Prostaglandin-concentration-dependent desensitization of adenylate cyclase in human erythroleukaemia (HEL) cells is abolished by pertussis toxin and enhanced by induction by dimethyl sulphoxide

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Prostaglandin-regulated cyclic AMP metabolism in human erythroleukaemia (HEL) cells was similar to that previously described in platelets [Ashby (1989) *Mol. Pharmacol.* **36**, 866–873], displaying prostaglandin-concentration-dependent desensitization that could be explained by the presence of separate stimulatory and inhibitory prostaglandin receptors. Pertussis toxin abolished prostaglandin-concentration-dependent desensitization, indicating that the process is mediated through a pertussis toxin-sensitive GTP-binding protein. Treatment of HEL cells for 4 days with the inducer dimethyl sulphoxide enhanced prostaglandin-concentration-dependent desensitization, but did not alter the initial rate of cyclic AMP synthesis or the amount of $G_{12\alpha}$ measured by immunoblotting, suggesting that the inhibitory receptor was selectively induced by changing the cells to a more platelet-like form.

INTRODUCTION

Previous work from this laboratory has provided a detailed analysis of prostaglandin-regulated cyclic AMP metabolism in human blood platelets [1–4]. Prostaglandins induce time-dependent inhibition that shows a different prostaglandin-concentration-dependence from the rapid activation of platelet adenylate cyclase by prostaglandins, leading us to suggest a mechanism of regulation involving separate stimulatory and inhibitory prostaglandin receptors [1–4]. The mechanism gives rise to prostaglandin-concentration-dependent desensitization of cyclic AMP formation that is due to slow turn-off of adenylate cyclase through the inhibitory prostaglandin receptor rather than activation of phosphodiesterase activity, as time-dependent inhibition of cyclic AMP formation occurs in the presence of phosphodiesterase inhibitors [3]. The mechanism is supported by radioligand binding studies which indicate that there are two classes of prostaglandin binding site on platelet membranes [5–7]. The proposed mechanism is further supported by our ability to mathematically model platelet cyclic AMP metabolism over a wide range of prostaglandin concentrations using a simple scheme involving rapid activation of adenylate cyclase, followed by slow, reversible transition of adenylate cyclase to an inactive form (desensitization) through a distinct inhibitory receptor [3]. Different prostaglandins bind to the stimulatory and inhibitory receptors with different affinities, and the model gives rise to heterologous desensitization such that prostaglandin E_1 (PGE_1) readily inhibits PGI_2 - and PGD_2 -stimulated cyclic AMP formation, since it has high affinity for the inhibitory receptor [4]. In all of our work, PGI_2 was replaced by its stable analogue iloprost, which gave similar results to authentic PGI_2 .

Implicit in our model is the assumption that the stimulatory receptor acts through the G-protein G_s and the inhibitory receptor acts through G_i . In most cells the role of G_i can be examined by use of pertussis toxin; however, the toxin is not effective against intact platelets, which apparently lack the appropriate ganglioside receptor for toxin entry [8]. Jakobs and coworkers [9–11] showed that phorbol ester treatment of platelets

appears to lead to protein kinase C-mediated phosphorylation of G_i and impairment of its function. We [3], and others [12], have shown that phorbol ester also abolishes time-dependent inhibition induced by prostaglandins, suggesting that the putative inhibitory receptor for prostaglandins acts through G_i . However, Carlson *et al.* [13] have shown that phorbol ester does not lead to phosphorylation of platelet G_i , but rather phosphorylates the pertussis toxin-insensitive protein G_{12} , which has no known function but is unlikely to play a role in inhibition of adenylate cyclase. In fact, recent work by Pfeuffer and coworkers [14] shows that phorbol ester also leads to phosphorylation of the catalytic unit of platelet adenylate cyclase and may impair its interaction with G_i . Overall, then, it appears that phorbol ester is not an appropriate tool for examining the role of G_i itself in regulation of platelet adenylate cyclase.

Human erythroleukaemia (HEL) cells provide a useful system with which to examine and extend our studies with platelets. HEL cells possess some properties of erythrocytes and some properties of megakaryocytes and platelets [15]. Unlike platelets, intact HEL cells are targets for ADP-ribosylation by pertussis toxin, so that the role of G_i in these cells can be more readily examined. In addition, HEL cells can be induced by treatment with phorbol ester or dimethyl sulphoxide (DMSO) to express more platelet-like characteristics [15], including an increase in both thromboxane A_2 [16] and PGI_2 [17] receptor expression. In the present paper we have used HEL cells to investigate the role of the pertussis toxin-sensitive G_i in prostaglandin-induced desensitization, and the effect of induction by DMSO on cyclic AMP metabolism.

MATERIALS AND METHODS

Materials

HEL cells were obtained from the Cell Culture Facility of the University of California, San Francisco, CA, U.S.A. [2,8- 3H]Adenine and cyclic [^{14}C]AMP were obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). The stable PGI_2 analogue iloprost was a gift from Berlex Laboratories, Inc. (Cedar Knolls,

Abbreviations used: HEL, human erythroleukaemia; DMSO, dimethyl sulphoxide; G-protein, GTP-binding protein; G_s , stimulatory GTP-binding protein; G_i , inhibitory GTP-binding protein; PGE_1 , prostaglandin E_1 ; PGI_2 , prostaglandin I_2 ; PGD_2 , prostaglandin D_2 .

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NJ, U.S.A.). Antiserum, designated AS/7 and specific to $G_{11\alpha}$, $G_{12\alpha}$ and the α subunit of transducin, was obtained from New England Nuclear. Adrenaline, pertussis toxin and other chemicals and reagents were from Sigma.

HEL cells

HEL cells were grown in RPMI 1640 medium (Hyclone Laboratories, Logan, UT, U.S.A.) supplemented with 10% fetal calf serum and antibiotics in 75 cm² culture flasks in an atmosphere of 95% air/5% CO₂ at 37°C. Cell viability was assessed by Trypan Blue exclusion and was greater than 95% in all of the experiments described here.

Measurement of cyclic AMP formation

In general, three to four flasks of HEL cells were used for each set of curves. Cells were harvested, pooled and centrifuged for 5 min at 1000 *g*. Cyclic AMP formation in intact HEL cells was measured by incubating a suspension of the cells in serum-free RPMI for 1 h at 37°C with 3 μ Ci of [³H]adenine/ml prior to washing. Time courses were generated at 37°C by addition of iloprost to a suspension of radiolabelled washed cells containing 1.5×10^7 cells/ml, and withdrawing 0.5 ml samples at appropriate times into 0.5 ml of stopping solution containing 0.5 M-HCl, with cyclic [¹⁴C]AMP as a recovery standard. Within a particular experiment all curves were obtained simultaneously by sampling at 10 s intervals. The samples were boiled for 15 min before measurement of the cyclic AMP content by the two-column method of Salomon [18]. Results are expressed as percentages of total radioactivity appearing as cyclic AMP.

Induction of HEL cells

HEL cells were induced by growing them for 4 days in medium containing 1.25% DMSO [15]. Cells were grown in parallel in the absence of DMSO for use in control experiments.

Treatment with pertussis toxin

HEL cells were treated for 16 h with 50 ng of pertussis toxin/ml before harvest, radiolabelling and washing. Untreated cells were grown in parallel and adjusted to the same cell count for use in control experiments.

Immunoblotting

Proteins from non-induced and DMSO-induced HEL cell freeze/thaw lysates were resolved by SDS/PAGE on a non-reduced 10% gel. The proteins were transferred to poly(vinylidene fluoride) (Immobilon-P; Millipore Corporation) for immunostaining using AS/7 antiserum and a secondary alkaline phosphatase-conjugated antibody. Blots were developed using 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium as substrates.

RESULTS

Pertussis toxin reverses adrenaline-mediated inhibition of iloprost-stimulated HEL cell cyclic AMP formation

HEL cells possess α_2 -adrenergic receptors [19] acting through G_i , so that adrenaline can be used to inhibit prostaglandin-stimulated cyclic AMP formation in the same manner as in platelets. Fig. 1 shows the effect of 50 μ M-adrenaline on the time course of cyclic AMP formation elicited by 1 μ M of the stable PGI₂ analogue iloprost, resulting in almost complete inhibition. Pretreatment of the cells with pertussis toxin resulted in significant reversal of adrenaline-mediated inhibition, indicating that pertussis toxin was effective in abolishing G_i function in HEL cells.

Effects of DMSO and pertussis toxin on iloprost-regulated cyclic AMP metabolism

Patterns of cyclic AMP formation in response to various concentrations of iloprost in HEL cells treated with or without DMSO and pertussis toxin are shown in Fig. 2. The most dramatic effects were at 10 μ M-iloprost and are emphasized with a bold line.

Results obtained with HEL cells that had not been treated with DMSO or pertussis toxin are shown in Fig. 2(a). At low concentrations of iloprost, the time course rose to a plateau representing the steady state between formation of cyclic AMP by iloprost-stimulated adenylate cyclase and hydrolysis of cyclic AMP by phosphodiesterase activity. The initial rate of cyclic AMP formation increased as a saturable function of iloprost concentration. However, as the concentration of iloprost increased, so did the degree of desensitization represented by a fall in cyclic AMP levels to a new steady state. As a consequence, the response to iloprost was biphasic and peak levels of cyclic AMP obtained at 3 and 10 μ M-iloprost were lower than those obtained at 1 μ M. The family of curves obtained with HEL cells resembles that obtained with platelets [2–4], although higher concentrations of iloprost are necessary to saturate the stimulatory effect and to elicit desensitization. Detailed studies of cyclic AMP metabolism in platelets indicate that the fall is primarily due to a time-dependent turn-off of adenylate cyclase activity rather than a time-dependent increase in phosphodiesterase activity [3]. Because desensitization occurs with a different iloprost-concentration-dependence from stimulation, we have interpreted the patterns in terms of separate stimulatory and inhibitory receptors differing in their affinity for prostaglandins [2–4].

Treatment of HEL cells for 4 days with 1.25% DMSO led to a marked change in the pattern of iloprost-regulated cyclic AMP formation (Fig. 2b). In particular, the degree of desensitization observed at high concentrations of iloprost was markedly increased, so that the pattern of curves was much more similar to that observed in platelets [3]. In control experiments, short-term exposure to DMSO (30–60 min) had no effect on the kinetics of cyclic AMP formation (results not shown), indicating that responses to DMSO were not due to non-specific effects on the cyclase system.

The effect of the treatment of HEL cells with pertussis toxin on iloprost-dependent cyclic AMP metabolism is shown for both non-induced (Fig. 2c) and DMSO-induced (Fig. 2d) cells. In each case, iloprost-concentration-dependent desensitization was

Fig. 1. Effect of pertussis toxin treatment on adrenaline-induced inhibition of iloprost-mediated cyclic AMP formation in HEL cells

Curves were obtained as described in the Materials and methods section. ●, Control; □, +adrenaline; △, pertussis toxin; ▲, pertussis toxin + adrenaline. Cells were challenged with 1 μ M-iloprost and the adrenaline concentration was 50 μ M.

Fig. 2. Effect of iloprost concentration on cyclic AMP metabolism in HEL cells

(a) Non-induced cells; (b) cells induced for 4 days with 1.25% DMSO; (c) non-induced cells treated for 16 h with pertussis toxin; (d) DMSO-induced cells treated for 16 h with pertussis toxin. Iloprost concentrations were as follows (μM): $\bullet$, 0.03; $\square$, 0.1; $\blacktriangle$, 0.3; $\triangle$, 1.0; $\blacklozenge$, 3.0; $\circ$, 10. Results obtained at 10 μM -iloprost are emphasized with a bold line. All sets of curves were obtained within a total time period of 45 min. The cell count was 1.5×10^7 cells/ml in each case, and each set of cells contained a virtually identical amount of [^3H]adenine nucleotide label. The Figure is representative of three separate experiments performed on different days. There was some variation in the time courses between experiments, but the relative position of each curve and the nature of the changes engendered by DMSO and pertussis toxin were the same in each experiment.

markedly decreased by pertussis toxin treatment. The result indicates that this form of desensitization is mediated through G_i , and supports our model of dual regulation of adenylate cyclase in the same cell type occurring through separate receptors coupled to G_s and G_i .

Effects of DMSO and pertussis toxin on PGE_1 -regulated cyclic AMP metabolism

Fig. 3 shows the time course of cyclic AMP metabolism for a single concentration of PGE_1 . Again, the degree of prostaglandin inhibition increased following DMSO induction, and the inhibition was reversed by pertussis toxin. The curves represent non-linear regression fits to an integrated rate equation (given in the legend to Fig. 3) describing the time course of cyclic AMP formation when adenylate cyclase is desensitized in a reversible manner [20,21]. Using this equation it can be calculated that the rate of cyclic AMP synthesis is identical in each curve shown in Fig. 3, indicating that there was no change in the amount of stimulatory receptor or adenylate cyclase associated with growth in the presence of DMSO or pertussis toxin.

Immunoblots of HEL cell lysates with antiserum specific to G_{12x}

Immunoblots of HEL cell lysates prepared from non-induced and DMSO-induced cells using antiserum to G_{12x} showed no change in the amount of this protein, as judged from the intensity of the bands, at several different concentrations of the detergent-solubilized lysates (Fig. 4). The same antiserum, designated AS, has been used by Simonds *et al.* [22] to show that G_{12x} mediates α_2 -adrenergic inhibition of adenylate cyclase in platelet membranes.

Fig. 3. Effect of DMSO and pertussis toxin on PGE_1 -stimulated cyclic AMP metabolism in HEL cells

The curves represent fits to the following equation [18,19] describing the time course of cyclic AMP (cAMP) formation when adenylate cyclase activity decays in a time-dependent reversible manner:

$$[\text{cAMP}] = \frac{k_s k_n (1 - e^{-k_d t})}{(k_m + k_n) k_d} + \frac{k_m [e^{-(k_m + k_n)t} - e^{-k_d t}]}{(k_m + k_n) (k_d - k_m - k_n)}$$

where k_s = rate constant for cyclic AMP synthesis; k_d = rate constant for cyclic AMP degradation; k_m = rate constant for time-dependent fall in adenylate cyclase activity (engendered through the inhibitory receptor); and k_n = rate constant for reversal of adenylate cyclase inhibition. Values of k_s for the four curves were as follows: control ($\bullet$), 1.01; + pertussis toxin ($\blacktriangle$), 0.94; + DMSO ($\circ$), 1.04; + pertussis toxin + DMSO, 0.99.

Fig. 4. Immunoblots of solubilized lysates from DMSO-induced and non-induced HEL cells

Samples were run on SDS/10%-PAGE under non-reduced conditions. Proteins were transferred to poly(vinylidene fluoride) for immunostaining with a 1:1000 dilution of antibody AS/7 directed at the C-terminus of $G_{12\alpha}$. Samples were visualized using a 1:500 dilution of goat anti-(rabbit IgG)-alkaline phosphatase conjugate. Samples from DMSO-treated cells are in lanes 1, 3, 5 and 7; samples from non-treated cells are in lanes 2, 4, 6 and 8. Samples represent the following amounts of protein: lanes 1 and 2 each contain 100 μ g, lanes 3 and 4 contain 65 μ g, lanes 5 and 6 contain 50 μ g and lanes 7 and 8 contain 35 μ g.

DISCUSSION

We have shown that prostaglandin regulation of cyclic AMP metabolism in HEL cells is similar to that obtained in platelets. In common with platelets, HEL cells display prostaglandin-concentration-dependent desensitization that can be explained by the existence of separate stimulatory and inhibitory prostaglandin receptors linked to adenylate cyclase, as stimulation and time-dependent inhibition of adenylate cyclase show different dependences on prostaglandin concentration. Studies with HEL cells provide evidence for the involvement of G_i in prostaglandin-concentration-dependent desensitization, because time-dependent inhibition can be abolished by pertussis toxin, which has no effect on G_i in intact platelets.

HEL cells can be induced by treatment with DMSO to express a variety of platelet markers, including platelet-specific surface glycoproteins and granule proteins including von Willebrand factor [15]. We show here that induction by DMSO has no effect on prostaglandin stimulation of adenylate cyclase, but leads to an increase in the extent of prostaglandin-concentration-dependent desensitization. Hence determination of the initial rate of cyclic AMP formation indicates that prostaglandin stimulation of adenylate cyclase is unaffected by DMSO treatment, whereas the amount of prostaglandin-induced inhibition, mediated through the putative inhibitory receptor, increases following DMSO induction. The increase in prostaglandin inhibition is not due to an increase in the amount of $G_{12\alpha}$, the G-protein that mediates hormonal inhibition of adenylate cyclase in platelet membranes [22], as the level of this protein was the same in immunoblots of induced and non-induced HEL cell extracts. We conclude that DMSO treatment selectively increases the amount of the prostaglandin inhibitory receptor. This conclusion is supported by direct binding studies [17] showing that induction of HEL cells is accompanied by an increase in prostacyclin binding.

The fact that transformation of HEL cells to a more platelet-like form is accompanied by apparent induction of the inhibitory receptor indicates that the inhibitory receptor is characteristic of

platelets and plays a physiological role in prostaglandin-regulated cyclic AMP metabolism. The presence of a distinct inhibitory receptor may represent a general mechanism of autocrine or paracrine regulation, buffering cellular responses to transient extremes of concentration when agonists are produced close to their sites of action [4]. We have suggested that the release of dopamine into the synaptic cleft may represent an example of this type of regulation, because dopamine may interact with D_1 - and D_2 -dopamine receptors on the same post-synaptic membrane, maintaining neuronal cyclic AMP at a constant level despite wide variations in dopamine concentration [23]. Loss of control of the dopamine buffering mechanism may lead to wild swings in cyclic AMP and clinical manifestations such as those seen in schizophrenics [23]. In the case of platelets, PGI_2 released from vessel walls has been postulated to maintain a non-thrombogenic surface by maintaining platelets in a non-active state [24], as cyclic AMP inhibits platelet activation. The PGI_2 level is further elevated in response to inflammatory and other agents. We believe that the inhibitory receptor serves a homeostatic function in maintaining the steady-state level of cyclic AMP within reasonable limits so that the platelet can still be activated in response to appropriate challenges.

This work was supported by National Institutes of Health Grants HL 36579 and BRSG 7RR05417.

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