MODULATION OF CARDIAC MEMBRANE CURRENTS

BY PHORBOL ESTERS

by

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Submitted in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

at

Dalhousie University

Halifax, Nova Scotia, Canada

December, 1995

Canada

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ABSTRACT

Protein kinase C (PKC) and protein kinase A (PKA) are important regulators of cardiac cell function. I have used PKC-activating phorbol 12-myristate 13-acetate (PMA) and PKA-activating forskolin (FSK), to investigate the regulation of membrane channels in guinea pig ventricular myocytes.

The whole-cell patch-clamp technique was used to measure currents through Cl$^-$ channels (I_{Cl}), delayed-rectifying K$^+$ channels (I_K), inward-rectifying K$^+$ channels (I_{K1}), and L-type Ca^{2+} channels ($I_{Ca,L}$, $I_{Na,L}$). In myocytes dialyzed with standard pCa 9 solutions, the active phorbol ester PMA had no effect on I_{K1}, moderately increased I_K and I_{Cl} in a concentration-dependent manner (EC$_{50}$ 4 and 10 nM, respectively), and frequently depressed $I_{Ca,L}$.

I_{Cl} activated by PMA was time-independent, displayed marked outward rectification when the dialysate contained 30 mM Cl$^-$, and became almost linear when dialysate Cl$^-$ concentration was increased to 140 mM. The current was strongly depressed by the Cl$^-$ channel blocker, anthracene-9-carboxylic acid.

Stimulation of I_{Cl} and I_K by PMA was suppressed when myocytes were pretreated with PKC inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) or dialyzed with pCa ~11 solution. In addition, stimulation by PMA was not duplicated by the inactive phorbol esters, 4α-phorbol 12,13-didecanoate (αPDD) and 4α-phorbol (αPHR). These results strongly suggest that the stimulatory effects of PMA on Cl$^-$ and K$^+$ channels are mediated by PKC.

In contrast to the foregoing, the depression of $I_{Ca,L}$ induced by PMA (often co-monitored with I_K in the same cell) occurred ~3 times faster than I_K stimulation, and was unaffected by the two interventions that inhibited I_K stimulation (pretreatment with H-7 and pCa ~11 dialysate). Inactive phorbol ester αPDD, but not αPHR, had PMA-like effects on $I_{Ca,L}$. Similar inhibitory effects of phorbol esters were also observed when Na$^+$ was used as a charge-carrier for L-type current. These results suggest that phorbol esters depress Ca^{2+} channel currents by a PKC-independent action.

A final objective was to investigate the effects of co-application of maximally-effective concentrations of PMA and FSK on the activation of I_{Cl} and stimulation of I_K. Mutual occlusionary effects of these agents were not detected; rather, there were hints of a synergistic activation of I_{Cl} by FSK in PMA-treated myocytes, and mutually additive effects of PMA and FSK on I_K.

LIST OF ABBREVIATIONS AND SYMBOLS

9-AC	anthracene-9-carboxylic acid
A	ampere
A/D	analog-to-digital
Ag-AgCl	silver-silver chloride
ANOVA	one-way analysis of variance
ATP	adenosine triphosphate
αPDD	4α-phorbol 12,13-didecanoate
αPHR	4α-phorbol
BAPTA	1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid
cAMP	adenosine 3',5'-cyclic monophosphate
CFTR	cystic fibrosis transmembrane conductance regulator
DIDS	4,4'-diisothiocyanostilbene-2,2'-disulfonic acid
DNDS	4,4'-dinitrostilbene-2,2'-disulfonic acid
DMSO	dimethyl sulfoxide
E_{max}	maximal response
E_{rev}	reversal potential
E_X	equilibrium potential for an ion X
EC_{50}	concentration of an agonist that produces 50% of the maximal response
EGTA	ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
F	farad
FSK	forskolin
g	gram
g	chord conductance
GHK	Goldman-Hodgkin-Katz

G protein	guanine-nucleotide binding protein
h	hour
H-7	1-(5-isoquinolinesulfonyl)-2-methylpiperazine
H-8	N-(2-[methylamino]ethyl)-5-isoquinolinesulfonamide
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Hz	hertz
i	single-channel current
I	whole-cell current
IBMX	3-isobutyl-1-methylxanthine
$I_{Ca,L}$	L-type calcium current
$I_{Ca,T}$	T-type calcium current
I_{Cl}	chloride current
I_K	delayed-rectifying potassium current
I_{K1}	inward-rectifying potassium current
$I_{Na,L}$	sodium current through L-type calcium channels
ISO	isoproterenol
i-V	single-channel current-voltage
I-V	whole-cell current-voltage
k	slope factor
K_1 channels	inward-rectifying potassium channels
M	moles per liter
m	meter
min	minute
n	Hill coefficient
n	number of experiments
p	probability (significance level in a statistical test)
PDBu	phorbol 12,13-dibutyrate

pCa	negative logarithm of the calcium ion concentration
pH	negative logarithm of the hydrogen ion concentration
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
P_o	single-channel open probability
Rp-cAMPS	Rp-adenosine 3',5'-cyclic monophosphothioate
S	siemens
s	second
S.E.M.	standard error of the mean
SITS	4-acetamido-4'-isothiocyanostilbene-2,2'disulfonic acid
TTX	tetrodotoxin
V	volt
V_{half}	half-maximum potential
V_{peak}	potential at which whole-cell current amplitude is maximum
[X]	concentration of an ion X
$[X]_i$	intracellular concentration of an ion X
$[X]_o$	extracellular concentration of an ion X
~	approximately
°C	degree Celsius
Ω	ohm
%	per cent

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, Dr. Terence F. McDonald for his advice, guidance, and encouragement, and also for his patience throughout the course of my study.

I am indebted to Ms. Lesya Shuba for contributions to the chloride current data reported here, and to Drs. Dieter Pelzer, Siegried Pelzer, Yaroslav Shuba, Kyoichi Ono, and Toshitsugu Ogura for invaluable advice and assistance.

I would like to thank Mrs. Jean Crozsman, Mr. Darren Cole, Mr. Brian Hoyt, and Mr. Bob Potter for their excellent technical assistance.

I would also like to extend thanks to my Supervisory Committee members (Drs. Alan Wong and Nik Morgunov) for their guidance, with special thanks to the past Graduate Student Coordinator Dr. Paul Murphy for his support.

Finally, I am especially grateful to my wife, Junko, for her constant encouragement, support, and understanding throughout this endeavour.

I. INTRODUCTION

A. BRIEF DESCRIPTION OF I_{Cl}, I_K, I_{K1}, AND $I_{Ca,L}$ AND THEIR ROLES IN THE CARDIAC VENTRICULAR ACTION POTENTIAL

1. I_{Cl} and its role in the cardiac ventricular action potential

The initial reports on protein kinase A (PKA)-activated Cl^- current (I_{Cl}) in guinea pig and rabbit ventricular myocytes (Harvey and Hume, 1989; Bahinski et al., 1989) have been followed by descriptions of several types of cardiac I_{Cl}. These include Ca^{2+}-activated I_{Cl} (Zygmunt and Gibbons, 1991, 1992; Zygmunt, 1994), I_{Cl} activated by protein kinase C (PKC) (Walsh, 1991; Walsh and Long, 1994; Zhang et al., 1994a), I_{Cl} activated by swelling (Duan et al., 1992; Hagiwara et al., 1992; Sorota, 1992; Tseng, 1992; Zhang et al., 1993; Vandenberg et al., 1994; Shuba et al, 1995), and I_{Cl} activated by purinergic stimulation (Matsuura and Ehara, 1992). I_{Cl} appears to be very small under basal conditions in guinea pig ventricular myocytes (e.g. Gadsby et al., 1995), but detectable (10's–100's pA per cell) in rabbit ventricular myocytes (Duan et al., 1992). PKA-activated I_{Cl} is the best studied of these Cl^- currents, and it strongly resembles the current that is transported by cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels in epithelial cells (Ehara and Ishihara, 1990; Nagel et al., 1992; Ehara and Matsuura, 1993; for review, see Gadsby et al., 1995; also see Section I.B.1. below). When activated by PKA, whole-cell I_{Cl} is time-independent, and its single-channel open probability (P_o) is approximately voltage-independent.

Whole-cell current-voltage (I-V) relationships of PKA-activated I_{Cl} are nearly linear with approximately symmetrical ~150 mM Cl^-, but rectify in the

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outward direction with physiological [Cl^-] gradients (~150 mM external and 20–30 mM internal) (Bahinski et al., 1989; Harvey and Hume, 1989; Matsuoka et al., 1990). The outward rectification can be well described by the Goldman-Hodgkin-Katz (GHK) current equation (Hwang et al., 1992b, 1993; Overholt et al., 1993). However, Overholt et al. (1993) have suggested that a one-site, two-barrier model based on Eyring rate theory can also account for the outward rectification with symmetrical low [Cl^-]. In agreement with whole-cell I_{Cl} data from cardiac myocytes, single-channel current-voltage (i-V) relationships are approximately linear (Nagel et al., 1992) or slightly outwardly rectified (Ehara and Matsuura, 1993) with nearly symmetrical high [Cl^-], and show outward rectification with roughly physiological low internal [Cl^-] (Ehara and Ishihara, 1990; Nagel et al., 1992). The unitary conductance of single cardiac Cl^- channels is ~12 pS in roughly symmetrical ~150 mM Cl^- conditions at 25–35°C (Ehara and Ishiha ', 1990; Nagel et al., 1992; Ehara and Matsuura, 1993).

The relative permeability of Cl^- channels to various anions can be estimated from shifts of the reversal potential (E_{rev}) of whole-cell I_{Cl} when extracellular Cl^- is replaced by test anions; the sequence for cardiac CFTR is $NO_3^- > Br^- \geq Cl^- \geq I^- >$ isethionate$^- >$ glutamate$^-$ (Overholt et al., 1993). The corresponding conductance sequence, estimated from the slope conductance of the outward currents, is $NO_3^- = Cl^- >$ glutamate$^- = Br^- \geq$ isethionate$^- > I^-$ (Overholt et al., 1993).

The stilbene derivatives DNDS, DIDS, and SITS, are well-known inhibitors of Cl^- transporters that also block a variety of Cl^- channels (Gadsby et al., 1995). In cardiac cells, relatively high concentrations of DNDS (Bahinski et al., 1989; Matsuoka et al., 1990; Takano and Noma, 1992), DIDS (Hwang et al., 1992a), and SITS (Bahinski et al., 1989) have been shown to block I_{Cl} under certain conditions (Hwang et al., 1992a). However, DNDS was found to have no

direct effect on cardiac CFTR channels, and SITS and DIDS can actually increase I_{Cl} via effects on the β-adrenoceptor (Harvey, 1993). Anthracene-9-carboxylic acid (9-AC), an effective inhibitor of I_{Cl} in skeletal muscle, reduces isoproterenol (ISO)-activated I_{Cl} in cardiac myocytes (Levesque et al., 1993) in a voltage-independent manner (Harvey et al., 1990). Sulfonylureas such as ATP-sensitive-K^+-channel blocker glibenclamide may also inhibit PKA-activated I_{Cl} (Sturgess et al., 1985; Tominaga et al., 1995).

Role in the cardiac ventricular action potential

Because CFTR channel gating is approximately voltage-independent, and the equilibrium potential for Cl^- (E_{Cl}) is normally around −50 mV (Vaughan-Jones, 1982; Désilets and Baumgarten, 1986), an outward I_{Cl} flows throughout the plateau phase of the action potential. This outward I_{Cl} will shorten action potential duration (although the shortening is likely to be significant only after receptor-stimulation, swelling, or other perturbation). There is also a constant inward flow of I_{Cl} between action potentials, and this constitutes a depolarizing influence on the resting membrane potential. A shortening of the action potential duration upon activation of I_{Cl} by β-adrenergic stimulation has been demonstrated (Harvey and Hume, 1989; Harvey et al., 1990; Takano and Noma, 1992; Tominaga et al., 1995), as has a depolarization of the resting membrane potential (Harvey et al., 1990; unpublished observation). It is not clear whether the action potential shortening caused by activated I_{Cl} is antiarrhythmic or arrhythmogenic (cf. Harvey et al., 1990; Ackerman and Clapham, 1993; Levesque et al., 1993). However, an increase in the inward flow of I_{Cl} during diastole is clearly arrhythmogenic.

2. I_K and its role in the cardiac ventricular action potential

The delayed-rectifying K^+ current (I_K) is one of several K^+ currents found in heart (for review, see Sanguinetti and Jurkiewics, 1994). At positive potentials, the current is activated with a halftime approaching several hundred milliseconds; after repolarization to negative potentials, the deactivation of K^+ channels is indicated by a slowly decaying tail current (McDonald and Trautwein, 1978). The activation of I_K follows a sigmoidal time course (Matsuura et al., 1987; Sanguinetti and Jurkiewics, 1990), and the deactivation time course of the tail current has been described by a single-exponential (Matsuura et al., 1987; Walsh and Kass, 1988; Walsh et al., 1989) or a double-exponential (Matsuura et al., 1987; Yazawa and Kameyama, 1990; Tohse et al., 1992) function with time constants of 100-500 ms.

The voltage-dependence of I_K activation can be fitted with a Boltzmann distribution equation, $y = 1 / (1 + \exp (V_{half} - V) / k)$, where V is the test potential, V_{half} is the half-maximum potential, and k is the slope factor. The relation rises sigmoidally from 0 to 1 over the range -40 to +50 mV with V_{half} ranging from -10 to +20 mV and k of ~12 mV (Matsuura et al., 1987; Sanguinetti and Jurkiewics, 1990; Yazawa and Kameyama, 1990; Tohse et al., 1992). The fully-activated I_K-voltage relation displays inward rectification and no inactivation (McDonald and Trautwein, 1978; Hume et al., 1986; Matsuura et al., 1987: Clay et al., 1988).

The permeability sequence for the K^+ channel, determined from the shifts of E_{rev}, is $K^+ = Rb^+ > NH_4^+ = Cs^+ > Na^+$ (Hadley and Hume, 1990), and the relative permeability (P_{Na}/P_K) is ~0.015 (Matsuura et al., 1987; Hadley and Hume, 1990).

Recent studies indicate that I_K is composed of two distinct K^+ current components, rapid $I_{K,r}$ and slow $I_{K,s}$ (Sanguinetti and Jurkiewics, 1990; Horie et al., 1990; Chinn, 1993; Liu and Antzelevitch, 1995; for review, see Sanguinetti and Jurkiewics, 1994). $I_{K,r}$ displays fast activation and prominent inward rectification (Sanguinetti and Jurkiewics, 1990; Horie et al., 1990; Ito and Ono, 1995; Liu and Antzelevitch, 1995), and is sensitive to blockers such as E-4031 or sotalol (Sanguinetti and Jurkiewics, 1990; Komeichi et al., 1990; Chinn, 1993; Ito and Ono, 1995; Liu and Antzelevitch, 1995). $I_{K,s}$ is similar to the classically described I_K and is characterized by slower activation kinetics (Sanguinetti and Jurkiewics, 1990). The single-channel conductance for $I_{K,r}$ is 10-14 pS, while that for $I_{K,s}$ is <1-3 pS with ~150 mM $[K^+]_o$ (Horie et al., 1990; Walsh et al., 1991; Veldkamp et al., 1993; Veldkamp et al., 1994; Ito and Ono, 1995). However, the relative magnitude of the two components in guinea pig ventricular myocytes is $I_{K,r} / I_{K,s} \approx 0.1$ (Sanguinetti and Jurkiewics, 1990; Chinn, 1993; unpublished observation).

Role in the cardiac ventricular action potential

I_K is thought to play an important role in determining the shape and duration of the action potential (McDonald and Trautwein, 1978; Matsuura et al., 1987; Sanguinetti and Jurkiewicz, 1990; Clay et al., 1995; for reviews, see Pelzer and Trautwein, 1987; Carmeliet, 1993). An increase in I_K shortens the action potential duration, and will therefore restrict the influx of Ca^{2+} via L-type channels and reverse-mode Na^+-Ca^{2+} exchange. In sinoatrial node cells, I_K plays a crucial role in determining the time course of the pacemaker depolarization (Nakayama et al., 1984; Anunonwo et al., 1992; Ono and Ito, 1995; for review, see Irisawa et al., 1993).

I_K can be separated into two components, $I_{K,r}$ and $I_{K,s}$ (see above). Their respective roles and relative importance in plateau repolarization and in determining action potential duration remain to be fully determined. Computer simulation of the ventricular action potential suggests that $I_{K,s}$ is a major outward current during the plateau repolarization, and that a specific block of either $I_{K,r}$ or $I_{K,s}$ can effectively prolong action potential duration (Sanguinetti and Jurkiewicsz, 1990; Komeichi et al., 1990; Zeng et al., 1995). The importance of one component versus the other is clearly species- and tissue-dependent (c.f. Sanguinetti and Jurkiewicsz, 1994; Liu and Antzelevitch, 1995).

3. I_{K1} and its role in the cardiac ventricular action potential

Inward-rectifying K^+ (K_1) channels are found in both cardiac and non-cardiac cells (for review, see Vandenberg, 1994). These channels conduct inward current more easily than outward current, and the I-V relationships are therefore inwardly-rectifying (e.g. Noma et al., 1984; Sakmann and Trube, 1984a; Hume and Uehara, 1985; Tourneur et al., 1987; Giles and Imaizumi, 1988). Single-channel i-V relationships also show strong inward rectification (Kameyama et al., 1983; Sakmann and Trube, 1984a; Trube and Hescheler, 1984; Kurachi, 1985) which is thought to be primarily due to block of the K_1 channels by internal Mg^{2+} and Ca^{2+} (Matsuda et al., 1987; Vandenberg, 1987; Matsuda, 1988).

The slope conductance, determined from the linear portion of the whole-cell I-V relationship at potentials negative to E_{rev}, increases approximately proportional to the square root of $[K^+]_o$ (Sakmann and Trube, 1984a; Harvey and Ten Eick, 1988). The single-channel conductance in cardiac cells is about 25 to 32 pS in approximately symmetrical 150 mM $[K^+]$ at room temperature (e.g.

Noma et al., 1984; Sakmann and Trube, 1984a; Hume and Uehara, 1985; Payet et al., 1985; Josephson and Brown, 1986; Chen et al., 1991), and depends roughly on the square root of $[K^+]_o$ (Kameyama et al., 1983; Sakmann and Trube, 1984a; Payet et al., 1985; Josephson and Brown, 1986; Matsuda, 1988, 1991; Chen et al., 1991).

Although I_{K1} was initially considered to be a time-independent current, it is now clear that it undergoes time-dependent activation, deactivation, and inactivation; activation kinetics are in the millisecond range, inactivation is much slower, and both are dependent on the equilibrium potential for K^+ (E_K) (Kameyama et al., 1983; Sakmann and Trube, 1984b; Kurachi, 1985; Josephson and Brown, 1986; Biermans et al., 1987; Tourneur et al., 1987; Harvey and Ten Eick, 1988 ; Saigusa and Matsuda, 1988; Cohen et al., 1989; Ishihara et al., 1989; Oliva et al., 1990).

I_{K1} is blocked in a time- and voltage-dependent manner by monovalent cations such as Cs^+ and Na^+ (Kameyama et al., 1983; Sakmann and Trube, 1984b; Biermans et al., 1987; Tourneur et al., 1987; Harvey and Ten Eick, 1988, 1989b; Matsuda et al., 1989), and by divalent cations such as Ba^{2+} and Sr^{2+} (Bechem et al., 1983; Kameyama et al., 1983; Sakmann and Trube, 1984b; Tourneur et al., 1987; Chen et al, 1991; Mitra and Morad, 1991).

Role in the cardiac ventricular action potential

I_{K1} plays an important role in the cardiac action potential. The relatively large conductance of the channels at membrane potentials around E_K helps to keep the resting membrane potential close to E_K, and contribute to repolarization of the action potential (Shimoni et al., 1992; Ibarra et al., 1991). Moderate block of I_{K1} by low concentrations of Ba^{2+} slows the final phase of

repolarization and produces a small depolarization of the resting potential in cardiac ventricular myocytes (e.g. Giles and Imaizumi, 1988).

4. $I_{Ca,L}$ and its role in the cardiac ventricular action potential

Two types of Ca^{2+} channels exist in cardiac cells (T-type and L-type; see Bean, 1985; Nilius et al., 1985; for review, see McDonald et al., 1994); the present study deals exclusively with L-type channels.

Step depolarization from relatively negative potentials triggers the opening (activation) of L-type Ca^{2+} channels and allows an influx of Ca^{2+} down its concentration gradient. After reaching a peak, $I_{Ca,L}$ inactivates with a distinctly slower time course. The activation time course of $I_{Ca,L}$ is sigmoidal (Lee and Tsien, 1982, 1984; Pelzer et al., 1986; Richard et al., 1993), and speeds up with more positive voltage (McDonald et al., 1986; Bean and Ríos, 1989; Richard et al., 1993). The time to peak at 0 mV is 2-4 ms at 35-37°C (e.g. McDonald et al., 1986). The voltage dependence of activation of $I_{Ca,L}$ (often labelled d_∞) is generally described by a Boltzmann equation. The relation rises sigmoidally from 0 to 1 over the range from -40 to +10 mV with V_{half} near -15 mV and k of ~7 mV (Isenberg and Klöckner, 1982; Osaka and Joyner, 1991; Tseng et al., 1987). The $I-V$ relation is bell-shaped, with a maximum near 0 mV in myocytes superfused with solution that contains millimolar divalent cation charge-carrier.

The inactivation time course of $I_{Ca,L}$ near V_{peak} (potential at which $I_{Ca,L}$ amplitude is maximum) in cardiac cells has been fitted with single-exponential (Campbell et al., 1988; Tseng et al., 1987), double-exponential (Isenberg and Klöckner, 1982; Kass and Sanguinette, 1984; Imoto et al., 1985; Tseng et al., 1987; Richard et al., 1993), or other functions (Bean, 1985; Bechem and Pott, 1985;

McDonald et al., 1986). Where $I_{Ca,L}$ in guinea pig ventricular myocytes has been described by two exponentials, the time constants are 3-7 ms and 30-80 ms near 35°C. The voltage dependence of inactivation time constants is U-shaped with a minimum near V_{peak} and several-fold larger values at $V_{peak} \pm 30$ mV (Isenberg and Klöckner, 1982; Josephson et al., 1984; Iijima et al., 1985; Uehara and Hume, 1985; Tseng et al., 1987; Campbell et al., 1988). Inactivation 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; Osaka and Joyner, 1991). In large part, this is probably due to the removal of a strong voltage-dependent inactivation related to the influx of Ca^{2+} (for reviews see Pelzer et al., 1992; McDonald et al., 1994).

The voltage dependence of steady-state inactivation of $I_{Ca,L}$ (often labelled f_∞) is usually sigmoidal and described by a Boltzmann function. In cardiac myocytes, V_{half} is around -30 mV and k ~7 mV (Isenberg and Klöckner, 1982; Josephson et al., 1984; Tseng et al., 1987; Campbell et al., 1988; Hirano et al., 1989a).

Depolarization-induced inactivation of Ca^{2+} channel can be removed by repolarizing the membrane to negative potentials (i.e. reactivation). The time course of $I_{Ca,L}$ recovery in cardiac cells has been described as single-exponential, double-exponential, oscillatory, or sigmoidal (McDonald et al., 1994). Reactivation is accelerated at more negative potentials with a half-time near 100 ms at -80 to -100 mV and 200-400 ms near -50 mV (Fischmeister and Hartzell, 1986; Hardly and Hume, 1987, 1988; Campbell et al., 1988; Hirano et al., 1992; Wetzel et al., 1993).

Single L-type Ca^{2+} channels have a unitary conductance of ~25 pS with 90-110 mM $[Ba^{2+}]_o$; the i-V relation is essentially ohmic over most of the

potential range, with gradual inward rectification as the potential approaches E_{rev} (Cavalié et al., 1983; Pelzer et al., 1992).

Role in the cardiac ventricular action potential

$I_{Ca,L}$ plays an important role in determining action potential duration in ventricular cells. An increase in $I_{Ca,L}$ lengthens the action potential plateau phase, whereas a decrease depresses plateau amplitude and duration (Reuter, 1983; Carmeliet, 1993). Ca^{2+} ions flowing inward through L-type Ca^{2+} channels are essential for excitation-contraction coupling as they trigger the release of Ca^{2+} from the sarcoplasmic reticulum, and also fill the stores for subsequent release; thus, a stimulation of $I_{Ca,L}$ causes positive inotropy, whereas an inhibition causes negative inotropy (for review, see Shuba and McDonald, 1995).

B.   STRUCTURE AND REGULATION OF CARDIAC CL^-, K^+, AND CA^{2+} CHANNELS

1.   CFTR Cl^- channels

CFTR Cl^- channels are composed of two similar subunits, each comprising six putative transmembrane stretches (Riordan et al., 1989; Gadsby et al., 1995). On the intracellular side, there are two nucleotide-binding domains attached to each subunit, and these are the presumed sites of the ATP hydrolysis; a large intracellular regulatory domain links the subunits, and contains multiple sites for phosphorylation by PKA and PKC.

In the heart, stimulation of β-adrenoceptors results in the activation of adenylate cyclase and the PKA pathway, leading to modulation of the activity of a number of ion channels (Hartzell, 1988) including CFTR Cl⁻ channels (Gadsby et al., 1995). This is confirmed by the direct activation of channels in excised inside-out patches upon application of MgATP and the catalytic subunit of PKA (Nagel et al., 1992). A property that sets Cl⁻ channels apart from other PKA-regulated channels is that I_{Cl} (in most types of myocytes) is not evident before PKA stimulation (Gadsby et al., 1995), whereas there is a substantial basal $I_{Ca,L}$ (Kameyama et al., 1986; Parsons et al., 1991) and basal I_K (Duchatelle-Gourdon et al., 1991). Recent evidence suggests that phosphorylation alters the conformation of the regulatory domain of CFTR Cl⁻ channels (Dulhanty and Riordan, 1994), and thereby relieves occlusion of the channel. Following this phosphorylation by PKA, there is a requirement for ATP hydrolysis, most likely at nucleotide-binding domain 1, for opening of the channels (Anderson et al., 1991; Gadsby et al., 1995). Dephosphorylation by phosphatases deactivates the channels (Gadsby et al., 1995).

Activation of PKC by phorbol esters has been shown to elicit a time-independent I_{Cl} in cardiac cells with (Walsh, 1991; Walsh and Long, 1994) or without (Walsh, 1991; Zhang et al., 1994a; Asai et al., 1995) the inclusion of purified PKC in the pipette. Phorbol esters also activate single Cl⁻ channels in cell-attached patches of guinea pig ventricular myocytes (Collier and Hume, 1995). Whether PKC activates the CFTR Cl⁻ channel or another species of Cl⁻ channel remains to be proven (cf. Zhang et al., 1994a; Collier and Hume, 1995).

2. The delayed-rectifying K$^+$ channel

The delayed-rectifying cardiac K$^+$ channel is composed of four subunits, and these subunits associate as homo- or hetero-tetramers (for reviews, see Pongs, 1992; Jan and Jan, 1994). Each subunit has six segments (S1–S6) which are thought to form membrane-spanning domains. Five of these segments (S1, S2, S3, S5, and S6) are highly hydrophobic, whereas the hydrophilic S4 segment of each repeat is positively charged, having a basic amino acid (lysine or arginine) every third residue; this S4 segment is thought to be an important component of the channel voltage-sensor. The permeation pathway is thought to be formed, in part, by the region linking the S5 and S6 transmembrane sequences, and this is called the H5 or pore region. K$^+$ channel proteins whose properties are similar to either $I_{K,r}$ or $I_{K,s}$ have been cloned and expressed from human (Sanguinetti et al., 1995) and guinea pig (Freeman and Kass, 1993; Zhang et al., 1994b) heart.

I_K in cardiac cells is augmented following the activation of PKA (Giles and Shibata, 1985; Bennett et al., 1986; Bennett and Begenisich, 1987; Matsuura et al., 1987; Wash and Kass, 1988; Walsh et al., 1988; Yazawa and Kameyama, 1990). Cardiac I_K is also affected by the activation of PKC, with directional effects (stimulation, inhibition) being species-dependent (cf. Zhang et al., 1994b). Activation of PKA generally produces a leftward shift in the I-V relationships (Duchatelle-Gourdon et al., 1989; Giles et al., 1989; Walsh and Kass, 1991). However, activation of PKC produces little change in the kinetics of I_K. (Walsh and Kass, 1988, 1991).

I_K has been found to be sensitive to [Ca^{2+}]$_i$ (Tohse et al., 1987; Tohse, 1990; Nitta et al., 1994). [Ca^{2+}]$_i$ above 10 nM increases I_K amplitude and also slows the deactivation of the tail current (Tohse et al., 1987; Tohse, 1990). Since

PKC is Ca^{2+}-dependent, the increase in I_K by the $[Ca^{2+}]_i$ elevation may be mediated by activation of PKC. Although activation of PKC appears to cause a leftward shift in the concentration-response curve for effects of $[Ca^{2+}]_i$ on I_K (Tohse et al., 1990), the mechanism of stimulation of I_K by Ca^{2+} seems to be different than that of stimulation by PKC activation (Tohse et al., 1987, Tohse, 1990; Tohse and Kanno, 1995), and it has been suggested that it may involve activation of Ca^{2+}/calmodulin-dependent protein kinase (Nitta et al., 1994).

3.   The inward-rectifying K^+ channel

The inward-rectifying cardiac K^+ channel is composed of four subunits (for review, see Jan and Jan, 1994). These subunits contain only two hydrophobic segments separated by pore forming region H5. These segments show similarity in sequence with the S5, S6, and H5 segments of the delayed-rectifying K^+ channel. It appears that the two classes of K^+ channels share the same basic pore design, but unlike the K^+ channel, the K_1 channels lacks a voltage sensor. cDNA encoding the K_1 channels has been isolated and expressed from a mouse macrophage cell line (Kubo et al., 1993) and human heart (Raab-Graham et al., 1994; Ashen et al., 1995; Wible et al., 1995).

In contrast to other cardiac voltage-dependent channels, little is known about the intracellular regulation of I_{K1} (Clapham, 1990). In the limited number of reports, ISO was shown to either increase I_{K1} (Gadsby, 1983; Boyden et al., 1983) or inactivate I_{K1} via β-adrenergic-independent mechanism (Tromba and Cohen, 1990).

Recently, Koumi et al. (1995a,b) found that ISO inhibits I_{K1} through activation of PKA, and that this inhibition was antagonized by acetylcholine. A

low (1 nM) concentration of PMA had no effect on I_{K1} in guinea pig ventricular myocytes (Tohse et al., 1990).

4.   L-Type Ca^{2+} channels

The L-type Ca^{2+} channel is a complex of five polypeptide chains termed α_1-, α_2-, β-, γ-, δ-subunits (for reviews, see Catterall, 1988; Glossmann and Striessnig, 1990). The α_1-subunit is the major transmembrane component, the α_2/δ-subunit is the major extracellular-facing glycoprotein, the β-subunit is mainly cytoplasmic, and the γ-subunit is embedded in the membrane with carbohydrate residues to the outside. The α_1-subunit has a basic structure similar to the delayed-rectifying K^+ channel (see Section I.B.2), comprised of four repeated homologous units, each with five hydrophobic segments (S1, S2, S3, S5, and S6) and one positively charged segment (S4) (Mikami et al., 1989). Unlike K^+ channels, however, each Ca^{2+} channel is a single molecule rather than an aggregation of four units. L-type Ca^{2+} channels contain several potential phosphorylation sites for PKA, PKC and other kinases on the cytoplasmic side.

Activation of PKA increases cardiac $I_{Ca,L}$, with maximal increases of 100–400% at V_{peak}, and the enhancement of $I_{Ca,L}$ has been recorded from a wide variety of species and preparations (McDonald et al., 1994). In contrast, responses to PKC activation are variable, perhaps due to species variability (McDonald et al., 1994; Asai et al., 1995a). Other kinases, including Ca^{2+}/calmodulin-dependent protein kinase II, are also thought to be important regulators of $I_{Ca,L}$ in heart cells (Gurney et al., 1989; Anderson and Braun, 1994; Xiao et al., 1994). (Features of $I_{Ca,L}$ regulation by PKA and PKC kinases are provided in more detail at later points in the thesis.)

C. CROSS-TALK OF PKC AND PKA AFFECTING CHANNEL REGULATION

There are numerous possibilities for cross-talk between the PKC and PKA pathways, with resultant effects on target proteins, including ion channels (Nishizuka, 1988; Kaczmarek, 1987). Kinase pathway interaction is usually investigated by use of a phorbol ester to activate PKC and FSK to activate adenylate cyclase (and PKA). Examples of "upstream" interaction of the PKC and PKA pathways include PKC-mediated desensitization of β-adrenergic receptors (Pitcher et al., 1992) and cAMP-mediated inhibition of phospholipase C (Takai et al., 1982; Teitelbaum, 1990). At the upstream adenylate cyclase level, there is considerable complexity regarding modulation of adenylate cyclase (i.e. PKC has no effect, stimulates, or inhibits enzyme activity) (Yoshimasa et al., 1987; Nordstedt et al., 1989; Gusovsky and Gutkind, 1991; Cooper et al., 1995). This situation may be due to the existence of a variety of PKC isoenzymes, some of which may stimulate, inhibit or have no effect on adenylate cyclase (Cooper et al., 1995). However, a primary reason is that various adenylate cyclase isoenzymes react differently to PKC (Gusovsky and Gutkind, 1991; Cooper et al., 1995).

There are many examples of cellular responses being synergistically affected by co-application of phorbol ester and FSK (e.g. secretion in a variety of cells: see Forstner et al., 1994). While other factors are no doubt involved (see above), it is possible that modified cellular responses are related to cross-talk at the kinase level (e.g. PKC activation of PKA) and/or to joint regulation of target proteins.

1. Non-cardiac CFTR Cl⁻ channels

Studies on the interaction of PKC and PKA in the activation of CFTR Cl⁻ channels have produced variable results. In a recent investigation on rat pancreatic duct cells, FSK/cAMP/IBMX-activated CFTR I_{Cl} amplitude was unaffected by pretreatment with 100 nM phorbol 12,13-dibutyrate (PDBu) (McAlroy et al. 1994). On the other hand, there are a number of examples of synergistic or facilitatory action by PKC and PKA on epithelial cell CFTR Cl⁻ channels. Tabcharani et al. (1991) reported that CFTR open probability (P_o) in excised membrane patches from CHO-K1 cells transfected with the CFTR gene was potentiated by PKC. They concluded that there was a synergism between converging kinase regulatory pathways on CFTR. In T84 cells, PMA-activated PKC appeared to exert a permissive influence on PKA activation of CFTR; when PKC was down-regulated, a cAMP analogue failed to activate fluorescence-monitored Cl⁻ conductance (Dechecchi et al. 1992). Conversely, the apical membrane of human colon carcinoma HT-29cl.19A cells responded with an increase in Cl⁻ conductance to either PDBu or FSK, and addition of PDBu augmented maximal FSK-induced conductance; in cell-attached patches, channel incidence with FSK + PDBu was 16 times greater than with FSK alone, and addition of PDBu provoked noisy current traces that subsequently settled down (Bajnath et al. 1993a,b). The authors surmised that the noise might be due to recruitment of cAMP-dependent channels, and subsequently reported that 1–8 h incubations with protein-trafficking inhibitor brefeldin A had no effect on FSK action but depressed the PDBu increments by about 50% (Bajnath et al. 1994).

In summary, there are a number of examples of synergistic activation of epithelial CFTR by PKC and PKA. In each case, activation by PKC alone was absent or quite weak in comparison to PKA activation. It can be argued that this

is a situation better suited for a controlling influence by PKC on PKA-activated channel opening than if, for example, the two kinases had more equivalent modulatory influences.

2. Cardiac Cl⁻ channels

There is very little known about possible interactive regulatory effects of PKC and PKA on cardiac Cl⁻ channels. Walsh and Long (1994) observed that activation of guinea pig myocyte I_{Cl} by internal dialysis with bovine brain PKCα and external application of PDBu significantly reduced the degree of activation produced by addition of FSK. They suggested that this may have been due to a PKC-induced desensitization of adenylate cyclase. Zhang et al. (1994a) reported that (i) submaximal concentrations of PMA and FSK had additive stimulatory effects on I_{Cl} activation and (ii) maximally-effective concentrations of PMA precluded further activation by FSK. For comparison, Collier and Hume (1995) reported that (i) PKA activation (with IBMX) further increased the P_O of channels opened by PKC activation (with PMA or PDBu), and (ii) PKA activation induced channel opening even when prior PKC activation did not. Their results did not establish whether the activation of one of these kinases partially occluded, added to, or enhanced the induction of Cl⁻ channel activity produced by activation of the other kinase.

3. Cardiac Ca^{2+} and K^+ channels

The best characterized of Ca^{2+} and K^+ channels in regard to PKC-PKA interaction are guinea pig ventricular delayed-rectifying K^+ channels and rat ventricular L-type Ca^{2+} channels. Phorbol ester increments PKA-stimulated I_K and vice versa (Walsh and Kass, 1988, 1991; Yazawa and Kameyama, 1990; Asai

et al. 1995a), and Yazawa and Kameyama (1990) concluded that joint treatment caused a simple additive stimulation of I_K. In a study on rat ventricular Ca^{2+} channels expressed in oocytes, Bourinet et al. (1992) reported that co-stimulation of Ba^{2+}-carried Ca^{2+} channel current by PMA and FSK was incremental, but considerably less than expected from additive stimulatory processes.

D.   RATIONALE AND OBJECTIVES OF THIS STUDY

Compared with information available on the modulation of cardiac membrane currents by the activation of PKA, there have been very few studies on the modulation of I_{Cl} and I_{K1} by PKC, and there are contrasting reports on responses of L-type Ca^{2+} channel to phorbol ester and their PKC involvement. In addition, there is limited information available on cross-talk in the regulation of cardiac membrane currents by PKC and PKA.

In regard to phorbol ester action on I_{Cl} in voltage-clamped cardiomyocytes, the time course and the concentration dependence of phorbol ester activation of I_{Cl} have not been established. One group (Walsh, 1991; Walsh & Long, 1994) has provided limited data on the effects of 20 nM PMA and PDBu on I_{Cl} in guinea pig ventricular myocytes and noted the onset of activation was within 2 to 5 min, whereas a second group (Zhang et al., 1994a) primarily investigated responses to 6 μM PMA in feline ventricular myocytes. Whether due to differences in cell type, phorbol ester concentration, and/or other experimental conditions, the I_{Cl} activated by PMA in the guinea pig myocytes differed in two respects from that in the feline myocytes: (i) it had a linear I-V relationship, compared to an outward-rectifying relationship in feline

myocytes, and (ii) it appeared to have a several-fold lower density than in feline myocytes. In fact, the magnitude of the I_{Cl} activated by PMA in the feline myocytes rivalled that of I_{Cl} activated by micromolar FSK or ISO (Zhang et al., 1994a).

The literature on cardiac L-type Ca^{2+} channel responses to phorbol ester is replete with contrasting results, whether due to species differences, multiple effects of PKC activation, or PKC-independent effects of the experimental probes (cf. McDonald et al., 1994; Asai et al., 1995a). Active phorbol esters have been reported to stimulate L-type Ca^{2+} channel current, depress it, have no effect, or have a biphasic action (see Section IV.D.2). By contrast, I_K in guinea pig ventricular myocytes appears to be consistently stimulated by phorbol ester activation of PKC (Tohse et al., 1987, 1990; Walsh and Kass, 1988), but the time course and the concentration dependency of the I_K responses have not been characterized.

The objectives of this study included (i) determination of (a) the time courses of PMA modulation of cardiac membrane currents, and (b) the PMA concentration-response relationships, (ii) assessment of the involvement of PKC activation in these PMA responses using inactive phorbol esters, αPDD and 4α-phorbol, and kinase inhibitor H-7, and (iii) examination of possible interaction between PKA and PKC regulation of cardiac membrane currents.

II. METHODS

A. MYOCYTE PREPARATION, ELECTROPHYSIOLOGICAL RECORDING AND ANALYSIS, EXTERNAL SOLUTIONS, DIALYSATES, AND DRUGS

1. Myocyte preparation

Guinea pigs weighing 250–400 g were sacrificed by cervical dislocation in accord with national and local regulations on animal experimentation. The heart was excised, attached to the base of a Langendorff column, and retrogradely perfused with normal Tyrode's solution for ~1 min, Ca^{2+}-free supplemented Tyrode's for 4–6 min, Ca^{2+}-free Tyrode's containing collagenase (0.05–0.1 mg/ml; Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan) for 8–12 min, and finally a high-K^+, low-Cl^- storage solution for 6 min. All the perfusates were oxygenated with 100% O_2 and maintained at 37°C. The ventricles were cut into chunks, 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 12 h.

2. Electrophysiological recording and analysis

Electrophysiology

An aliquot of storage solution containing myocytes was transferred to the experimental chamber (~0.1 ml) positioned on top of an inverted microscope stage (Nikon Diaphot, Tokyo, Japan). After the cells had settled on the Perspex bottom (Sarstedt, Inc., Newton, NC, USA), the chamber was

perfused with normal Tyrode's solution at a rate of about 2 ml/min. All the experiments were performed with solutions preheated to 34-36°C.

Single ventricular cells 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 (Jencons Scientific, Bedfordshire, UK) in the usual two-step process. They 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. 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 a 150-mM NaCl-agar bridge connected to an Ag–AgCl wire. Series resistance was 3-5 MΩ, and compensated (60-80%) in most of the experiments.

The voltage-clamp amplifier was an EPC-7 (List Medical Electronic, Darmstadt, Germany). Currents and voltages were displayed on a storage oscilloscope (Tektronix 5113), and recorded on an FM tape-recorder (Hewlett-Packard 3964) or on a video cassette recorder (Sony SLV-400) through an A/D VCR adapter PCM-2-B (Medical Systems Corp. Greenvale, NY, USA) after low-pass filtering at 3 kHz (EPC-7). The currents and voltages were analyzed off-line. Data were digitized at sampling rates of 4 to 10 kHz, and analyzed using a Compaq Deskpro 386/20e computer with custom-designed programs. Data were stored on an external storage device (Bernoulli Box II 44, Iomega, Roy, UT, USA).

Statistics

Results are expressed as means ± S.E.M., with n indicating the number of experiments. Comparisons were made using the unpaired Student's t test or the

one-way analysis of variance (ANOVA); a difference was considered significant when $p < 0.05$.

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) 10 (pH 7.4 with NaOH); (ii) Cs$^+$ Tyrode's solution (KCl replaced with equimolar CsCl); (iii) Ca^{2+}-free, EGTA Tyrode's solution (CaCl$_2$ and KCl omitted, MgCl$_2$ reduced to 0.2 mM, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) added, pH 7.4 with NaOH); (iv) K$^+$-, Ca^{2+}-free Tyrode's solution (KCl and CaCl$_2$ omitted, 0.2 mM CdCl$_2$, 1 mM BaCl$_2$, and 5 mM NiCl$_2$ added, pH 7.4 with NaOH); (v) Na$^+$-, K$^+$-, Ca^{2+}-free Tyrode's solution (NaCl replaced with equimolar tetramethylammonium (TMA) chloride, KCl and CaCl$_2$ omitted, 0.2 mM CdCl$_2$ and 1 mM BaCl$_2$ added, pH 7.4 with TMAOH).

The Ca^{2+}-free supplemented Tyrode's solution used for cell isolation contained (in mM): NaCl 125, KCl 5.4, MgCl$_2$ 1, taurine 20, glucose 20, and HEPES 10 (pH 7.4 with NaOH). Storage solution contained (in mM): KCl 30, KOH 90, glutamic acid 50, KH$_2$PO$_4$ 30, MgSO$_4$ 3, taurine 20, glucose 10, EGTA 0.5, and HEPES 10 (pH 7.4 with KOH).

4. Dialysates

Two types of dialysates were used, those that contained K$^+$, and those with K$^+$ replaced by Cs$^+$. Free Ca^{2+} concentration in these solutions was modified by addition of CaCl$_2$ calculated after Fabiato and Fabiato (1979) with

the correction of Tsien and Rink (1980) to give nominal pCa 9 (standard), pCa 7, or pCa 11.

K^+ pipette solution (pH 7.4 with KOH) contained (in mM) KCl 50, KOH 80, aspartic acid 80, KH_2PO_4 10, $MgSO_4$ 1, Na_2ATP 5, HEPES 5, and (a) EGTA 5 + $CaCl_2$ 0.08 (pCa 9) or (b) EGTA 10 (pCa 11). Cs^+ pipette solution (pH 7.4 with CsOH) contained either high or low Cl^-: (i) high Cl^- solution was (in mM) CsCl 120, CsOH 30, $MgCl_2$ 1, MgATP 5, HEPES 10, with (a) EGTA 10 + $CaCl_2$ 0.16 (pCa 9), (b) EGTA 10 + $CaCl_2$ 6.1 (pCa 7), or (c) EGTA 10 (pCa 11); (ii) low Cl^- pipette solution contained (in mM) CsCl 30, CsOH 110, aspartic acid 110, MgATP 5, HEPES 5, and EGTA 5 + $CaCl_2$ 0.08 (pCa 9). In regard to the use of these dialysates, dialysate pCa is unlikely to be fully translated into cytoplasmic pCa, and EGTA dialysates are unlikely to "clamp" myocyte subsarcolemmai Ca^{2+} concentration during surges of $I_{Ca,L}$.

5.   Drugs

Phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate (αPDD), 4α-phorbol (αPHR), forskolin (FSK), and 9-anthracenecarboxylic acid (9-AC) were dissolved in dimethyl sulfoxide (DMSO) and stored in small aliquots as stock solutions at $-20°C$. 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), D600, and tetrodotoxin (TTX) were dissolved in water and also kept in small aliquots as stock solutions at $-20°C$. Appropriate amounts of stock solutions were added to the external solution, and corresponding amounts of DMSO ($\leq0.2\%$) were also added to the control external solution for the experiments in order to avoid a moderate decrease in I_K upon addition of DMSO (Ogura et al., 1994). Rp-adenosine 3',5'-cyclic monophosphothioate (Rp-cAMPS) was added directly to the pipette solution.

H-7 was purchased from Calbiochem (La Jolla, CA, USA), Rp-cAMPS was from Research Biochemicals International (Natrick, MA, USA), and all other drugs were from Sigma (St. Louis, MO, USA).

B. MEASUREMENT AND CONTROL CHARACTERIZATION OF MEMBRANE CURRENTS

This section of the Methods describes how the five currents were measured, their stability over time, and typical kinetic features and current-voltage (I-V) relationships. The responses of three of these currents (I_{Cl}, I_K, $I_{Ca,L}$) to FSK are also presented to illustrate their regulation by PKA, and to provide a baseline for comparison with responses to PMA in the Results.

In most of the experiments, myocytes were held at -80 mV, and selected test pulses of constant amplitude were applied at 0.2 Hz after 100-ms prepulses to -40 mV. This pulsing routine was periodically interrupted for the collection of data for I-V relationships over the range -110 to +60 mV.

1. Measurement and control characterization of I_{Cl}

Myocytes were superfused with K^+-, Ca^{2+}-free or Na^+-, K^+-, Ca^{2+}-free Tyrode's solution and dialyzed with Cs^+ pipette solution. These conditions were expected to eliminate or minimize electrogenic cation movements through ion channels, pumps, and exchangers; K^+ currents (K^+-free, Ba^{2+}-containing superfusate; K^+-free, Cs^+ dialysate), Na^+ current (Na^+-free superfusate, voltage protocol), Ca^{2+} currents (Ca^{2+}-free, Cd^{2+}-containing

superfusate), Na^+-K^+ pump current (K^+-free superfusate; Na^+-free dialysate), and Na^+-Ca^{2+} exchange current (Na^+-free superfusate or Ni^{2+}-containing Na^+ superfusate; low Ca^{2+} dialysate). In most experiments, the external Cl^- concentration ([Cl^-]$_o$) was 144 mM, and the dialysate concentration was 30 mM, giving a calculated Cl^- equilibrium potential (E_{Cl}) of -42 mV. Currents were elicited by 100-200 ms depolarizations or hyperpolarizations applied at 0.1-0.2 Hz from prepulse -40 mV (-80 mV holding potential), and I-V relationships were generated from mean current amplitudes measured as the average current flowing during the last 10-20 ms of test pulses by reference to zero current. I_{Cl} was measured as a difference current from basal background current.

Sorota (1992) observed that I_{Cl} in canine atrial myocytes superfused and dialyzed with isosmotic solution can activate with time after patch breakthrough, and concluded that this was due to osmotic swelling caused by whole-cell dialysis. Similar activation of volume-sensitive I_{Cl}, despite the use of isosmotic external and pipette-filling solutions, has been recorded in non-cardiac cells (e.g. McCann et al., 1989). Since hyposmotic swelling of guinea pig ventricular myocytes activates a large I_{Cl} (Vandenberg et al., 1994; Shuba et al., 1995), unintentional swelling and turn-on of I_{Cl} is a concern in evaluating responses of I_{Cl} to pharmacological interventions. However, neither visually-detectable swelling nor activation of I_{Cl} over time occurred in myocytes investigated under isosmotic conditions in the present study.

Figure 1 illustrates the characteristics of background current and its stability under the conditions used to isolate I_{Cl}. Current amplitudes monitored at a holding potential of -80 and a test potential of +40 mV are plotted against post-patch breakthrough time in Figure 1A. The I-V relationships were collected at 11, 21 and 29 min, and original current traces and I-V relationships at 11 and 29 min are shown in Figures 1B and 1C, respectively. The background
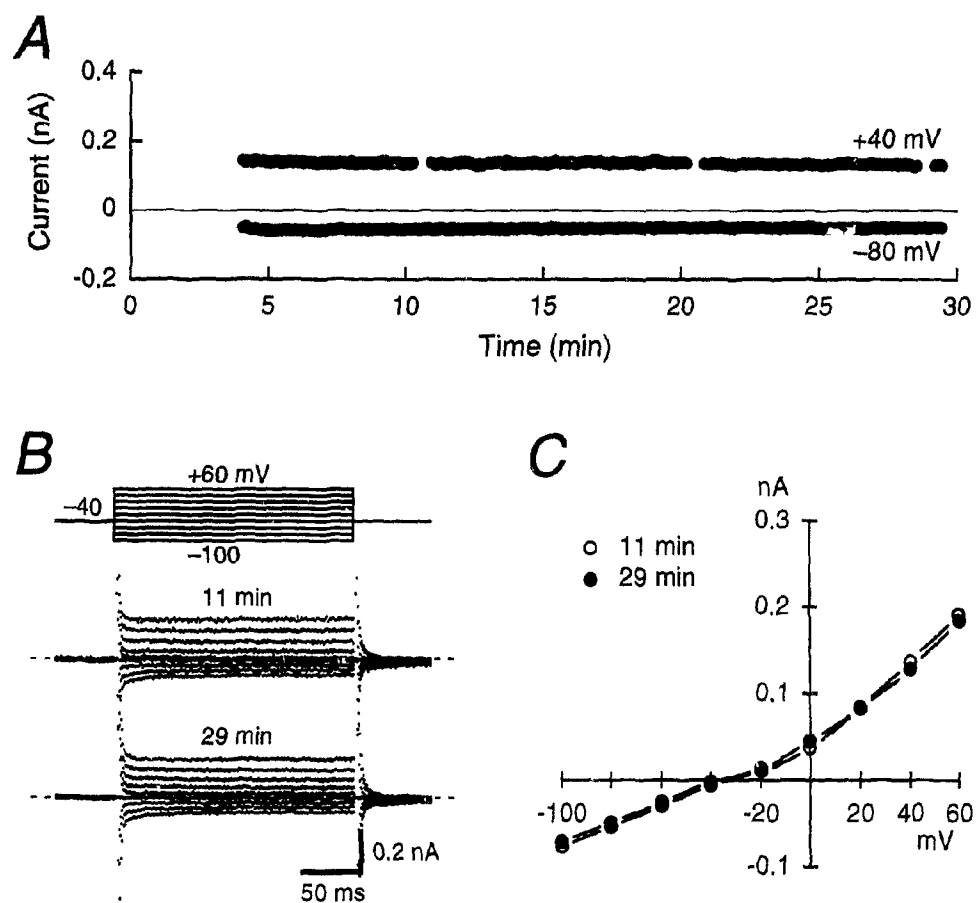
Figure 1. Background current in a guinea pig ventricular myocyte

The myocyte was superfused with Na$^+$-, K$^+$-, Ca^{2+}-free Tyrode's solution and dialyzed with Cs$^+$ pipette solution. Currents were elicited by 200-ms test pulses to +60 mV applied at 0.2 Hz from prepulse −40 mV (−80 mV holding potential). Regular pulsing was periodically interrupted to record currents at other potentials for determination of I-V relationships. **A.** Background current amplitudes monitored at −80 mV and test potential +40 mV plotted against post-patch breakthrough time. **B,C.** Families of current records (**B**) and I-V relationships (**C**) over the test potential range −100 (*bottom trace*) to +60 mV (*top trace*) in 20 mV increments collected at 11 min and 29 min. The horizontal dashed line in **B** (and in subsequent thesis figures) indicate the zero current level.

current was small (190 ± 17 pA at +40 mV, $n = 12$) and relatively time-independent, with an *I-V* relationship that rectified slightly in the outward direction and crossed the voltage axis near -35 mV. During the 30-min observation period, the background current amplitude was quite stable and the *I-V* relationship was essentially unchanged (also see Figure 22 for similarly stable background current). The background current and current activated by PMA or FSK were usually expressed in terms of chord conductances. A chord conductance was defined as the slope of the whole-cell *I-V* relationship between two potentials (e.g. from E_{rev} to +40 mV) i.e. $\Delta I / \Delta V$. (Unsatisfactory experiments (data discarded) featured large rapid increases in unstable currents that were evident from patch formation on, or suddenly arose upon loss of seal during an experiment.)

Responses to PKA activation by FSK

Myocytes superfused with Na^+-, K^+-, Ca^{2+}-free Tyrode's solution were pulsed with 100-ms steps to potentials between -110 and +50 mV before and during 5-min applications of 5 µM FSK (Figure 2). FSK activated inward- and outward-directed current, both of which were time-independent (Figure 2A). The *I-V* relationships of the stimulated currents were outwardly-rectifying and crossed the pre-FSK relationships at about -37 mV, which is near the E_{Cl} of -42 mV calculated from superfusate (144 mM) and dialysate (30 mM) Cl^- concentrations (Figure 2B). When myocytes were dialyzed with 140 mM Cl^- solution, the *I-V* relationships were nearly linear and the cross-over potentials were near 0 mV (E_{Cl} = -5 mV) (Figures 2C and D). In additional experiments, replacement of external Cl^- with relatively impermeant aspartate (cf. Matsuoka et al., 1990) markedly reduced the FSK-induced current, and dialysis with 450
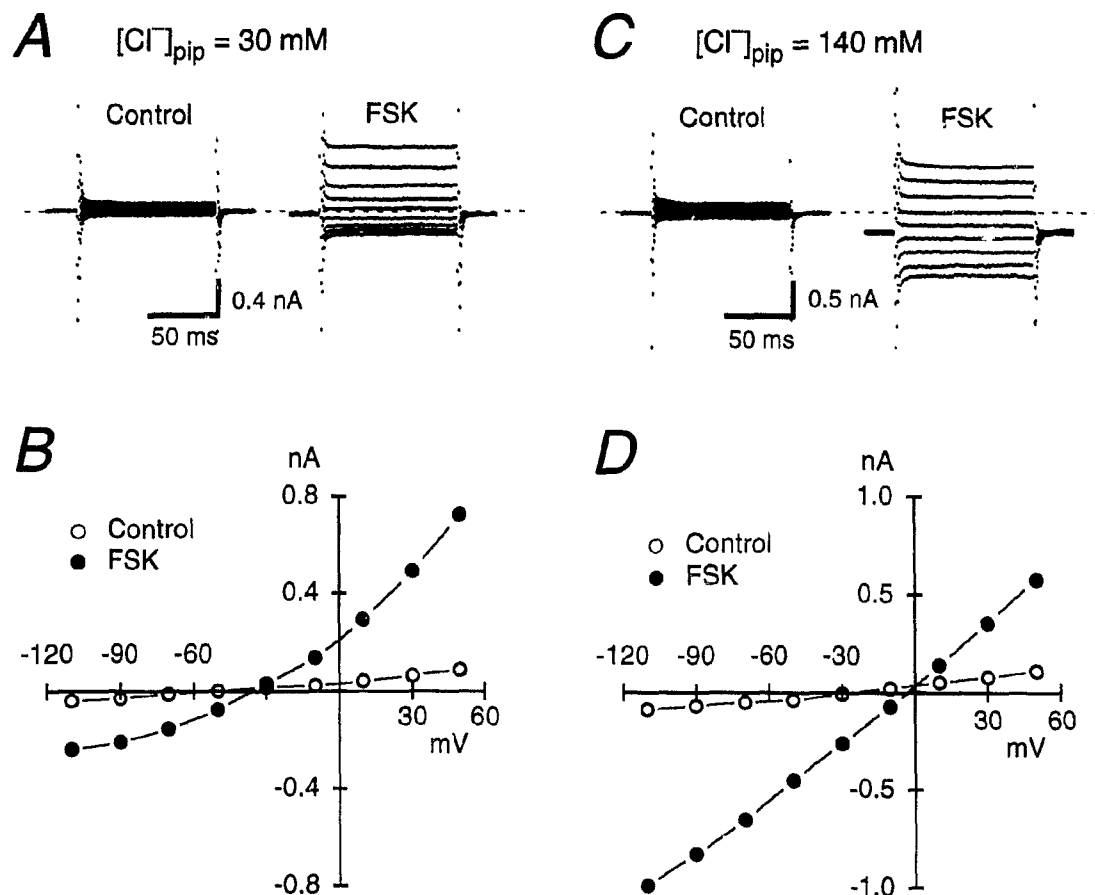
Figure 2. Activation of Cl⁻ current by FSK

A,B. Families of current records (*A*), and *I-V* relationships (*B*), elicited over the range −110 (*bottom trace*) to +50 mV (*top trace*) by 100-ms test pulses in 20 mV increments. Currents were obtained before (*Control*) and during application of 5 µM FSK (*FSK*). The myocyte was dialyzed with standard Cs⁺ pipette solution containing 30 mM Cl⁻. *C,D.* Families of current records (*C*), and *I-V* relationships (*D*), obtained before (*Control*) and during application of 5 µM FSK (*FSK*) to a myocyte was dialyzed with Cs⁺ pipette solution containing 140 mM Cl⁻.

µM Rp-cAMPS for 12-16 min almost completely inhibited the FSK stimulation (see Figure 8B). These characteristics of the currents stimulated by FSK identify the activated current as I_{Cl} (cf. Bahinski et al., 1989; Harvey and Hume, 1989a; Harvey et al., 1990; Matsuoka et al., 1990).

2. Measurement and control characterization of I_K

I_K was recorded from guinea pig ventricular myocytes superfused with normal Tyrode's solution and dialyzed with K^+ pipette solution. In these cells, depolarizing 500-2000 ms test pulses from -40 mV elicited rising outward currents. These outward currents were primarily, but not exclusively, caused by the activation of delayed-rectifying I_K. The corresponding tail current upon repolarization to -40 mV reflects the deactivation of total I_K turned on during the depolarization (cf. Walsh et al., 1989), and we used this parameter (measured as the difference between tail current peak and steady-state current at -40 mV) to monitor changes in I_K. Isochronic activation relationships for I_K were obtained by applying test pulses between -30 and +50 mV in 10 mV increments, and plotting the tail current amplitudes against the test potentials.

The current was identified as I_K based on the following properties: (i) it was activated by depolarizing pulses at potentials more positive than -20 mV; (ii) its tail current reversed at around -80 mV, which is near the K^+ equilibrium potential (E_K) of -86 mV, calculated from superfusate (5.4 mM) and dialysate (140 mM) K^+ concentrations; (iii) it was almost completely abolished by dialysis with Cs^+ pipette solution. In this study, I_K refers to total I_K; no effort was made to subdivide it into its relatively small fast and relatively large slow components (cf. Sanguinetti and Jurkiewics, 1990; Chinn, 1993).

Figure 3. I_K in guinea pig ventricular myocytes

Myocytes were superfused with normal Tyrode's solution and dialyzed with K^+ pipette solution. I_K was elicited by 1000-ms test pulses to +50 mV applied at 0.2 Hz from prepulse –40 mV (–80 mV holding potential) (see pulse protocol illustrated on top of the records in *B*); regular pulsing was periodically interrupted to record currents at other potentials for determination of *I-V* relationships. *A.* I_K tail current amplitude recorded at –40 mV (peak current *minus* steady-state level at –40 mV) in a myocyte test-pulsed to +50 mV plotted against post-patch breakthrough time. *B,C.* Superimposed current records (*B*) and *I-V* relationships (*C*) obtained at 11 min and 18 min. *D.* Average time diary of I_K tail amplitude (*n* = 12 myocytes) after test pulses to +50 mV. The amplitude at 2 min after patch breakthrough was designated as 100%, and all other values were expressed accordingly. The S.E.M. falls within the symbols.
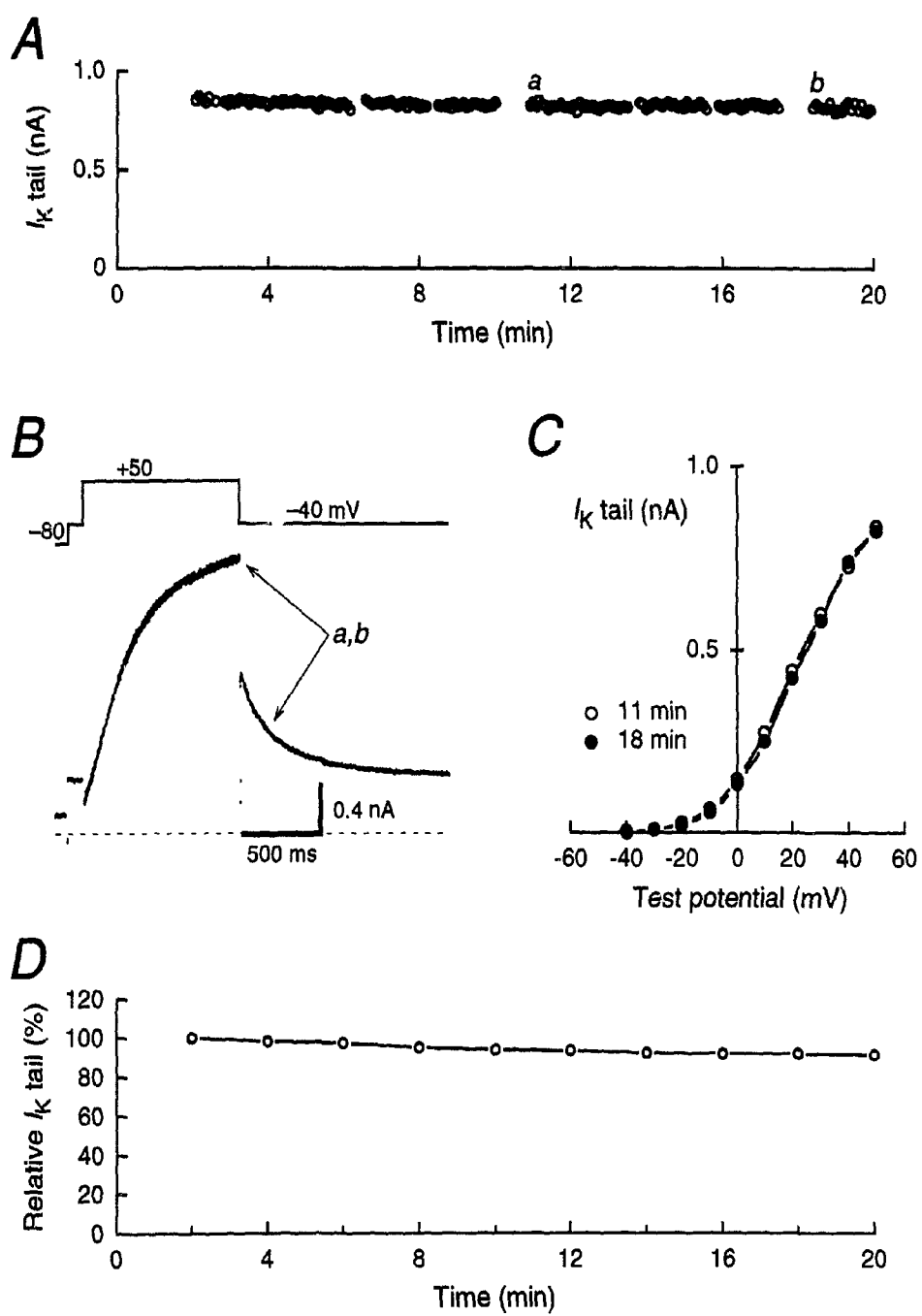
Figure 3. I_K in guinea pig ventricular myocytes

Figure 3A shows a time diary of I_K tail current amplitude activated by 1000-ms test pulses to +50 mV. Original current traces collected at times indicated in the time diary are shown in Figure 3B, and I-V relationships determined at 11 and 18 min are shown in Figure 3C. I_K tail current amplitude was relatively stable during the 20-min observation period. I_K activation and I_K tail deactivation kinetics, and the shape of the I-V relationships, remained unchanged between 11 and 18 min. Average changes in I_K tail current amplitude at +50 mV are shown in Figure 3D. The amplitude at 2 min after patch breakthrough was designated as 100%, and the amplitudes at later times were normalized accordingly. After 20 min, the amplitude had declined to 91 ± 2% ($n = 12$).

Responses to PKA activation by FSK

Application of 1 µM FSK increased rising outward currents and associated I_K tail amplitudes to new steady-states within 4-5 min (Figure 4A). The records and I-V relationships obtained from the example experiment before and 4 min after application of FSK (Figures 4B and C) illustrate that the drug increased tail amplitude by about 90%. On average, 1 µM FSK stimulated the tail amplitude by 72 ± 14% ($n = 5$). In additional experiments, FSK stimulation of I_K was strongly suppressed by addition of 450 µM Rp-cAMPS in the pipette solution ; the increase with 1 µM FSK was only 6 ± 6% ($n = 3$) (see Figure 8B).
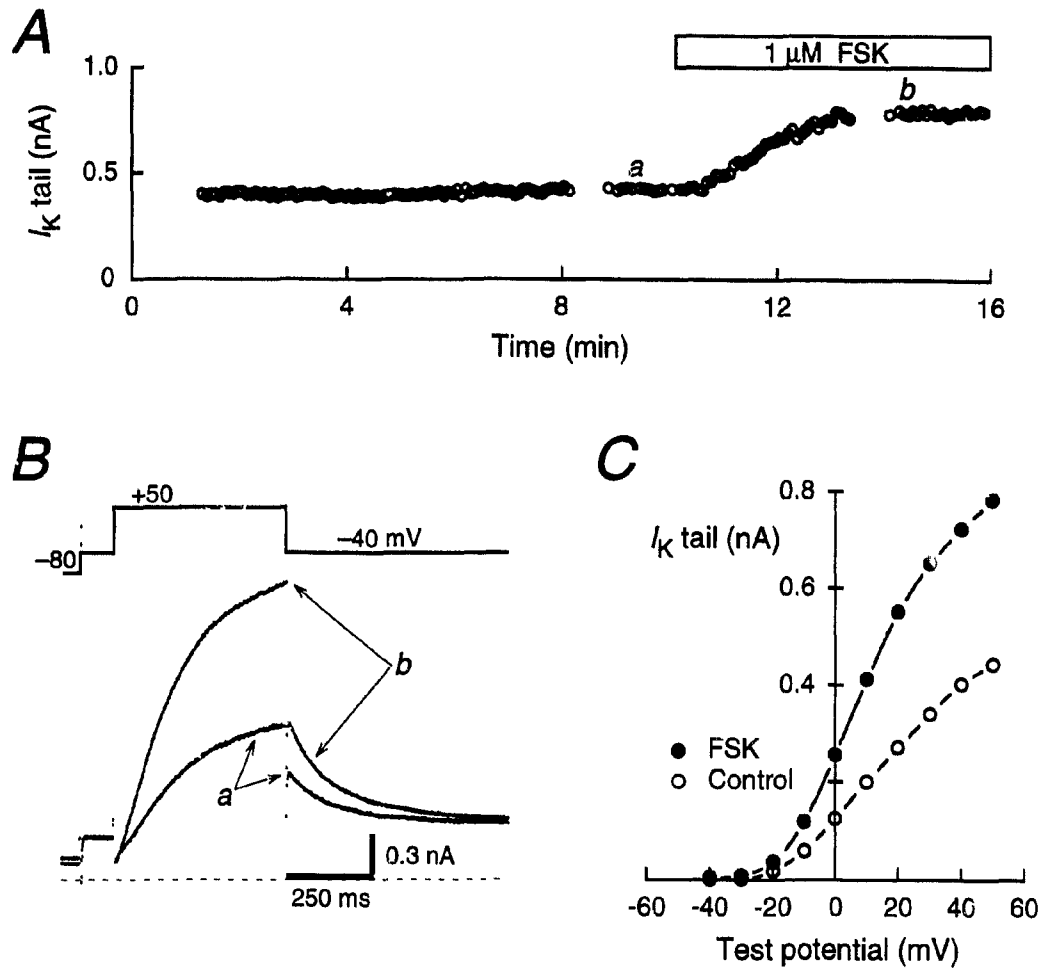
Figure 4. Stimulation of I_K by FSK

A. Time diary of I_K tail current amplitudes recorded at -40 mV following 500-ms test pulses to +50 mV. 1 µM FSK was bath-applied as indicated by the bar above the diary. **B.** Superimposed current records taken at the times indicated in **A.** **C.** *I-V* relationships obtained before (*Control*) and after 4 min application of 1 µM FSK (*FSK*).

3. Measurement and control characterization of I_{K1}

I_{K1} was recorded under conditions similar to those for I_K measurement. Myocytes were superfused with normal Tyrode's solution and dialyzed with K^+ pipette solution. I_{K1} was elicited by 500-ms test pulses to -30 mV and more negative potentials applied at 0.2 Hz from prepulses -40 mV (-80 mV holding potential), and I-V relationships were generated from measurements of the average current flowing during the last 10 ms of test pulses by reference to zero current.

Figure 5A shows families of current records at test potentials between -30 and -50 mV (*top traces*) and between -60 and -110 mV (*bottom trace.*). I_{K1} recorded between these potentials was relatively time-independent. I-V relationship in Figure 5C illustrates that I_{K1} reversed the direction at around -83 mV, which is close to E_K of -86 mV. The current rectified strongly in the inward, creating a negative slope region in the I-V relationship at potentials between -30 and -50 mV. As shown previously in guinea pig ventricular myocytes (Sakmann and Trube, 1984; Tourneur et al., 1987; Ishihara et al., 1989), both inward and outward components of I_{K1} were blocked by addition of 0.5-1 mM Ba^{2+} to the superfusate (Figure 5B), or by omission of extracellular K^+ (data not shown).

4. Measurement and control characterization of $I_{Ca,L}$

$I_{Ca,L}$ was recorded from myocytes that were either (i) superfused with normal Tyrode's solution and dialyzed with K^+ pipette solution, or (ii) superfused with Cs^+-Tyrode's solution and dialyzed with Cs^+ (high Cl^-) pipette solution. Myocytes were prepulsed to -40 mV for 80-100 ms to inactivate Na^+ current and any T-type Ca^{2+} current, and then depolarized to 0 mV at 0.2 Hz to

A   Control

−40   −50 mV
         −30

         −60

         −110

1 nA

150 ms

B   Ba²⁺

−40   −50 mV
         −30

         −60

         −110

C   nA
                                        1

         -120   -60   -30 mV

                                        -1

              o Control
              ● Ba²⁺   -2
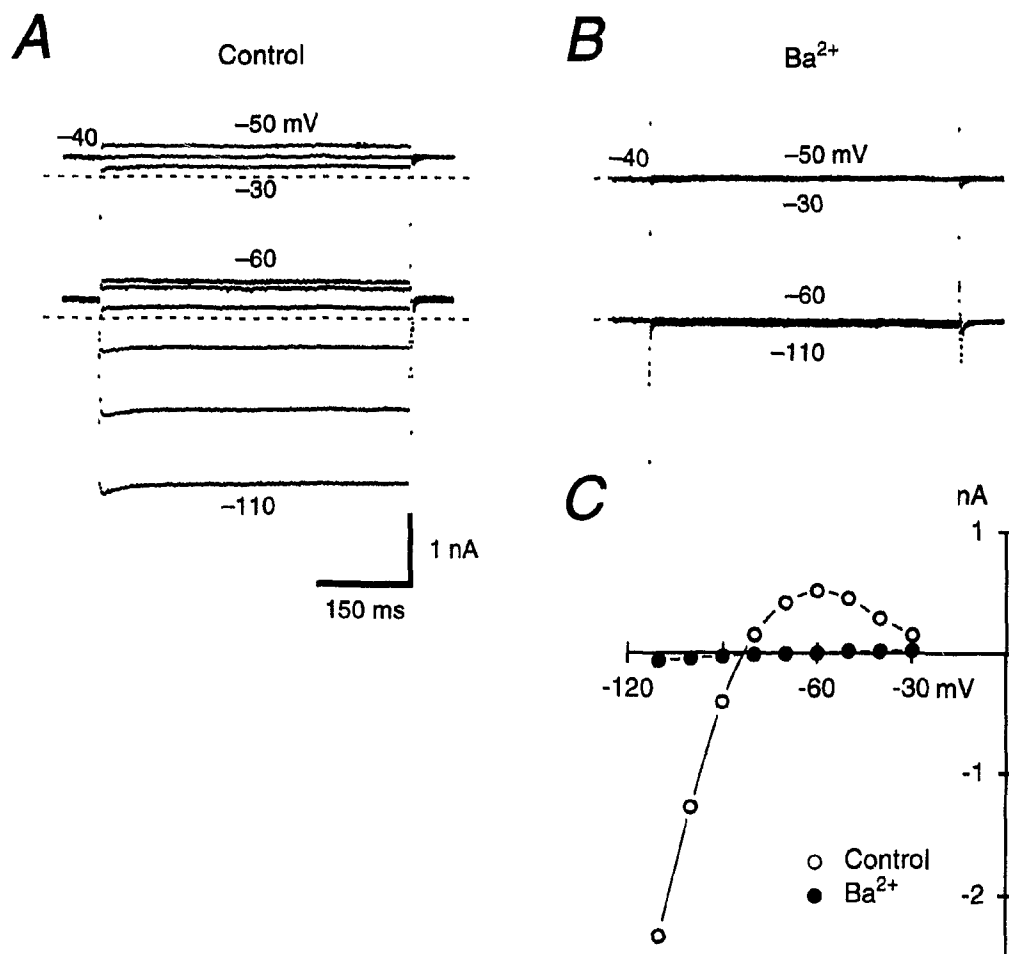
Figure 5. I_{K1} in guinea pig ventricular myocytes

The myocyte was superfused with normal Tyrode's solution and dialyzed with K⁺ pipette solution. I_{K1} was elicited by 500-ms depolarizations and hyperpolarizations applied at 0.2 Hz from prepulse −40 mV (−80 mV holding potential) *A,B*. Families of current records between −50 and −30 mV (*top panels*) and −110 and −60 mV (*bottom panels*) in 10 mV increments. The records were obtained before (*A*) and after 2 min application of 0.5 mM Ba²⁺ (*B*). *C. I-V* relationships from *A* and *B*.

elicit $I_{Ca,L}$. Its amplitude was measured as peak inward current by reference to zero current. $I_{Ca,L}$ measured under these conditions was completely blocked by 1 mM Cd^{2+} or 1 µM D600 (data not shown).

Figure 6 presents records and measurements from a typical myocyte superfused for 20 min with normal Tyrode's solution. The time diary of peak $I_{Ca,L}$ amplitude activated by 500-ms test pulses to 0 mV illustrates that there was moderate rundown of the current during this observation period. Original current traces collected at times indicated in the time diary are shown in Figure 6B, and I-V relationships determined at 9 and 18 min in Figure 6C. There was little change in the time to peak and inactivation time course of $I_{Ca,L}$, and the shape of the I-V relationship was also quite stable. Average changes in peak $I_{Ca,L}$ amplitude at 0 mV are shown in Figure 6D. Current amplitude at 2 min after patch breakthrough was designated as 100%, and other amplitudes normalized accordingly. The rundown of $I_{Ca,L}$ was larger than with that of the I_K tail; after 20 min, the amplitude had declined to 78 ± 3% (n = 13).

Responses to PKA activation by FSK

In agreement with previous studies (e.g. Hescheler et al., 1986; West et al., 1986; Yuan and Bers, 1995; also see McDonald et al., 1994), 1 µM FSK caused a 2-fold increase in $I_{Ca,L}$, that reached a steady-state in 3-4 min (Figure 7). The peak of the $I_{Ca,L}$-V relationship measured during treatment with FSK was often shifted to the left by 5-10 mV compared to the control (Figure 7C). These characteristics of FSK stimulation of $I_{Ca,L}$ were suppressed by dialysis with 450 µM Rp-cAMPS as shown in example current records in Figure 8A. Figure 8B summarizes responses of I_{Cl}, I_K, and $I_{Ca,L}$ to FSK after 10-12 min dialysis with Rp-cAMPS.

Figure 6. $I_{Ca,L}$ in guinea pig ventricular myocytes

Myocytes were superfused with normal Tyrode's solution and dialyzed with K^+ pipette solution. $I_{Ca,L}$ was elicited by 5ᐱ0-ms test pulses to +0 mV applied at 0.2 Hz from prepulse −40 mV (−80 mV holding potential) (see pulse protocol illustrated on top of the records in *B*); regular pulsing was periodically interrupted to record currents at other potentials for determination of *I-V* relationships. **A.** Peak $I_{Ca,L}$ amplitude at 0 mV (measured by reference to zero current) plotted against post-patch breakthrough time. **B.** Superimposed current records taken at the times indicated in **A**. Only the initial 100-ms portion of the inward $I_{Ca,L}$ is shown. **C.** *I-V* relationships obtained at 9 min and 18 min. **D.** Average time diary of $I_{Ca,L}$ amplitude at 0 mV ($n = 13$ myocytes). The amplitude at 2 min after patch break-through was designated as 100%, and all other values were expressed accordingly. The S.E.M. generally falls within the symbols.

A

2.5

2.0

1.5

1.0

0.5

0

$I_{Ca,L}$ (nA)

a

b

0   4   8   12   16   20

Time (min)

B

0 mV

−40

b
a

0.5 nA

25 ms

C

nA

1

−40   20   60 mV

−1

−2

○ 9 min
● 18 min

D

120

100

80

60

40

20

0

Relative $I_{Ca,L}$ (%)

0   4   8   12   16   20

Time (min)
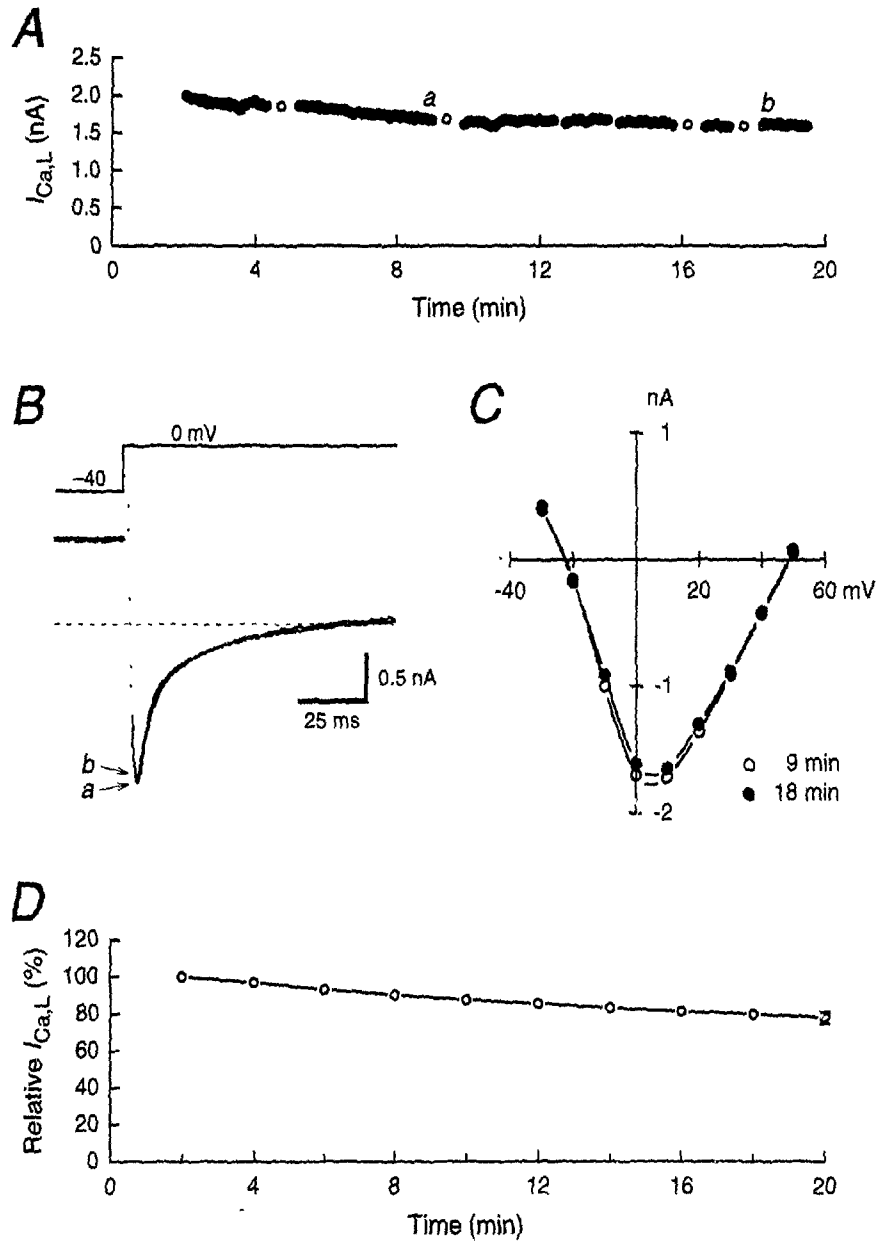
Figure 6. $I_{Ca,L}$ in guinea pig ventricular myocytes
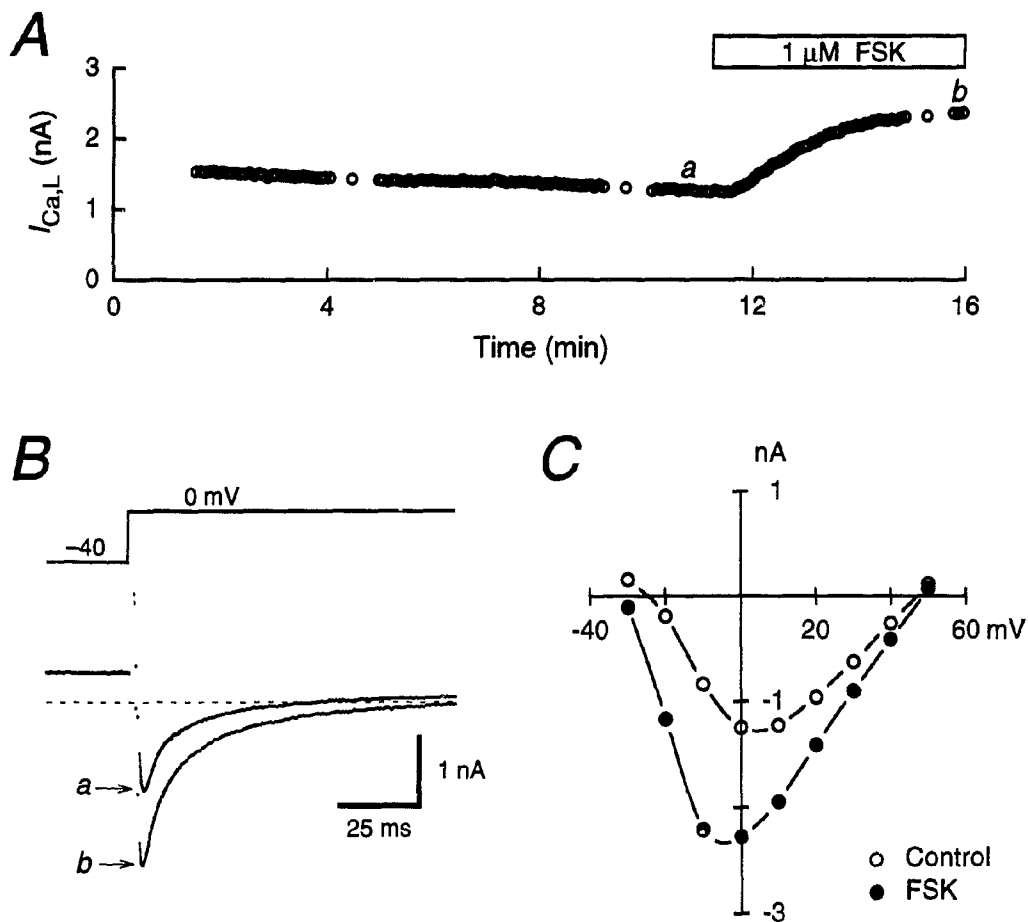
Figure 7. Stimulation of $I_{Ca,L}$ by FSK

A. Time diary of peak $I_{Ca,L}$ amplitude elicited by test pulses to 0 mV from pre-pulse –40 mV. 1 µM FSK was applied as indicated by the bar above the diary. **B.** Superimposed current records taken at the times indicated in **A**. **C.** I-V relationships obtained before (*Control*) and after 4 min application of 1 µM FSK (*FSK*).
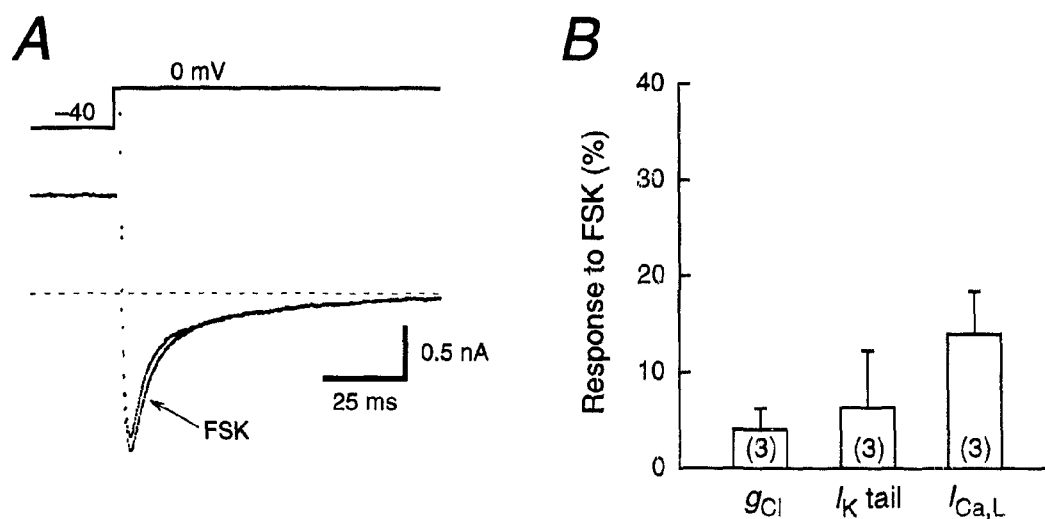
Figure 8. Effect of dialysis with Rp-cAMPS on the stimulation of membrane currents by FSK

A. Original records of $I_{Ca,L}$ obtained before and 4.5 min after application of 1 μM FSK (*FSK*) to a myocyte dialyzed with 450 μM Rp-cAMPS for 16 min. Only the initial 100-ms portion of the depolarization to 0 mV is shown. **B.** Summary of effects of dialysis with Rp-cAMPS on the activation of g_{Cl} (% background conductance), stimulation of I_K tail (+50 mV test pulse), and stimulation of $I_{Ca,L}$ (test pulse to 0 mV) by FSK. Concentrations of FSK were 5 μM for g_{Cl} and 1 μM for I_K tail and $I_{Ca,L}$. Number of myocytes in brackets.

5.   Measurement and control characterization of $I_{Na,L}$

$I_{Na,L}$ was recorded from myocytes superfused with K^+-free, EGTA Tyrode's solution and dialyzed with Cs^+ (high Cl^-) pipette solution. Figure 9 illustrates how $I_{Na,L}$ developed upon switching from Ca^{2+}-containing to Ca^{2+}-free, EGTA-containing solution. Voltage clamp pulses applied from a holding potential of -40 to 0 mV for 100 ms at 0.2 Hz activated $I_{Ca,L}$ during the initial superfusion with Cs^+-Tyrode's solution. The current had an amplitude of 2.7 nA and largely inactivated during the 100-ms test pulses (Figure 9A, left hand trace). The superfusate was then switched to Ca^{2+}-free, EGTA Tyrode's solution. As previously reported (e.g. Matsuda, 1986), the reduction of external Ca^{2+} to submicromolar concentration produced an immediate suppression of $I_{Ca,L}$ (Figure 9A, second and third traces) followed by development of a slowly-inactivating inward current (Figure 9A, fourth and fifth traces). The latter was carried by Na^+ through L-type Ca^{2+} channels since Na^+ channels and T-type Ca^{2+} channels were inactivated by the -40 mV holding potential. In addition, the current was insensitive to 50 μM tetrodotoxin (TTX), but sensitive to external Na^+ concentration ($[Na^+]_o$), and abolished by 1 μM D600 (Figure 9B). At normal $[Na^+]_o$ and low external divalent cation concentration, $I_{Na,L}$ is many times larger than preceding $I_{Ca,L}$ (Matsuda 1986; Hadley and Hume, 1987) and this large current results in poor voltage control during the first 5-10 ms of a clamp pulse (Shuba et al., 1990). This situation was avoided by partial block of $I_{Na,L}$ with 0.2 mM external Mg^{2+} (cf. Matsuda, 1986; McDonald et al., 1994), which restricted $I_{Na,L}$ to $I_{Ca,L}$-like amplitudes (Figure 9A). The addition of Mg^{2+} also reduced a leak current which can develop following the removal of divalent cations (Matsuda, 1986); the remaining leak current was analogue

compensated (cf. Kostyuk and Krishtal, 1977). $I_{Na,L}$ amplitude was measured as peak inward current by reference to zero current.
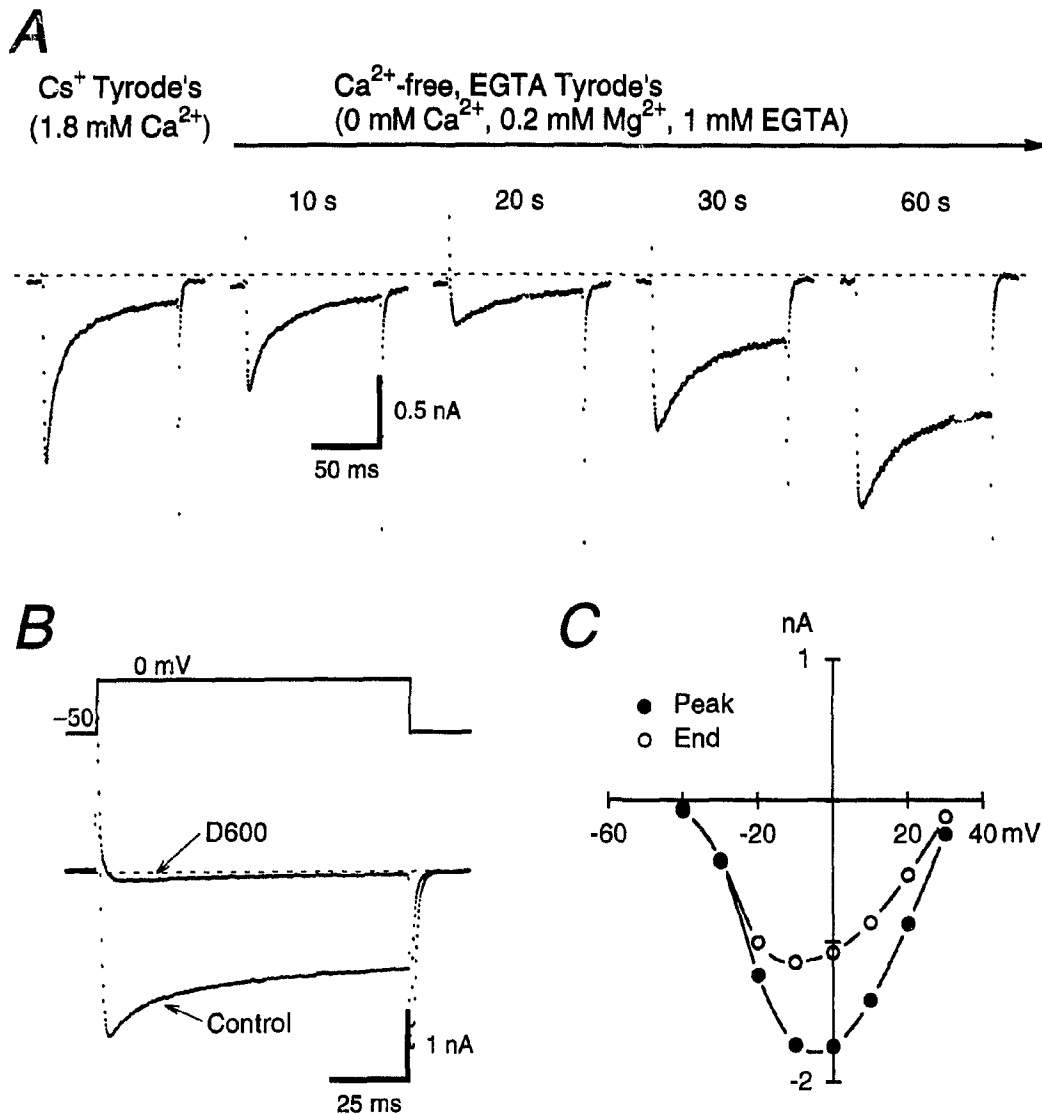
Figure 9. $I_{Na,L}$ in guinea pig ventricular myocytes

A. Development of $I_{Na,L}$. The myocyte was initially superfused with Cs^+ Tyrode's solution and dialyzed with Cs^+ (high Cl^-) pipette solution, and then superfused with Ca^{2+}-free, EGTA Tyrode's. $I_{Ca,L}$ and $I_{Na,L}$ were elicited by 100-ms test pulses to 0 mV applied at 0.2 Hz from a holding potential of –40 mV. ***B.*** Superimposed current records obtained before (*Control*) and 1 min after application of 1 µM D600 (*D600*). ***C.*** *I-V* relationships for peak $I_{Na,L}$ and current at the end of 100-ms test pulses.

III. RESULTS

A. EFFECTS OF PMA ON CARDIAC MEMBRANE CURRENTS

The time courses and concentration dependencies of PMA action on I_{Cl}, I_K, I_{K1}, and $I_{Ca,L}$ were investigated. The results are described in the four sections below.

1. Activation of I_{Cl} by PMA

Figure 10 shows families of current traces and I-V relations obtained from a myocyte superfused with Na^+-, K^+-, Ca^{2+}-free Tyrode's solution and dialyzed with Cs^+ pipette solution. Addition of 100 nM PMA for 6 min increased both outward and inward current by approximately 70% (Figures 10A and B). Control currents were subtracted from PMA-stimulated currents to isolate PMA-activated currents. The activated currents had time-independent waveforms, reversed direction at -30 mV, and had an outwardly-rectifying dependence on voltage (Figures 10C and D). The increment in current at +40 mV was ~130 pA (~1 pA/pF), corresponding to an induced chord (E_{rev} to +40 mV) conductance of ~1.9 nS (one of the largest recorded in this study).

Time course and concentration dependence of PMA stimulation

Figure 11 shows plots of current amplitudes monitored at -80 and +40 mV from representative myocytes treated with one of three concentrations (10 nM, 100 nM, and 5 µM) of PMA for 10-15 min. There was a small increase in current during the 10 nM application, and larger increases with the higher concentrations. The latter were more pronounced at +40 mV than at -80 mV,
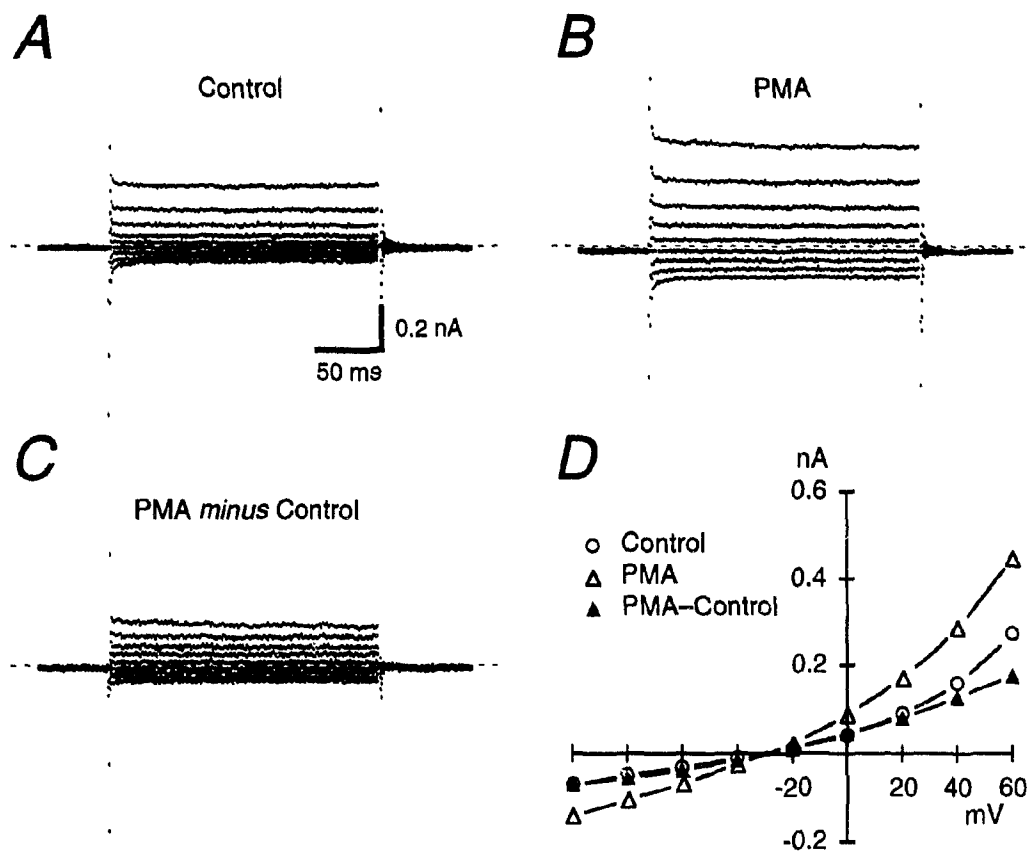
Figure 10. Activation of current by PMA

A,B. Families of currents elicited by 200-ms test pulses to potentials between -100 mV (*bottom trace*) and +60 mV (*top trace*) in 20 mV increments were obtained before (*A*) and 12 min after application of 100 nM PMA (*PMA*). ***C.*** PMA-activated currents isolated after subtraction of control from PMA-stimulated currents. ***D.*** Corresponding *I-V* relationships from ***A-C***.
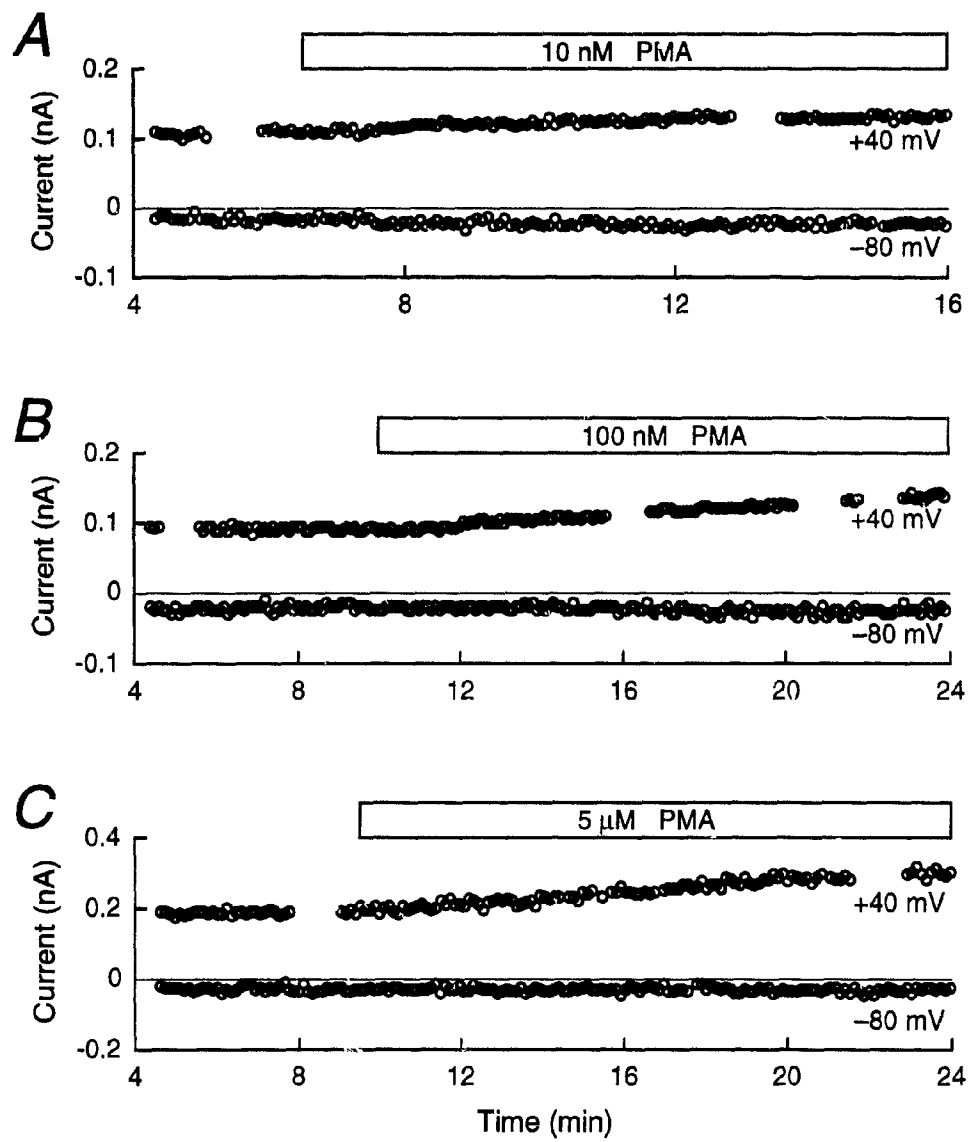
Figure 11. Time diaries of currents stimulated by PMA

A-C. Time diaries of current amplitudes at −80 mV and +40 mV measured from three myocytes before and during application of 10 nM (*A*), 100 nM (*B*), and 5 μM PMA (*C*).

and were usually not reversed by 5-10 min washouts with control solution, a difficulty previously reported in regard to other cardiac effects of phorbol esters (e.g. Yuan et al., 1987; Dösemeci et al., 1988; Tseng and Boyden, 1991; Walsh & Kass, 1991). However, stimulations produced by shorter treatments with moderate concentrations of PMA were reversible on occasion (data not shown).

The time courses of the stimulations caused by PMA, and their magnitudes, were evaluated by referencing PMA-induced increases in chord conductance (-80 mV to +40 mV) to the pre-PMA basal membrane conductances of the myocytes. A plot of the time courses of the conductance increments in myocytes treated with 100 nM or 5 µM PMA indicates that steady-state was generally reached within 6-9 min of drug application (Figure 12A). The concentration-response curve (Figure 12B) fitted to data from a large number of myocytes treated with single concentrations of PMA between 1 nM and 5 µM for 8 to 19 min indicates that the threshold concentration for stimulation was ≤ 1 nM, and the EC_{50} was ~10 nM. There were no significant differences between the increases produced by 100 nM ($40 \pm 9\%$, $n = 21$), 300 nM ($34 \pm 10\%$, $n = 8$), 1 µM ($39 \pm 5\%$, $n = 14$), and 5 µM ($35 \pm 7\%$, $n = 19$) PMA. These large standard errors indicate that there was a significant proportion of relatively insensitive myocytes in each of these four test groups; on a pooled basis, 12 of 62 (19%) myocytes responded with $\leq 15\%$ increase in conductance, and a similar proportion registered a $\geq 60\%$ stimulation.
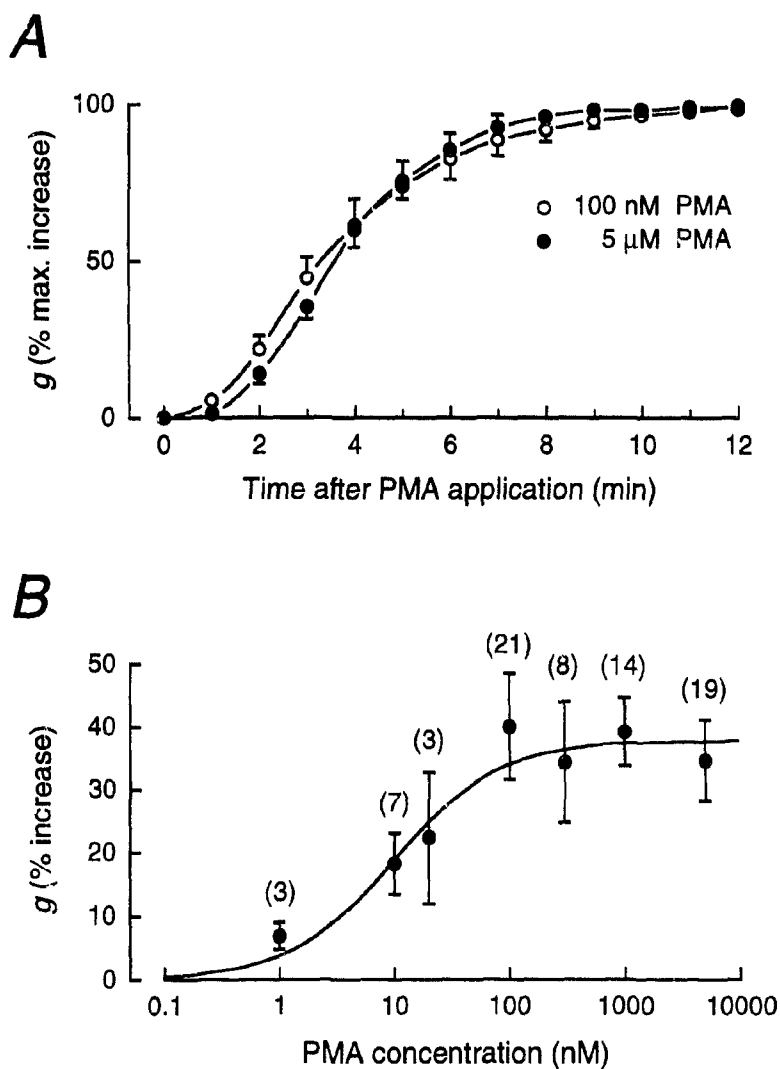
Figure 12. Time course of PMA stimulation and steady-state concentration-response relations

A. Normalized time courses of the increases in chord conductance (g, −80 to +40 mV) induced by 100 nM ($n = 9$) and 5 µM ($n = 8$) PMA. **B.** Concentration-g relationship. The data are from myocytes treated for 8–19 min with 1 nM, 10 nM, 20 nM, 100 nM, 300 nM, 1 µM, or 5 µM PMA. Data are expressed as percentage increase over pre-PMA g (−80 to +40 mV), and the curve is drawn according to $y = E_{max} / \{1 + (EC_{50} / [PMA])^n\}$, with $EC_{50} = 10.1$ nM, $E_{max} = 37.9\%$, and n = 0.95.

Anion and cation dependence

Control currents elicited by pulses to potentials between -100 and +60 mV were subtracted from currents elicited after 10-15 min treatment of myocytes with 100 nM or 5 μM PMA to isolate PMA-activated currents. After normalization by reference to the amplitude of current activated at +40 mV, the mean *I-V* relationships were superimposable, with common E_{rev} near -33 mV (Figure 13*A*). The other main feature emerging from this normalization is the strong degree of outward rectification. Chord conductance over the potential range E_{rev} to E_{rev} + 40 mV was twice as large as the conductance measured over the E_{rev} to E_{rev} - 40 mV range.

All of the results on PMA-treated myocytes described to this point were obtained from cells superfused with 144 mM Cl⁻ solution and dialyzed with 30 mM Cl⁻, 110 mM aspartate solution. To investigate the dependence of PMA-activated I_{Cl} on intracellular [Cl⁻], a group of myocytes was dialyzed with 140 mM Cl⁻, aspartate free solution (155 mM Cl⁻ superfusate, calculated E_{Cl} = -3 mV). Figure 13*B* illustrates the normalized, mean *I-V* (+40 mV = 1.0) of PMA-activated current obtained under the near symmetrical Cl⁻ condition. The *I-V* relationship crossed the voltage axis at about -5 mV. The ratio of the outward chord conductance (0 to +40 mV) to the inward chord conductance (0 to -40 mV) was 1.2 ± 0.1 (n = 5), considerably smaller than the 1.9 ± 0.03 (n = 16) value measured in low Cl⁻ dialyzed myocytes, and in rough accord with predictions of the Goldman-Hodgkin-Katz (GHK) current equation (see Discussion).

The E_{rev} of PMA-activated I_{Cl} was within a few millivolts of calculated E_{Cl} in the 5 myocytes dialyzed with high Cl⁻ solution (-5 ± 1.4 mV), and E_{rev} in one myocyte superfused with 75 mM Cl⁻, sucrose solution and dialyzed with 30 mM Cl⁻ solution was -20 mV (calculated E_{Cl} = -24 mV). These results indicate

A [Cl⁻]$_{pip}$ = 30 mM

Relative I_{Cl}

o   100 nM PMA
●   5 μM PMA

B [Cl⁻]$_{pip}$ = 140 mM

Relative I_{Cl}

o   100 nM PMA
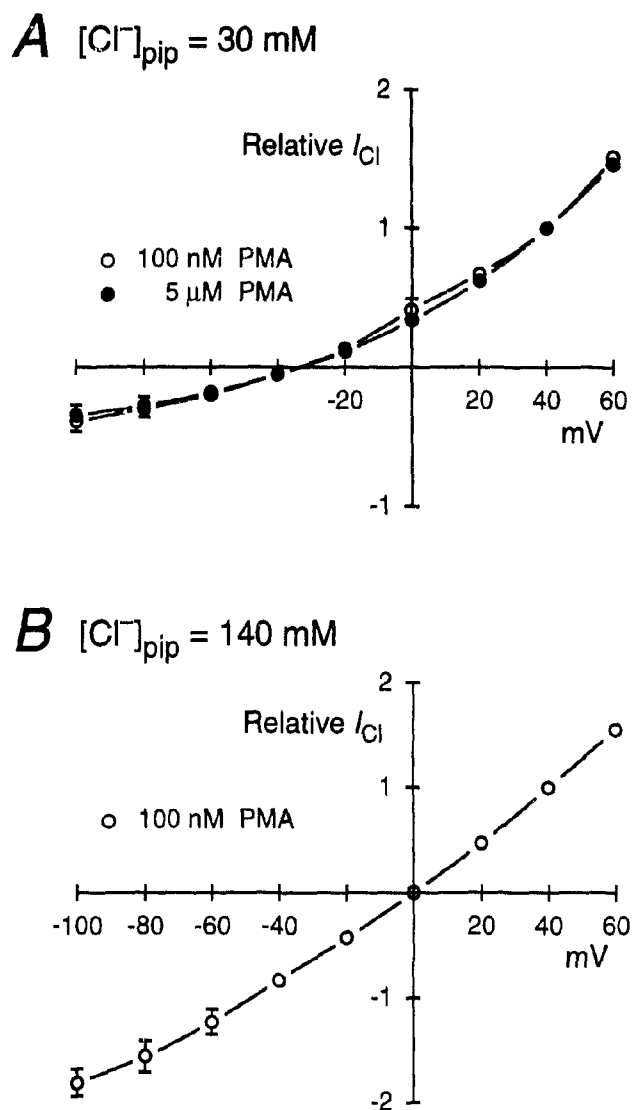
Figure 13. Normalized I-V relationships of PMA-activated I_{Cl}

A. I-V relationships of PMA-activated I_{Cl} from myocytes dialyzed with standard Cs^+ pipette solution containing 30 mM Cl^-. Pre-PMA currents were subtracted from PMA-stimulated currents (see Figure 11), normalized relative to the current activated at +40 mV, and averaged. n = 6 myocytes for each relationship. **B.** I-V relationships of PMA-activated I_{Cl} from myocytes dialyzed with Cs^+ pipette solution containing 140 mM Cl^- (n = 4).

that the current induced by PMA was a Cl^--dominated current. In two other PMA-treated myocytes, replacement of 140 mM of total 145 mM external Cl^- by I^- for 2 min shifted the E_{rev} of the activated current by -5 and -7 mV, and increased outward current at +40 mV by 20-30% (data not shown).

The absence of external Na^+ (particularly when replaced by TMA^+) markedly depressed cAMP-dependent activation of I_{Cl} in guinea pig ventricular myocytes (Harvey et al., 1991; Zakharov et al., 1995). The role of Na^+ in the PMA activation of I_{Cl} was investigated by conducting a series of experiments with a superfusate that contained 140 mM Na^+ instead of TMA^+. Figure 14A illustrates that 100 nM PMA incremented the current at +40 mV in a manner similar to that observed in myocytes superfused with TMA^+ solution. In 10 experiments, the stimulation in myocytes superfused with Na^+ solution averaged $40 \pm 7\%$ compared to $40 \pm 9\%$ ($n = 21$) with TMA^+ solution (Figure 14B).

Inhibition by 9-AC

Anthracene-9-carboxylic acid (9-AC) is a widely-used anion transport inhibitor (Cabantchik and Greger, 1992) that has a moderate to strong inhibitory action on PKA-activated cardiac I_{Cl} (e.g. Harvey et al., 1990; Levesque et al., 1993; Gadsby et al., 1995). Attempts to measure the effects of 1 mM 9-AC on PMA-activated I_{Cl} were complicated by what appeared to be rapid development of a significant leak current or other membrane destabilizing effect in about 30% of the myocytes tested. (9-AC-like Cl^- channel inhibitors are known to cause depolarization/destabilization in other preparations (Cabantchik and Greger, 1992); also see Harvey (1993) for anomalous activation of current by Cl^- transport-inhibiting stilbene derivatives.) This problem was
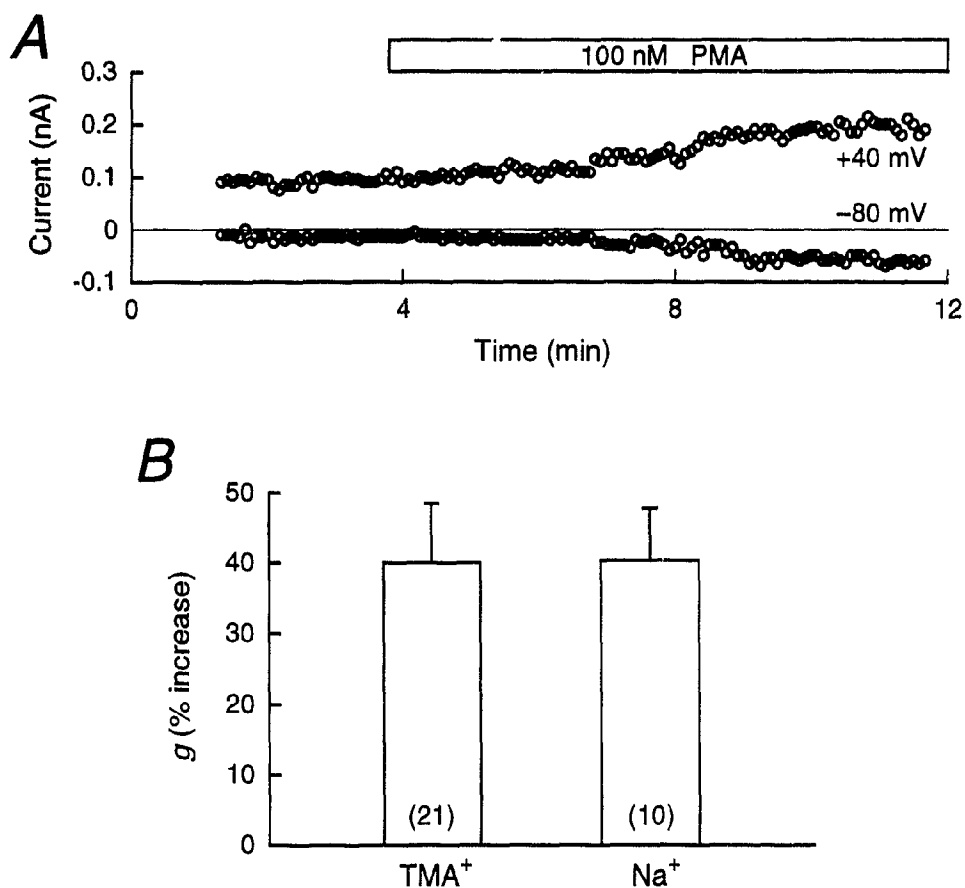
Figure 14. Activation of current by PMA in myocytes superfused with K^+-, Ca^{2+}-free Tyrode's solution containing 140 mM Na^+

A. Time diary of current amplitude at –80 and +40 mV. **B.** Comparison of stimulation (% increase in g (–80 to +40 mV)) in myocytes superfused with Na^+-containing and TMA^+-containing solutions. Number of myocytes in brackets.

minimized by measuring the Cl⁻ conductance that was (i) *inhibited* by *addition* of 9-AC to 7 of 10 PMA-treated myocytes in which there was no obvious 9-AC-induced increase in leak current, or (ii) *disinhibited* by the *removal* of 9-AC from (PMA + 9-AC)-treated myocytes.

Figure 15A shows an example of the first type of measurement in myocytes superfused with K^+-, Ca^{2+}-free (Na^+-containing) Tyrode's solution. The current was measured at 0 mV to minimize the influence of any nonspecific leak conductance centred around 0 mV. Application of 100 nM PMA induced a significant current, and 1 mM 9-AC rapidly abolished it. In 7 myocytes, 62 ± 15% of PMA-activated g_{Cl} was inhibited by 9-AC (Figure 15B). For comparison, Zhang et al. (1994a) reported that 0.1 mM 9-AC blocked 48% ($n = 5$) of the PMA-activated current in feline ventricular myocytes, and Walsh and Long (1994) found that 1 mM 9-AC blocked 52 ± 9% ($n = 3$) of the current activated by phorbol ester in guinea pig ventricular myocytes dialyzed with exogenous PKC.

An example of the second type of measurement is provided in Figure 15C. The membrane conductance of the myocyte was stable for the 3 min prior to co-application of 100 nM PMA and 1 mM 9-AC. It remained stable for the ensuing 5 min, but then promptly increased by approximately 2 nS when 9-AC was removed from the bath. In four experiments of this type, relief from 9-AC inhibition elicited a 42 ± 10% increase in conductance (Figure 15D), a value which is not statistically different than the control stimulation by 100 nM PMA in myocytes superfused with Na^+ solution (40 ± 7% ($n = 10$), Figure 14B).
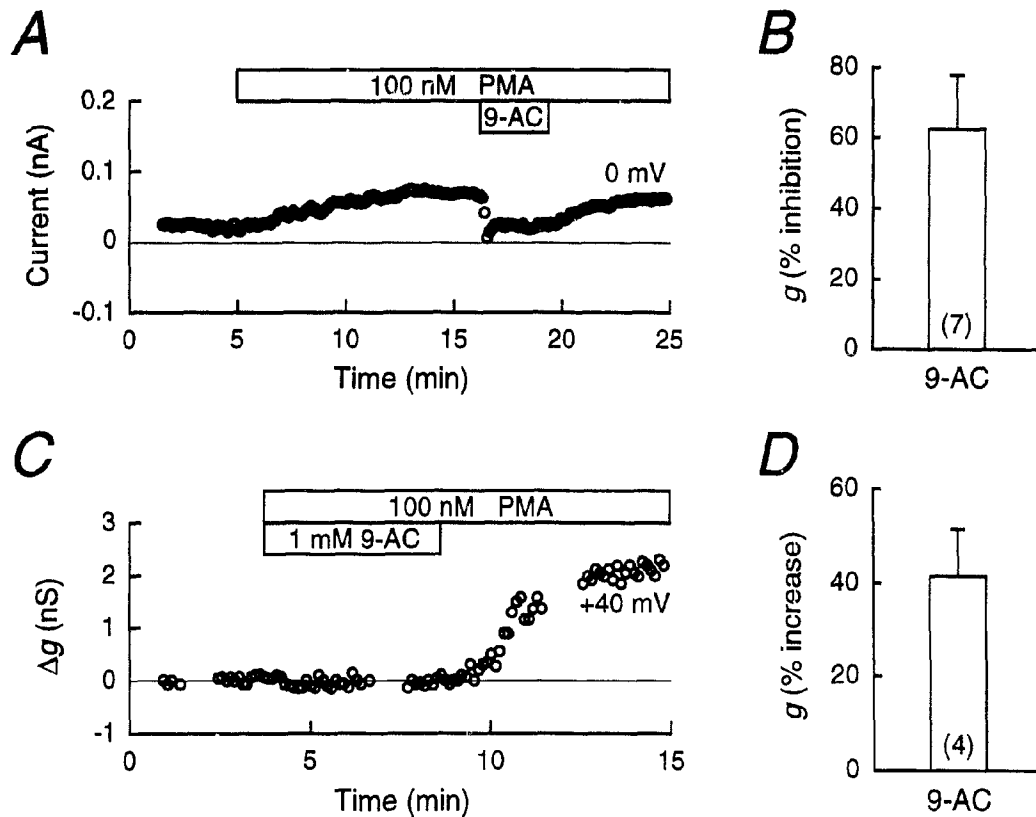
Figure 15. Effect of 1 mM 9-AC on PMA-activated Cl⁻ conductance

A. Inhibition by 9-AC of PMA-induced outward current measured on 200-ms pulses to 0 mV. **B.** Summary of inhibition by 9-AC in experiments similar to **A**. The data are expressed as percentage decrease in PMA (100 nM, >6 min)-induced chord conductance (E_{rev} to +40 mV) measured ~3 min after addition of 1 mM 9-AC. Number of myocytes in brackets. **C.** Disinhibition of the conductance (E_{rev} to +40 mV) induced by 100 nM PMA, upon removal of external 9-AC, expressed as difference (Δg) from background conductance. **D.** Increase in conductance expressed as a percentage of background conductance in experiments similar to that in **C**. Na⁺ superfusates were used in **A**, **C**, **D**, and partly in **B**.

2. Stimulation of I_K by PMA

Figure 16 illustrates the effects of PMA on I_K in a myocyte dialyzed with K^+ pipette solution and pulsed at 0.2 Hz with 500-ms test depolarizations to +50 mV followed by repolarizations to -40 mV. Application of maximally-effective 100 nM PMA (cf. Tohse et al., 1987; see below) increased rising outward currents and associated I_K tail amplitudes to new steady-states within 5-6 min (Figure 16A). The records and I-V relationships obtained from the example experiment before and 7 min after application of PMA illustrate that the increase in I_K tail amplitude was about 35%, and that the tail amplitude was enhanced over the entire activation range during exposure to PMA (Figures 16B and C).

Time course and concentration dependence of PMA stimulation

The example time diary in Figure 16A shows that, after a lag of ~1 min, PMA increased I_K over the next 5-6 min. An analysis of the time courses of the I_K responses to 10-100 nM PMA in experiments of this type indicates that the time course of I_K stimulation by PMA was similar to that of I_{Cl} activation by the phorbol ester. The delay prior to I_K tail stimulation was about 1 min, and the half-time to maximal stimulation was 2.1 ± 0.2 min (n = 6) (Figure 17A).

The effects of 0.1, 1, 10, 100, and 1000 nM PMA on steady-state I_K responses are shown in Figure 17B. I_K was unchanged by 0.1 nM PMA (n = 3), and stimulated by 6 ± 3% (n = 5), 23 ± 5% (n = 13), and 31 ± 4% (n = 13) with 1, 10, and 100 nM PMA, respectively. There was a downturn in I_K stimulation at higher concentrations of PMA; 1 μM PMA only increased I_K by 21 ± 4% (n = 6), and, the increment was 15% and 28% in two cells treated with 5 μM PMA. Smaller stimulation of I_K with higher than 100 nM concentrations of PMA has been observed in guinea pig ventricular myocytes (Tohse et al., 1987).
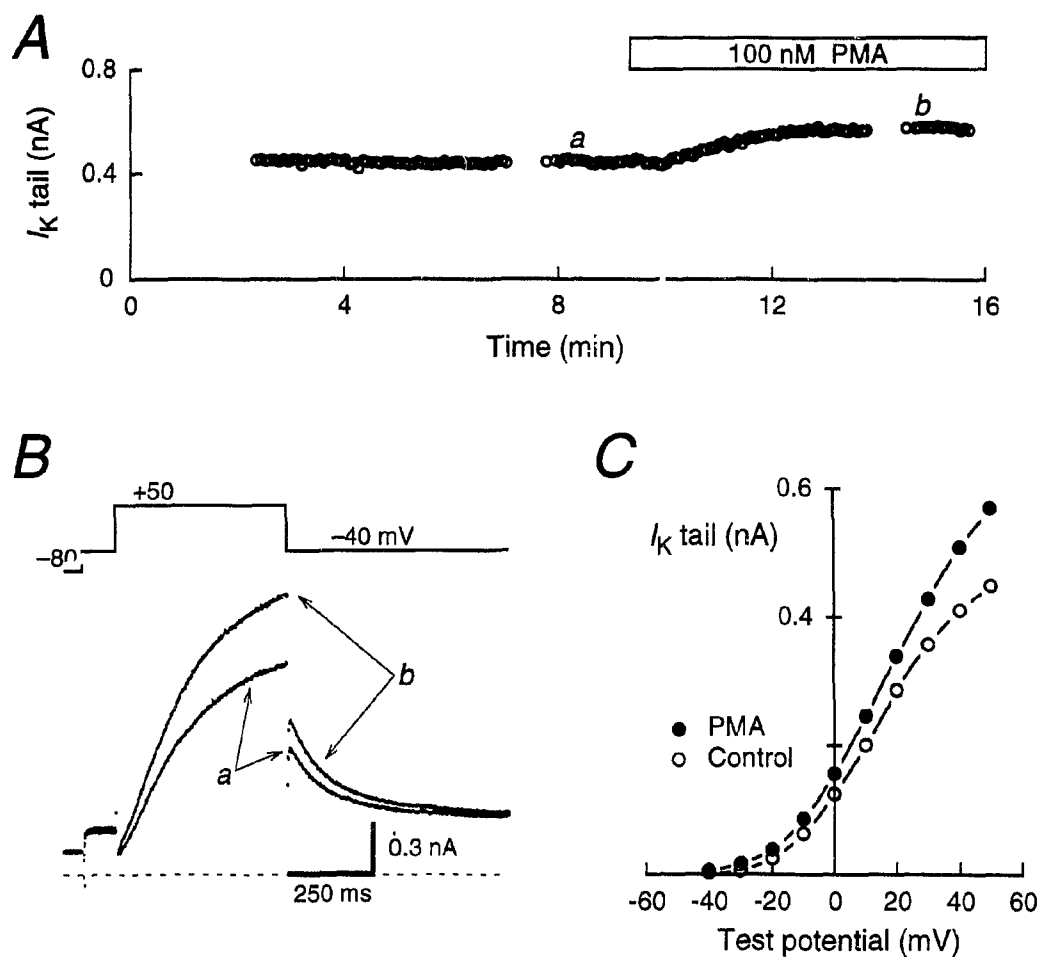
Figure 16. Stimulation of I_K by PMA

A. Time diary of I_K tail current amplitudes recorded at –40 mV following 500-ms test pulses to +50 mV. 100 nM PMA was applied extracellularly as indicated by the bar above the diary. **B.** Superimposed current records taken at the times indicated in **A.** **C.** *I-V* relationships obtained before (*Control*) and after 5 min application of 100 nM PMA (*PMA*).
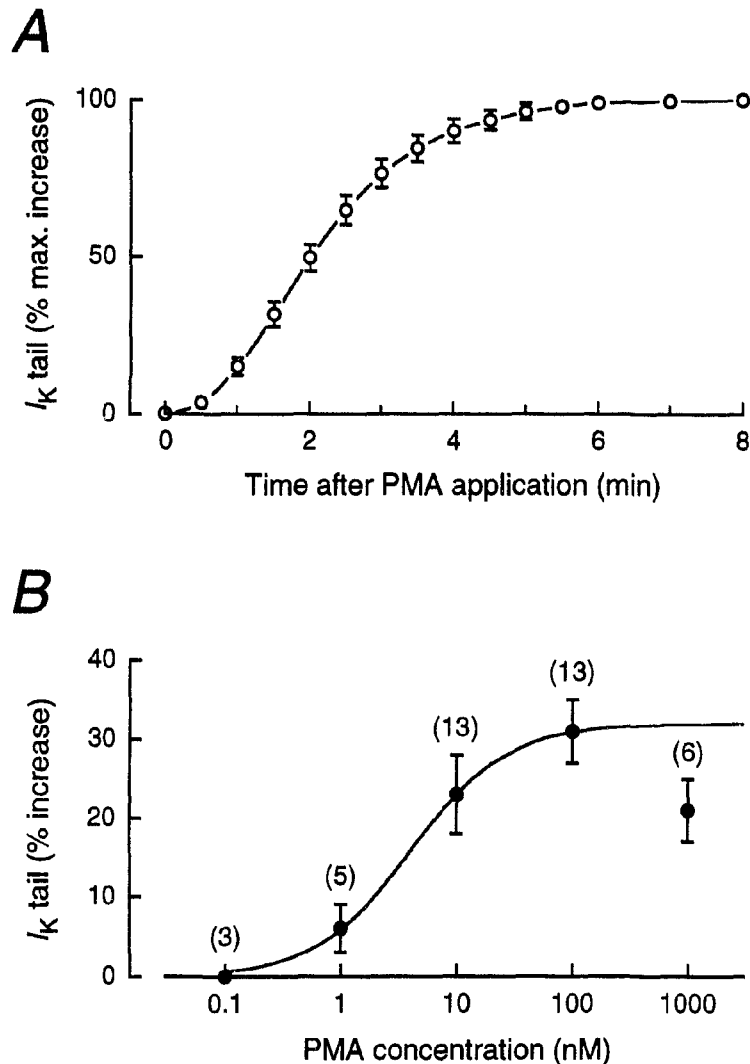
Figure 17. Time course of PMA stimulation of I_K and steady-state concentration-response relations

A. Normalized time course of the increase in I_K tail amplitude induced by 100 nM PMA ($n = 6$). **B.** Concentration-response relationship for I_K tail. The data are from myocytes treated for 5–8 min with 0.1 nM, 1 nM, 10 nM, 100 nM, or 1 µM PMA. Data are expressed as percentage increase over pre-PMA I_K tail following test pulses to +50 mV, and the curve is drawn from 0.1–100 nM PMA data according to $y = E_{max} / \{1 + (EC_{50} / [PMA])^n\}$, with $EC_{50} = 4.1$ nM, $E_{max} = 32.0\%$, and n = 1.06. Stimulation with 1 µM PMA was not significantly smaller than with 100 nM.

3.   Lack of effect of PMA on I_{K1}

I_{K1} was measured in myocytes superfused with normal Tyrode's solution and dialyzed with K$^+$ pipette solution. From a holding potential of -60 mV, 500-ms test pulses to quite negative potentials (-110 to -90 mV) elicited inward-going I_{K1}, whereas test pulses to potentials between -70 and -30 mV elicited outward-going I_{K1}. Original current records and I-V relationships collected before and 5 min after application of 100 nM PMA are shown in Figure 18A-C. PMA had little effect on either inward or outward I_{K1} activated by test potentials between -110 and -30 mV. Figure 18D shows a summary of the effects of 100 nM PMA and inactive phorbol ester 4α-phorbol 12,13-didecanoate (αPDD) on inward (measured at -100 mV) and outward (measured at -40 mV) components of I_{K1}. Both components of I_{K1} were essentially insensitive to the phorbol esters.

4.   Inhibition of $I_{Ca,L}$ by PMA

Since the literature on cardiac L-type Ca^{2+} channel responses to PKC activation is replete with contrasting results (see Introduction), and since variable responsiveness to phorbol esters (e.g. Tseng and Boyden, 1991; Zhang et al., 1994a; and this study) could lead to mixed results, $I_{Ca,L}$ was frequently measured with I_K in the same myocyte using similar pulsing conditions as to that used for the I_K experiments in the previous section. Figure 19 shows the effects of 100 nM PMA on a myocyte pulsed with 500-ms test depolarizations from prepulse -40 mV. Current records obtained from pulses to 0 mV before and 5 min after application of PMA indicate that the phorbol ester slightly depressed the inward $I_{Ca,L}$ peak and moderately enhanced the late time-dependent outward current (Figure 19A). An analysis of currents recorded from
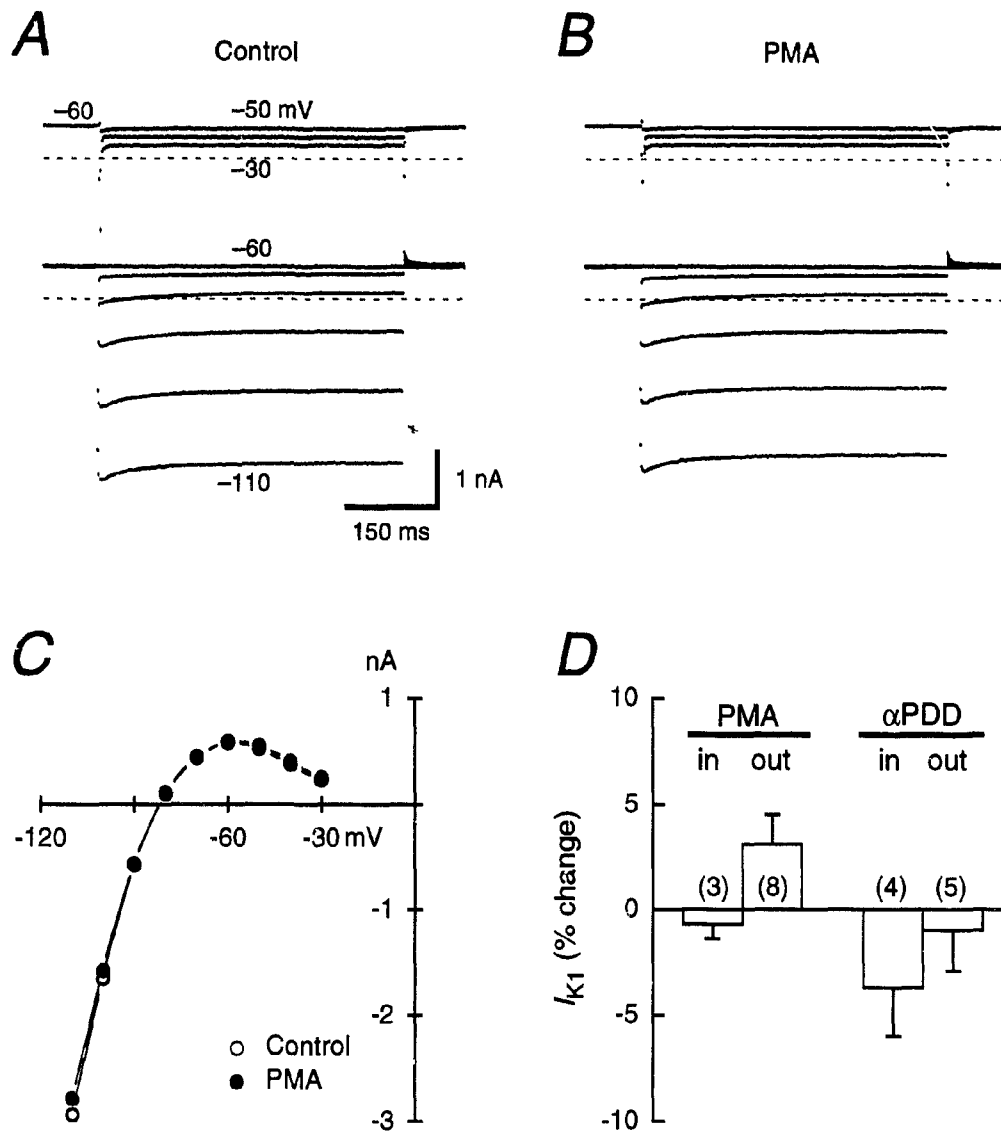
Figure 18. Lack of effect of phorbol esters on I_{K1}

A,B. Families of current records elicited by 500-ms test pulses to potentials between –110 and –30 mV in 10 mV increments from a holing potential of –60 mV were obtained before (*A*) and after 5 min application of 100 nM PMA (*B*). *C. I-V* relationships from *A* and *B. C.* Summary of changes in inward (at –100 mV) and outward (at –40 mV) components of I_{K1} induced by 100 nM PMA (*left two columns*) and 100 nM αPDD (*right two columns*). Number of myocytes in brackets.
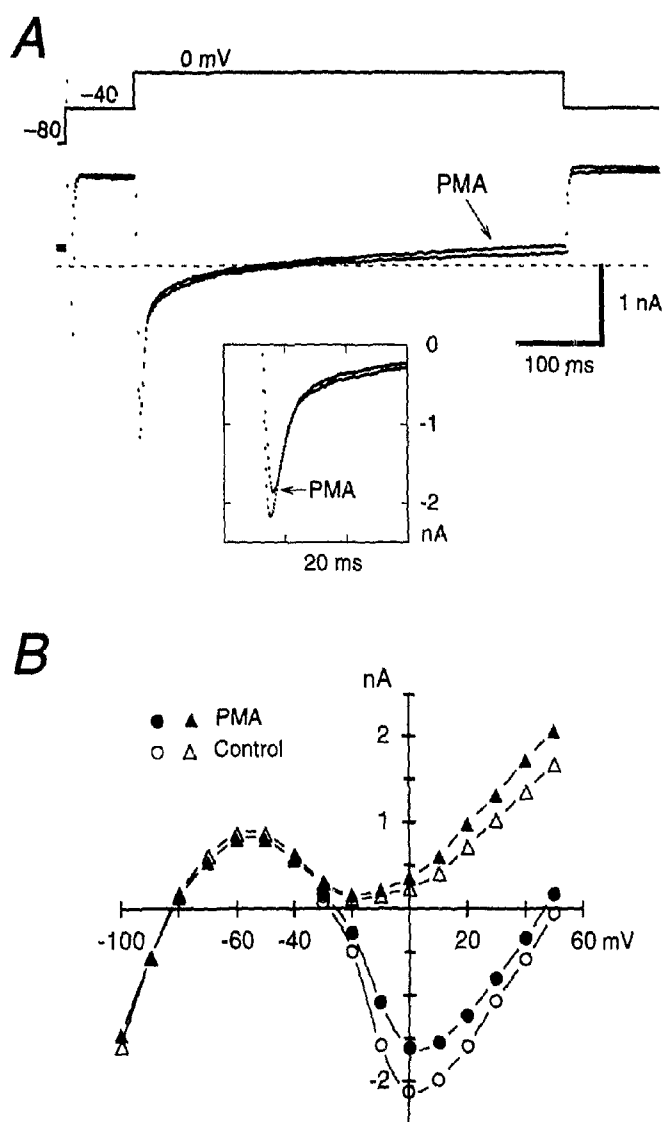
Figure 19. Effect of PMA on $I_{Ca,L}$ and K^+ currents

A. Superimposed current records obtained on test depolarizations to 0 mV before and after 5 min application of 100 nM PMA. The inset shows the inward-going $I_{Ca,L}$ transients on an expanded time scale. ***B.*** *I-V* relationships from a different myocyte. Peak inward currents (circles) and currents at the end of 500-ms test pulses (triangles) were measured before (open symbols) and 6–7 min after the addition of 100 nM PMA (closed symbols), with reference to zero current.

another myocyte treated with 100 nM PMA for 6–7 min is shown in Figure 19B. On test depolarizations between −20 and +20 mV, the phorbol ester caused a significant depression of $I_{Ca,L}$ (circles) (o..e of the largest recorded) and a stimulation of the delayed outward current at positive potentials (triangles), primarily due to an increase in I_K. In contrast, PMA caused little change in I_{K1} at potentials between −100 and −40 mV, confirming the lack of PMA responses on I_{K1}, even when I_K was stimulated by PMA.

Two pertinent observations at this point are as follows. (i) After application of 10–100 nM PMA for 5–15 min, washout for 10–15 min rarely produced a reversal of $I_{Ca,L}$ inhibition. (ii) On average, 10–100 nM PMA depressed the amplitude of $I_{Ca,L}$ by ~7%, i.e. an inhibitory effect was absent or quite small in m·ny of the myocytes studied. A full listing of these data is provided in Fig· ·e 28, but I point out here that the examples presented in the figures are generally from myocytes with $I_{Ca,L}$ "sensitive" to inhibition by PMA.

Earlier reports have suggested that the PKC-dependent effects of phorbol esters on Ca^{2+} channel currents may be voltage-dependent (Lacerda et al., 1988; Tseng and Boyden, 1991). However, the I-V relationships in Figure 19B show that PMA did not affect the shape of the $I_{Ca,L}$-V relationship. This apparent lack of effect on voltage-dependency was more closely studied in two ways, first by varying the holding potential, and second by measuring the degree of inhibition at various test potentials. Figure 20A indicates that the average response at a constant test potential of 0 mV was independent of the holding potential (−80 or −40 mV). Figure 20B shows that, at a constant holding potential of −80 mV, the degree of $I_{Ca,L}$ inhibition on test pulses tended to be larger at −20 mV than at more positive potentials. However, the latter effect was not significant at the $p < 0.05$ level. Thus, there as no indication that the
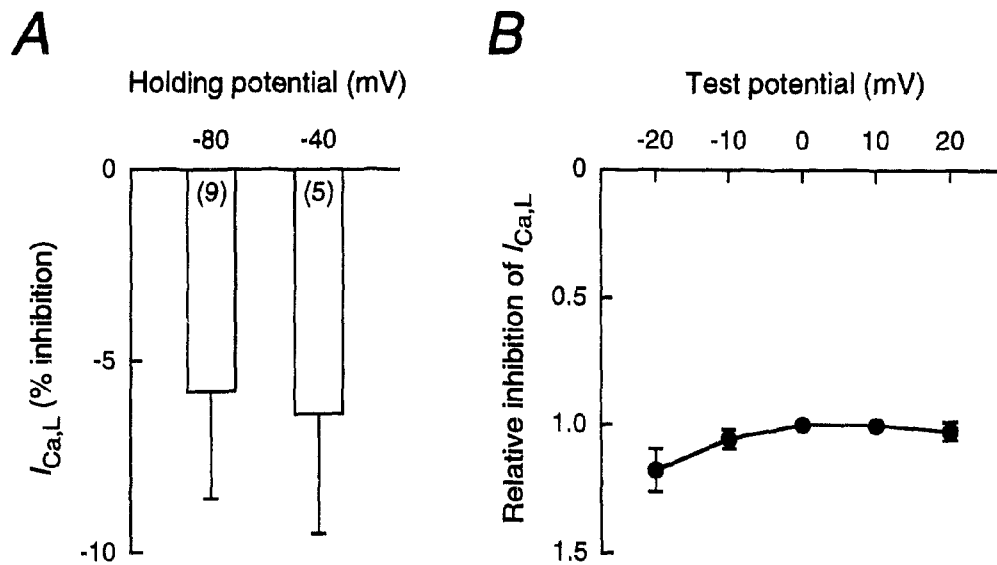
Figure 20. Voltage dependence of $I_{Ca,L}$ inhibition by PMA

All myocytes were dialyzed with Cs^+ (high Cl^-) solution. **A.** Dependence on holding potential. The percentage change in $I_{Ca,L}$ amplitude 3 min after addition of 10–100 nM PMA was measured from currents elicited on test pulses to 0 mV applied after 80-ms prepulses to –40 mV from –80 mV holding potential, or applied from a –40 mV holding potential. Number of myocytes is given in brackets. **B.** Degree of inhibition of $I_{Ca,L}$ at different test potentials between –20 and +20 mV with reference to the inhibition at 0 mV. Holding potential –80 mV; $n = 6$ myocytes.

inhibitory effect of PMA on $I_{Ca,L}$ was voltage-dependent. Dösemeci et al. (1988) observed that PMA stimulation of rat cardiac $I_{Ca,L}$ had no effect on the shape of the $I_{Ca,L}$-V relationship.

Time courses of PMA effects on $I_{Ca,L}$ in myocytes in which I_K was co-monitored

As indicated above, the effects of 10–100 nM PMA were examined on a number of myocytes in which both peak $I_{Ca,L}$ and I_K tail amplitudes were monitored. The example time diaries in Figures 21A and B show that, after a lag of ~1 min, PMA increased I_K by 30–60% over the next 4–6 min. This result is quantitatively similar to that obtained in experiments on I_K alone (see Figure 17A). By contrast, the phorbol ester had a moderate inhibitory effect on $I_{Ca,L}$ that reached steady-state within 2 min. An analysis of the time courses of the I_K and $I_{Ca,L}$ responses to 10–100 nM PMA in experiments of this type shows that the delay prior to I_K tail stimulation was about 1 min, and the half-time to maximal stimulation was 2.2 ± 0.2 min (n = 5) (Figure 21C). $I_{Ca,L}$ (from myocytes with ≥10% depression) began to decline almost immediately and reached 50% of maximum in a significantly-shorter 0.7 ± 0.2 min (n = 5). For reference, the lag to stimulation of I_{Cl} by 100 nM PMA was 1–2 min, and the time to half-maximal response was 3.3 ± 0.4 min (n = 9) (see Figure 12A).

Figure 21. Time courses of changes in $I_{Ca,L}$ and I_K induced by PMA

A,B. Time diaries of I_K tail amplitudes measured at −40 mV and peak $I_{Ca,L}$ measured at 0 mV from two myocytes pulsed with 500-ms test pulses to 0 mV. The pulses to 0 mV were periodically interrupted for a set of test pulses to +30, +40, and +50 mV to monitor I_K activation. **C.** The time courses of the changes in $I_{Ca,L}$ and I_K induced by 10–100 nM PMA. $I_{Ca,L}$ was measured on pulses to 0 mV, and I_K on repolarization to −40 mV after pulses to +50 mV in 5 myocytes.
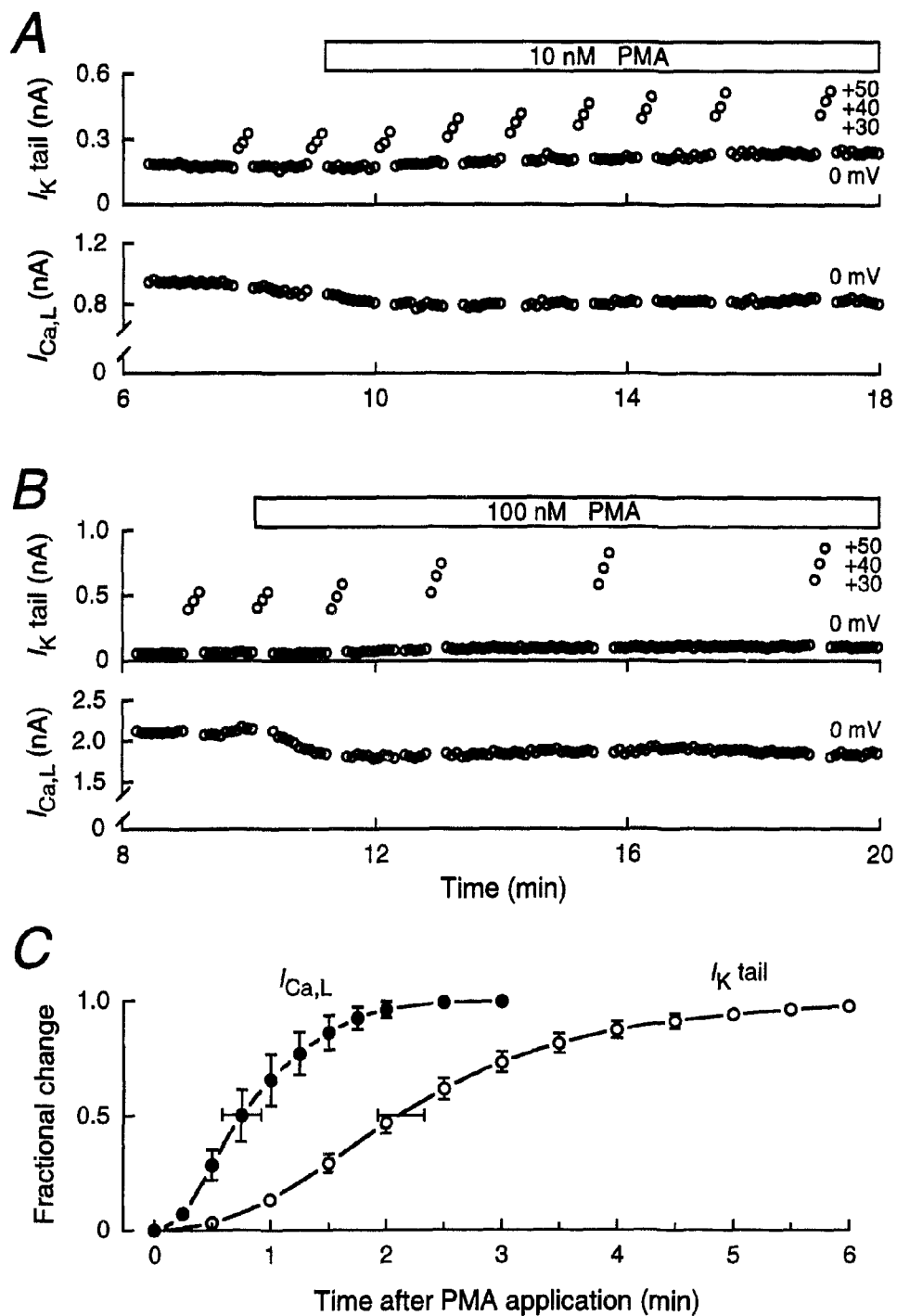
Figure 21. Time courses of changes in $I_{Ca,L}$ and I_K induced by PMA

B.   INVESTIGATION OF PKC INVOLVEMENT IN PMA RESPONSES

The possible role of PKC activation in the PMA stimulation/inhibition of I_{Cl}, I_K, and $I_{Ca,L}$ was investigated by (i) determining whether pretreatment with 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) suppressed the effects of PMA, (iii) examining whether dialysate pCa influenced the response to PMA, and (ii) comparing the effects of the inactive phorbol esters αPDD and α-phorbol (αPHR) with those of PMA.

1.   PKC is involved in the stimulation of I_{Cl} by PMA

Effects of protein kinase inhibitor H-7 on PMA activation of I_{Cl}

The protein kinase inhibitor H-7 (Hidaka et al., 1984) was used to inhibit PKC activity (e.g. Tohse et al., 1987; Conn et al., 1989; Doerner et al., 1990; Honoré et al., 1991; Forstner et al., 1994; for recent review, see Hidaka and Kobayashi, 1992). In most experiments, 20 μM H-7 was included in the superfusate, and 200 μM H-7 was added to the pipette solution. Neither the amplitude of basal current nor the shape of the basal I-V relationship was affected by 20-min application of this protocol (data not shown).

Figure 22A shows a time diary of current amplitude monitored at -80 and +40 mV from a myocyte pretreated with H-7 for 20 min prior to application of 5 μM PMA. PMA failed to elicit a stimulation over the 9 min observation period. This result suggests that stimulation of I_{Cl} by PMA occurs via activation of PKC.
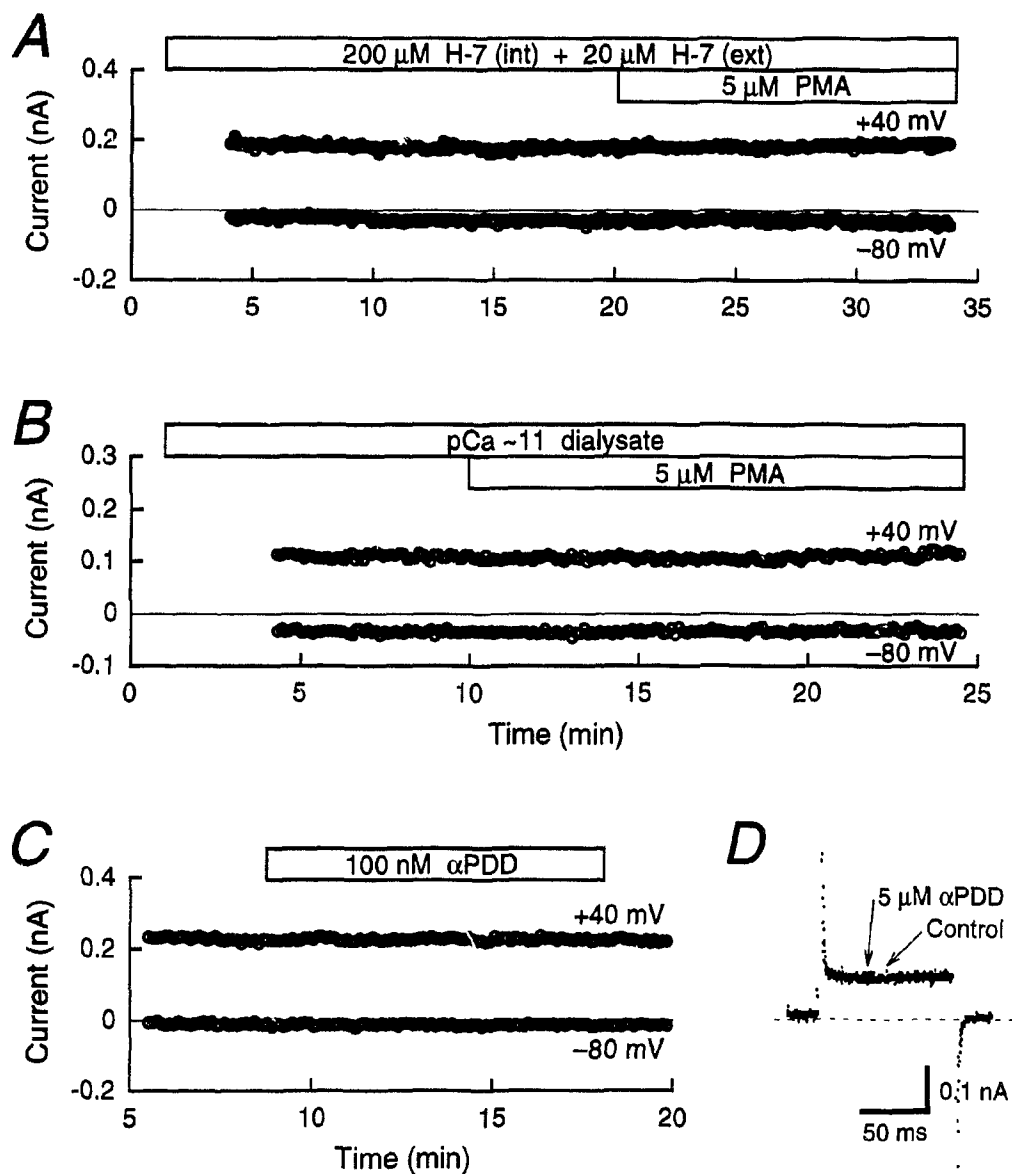
Figure 22. Experiments with H-7, pCa ~11 dialysate, and αPDD

A. Lack of stimulation by 5 μM PMA in a myocyte pretreated for 20 min with 20 μM H-7 superfusate/200 μM H-7 (pCa 9) dialysate. ***B.*** Lack of stimulation by 5 μM PMA in a myocyte dialysed with pCa ~11 solution. ***C,D.*** Lack of stimulation by 100 nM (*C*) and 5 μM (*D*) αPDD; the superimposed current records in *D* were obtained before (*Control*) and 9 min after addition of αPDD. pCa 9 dialysate.

Effects of high pCa dialysates on PMA activation of I_{Cl}

The experiments on I_{Cl} described to this point were carried out on myocytes dialyzed with a pCa 9 solution. Dialysis with pCa 11 solution instead of pCa 9 solution has been shown to inhibit the activation of I_K by PMA in guinea pig ventricular myocytes (Tohse et al., 1987, 1990). The dependence of PMA stimulation of I_{Cl} on dialysate pCa was investigated using pCa ~11 solution. Figure 22B indicates that there was also little stimulation of I_{Cl} when PMA was applied to a myocyte dialyzed with pCa ~11 solution.

Effects of inactive phorbol ester on I_{Cl}

Figure 22C shows measurements of current amplitude at -80 mV and +40 mV from a representative myocyte treated with the inactive phorbol ester αPDD (100 nM); the 10-min application of the inactive phorbol ester had little effect. Records from a different myocyte (Figure 22D) indicate that a 50-fold larger concentration of this phorbol ester was equally ineffective in activating Cl⁻ current.

Summary of results

A summary of the data from experiments investigating PKC involvement in the PMA stimulation (Figure 23) indicates that (i) compared to control stimulation with 5 µM PMA ($34.6 \pm 6.5\%$, $n = 19$), stimulation was absent ($1 \pm 3\%$, $n = 5$) in myocytes pretreated with H-7, (ii) compared to standard pCa 9 dialysate conditions, stimulation with 5 µM PMA was minimal ($3 \pm 3\%$, $n = 7$) in myocytes dialyzed with pCa 11 solution, and reduced ($15 \pm 7\%$, $n = 6$) in myocytes dialyzed with pCa 7 solution, and (iii) compared to control
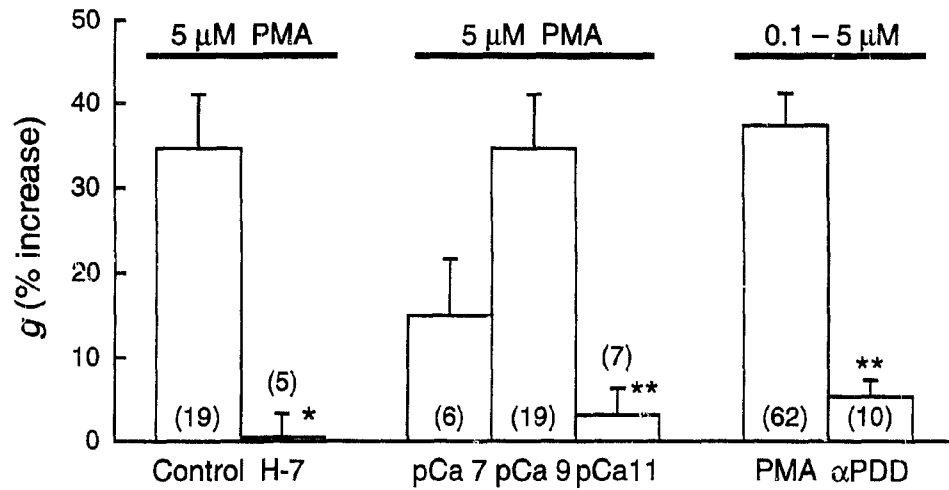
Figure 23. Summary of results from experiments on PKC involvement in PMA action

Data from experiments with H-7, pCa 7–11 dialysates, αPDD. The H-7 and αPDD experiments were conducted with pCa 9 dialysate. The data are expressed as percentage increase in g (−80 to +40 mV) over pre-PMA values. H-7, αPDD, and pCa ~11 data are significantly different than control (*, $p < 0.05$; **, $p < 0.01$); pCa 7 (versus 9) is not significantly different. Number of myocytes in brackets.

stimulation with 0.1–5 µM PMA (37 ± 4%, n = 62), there was little stimulation (5 ± 2%, n = 10) with a similar range of αPDD concentrations.

2.   PKC involvement in the responses of I_K and $I_{Ca,L}$ to PMA

Effects of protein kinase inhibitor H-7 on I_K and $I_{Ca,L}$ responses

In H-7 experiments on I_K and $I_{Ca,L}$, a 50 µM concentration of the inhibitor was added to the external solution for up to 15 min prior to the addition of PMA. Addition of the kinase inhibitor alone had distinct effects on basal I_K and basal $I_{Ca,L}$. These are described first.

The time diary in Figure 24A shows that 50 µM external H-7 reduced the basal I_K tail current amplitude by about 50%. The effect was rapid, with new steady-state being reached within 2–3 min and amplitude changing very little thereafter. Original currents taken at the times indicated in the time diary are illustrated in Figure 24B, and I-V relationships measured before and 4 min after application of H-7 are shown in Figure 24C. H-7 caused little change in the shape of the I-V relationship. On average, 50 µM H-7 caused a 42 ± 3% (n = 19) inhibition of the I_K tail current measured upon repolarization from +50 mV (Figure 24D).

Figure 25 describes the effects of 50 µM H-7 on basal $I_{Ca,L}$. As indicated by the time diary in Figure 25A, $I_{Ca,L}$ elicited by pulses to 0 mV declined quickly after the addition of H-7, although the degree of inhibition was much smaller than for I_K. Original currents obtained at the times marked in the time diary are presented in Figure 25B, and these indicate that the time to peak and inactivation phase were little affected by the inhibitor. The I-V relationships measured before and 5 min after application of H-7 indicate that H-7 caused almost no change in the shape of the I-V relationship (Figure 25C). Average

Figure 24. Effect of H-7 on basal I_K

A. Time diary of I_K tail amplitudes recorded at −40 mV following 500-ms depolarizations to +50 mV at 0.2 Hz (see pulse protocol illustrated on top of the records in **B**). 50 μM H-7 was bath-applied as indicated by the bar above the diary. **B.** Superimposed original currents taken at the times indicated in **A**. Prepulse duration was 50 ms in this experiment. **C.** I-V relationships measured before and 4 min after application of H-7. **D**: Summary time diary of I_K tail amplitude in control myocytes (o, n = 12) and in myocytes treated with H-7 (●, n = 17); the average application time for H-7 was 6.1 min. The control curve was taken from Figure 3D.
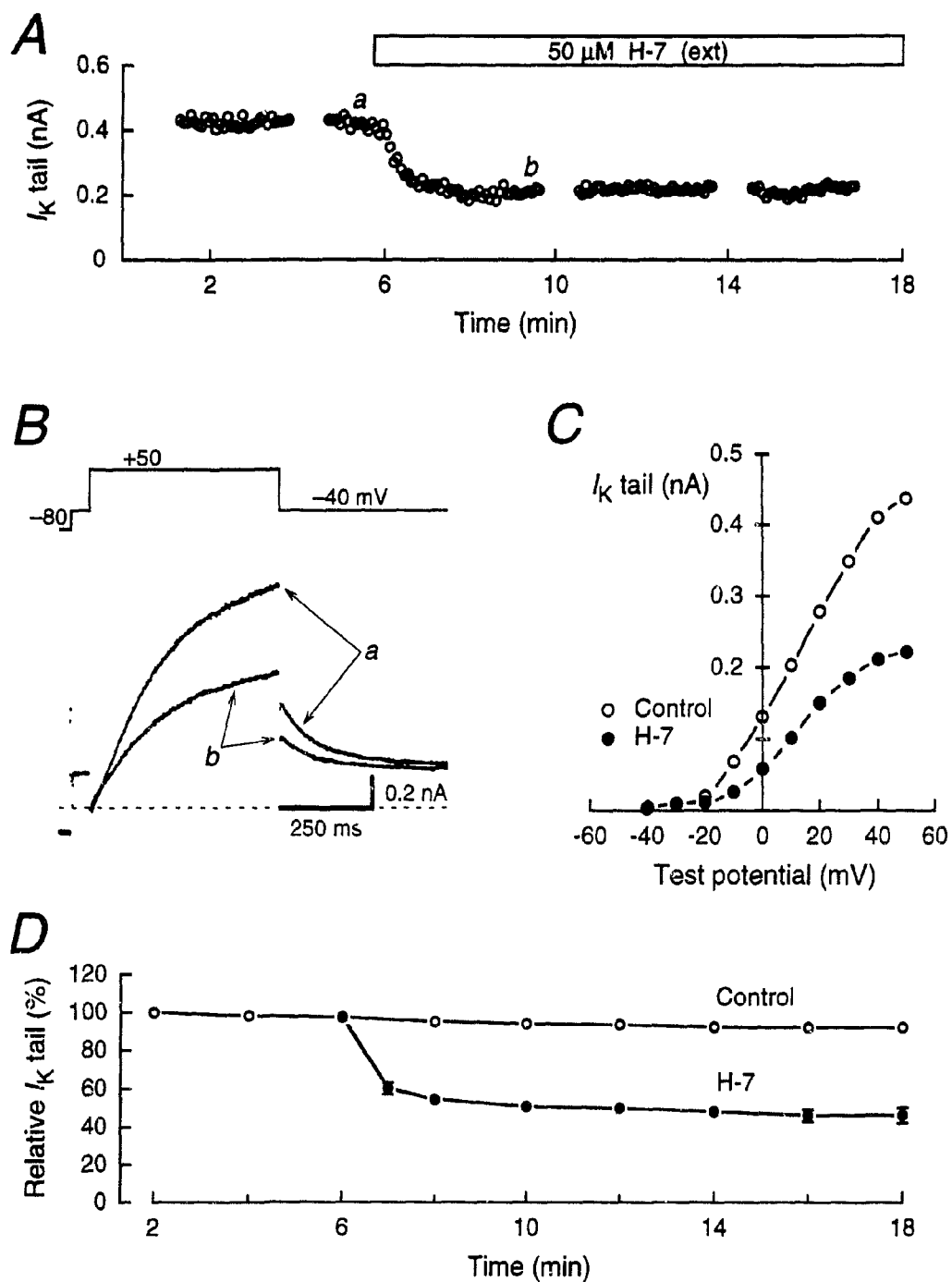
Figure 24. Effect of H-7 on basal I_K

Figure 25. Effect of H-7 on basal $I_{Ca,L}$

A. Time diary of peak $I_{Ca,L}$ amplitudes elicited at 0.2 Hz by test pulses to 0 mV. 50 μM H-7 was applied extracellularly as indicated by the bar above the diary. **B.** Superimposed original currents taken at the times indicated in **A.** Only the initial 100-ms portion of the depolarization to 0 mV is shown. **C.** I-V relationships measured before and 5 min after application of H-7. **D.** Summary time diary of $I_{Ca,L}$ amplitude in control myocytes (o, n = 13) and in myocytes treated with H-7 (●, n = 16); H-7 was applied for an average 6.0 min. The control curve was taken from Figure 5D.

A

50 μM H-7 (ext)

$I_{Ca,L}$ (nA)

a

b

Time (min)

B

0 mV

−40

b

a

0.5 nA

25 ms

C

nA

0.5

−40   20   60 mV

−0.5

−1.5

● H-7
○ Control

D

Relative $I_{Ca,L}$ (%)

Control

H-7

Time (min)
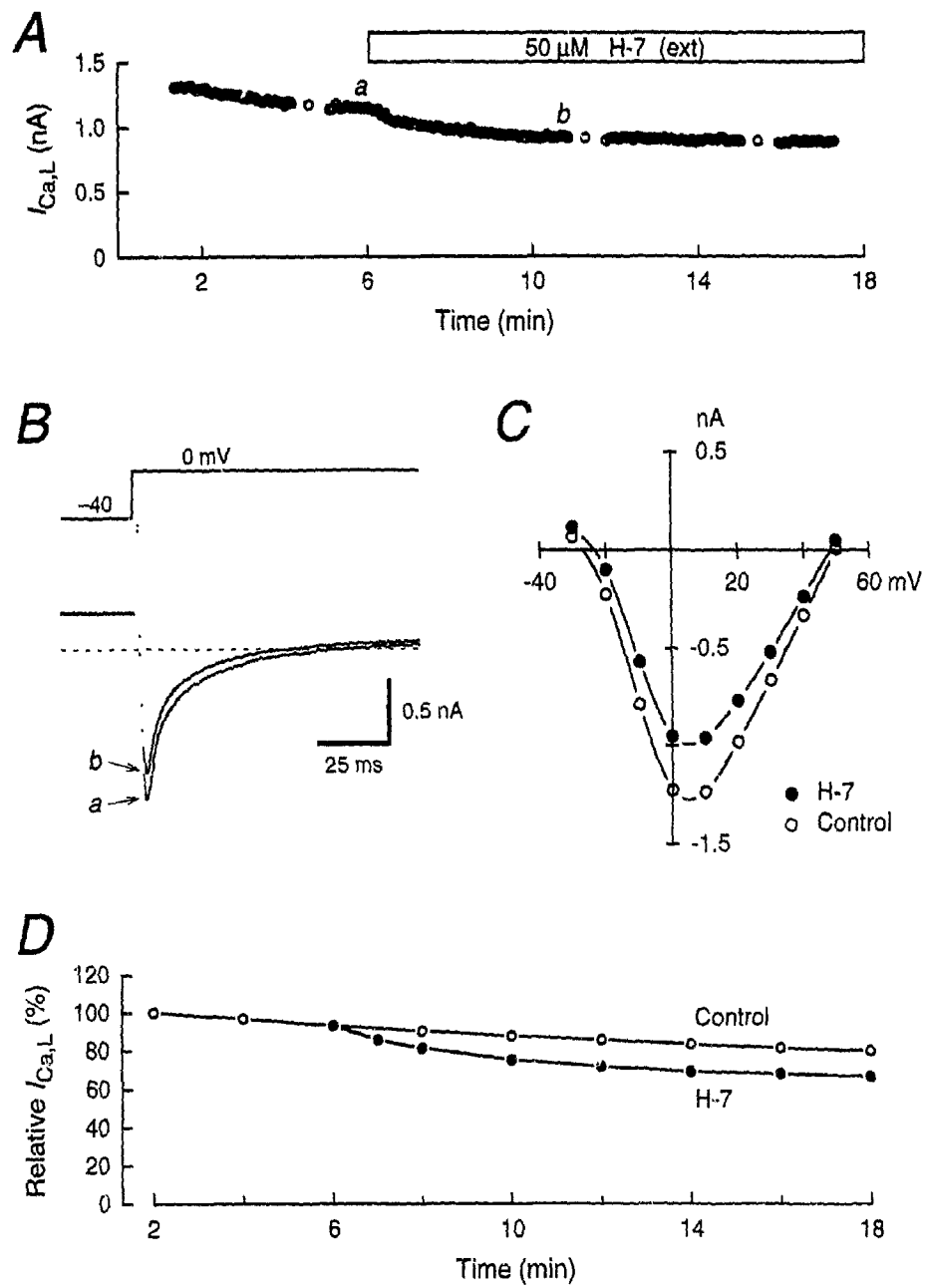
Figure 25. Effect of H-7 on basal $I_{Ca,L}$

results in Figure 25D show that $I_{Ca,L}$ was inhibited by $10 \pm 2\%$ ($n = 17$) after 2–3 min with H-7 treatment. There was no detectable effect on the rate of $I_{Ca,L}$ rundown after the initial rapid effect of H-7; with respect to $I_{Ca,L}$ at 2 min after application of H-7, $I_{Ca,L}$ declined by $18 \pm 2\%$ ($n = 16$) in 10 min, versus a decline of $12 \pm 3\%$ ($n = 13$) during the same time period under control conditions.

Effects of H-7 on PMA action on I_K and $I_{Ca,L}$

Figure 26A shows a time diary from an experiment in which I_K and $I_{Ca,L}$ were co-monitored during H-7 pretreatment and subsequent test with 10 nM PMA. After 10 min pretreatment with 50 µM H-7, 10 nM PMA produced little stimulation of I_K tail amplitude but a small, typically-rapid inhibition of $I_{Ca,L}$. This suggests that the effect of PMA on I_K is mediated by PKC, whereas that of PMA on $I_{Ca,L}$ is not.

Effects of high pCa dialysates on PMA action on I_K and $I_{Ca,L}$

The myocyte in the experiment of Figure 26A was dialyzed with a pCa 9 solution. High pCa dialysates have been shown to inhibit PMA stimulation of I_K in guinea pig ventricular myocytes (Tohse et al., 1987, 1990), and PMA stimulation of rat cardiac $I_{Ca,L}$ expressed in oocytes was prevented by injection of the eggs with BAPTA (Bourinet et al., 1992). Figure 26B indicates that dialysis of a myocyte with pCa ~11 solution largely prevented the stimulatory effect of 100 nM PMA on I_K tail amplitude. However, the high pCa dialysate did not prevent the rapid inhibition of $I_{Ca,L}$ induced by the phorbol ester.
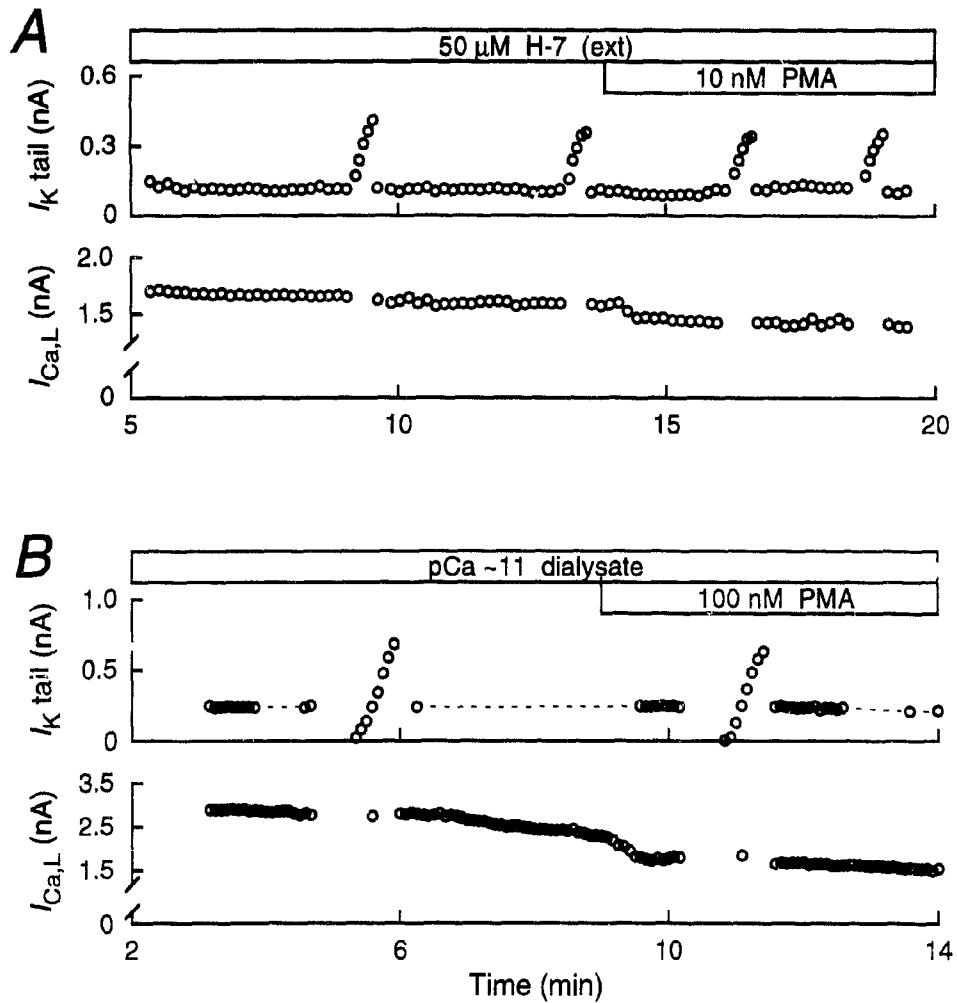
Figure 26. Experiments with H-7 and pCa ~11 dialysate

A. External 50 μM H-7 applied at 3.5 min post-patch breakthrough prevented stimulation of I_K tail current by 10 nM PMA in a myocyte dialyzed with pCa 9 solution. The pulses to 0 mV were periodically interrupted for a set of test pulses between +10 and +50 mV to monitor I_K activation. Pulsing rate was 0.1 Hz. **B.** Time diary showing I_K tail and $I_{Ca,L}$ responses to 100 nM PMA in a myocyte dialyzed with pCa 11 solution. The pulses to 0 mV were periodically interrupted for a set of test pulses between −30 and +40 mV to monitor I_K activation.

Effects of inactive phorbol ester on I_K and $I_{Ca,L}$

In other groups of myocytes, the responses of I_K and $I_{Ca,L}$ to the inactive phorbol ester αPDD were recorded. Figures 27A and B document the results from an experiment in which a 6-min application of 100 nM αPDD preceded an application of 100 nM PMA. Although the inactive phorbol ester had little effect on I_K tail amplitude, it evoked a small, rapid PMA-like inhibition of $I_{Ca,L}$. Subsequent replacement of inactive αPDD by active PMA stimulated I_K tail amplitude, but had no detectable effect on $I_{Ca,L}$.

Summary of results investigating PKC involvement

The plots in Figure 28 summarize the findings on I_K and $I_{Ca,L}$. The top panel (I_K tails) presents a profile that is suggestive of a PKC-dependent action by PMA, i.e. stimulation was blocked in myocytes pretreated with H-7 or dialyzed with pCa 11 solutions, and there was little stimulation when PMA was substituted by inactive αPDD and αPHR. By contrast, the profile of $I_{Ca,L}$ responses (*bottom panel*) points to a PKC-independent action by PMA, i.e. neither H-7 pretreatment nor pCa 11 dialysate affected the outcome, and αPDD data were indistinguishable from PMA data. Surprisingly, application of the inactive phorbol ester αPHR (100 nM) had little effect on $I_{Ca,L}$ ($p < 0.05$ versus PMA or αPDD).

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Figure 27. Experiments with αPDD

A. Co-monitored $I_{Ca,L}$ and I_K tail amplitudes during sequential application of 100 nM αPDD and 100 nM PMA. The pulses to 0 mV were periodically interrupted for a set of test pulses to potentials between −30 and +50 mV to monitor I_K activation. pCa 9 dialysate. *B.* Superimposed current records collected at the times (a,b,c) indicated in *A*. The inset shows the superimposed $I_{Ca,L}$ transients on an expanded time scale.

Figure 28. Summary plots showing the changes in I_K and $I_{Ca,L}$ induced by phorbol esters

Top, changes in I_K (+50 mV tails) measured 5–10 min after application of PMA, αPDD and αPHR under the conditions listed below the data. Each point represents a different myocyte. *Bottom*, changes in $I_{Ca,L}$ measured 2–3 min after application of phorbol ester under the conditions listed above the data. Each point represents a different myocyte; holding potential –80 mV.

3. Effects of phorbol esters on $I_{Na,L}$

Several groups of myocytes were superfused with K^+-free, EGTA Tyrode's solution, and dialyzed with Cs^+ (high Cl^-) pipette solution. A major reason for examining the effects of PMA on $I_{Na,L}$ under these conditions was that a biphasic (early stimulation, delayed inhibition) response of neonatal rat ventricular Ca^{2+} channel current to PMA has consistently been observed when Na^+ or Ba^{2+} served as the charge-carrier (Lacerda et al., 1988; Bourinet et al., 1992; Liu et al., 1993). A second reason was that the experimental conditions employed to measure $I_{Na,L}$ eliminated any concerns about surges in subsarcolemmal Ca^{2+}, interference from K^+ channel currents, and measurement complications at 0 mV related to I_{Cl} activation by PMA (i.e. $E_{Cl} = -4$ mV).

Figures $29A-C$ show that 10-100 nM PMA applied for 3-6 min had a distinct inhibitory effect on $I_{Na,L}$ in myocytes dialyzed with pCa 9 or 11 solution. In addition, the inactive phorbol ester αPDD (100 nM) also caused a rapid inhibition of the current (Figure 29D). The percentage inhibition caused by 10-100 nM PMA was $7 \pm 1\%$ ($n = 17$) with pCa 9 dialysate, $12 \pm 6\%$ ($n = 3$) with pCa 11 dialysate, and $13 \pm 6\%$ ($n = 5$) with pCa 7 dialysate. In myocytes dialyzed with pCa 9 solution, the inhibition after H-7 pretreatment was $10 \pm 6\%$ ($n = 4$), and that caused by 10-100 nM αPDD was 12 ± 3 % ($n = 6$). In summary, $I_{Na,L}$ was affected by active and inactive phorbol esters in the same manner as $I_{Ca,L}$.

I
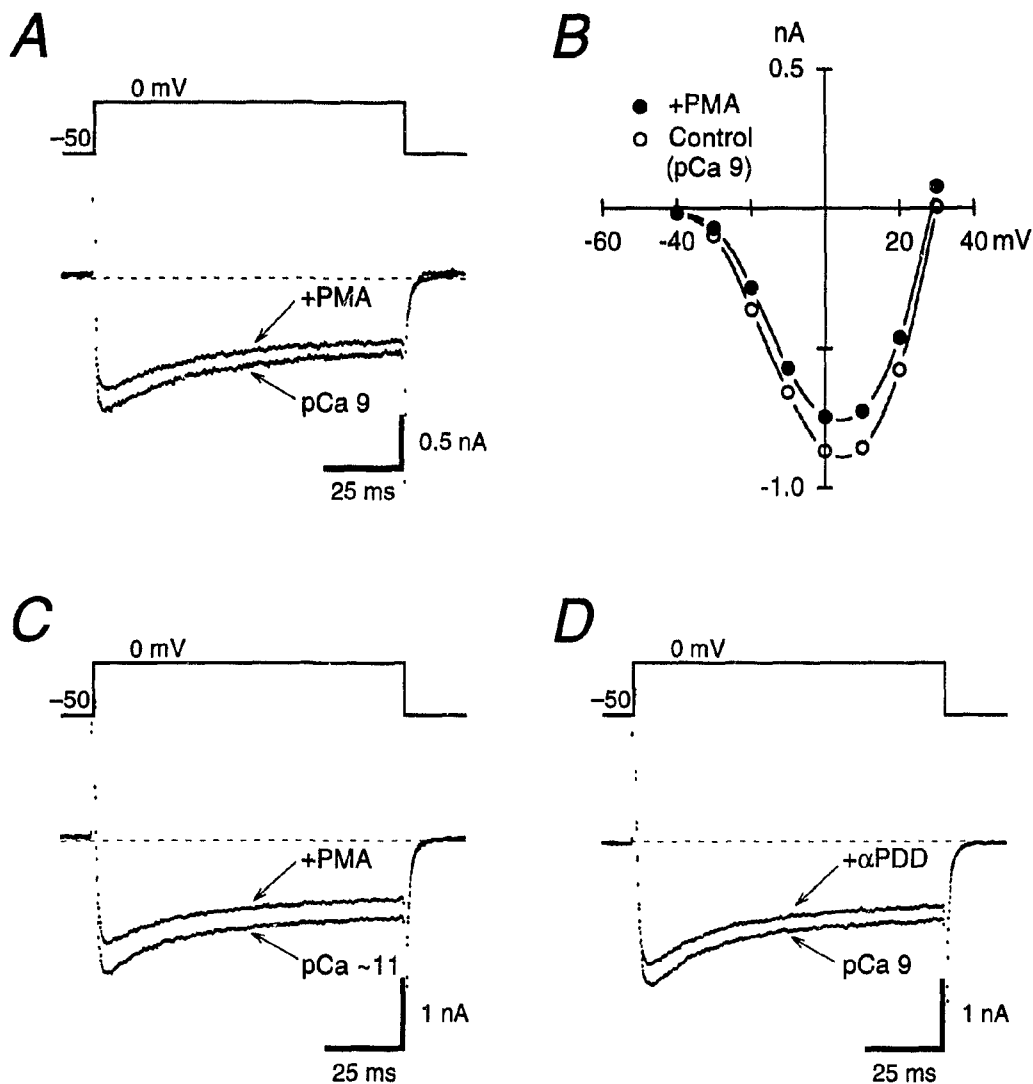
Figure 29. Inhibition of $I_{Na,L}$ by PMA and αPDD

A. Superimposed current records obtained before and 2 min after application of 10 nM PMA (*+PMA*). The myocyte was dialyzed with pCa 9 pipette solution. Inward $I_{Na,L}$ was elicited by 100-ms pulses from −50 to 0 mV at 0.2 Hz. *B.* *I-V* relations of peak inward $I_{Na,L}$ obtained from a different myocyte dialyzed with pCa 9 solution before (o) and 5 min after the addition of 10 nM PMA (●). *C.* Superimposed current records at 0 mV obtained on test depolarizations to 0 mV before and 2 min after application of 100 nM PMA (*+PMA*) to a myocyte dialyzed with pCa ~11 solution. *D.* Records before and 2 min after application of 100 nM αPDD (*+αPDD*) to a myocyte dialyzed with pCa 9 solution.

C.   Co-Activation of Membrane Currents by PMA and FSK

There are several examples of synergistic responses of CFTR Cl⁻ channels by PKC and PKA in non-cardiac cells (e.g. Tabcharani et al., 1991); Dechecchi et al., 1992), and there are numerous possibilities for cross-talk between the PKC and PKA pathways, with resultant effects on target proteins, including ion channels (Nishizuka, 1988; Kaczmarek, 1987). These possibilities were investigated by measuring currents in myocytes co-treated with PMA and FSK.

1.   Effects of co-application of PMA and FSK on I_{Cl}

Eighteen myocytes superfused with Na^+-, K^+-, Ca^{2+}-free Tyrode's solution and dialyzed with Cs^+ pipette solution were treated with maximally-effective concentrations (100 nM to 5 µM) of PMA first, and then with a maximally-effective concentration (5 µM) of FSK in the continued presence of PMA. Figure 30 illustrates that responses in this series of experiments fell into one of two categories. First, there was a group of myocytes in which PMA failed to activate a significant I_{Cl} (<10% increase in current over background amplitude); however, these myocytes were fully responsive to FSK (e.g. Figure 30A). Second, there was a group of myocytes with I_{Cl} responsive to both PMA and FSK (e.g. Figure 30B).

To determine whether FSK stimulation of I_{Cl} was additive to that induced by PMA, the increment in I_{Cl} elicited by FSK in PMA-pretreated myocytes was compared with I_{Cl} induced by separate applications of the activators to other control myocytes. The control FSK stimulation was 366 ± 29% (n = 14 other myocytes) when referenced to the amplitude of background

Figure 30. Effect of FSK on I_{Cl} in PMA-treated myocytes

A. Time diary of current amplitudes at –80 and +40 mV. I_{Cl} stimulation by 5 μM FSK in a myocyte unresponsive to 5 μM PMA. *B.* Time diary of current amplitudes at –80 and +40 mV. I_{Cl} stimulation by 5 μM FSK after typical increase of current with 5 μM PMA. *C.* Summary of data expressed as percentage increase in *g* (–80 to +40 mV) over control value. 5 μM FSK was applied for 5 min under control conditions (*left column*) and after pre-stimulation with 5 μM PMA for ≥9 min (*right column*). Number of myocytes in brackets. PMA+FSK value is significantly larger than that for FSK alone ($p < 0.01$); post-PMA FSK increment versus FSK ($p < 0.24$).
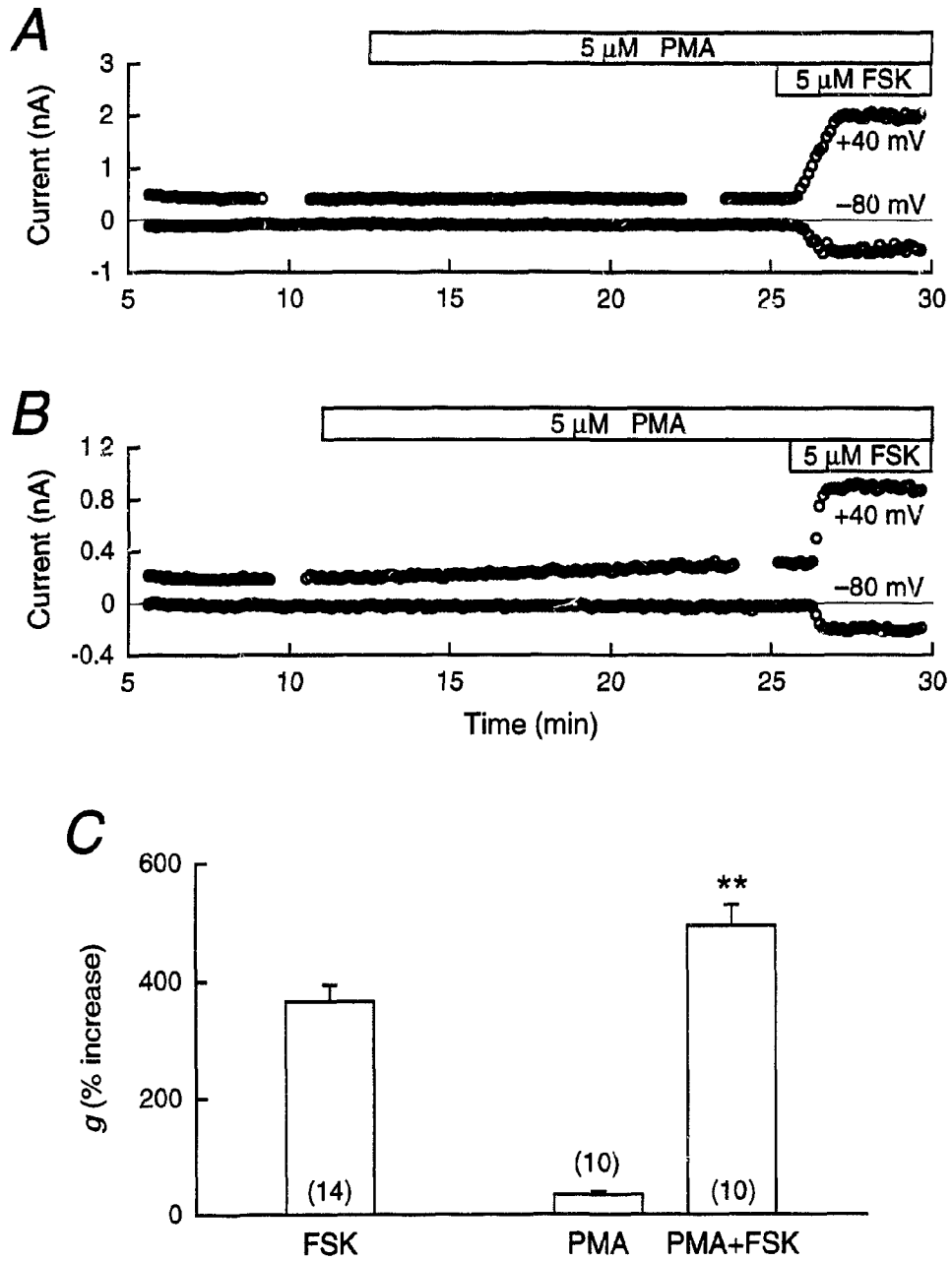
Figure 30. Effect of FSK on i_{Cl} in PMA-treated myocytes

current at +40 mV. The total stimulation in the PMA + FSK trials was $494 \pm 35\%$ (cells with $\geq 10\%$ increase, $n = 10$) (Figure 30C). The latter was comprised of relatively small PMA stimulation of $34 \pm 7\%$, and FSK stimulation of $460 \pm 37\%$ (both referenced to basal current). Thus, the combined treatment produced a larger stimulation than the single FSK treatment ($p < 0.01$). The degree of FSK stimulation was larger in PMA-treated myocytes than in untreated myocytes and just barely failed the significance test ($p < 0.06$).

2. Effects of co-application of PMA and FSK on I_K

A similar experimental protocol was employed for co-activation of I_K. A group of myocytes ($n = 5$) was treated with maximally-effective concentrations (100 nM) of PMA first, and then with a near-maximally-effective concentration (1 μM) of FSK in the continued presence of PMA. Figure 31A shows the results from a typical experiment. 100 nM PMA stimulated the I_K tail (measured on repolarization from +50 mV) by 43%, and subsequent application of 1 μM FSK further enhanced the I_K tail amplitude to 90% above control value. Original current records taken from the time diary are shown in Figure 31B, and a summary of the experimental result is shown in Figure 31C. The control FSK stimulation was $72 \pm 14\%$ ($n = 5$), and the total stimulation in the PMA + FSK trials was $82 \pm 9\%$ ($n = 5$). The latter was comprised of PMA stimulation of $30 \pm 5\%$, which is near the average response recorded in other experiments with 100 nM PMA (see Results A.2.), and FSK stimulation of $52 \pm 37\%$ (both referenced to control current). Thus, the combined treatment produced a slightly larger stimulation than the single FSK treatment, but FSK stimulation was smaller in PMA-treated myocytes than in untreated myocytes. However, neither of these results was statistically significant.

Figure 31. Co-activation of I_K by PMA and FSK

A. Time diary of I_K tail amplitudes recorded at −40 mV following 500-ms test pulses to +50 mV. I_K stimulation by 1 μM FSK after typical increase of current with 100 nM PMA. **B.** Superimposed current records obtained at the times (a,b,c) indicated in **A.** **C.** Summary of data expressed as percentage increase in I_K tail (at +50 mV) over control value. 1 μM FSK was applied for 5 min under control conditions (*left column*) and after pre-stimulation with 100 nM PMA for 6–9 min (*right column*). +FSK indicates PMA+FSK. Number of myocytes in brackets. PMA+FSK value is not significantly different from control FSK value. **D.** Time diary of I_K tail amplitudes recorded at −40 mV following 500-ms test pulses to +50 mV. I_K stimulation by 100 nM PMA after typical increase of current with 1 μM FSK.
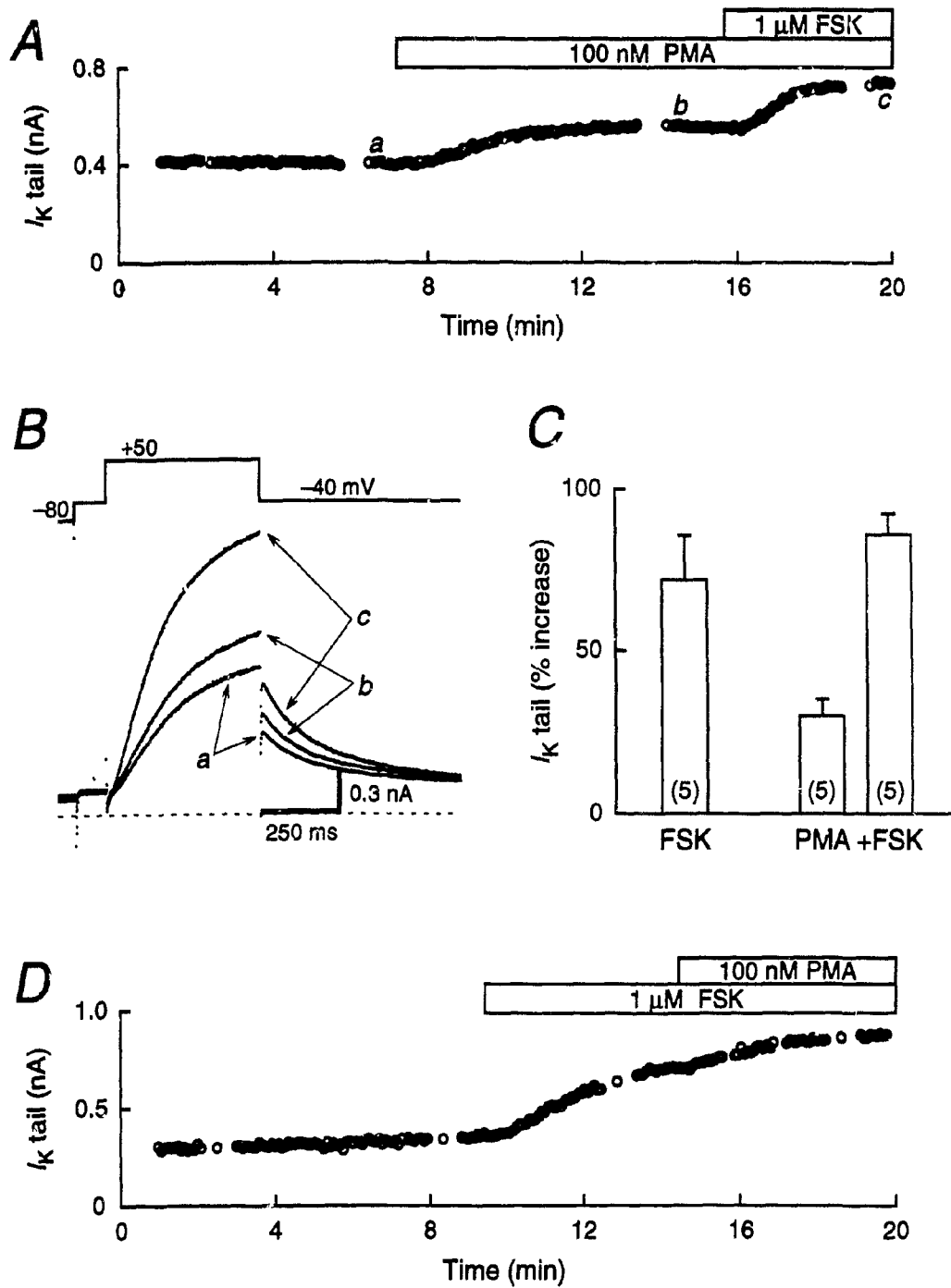
Figure 31. Co-activation of I_K by PMA and FSK

Yazawa and Kameyama (1990) observed that in guinea pig ventricular myocytes treated with 10 μM FSK, an additional application of 10 nM PMA produced a further increase in I_K; the increase elicited by PMA was similar to that achieved in control FSK-untreated myocytes. A similar result was obtained in the present study. After 1 μM FSK had increased I_K in a myocyte, application of 100 nM PMA increased it by an additional 38% of control amplitude (Figure 31D). In a second myocyte pretreated with 1 μM FSK, PMA incremented the current by 18%. Taken together these results do not suggest that a synergistic effect is achieved when I_K is co-activated by maximally-effective concentrations of PMA and FSK.

3. Effects of co-application of PMA and FSK on $I_{Ca,L}$

Although $I_{Ca,L}$ does not seem to be regulated by PKC activation, possible effects of PMA on FSK activation of $I_{Ca,L}$ were investigated. 1 μM FSK was applied to myocytes pretreated with 100 nM PMA for 5–8 min. Figure 32A shows a time diary of an example experiment, and Figure 32B the original current records obtained at the times indicated on the diary. In this cell, 100 nM PMA caused an immediate small reduction of $I_{Ca,L}$ amplitude, and had little effect thereafter. Application of 1 μM FSK stimulated $I_{Ca,L}$ in the usual manner. On average, the stimulation of $I_{Ca,L}$ by FSK in PMA-treated myocytes was similar to that in control myocytes (Figure 32C).
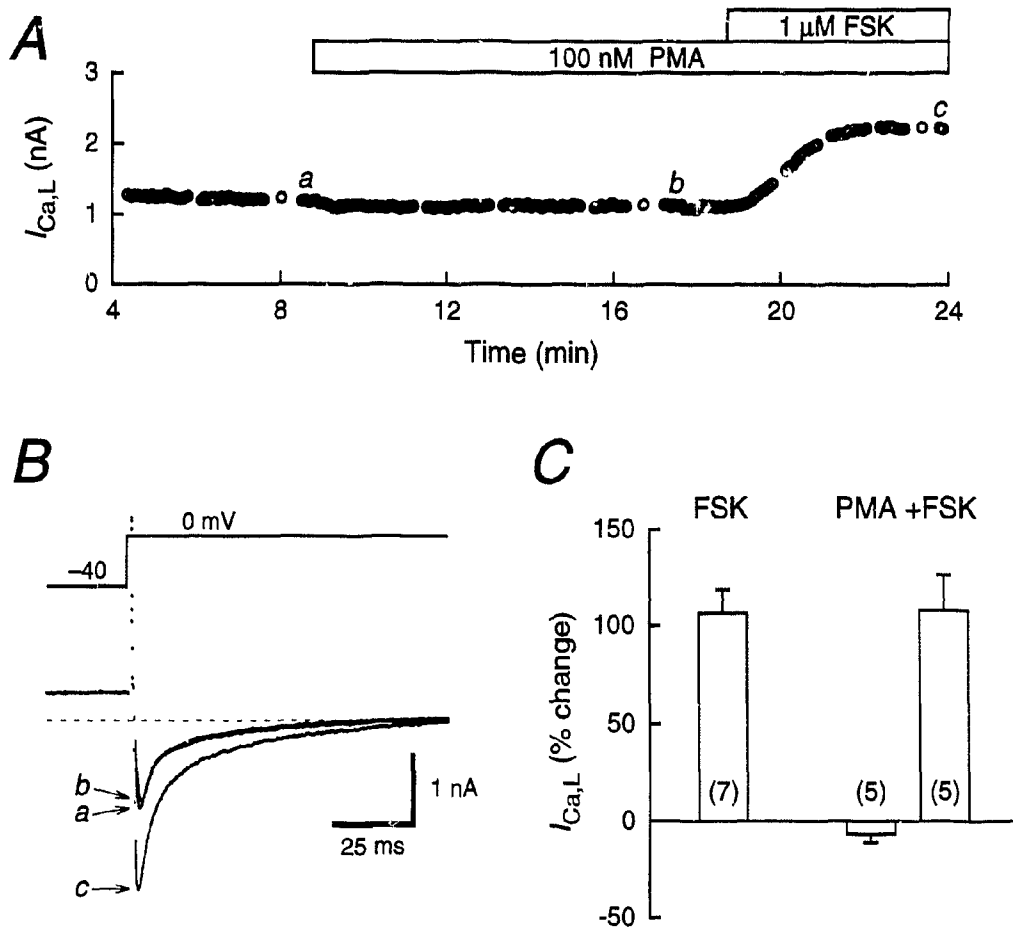
Figure 32. Effect of FSK on $I_{Ca,L}$ in PMA-treated myocytes

A. Time diary of peak $I_{Ca,L}$ amplitudes elicited by test pulses to 0 mV from prepulse −40 mV at 0.2 Hz. 100 nM PMA and 1 μM FSK were applied as indicated by the bars above the diary. **B.** Current records obtained at the times (a,b,c) indicated in *A* are superimposed. **C.** Summary of data expressed as percentage change in $I_{Ca,L}$ (at 0 mV). 1 μM FSK was applied for 5 min under control conditions (*left column*) or after pretreatment of myocytes with 100 nM PMA for 6–10 min (*right column*). +FSK indicates PMA+FSK. Number of myocytes in brackets. PMA+FSK value is not significantly different from control FSK value.

IV. DISCUSSION

The discussion below is organized under the following seven major sections: (A) biophysical characteristics of I_{Cl} activated by PMA, (B) concentration dependence, time course, and variability of the PMA-induced activation of I_{Cl} and I_K, (C) involvement of Ca^{2+}-dependent PKC in the activation of I_{Cl} and I_K by PMA, (D) species/cell type variation in responses to phorbol ester, (E) PKC-independent effect of phorbol esters on $I_{Ca,L}$, (F) relation between PKC-activated and PKA-activated I_{Cl} and I_K, and (G) responses during co-activation of PKC and PKA.

A. BIOPHYSICAL CHARACTERISTICS OF I_{CL} ACTIVATED BY PMA

Subtraction of basal current from PMA-stimulated current isolated a PMA-activated current whose E_{rev} shifted as expected for a Cl^--dominated current (see below). The $I\text{-}V$ relationship of PMA-activated I_{Cl} rectified in the outward direction. The rectification was strong in myocytes dialyzed with low Cl^- solution, and relatively weak in myocytes dialyzed with high Cl^- solutions. These features of PMA-activated I_{Cl} are discussed below in relation to earlier findings on cardiac I_{Cl} stimulated by activation of PKC or PKA.

1. Rectification

The results of the investigation of the rectification properties of PMA-activated I_{Cl} address an uncertainty about the properties of PMA-activated I_{Cl} in guinea pig ventricular myocytes dialyzed with low Cl^- solution. In an earlier study on these myocytes (Walsh, 1991), 20 nM PMA (or PDBu) activated a

current that had a linear dependence on voltage. However, the same group (Walsh and Long, 1994) subsequently found that the I_{Cl} activated by PDBu had an outwardly-rectifying I-V relationship in myocytes dialyzed with solution that contained rabbit brain PKCα. They speculated that the discrepancy between the two findings on I_{Cl} rectification may have been related to PDBu activation of *endogenous* PKC in the control myocytes, versus PDBu activation of *exogenous* PKC in the PKCα-dialyzed myocytes. The present study indicates that activation of endogenous PKC turns on outwardly-rectifying whole-cell I_{Cl} in guinea pig ventricular myocytes dialyzed with low Cl⁻ solution. This result is in accord with reports of outward-rectifying relationships for (i) PDBu-activated average single-channel Cl⁻ current in cell-attached patches of non-dialyzed guinea pig ventricular myocytes (Collier and Hume, 1995), and (ii) PMA-activated I_{Cl} in feline ventricular myocytes dialyzed with low Cl⁻ solution (Zhang et al., 1994a).

In feline myocytes dialyzed with 150 mM Cl⁻ solution, PMA activated an I_{Cl} that had a near-linear I-V relationship (Zhang et al., 1994a). A linearizing effect of high internal Cl⁻ was also observed on PMA-activated I_{Cl} in the guinea pig ventricular myocytes examined here, i.e. the ratio of the chord conductance measured above E_{rev} to the conductance measured below E_{rev} declined from 1.9 with 30 mM Cl⁻ dialysate, to 1.2 with 150 mM Cl⁻ dialysate. A similar linearization with high internal Cl⁻ has been observed in studies on PKA-activated I_{Cl} in guinea pig ventricular myocytes (Bahinski et al., 1989; Harvey and Hume, 1989; Harvey et al., 1990; Overholt et al., 1993; Vandenberg et al., 1994; this study). The linearization is predicted by the Goldman-Hodgkin-Katz (GHK) current equation where the degree of rectification is determined by the ratio of extracellular to intracellular concentration for a permeating ion (cf.

Hille, 1992; Overholt et al., 1993), as well as by other theoretical formulations (Overholt et al., 1993).

I_{Cl} in PMA-pretreated myocytes dialyzed with 140 mM Cl⁻ solution was not as linear as expected from the GHK current equation (i.e. it still rectified in the outward direction). However, single-channel Cl⁻ current activated by PKA can also exhibit slight rectification under near-symmetrical Cl⁻ conditions in these myocytes (Ehara and Matsuura, 1993). In accord with the explanation offered by Overholt et al. (1993) for the outward rectification of isoproterenol-activated I_{Cl} in guinea pig ventricular myocytes dialyzed with low Cl⁻ solution, it is concluded that this aspect of PMA-activated I_{Cl} is at least in part due to a partial blocking action (at negative potentials) of the replacement anion (glutamate in their experiments; aspartate here) included in low Cl⁻ dialysates. Under near symmetrical Cl⁻ conditions (no added aspartate), the slight outward rectification may be due to the blocking action (at negative potentials) of an endogenous organic anion.

2.   Permeability

Partial block by internal aspartate would be consistent with tight binding and limited permeation of this anion compared to internal Cl⁻. This type of behaviour would help explain the deviation of the E_{rev} of PMA-activated I_{Cl} (-33 mV) from calculated E_{Cl} (-42 mV) when the dialysate contained 30 mM Cl⁻ and 110 mM aspartate. Application of the GHK voltage equation, $E_{rev} = 61 \log \{([Cl^-]_i + P_{Asp}/P_{Cl} [Asp^-]_i) / [Cl^-]_o\}$, where P is permeability, yields $P_{Asp} \approx 0.1\ P_{Cl}$. This estimate of P_{Asp} for PMA-activated Cl⁻ channels is somewhat lower than the $P_{Asp} \approx 0.45\ P_{Cl}$ reported for swelling-activated anion channels in canine ventricular myocytes (Tseng, 1992) and for

swelling- and PKA-activated anion channels in guinea pig ventricular myocytes (Vandenberg et al., 1994).

I have also performed preliminary experiments ($n = 3$) on the effect of I^- replacement of external Cl^-. The results (-6 mV shift in E_{rev}) suggest $P_I \approx 1.2$ P_{Cl}, a value in good accord with the 1.4 P_{Cl} determined by Walsh and Long (1994) from myocytes dialyzed with PKCα. The P_I/P_{Cl} of cardiac PKA-activated Cl^- channels is not fully resolved (see Gadsby et al., 1995), with reported values in guinea pig ventricular myocytes ranging from 1.67 (Vandenberg et al., 1994) to 0.88 (Overholt et al., 1993).

B.   CONCENTRATION DEPENDENCE, TIME COURSE, AND VARIABILITY OF THE PMA-INDUCED ACTIVATION OF I_{CL} AND I_K

1.   Concentration dependence of PMA stimulation

The increase in I_{Cl} provoked by PMA had a threshold near 1 nM, was half-maximal at ~10 nM, and maximal near 100 nM. There are no other accounts of the concentration dependence of cardiac I_{Cl}. However, the Walsh group has employed 20-50 nM PMA to activate I_{Cl} in guinea pig ventricular myocytes (Walsh, 1992), and Collier and Hume (1995) used similar concentrations to activate single-channel currents in these myocytes, whereas Zhang et al. (1994a) used two orders higher concentrations to activate I_{Cl} in feline ventricular myocytes (see below).

The stimulation of I_K by PMA had a concentration dependence (threshold 0.1-1 nM, half-maximal at ~4 nM, and maximal at 100 nM) that was similar to that of I_{Cl}. This "effective" concentration range of PMA is also similar to that

determined in earlier studies on PMA stimulation of I_K in guinea pig ventricular myocytes (Tohse et al., 1987, 1990), PMA stimulation of L-type Ca^{2+} channel current in neonatal rat ventricular myocytes (Dösemeci et al., 1988; Lacerda et al., 1988; Liu et al., 1993), and PMA stimulation of "slow inward current" in rabbit sinoatrial node cells (Satoh and Hashimoto, 1988). Furthermore, this concentration range is also compatible with the dissociation constant of PMA binding to PKC (0.1–2.5 nM) (Dunphy et al., 1980; Castagna et al., 1982; Ashendel et al., 1983).

High concentrations of phorbol esters can have unpredictable effects. Tohse et al. (1987) observed that the stimulation of cardiac I_K declined at PMA concentrations >100 nM, and transient outward current that was stimulated by 50–200 nM PMA in rabbit atrial cells was inhibited by >1 µM PMA (Braun et al., 1990). Tohse et al. (1987) attributed the reduced stimulation of I_K at high concentrations of PMA to the "membrane perturbation" that may be caused by phorbol esters at concentrations >100 nM (cf. Yamanishi et al., 1983; Nishizuka, 1984) [or >1.5 µM: cf. Ryves et al., 1991]. However, the reduced stimulation of I_K may also have been due to K^+-channel block (cf. Hockberger et al., 1989; Doerner et al., 1990; and see below) and/or to desensitization/downregulation of PKC. Bourinet et al. (1992) invoked desensitization/downregulation to explain the marked (post-stimulation) inhibition of cardiac Ca^{2+} channel current that begins ~5 min after addition of a moderate concentration of PMA to rat ventricular myocytes (Lacerda et al., 1988; Liu et al., 1993).

In the present study, there was also a small reduction in the stimulation of I_K when high concentrations (1–5 µM) of PMA were employed. This may have been due to any of the foregoing effects. In regard to a possible block of K^+ channels by higher concentrations of the phorbol ester, it may be pertinent that two other diterpenes, FSK and inactive 1,9-dideoxyforskolin at

concentrations ≥ 10 μM, also block I_K in guinea pig ventricular myocytes (unpublished observations; also see Walsh and Kass, 1991) but do not affect I_{Cl} (see below) .

There were no indications that any of the factors mentioned above (membrane perturbation, channel block, desensitization/downregulation) had a major impact on I_{Cl} activated by higher concentrations of PMA. I_{Cl} activated by 5 μM PMA was not significantly smaller than I_{Cl} activated by 100 nM PMA, macroscopic properties of the current (rectification, E_{rev}) were not detectably affected, activation time courses remained monotonic, and delayed inhibitory phases following activation were not evident. Zhang et al. (1994a) reported that they required 6 μM PMA to achieve maximal activation of I_{Cl} in feline ventricular myocytes. This is a very high concentration of PMA, necessity of which may indicate that activation of a PKC isoform relatively insensitive to PMA (cf. Nishizuka, 1988; Ryves et al., 1991) is important for stimulation of I_{Cl} in feline myocytes. As noted above, activation of I_{Cl} in guinea pig ventricular myocytes was not larger with 5 μM PMA than with a 100 nM concentration.

2. Time course of PMA stimulation

Although there are no other published descriptions of the time course of I_{Cl} activated by phorbol ester, those of I_{Cl} and I_K measured here are in good agreement with those noted on I_K in rabbit sinoatrial node cells (Satoh and Hashimoto, 1988) and in guinea pig ventricular myocytes (Tohse et al., 1990; Yazawa and Kameyama, 1990; Tohse et al., 1992). The time courses also agree with those of phorbol ester stimulation of rat ventricular Ca^{2+} channel currents (Dösemeci et al., 1988; Lacerda et al., 1988; Bourinet et al., 1992; Liu et al., 1993).

3. Variability of responses to PMA

Walsh (1991) has presented example records indicating a 100% increase of I_{Cl} in guinea pig ventricular myocytes treated with 20 nM PMA. The largest turn-on of I_{Cl} recorded in the present study was ~40% (reference: basal background current) with 10–20 nM PMA, although increases >60% were obtained in about 20% of the myocytes treated with $\geq$100 nM concentrations. Zhang et al. (1994a) noted that activation of I_{Cl} by PMA in guinea pig ventricular myocytes was much weaker than in feline ventricular myocytes. Although they provided no comparative data, the average size of the I_{Cl} induced in the feline myocytes they investigated was approximately 100% (reference: basal background current) after application of a maximally-effective 6 μM concentration. As noted above, activation of I_{Cl} in the guinea pig ventricular myocytes investigated in the present study was not larger with 5 μM PMA than with 100 nM. However, independent of the PMA concentration (0.1–5 μM) applied, approximately 20% of the guinea pig myocytes tested in the present study responded with rather small activation of I_{Cl}. Zhang et al. (1994a) have previously reported that there was little activation of I_{Cl} in 24% of feline ventricular cells treated with 6 μM PMA.

In regard to I_K, 15% of the myocytes treated with 10–100 nM PMA responded with stimulation that was <10% of control amplitude. Earlier publications have noted that a sizeable fraction of sampled cardiac cells fail to respond to phorbol ester treatment in the same manner as the remainder. Braun et al. (1990) found that transient outward K^+ current did not respond to 100–200 nM PMA in 4 out of 15 rabbit atrial cells. PMA caused only depression of $I_{Ca,L}$, instead of a biphasic response (moderate stimulation and inhibition), in 1 out of 7 canine ventricular cells and 4 out of 7 Purkinje cells (Tseng and Boyden, 1991).

Variable responses to PMA have been also observed in non-cardiac cells. For example, the inhibition of $I_{Ca,L}$ commonly observed during application of PMA to rat GH_3 anterior pituitary cells was absent in about 10% of cells (Haymes et al., 1992). The reason for an absence or minor response of current systems to phorbol esters is not clear (cf. Haymes et al., 1992). In the case of guinea pig ventricular myocytes, it may be that PKC isoforms responsible for the activation of I_K and/or I_{Cl} are poorly expressed in some myocytes. An additional possibility in regard to PMA activation of I_{Cl} is that variation in responses may be due to regional differences in the expression of PKA-regulated Cl^- channels in guinea pig heart (cf. James et al., 1995) (also see Section IV.F. below).

C. INVOLVEMENT OF Ca^{2+}-DEPENDENT PKC IN THE ACTIVATION OF I_{CL} AND I_K BY PMA

Earlier studies on cardiomyocytes have provided evidence that phorbol ester activation of I_{Cl} is mediated by PKC. Walsh and Long (1994) observed that PDBu applied to guinea pig ventricular myocytes dialyzed with bovine brain $PKC\alpha$ activated I_{Cl}, and that kinase-inhibiting staurosporine abolished activation. Collier and Hume (1995) found that staurosporine reduced the open probability of single Cl^- channels activated by PDBu in guinea pig cells, and Zhang et al. (1994a) observed that PMA stimulation of feline ventricular I_{Cl} was blocked by staurosporine and calphostin C. The latter group also reported that PMA stimulation was not duplicated by αPDD and 4β-phorbol.

PKC activation has also been implicated in the stimulation of I_K by phorbol ester in guinea pig ventricular cells. Tohse et al. (1987, 1990) observed

that PMA stimulation of I_K was blocked by pCa 11 dialysates and H-7 pretreatment. PMA stimulation of I_K was mimicked by diacylglycerol analogue (Tohse et al., 1990), but not by inactive phorbol ester β-phorbol (Yazawa and Kameyama, 1990). The finding of enhanced stimulation by PMA after dialysis of bovine brain PKCα (Tohse et al., 1990) adds to the case for PKC-mediated I_K stimulation by PMA in guinea pig ventricular myocytes.

The primary evidence for PKC involvement in the PMA activation of I_{Cl} and I_K in the guinea pig ventricular myocytes investigated in the present study is that pretreatment with PKC-inhibitor H-7 (Hidaka et al., 1984; Hidaka et al., 1991; Hidaka and Kobayashi, 1992) blocked PMA stimulation (also see below), that αPDD failed to activate I_{Cl} or I_K, and that αPHR failed to activate I_K. In addition, PMA failed to activate I_{Cl} or stimulate I_K in myocytes that were dialyzed with pCa 11 pipette solution. While this is a novel finding in regard to cardiac I_{Cl}, Bourinet et al. (1992) found that PMA stimulation of expressed rat cardiac Ca^{2+} channels was absent in oocytes injected with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), and Tohse et al. (1990) have reported that PMA stimulation of I_K was completely inhibited in guinea pig ventricular myocytes dialyzed with pCa 11 (versus pCa 9) solution. The latter authors also found that I_K stimulation by PMA was occluded in myocytes dialyzed with pCa 7 solution. In the present study, the effects of pCa 7 dialysate on PMA stimulation of I_K were not examined, but there was a smaller activation of I_{Cl} by PMA in myocytes dialyzed with pCa 7 versus pCa 9 solution. By reference to the I_K study of Tohse et al. (1990), a suppressed activation of I_{Cl} with pCa 7 dialysate might have been due to a partial occlusion of the PMA effect, i.e., high Ca^{2+} dialysate had already activated PKC (and a fraction of PMA-responsive I_{Cl}), before the PMA was applied. Since the activation of I_{Cl} by PMA is relatively small, the latter possibility would be

difficult to establish with any degree of certainty. Although more refined studies incorporating measurement of intracellular Ca^{2+} concentration are required for firmer conclusions, these findings suggest that both Cl^- and delayed-rectifying K^+ channels in these myocytes are regulated by one or more Ca^{2+}-dependent rather than Ca^{2+}-independent PKC isoforms. In this regard, there are recent investigations suggesting that H-7, the kinase inhibitor used in the present study, preferentially inhibits PKC that is Ca^{2+}-dependent (Ison et al., 1993) and membrane-bound (Budworth and Gescher, 1995).

D.   SPECIES/CELL TYPE VARIATION IN RESPONSES TO PHORBOL ESTER

1.   Responses of cardiac I_K to phorbol ester

The PKC-mediated *stimulation* of I_K in guinea pig ventricular myocytes (Tohse et al., 1987, 1990; Walsh and Kass, 1988; Yazawa and Kameyama, 1990; present study) contrasts with reported PKC-mediated *depression* of I_K in rabbit sinoatrial node cells (Satoh and Hashimoto, 1988) and in neonatal mouse ventricular myocytes (Honoré et al., 1991). These divergent effects of PKC activation on cardiac K^+ currents appear to be related to species-dependent variation in K^+ channel protein. Zhang et al. (1994b) have cloned guinea pig cardiac delayed-rectifier K^+ channel protein and expressed it in oocytes. They found that expressed channel activity was stimulated by phorbol ester. In addition, they reported that mutagenesis of the protein at four cytoplasmic amino acid residues converted the response to PKC activation from stimulation to inhibition. These results led them to conclude that the K^+ channel protein in

guinea pig heart lacks a residue found in other variants (e.g. rat: Busch et al., 1992b) which, when phosphorylated by PKC, results in a decrease in I_K.

2. Responses of cardiac $I_{Ca,L}$ to phorbol ester

Active phorbol esters have produced highly variable effects on cardiac L-type whole-cell and single Ca^{2+} channel currents. $I_{Ca,L}$ in guinea pig ventricular myocytes was unaffected by 1 nM PMA (Tohse et al., 1990) and 10–50 nM PDBu (Walsh and Kass, 1988), but depressed by 100 nM PMA (Satoh et al., 1992). In other studies, 150 nM PMA had no effect on $I_{Ca,L}$ in feline ventricular myocytes (Hartmann et al., 1988), 100–1000 nM moderately stimulated $I_{Ca,L}$ in rabbit sinoatrial node cells (Satoh and Hashimoto, 1988), and 65 nM enhanced $I_{Ca,L}$ by about 40% in cultured neonatal rat ventricular cells (Dösemeci et al., 1988). Biphasic effects of phorbol ester on $I_{Ca,L}$ were common in Tseng and Boyden's (1991) study on canine myocytes. They reported that 100 nM PMA moderately (13%) stimulated $I_{Ca,L}$ for 5–15 min prior to inhibition in 6 of 7 ventricular cells and 3 of 7 Purkinje cells; pure inhibition was recorded in the remaining cells. They noted that $I_{Ca,L}$ responses were temperature-dependent (frequent stimulation at 36°C, only inhibition at 28°C), independent of dialysate EGTA (1–10 mM), and blocked by the protein kinase inhibitor N-(2-[methylamino]ethyl)-5-isoquinolinesulfonamide (H-8). A further finding was that 100 nM αPDD depressed $I_{Ca,L}$ in 4 of 4 cells. In the present study, there was not a single instance of $I_{Ca,L}$ stimulation >5% with 1–100 nM PMA, nor a single biphasic event; 10–100 nM phorbol ester had essentially no effect, or (in 20 of 44 myocytes tested) caused inhibition that was >5%.

A consistent pattern of stimulation (peak at ~5 min after drug application) followed by marked inhibition has emerged from studies of PMA

action on rat ventricular L-type Ca^{2+} channel currents carried by Na^+ or Ba^{2+}. These studies involved measurements on single-channel currents (Lacerda et al., 1988) in neonatal cells, whole-cell currents (Liu et al., 1993) in neonatal cells, and/or whole-cell currents carried by channels expressed in oocytes (Bourinet et al., 1992). The results with Na^+ or Ba^{2+} as charge-carrier can be compared with the results obtained here from guinea pig myocytes superfused with low Ca^{2+} solution to permit Na^+ passage through L-type channels. These experiments on $I_{Na,L}$ did not uncover a PMA-induced phase of stimulation similar to that found in the foxgoing studies. Since stimulation of the magnitude recorded in studies on rat cardiac currents (46-60%) (Lacerda et al., 1988; Bourinet et al., 1992; Liu et al., 1993) could not have gone undetected in this study, it seems that either guinea pig ventricular myocytes lack a type of PMA-sensitive PKC found in rat myocytes (and oocytes), or that L-type Ca^{2+} channel protein in guinea pig ventricular cells lacks an amino acid residue (or residues) critical for PKC phosphorylation or its translation into stimulatory activity.

E.   PKC-INDEPENDENT EFFECT OF PHORBOL ESTERS ON $I_{Ca,L}$

The results on I_K and I_{Cl} indicate that these currents are enhanced by PMA, and suggest that the stimulation is mediated by activation of PKC. The results on $I_{Ca,L}$ were quite different; PMA caused a moderate depression of $I_{Ca,L}$ in about 50% of myocytes tested. This inhibition is discussed below in regard to possible measurement errors, comparison with earlier findings, and the case for PKC-dependent versus PKC-independent action of PMA.

1.   Possible measurement errors

The PMA-induced inhibition of $I_{Ca,L}$, which was generally assessed by measuring the reductions in peak inward $I_{Ca,L}$ at 0 mV, was not an artefact created by the concomitant PMA-induced stimulation of outward-directed I_{Cl}. In myocytes dialyzed with low Cl^- solution, the magnitude of the PMA-induced increment in outward-directed I_{Cl} at 0 mV was negligible (about 30 pA) in comparison to $I_{Ca,L}$ amplitude (about 1 to 2 nA) (see Figures 10 and 19). In myocytes dialyzed with high Cl^- solution, any impact of activated I_{Cl} was negated by the near-zero driving force on Cl^- at 0 mV. The latter situation also applied in all experiments in which Na^+ was the charge-carrier for the L-type Ca^{2+} channel current. In addition, the degree of the PMA-induced inhibition of $I_{Ca,L}$ was not significantly different at test potentials between -20 and +20 mV. Finally, possible complications related to activation of I_{Cl} were inapplicable in the experiments with inactive αPDD.

Although $I_{Ca,L}$ was frequently measured in the presence of K^+ currents (I_{K1} and I_K), the PMA-induced inhibition of $I_{Ca,L}$ was not caused by interference from these currents. In regard to I_{K1}, PMA (1-100 nM) had no significant effects on I_{K1} amplitude (which, in any case, has very small magnitude at 0 mV). In regard to I_K, the activation of this current within 4 ms of test pulses to 0 mV (time of $I_{Ca,L}$ measurement) was minimal, i.e. the activation time constants around these potentials are in the range 10's-100's of milliseconds (Matsuura et al., 1987; Giles et al., 1989; Chinn, 1993; Clay et al., 1995). In addition, the inhibition of $I_{Ca,L}$ was also observed in myocytes in which I_K was blocked , i.e. during dialysis with Cs^+ pipette solution and superfusion with K^+-free solution. This condition (inhibition of I_K) also applied in all experiments that involved measurement of phorbol ester effects on $I_{Na,L}$.

Another argument against contamination measurements of PMA inhibition of $I_{Ca,L}$ by concomitant stimulation of I_{Cl} and I_K is based on differences in the time courses of phorbol ester action. The Ca^{2+}-channel inhibition had a rapid onset, and was complete within ~1.5 min, whereas I_K and I_{Cl} stimulation had short lags (~1 min) and slower progression to full effect. This disparity was evident on an average basis, as well as when $I_{Ca,L}$ and I_K were simultaneously measured in a given cell.

2.   PKC-independent effects of phorbol esters

The results suggest that PMA stimulation of I_{Cl} and I_K was mediated via an activation of PKC; the stimulation was inhibited by H-7 pretreatment, and not duplicated by inactive αPDD or αPHR. The outcome of a similar investigation of PMA-induced inhibition of Ca^{2+} channel current was quite different. The inhibition was unaffected by H-7 pretreatment and fully duplicated by αPDD. There have been other accounts of αPDD action on cardiac preparations, including negative chronotropy (Satoh and Hashimoto, 1988) and negative inotropy (Watson and Karmazyn, 1991; Ward and Moffat, 1992) with 100 nM concentrations, as well as inhibition of $I_{Ca,L}$ with 10-1000 nM concentrations (Tseng and Boyden, 1991). In addition, studies on high-threshold Ca^{2+} channel current in neurons have documented phorbol ester inhibition that was unaffected by protein kinase inhibition with H-7 or staurosporine (Hockberger et al., 1989; Doerner et al., 1990).

The first of two other features that distinguished PMA effects on L-type Ca^{2+} channel current from those on I_{Cl} and I_K was the effect of dialysate pCa. In myocytes dialyzed with pCa ~11 solution, the stimulatory effects of PMA on I_{Cl} and I_K were inhibited. By contrast, PMA-induced inhibition of Ca^{2+} channel

current was indifferent to dialysate pCa. In regard to the foregoing, Bourinet et al. (1992) observed that the stimulation of expressed rat ventricular Ca^{2+} channels by diacylglycerol analogues was completely suppressed in oocytes injected with BAPTA.

The second distinguishing feature was the time course of phorbol ester action. Ca^{2+} channel inhibition occurred almost immediately, reached 50% of maximum in ~0.7 min, and was complete within ~1.5 min. In contrast, the delay prior to I_K and I_{Cl} stimulation was ~1 min, and the full effect was reached after 5-8 min. Interestingly, other reports of PKC-independent activity by phorbol esters note fast time courses (e.g. Hockberger et al., 1989; Doerner et al., 1990) compared to accounts of PMA responses attributed to PKC activation (i.e. short lags and full effect many minutes later: e.g. Dösemeci et al., 1988; Lacerda et al., 1988; Doerner et al., 1990; MacLeod and Harding, 1991; Walsh, 1991; Tohse et al., 1992; Liu et al., 1993; Zhang et al., 1994a).

3.   Possible mechanisms of PKC-independent effects of phorbol esters

A dangling issue is the mechanism by which PMA and αPDD can cause PKC-independent inhibition of the L-type Ca^{2+} channels. Direct channel block as proposed for inhibition of high threshold Ca^{2+} channel current by micromolar phorbol ester in neurons (Hockberger et al., 1989; Doerner et al., 1990) is one possibility. Another is that the inhibition involves arachidonic acid since (i) externally-applied arachidonic acid has been shown to inhibit $I_{Ca,L}$ in guinea pig atrial myocytes (Cohen et al., 1990), and (ii) phorbol esters can promote a rapid release of arachidonic acid from membrane phospholipids (e.g. Tao et al., 1989; Carlson and Levitan, 1990; Thomson et al., 1991; Lin et al., 1992). It may also be relevant that PMA and αPDD, but not αPHR, caused PKC-

independent, (likely) arachidonic acid-mediated, prostaglandin release in rat hearts (Watson and Karmazyn, 1991). In contrast to PMA and αPDD, αPHR did not inhibit $I_{Ca,L}$ in the present study.

F.   RELATION BETWEEN PKC-ACTIVATED AND PKA-ACTIVATED I_{Cl} AND I_K

This section discusses the relationship between PKC-activated and PKA activated I_{Cl} and I_K by addressing the following two questions: (i) do PKC and PKA activate the same Cl⁻ channel?, and (ii) is PMA activation of I_{Cl} and I_K due to activation of PKA?

1.   Do PKC and PKA activate the same Cl⁻ channel?

There is considerably more information available on the activation of cardiac Cl⁻ channels by PKA than on the activation of Cl⁻ channels by PKC (Gadsby et al., 1995). Hume and colleagues (Levesque et al., 1993; Hume and Horowitz, 1995) have identified the cardiac Cl⁻ channel activated by PKA as an alternatively spliced variant of the cystic fibrosis transmembrane conductance regulator protein (CFTR) that is commonly found in epithelial cell membranes (e.g. Berger et al., 1991; Welsh et al., 1992; Riordan, 1993). This epithelial Cl⁻ channel can be phosphorylated by either PKA or PKC (Berger et al., 1993), but the functional effect of PKC activation by phorbol ester on Cl⁻ current is often relatively small (Tabcharani et al., 1991; Dechecchi et al., 1992; Bajnath et al., 1994; McAlroy et al., 1994).

The tentative conclusion reached in recent studies on cardiac cells is that PKC enhances I_{Cl} by activating CFTR rather than by activating a different set of

Cl^- channels (Walsh and Long, 1994; Zhang et al., 1994a; Collier and Hume, 1995). In cat ventricular myocytes, I_{Cl} stimulated by phorbol ester had similar macroscopic time-independent "kinetics", and (with low Cl^- dialysate) similar outwardly-rectifying I-V relations, as FSK-stimulated current (Zhang et al., 1994a). Furthermore, phorbol-ester-activated single-channel Cl^- currents in cell-attached guinea pig myocyte membrane patches were indistinguishable from the single-channel currents activated by FSK (Collier and Hume, 1995). In the latter study, the activation of PKA with 3-isobutyl-1-methylxanthine (IBMX) further increased the open-state probability of channels opened by application of PMA or PDBu, and PKA activation induced channel opening even when prior PKC activation did not. As Collier and Hume (1995) stated, their results were not conclusive, but they favoured the interpretation that both PKC and PKA regulate cardiac CFTR Cl^- channels.

The present study does not provide any evidence to the contrary. The characteristics of the macroscopic I_{Cl} induced by PMA in guinea pig ventricular myocytes are quite consistent with those of PKA-activated I_{Cl} previously described for these myocytes (cf. Harvey and Hume, 1989; Overholt et al., 1993; Gadsby et al., 1995).

2. Does PMA stimulate I_{Cl} and I_K through activation of PKA?

In the present study on guinea pig ventricular myocytes, the characteristics of whole-cell I_{Cl} and I_K induced/stimulated by PMA are quite consistent with those of PKA-activated I_{Cl} and I_K previously described for these myocytes (Harvey and Hume, 1989; Overholt et al., 1993; Yazawa and Kameyama, 1990). This raises the issue of whether PMA induction of I_{Cl} might not have been due to an activation of PKA.

This possibility can be discounted on several grounds. The most likely mechanism for activation of PKA by phorbol ester is via a stimulation of adenylate cyclase. This matter has been studied in a wide variety of cells, with varying results (e.g. Sibley et al., 1986; Phaneuf et al., 1988; Gusovsky and Gutkind, 1991; Haymes et al., 1992; Forstner et al., 1994) that were likely to have been dictated by the predominant types of adenylate cyclase present in the cells (cf. Cooper et al., 1995). The predominant isoforms of adenylate cyclase present in cardiac cells are thought to be insensitive to PKC activated by phorbol esters (Cooper et al., 1995). In support of this, there was no elevation of cAMP in rat ventricular myocytes treated with PMA (Lacerda et al., 1988). Furthermore, in guinea pig ventricular myocytes, phorbol esters failed to stimulate $I_{Ca,L}$ (Walsh and Kass, 1988; Tohse et al., 1990; Asai et al., 1995a), a current well-known for its stimulatory response to PKA activation (Hescheler et al., 1987; McDonald et al., 1994) (Figure 7). The present study confirms the lack of a stimulatory $I_{Ca,L}$ response to PMA even in guinea pig myocytes in which co-monitored I_K was stimulated by PMA, and $I_{Ca,L}$ was fully responsive to subsequent FSK.

There are several reports indicating that there are differences in the properties of currents that are activated by PKC and compared to the properties of the same currents activated by PKA. (i) Walsh and Long (1994) reported that total replacement of Cl^- by I^- strongly reduced the activation of I_{Cl} (and $I_{Ca,L}$) by PKA, while a similar substitution slightly augmented the activation of I_{Cl} by PKC in guinea pig ventricular myocytes. They also noted that there was a difference in the relative permeability ratio of I^- to Cl^- for PKA-activated ($Cl^- \geq I^-$) and PKC-activated ($I^- > Cl^-$) Cl^- channels. Preliminary results in the present study on Cl^- substitution with I^- have confirmed their findings; replacement of most of the external Cl^- with I^- increased outward I_{Cl} at +40 mV by 20-30% in PMA-treated myocytes. (ii) The kinetic effects accompanying phorbol ester

stimulation of PKA-sensitive guinea pig ventricular I_K (Walsh and Kass, 1991) and rat ventricular L-type Ca^{2+} current ($I_{Ca,L}$) (Bourinet et al., 1992) are different than those elicited by PKA stimulation. These results, and the fact that PMA fails to stimulate PKA-sensitive $I_{Ca,L}$ in guinea pig ventricular myocytes, suggest the absence of a convergence at, or upstream, of PKA.

Two experimental details related to the foregoing deserve mention. First, Na^+-substituted TMA^+ superfusate was routinely used for I_{Cl} experiments. TMA^+ has been shown to stimulate muscarinic receptors and dampen FSK activation of adenylate cyclase and I_{Cl} in guinea pig ventricular myocytes (Zakharov et al., 1995). Secondly H-7 pretreatment in the present study had no significant inhibitory effect on the stimulation of $I_{Ca,L}$ by 1 μM FSK (Asai et al., 1995b). This suggests that, as in other cells (Conn et al., 1989; Forstner et al., 1994), H-7 is a relatively ineffective inhibitor of PKA under 'isolated in vivo" conditions, and that its inhibition of PMA-mediated stimulation of I_{Cl} and I_K in guinea pig ventricular myocytes was not due to spill-over inhibition of PKA.

G.   RESPONSES DURING CO-ACTIVATION OF PKC AND PKA

1.   Co-activation of I_{Cl}

Walsh and Long (1994) observed that activation of guinea pig myocyte I_{Cl} by internal dialysis with $PKC\alpha$ and external application of PDBu significantly reduced the degree of stimulation produced by subsequent application of FSK. In addition, Zhang et al. (1994a) reported that (i) submaximal concentrations of PMA and FSK had additive stimulatory effects on I_{Cl}, (ii) maximally-effective concentrations of PMA precluded further

activation by FSK, and (iii) cells with I_{Cl} unresponsive to PMA were similarly unresponsive to FSK or isoproterenol.

The results with co-application of maximally-effective concentrations of PMA and FSK differ from the foregoing. First, FSK activated I_{Cl} in all of the 8 cells in which 0.1-5 μM PMA failed to stimulate I_{Cl}. Secondly, in PMA-responsive myocytes, maximal activation of I_{Cl} by the phorbol ester did not occlude further stimulation by FSK. In fact, the degree of FSK stimulation in the latter myocytes was slightly larger ($p < 0.06$) than in control myocytes. While other interpretations are possible, this result is consistent with a small potentiating effect of PKC on PKA phosphorylation of cardiac Cl^- channels, as previously suggested for CFTR Cl^- channels in some non-cardiac cells (e.g. Dechecchi et al., 1992; Tabcharani et al., 1991).

The effects of PMA and FSK on macroscopic I_{Cl} in guinea pig ventricular myocytes measured in the present study complement the observation of Collier and Hume (1995) on single-channel Cl^- currents in cell-attached membrane patches of these cardiac cells. They found that (i) PKA activation (with IBMX) further increased the channel open-state probability after PKC activation (with PMA/PDBu), and (ii) PKA activation enhanced open-state probability even when PKC activation did not.

2.   Co-activation of I_K

Walsh and Kass (1988) found that the cAMP analogue, 8-(4-chlorophenylthio)-cAMP, had an additive stimulatory effect on I_K during application of a maximally-effective concentration (10 nM) of PDBu to guinea pig ventricular myocytes. In similar myocytes, Yazawa and Kameyama (1990) found that PMA could enhance I_K even after I_K had been maximally stimulated

by either FSK (10 μM), cAMP (100 μM), or catalytic subunit of PKA (5 μM). Based on these results, the two groups of authors suggested that the active sites of PKA and PKC on the delayed-rectifying K^+ channel are different (Walsh and Kass, 1988; Yazawa and Kameyama, 1990) and may be independent of each other (Yazawa and Kameyama, 1990).

The results with co-application of PMA and FSK in the present study confirmed the observations cited above. 1 μM FSK incremented the stimulation of I_K that had been induced by a maximal concentration (100 nM) of PMA. Although the degree of stimulation by FSK in PMA-treated myocytes was slightly less than that in control myocytes, the difference was not statistically significant. The reverse sequence of drug application (FSK followed by PMA) also resulted in an additive increase in I_K. These results, together with these of Walsh and Kass (1988) and Yazawa and Kameyama (1990), suggest that simultaneous activation of the two kinases does not lead to either potentiation or occlusion of individual effects on I_K. The roughly additive effects of PKC and PKA activation on I_K also suggest that PMA stimulation of I_K is not mediated by activation of PKA (and that FSK stimulation is not mediated by activation of PKC) (see Section IV.F.2. above)

3. Co-activation of $I_{Ca,L}$

A possible interaction of the PKC and PKA systems is not readily open to investigation in regard to $I_{Ca,L}$ in guinea pig ventricular myocytes because in these cells $I_{Ca,L}$ does not seem to be modulated by PKC activation (see Section IV.D.2). Nevertheless, FSK stimulation of $I_{Ca,L}$ in PMA-treated cells was compared with control cells. Stimulation of $I_{Ca,L}$ by 1 μM FSK was unaffected by pretreatment with 100 nM PMA.

4. Summary of modulation by PMA + FSK

The results on the effects of PMA and FSK on I_K and $I_{Ca,L}$ do not provide any indication of an interaction of PKC and PKA pathways (although interaction at the receptor level with other agonists cannot be ruled out; i.e. effects of PKC activation on β-adrenergic receptors or G proteins or effects of PKA activation on phospholipase C: Takai et al., 1982; Sibley et al., 1984; Teitelbaum, 1990; Pitcher et al., 1992). The small potentiating effect of co-applied PMA and FSK on I_{Cl} suggests that there may be an interaction between PKC and PKA in regard to phosphorylation and opening of the CFTR Cl⁻ channel. It is important to point out that interaction of the two enzyme pathways was investigated using maximally-effective concentrations of PMA and FSK; submaximally-effective concentrations may well turn up different effects that lead to different conclusions from those reached here.

F. CONCLUDING REMARKS

I have investigated phorbol ester modulation of four different membrane currents, I_{Cl}, I_K, I_{K1}, and L-type Ca^{2+} channel current ($I_{Ca,L}$, $I_{Na,L}$), in guinea pig ventricular myocytes. The results indicate that two of the four currents, I_{Cl} and I_K, were activated by phorbol ester PMA via the activation of PKC, and that $I_{Ca,L}$ and $I_{Na,L}$ were inhibited by PMA via a PKC-independent action. Although changes in I_{Cl} and I_K by the activation PKC were relatively small compared with those induced by the activation of PKA, these were expected to shorten action potential duration. Shortening of action potential

duration by PDBu and α_1-adrenergic stimulation has been observed in guinea pig papillary muscle (Dirksen and Sheu, 1990).

The results on I_K and L-type Ca^{2+} channel current were different from other reports on PMA inhibition of I_K (Apkon and Nerbonne, 1988; Zhang et al., 1994b) and PMA stimulation of L-type Ca^{2+} channel current (Lacerda et al., 1988; Bourinet et al., 1992; Liu et al., 1993) in (or expressed from) rat ventricular myocytes. Lack of stimulation of L-type Ca^{2+} channel current was also observed when Na^+ was used as the charge-carrier in guinea pig ventricular myocytes. This result established that charge-carrier is not the cause of the difference in PMA responses between rat and guinea pig myocytes. These results may suggest species difference in the modulation of I_K and L-type Ca^{2+} channel current by PKC.

Since I_{Cl} and I_K are also activated by the activation of PKA, these currents are under dual regulation by PKC and PKA. I have examined possible cross-talk between the two kinase systems by using maximally-effective concentrations of PMA and FSK. Although these experiments did not indicate that there was a synergistic activation of I_K by co-application of PMA and FSK, there were hints of a synergistic activation of I_{Cl} by FSK in PMA-treated myocytes. However, I_K may be activated synergistically under more physiological conditions since I_K appears to become more sensitive to an increase in $[Ca^{2+}]_i$ when the current has been stimulated by PKC (Tohse et al., 1990) and $[Ca^{2+}]_i$ may be increased during PKA activation via an increase in influx of Ca^{2+} through L-type Ca^{2+} channels. There may also be a synergistic stimulation of contraction by PKC and PKA since α_1-adrenoceptor stimulation (which activates PKC) and β-adrenoceptor stimulation cause positive inotropy via myofibrillar sensitization (Pucéat et al., 1992) and an increase in $I_{Ca,L}$,

respectively. These observations indicate that PKC and PKA are complementary under physiological situation.

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