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Rat platelets are deficient in internal Ca^{2+} release and require influx of extracellular Ca^{2+} for activation

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Calcium fluxes were studied in fura-2-labeled rat platelets. Thrombin, ADP and ionomycin induced rapid mobilization of internally stored Ca^{2+}, which resulted in only a moderate increase of cytosolic $[Ca^{2+}]_i$. Thrombin and ADP stimulated influx of extracellular Ca^{2+}, which was monitored as uptake of $^{45}Ca^{2+}$ and of Mn^{2+}. With either agonist, the influx of Ca^{2+} magnified the initial increase of $[Ca^{2+}]_i$. Since responses of rat platelets were dependent on external $[Ca^{2+}]$, we conclude that Ca^{2+} influx complements the mobilization of internal stores to reach sufficiently high $[Ca^{2+}]_i$ for full activation. A regulatory effect of protein kinase C modulators was observed on both agonist-induced elevation of $[Ca^{2+}]_i$ and receptor-mediated Ca^{2+} entry.

ADP; Calcium channel; Fura-2; Platelet; Thrombin; (Rat)

1. INTRODUCTION

Elevation of cytosolic $[Ca^{2+}]_i$ is a requirement for the activation of blood platelets and can be effected in 2 different ways [1,2]. Stimulation of phospholipase C generates inositol 1,4,5-trisphosphate ($InsP_3$), which induces the mobilization of Ca^{2+} from intracellular stores. In addition, extracellular Ca^{2+} can enter the cytosol via receptor-mediated gatings in the plasma membrane [3–5]. The nature of the latter calcium channels is virtually unknown, but several papers point to a diversity in the Ca^{2+} entry mechanism, proposing that thrombin and ADP each stimulate the opening of a different subtype of calcium channels [2,5,6]. However, all studies so far have been carried out with human platelets, in which quantification of the contribution of Ca^{2+} entry to $[Ca^{2+}]_i$ was hindered by a relatively large mobilization of internal Ca^{2+}.

It is known that rat platelets require external Ca^{2+} for full activation [7]. Here, we present evidence that platelets from these animals have relatively small Ca^{2+} stores. Our results suggest that, with thrombin and ADP as agonist, additional entry of Ca^{2+} is necessary to reach sufficiently high $[Ca^{2+}]_i$ to allow platelet activation. The high contribution of receptor-mediated Ca^{2+} entry in rat platelets makes these cells attractive for studying calcium channels. Additionally, we collected evidence in favour of a coordinated inhibitory effect of protein kinase C on agonist-induced elevation of $[Ca^{2+}]_i$, by inhibiting Ca^{2+} influx and by stimulating Ca^{2+} efflux.

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2. EXPERIMENTAL

Platelets isolated from Wistar rats [8] were washed and suspended in buffer (pH 7.4), containing 136 mM NaCl, 5.6 mM glucose, 5 mM HEPES, 2.7 mM KCl, 2 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 5% (w/v) bovine serum albumin, 2.5 μg/ml apyrase and 2 μM prostaglandin E_1. Platelets ($1 \cdot 10^9$/ml) were loaded with fura-2/AM (1 μM) in the presence of pluronic F-127 (0.5 mg/ml) (both from Molecular Probes) under slow rotation at 18°C for 45 min. These conditions prevented sequestration of dye into extra-cytosolic compartments. After spinning down, the platelets were resuspended in modified buffer, pH 7.4, where apyrase and prostaglandin were omitted and albumin was reduced to 0.05% (w/v). Human platelets [9] were treated similarly. Activations were carried out with stirring at 100 rpm (37°C). Data given are representative of 3 or more experiments.

Fluorescence was measured with the equipment described previously [9]. Emission wavelength was 500 nm and the excitation was switched continuously between 340 and 380 nm. Fluorescence data were collected for 2 s and processed by a personal computer. Calibration of $[Ca^{2+}]_i$ [10] was by the addition of 0.1% (w/v) Triton X-100 in the presence of 1 mM $CaCl_2$ or 10 mM EGTA, 50 mM Tris (pH 8.3). Fluorescence signals were corrected for leakage of dye by the addition of 2 mM $NiCl_2$ or according to [3]. Influx of Mn^{2+} was detected as the quenching of fluorescence of cytosolic fura-2 by externally added Mn^{2+} [4], and was measured at a fixed excitation wavelength of 360 nm.

Influx of $^{45}Ca^{2+}$ was measured with washed platelets ($2 \cdot 10^8$/ml), suspended in low albumin buffer. To the platelet suspension, $^{45}CaCl_2$ (0.5 mM, 11 kBq/nmol) was added 1 min before activation. Samples (1 ml), taken just before and 2 min after the addition of agonist, were allowed to equilibrate with 20 mM EGTA for 1 min, filtered through a Whatman GF/C filter, and rinsed 3 times with 6 ml of buffer (pH 7.4) containing 2 mM EGTA. The filters were counted for radioactivity.

3. RESULTS AND DISCUSSION

The aggregation response of washed rat platelets (Fig. 1) and of whole rat blood (data not shown) appeared strongly dependent on the presence of external Ca^{2+}. With thrombin as activator, at least 20 μM extracellular Ca^{2+} was required to induce aggregation and secretion (Fig. 1). The weak agonist ADP required similar Ca^{2+} concentrations for aggregation, but did not induce secretion. This is strikingly different from the situation in human platelets, in which thrombin-induced secretion is not notably influenced by external Ca^{2+} [5,11].

Rat platelets were rather difficult to load with fura-2, since the dye tended to sequestrate in extracytosolic compartments (data not shown). When loaded adequately (see section 2), platelet stimulation with ADP (20 μM) or thrombin (2 nM) in the presence of EGTA resulted in a rapid, but slight and transient, increase of $[Ca^{2+}]_i$ (Fig. 2A), raising from a resting level of 50 ± 9 nM to 169 ± 11 and 161 ± 6 nM, respectively ($\pm$SEM, $n = 8$). With human platelets, using the same experimental settings, these agonists increased $[Ca^{2+}]_i$ to 194 ± 25 and 424 ± 59 nM ($\pm$SEM, $n = 4$), respectively. Under similar conditions, Pollock et al. [3] have measured in thrombin-activated human platelets even higher (up to 600 nM) $[Ca^{2+}]_i$, probably because of their more rapid recording of fura-2 fluorescence.

We estimated the size of internal Ca^{2+} pools in rat platelets by activation with ionomycin in the presence of EGTA, in which case all internally stored Ca^{2+} becomes translocated to the cytosol [2,3]. Ionomycin (1.5 μM) raised $[Ca^{2+}]_i$ to only 219 ± 15 nM ($\pm$SEM, $n = 5$) (Fig. 2B), which is much lower than the micromolar level reached in human platelets [3]. From these observations, we conclude that in rat platelets intracellular Ca^{2+} stores are relatively small and, therefore, $InsP_3$-induced discharge of Ca^{2+} from these stores is limited.

It might be possible that the internal Ca^{2+} pool in rat platelets had been artificially lowered during isolation or dye-loading. To check this possibility, we tried to increase the pool by: (i) preincubation of the platelets with external $CaCl_2$ or (ii) preactivation with ADP in the presence of $CaCl_2$. However, when such pretreated platelets subsequently were activated with thrombin or ionomycin in the presence of excess EGTA, the maximal $[Ca^{2+}]_i$ was increased by no more than 0–15%, compared to untreated platelets (data not shown).

In the presence of extracellular Ca^{2+} (1 mM), ADP (20 μM) and thrombin (2 nM) induced a rapid and high elevation of $[Ca^{2+}]_i$ (Fig. 3A), amounting to 619 ± 52 and 1572 ± 122 nM ($\pm$SEM, $n = 8$), respectively. The level of $[Ca^{2+}]_i$ reached depended on extracellular $[Ca^{2+}]$ (data not shown). The high Ca^{2+} response is likely to be due to Ca^{2+} influx, because of the minor internal mobilization (see above). Indeed, receptor-

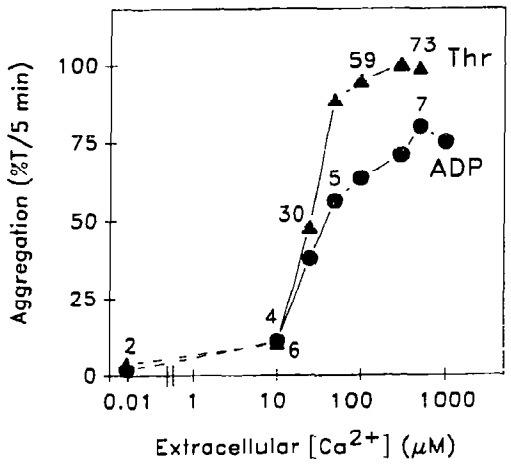

Fig. 1. Effect of extracellular Ca^{2+} on aggregation and secretion of rat platelets, activated by ADP (20 μM) plus fibrinogen (1 mg/ml) or thrombin (Thr, 2 nM). Numbers represent percentages of [^{14}C]serotonin, secreted after 5 min of activation.

mediated influx of external Ca^{2+} was measured by 2 methods. Uptake of $^{45}Ca^{2+}$ into rat platelets was stimulated not only by thrombin, in agreement with the data of Blache et al. [7], but also by ADP (Table I). The uptake with either agonist was inhibited by Ni^{2+}, a putative calcium channel blocker [4,7], and by Mn^{2+}.

An alternative way of monitoring Ca^{2+} influx is by following the quenching of fura-2 fluorescence by externally added Mn^{2+}, which is thought to enter through receptor-mediated calcium channels [4,6]. In the rat platelets, both thrombin and ADP stimulated influx of Mn^{2+} (Fig. 4), and Ni^{2+} was inhibitory (data not shown). Interestingly, both types of measurements revealed a non-zero basal entry rate in the absence of agonist (Table I and Fig. 4), pointing to sizeable calcium fluxes over the plasma membrane in resting, apparently non-activated, platelets. Taking together the

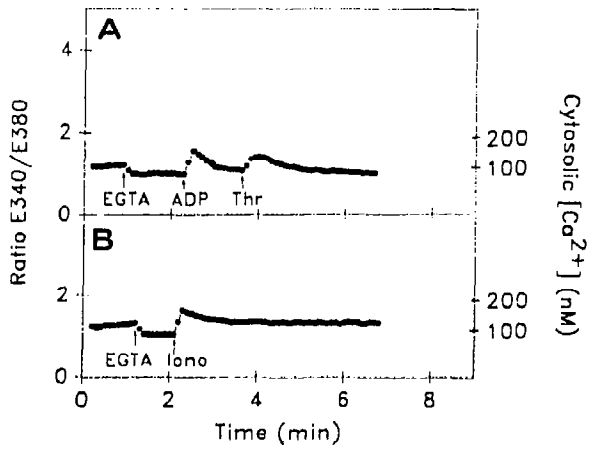

Fig. 2. Ratios of fura-2 fluorescence in rat platelets in the presence of EGTA (1 mM). Platelets were activated with ADP (20 μM), thrombin (Thr, 2 nM) and ionomycin (Iono, 1.5 μM), as indicated.

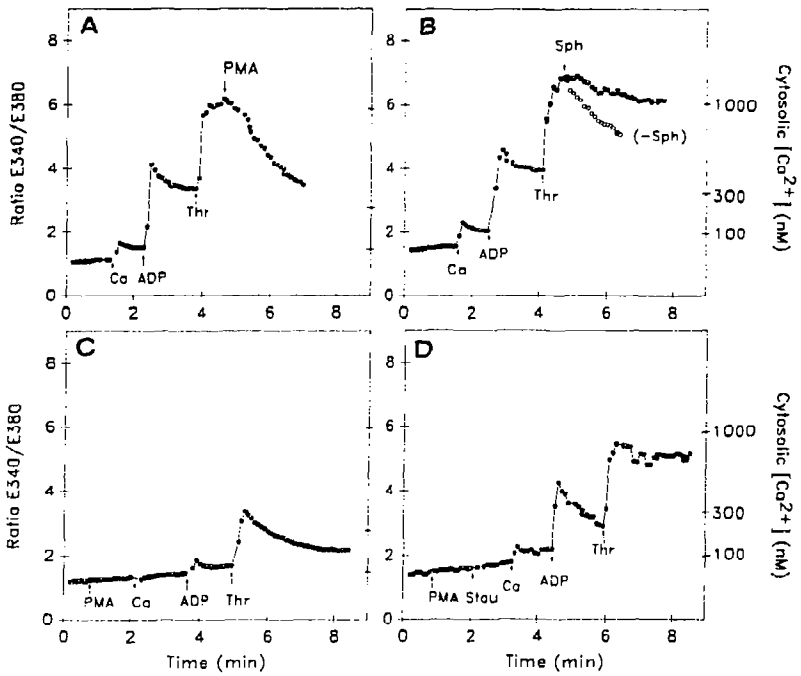

Fig. 3. Ratios of fura-2 fluorescence in rat platelets. Given were $CaCl_2$ (1 mM), ADP (20 μM), thrombin (Thr, 2 nM), PMA (100 nM), sphingosine (Sph, 10 μM) and staurosporine (Stau, 500 nM), as indicated.

requirement of extracellular Ca^{2+} for platelet responses and the high contribution of Ca^{2+} influx to elevation of $[Ca^{2+}]_i$, receptor-mediated Ca^{2+} entry seems to be crucial for activation of rat platelets.

In human platelets, it has been shown that the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) stimulates Ca^{2+} efflux out of the platelets [3,12]. In rat platelets, protein kinase C apparently modulated Ca^{2+} efflux in a similar way. This was concluded from (i) the PMA-induced enhancement of decrease in $[Ca^{2+}]_i$, following the initial thrombin-mediated elevation (Fig. 3A), and (ii) the contrary effect here of kinase C inhibitor sphingosine (Fig. 3B).

Pretreatment of rat platelets with PMA gave a substantial attenuation of initial ADP- and thrombin-induced increase of $[Ca^{2+}]_i$ (Fig. 3C). This effect of PMA was reversed by the potent protein kinase C inhibitor [13] staurosporine (Fig. 3D). PMA not only influenced Ca^{2+} extrusion (as concluded above) but also Ca^{2+} influx, as was inferred from its inhibition of ADP- and thrombin-dependent Mn^{2+} entry (Fig. 4), also being reversed by staurosporine (data not shown). Apparently, activation of protein kinase C suppresses (agonist-induced) elevation of $[Ca^{2+}]_i$ in an well-organized way, by increasing Ca^{2+} efflux and by reducing Ca^{2+} influx. Evidence for a similar control of $[Ca^{2+}]_i$ by protein kinase C has been found in human neutrophils [14], so that this may be a more wide-spread phenomenon.

Table I

Influx of $^{45}Ca^{2+}$ in rat platelets

	Influx of $^{45}Ca^{2+}$ (nmol $Ca^{2+}/10^9$ platelets/2 min)		
Pre-incubation	Control	ADP	Thrombin
$^{45}Ca^{2+}$	0.18 ± 0.02	0.54 ± 0.05	0.64 ± 0.05
$^{45}Ca^{2+}$ + Mn^{2+} (1 mM)	0.12 ± 0.03	0.18 ± 0.03	0.22 ± 0.08
$^{45}Ca^{2+}$ + Ni^{2+} (5 mM)	0.08 ± 0.01	0.11 ± 0.05	0.14 ± 0.07

Platelets were pre-incubated with $^{45}Ca^{2+}$ (0.5 mM) for 1 min, and activated with ADP (20 μM) or thrombin (2 nM), as indicated. Uptake of $^{45}Ca^{2+}$ is given relative to the start of activation. Data are mean values ± SEM (n = 3-6).

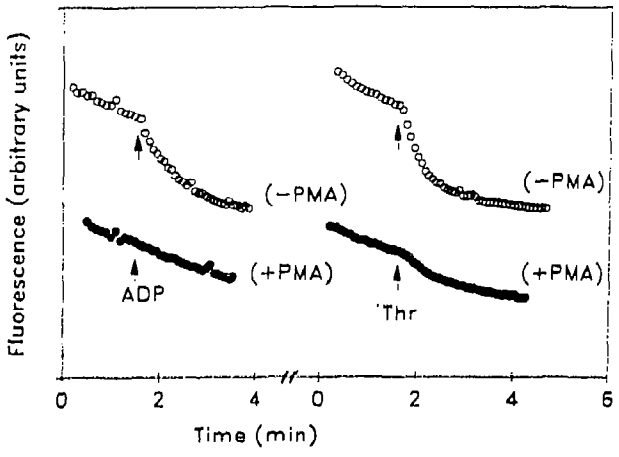

Fig. 4. Quenching of fura-2 fluorescence in rat platelets by Mn^{2+}. Platelets were activated with ADP (20 μM) or thrombin (Thr, 2 nM) in the presence of $MnCl_2$ (0.1 mM) and $CaCl_2$ (0.5 mM). Closed symbols indicate pre-incubation with PMA (100 nM) for 2 min.

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