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European Journal of Pharmacology 399 (2000) 65–73

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Effects of phorbol 12,13-diacetate on human isolated bronchus

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Received 27 January 2000; received in revised form 20 April 2000; accepted 2 May 2000

Abstract

Protein kinase C appears to be involved in the regulation of airway contractility. Phorbol 12,13-diacetate (PDA; 0.01–10 μM), a protein kinase C activator, produced a transient relaxation followed by a sustained contraction of human isolated bronchus. Different protein kinase C inhibitors (calphostin C, staurosporine and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine) (H-7), nifedipine (NIF; 1 μM) or incubation with Ca^{2+}-free medium, inhibited the spasmogenic response to phorbol, while ouabain (10 μM) suppressed only the initial relaxation. These results indicate that the initial relaxation, in response to PDA, is related to the activation of Na^{+}/K^{+}-ATPase, while the ensuing contraction depends on extracellular Ca^{2+} entry.

Incubation with PDA (1–5 μM) depressed the maximal relaxation to theophylline and caffeine obtained at 37°C but augmented the spasmogenic responses to methylxanthines (10 mM) obtained in cooled preparations. These effects do not result apparently from increased extracellular entry of Ca^{2+}, but instead, from facilitation of the release of Ca^{2+} from intracellular stores.

Keywords: Bronchus, human, isolated; Phorbol 12,13-diacetate; Theophylline; Caffeine

1. Introduction

A considerable body of evidence suggests that protein kinase C has a role in the regulation of airway smooth muscle contractility (Hakonarson and Grunstein, 1998). Most of this evidence stems from the use of active phorbol esters, which directly bind to, and activate, protein kinase C (Castagna et al., 1982). The results from in vitro experiments conducted in airway smooth muscle from a variety of animal species, including bovine (Park and Rasmussen 1985; Knox et al., 1993), guinea pig (Menkes et al., 1986; Huang et al., 1987; Obianime et al., 1989; Cortijo et al., 1994), rabbit (Schramm and Grunstein, 1989), swine (Baba et al., 1989) and canine (Gunst et al., 1994), indicate that the effects of phorbol esters are tissue- and species-dependent, comprising both contractile and relaxing responses, and that they are of complex nature since different mechanisms appear to contribute to their effects.

Compared to animal studies, little work has so far been carried out on the effects of phorbol esters in airway smooth muscle from humans. Rossetti et al. (1995) found that phorbol 12,13-dibutyrate (1 nM–3 μM) produces a concentration-dependent contraction of human isolated bronchus, and Yang and Black (1995) reported that phorbol 12,13-dibutyrate (10 μM) produced a biphasic response (relaxation followed by contraction). These two studies concluded that phorbol-induced contraction of human bronchus is largely dependent on extracellular calcium, and that protein kinase C activation plays a role in the maintenance of contraction in human airway smooth muscle.

Several authors have investigated the interaction between phorbol esters and spasmogenic and relaxant agents acting on airway smooth muscle (Menkes et al., 1986; Huang et al., 1987; Schramm and Grunstein, 1989; Cortijo et al., 1994; De Diego et al., 1995; Pype et al., 1998), but little research has been conducted in human airway smooth muscle.

The object of the present work was, therefore, to determine the effects of acute activation of protein kinase C by phorbol 12,13-diacetate (PDA) on the spontaneous tone of human isolated bronchus, and the influence of different

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00351-4

pharmacological interventions on the phorbol-induced responses. This active phorbol ester was selected based on previous studies (Cortijo et al., 1994; De Diego et al., 1995). In addition, we examined the influence of PDA on the relaxant and spasmogenic responses to methylxanthines obtained in human isolated bronchus under the appropriate experimental conditions. Isoprenaline and levcromakalim were used as reference relaxant drugs.

2. Methods

2.1. Human bronchial tissue preparation

Lung tissue was obtained from patients who were undergoing surgery for lung carcinoma. None of the patients had a history of asthma. The experimental protocol of this study was approved by the local ethics committee and conforms to the recommendations of the Declaration of Helsinki. After the resection of one or more lung lobes, a piece of macroscopically normal tissue was supplied by the hospital pathologist, submerged in physiological salt solution (composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and dextrose 11.1, equilibrated with 5% CO_2 in O_2, at 4°C) and transported to the laboratory. Then, parts of the bronchus were dissected free from parenchymal lung tissue and preparations cut (3–4 mm length × 3–4 mm internal diameter) as previously described (Cortijo et al., 1992b). Preparations were stored at 4°C in oxygenated physiological salt solution until used. Experiments were routinely completed within 24 h of initiating storage. Previous experience in this laboratory and published data has demonstrated that overnight storage of tissues does not alter its reactivity (Sarria et al., 1995).

Bronchial rings were suspended on tissue hooks in 10 ml organ baths containing physiological salt solution, gassed with 5% CO_2 in O_2 at 37°C (pH 7.4). Each preparation was connected to a force displacement transducer (Grass FTO3) and isometric tension changes recorded on a computer by means of the software Proto5® (Letica, Barcelona, Spain). The preparations were equilibrated for 60–90 min with changes in bath solution every 20 min before any pharmacological intervention occurred. A load of 2 g was maintained throughout the equilibrium period and a stable resting level of spontaneous tone was present in all preparations at the end of this period (Cortijo et al., 1997; Watson et al., 1998). In all experiments, human bronchi were first contracted with acetylcholine (1 mM) to obtain reference values to normalise the tension changes of the preparations. The level of spontaneous tone after the equilibration period was 1.89 ± 0.12 g, and the additional increase in tension generated by acetylcholine (1 mM) was 1.67 ± 0.09 g (64 preparations from 27 patients). These values are within the range of those previously reported (Cortijo et al., 1997). When the increase in tension produced by acetylcholine (1 mM) was less than 0.5 g, the bronchi were considered to be abnormal and they were not used for the experiments (Björck and Dahlén, 1993).

2.2. Assessment of the effect of PDA on the spontaneous bronchial tone and its modification by different pharmacological interventions

Following tissue equilibration with physiological salt solution, PDA (0.01, 0.1, 1, 5 or 10 μM) or its vehicle was added to the tissue bath and changes in tissue tone were monitored for up to 30 min. In separate experiments, the response to PDA (1 μM) was obtained in time-matched control tissues and in tissues subjected to different drug treatments: a combination of atropine, phentolamine and mepyramine (each at 1 μM), indomethacin (2.8 μM), zileuton (10 μM), calphostin C (1 μM), staurosporine (0.1 μM), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine) (H-7; 10 μM), nifedipine (NIF; 1 μM), amiloride (10 μM) or ouabain (10 μM). The response to PDA was obtained after 30 min of incubation and in the presence of the mentioned drug treatments. In a group of bronchial rings, the epithelium was removed as previously outlined (Iriarte et al., 1990), and the response to PDA (1 μM) was obtained and compared to that produced in paired intact preparations. The response to PDA (1 μM) was also obtained in preparations bathed in Ca^{2+}-free medium prepared by substituting ethyleneglycol-*bis* (β-amino-ethylether)-*N*,*N*′-tetraacetic acid (EGTA; 0.1 mM) for $CaCl_2$. The period of exposure to the Ca^{2+}-free, EGTA-containing solution was for 30 min before and during the generation of the response to PDA.

2.3. Assessment of the effects of PDA on the contractile responses to $CaCl_2$ and on precontracted human isolated bronchus

Cumulative concentration–effect curves to $CaCl_2$ (0.01–30 mM) were constructed in preparations equilibrated with K^+-rich (40 mM), Ca^{2+}-free medium, as essentially described by Advenier et al. (1986), in the absence (control tissues) and presence of PDA (1 or 5 μM, 30 min incubation). In separate experiments, after equilibration in physiological salt solution, the preparations were contracted by the addition of equieffective concentrations of KCl (75 mM) or acetylcholine (50 μM), and PDA (1 μM) or its vehicle was added on top of the plateau contraction obtained in response to these spasmogens. The K^+-rich (40 or 75 mM) solutions were prepared by raising the KCl concentration to the desired level and reducing the concentration of NaCl to preserve the osmolarity of the solution, as previously outlined (Cortijo et al., 1997).

2.4. Assessment of the effects of PDA on the actions of caffeine, theophylline and isoprenaline in suppressing the spontaneous and histamine-induced tone of the human isolated bronchus

Following equilibration, the tissues were allocated randomly in equal numbers to test and control groups and incubated with PDA (1 or 5 μM; test tissues) or vehicle (time-matched control tissues); 30 min later, a cumulative concentration–relaxation curve for caffeine (10 μM–3 mM), theophylline (10 μM–3 mM), isoprenaline (1 nM–1 μM) or levcromakalim (10 nM–30 μM) was constructed in each tissue. In a further series of experiments, test tissues were treated with PDA (1 μM) and NIF (1 μM) for 30 min before and during construction of the log concentration–effect curve to theophylline. Time-matched control tissues were treated identically to test tissues but were not exposed to PDA and NIF.

In separate experiments, following equilibration, the tissues were treated with PDA (1 μM) and after 20 min, histamine (50 μM) was added, and 10 min later, a cumulative concentration–response curve for theophylline (10 μM–3 mM) or isoprenaline (1 nM–1 μM) was constructed on the plateau contraction to histamine. Time-matched control tissues were treated identically to test tissues but were treated with vehicle instead of PDA.

2.5. Assessment of the effect of PDA on the spasmogenic effects of caffeine and theophylline in human bronchus maintained at low temperature

In these experiments, the bath temperature was lowered to 10°C by a circulator (Selecta 398, Barcelona, Spain) as previously described (Cortijo et al., 1992a). The temperature and the pH of the bath solution were monitored as previously described (Ortiz et al., 1991). Cooled control tissues were incubated for 30 min with physiological salt solution and the response to caffeine or theophylline (each at 10 mM) was obtained. Bath temperature and concentration of methylxanthines were selected from previous results obtained in this laboratory (data not shown). For the present study, responses to methylxanthines were obtained in the absence (control tissues incubated for 30 min with physiological salt solution) and presence of NIF (1 μM) or Ca^{2+}-free, EGTA (0.1 mM)-containing medium (30 min incubation). In additional experiments, PDA (1 μM) was added to test tissues bathed in physiological salt solution 30 min before challenge with methylxanthines, while time-matched control tissues were treated identically to test tissues, but were not exposed to PDA. Further experiments were carried out to obtain responses to methylxanthines in the combined presence of PDA and NIF (each at 1 μM).

2.6. Statistical analysis of results. Drugs and solutions

Contractile and relaxant responses are expressed in absolute values (g) or as percentage of the response to acetylcholine (1 mM). Each cumulative concentration–relaxation curve was computer fitted (GraphPad Prism version 3.00) by using a four parameter logistic equation as follows: $Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10^{((\log EC_{50} - X) * n_H)})$, where X is the logarithm of the agonist concentration and Y is the response, bottom is the starting level and top is the maximum response developed to the agonist, EC_{50} is the molar concentration producing half maximal response, and n_H is the apparent Hill slope. The maximum relaxation and potency (expressed as $-\log EC_{50}$ values) of agonists were calculated by this procedure for each response curve. Data are presented as means ± S.E.M. of n/p experiments, where n represents the number of preparations examined and p is the number of patients from which those tissues were derived. The significance of differences between means was assessed by the application of analysis of variance followed by Bonferroni multiple comparison test or by Student's t-test as appropriate. Differences were considered significant when $P < 0.05$.

The following drugs were used: atropine sulphate, caffeine, EGTA, H-7, histamine hydrochloride, indomethacin, (−)-isoprenaline hydrochloride, mepyramine maleate, PDA, theophylline (each from Sigma, Madrid, Spain), NIF (Bayer, Madrid, Spain), and zileuton (gift from Lab. Dr. Esteve, Barcelona, Spain). A stock solution of PDA was prepared in dimethylsulphoxide and stored at −20°C until used. A stock solution of isoprenaline was prepared in 0.1 M HCl and dilutions made into physiological salt solution containing ascorbic acid (0.57 mM). Stock solutions of NIF and zileuton were prepared in absolute ethanol. Final bath concentrations of the solvents did not themselves affect the mechanical activity of the tissues. Other substances were dissolved in physiological salt solution immediately before use. Drug concentrations are expressed throughout as the bath concentrations of the active species. Calphostin C, NIF and staurosporine were protected from light exposure.

3. Results

3.1. The effects of PDA on the spontaneous tone of human isolated bronchus and the influence of different pharmacological interventions

At 37°C, PDA produced a small and transient (~5 min) reduction in the spontaneous tone of the bronchus, followed by a sustained contraction to a level above that existing at the beginning of the experiment. This biphasic response was concentration-dependent (Fig. 1A). Preincubation of bronchial rings with a combination of atropine, phentolamine and mepyramine (each at 1 μM), indomethacin (2.8 μM) or zileuton (10 μM), or removal of the epithelium did not alter the effects of PDA on bronchial tone ($n/p = 3/3$ for each group; data not shown).

Protein kinase C inhibitors produced inhibition of the spontaneous tone of preparations. The relaxation was small

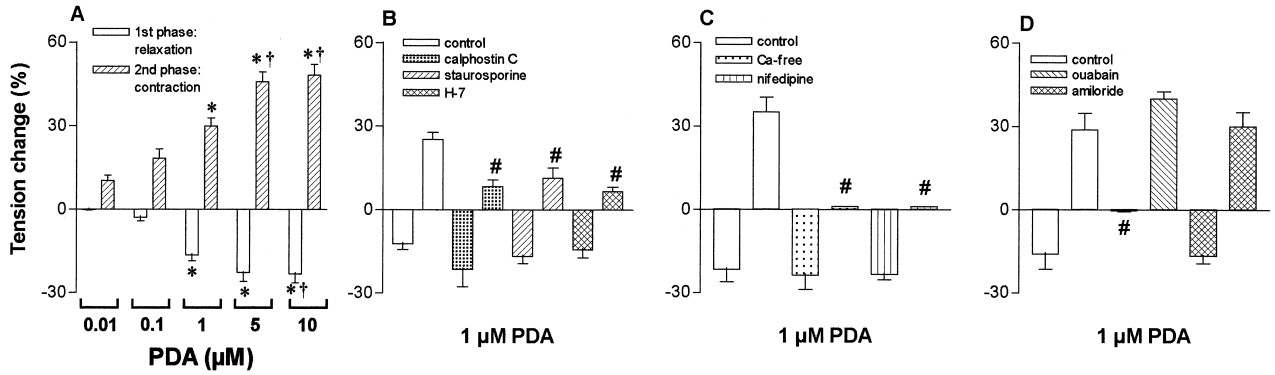

Fig. 1. Effect of PDA on the mechanical activity of human isolated bronchus. Panel A: biphasic response to PDA (0.01–10 μM as indicated) consisting of an initial relaxation followed by contraction. Panels B to D show the effect of protein kinase C inhibitors (1 μM calphostin C, 0.1 μM staurosporine, and 10 μM H-7), Ca^{2+}-free, EGTA (0.1 mM)-containing medium, NIF (1 μM), ouabain (10 μM) and amiloride (10 μM) on the response to PDA (1 μM). Tension changes are expressed as percentages of the reference response to acetylcholine (1 mM). Data are presented as mean ± S.E.M. of $n/p = 5/3$ for each group. Panel A: $^{*}P < 0.05$ compared to 0.1 μM PDA; $^{\dagger}P < 0.05$ compared to 1 μM PDA. Panels B to D: $^{\#}P < 0.05$ compared to control tissues. Analysis of variance showed no statistically significant difference among the control responses to 1 μM PDA in the different experimental groups (panels A–D).

for 0.1 μM staurosporine (5 ± 2% of acetylcholine 1 mM, $n/p = 5/3$) and 1 μM calphostin C (12 ± 3% of acetylcholine 1 mM; $n/p = 5/3$) while 10 μM H-7 produced a marked relaxation (49 ± 10% of acetylcholine 1 mM; $n/p = 5/3$). In tissues incubated with these protein kinase C inhibitors for 30 min, the subsequent addition of PDA produced an additional relaxation, followed by a contractile response that was significantly reduced (Fig. 1B).

Change of the physiological salt solution by a Ca^{2+}-free, EGTA (0.1 mM)-containing medium produced a significant reduction of spontaneous tone (63 ± 12% of acetylcholine 1 mM; $n/p = 5/3$), while incubation with NIF (1 μM) produced a small inhibition of baseline tone (11 ± 6% of acetylcholine 1 mM; $n/p = 5/3$). In tissues incubated with Ca^{2+}-free medium or NIF for 30 min, the subsequent addition of PDA produced an additional relaxation, but the contractile response was virtually abolished (Fig. 1C).

Ouabain (10 μM) produced a sustained increase of baseline tone of human bronchus (62 ± 7% of acetylcholine 1 mM; $n/p = 5/3$). In tissues incubated with ouabain for 30 min, the initial relaxation in response to PDA was abolished, but the subsequent contraction was unaltered (Fig. 1D). Amiloride (10 μM) had no effect on the spontaneous tone of preparations and did not modify the subsequent biphasic response to PDA (Fig. 1D).

3.2. The effect of PDA on the concentration–response curve to $CaCl_2$ and on precontracted human isolated bronchus

The addition of $CaCl_2$ (0.01–30 mM) to the K^+-rich (40 mM), Ca^{2+}-free medium, caused concentration-dependent contractions as reported by Advenier et al. (1986). The values for potency and maximal effect, as well as the shape of the log concentration–response curve for $CaCl_2$, were not altered by the presence of PDA ($-\log EC_{50}$ values were 2.75 ± 0.09, 2.88 ± 0.12 and 2.79 ± 0.11 in control, 1 and 5 μM phorbol-treated tissues, respectively; $n/p = 3/3$ for each group; $P > 0.05$).

Acetylcholine (50 μM) and KCl (75 mM), each produced a sustained contraction of human isolated bronchus of approximately the same size (0.89 ± 0.12 g for acetylcholine and 0.74 ± 0.13 g for KCl; $n/p = 5/3$ for each group; $P > 0.05$ acetylcholine vs. KCl). Addition of PDA to precontracted tissues produced a contractile response not preceded by relaxation. The PDA-induced contraction was significantly larger in KCl-contracted tissues (0.53 ± 0.11 g; $n/p = 5/3$) than in those contracted by acetylcholine (0.24 ± 0.07 g; $n/p = 5/3$; $P < 0.05$ from KCl).

3.3. Influence of PDA on the relaxant and spasmogenic responses to methylxanthines

At 37°C, theophylline and caffeine produced concentration-related relaxations of human isolated bronchus, either with spontaneous tone (Fig. 2A,B) or precontracted with histamine (50 μM; Fig. 3A). The values for maximal relaxation produced by theophylline and caffeine in preparations with spontaneous tone were 1.79 ± 0.08 and 1.71 ± 0.08 g, respectively ($n/p = 5/3$). In tissues incubated with PDA for 30 min, the maximal relaxant activity of methylxanthines on the spontaneous tone was significantly reduced (Fig. 2A,B), although the potency was not changed ($-\log EC_{50}$ values were 3.73 ± 0.05, 3.83 ± 0.08 and 3.95 ± 0.07 for theophylline, and 3.14 ± 0.04, 3.19 ± 0.10 and 3.28 ± 0.06 for caffeine, in control, 1 and 5 μM phorbol-treated tissues, respectively; $n/p = 5/3$ for each group; $P > 0.05$ control vs. treated for each xanthine). In preparations contracted with histamine (50 μM; 1.41 ± 0.07 and 1.44 ± 0.05 g in control and phorbol-treated tissues, respectively), the maximal relaxation produced by theophylline (3.13 ± 0.11 g) was also reduced in the pres-

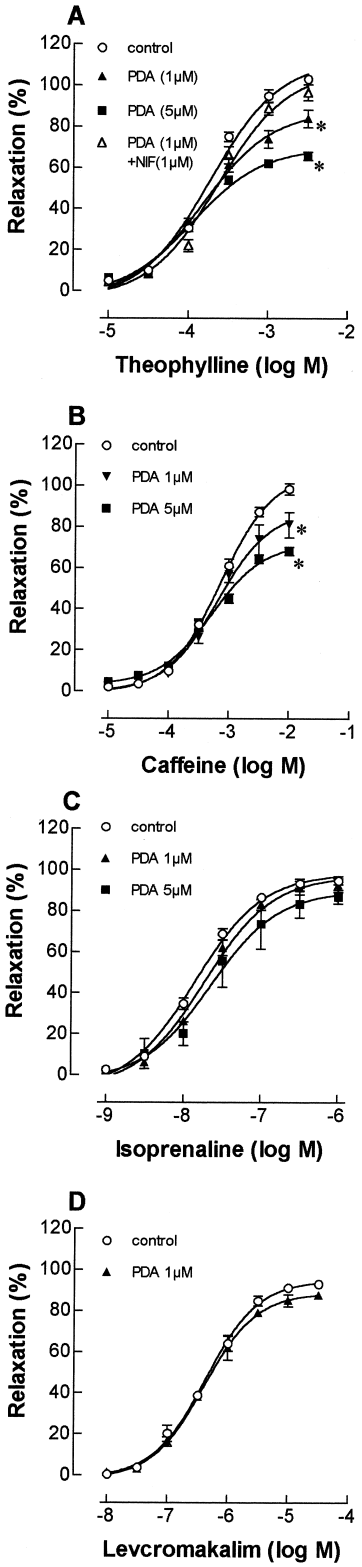

Fig. 2. Influence of PDA on the relaxant responses to theophylline (panel A), caffeine (panel B), isoprenaline (panel C) and levcromakalim (panel D) in human isolated bronchus with spontaneous tone. PDA depressed the maximal relaxation of the methylxanthines without affecting those of isoprenaline and levcromakalim. NIF (1 μM) reversed the inhibitory effect of PDA (1 μM) on the relaxation to theophylline (panel A). Data are mean ± S.E.M. of $n/p = 5/3$ for each group; $^{*}P < 0.05$ compared to control tissues.

ence of PDA (1 μM) as shown in Fig. 3A, without altering $-\log EC_{50}$ values (3.66 ± 0.09 and 3.60 ± 0.11 in control and phorbol treated tissues, respectively). Pretreatment with NIF (1 μM) restored the relaxation of PDA (1 μM)-treated tissues in response to theophylline to levels similar to those seen in control tissues (Fig. 2A).

Isoprenaline produced concentration-related relaxation of preparations with spontaneous and histamine-induced tone giving maximal values of 1.82 ± 0.15 and 3.09 ± 0.11 g, respectively ($n/p = 5/3$), which are similar to maximal relaxations obtained for methylxanthines. Neither the maximal relaxation nor the potency values (data not shown) of isoprenaline were altered in tissues treated with PDA (Figs. 2C and 3B).

The maximal relaxation of spontaneous tone produced by levcromakalim was 1.37 ± 0.06 g ($n/p = 5/3$). This value is significantly ($P < 0.05$) smaller than maximal relaxation obtained for methylxanthines and isoprenaline in this preparation as previously reported by others (Black et al., 1990; Cortijo et al., 1992b). The maximal relaxation

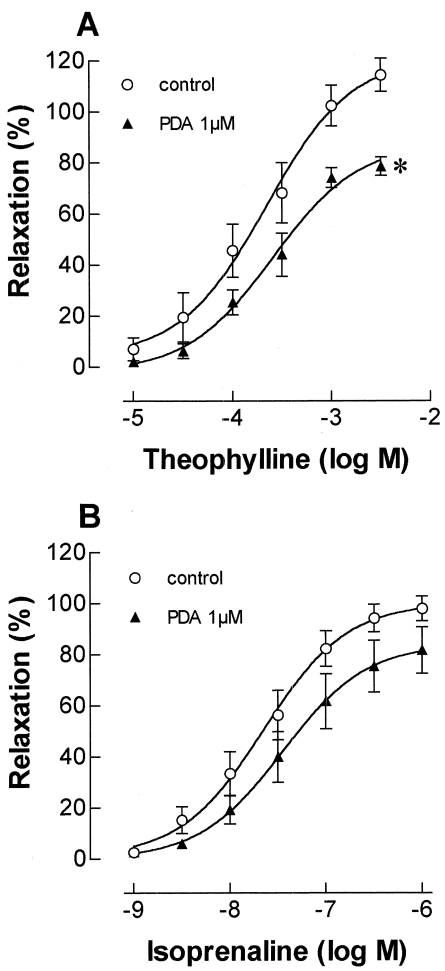

Fig. 3. Influence of PDA on the relaxant responses to theophylline (panel A) and isoprenaline (panel B) in human isolated bronchus contracted with histamine (50 μM). PDA depressed the maximal relaxation of theophylline without affecting that of isoprenaline. Data are mean ± S.E.M. of $n/p = 5/3$ for each group; $^{*}P < 0.05$ compared to control tissues.

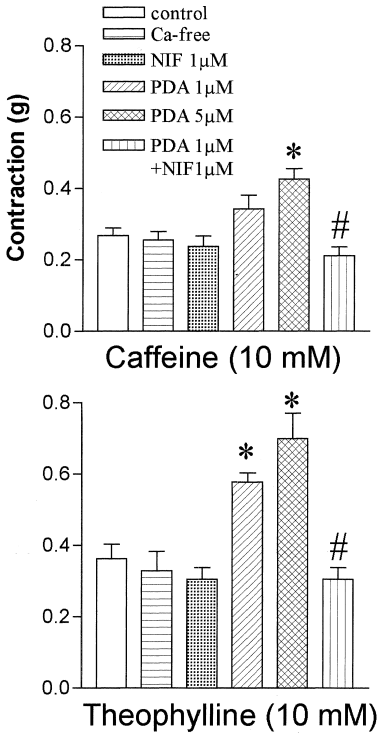

Fig. 4. Spasmogenic responses to caffeine and theophylline in human isolated bronchus at 10°C. The contractile responses to methylxanthines were resistant to Ca^{2+}-free, EGTA (0.1 mM)-containing medium and NIF (1 μM). Spasm to methylxanthines was augmented in the presence of PDA (1–5 μM) and this enhancing effect was reversed by NIF (1 μM). Data are mean ± S.E.M. of $n/p = 5/3$ for each group; $^{*}P < 0.05$ compared to control responses; $^{\#}P < 0.05$ compared to 1 μM PDA.

and potency values (data not shown) of levcromakalim remained unaltered in PDA-treated tissues (Fig. 2D).

When caffeine (10 mM) or theophylline (10 mM) were applied to preparations maintained at 10°C, the two methylxanthines each produced contraction (Fig. 4). This contraction was unaltered when obtained after 30 min incubation with Ca^{2+}-free, EGTA (0.1 mM)-containing medium or in the presence of NIF (1 μM). Tested in cooled preparations, PDA (1 and 5 μM) produced spasm (0.19 ± 0.05 and 0.31 ± 0.06 g, respectively; $n/p = 9/5$ for each group). The contraction evoked by PDA was not preceded by relaxation. When phorbol-treated tissues were challenged with caffeine or theophylline (each at 10 mM), the spasmogenic response to these methylxanthines was significantly increased compared with the equivalent response observed in time-matched control tissues; NIF (1 μM) reduced the ability of PDA (1 μM) to enhance the spasm evoked by caffeine and theophylline (Fig. 4).

4. Discussion

4.1. The biphasic response to PDA in human isolated bronchus

The present observation that PDA (1–5 μM) transiently inhibits but then increases the spontaneous tone of human isolated bronchus, confirms and extends the findings of earlier workers with a different active phorbol ester, phorbol 12,13-dibutyrate (Rossetti et al., 1995; Yang and Black 1995). The biphasic response, relaxation followed by contraction, elicited by active phorbols in human airway smooth muscle differs from the pattern of responses described in non-human airway smooth muscle preparations (Park and Rasmussen, 1985; Menkes et al., 1986; Huang et al., 1987; Schramm and Grunstein, 1989; Knox et al., 1993; Cortijo et al., 1994; Gunst et al., 1994), as well as from responses observed in human pulmonary artery (Savineau et al., 1991), thereby confirming the existence of tissue- and species-dependent differences in the responsiveness to phorbol esters.

The lack of effect of blockade of muscarinic receptors, histamine H_1-receptors, and α-adrenoceptors on PDA-induced contraction indicated that it did not act via these receptors or indirectly by releasing the corresponding mediators. Inhibition of prostaglandin synthesis by indomethacin or inhibition of 5-lipoxygenase with zileuton had no effect on PDA responses, indicating that they were not due to prostaglandin or leukotriene production. The response to PDA was also independent of the presence of epithelium; therefore, epithelium-derived factors are not presumably involved.

Since phorbol esters directly activate protein kinase C by binding to the enzyme (Castagna et al., 1982), we decided to study the effects of three structurally unrelated inhibitors of this kinase. Staurosporine and H-7 are widely used as inhibitors of protein kinase C, but may not be specific inhibitors (Wilkinson and Hallam, 1994); hence, calphostin C, a more specific inhibitor with less effect on other kinases (Kobayashi et al., 1989), was also used in these experiments. Calphostin C and staurosporine had little effect on spontaneous tone of human bronchus compared to relaxation promoted by H-7, a finding that confirms and extends previous results from Yang and Black (1995) and Rossetti et al. (1995). Incubation with each of these three protein kinase C inhibitors significantly reduced the contractile response to PDA, thus, suggesting that activation of protein kinase C is involved in this response. These results are consistent with those reported by Yang and Black (1995) and Rossetti et al. (1995) for staurosporine against responses to phorbol 12,13 dibutyrate. The specificity of action of the active phorbol esters on protein kinase C activation is further supported by the finding that the inactive phorbol ester 4α-phorbol 12,13 didecanoate had no contractile or relaxant effect in human isolated bronchus (Rossetti et al., 1995).

The contractile response to PDA was virtually abolished in Ca^{2+}-free medium or in the presence of NIF (1 μM). These findings are consistent with a reduced contraction of human bronchus in response to phorbol 12,13 dibutyrate after removal of external Ca^{2+} or addition of verapamil (Rossetti et al., 1995; Yang and Black, 1996). Therefore, the initiation of contraction of human airway smooth mus-

cle that follows acute activation by phorbol esters of protein kinase C, appears supported by Ca^{2+}-influx through L-type voltage-dependent channels. The finding that the contraction promoted by PDA in KCl-contracted tissues was greater than the contraction observed on acetylcholine-contracted tissues (this study) is in agreement with a similar observation of Yang and Black (1996) for phorbol 12,13-dibutyrate in human bronchi and may be interpreted as giving further support to this notion.

However, the sensitivity of PDA-induced spasm to inhibitors of L-type Ca^{2+}-channels does not imply that the source of activator Ca^{2+} for contraction is exclusively of extracellular origin. Bourreau et al. (1993) have suggested that in airway smooth muscle, spasmogenic agents which promote the release of Ca^{2+} from intracellular stores are also sensitive to inhibitors of L-type Ca^{2+} channels due to refilling of intracellular stores by extracellular Ca^{2+} entry through such channels.

Neither of the pharmacological intervention cited above reduced the initial relaxation observed in response to PDA. Na^+/K^+-ATPase is a substrate for protein kinase C (Nishizuka, 1986), and it may be that, by activating protein kinase C, phorbol esters can stimulate the activity of Na^+/K^+-ATPase, thus, producing hyperpolarisation, closure of voltage-dependent channels and relaxation. The presence of an active electrogenic Na^+ pump has been described in human airway smooth muscle, and ouabain (10 μM) is a selective inhibitor of this mechanism (Chideckel et al., 1987; Cortijo et al., 1997). Cooling of preparations also results in inhibition of the Na^+ pump (Souhrada and Souhrada, 1981). The finding that PDA produced a contractile response not preceded by relaxation in ouabain-treated, as well as in cooled preparations, indicated that activation of Na^+/K^+-ATPase is likely to be the mechanism involved in the relaxation observed immediately after addition of PDA to human isolated bronchus.

In different human cells, activation of protein kinase C by phorbol esters stimulates an amiloride sensitive Na^+/H^+ antiport (Besterman et al., 1985). However, amiloride (10 μM) did not alter the relaxation or the contraction in response to PDA, suggesting that the Na^+/H^+ exchange system is not involved. This finding would be consistent with results reported for other phorbol esters in rabbit and bovine trachea (Schramm and Grunstein, 1989; Knox et al., 1993).

4.2. Effects of PDA on the relaxant and spasmogenic responses of methylxanthines

Treatment of human bronchus with PDA (1–5 μM) resulted in a reduction of the maximal relaxation produced by caffeine and theophylline in preparations with spontaneous tone at 37°C. The inhibition of the maximal relaxation in response to methylxanthines produced by PDA was not a simple consequence of PDA having increased the tissue tone, since this reduced relaxation by PDA was not observed for isoprenaline and levcromakalim under similar experimental conditions. Furthermore, the differential effects of PDA on the relaxations produced by theophylline and isoprenaline were also observed in preparations precontracted with histamine.

Methylxanthines, but not isoprenaline, have been shown capable of releasing Ca^{2+} from intracellular stores in airway smooth muscle (Chopra et al., 1991; Takuwa et al., 1987). Therefore, we considered the possibility that PDA reduced the relaxant activity of methylxanthines by facilitating the methylxanthine-induced release of Ca^{2+} from intracellular stores, as previously suggested to occur in guinea pig tracheal muscle (Cortijo et al., 1994). As mentioned above, the susceptibility of the interaction between PDA and the methylxanthines to inhibition by NIF found in this study may be a reflection of the fact that refilling of the intracellular Ca^{2+} store requires the entry of extracellular Ca^{2+} directly into the store via L-type Ca^{2+}-channels (Bourreau et al., 1993). Alternatives, such as augmented influx of Ca^{2+} through L-type channels or increased sensitivity to Ca^{2+} of the intracellular contractile machinery (Bremerich et al., 1998; Yoshii et al., 1999) appear unlikely since PDA neither potentiates the contractile responses to Ca^{2+} acting on K^+ depolarized human bronchus (this study) nor sensitizes Triton X-100 skinned trachealis muscle fibres to Ca^{2+} (Cortijo et al., 1992a). In the look for a further support for the hypothesis of facilitated release of intracellular Ca^{2+} by phorbol esters, we explore the influence of PDA on the spasmogenic responses to methylxanthines in human airway smooth muscle.

By contrast with the relaxant effects obtained at 37°C, Small et al. (1988) and Ortiz et al. (1988, 1991) have shown that methylxanthines evoke spasm of cooled guinea pig trachea and that such spasmogenic effect results from methylxanthine-induced release of Ca^{2+} from intracellular stores. In the present study, we have extended these observations to show that relaxation to methylxanthines at 37°C was reversed to a contraction in cooled human airway preparations. The spasmogenic response produced by caffeine or theophylline (10 mM) at 10°C was not reduced by incubation in Ca^{2+}-free, EGTA (0.1 mM)-containing medium, or in the presence of NIF (1 μM), thus, indicating that activator Ca^{2+} was mainly of intracellular origin. This finding would be consistent with the report by Chideckel and Anireddy (1991) that caffeine (25 mM) releases intracellular Ca^{2+} from a ryanodine-sensitive pool, thus, producing a very small and transient contraction, followed by relaxation of the human trachealis at 37°C.

PDA augmented the spasmogenic response to methylxanthines in cooled preparations (this study). As suggested above, this enhancing effect of PDA on methylxanthines-induced contraction may be due to the facilitation by phorbol esters of the intracellular Ca^{2+} release triggered by methylxanthines. This would be consistent with the finding that PDA augments the spasmogenic responses to

methylxanthines and ryanodine in guinea pig isolated trachea (Cortijo et al., 1994). Also, the inhibition by NIF of the enhancing effect of PDA suggests that refilling of internal Ca^{2+} stores requires Ca^{2+} entry via L-type channels, as mentioned above (Bourreau et al., 1993).

In summary, these findings indicate that protein kinase C activation modulates the tone of human airways and alters their responsiveness to therapeutically relevant drugs, such as theophylline. An inappropriate protein kinase C activation may be involved in the pathogenesis of asthma (Bradshaw et al., 1993), but the potential value of protein kinase C inhibitors in asthma treatment is presently uncertain.

Acknowledgements

This work was supported by grants SAF97-0047 and SAF99-0111 from ''Comisión Ministerial de Ciencia y Tecnología'' (CICYT) of the Spanish government, by fellowships of the Ministry of Education, and by grants from ''Generalitat Valenciana'' (local government of the ''Comunidad Valenciana'', Valencia, Spain). The authors are indebted to the surgical team of the Services of Thoracic Surgery of the Hospital La Fe and Hospital Clínico, and to the staff of the Pathology Services of these two hospitals of the University of Valencia for kindly making the human lung tissue available to us. The authors also wish to thank P. Santamaria and S. Marti for technical assistance.

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