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Effects of sodium removal on calcium mobilization and dense granule secretion induced by thrombin in human platelets

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Removal of extracellular sodium decreased calcium mobilization from intracellular stores induced by thrombin in aspirin-treated human platelets. ATP and serotonin secretion were also significantly reduced. Secretion was positively correlated with calcium mobilization, but the presence or absence of sodium did not modify the slope of the regression line. Half-maximal secretion was reached when $[Ca^{2+}]_i$ was increased by about 0.1 μM. Calcium mobilization induced by the divalent cation ionophore ionomycin was not modified by sodium removal. Secretion induced by ionomycin was much smaller than the thrombin-induced one for the same increases of $[Ca^{2+}]_i$. These results suggest that the presence of external sodium is required for normal thrombin-induced calcium release from the intracellular stores and hence for dense granule secretion. However, secretion cannot be only attributed to the increase of cell $[Ca^{2+}]_i$ but also to other process(es) which are not affected by external sodium.

Removal of extracellular sodium interferes with aggregation and secretion induced by several agonists in human platelets [1]. Much of this effect can be attributed to actions on the cyclooxygenase pathway [1,2], responsible for 'secondary' activation. A more direct link has been proposed recently by postulating that a Na^+/H^+ exchange-mediated cell alkalinization is necessary for both (i) calcium mobilization from intracellular stores and (ii) calcium influx from the extracellular medium [3–5]. The facilitating effect of cell alkalinization on calcium influx has been confirmed and extended by recent evidence [6], whereas several papers argue against an essential role of Na^+/H^+ exchange in calcium mobilization [7–10].

In the present paper we have studied the effects of Na^+ removal on calcium mobilization from the intracellular stores and secretion from dense granules (ATP and serotonin) induced by thrombin in human platelets. Experiments were performed in aspirin-treated cells in order to eliminate the cyclooxygenase pathway. Calcium and pH measurements were performed using the fluorescent dyes fura-2 [11] and 2',7'-bis(2-carboxyethyl)-5 (and -6) carboxyfluorescein (BCECF) [12], respectively. All the experiments were performed in Ca^{2+}-free medium (containing 1 mM EGTA) in order to evidence only rises of $[Ca^{2+}]_i$ consecutive to the release of calcium from the intracellular stores. Other experimental details are given with the figure legends.

Fig. 1 shows the effects of Na^+ removal on the calcium mobilization induced by several concentrations of either thrombin (A) or the divalent cation ionophore ionomycin (B). As reported before [13], the increase of $[Ca^{2+}]_i$ induced by thrombin was fast and transient, whereas the one induced by ionomycin was more sustained. This suggests that the ionophore acts on additional calcium stores (the secretion granules), which release their calcium content very slowly, probably because most of the calcium in this compartment is bound and dissociates slowly. Neither the extent of the peak nor the slope of the clearance was modified by sodium removal in ionophore-treated cells (Figs. 1B and 1D). On the contrary, the shape of the $[Ca^{2+}]_i$ transient induced by thrombin was deeply modified by sodium removal. The maximum $[Ca^{2+}]_i$ reached at the peak was decreased, the peak itself was delayed, and the clearance of calcium was slowed down resulting in a $[Ca^{2+}]_i$ plateau lasting for 1–2 minutes after stimulation (Figs. 1A and 1C). The effects were more marked at the smaller concentrations of thrombin.

Siffert and Akkerman have postulated that cytoplasmic alkalinization mediated by Na^+/H^+ exchanger

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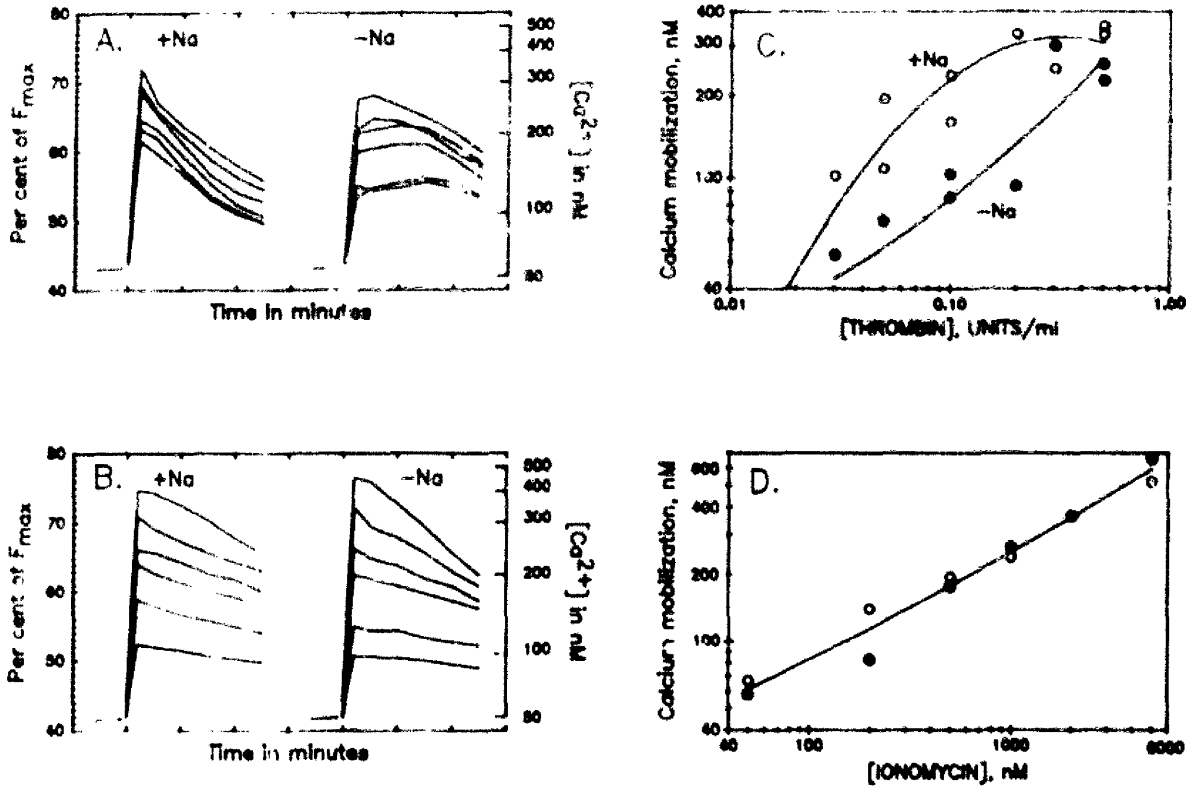

Fig. 1. Effects of sodium removal on changes of $[Ca^{2+}]_i$ induced by thrombin (upper panels) and ionomycin (lower panels) in human platelets. Platelet-rich plasma was obtained from freshly drawn citrated blood and centrifuged for 20 min at 350 × g. Platelets were resuspended at $3 \cdot 10^8$ cells/ml in nominally Ca^{2+}-free medium of the following composition (in mM): NaCl, 145; KCl, 5; $MgSO_4$, 1; sodium-Hepes, 10 (pH 7.4); glucose, 10. Cells were loaded with fura-2 by incubation with the acetoxymethyl ester (Molecular Probes, Eugene, OR, U.S.A.) at 2 μM concentration for 45 min at 37°C. Aspirin was added during the last 10 min of incubation in order to inactivate cyclooxygenase. The cell suspension was then diluted with two volumes of standard medium containing albumin (1 mg/ml) and acid-citrate-dextrose solution (2%), sedimented by centrifugation and resuspended in standard medium at $5 \cdot 10^8$ cells/ml. At the beginning of each experiment the cells were diluted with 50 volumes of either standard Na^+-containing medium or Na^+-free medium (sodium substituted equimolecularly by *N*-methylglucamine) in a fluorescence spectrophotometer cuvette at 37°C under magnetic stirring. After about 1 min EGTA (1 mM) was added, and 30 s later the additions of thrombin (human, Ortho Diagnostic Systems, New Jersey, U.S.A.; final concentrations: 0.03, 0.05, 0.1, 0.2, 0.3 and 0.5 U/ml) or ionomycin (Calbiochem-Behring, La Jolla, CA, U.S.A.; final concentrations: 0.05, 0.2, 0.5, 1, 2 and 5 μM) were made from concentrated stocks at $t = 0$. Measurements of specific fluorescence and calibration of the fura-2 signal were performed as described before [8,15]. Panels on the left (A and B) show data from the crude records digitalized at −0.8, 0, 0.2, 0.5, 1, 1.5, 2 and 2.5 minutes. Panels on the right (C and D) shows plots of the calcium mobilization (as increase of $[Ca^{2+}]_i$ at the peak) versus the concentrations of thrombin or ionomycin. Open symbols, with Na^+; closed symbols, without Na^+.

is required for thrombin-induced release of calcium from intracellular stores [3–5]. This would explain why calcium mobilization is reduced by sodium removal. However, Fig. 2 shows that this could not be the case since: (i) the calcium peak coincides in time with an intracellular acidification, and (ii) inhibition of Na^+/H^+ exchanger with ethylisopropylamiloride (EIPA) had little effect on the $[Ca^{2+}]_i$ peak. The efficiency of this inhibitor was demonstrated in the same experiment by the blocking of the intracellular alkalinization which follows, with some delay, thrombin activation (Fig. 2). Other evidence against the role of Na^+/H^+ exchange on calcium mobilization from intracellular stores has been published previously [7–10]. Thrombin has been reported to accelerate $[Ca^{2+}]_i$ clearance in cells treated with Ca^{2+} ionophores [13]. The fact that the clearance of $[Ca^{2+}]_i$ is also slowed down by Na^+ removal could suggest that a more general step of thrombin-platelet interaction is affected by external sodium. In any case we must conclude that the interference of Na^+ removal with thrombin-induced calcium mobilization from intracellular stores cannot be attributed to inhibition of the Na^+/H^+ exchanger. Note that the experiments presented here were not addressed to evidence effects on calcium influx from the extracellular medium, which has also been reported to increase upon cellular alkalinization [3,6].

Thrombin-induced dense granule secretion was also impaired by Na^+ removal. ATP secretion induced by low doses of thrombin (0.03–0.3 U/ml) was reduced by an average of 25%. This effect was statistically significant ($p < 0.02$; paired *t*-test; seven pairs of values). ATP secretion at 0.5 U/ml thrombin was not significantly modified. In similar experiments [^{14}C]serotonin

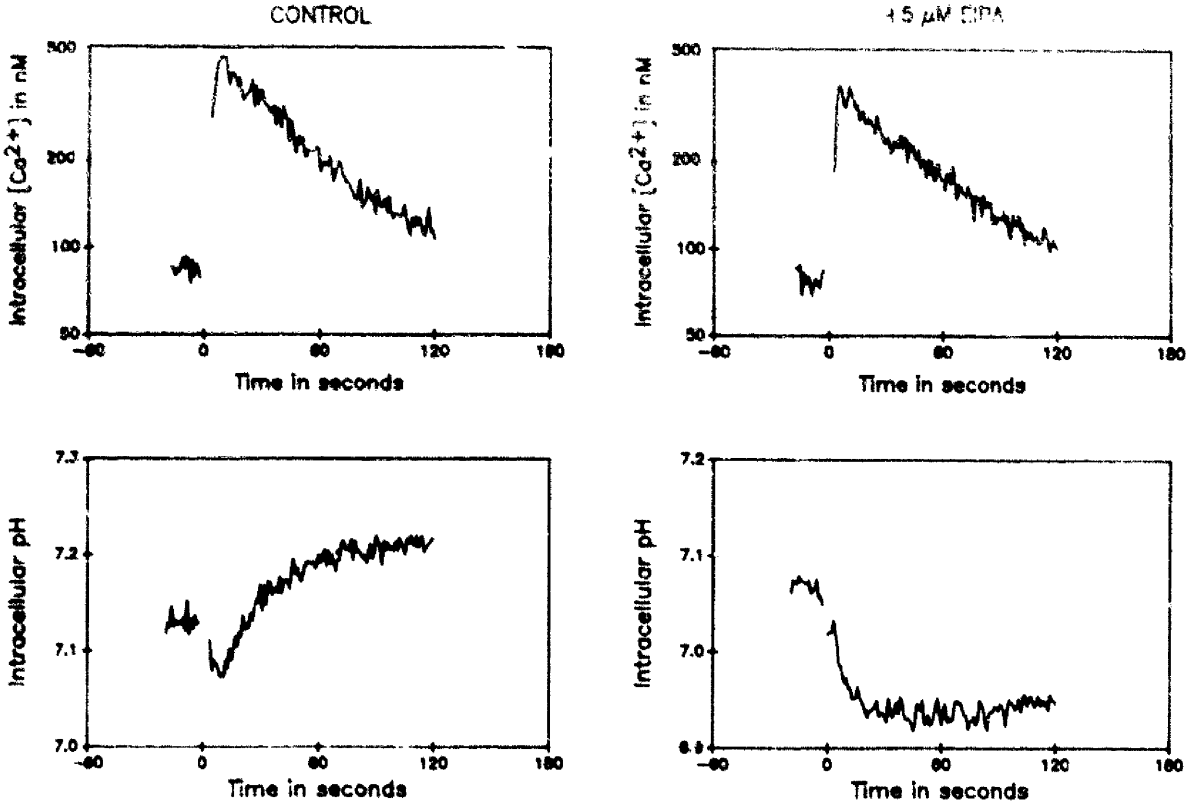

Fig. 2. Effects of ethylisopropylamiloride (EIPA) on the changes of $[Ca^{2+}]_i$ any pH_i induced by thrombin (0.2 U/ml) in human platelets. Platelets were loaded simultaneously with fura-2 and BCECF by incubation with the corresponding esters (Molecular Probes, Eugene, OR, U.S.A.) at concentrations of 2 and 0.25 μM, respectively, for 45 min at 37°C. Fluorescence measurements were performed at alternating excitations (32 Hz) of 340, 380, 430, 450 and 500 nm and fixed emission at 530 nm using a rotating filter wheel (Cairn Research, Newnham, Sittingbourne, U.K.). The first two excitation wavelengths were used for Ca^{2+} measurements and the last three for pH measurements. Signals were calibrated as described before [8,15,16]. EIPA (a generous gift of Drs. T. Friedrich and G. Burkhardt, Max-Planck-Institut, Frankfurt, F.R.G.) was added 1 min before thrombin at 5 μM final concentration in the right-hand side experiments. This concentration produced more than 90% of maximal inhibition of the pH_i changes. Other details as in Fig. 1.

secretion was decreased by 10% by sodium removal ($p < 0.01$; paired t-test; seven pairs of values). The impairment of secretion could be explained directly by the decrease of calcium mobilization. Fig. 3 shows a plot of secretion (ATP or serotonin) versus calcium mobilization in cells treated with several concentrations of either thrombin or ionomycin in Na^+-containing and Na^+-free medium. There was a positive correlation between secretion and calcium mobilization, but the slopes of the regression lines obtained in Na^+-containing and Na^+-free media were not significantly different. These results suggest that the secretion process itself is not impaired by sodium removal.

Fig. 3 also shows that secretion induced by both thrombin and ionomycin is related to the increase of $[Ca^{2+}]_i$. However, the Ca^{2+} sensitivity is very different in both cases. While thrombin-induced secretion is half-maximal when $[Ca^{2+}]_i$ increases by about 0.1 μM, an increase of $[Ca^{2+}]_i$ of 0.7 μM induced by ionomycin produced less than 30% secretion. These results are consistent with previous observations in permeabilized

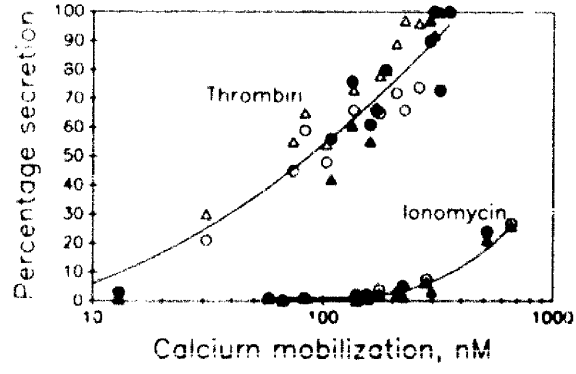

Fig. 3. Dependence of the secretion of ATP (circles) and serotonin (triangles) on calcium mobilization. Data obtained with thrombin (0.03–0.5 U/ml) and with ionomycin (0.05–5 μM) in either Na^+-containing (filled symbols) or Na^+-free (open symbols) medium are shown. Secretion of ATP [17] or serotonin [18] was measured 2 min after the addition of the agonist. Secretion is expressed as percentage of the value obtained with the highest thrombin concentration in Na^+-containing medium. Other details as in Fig. 1.

platelets [14] and support the idea that thrombin increases the sensitivity to Ca^{2+} of the secretory mechanism.

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