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POTENTIATION OF ADENYLYL CYCLASE IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS BY CELL-ACTIVATING STIMULI

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Abstract—The isoprenaline-induced production of cAMP in human peripheral blood mononuclear cells (PBMC) was potentiated significantly by incubating PBMC with isoprenaline in the presence of phytohaemagglutinin (PHA), Concanavalin A (Con A) or A23187. This potentiation, that proved to be dependent on the concentration of PHA, Con A or A23187, increased the maximal response but did not cause a change in the potency of isoprenaline. Potentiation could not be induced by the phorbol ester phorbolmyristate acetate, suggesting that protein kinase C-dependent pathways are not likely to be involved in potentiation of adenylyl cyclase. Potentiation could be inhibited by chelating extracellular Ca^{2+} with EGTA and also by *N*-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamine, an inhibitor of calmodulin. Potentiation could not be induced by preincubation of PBMC with PHA, suggesting that transient biochemical changes are involved. It was concluded from these results that potentiation in PBMC probably involves the activation of Ca^{2+}/calmodulin-dependent adenylyl cyclase subtypes. Potentiation of the adenylyl cyclase activity could be an important physiological mechanism *in vivo* preventing cells from becoming "over stimulated".

The intracellular second messenger cAMP is important in the regulation of cellular activities. In leucocytes in particular, elevation to cAMP levels is associated with inhibition of responses like mitogen-induced proliferation, mediator release and cytokine production [1–6]. The production of cAMP is regulated by the adenylyl cyclase system which consists of different stimulatory or inhibitory receptors, heterotrimeric (α-β-γ) GTP binding proteins (G-proteins) and adenylyl cyclase itself [7–9]. It has become clear that activation of the adenylyl cyclase system is a negative regulator for signal transduction systems activated by mitogens, e.g, activation of phospholipase C (PLC†) and protein kinase C (PKC) [10–13]. Conversely, activation of adenyly cyclase can be negatively influenced by the activity of PLC and PKC [14–16], so these two second messenger systems are mutually inhibitory [17]. However, positive modification of adenylyl cyclase activity by stimuli that activate leucocyte responses was recently shown in several cell types. This has been referred to as "potentiation". For example, adenylyl cyclase activity in human mononuclear blood cells was found to be potentiated by lectins, activators of the T-cell receptor, or by non-specific activation with calcium ionophore and phorbol esters [18–21]. However, contradictory results were found with respect to the stimuli capable of inducing potentiation of adenylyl cyclase in leucocytes, while in some studies no potentiation of adenylyl cyclase was found at all [13, 18, 19, 22, 23]. To obtain more insight into the conditions for potentiation in PBMC and the mechanisms involved, we studied stimuli capable of inducing or inhibiting potentiation, and examined whether potentiation of the adenylyl cyclase system was dependent on the type of receptor used to stimulate adenylyl cyclase. Furthermore, the effect of preincubation of PBMC with these stimuli on potentiation of adenylyl cyclase was studied to see whether the changes induced by stimuli capable of potentiation are transient.

MATERIALS AND METHODS

Reagents. Lymphoprep was obtained from Nycomed Pharma (Oslo, Norway). L-Isoproterenol, histamine, prostaglandin E_1 (PGE), A23187, 1-methyl-3-isobutyl-xanthine, Concanavalin A (Con A), phorbol myristate acetate (PMA) and *N*-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamine (W-7) were obtained from the Sigma Chemical Co. (St Louis, U.S.A.). Phytohaemagglutinin (PHA) was obtained from Wellcome Diagnostics, (Dartford, U.K.). cAMP kits were from the Radiochemical Centre (Amersham, U.K.).

Measurements of cAMP production. Blood from healthy control subjects was collected in tubes with EDTA as anticoagulant. PBMC were isolated by density-gradient centrifugation on Lymphoprep as described by Böyum [24]. The mononuclear cell fraction contained approximately 90% lymphocytes, 10% monocytes and <1% polymorphonuclear leucocytes. Cells were washed twice in Tris buffer containing 120 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl,

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† PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; Con A, Concanavalin A; PMA, phorbol myristate acetate; PGE, prostaglandin E_1; PKC, protein kinase C; PLC, phospholipase C; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamine.

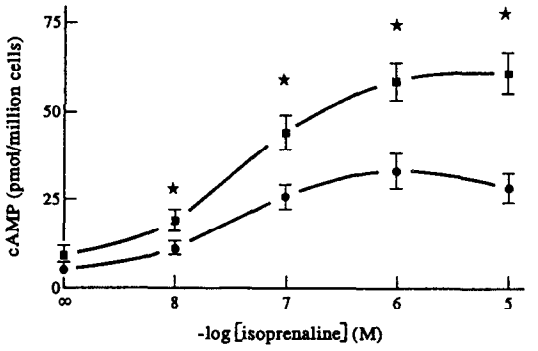

Fig. 1. Potentiation of isoprenaline-induced cAMP production by PHA. PBMC were incubated for 10 min with various concentrations of isoprenaline in the absence (circles) or presence (squares) of PHA (45 μg/mL). cAMP concentrations were measured as described in Materials and Methods. Values are means of six separate experiments, each performed in duplicate. * $P < 0.05$ cAMP versus cAMP production induced by isoprenaline alone.

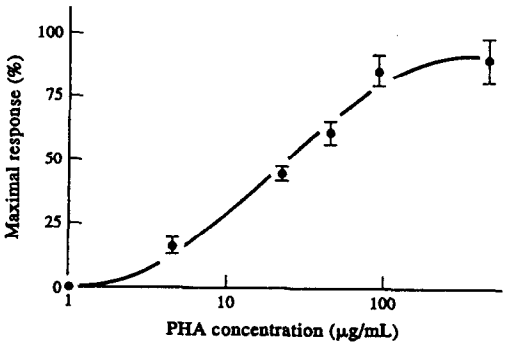

Fig. 2. Concentration dependence of PHA-induced potentiation of adenylyl cyclase activity. PBMC were incubated for 10 min with 1 μM isoprenaline in the presence of various concentrations of PHA. Potentiation was expressed as a percentage of the maximal effect. EC_{50} for PHA was 22.7 ± 2.1 μg/mL. Maximal effect (PHA 450 μg/mL) was 235 ± 35% of control (isoprenaline alone). Values are means of three separate experiments, each performed in duplicate.

0.6 mM $CaCl_2$, 25 mM Tris, 5 mM glucose and 0.1% human albumin, adjusted to pH 7.4 with HCl. Cells were suspended in Tris buffer to a final density of 1.25–2.50 × 10^6 cells/mL. Samples of 0.8 mL were preincubated with 0.1 mL 1-methyl-3-isobutyl-xanthine (0.5 mM final concentration) for 10 min. After preincubation the samples were stimulated for 10 min with 0.1 mL stimulus solution as indicated in the experiments. Reactions were terminated by adding 0.1 mL of 2 N HCl/0.1 M EDTA followed by boiling the samples for 5 min. After centrifugation of precipitated protein the samples were neutralized by $CaCO_3$ and cAMP was determined using the cAMP binding protein assay as described by Meurs *et al.* [25].

Responses are given as pmol cAMP/10^6 cells or expressed as percentage of control.

Statistical analysis. Statistical analysis was performed with the Student's *t*-test for paired and unpaired observations and by using two-way analysis of variance. Values refer to means ± SEM.

RESULTS

PBMC were stimulated with various concentrations of isoprenaline in the absence or presence of PHA (45 μg/mL). The production of cAMP induced by isoprenaline was potentiated significantly by co-stimulation of the cells with PHA, while PHA alone induced very little cAMP production (Fig. 1). The low cAMP production induced by PHA alone is probably due to endogenous production of histamine induced by PHA [26]. Potentiation of adenylyl cyclase activity with PHA proved to be concentration-dependent with a calculated EC_{50} value (concentration that is 50% effective) of 22.9 ± 2.1 μg/mL (Fig. 2).

Potentiation of adenylyl cyclase activity could also be induced by Con A or the calcium ionophore A23187 (Fig. 3). Potentiation of isoprenaline-induced cAMP production by these agents was also concentration dependent (EC_{50} values for Con A and A23187 were 10.7 ± 4.4 μg/mL and 0.30 ± 0.6 μM, respectively). No potentiation of adenylyl cyclase activity, however, was found by incubation of PBMC with the phorbol ester PMA (Fig. 3).

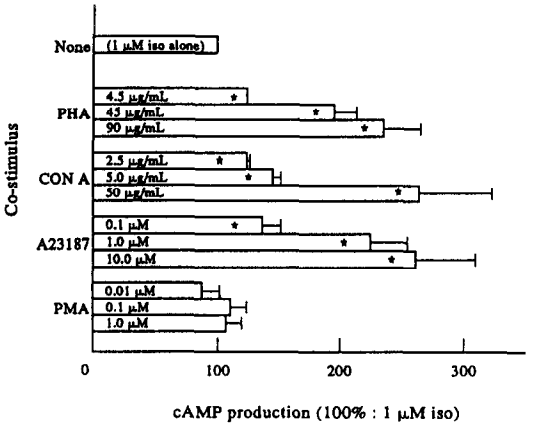

Fig. 3. Effect of PHA, Con A, A23187 and PMA on isoprenaline-induced cAMP production. PBMC were incubated for 10 min with 1 μM isoprenaline in the absence or presence of various concentrations of PHA, Con A, A23187 or PMA. Potentiation is expressed as percentage of control (isoprenaline alone). Values are means of four separate experiments, each performed in duplicate. * $P < 0.05$ versus cAMP production induced by isoprenaline alone.

Potentiation of isoprenaline-induced cAMP production by PHA, Con A or A23187 increased maximal stimulation; however, no significant change in the potency of isoprenaline could be observed. EC_{50} values for isoprenaline were calculated from potentiated and non-potentiated dose–response

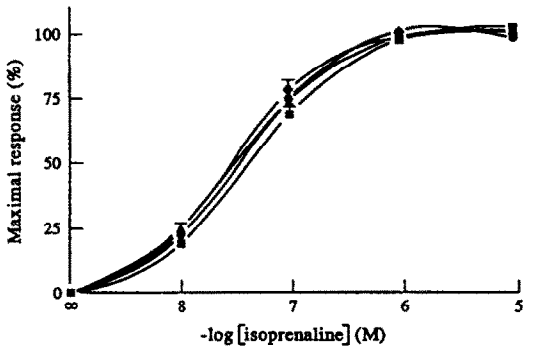

Fig. 4. Concentration–response curves for isoprenaline-induced cAMP production. PBMC were incubated for 10 min with various concentrations of isoprenaline in the absence (circles) or presence of PHA (45 μg/mL, squares), Con A (5.0 μg/mL, triangles) or A23187 (0.1 μM, diamonds). cAMP production was expressed as percentage of maximal response (isoprenaline 10 μM) for each concentration–response curve. Values are the means of at least three separate experiments, each performed in duplicate. EC_{50} values were 35.9 ± 2.5, 46.0 ± 5.2, 34.6 ± 3.5 and 32.4 ± 4.9 nM, respectively.

curves (Fig. 4). EC_{50} values were 35.9 ± 2.5, 46.0 ± 5.2, 34.6 ± 3.5 and 32.4 ± 4.9 nM for the non-potentiated isoprenaline curve, PHA (45 μg/mL)-potentiated curve, Con A (5.0 μg/mL)-potentiated curve and A23187 (0.1 nM)-potentiated isoprenaline curve, respectively. (The potency of isoprenaline was changed by none of the other concentrations of PHA, Con A and A23187 tested).

To see whether potentiation of adenylyl cyclase activity is specific for cAMP production induced by β-adrenergic receptor activation, cells were stimulated with histamine and PGE, two other stimuli that activate adenylyl cyclase in PBMC. It appeared that the histamine-induced cAMP response was not potentiated by PHA, whereas the PGE-induced cAMP response was potentiated significantly (Table 1). However, the relative potentiation (expressed as percentage of control) was significantly less for PGE-induced cAMP production than for isoprenaline-induced cAMP production [136.15 ± 9.26% and 186.99 ± 8.99%, respectively (N = 7), $P < 0.05$ Student's *t*-test].

Table 2. Effect of preincubation of PBMC with PHA on cAMP production

Stimulus	cAMP (pmol/10^6 cells)	
	Buffer	PHA (45 μg/mL)
Buffer	1.93 ± 0.59	2.42 ± 1.68
Isoprenaline (1 μM)	14.15 ± 0.99	17.50 ± 2.77
PHA (45 μg/mL)	3.56 ± 1.75	4.26 ± 2.44
Isoprenaline + PHA	29.95 ± 6.65*	30.19 ± 10.36*

PBMC were preincubated for 10 min with buffer or PHA. After washing the cells with buffer PBMC were stimulated for 10 min with buffer, isoprenaline, PHA or isoprenaline together with PHA.

Values are the means of three separate experiments, each performed in duplicate. * $P < 0.05$ versus cAMP production by isoprenaline alone.

To test whether the biochemical changes induced by the potentiating agent are reversible, PBMC were preincubated for 10 min with PHA and, after washing, stimulated with isoprenaline. Preincubation with PHA (45 μg/mL) for 10 min had no effect on isoprenaline-induced cAMP production nor on the potentiation of isoprenaline-induced cAMP production by PHA (Table 2).

The results presented above suggested intracellular calcium mobilization and subsequent activation of the calcium-dependent enzyme calmodulin to be a possible mechanism in potentiation (see Discussion). Therefore, we tested the effect of the calmodulin inhibitor W-7 and the effect of chelating extracellular calcium with EGTA on PHA-induced potentiation. Potentiation of isoprenaline-induced cAMP production was inhibited almost completely by W-7, and 5 mM EGTA completely inhibited potentiation (Fig. 5).

DISCUSSION

Potentiation of adenylyl cyclase activity by mitogens was reported in several types of leucocyte. However, contradictory results were found with respect to the stimuli capable of inducing potentiation

Table 1. Effect of PHA on cAMP production induced by isoprenaline, histamine and PGE

Stimulus	cAMP (pmol/10^6 cells)		
	− PHA	+ PHA (45 μg/mL)	
Isoprenaline (1 μM)	31.09 ± 3.19 (100%)	56.72 ± 4.75*	(186.69 ± 8.99%)†
Histamine (1 μM)	19.87 ± 2.20 (100%)	21.47 ± 2.38	(108.43 ± 6.62%)
PGE (0.1 μM)	48.54 ± 7.46 (100%)	63.69 ± 8.91*	(136.15 ± 9.26%)*

PBMC were incubated for 10 min with isoprenaline, histamine or PGE alone in the presence or absence of PHA (45 μg/mL).

Values are means of five separate experiments, each performed in duplicate.

* $P < 0.01$ versus cAMP production in absence of PHA, † $P < 0.05$ versus potentiation of PGE-induced cAMP production.

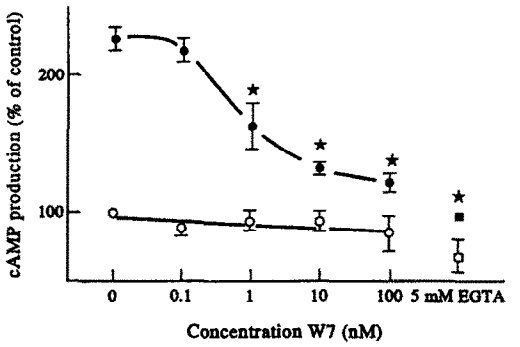

Fig. 5. Effect of W-7 and EGTA on PHA-induced potentiation of adenylyl cyclase activity. PBMC were preincubated for 10 min with various concentrations of W-7 (circles) or 5 mM EGTA (squares). After preincubation PBMC were stimulated for 10 min with isoprenaline (1 μM) in the absence (open symbols) or presence (closed symbols) of PHA (45 μg/mL). cAMP production was expressed as percentage of control (1 μM isoprenaline alone). * $P < 0.05$ versus cAMP production induced by isoprenaline and PHA in the absence of W-7.

of adenylyl cyclase in leucocytes, while in some studies no potentiation of adenylyl cyclase was found at all [13, 18, 19, 22, 23]. Here we show that adenylyl cyclase activity in PBMC can be potentiated concentration-dependently by PHA, Con A and A23187 (Figs 1–3). This is in agreement with findings in T-cells [18, 19, 21], B-cells [20, 27] and neutrophils [22, 23, 28]. However, in contrast to our results van Tits *et al.* did not find any potentiation of adenylyl cyclase activity in PBMC at all [13].

The data presented in Fig. 4 show that, although the maximal response to isoprenaline was increased, potentiation of adenylyl cyclase activity by mitogens did not cause a change in the potency of isoprenaline. This suggests that potentiation does not involve changes in the affinity of the β-adrenergic receptor on PBMC for isoprenaline, but more likely involves changes distal to the coupling of receptor and ligand. This is in agreement with findings in T-cells, B-cells and astrocytoma cells that forskolin-induced cAMP production can be potentiated [20, 21, 29]. (Forskolin activates adenylyl cyclase directly at the post-receptor level.)

The intracellular pathways that induce the potentiation of adenylyl cyclase activity are not yet fully understood. In PBMC, stimulation with PHA and Con A is known to cause hydrolysis of phosphatidyl-inositol-bisphosphate, resulting in activation of PKC and an increase in the intracellular calcium concentration [13, 30, 31]. Therefore, both pathways of this bifurcating signal transduction system could be involved in the mechanism of potentiation. In the experiments described here, no potentiation occurred when PKC was activated directly by the phorbol ester PMA (Fig. 3), in contrast to some of the studies done in T-cells and B-cells [18, 20]. It therefore seems unlikely that activation of PKC is an important mechanism in potentiation in PBMC. The role of PKC in the regulation of cAMP production, however, is multiple. Activation of PKC was shown to induce potentiation of adenylyl cyclase in some studies [18, 20, 22, 27, 29] as well as desensitization of adenylyl cyclase in others [15, 19, 21, 23, 32]. A possible explanation for this paradox is suggested by data from Gusovsky and Gutkind [33] who show that the effect of PKC on cAMP production is dependent on the subset of isozymes of PKC that are present in the cell.

A common effect of the stimuli found capable of inducing potentiation in PBMC is increase in the intracellular calcium concentration. Moreover, the EC_{50} found for PHA-induced potentiation of adenylyl cyclase (Fig. 2) is identical to the EC_{50} for PHA-induced intracellular calcium mobilization in PBMC [13]. This indicates that in PBMC the increase in intracellular calcium and the subsequent activation of the calcium-dependent enzyme calmodulin could be an important molecular mechanism in potentiation. Additional support for a role for calmodulin was presented by the inhibitory effect of W-7 and EGTA on potentiation (Fig. 5). These findings are in agreement with studies in other cell types showing potentiation of adenylyl cyclase by raising the intracellular calcium concentration as well as inhibition of potentiation by inhibitors of calmodulin [21, 23, 28, 34]. A role for calmodulin in potentiation is also in keeping with the recent discovery of four different forms of adenylyl cyclase [35–38]. The enzymatic activity of two types (type I and type III) of adenylyl cyclase can be greatly enhanced by the addition of Ca^{2+} and calmodulin [35, 37], whereas the activity of the other two types (type II and type IV) is not. Therefore, activation of calmodulin-sensitive adenylyl cyclase subtypes could be a mechanism involved in potentiation. Furthermore, PMA, which does not activate calmodulin, would not induce potentiation according to this model, which is in agreement with our data (Fig. 3).

The presence of multiple forms of adenylyl cyclase could also provide an explanation for the difference in potentiation we found between isoprenaline-induced, PGE-induced and histamine-induced cAMP production (Table 1). If β-adrenergic receptors predominantly activated calmodulin-sensitive adenylyl cyclases (type I and/or type III), histamine receptors predominantly calmodulin-insensitive adenylyl cyclases (type II and type IV) and PGE receptors both types, potentiation caused by activation of calmodulin would be more effective for isoprenaline-induced cAMP production than for PGE-induced cAMP production and have no effect on histamine-induced cAMP production. However, currently there are no data available on adenylyl cyclase subtypes in PBMC to support this hypothesis.

An alternative model for the mechanism of potentiation was presented very recently by Federman *et al.* [39]. They showed that production of cAMP by adenylyl cyclase type II in human embryonic kidney cells (HEK-293) can be potentiated by β-γ subunits released from an activated G_i-protein [39]. Similarly, the release of β-γ subunits from a G_q-protein activated by PHA or Con A in PBMC could result in an enhancement of the activity of adenylyl cyclase type II [40, 41]. However, this

model can not explain the potentiation induced by the calcium ionophore A23187 (which raises intracellular calcium without activating a G-protein).

Potentiation of isoprenaline-induced cAMP production in PBMC with PHA was found when PHA and isoprenaline were added simultaneously to the cells, whereas preincubation of PBMC with PHA was not effective (Table 2). This indicates the necessity of both potentiating agent and stimulus for adenylyl cyclase to be present to induce potentiation. Furthermore, it can be concluded from these data that the molecular changes induced by the potentiating agent are rapidly reversible. This, again, is in keeping with the above-described model for potentiation by an increase in intracellular calcium and the activation of calmodulin. It is worth mentioning that, in accordance with these findings, we were not able to detect potentiation of cAMP production in PBMC *membranes* by co-stimulation with PHA and isoprenaline (data not shown), a phenomenon also reported in human astrocytoma cells [42] and PMA [23]. This suggests the involvement of cytosolic components in the mechanism of potentiation and is also in agreement with the above-described role for calmodulin in potentiation.

Activation of the adenylyl cyclase system in leucocytes in general leads to a damping of cellular events (e.g. inhibition of proliferation and cytokine production) by means of a negative feedback on the activation of PLC and PKC [10, 11, 13, 17, 43, 44]. It now appears that stimuli that activate PLC (and subsequently activate PKC) simultaneously cause potentiation of the adenylyl cyclase system. This means that cell-activating stimuli increase the capacity of cells to react to cell-deactivating stimuli. This suggests that potentiation of adenylyl cyclase activity could be an important physiological mechanism to prevent cells from becoming "overstimulated".

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