Desensitization of α_2 -adrenergic receptors in NG 108 15 cells by (–)-adrenaline and phorbol 12-myristate 13-acetate

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α_2 -Adrenergic receptors on NG 108 15 cell membranes were identified by [3 H]rauwolscine binding: $B_{\max} = 661 \pm 81$ fmol/mg of protein, $K_d = 6.9 \pm 2.5$ nM (means $\pm$ S.E.M., $n = 6$). On intact cells, stimulation of these receptors by (–)-adrenaline inhibited the prostaglandin- E_1 -stimulated adenylate cyclase activity by about 60 %. The effect of (–)-adrenaline was pertussis-toxin-sensitive, indicating the involvement of an inhibitory G protein. (–)-Adrenaline/[3 H]rauwolscine competition-binding experiments revealed that only 50 % of the α_2 receptors were coupled to G proteins (i.e. displayed high agonist affinity). Pre-treatment of the cells with 20 μ M-(–)-adrenaline provoked homologous desensitization of the α_2 receptors. The α_2 -adrenergic response decreased after a time lag of about 2 h, to reach a minimum after 12 h. The bradykinin and muscarinic responses were not affected. The α_2 -receptor concentration decreased without time lag. The high-agonist-affinity sites disappeared more rapidly ($t_{1/2} = 42$ min) than did the low-affinity uncoupled sites ($t_{1/2}$ approx. 20 h). In contrast, pertussin-toxin-mediated [32 P]ADP-ribosylation of inhibitory G proteins was unaffected by the pre-treatment. Pretreatment of intact NG 108 15 cells with 1 μ M-phorbol 12-myristate 13-acetate (PMA) provoked a rapid decrease of the α_2 -adrenergic response. The effect was nearly complete after 40 min. PMA also decreased the bradykinin response, suggesting a heterologous desensitization process. The α_2 -receptor concentration, the (–)-adrenaline competition-binding curves and the pertussis- and cholera-toxin-mediated [32 P]ADP-ribosylation of their respective G proteins were not affected.

INTRODUCTION

G proteins are required for the transfer of information between hormone-stimulated receptors and the adenylate cyclase enzyme: G_s for stimulatory receptors, and G_i and possibly also G_o for inhibitory receptors [1]. They are heterotrimeric, with interchangeable $\beta\gamma$ subunits, but with distinct α subunits possessing a guanine-nucleotide-binding site. The agonist–receptor complex facilitates the exchange of bound GDP with cytosolic GTP, resulting in the separation of the active GTP-bound α subunit from $\beta\gamma$. Whereas for G_s , α_s -GTP is known to stimulate the adenylate cyclase, the mechanism of adenylate cyclase inhibition by the activated G_i is still controversial.

Prolonged treatment of target cells with agonists results in the blunting or desensitization of the adenylate cyclase response. The regulation of stimulatory receptors such as the β -adrenergic receptor has been elucidated to a great extent [2,3], but desensitization studies with the inhibitory α_2 -adrenergic receptors have led to controversial results [3–7]. Heterologous regulation of the α_2 receptors is also evidenced by the ability of PMA to desensitize the α_2 -receptor-mediated inhibition of adenylate cyclase in platelets [8,9], presumably via activation of a Ca^{2+} - and phospholipid-dependent protein kinase C [10].

NG 108 15 cells represent an excellent model system for the investigation of neuronal cells [11]. In the present study, we have compared the regulation of the α_2 -adrenergic receptors by (–)-adrenaline and PMA in these cells, using adenylate cyclase activity measurements,

radioligand binding and pertussis- and cholera-toxin-mediated [32 P]ADP-ribosylation experiments.

MATERIALS AND METHODS

Materials

Culture media and supplements were purchased by Gibco. [3 H]Rauwolscine (74 Ci/mmol), cyclic [3 H]AMP (29.4 Ci/mmol) and [32 P]NAD $^+$ (35 Ci/mmol) were obtained from New England Nuclear. (–)-Adrenaline, PMA, nicotinamide, forskolin, cholera toxin and pargyline hydrochloride were from Sigma. Thymidine, EDTA, trichloroacetic acid and the premixed low- M_r protein standard kit were from Merck. ATP, GTP and p[NH]ppG were obtained from Boehringer Mannheim. Charcoal (Norit A) and electrophoresis reagents were from Serva. Theophylline was from Calbiochem, Hepes from U.S. Biochemical Corp., rauwolscine from Carl Roth K.G., and ascorbic acid from Carlo Erba. The following were obtained as generous gifts: UK 14304 [5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline] (Pfizer Central Research), guanafacine hydrochloride (Sandoz), PGE $_1$ (Upjohn), pertussis toxin (R. Verschuere, Pasteur Instituut van Brabant, Brussels). All other compounds were of analytical purity.

Cell growth and harvesting

NG 108 15 cells of passage numbers 14 and 20 were kindly provided by Dr. D. M. Nirenberg (National Institutes of Health, Bethesda, MD, U.S.A.) and Dr. S.

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; G protein, guanine-nucleotide-binding protein; p[NH]ppG, guanylyl imidodiphosphate; PGE $_1$, prostaglandin E_1 .

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Bottari (Sandoz, Basel, Switzerland) respectively. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal-calf serum, 10% (v/v) HAT (100 μ M-hypoxanthine, 1 μ M-aminopterin, 16 μ M-thymidine), 2 mM-glutamine and 1 mM-sodium pyruvate, at 37 °C in a 7% CO_2 incubator [12]. Confluent monolayers in 75 cm² culture flasks were detached with 0.004% EDTA in phosphate-buffered saline (10 mM-potassium phosphate/145 mM-NaCl, pH 7.4) and subcultured in 6 mm-diam. Petri dishes for cyclic AMP determinations or in 140 mm Petri dishes for membrane preparations.

(-)-Adrenaline and PMA pre-treatment

Pre-treatment experiments were performed on monolayers in culture medium. Agonist pre-treatment was initiated by addition of (final concns.) 20 μ M-(-)-adrenaline, 100 μ M-pargyline to inhibit monoamine oxidase and 100 μ M-ascorbic acid to inhibit catecholamine oxidation. In control experiments vehicle contained 100 μ M-pargyline and 100 μ M-ascorbic acid. Care was taken to prevent the pH of the medium from exceeding 7.4. The medium was replaced every 10 h for long exposures. Phorbol ester pre-treatment was performed by adding 1 μ M-PMA in 0.5% dimethyl sulphoxide to the monolayers, or for control experiments only the vehicle.

The pre-treatment was stopped by aspiration of the medium. The monolayers were then washed once with ice-cold Dulbecco's medium. For membrane preparations, the cells were quickly harvested with phosphate-buffered saline/0.004% EDTA and stored in liquid N₂.

For cyclic AMP determinations, harvested cells were centrifuged (1000 g) for 3 min at 4 °C in Krebs-Ringer-Hepes buffer (110 mM-NaCl, 5 mM-KCl, 1 mM-MgCl₂, 1.8 mM-CaCl₂, 25 mM-glucose, 1 mg of bovine serum albumin/ml and 10 mM-Hepes, pH 7.4). The pelleted cells were resuspended in Krebs-Ringer-Hepes buffer, and the cyclic AMP determination was immediately started. The cells could easily be detached from the culture dishes without using the proteolytic enzyme trypsin. Experiments could thus be performed in cell suspension, leading to more accurate results.

Cyclic AMP determination

Krebs-Ringer-Hepes buffer (100 μ l), containing the indicated drugs, was added to 100 μ l of cell suspension (5×10^5 cells/ml) and incubated at 37 °C. All incubation mixtures contained 0.5 mM-theophylline to inhibit phosphodiesterase activity. After 15 min, the incubation was stopped by adding 800 μ l of 4 mM-EDTA (pH 7.4) and boiling for 6 min. Denatured proteins were discarded by centrifugation at 13000 g for 2 min, and the supernatants were stored at -20 °C. Cyclic AMP concentrations were determined by the method of Gilman [13], by using protein kinase A (kindly provided by Dr. A. P. IJzerman, Center for Bio-Pharmaceutical Sciences, Leiden, The Netherlands).

Membrane preparation

NG 108 15 cells, stored in liquid N₂, were thawed in 10 vol. of 10 mM-Tris/HCl (pH 7.5)/10 mM-MgCl₂/0.25 M-sucrose (sucrose buffer). This suspension was homogenized with a motor-driven Potter/Elvehjem homogenizer (15 strokes at maximum speed). The homogenate was centrifuged at 2000 g for 10 min, and the resulting supernatant was centrifuged at 29000 g for

20 min. The pellets were washed three times by centrifugation as above, suspended in 50 mM-Tris/HCl (pH 7.5)/10 mM-MgCl₂ containing 10% (v/v) glycerol, and stored in liquid N₂. Protein concentrations were determined by the method of Lowry *et al.* [14], with serum albumin as the standard.

Binding of [³H]rauwolscine

The binding was performed as described previously for calf retina membranes [15]. Briefly, membrane protein (0.3–0.6 mg/ml) was incubated with the indicated concentration of [³H]rauwolscine (1–20 nM for saturation binding, 5 mM for competition binding) for 15 min at 37 °C in 50 mM-Tris/HCl (pH 7.5)/10 mM-MgCl₂ in a final volume of 500 μ l. At the end of the incubation, the samples were filtered under reduced pressure through glass-fibre filters (Whatman GF/B) and rapidly washed with 4 $\times$ 4 ml of ice-cold buffer. The amount of radioligand remaining on the filters was determined by liquid-scintillation counting. Non-specific binding was determined with 10 μ M-phentolamine. Binding isotherms were analysed by non-linear least-squares curve fitting with the program LIGAND [16] to determine the maximum number of sites (B_{max}) and equilibrium dissociation constants.

ADP-ribosylation

ADP-ribosylation was performed as described by Ribeiro-Neto *et al.* [17]. Membranes (1–5 mg of protein/ml) were incubated with 1 mM-ATP, 0.1 mM-GTP, 1 mM-EDTA, 10 mM-thymidine, 10 μ M-[³²P]NAD⁺ and 10 μ g of activated pertussis toxin/ml in a final volume of 60 μ l for 45 min at 30 °C. For cholera-toxin-mediated ADP-ribosylation, 300 mM-potassium phosphate (pH 7.0), 10 mM-MgCl₂ and 100 μ g of activated cholera toxin/ml were added instead of activated pertussis toxin. The reaction was stopped by addition of 1 ml of ice-cold 20% (w/v) trichloroacetic acid, kept on ice for 20 min and then centrifuged at 1000 g for 20 min at 4 °C. The pellets were washed once with 1.5 ml of diethyl ether and collected by centrifugation at 1000 g for 30 min. The membranes in the pellet were then subjected to polyacrylamide-gel electrophoresis as described by Laemmli [18], using 10% acrylamide slab gels. The dried gels were exposed for 24 h to Kodak X-Omat AR films.

RESULTS

The basal adenylate cyclase activity in intact NG 108 15 cells was measured in the presence of the phosphodiesterase blocker theophylline (0.5 mM). A 15 min incubation at 37 °C resulted in the accumulation of 1.2 ± 0.2 pmol of cyclic AMP/10⁴ cells (mean $\pm$ S.E.M., $n = 4$). Addition of 10 μ M-PGE₁ to the incubation mixture increased the cyclic AMP concentration about 10-fold (Table 1). The α_2 -adrenergic agonists (-)-adrenaline (20 μ M; Table 1) and UK 14304 (1 μ M; cyclic AMP content = 3.0 ± 1.6 pmol/10⁴ cells) provoked a 60% inhibition in the PGE₁ response. This maximal inhibition was completely reversed upon addition of the α_2 -adrenergic antagonist rauwolscine (100 μ M) to the incubation mixture (Table 1). Bradykinin (10 μ M) and carbachol (100 μ M) inhibited the PGE₁ response to a similar extent as (-)-adrenaline. Pre-treatment of NG 108 15 cells with 500 ng of pertussis toxin/ml for 12 h in culture medium abolished these inhibitory responses

Table 1. Adenylate cyclase activity in intact NG 108 15 cells

Cells were pre-treated with 500 ng of pertussis toxin/ml or 20 μ M-(−)-adrenaline (ADR) for 12 h and with 1 μ M-PMA for 40 min. In experiments where (−)-adrenaline and pertussis toxin were co-incubated, 1 μ g of the toxin/ml was added 3 h before the end of the pre-treatment. Cyclic AMP determinations were performed as described in the Materials and methods section. A concentration of 10 μ M was used for (−)-adrenaline and bradykinin, and 100 μ M for rauwolscine (RAUW) and carbachol. Values are means $\pm$ S.E.M. of duplicate determinations in three experiments.

Pretreatment	Cyclic AMP content (pmol/10 ⁴ cells) in presence of 10 μ M-PGE ₁ + :				
	Buffer	ADR + RAUW	ADR + RAUW	Bradykinin + RAUW	Carbachol
Vehicle	13.6 $\pm$ 1.4	5.1 $\pm$ 1.7	12.7 $\pm$ 1.8	4.3 $\pm$ 2.0	3.4 $\pm$ 1.5
Pertussis toxin (500 ng/ml)	27.0 $\pm$ 3.0	24.2 $\pm$ 4.0	29.2 $\pm$ 2.3	24.3 $\pm$ 4.6	28.3 $\pm$ 2.8
ADR (20 μ M)	23.6 $\pm$ 3.6	19.4 $\pm$ 2.9	24.5 $\pm$ 4.8	6.8 $\pm$ 2.0	6.3 $\pm$ 2.6
PMA (1 μ M)	26.5 $\pm$ 3.2	21.9 $\pm$ 3.8	24.7 $\pm$ 3.9	20.7 $\pm$ 5.9	4.6 $\pm$ 1.3
ADR (20 μ M) + pertussis toxin (1 μ g/ml)	22.4 $\pm$ 2.1	2.5 $\pm$ 3.5	23.0 $\pm$ 3.6	21.3 $\pm$ 1.8	22.5 $\pm$ 3.5
PMA (1 μ M) + pertussis toxin (1 μ g/ml)	51.0 $\pm$ 3.2	45.0 $\pm$ 3.8	44.2 $\pm$ 5.9	51.4 $\pm$ 5.4	48.9 $\pm$ 3.9

(Table 1). Accordingly a pertussis-toxin-sensitive G protein was involved in the adenylate cyclase inhibition by all three receptor types. Pertussis toxin also potentiated the PGE₁ response by about 90 % (Table 1).

To study agonist-mediated desensitization of the α_2 -adrenergic receptors, NG 108 15 cells were pre-treated with 20 μ M-(−)-adrenaline for different periods of time up to 24 h. (−)-Adrenaline is a more suitable ligand for long pre-treatments than UK 14304, for the latter is more lipophilic and is strongly retained in the cells [19]. The (−)-adrenaline-pre-treated cells were quickly washed by centrifugation (3 min at 4 °C) and resuspension in fresh buffer, and immediately used for cyclic AMP determinations. Similar amounts of cyclic AMP were obtained when the pre-treated cells were incubated with PGE₁ either alone or in the presence of 100 μ M-rauwolscine (results not shown). Hence possible adenylate cyclase inhibition by residual (−)-adrenaline was excluded.

(−)-Adrenaline pre-treatment of the cells provoked a time-dependent decrease in the α_2 -adrenergic response (Fig. 1). After an initial time lag of 1–2 h, the response decreased rapidly until a plateau value was reached at about 6 h of pre-treatment. No further decrease could be observed by prolonging the preincubation time up to 24 h (Fig. 1a) or by increasing the (−)-adrenaline concentration to 50 μ M (results not shown). Similar results were obtained when washing and incubation were carried out in the continuous presence of (−)-adrenaline (Fig. 1b). In these experiments, the α_2 -adrenergic response was evaluated by comparing cyclic AMP levels in the presence of 10 μ M-PGE₁ + 100 μ M-rauwolscine and PGE₁ alone. Accordingly, the incomplete desensitization of the α_2 response could not be attributed to α_2 -receptor resensitization in (−)-adrenaline-free incubation medium. The bradykinin and carbachol responses were not affected by (−)-adrenaline pre-treatment (Table 1), suggesting that this agonist produces a receptor-specific homologous desensitization.

(−)-Adrenaline pre-treatment potentiated the PGE₁ response to the same degree as did pertussis toxin. This (−)-adrenaline effect occurred without time lag and with a $t_{1/2}$ of approx. 1.75 h (Fig. 1a). The (−)-adrenaline and pertussis-toxin effects were not additive: incubation of (−)-adrenaline-pre-treated cells (20 μ M; 12 h) with 1 μ g

of pertussis toxin/ml for 3 h did not further increase the PGE₁ response.

The phorbol ester PMA (1 μ M) also induced a time-dependent decline of the α_2 -adrenergic response as well as a concomitant potentiation of the PGE₁ response. Both effects occurred with a $t_{1/2}$ of about 4 min and were complete within 40 min (Fig. 2). Incubation of the cells with PMA for 30 min also decreased the bradykinin response, but not the carbachol response (Table 1). In contrast with (−)-adrenaline regulation, the PMA regulation appears to be heterologous. The difference between both mechanisms is further stressed by the additivity of the PMA- and pertussis-toxin-mediated potentiation of the PGE₁ response (Table 1).

α_2 -Adrenergic receptors were characterized in NG 108 15 cell membranes by binding of the radiolabelled antagonist [³H]rauwolscine. Non-linear regression analysis of the saturation-binding experiments revealed the presence of one class of non-co-operative high-affinity binding sites (Fig. 3). These sites have previously been described as α_2 -adrenergic receptors. Pre-treatment of the cells with 20 μ M-(−)-adrenaline provoked a time-dependent ($t_{1/2}$ = 1.75 h) decline in the α_2 -adrenergic-receptor number (Figs. 3a and 4); a maximal decline of 60 % was reached after 12 h of preincubation (Fig. 4). The K_d values of [³H]rauwolscine for the remaining sites were not affected by the pre-treatment. p[NH]ppG (0.5 mM), which decreases the affinity of α_2 receptors for agonists [20], did not affect the [³H]rauwolscine saturation-binding parameters in membrane preparations from control or (−)-adrenaline pre-treated cells (results not shown). These data indicate that the decreased α_2 -receptor number in membranes from (−)-adrenaline-pre-treated cells is unrelated to 'tight binding' of residual agonist.

(−)-Adrenaline/[³H]rauwolscine competition-binding experiments displayed shallow curves (h = 0.65). This can be attributed to the existence of receptor populations with different agonist affinity: the high-affinity population corresponding to the receptors which can undergo functional coupling to G proteins, the low-affinity sites corresponding to the free receptors [20]. Computer-assisted analysis of these curves, following a two-site model, revealed that 50 % of the receptors displayed high affinity for the agonist (Table 2). p[NH]ppG (0.5 mM),

Fig. 1. Effect of (–)-adrenaline pre-treatment on the response to PGE_1 and the α_2 -adrenergic response

(a) Cells were pre-treated with $20 \mu\text{M}$ (–)-adrenaline for the times indicated, washed with Krebs–Ringer–Hepes buffer and incubated for 15 min with $10 \mu\text{M}$ - PGE_1 or $10 \mu\text{M}$ - PGE_1 + $10 \mu\text{M}$ (–)-adrenaline, after which the cyclic AMP content was measured. Adenylate cyclase (AC) inhibition by (–)-adrenaline (ADR; $\square$) is expressed as a percentage of the cyclic AMP level in cells incubated with PGE_1 alone (for corresponding pre-treatment times). Cyclic AMP levels in cells incubated with PGE_1 alone ($\blacksquare$) are expressed as a percentage of control (i.e. the cyclic AMP level when the pre-treatment was omitted). (b) (–)-Adrenaline-pre-treated cells were washed in the continuous presence of $10 \mu\text{M}$ (–)-adrenaline and incubated for 15 min with $10 \mu\text{M}$ - PGE_1 + $10 \mu\text{M}$ (–)-adrenaline either in the absence ($\square$) or presence (hatched) of $100 \mu\text{M}$ -rauwolscine ($\blacksquare$), after which the cyclic AMP content was measured. Values are means $\pm$ S.E.M. of duplicate determinations in six experiments.

known to induce receptor–G-protein dissociation, provokes a rightward shift and steepening of the curves (Table 2).

Pretreatment of the NG 108 15 cells with (–)-adrenaline provoked a time-dependent decrease in the proportion of high-affinity sites with $t_{1/2}$ of about 1.75 h (Table 2). These sites were completely lost after 9 h of pre-treatment, the (–)-adrenaline competition-binding curves for these membranes being steep ($h = 0.93$) and displaying only low affinity (Table 2). In addition, 0.5 mM -p[NH]ppG no longer shifted curves to low affinity. These curves are superimposable on those of control membranes in the presence of nucleotide.

PMA pre-treatment of the cells ($1 \mu\text{M}$, 40 min) did not provoke a decline in the amount of α_2 receptors (Fig. 3). The (–)-adrenaline competition-binding parameters, as

Fig. 2. Effect of PMA on the response to PGE_1 and the α_2 -adrenergic response

Cells were incubated for 15 min at 37°C with $10 \mu\text{M}$ - PGE_1 (a, $\blacksquare$; b, $\square$), with $10 \mu\text{M}$ - PGE_1 + $10 \mu\text{M}$ (–)-adrenaline (b, hatched) or with $10 \mu\text{M}$ - PGE_1 + $10 \mu\text{M}$ (–)-adrenaline + $100 \mu\text{M}$ -rauwolscine (b, hatched), after which $1 \mu\text{M}$ -PMA was added and the cyclic AMP content measured after the indicated periods of time. In (a), the adenylate cyclase (AC) inhibition by (–)-adrenaline (ADR; $\square$) is expressed as a percentage of the cyclic AMP level in cells incubated with PGE_1 (for corresponding periods of PMA treatment). Cyclic AMP levels in cells incubated with PGE_1 ($\blacksquare$) are expressed as a percentage of control (i.e. the cyclic AMP level when the PMA treatment was omitted). Values are means $\pm$ S.E.M. of duplicate determinations in three experiments.

well as the ability of these curves to undergo a rightward shift in the presence of p[NH]ppG, were not affected either.

Modification of pertussis-toxin-sensitive G proteins by endogenous factors was assessed by comparing the toxin-catalysed incorporation of [^{32}P]ADP-ribose into the various membrane preparations [21,22]. SDS/polyacrylamide-gel electrophoresis and autoradiography revealed ^{32}P -labelling of a single band with a M_r of about 40000. As shown in Fig. 5, the pertussis-toxin-catalysed ADP-ribosylation occurred to a similar extent in control, (–)-adrenaline- and PMA-preincubated membranes. The K_m for [^{32}P]NAD $^+$ was also similar in all three membrane preparations, i.e. 11, 14 and $10 \mu\text{M}$ ($n = 2$). Endogenous ADP-ribosylation of G_s has recently [22] also been described as a desensitization mechanism for adenylate cyclase inhibitory receptors. Cholera toxin catalysed the [^{32}P]ADP-ribosylation of a single peptide band with an M_r of 48000 on SDS/polyacrylamide-gel electrophoresis. The autoradiograph showed no differences in the extent of [^{32}P]ADP-ribosylation between

Fig. 3. Scatchard plots of [3 H]rauwolscine binding to membranes of (–)-adrenaline- and PMA-pre-treated NG 108 15 cells

Cells were pre-treated for 24 h in the absence (●) or presence (○) of 20 μ M (–)-adrenaline (a), or for 40 min in the absence (■) or presence (□) of 1 μ M-PMA (b), after which membranes were prepared and specific [3 H]rauwolscine binding was determined as described in the Materials and Methods section. The saturation binding curves were analysed by non-linear least-squares fitting by using LIGAND. All curves were significantly fitted by a one-site model ($P < 0.01$). The receptor concentrations ($B_{\max}$, in fmol/mg of protein) and the equilibrium dissociation constants (K_d , in nM) are: (●), 661 ± 81 and 6.9 ± 2.5 ; (○), 299 ± 22 and 7.1 ± 2.7 ; (■), 641 ± 38 and 4.6 ± 1.8 ; (□), 657 ± 35 and 5.9 ± 1.0 (means $\pm$ S.E.M. of 3–6 experiments). The Scatchard plots shown (drawn according to K_d and $B_{\max}$ values calculated by LIGAND) are those of representative experiments.

control membranes and membranes of (–)-adrenaline- and PMA-pre-treated cells (Fig. 5). Furthermore, addition of 25 mM-nicotinamide to the preincubation mixture did not influence the (–)-adrenaline- and PMA-mediated changes in adenylate cyclase activity (results not shown). These data confirm the absence of putative endogenous ADP-ribosylation of α_s .

DISCUSSION

(–)-Adrenaline and the phorbol ester PMA desensitize the α_2 -receptor-mediated inhibition of the adenylate cyclase activity in NG 108 15 cells by different mechanisms.

(–)-Adrenaline provokes homologous desensitization of the α_2 receptors. This conclusion is based on the agonist's ability to decrease in the α_2 -receptor-mediated inhibition of the adenylate cyclase activity without affecting the analogous muscarinic and bradykinin responses

Fig. 4. Time-dependent changes of the [3 H]rauwolscine-binding characteristics for membranes of (–)-adrenaline-pre-treated cells

Cells were pre-treated with 20 μ M (–)-adrenaline for the times indicated, and membranes were prepared. Specific [3 H]rauwolscine saturation binding (2–10 nM) was determined as described in the Materials and Methods section. K_d values (upper panel) and $B_{\max}$ values (lower panel, expressed as a percentage of the $B_{\max}$ value for non-pre-treated membranes) were calculated by non-linear regression analysis. Values are means $\pm$ S.E.M. of three experiments.

(Table 1). The observation that (–)-adrenaline pre-treatment did not affect the pertussis-toxin-mediated [32 P]ADP ribosylation of its M_r -41 000 substrate also indicates that no major modification of the inhibitory G proteins took place.

Desensitization of the α_2 -adrenergic response was accompanied by a decrease in the total receptor number (i.e. specific [3 H]rauwolscine-binding sites). This decrease can be divided into two components, each affecting half of the receptor population. The fast component ($t_{1/2} = 42$ min) corresponds to the loss of high-agonist-affinity sites. After 6 h, coupling between the receptors and G proteins (i.e. GTP-sensitive high-agonist-affinity sites) could no longer be detected (Fig. 6). The slow component ($t_{1/2}$ of approx. 20 h) corresponds to the loss of low-agonist-affinity sites. The conversion of high- into low-agonist-affinity sites could not be detected by the present experiments. This indicates either that the coupled receptors are down-regulated directly, or that the loss of functional coupling between the receptors and G proteins is immediately followed by their down-regulation. The demonstration by Thomas & Hoffman [7] that down-regulation of α_2 receptors still occurs in pertussis-toxin-treated NG 108 15 cells is compatible with this last-mentioned mechanism.

Table 2. (–)-Adrenaline/[³H]rauwolscine competition-binding parameters as a function of pre-treatment time with (–)-adrenaline and PMA

Cells were preincubated with 20 μ M-(–)-adrenaline (ADR) or with 1 μ M-PMA for the indicated periods of time. Membranes were prepared and incubated with 5 nM-[³H]rauwolscine and increasing concentrations of (–)-adrenaline (0.3 nM–0.1 mM) in either the absence or the presence of 0.5 mM-p[NH]ppG. The competition-binding data were analysed by non-linear regression analysis (LIGAND) to yield the percentage of high-affinity sites (R_h) and the agonist K_i values from the high- (K_h) and low-affinity (K_l) sites. Values are means $\pm$ S.E.M. of three experiments.

Pretreatment conditions		Competition binding parameters for ADR			
Drug	p[NH]ppG	Time (h)	K_h (nM)	K_l (nM)	R_h (%)
ADR (20 μ M)	–	0	10 $\pm$ 0	630 $\pm$ 10	49 $\pm$ 1
	–	1	17 $\pm$ 8	770 $\pm$ 40	31 $\pm$ 7
	–	2	15 $\pm$ 11	720 $\pm$ 30	22 $\pm$ 3
	–	3	11 $\pm$ 6	510 $\pm$ 80	8 $\pm$ 3
	–	6	–	630 $\pm$ 60	0
	–	9	–	590 $\pm$ 60	0
	–	12	–	290 $\pm$ 00	0
	–	24	–	230 $\pm$ 70	0
	0.5 nM	0	–	780 $\pm$ 70	0
	0.5 nM	12	–	940 $\pm$ 80	0
PMA (1 μ M)	–	0.75	11 $\pm$ 2	840 $\pm$ 400	54 $\pm$ 5
	0.5 nM	0.75	–	830 $\pm$ 200	0

Fig. 5. Autoradiograph of pertussis- and cholera-toxin-catalysed [³²P]ADP-ribosylation of membranes

Cells were pre-treated with vehicle, 20 μ M-(–)-adrenaline (ADR) or 1 μ M-PMA. Membranes were prepared and incubated with 10 μ M-[³²P]NAD⁺ in the presence of pertussis toxin (a) or cholera toxin (b) as described in the Materials and methods section. SDS/PAGE and autoradiography showed a band of M_r 40 000–41 000 and 48 000 for the pertussis- and cholera-toxin-catalysed ADP-ribosylation, respectively. The bands were cut out from the gel and counted for radioactivity by liquid-scintillation counting. Control, (–)-adrenaline and PMA bands contained respectively 6784, 6666 and 6802 c.p.m. for the pertussis-toxin-mediated ADP-ribosylation, and 1117, 1247 and 1384 c.p.m. for cholera-toxin-mediated ADP-ribosylation. The data are representative of those obtained in three similar experiments.

Benovic *et al.* [23] recently demonstrated that the cyclic-AMP-independent β -adrenergic-receptor kinase, an enzyme known to be involved in the homologous desensitization of β -receptors, is also able to catalyse the phosphorylation of purified human platelet α_2 receptors.

Fig. 6. (–)-Adrenaline-mediated desensitization of the total α_2 -receptor concentration, the high- and low-affinity sites and the α_2 -adrenergic inhibition of the adenylate cyclase

Total α_2 -receptor concentration, expressed as a percentage of the receptor concentration of untreated membranes ($\square$), was obtained from Fig. 4. The number of high- ($\blacksquare$) and low- ($\bullet$) affinity sites was calculated by multiplication of the total receptor concentration by, respectively, the fraction of R_h and R_l sites (Table 2) for the corresponding times of agonist pre-treatment. (–)-Adrenaline (ADR)-mediated inhibition of the adenylate cyclase (AC) (%; $\circ$) was calculated from the values in Fig. 1 by normalization of the percentage inhibition in untreated cells to 100 %.

Whether such a mechanism intervenes in the down-regulation of the α_2 receptors in NG 108 15 cells remains to be investigated.

After 12 h of (–)-adrenaline pre-treatment, this agonist still produced a 30 % inhibition of the adenylate cyclase. This response was mimicked by the synthetic agonist UK 14304 (results not shown) and inhibited by rauwolscine, indicating the involvement of α_2 receptors. This incomplete decrease in the α_2 -adrenergic response is in apparent contradiction with the loss of detectable

coupling between the α_2 receptors and G proteins. The 'spare receptor' concept provides a plausible explanation for this apparent discrepancy [24]. The ability of a single β -adrenergic receptor to stimulate multiple G_s proteins has been clearly demonstrated by reconstitution experiments [25]. Accordingly, it is quite possible that, after desensitization, a restricted (i.e. no longer detectable by agonist competition-binding studies) number of α_2 receptors remain able to stimulate a sufficient amount of G proteins to provoke perceptible adenylate cyclase inhibition. The 'spare receptor' concept also provides an explanation for the time lag of approx. 2 h, after which the α_2 -adrenergic response starts to decline in (–)-adrenaline-pre-treated cells (Fig. 6).

PMA treatment of the NG 108 15 cells provoked rapid ($t_{1/2} = 4$ min) desensitization of the α_2 -adrenergic response. This phenomenon clearly involved stimulation of the protein kinase C enzyme, since it was inhibited by the protein kinase C blocker staurosporine (results not shown) [26]. In contrast with the (–)-adrenaline pre-treatment, desensitization of the α_2 -adrenergic response by PMA was not associated with a decrease in total α_2 -receptor number, or with a decrease in their ability to couple to G proteins. These findings suggest that the α_2 -receptor proteins themselves are not affected by protein kinase C activation. Instead, protein kinase C-mediated phosphorylation of G proteins has already been reported to be responsible for the decreased transduction of inhibitory signals to the adenylate cyclase enzyme in human blood platelets and in the cyc[−] S49 lymphoma cells [8,9]. The ability of PMA to decrease the (–)-adrenaline- as well as the bradykinin-mediated cyclase inhibition is also compatible with a modification of G proteins in NG 108 15 cells. However, this modification does not affect the receptor-binding capability of these proteins, since the α_2 receptors still display GTP-sensitive high agonist affinity. Their site for pertussis-toxin-mediated [³²P]ADP-ribosylation is not affected, either.

(–)-Adrenaline and PMA pre-treatment of the NG 108 15 cells also potentiated the PGE₁ stimulation of the adenylate cyclase. This effect of PMA has also been observed in human blood platelets, and is compatible with the proposed decline in G_i activity. The effect of (–)-adrenaline is more difficult to explain: it can be attributed to an increase in the amount of adenylate cyclase catalytic units, a decreased inhibition by G_i or, as proposed by Jacquemin *et al.* [22], an increased stimulation by G_s . If so, this last mechanism does not seem to involve endogenous ADP-ribosylation of G_s , since cholera toxin catalyses the [³²P]ADP-ribosylation of α_s to a similar extent in membranes from control and (–)-adrenaline-treated NG 108 15 cells.

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