane currents or intracellular processes, interpretation of results will be complicated. To what extent can the conclusions

obtained from internally dialysed single cells be applied to Purkinje fibres whose intracellular solution is more physiological? In the studies cited from one group (Mechmann & Pott; 1986; Lipp & Pott, 1988), the tonicity of the pipette solution was much lower than the extracellular solution. Will this affect the results and therefore the interpretation of the results? Answers to these questions are not clear.

V. MECHANISMS OF ADRENERGIC STIMULATION OF TI

1. General Considerations

Sympathetic regulation of cardiac contractility is mediated by release of noradrenaline and adrenaline which act by binding to adrenergic receptors. The positive inotropic effect can be mediated by both α- and β-adrenoceptors, but mediation by the latter is more important (Endoh & Blinks, 1988). It is generally accepted that β-adrenergic stimulation leads to the formation of cyclic AMP (cAMP) which functions as an intracellular second messenger to activate a cAMP-dependent protein kinase and thereby induce phosphorylation of proteins in the sarcolemma, SR, and thin filaments (Tsien, 1977; Tada & Katz, 1982; Reuter, 1983; Stiles & Lefkowitz, 1984; Solaro, 1986). One of the most important consequences of protein phosphorylation at these sites is an elevation of free intracellular Ca^{2+}. In contrast to the relatively well-established role of cAMP-dependent phosphorylation in mediating β-adrenergic effects, α-adrenergic inotropism does not involve the formation of cAMP and therefore cAMP-dependent phosphorylation (Schumann et al, 1975; Tahiliani et al, 1982). On the other hand, increased $[Ca^{2+}]_i$ during α-adrenergic stimulation is currently believed to be mediated by an increase in myocardial inositol phosphate levels such as inositol trisphosphate (IP_3) and diacylglycerol (DAG). These agents can act as second messengers to mobilize Ca^{2+} from intracellular stores (IP_3) or to increase Ca^{2+} influx (DAG) through voltage-gated membrane Ca^{2+} channels (Minneman, 1988; Mouton et al, 1991). The effect of DAG is thought to be mediated by protein kinase C (Wise et al, 1982). However, inhibition on isoproterenol stimulated L-type I_{Ca} also has been reported in frog atrial myocytes with DAG analogues (Shibata et al, 1987).

While an increase in $[Ca^{2+}]_i$ is commonly viewed as the major mechanism responsible for the positive inotropic effects of both α- and β-adrenergic stimulation,

Ca^{2+}-overloaded cardiac cells also manifest many abnormal electrophysiological and mechanical alterations which in turn result in arrhythmias. As discussed earlier, stimulation of β-adrenoceptors has been shown to induce OAP in both multicellular and single cell preparations from the heart (Wit & Cranefield, 1977; Priori & Corr, 1990). The amplitude of OAP induced by ouabain in isolated canine Purkinje fibers (Hewett and Rosen, 1984), spontaneous OAP in isolated ventricular myocytes (Belardinelli and Isenberg, 1983), as well as OAP occurring in feline Purkinje fibres surviving in 2 to 4 month old infarcts (Kimura et al, 1987) were all increased by β-adrenergic stimulation. Stimulation of α-adrenoceptors also has been reported to have arrhythmogenic effects during ischemia and reperfusion (Penny, 1984; Culling et al, 1987). The protective effect of α-adrenergic antagonists against ischemia- and reperfusion-induced arrhythmias is well documented (Corbalan et al, 1976; Sheridan et al, 1980; Stewart et al, 1980). Under certain pathological conditions, the arrhythmogenic effect of α-adrenergic stimulation has been attributed to enhancement of OAP (Sheridan, 1986; Kimura et al, 1984; 1987).

β-adrenergic stimulation can increase the peak TI in atrial cells (Boller & Pott, 1989), coronary sinus (Tseng and Wit, 1987a), and sino-atrial node cells (Satoh and Hashimoto, 1988). Stimulation of α-adrenoceptors with phenylephrine has been shown to inhibit digitalis-induced TI but enhance TI induced by elevated $[Ca^{2+}]_o$ (Ferrier & Carmeliet, 1990).

Enhancement of TI by β-adrenergic stimulation has been generally attributed to an increase in I_{Ca}, however, the exact mechanism(s) is far from being clear. In part, this is because the ionic basis underlying TI is still a subject of much debate (Giles & Shimoni, 1989a; Wit & Rosen, 1991). Information regarding the mechanism of α-adrenergic stimulation of TI also is largely unknown. Therefore, the following literature review will mainly concentrate on the adrenoceptor-initiated biochemical and

electrophysiological processes leading to changes of $[Ca^{2+}]_i$, with brief comments on any possible relevance to the generation of TI.

2. α-Adrenergic Stimulation

A. Classification

Ahlquist (1948) began the modern era in adrenergic pharmacology by recognizing that responses to adrenergic stimuli could be divided into two major categories. He proposed that the two major receptor subtypes, which he named α and β-, should be classified on the basis of their pharmacological properties rather than on the type of tissue response. During the 1970s, it became clear that α-adrenergic receptors in different tissues do not have identical pharmacological properties. Based on differences in the potency of phenoxybenzamine to block presynaptic increases in noradrenaline release and postsynaptic increases in contractility in cat spleen (Cubeddu et al, 1974), Langer (1974) proposed that postsynaptic α-adrenergic receptors be referred as α_1, and presynaptic receptors as referred as α_2. Berthelsen and Pettinger (1977) suggested that α-receptors should also be classified pharmacologically rather than on the basis of their anatomical localization. This suggestion subsequently was supported by variety of studies which showed that both α_1- and α_2-receptors could exist both presynaptically and postsynaptically (Minneman, 1988) and could mediate the same type of response such as activation of contraction and regulation of transmitter release.

Cardiac α-receptors are primarily of α_1-subtype. However, many investigators now agree that α_1-adrenergic receptors in different tissues do not have identical properties (Drew, 1985; Flavahan & Vanhoutte, 1986; Hieble et al, 1986). Binding studies with 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane HCl (WB4101) and chlorethylclonidine (CEC) have indicated that the α_1-receptors can be subclassified into α_{1a} and α_{1b} subtypes (Minneman, 1988; McGrath & Wilson, 1988).

The functional significance of these subtypes of α_1-receptor awaits further investigation.

B. Signal Transduction

α_1-Adrenergic receptors utilize changes in $[Ca^{2+}]_i$ as their primary signal transduction mechanism (Capogrossi et al, 1991) Activation of α_1 adrenergic receptors increases the hydrolysis of a specific membrane lipid to release diffusible second messengers which may control both the mobilization of stored intracellular Ca^{2+} and the Ca^{2+} influx from extracellular spaces.

For many years, numerous studies linked stimulation of α-receptors to increases in turnover of the membrane lipid phosphatidylinositol (PI, Hokin & Sherwin, 1957; Jones & Michell, 1974). Increased accumulation of [^{3}H]inostitol phosphates in response to α_1-adrenergic stimulation in the presence of Li^+, which inhibits the breakdown of inositol monophosphates (Berridge et al, 1982), has been demonstrated in cardiac myocytes (Brown et al, 1985). Recently, other studies also provided evidence that PI metabolism is stimulated in the heart through activation of α_1-adrenergic receptors (Renard & Poggioli, 1987; Otani et al, 1988; Woodcock et al, 1988; Mouton et al, 1991).

The mechanism by which α_1-adrenergic receptor stimulation activates PI breakdown is not fully understood. Two different guanine nucleotide regulatory proteins (G-protein) are possibly involved (Minneman, 1988) in the actions of α_1-receptors to activate phospholipase C which catabolizes the breakdown of phosphatidylinositol-4,5,-bisphosphate (PIP_2).

C. Modification of $[Ca^{2+}]_i$

Two important second messengers, $Ins(1,4,5)P_3$ and DAG, are released from the hydrolysis of PIP_2. It is now well established that $Ins(1,4,5)P_3$ plays a crucial role in maintaining intracellular Ca^{2+} homeostasis by releasing Ca^{2+} from non-mitochondrial

stores in various tissues (Berridge & Irving, 1984; 1989; Berridge, 1987). DAG activates protein kinase C (Nishizuka, 1984) which may lead to phosphorylation of specific cellular proteins (Berridge, 1981). Indeed, phosphorylation of a 15-kd protein in the cardiac sarcolemma has been implicated in the increase of Ca^{2+} inward current caused by α_1-adrenergic stimulation (Lindemann, 1985).

In addition to what has been discussed above, increasing evidence suggests that a variety of other mechanisms may be involved in signal transduction by α_1-adrenergic receptors in some tissues including the heart (Minneman, 1988). For instance, activation of α_1-receptors in the heart has been shown to cause degradation of cAMP by activating a cyclic nucleotide phosphodiesterase (Buxton & Brunton, 1985). In cardiomyocytes cocultured with neurons, Steinberg et al (1985) have observed both positive and negative effects on the rate of contraction caused by α_1 receptor stimulation. The negative effect can be blocked by treatment with pertussis toxin. More recently, del Balzo et al (1990) have suggested that at least two distinct α_1-adrenergic receptor subtypes are presented in neonatal and adult cardiac Purkinje tissue. The CEC-sensitive subtype is linked to a decrease in automaticity via a pertussis toxin-sensitive substrate and the WB 4101-sensitive subtype is linked to an increase in automaticity. Nevertheless, the significance of these alternate pathways still requires further investigation.

D. Direct Effect on TI

Despite numerous studies discussed above, there have been few direct observations of the effects of α_1-adrenergic stimulation on OAP and especially TI in cardiac tissues. Controversial reports over whether α_1-adrenergic agonists could affect OAP have led us to compare the effects of α-adrenergic agents on OAP and triggered activity induced by two different agents, digitalis and high Ca^{2+}, in cardiac Purkinje fibres. This will be presented in detail in the Results section of the thesis. In rabbit

Purkinje fibres, Ferrier and Carmeliet (1990) recently demonstrated that under voltage-clamped condition, TI induced by digitalis intoxication could be inhibited by phenylephrine whereas TI induced by high $[Ca^{2+}]_o$ was enhanced. These effects seemed to be α-adrenergic specific because they were abolished by prazosin, a specific α_1-adrenergic blocker. The cellular mechanism by which α_1-receptor stimulation did so remains to be determined.

3. β-Adrenergic Stimulation

A. Classification

In the 1960s it became clear that β-adrenergic receptors in different tissues showed different pharmacological properties. Lands and coworkers proposed that these receptors be divided into two subclasses called β_1- and β_2-adrenergic receptors (Lands et al, 1967a; 1967b). Recent evidence from radioligand binding assays has made it clear that these receptor subtypes can coexist in the same tissue and on the same cell (Minneman et al, 1981). While cardiac tissues may contain both β_1- and β_2-adrenergic receptors, the exact distribution of the two receptor subtypes depends on animal species. For example, it has been reported that canine ventricular muscles contain 85% β_1- and 15% β_2-adrenoceptors (Manalan et al, 1981), and guinea pig ventricular cells contain only β_1-receptors (Hedberg et al, 1980; Engel et al, 1981; Iijima & Taira, 1989). However, rat atrioventricular node contains 56% β_1- and 44% β_2-receptors (Saito et al, 1988), canine sinoatrial node has the same proportion of β_1- and β_2-receptors (Muntz, 1992), and frog hearts contain only β_2-receptors. Differences in distribution of β-receptor subtypes also exist in different parts of the heart (Muntz, 1992). β-adrenergic receptor subtypes show many similarities and only a few differences: both β_1- and β_2-receptors stimulate formation of cAMP as their primary mechanism for signal transduction in cells (as will be discussed later); and very few drugs show more than a 20- to 50-fold difference in potency in binding to

the two different subtypes. However, the use of molecular biological techniques has demonstrated that the two receptor subtypes are not produced by alternative mRNA splicing or cell-specific posttranslational modifications, but that the properties of each subtype are intrinsic to the receptor gene products (Strader et al, 1987).

B. Signal Transduction

Pelzer et al (1990) have summarized briefly the intracellular enzymatic responses to β-adrenoceptor activation. Agonist binding to the β-adrenoceptor catalyses conversion of the inactive form of G-protein (GDP-bound) to the active form (GTP-bound) which dissociates its $\beta\gamma$-subunit with the α-subunit. The activated α-subunit of the G-protein (Gsα) activates the catalytic subunit of adenylate cyclase which then triggers an increase in the production of cAMP. cAMP activates the catalytic subunit of protein kinase A (PKA). In the presence of ATP, the activated PKA catalytic subunit phosphorylates a number of membrane and intracellular proteins. Forskolin, which directly activates the catalytic subunit of adenylate cyclase (Daly, 1984), also can increase cAMP content, and therefore cAMP-dependent phosphorylation activity, in the absence of agonist-receptor binding.

C. Phosphorylation-Dependent Modification of $[Ca^{2+}]_i$

β-Adrenergic receptor-stimulated cAMP-dependent phosphorylation of a variety of cellular proteins in the sarcolemma, SR, and thin filaments of cardiac cells has been a subject of numerous investigations over the past two decades. The most important consequences of phosphorylation are thought to be, respectively, 1) phosphorylation of sarcolemmal L-type Ca^{2+} channel results in an increase in Ca^{2+} influx by increasing the availability of the channel (Tsien et al, 1986; Pelzer et al, 1991), 2) an increase in the rate of Ca^{2+} uptake into the SR (Tada & Katz, 1982), and 3) a decrease in myofilament Ca^{2+} sensitivity (Winegrad, 1984; Solaro, 1986). Recently, direct G-protein (Gs) coupled stimulation of the Ca^{2+} channel in the heart has been

reported (Yatani & Brown, 1989; Shuba et al, 1990). However, conflicting results also have been reported (Hartzell et al, 1991). The net effect of these changes is an increase in myocardial contractility associated with accelerated relaxation and abbreviation of contraction. Direct measurements of the $[Ca^{2+}]_i$ transient and contraction has shown that both peak aequorin signal and peak tension are increased but time to peak and duration of both the aequorin signals and contraction are decreased (Endoh & Blinks, 1988).

D. Possible Mechanisms Underlying Stimulation of TI

Given that TI is a $[Ca^{2+}]_i$ activated current through either a non-specific cation channel or the electrogenic Na^+-Ca^{2+} exchange, the mechanism of β-adrenergic stimulation of TI can be considered at two stages: Ca^{2+} loading and charge-carrying. It seems less likely that stimulation of TI is mediated at the level of charge-carrying systems because TI can not be induced by β-adrenergic stimulation alone in cardiac sino-atrial (SA) node cells (Satoh & Hashimoto, 1988). However, experimental evidence to support this is weak. In isolated single myocytes from the guinea pig hearts, Brum et al (1983) demonstrated that intracellular injection of a catalytic subunit of cAMP--dependent protein kinase could induce TI. This appears to indicate that β-adrenergic stimulation of TI may be phosphorylation dependent. Due to the difficulties in distinguishing between the two change-carrying mechanisms, we did not test the effect of ISO on either the non-selective cation channel or the Na^+-Ca^{2+} exchange directly. Failure to generate TI in SA node cells can be equally well explained by assuming that the degree of Ca^{2+} overload through increased I_{Ca} is not high enough to activate the current. If TI has been activated already by pre-existing Ca^{2+} overload through inhibiting the Na^+-K^+ pump or modifying the Na^+-Ca^{2+} exchange directly, stimulation of the β-adrenoceptor may enhance TI by further increasing the $[Ca^{2+}]_i$ through 1) I_{Ca}, 2) Na^+-Ca^{2+} exchange, and 3) modifying SR

function. Since it is known that β-adrenoceptor stimulation can speed up the reuptake of Ca^{2+} by the SR and therefore shorten the time course of free $[Ca^{2+}]_i$ transient (Endoh & Blinks, 1988), the most likely mechanisms will be the first two. However, experimental test of Na^+-Ca^{2+} exchange as the pathway which mediates effects of β-adrenergic stimulation of TI has not been published.

VI. OBJECTIVES

The overall goals of this study were to determine the ionic basis of TI, to identify the charge-carrying systems of TI, and to determine the mechanisms of adrenergic effects on both OAP and TI. The specific objectives were:

1. To assess effects of α-adrenergic agonists and blockers on induction of OAP and triggered activity by digitalis or elevated $[Ca^{2+}]_o$.

2. To determine if β-adrenergic stimulation can induce TI and to evaluate possible mechanisms of β-adrenergic stimulation of TI induced by digitalis intoxication or elevated $[Ca^{2+}]_o$.

3. To determine: a) whether TI can be induced in the absence of Na^+-Ca^{2+} exchange, b) whether a clear-cut reversal potential of TI can be rvealed, and c) which ionic components contribute to the generation of TI in the absence of Na^+-Ca^{2+} exchange.

4. To determine: a) which ionic components contribute to the generation of TI in the presence of Na^+-Ca^{2+} exchange, b) if cationic and anionic channels are both involved in the generation of TI, can they be preferentially blocked, and c) whether Na^+-Ca^{2+} exchange participates in the generation of TI in the presence of $[Na^+]_o$.

5. To determine: a) whether β-adrenergic stimulation of TI is secondary to the elevation of $[Ca^{2+}]_i$ or a direct effect at the TI channels, b) which is more important in mediating β-adrenergic stimulation of TI, L-type Ca^{2+} channels or Na^+-Ca^{2+} exchange, and c) whether the effects of β-adrenergic stimulation on TI can be mimicked by forskolin, a direct activator of adenylate cyclase.

MATERIALS AND METHODS

I. TISSUE PREPARATION

1. Conventional Microelectrode Recordings

All studies were conducted on isolated rabbit cardiac Purkinje fibres. Male New Zealand white rabbits (1.9-3.2 kg) were stunned and exsanguinated via the carotid vessels. Hearts were quickly excised and both the left and the right ventricles were opened through an incision in the free wall, while immersed in modified Tyrode's solution whose composition is shown in table 1. The solution was bubbled with 95% O_2, 5% CO_2 and was maintained at 37 $\pm$ 0.5° C. The use of this solution for dissection of rabbit cardiac Purkinje fibres has been described by Mubagwa and Carmeliet (1983). Purkinje fibres with muscle attached at both ends were mounted in a 2.5 ml tissue bath. This was done with small (0.5 cm in length) stainless steel pins to fix the muscles to a wax tissue bath in such a way that solution superfuses the Purkinje fibres on all surfaces including the lower side.

B. Voltage Clamp Studies

All studies were conducted on isolated rabbit cardiac Purkinje fibres. Male rabbits (1.9-3.2 kg) were stunned and exsanguinated via the carotid vessels. Hearts were quickly removed through a parasternal incision and rinsed in the high K^+ dissecting solution as described in table 1. Purkinje fibres then were dissected from both ventricles. A Purkinje fibre bundle consists of a cluster of 2 to 10 cells, with an individual diameter of about 10 μm. Adjacent cells are separated laterally by cleft spaces roughly 1 μm wide. The large intercellular cleft spaces of the rabbit Purkinje fibres greatly minimize any possible accumulation or depletion of ions during voltage clamp (Colatsky & Tsien, 1979). Short segments (0.8-1.0 mm) of Purkinje fibres

were obtained by crushing the isolated fibres with a wire grid as described by Aronson et al (1973). After healing over, high membrane resistance was formed at both crushed ends. More than 90% of the shortened Purkinje fibre segments survived well from the crushing after they were equilibrated with the dissecting solution for 30 to 60 min . The short Purkinje segments showed a uniform membrane potential as checked by introduction of two microelectrodes into the same segment. The short Purkinje segments also had a low length constant (less than 1 mm), thus, they were very suitable for voltage clamp (Aronson et al, 1973). Preparations surviving impalement with two microelectrodes were remarkably stable; action potentials and good electrical coupling between cells could be observed for periods of 4-5 h in normal Tyrode's solution (table 2). The composition of modified Tyrode's solution (table 1) used for dissection has been reported by Mubagwa and Carmeliet (1983). Only unbranched healthy-looking Purkinje fibres with normal resting membrane potentials and action potential configurations were used for the voltage clamp study.

II. ELECTRODES AND RECORDING SETUP

1. Conventional Microelectrode Recording

Transmembrane action potentials of Purkinje fibres were recorded using microelectrodes filled with filtered 2.7 M KCl. The tip resistances of the microelectrodes were in the range of 15 - 30 MΩ in Tyrode's solution. Microelectrodes were made from glass pipettes (World Precision instruments, inc., New Haven, CT, U.S.A.) having outer diameter of 1.0 mm and inner diameter of 0.58 mm. A two-stage microelectrode puller (MI Micropipette Puller, Industrial Science Associates Inc., Flushing, N.Y., U.S.A.) was used to pull the microelectrodes. WPI microelectrode holders (MEH-1S, W.P. Instruments) filled with 2.7 M KCl were used to connect both the microelectrodes and the input of a miniature probe of WPI pre-amplifier

(gain = X1, input impedance = 10^{11} ohms; model 750, Dual Microprobe, W.P. instruments). The cell was impaled with a microelectrode held in a micromanupulator (MP1, Narishige). The output of the pre-amplifier was connected to a Tektronix 5110 oscilloscope (Tektronix, Inc., Beaverton, Oregon, U.S.A.) and a Gould Brush 2200S pen recorder (Gould Inc., Cleveland, Ohio, U.S.A.) to display transmembrane action potentials. In experiments where the maximum upstroke velocity of the action potentials were measured, the output of the WPI pre-amplifier was also connected to the 5A18N Dual Trace Amplifier (frequency response: DC, at least 2 megahertz; AC, 2 hertz to 2 megahertz) which is a plug-in unit for use with Tektronix 5110-series oscilloscopes. The amplified signal was then connected to a dV/dt differentiator which was built by the Departmental electronics shop (Department of Pharmacology, Dalhousie University, Canada). The dV/dt signal was linear within the range of 50 to 2000 volts/sec. Both the transmembrane potentials and the dV/dt signals were stored on magnetic tape Vetter Model D (A.R. Vetter Co., Rebersburg, Pennsylvania, U.S.A.) for off-line analysis. A battery driven calibration box which generates a 100 mV voltage with reference to ground was used as calibration signal for membrane potential. Calibration was tested before recording the electrical activity from the preparation. The ground electrode was fabricated using a silver wire placed in an electrolytic solution containing 0.1 N HCl. Chloride was electroplated on the silver wire using a 1.5 V dry cell. This electrode was placed inside the superfusate in the tissue bath and served as a reference for intracellular membrane potential measurements. A schematic of the setup for conventional microelectrode recording is shown in figure 2.

2. Voltage Clamp Studies

The two microelectrode voltage clamp technique initially described by Deck et al

(1964) was used. The glass micropipettes used for making microelectrodes were the same as described above except having an outer diameter of 1.2 mm and inner diameter of 0.78 mm. The two-stage microelectrode puller (MI Micropipette Puller, Industrial Science Associates Inc., Flushing, N.Y., U.S.A.) was adjusted at settings to produce microelectrodes having tip resistances of approximate 12-15 MΩ. Some microelectrodes were bevelled with a Micropipette Beveler (Model 1300M, World Precision Instruments, U.S.A.) to decrease the tip resistance to 5-8 MΩ when filled with 2.7 M KCl (in Tyrode's solution) and they were used for current passage. Those microelectrodes with a higher tip resistance (about 12 MΩ when filled with 2.7 M KCl) were used for voltage recording. The current passing microelectrode was placed in the centre of the short segment of Purkinje strand. The voltage recording microelectrode was placed at approximately half of the distance from the centre to the terminal end of the Purkinje segment. A grounded shield was positioned in between the two microelectrodes and was placed at about 0.5-1.0 mm away from the surface of the perfusing solution in the tissue bath. This method greatly minimized large capacitance transients due to the current injection during the voltage clamp. The reference electrode was Ag-AgCl wire immersed in a glass pipet filled with 2.7 mM KCl. The tip of the pipet was plugged with 2.7 M KCl-agar to minimize the liquid junction potentials that might occur when changing the perfusing solutions, and was placed downstream from the preparations. In some experiments, asbestos instead of agar was used to plug the tip of the pipet. This was found to have the same effect as agar-plugged reference electrode in reducing the liquid junction potentials. In experiments where extensive changes of the components of the perfusing solution were used, junction potentials between Tyrode's solution and the Cl^--poor solution (60 mM Cl^-) was measured, and was approximately 2.5 mV. Therefore, this small change in liquid junction potential was not a major concern in these experiments.

The Axoclamp 2A was set in two-electrode voltage clamp (TEVC) mode. The maximum gain in TEVC mode was 10000 mV/mV and the rise time was 30 μsec. The headstage which was connected to the current injection microelectrode had a gain of X10 (input resistance = 10^{10} Ω). The headstage which was connected to the membrane potential recording microelectrode had a gain of X1 (input resistance = 10^{11} Ω). The ionic currents generated by voltage clamp were filtered at 3 kHz with a second-order variable frequency filter (Model AP255-5, A. P. Circuit Corp., New York). The output of the filter was then connected to a Gould Brush (RS 3400) rectilinear pen recorder (Gould Inc., Cleveland, Ohio, U.S.A.) and displayed on a Tektronix 5111A storage oscilloscope (Tektronix, Inc., Beaverton, Oregon, U.S.A.). Both the currents and the membrane potentials were also acquired and stored simultaneously by an IBM-AT (Multitech Industrial Corp) microcomputer interfaced with an Acquisition Logic board (16-channel multiplexed analog to digital conversion board, Axon Instruments). This was done by connecting the output of the filtered currents from the second-order filter and the output of the membrane potentials from the Axoclamp 2A to the Acquisition Logic board. The maximum acquisition rate was 110 kHz and the resolution was 12 bits for A/D conversion. The program used for data acquisition and storage was CLAMPEX (Axon Instruments) in which the data acquisition rate (sampling rate) was set at 1000 Hz (1 sample per ms). Due to the slow decay of capacitance transients (about 5 ms) for two-electrode voltage clamp, fast current like I_{Na} was not recorded regularly at a sampling rate of 1000 Hz. However, currents with a time course more than 10 ms (i.e. TI, Na^+-Ca^{2+} exchange current) could be recorded accurately. In most experiments where TI was generated, the magnitude of the peak TI was measured from the maximum inward or outward deflection to a line joining the preceding and following minima.

The perfusion bath had a volume of 1.5 ml. A constant flow rate of 4.5 ml/min

was used to enable a quick change of solutions. After changing the perfusing solution there usually was an equilibration period during which the background current (I_b) shifted, in either inward or outward direction depending on the compositions of the superfusates. This period took about 3 min until the steady-state was reached. Therefore unless otherwise indicated, both the TI magnitude and the reversal potential were obtained only after I_b had stabilized.

III. STIMULATION PROTOCOL

1. Conventional Microelectrode Recordings

Electrical stimulation at a basic cycle length (BCL, interval between stimuli) of 500 msec was initiated 30 min after switching to "normal" Tyrode's solution. Rectangular stimuli, 1.5 times diastolic threshold and 3 msec in duration, were delivered through bipolar AgCl electrodes to the Purkinje fibres near the site of attachment to the muscles. Pulses were obtained from digital stimulators (Pulsar 4i and 6i, Frederickhaer and Co., Brunswick, Maine). A pause of 3 seconds duration was interpolated after every 15 stimuli. Such interruption of stimulation will allow any spontaneous or triggered diastolic membrane potential changes to be observed. The BCL during the train of stimuli was set using a Pulsar 4i. After 60 min of equilibration with normal Tyrode's solution, the BCL was changed from 200 to 1000 msec in 50 or 100 msec steps to determine if spontaneous OAP could be observed.

2. Voltage Clamp Studies

After 30 min perfusion with normal Tyrode's solution, preparations were stimulated through intracellular microelectrodes. Rectangular stimuli, 1.5 times diastolic threshold and 3 msec in duration, were delivered from Axoclamp-2A (Axon Instruments) to the preparation through a glass microelectrode impaled into the Purkinje

fibres. Pulses were triggered by digital stimulators (Pulsar 4i and 61, Frederick Haer and Co., Brunswick, Maine) which were connected to the Axoclamp-2A. When Axoclamp 2A was set in bridge mode, stimulation pulses could be triggered by an external digital stimulator (Pulsar 4i). Only those Purkinje fibres showing normal action potential configuration (maximum diastolic potential -85 to -94 mV, APD_{90} 250 to 450 msec) were used in the voltage clamp studies. Voltage steps were generated by pCLAMP software (Axon Instruments) running on an IBM-AT microcomputer (ACER 900, Multitech Industrial Corp) which controlled the command voltage of the voltage clamp feed-back circuit (Axoclamp-2A, Axon Instruments) via a Waveform Generator Logic (digital to analog conversion, D/A) board (Axonlab-1A, Axon Instruments). The maximum settling time was 2 μs and the resolution was 16 bits for D/A conversion. Holding potentials were either -80, -55 or -50 mV according to the specific experimental requirements. The duration of depolarization was either 3 or 1 sec, as indicated in the appropriate text. TI usually was elicited upon repolarizing to different test potential levels from a previous depolarizing voltage step. Activating steps were repeated every 15 to 20 sec except for the I-V relationship study in which they were repeated every 10 sec.

IV. SOLUTIONS

In conventional microelectrode studies, preparations were initially superfused with the dissecting solution described in table 1. After one hour, the superfusate was changed to normal Tyrode's solution with the components shown in table 2. Normal Tyrode's solution was bubbled continuously with 95% O_2 and 5% CO_2 gas mixture to keep the pH at 7.35 $\pm$ 0.05. In experiments to assess the effects of α-adrenergic agents on high Ca^{2+}-induced OAP, the superfusate was changed to HEPES-NaOH

buffer Tyrode's solution (pH 7.35) as described in table 3. This solution was gassed with 100% O_2. The K^+ concentration was decreased to 2 mM in this solution to increase the amplitude of Ca^{2+}-induced OAP. The rationale for choosing HEPES-NaOH buffer was to minimize any possible precipitation due to the high Ca^{2+} concentration (8 mM) in this solution.

All solutions were placed in glass perfusion bottles. The solutions were delivered by a Peristaltic Metering pump (Fred A. Dungey Incorporated, Ontario, Canada) through a very thin plastic tubing placed in a water bath. The preparations were superfused at a flow rate of 15 ml/min by adjusting the flow rate control of the pump. The temperature of the water bath was maintained by a YSI Model 73A Instrument Temperature Controller (Yellow Springs Instrument Co., Ohio, U.S.A.). The temperature of the superfusate entering the tissue bath was monitored by a YSI series 400 thermistor temperature probe (Yellow Springs Instrument Co.). The thermistor temperature probe was attached to the instrument temperature controller. The temperature of the superfusate in the tissue bath was maintained at 37°C. Therefore, the solutions were heated to 37°C as they passed through the plastic tubing coil in the water bath.

In the voltage clamp experiments, after 30 min to 1 hr perfusion with dissecting solution, preparations were perfused with normal Tyrode's solution (table 2) or modified Tyrode's solution (table 4) depending on the specific experimental requirements. The resting membrane potential and the action potential parameters of the Purkinje fibres did not change with either solution. This will be further described in the appropriate results sections. The solution shown in table 2 was bubbled with 95% O_2 and 5% CO_2 gas mixture. The solution shown in table 4 was bubbled with 100% O_2. The pH of these solutions was kept at 7.35 $\pm$ 0.05. The temperature of the tissue bath was maintained at 37° $\pm$ 0.05 C. The tissue bath had a volume of 1.5 ml

and a constant flow rate of 4.5 ml/min. The flow rate was set by adjusting the flow rate control of the Peristaltic Metering pump. In the studies of the ionic basis of TI, various high Ca^{2+} solutions were used to superfuse the Purkinje fibres after at least 40 min perfusion with the modified Tyrode's solution shown in table 4. The composition of the high Ca^{2+} solutions (also bubbled with 100% O_2) is shown in table 5. The rationale for using the solutions of table 4 and 5 in which HCO_3^-/CO_2 were replaced by HEPES-Tris base buffer was to minimize any possible precipitation due to the high Ca^{2+} concentrations (5-105 mM) or the reaction of bicarbonate with Mn^{2+}, Cd^{2+}, and Ba^{2+} which were used in some experiments. The osmolarity of all except the desecting solution was maintained at about the same level as normal Tyrode's solution (322 mosm/L). Alterations in osmolarity among these solutions were less than 5%. The desecting solution had a higher tonicity than normal Tyrode's solution. However, it was shown that short time superfusion with the high K^+ desecting solution preserved more healthy Purkinje fibres (Mubagwa & Carmeliet, 1983).

V. DRUGS AND REAGENTS

The drugs used in this study include: 3-acetylstrophanthidin (Eli Lilly Co., Indianapolis, Ind., U.S.A), dl-propranolol hydrochloride (Sigma Chemical Co., St. Louis, Mo., U.S.A.), l-phenylephrine hydrochloride (Sigma), prazosin hydrochloride (Sigma), clonidine hydrochloride (Sigma), yohimbine hydrochloride (Sigma), HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma), Tris base (Sigma), isoproterenol hydrochloride (Sigma), atenolol hydrochloride (Sigma), quinacrine (Sigma), amiloride (Sigma), verapamil (Sigma), ryanodine (Calbiochem Corp.), N-methyl-D-glucamine (NMG, Sigma), 4,4'-diisothiocyanatostilbene-2,2'disulfonic acid (DIDS, Sigma), 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS, Sigma), choline chloride (Sigma), forskolin (Sigma), dodecylamine (Aldrich Chemical

Company, Inc.). All drugs except 3-acetylstrophanthidin were prepared freshly as aqueous stock solutions (10^{-3} or 10^{-4}M). 3-acetylstrophanthidan was dissolved in 6% ethanol in water to yield a stock concentration of 10^{-4}M. Forskolin and ryanodine were dissolved in 100% ethanol. The final ethanol concentration superfusing the tissue never exceeded 0.001% in the superfusing medium. It was shown that this concentration of ethanol had no effect on the tissue. NMG chloride was made by titrating the stock solution with HCl to pH 7. Since DIDS, SITS, quinacrine and amiloride all demonstrate photolytic degradation, preparation of stock solutions for these agents were performed in a darkened environment. Also, all glassware and tubing were covered with aluminum foil.

VI. DRUG ADDITION PROTOCOLS

In general, all aliquots of stock solutions were diluted directly in the Tyrode's superfusate to achieve final concentrations. In experiments designed to assess the effects of α-adrenergic agents on digitalis-induced OAP, 0.5 μM propranolol was used to eliminate any β-adrenergic stimulation of phenylephrine. Acetylstrophanthidin was then added to the superfusate. After OAP or triggered activity was induced, α-adrenergic agonists and/or antagonists were added to the superfusate to determine their effects. Dose-response curves of phenylephrine and prazosin were cumulative. Concentrations and details of specific protocols are also indicated in the appropriate result sections. When experiments were designed to study the effects of β-adrenergic stimulation on AS-induced TI, the effects of isoproterenol (ISO) on Purkinje fibres were first determined in the absence of AS by cumulative addition of ISO (10^{-8} to $5x10^{-6}$ M) to the Tyrode's solution. After TI was induced by AS, stock ISO solution was diluted directly into the superfusate to achieve a final concentration of 10^{-7}M.

VII. ANALYSIS OF RECORDS

Only those experiments where continuous impalement was maintained or when the impalement was lost and a cell with identical electrophysiological characteristics was obtained, were analyzed. Also, only those preparations which displayed normal action potential characteristics were used for subsequent studies. For the conventional microelectrode study, a preparation was considered to display normal action potential characteristics when the maximum diastolic potential of the Purkinje tissue was between -89 and -94 mV, and when there was no automatic activity. For voltage clamp studies, the short Purkinje segment was considered to be "normal" when its MDP was between -85 and -94 mV, irrespective of its automaticity. Since two-electrode voltage clamp technique was used, voltage clamp was initiated only after both microelectrodes displayed identical action potential parameters. In experiments where extensive changes of the superfusate composition (i.e. various high Ca^{2+} solutions) were used, data collection were limited to those preparation which displayed normal action potential characteristics after return to normal Tyrode's solution. Data from conventional microelectrode studies were measured using a Hipad, Model A digitizer (DT-11 Hipad Digitizer, Bausch and Lomb, Texas, U.S.A.). Analysis of action potential and OAP characteristics was performed on an Apple IIe dual disc drive microcomputer with an Apple IIe Board Interface (RS-232) for the Hipad Digitizer. Records from voltage clamp study were either measured by hand or by pCLAMP software, CLAMPAN and CLAMPFIT (Axon Instruments, CA, U.S.A.), running on an IBM-AT 80286 microcomputer.

VIII. STATISTICAL ANALYSIS

All data are presented as means $\pm$ SEM unless otherwise indicated. Whenever experiments consisted of two groups, statistical analyses were performed by Student's

t-test for paired or unpaired data, as appropriate. For comparisons consisting of more than two groups, analysis of variance with multiple comparison test of Student Neuman Keuls (SNK) was applied. Differences with p values < 0.05 were considered significant. S.E.M. bars are shown where n (n is the number of Purkinje fibres) is at least from 3 hearts. In data presented as percentage, statistical analysis was performed on raw data. The name of the computer program used for performing statistical analysis was STATISTIX (Ver 3.1, Analytical Software).

Figure 2. Schematic representation of the set-up used for stimulation and intracellular recording from isolated cardiac Purkinje fibres.

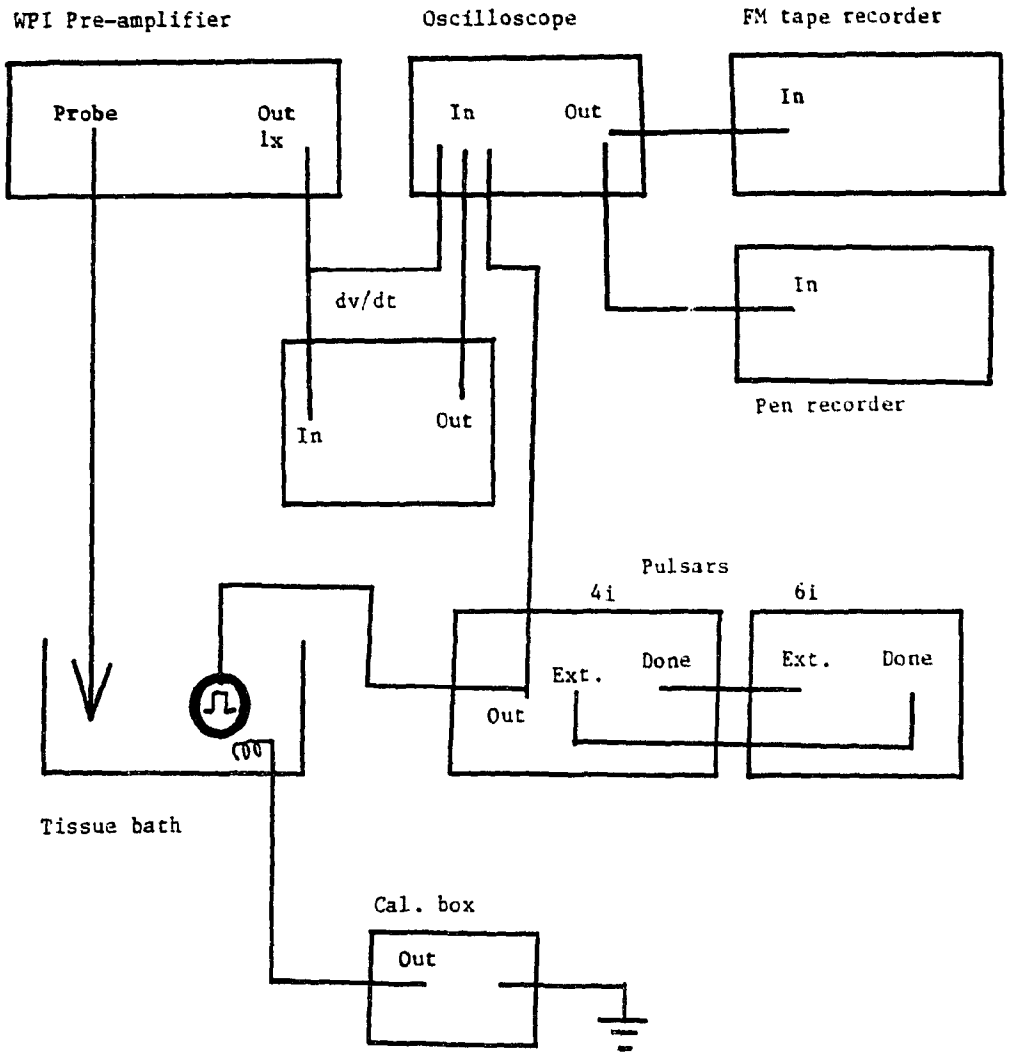

WPI Pre-amplifier
Oscilloscope
FM tape recorder
Probe
Out
1x
In
Out
In
dv/dt
In
Out
In
Pen recorder
Pulsars
4i
6i
Ext.
Done
Ext.
Done
Out
Tissue bath
Cal. box
Out

Figure 2.

Table 1. Composition of Modified Tyrode's Solution Used for Dissection (mM)

NaCl	129
KCl	27
$MgSO_4$	0.5
$CaCl_2$	2.5
$NaHCO_3$	20
NaH_2PO_4	0.9
Glucose	55

Table 2. Composition of Normal Tyrode's Solution (mM)

NaCl	129
KCl	4
$MgSO_4$	0.5
$CaCl_2$	2.5
$NaHCO_3$	20
NaH_2PO_4	0.9
Glucose	5.5

Table 3. Composition of HEPES-NaOH Buffer Tyrode's Solution (mM)

NaCl	137
KCl	2
$MgSO_4$	0.5
$CaCl_2$	8
HEPES	1.5
Glucose	5.5

Titriated with 1 N NaOH to pH 7.4

Table 4. Composition of HEPES-Tris Base Buffer Tyrode's Solution (mM)

NaCl	145
KCl	4
$MgCl_2$	0.5
$CaCl_2$	2.5
HEPES	10
Tris Base	5
Glucose	5.5

Table 5. Composition of Various High Ca^{2+} Solutions (mM)

NaCl	0 - 145
Choline Chloride	0 - 145
NMG Chloride	0 - 145
LiCl	0 - 145
Sucrose	0 - 210
KCl	0 - 4
$MgCl_2$	0 - 0.5
$CaCl_2$	0 - 105
HEPES	10
Tris Base	5
Glucose	10

The exact concentrations of various substances were indicated in the appropriate text. Osmotic pressure was maintained at the same level as normal Tyrode's solution by adjusting the concentrations of NaCl or its substitutes (choline chloride, NMG chloride, LiCl, Sucrose) and $CaCl_2$.

RESULTS

I. RESEARCH PLAN

We first wanted to assess effects of α-adrenergic agents on induction of OAP and triggered activity. To do this, we carried out study in isolated rabbit cardiac Purkinje fibres with standard intracellular microelectrode recording techniques. OAP and triggered activity were induced by either digitalis intoxication or elevated $[Ca^{2+}]_o$. Then the effects of α_1- and α_2-adrenergic agonists and blockers were assessed separately.

Because TI was the current responsible for OAP, we next wanted to determine whether β-adrenergic stimulation could generate or enhance TI. If TI could be induced or increased, what was (were) the possible mechanism(s). We performed these experiments on isolated rabbit cardiac Purkinje fibres with conventional two-microelectrode voltage clamp techniques. We first determined whether TI could be induced by ISO, a β-adrenergic agonist. In a separate series of experiments, we then induced TI with toxic concentration of AS and determined whether AS-induced TI could be stimulated by ISO. Since TI was stimulated by ISO, we evaluated possible mechanism(s) of stimulation.

The ionic basis and the charge-carrying systems of TI are not yet fully understood. To study these, we first tried to induce TI by elevating $[Ca^{2+}]_o$ in the absence and presence of $[Na^+]_o$. The I-V relations of TI thus induced were determined. Also, effects of changing concentrations of extracellular ions (i.e. Ca^{2+}, Cl^-) on the I-V relations of TI were determined. Since both cationic and anionic conductances were involved in the generation of TI, we also determined whether they could be blocked selectively with certain agents. Information derived from these studies would reveal: 1) which, TI channel or Na^+-Ca^{2+} exchange, was the charge-carrying system for TI; 2) what was the contribution of individual ions (Na^+, Ca^{2+}, Cl^-) to the

generation of TI.

Finally, we determined whether β-adrenergic stimulation of TI was caused by phosphorylation of the TI channels or phosphorylation of other membrane proteins, of particular, the Na^+-Ca^{2+} exchanger, which might increase $[Ca^{2+}]_i$ overload. Thus, TI was induced first by elevation of $[Ca^{2+}]_o$ in the absence and presence of $[Na^+]_o$. Effects of ISO then were assessed under these two different conditions. Effects of forskolin, a direct activator of adenylate cyclase, were also assessed and compared with ISO under the same conditions.

II. EFFECTS OF α-ADRENERGIC AGENTS ON GENERATION OF OAP AND TRIGGERED ACTIVITY

1. Induction of OAP in Rabbit Cardiac Purkinje Fibres by Exposure to Acetylstrophanthidin (AS)

OAP have not been extensively studied in rabbit tissues. Therefore we first examined the induction and characteristics of AS-induced OAP. Spontaneous OAP were not observed at any BCL from 200 to 1000 msec in untreated rabbit Purkinje fibers perfused with propranolol-containing Tyrode's solution. OAP also were not observed at any BCL at concentrations of AS less than 0.22 μM (0.1 μg/ml). Twenty min after adding 0.45 μM AS to the superfusate, OAP could be observed in 8 of 10 preparations but only at short BCL (200 msec). After 40 min, OAP appeared in all 10 preparations at a BCL of 500 msec. Effects of AS in a representative experiment are shown in Figure 3. Both the occurrence and the amplitude of OAP were strongly influenced by the preceding BCL. Figure 3A shows the influence of BCL on OAP. In the presence of 0.45 μM AS, prominent OAP could be evoked at a BCL of 500 msec or less, but the amplitude tended to decrease when the preceding BCL was

longer than 600 msec. When the concentration of AS was increased to 0.9 μM (panel B), the amplitude of OAP increased and triggered activity developed in 6 out of 10 preparations at a BCL of 500 msec, and 8 of 10 preparations at a BCL of 200 msec. Triggered activity did not occur at longer BCL. These observations demonstrated that OAP induced by AS in rabbit Purkinje fibers had characteristics similar to those induced in other species (Ferrier, 1977).

2. Effect of Propranolol on the Amplitudes of AS-Induced OAP

Propranolol was used in this study to eliminate ß-adrenergic activity. Therefore, we first determined in 5 preparations if OAP induced by AS in the absence of propranolol were affected by subsequent exposure to propranolol for 1 hr. As shown in figure 4, 0.5 μM propranolol had no effect on OAP amplitude. However, OAP were suppressed when the concentration was increased to 2 μM. This effect was statistically significant (table 6). Therefore, 0.5 μM propranolol was used in subsequent experiments since it exerts ß-antagonistic action at this concentration (Hewett and Rosen, 1984) but has little effect on OAP. The observation that propranolol did not alter the amplitude of OAP, at a concentration known to block enhancement of OAP by β-adrenergic agonists, also implies that little or no release of endogenous norepinephrine was occurring under the experimental conditions used in this study.

3. Effects of Phenylephrine and Prazosin on AS-Induced OAP

Effects of PE on AS-induced OAP were determined in 8 preparations in the presence of propranolol. As shown in figure 5A, PE (5 μM) decreased the amplitude of OAP induced by 0.45 μM AS. This effect was dose related, as shown in figure 6, and was statistically significant at PE concentrations of 4 μM or greater.

In 2 experiments, we observed tachyphylaxis when we determined cumulative

dose response curves by increasing the concentration of PE in the following sequence: 2.5 μM, 5 μM, 7.5 μM, 10 μM. Following each addition of PE the OAP amplitude showed a decrease in the first 4 min, and then showed a slight return. However, at 10 μM PE, OAP were permanently suppressed. The values plotted in figure 6 were taken after 15 min of exposure to PE and therefore, after the responses to addition of PE had stabilized.

The inhibitory effects of PE on OAP occurred at concentrations at which PE exerts α-adrenergic agonism, therefore, we determined if PE's effect could be antagonized by prazosin, a specific α_1-adrenergic antagonist. Figure 5 (panel B) shows an example in which OAP were induced by AS (0.4 μg/ml) in the presence of both propranolol and prazosin (0.5 μM). In the presence of prazosin, OAP could not be suppressed by PE at 10 μM as shown in figure 5B. Thus the inhibitory effect of PE on AS-induced OAP is likely mediated by α_1-adrenergic stimulation. Blockade of the inhibitory effect of PE by prazosin was confirmed in 6 preparations.

Previous voltage clamp studies (Ferrier and Carmeliet, 1990) indicated that prazosin exerted a direct inhibitory effect on the TI. This observation suggested that prazosin might suppress OAP and exert antiarrhythmic efficacy, at least at higher concentrations. To test this possibility, 8 experiments were conducted with ß-receptor blockade and toxic concentration of digitalis. As shown in figure 5C, 5 μM prazosin strongly inhibited AS-induced OAP. The dose-response curve for prazosin showed that the inhibitory effect of prazosin was statistically significant at concentrations of 1 μM or greater (Figure 6).

4. Effects of Phenylephrine and Prazosin on Triggered Activity

These observations imply that both PE and prazosin should have antiarrhythmic efficacy. Therefore we studied the effects of PE (n = 8) and prazosin (n = 5) on

triggered activity induced by AS. Figure 7 shows the typical responses observed in two representative experiments. Panel A (left) shows triggered activity induced by AS. Before addition of PE, triggered activity continued throughout pauses in stimulation (panel A, left). Following 10 μM PE perfusion, the frequency and number of triggered action potentials gradually decreased (panel A, middle) and finally stopped as shown in Figure 7 (panel A, right). AS-induced triggered activity was terminated by PE by 30 min in all 8 observations. Figure 7, panel B shows that prazosin also slowed and terminated triggered activity induced by AS. Triggered activity was similarly suppressed by prazosin (5 μM) within 20 min in 5 of 5 preparations.

5. Effects of Clonidine and Yohimbine on AS-Induced OAP

Although their functional role is not clear, α_2-adrenoceptors have been demonstrated in the heart, and the α_2 antagonist yohimbine has been reported to decrease norepinephrine induced triggered activity in hypoxic glucose-free solution in sheep Purkinje fibers (Mugelli et al., 1986). We therefore determined whether α_2-adrenergic influence might also affect OAP in our isolated tissue model (n = 4). OAP induced by AS were neither enhanced nor suppressed by 0.5 to 5 μM of α_2 agonist clonidine (Figure 8A and table 6). Therefore we concluded that α_2-adrenoceptors did not play major role in response to catecholamines under our experimental conditions. On the other hand, yohimbine at a concentration of 2 μM strongly suppressed OAP induced by 0.45 μM AS in 4 preparations (Figure 8B). The decrease in amplitude of OAP was statistically significant (table 6). Since this effect occurred in the absence of exogenous α_2-agonist, it is likely related to an action other than α_2-antagonism.

6. Induction of OAP by High $[Ca^{2+}]_o$ (8 mM) and Low $[K^+]_o$ (2 mM)

In the present study, OAP and triggered activity also were induced in rabbit Purkinje fibers by 8 mM Ca^{2+} in the presence of 2 mM K^+. Elevation of Ca^{2+} concentration by itself only induced a very small OAP, and 2 mM K^+ by itself was ineffective. However, the combination quickly induced prominent OAP (figure 9).

7. Effects of Phenylephrine and Prazosin on High Ca^{2+}, Low K^+ Induced OAP

Effects of PE and prazosin on high Ca^{2+}, low K^+ induced OAP also were evaluated in the presence of 0.5 μM propranolol. In contrast to its effects on AS-induced OAP, PE increased the amplitude of OAP at concentrations ranging from 0.5 to 10 μM in 5 experiments. This stimulatory effect caused OAP to reach threshold and give rise to triggered action potentials (Figure 10A). These effects reversed upon washout of PE (not illustrated).

The observations above raise the question of whether both inhibitory and stimulatory effects of PE on OAP could be mediated by α_1-receptors. If this were true, induction of triggered activity also should be blocked by prazosin. Preparations were pretreated with 0.5 μM prazosin. This concentration of prazosin did not prevent induction of OAP (figure 10B), however it completely blocked the effect of PE to increase OAP amplitude and induce triggered activity in 5 preparations.

Since prazosin by itself inhibited AS-induced OAP, the effect of prazosin on high Ca^{2+}- induced OAP was further investigated in 5 preparations. We found 0.5 μM prazosin to be without effect on OAP amplitude. However, when the prazosin concentration was increased to 2 μM, OAP gradually disappeared in all 5 experiments. A representative experiment is illustrated in figure 10C.

8. Effects of Clonidine and Yohimbine on High Ca^{2+}, Low K^+ Induced OAP

Possible α_2-adrenergic influences on high Ca^{2+} induced OAP were assessed in 4

preparations with clonidine and yohimbine. Three concentrations were chosen for clonidine: 0.5, 2, and 5 μM. Exposure to the drug for 1 hr did not cause significant change in the OAP amplitude (Figure 11A). Quantitative data are summarized in table 6. After 1 hr wash-out with normal Tyrode's solution, reexposure to high Ca^{2+}, low K^+ again induced OAP. However, OAP amplitude decreased progressively with exposure to 2 μM yohimbine for 15 min (Figure 11B, table 6).

9. Effect of Phenylephrine on the Maximum Upstroke Velocity of the Action Potential of Rabbit Purkinje Fibres

Drugs that inhibit Na^+ influx (I_{Na}) during the upstroke of action potential decrease the amplitude of OAP (Rosen and Danilo, 1980). PE is known to affect intracellular Na^+ activity (Shah et al.,1988). This led us to ask if the actions of PE on AS-induced OAP might involve inhibition of Na^+ influx. Maximum upstroke velocity of the action potential (Vmax) is a useful index of changes in the magnitude of the sodium current (Sheets et al., 1988). Therefore an electronic differentiator was used to measure Vmax of action potentials initiated at different membrane potentials during repolarization of a previous action potential. Possible effects on Vmax were determined under three consecutive conditions in two series of experiments: (1) perfusion with normal Tyrode's solution; (2) with AS or high Ca^{2+}, low K^+ Tyrode's solution; (3) with the addition of 10 μM PE in AS or high Ca^{2+} low K^+ containing Tyrode's solution. As shown in figure 12A, in AS-intoxicated preparations PE did not cause any change of Vmax at any membrane potential tested (n = 6). In another series of 6 experiments, superfusion with high Ca^{2+} low K^+ containing Tyrode's solution caused a significant increase in Vmax at lower membrane potentials. However, addition of PE failed to produce any further change of Vmax (Figure 12B). These results indicate that change in I_{Na} is not likely involved in the of action of PE.

Figure 3. Induction of OAP by acetylstrophanthidin in rabbit Purkinje fibers. **Panel A:** Amplitude of OAP induced by 0.45 μM AS in rabbit Purkinje tissue changes with the basic cycle length of stimulation. **Panel B:** At 0.9 μM AS, the amplitude of OAP increased and triggered activity occurred at short cycle lengths. BCL = basic cycle length. AS = acetylstrophanthidin.

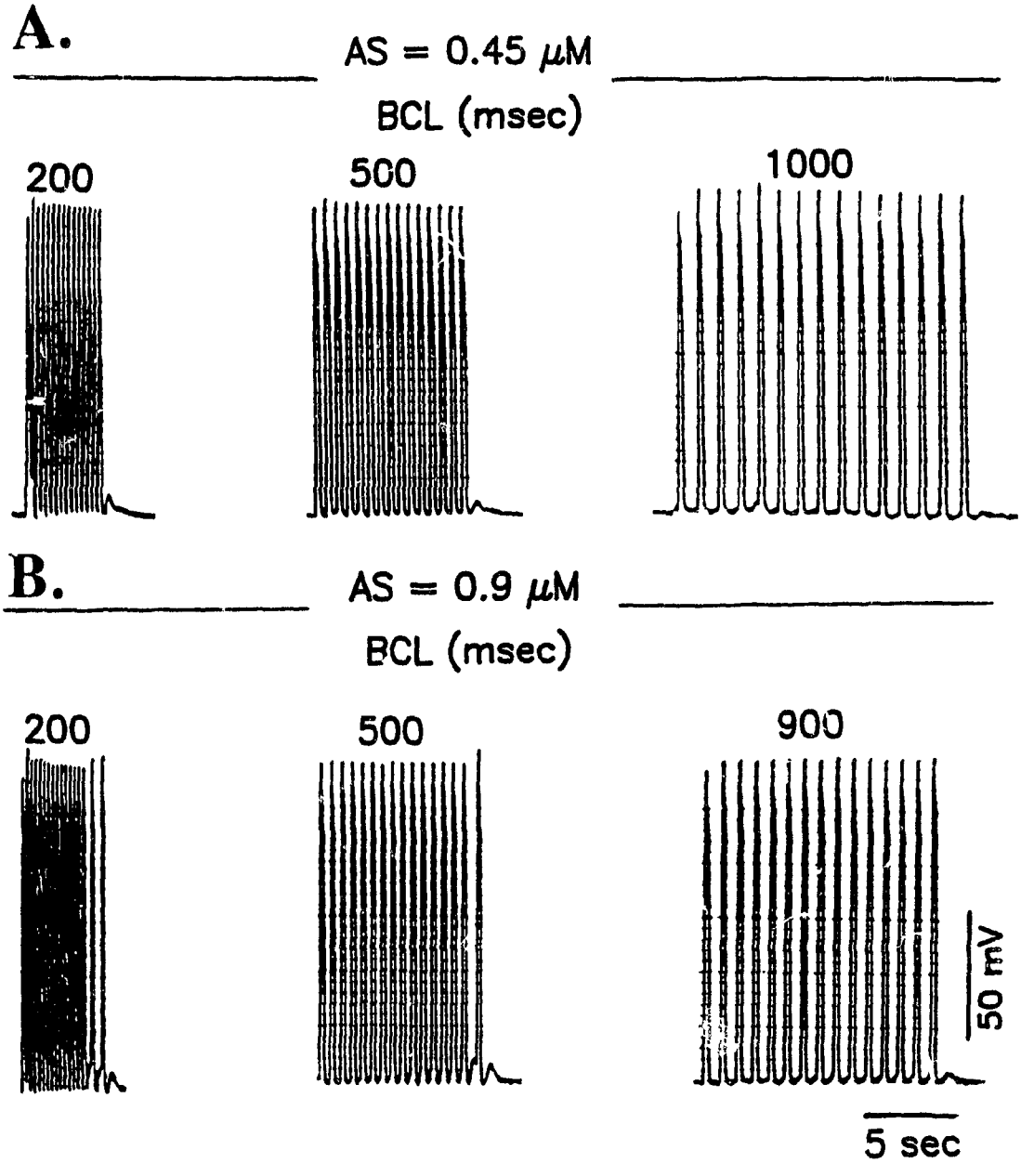

A.
AS = 0.45 μM
BCL (msec)
200
500
1000
B.
AS = 0.9 μM
BCL (msec)
200
500
900
50 mV
5 sec

Figure 3.

Figure 4. Effect of propranolol on the amplitudes of OAP induced by 0.45 μM acetylstrophanthidin. Propranolol (Prop) at a concentration of 0.5 μM had no measurable effect on OAP amplitude, whereas 2.0 μM propranolol abolished OAP.

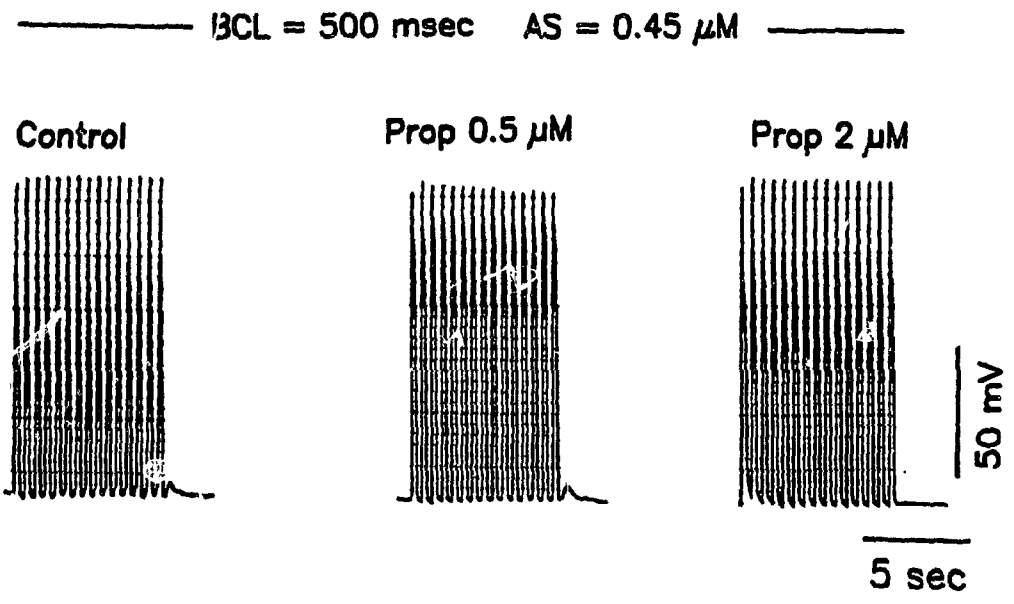


Figure 4.

Figure 5. Effects of phenylephrine and prazosin on AS-induced OAP. **Panel A:** Inhibition of AS-induced OAP by PE. **Panel B:** Block of PE's inhibitory effect on OAP by 0.5 μM prazosin. **Panel C:** Direct inhibitory effect of a higher (5.0 μM) concentration of prazosin on OAP in the absence of PE. BCL was 500 ms. Propranolol (0.5 μM) was present throughout. PE = phenylephrine. PZ = prazosin.

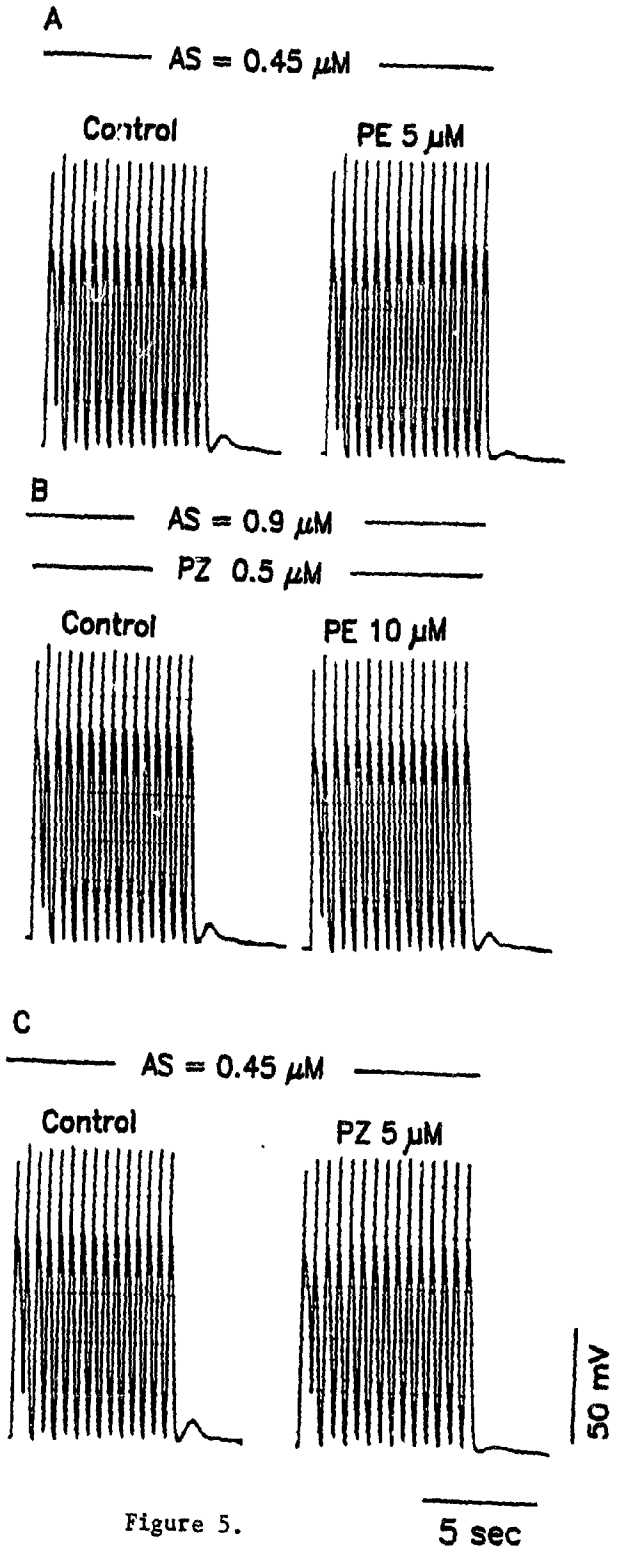

A
AS = 0.45 μM
Control
PE 5 μM
B
AS = 0.9 μM
PZ 0.5 μM
Control
PE 10 μM
C
AS = 0.45 μM
Control
PZ 5 μM
50 mV
5 sec

Figure 5.

Table 6. Effects of propranolol, clonidine and yohimbine on acetylstrophanthidin and 8 mM Ca^{2+} plus 2 mM K^+ induced oscillatory afterpotentials.

		AS induced OAP (mV)	8 mM Ca^{2+} + 2 mM K^+ induced OAP (mV)
Propranolol	0.0 μM	7.0 ± 1.8	9.6 ± 3.1
(n = 5)	0.5 μM	7.1 ± 2.1	10.1 ± 3.9
	2.0 μM	1.1 ± 0.4*	1 ± 0.6*
Clonidine	0.0 μM	8.2 ± 1.7	10.5 ± 2.9
(n = 4)	0.5 μM	8.1 ± 2.1	11.2 ± 3.7
	2.0 μM	ND	11.0 ± 3.3
	5.0 μM	8.0 ± 2.2	10.2 ± 4.3
Yohimbine	0.0 μM	8.4 ± 2.3	11.4 ± 3.5
(n = 4)	0.5 μM	8.0 ± 2.6	10.5 ± 3.7
	2.0 μM	1.2 ± 0.5*	1.4 ± 0.4*

Data expressed as mean ± SEM. *: $p < 0.05$ compared with corresponding zero concentration value. AS, acetylstrophanthidin; ND, not determined.

Figure 6. Normalized dose response curves demonstrating inhibitory effects of phenylephrine (PE) and prazosin (PZ) on the amplitude OAP induced by AS (0.2μg/-ml). BCL was 500 ms for these experiments. n = number of replicates. Statistical analysis performed on raw data; * = $p < 0.05$.

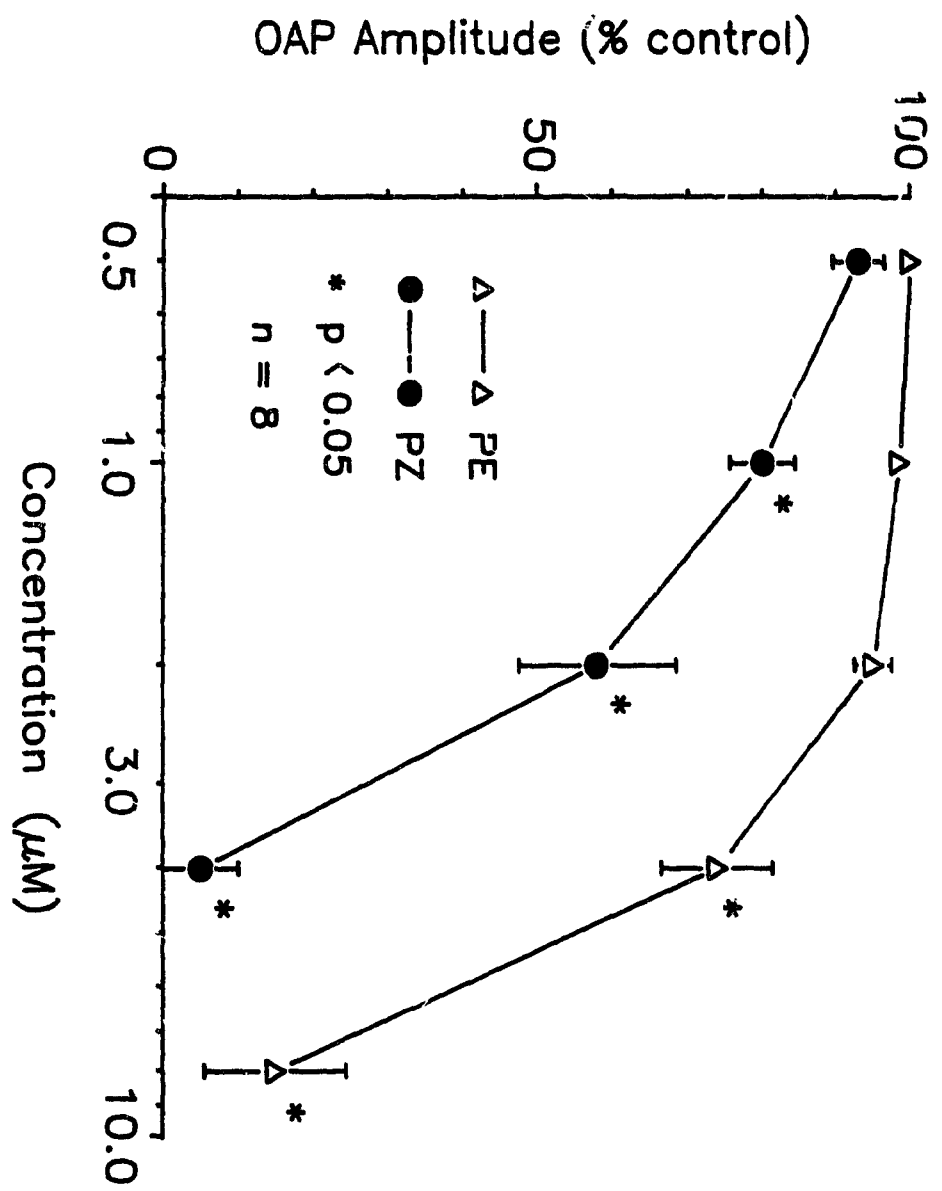

OAP Amplitude (% control)
100
50
0
0.5
1.0
3.0
10.0
Concentration (μM)
Δ—Δ PE
●—● PZ
* p < 0.05
n = 8

Figure 6.

Figure 7. Effects of phenylephrine and prazosin on triggered activity induced by AS. **Panel A:** Driven trains of action potentials were followed by triggered activity induced by 0.9 μM AS (Control). PE caused the frequency and number of triggered action potentials to decrease (10 min) and stop (20 min). **Panel B:** Triggered activity was also suppressed by prazosin in the absence of PE. BCL was 500 ms. Abbreviations are as in figure 4 and 5.

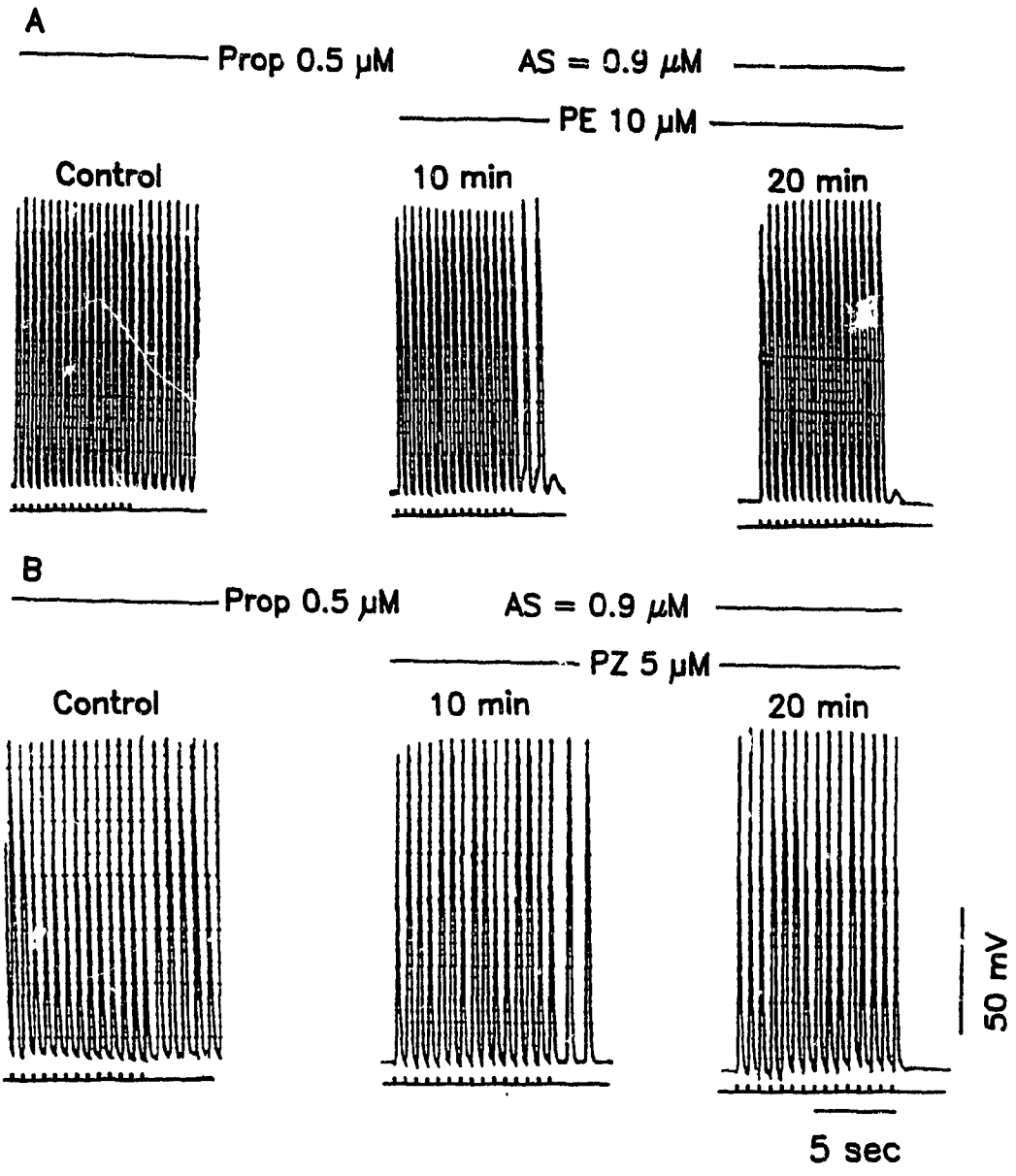

A
Prop 0.5 μM
AS = 0.9 μM
PE 10 μM
Control
10 min
20 min
B
Prop 0.5 μM
AS = 0.9 μM
PZ 5 μM
Control
10 min
20 min
50 mV
5 sec

Figure 7.

Figure 8. Effects of α_2-adrenergic agents on acetylstrophanthidin-induced oscillatory afterpotentials. **Panel A, Left:** Action potentials and coupled OAP recorded before exposure to clonidine. **Panel A, Right:** Clonidine failed to alter the amplitude of OAP. **Panel B, Left:** Control recording of OAP. **Panel B, Right:** Yohimbine decreased the amplitude of AS-induced OAP. BCL was 500 ms.

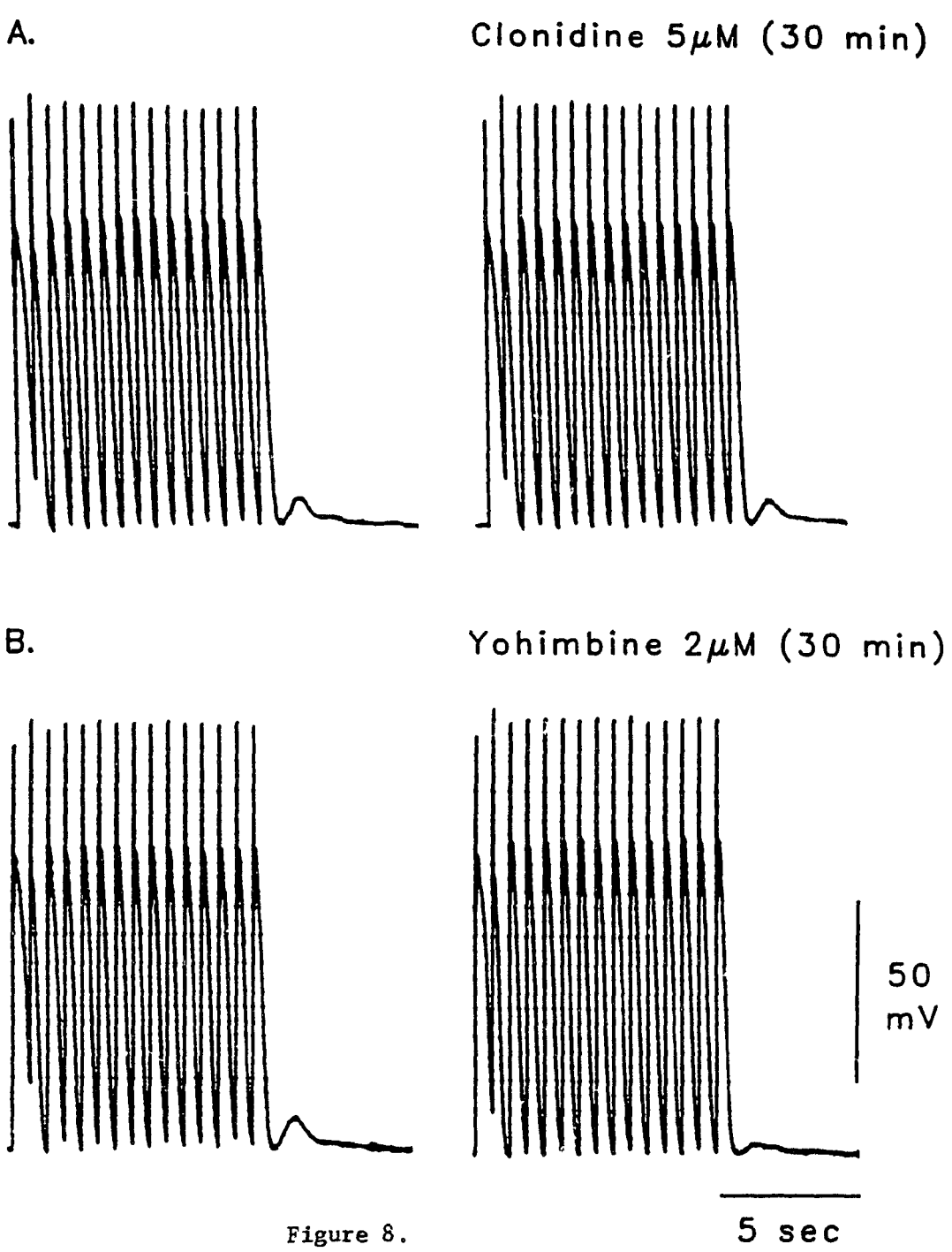

AS = 0.45 μM
A.
Clonidine 5μM (30 min)
B.
Yohimbine 2μM (30 min)
50
mV
5 sec

Figure 8.

Figure 9. Induction of OAP by high Ca^{2+} and low K^+ concentrations. Elevation of Ca^{2+} concentration induced only small OAP and 2 mM K^+ alone failed to induce OAP. However, 8 mM Ca and 2 mM K in combination induced prominent OAP (right panel). BCL = basic cycle length.

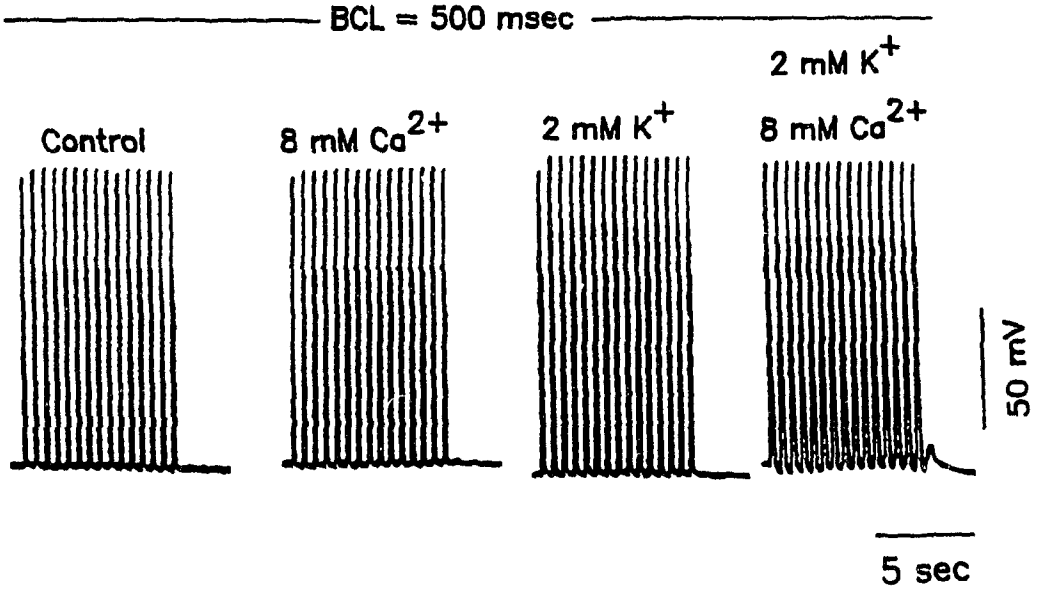


Figure 9.

Figure 10. Effects of phenylephrine and prazosin on OAP induced by 8 mM Ca^{2+} plus 2 mM K^+. **Panel A:** PE increased the amplitude of OAP and induced triggered activity. **Panel B:** Pretreatment with prazosin (0.5 μM) blocked the stimulatory effect of PE. **Panel C:** Prazosin (2 μM) inhibited high Ca^{2+}, low K^+-induced OAP in the absence of PE. BCL was 500 ms. Abbreviations are as in figure 4 and 5.

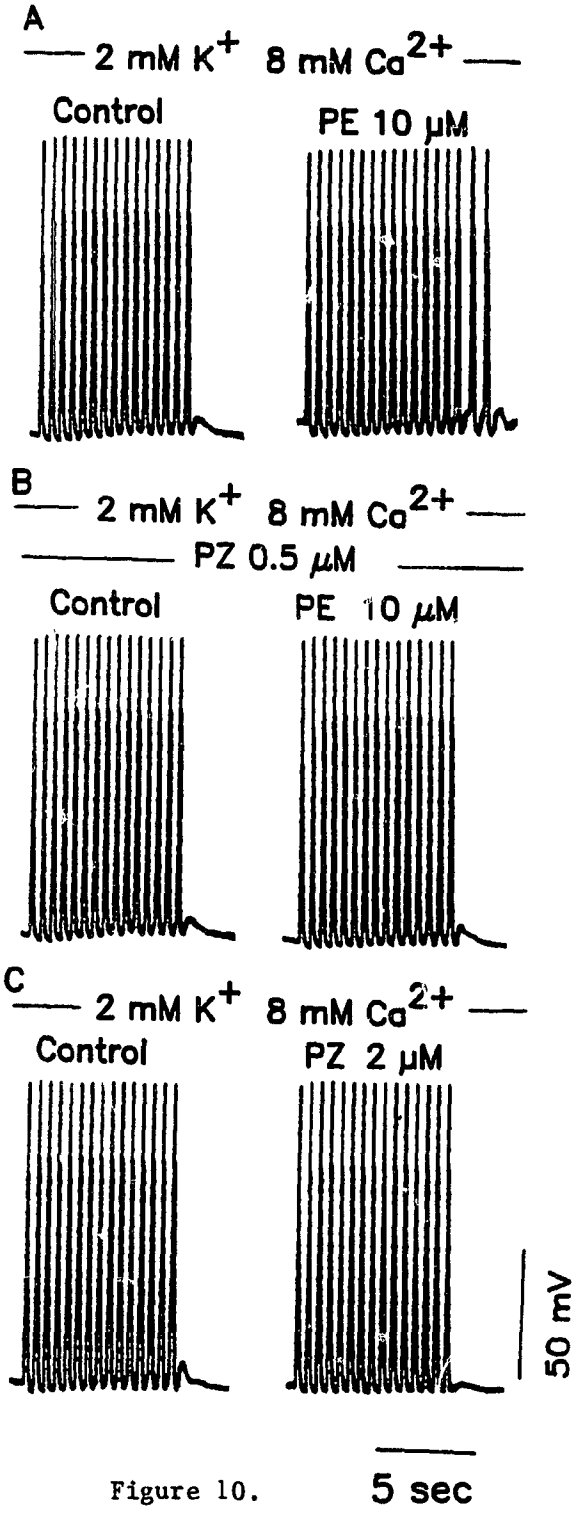

A
2 mM K+ 8 mM Ca2+
Control
PE 10 μM
B
2 mM K+ 8 mM Ca2+
PZ 0.5 μM
Control
PE 10 μM
C
2 mM K+ 8 mM Ca2+
Control
PZ 2 μM
50 mV
5 sec

Figure 10.

Figure 11. Effects of α_2-adrenergic agents on high Ca^{2+} low K^+-induced OAP. **Panel A:** Clonidine (right) failed to decrease the amplitude of OAP relative to control (left). **Panel B:** Yohimbine (right) decreased the amplitude of OAP relative to control (left). Effects of drug treatments were recorded after 30 min of drug exposure. CLD = clonidine. YH = yohimbine.

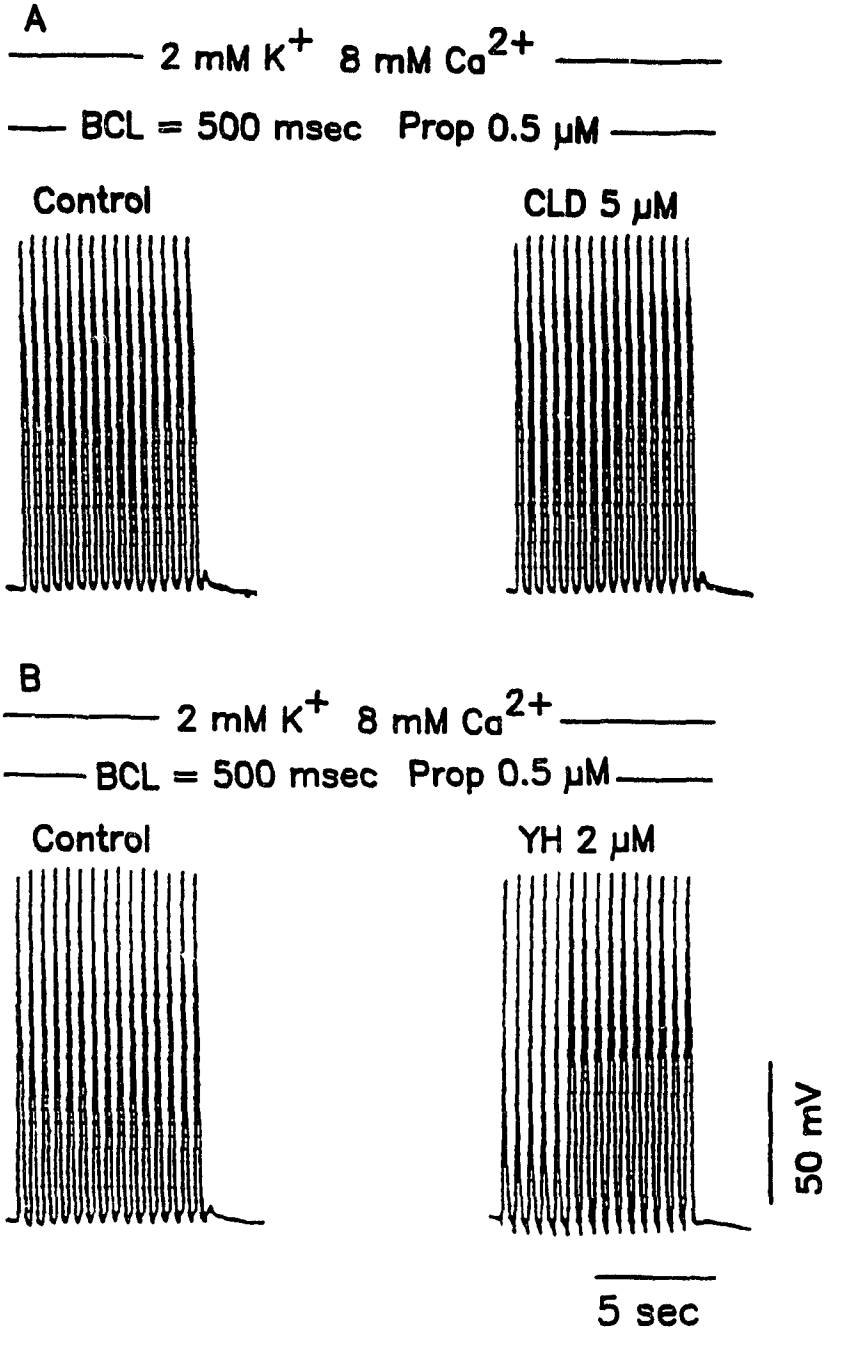
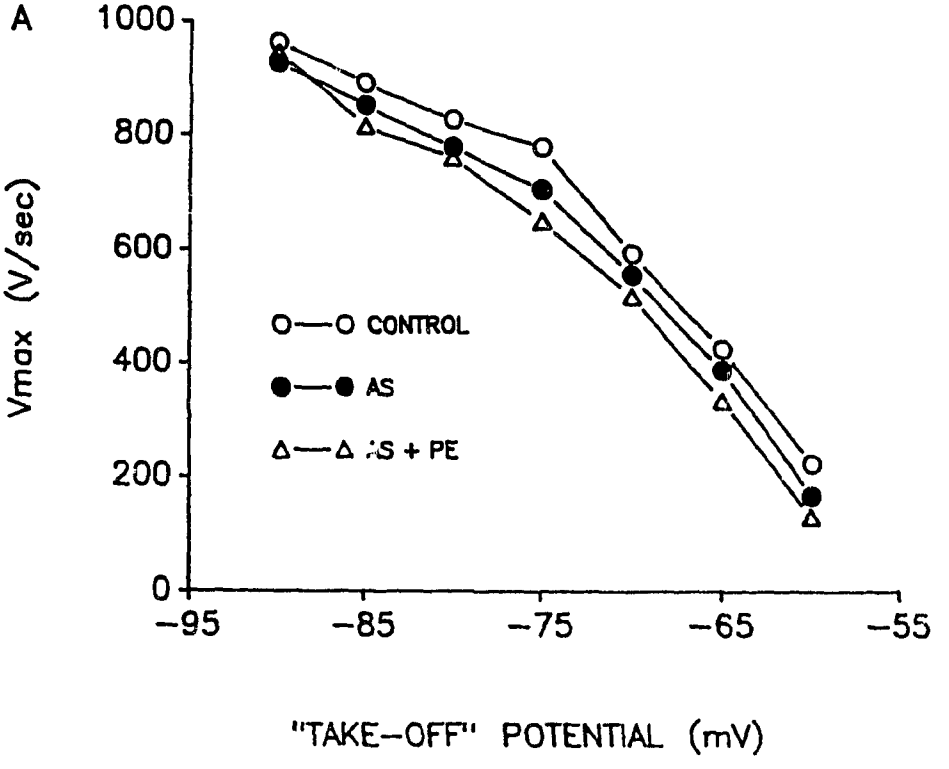

A
2 mM K+ 8 mM Ca2+
BCL = 500 msec Prop 0.5 µM
Control
CLD 5 µM
B
2 mM K+ 8 mM Ca2+
BCL = 500 msec Prop 0.5 µM
Control
YH 2 µM
50 mV
5 sec

Figure 11.

Figure 12. Lack of effect of phenylephrine on Na^+ influx measured by changes in maximum upstroke velocity of action potentials initiated at different membrane potentials. **Panel A:** Relationship between maximum upstroke velocity and take-off potential in control (open circles), AS-intoxicated (filled circles), and PE treated AS-intoxicated preparations (triangles). **Panel B:** Relationship between maximum upstroke velocity and take-off potential in control (open circles), high Ca^{2+}, low K^+ treated (filled circles), and PE plus high Ca^{2+}, low K^+ treated preparations (triangles). Vmax = maximum upstroke velocity. * = $p < 0.05$.


A
1000
800
600
400
200
0
Vmax (V/sec)
O—O CONTROL
●—● AS
Δ—Δ AS + PE
-95
-85
-75
-65
-55
"TAKE-OFF" POTENTIAL (mV)

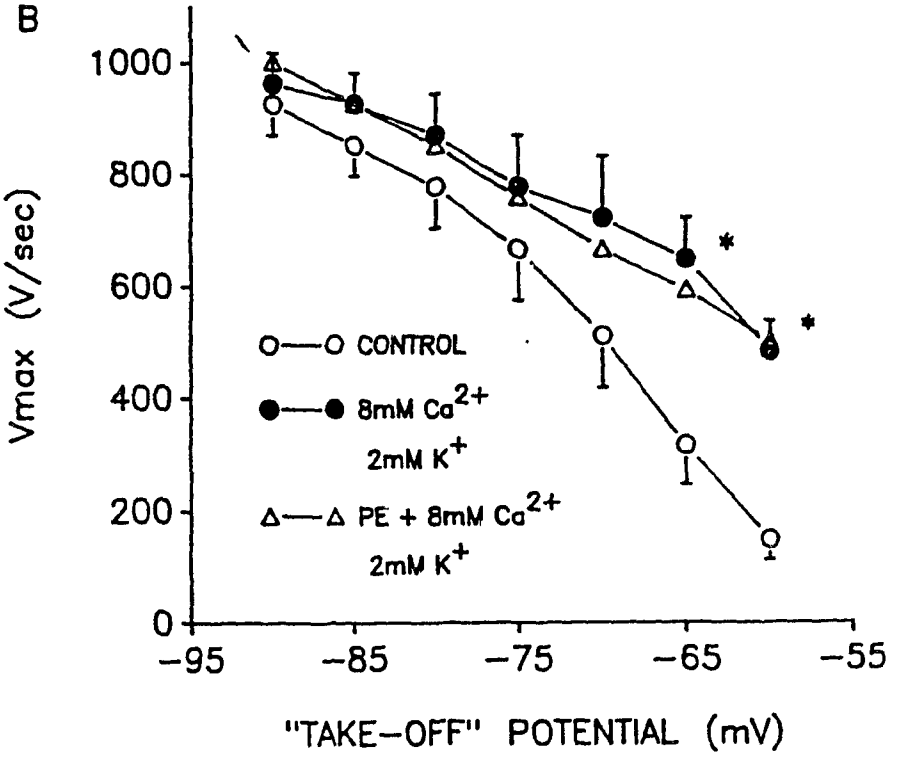

B
1000
800
600
400
200
0
Vmax (V/sec)
O—O CONTROL
●—● 8mM Ca2+
2mM K+
Δ—Δ PE + 8mM Ca2+
2mM K+
*
*
-95
-85
-75
-65
-55
"TAKE-OFF" POTENTIAL (mV)

Figure 12.

III. EFFECTS OF β-ADRENERGIC AGENTS ON GENERATION OF TRANSIENT INWARD CURRENT (TI)

1. Effects of Isoproterenol (ISO) on Transmembrane Currents in Rabbit Cardiac Purkinje Fibres

We first determined whether ISO could induce the TI in isolated rabbit Purkinje fibers. Initial experiments were conducted in preparations from 5 different animals in which spontaneous TI could not be detected. The holding potential was -80 mV. The membrane potential was stepped to -10 mV for 3 sec and then returned to the holding potential as shown in figure 13A (right). In a previous study, this protocol was found to be effective in eliciting a large TI following return to the holding potential in preparations exposed to a toxic concentration of acetylstrophanthidin or 8 mM Ca^{2+} (Ferrier and Carmeliet, 1990). This activating step was repeated every 20 sec. The transmembrane currents elicited by this voltage clamp sequence in the absence of drug are shown in figure 13A (control). Preparations were then exposed to 4 concentrations of ISO: 10^{-8}M, 10^{-7}M, 10^{-6}M, and 5 x 10^{-6}M for 10 min each. Figure 13A illustrates the effects of 1 and 5 x 10^{-6}M ISO. Both concentrations increased the net outward current at the end of the 3 sec activating step but failed to induce a TI.

In approximately 20% of rabbit Purkinje fibers, a small TI can be elicited by repolarization from a depolarizing step in the absence of an inducing agent. An example is shown in figure 13B (left). Following the return to -80 mV a small but distinct TI can be seen. Addition of 1 x 10^{-7}M ISO to the Tyrode's solution caused the mean peak TI to increase from -4.9 ± 1.6 to -8.8 ± 1.9 nA (79.6% increase; n = 5; $p < 0.05$). No further increase was observed following 20 min exposure to 10^{-6}M ISO (figure 13B).

2. Effects of ISO on AS-Induced TI

A. Effect of ISO on TI Amplitude

We also determined the effects of ISO on TI induced by AS. Experiments were conducted in preparations in which the TI did not occur spontaneously and ISO alone did not induce the TI. Traces from a representative experiment are shown in figure 14. Control records are shown at the left. AS (0.4μg/ml = 0.9 x 10^{-6}M) induced a large TI (center panel) which increased dramatically with exposure to 10^{-7}M ISO (right).

B. Time Course of Stimulatory Effect of ISO on TI

The time course of this effect is shown in figure 15. In experiments in 5 preparations, 10^{-7}M ISO increased peak TI induced by acetylstrophanthidin from -28.1 $\pm$ 5.8 to -48.5 $\pm$ 8.3 nA (mean $\pm$ S.D.; 72.6% increase; n = 5; $p < 0.05$). The maximum effect reached within 5 min and reversed when ISO was washed from the bath (figure 15).

C. Effects of ISO on "Activation Curves" and I-V Curves of AS-Induced TI

Although the TI is not activated through classic gating mechanisms, the magnitude of the TI varies with the voltage of the activating step (Lederer and Tsien, 1976). The "activation curves" for the TI induced by AS were determined before and after exposure to 10^{-7}M ISO. The peak magnitude of the TI was again measured at the holding potential, however the voltage of the preceding activating step was varied systematically from -80 to between 0 and +20 mV. A representative control activation curve is shown in figure 16A (open circles). ISO increased the peak TI induced by all activating steps (closed circles). A similar enhancement of TI was seen in 5 of 5 preparations. ISO also shifted the minimum activating step (defined as the most negative step to elicit a TI with a peak magnitude greater than 5 nA), 10 mV negative. The mean values for the minimum activation step were -38 mV $\pm$ 4.5 S.D.

with AS alone, and -48 mV ± 4.5 S.D. in the presence of AS plus 10^{-7}M ISO ($p<0.05$). Thus, the TI was "activated" by a significantly smaller depolarizing step. The shift was also seen clearly in figure 16B, in which the "activation curves" were normalized by plotting I/I_{max} vs the corresponding activation voltages.

The effects of ISO on the current-voltage (I-V) relationship of the TI induced by AS also were determined. For these experiments the TI was activated by a voltage step to +15 mV for 3 sec from the holding potential of -80 mV. The peak TI was measured upon return of the membrane potential to various test potentials between -110 and +10 mV. The test potential was maintained for 1 sec before returning to the holding potential. ISO significantly increased peak TI at all repolarization voltages (figure 16C). The increase was proportionately large towards the extremes of the range of test voltages examined. This is clearly seen in figure 16D, in which the I-V relationship was normalized by plotting I/I_{max} vs the corresponding repolarization voltages. In 5 preparations the minimum (least negative) repolarization step required to elicit a TI greater than 5 nA was shifted significantly by ISO from -33 ± 4.5 mV to -19 ± 4.2 mV ($p<0.05$). Similarly, TI with large magnitudes persisted at more negative voltages near the normal resting potential of Purkinje tissue. The I-V relationship in figure 16C also shows a small shift in the test voltage at which the maximum peak TI occurred. In 5 preparations the mean test voltage corresponding to maximum peak current shifted from -69 ± 4.2 mV to -75 ± 3.5 mV. This difference was not statistically significant ($p>0.05$).

Similar determinations of "activation curves" and I-V relations were repeated after pretreatment of preparations with β-adrenergic antagonists. The effects of ISO on TI were abolished by 10^{-7} M propranolol (n=4) or atenolol (n=2) (not illustrated).

3. Effects of Mn^{2+} and Verapamil on ISO Stimulation of Ca^{2+} Currents (I_{Ca})

Since β-agonists can enhance the slow inward current (I_{Ca}) and TI is activated by an increase in intracellular Ca^{2+} activity, it would seem likely that β-adrenergic agents would enhance the TI indirectly by stimulating I_{Ca}. If this hypothesis is correct, the effects of ISO on TI should be blocked by agents that block I_{Ca}. Therefore, we determined dose response curves for the effects of Mn^{2+} and verapamil on I_{Ca}, in order to identify appropriate concentrations of these agents that strongly block the I_{Ca} (figure 17A and B). Since we wanted to measure both I_{Ca} and the TI with the same protocol in later experiments, we used voltage steps to -20 mV from a holding potential of -80 mV. I_{Ca} would be induced by the depolarizing step and TI upon return to -80mV. The I_{Ca} was taken as the major slow inward deflection seen upon depolarization to -20 mV. With this protocol, I_{Ca} is not sharply separated from all contaminating currents and the dose response curves asymptote toward a value greater than zero. The lowest concentrations of the agents causing close to maximal inhibition were 2.0 mM Mn^{2+} and 2.0 μM verapamil.

Figure 17C shows that, although 2 mM Mn^{2+} dramatically inhibited the peak I_{Ca} activated by depolarizing to -20mV from a previous holding potential of -80mV, there was still a downward deflection that could not be abolished. The downward deflection may represent a combination of decay of transient outward current, slowly inactivating Na^+ current, and activation of the delayed rectifier potassium current (I_k). That I_{Ca} was truly inhibited was confirmed in another series of 5 experiments. These experiments were conducted in the presence of 4 mM CsCl to partially inactivate I_k and used a holding potential of -50 mV to inactivate I_{Na}. The concentrations of blocking agents that were tested (2.0 mM Mn^{2+} or 2.0 μM verapamil) corresponded to those that caused near maximal inhibition of I_{Ca} in the dose-response relations shown in figure 17A and B. Traces from a representative experiment with Mn^{2+} are shown in figure 17D. Under these conditions 2 mM Mn^{2+} completely abolished the

inward deflection corresponding to I_{Ca}. Observations made with 2.0 μM verapamil were identical except that the development of inhibition usually took slight longer (10 to 15 min compared to 5 min for Mn^{2+}). These concentrations of blocking agents also were used in experiments to determine whether ISO could stimulate TI in the presence of blockade of I_{Ca}. Under these conditions, 2 mM Mn^{2+} completely blocked stimulation of I_{Ca} by 10^{-7}M ISO (figure 17D). Verapamil (2μM) also totally abolished the stimulatory effect of 10^{-7}M ISO on I_{Ca} (data not shown). Partial stimulation could be observed by ISO at concentrations several times higher than used for the present study (not illustrated).

4. Time Course of Inhibition of I_{Ca} and TI by Mn^{2+}

The TI and I_{Ca} show a different time course of inhibition by Mn^{2+} and verapamil (Kass et al, 1978a). This was confirmed in the present study (figure 18A). Maximal blockade of I_{Ca} occurred between 6 and 10 min after exposure to Mn^{2+}, whereas TI was only reduced to about 75% of its previous magnitude at this time. The effects of ISO were determined by adding this agent 6 min after Mn^{2+}. As shown in figure 18B, ISO still enhanced TI with a time course similar to that observed in the absence of blocker (figure 15). Quantitative results are summarized in figure 20. The peak TI was measured 5 min after addition of ISO, and was compared to the value measured 1 min before onset of action. ISO increased peak TI from -18 $\pm$ 3.5 to -27.8 $\pm$ 4.1 nA (mean $\pm$ S.D.; $p<0.05$) in the presence of Mn^{2+}. Similarly ISO increased TI from -20.6 $\pm$ 4.4 to -29.8 $\pm$ 2.9 nA (mean $\pm$ S.D.; $p<0.05$) in the presence of 2μM verapamil in 5 experiments (not illustrated). Thus, ISO can still significantly stimulate TI in the presence of blockade of I_{Ca}. This observation suggests that ISO exerts its stimulatory effect, at least in part, by some other action.

5. Effects of Quinacrine on AS-Induced TI and on ISO Stimulation of TI

One other possibility for the mechanism of action of ISO on TI is stimulation of Na^+-Ca^{2+} exchange. We tested this possibility in experiments similar to those with Ca^{2+} channel blockers. Quinacrine was used to block Na^+-Ca^{2+} exchange (Bielefeld et al, 1986; Satoh et al, 1989). Because Na^+-Ca^{2+} exchange is involved in the generation of Ca^{2+} overload by AS, quinacrine (20 μM) also caused a gradual decline in the magnitude of the peak TI. The mean response of 6 preparations to quinacrine are illustrated in figure 19A. In 5 additional experiments 10^{-7}M ISO was added 6 min after quinacrine. A representative experiment is illustrated in figure .9B. Quinacrine completely abolished stimulation of TI by ISO. Mean data for 5 experiments are summarized in figure 20. The peak magnitude of the TI in the presence of quinacrine, immediately before addition of ISO was -18.4 nA. Five minutes after addition of ISO, the peak TI declined significantly. However, the mean value was not significantly different from that observed after the same total elapsed time in 6 preparations in which ISO was not added. Similar blockade of ISO (10^{-7}M) stimulation of TI was also observed in another 3 preparations in which 2 mM amiloride was substituted for quinacrine.

Presently there is no specific Na^+-Ca^{2+} exchange blocker available (Giles and Shimoni, 1989a). Thus, it is possible that quinacrine might exert its effect on TI by inhibiting I_{Ca}. Therefore, in 5 preparations both TI and I_{Ca} were generated in the presence of 0.9 x 10^{-6}M AS by a 500 msec voltage step to -20 mV from a holding potential of -45mV or -50mV. As shown in figure 21A both I_{Ca} and TI were stimulated strongly by 10^{-7}M ISO. Once maximal stimulation of both currents had developed, addition of 20μM quinacrine caused a dramatic inhibition of TI while the I_{Ca} amplitude was basically unchanged (figure 21B). Identical observations were made in 5 preparations. Our results suggest that the stimulatory effect of isoproterenol on TI induced by digitalis is mediated by Na^+-Ca^{2+} exchange.

Figure 13: **A.** Effects of ISO on transmembrane currents in a preparation not exhibiting spontaneous I_{ti}. The voltage clamp step applied to the preparation is illustrated schematically at the right. ISO increased net outward current at the end of the voltage step to -10 mV, but failed to induce a I_{ti} upon return to the holding potential. **B.** Effects of ISO on spontaneously occurring I_{ti}. A I_{ti} was observed following return to the holding potential in the absence of drug treatment (left). ISO (10^{-7}M) increased the magnitude of the peak I_{ti}. A higher concentration of ISO produced no additional increase in the I_{ti}. ISO = isoproterenol.

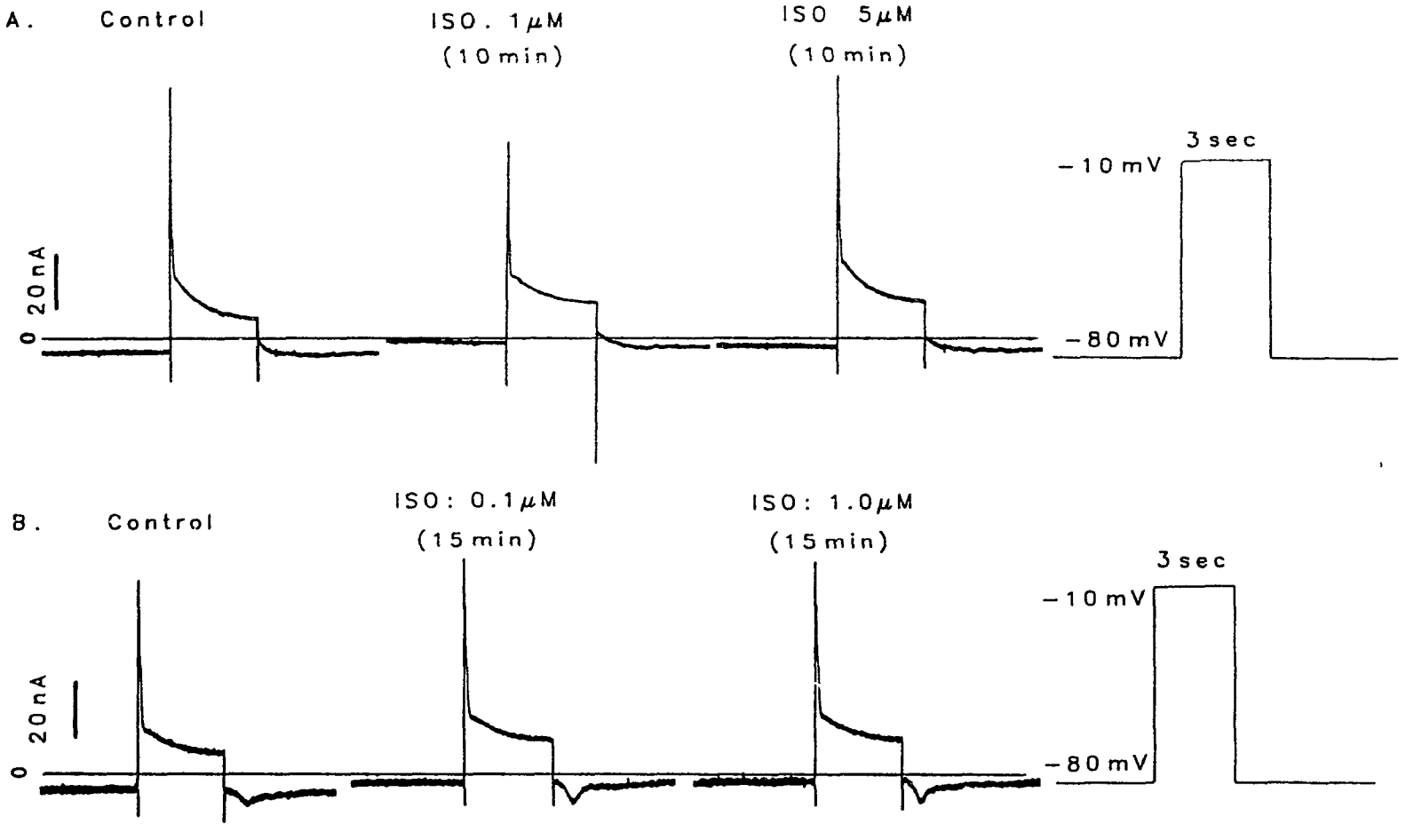

A.
Control
ISO. 1μM
(10 min)
ISO 5μM
(10 min)
3 sec
-10 mV
-80 mV
20 nA
0
B.
Control
ISO: 0.1μM
(15 min)
ISO: 1.0μM
(15 min)
3 sec
-10 mV
-80 mV
20 nA
0

Figure 13.

Figure 14: Effects of ISO on I_{ti} induced by AS. Voltage steps are shown below corresponding current traces. The left panel shows that the voltage step failed to elicit a I_{ti} upon return to the holding potential in the absence of drug. The same voltage steps elicited a large I_{ti} after exposure of the preparation to AS (center traces). ISO caused a marked increase in the magnitude of the peak I_{ti} (right). AS = acetyl-strophanthidin.

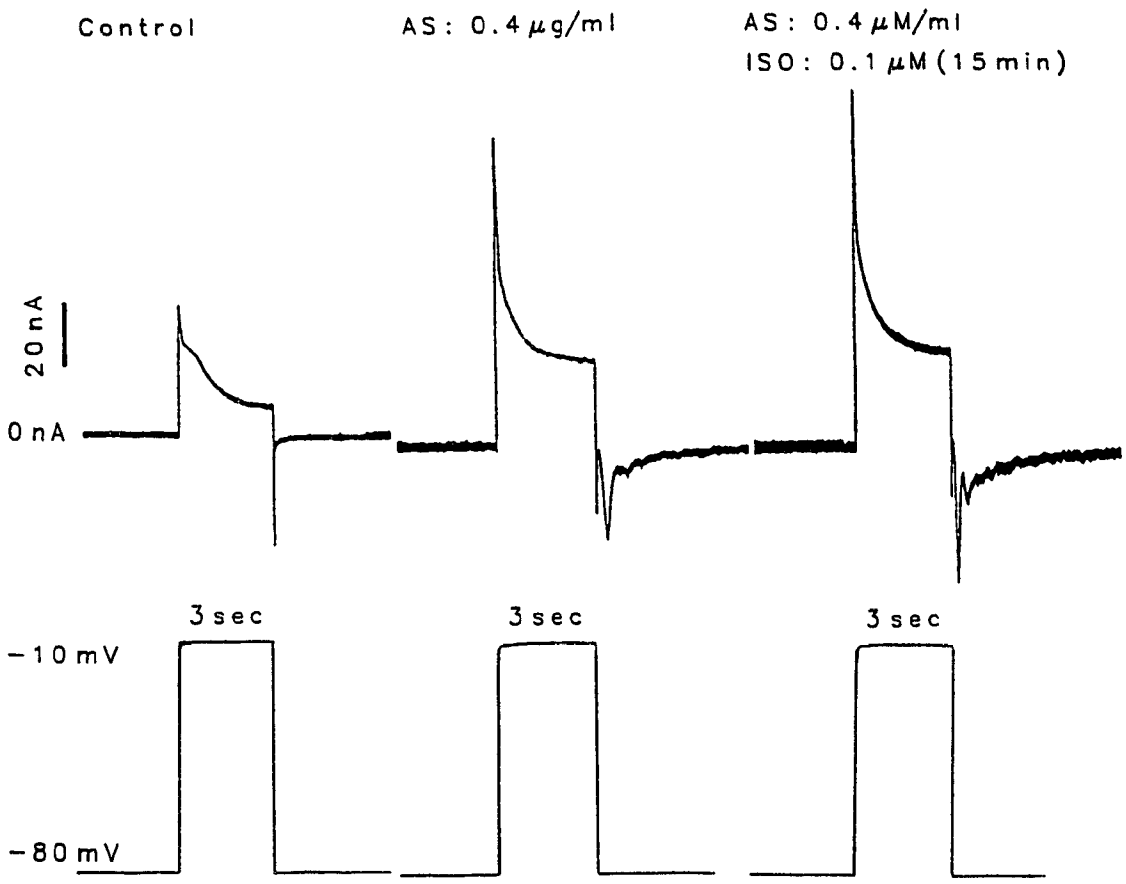

Control
AS : 0.4 μg/ml
AS : 0.4 μM/ml
ISO : 0.1 μM (15 min)
20 nA
0 nA
3 sec
3 sec
3 sec
−10 mV
−80 mV

Figure 14.

Figure 15: Time course of stimulatory effect of ISO on I_{tr} induced by AS. Mean data are shown for preparations from 5 hearts. Bars represent standard deviations. ISO was added at time zero, and was washed from the tissue bath after 10 min. Peak effect was achieved by 5 min, and the stimulatory effect reversed when ISO was removed.

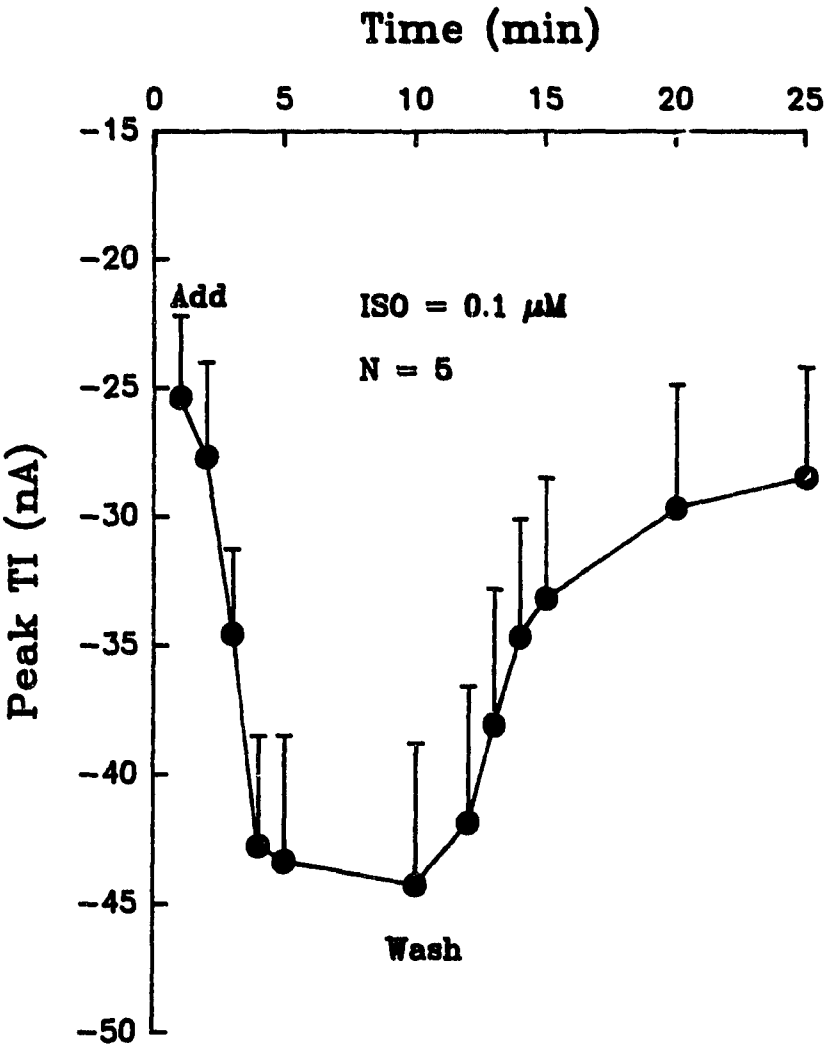

Time (min)
0
5
10
15
20
25
-15
-20
-25
-30
-35
-40
-45
-50
Peak TI (nA)
Add
ISO = 0.1 μM
N = 5
Wash

Figure 15.

Figure 16: **A.** Effect of ISO on "activation curves" for I_{ti}. The magnitude of the peak I_{ti} (ordinate) was measured at the holding potential, and was activated by 3 sec steps to various activation voltages (abscissa). ISO increased the magnitude of the I_{ti} for all activation voltages that elicited measurable I_{ti}. ISO also shifted the minimum depolarization required to activate an I_{ti} with peak magnitude greater than 5 nA. **B.** Effect of ISO on normalized "activation curves" for I_{ti}. Data applied were the same as in **A** except by plotting I/I_{max} vs corresponding activation voltages. **C.** Current-voltage relationship for I_{ti} induced by AS in the absence and presence of ISO. The I_{ti} was measured at various test voltages following an activating step to +15 mV. ISO increased the peak current at all test voltages and decreased the minimum repolarization step required to elicit an I_{ti} with a peak magnitude greater than 5 nA. **D.** Normalized I-V relationship for I_{ti} before and after ISO exposure. Data applied were the same as in **C** except by plotting I/I_{max} vs corresponding repolarization voltages.

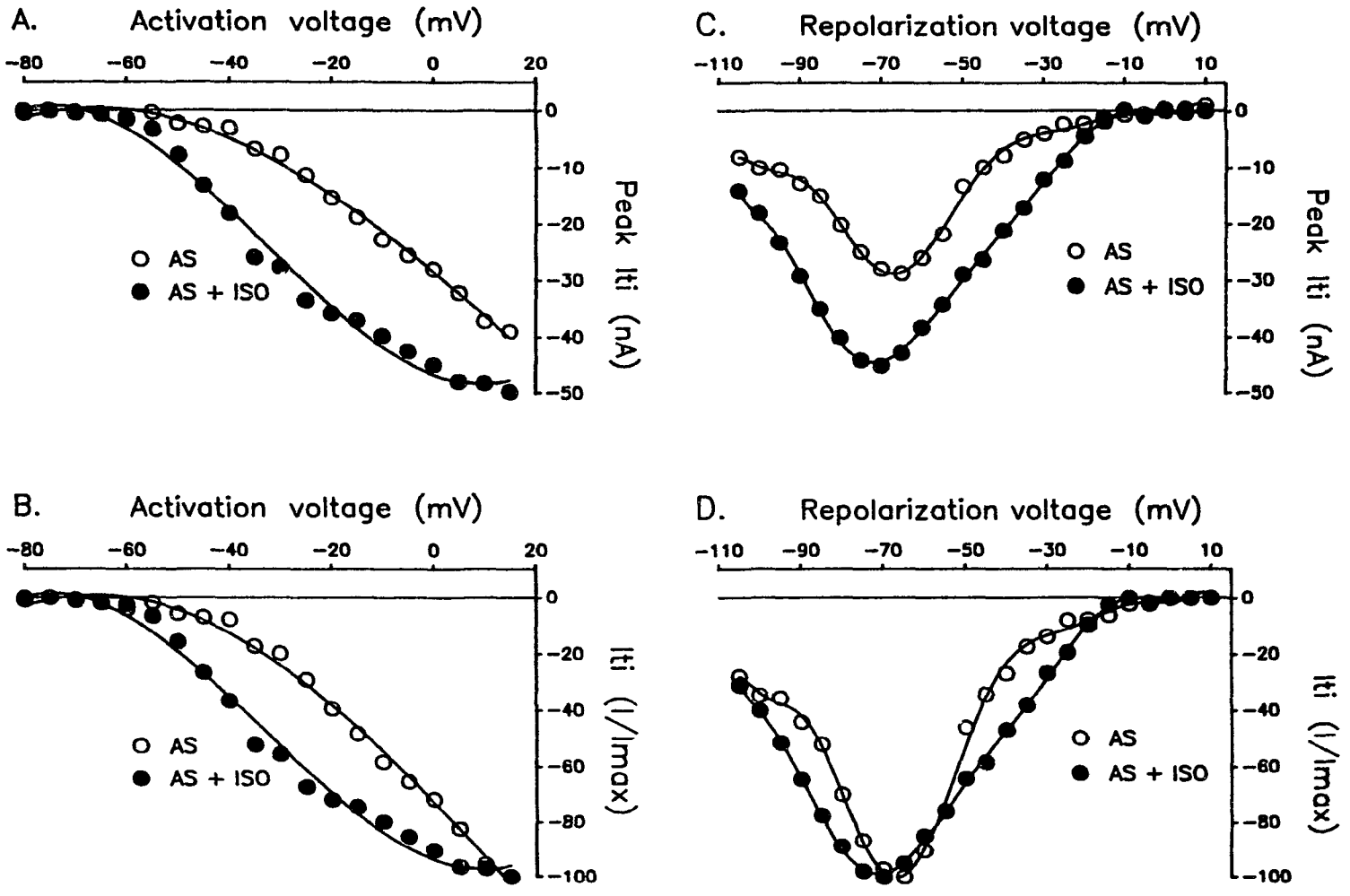

A.
Activation voltage (mV)
-80 -60 -40 -20 0 20
0 -10 -20 -30 -40 -50
Peak Iti (nA)
AS
AS + ISO
C.
Repolarization voltage (mV)
-110 -90 -70 -50 -30 -10 10
0 -10 -20 -30 -40 -50
Peak Iti (nA)
AS
AS + ISO
B.
Activation voltage (mV)
-80 -60 -40 -20 0 20
0 -20 -40 -60 -80 -100
Iti (I/Imax)
AS
AS + ISO
D.
Repolarization voltage (mV)
-110 -90 -70 -50 -30 -10 10
0 -20 -40 -60 -80 -100
Iti (I/Imax)
AS
AS + ISO

Figure 16.

Figure 17: Blockade of I_{Ca} by Mn^{2+} and verapamil. **A and B.** Dose response relations for inhibition of I_{Ca} by Mn^{2+} and verapamil. A voltage step to -20 from -80 mV was used to activate I_{Ca}. The magnitude of I_{Ca} is measured by taking the difference between the maximum downward deflection and the level at the end of 3 sec depolarizing pulse. Data points represent means plus standard errors for 5 experiments. **C.** Inhibition of I_{Ca} by 2 mM Mn^{2+}. Note that the protocols used for the induction of I_{Ca} are identical to those in **A** and **B.** Traces are identified as follows: a = Control; b = Mn^{2+}. **D.** Inhibition of I_{Ca} and blockade of the stimulatory effect of ISO (10^{-7}) on I_{Ca} by 2 mM Mn^{2+}. Parameters used to induce I_{Ca} are shown on the top. Traces are identified as follows: a = Control; b = Mn^{2+}; c = Mn^{2+} + ISO.

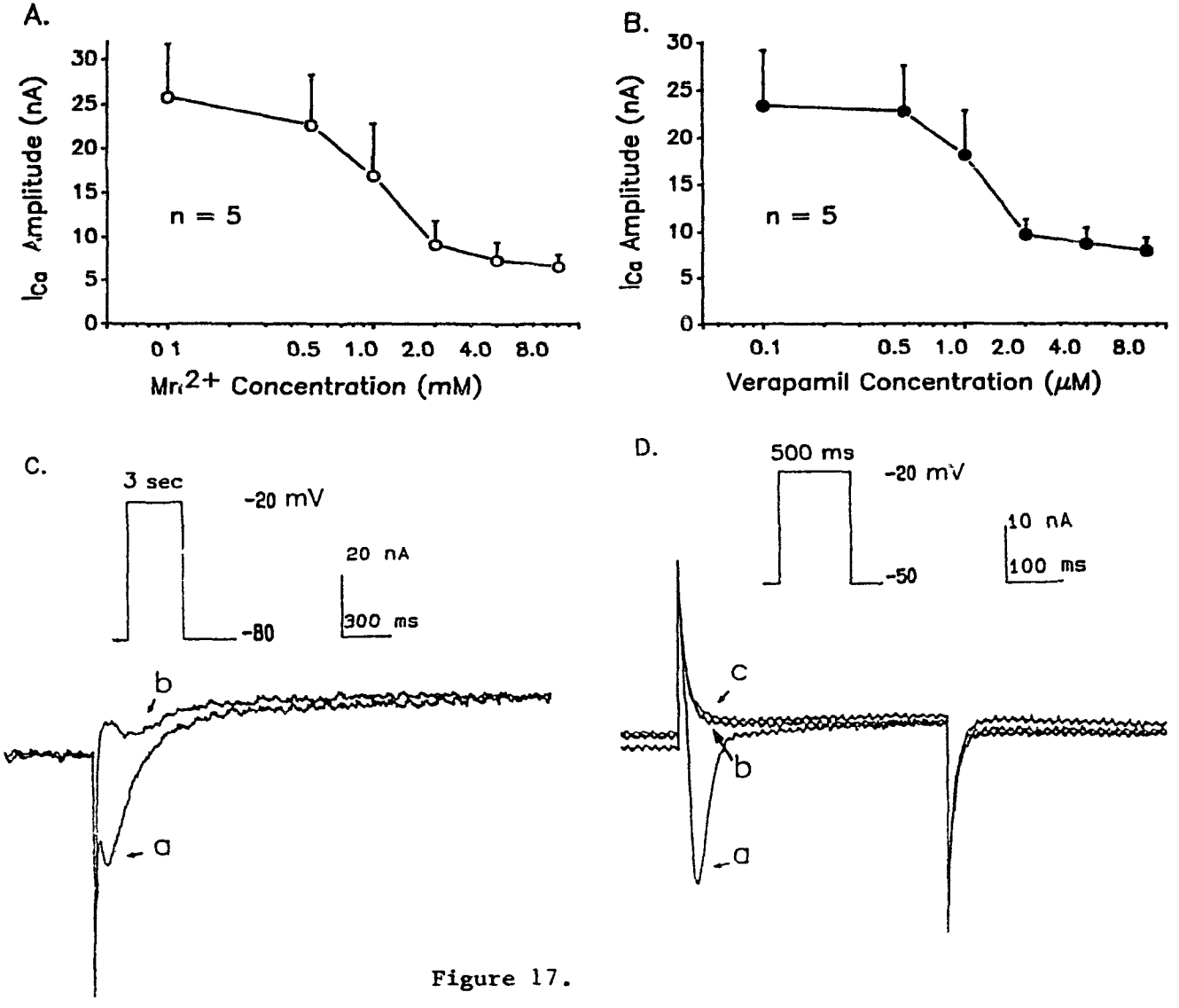

A.
I_{Ca} Amplitude (nA)
30
25
20
15
10
5
0
n = 5
0 1
0.5
1.0
2.0
4.0
8.0
Mn^{2+} Concentration (mM)
B.
I_{Ca} Amplitude (nA)
30
25
20
15
10
5
0
n = 5
0.1
0.5
1.0
2.0
4.0
8.0
Verapamil Concentration (μM)
C.
3 sec
-20 mV
-80
20 nA
300 ms
b
a
D.
500 ms
-20 mV
-50
10 nA
100 ms
c
b
a

Figure 17.

Figure 18: **A.** Comparison of time course of inhibition of I_{Ca} by 2 mM Mn^{2+} and the more gradual decay of I_{ti}. Inhibition of I_{Ca} approached maximum at a time when I_{ti} was only partially inhibited. **B.** Stimulation of I_{ti} by ISO in a preparation in which I_{Ca} was inhibited by Mn^{2+}. Addition of ISO after 6 min exposure to Mn^{2+} resulted in an increase in the magnitude of peak I_{ti} with a time course similar to that seen in preparations in which I_{Ca} was not blocked.

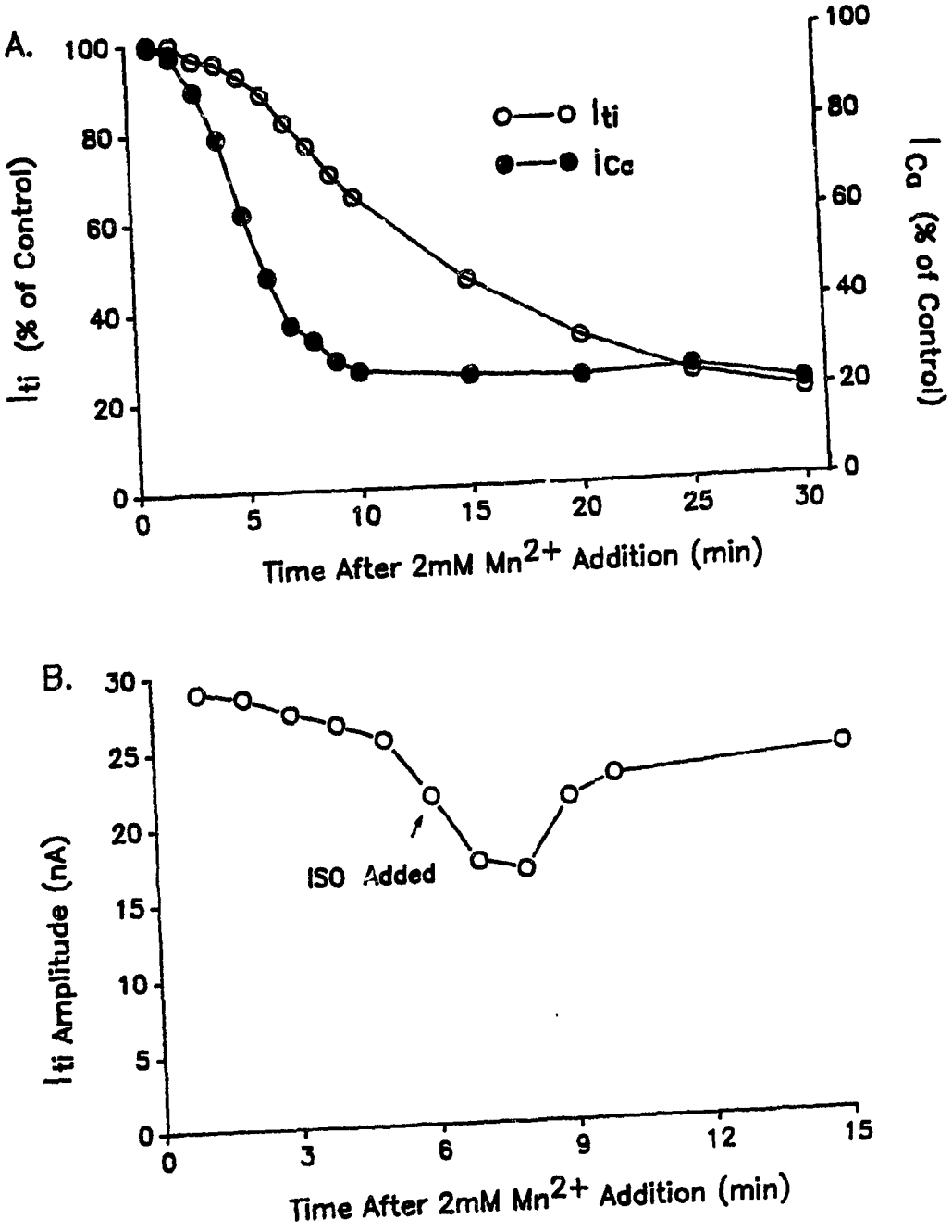

A.
100
80
60
40
20
0
I_{ti} (% of Control)
I_{ti}
I_{Ca}
100
80
60
40
20
0
I_{Ca} (% of Control)
0
5
10
15
20
25
30
Time After 2mM Mn^{2+} Addition (min)
B.
30
25
20
15
10
5
0
I_{ti} Amplitude (nA)
ISO Added
0
3
6
9
12
15
Time After 2mM Mn^{2+} Addition (min)

Figure 18.

Figure 19: **A.** Time course of inhibition of I_{ti} by quinacrine, an inhibitor of Na^{+}-Ca^{2+} exchange. Data represent means and standard deviations from determinations made in preparations from 6 hearts. After exposure to quinacrine for 6 min, the mean peak I_{ti} had decreased to 75% of its value measured at time zero. **B.** Block of stimulation of I_{ti} by ISO in a representative experiment in which the Purkinje fiber was pretreated with quinacrine. ISO was added 6 min after exposure of the tissue to quinacrine. No stimulation of I_{ti} was observed.

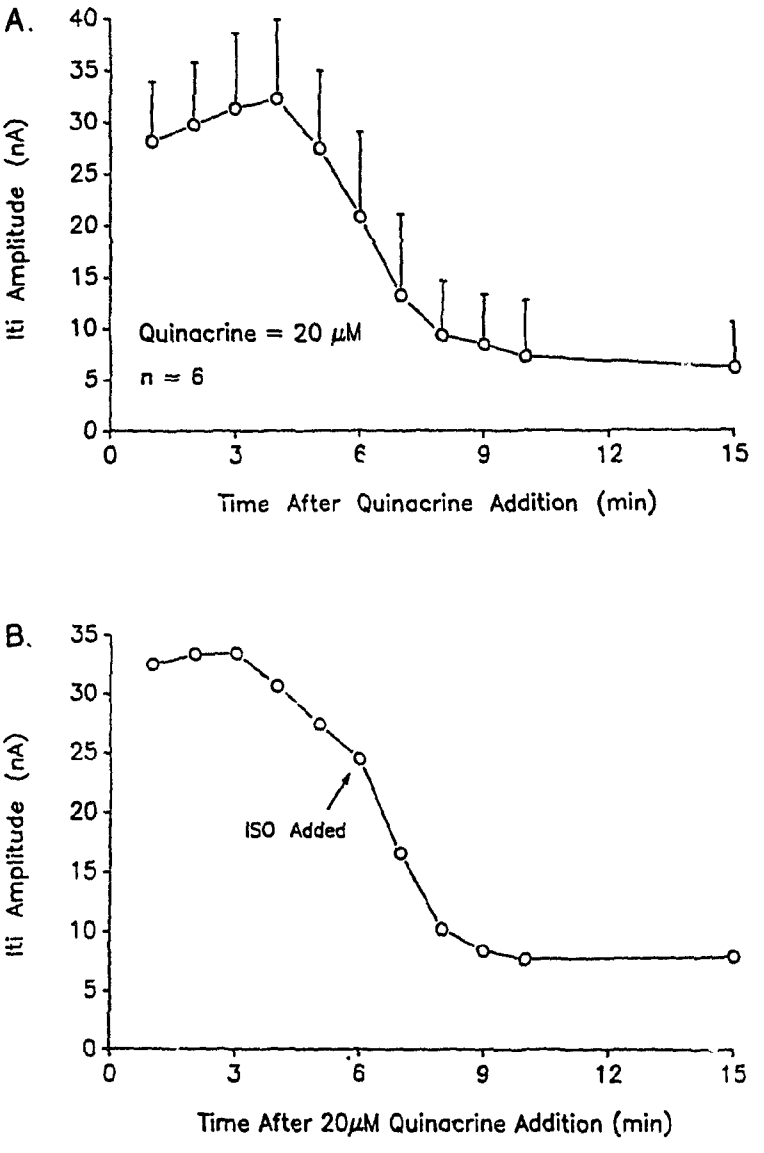

A.
Iti Amplitude (nA)
Quinacrine = 20 μM
n = 6
Time After Quinacrine Addition (min)
B.
Iti Amplitude (nA)
ISO Added
Time After 20μM Quinacrine Addition (min)

Figure 19.

Figure 20: Summary of data showing stimulation of I_{ti} induced by AS in control preparations and preparations pretreated with Mn^{2+}, and block of stimulation in preparations pretreated with quinacrine. Data represent means $\pm$ S.D. The increase in peak I_{ti} caused by ISO in control and Mn^{2+} pretreated preparations was statistically significant ($p<0.05$). The effects of ISO in preparations pretreated with either Mn^{2+} or quinacrine are compared to the magnitude of the peak I_{ti} 1 min before onset of action of ISO. A time control for the effect of quinacrine alone without addition of ISO is also shown. The value for the magnitude of the peak I_{ti} after 5 min exposure to ISO in quinacrine preparations was significantly less than the value 1 min before addition of ISO. However, the peak I_{ti} at 5 min exposure to ISO was not significantly different from the value for the corresponding time control.

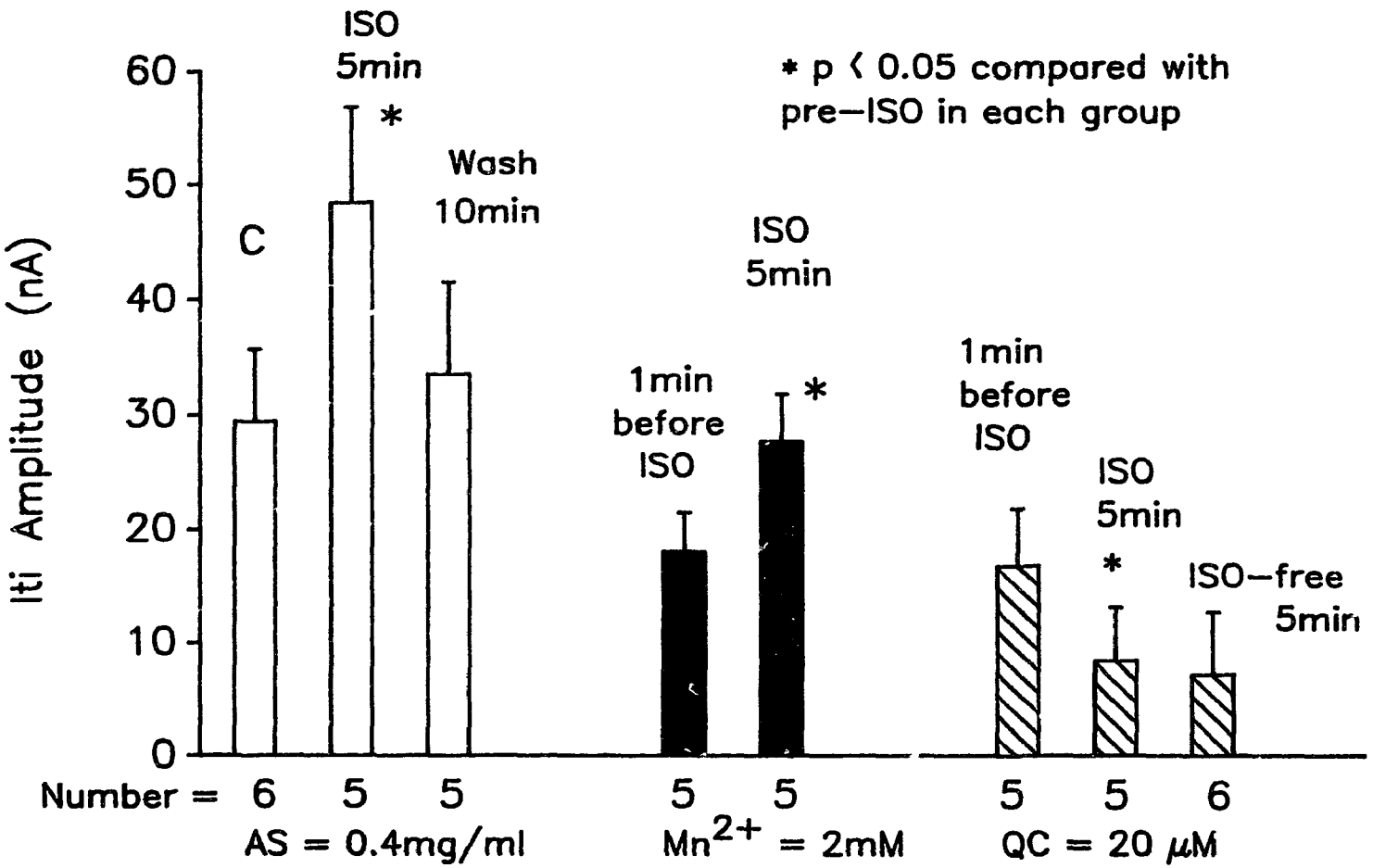

ISO
5min
*
* p ⟨ 0.05 compared with
pre—ISO in each group
Wash
10min
C
ISO
5min
1min
before
ISO
*
1min
before
ISO
ISO
5min
*
ISO—free
5min
Iti Amplitude (nA)
60
50
40
30
20
10
0
Number = 6 5 5 5 5 5 5 6
AS = 0.4mg/ml
Mn2+ = 2mM
QC = 20 μM

Figure 20.

Figure 21: **A.** Effects of quinacrine on I_{Ca} and I_{ti} in the presence of ISO. I_{ti} was induced by 0.9 μM of AS. Membrane potential was clamped to -20 mV for 1.5 sec from a holding potential of -50mV every 20 sec. Traces are identified as follows: a = I_{Ca} and I_{ti} in the presence of AS alone; b = developing stimulation of I_{Ca} and I_{ti} by 10^{-7}M ISO; c = Maximum stimulation of I_{Ca} and I_{ti} by 10^{-7}M ISO. **B.** Blockade of the stimulatory effect of ISO on I_{ti} but not I_{Ca} by 20μM quinacrine in the same preparation as in **A.** a = I_{ti} recorded in the presence of AS and ISO; b and c = inhibition of the stimulatory effect of ISO on I_{ti}. The peak amplitude of I_{Ca} was unaffected by quinacrine.

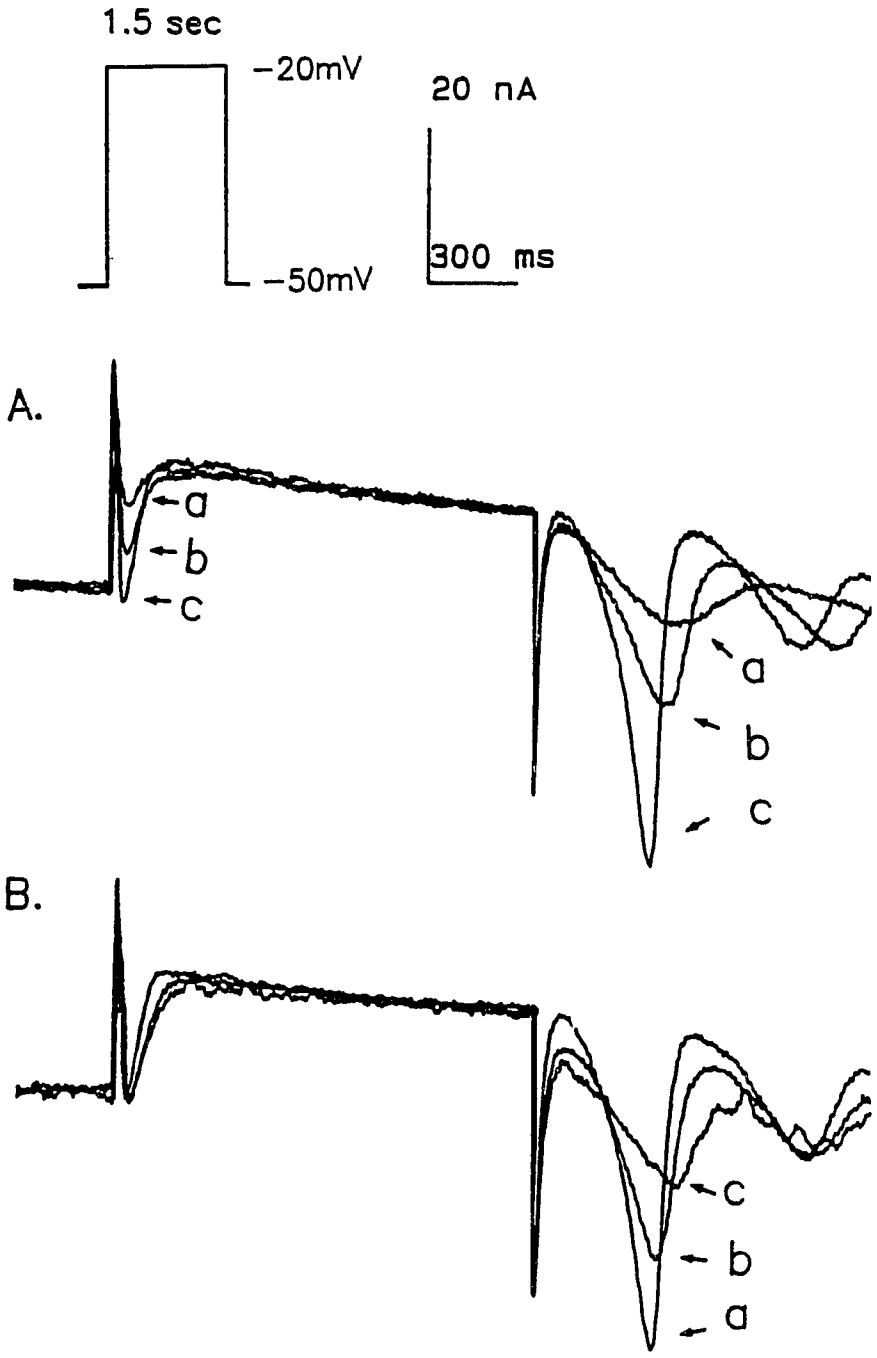


Figure 21.

IV. IONIC MECHANISMS OF HIGH $[Ca^{2+}]_o$ INDUCED TI IN THE ABSENCE OF Na^+-Ca^{2+} EXCHANGE

1. Induction of TI in the Absence of $[Na^+]_o$

TI has been previously induced by various treatments. In all but one study (Cannell & Lederer, 1986) Na^+ was present when TI was generated. This makes it difficult to distinguish the mechanism because Na^+ is known both to permeate the non-selective cationic channel and to participate in electrogenic Na^+-Ca^{2+} exchange. Since it is believed that the absence of extracellular Na^+ can exclude Na^+-Ca^{2+} exchange (Cannell & Lederer, 1986; Lipp & Pott, 1988), we initially tried to use isotonic Ca^{2+} (105mM) as the Na^+ substitute to induce TI in rabbit Purkinje fibres as described by Cannell & Lederer (1986).

Two problems were met when we exposed rabbit Purkinje fibres to isotonic Ca^{2+}. The first was that the resistence of the microelectrode-tissue circuit increased too quickly to permit the protocols needed for the present study and resulted in an inadequate voltage clamp during the depolarizing pulse. The second was a high failure rate of experiments (14 out of 15) due to loss of impalement or deterioration of the preparations. We attributed these problem to marked Ca^{2+} overload. We therefore tried to select an appropriate Ca^{2+} concentration which would be high enough to generate TI without causing marked loss of voltage control. To do this, NaCl was replaced by a combination of $CaCl_2$ and cholineCl.

Figure 22 shows original recordings of TI induced by 5 mM $[Ca^{2+}]_o$ in one of four Purkinje fibres. The inward TI which was activated upon repolarizing to -55 mV declined to zero at membrane potentials positive to -35 mV. However, no outward TI was observed even with repolarization to -5 mV, 0 and +5 mV (traces not shown). On returning to high membrane potentials, the oscillatory inward current

(TI) was superimposed on the declining outward tail current which most likely represents inactivation of I_K. The observations that TI was large at negative potentials and had no reversal potential at -5 to +5 mV resemble results reported in muscle preparations in which Na^+-Ca^{2+} exchange was implicated as the charge-carrying system (Brown et al, 1986; Arlock & Katzung, 1985). However, there was no $[Na^+]_o$ in this study. The disappearence of TI at more positive potentials and lack of apparent reversal might indicate that the TI channel conductance was not activated equally at all membrane potentials with 5 mM $[Ca^{2+}]_o$. Therefore, we determined the effects of changing $[Ca^{2+}]_o$ on the generation of TI.

2. Effect of $[Ca^{2+}]_o$ on TI Induced in the Absence of $[Na^+]_o$

Figure 23 shows experimental results typical of those obtained in 4 preparations. In panel A, trace a was the current recorded in normal Tyrode's solution. Trace b shows that TI could be generated with as little as 5 mM $[Ca^{2+}]_o$, just as shown in figure 22. An increase of $[Ca^{2+}]_o$ from 5 to 15 mM significantly increased the magnitude of peak TI (trace c). A further increase in $[Ca^{2+}]_o$ from 15 to 30 mM caused only a slight increase of peak TI (not shown). Panel B shows recordings from one of two preparations in which TI was initially induced by 30 mM $[Ca^{2+}]_o$ (trace a). An abrupt increase to 50 mM $[Ca^{2+}]_o$ resulted in a decrease of peak TI (trace b). TI could also be induced with 15 and 30 mM $[Ca^{2+}]_o$ when either sucrose (n=3), NMG chloride (n=2), or LiCl (n=2) was the substitute for NaCl.

These experiments demonstrated that we could routinely induce TI in the absence of extracellular Na^+. We next conducted experiments to confirm that: 1) Preparations thus treated were not seriously damaged; 2) TI was Ca^{2+} dependent. The first question was tested in 5 Purkinje fibres which were exposed to 30 mM $[Ca^{2+}]_o$ and observations were made for at least 30 min. After 30 min washout of high Ca^{2+}, no

apparent alterations in either the configuration or the rate of non-driven action potentials could be observed. In two experiments in which 50 mM $[Ca^{2+}]_o$ was applied, after 30 min of high Ca^{2+} treatment the preparations were persistently depolarized and only action potentials similar to those described as depolarization-induced-automaticity (Mohabir & Ferrier, 1988; Ferrier, 1991) were observed even after 1 hr perfusion with Tyrode's solution containing normal Ca^{2+}. These observations suggest that the cellular mechanisms responsible for maintaining the basic electrophysiological properties of the Purkinje fibres were not irreversibly affected by elevation of $[Ca^{2+}]_o$ up to 30 mM.

The second question was tested in 6 preparations by either substituting Ca^{2+} with Ba^{2+} (n=3) or using zero or low $[Ca^{2+}]_o$ (2.5 mM) in the absence of $[Na^+]_o$. Results from two experiments are shown in figure 24. TI appeared only transiently upon introduction of solution containing 30 mM Ba^{2+} (trace b). However, with further equilibration, no TI could be generated when $[Ca^{2+}]_o$ was replaced by $BaCl_2$, even though there was a marked increase in I_{Ca} upon depolarization and a significant suppression of the outward current at the end of depolarizing pulse (figure 24A, trace c). When identical protocols were repeated in another 3 preparations perfused with 2.5 mM $[Ca^{2+}]_o$ (figure 24B) or 0 mM $[Ca^{2+}]_o$ (data not shown), TI also was absent.

3. Reversal Potential and the I-V Relationship of TI

The TI induced by high Ca^{2+} in the absence of Na^+ in rabbit Purkinje fibres resembled TI induced by other means (Lederer & Tsien, 1976; Kass et al, 1978a, b; Brown et al, 1986) in terms of the activation parameters (activated upon repolarization) and activation/inactivation time course. To determine whether TI generated in our experiments had a distinct E_{REV} and whether E_{REV} was sensitive to Ca^{2+} concentration, we next determined I-V relations for TI at different concentrations of Ca^{2+} from

5 to 105 mM. The tonicity was maintained by adjusting the concentrations of either choline chloride or LiCl (LiCl substitution was tested only with 30 mM $[Ca^{2+}]_o$).

Figure 25 shows original recordings from one of five experiments in which 30 mM $[Ca^{2+}]_o$ was tested and tonicity was maintained by cholineCl. Neither inward nor outward TI was seen at the test potential of -25 mV (arrow). Inward TI was seen at all testing potentials negative to -25 mV. Peak inward TI monotonically declined as the test potential became less negative. However, there was no apparant shift of the time to peak TI. We also noticed that the peak outward TI increased to maximum as the test potential became more positive, and then decreased in amplitude. Nevertheless, the TI remained coupled to the step repolarization and the time to peak outward TI only shortened slightly with the least negative steps. In another 3 preparations we observed that when tonicity was maintained by LiCl and the $[Ca^{2+}]_o$ was 30 mM, E_{REV} occurred at -21 mV and there was no apparant shift of the time to peak TI (data not shown). Since Li^+ is not an inert substitute for Na^+, we did not pursue further studies with this substitute. However, experiments with Li^+ further confirmed that both inward and outward TI generated under our experimental conditions did not involve Na^+-Ca^{2+} exchange (Baker, 1972).

Since both inward and outward TI were induced by elevating $[Ca^{2+}]_o$ in the absence of $[Na^+]_o$, a question that should be addressed is whether both currents are activated by the oscillatory changes of $[Ca^{2+}]_i$. Ryanodine is known to block Ca^{2+} release from the sarcoplasmic reticulum, thereby abolishing the $[Ca^{2+}]_i$ transient. Figure 26 shows the effects of ryanodine on TI induced by 30 mM $CaCl_2$ with cholineCl as the NaCl substitute. After 5 min exposure to 1 μM ryanodine both inward and outward TI were totally abolished. Beat by beat decrease in the amplitudes of both inward and outward TI was seen before TI was completely abolished. Identical observations were made in 4 preparations. These results suggest that both

inward and outward TI are activated by an oscillatory release of $[Ca^{2+}]_i$ from the SR.

The time to peak TI was virtually constant near the reversal potential. Thus a distinct reversal potential could be demonstrated. The induction of TI was reproducible and stable, and this made it possible to study the I-V relation of TI in detail.

Figure 27 shows the I-V relations of TI induced by 5, 10, 20, and 30 mM $[Ca^{2+}]_o$. As already indicated, no outward TI was induced by 5 mM $[Ca^{2+}]_o$ (open circles). When $[Ca^{2+}]_o$ was elevated to 10 mM, inward TI declined to zero at -19.5 mV (filled circles), and reversed when the test potential was made more positive. When $[Ca^{2+}]_o$ was elevated to 20 mM, the peak inward and outward TI were both increased (especially the outward TI). The reversal potential shifted only slightly in the negative direction (open triangles). A larger shift in $E_{RI:V}$ was seen when $[Ca^{2+}]_o$ was increased to 30 mM. In the presence of 30 mM $[Ca^{2+}]_o$, $E_{RI:V}$ was -25 mV (filled triangles).

A further shift of $E_{RI:V}$ in the negative direction in response to the elevation of $[Ca^{2+}]_o$ was observed when $[Ca^{2+}]_o$ was elevated to 50 and 105 mM. Although we showed that high $[Ca^{2+}]_o$, beyond 30 mM, depressed recovery of action potential configuration of Purkinje fibres, short exposures might not seriously damage the fundamental characteristics of the TI conducting channels (Cannell & Lederer, 1986). Representative results are shown in figure 28. Panel A shows original recordings from one of three experiments in which 50 mM $[Ca^{2+}]_o$ was used and stable impalement was maintained. $E_{RI:V}$ was shifted even more negative, compared to 30 mM $[Ca^{2+}]_o$ (figure 27). The average of $E_{RI:V}$ from 3 experiments (5 repeated runs) was -28.5 ± 2.5 mV (mean $\pm$ SD). Panel B shows the original recordings obtained in one experiment in which isotonic Ca^{2+} (105 mM) was applied. The preparation was clamped at -55 mV and repolarized to different test potentials between -55 and +5 mV in 10 mV steps following a 200 ms depolarization to +15 mV. The reversal

potential occurred between -35 and -45 mV in three repeated runs. The shift with sequential runs might represent inability to reach equilibrium. The I-V relations for 50 (open circles) and 105 mM Ca^{2+} (filled circles) are shown in panel C.

4. Effect of Ionic Substitution on the Reversal Potential of TI

The behaviour of the $E_{RI,V}$ shift of TI can not be easily explained by a non-selective "cationic" conductance change, because elevation of Ca^{2+} concentration would be expected to shift $E_{RI,V}$ to more positive potentials. In figure 29, $E_{RI,V}$ is plotted as a function of $[Ca^{2+}]_o$. Even if one overlooks the $E_{RI,V}$ obtained in the single experiment with isotonic (105 mM) $[Ca^{2+}]_o$, there was still a 9 mV shift in the negative direction with elevation of $[Ca^{2+}]_o$ from 10 to 50 mM. Statistically, this shift was also significant ($p < 0.05$). This shift could not be explained by Na^+-Ca^{2+} exchange because 1) Na^+-Ca^{2+} exchange is not activated in the absence of $[Na^+]_o$ (Cannell & Lederer, 1986; Sheu & Blaustein, 1991); 2) the presence of a clear-cut reversal potential is not predicted for Na^+-Ca^{2+} exchange (Mullins, 1981; Eisner & Lederer, 1985; DiFrancesco & Noble, 1985); 3) Li^+ cannot substitute for Na^+ in Na^+-Ca^{2+} exchange and yet there was still an $E_{RI,V}$ for TI induced by Li^+-containing high Ca^{2+} solution (Baker, 1972; Hume et al, 1991).

To explain the negative shift of $E_{RI,V}$ with elevation of $[Ca^{2+}]_o$, it was necessary to determine: 1) which ion(s) was responsible for the inward TI and 2) which ion(s) was the major charge carrier for the outward TI. Under our experimental conditions two cations, Ca^{2+} and choline$^+$, might carry the inward TI. Evidence that choline$^+$ did not contribute to the generation of inward TI is shown in figure 30, which illustrates typical observations from 6 experiments in which the concentration of $[Ca^{2+}]_o$ was fixed at 10 (panel A) or 30 (panel B) mM and the tonicity was maintained by either cholineCl or sucrose. As shown in panel A, the peak inward TI did

not change when the perfusate was switched from cholineCl (trace b) to sucrose containing solutions (trace c). Similarly, no significant alteration could be seen when changing solution from sucrose (panel B, trace b) to cholineCl substitution (trace c). However, we consistently observed a significant decrease of outward current during the depolarization step with sucrose as the substitute, no matter which solution was superperfused first. These results demonstrated that the inward TI was not carried by choline$^+$, and was most likely carried by Ca^{2+}.

There were also two ions which might be responsible for the outward TI: intracellular K^+ and extracellular Cl^-. Indeed, either an efflux of K^+ or an influx of Cl^- or both could theoretically generate the outward TI at the repolarization potentials tested in this study. To differentiate between these possibilities, we fixed the $CaCl_2$ concentration at 30 mM and the tonicity was maintained by adjusting the concentrations of cholineCl or sucrose or both. In each case extracellular K^+ was zero. If the major outward TI was from the outflow of K^+, one would expect no apparent shift of E_{REV} when switching between these solutions because the K^+ gradient was not changed.

In six experiments the preparations were initially exposed to sucrose. A small outward TI was consistantly observed when the membrane was repolarized positive to +5 mV from a previous step to +25 mV. When the solution was changed to cholineCl with no sucrose, E_{REV} shifted to -25 mV (as shown in figure 27). Furthermore, if the osmotic pressure was maintained by half cholineCl (52.5 mM) and half sucrose (105 mM), outward TI was found at all testing potentials positive to -12.5 mV. The complete I-V relations seen with changes of cholineCl concentration are shown in the panel A of figure 31. When we plotted the mean values of E_{REV} vs corresponding log $[Cl^-]_o$ (panel B, open circle), we found that the shift in E_{REV} was compatible with the shift expected if Cl^- was an important charge carrier for TI.

Assuming the intracellular Cl^- concentration is 30 mM (Vaughan Williams, 1990), the slope of the relation between E_{REV} and $[Cl^-]_o$ calculated by Nernst equation for the $[Cl^-]_o$ tested (filled circle) is indeed very close to the measured value. For each 10 fold change in $[Cl^-]_o$, the shifts in E_{REV} were 62 mV according to the Nernst equation (filled circles), and 67 mV as calculated from the curve of measured data (open circles). At all $[Cl^-]_o$ E_{REV} was shifted by a constant value in the positive direction from that predicted by the Nernst equation. This shift is compatible with a constant contribution from a conductance with a positive E_{REV}, such as Ca^{2+}.

5. Effects of DIDS and SITS on TI

If the outward TI is carried by Cl^-, it should be inhibited by agents that inhibit anionic currents. Recently some stilbene derivatives have been used to block the Cl^- conductance in cardiac myocytes (Bahinski, Nairn, Greengard & Gadsby, 1989; Matsoka, Ehara & Noma, 1990; Zygmunt & Gibbons, 1991). Two common anion transporter blockers, DIDS and SITS, are among these derivatives. Figure 32 shows the effect of DIDS on TI induced by 30 mM $[Ca^{2+}]_o$ and 105 mM cholineCl. After 4 min exposure to 10 μM DIDS, peak outward TI was virtually abolished while peak inward TI was just slightly decreased. This effect easily reversed following 5 min perfusion with DIDS-free high $[Ca^{2+}]_o$ solution. Eight identical observations were made in 4 preparations. Compared to the pre-drug control, the inhibition was 85.5 $\pm$ 4.7% for outward TI and 10.5 $\pm$ 2.5% for inward TI (mean $\pm$ SEM). Similar effects also were observed with 0.1 to 0.2 mM SITS in two other Purkinje fibres when 30 mM $[Ca^{2+}]_o$ and 105 mM NMG chloride were used to induce TI (data not shown). These results provide further evidence indicating that outward TI is indeed largely a Cl^- current. The results also suggest that a small portion of inward TI may be generated by an efflux of Cl^- at potentials negative to E_{Cl}.

Figure 22. Induction of TI by 5 mM $[Ca^{2+}]_o$ in the absence of $[Na^+]_o$ (NaCl was substituted with choline chloride). Current traces are separated (by 6.2 nA) for clear view. Voltage steps used for generation of TI are shown at the top. Inward TI was seen at the test potential of -55 and disappeared at test potentials positive to -35 mV. At test potentials between -25 and -5 mV, neither inward nor outward TI could be seen.

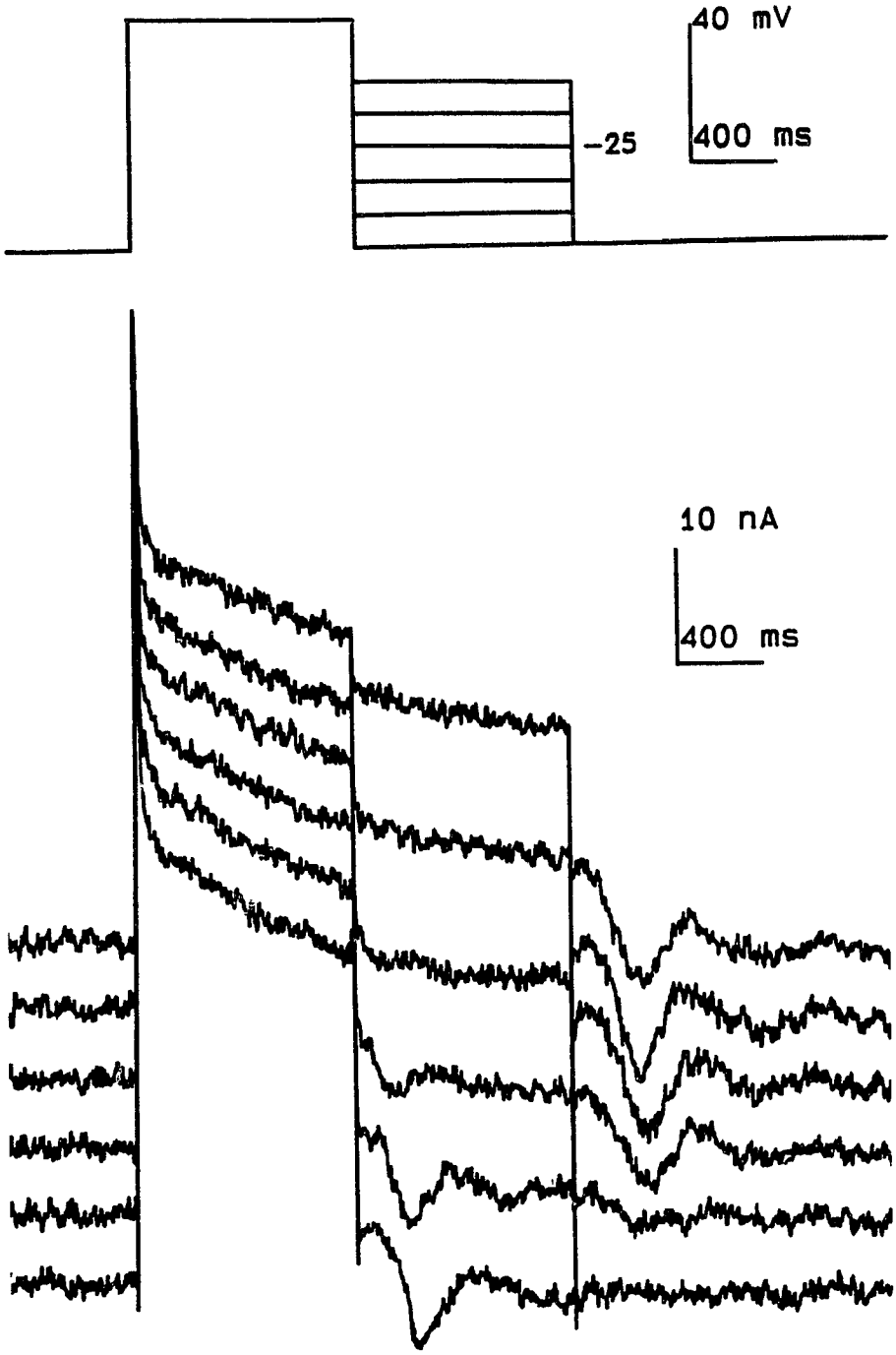


Figure 22.

Figure 23. Effect of changing $[Ca^{2+}]_o$ on induction of TI in the absence of $[Na^+]_o$. The voltage clamp steps applied to the preparation are illustrated schematically at the top. **Panel A:** Current traces were recorded during perfusion with a) normal Tyrode's solution, b) modified solution with 5 mM $CaCl_2$ and 140 mM CholineCl, c) modified solution with 15 mM $CaCl_2$ and 125 mM CholineCl. Perfusion with 15 mM Ca^{2+} increased TI amplitude. **Panel B:** Elevation of $[Ca^{2+}]_o$ from 30 to 50 mM resulted in a slight decrease of peak TI. Traces were obtained from a different preparation from those in panel A. Traces were recorded during perfusion with a) modified solution with 30 mM $CaCl_2$ 30 and 102.5 mM CholineCl, b) modified solution with 50 mM $CaCl_2$ and 72.5 mM CholineCl.

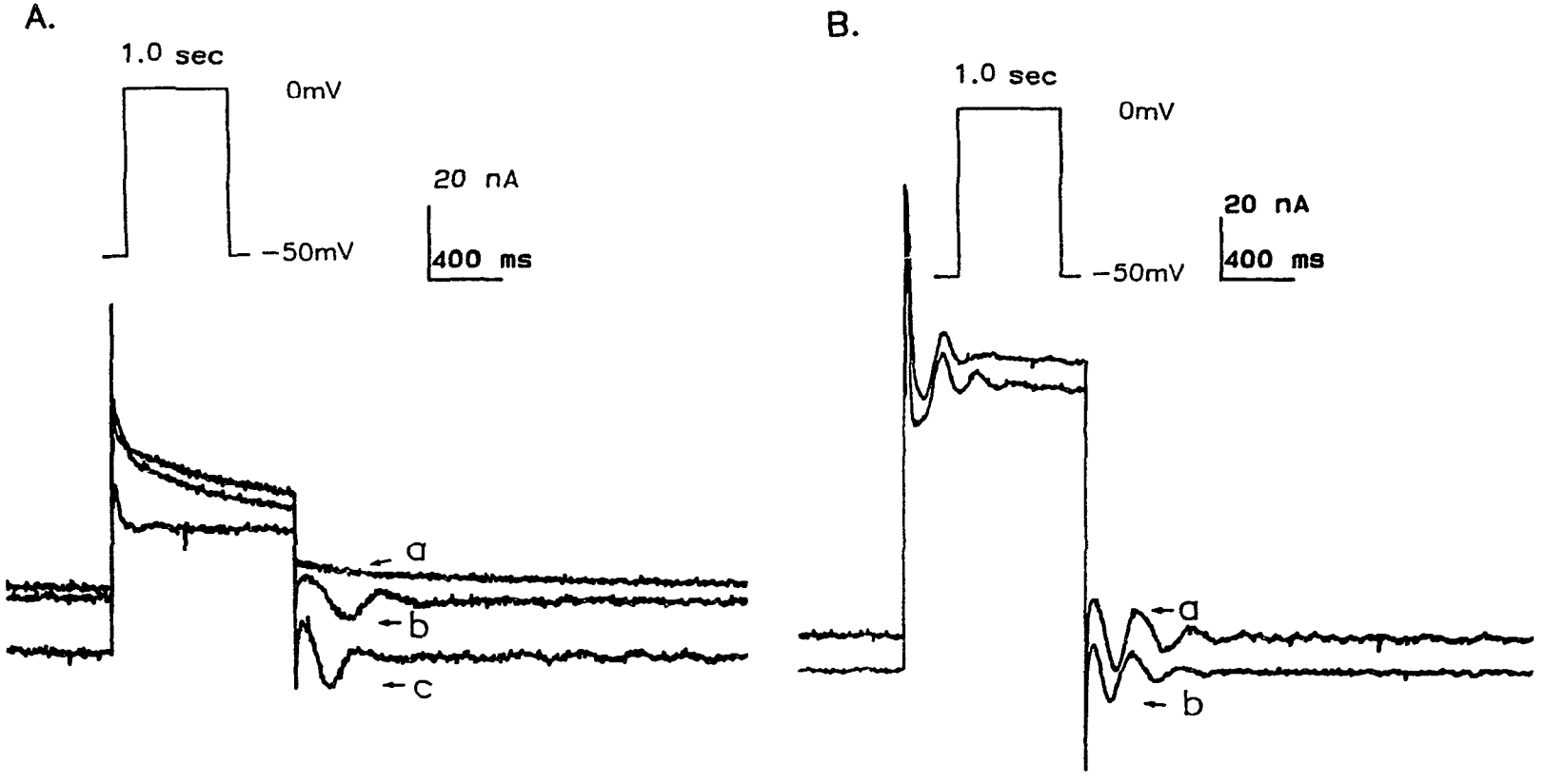


Figure 23.

Figure 24. Absence of TI with Ba^{2+} substitution or low $[Ca^{2+}]_o$. The voltage step applied to the preparation is illustrated schematically at the top. **Panel A:** Current tracings recorded a) during perfusion with normal Tyrode's solution, b) 30 sec after perfusing with solution containing (mM) $BaCl_2$ 30, Choline Cl 102.5, HEPES 10, Tris base 5, glucose 10, and c) 2 min after perfusing with the same solution. TI transiently appeared with the onset of solution change and quickly disappeared with continued exposure. With Ba^{2+} as the Ca^{2+} substitute, there also was a significant increase of I_{Ca}, and a dramatic suppression of outward current at the end of the depolarization pulse. **Panel B:** a) control recording of current in normal Tyrode's solution; b) recordings made in the presence of modified solution containing (mM): $CaCl_2$ 2.5, CholineCl 145. There was a significant increase in outward current during the depolarizing pulse, but no TI occurred upon repolarizing to the holding potential.

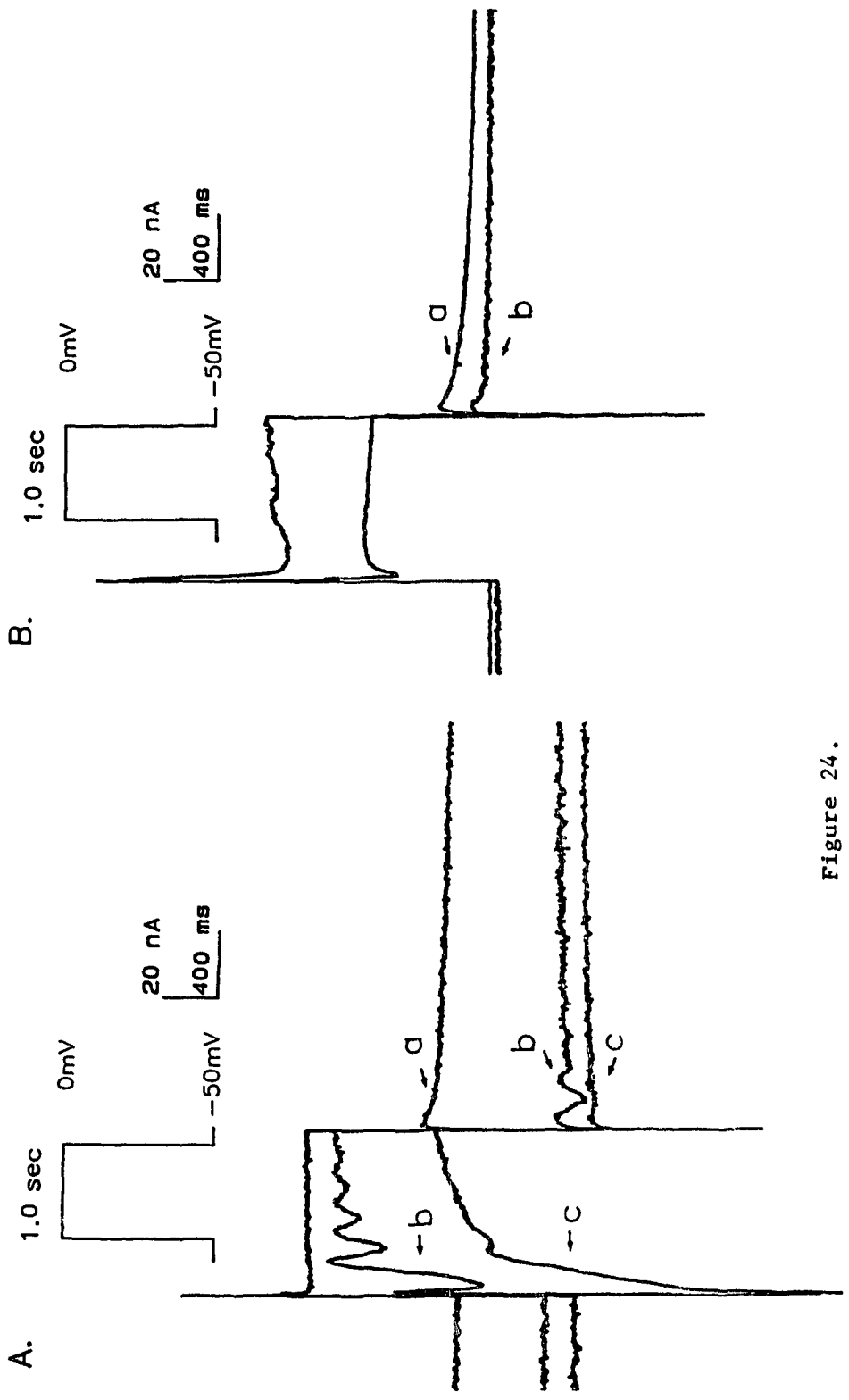


Figure 24.

Figure 25. Occurrence of a distinct reversal potential for TI induced by 30 mM $[Ca^{2+}]_o$ in the absence of $[Na^+]_o$ (NaCl was substituted with choline chloride). Test potentials applied to the preparation are shown on the bottom. The holding potential was -55 mV. Current trace corresponding to a test potential of -25 mV (arrow) showed neither inward nor outward deflection, but a large outward TI was seen at all test potentials positive to -25 mV. Also, when TI reversed its direction, there was little or no apparent shift of time to peak TI (inward and outward).

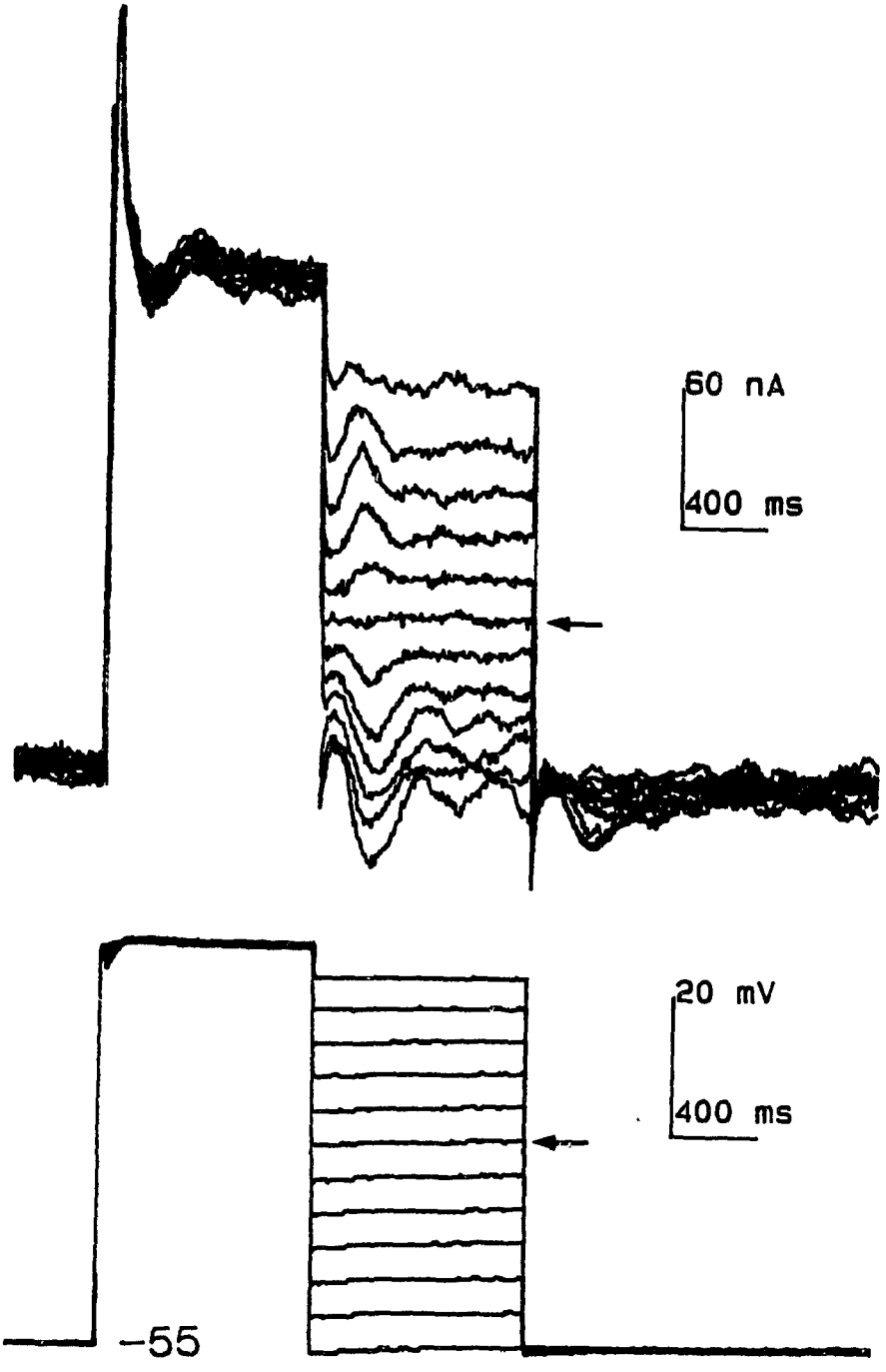


Figure 25.

Figure 26. Effects of ryanodine on TI induced by elevation of $[Ca^{2+}]_o$. Solutions used for the induction of TI were described in the text. Holding potential was -55 mV. Panel A: inward and outward TI induced in the absence of ryanodine. Panel B: both inward and outward TI were abolished by exposure to 1 μM ryanodine for 5 min.

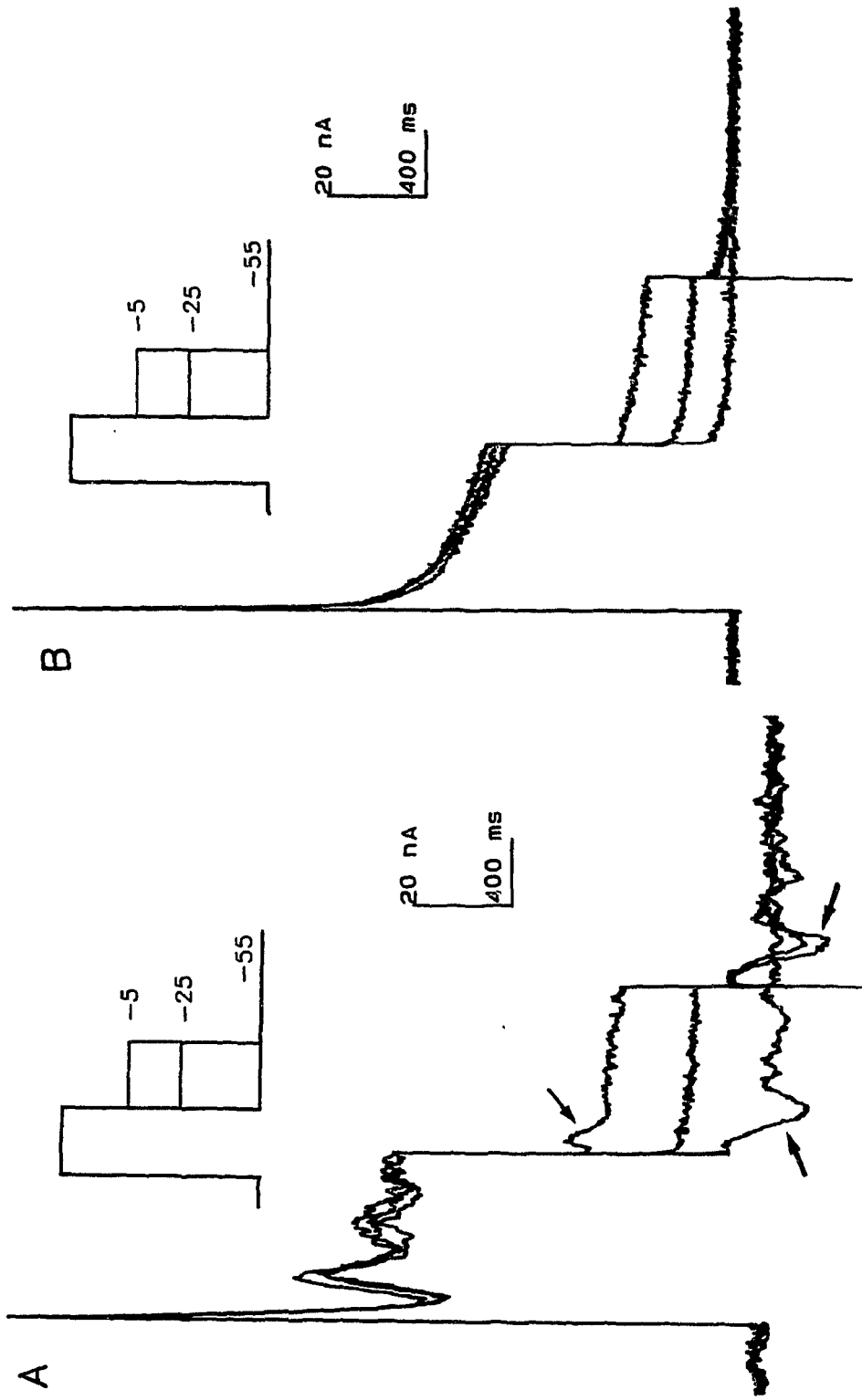


Figure 26.

Figure 27. Effects of $[Ca^{2+}]_o$ on the I-V relations of TI. Holding potential was -55 mV. Peak TI was measured upon repolarization to various test potentials after depolarizing to +15 mV. E_{REV} was not seen with 5 mM $[Ca^{2+}]_o$. However, TI reversed from inward to outward at -15 mV in 10, 20, and 30 mM $[Ca^{2+}]_o$. Peak inward and outward TI increased with increasing $[Ca^{2+}]_o$.

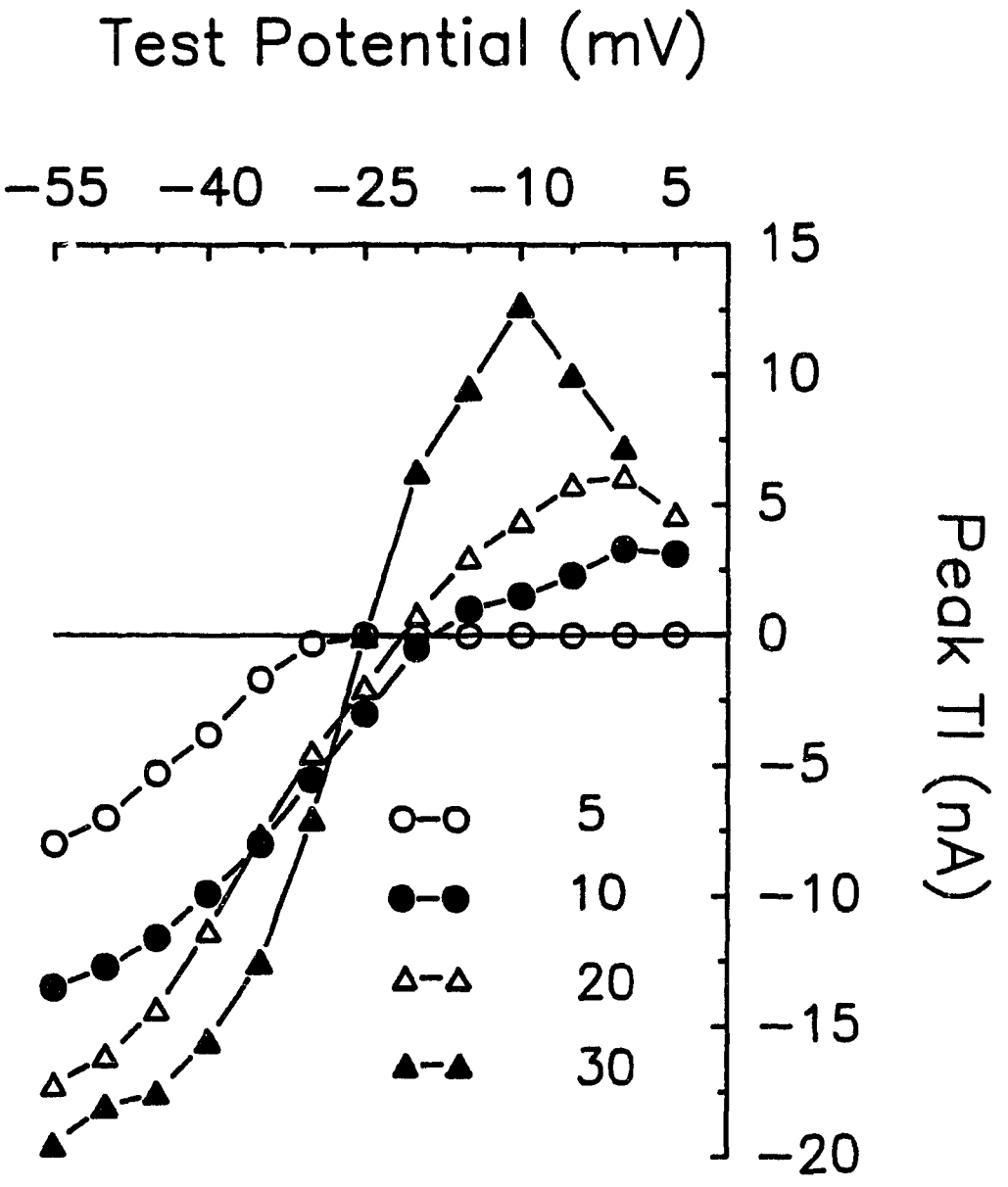

Test Potential (mV)
−55 −40 −25 −10 5
15
10
5
0
−5
−10
−15
−20
Peak TI (nA)
5
10
20
30

Figure 27.

Figure 28. Effects of 50 and 105 mM (isotonic calcium) $[Ca^{2+}]_o$ on the I-V relations of TI. **Panel A:** current traces recorded in the presence of 50 mM $[Ca^{2+}]_o$. **Panel B:** current traces recorded in the presence of 105 mM $[Ca^{2+}]_o$. **Panel C:** I-V relations of TI induced by 50 (open circles) and 105 mM $[Ca^{2+}]_o$ (filled circles). E_{REV} was about -29 mV in the presence of 50 mM $[Ca^{2+}]_o$, which was more negative than seen at lower $[Ca^{2+}]_o$ (compare with figure 4, 6). The reversal potential resolved from the isotonic calcium experiments was about -40 mV. Induction of TI was only successful in 1 out of 15 experiments because of severe uncoupling of cells.

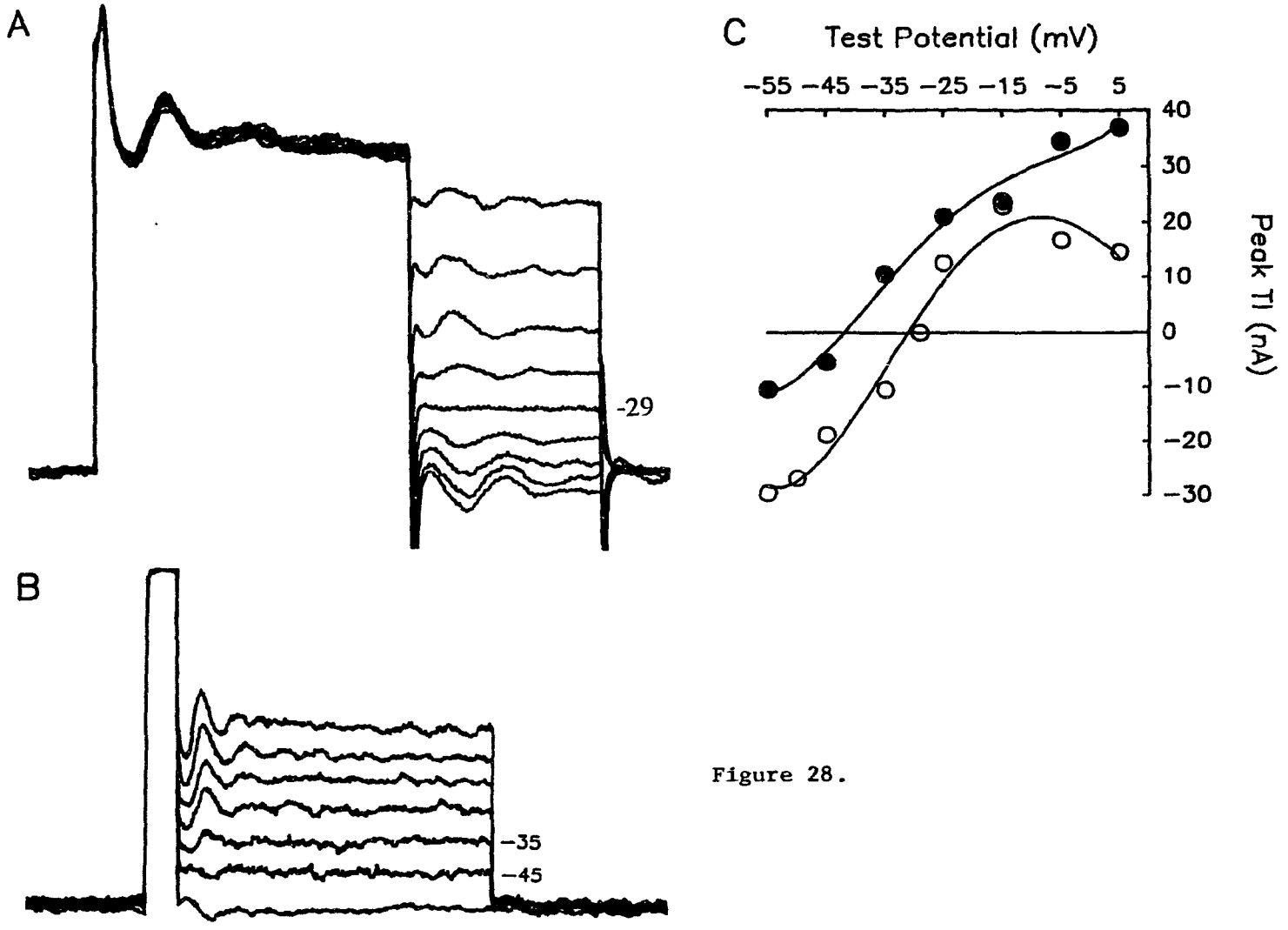

A
-29
B
-35
-45
C
Test Potential (mV)
-55 -45 -35 -25 -15 -5 5
40
30
20
10
0
-10
-20
-30
Peak TI (nA)

Figure 28.

Figure 29: Effect of elevating $[Ca^{2+}]_O$ on E_{REV}. Tonicity was maintained by adjusting the concentrations of cholineCl. If Ca^{2+} serves as a major charge carrier of the TI, E_{REV} would be expected to shift in the positive direction with elevation of $[Ca^{2+}]_O$. However, E_{REV} was consistently shifted in the negative direction. n = number of preparations; R = number of repeated runs. Vertical bars represent standard errors. E_{REV} at 50 mM $[Ca^{2+}]_O$ was statistically different from that at 10 mM $[Ca_{2+}]_O$ ($p < 0.05$).

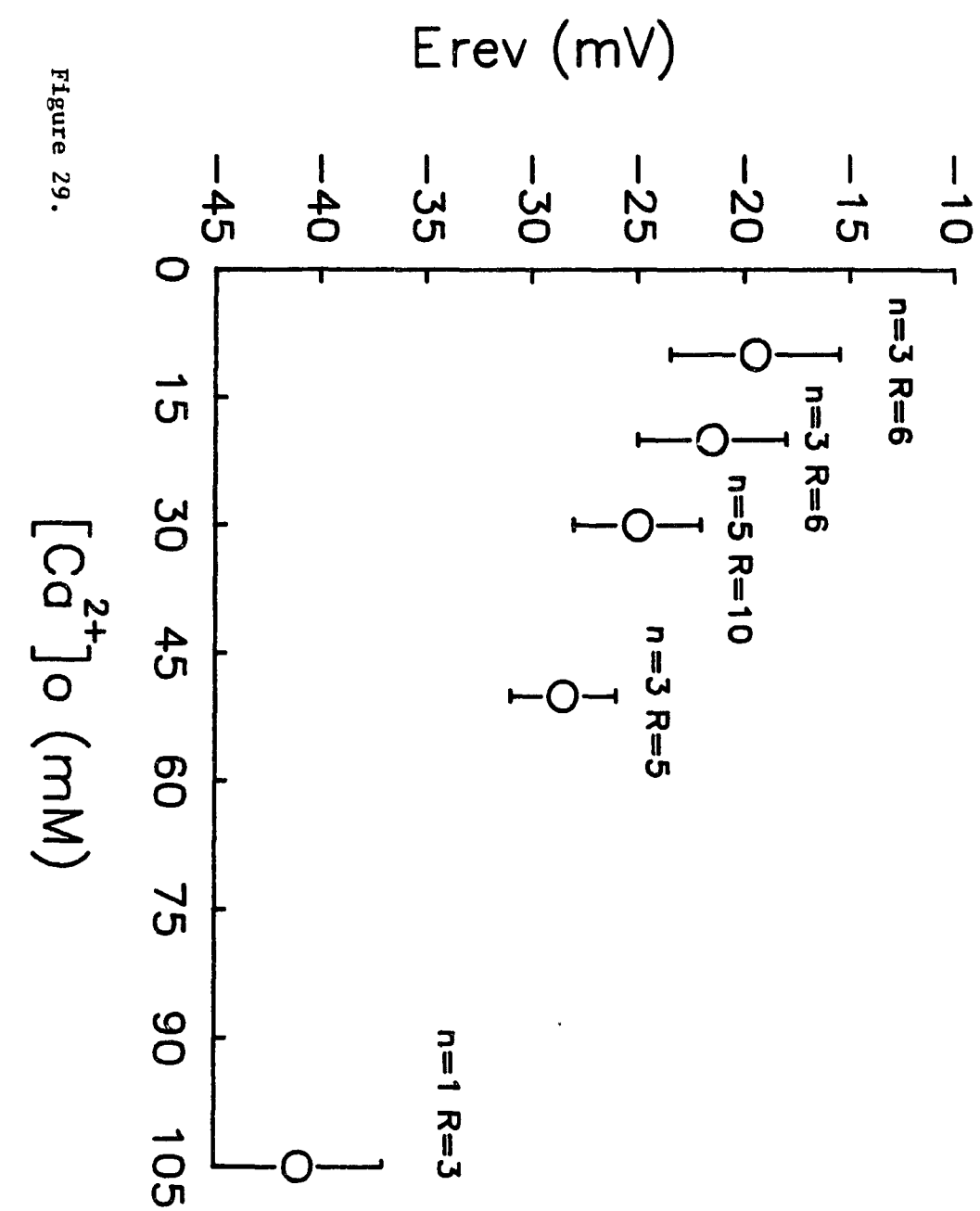

Erev (mV)
−10
−15
−20
−25
−30
−35
−40
−45
0
15
30
45
60
75
90
105
[Ca2+]o (mM)
n=3 R=6
n=3 R=6
n=5 R=10
n=3 R=5
n=1 R=3

Figure 29.

Figure 30. Effects of switching between CholineCl and sucrose as the sodium substitute on the generation of inward TI. The voltage step applied to the preparation is shown at the bottom of each panel. The holding potential was -55 mV. **Panel A:** The current trace recorded in normal Tyrode's solution was labelled a). When TI was induced by 10 mM $[Ca^{2+}]_o$, changing the superfusate from cholineCl (132.5 mM, trace b) to sucrose (265 mM, trace c) containing solution did not alter the magnitude of the peak TI. **Panel B:** The current trace recorded in normal Tyrode's solution was labelled a). When TI was induced by 30 mM $[Ca^{2+}]_o$, switching the superfusate from sucrose (205 mM, trace b) to cholineCl (102.5 mM, trace c) did not produce any significant change of peak TI.

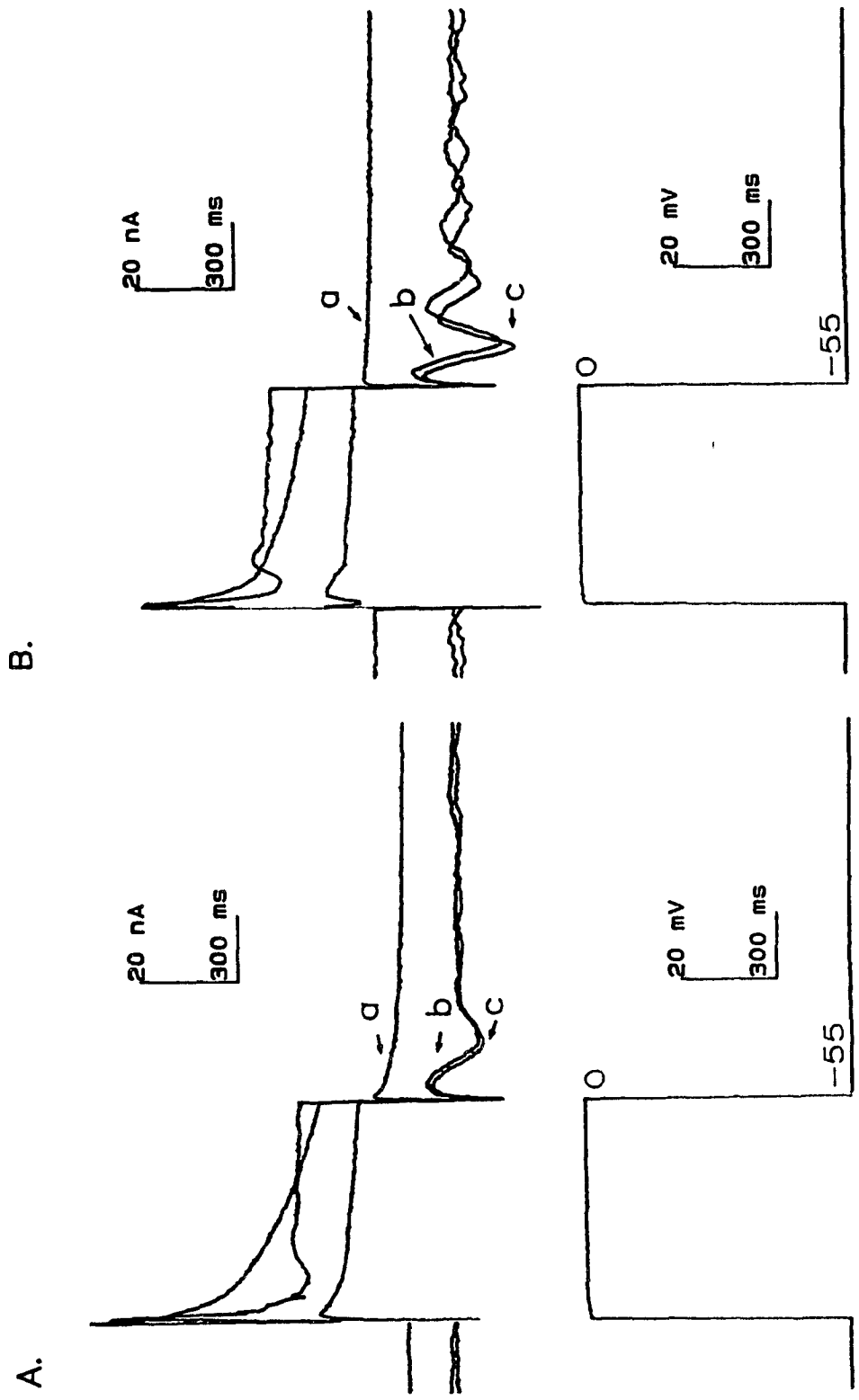


Figure 30.

Figure 31. Panel A: Effect of changing $[Cl^-]_o$ on the I-V relation of TI induced by 30 mM $[Ca^{2+}]_o$. Open circle = 0 mM CholineCl; Filled circle = 52.5 mM CholineCl + 100 mM sucrose; Open triangle = 105 mM CholineCl. With the decrease in cholineCl concentration, the reversal potential of TI was significantly shifted in the positive direction. **Panel B:** Effect of changing $[Cl^-]_o$ on E_{RLV}. Open circle shows measured data. Filled circles show values predicted by the Nernst equation. The slopes indicate that for each 10-fold changes in $[Cl^-]_o$, the shifts of E_{RLV} calculated from our experiments were 67 mV, which is very close to the value predicated by the Nernst equation (62 mV).

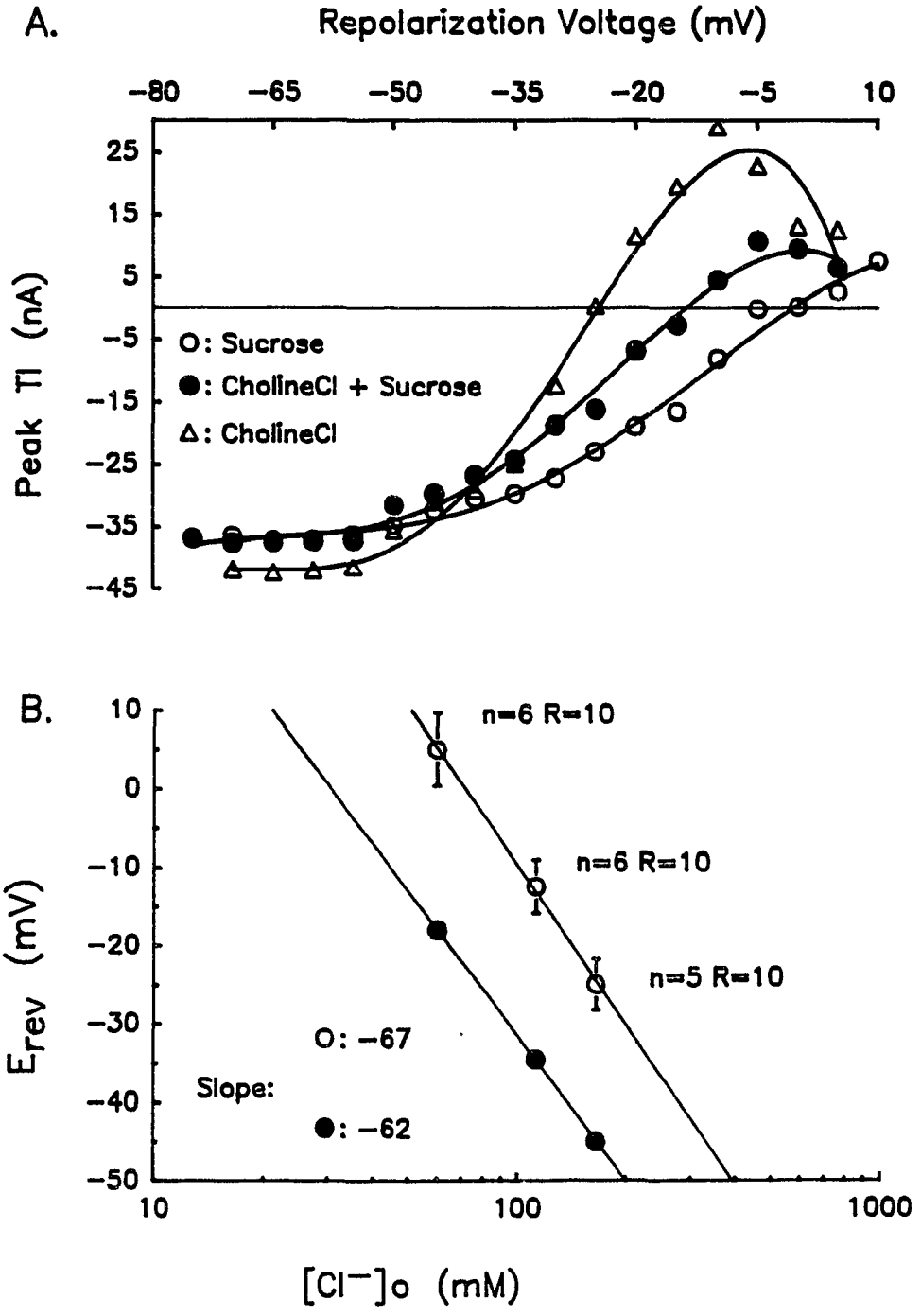

A.
Repolarization Voltage (mV)
-80
-65
-50
-35
-20
-5
10
25
15
5
-5
-15
-25
-35
-45
Peak Tl (nA)
O: Sucrose
●: CholineCl + Sucrose
△: CholineCl
B.
10
0
-10
-20
-30
-40
-50
E_{rev} (mV)
n=6 R=10
n=6 R=10
n=5 R=10
O: -67
Slope:
●: -62
10
100
1000
$[Cl^-]o$ (mM)

Figure 31.

Figure 32. Effect of DIDS on TI induced by a combination of 30 mM $[Ca^{2+}]_o$ and 105 mM cholineCl. **Panel A:** Voltage steps applied to the preparation. **Panel B:** Current traces showing inward and outward TI in the absence of DIDS (a), 4 min after addition of 10 μM DIDS (b), and 5 min washout in DIDS-free high Ca^{2+} solution (c). After 4 min exposure to 10 μM DIDS, peak outward TI was dramatically decreased (arrow) while inward TI was just slightly inhibited. Outward TI recovered following 5 min washout with DIDS-free high Ca^{2+} solution. The outward current activated upon depolarization also was suppressed by DIDS.

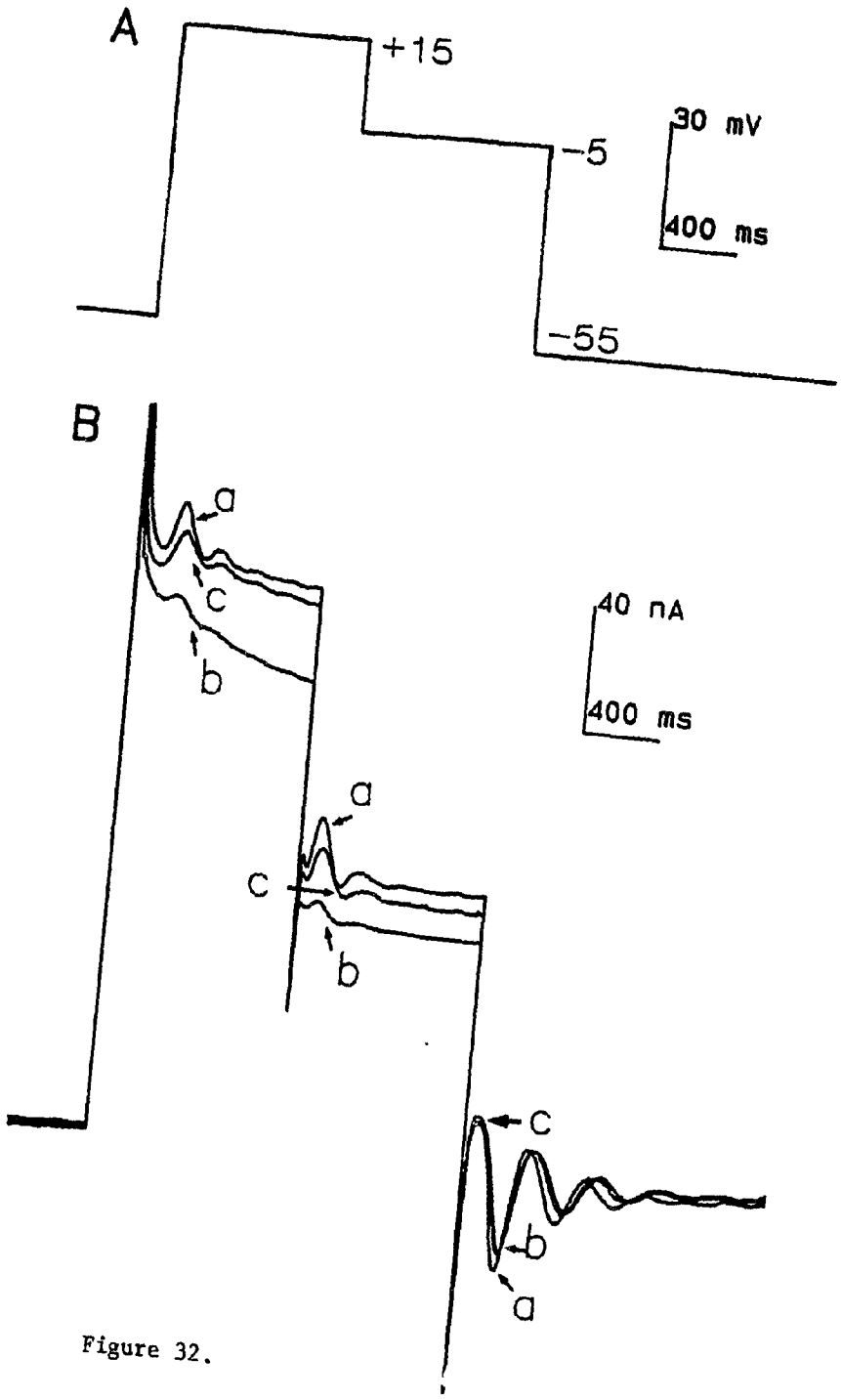


Figure 32.

V. IONIC MECHANISMS OF HIGH $[Ca^{2+}]_o$ INDUCED TI IN THE PRESENCE OF $[Na^+]_o$

1. TI Induced by High $[Ca^{2+}]_o$ in the Presence of $[Na^+]_o$

Induction of TI by high Ca^{2+} in the presence of Na^+ has been previously reported (Kass et al, 1978a). However, the ionic mechanisms of this high-Ca^{2+} induced TI have not been studied extensively. It seems possible that the mechanisms leading to generation of TI by high-Ca^{2+} may be different from digitalis, since the amplitudes of the two types of TI and OAP are affected by catecholamines in opposite directions (Ferrier & Carmeliet, 1990; Han & Ferrier, 1990). Figure 33 shows representative recordings from one of six Purkinje fibres in which TI was induced by a combination of 30 mM Ca^{2+} and 105 mM Na^+. $[K^+]_o$ was zero for all experiments in the absence of $[Na^+]_o$. The voltage clamp protocol for determining the I-V relation of the TI is illustrated at the top of the figure. Following repolarization to the holding potential of -55 mV, an inward TI developed and peaked about 250 ms after the repolarization step (arrow). The time to peak inward TI shortened as the test potential was made less negative. Meanwhile, the peak inward TI also decreased until no obvious inward deflection could be seen when the potential upon repolarization was -20 mV. We consistently detected an outward TI at all test potentials positive to -20 mV. The voltage-dependent shift of time to peak inward TI has been reported previously (Lederer & Tsien, 1976; Kass et al, 1978; Brown et al, 1986). As shown in this figure, the shift in time to peak outward TI was not apparent. The relatively constant time to peak outward TI made the reversal potential (E_{REV}) unambiguous.

Figure 34 shows a plot of the I-V relation from the experiment illustrated by figure 33. E_{REV} occurred at approximately -20 mV. The average value of E_{REV} calculated from 6 experiments was 20.5 $\pm$ 2.5 mV (Mean $\pm$ SD). The existence of

a clear-cut reversal potential argues strongly for a channel mechanism for TI generation, but does not eliminate the possibility, in the presence of $[Na^+]_o$, that Na^+-Ca^{2+} exchange might also contribute to TI generation.

2. Effects of Ni^{2+} on High Ca^{2+} Induced TI in the Presence of $[Na^+]_o$

Na^+-Ca^{2+} exchange might contribute to generation of TI in two ways: Ca^{2+} loading during depolarization and Ca^{2+} extrusion following repolarization (Beuckelmann & Wier, 1989; Wier, 1990; Han & Ferrier, 1991). The latter also generates an inwardly directed current which might cause or contribute to the TI. If TI is generated by a single carrier, agents known to block the exchanger should inhibit both inward and outward TI. Presently, there is no Na^+-Ca^{2+} exchange blocker available that is specific only for the exchanger (Giles & Shimoni, 1989b). Ni^{2+} successfully blocks the current attributable to Na^+-Ca^{2+} exchange (Kimura, Miyamae & Noma, 1987; Beuckelmann & Wier, 1989). We therefore determined the effects of Ni^{2+} on high-Ca^{2+} induced TI in our rabbit Purkinje preparations.

Figure 35 shows representative traces from one of five experiments in which 30 mM Ca^{2+} and 105 mM Na^+ were used to induce TI. The holding potential was -55 mV. Following depolarization to +20 mV for 1 sec, two steps of repolarization were made to 0 mV and -55 mV, respectively. An outward TI occurred upon repolarization to 0 mV, and an inward TI at -55 mV (panel A). Three minutes after adding 2.5 mM $NiCl_2$ to the above solution, the amplitude of inward TI was significantly decreased while the peak outward TI increased by about 50% (panel B). Complete recovery of both inward and outward TI was observed in all five preparations following 10 minutes of superfusion with Ni^{2+}-free high-Ca^{2+} containing solution.

A more detailed analysis of the effects of Ni^{2+} on TI and its I-V relation is shown in figure 36. The amplitude of TI became constant at potentials negative to -55 mV,

therefore, current traces corresponding to these potentials have been omitted for clarity. The voltage steps applied to the Purkinje fibre are shown schematically at the bottom of panel C. The outward currents activated upon the initial depolarization are not central to the issue of this paper and are not illustrated. Three minutes after addition of 2.5 mM Ni^{2+}, strong inhibition of inward TI was seen at test potentials negative to E_{REV} (arrow). Significant increase in peak amplitudes of outward TI was seen at test potentials positive to E_{REV}. The average inhibition for inward TI at a test potential of -55 mV was 57 $\pm$ 3% (n=5, $p < 0.01$). The average increase for outward TI at a test potential of 0 mV was 85 $\pm$ 9% (n=5, $p < 0.01$). The slight shift in reversal potential (about -3 mV) in response to Ni^{2+} treatment was within the range of experimental error.

The effects of Ni^{2+} strongly suggest that multiple components may be involved in generation of TI, because neither the non-selective cationic channel nor the Na^{+}-Ca^{2+} exchange can explain the opposite effects of Ni^{2+}. However, if the outward TI is carried by an additional mechanism, the inhibitory effect of Ni^{2+} on inward TI can still be interpreted as inhibition of either a non-selective cationic channel conductance (Kass et al, 1978b; Ehara, Noma & Ono, 1988; Hill, Coronado & Struss, 1988) or Na^{+}-Ca^{2+} exchange (Kass et al, 1978b; Brown et al, 1986; Kimura et al,1987), or both.

To assess whether the Na^{+}-Ca^{2+} exchange current contributes to the inward TI, we initially attempted to induce the inward tail current attributed to the exchanger ($I_{i,\backslash}$) by other investigators (Fedida, Noble, Shimoni & Spindler, 1987b; Giles & Shimoni,1989a; Egan et al, 1989). In five Purkinje fibres, we were unable to generate the inward tail current under our experimental conditions. However, when we omitted the K^{+} from the Na^{+}-containing high Ca^{2+} solution, inward TI disappeared and an inward tail current was easily induced upon repolarization. Results

illustrated in figure 37 are typical of observations in 5 Purkinje fibres. After omission of K^+, the peak inward TI initially increased significantly (panel C), then decreased as inward tail current developed (panel D). Approximately 5 min after 0 K^+ perfusion, only a large tail current was seen following repolarization (panel E). Once K^+ was re-introduced, the inward tail current quickly disappeared and the inward TI was partially restored (panel F).

That the inward tail current induced in 0 $[K^+]_o$ solution was also very sensitive to Ni^{2+} is illustrated in figure 38. Here the tail current (large arrow) was induced by the same treatment as stated in figure 37, except with a shorter activation pulse (400 ms). Two min following addition of 2.5 mM $NiCl_2$ to the solution, the inward tail current was totally abolished (small arrow).

Results shown in figure 37 and 38 suggest that while Ni^{2+} has the ability to abolish the Na^+-Ca^{2+} exchange current, this inward tail current is not normally generated under our experimental conditions (4 mM K^+) in cardiac Purkinje fibres. Therefore, the inhibitory effect of Ni^{2+} on inward TI might be mediated by a direct inhibition on the non-selective cationic channel.

The absence of $[Na^+]_o$ is known to minimize or abolish the influence of Na^+-Ca^{2+} exchange (Cannell & Lederer, 1986; Kimura et al, 1987), thus leaving only the channel mechanism operative. To test this, we studied the effect of Ni^{2+} on TI induced by high Ca^{2+} in the absence of $[Na^+]_o$ (to eliminate the Na^+-Ca^{2+} exchange mechanism) in the following experiments.

3. Effects of Ni^{2+} on TI Induced in the Absence of $[Na^+]_o$

The following experiments were performed in the absence of extracellular Na^+, with NMG chloride, choline chloride or sucrose as the substitute for NaCl. Figure 39 illustrates a representative example of the effects of 2.5 mM Ni^{2+} observed in four

preparations. Voltage steps applied to the preparation are shown at the bottom of the figure. The solution used to induce TI contained (mM): $CaCl_2$ 30, NMG chloride 105, KCl 0, $MgCl_2$ 0.5, glucose 10, tris base 5, HEPES 10, and was gassed with 100% O_2 (pH = 7.4 at 37°C). An outward TI was seen at a test potential of 0 mV and an inward TI at -55 mV (panel A). The inward TI was decreased markedly but the outward TI was increased by about 60% three min after addition of 2.5 mM Ni^{2+} to the Ca^{2+}-rich Na^+-free solution (panel B).

The qualitatively similar effects of Ni^{2+} on TI induced by high Ca^{2+} in the absence and presence of $[Na^+]_o$ initially led us to consider the possibility of NMG^+-Ca^{2+} exchange similar to that reported by Mullin (1981). Therefore in 3 preparations we determined the effects of Ni^{2+} on TI induced by a combination of 30 mM $CaCl_2$ and 210 mM sucrose. The other components of the solution were the same as described for figure 39 except for the absence of NMG chloride. Sucrose is electrically neutral and should not contribute to the net charge movement through the exchanger (Arlock & Katzung, 1985). Recordings from a representative experiment are shown in figure 40. Substitution of NaCl with sucrose also greatly reduces the Cl^- concentration in the superfusates. As shown earlier, Cl^- is the major charge carrier for outward TI. Therefore, outward TI was not seen at a test potential of -5 mV but prominent inward TI was always seen following repolarization to -55 mV (large arrow). Three minutes after adding 5 mM Ni^{2+} to the solution, inward TI was totally abolished (small arrow). Thus, the results shown in figure 39 and 40 strongly suggest that the inhibitory effects of Ni^{2+} on inward TI may be mediated by the inhibition on the non-selective cationic channel.

4. Effects of Cd^{2+} and Mn^{2+} on TI Induced by High $[Ca^{2+}]_o$.

It is not yet known whether inward and outward TI will have the same response

to changes in intracellular Ca^{2+} concentration. We therefore compared the effects of Cd^{2+} and Mn^{2+} on TI generated by high Ca^{2+} in the presence of $[Na^+]_o$. Figure 41 illustrates the effect of Cd^{2+}. The solution as well as the voltage steps used to induce TI was the same as described for figure 35. Outward TI was seen upon repolarization to -5 mV (arrow) and inward TI at -55 mV (arrow). Both inward and outward TI were simultaneously decreased by approximately the same extent after 5 min addition of 0.5 mM Cd^{2+} to the high Ca^{2+} solution. The mean values of inhibition averaged from 4 experiments were 48 $\pm$ 8% for inward TI and 44 $\pm$ 9% for outward TI (mean $\pm$ SE). The gradual and simultaneous decrease of both inward and outward TI suggests that this effect may be secondary to the inhibition of Ca^{2+} influx through the Ca^{2+} channel.

Effects of Mn^{2+} on high Ca^{2+} induced TI are shown in figure 42. Traces in this figure were obtained under the same conditions as described for figure 41. Panel A shows controls for both inward and outward TI. After adding 5 mM Mn^{2+} to the solution, inward TI was markedly inhibited while outward TI just showed a slight decrease (panel B). The average inhibition from 4 experiments was 80 $\pm$ 15% for inward TI and 15 $\pm$ 7% for outward TI (mean $\pm$ SE). The difference in extent of inhibition for inward and outward TI indicates that Mn^{2+} may directly inhibit the TI channel responsible for the inward TI.

5. Effects of DIDS and SITS on High $[Ca^{2+}]_o$ Induced TI in the Presence of $[Na^+]_o$

In the experiments shown above, we demonstrated that in the absence of $[Na^+]_o$ the outward TI could be virtually abolished by two anion transporter blockers, DIDS and SITS. In the presence of $[Na^+]_o$, it is possible that a Na^+ conductance through the non-selective cationic channel or Na^+-Ca^{2+} exchange might be superimposed on

the Cl^- conductance. We therefore determined whether DIDS and SITS could still completely abolish outward TI in the presence of $[Na^+]_o$, and whether block could be accompanied by a shift in apparent reversal potential of the TI.

Figure 43 illustrates the effects of DIDS. Panels A and B show the original recordings of TI current before and after the addition of DIDS, respectively. For clarity, current traces corresponding to test potentials negative to -55 mV, at which the amplitude of TI changed little, are not shown. Five min after adding 10 μM DIDS to the perfusing solution, both inward and outward TI decreased (panel B). Eight similar observations were obtained in total four preparations. However, the average inhibition on outward TI at 0 mV was 80 $\pm$ 7% (n=8, mean $\pm$ SE, $p < 0.01$), whereas the average inhibition of inward TI at -55 mV was only 20 $\pm$ 4% (n=8, mean $\pm$ SE, $p < 0.05$). The I-V relation (panel C) showed that the residual outward current was constant over the range of -15 to +15 mV and that E_{RIV} was not affected significantly. The effects of DIDS reversed following a 10 min washout in DIDS-free solution containing high Ca^{2+}. Under the same experimental conditions, we also observed in 2 additional Purkinje fibres that 0.2 mM SITS greatly decreased the outward TI when the inward TI was only slightly inhibited (data not shown). These results suggest that the TI conductance responsible for outward TI is carried almost entirely by Cl^- even in the presence of $[Na^+]_o$.

Figure 33. Induction of TI by 30 mM Ca^{2+} in the presence of extracellular Na^{+}. **A**: Voltage steps applied to the preparation. **B**: Currents generated in response to the changes in transmembrane potential. The amplitude of peak inward TI was decreased when the test potentials became less negative. The time to peak inward TI also shortened. Prominent outward TI was seen at all test potentials positive to -20 mV and the outward TI only shifted slightly in response to the changes of test potential.

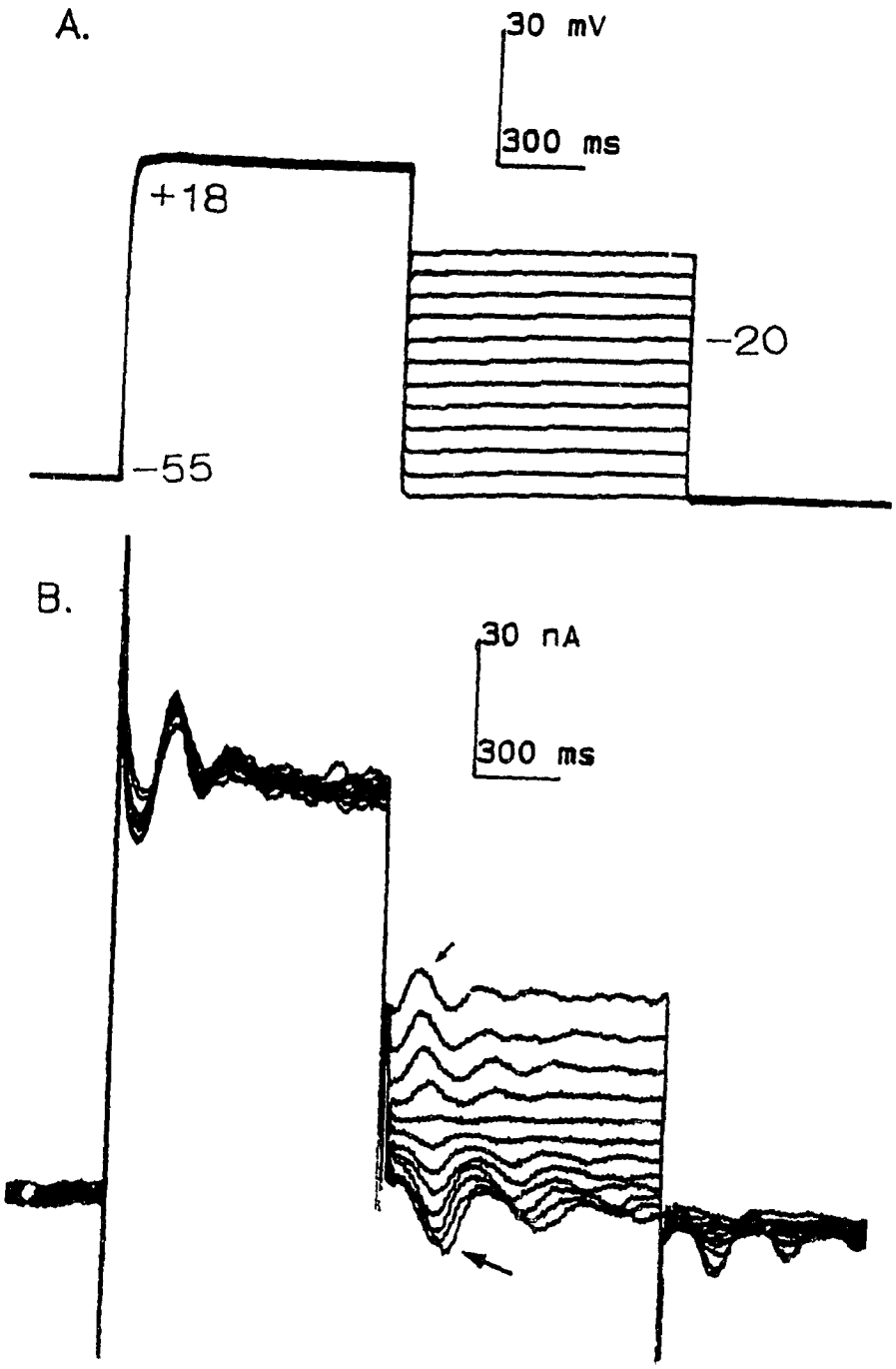


Figure 33.

Figure 34. Current-voltage (I-V) relation of TI induced by high $[Ca^{2+}]_o$ in the presence of $[Na^+]_o$. Data were taken from the same preparation as figure 1. TI clearly reversed polarity at about -20 mV (E_{REV}).

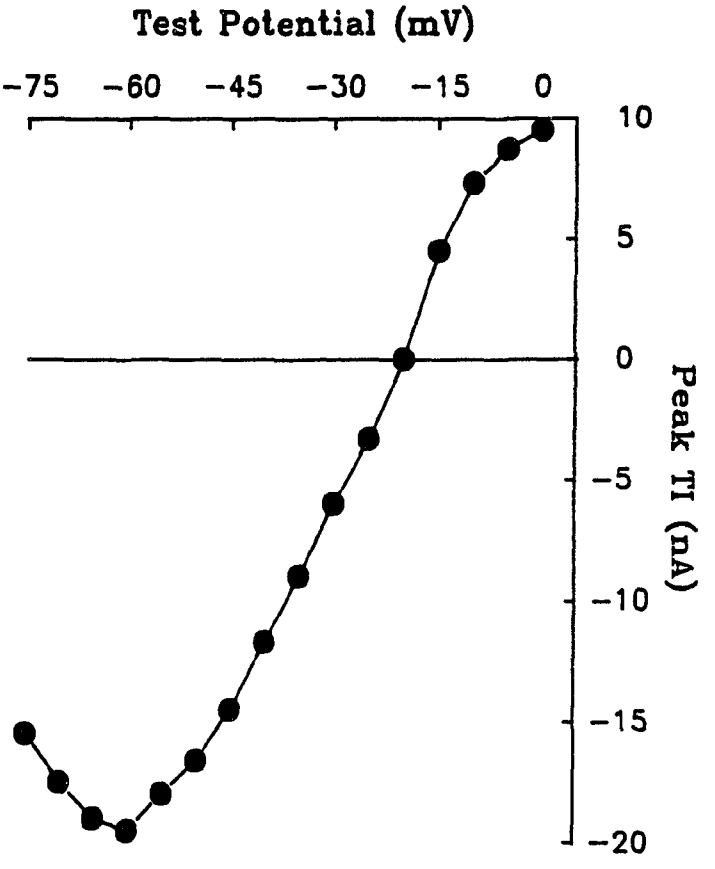


Figure 34.

Figure 35. Opposite effects of Ni^{2+} on inward and outward TI induced by high $[Ca^{2+}]_o$ in the presence of $[Na^+]_o$. Recorded voltage steps applied to the preparation are shown at the bottom of each panel. **A**: Inward (large arrow) and outward TI (small arrow) induced by high Ca^{2+} before Ni^{2+} treatment. **B**: Current traces obtained after 5 min of Ni^{2+} (2.5 mM) treatment. The inward TI was significantly inhibited by Ni^{2+}, however, the peak outward TI increased.

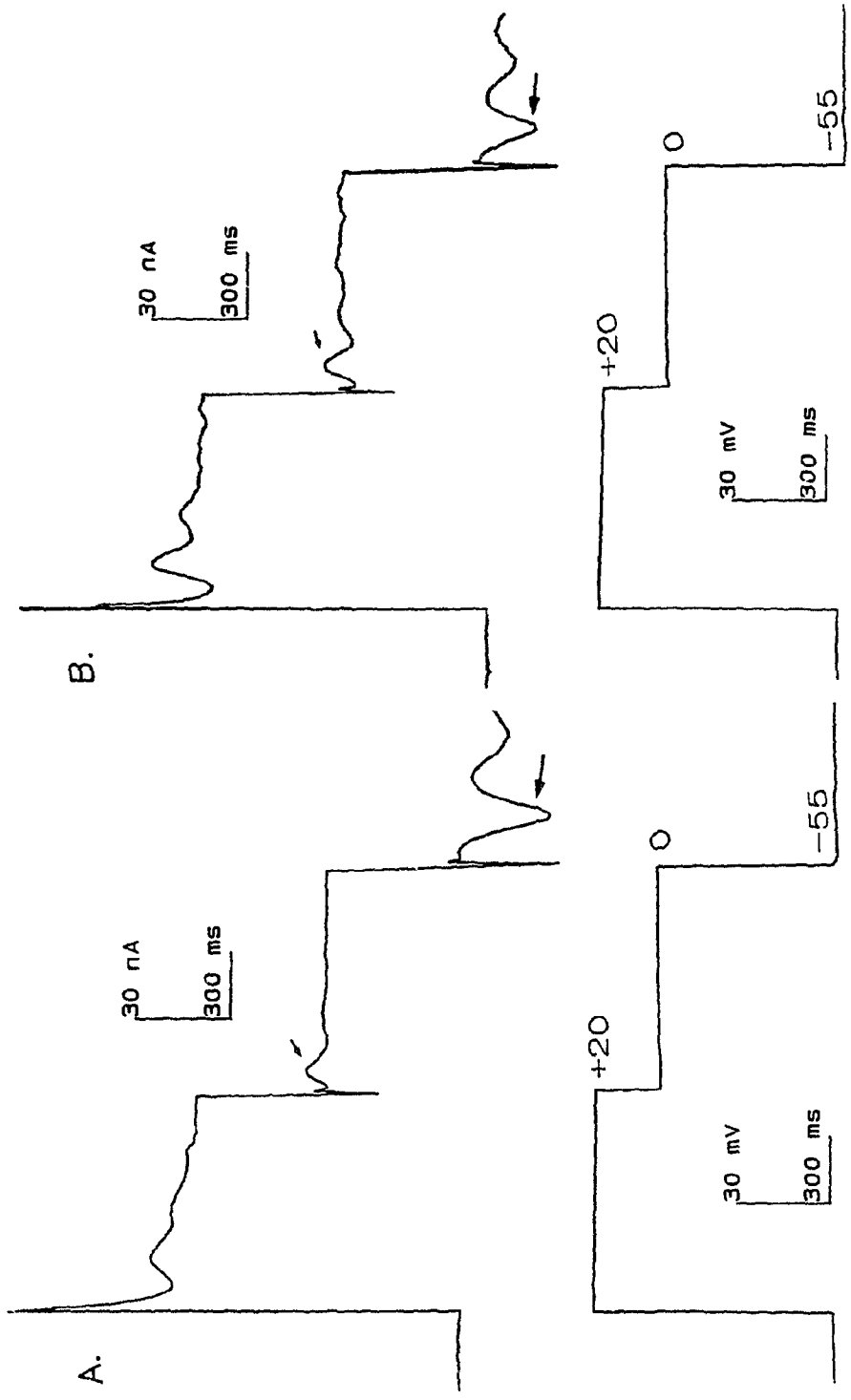


Figure 35.

Figure 36. Effects of Ni^{2+} on inward and outward TI induced by high Ca^{2+} at different test potentials. **Panel A**: Current traces recorded before the addition of Ni^{2+}. **Panel B:** Current traces recorded 4 min after the addition of 2.5 mM Ni^{2+}. **Panel C:** I-V relations of TI in the absence and presence of Ni^{2+}. Voltage steps applied to the preparation are shown schematically in panel C. The holding potential was -55 mV. For clarity, current traces corresponding to test potentials negative to -55 mV are not shown. The large outward currents activated upon the initial depolarization are off scale. The arrows in panel A and B indicate the current obtained at -20 mV. All inward TI were decreased and outward TI were increased after Ni^{2+} treatment (panel B). Also, there was a slight shift (-3 mV) of E_{REV} in the negative direction (panel C).

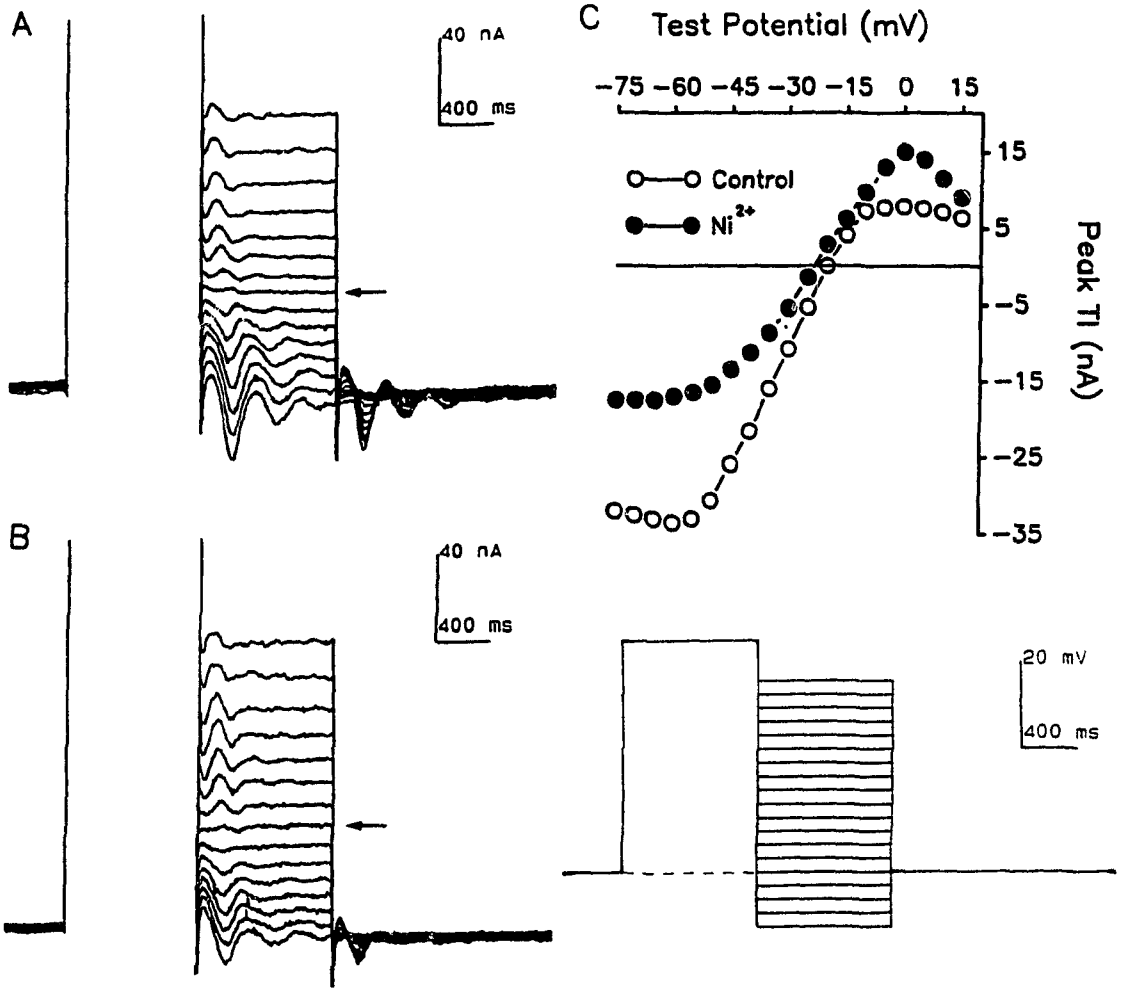

A
40 nA
400 ms
B
40 nA
400 ms
C
Test Potential (mV)
-75 -60 -45 -30 -15 0 15
Control
Ni2+
15
5
-5
-15
-25
-35
Peak TI (nA)
20 mV
400 ms

Figure 36.

Figure 37. Induction of I_{EX} in the absence of $[K^+]_o$. **A**: Voltage step applied to the preparation. **B**: TI was induced by the same solution described in figure 33. **C:** Initially switching to 0 $[K^+]_o$ caused an increase in peak inward TI. **D**: Prolonged perfusion with 0 $[K^+]_o$ resulted in decrease of peak inward TI and appearance of I_{EX}. **E**: Only a tail current (I_{EX}) was apparent 5 min after 0 $[K^+]_o$ perfusion. **F**: Return to 4 mM KCl caused the disappearance of I_{EX} and the reappearance of TI.

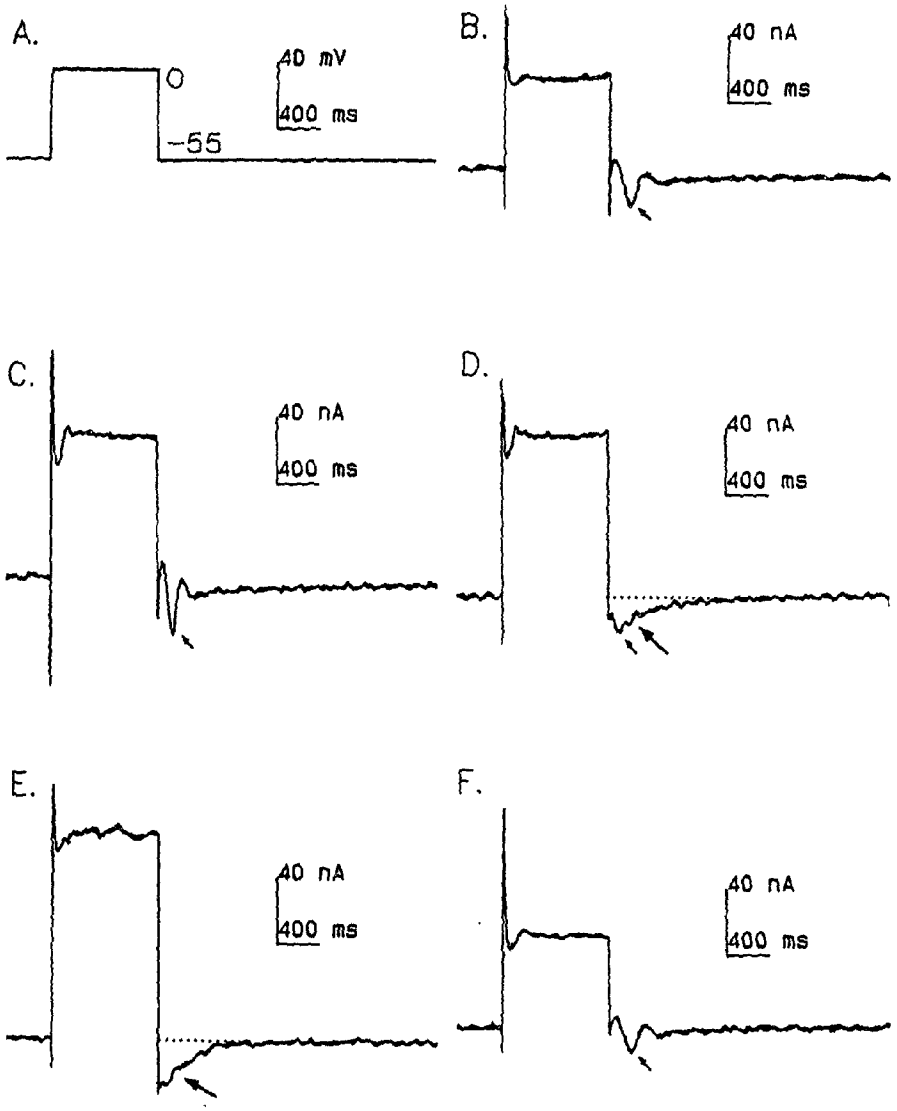

A.
0
-55
40 mV
400 ms
B.
40 nA
400 ms
C.
40 nA
400 ms
D.
40 nA
400 ms
E.
40 nA
400 ms
F.
40 nA
400 ms

Figure 37.

Figure 38. Effect of Ni^{2+} on $I_{I,X}$. $I_{I,X}$ was induced by the same treatment described in figure 5. **A**: Voltage steps. **B**: Superimposed current traces recorded before (large arrow) and after (small arrow) addition of 2.5 mM Ni^{2+} to the superfusate. $I_{I,X}$ was abolished by 2 min exposure to 2.5 mM Ni^{2+}.

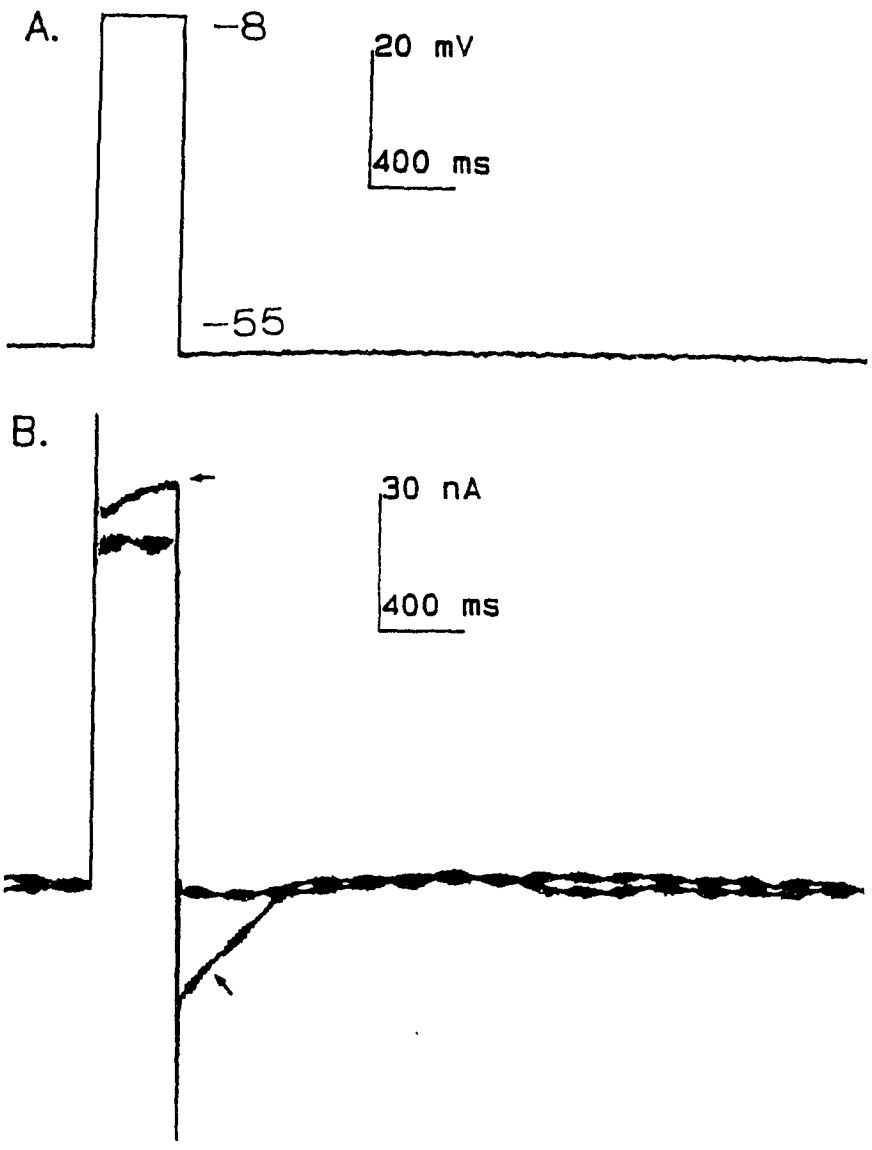


Figure 38.

Figure 39. Opposite effects of Ni^{2+} on high $[Ca^{2+}]_o$ induced TI in the absence of $[Na^+]_o$. The solution used for TI induction contained 30 mM Ca^{2+} with 105 mM NMG chloride replacing NaCl. Test potentials applied to the preparation are shown at the bottom of each panel. **A**: Inward (large arrow) and outward TI (small arrow) induced by high Ca^{2+} in the absence of $[Na^+]_o$. **B**: Current trace recorded after Ni^{2+} (2.5 mM) treatment. Outward TI was significantly increased at a time when inward TI was significantly inhibited by Ni^{2+}.

Figure 39.

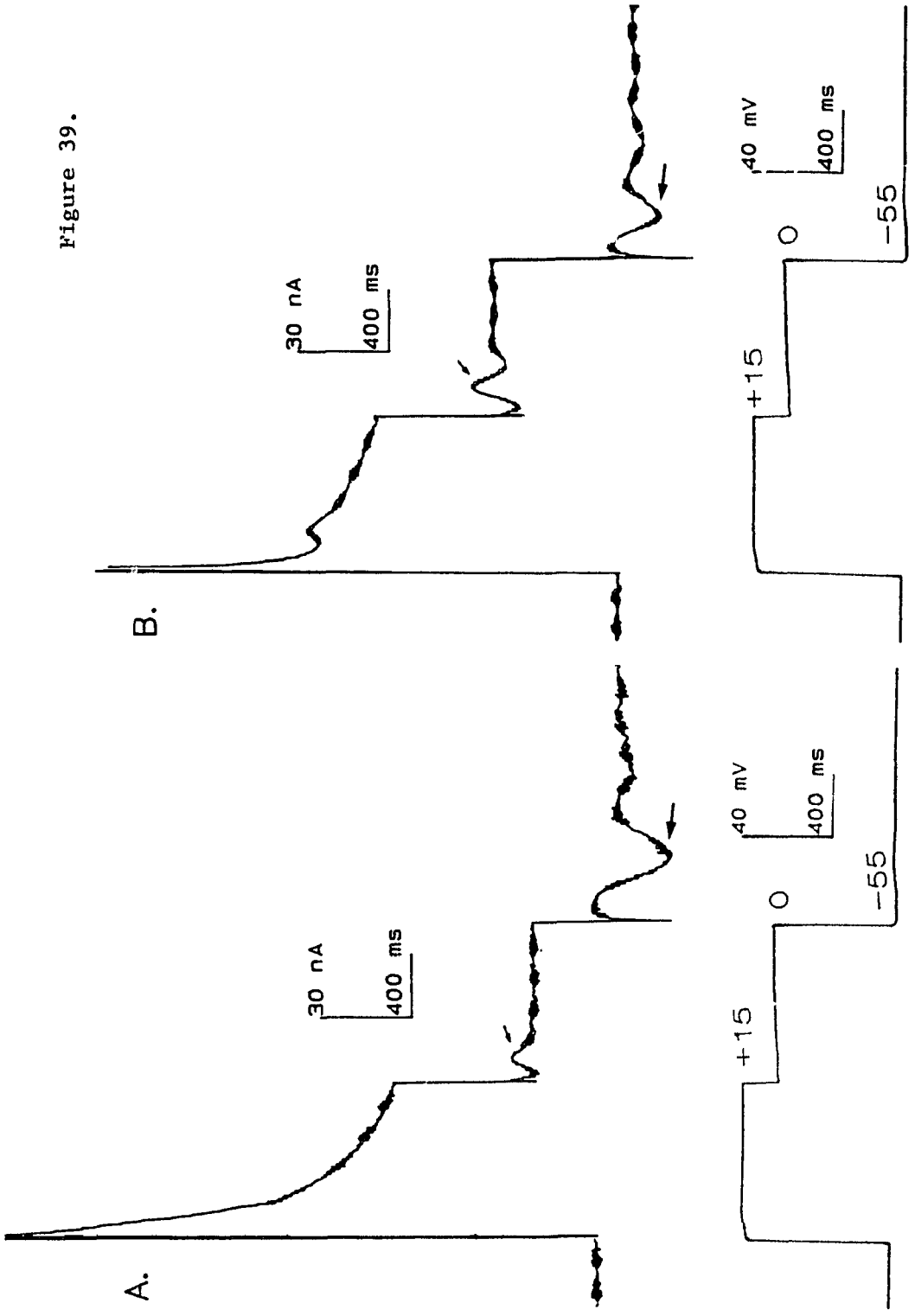


Figure 40. Effect of Ni^{2+} on TI induced by 30 mM $[Ca^{2+}]_o$, and with NaCl substituted with 210 mM sucrose. The voltage steps are shown at the bottom. The protocol only induced an inward TI (large arrow) which was abolished by exposure to 5 mM Ni^{2+} (small arrow).

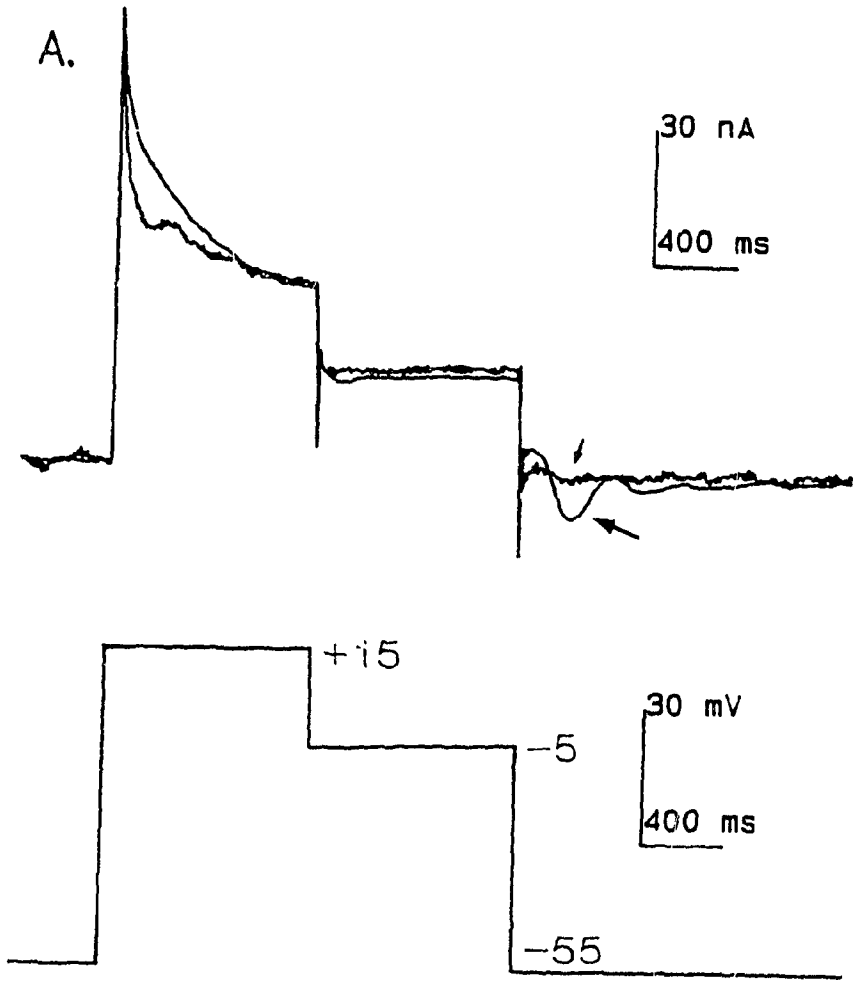


Figure 40.

Figure 41. Effects of Cd^{2+} on TI. Voltage steps applied to the preparation are shown at the bottom. Superimposed current traces were obtained before (arrows) and after the addition of 0.5 mM Cd^{2+}. Following 5 min exposure to 0.5 mM Cd^{2+} inward and outward TI were simultaneously decreased.

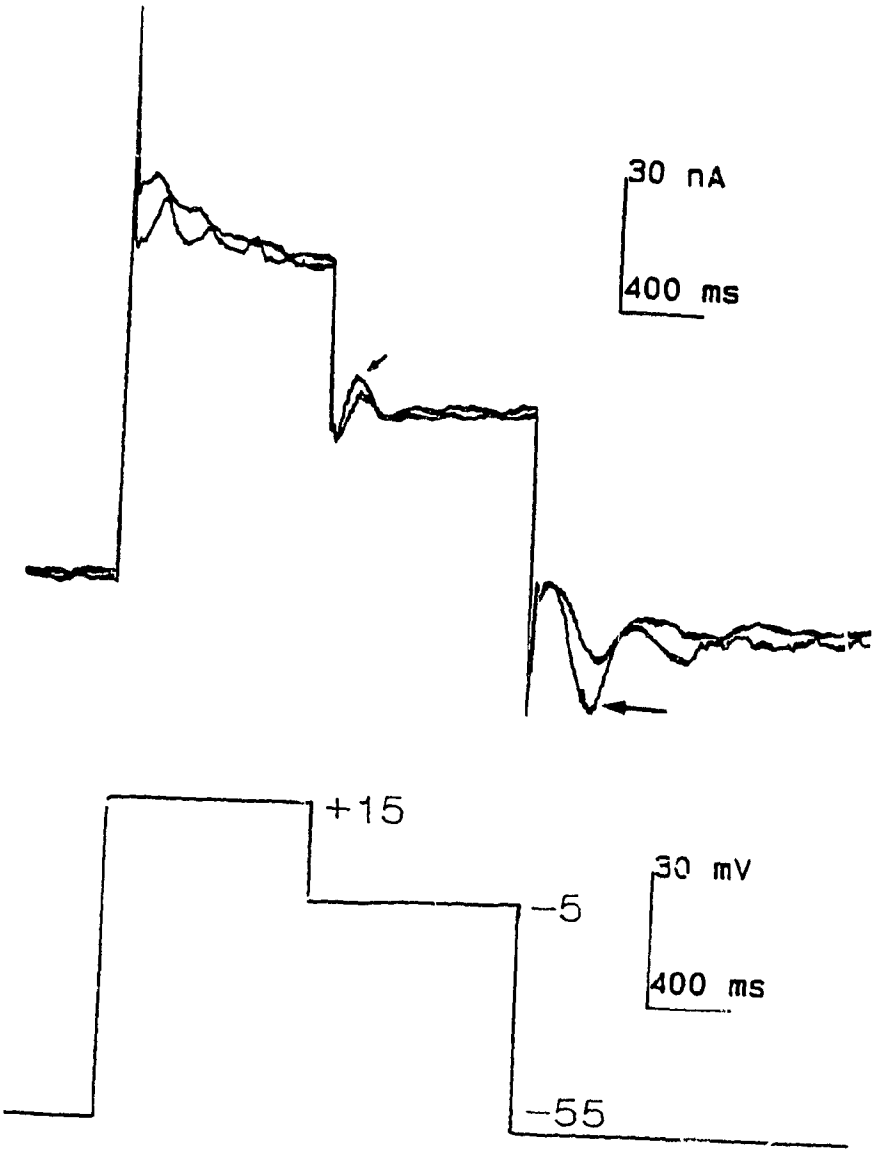


Figure 41.

Figure 42. Effects of Mn^{2+} on TI. Voltage steps are shown at the bottom of each panel. **A:** Current trace recorded in the absence of Mn^{2+}. **B:** Current trace obtained after addition of 5 mM Mn^{2+}. After addition of Mn^{2+} to the high Ca^{2+} solution, inward TI (large arrow) was inhibited by approximate 80% while the outward TI (small arrow) was inhibited by only 15%.

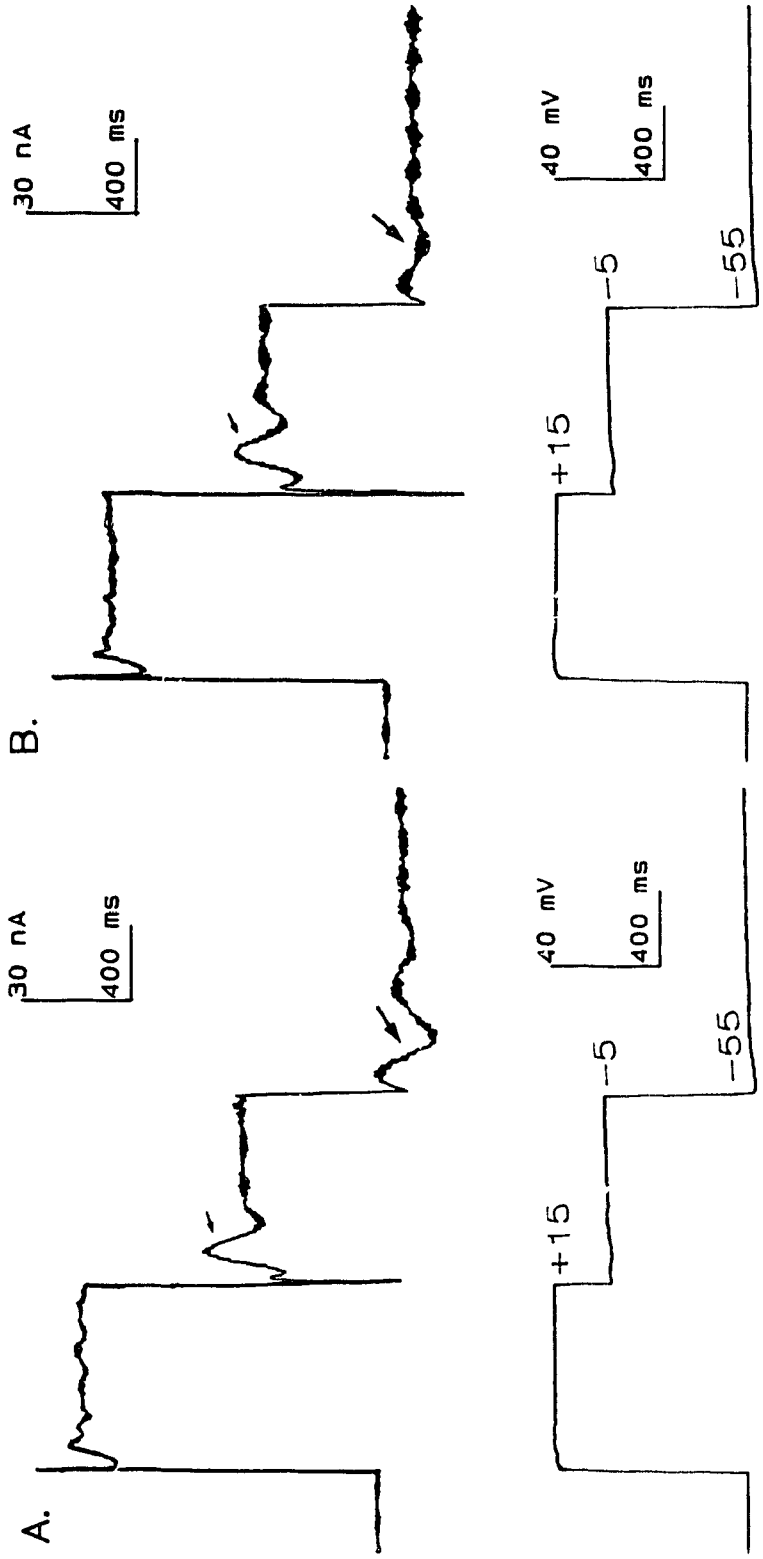


Figure 42.

Figure 43. Effects of DIDS on inward and outward TI induced by 30 mM Ca^{2+} at different test potentials. **Panel A**: Current traces recorded before DIDS treatment. **Panel B:** Current traces recorded 4 min after the addition of 10 μM DIDS. **Panel C:** I-V relations of TI in the absence and presence of DIDS. Voltage steps applied to the preparation are shown schematically in panel C. The holding potential was -55 mV. For clarity, current traces corresponding to test potentials negative to -55 mV are omitted. The arrows in panel A and B indicate the current obtained at test potential -20 mV. Outward TI was greatly suppressed but inward TI was only slightly decreased after treatment with DIDS (panel B). E_{REV} of TI was not changed significantly by DIDS (panel C).

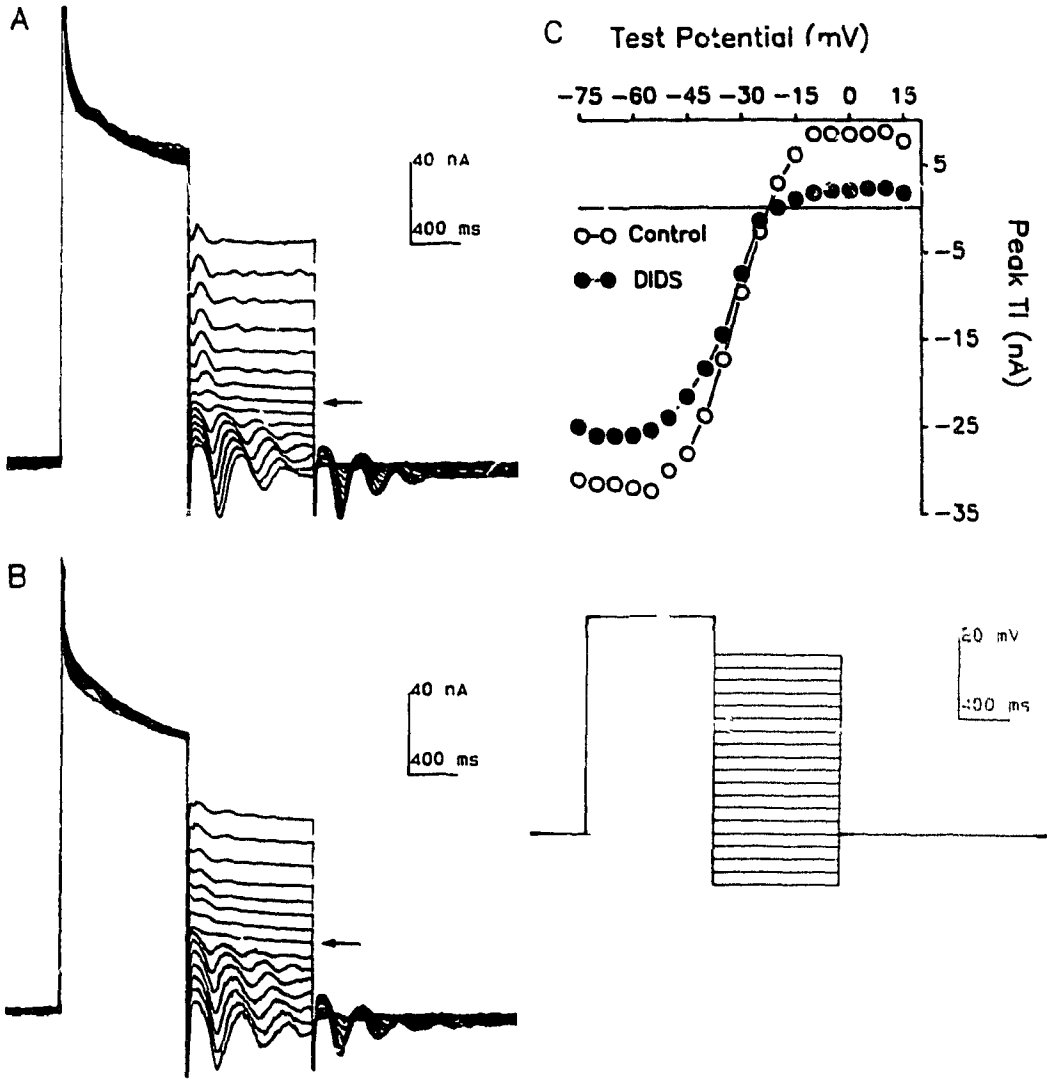

A
40 nA
400 ms
B
40 nA
400 ms
C
Test Potential (mV)
-75 -60 -45 -30 -15 0 15
5
-5
-15
-25
-35
Control
DIDS
Peak TI (nA)
20 mV
400 ms

Figure 43.

VI. CONTRIBUTION OF Na^+-Ca^{2+} EXCHANGE TO THE β-ADRENERGIC STIMULATION OF TI

1. Characteristics of TI Induced by High $[Ca^{2+}]_o$ in the Absence of $[Na^+]_o$

Previously we have shown that the β-adrenergic agonist isoprenaline (ISO) strongly stimulates TI induced by acetylstrophanthidin. We presented evidence that the stimulation was likely mediated by Na^+-Ca^{2+} exchange. If this is true, one would expect that TI could not be stimulated by ISO if the Na^+-Ca^{2+} exchange mechanism was abolished. One way to do this is to induce TI in the absence of $[Na^+]_o$, and thereby make the Na^+-Ca^{2+} exchange mechanism inoperative. Recently, NMG has been used satisfactorily as a Na^+ substitute (Zygmunt & Gibbons, 1991). We then studied the characteristics of TI induced by elevation of $[Ca^{2+}]_o$ and with NMG as the Na^+ substitute.

Figure 44 shows an example of TI induced by elevating $[Ca^{2+}]_o$ in the absence of $[Na^+]_o$. The membrane was clamped at -55 mV and depolarized to different test potentials between -20 to +20 mV to generate the TI current. Panel A shows currents recorded in normal Tyrode's solution. Panel B shows currents generated by elevating $[Ca^{2+}]_o$ to 30 mM with $CaCl_2$ and substituting $[Na^+]_o$ with NMG chloride. Prominent TI was generated upon repolarization to -55 mV from a previous depolarization to -20 mV. The peak amplitude of TI was increased when the depolarization pulse was increased to 0 mV (Panel C). Further depolarization to +20 mV did not result in any increase of the TI amplitude (Panel D). However, the current activated at the end of the depolarization pulse (small arrow) increased as depolarizations became progressively more positive. Similar observations were made in 5 Purkinje fibres. Similar observations have been described previously when TI was induced by digitalis (Lederer & Tsien, 1976; Kass et al, 1978a).

TI thus induced was very stable and reproducible, and we next studied the I-V relation. Figure 45 shows superimposed current traces (Panel A) and corresponding voltage protocols (Panel B). The I-V relation for the TI is shown in Panel C. From panel A it is clear that inward TI decreased monotonically and reversed its direction from inward to outward as the test potentials were made less negative. Thus, a distinct reversal potential (E_{REV}) was resolved at -25 mV (Panel C). Similar results were obtained in 4 Purkinje fibres. The E_{REV} was identical to that observed when cholineCl was used to substitute NaCl.

2. Effect of ISO or TI Induced in the Absence of $[Na^+]_o$

It would be advantagous to simultaneously observe whether β-stimulation could affect both inward and outward TI. The superimposed current traces shown in figure 45 actually indicate that both significant inward and outward TI can be generated simultaneously with certain activation protocols. Figure 46 illustrates TI generated with such protocol. Panel A shows superimposed current traces recorded before and after addition of 0.1 μM ISO. The membrane potential was clamped at -55 mV. Apparent outward and inward TI were induced (panel A, trace a) upon repolarization to test potentials corresponding to -5 (for 1 sec) and -55 mV from a previous depolarization to +20 mV (for 1 sec) (panel B). Three concentrations of ISO (10 nM, 0.1 μM, 1 μM) were tested and no significant effects on either inward or outward TI were seen following 5 to 10 min exposure to drug. Trace b shows inward and outward TI recorded after administration of 1 μM ISO. However, the outward current activated at the end of depolarizing pulse which probably reflects I_K was greatly increased (large arrow). Identical observations have been made in 5 preparations.

Effects of ISO (1 μM) on the I-V relation of TI generated in the absence of $[Na^+]_o$ is shown in figure 47. Results represent means obtained from 5 Purkinje

fibres. The voltage steps applied to the preparations were the same as shown in figure 45. Peak amplitudes of both inward and outward TI were not affected at all potentials tested. Also, no significant shift in E_{REV} was seen after addition of ISO.

The absence of stimulation of TI by ISO might be related to interruption of β-adrenoceptor-initiated phosphorylation following withdrawal of $[Na^+]_o$ (Hume & Harvey, 1991). If it is the phosphorylation process which is affected in our system, one would expect that some other currents, for example, I_{Ca} and I_K, should not be stimulated by ISO. It is very difficult to measure I_{Ca} quantitatively due to the presence of a large transient outward current (I_{TO}) which overlaps I_{Ca}. However, the current activated at the end of 1 sec depolarization (tentatively termed I_{step} by Ferrier and Carmeliet, 1990), which roughly reflects I_K activation, may be a useful parameter because it is well known that β-adrenergic stimulation of I_K is mediated by the phosphorylation of I_K channels (Bennett et al, 1986; Bennett & Begenisich, 1987; Walsh et al, 1988). Thus, records in figure 46 showing that the current activated at the end of the depolarization pulse (+20 mV, 1 sec) was significantly increased by ISO indicate that the phosphorylation process can still be initiated by ISO via stimulation of β-adrenoceptors in the absence of $[Na^+]_o$. When Purkinje fibres (n = 2) were pretreated with 0.2 μM propranolol, a β-adrenoceptor blocker, no stimulation of the current at the end of the depolarizing pulse was seen even when ISO was increased to 5 μM (data not shown).

3. Effects of ISO on TI Induced in the Presence of $[Na^+]_o$

If ISO still can initiate the phosphorylation process, it is quite unlikely that the previously demonstrated β-adrenergic stimulation of digitalis-induced TI is caused by phosphorylation of the TI channels. L-type Ca^{2+} current can be stimulated by ISO in the absence of $[Na^+]_o$ (Hume, 1987). In fact, L-type I_{Ca} also can be stimulated directly by ISO-β-adrenoceptor activated G-protein even in the absence of phospher-

ylation (Yatani & Brown, 1989; Shuba et al, 1990; Trautwein & Hescheler, 1990). Thus, results demonstrated in figure 47 are not compatible with the hypothesis that L-type Ca^{2+} channel is the major pathway which mediates β-adrenergic stimulation of TI. On the other hand, Na^{+}-Ca^{2+} exchange which can cause Ca^{2+} loading during depolarization (Bielefeld et al, 1986, Brill et al, 1987) is probably the pathway which mediats the β-adrenergic stimulation of TI. If this is true, one may expect to see stimulation of high Ca^{2+} induced TI by ISO in the presence of $[Na^{+}]_o$.

Figure 48 shows the effects of ISO on TI induced by elevation of $[Ca^{2+}]_o$ (30 mM) in the presence of $[Na^{+}]_o$ (105 mM). Previously we have demonstrated that even in the presence of $[Na^{+}]_o$ TI is still conducted through TI channels (Han & Ferrier, 1992b). There is a distinct E_{REV} of -20 mV for TI induced by such treatment. Therefore, an activation protocol identical to that shown in figure 46 was used to generate both outward and inward TI (Panel B). Panel A shows superimposed current traces recorded in the absence (trace a) and presence (trace b) of ISO. Prominent outward and inward TI were seen following repolarizations to test potentials of -5 and -55 mV, respectively. Five min after addition of 0.1 μM ISO both outward and inward TI were dramatically increased. The average increase of the peak TI amplitudes in 5 preparations was 90 $\pm$ 20% for outward TI and 100 $\pm$ 25% for inward TI.

The effects of ISO on the I-V relation of TI in the presence of $[Na^{+}]_o$ is shown in figure 49 which is representative of observations made in 5 Purkinje fibres. Voltage steps applied to the preparations were the same as shown in figure 45 and are schematically shown in panel D. Panel A shows the superimposed current traces recorded in the absence of ISO. Five min after addition of 0.1 μM ISO both outward and inward TI generated at all test potentials were greatly increased (Panel B). Maximum stimulation on TI was often seen within 5 min and no significant changes

in peak TI (both inward and outward) were observed with further drug exposure (15 min). The I-V relations (Panel C) clearly indicated that increases in peak TI were greater at test potentials away from the reversal potential. E_{REV} (-20 mV) was not significantly affected.

The stimulatory effects of ISO on both inward and outward TI were totally abolished by pretreating the preparation with 0.2 μM propranolol (n = 2) or atenolol (n = 2), suggesting that the effects are mediated by the β-adrenoceptors. However, once the preparation was stimulated by ISO, β-adrenoceptor antagonist only partially reversed the stimulation. Figure 50 shows such an experiment. Superimposed current traces recorded in the absence (trace a), presence (trace b) of ISO, and after addition of propranolol (trace c) are shown in panel A. Voltage steps applied to the preparation are illustrated in panel B. Inward and outward TI were generated upon repolarizations to -5 and -55 mV, respectively. Following addition of 0.1 μM ISO, marked stimulation of both inward and outward TI developed within 2 min (trace b). Propranolol (0.2 μM) was added to the perfusing solution at the time when apparent increase in peak TI was observed. Five min after addition of propranolol, both inward and outward TI were significantly decreased (trace c). However, both inward and outward TI were still much greater than those generated in the absence of ISO (trace a). Observations similar to those illustrated in figure 50 were obtained in 4 preparations.

4. Effects of Forskolin on TI Induced in the Presence and Absence of $[Na^+]_o$

Propranolol is a competitive β-adrenergic antagonist. Its action is believed to prevent agonist-receptor binding or to displace the agonist from receptors. Therefore partial reversal of ISO stimulation on TI suggests that the agonist-receptor binding has already triggered a cascade process which is less easily reversed than drug receptor interactions. These might be activation of G-protein, adenylate cyclase, cAMP,

cAMP-dependent protein kinase, and protein phosphorylation. If this is true, forskolin, a direct activator of adenylate cyclase might also stimulate TI by phosphorylation of the Na^+-Ca^{2+} exchanger.

Results demonstrating the effects of forskolin (1μM) on high Ca^{2+} induced TI in the presence of $[Na^+]_o$ are shown in figure 51. Experiments were performed and recorded under identical conditions as described for figure 49. Five min after exposure to forskolin, both inward and outward TI were significantly increased at all test potentials (Panel B). The increases in TI were greater at the extremes of test potentials but the E_{REV} was not affected (Panel C). These effects of forskolin on TI were also identical to those of ISO. Pretreatment of the preparation with propranolol (0.2 μM) had no effect on stimulation of TI by forskolin (data not shown), suggesting that the effects of forskolin were independent of agonist-adrenoceptor binding. Results similar to that shown in figure 51 were observed in 4 Purkinje fibres.

If indeed forskolin acts by phosphorylation of the Na^+-Ca^{2+} exchanger protein and thereby promoting $[Ca^{2+}]_i$ overload, absence of $[Na^+]_o$ should abolish the stimulatory effects of forskolin. The absence of stimulatory effects of forskolin on TI in Na^+-free solution is illustrated in figure 52 which is representative of observations made in 3 Purkinje fibres. Here the experiments were performed and recorded under identical conditions to those described for figure 46. Panel A shows superimposed current traces recorded before (trace a) and after (trace b) addition of forskolin. Panel B illustrates the voltage steps applied to the preparation. Neither the inward nor outward TI was changed after administration of forskolin. However, outward current at the end of depolarization pulse was significantly increased by forskolin in the absence of $[Na^+]_o$.

5. Effects of Quinacrine and Dodecylamine on the Stimulation of TI by ISO

Experiments presented so far suggest that the Na^+-Ca^{2+} exchanger protein may be

phosphorylated and thereby mediate stimulation by ISO and forskolin. If this indeed occurs, agents known to block the transporting process of the exchanger may also prevent stimulation by ISO and forskolin. Quinacrine and dodecylamine are putative Na^+-Ca^{2+} exchange blocker (de la Pena & Reeves, 1987) which are able to block both the current and the contraction generated by the Na^+-Ca^{2+} exchange (Bielefeld et al, 1986; Satoh et al, 1989; Terrar & White, 1989). Figure 53 shows the effects of quinacrine on the ISO stimulation of high Ca^{2+} induced TI. Voltage steps applied to the preparation are illustrated in panel A. Inward and outward TI induced in the absence of ISO are shown in panel B. Three min after addition of ISO (0.1 μM), both inward and outward TI were dramatically increased (Panel C). At this time, quinacrine (10 μM) was added to the perfusing solution. TI was decreased after 5 min exposure to quinacrine (Panel D). The decrease in ISO-stimulated TI by quinacrine was observed in three Purkinje fibres. Inhibition of TI by quinacrine was also observed in other two preparations in which forskolin (2 μM) was used to stimulate TI (data not shown). In additional 5 preparations, preteatment of the Purkinje fibres with 20 μM dodecylamine abolished ISO stimulation of TI. After TI was stimulated by ISO (1 μM), addition of dodecylamine (20 μM) to the superfusate also resulted in quick inhibition of TI. These results further suggest that the Na^+-Ca^{2+} exchanger protein is most likely the target which mediates the β-adrenergic stimulation on TI.

Figure 44. Induction of TI by 30 mM $[Ca^{2+}]_o$ in the absence of $[Na^+]_o$. NMG chloride was used to substitute NaCl. The holding potential was -55 mV. The duration of the depolarization was 1 sec. **Panel A:** Current activated by depolarization to 0 mV in normal Tyrode's solution. **Panel B, C, D:** Currents generated by depolarization to -20, 0, and +20 mV, respectively, in the presence of 30 mM $[Ca^{2+}]_o$. Prominent TI was seen upon repolarization to -55 mV (large arrows). The amplitude of TI was the largest when the activation voltage was 0 mV (panel C). The current activated at the end of depolarization pulse (I_{step}, small arrows) was increased as depolarization pulse was made further positive (panel D).

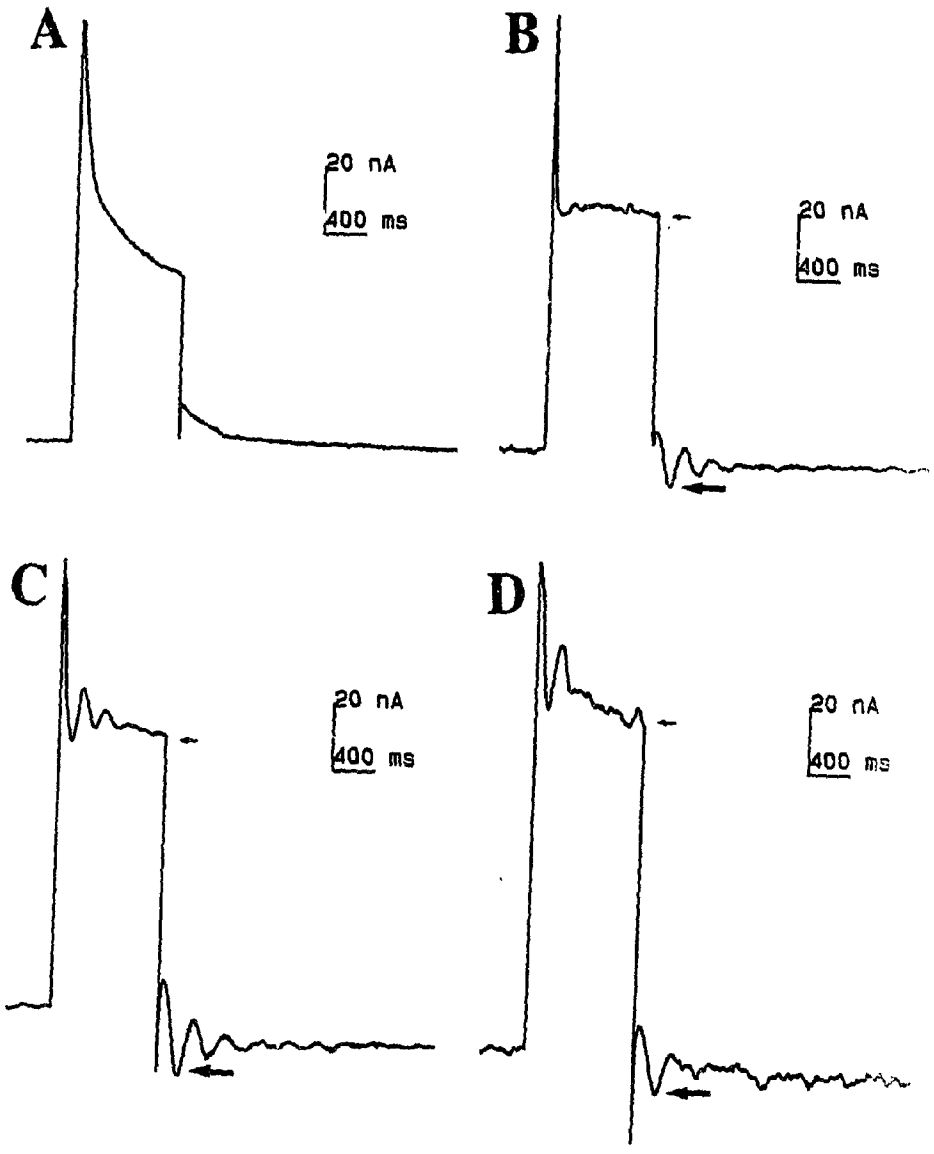


Figure 44.

Figure 45. I-V relation of TI induced by 30 mM $[Ca^{2+}]_o$ and 105 mM NMG chloride. **Panel A:** Superimposed current traces. **Panel B:** Voltage steps applied to the preparation. **Panel C:** I-V relation of TI. Inward TI monotonically decreased and reversed its direction from inward to outward as the test potentials were made less negative (panel A). Therefore, a distinct reversal potential (E_{REV}) was clearly resolved at -25 mV (panel C).

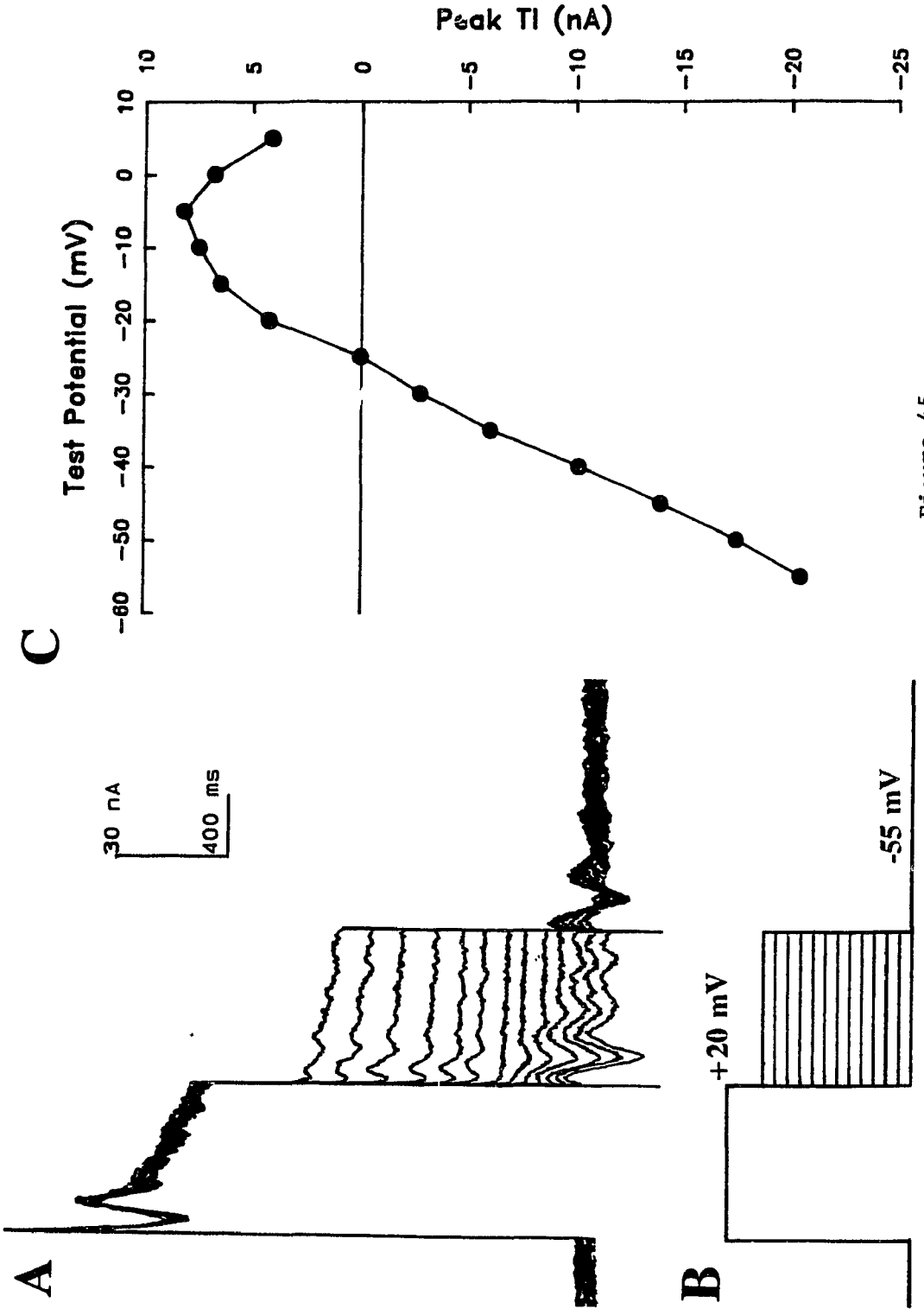

A
30 nA
400 ms
B
+20 mV
-55 mV
C
Peak TI (nA)
10
5
0
-5
-10
-15
-20
-25
Test Potential (mV)
10
0
-10
-20
-30
-40
-50
-60

Figure 45.

Figure 46. Lack of effect of ISO on TI induced in the absence of $[Na^+]_o$. **Panel A:** Superimposed current traces recorded before (trace a) and after (trace b) addition of 0.1 μM ISO. **Panel B:** Voltage steps applied to the preparation. Outward and inward TI were induced at test potentials corresponding to -5 and -55 mV, respectively. No significant changes in both inward and outward TI were seen after administration of 1 μM ISO (trace b). However, the outward current activated at the end of depolarization pulse (I_{step}) was greatly increased (large arrow).

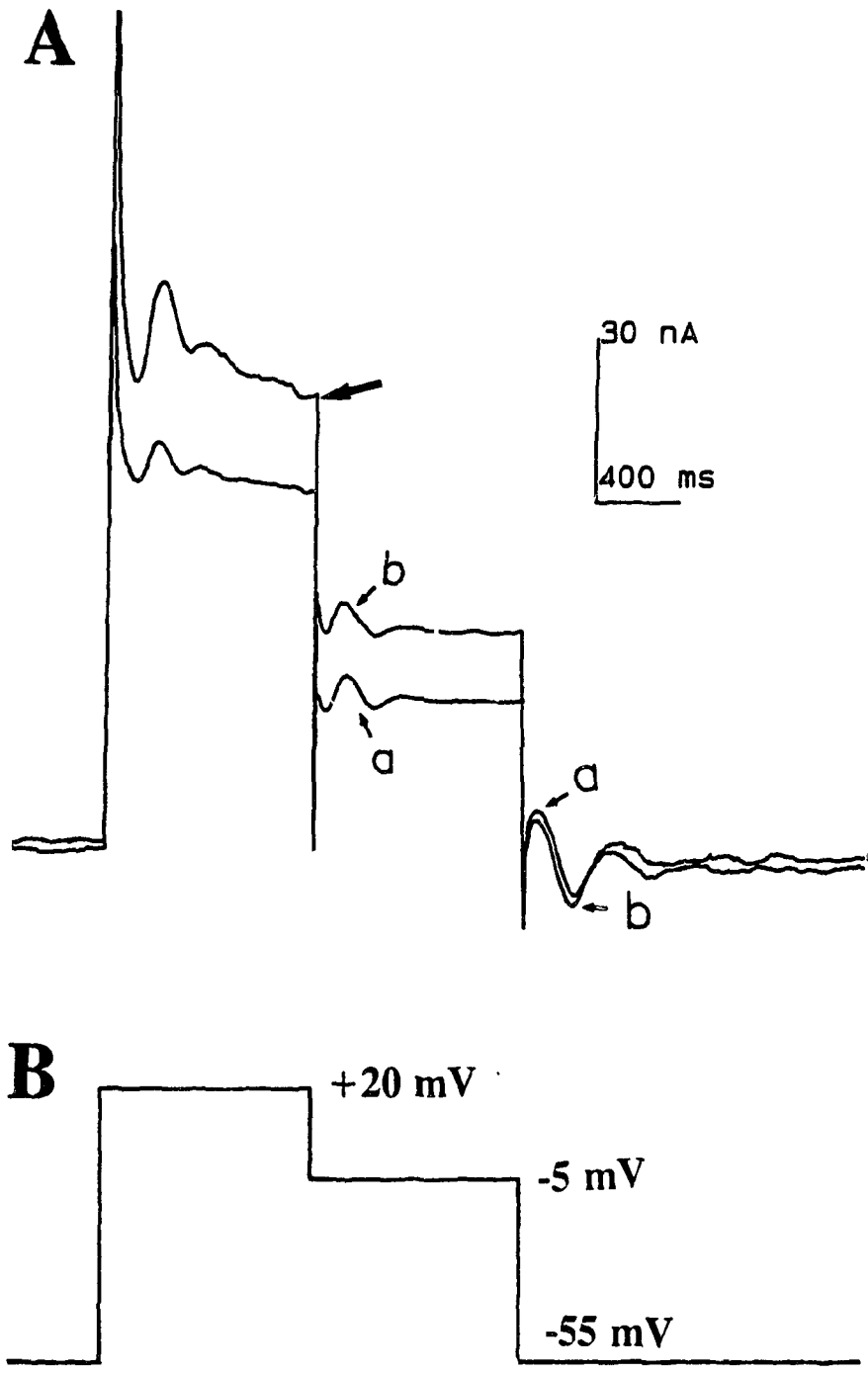


Figure 46.

Figure 47. Lack of effect of ISO (1 μM) on the I-V relations of TI induced by high Ca^{2+} in the absence of $[Na^+]_o$. The voltage steps applied to the preparations were the same as shown in figure 45. Each point represents 4 to 7 determinations (mean $\pm$ SEM) made in 5 Purkinje fibres. The peak amplitudes of both inward and outward TI were not affected at all potentials tested. Also, no significant shift in E_{REV} was seen after addition of ISO.

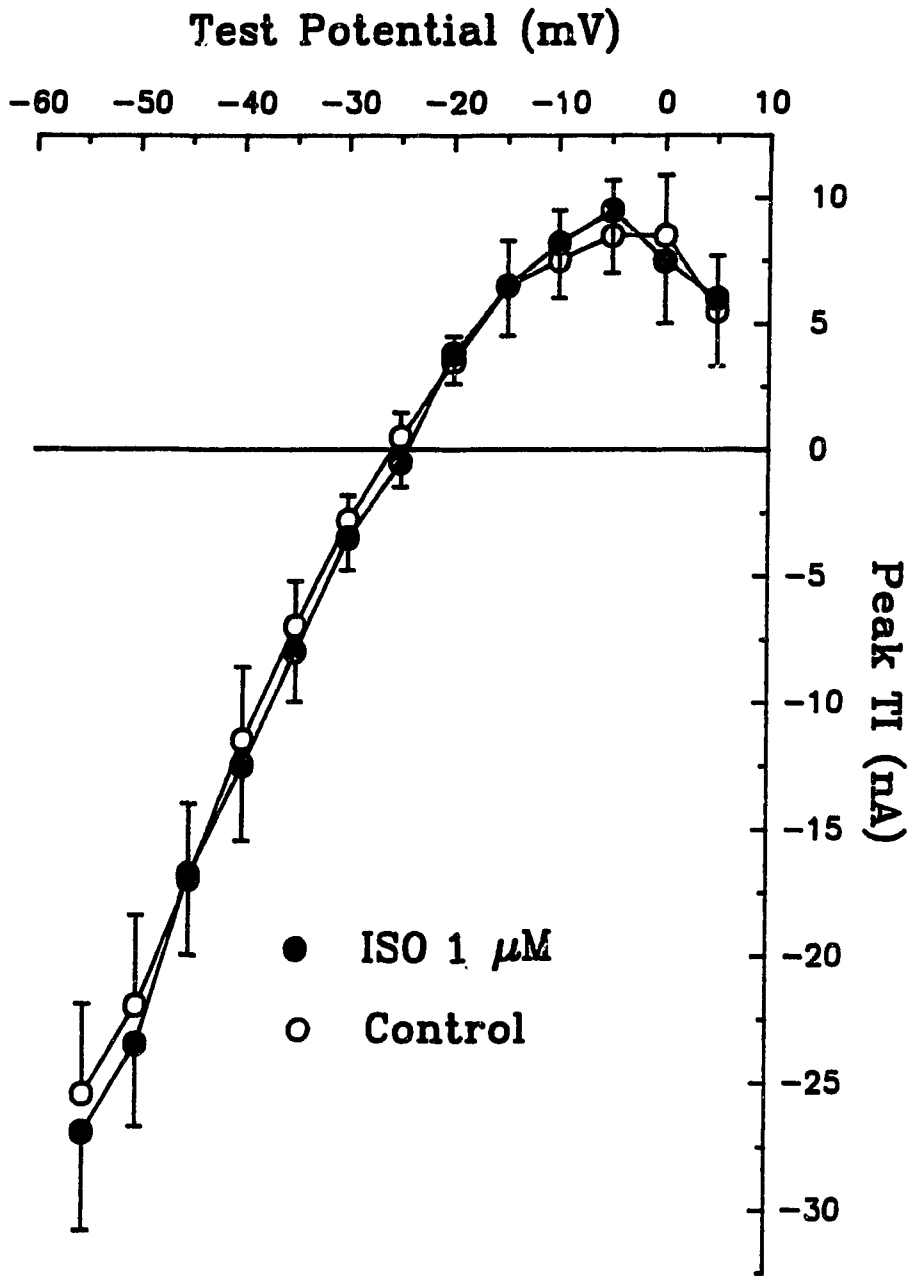

Test Potential (mV)
-60
-50
-40
-30
-20
-10
0
10
10
5
0
-5
-10
-15
-20
-25
-30
Peak TI (nA)
ISO 1 μM
Control

Figure 47.

Figure 48. Effect of ISO on TI induced by elevation of $[Ca^{2+}]_o$ (30 mM) in the presence of $[Na^+]_o$ (105 mM). **Panel A:** Superimposed current traces recorded in the absence (trace a) and presence (trace b) of ISO. **Panel B:** Voltage steps applied to the preparation. Prominent outward and inward TI were seen following repolarizations to test potentials of -5 and -55 mV, respectively. Five min after addition of 0.1 μM ISO both outward and inward TI were dramatically increased.

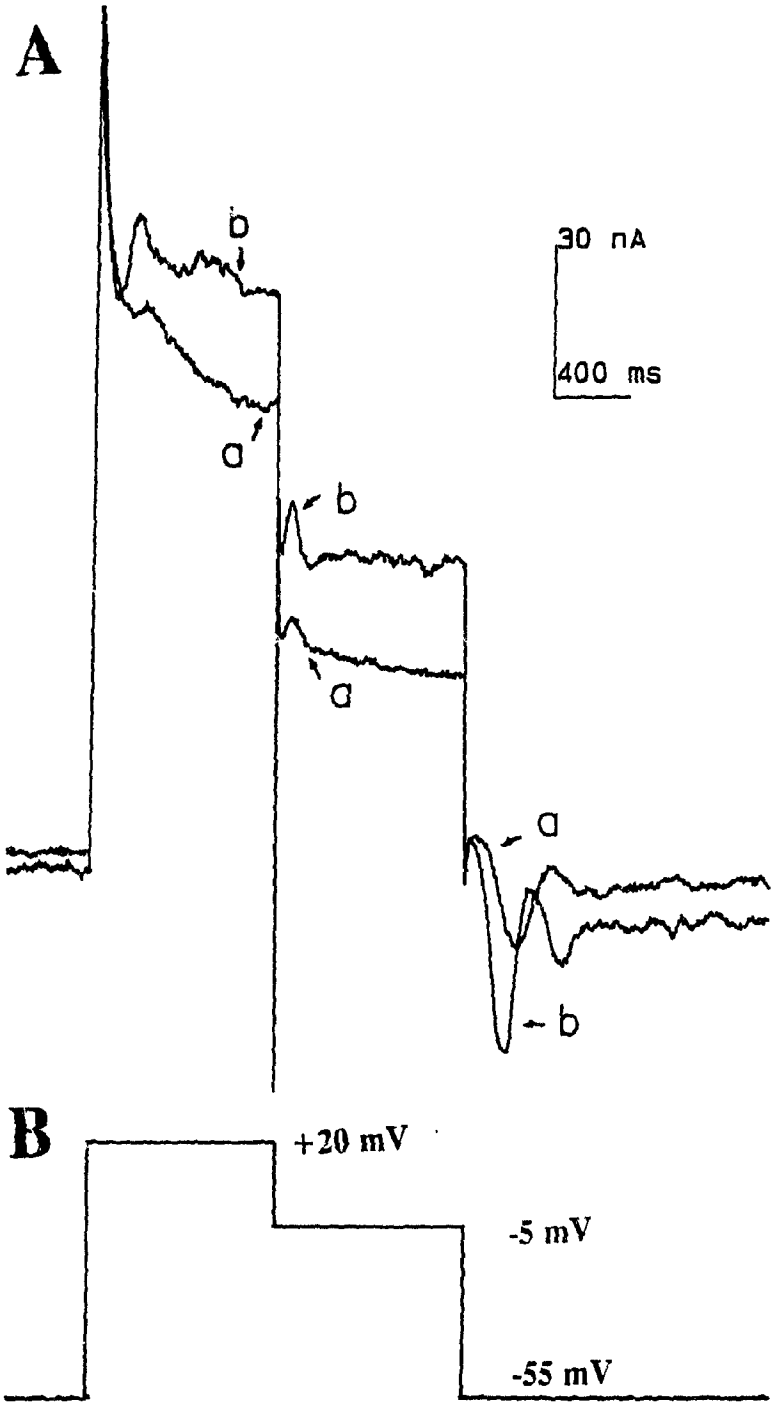


Figure 48.

Figure 49. Effects of ISO on the I-V relation of TI in the presence of $[Na^+]_o$. Voltage steps applied to the preparations are schematically shown in panel D. **Panel A:** Superimposed current traces recorded in the absence of ISO. **Panel B:** Superimposed current traces recorded in the presence of ISO. Five min after addition of 0.1 μM ISO both outward and inward TI generated at all test potentials were greatly increased. **Panel C:** I-V relations. The increases in peak TI were greater at test potentials away from the reversal potential. E_{REV} (-20 mV) was not significantly affected.

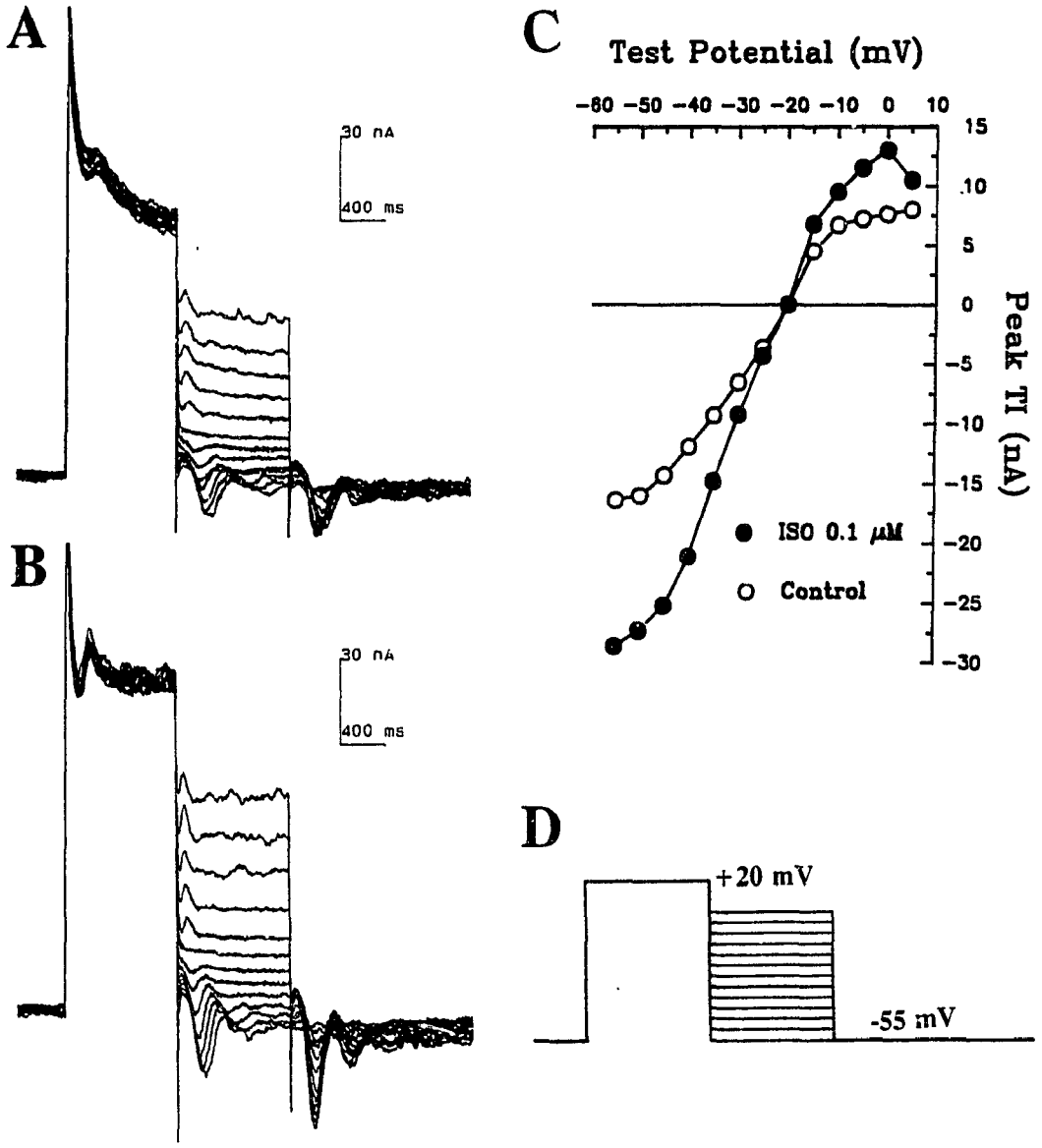

A
30 nA
400 ms
B
30 nA
400 ms
C
Test Potential (mV)
-60 -50 -40 -30 -20 -10 0 10
15
10
5
0
-5
-10
-15
-20
-25
-30
Peak TI (nA)
ISO 0.1 μM
Control
D
+20 mV
-55 mV

Figure 49.

Figure 50. β-adrenoceptor antagonist propranolol partially reversed the ISO stimulation on TI. **Panel A:** Superimposed current traces recorded in the absence (trace a), presence (trace b) of ISO, and after addition of propranolol (trace c) are shown in panel A. **Panel B:** Voltage steps applied to the preparation. Inward and outward TI were generated upon repolarizations to -5 and -55 mV, respectively. Following addition of 0.1 μM ISO, marked stimulations of both inward and outward TI were seen (trace b). Propranolol (0.2 μM) was added to the perfusing solution right at the time when submaximal effects were observed. Five min after addition of propranolol, both inward and outward TI were significantly decreased (trace c). However, both inward and outward TI were still much greater than those generated in the absence of ISO (trace a).

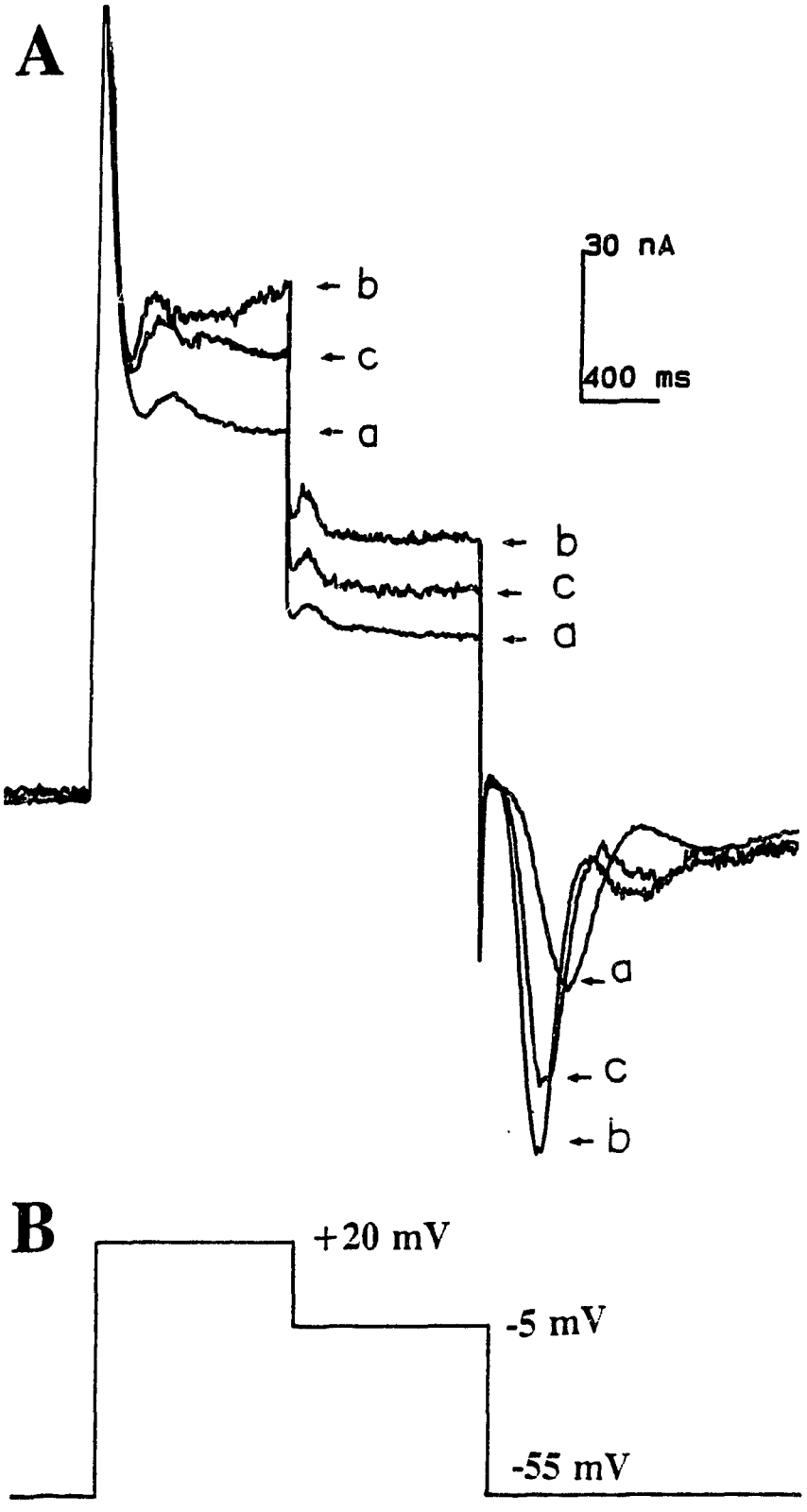


Figure 50.

Figure 51. Effects of forskolin (1μM) on high Ca^{2+} induced TI in the presence of $[Na^+]_o$. Experiments were performed and recorded under identical conditions as described for figure 49. **Panel A:** Superimposed current traces recorded before addition of forskolin. **Panel B:** Superimposed current traces recorded after addition of forskolin. **Panel C:** I-V relations of TI. **Panel D:** Voltage steps applied to the preparation. Five min after exposure to forskolin, both inward and outward TI were significantly increased at all test potentials (Panel B). The increases of peak TI were greater at the extremes of test potentials but the E_{REV} (-20 mV) was not affected (Panel C). Abbreviation: Forsk = Forskolin.

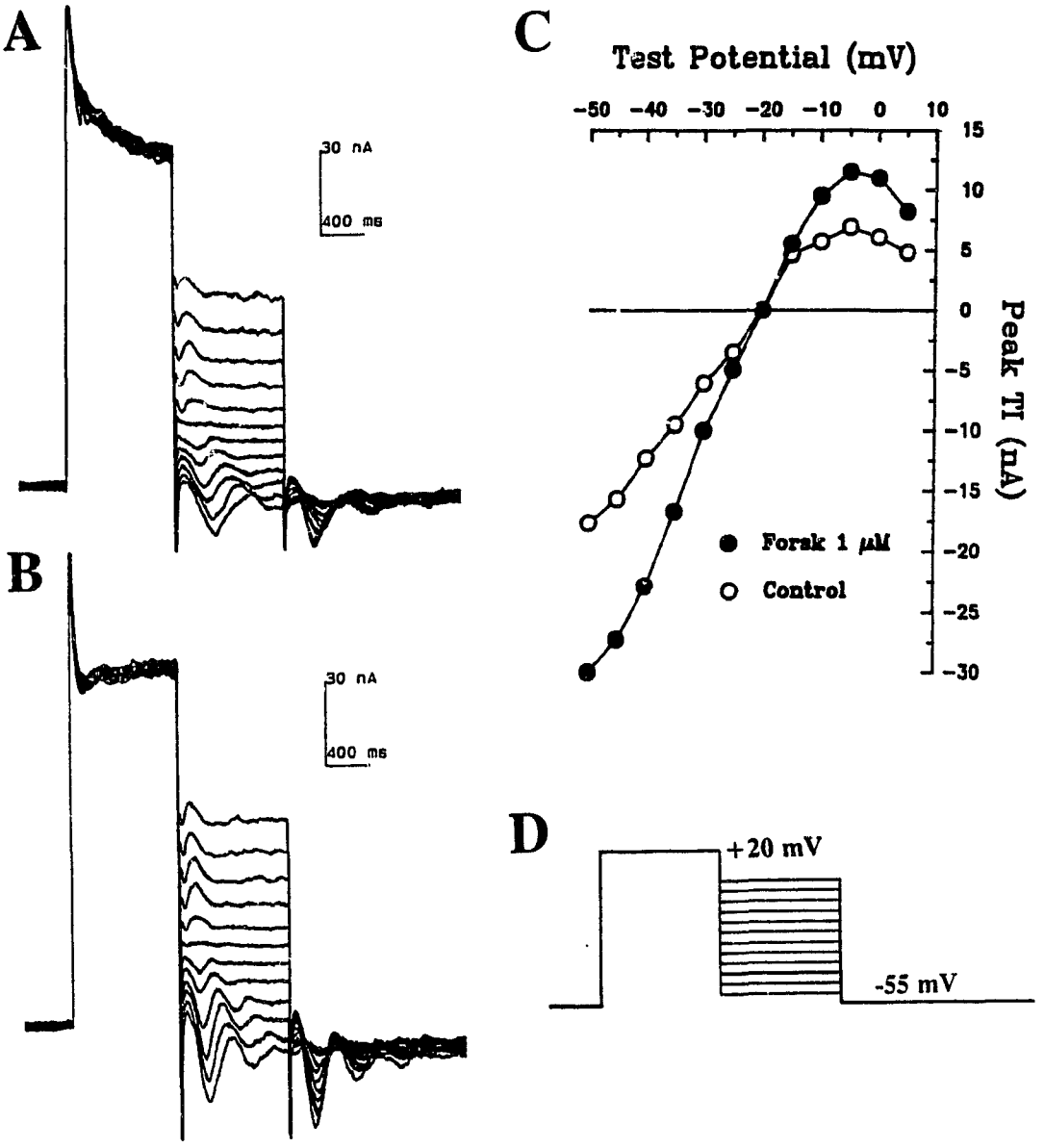

A
30 nA
400 ms
B
30 nA
400 ms
C
Test Potential (mV)
-50 -40 -30 -20 -10 0 10
15
10
5
0
-5
-10
-15
-20
-25
-30
Peak TI (nA)
Forsk 1 μM
Control
D
+20 mV
-55 mV

Figure 51.

Figure 52. Absence of stimulatory effects of forskolin (1 μM) on TI in Na^+-free solution. Experiments were performed and recorded under identical conditions to those described for figure 46. **Panel A:** Superimposed current traces recorded before (trace a) and after (trace b) addition of forskolin. **Panel B:** Voltage steps applied to the preparation. Neither the inward nor outward TI was changed after administration of forskolin. However, I_{step} was significantly increased by forskolin in the absence of $[Na^+]_o$.

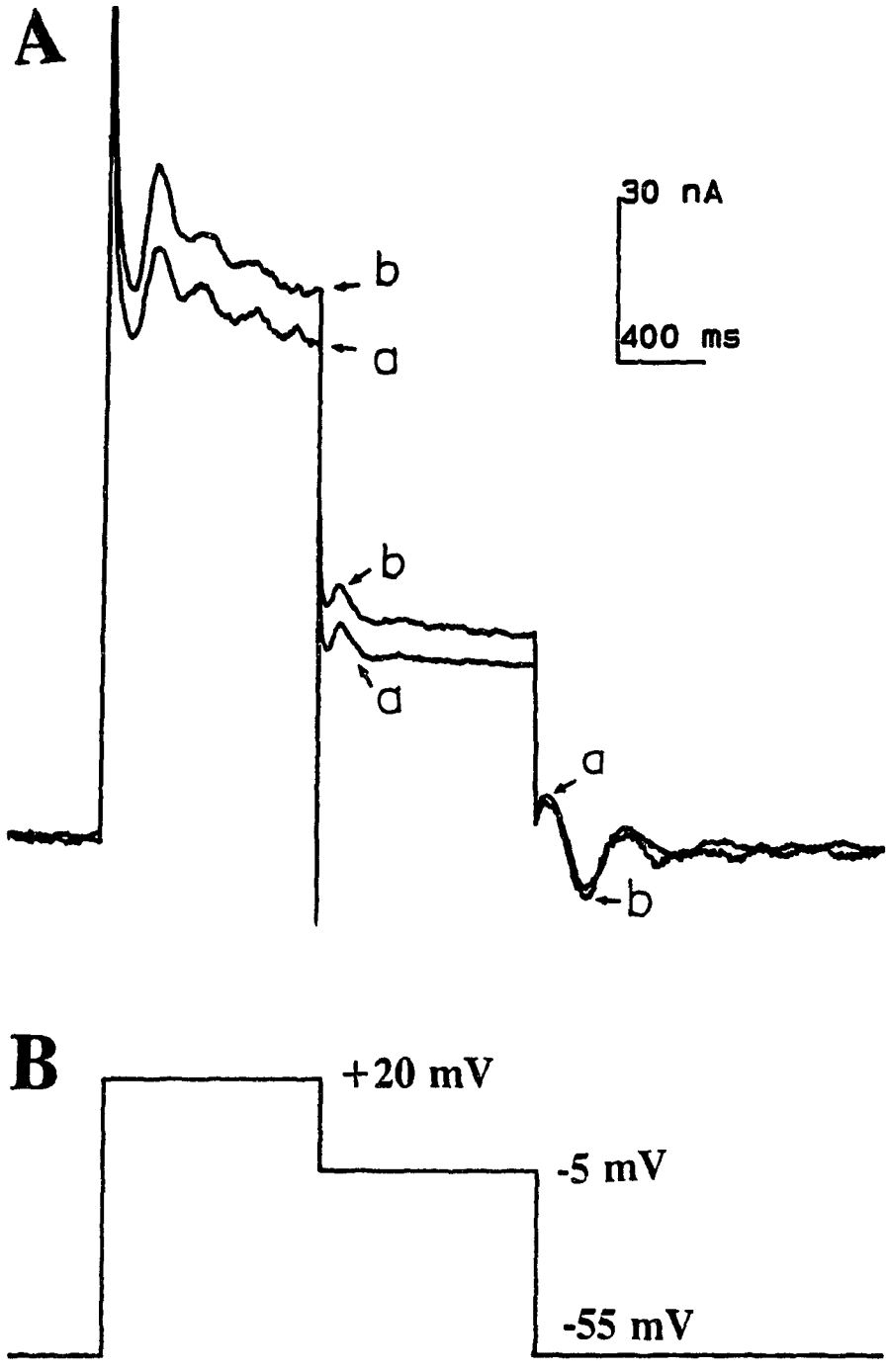


Figure 52.

Figure 53. Effects of quinacrine on the ISO (0.1 μM) stimulation of TI. **Panel A:** Voltage steps applied to the preparation. **Panel B:** Inward (large arrow) and outward (small arrow) TI induced in the absence of ISO. **Panel C:** Inward and outward TI generated in the presence of ISO. **Panel D:** Blockade of the ISO stimulation of TI by quinacrine. Three min after addition of ISO, both inward and outward TI were dramatically increased (Panel C). At this time, quinacrine (10 μM) was added into the perfusing solution. Both inward and outward TI were drastically decreased following 5 min exposure to quinacrine (Panel D).

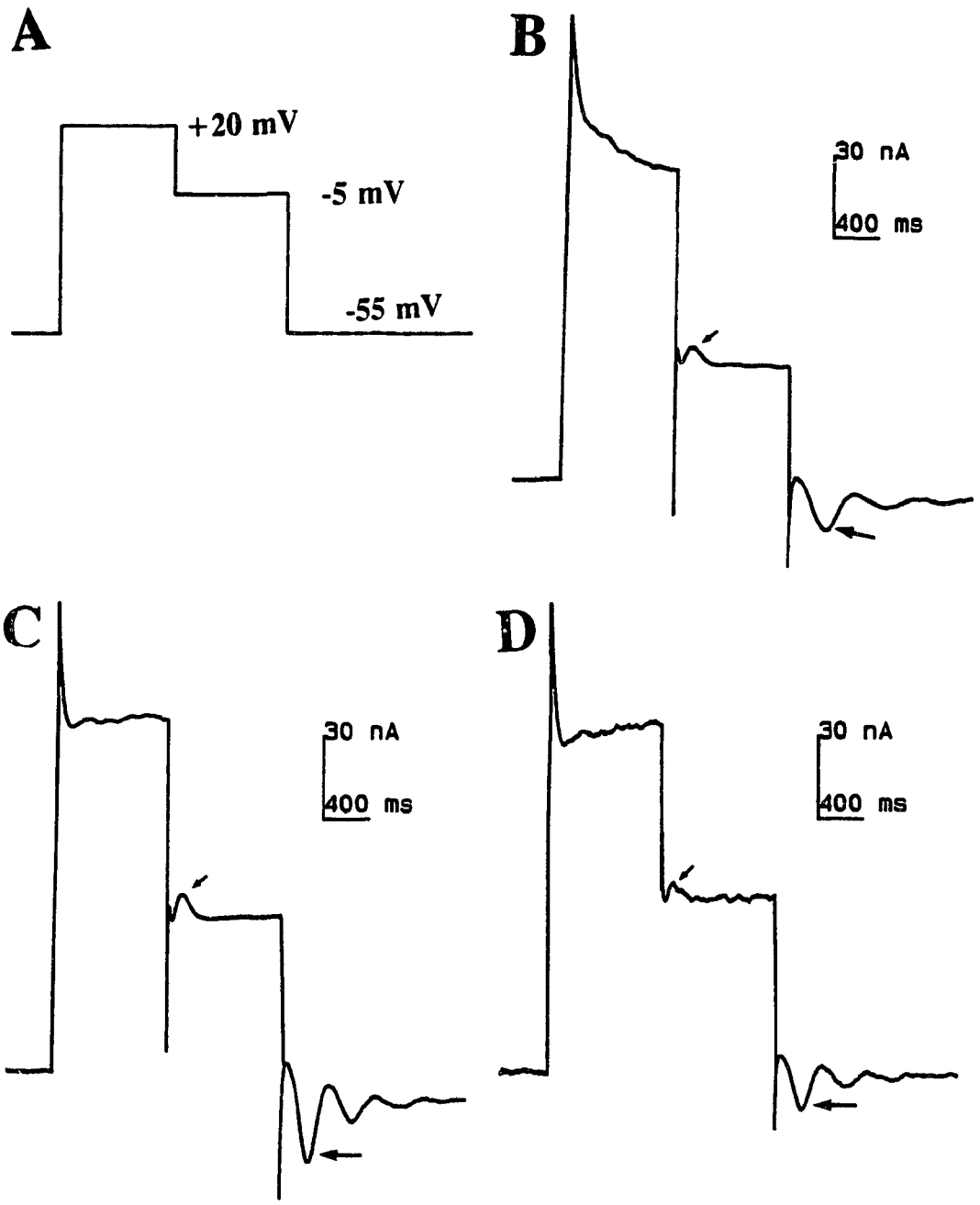


Figure 53.

DISCUSSION

The present projects have been designed and carried out to address two major questions: 1) what is/are the mechanism(s) of adrenergic arrhythmogenesis? 2) what is the ionic basis and what are the charge-carrying systems of the arrhythmogenic TI? These questions are closely related, and are of clinical importance. Therefore, they are the focus of research interest for many basic and clinical cardiologists. Adrenergic stimulation may initiate cardiac arrhythmias by promoting reentry or by potentiating impulse formation (Vaughan Williams, 1989). This thesis is concerned with effects on impulse formation; especially those mediated by stimulation of OAP and TI. The following discussion will be concentrated on experimental evidence related to effects of adrenergic stimulation on induction of OAP and TI and the ionic mechanisms underlying generation of TI.

I. MECHANISMS OF ACTION OF α-ADRENERGIC AGENTS ON GENERATION OF OAP AND TRIGGERED ACTIVITY

OAP are believed to be important in the genesis of cardiac arrhythmias in a wide variety of settings (Ferrier, 1977; January and Fozzard, 1988; Ferrier, 1991). Conflicting observations have been reported in different animal species and under different experimental conditions, regarding whether α_1-adrenergic stimulation can initiate arrhythmias by promoting induction of OAP (see Introduction). In voltage clamped rabbit cardiac Purkinje fibres, Ferrier and Carmeliet (1990) demonstrated that phenylephrine could either inhibit or stimulate the TI, believed to generate OAP, depending on whether the current was induced by digitalis or elevated Ca^{2+}. Both effects were mediated by α_1-adrenocepter stimulation. These observations suggest that α_1-adrenergic agonists may exert pro- or antiarrhythmic effects under non-voltage clamp conditions. It is essential to test this hypothesis experimentally. Therefore, the

first part of the present study assessed effects of α_1- and α_2-adrenergic agonists and blockers on induction of OAP and triggered activity by acetylstrophanthidin and elevated Ca^{2+} concentration in a single species.

In the present experiments, the following effects were demonstrated: (1) PE, a relatively specific alpha-1 adrenergic receptor agonist, suppressed AS-induced OAP and triggered activity; (2) the same agonist, PE, increased the amplitude of OAP and promoted triggered activity induced by high Ca^{2+} low K^+ Tyrode's solution; (3) both the stimulatory and the inhibitory effects of PE were blocked by pretreatment with a specific α_1-adrenergic blocker, prazosin; (4) 3 adrenergic antagonists (prazosin, yohimbine and propranolol) each with a different specific adrenergic blocking action, strongly suppressed OAP and triggered activity induced by either acetylstrophanthidin or high Ca^{2+}, low K^+ Tyrode's solution. The inhibitory effects of these agents on OAP occurred in the absence of exogenous agonist.

This study also demonstrated that rabbit Purkinje fibres provided a suitable model for the study of OAP and agents that affect induction of OAP. Results showed that generation of OAP in rabbit Purkinje fibres occurred over a narrow range of AS concentrations (0.1 to 0.4 μg/ml). A slight increase in AS caused triggered activity, but a slight decrease resulted in failure to induce OAP. OAP induced in rabbit Purkinje fibres also showed a dependence on preceding BCL similar to that reported in other preparations (Ferrier, 1977; Ferrier et al., 1973). When the preceding BCL was decreased, the amplitude of the most prominent OAP increased. Thus, induction of OAP in this model was very stable and reproducible and allowed detailed study of agents affecting generation of OAP and triggered activity.

The mechanism by which α_1-adrenergic stimulation by phenylephrine exerted both an inhibitory effect on AS-induced OAP and a stimulatory effect on high Ca^{2+} induced OAP is a question which deserves further investigation. Either effect could

be prevented by prazosin at a concentration that blocked α_1-receptors but which exerted little direct inhibition of OAP. This would suggest that differences in effect must be related to events downstream from drug-receptor interaction. The opposite effects of PE could depend on involvement of different steps in the chain of events leading to generation of OAP with the two methods used to induce OAP.

The mechanisms underlying generation of OAP are still under active investigation. TI has been shown to be the current responsible for OAP. The ionic basis of TI will be discussed in detail in the later part of Discussion. However, it is generally accepted that digitalis agents cause an accumulation of intracellular Na^+ by inhibiting the sarcolemmal Na^+/K^+ ATPase, and that intracellular Ca^{2+} accumulation then results by way of Na^+/Ca^{2+} exchange. Increased intracellular Ca^{2+} has been hypothesized to trigger oscillatory release of Ca^{2+} from the sarcoplasmic reticulum intracellularly (Fabiato, 1983; 1985c). The oscillations in Ca^{2+} may then act upon the sarcolemma to activate the TI which is the immediate cause of OAP (January and Fozzard, 1988; Ferrier, 1991).

Suppression of AS-induced OAP by PE could be mediated by actions of PE on one or more of the above processes. A decrease in intracellular Na^+ concentration would be expected to suppress OAP caused by the chain of events described above. It has recently been reported that α-adrenoceptor stimulation can decrease intracellular Na^+ concentration possibly by increasing Na^+/K^+ pump activity (Shah et al., 1988). Intracellular Na^+ concentration also could be influenced by changes in influx. However, our observations indicated that suppression of OAP by PE was not accompanied by inhibition of Na^+ influx (I_{Na}) during the action potential upstroke, since Vmax at various "take-off" potentials was not changed by PE. Thus, decrease in intracellular Na^+ most likely occurred secondarily to stimulation of the Na^+/K^+ pump. This action might be one mechanism by which PE exerted its inhibitory effect

on digitalis induced OAP since the primary event in induction of OAP by digitalis was inhibition of this transport system. The relatively slow development of the inhibitory effect illustrated in figure 7 was compatible with an indirect effect that involved a gradual change in steady state intracellular ionic concentrations.

Exposure of Purkinje tissue to high Ca^{2+} concentrations also induced OAP (Ferrier, 1977), most likely by directly increasing Ca^{2+} currents (I_{Ca}, Reuter, 1983; Tsien et al, 1986; Pelzer et al, 1990; 1991) and/or stimulating Na^+/Ca^{2+} exchange (Deitmer and Ellis, 1978). Since inhibition of the Na^+/K^+ pump was bypassed by this method, one might hypothesize that alpha adrenergic stimulation of Na^+/K^+ pump would not offset the intracellular Ca^{2+} overload caused by elevation of extracellular Ca^{2+}. Indeed, although intracellular Na^+ activity was increased by digitalis (Eisner and Lederer, 1979), it was decreased by high Ca^{2+} perfusion (Deitmer and Ellis, 1978). These considerations might explain attenuation or absence of the inhibitory effect of PE on high Ca^{2+}-OAP. On the other hand, other actions of PE might contribute to a stimulatory effect observed with high Ca^{2+}. Alpha-adrenergic stimulation has been shown to induce Ca^{2+} dependent action potentials and increase the slow inward Ca^{2+} current (Bruckner and Scholz, 1984). In addition, effects on phosphoinositide metabolism have been reported to mobilize intracellular Ca^{2+} (Otani et al, 1988). In either case, an increase in intracellular Ca^{2+} concentration might promote induction of OAP.

The opposing effects of phenylephrine also may be related to the involvement of different α_1-adrenergic receptor subtypes (Minneman, 1988). At least two distinct α_1-adrenergic receptor subtypes have been demonstrated in adult cardiac tissue (del Balzo et al, 1990). One subtype which can be selectively antagonized by chloroethyl-clonidine (CEC) is linked to a decrease in automaticity via a pertussis toxin-sensitive substrate (del Balzo et al, 1990). Activation of this subtype of α_1-adrenoceptor also

stimulates the Na^+-K^+ pump current (Shah et al, 1988; Zaza et al, 1990) which may oppose the effect of digitalis and thereby decrease the amplitude of digitalis-induced OAP. Another subtype which can be selectively blocked by WB 4101 is linked to the stimulation of inositol phosphate accumulation (del Balzo et al, 1990; Otani et al, 1988). Mobilization of $[Ca^{2+}]_i$ by inositol trisphosphate (IP_3) may be an important mechanism underlying the stimulation of OAP induced by high Ca^{2+}.

Because the K^+ concentration was reduced to 2 mM in experiments utilizing elevated Ca^{2+}, one may question whether the test system is clearly different from that generated by AS. It is known that extreme hypokalemia ([K] = 0 - 0.5 mM) by itself can induce OAP, presumably by inhibiting the Na^+-pump (Eisner and Lederer, 1979a,b). The combination of 8 mM Ca^{2+} and 2 mM K^+ was chosen in order to use the same system utilized in earlier voltage clamp studies (Ferrier and Carmeliet, 1990). In the present study 8 mM Ca^{2+} induced only very small OAP when the K^+ concentration was 4 mM, but large OAP when the concentration was reduced to 2 mM. The increase in amplitude of OAP might result from inhibition of the Na^+-pump or by a decrease in potassium currents opposing the TI. Voltage clamp studies by Kass et al (1978) have demonstrated that decreasing the K^+ concentration from 4 to 1 mM has no effect on the magnitude of the TI in Purkinje fibres at any given membrane potential. This observation suggests that reducing the K^+ to 2 mM increases the amplitudes of OAP primarily by reduction of the background membrane conductance and polarizing currents rather than by an additive effect on the TI mediated by inhibition of the Na^+-pump. Aronson and Nordin (1988) reached similar conclusions from observations made in experiments on isolated ventricular myocytes.

The antiarrhythmic activity of α_1-adrenergic antagonists has been observed both in vivo and in vitro in many Ca^{2+}-loaded preparations, including, ischemia and reperfusion (Sheridan et al., 1980; Stewart et al., 1980). The antiarrhythmic effect has been

attributed to α_1-adrenergic antagonism, although Na^+ channel blocking activity (Type I antiarrhythmic activity) has long been known for phentolamine (Northover, 1983). This study showed that OAP and triggered activity induced by either digitalis intoxication or high Ca^{2+} could be suppressed directly by prazosin, yohimbine, or propranolol at concentrations slightly higher than that giving only specific receptor blockade. The significance of the direct inhibitory effects was underscored by the observation that OAP induced by AS and high Ca^{2+} perfusion showed different responses to PE but the same response to prazosin.

Prazosin, yohimbine, and propranolol can directly block the fast Na^+ channel without involvement of alpha adrenergic antagonism (Azuma et al., 1978; Northover, 1983). Thus local anaesthetic action may explain at least part of the antiarrhythmic efficacy of these agents. However, prazosin inhibited the TI at a concentration that did not affect Na^+ influx (Ferrier and Carmeliet, 1990).

In the present study, the α_2-agonist clonidine had no effect on OAP. Mugelli et al (1986) reported that yohimbine inhibited automaticity induced by norepinephrine in hypoxic and glucose free solution. The latter observations suggest a possible role of α_2-adrenoceptor stimulation in this setting. The differences between the observations in the two studies might be related to a change in receptor availability with hypoxic conditions or possibly different mechanisms of underlying the induction of spontaneous beats in the two studies.

These results clearly demonstrate that studies reporting antiarrhythmic effects of adrenergic blocking drugs, but not experimentally addressing the possibility of non-adrenergic actions, can not be accepted as reliable evidence of a proarrhythmic role for α-adrenoceptor stimulation. Identification of specific α-adrenergic mechanisms with blocking agents requires that α-adrenergic stimulation is tested after pretreatment with the blocking agent, rather than adding the blocker in the continued presence of

agonist. This is important in order not to confuse nonspecific actions with blockade of specific adrenergic receptors. Furthermore, the present observations indicate that α-adrenergic agonists may, in fact, exert either pro- or antiarrhythmic actions on OAP and triggered activity depending on the agent or conditions that induced these phenomena. These observations further suggest that α_1-adrenergic blocking agents also might exert either pro- or antiarrhythmic effects depending on the effect that α_1-adrenoceptor stimulation has under a given set of conditions.

II. MECHANISMS OF ACTION OF β-ADRENERGIC AGENTS ON TI

Stimulation of β-adrenoceptors with isoproterenol has been shown to increase the amplitude of OAP induced by ouabain in isolated canine Purkinje fibres (Hewett and Rosen, 1984), spontaneous OAP in isolated ventricular myocytes (Belardinelli and Isenberg, 1983), as well as OAP occurring in feline Purkinje fibres surviving in 2 to 4 month old infarcts (Kimura et al, 1987). Catecholamines can increase the peak TI in atrial cells of the coronary sinus (Tseng and Wit, 1987), sino-atrial node cells (Satoh and Hashimoto, 1988), and in sheep Purkinje fibres (Vassalle and Mugelli, 1981), but it is not clear whether catecholamines actually induce the current, if it is not already present. Although it has been shown that OAP and TI may be generated by intracellular injection of catalytic subunit of cAMP-dependent protein kinase (Brum et al, 1983), it is not clear whether induction of TI can occur through β-adrenoceptor stimulation.

Beta-adrenergic stimulation enhances the inward Ca^{2+} current (I_{Ca}) (Reuter and Scholz, 1976; Tsien, 1977; Pelzer et al, 1991). It has been assumed that enhancement of OAP by β-adrenoceptor stimulation is secondary to stimulation of I_{Ca} with consequent elevation of intracellular Ca^{2+} (Belardinelli and Isenberg, 1983). Therefore, the second part of the present study was aimed at determining if the β-adren-

ergic agonist, isoproterenol (ISO) can induce the TI in rabbit Purkinje fibres, and to determine the mechanism by which this agent promotes induction of this current.

The present study demonstrated that the β-adrenergic agonist ISO could strongly increase the magnitude of the peak TI after the current had been induced by some other means. However, the TI could not be induced *de novo* by ISO. Approximately 15% of rabbit Purkinje fibres showed a spontaneous TI similar to that reported by Vassalle and Mugelli (1981) and termed I_{os}. ISO potentiated this spontaneous current. Under non-voltage clamp conditions, outward potassium currents would be expected to oppose depolarization by the TI. Thus, it was possible that the magnitude of the spontaneous TI, in the absence of catecholamine, might not be sufficient to produce a measurable OAP. Apparent induction of OAP by β-adrenergic agonists especially in larger multicellular preparations, such as the coronary sinus, might actually represent potentiation of spontaneous but weak TI in some or all of the cells (Tseng and Wit, 1987a). Indeed, Tseng and Wit (1987a) reported the occurrence of spontaneous TI in small voltage clamp preparations from the coronary sinus. However, it is not clear from either their study or the present one, whether the spontaneous TI represents a normal physiological phenomenon, or a phenomenon induced by dissection and mounting of the preparations.

Potentiation of the TI by ISO was not limited to increase in peak current. The activation voltage shifted so that weaker depolarizing steps could elicit the TI, and the I-V relationship was extended so that the TI was observed over a wider range of membrane potentials. In addition there was a trend for the maximum peak current to occur at potentials closer to physiological MDP, although this trend did not reach statistical significance.

The shift in minimum repolarization to elicit TI might represent a shift in the reversal potential or an increase in magnitude of the current seen at membrane

potentials approaching 0 mV. The latter appeared more likely since examination of all 5 I-V relationships revealed that the control and ISO curves converged as the test voltage steps approached 0 mV. This was close to the reversal potential for the TI reported in earlier studies on calf Purkinje fibres (Kass et al, 1978a and b). The clamp protocol used to determine the I-V relationships in the present study did not use sufficiently positive activation voltages to measure currents at potentials positive to the reversal potential, and therefore to clearly demonstrate the occurrence of a true reversal potential. Earlier studies in calf Purkinje fibres utilized activating steps to +25 mV (Kass et al, 1978a and b). Frequent steps to these positive levels with electrodes like those used in this study for rabbit Purkinje fibres (5 - 8 MΩ and filled with 2.7 M KCl), often caused deterioration of the current passing properties of the electrodes. Therefore, steps to very positive voltages were avoided at this stage of experiments.

Agents that block Ca^{2+} influx through I_{Ca} channels result in a gradual decline in the magnitude of the TI (Kass et al, 1978a). The time course of this effect is much slower than the blockade of I_{Ca}. Therefore, it is believed that the inhibitory effect on TI is indirect and is mediated by a gradual decrease in intracellular Ca^{2+} levels (Kass et al, 1978a). In contrast, β-adrenergic stimulation enhances I_{Ca} (Reuter and Scholz, 1976; Tsien et al, 1986; Sperelakis, 1988; Shuba et al, 1990). This occurs through elevation of cAMP levels and phosphorylation of I_{Ca} channels, with subsequent increases in density of available channels and increases in opening probability (Tsien et al, 1986). It therefore would seem logical that β-adrenoceptor stimulation would increase the magnitude of the TI by increasing intracellular Ca^{2+} levels (Belardinelli and Isenberg, 1983). However, the present investigation demonstrated that blockade of I_{Ca} did not block the ability of ISO to cause a rapid increase in magnitude of the TI. This observation does not eliminate the possibility that modulation of the I_{Ca}

channel by β-adrenoceptor stimulation can cause gradual changes in the magnitude of the TI, but it does indicate that another mechanism must contribute or be responsible for the rapid response to ISO.

Quinacrine, a putative inhibitor of Na^+-Ca^{2+} exchange (De La Pena and Reeves, 1987; Kaczorowski et al, 1989), abolished stimulation of AS-induced TI by ISO. One may question the specificity of quinacrine, and therefore, whether blockade of ISO was mediated specifically by inhibition of Na^+-Ca^{2+} exchange. Several observations strongly favoured inhibition of Na^+-Ca^{2+} exchange. Amiloride, a second inhibitor of Na^+-Ca^{2+} exchange, that is chemically unrelated to quinacrine, also prevented the stimulatory effect of ISO on TI. In addition, it is highly unlikely that quinacrine acted indirectly by inhibiting I_{Ca} since, when both currents were stimulated by ISO, quinacrine preferentially reversed stimulation of TI without affecting I_{Ca} (figure 21). With longer exposure to quinacrine (10 to 20 min), we did observe a slight decrease in peak amplitude of I_{Ca}, but this occurred well after marked inhibition of TI. Furthermore, inhibition of I_{Ca} by Mn^{2+} or verapamil failed to block stimulation of TI by ISO. An effect on I_{Na} also is unlikely to have contributed to blockade of ISO. Firstly, ISO itself inhibits I_{Na} (Schubert et al, 1990; Hisatome et al, 1985). Secondly, in experiments in which I_{Na} was inactivated with a holding potential of -50 mV, quinacrine still abolished the stimulatory effect. These observations strongly suggests that rapid stimulation of the TI by ISO is mediated by stimulation of Na^+-Ca^{2+} exchange.

Inhibition of the Na^+-K^+ ATPase by digitalis will result in accumulation of $[Na^+]_i$. Elevation of $[Na^+]_i$ favours Ca^{2+} load through Na^+-Ca^{2+} exchange during depolarization (Sheu et al, 1986; Brill et al, 1987; Blaustein, 1988). Ca^{2+} overloaded cardiac cells exhibit oscillatory release of Ca^{2+} from the SR and consequently oscillatory activation of the TI (Kass et al, 1978a; 1978b; Ferrier, 1991). Stimulation of

Na^+-Ca^{2+} exchange by ISO might further increase intracellular Ca^{2+} levels and thereby cause stronger activation of the TI. The inhibitory effect of quinacrine also could be mediated by actions on intracellular Ca^{2+} load through inhibition of Na^+-Ca^{2+} exchange. This is most likely the mechanism for the action of quinacrine since TI was only partly inhibited at a time when the effects of ISO were completely abolished.

If β-adrenergic agonists stimulate Na^+-Ca^{2+} exchange, one can suggest an explanation for the observation that ISO can stimulate TI, once induced, but cannot itself induce the current. In the absence of elevated intracellular Na^+ levels, stimulation of Na^+-Ca^{2+} exchange would not be expected to increase intracellular Ca^{2+} levels if Na^+ and Ca^{2+} levels are already at steady state. However, if intracellular Na^+ accumulation is induced by digitalis, and if Na^+-Ca^{2+} exchange has not reached steady state, stimulation of Na^+-Ca^{2+} exchange may further increase Ca^{2+} load and thereby potentiate the TI and OAP.

III. IONIC MECHANISMS UNDERLYING GENERATION OF TI BY ELEVATION OF $[Ca^{2+}]_o$

The next part of the present study was designed to address the following questions in the absence and presence of $[Na^+]_o$: 1) Whether TI can be routinely induced by high Ca^{2+}. 2) Whether a clear-cut reversal potential of TI can be routinely revealed. 3) Which ionic components contribute to the TI. 4) If both cationic and anionic channels were involved in the generation of TI, could they be preferentially blocked with certain agents. 5) Whether Na^+-Ca^{2+} exchange participates in the generation of TI in the presence of $[Na^+]_o$. Experiments were performed in voltage-clamped rabbit cardiac Purkinje fibres. Our experiments to address these questions presented evidence that the TI induced by elevation of $[Ca^{2+}]_o$ in Purkinje fibres was carried by

ionic conducting channels rather than Na^+-Ca^{2+} exchange, even in the presence of $[Na^+]_o$, and that TI channels consisted of at least two different populations, cationic and anionic. Information derived from this study may be helpful in evaluating the mechanisms by which a number of arrhythmogenic (in particular, adrenergic agonists as discussed above) and antiarrhythmic agents exert their actions.

1. Induction and Modulation by Calcium

An oscillatory membrane current which could be routinely induced in rabbit cardiac Purkinje fibres exposed to elevated $[Ca^{2+}]_o$ in the absence of Na^+ was demonstrated in the present investigation. That this current was a true current can be inferred from observations that: 1) it had a distinct reversal potential; 2) it was inducible by moderately elevated $[Ca^{2+}]_o$; and 3) the preparations were not seriously damaged during its induction. However, since TI was induced in the absence of $[Na^+]_o$ under our experimental conditions, Na^+-Ca^{2+} exchange could not be the charge carrier of this current. This current was similar to the transient inward current described previously (Lederer & Tsien, 1976; Kass et al, 1978a, b; Eisner & Lederer, 1979a, b; Karagueuzian & Katzung, 1982; Noble, 1984; Arlock & Katzung, 1985; Cannell & Lederer, 1986; Noble, 1986; Brown et al, 1986; Fedida et al, 1987; Lipp & Pott, 1988; Han & Ferrier, 1991) in that it was generated by repolarization from a previous depolarizing pulse and followed the similar time course, and occurred in response to Ca^{2+} overload.

Various studies have shown that Ca^{2+} overload must be present in order to induce TI. In the present study we demonstrated that either zero $[Ca^{2+}]_o$ or substitution of Ca^{2+} by Ba^{2+} led to failure of TI generation. We also confirmed that both inward and outward TI were activated by the transient release of Ca^{2+} from the SR, because both inward and outward TI were abolished by ryanodine. Ca^{2+} overload can be achieved through two possible pathways under our experimental conditions. First, elevation of

$[Ca^{2+}]_o$ may cause increased Ca^{2+} influx through both L- and T-type calcium channels (I_{Ca}). Second, the concentration of Ca^{2+} required to cause intracellular overload may be reduced in the absence of $[Na^+]_o$, because Ca^{2+} extrusion by Na^+-Ca^{2+} exchange was eliminated (Chapman, 1983; Bridge et at, 1990). Thus 5 mM $[Ca^{2+}]_o$ was sufficient to activate the inward TI (figure 22). When $choline^+$ was substituted for Na^+, the major cation which could result in inward TI was Ca^{2+} (see later). Under these conditions elevation of $[Ca^{2+}]_o$ would be expected to affect not only the degree of Ca^{2+} overload, which may roughly determine the TI channel conductance, but also the transmembrane Ca^{2+} gradient which contributes to the influx of positive charges for TI. The absence of TI in 2.5 mM Ca^{2+} solution may thus be explained by a lower channel conductance and a smaller concentration gradient for Ca^{2+}.

The effect of elevating $[Ca^{2+}]_o$ on peak TI was biphasic. A sudden rise of Ca^{2+} concentration from 5 to 15 mM resulted in significant increase of peak TI while further elevation of $[Ca^{2+}]_o$ from 30 to 50 mM resulted in a decrease in TI amplitude (figure 23). In many tissues including heart, Ca^{2+} current saturates at about 30 mM $[Ca^{2+}]_o$ (Pelzer et al, 1990). This indicates that elevation of $[Ca^{2+}]_o$ within the concentration range between 2.5 to 30 mM may increase Ca^{2+} influx through I_{Ca}. Elevating $[Ca^{2+}]_o$ to 50 mM may not increase the degree of Ca^{2+} overload through I_{Ca} due to its saturable nature. On the other hand, high concentrations of $[Ca^{2+}]_o$ may decrease the sensitivity of the channel protein to the activation voltage, and therefore decrease the conductance of TI channels (Hille, 1992).

2. Which Ions Can Carry TI

Previous studies, either at the single channel level (Ehara et al, 1988; Hill et al, 1988) or at the multicellular level (Kass et al, 1978b; Cannell & Lederer, 1986) supported the idea that only cations carry TI. In experiments in which a clear-cut reversal potential had been demonstrated, the inward TI was believed to be carried

mainly by Na^+ (Kass et al, 1978b) or in the case of isotonic Ca^{2+}, by Ca^{2+} (Cannell & Lederer, 1986), and the outward TI was attributed to K^+ efflux. Our study with 30 mM Ca^{2+} (sucrose and cholineCl substitutions) supported the experimental findings of Cannell and Lederer (1986) that Ca^{2+} could pass through the non-selective channel to carry inward TI with a peak amplitude comparable to that of TI observed in Na^+-containing solutions. It was possible that substitution of sucrose for NaCl might facilitate Ca^{2+} movements by minimizing any competition between Ca^{2+} and Na^+ ions for channel binding sites and by reducing ionic strength and increasing negative surface potential. We were aware that $choline^+$ might be moderately permeable in some cardiac cells at rest. However, it had no effect on membrane potential and resistance in Purkinje fibres (Hall et al, 1963). The effect of sucrose was thought to be primarily osmotic in nature (Arlock & Katzung, 1985). The fact that peak inward TI was not altered by switching between cholineCl and sucrose suggested that the contribution of $choline^+$ to the inward TI was negligible.

A major observation of this study was that the TI conducting channels permit Cl^- to pass. It was clear that E_{REV} was much less positive (-21 mV to -25 mV) when LiCl or cholineCl was the substitute for Na^+ than when sucrose was the substitute. When sucrose was used as the substitute, E_{REV} shifted in the positive direction and the outward TI magnitude was markedly decreased (figure 31). This shift could not be explained if K^+ was the only source of outward TI since the concentration of extracellular K^+ was zero with either ion substitution. For the same reason, intracellular Na^+ was highly unlikely to be the charge carrier of outward TI. One might argue that sucrose substitution may shift the I-V relation due to the decreased ionic strength. However, in this case the I-V relationship would be expected to shift in the negative direction (Hille, 1992).

The consistent shift in E_{REV} provided additional evidence against the possible

participation of choline$^+$ as a carrier of inward current, because if choline was a charge carrier, E_{REV} would be expected to shift in the positive direction. The possibility that Ca^{2+} might also carry the outward TI could be readily ruled out because E_{Ca} lay much more positive to E_{REV} observed in this study. In light of the consideration above, the only ion left which could carry outward TI was Cl^-. In fact, the shift of E_{REV} observed in the presence of 30 mM $[Ca^{2+}]_o$ was roughly in accord with the changes in Cl^- gradient (figure 31). Assuming $[Cl^-]_i$ is 30 mM (Vaughan Williams, 1990), at 37°C (30 mM $CaCl_2$, 105 mM cholineCl) the E_{Cl} calculated from Nernst equation would be: $E_{Cl} = RT/ZF \times Ln[Cl^-]_i/[Cl^-]_o = 60.5 \times \log_{10}30/60+105 = -45$ mV. When the concentration of cholineCl was decreased to 52.5 and 0 mM, E_{Cl} will be $60.5 \times \log_{10}30/60+52.5 = -34$ mV and $60.5 \times \log_{10}30/60 = -18$ mV, respectively. The net shifts of E_{Cl} caused by the changes of $[Cl^-]_o$ would be 11 and 16 mV, which were very close to what was observed in our experiments (figure 31). E_{REV} of TI, which was about -25 mV in the presence of 105 mM cholineCl, lay 20 mV positive to E_{Cl}, suggesting net inward current (mainly carried by Ca^{2+}) might contribute to the net TI amplitude, and to the absolute value of E_{REV}. That the magnitude of peak outward TI was dramatically and selectively reduced by two common anion transporter blockers, DIDS and SITS, suggested that the Cl^- conductance might represent a different population of channels from those mediating the inward TI.

There was a small shift (about 5 mV) in E_{REV} in the negative direction on changing $[Ca^{2+}]_o$ from 10 to 30 mM. Statistically, the shift was not significant ($p = 0.055$). However, when $CaCl_2$ was elevated to 50 mM, E_{REV} shifted further in the negative direction ($p < 0.05$ compared to E_{REV} obtained with 10 mM $CaCl_2$). This shift was not in the positive direction as one would predict if Ca^{2+} was the primary charge carrier. It was important to note that $[Ca^{2+}]_o$ was increased by addition of

$CaCl_2$. Thus, both $[Ca^{2+}]_o$ and $[Cl^-]_o$ were increased. While the effects on the two reversal potentials tend to cancel, shift in E_{REV} in the positive direction as expected with elevation of $[Ca^{2+}]_o$ might be minimal due to the simultaneous increase in $[Ca^{2+}]_i$. In addition, elevation of $[Ca^{2+}]_o$ in this range activated outward (Cl^-) TI more than inward (Ca^{2+}) TI (see fig. 27). Therefore, one would expect a net negative shift in apparent reversal. This corresponded well with our experimental observations. However, since $[Ca^{2+}]_i$ and $[Cl^-]_i$ could not be measured in the present experiments, the quantitative contributions of $[Ca^{2+}]_o/[Ca^{2+}]_i$ and $[Cl^-]_i/[Cl^-]_o$ to the E_{REV} remained to be determined.

In normal physiological solutions, E_{REV} may reflect a balance among E_{Na}, E_{Ca}, E_{Cl}, and E_K. Since E_{Cl} was -45 mV in the presence of 105 mM cholineCl, the contribution of Cl^- to inward TI at a test potential of -55 mV was very small due to the small driving force (10 mV). Indeed, 10 μM DIDS only slightly decreased inward TI at -55 mV (figures 32, 43). It was also possible that the channels which permit Cl^- to pass had a strong rectifying property at potentials negative to E_{Cl}. This might be why we did not detect a difference in peak inward TI when switching from sucrose to cholineCl and vice versa (figure 30). However, the exact contribution of K^+ to outward TI, or the influence of E_K on E_{REV} could not be determined by the present study. That E_{REV} lay positive to both E_{Cl} and E_K indicated that both ions might be charge carriers for outward TI (Cl^- influx, K^+ efflux). The fact that E_{REV} shifted in response to changes in $[Cl^-]_o$ did not exclude the possible influence of E_K on E_{REV}.

The identity of the channels that permit Cl^- to pass has not yet been resolved. Outward TI responded quite differently from inward TI to DIDS and SITS. This suggests that more than one type of TI channel may be involved in the generation of TI. There are several possible conductances that might account for the outward TI:

Cl^- channel; I_{TO} channel; non-selective cationic channel. The possible contributions of these conductances to outward TI are discussed below.

1) Cl^- Channel: The existence of Cl^- conductance in cardiac myocytes has been the topic of many studies. Early studies on multicellular preparations (Carmeliet, 1961; Hutter & Noble, 1961) indicated the presence of a Cl^- conductance in cardiac sarcolemma. A distinct single-channel activity representing Cl^- current has been recorded in cardiac sarcolemmal vesicles incorporated into lipid bilayer membrane (Coronada & Latorre, 1982). Also, a cyclic AMP-dependent protein kinase-regulated Cl^- conductance which is activated by β-adrenergic stimulation and suppressed by acetylcholine has been identified in isolated guinea-pig ventricular myocytes (Bahinski et al 1989; Harvey & Hume, 1989; Matsuoka et al, 1990). More recently, Zygmunt and Gibbons (1991) reported a Ca^{2+}-activated Cl^- current in rabbit ventricular myocytes. It is very possible that this current could carry TI in the outward direction. In this case two different channels would be activated by the same oscillatory change in free intracellular Ca^{2+}. Thus, inward and outward components would have the same, or similar time courses.

2) I_{TO} Channel: Transient outward current (I_{TO}) has been characterized by voltage clamp experiments in Purkinje fibre preparations, atrial cells, and atrioventricular cells. The ionic nature of I_{TO} is still debateable. Initially it was attributed to Cl^- and later to K^+. This current may have more than one component. One component of I_{TO} in Purkinje fibres may be attributable to Cl^- (Kenyon & Gibbons, 1977, 1979a, b) and may be activated by intracellular Ca^{2+} (Siegelbaum & Tsien, 1980). This component of I_{TO} could contribute to TI in the outward direction if it is also activated by the TI generating protocols.

3) Non-selective Cationic Channel: Initially suggested as a possible mechanism of the TI by Kass et al (1978b), this type of channel has been identified at the single-

channel level in myocytes (Colquhoun et al, 1981; Ehara et al, 1988; Hill et al, 1988). It is generally believed that inward TI is mediated by this type of channel. The non-selective cationic current might also be responsible for outward current if it shows reversal. However, in the absence of Na^+ and K^+ the reversal potential would be much too positive to account for reversal of the TI observed in the present study. In addition, it is unlikely that this type of channel also conducted the outward Cl^- components, because DIDS and SITS differentially blocked the outward component. Thus, our results suggest that the cationic component contributes little to outward TI.

3. The Charge Carrying Systems of TI Induced by High Ca^{2+}

Several lines of evidence support our conclusion that high $[Ca^{2+}]_o$ induced TI in Purkinje fibres is mainly due to activation of TI-conducting channels, rather than electrogenic Na^+-Ca^{2+} exchange: 1) TI could be induced in the absence of $[Na^+]_o$ which eliminated the Na^+-Ca^{2+} exchange; 2) Even in the presence of $[Na^+]_o$, TI had a clear-cut reversal potential which supported the channel-gated current theory; 3) The Na^+-Ca^{2+} exchange current, I_{EX} (Giles & Shimoni, 1989b), could not be generated by an established protocol in the presence of high $[Ca^{2+}]_o$ without removing $[K^+]_o$ (inhibiting Na^+-K^+ pump and/or I_K conductance); 4) Ni^{2+} at a concentration high enough to block the current attributable to Na^+-Ca^{2+} exchange did not inhibit outward TI.

If TI is conducted through ion channels, at least two different populations of TI channels are involved. Inward and outward TI would be expected to be uniformly affected if both were generated through activation of a single population of channels. That Ni^{2+} exerted an inhibitory effect on inward TI at a time when outward TI was actually increased was not easily explained by the hypothesis that only the non-selective cationic channel was involved in the generation of TI. The observations made with DIDS and SITS further suggested that the channel properties responsible

for outward and inward TI were indeed different. Based on our experimental findings with Ni^{2+}, DIDS and SITS, we believe that 1) the non-selective cationic channel (Kass et al, 1978) is mainly responsible for the inward TI which can be blocked preferentially by Ni^{2+} and Mn^{2+}; 2) Cl^- channels which are inhibited by DIDS and SITS mediate the major part of outward TI and a minor portion of inward TI. Assuming the intracellular Cl^- concentration is 30 mM (Vaughan Williams, 1990), the calculated E_{Cl} will be -45 mV under our experimental conditions. This indicates that Cl^- may indeed participate in the generation of inward TI at test potentials negative to its equilibrium potential and is compatible with the partial inhibition of inward TI observed with DIDS and SITS.

4. Nature of the Reversal Potential of TI

Whether TI has a reversal potential in Na^+-containing solution has been a question of debate (Brown et al, 1986; Fedida et al, 1987a), possibly due to the shifts in time to peak TI observed with some protocols and differences between Purkinje and muscle tissues. In Na^+-free Ca^{2+}-rich solution a shift in time to peak TI is not apparent (Cannell & Lederer, 1986; Han & Ferrier, 1992a). However, in Na^+-containing solution, a voltage-dependent shift of time to peak inward TI has been observed by many investigators (Lederer & Tsien, 1976; Kass et al, 1978b; Brown et al, 1986) and also in the present study (figure 33). However, in the present study, outward TI did not exhibit a voltage-dependent shift in time to peak (figure 33). Although we did not concentrate on this issue, the difference in time to peak inward v.s. outward TI may be taken as additional evidence suggesting the existence of different populations of TI channel. Nevertheless, we emphasize that even though there were apparent shifts in time to peak inward TI, a clear-cut reversal potential (E_{REV}) was easily discernable in all of our experiments.

A distinct E_{REV} for TI cannot be interpreted as evidence for involvement of just a

single class of TI channel. E_{REV} most likely reflects a balance determined not only by ionic gradients, but also by the conductances of both inward TI channels and outward TI channels to different ions. Involvement of more than one population of TI channel, activated by the same oscillatory change in $[Ca^{2+}]_i$, makes it difficult to estimate the relative permeabilities of TI channels to individual ions from ionic gradients and the E_{REV}. Resolution of this question should be facilitated by studies at single channel level.

5. Possible Role of Na^+-Ca^{2+} Exchange in the Generation of TI

The influence of Na^+-Ca^{2+} exchanger on intracellular calcium levels during depolarization is a subject currently under active investigation. In ventricular myocytes, the exchanger may generate an inward tail current in parallel with loss of $[Ca^{2+}]_i$ during most of the action potential (Egan et al, 1989). However, at more positive membrane potentials, loading of $[Ca^{2+}]_i$ by Na^+-Ca^{2+} exchange has also been demonstrated (Terrar & White, 1989; Beuckelmann & Wier, 1989). Therefore, the effects of Na^+-Ca^{2+} exchange on $[Ca^{2+}]_i$ in Purkinje fibres can reverse with changes in membrane potentials (Brill et al, 1987). Net Ca^{2+} influx and net Na^+ efflux occur at potentials positive to -20 mV, and the reverse at more negative potentials. Na^+-Ca^{2+} exchange may contribute, at least partially, to the $[Ca^{2+}]_i$ overload in the present study, and thus facilitate the generation of TI.

Our observations do not imply that the exchanger current is also absent when the TI is induced by other conditions such as digitalis toxicity. In fact, concurrence of TI and I_{EX} (Shimoni & Giles, 1987; Giles & Shimoni, 1989b) and overlapping of TI with I_{EX} (Satoh et al, 1989) have been documented. Under these conditions, the channel mediated TI and the exchanger mediated I_{EX} may both contribute to the generation of OAP (Ferrier, 1977; 1991; January & Fozzard, 1988).

6. Mechanisms of Action of Cd^{2+}, Mn^{2+}, and DIDS on TI Generation

Intracellular Ca^{2+} overload may be associated with oscillatory release of Ca^{2+} from the SR. Oscillatory release in $[Ca^{2+}]_i$ activates two simultaneous conductances to various degrees: inward and outward TI. Inward TI channel might be the same as the non-selective cationic channels documented by Colquhoun et al (1981), Hill et al (1988) and Ehara et al (1988). In Purkinje fibres, either very high $[Ca^{2+}]_i$ or disruption of $[Ca^{2+}]_i$ homeostasis may decrease the inward TI as demonstrated in this study (figure 37, high $[Ca^{2+}]_o$ + 0 $[K^+]_o$) and others (Satoh et al, 1989; digitalis + caffeine). Once activated, the overall ionic gradients will favour net influx of cations through the inward TI channel at negative membrane potentials, and anions through outward TI channel at more positive potentials. These ionic fluxes will produce the major parts of inward and outward TI, respectively.

Based on the above hypothesis, the effect of Cd^{2+} can be explained by a predominant inhibition of I_{Ca} (Lansman et al, 1986) which would decrease the $[Ca^{2+}]_i$, and thus the $[Ca^{2+}]_i$-activated inward and outward TI channel conductances. Any possible inhibition of Na^+ current (and thereby decrease in the steady-state $[Na^+]_i$ level) is unlikely to underlie TI inhibition by Cd^{2+} since both the fast and the slowly inactivating Na^+ currents (Carmeliet, 1987) were largely voltage inactivated with a holding potential of -55 mV. The differential effects of Mn^{2+} on TI can be expected if one assumes that the inhibition of outward TI is only secondary to I_{Ca} blockade (Lansman et al, 1986), but the decrease of inward TI is through a direct inhibition of inward TI channel conductance as well as I_{Ca} blockade. Inhibition of inward TI by Ni^{2+} in the absence of $[Na^+]_o$ strongly suggests that its action is mediated by inhibiting the inward TI channel. Simultaneous inhibition of inward TI would reveal an increment of outward TI in the presence of Ni^{2+}. It is unlikely that an increase in outward TI is due to I_{Ca} blockade. Finally, the dramatic inhibition on outward TI and a minor suppression on inward TI by DIDS and SITS is likely to reflect selective blockade of

Cl^- conductance.

7. Comparisons with Earlier Results

Kass et al (1978b) demonstrated that substitution of Cl^- by methylsulphate failed to show any change of E_{REV} (figure 7 in their study) although the solution change did reduce both the amplitudes of TI and the aftercontractions induced by strophanthidin. Therefore, they concluded that Cl^- did not contribute to the TI. However, several lines could complicate interpretation of their observations. First, when the Na^+-K^+ pump is inhibited, Na^+-Ca^{2+} exchange current is activated (Giles & Shimoni, 1989a, b) and may contribute to the TI (Egan et al, 1989). This component would be relatively insensitive to changes in concentrations of other ions. Secondly, total replacement of Cl^- may change the intracellular pH (Mullin, 1981), which may also lead to the changes in intracellular Ca^{2+} through Na^+-H^+ exchange and Na^+-Ca^{2+} exchange. Thirdly, the "impermeant" Cl^- replacement, methylsulphate, may bind to Ca^{2+} and thus reduce $[Ca^{2+}]_o$, as proposed in a study of I_{To} by Kenyon & Gibbons (1977, 1979a, b). The reduction of $[Ca^{2+}]_o$ and $[Ca^{2+}]_o$ may lead to inhibition of TI, which would make it difficult to measure outward TI of small amplitude. Activation of outward TI seems to require greater Ca^{2+} overload than inward TI (figure 22). Therefore, the different observations in these two studies may be attributed to the different experimental conditions.

IV. CONTRIBUTION OF Na^+-Ca^{2+} EXCHANGE IN THE STIMULATION OF TI BY β-ADRENERGIC AGONIST

Na^+-Ca^{2+} exchange has little or no role as the charge-carrying system for TI under our experimental conditions, whether $[Na^+]_o$ is absent or present. However, Ca^{2+} loading can be achieved by influx of Ca^{2+} via both L- and T-type Ca^{2+} channels and electrogenic Na^+-Ca^{2+} exchange (Bielefeld et al, 1986; Brill et al, 1987; Sheu et

al, 1986; Sheu & Blaustein, 1991; Terrar & White, 1989). Indeed, our earlier experiments also suggested that Na^+-Ca^{2+} exchange might be an important mechanism for the stimulatory effects of β-adrenergic agonists on TI. Nevertheless, it is still not clear whether β-adrenergic stimulation of TI is mediated at the Ca^{2+}-loading stage (elevation of $[Ca^{2+}]_i$) or by the TI channels. Therefore, the final part of the present study was aimed at addressing the following questions: 1) is β-adrenergic stimulation of TI secondary to elevation in $[Ca^{2+}]_i$ or a direct effect TI channels? 2) Which pathway, I_{Ca} or Na^+-Ca^{2+} exchange, is more important in mediating the β-adrenergic stimulation of TI? 3) can the effect of β-adrenergic stimulation be mimicked by forskolin, a direct activator of adenylate cyclase which activates protein kinase A in the absence of agonist-receptor binding and G-protein activation (Seamon et al, 1981)?

An important conclusion drawn from this study is that the Na^+-Ca^{2+} exchanger may be a major pathway which mediates the β-adrenergic stimulation of the arrhythmogenic transient inward current (TI) under a variety of conditions. Furthermore. the stimulatory effect is secondary to the elevation in $[Ca^{2+}]_i$, not a direct effect on the TI channels. All effects of ISO can be mimicked by forskolin, a direct activator of adenylate cyclase (Seamon et al, 1981). This is taken to mean that cAMP-dependent protein kinase activation and phosphorylation processes are involved in the β-adrenergic stimulation of TI.

Clarification of the ionic basis for the TI allows us to address the following question: what is the mechanism(s) by which β-adrenergic stimulation enhances TI and thereby promotes the OAP initiated arrhythmias (Ferrier, 1977; Wit & Rosen, 1991). It is well known that β-adrenergic stimulation can lead to consecutive activations of a stimulatory guanine nucleotide-binding regulatory protein (G protein), adenylate cyclase which synthesizes cAMP from ATP, and cAMP-dependent protein kinase which phosphorylates a number of membrane proteins including the L-type

Ca^{2+} channels and the delayed rectifier K^+ channels. Phosphorylation of these channels results in increased conductances and current amplitudes (I_{Ca}, I_K). It is conceivable that β-adrenergic enhancement of TI might be mediated by a similar mechanism, i.e. phosphorylation of the TI-conducting channels. Our results clearly do not support this explanation because when TI was induced by high Ca^{2+} in the absence of Na^+, no stimulation was observed with ISO (figures 46,47). Under the same experimental conditions the current activated at the end of the depolarizing pulse (I_{step}, which roughly reflects I_K) was increased by ISO (figure 46), suggesting that the β-adrenergic dependent phosphorylation was still present.

Recently it has been reported that there is a β-adrenoceptor stimulated, cAMP-dependent Cl^- current ($I_{Cl(cAMP)}$) in heart (Hume & Harvey, 1991; Harvey et al, 1991) and that this current is inhibited by removal of $[Na^+]_o$. Thus, in the absence of $[Na^+]_o$, failure of ISO to stimulate TI, especially the outward TI which is mainly conducted through a Cl^- channel, seems to indicate an inability of ISO to initiate the phosphorylation of the TI channels. Although we cannot totally eliminate this possibility, we believe that this is very unlikely because of the fundamental differences between the TI-conducting Cl^- channels found in our experiments and the $I_{Cl(cAMP)}$. First, the Cl^- channels which mainly conduct the outward TI under our experimental conditions do not require the presence of β-adrenergic agonist or cAMP to be activated. In contrast, very little basal channel activity for $I_{Cl(cAMP)}$ can be found in the absence of β-receptor stimulation (Bahinski et al, 1989; Harvey & Hume, 1989). Second, the Cl^- channels that conduct outward TI are activated by transient elevation of $[Ca^{2+}]_i$ because ryanodine which abolishes $[Ca^{2+}]_i$ transient totally eliminates TI (Han & Ferrier, 1992a), whereas $I_{Cl(cAMP)}$ is not affected by buffering $[Ca^{2+}]_i$ (Harvey et al, 1991).

Since TI is a Ca^{2+}-activated current, any intervention that can promote $[Ca^{2+}]_i$

overload should increase the TI (January & Fozzard, 1988). T-type Ca^{2+} current is relatively small in heart (Mitra & Morad, 1986; Tseng & Boyden, 1989; Hirano et la, 1989) and may be insensitive to β-adrenergic stimulation (Tytgat et al, 1988; Tseng & Boyden, 1989). B-type Ca^{2+} channel current also is very small and even may be absent in intact cells (Rosenberg et al, 1988). Therefore, the most likely candidates are the L-type Ca^{2+} channel and the Na^+-Ca^{2+} exchanger.

While L-type I_{Ca} can be strongly stimulated by both a G protein and cAMP-dependent phosphorylation, experimental evidence presented in this study does not seem to support L-type I_{Ca} as the major contributor to the effect of β-adrenergic stimulation on TI. This could be inferred from the observation that no significant increase in TI was observed with ISO (figures 46, 47). Caution has to be taken in interpreting these data because we did not measure L-type I_{Ca} directly. Measurement of I_{Ca} would require blockade of other interfering membrane currents such as I_{to}, I_{Na}, and I_{EX}. This may affect the induction of TI in our experiments. However, the increase in I_{Ca} may be minimal. In the presence of elevated $[Ca^{2+}]_i$, Ca^{2+}-dependent inactivation of I_{Ca} (Pelzer et al, 1990; 1991) may counterbalance the ISO-stimulated increase in I_{Ca}.

On the other hand, the absence of stimulation of TI in Na^+-free solution (figures 46, 47) and the presence of strong stimulation in Na^+-containing solution (figures 48-50) for TI are consistent with a role for Na^+-Ca^{2+} exchange in ISO stimulation of Ca^{2+} load. Ca^{2+} entry through this pathway has been well established under conditions of elevated $[Na^+]_i$ (Bers, 1987; Bers et al, 1988; Philipson & Ward, 1986). Convincing evidence also exists for the direct contribution of Na^+-Ca^{2+} exchange to cytosolic Ca^{2+} elevation with membrane depolarization (Sheu et al, 1986; Brill et al, 1987; Terrar & White, 1989). In support of this, we also observed that two putative Na^+-Ca^{2+} exchange blockers, quinacrine and dodecylamine (Bielefeld et al, 1986; de

la Pena & Reeves, 1987; Terrar & White, 1989), could stop the ISO stimulation of TI induced by either digitalis toxicity or high Ca^{2+} (figure 53).

Phosphorylation/dephosphorylation of Na^+-Ca^{2+} exchanger of isolated heart sarcolemma by a calmodulin-dependent kinase has been reported previously (Caroni & carafoli, 1983). The phosphorylation process increases both the affinity of the exchanger for Ca^{2+} and the maximal velocity of Ca^{2+} transport. It is therefore possible that β-adrenergic stimulation potentiates Ca^{2+} entry during depolarization by regulating phosphorylation of the Na^+-Ca^{2+} exchanger protein. Several lines of evidence support this hypothesis: 1) the ISO stimulation of TI is abolished by pretreating the preparations with propranolol, suggesting that this effect is mediated by β-adrenoceptors. 2) propranolol can only partially reverse the ISO stimulation of TI once the increase in TI has been estabolished. 3) forskolin, a direct adenylate cyclase activator which can generate cAMP directly and initiates protein phosphorylation, mimics the effects of ISO (e.g. absence of stimulatory effect of TI in Na^+-free solution and presence of stimulatory effect of TI in Na^+-containing solution). 4) propranolol has no effect on forskolin-stimulated TI increase, further suggesting that the effect of propranolol is specific. Under physiological conditions, phosphorylation of the Na^+-Ca^{2+} exchanger may not cause significant Ca^{2+} loading (Rinaldi et al, 1982). Indeed, TI could not be induced by ISO itself in both Purkinje fibres (this study) and the sino-atrial node cells (Satoh & Hashimoto, 1988) perfused with normal Tyrode's solution. However, if the transmembrane Na^+ and Ca^{2+} gradients were greatly altered to activate Na^+-Ca^{2+} exchange, β-adrenergic stimulation would cause greater $[Ca^{2+}]_i$ overload and therefore potentiate TI and OAP-initiated cardiac arrhythmias.

V. SIGNIFICANCE OF THE PRESENT STUDY

Cardiac arrhythmias may be promoted by a rise of $[Ca^{2+}]_i$. Many pathological conditions, such as "calcium paradox" (Zimmerman & Hulsmann, 1966; Han & Tang, 1986; Bhojani & Chapman, 1990), "oxygen paradox" (Hearse, Humphrey & Chain, 1973; Hayashi, Ponnambalam & McDonald, 1987), and ischemia and reperfusion (Manning & Hearse, 1984), may produce calcium overload without Na^+-K^+ pump inhibition. Thus, the ionic current responsible for OAP under such conditions may be conducted through TI channels, rather than generated via electrogenic Na^+-Ca^{2+} exchange. The deteriorating effects of increased sympathetic tone during cardiac ischemia and reperfusion may be mediated by stimulation of Na^+-Ca^{2+} exchange which causes further Ca^{2+} overload. Thus, the present study may further our understanding of cellular mechanisms of arrhythmias. This study also provides useful information relevant to the investigation of novel antiarrhythmic drugs. For instance, drugs which can selectively block the inward TI channel and/or Na^+-Ca^{2+} exchange in the heart should be developed and evaluated.

VI. SUMMARY AND FUTURE WORK

Work presented in this thesis has demonstrated that α-adrenergic stimulation can exert either proarrhythmic or antiarrhythmic effects, depending on the method to induce OAP. The arrhythmogenic effect of α-adrenergic stimulation during ischemia and reperfusion has been well documented in experimental animals (Sheridan, 1986; Sheridan et al, 1980). However, the antiarrhythmic effect of α-adrenergic stimulation on digitalis-induced OAP has not been tested in experimental animals. Therefore, it would be of clinical importance to test whether arrhythmias generated by digitalis, either under Langendorff perfusion or in situ, can be affected by α-adrenergic stimulation and blockade.

β-Adrenergic stimulation cannot induce, but can enhance TI induced by other

means (i.e. spontaneous TI, digitalis-induced TI, and high Ca^{2+}-induced TI). This stimulatory effect is thought to be mediated by Na^{+}-Ca^{2+} exchange which increases Ca^{2+} overload during depolarization. This point would be greatly strengthened if ISO can directly stimulate the Na^{+}-Ca^{2+} exchange current in the absence of other interfering currents.

In Purkinje fibres, high $[Ca^{2+}]_o$ induced TI is conducted through TI channels, not Na^{+}-Ca^{2+} exchange. TI channels consist of two different populations of which one is anionic (Cl^{-}). That Cl^{-} contributes to generation of TI may open a new area for studies of actions of antiarrhythmic agents. Will Cl^{-} channel blockers such as those used in this study also affect OAP induced by digitalis or high Ca^{2+}? Can these blockers affect arrhythmias initiated by digitalis or ischemia and reperfusion in Langendorff preparations or in situ?

Finally, work presented in this thesis might be extended to single channel preparations with patch clamp techniques. Strong evidence to support involvement of Cl^{-} in the generation of TI would be provided if single channel conductance selective to Cl^{-} could be recorded under conditions used to generate TI in the present study. To our knowledge, a clear-cut reversal of TI in muscle preparations has not been recorded. Can the present experimental conditions generate TI with a distinct reversal in single myocytes? This question would help us determine whether TI is distinctly different in Purkinje and muscle tissues, and would provide a clear understanding of arrhythmogenesis in these two cardiac tissues.

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