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The involvement of protein kinase C in the contraction of human airway smooth muscle

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Abstract

The involvement of protein kinase C in the contraction of airway smooth muscle has been investigated in human isolated bronchus. Phorbol 12,13-dibutyrate (PDB) (10 μM) produced a biphasic response – relaxation followed by contraction. The protein kinase C inhibitor, staurosporine (0.1 μM) reduced the contractile response to PDB from 89 ± 2.9% to 53 ± 4.5% of the response to 1 mM acetylcholine ($P < 0.05$, $n = 6$) but increased the relaxation response from 12 ± 6.1% to 29 ± 5% ($P < 0.05$, $n = 6$). Staurosporine also reduced the maximal contractile response to a single dose of histamine (10 μM) from 121 ± 13% to 91 ± 10% ($P < 0.05$, $n = 4$) and the sustained phase tension from 94 ± 4% to 85 ± 5% at 30 min ($P < 0.05$, $n = 4$). However, GF 109203X, a more selective inhibitor of protein kinase C at 0.1 μM, 1 μM and 10 μM had no effect on the maximal contractile response and reduced only the sustained phase of the contraction to histamine. These results suggest that protein kinase C plays a role in maintenance of contraction in human airway smooth muscle.

Keywords: Contraction; Histamine; Bronchus, human; Protein kinase C; GF 109203X; Phorbol ester

1. Introduction

Protein kinase C was first found in 1977 as a proteolytically activated protein kinase in many tissues (Inoue et al., 1977). Later, it was shown to be a Ca^{2+}-activated, phospholipid-dependent enzyme (Takai et al., 1979). Since Kuo and colleagues (1980) identified protein kinase C in animal vascular and tracheal smooth muscle, the function of protein kinase C in smooth muscle has been investigated. A cellular model of Ca^{2+}-dependent regulation of smooth muscle contraction has been developed (Dillon et al., 1981; Kamm and Stull, 1985) and this consists of two phases (Rasmussen et al., 1987). The initial phase is mediated by an activation of myosin light chain kinase resulting in the phosphorylation of the 20000 Da myosin light chain; the second phase is a sustained phase in which activation of protein kinase C results in the phosphorylation of both structural and regulatory components of the filamin-actin-desmin fibrillar domain. Protein kinase C also phosphorylates myosin light chain at different sites to those phosphorylated by myosin light chain kinase (Kamm et al., 1989).

Phorbol esters, which have a diacylglycerol-like structure, are tumour promoters and directly activate protein kinase C both in vitro and in vivo by binding to the enzyme (Castagna et al., 1982). Phorbol esters induce contractile responses in vascular smooth muscle in rabbit aorta (Sybertz et al., 1986; Gleason and Flaim 1986), rat aorta (Litter et al., 1987), canine saphenous vein (Chiu et al., 1988), rat and human pulmonary arteries (Savineau et al., 1991). In airway smooth muscle studies, phorbol 12-myristate 13-acetate (PMA) alone failed to induce contraction in calf tracheal smooth muscle, although a contractile response was induced by PMA with Ca^{2+} ionophore (Park and Rasmussen, 1985). Phorbol 12,13-dibutyrate (PDB) induced contraction in bovine bronchial rings (Knox et al., 1993). There has been, however, no study carried out in human airway smooth muscle.

Histamine is a potent bronchoconstrictor released from mast cells, which acts on airway smooth muscle to produce airway narrowing. Recent studies suggest that histamine binding to histamine H_1 receptors causes internal Ca^{2+} release resulting in raised cytosolic Ca^{2+}

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concentration (Kotlikoff et al., 1987). Thus histamine-induced activation of smooth muscle would be expected to involve inositol 1,4,5-trisphosphate (IP_3)-triggered release of Ca^{2+} from the sarcoplasmic reticulum (Berridge and Irvine, 1984; Van Amsterdam et al., 1990) and diacylglycerol-stimulated activation of protein kinase C. Protein kinase C causes a late phosphorylation of myosin light chain in bovine carotid arteries after 60 min incubation with histamine and this suggests that protein kinase C may play a role in the sustained phase of the contractile response to histamine (Takuwa et al., 1988). There has been, however, no such functional study carried out in human airway smooth muscle.

The aims of the present study were to investigate the effect of stimulation of protein kinase C by phorbol ester and its inhibition. In addition, the effect of protein kinase C inhibition on the contractile response to histamine was investigated. PDB was used to stimulate protein kinase C and three inhibitors of protein kinase C – staurosporine, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) (Gschwendt et al., 1984; Hidaka et al., 1984) and a more specific inhibitor of protein kinase C, bisindolylmaleimide (GF 109203X) (Toullec et al., 1991) were used to estimate the role of protein kinase C activation in the contractile response to histamine.

2. Materials and methods

2.1. Tissue preparation

Samples of human lung were obtained from patients undergoing resection of pulmonary carcinoma. The protocol had been approved by the Human Ethical Review Committee of The University of Sydney. Macroscopically normal lung tissue was transported to the laboratory in Krebs-Henseleit solution (composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and D-glucose 11.1) at 4°C. Bronchi were dissected free from surrounding parenchyma and cut into rings measuring 2–4 mm in internal diameter and 4–5 mm in length. Paired rings of bronchus were mounted on stainless steel hooks and 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 by Black et al. (1988). Bronchial rings were allowed to equilibrate against a 1–1.5 g of tension for 1–2 h during which time the Krebs-Henseleit solution was exchanged at 15–20 min intervals. When stable tone had been established, the following experiments were commenced. Changes in tension were measured isometrically with Grass FTO3 transducers and recorded on Grass polygraphs.

2.2. The effects of H-7 and staurosporine on the baseline tone of human isolated bronchus

Cumulative concentration-response curves to H-7 (over the range 1 nM–300 μM) and staurosporine (over the range 1 nM–300 μM) were obtained in the tissues. In separate experiments, when the baseline tone was stable, one tissue from each pair was incubated in staurosporine (0.1 μM) for 10 min before cumulative concentration-response curves were elicited to H-7 (1 nM–300 μM).

2.3. The effects of PDB in the presence and absence of staurosporine

In separate experiments, when the baseline tone was stable, a single dose of acetylcholine (1 mM) was added to each tissue. After the response to acetylcholine had reached a plateau (which took less than 5 min), the tissues were washed repeatedly at 15 min intervals until a stable baseline tone was re-established. One tissue from each pair was incubated in staurosporine (0.1 μM) for 10 min, before a single dose of PDB was added to each tissue, at a final bath concentration of 10 μM. Changes in tension in response to the addition of PDB to the bath were monitored over a period of 30 min.

2.4. The effects of staurosporine on the contractile response to histamine

In a separate series of experiments, when stable tone was established, a supramaximal dose of acetylcholine (1 mM) was added to the baths and the contractile response was allowed to plateau before the acetylcholine was removed from the baths by repeated washing of the tissues. When baseline tone was re-established, half of the tissues were incubated in 0.1 μM staurosporine for 10 min. Histamine cumulative concentration-response curves (10 nM–300 μM) were then elicited in each tissue. In a separate series of experiments, after the acetylcholine was washed out, a single bolus dose of histamine (10 μM) was added to the tissue instead of the construction of cumulative concentration-response curves. Isometric changes in tension in response to histamine in the presence and absence of staurosporine were monitored over a period of 90 min.

2.5. The effects of GF 109203X on the contractile response to histamine

In a separate series of experiments, when stable tone was established, a supramaximal dose of acetylcholine (1 mM) was added to the baths and the contractile response was allowed to plateau before the

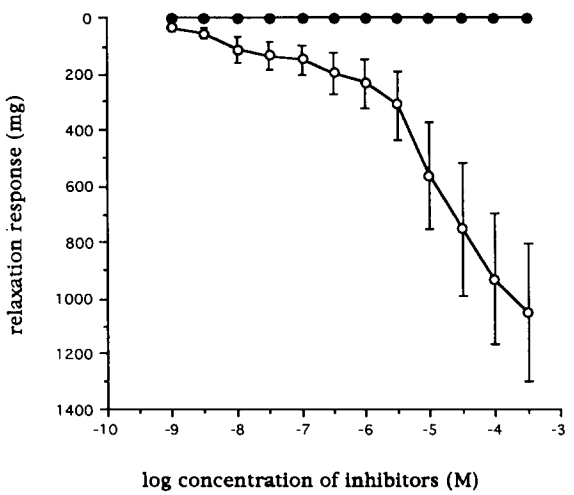

Fig. 1. Mean cumulative concentration-response curves to staurosporine (●) for 3 experiments and H-7 (○) for 8 experiments. Relaxation response is expressed as tension in mg. Vertical bars indicate the standard error of the mean.

acetylcholine was removed from the baths by repeated washing of the tissues. When the tension returned to baseline levels, three of the four tissues from each patient received a different concentration of GF 109203X. The fourth tissue acted as a control. The concentrations used were 0.1 μM, 1 μM and 10 μM and the tissues were incubated in GF 109203X for 20 min before 1 mM histamine was added to each tissue. Changes in tension in response to histamine in the presence and absence of GF 109203X were monitored over a period of 90 min.

2.6. Analysis of results

In experiments in which H-7 and staurosporine cumulative concentration-response curves were studied, the responses at each concentration of H-7 and staurosporine were expressed as tension changes in mg from the baseline level. In experiments in which histamine cumulative concentration-response curves were studied, the response at each concentration of histamine was expressed as a percentage of the response to 1 mM acetylcholine. Where a single dose of PDB or histamine was administered, tension was measured at 5 min intervals and was expressed as a percentage of the initial response to 1 mM acetylcholine. A geometric mean EC_{50} value and pD_2 value for each drug in the cumulative concentration-response curves studies was derived. Where duplicate preparations were studied, a mean response curve was constructed for each experiment. Two-tailed paired Student's t-tests and analysis of variance (ANOVA) with Fisher probability test were used to compare the mean results for treated and untreated tissues and differences were considered as statistically significant at $P \leq 0.05$.

2.7. Compounds used

Acetylcholine, histamine acid phosphate, isoprenaline, phorbol 12,13-dibutyrate (PDB), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) and staurosporine were purchased from Sigma Chemical Co., St. Louis, MO, USA. Bisindolylmaleimide GF 109203X was purchased from Calbiochem. All compounds (except isoprenaline and PDB) were dissolved in distilled water. Isoprenaline was dissolved in 0.01 M HCl and stored at −20°C. PDB was dissolved in 10% dimethyl sulphoxide (DMSO) and stored at −70°C. The maximum concentration of DMSO added to the bath was < 0.1% and at this concentration had no effect itself on the tone of bronchial rings. Serial dilutions were made on the day of the experiment using Krebs-Henseleit solution and were kept on ice.

3. Results

3.1. The effects of H-7 and staurosporine on the baseline tone of human isolated bronchus

Staurosporine (1 nM–300 μM) had no effect on the baseline tone of human isolated bronchus. However, H-7 (1 nM–300 μM) caused dose-related relaxation in the bronchial rings. The mean maximal relaxation response to H-7 was 1050 ± 247 mg (n = 8) (Fig. 1). The mean pD_2 value was 5.02 ± 0.18 (n = 8). In the tissues which were pretreated with 0.1 μM staurosporine, H-7-induced relaxation was significantly inhibited at lower concentrations (Fig. 2). However, staurosporine did not decrease the maximal relaxation response to

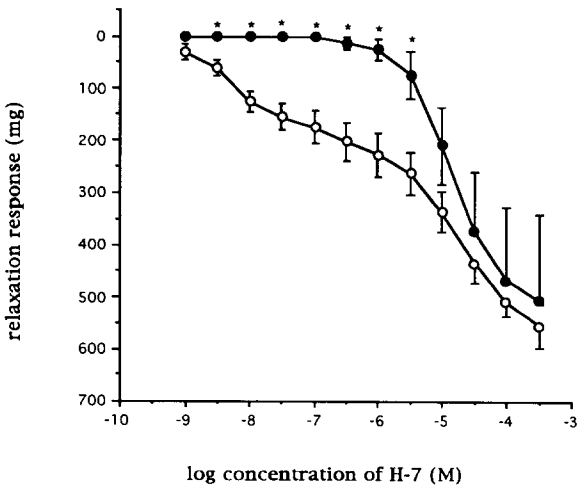

Fig. 2. Mean cumulative concentration-response curves to H-7 in the presence (●) and absence (○) of staurosporine (0.1 μM) for 3 experiments. Relaxation response is expressed as tension in mg. Vertical bars indicate the standard error of the mean. * Significant difference at $P < 0.05$, Student's t-test.

H-7 which was 506 ± 164 mg compared with 555 ± 42 mg in control tissue ($P > 0.05$, $n = 3$). Since H-7 exhibited such marked effects on baseline tone, only staurosporine was used in subsequent experiments.

3.2. The effects of PDB in the presence and absence of staurosporine

PDB at a concentration of 10 μM produced a biphasic response – a relaxation followed by a contraction. Initially, after PDB was added, a small relaxation response was induced over the first 5 min. The magnitude of the mean maximum relaxation response was 12 ± 6.1% ($n = 6$) of the response to acetylcholine (1 mM). After the relaxation response had plateaued, the contractile response developed over the next 30 min. The maximum contraction was equal to 89 ± 2.9% ($n = 6$) of that produced by acetylcholine (1 mM). In the tissues which were incubated with 0.1 μM staurosporine for 10 min, the initial relaxant response was significantly increased to 29 ± 5% ($P < 0.05$, $n = 6$). Furthermore, the subsequent contractile response was significantly decreased to 53 ± 4.5% ($P < 0.05$, $n = 6$) (Fig. 3).

3.3. The effects of staurosporine on the contractile response to histamine

When histamine was studied in cumulative concentration-response curves (10 nM–300 μM), staurosporine had no effect on contractile responses, in that the maximal response to histamine was unaffected by prior incubation of the tissues in 0.1 μM staurosporine (Fig. 4). The mean values were 102 ± 8.1% of the response to acetylcholine in the control tissues and 100.4 ± 9.4% in tissues pre-incubated in staurosporine ($P > 0.05$, $n = 5$). Moreover, there was no significant shift in the cumulative concentration-response curves with mean pD_2 values of 5.40 ± 0.27 and 5.41 ± 0.29 respectively ($P > 0.05$, $n = 5$). However, when histamine was added as a single dose (10 μM) and contraction monitored over time, tissues treated with staurosporine contracted significantly less to histamine. The maximal contraction was 91 ± 10% of the response to acetylcholine compared to 121 ± 13% in control tissues ($P < 0.05$, $n = 4$) (Fig. 5). In addition, when the contractile responses to 10 μM histamine were examined over time, significant tension decreases were thus seen in the sustained phase of the response to histamine in the presence of staurosporine. At 30 min after histamine administration, tone was 81 ± 10% compared with 116 ± 12% in control tissues ($P < 0.05$, $n = 4$) and, at 60 min, 64 ± 11% in staurosporine-treated compared with 97 ± 13% in control tissues ($P < 0.05$, $n = 4$).

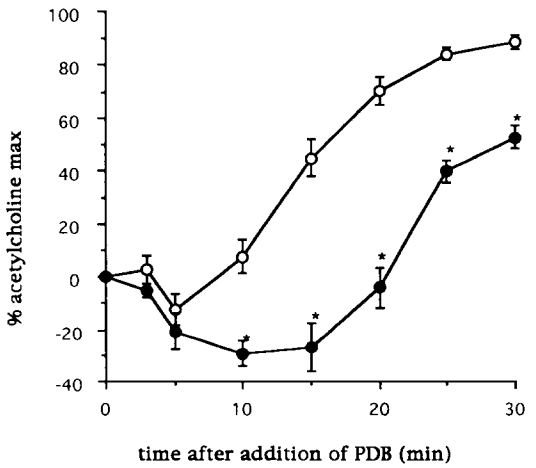

Fig. 3. Mean contractile responses to PDB (10 μM) single dose in the absence (○) and presence (●) of staurosporine (0.1 μM) for 6 experiments were expressed as a percentage of the initial maximal response to acetylcholine (1 mM) over time (min). Vertical bars indicate the standard error of the mean. * Significant difference at $P < 0.05$, Student's t-test.

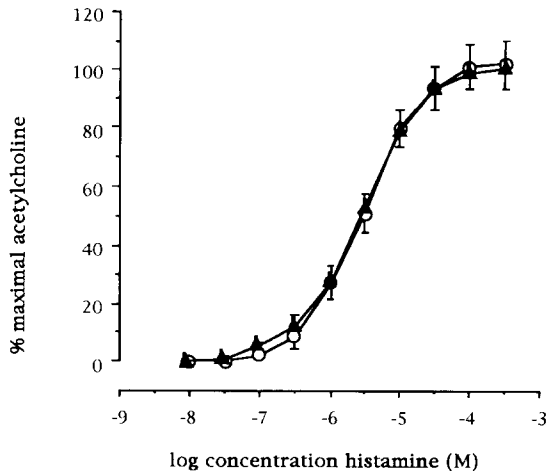

Fig. 4. Mean cumulative concentration-response curves for histamine in the presence (●) and the absence (○) of staurosporine (0.1 μM) in human bronchus. Mean responses for 5 experiments are expressed as a percentage of the reference response obtained to 1 mM acetylcholine. Vertical bars indicate the standard error of the mean.

3.4. The effects of GF 109203X on the contractile response to histamine

Histamine at a concentration of 1 mM induced a contractile response which was 110 ± 6% of the response to 1 mM acetylcholine ($n = 4$), and this was unaffected by preincubation of the tissues in 0.1 μM, 1 μM or 10 μM GF 109203X, where the responses were 101 ± 2%, 100 ± 6% and 103 ± 5% ($n = 4$) respectively. However, when the contractile responses to 1 mM histamine were examined over time, tone in treated

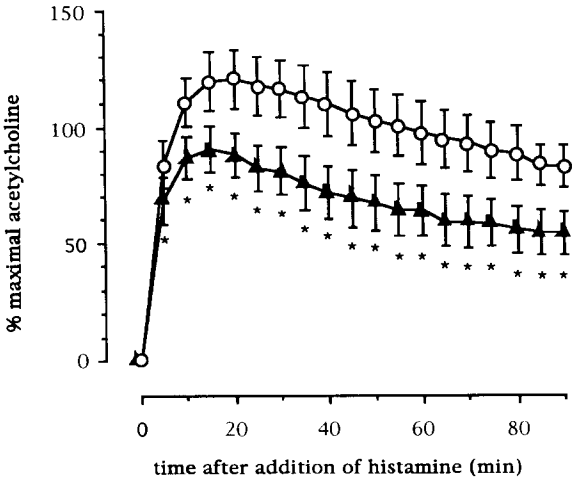

Fig. 5. Mean contractile response to histamine (10 μM) single dose in the presence (▲) and the absence (○) of staurosporine (0.1 μM) in human bronchus. Mean responses for 4 experiments are expressed as a percentage of the maximal response to acetylcholine (1 mM). Vertical bars indicate the standard error of the mean. * Significant difference at $P < 0.05$, ANOVA.

tissues returned to baseline levels more rapidly. Significant tension decreases were thus seen in the sustained phase of the response to histamine in the presence of all 3 concentrations of GF 109203X. At 30 min after histamine administration, tone was 101 ± 2% in control tissues compared with that in the tissues pretreated with GF 109203X at a concentration 0.1 μM which was 90 ± 3% ($P < 0.05$, $n = 4$). At 1 μM tone was 87 ± 5% ($P < 0.05$, $n = 4$) and at 10 μM it was 81 ± 3% ($P < 0.05$, $n = 4$). At 60 min, these values were 91 ± 2% in control compared with 77 ± 4%, 76 ± 2% and 54 ± 6% in the tissues treated with GF 109203X 0.1 μM, 1 μM and 10 μM ($P < 0.05$, $n = 4$) (Fig. 6).

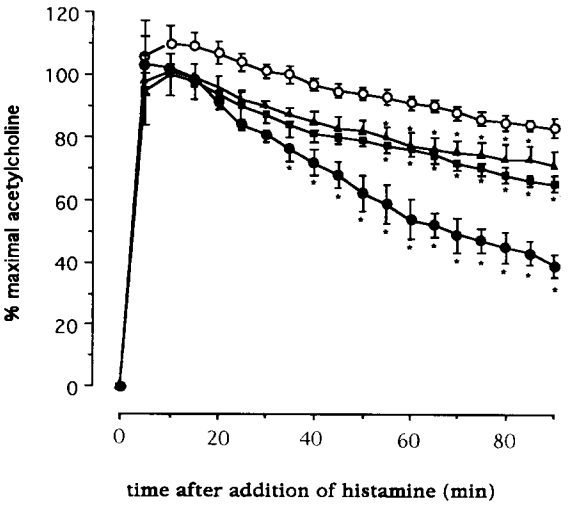

Fig. 6. Mean contractile response to histamine (1 mM) single dose in the absence (○) and the presence of GF 109203X 0.1 μM (▲), 1 μM (■) and 10 μM (●) in human bronchus. Mean responses for 4 experiments are expressed as a percentage of the maximal response to acetylcholine (1 mM). Vertical bars indicate the standard error of the mean. * Significant difference at $P < 0.05$, ANOVA.

4. Discussion

These studies show that, in human airway smooth muscle, activation of protein kinase C by the phorbol ester, PDB, produced a biphasic response – relaxation followed by contraction. The contractile response was greater than the relaxation. Inhibition of protein kinase C activity, by preincubation of the tissues in staurosporine, significantly reduced the contractile responses to PDB and histamine, as well as the sustained phase of the contractile response to histamine. Staurosporine over the range of 1 nM–300 μM had no effect on the baseline tone of human bronchial rings. H-7 induced a relaxation response in human airway smooth muscle and staurosporine partially inhibited this response. The H-7-induced relaxation could perhaps have been due to inhibition of tonic protein kinase C activity. However, it is difficult to interpret the effects of staurosporine on this relaxation since both these compounds have nonspecific actions against a number of protein kinases which may be unrelated to protein kinase C.

The fact that a protein kinase C activator, PDB, induced a contractile response in human isolated bronchus indicates that this enzyme may play a role in the regulation of airway smooth muscle contraction. Studies of vascular smooth muscle reveal that another protein kinase C activator, PMA, induces a slowly developing and sustained contractile response in the rabbit ear artery (Forder et al., 1985) and in rabbit aorta (Khalil and Van Breemen, 1988). Merkel et al. (1991) used PDB in canine artery and found a similar result. However, in airway smooth muscle studies, PMA alone failed to induce contraction in calf tracheal smooth muscle, although a contractile response was induced by PMA in the presence of Ca^{2+} ionophore (Park and Rasmussen, 1985). The results of the present study provide evidence for the involvement of protein kinase C in the contraction of human airway smooth muscle.

A finding of interest in our studies was that PDB induced a biphasic response – a relaxation response followed by a contractile response in human isolated bronchus. We found that the PDB relaxation response was enhanced by staurosporine, whereas the contractile component was reduced. A possible explanation for this could be that the contractile component of the PDB response is related to PKC stimulation, whereas the relaxation component is unrelated. If the response to PDB is a result of these 2 opposite effects, then a diminution in the contraction by staurosporine could

result in an apparent augmentation of the relaxation. Souhrada and Souhrada (1991) also reported a similar biphasic response in guinea pig tracheal smooth muscle by stimulation with PMA (10 μM) and the relaxation response was blocked by amiloride (10 μM), a specific inhibitor of Na^+/H^+ exchange. This result suggests that phorbol esters can enhance the amiloride-sensitive plasma membrane Na^+/H^+ exchange system in airway smooth muscle. Whether this mechanism explains the relaxation response to PDB in the present study remains to be investigated. Knox et al. (1993) examined the effects of PDB in bovine bronchus. They reported only a contractile response. This is not surprising, however, since they elicited only cumulative responses with which it is difficult to observe a biphasic response to PDB.

Staurosporine and H-7 are widely used as inhibitors of protein kinase C (Gschwendt et al., 1984; Hidaka et al., 1984). Staurosporine is a more potent inhibitor than H-7. The IC_{50} value (concentration causing a 50% inhibition) for staurosporine is 30 nM and for H-7 is 4.5 μM (Herbert et al., 1990). In this study, H-7 caused a concentration-dependent relaxation of human isolated airway tissue, whereas staurosporine had no effect on the baseline tone. It was therefore decided to further study protein kinase C inhibition with staurosporine and not H-7.

Staurosporine binds to the ATP binding site on protein kinase C, and as this exhibits significant homology with the ATP binding site of other enzymes, it has been suggested that staurosporine may not be a specific protein kinase C inhibitor (Rüegg and Burgess, 1989). Myosin light chain kinase is one enzyme with an ATP binding site similar to protein kinase C, and causes phosphorylation of myosin light chain. According to Rasmussen's model (Rasmussen et al., 1987), the initial contraction is mediated by an activation of myosin light chain kinase and the phosphorylation of 20000 Da myosin light chain. During the sustained phase, at which time myosin light chain is dephosphorylated, simultaneously, several proteins which are also involved in the regulation of smooth muscle contraction, such as caldesmon, are phosphorylated and that phosphorylation is mediated via an activation of protein kinase C (Rasmussen et al., 1987). It is possible that the maximum response to histamine in the present study was caused by the activation of myosin light chain kinase and inhibited by staurosporine. However, the sustained phase of the contractile response to histamine was significantly decreased in the tissues preincubated with staurosporine. Compared with staurosporine, GF 109206X is a more specific inhibitor of protein kinase C and has less effect on myosin light chain kinase (Toullec et al., 1991). It reduced the sustained phase response to histamine in a dose-related manner without affecting the maximal contraction. These results strongly suggest that protein kinase C plays a role in the maintenance of contraction in human airway smooth muscle.

Although staurosporine had no effects on the cumulative concentration-response curves to histamine, it did inhibit the initial contraction and the sustained phase of the response to a single dose of histamine (10 μM). Hay (1990) has reported that staurosporine at concentrations of 10 nM and 0.1 μM had no effect on the cumulative concentration-response to endothelin-1 in guinea pig isolated trachea, although the contractile response in rat aorta was inhibited. However, the contractile response to 0.1 μM endothelin-1 (single dose) was reduced by staurosporine at 200 nM and 2 μM in human isolated airways (McKay, personal communication, unpublished data). These results indicate that the response to the same drug can be different in vascular and airway smooth muscle. In this study, there is no apparent explanation for the fact that cumulative concentration-response curves to histamine were unaffected by staurosporine in spite of an effect on a single dose, other than the difference in experimental protocol. However, the fact that GF 109203X inhibited only the sustained phase of the response to histamine and not the initial response could suggest that the staurosporine effect on the initial response could be a result of inhibition of myosin light chain kinase.

In summary, stimulation of protein kinase C by the phorbol ester PDB causes modulation of tone in human isolated bronchial smooth muscle. This response is inhibited by staurosporine, a protein kinase C inhibitor, which also decreases both maximal and sustained phase contractile response to histamine. GF 109203X only decreases the sustained phase contractile response. These findings implicate protein kinase C in the regulation of tone in human airways.

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