Protein kinase C potentiates homologous desensitization of the β2-adrenoceptor in bovine tracheal smooth muscle

Mark Boterman
Steven R.J.G. Smits
Herman Meurs
Johan Zaagsma

Chapter 3

Summary

Preincubation (30 min) of bovine tracheal smooth muscle with various concentrations (0.1, 1 and 10 μM) of fenoterol decreased isoprenaline-induced maximal relaxation (E_max) of methacholine-contracted preparations in a concentration dependent fashion, indicating desensitization of the β2-adrenoceptor. Preincubation with 1 μM of the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) caused a small but significant decrease in isoprenaline-induced E_max, indicating activated PKC-mediated heterologous β2-adrenoceptor desensitization. To investigate the capacity of activated PKC to regulate homologous desensitization, we incubated the smooth muscle strips with the combination of both 1 μM PMA and 1 μM fenoterol. This combined treatment synergistically decreased the isoprenaline-induced maximal relaxation, as compared to the individual effects of PMA and fenoterol alone, indicating a common pathway for heterologous and homologous desensitization. Moreover, the specific PKC- inhibitor 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl) maleimide (GF 109203X) markedly increased the potency and E_max of isoprenaline for all conditions used, including control conditions, and the synergistic effects of PMA and fenoterol were completely prevented. In conclusion, the present study demonstrates that homologous desensitization of the β2-adrenergic receptor can be enhanced by PKC activation. For the first time we have provided evidence that this concept is functionally operative in airway smooth muscle, and it may explain the reduced bronchodilator response to β2-adrenoceptor agonists in patients with asthma during a severe exacerbation.

Introduction

Inhaled β2-adrenoceptor agonists are by far the most effective bronchodilators used for the relief of symptoms in patients with asthma [1]. However, it has been shown that chronic β-agonist therapy can diminish the efficacy of these drugs [2,3] and it is well known that patients with asthma have a reduced bronchodilator response to β-agonists during a severe exacerbation [4,5]. Possible mechanisms, which could account for these effects, are the development of homologous and/or heterologous desensitization of the β2-adrenoceptor. Various roles for intracellular kinases, such as G protein-coupled receptor kinases (GRKs), protein kinase A (PKA) and protein kinase C (PKC) have been described in modulating the function of the β2-adrenoceptor. Homologous desensitization of the β2-adrenoceptor is characterized by a rapid loss in agonist-stimulated cAMP generation, primarily mediated by GRKs, which recognise the agonist-occupied form of the receptor, and involves phosphorylation of the receptor. Such phosphorylation results in the binding of β-arrestin which prevents coupling of the receptor to its stimulatory G protein (Gₛ), subsequently followed by sequestration and/or downregulation of the receptor [6,7]. Heterologous desensitization is initiated by second messenger dependent kinases such as PKA and PKC. Cyclic AMP-activated PKA can phosphorylate the β2-adrenoceptor, thereby
causing uncoupling of the receptor from its Gs protein, which results in a diminished adenylyl cyclase response [6]. Agents which activate PKC, such as muscarinic agonists and phorbol esters, have also been shown to attenuate responses to β-agonists [8], presumably by phosphorylation of the β2-adrenoceptor and subsequent uncoupling from Gs [9]. Recently, we have presented direct functional evidence for the involvement of PKC in the cross-talk between the muscarinic M3 receptor and β2-adrenoceptor in bovine tracheal smooth muscle [10] and rat oesophagus smooth muscle [11]. Heterologous desensitization does not promote β-arrestin binding to the receptor and subsequent rapid internalization [12,13].

Interestingly, heterologous and homologous desensitization are not necessarily completely independent processes. Thus, it has been shown that PKC can mediate changes in the cellular expression and activity of GRKs [14]. PKC-mediated phosphorylation not only upregulates the activity of GRK2 [15] but also targets this kinase to the plasma membrane [16]. In addition, it has been suggested that PKA can induce β-arrestin1 expression [17,18] and PKA-mediated phosphorylation has been also shown to promote the translocation of GRK2 to the plasma membrane [19]. Little is known, however, about the functional consequences of these interactions. In this study, we present direct evidence for the capacity of PKC to regulate homologous β2-adrenoceptor desensitization in bovine tracheal smooth muscle, using the specific PKC activator phorbol 12-myristate 13-acetate (PMA) and a specific nonselective PKC inhibitor 2-(1-(3-dimethylaminopropyl)-1H-indol-3-yl)-3-(1H-indol-3-yl) maleimide (GF 109203X) [20]. GF 109203X inhibits all conventional (α, β1 and β2) and novel (δ, ε and θ) PKC isozymes present in bovine tracheal smooth muscle [21], and is 200-fold more selective in inhibiting PKC compared to PKA [20].

Materials and methods

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 calf serum at 37°C (55 rpm).

Experimental procedure

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 20 ml organ baths containing gassed KH-buffer (37°C) for isotonic recording, using a preload of 500 mg.
After a 60 min equilibration period the strips were precontracted twice with cumulatively administered methacholine (0.1, 1, 10 and 0.1, 1, 10, 100 μM, respectively) with a 60 min washing period in between. After washing, basal tone was established with isoprenaline (0.1 μM), immediately followed by a 30 min washing period.

After preincubation for 45 min in the absence or presence of 10 μM GF 109203X, smooth muscle strips were incubated with KH-buffer (35 min), 1 μM PMA (35 min), 0.1, 1 or 10 μM fenoterol (30 min), or 1 μM PMA (35 min) plus 1 μM fenoterol (30 min), immediately followed by a thorough washing period of 45 min. 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 (data not shown). Subsequently, smooth muscle tone was raised with methacholine at 1 μM, which concentration was built up gradually in 3 concentration steps. Cumulative concentration-relaxation-curves were constructed using (-)-isoprenaline (0.1 nM – 100 μM), added in 0.5 log increments. At the end of each experiment the smooth muscle strips were washed twice and basal tone was re-established with 10 and 100 μM isoprenaline. In all experiments, the reduction of methacholine-induced smooth muscle tone, as a consequence of the preincubation condition, was carefully compensated for by additional administration of small amounts of the contractile agonist, before isoprenaline relaxation curves were obtained.

**Data Analysis**

Responses were expressed as percentages of the response to 100 μM methacholine in the second preconstriction in each experiment, with reference to basal tone as established at the end of each experiment. All data are presented as mean ± S.E.M. Curves were fitted using the logistic 4-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.

**Materials**

Dulbecco’s modification of Eagle’s Medium (DMEM), foetal 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, U.K.). Methacholine chloride, (-)-isoprenaline hydrochloride and PMA (phorbol 12-myristate 13-acetate) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), GF 109203X (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl) maleimide) was purchased from Boehringer (Mannheim, Germany) and fenoterol hydrobromide was a generous gift from Boehringer-Ingelheim (Ingelheim, Germany). All other chemicals were of analytical grade.
Results

Effect of fenoterol treatment on isoprenaline-induced relaxation

In order to establish fenoterol-induced desensitization of the isoprenaline-induced relaxation of bovine tracheal smooth muscle, contracted by 1 µM methacholine, smooth muscle strips were preincubated with various concentrations of fenoterol for 30 min. (Table 1). Both 1 and 10 µM fenoterol significantly reduced the maximal response (E_max) of the isoprenaline-induced relaxation from 71.4 ± 2.8% to 64.7 ± 4.2% (P<0.05) and 34.6 ± 2.9% (P<0.001), respectively (Fig. 1, Table 1), whereas 0.1 µM fenoterol was ineffective. In contrast, the potency (pD2) of isoprenaline to induce relaxation remained unchanged (Table 1).

Table 1. Maximal effect (E_max) and potency (pD2) of (-)-isoprenaline-induced relaxation of bovine tracheal smooth muscle contracted by 1 µM methacholine, after preincubation with vehicle (control) or 0.1, 1 and 10 µM fenoterol (Feno).

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>E_max (%)</th>
<th>pD2 (-log M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71.4 ± 2.8</td>
<td>6.1 ± 0.1</td>
</tr>
<tr>
<td>0.1 µM Feno</td>
<td>73.9 ± 5.1</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td>1 µM Feno</td>
<td>64.7 ± 4.2a</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>10 µM Feno</td>
<td>34.6 ± 2.9b</td>
<td>5.9 ± 0.2</td>
</tr>
</tbody>
</table>

Data represent means ± S.E.M. of 3-14 experiments, each performed in duplicate. Significantly different from control: a P<0.05; b P<0.001.
Figure 2 (-)-Isoprenaline-induced relaxation of bovine tracheal smooth muscle preparations precontracted by 1 μM methacholine after preincubation with vehicle (control) and 1 μM PMA (A) or with vehicle (control), 1μM fenoterol (Feno) and 1 μM PMA + 1 μM fenoterol (B) in the absence and presence of 10 μM GF 109203X. Results are means ± S.E.M. of 4-11 experiments, each performed in duplicate.

Effect of PMA treatment on isoprenaline-induced relaxation

We also examined the effect of pretreatment with 1 μM PMA, an activator of PKC, on the isoprenaline-induced relaxation. Figure 2A shows a small but significant reduction of the isoprenaline-induced E\textsubscript{max} from 71.2 ± 3.2% to 64.2 ± 3.4% (P < 0.05). No change was found in the pD\textsubscript{2} value of isoprenaline (Table 2).

Table 2. Maximal effect (E\textsubscript{max}) and potency (pD\textsubscript{2}) of (-)-isoprenaline-induced relaxation of bovine tracheal smooth muscle contracted by 1 μM methacholine, in the absence (n = 11) and presence (n = 4) of 10 μM GF 109203X (GF) and after preincubation with vehicle (control), 1 μM PMA, 1 μM fenoterol (Feno) or 1 μM PMA + 1 μM fenoterol.

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>- GF</th>
<th>+ GF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E\textsubscript{max} (%)</td>
<td>pD\textsubscript{2} (-log M)</td>
</tr>
<tr>
<td>Control</td>
<td>71.2 ± 3.2</td>
<td>6.1 ± 0.1</td>
</tr>
<tr>
<td>1 μM PMA</td>
<td>64.2 ± 3.4\textsuperscript{a}</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>1 μM Feno</td>
<td>64.7 ± 4.2\textsuperscript{a}</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>1 μM PMA + 1 μM Feno</td>
<td>48.0 ± 4.4\textsuperscript{b}</td>
<td>6.1 ± 0.1</td>
</tr>
</tbody>
</table>

Data represent means ± S.E.M. of 4-11 experiments, each performed in duplicate. (* P < 0.05; † P < 0.001 compared to Control; ‡ P < 0.05; ‡‡ P < 0.01; ‡‡‡ P < 0.001 compared to the absence of GF 109203X)
**Effect of the combination of PMA and fenoterol on isoprenaline-induced relaxation in the absence and presence of GF 109203X**

In Figure 2B it is shown that combined pretreatment of PMA (1 μM) and fenoterol (1 μM) caused a significant potentiation of the desensitization of isoprenaline-induced relaxation by fenoterol (Fig. 2B) or PMA (Fig. 2A) alone. The decrease in E\textsubscript{max} for the combined treatment was significantly (P<0.05) different from the sum of the individual effects of fenoterol and PMA preincubation (Table 2). Again, the potency of isoprenaline was not significantly changed.

In the presence of GF 109203X, isoprenaline-induced relaxation was potentiated for all conditions studied, as visualised by the marked left shifts of the concentration-relaxation-curves of isoprenaline and the enhancement of E\textsubscript{max} (Figs 2A, B). Both E\textsubscript{max} and pD\textsubscript{2} values were significantly increased compared to their respective controls in the absence of GF 109203X (Table 2). Interestingly, after pretreatment of 1 μM fenoterol with or without 1 μM PMA, the potency of isoprenaline in the presence of GF 109203X was significantly decreased (Fig. 2B, Table 2), whereas after pre-treatment with 1 μM PMA alone the concentration-relaxation-curve of isoprenaline in the presence of GF 109203X was identical to the control (non-PMA-pretreated) curve (Fig. 2A, Table 2).

**Discussion**

The present study has established that activation of PKC by PMA causes a marked aggravation of β\textsubscript{2}-agonist-induced β\textsubscript{2}-adrenoceptor desensitization. Preincubation of bovine tracheal smooth muscle with 1 μM fenoterol for 30 minutes caused a small but significant decrease of the isoprenaline-induced maximal relaxation of the methacholine-induced contraction, whereas no effect was found on the potency of isoprenaline. With 10 μM fenoterol the E\textsubscript{max} of isoprenaline was markedly reduced, indicating a concentration-dependent desensitization of the β\textsubscript{2}-adrenoceptor. Both PKA- and GRK-mediated phosphorylation of the β\textsubscript{2}-adrenoceptor could account for this effect. For isoprenaline, it has been established that at low receptor occupancy (agonist concentration <10 nM) the PKA pathway is selectively activated [22], whereas with higher agonist concentrations (>50 nM), GRK-induced desensitization is quantitatively more important [23-25]. This would imply that at both concentrations used, fenoterol caused a desensitization of the β\textsubscript{2} adrenoceptor with a substantial GRK-mediated contribution. To date seven members of the GRK family have been identified, but only GRK2 (βARK 1), GRK3 (βARK 2) and GRK5 appear to be involved in agonist-induced β\textsubscript{2}-adrenoceptor phosphorylation [26-28]. To investigate a putative contribution of PKC-activation in this homologous desensitization by GRKs, we considered 1 μM fenoterol to be most appropriate, as the desensitization induced by this concentration was only small. Moreover, in separate experiments we found that this concentration of fenoterol causes a submaximal (85.7% ± 2.0) relaxation of 1 μM methacholine-induced tone (data not shown), indicating that this concentration is relevant with regard to the relief of bronchoconstriction in severe asthma.
Exposure of bovine tracheal smooth muscle to 1 μM PMA for 35 minutes caused a small but significant decrease in isoprenaline-induced maximal relaxation, with no effect on the pD2. Activated PKC is most likely responsible for this heterologous desensitization, as it has been demonstrated in various cells and tissues that activation of PKC via agonist-induced PI metabolism or phorbol esters may lead to uncoupling of the β-adrenoceptor, presumably as a result of phosphorylation of the receptor and/or Gs [8,29-35]. In addition, we have recently demonstrated the functional relevance of this receptor cross-talk in bovine tracheal smooth muscle by the marked potentiation of β-agonist-induced relaxation of cholinergic smooth muscle tone in the presence of the specific PKC inhibitor GF 109203X [10]. To investigate the capacity of activated PKC to regulate homologous desensitization, we incubated the bovine tracheal smooth muscle preparations with the combination of both 1 μM PMA and 1 μM fenoterol. This combined treatment synergistically attenuated the isoprenaline-induced maximal relaxation, as compared to the individual effects of PMA and fenoterol, indicating a common pathway for heterologous and homologous desensitization in bovine tracheal smooth muscle. Although in Chinese hamster ovary cells, coexpressing the M3-muscarinic and β2-adrenoceptor, activation of the PLC pathway appeared to have no effect on homologous β2-adrenoceptor desensitization [35], it has been shown in mononuclear leukocytes that activated PKC not only increased the expression of GRK2 [14], but also increased its phosphorylation and activation, resulting in an enhanced homologous desensitization of the β2-adrenoceptor. Thus, pretreatment of mononuclear leukocytes with 1 μM PMA significantly potentiated the degree of homologous desensitization induced by the incubation of 10 μM isoprenaline for 5 minutes [15]. Our results are in agreement with these findings. Moreover, we have found that GF 109203X markedly increased the potency and maximal relaxation of isoprenaline for all conditions used, including control conditions. As expected, the 1 μM PMA-induced desensitization, due to PKC-mediated phosphorylation of the β2-adrenoceptor and/or Gs, was completely prevented in the presence of GF 109203X, the two concentration-relaxation-curves of isoprenaline becoming superimposed (Fig. 2A). In contrast, the fenoterol-induced homologous desensitization as well as the synergistic effects of PMA and fenoterol were incompletely prevented, the positions of the two concentration-relaxation-curves in the presence of GF 109203X being significantly right from the corresponding control curve (Fig. 2B, Table 2). These findings indicate that part of the GRK-mediated desensitization induced by fenoterol, alone or in combination with PMA, is not susceptible to potentiation by PKC. It is also possible, however, that this PKC-insensitive component represents the PKA-mediated desensitization. Interestingly, PKA-mediated phosphorylation of GRK2, enhancing β2-adrenoceptor phosphorylation and desensitization, was recently found in HEK 293 cells [19]. This opens the fascinating possibility that both PKC and PKA are able to potentiate desensitization by phosphorylation of GRKs.

In conclusion, the present study not only confirms our previous findings, showing that PKC is directly involved in the acute functional cross-talk between the muscarinic M3 receptor and β2-adrenoceptor in bovine tracheal smooth muscle [10], but also that homologous desensitization of the β2-adrenoceptor can be enhanced by PKC-activation, presumably by
PKC potentiates homologous desensitization of the $\beta_2$-adrenoceptor

phosphorylation and activation of GRKs, indicating that the efficacy of homologous desensitization indeed can be modulated heterologously. For the first time we have provided evidence that this concept is functionally operative in airway smooth muscle. It may be of great functional importance for the development of a reduced bronchodilator response in patients with severe asthma. Thus, various inflammatory mediators and neurotransmitters released during a severe exacerbation are known to cause activation of PKC in airway smooth muscle cells [36]. Prolonged inhalation of $\beta_2$-agonists during such an exacerbation may therefore lead to aggravation of the homologous $\beta_2$-agonist-induced desensitization due to the mediator- and neurotransmitter-induced activation of PKC, causing further activation of GRKs.

Acknowledgements

This work was financially supported by the Stichting Astma Bestrijding, grant 99.03.

References