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Modulation of β_2- and β_3-adrenoceptor-mediated relaxation of rat oesophagus smooth muscle by protein kinase C

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Abstract

Although a prominent role for protein kinase C (PKC) in the cross-talk between the phosphoinositide pathway and β_2-adrenoceptor signalling has been indicated, modulation of β_3-adrenoceptor function by PKC has not been studied thus far. In the present study, we have compared the relative capacity of PKC in modulating β_2- and β_3-adrenoceptor-mediated relaxation of methacholine-contracted rat oesophagus smooth muscle. To this purpose the effects of the PKC-inhibitor GF 109203X (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide) on relaxation induced by fenoterol, formoterol, (−)-noradrenaline, BRL 35135 (4-[2-[(2-hydroxy-2-(chlorophenyl)ethyl)amino]-propyl]-phenoxyacetic-acidmethylester) and IBMX (3-isobutyl-1-methyl-xanthine) were studied, in the absence and presence of the selective β_2-adrenoceptor antagonist ICI 118,551 (erythro-1(7-methylindan-4-yloxy)-3-(isopropylamin)-butan-2-ol). Our results show that inhibition of PKC resulted in differential augmentation of both β_2- and β_3-adrenoceptor-mediated relaxation. In contrast, relaxation induced by IBMX was not influenced at all by GF 109203X. The β_2-adrenoceptor bears phosphorylation sites for several kinases, including PKC. Since the β_3-adrenoceptor lacks these consensus sites, the results may also indicate that PKC-mediated $G\alpha_s$ phosphorylation is involved in the cross-talk between the muscarinic receptor-mediated phosphoinositide pathway and β_2- and, particularly, β_3-adrenoceptor signalling.

Keywords: Oesophagus muscularis mucosae; β_2-Adrenoceptor; β_3-Adrenoceptor; Protein kinase C; Receptor cross-talk

1. Introduction

In several cell types, including airway smooth muscle and peripheral blood mononuclear cells, it has been demonstrated that activation of protein kinase C (PKC), through phosphoinositide metabolism or phorbol ester administration, may lead to desensitization of β-adrenoceptor-mediated adenylyl cyclase activity (Houslay, 1991; Meurs and Zaagsma, 1991; Zaagsma et al., 1997). In a recombinant system, activation of PKC has been shown to phosphorylate purified hamster lung β_2-adrenoceptors, resulting in a decreased ability to couple to the $G\alpha_s$ protein (Pitcher et al., 1992). Moreover, recombinant Chinese hamster ovary cells, coexpressing muscarinic M_3-receptors and β_2-adrenoceptors, did show phosphorylation of the β_2-adrenoceptor after muscarinic receptor stimulation, which correlated with a loss in GTP-sensitive high affinity agonist binding and a reduction in maximal isoprenaline-induced cAMP accumulation (Budd et al., 1999). Accordingly, site-directed mutagenesis of protein kinase A (PKA) and PKC consensus sites ($Ser \rightarrow Ala^{261}$ and $Ser \rightarrow Ala^{262}$) in the third intracellular loop of the β_2-adrenoceptor resulted in decreased phorbol ester-induced, PKC-mediated desensitization of adrenaline-induced adenyl cyclase activity (Yuan et al., 1994). Direct functional evidence for receptor cross-talk between the muscarinic receptor-mediated phosphoinositide pathway and β_2-adrenoceptor signalling via PKC has been obtained in bovine tracheal smooth muscle. Specific inhibition of PKC with GF 109203X ((2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide) (Toullec et al., 1991) markedly potentiated isoprenaline-induced relaxation of methacholine-contracted

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bovine tracheal smooth muscle, without affecting forskolin- and 8-Br-cAMP-induced relaxations (Zaagsma et al., 1997). However, modulation of β_3-adrenoceptor-mediated smooth muscle relaxation by PKC has not been studied thus far.

Previous relaxation studies have revealed that the functional β-adrenoceptor population of rat oesophagus smooth muscle predominantly consists of the β_3-, and also of the β_2-subtype (De Boer et al., 1993, 1996). Therefore, isolated rat oesophagus smooth muscle strips are attractive for comparing the relative susceptibilities of the β_2- and the β_3-adrenoceptor for PKC-mediated functional cross-talk. To investigate possible modulation by PKC, β_2- and β_3-adrenoceptors were selectively stimulated in the absence and presence of the specific PKC-inhibitor GF 109203X.

Our results show that inhibition of PKC resulted in a differential augmentation of the β_2- and the β_3-adrenoceptor-mediated relaxation of methacholine-contracted rat oesophagus smooth muscle.

2. Material and methods

2.1. Drugs

Methacholine, fenoterol, (−)-noradrenaline, IBMX (3-isobutyl-1-methyl xanthine) and (−)-isoprenaline were from Sigma (St. Louis, MO, USA). Formoterol was kindly donated by Dr. B. Waldeck, Astra Zeneca (Lund, Zweden). BRL 35135 (4-[2-[(2-hydroxy-2-(chlorophenyl)ethyl)amino]-propyl]phenoxyacetic-acidmethylester) was a generous gift from Dr. J.R.S. Arch, Glaxo SmithKline (Epsom, UK). ICI 118,551 (erythro-1(7-methylindan-4-yloxy)-3-(isopropylamin)-butan-2-ol) was kindly donated by ICI (Macclesfield, UK). Cocaine was purchased from Brocacef (Maarsen, The Netherlands) and corticosterone was from Organon (Oss, The Netherlands). GF 109203X (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide) was purchased from Boehringer (Mannheim, Germany).

2.2. Animals and tissue preparation

Animals were housed in individual cages in climate-controlled animal quarters according to the University of Groningen Committee for Animal Experimentation, which is responsible for the care and proper use of experimental animals. Oesophagus muscularis mucosae strips from male Wistar rats (228–330 g) (Harlan, Heathfield, UK) were prepared as described (De Boer et al., 1993). For isotonic recording under 0.2 g load, smooth muscle strips were mounted in 20 ml water-jacketed organ baths, filled with Krebs–Henselheit solution at 37 °C, composed of (mM): NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2$ 2.52, NaH_2PO_4 1.28, $NaHCO_3$ 25.0, glucose 5.5, gassed with 95% O_2 and 5% CO_2, pH 7.4. After an equilibration period of at least 30 min smooth muscle strips showed neither spontaneous activity nor resting tone.

2.3. Concentration–response curves

An initial methacholine concentration–response curve (0.1, 1, 10 μM) was constructed for each preparation, which, after a washing period of at least 30 min, was followed by a second methacholine concentration–response curve (0.1, 1, 10, 100 μM) to establish maximal contraction. The preparations were then washed twice and, before the cumulative addition of β-adrenoceptor agonists or IBMX, contracted stepwise with two cumulative concentrations of methacholine (0.1 and 1 μM) to gain approximately 50% of the maximal contraction level. When studying the effects of the selective β_2-adrenoceptor antagonist ICI 118,551 and/or the PKC-inhibitor GF 109203X, at least two untreated strips served as a control for every individual experiment. ICI 118,551 (1 μM) was added 30 min, and the PKC inhibitor GF 109203X (10 μM) 45 min, prior to elevation of muscle tone with methacholine. In case of GF 109203X incubations, additional concentrations of methacholine were carefully given in small increments (0.1–0.2 log units) to gain similar half maximal contraction levels when compared to control preparations (50.5 ± 2.0%, $n = 35$ for control versus 48.2 ± 1.6%, $n = 31$ for GF 109203X treated smooth muscle, not significantly different).

Fenoterol, formoterol, (−)-noradrenaline and IBMX were administered in 0.5 log increments. BRL 35135 was added in whole log intervals, since relaxations developed more slowly. Cocaine (10 μM) and corticosterone (10 μM) were added 30 min prior to (−)-noradrenaline concentration–response curves to prevent neuronal and extraneuronal uptake, respectively.

After each concentration–response curve of a β-adrenoceptor agonist or IBMX, maximal relaxation was assessed by adding 0.1 mM (−)-isoprenaline.

All experiments were performed in duplicate each day, using strips from the same animal, providing one data-set for the mean results.

2.4. Data analysis

Concentration–response curves of β-adrenoceptor agonists and IBMX were expressed as percentages of methacholine-induced half-maximal contractions. All data are expressed as the mean ± S.E.M. of (n) experiments. Groups were assessed and tested for significance with (paired) Student's t-test at $P < 0.05$. To determine if the data were fitted significantly better with either one or two binding sites, concentration response curves were fitted by the following sigmoidal model: $Y = V_1 + V_2 \times X\hat{}V_3/(X\hat{}V_3 + V_4\hat{}V_3) + V_5 \times X\hat{}V_6/(X\hat{}V_6 + V_7\hat{}V_6)$, as described previously (Filipeanu et al., 2001). In this equation Y represents % contraction, X the agonist concentration and V_2, V_3, V_4 and V_5, V_6, V_7 represent E_{max}, Hill-coefficient and pEC_{50} value

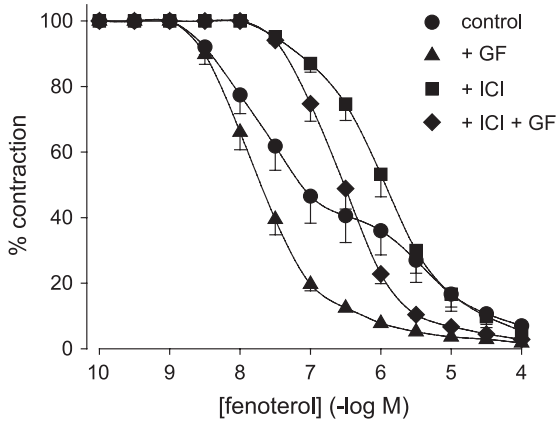

Fig. 1. Fenoterol-induced relaxation of methacholine-contracted rat oesophagus muscularis mucosae preparations in the absence and presence of 1 μM ICI 118,551 (ICI) and/or 10 μM GF 109203X (GF). Data represent means ± S.E.M. from four to five experiments performed in duplicate.

of the first and the second binding site, respectively. The last term (V_5, V_6 and V_7) was omitted for the single site fit. For fitting two binding sites, the Hill-coefficient (V_3 or V_6) for the smallest compartment was fixed at 1. $V_1 = 0$, since concentration–response curves were expressed as percentage contraction. To determine if the data were fitted significantly better with one or two binding sites, the variance was calculated using the F-test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Modulation of fenoterol- and formoterol-induced relaxation by PKC

The selective β_2-adrenoceptor agonist fenoterol caused a biphasic concentration-dependent relaxation of methacholine precontracted rat oesophageal smooth muscle strips (Fig. 1). Data analysis showed that the concentration–response curve to fenoterol was best described using a model with two binding site kinetics (Table 1), consistent with involvement of β_2- and β_3-adrenoceptors. The selective β_2-antagonist ICI 118,551 (1 μM) caused a substantial shift to the right and clear steepening of the fenoterol concentration–response curve, resulting in a monophasic, β_3-adrenoceptor-mediated relaxation (Fig. 1, Table 1). Treatment with the specific PKC-inhibitor GF 109203X (10 μM) caused a significant increase in the maximal relaxation (E_{max}) of the first phase of the fenoterol concentration–response curve. In contrast, the pEC_{50} value of this β_2-adrenoceptor component did not change after PKC-inhibition by GF 109203X (Fig. 1, Table 1). However, in the presence of ICI 118,551, GF 109203X did increase the potency of fenoterol-induced relaxation of rat oesophageal smooth muscle, resulting in a significant left shift of the monophasic, β_3-adrenoceptor-mediated relaxation (Fig. 1, Table 1).

Similar to fenoterol, formoterol-induced relaxation was best described using a model of two binding site kinetics. The E_{max} value reached by the first phase of the shallow concentration–response curve was lower than for fenoterol (Fig. 2, Table 2). Interestingly, the pEC_{50} values of both the first and the second phase of formoterol were higher than for fenoterol (Tables 1 and 2). In the presence of ICI 118,551, the β_2-component was completely suppressed, resulting in a monophasic, β_3-adrenoceptor-mediated relaxation (Fig. 2, Table 2). The higher potency of formoterol on the β_3-adrenoceptor when compared to fenoterol was confirmed in the presence of ICI 118,551 (Tables 1 and 2). Treatment with GF 109203X resulted in an increased E_{max} value of the first phase, whereas the pEC_{50} value did not change, as was the case with fenoterol (Fig. 2, Table 2). In the presence of ICI 181,551, GF 109203X caused a significant left shift of the β_3-adrenoceptor-mediated monophasic concentration–response curve of formoterol (Fig. 2, Table 2).

Table 1
Effects of 1 μM ICI 118,551 (ICI) and 10 μM GF 109203X (GF) alone and in combination on pEC_{50} and maximal effect (E_{max}) of fenoterol-induced relaxation of rat oesophagus smooth muscle

	Fenoterol			
	Control ($n = 4$)	+ ICI ($n = 5$)	+ GF ($n = 4$)	+ ICI + GF ($n = 4$)
Binding sites	2	1	2	1
E_{max} (%) (V_2)	61.2 ± 6.6	96.0 ± 3.7	86.6 ± 5.4[a]	95.9 ± 1.6
Hill-coefficient (V_3)	1.07 ± 0.32	0.84 ± 0.10	1.26 ± 0.14	1.10 ± 1.6
pEC_{50} ($-\log M$) (V_4)	7.74 ± 0.11	6.00 ± 0.12[a]	7.82 ± 0.10	6.57 ± 0.10[b,c]
E_{max} (%) (V_5)	40.0 ± 7.5	–	11.9 ± 5.1	–
Hill-coefficient (V_6)	1 (fixed)	–	1 (fixed)	–
pEC_{50} ($-\log M$) (V_7)	5.07 ± 0.11	–	5.96 ± 0.08	–
P-value[d]	0.0079		0.0216	

Concentration–response curves were fitted using the equation: $Y = V_1 + V_2 \times X\hat{}V_3/(X\hat{}V_3 + V_4\hat{}V_3) + V_5 \times X\hat{}V_6/(X\hat{}V_6 + V_7\hat{}V_6)$. The last term was omitted for the single site fit. $V_1 = 0$, since effects were expressed as % contraction.

[a] $P < 0.05$ compared to control.

[b] $P < 0.05$ compared to ICI 118,551 (ICI) treatment.

[c] $P < 0.05$ compared to GF 109203X (GF) treatment.

[d] Two site model fitted significantly better than single site model.

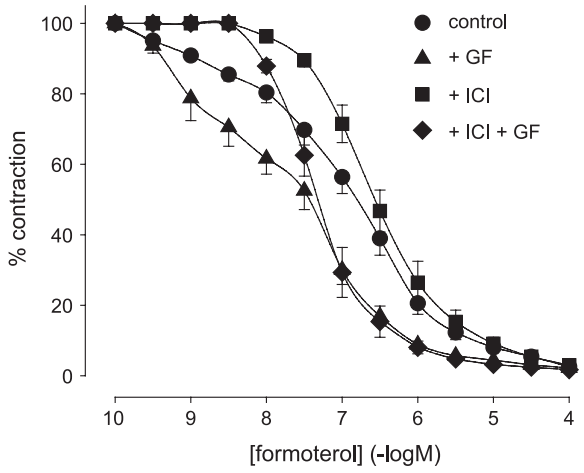

Fig. 2. Formoterol-induced relaxation of methacholine-contracted rat oesophagus muscularis mucosae preparations in the absence and presence of 1 μM ICI 118,551 (ICI) and/or 10 μM GF 109203X (GF). Data represent means ± S.E.M. from five experiments performed in duplicate.

3.2. β_3-Adrenoceptor-mediated relaxation

As previously described, (−)-noradrenaline-induced relaxation of rat oesophageal smooth muscle is solely mediated by β_3-adrenoceptors (De Boer et al., 1995). After treatment with GF 109203X (10 μM), (−)-noradrenaline-induced relaxation was potentiated, resulting in a small but significant shift to the left of the concentration–response curve (Fig. 3A, Table 3).

The selective β_3-adrenoceptor agonist BRL 35135 caused a concentration-dependent, submaximal relaxation (Fig. 3B). In the presence of GF 109203X (10 μM) the maximal BRL 35135-induced relaxation was slightly, but significantly increased, whereas the pEC_{50} value did not change (Fig. 3B, Table 3).

3.3. β-Adrenoceptor-independent relaxation

The non-selective phosphodiesterase-inhibitor IBMX was used to investigate the effects of GF 109203X on β-adrenoceptor-independent relaxation. As expected, PKC-inhibition by GF 109203X (10 μM) did not exert any effect on the potency of IBMX-induced relaxation of rat oesophagus smooth muscle (Fig. 4, Table 3).

4. Discussion

Pharmacological as well as biochemical data have indicated a prominent role for PKC in the cross-talk between the phosphoinositide pathway and β_2-adrenoceptor signalling (Budd et al., 1999; Houslay, 1991; Meurs and Zaagsma, 1991; Pitcher et al., 1992; Yuan et al., 1994; Zaagsma et al., 1997). In the present study, we have compared the capacity of PKC in modulating β_2- and β_3-adrenoceptor-mediated relaxation of rat oesophagus smooth muscle, precontracted by the muscarinic receptor agonist methacholine. Pretreatment with the selective PKC-inhibitor GF 109203X was found to differentially augment both the β_2- and the β_3-adrenoceptor-mediated relaxation.

The biphasic shape of the concentration–response curves of fenoterol and formoterol confirms previous findings that both β_2- and β_3-adrenoceptors are involved in relaxation of oesophagus smooth muscle (De Boer et al., 1993, 1996). GF 109203X caused a significant enhancement of maximal β_2-adrenoceptor-mediated relaxation both with fenoterol and formoterol as the agonist, with no effect on the pEC_{50} values. In contrast, GF 109203X did enhance the potencies of the fenoterol- and formoterol-induced relaxation mediated through the β_3-adrenoceptor, i.e. in the presence of the selective β_2-adrenoceptor antagonist ICI 118,551.

The lower efficacy and the higher potency of formoterol at the β_2-adrenoceptor of rat oesophagus smooth muscle

Table 2
Effects of 1 μM ICI 118,551 (ICI) and 10 μM GF 109203X (GF) alone and in combination on pEC_{50} and maximal effect (E_{max}) of formoterol-induced relaxation of rat oesophagus smooth muscle

	Formoterol			
	Control ($n=5$)	+ICI ($n=5$)	+GF ($n=5$)	+ICI+GF ($n=5$)
Binding sites	2	1	2	1
E_{max} (%) (V_2)	13.9 ± 5.9	96.3 ± 1.8	36.1 ± 8.1[a]	96.8 ± 1.6
Hill-coefficient (V_3)	1 (fixed)	0.90 ± 0.07	1 (fixed)	1.20 ± 0.10
pEC_{50} (−log M) (V_4)	9.08 ± 0.08	6.58 ± 0.15[a]	9.01 ± 0.11	7.34 ± 0.12[b,c]
E_{max} (%) (V_5)	83.8 ± 6.8	–	61.0 ± 8.6	–
Hill-coefficient (V_6)	0.75 ± 0.10	–	1.04 ± 0.24	–
pEC_{50} (−log M) (V_7)	6.68 ± 0.09	–	7.04 ± 0.04[a]	–
P-value[d]	0.042		0.007	

Concentration–response curves were fitted using the equation, as given in Table 1.
[a] $P<0.05$ compared to control.
[b] $P<0.05$ compared to ICI 118,551 (ICI) treatment.
[c] $P<0.05$ compared to GF 109203X (GF) treatment.
[d] Two site model fitted significantly better than single site model.

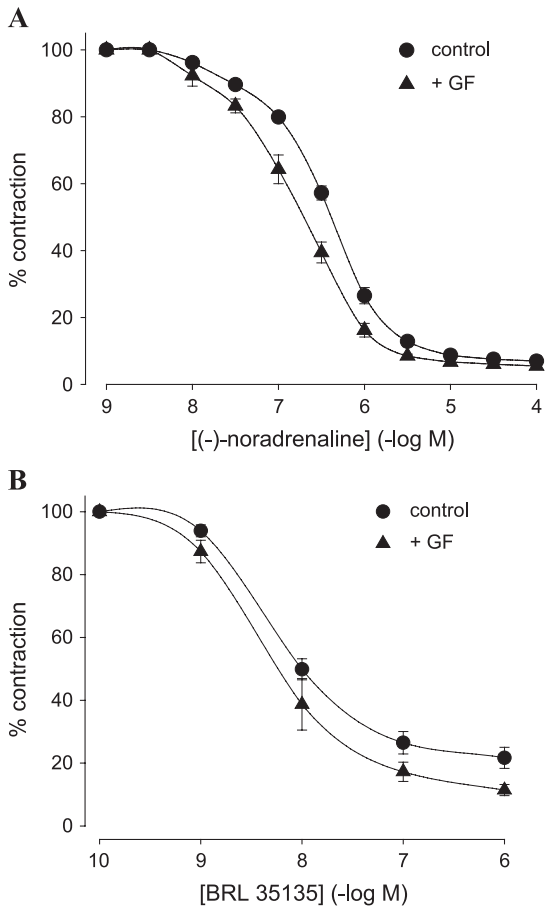

Fig. 3. Relaxation of methacholine-contracted rat oesophagus muscularis mucosae preparations by (−)-noradrenaline (A) and BRL 35135 (B) in the absence and presence of 10 μM GF 109203X (GF). Data represent means ± S.E.M. from four experiments performed in duplicate.

when compared to fenoterol is in accordance with results obtained in contracted human bronchial smooth muscle (Molimard et al., 1998). Interestingly, with respect to β_3-adrenoceptor-mediated relaxation, our results clearly show that formoterol again is more potent than fenoterol.

We also investigated the effect of GF 109203X on selective β_3-adrenoceptor agonist-induced relaxation. As previously described, (−)-noradrenaline-induced relaxation of rat oesophageal smooth muscle is solely mediated by β_3-adrenoceptors (De Boer et al., 1995). Pretreatment with GF 109203X resulted in an increased potency of the concentration–response curve to (−)-noradrenaline. The selective β_3-adrenoceptor agonist BRL 35135 acted as a partial agonist, as was the case in guinea pig taenia caecum (Koike et al., 1997). Pretreatment with GF 109203X led to increased efficacy of BRL 35135 rather than to increased relaxant potency.

Whereas pretreatment of GF 109203X increased the efficacy of the minor β_2-adrenoceptor-mediated response, it resulted in a leftward shift of the β_3-adrenoceptor-mediated relaxation. This differential effect of GF 109203X may be explained by a difference in functional expression levels of the β_2- and β_3-adrenoceptors. In recombinant systems, it has been shown that the efficacy and potency of β-adrenoceptor agonists are largely dependent on the receptor expression level (Whaley et al., 1994, Rousseau et al., 1997). It has been predicted that, at high receptor levels, loss of coupling efficacy due to desensitization would first manifest itself in a decrease in $pEC_{50,}$ with little or no change in E_{max}, while at low receptor levels, desensitization would rather lead to a decreased E_{max} value (Whaley et al., 1994, Rousseau et al., 1997). In line with this hypothesis, we found that PKC-inhibition increased the efficacy (E_{max}) of the fenoterol- and formoterol-induced relaxation mediated by the β_2-adrenoceptor subtype (low functional expression), whereas pretreatment with GF 109203X lead to an increased potency (pEC_{50}) of fenoterol-, formoterol- and (−)-noradrenaline-induced relaxations mediated by the β_3-adrenoceptor (high functional expression). These results would

Table 3
Effects of 10 μM GF 109203X (GF) on pEC_{50} and maximal effect (E_{max}) of BRL 35135-, (−)-noradrenaline- and IBMX-induced relaxation of rat oesophagus smooth muscle, respectively

	E_{max} (%) (V_2)	Hill-coefficient (V_3)	pEC_{50} (−log M) (V_4)	n
BRL 35135	77.1 ± 2.1	1.30 ± 0.20	8.20 ± 0.03	4
BRL 35135 + GF	87.3 ± 1.7[a]	1.13 ± 0.19	8.32 ± 0.09	4
(−)-Noradrenaline	93.9 ± 1.0	1.06 ± 0.05	6.48 ± 0.03	4
(−)-Noradrenaline + GF	95.3 ± 1.2	0.98 ± 0.06	6.80 ± 0.04[b]	4
IBMX	100.0 ± 2.5	0.90 ± 0.06	5.26 ± 0.08	6
IBMX + GF	98.8 ± 4.0	0.86 ± 0.10	5.38 ± 0.14	6

Concentration–response curves were fitted using the equation, as given in Table 1.

[a] $P < 0.05$ compared to BRL 35135.

[b] $P < 0.01$ compared to (−)-Noradrenaline.

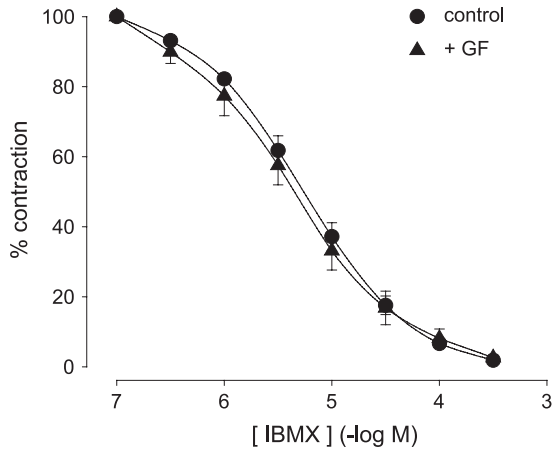

Fig. 4. IBMX-induced relaxation of methacholine-contracted rat oesophagus muscularis mucosae preparations in the absence and presence of 10 μM GF 109203X (GF). Data represent means ± S.E.M. from six experiments performed in duplicate.

also suggest that submaximal relaxation, whether induced by partial agonists acting through a high-density β-adrenoceptor population or by full agonists acting through a minor β-adrenoceptor population, would be potentiated similarly by inhibition of PKC. The observation that GF 109203X increased the efficacy rather than the potency of BRL 35135 may thus be explained by the partial agonism of BRL 35135 at the β_3-adrenoceptor (Koike et al., 1997).

Since PKC activation may contribute to smooth muscle contraction (Zaagsma et al., 1997), it could be argued that the observed increase in the efficacy or potency of β_2- and β_3-adrenoceptor agonist-induced relaxation of precontracted tissue in the presence of GF 109203X was due to a lower level of developed tension. However, additional small amounts of methacholine were carefully applied to gain similar half-maximal precontraction levels in GF 109203X treated preparations when compared to control preparations. Moreover, β-adrenoceptor-independent relaxation of oesophagus smooth muscle by IBMX was not modulated by GF 109203X at all.

Putative consensus sites for PKA- and PKC-induced phosphorylation (Ser^{261} and Ser^{262}) are located in the third intracellular loop of the β_2-adrenoceptor (Yuan et al., 1994). Interestingly, the β_3-adrenoceptor lacks consensus sites for PKA and PKC phosphorylation in its third intracellular loop (Nantel et al., 1993). Chuang et al. (1995) have reported that PKC is also able to phosphorylate and activate β-adrenergic receptor kinase (β-ARK), which might indicate an additional pathway for PKC in the cross-talk between phosphoinositide metabolism and β-adrenoceptor signalling. Sites of β-ARK phosphorylation reside in serine/threonine residues in the C-cytoplasmatic tail of the human β_2-adrenoceptor (Liggett et al., 1993). In contrast, the β_3-adrenoceptor lacks most of the serine/threonine residues and is not prone to β-ARK induced phosphorylation (Liggett et al., 1993).

Since the presence and absence of PKA, PKC and β-ARK consensus sites is rather conserved among mammalian β_2- and β_3-adrenoceptors (Lenzen et al., 1998; Oostendorp et al., 2002), the effect of GF 109203X on β_3-adrenoceptor-mediated relaxation of rat oesophagus smooth muscle can hardly be explained by prevention of β_3-adrenoceptor phosphorylation and/or β-ARK activation by PKC. Therefore, we hypothesize that the $G\alpha_s$ protein is an additional target for PKC-mediated phosphorylation, as has been demonstrated in a recombinant system (Pyne et al., 1992).

In conclusion, we have shown that relaxation of methacholine-contracted oesophagus smooth muscle via both the minor population of β_2-adrenoceptors and the major population of β_3-adrenoceptors is augmented after selective PKC-inhibition by GF 109203X. The β_2-adrenoceptor bears phosphorylation sites for PKC and β-ARK. Since the β_3-adrenoceptor lacks both consensus sites, PKC-mediated $G\alpha_s$ phosphorylation is most probably involved in the cross-talk between the muscarinic receptor-mediated phosphoinositide pathway and β_3- (and β_2-) adrenoceptor signalling.

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