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CORRELATION BETWEEN CHANGES IN Na^+/H^+-EXCHANGE AND CYTOPLASMIC Ca CONCENTRATION DURING PLATELET ACTIVATION

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UDC 612.111.7.014.46].06:612.015.31:
[546.33+546.41].08

KEY WORDS: platelet activation, agonists, Na^+/H^+-exchange.

Stimulation of platelets by various agonists (physiological and artificial) isknown to induce the passage of Na^+ ions into the cytoplasm in exchange for intracellular H^+ ions, an indication of activation of Na^+/H^+-exchange [3, 4, 11-13]. At the same time there is an increase in the concentration of intracellular free Ca (Ca_i^{++}), which plays a key role in the triggering of most intracellular processes [5]. The writers showed previously that, on the one hand, an increase in the Na^+ ion concentration in the platelets (for example, by the aid of monensin) leads to a rise of Ca_i^{++} level, but on the other hand, replacement of Na^+ by a nonpenetrating organic cation (choline) does not cause any significant changes in the Ca_i^{++} concentration in response to inducers such as PAF [3]. This fact, together with data in the literature [10], have led to the suggestion that correlation exists between Na^+/H^+-exchange and the Ca_i^{++} level during platelet activation.

This paper describes the further study of these relationships by analyzing data on changes in the intracellular pH (a marker of Na^+/H^+-exchange activity) and [Ca_i^{++}] during the action of agonists on platelets.

EXPERIMENTAL METHOD

Venous blood from healthy blood donors or from Wistar rats, anesthetized with ether, mixed in the ratio of 6:1 with an anticoagulant of the following composition: sodium citrate 93 mM, citric acid 7.7 mM, glucose 140 mM (pH 6.5) — was used in the experiments. Platelet-enriched plasma (PEP) was obtained by centrifuging the blood for 15 min at 100g. Platelets were isolated by centrifuging the mixture of anticoagulants and PEP (1:1) for 10 min at 350g and resuspension in buffer solution containing 138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 0.2% bovine serum albumin, 0.2 U/ml apyrase, and 10 mM HEPES, pH 6.5 (buffer A) up to a con-

Research Institute of Physicochemical Medicine, Ministry of Health of the RSFSR. Research Institute of Pediatrics, Academy of Medical Sciences of the USSR. A. V. Vishnevskii Institute of Surgery, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR M. Ya. Studenikin.) Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 106, No. 9, pp. 299-302, September, 1988. Original article submitted November 3, 1987.

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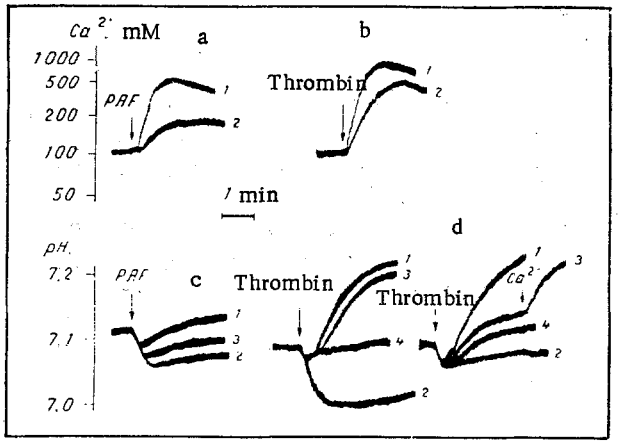

Fig. 1. Changes in intracellular Ca^{++} concentration in platelets (a, b) and intracellular pH (c, d) under the influence of 10 μM PAF and 0.1 U/ml thrombin. Measurements made at 22-24°C either in control medium, containing 138 mM Na^+ and 1 mM $CaCl_2$ (1) or in medium not containing Na^+ (2) or Ca^{++} (3) ions. 4) Measurements made in medium containing 1 mM Ca^{++} and 1 mM Ni^{++}. Typical results for human platelets shown in a-c (the character of the changes for rat platelets was similar). For pH changes under the influence of thrombin (d) results are given separately for human (left) and rat (right) platelets.

centration of $(3-5)\cdot10^8$ cells in 1 ml. Fluorescent probes quin-2/AM for determination of Ca_i^{++} and BCECF-2/AM to record pH_i were added to the platelet suspension up to a final concentration of 10 and 3-5 μM respectively, and incubated for 30 min at 37°C. The platelet suspension, diluted with anticoagulant (1:1) was then again centrifuged for 10 min at 350g and resuspended in buffer solution A up to a concentration of $(0.8-1.0)\cdot10^{10}$ cells/ml. Immediately before the measurements 10 μl of suspension was added to 1 ml of buffer solution A (without albumin and apyrase, pH 7.4), or to 1 ml of buffer solution in which the NaCl was replaced by an equimolar quantity of choline chloride. Experiments in medium not containing Ca^{++} ions were carried out either in buffer solutions made up in deionized water (Ca^{++} concentration not above 250 nM) or, in cases when no extra $CaCl_2$ was added, in the presence of 1 mM EGTA. Fluorescence was measured on a Hitachi 650-60 spectrofluorometer (Japan) at 22-23 or 37°C. The excitation and emission wavelengths were 339 and 490 nm for quin-2 and 500 and 535 nm respectively for BCECF, and the width of the slits was 5 nm. The intracellular Ca concentration was determined by the method in [14] and the intracellular pH by the method in [9]. The following reagents were used: thrombin, PAF, A23187, choline chloride, HEPES, apyrase, albumin, and phorbol ester (PMA) were all from Sigma (USA), the quin-2/AM was from Amersham International (England), digitonin from Merck (West Germany), BCECF-2/AM from "Molecular Probe," and the remaining reagents were of Soviet origin.

EXPERIMENTAL RESULTS

Changes observed in the Ca_i^{++} level and value of pH_i are given in Fig. 1. As was noted above, human and rat platelets were used in the experiments. In cases when the character and magnitude of the changes in platelets of both species coincided, common curves are shown, whereas if they did not coincide (for example, under the influence of thrombin) data for human and rat platelets were analyzed separately.

Changes in dependence of $[Ca_i^{++}]$ on the presence of Na^+ in the extracellular medium, induced by activators, are given in Fig. 1a, b. PAF (0.1 μM) caused a much smaller increase in the Ca_i^{++} level in the absence of Na^+ in the extracellular medium [3]. Under the influence of thrombin (0.1 U/ml) these differences were less marked but were still found, in agreement with data obtained by other workers [9, 15]. The results thus indicate that Na^+/H^+-exchange participates in the mechanisms in the increase in $[Ca_i^{++}]$, especially under the influence of weak aggregation inducers such as PAF.

Data on changes in pH_i under the influence of PAF and thrombin are given in Fig. 1c, d. After the first short phase of acidification, pH_i increased. The fact will be noted that thrombin induced a longer and more marked rise of pH_i (by 0.1-0.2 U), whereas in the case

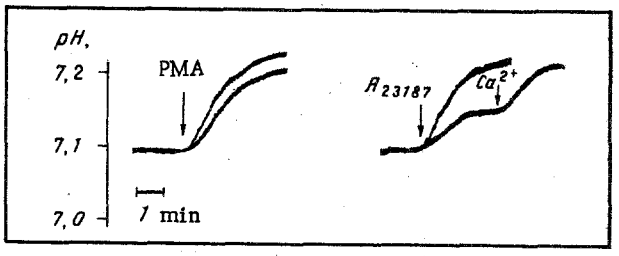

Fig. 2. Changes in pH_i induced in human platelets by 1 nM PMA (a) and 0.1 μM A23187 (b). Action of PMA illustrated at 37°C. Top curves were obtained in medium containing 1 mM Ca^{++}, bottom curves in calcium-free medium.

of PAF the value of pH_i did not significantly exceed the basal level. In individual experiments with PAF as activator, alkalification after the acidification stage led only to a slow return of pH_i to its basal level.

If Na^+ ions were replaced by choline chloride the activators induced more considerable acidification without any subsequent rise of pH_i (Fig. 1c, d, 2), evidence of the absence of Na^+/H^+-exchange under these conditions. Similar data, but only for activation by thrombin, were obtained in [15]. In medium not containing Ca^{++} less activity of Na^+/H^+-exchange was observed under the influence of PAF (Fig. 1a, b, 3). The data described above were similar for human and rat platelets. Differences were found only in the case of thrombin (0.1 U/ml). It will be clear from Fig. 1 that whereas changes in pH_i in human platelets were only weakly dependent on the presence of Ca^{++} in the external medium, for rat platelets they were much less in the absence of Ca^{++}, but were fully restored on the addition of Ca^{++} to the extracellular medium.

Comparison of the time course of the curves showing changes in Ca_i^{++} and pH_i in Fig. 1, reveals that the rise of the Ca_i^{++} level under the influence of the activators preceded the rise of pH_i. In other words, it can be postulated that changes in the Ca_i^{++} concentration somehow facilitate activation of Na^+/H^+-exchange. This is shown also by data [6, 7] obtained in experiments on thymocytes.

Considering the important role of Ca^{++} in regulation of the function of the Na^+/H^+-exchanger, it appeared essential to determine whether activation of Na^+/H^+-exchange is connected with the Ca_i^{++} level or (and) with the presence of this cation on the outer surface of the membrane. Accordingly experiments were set up in which Ni^{++} ions (1 mM) were added to the extracellular medium (incubation medium). This cation blocks the entry of Ca^{++} into the cell [8]. If the leading role in activation of Na^+/H^+-exchange is played by external Ca^{++} ions, inhibition of Ca channels by Ni^{++} ions ought not to have any significant effect on the pH_i changes. However, as Fig. 1 show, pH_i fell in medium containing 1 mM Ni^{++} and 1 mM Ca^{++} by almost the same degree as in medium not containing Ca^{++}. Consequently, to initiate Na^+/H^+-exchange, an intracellular rise of the Ca^{++} level is essential. In turn, Na^+/H^+-exchange stimulates the entry of Ca^{++} into the cell in full measure, probably on account of an increase in the intracellular Na^+ concentration [3]. In the case of human platelets, the weaker dependence of the increase in pH_i on the extracellular Ca^{++} concentration during activation by thrombin may perhaps be connected with the fact that thrombin releases more intracellular Ca^{++} than PAF [2]. The differences between the sensitivity of human and rat platelets in response to thrombin may probably be due to the stronger dependence of the Na^+/H^+-exchanger of rat platelets on Ca^{++}.

Analysis of the data also suggested that pathways of activation of Na^+/H^+-exchange that depend to different degrees on Ca^{++} exists. To test this hypothesis experiments were carried out with PMA, which activates protein kinase C [1] and increases pH_i [10]. It will be clear from Fig. 2 that PMA caused a rise of pH_i both in medium containing Ca^{++} and in calcium-free medium. Absence of dependence on Ca would be expected, for under the influence of PMA neither intracellular mobilization of Ca^{++} nor entry of Ca^{++} into platelets is observed [1]. Changes in pH_i under the influence of PMA, incidentally, were clearly observed only at 37°C. Since, however, thrombin and PAF induced changes in pH_i at 22°C also, the presence of different pathways of activation of Na^+/H^+-exchange independent of protein kinase C must be postulated under these conditions.

Different relationships between pH_i and Ca^{++} were observed during activation of platelets by A23187, which acts directly through elevation of the Ca^{++} level. As Fig. 2 shows, this ionophore increased pH_i only in the presence of extracellular Ca^{++}. Removal of the latter abolished the effect of the ionophore on pH_i, although mobilization of Ca^{++} continued under these circumstances. The effect of the ionophore on platelets differed in this respect from the action of thrombin, which, as was shown above, increases pH in calcium-free medium also (human platelets). Comparison of these data shows that the increase in pH_i produced by thrombin in calcium-free buffer is due to some Ca^{++}-independent mechanism. In this connection it is tempting to suggest that activation of Na^+/H^+-exchange through the action of thrombin (and also, perhaps, of other inducers) takes place directly through transmission of the signal from the receptor to this exchanger through regulatory proteins (N-proteins).

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