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Contractile and relaxant effects of phorbol ester in the intestinal smooth muscle of guinea-pig taenia caeci

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1 Effects of phorbol esters on the cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) and muscle tension in the intestinal smooth muscle of guinea-pig taenia caeci were examined.

2 12-Deoxyphorbol 13-isobutyrate (DPB, 1 μM) did not change the $[Ca^{2+}]_i$ and tension in resting muscle.

3 In high K^+-stimulated muscle, 1 μM DPB transiently augmented the contraction and decreased $[Ca^{2+}]_i$. 12-Deoxyphorbol 13-isobutyrate 20-acetate (1 μM) and phorbol 12, 13-dibutyrate (1 μM) showed similar effects to DPB whereas phorbol 12-myristate 13-acetate (1 μM) and phorbol 12, 13-didecanoate (1 μM) were ineffective.

4 DPB (1 μM) inhibited both $[Ca^{2+}]_i$ and tension stimulated by 300 nM carbachol or 3 μM histamine. In the presence of a higher concentration of carbachol (1 μM), DPB decreased $[Ca^{2+}]_i$ and transiently increased muscle tension.

5 In the muscle strips permeabilized with bacterial α-toxin, 1 μM DPB shifted the Ca^{2+}-tension curve to the left. An inhibitor of protein kinase C, H-7 (30 μM), inhibited the effect of DPB.

6 DPB did not change the high K^+-induced contraction in the muscle strips pretreated with 3 μM phorbol 12-myristate 13-acetate for 24 h.

7 These results suggest that activation of protein kinase C has dual effects; it augments contraction by increasing the Ca^{2+} sensitivity of the contractile elements and it inhibits contraction by decreasing $[Ca^{2+}]_i$.

Keywords: Smooth muscle (intestinal); phorbol ester; cytosolic Ca^{2+} levels; protein kinase C; permeabilized smooth muscle

Introduction

Since Nishizuka (1984) reported the biological effect of tumour promoting phorbol esters, these esters have been widely used to examine the role of protein kinase C in various biological systems. In the intestinal smooth muscle, conflicting results have been reported on the effects of phorbol esters on contractility. Phorbol esters potentiated the contractions induced by high K^+ (Menkes *et al.*, 1986; Sasaguri & Watson, 1989; Xu *et al.*, 1991) and histamine (Holzer & Lippe, 1989) possibly by activating Ca^{2+} channel (Menkes *et al.*, 1986; Xu *et al.*, 1991). In contrast, phorbol esters inhibited the contractions induced by histamine (Baraban *et al.*, 1985; Holzer & Lippe, 1989), carbachol (Menkes *et al.*, 1986; Sasaguri & Watson, 1989) and substance P (Holzer & Lippe, 1989) possibly by the activation of Na^+-K^+-ATPase (Sasaguri & Watson, 1990) or a negative feedback mechanism (Baraban *et al.*, 1985; Menkes *et al.*, 1986).

In order to understand the role of protein kinase C in the intestinal smooth muscle, we examined the effects of phorbol esters on muscle tension and cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) in the guinea-pig taenia caeci.

Methods

Measurement of contractions in isolated muscle strips

Male guinea-pigs, weighing 250–300 g, were killed by a blow on the neck and bled. A section of taenia (5–10 mm in length) was dissected from the caecum. The normal physiological salt solution contained (mM): NaCl 136.9, KCl 5.4, glucose 5.5, $NaHCO_3$ 23.8, $CaCl_2$ 1.5, $MgCl_2$ 1.0 and EDTA 0.01. High K^+ (45 mM) solution was made by adding KCl to the PSS. In some experiments, all the NaCl was substituted with KCl, and pyruvate (5 mM) and indomethacin (10 μM) were added because Na^+ deficiency may interfere with glucose uptake (Karaki *et al.*, 1982) and release endogenous prostaglandins (Satoh & Karaki, 1988). These solutions were aerated with a 95% O_2 and 5% CO_2 mixture at 37°C (pH 7.4). Muscle tension was recorded isometrically with a force-displacement transducer. Passive tension of 2 mN was initially applied and tissues were allowed to equilibrate for 60–90 min until the contractile response to 45 mM K^+ solution became stable.

Measurement of cytosolic Ca^{2+} concentration

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured simultaneously with muscle contraction as described previously (Ozaki *et al.*, 1987; Sato *et al.*, 1988; Mitsui & Karaki, 1990). Muscle strips were treated with 10 μM acetoxymethyl ester of fura-2 (fura-2/AM) for approximately 12 h at 4°C. The non-cytotoxic detergent, cremophor EL (0.02%), was added to increase the solubility of fura-2/AM. The muscle strip was illuminated alternately (48 Hz) with two excitation wave lengths (340 nm and 380 nm), and the intensity of the 500 nm fluorescence (F340 and F380) was measured with a fluorimeter (CAF-100, JASCO, Tokyo, Japan). High K^+ (45 mM) solution for this experiment was made by substituting appropriate concentration of NaCl with equimolar KCl in PSS, because hypertonicity interferes with the fura-2-Ca^{2+} fluorescence (Himpens & Somlyo, 1988; Williams & Fay, 1990). Absolute Ca^{2+} concentration was not calculated because the dissociation constant of fura-2 for Ca^{2+} may be different from that obtained *in vitro* (Karaki, 1989). In the present study, the ratio of F340 to F380 (R340/380) was used as an indicator of $[Ca^{2+}]_i$, and the quantitative comparison of

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the $[Ca^{2+}]_i$ was made by taking the resting and high K^+ stimulated $[Ca^{2+}]_i$ as 0% and 100%, respectively (Ozaki *et al.*, 1987; Sato *et al.*, 1988). It was not possible to measure $[Ca^{2+}]_i$ for a period longer than 90 min, because of the leakage of fura-2 from the cell.

Measurement of contractions in permeabilized smooth muscle

Permeabilized muscle was made by treating the isolated tissue with *Staphylococcus aureus* α-toxin as described by Nishimura *et al.* (1988) and Kitazawa *et al.* (1989). A thin bundle of muscle strip (0.1 mm wide and 2 mm long) was treated for 30 min with 100 μg ml^{-1} α-toxin in a relaxing solution containing (mM): K^+-propionate 130.0, $MgCl_2$ 4.0, Na_2ATP 5.0, Tris-maleate 20.0, creatine phosphate 2.0, creatine phosphokinase 10 u ml^{-1} and EGTA 2.0 (pH 6.8). Ca^{2+} concentrations were changed by adding an appropriate amount of $CaCl_2$. The apparent binding constant of EGTA for Ca^{2+} was considered to be 1×10^6 M^{-1} at pH 6.8 (Harafuji & Ogawa, 1980). The contractile tension of the permeabilized muscle was recorded isometrically.

Down-regulation of protein kinase C

Long-term exposure to phorbol esters has been shown to reduce the protein kinase C activity and also the amount of protein kinase C molecules in the cell (Jaken *et al.*, 1981; Collins *et al.*, 1982). In order to down-regulate the protein kinase C activity, taenia strips were incubated in Dulbecco's modified Eagle medium with 10% foetal calf serum for 24 h at 37°C in a CO_2 incubator in the presence of phorbol ester (3 μM phorbol 12-myristate 13-acetate or 3 μM DPB).

Chemicals

Chemicals used were 12-deoxyphorbol 13-isobutyrate (DPB), 12-deoxyphorbol 13-isobutyrate 20-acetate, phorbol 12, 13-dibutyrate, phorbol 12, 13-didecanoate, phorbol 12-myristate 13-acetate (Funakoshi, Tokyo, Japan), carbachol, indomethacin (Sigma Chemicals, St. Louis, MO, U.S.A.), histamine (Wako Pure Chemicals, Tokyo, Japan), clemophor EL (Nacalai Tesque, Kyoto, Japan), EGTA, fura-2/AM (Dojindo Laboratories, Kumamoto, Japan), H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperidine, Seikagaku, Corp., Tokyo, Japan). *Staphylococcus aureus* α-toxin was donated by Dr Iwao Kato.

Statistics

Results of the experiments are expressed as the mean ± s.e.mean. Student's *t* test was used for statistical analysis of the results and $P<0.05$ was considered to indicate a significant difference.

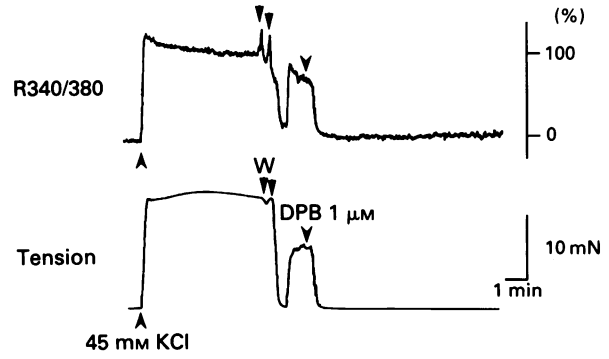

Figure 1 Effect of 45 mM KCl and 1 μM 12-deoxyphorbol 13-isobutyrate (DPB) on $[Ca^{2+}]_i$ and muscle tension in fura-2 loaded taenia caeci. Traced from a typical result of 4 experiments.

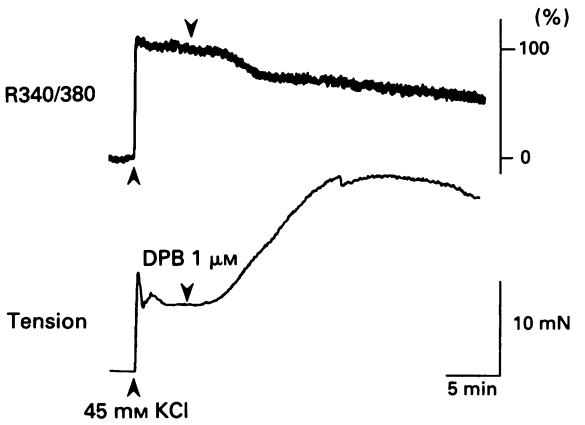

Figure 2 Effect of 1 μM 12-deoxyphorbol 13-isobutyrate (DPB) on $[Ca^{2+}]_i$ and muscle tension in the presence of 45 mM KCl. Traced from a typical result of 4 experiments.

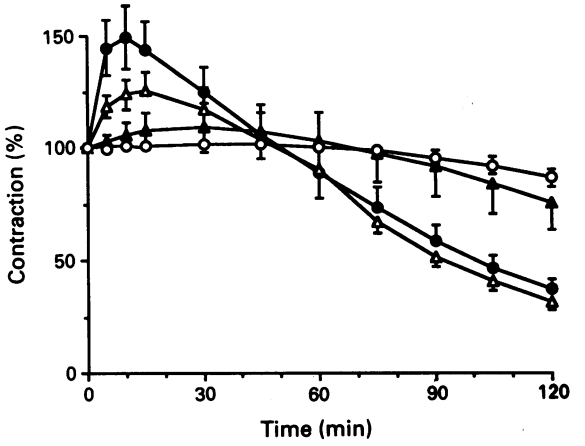

Figure 3 Time course of the effects of the various concentrations of 12-deoxyphorbol 13-isobutyrate (DPB) on 45 mM KCl-induced contractions. A single dose of DPB (10 nM, ▲; 100 nM, △; 1 μM, ●) was added after the contraction reached a steady level. (○) In the absence of DPB. Each point represents mean ± s.e.mean of 4 experiments.

Results

Effects of phorbol esters on $[Ca^{2+}]_i$ and muscle tension

As shown in Figure 1, addition of high K^+ induced sustained increase of $[Ca^{2+}]_i$ and muscle tension in the taenia. After removal of high K^+, spontaneous rhythmic changes of $[Ca^{2+}]_i$ and muscle tension were observed. In the presence of 1 μM DPB, both muscle tension and $[Ca^{2+}]_i$ stayed at their respective resting levels.

The effects of DPB on high K^+-induced increase of $[Ca^{2+}]_i$ and contraction are shown in Figures 2 and 3. DPB (1 μM) decreased high K^+-stimulated $[Ca^{2+}]_i$, although it gradually increased muscle tension reaching the maximum within 10–15 min followed by a decrease. Because of the leakage of fura-2 from the cells, it was possible to examine the effects of DPB for only 30 min. 12-Deoxyphorbol 13-isobutyrate 20-acetate (1 μM) and 1 μM phorbol 12, 13-dibutyrate showed similar effects to DPB, although 1 μM phorbol 12-myristate 13-acetate and 1 μM phorbol 12, 13-didecanoate were ineffective ($n = 4$ each).

Figure 3 shows the concentration-response relationships for the effects of DPB. DPB at a concentration of 100 nM and 1 μM induced augmentation followed by inhibition of the

high K^+-induced contraction whereas 10 nM DPB was ineffective. DPB, 1 μM, also transiently augmented and then inhibited the contraction induced by Na^+-free, 166.1 mM K^+-solution to 16.5 ± 6.7% at 120 min ($n = 4$).

We also examined the effects of DPB on the contraction and $[Ca^{2+}]_i$ stimulated by carbachol and histamine. DPB (1 μM) rapidly inhibited $[Ca^{2+}]_i$ whereas it augmented the contraction for only a short period of time in the muscle stimulated by 300 nM carbachol (Figure 4a) or 3 μM histamine (Figure 4b). In the presence of a higher concentration of carbachol (1 μM), 1 μM DPB gradually decreased $[Ca^{2+}]_i$ and transiently increased and then decreased muscle tension (Figure 4c).

Effect of 12-deoxyphorbol 13-isobutyrate on permeabilized muscles

As shown in Figure 5a, 1 μM Ca^{2+} induced transient increase followed by a sustained increase of muscle tension in permeablilized taenia. DPB (1 μM) potentiated the Ca^{2+}-induced contraction to 391.0 ± 76.7% ($n = 4$). An inhibitor of protein kinase C, 30 μM H-7 (Hidaka *et al.*, 1984), inhibited the contraction to 173.5 ± 31.2% ($n = 3$, $P < 0.01$). In contrast, 30 μM H-7 did not inhibit the contraction induced by 1 μM Ca^{2+} in the absence of DPB ($n = 3$, Figure 5b). Figure 5c shows that DPB (1 μM) shifted the Ca^{2+}-tension relationships to the left in permeabilized muscle strips.

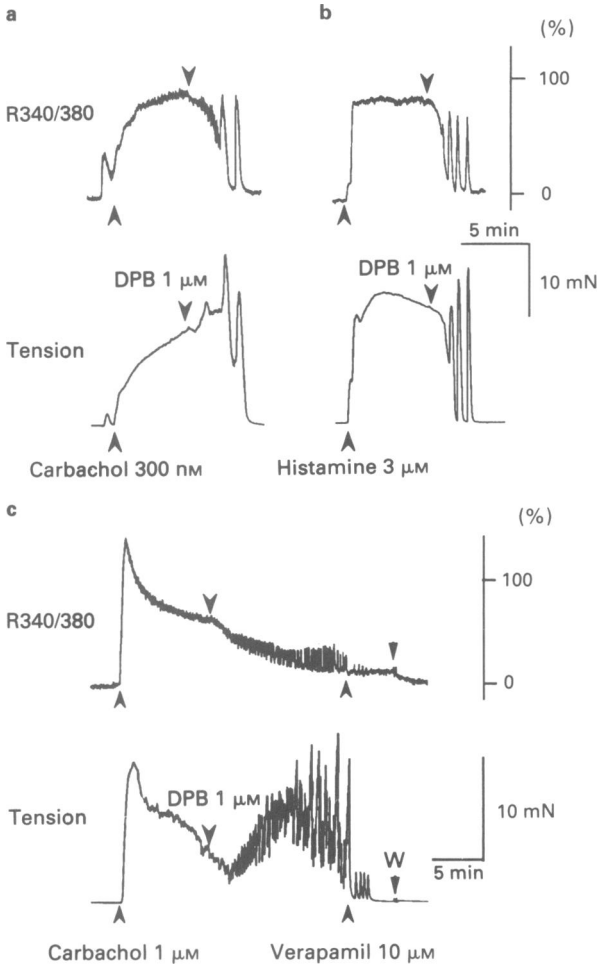

Figure 4 Effects of 1 μM 12-deoxyphorbol 13-isobutyrate (DPB) on $[Ca^{2+}]_i$ and muscle tension in the presence of carbachol (a, 300 nM; c, 1 μM) and histamine (b, 3 μM). Traced from typical results of 4 experiments.

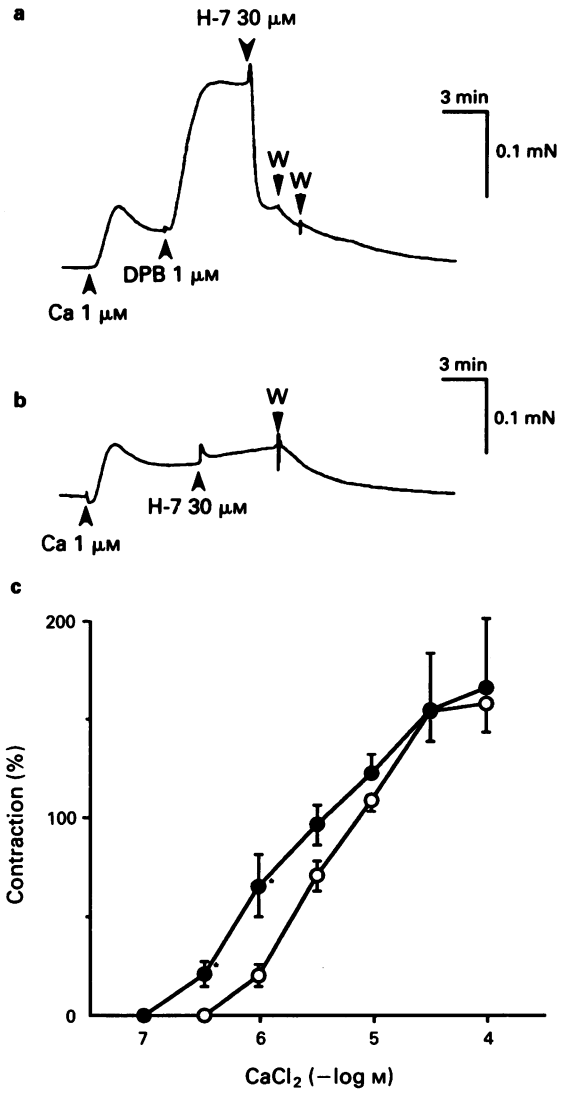

Figure 5 Effects of 1 μM 12-deoxyphorbol 13-isobutyrate (DPB) (a) and 30 μM H-7 (b) on the Ca^{2+}-induced contraction in α-toxin permeabilized taenia strips. Traced from typical results of 3–4 experiments. (c) Ca^{2+}-tension relationships in the absence (○) or presence of 1 μM DPB (●). Ca^{2+} was added cumulatively in the absence or presence of 1 μM DPB. Each point represents mean ± s.e.mean of 6–11 experiments; 100% represents the magnitude of contraction induced by a single application of 10 μM Ca^{2+} performed before the experimental procedure.

Effects of pretreatment with phorbol esters

In muscle strips incubated for 24 h at 37°C, 1 μM DPB increased the high K^+-induced contraction to 186.9 ± 12.0% ($n = 6$, Figure 6a). In muscle pretreated with 3 μM phorbol 12-myristate 13-acetate for 24 h at 37°C, 1 μM DPB did not augment the high K^+-induced contraction ($n = 9$, Figure 6b). Pretreatment with 3 μM DPB for 24 h at 37°C partially inhibited the ability of 1 μM DPB to augment the high K^+-induced contraction (120.7 ± 3.0%, $n = 8$, $P < 0.01$ vs the value without phorbol ester-pretreatment, Figure 6c).

Discussion

Phorbol ester-induced augmentation of contraction

In the present experiments, we found that the effects of DPB on muscle tension and $[Ca^{2+}]_i$ are different. DPB transiently augmented the contractions induced by high K^+ or a high

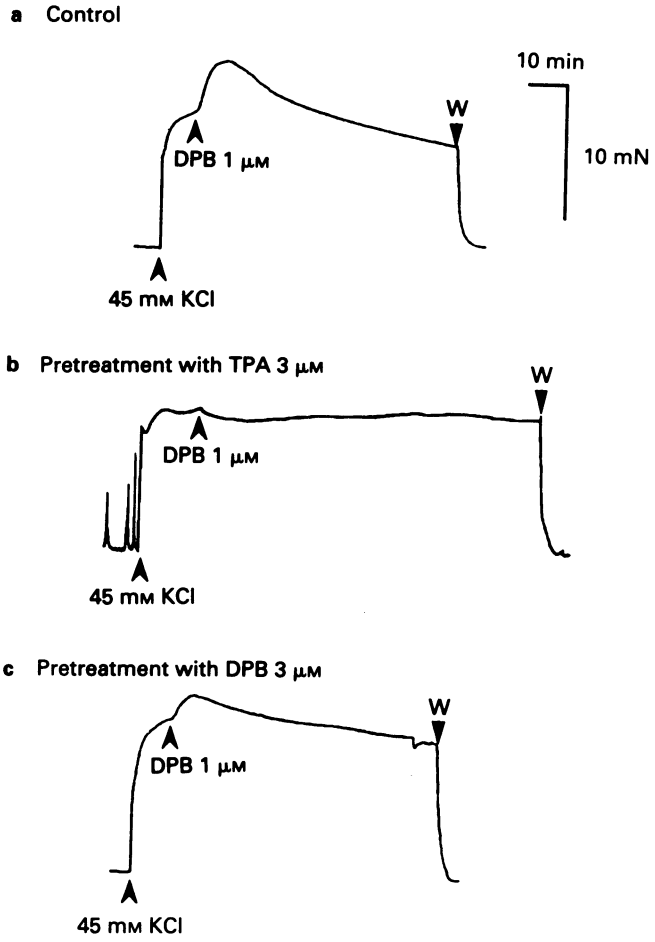

Figure 6 Effect of 12-deoxyphorbol 13-isobutyrate (DPB) on 45 mM KCl-induced contraction in the taenia strip without (a, control) or with preincubation with phorbol ester (b, phorbol 12-myristate 13-acetate (TPA) 3 μM and c, DPB 3 μM) for 24 h. DPB 1 μM was added when the muscle tension reached a steady level. Traced from typical results of 6–9 experiments.

concentration of carbachol (1 μM) whereas it decreased $[Ca^{2+}]_i$ (Figures 2 and 4c). The augmentation of contraction may be explained by an increase in Ca^{2+} sensitivity, as it has been shown that phorbol esters increase Ca^{2+} sensitivity of the contractile elements in vascular and intestinal smooth muscles (Itoh *et al.*, 1988; Nishimura & Van Breemen, 1989; Sato *et al.*, 1992; Ozaki *et al.*, 1993). In order to confirm this possibility, we examined the effect of DPB on the Ca^{2+}-induced contraction in permeabilized muscle strips. It was found that 1 μM DPB shifted the Ca^{2+}-tension relationship to the left (Figure 6c), supporting the suggestion that DPB augmented contraction by increasing the Ca^{2+} sensitivity of the contractile elements. In the resting muscle, DPB did not induce contraction (Figure 1) possibly because resting $[Ca^{2+}]_i$ was lower than threshold level even when the Ca^{2+} sensitivity was increased. Although we did not examine the mechanism of Ca^{2+} sensitization, it has been shown that DPB increases Ca^{2+} sensitivity of myosin light chain phosphorylation in vascular smooth muscle (Sato *et al.*, 1992; Hori *et al.*, 1992).

DPB induced similar but much faster changes in $[Ca^{2+}]_i$ and muscle tension in the taenia stimulated by a lower concentration of carbachol (300 mM) or histamine (3 μM) (Figure 5a,b). This result may be due to the ability of DPB to decrease $[Ca^{2+}]_i$ more rapidly under these conditions than in the presence of higher concentrations of stimulants.

Inhibitory effect of phorbol esters on $[Ca^{2+}]_i$

On the mechanism of DPB-induced decrease in $[Ca^{2+}]_i$, it has been reported that phorbol ester activates Na^+-K^+-ATPase in tracheal (Schramm & Grunstein, 1989; Souhrada & Souhrada, 1989) and intestinal smooth muscles (Sasaguri & Watson, 1990) which results in a membrane hyperpolarization and inhibition of the voltage-dependent Ca^{2+} channels. The second possibility is an inhibition of Ca^{2+} channels mediated more directly by the activation of protein kinase C (Shearman *et al.*, 1989). We have reported that a Ca^{2+} channel blocker, verapamil, inhibited the contraction induced by a higher concentration of carbachol more strongly than that induced by a lower concentration in the guinea-pig taenia caeci (Karaki & Mitsui, 1988). In the present experiments, we found that DPB inhibited the contractions induced by lower concentrations of carbachol and histamine more strongly than those induced by higher concentrations. Since the lower concentrations of agonists are expected to induce smaller membrane depolarization than those induced by higher concentrations, membrane hyperpolarization resulting from activation of Na^+-K^+-ATPase may be responsible for the inhibitory effect of phorbol ester on the contractions induced by carbachol and histamine. However, we also found that 1 μM DPB inhibited the contraction induced by Na^+-free, 166.1 mM K^+-solution in which stimulation of Na^+-K^+ATPase activity would not be possible.

Detailed analysis is necessary to determine whether there is an inhibitory effect of phorbol ester on Ca^{2+} channels.

Role of protein kinase C

It has been reported that an inhibitor of protein kinase C, H-7, non-selectively inhibits smooth muscle contraction in intact vascular smooth muscle (Ratz *et al.*, 1990). In a preliminary experiment, we confirmed this in the taenia. In the permeabilized taenia caeci, however, H-7 inhibited the DPB-induced augmentation of the Ca^{2+}-induced contraction without changing the contraction in the absence of DPB (Figure 5). Furthermore, pretreatment of the muscle with phorbol 12-myristate 13-acetate or DPB, which may cause down-regulation of protein kinase C, inhibited the effects of DPB (Figure 6b,c). These results suggest that effects of DPB are due to activation of protein kinase C.

Spedding (1987) showed that phorbol 12-myristate 13-acetate had no effects on the Ca^{2+}-induced contraction in the high K^+depolarized taenia caeci. In the present experiments, we also found that phorbol 12-myristate 13-acetate had no effect on the high K^+-induced contraction. However, pretreatment of the muscle with phorbol 12-myristate 13-acetate seemed to down-regulate the protein kinase C activity more strongly than DPB (Figure 6b,c). Mattingly *et al.* (1987) also reported that down-regulation of protein kinase C by phorbol 12-myristate 13-acetate was stronger than phorbol 12,13-dibutyrate. The reason for this difference has not been examined in the present experiments.

In conclusion, it is suggested that phorbol esters decrease $[Ca^{2+}]_i$ and increase Ca^{2+} sensitivity of the contractile element (shifting the Ca^{2+}-tension relationship to the left) in the taenia.

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