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Role of Na^+/H^+ exchange in thrombin- and arachidonic acid-induced Ca^{2+} influx in platelets

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Platelet activation is accompanied by an increase of cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, (due to both extracellular Ca^{2+} influx and Ca^{2+} movements from the dense tubular system) and an Na^+ influx associated with H^+ extrusion. The latter event is attributable to the activation of Na^+/H^+ exchange, which requires Na^+ in the extracellular medium and is inhibited by amiloride and its analogs. The present study was carried out to determine whether a link exists between Ca^{2+} transients (measured by the quin2 method and the $^{45}CaCl_2$ technique) and Na^+/H^+ exchange activation (studied with the pH-sensitive intracellular probe, 6-carboxyfluorescein) during platelet stimulation. Washed human platelets, stimulated with thrombin and arachidonic acid, showed: (1) a large and rapid $[Ca^{2+}]_i$ rise, mostly due to a Ca^{2+} influx through the plasma membrane; (2) a marked intracellular alkalinization. Both phenomena were markedly inhibited in the absence of extracellular Na^+ or in the presence of an amiloride analog (EIPA). Monensin, a cation exchanger which elicits Na^+ influx and alkalinization, and NH_4Cl, which induces alkalinization only, were able to evoke an increase in $[Ca^{2+}]_i$, mostly as an influx from the extracellular medium. Our results suggest that Ca^{2+} influx induced by thrombin and arachidonic acid in human platelets is strictly dependent on Na^+/H^+-exchange activation.

Introduction

Most known excitatory platelet agonists cause an elevation in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) [1–3]. The respective contributions of the release of sequestered intracellular Ca^{2+} and the influx of extracellular Ca^{2+} have not been precisely determined, and may vary with the different membrane stimuli. As regards the discharge from intracellular stores, by acting on a cell-surface receptor coupled with the activation of phospholipase C, most agonists (including thrombin and arachidonic acid) elicit the hydrolysis of phosphatidylinositol 4,5-bisphosphate to give inositol 1,4,5-trisphosphate and diacylglycerol [4–7]: inositol 1,4,5-trisphosphate induces the rapid release of Ca^{2+} from the dense tubular membrane system triggering the secretion of the contents of granules [8,9]. As regards the Ca^{2+} influx from the extracellular medium, the evidence available rules

Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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out the presence of voltage-gated calcium channels in platelet membranes [2,3,10]. Ligand-operated channels have been proposed, but the mechanisms by which agonists induce Ca^{2+} influx are still unknown. Platelet activation is accompanied by increased Na^+ influx [11,12], and removal of extracellular Na^+ has been reported to decrease platelet aggregation and serotonin release [13,14]. Previous studies have suggested the presence of a Na^+/H^+ exchanger in platelets, in the light of the inhibition by amiloride of the early phase of H^+ ejection caused by thrombin or A23187 [15,16], the Na^+-dependent cellular swelling caused by influx of a permeant acid [17], and thrombin-induced alkalinization [18].

Measurements of stimulus-evoked changes in cytoplasmic pH with intracellular fluorescent probes, and the use of much more potent and selective amiloride analogs [14,19,20] have confirmed the presence of a Na^+/H^+ exchange in platelet plasma membrane. The present studies were designed to determine whether changes in platelet cytoplasmic pH induced by thrombin, arachidonic acid and monensin are correlated with Ca^{2+} influx from extracellular medium.

Materials and Methods

Reagents. Thrombin (human), arachidonic acid, prostaglandin I_2, nigericin and monensin were from Sigma. Quin2 and quin2 tetraacetoxymethyl ester were obtained from Amersham. Ionomycin and 6-carboxyfluorescein diacetate were from Calbiochem-Behring. $^{45}CaCl_2$ (2 mCi/ml, 93 μg/ml) was from New England Nuclear. EGTA was from Fluka, top purity grade. 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) was a generous gift from Dr. T. Friedrich (Max-Planck-Institut für Biophysik, Frankfurt, F.R.G.). Other reagents were Analar grade or of the highest purity available. Arachidonic acid was dissolved in nitrogen-bubbled absolute ethanol and stored at $-20\,^\circ$C as 50 mM stock solution: dilutions in isotonic saline (pH 7.4) were freshly prepared before each experiment. 6-Carboxyfluorescein diacetate, quin2 acetoxymethyl ester and ionomycin were prepared in dimethylsulfoxide and stored at $-20\,^\circ$C. Stock solutions of nigericin and monensin were prepared in ethanol. Control and experimental samples received the same volume addition of solvent, and the final solvent concentration never exceeded 0.1%. The Na^+ solution contained 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Hepes, 10 mM glucose (pH 7.4 at 37 °C). Choline chloride and *N*-methyl-D-glucamine$^+$ solutions were prepared by partial or total isoosmotic replacement of NaCl by choline chloride and the chloride salt of *N*-methyl-D-glucamine$^+$, respectively, but were otherwise identical.

Measurements of $[Ca^{2+}]_i$ and ^{45}Ca influx. Platelets were prepared as described [1] from fresh human blood anticoagulated with 0.15 volumes of ACD (85 mM trisodium citrate, 111 mM dextrose, 71 mM citric acid). Blood donors had not received any medication in the previous 4 weeks. Platelet-rich plasma was obtained by centrifugation at $200 \times g$ for 15 min at room temperature. The platelets were loaded with quin2 by incubating the platelet-rich plasma at 37 °C for 30 min with 15 μM quin2 acetoxymethyl ester [1]. The platelet-rich plasma was then adjusted to pH 6.8 with ACD and centrifuged at $800 \times g$ for 12 min at room temperature. The pellet was gently resuspended in Na^+ solution, or in solutions where Na^+ had been differently replaced. The platelet suspension was adjusted to a density of approx. $2 \cdot 10^8$ cells/ml and kept at room temperature in a plastic tube until use. The measurements were carried out within 30 min after final resuspension, as platelet responsiveness was constant during that period. 1 mM $CaCl_2$ was added and the cells were equilibrated at 37 °C for about 3 min before agonists. When required, the cells were kept at 37 °C for about 3 min in Ca^{2+}-free Na^+ solution and 1 mM EGTA was added 30 s before the agonist. Measurement of $[Ca^{2+}]_i$ from the fluorescence of intracellular quin2 was performed as described [1] in a Perkin-Elmer LS-5 spectrofluorimeter (Perkin-Elmer Corp., Norwalk, CT) at 37 °C; quin2 release into the medium under various incubation conditions was negligible. Quin2-loaded washed platelets showed a normal aggregation pattern. For ^{45}Ca influx studies, platelet suspensions ($2 \cdot 10^8$ cells/ml) in different solutions were incubated with 1 mM $CaCl_2$ for 30 min at 37 °C, then $^{45}CaCl_2$ was added (10 μCi/ml of platelets). After an additional 20 min (during which two control samples were removed), agonist was added and

0.02-ml aliquots of the platelet suspension were removed at intervals over 180–240 s. Each aliquot was diluted in 1 ml cold wash buffer containing 2 mM $CaCl_2$ and filtered through 0.22 μM Millipore filters (Millipore Corp., Bedford, MA). These were then rinsed with at least two additional 5-ml aliquots of Ca^{2+}-containing cold wash buffer to remove nonincorporated tracer. This process took approx. 1 min. Platelet-associated $^{45}Ca^{2+}$ retained by the filters was measured by scintillation counting, using Instagel solution (Amersham Corp., U.K.).

Cytoplasmic pH determination. Platelets were pelleted from platelet-rich plasma after addition of 1 μM prostaglandin I_2 by centrifugation at $800 \times g$ for 12 min at room temperature. The pellet was resuspended in Na^+ solution (pH 7.4) ($2 \cdot 10^8$ cells/ml) and incubated with 15 μM 6-carboxyfluorescein diacetate for 15 min at 20 °C. The permeable diacetate is hydrolyzed by cellular esterases and the resulting impermeable 6-carboxyfluorescein is trapped in the cytoplasm. When excited at 490 nm, the intensity of its emission at 520 nm is a function of pH. After loading, platelets were pelleted again and washed once in prostaglandin I_2-containing Na^+ solution, followed by resuspension ($2 \cdot 10^9$ cells/ml) in the same medium without prostaglandin I_2. The 6-carboxyfluorescein-loaded platelets were fully responsive to thrombin (0.01–0.1 U/ml) and arachidonic acid (1–10 μM), as tested in the aggregometer, and were stored at 37 °C until used. 20 μl cell suspension was employed for fluorescence determinations in 2 ml of the indicated medium (final cell count: $2 \cdot 10^7$ cells/ml), using a Perkin-Elmer LS-5 spectrofluorimeter, with excitation at 490 nm and emission at 520 nm, 5 nm slit width at 37 °C. The nigericin/K^+ method of Thomas et al. [21] was used for calibration of cytoplasmic pH. Leakage of dye during the time of measurement was negligible: it was estimated by comparing the fluorescence of digitonin-lysed cells with that of the supernatant after removal of the cells by centrifugation.

Statistics. The data are presented as means ± S.D. of the number of determinations indicated (n).

Results

$[Ca^{2+}]_i$ measurements in thrombin- and arachidonic acid-stimulated platelets

Washed human platelets, preloaded with quin2 and resuspended in Na^+ solution containing 1 mM Ca^{2+}, were exposed to 0.1 U/ml thrombin and 10 μM arachidonic acid (Table I, A). Both agonists evoked a large increase in $[Ca^{2+}]_i$ within a few seconds. In the presence of 1 mM extracellular calcium, $[Ca^{2+}]_i$ rose from the basal level of 0.115 ± 0.008 μM to 1.012 ± 0.237 μM ($n = 6$) with thrombin, and from 0.113 ± 0.007 μM to 0.653 ± 0.107 μM ($n = 9$) with arachidonic acid. This increase was transient: it reached its maximum within about 20–30 s and then fell within a few minutes to below 0.300 μM, and often to baseline-like values. In Ca^{2+}-free medium with 1

TABLE I

EFFECT OF THROMBIN AND ARACHIDONIC ACID ON $[Ca^{2+}]_i$ IN PLATELETS SUSPENDED IN VARIOUS MEDIA

Quin2-loaded washed human platelets ($2 \cdot 10^8$ cells/ml) were stimulated with 0.1 U/ml thrombin and 10 μM arachidonic acid, after resuspension in the following media (pH 7.4 at 37 °C) (mM): A = 145 NaCl, 5 KCl, 1 $MgSO_4$, 10 Hepes, 10 glucose, 1 $CaCl_2$ (Na^+ solution); B = Na^+ solution with 1 EGTA instead of 1 $CaCl_2$; C = 30 NaCl, 115 choline chloride, 5 KCl, 1 $MgSO_4$, 10 Hepes, 10 glucose, 1 $CaCl_2$; D = 145 choline chloride, 5 KCl, 1 $MgSO_4$, 10 Hepes, 10 glucose, 1 $CaCl_2$. Values are means of the indicated number of experiments (n) ± S.D.

Medium	Thrombin			Arachidonic acid		
	basal $[Ca^{2+}]_i$ level (μM)	$[Ca^{2+}]_i$ maximal response (μM)	n	basal $[Ca^{2+}]_i$ level (μM)	$[Ca^{2+}]_i$ maximal response (μM)	n
A	0.115 ± 0.008	1.012 ± 0.237	6	0.113 ± 0.007	0.653 ± 0.107	9
B	0.100 ± 0.011	0.324 ± 0.070	6	0.090 ± 0.010	0.180 ± 0.017	9
C	0.112 ± 0.007	0.542 ± 0.100	6	0.115 ± 0.013	0.385 ± 0.070	9
D	0.120 ± 0.011	0.374 ± 0.052	6	0.123 ± 0.017	0.231 ± 0.035	9

mM EGTA, the $[Ca^{2+}]_i$ increase was much smaller (Table I, B) both with thrombin (25% of control value) and with arachidonic acid (17%). When washed platelets were resuspended in a solution containing 30 mM Na^+ and 115 mM choline chloride, in the presence of 1 mM extracellular Ca^{2+}, a 50% inhibition of $[Ca^{2+}]_i$ rise was observed for both agonists (Table I, C). When Na^+ was completely and isoosmotically substituted by choline chloride (145 mM), a much higher $[Ca^{2+}]_i$ transient inhibition was observed (Table I, D).

Cytoplasmic pH measurements in thrombin- and arachidonic acid-stimulated platelets

In five experiments, the cytoplasmic pH of 6-carboxyfluorescein-loaded platelets suspended in Na^+ solution at 37 °C averaged 7.22 ± 0.02. Cytoplasmic pH was then measured in 6-carboxyfluorescein-loaded platelets stimulated by thrombin and arachidonic acid (Fig. 1). Cells suspended in Na^+-containing medium underwent a transient cytoplasmic acidification, followed by a sustained alkalinization (about + 0.2 pH units). The acidification phase became more pronounced and sustained (about − 0.30 pH units) when the Na^+/H^+ exchange was impaired by removal of extracellular Na^+ (choline chloride substitution) or by addition of EIPA: the alkalinization response was inhibited in both cases.

Cytoplasmic pH and $[Ca^{2+}]_i$ measurements in monensin- and NH_4Cl-treated platelets

Countertransport of external Na^+ for internal H^+ can also be obtained by addition of the exogenous cation exchanger monensin. When added to 6-carboxyfluorescein-loaded platelets suspended in Na^+ solution, monensin induced a dose-dependent alkalinization (0.19 ± 0.04 increase of pH units ($n = 8$) with 5 μM monensin, 0.31 ± 0.06 increase of pH units ($n = 8$) with 10 μM monensin), which reached a maximum after 1–2 s (Fig. 2, left panel). When added to quin2-loaded platelets suspended in Na^+ solution, monensin was found to elicit a dose-dependent increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$: 0.180 ± 0.040 μM ($n = 9$) with 5 μM monensin, 0.420 ± 0.06 μM ($n = 9$) with 10 μM monensin), which reached a maximum after 20–30 s. Interestingly enough, $[Ca^{2+}]_i$ level did not fall to baseline-like values, but remained near to maximum for a prolonged period. Monensin did not increase $[Ca^{2+}]_i$ when choline chloride replaced Na^+ (not shown). The monensin-induced $[Ca^{2+}]_i$ rise was largely (85%) eliminated when external Ca^{2+} was removed (Ca^{2+}-free medium plus 1 mM EGTA). Monensin-dependent Na^+/H^+ exchange activation results in increased intracellular Na^+ concentration and cytoplasmic alkalinization. In order to understand which of the two events was the likely trigger of extracellular Ca^{2+} influx, 20

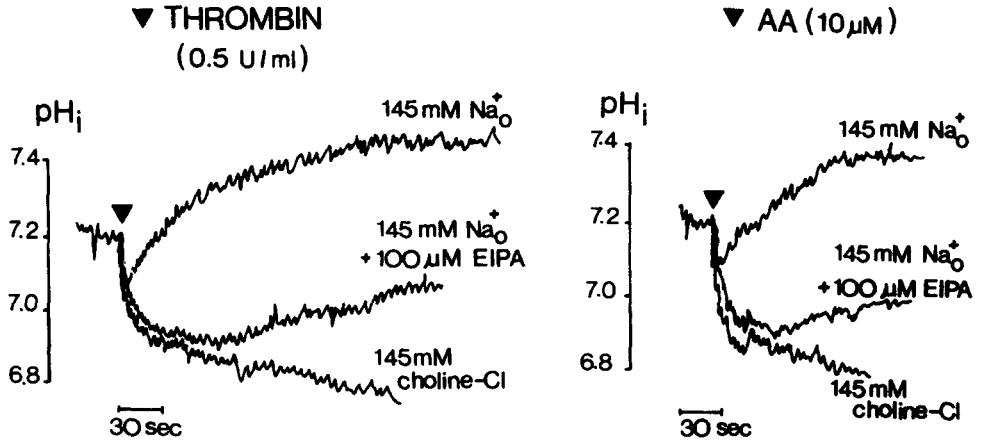

Fig. 1. Effect of thrombin and arachidonic acid on platelet cytoplasmic pH (pH_i). 6-Carboxyfluorescein-loaded human platelets ($2 \cdot 10^7$ cells/ml) were stimulated with 0.5 U/ml thrombin and 10 μM arachidonic acid (AA) under different conditions. 145 mM Na_o^+, Na^+ solution (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Hepes, 10 mM glucose, 1 mM $CaCl_2$, pH 7.4 at 37 °C); 100 μM EIPA, added 3 min before stimulation; 145 mM choline-Cl, choline chloride solution prepared by total isoosmotic replacement of NaCl by choline chloride. The experiment shown is representative of four performed with similar results.

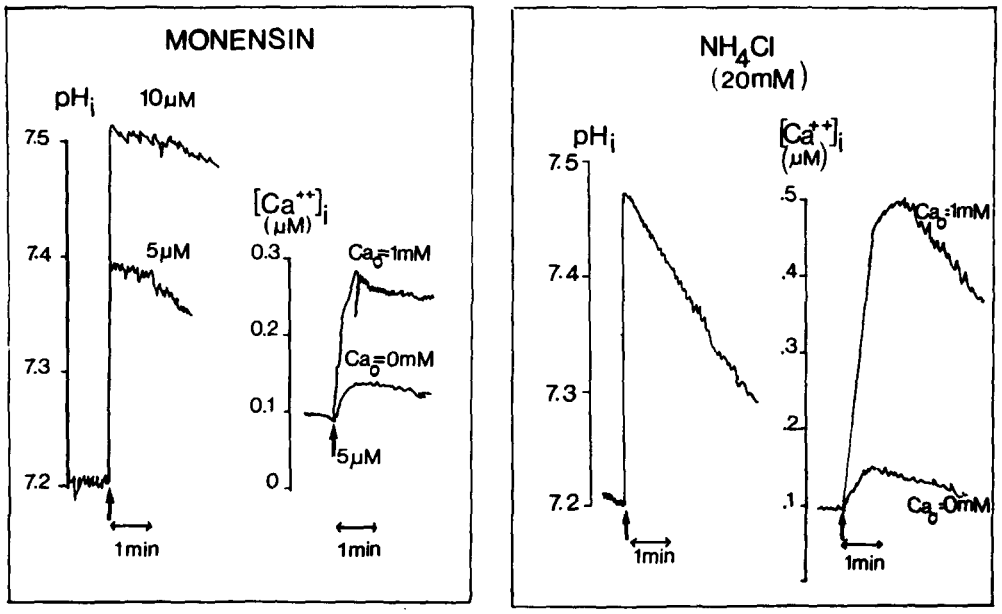

Fig. 2. Effect of monensin and NH_4Cl on platelet cytosolic pH (pH_i) and $[Ca^{2+}]_i$. 5 and 10 μM monensin (left panel) and 20 mM NH_4Cl (right panel) were added to human platelets ($2 \cdot 10^8$ cells/ml) loaded with 6-carboxyfluorescein (for pH_i measurements) and quin2 (for $[Ca^{2+}]_i$ measurements) (representative tracings; only 5 μM monensin-evoked $[Ca^{2+}]_i$ transient is shown). $[Ca^{2+}]_i$ was measured in the presence (1 mM $CaCl_2$) and in the absence (0 mM $CaCl_2$, 1 mM EGTA) of extracellular Ca^{2+} (Ca_0). The $[Ca^{2+}]_i$ is plotted on a linear scale. Extracellular medium: Na^+ solution (see legend to Fig. 1).

mM NH_4Cl (pH 7.4) was used to elevate intracellular pH independently of Na^+ translocation (Fig. 2, right panel). NH_4^+, which is in equilibrium with the permeating weak base NH_3 [22], elicited a very rapid alkalinization in 6-carboxyfluorescein-loaded platelets suspended in Na^+ solution (increase of 0.273 ± 0.07 pH units; $n = 8$). When added to quin2-loaded platelets suspended in Ca^{2+}-containing Na^+ solution, NH_4^+ evoked a $[Ca^{2+}]_i$ transient ($\Delta[Ca^{2+}]_i$: 0.400 ± 0.076 μM; $n = 8$), which was decreased by 88% in Ca^{2+}-free medium plus 1 mM EGTA.

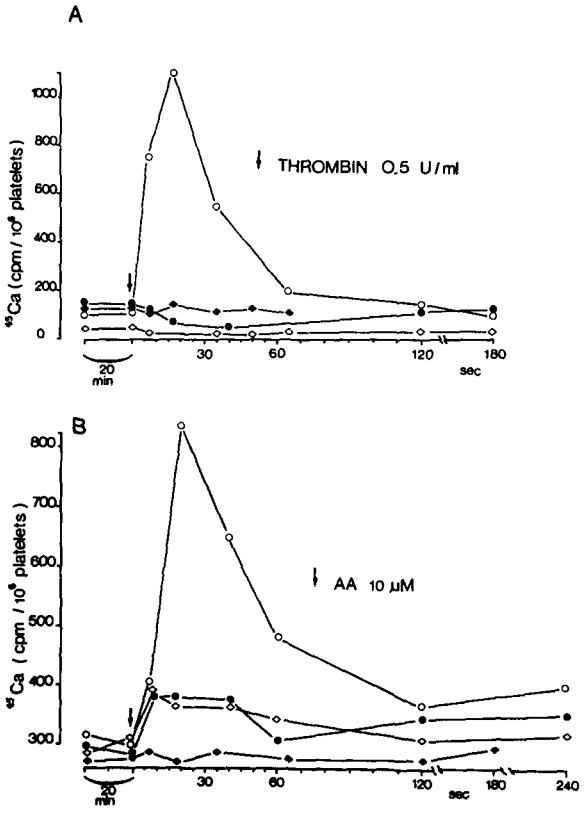

Fig. 3. Time course of $^{45}Ca^{2+}$ influx after platelet stimulation with thrombin and arachidonic acid. Human platelets ($2 \cdot 10^8$ cells/ml) were stimulated (arrow) with 0.5 U/ml thrombin (panel A) and 10 μM arachidonic acid (AA) (panel B). Extracellular medium: (○— — —○) Na^+-solution (see legend to Fig. 1); (●— — —●) choline chloride and (◇— — —◇) *N*-methyl-D-glucamine solutions prepared by total isoosmotic replacement of NaCl by choline chloride and *N*-methyl-D-glucamine chloride; (◆— — —◆) Na^+ solution + 100 μM EIPA (3 min preincubation). The experiment shown is representative of four experiments performed with similar results.

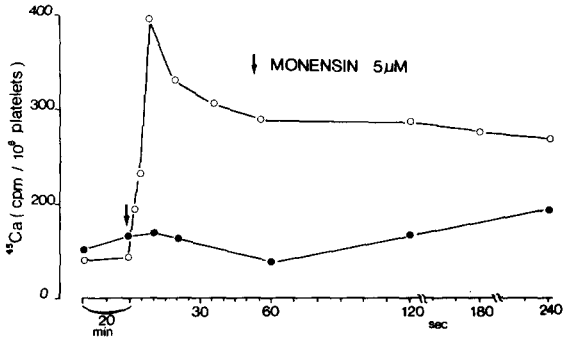

Fig. 4. Time course of $^{45}Ca^{2+}$ influx into human platelets ($2 \cdot 10^8$ cells/ml) after addition of 5 μM monensin. Extracellular medium: (○— — —○) Na^+ solution (see legend to Fig. 1); (●— — —●) *N*-methyl-D-glucamine solution prepared by total isoosmotic replacement of NaCl by *N*-methyl-D-glucamine chloride. The experiment shown is representative of four experiments performed with similar results.

^{45}Ca studies

In order to overcome the interference caused by EIPA fluorescence on quin2 measurements, the time course of Ca^{2+} influx into platelets was measured by observing $^{45}Ca^{2+}$ uptake after thrombin and arachidonic acid stimulation in platelets pre-equilibrated with $^{45}Ca^{2+}$ at 37 °C (Fig. 3). Platelet-associated $^{45}Ca^{2+}$ peaked within 5–25 s after stimulation by both agonists, and fell to baseline values within 120 s. The peak was almost completely abolished when Na^+ was totally replaced by choline and *N*-methyl-D-glucamine, and when Na^+/H^+ exchange was inhibited by addition of EIPA. 5 μM monensin evoked a rapid (5 s peak) and prolonged $^{45}Ca^{2+}$ influx that was eliminated in *N*-methyl-D-glucamine medium (Fig. 4).

Discussion

The presence of the Na^+/H^+ exchanger in platelets and its role in regulating cytoplasmic pH after thrombin stimulation have recently been confirmed by direct measurements of stimulus-evoked changes in cytoplasmic pH under conditions in which Na^+/H^+ exchange was impaired by Na^+-free media, or by the amiloride analog EIPA, which shows a potency 140-fold higher than that of amiloride [23] and a better selectivity [14,19,20,24]. Na^+/H^+ exchange activation has already been described for the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate, synthetic diacylglycerol 1-oleoyl-2-acetylglycerol, growth factors and a variety of extracellular signals on different cell types (for reviews see Refs. 25, 26): most of these agonists (as well as thrombin and arachidonic acid in platelets) act through the phospholipase C-mediated hydrolysis of inositol phospholipids and the formation of endogenous diacylglycerol, which activates protein kinase C. Activation of Na^+/H^+ exchange in platelets by 12-*O*-tetradecanoylphorbol 13-acetate and 1-oleoyl-2-acetylglycerol has recently been described [27] through measurement of stimulus-induced, amiloride-sensitive extracellular H^+ release.

Our results show that thrombin (in agreement with Siffert and Akkerman) [14] and arachidonic acid, two agonists known to activate phospholipase C, elicit a sustained alkalinization in human platelets (Fig. 1): the involvement of Na^+/H^+ exchanger in this response is stressed by the dramatic acidification observed when exchange is impaired by substituting choline chloride for Na^+, or by addition of the specific inhibitor EIPA. The cytoplasmic acidification of stimulated platelets is probably due to the early hexose monophosphate shunt activation observed after addition of thrombin [28] and arachidonic acid [29].

In washed human platelets, thrombin and arachidonic acid produce a rise in $[Ca^{2+}]_i$ in both the presence and the absence of external Ca^{2+} (Table I). The much larger increase in the presence of external Ca^{2+} indicates a substantial Ca^{2+} influx across the plasma membrane, additional to the release from internal stores observed in its absence: this conclusion is supported by Mn^{2+} influx studies in quin2-loaded cells [3]. While it is demonstrated that inositol 1,4,5-trisphosphate generated at the plasma membrane after thrombin and/or arachidonic acid stimulation diffuses to dense tubular membrane system to trigger Ca^{2+} release [8,9], so far no evidence exists to link inositol 1,4,5-trisphosphate (and other cyclic or more phosphorylated intermediates) to receptor-mediated Ca^{2+} influx in platelets. The evidence available does not support the presence of voltage-gated Ca^{2+} channels in platelet membranes: (a) organic calcium channel blockers are ineffective in blocking $[Ca^{2+}]_i$ rises evoked by ADP and thrombin in quin2-loaded platelets, (b)

depolarization of the membrane by gramicidin or K^+-rich solutions does not increase $[Ca^{2+}]_i$ [2,3,10]. Our experiments show that thrombin- and arachidonic acid-induced Ca^{2+} influx in human platelets closely depends on the presence of Na^+ in the extracellular medium: in quin2-loaded platelets, the influx is inhibited by 50% when Na^+ is reduced to 30 mM and almost completely eliminated when Na^+ is totally replaced by choline chloride (Table I). These results are confirmed by $^{45}Ca^{2+}$ experiments, in which only $^{45}Ca^{2+}$ influx from extracellular medium is measured (Fig. 3). The influx elicited by thrombin and arachidonic acid in the presence of 145 mM extracellular Na^+ is completely blocked when Na^+ is totally replaced by choline chloride and *N*-methyl-D-glucamine; moreover, EIPA, the specific inhibitor of Na^+/H^+ exchange, abolishes the $^{45}Ca^{2+}$ peak in both thrombin- and arachidonic acid-stimulated platelets. .This strongly suggests that, under the conditions of our experiments, Na^+/H^+ exchange activation by thrombin and arachidonic acid is a prerequisite for Ca^{2+} influx in human platelets. We cannot exclude the possibility that other mechanisms may be involved when higher concentrations of the agonists (or other activators) are used.

To acquire further evidence that Na^+/H^+ exchange activation may be the trigger of Ca^{2+} influx, monensin was used to induce countertransport of external Na^+ for internal H^+ and cytoplasmic alkalinization in quin2- and 6-carboxyfluorescein-loaded platelets. Monensin is known to induce a $[Ca^{2+}]_i$ transient in rat lymphocytes [30], in a transformed murine pre-B lymphocyte cell line [31] and in sea urchin sperm cells [32]. Moreover, it induces thromboxane B_2 formation in platelets [33] and augments platelet aggregation elicited by norepinephrine [34] and thrombin [14], as would be expected if the cytosolic free Ca^{2+} concentration were increased to a level closer to the threshold for aggregation. Our experiments, in fact, show (Fig. 2) that monensin elicits a dose-dependent increase in $[Ca^{2+}]_i$, that is well correlated with a dose-dependent alkalinization: the major component of monensin-induced $[Ca^{2+}]_i$ rise is a Ca^{2+} influx from extracellular medium. The results obtained with monensin in the absence of extracellular Ca^{2+} rule out the possibility that the increase in $[Ca^{2+}]_i$ observed in the presence of 1 mM Ca^{2+} merely reflect pH-dependent changes in the affinity of the indicator; moreover it has already been demonstrated [30] that quin2 fluorescence increases by no more than 2% when cytoplasmic pH increases from 7.0 to 7.5. Monensin evokes a rapid $^{45}Ca^{2+}$ influx, which is abolished in *N*-methyl-D-glucamine medium (Fig. 4). Both monensin-elicited Ca^{2+} transients (i.e., in quin2- and in $^{45}Ca^{2+}$-loaded platelets) are long lasting, as would be expected in the presence of a ionophore-induced Na^+/H^+ countertransport. When NH_4Cl is used in quin2-loaded platelets to elevate intracellular pH independently of Na^+ translocation, a rapid $[Ca^{2+}]_i$ transient is observed. This is 88% decreased in Ca^{2+}-free medium. So far, Ca^{2+} influx is linked to cytoplasmic alkalinization and not to the concomitant Na^+ influx.

When taken as a whole, our results suggest that: (a) the strong cytoplasmic acidification elicited by thrombin and arachidonic acid, which may result in protonation of an allosteric regulatory or 'modifier site' [25,26,35], could initially activate Na^+/H^+ exchanger; (b) H^+ extrusion, which tends to restore the initial cytoplasmic pH, and the resulting alkalinization (which is totally equivalent to about 0.45–0.5 pH units) are the likely triggering mechanisms for Ca^{2+} influx from extracellular medium. Our data are in agreement with recent reports [14,36] demonstrating that activation of Na^+/H^+ exchange is a prerequisite for thrombin-induced Ca^{2+} mobilization in human platelets. Moreover, they fit recent observations [37,38] showing that epinephrine and ADP cause mobilization of a small pool of arachidonic acid by a pathway involving Na^+/H^+ exchange; both agonists stimulate a phosphatidylinositol-hydrolyzing Ca^{2+}-dependent phospholipase A_2 activity and alkalinization of platelet cytoplasm secondary to increased Na^+/H^+ exchange plays an important role in the activation of this enzyme.

Acknowledgments

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