Acute desensitization of isoprenaline-mediated inhibition of Ca$^{2+}$-influx in airway smooth muscle cells is potentiated by agonist-induced protein kinase C activation

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Summary

It has been shown that patients with asthma have a reduced bronchodilator response to β-agonists during a severe exacerbation and that chronic β-agonist therapy can diminish the efficacy of these drugs. In the present study, we considered the possibility that a novel form of transductional cross-talk, in which contractile agonist-induced protein kinase C (PKC) activation enhances homologous β-agonist-induced desensitization, could contribute to these effects. In isolated bovine tracheal smooth muscle cells we investigated the effect of the specific PKC inhibitor 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl) maleimide (GF 109203X) on isoprenaline-mediated inhibition of methacholine-induced extracellular Ca^{2+}-influx, using the fluorescent dye Fura-2/AM. Single concentrations of isoprenaline (1 nM - 10 μM) were administered on the methacholine (100 μM)-induced Ca^{2+}-plateau at t = 150 or 340 seconds after methacholine.

Isoprenaline caused a rapid and transient inhibition of the methacholine-induced Ca^{2+}-influx at all concentrations of the β-agonist, indicating rapid desensitization of this response. This β₂-adrenoceptor desensitization was markedly reduced in the presence of GF 109203X, indicating that methacholine-induced activation of PKC was importantly involved. GF 109203X had no significant effect on the isoprenaline-induced peak-inhibition of Ca^{2+}-influx and no differences were found between the two different application times of isoprenaline (t = 150 or 340 seconds), suggesting that direct heterologous desensitization of the β₂-adrenoceptor induced by PKC was not involved in the short lasting effect of isoprenaline.

The results indicate that acute β-agonist-induced homologous desensitization of the β₂-adrenoceptor in bovine tracheal smooth muscle cells is markedly amplified by muscarinic agonist-induced PKC activation.

Introduction

Inhaled β₂-adrenergic agonists are by far the most effective bronchodilators used to control airway function in patients with asthma [1-3]. The efficacy of these drugs is mainly due to functional antagonism, counteracting the bronchoconstrictor effects of neurotransmitters and mediators released in airway inflammation [4]. However, it is well known that patients with asthma have a reduced bronchodilator response to β-agonists during a severe exacerbation [5,6]. Moreover, it has been shown that chronic β-agonist therapy can diminish the efficacy of these drugs [7,8], resulting in a loss of bronchoprotection against contractile stimuli and allergens [9].

Several protein kinases, including G protein-coupled receptor kinases (GRKs), protein kinase A (PKA) and protein kinase C (PKC) have been implicated in modulating β₂-adrenoceptor function. Homologous desensitization of the β₂-adrenoceptor is mediated by activation of GRKs. GRKs phosphorylate the agonist-occupied form of the receptor, after translocation to the plasma membrane by anchoring to free G_{i,γ} subunits generated upon receptor activation. This results in binding of β-arrestins and subsequent uncoupling of the
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PKC potentiates β-agonist induced desensitization from its stimulatory G protein (G_s), which is followed by sequestration and/or downregulation of the receptor [10,11]. The GRK family consists of seven isoforms that share a number of structural and functional similarities and GRKs 1-6 have been shown to phosphorylate the β_2-adrenoceptor in its C-terminal cytoplasmatic tail [12,13]. Another possible mechanism of reduced β_2-adrenoceptor function is heterologous desensitization, which may be induced by PKC and PKA. Agents which activate PKC, including muscarinic agonists and phorbol esters, have been shown to attenuate β-agonist-induced cAMP accumulation in airway smooth muscle slices [14], presumably involving phosphorylation of the third intracellular loop of the β_2-adrenoceptor and subsequent uncoupling from G_s [15]. Recently, we have presented direct functional evidence for the involvement of PKC in the cross-talk between muscarinic M_3 receptor stimulation and β_2-adrenoceptor-induced relaxation in bovine tracheal smooth muscle [16] and rat oesophagus smooth muscle [17]. PKA, which is activated by agents that increase intracellular cAMP levels, has also been identified as an important mediator of heterologous desensitization by phosphorylating the β_2-adrenoceptor in the third intracellular loop [10,18,19]. Interestingly, some studies have indicated that homologous and heterologous desensitization are not completely independent processes. Thus, in mononuclear leukocytes, it has been shown that activation of PKA and PKC can induce changes in the cellular expression and activity of GRKs and β-arrestins [20]. Moreover, it has been demonstrated that PKC-induced phosphorylation increased the activity of GRK2 by targeting the kinase to the plasma membrane [21]. Importantly, this novel form of cross-talk could contribute to the reduced bronchodilator response to β-agonists in patients with asthma during a severe exacerbation. We have recently demonstrated that PKC-activation by the phorbol ester PMA (phorbol 12-myristate 13-acetate) indeed enhances β-agonist-induced desensitization of bovine tracheal smooth muscle relaxation by isoprenaline [22]. In the present study we explored this concept into further detail at the level of intracellular Ca^{2+}-homeostasis in enzymatically dispersed bovine tracheal smooth muscle cells. Investigating the effect of the specific PKC-inhibitor GF 109203X on the inhibition by isoprenaline of methacholine-induced Ca^{2+}-influx, the present study revealed that the acute homologous β_2-adrenoceptor desensitization is being amplified heterologously by PKC. Therefore, this finding may represent a novel target for therapeutic strategy in patients with asthma.

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, MgSO_4 1.2, CaCl_2 2.5, Na_2HPO_4 1.3, NaHCO_3 25.0, glucose 5.5, pregassed with 95% O_2 and 5% CO_2; pH 7.4. The tracheal smooth muscle was dissected carefully and prepared free of mucosa and serosal connective
tissue in KH buffer gassed with 95% O₂/5% CO₂ at room temperature. Subsequently, the tracheal smooth muscle was chopped, using a McIlwain tissue chopper, two times at the size of 300 μm and three times at 100 μm. Tissue fragments were washed under sterile conditions (three times) and maintained overnight in Dulbecco’s modified Eagle’s medium (DMEM; 2.5 mg/ml) supplemented with 10 mM NaHCO₃, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS) at 37°C (55 rpm).

**Isolation of tracheal smooth muscle cells**

Tissue fragments were washed three times in Krebs-Ringer-Henseleit (KRH) buffer containing (mM): NaCl 125.0, KCl 6.0, MgCl₂ 2.5, CaCl₂ 1.2, NaH₂PO₄ 1.2, NaHCO₃ 25.0, glucose 11.0, HEPES 25.0, pH 7.4, supplemented with 2.0 mM (±)-dithiothreitol (DTT) and were then resuspended in a digestion mixture of collagenase P (0.75 mg/ml), papain (1 mg/ml) and trypsin inhibitor (1 mg/ml) in KRH buffer. The suspension was incubated for 20 min at 37°C (55 rpm) and then gently dispersed with a wide-bored pipette. After another 10 min of incubation at 37°C (70 rpm), the suspension was gently dispersed again and filtered over a 50 μm gauze and the cells were collected by centrifugation (1000g, 10 min). The isolated cells were washed three times with KRH, pH 7.4, supplemented with 2 mg ml⁻¹ fetal bovine serum (KRH/FBS) and were allowed to regenerate for 1h at 37°C (55 rpm). Subsequently, the cells were incubated with the fluorescent dye, Fura-2/AM (3 μM) for 30 min at 37°C (55 rpm). The loaded cells were washed three times in KRH/FBS and resuspended to a density of 1 x 10⁶ cells/ml. The cells were kept at room temperature on a Rock-N-Roller (Breda Scientific, The Netherlands) and used within 2-4 h, during which they remained viable and responsive.

**Intracellular Ca²⁺ measurements**

Fura-2 fluorescence of the cells (excitation wavelengths: 340 and 380 nm; emission wavelength: 510 nm) was measured at 37°C with a Perkin Elmer Spectrometer (LS-50B). Each cuvet contained 2 ml of magnetically stirred cell suspension. In all experiments, Ca²⁺- influx and -mobilisation was induced by 100 μM methacholine, added at t = 0 s. To establish the inhibitory effect of isoprenaline on Ca²⁺-influx, single doses of isoprenaline (1 nM – 10 μM) were administered on the methacholine-induced sustained rise in intracellular Ca²⁺ concentration (Ca²⁺-plateau) at t = 150 or 340 s. When used, GF109203X (10 μM) was added 45 min prior to the methacholine administration. The intracellular Ca²⁺-concentration ([Ca²⁺]) was calculated every 0.2 s according to Grynkiewicz [23]. At the end of the experiment, the maximal fluorescence ratio (R_max) was determined after adding 0.01% of Triton-X-100 as a permeabilizing agent. The minimal fluorescence ratio (R_min) was determined by addition of 5 mM EGTA to the permeabilized cells.
**Data analysis**

The methacholine-induced transient rise of \([\text{Ca}^{2+}]_i\), representing \(\text{Ca}^{2+}\)-mobilisation from internal stores, was expressed as the maximal increase of \([\text{Ca}^{2+}]_i\) above basal after the addition of methacholine. The plateau level of \([\text{Ca}^{2+}]_i\), representing \(\text{Ca}^{2+}\)-influx, was expressed as the value obtained 2 min after contractile agonist addition.

The acute inhibitory effect of isoprenaline was expressed as the peak-inhibition of \([\text{Ca}^{2+}]_i\)-influx immediately after the administration of isoprenaline. The inhibitory effect of isoprenaline on the methacholine-induced \(\text{Ca}^{2+}\)-plateau was also expressed as the area under the \([\text{Ca}^{2+}]_i\)-time curve above basal during 190 s after the addition of isoprenaline (AUC\(_{150-340}\) and AUC\(_{340-530}\)), calculated by trapezoid integration over 0.2 s periods, compared to control in the absence of the \(\beta\)-agonist. Due to isoprenaline-induced desensitization, this inhibition was time-dependently reversed. The rate of this \(\text{Ca}^{2+}\)-influx reversal was expressed as its maximal slope. All data are presented as means ± S.E.M. Statistical analysis was performed by means of the two-tailed Student’s \(t\)-test for paired observations. \(P\) values < 0.05 were considered statistically significant.

**Materials**

Dulbecco’s modification of Eagle’s Medium (DMEM), \(\text{NaHCO}_3\) solution (7.5%), penicillin/streptomycin solution (5000 U/ml; 5000 \(\mu\)g/ml) and HEPES solution (1 M) were obtained from Gibco BRL Life Technologies (Paisley, U.K.). Fetal bovine serum (FBS), dithiothreitol (DTT), trypsin inhibitor (type II-S), Fura-2/AM, methacholine chloride and (-)-isoprenaline hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and GF 109203X (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl) maleimide), papain and Collagenase P were purchased from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade.

**Results**

Figure 1a shows a typical intracellular \(\text{Ca}^{2+}\)-response in bovine tracheal smooth muscle cells to 100 \(\mu\)M methacholine, characterised by a transient-rise in \([\text{Ca}^{2+}]_i\), followed by a sustained plateau level of \([\text{Ca}^{2+}]_i\). As is also demonstrated in this figure, 10 \(\mu\)M isoprenaline caused a rapid but short-lasting inhibition of the methacholine-induced \(\text{Ca}^{2+}\)-plateau, which was almost fully reversed at \(t = 340\) s, i.e. 190 s after \(\beta\)-agonist administration, indicating a rapid desensitization of the isoprenaline-induced response.

In the presence of GF 109203X, basal \(\text{Ca}^{2+}\) level as well as the methacholine-induced transient and the sustained rise in \([\text{Ca}^{2+}]_i\), were slightly inhibited (Fig. 1b and 2). Remarkably, the isoprenaline-induced inhibition of \([\text{Ca}^{2+}]_i\)-influx was only partially reversed at \(t = 340\) s in the presence of the PKC-inhibitor (Fig. 1b), indicating that GF 109203X reduced the rate of \(\text{Ca}^{2+}\)-recovery after the administration of the \(\beta\)-agonist.
Figure 1 Typical intracellular Ca\(^{2+}\)-responses in bovine tracheal smooth muscle cells, showing the effect of 10 μM (-)-isoprenaline (Iso) on 100 μM methacholine (MCh)-induced Ca\(^{2+}\)-plateau in the absence (a) and presence (b) of 10 μM GF 109203X.

Using the AUC\(_{150-340}\) as a parameter, it is demonstrated in Figure 3 that isoprenaline dose-dependently inhibited the methacholine-induced Ca\(^{2+}\)-plateau, which was significantly potentiated by GF 109203X. Maximal (E\(_{\text{max}}\)) values of isoprenaline-induced inhibition of
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Ca^{2+}-influx in the absence and presence of GF 109203X were 34.5 ± 7.6% and 66.3 ± 5.0%, respectively ($P = 0.02$). No differences were found in the potency of isoprenaline (pEC_{50} values of 7.8 ± 0.4 and 7.6 ± 0.3 in the absence and presence of GF 109203X, respectively).

**Figure 3** Effect of (-)-isoprenaline on 100 μM methacholine-induced Ca^{2+}-plateau, measured as AUC_{150-340}, compared to control, in the absence (open symbols) and presence (closed symbols) of 10 μM GF 109203X in bovine tracheal smooth muscle cells. Results are means ± S.E.M. of 6 experiments.

The difference in $E_{\text{max}}$ of the isoprenaline-induced inhibition of Ca^{2+}-influx, using the AUC_{150-340} as a parameter, may well be explained by the different recovery rates of this inhibition in the absence and presence of GF 109203X. Figure 4 shows that, after an initial rise at 10 nM, compared to 1 nM isoprenaline, the rate of recovery decreased dose-dependently, both in the presence as well as in the absence of GF 109203X. For all isoprenaline concentrations used, GF 109203X significantly reduced this rate of recovery compared to control, resulting in a beneficial effect on the isoprenaline-induced inhibition of [Ca^{2+}]_i-influx. Interestingly, from 10 nM isoprenaline on, the fractional reduction by GF 109203X on the rate of recovery increased with higher concentrations of the β-agonist.

We also investigated the effect of GF 109203X on the maximal inhibition of the Ca^{2+}-influx, immediately after administration of the β-agonist (Fig. 5). This effect was concentration-dependent and maximal at 10^{-7} M isoprenaline. Interestingly, GF 109203X had only a minor effect on this peak-inhibition; $E_{\text{max}}$ values in the absence and presence of GF 109203X were not significantly different (85.5 ± 4.5 and 101.6 ± 5.9, respectively; $P = 0.09$). No significant differences were found in the potency of isoprenaline (pEC_{50} values of 8.7 ± 0.2 and 8.5 ± 0.1 in the absence and presence of GF 109203X, respectively).
To assess the role of methacholine-induced PKC activation in heterologous desensitization of the β₂-adrenoceptor, independent of its effect on the acute β-agonist induced desensitization, we compared two different application times of isoprenaline. If a direct interaction of activated PKC with the β₂-adrenoceptor would be responsible for the observed desensitization of the isoprenaline-induced inhibition of Ca²⁺-influx, this would imply that isoprenaline could hardly or not be able to exert an effect anymore when added at 340 s instead of at 150 s.

Figure 4 Effect of 10 μM GF 109203X on the recovery of isoprenaline-induced Ca²⁺-influx inhibition. The rate of [Ca²⁺]-recovery (nM/s) was expressed as its maximal slope. Results are means ± S.E.M. of 6 experiments. Significantly different from control: * P<0.05; ** P<0.01; *** P<0.001

Figure 5 Effect of (−)-isoprenaline on 100 μM methacholine-induced Ca²⁺-plateau, measured as maximal inhibition of [Ca²⁺], immediately after administration of the β-agonist, in the absence (open symbols) and presence (closed symbols) of 10 μM GF 109203X. Results are means ± S.E.M. of 6 experiments.
Typical intracellular Ca²⁺-responses, showing the effects of 10 μM (-)-isoprenaline added at t = 150 s (a) or 340 s (b) on 100 μM methacholine-induced Ca²⁺-plateau, in the absence (upper panels) and presence (lower panels) of 10 μM GF 109203X. Using the AUC150-340 and AUC340-530, we also found almost identical effects of isoprenaline, both in the presence and absence of GF 109203X (Fig. 7). Similar results were obtained for 1 and 10 μM isoprenaline (Fig. 7). Moreover, no differences were found in the immediate inhibitory effects of isoprenaline (1 and 10 μM) at t = 150 s and t = 340 s (not shown).
Figure 7 Effect of 1 µM (a) and 10 µM (b) (-)-isoprenaline added at \( t = 150 \) or 340 s on 100 µM methacholine-induced \( \text{Ca}^{2+} \)-plateau, measured as AUC$_{150-340}$ and AUC$_{340-530}$, compared to control, in the absence (open bars) and presence (filled bars) of 10 µM GF 109203X. Results are means ± S.E.M. of 4 separate experiments. Significantly different from control: * \( P<0.05; \) *** \( P<0.001 \).

Discussion

The present study indicates that muscarinic agonist-induced PKC activation markedly contributes to \( \beta \)-agonist induced \( \beta_2 \)-adrenoceptor desensitization in bovine tracheal smooth muscle cells. Thus, it was shown that isoprenaline caused a transient inhibition of the methacholine-induced \( \text{Ca}^{2+} \)-influx, indicating a rapid agonist-induced desensitization of this response. This \( \beta_2 \)-adrenoceptor desensitization was markedly attenuated in the presence of the PKC-inhibitor GF 109203X, indicating major involvement of methacholine-induced activation of PKC, following phosphoinositide metabolism and generation of diacylglycerol.

The heterologous regulation of \( \beta \)-agonist induced desensitization by PKC might involve phosphorylation-induced activation of GRK2. Thus, in mononuclear leukocytes it has been found that PKC-dependent increase in GRK2 activity enhanced the isoprenaline-induced homologous \( \beta_2 \)-adrenoceptor desensitization [20]. In addition, it has been shown that GRK2 activity indeed can be increased by PKC-induced phosphorylation, possibly by increasing the ability of GRK2 to bind to the plasma membrane [21,24] or by relieving tonic inhibition of GRK2 by calmodulin, allowing GRK2 to bind to the \( \beta \)-adrenoceptor [25]. Not only GRK2 but also GRK5 has been shown to be a substrate for PKC. However, in contrast to GRK2, PKC-induced phosphorylation of GRK5 strongly inhibits its activity [26], indicating that this mechanism is not involved in the observed PKC-induced potentiation of acute agonist-induced \( \beta_2 \)-adrenoceptor desensitization. However, irrespective of the GRK(s) involved, we very recently demonstrated that direct activation of PKC by the phorbol ester PMA potentiates the homologous \( \beta \)-agonist-induced desensitization of intact...
bovine tracheal smooth muscle relaxation by isoprenaline [22]. Presently it is unclear which PKC-isozyme is involved in the modulation of β2-adrenoceptor desensitization, since GF 109203X inhibits all conventional (α, β1 and β2) and novel (δ, ε and θ) PKC isozymes present in bovine tracheal smooth muscle [27].

Remarkably, a possible direct heterologous effect of methacholine-induced PKC activation on β2-adrenoceptor function was only small or even absent at t = 150 s, as indicated by the minor effect of GF 109203X on the peak-inhibition of Ca2+-influx immediately after the administration of isoprenaline, and by the equal levels of inhibition of Ca2+-influx by isoprenaline at different time points after methacholine addition. In contrast, we have previously presented direct evidence for the involvement of PKC in the cross-talk between muscarinic M4 receptor stimulation and β2-adrenoceptor function, strongly diminishing β2-agonist-induced bovine tracheal smooth muscle relaxation [16]. However, these effects were observed after longer incubation times with methacholine, i.e. after establishment of tonic contraction. This strongly suggests that PKC-induced activation of GRK(s) is the main cause of the rapid β-adrenoceptor homologous desensitization and that this process precedes PKC-induced β2-adrenoceptor (and/or Gs) phosphorylation.

From 10 nM isoprenaline on, the rate of Ca2+-recovery decreased dose-dependently with higher concentrations of isoprenaline. Although GRK is particularly activated at high β-agonist concentrations, the GRK-induced desensitization may be partially overruled by the efficacy of the agonist to increase cAMP accumulation inhibiting Ca2+-influx. GF 109203X significantly inhibited the rate of recovery for all concentrations of isoprenaline used, which is fully in line with the observed effects of GF 109203X on the isoprenaline-induced inhibition of Ca2+-influx, using the AUC150-340 as a parameter. Assuming that PKC enhances GRK-induced desensitization, this observation is in line with the fact that GRK-induced desensitization becomes quantitatively more important at higher concentrations of isoprenaline [13,18,28].

It was demonstrated that isoprenaline-induced desensitization was not fully reversed by GF 109203X, indicating PKC-independent contribution of GRKs and/or PKA to the agonist-induced desensitization. Notably, in previous studies in bovine tracheal and rat oesophagus smooth muscle it has been demonstrated that PKA-dependent, but β-adrenoceptor-independent, relaxation induced by forskolin and 3-isobutyl-1-methyl-xanthine (IBMX) was not inhibited by 10 μM GF109203X [16,17], indicating that PKA-activity is not affected by the PKC inhibitor. As shown in Figures 1 and 2, GF 109203X slightly diminished both the methacholine-induced Ca2+-transient and the Ca2+-plateau, which is in agreement with previous observations indicating that methacholine-induced PKC activation may potentiate agonist-induced mobilization and influx of Ca2+ in bovine tracheal smooth muscle cells [29]. This effect is relatively small at high concentrations of methacholine [29], hence all experiments were performed with 100 μM of the agonist.

In conclusion, the present study was undertaken to explore the concept of heterologous regulation of homologous β2-adrenoceptor desensitization by contractile agonists in airway smooth muscle cells. The results indicated that methacholine-induced PKC-activity is markedly involved in the acute and rapid desensitization of the β-agonist-induced inhibition
of Ca\textsuperscript{2+}-influx in bovine tracheal smooth muscle. Since it was demonstrated that direct heterologous desensitization of the β\textsubscript{2}-adrenoceptor by PKC was hardly or not involved under the conditions used, muscarinic receptor-mediated PKC-activation may indeed potentiate the β-agonist-induced (homologous) desensitization, presumably by enhancing GRK activity. As many PKC-activating mediators and neurotransmitters are being released during an asthma exacerbation, the cross-talk between PKC and GRKs may be of great functional importance for the development of a β\textsubscript{2}-adrenoceptor dysfunction in patients with severe asthma, who are frequently using high doses of β-adrenergic drugs to relieve bronchoconstriction.

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**References**

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