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EFFECTS OF PHORBOL ESTER ON CONTRACTION, INTRACELLULAR pH AND INTRACELLULAR Ca^{2+} IN ISOLATED MAMMALIAN VENTRICULAR MYOCYTES

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SUMMARY

1. We have investigated the actions of certain phorbol esters on the intracellular pH, intracellular Ca^{2+} and contractility of isolated rat and guinea-pig cardiac myocytes. Intracellular pH was measured using 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and intracellular Ca^{2+} was measured using Fura-2.

2. Application of the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (also called phorbol 12-myristate 13-acetate) (TPA) (which activates protein kinase C) to rat cardiac myocytes significantly increased cell shortening by $116 \pm 34\%$ ($n = 8$) ($p < 0{\cdot}02$). The rate of change of cell length during contraction (i.e. $+dL/dt$) increased from $67{\cdot}2 \pm 8{\cdot}7$ μm/s to $127{\cdot}7 \pm 14{\cdot}1$ μm/s ($n = 7$). The rate of change of cell length during relaxation ($-dL/dt$) increased from $55{\cdot}8 \pm 7{\cdot}4$ μm/s to $118{\cdot}9 \pm 12{\cdot}1$ μm/s ($n = 7$). Time to peak shortening was unchanged.

3. Application of 4α-phorbol 12,13-didecanoate, which does not activate protein kinase C, did not affect rat myocyte contractility. An insignificant decrease in contractility (by $7{\cdot}5 \pm 7{\cdot}5\%$) was observed ($n = 5$). The positive inotropic effect of TPA may therefore be evoked through an activation of protein kinase C.

4. In rat myocytes we have measured the changes of pH_i and contractility (cell shortening) during an alkalosis and acidosis induced by exposure to and subsequent removal of NH_4Cl both in the presence and absence of TPA. Recovery times from an acid load were significantly ($p < 0{\cdot}05$) enhanced by $15{\cdot}1 \pm 6{\cdot}9\%$ ($n = 13$) in the presence of TPA. Recovery times of cell shortening were also more rapid ($p < 0{\cdot}05$) by an average of $59{\cdot}1 \pm 10{\cdot}6\%$ ($n = 5$) in the presence of TPA. Recovery times were unchanged in the presence of 4-phorbol 12,13-didecanoate (which does not activate protein kinase C).

5. Since pH_i recovery of an isolated myocyte from an acid load is partially inhibited by the presence of 1 mM-amiloride and inhibited by removing extracellular Na^+ then it is suggested that, like pH_i regulation in sheep heart Purkinje fibres, pH_i recovery in rat cardiac ventricular myocytes is mainly through sarcolemmal Na^+–H^+ exchange. We suggest that in the presence of TPA the Na^+–H^+ exchange is stimulated.

6. The relationship between pH_i and cell shortening is non-linear as has been

observed by others in whole tissue preparations. The presence of TPA shifts the relationship upwards such that at any one pH_i, cell shortening is greater.

7. Addition of the phorbol ester did not change steady-state pH_i so the positive inotropic effect cannot simply be due to pH_i becoming more alkaline.

8. In rat and guinea-pig myocytes TPA increased peak systolic $[Ca^{2+}]$ but did not alter resting (diastolic) $[Ca^{2+}]$. Fura-2 ratios increased by $21 \pm 4\%$ ($n = 5$). We conclude that some of the positive inotropic effect produced by phorbol esters which activate protein kinase C is caused by an increase in systolic Ca^{2+}.

INTRODUCTION

Protein kinase C may have an important role in signal transduction for a variety of biological substances which activate cellular functions. When cells are stimulated by certain agonists binding to receptors there is a transient activation of protein kinase C by diacylglycerol which is produced in the membrane as a result of receptor-mediated inositol phospholipid breakdown. Many substances appear to activate protein kinase C, notably growth factors and some tumour-promoting phorbol esters. In recent years convincing evidence has accumulated for the action of extracellular growth stimuli being mediated through changes in intracellular pH (pH_i). The first direct evidence for this possibility came from flux studies on serum-stimulated mouse neuroblastoma cells (Moolenaar, Boonstra, van der Saag & de Laat, 1981) and later from a fluorescent study on human fibroblasts (Moolenaar, Tsien, van der Saag & de Laat, 1983). In the latter work, activation by epidermal growth factor or fetal calf serum resulted in a rapid and persistant increase in pH_i which could be inhibited by amiloride and could be reversed in Na^+-free medium (i.e. when the driving force for H^+ expulsion is reversed) suggesting that growth factors somehow activated Na^+–H^+ exchange.

In many tissues evidence is accumulating for there being a synergistic role of protein kinase C with Ca^{2+}. Stimulation of the receptors simultaneously mobilises Ca^{2+} *and* activates protein kinase C leading to full physiological responses which are not observed when either pathway is activated alone (see Nishizuka, 1984 for review). It has been suggested that one important consequence of having a parallel signal pathway is that it provides scope for subtle variations in control mechanisms (Berridge, 1984). This subtle control may involve interaction between H^+ and Ca^{2+}. In heart, the control of pH_i and intracellular Ca^{2+} concentration ($[\text{free } Ca^{2+}]_i$) are closely interlinked (Bers & Ellis, 1982; Vaughan-Jones, Lederer & Eisner, 1983) and both ions exert powerful influences on the contraction process. The action of protein kinase C in cardiac muscle remains unclear but if protein kinase C activation alters either pH_i or $[\text{free } Ca^{2+}]_i$ then this could be another mechanism whereby agonists and antagonists could alter the contractility of heart cells.

The physiological catecholamines, adrenaline and noradrenaline, increase the force of the heart beat through α- and β-adrenoreceptors. The maximum effect of an α-agonist is usually small when compared with that of a full β-agonist (Jakob, Nawrath & Rupp, 1988), but it is possible that the contribution may become significant under certain circumstances. In the failing heart for example, the effectiveness of β-adrenoceptor stimulation is reduced (Bristow, Ginsburg, Minobe, Cubicciotti, Sageman, Lurie, Billingham, Harrison & Stinson, 1982), which may lead to a greater

dependence on α-adrenoreceptors (Bohm, Diet, Feiler, Kemkes & Erdmann, 1988). It is known that α-adrenoceptor stimulation results in inositol phospholipid breakdown in mammalian heart (Woodcock, McLeod, Smith & Clark, 1987). Diacylglycerol production is also enhanced (Okamura, Kawai, Hashimoto, Ito, Ogawa & Satake, 1988), which will result in activation of protein kinase C. If protein kinase C stimulates Na^+–H^+ exchange, as suggested above, α-adrenoceptor activation may result in an intracellular alkalinisation which could underlie the increase in force.

The present work describes the effects of two phorbol esters upon pH_i and [free $Ca^{2+}]_i$ and how they change myocyte contractility. One phorbol ester activates protein kinase C, the other does not. Intracellular pH and [free Ca^{2+}] were measured using the fluorescent indicators 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (Paradiso, Tsien & Machen, 1984) and Fura-2 (Grynkiewicz, Poenie & Tsien, 1985) respectively. Some of this work has already been communicated to the Physiological Society.

METHODS

Cell isolation

Male Sprague–Dawley rats (100–300 g) or guinea-pigs (350–650 g) were heparinized and after being killed by cervical dislocation, the heart was rapidly removed from the animal and placed in Krebs–Henseleit (K–H) solution containing (mM) NaCl, 119; KCl, 4·2; $CaCl_2$, 1·0; $MgSO_4$, 0·94; KH_2PO_4, 1·2; $NaHCO_3$, 25; glucose, 11·5 and equilibrated with 95% O_2/5% CO_2 at 35 °C giving a pH of 7·4. The aorta was cannulated and the heart retrogradely perfused on a Langendorff apparatus with fresh K–H solution for 5 min. Perfusion was then changed to a modified, low-Ca^{2+} (L–C) Tyrode solution containing (mM) NaCl, 120; KCl, 5·4; $MgSO_4$, 5·0; pyruvate, 5; glucose, 20; taurine, 20; HEPES, 10; nitrolotriacetic acid (NTA), 5; pH 7·0 at 35 °C. $CaCl_2$ was added to this solution so that the [free Ca^{2+}] was ≈ 12 μM. Approximately 5 min later a mixture of 1·0 mg/ml collagenase (Worthington) and 1·0 mg/ml hyaluronidase (Sigma) was added to a L–C solution (this time lacking NTA but to which Ca^{2+} had been added to bring the [free Ca^{2+}] to 50 μM) and perfused through the heart for 2 min. For guinea-pig hearts this solution also contained 4 units/ml protease–type XXIV (Sigma). Thereafter, the heart was cut down, the ventricles chopped and incubated for two periods of 20 min in fresh L–C solution containing collagenase and hyaluronidase but again lacking NTA. The medium was shaken gently at 35 °C throughout the incubation and kept under an atmosphere of 100% O_2. The dispersed cells were then strained through a 300 μm gauze and centrifuged at 400 rev/min for 30 s–1 min. The pellet was resuspended and stored in K–H solution at room temperature.

Cell loading

Cells were loaded with fluorescent indicator using their acetoxymethyl ester forms (Molecular Probes, Eugene, Oregon, USA). BCECF or Fura-2 was added to a suspension of cells to give a final concentration of 5 μM and incubated for 20–25 min at room temperature. The cells were then pelleted, washed and re-pelleted with fresh K–H solution and transferred to the superfusion chamber. The experiments were started approximately 1–2 h after loading with indicator.

Apparatus

Cells were placed in a Perspex superfusion chamber (volume ≈ 60 μl), mounted on the stage of an epifluorescence microscope (Nikon, Diaphot). The base of the chamber was a glass cover-slip. Unless otherwise specified the cells were field stimulated at 0·5 Hz using two platinum wires lying along the edges of the chamber. Solutions were carried to the chamber via gas-impermeable tubing. Light from a 75 W xenon lamp passed through two narrow bandpass (10 nm) filters at 440 and 490 nm for BCECF and 340 and 380 for Fura-2. The filters were mounted in a spinning wheel (Cairn Research, Sittingbourne, Kent, UK) which was rotated at up to 120 Hz. Excitation light from the filter wheel was directed to the cells via a dichroic mirror block (with half-pass wavelengths of

510 nm for BCECF and 430 nm for Fura-2) and objective lens (Nikon ×40 CF Fluor DL) of numerical aperture 0·85. Red light (> 630 nm) illuminated the cells in a conventional brightfield manner and was used to carry an image of the cells to a video camera or photodiode array for measurement of cell length. Light emitted by the cells containing the appropriate fluorescent

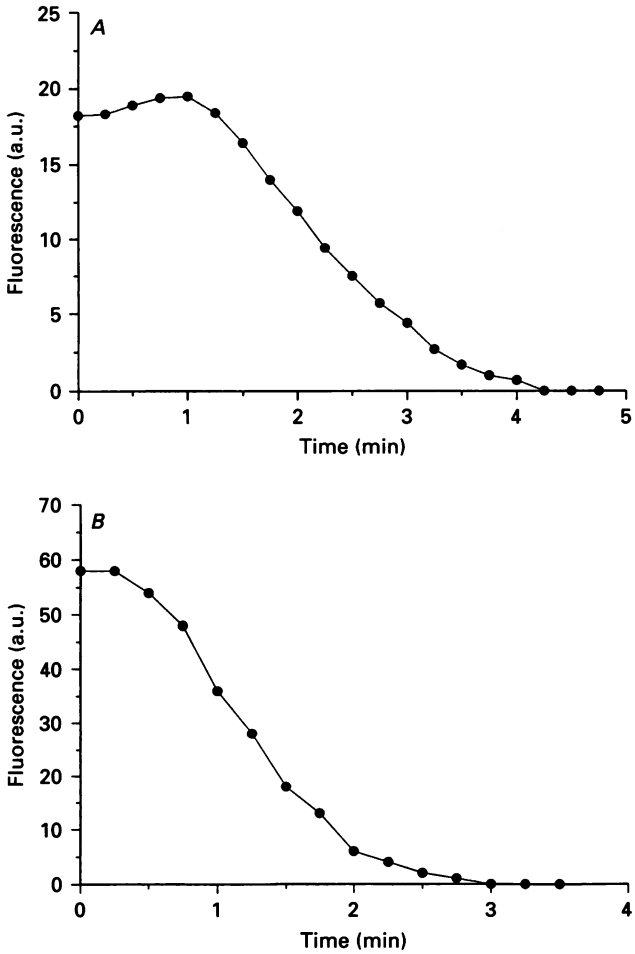

Fig. 1. Release of fluorescence (arbitary units, a.u.) from cells loaded with (*A*) Fura-2-acetoxymethyl ester and (*B*) BCECF-acetoxymethyl ester when exposed to the skinning agent β-escin.

indicator, together with the transmitted (red) light, passed through a variable diaphragm which was adjusted to allow light to pass only from the region occupied by the cell under investigation. This light was then split using a dichroic mirror block centred at 580 nm so that shorter wavelengths of light (emitted from the fluorescent indicators) passed to a photomultiplier tube and longer wavelengths passed to the cell length detector. Either a bandpass, centred at 515 nm (bandwidth $\pm$10 nm), or a low pass filter (540 nm cut-off) was placed in front of the photomultiplier tube to measure fluorescent light from the cells. Changes in cell length were measured either using a video camera and contrast enhancement edge-detection system (Harding, Vescovo, Kirby, Jones, Gurden & Poole-Wilson, 1988) or a Reticon S-series photodiode array (E. G. & G. Reticon, Sunnyvale, California, USA) (Boyett, Moore, Jewell, Montgomery & Kirby, 1988).

Calibration of fluorescence

The amount of light emitted by the preparation loaded with BCECF when excited by 490 nm light was divided by the amount of light emitted when excited by 440 nm to form a ratio measurement indicative of intracellular pH. Similarly, light emitted from preparations loaded with Fura-2 while excited by 340 nm was divided by that emitted when excited by 380 nm to give a ratio indicative of the changes in intracellular [free Ca^{2+}]. Calibration of Fura-2 fluorescence changes is difficult. Possible reasons for this have been reviewed recently by Roe, Lemasters & Herman (1990) and include (a) incomplete hydrolysis of Fura-2-acetoxymethylester so forming Ca^{2+}-insensitive but fluorescent compounds; (b) sequestration of Fura-2 in non-cytoplasmic compartments; (c) dye loss; (d) photobleaching and photochemical formation of fluorescent Ca^{2+}-insensitive forms and (d) shifts in excitation and emission spectra and dissociation constant (K_d) for Ca^{2+} due to changes in ionic strength and viscosity. For these reasons we have chosen not to quantify the ratios due to Fura-2 fluorescence but to use them as a qualitative indicator of changes in [free $Ca^{2+}]_i$. The sequestration of Fura-2 or BCECF in non-cytoplasmic compartments could present difficulties in interpretation of cytoplasmic Ca^{2+} or pH changes as a portion of the observed signal could come from organelles. In order to obtain a qualitative estimate of the extent of accumulation of indicator in cellular organelles we have chemically skinned the preparations and followed the release of fluorescence from the cells. We used the skinning agent β-escin (Sigma), a saponin ester, which makes the cell membrane permeable to high molecular weight solutes but leaves sarcolemmal receptors and the excitation–contraction coupling system intact (Kobayashi, Kitazawa, Somlyo & Somlyo, 1989). β-Escin (20 μM) was added to a solution containing (in mM): KCl, 160; imidazole, 20; ATP, 5·5; PIPES (piperazine-*N*,*N*′-bis(2-ethanesulphonic acid)), 10; creatine phosphate, 5; EGTA, 10; $CaCl_2$ and $MgCl_2$ added to produce [free Ca^{2+}] of 0·1 μM and [free Mg^{2+}] of 1 mM; pH = 7·2. This produced the effect on Fura-2 fluorescence monitored at 360 nm as shown in Fig. 1*A*. The release of Fura-2 took place within 4·5 min and the fluorescence decreased to average background values within this time suggesting that all Fura-2 could be released and so is not contained within organelles. Figure 1*B* shows the effect of 20 μM-β-escin on the fluorescence of BCECF measured at 440 nm. Again, release of the fluorescent molecule was complete. Release of BCECF was faster than release of Fura-2. In four experiments the average fluorescence remaining after skinning treatment was 4·75 ± 3·5 %. We conclude that at least 95 % of indicator is located in the cytoplasm.

Calibration of BCECF was done using the K^+–H^+ exchanger nigericin to equilibrate pH_i with pH_o. At the end of an experiment the superfusing solution was changed to one similar to that used by Eisner, Nichols, O'Neill, Smith & Valdeolmillos, (1989) containing (mM): KCl, 140; $MgSO_4$, 1·0; KH_2PO_4, 1·2; pH buffer, 10; and 10 μM-nigericin. The pH buffer was 2-(*N*-morpholino)-ethanesulphonic acid (MES) for solutions of pH 5·8 and 6·5, PIPES for solutions of pH 6·8 and 7·0 and HEPES for solutions of pH 7·5 and 8·2. Calibration was performed at the experimental temperature. Some rounding of the cells occurred in solutions of high pH. However, with similar calibration solutions but with 100 μM-EGTA added, fewer contractures occurred. Under these conditions the calibration curve of ratio *vs.* pH corresponded closely to that observed in the solutions without EGTA so we assume that cell rounding did not affect the calibrations.

Solutions

The cells were superfused at a rate of 1·2 ml/min with K–H solution similar to that above but titrated with $NaHCO_3$ to give a pH = 7·4 at 30 °C. All experiments were performed between 30–32 °C with the temperature during any one experiment not varying by more than ±0·5 °C. The exchange of solutions in the superfusion chamber was complete within 20 s. Phorbol esters (Sigma) were dissolved in dimethylsulphoxide to form a 1 mM stock solution which was kept at −20 °C until required. Dimethylsulphoxide alone had no significant effect on cell contractility or cell fluorescence at the dilutions used. Amiloride was a gift from Merck, Sharpe and Dohme and was added as solid to the superfusate just before use. No immediate change in fluorescence was observed when amiloride was present indicating that the compound did not alter the measurement of pH_i *per se*.

Statistics

Where applicable data are presented as the mean ± standard error of the mean (S.E.M.). The Student's *t* test was used to calculate significance between means.

RESULTS

Many substances appear to activate protein kinase C, notably growth factors and some tumour-promoting phorbol esters. One such phorbol ester is 12-*O*-tetradecanoylphorbol 13-acetate (also called phorbol 12-myristate 13-acetate) (TPA) and its effect on rat myocyte contractility is shown in Fig. 2. Addition of 10^{-7} M-TPA increased cell shortening by 3·2 times after 22 min when shortening reached a plateau. This cell showed the greatest effect on cell shortening. Overall rat cardiac myocyte shortening ($n = 8$) was significantly increased from control by $116 \pm 34\%$ ($p < 0{\cdot}02$). The rate of change of cell length during contraction (i.e. $+dL/dt$) increased from $67{\cdot}2 \pm 8{\cdot}7$ μm/s to $127{\cdot}7 \pm 14{\cdot}1$ μm/s ($n = 7$). The rate of change of cell length during relaxation ($-dL/dt$) increased from $55{\cdot}8 \pm 7{\cdot}4$ μm/s to $118{\cdot}9 \pm 12{\cdot}1$ μm/s ($n = 7$). Time to peak shortening was unchanged.

Application of 10^{-7}–10^{-6} M 4α-phorbol 12,13 didecanoate, which does not activate protein kinase C, did not affect rat myocyte contractility as shown in Fig. 3. An insignificant decrease in contractility (by $7{\cdot}5 \pm 7{\cdot}5\%$) was observed in cells from four other different hearts. The positive inotropic effect of TPA would therefore appear to be evoked through an activation of protein kinase C. Two likely candidates for promoting the increase in twitch are (1) an intracellular alkalinisation which sensitizes the myofilaments to Ca^{2+} (Fabiato & Fabiato, 1978) and/or (2) an increase in [free Ca^{2+}]$_i$.

We have measured pH_i using the fluorescent indicator BCECF (Paradiso *et al.* 1984). In isolated rat cardiac myocytes we have been unable to find any consistent change in pH_i in the presence of TPA (see Fig. 6). In thirteen cells from different hearts exposed to TPA pH_i was unchanged in eight, became acid in three and alkaline in two. Thus the positive inotropic effect seen with phorbol esters which activate protein kinase C does not seem to be due to a change in pH_i.

Intracellular Ca^{2+} may be increased when protein kinase C is activated so we have investigated this in experiments of the type shown in Fig. 4. Intracellular Ca^{2+} concentration was measured by Fura-2 (Grynkiewicz *et al.* 1985). Figure 4*A* shows that, in rat, TPA increased peak systolic [Ca^{2+}] and resting (diastolic) [Ca^{2+}]. This would agree with the fact that in this case, though not a consistent finding, resting cell length also decreased in the presence of TPA. Figure 4*B* shows that guinea-pig cardiac myocytes display similar responses to TPA. The time course of the changes in Ca^{2+} are shown in this illustration. Fura-2 ratios increased by $21 \pm 4\%$ ($n = 5$).

Our results so far suggest that activation of protein kinase C produces a positive inotropic effect by increasing systolic [Ca^{2+}] and not by altering pH_i. This may imply that protein kinase C activation does not enhance the activity of Na^+–H^+ exchange. However, an alternative explanation is that at normal pH_i, the exchanger is relatively inactive. Kaila and Vaughan-Jones (1987) have shown that the Na^+–H^+ exchanger becomes increasingly more activated as pH_i decreases from 7·2 to 6·2 and so an effect of protein kinase C on the exchange may only be apparent at more acid intracellular pH. Consequently, we have examined the recovery of pH_i and cell shortening from acid loading. The acid loading was accomplished by exposure of the cells to, then subsequent removal of NH_4Cl at constant pH_o. The transmembrane movements of NH_3 and NH_4^+ responsible for the intracellular acid load have been

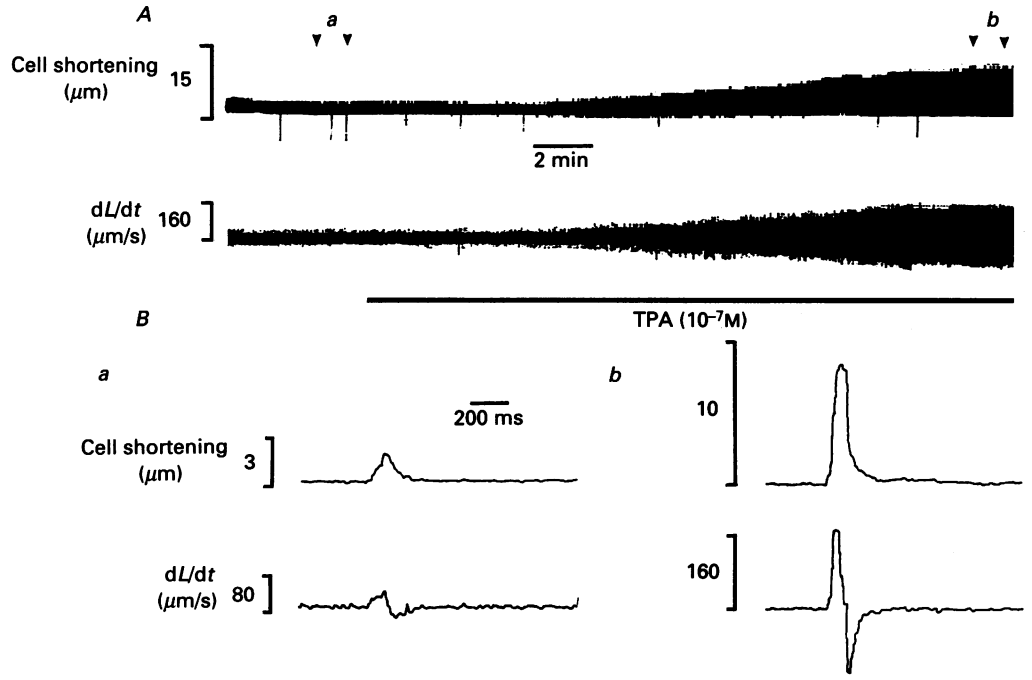

Fig. 2. *A*, continuous measurement of cell shortening (top trace) and the differential of the shortening signal (bottom trace) during application of 10^{-7} M-phorbol ester, TPA. *B* shows averaged sweeps during the periods *a* and *b* shown in the upper panel. The cell shortening was measured by a video camera technique (see Methods) and this gives a slightly stepped appearance to the records. Increased cell shortening produces a larger upward deflection.

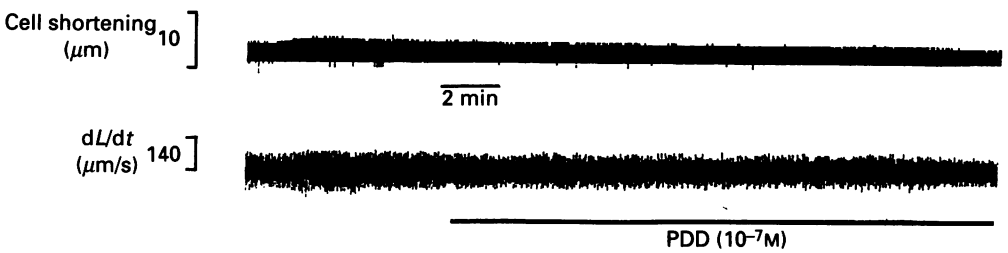

Fig. 3. Continuous measurement of cell shortening (top trace) and the differential of the shortening signal (bottom trace) during application of 10^{-7} M-4α-phorbol 12,13 didecanoate (PDD).

described by Boron and De Weer (1976). Figure 5 shows that the recovery of an isolated rat cardiac myocyte from the acid load is partially inhibited by the presence of 1 mM-amiloride, a Na^+–H^+ exchange inhibitor, (Fig. 5*A*) and inhibited by removing Na_o^+ in the absence of HCO_3^- (Fig. 5*B*). This confirms previous work

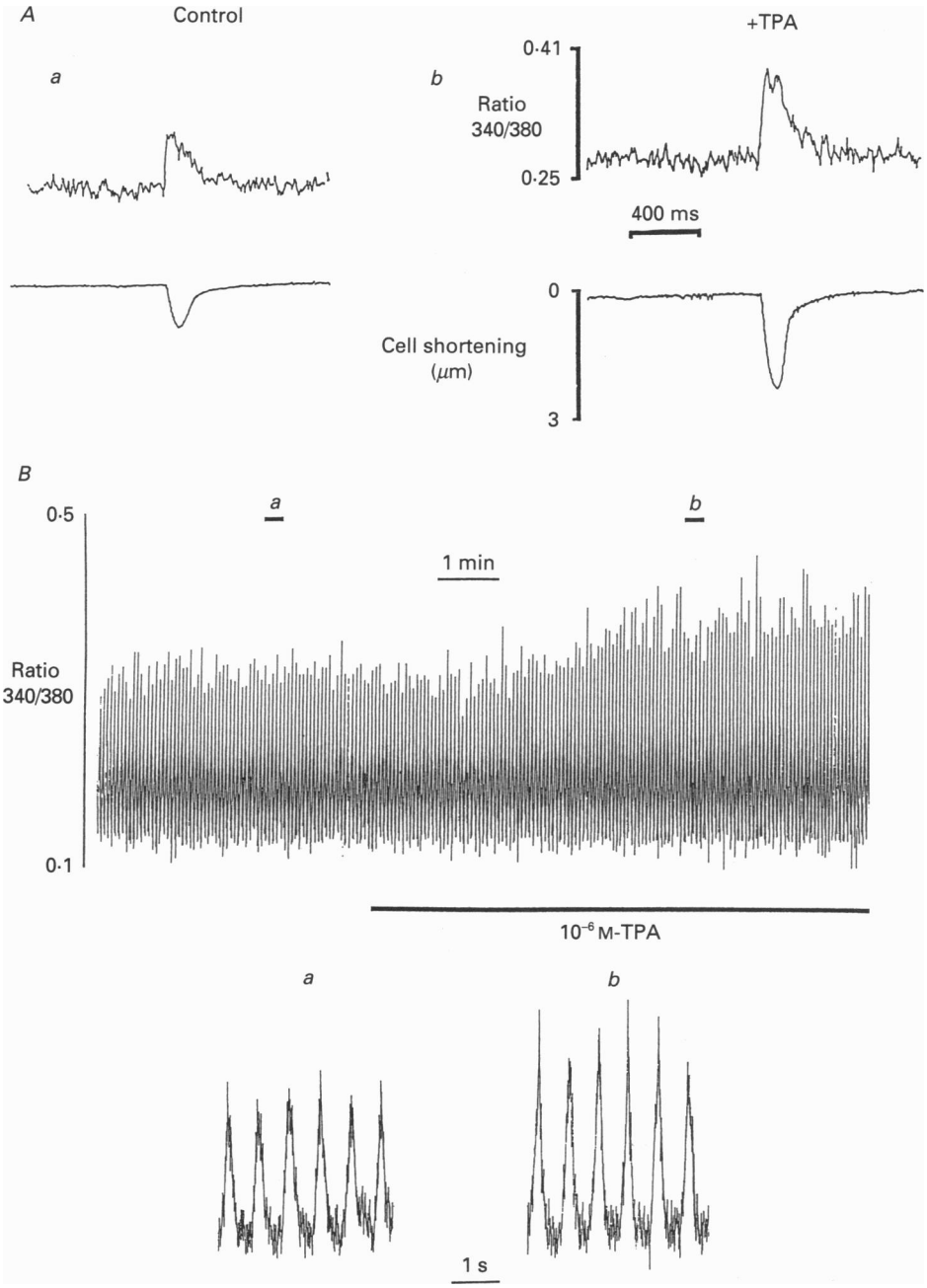

Fig. 4. Recording of intracellular Ca^{2+} changes and cell shortening in rat (*A*) and guinea-pig (*B*) cardiac myocytes. Increased shortening is shown as a greater downward deflection. *A* shows averaged records ($n = 16$) under control conditions (*a*) and in the presence of 10^{-7} M-TPA (*b*). *B* shows a trace recorded at a slow chart speed and below it isolated portions (*a* and *b*) played at a faster time base.

(Wallert & Frohlich, 1989) that, like pH_i regulation in sheep heart Purkinje fibres, pH_i recovery in rat cardiac ventricular myocytes is mainly through sarcolemmal Na^+–H^+ exchange using the entry gradient for Na^+ to expel H^+ from the cell.

The contractility changes which the cells undergo during the acid–base changes are shown in Fig. 6. As pH_i became more alkaline, cell shortening transiently increased

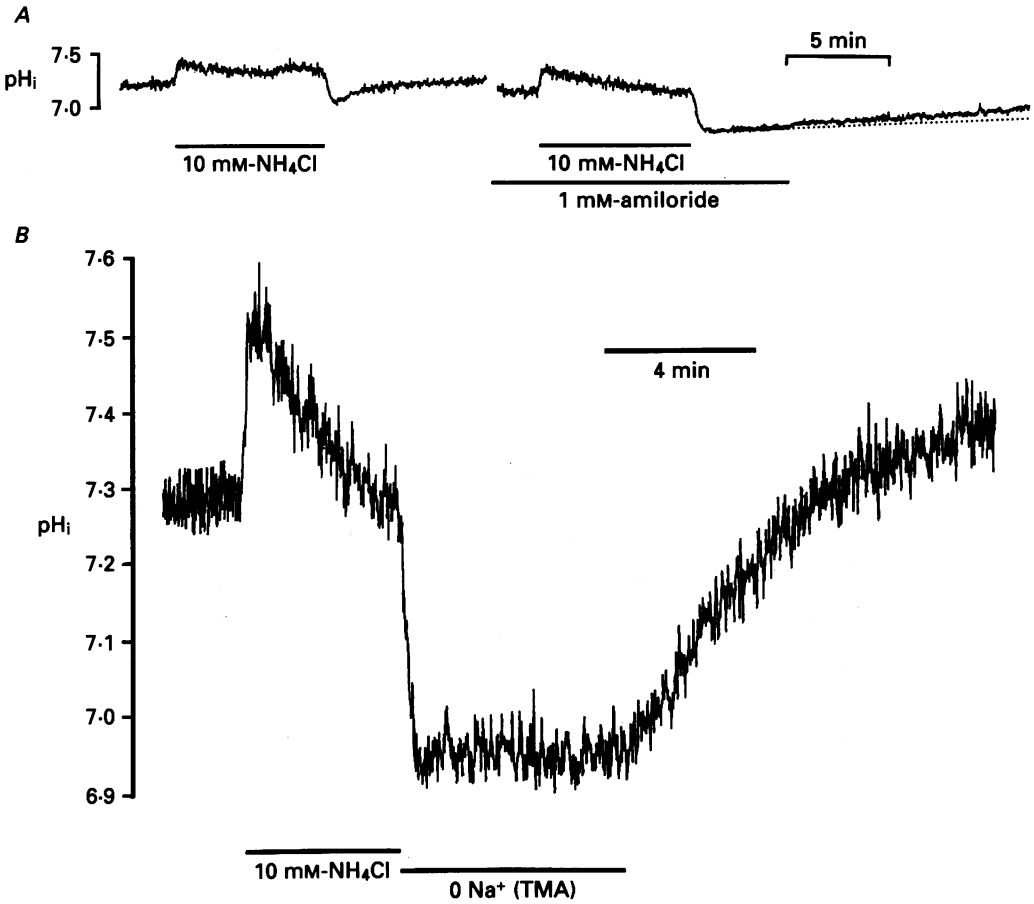

Fig. 5. *A*, effect of 1 mM-amiloride on the recovery of pH_i from an acidosis induced by application and subsequent removal of NH_4Cl. *B*, the effect of removal of extracellular Na^+ (replacement cation was tetramethyl ammonium (TMA^+)) on the recovery of pH_i from an acidosis induced by removing NH_4Cl. HCO_3^- was replaced by 10 mM-HEPES for the duration of the experiment. $pH_o = 7{\cdot}4$.

and declined again as pH_i recovered *during* the NH_4Cl exposure (Fig. 6*A*). Notice also the small degree of tonic shortening of the cell during the alkalinisation. When NH_4Cl was removed the intracellular pH became acid and cell shortening decreased. Under these conditions there is no transient *increase* in cell shortening during the acidosis as observed by Bountra & Vaughan-Jones (1989) suggesting that the intracellular sodium activity (a^i_{Na}) does not increase greatly during the recovery of the acidosis.

We have measured pH_i and contractility (cell shortening) recoveries from an acidosis induced by removal of NH_4Cl in the presence of TPA in experiments of the type shown in Fig. 6. This experiment is carried out on the same cell and shows how pH_i recovery is typically speeded by the presence of TPA. Again, addition of the

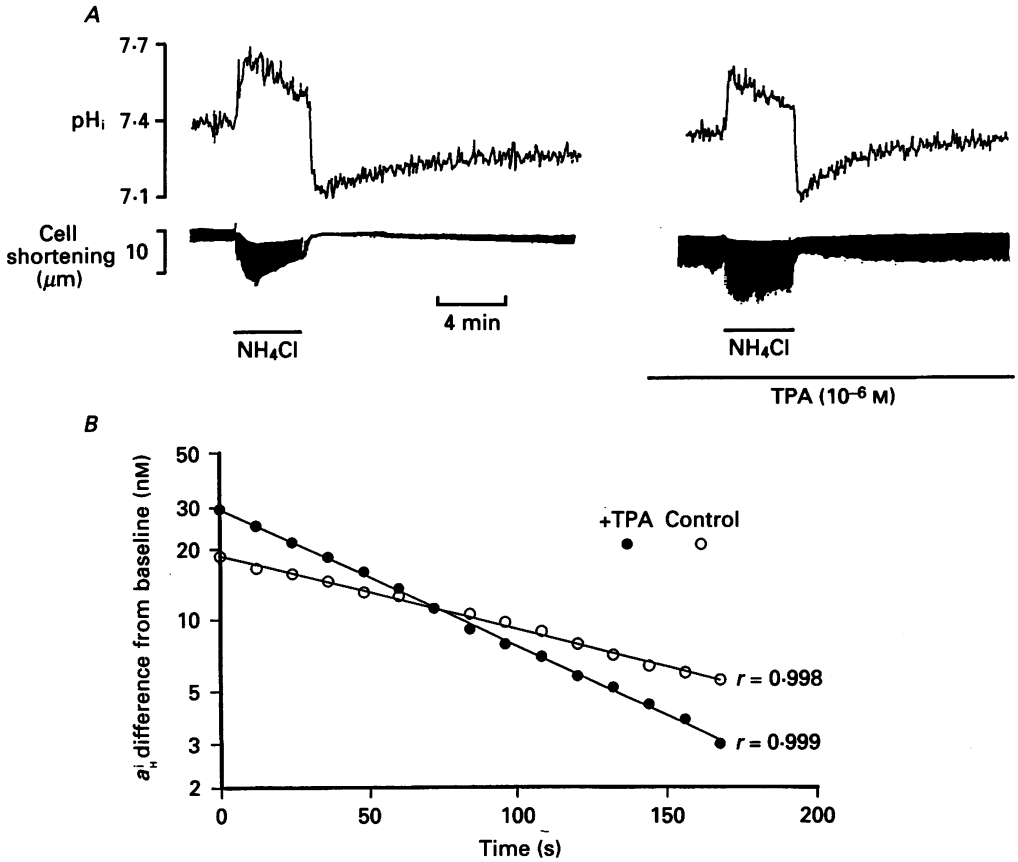

Fig. 6. The effect of application of TPA on recovery of pH_i and cell shortening. *A*, pH_i and cell shortening changes during application and subsequent removal of 10 mM-NH_4Cl. Cell shortening was measured by a photo-diode array (see Methods) and increases in the signal are shown as greater downward deflections of the trace. *B*, the recovery of a^i_H back to baseline values in the presence and absence of TPA. Data are taken from the same experiment. *r* is the correlation coefficient.

phorbol ester did not change steady-state pH_i. The rates of recovery from this experiment are shown in Fig. 6*B*. This process was found to be exponential and in this case (the greatest difference observed) the gradients were −0·311 (control) and −0·581 (+TPA) i.e. an increase in recovery rate of 87%. Recovery *times* from an acid load were significantly ($p < 0·05$) enhanced by 15·1 ± 6·9% ($n = 13$) in the presence of TPA. Recovery times of cell shortening from the same type of acid loading were always more rapid ($p < 0·05$) by an average of 59·1 ± 10·6% ($n = 5$) in the presence of TPA. Recovery times were unchanged in the presence of 4α-phorbol 12,13-didecanoate (which does not activate protein kinase C).

Total buffering power (β_T) in these cells was 40 mmol/l in the presence and 42 mmol/l in the absence of TPA. We have calculated β_T as CO_2 was present in our system. Assuming intracellular P_{CO_2} equals extracellular P_{CO_2} then:

$$[HCO_3^-]_i = 10^{(pH_i - pH_o)} \times [HCO_3^-]_o \quad \text{and} \quad \beta_{CO_2} = 2{\cdot}3[HCO_3^-]_i.$$

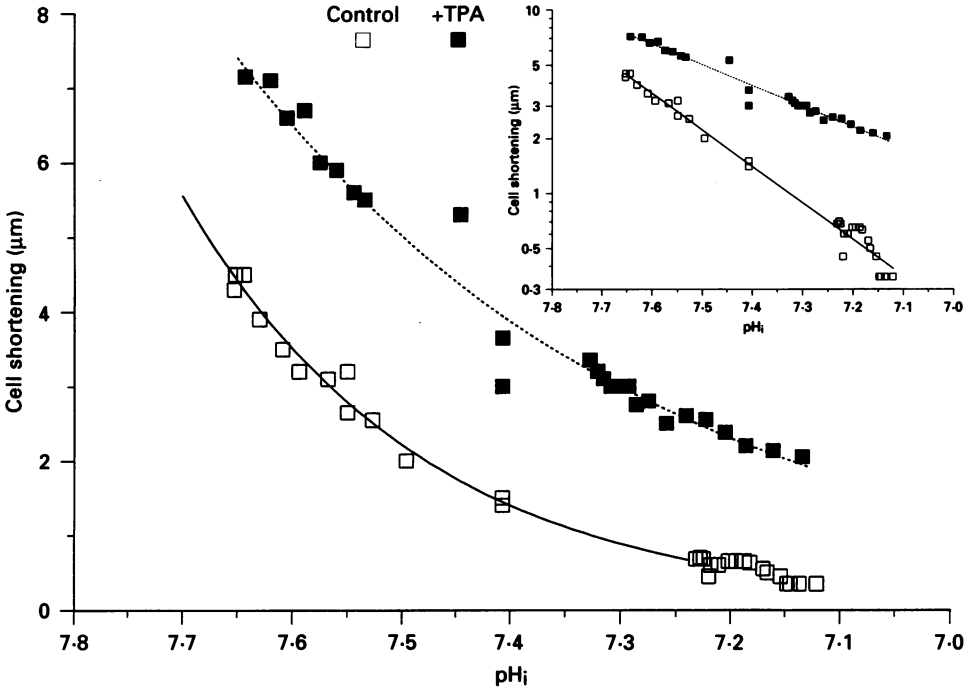

Fig. 7. The relationship between pH_i and cell shortening from the experiment in Fig. 6. The lines are fitted by an exponential regression and have slope 1·99 in control and 1·13 in the presence of TPA. The values were obtained during and after the NH_4Cl exposure.

This yields a value of 25 mmol for β_{CO_2} and assuming that $\beta_T = \beta_i + \beta_{CO_2}$, this gives a value of 17 mmol/l for the intrinsic buffering power (β_i). This compares favourably with 20 mmol/l quoted by Bountra, Powell & Vaughan-Jones, (1990) and 25 mmol/l by Eisner *et al.* (1989).

Figure 7 illustrates the relationship between pH_i and cell shortening. Contractility decreases in a non-linear manner as pH_i becomes more acidic. Throughout this range of pH_i the plot of logarithmic cell shortening is linear (Fig. 7 inset) having a slope of 1·99 in control and 1·13 in the presence of TPA. The presence of TPA shifts the relationship upwards such that at any one pH_i, cell shortening is greater.

Two phenomena are apparent from these data and are demonstrated more clearly in Fig. 8. Firstly, for a given pH_i change in the alkaline direction, the change in the size of the twitch is much larger than for the same pH_i excursion in the acid direction. This is the case for control experiments as well as in the presence of TPA. Secondly, in the presence of TPA, a similar change in pH_i as in control produces less effect on twitch.

The data suggest that the effect of activating protein kinase C is to increase systolic [Ca^{2+}] and stimulate the Na^+–H^+ exchanger. Although steady-state pH_i is unaffected, recovery from an acidification is enhanced. Thus the positive inotropic effect seen with phorbol esters which activate protein kinase C does not seem to be due to a stimulated exchanger.

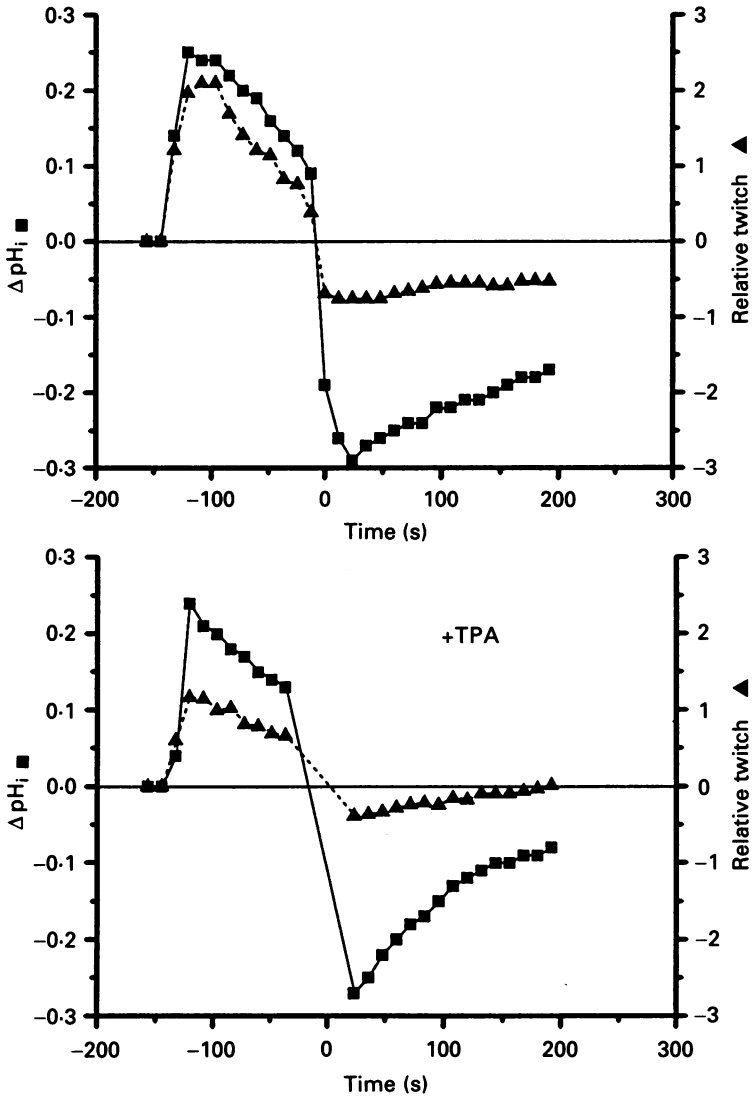

Fig. 8. A plot of the change in pH_i (ΔpH_i) and cell shortening (relative twitch) during alkalosis and acidosis produced by application and removal of 10 mM-NH_4Cl. NH_4Cl was removed at time zero. Top panel is control and the bottom panel is in the presence of 10^{-7} M-TPA.

DISCUSSION

Effects of TPA on contraction

Good evidence exists that application of certain phorbol esters, and compounds like diacylglycerol, stimulate protein kinase C (Nishizuka, 1984). Superfusion of myocytes isolated from rat heart with TPA causes an increase in contraction whilst superfusion with solutions which contain the analog 4α-phorbol 12,13-didecanoate cause no change in contraction. 4α-phorbol 12,13-dideanoate does not stimulate protein kinase C but TPA does and so this suggests that the positive inotropic effect which we observe is brought about by protein kinase C stimulation and not by non-specific effects of phorbol ester. The onset of the positive inotropic effect takes place over 3–15 min. Thus the time to peak effects of TPA are noticeably longer than the positive inotropic processes occurring when, for example, $[Ca^{2+}]_o$ is increased. The effects of TPA have a time course of action similar to that of the α_1-agonist, phenylephrine. In experiments carried out by us on similarly isolated cells and similar equipment, an approximate doubling of cell shortening was observed in response to 100 μM-phenylephrine. This increase in cell shortening took around 6–8 min for full effects to occur (Moody, Dashwood, Sykes, Chester, Jones, Yacoub & Harding, 1990). The effects of α_1-agonists also appear to be mediated through phosphoinositol breakdown and possible activation of protein kinase C (Henrich & Simpson, 1988).

The effects of TPA which we observe are opposite to those observed by Leatherman, Kim & Smith, (1987) using cultured chick heart cells, Dosemeci, Dhallan, Cohen, Lederer & Rogers, (1988) using cultured neonatal rat heart cells and Capogrossi, Kaku, Filburn, Pelto, Hansford, Spurgeon & Lakatta (1990) using adult rat myocytes. All these groups found that TPA decreased contraction. The method Leatherman *et al.* (1987) used to produce their cultures was derived from that of Barry & Smith (1982) where fetal calf serum was present in the balanced salt solution in which the cells were incubated. Dosemeci *et al.* (1988) also used fetal calf serum in their growth medium. Fetal calf serum may activate protein kinase C (Moolenaar *et al.* 1981; 1983) and so desensitization of receptors may occur which alters the contractile response. TPA may inhibit diacylglycerol formation by epidermal growth factor (Smith, Losonczy, Sahai, Pannerselvam, Fehnel & Salomon, 1983) and TPA may attenuate the stimulation of Na^+–H^+ exchange by epidermal growth factor (Whiteley, Cassel, Zhuang & Glaser, 1984). Capogrossi *et al.* (1990) also had fetal calf serum present in the cocktail used to load cells with fluorescent indicator which were subsequently used in contraction studies. Rat cardiac myocytes are not the only species in which we observe a positive inotropic effect. Two experiments on rabbit ventricular myocytes showed that, in the presence of TPA, contraction also increased (by an average of 165%) (MacLeod & Harding, unpublished observations).

Ca^{2+} phorbol esters and contraction

It is clear that there are increases in systolic $[Ca^{2+}]$ in both rat and guinea-pig heart cells in the presence of TPA. This increase may account for some of the positive inotropy seen under these conditions but the twitch increases by 100% whilst Fura-2 ratios only increase by about 20%. Is the increase in [free $Ca^{2+}]_i$ great enough to

account for the increase in twitch? Data from Fabiato (1983) would indicate that rat cells require approximately 50% increase in myoplasmic [Ca^{2+}] to double force. This relationship may be overestimated and also may not hold true for unloaded cells i.e. in unloaded cells small increments in myoplasmic [Ca^{2+}] may produce larger increases in twitch than expected from loaded (isometrically contracting) cells. Thus the increase in twitch seen in the presence of TPA may not be acounted for solely on the basis that systolic [Ca^{2+}] is increased. The possibility remains that activation of protein kinase C is increasing the sensitivity of the contractile proteins for Ca^{2+}.

The mechanism(s) by which systolic [free Ca^{2+}] increases are unknown. Activation of protein kinase C may enhance the currents responsible for promoting Ca^{2+} influx during the action potential. The fast component ($I_{Ca,f}$) activating fully within about 3 ms, is probably responsible for the fast influx of Ca^{2+} into the cell which may serve as the trigger for the contraction process (Fabiato, 1983). Dihydropyridine-sensitive Ca^{2+} channels are believed to be regulated by phosphorylation/dephosphorylation reactions catalysed by protein kinase A which is activated by increased levels of cyclic AMP. Phosphorylation of the dihydropyridine-sensitive Ca^{2+} channel increases the probability of channel opening and when the channel is open it remains open for longer (Brum, Osterrieder & Trautwein, 1984). In principle other kinases within the cell could phosphorylate the channel e.g. Ca^{2+}-calmodulin dependent protein kinase or protein kinase C requiring diacylglycerol. O'Callahan, Ptasienski & Hosey (1988) showed that a skeletal muscle peptide of 165 kDa – known to contain receptors for dihydropyridines, phenylalkylamines and other Ca^{2+} channel effectors – is an efficient substrate for protein kinase C. Phosphorylation of the peptide by protein kinase C was not additive with phosphorylation by cyclic AMP-dependent kinase. Prior phosphorylation by cyclic AMP-dependent kinase prevented subsequent phosphorylation by protein kinase C. Satoh & Hashimoto (1988) found that TPA increased action potential duration and $I_{Ca,f}$ in rabbit sino-atrial node cells. Work by Dosemeci *et al.* (1988) and Lacercda, Rampe & Brown (1988) provides strong evidence that, in cultured neonatal tissue, activated protein kinase C enhances I_{Ca}. Together, these results suggest that protein kinase C can regulate dihydropyridine-sensitive Ca^{2+} channels perhaps by altering their gating or by recruiting dormant channels (Strong, Fox, Tsien & Kaczmarek, 1987). Protein kinase C activation may promote the appearance of dihydropyridine receptors in sarcolemmal membranes (Navarro, 1987). However, it should be noted that Walsh & Kass (1988) failed to find any evidence of phorbol esters increasing I_{Ca} in guinea-pig cardiac myocytes.

Activated kinases may also phosphorylate the sarcoplasmic reticulum (SR) Ca^{2+}-release channel so altering gating and therefore release of Ca^{2+} from the reticular stores. Takasago, Imagawa & Shigekawa, (1989) demonstrated that the channel isolated from cardiac SR can be phosphorylated by cyclic AMP-dependent kinase and this occurs with an increase in [^{3}H]ryanodine binding. Timerman, Chadwick & Fleischer, (1990) reported that the skeletal SR Ca^{2+}-release channel can be phosphorylated by protein kinase A and protein kinase C.

pH_i, phorbol esters and contraction

It is now widely recognised that pH_i in a variety of tissues can be altered by growth factors (Moolenaar *et al.* 1981), insulin (Moore, 1981) and phorbol esters

(Moolenaar *et al.* 1983). The important intermediary would seem to be protein kinase C but evidence for stimulated protein kinase C activating Na^+–H^+ exchange is mostly indirect. Since stimulation of protein kinase C may stimulate Na^+–H^+ exchange in heart cells also (for review see Frelin, Vigne, Ladoux & Lazdunski (1988) then this might be the mechanism for the positive inotropy observed in the presence of TPA. An alkalinisation would alter the Ca^{2+}-sensitivity of the myofilaments and shift the pCa *vs.* tension relationship to the left (Fabiato & Fabiato, 1978). However, we have been unable to find any clear change in pH_i in the presence of TPA. This may imply that protein kinase C activation does not stimulate the exchanger in heart muscle. An alternative explanation is that at normal pH_i (7·0–7·3) the Na^+–H^+ exchange is relatively inactive. Kaila & Vaughan-Jones (1987) have shown that the exchanger becomes increasingly more activated as pH_i decreases from 7·2–6·2. They noticed that amiloride-induced changes in a^i_{Na} were ten times larger when pH_i was 6·95 than those observed when pH_i was 7·30. They also applied amiloride at successively lower pH_i's and found that pH_i decreased at progressively faster rates. It was clear from their experiments that exchanger activation is steeply dependent upon pH_i. Activation of the Na^+–H^+ exchange is low when pH_i is 7·2–7·3, but as pH_i decreases to pH 6·7 the exchanger is about ten times more active. Our experiments show that the exchanger can indeed be stimulated by activated protein kinase C. The presence of TPA also shifts the non-linear relationship of pH_i *vs.* twitch upwards so that at any one pH_i, the twitch is greater.

The general exponential relationship between pH_i and twitch has already been observed in isolated tissue preparations by Vaughan-Jones, Eisner & Lederer (1987) (sheep Purkinje fibre), Bountra & Vaughan-Jones (1989) (guinea-pig papillary muscle) and in isolated rat cardiac myocytes by Eisner *et al.* (1989). Like these earlier studies, the results reported here differ from those of Jacobus, Pores, Lucas, Kallman, Weisfeldt & Flaherty, (1982) and Poole-Wilson & Seabrooke (1985) who found linear relationships between pH_i and left ventricular developed pressure in the former, and twitch force in the latter. As pointed out by Bountra & Vaughan-Jones (1989), it is possible that the pH_i excursions in these studies were small and so the non-linearity would not be as apparent. In addition, the steep relationship between pH_i and twitch only becomes apparent at alkaline pH_i's ($pH_i \geqslant 7{\cdot}3$) and so if interventions were not designed to impose pH_i changes more alkaline than this then the non-linearity would, again, not be as apparent. The slope of the relationship we find to be $\approx 2{\cdot}0$ which is similar to that found by Vaughan-Jones *et al.* (1987) and Bountra & Vaughan-Jones (1989). This similarity is interesting since our observations were carried out on unloaded cells, contracting isotonically whilst Vaughan-Jones *et al.* (1987) and Bountra & Vaughan-Jones (1989) were measuring isometrically derived force from their intact preparations. Using data from Fabiato & Fabiato (1978) where they have plotted tension *vs.* pCa at various bathing pH_i's in skinned rat heart cells, the pH *vs.* log tension relationship has a slope of $\approx 1{\cdot}7$. As pointed out by Vaughan-Jones *et al.* (1987), this value is close to that obtained in intact preparations. Given that alterations in pH_i may alter other aspects of excitation–contraction coupling in intact cells or preparations whereas the Fabiato's work demonstrates the sensitivity to pH of the myofilaments alone, it is surprising there is such close agreement. The slope of the pH_i *vs.* twitch relationship was smaller in the presence of phorbol ester implying that the shortening of the cells was less

sensitive to pH_i changes when protein kinase C had been stimulated. This appears not to be due to an increase in buffering capacity of the cells as intracellular buffering power appears to be unchanged in the presence of TPA. This result may reflect an alteration in the sensitivity of troponin C to Ca^{2+} and not to H^+ and/or a phosphorylation of other troponin complexes such that they are less affected by H^+. It is also possible that myosin may be phosphorylated (Ruegg, 1988) by protein kinase C stimulation leading to an enhanced twitch. Troponin T has been shown to be phosphorylated by TPA addition (Liu, Wood, Raynor, Wang, Noland, Ansari & Kuo, 1989).

An alternative possibility may arise from pH-induced changes in [free $Ca^{2+}]_i$. Increasing pH_i leads to a decrease in [free $Ca^{2+}]_i$ and vice versa (Bers & Ellis, 1982; Kohmoto, Spitzer, Movsesian & Barry, 1990) so if Ca^{2+} influx is stimulated by TPA, any decrease in pH may induce a larger increase in intracellular Ca^{2+} compared with control conditions. This may attenuate the effect of acidosis on myofilament sensitivity.

In summary, we have observed that certain phorbol esters can stimulate Na^+–H^+ exchange and increase [free $Ca^{2+}]_i$ in adult cardiac myocytes possibly by increasing activation of protein kinase C. By this mechanism agonists and antagonists can alter the contractility of heart cells.

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