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Protein kinase C induced changes in human airway smooth muscle tone: the effects of Ca^{2+} and Na^{+} transport

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Abstract

Activation of protein kinase C by phorbol 12,13-dibutyrate (PDB) (1 nM–3 μM) caused a concentration-dependent contractile response in human isolated bronchus. The mean maximal contraction was 26 ± 4.4% ($n = 11$) of that induced by acetylcholine (1 mM). The contraction was increased by the presence of the Ca^{2+} ionophore (A23187) to 47 ± 6% ($n = 7$, $P < 0.05$) by the Ca^{2+} channel agonist, Bay K 8644 to 59.5 ± 4.5% ($n = 4$, $P < 0.05$) and by KCl to 47.4 ± 6%, while it was unaffected by carbachol (28.7 ± 6.8%, $n = 4$, $P > 0.05$). The Ca^{2+} channel antagonist, verapamil (1 μM) significantly reduced the contraction from 32.3 ± 4.9 to 12.5 ± 1% ($n = 4$, $P < 0.05$) and in the presence of nifedipine (1 μM), the contractile response was abolished. A single concentration of 10 μM PDB produced a biphasic response-relaxation (6 ± 1%) followed by contraction (76 ± 4%, $n = 4$) which was greater than that produced when responses were obtained cumulatively. The relaxation response was inhibited by the addition of a Na^{+}/K^{+} exchange antagonist, ouabain (10 μM) which also markedly potentiated the contractile response to 110 ± 10% ($n = 4$, $P < 0.05$). These results suggest that the protein kinase C-mediated contraction in human airway smooth muscle is dependent on extracellular Ca^{2+} influx. Protein kinase C may also phosphorylate Na^{+}/K^{+}-ATPase resulting in a relaxation response.

Keywords: Contraction; Human bronchus; Protein kinase C; Na^{+}/K^{+}-ATPase; Ca^{2+} channel

1. Introduction

In airway smooth muscle, the contraction-relaxation cycle is dependent largely upon the regulation of the cytosolic free Ca^{2+} concentration (Ito and Itoh, 1994). Contraction of smooth muscle in response to stimulation by hormones and neurotransmitters is thought to be mediated by an increase in the concentration of cytosolic free Ca^{2+} and subsequent formation of a Ca^{2+}-calmodulin complex, resulting in activation of myosin light chain kinase causing phosphorylation of myosin light chain. This phosphorylation reaction increases the actin-activated MgATPase activity and hence initiates force development in smooth muscle (Kamm and Stull, 1985; Stull et al., 1988). The Ca^{2+} contributing to the activation of contractile proteins is of both extra- and intracellular origins. In the resting state, the cytosolic free Ca^{2+} concentration is in the region of 0.05–0.35 μM (Rasmussen and Barrett, 1984; Kotlikoff et al., 1987; Takuwa and Rasmussen, 1987). In contrast, upon stimulation of receptors by various agonists, the cytosolic free Ca^{2+} concentration rises to 0.5–2.0 μM (Morgan and Morgan, 1984; Rasmussen and Barrett, 1984). This can occur as a consequence of Ca^{2+} arriving either from extra- or intracellular sources. Extracellular Ca^{2+} may enter the cell through voltage-dependent channels, receptor-operated channels or through the cell membrane ('leak' pathway) (Triggle, 1985; Rodger, 1987). Intracellular Ca^{2+} may be released from intracellular Ca^{2+} stores to the cytosol (Rodger, 1989).

On the other hand, Ca^{2+}-mobilizing hormones or neurotransmitters stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (Nishizuka, 1984). IP_3 may serve as the signal to release Ca^{2+} from intracellular storage sites sequestered in intracellular compartments (Nishizuka, 1984; Singer and Baker, 1987). Diacylglycerol dramatically increases the apparent affinity of protein kinase C for Ca^{2+}, fully activating this enzyme (Nishizuka, 1984).

Our previous studies in human airway smooth muscle have shown that protein kinase C plays a role in the sustained phase, rather than the initial phase of the contrac-

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tion and that the activation of protein kinase C by a single concentration of 10 μM phorbol 12,13-dibutyrate (PDB) induced a biphasic response (relaxation followed by contraction) (Yang and Black, 1995). While the contractile response is believed to be related to the activation of protein kinase C, the mechanism of the relaxation response however is unclear. Souhrada and Souhrada (1991) demonstrated similar biphasic responses in guinea-pig airway smooth muscle cells, which appeared to be caused primarily by alterations in Ca^{2+} and Na^+ transport systems in the cell membrane. In bovine tracheal smooth muscle, phorbol esters induced slowly developing contractile responses (Park and Rasmussen, 1985; Knox et al., 1993). This contraction is reduced by a low Ca^{2+} concentration in the medium or Ca^{2+} channel antagonist, indicating a Ca^{2+} dependence. The roles of different cell membrane sodium transport systems in the contraction of airway smooth muscle have also been considered. Phorbol ester activates both Na^+/H^+ exchange and Na^+/K^+ ATPase in guinea-pig trachea (Souhrada and Souhrada, 1989). Na^+/K^+-ATPase is crucial for the maintenance of ionic gradients across the cell membrane (Glynn and Karlish, 1975; Jorgensen, 1982; Kaplan, 1985) and its activation causes hyperpolarization of the membrane (Bolton, 1973). This action leads to the relaxation of excitable smooth muscle (Sasaguri and Watson, 1990).

The purpose of these studies was to elicit cumulative concentration-response curves to the phorbol ester, PDB in human isolated bronchus. In addition, the effects of both extra- and intracellular Ca^{2+} on the PDB-induced contraction were also examined, in addition to the relationship between Na^+ transport and PDB-induced biphasic responses.

2. Materials and methods

2.1. Tissue preparation

Human bronchus was obtained from the macroscopically normal portion of lung tissue resected from patients with pulmonary carcinoma. The protocol had been approved by the Human Ethical Review committee of The University of Sydney. The specimen was transported to the laboratory in Krebs-Henseleit solution (composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2 \cdot 2\ H_2O$ 2.5, $MgSO_4 \cdot 7\ H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and D-glucose 11.1) at 4°C. Lengths of bronchus were dissected from the specimen and the surrounding tissue was carefully removed. Ring segments, each measuring 2–4 mm in i.d. and 4–5 mm in length, were cut from the bronchial tissue and mounted on stainless steel hooks in 5-ml water-jacketed organ baths containing Krebs-Henseleit solution maintained at 37°C and aerated with 5% carbon dioxide in oxygen as described previously (Black et al., 1988). Bronchial rings were allowed to equilibrate under 1–1.5 g of tension for 1–2 h during which time the Krebs-Henseleit solution was replaced at 15–20-min intervals. The contractile response to 1 mM acetylcholine was determined at the start of each experiment, to standardize contraction in each bronchial ring. The tissue was then washed repeatedly and left for 1 h. Further washings were performed if required, until tension returned to baseline. Changes in tension were measured isometrically with Grass FTO3 transducers and recorded on Grass polygraphs.

2.2. Experiment protocols

In order to study the role of protein kinase C and Ca^{2+} in activating human airway contraction, a cumulative concentration-response curve to PDB (1 nM–3 μM) was initially performed. In addition, the effects of Ca^{2+} ions on contractions caused by PDB were studied. Bronchial rings were pre-treated with the Ca^{2+} ionophore A23187, Bay K 8644, potassium chloride (KCl) and carbachol to create a contraction of approximately 25–30% of the response to the 1 mM acetylcholine. This indicated that the level of intracellular free Ca^{2+} was increased. When the contractile responses had reached a plateau, cumulative concentration-response curves to PDB (1 nM–3 μM) were commenced. In other experiments, Ca^{2+} channel inhibitors – verapamil (1 μM) and nifedipine (1 μM) – were added for 20 min before the addition of PDB. In further experiments, the response to a single concentration of PDB (10 μM) was also examined in the presence and absence of an inhibitor of Na^+/H^+ exchange, amiloride (10 μM) and an inhibitor of Na^+/K^- exchange, ouabain (10 μM). The changes in tension in response to PDB were observed over 90 min.

2.3. Analysis of results

In each tissue preparation, contractile responses to each concentration of PDB were expressed as a percentage of the response (% response) to 1 mM acetylcholine. The contractions caused by A23187, Bay K 8644, KCl or carbachol were also expressed as a percentage of the response to acetylcholine. Responses to PDB in the presence of A23187, Bay K 8644, KCl or carbachol-induced tone were calculated as though this level of tone was now the baseline. Where duplicate preparations were studied, a mean response curve was constructed for each experiment. Paired or unpaired two-tailed Student's *t*-tests and analysis of variance (ANOVA) with Fisher probability test were used to compare the mean results for treated and control tissues and differences were considered as having statistical significance at $P \leq 0.05$.

2.4. Compounds used

Acetylcholine, Ca^{2+} ionophore (A23187), carbachol (carbamylcholine chloride), amiloride hydrochloride,

ouabain, verapamil, nifedipine and phorbol 12,13-dibutyrate (PDB) were purchased from Sigma (USA); methyl-1, 4-dihydro-2, 6-dimethyl-3-nitro-4 (2-trifluoromethylphonyl)-pyridine-5-carboxylate (Bay K 8644) from Calbiochem (USA); potassium chloride (KCl) from AJAX (Australia). Stock solutions of acetylcholine, carbachol, amiloride, ouabain and KCl were dissolved in distilled water and stored at −20°C. A23187, Bay K 8644, verapamil and nifedipine were dissolved in absolute ethanol and stored at −20°C. PDB was dissolved in 10% dimethyl sulphoxide (DMSO) and stored at −70°C. The maximum concentrations of ethanol and DMSO in the bath were < 0.1% and had no effects alone on the tone of human bronchus. Serial dilutions were made on the day of the experiment using Krebs-Henseleit solution and kept on ice.

3. Results

3.1. The effects of agonists on the cumulative concentration-response curve to PDB

A contraction was elicited by the addition of acetylcholine (1 mM). The range of contractile responses was 390–5115 mg in 11 patients, dependent on the size of the human isolated bronchial rings, and the mean contractile response was 1443 ± 417 mg ($n = 11$). PDB, over the range of 1 nM–3 μM, produced a dose-dependent contractile response. The mean maximum contraction was 26 ± 4.4% ($n = 11$) of the response to acetylcholine (1 mM). A23187 (0.1 μM–3 μM) caused a 25 ± 6.5% ($n = 7$) contraction of that produced by acetylcholine (1 mM). The cumulative concentration-response curve to PDB was commenced at the top of the contraction to A23187. The maximum contraction to PDB in the presence of A23187 was significantly increased to 47 ± 6% ($P < 0.05$, $n = 7$) (Fig. 1). Bay K 8644 (5 μM–20 μM) produced a contraction of 26.2 ± 10.2% ($n = 4$). The maximum contractile response to the PDB cumulative concentration-response curves, which started at the top of the contraction to Bay K 8644, was significantly increased to 59.5 ± 4.5% ($P < 0.05$, $n = 4$) (Fig. 1). KCl (1 mM–30 mM) induced a contractile response of 32.3 ± 3.2% ($n = 7$). The maximum contraction to PDB in the presence of KCl was 47.4 ± 6%, which was significantly increased ($P < 0.05$, $n = 7$) (Fig. 1). Carbachol (30 nM–100 nM) created a contraction of 24 ± 5% ($n = 4$), however, the maximum contractile response to the PDB cumulative concentration-response curve was 28.7 ± 6.8% which not significantly different from that in the control group ($P > 0.05$, $n = 4$) (Fig. 1).

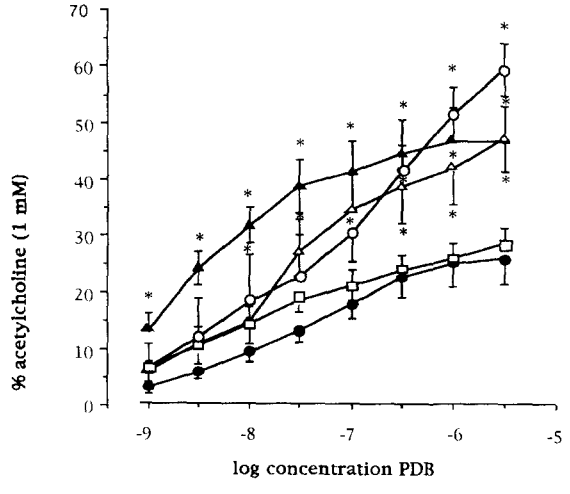

Fig. 1. Mean cumulative concentration-response curves to PDB in human bronchus in control tissue from 11 patients (●) and in the presence of Bay K 8466 (○) ($n = 4$), A23187 (▲) ($n = 7$), KCl (△) ($n = 8$) and carbachol (□) ($n = 4$). Mean responses are expressed as a percentage of the response to acetylcholine (1 mM). Vertical bars, S.E.M. * Significance difference from control at $P < 0.05$, unpaired Student's *t*-test.

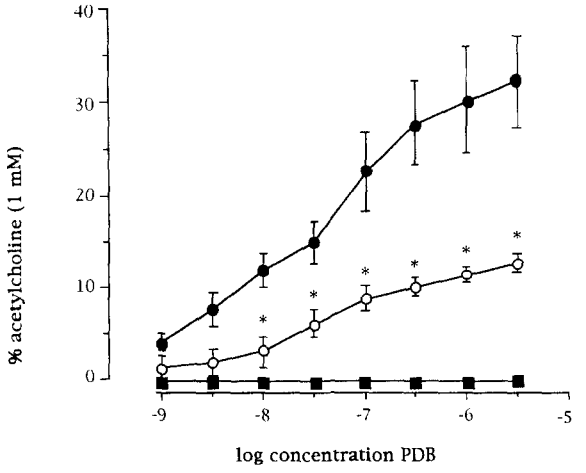

Fig. 2. Mean cumulative concentration-response curves to PDB in human bronchus in control tissues (●) ($n = 4$) and in the presence of 1 mM verapamil (○) ($n = 4$) and 1 mM nifedipine (■). Mean responses are expressed as a percentage of the response to acetylcholine (1 mM). Vertical bars, S.E.M. * Significance difference from control at $P < 0.05$, paired Student's *t*-test.

3.2. The effects of Ca^{2+} channel inhibitors on the cumulative concentration-response curve to PDB

The cumulative concentration contractile response induced by PDB (1 nM–3 μM) was reduced in tissues pre-incubated with a Ca^{2+} channel inhibitor, verapamil (1 μM). The maximal contraction was 12.5 ± 1% of the response to 1 mM acetylcholine which was significantly different to control 32.3 ± 4.9% ($P < 0.05$, $n = 4$) (Fig. 2). In the tissues which were pre-incubated with 1 μM nifedipine, the contractile response to PDB was abolished ($n = 4$) (Fig. 2).

3.3. The effects of Na^{+} inhibitors on the biphasic response to a single concentration of PDB

A single concentration of PDB (10 μM) produced a biphasic response – a relaxation followed by a contractile response. An initial small relaxation was induced in the

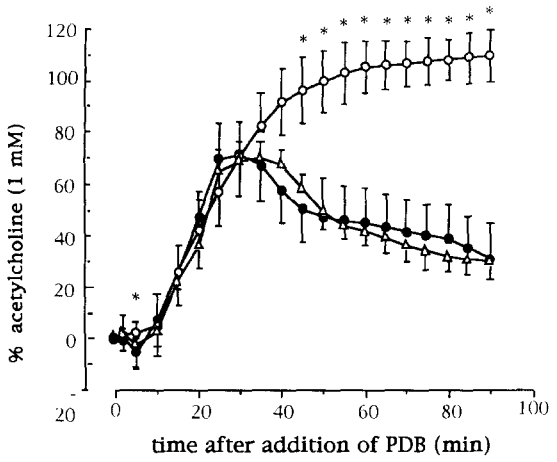

Fig. 3. Mean responses to a single concentration of PDB (10 μM) in human bronchus in control tissues (●) and in the presence of amiloride (△) ($n = 4$) and ouabain (○) ($n = 4$). Mean responses are expressed as a percentage of the response to acetylcholine (1 mM). Vertical bars, S.E.M. * Significance difference from control at $P < 0.05$, ANOVA.

first 5 min. The maximum relaxation response was 6 ± 1% of the contractile response to 1 mM acetylcholine ($n = 4$). After the relaxation response had plateaued, the contractile response developed over the next 30 min. The maximum contraction was 76 ± 4% ($n = 4$) of that produced by 1 mM acetylcholine. After the contractile response had plateaued, the tension slowly decreased. At 90 min after PDB administration, the tone was 38.8 ± 9% ($n = 4$) (Fig. 3). In the tissues treated with 10 μM amiloride, neither the relaxation nor the contraction to PDB was affected. The relaxation response was unaffected and the contractile response was 70 ± 6% which was not significantly different from control values ($P > 0.05$, $n = 4$). In the tissues which were incubated with 10 μM ouabain, the first-phase relaxation response induced by 10 μM PDB was abolished. The second-phase contractile response was the same as in the control tissues for the first 30 min, in that the contractile response at 30 min was 71 ± 15% which was not significantly different from control ($P > 0.05$, $n = 4$). However, after 30 min, the contraction continued to develop over 90 min. The mean maximum contractile response was 110 ± 10% of the response to 1 mM acetylcholine which was significantly greater than control ($P < 0.05$, $n = 4$) (Fig. 3).

4. Discussion

The results of this study have shown that activation of protein kinase C by PDB in the range of 1 nM–3 μM produced a concentration-dependent contractile response in human isolated bronchus. This contractile response was increased in the presence of the Ca^{2+} ionophore-A23187 and by Bay K 8644 and KCl but decreased in the presence of Ca^{2+} channel inhibitors, nifedipine and verapamil. However, carbachol did not affect the dose-dependent contraction to PDB. A single concentration of PDB (10 μM) produced a relaxation followed by a contractile response. Ouabain inhibited the relaxation but increased the contractile response while amiloride had no effect on this biphasic response.

The increased contractile response to PDB can be attributed to the influx of extracellular Ca^{2+} through Ca^{2+} channels. Bay K 8644 is a Ca^{2+} voltage-dependent channel agonist (Schramm et al., 1983) and activates these channels in human airways (Marthan et al., 1987). KCl also has been identified to produce stimulation of Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Black et al., 1986), causing contraction by depolarization of the cell membrane in human airway smooth muscle (Kotlikoff, 1987). Studies in vascular smooth muscle have shown that phorbol ester-activated protein kinase C causes a contraction dependent on extracellular Ca^{2+} in the rabbit thoracic aorta (Danthuluri and Deth, 1984), rat aorta (Litten et al., 1987), human and rat pulmonary arteries (Savineau et al., 1991). However, the contraction was independent of extracellular Ca^{2+} in porcine coronary artery (Itoh et al., 1986) and rabbit aorta (Sybertz et al., 1986). These results indicate that there may be important species- and/or tissue-specific differences in the actions of phorbol ester on Ca^{2+} flux. The present studies provide evidence that, in human airway smooth muscle, movement of extracellular Ca^{2+} across the cell membrane, through voltage-dependent channels (Bay K 8644 and KCl) into the cytosol forms an important component of the smooth muscle contraction to the activation of protein kinase C by PDB. It is consistent with the finding of the studies of bovine tracheal smooth muscle preparations (Park and Rasmussen, 1985; Knox et al., 1993). An interesting finding in this study was that carbachol had no effect on the PDB-induced contraction. Carbachol produces a contractile response in human airway by increasing the intracellular Ca^{2+} concentration (Black et al., 1986). However, the Ca^{2+} is released from intracellular Ca^{2+} stores (Black et al., 1986; Marthan et al., 1987; Komori et al., 1995). PDB does not cause Ca^{2+} release from intracellular stores (Chiu et al., 1988). This indicates that the Ca^{2+} released from intracellular stores by carbachol is not involved in the activation of protein kinase C in human airway smooth muscle.

The Ca^{2+} ionophore A23187, which increases intracellular Ca^{2+} concentrations by transferring extracellular Ca^{2+} across the cell membrane (Murray et al., 1975) also increased the contraction to PDB. In bovine airways, phorbol myristate acetate (PMA) induces a contractile response in the presence of A23187 (Park and Rasmussen, 1985). A23187 combined with PMA activates protein kinase C to cause phosphorylation of a contractile protein – the 20000-Da myosin light chain (Ludowyke et al., 1989). These data suggest that phorbol ester-induced contraction in human airway smooth muscle requires influx of extra-

cellular Ca^{2+} which can occur not only through voltage-dependent channel, but also via other modes of entry.

Further evidence that PDB-induced contraction in human airway smooth muscle is dependent on the influx of extracellular Ca^{2+} through Ca^{2-} channels, arose from the inhibition of the contraction by two classic inhibitors of voltage-dependent Ca^{2+} channels, nifedipine and verapamil. These antagonists have also been shown to inhibit phorbol ester-induced contractions in bovine (Knox et al., 1993), guinea-pig (Souhrada and Souhrada, 1989) and rabbit airways (Schramm and Grunstein, 1989). Our results are also consistent with those of Rossetti et al. (1995). They demonstrated that addition of verapamil or removal of Ca^{2+} from the Krebs-Henseleit solution reduced the contractile response to PDB in human isolated bronchus. Protein kinase C is a Ca^{2+}-dependent phosphorylation enzyme (Kishimoto et al., 1980), although some isoforms are Ca^{2+} independent (Nishizuka, 1992). The data in this study suggest that the Ca^{2+}-dependent protein kinase C may be involved in the contractile response in human airways. Thus, it is possible to conclude that the mechanism by which activation of protein kinase C by phorbol ester leads to contraction of airway smooth muscle is associated with the increase of intracellular Ca^{2+} via influx from extracellular sources.

The contractile response to PDB gradually declined over time and the pattern of this response was similar to that which we observed in a previous study, in which we examined the decline in contraction to a single concentration of histamine (Yang and Black, 1995). In that study, at 90 min after histamine administration, the response was about 66% of the maximum whereas in this study the tension at 90 min after PDB was approximately 50% of the maximum. The speed with which the contractile response is reversed is presumably dependent on the mechanism underlying the contraction and, in the case of histamine and PDB, stimulation of PKC is involved.

An aim of this study was to investigate the mechanism of the biphasic response-relaxation followed by contraction which was produced by a single concentration of 10 μM PDB. Souhrada and Souhrada (1991) reported a similar finding in guinea-pig airway smooth muscle cells using 10 μM PMA. However, others have reported that 1 μM PDB produced a pure contractile response in bovine airway smooth muscle (Knox et al., 1993). Furthermore, Schramm and Grunstein (1989) found that the phorbol ester 12-deoxphorbol-13-isobutyrate (DPB), at a concentration of < 1 μM, induced contraction and, at high concentrations of > 1 μM, induced relaxation in rabbit tracheal smooth muscle. These results indicate that the relaxation phase of the biphasic response may be induced by the high concentration of phorbol esters in airway smooth muscle. The results of the present study showed that ouabain, an inhibitor of the Na^+/K^+-ATPase, inhibited the first-phase relaxation response and increased the second-phase contractile response. Na^+/K^+-ATPase is stimulated by phorbol esters (Lynch et al., 1986) and believed to be a substrate of protein kinase C (Greene and Lattimer, 1986; Nishizuka, 1986). Ouabain induces a weak contraction in smooth muscle on its own, presumably mediated through depolarization, which leads to opening of the voltage-dependent Ca^{2+} channels (Komori et al., 1995). This could therefore explain the potentiation of the contractile response to PDB which we observed in the present study. However, amiloride did not alter either the relaxation or contractile responses to PDB. This is consistent with the finding of Schramm and Grunstein (1989). They reported that, in rabbit tracheal smooth muscle, phorbol esters (PMA and DPB) at a concentration of > 1 μM, produced a relaxation response which was inhibited by ouabain but not amiloride.

Another interesting finding was that a single concentration of PDB at 10 μM produced a contractile response that was even greater than the maximum contraction induced by the cumulative concentration-response curves of PDB (1 nM–3 μM). Increasing concentrations in the cumulative response curves did not create further contraction. This is consistent with the finding in our previous studies in which the contraction to a single concentration of histamine (10 μM) was also greater than the maximum contraction in the histamine cumulative concentration-response curves (10 nM–300 μM) (Yang and Black, 1995). Van Rossum (1963) demonstrated that cumulative concentration-response curves produced the same responses when compared with those from bolus doses. Haye-Legrand et al. (1986) also reported that the maximal response to histamine in human isolated bronchus, using the individual concentration method, was similar to that obtained using the cumulative method. However, those studies compared cumulative concentration-response curves with multiple increasing bolus doses. In our study, a single bolus dose was given without preceding lower concentrations. It is possible that frequent stimulation of target receptors may have desensitised the receptors resulting in the decrease of the response, i.e. tachyphylaxis. We could not test this hypothesis in the present experiments, since the response to a single concentration of PDB was non-reproducible and difficult to wash out.

In summary, we have shown that activation of protein kinase C by the phorbol ester PDB caused a concentration-dependent contractile response in human isolated bronchus. Both the augmentation of the contraction and the contraction itself can be attributed to Ca^{2+} influx from extracellular sources but not to intracellular Ca^{2+} release from the carbachol-stimulated Ca^{2+} stores. The initial relaxation response to a single concentration of PDB (10 μM) may be caused by the phosphorylation of Na^+/K^+-ATPase, since it was inhibited by oaubain, which also potentiated the subsequent contractile response. This study provides further evidence to support a role for Ca^{2+}-dependent isoforms of protein kinase C in regulation of tone in human isolated airways.

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