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Thrombin-induced changes of intracellular [Ca^{2+}] and pH in human platelets. Cytoplasmic alkalinization is not a prerequisite for calcium mobilization

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We have studied the effects of thrombin (0.1 U/ml) on intracellular Ca^{2+} ($[Ca^{2+}]_i$) and pH (pH_i) in human platelets loaded with fluorescent indicators. Thrombin produced a transient decrease of pH_i which reached its maximum within 15–25 seconds (s) and was followed by a sustained alkalinization which brought pH_i above the resting value. $[Ca^{2+}]_i$ increased transiently peaking at 5–10 s. The late alkalinization induced by thrombin was antagonized by ethylisopropylamiloride, an inhibitor of Na^+-H^+ exchange, and by sphingosine, an inhibitor of protein kinase C, with little effect on the $[Ca^{2+}]_i$ transient. The early acidification was not inhibited by these treatments. We conclude that the thrombin-induced changes of $[Ca^{2+}]_i$ and pH_i are mediated by different mechanisms. The late alkalinization is due to activation of Na^+/H^+ exchange mediated by protein kinase C and, contrarily to previous proposals (Siffert, W. and Akkerman, J.W.N. (1987) Nature 325, 456–458), it is not necessary for calcium mobilization from intracellular stores.

Phosphodiesterasic cleavage of phosphatidylinositol bisphosphate leading to the formation of inositol trisphosphate, which mobilizes calcium from intracellular stores, and 1,2-diacylglycerol, which activates protein kinase C, seems to be the earliest event in the stimulation of platelets by thrombin [1]. Removal of extracellular Na^+ and treatment with amiloride have been reported to inhibit several aspects of platelet activation induced by different agonists [2–4]. Since both manoeuvres prevent the operation of Na^+/H^+ exchanger, these results have been interpreted to indicate some involvement of this mechanism in the stimulus-response coupling. Thrombin has been documented to stimulate Na^+-H^+ exchange in human platelets [5,6]. On the basis of the recently reported inhibition of the thrombin-induced rise in cytoplasmic Ca^{2+} by either Na^+ removal or amiloride it has been postulated that cytoplasmic alkalinization mediated by Na^+/H^+ exchanger is a prerequisite for Ca^{2+} release from intracellular stores [7], although this proposal has been controversial [8,9]. The aim of this work is to study in some detail the changes of pH_i induced by thrombin and their relation to the changes of $[Ca^{2+}]_i$.

Utilization of the recently developed Ca^{2+} probe fura2 [10] made possible to follow changes of $[Ca^{2+}]_i$ avoiding the strong Ca^{2+} buffering effect observed in cells heavily loaded with the less sensitive indicator quin2 [11]. In parallel experiments changes of pH_i were followed in cells loaded

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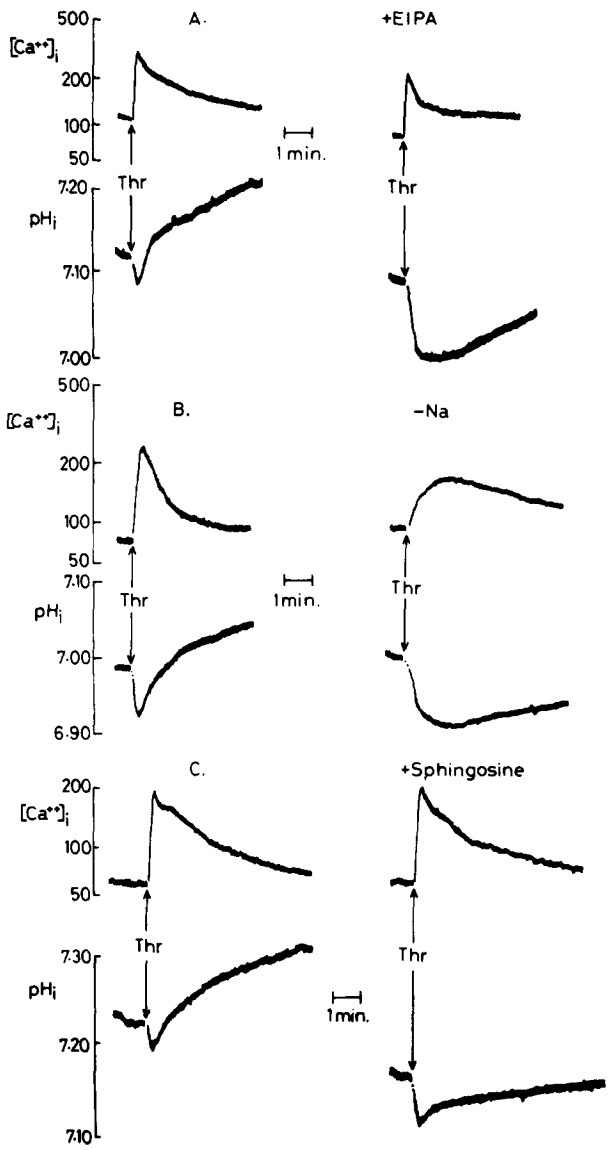

Fig. 1. Time course of changes of $[Ca^{2+}]_i$ (upper traces) and pH_i (lower traces) after addition of thrombin (0.1 U/ml). Effects ethylisopropylamiloride (EIPA, A), Na^+ removal (B) and sphingosine (C). Platelet-rich plasma was obtained from freshly drawn citrated blood and centrifuged for 20 min at $350 \times g$. Platelets were resuspended at $3 \cdot 10^8$ cells/ml in nominally Ca^{2+}-free standard medium of the following composition (in mM): NaCl, 145; KCl, 5; $MgSO_4$, 1; Na-Hepes, 10 (pH 7.4), glucose 10. This suspenssion was split in two halves and loaded with either fura2 or BCECF by incubation with the corresponding esters (Molecular Probes, Junction City, OR, U.S.A.) at 2 μM concentration for 45 min at 37 °C. Aspirin (100 μM) was added during the last 10 minutes of incubation in order to inactivate cyclo-oxygenase. The cell suspensions were then diluted with two volumes of standard medium containing albumin (1 mg/ml) and acid citrate dextrose solution (2%). The cells were sedimented by centrifugation and resuspended in fresh standard medium. Specific fluorescence was measured at 10^8 cells/ml and 37 °C under magnetic stirring. Calibration of the fura2 signal was performed as described before [21]. Calibration of the BCECF signal was performed in high-K medium containing 2 μM nigericin (a gift of Lilly Indiana de España) as described previously [22]. Thrombin (human, Ortho Diagnostic Systems Inc., New Jersey, U.S.A.) was added from a 10 U/ml stock solution in Na^+-free medium. EGTA (1 mM) was added always 1 min before thrombin addition. Concentrations of Ca^{2+} are given in nM on the scales. Traces on the left-hand side are controls, and those on the right experimentals carried out with the same cell batch. (A) EIPA (a generous gift of Drs. T. Friedrich and G. Burckhardt, Max-Planck-Institut, Hamburg, F.R.G.), 40 μM, was added from a 10 mM stock solution in ethanol. (B) The protocol was changed in these experiments in order to avoid deleterious effects of Na^+ removal. Loaded platelets were first suspended in medium containing 43 mM Na^+ at 10^{10} cells/ml and diluted 100-times in the cuvette with either standard (control) or Na^+ free medium (Na^+ replaced isoosmotically by *N*-methylglucamine, experimental). Thrombin was added 1 min after dilution. The real extracellular Na^+ concentration in the 'Na^+-free' suspension, measured by flame photometry, was 0.5 mM. (C) Sphingosine (free base, Sigma London, U.K.), 2 μM, was added from a 5 mM stock in dimethylsulfoxide 1 min before thrombin addition. Concentrations of dimethylsulfoxide above 0.05% inhibited significantly the thrombin-induced alkalinization.

with the well-known pH probe 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF) [12]. All the experiments were performed in Ca^{2+}-free media in order to evidence only the rises of $[Ca^{2+}]_i$ consecutive to the release of Ca^{2+} from the intracellular stores. Other experimental details are given with the figure legends.

Left-hand side records in Fig. 1 show the changes of $[Ca^{2+}]_i$ and pH_i observed in human platelets stimulated with thrombin (0.1 U/ml). Thrombin addition was followed by a sharp and transient rise of cytoplasmic $[Ca^{2+}]$. Parallel recording of pH_i showed a biphasic response with an early acidification step followed by a more sustained alkalinization of the cytoplasm that brought pH_i above the resting value. In 13 similar experiments the following values were obtained (mean $\pm$ S.E.): resting pH_i 7.16 ± 0.02; resting $[Ca^{2+}]_i$, 95 ± 9 nM. On addition of thrombin a peak $[Ca^{2+}]_i$ of 219 ± 20 nM was reached within 5–10 s. The early acidification amounted 0.055 ± 0.008 pH units and was completed within 15–25 s

and the late alkalinization brought pH_i 0.052 ± 0.009 units above the resting value within 5 min. The increase of $[Ca^{2+}]_i$ induced by thrombin was then faster than changes in pH_i. This shows that the rise in $[Ca^{2+}]_i$ may perhaps cause cell acidification, but cannot be the result of the alkalinization it precedes.

Treatment with ethylisopropylamiloride (EIPA), a powerful inhibitor of Na^+-H^+ exchange [13], antagonized the thrombin-induced alkalinization with little modification of the $[Ca^{2+}]_i$ peak (Fig. 1A). Treatment with 1 mM amiloride had the same effects on the pH_i shifts, but strong interferences with fura2 fluorescence prevented estimation of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ rise obtained after thrombin treatment tended to disappear within a few minutes after suspension of the platelets in low-Na medium (Na^+ replaced by either choline or *N*-methyl-D-glucamine, data not shown). However, when low Na^+ levels were obtained by fast dilution of a concentrated platelet suspension with Na^+-free medium, the addition of thrombin immediately after dilution still produced a substantial rise of $[Ca^{2+}]_i$, even though the late cytoplasmic alkalinization was almost fully abolished (Fig. 1B). The early and delayed effects of external Na^+ replacement may help to explain previous discrepances [7,14], although differences in the concentrations of thrombin used should also be considered. The slopes for the $[Ca^{2+}]_i$ rise and the early acidification of the cells became both smaller in low-Na medium, suggesting that acidification is a consequence of the increase of $[Ca^{2+}]_i$.

Ionomycin had the same qualitative effects as thrombin (Fig. 2). The $[Ca^{2+}]_i$ rise declined more slowly, as noted before [15], and again a biphasic change of pH_i was observed. The cytoplasmic alkalinization, however, was weaker than that observed after thrombin, the cell pH never exceeding the resting value. In nine similar experiments treatment with 100 nM ionomycin produced an early decrease of pH_i of (mean ± S.E.) 0.048 ± 0.006 units and the late alkalinization returned pH_i to only 0.018 ± 0.006 units below the resting value. Peak $[Ca^{2+}]_i$ during ionomycin treatment was (mean ± S.E.) 483 ± 42 nM, compared to 92 ± 3 nM before ionophore addition. It is important to note that the cell pH changes observed on ionomycin treatment cannot be attributed to H^+

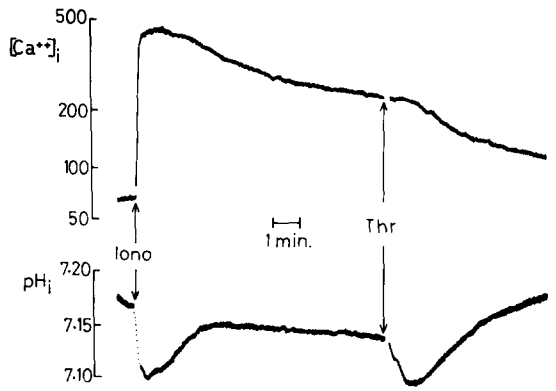

Fig. 2. Time course of the changes of $[Ca^{2+}]_i$ (upper traces) and pH_i (lower traces) induced by the addition of ionomycin (100 nM) to human platelets suspensions. Effect of the subsequent addition of 0.1 U/ml thrombin. EGTA (1 mM) was added 1 min before ionomycin (Calbiochem), which was added from a 0.1 mM stock solution in ethanol. Other details as in Fig. 1.

shifts mediated by the Ca^{2+} ionophore itself. The early acidification of the cytoplasm is the effect contrary to that expected for ionophore-mediated Ca^{2+}-H^+ exchange, and the alkalinization was abolished in Na^+-free medium (with little change in the $[Ca^{2+}]_i$ transient) or by the presence of 1 mM amiloride (data not shown).

A possible reason for the weaker alkalinization observed with ionophore versus that observed with thrombin could be that the thrombin effect on Na^+-H^+ exchange included mechanisms other than the increase of cell $[Ca^{2+}]$, the only effect one would expect with the ionophore. In other cell systems Na^+/H^+ exchanger is activated by protein kinase C [16], and thrombin is known to activate this enzyme in platelets [17,18]. Fig. 2 shows that thrombin reinforces the final cytoplasmic alkalinization in ionomycin-treated platelets after a lag period in which the pH_i drops. This was the only instance in which cell acidification did not match with an increase in cytoplasmic $[Ca^{2+}]$. In order to test more directly the mediation of protein kinase C in the thrombin-induced stimulation of Na^+-H^+ exchange we studied the effects of sphingosine, a powerful and specific inhibitor of this enzyme [19]. Fig. 1C shows that sphingosine inhibited the cytoplasmic alkalinization induced by thrombin without affecting the $[Ca^{2+}]_i$ rise.

Our results indicate that thrombin-induced stimulation of Na^+-H^+ exchange is mediated by activation of protein kinase C. The involvement of this enzyme on activation of Na^+-H^+ exchange has been proposed in many cells [16], including platelets [4], on the basis of the effects of phorbol esters. In some cell preparations the increase of cell $[Ca^{2+}]$ has also been reported to activate Na^+-H^+ exchange, although this behaviour is not general [20]. The results obtained with ionomycin suggest that the effect of intracellular Ca^{2+} on Na^+-H^+ exchange is rather poor, and that thrombin is able to stimulate the exchanger without increasing $[Ca^{2+}]_i$ (Fig. 2).

The thrombin-induced cytoplasmic alkalinization is preceded by an early acidification which is also obtained with ionomycin. Although its mechanism is not clear at present, similar observations have been reported earlier in platelets [6] and cytoplasmic acidification on increase of $[Ca^{2+}]_i$ has been documented in other cells (see Table I in Ref. 20). What is pertinent now is that the temporal coincidence of cellular acidification with Ca^{2+} release from the intracellular stores is inconsistent with the recent proposal that Na^+/H^+ exchanger-mediated alkalinization is essential for calcium mobilization [7]. On the other hand, we show here that calcium mobilization and cytoplasmic alkalinization can be dissociated under appropriate conditions (EIPA or sphingosine treatments, Fig. 1A and C). On the basis of the evidence available at present we find it difficult to decide whether Na^+-H^+ exchange-mediated cytoplasmic pH changes are a central mechanism in platelet activation or only the expression of homeostatic side-compensations.

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