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ANALYSIS OF LYMPHOCYTE CELL MEMBRANE CYCLASE ACTIVITY AFTER STIMULATION BY A MITOGENIC POLYANION

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UDC 612.112.94.017.1-063

KEY WORDS: polyanion; lymphocyte membrane; cyclase enzymes.

The molecular mechanism of lymphocyte stimulation by ligands with different chemical structure and properties is not yet explained. However, there are now serious grounds for considering that the key systems concerned in the formation of the signal at the plasma membrane level are as follows: 1) the system of cyclase enzymes of the membrane, 2) the membrane ionic transport system, and 3) the system of the lipid matrix of the membrane, which must be regarded along with membrane enzymes as controlling the chemical and physicochemical properties of the matrix [7, 8].

We have studied the molecular mechanisms of triggering the response of the lymphocytes by polyelectrolytes and, in particular, by the mitogenic polyanion polyacrylic acid (PAA) [1, 2, 6, 8]. Most attention was paid to the state of the above-mentioned "key" systems of the plasma membrane. PAA has already been shown to have a strong modulating action on permeability of the plasma membrane and on activity of ion-transporting ATPase [1, 2].

The aim of this investigation was to study activity of adenylate and guanylate cyclases in the plasma membrane of lymphocytes before and after activation by mitogenic doses of PAA. Since it was shown previously that PAA activates division of B lymphocytes [9], the action of PAA was compared with that of another B-cell mitogen, namely the lipopolysaccharide (LPS) from *Escherichia coli*.

EXPERIMENTAL METHOD

Cultures of splenic lymphocytes from (CBA × C57BL)F_1 mice were prepared in accordance with recommendations described previously [5, 7]. Division of B lymphocytes was activated by PAA according to the optimal "pulsed" scheme [6]. A preparation of PAA with molecular weight of 80,000 daltons, which was added to the lymphocyte suspension for 10 min in a final concentration of 20-40 μg/ml, was used. The lymphocytes were washed free from the polyanion by triple centrifugation, after which the cells were resuspended in enriched culture medium. LPS of *E. coli* (from Sigma, USA) activated B-cell proliferation in a final concentration of

Institute of Biophysics, Ministry of Health of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR R. V. Petrov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 97, No. 5, pp. 567-569, May, 1984. Original article submitted June 23, 1983.

 0007-4888/84/9705-0604$08.50

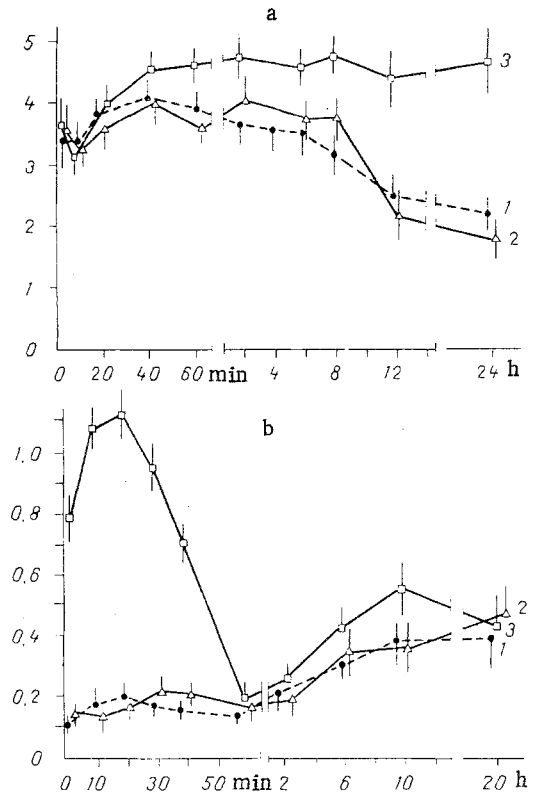

Fig. 1. Dynamics of intracellular cAMP (a) and cGMP (b) concentrations in lymphocytes after activation by mitogen. Abscissa, time after addition of cells to culture; ordinate, concentration (in pM). 1) Cultures without mitogen, 2) cells activated by PAA (50 µg/ml), 3) cells activated by LPS (50 µg/ml). Values of nucleotide concentrations calculated per 10^6 cells.

20-50 µg/ml. Activity of adenylate and guanylate cyclases was determined from the change in intracellular concentration of cyclic AMP and cyclic GMP respectively. The concentration of cyclic nucleotides was measured by radioimmunoassay on the basis of competition with labeled [^{3}H]-cAMP or [^{3}H]-cGMP for binding with the specific receptor protein [11]. To analyze the cAMP concentration, cAMP-dependent protein kinase from bovine muscle was used as receptor protein, and specific antibodies against cGMP were used to measure the cGMP concentration. Commercial reagents from Amersham Corporation (England) and Boehringer (West Germany) were used. To determine the intracellular concentrations of the cyclic nucleotides, extracts were prepared from the solid residue of 10^7 lymphocytes obtained after centrifugation at 1500 rpm for 15 min. The supernatant was completely removed and the cell residue broken up by shaking. To destroy the cells and extract the intracellular contents, 300 µl of 0.5 M perchloric acid was poured into a test tube with the cells and vigorous shaking continued for 30-60 sec. The extracts were than clarified by centrifugation at 1000 rpm for 10 min and neutralized with 10-20 µl of a saturated solution of K_2CO_3. The dynamics of the cyclic nucleotide concentrations after activation of the lymphocytes by mitogenic doses of PAA was studied after 15, 20, 30, 40, and 60 min and 2, 4, 6, 8, 10, 12, and 24 h. To determine the cyclic nucleotide concentration, 1, 2, 5, and 10 min after activation by the mitogen the cell residue was obtained not by centrifugation, but by ultrafiltration (for 30-40 sec) through "Synpor" filters (Czechoslovakia) with pore diameter of 2.5 µm. The cells settling on the filter were then transferred together with the filter into a tube with perchloric acid for extraction of the intracellular contents.

EXPERIMENTAL RESULTS

The time course of the cAMP concentration in control and mitogenic-stimulated lymphocytes is shown in Fig. 1. In the course of culture *in vitro* a gradual fall of the intracellular cAMP level took place in the control splenic lymphocytes, and 8-12 h after the beginning of incubation it was significantly lower than initially. In lymphocytes subjected to

the mitogenic action of PAA no significant changes were found in the cAMP concentration for 24 h after activation. In cultures stimulated by LPS, the intracellular cAMP concentration coincided with the control for the first 30 min. Later, 2-8 h after mitogenic activation, the cAMP concentration in cells activated by LPS was 30-40% higher than the cAMP level in the control lymphocytes. A marked increase in the intracellular cAMP concentration (up to 250% of the control) under the influence of LPS was recorded 12-24 h after activation by the mitogen.

The time course of the cGMP level in the control cells differed from that of the cAMP level (Fig. 1b). A significant increase in the cGMP concentration compared with initially was observed in the intact lymphocytes 6-20 h after the beginning of incubation compared with the initial level (at the beginning of incubation). The character of the change of cGMP concentration on the whole in cells activated by PAA was the same as in the control. In the course of measurement of the cGMP level during the 24-h period no significant differences were found between concentrations of the cyclic nucleotide in intact and polyanion-stimulated lymphocytes. Conversely, under the influence of LPS there was a rapid "pulsed" increase in the intracellular cGMP concentration in the splenic lymphocytes. In the first 5-20 min the cGMP level in cells activated by LPS was five to eight times higher than the cGMP level in the control lymphocytes. The cGMP level 40-60 min after addition of LPS fell to the control values. During the next 20 h of incubation the cGMP concentration in LPS-activated lymphocytes was virtually identical with the cGMP level in the control. Not until the 10th hour of culture was a tendency observed for the cGMP level to rise again in lymphocytes stimulated by LPS.

It will be clear from the data given above that during induction of division of B lymphocytes by PAA there was no significant change in the intracellular cAMP and cGMP concentrations. Consequently, the molecular mechanism of activation of the lymphocytic response by this polyanionic mitogen does not involve the cyclase system in the cell membrane. The writers showed previously that during the first 1-2 min after addition of mitogenic doses of PAA *in vitro* marked changes take place in the membrane ionic transport system: Membrane permeability for mono- and bivalent cations, and also for nucleosides, increase strongly [1, 2], and membrane Na^+, K^+ - and Ca^{++} -/ATPases are activated [1, 8]. At the same time we showed that under the influence of mitogenic doses of PAA no significant change is observed in the physicochemical properties of the lipid matrix of the lymphocyte membrane [3]. All these facts taken together suggest that the principal change which plays the "key" role in triggering the response of the B cells is increased permeability of the plasma membrane induced by the polyanion.

It was stated previously that the kinetics of response of B lymphocytes to PAA and LPS differs [9]. The present investigation also reveals differences in the molecular mechanism of triggering of B-cell division by these mitogens. In fact, under the influence of LPS a marked increase in the cGMP concentration took place during the first 5-20 min, only to fall again to the control level after 40-60 min. Later, in LPS-activated lymphocytes the intracellular cAMP concentration began to rise, to reach 200-250% relative to the control after 12-24 h. These results, incidentally, are in complete agreement with those of measurement of the dynamics of the cyclic nucleotide level after activation by LPS, described by other workers [12, 14]. We consider that the principal factor relevant to triggering the lymphocyte response (passage from the G_0-phase to the G_1-phase of the cell cycle) is an increase in the cGMP concentration at the initial moment after addition of LPS. The later rise in the cAMP concentration more probably reflects changes in the cyclic nucleotide level in phases of the cell cycle [8].

Quite possibly early activation of cell membrane guanylate cyclase under the influence of LPS is linked in a particular manner with the increase in viscosity of the lipid matrix of the membrane which we have found. This latter increase can be recorded at about the same time (20-30 min) after addition of the same mitogenic concentrations (50 μg/ml) of *E. coli* LPS *in vitro* [3]. An important role in the functioning of cyclases associated with the cell membrane has been ascribed to the lipid microenvironment [13]. We know that a change in the phase state of the membrane lipids can facilitate the transition of the cyclase from the active into the inactive state and vice versa [4]. It can accordingly be postulated that LPS or, more precisely, its active fragment, namely lipid A, when "built" into the lymphocyte membrane, alters the viscosity of its lipid matrix. This, in turn, is reflected in the state and activity of the cyclase enzymes. The precise mechanism of this effect of LPS on the lipid component of the membrane and, in particular, on the lipid microenvironment of the cyclases,

is still unknown. However, the results of the present investigation suggest that LPS activates B-cell division through activation of guanylate cyclase in the cell membrane. The action of LPS, under these circumstances, evidently does not involve the ion-transporting system of the membrane. Conversely, the mitogenic polyanion PAA activates B-cell division by increasing ionic permeability of the plasma membrane, without changing cyclase activity.

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USE OF LIPOSOMES AS A NONSPECIFIC DETOXICATOR IN THE CRUSH SYNDROME

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UDC 617-001.32-008.6-092.9-085.451.234

KEY WORDS: liposomes; crush syndrome; myocardial contractility; hemodynamics.

Phospholipids of the plasma membrane of cells play an important role in recognition and binding of various biologically active substances and toxins [8, 9]. It has been shown, for instance, that cholera toxin interacts with the ganglioside G_{JM}, isolated from the epithelium of small intestine, with a binding constant of 10^{-9} M. Addition of exogenous gangliosides sharply reduced the pathogenic action of cholera toxin, evidently by preventing it from binding with the cell membranes [10]. Artificial phospholipid vesicles (liposomes) are analogs of cell membranes and, because of their small size, they have a large total surface area. The high adsorptive activity of liposomes relative to proteins has often been described [7].

Considering that toxic metabolites appearing in the blood stream in ischemia are substances of protein nature and have high affinity for cell membranes [4], the writers postulated that injection of liposomes into animals with a model of endogenous poisoning would enable an additional target to be created for toxins with affinity for the membrane and would prevent or alleviate the course of toxic shock. To test this hypothesis a model of the crush

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Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 97, No. 5, pp. 569-572, May, 1984. Original article submitted April 29, 1983.

0007-4888/84/9705-0607$08.50