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Mechanisms for cardiac depression induced by phorbol myristate acetate in working rat hearts

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1 The effects of the phorbol ester, phorbol myristate acetate (PMA) were examined on function and energy metabolism in the isolated working heart of the rat.

2 At a concentration of 10^{-9} M PMA produced a rapid loss in cardiac function in terms of aortic flow rate (AFR) and coronary flow rates (CFR) whereas a similar concentration of 4α-phorbol 12,13-didecanoate was ineffective. At a concentration of 10^{-10} M, the PMA-induced depression was more gradual but nevertheless very pronounced with an almost total loss in AFR after 30 min perfusion. The reduction in CFR was more moderate than that observed with respect to AFR.

3 The protein kinase C (PKC) inhibitor (±)-1-O-hexadecyl-2-O-acylglycerol significantly attenuated the loss in AFR and CFR following addition of PMA.

4 Two inhibitors of Na^+/H^+ exchange, amiloride and quinacrine, totally prevented the reduction in AFR. Although the PMA-induced depression in CFR was also attenuated by both amiloride and quinacrine, these effects were not significant, probably reflecting the less pronounced effect of PMA on this parameter.

5 Nifedipine, a dihydropyridine calcium channel blocker reduced PMA toxicity to a similar degree as Na^+/H^+ exchange inhibition whereas the calcium channel agonist Bay K 8644 was without effect.

6 Tissue content of energy metabolites including high energy phosphates, total adenine nucleotides or lactate were not significantly affected by PMA perfusion.

7 We conclude that PKC activation is necessary for phorbol ester-induced cardiac dysfunction. The consequence of PKC stimulation includes (1) Na^+/H^+ exchange activation and a subsequent elevation in intracellular calcium $[Ca^{2+}]_i$ via Na^+/Ca^{2+} exchange and (2) PKC-dependent phosphorylation of the calcium channel, both of which would produce toxicity by elevation of $[Ca^{2+}]_i$. Pharmacological manipulation of any of these steps prevents PMA toxicity by virtue of a reduction in the accumulation of $[Ca^{2+}]_i$. PMA effects or their prevention are unrelated to any changes in energy metabolism.

Introduction

Tumour promoting phorbol esters are potent activators of protein kinase C (PKC) (Nishizuka, 1984; Bazzi & Nelsestuen, 1989). In isolated hearts as well as cultured myocytes, phorbol esters have been shown to exert a potent negative inotropic effect (Leatherman *et al.*, 1987; Yuan *et al.*, 1987; Dosemeci *et al.*, 1988). The mechanism(s) which accounts for phorbol ester-induced cardiac depression is not known although a number of cellular events occurring following phorbol-ester treatment have been suggested as possible mechanisms. For instance, treatment of membranes with phorbol esters reduces the affinity of β-adrenoceptors to agonists and decreases their content (Limas & Limas, 1985). In cultured chick heart cells the phorbol ester phorbol 12-myristate 13-acetate (PMA) has been shown to depress transsarcolemmal calcium influx, a phenomenon associated with reduced contractility of the cells (Leatherman *et al.*, 1987). Similarly, this phorbol ester depressed contractility in neonatal rat heart myocytes although this effect was associated with increased calcium entry (Dosemeci *et al.*, 1988). Irrespective of the precise mechanism it is most likely that initial activation of PKC produces intracellular changes resulting in a negative inotropic effect. Indeed, phorbol esters which do not activate PKC fail to depress contractility and, moreover, concentrations of phorbol esters which are effective in depressing contractility are also effective in activating PKC in membrane fractions (Yuan *et al.*, 1987). Activation of PKC can produce numerous intracellular changes such as activation of the Na^+/H^+ exchanger (Besterman *et al.*, 1985) or the phosphorylation and activation of calcium channels both of which can profoundly affect cardiac function (Hosey *et al.*, 1989). In the present study we used specific pharmacological approaches to modulate PKC-dependent mechanisms in rat isolated working hearts exposed to the potent phorbol ester PMA. This preparation is particularly advantageous as it responds to very low PMA concentrations. Since the heart relies on a continuous availability of high energy compounds for the maintenance of contractility, we also examined whether contractile changes observed in the present study could be related to alterations in energy metabolism.

Methods

Experiments were carried out on isolated working rat hearts as described by Neely & Rovetto (1975) with some modification (Karmazyn & Neely, 1989). Male Sprague-Dawley rats (Charles-River Canada Ltd., 275–300 g) were killed by decapitation and the heart was immediately excised and placed in a small crucible containing ice-cold Krebs-Henseleit buffer (composition noted below) to produce an immediate cessation of contraction. The heart was squeezed a few times to dislodge any clotted blood and immediately mounted by the aorta and arranged for retrograde perfusion through the coronary arteries with a hydrostatic pressure of 60 mmHg. The left atrial appendage was cannulated via the pulmonary vein and after 10 min retrograde perfusion the Langendorff column was clamped and buffer was introduced from an oxygenated bubble trap positioned 12.5 cm above the atrium. The hearts were perfused in a non-circulating fashion and the perfusate was pumped against an afterload of 60 mmHg hydrostatic pressure. A side arm of the aortic outflow tract was connected to a Spectromed P23XL pressure transducer to obtain the aortic pressure which was used for the determination of heart rate; however, there were no effects of any treatment on heart

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rate. The perfusion medium was Krebs-Henseleit buffer containing (in mM): NaCl 120, KCl 4.63, KH_2PO_4 1.17, $CaCl_2$ 1.25, MgCl 120, $NaHCO_3$ 20, glucose 11 and pyruvic acid 5. The buffer was continuously gassed with a 95% O_2/5% CO_2 mixture both in the main perfusion reservoir as well as in the left atrial oxygenating chamber. The pH of the buffer was 7.4 and the entire system was regulated at 37°C by a water jacket system. Left ventricular performance was assessed by timed determination of both aortic and coronary flow rates.

The following drugs were employed; PMA (10^{-9} and 10^{-10} M), 4α-phorbol 12,13-didecanoate (PDD, 10^{-9} M), amiloride (174 μM), quinacrine (10 μM) (all from Sigma Chemical Co., St. Louis, MO, U.S.A.), nifedipine (10 nM, gift of Miles Laboratories, Rexdale, Ontario, Canada), 5-(N,N-hexamethylene)-amiloride (200 nM, Research Biochemical Inc., Natick, MA, U.S.A.), (±)-1-O-hexadecyl-2-O-acetylglycerol (HAG, 40 μM, Biomol Research Laboratories, Plymouth Meeting, PA, U.S.A.) and Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, 10 μM, Calbiochem, San Diego, CA, U.S.A.). Pretreatments were administered 15 min before the addition of PMA and were allowed to remain for the duration of the perfusion sequence. All drugs and their vehicles were tested for their direct effects over a 45 min perfusion in the absence of PMA. None significantly affected cardiac function when perfused alone.

At the end of 30 min perfusion the hearts were rapidly clamped between tongs pre-cooled in liquid nitrogen while on the perfusion cannula. Tissue metabolites were measured in 6% perchloric acid extracts as described previously (Bergmeyer, 1963).

Data were analysed either by analysis of variance followed by Student-Newman-Keuls test or Student's t test for unpaired data. A value of $P < 0.05$ was considered to represent a significant difference between treatment groups.

Results

The addition of PMA 10^{-9} M produced a rapid decrease in cardiac function in terms of both AFR and CFR as characterized by a 50% reduction in AFR after only 5 min treatment whereas a total absence of AFR was evident after 20 min perfusion (Figure 1). In contrast, PDD, a phorbol ester devoid of PKC activating ability produced no effect on cardiac function at this concentration (Figure 1). To obtain a less severe and abrupt cardiac depression, we tested the effects of 10^{-10} M PMA and, as shown in Figure 2, this concentration produced a markedly more progressive reduction in function in terms of AFR and CFR. Nevertheless, AFR was almost totally abolished after 25–30 min of PMA perfusion (Figure 2); in contrast CFR was decreased to only 40% of pre-PMA levels. We initially examined the effect of amiloride on PMA-induced depression of cardiac function by addition 15 min prior to PMA. As can be seen from Figure 2 (left column), amiloride almost completely prevented the cardiodepressant effects of PMA. Thus, 30 min following PMA addition AFR was maintained at 85% of pre-PMA levels; a similar protection was seen with respect to CFR. To assess whether the effect of amiloride may have been due to an inhibition of the Na^+/H^+ exchanger we carried out experiments with the structurally dissimilar but markedly more potent Na^+/H^+ exchange inhibitor, quinacrine. These results (Figure 2, right column) show an equal ability of quinacrine to prevent PMA-induced depression. Thus, in the presence of quinacrine there was only a 20% loss in both AFR and CFR after 30 min of PMA treatment. In addition, 5-(N,N,-hexamethylene)-amiloride, a much more potent Na^+/H^+ exchange inhibitor than its parent compound also totally prevented the PMA-induced depression (data not shown).

The controversy regarding the potential role of calcium in PMA-induced cardiac injury was addressed by examining the effects of nifedipine, a dihydropyridine calcium channel

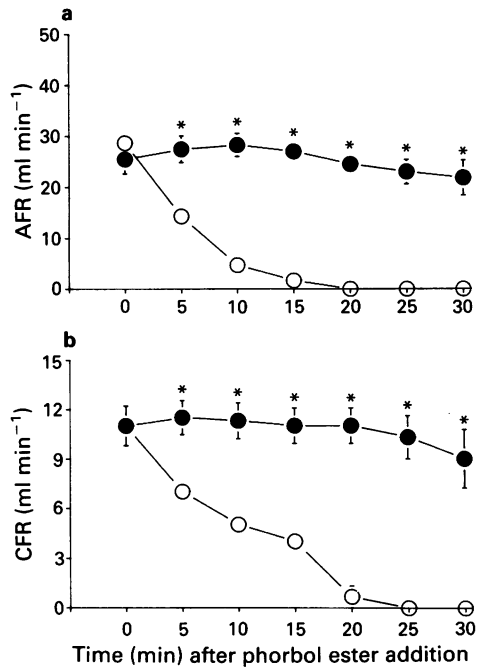

Figure 1 The effect of phorbol myristate acetate (PMA) 10^{-9} M (○) or 4α-phorbol 12,13-didecanoate (PDD) (●) on aortic flow rate (a) and coronary flow rate (b) in isolated working rat hearts. Values indicate mean of $n = 4$; s.e.mean shown by vertical bars. * $P < 0.05$ from PMA values. Where absent s.e.bars were smaller than the size of the symbols.

blocker and Bay K 8644, a dihydropyridine calcium channel agonist which prolongs the opening of the calcium channel. The concentrations of both drugs used in the PMA studies were selected from initial titrations on normal hearts and represent threshold concentrations of nifedipine and Bay K 8644 which produced a decrease or increase, respectively, in AFR. By using minimum effective concentrations of these cardioactive compounds, we hoped to avoid the possibility that basal cardiac function affected the subsequent response to PMA. The influence of nifedipine and Bay K 8644 on effects of PMA is shown in Figure 3. It is evident that in the presence of nifedipine PMA failed to exert any cardiodepressant effects at the end of a 30 min perfusion (approximately 5% change in AFR). Interestingly, in 4 of 5 hearts studied the addition of

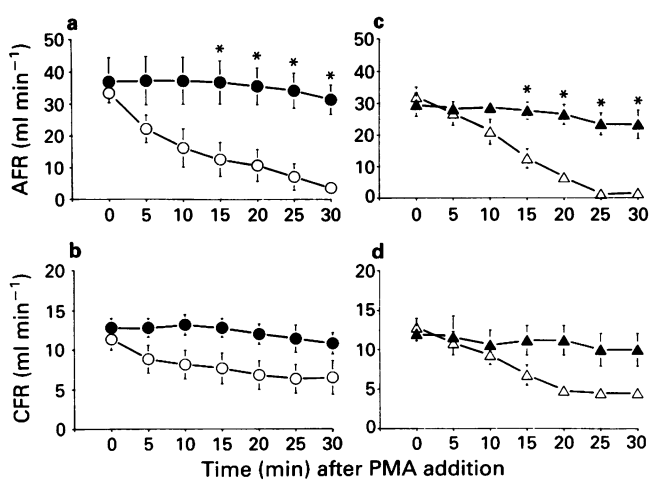

Figure 2 Effect of 174 μM amiloride (●, a,b) or 10 μM quinacrine (▲, c,d) on the phorbol myristate acetate (PMA, 10^{-10} M)-induced effects on aortic flow rate (AFR) and coronary flow rate (CFR) in isolated working hearts. Respective PMA only controls are shown by open circles or triangles. Values indicate mean of $n = 5$; vertical bars show s.e.mean. * $P < 0.05$ from respective PMA control. Where absent s.e. bars were smaller than the size of the symbols.

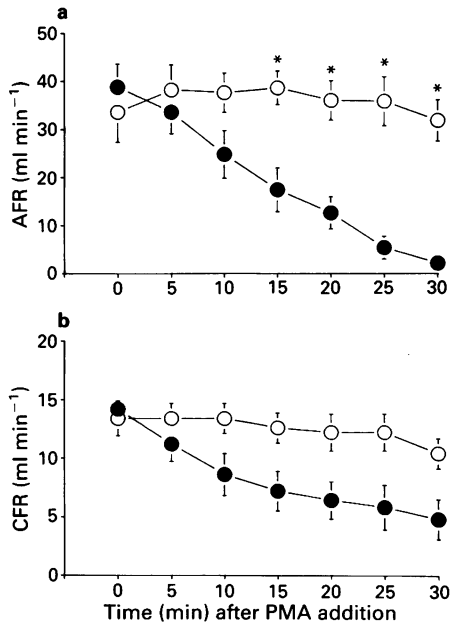

Figure 3 Effect of 10^{-10} M phorbol myristate acetate (PMA) on aortic flow rate (AFR) (a) and coronary flow rate (CFR) (b) in rat isolated working hearts in the presence of either 10 nM of the calcium channel blocker nifedipine (○) or a similar concentration of Bay K 8644 (●). Values indicate mean of $n = 5$; s.e.mean shown by vertical bars. *$P < 0.05$ between treatment groups. Where absent s.e.bars were smaller than the size of the symbols.

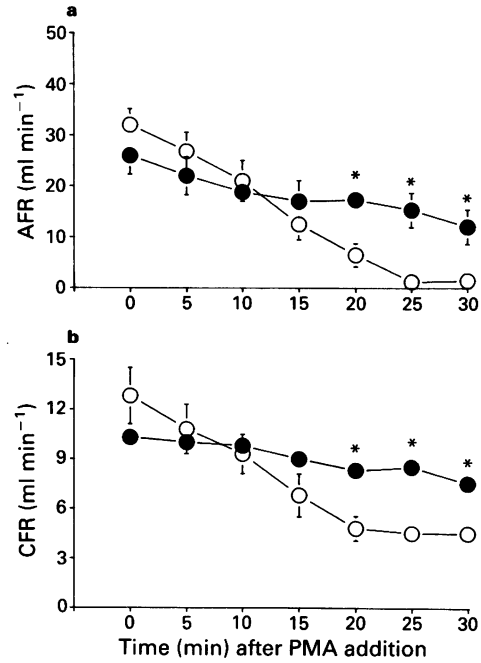

Figure 4 Effect of 10^{-10} M phorbol myristate acetate (PMA) either alone (○) or after pretreatment with 40 μM (±)-1-O-hexadecyl-2-O-acetylglycerol (●) on aortic flow rate (AFR) (a) or coronary flow rate (CFR) (b) in rat isolated working hearts. Values indicate mean of $n = 5$; s.e.mean shown by vertical bars. *$P < 0.05$ from PMA only values. Where absent s.e.bars were smaller than the size of the symbols.

PMA produced a slight increase (13% maximum) in left ventricular function as assessed by AFR which persisted up to 25 min after PMA addition (Figure 3). In contrast to the salutary effect of nifedipine, in the presence of Bay K 8644, PMA produced similar depression in cardiac performance as observed in control PMA-treated hearts (Figure 3).

A series of experiments was also carried out to determine the influence of the PKC inhibitor HAG on PMA toxicity. As shown in Figure 4, HAG provided substantial protection against PMA effects in terms of preventing both the reduction in AFR and CFR after PMA addition. The protective effect of HAG was somewhat less than that observed with either Na^+/H^+ exchange inhibitors or nifedipine since in the presence of this compound, hearts perfused with PMA exhibited a 40% reduction in AFR after 30 min treatment. Nevertheless, these values were significantly higher than the respective control values after 20–30 min perfusion (Figure 4). A similar protective effect of HAG was seen with respect to preservation of coronary flow rate following PMA administration (Figure 4).

Table 1 summarizes energy metabolite data for hearts subjected to PMA perfusion with various pretreatments. Hearts perfused for 30 min with PMA only or with PMA following Bay K 8644 pretreatment demonstrated somewhat reduced levels of high energy phosphates and an increase in tissue lactate compared to other groups. However, as is evident from Table 1, we found substantial variability in this regard and none of these differences was significant. Thus, there was no overall effect of any treatment on tissue energy metabolites.

Discussion

Biologically active phorbol esters represent widely used tools for the study of protein kinase C (PKC)-dependent reactions by virtue of the fact that they are potent activators of PKC activity and PKC appears to represent the physiological receptor for these compounds (Nishizuka, 1984; Bazzi & Nelsestuen, 1989). Studies with isolated perfused hearts or cultured heart cell preparations have revealed a potent ability of many phorbol esters to depress contractility (Leatherman *et al.*, 1987; Yuan *et al.*, 1987; Dosemeci *et al.*, 1988). The mechanism(s) for the phorbol ester-induced negative inotropic effect is not known, however it is likely that it is mediated by initial PKC activation since those phorbol esters which have been shown not to activate PKC fail to elicit cardiac depression (Yuan *et al.*, 1987). Our results confirm these findings since 10^{-9} M PDD, an inactive phorbol ester, failed to produce a negative inotropic effect at a concentration that resulted in a rapid loss in function following equimolar

Table 1 Myocardial tissue metabolites after 30 min perfusion with various treatments

	ATP	*CrP*	*ADP*	*AMP*	*Lactate*
Control	19.7 ± 2.3	29.9 ± 6.7	6.9 ± 1.4	2.7 ± 0.9	1.2 ± 0.4
PMA only	13.3 ± 6.1	23.4 ± 8.9	7.4 ± 2.6	3.2 ± 1.1	10.3 ± 4.7
PMA + Amiloride	23.7 ± 3.1	37.4 ± 4.3	6.7 ± 0.9	2.5 ± 0.2	0.9 ± 0.3
Quinacrine	22.8 ± 1.6	33.4 ± 4.9	6.3 ± 0.4	2.1 ± 0.4	0.8 ± 0.3
HAG	19.7 ± 3.1	25.4 ± 4.7	7.3 ± 1.2	3.0 ± 0.7	2.4 ± 0.7
Nifedipine	24.6 ± 1.7	33.1 ± 4.6	6.9 ± 2.5	3.3 ± 0.4	3.0 ± 0.8
Bay K 8644	13.7 ± 4.9	21.8 ± 6.7	8.2 ± 3.0	3.7 ± 0.9	22.3 ± 6.8

Values are given in μmol g^{-1} dry weight and indicate mean ± s.e.mean of $n = 5$. ATP = adenosine triphosphate; CrP = creatine phosphate; ADP = adenosine diphosphate; AMP = adenosine monophosphate; PMA = phorbol myristate acetate; HAG = (±)-1-O-hexadecyl-O-acetylglycerol.

administration of PMA. The requirement for PKC activation is further supported by the ability of HAG, a PKC inhibitor, to attenuate the depressant effects of PMA. However, it is noteworthy that a total inhibition of PMA effects was not observed. This may be due to an incomplete ability of HAG to inhibit PKC activity; indeed, in human leukaemic (HL-60) cells, HAG concentrations of up to 100 μM inhibited PKC activity by only 60% (Daniel *et al.*, 1988). Although a variety of other PKC inhibitors, including phloretin and sphingosine, were also initially investigated these produced a direct negative inotropic and chronotropic influence such that a meaningful evaluation of their ability to modify PMA-dependent effects was not possible.

Our results support the findings of other laboratories that phorbol esters are potent cardiodepressants. However, the isolated working heart of the rat, which differs from Langendorff-perfused hearts by performing left ventricular filling and ejection demonstrates a marked sensitivity to PMA in that 10^{-10} M of this phorbol ester produced a marked time-dependent loss of left ventricular function. The addition of a similar concentration of PMA to non-working Langendorff heart preparations failed to produce any depression in contractility (Watson & Karmazyn, 1989). The reasons for the substantially greater sensitivity in working hearts are uncertain although they may be related to well-known differences in energy substrate utilization and oxygen demand between the two preparations (see Williamson & Kobayashi, 1984 for review). However, it is noteworthy that the PMA-induced depression in function was seemingly unrelated to defective energy production unless coronary flow was severely compromised following PMA addition such that ischaemic conditions were produced. Nevertheless, our results suggest that the working heart represents an excellent model for the study of cardiac response to very low concentrations of phorbol esters.

PKC represents an important intracellular regulatory enzyme and numerous PKC-dependent intracellular reactions have been identified, one of which is the phosphorylation and activation of the Na^+/H^+ exchanger, an electroneutral cotransport mechanism which extrudes protons against an inward-directed Na^+ gradient (Besterman *et al.*, 1985). This system, which has been identified in the cardiac cell, is likely to be of major importance in the regulation of intracellular pH as it represents the only route of proton extrusion following their generation during normal metabolic processes or after an acid load such as may occur during ischaemia (Lazdunski *et al.*, 1985; Wallert & Frohlich, 1989). In addition, the Na^+/H^+ exchanger represents a major mechanism for Na^+ influx into the cardiac cell (Lazdunski *et al.*, 1985; Wallert & Frohlich, 1989). Inhibitors of Na^+/H^+ exchange such as amiloride have been shown to exert a protective effect against digitalis-induced cardiac arrhythmias probably as a result of the inhibition of Na^+ influx and thus a prevention of the elevation in intracellular Ca^{2+} through Na^+/Ca^{2+} exchange (Kim & Smith, 1986). Amiloride has also been shown to reduce reperfusion-induced myocardial injury, possibly also through a reduction in intracellular Ca^{2+} accumulation following reflow (Karmazyn, 1988). The ability of amiloride as well as the structurally dissimilar but markedly more potent Na^+/H^+ exchange inhibitor quinacrine effectively to prevent the toxic effect of PMA suggests the involvement of the transporter in PMA-induced toxicity. It should be noted that both amiloride and quinacrine possess pharmacological properties unrelated to Na^+/H^+ exchange inhibition. For instance amiloride inhibits numerous membrane bound enzymes systems such as Na^+/K^+ ATPase and also inhibits Na^+/Ca^{2+} exchange; however these effects occur at substantially higher concentrations than those used in the present study (Floreani *et al.*, 1987). Quinacrine, on the other hand, has been shown to inhibit phospholipase A_2 (Otani *et al.*, 1986); however, we have been unable to demonstrate any ability of similar concentrations of quinacrine as used in the present study to modulate the stimulated prostaglandin production in isolated hearts subjected to various conditions of phospholipase A_2 activation including ischaemia and reperfusion or induction of severe Ca^{2+} overload with either the 'Ca^{2+} paradox' or by addition of the Ca^{2+} ionophore A23187 (unpublished observations). Indeed, in canine cardiac sarcolemmal vesicles, quinacrine was found to be the most potent inhibitor of Na^+/H^+ exchange compared to amiloride and various of its analogues (Seiler *et al.*, 1985). To implicate Na^+/H^+ exchange further in PMA-mediated cardiotoxic effects we have recently observed that 0.2 μM of 5-(N,N-hexamethylene)-amiloride, which has been shown to be about 500 fold more potent in inhibiting Na^+/H^+ exchange in neutrophils than its parent compound (Simchowitz & Cragoe, 1986), also totally prevented PMA-induced depression in function (data not shown).

It has been suggested that inhibition of the Ca^{2+} current (I_{si}) may represent the mechanism for phorbol ester-induced cardiac depression (Leatherman *et al.*, 1987), based on the observation that 1 μM PMA inhibited transarcolemmal Ca^{2+} influx and depressed contractility in chick cultured heart cells. Our results do not support this hypothesis but instead suggest that an increase in Ca^{2+} influx is required for the toxicity in view of the fact that very low concentrations of nifedipine, a dihydropyridine Ca^{2+} antagonist were extremely effective in preventing PMA-induced depression. The reason for this difference is unknown although it is possible that chick heart cells respond differently to phorbol ester treatment in terms of Ca^{2+} homeostasis from cardiac cells of other species. Since the protective effect of nifedipine occurred under conditions of activated PKC and Na^+/H^+ exchange following PMA administration, it is likely that inhibiting the Ca^{2+} channel served to prevent $[Ca^{2+}]_i$ from reaching a critically elevated level which would have produced a toxic effect. Therefore, taken together, our results suggest that PMA toxicity can be prevented through specific approaches such as inhibition of Na^+/H^+ exchange or blocking of the Ca^{2+} channel, each of which would attenuate an elevation of $[Ca^{2+}]_i$. In support of our contention that PMA-induced cardiodepression is not mediated by a decrease in Ca^{2+} entry, the dihydropyridine Ca^{2+} channel agonist Bay K 8644 did not antagonize the cardiac response to PMA as would have been expected if the depression in contractility was due to a decrease in Ca^{2+} entry.

Based on our observations, we propose that the cardiodepressant effects of PMA, at least at low concentrations of the compound, are unlikely to be mediated by one mecha-

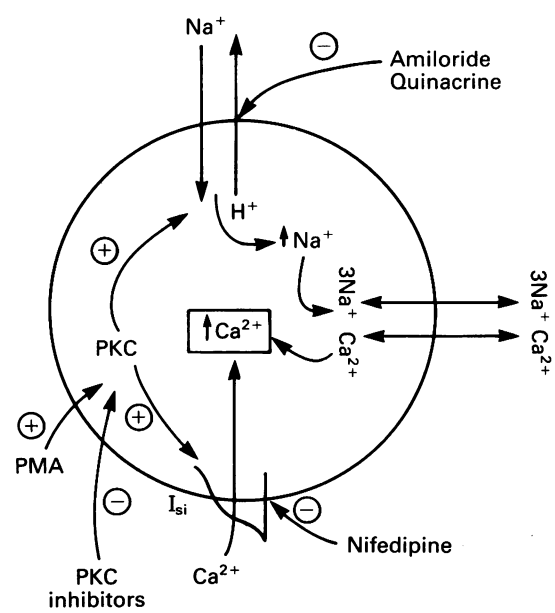

Figure 5 Hypothesis for phorbol myristate acetate (PMA)-induced depression in cardiac function. See Discussion for details.

nism, but are more likely to involve a number of intracellular events acting in concert and linked to initial PKC activation. This is based on the fact that PMA toxicity can be dramatically reduced by drugs acting at different cellular sites. A summary of our hypothesis including the site of action of pharmacological agents used in our study is presented in Figure 5. PMA activates PKC which can result in a number of cellular events. PKC is known to increase the activity of the Na^+/H^+ exchanger which would result in an increase in $[Na^+]_i$. This in turn would increase $[Ca^{2+}]_i$ *via* the Na^+/Ca^{2+} exchange mechanism (represented by its stoichiometric relationship in Figure 5). Similarly, PKC has been shown to phosphorylate and activate the L-type Ca^{2+} channel which is the major component of I_{si}, the predominant route of Ca^{2+} entry in ventricular myocytes (Hosey *et al.*, 1989). Thus it is likely that PMA treatment results in a PKC-dependent phosphorylation and activation of the Ca^{2+} channel. Although extrapolation from other studies (using different preparations) to our results should be done cautiously, it is interesting to point out that phorbol esters have recently been shown to cause a small increase in I_{si} in rabbit sino-atrial node cells (Satoh & Hashimoto, 1988). Moreover, it has recently been shown that PMA can increase $[Ca^{2+}]_i$ in non-cardiac (rat anterior pituitary) cells through a mechanism involving Ca^{2+} channel activation, a phenomenon prevented by nifedipine (Schöfl *et al.*, 1989).

Taken together, our results suggest that PKC activation results in an amplification of Ca^{2+} influx such that toxicity would occur. The ability of individual manipulations targeted against a specific route of Ca^{2+} entry to reduce effectively PMA toxicity is likely to reflect an ability of such treatment to maintain $[Ca^{2+}]_i$ at subtoxic levels. Confirmation of this hypothesis will require the determination of $[Ca^{2+}]_i$ following various manipulations, ideally in myocytes where intracellular events can be monitored directly.

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