The phorbol ester TPA stimulates the expression of functional β -adrenoceptors in human T lymphoblasts Molt 3

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In crude membranes from human T lymphoblasts Molt 3 cultured under standard conditions, the adenylate cyclase system was stimulated by GTP, its $\beta\gamma$ -imido analogue (p[NH]ppG,) NaF and forskolin, but not by isoprenaline, prostaglandin E_1 and vasoactive intestinal peptide. TPA (tumour-promoting agent phorbol ester) added at low concentration (3.2 nM) to the culture medium induced a marked increase in functional β_2 -adrenoceptors. Competition curves of [125 I]cyanopindolol with the antagonist ICI 118.551 and four β -adrenergic agonists indicated that the emergence of functional β_2 -adrenoceptors corresponded to one class of binding sites, shifting from a high-affinity state for agonists to a low-affinity state in the presence of p[NH]ppG. This expression of β_2 -adrenoceptors after a 4 h lag period depended on newly formed mRNA and protein synthesis as judged by the inhibitory effects of actinomycin D and cycloheximide. Further effects of TPA included alterations of the stimulatory G-protein G_s and/or the catalytic unit of adenylate cyclase.

INTRODUCTION

It was claimed recently that the penetration of the human immunodeficiency virus (HIV) into human T lymphocytes can be inhibited by low concentrations of VIP, and VIP receptors in lymphocytes were considered as directly involved in the recognition of the viral surface gp 120 env glycoprotein through a threonine-rich 'peptide T' epitope (ASTTTNYT) structurally related to the 4–11 (A-T-NYT) sequence of VIP (Pert *et al.*, 1986; Sacerdote *et al.*, 1988).

We previously documented the presence of an original subclass of VIP receptors in human CD_4^+ SUP-T1 lymphoma cells, and were unable to observe any interaction of a 'peptide T' analogue with these receptors (Robberecht *et al.*, 1988, 1989). We then explored VIP receptors in other CD_4^+ lymphoblast cell lines currently utilized for testing syncytium formation after HIV invasion. We came across the human Molt 3 cell line, whose adenylate cyclase system is stimulated by guanine nucleotides, NaF and forskolin, but not by VIP, β -adrenergic agonists and prostaglandins (three typical stimulants of lymphoid cells). Accordingly, we were also unable to identify binding sites for these hormonal ligands. We then found that the tumour-promoting agent phorbol ester TPA induces, at low concentration, a marked expression of β -adrenoceptors coupled to adenylate cyclase. Such an effect of TPA on the β -adrenergic system has not been observed previously, to the best of our knowledge, and is detailed in the present paper.

MATERIALS AND METHODS

Chemicals

All solutions used for culture were purchased from Gibco Europe (Gent, Belgium). ATP, cyclic AMP, GTP,

p[NH]ppG, phosphoenolpyruvate, pyruvate kinase, phorbol esters (TPA and 4 α -phorbol 12,13-dibutyrate), PGE_1 , DL-isoprenaline, adrenaline, noradrenaline, epinephrine and cycloheximide were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Actinomycin D was from Janssen Chimica (Beerse, Belgium), and ICI 118.551 from I.C.I. (Macclesfield, Cheshire, U.K.). Cyclic [8- 3H]-AMP (27 Ci/mmol) and [125 I]cyanopindolol (2000 Ci/mmol) were from Amersham International (Amersham, Bucks., U.K.); [α - ^{32}P]ATP (20 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.). All other reagents were of the highest grade available.

Cell culture

Human Molt 3 lymphoblasts were obtained from Dr. N. Van Tieghem (Department of Virology, Medical School of the Université Libre de Bruxelles) and cultured at 37 °C in a humidified incubator containing air/CO₂ (19:1) in RPMI-1640 medium supplemented with 7.5 % (v/v) inactivated fetal-calf serum, antibiotics (40 units of penicillin + streptomycin/ml), 1 mM-sodium pyruvate, 2 % (w/v) essential amino acid solution in Minimum Eagle's Medium (MEM) and 1 % MEM with non-essential amino acid solution.

Phorbol esters were dissolved in dimethyl sulphoxide, but the final concentration of dimethyl sulphoxide in the culture medium never exceeded 0.02 %, a concentration without effect on cells. Actinomycin D was dissolved in ethanol (final ethanol concn. 0.1 %) and the solvent was added in control cultures.

Membrane preparation

Cells were pelleted by centrifugation at 100 g for 5 min at 20 °C, washed in the culture medium, lysed in 1 mM-NaHCO₃ and frozen in liquid N₂. After thawing and centrifugation at 400 g for 10 min at 4 °C, the supernatant

Abbreviations used: VIP, vasoactive intestinal peptide; PGE_1 , prostaglandin E_1 ; TPA, tumour-promoting agent phorbol ester (12-*O*-tetradecanoylphorbol 13-acetate); K_d , concentration required for half-maximal inhibition of tracer binding to a given class of receptors; p[NH]ppG, guanosine 5'-[β,γ -imido]triphosphate; G_s , guanine-nucleotide-binding stimulatory protein responsible for adenylate cyclase activation.

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was centrifuged at 10000 *g* for 15 min. The final pellet was resuspended in 1 mM-NaHCO₃ and immediately tested for adenylate cyclase activity and for the presence of β -adrenoceptors. Protein concentration was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Adenylate cyclase assay

Adenylate cyclase activity was determined by the procedure of Salomon *et al.* (1974) with minor modifications. Membrane protein (5–20 μ g) was incubated in a total volume of 60 μ l containing 0.5 mM-[α -³²P]ATP, 5 mM-MgCl₂, 0.5 mM-EDTA, 1 mM-cyclic AMP, 0.5 mM-theophylline, 10 mM-phosphoenolpyruvate, 30 μ g of pyruvate kinase/ml and 30 mM-Tris/HCl, final pH 7.5, and, when hormones were tested, 10 μ M-GTP. The reaction was initiated by addition of membranes and was terminated after incubation for 15–30 min at 37 °C by adding 0.5 ml of 0.5% SDS solution containing 0.5 mM-ATP, 0.5 mM-cyclic AMP and 20000 c.p.m. of cyclic [8-³H]AMP (for determination of cyclic AMP recovery). Cyclic AMP was separated from ATP by two successive chromatographies on Dowex 50W X8 and neutral alumina. Under all conditions tested, cyclic AMP production was linear for at least 40 min.

Identification of β -adrenoceptors

Binding of [¹²⁵I]cyanopindolol (50000 c.p.m.) was carried out in a buffer consisting of 40 mM-Tris/HCl, 150 mM-NaCl, 5 mM-MgCl₂, 1 mM-ascorbic acid and 0.08% bovine serum albumin (pH 7.4). The total assay volume was 2 ml. Non-specific binding was determined in the presence of 1 mM-DL-isoprenaline. The binding was initiated at 37 °C by addition of 30 μ g of membrane protein, and the reaction was stopped 60 min later by rapid filtration through glass-fibre filters (GF/C; Whatman, Maidstone, Kent, U.K.) presoaked for 24 h in 0.1% poly(ethyleneimine). The filters were rinsed at room temperature with 3 $\times$ 4 ml of 20 mM-Tris/HCl (pH 7.4)/150 mM-NaCl. Radioactivity was counted in a multidetector γ -radiation counter.

RESULTS

Adenylate cyclase activity

Adenylate cyclase activity in membranes from human T lymphoblasts Molt 3 was stimulated by GTP (4-fold), p[NH]ppG (20-fold), NaF (40-fold) and forskolin (15-fold) (Table 1 and Figs. 1 and 2). In the presence of GTP (a prerequisite for testing receptor-mediated activation), there was no significant effect of isoprenaline, PGE₁, VIP and its parent peptide helodermin (Table 1, and results not shown).

Cells incubated in the presence of 3.2 nM-TPA (but not with 3 nM and 30 nM α -phorbol esters) underwent complex changes in the profile of their adenylate cyclase activity. A rapid decrease (within 4 h) in basal and GTP-stimulated activities was followed, after a 4 h lag period, by a slower decrease in p[NH]ppG and NaF-stimulated activities, whereas an initial increase in forskolin stimulation was followed by a 40% decrease (Figs. 1 and 2 and Table 1).

After incubation for 5 h in the presence of TPA, adenylate cyclase became sensitive to isoprenaline (results not shown). The β -adrenergic agonist was maximally

Table 1. Effects of a 24 h preincubation of human Molt 3 lymphoblasts on the adenylate cyclase activity of their membranes

Results are means $\pm$ S.E.M. for five experiments performed in duplicate.

Addition	TPA pretreatment ...	Adenylate cyclase (pmol of cyclic AMP produced/min per mg of protein)	
		–	+
None		4.7 $\pm$ 0.5	2.5 $\pm$ 0.8
100 μ M-GTP		20.9 $\pm$ 1.5	4.9 $\pm$ 0.5
100 μ M-p[NH]ppG		92.2 $\pm$ 3.2	41.1 $\pm$ 2.0
10 mM-NaF		184.4 $\pm$ 8.4	80.0 $\pm$ 3.1
10 μ M-forskolin		70.6 $\pm$ 2.0	46.1 $\pm$ 2.4
10 μ M-GTP		15.4 $\pm$ 0.9	5.3 $\pm$ 0.6
10 μ M-GTP + 10 μ M-PGE ₁		17.3 $\pm$ 0.8	10.8 $\pm$ 1.0
10 μ M-GTP + 100 μ M-isoprenaline		15.7 $\pm$ 0.8	24.6 $\pm$ 0.8
10 μ M-GTP + 1 μ M-VIP		15.3 $\pm$ 0.7	6.0 $\pm$ 0.6

Fig. 1. Effects of forskolin, p[NH]ppG and NaF on adenylate cyclase

Human Molt 3 lymphoblasts were exposed for 0–24 h to 3.2 nM-TPA. At indicated times cells were lysed, membranes were prepared, and adenylate cyclase activity was determined in the presence of 100 μ M-p[NH]ppG (Δ), 10 μ M-forskolin ($\bullet$) or 10 mM-NaF ($\circ$). The values, expressed in pmol of cyclic AMP produced/min per mg of protein, are means of five separate experiments: * indicates values different ($P < 0.05$ by the Student's *t* test) from values observed at zero time.

Fig. 2. Effects of GTP and isoprenaline on adenylate cyclase

Experimental conditions were the same as in Fig. 1. Adenylate cyclase activity was determined in the presence of 10 μ M-GTP, without ($\circ$) or with ($\bullet$) 100 μ M-isoprenaline. The values, expressed in pmol of cyclic AMP produced/min per mg of protein, are means of five separate experiments performed in duplicate: * indicates values different ($P < 0.05$ by Student's t test) from values observed at zero time; † indicates values observed in the presence of isoprenaline differing significantly from corresponding control values.

Fig. 3. Dose/response curves of adenylate cyclase activation in membranes from human Molt 3 lymphoblasts by the β -adrenergic agonists isoprenaline ($\circ$), adrenaline ($\bullet$), noradrenaline (Δ) and epinine ($\blacktriangle$)

Cells were preincubated for 24 h in the presence of 3.2 nM-TPA. Results, expressed in pmol of cyclic AMP produced/min per mg of protein, are means of three experiments performed in duplicate.

efficient after 8 h, its efficacy remaining stable for at least 24 h. PGE₁ (but neither VIP nor helodermin) also proved to be an effective adenylate cyclase stimulant (Table 1); the amplitude of the response was lower, however, and was not further explored.

After exposure for 24 h to 3.2 nM-TPA, adenylate cyclase activation by β -adrenergic agonists was typical of a β_2 -adrenoceptor-mediated response (Fig. 3), the order of potency decreasing in the order isoprenaline > adrenaline $\gg$ noradrenaline > epinine, with noradrenaline showing a lower intrinsic activity than isoprenaline and adrenaline.

β_2 -adrenergic receptors

Specific binding of [¹²⁵I]cyanopindolol to membranes from control human Molt 3 lymphoblasts was barely detectable (Fig. 4). It remained very low during the first 4 h after addition of 3.2 nM-TPA, but increased markedly during the next 8 h. Maximal specific binding was attained after 16 h and remained stable for at least another 8 h. This emergence of specific binding sites was

Fig. 4. Effects of TPA, cycloheximide and actinomycin D on [¹²⁵I]cyanopindolol specific binding

Human Molt 3 lymphoblasts were preincubated in the presence of 3.2 nM-TPA from 0 to 24 h ($\bullet$) or for 8 h only in the presence of 3.2 nM-TPA and either 1 μ g of cycloheximide/ml (Δ) or 5 μ g of actinomycin D/ml ($\circ$). Binding was performed in the presence of saturating concentrations of [¹²⁵I]cyanopindolol, so that specific binding of the tracer per mg of protein was directly related to receptor density. Results are expressed as a percentage of the receptor density observed after 24 h exposure to TPA, and are means $\pm$ S.E.M. for four experiments.

completely blunted by both cycloheximide (1 μ g/ml) and actinomycin D (5 μ g/ml) (Fig. 4).

These binding sites were further characterized after a 24 h preincubation in the presence of 3.2 nM-TPA, as follows.

(a) A saturation curve with the antagonist [125 I]cyanopindolol revealed only one class of binding site, with a K_d of 13 ± 1 pM and a maximum binding capacity of 160 ± 30 fmol/mg of protein (means $\pm$ S.E.M. for three determinations).

(b) Competition curves (Fig. 5) with the antagonist ICI 118,551 and four β_2 -adrenergic agonists were typical of β_2 -adrenergic receptors, and suggested that agonists were able to discriminate between low- and high-affinity states or classes (Fig. 5).

(c) The competition curve with the agonist isoprenaline became steeper in the presence of 10 μ M-p[NH]ppG (Fig. 6). For both curves, a computer analysis by the Ligand program of Richardson & Humrich (1984) gave the best fit when two receptor classes were considered.

Fig. 5. Inhibition of [125 I]cyanopindolol binding to membranes from human Molt 3 lymphoblasts by the β_2 -adrenergic antagonist ICI 118,551 ($\square$) and the β_2 -adrenergic agonists isoprenaline ($\circ$), adrenaline ($\bullet$), noradrenaline ($\triangle$) and epinephrine ($\blacktriangle$).

Molt 3 cells were preincubated for 24 h in the presence of 3.2 nM-TPA. The data are expressed as a percentage of [125 I]cyanopindolol specifically bound in the presence of unlabelled ligand, and are means of three experiments.

Fig. 6. Inhibition of [125 I]cyanopindolol binding to membranes from human Molt 3 lymphoblasts by isoprenaline in the absence ($\circ$) or presence ($\bullet$) of 10 μ M-p[NH]ppG.

Experimental conditions were the same as in Fig. 5.

Under control conditions the high-affinity class had a K_d of 3.0 ± 0.4 nM and a maximum binding capacity that corresponded to 50 % of total binding sites, whereas the low-affinity class had a K_d of 0.4 ± 0.1 μ M and showed an equal binding capacity. In the presence of p[NH]ppG the high-affinity class had a K_d of 3.8 ± 1.0 nM and a maximum binding capacity decreased to 15 % of total binding sites, whereas the low-affinity sites had a K_d of 0.3 ± 0.1 μ M and a maximum binding capacity increasing to 85 % of total binding sites. This strongly suggests that p[NH]ppG induced the conversion of β_2 -adrenoceptors from a high-affinity state for agonists into a low-affinity state.

DISCUSSION

TPA, a well-known activator of protein kinase C, acting as a substitute for diacylglycerol, also influences adenylate cyclase activity, the coupling of receptors to adenylate cyclase and, in some cases, receptors themselves (Nishizuka, 1986). Opposite effects have been reported depending on TPA concentration, the duration of exposure, the cell type examined, and the nature of the receptor investigated. In the human T lymphoblastic cell line Jurkat, TPA enhances the increase in cyclic AMP in response to adenosine, but decreases the response to prostaglandin (Nordstedt *et al.*, 1987). TPA inhibits dose-dependently the glucagon-stimulated rat liver adenylate cyclase activity without altering glucagon receptors (Heyworth *et al.*, 1984). The phorbol ester intensifies the VIP-induced increase in cyclic AMP in vascular smooth-muscle cells (Nabika *et al.*, 1985), but provokes a loss of VIP-stimulated adenylate cyclase activity and VIP-binding sites in HT 29 cells (Bozou *et al.*, 1987).

The response to TPA of β -adrenoceptors coupled to adenylate cyclase has also been extensively studied.

(a) TPA promotes the phosphorylation of β -adrenoceptors and adenylate cyclase desensitization in duck erythrocytes (Sibley *et al.*, 1984). It desensitizes rat reticulocytes (Yamashita *et al.*, 1988), human astrocytoma cells (Toews *et al.*, 1987) and rat glioma C6 cells (Kassis *et al.*, 1985). This desensitization process differs clearly from that induced by a β -adrenergic agonist, and does not correlate with a decreased number of receptors.

(b) TPA increases the isoprenaline-mediated cyclic AMP production in frog erythrocytes (Nambi *et al.*, 1985), mouse S49 lymphoma cells (Bell *et al.*, 1985; Bell & Brunton, 1986, 1987), mouse myeloid leukaemic cells (Simantov & Sachs, 1982), vascular smooth-muscle cells (Nabika *et al.*, 1985), a murine macrophage cell line J774 (Chambaut-Guerin & Thomopoulos, 1987) and pinealocytes (Sugden *et al.*, 1985). Several mechanisms can be evoked to account for these findings, including a better coupling of receptors to adenylate cyclase and a loss of adenylate cyclase inhibition, but an increased number of β -adrenoceptors never appeared to be involved.

The present results constitute the first evidence that TPA, at least in human T lymphoblasts Molt 3, induces the expression of β -adrenoceptors, probably through protein kinase C activation. The newly expressed receptors displayed the characteristics of functional β_2 -adrenoceptors, in terms of the K_d for β -adrenergic agonists and antagonists, the effects of p[NH]ppG on

isoprenaline binding, and the relative potency of agonists to stimulate adenylate cyclase.

The molecular basis for the stimulation of β -adrenoceptor expression in human lymphoblasts Molt 3 is unclear. The 4 h lag period before the number of receptors increased argues against the rapid externalization of a latent pool of intracellular receptors, and the effects of actinomycin D and cycloheximide indicate a requirement for protein synthesis depending on newly formed mRNA. In this respect, protein kinase C can be translocated to the cell nucleus (Kiss *et al.*, 1988), where it may directly bind to DNA motifs and modulate gene expression (Rosenthal *et al.*, 1987). Consensus sequences for upstream regulatory elements have been proposed for TPA-stimulated transcription for several genes (Angel *et al.*, 1987; Lee *et al.*, 1987). These elements could bind the transcription factor AP-1, whose activation might follow phosphorylation by a protein kinase C. It is therefore tempting to speculate that one of the regulatory elements in the human β_2 -adrenoceptor gene was indeed the site of action of protein kinase C (Emorine *et al.*, 1987).

The temporal sequence of TPA effects on adenylate cyclase activity in human T lymphoblasts Molt 3 intimates multiple targets: the immediate decrease of GTP efficacy, followed 4 h later by a decrease in stimulations by p[NH]ppG and NaF, suggests an alteration of G_s , whereas the increase in forskolin stimulation implied, as in other tissues (Yoshimasa *et al.*, 1987; Summers & Cronin, 1988), an effect on the catalytic unit. Moreover, comparison of Figs. 2 and 4 indicates that isoprenaline-stimulated adenylate cyclase activity was not proportional to the number of β -adrenoceptors in Molt 3 cells, hinting, as discussed above for other β -adrenoceptors, at a possible desensitization of newly appearing receptors.

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