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Phorbol esters inhibit smooth muscle contractions through activation of Na^+-K^+-ATPase

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1 The role of protein kinase C (PKC) in agonist-induced contractions of guinea-pig ileum longitudinal smooth muscle has been investigated.

2 The phorbol esters, phorbol 12,13-dibutyrate (PDBu), phorbol 12,13-diacetate (PDA) and phorbol 12-myristate 13-acetate (PMA), relaxed tissues precontracted by submaximal concentrations of carbachol, histamine or substance P.

3 This inhibitory action of the phorbol esters was reversed following the application of ouabain, a specific inhibitor of Na^+-K^+-ATPase. Similarly, pretreatment with ouabain inhibited the ability of phorbol esters to relax tissues precontracted by the above agonists.

4 The slow relaxation of the tonic component of contraction induced by submaximal concentrations of carbachol and histamine, and all concentrations of substance P, was abolished in the presence of ouabain.

5 In Na^+-loaded tissues, PDBu and carbachol caused a concentration-dependent increase of Na^+-K^+-ATPase activity, assessed by ouabain-sensitive $^{86}Rb^+$-uptake. Extrusion of Na^+, assessed by the cellular content of the ion, was also stimulated by PDBu (the effect of carbachol was not investigated).

6 We conclude that phorbol esters inhibit the tonic component of contractions induced by submaximal concentrations of these agonists through activation of Na^+-K^+-ATPase. We suggest that PKC may exert feedback control over the tonic component of agonist contractions through stimulation of the pump.

Introduction

Ca^{2+}-mobilizing hormones or neurotransmitters stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate generating two second messengers inositol 1,4,5-trisphosphate ($InsP_3$), which releases Ca^{2+} from non-mitochondrial intracellular stores, and 1,2-diacylglycerol (DAG), which stimulates a Ca^{2+}-activated, phospholipid-dependent enzyme, protein kinase C (PKC; for review see Nishizuka, 1984; Berridge, 1987). In smooth muscles such as porcine coronary artery and guinea-pig ileum, Ca^{2+} released by $InsP_3$ has been proposed to contribute to the formation of agonist-induced contractions (Hashimoto *et al.*, 1986; Watson *et al.*, 1988). In contrast, the role of the DAG/PKC system in the regulation of contraction is unclear in spite of a growing number of investigations (Watson & Godfrey, 1988).

PKC is activated by tumour promoting phorbol esters (Castagna *et al.*, 1982). In our recent study on the guinea-pig ileum longitudinal smooth muscle (Sasaguri & Watson, 1989a), we found that phorbol esters enhance the phasic component of carbachol-induced contractions when added before the agonist, but quickly inhibit the tonic component of contraction when added after the phasic component. Further, the long-term exposure of tissue to a high-concentration of phorbol 12-myristate 13-acetate (PMA) which leads to a down-regulation (or loss) of PKC, has little effect on the phasic component of the carbachol contraction but markedly enhances the tonic component. Taken together, we concluded that PKC may have an important feedback role in limiting the tonic component of contraction but that it plays little part during the phasic component, since this was unchanged following the down-regulation of PKC. The mechanism of inhibition of the tonic component of contraction by PKC was unrelated to an effect on phosphoinositide hydrolysis (Sasaguri & Watson, 1989a).

Na^+-K^+-ATPase is crucial for the maintenance of ionic gradients across biological membranes (for review, see Glynn & Karlish, 1975; Jorgensen, 1982; Kaplan, 1985) and its activation causes hyperpolarization of the membrane (Bolton, 1973a). This action leads to the relaxation of excitable smooth muscles such as the guinea-pig ileum. Since Na^+-K^+-ATPase is believed to be a substrate for PKC (Greene & Lattimer, 1986; Nishizuka, 1986; Yingst, 1988) and is stimulated by phorbol esters in rat hepatocytes (Lynch *et al.*, 1986), guinea-pig pancreatic acinar cells (Hootman *et al.*, 1987) and rat aortic smooth muscle (Moisey & Cox, 1987), we have investigated the possibility that relaxation of ileal smooth muscle by phorbol esters results from stimulation of this enzyme. We conclude that phorbol esters inhibit the tonic component of contractions induced by submaximal concentrations of these agonists through activation of Na^+-K^+-ATPase. We suggest that PKC may exert a feedback control over the tonic component of agonist contractions through the stimulation of this pump. Part of this work has been published in a preliminary form (Sasaguri & Watson, 1989b).

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Methods

Hartley-strain guinea-pigs of either sex (300–500 g) were killed by a blow on the head. The whole length of small intestine (except for the duodenum and the ileocaecal region) was quickly dissected and put into Krebs-Henseleit solution gassed with 95% O_2/5% CO_2 at room temperature. Longitudinal smooth muscle was carefully removed as described previously (Sasaguri & Watson, 1988).

Measurement of contractions

A longitudinal muscle strip (approximately 10 mm length without tension) was set up in a 3 or 5 ml glass organ bath to record isotonic contractions under a tension of 1.5 g. Experiments were carried out following 1 h equilibration in HEPES-buffered solution gassed with 100% O_2 at 37°C. When substance P-induced contractions were recorded, atropine (3 μM) was added 5 min before substance P to eliminate the influence of muscarinic neuromuscular transmission (Holzer & Lembeck, 1980). All contraction records, if not otherwise indicated, are shown as percentage response to a maximally effective concentration (1 μM) of carbachol, which was routinely measured at the beginning of each experiment.

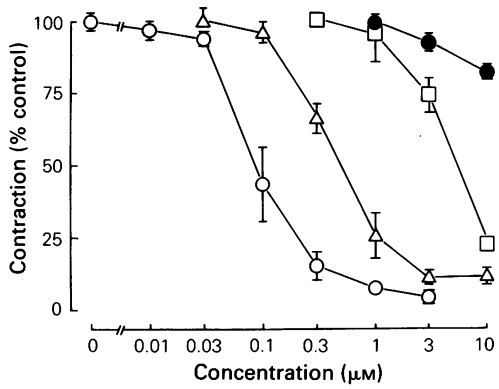

Figure 1 Relaxation by phorbol esters of carbachol-induced contractions. Phorbol 12,13-dibutyrate (○), phorbol 12,13-diacetate (△), phorbol 12-myristate 13-acetate (□) or 4-α-phorbol (●) was added 2 min after the application of 100 nM carbachol (i.e. during the tonic component). Contractions were measured 3 min after the addition of carbachol (1 min after phorbol esters or phorbol) and are demonstrated as percentages of the control (vehicle-treated) contraction induced by 100 nM carbachol. Data represent mean of four separate experiments; vertical lines show s.e.mean.

Measurement of $^{86}Rb^+$-uptake

These studies were carried out essentially as described by Brading & Widdicombe (1974). After 1 h equilibration in normal HEPES-buffered solution, muscle strips were incubated for 3 h in K^+-free solution bubbled with 100% O_2 at 37°C, to increase intracellular Na^+ concentration through inhibition of the Na^+-K^+-pump. Tissues were then placed in low-K^+ solution (K^+ = 1 mM) containing $^{86}Rb^+$ (approximately 5 μCi ml^{-1}) with other reagents as indicated. Four minutes later, they were removed and washed for 5 min in ice-cold low-K^+ solution to remove extracellular radioactivity. Tissues were then blotted, weighed and placed into tubes containing 2 ml of distilled water and counted for gamma radioactivity. When ouabain-insensitive uptake was determined, tissues were pre-incubated with 0.1 mM ouabain for the last 10 min of the incubation in K^+-free solution and the same concentration of ouabain was added to the reaction medium. Ouabain-sensitive $^{86}Rb^+$-uptake was determined by subtracting ouabain-insensitive uptake from total uptake.

Determination of intracellular Na^+ and K^+ content

This assay was based on the method described by Brading (1975). The content of Na^+ and K^+ in smooth muscles was measured by atomic absorption flame photometry. Muscle pieces (approximately 20 mg), treated as indicated in the legend to Table 1, were extracted for 24 h in tubes containing 3 ml of 'diluting fluid' which consisted of 1 N HNO_3 containing 18 mM La^{3+} (La_2O_3) and 10 mM Li^+ (Li_2CO_3) to minimize ionic interactions during the flame photometry and to avoid precipitation of Ca^{2+}. Standard solutions were prepared in the same diluting fluid.

Solutions

Krebs-Henseleit solution (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10. The solution was bubbled with 95% O_2 and 5% CO_2; the pH was 7.4 at room temperature.

HEPES-buffered solution (mM): HEPES/Tris buffer 5, NaCl 135, KCl 4, $MgCl_2$ 2, $CaCl_2$ 2, KH_2PO_4 1 and glucose 12. The solution was bubbled with 100% O_2; the pH was adjusted to 7.4 at 37°C. HEPES/Tris buffer was made by the method described previously (Sasaguri & Watson, 1989a). For K^+-free and low-K^+ solutions NaCl substituted for KCl and KH_2PO_4 was replaced with NaH_2PO_4.

Chemicals

Phorbol 12,13-dibutyrate (PDBu), phorbol 12,13-diacetate (PDA), PMA, 4-α-phorbol, ouabain (G-strophanthin), atropine, tetrodotoxin, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), hydroxymethylaminomethane (Tris), histamine and nifedipine were all purchased from Sigma. Carbamylcholine chloride (carbachol) was from BDH. Substance

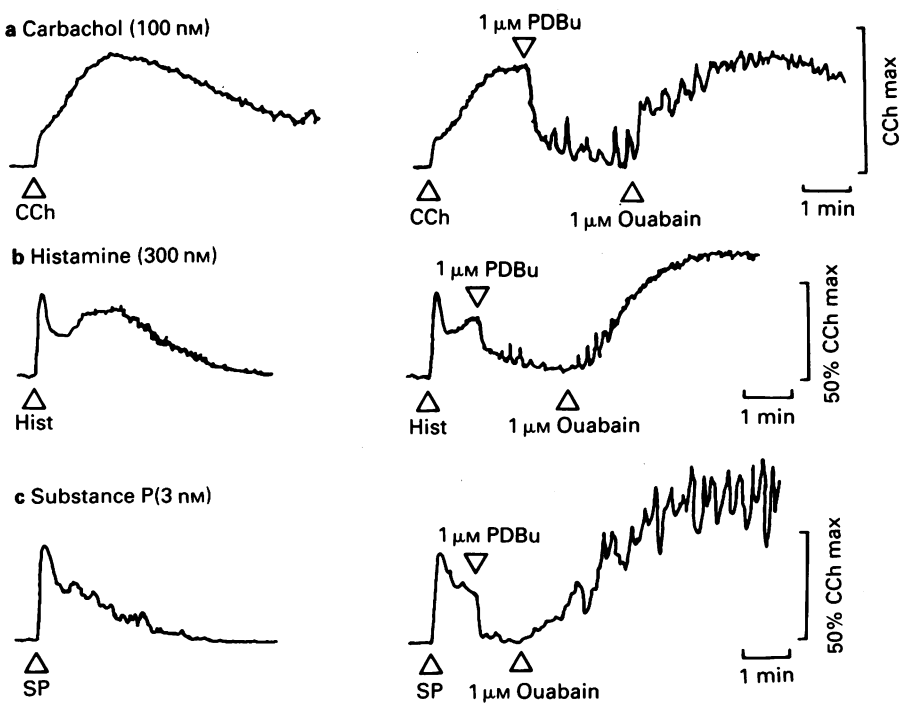

Figure 2 Effect of ouabain on phorbol 12,13-dibutyrate (PDBu)-induced relaxation. Right-hand contractions were recorded 30 min after the control (left). PDBu (1 μM) was applied when the contraction reached a plateau and ouabain (1 μM) was added after maximal relaxation was achieved. Substance (SP)-induced contractions were recorded in the presence of 3 μM atropine. Traces are representative of four independent experiments. CCh = carbachol; Hist = histamine.

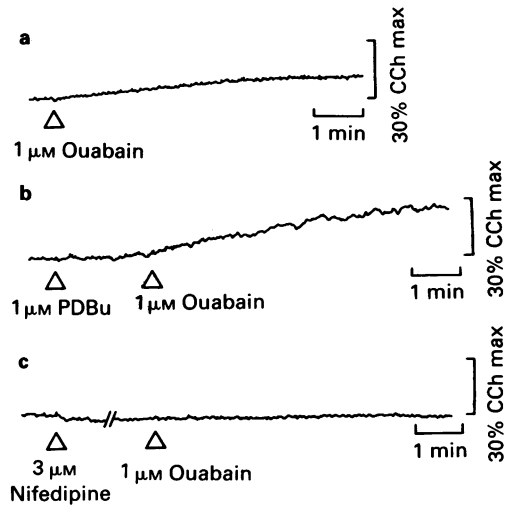

Figure 3 Ouabain-induced contractions. (a) Contraction induced by ouabain (1 μM) on its own. (b) Effect of phorbol 12,13-dibutyrate (PDBu, 1 μM) added 2 min before ouabain. (c) Effect of nifedipine (3 μM) added 5 min before ouabain. These are representative traces from three separate experiments. CCh = carbachol.

P was purchased from Peninsula Laboratories. Rubidium chloride ($^{86}Rb^+$; 0.171 Ci mmol^{-1}) was from Amersham International.

Statistics

The results are shown as the mean ± s.e.mean of the number of observations (n). The statistical significance was assessed by use of Student's t test for paired or unpaired values. P values less than 0.05, were considered to be significant.

Results

Phorbol esters inhibit the tonic component of contractions induced by carbachol, histamine and substance P

We have shown that PDBu quickly inhibits the tonic component of contractions induced by submaximal concentrations of carbachol (30–300 nM), although oscillatory contractions are enhanced (Sasaguri & Watson, 1989a). In the present study we observed similar effects with two further phorbol esters, PDA and PMA. All three compounds relaxed tissues precontracted by 100 nM carbachol in a concentration-dependent manner and exhibited parallel concentration-response curves (Figure 1). The EC_{50} values for PDBu, PDA and PMA were 0.086 μM, 0.53 μM and 3.5 μM, respectively. 4-α-Phorbol, which is unable to activate PKC, produced little relaxation (Figure 1).

The three phorbol esters also induced a similar relaxation of the tonic component of contraction induced by submaximal concentrations of histamine and substance P. Example traces of the effect of PDBu on contractions induced by these agonists in comparison with carbachol are shown in Figure 2.

Ouabain reverses inhibition by phorbol esters

Contractions induced by carbachol, histamine and substance P consisted of a rapid phasic component followed by a slower tonic component which could be inhibited by PDBu (see Figure 2 for example). The addition of ouabain (1 μM) after PDBu caused a rapid reversal of this inhibition and tension quickly rose to a level exceeding that in the control tissues (Figure 2). Higher concentrations of ouabain (3–10 μM) produced a more rapid reversal of PDBu inhibition (not shown).

Ouabain (1 μM) produced a small, slowly-developing contraction which levelled after 5–7 min at 13 ± 3% (n = 10) of the maximal tension generated by carbachol (Figure 3a).

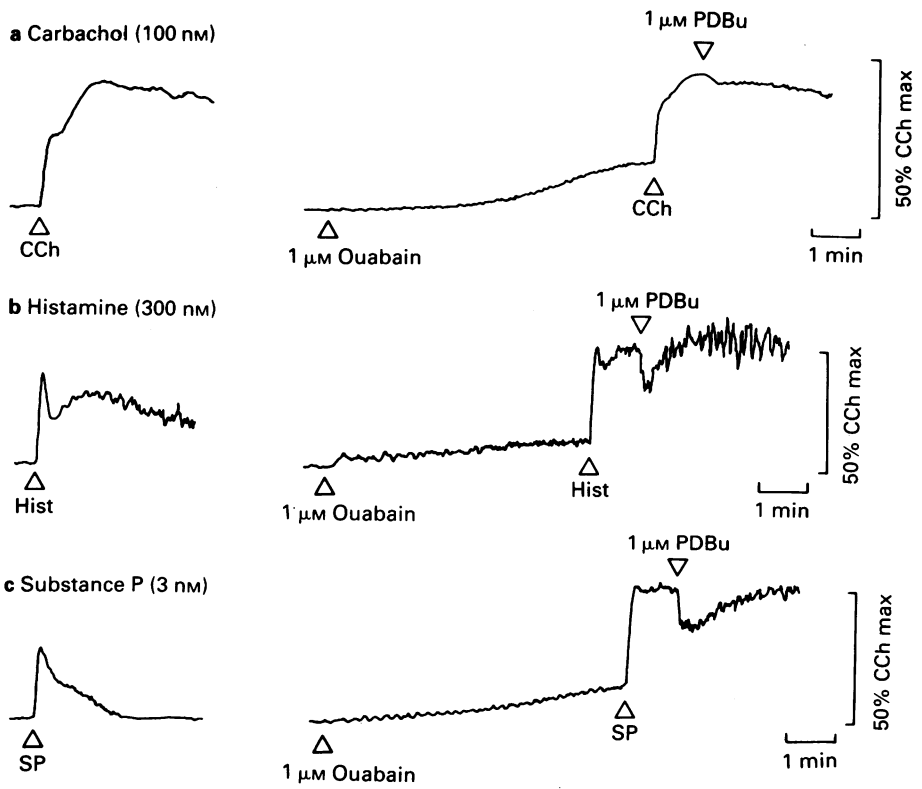

Figure 4 Effect of pretreatment with ouabain on the relaxation induced by phorbol 12,13-dibutyrate (PDBu). Right-hand traces were recorded 30 min after the control (left). Agonist was applied after ouabain-induced contraction reached a plateau and PDBu (1 μM) was added during the tonic component of contractions. Atropine (3 μM) was present during experiment. Traces are representative from three separate experiments. CCh = carbachol and SP = substance P.

Pretreatment of the tissue with PDBu (1 μM) for 2 min caused a small potentiation of the ouabain-induced contraction to 21 ± 4% ($n = 3$, $P < 0.05$) (Figure 3b). However, the size of the contraction generated by ouabain in the presence of PDBu was considerably less than the reversal of the PDBu-induced inhibition of agonist-induced contractions by ouabain, which, in some cases, amounted to almost 75% of the maximal contractile force (e.g. see Figure 2). Contraction to ouabain was inhibited completely in the presence of 3 μM nifedipine (Figure 3c), indicating that it involves the entry of Ca^{2+} through voltage-operated channels consistent with the observation that ouabain induces depolarization in this tissue (Bolton, 1973a).

To look at the effect of pretreatment with ouabain on the phorbol ester-induced relaxation, tissues were incubated with ouabain (1 μM) until the contractions reached a plateau (5 to 7 min) before application of the agonist. Under these conditions, the tonic component of the contractile response to these agonists was sustained and PDBu (1 μM) induced only a small and transient relaxation of the tonic component of contractions (Figure 4). Similarly, if ouabain was given during the tonic component of contraction induced by submaximal concentrations of these agonists (Figure 5) and also maximal concentrations of substance P (up to 1 μM; not shown), the relaxation of the tonic component was inhibited. However, ouabain had little effect on the decay of the tonic component induced by maximally effective concentrations of carbachol (1 μM) and histamine (3 μM). Essentially the same results to those described above were obtained in the presence of tetrodotoxin (300 nM; not shown).

PDBu stimulates the Na^+-K^+-pump

The above data suggest the involvement of the Na^+-K^+-ATPase pump in the inhibition of the tonic component of contractions by phorbol esters. As a test of this, we investigated the action of PDBu on Na^+-K^+-ATPase activity by analysing ouabain-sensitive $^{86}Rb^+$ uptake and the cellular content of Na^+ and K^+ (it should be borne in mind though that these changes may occur partially within neuronal tissue in the preparation). $^{86}Rb^+$ behaves as an isotope of K^+ and so its movement across the membrane is believed to reflect that of K^+.

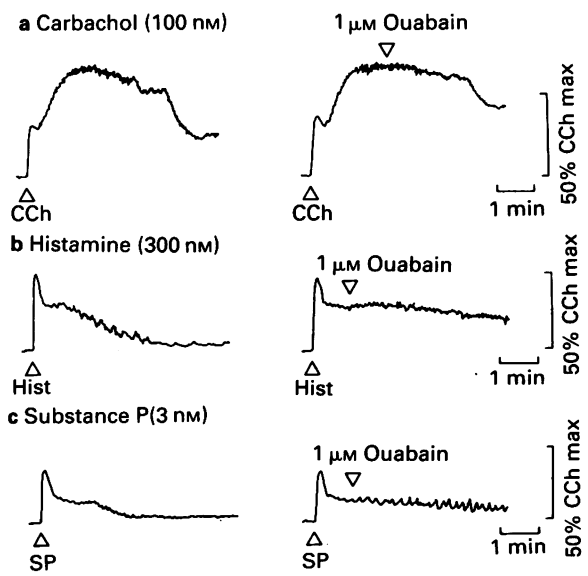

Figure 5 Effect of ouabain on the tonic component of contraction. Ouabain was added before decay of the tonic component began, 2 min after carbachol (CCh) or 1 min after histamine (Hist) and substance P (SP). In (c), atropine (3 μM) was added 5 min before substance P. These are representative traces from three independent experiments.

Table 1 Effect of phorbol 12,13-dibutyrate (PDBu) on the cellular content of Na^+ and K^+

	Na^+ (nmol mg^{-1} wet weight)	K^+ (nmol mg^{-1} wet weight)
Control	70.0 ± 1.8	90.1 ± 4.5
K^+-free	139.9 ± 1.6	1.0 ± 0.1
1 mM K^+	137.4 ± 1.3	4.3 ± 0.3**
1 mM K^+ + 3 μM PDBu	129.4 ± 2.5*	5.0 ± 0.3**

Longitudinal muscle strips were incubated in normal HEPES-buffered solution (control) or K^+-free solution bubbled with 100% O_2 for 3 h at 37°C. Some of the strips treated with K^+-free solution were further incubated for 4 min in the medium containing 1 mM K^+ in the presence or absence of 3 μM PDBu. After being blotted and weighed, the tissues were placed in tubes containing 'diluting fluid' (see Methods). After 24 h, concentrations of Na^+ and K^+ in the solution were measured with an atomic absorption spectrophotometer. Data are expressed as mean ± s.e.mean of 6–12 observations from two or three independent experiments. * $P < 0.02$, ** $P < 0.01$ (vs K^+-free; unpaired t test).

However, preliminary experiments revealed that more than 90% of the basal $^{86}Rb^+$ uptake was insensitive to ouabain. In order to increase the size of the ouabain-sensitive component, therefore, the intracellular Na^+ concentration was elevated by pre-incubation of the tissue in K^+-free solution for 3 h. Confirmation of this increase in cellular Na^+ content is shown in Table 1. The extracellular K^+ concentration during the $^{86}Rb^+$-uptake was maintained at 1 mM to avoid maximal pump activation (Brading & Widdicombe, 1974).

Under these conditions, the ouabain-sensitive portion of the uptake of $^{86}Rb^+$ was between 70–80% of total uptake. A maximally effective concentration of PDBu (3 μM) increased the pump activity by 24 ± 4% above basal (Figure 6). The EC_{50} for this action of PDBu was 225 nM, which is of the same order as the value for inhibition of the tonic component of contraction (86 nM). In contrast, 4-α-phorbol did not stimulate $^{86}Rb^+$-uptake (Figure 6). Carbachol (100 μM) also increased the pump activity by 16 ± 4% above basal, although lower concentrations had no significant effect.

As shown in Table 1, pre-incubation of tissues for 3 h in K^+-free medium almost totally depleted the tissues of K^+, and loaded them with Na^+. A subsequent 4 min incubation in the presence of 1 mM K^+ resulted in a significant cellular

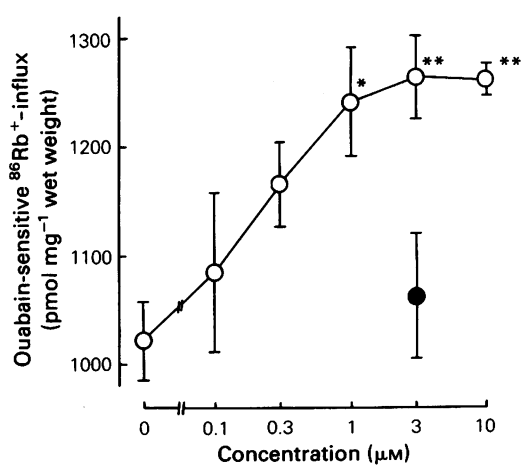

Figure 6 Effect of phorbol 12,13-dibutyrate (PDBu) on ouabain-sensitive $^{86}Rb^+$-uptake. After being loaded with Na^+ by exposure to K^+-free soltuion for 3 h, the tissues were incubated with $^{86}Rb^+$ and the indicated concentration of PDBu (○) or 4-α-phorbol (●) in HEPES-buffered solution containing 1 mM K^+ for 4 min with and without 0.1 mM ouabain. Vertical bars show s.e.mean of 6–12 observations from two or three independent experiments. * $P < 0.05$, ** $P < 0.01$.

uptake of K^+, but the reduction in Na^+ content was not significant. When PDBu was given during incubation with 1 mM K^+, the uptake of K^+ was slightly enhanced, and there was a larger, and significant decrease in cellular Na^+. These results are consistent with the suggestion that there was a greater stimulation of Na^+-K^+-ATPase in the presence of PDBu.

Discussion

Phorbol esters inhibit the tonic component of agonist-induced contractions

The present study provides several lines of evidence to suggest that the activation of PKC leads to an inhibition of the tonic component of agonist-induced contractions of the longitudinal smooth muscle of guinea-pig ileum through stimulation of Na^+-K^+-ATPase.

A number of phorbol esters inhibit the tonic component of contraction induced by a range of agonists. In contrast, 4-α-phorbol, which is unable to activate PKC, has little effect on contraction. The ability of the active phorbol esters to relax precontracted muscle seems to be more closely related to their hydrophilicity than to their potency against PKC. PMA, the most lipophilic of the esters, is the most potent activator of PKC (Nishizuka, 1984) yet is the weakest in inducing relaxation. It seems likely, therefore, that the inhibitory effect of the phorbol esters in influenced by their ability to diffuse through the smooth muscle cell layers, a process which will favour the more hydrophilic agents. A relaxing effect of phorbol esters on the resting tension or on agonist-induced contractions has also been observed in other smooth muscles, including the guinea-pig trachea and rat uterus (Baraban *et al.*, 1985; Menkes *et al.*, 1986; Jackson *et al.*, 1988).

The ability of the phorbol esters to suppress the tonic component of contraction is inhibited by the presence, or reversed by the addition, of the Na^+-K^+-ATPase inhibitor ouabain. Since ouabain induces a weak contraction on its own, mediated through depolarization which leads to the opening of voltage-operated Ca^{2+} channels, it is possible that this effect could account for the reversal of the phorbol ester-induced relaxation. However, this seems unlikely, since the onset of contraction by ouabain is very slow (several minutes) and also small. In contrast, the ability of ouabain to reverse the relaxation of the tonic component induced by phorbol esters is both rapid (significant recovery occurs with 60 s) and powerful, with the final contractile force being greater than in controls (see next section). The possibility that the release of neurotransmitters by ouabain accounts for its ability to reverse the inhibitory action of phorbol esters also appears unlikely. The major transmitter innervating the longitudinal muscle is acetylcholine, but ouabain causes a similar reversal of the responses to substance P, histamine and carbachol despite the fact that the experiments with substance P were carried out in the presence of atropine. Further, similar results were also observed in the presence of tetrodotoxin. Jackson *et al.* (1988) suggested that phorbol esters may relax guinea-pig airways through an increase in cyclic AMP, but this mechanism offers no explanation as to why contraction recovers in the presence of ouabain. However, it is possible that relaxation results from a stimulation of the Na^+-Ca^{2+} exchanger by protein kinase C and that this is reversed by ouabain through an alteration of the Na^+ gradient across the membrane. Nevertheless this does not account for the ability of phorbol esters to increase ouabain-sensitive K^+ uptake into the tissue under conditions of high Na^+ loading.

As discussed in the Introduction, there are a number of studies which have described the activation of Na^+-K^+-ATPase by protein kinase C (Greene & Lattimer, 1986; Nishizuka, 1986; Yingst, 1988). Stimulation of this pump by protein kinase C in guinea-pig ileum would lead to a recovery of membrane potential and relaxation of agonist-induced contractions. Indeed, direct measurements of Na^+-K^+-ATPase activity support this suggestion. The phorbol ester, PDBu, was demonstrated to stimulate the pump over a similar concentration range to that at which it inhibits the tonic component of contraction. Moreover, the observation that PDBu stimulated extrusion of Na^+ confirms this observation. Therefore, taken together, the results obtained provide preliminary evidence that the activation of protein kinase C by phorbol esters plays a role in inhibiting the tonic component of agonist-induced contraction through the stimulation of Na^+-K^+-ATPase. Other actions of protein kinase C (as discussed above may also contribute to this relaxation.

Phorbol esters also enhance contraction

Phorbol esters enhance both the phasic and tonic components of contractions induced by K^+, but only potentiate the phasic component of contractions induced by carbachol and inhibit the tonic component (Sasaguri & Watson, 1989a). When ouabain was added in the presence of phorbol ester (Figure 2, right), the tonic component of the agonist induced contractions exceeded that in control tissues (Figure 2, left) or the tonic component produced in the presence of ouabain (Figure 5, right). This suggests that it is the phorbol ester and not ouabain which is potentiating the tonic component of contraction under these conditions. Thus it appears that, as with K^+, phorbol esters stimulate a pathway which is able to enhance both the phasic and tonic components of agonist-induced contraction, but that the stimulation of Na^+-K^+-ATPase masks this potentiation. The inability of phorbol esters to inhibit the tonic component of contractions induced by high-K^+ may have been due to the large concentrations of K^+ rendering the Na^+-K^+-pump less able to hyperpolarize the cells. The mechanism of enhancement of contraction by phorbol esters is not known.

Physiological significance?

The normal time course of contraction to the above agonists includes a slowly decaying tonic phase. One explanation for this decline therefore is the stimulation of Na^+-K^+-ATPase through the activation of protein kinase C. The observation that ouabain prevents the decay of the tonic component of contraction to submaximal concentrations of these agonists supports this possibility. Under normal conditions an increase in intracellular Na^+, resulting from Na^+-influx following muscarinic receptor stimulation, has been suggested to contribute to activation of Na^+-K^+-ATPase (Bolton, 1973b). However, carbachol stimulated Na^+-K^+-ATPase in Na^+-loaded tissues consistent with the suggestion that carbachol also stimulates pump activity through the activation of PKC.

In contrast with its effects on the tonic component of contraction to submaximal concentrations of the agonists, ouabain had little effect on the decline of the tonic component induced by maximally effective concentrations of carbachol and histamine. It appears therefore that other mechanisms, e.g. receptor desensitization, are responsible for this decay.

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