Potentiation of β-adrenoceptor function in bovine tracheal smooth muscle by inhibition of protein kinase C

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Abstract

To examine the role of contractile agonist-induced activation of protein kinase C (PKC) in functional antagonism of airway smooth muscle contraction by β-adrenoceptor agonists, we examined the effects of the specific PKC-inhibitor GF 109203X (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl) maleimide) on isoprenaline-induced relaxation of bovine tracheal smooth muscle contracted by various concentrations of methacholine and histamine. In the absence of GF 109203X, the potency of isoprenaline ($p\mathrm{D}_2$) was gradually reduced at increasing methacholine- and histamine-induced smooth muscle tones, but the maximal relaxation (E_{max}) was decreased only at higher concentrations of methacholine. In the presence of GF 109203X, $p\mathrm{D}_2$ values were significantly increased for both methacholine- and histamine-induced contractions. Moreover, isoprenaline E_{max} values in the presence of high concentrations of methacholine were also increased. Although both methacholine- and histamine-induced contractions were slightly reduced by GF 109203X, the changes in isoprenaline $p\mathrm{D}_2$ could only partially be explained by reduced contractile tone. In contrast to isoprenaline, forskolin-induced relaxations were not affected by GF 109203X. The results indicate that PKC activation contributes to the reduced β-adrenergic responsiveness induced by methacholine and histamine, which may involve uncoupling of the β-adrenoceptor from the effector system. Since many mediators and neurotransmitters in allergic airway inflammation can activate PKC, this cross talk may be important in the reduced bronchodilator response of patients with severe asthma.
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1. Introduction

Inhaled β$_2$-adrenergic agonists are effective bronchodilators and widely used to control airway function in asthma and chronic obstructive pulmonary disease (COPD) (Larj and Bleecker, 2002; Waldeck, 2002). The efficacy of these drugs in asthma is mainly due to the functional antagonism counteracting the bronchoconstrictor effects of neurotransmitters and mediators released in airway inflammation (Barnes, 1998).

However, it is well known that patients during a severe exacerbation of asthma have a reduced bronchodilator response to β$_2$-adrenoceptor agonists (Torphy, 1988). Most bronchoconstrictive receptors, including muscarinic M$_3$- and histamine H$_1$-receptors, activate G$_q$-protein-coupled receptors and cause bronchoconstriction through phosphatidyl inositide hydrolysis, resulting in the formation of inositol trisphosphate which releases Ca^{2+} from internal stores to initiate contraction. Subsequently, a sustained influx of extracellular Ca^{2+} and a sn-1,2-diacylglycerol (DAG)-induced activation of protein kinase C (PKC) follows, which are both implicated in the tonic phase of contraction (Hoiting et al., 1996; Yang and Black, 1995).

β$_2$-Adrenergic agonists mediate relaxation of airway smooth muscle by stimulation of G$_s$-coupled β$_2$-adrenocep-

tors, which results in the activation of adenylyl cyclase (AC) to generate cyclic adenosine $3',5'$-monophosphate (cAMP), which in turn activates cAMP-dependent protein kinase A (PKA) (Torphy et al., 1985). Phosphorylation of specific target proteins by PKA results in various biochemical responses that induce smooth muscle relaxation, by reducing intracellular $[Ca^{2+}]$ and diminishing the Ca^{2+} sensitivity of the contractile elements.

Both signalling pathways, however, do not appear to be independent, but are likely to interact at different homeostatic levels. It is well known that contractions induced by muscarinic agonists are relatively resistant to relaxation by β_2-agonists compared with contractions induced by histamine. Thus, in canine and bovine tracheal smooth muscle it has been demonstrated that β_2-adrenoceptor-mediated and nonreceptor-mediated elevation of cAMP potently inhibited histamine- but not methacholine-induced accumulation of inositol phosphates (Hall and Hill, 1988; Madison and Brown, 1988). Furthermore, several studies have shown that exaggerated cholinergic stimulation of airway smooth muscle causes a reduced relaxability of the muscle by β_2-adrenoceptor agonists. In human (Raffestin et al., 1985; Van Amsterdam et al., 1990) and animal (Roux et al., 1995; Russell, 1984; Torphy et al., 1985; Van Amsterdam et al., 1989) airway smooth muscle, it has been found that both the potency and the maximal relaxation are gradually reduced in the presence of increasing concentrations of contractile agonists. Such diminished functional antagonism has also been observed in vivo (Jenne et al., 1987), which may explain why β_2-adrenoceptor agonists become less effective during severe asthmatic episodes, whereas their efficacy is unchanged in patients with mild or asymptomatic asthma (Barnes and Pride, 1983; Tattersfield et al., 1983).

Although it has been found in various cells and tissues that activation of PKC may lead to uncoupling of the β_2-adrenoceptor, presumably via phosphorylation of the β_2-adrenoceptor and/or G_s (Abdel-Latif, 1991; Grandordy et al., 1994; Xu et al., 1997), the specific role of contractile agonist-induced PKC activation in functional antagonism has never been addressed in the airways. In this study we present direct evidence for the involvement of PKC in the (acute) functional antagonism of contractile agonist-induced bovine airway smooth muscle contraction by β_2-adrenoceptor agonists, using a specific, nonselective PKC inhibitor GF 109203X (2-[1-(3-dimethylamino-propyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl) maleimide) (Toullec et al., 1991), which inhibits all conventional (α, β_1 and β_2) and novel (δ, ϵ and θ) PKC isozymes present in bovine tracheal smooth muscle (Webb et al., 1997). The marked involvement of PKC in the cross talk between contractile agonist-induced contraction and β_2-adrenoceptor agonist-induced relaxation of airway smooth muscle may be of considerable importance by reducing the bronchodilator response of patients with severe asthma.

2. Materials and methods

2.1. Reagents

Dulbecco's modification of Eagle's medium (DMEM), fetal bovine serum, NaHCO₃ solution (7.5%), penicillin/streptomycin solution (5000 U/ml; 5000 μg/ml) and HEPES solution (1 M) were obtained from Gibco BRL Life Technologies (Paisley, UK). Methacholine chloride, histamine dihydrochloride, (−)-isoprenaline hydrochloride and forskolin were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and GF 109203X (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl) maleimide) was purchased from Boehringer (Mannheim, Germany).

All other chemicals were of analytical grade.

2.2. Tissue preparation

Fresh bovine tracheas were obtained from the slaughterhouse and were transported to the laboratory within 30 min at room temperature in Krebs–Henseleit (KH) buffer of the following composition (nM): NaCl 177.5, KCl 5.6, MgSO₄ 1.2, CaCl₂ 2.5, NaH₂PO₄ 1.3, NaHCO₃ 25.0, glucose 5.5, pregassed with 95% O₂ and 5% CO₂; pH 7.4. The tracheal smooth muscle was dissected carefully and smooth muscle strips (12x3 mm) were prepared free of mucosa and serosal connective tissue in KH buffer gassed with 95% O₂/5% CO₂ at room temperature. Subsequently, all strips were maintained overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 mM NaHCO₃, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum at 37 °C (55 rpm).

2.3. Mechanical responses

After washing in several volumes of KH buffer, gassed with 95% O₂ and 5% CO₂, pH 7.4 at 37 °C, the bovine tracheal smooth muscle strips were mounted in 15 ml organ baths containing gassed KH buffer (37 °C) for isotonic recording, using a preload of 500 mg. No basal myogenic tone was observed in bovine tracheal smooth muscle. After a 60-min equilibration period the strips were precontracted twice with methacholine (0.1, 1, 10 and 0.1, 1, 10, 100 μM, respectively) with a 60-min washing period in between. Maximal relaxation was established with isoprenaline (0.1 μM), immediately followed by a 30-min washing period.

Cumulative concentration response curves were made with methacholine (1 nM–100 μM) and histamine (10 nM–100 μM) in the absence and presence of 10 μM GF 109203X. In separate experiments it was found that this concentration of GF 109203X caused complete inhibition of 10 μM phorbol 12-myristate 13-acetate (PMA)-induced contraction.

For relaxation studies, the preparations were precontracted with different concentrations of methacholine (100 nM–100 μM) or histamine (3 μM–1 mM) in the absence or presence of 10 μM GF 109203X, increasing smooth muscle tone gradually in 2–4 concentration steps. Subsequently, cumulative concentration–relaxation curves were obtained with isoprenaline (using 1 nM–100 μM for methacholine-induced tone and 0.1 nM–10 μM for histamine-induced tone) or forskolin (0.1 nM–10 μM). After the maximal relaxation had been obtained, the preparations were washed twice and maximal relaxation of the smooth muscle strips was re-established with 10 and 100 μM isoprenaline.

When used, GF 109203X was administered 45 min before building up smooth muscle tone with methacholine or histamine.

In separate experiments, the reduction of methacholine- and histamine-induced contractile tone in the presence of GF 109203X was carefully compensated for by additional administration of small amounts of agonist, before isoprenaline relaxation curves were obtained.

2.4. Data analysis

Contractile responses were expressed as percentages of the response to 100 μM methacholine in the second precontraction curve in each experiment. Maximal relaxant effects of isoprenaline were expressed as percentages of contractile agonist-induced tone.

All data are presented as mean ± S.E.M. Curves were fitted using the logistic four-parameter model (Sigmaplot 8.0). Statistical analysis was performed by means of the two-tailed Student's t-test for paired or unpaired observations. P values <0.05 were considered statistically significant.

3. Results

3.1. Effect of GF 109203X on methacholine- and histamine-induced contraction

GF 109202X caused a small but significant shift to the right of the cumulative concentration–contraction curve of methacholine, with a reduction in pD_2 (i.e. $-\log EC_{50}$) from 7.05 ± 0.07 to 6.67 ± 0.06 ($P < 0.05$), while the maximal contractile effect (E_{max}) was unchanged (Fig. 1). For histamine-induced contraction, a pD_2 change from 5.88 ± 0.10 to 5.36 ± 0.11 ($P < 0.05$) was observed, which was accompanied by a small but significant decrease in E_{max}, from 107 ± 3 to $95 \pm 5\%$ ($P < 0.05$) (Fig. 1).

3.2. Functional antagonism of methacholine- and histamine-induced contraction by isoprenaline

Both for methacholine and histamine, it was found that at increasing smooth muscle tone isoprenaline-induced relaxation was gradually reduced (Figs. 2A and B, open symbols). However, the maximal changes in isoprenaline pD_2 and E_{max} values were considerably larger for methacholine than for histamine. Thus, for methacholine, a gradual fall of the pD_2 value of 3.4 log units was observed at contraction levels increasing from 24.9% (at 30 nM) to 108.9% (at 100 μM methacholine), while for histamine the pD_2 decrease amounted to 2.2 log units at smooth muscle tone ranging from 28.9% (1 μM) to 102.6% (1 mM histamine). In addition, at high contraction levels, the E_{max} of isoprenaline-induced relaxation was gradually reduced to only 5.6% in the presence of 100 μM methacholine, while with histamine only at the highest concentration a very small but significant decrease in E_{max} to 95.3% was observed (Table 1; Figs. 2A and B). Figs. 3A and B (open symbols) show that the isoprenaline pD_2 values were lower with methacholine than with histamine at equal levels of contractile tone.

3.3. Effect of GF 109203X on isoprenaline-induced relaxation

In the presence of the specific PKC inhibitor, both the pD_2 and the E_{max} values of isoprenaline-induced relaxations were
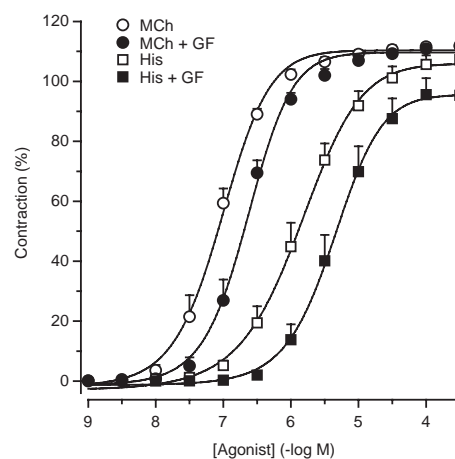

Fig. 1. Cumulative concentration–response curves of methacholine (MCh)- and histamine (His)-induced bovine tracheal smooth muscle contraction in the absence and presence of 10 μM GF 109203X (GF). Results are means ± S.E.M. of 4–6 experiments each performed in duplicate.

significantly enhanced at the different concentrations of methacholine used (Figs. 2A and 3A). Similarly, at 3, 10 and 100 μM histamine-induced contractile tones, significant increases in the isoprenaline pD_2 values were found (Figs. 2B and 3B). Although methacholine- and histamine-induced contractile tones were reduced in the presence of GF 109203X, the increased sensitivity to the β-agonist could not solely be explained by the reduced contraction levels. Thus, both for methacholine and histamine, isoprenaline pD_2 values were higher in the presence of the PKC inhibitor compared to control at equal levels of contractile tone (Figs. 3A and B). To overcome the effects of a reduced methacholine-induced contraction in the presence of GF 109203X on the potency and maximal relaxation of isoprenaline, in separate experiments the reduced contractile tones were compensated for by additional agonist administration.

3.4. Effect of GF 109203X on isoprenaline-induced relaxation after readjustment of contractile tone

In accordance with the results described above, both at methacholine- and histamine-induced contractions significantly higher isoprenaline pD_2 values were obtained in the presence of GF 109203X, when reduced contraction levels were readjusted by additional agonist administration (Figs. 4A and B; Table 2). However with histamine, the effects of GF 109203X were considerably smaller than with methacholine (Figs. 4A and B; Table 2). Fig. 4A also indicates that the potentiating effect of GF 109203X on the maximal isoprenaline-induced relaxation increased with increasing levels of the adjusted methacholine-induced smooth muscle tone. As before, with all histamine-induced contraction levels, both in the absence and presence of GF 109203X, maximum relaxation by isoprenaline was achieved.

3.5. Effect of GF 109203X on forskolin-induced relaxation

In contrast to isoprenaline, forskolin-induced relaxation of 1 μM methacholine-induced contraction was not affected by GF 109203X
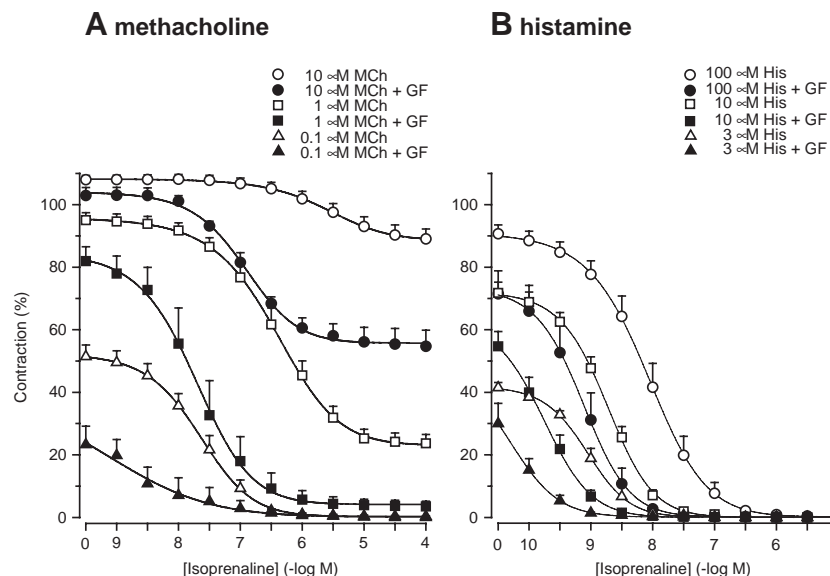
A methacholine   **B histamine**

Fig. 2. (−)-Isoprenaline-induced relaxation of bovine tracheal smooth muscle preparations precontracted with various concentrations of methacholine (MCh) and histamine (His) in the absence and presence of 10 μM GF 109203X. Results are means±S.E.M. of 4–14 experiments each performed in duplicate.

(Fig. 5), indicating a specific effect of methacholine-induced PKC activation on the β-adrenoceptor system.

4. Discussion

Stimulation of muscarinic M_3-receptors and histaminic H_1-receptors in bovine, human and canine tracheal smooth muscle activate phospholipase C (PLC), producing a rapid and dose-related increase of inositol phosphates including inositol 1,4,5-trisphosphate (IP_3) (Challiss et al., 1992; Chilvers et al., 1989; Grandordy et al., 1986; Hall and Hill, 1988; Hoiting et al., 1996; Madison and Brown, 1988; Meurs et al., 1989). The

formation of IP_3 is accompanied by a parallel increase in sn-1,2-diacylglycerol (DAG) (Roux et al., 1995). It has been reported that activation and translocation of PKC by DAG and Ca^{2+} is involved in the tonic phase of smooth muscle contraction (Langlands and Diamond, 1992; Park and Rasmussen, 1985). Thus, concentration-dependent methacholine-induced contraction of bovine tracheal smooth muscle is correlated with the translocation of PKC from the cytosol to the plasma membrane (Langlands and Diamond, 1992). Furthermore, in human airway smooth muscle PKC is involved in the sustained phase of histamine-induced contraction (Yang and Black, 1995). Overall, these data indicate that methacholine- and histamine-induced inositol phosphates

Table 1

(−)-Isoprenaline pD_2 and E_{max} values obtained after contraction of bovine tracheal smooth muscle strips with different concentrations of methacholine (MCh) and histamine (His) in the absence and presence of 10 μM GF 109203X (GF)

	Contraction level (%)		pD_2 ($-\log M$)		ΔpD_2	E_{max} (%)	
	Control	+GF	Control	+GF		Control	+GF
MCh (μM)							
0.1	51.4±3.7	21.6±8.1	7.69±0.08	8.70±0.04[b]	1.01	99.4±0.4	100
0.3	90.9±1.6	73.9±5.9	6.59±0.10	8.11±0.19[b]	1.52	95.2±0.9	100
1	95.1±2.4	82.0±4.6	6.39±0.10	7.72±0.20[b]	1.43	75.5±2.6	96.2±1.8[b]
3	103.1±2.1	93.9±1.2	5.97±0.10	7.39±0.13[b]	1.42	33.2±4.4	64.4±7.8[a]
10	108.0±1.4	102.9±2.6	5.57±0.06	6.93±0.14[b]	1.36	17.8±2.2	47.2±4.4[b]
100	108.9±1.7	103.3±1.4	5.06±0.13	6.86±0.12[b]	1.80	5.62±1.3	22.0±4.3[a]
His (μM)							
3	41.5±1.7	30.0±6.5	9.06±0.08	9.84±0.06[b]	0.78	100	100
10	71.9±3.4	54.7±4.7	8.72±0.08	9.61±0.07[b]	0.89	100	100
100	90.7±2.9	71.4±2.6	8.08±0.13	9.17±0.13[b]	1.09	99.6±0.1	100
1000	102.6±2.6	–	7.33±0.05	–		95.3±1.5	–

Date represent means±S.E.M. of 4–14 experiments each performed in duplicate. Significantly different from control: [a]$P<0.01$; [b]$P<0.001$.
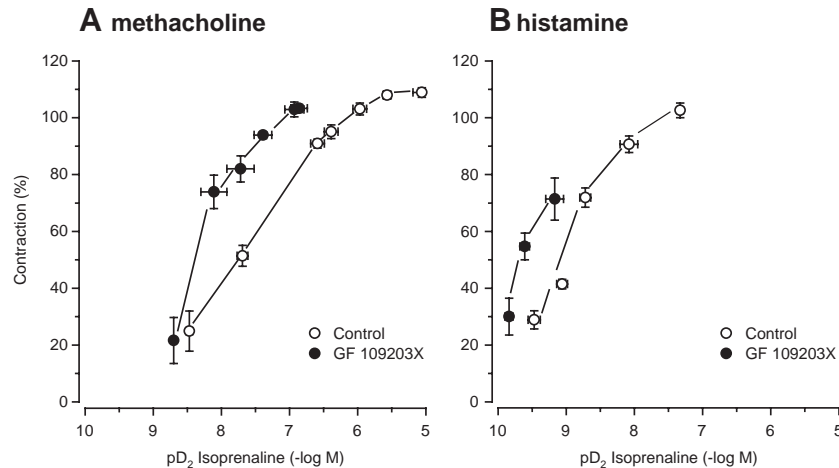
Fig. 3. Relationship between contraction levels of bovine tracheal smooth muscle induced by various concentrations of methacholine (A) and histamine (B) and (−)-isoprenaline relaxation potencies (pD_2) in the absence and presence of 10 μM GF 109203X.

production, over a wide concentration range, is paralleled by the increase in DAG, resulting in the activation of PKC.

Using the specific PKC inhibitor GF 109203X, we found that the cumulative concentration–contraction curve of methacholine in bovine tracheal smooth muscle strips slightly but significantly shifted to the right, with no changes in maximal effect (Fig. 1), whereas for histamine also a significant decrease in maximal effect in the presence of GF 109203X was observed. This reduced contraction confirms that PKC activation by DAG is implicated in the tonic phase of the contraction and that PKC exerts a feedforward control of agonist-induced Ca^{2+} mobilization and influx (Hoiting et al., 1995), the more

pronounced attenuation of the histamine-induced response by GF 109203X being in line with the lower phosphoinositide metabolism seen with this agonist (Hoiting et al., 1996; Van Amsterdam et al., 1989). In addition, we found that at increasing concentrations of the contractile agonists, the isoprenaline-induced relaxation was gradually reduced (Figs. 2A and B). Similar findings were shown in other studies, using human (Van Amsterdam et al., 1990) and animal (Roux et al., 1995; Torphy et al., 1985; Van Amsterdam et al., 1989) airway smooth muscle. Remarkably, in the presence of increasing concentrations of methacholine, in contrast to histamine, not only the potency (pD_2) of isoprenaline but also the maximal relaxation was gradually reduced. Again, this is
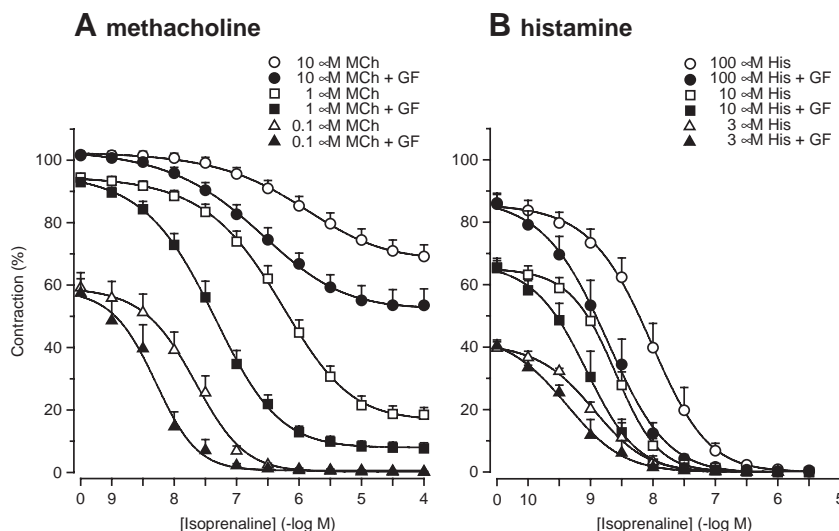

Fig. 4. (−)-Isoprenaline-induced relaxation of bovine tracheal smooth muscle strips following contraction by various concentrations of methacholine (MCh) (A) and histamine (His) (B) in the absence and presence of 10 μM GF 109203X, with readjustments of smooth muscle tone, if necessary (see Materials and methods). Results are means ± S.E.M. of 3–14 experiments each performed in duplicate.

Table 2
$(-)$-Isoprenaline pD_2 and E_{max} values obtained after contraction of bovine tracheal smooth muscle strips with different concentrations of methacholine (MCh) and histamine (His) in the absence and presence of 10 μM GF 109203X (GF), after readjustment of contractile tone if necessary (see Materials and methods)

| | Contraction level (%) | | pD_2 $(-\log M)$ | | ΔpD_2 | E_{max} (%) | |
	Control	+GF	Control	+GF		Control	+GF
MCh (μM)							
0.1	59.1 ± 4.9	57.5 ± 4.6	7.75 ± 0.10	8.36 ± 0.14[b]	0.61	99.7 ± 0.2	99.9 ± 0.1
1.0	94.5 ± 1.3	93.0 ± 1.1	6.32 ± 0.10	7.39 ± 0.09[c]	1.07	80.9 ± 2.4	91.9 ± 1.9[b]
10	101.7 ± 1.2	101.5 ± 1.2	5.94 ± 0.14	6.61 ± 0.12[b]	0.67	32.3 ± 3.6	48.5 ± 4.9[a]
His (μM)							
3	39.7 ± 2.0	40.4 ± 1.9	8.85 ± 0.21	9.22 ± 0.16[a]	0.37	100	100
10	65.6 ± 2.9	65.3 ± 2.4	8.70 ± 0.05	9.04 ± 0.15[a]	0.34	100	100
100	86.2 ± 3.3	85.8 ± 3.1	8.08 ± 0.14	8.74 ± 0.14[b]	0.66	100	100

Data represent means $\pm$ S.E.M. of 3–14 experiments each performed in duplicate. Significantly different from control: [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$.

in line with the markedly higher inositol phosphates responses of bovine tracheal smooth muscle seen with methacholine compared to histamine, at all concentrations used (Van Amsterdam et al., 1989). The resulting Ca^{2+} mobilization and influx induced by methacholine have shown to be less susceptible towards cAMP elevation indeed (Hoiting et al., 1996). Interestingly, in human bronchial smooth muscle, showing similar inositol phosphates responses with histamine and methacholine, very similar correlations between inositol phosphates production induced by various concentrations of the two agonists and the reduction of isoprenaline pD_2 and E_{max} values were found (Van Amsterdam et al., 1990).

In addition to cAMP-induced PKA mediated inhibition of the Ca^{2+} responses (Hoiting et al., 1996), direct interference by phosphoinositide metabolism of β_2-adrenoceptor function through DAG-induced PKC activation
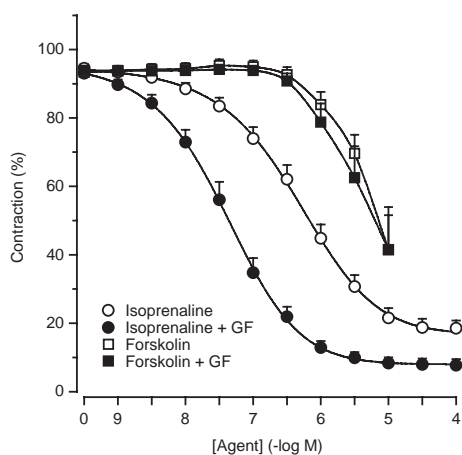

Fig. 5. $(-)$-Isoprenaline- and forskolin-induced relaxation of bovine tracheal smooth muscle strips following contraction by 1 μM methacholine in the absence and presence of 10 μM GF 109203X, with readjustment of smooth muscle tone (see Materials and methods). Results are means $\pm$ S.E.M. of 4–14 experiments each performed in duplicate.

may also play an important role. It has been demonstrated that PKC may desensitise β-adrenoceptors, presumably via phosphorylation of the β-adrenoceptor and/or G_s-protein (Houslay, 1991). Thus, phorbol-ester-induced PKC activation attenuates β-adrenoceptor function in human lymphocytes because of uncoupling of the receptor from the G_s-protein (Meurs et al., 1987). In the present study we found a significant increase in sensitivity to isoprenaline when PKC was inhibited by GF 109203X, with bovine tracheal smooth muscle strips precontracted with methacholine and histamine (Fig. 2). Although methacholine- and histamine-induced contractions were reduced by GF 109203X to some extent, the enhanced responsiveness to the β-agonist could only partially be explained by the reduced smooth muscle tone. When the reduced contractile tone in the presence of GF 109203X was carefully compensated for by additional agonist administration to reach the same contraction levels compared to controls (Fig. 4; Table 2), still significantly enhanced pD_2 values were obtained, both for methacholine and histamine. In addition, at all histamine-evoked contraction levels, in the absence and presence of GF 109203X, maximal relaxation by isoprenaline was achieved, whereas with methacholine the E_{max} values for isoprenaline were consistently enhanced in the presence of GF 109203X. This enhancement increased with increasing levels of contraction, supporting the idea that β-adrenoceptor function worsens in parallel with increasing DAG-induced PKC activation. Remarkably, in contrast to isoprenaline, forskolin-induced relaxation of preparations precontracted by methacholine was not affected at all by GF 109203X (Fig. 5), strongly suggesting that uncoupling of the β-adrenoceptor from G_s by PKC-induced phosphorylation of the receptor- and/or the G_s-protein is indeed involved. In line with these results, it has been recently demonstrated in rat oesophagus smooth muscle that the PKA-dependent but β-adrenoceptor-independent relaxation by IBMX (3-isobutyl-1-methyl-xanthine) is not modulated by 10 μM

GF109203X either (Oostendorp et al., 2004). These findings demonstrate that in intact airway and oesophagus smooth muscle PKA activity is not inhibited by 10 μM GF 109203X.

In conclusion, using the specific PKC inhibitor GF 109203X, we found direct evidence for the involvement of PKC in the acute functional antagonism of contractile agonist-induced bovine airway smooth muscle contraction by β-adrenoceptor agonists. Since PKC, via receptor-mediated phosphoinositide metabolism, is activated by many mediators and neurotransmitters in airway inflammation, this could lead to reduced airway smooth muscle responsiveness toward β-adrenoceptor agonists in asthma. Therefore, this cross talk may be of considerable importance in the reduced bronchodilator response of patients with severe asthma.

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