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BETA-ADRENERGIC RESPONSIVENESS OF HUMAN PERIPHERAL LYMPHOCYTES AFTER MITOGENIC TRANSFORMATION WITH PHYTOHEMAGGLUTININ*

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Abstract—*In vitro* transformation of human peripheral blood lymphocytes with the mitogen phytohemagglutinin did not alter the total number of beta-adrenergic binding sites for (±)-^{125}iodocyanopindolol on the surface of intact cells, whereas binding to membrane fragments of transformed cells appeared to be diminished. In isolated membranes, there was also a marked decrease in basal, fluoride- and hormone-stimulated adenylate cyclase activity after phytohemagglutinin treatment. In whole cells, however, a lowering effect of phytohemagglutinin on levels of cyclic adenosine 3′,5′-monophosphate was not apparent. The discrepancy between data on intact and broken cells indicates that the transformed cells do not acquire additional beta-adrenergic receptors or catalytic adenylate cyclase as their cell surface expands due to blastogenesis. It is therefore concluded that mitogenic transformation of human peripheral lymphocytes does not cause specific changes in the beta-adrenergic/adenylate cyclase system.

Exposure of human peripheral lymphocytes to mitogens such as the plant lectin phytohemagglutinin (PHA)† initiates a series of biochemical reactions culminating in morphologically transformed and metabolically highly activated blast cells which have been triggered from quiescent to growing cells [1–3]. As the primary event of lectin stimulation, the mitogen interacts with glycoproteins on the cell surface and there is strong evidence for the existence of intracellular mediators of blastogenesis [1, 4]. Because of the marked membranous alterations occurring during PHA stimulation, e.g. increased membrane protein phosphorylation [5], numerous receptor proteins or membrane-related enzymes could be involved in the activation process. The enzyme adenylate cyclase (AC, ATP pyrophosphate lyase, cyclizing, EC 4.6.1.1.), the generator of cyclic adenosine 3′,5′-monophosphate (cAMP), for instance, has been found to be affected by PHA but, as for cAMP itself [1, 5–11], the role of the AC in lymphocyte stimulation is still controversial [9, 10, 12]. The complexity of the AC system with specific hormone receptors on the cell surface, a catalytic unit facing the cytoplasm, and a guanine nucleotide binding protein communicating between hormone-occupied receptors and the catalyst [13], offers diverse parameters to be measured in addition to the result of enzyme activation, i.e. the intracellular cAMP concentration. In view of the marked increase in the synthesis and insertion of membrane glycoprotein components discussed during PHA-transformation [14], enhanced expression of hormone-receptor proteins could represent an important effect of the mitogen. Augmentation of the hormonal sensitivity would contribute to the triggering of resting lymphocytes into the proliferative cycle. Therefore, a study comparing hormone-receptor density in addition to AC activity and whole cell cAMP levels in non-stimulated and PHA-activated cells should add substantial information to our knowledge of mitogen action.

In this communication, we will document that PHA-induced transformation of human peripheral lymphocytes does not affect the beta-receptor outfit of the cells. A decrease in both beta-adrenergic receptor density and AC activity as suggested by our data on membrane preparations of transformed cells has to be accounted for by the increase in cell surface and total amount of membrane protein associated with blastogenesis.

MATERIALS AND METHODS

Cell isolation. Lymphocytes were isolated from the blood of voluntary blood donors by Ficoll–Hypaque density gradient centrifugation [15]. Final cell purity ranged from 90 to 95% lymphocytes, 2 to 10% monocytes, and 0 to 2% granulocytes by morphological criteria. Aliquots of the cells (1×10^6 cells/ml) were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.4 μmol/ml L-glutamine at 37°C in humidified atmosphere of 5% CO_2 in air.

Mitogenesis was induced by addition of bacto-phytohemagglutinin P (PHA) at a final concentration of 20 μg/ml to the culture medium. After 48 hours of PHA-stimulation, 60–70% of the cells presented with typical blast cell morphology (large vacuolated cells with basophilic cytoplasm). Furthermore, at this time, ^{3}H-thymidine incorporation as determined

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† Abbreviations: AC, adenylate cyclase; Na-HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, sodium salt; INE, (±)-isoproterenol; PROP, (±)-propranolol; PGE_1, prostaglandin E_1; HIS, histamine; cAMP, cyclic adenosine 3′,5′-monophosphate; ICYP, (±)-^{125}iodocyanopindolol; PHA, bacto-phytohemagglutinin P.

according to Aye *et al.* [16] was 20–30-fold higher than in the non-stimulated control cells indicating that a considerable part of the PHA-treated cells were in the replicative phase of the cell cycle. Viability of the cells (trypan blue exclusion) after 48 hr of PHA was above 95% but decreased with longer *in vitro* incubation. Morphology of lymphocytes incubated for 48 hr in the absence of PHA (control cells) was undistinguishable from that of the cells prior to culture. Viability of the lymphocyte population prior to and after 48 hr of culture was at least 95%.

Cyclic AMP-determination. Cyclic AMP-containing extracts were prepared from lymphocytes incubated for 48 hr with and without PHA after exposure of the cells to the following agents at the stated final concentrations: sodium fluoride (NaF) 10 mM; (±)-isoproterenol (INE) 10 μM with or without (±)-propranolol (PROP) 50 μM; prostaglandin E_1 (PGE_1) 50 μM. To prevent cAMP-degradation, the phosphodiesterase inhibitor R020–1724 was simultaneously added (0.1 mM). After 1, 5 or 10 min of exposure, the reaction was stopped with concentrated $HClO_4$ (final 1 N), the denatured protein discarded by centrifugation at 2500 *g*, the acidic supernatant neutralized with 9 N KOH, and the $KClO_4$ eliminated by centrifugation as described by Cailla *et al.* [17]. The cAMP radioimmunoassay was based on sensitive acetylation as provided by New England Nuclear (Boston, MA).

Preparation of plasma membranes. To prepare particulate membrane fractions [18], cells were homogenized in hypotonic HME-buffer (20 mM Na–HEPES, pH 8, 2 mM $MgCl_2$, 1 mM EDTA) using a tight-fitting glass homogenizer (10 strokes). Intact cells and nuclei were removed by sequential centrifugation, and from the resulting supernatant, the 3000 *g* pellet was obtained.

Protein was assayed following the precipitation of membraneous material with 8% trichloroacetic acid by the method of Lowry *et al.* [19].

Adenylate cyclase assay. Adenylate cyclase (AC) activity was quantified as described [20] by incubating 10–50 μg of membrane protein with 0.5 mM [α-^{32}P]adenosine 5′ triphosphate, 10 mM $MgCl_2$, 1 mM EDTA (manganese chelating agent), 3 mM K_2-phosphoenolpyruvate, 10 μg/ml pyruvate kinase (rabbit muscle), 0.01 mg/ml bovine serum albumin (fraction V), 0.1 mM Na-ascorbate (antioxidant), 50 mM Na–HEPES, pH 8, and 0.1 mM R020–1724. When indicated, the following chemicals and hormones were present at the stated final concentrations: NaF, 10 mM; INE, 10 μM (with or without 50 μM PROP); PGE_1, 50 μM; histamine (HIS), 100 μM (with or without 200 μM cimetidine). When hormone effects on the AC were tested, guanosine-5′-triphosphate (GTP), 10 μM, was also included. The AC reaction was terminated after 15 min at 30°C by addition of 0.25% sodium dodecylsulfate, 5 mM ATP and 0.175 mM cAMP, pH 7.5 ^{32}P-labeled cAMP was separated by sequential chromatography on Dowex AG 50WX4 resin and neutral alumina after addition of ^{3}H-cAMP (approx. 30,000 cpm) to estimate the recovery. Radioactivity in the column eluate was measured in a Packard Tri-Carb scintillation counter using a toluene-based scintillation cocktail. A blank containing all components of the reaction mixture except for membrane protein was run with each experiment and subtracted from the experimental values. AC activity was expressed as picomol ^{32}P incorporated per milligram protein per minute.

Beta-adrenergic receptor assay. Beta-adrenergic binding sites on particulate membrane fragments were assessed according to Maguire *et al.* [21] using (±)-^{125}iodocyanopindolol (ICYP) as highly specific beta-adrenoceptor ligand [22]. Membranes (5–10 μg) were incubated with 5–250 pM ICYP for 60 min at 30°C in 50 mM Na–HEPES, pH 8, 4 mM $MgSO_4$ using disposable polypropylene tubes. Bound and free ligand were separated by dilution of the samples with 3 ml of 37°C wash buffer (20 mM potassium phosphate, pH 8, 1 mM $MgSO_4$) containing 100 μM PROP, followed by rapid filtration through nitrocellulose Millipore filters (HAWP 02400, pore size 0.45 μm). Each filter was washed with additional 25 ml of wash buffer without PROP and counted in an Auto-Logic gamma counter (Abbot Lab). In the radioligand batches used, [^{125}I]iodide was below 5% as checked by thin layer electrophoresis. Specific binding was determined in the presence and absence of 2 μM PROP (3 orders of magnitude above the dissociation constant of this beta-adrenoceptor antagonist). Non-specific binding averaged 30–50% of the total counts bound. The data were analyzed by the method of Scatchard [23] for maximal binding capacity and the dissociation constant (K_D) of the ligand.

Beta-adrenergic binding sites on the surface of intact cells were determined following a modification of the above binding procedure [24]. After incubation of $1–2\times10^6$ cells for 60 min at 30°C and separation of bound and free ligand, the samples were centrifuged at 200 *g* for 5 min (4°C), the supernatant was discarded, and the cell pellet was resuspended in 3 ml of ice-cold HME-buffer without PROP for 10 min at 4°C. This treatment caused swelling of the cells without lysis. After 10 min in HME, the samples were filtered, washed with 25 ml of 37°C wash buffer and counted for radioactivity. The efficiency of these modified binding conditions in reversing the cellular uptake of beta-adrenoceptor ligands has been checked in experiments involving the very slowly dissociable beta-adrenoceptor blocker FM 24 together with the highly lipophilic radioligand ^{125}I-iodohydroxybenzylpindolol (IHYP). In the presence of FM 24, a considerable amount of IHYP was still measured on FM 24-treated cells when standard binding conditions were applied, i.e. binding conditions lacking hypotonic treatment of IHYP-loaded cells. This IHYP-binding to FM 24-treated cells was due to non-specific binding and/or uptake of the ligand by the cells as confirmed by the absence of IHYP-binding displaceable by PROP. Under modified binding conditions, however, the FM 24-treated cells were completely depleted of intracellularly stored IHYP indicating that HME-exposure of the IHYP-loaded cells had reversed the non-specific uptake of the radioligand by the intact cells. With regard to the new radioligand ICYP used in the present study, very recent results by Molinoff *et al.* (personal communication) have shown linearity of the off-reaction of ICYP-binding pointing towards

an infinitesimal degree of non-specific cellular uptake occurring with this ligand. The use of this highly stereospecific beta-adrenoceptor ligand ICYP [25–27] together with a binding technique shown to minimize intracellular ligand trapping allows us to assume the lack of interference of non-specific radioligand uptake with specific binding data in the present study.

Specific binding data were converted from fmol ICYP bound per 10^6 cells or the corresponding milligrams of membrane protein to molecules of ICYP bound using the Avogadro's number (6.023×10^{23}) thereby estimating the number of receptor sites per cell.

Statistics. Experimental data, presented as mean values and the standard error of the mean, were compared using Student's *t*-test; P values of 0.05 and less were considered significant.

Materials. [α-^{32}P]adenosine-5′-triphosphate (triethylammonium salt; 12.5–18 Ci/mmol), [8-^{3}H]cAMP (20–30 Ci/mmol), and ICYP (~2000 Ci/mmol) were from the Radiochemical Centre, Amersham, Buckinghamshire, U.K. PHA-P was purchased from Difco Lab., Detroit, U.S.A. Sources of other materials have been previously described [20].

RESULTS

Beta-adrenergic binding sites. The density of beta-adrenergic binding sites on intact lymphocytes was not affected by *in vitro* incubation of the cells for 48 hr in the absence or presence of PHA (Table 1). When plasma membrane preparations of the lymphocytes were used, the incubation procedure *per se* again lacked any effect on the binding properties of the ligand. After PHA-transformation, however, the amount of ICYP bound per milligram protein was significantly ($P < 0.05$) decreased (Table 2). Under the various experimental conditions, Scatchard analysis of the specific binding data yielded linear plots indicative for a single high-affinity binding site for ICYP.

In order to compare beta-receptor findings in intact cells and cell membranes, the protein content of membranes derived from non-stimulated and stimulated cells was determined. Membrane material from 10^6 non-stimulated lymphocytes contained 1.01 ± 0.1 μg protein vs 2.23 ± 0.1 μg protein measured in membranes obtained from the same number of stimulated cells ($P < 0.025$). After recalculation of the amount of ICYP bound per milligram protein in terms of binding sites per cell, no significant difference between controls and PHA-treated cells was apparent ($P > 0.5$). On the other hand, a considerable loss or inactivation of ICYP binding sites during the preparation of lymphocyte membrane particles became obvious.

Adenylate cyclase. Basal levels of AC activity were identical in plasma membranes from lymphocytes prior to *in vitro* incubation and from controls which had been incubated for 48 hr without PHA (Table 3). Fluoride, a direct, receptor-independent stimulator of the catalytic AC unit [13], increased the enzyme activity equally strong in both cell types. Hormone-stimulated AC activity appeared to be slightly, but not significantly ($P > 0.1$), higher in cultured lymphocytes. After PHA-stimulation, basal AC activity was low and responses of the enzyme to both fluoride and to the hormones INE, PGE_1, and HIS were markedly weaker than in the control ($P < 0.05$). The stimulating effect of INE was generally completely blocked after addition of the beta-adrenergic antagonist PROP, and the effect of HIS could be prevented by addition of the H_2-antagonist cimetidine (data not shown).

When relating these data obtained in isolated plasma membranes to AC activity in the total cells, the twofold higher membrane content of stimulated lymphocytes has to be considered. As a consequence, the AC activity expressed in terms of total membrane protein does not differ significantly between untransformed and transformed cells.

Whole cell cAMP. The basal intracellular cAMP levels did not differ in lymphocytes cultured for 48 hr with or without PHA provided that the cyclic nucleotide concentration was expressed per cell number. Exposure of the lymphocytes to fluoride or to the hormones INE and PGE_1 for 1 min resulted in comparable rises of cAMP in the two cell types (Fig. 1). Use of the beta-adrenoceptor antagonist PROP completely abolished the INE-effect (data not shown). Longer exposure times (5 or 10 min) yielded identical results.

DISCUSSION

In vitro transformation of human circulating lymphocytes with concanavalin A has been shown to result in the emergence of binding sites for insulin on the cell surface [28], alloantigenic stimulation of

Table 1. Beta-adrenergic binding sites on intact peripheral lymphocytes prior to culture and after 48 hr of incubation with or without phytohemagglutinin (PHA) using the ligand (±)-^{125}iodocyanopindolol (ICYP).* Data are means ± S.E.M. from 8 experiments

Experimental condition	Maximal binding		K_D (pM)
	fmol ICYP bound/10^6 cells	Receptors/cell	
Prior to culture	2.03 ± 0.1	1222 ± 82	25 ± 7
48 hr culture			
− PHA	2.66 ± 0.7	1603 ± 435	28 ± 9
+ PHA	2.25 ± 0.5	1353 ± 316	23 ± 5

* Intact lymphocytes were incubated at pH 8 (30°C) for 60 min with increasing concentrations of (±)-^{125}iodocyanopindolol (ICYP; 5–250 pM). For details of the binding method used see Materials and Methods. Maximal binding capacity and K_D-values were calculated from Scatchard analysis of the specific binding data.

Table 2. Beta-adrenergic binding sites on plasma membranes from peripheral lymphocytes prior to culture and after 48 hr of incubation with and without phytohemagglutinin (PHA) using the ligand (±)-^{125}iodocyanopindolol (ICYP).* Data are means ± S.E.M. from 8 experiments

Experimental condition	Maximal binding		K_D (pM)
	fmol ICYP bound/mg protein	Receptors/cell	
Prior to culture	309.5 ± 26	186 ± 16	22 ± 5
48 hr culture			
− PHA	314.8 ± 61	190 ± 37	18 ± 3
+ PHA	120.8 ± 25	162 ± 33	23 ± 2

* Plasma membranes from lymphocytes were incubated at pH 8 (30°C) for 60 min with increasing concentrations of (±)-^{125}iodocyanopindolol (ICYP; 5–250 pM). Maximal binding capacity and K_D-values of ICYP were calculated from Scatchard analysis of the specific binding data. After 48 hr of incubation of lymphocytes in the presence of PHA, the amount of ICYP bound specifically per milligram of membrane protein was significantly decreased as compared to control cells ($P < 0.05$).

Table 3. Adenylate cyclase activity in plasma membranes from lymphocytes prior to culture and after 48 hr of incubation with or without phytohemagglutinin (PHA).* Data are means ± S.E.M. from 7 experiments

Experimental condition	pmol ^{32}P incorporated/mg protein/min				
	Basal	NAF	INE + GTP	PGE_1 + GTP	HIS + GTP
Prior to culture	21.9 ± 8	145.0 ± 44	138.7 ± 30	445.0 ± 17	145.3 ± 38
48 hr culture					
− PHA	22.0 ± 7	183.3 ± 43	330.8 ± 87	514.9 ± 100	314.3 ± 81
+ PHA	13.3 ± 3	106.3 ± 25	125.3 ± 27	276.8 ± 51	122.1 ± 30

* Adenylate cyclase activity was assessed by incubating isolated lymphocyte membranes for 15 min (30°C) in [α-^{32}P]ATP-containing reaction mixture provided with an ATP-regenerating system. Enzyme activity levels in membranes from lymphocytes cultured for 48 hr in the absence of PHA were not significantly different from those obtained in the cells prior to culture ($P > 0.1$). The decrease in enzyme activity due to PHA-treatment was significant ($P < 0.05$) when compared to lymphocytes incubated for 48 hr in the absence of the mitogen.

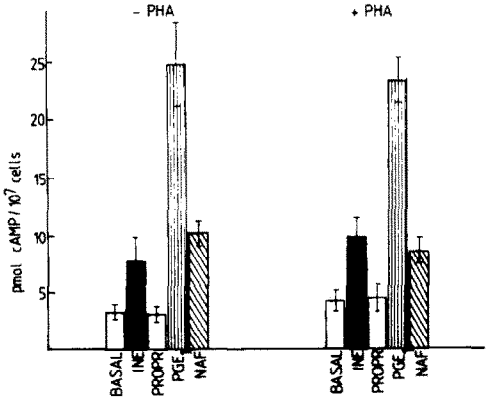

Fig. 1. Intracellular cyclic adenosine 3',5'-monophosphate (cAMP)-levels in untransformed and PHA-transformed lymphocytes in response to isoproterenol (INE, 10 μM) alone and together with the beta-adrenoceptor antagonist propranolol (PROP, 50 μM), prostaglandin E_1 (PGE_1, 50 μM), and sodium fluoride (NaF, 10 mM) were determined. Following the incubation of the lymphocytes for 48 hr in the absence (−PHA) or presence (+PHA) of phytohemagglutinin (PHA), aliquots of the cell cultures (10^7 cells/ml) were exposed to above listed ligands for 1 min (37°C), reaction was stopped, and cAMP-radioimmunoassays were performed as described in Materials and Methods.

mouse splenocytes has been described to increase the appearance of histamine receptors [29], and the addition of PHA to human lymphocyte cultures has been reported to prevent the loss of receptors for prostaglandin E_2 during the *in vitro* incubation [30]. These observations suggest that mitogen stimulation of lymphocytes might cause enhanced expression of various hormone receptors. Therefore, the present study was designed to investigate the behaviour of beta-adrenergic receptors in PHA-transformed peripheral human lymphocytes.

Our results indicate that after 48 hr of mitogen treatment, the total number of binding sites for the highly specific beta-adrenoceptor ligand ICYP on intact cells is not significantly altered as compared to non-stimulated cells. On membrane preparations of PHA-activated lymphocytes, however, we found the beta-adrenergic binding sites to be markedly decreased. This discrepancy can be explained by the fact that the increase in cell size during PHA-transformation is not accompanied by a proportional rise in the number of beta-receptors. Therefore, the concentration of the receptors as calculated per milligram membrane protein in the blast cells appears to be lowered.

Comparing the maximal binding of ICYP on intact and broken cells from either the untransformed or transformed lymphocytes, a smaller number of bind-

ing sites is detected on membrane fragments. The fact that the binding affinity of ICYP is the same whether cell membranes or intact cells are considered suggests that the receptors identified are qualitatively identical. A similar loss or inactivation of binding sites for the ligand (±)-^{125}iodohydroxybenzylpindolol has been described in disrupted S 49 mouse lymphoma cells by Insel and Stoolman [31]. These observations that disruption of cells is accompanied by a reduction of hormone receptors underline the importance of studying specific receptor-ligand binding on both broken and intact cells.

In order to correlate the binding data with the physiological cell response, we investigated the effectiveness of the beta-agonist INE to stimulate the AC. It turned out that in isolated plasma membranes from PHA-transformed lymphocytes, INE-induced AC activation was considerably weaker than in untransformed cells. Furthermore, basal AC and fluoride-stimulated enzyme activity in transformed cells appeared to be diminished. With regard to the increase in cell surface area and total membrane protein occurring during mitogenesis, the reduced AC activities signify that catalytic enzyme units do not increase with cell enlargement. This assumption is strongly supported by our finding of identical whole cell cAMP levels in non-stimulated and stimulated cells in response to various enzyme effectors when calculated per cell number.

Interestingly, similar to INE, also the hormones PGE_1 and HIS are less effective on the AC in membranes from PHA-induced blast cells. If one assumes that the magnitude of AC activation is a direct function of the number of receptors occupied (for review see Ref. [32]) then this could be interpreted as a result of reduced receptor sites for PGE_1 and HIS per milligram membrane protein. In contrast to the obvious failure of transformed lymphocytes to acquire additional receptors for INE, PGE_1 and HIS as their cell surface area increases, the cells have been shown to bind insulin in proportion to their cell size [28]. Further evidence for an opposite involvement of these hormone receptors in processes of cell growth and division come from studies on leukemic lymphoblasts. While marked display of insulin receptors that are virtually absent on normal lymphocytes could be identified on leukemic blast cells from chronic and above all acute lymphoblastic leukemia [28, 33], a considerable loss of beta-adrenergic binding sites was found on these cells parallelled by a decrease in the AC responsiveness to INE, PGE_1 and HIS when compared to their normal counterparts [20]. Such considerations become especially meaningful in view of the fact that both *in vitro* transformed lymphocytes and malignant cells are completely independent of exogenous hormonal signals with respect to growth and maturation capacity. This makes the lectin stimulation a valuable model for studying mechanisms fundamental to unregulated cell proliferation.

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