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"THE EFFECTS OF TERODILINE AND OXYBUTYNINS ON CARDIAC MEMBRANE CURRENTS"

by

Stephen E. Jones

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
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DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "The Effects of Terodiline and Oxybutynins on Cardiac Membrane Currents"

by Stephen E. Jones

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

This thesis is dedicated to my mother Dr. Pauline Alice Jones who passed away unexpectedly during the course of my thesis research.

I want to thank her for the years of unconditional love, and all that she has done in my development and for making me aware of the tools that are required for living a full and rewarding life.

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ABSTRACT

1. The objective of this electrophysiological study on guinea pig and rabbit ventricular myocytes was to investigate the effects of three urinary-dysfunction drugs (terodiline, oxybutynin, and S-oxybutynin) on ionic currents that govern repolarization of the cardiac action potential. Terodiline is presently restricted due to its adverse effects on cardiac function, oxybutynin has replaced terodiline as the drug of choice for management of bladder dysfunction, and S-oxybutynin is currently in Phase 3 trials.

2. Membrane currents were recorded from myocytes in the whole-cell patch clamp configuration. The currents included L-type Ca^{2+} current ($I_{Ca,L}$), the inwardly-rectifying K^+ current (I_{K1}), and three delayed-rectifier K^+ currents (rapidly-activating inactivating I_{Kr}, slowly-activating noninactivating I_{Ks}, and transient outward current I_{to}).

3. Terodiline inhibited $I_{Ca,L}$ with an IC_{50} of 12-15 μM; inhibition was use-dependent, with block increasing with pulsing rate and more positive holding potential. The drug accelerated the decay of $I_{Ca,L}$, and slowed its recovery from inactivation.

4. Terodiline preferentially inhibited I_{Kr} over the two other delayed-rectifier K^+ currents. The IC_{50} for I_{Kr} was 0.5 μM, versus 6 μM for I_{to}, 7 μM for I_{K1}, and 26 μM for I_{Ks}. Inhibition of I_{K1} was independent of current direction, and persisted after removal of the drug. Block of I_{Ks} appeared to be voltage-dependent, and was fully attained on the first pulse following a rest period.

5. Membrane currents were unaffected by ≤ 1 μM oxybutynins, and inhibited by higher concentrations. Compared to terodiline, IC_{50} values were up to 2.6-fold higher for $I_{Ca,L}$, I_{Ks} and I_{K1}, and > 10-fold higher for I_{to} and I_{Kr}.

6. The results obtained with clinically relevant concentrations (< 10 μM) of terodiline help explain its cardiotoxic profile (bradycardia; nodal conduction disturbance; QT prolongation; ventricular tachyarrhythmia); whereas the results obtained with clinically relevant concentrations (<< 1 μM) of the oxybutynins indicate that clinical use of these drugs is highly unlikely to result in terodiline-like cardiotoxicity.

LIST OF ABBREVIATIONS AND SYMBOLS

A	ampere
A/D	analog-to-digital
Ag-AgCl	silver-silver chloride
APD	action potential duration
ATP	adenosine triphosphate
C_{max}	peak plasma concentration
d_{∞}	steady-state activation variable
DMSO	dimethyl sulfoxide
ECG	electrocardiogram
E_{rev}	reversal potential
E_K	equilibrium potential for K^+
EGTA	ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid
F	farad
g	gram
h	hour
HEPES	*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid
Hz	hertz
I	whole-cell current
IC_{50}	concentration of an agonist that produces 50% of the maximal inhibition
$I_{Ba,L}$	barium-carried current through L-type calcium channels
$I_{Ca,L}$	L-type calcium current
I_{K1}	inward-rectifying potassium current
I_K	delayed-rectifier potassium current

I_{Kr}	rapidly-activating delayed-rectifier potassium current
I_{Ks}	slowly-activating delayed-rectifier potassium current
I_{Na}	sodium current
I_{to}	transient outward potassium current
I-V	whole-cell current-voltage
k	slope factor of activation curve
Kir	inward-rectifying potassium channel
Kr	rapidly-activating delayed-rectifier potassium channel
Ks	slowly-activating delayed-rectifier potassium channel
Kto	transient outward potassium channel
M	moles per liter
m	meter
min	minute
n	Hill coefficient
n	number of experiments
p	probability (significance level in a statistical test)
pH	negative logarithm of the hydrogen ion concentration
RMP	resting membrane potential
Q_{10}	temperature coefficient per $10^{o}C$
S	slope factor of Boltzmann equation
s	second
S.E.M.	standard error of the mean
τ	time constant
$t_{½}$	time to one-half decay
V	volt
$\dot{V}_{max}$	maximal upstroke velocity of the action potential
$V_{½}$	half-maximal potential

$[X]$	concentration of an ion X
$[X]_o$	extracellular concentration of an ion X
~	approximately
^oC	degree Celsius
Ω	ohm
%	per cent
Δt	time interval
$\leq$	less than or equal to
$\geq$	greater than or equal to
$>$	greater than

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I. INTRODUCTION

This research was undertaken to investigate the effects of three drugs used in the management of bladder dysfunction, terodiline, oxybutynin, and S-oxybutynin on five membrane currents in cardiac ventricular cells: the inwardly-rectifying K^+ current (I_{K1}), L-type Ca^{2+} current ($I_{Ca,L}$), rapidly-activating delayed-rectifier K^+ current (I_{Kr}), slowly-activating delayed-rectifier K^+ current (I_{Ks}), and transient outward delayed-rectifier K^+ current (I_{to}). The material presented in the sections below is intended to provide a suitable background for the experimental results and their implications. It is organized under the following headings: (i) ionic currents that govern repolarization of the ventricular action potential, (ii) use of anticholinergic / antispasmodic agents in managing unstable bladder, and (iii) rationale and objectives of the study.

SECTION A. IONIC CURRENTS THAT GOVERN REPOLARIZATION OF THE VENTRICULAR ACTION POTENTIAL

The shape and duration of the action potential in ventricular muscle cells is determined by a fine balance between inward-directed and outward-directed membrane currents that flow during the several-hundred-millisecond period that follows the action potential upstroke. Any change in this balance results in an alteration of the configuration of the action potential. Depolarizing inward current following the action potential upstroke is primarily carried through L-type Ca^{2+} channels, whereas repolarizing outward current is primarily carried by multiple types of K^+ channels. The outward K^+ currents important for repolarization include the following: (i) the transient outward K^+

current (I_{to}) that underlies the early phase of rapid repolarization (phase 1), (ii) two components of the delayed-rectifier K^+ current I_K (I_{Kr} and I_{Ks}) that increase with time during the plateau (phase 2) and subsequent repolarization period (phase 3), and (iii) the inward rectifier K^+ current (I_{K1}) that drives late repolarization and ensures a strongly negative resting potential between action potentials (phase 4) (see Figure 1).

A brief description of each of these currents and their roles in the cardiac ventricular action potential are presented in the subsections that follow. In addition, a graphic representation is provided in the Appendix.

(1) Inwardly-rectifying K^+ current (I_{K1})

Inwardly-rectifying K^+ (Kir) channels are so-named because they conduct inward-directed current more easily than outward-directed current; consequently, the current-voltage (I-V) relationship is described as inwardly-rectifying (Hume and Uehara, 1985; Giles and Imaizumi, 1988).

Hyperpolarizations to potentials negative to the reversal potential of the current (E_{rev}) (i.e., the potential at which the current reverses in direction) elicit large inward currents. The slope conductance measured over potential ranges negative to E_{rev}, increases as the concentration of extracellular K^+ ($[K^+]_o$) is increased (Dudel *et al.*, 1967; Noble and Tsien, 1968; McDonald and Trautwein, 1978). When the membrane potential is shifted to progressively more positive levels than E_{rev}, the now outward current is progressively smaller due to a voltage dependent block of the Kir channels by intracellular Mg^{2+} (Vandenberg, 1987; Matsuda, 1991; Lu and MacKinnon, 1994; Nichols *et al.*, 1994), polyamines (Ficker *et al.*, 1994; Lopatin *et al.*, 1994), and perhaps Ca^{2+} (Mazzanti and DiFrancesco, 1989); these blocking actions are the basis for the inward rectification of the current.

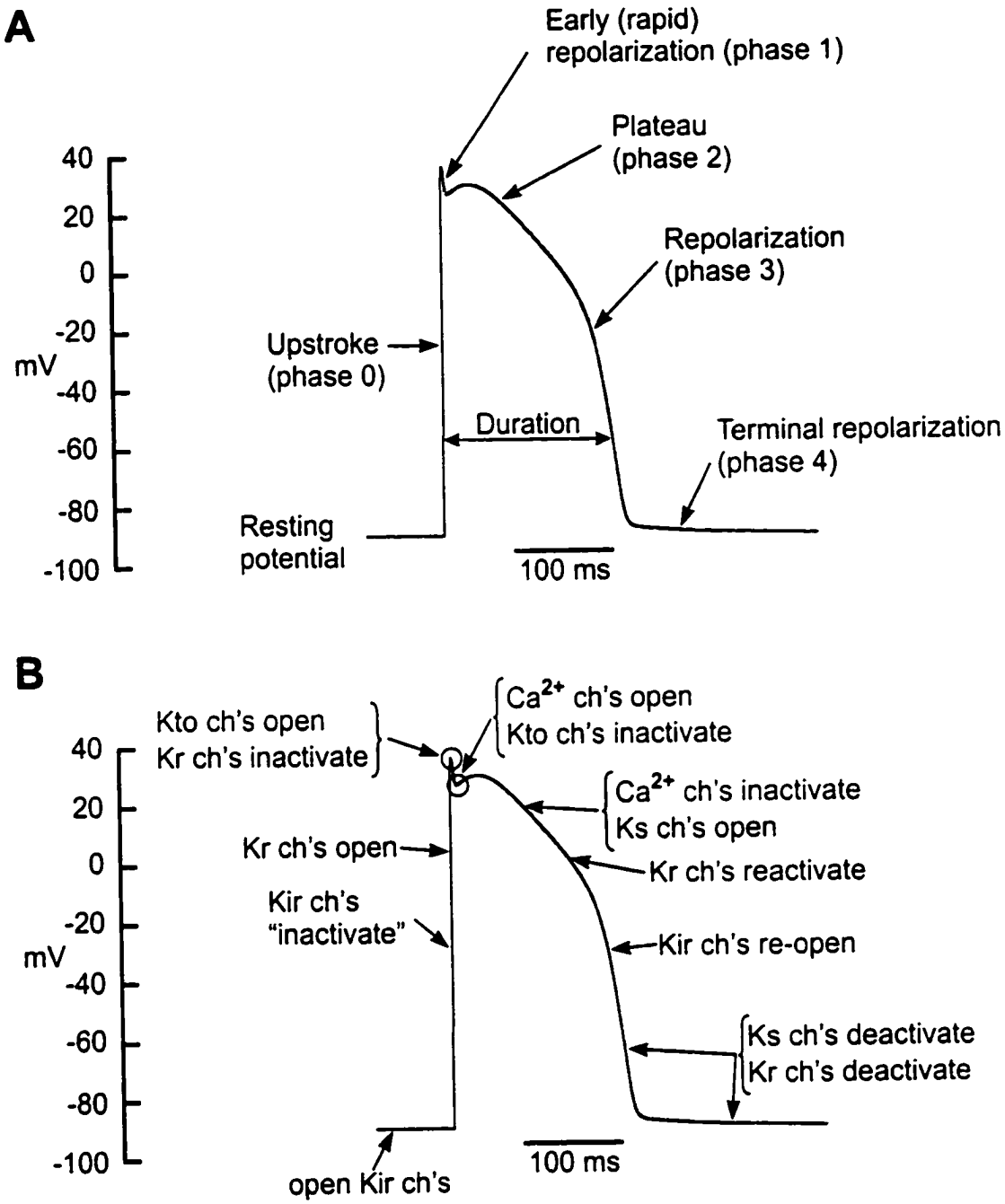


Figure 1. Description of the cardiac ventricular action potential. (A) Terms used to describe the various phases of the action potential. (B) Changes in ionic channel activity that underly the plateau and repolarization phases of the action potential. Abbreviations used: Na^+ ch's, voltage-dependent Na^+ channels; Kir ch's, inwardly-rectifying K^+ channels; Kto ch's, K^+ channels that carry transient outward current; Kr ch's, rapidly-activating inactivating delayed-rectifier K^+ channels; Ks ch's, slowly-activating non-inactivating delayed-rectifier K^+ channels.

Role in the ventricular action potential

The role of I_{K1} in the repolarization of the ventricular action potential is determined by a number of the characteristic features of the current. Little or no current is detected during most of the plateau phase because the Kir channels are fully blocked. However, voltage-dependent unblock of the strong Kir channel system results in large outward I_{K1} between -20 and -80 mV. This large current accelerates the rate of repolarization over this potential range, with the rate slowing as the driving force (membrane potential minus E_{rev}) for K^+ ions declines (late phase 3). Because E_{rev} is very close to the K^+ equilibrium potential (E_K), and Kir conductance is far larger than other ionic conductances at this potential, the asymmetrical conductance through Kir channels sets / maintains the resting membrane potential (RMP) around E_K, and offsets excitable stimuli caused by various non-K^+ inward-directed currents. Moderate block of I_{K1} in cardiac ventricular myocytes (for example, by submicromolar concentrations of Ba^{2+}) slows the rate of phase 3 repolarization, and produces a small depolarization of the resting membrane potential (e.g., Giles and Imaizumi, 1988).

(2) L-type Ca^{2+} current ($I_{Ca,L}$)

Depolarization from relatively negative potentials quickly activates (opens) L-type Ca^{2+} channels, and this permits the flow of inward Ca^{2+} current. Activation of $I_{Ca,L}$ has a distinct sigmoidal time course (Lee and Tsien, 1982; Pelzer *et al.*, 1986) that speeds up as the potential becomes more positive (McDonald *et al.*, 1986). When $I_{Ca,L}$ is activated by a step depolarization to 0 mV, the time for the current to reach a peak is 2-4 ms at ca. 35°C (Isenberg and Klöckner, 1982; McDonald *et al.*, 1986). The degree of L-type Ca^{2+} channel activation depends on the potential of the depolarization. The threshold for activation in cardiac myocytes is near -40 mV (Isenberg and Klöckner,

1982; Campbell *et al.*, 1988), and the steady-state activation variable (d_∞) ranges from 0 at threshold to 1 near +10 mV; the dependence of the activation variable on voltage has been described by a Boltzmann distribution equation, $I/I_{max} = 1/[1+\exp(V_{½}-V)/k]$, with a half-maximal activation at a voltage ($V_{½}$) near -15 mV and a slope factor (k) of ca. 7 mV (Kass and Sanguinetti, 1984; Cohen and Lederer, 1987; Tseng *et al.*, 1987).

After reaching a peak, $I_{Ca,L}$ inactivates (decays) with a distinctly slower time course than that required for full activation. In studies on cardiac cells, the time course of the decay phase has generally been described as monoexponential (Campbell *et al.*, 1988), or biexponential (Isenberg and Klöckner, 1982; Irisawa, 1984; Imoto *et al.*, 1985; Tseng *et al.*, 1987). When described by the sum of two exponentials, the first time constant is usually 5-10-fold shorter than the second time constant. For example, rough average time constants from guinea pig ventricular myocytes at 35°C are 3-7 ms and 30-80 ms (Imoto *et al.*, 1985; Ogura *et al.*, 1997). A number of studies suggest that the rapid phase of inactivation is related to inactivation caused by Ca^{2+} entry (McDonald *et al.*, 1994).

Inactivated L-type Ca^{2+} channels can be restored to a closed configuration by repolarization of the membrane to negative potentials. This recovery process takes time, and is accelerated at more negative potentials. For example, at room temperature the half-time of recovery from inactivation is reported to be 200-400 ms near -50 mV (Isenberg and Klöckner, 1982; Tseng, 1988), and 100-200 ms at -80 to -100 mV (Fischmeister and Hartzell, 1986; Campbell *et al.*, 1988; Hadley and Hume, 1987). Faster rates of $I_{Ca,L}$ restoration are evident at higher temperatures, in accord with a temperature coefficient per 10°C (Q_{10}) of ca. 3 for Ca^{2+} channel kinetics (Mitchell *et al.*, 1983; Cavalié *et al.*, 1985). The actual time course of $I_{Ca,L}$ restoration in cardiac cells is unclear. It has been

described as monoexponential, biexponential, oscillatory, or sigmoidal (Pelzer *et al.*, 1990; McDonald *et al.*, 1994)

Role in the ventricular action potential

$I_{Ca,L}$ plays an important role in determining the action potential duration (APD) in ventricular cells. An increase in $I_{Ca,L}$ lengthens the action potential plateau phase, whereas a decrease depresses the amplitude and duration of the plateau (Carmeliet, 1993). In some species, activation of $I_{Ca,L}$ early in the action potential contributes inward current that offsets outward I_{to}, and thereby moderates the extent of the early repolarization caused by I_{to}.

(3) Delayed-rectifier K^+ current (I_K)

Early work on cardiac tissues established that the K^+ current (I_X; I_K) that develops during longer depolarizations is a time- and voltage-dependent outward current that is comprised of two components (Noble and Tsien, 1969; McDonald and Trautwein, 1978). More recent work on cardiac myocytes indicates that these components can be attributed to distinct types of K^+ channels as shown by distinct kinetics and responses to pharmacological interventions. One component is "rapidly-activating inactivating" and is termed I_{Kr}, whereas the other is "slowly-activating noninactivating" and is termed I_{Ks} (Sanguinetti and Jurkiewicz, 1990, 1991; Heath and Terrar, 1996a,b). Both I_K components activate in response to membrane depolarization, with I_{Kr} activating at more negative potentials than I_{Ks} (see below). Post-activation repolarizations to more negative potentials results in the deactivation of I_K; in voltage-clamp experiments, the

deactivation is observed as a slowly decaying tail current that is outward at potentials positive to E_K (McDonald and Trautwein, 1978; Sanguinetti and Jurkiewicz, 1990).

The actual composition of I_K is dependent on both cell-type and species. For example, both currents are present in guinea pig atrial and ventricular myocytes (Sanguinetti and Jurkiewicz, 1990, 1991; Chinn, 1993; Turgeon *et al.*, 1994; Valenzuela *et* al., 1994; Daleau *et al.*, 1997; Pascarel *et al.*, 1998), canine atrial and ventricular myocytes (Gintant, 1996; Liu and Antzelevitch, 1995), and human atrial and ventricular myocytes (Wang *et al.*, 1994; Li *et al.*, 1996). On the other hand, I_K in cells from rabbit heart (nodal, Purkinje and ventricular cells) (Furukawa *et* al., 1989; Shibasaki, 1987; Carmeliet, 1992) and cat heart ventricle (Follmer and Colatsky, 1990) is dominated by I_{Kr}.

I_{Kr}-conducting channels (Kr) activate during depolarizations, the current is fully activated within 0.1-0.5 s and reaches a maximum at 0 to +10 mV (Sanguinetti and Jurkiewicz, 1990; Heath and Terrar, 1996a). The activation of the current in guinea pig ventricular myocytes is usually well fit by a single exponential function; the activation threshold is near -35 mV, and the voltage for half-activation ($V_{½}$) has been reported to range from -15 to -21 mV (Sanguinetti and Jurkiewicz, 1990; Heath and Terrar, 1996a). Although the activation of Kr channels is fairly quick at positive potentials (e.g., τ~30 ms at +10 mV), the channels fail to conduct significant outward current at more positive potentials because they undergo a rapid inactivation (Sanguinetti and Jurkiewicz, 1990; Ito and Ono, 1995; Liu and Antzelevitch, 1995). This inactivation is due to an intrinsic voltage-dependent mechanism that is extremely rapid, relative to the rate of channel activation (Sanguinetti and Jurkiewicz, 1991; Spector *et al.*, 1996). The rate of Kr channel recovery from inactivation is also very fast relative to deactivation, such that tail currents with short times to peak are evident when pulses to positive potentials are

followed by repolarizations to negative potentials between -20 and -70 mV (Heath and Terrar, 1996a). Deactivation of I_{Kr} at -40 mV is reported to have both fast and slow decay phases, with the fast phase having only a slightly larger amplitude than the slow phase (Heath and Terrar, 1996b).

In contrast to Kr channels, I_{Ks}-conducting channels (Ks) slowly activate during depolarizations to positive potentials, and the activation time course has been described as sigmoidal (Sanguinetti and Jurkiewicz, 1990; Heath and Terrar, 1996a). The activation threshold for Ks channels is near 0 mV, and reported $V_{½}$ values range from +20 to +38 mV, depending on the duration of the activating depolarization; thus, maximal activation is reached near +70 mV (Sanguinetti and Jurkiewicz, 1990; Heath and Terrar, 1996a). Unlike Kr channels, Ks channels do not inactivate at positive potentials. However, the channels do deactivate when the membrane is repolarized from positive potentials to more negative potentials. Like Kr channels, this process is accelerated at potentials more negative than -20 mV. I_{Ks} deactivates in a biphasic manner, with the fast phase having a much greater amplitude than the slow phase (Heath and Terrar, 1996b).

Role of I_{Kr} in the ventricular action potential

I_K plays a major role in controlling the shape and duration of the plateau phase and in repolarizing the cardiac cell membrane (Noble, 1984; Arena *et al.*, 1990). The importance of I_{Kr} in governing repolarization of the cardiac action potential is best revealed through the effects of class III, I_{Kr} blocking antiarrhythmic agents (e.g., E4031 (N-[4-[[1-[2-(6-methyl-2-pyridinyl)ethyl]-4-piperidinyl]carbonyl]phenyl] methanesulfonamide dihydrochloride dihydrate); sotalol; dofetilide) which selectively inhibit this component of I_K (Sanguinetti and Jurkiewicz, 1990; Carmeliet, 1992). For example, a full-I_{Kr}-blocking concentration (5 μM) of E4031 can lengthen the action potential in

guinea pig papillary muscles by ca. 30% (Jones *et al.*, 1998). Inhibition of I_{Kr} has a lengthening effect on the action potential duration because Kr channels begin to recover from inactivation at ca. ≤ +20 mV and significant outward current is conducted as repolarization proceeds between 0 and -40 mV.

Role of I_{Ks} in the ventricular action potential

Outward I_{Ks} develops in a slow manner during the plateau phase of the action potential. As the magnitude of the current increases, its repolarizing influence outweighs the depolarizing influence of slowly-inactivating inward $I_{Ca,L}$, and the plateau voltage falls to less positive values. This is one aspect of the contribution of I_{Ks} to repolarization. The other is that its repolarizing action during the plateau moves the membrane potential to levels that allow re-opening of Kr channels.

The contribution of I_{Ks} to action potential repolarization may be especially important at high heart rates. The reason is that during rapid stimulation, I_{Ks} is likely to grow over time because the Ks channels may not have sufficient time to fully close during the abbreviated interpulse intervals. This incomplete deactivation and build up of repolarizing I_{Ks} should have a shortening effect on the action potential duration.

Incomplete deactivation of I_{Ks} at high heart rates is also an attributing factor in the "reverse" rate-dependent prolongation of the action potential by class III (I_{Kr} blocking) antiarrhythmic agents (i.e., the agents have a greater lengthening effect at low versus high rates of stimulation) (Hondeghem and Snyders, 1990; Tande *et al.*, 1990; Sanguinetti and Jurkiewicz, 1991; Jurkiewicz and Sanguinetti, 1993). This frequency-dependent action of class III antiarrhythmic drugs is an unfavourable feature because it limits the effectiveness of these agents in controlling tachyarrhythmias and has been

implicated in acquired long QT syndromes, torsades de pointes, and bradycardia-associated arrhythmias (Roden *et al.*, 1996; Nair and Grant, 1997).

(4) Transient outward K^+ current (I_{to})

I_{to}-conducting channels (Kto) are rapidly-activating, voltage-gated K^+ channels that conduct outward current in response to depolarizations. The threshold for channel activation is ca. -30 mV, with full activation occurring near +30 mV (Clark *et al.*, 1988; Wettwer *et al.*, 1992). Activation at positive potentials is quite fast ($\tau \sim 1$ ms at 35°C (Jahnel *et al.*, 1994)), and is followed by inactivation that proceeds along a monoexponential or biexponential time course (Lefevre *et* al., 1991; Jahnel *et* al., 1994). Biexponential descriptions have variable fast (20 to 50 ms) and slow (several hundred ms) time constants, depending on the test potential and experimental temperature (Lefevre *et al.*, 1991; Jahnel *et al.*, 1994; Wettwer *et* al., 1994; Yeola and Snyders, 1997).

Once inactivated, Kto channels require time at negative potentials before being restored to the "available" closed state. The time required for Kto channels to recover from inactivation is variable; the recovery rate has been described by single exponential functions with time constants ranging from 20 to 50 ms, or by biexponentials with slow time constants as long as 900 ms (Lefevre *et al.*, 1991; Li *et al.*, 1998; Sanchez-Chapula, 1999). In a study involving human cardiac myocytes from different regions of the ventricle, inactivation recovery times varied depending on the regional origin of the myocyte (Wettwer *et al.*, 1994). At -100 mV, subepicardial cells recovered from inactivation with a time constant of 25 ms at 22°C, whereas subendocardial cells recovered in a biphasic manner (τ=25 ms; τ=330 ms).

Role in the ventricular action potential

I_{to} is an important determinant of the configuration of the early phase of the cardiac action potential. In cardiac cells that express Kto channels, the upstroke of the action potential is followed by a characteristic repolarizing downward spike (Ravens *et al.*, 1996). This brief period of early rapid repolarization (phase 1) is due to I_{to}. Outward I_{to} is offset to varying degrees by inward $I_{Ca,L}$, and, depending on the relative strength of I_{to}, phase 1 of the action potential may or may not be followed by a secondary depolarization related to the influx of Ca^{2+} ions. For example, guinea pig ventricle has a negligible density of Kto channels and the action potential lacks an early rapid repolarization phase (Hiraoka and Kawano, 1987; McDonald *et al.*, 1989; Carmeliet, 1993), whereas rat ventricle has a high density of Kto channels and action potentials are shorter in duration than those from guinea pig and lack a well-defined plateau phase (phase 2).

The contribution of I_{to} to action potential repolarization is rate-dependent. Although there are interspecies variations in the extent of the rate-dependence of I_{to}, the amplitude of I_{to} generally decreases at high heart rates as a result of incomplete recovery of Kto channels from inactivation during the shorter diastolic intervals (Fermini *et al.*, 1992). The reduction in outward I_{to} will translate into a lengthening of the action potential duration if its influence outweighs other possible changes induced by increasing the driving rate (e.g., decrease in $I_{Ca,L}$, increase in I_K: Hiraoka and Kawano, 1987; Boyett *et al.*, 1994).

SECTION B. USE OF ANTICHOLINERGIC / ANTISPASMODIC AGENTS IN MANAGING UNSTABLE BLADDER

Unstable bladder disorders are major medical and social problems that have a particularly high incidence in young children and older adults (Thomas *et* al., 1980; Fonda, 1989; Caione *et* al., 1997; Hjalmas, 1997; Theodorou *et al.*, 1998). The disorders result from involuntary contractions of the detrusor muscle during bladder filling. The characteristic symptoms are frequency of micturition, a strong and sudden desire to micturate resulting from involuntary detrusor contractility (urgency) and, if the contraction cannot be suppressed, involuntary urine loss (urge incontinence) (Yarker *et* al., 1995; Wein, 1998).

Bladder contraction, both in normal subjects and in patients with unstable bladder, is primarily controlled through parasympathetic stimulation of cholinergic muscarinic receptors (Andersson, 1993). Administration of anticholinergic drugs, which decrease detrusor muscle activity and may result in an increased bladder capacity, once formed the mainstay of medical treatment for the management of symptoms of the unstable bladder (Abrams *et al.*, 1998). The majority of these anticholinergics are quaternary ammonium compounds which do not readily penetrate biological membranes, and generally have a low and variable absorption (Andersson and Ulmsten, 1980). Thus, the degree of clinical effectiveness of anticholinergics vary, and quite often the anticholinergic effects are not specific for the bladder. This results in a high incidence of systemic antimuscarinic side effects such as dry mouth, blurred vision, constipation, and nausea, which are major limiting factors in restricting patient compliance and treatment efficacy (Abrams, 1984; Langtry and McTavish, 1990; Yarker *et al.*, 1995; Buyse *et al.*, 1998a,b).

Due in part to reports of anticholinergic drugs being only modestly effective in treating urinary incontinence (Bonnesen *et* al., 1984; Zorzitto *et* al., 1989), alternative approaches were needed for increasing therapeutic actions and decreasing the side effects seen with effective dosages of anticholinergics. A current method of therapy is focused on administering drugs that inhibit bladder contractions through synergistic anticholinergic and Ca^{2+} entry blocking actions. Theoretically, such dual-action drugs should be more effective in treating overactive bladders than drugs that have purely anticholinergic activity. Evidence for this stems from investigations that have shown that contractions of normal human bladder can be completely blocked by atropine, whereas atropine-resistant contractions remain in unstable bladders and require further addition of Ca^{2+} antagonists for complete suppression (Sjögren *et al.*, 1982).

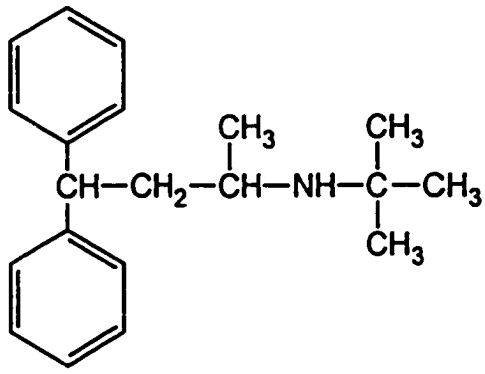
(1) Terodiline

Drug profile and clinical usage

Terodiline (N-tert-butyl-3,3-diphenyl-1-methyl-propylamine) is an antispasmodic drug with anticholinergic properties (Husted *et al.*, 1980). The drug is classified as a secondary amine (Figure 2), and is well absorbed from the gastrointestinal tract, with a bioavailability of 90% (Macfarlane and Tolley, 1984; Peters, 1984). Terodiline is about 85% bound to plasma proteins and has a mean elimination half-life of ca. 60 hours (Andersson, 1984), enabling steady-state plasma concentrations to be reached after ca. 10 days of treatment (Peters, 1984).

Terodiline was first registered as a prophylactic agent in the management of angina pectoris because the drug has a relaxing effect on coronary arteries (Wibell, 1968). The first suggestion that terodiline had anticholinergic properties stemmed from results of studies on isolated bronchial muscle (Iravani and Melville, 1975) and on

Terodiline



Oxybutynin

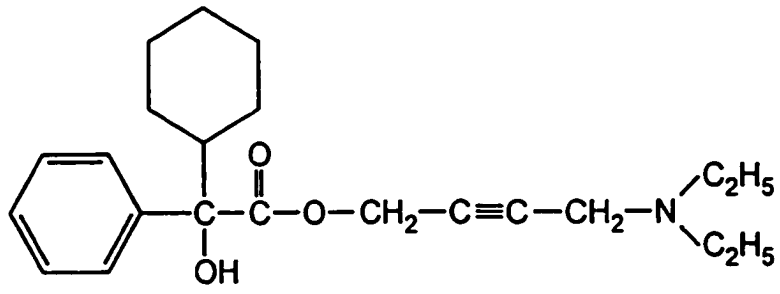


Figure 2. Chemical structures of terodiline and oxybutynin.

patients with obstructive pulmonary disease (Castenfors *et* al., 1975). Subsequent investigations on isolated rabbit detrusor (Husted *et al.*, 1980), guinea pig (Nilvebrant and Sparf, 1983), and human (Rud *et* al., 1980) urinary bladder not only confirmed the anticholinergic activity of terodiline, but also documented that the drug antagonizes Ca^{2+}-dependent contractions in smooth muscle tissues. Based on this spectrum of activity, terodiline was investigated for the treatment of detrusor instability and urinary incontinence in adults and of nocturnal enuresis in children (Andersson and Ulmsten, 1980; Elmér, 1984; Peters, 1984). The outcome of these trials was withdrawal of the drug as an antianginal agent in 1985 and reintroduction in over 20 countries in 1986 as an effective treatment for urinary incontinence. Terodiline eventually became the preferred drug throughout Europe for the treatment of urinary incontinence and other smooth muscle disorders (Andersson, 1984; Macfarlane and Tolley, 1984; Hallén *et al.*, 1989; Langtry and McTavish, 1990).

Cardiotoxicity

Terodiline was withdrawn worldwide in 1991 following reports of serious adverse effects on the heart, including bradycardia, atrioventricular block, lengthening of the QT interval (the time from the earliest activation of the ventricular myocardium to the latest ventricular repolarization) of the electrocardiogram (ECG), and ventricular tachyarrhythmia (torsades de pointes) (Connolly *et al.*, 1991; Davies *et* al., 1991; McLeod *et* al., 1991; Stewart *et al.*, 1992; Thomas *et al.*, 1995). The drug is currently authorized for clinical investigation and is a prototype structure for chemical derivatives that are either under development for possible therapeutic use (e.g., Taniguchi *et al.*, 1994; Take *et al.*, 1996; Koch *et al.*, 1998; Smith *et al.*, 1998) or have already entered the marketplace (Abrams *et al.*, 1998).

In all likelihood, the cardiotoxic profile of terodiline stemmed from drug-induced alteration(s) of key membrane ionic currents that govern cardiac action potential configuration and excitability. For example, experimental and clinical investigations have suggested that torsades de pointes results from an abnormal repolarization of the action potential either due to block of outward repolarizing K^+ currents or to an increase in inward depolarizing Ca^{2+} and Na^+ currents (Roden *et al.*, 1996). These possibilities recently gained support through the linkage of candidate genes with genetically inherited forms of the long-QT syndromes (LQTSs). All of the genes currently identified that are responsible for congenital LQTSs, encode ion channels or the regulatory (β) subunits involved in controlling the duration of the cardiac action potential. These include the Na^+ channel gene, SCN5A (Wang *et al.*, 1995), and four K^+ channel genes, HERG (Curran *et al.*, 1995), KCNE2 (Abbott *et al.*, 1999), KCNQ1 (previously designated KvLQT1) (Wang *et al.*, 1996), and KCNE1 (Splawski *et al.*, 1997). HERG encodes the pore-forming α-subunits of the rapidly-activating delayed rectifier K^+ channel, KCNE2 encodes MiRP1 which is a regulatory subunit for the same channel, while the latter two K^+ channel genes encode KvLQT1 and IsK (also called minK) proteins, two subunits which combine to form the slowly-activating delayed rectifier K^+ (Ks) channel (Sanguinetti *et al.*, 1996; Barhanin *et al.*, 1996).

Torsades de pointes has also been closely associated with a wide variety of drugs that prolong the QT interval (Zipes, 1987; Benedict, 1993; Jackman *et al.*, 1988), primarily through inhibition of the delayed-rectifier K^+ current (I_K) in ventricular muscle cells (Roden, 1993; Thomas, 1994; Woosley, 1996). Of the two components comprising I_K, rapidly activating I_{Kr} and slowly activating I_{Ks} (Sanguinetti and Jurkiewicz, 1990), torsades de pointes has primarily been associated with drugs that preferentially block I_{Kr} over I_{Ks}. These drugs include class Ia or class III antiarrhythmic

agents (Colatsky, 1982; Carmeliet, 1985; Sanguinetti, 1992) such as quinidine (Balser *et al.*, 1991), E4031 and sotalol (Sanguinetti and Jurkiewicz, 1990; Wettwer *et al.*, 1992), and dofetilide (Carmeliet, 1992; Williams and Beatch, 1997). Various non-antiarrhythmic drugs such as psychotropic, antimicrobial, antifungal, and antihistaminic agents can also induce torsades de pointes (Stratmann and Kennedy, 1987; Benedict, 1993), and some of these compounds have been shown to inhibit cardiac K^+ currents (Valenzuela *et al.*, 1994; Berul and Morad, 1995; Daleau *et al.*, 1995; Ming and Nordin, 1995; Salata *et al.*, 1995).

It is unclear why so many structurally diverse compounds preferentially block Kr channels over other cardiac K^+ channels. A possible structural explanation involving three critical aromatic residues located on the S6 transmembrane domain (G648, Y652, and F656) and two residues within the pore helix (T623 and V625) has been suggested (Mitcheson *et al.*, 2000).

(2) Oxybutynin

Subsequent to the withdrawal of terodiline, oxybutynin emerged as the most commonly prescribed drug for the relief of symptoms related to urinary disorders, including urinary urge with or without incontinence, nocturia, enuresis, postsurgical bladder dysfunction, neurogenic spastic bladder, and detrusor muscle instability in both children and adults (Yarker *et al.*, 1995; Abrams *et* al., 1998; Åmark *et al.*, 1998; Wein, 1998). Oxybutynin is a tertiary amine ester (Figure 2) with a similar pharmacological profile to terodiline. The drug is characterized as a muscarinic M1/M3-selective antagonist (Nilvebrant and Sparf, 1986; Noronha-Blob and Kachur, 1991), with direct relaxant effects on smooth muscle tissues (Lish *et al.*, 1965; Fredericks *et al.*, 1975; Tonini *et al.*, 1987; Smith *et al.*, 1998).

Although patients taking oxybutynin have complained of classical systemic anticholinergic side effects (e.g., dry mouth), the effectiveness of the drug has been demonstrated in several controlled clinical studies (Yarker *et* al., 1995; Burgio *et* al., 1998; Buyse *et* al., 1998a,b). To date, there have been no reports of terodiline-like cardiac complications in patients receiving oxybutynin, and Hussain *et al.* (1996) reported that there was little change in the QT intervals of a small cohort of elderly patients treated with low therapeutic dosages of oxybutynin.

(3) S-oxybutynin

Compared to the incidence of anticholinergic side effects with oxybutynin, a lower incidence is the major advantage claimed for S-oxybutynin, a stereoisomer that is currently undergoing evaluation as a urinary incontinence agent (Koch *et al.*, 1998; Smith *et al.*, 1998). As part of that process, one of the goals of the present study was to investigate the effects of S-oxybutynin on cardiac membrane currents, and compare them with those of oxybutynin and terodiline.

SECTION C. RATIONALE AND OBJECTIVES OF THE STUDY

The primary objective of the present research was to compare the effects of terodiline, oxybutynin, and S-oxybutynin on membrane currents that govern repolarization of the ventricular action potential. The membrane currents that were studied include I_{K1}, $I_{Ca,L}$, I_{Kr}, and I_{Ks}, all in guinea pig ventricular myocytes, as well as I_{to} in rabbit ventricular myocytes.

The thesis research was performed against a background of clinical reports that detailed the involvement of terodiline in a variety of adverse cardiac effects. On the other hand, there were no reports of adverse cardiac effects related to the clinical use of oxybutynin.

On the basis of the cardiotoxic profile of terodiline, it seemed highly likely that the drug would have marked effects on at least one of the primary membrane currents governing action potential repolarization. However, there were no published studies on this subject at the time the present research was initiated. In the interim, there has been a study of the effects of terodiline on guinea pig ventricular myocytes (Hayashi *et al.*, 1997), but the results have left a number of unanswered questions. For example, these investigators measured I_K tail current ($I_{K,tail}$) at -40 mV after 500-ms depolarizations to positive potentials, and reported that 10 μM terodiline reduced the amplitude by ca. 20%. In the absence of further information, they were unable to conclude whether terodiline has a preferential blocking action on one or the other components of I_K. Although inhibition of either I_{Kr} or I_{Ks} will tend to lengthen the cardiac action potential, there is a strong correlation between drugs that selectively block I_{Kr} over I_{Ks} and QT lengthening / torsades de pointes.

Recent studies have shown that clinically relevant concentrations of terodiline (< 10 μM) induce a moderate lengthening (peak 12% at 3 μM) of the action potential in guinea pig papillary muscles (Shuba *et al.*, 1999). This result is in good agreement with the lengthening effect of terodiline on the monophasic action potential in dog ventricle (Pressler *et al.*, 1995), and with the degree of lengthening of the rate-corrected QT interval in dogs (Natsukawa *et al.*, 1998) and humans (Stewart *et al.*, 1992; Thomas *et al.*, 1995) treated with the drug.

In regard to the oxybutynin compounds, prior to the present research there were no studies on the effects these drugs may have on specific cardiac membrane currents. Recent investigations of the effects of clinically relevant concentrations of oxybutynin compounds on guinea pig and rabbit papillary muscles indicate that these drugs have little or no effect on the action potential configuration (see below, and Jones *et al.*, 2000a,b).

All three of the antispasmodic / anticholinergic agents had concentration-dependent effects on the membrane currents that were examined, with terodiline being the most potent. The results of this investigation, in conjunction with those that studied the drug effects on guinea pig and rabbit papillary muscles, lead to plausible explanations for the adverse cardiac effects of terodiline, and suggest that therapeutic levels of the oxybutynin compounds are unlikely to produce similar cardiac complications.

II. METHODS

The Methods are divided into two major sections. The first section is concerned with myocyte preparation, electrophysiology, solutions, drugs, and statistics. The second major section is concerned with measurement and brief characterization of the membrane currents pertinent to this study.

SECTION A. MYOCYTE PREPARATION, ELECTROPHYSIOLOGY, SOLUTIONS, DRUGS, POTENCY AND STATISTICS

(1) Myocyte preparation

Guinea pigs weighing ca. 250 g were sacrificed by cervical dislocation in accord with national and local regulations on animal experimentation. Male New Zealand white rabbits (ca. 1.5 kg) were anaesthesized by injection of sodium pentobarbitone (40 mg / kg) into a marginal ear vein. Hearts were excised, attached to the base of an 80-cm Langendorff column, and retrogradely perfused through the aorta with normal Tyrode's solution for 1-2 min, Ca^{2+}-free supplemented Tyrode's solution for 4-6 min, Ca^{2+}-free supplemented Tyrode's solution containing collagenase (0.08-0.12 mg/ml: Yakult Pharmaceutical Co., Tokyo, Japan) for 10-15 min, and finally a high-K^{+}, low-Na^{+} storage solution supplemented with 50 mM glutamic acid and 20 mM taurine for 5 min. The full composition of the solutions are provided below (section A3). All perfusates were oxygenated with 100% O_2 and maintained at 37°C. The ventricles were cut into

small pieces, and cells dispersed by mechanical agitation. The cell solution was filtered through a 220-μm polyethylene mesh, and the filtrate placed in storage solution at room temperature prior to experiments within 10 h.

(2) Electrophysiological recording and analysis

An aliquot of storage solution containing myocytes was placed in a 0.3-ml perfusion chamber mounted on top of an inverted microscope stage (Nikon Diaphot, Tokyo, Japan). After the cells had settled to the glass bottom (Sarstedt, Inc., Newton, NC, USA), the chamber was perfused with normal Tyrode's solution at a rate of 2 ml/min. All of the experiments on guinea pig ventricular myocytes were conducted with normal or modified Tyrode's solution warmed to 36°C. The experiments on rabbit ventricular myocytes were conducted with a modified Tyrode's solution warmed to either 24°C or 36°C.

Single ventricular myocytes were voltage clamped using the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). Pipettes were pulled from thick-walled borosilicate glass capillaries (H15/10/137, Jencon's Scientific, Bedfordshire, UK) in the usual two step process. The pipettes had an inside tip diameter of 2-4 μm, and a resistance of 2-3 MΩ when filled with pipette solution and immersed in normal Tyrode's solution. Liquid junction potentials between external and pipette-filling solution were nulled prior to patch formation. After establishing a gigaohm seal, a brief strong negative pressure was applied to rupture the patch membrane under the pipette tip. The reference electrode was an Ag-AgCl wire. Series resistance ranged between 3 and 7

MΩ and was compensated by 60-80% in most experiments. Cell capacitance generally ranged from 80 to 130 pF.

The voltage-clamp amplifier was an EPC-7 (List Medical Electronic, Darmstadt, Germany). Membrane currents were displayed on a storage oscilloscope (Tektronix 5111), filtered at 3 kHz (EPC-7), and digitized with an A/D converter (Digidata 1200A, Axon Instruments) and pCLAMP software (Axon Instruments) at a sampling rate of 8 kHz prior to analysis.

(3) External solutions

The following superfusates were used: (i) normal Tyrode's solution containing (in mM) NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 10, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) 5 (pH 7.4 with NaOH); (ii) K^+-free Tyrode's solution (normal Tyrode's with KCl omitted; pH 7.4 with NaOH), (iii) K^+-, Ca^{2+}-free Tyrode's solution (normal Tyrode's with KCl and $CaCl_2$ omitted, 0.4 mM $CdCl_2$ added; pH 7.4 with NaOH), and (iv) modified Tyrode's solution containing 0.2 mM $CdCl_2$, 0.1 mM Ba^{2+}, 3 μM E4031, and 20 μM tetrodotoxin.

The Ca^{2+}-free supplemented Tyrode's solution used for cell isolation contained (in mM): NaCl 125, KCl 4.6, $MgCl_2$ 1.15, taurine 20, glucose 20, and HEPES 5 (pH 7.4 with NaOH). Storage solution contained (in mM): KCl 30, KOH 80, KH_2PO_4 30, glutamic acid 50, $MgSO_4$ 3, taurine 20, glucose 10, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) 0.5, and HEPES 10 (pH 7.4 with KOH).

(4) Dialysates

The standard pipette solution contained (in mM): KCl 40, potassium aspartate 106, Mg-ATP 1, K_2-ATP 5, EGTA 5, and HEPES 5 (pH 7.2 with KOH). A high Mg^{2+} solution contained (in mM): KCl 30, KOH 110, aspartic acid 110, Mg-ATP 5, EGTA 5, HEPES 5 (pH 7.2 with KOH). The K^+-free pipette solution contained (in mM): CsCl 30, CsOH 110, aspartic acid 110, Mg-ATP-5, EGTA 5, and HEPES 5 (pH 7.2 with CsOH).

(5) Drugs

Terodiline, oxybutynin, and S-oxybutynin were supplied as hydrochloride salts by Sepracor Inc. (Marlborough, MA, USA), daidzein was supplied by Calbiochem (La Jolla, CA, USA), and E4031 was supplied by Eisai (Tokyo, Japan). Nisoldipine was kindly provided by Bayer Inc. (Etobicoke, ON, Canada). These agents were dissolved in dimethyl sulphoxide (DMSO) (Sigma Chemical Co., St. Louis MO, USA) immediately prior to addition to the superfusate. The highest final concentration of DMSO in the superfusate was 0.1%, a concentration of DMSO that has no significant effect on electrical activity or membrane currents in guinea pig ventricular cells (Ogura *et al.*, 1995). Nevertheless, the vehicle was included in control solutions when the drug solution contained vehicle > 0.01%. Tetrodotoxin (Calbiochem, La Jolla, CA, USA), 4-aminopyridine (Sigma), and verapamil (Sigma-Aldrich, Oakville, ON, Canada) were dissolved in distilled water. Stock solutions of nisoldipine and verapamil (0.1 M) were stored in the dark at -20°C, freshly added to bathing solutions, and protected from the light during experiments.

(6) Potency of drug action

The concentration-dependent inhibitory effects of terodiline, oxybutynin, and S-oxybutynin on membrane currents were expressed as IC_{50} values (concentration of drug resulting in half-maximal inhibition of current amplitude, in relation to the pre-drug control amplitude). The IC_{50} values were obtained by the Hill equation describing the data.

(7) Statistics

Results are expressed as means ± S.E.M., with n indicating the number of experiments. Single comparisons were made using Student's t-test, and multiple comparisons were made using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test. Differences were considered significant when $p < 0.05$.

SECTION B. MEASUREMENT AND BRIEF CHARACTERIZATION OF PERTINENT MEMBRANE CURRENTS

The membrane currents measured in this study included the inwardly-rectifying K^+ current (I_{K1}), L-type Ca^{2+} current ($I_{Ca,L}$), rapidly-activating inactivating delayed-rectifier K^+ current (I_{Kr}), slowly-activating noninactivating delayed-rectifier K^+ current (I_{Ks}), and transient outward delayed-rectifier K^+ current (I_{to}). This section of the Methods describes how these five cardiac currents were recorded and measured, and presents examples that illustrate voltage-dependent and kinetic features of the currents.

(1) Inwardly-rectifying K^+ current

I_{K1} was recorded from myocytes that were superfused with normal Tyrode's solution and dialyzed with K^+ pipette solution. The myocytes were generally held at -80 mV and depolarized for 500 ms to prepulse -40 mV. Full current-voltage (I-V) relationships for I_{K1} were generated from measurements of the (quasi-steady) current flowing at the end of 500- or 1000-ms pulses from the prepulse potential to potentials ranging from ca. -120 mV to 0 mV.

An example of the type of current records obtained on 500-ms pulses to potentials between -120 and 0 mV is shown in Figure 3A. The associated I-V relationship in Figure 3B illustrates the following well-known features of I_{K1}: (i) the current reverses in direction close to the K^+ equilibrium potential (E_K) (in this case, -86 mV, calculated from superfusate (5.4 mM) and dialysate (140 mM) K^+ concentrations), (ii) pulses to

potentials negative to E_K evoke large inward-directed currents, whereas pulses to potentials positive to E_K elicit smaller outward currents (e.g.,Trautwein and McDonald, 1978; Giles and Imaizumi, 1988), and (iii) as the potential becomes progressively more positive than E_K, outward I_{K1} decreases in amplitude due to voltage-dependent inward rectification.

Two sets of control experiments were conducted to verify that the inward- and outward-directed currents recorded using the voltage-clamp protocols above, are primarily comprised of inward-rectifier K^+ current. Data from the first of these (Figure 4A) indicates that the inward-directed current at -90 mV and the outward-directed current at -50 mV both decline to near zero when inward-rectifier K^+ channel (Kir) conductance is suppressed by removal of external K^+. Results from the second set of experiments (Figure 4B) indicate that Ba^{2+}, a classic blocker of Kir channels (Giles and Imaizumi, 1988; Kamouchi *et al.*, 1997; Gillis *et al.*, 1998), suppresses nearly 100% of the late inward- and outward-directed currents over the potential range of -120 to -10 mV.

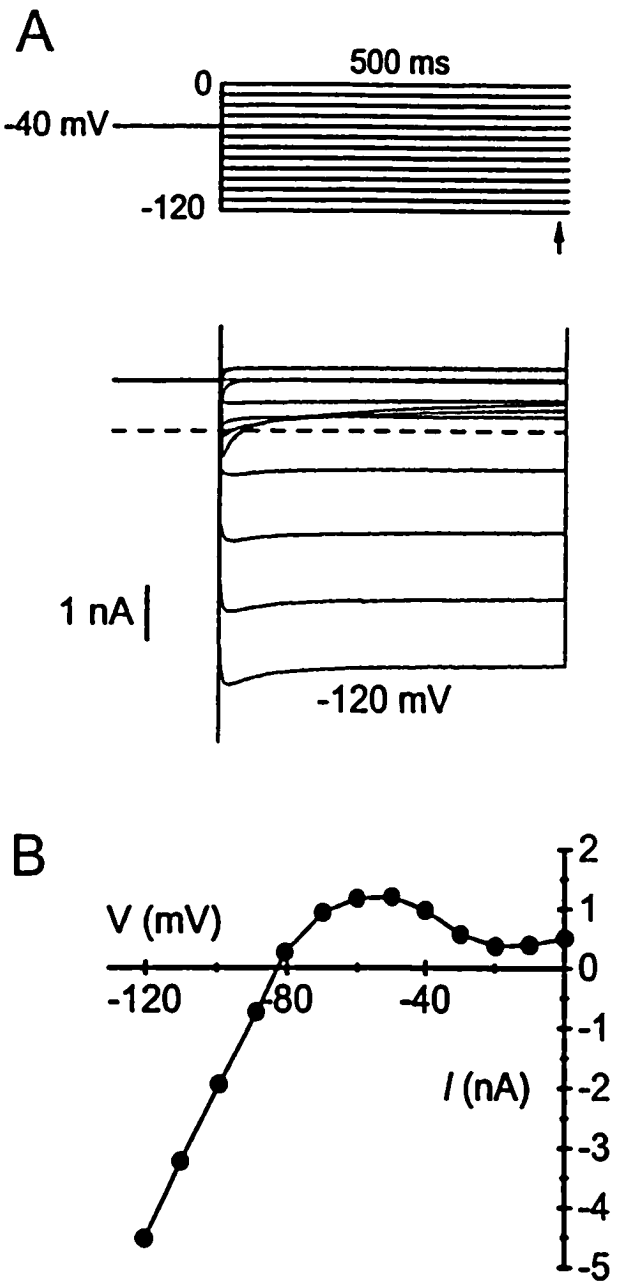


Figure 3. I_{K1} in guinea pig ventricular myocytes superfused with normal Tyrode's solution and dialyzed with K^+ pipette solution. The holding potential was -80 mV, and I_{K1} was elicited by 500-ms depolarizations and hyperpolarizations applied at 0.1 Hz from prepulse -40 mV. (A) Family of current records obtained on pulses to potentials between -120 mV and 0 mV (10-mV steps). The dashed line indicates the zero-current level. (B) The I-V relationship generated from the results in A. The amplitude of I_{K1} was measured as the current at 500 ms (arrow, panel A) relative to zero current.

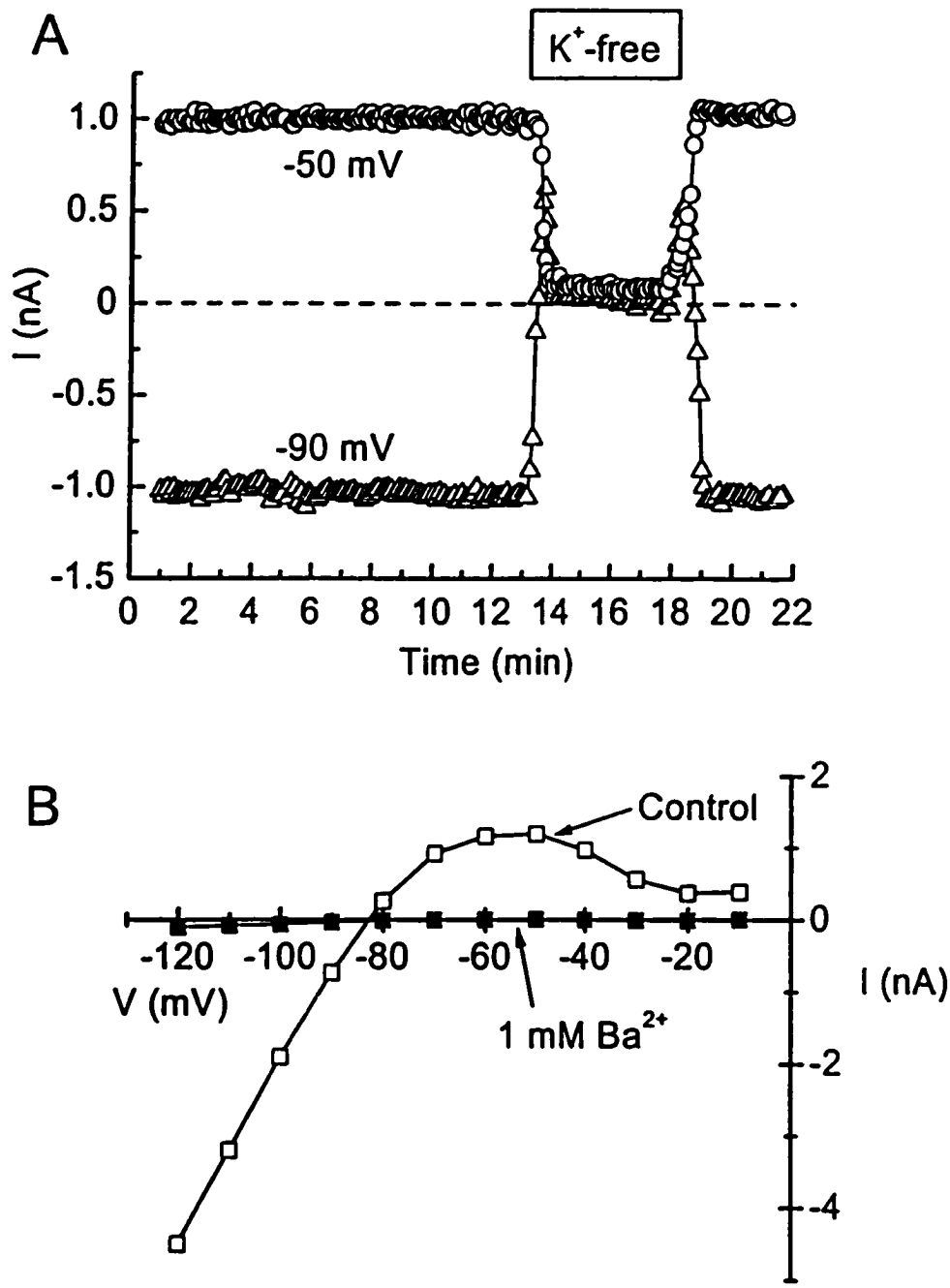


Figure 4. Control experiments related to the identification and measurement of I_{K1}. (A) Inward-directed current at -90 mV and outward-directed current at -50 mV were almost completely suppressed by the removal of external K^+ from the Tyrode's solution (suggesting that the current at these potentials is almost exclusively I_{K1}). (B) Abolition of current over the potential range -120 to -10 mV after addition of I_{K1} inhibitor Ba^{2+} (1 mM) to the Tyrode's solution.

(2) L-type Ca^{2+} current

$I_{Ca,L}$ was recorded from guinea pig ventricular myocytes that were superfused with either normal Tyrode's solution or K^+-free Tyrode's solution, and dialyzed with K^+ or Cs^+ pipette solution. The myocytes were generally held at -80 mV and prepulsed to -40 mV for 100-500 ms to inactivate Na^+ current and any T-type Ca^{2+} current, and then depolarized to 0 mV for 200 ms to elicit $I_{Ca,L}$. A record of $I_{Ca,L}$ obtained from a myocyte bathed and dialyzed in K^+-containing solutions is displayed in Figure 5A. The inward current elicited upon depolarization quickly activates, reaches a peak, and then slowly declines (inactivates) with continued depolarization. The amplitude of $I_{Ca,L}$ was most often measured as peak inward current with reference to zero current (I_{in}). In some experiments, the $I_{Ca,L}$-V relationship was determined by measuring the peak inward current elicited by a series of 200-ms depolarizations from -40 to +50 mV. An example $I_{Ca,L}$-V relationship obtained from a myocyte bathed and dialyzed with K^+-free conditions is displayed in Figure 5B. The relationship has an inverted bell shape with a peak near 0 mV.

To verify that the peak inward current elicited by 200-ms depolarizations from -40 to 0 mV is $I_{Ca,L}$, myocytes were exposed to organic and inorganic Ca^{2+} channel blockers. $I_{Ca,L}$ was nearly completely blocked by 0.2 mM Cd^{2+} and by commonly used Ca^{2+} antagonists such as the dihydropyridine nisoldipine (1 μM) and the phenylalkylamine verapamil (10 μM) (Figure 5C).

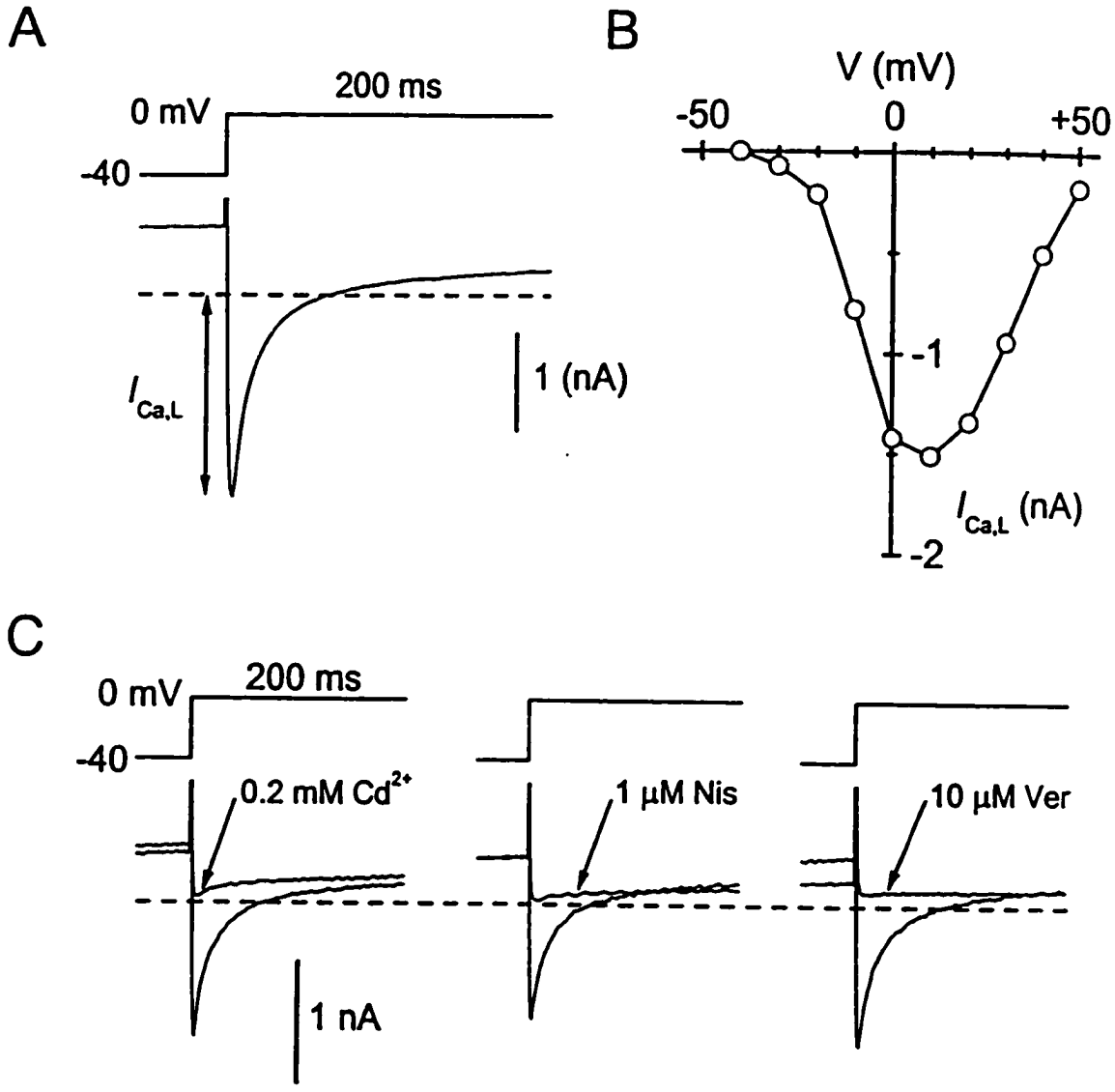


Figure 5. $I_{Ca,L}$ in guinea pig ventricular myocytes. The myocytes were depolarized for 200 ms from -80 to -40 mV, and pulsed to 0 mV for 200 ms at 0.1 Hz except for the determination of the $I_{Ca,L}$-V relationship. (A) Example record from a myocyte superfused with normal Tyrode's solution and dialyzed with K^+ pipette solution. (B) Plot of a representative $I_{Ca,L}$-V relationship from an experiment conducted under K^+-free conditions. The myocyte was held at -40 mV and depolarized for 200 ms to potentials ranging from -40 to +50 mV. (C) Inhibition of $I_{Ca,L}$ by 0.2 mM Cd^{2+} (left), 1 μM nisoldipine (middle), and 10 μM verapamil (right). The dashed lines in A and C indicate the zero-current levels.

(3) Delayed-rectifier I_K

Total delayed-rectifier K^+ current (I_K) in guinea pig ventricular myocytes is a time- and voltage-dependent current comprised of two kinetically distinct components, rapidly activating I_{Kr} and slowly activating I_{Ks} (Sanguinetti and Jurkiewicz, 1990). I_{Kr} is absent at potentials more negative than -40 mV, reaches full activation at about +10 mV, and rapidly inactivates at more positive potentials; the current is dependent on external K^+ concentration, and is inhibited by the removal of external K^+. On the other hand, I_{Ks} activates over a more positive potential range, does not inactivate at positive potentials, and is enhanced by the removal of external K^+. These properties, and the availability of a selective I_{Kr} inhibitor (the methanesulfonanilide class III antiarrhythmic compound E4031 (IC_{50} ~ 0.4 μM: Sanguinetti and Jurkiewicz, 1990)), provide tools for the separation of I_{Kr} from I_{Ks} in voltage clamp experiments on guinea pig ventricular myocytes (Sanguinetti and Jurkiewicz, 1990, 1992; Wettwer *et al.*, 1992; Heath and Terrar, 1996a,b).

Myocytes were repolarized to -40 mV for measurement of $I_{K,tail}$ elicited after 500-ms I_K-activatory depolarizations to test potentials between -30 and +70 mV. Over this potential range, the depolarizations were long enough to activate both I_K components. As a result, when $I_{K,tail}$ amplitude was plotted against the voltage of the I_K-activating depolarization ($I_{K,tail}$-V), the shape of the relationship was biphasic; there was one ascending phase between ca. -30 and +10 mV, and a second ascending phase at more positive potentials (Figure 6A). The ascending phase at low voltages is primarily due to $I_{Kr,tail}$, whereas the ascending phase at high voltages reflects the recruitment of $I_{Ks,tail}$

(Sanguinetti and Jurkiewicz, 1990; Liu and Antzelevitch, 1995; Heath and Terrar, 1996a,b). The relative amplitudes of these two phases varied from myocyte to myocyte.

The results obtained from five experiments with a saturating concentration (3 μM) of the selective I_{Kr} inhibitor E4031 are shown in Figure 6B. E4031 selectively inhibited low-voltage $I_{K,tail}$ with a negligible effect on the increments in $I_{K,tail}$ amplitude produced by pulses to potentials above +10 mV.

Rapidly activating delayed-rectifier K^+ current

I_{Kr} was recorded from myocytes that were dialyzed with K^+ pipette solution and superfused with either normal Tyrode's solution or Ca^{2+}-free Tyrode's solution. Based on the voltage-dependence of the inhibition of $I_{K,tail}$ by E4031 (Figure 6B), I_{Kr} was measured as the amplitude of the $I_{K,tail}$ elicited after depolarizations from -40 to 0 mV at 0.1 Hz. The depolarizations were of relatively short duration (200 ms) to allow for near complete activation of I_{Kr}, and to minimize any concomitant activation of slowly-activating I_{Ks}. Figure 7 shows voltage-clamp records and the time course of changes in $I_{Kr,tail}$ amplitude from an experiment with 3 μM E4031. The drug almost completely inhibited $I_{K,tail}$ amplitude elicited after 200-ms depolarizations. In eighteen experiments of this type, 3-5 μM E4031 reduced $I_{K,tail}$ amplitude to 11 ± 2% of control.

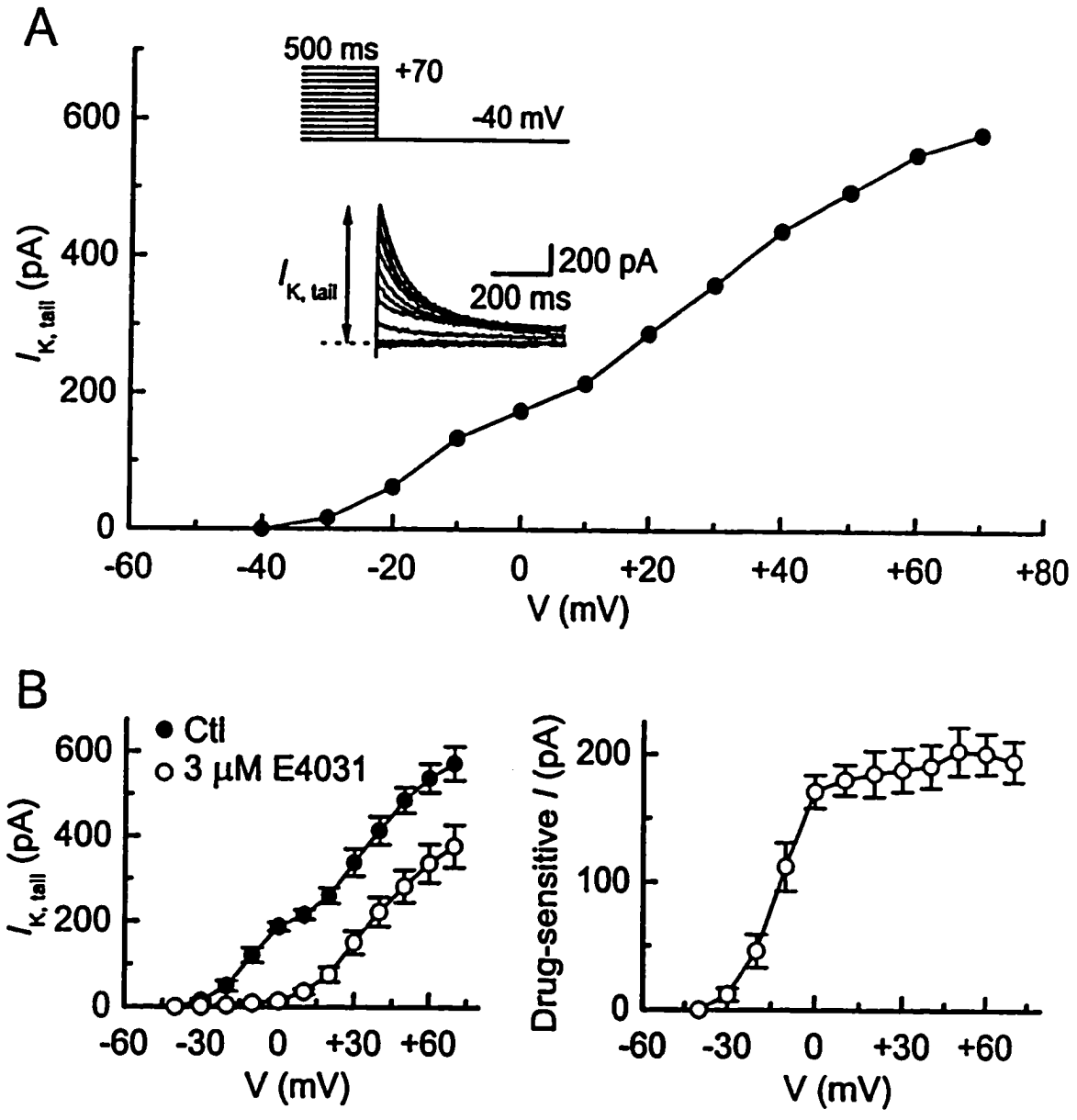


Figure 6. $I_{K,tail}$-V relationships from myocytes bathed in normal Tyrode's solution. Tail currents were measured on repolarizations to -40 mV after 500-ms pulses to potentials as positive as +70 mV. (A) Representative tail currents and $I_{K,tail}$-V relationship. The dotted line indicates the level of the steady-state current at -40 mV. (B) Effects of the selective I_{Kr} inhibitor E4031 (3 μM). Left: mean $I_{K,tail}$-V relationships from five experiments. $p <$ 0.01-0.001 at all potentials > -20 mV. Right: E4031-sensitive current.

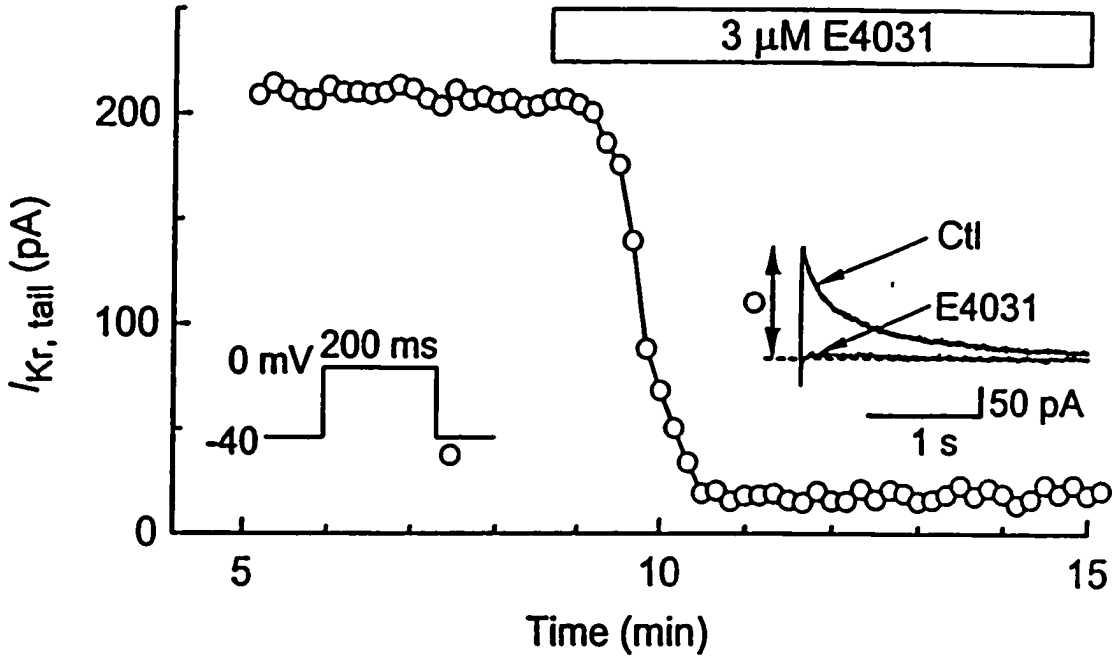


Figure 7. Inhibition of $I_{K,tail}$ by E4031 in a myocyte bathed in normal Tyrode's solution. $I_{K,tail}$, measured after 200-ms depolarizations to 0 mV, was identified as predominantly $I_{Kr,tail}$ based on its sensitivity to 3 μM E4031.

Slowly activating delayed-rectifier K^+ current

The amplitude of slowly activating I_{Ks} was measured in three different ways: (i) as the amplitude of the time-dependent outward current elicited by 500-ms depolarizations to +60 mV in myocytes that were bathed in normal Tyrode's solution, (ii) as the amplitude of $I_{K,tail}$ on repolarization to -40 mV after 500-ms depolarizations to +30 mV in myocytes bathed in K^+-, Ca^{2+}-free Cd^{2+} solution, and (iii) as the amplitude of $I_{K,tail}$ on repolarization to -40 mV after 2-s depolarizations to positive potentials in myocytes bathed in K^+-, Ca^{2+}-free Cd^{2+} solution. The rationale and justification for using each of these protocols are presented below.

(i) Measurement of I_{Ks} in experiments on myocytes superfused with normal Tyrode's conditions. When myocytes were superfused with normal Tyrode's solution, the amplitude of I_{Ks} was estimated as the time-dependent current elicited by 500-ms depolarizations from a prepulse of -40 mV to a test potential of +60 mV. Under these conditions, contaminating contributions from other time-dependent currents that may overlap I_{Ks}, such as I_{Kr}, $I_{Ca,L}$, and rapid Na^+, were likely to have been relatively small; I_{Kr} undergoes rapid inactivation at high positive potentials (Sanguinetti and Jurkiewicz, 1990), $I_{Ca,L}$ is close to its reversal potential (McDonald *et al.*, 1994), and Na^+ current, which is strongly inactivated by the prepulse to -40 mV, is also near its reversal potential.

Figure 8 shows that 5 μM E4031 had a negligible effect on the time course and amplitude of the outward time-dependent current at +60 mV. Although small reductions in amplitude were recorded in some cells, the overall result in a series of control experiments with 5-8 min exposures to E4031 (reduction of 8 ± 4%, n=16) suggests that

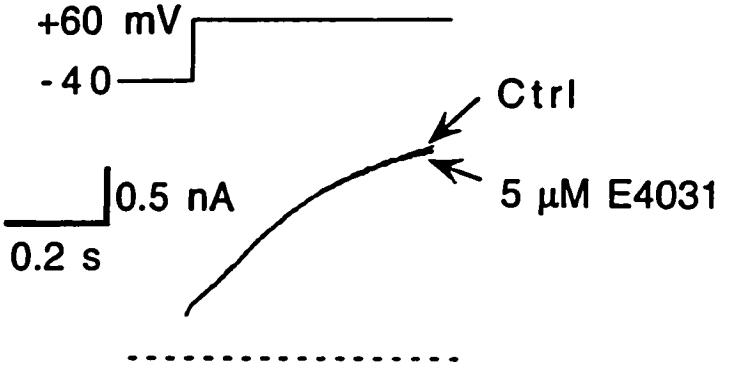


Figure 8. Activation of time-dependent I_{Ks} at +60 mV in a guinea pig ventricular myocyte bathed in normal Tyrode's solution. The myocyte was depolarized from -40 to +60 mV for 500 ms before and after addition of 5 μM E4031 for 6 min. The dotted line indicates zero-current level.

there was little residual I_{Kr} in the time-dependent current at +60 mV (and that there was little rundown of I_{Ks} during the observation period).

(ii) Measurement of I_{Ks} in myocytes superfused with K^+-, Ca^{2+}-free Cd^{2+} solution. Myocytes were superfused with K^+-, Ca^{2+}-free Cd^{2+} solution to (i) minimize the amplitudes of Na^+, Ca^{2+}, Ca^{2+}-dependent, Na^+ pump, and exchanger currents, (ii) minimize the activation of (external-K^+-dependent) I_{Kr}, and (iii) enhance the activation of I_{Ks} (Sanguinetti and Jurkiewicz, 1992; Gintant 1996). As previously shown (Daleau *et al.*, 1997), inclusion of Cd^{2+} appears to have little effect on I_{Ks} (data not included).

When myocytes were bathed in K^+-, Ca^{2+}-free Cd^{2+} solution, the amplitudes of the tail currents elicited after 500-ms depolarizations increased with the amplitude of the depolarization (Figure 9A). The $I_{K,tail}$-V relationship had a single ascending phase that originated at about 0 mV. This indication of a near-exclusive I_{Ks} condition was confirmed by the negligible effect of 5 μM E4031 on the $I_{K,tail}$-V relationship (Figure 9A). Likewise, the current records in Figure 9B illustrate that 5 μM E4031 had a negligible effect on the amplitude and time course of both the time-dependent outward I_K elicited during 500-ms depolarizations to +30 mV, and the associated $I_{K,tail}$ recorded after repolarization to -30 mV.

In many of the experiments in which myocytes were superfused with a K^+-, Ca^{2+}-free Cd^{2+} solution, I_{Ks} was measured as the amplitude of the tail current elicited after relatively long (2 s) depolarizations to positive potentials. The long depolarizations permitted slowly-activating I_{Ks} to activate to a greater extent than after 500-ms

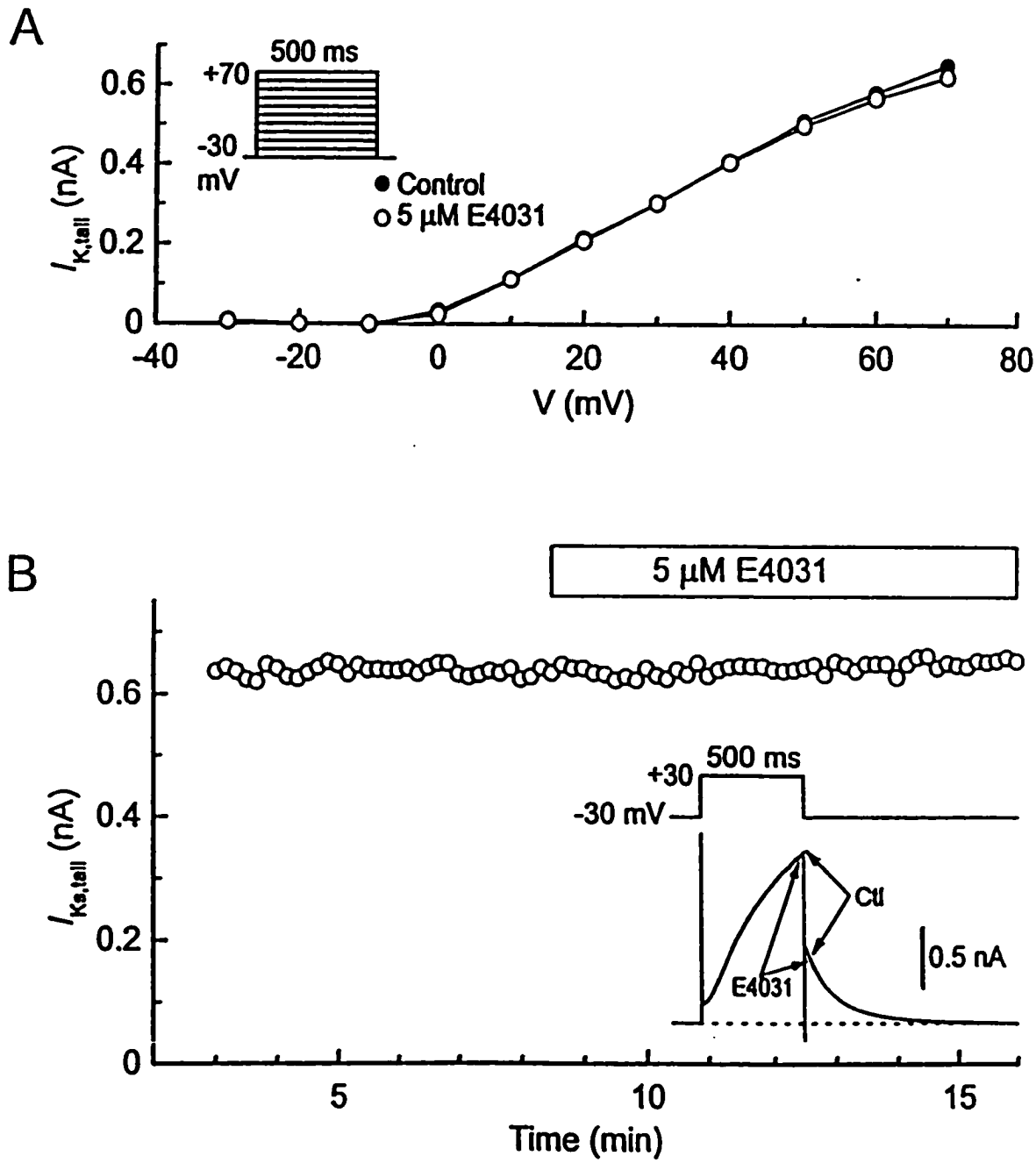


Figure 9. Measurement of I_{Ks} under K^+-, Ca^{2+}-free Cd^{2+} conditions. I_{Ks} was activated by sequences of 500-ms depolarizations from -30 mV to +70 mV at 0.1 Hz. (A,B) Results from a representative myocyte illustrating the $I_{K,tail}$-V relationship and the lack of effect of the I_{Kr} inhibitor E4031. Similar results were obtained in four other experiments with 5 µM E4031.

depolarizations (Sanguinetti and Jurkiewicz, 1990; Heath and Terrar, 1996a), and further marginalized any contribution from residual rapidly-activating I_{Kr}.

(4) Transient outward K^+ current

I_{to} was recorded from rabbit ventricular cells because, in contrast to guinea pig ventricular myocytes, the rabbit myocytes have a prominent I_{to} (Hiraoka and Kawano, 1987; McDonald *et al.*, 1989; Carmeliet, 1993). To separate I_{to} from other membrane currents, rabbit myocytes were dialyzed with K^+ pipette solution and superfused with a modified Tyrode's solution that contained 0.2 mM Cd^{2+} to block $I_{Ca,L}$, 0.1 mM Ba^{2+} to block I_{K1}, 3 μM E4031 to block I_{Kr}, and 20 μM tetrodotoxin to block I_{Na}. The temperature was either 24°C or 36°C and the myocytes were generally held at -80 mV, and depolarized to +30 mV for 400 ms at 0.1 Hz.

A record of the membrane current obtained from a myocyte bathed with 24°C solution (to slow down current kinetics and improve resolution) is displayed in Figure 10. The outward current quickly reached a peak following depolarization and then decayed to a near steady-state level by the end of the 400-ms pulse. The amplitude of I_{to} was measured as the time-dependent component of the current, i.e., the difference between the outward peak at ~10 ms, and the level at the end of the pulse. In many experiments, the I_{to} blocker, 4-aminopyridine (5 mM), was added at the end of the drug treatment period to establish background zero- I_{to} (see Castle *et al.*, 1993; Jahnel *et al.*, 1994).

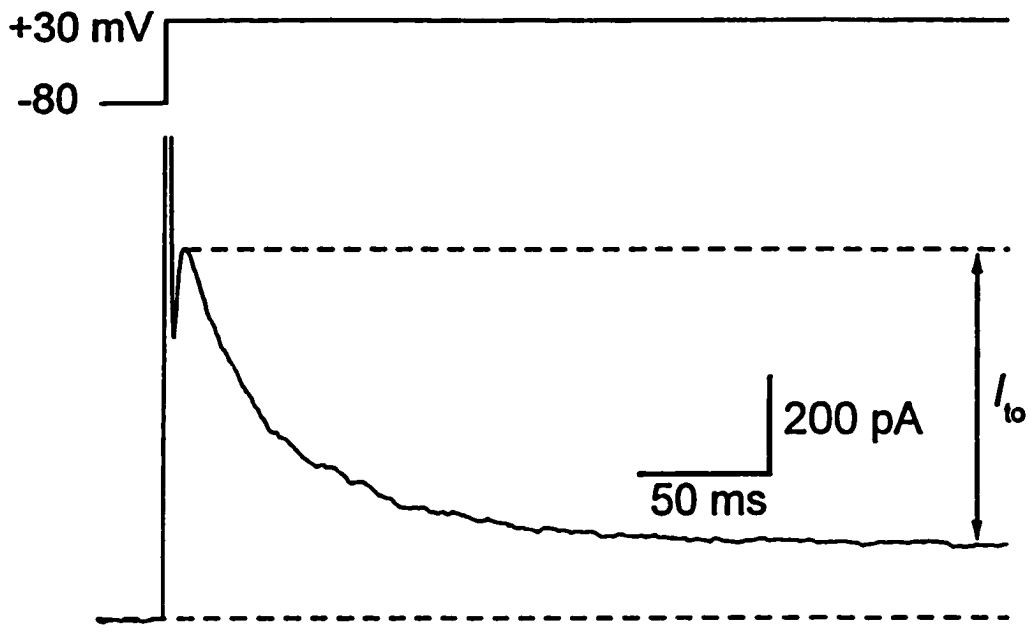


Figure 10. Measurement of I_{to}. The rabbit ventricular myocyte was superfused with a modified Tyrode's solution that contained 0.2 mM Cd^{2+}, 0.1 mM Ba^{2+}, 3 μM E4031, and 20 μM tetrodotoxin. The myocyte was held at -80 mV, and I_{to} was elicited by 400-ms depolarizations to +30 mV at 0.1 Hz. The amplitude of outward I_{to} was measured as the time-dependent current between the early outward peak and the near-steady-state late outward current. The bottom dashed line indicates the zero-current level. Temperature 24°C.

III. RESULTS

The results of this study are organized into two main sections. The first section is concerned with the concentration-dependent inhibitory effects of terodiline on four membrane currents in guinea pig ventricular myocytes (I_{K1}, $I_{Ca,L}$, I_{Kr}, and I_{Ks}), and on an additional membrane current (I_{to}) in rabbit ventricular myocytes. The second major section is concerned with the concentration-dependent inhibitory effects of oxybutynin and S-oxybutynin on the same five membrane currents, with an emphasis on how these effects compare with those of terodiline.

SECTION A. EFFECTS OF TERODILINE ON MEMBRANE CURRENTS

Figure 11 shows records of membrane currents obtained from a representative myocyte that was bathed in normal Tyrode's solution and held at -80 mV. The myocyte was depolarized for 500 ms from a prepulse potential of -40 mV to potentials up to +70 mV. The depolarizations elicited four membrane current components: outward I_{K1} at prepulse -40 mV prior to depolarization, inward $I_{Ca,L}$ that reached a peak shortly after the onset of depolarization, time-dependent outward I_K that was most prominent at large positive potentials, and decaying outward K^+ tail current ($I_{K,tail}$) upon repolarization to -40 mV. Applications of 10 and 50 μM terodiline caused reductions in the amplitudes of all four membrane current components, with 50 μM drug having a larger effect than 10 μM drug.

The concentration-dependent inhibitory effects of terodiline on these four cardiac membrane currents are examined in more detail in the subsections below.

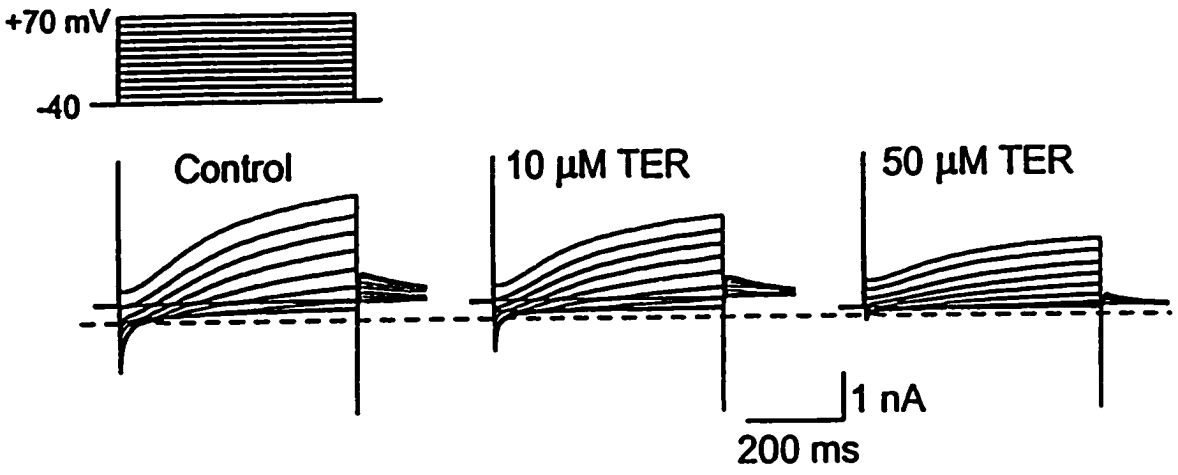


Figure 11. Inhibition of whole-cell membrane currents by terodiline (TER). The guinea pig ventricular myocyte bathed and dialyzed with K^+-containing solutions was held at -80 mV, and pulsed at 0.1 Hz for 500-ms depolarizations up to +70 mV following 200-ms prepulses to -40 mV. Records were obtained before (control) and 8-10 min after additions of 10 and 50 µM terodiline. The dashed line indicates zero-current level.

(1) Effects on I_{K1}

Concentration-dependent inhibition

To determine the effect of terodiline on the current-voltage relationship of I_{K1} (I_{K1}-V), myocytes were pulsed for 500 ms from -40 mV to potentials between -120 and -20 mV, and the late (end-of-pulse) current amplitudes were measured with respect to zero-current level.

I_{K1} in mammalian ventricular myocytes is large and inward at very negative potentials, reverses at a potential close to the calculated K^+ equilibrium potential, and is outward and progressively smaller at more positive potentials (Giles and Imaizumi, 1988). The current is by far the primary determinant of the quasi-steady-state I-V relationship between -120 and -20 mV (see Figures 3 and 4 in methods). The results from a representative experiment indicate that terodiline had little effect on the reversal potential of I_{K1}, but inhibited the amplitudes of both inward- and outward-directed current by approximately 20% (Figure 12A). The mean I-V relationship of the difference (control minus terodiline) current obtained from six myocytes treated with 3 μM terodiline is shown in Figure 12B. The terodiline-sensitive I_{K1}-V relationship has the same general features as the control and 3 μM terodiline I_{K1}-V relationships, suggesting that the inhibition of I_{K1} is voltage-independent.

The effects of terodiline on I_{K1} were primarily assessed by measuring drug-induced changes in the steady (or late) outward-directed current at ca. -40 mV. Aside from a small run-up during the first 5 min after patch breakthrough, this current was quite stable under control conditions (Figure 13A). The panels in Figure 13A-D illustrate that outward I_{K1} at ca. -40 mV was insensitive to submicromolar concentrations of terodiline, but markedly inhibited by high micromolar concentrations. The half-time to maximal inhibitory effect was approximately 3 min (e.g., Figure 13C), and was apparently

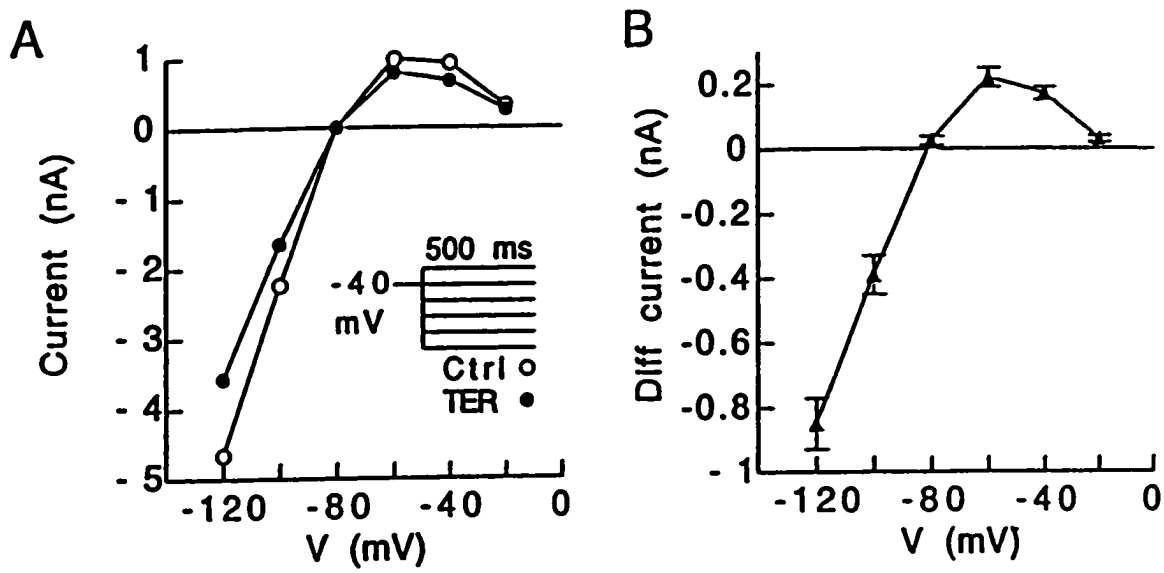


Figure 12. Effects of terodiline on the I_{K1}-V relationship. (A) I_{K1}-V relationships were determined from measurements of end-of-pulse currents elicited by 500-ms depolarizing and hyperpolarizing pulses from prepulse -40 mV; these pulses were applied before and 12 min after addition of 3 μM terodiline. (B) Mean I_{K1}-V relationship of the difference current (control minus terodiline) measured from six myocytes investigated as in A.

independent of (i) voltage across the membrane, and (ii) direction of the current (Figure 13D). Very high (100-300 μM) concentrations of terodiline blocked about 60% of outward (and inward) I_{K1}, and the remaining current in the presence of the drug declined to near zero when inward-rectifier channel conductance was suppressed by removal of external K^+ (e.g., Figure 13D).

Terodiline-induced effects on outward I_{K1} were evaluated in experiments on a total of 88 myocytes. The data are well described by the Hill equation, I_{K1} (% control) = $(Max/(1+(TER/IC_{50})^{n_H})) + (100-Max)$ where Max refers to maximal percentage inhibition. The projected Max is 60% and the IC_{50} is estimated to be 7 μM, with a Hill coefficient of 0.82 (Figure 13E).

It was possible that the measurements of I_{K1} at ca. -40 mV were "contaminated" due to a superimposed, terodiline-induced, inhibition of steady-state outward I_{Kr}. To assess this possibility, eight myocytes were pretreated with I_{Kr} inhibitor E4031 (1 μM) for ≥ 5 min and then exposed to terodiline. E4031 had little effect on the current amplitude at -40 mV, and the extent of the subsequent inhibition of I_{K1} by terodiline in these myocytes was as follows: 1 μM: 10 ± 2% inhibition (n=4); 10 μM: mean 27% inhibition (n=2); 30 μM: mean 45% inhibition (n=2). Since the degree of inhibition was not significantly different than that measured in non-E4031-pretreated myocytes (see Figure 13E), it can be concluded that the measurements of outward I_{K1} were not contaminated by changes in I_{Kr}.

Delayed recovery of outward I_{K1} from inhibition

Terodiline-induced inhibition of I_{K1} was not easily reversed upon drug removal. For example, washout for 13 min only restored the current to 70% control after a 4-min treatment with 10 μM terodiline had reduced it to 63% control (Figure 14B). Additional

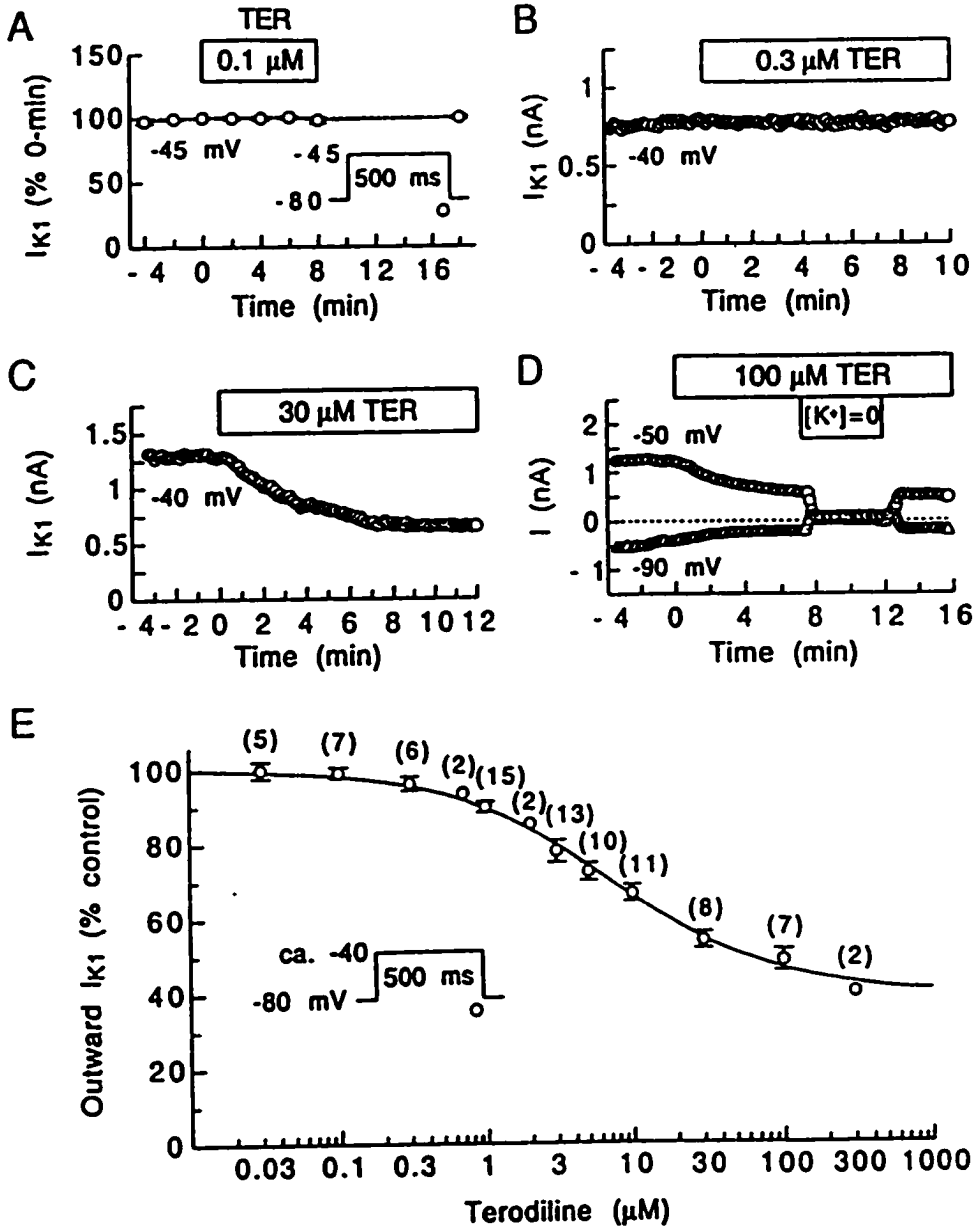


Figure 13. Terodiline-induced inhibition of I_{K1}. (A) Stable outward I_{K1} at -45 mV before, during, and after exposure to 0.1 μM terodiline. Current amplitudes were normalized to the current measured at 0 min (approximately 8 min post-patch-breakthrough); n=7 myocytes up to 8 min, n=4 thereafter. (B) Outward I_{K1} amplitude at -40 mV in a myocyte exposed to 0.3 μM drug. (C) Inhibition by 30 μM drug in a myocyte pretreated with 1 μM E4031. (D) Effects of 100 μM drug on outward I_{K1} at -50 mV and inward I_{K1} at -90 mV. Removal of external K^+ ($[K^+]=0$) reversibly suppressed terodiline-insensitive current. (E) Concentration-response relationship. The amplitude of steady-state outward I_{K1} at ca. -40 mV in the presence of the drug was expressed as a percentage of the pre-drug control amplitude. The data are fitted with the Hill equation with an IC_{50} of 7 μM and a coefficient of 0.82. Number of myocytes are shown in parentheses.

long-washout experiments with 3 μM (n=4), 10 μM (n=2), 30 μM (n=2), and 100 μM (n=2) terodiline confirmed this pattern. The slow reversibility was not simply a consequence of current rundown because inhibition of I_{K1} by a 'reference' K^+ channel blocker (150 μM daidzein: Smirnov and Aaronson, 1995) was promptly reversed upon drug removal (Figure 14C).

Not all of the cardiac membrane currents inhibited by terodiline were slow to recover after the drug was removed. For example, the time plots in Figure 16B-D illustrate that there was prompt recovery of $I_{Ca,L}$ after removal of 3-100 μM drug. Similarly, both the time-dependent I_{Ks} at +40 mV and the associated tail current at -40 mV quickly recovered after washout of 100 μM terodiline (see Figure 29A). These results suggest that there is a temporal pattern of recovery of cardiac membrane currents after inhibition with terodiline, with $I_{Ca,L}$ and I_K recovering faster and to a larger extent than I_{K1}.

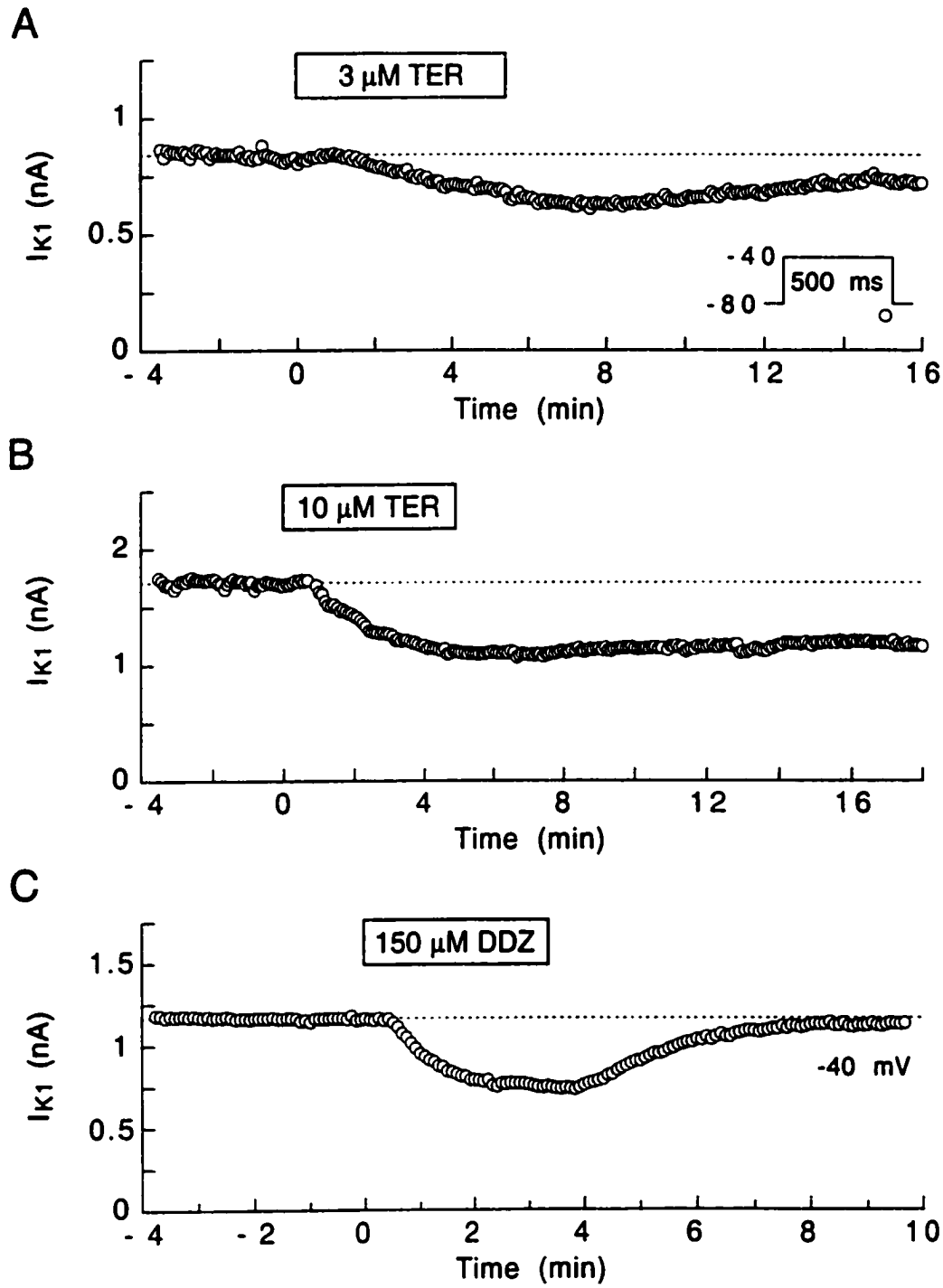


Figure 14. Recovery of outward I_{K1} after drug treatment. Examples of slow recovery after removal of 3 μM terodiline (A) and 10 μM terodiline (B). (C) Prompt recovery on washout of a reference inhibitory drug (daidzein; DDZ).

(2) Effects on Ca^{2+} Channel Currents

Concentration-dependent inhibition

The initial series of experiments designed to evaluate the effects of terodiline on $I_{Ca,L}$ in guinea pig ventricular myocytes were conducted on myocytes that were bathed and dialyzed with K^+-containing solutions. $I_{Ca,L}$ was elicited by 200-ms depolarizations from -40 to 0 mV, and peak $I_{Ca,L}$ was generally measured as peak inward current (reference to zero-current). Time plots and current records from a representative experiment with 3 μM terodiline illustrate that the drug inhibited peak $I_{Ca,L}$, but had a negligible effect on the end-of-pulse outward current (I_{200}) level (Figure 15A) (which is likely to be due to offsetting inhibitions of noninactivated inward $I_{Ca,L}$ and outward I_{Kr} (see below)). In five myocytes treated with 3 μM terodiline, peak $I_{Ca,L}$ at 0 mV was reduced by 26 ± 4% ($p < 0.005$). The $I_{Ca,L}$-V relationship in Figure 15B illustrates that the inhibition was independent of test potential between -20 and +40 mV.

To examine the dependence of the inhibition of peak $I_{Ca,L}$ on terodiline concentration, myocytes were exposed to concentrations that ranged from 0.1 to 100 μM. Time plots of peak $I_{Ca,L}$ amplitude measured in representative myocytes illustrate that 0.3 μM terodiline had little effect, whereas 3, 10, and 100 μM drug reversibly inhibited the amplitude by 25, 40, and 95%, respectively (Figure 16A-D).

The results obtained from these and similar experiments are summarized in the concentration-response relationship shown in Figure 16E. $I_{Ca,L}$ amplitude was expressed as a percentage of pre-drug control, and the data are well described by the Hill equation, $I_{Ca,L}$ (% control) $=100/\{1+([TER]/IC_{50})^{n_H}\}$ in which the IC_{50} is 15.2 μM and the Hill coefficient is 0.84.

A similar series of experiments was conducted on myocytes that were superfused and dialyzed with K^+-free solutions to minimize the influence of K^+ currents on the

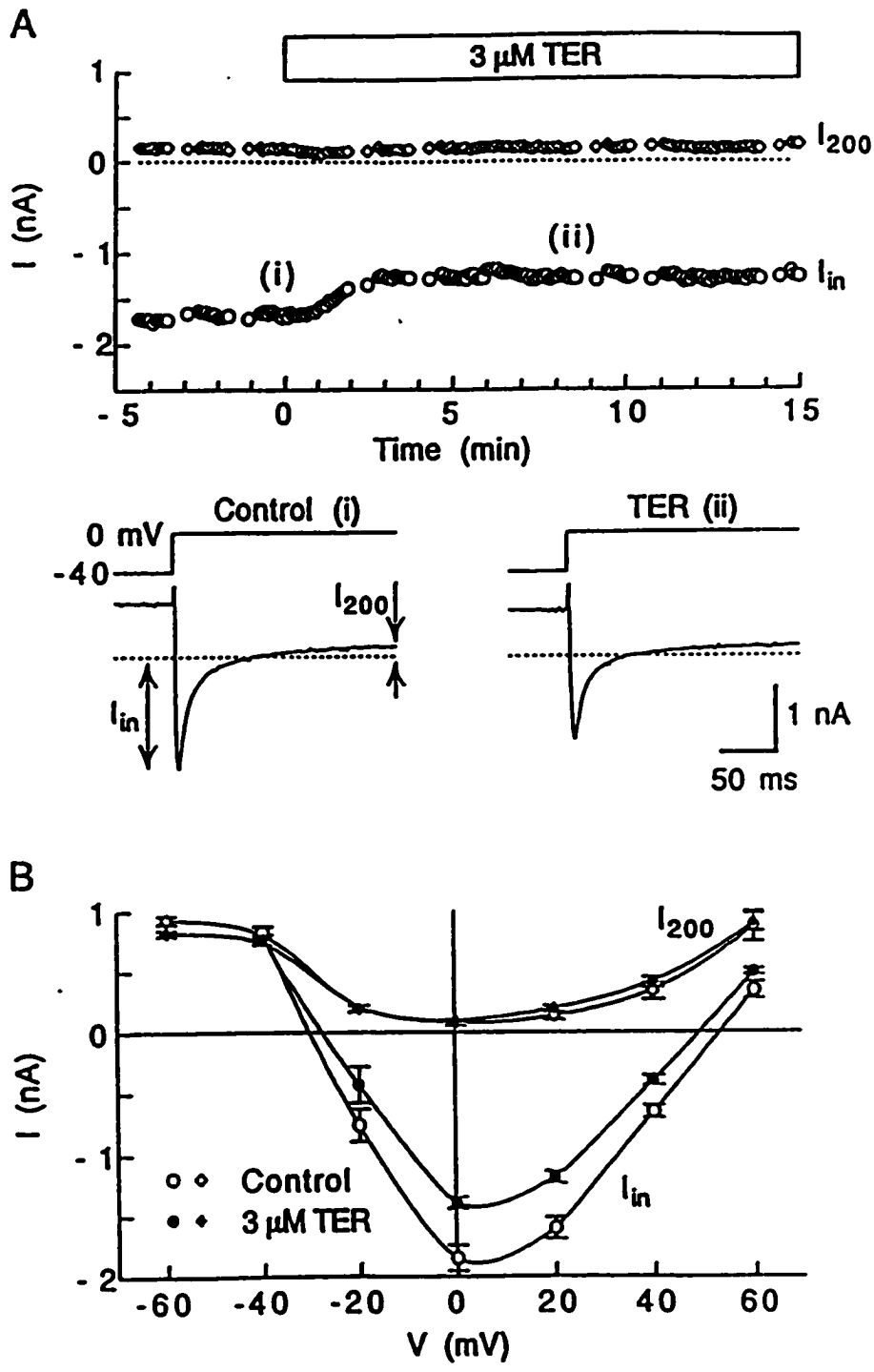


Figure 15. Effects of 3 μM terodiline on $I_{Ca,L}$. (A) Data from a representative experiment. Top: time course of changes in peak inward (I_{in}) and end-of-pulse current (I_{200}) amplitudes at 0 mV, measured with respect to zero current. Bottom: records obtained at times indicated in the time plot. (B) Average $I_{Ca,L}$-V relationships from five myocytes obtained before (control) and 9-12 min after addition of 3 μM drug.

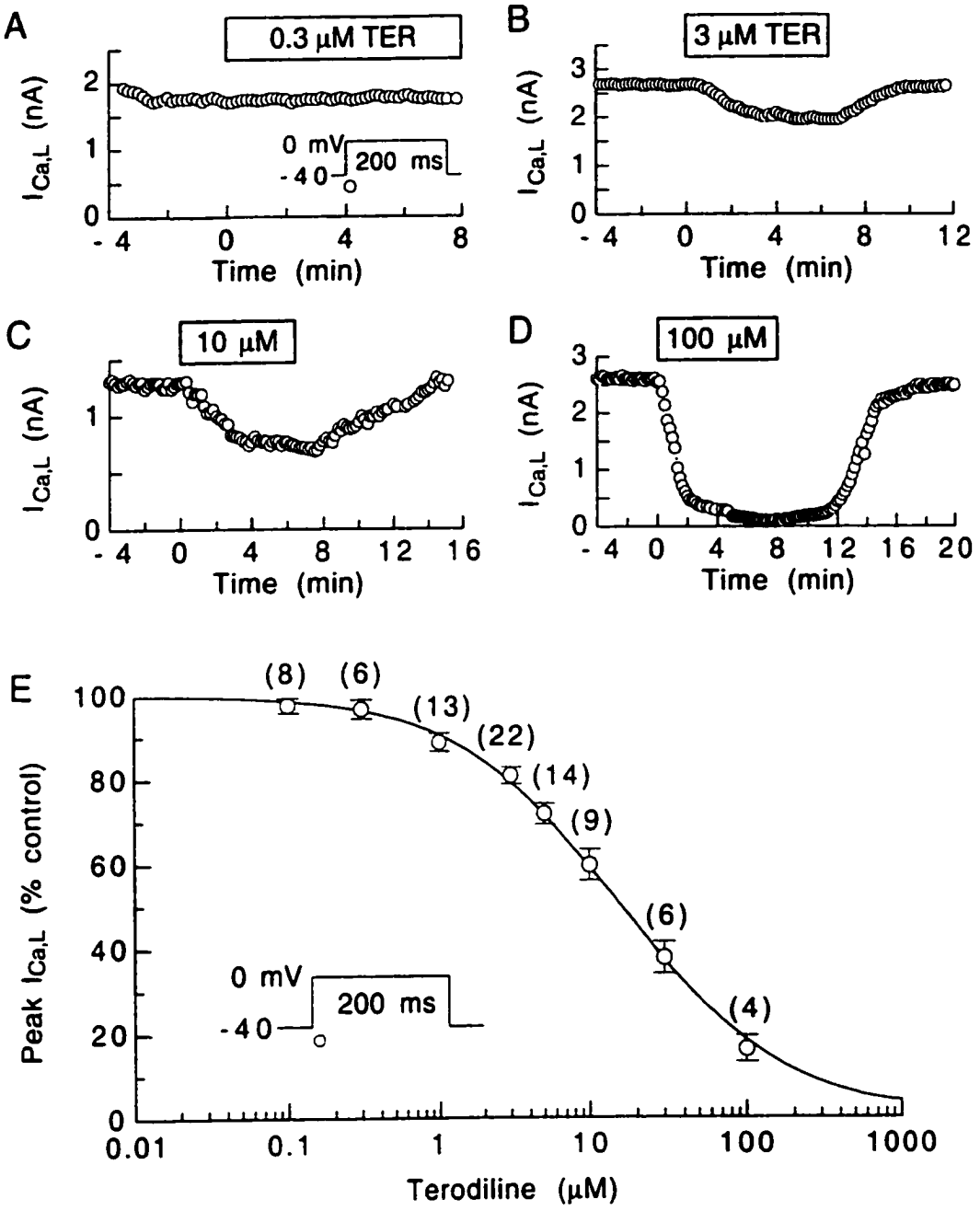


Figure 16. Concentration-dependent inhibition of $I_{Ca,L}$ amplitude by terodiline. The myocytes were bathed and dialyzed with K^+ solutions, and $I_{Ca,L}$ was elicited by 200-ms pulses from -40 mV to 0 mV at 0.1 Hz. (A-D) Time courses of $I_{Ca,L}$ amplitude measured from representative myocytes exposed to 0.3, 3, 10, and 100 μM drug. (E) Concentration-response relationship. Most (>85%) of the myocytes were treated with a single concentration for 6-12 min, and $I_{Ca,L}$ amplitude was expressed as a percentage of pre-drug control. The data are described by the Hill equation with an IC_{50} of 15.2 μM and a coefficient of 0.84. Number of myocytes in parentheses.

measurement of the amplitude of $I_{Ca,L}$. Figure 17A displays current records obtained from a myocyte depolarized for 200 ms from -40 to 0 mV, treated with 5 and 20 μM terodiline, and then exposed to 0.4 mM Cd^{2+} to establish the zero-$I_{Ca,L}$, background current level. Terodiline reduced the amplitude of $I_{Ca,L}$ in a concentration-dependent manner, with 5 μM lowering the current to 73% control and 20 μM lowering it to 28% control. The time plot of peak $I_{Ca,L}$ from an experiment with 1, 10, and 50 μM terodiline provides a further example of concentration-dependent inhibition (Figure 17B).

The overall data on peak $I_{Ca,L}$ in myocytes bathed and dialyzed in K^+-free conditions are well described by the Hill equation with an IC_{50} of 12.2 μM and a coefficient of 0.9 (Figure 17C). This result is not very different than that obtained from myocytes bathed and dialyzed with K^+-containing solutions (IC_{50} 15.2 μM, coefficient 0.84).

Dependence of inhibition on holding potential

To investigate whether inhibition of peak $I_{Ca,L}$ by terodiline is sensitive to changes in holding potential, the holding potential in myocytes pulsed to 0 mV for 200 ms at 0.1 Hz was changed from ca. -43 mV to more positive potentials for short (2-3 min) test periods before and after application of the drug. It is important to note that a lowering of the holding potential that reduces Ca^{2+} channel availability can depress $I_{Ca,L}$ even in the absence of an inhibitory drug (McDonald *et al.*, 1994). The records in Figure 18A (left) depict such an effect when the holding potential is shifted from -43 to -20 mV. The same records also highlight a measurement problem that arises when the holding potential is adjusted to less positive potentials, i.e., the change causes an outward shift in the end-of-pulse current, and this needs to be taken into account when $I_{Ca,L}$ amplitude is measured. In order to minimize the effects of end-of-pulse current shifts on the

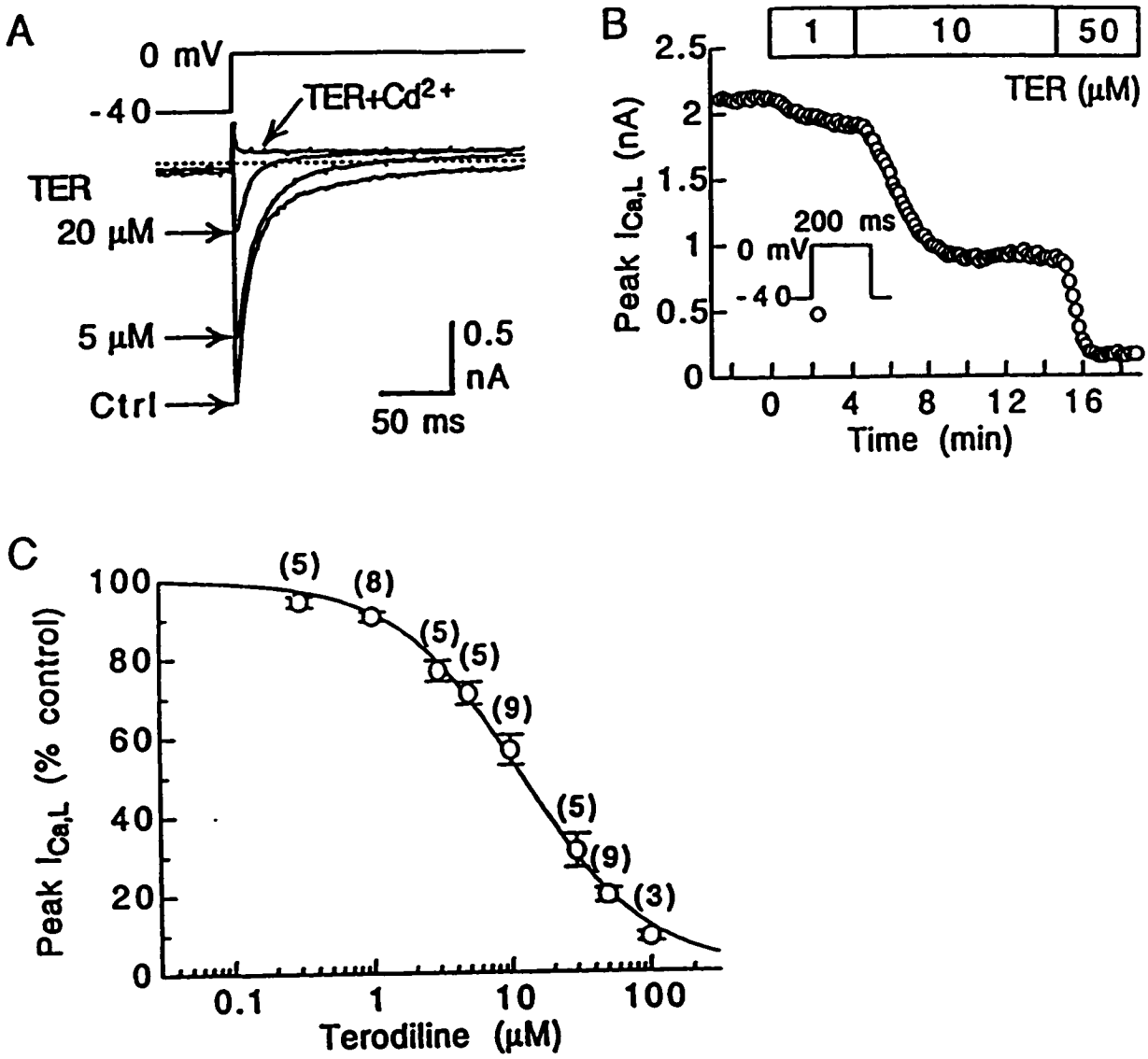


Figure 17. Inhibition of $I_{Ca,L}$ by terodiline in myocytes bathed in K^+-free solution and dialyzed with Cs^+ solution. The myocytes were depolarized from -40 to 0 mV for 200 ms at 0.1 Hz. Exposures to terodiline were followed by additions of 0.4 mM Cd^{2+} to establish background current levels at 0 mV for measurement of peak $I_{Ca,L}$. (A) Current records from a representative myocyte treated with 5 and 20 µM drug, and then with 0.4 mM Cd^{2+}. (B) Time course of peak $I_{Ca,L}$ from a representative myocyte treated with 1, 10, and 50 µM terodiline. (C) Concentration-response relationship. The data are fitted with the Hill equation with an IC_{50} of 12.2 µM and a coefficient of 0.9. Number of myocytes in parentheses.

measurement of $I_{Ca,L}$ amplitude, $I_{Ca,L}$ was measured as the difference between the end-of-pulse current level and the inward peak. The current records in the right hand panel of Figure 18A, and the time plot of $I_{Ca,L}$ amplitude in Figure 18B, illustrate that the changes in $I_{Ca,L}$ amplitude elicited by shifts in the holding potential were larger in the presence of 1 μM terodiline than in its absence.

In trials on 23 myocytes treated with 1 or 5 μM terodiline, the degree of drug-induced inhibition was assessed at holding potentials of -43, -35, -30, and -25 mV. Each reduction in holding potential accentuated inhibition by the drug (Figure 18C). For example, inhibition by 1 μM terodiline (expressed relative to pre-drug current elicited at 0 mV from that holding potential) was 12 ± 2% (n=11 observations) at -43 mV holding potential, 33 ± 5% (n=5) at -35 mV, and 56 ± 4% (n=8) at -25 mV. These results indicate that inhibition of $I_{Ca,L}$ by terodiline is favoured at holding potentials that cause partial steady-state inactivation.

Dependence of inhibition on pulsing frequency

To investigate whether inhibition of $I_{Ca,L}$ by terodiline is affected by the frequency of voltage-clamp pulsing, myocytes were regularly depolarized with 200 ms pulses from -40 to 0 mV at 0.1 Hz, and periodically probed with trains of 25 pulses at 3 Hz. At this increased pulsing rate, the interpulse interval is only 133 ms long, and therefore too short to allow for full recovery of $I_{Ca,L}$ from depolarization-induced inactivation. As a consequence, $I_{Ca,L}$ amplitude under pre-drug control conditions is decreased when the pulsing frequency is increased to ca. 3 Hz (Boyett *et al.*, 1994; Asai *et al.*, 1996), and an example of this modulation is shown in the left hand panel of Figure 19A.

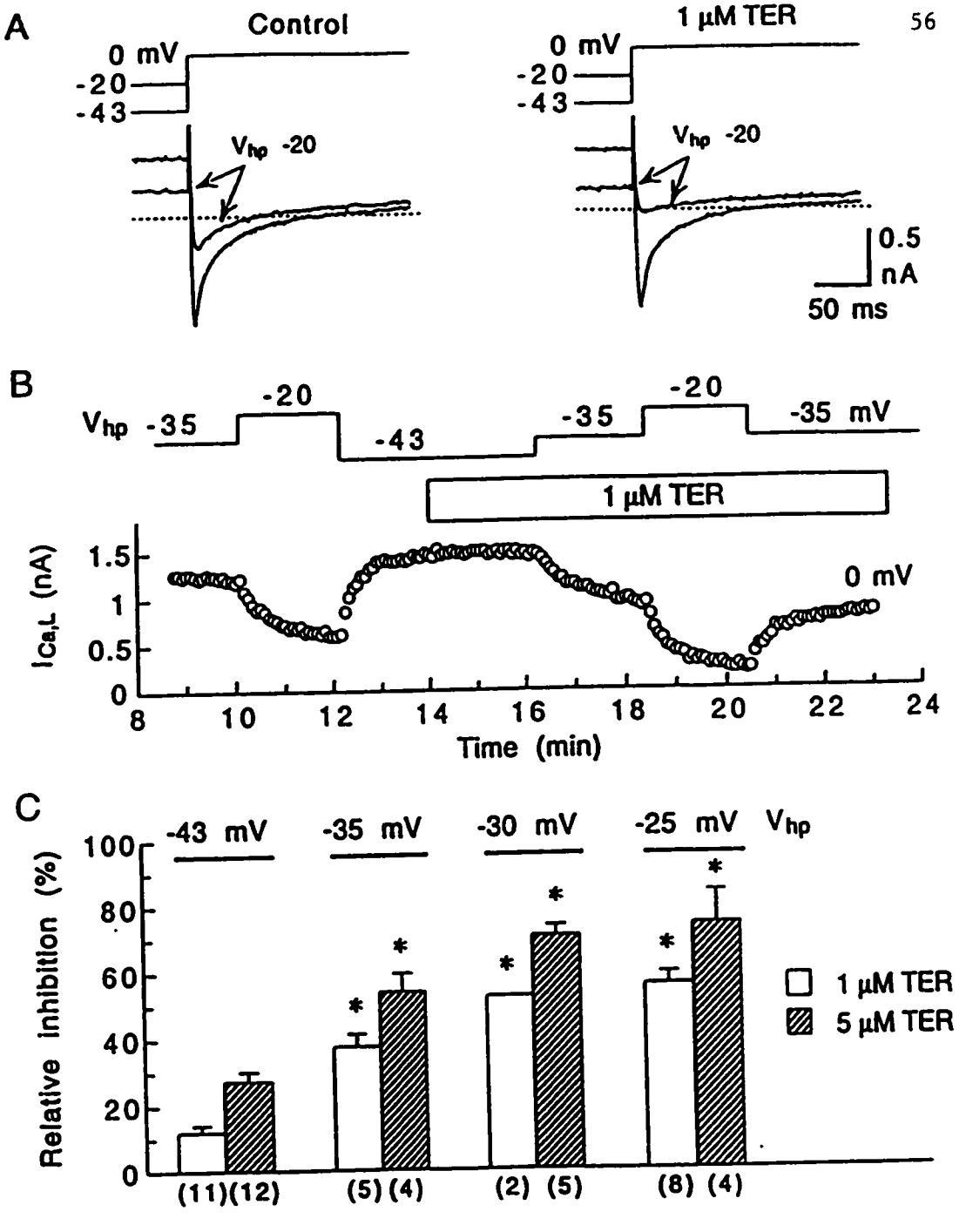


Figure 18. Effect of holding potential on the inhibition of $I_{Ca,L}$ by terodiline. Myocytes bathed and dialyzed with K^+ solutions were held at different potentials and pulsed to 0 mV at 0.2 Hz. (A) Records showing the steady-state effects of changing the holding potential (V_{hp}) from -43 to -20 mV for 3 min before (left) and 6 min after addition of 1 μM drug (right). (B) Time course of the changes in $I_{Ca,L}$ amplitude induced by changes in holding potential before and during exposure to 1 μM drug. (C) Summary of data obtained from myocytes treated with 1 or 5 μM drug. Inhibition at each holding potential is expressed relative to pre-drug current elicited at 0 mV from that holding potential. Numbers of myocytes in parentheses. $^*p < 0.001$ versus relative inhibition at -43 mV.

When measured relative to $I_{Ca,L}$ amplitude at 0.1 Hz just prior to a 3-Hz train, the reductions caused by the increased stimulation rate were more severe in the presence of 5 μM terodiline than under pre-drug conditions (Figure 19A,B). In seven myocytes, $I_{Ca,L}$ at 3 Hz declined to 32 ± 5% of its 0.1-Hz amplitude after treatment with 5 μM terodiline, and this reduction was significantly ($p < 0.001$) greater than that observed under pre-drug conditions (decline to 68 ± 5%). Smaller drug-related effects were observed with lower terodiline concentrations and lower test frequencies. For example, an increase from 0.1 to 2 Hz in the presence of 1 μM terodiline reduced $I_{Ca,L}$ to 74 ± 5% (n=4) compared to the pre-drug reduction to 87 ± 5% ($p < 0.01$).

In summary, these results indicate that there is a frequency-dependent component of terodiline action on $I_{Ca,L}$ such that drug-induced inhibition becomes more pronounced as the stimulation rate is increased.

Slowed recovery from inactivation

Frequency-dependent block of $I_{Ca,L}$ by organic compounds is commonly associated with a slowing of the recovery of $I_{Ca,L}$ from inactivation (McDonald *et al.*, 1994). To investigate whether this occurs in myocytes treated with terodiline, a double-pulse protocol was employed. The conditioning 200-ms pulse from ca. -40 mV to 0 mV served to inactivate $I_{Ca,L}$, and the 200-ms test pulse applied at various intervals (Δt) thereafter served to monitor the time course of recovery of $I_{Ca,L}$ from inactivation. Consecutive pairs of pulses were separated by an interval of 20 s.

In ten myocytes held at -45 mV and depolarized with 200-ms double pulses to 0 mV with recovery time (Δt) ranging from 10 ms to 5 s, the recovery of $I_{Ca,L}$ prior to drug treatment was adequately described by an exponential with a time constant of 82 ms. In these same myocytes, recovery from inactivation determined after ca. 5-min exposure to

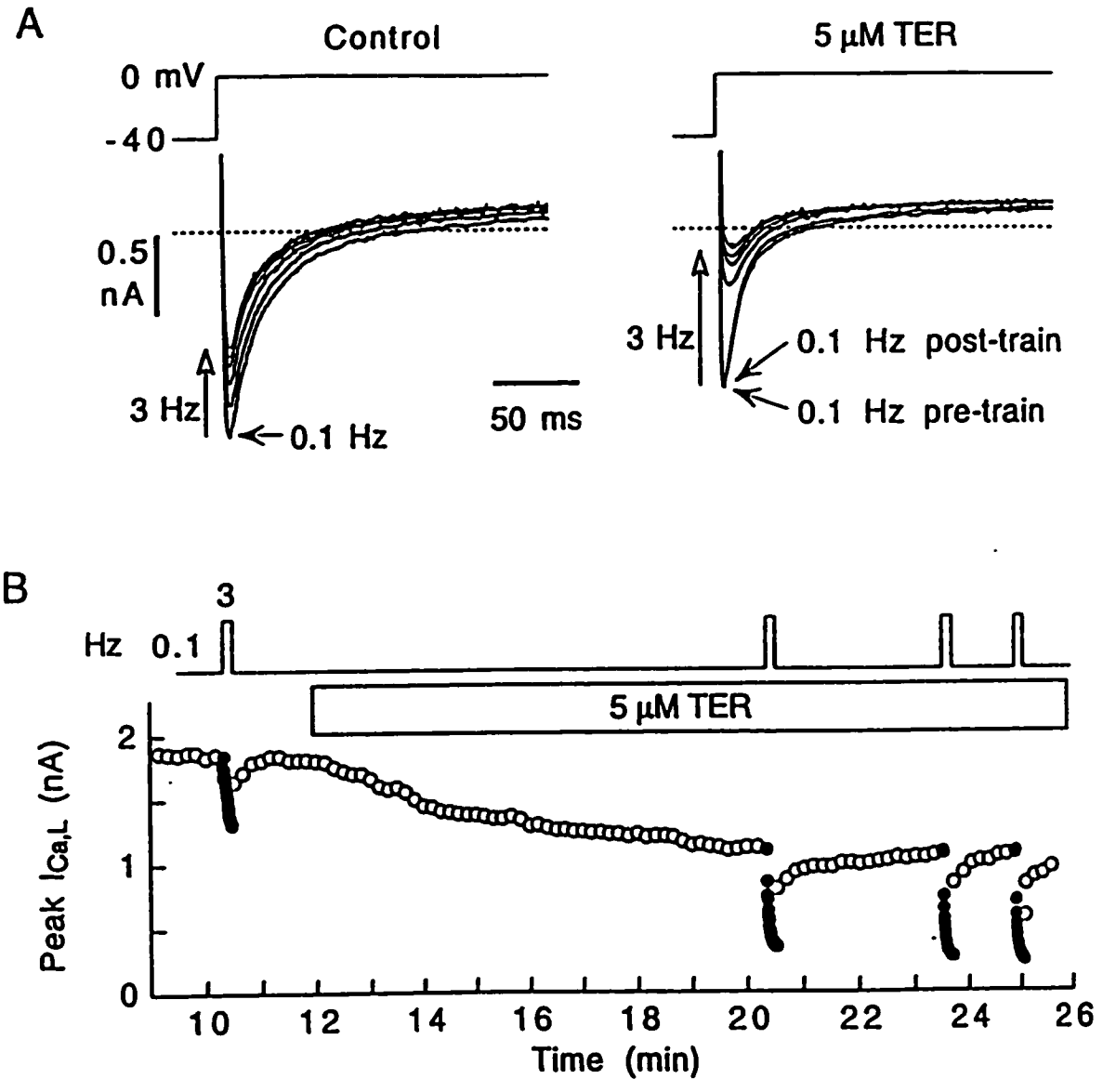


Figure 19. Terodiline enhances frequency-dependent reduction of $I_{Ca,L}$. Myocytes were regularly depolarized from -40 to 0 mV for 200 ms at 0.1 Hz with periodic 25 pulse trains at 3 Hz. (A) Representative records from a myocyte bathed and dialyzed with K^+ solutions, illustrating the reduction of $I_{Ca,L}$ induced by 3-Hz trains before and 6 min after addition of 5 μM terodiline. (B) Time course of peak $I_{Ca,L}$ from a myocyte bathed in K^+-free solution and dialysed with Cs^+ solution. Trains of 25 pulses at 3 Hz (•) inhibited $I_{Ca,L}$ to a larger degree in the presence of the drug than in its absence.

3 μM terodiline was slower and no longer monoexponential (Figure 20A). Analysis of the data (Tukey-Kramer multiple comparisons test) indicates that the extent of the recovery of $I_{Ca,L}$ from inactivation was significantly smaller in the presence of the drug when the Δt values were 20 ms ($p < 0.05$ versus predrug), 50 ms ($p < 0.001$), 133 ms ($p < 0.001$), and 200 ms ($p < 0.01$). When the time course of recovery was characterized in terms of time to half-recovery, 3 μM terodiline slowed the process by a factor of 1.9. In four other myocytes held at -35 mV and tested with recovery intervals of 20, 100, 300, and 500 ms, 10 μM terodiline slowed the half-time of recovery from 105 ms to 340 ms. A multiple comparison analysis of the data indicates that the degree of recovery of $I_{Ca,L}$ from inactivation in the presence of 10 μM terodiline was significantly smaller than the degree of recovery of $I_{Ca,L}$ under control conditions (recovery intervals 300 ms ($p < 0.05$) and 500 ms ($p < 0.01$)).

These results indicate that terodline lengthens the time required for $I_{Ca,L}$ to recover from inactivation, and help explain the increase in drug-induced inhibition when the stimulation rate was increased. Under control predrug conditions, high pulsing rates result in incomplete recovery from inactivation during interpulse intervals, and thereby reduce the amplitude of $I_{Ca,L}$; slowed recovery induced by terodiline depresses interpulse recovery even more, and thereby accentuates the reduction in $I_{Ca,L}$ amplitude.

Drug-induced acceleration of current decay

In addition to slowing the recovery of $I_{Ca,L}$ from inactivation, terodiline accelerated the time course of current decay during 200-ms depolarizations to 0 mV. This effect was evident even with low micromolar concentrations (0.3 and 1 μM) that had little effect on $I_{Ca,L}$ amplitude in myocytes bathed in normal Tyrode's and dialyzed with K^+ pipette solution (Figure 21A, left). The speeding up of current decay was more

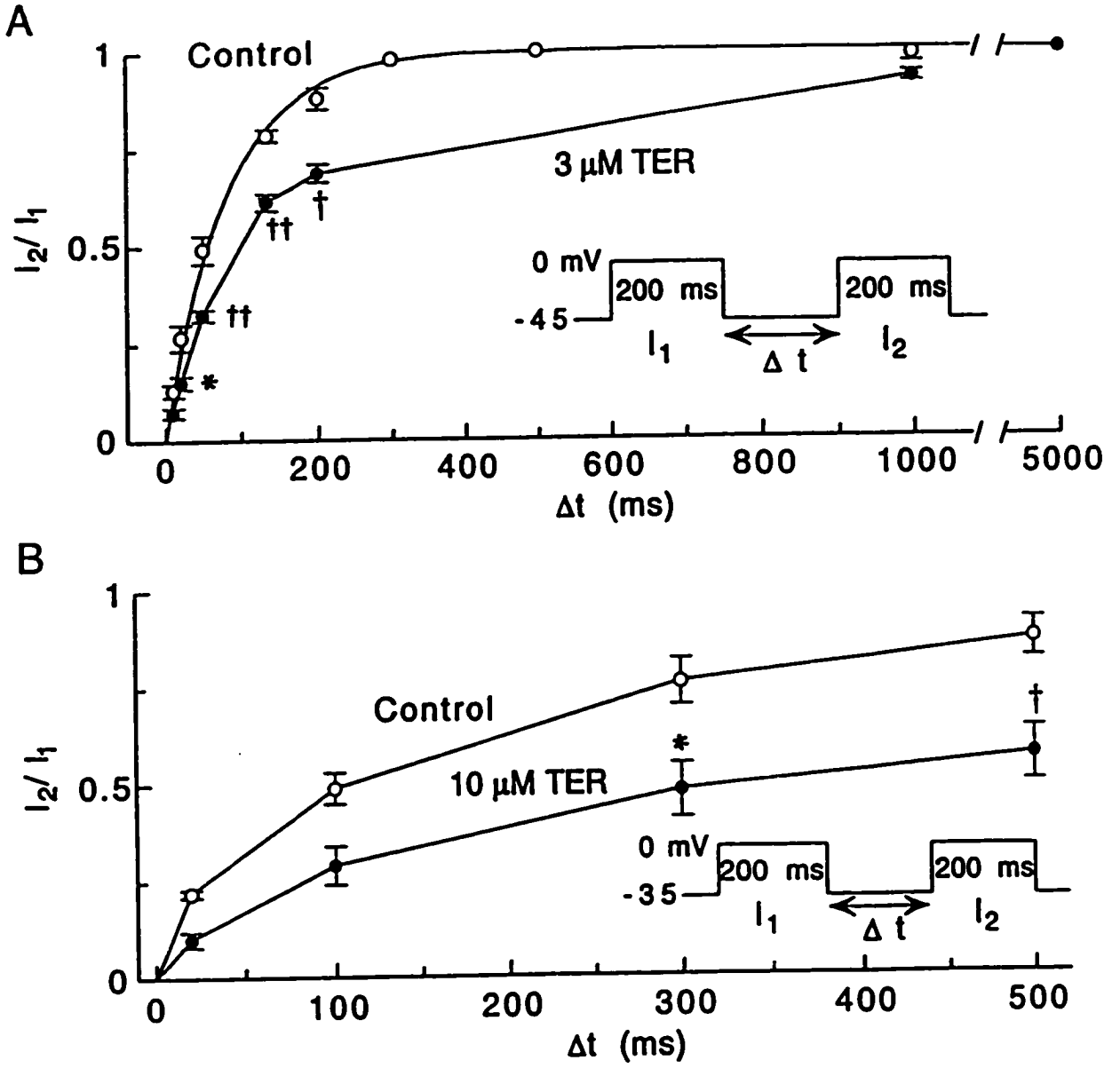


Figure 20. Terodiline slows the recovery of $I_{Ca,L}$ from inactivation. (A) Time courses of recovery of $I_{Ca,L}$ (measured as I_2/I_1) from 200-ms inactivating pulses before and 5 min after addition of 3 μM terodiline to 10 myocytes. The exponential function that fits the control data has τ = 82 ms. A point-to-point line is used to connect the terodiline data since they are poorly described by a single exponential. (B) Recovery from inactivation in four myocytes held at -35 mV and treated with 10 μM terodiline. All data are from myocytes bathed and dialyzed with K^+ solutions. Significance was assessed using the Tukey-Kramer multiple comparisons test: * $p < 0.05$, † $p < 0.01$, †† $p < 0.001$.

pronounced at higher drug concentrations (e.g., 5 μM: Figure 21A, right). Using I_{200} as an estimate of the zero-$I_{Ca,L}$ current level, the time to half-decay of $I_{Ca,L}$ declined from 10.2 ± 0.7 ms to 8.0 ± 0.7 ms (n=8) ($p < 0.01$) after exposure to 5 μM terodiline.

To investigate the acceleratory effects of terodiline on $I_{Ca,L}$ decay in greater detail, K^+-free internal and external solutions were used to minimize K^+ currents, and 0.4 mM Cd^{2+} was applied at the end of the terodiline treatment to establish a zero-$I_{Ca,L}$, background-current reference level for measurement of $I_{Ca,L}$. In the first series of experiments, $I_{Ca,L}$ elicited by 200-ms pulses to 0 mV was recorded before and during exposure to concentrations of terodiline between 0.3 and 100 μM. The representative results in Figure 21B indicate that terodiline inhibited both the peak and end-of-pulse (late) $I_{Ca,L}$. The IC_{50} for inhibition of peak $I_{Ca,L}$ under these recording conditions was 12.2 μM (from Figure 17C), whereas the IC_{50} for inhibition of late $I_{Ca,L}$ was a much lower 6 μM. This discrepancy in IC_{50} values is consistent with an enhanced rate of decay of $I_{Ca,L}$ in the presence of terodiline.

It has previously been reported that the inactivation of Ca^{2+} channel current is greatly slowed when Ca^{2+} is replaced as charge-carrier by Ba^{2+}, Sr^{2+}, or Na^+ (Kass and Sanguinetti, 1984; Matsuda, 1986; McDonald *et al.*, 1986; Hadley and Hume, 1987). This slowing is due to the removal of a Ca^{2+}-dependent speeding up of the inactivation of the Ca^{2+} channel current (see reviews by Pelzer *et al.*, 1992; McDonald *et al.*, 1994).

To investigate the effects of terodiline on Ba^{2+}-carried current ($I_{Ba,L}$), external Ca^{2+} was replaced by 1.5 mM Ba^{2+}. The current records and time courses of changes in $I_{Ba,L}$ amplitude from a representative experiment illustrate that 5 μM terodiline had a pronounced inhibitory effect on peak $I_{Ba,L}$, and an even greater effect on late (200 ms) $I_{Ba,L}$ (Figure 22A). Results from eleven myocytes treated with 5 μM terodiline indicate that the drug reduced peak Cd^{2+}-sensitive $I_{Ba,L}$ to 43 ± 2% control, and reduced late

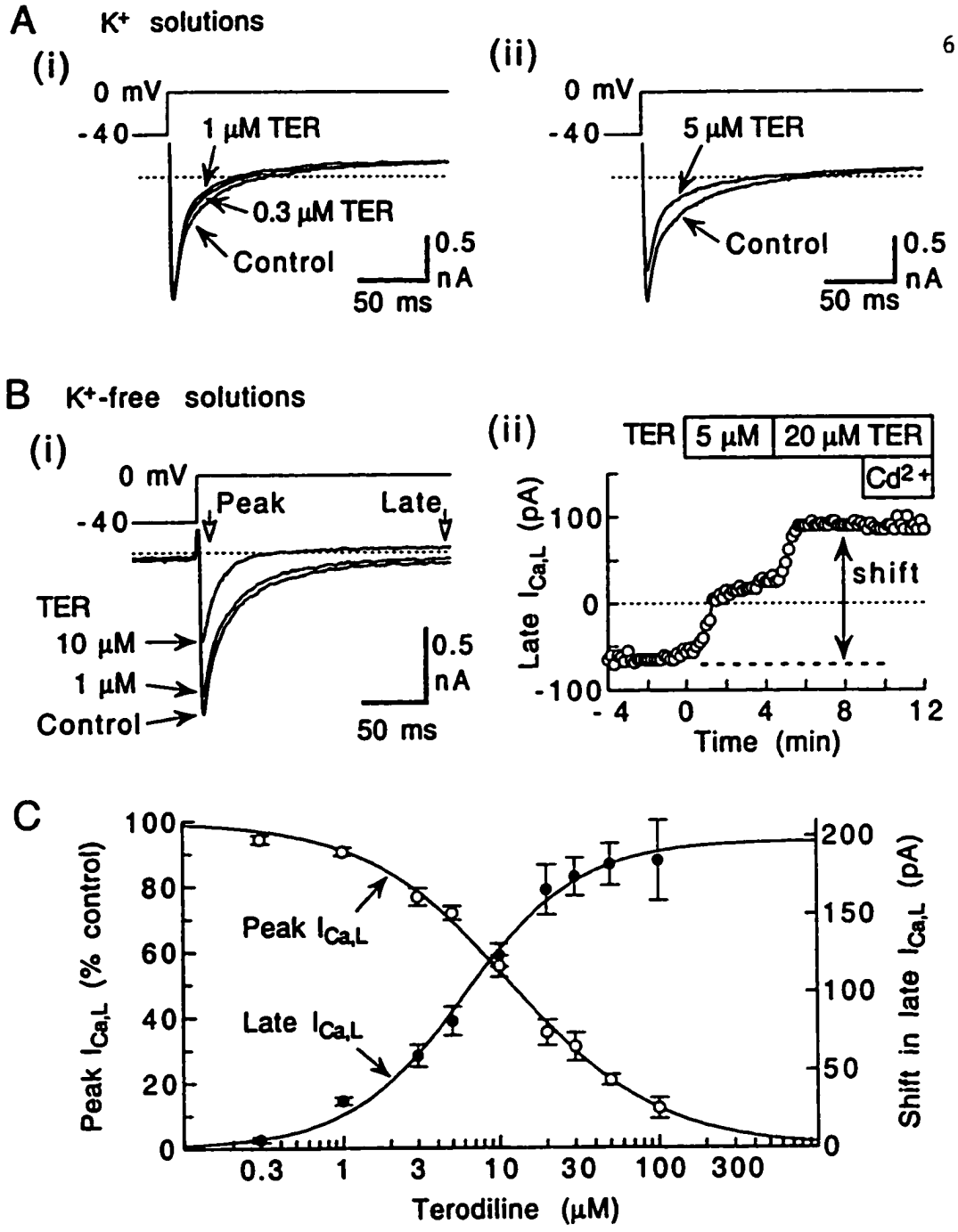


Figure 21. Acceleration of the decay of $I_{Ca,L}$ by terodiline. (A) Records from myocytes superfused and dialyzed with K^+ solutions, and pulsed from -40 to 0 mV at 0.1 Hz. (i) Acceleration of $I_{Ca,L}$ decay in a myocyte treated with 0.3 μM terodiline for 5 min and 1 μM for a further 5 min. (ii) Acceleration of $I_{Ca,L}$ decay in a myocyte treated with 5 μM for 3 min. (B) Results from experiments in K^+-free solutions. (i) Records from a representative myocyte treated for 6 min with 1 μM terodiline, and a further 4 min with 10 μM drug. (ii) Time course of shifts in late $I_{Ca,L}$ during successive applications of 5 μM drug, 20 μM drug, and 0.4 mM Cd^{2+}. (C) Concentration-response relationships for Ca^{2+}-sensitive peak $I_{Ca,L}$ (o) and shift in late $I_{Ca,L}$ (•); 4-14 myocytes at each concentration.

Cd^{2+}-sensitive $I_{Ba,L}$ to 24 ± 1% control. This significant difference ($p < 0.001$) can be attributed to an acceleration of the inactivation of $I_{Ba,L}$ by the drug.

$I_{Ba,L}$ is a long-lasting current that inactivates with a multi-exponential time course (Boyett *et al.*, 1994). Although the experiments on $I_{Ba,L}$ described above clearly indicate that 5 μM terodiline accelerates apparent inactivation of the current, the full extent of this effect could not be observed because full inactivation was not attained within the 200-ms pulse duration. To overcome this limitation, five myocytes that were otherwise pulsed to 0 mV for 200 ms at 0.1 Hz, were probed with single 20-s-long depolarizations to 0 mV at three times: (i) before the addition of 5 μM terodiline, (ii) after steady-state inhibition by the drug, and (iii) after subsequent nulling of $I_{Ba,L}$ with an exposure to 0.4 mM Cd^{2+}. Although $I_{Ba,L}$ failed to inactivate completely during 20-s depolarizations applied prior to the addition of terodiline, it decayed to the Cd^{2+}-defined, background current level within the first 5-s of depolarization in the presence of the drug (Figure 22B).

As previously reported by Boyett *et al.* (1994), the time course of inactivation of Cd^{2+}-sensitive $I_{Ba,L}$ during long depolarizations was best-described by fits of up to four exponential functions. However, the important features were captured by the first three exponentials (time constants 25 ± 1 ms, 171 ± 9 ms, and 1982 ± 38 ms), each of which was significantly ($p < 0.05$) shortened by 5 μM terodiline (time constants 17 ± 2 ms, 87 ± 19 ms, and 749 ± 290 ms) (Figure 22C).

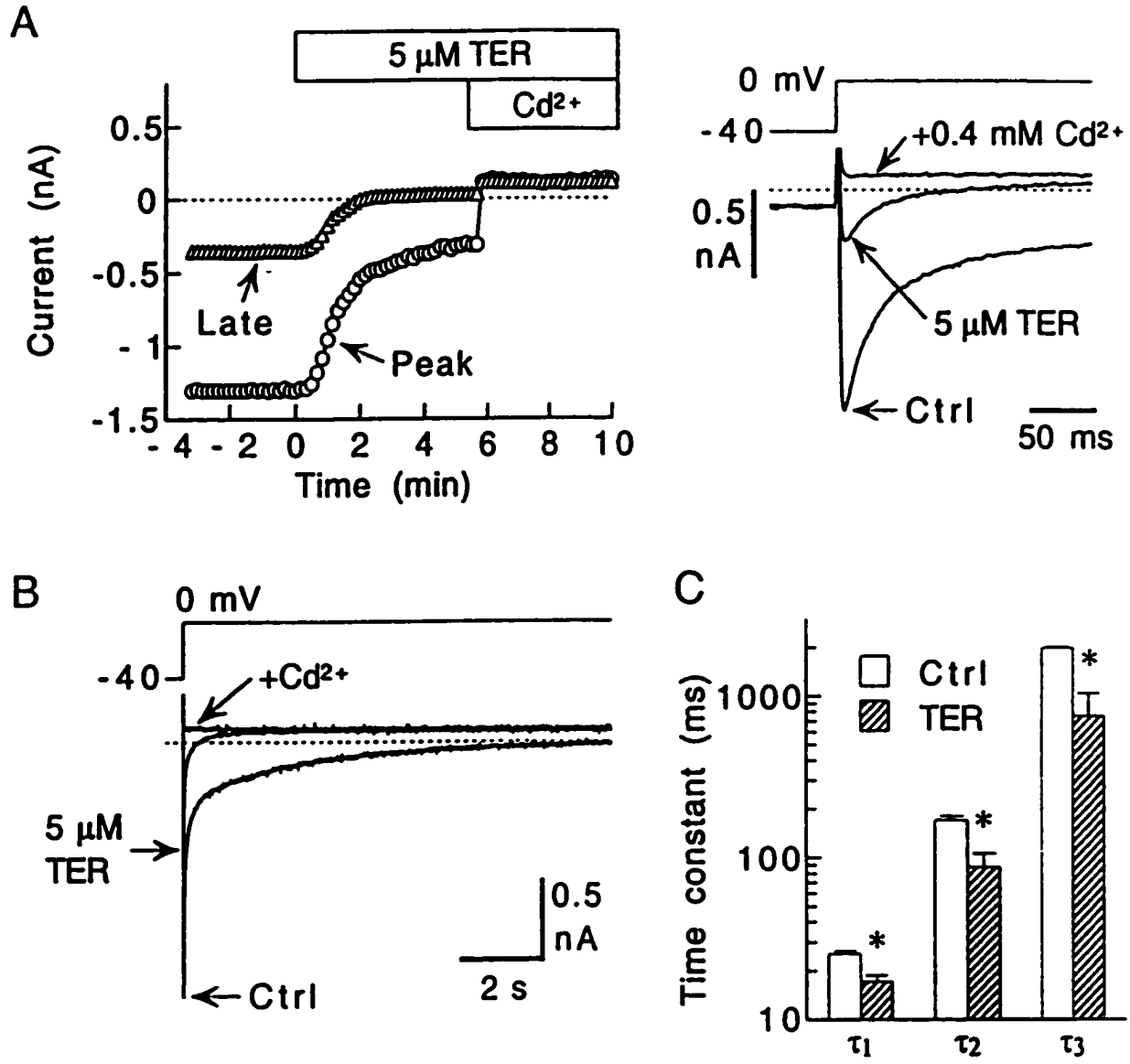


Figure 22. Inhibition of $I_{Ba,L}$ by terodiline. (A) Results from a representative myocyte that was depolarized with 200-ms pulses from -40 to 0 mV. The cell was treated with 5 μM drug alone, and then with 0.4 mM Cd^{2+}. Both peak and late $I_{Ba,L}$ were inhibited, with late $I_{Ba,L}$ being reduced to a larger extent than peak $I_{Ba,L}$. (B) Superimposed records showing the first 10 s of currents elicited by 20-s depolarizations before, 6 min after addition of 5 μM terodiline, and 3 min after subsequent addition of 0.4 mM Cd^{2+}. Note the marked acceleration of the decay of $I_{Ba,L}$ in the presence of the drug (horizontal arrow (left) indicates peak $I_{Ba,L}$). (C) Time constants obtained from multiexponential analysis of Cd^{2+}-sensitive $I_{Ba,L}$ recorded from five myocytes investigated as in B. $*p < 0.05$. Note the logarithmic scale.

(3) Effects on I_{Kr}

I_{Kr} is the primary I_K component inhibited by low micromolar terodiline

The standard protocol used to examine the effects of terodiline on I_{Kr} was to pulse myocytes from -40 mV to 0 mV for 200 ms at 0.1-0.2 Hz to permit measurement of drug-induced changes in the amplitude of $I_{K,tail}$ elicited on the repolarizations to -40 mV. This pulsing protocol was routinely interrupted during the predrug period and after steady-state drug action to examine the dependence of global $I_{K,tail}$ ($I_{Kr,tail}$ and $I_{Ks,tail}$) amplitude on test voltage ($I_{K,tail}$-V), by applying depolarizations (500 ms to 1 s) from -40 mV to potentials as positive as +70 mV.

Results from a representative myocyte (Figure 23A) illustrate that 3 μM terodiline rapidly inhibited the amplitude of $I_{K,tail}$ after depolarizations to 0 mV, and that the current recovered to a large extent when the drug was removed. In this experiment, $I_{K,tail}$-V relationships were determined by applying sequences of 1-s depolarizations to potentials between -40 and +60 mV. Subtraction of the tail currents (control minus terodiline) (Figure 23B), indicate that nearly all of the terodiline-sensitive tail current occurred in response to depolarization to potentials between -40 and +20 mV.

Average $I_{K,tail}$-V relationships determined from this and five similar experiments are shown in Figure 23C. The main result is that the I_{Kr}-dominated phase at low test voltages (see Methods, page 29) was inhibited to a larger extent than the I_{Ks}-dominated phase at more positive potentials. These results suggest that 3 μM terodiline preferentially inhibits I_{Kr} over I_{Ks}.

A more definitive answer on whether low micromolar concentrations of terodiline selectively inhibit the I_{Kr} component of I_K was sought by pretreating myocytes with 3 μM terodiline and then assessing the extent of I_{Kr} inhibition from responses to subsequent 5 μM E4031 (a saturating I_{Kr}-blocking concentration). The $I_{K,tail}$-V

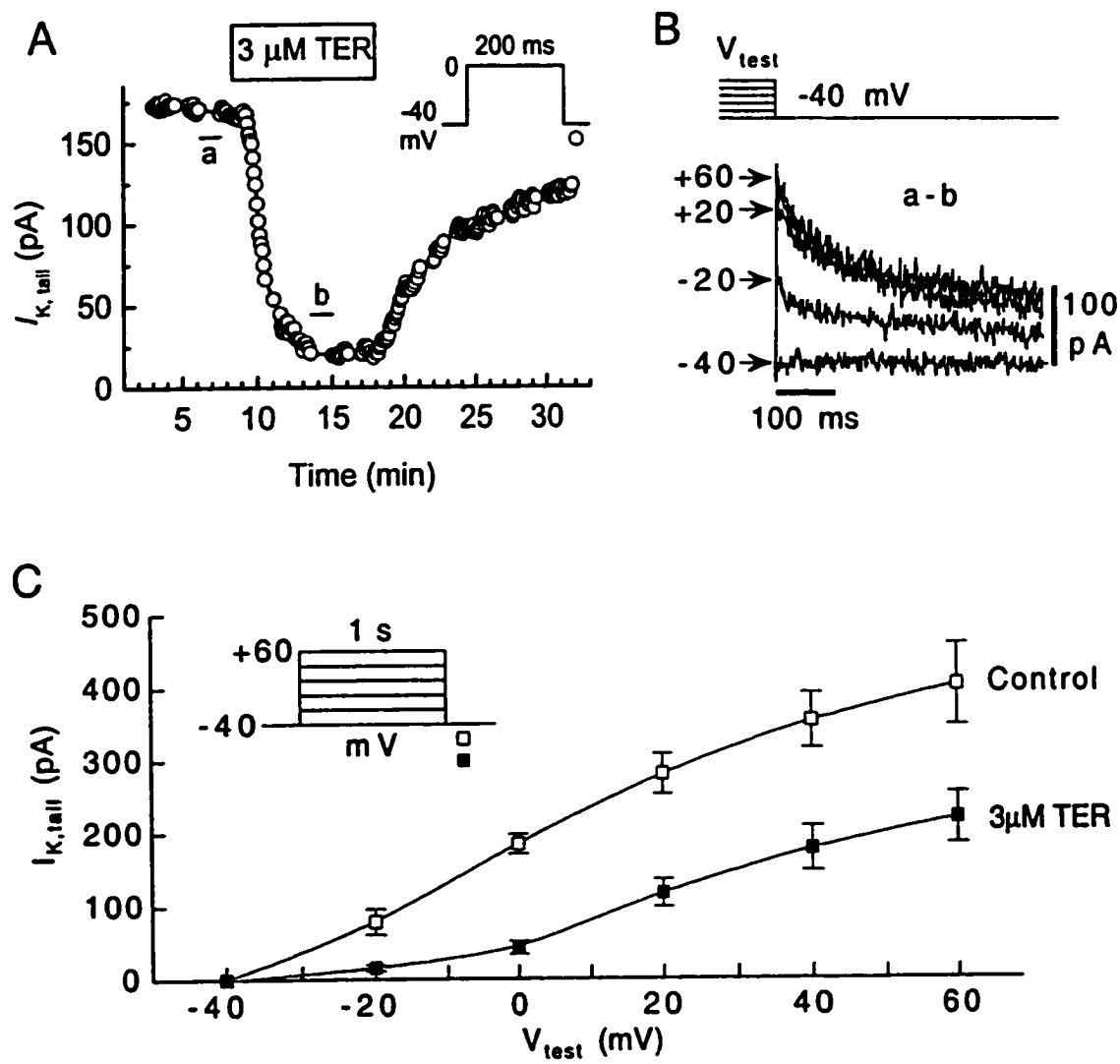


Figure 23. $I_{K,tail}$ in myocytes treated with 3 μM terodiline. The myocytes were held at -80 mV and depolarized from prepulse -40 to 0 mV for 200 ms at 0.2 Hz except for sequences of 1-s pulses to potentials between -40 and +60 mV. (A) Time course of changes in $I_{K,tail}$ amplitude at -40 mV during regular pulsing to 0 mV. (B) Terodiline-sensitive $I_{K,tail}$ obtained by subtraction of records obtained on I-V runs before and during drug treatment (see A). (C) Average $I_{K,tail}$-V relationships; n = six myocytes.

relationships in Figure 24 illustrate that inhibition of $I_{K,tail}$ by 5 μM E4031 was almost completely occluded by 3 μM terodiline. In five experiments of this type, the amplitude of $I_{K,tail}$ elicited by 500-ms pulses to 0 mV was unaffected by the addition of E4031. The addition also had no significant effect on $I_{K,tail}$ after 500-ms depolarizations to +60 mV. Tail amplitude was lowered to 58 ± 6% control by the terodiline treatment, and to 53 ± 7% by subsequent E4031. These results indicate that 3 μM terodiline and 5 μM E4031 inhibit I_{Kr} to the same extent, and solidify the previous results that suggested that low micromolar concentrations of terodiline preferentially inhibit the I_{Kr} component of I_K.

The voltage-dependencies of $I_{K,tail}$ sensitive to 3 μM terodiline and 5 μM E4031 were compared by analyzing $I_{K,tail}$ data from myocytes that were depolarized for 500 ms to potentials up to +70 mV. Drug-sensitive tails were computed, and their amplitudes normalized by reference to amplitudes elicited after pulses to +20 mV (Figure 25). The normalized E4031 data (n=12) are described by the Boltzmann equation with half-activation voltage ($V_{½}$) of -12.2 ± 1.6 mV and slope factor (S) of 8.0 ± 0.4 mV, and the 3 μM terodiline data (n=10) are described by $V_{½}$ = -14.5 ± 1.7 mV and S = 8.7 ± 1.0 mV. However, it is important to point out that (unlike the E4031 data) the 3 μM terodiline data diverge from the Boltzmann description at potentials above +20 mV. This divergence of normalized data was larger with 10 μM terodiline (n=4) and absent with 0.3 μM terodiline (n=4) (Figure 25). These findings suggest that ≥ 3 μM concentrations of terodiline inhibit the I_{Ks} component of I_K (that is activated at potentials above +20 mV).

Concentration-dependent inhibition

The dependence of I_{Kr} inhibition on terodiline concentration was evaluated from measurements of $I_{K,tail}$ recorded from myocytes pulsed from -40 to 0 mV. The pulses

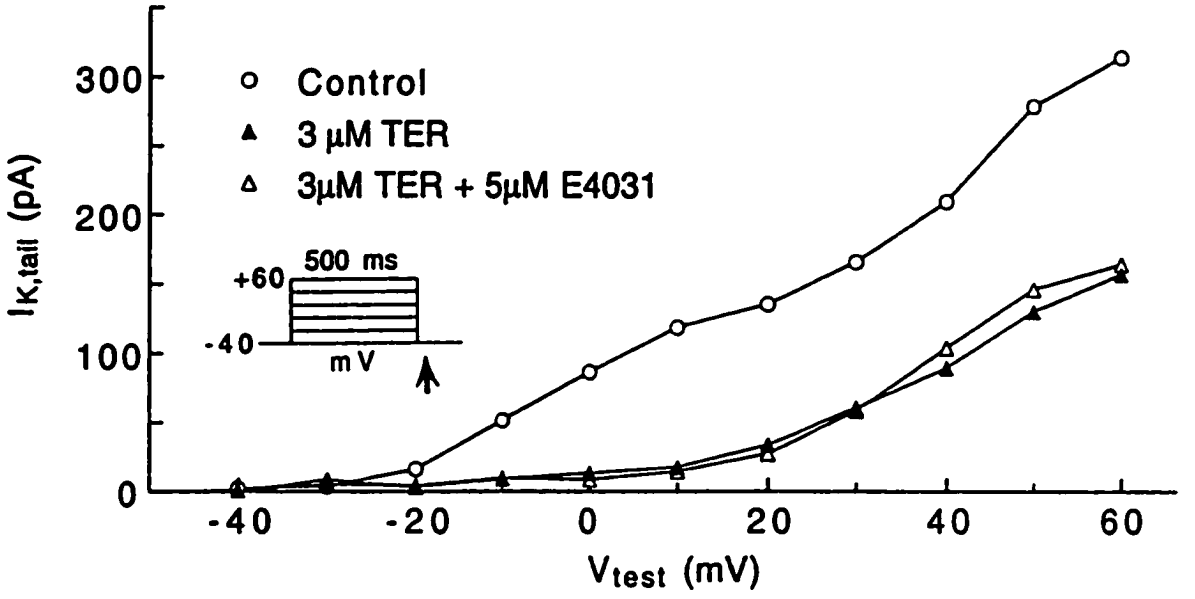


Figure 24. Occlusion of E4031 action by terodiline. $I_{K,tail}$-V relationships (500-ms test pulses) obtained from a myocyte before, 6-min after addition of 3 μM terodiline, and 5-min after further addition of 5 μM E4031.

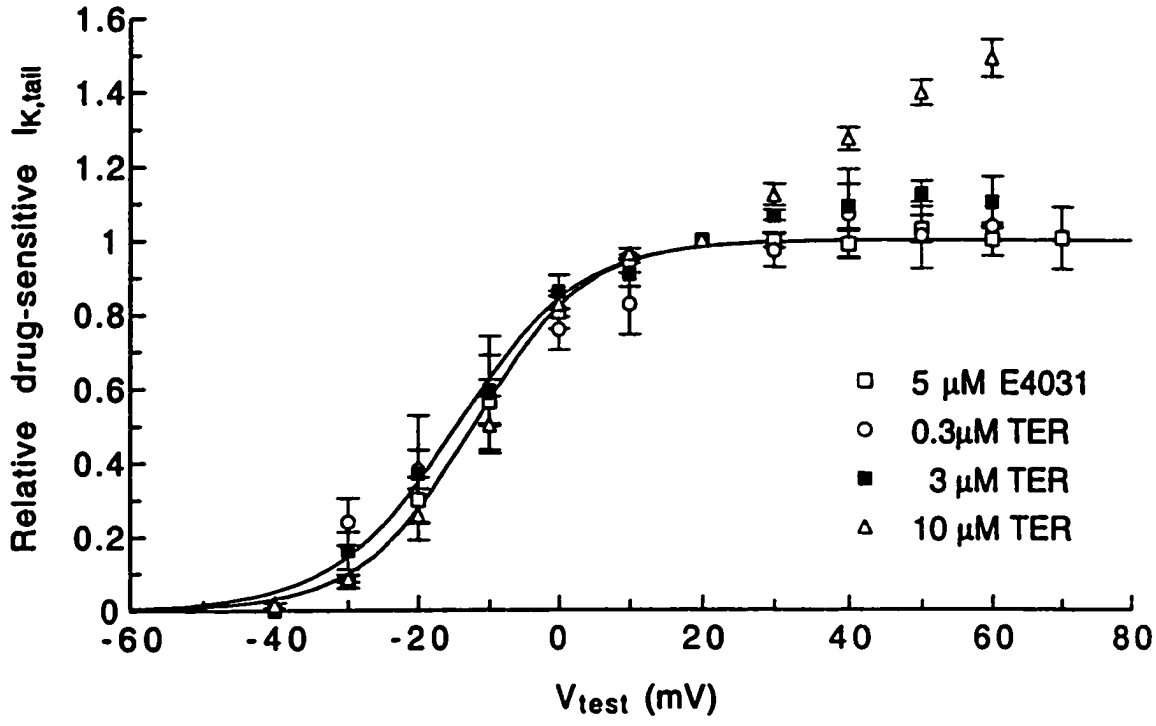


Figure 25. The voltage dependencies of $I_{K,tail}$ sensitive to terodiline and 5 μM E4031. Plot of the differences between control and treatment tail currents measured from myocytes treated with either 3 μM terodiline (n=10) or 5 μM E4031 (n=12). The data are described by the Boltzmann equation, $I/I_{+20}=1/[1+\exp((V_{0.5}-V_t)/S)]$, where I_{+20} is the tail amplitude after a 500-ms pulse to +20 mV, $V_{0.5}$ is the test voltage eliciting half-activation, and S is the slope factor. The $V_{0.5}$ and S values are -12.2 ± 1.6 and 8.0 ± 0.4 mV, respectively, for the E4031 data, and -14.5 ± 1.7 mV and 8.7 ± 1.0 mV, respectively, for the 3 μM terodiline data. Also shown are normalized data from experiments with 0.3 μM (n=4) and 10 μM (n=4) terodiline.

were relatively short (200 ms) in duration to allow for near complete activation of I_{Kr} with minimal activation of any more slowly activating I_{Ks}. The time plot in Figure 26A illustrates that even submicromolar concentrations of terodiline had rapid inhibitory effects on the tail current recorded with this pulsing protocol.

Myocytes pulsed as described above were treated with a single concentration of terodiline for ≥ 5 min to determine the dependence of I_{Kr} inhibition on the concentration of the drug. The steady-state data provided in Figure 26B indicate that terodiline had significant inhibitory effects at concentrations as low as 0.3 μM (reduction to 65 ± 5% of predrug control (n=10), $p < 0.01$, t-test). The overall data are well-described by the Hill equation with an IC_{50} of 0.5 μM and a coefficient of 1.04.

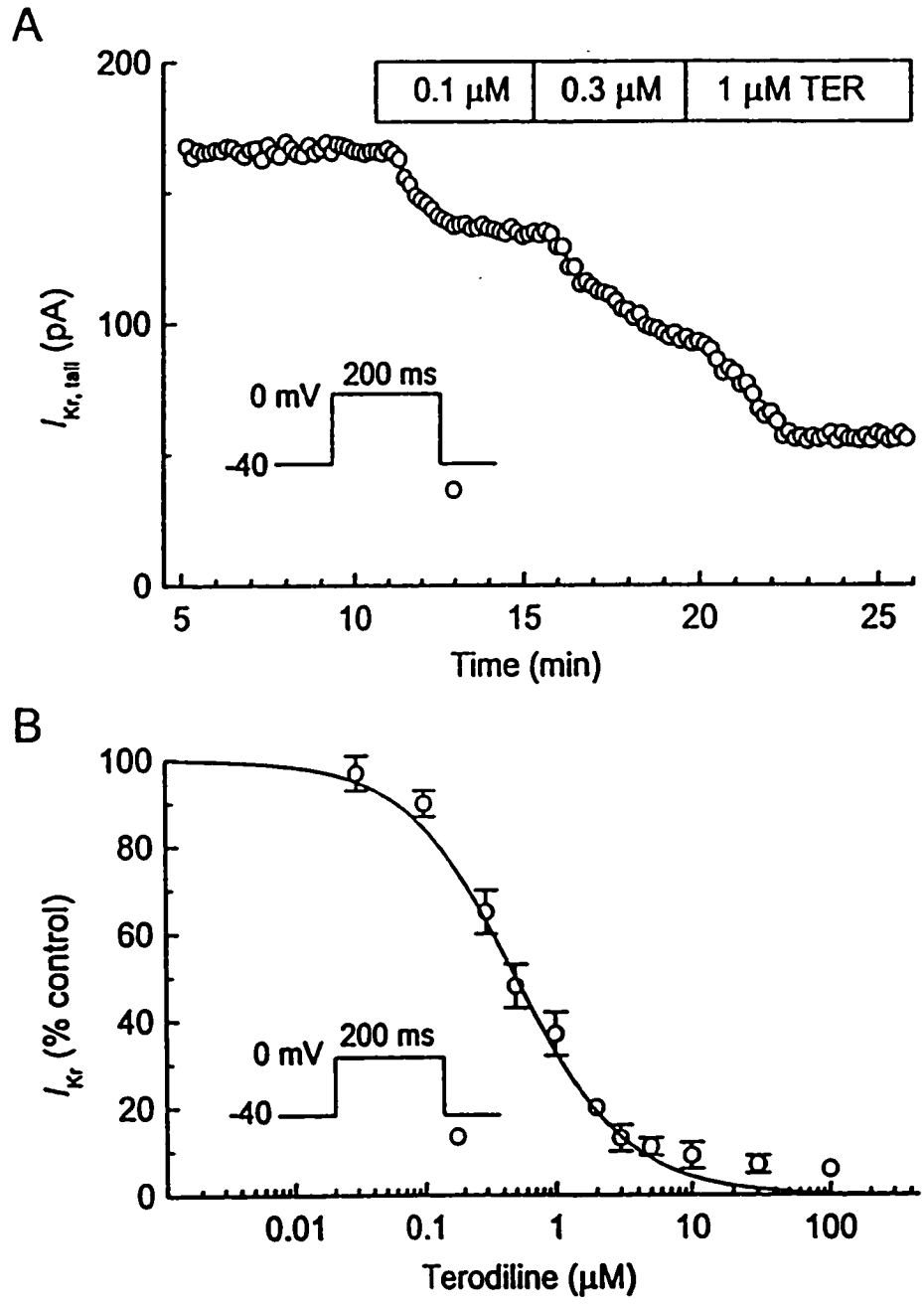


Figure 26. Dependence of I_{Kr} inhibition on terodiline concentration. Myocytes were depolarized to 0 mV for 200 ms at 0.1 Hz, and $I_{Kr,tail}$ was measured on the repolarizations to -40 mV. (A) Marked inhibition of $I_{Kr,tail}$ during sequential applications of 0.1, 0.3, and 1 μM terodiline. (B) Concentration-response relationship. $I_{Kr,tail}$ was measured before and during treatment with a single concentration of the drug. The Hill equation fitting the data (n=1 at 2 μM and 100 μM; otherwise n=3-18) has an IC_{50} of 0.5 μM and a Hill coefficient of 1.04.

(4) Effects on I_{Ks}

In order to quantify the effects of terodiline on I_{Ks}, the magnitude of the current was estimated in three ways: (i) as the amplitude of the time-dependent outward current elicited when 500-ms depolarizations to +60 mV were applied to myocytes superfused with normal Tyrode's solution (protocol 1), (ii) as the amplitude of the time-dependent current during 500-ms depolarizations to +30 mV in myocytes superfused with K^+-, Ca^{2+}-free Cd^{2+} solution (protocol 2), and (iii) as the amplitude of the tail current on repolarizations to -40 mV following long (2-s) depolarizations to more positive potentials in myocytes superfused in K^+-, Ca^{2+}-free Cd^{2+} solution (protocol 3). Examples of the effects of terodiline on I_{Ks} estimated with these protocols are provided in the next section.

Representative results

The rationale for using the amplitude of the time dependent current at +60 mV as a means of estimating the amplitude of I_{Ks} was that at this potential, currents that overlap I_{Ks} are expected to be relatively small. It would be difficult to estimate the extent of I_{Ks} inhibition by terodiline if, for example, there were a superimposed concentration-dependent inhibition of I_{Kr}. However, at +60 mV, time-dependent outward I_{Kr} is small due to the rapid voltage-dependent inactivation of the I_{Kr}-carrying channels (Sanguinetti and Jurkiewicz, 1990).

(i) Protocol 1. The records shown in Figure 27A indicate that the amplitude of the time-dependent current at +60 mV was inhibited when myocytes were exposed to relatively high concentrations of terodiline. In seven myocytes, treatment with 10 μM terodiline for ≥ 5 min reduced the amplitude of the time-dependent current to 77 ± 3% of

predrug value ($p < 0.001$). In three other myocytes, 100 μM terodiline reduced the amplitude of the current to 34 ± 5% of predrug value ($p < 0.01$).

The effect of terodiline was also determined in myocytes that were pretreated with the I_{Kr} inhibitor E4031. The myocytes were repolarized to -40 mV for measurement of $I_{K,tail}$ elicited after 500-ms depolarizations to test potentials between -40 and +60 mV. The $I_{K,tail}$-V relationships shown in Figure 27B indicate that 5 μM E4031 almost completely abolished the $I_{K,tail}$ elicited after depolarizations between -40 and +10 mV, with little effect on the increment in $I_{K,tail}$ induced by depolarizations to more positive potentials. Subsequent exposure to 15 μM terodiline depressed the monophasic ascending limb of the I-V relation (isolated I_{Ks}) at positive potentials. Records of the time-dependent current elicited by the depolarizations to +60 mV (Figure 27C) indicate that 15 μM terodiline inhibited the amplitude of the time-dependent outward current by ca. 30% (a result that is in line with those obtained from the non-E4031-pretreated myocytes of Figure 27A). The similar extent of terodiline-induced inhibition of I_{Ks} in myocytes with and without prior E4031 exposure was expected because E4031 by itself had a negligible effect on I_{Ks} elicited during 500-ms depolarizations to +60 mV under normal Tyrode's conditions (see Figure 8 in Methods).

(ii) Protocol 2. The rationale for measuring I_{Ks} from myocytes superfused with K^+-, Ca^{2+}-free Cd^{2+} solution was that this condition is designed to enhance I_{Ks} and to suppress I_{Kr} and Ca^{2+}-dependent currents (Sanguinetti and Jurkiewicz, 1992; Daleau *et al.*, 1997). Myocytes bathed in this solution were depolarized with 500-ms pulses from -40 to +30 mV at 0.1 Hz, and the amplitude of the time-dependent current activated during the pulse was monitored before and during steady-state terodiline action. The time course and records obtained from an example experiment are shown in Figure 28. They

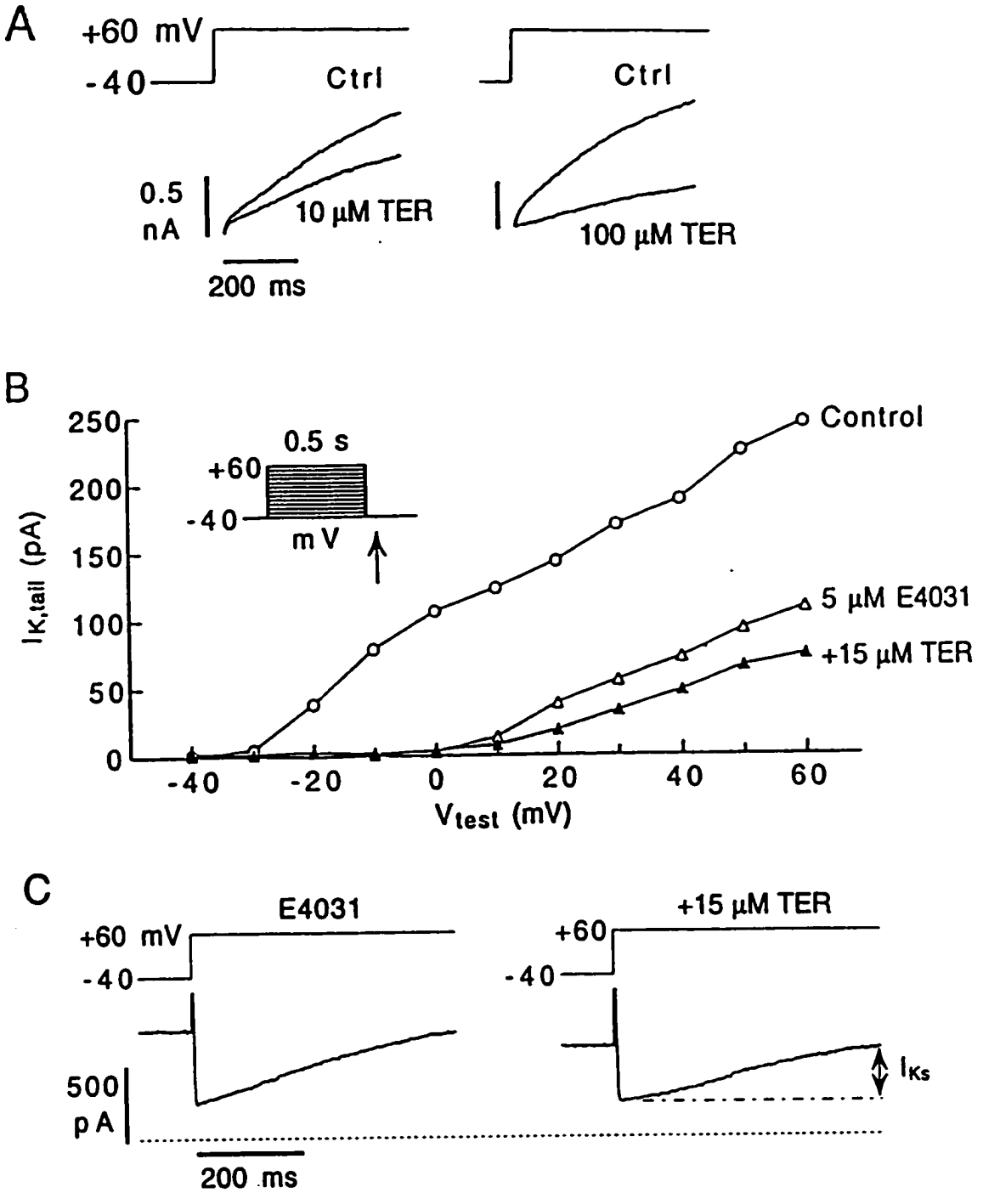


Figure 27. Effects of terodiline on I_{Ks} in myocytes bathed in normal Tyrode's solution. (A) Records from two experiments in which myocytes were exposed to 10 or 100 μM terodiline for 8-10 min. The myocytes were depolarized from -40 mV to +60 mV for 500 ms at 0.1 Hz. (B) $I_{K,tail}$-V relationships determined from a myocyte exposed to 5 μM E4031 for 5 min, and additional 15 μM terodiline for 8 min. $I_{K,tail}$ was elicited at -40 mV after 500-ms pulses to more positive potentials. (C) Reduction of activating I_{Ks} (+60 mV) by 15 μM terodiline. Same experiment as in B. Dashed line indicates zero-current level.

indicate that the time-dependent current at +30 mV was inhibited by ca. 15% and 70% during sequential exposures to 5 and 100 μM terodiline. This degree of inhibition is in line with that observed when drug action on I_{Ks} was evaluated using protocol 1.

(iii) Protocol 3. With this protocol, myocytes were bathed with K^+-, Ca^{2+}-free Cd^{2+} Tyrode's solution and I_{Ks} was measured as (i) the amplitude of time-dependent current activated during 2-s depolarizations to potentials as positive as +80 mV, and (ii) the amplitude of the subsequent tail current on the repolarizations to -40 mV. Time plots of the amplitudes of these two currents in Figure 29A indicate that the highest concentration of terodiline tested (100 μM) caused reversible 75% reductions in I_{Ks} as measured by both methods. Records from a myocyte depolarized with 2-s pulses indicate that I_{Ks} was markedly inhibited at all test potentials between 0 and +80 mV (Figure 29 B,C).

Concentration-dependent relationship

The extent of I_{Ks} inhibition by terodiline was independent of the protocol used to estimate the amplitude of I_{Ks}. For this reason, the dependence of I_{Ks} inhibition on terodiline concentration (1 to 100 μM) was assessed using data pooled from myocytes bathed with normal Tyrode's and with K^+-, Ca^{2+}-free Cd^{2+} solutions. Results from a total of 62 myocytes are shown in Figure 30. The pooled data are well described by the Hill equation with an IC_{50} of 26 μM and a coefficient of 1.11.

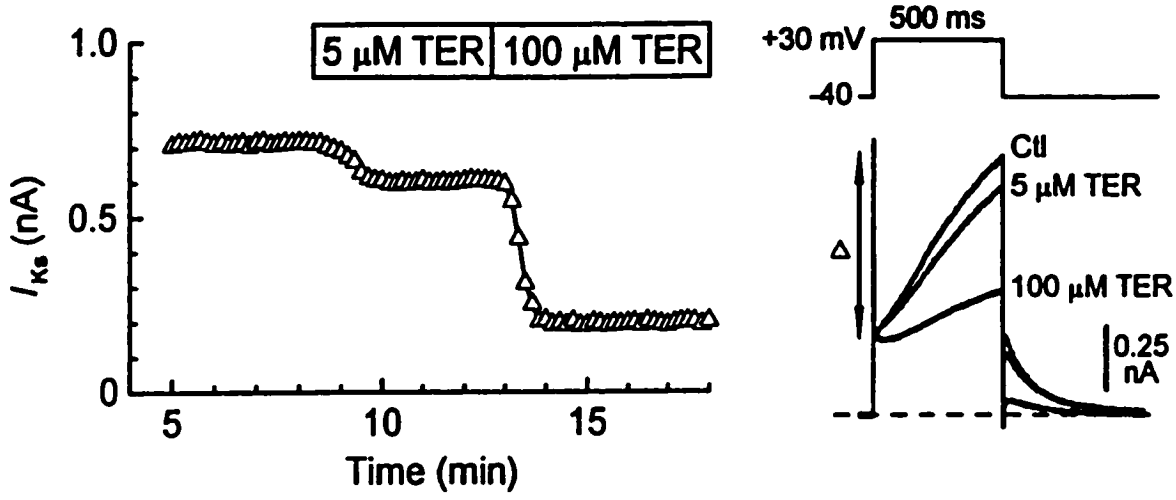


Figure 28. Inhibition of I_{Ks} by terodiline. Effects of 5 and 100 μM terodiline on I_{Ks} in a representative myocyte superfused with K^+, Ca^{2+}-free Cd^{2+} solution. The myocyte was depolarized from -40 to +30 mV for 500 ms at 0.1 Hz, and the amplitude of I_{Ks} was estimated as indicated by the vertical arrow on the records.

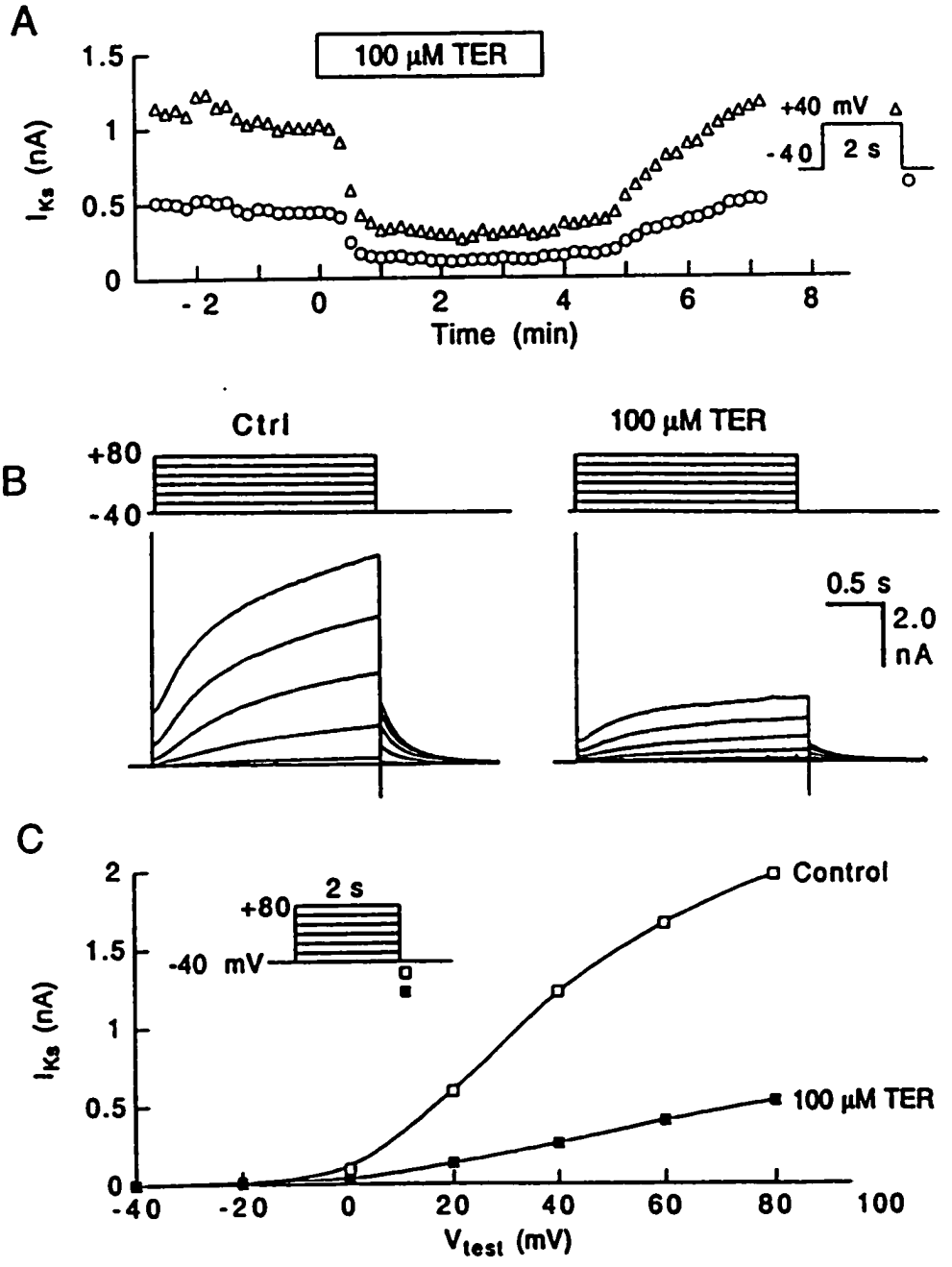


Figure 29. Effects of 100 μM terodiline on I_{Ks} in representative myocytes superfused with K^+-, Ca^{2+}-free Cd^{2+} solution. (A) Time courses of changes in the amplitudes of I_{Ks} activated during 500-ms pulses to +40 mV and of $I_{Ks,tail}$ elicited by the repolarizations to -40 mV. (B) Records from a representative myocyte depolarized with sequences of 2-s pulses from -40 to +80 mV. The records were obtained before and 5 min after the addition of 100 μM terodiline. (C) $I_{Ks,tail}$-V relationships from the data in B.

Dependence of inhibition on voltage

The dependence of terodiline-induced inhibition of I_{Ks} on the voltage of the test pulse was evaluated in myocytes bathed with K^+-, Ca^{2+}-free Cd^{2+} solution and treated with 5, 30, or 100 μM drug. As shown in Figure 31A (left), $I_{Ks,tail}$ expressed as a percentage of predrug control was not significantly different on 2-s pulses to +20 mV than on 2-s pulses to +80 mV in myocytes exposed to 5 μM terodiline. However, a similar comparison of data from myocytes treated with 100 μM terodiline indicates that block at high concentrations was significantly larger at +20 mV than at +80 mV ($p < 0.001$) (Figure 31A, right). Similarly, normalized $I_{Ks,tail}$-V relationships (by reference to the amplitudes of the tail currents following pulses to +80 mV under control conditions) from five myocytes depolarized with 2-s pulses before and ca. 5-min after addition of 30 μM terodiline point to a moderate relief of inhibition with increasing positive potential (Figure 31B). Fits of the normalized data with the Boltzmann function indicate that the test potential eliciting half-maximal tail current amplitude shifted from +26 mV to +34 mV during exposure to 30 μM terodiline.

Dependence of inhibition on channel configuration

To determine whether channel opening is a requirement for inhibition by terodiline, myocytes bathed with K^+-, Ca^{2+}-free Cd^{2+} solution were depolarized for 500 ms from a holding potential of -40 mV to a test potential of +40 mV at 0.1 Hz , rested at -40 mV in the presence of 20 μM drug, and re-stimulated 4 min later. The records in Figure 32A indicate that control rests had negligible effects on I_{Ks} activated during the pulse and $I_{Ks,tail}$ on repolarization to -40 mV (i.e., currents elicited by the first post-rest pulse were similar to the currents registered prior to the rest). However, application of 20

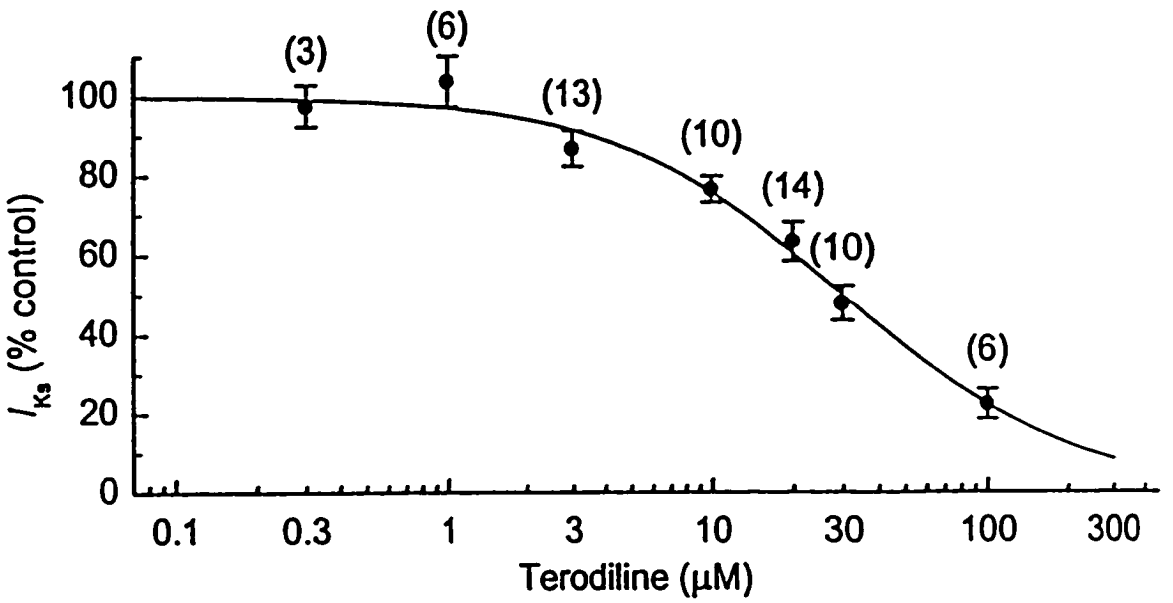


Figure 30. Dependence of I_{Ks} inhibition on terodiline concentration. I_{Ks} was measured as (i) the time-dependent outward current during 500-ms pulses to +60 mV when myocytes were superfused in normal Tyrode's solution, (ii) the time-dependent current during 500-ms pulses to +30 mV when myocytes were superfused with K^+-, Ca^{2+}-free Cd^{2+} solution, and (iii) the tail current on repolarizations to -40 mV following 2-s depolarizations to +60 mV. The Hill equation fitted to the pooled data has an IC_{50} of 26 μM and a Hill coefficient of 1.11. Number of myocytes in parentheses.

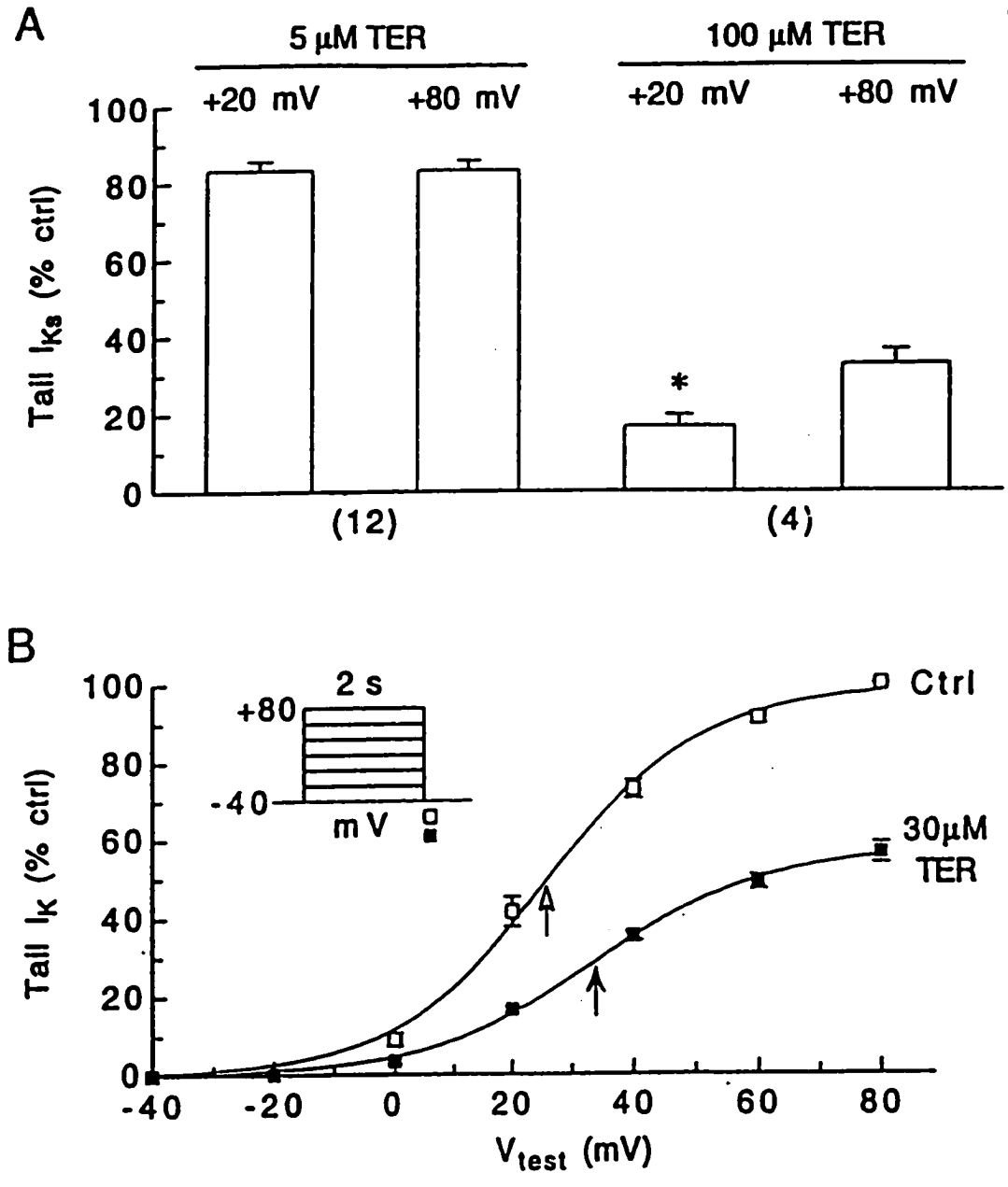


Figure 31. Examination of the voltage dependence of I_{Ks} block by terodiline. Myocytes were bathed in K^+-, Ca^{2+}-free Cd^{2+} solution and depolarized from -40 mV for 2-s at 0.1 Hz. (A) $I_{Ks,tail}$ amplitude following pulses to +20 mV and +80 mV in myocytes treated with 5 μM (n=12) or 100 μM (n=4) drug. *$p < 0.001$, paired t-test. (B) Normalized $I_{Ks,tail}$-V relationship derived from the responses of five myocytes depolarized with 2-s pulses at 0.1 Hz before and ca. 5-min after addition of 30 μM terodiline. Data were normalized by reference to the amplitudes of the tail currents following pulses to +80 mV under control conditions. Fits of the data with the Boltzmann function indicate that the potential that resulted in half-maximal tail amplitude was shifted to the right by 8 mV (arrows).

μM terodiline at the onset of a subsequent 4-min rest resulted in a substantially smaller, more slowly activating I_{Ks} on the first post-rest pulse (Figure 32B).

$I_{Ks,tail}$ amplitudes on post-rest pulses were normalized by reference to amplitudes on the last pulses before control rests, and the resultant data from three myocytes indicate that the full extent of inhibition by terodiline was reached in the absence of channel-opening depolarizations (Figure 32B). Similar results were obtained from three other myocytes exposed to 20 μM terodiline without predrug rest trials.

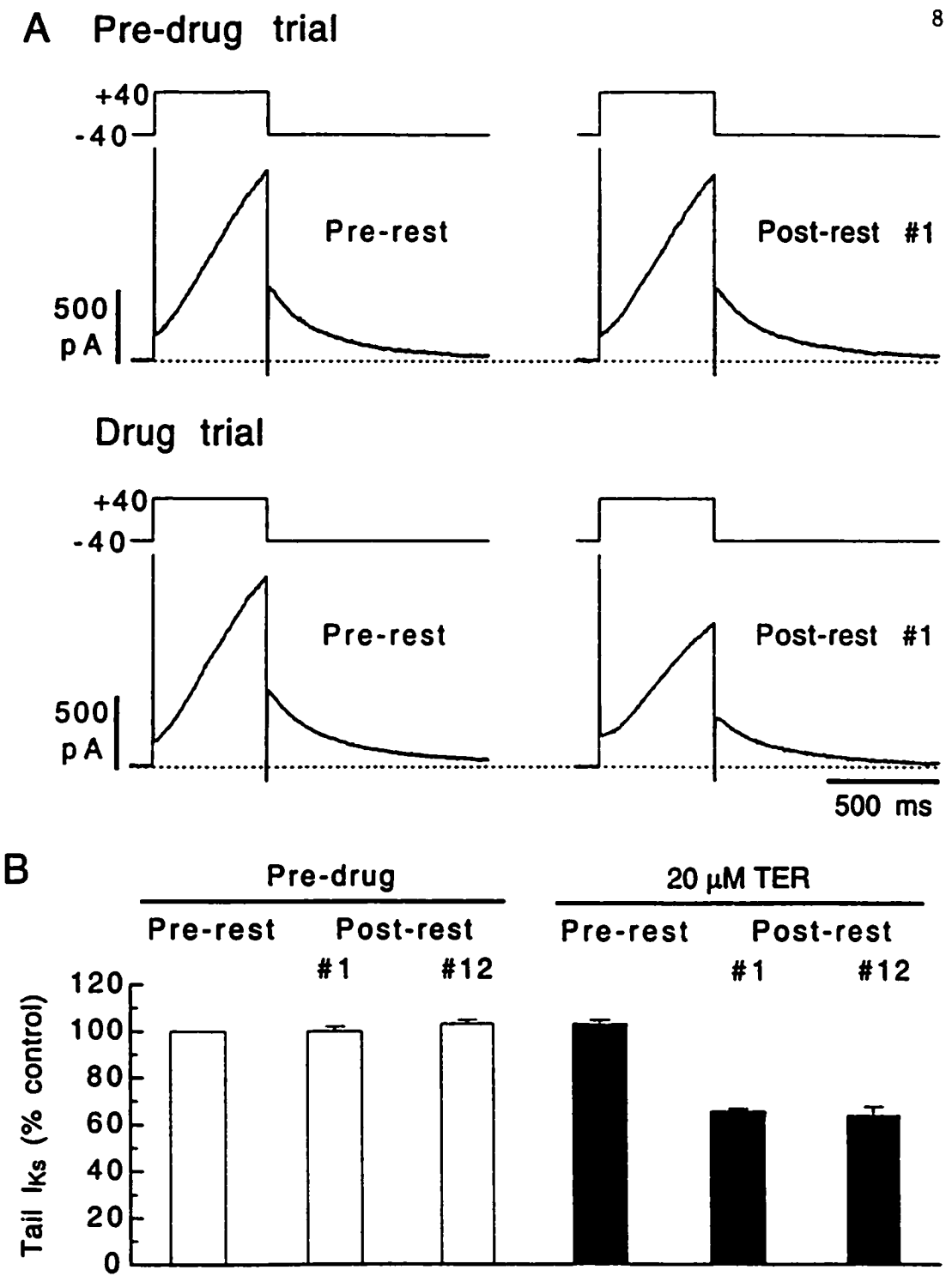


Figure 32. Inhibition of I_{Ks} by 20 μM terodiline during 4-min rests at -40 mV. (A) Currents elicited on 500-ms depolarizations to +40 mV at 0.1 Hz immediately before and after rests under pre-drug (top) and drug (bottom) conditions. (B) Summary of changes in I_{Ks} amplitudes measured from trials on three myocytes. All $I_{Ks,tail}$ amplitudes were normalized by reference to amplitudes on the last pre-rest pulses on pre-drug trials. Full inhibition was reached in the absence of pulsing.

(5) Effects on I_{to}

Differences in the responses of the action potential in rabbit and guinea pig papillary muscles to terodiline (Jones *et al.*, 2000a,b) suggested that the drug might have concentration-dependent inhibitory effects on I_{to}, a repolarizing current present in rabbit but not in guinea pig papillary muscles. This possibility was examined by measuring whole-cell I_{to} in rabbit ventricular myocytes. The myocytes were bathed in a modified Tyrode's solution that contained 0.2 mM Cd^{2+} to block $I_{Ca,L}$, 0.1 mM Ba^{2+} to block I_{K1}, 3 μM E4031 to block I_{Kr}, and 20 μM tetrodotoxin to block Na^{+} current.

Concentration-dependent inhibition at 36°C

In a series of experiments conducted at 36°C, rabbit ventricular myocytes were held at -80 mV and depolarized to +50 mV for 400 ms at 0.1 Hz. This voltage-clamp pulsing protocol was interrupted for a series of depolarizations to test potentials between -40 to +50 mV before and approximately 6 min after exposures to 3 and/or 30 μM terodiline. The depolarizations elicited inactivating outward currents whose amplitude increased with test potential (Figure 33A). The data in Figure 33B indicate that I_{to} amplitude measured on pulses to +50 mV was reduced by 31 ± 3% (n=11), and 87 ± 3% (n=8) by 3 μM and 30 μM terodiline, respectively (both $p < 0.001$). Based on these results, the IC_{50} for terodiline inhibition of I_{to} is on the order of 6 μM.

Concentration-dependent inhibition at 24°C

A series of experiments was conducted at 24°C to slow the kinetics of I_{to} and provide better resolution of the current. In these experiments, 5 mM 4-aminopyridine (which maximally inhibits peak I_{to}) was added at the end of the terodiline exposures to

allow measurement of peak I_{to} on pulses to +30 mV (0.1 Hz) as 4-aminopyridine-sensitive current. Using this protocol, current records obtained from a representative myocyte (Figure 33C, left) indicate that sequential exposures to 3 and 30 μM terodiline markedly reduced the amplitude of time-dependent I_{to} (difference between the current levels at 10 and 400 ms depolarization) in a concentration-dependent manner. Subtraction of the currents in the presence of the drugs from the currents under predrug control conditions, isolated the drug-sensitive current component (Figure 33C, right). A time plot of the amplitude of 4-aminopyridine-sensitive current (i.e., I_{to}) measured in this experiment is shown in Figure 33D. Applications of 3 and 30 μM terodiline lowered the current amplitude by ca. 35% and 90%, respectively. Inhibition by the drug was reversible because most of the current recovered after removal of terodiline and 4-aminopyridine.

A total of eight myocytes were superfused with 24°C solution, treated with 3 and 30 μM terodiline, and then treated with 4-aminopyridine. These concentrations of terodiline inhibited the 4-aminopyridine-sensitive current by 35 ± 3% and 88 ± 3%, respectively (both $p < 0.001$). Thus, the results are similar to those obtained when myocytes were bathed with solution warmed to 36°C, and pulsed to +50 mV instead of +30 mV. Thus, there was no evidence of voltage dependent inhibition over this potential range.

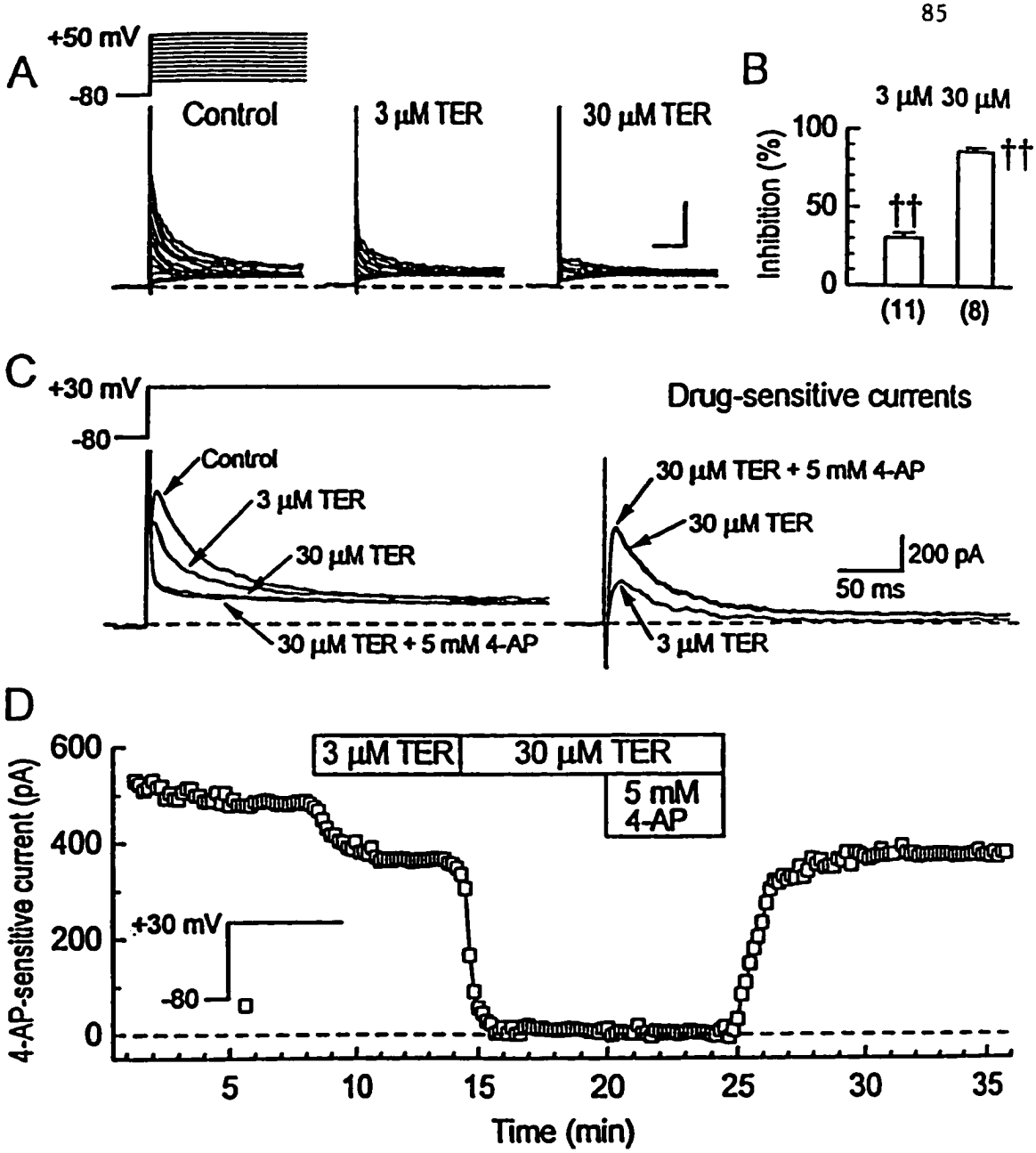


Figure 33. Effects of terodiline on I_{to} in rabbit ventricular myocytes. The myocytes were superfused with Tyrode's solution that contained 0.2 mM Cd^{2+}, 0.1 mM Ba^{2+}, 3 μM E4031, and 20 μM tetrodotoxin. (A) Records from an experiment conducted at 36°C. The myocyte was held at -80 mV, and depolarized to more positive potentials for 400 ms at 0.1 Hz before and ca. 6 min after the addition of 3 and 30 μM drug. The calibration bars indicate 0.5 nA and 100 ms. (B) Percentage inhibition of I_{to} amplitude. The amplitude was measured as the time-dependent current (10 ms to 400 ms) elicited on pulses to +50 mV. Numbers of myocytes in parentheses. Significance was assessed with the *t*-test: †† $p < 0.001$. (C,D) Results from representative experiments conducted at 24°C. The myocytes were held at -80 mV and depolarized to +30 -mV for 400 ms at 0.1 Hz. I_{to} at +30 mV was measured as the current sensitive to 5 mM 4-aminopyridine (4-AP).

SECTION B. EFFECTS OF OXYBUTYNIN AND S-OXYBUYNIN ON MEMBRANE CURRENTS

Figure 34A,B provides an overview of the concentration-dependent inhibitory effects of oxybutynin and S-oxybutynin on membrane currents in guinea pig ventricular myocytes. The myocytes were bathed in normal Tyrode's solution, dialyzed with K^+ pipette solution, and depolarized for 500 ms from prepulse -40 mV to more positive potentials before at 0.1 Hz. The records show that 10 to 100 μM concentrations of the drugs inhibited outward I_{K1} at -40 mV, inward $I_{Ca,L}$ on depolarizations, time-dependent outward I_K at positive potentials, and deactivating outward $I_{K,tail}$ on repolarizations to -40 mV. These inhibitory effects are examined more closely in the subsections below.

(1) Concentration-dependent inhibition of I_{K1}

To determine the effects of oxybutynin and S-oxybutynin on the I_{K1}-V relationship, myocytes were bathed in normal Tyrode's solution that contained 0.2 mM Cd^{2+} (to block $I_{Ca,L}$), and pulsed for 1 s from prepulse -40 mV to potentials between -130 and 0 mV for measurement of end-of-pulse current amplitude. The example results in Figure 35 indicate that the drugs lowered the amplitude of both inward- and outward-directed currents. The IC_{50} values for outward I_{K1} estimated by reductions in the amplitude of the current at -50 mV were 18.2 μM for oxybutynin, and 35.8 μM for S-oxybutynin. Thus, the oxybutynins have less potent inhibitory effects on I_{K1} than terodiline (IC_{50} = 7 μM). However, like terodiline, the inhibition was long-lasting and not easily reversed.

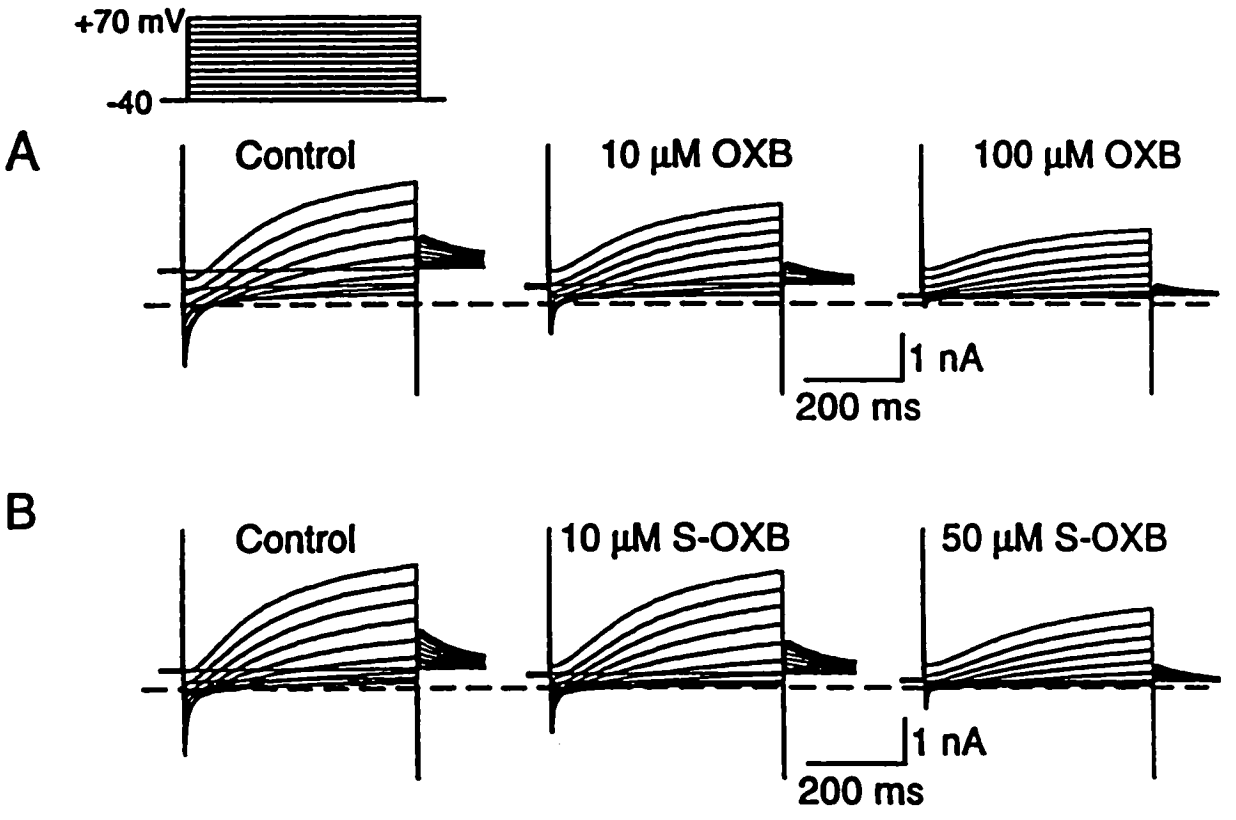


Figure 34. Effects of oxybutynin and S-oxybutynin on membrane currents in guinea pig ventricular myocytes. The myocytes were bathed in normal Tyrode's solution, held at -80 mV, and depolarized for 500 ms at 0.1 Hz from prepulse -40 mV to more positive potentials before (control) and after attainment of steady-state drug action. (A) Records obtained from a myocyte exposed to 10 and 100 μM oxybutynin (OXB). (B) Records obtained from a myocyte exposed to 10 and 50 μM S-oxybutynin (S-OXB). The dashed lines indicate zero-current levels.

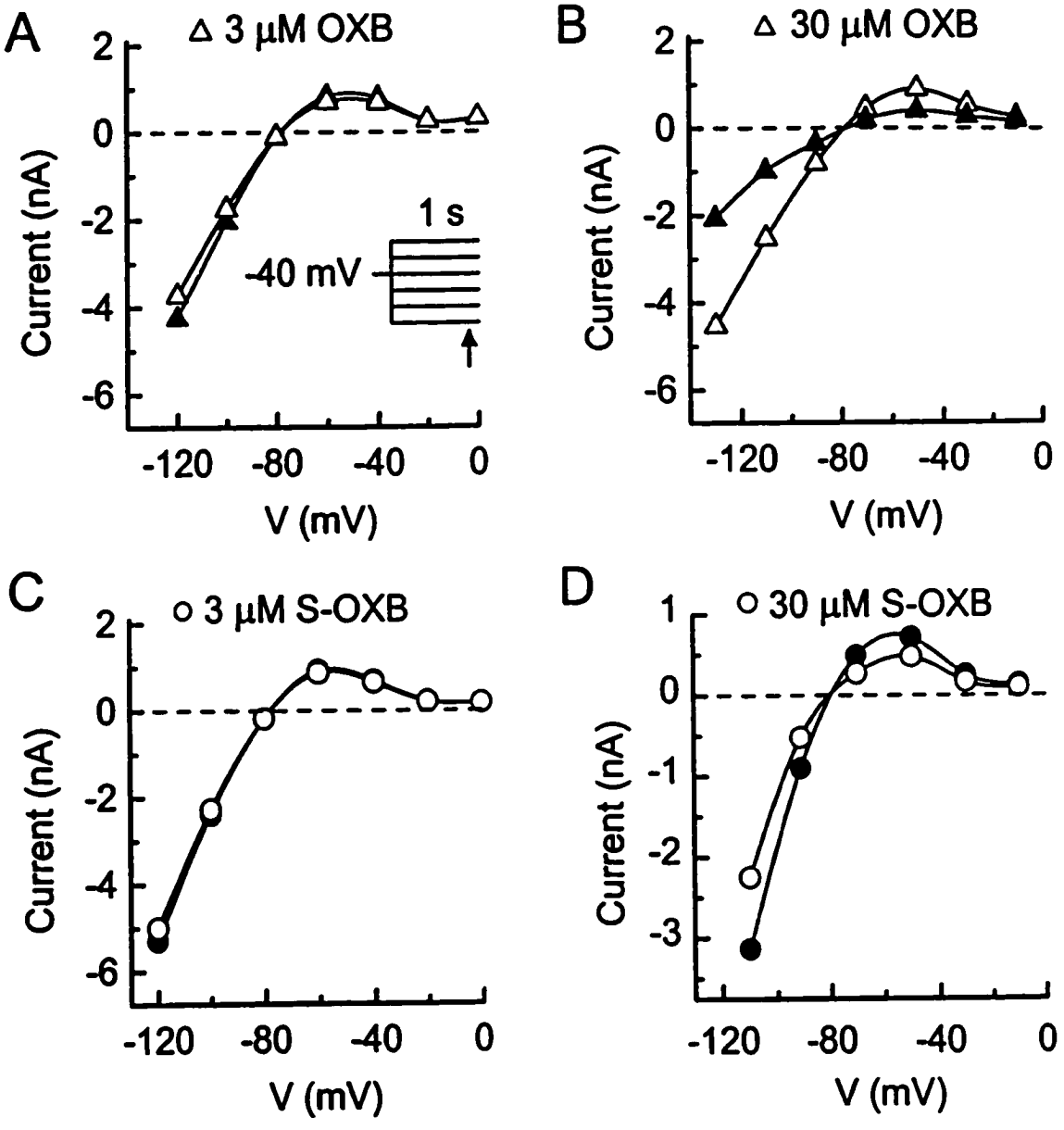


Figure 35. Effects of oxybutynin and S-oxybutynin on the I_{K1}-V relationship. Myocytes were bathed in normal Tyrode's solution, pretreated with 0.2 mM Cd^{2+}, and pulsed from prepulse -40 mV to potentials between -130 and 0 mV for measurement of end-of-pulse current amplitudes. Exposures to the drugs lasted for 10 to 15 min.

(2) Concentration-dependent inhibition of $I_{Ca,L}$

The effects of oxybutynin and S-oxybutynin on peak $I_{Ca,L}$ were examined in myocytes that were bathed and dialyzed with either K^+-containing or K^+-free solutions. The myocytes were held at -80 mV and depolarized at 0.1 Hz from prepulse -40 to 0 mV for 200 ms to monitor peak $I_{Ca,L}$; in some myocytes, regular pulsing was interrupted for sequences of depolarizations to other potentials for measurement of $I_{Ca,L}$-V relationships. The results in Figure 36 indicate that low micromolar concentrations of the drugs had relatively small inhibitory effects on peak $I_{Ca,L}$, whereas higher concentrations rapidly inhibited the current in a manner that was relatively independent of voltage and reversible on washout of the drugs. The Hill equations fitted to the steady-state data have IC_{50} values of 16.1 μM for oxybutynin and 17.8 μM for S-oxybutynin, and coefficients of 0.95 and 0.99, respectively (Figure 37).

These results indicate that peak $I_{Ca,L}$ is inhibited to a similar extent by oxybutynin and S-oxybutynin, and that the IC_{50} values of these drugs are only slightly higher than that of terodiline (IC_{50} 12.2-15.2 μM).

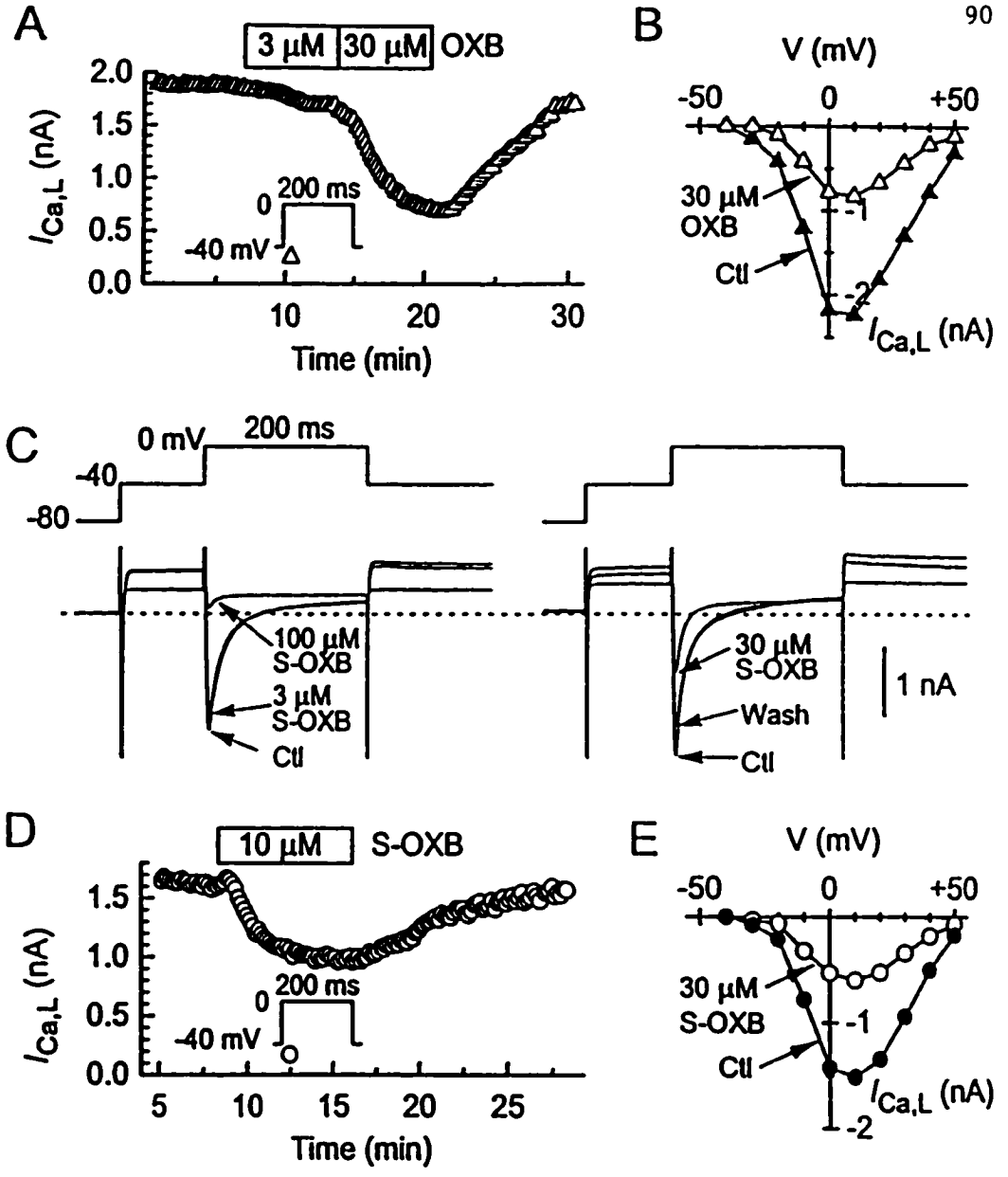


Figure 36. Inhibition of peak $I_{Ca,L}$ by oxybutynin compounds. Myocytes were held at -80 mV, depolarized to prepulse -40 mV for 100 ms, and then pulsed to 0 mV for 200 ms at 0.1 Hz. (A) Time course of peak $I_{Ca,L}$ in a myocyte bathed and dialyzed with K^+-containing solutions and exposed to 3 and 30 μM oxybutynin. (B) $I_{Ca,L}$-V relationships determined before and 9 min after addition of 30 μM oxybutynin. The myocyte was bathed and dialyzed with K^+-free solutions. (C) Records obtained from representative experiments. Left: effects of sequential applications of 3 μM (10 min) and 100 μM (5 min) S-oxybutynin. Right: reversible effects of 30 μM S-oxybutynin (drug treatment 12 min; washout 8 min). The experiments were performed using K^+-containing external and internal solutions. The dashed lines indicate zero-current levels. (D) Time course of reversible inhibition of peak $I_{Ca,L}$ by 10 μM S-oxybutynin in a myocyte bathed and dialyzed with K^+-free solutions. (E) $I_{Ca,L}$-V relationships determined before and 6 min after addition of 30 μM S-oxybutynin. K^+-free solutions.

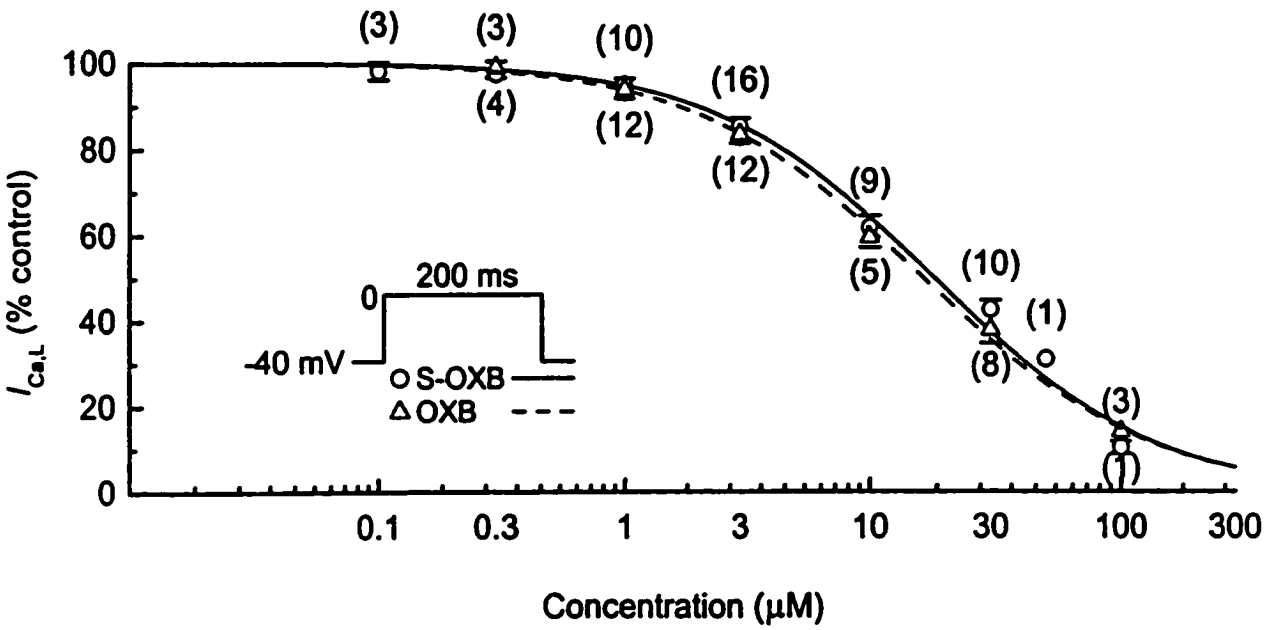


Figure 37. Dependence of the inhibition of peak $I_{Ca,L}$ on the concentration of oxybutynin and S-oxybutynin concentration. Myocytes were held at -80 mV, depolarized to prepulse -40 mV for 100 ms, and then pulsed to 0 mV for 200 ms at 0.1 Hz. The Hill equation describing the data has an IC_{50} of 16.1 μM for oxybutynin and 17.8 μM for S-oxybutynin; the respective coefficients are of 0.95 and 0.99. The numbers of myocytes are given in parentheses.

(3) Concentration-dependent inhibition of I_{Kr}

The effects of oxybutynin and S-oxybutynin on I_{Kr} were investigated in myocytes that were bathed in normal Tyrode's solution, depolarized to 0 mV for 200 ms, and repolarized to -40 mV to measure the amplitude of $I_{Kr,tail}$. Representative results indicate that $I_{Kr,tail}$ was relatively insensitive to low micromolar concentrations of oxybutynin (e.g., 3 μM: Figure 38A) and S-oxybutynin (e.g., 1 μM: Figure 38B), and reversibly inhibited by higher concentrations of the drugs (Figure 38A,B).

The results obtained from these and similar experiments are summarized in the concentration-response relationships shown in Figure 38C. The data are described by the Hill equation with an IC_{50} value of 11.4 μM and a coefficient of 0.97 for oxybutynin, and an IC_{50} of 12 μM and a coefficient of 1.01 for S-oxybutynin. In summary, the oxybutynin compounds were equipotent in their inhibitory actions on I_{Kr}, and approximately 25-fold less potent than terodiline (IC_{50} = 0.5 μM).

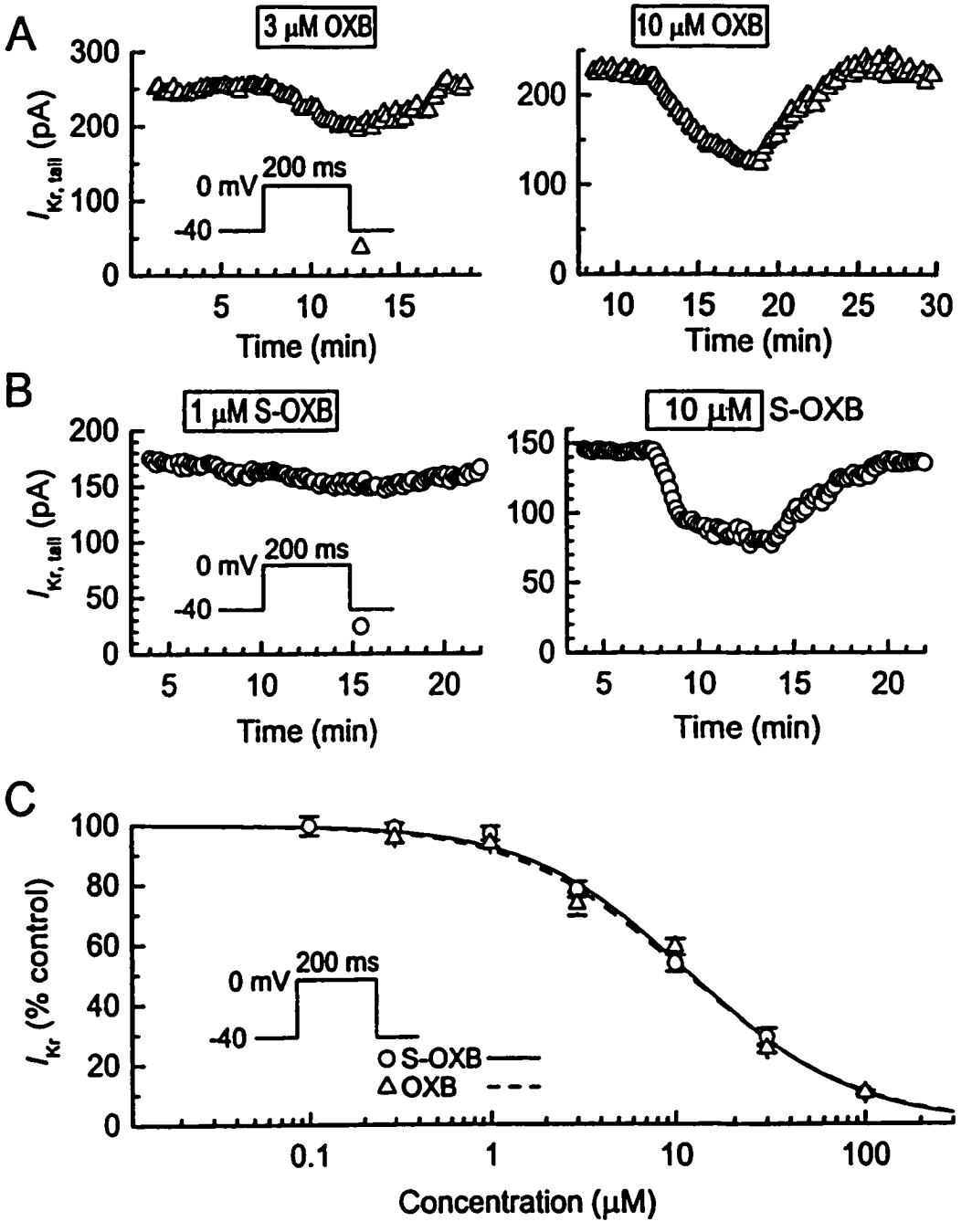


Figure 38. Inhibition of I_{Kr} by oxybutynin and S-oxybutynin. The myocytes were depolarized from -40 to 0 mV for 200 ms at 0.1 Hz, and repolarized to -40 mV for measurement of $I_{K,tail}$ amplitude. (A) Data from representative experiments with 3 μM (left) and 10 μM (right) oxybutynin. (B) Data from representative experiments with 1 μM (left) and 10 μM (right) S-oxybutynin. (C) Concentration-response relationships. The Hill equation fitting the oxybutynin data (n=3-10) has an IC_{50} of 11.4 μM and a Hill coefficient of 0.97, and that fitting the S-oxybutynin data (n=3-16) has an IC_{50} of 12 μM and a Hill coefficient of 1.04.

(4) Concentration-dependent inhibition of I_{Ks}

To evaluate the effects of oxybutynin and S-oxybutynin on I_{Ks}, the current was measured in three ways: (i) as the time-dependent current elicited by 500-ms depolarizations to +60 mV in myocytes that were bathed in normal Tyrode's solution, (ii) as $I_{K,tail}$ after 500-ms depolarizations to +30 mV in myocytes that were bathed in K^+-, Ca^{2+}-free Cd^{2+} solution, and (iii) as $I_{K,tail}$ after 2-s depolarizations to +80 mV under similar K^+-free conditions. Representative results obtained with these protocols indicate that high micromolar concentrations of oxybutynin (Figure 39A,C) and S-oxybutynin (Figure 39B) inhibited I_{Ks}. There were no obvious differences between the results with the three protocols, and the pooled data are described by the Hill equation with an IC_{50} of 28.7 μM and a Hill coefficient of 0.87 for oxybutynin, and an IC_{50} of 43 μM and a Hill coefficient of 0.98 for S-oxybutynin (Figure 39D).

These results indicate that oxybutynin (IC_{50} 28.7 μM) and terodiline (IC_{50} 26 μM) are equipotent inhibitors of I_{Ks}, and that both of these drugs are significantly more potent ($p < 0.01$) than S-oxybutynin.

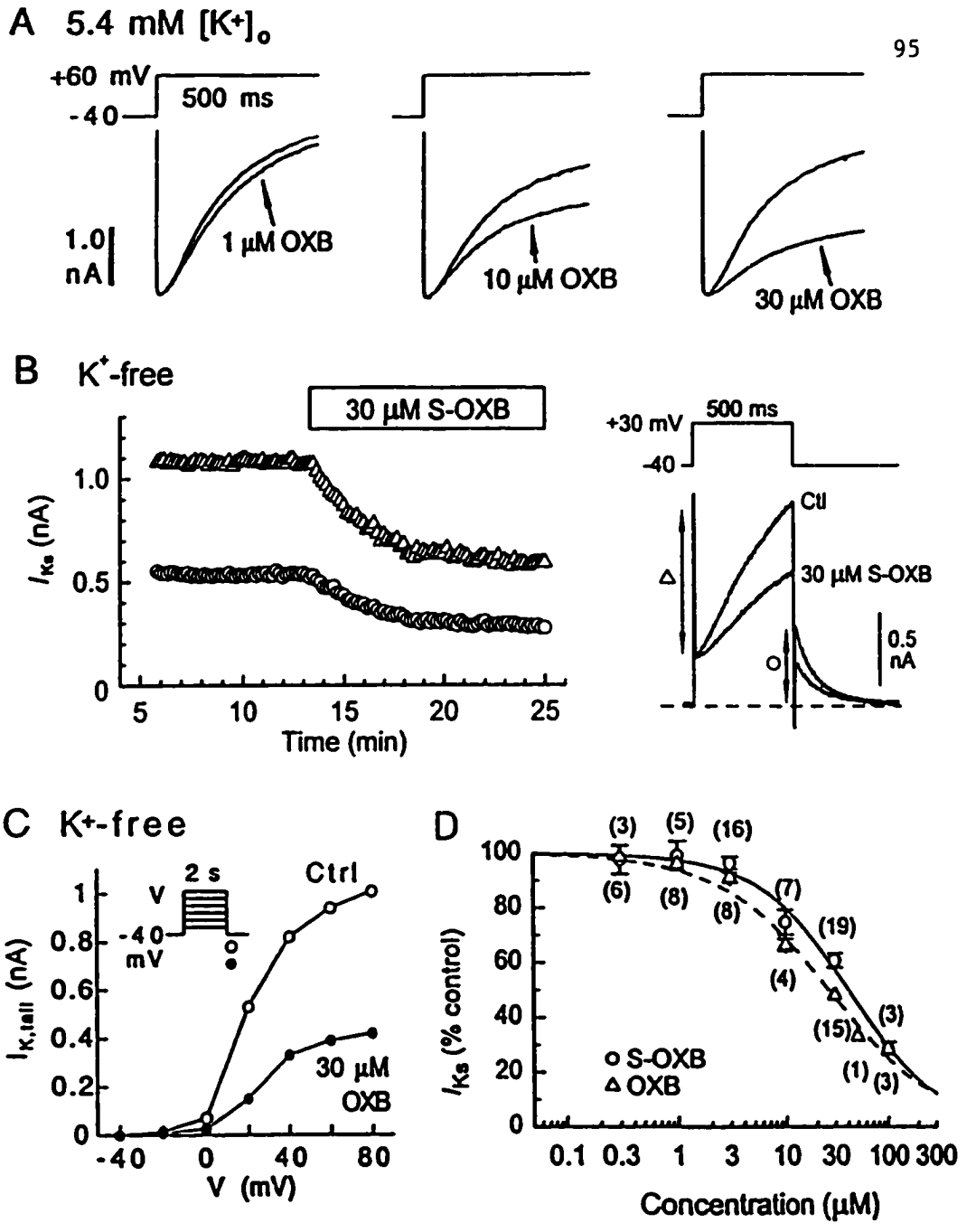


Figure 39. Inhibition of I_{Ks} by oxybutynin compounds. Myocytes were dialyzed with K^+ solution and superfused with normal Tyrode's (A) or K^+-, Ca^{2+}-free Cd^{2+} solution (B,C). (A) Effects of 1, 10, and 30 μM oxybutynin treatments (≥ 5 min) on time-dependent current activated by 500-ms pulses to +60 mV. (B) Effect of 30 μM S-oxybutynin on I_{Ks} measured as the change in amplitude of the time-dependent outward current elicited during 500-ms depolarizations to +30 mV, and as the change in amplitude of $I_{K,tail}$ upon repolarization to -40 mV. (C) $I_{K,tail}$-V relationships determined before and during treatment with 30 μM oxybutynin. (D) Concentration-response relationship. The Hill equation fitting the pooled data from the three protocols above has an IC_{50} of 28.7 μM and a coefficient of 0.87 for oxybutynin, and an IC_{50} of 43 μM and coefficient of 0.98 for S-oxybutynin. Number of myocytes in parentheses.

(5) Concentration-dependent inhibition of I_{to}

The effects of S-oxybutynin on I_{to} were examined in rabbit ventricular myocytes that were bathed in Tyrode's solution that contained 0.2 mM Cd^{2+}, 0.1 mM Ba^{2+}, 3 μM E4031, and 20 μM tetrodotoxin (temperature 36°C). The myocytes were held at -80 mV, and regularly depolarized to +50 mV for 400 ms at 0.1 Hz. This pulsing protocol was interrupted for a series of 400-ms depolarizations to potentials between -40 to +50 mV before and 6-8 min after addition of 3 and/or 30 μM oxybutynin to the bath (Figure 40A).

I_{to} amplitude was measured as the time-dependent current (difference between the current levels at 10 and 400 ms depolarization) elicited on depolarizations to +50 mV. Figure 40B indicates that the amplitude was decreased by 9 ± 2% (n=6) ($p < 0.05$) in the presence of 3 μM S-oxybutynin, and by 35 ± 3% (n=4) ($p < 0.001$) in the presence of 30 μM S-oxybutynin. Based on these results, the IC_{50} for S-oxybutynin appears to be approximately 70 μM, or roughly 12 times larger than the IC_{50} for terodiline.

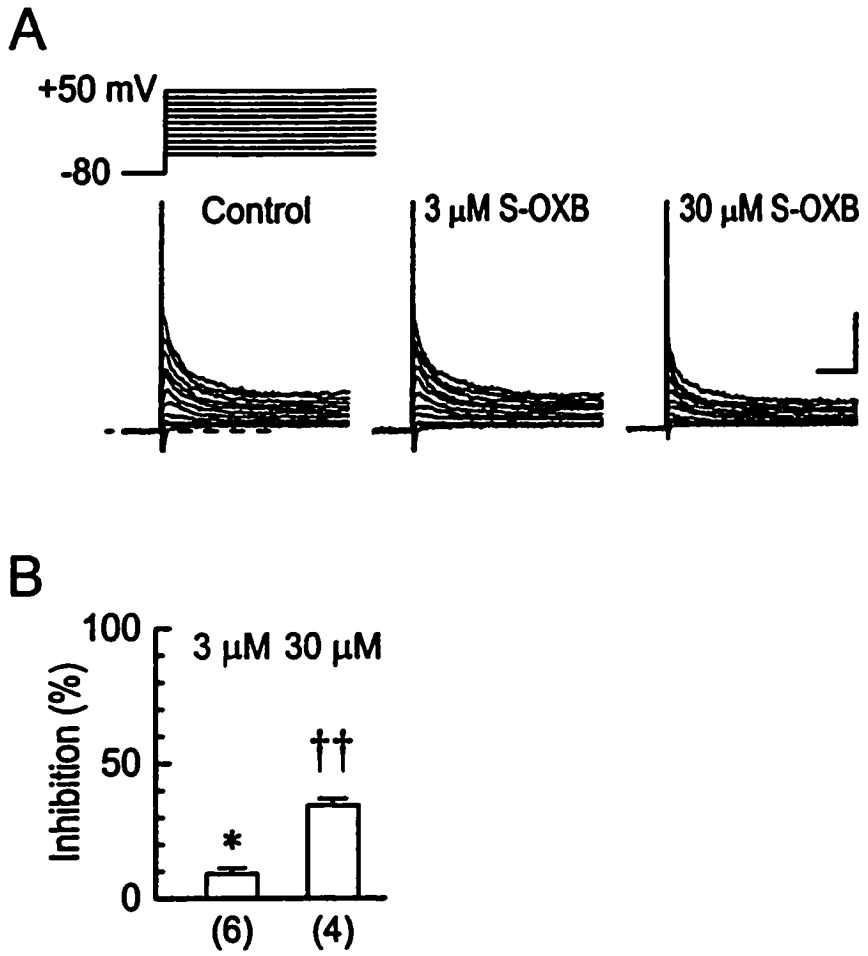


Figure 40. Effect of S-oxybutynin on I_{to} in rabbit ventricular myocytes. The myocytes were superfused with Tyrode's solution that contained 0.2 mM Cd^{2+}, 0.1 mM Ba^{2+}, 3 μM E4031, and 20 μM tetrodotoxin. Temperature 36°C. (A) Records from a representative experiment. The myocyte was held at -80 mV, and depolarized to more positive potentials for 400 ms at 0.1 Hz before and approximately 6 min after application of 3 and 30 μM drug. The calibration bars indicate 0.5 nA and 100 ms. (B) Percentage inhibition of I_{to} amplitude. The amplitude of I_{to} was measured as the time-dependent current (10 ms to 400 ms) elicited on pulses to +50 mV. Numbers of myocytes in parentheses. Significance was assessed with the *t*-test: * $p < 0.05$, †† $p < 0.001$.

IV. DISCUSSION

The discussion of the results is divided into three main sections: (1) evaluation of the effects of the investigated drugs on cardiac membrane currents in relation to pertinent literature; (2) interpretation of the present results with terodiline, oxybutynin, and S-oxybutynin within the context of previous electrophysiological studies on guinea pig and rabbit papillary muscles, and (3) consideration of the results in relation to the clinical safety of the drugs.

SECTION A. EFFECTS OF TERODILINE, OXYBUTYNIN, AND S-OXYBUTYNIN ON CARDIAC MEMBRANE CURRENTS

The effects of terodiline, oxybutynin, and S-oxybutynin on membrane currents in guinea pig and rabbit ventricular myocytes have been examined. Although all three compounds inhibited Ca^{2+} and K^{+} currents, terodiline had the most potent effects, especially on I_{Kr} and I_{to} (Table 1). These effects and their relation to previous studies are discussed in the five subsections that follow.

Table 1. Summary of the concentration-dependent effects of terodiline, oxybutynin, and S-oxybutynin on membrane currents in ventricular myocytes.

Current	Species	Terodiline IC_{50}	Oxybutynin IC_{50}	S-oxybutynin IC_{50}
I_{K1}	Guinea Pig	7 μM	18.2 μM	35.8 μM
$I_{Ca.L}$	Guinea Pig	12.2-15.2 μM	16.1 μM	17.8 μM
I_{Kr}	Guinea Pig	0.5 μM	11.4 μM	12 μM
I_{Ks}	Guinea Pig	26 μM	28.7 μM	41 μM
I_{to}	Rabbit	6 μM	———	70 μM

(1) Inwardly-Rectifying I_{K1}

The effects of the drugs on I_{K1} in guinea pig ventricular myocytes can be summarized as follows: (i) terodiline reduced outward-directed I_{K1}, the IC_{50} was 7 μM, and maximal reductions of ca. 60% were observed with concentrations of 100-300 μM; (ii) oxybutynin and S-oxybutynin were ca. 2.5-fold less potent than terodiline; (iii) drug-induced inhibition was independent of the direction of the current; and (iv) recovery from inhibition was slow, and incomplete, after removal of the drugs.

Concentration-dependent inhibition by terodiline

The degree of terodiline-induced inhibition of I_{K1} was more pronounced than that recently reported by Hayashi *et al.* (1997). In that study, the inhibition of inward I_{K1} (-100 to -140 mV) by 10 μM terodiline was on the order of 10-15%, considerably smaller than the 33 ± 2 % inhibition measured in the present study. There are no apparent experimental differences that might account for this discrepancy; the cell type, temperature, external K^+ concentration, and voltage-clamp pulsing rate in that study were comparable to those employed here. However, one difference between the two studies concerns the drug itself. The terodiline used in the present study was synthesized by Sepracor Inc. (Marlborough, MA, U.S.A.), provided as a hydrochloride salt, and freshly dissolved in DMSO immediately before use. In the study by Hayashi *et* al. (1997) the drug was synthesized in their own laboratory, and the storage and experimental usage conditions were not provided in their paper.

A second factor that needs to be considered in comparing the results of the two studies is the duration of the exposures of myocytes to the drug. It is possible that the extent of inhibition produced by terodiline may have been underestimated (in both

studies) if the drug-application times were not long enough to achieve full steady-state effects. The mean duration of the terodiline exposures in the present study was 12.2 min, or approximately four times the estimated time to half maximal inhibition; however neither representative time courses of drug action nor duration of exposures were provided by Hayashi *et* al. (1997).

The present results indicate that terodiline is a relatively potent inhibitor of I_{K1}. It is comparable in potency with RP58866 (IC_{50} ~ 6 μM: Jurkiewicz *et al.*, 1996) and its active enantiomer, terikalant (IC_{50} ~ 8 μM: Jurkiewicz *et al.*, 1996; maximal inhibition ~ 73%: McLarnon and Xu, 1995), and appears to be considerably more potent than quinidine (30% inhibition at 20-40 μM: Salata and Wasserstrom, 1988), a cardioactive drug that is often referred to as a potent I_{K1}-inhibiting drug.

Proportional inhibition of inward and outward current

The finding that the degree of inhibition of I_{K1} by terodiline, oxybutynin, and S-oxybutynin was independent of the direction of the current is interesting because it is becoming apparent that I_{K1}-inhibitory compounds can have varying directional effects on I_{K1}. For example, both RP58866 and terikalant have similar-sized inhibitory effects on inward- and outward-directed I_{K1} (Jurkiewicz *et al.*, 1996; McLarnon and Xu, 1995), whereas taurine appears to have a selective inhibitory action on inward-directed I_{K1} (Satoh, 1998), and the inhalational anesthetic sevoflurane has a preferential inhibitory effect on inward-directed I_{K1} (Stadnicka *et al.*, 1997).

Slow and incomplete recovery

The reversal of the inhibition of I_{K1} after exposures to terodiline or oxybutynin compound was slow and incomplete compared to reversal of $I_{Ca,L}$ or I_{Ks} inhibition. The

absence of a rapid recovery upon drug washout appears to be a common finding with drugs that block inwardly-rectifying K^+ channels. For example, Balser *et al.* (1991) observed that both the onset and washout of single Kir channel block were relatively slow processes, and Hiraoka *et al.* (1986) reported that quinidine-induced inhibition of I_{K1} was 'irreversible' (whereas inhibitions of $I_{Ca,L}$ and I_K in the same cells were quickly reversible). Arena and Kass (1998) reported that recovery from block of guinea pig ventricular I_{K1} by externally-applied tertiary clofilium was often slow and incomplete after 10-min washout periods, in contrast to that of I_K which was rapid and approached 100%. They suggested that the different recovery patterns arose as a result of different off-rates for binding to the drug receptors responsible for inhibition of the two currents.

The temporal pattern of recovery of I_{K1}, $I_{Ca,L}$, and I_K after terodiline removal can explain the ca. 10% lengthening of the action potential that was observed upon washout of terodiline from guinea pig papillary muscles (Jones *et al.* 1999) because the relatively quick recovery of plateau currents such as $I_{Ca,L}$ and I_K should unmask the full lengthening consequences of inhibited I_{K1}. Similar post-washout lengthening effects have also been observed during washout of quinidine from canine Purkinje fibres (Nattel and Bailey, 1983) and canine papillary muscles (Salata and Wasserstrom, 1988), as well as during washout of millimolar sotalol from guinea pig papillary muscles (Carmeliet, 1985). In the latter studies, the extra lengthening of the action potential during washout was also attributed to rapid recovery of plateau currents and maintained inhibition of I_{K1}.

(2) L-Type Ca^{2+} Current

Terodiline, oxybutynin, and S-oxybutynin had reversible concentration-dependent inhibitory effects on peak $I_{Ca,L}$. However, the actions of terodiline were investigated in

much more depth than those of the oxybutynin compounds, and the discussion is focused on that material.

The effects of terodiline on L-type Ca^{2+} channel currents can be summarized as follows: (i) inhibition of $I_{Ca,L}$ was concentration-dependent, with IC_{50} values of 15.2 μM in K^+-dialyzed cells and 12.2 μM in Cs^+-dialyzed cells; (ii) inhibition of $I_{Ca,L}$ was use-dependent (block increased when the voltage-clamp pulsing rate was increased and when the holding potential was lowered); (iii) the drug had a more potent inhibitory effect on peak $I_{Ba,L}$ than on peak $I_{Ca,L}$; (iv) the apparent rate of inactivation of both $I_{Ca,L}$ and $I_{Ba,L}$ was markedly accelerated in the presence of the drug; and (v) the time required for Ca^{2+} channels to recover from inactivation was lengthened in the presence of the drug. These results are discussed in the subsections below.

Concentration-dependent inhibition of peak $I_{Ca,L}$

The only other study that examined the effects of terodiline on $I_{Ca,L}$ in cardiac cells was conducted by Hayashi *et al.* (1997). They reported that peak $I_{Ca,L}$ in guinea pig ventricular myocytes was inhibited with an IC_{50} of 34 μM, a value that is about 2.5 times higher than that reported here. The reason for this difference is unclear, especially since the two studies were conducted on guinea pig ventricular myocytes under broadly similar experimental conditions (external K^+ concentration, temperature, voltage-clamp pulsing rate). However, it is worth noting that Hayashi *et* al. (1997) measured the effects of terodiline after relatively short (~3-min) cumulative exposures to six concentrations between 1 and 300 μM, whereas myocytes in the present study were primarily treated with one concentration of the drug for up to 10 min. It is conceivable that the short, cumulative-exposure protocol may have skewed the results of Hayashi *et* al. (1997) to higher concentrations.

A comparison of the present results with those from an earlier study on smooth muscle cells from guinea pig bladder suggests that terodiline has a much weaker action on cardiac $I_{Ca,L}$ than on bladder $I_{Ca,L}$. In the study on smooth muscle cells, Kura *et* al. (1992) found that terodiline inhibited $I_{Ca,L}$ with an IC_{50} of 1.7 μM, i.e., approximately 10-fold lower than the IC_{50} determined for cardiac $I_{Ca,L}$. Such a large difference points to a cell-type-related difference in sensitivity to terodiline, although other factors such as experimental design and data analysis may also be involved. The smooth muscle study was conducted at 22°C, and slowed recovery from inactivation at this temperature may explain why (unlike $I_{Ca,L}$ in cardiac myocytes) bladder myocyte $I_{Ca,L}$ declined by 40% during post-rest pulsing. Therefore, these investigators faced a tricky measurement situation in calculating the degree of terodiline inhibition by relating post-rest inhibition in the presence of the drug to post-rest inhibition prior to the drug. Any measurement difficulty that led to over-estimates of the effects of 1 and 10 μM drug would have greatly modified the IC_{50} because the latter was estimated from the responses to just three concentrations (1, 10, and 100 μM).

Use-dependent inhibition of $I_{Ca,L}$

In the study on guinea pig bladder smooth muscle cells referred to above, Kura *et al.* (1992) observed that inhibition of $I_{Ca,L}$ by 10 μM terodiline developed more quickly when the stimulation rate was 1 Hz rather than 0.1 Hz. The present results indicate that terodiline-induced block of $I_{Ca,L}$ in guinea pig ventricular myocytes is also enhanced by increases in pulsing frequency. In addition, they establish that inhibition by terodiline is augmented by more depolarized holding potentials, and that recovery of $I_{Ca,L}$ from inactivation is slowed in the presence of the drug. These indications of use-dependent channel block have been observed with a number of organic Ca^{2+} channel blockers,

including verapamil, diltiazem, and D600 (Ehara and Kaufmann, 1978; Kanaya *et al.*, 1983; McDonald *et al.*, 1994; Pelzer *et* al., 1992).

Enhanced inhibition of $I_{Ca,L}$ by terodiline at lower holding potentials may have been an important factor in its effectiveness as a therapeutic agent for the treatment of unstable bladder disorders. Electrophysiological studies using multicellular strips of guinea pig urinary bladder have shown that the resting potential of bladder smooth muscle cells is ca. -37 mV (Creed, 1971; Kurihara and Creed, 1972; Creed *et al.*, 1983). Based on the present findings, terodiline is expected to be a potent blocker of $I_{Ca,L}$ in these cells through its voltage-dependent inhibitory action. Furthermore, use-dependent block of L-type Ca^{2+} channels may have been a factor in the treatment of unstable bladder with terodiline because strips of unstable urinary bladder exhibit spontaneous electrical activity (Anderson, 1993), and individual muscle cells from unstable bladders are often in a hyperactive state (Brading and Turner, 1994).

Inhibition of peak $I_{Ba,L}$ versus peak $I_{Ca,L}$

Terodiline had a more potent action on peak $I_{Ba,L}$ than on peak $I_{Ca,L}$. For example, 5 μM drug reduced peak $I_{Ba,L}$ to 43 ± 2% of control (n=11), but only reduced peak $I_{Ca,L}$ to 72 ± 2% of control (n=7) ($p < 0.001$). In a recent study on fendiline, a drug that is structurally similar to terodiline, Nawrath *et al.* (1998) found that the IC_{50} values for peak $I_{Ba,L}$ and peak $I_{Ca,L}$ were 8 and 13 μM, respectively. They suggested that the more potent inhibition of $I_{Ba,L}$ was due to enhanced binding of fendiline as a result of the enhanced openness (i.e., slowed inactivation) of Ca^{2+} channels during depolarizations when Ba^{2+} is the charge-carrier. Since it is likely that terodiline also preferentially binds to the open configuration of Ca^{2+} channels, a similar reasoning can be advanced to explain the enhanced sensitivity of $I_{Ba,L}$ to terodiline.

Accelerated decay of Ca^{2+} channel current

Terodiline markedly accelerated the decay of $I_{Ca,L}$ during depolarizations to 0 mV. The acceleration was evident in myocytes treated with low micromolar concentrations that had little effect on current amplitude, but was more pronounced with higher concentrations. Terodiline also accelerated the decay of $I_{Ba,L}$ during long depolarizations. The current inactivated along a multi-exponential time course (cf. Boyett *et* al., 1994), and terodiline shortened the time constant of the three most prominent phases of inactivation.

There is evidence that some organic Ca^{2+} channel blockers accelerate the decay of Ca^{2+} channel currents, while others do not (McDonald *et al.*, 1994). In that regard, Nawrath *et al.* (1998) have recently reported that 10 μM fendiline accelerated the decay of $I_{Ba,L}$ in guinea pig ventricular myocytes, whereas 10 μM verapamil did not. They postulated that the ineffectiveness of verapamil was due to relatively weak open-channel block by the drug. By contrast, strong open-channel block by fendiline caused time-dependent removal of channels from the conducting pool, and this was observed as a speeding up of the decay of $I_{Ba,L}$. To estimate the time course of this block, they analyzed the 'fractional block' of $I_{Ba,L}$ elicited by 300-ms pulses to +10 mV, and concluded that block occurred along a monoexponential time course with $\tau \sim 100$ ms.

The present results are not as tidy as those with fendiline because a similar type of fractional block analysis failed to identify a monoexponential process. One could argue that the requirement for a multiexponential description for the fractional block reflects differing rates of binding to multiple open states of Ca^{2+} channels conducting Ba^{2+}. Alternatively, acceleration of the multiple phases of decay of $I_{Ba,L}$ may reflect drug-induced acceleration of multiple inactivation processes. The present results do not

warrant any additional speculation, especially since the physical meaning of the multiexponential decay of $I_{Ba,L}$ is obscure (Boyett *et al.*, 1994).

(3) Delayed-rectifying I_K

Total I_K was more sensitive to inhibition by terodiline than to oxybutynin or S-oxybutynin. This difference in sensitivity was primarily due to selective inhibition of I_{Kr} by terodiline, and the implications of this finding are considered at a later point in relation to studies on guinea pig and rabbit papillary muscle action potentials, and in relation to the clinical safety of the drugs.

The effects of terodiline on guinea pig ventricular myocytes indicated that concentrations ≤ 3 μM preferentially inhibited the I_{Kr} component of I_K. Identification of this selective property expands on recent findings of Hayashi *et al.* (1997) concerning terodiline action on I_K in guinea pig ventricular myocytes. These investigators measured $I_{K,tail}$ at -40 mV after 500-ms depolarizations to positive potentials, and determined that 10 μM terodiline reduced the amplitude by ca. 20%. In the absence of any additional information, the authors suggested that the drug might inhibit both I_{Kr} and I_{Ks}. The present results establish that terodiline does in fact inhibit the two components, and that the IC_{50} for inhibition of I_{Kr} is about 50 times lower than that for I_{Ks} (see Table 1).

Inhibition of I_{Kr} by terodiline

Preferential inhibition of I_{Kr} over I_{Ks} appears to be a property of a growing list of "cardiac" and "non-cardiac" drugs. Examples include E4031 and sotalol (Sanguinetti and Jurkiewicz, 1990; Wettwer *et* al., 1992), dofetilide (Carmeliet, 1992; Williams and

Beatch, 1997), quinidine (Balser *et* al., 1991), terfenadine (Ming and Nordin., 1995; Salata *et* al., 1995; Berul and Morad, 1995), and flecainide (Follmer and Colatsky, 1990).

Of these drugs, E4031 is widely used as a standard selective inhibitor of I_{Kr} in guinea pig ventricular myocytes (Sanguinetti and Jurkiewicz, 1990; Wettwer *et al.*, 1992; Heath and Terrar, 1996a,b), and Sanguinetti and Jurkiewicz (1990) have reported that the IC_{50} is 0.4 μM. Accordingly, I_{Kr} in this study was identified as the $I_{K,tail}$ component sensitive to a full blocking concentration (3-5 μM) of E4031. The results indicate that the normalized E4031-sensitive $I_{K,tail}$-V relationship can be described by the Boltzmann equation with a half-activation voltage ($V_{½}$) of -12.2 mV and a slope factor (S) of 8.0 mV. This finding is in good agreement with earlier estimates of the $V_{½}$ and S for I_{Kr} in guinea pig ventricular myocytes ($V_{½}$ = -14.3 to -18.7 mV; S = 6.6 to 8.7 mV) (Heath and Terrar, 1996a) and in human atrial and ventricular myocytes ($V_{½}$ = -14.0 mV; S = 6.5 to 7.7 mV) (Wang *et al.*, 1994; Li *et al.*, 1996).

The similarity of the voltage dependence of $I_{K,tail}$ sensitive to 3 μM terodiline ($V_{½}$ = -14.5 mV; S = 8.7 mV) to that of E4031-defined $I_{Kr,tail}$ is strong evidence that terodiline inhibits I_{Kr}. In fact, $I_{K,tail}$ sensitive to 0.3 μM terodiline was indistinguishable from $I_{K,tail}$ sensitive to E4031 (Figure 27), suggesting that like E4031 (Sanguinetti and Jurkiewicz, 1990), but unlike other I_{Kr} inhibitors such as dofetilide (Carmeliet, 1992) and WAY 123,398 (Spinelli *et al.*, 1993), inhibition of I_{Kr} by terodiline is relatively independent of the activating voltage.

At concentrations of terodiline higher than 3 μM, the voltage dependence of inhibition of normalized $I_{K,tail}$ sensitive to the drug diverged from the saturating-type of voltage-dependent relationship found with E4031 (Figure 27). A straightforward explanation to account for this result (larger drug-sensitive $I_{K,tail}$ after 500-ms pulses to

high positive potentials than after pulses to +20 mV) is that high terodiline also inhibits I_{Ks}.

Further support for inhibition of I_{Kr} by terodiline was obtained from experiments designed to test for mutual occlusion of 3-5 μM terodiline and E4031 actions. Not only was inhibition of $I_{K,tail}$ at 0 mV by 5 μM E4031 fully occluded in terodiline-pretreated myocytes, but inhibition by 3-5 μM terodiline was occluded in E4031-pretreated myocytes.

In summary, the results discussed above indicate that 3-5 μM terodiline completely inhibits I_{Kr}, whereas concentrations ≥ 3 μM also inhibit I_{Ks}.

Concentration-dependent inhibition of I_{Ks}

The effects of terodiline on I_{Ks} were evaluated in myocytes superfused with one of three solutions: normal Tyrode's, Tyrode's that contained 3-5 μM E4031, and K^+-, Ca^{2+}-free Cd^{2+} Tyrode's. Under the first two conditions, I_{Ks} was measured as the time-dependent current elicited by 500-ms pulses to +60 mV. Any contribution by rapidly-inactivating I_{Kr} to the measurement of I_{Ks} at +60 mV was expected to be relatively small when myocytes were bathed in normal Tyrode's solution, and essentially zero when E4031 was also present (Sanguinetti and Jurkiewicz, 1990; Jurkiewicz and Sanguinetti, 1993). Although I_{Ks} inhibition by terodiline was similar in myocytes superfused with normal Tyrode's and normal Tyrode's *plus* E4031, the I_{Ks} evaluation protocol is nevertheless open to the criticism that unidentified overlapping non-I_{Ks} currents present in the time-dependent current may have been affected to an unknown extent by the drug, and thereby have influenced the results.

To circumvent this possible source of error, measurements of drug effects were also obtained under conditions designed to enhance the amplitude of I_{Ks} and to suppress

I_{Kr} and Ca^{2+}-dependent currents (K^+-, Ca^{2+}-free Cd^{2+} solution; 0.5- to 2-s-long depolarizations). $I_{K,tail}$-V relationships obtained under these conditions lacked an E4031-sensitive component, and the degree of drug-induced changes in the amplitude of time-dependent I_K was similar to the degree of drug-induced changes in the amplitude of $I_{K,tail}$. The latter observation indicates that just one species of the time-dependent current was being measured under these K^+-free conditions.

The effects of terodiline on I_{Ks} were independent of the protocol used to measure I_{Ks}, and the IC_{50} estimated from the pooled data was 26 μM. This result is of additional interest because it suggests that, in contrast to reports suggesting that external K^+ can modulate the inhibitory potency of K^+ channel blockers (Armstrong, 1971; Yang *et al.*, 1997), block of Ks channels by terodiline was unaffected by K^+ withdrawal.

Characteristics of I_{Ks} block

Block of I_{Ks} by terodiline had the following features: (i) it resulted in a slowed activation of I_{Ks} during depolarizations to positive potentials, (ii) it was fully established on the first pulse after a rest in the presence of the drug; and (iii) it was dependent on the voltage of the activating depolarization. These results are discussed in the context of the possible mechanisms of Ks channel block by the drug.

(i) State-dependent block of K^+ channels. As first detailed by Armstrong (1971) in regard to block of squid axon delayed-rectifier K^+ channels by tetraethylammonium ions, block of delayed-rectifier K^+ channels by organic compounds appears to occur primarily via the open channel route. For native K^+ currents, this mode of block appears to be implicated in the inhibition of cardiac ventricular I_{Kr} by dofetilide (Carmeliet, 1992) and almokalant (Carmeliet, 1993), cardiac nodal I_K by quinidine (Furukawa *et al.*,

1989), cardiac atrial I_{to} by quinidine (Slawsky and Castle, 1994; Clark *et al.*, 1995; Wang *et al.*, 1995), and noncardiac epithelial I_K by phenylalkylamine (DeCoursey, 1995). Similarly, almost all accounts of inhibition of cloned delayed-rectifier K^+ channels by organic compounds suggest that the compounds preferentially bind to channels that are in the open configuration (e.g., Snyders *et al.*, 1992; Avdonin *et al.*, 1997; Zhang *et al.*, 1997). Nevertheless, there are a few reports concluding that inhibitory organic compounds can bind in a preferential manner to the closed configuration of delayed-rectifier K^+ channels. These include studies on 4-aminopyridine inhibition of I_{to} in ferret ventricular (Campbell *et al.*, 1993) and human atrial (Wang *et al.*, 1995) myocytes, and quinidine inhibition of I_{to} in human ventricular myocytes (Tseng *et al.*, 1996). In the latter study, the investigators also found that quinidine preferentially blocks closed Kv 4.2 channels, and Yao *et* al. (1996) proposed a similar mechanism to account for quinidine block of an I_{Ks}-like current induced by a human cDNA (hisK) in oocytes.

(ii) Block by terodiline. The most straightforward interpretation of the terodiline-induced, marked delay in the activation of I_{Ks} during depolarizations to positive potentials is that (i) a strong block is present at -40 mV just before each depolarization, (ii) block weakens with time when the membrane is depolarized to positive potentials, and (iii) block is restored again following repolarization. In regard to the restoration of block, the most likely mechanisms are (i) block of open channels at early times after repolarization to -40 mV when Ks channels are undergoing deactivation from the open to the closed state, and (ii) block of closed channels as the Ks channels quickly accumulate in that state during the long interpulse interval at -40 mV. Fast open-channel block at -40 mV might have been manifested in a speeding up of tail deactivation; however, the results showed no indication of this effect. On the other hand, restoration of block via a

closed channel pathway would be consistent with the finding that maximal block in rest/postrest trials was imposed at negative potentials that restricted Ks channels to the closed state.

Block of I_{Ks} by 30-50 μM terodiline was stronger on depolarizations to less positive potentials than on those to more positive potentials. This is not a novel finding in regard to blocking mechanisms, i.e., voltage-dependent block by organic compounds has previously been documented in studies on cardiac delayed-rectifier K^+ channels. For example, Hume (1985) observed that inhibition of frog atrial I_K by 10 μM nisoldipine was weaker on depolarizations to positive potentials than to negative potentials, and Zhang *et al.* (1997) reported that block of hKv1.5 channels by 30 μM nifedipine weakened at potentials above +20 mV. In addition, Valenzuela *et al.* (1994) found that imipramine-induced inhibition of the La^{3+}-insensitive $I_{K,tail}$ weakened with increased duration and amplitude of test pulses applied to guinea pig ventricular myocytes. Although Valenzuela *et* al. (1994) concluded that imipramine preferentially binds to closed Ks channels, they also left open the possibility that the drug interacts with open channels as well.

The details of the mechanism underlying the voltage-dependency of I_{Ks} block by terodiline are not known. One possibility is that the drug causes a shift of the voltage dependence of I_{Ks} activation to much more positive potentials. Alternatively, the voltage dependency may arise from a coupling of the drug binding site to a voltage-sensitive process such that unbinding is enhanced at more positive potentials. More refined experiments are required to distinquish between these and other possible blocking schemes (for example, see Woodhull, 1973; Hondeghem and Snyders, 1990).

Block of closed Ks channels and reverse frequency-dependence

The strong likelihood that terodiline inhibits I_{Ks} by preferentially binding to closed Ks channels has implications for the behaviour of the action potential in cardiac tissue exposed to the drug. The reason for this view is that closed-channel block of delayed-rectifier K^+ channels is closely connected with the phenomenon of reverse frequency-dependence of action potential lengthening (see Hondeghem and Snyders, 1990) in which the lengthening induced by antiarrhythmic drugs is larger at low stimulation rates than at high ones. This behaviour of drug-treated cardiac preparations is attributed to a "reverse use-dependence" of block of delayed-rectifier K^+ channels by the drug (Hondeghem and Snyders, 1990; Balser *et al.*, 1991). The concept of a drug that preferentially binds to closed channels and unbinds from open channels offers an explanation for relief from drug-induced action potential lengthening when the stimulation rate is raised, i.e., at higher stimulation rate, there is a reduced time for binding to the closed state, and an increased time for unbinding from the open state. It has been proposed that this type of drug binding/unbinding is involved in the block of cardiac Kr channels by class III antiarrhythmic agents such as sotalol, E4031, dofetilide, and UK-66,914 (Hondeghem and Snyders, 1990; Hafner *et al.*, 1988; Tande *et al.*, 1990; Gwilt *et al.*, 1991; Baskin *et al.*, 1991). The importance of reverse frequency-dependence, as it pertains to terodiline action, is discussed at a later point when the voltage-clamp results are related to action potential changes and cardiotoxicity.

(4) Transient outward K^+ current

I_{to} is a delayed-rectifier K^+ current that promotes early repolarization of the action potential in rabbit ventricular tissue, but is reportedly absent in guinea pig ventricular tissue (Hiraoka and Kawano, 1987; McDonald *et* al., 1989; Carmeliet, 1993).

The results of the present study on rabbit ventricular myocytes indicate that terodiline is much more potent than S-oxybutynin at inhibiting I_{to} (IC_{50} values of 6 μM and 70 μM, respectively). This finding helps explain differences reported by Jones *et al.* (2000b) in the responses of the action potential in guinea pig and rabbit papillary muscles to treatments with these drugs. The results of that study are considered at a later point in the Discussion in relation to the present voltage-clamp analysis of I_{to} inhibition by terodiline and S-oxybutynin.

The relatively low IC_{50} of terodiline is comparable to the IC_{50} values of a number of other cardioactive drugs that block I_{to}. These include the putative class III antiarrhythmic drug, terikalant (IC_{50} ~ 5 μM: McLarnon and Xu, 1995), the K^+ channel blocker, tedisamil (IC_{50} ~ 6 μM: Dukes and Morad, 1989), the Ca^{2+}-channel blocking diphenylalkylamine, fendiline (IC_{50} ~ 3 μM: Fassbender *et al.*, 1999), and the Ca^{2+}-channel activating dihydropyridine, BAY K 8644 (IC_{50} ~ 7 μM: Gotoh *et al.*, 1991). Terodiline is more potent than the experimental class III antiarrhythmic agent, ambasilide (IC_{50} ~ 23 μM: Feng *et al.*, 1997), and less potent than the dihydropyridine, nicardipine (IC_{50} ~ 630 nM: Gotoh *et al.*, 1991).

SECTION B. INTERPRETATION OF RESULTS IN RELATION TO STUDIES ON PAPILLARY MUSCLE ACTION POTENTIALS

In studies on guinea pig and rabbit papillary muscles, action potentials were unaffected by ≤ 10 μM oxybutynin and S-oxybutynin, and moderately shortened by higher concentrations of the drugs (Shuba *et* al., 1999; Jones *et* al., 2000a,b). By contrast, ≤ 10 μM terodiline lengthened ventricular action potentials, and higher concentrations either shortened (guinea pig) or lengthened (rabbit) them. An additional finding was that rate-dependent changes in rabbit ventricular action potentials were more strongly modified by terodiline than by the oxybutynin compounds (Jones *et al.*, 2000b).

The effects of terodiline, oxybutynin, and S-oxybutynin on membrane currents in guinea pig and rabbit ventricular myocytes are consistent with the foregoing results. To provide a helpful visual aid for the discussion on the correlation between changes in membrane currents and changes in ventricular action potentials, Appendix A shows figures that illustrate the effects of terodiline, oxybutynin, and S-oxybutynin on action potentials in guinea pig and rabbit papillary muscles.

(1) Guinea pig papillary muscles

Terodiline

Micromolar concentrations of terodiline inhibited $I_{Ca,L}$ and multiple K^+ currents in ventricular cells. This spectrum of action appears to be quite similar to that of micromolar quinidine in cardiac ventricular preparations (Nawrath, 1981; Hiraoka *et al.*, 1986). Depending upon the species and experimental conditions, the net effect of quinidine on the action potential duration may be a shortening, lengthening, or little change (Nawrath, 1981; Hiraoka *et al.*, 1986; Imaizumi and Giles, 1987; Salata and

Wasserstrom, 1988). In recent studies on guinea pig papillary muscles (Shuba *et al.*, 1999; Jones *et al.*, 2000b), submicromolar concentrations of terodiline induced small but significant lengthenings of the action potential that were almost certainly due to partial inhibition of I_{Kr} (I_{Kr} IC_{50} = 0.5 μM), and micromolar concentrations induced additional lengthening as expected from more complete inhibition of I_{Kr}. However, the average maximal degree of lengthening (ca. 10% with 3-10 μM drug) was considerably smaller than the average ca. 25% lengthenings recorded from guinea pig papillary muscles treated with saturating concentrations of I_{Kr}-inhibiting E4031 (Sanguinetti and Jurkiewicz, 1990; Shuba *et al.*, 1999). Based on the inhibitory profile of terodiline on ventricular membrane currents, the most likely explanation for its smaller maximal lengthening effect is that the lengthening influence related to its inhibition of I_{Kr} was partially offset by the shortening influence related to a lowering of peak $I_{Ca,L}$ ($I_{Ca,L}$ IC_{50} = 15.2 μM). In accord with this interpretation, Shuba *et* al. (1999) reported that 3 μM terodiline shortened rather than lengthened the action potential duration when muscles were pretreated with E4031.

Shuba *et* al. (1999) have shown that higher concentrations of terodiline (≥ 10 μM) strongly reduced plateau duration but also lengthened the action potential at later stages of repolarization ("triangularization" of the action potential). It seems likely that the plateau abbreviation was due to pronounced inhibition of $I_{Ca,L}$ (that was only partially counterbalanced by a lengthening influence related to near complete inhibition of I_{Kr} and partial inhibition of I_{Ks}), whereas the phase 3 lengthening was due to inhibition of $I_{Kr,tail}$, $I_{Ks,tail}$, and I_{K1}. The triangularization of the action potential induced by high terodiline concentrations is a configurational change that has also been observed in the action potential of canine Purkinje fibers exposed to high concentrations of terodiline (Pressler *et al.*, 1995).

Oxybutynin compounds.

Unlike terodiline, oxybutynin and S-oxybutynin are relatively weak and nonselective inhibitors of cardiac membrane currents. At submicromolar concentrations, neither drug significantly inhibited any of the currents examined. Their IC_{50} values were relatively high in comparison to terodiline, and, with the exception of I_{to}, were generally clustered within a relatively narrow range (11.4 to 28.7 μM for oxybutynin, and 12 to 41 μM for S-oxybutynin). This inhibitory profile correlates with the finding that low micromolar concentrations (≤ 3 μM) of oxybutynin and S-oxybutynin had a negligible effect on the configuration of action potentials in guinea pig papillary muscles (Shuba *et* al., 1999; Jones *et al.*, 2000a,b). The lowest concentration of oxybutynin that resulted in any significant change in configuration was 10 μM (7% shortening of the action potential plateau duration). This concentration of oxybutynin inhibits both $I_{Ca,L}$ and global I_K, and the shortening effect likely arose from a relatively larger of reduction of $I_{Ca,L}$ than I_K. Higher concentrations of oxybutynin selectively shortened the plateau by as much as 25% (100 μM), suggesting an accentuation of this imbalance (shortening > lengthening influence) at plateau potentials.

(2) Rabbit papillary muscles

Drug effects on the configuration of the action potential

In agreement with the results on guinea pig papillary muscles, low micromolar concentrations (≤ 3 μM) of oxybutynin and S-oxybutynin had no significant effect on action potentials in rabbit muscles (Shuba *et* al., 1999; Jones *et* al., 2000a,b). This implies that action potential shortening and lengthening influences caused by inhibitions of Ca^{2+} and K^+ currents, respectively, were in balance at these concentrations. In contrast to the oxybutynin compounds, ≤ 3 μM terodiline prolonged rabbit muscle action potentials,

suggesting relatively larger inhibition of K^+ currents by this drug. The IC_{50} values for terodiline and the oxybutynin compounds (see Table 1) support this conclusion.

Terodiline and oxybutynin have species-related effects on the action potential configuration in guinea pig and rabbit papillary muscles (Shuba *et* al., 1999; Jones *et al.*, 2000a). For example, (i) 30 μM oxybutynin, which shortened the plateau duration in guinea pig muscles by 20%, only caused a 5% shortening in rabbit muscles, and (ii) terodiline-induced lengthening of the action potential was larger and peaked at a higher drug concentration in rabbit (10 μM) than in guinea pig (1-3 μM) papillary muscles.

These interspecies differences in action potential responses are likely to be related to the interspecies differences in membrane currents responsible for action potential repolarization. For example, the relative importance of I_{Kr} versus I_{Ks} for repolarization may be much larger in rabbit than in guinea pig papillary muscles (Carmeliet, 1993). Therefore, slight preferential inhibition of I_{Kr} by oxybutynin (IC_{50} of 11.4 μM versus 28.7 μM for I_{Ks}), and marked preferential inhibition of I_{Kr} by terodiline (IC_{50} of 0.5 μM versus 26 μM for I_{Ks}), will contribute larger lengthening influences in rabbit than in guinea pig muscles.

I_{to} is an additional factor in the interspecies differences in action potential responses to the drugs. The current, which is prominent in rabbit but reportedly absent in guinea pig ventricular cells (McDonald *et* al., 1989; Carmeliet, 1993), is inhibited by both terodiline and oxybutynin. Therefore, shortening influences related to concentration-dependent inhibition of $I_{Ca,L}$ in rabbit muscles will be counterbalanced by lengthening influences related to concentration-dependent inhibition of I_{to}.

Reverse frequency-dependence

In control, non-drug-treated rabbit papillary muscles, slowing the driving rate from 1 to 0.4 Hz shortened the action potential plateau duration by an average of 23% (i.e., net outward plateau current was strongly enhanced at the slower driving rate) (Jones *et al.*, 2000b). This enhancement of net outward plateau current is likely to be due to an increase in I_{to} that outweighs other possible changes induced by slowing of the driving rate (e.g., increase in $I_{Ca,L}$, reduction in I_K: Hiraoka and Kawano, 1987; Boyett *et al.*, 1994; Ogura *et al.*, 1999).

In drug-treated rabbit papillary muscles, the rate-related abbreviation of the plateau was unaffected by 3 μM S-oxybutynin, moderately depressed by 30 μM S-oxybutynin and 3 μM terodiline, and strongly depressed by 30 μM terodiline (Jones *et al.*, 2000b). This set of results is consistent with the concentration-dependent effects of terodiline and S-oxybutynin on I_{to} (see Table 1).

Jones *et* al. (2000b) found that when control and S-oxybutynin-treated rabbit muscles were driven at 3 Hz, the duration of the plateau was only slightly shorter than the duration at 1 Hz, indicating that rate-induced shortening influences (smaller $I_{Ca,L}$, larger I_K) were almost fully offset by the lengthening influence of smaller I_{to} (weaker recovery from inactivation due to the shorter diastolic intervals). In marked contrast, the plateau duration in terodiline-treated muscles was 15% (3 μM) to 26% (30 μM) shorter at 3 Hz than at 1 Hz, suggesting involvement (at least in part) of the concentration-dependent removal of the I_{to}-related lengthening influence. A rough indication of the normal magnitude of the latter lengthening influence is that the plateau duration in I_{to}-deficient guinea pig papillary muscles shortens by 27% when the stimulation rate is increased from 1 to 3 Hz (Shuba *et al.*, 1999). These results suggest that I_{to} is a major factor in the

terodiline-induced, reverse rate-dependence in rabbit papillary muscles (expressed as accentuation of the degree of action potential lengthening at lower stimulation rates).

It is worth mentioning that although terodiline inhibition of I_{Ks} may have a bearing on the reverse frequency-dependence of action potential lengthening, I_{Ks} can be excluded as a factor in the rate-dependent results observed on rabbit papillary muscles. This reasoning is based on the following observations: (i) relative to outward K^+ currents such as I_{to} and I_{Kr}, I_{Ks} is not a dominant factor in repolarization of rabbit papillary muscles (Carmeliet, 1993), and (ii) terodiline is a much more potent inhibitor of I_{to} than I_{Ks} (IC_{50} values of 6 and 26 μM, respectively).

SECTION C. RELATION TO CLINICAL OBSERVATIONS

The adverse cardiac effects of terodiline include slowing of the heart rate, atrioventricular conduction disturbances, QT prolongation, and malignant ventricular tachycardia (torsades de pointes) (Connolly *et al.*, 1991; McLeod *et* al., 1991; Stewart *et al.*, 1992; Pressler *et al.*, 1995). To date, there have been no reports of similar cardiac complications in patients receiving oxybutynin, and Hussain *et* al. (1996) observed that there was little change in the QT intervals of elderly patients treated with low therapeutic concentrations of oxybutynin.

The first two subsections below relate the terodiline-induced effects on specific cardiac membrane currents to particular cardiotoxic side effects of the drug, focusing first on QT lengthening and torsades de pointes, and then on bradycardia and atrioventricular block. In the final subsection, the results obtained from the investigation of oxybutynin and S-oxybutynin are discussed in the context of possible implications for the clinical safety of these drugs.

(1) Terodiline, QT lengthening, and torsades de pointes

Torsades de pointes is a malignant ventricular arrhythmia associated with a wide spectrum of chemically and therapeutically unrelated drugs that are either administered for their antiarrhythmic properties (e.g., quinidine, sotalol, amiodarone) or for entirely different purposes (e.g., terfenadine, erythromycin, terodiline). A common finding with drugs that cause torsades de pointes is that they lengthen the QT interval (Zipes, 1987; Jackman *et* al., 1988; Benedict, 1993; Roden *et al.*, 1996; Woosley, 1996). The precise electrophysiological alterations responsible for the arrhythmia have not been fully characterized, but it has been postulated that bradycardia, action potential prolongation,

and early afterdepolarizations are crucial factors (Jackman *et al.*, 1988; Thomas *et al.*, 1995; Roden *et al.*, 1996).

The inhibitory profile of terodiline on several cardiac ionic currents suggest that the drug could lead to QT lengthening and torsades de pointes through both direct and indirect mechanisms. The particular ionic currents that may be involved in the development of these cardiotoxic side effects are discussed below.

Involvement of I_{Kr}

Drugs that cause QT lengthening and torsades de pointes commonly inhibit I_K in ventricular cells (Roden, 1993; Thomas, 1994; Woosley, 1996). To date, torsades de pointes has been associated with drugs that selectively block I_{Kr} over I_{Ks} (e.g., quinidine (Balser *et* al., 1991); E4031 and sotalol (Sanguinetti and Jurkiewicz, 1990; Wettwer *et* al., 1992); dofetilide (Carmeliet, 1992; Williams and Beatch, 1997); terfenadine (Ming and Nordin, 1995; Salata *et* al., 1995; Berul and Morad, 1995). Based on the results presented here, it seems certain that the primary mechanism underlying the reported QT lengthening effects for terodiline is a lengthening of the ventricular action potential due to inhibition of I_{Kr}.

Involvement of I_{Ks}

QT lengthening increases with increasing plasma concentrations of terodiline (Thomas *et* al., 1995), and concentrations as high as 9.3 μM have been measured in adversely affected patients (Connolly *et* al., 1991). In such cases, it is likely that concentration-dependent inhibition of I_{Ks} (IC_{50} 26 μM) will contribute to QT lengthening (and possible emergence of torsades de pointes). Inhibition of I_{Ks} by terodiline may also encourage the onset of torsades de pointes through an indirect

mechanism if, as in guinea pig heart, a reduction of I_{Ks} in the sinoatrial node (Guo *et al.*, 1997) slows the pacemaker rate (Irisawa *et al.*, 1993).

Involvement of I_{K1}

Administration of the antihistamine terfenadine can cause QT lengthening and torsades de pointes (Monahan *et al.*, 1990; Woosley, 1996), and it has been suggested that, aside from block of I_K, block of ventricular I_{K1} may be an important element in this adverse reaction (Berul and Morad, 1995). In addition, a number of the predisposing factors frequently associated with acquired QT lengthening may be linked to suppression of I_{K1}; these factors include co-prescription of I_{K1}-inhibiting drugs such as quinidine (Hiraoka *et al.*, 1986), heart disease (Lue and Boyden, 1992; Beuckelmann *et al.*, 1993; Kääb *et al.*, 1996; McIntosh *et al.*, 1998), and hypokalemia (e.g., Harvey and Ten Eick, 1988). These considerations, and the relatively low I_{K1} IC_{50} of 7 μM, suggest that inhibition of I_{K1} is likely to be a factor in the concentration-dependent cardiotoxicity (Thomas *et al.*, 1995) of terodiline.

Involvement of I_{to}

Transient outward K^+ current contributes to early repolarization in the human myocardium (Beuckelmann *et al.*, 1993). Based on the inhibitory potency of terodiline for I_{to} (IC_{50} 6 μM), it is likely that inhibition of this current was a factor in the reported lengthening of the QT interval in adversely affected patients being treated with the drug.

(2) Bradycardia and atrioventricular block

In clinical studies on the treatment of urinary incontinence in elderly patients, terodiline was reported to cause bradycardia and atrioventricular conduction disturbances that appeared to be directly linked to high plasma concentrations of the drug (Hallén *et* al., 1989; Connolly *et* al., 1991). Based on the present results, drug-induced inhibition of $I_{Ca,L}$ and I_{Kr} are likely to be responsible for these adverse effects.

Involvement of $I_{Ca,L}$

$I_{Ca,L}$ is essential for normal pacemaking and nodal conduction (Kokubun *et al.*, 1982; Hagiwara *et* al., 1988; Hancox and Levi, 1994). Inhibition of $I_{Ca,L}$ by terodiline (IC_{50} 15.2 μM), may well be a contributing factor in the slowing of heart rate and nodal conduction observed in patients with high plasma concentrations of this drug (up to 9.3 μM: Connolly *et* al., 1991; Stewart *et* al., 1992).

Involvement of I_{Kr}

Inhibition of I_{Kr} by terodiline may also contribute to the bradycardia and atrioventricular block in both a direct and indirect manner if, as in rabbit nodal tissue (Shibasaki, 1987; Verheijck *et al.*, 1995; Ono and Ito, 1995; Lei and Brown, 1995), partial inhibition of I_{Kr} in human nodal tissues lengthens the action potential, slows the pacemaker rate, and shifts the maximum diastolic potential to more positive potentials. Since the inhibition of $I_{Ca,L}$ by terodiline is voltage-dependent, I_{Kr}-related depolarization of diastolic potential could potentiate terodiline-induced inhibition of $I_{Ca,L}$ and consequent disturbance of nodal pacing and conduction. In turn, the bradycardia may be an important element in QT lengthening and ventricular tachyarrhythmias (Stewart *et al.*, 1992; Thomas *et al.*, 1995).

(3) Implications of the results for the clinical safety of oxybutynin compounds

The results of this study have identified I_{Kr} as a target for inhibition by both oxybutynin and S-oxybutynin. To evaluate the likelihood of whether this inhibition will lead to adverse cardiac effects in patients, the action of the drugs should be placed in proper context (i.e., in relation to the therapeutic plasma concentration of the drugs).

Oxybutynin is administered orally (up to four 5-mg tablets daily, or one 10- to 30-mg sustained action tablet daily) (Holmes *et* al., 1989; Anderson *et* al., 1999) or intravesically (Yarker *et* al., 1995; Åmark *et* al., 1998; Buyse *et* al., 1998a,b). Although there is significant interindividual variation in the C_{max} (peak plasma concentration) (Hughes *et al.*, 1992), average values near 0.01 μM have been reported in recent studies in adults (Lukkari *et al.*, 1997a,b), and average values of 0.07 μM (oral) and 0.15 μM (intravesical) have been reported in children (Buyse *et al.*, 1998b) A recent pharmacokinetic study on human volunteers (Koch *et al.*, 1998) indicated that the C_{max} of orally-administered S-oxybutynin was similar to that of oxybutynin.

Taking the C_{max} of the drugs in adults as 0.01 μM, the ratio of C_{max} to the IC_{50} for I_{Kr} (ca. 12 μM) is approximately 0.001. A similar calculation for terodiline yields a C_{max} / IC_{50} (I_{Kr}) value of 2 (C_{max} ~ 1 μM (Connolly *et al.*, 1991); IC_{50} (I_{Kr}) = 0.5 μM). For comparitive purposes, the ratio for chlorpheniramine, a first-generation antihistamine with a good cardiac safety record, appears to be about 0.02 (C_{max} of 0.03 μM (Yasuda *et al.*, 1995); IC_{50} for I_{Kr} of 1.6 μM (Salata *et al.*, 1995)), whereas the approximate ratio for the second-generation antihistamine, torsades-inducing terfenadine, is a much larger 0.4 (C_{max} ~ 0.02 μM (Woosley *et al.*, 1993); IC_{50} (I_{Kr}) ~ 0.05 μM (Salata *et al.*, 1995)).

The majority of patients taking incontinence drugs are elderly, making it all the more important that drugs used to manage incontinence have a low C_{max}-to-IC_{50} (I_{Kr}) ratio. The caution is not connected to age-related higher C_{max} values, but to the fact that

many of the patients taking incontinence drugs may have aging-related cardiac hypertrophy (Svanborg, 1997) and, consequently, a reduced density of repolarizing K^+ current in the heart (Beuckelmann *et al.*, 1993; McIntosh *et al.*, 1998).

In previous studies by Jones *et al.* (2000a,b), concentrations of oxybutynin and S-oxybutynin ($\leq$ 10 μM) have been reported to have negligible effects on the maximal upstroke velocity ($\dot{V}_{max}$) of action potentials in guinea pig and rabbit papillary muscles. As a result, there is little reason to suspect that concentrations of these compounds in the plasma of patients would affect cardiac impulse generation and conduction that is dependent on Na^+ channel activity. Similarly, $I_{Ca,L}$ was relatively insensitive to the drugs (IC_{50} ~ 17 μM), suggesting that cardiac function that is sensitive to altered Ca^{2+} channel activity (e.g., sinus node pacemaker activity, atrioventricular transmission, and muscle cell contraction) is unlikely to be compromised.

V. APPENDIX

The contribution of each of the five membrane currents examined in this study during the cardiac action potential are illustrated in Figure 41. The figure is a variation of a figure from Snyders (1999).

The effects of terodiline, oxybutynin, and S-oxybutynin on action potentials in guinea pig and rabbit papillary muscles are depicted in Figures 42 and 43. The data are taken from Jones *et al.* (2000a,b).

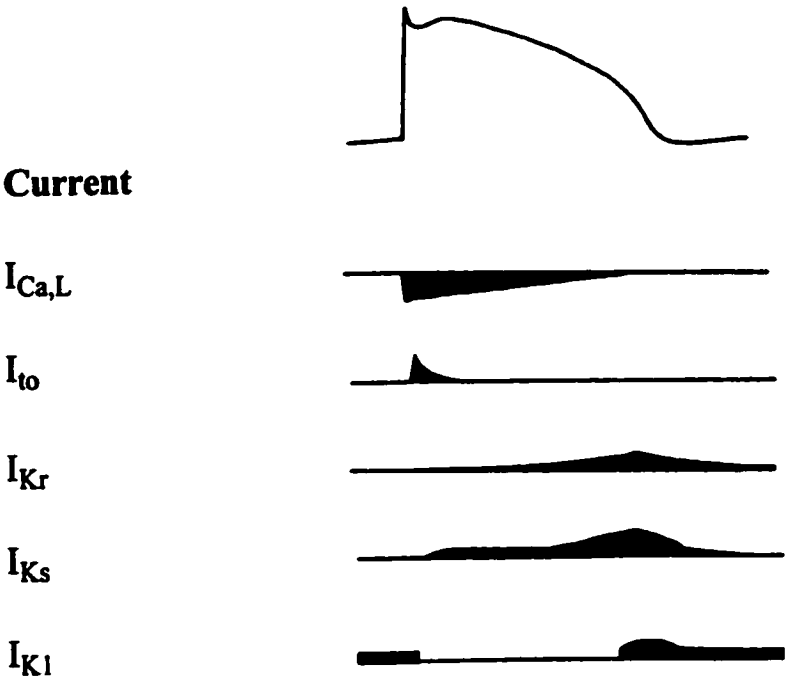


Figure 41. Schematic indication of the time course of five membrane currents that govern repolarization of the cardiac action potential; depolarizing inward current (downward) and repolarizing outward currents (upward). Abbreviations: see text.

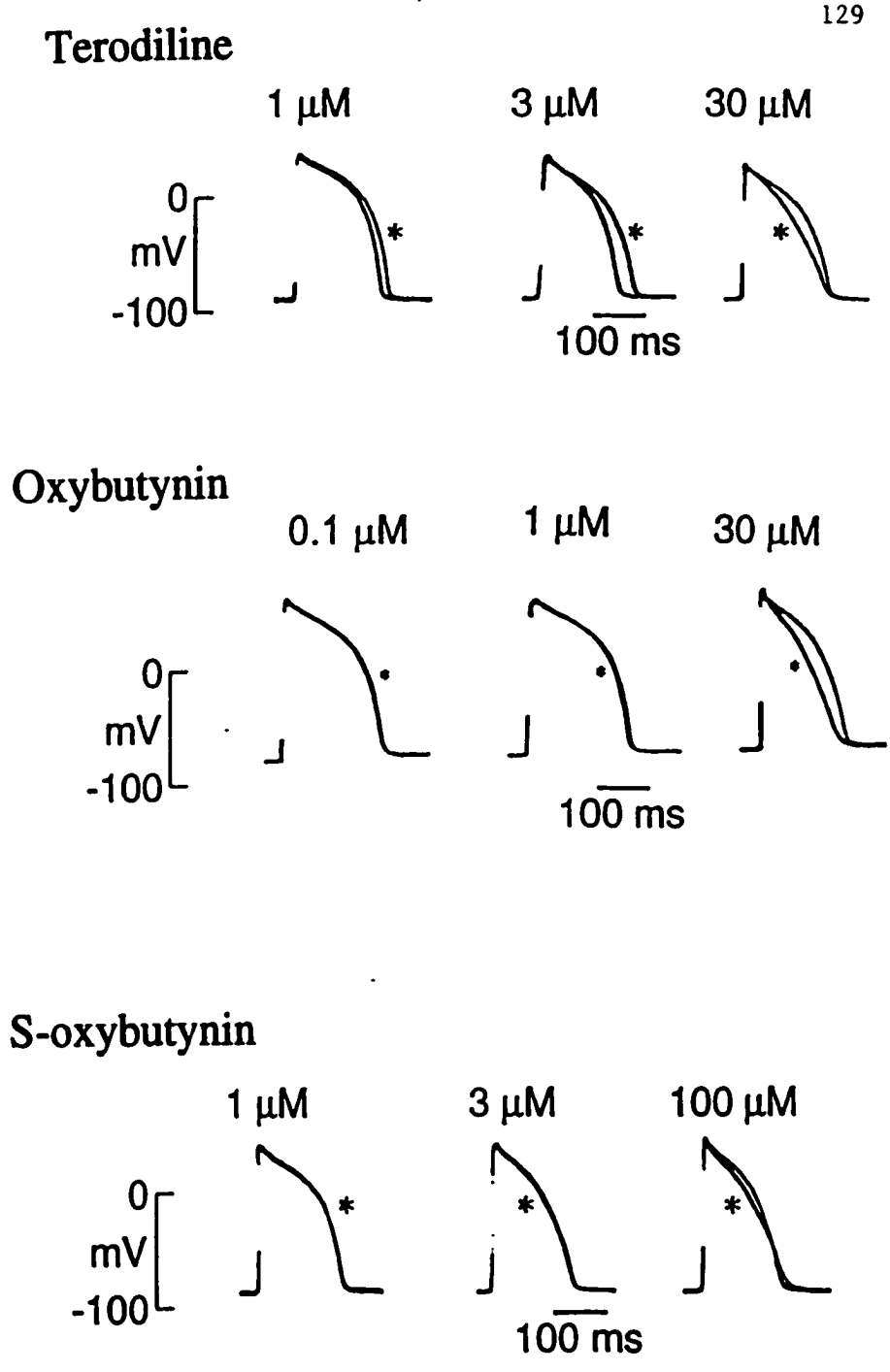


Figure 42. Effects of terodiline, oxybutynin, and S-oxybutynin on action potentials in guinea pig papillary muscles driven at 1 Hz. Records obtained from muscles before and 30 min after each addition of drug have been superimposed. Action potentials recorded in the presence of the drugs are indicated by an asterisk.

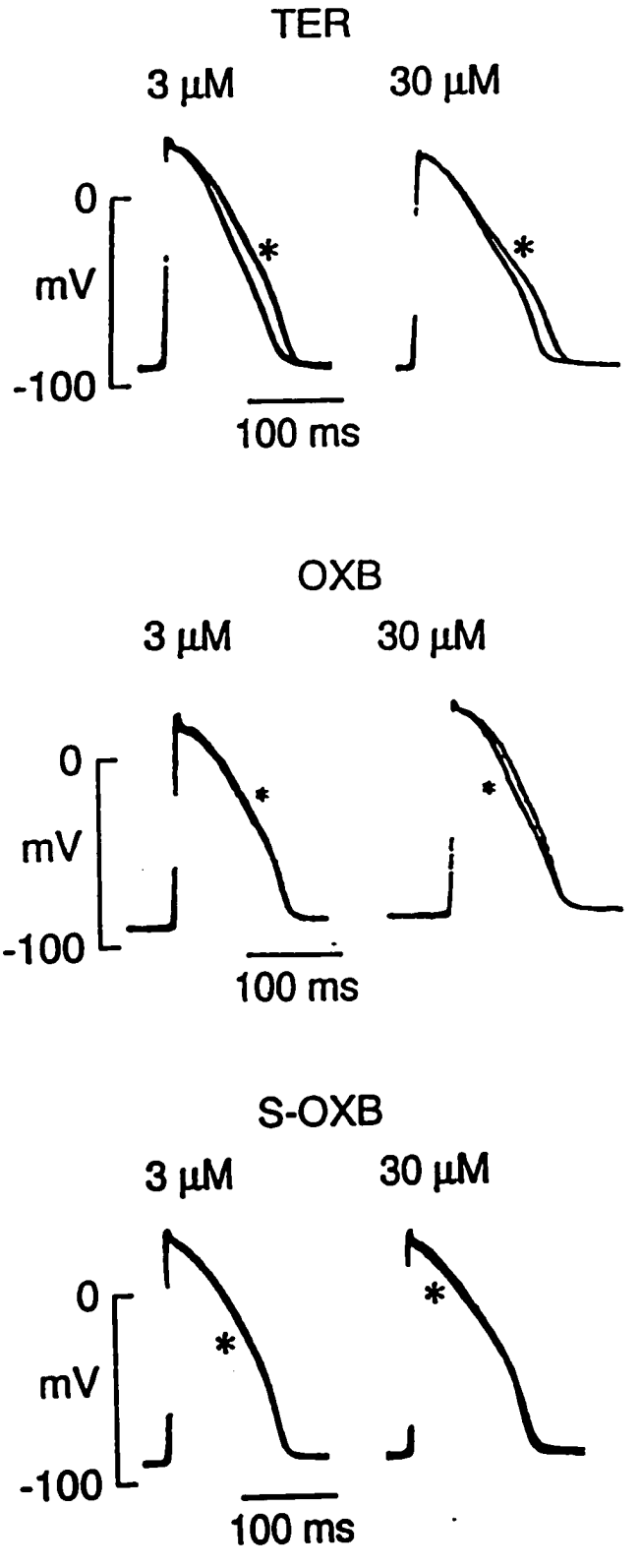


Figure 43. Effects of terodiline, oxybutynin, and S-oxybutynin on action potentials in rabbit papillary muscles driven at 1 Hz. Records obtained from muscles before and 30 min after addition of 3 or 30 μM drug (asterisk) have been superimposed.

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