Two classes of agonist-sensitive Ca^{2+} stores in platelets, as identified by their differential sensitivity to 2,5-di-(tert-butyl)-1,4-benzohydroquinone and thapsigargin

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In the absence of extracellular Ca^{2+} , extensive Ca^{2+} release from the platelet intracellular stores [monitored as an increase of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)] is produced by the combined action of the endomembrane Ca^{2+} -ATPase inhibitor thapsigargin and 2 nM ionomycin. The titration of Ca^{2+} unloading with thapsigargin (plus ionomycin) shows that a substantial fraction of the store-associated Ca^{2+} is released by 8–10 nM thapsigargin, but that 100–200 nM thapsigargin is required for the complete release. The store depletion obtained in similar conditions with a different endomembrane Ca^{2+} -ATPase

inhibitor, 2,5-di-(tert-butyl)-1,4-benzohydroquinone (TBHQ), is always incomplete. It is completed by thrombin or by 10 nM thapsigargin. We conclude that two different types of Ca^{2+} pumps exist in platelets, one sensitive to TBHQ and to high thapsigargin, the other insensitive to TBHQ and sensitive to low thapsigargin. They are distributed separately in discrete subpopulations of the agonist-sensitive stores. The influx of external Ca^{2+} is maximal when both types of stores are Ca^{2+} -depleted, either by high thapsigargin or by the combined action of low thapsigargin and TBHQ.

INTRODUCTION

High amounts of Ca^{2+} are present in platelets (about 20 nmol per 10^8 cells). Most of this Ca^{2+} is associated with the exocytosable granules and does not appear to communicate with the cytosolic compartment. Only a minor fraction (about 1%) of the total platelet Ca^{2+} is retained within the endoplasmic reticulum storage sites and is sensitive to the action of the agonists [1,2].

Following the increase of the Ca^{2+} -release mediator InsP_3 , the agonist-sensitive deposits release their Ca^{2+} content into the cytosol. The depletion of the stores activates the opening of Ca^{2+} influx channels in the plasma membrane [3,4]. Furthermore, agonists such as ADP and thrombin also activate directly the opening of Ca^{2+} influx channels [5–7].

Selective inhibitors [thapsigargin and 2,5-di-(tert-butyl)-1,4-benzohydroquinone (TBHQ)] of the endomembrane Ca^{2+} -ATPase responsible for the accumulation of Ca^{2+} within the agonist-sensitive stores have been introduced recently [8–13]. In aspirinated platelets they activate the release into the cytosol of some 50% of the agonist-releaseable Ca^{2+} [14].

In platelet membrane vesicles, the ATP-dependent Ca^{2+} uptake exhibits a dual sensitivity to thapsigargin, a fraction of the activity being inhibited at low concentrations of the inhibitor, but full inhibition being achieved at comparatively much higher concentrations. This led to the identification of two distinct endomembrane sarco-endoplasmic reticulum Ca^{2+} -ATPases (SERCA), with molecular masses of 100 and 98 kDa [15]. Low concentrations of thapsigargin mainly inhibited phosphoenzyme formation of the 100 kDa pump while TBHQ was active on the 98 kDa species [16]. The 100 and 98 kDa proteins were characterized as SERCA 2b and SERCA 3 [17,18].

The present research was undertaken to determine whether

functional signs of the activity of the two Ca^{2+} pumps could be detected in whole platelets.

MATERIALS AND METHODS

Materials

Thrombin, prostacyclin (PGI_2), apyrase, hirudin, Indo 1-AM, ionomycin, thapsigargin, and TBHQ were purchased from Sigma or Calbiochem. All other reagents were of analytical grade.

Platelet preparation

Platelet-rich plasma (PRP) and washed platelets were prepared as previously reported [19] from freshly drawn blood obtained from healthy volunteers and mixed with acid citrate–dextrose anticoagulant supplemented with hirudin (50 munits/ml), apyrase (80 munits/ml) and PGI_2 (0.2 $\mu\text{g}/\text{ml}$).

Determination of cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)

The $[\text{Ca}^{2+}]_i$ was determined with Indo 1-AM essentially according to Pollock and Rink and by using a K_d value of 250 nM [20]. The Ca^{2+} fluorescent probe (2 μM) was added to aspirinated PRP, and loading was performed for 30 min at 37 °C. After centrifugation the pellet was resuspended in 145 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 20 mM Na-Hepes, 10 mM glucose, pH 7.4, in the presence of 0.1 mM sulphinpyrazone [21], 40 munits/ml of apyrase and 5 munits/ml of hirudin. The platelets were used at a concentration of $(5\text{--}7) \times 10^7$ cells/ml. All the experiments were

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; InsP_3 , myo-inositol 1,4,5-trisphosphate; PGI_2 , prostacyclin; PRP, platelet-rich plasma; SERCA, sarco-endoplasmic reticulum Ca^{2+} -ATPase; TBHQ, 2,5-di-(tert-butyl)-1,4-benzohydroquinone.

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performed in the presence 0.5 mM EGTA. Fluorescence was measured at 37 °C in a thermostatically controlled, magnetically stirred cuvette, in a Shimadzu RL-5000 spectrofluorimeter with excitation and emission wavelengths set at 340 nm and 400 nm.

RESULTS AND DISCUSSION

Extensive depletion of the Ca^{2+} stores is induced by the combined action of thapsigargin and low ionomycin

The treatment of aspirinated platelets incubated in EGTA-supplemented, Ca^{2+} -free media with maximally effective concentrations of the endomembrane Ca^{2+} -ATPase inhibitor thapsigargin induces the relatively rapid release into the cytosol of a fraction (about 50%) of the Ca^{2+} associated with the intracellular Ca^{2+} stores [14]. The remaining Ca^{2+} is released by the subsequent treatment with thrombin or with the Ca^{2+} ionophore ionomycin [2,7]. These observations were taken as evidence that two types of Ca^{2+} stores exist in platelets, exhibiting high and low Ca^{2+} leakages in basal conditions. The low-leak stores do not release their Ca^{2+} content readily upon inhibition of their Ca^{2+} pump, unless their permeability to Ca^{2+} is increased by the agonist (which produces InsP_3) or by ionomycin.

If the concentration of ionomycin is sufficiently low, the increased leakage from the stores is small. In these conditions ionomycin *per se* increases only slightly the platelet basal $[\text{Ca}^{2+}]_i$. It induces, however, an extensive discharge of the stores-associated Ca^{2+} upon inhibition of the endomembrane Ca^{2+} -ATPase. This is shown in Figure 1(a), where a complete discharge of the stores-associated Ca^{2+} was induced by a very low concentration of ionomycin (2 nM), added after the partial elevation of $[\text{Ca}^{2+}]_i$ induced by maximal (200 nM) thapsigargin. That the discharge was complete was shown by the lack of any further increase of $[\text{Ca}^{2+}]_i$ upon addition of thrombin. In control experiments, 2 nM ionomycin alone increased $[\text{Ca}^{2+}]_i$ by only 30–40 nM (results not shown).

Two different subsets of Ca^{2+} deposits are distinguished by their sensitivity to thapsigargin and TBHQ

Ca^{2+} release, and its stimulation by 2 nM ionomycin, are visible with thapsigargin concentrations as low as 2 nM (Figure 1b). The increment of $[\text{Ca}^{2+}]_i$ induced by the combined action of thapsigargin and ionomycin increases sharply at 5 and 12 nM

Figure 1 The action of thapsigargin (Tg) and ionomycin on the unloading of Ca^{2+} from the intracellular stores

When indicated ionomycin (Iono) was 2 nM and thrombin (Thr) 0.25 unit/ml. The traces are representative of duplicate experiments of at least five different preparations with similar results.

Figure 2 Titration of the agonist-sensitive Ca^{2+} stores by thapsigargin

Data are taken from a number of traces as in Figure 1, obtained from four different platelet preparations. The points represent the values of $[\text{Ca}^{2+}]_i$ observed after the addition of thapsigargin (■) followed by 2 nM ionomycin (●) and finally by 0.25 unit/ml thrombin (▲).

Figure 3 Partial unloading of the platelet Ca^{2+} stores by TBHQ and its completion by thrombin or thapsigargin

Conditions as in Figure 1.

thapsigargin (Figures 1c and 1d). It is not maximal, however, since further Ca^{2+} release is induced by the subsequent addition of thrombin. As much as 200 nM thapsigargin is required to promote the complete depletion of the stores (Figure 1a). This is best depicted in Figure 2 which reports the values of $[\text{Ca}^{2+}]_i$ obtained from a number of traces as in Figure 1. From these results it appears that a fraction of the thapsigargin-sensitive stores is sensitive to very low concentrations of the inhibitor, while the rest of the stores releases Ca^{2+} only at significantly higher concentrations. These observations are in agreement with the report [16] that a fraction of the platelet membrane vesicles-associated Ca^{2+} -ATPase activity is inhibited only at relatively high thapsigargin concentrations.

When the release of Ca^{2+} from the stores is induced with TBHQ rather than thapsigargin as the inhibitor of the endomembrane Ca^{2+} -ATPase activity, the increment of $[\text{Ca}^{2+}]_i$ induced by the combined addition of TBHQ and 2 nM ionomycin increases progressively to a maximum (at 10–12 μM TBHQ) which is about 150 nM lower than that reached with 200 nM thapsigargin. Furthermore, with TBHQ the subsequent addition of thrombin invariably induces a further increase of $[\text{Ca}^{2+}]_i$ (Figure 3c). That a fraction of the agonist-sensitive stores is

Figure 4 Titration of the agonist-sensitive Ca^{2+} stores by TBHQ

Data are taken from a number of traces as in Figure 3, obtained from four different platelet preparations. The points represent the values of $[\text{Ca}^{2+}]_i$ observed after the addition of TBHQ (■) followed by 2 nM ionomycin (●) and finally by 0.25 unit/ml thrombin (▲).

Figure 5 Ca^{2+} influx after the depletion of the stores with low thapsigargin, TBHQ, and their combination

Conditions are as in Figure 1. The first addition of ionomycin (lono) was 2 nM and the second was 100 nM; CaCl_2 was 0.8 mM.

insensitive to the action of TBHQ is also indicated by the finding that after treatment with maximal TBHQ (plus ionomycin) a further increase of $[\text{Ca}^{2+}]_i$ is induced by adding low concentrations (10 nM) of thapsigargin (Figure 3d). The thapsigargin-induced increase of $[\text{Ca}^{2+}]_i$ is similar to that observed with thrombin and is not increased further by thrombin. A complete dose-response curve of the TBHQ titration of the platelet Ca^{2+} stores is reported in Figure 4.

These results show that TBHQ releases Ca^{2+} from a discrete subfraction of the platelet agonist-sensitive Ca^{2+} stores. Ca^{2+} release from this fraction is also induced by high, but not by low, concentrations of thapsigargin. On the contrary, low concentrations of thapsigargin promote Ca^{2+} release from the TBHQ-insensitive stores.

Taking into account the findings in [15–17] it is likely that Ca^{2+} pumping in the TBHQ-sensitive stores is operated by the 97 kDa SERCA 3 isoform, while the 100 kDa SERCA 2b pump should be involved in Ca^{2+} pumping into the TBHQ-insensitive (and highly thapsigargin-sensitive) deposits.

Our results also imply that the two different types of Ca^{2+} pumps are largely distributed separately in discrete subpopulations of the agonist-sensitive stores.

In platelet membrane vesicles the 100 kDa Ca^{2+} pump was reported to accumulate Ca^{2+} in an InsP_3 -resistant pool [16]. Since both stores are readily depleted by thrombin in whole platelets, this may indicate that another Ca^{2+} -release signal molecule besides InsP_3 is formed by this agonist, specifically involved in Ca^{2+} release from the 100 kDa pump-containing deposits.

Both types of stores, as discriminated by their sensitivity to thapsigargin and TBHQ, release some Ca^{2+} when their Ca^{2+} -ATPase is inhibited (by TBHQ or by low thapsigargin respectively) in the absence of ionomycin. Therefore, they both exhibit an ionomycin-independent and an ionomycin-dependent component. This may indicate that each type of store is in fact further subdivided into two subcomponent vesicle species, i.e. high leak and low leak. Alternatively, and maybe more likely, the Ca^{2+} leakage from each type of store may be controlled by the filling state of the store itself, being relatively high when the store is full and decreasing readily once the intravesicular concentration falls below a certain limiting value.

Ca^{2+} influx is controlled by the filling state of both Ca^{2+} deposits

In platelets, the influx of Ca^{2+} from the suspending medium is activated by the agonists as well as by the depletion of the stores. ADP opens, in less than 20 ms, a Ca^{2+} influx pathway [5]. Likewise, thrombin induces, though on a longer time scale, a Ca^{2+} influx that precedes by only a few milliseconds the depletion of the stores [6]. Such an influx, which is sensitive to elevations of cyclic AMP and cyclic GMP, may also be observed in situations when the stores are not depleted [7]. In the absence of agonists, the depletion of the stores can be induced by the inhibition of the endomembrane Ca^{2+} -ATPase. In these conditions, the store-controlled Ca^{2+} influx is the sole mode of Ca^{2+} entry. Such an entry is insensitive to PGI_2 and the nitric oxide generator sodium nitroprusside [7].

The experiments reported in Figure 5 were performed to determine how the two types of platelet Ca^{2+} deposits, as identified above, participate in the control of the influx of Ca^{2+} . To this end external Ca^{2+} was supplemented after depletion of the highly thapsigargin-sensitive and the TBHQ-sensitive stores, separately (Figure 5, traces a and b) and in combination (trace c). To better discriminate the differential effect of the selective depletion of the deposits, external Ca^{2+} was added at a relatively low concentration (300 μM free Ca^{2+}). As shown in Figure 5, the depletion of each type of store individually activates a fraction of the overall Ca^{2+} influx activity, and the maximal Ca^{2+} influx is observed when TBHQ and low (10 nM) thapsigargin are present together. In the latter condition, no further increment of $[\text{Ca}^{2+}]_i$ is observed upon addition of excess of ionomycin (trace c).

The mechanism by which the depletion of the deposits controls Ca^{2+} influx is presently unknown. A possible candidate could be a small phosphorylated diffusible mediator which was proposed to move in and out of the deposits together with Ca^{2+} . This mediator would open the Ca^{2+} influx channel directly [22–24]. Small monomeric GTP-binding proteins [25,26] and cyclic GMP [27] have also been implicated. Alternatively, the link could be via a direct protein–protein interaction between a plasma membrane Ca^{2+} channel and a regulatory protein situated in the stores [28,29].

Whatever the mechanism of the control of the Ca^{2+} influx channel by the depletion of the stores, it may be concluded that the emptying of each type of Ca^{2+} deposit activates a fraction of the overall store-controlled Ca^{2+} -influx activity.

Conclusion

In summary, the results presented in this study show that human platelets possess two distinct types of agonist-sensitive Ca^{2+} deposits, one depleted by TBHQ (or by high thapsigargin concentrations), the other by low concentrations of thapsigargin, but not by TBHQ. This is in accordance with the notion that platelets contain two distinct types of Ca^{2+} pumps of 100 and 98 kDa, characterized by a differential sensitivity to the inhibitors TBHQ and thapsigargin. Each Ca^{2+} -storage vesicle possesses only (or prevalently) one type of Ca^{2+} -ATPase. The depletion of both types of Ca^{2+} deposits is required to activate Ca^{2+} influx maximally.

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