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INCREASED PERMEABILITY OF THE LYMPHOCYTE PLASMA MEMBRANE FOR MONO- AND BIVALENT CATIONS AND LOW-MOLECULAR-WEIGHT METABOLITES CAUSED BY MITOGENIC POLYANIONS

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UDC 612.112.94.017.1.063

KEY WORDS: polyanions; lymphocytes; membrane permeability.

The immunostimulating properties of polyanions such as polyacrylic acid (PAA) or dextran sulfate (DS) were described quite a long time ago. However, until recently the immunocompetent cells on which these agents acted, and how they acted, were unknown. Recently the writers determined the nature of lymphocytes which respond to PAA and described the detailed kinetics of their response with respect to parameters of cellular proliferation and differentiation [2, 3, 5, 6]. We are now investigating molecular changes taking place in the lymphocyte membrane under the influence of a polyanionic activator. Attention is being concentrated on functional systems in the plasma membrane which may be involved in the formation of the membrane-dependent signal that activates cell metabolism [4]. One such system is the ionic transport system. It consists of "channels" through which ions are transported along their concentration gradient, and of ion-transporting membrane enzymes, responsible for carrying ions against their concentration gradient, utilizing for this purpose the energy of the chemical bonds of ATP (for example, Ca^{++}-ATPase, Na^+, K^+-ATPase, etc.).

This paper describes the study of transmembrane flows of K^+ and Ca^{++} ions and also of nucleoside molecules before and after treatment of lymphocytes with immunostimulating doses of polyanions.

EXPERIMENTAL METHOD

In vitro cultures of splenic lymphocytes from (CBA × C57Bl)F_1 mice were used. To activate the lymphocytes mitogenic concentrations of PAA with molecular weight of 80,000 or 16,000 daltons [2] were added to the cultures. DS was added up to a final concentration of 200-500 μg/ml.

Permeability of the cell membrane for Ca^{++} was measured by a radio-indicator method. The isotope ^{45}Ca was used and was added to a suspension of lymphocytes (5×10^6 to 10×10^6 cells/ml), kept under optimal cultural conditions [2, 7]. Samples of 0.2-1.0 ml were taken from the suspension 1 h after addition of ^{45}Ca (final concentration 0.1 μCi/ml). Cells contained in the

Research Institute of Immunology, 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. 98, No. 7, pp. 81-84, July, 1984. Original article submitted August 12, 1983.

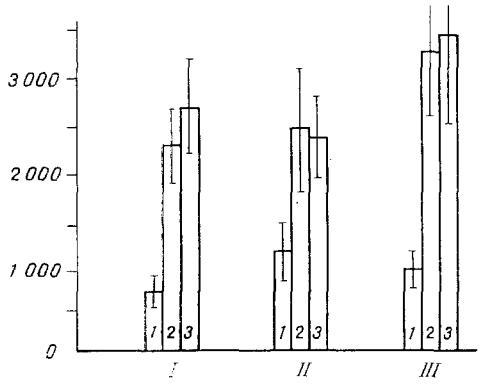

Fig. 1. Increase in penetration of exogenous ^{45}Ca into lymphocytes *in vitro*. Ordinate, radioactivity (in cpm) of extracts from equivalent number of cells taken from cultures with PAA with molecular weight of 80 kilodaltons (2) or 16 kilodaltons [3], or from control cultures (1) without mitogen. Results of three independent experiments are shown (I-III). Each value in a separate experiment for a given experimental group is the arithmetic mean of the results for 4-5 identical samples.

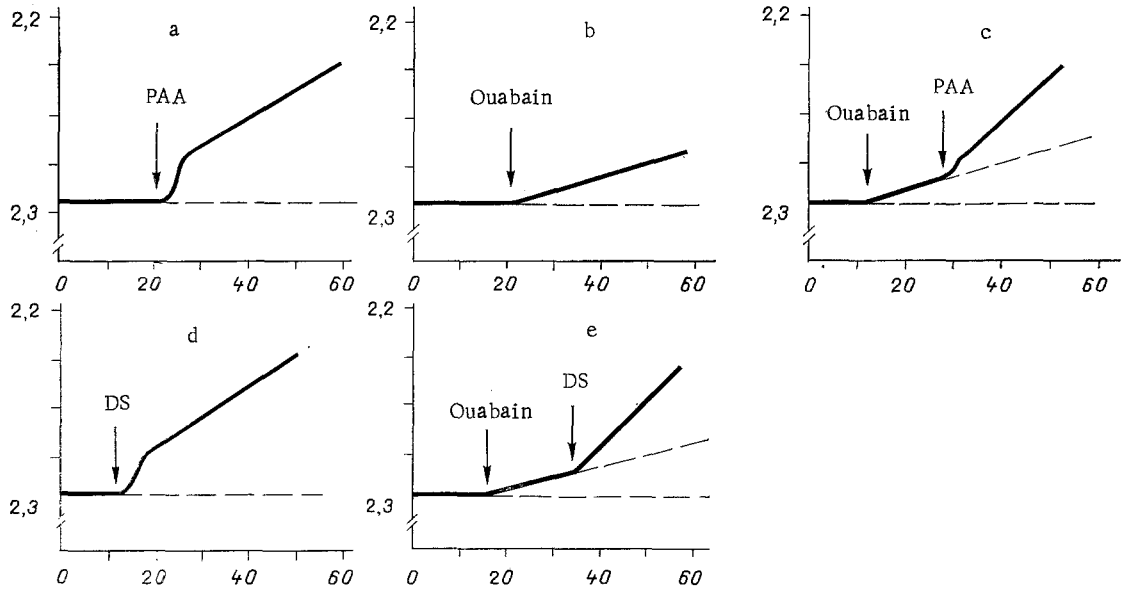

Fig. 2. Outflow of K^+ from lymphocytes under the influence of polyanions and (or) ouabain *in vitro*. Abscissa, incubation time of lymphocytes (in min); ordinate, pK^+. Effectors and times of their injection into cell cultures indicated by arrows. Explanation in text.

samples were washed to remove culture fluid by centrifugation (1000 rpm, 10 min) twice or three times in 20-50 volumes of medium 199. The dense cellular residue obtained by the last centrifugation was then treated with 300 µl of 5% perchloric acid to destroy the cells and to extract the intracellular contents. Extraction continued for 1-2 min with constant shaking. The extracts were then "clarified" by centrifugation at 500 rpm for 10 min. The supernatant was neutralized with 5-10 µl of a saturated solution of K_2CO_3. Radioactivity of the cell extracts was measured on an SL-30 β-spectrometer (from Intertechnique, France). Samples of cell extracts were dissolved in the ratio of 1:50 in dioxan scintillator (900 ml of dioxane, 4.0 g of PPO,

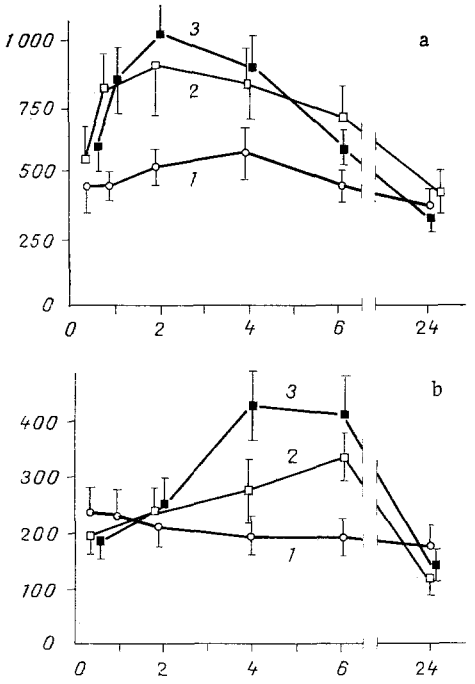

Fig. 3. Accumulation of exogenous [^{3}H]uridine (a) and [^{3}H]thymidine (b) in cytoplasm of lymphocytes incubated *in vitro* in the presence of mitogenic concentrations of PAA with molecular weight of 80 kilodaltons (2) or 16 kilodaltons (3), compared with control cells (1) without mitogen. Abscissa, time after addition of PAA *in vitro* (in h); ordinate, δ-pool for corresponding nucleoside.

0.2 g of POPOP, and 60 g of naphthalene). The radioactivity of extracts from cultures activated by mitogenic doses of PAA was compared with the radioactivity of extracts from control, unactivated lymphocytes. In this way the effect of the mitogen on penetration of ^{45}Ca from the culture medium inside the cells could be estimated.

Permeability of the cell membrane for K^+ was measured as follows. Suspensions of living lymphocytes containing 30×10^6 to 50×10^6 cells/ml were made up in Hanks' solution, with the addition of 20 mM HEPES-buffer and 50 Units of penicillin (sodium salt). The suspension was introduced in a volume of 0.5 ml into a cell in which was mounted a valinomycin membrane electrode, the design of which was worked out by the "Kvant" Research-Production Combine, and a double electrolytic bridge, connecting the measuring cell with an ÉVL-1MZ comparison electrode. The compartment of the electrolytic bridge which was in contact with the test suspension was filled with a 0.5-1 M solution of choline chloride. The steepness of the characteristic curve of the electrode in the potassium concentration zone from 10^{-4} to 1 M was 59-60 mV per pK^+ unit. Na^+/K^+ selectivity was not below 10^{-4}. Selectivity relative to the remaining ions was better still. The results were recorded with the ÉV-74 ionometer with N-339 automatic writer. With this apparatus the K^+ concentration in the culture medium could be recorded continuously. Any increase in permeability of the cell membrane for K^+ must lead to "leaking" of K^+ from the cells and to an increase in the K^+ concentration in the culture medium.

Permeability of the cell membrane for nucleosides was measured by the method suggested by Gurvich et al. [1]. To a suspension containing 5×10^6 cells/ml, 1 μCi/ml of [^{3}H]thymidine or [^{3}H]uridine was added. After incubation in the presence of radioactive nucleoside, samples (50-200 μl) were taken from the suspension and applied to "Synpor" filters (pore diameter 2.5 μ). Ultrafiltration was carried out in an apparatus (Millipore, USA) for simultaneous filtration in 12 wells. Cells which settled on the filters were washed to remove remains of culture medium with 40-50 ml of Hanks' solution. To measure the quantity of nucleoside taken up into the cell, samples of washed cells were fixed on the filter with 10 ml of 96° ethanol. To measure the quantity of nucleoside incorporated into newly synthesized cellular DNA or RNA, before

ethanol fixation the samples were treated with 20 ml of 5% TCA to produce lysis of the cells and to remove acid-soluble material. The samples thus prepared were dried and their radioactivity determined on an SL-30 liquid scintillation spectrometer. The intensity of penetration of the nucleoside through the cell membrane was estimated from the size of the δ-pool of the corresponding nucleoside in the cytoplasm. The δ-pool was used as the indicator of the quantity of nucleoside which penetrated into the cell, but had not yet been incorporated into nucleic acid. The δ-pool was calculated by the equation

$$\delta = \alpha - \beta,$$

where δ denotes the δ-pool for [^{3}H]thymidine or [^{3}H]uridine; α the radioactivity of whole cells which penetrated in the presence of labeled nucleoside; β the radioactivity of the acid-insoluble material of the same cells.

EXPERIMENTAL RESULTS

Addition of mitogenic concentrations of PAA to lymphocyte cultures led to a considerable increase in ^{45}Ca accumulation in the cell cytoplasm (Fig. 1). The radioactivity of the intracellular contents from lymphocytes activated with PAA was 2.5-4 times greater than the radioactivity of extracts from the equivalent number of unactivated control cells. An increase in the ^{45}Ca content in activated lymphocytes 1 h after addition of ^{45}Ca and PAA to the cell cultures evidently indicated a considerable increase in permeability of the cell membrane for ^{45}Ca under the influence of PAA.

The results of experiments to measure the outflow of K^+ from lymphocytes (Fig. 2) clearly demonstrated the great increase in cell membrane permeability for K^+ under the influence of mitogenic doses of PAA (Fig. 2a). The "outflow" of K^+ in this case was not the result of inhibition of Na^+, K^+-ATPase, as is the case on the addition of 10^{-4} M ouabain (Fig. 2b). Even after blocking of Na^+, K^+-ATPase by ouabain, the addition of PAA led to a further increase in the rate of "outflow" of K^+ from the cells (Fig. 2c). It is an interesting fact that another B-cell mitogen of polyanionic nature, namely dextran sulfate (DS), exhibited properties similar to PAA. Under the influence of DS the outflow of K^+ from the cells was significantly increased, independently of inhibition of Na^+, K^+-ATPase by ouabain (Fig. 2, d and e). The method used to record permeability of the cell membrane for K^+, incidentally, was much more sensitive than the method of recording membrane permeability for ^{45}Ca described above. The radioisotope method was more laborious and necessitated incubation of the cells with the isotope ^{45}Ca for at least 30-60 min, so that the time of action of PAA on membrane permeability could not be precisely determined. By the method of measuring K^+ "outflow" from the cells it was possible to show that permeability of the cell membrane for K^+ is increased as early as during the first few minutes after addition of PAA or DS to the lymphocyte culture.

Increased membrane permeability caused by the action of PAA was recorded both for ^{45}Ca and for K^+. This evidently indicates absence of selectivity (as regards the nature and valency of the cation) in the modification of membrane permeability. Moreover, special experiments showed that the effect of PAA is not restricted to increased permeability for cations only. The rate of penetration of small molecules such as nucleotides into the cell also was shown to be increased.

The results of measurement of the cytoplasmic pool of labeled uridine and thymidine are given in Fig. 3. They clearly demonstrate an increase in the [^{3}H]nucleoside content under the influence of mitogenic doses of PAA. The effect of an increase in the δ-pool for [^{3}H]uridine could be recorded as early as 30-60 min after activation of the cells by PAA. Increased accumulation of [^{3}H]thymidine in the cytoplasm of the control, unactivated cells was found much later, not until 4-6 h after addition of PAA. In our view the differences between the times of recording increased accumulation of ^{45}Ca, [^{3}H]uridine, or [^{3}H]thymidine and the time of recording increased K^+ "leakage" are connected with differences in sensitivity of the recording methods used. Meanwhile it is perfectly possible that under the influence of the polyanionic mitogen the permeability of the cell membrane for K^+ and Ca^{++} cations increased rapidly (in the first minutes). This change is directly related to "triggering" of the cell response. On the other hand, the later (after 30-60 min) increase in nucleoside flows is unrelated to the initiation of the cell reaction, but is a sign of already activated cell metabolism.

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