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EFFECT OF ACTIVATION OF THE ADENYLATE CYCLASE SYSTEM ON Na^+/H^+-EXCHANGE IN HUMAN PLATELETS

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Substances potentiating activity of the adenylate cyclase system in platelets inhibit the cell response to the action of various agonists [6]. An increase in the intracellular cAMP concentration depresses both the agonist-induced rise in the cytoplasmic Ca^{++} level [1, 2] and hydrolysis of phosphatidylinositol, leading to the formation of secondary messengers: inositol-1,4,5-triphosphate and diacylglycerol [9]. An essential role in the mechanism of platelet activation has been shown to be played by transmembrane Na^+/H^+-exchange, which is involved in elevation of the intracellular Ca^{++} level, the circulation of phosphatidylinositol, and aggregation and secretion [1, 4, 11, 12].

What is the effect of the adenylate cyclase system on Na^+/H^+-exchange in platelets? To solve this problem, we conducted an investigation on human platelets, in which the state of Na^+/H^+-exchange was assessed (by reference to changes in intracellular pH) in response to the action of aggregation inducers, phorbol ester (PMA, an activator of protein kinase C), and ionophores of monovalent and bivalent cations, before and after administration of carbacyclin (a stable analog of prostacycline), which raises the cAMP level in the cell [2].

EXPERIMENTAL METHOD

Venous blood from healthy donors, taken in a ratio of 6:1 with an anticoagulant of the following composition (in mM): sodium citrate 93, citric acid 7.7, glucose 140 (pH 6.5) — was used in the experiments. Platelet-enriched plasma (PEP) was obtained by centrifugation of the blood for 15 min at 150g. Platelets were isolated by centrifuging the mixture of PEP and anticoagulants (1:1) for 10 min at 350g and resuspended in buffer solution containing 138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 0.2 U/ml of apyrase, 10 mM HEPES, and 0.2% bovine serum albumin, pH 6.5 (buffer A) up to a concentration of $(3\text{-}5)\cdot10^8$ cells/ml. The intracellular pH was measured by means of the fluorescent probe 2,7-bis-(carboxyethyl)-5,6-carboxyfluorescein (BCECF) by the method in [11]. For this purpose the pH-probe, in the form of the acetoxymethyl ester (BCECF/AM), was added to the platelet suspension up to a final concentration of 2.5-4 mM and incubated for 30 min at 37°C. The platelet suspension, diluted with anticoagulants (1:1), was recentrifuged for 10 min at 350g and resuspended in buffer A solution to a concentration of $(0.8\text{-}1.0)\cdot10^{10}$ cells/ml. Immediately before measurement, 10 µl of platelet suspension was added to 1 ml of buffer A solution (without albumin and apyrase, pH 7.4), or to 1 ml of a solution in which the NaCl was replaced by an equimolar concentration of choline chloride. Fluorescence was measured on a Hitachi 650-60 spectrofluorometer (Japan) at 22-24°C or 37°C. The wavelengths of excitation and emission

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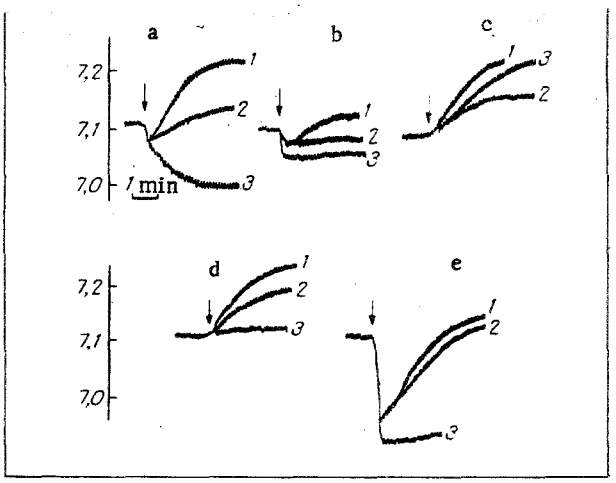

Fig. 1. Effect of carbacycline and sodium-free incubation medium on changes in intracellular pH (pH_i) in platelets under the influence of agonists. Ordinate, changes in pH_i. 1) Effect of agonist, 2) agonist + carbacyclin (20 mg/ml): 1 and 2) in incubation medium with 140 mM Na^+; 3) in incubation medium without Na^+. a) thrombin (0.1 unit/ml), b) PAF (1 μM), 3) A23187 (0.1 μM), d) phorbol ester (PMA, 2 nM), e) nigericin (20 μM).

were 500 ± 5 and 535 ± 5 nm respectively. The intracellular pH was determined without correction by the nigericin method [11]. Platelets were lysed with digitonin (10 mM) and their pH was recorded by means of a pX-250 pH-meter (USA) with an accuracy of 0.01 unit, using a LOT-405-M3 microelectrode (Ingold, West Germany). The following reagents were used: thrombin, ionophore A23187, 4-phorbol-12-myristate-13-acetate (PMA), nigericin, choline chloride, platelet activation factor (PAF), from the firm "MA," carbacycline, from Upjohn Company (Belgium), digitonin, from Merck, West Germany, BCECF/AM from "Molecular Probe" (USA), and the remaining reagents were of Soviet origin.

EXPERIMENTAL RESULTS

Transmembrane antiport of sodium ions and protons is effected by an exchanger, located in the plasma membrane of the cells [4]. In resting cells it does not function, and is "activated" only if the pH falls below physiological values (7.0-7.1). Under the influence of agonists inducing activation of protein kinases, the Na^+/H^+-exchanger is modified and its sensitivity is changed, as a result of which the antiporter begins to work at physiological values of pH. In the present investigation we studied changes in the intracellular pH caused by the action of both physiological (thrombin and PAF) and artificial (A23187 and the protein-kinase C-activator PMA). In addition, to assess the working of the Na^+/H^+-antiporter in the unstimulated platelets, artificial acidification of the cytoplasm was induced by nigericin. It will be clear from Fig. 1 that under the influence of activators on the platelets an increase in pH_i (curve 1) was observed. In the case of PAF and thrombin this was preceded by slight acidification of the cytoplasm. The fact will be noted that at room temperature PMA, unlike other activators, did not lead to a change in pH_i in the course of 5 min. Meanwhile at 37°C definite alkalification of the cytoplasm of the platelets was discovered (Fig. 1d). In resting cells rapid acidification with nigericin stimulated Na^+/H^+-exchange, as a result of which the pH returned to its basal level (Fig. 1e).

When Na^+ ions in the extracellular medium were replaced by the nonpenetrating organic cation choline, the stage of alkalification was eliminated in all cases (Fig. 1 curve 3). This confirms that the pH changes observed in the present investigation are the result of activity of the Na^+/H^+-exchanger, as data obtained by other workers also show [1, 12, 14]. The exception was ionophore A23187, which caused alkalification of the cytoplasm even in the absence of Na^+ ions in the external medium. This may perhaps be connected with $Ca^{++}/2H^+$-exchange, carried out by the ionophore [8]. A similar effect has been described as a result of the action of ionomycin on thymocytes [5].

Incubation of the platelet suspension with carbacyclin in a dose of 20 mg/ml for 2 min led to a fall of the pH-response to all agonists used (Fig. 1:2). Meanwhile carbacyclin did not affect restoration of pH in the platelets when depressed by nigericin.

The results of this investigation thus showed that elevation of the cAMP level in the cell reduces Na^+/H^+-exchange activity, stimulated by various activators, i.e., in cells in which the Na^+/H^+-exchanger was modified as a result of activation of biochemical processes. In resting cells the adenylate cyclase system does not affect unmodified Na^+/H^+-exchange.

There is evidence that an increase in activity of the adenylate cyclase system may uncouple the common paths of intracellular activation stimulating Na^+/H^+-exchange [6, 9] and (or) may directly inhibit phosphorylation of the Na^+/H^+-exchanger. For example, the influence of protein kinase C on functioning of the Na^+/H^+-antiporter has been established in thymocytes and lymphocytes. Further evidence in support of this conclusion also are given by the results of the present investigation, when an increase in pH was observed by PMA — a protein kinase C activator. Activation of this enzyme was known to take place as a result of the action of the endogenous secondary messenger diacylglycerol, formed on hydrolysis of phosphatidylinositol [9]. Since it has been shown that elevation of the cAMP level leads to uncoupling of the phosphoinositol cycle and to inhibition of phosphoinositol resynthesis [3, 15], this may also explain the reduction of Na^+/H^+-exchange, discovered in the present investigation, during activation of the adenylate cyclase system by carbacyclin. However, another sequence of events also is possible. For instance, a study of Na^+/H^+-exchange in the platelets showed [13, 14] that activation of phospholipase C by the action of weak inhibitors is preceded by the formation of arachidonic acid metabolites (prostaglandin endo- and hydroperoxides), released by phospholipase A_2. For the latter to work, preliminary initiation of Na^+/H^+-exchange is essential. In other words, according to this scheme Na^+/H^+-exchange is one of the first links in the chain of internal processes, whereas activation of protein kinase C by diacylglycerol is the last stage. Under these circumstances the authors cited above did not state how Na^+/H^+-exchange itself is initiated. Nevertheless, if this hypothesis is true, the inhibitory action of the adenylate cyclase system must evidently be explained by a direct reduction of the degree of modification (phosphorylation) of the Na^+/H^+-exchanger. An example of such an action has been described: an increase in the cAMP concentration has been shown to depress the degree of phosphorylation of the Na^+/H^+-exchanging protein in vesicles of the brush border of the kidneys [7].

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