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Interaction of phorbol esters with Ca^{2+} channels in smooth muscle

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1 The phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA), a selective activator of protein kinase C, had no effect on the sensitivity to Ca^{2+} or verapamil of K^+-depolarized taenia preparations from the guinea-pig caecum, despite the use of high concentrations (1 μM for 3 h); this preparation is sensitive to Ca^{2+} channel activators and antagonists.

2 TPA (0.03–3 μM) caused a slow contraction of rat aorta preparations; the contractions were resistant to the calcium-antagonists nifedipine (0.01 μM), verapamil (10 μM), diltiazem (10 μM) and cinnarizine (10 μM), but were antagonized by N-(6-aminohexyl)-5-chloro-1-naphthalensulphonamide (W-7, 50–200 μM). Prolonged exposure to TPA (> 2 h) resulted in spontaneous contractions which were sensitive to verapamil (1 μM).

3 Isoprenaline and sodium nitroprusside relaxed phenylephrine-induced contractions in rat aorta preparations. TPA (0.3 μM) blocked the maximal response to isoprenaline but not to sodium nitroprusside indicating that TPA did selectively activate protein kinase C under these experimental conditions.

4 These findings indicate that protein kinase C activation does not result in direct effects on Ca^{2+} channel function, but may exert effects indirectly (e.g. by modifying intracellular sensitivity to Ca^{2+}, Ca^{2+} extrusion, or cellular depolarization).

Introduction

Although voltage-operated Ca^{2+} channels (VOCs) play key roles in cellular activation, few control mechanisms modulating VOC activity have been described. Thus, although there are many binding sites for exogenous substances (dihydropyridines, phenylalkylamines) on VOCs there are few endogenous ligands for these sites (but see Mir & Spedding, 1986). VOC activation appears to be primarily dependent on membrane potential, surface charge and phosphorylation (Reuter, 1983; Brum *et al.*, 1984; Hess *et al.*, 1984; Spedding, 1985; Flockerzi *et al.*, 1986). Myocardial VOCs are phosphorylated by cyclic AMP-dependent protein kinase (Brum *et al.*, 1986) and this phosphorylation appears to be the principle cause of the positive inotropic effects of β-adrenoceptor stimulants (Reuter, 1983).

However, control systems for activation of VOCs in smooth muscle are not well defined, with the exception of the evident effects of cellular depolarization (Durbin & Jenkinson, 1961) and surface charge (Spedding, 1984). β-Adrenoceptor stimulants do not appear to modify directly Ca^{2+} entry into smooth muscle, although agonists may have marked effects on Ca^{2+} sequestration. Phosphorylation of Ca^{2+} channels by protein kinase C might therefore be considered as a possible modulator of VOC activity and Ca^{2+} entry in smooth muscle, particularly as this enzyme is activated following receptor stimulation, via diacylglycerol (Berridge, 1984; Nishizuka, 1984); it might therefore be expected that stimuli which increase the tissue content of inositol phosphates might, as a consequence of the diacylglycerol formation, activate protein kinase C, modulating VOC activity.

Some evidence for such a mechanism has been advanced because phorbol esters, which activate protein kinase C with some selectivity (Blumberg *et al.*, 1984; Nishizuka, 1984; Wolf *et al.*, 1985), contract smooth muscle directly (Danthuluri & Deth, 1984; Rasmussen *et al.*, 1984; Baraban *et al.*, 1985; Dale & Obianime, 1985; Forder *et al.*, 1985; Gleason & Flaim, 1986; Menkes *et al.*, 1986; Miller *et al.*, 1986; Sybertz *et al.*, 1986) although inhibitory effects have also been reported (Baraban *et al.*, 1985; Menkes *et al.*, 1986). Phorbol esters also increase responsiveness to Ca^{2+} channel activators, such as Bay K 8644 (Forder *et al.*,

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1985). However, activation of protein kinase C leads to many diverse effects (Nishizuka, 1984) so I have investigated whether phorbol esters cause a direct activation of VOCs in isolated blood vessel preparations and in K^+-depolarized taenia preparations from the guinea-pig caecum; the latter preparation is especially sensitive to Ca^{2+} channel activators and inactivators (Spedding, 1982; 1985). Some of these results have been communicated previously to the British Pharmacological Society (Spedding, 1986).

Methods

K^+-depolarized taenia preparations

Taenia preparations from the caecum of male guinea-pigs (200–350 g) were set up in 10 ml isolated organ baths containing Ca^{2+}-free K^+-Tyrode solution, of the following composition (mM): NaCl 97, KCl 40, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5. The solution was gassed wth 95% O_2: 5% CO_2 and maintained at 35°C. Contractions were measured with Bioscience isotonic transducers, with 1 g load. Cumulative concentration-response curves for Ca^{2+} (0.1, 0.3, 1, 3, 10 mmol l^{-1}) were obtained by increasing the Ca^{2+} concentration at 3–5 min intervals in logarithmic increments; the curves were obtained at 40 min intervals. The first Ca^{2+} concentration-response curve was used only to position the baseline and maximum response on the chart paper. With this technique the sensitivity to Ca^{2+} does not change for 5 h (Spedding, 1982).

Rat aorta preparations

Rings, 5 mm in length, taken from the thoracic aorta of male Sprague-Dawley rats (220–280 g) were set up in 10 ml baths containing Tyrode solution (mM: NaCl 140, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 5.5) which was gassed with 95% O_2: 5% CO_2 maintained at 35°C. The rings were denuded of endothelium by gentle rubbing and suspended between two horizontal steel wires, one of which was used to anchor the preparation and the other to record isometric tension. Resting tension was set at 1 g. After a 60 min equilibration period, a response to phenylephrine (1 μM) was obtained. Subsequent contractions to phorbol esters were calculated as a percentage of this response, which was 95–100% of the tissue maximum response to phenylephrine, in the absence of endothelium.

Rat portal vein preparations

Preparations of rat portal vein were set up under similar conditions to those used for the rat aorta, except that the preparations were suspended longitudinally for measurement of isometric contractions.

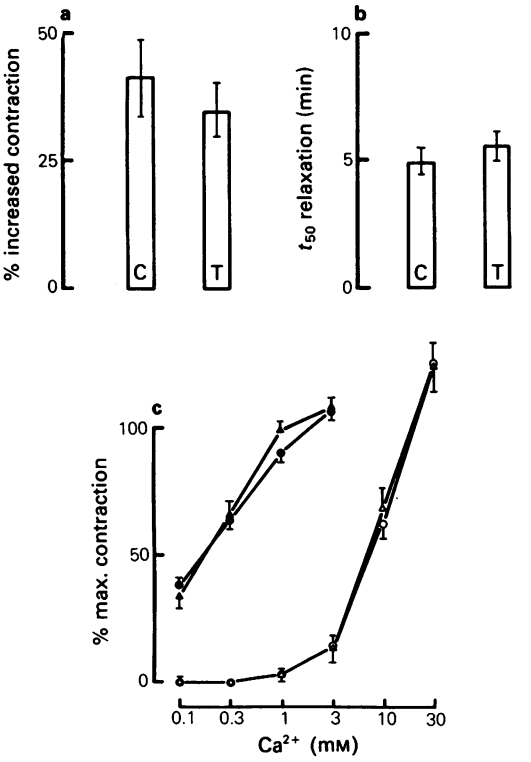

Figure 1 Failure of 12-O-tetradecanoylphorbol-13-acetate (TPA) to affect Ca^{2+}-induced contractions in K^+-depolarized taenia preparations from the guinea-pig caecum. The taenia were submaximally contracted with Ca^{2+} (0.1 mM) and after the contractions had initially stabilized the preparations were incubated with TPA (1 μM, T) or the control solvent (0.1% dimethylsulphoxide, C) for 3 h. The tone of the preparations slowly increased over the 3 h period (a). The preparations were then washed with Ca^{2+}-free Tyrode and the time for 50% relaxation measured (t_{50}) b). Following maximal relaxation TPA was readded (1 μM, ▲; solvent controls, ●) and cumulative concentration-response curve to Ca^{2+} obtained (c). Following washout, and readdition of TPA, the preparations were incubated with verapamil (0.2 μM for 30 min) and a subsequent Ca^{2+} concentration-response curve obtained (TPA, Δ; controls, O). Vertical lines represent s.e.mean of 5 experiments.

Drugs

The following drugs were used: Bay K 8644 (Bayer AG, a gift from Dr M. Schramm), cinnarizine tartrate (Janssen Pharmaceutical), diltiazem hydrochloride

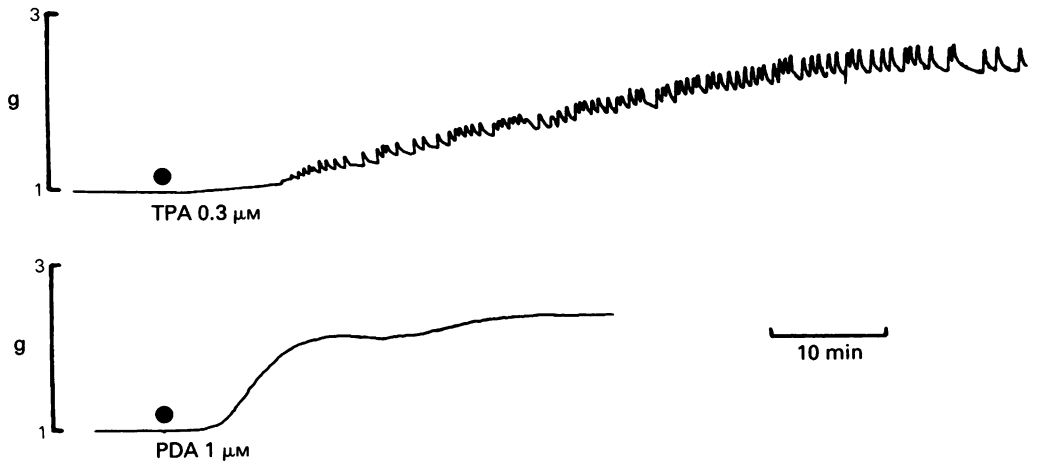

Figure 2 Contractile effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) and phorbol-12, 13-dibutyrate (PDA) in rat aorta preparations.

(Synthelabo), idazoxan hydrochloride (Reckitt & Coleman), (−)-isoprenaline sulphate (Sigma), nifedipine (Bayer), phenylephrine hydrochloride (Sigma), prazosin hydrochloride (Pfizer), sodium nitroprusside (Sigma), (±)-verapamil hydrochloride (Knoll, Isoptin), W7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide; Sigma). The phorbol esters TPA (12-O-tetradecanoylphorbol-13-acetate) and PDA (phorbol-12, 13-dibutyrate) were from Sigma and dissolved in the minimal quantity of dimethylsulphoxide. Final concentrations of dimethylsulphoxide in the Tyrode solution were $<0.1\%$ and did not affect contractility. Bay K 8644 and nifedipine were dissolved in ethanol at a concentration of 1 mM; experiments involving these drugs were performed in a darkened room. Data are expressed as mean ± s.e.mean and statistical significance was assessed by use of Student's *t* test.

Phorbol esters are tumour promoters and appropriate precautions were taken. In isolated tissue experiments organ baths were not fully filled, minimally gassed and sealed with Parafilm.

Results

K^+-depolarized taenia

Taenia preparations from the guinea-pig caecum relaxed fully after 30 min incubation in K^+-depolarizing Tyrode solution which was Ca^{2+}-free. Cumulative addition of Ca^{2+} (0.1–3 mM) contracted the preparations. Submaximal contractions to Ca^{2+} initially stabilized at 40–55% of the maximal response but tone slowly increased by 30–45% over a 3 h period. Addition of TPA (1 μM) to preparations which were submaximally contracted with Ca^{2+} (0.1 mM) did not affect the contractions over the 3 h period (Figure 1). Lower concentrations were also ineffective (0.01 μM, $n = 2$). Furthermore, relaxation processes were unaffected by TPA in that the tissues relaxed rapidly upon washout of the Ca^{2+} and the time for 50% relaxation (t_{50}) was not significantly different between preparations treated with TPA and control preparations which were run in parallel (Figure 1). Readdition of TPA did not change the sensitivity of the taenia to Ca^{2+}, nor to the subsequent inhibitory effects of verapamil (Figure 1). Thus TPA (1 μM for >3 h) had no effect on Ca^{2+}-induced contractions or on the sensitivity to verapamil in K^+-depolarized taenia preparations.

Rat aorta

TPA (0.3 μM) caused a slowly developing contraction of rat aorta, sometimes with superimposed rhythmic contractions. There was always a 5–20 min threshold period following addition of TPA (0.03–3 μM) before development of the contraction. The related phorbol ester, PDA (0.3–1 μM), caused a contraction that was more rapid in onset (Figure 2), although the preparations were less sensitive to PDA than to TPA (threshold contraction for TAP 3–30 nM, threshold for PDA 30–100 nM). Rhythmic contractions were sometimes superimposed on the sustained contraction induced by PDA. The concentration-response relationship to TPA is shown in Figure 3. A submaximal contraction to TPA (0.3 μM) was not altered by

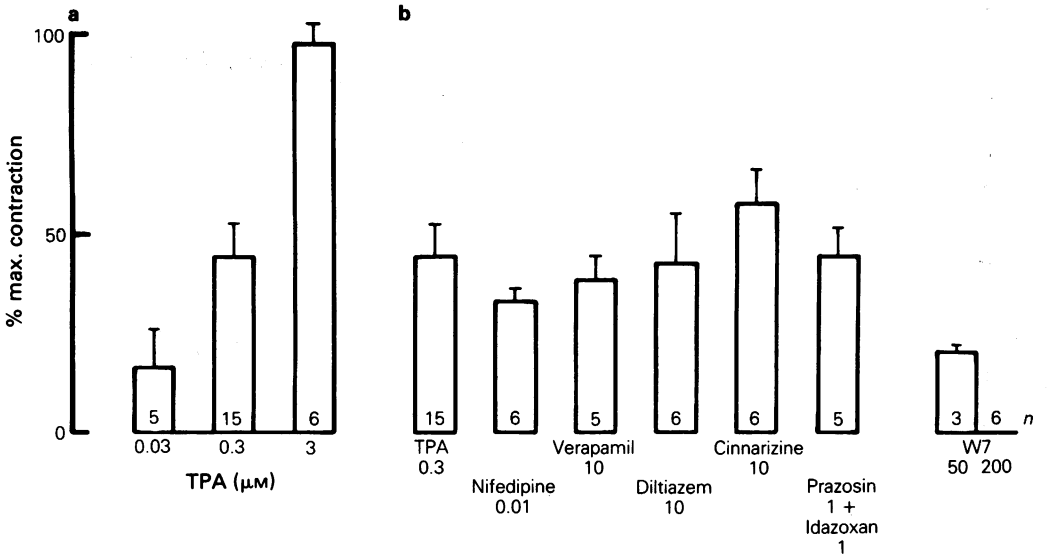

Figure 3 Concentration-response relationship of the contractile effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) in rat aorta (a) expressed as a percentage of the response to phenylephrine (1 μM). The effects of a 30 min preincubation with nifedipine (10 nM), verapamil (10 μM), diltiazem (10 μM), cinnarizine (90 min incubation, 10 μM), prazosin (1 μM) and idazoxan (1 μM) or W-7 (50 and 200 μM) are shown in (b). Vertical lines represent s.e.mean, *n* is as indicated.

preincubation of the preparations with the calcium-antagonists nifedipine (10 nM), verapamil (10 μM), diltiazem (10 μM) or cinnarizine (10 μM) (Figure 3). The TPA-induced contraction was not secondary to noradrenaline release because it was also resistant to preincubation with prazosin (1 μM) and idazoxan (1 μM). However, the putative calmodulin inhibitor W-7 caused a concentration-dependent inhibition of the contractile-response to TPA (Figure 3).

The contraction induced by TPA (0.3 μM) was sustained over a 2–4 h period but then declined slowly and was followed by regular slow contractions, which persisted for up to 16 h (Figure 4). These contractions, in contrast to the initial TPA-induced contraction, were sensitive to verapamil (1 μM; Figure 4).

The phorbol esters also increased the frequency and amplitude of the spontaneous contractions in rat portal vein (Figure 5); at the highest concentration of PDA tested (1 μM, Figure 5) a transient direct contraction occurred. PDA (0.1 and 1 μM) caused similar effects in guinea-pig portal vein ($n = 3$).

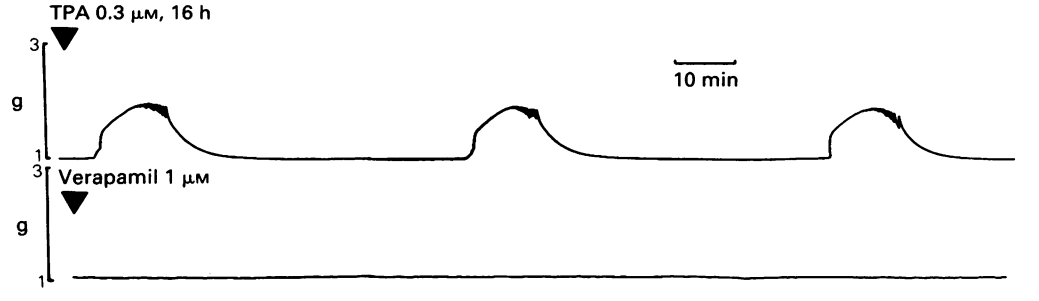

Figure 4 Rhythmic contractions in rat aorta preparations after exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA) (0.3 μM for 16 h, upper trace). The contractions were abolished by incubation with verapamil (1 μM, lower trace).

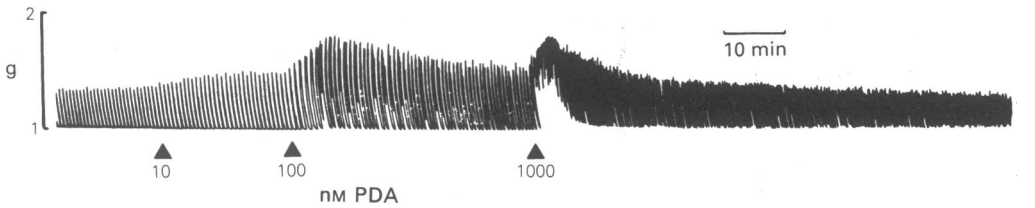

Figure 5 Effects of phorbol-12, 13-dibutyrate (PDA) on the spontaneous contractions of the rat portal vein.

Selectivity and phorbol esters

Isoprenaline (0.1–3.0 μM) and sodium nitroprusside (0.3–100 nM) relaxed phenylephrine-induced contractions of rat aorta (Figure 6). Pretreatment with TPA (0.3 μM for 20 min) partially contracted the tissues; addition of phenylephrine (1 μM) caused a further contraction, although this was not significantly greater than the initial responses of the tissues to phenylephrine added alone. In the presence of phenylephrine (1 μM) and TPA (0.3 μM) the relaxant responses to sodium nitroprusside were similar to the responses in the presence of phenylephrine alone. In contrast, TPA (0.3 μM) markedly reduced the relaxant responses of higher (> 1 μM) but not lower (< 1 μM) concentrations of isoprenaline.

Discussion

The phorbol ester TPA was found to be without effect on Ca^{2+}-induced contractions in K^+-depolarized taenia preparations from the guinea-pig caecum despite the use of high concentrations and a long incubation period. Taenia preparations are exquisitely sensitive to both Ca^{2+} channel activators and antagonists (Spedding, 1982; 1984; 1985) yet TPA influenced neither the sensitivity to Ca^{2+} nor that to verapamil. This finding represents strong evidence that protein kinase C does not directly modulate Ca^{2+} channel function in smooth muscle, at least under constant K^+-depolarization. Further experiments in rat aorta substantiated the findings in guinea-pig taenia.

Although TPA contracted the rat aorta directly as

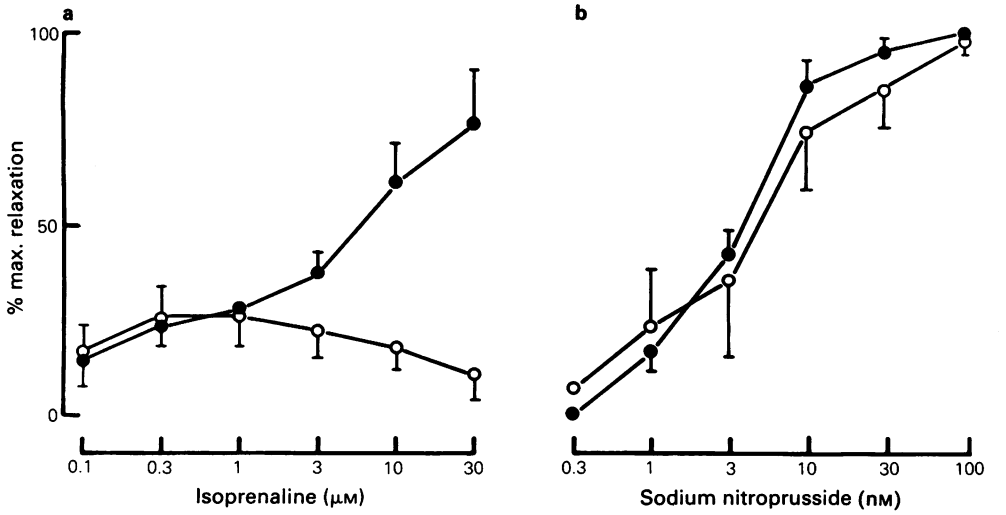

Figure 6 Effects of 12-O-tetradecanoylphorbol-13-acetate (TPA 0.3 μM for 30 min) on cumulative concentration-response curves to isoprenaline (a) and sodium nitroprusside (b) in rat aorta. Control relaxations (●) were taken from preparations that were run in parallel to those pretreated with TPA (○); all preparations were contracted with phenylephrine (1 μM). Vertical lines represent s.e.mean, $n = 5$.

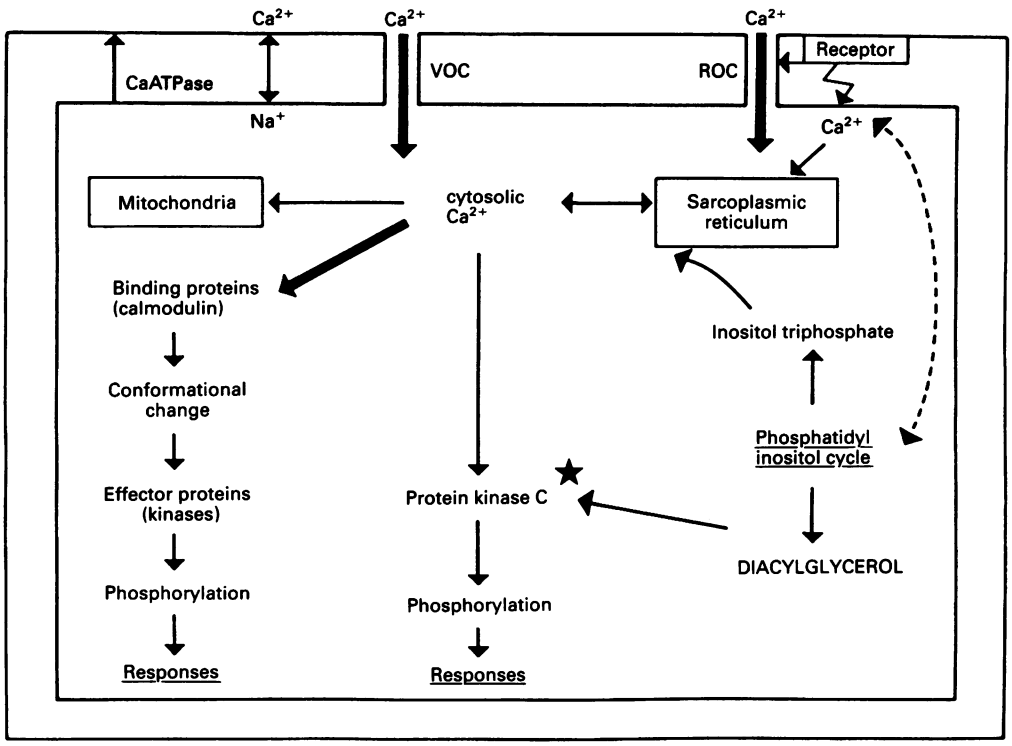

Figure 7 Schematic diagram of the activation processes in smooth muscle showing two main branches of activation: voltage-operated calcium channels (VOCs), calmodulin; receptor-operated channels; 'ROCs', the phosphatidyl inositol cycle, protein kinase C). Ca^{2+} channel activators and antagonists have some selectivity for VOCs, whereas phorbol esters would activate protein kinase C (*) inducing a distinct pattern of responses, which may be different from simple elevation of cytosolic Ca^{2+}. Different tissues show differing response patterns depending on the degree of activation of each 'branch'. The taenia would presumably represent a tissue more dependent on VOC and calmodulin activation whereas receptor stimulation of the aorta would involve both branches to different extents. This scheme presumably reflects the different Ca^{2+}-dependence of tissues reported earlier (e.g. the P-T system, Golenhofen *et al.*, 1977). There is some overlap between the patterns of activation in that K^+ depolarization may activate the phosphatidyl cycle, but in a manner resistant to calcium-antagonists and unrelated to contractile events (Best & Bolton, 1986). Activation of VOCs (e.g. by Bay K 8644) might enhance responsiveness via the protein kinase C branch indirectly by an increase in cytosolic Ca^{2+} allowing potentiation of the responses to phorbol esters by Bay K 8644 (Forder *et al.*, 1985). A strong temporal interaction between activation of the branches allows facilitation, potentiation or priming of responses to some activators following exposure to others, as elegantly demonstrated by Barrett *et al.* (1986). Nevertheless, there does not appear to be a direct interrelationship between protein kinase C and VOCs.

reported previously (Danthuluri & Deth, 1984), the contraction was resistant to high concentrations of calcium-antagonists, implying that it was not secondary to Ca^{2+} channel activation. Thus in two tissues from two different species, protein kinase C does not appear to be involved in the control of Ca^{2+} channel function under classical pharmacological conditions.

Nevertheless, TPA and PDA did contract rat aorta. Such an effect might well be expected in this tissue, for protein kinase C would play a role in modulating contractions consequent to inositol phosphate liberation and intracellular Ca^{2+} release (Best & Bolton, 1986). In this respect, in rat and rabbit aorta, but not in the guinea-pig taenia, the events following receptor activation are relatively resistant to Ca^{2+} channel antagonists (Spedding & Cavero, 1984) and involve 'receptor-operated channels' (ROCs) or release of intracellular Ca^{2+} which may be consequent to liberation of inositol phosphates (Berridge, 1984). An effect of TPA, via protein kinase C, on the contractile proteins (Miller *et al.*, 1986), or on Ca^{2+} sequestration or extrusion (Sybertz *et al.*, 1986) would therefore account for the contractions; direct activation of ROCs (see Gleason & Flaim, 1986) cannot be excluded.

There is good evidence supporting the hypothesis

that TPA activated protein kinase C in the aorta under the present experimental conditions. The only agent found to antagonize the contractile effects of TPA in rat aorta was W-7, a compound which binds to calmodulin, but which also has been shown to inhibit protein kinase C activation (Sanchez *et al.*, 1983). This finding is therefore consistent with the TPA-induced contraction being secondary to activation of protein kinase C. The finding that PDA was slightly less potent, yet had a more rapid onset of action is also consistent with such a site of action, because although PDA has a lower affinity for the enzyme (Blumberg *et al.*, 1984) it is a considerably smaller molecule and penetrates cell membranes more easily, resulting in a more rapid onset of action (Baraban *et al.*, 1985). Inhibition of the maximal effects of isoprenaline, but not sodium nitroprusside, by TPA represents strong evidence that protein kinase C was activated in rat aorta, because protein kinase C is involved in β-adrenoceptor phosphorylation, down regulation, and possibly internalization (Kelleher *et al.*, 1984). TPA would therefore appear to be a useful tool to analyse β-adrenoceptor down regulation in smooth muscle.

Thus, although the phorbol esters appeared to activate protein kinase C under the present experimental conditions, no evidence for a direct activation of VOCs was obtained, which indicates that VOCs may not be phosphorylated by protein kinase C. This finding does not mean that protein kinase C activation by phorbol esters will not modify VOC activity in general, because following prolonged incubation (Figure 4) or in tissues with multiple activator systems (Figures 5 and 7), activation of protein kinase C would result in many effects (Na^+ accumulation, depolarization, intracellular Ca^{2+} sequestration) that would indirectly modify VOC activity. These results therefore allow a general scheme for smooth muscle activation to be advanced, whereby activation can occur via two main branches, with different susceptibility to calcium-antagonists (Figure 7). It remains to be seen whether neuronal and chromaffin Ca^{2+} channels are directly modified by protein kinase C as claimed (Harris *et al.*, 1986; Messing *et al.*, 1986; Osugi *et al.*, 1986; Wakade *et al.*, 1986) or whether these effects are indirect, as in smooth muscle.

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