

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

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Received 15 April 1991

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

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

## 1. INTRODUCTION

Elevation of cytosolic  $[\text{Ca}^{2+}]_i$  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 ( $\text{InsP}_3$ ), which induces the mobilization of  $\text{Ca}^{2+}$  from intracellular stores. In addition, extracellular  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  entry to  $[\text{Ca}^{2+}]_i$  was hindered by a relatively large mobilization of internal  $\text{Ca}^{2+}$ .

It is known that rat platelets require external  $\text{Ca}^{2+}$  for full activation [7]. Here, we present evidence that platelets from these animals have relatively small  $\text{Ca}^{2+}$  stores. Our results suggest that, with thrombin and ADP as agonist, additional entry of  $\text{Ca}^{2+}$  is necessary to reach sufficiently high  $[\text{Ca}^{2+}]_i$  to allow platelet activation. The high contribution of receptor-mediated  $\text{Ca}^{2+}$  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  $[\text{Ca}^{2+}]_i$ , by inhibiting  $\text{Ca}^{2+}$  influx and by stimulating  $\text{Ca}^{2+}$  efflux.

## 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  $\text{MgCl}_2$ , 0.4 mM  $\text{NaH}_2\text{PO}_4$ , 5% (w/v) bovine serum albumin, 2.5  $\mu\text{g}/\text{ml}$  apyrase and 2  $\mu\text{M}$  prostaglandin  $\text{E}_1$ . Platelets ( $1 \cdot 10^9/\text{ml}$ ) were loaded with fura-2/AM (1  $\mu\text{M}$ ) 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  $[\text{Ca}^{2+}]_i$  [10] was by the addition of 0.1% (w/v) Triton X-100 in the presence of 1 mM  $\text{CaCl}_2$  or 10 mM EGTA, 50 mM Tris (pH 8.3). Fluorescence signals were corrected for leakage of dye by the addition of 2 mM  $\text{NiCl}_2$  or according to [3]. Influx of  $\text{Mn}^{2+}$  was detected as the quenching of fluorescence of cytosolic fura-2 by externally added  $\text{Mn}^{2+}$  [4], and was measured at a fixed excitation wavelength of 360 nm.

Influx of  $^{45}\text{Ca}^{2+}$  was measured with washed platelets ( $2 \cdot 10^8/\text{ml}$ ), suspended in low albumin buffer. To the platelet suspension,  $^{45}\text{CaCl}_2$  (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.

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### 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  $\text{Ca}^{2+}$ . With thrombin as activator, at least  $20 \mu\text{M}$  extracellular  $\text{Ca}^{2+}$  was required to induce aggregation and secretion (Fig. 1). The weak agonist ADP required similar  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  [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 ( $20 \mu\text{M}$ ) or thrombin ( $2 \text{ nM}$ ) in the presence of EGTA resulted in a rapid, but slight and transient, increase of  $[\text{Ca}^{2+}]_i$  (Fig. 2A), raising from a resting level of  $50 \pm 9 \text{ nM}$  to  $169 \pm 11$  and  $161 \pm 6 \text{ nM}$ , respectively ( $\pm \text{SEM}$ ,  $n=8$ ). With human platelets, using the same experimental settings, these agonists increased  $[\text{Ca}^{2+}]_i$  to  $194 \pm 25$  and  $424 \pm 59 \text{ nM}$  ( $\pm \text{SEM}$ ,  $n=4$ ), respectively. Under similar conditions, Pollock et al. [3] have measured in thrombin-activated human platelets even higher (up to  $600 \text{ nM}$ )  $[\text{Ca}^{2+}]_i$ , probably because of their more rapid recording of fura-2 fluorescence.

We estimated the size of internal  $\text{Ca}^{2+}$  pools in rat platelets by activation with ionomycin in the presence of EGTA, in which case all internally stored  $\text{Ca}^{2+}$  becomes translocated to the cytosol [2,3]. Ionomycin ( $1.5 \mu\text{M}$ ) raised  $[\text{Ca}^{2+}]_i$  to only  $219 \pm 15 \text{ nM}$  ( $\pm \text{SEM}$ ,  $n=5$ ) (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  $\text{Ca}^{2+}$  stores are relatively small and, therefore,  $\text{InsP}_3$ -induced discharge of  $\text{Ca}^{2+}$  from these stores is limited.

It might be possible that the internal  $\text{Ca}^{2+}$  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  $\text{CaCl}_2$  or (ii) preactivation with ADP in the presence of  $\text{CaCl}_2$ . However, when such pretreated platelets subsequently were activated with thrombin or ionomycin in the presence of excess EGTA, the maximal  $[\text{Ca}^{2+}]_i$  was increased by no more than 0–15%, compared to untreated platelets (data not shown).

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

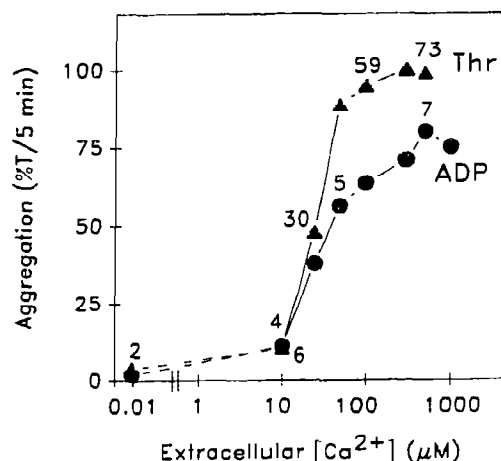


Fig. 1. Effect of extracellular  $\text{Ca}^{2+}$  on aggregation and secretion of rat platelets, activated by ADP ( $20 \mu\text{M}$ ) plus fibrinogen ( $1 \text{ mg/ml}$ ) or thrombin (Thr,  $2 \text{ nM}$ ). Numbers represent percentages of  $[^{14}\text{C}]$ serotonin, secreted after 5 min of activation.

mediated influx of external  $\text{Ca}^{2+}$  was measured by 2 methods. Uptake of  $^{45}\text{Ca}^{2+}$  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  $\text{Ni}^{2+}$ , a putative calcium channel blocker [4,7], and by  $\text{Mn}^{2+}$ .

An alternative way of monitoring  $\text{Ca}^{2+}$  influx is by following the quenching of fura-2 fluorescence by externally added  $\text{Mn}^{2+}$ , which is thought to enter through receptor-mediated calcium channels [4,6]. In the rat platelets, both thrombin and ADP stimulated influx of  $\text{Mn}^{2+}$  (Fig. 4), and  $\text{Ni}^{2+}$  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

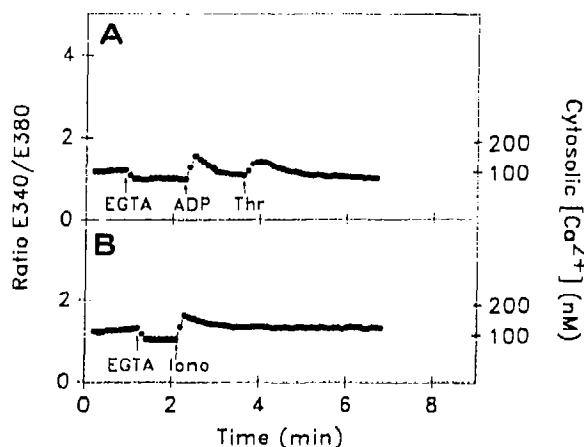


Fig. 2. Ratios of fura-2 fluorescence in rat platelets in the presence of EGTA ( $1 \text{ mM}$ ). Platelets were activated with ADP ( $20 \mu\text{M}$ ), thrombin (Thr,  $2 \text{ nM}$ ) and ionomycin (Iono,  $1.5 \mu\text{M}$ ), as indicated.

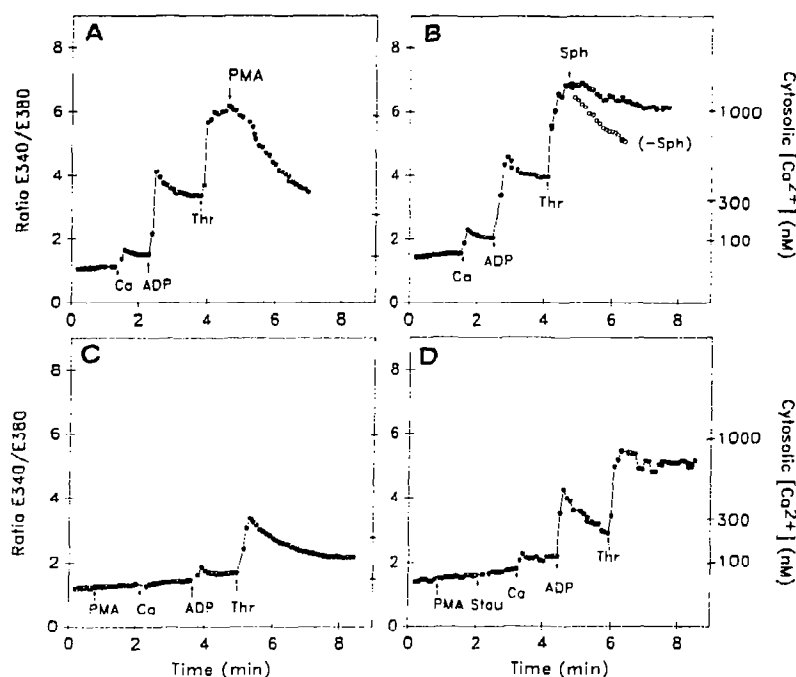


Fig. 3. Ratios of fura-2 fluorescence in rat platelets. Given were  $\text{CaCl}_2$  (1 mM), ADP (20  $\mu\text{M}$ ), thrombin (Thr, 2 nM), PMA (100 nM), sphingosine (Sph, 10  $\mu\text{M}$ ) and staurosporine (Stau, 500 nM), as indicated.

requirement of extracellular  $\text{Ca}^{2+}$  for platelet responses and the high contribution of  $\text{Ca}^{2+}$  influx to elevation of  $[\text{Ca}^{2+}]_i$ , receptor-mediated  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  efflux out of the platelets [3,12]. In rat platelets, protein kinase C apparently modulated  $\text{Ca}^{2+}$  efflux in a similar way. This was concluded from (i) the PMA-induced enhancement of decrease in  $[\text{Ca}^{2+}]_i$ , 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  $[\text{Ca}^{2+}]_i$  (Fig. 3C). This effect of PMA was reversed by the potent protein kinase C in-

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

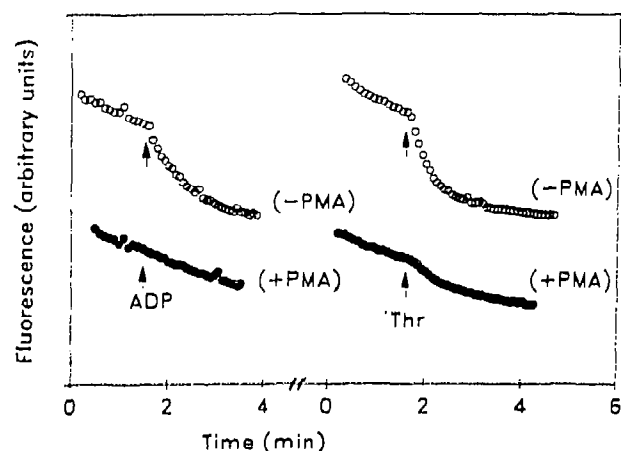


Fig. 4. Quenching of fura-2 fluorescence in rat platelets by  $\text{Mn}^{2+}$ . Platelets were activated with ADP (20  $\mu\text{M}$ ) or thrombin (Thr, 2 nM) in the presence of  $\text{MnCl}_2$  (0.1 mM) and  $\text{CaCl}_2$  (0.5 mM). Closed symbols indicate pre-incubation with PMA (100 nM) for 2 min.

Table 1

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

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

*Acknowledgements:* This work was partially supported by the Netherlands Heart Foundation (NHS 84.060) and Hoffmann-La Roche (Basle, Switzerland).

## REFERENCES

- [1] Davies, T.A., Drotts, D.L., Weil, G.J. and Simons, E.R. (1989) *J. Biol. Chem.* 264, 19600-19606.
- [2] Rink, T.J. and Sage, S.O. (1990) *Annu. Rev. Physiol.* 52, 431-449.
- [3] Pollock, W.K., Sage, S.O. and Rink, T.J. (1987) *FEBS Lett.* 210, 132-136.
- [4] Merritt, J.E. and Hallam, T.J. (1988) *J. Biol. Chem.* 263, 6161-6164.
- [5] Magócsi, M., Sarkadi, B., Kovács, T. and Gárdos, G. (1989) *Biochim. Biophys. Acta* 984, 88-96.
- [6] Sage, S.O., Merritt, J.E., Hallam, T.J. and Rink, T.J. (1989) *Biochem. J.* 258, 923-926.
- [7] Blache, D., Ciavatti, M. and Ojeda, C. (1987) *Biochim. Biophys. Acta* 923, 401-412.
- [8] Heemskerk, J.W.M., Feijge, M.A.H., Kalafusz, R. and Hornstra, G. (1989) *Biochim. Biophys. Acta* 1004, 252-260.
- [9] Feijge, M.A.H., Heemskerk, J.W.M. and Hornstra, G. (1990) *Biochim. Biophys. Acta* 1025, 173-178.
- [10] Cobbold, P.H. and Rink, T.J. (1987) *Biochem. J.* 248, 313-328.
- [11] Tsunoda, Y., Matsuno, K. and Tashiro, Y. (1988) *Biochem. Biophys. Res. Commun.* 156, 1152-1159.
- [12] Rink, T.J. and Sage, S.O. (1987) *J. Physiol.* 393, 513-524.
- [13] Watson, S.P., McNally, J., Shipman, L.J., Godfrey, P.P. (1988) *Biochem. J.* 249, 345-350.
- [14] McCarthy, S.A., Hallam, T.J. and Merritt, J. (1989) *Biochem. J.* 264, 357-364.