Ca²⁺ influx in platelets: activation by thrombin and by the depletion of the stores

Effect of cyclic nucleotides

Maria G. DONI,* Lucia CAVALLINI† and Adolfo ALEXANDRE†‡

†Department of Biological Chemistry, C.N.R. Unit for the Study of Mitochondrial Physiology, and *Institute of Human Physiology, University of Padova, Padova, Italy

In aspirin-treated platelets the thrombin-induced increase of cytosolic Ca²⁺ ([Ca²⁺]_i) associated with the release from the intracellular stores is followed by a decrease to the baseline which is largely dependent on the re-uptake into the stores. This is shown by the further increase of [Ca²⁺]_i upon inhibition of the endomembrane Ca²⁺-ATPase with thapsigargin. The re-uptake of Ca²⁺ into the stores is accelerated by sodium nitroprusside (SNP) or prostacyclin (PGI₂). In all cases, after store depletion with thapsigargin the influx of external Ca²⁺ is maximal. After a thrombin-induced cycle of Ca²⁺-release re-uptake the stores are partly full: in these conditions the addition of external Ca²⁺ elicits a significant increment of [Ca²⁺]_i and a further filling of the stores. Both are strongly reduced if Ca²⁺ addition is preceded by SNP or PGI₂. Similar results are obtained also if (by supplementing and then depleting Ca²⁺) the stores are as full as in native

platelets at the moment of adding Ca²⁺. The thrombin-activated Ca²⁺ influx is reversed by hirudin. A PGI₂- and SNP-sensitive Mn²⁺ influx is observed if Mn²⁺ is added in place of Ca²⁺. It is concluded that thrombin activates a cyclic nucleotide-sensitive Ca²⁺ (and Mn²⁺) influx pathway dependent on the occupancy of the thrombin receptor and independent of the filling state of the stores. In the absence of thrombin, thapsigargin releases Ca²⁺ relatively rapidly from a fraction of the stores; the remaining deposits are discharged much more slowly. This may indicate that platelets contain two distinct classes of agonist-sensitive stores. The addition of external Ca²⁺ (or Mn²⁺) at short or long incubation times with thapsigargin monitors the influx of Ca²⁺ activated by the depletion of one or both types of stores. The depletion of each type of store activates Ca²⁺ (Mn²⁺) influx. This type of cation influx is not inhibited by the cyclic nucleotides.

INTRODUCTION

Like other cells, platelets have a complex mechanism of Ca²⁺ signalling, involving the operation and interaction of Ca²⁺ sequestration into, and release from, the intracellular stores, and of Ca²⁺ influx from, and release to, the extracellular environment. In the absence of extracellular Ca²⁺, the Ca²⁺ released from the stores by the agonist-generated inositol 1,4,5-trisphosphate (IP₃) is readily re-accumulated into the stores by an ATP-energized Ca²⁺ pump [1,2], which is inhibited by thapsigargin [3–5]. By inhibiting the endomembrane Ca²⁺-ATPase, thapsigargin promotes Ca²⁺ release from the stores also in the absence of an agonist. If the cyclo-oxygenase pathway is inhibited, the thapsigargin-promoted Ca²⁺ release is not accompanied by an increase of IP₃ [6]; in these conditions, however, the Ca²⁺ released from the intracellular stores is 50% or less of the total agonist-dischargeable Ca²⁺ and an agonist like thrombin is required for full discharge [1,7]. These observations led to the proposal of a two-pool model for Ca²⁺ in platelets, a thapsigargin-sensitive store and an agonist-releasable compartment [1,2,6].

In the presence of extracellular Ca²⁺, platelet activation is also accompanied by the influx of Ca²⁺ from the extracellular environment. The modes of Ca²⁺ influx in cells have been the object of considerable attention in recent years. In general, it was established that Ca²⁺ entry can be activated either directly by the interaction of agonists with their receptors, or indirectly by the depletion of the intracellular stores [8,9].

In platelets it was shown with stopped flow fluorimetry that ADP evokes a biphasic elevation of intracellular Ca²⁺ con-

centration ([Ca²⁺]_i). The first phase, which is observed only with external Ca²⁺, commences without measurable delay and precedes the second by over 200 ms [10]. It appears to be conducted by an ADP-receptor-operated non-selective cation channel [11,12]. The second phase appears to coincide with and be caused by the release of Ca²⁺ from the intracellular stores [10]. Both phases are associated with the entry of Mn²⁺ and therefore, it was assumed, with Ca²⁺ entry. Depleting the intracellular stores with the endomembrane Ca²⁺-ATPase inhibitor thapsigargin or 2,5-di-(*t*-butyl)-1,4-benzohydroquinone, in the absence of an agonist, promotes Ca²⁺ and Mn²⁺ entry, proving directly that Ca²⁺ influx is activated by store depletion [13–15].

Upon stimulation with agonists other than ADP, the onset of the increase of [Ca²⁺]_i was reported to be much less affected by the presence of extracellular Ca²⁺ [15,16]. With thrombin, it was initiated some 280 ms after the addition of the agonist and it appeared to be anticipated only by 50–100 ms in the presence of extracellular Ca²⁺. These observations did not allow us to establish clearly whether Ca²⁺ influx with these agonists was dependent solely on the depletion of the intracellular stores (the onset of which might occur a few milliseconds earlier with extracellular Ca²⁺) or whether it was to some extent linked to a receptor-operated Ca²⁺ influx pathway. The idea that all Ca²⁺ entry was controlled solely by the filling state of the stores was put forward by Alonso et al. [14] based on the properties of Mn²⁺ influx and the inhibitory action of the antifungal drugs, cytochrome *P*-450 inhibitors econazole and miconazole. With agonists like thrombin, a clear-cut Mn²⁺ entry was reported in stopped-flow experiments only coincident with the depletion of

Abbreviations used: IP₃, inositol 1,4,5-trisphosphate; PRP, platelet-rich plasma; PGI₂, prostacyclin; SNP, sodium nitroprusside; [Ca²⁺]_i, intracellular Ca²⁺ concentration.

‡ To whom correspondence should be addressed: Dipartimento di Chimica Biologica, Via Trieste 75, 35121 Padova, Italy.

the stores [15]. More recently, the thrombin-evoked Ca^{2+} entry in platelets was shown to be inhibited by inhibitors of protein tyrosine kinases [17].

The observations reported in this study help to clarify the relative contributions of the agonist *per se* and of the filling state of the stores to Ca^{2+} - and Mn^{2+} -entry in platelets, and provide new information on the control exerted by the cyclic nucleotides on Ca^{2+} movements.

EXPERIMENTAL

Materials

Thrombin, prostacyclin (PGI_2), apyrase, hirudin, Fura 2-AM, ionomycin, thapsigargin, were purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

Platelet preparation

Platelet-rich plasma (PRP) and washed platelets were treated with aspirin and prepared as previously reported [18] from fresh blood drawn from healthy volunteers and mixed with acid citrate-dextrose anticoagulant supplemented with apyrase (80 m-units/ml) and prostacyclin (0.8 $\mu\text{g}/\text{ml}$).

Determination of $[\text{Ca}^{2+}]_i$

The cytosolic Ca^{2+} concentration was determined with Fura-2AM essentially according to Pollock and Rink [19]. The probe (2 μM) was added to aspirinated PRP, and loading was performed for 40 min at 37 °C. After centrifugation, platelets were resuspended in 150 mM NaCl, 5 mM KCl, 2 mM MgCl_2 , 10 mM glucose, 20 mM Hepes, pH 7.4, at a concentration of 1×10^8 cells/ml in the presence of 0.1 mM sulphhydrylpyrazole [20], 40 m-units/ml of apyrase and 5 m-units/ml of hirudin. The experiments were performed in the presence of 0.3 mM EGTA. Fluorescence was measured at 29 °C in a thermostatically controlled, magnetically stirred cuvette in a Shimadzu RL-5000 or a Perkin-Elmer LS3 spectrofluorimeter with excitation and emission wavelengths set at 340 nm and 505 nm respectively. $[\text{Ca}^{2+}]_i$ was calculated as recommended by Grynkiewicz et al. [21].

Mn^{2+} influx

This was monitored as the quenching of Fura-2 fluorescence, with the excitation and emission wavelength set at 360 and 505 nm as indicated by [22]. Platelets were treated with 0.2 mM CaCl_2 for 2 min, followed by 0.5 mM EGTA before the addition of the effectors.

When statistical data are reported, the values are indicated as mean values (number of experiments with different platelet preparations) $\pm$ S.D.

RESULTS

Effect of thapsigargin on the PGI_2 - and sodium nitroprusside (SNP)-stimulated re-uptake into the stores of Ca^{2+} released by thrombin

The addition of the agonist thrombin to aspirin-treated platelets suspended in an EGTA-supplemented medium elicits a rapid increase of $[\text{Ca}^{2+}]_i$ followed by a relatively rapid decrease close to the baseline, which is completed in 3–5 min (Figure 1a, [1]). This

decay of $[\text{Ca}^{2+}]_i$ is attributable to a relatively large extent to a net re-uptake into the intracellular stores, since the subsequent addition of thapsigargin, a specific inhibitor of the stores-associated Ca^{2+} -ATPase, induces the release into the cytosol of a significant proportion of the Ca^{2+} which disappeared. The results are unchanged by further increasing the thapsigargin concentration (up to 1.5 μM , see also [1]).

The decay of $[\text{Ca}^{2+}]_i$ elevated by thrombin is accelerated if PGI_2 or the nitroxide radical generator SNP, which mediate the production of cyclic AMP and cyclic GMP respectively, are supplemented at the peak of the thrombin-induced Ca^{2+} transient (see also [2,23]). This appears to be the consequence of a stimulated re-uptake into the stores, since: (1) a large increase of $[\text{Ca}^{2+}]_i$ is promoted by thapsigargin added soon after $[\text{Ca}^{2+}]_i$ has returned to the baseline level and (2) the effect of the cyclic nucleotides is abolished in the presence of thapsigargin. Specifically, if thapsigargin is added together with thrombin, the increase of $[\text{Ca}^{2+}]_i$ is essentially unmodified, but the increased decay-rate induced by the subsequent addition of PGI_2 or SNP is abolished (results not shown).

Figure 1(a) also shows that a large further increase of $[\text{Ca}^{2+}]_i$ is observed, both in the absence and presence of PGI_2 and SNP, when Ca^{2+} is supplemented to the incubation medium after thapsigargin treatment, that is when the stores are depleted. This is expected, since the emptying of the agonist-sensitive Ca^{2+} deposits has been reported to stimulate Ca^{2+} influx [13,14]. On the contrary, if Ca^{2+} is supplemented to the suspending medium before the addition of thapsigargin, i.e. when the stores are partially refilled after their transient depletion by thrombin (Figure 1b), the increment of $[\text{Ca}^{2+}]_i$ is reduced. Specifically, the increase of $[\text{Ca}^{2+}]_i$ is almost completely prevented in the presence of PGI_2 or SNP; however, it is still relatively large in their absence. Interestingly, the filling state of the stores is not significantly modified by the cyclic nucleotides (as judged by the increment of $[\text{Ca}^{2+}]_i$ induced by thapsigargin, Figure 1a). This raises the possibility that the cyclic nucleotide-sensitive Ca^{2+} influx observed after a cycle of store depletion–re-replenishment

Figure 1 Action of PGI_2 and SNP on the thrombin-induced increase of $[\text{Ca}^{2+}]_i$ and on Ca^{2+} influx

Platelets were stimulated with thrombin (THR) (0.2 unit/ml). PGI_2 (—) (1 $\mu\text{g}/\text{ml}$) or SNP (---) (60 μM) were added where indicated by the arrow. Thapsigargin (TG) was 0.4 μM and CaCl_2 0.8 mM. The traces are representative of duplicate experiments with at least five different preparations with similar results and are corrected for the small interference signal originating from extracellular Fura 2.

Figure 2 PGI_2 inhibits the thrombin-dependent Ca^{2+} influx

Conditions are as in Figure 1. PGI_2 was added as indicated (---). Where indicated by the arrow, EGTA (1.5 mM) was added, followed a few seconds later (as soon as the signal was stable) by thapsigargin (0.4 μM) plus ionomycin (0.05 μM). The trace is representative of duplicate experiments with at least five different preparations.

with thrombin is not controlled by the filling state of the stores but directly by the agonist.

Thrombin activates a PGI_2 - and SNP-sensitive Ca^{2+} (and Mn^{2+}) influx independent of the filling state of the stores

This possibility was tested in greater detail in the experiment depicted in Figure 2. It is shown that the inhibition of the thrombin-activated Ca^{2+} influx is also observed when PGI_2 is added after the completion of the decay curve of $[\text{Ca}^{2+}]_i$ promoted by thrombin, 1 min before the addition of Ca^{2+} . In these conditions PGI_2 promotes only a negligible further decrease of $[\text{Ca}^{2+}]_i$, which does not affect significantly the overall filling of the stores. The latter was measured directly as the $\Delta[\text{Ca}^{2+}]_i$ induced by the addition of thapsigargin (together with a small concentration of ionomycin, to ensure a complete depletion of the stores, see below): it was $350 (5) \pm 30$ nM and $370 (5) \pm 30$ nM in the absence and presence respectively of PGI_2 .

A possible objection to the interpretation of these results derives from the fact that the Fura 2 method provides information on $[\text{Ca}^{2+}]_i$, it does not measure the actual fluxes of Ca^{2+} . It follows that the small increment of $[\text{Ca}^{2+}]_i$ upon addition of Ca^{2+} in the presence of PGI_2 could depend on a more active transfer to the storage sites rather than on a decreased influx through the plasma membrane. In fact (as described in [2] and Figure 1a) a stimulation of Ca^{2+} transfer into the storage sites appears to be one of the actions exerted in platelets by cyclic AMP and cyclic GMP. To obtain reliable information on Ca^{2+} influx it was therefore necessary to monitor also the filling state of the stores after the addition of Ca^{2+} . This was done by first chelating the extracellular Ca^{2+} with excess EGTA, followed a few seconds later by thapsigargin (reinforced with a low concentration of ionomycin), to release Ca^{2+} from the deposits. When EGTA/

	$\Delta[\text{Ca}^{2+}]_i$ (nM)	
	*	**
Control	550(8)±80	1700(5)±400
PGI_2	570(5)±80	650(5)±120
SNP	560(5)±70	940(5)±180

Figure 3 Thrombin also activates Ca^{2+} influx when the Ca^{2+} content of the stores is high: inhibition by PGI_2 and SNP

Conditions are as in Figure 1. Where indicated, CaCl_2 was 0.6 and 2.5 mM, and EGTA 1 mM. Enough NaOH was added at the second addition of Ca^{2+} to compensate for the H^+ generated at the moment of complexation. Where indicated, PGI_2 (---) was 0.7 $\mu\text{g}/\text{ml}$ and SNP (····) 70 μM . * and ** represent the $\Delta[\text{Ca}^{2+}]_i$ observed before and after the addition of Ca^{2+} upon addition of thapsigargin plus ionomycin (*) and of EGTA (5 mM, plus NaOH) followed by thapsigargin plus ionomycin (**). The $\Delta[\text{Ca}^{2+}]_i$ induced by thrombin in unstimulated platelets was $560 (10) \pm 70$ nM. The traces were repeated with similar results in at least five different preparations from different donors.

thapsigargin/ionomycin were added 1 min after Ca^{2+} , they induced an increment of $[\text{Ca}^{2+}]_i$ which was much larger in the absence than in the presence of PGI_2 [$1150 (5) \pm 200$ nM versus $650 (5) \pm 80$ nM]. Although $\Delta[\text{Ca}^{2+}]_i$ values cannot be used to measure actual amounts of Ca^{2+} released, an approximate estimate of the increment of the filling state of the stores after supplementing Ca^{2+} can be obtained by comparing the $\Delta[\text{Ca}^{2+}]_i$ induced by thapsigargin (plus ionomycin) before and after Ca^{2+} addition. This increased from 350 to 1150 nM in the absence of PGI_2 and only from 370 to 650 in its presence. It can be concluded that after supplementing extracellular Ca^{2+} the overall increment of the platelet-associated Ca^{2+} is strongly depressed by PGI_2 . Similar results were obtained with SNP (results not shown).

A possible objection to the conclusions reached in Figure 2 is that after the thrombin-induced cycle of Ca^{2+} release-re-uptake, the Ca^{2+} content of the stores is significantly less than that of unstimulated platelets. This makes a contribution of the store-dependent activation of Ca^{2+} influx likely. Indeed, in Figure 2, a sizeable increment of the stores-associated Ca^{2+} was observed, after the addition of Ca^{2+} , in the presence of PGI_2 also. This could represent Ca^{2+} influx activated by residual store depletion. The experiment of Figure 3 was performed to show that thrombin can induce a PGI_2 /SNP-sensitive Ca^{2+} influx also if the Ca^{2+}

Figure 4 Hirudin counteracts the thrombin-induced Ca^{2+} influx

Conditions are as in Figure 1. Where indicated hirudin (HIR) (---) was 0.5 unit/ml and CaCl_2 0.8 mM. The trace is typical of duplicate determinations, with four platelet preparations.

Figure 5 PGI_2 and SNP inhibit the thrombin-dependent influx of Mn^{2+}

Conditions are as described in the Experiment section. When present PGI_2 and SNP were added at the arrow. MnCl_2 was 0.5 mM. The traces are corrected for the small interference signal originating from extracellular Fura 2. The trace is typical of duplicate determinations with four preparations.

content of the stores at the moment of adding Ca^{2+} is comparable with that of unstimulated platelets. Ca^{2+} loading of the deposits was performed by adding Ca^{2+} after a cycle of thrombin-induced store depletion–refilling (as in Figure 2). External Ca^{2+} was then chelated with EGTA. This determined a strong decay of $[\text{Ca}^{2+}]_i$. After EGTA treatment (4 min) the Ca^{2+} content of the stores (monitored as the thapsigargin plus ionomycin-induced $\Delta[\text{Ca}^{2+}]_i$) was about the same as that of unstimulated platelets. Also in this situation, the addition of external Ca^{2+} was followed by a rapid increase of $[\text{Ca}^{2+}]_i$ as well as of the Ca^{2+} associated with the stores. Both were strongly depressed when PGI_2 was supplemented before Ca^{2+} : in particular, the increase of the store-associated Ca^{2+} was negligible in this protocol. Similar results were obtained with SNP.

The existence of a thrombin-induced mode of Ca^{2+} influx independent of the filling state of the stores can also be shown using hirudin to terminate the action of thrombin. This is shown

Figure 6 Thapsigargin (TG) plus ionomycin promote extensive depletion of the agonist-releasable stores

Conditions are as in Figure 1. Ionomycin (IONO) concentration was 0.05 μM . The traces are typical of duplicate determinations with five platelet preparations. Abbreviation: THR, thrombin.

in Figure 4 where, after a cycle of store depletion–refilling induced by thrombin, the increase of $[\text{Ca}^{2+}]_i$ was strongly reduced if hirudin was supplemented 5 min before the addition of Ca^{2+} .

Taken together, these findings show that thrombin does indeed activate a type of Ca^{2+} influx which is independent of the filling state of the stores and is inhibited by the removal of thrombin, as well as by PGI_2 and SNP.

The influx of Mn^{2+} has been used as a tracer for Ca^{2+} entry in several cell types, including platelets. At the excitation wavelength of 360 nm, the Mn^{2+} -induced quenching of the Fura 2 fluorescence is not affected by $[\text{Ca}^{2+}]_i$. As shown in Figure 5 the addition of Mn^{2+} after a cycle of thrombin-induced depletion–refilling of the Ca^{2+} stores promotes a quenching of the Fura 2 fluorescence, which is inhibited by PGI_2 and SNP. This experiment indicates that the thrombin-activated, cyclic nucleotides-sensitive Ca^{2+} -entry system described above is also permeable to Mn^{2+} .

The sequential depletion of the two types of Ca^{2+} stores activates a progressively increased, PGI_2 - and SNP-insensitive influx of Ca^{2+} and Mn^{2+}

It is generally agreed that the increase of $[\text{Ca}^{2+}]_i$ promoted by thapsigargin is due to a leak from the stores the Ca^{2+} -ATPase of which is inhibited. As already described [1,2,6,13], thapsigargin depletes the agonist-sensitive stores only partially in aspirin-treated platelets. The emptying of the deposits is completed by thrombin. On the contrary, the thapsigargin-induced depletion is complete if it is added after the thrombin-promoted cycle of store depletion–re-replenishment [1,2]. These properties were taken as evidence that two types of Ca^{2+} stores exist in platelets, one discharged by thapsigargin and the other by the agonist [1,2,6].

In Figure 6 we report experiments showing that a low concentration (50 nM) of ionomycin can substitute for thrombin in completing the release into the cytosol of the Ca^{2+} associated with the agonist-sensitive stores promoted by thapsigargin. In fact, the addition of thrombin in the presence of both thapsigargin and ionomycin (added in either order) elicits no further increase of $[\text{Ca}^{2+}]_i$. (At these low concentrations ionomycin releases Ca^{2+} preferentially from the agonist-sensitive stores and only marginally from the secretory granules [24], which contain most of the platelet Ca^{2+} [25].) A likely explanation for these observations is

Figure 7 The thapsigargin (TG)-activated Ca^{2+} influx increases with the progression of the depletion of the deposits

Conditions are as in Figure 1. The traces are typical of duplicate determinations with four different preparations. Abbreviation: THR, thrombin.

that the stores which are not readily discharged by thapsigargin are characterized by a low Ca^{2+} leak in the absence of a suitable agonist, so that the inhibition of their Ca^{2+} pump does not lead to a ready Ca^{2+} release, unless an artificial leak is introduced. Indeed, a sizeable leak is present also in the absence of ionomycin, since increasing the time of incubation after thapsigargin addition leads to a progressive discharge of the thapsigargin-insensitive stores also [2]. This is shown in Figure 7(a) where thrombin added at the top of the thapsigargin-induced increase of $[\text{Ca}^{2+}]_i$ releases a large further amount of Ca^{2+} ; it releases much less Ca^{2+} if it is added 20 min later. It is therefore possible to decrease sequentially the Ca^{2+} content of the two types of agonist-sensitive stores, simply by incubating platelets in the presence of thapsigargin for long enough times. This allows us to study Ca^{2+} (and Mn^{2+}) influx when only the thapsigargin-sensitive stores are empty, as well as when the other stores also (less readily discharged by thapsigargin) are depleted.

As shown in Figure 7(b) the addition of Ca^{2+} 1 min after thapsigargin, i.e. when only the first class of Ca^{2+} deposits is empty, is followed by an increase of $[\text{Ca}^{2+}]_i$ which is much slower and less extensive than that observed when Ca^{2+} is added 20 min after thapsigargin, when the second type of Ca^{2+} deposits is also largely depleted. This indicates that the filling state of each type of Ca^{2+} store controls the activation of Ca^{2+} influx. In this case, the addition of PGI_2 or SNP before Ca^{2+} is without effect on the increase of $[\text{Ca}^{2+}]_i$. The insensitivity to cyclic nucleotides differentiates the Ca^{2+} -influx pathway that is activated by the depletion of the stores from that controlled by the occupancy of the thrombin receptor.

The dependence of Mn^{2+} influx on the differential depletion of the stores was studied by adding Mn^{2+} to the suspending medium, in the place of Ca^{2+} , after a short or a long incubation time with thapsigargin (Figure 8). The properties of Mn^{2+} influx were

Figure 8 The thapsigargin-activated Mn^{2+} influx increases with the progression of the depletion of the deposits

Conditions are as in Figure 5. The traces are typical of duplicate determinations with at least four platelet preparations.

similar to those just described for Ca^{2+} , i.e. a slow entry after the depletion of the first stores, which was strongly increased by the depletion of the second stores. PGI_2 and SNP had no effect on this type of Mn^{2+} entry.

DISCUSSION

The pathways of Ca^{2+} influx in platelets with agonists other than ADP are controversial. With thrombin, stopped-flow experiments indicate that the increase of $[\text{Ca}^{2+}]_i$, in the presence of extracellular Ca^{2+} , precedes, but only by 50–100 ms, that observed without Ca^{2+} in the suspending medium [15]. This was taken as an indication of the existence of a receptor-activated Ca^{2+} -influx pathway operating prior to the store depletion-activated entry. This view was not shared by Alonso et al. [14]. The observations reported in the present research prove the existence of a Ca^{2+} -entry pathway activated by the occupancy of the thrombin receptor and independent of the filling state of the stores. This conclusion was reached taking advantage of the property of thrombin to promote a rapid Ca^{2+} release from the stores into the cytosol, followed readily by the re-uptake into the deposits of part of the Ca^{2+} released. After this cycle of Ca^{2+} release–re-uptake, the addition of Ca^{2+} is followed by Ca^{2+} influx. The latter is largely unrelated to the store-controlled Ca^{2+} entry, since (1) it is also observed if the Ca^{2+} content of the stores at the moment of supplementing Ca^{2+} is increased to a level comparable with that of unstimulated platelets, and (2) it is prevented by hirudin, added before the addition of Ca^{2+} to terminate the action of thrombin. The thrombin-dependent Ca^{2+} influx is inhibited by PGI_2 as well as by SNP. These agents are without effect on Ca^{2+} influx activated by the depletion of the stores. Also Mn^{2+} is permeable through the thrombin-activated Ca^{2+} -entry pathway. The PGI_2 - and SNP-sensitive Mn^{2+} entry is relatively slow. This may have rendered its detection difficult in stopped-flow experiments [15]. Interestingly, PGI_2 and SNP, which inhibit the thrombin-receptor-activated Ca^{2+} (Mn^{2+}) influx, are without effect on the ADP-receptor-activated influx [16,26]. The present experiments do not allow us to propose a mechanism for the inhibition by cyclic AMP and cyclic GMP of the thrombin-dependent Ca^{2+} (Mg^{2+}) influx. As the action of thrombin requires a continuous receptor occupancy, being counteracted by hirudin, the cyclic nucleotides may inhibit the generation of continuously metabolized signal molecules that cause Ca^{2+} entry. Alternatively,

thrombin may activate Ca^{2+} influx more directly with the mediation of a (cyclic nucleotide-sensitive) G protein.

Cyclic nucleotides inhibit platelet activation at multiple sites. Cyclic AMP interferes with the interaction between thrombin and its receptor [27,28] and with the activation of phospholipase C [29,30]. It also increases the incorporation of diacylglycerol into phosphatidylinositol [31] and it inhibits events distal to Ca^{2+} mobilization [32,33] and the activation of protein kinase C [18,34]. Similarly cyclic GMP interferes with the activation of phospholipase C [26,35–38] and with distal processes [39,40]. Furthermore, cyclic GMP potentiates the action of cyclic AMP-elevating stimuli by inhibiting the cyclic AMP phosphodiesterase [41]. The cyclic nucleotides increase the rate of Ca^{2+} re-uptake into the stores after their discharge with thrombin ([1,2,23] and Figure 1). The present experiments do not allow us to establish the mechanism of the cyclic nucleotides-stimulated Ca^{2+} re-uptake. Factors involved could be a decreased level of IP_3 , an inhibition of the IP_3 -activated Ca^{2+} release (as reported for cyclic AMP [42–44]) or an activation of the Ca^{2+} -ATPase (as again reported for cyclic AMP [23,45–50], but see [51,52]).

Recent studies with the endomembrane Ca^{2+} -ATPase inhibitor thapsigargin led to the hypothesis that platelets contain two distinct types of agonist-sensitive Ca^{2+} storage sites, one discharged by thapsigargin and the second by thrombin [1,2,6,53]. In fact, in platelets treated with aspirin or with a thromboxane A_2 -receptor inhibitor, and in the absence of extracellular Ca^{2+} , thapsigargin promotes the release into the cytosol of only a fraction of the stores-associated Ca^{2+} . The remaining Ca^{2+} is released only by an agonist like thrombin. On the other hand, thrombin promotes a fast release into the cytosol of the Ca^{2+} associated with the stores, and most of the Ca^{2+} released is then rapidly re-accumulated into the deposits. Interestingly, after a cycle of thrombin-induced depletion–refilling of the deposits, thapsigargin promotes the complete discharge into the cytosol of the Ca^{2+} re-accumulated [1]. This could mean that, after thrombin, Ca^{2+} is re-accumulated exclusively into the thapsigargin-sensitive stores, or alternatively that the presence of the agonist enables thapsigargin to stimulate the release of Ca^{2+} from both types of stores. We favour the latter possibility because, as shown in Figure 6, ionomycin can substitute for thrombin in potentiating the Ca^{2+} -releasing effect of thapsigargin. This indicates that, in the absence of a suitable agonist, those stores that do not release Ca^{2+} readily upon treatment with thapsigargin do so because their leaks are low, such that inhibiting their Ca^{2+} -ATPase has no rapid effect on $[\text{Ca}^{2+}]_i$ unless the leaks are artificially increased with a Ca^{2+} -ionophore. On the contrary, the leaks from the stores that release Ca^{2+} readily upon treatment with thapsigargin are relatively high even in the absence of an agonist. It is therefore likely that the thapsigargin-promoted Ca^{2+} release is more complete after the thrombin-induced cycle of Ca^{2+} release–re-uptake, because thrombin maintains a higher degree of leaks from all of the intracellular stores, when Ca^{2+} has fallen back close to the baseline. In support of this view is the observation that the rate of increment of $[\text{Ca}^{2+}]_i$ upon addition of thapsigargin varies depending on whether it is preceded or not by the thrombin-induced cycle of Ca^{2+} release–re-uptake being significantly faster in the former situation (compare Figure 1a with Figure 6). Indeed, two types of endoplasmic reticulum Ca^{2+} pump isoforms have been described in platelets, which are both inhibited by sufficiently high thapsigargin concentrations [54,55]. Furthermore, as already suggested [2], the stores that do not release their Ca^{2+} readily after thapsigargin are not insensitive to its action, since they are also depleted, but in a longer time frame.

We utilized this property to show that the depletion of both types of stores is associated with the activation of Ca^{2+} influx

from the extracellular space: in fact, a short (1 min) incubation with thapsigargin before addition of Ca^{2+} to the suspending medium is associated with the depletion of the thapsigargin-sensitive stores and with a limited Ca^{2+} influx. The latter is much less than that observed if Ca^{2+} is added 20 min after thapsigargin, when the second class of Ca^{2+} deposits is also largely depleted.

The influx of Mn^{2+} follows the same pattern: the thapsigargin-induced depletion of the first class of stores activates a slow Mn^{2+} entry and the rate increases substantially when the second class of stores is also depleted.

The nature of the signal(s) leading to the activation of Ca^{2+} influx upon depletion of the stores is still not well understood. It was proposed to be linked to some cytochrome *P*-450-dependent function, as Ca^{2+} influx was reported to be inhibited by the antifungal agents, cytochrome *P*-450-inhibitors econazole and miconazole [14]. In platelets, these substances were reported to act on both types of Ca^{2+} influx [14], or alternatively only on the influx activated by thapsigargin, but not on that activated by thrombin [13]. Quite recently, it was proposed that Ca^{2+} efflux from the stores is accompanied by the release into the cytosol of a low-molecular-mass phosphorylated agent that would activate Ca^{2+} influx; the action of this agent was reported to be inhibited by econazole [56].

If these data are confirmed, this would indicate that the agent is released by the emptying of both types of Ca^{2+} storage granules associated with the platelets.

In summary the results reported in this study show that thrombin activates a store-depletion-independent Ca^{2+} influx in platelets, observed by adding Ca^{2+} after a cycle of store depletion–refilling. Such influx is inhibited by the removal of thrombin with hirudin. It is also inhibited by PGI_2 and SNP. A PGI_2 - and SNP-sensitive Mn^{2+} influx can also be observed in the same conditions. In the absence of the agonist, thapsigargin promotes the rapid depletion of a fraction of the Ca^{2+} stores and the slow depletion of the remaining stores. The influx of Ca^{2+} (and Mn^{2+}) is activated by the depletion of the stores: it is relatively limited if only the first type of Ca^{2+} deposits is emptied and much larger when extensive depletion of the stores is obtained after 20–30 min of incubation with thapsigargin. This type of Ca^{2+} and Mn^{2+} influx is insensitive to PGI_2 and SNP.

We are grateful to Mr. Massimo Cesaro, Dr. Luigi Toma and Miss Emanuela Bellotto for their technical assistance and to Dr. Gianni Cavatton and Professor Giuseppe Ongaro, of the Transfusional Center of Padova, for providing human blood. The research was supported by 60% and 40% funds from the Italian Ministry of Research and Technology, and CT.CNR N 93.04209.CT04 respectively.

REFERENCES

- Brüne, B. and Ullrich, V. (1991) *J. Biol. Chem.* **266**, 19232–19237
- Brüne, B. and Ullrich, V. (1992) *Eur. J. Biochem.* **207**, 607–613
- Thastrup, O., Dawson, A. P., Scharff, O., Foder, B., Cullen, P. J., Drobrak, B. K., Bjerrum, P. J., Christensen, S. B. and Hanley, M. R. (1989) *Agents Actions* **27**, 17–33
- Thastrup, O., Foder, B. and Scharff, O. (1987) *Biochem. Biophys. Res. Commun.* **142**, 654–660
- Jackson, T. R., Patterson, S. L. and Thastrup, O. (1988) *Biochem. J.* **253**, 81–86
- Heemskerk, J. W. M., Vis, P., Feijge, M. A. H., Hoyland, J., Mason, W. T. and Sage, S. O. (1993) *J. Biol. Chem.* **268**, 356–363
- Brüne, B. and Ullrich, V. (1991) *FEBS Lett.* **284**, 1–4
- Putney, J. W. (1990) *Cell Calcium* **11**, 611–624
- Meldolesi, J., Clementi, E., Fasolato, C., Zacchetti, D. and Pozzan, T. (1991) *Trends Pharmacol. Sci.* **12**, 289–291
- Sage, S. O., Reast, R. and Rink, T. J. (1990) *Biochem. J.* **265**, 675–680
- Mahaut-Smith, M. P., Sage, S. O. and Rink, T. J. (1990) *J. Biol. Chem.* **265**, 10479–10483
- Mahaut-Smith, M. P., Rink, T. J. and Sage, S. O. (1990) *J. Physiol. (London)* **434**, 38P

- 13 Sargeant, P., Clarkson, W. D., Sage, S. O. and Heemskerk, J. W. M. (1992) *Cell Calcium* **13**, 553–564
- 14 Alonso, M. T., Alvarez, J., Montero, M., Sanchez, A. and Garcia-Sancho, J. (1991) *Biochem. J.* **280**, 783–789
- 15 Sage, S. O., Merritt, J. E., Hallam, T. J. and Rink, T. J. (1989) *Biochem. J.* **258**, 923–926
- 16 Sage, S. O. and Rink, T. J. (1987) *J. Biol. Chem.* **262**, 16364–16369
- 17 Sargeant, P., Farndale, R. W. and Sage, S. O. (1993) *FEBS Lett.* **315**, 242–246
- 18 Doni, M. G., Deana, R., Bertocello, S., Zoccarato, F. and Alexandre, A. (1988) *Biochem. Biophys. Res. Commun.* **156**, 1316–1323
- 19 Pollock, W. K. and Rink, T. J. (1986) *Biochem. Biophys. Res. Commun.* **139**, 308–314
- 20 Di Virgilio, F., Fasolato, C. and Steinberg, T. H. (1988) *Biochem. J.* **256**, 959–963
- 21 Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- 22 Sage, S. O., Merritt, J. E., Hallam, T. J. and Rink, T. J. (1989) *Biochem. J.* **257**, 923–926
- 23 Yoshida, K. and Nachmias, V. T. (1989) *Cell Calcium* **10**, 299–307
- 24 Cavallini, L. and Alexandre, A. (1994) *Eur. J. Biochem.* **222**, 693–702
- 25 Brass, L. F. (1984) *J. Biol. Chem.* **259**, 12563–12570
- 26 Geiger, J., Nolte, C., Butt, E., Sage, S. O. and Walter, U. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1031–1035
- 27 Lerea, K. M., Glomset, J. A. and Krebs, E. G. (1987) *J. Biol. Chem.* **262**, 282–288
- 28 Lerea, K. M. and Glomset, J. A. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5620–5624
- 29 Lapetina, E. G., Billah, M. M. and Cuatrecasas, P. (1981) *Nature (London)* **292**, 367–369
- 30 Watson, S. P., McConnell, R. T. and Lapetina, E. G. (1984) *J. Biol. Chem.* **259**, 13199–13203
- 31 Lapetina, E. G. (1986) *FEBS Lett.* **195**, 111–114
- 32 Knight, D. E. and Scrutton, M. C. (1984) *Nature (London)* **309**, 66–68
- 33 Pannocchia, A. and Hardisty, R. M. (1985) *Biochem. Biophys. Res. Commun.* **127**, 339–345
- 34 Siess, W. and Lapetina, E. G. (1989) *Biochem. J.* **258**, 57–65
- 35 Takai, Y., Kaybuki, K., Matsubara, T. and Nishizuka, Y. (1981) *Biochem. Biophys. Res. Commun.* **101**, 61–67
- 36 Kawahara, Y., Yamanishi, J. and Fukuzaki, H. (1984) *Thromb. Res.* **33**, 203–209
- 37 Nakashima, S., Tohatsu, T., Hattori, H., Okano, Y. and Nozawa, Y. (1986) *Biochem. Biophys. Res. Commun.* **135**, 1099–1104
- 38 Deana, R., Ruzzene, M., Doni, M. G., Zoccarato, F. and Alexandre, A. (1989) *Biochim. Biophys. Acta* **1014**, 203–206
- 39 Doni, M. G., Deana, R., Padoin, E., Ruzzene, M. and Alexandre, A. (1991) *Biochim. Biophys. Acta* **1094**, 323–329
- 40 Doni, M. G., Alexandre, A., Padoin, E., Francesconi, M. A. and Deana, R. (1993) *Arch. Biochem. Biophys.* **301**, 431–438
- 41 Maurice, D. H. and Haslam, R. J. (1990) *Mol. Pharmacol.* **37**, 671–681
- 42 Moos, M., Jr. and Goldberg, N. D. (1988) *Second Messengers Phosphoproteins* **12**, 163–170
- 43 Tomatsu, T., Nichida, A., Nagao, S., Nakashima, S. and Nozawa, Y. (1989) *Biochim. Biophys. Acta* **1013**, 190–193
- 44 Quinton, T. M. and Dean, W. L. (1992) *Biochem. Biophys. Res. Commun.* **184**, 893–899
- 45 Kaser-Glanzmann, R., Gerber, E. and Lusher, E. (1979) *Biochim. Biophys. Acta* **558**, 344–347
- 46 Enouf, J., Bredoux, R., Boucheix, C., Mirshahi, M., Soria, C. and Levy-Toledano, S. (1985) *FEBS Lett.* **183**, 398–402
- 47 Aduniah, S. E. and Dean, W. L. (1987) *Biochim. Biophys. Acta* **930**, 401–409
- 48 Hettasch, J. M. and LeBreton, G. C. (1987) *Biochim. Biophys. Acta* **931**, 49–58
- 49 Enouf, J., Giraud, F., Bredoux, R., Bourdeau, N. and Levy-Toledano, S. (1987) *Biochim. Biophys. Acta* **928**, 76–82
- 50 Tao, J., Johansson, J. S. and Haynes, D. (1992) *Biochim. Biophys. Acta* **1105**, 29–39
- 51 White, G. C., Barton, D. W., White, T. E. and Fisher, T. (1989) *Thromb. Res.* **56**, 575–581
- 52 O'Rourke, F., Zavoico, G. B. and Feinstein, M. B. (1989) *Biochem. J.* **257**, 715–721
- 53 Authi, K. F., Bokkala, S., Patel, Y., Kakkar, V. V. and Muntonge, F. (1993) *Biochem. J.* **294**, 119–126
- 54 Papp, B., Enyedi, Á., Kovács, T., Sarkadi, B., Wuytack, F., Thastrup, O., Gárdos, G., Bredoux, R., Levy-Toledano, S. and Enouf, J. (1991) *J. Biol. Chem.* **266**, 14593–14596
- 55 Papp, B., Pászty, K., Kovács, T., Sarkadi, B., Gárdos, G., Enouf, J. and Enyedi, Á. (1993) *Cell Calcium* **14**, 531–538
- 56 Randriamampita, C. and Tsien, R. Y. (1993) *Nature (London)* **364**, 809–814