British Journal of Pharmacology (1996) 117, 1395–1404

Phorbol ester activation of chloride current in guinea-pig ventricular myocytes

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1 Although earlier studies with phorbol esters indicate that protein kinase C (PKC) may be an important regulator of Cl^- current (I_{Cl}) in cardiac cells, there is a need for additional quantitative data and investigation of conflicting findings. Our objectives were to measure the magnitude, time course, and concentration-dependence of I_{Cl} activated in guinea-pig ventricular myocytes by phorbol 12-myristate 13-acetate (PMA), evaluate its PKC dependence, and examine its modification by external and internal ions.

2 The whole-cell patch clamp technique was used to apply short depolarizing and hyperpolarizing pulses to myocytes superfused with Na^+-, K^+-, Ca^{2+}-free solution (36°C) and dialysed with Cs^+ solution. Stimulation of membrane currents by PMA (threshold $\leqslant$1 nM, $EC_{50} \approx 14$ nM, maximal 40% increase with $\geqslant$100 nM) plateaued within 6–10 min.

3 PMA-activated current was time-independent, and suppressed by 1 mM 9-anthracenecarboxylic acid (9-AC). Its reversal potential (E_{rev}) was sensitive to changes in the Cl^- gradient, and outward rectification of the current-voltage (*I-V*) relationship was more pronounced with 30 mM than 140 mM Cl^- dialysate.

4 The relative permeability of PMA-activated channels estimated from E_{rev} measurements was $I^- > Cl^- >>$ aspartate. Channel activation was independent of external Na^+.

5 PMA failed to activate I_{Cl} in myocytes pretreated with 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) or dialysed with pCa 10.5 solution. Lack of response to 4α-phorbol 12, 13-didecanoate (αPDD) was a further indication of mediation by PKC.

6 I_{Cl} induced by 2 μM forskolin was far larger than that induced by PMA, suggesting that endogenous protein kinase A is a much stronger Cl^- channel activator than endogenous PKC in these myocytes.

7 The macroscopic properties of PMA-induced I_{Cl} appear to be indistinguishable from those of PKA-activated I_{Cl}. We discount stimulation of PKA by PMA as an explanation, and conclude that endogenous PKC may activate PKA-regulated Cl^- channels in these myocytes.

Keywords: Heart cells; chloride channels; protein kinase C (PKC); phorbol 12-myristate 13-acetate (PMA); protein kinase A (PKA); 4α-phorbol 12, 13-didecanoate (αPDD); forskolin (FSK)

Introduction

The movement of Cl^- through sarcolemmal ion channels (I_{Cl}) in the heart influences the configuration of the action potential (Harvey *et al.*, 1990; Hume & Harvey, 1991), participates in cell volume regulation (Tseng, 1992; Sorota, 1992; Zhang *et al.*, 1993), and can promote arrhythmogenic activity (Ackerman & Clapham, 1993). Cardiac I_{Cl} is stimulated by interventions that cause hyposmotic swelling (Tseng, 1992; Sorota, 1992; Zhang *et al.*, 1993; Vandenberg *et al.*, 1994; Shuba *et al.*, 1996), protein kinase A (PKA) activation (Bahinski *et al.*, 1989; Harvey & Hume, 1989; Ehara & Matsuura, 1993), and protein kinase C (PKC) activation (Walsh, 1991; Walsh & Long, 1994; Zhang *et al.*, 1994). Of these, the regulation by PKC is the least well-characterized (for recent review, see Gadsby *et al.*, 1995).

Active phorbol esters such as phorbol 12-myristate 13-acetate (PMA) are widely employed to activate cell PKC. The usual concentration-range investigated in heart (e.g. Tohse *et al.*, 1987; Walsh, 1991) and other cell types (e.g. Castagna *et al.*, 1982; Nishizuka, 1984; 1988; McDonald *et al.*, 1994) is from 0.1 to 100 nM. In regard to phorbol ester action on I_{Cl} in voltage-clamped cardiomyocytes, there is little information on the extent and concentration-dependence of stimulation by PMA (or the other commonly-used active phorbol ester, phorbol 12, 13-dibutyrate (PDBu)). 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, whereas a second group (Zhang *et al.*, 1994) 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: (1) it had a linear current-voltage (*I-V*) relationship, compared to an outward-rectifying relationship in feline myocytes, and (2) 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 forskolin (FSK) or isoprenaline, and maximal activation by PMA occluded further stimulation by FSK (Zhang *et al.*, 1994).

The present study on I_{Cl} in guinea-pig ventricular myocytes was designed to (1) determine the time course of PMA activation, as well as the PMA concentration-response relationship, (2) measure the reversal potential (E_{rev}) and rectification of PMA-activated currents to evaluate the influence of Cl^- distribution, and calculate the permeability of activated channels to other anions relative to that of Cl^-, (3) assess the involvement of PKC in these PMA responses, and (4) compare the relative magnitudes of Cl^- currents activated by PMA and FSK in these myocytes. The results are compared with those from earlier studies, and discussed in relation to cardiac Cl^- currents activated by PKA.

Methods

Cell isolation

Guinea-pigs (ca. 300 g) were cervically-dislocated, and their excised hearts were sequentially perfused (37°C) with oxyge-

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nated normal Tyrode solution, Ca^{2+}-free Tyrode ($CaCl_2$ omitted), Ca^{2+}-free Tyrode containing collagenase (0.05–0.1 mg ml^{-1}; Yakult, Tokyo, Japan), and modified 'KB' solution (Isenberg & Klöckner, 1982). The ventricles were cut into chunks, and cells were dispersed by mechanical agitation and stored in KB solution at room temperature prior to the experiments.

Electrophysiology

An aliquot of KB solution containing myocytes was transferred to the experimental chamber positioned on top of an inverted microscope stage (Nikon Diaphot, Tokyo, Japan). The chamber was perfused with normal Tyrode solution heated to 35–36°C. Pipettes were pulled from thick-walled borosilicate glass capillaries (Jencons, Bedfordshire, U.K.) in the usual two-step process (Hamill *et al.*, 1981). They had an inside tip diameter of 2–4 μm and resistance 2–3 MΩ when filled with pipette solutions. The voltage-clamp amplifier was an EPC-7 (List Medical Electronic, Darmstadt, Germany), and a flowing 3 M KCl, Ag-Cl reference electrode was used to minimize changes in liquid junction potential. Series resistance (generally) 3–5 MΩ was compensated (60–80%) in most of the experiments. Currents and voltages were recorded on a video cassette recorder through an A/D PCM-2-B adapter (Medical Systems Corp., Greenvale, NY, U.S.A.) for off-line computer analysis with pCLAMP 6.0 software (Axon Instruments, Inc., Foster City, CA, U.S.A.) (sampling frequency 3–4 kHz).

Solutions

Myocytes were usually superfused with Na^+-, K^+-, Ca^{2+}-free Tyrode solution containing (mM) tetramethylammonium chloride (TMACl) 140, $MgCl_2$ 1.15, glucose 10, and HEPES 10 (pH 7.4 with TMAOH), as well as 0.2 mM $CdCl_2$ and 1 mM $BaCl_2$ to block residual activity of Ca^{2+} and inward-rectifying K^+ channels, respectively. Test superfusates had one of the following modifications: (i) TMACl elevated to give external Cl^- concentration (Cl^-_0) of 155 mM versus normal ≈145 mM; (ii) 70 mM TMACl replaced by 140 mM sucrose; (iii) TMACl replaced by NaCl; (iv) TMACl replaced by TMA iodide. Myocytes were usually dialysed with Cs^+ pipette solution containing (mM) CsCl 30, CsOH 110, aspartic acid 110, MgATP 5, EGTA 5, and HEPES 5 (pH 7.2 with CsOH), with addition of 0.03 mM $CaCl_2$ calculated (cf. Fabiato & Fabiato, 1979; Tsien & Rink, 1980) to give pCa≈9. Test pipette solutions had one of the following modifications: (i) pCa adjusted to 7 (by addition of $CaCl_2$); (ii) pCa adjusted to nominal 10.5 (Ca^{2+}-free, EGTA increased to 10 mM); (iii) Cl^- concentration elevated to 140 mM by increasing CsCl and omitting aspartic acid.

The KB solution contained (mM) KCl 30, KOH 80, glutamic acid 50, KH_2PO_4 30, $MgSO_4$ 3, taurine 20, glucose 10, EGTA 0.5, and HEPES 10 (pH 7.4 with KOH).

Drugs

Phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12, 13-didecanoate (αPDD), forskolin (FSK), and 9-anthracenecarboxylic acid (9-AC) were dissolved in dimethyl sulphoxide (DMSO) and stored as stock solutions at −20°C; 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) was dissolved in water and also stored at −20°C. Appropriate amounts of stock solutions were added to external and pipette-filling solutions, and corresponding amounts of DMSO (≤0.2%) were also added to the control external solutions. In control experiments ($n=7$), further addition of 0.2% DMSO had no

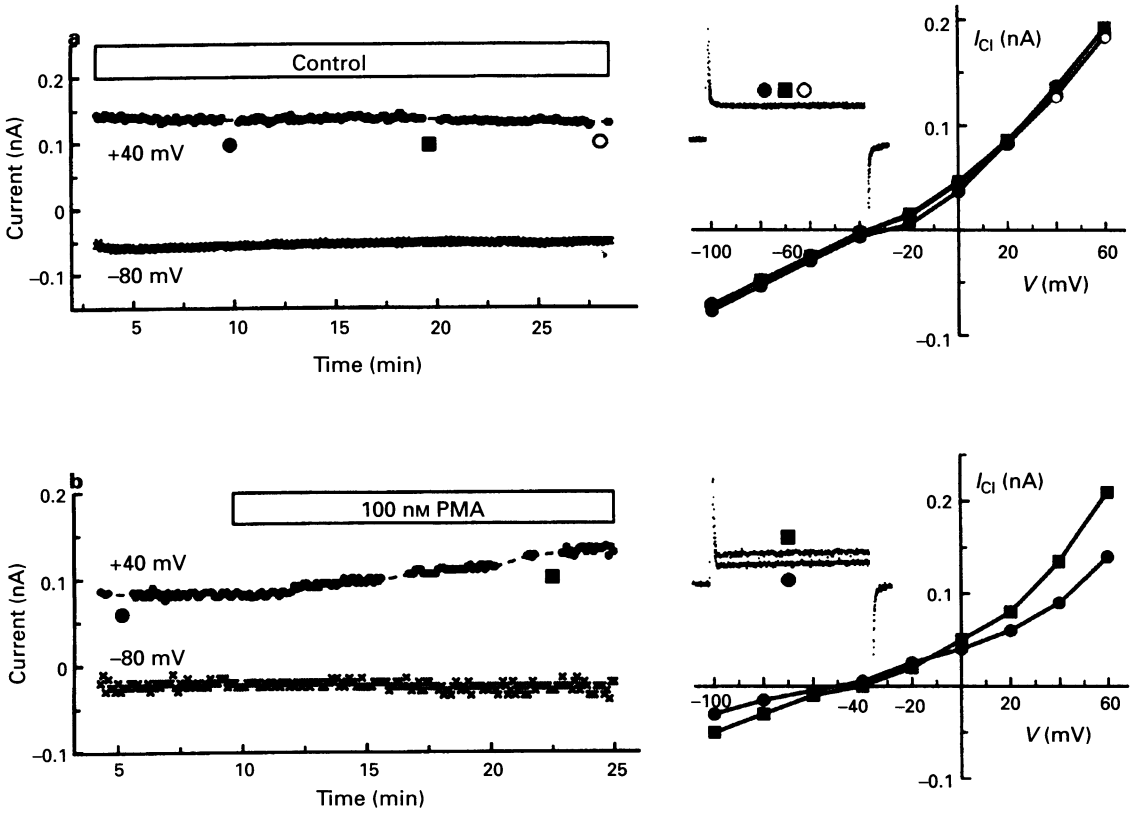

Figure 1 Stimulation of current by PMA. Myocytes held at −80 mV were prepulsed for 100 ms to −40 mV and test-pulsed to a constant potential for 200 ms at 0.2 Hz; regular pulsing was periodically interrupted to record currents at other potentials for determination of *I-V* relationships. (a) Stable currents at −80 and test potential +40 mV under control conditions. The 200 ms records (−40 to +40 mV) and the *I-V* relationships are referenced by symbols to the (post-patch) time graph. (b) Time diary showing stimulation of current (test pulse +40 mV) induced by 100 nM PMA, current records on pulses from −40 mV to +40 mV at the referenced times (inset), and corresponding *I-V* relationships.

effect on I_{Cl} activated by 100 nM PMA or 1–2 μM FSK. H-7 was purchased from Calbiochem (La Jolla, CA, U.S.A.) and 9-AC from Aldrich (St. Louis, MO, U.S.A.); all other agents were purchased from Sigma (St. Louis, MO, U.S.A.).

Statistics

Results are expressed as means ± s.e. mean. Comparisons were made using Student's unpaired *t* test or one-way analysis of variance (ANOVA). A difference was considered to be significant when $P<0.05$.

Results

Unless otherwise noted, the guinea-pig ventricular myocytes were investigated under conditions expected to minimize 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; low Ca^{2+} dialysate). In most experiments, the external Cl^- concentration (Cl^-_o) was 145 mM, and the dialysate concentration (Cl^-_i) was 30 mM (calculated $E_{Cl} = -42$ 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 during the last 20 ms of pulses.

Sorota (1992) observed that I_{Cl} in canine atrial myocytes superfused and dialysed 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.*, 1996), unintentional swelling and turn-on of I_{Cl} is a concern in evaluating responses of I_{Cl} to pharmacological interventions.

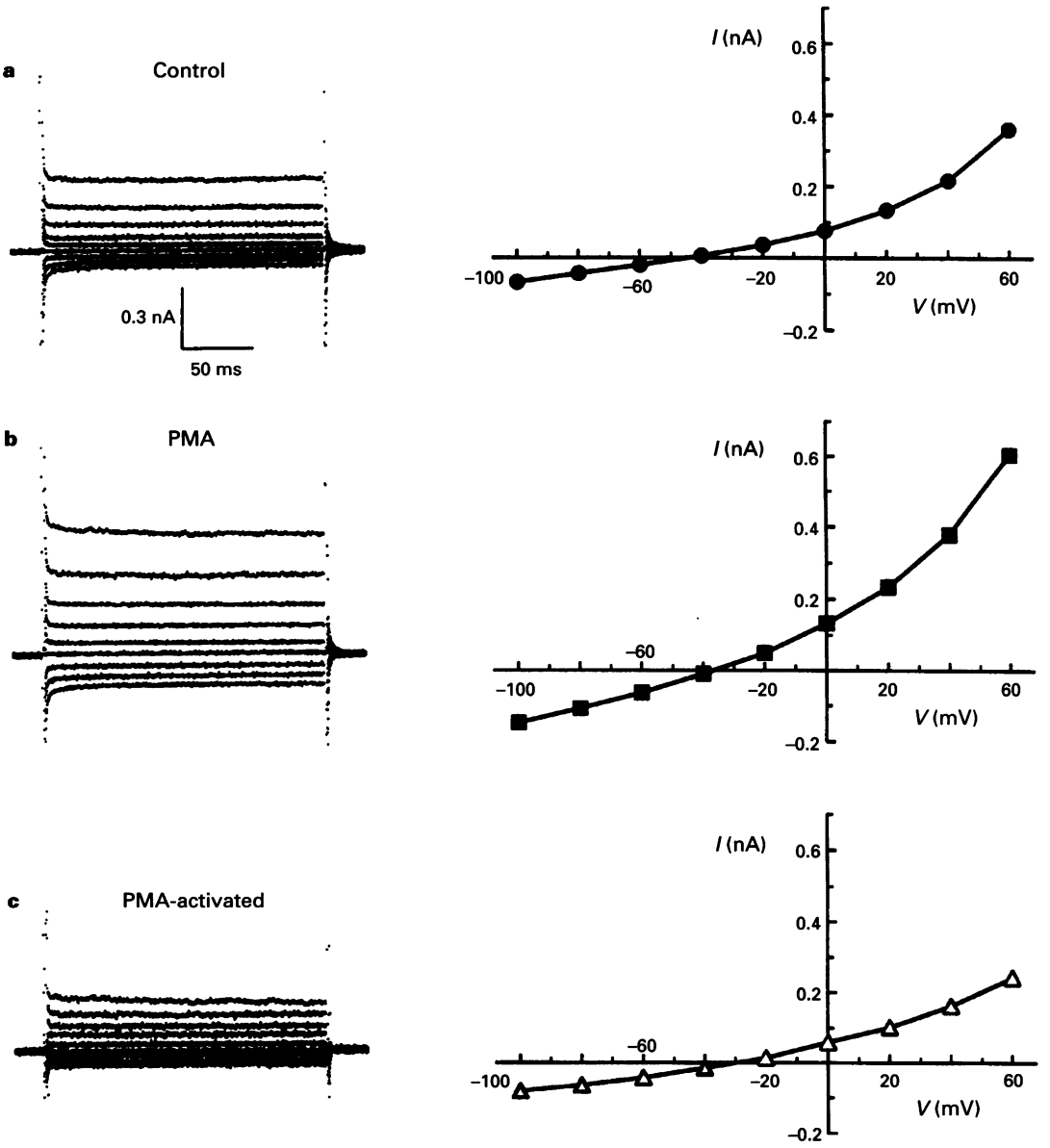

Figure 2 Current activated by PMA. Families of myocyte currents elicited by 200-ms pulses to potentials between −100 (bottom trace) to +60 mV (top trace) in 20 mV increments are shown on the left, and corresponding *I-V* relationships on the right. (a) Before, (b) 12 min after application of 100 nM PMA. (c) Currents and *I-V* obtained after subtraction of control from PMA-stimulated currents.

However, neither visually-detectable swelling nor activation of I_{Cl} over time occurred in myocytes investigated under isosmotic conditions in the present study. Figure 1a illustrates the stability of the basal current amplitude monitored at −80 and +40 mV, and the stability of the *I-V* relationships determined at 9, 20 and 28 min post-patch breakthrough (also see Figure 8). (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.)

Figure 1b depicts the results obtained from a myocyte treated with 100 nM PMA. The time diary shows that the current monitored from pulses to + 40 mV increased by about 40% during the 14-min treatment. In absolute terms, the increase in outward current at + 40 mV (≈45 pA) was considerably larger than the inward increment (≈12 pA) measured at −80 mV, and the induced chord conductance (*g*) between −40 and +40 mV was ≈0.6 nS. The PMA-stimulated current was relatively time-independent, and its *I-V* relationship crossed that of the basal current near −40 mV.

Families of current traces and *I-V* relations from a different myocyte indicate that addition of 100 nM PMA for 11 min increased both outward and inward current by approximately 70% (Figure 2a,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 (Figure 2c). The increment in current at +40 mV was ≈160 pA (≈1 pA/pF), corresponding to an induced chord (E_{rev} to +40 mV) conductance of ≈2.3 nS (one of the largest recorded in this study).

Time course and concentration-dependence of PMA stimulation

Figure 3 shows plots of current amplitudes monitored at −80 and +40 mV from representative myocytes treated with one of four concentrations (1 nM, 10 nM, 300 nM, 5 μM) of PMA for 10–15 min. There was little increase in current during the 1 nM application, a small increase with 10 nM, and larger increases with the higher concentrations. The latter were more pronounced at +40 mV than at −80 mV, 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; Tseng & Boyden, 1991). However, stimulations produced by shorter treatments with moderate concentrations of PMA were reversible on occasion (see Figure 5).

The time courses of the stimulations caused by PMA, and their magnitudes, were evaluated by relating PMA-induced increases in chord conductance (−80 mV to +40 mV) to the pre-PMA basal 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 8–10 min of drug application (Figure 4a). The concentration-response curve (Figure 4b) 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 ≈14 nM. There were no significant differences between the increases produced by 100 nM (40±8.5%, n=21), 300 nM (34.3±9.6%, n=8), 1 μM (39.2±4.6%, n=14), and 5 μM (34.6±6.5%, 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 ≤15% increase in conductance, and a similar proportion registered a ≥60% stimulation.

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, 1 μM or 5 μM PMA (n=4-6) 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 4c). 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.

Anion and cation-dependence

All of the results described to this point were obtained from myocytes superfused with 145 mM Cl^- solution and dialysed 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 dialysed with 140 mM Cl^-, aspartate-free solution (155 mM Cl^- superfusate, calculated

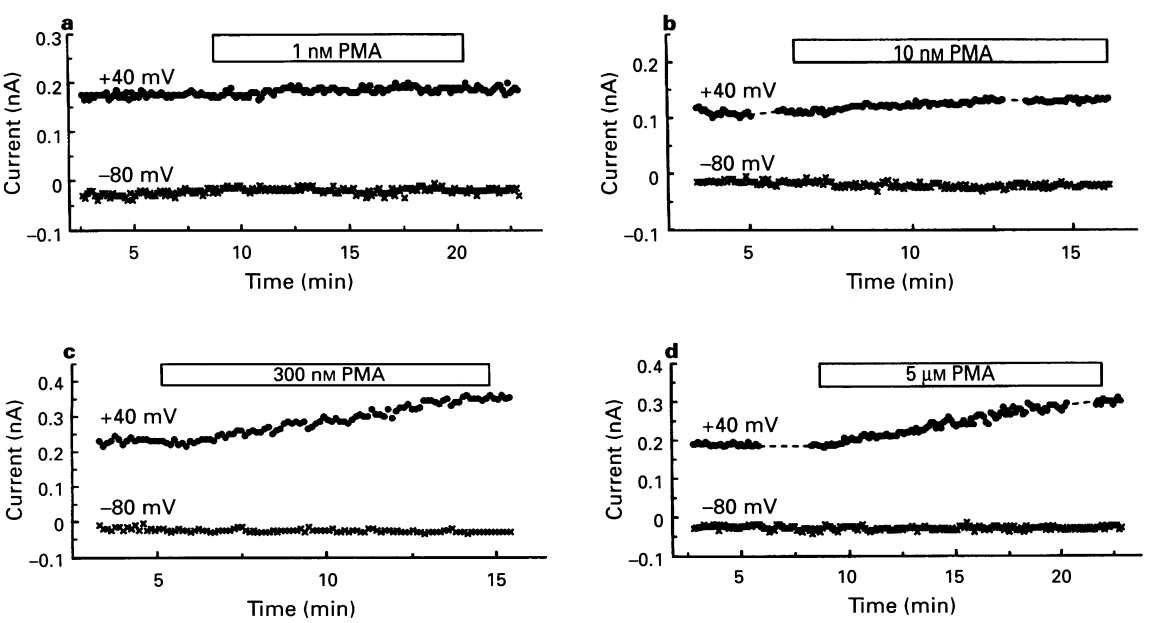

Figure 3 Stimulation by PMA: time plots of current amplitudes at −80 mV and +40 mV measured from four myocytes before and during application of (a) 1 nM, (b) 10 nM, (c) 300 nM and (d) 5 μM PMA.

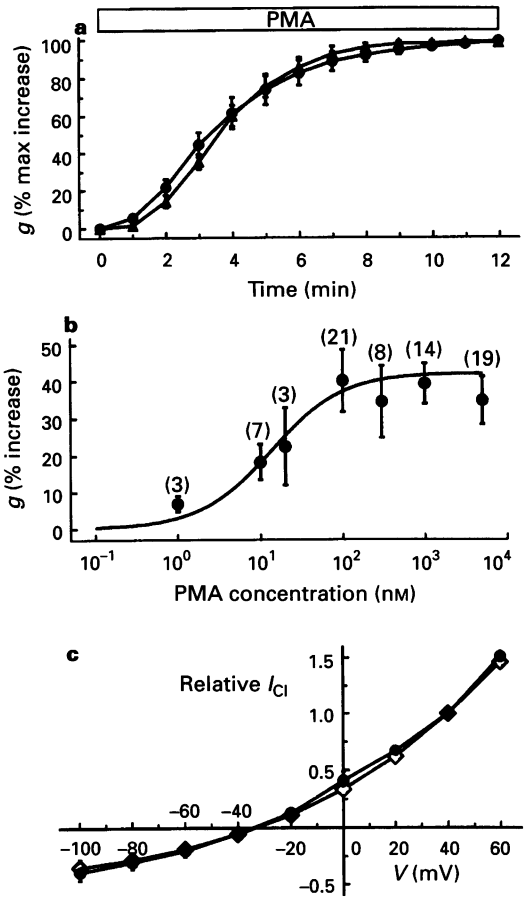

Figure 4 Time course of PMA stimulation, and steady-state concentration-response relations. (a) Normalized time courses of the increases in chord conductance (g, −80 to +40 mV) induced by 100 nM (n=9) (●) and 5 μM (n=8) (▲) PMA. (b) Concentration-g relationship. The data are from myocytes treated for 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}=14 nM, E_{max}=42%, and n=1. (c) I-V relationships of PMA-activated I_{Cl}. Pre-PMA currents were subtracted from PMA-stimulated currents (see Figure 2), normalized relative to the current activated at +40 mV, and averaged: (●) 100 nM; (▲) 1 μM; (◇) 5 μM; n=4–6 myocytes for each relationship.

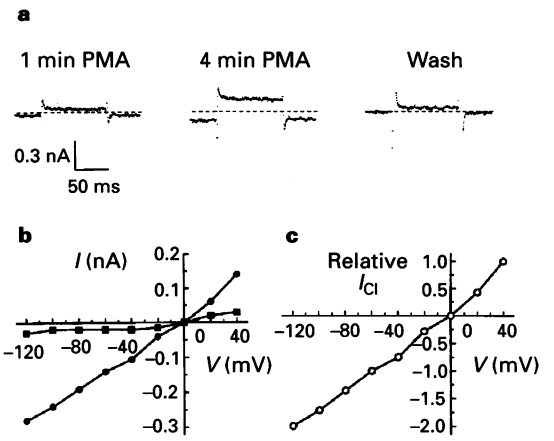

Figure 5 PMA-activated current in a myocyte dialysed with 140 mM Cl^- solution. (a) Difference currents (−40 to +40 mV pulses) activated 1 and 4 min after addition of 100 nM PMA decayed to near zero after 15 min washout. On each panel, the dashed line indicates zero difference from pre-PMA current, the leading and end currents are at −40 mV, and the middle current is at +40 mV. (b) I-V relationships of difference currents 4 min after application of PMA (●) and following 15 min wash (■). (c) PMA-activated current normalized to current activated at +40 mV.

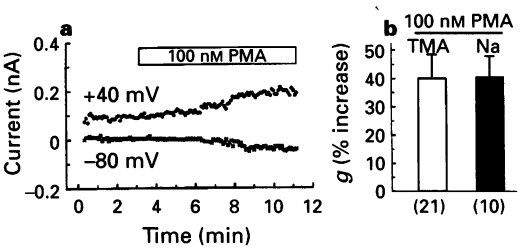

Figure 6 Activation of current by 100 nM PMA in myocytes superfused with 140 mM Na^+ solution. (a) Time diary of current amplitude at −80 and +40 mV. (b) Comparison of stimulation (% increase in g (−80 to +40 mV)) in myocytes superfused with Na^+-containing and TMA^+-containing solutions. Number of myocytes in parentheses.

E_{Cl} = −3 mV). Results from a representative myocyte indicate that the difference currents measured at −40 mV and +40 mV increased during the 4-min application of 100 nM PMA, and subsided to near zero following ≈15 min superfusion with drug-free solution (Figure 5a). The I-V relationship of the PMA-activated current crossed the voltage axis at about 0 mV (Figure 5b). Scrutiny of the normalized I-V (+40 mV = 1.0) indicates that the outward chord conductance (0 to +40 mV) was 1.3 times larger than the inward chord conductance (0 to −40 mV) (Figure 5c). On average, the ratio was 1.2±0.06 (n=5), considerably smaller than the 1.9±0.03 (n=16) value measured in low Cl^- dialysed myocytes (see Figure 4c), and in rough accord with predictions of the GHK equation (see Discussion).

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

The absence of external Na^+ (particularly when replaced by TMA^+) markedly depressed cyclic AMP-dependent activation of I_{Cl} in guinea-pig ventricular myocytes (Harvey *et al.*, 1991; Zakharov *et al.*, 1995). We investigated the role of Na^+ in the PMA activation of I_{Cl} by conducting a series of experiments with a superfusate that contained 140 mM Na^+ instead of TMA^+. Figure 6a 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±7.4% compared to 40±8.5% (n=21) with TMA^+ solution (Figure 6b).

Inhibition by 9-AC

9-AC is a widely-used Cl^- transport inhibitor (Cabantchik & 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 & Greger, 1992); also see Harvey (1993) for anomalous activation of current by Cl^- transport-inhibiting stilbene derivatives.) We minimized this problem 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 7a shows an example of the first type of measurement in myocytes superfused with Na^+ solution. The current was measured at 0 mV to minimize the influence of any non-specific 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, 62.3 ± 15% of PMA-activated g_{Cl} was inhibited by 9-AC (Figure 7b). For comparison, Zhang *et al.* (1994) reported that 0.1 mM 9-AC blocked 48% ($n = 5$) of the PMA-activated current in feline ventricular myocytes, and Walsh & 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 dialysed with exogenous PKC.

An example of the second type of measurement is provided in Figure 7c. 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 41.5 ± 10% increase in conductance (Figure 7d), a value which is not statistically different from the control stimulation by 100 nM PMA in myocytes superfused with Na^+ solution (40 ± 7.4% ($n = 10$), Figure 6b).

Investigation of PKC involvement

The possible role of PKC activation in the PMA stimulation of I_{Cl} was investigated by (1) determining whether pretreatment with H-7 suppressed stimulation by PMA, (2) comparing the effects of the inactive phorbol ester αPDD with those of PMA, and (3) examining whether dialysate pCa influenced the response to PMA. Since different PMA-activatable isoforms of PKC may require higher concentrations of phorbol ester for activation than others (Nishizuka, 1988; Ryves *et al.*, 1991), and since the lower intracellular Ca^{2+} concentration expected with pCa 10.5 versus 9 dialysate might desensitize PKC to phorbol ester action (Ryves *et al.*, 1991; Bourinet *et al.*, 1992), we used 5 μM PMA in these experiments.

H-7, a well-established inhibitor of PKC (e.g. Hidaka *et al.*, 1984; Conn *et al.*, 1989; Hidaka & Kobayashi, 1992; Forstner *et al.*, 1994), was added to both the superfusate (20 μM) and the dialysate (200 μM) for 22 ± 1.6 min prior to application of 5 μM PMA for 10–15 min. The example time diary in Figure 8a indicates that PMA failed to stimulate I_{Cl} under these conditions. Figure 8b shows measurements of current amplitude at −80 mV and +40 mV from a representative myocyte treated with 100 nM αPDD; the 10-min application of the inactive phorbol ester had little effect. Records from a different myocyte (Figure 8c) indicate that a 50 fold larger concentration of this phorbol ester was equally ineffective. Finally, there was also little stimulation when PMA was applied to a myocyte dialysed with pCa 10.5 solution (Figure 8d).

A summary of the data from experiments investigating PKC involvement in the PMA stimulation (Figure 9a) indicates that (1) compared to control stimulation with 5 μM PMA (34.6 ± 6.5%, $n = 19$), stimulation was absent (1 ± 3%, $n = 5$) in myocytes pretreated with H-7, (2) compared to control 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, and (3) compared to standard pCa 9 dialysate conditions, stimulation with 5 μM PMA was minimal (3 ± 3%, $n = 7$) in myocytes dialysed with pCa 10.5 solution, and reduced (15 ± 7%, $n = 6$) in myocytes dialysed with pCa 7 solution.

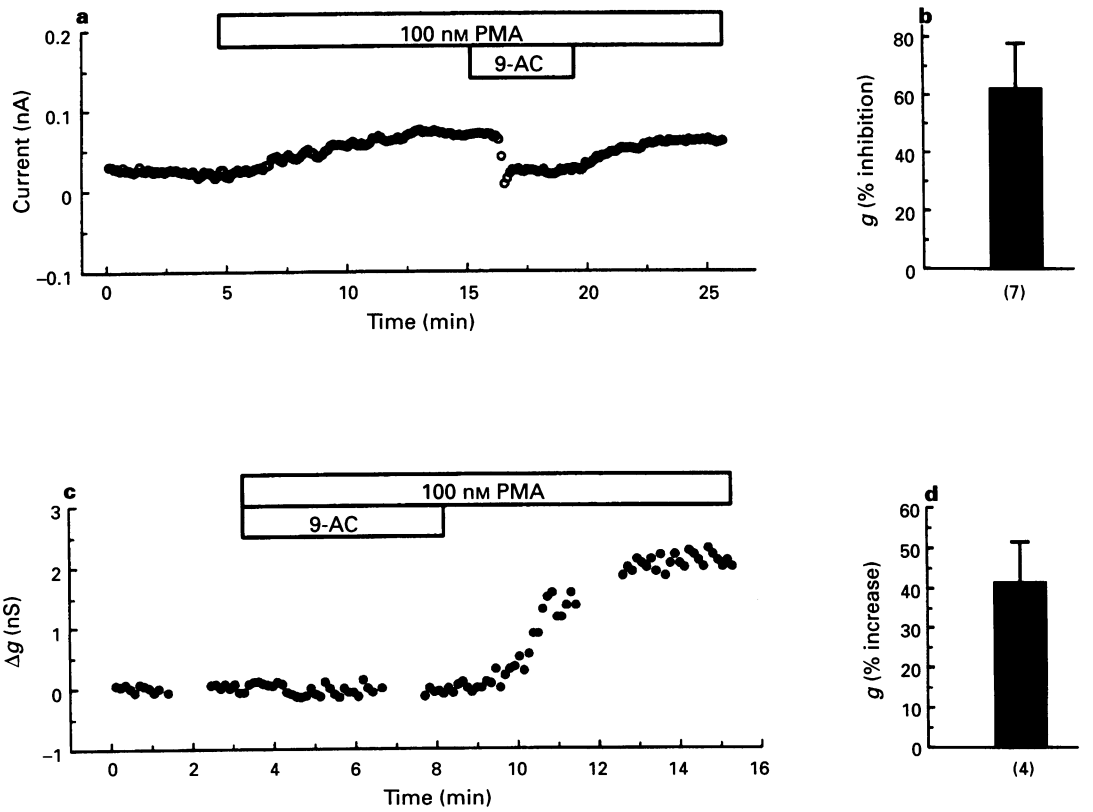

Figure 7 Effect of 1 mM 9-AC on PMA-activated Cl^- conductance. (a) Inhibition by 9-AC of PMA-induced outward current measured on 200 ms pulses to 0 mV. (b) Summary of inhibition by 9-AC in experiments similar to (a). The data are expressed as percentage decrease in PMA (100 nM, >6 min)-induced chord conductance (E_{rev} to +40 mV) measured ~3 min after addition of 1 mM 9-AC. (c) Disinhibition of conductance (E_{rev} to +40 mV) induced by 100 nM PMA upon removal of external 9-AC, expressed as difference ($\triangle g$) 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).

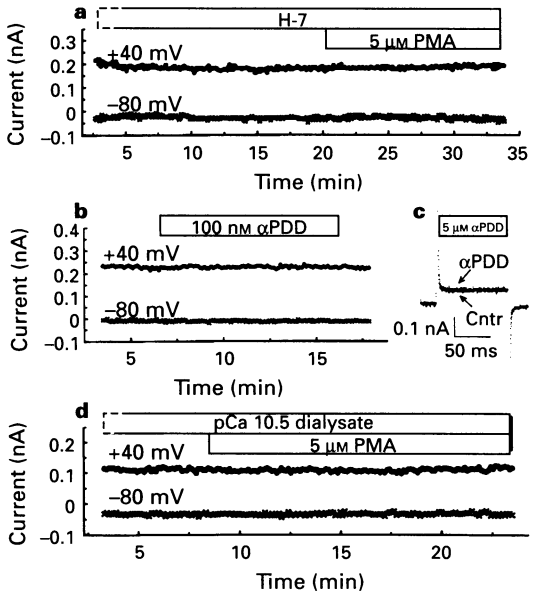

Figure 8 Experiments with H-7, αPDD and pCa 10.5 dialysate. (a) Lack of stimulation by 5 μM PMA in a myocyte pretreated for 20 min with 20 μM H-7 superfusate/200 μM H-7 (pCa 9) dialysate. (b), (c) Lack of stimulation by 100 nM (b) and 5 μM (c) αPDD; the superimposed records in (c) were obtained before (Cntr) and 9 min after addition of αPDD. pCa 9 dialysate. (d) Lack of stimulation by 5 μM PMA in a myocyte dialysed with pCa 10.5 solution.

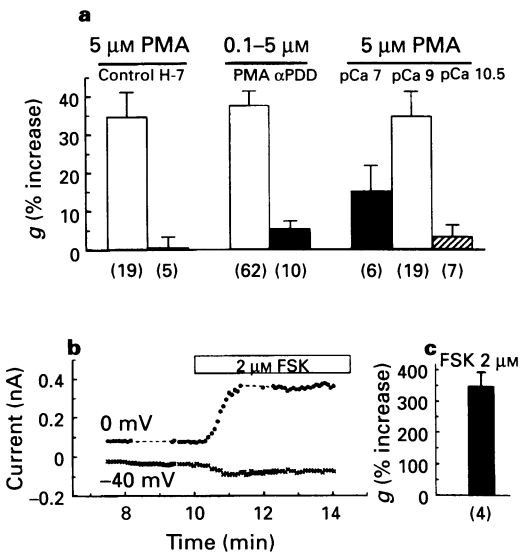

Figure 9 Summary of results from experiments on PKC involvement in PMA action, and comparison with FSK stimulation. (a) Data from experiments with H-7, αPDD, and pCa 7–10.5 dialysates. The H-7 and αPDD experiments were conducted with pCa 9 dialysate. The data are expressed as percentage increase in g (−80 to +40 mV) over pre-PMA values. H-7, αPDD, and pCa 10.5 data are significantly different from control; pCa 7 is not significantly different. (b) Stimulation by 2 μM FSK. Current was measured at −40 and 0 mV. Na^+ superfusate. (c) Average increase in g (−80 to +40 mV) measured after 3 min from experiments as in (b). Note the different amplitude scale in (c) compared to (a).

Stimulation with FSK

For comparison with the PMA results, we measured the magnitude of stimulation induced by activation of the PKA pathway. Myocytes superfused with 140 mM NaCl solution and dialysed with 30 mM CsCl solution were treated with 2 μM FSK for 3–5 min. The example time diary (Figure 9b) indicates that FSK increased outward current at 0 mV by several fold, and affected only slightly the inward current at −40 mV. The extra conductance (−40 to 0 mV) activated in this myocyte by FSK was 11 nS. This may be compared to some of the larger conductances activated by ⩾100 nM PMA; in the experiments of Figures 1b, 2, 4c, 4d, 6a and 7c, the induced conductances (E_{rev} to +40 mV) were 0.6, 2.3, 1.7, 1.4, 1.4 and 2 nS (1.57±0.24 nS, $n=6$). The data in Figure 9c allow a second comparison of the relative strengths of the two interventions. The percentage increase in g (−80 to +40 mV) induced by 2 μM FSK was 346±45% ($n=4$) which is approximately 9 times as large as the average increases recorded from myocytes treated with ⩾100 nM PMA (Figure 4b).

Discussion

In accord with earlier studies on guinea-pig (Walsh, 1991; Walsh & Long, 1994) and feline (Zhang *et al.*, 1994) ventricular myocytes, we found that PMA activates whole-cell I_{Cl}. When compared to basal membrane conductance, the average stimulation produced by 0.1–5 μM PMA was about 40%. In the discussion that follows, we begin by focusing on the concentration-dependence of PMA activation of I_{Cl}, and then examine the anion-dependence of the PMA-induced current, the evidence linking the current to an activation of PKC, and the relation of PMA-activated I_{Cl} to PKA-activated I_{Cl}.

Time course, concentration-dependence and variability of PMA stimulation

The increase in current provoked by PMA had a threshold near 1 nM, was half-maximal at 10–20 nM, and maximal near 100 nM. This 'effective' concentration-range of PMA is similar to that determined for PMA stimulation of delayed-rectifier K^+ current (I_K) in guinea-pig ventricular myocytes (Tohse *et al.*, 1987; 1990; Walsh & Kass, 1988) and L-type Ca^{2+} channel current in neonatal rat ventricular myocytes (Dösemeci *et al.*, 1988; Liu *et al.*, 1993). Although there are no other published descriptions of the time course of I_{Cl} activation by PMA, those measured here are in good agreement with those noted in the foregoing reports on I_K and Ca^{2+} channel currents.

Tohse *et al.* (1987) observed that stimulation of cardiac I_K declined at PMA concentrations >100 nM, and attributed this 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 (Ryves *et al.*, 1991). However, it may also have been due to channel block (cf. Hockberger *et al.*, 1989; Doerner *et al.*, 1990) and/or desensitization/down-regulation. Bourinet *et al.* (1992) invoked the latter to explain the marked (post-stimulation) inhibition of cardiac Ca^{2+} channel current that begins ≈5 min after addition of PMA (Lacerda *et al.*, 1988; Liu *et al.*, 1993). There were no indications that any of these factors had a major impact on our results; I_{Cl} activated by 5 μM PMA was not smaller than I_{Cl} activated by 100 nM, macroscopic properties of the current (rectification, E_{rev}) were not detectably affected, activation time courses were monotonic, and delayed inhibitory phases following activation were not evident.

Walsh (1991) has presented example records indicating a 100% increase of current in guinea-pig ventricular myocytes treated with 20 nM PMA. The largest stimulation we recorded with 10–20 nM PMA was ≈40%, although increases >60% were obtained in about 20% of the myocytes treated with ⩾100 nM concentrations. Zhang *et al.* (1994) 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 increase in the feline myocytes they investigated was approximately 100% after application of a maximally-effective 6 μM concentration. This is a very high concentration of PMA, the necessity for 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 myocytes was not larger with 5 μM than with 100 nM. However, independent of the PMA concentration (0.1–5 μM) which we applied, approximately 20% of the myocytes tested responded with rather small activation of I_{Cl}. Zhang *et al.* (1994) have previously reported that there was little activation of I_{Cl} in 24% of feline ventricular cells treated with 6 μM PMA. Perhaps PKC isoforms responsible for I_{Cl} activation are poorly expressed in some myocytes, or that variation in responses is due to regional differences in the expression of PKA-regulated Cl^- channels (see below) in guinea-pig heart (cf. James *et al.*, 1995).

Anion-dependence

Subtraction of basal current from PMA-stimulated current isolated an activated current whose E_{rev} shifted as expected for a Cl^- dominated current (see below). Activated I_{Cl} rectified in the outward direction in myocytes dialysed with low Cl^- solution. This result addresses an uncertainty about the properties of PMA-activated I_{Cl} in guinea-pig ventricular myocytes dialysed with low Cl^- solution. In one study (Walsh, 1991), 20 nM PMA (or PDBu) activated a current that had a linear dependence on voltage. However, the same group (Walsh & Long, 1994) subsequently found that I_{Cl} activated by PDBu had an outwardly-rectifying *I-V* relationship in myocytes dialysed with solution that contained rabbit brain PKCα. They speculated that the discrepancy may have been caused by activation of exogenous PKC. The present results indicate that activation of endogenous PKC turns on outwardly-rectifying whole-cell I_{Cl}, in accord with reports of outward-rectifying relationships for (1) PDBu-activated average single-channel Cl^- current in cell-attached patches of guinea-pig ventricular myocytes (Collier & Hume, 1995), and (2) PMA-activated I_{Cl} in feline ventricular myocytes dialysed with low Cl^- solution (Zhang *et al.*, 1994).

PMA-activated I_{Cl} in feline myocytes dialysed with 150 mM Cl^- solution had a near-linear *I-V* relationship (Zhang *et al.*, 1994). We have also observed a linearizing effect of high internal Cl^- on PMA-activated I_{Cl} in guinea-pig ventricular myocytes, i.e. the outward to inward conductance ratio 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 myocytes (Bahinski *et al.*, 1989; Harvey & Hume, 1989; Harvey *et al.*, 1990; Overholt *et al.*, 1993; Vandenberg *et al.*, 1994). The linearization is predicted by the Goldman-Hodgkin-Katz equation, as well as by other theoretical formulations (Overholt *et al.*, 1993). Although I_{Cl} in PMA-pretreated myocytes dialysed with 140 mM Cl^- solution still rectified in the outward direction, we note that single-channel Cl^- current activated by PKA can also exhibit slight rectification under near-symmetrical Cl^- conditions in these myocytes (Ehara & Matsuura, 1993). In accord with the explanation of outward rectification of isoprenaline-activated I_{Cl} in guinea-pig ventricular myocytes dialysed with low Cl^- solution, we conclude that this feature 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 of an endogenous organic anion.

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 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 swelling- and PKA-activated anion channels in guinea-pig (Vandenberg *et al.*, 1994) ventricular myocytes. Our preliminary data from experiments on I^- substitution of external Cl^- (−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 & Long (1994) from myocytes dialysed with PKCα. The P_I/P_{Cl} of cardiac PKA-activated Cl^- channels is not fully resolved (see Gadsby *et al.*, 1995), with values ranging from 1.67 (Vandenberg *et al.*, 1994) to 0.88 (Overholt *et al.*, 1993).

Indications of Ca^{2+}-dependent PKC involvement

Earlier studies on cardiomyocytes have provided evidence that phorbol ester activation of I_{Cl} is mediated by PKC. Walsh & Long (1994) observed that PDBu applied to guinea-pig ventricular myocytes dialysed with PKCα activated I_{Cl}, and that kinase-inhibiting staurosporine abolished activation. Collier & Hume (1995) found that staurosporine reduced the open probability of single Cl^- channels activated by PDBu in guinea-pig cells, and Zhang *et al.* (1994) 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.

The primary evidence for PKC involvement in the PMA activation of I_{Cl} in the guinea-pig ventricular myocytes investigated in the present study is that pretreatment with PKC-inhibitor H-7 (Hidaka *et al.*, 1984) blocked PMA stimulation (also see below), and that 0.1–5 μM αPDD was an ineffective substitute for PMA. In addition, we found that PMA failed to stimulate I_{Cl} in myocytes that were dialysed with pCa 10.5 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 BAPTA, and Tohse *et al.* (1990) have reported that PMA stimulation of I_K was completely inhibited in guinea-pig ventricular myocytes dialysed with pCa 11 (versus pCa 9) solution. The latter authors also found that I_K stimulation by PMA was occluded in myocytes dialysed with pCa 7 solution. In the present study, there was a smaller stimulation of I_{Cl} by PMA in myocytes dialysed with pCa 7 versus pCa 9 solution. By reference to the I_K study, we surmise that this was 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. More refined studies incorporating measurement of intracellular Ca^{2+} concentration are required to test this postulate. Nevertheless, these findings suggest that both Cl^- and delayed-rectifier K^+ channels in these myocytes are regulated by one or more Ca^{2+}-dependent rather than Ca^{2+}-independent PKC isoforms. In this regard, we note recent studies suggesting that H-7, the kinase inhibitor used in our tests, preferentially inhibits Ca^{2+}-dependent (Ison *et al.*, 1993), membrane-bound (Budworth & Gescher, 1995) PKC.

Relation between PMA-induced I_{Cl} and PKA-activated I_{Cl}

There is considerably more information available on the activation of cardiac Cl^- channels by PKA than by PKC (Gadsby *et al.*, 1995). Hume and colleagues (Levesque *et al.*, 1993; Hume & Horowitz, 1995) have identified the cardiac Cl^- channel activated by PKA as an alternatively spliced variant of the cystic fibrosis transmembrane conductance regulating 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 a different set of Cl^- channels (Walsh & Long, 1994; Zhang *et al.*, 1994; Collier & Hume, 1995). In cat ventricular myocytes, I_{Cl} stimulated by phorbol ester had similar macroscopic time-independent 'kinetics' and outwardly-rectifying (low Cl^-_i) *I-V* relations as FSK-stimulated current (Zhang *et al.*, 1994), and single-channel Cl^- currents activated in cell-attached guinea-pig myocyte membrane patches by phorbol ester were indistinguishable from the single-channel currents activated by FSK (Collier & Hume, 1995). In the latter study, the activation of PKA (with isobutylmethylxanthine) 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 & 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 & Hume, 1989; Overholt *et al.*, 1993; Gadsby *et al.*, 1995). This raises the issue of whether PMA induction of I_{Cl} might not have been due to an activation of PKA. We discount this possibility on several grounds. (1) 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. Gusovsky & Gutkind, 1991; 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, there was no elevation of cyclic AMP in rat ventricular myocytes treated with PMA (Lacerda *et al.*, 1988). (2) The kinetic effects accompanying phorbol ester stimulation of PKA-sensitive guinea-pig ventricular I_K (Walsh & Kass, 1991) and rat ventricular L-type Ca^{2+} current ($I_{Ca,L}$) (Bourinet *et al.*, 1992) are different from 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 (Asai *et al.*, 1996; also see McDonald *et al.*, 1994) suggest the absence of a convergence at, or upstream, of PKA.

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

We thank Jean Crozsman and Darren Cole for technical assistance. This work was supported by the Medical Research Council (Canada) and the Heart and Stroke Foundation of Nova Scotia. L.M.S. holds a Killam Memorial Scholarship.

References

ACKERMAN, M.J. & CLAPHAM, D.E. (1993). Cardiac chloride channels. *Trends Cardiovasc. Med.*, **3**, 23–28.

ASAI, T., SHUBA, L.M., PELZER, D.J. & MCDONALD, T.F. (1996). PKC-independent inhibition of cardiac L-type Ca^{2+} channel current by phorbol esters. *Am. J. Physiol.*, (in press).

BAHINSKI, A., NAIRN, A.C., GREENGARD, P. & GADSBY, D.C. (1989). Chloride conductance regulated by cyclic AMP-dependent protein kinase in cardiac myocytes. *Nature*, **340**, 718–721.

BAJNATH, R.B., GROOT, J.A., DE JONGE, H.R., KANSEN, M. & BIJMAN, J. (1993). Synergistic activation of non-rectifying small-conductance chloride channels by forskolin and phorbol esters in cell-attached patches of the human colon carcinoma cell line HT-29cl.19A. *Pflügers Arch.*, **425**, 100–108.

BERGER, H.A., ANDERSON, M.P., GREGORY, R.J., THOMPSON, S., HOWARD, P.W., MAURER, R.A., MULLIGAN, R., SMITH, A.E. & WELSH, M.J. (1991). Identification and regulation of the cystic fibrosis transmembrane conductance regulator-generated chloride channel. *J. Clin. Invest.*, **88**, 1422–1431.

BERGER, H.A., TRAVIS, S.M. & WELSH, M.J. (1993). Regulation of the cystic fibrosis transmembrane conductance regulator Cl channels by specific kinases and protein phosphatases. *J. Biol. Chem.*, **268**, 2037–2047.

BOURINET, E., FOURNIER, F., LORY, P., CHARNET, P. & NARGEOT, J. (1992). Protein kinase C regulation of cardiac calcium channels expressed in *Xenopus* oocytes. *Pflügers Arch.*, **421**, 247–255.

BUDWORTH, J. & GESCHER, A. (1995). Differential inhibition of cytosolic and membrane-derived protein kinase C activity by staurosporine and other kinase inhibitors. *FEBS Lett.*, **362**, 139–142.

CABANTCHIK, Z.I. & GREGER, R. (1992). Chemical probes for anion transporters of mammalian cell membranes. *Am. J. Physiol.*, **262**, C803–C827.

CASTAGNA, M., TAKAI, Y., KAIBUCHI, K., SANO, K., KIKKAWA, U. & NISHIZUKA, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.*, **257**, 7847–7851.

COLLIER, M.L. & HUME, J.R. (1995). Unitary chloride channels activated by protein kinase C in guinea pig ventricular myocytes. *Circ. Res.*, **76**, 317–324.

CONN, P.J., STRONG, J.A., AZHDERIAN, E.M., NAIRN, A.C., GREENGARD, P. & KACZMAREK, L.K. (1989). Protein kinase inhibitors selectively block phorbol ester- or forskolin-induced changes in excitability of *Aplysia* neurons. *J. Neurosci.*, **9**, 473–479.

COOPER, D.M.F., MONS, N. & KARPEN, J.W. (1995). Adenylyl cyclases and the interaction between calcium and cAMP signalling. *Nature*, **374**, 421–424.

DECHECCHI, M.C., ROLFINI, R., TAMANINI, A., GAMBERI, C., BERTON, G. & CABRINI, G. (1992). Effect of modulation of protein kinase C on the cAMP-dependent chloride conductance in T84 cells. *FEBS Lett.*, **311**, 25–28.

DOERNER, D., ABDEL-LATIF, M., ROGERS, T.B. & ALGER, B.E. (1990). Protein kinase C-dependent and -independent effects of phorbol esters on hippocampal calcium channel current. *J. Neurosci.*, **10**, 1699–1706.

DÖSEMECI, A., DHALLAN, R.S., COHEN, N.M., LEDERER, W.J. & ROGERS, T.B. (1988). Phorbol ester increases calcium current and simulates the effects of angiotensin II on cultured neonatal rat heart myocytes. *Circ. Res.*, **62**, 347–357.

EHARA, T. & MATSUURA, H. (1993). Single-channel study of the cyclic AMP-regulated chloride current in guinea-pig ventricular myocytes. *J. Physiol.*, **464**, 307–320.

FABIATO, A. & FABIATO, F. (1979). Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol., Paris*, **75**, 463–505.

FORSTNER, G., ZHANG, Y., MCCOOL, D. & FORSTNER, J. (1994). Regulation of mucin secretion in T84 adenocarcinoma cells by forskolin: relationship to Ca^{2+} and PKC. *Am. J. Physiol.*, **266**, G606–G612.

GADSBY, D.C., NAGEL, G. & HWANG, T-C. (1995). The CFTR chloride channel of mammalian heart. *Annu. Rev. Physiol.*, **57**, 387–416.

GUSOVSKY, F. & GUTKIND, J.S. (1991). Selective effects of activation of protein kinase C isozymes on cyclic AMP accumulation. *Mol. Pharmacol.*, **39**, 124–129.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HARVEY, R.D. (1993). Effects of stilbenedisulfonic acid derivatives on the cAMP-regulated chloride current in cardiac myocytes. *Pflügers Arch.*, **422**, 436–442.

HARVEY, R.D., CLARK, C.D. & HUME, J.R. (1990). Chloride current in mammalian cardiac myocytes. Novel mechanism for autonomic regulation of action potential duration and resting membrane potential. *J. Gen. Physiol.*, **95**, 1077–1102.

HARVEY, R.D. & HUME, J.R. (1989). Autonomic regulation of a chloride current in heart. *Science*, **244**, 983–985.

HARVEY, R.D., JUREVICIUS, J.A. & HUME, J.R. (1991). Intracellular Na^+ modulates the cAMP-dependent regulation of ion channels in the heart. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6946–6950.

HIDAKA, H., INAGAKI, M., KAWAMOTO, S. & SASAKI, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry*, **23**, 5036–5041.

HIDAKA, H. & KOBAYASHI, R. (1992). Pharmacology of protein kinase inhibitors. *Annu. Rev. Pharmacol. Toxicol.*, **32**, 377–397.

HOCKBERGER, P., TOSELLI, M., SWANDULLA, D. & LUX, H.D. (1989). A diacylglycerol analogue reduces neuronal calcium currents independently of protein kinase C activation. *Nature*, **338**, 340–342.

HUME, J.R. & HARVEY, R.D. (1991). Chloride conductance pathway in heart. *Am. J. Physiol.*, **261**, C399–C412.

HUME, J.R. & HOROWITZ, B. (1995). A plethora of cardiac chloride conductances: molecular diversity or a related gene family. *J. Cardiovasc. Electrophysiol.*, **6**, 325–331.

ISENBERG, G. & KLÖCKNER, U. (1982). Calcium tolerant ventricular myocytes prepared by preincubation in a 'KB medium'. *Pflügers Arch.*, **395**, 6–18.

ISON, A.J., MACEWAN, D.J., JOHNSON, M.S., CLEGG, R.A., CONNOR, K. & MITCHELL, R. (1993). Evidence for distinct H7-resistant form of protein kinase C in rat anterior pituitary gland. *FEBS Lett.*, **329**, 199–204.

JAMES, A.F., TOMINAGA, M., TOMINAGA, T. & OKADA, Y. (1995). Distribution of cAMP-dependent chloride current and CFTR mRNA in the guinea-pig heart. *Proc. Physiol. Soc.*, 87*P*.

LACERDA, A.E., RAMPE, D. & BROWN, A.M. (1988). Effects of protein kinase C activators on cardiac Ca^{2+} channels. *Nature*, **335**, 249–251.

LEVESQUE, P.C., CLARK, C.D., ZAKAROV, S.I., ROSENSHTRAUKH, L.V. & HUME, J.R. (1993). Anion and cation modulation of the guinea-pig action potential during β-adrenoreceptor stimulation. *Pflügers Arch.*, **424**, 54–62.

LIU, Q-L., KARPINSKI, E. & PANG, P.K.T. (1993). Comparison of the action of two protein kinase C activators on dihydropyridine-sensitive Ca^{2+} channels in neonatal rat ventricular myocytes. *Biochem. Biophys. Res. Commun.*, **191**, 796–801.

MCALROY, H.L., WINPENNY, J.P., GRAY, M.A. & ARGENT, B.E. (1994). Modulation of CFTR Cl^- currents in isolated rat pancreatic duct cells by protein kinase C. *J. Physiol.*, **480**, 61*P*.

MCCANN, J.D., LI, M. & WELSH, M.J. (1989). Identification and regulation of whole-cell chloride currents in airway epithelium. *J. Gen. Physiol.*, **94**, 1015–1036.

MCDONALD, T.F., PELZER, S., TRAUTWEIN, W. & PELZER, D.J. (1994). Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. *Physiol. Rev.*, **74**, 365–507.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature*, **308**, 693–698.

NISHIZUKA, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature*, **334**, 661–665.

OVERHOLT, J.L., HOBERT, M.E. & HARVEY, R.D. (1993). On the mechanism of the rectification of the isoproterenol-activated chloride current in guinea-pig ventricular myocytes. *J. Gen. Physiol.*, **102**, 871–895.

RIORDAN, J.R. (1993). The cystic fibrosis transmembrane conductance regulator. *Annu. Rev. Physiol.*, **55**, 609–630.

RYVES, W.J., EVANS, A.T., OLIVIER, A.R., PARKER, P.J. & EVANS, F.J. (1991). Activation of the PKC-isotypes α, β_1, γ, δ and ε by phorbol esters of different biological activities. *FEBS Lett.*, **288**, 5–9.

SHUBA, L.M., OGURA, T. & MCDONALD, T.F. (1996). Kinetic evidence distinguishing volume-sensitive chloride current from other types in guinea pig ventricular myocytes. *J. Physiol.*, (in press).

SOROTA, S. (1992). Swelling-induced chloride-sensitive current in canine atrial cells revealed by whole-cell patch-clamp method. *Circ. Res.*, **70**, 679–687.

TABCHARANI, J.A., CHANG, X-B., RIORDAN, J.R. & HANRAHAN, J.W. (1991). Phosphorylation-regulated Cl channel in CHO cells stably expressing the cystic fibrosis gene. *Nature*, **352**, 628–631.

TOHSE, N., KAMEYAMA, M. & IRISAWA, H. (1987). Intracellular Ca^{2+} and protein kinase C modulate K^+ current in guinea pig heart cells. *Am. J. Physiol.*, **253**, H1321–H1324.

TOHSE, N., KAMEYAMA, M., SEKIGUCHI, K., SHEARMAN, M.S. & KANNO, M. (1990). Protein kinase C activation enhances the delayed rectifier potassium current in guinea-pig heart cells. *J. Mol. Cell. Cardiol.*, **22**, 725–734.

TSENG, G-N. (1992). Cell swelling increases membrane conductance of canine cardiac cells: evidence for a volume-sensitive Cl channel. *Am. J. Physiol.*, **262**, C1056–C1068.

TSENG, G-N. & BOYDEN, P.A. (1991). Different effects of intracellular Ca and protein kinase C on cardiac T and L Ca currents. *Am. J. Physiol.*, **261**, H364–H379.

TSIEN, R.Y. & RINK, T.J. (1980). Neutral carrier ion-selective microelectrodes for measurement of intracellular free calcium. *Biochem. Biophys. Acta*, **599**, 623–638.

VANDENBERG, J.I., YOSHIDA, A., KIRK, K. & POWELL, T. (1994). Swelling-activated and isoprenaline-activated chloride currents in guinea pig cardiac myocytes have distinct electrophysiology and pharmacology. *J. Gen. Physiol.*, **104**, 997–1017.

WALSH, K.B. (1991). Activation of a heart chloride current during stimulation of protein kinase C. *Mol. Pharmacol.*, **40**, 342–346.

WALSH, K.B. & KASS, R.S. (1988). Regulation of a heart potassium channel by protein kinase A and C. *Science*, **242**, 67–69.

WALSH, K.B. & KASS, R.S. (1991). Distinct voltage-dependent regulation of a heart-delayed I_K by protein kinases A and C. *Am. J. Physiol.*, **261**, C1081–C1090.

WALSH, K.B. & LONG, K.J. (1994). Properties of a protein kinase C-activated chloride current in guinea pig ventricular myocytes. *Circ. Res.*, **74**, 121–129.

WELSH, M.J., ANDERSON, M.P., RICH, D.P., BERGER, H.A., DENNING, G.M., OSTEDGAARD, L.S., SHEPPARD, D.N., CHENG, S.H., GREGORY, R.J. & SMITH, A.E. (1992). Cystic fibrosis transmembrane conductance regulator: a chloride channel with novel regulation. *Neuron*, **8**, 821–829.

YAMANISHI, J., TAKAI, Y., KAIBUCHI, K., SANO, K., CASTAGNA, M. & NISHIZUKA, Y. (1983). Synergistic function of phorbol ester and calcium in serotonin release from human platelets. *Biochem. Biophys. Res. Commun.*, **112**, 778–786.

YUAN, S., SUNAHARA, F.A. & SEN, A.K. (1987). Tumor-promoting phorbol esters inhibit cardiac function and induce redistribution of protein kinase C in perfused beating rat heart. *Circ. Res.*, **61**, 372–378.

ZAKHAROV, S.I., WAGNER, R.A. & HARVEY, R.D. (1995). Muscarinic regulation of the cardiac CFTR Cl^- current by quaternary ammonium compounds. *J. Pharmacol. Exp. Ther.*, **273**, 470–481.

ZHANG, J., RASMUSSON, R.L., HALL, S.K. & LIEBERMAN, M. (1993). A chloride current associated with swelling of cultured chick heart cells. *J. Physiol.*, **472**, 801–820.

ZHANG, K., BARRINGTON, P.L., MARTIN, R.L. & TENEICK, R.E. (1994). Protein kinase-dependent Cl^- currents in feline ventricular myocytes. *Circ. Res.*, **75**, 133–143.

(Received June 29, 1995
Revised October 11, 1995
Accepted December 4, 1995)