Thapsigargin-induced Calcium Influx in the Absence of Detectable Tyrosine Phosphorylation in Human Platelets*

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Tyrosine phosphorylation is a potential mechanism for mediating store-operated calcium (SOC) influx in platelets and other nonexcitable cells. Thapsigargin induces calcium-dependent tyrosine phosphorylation and SOC influx in platelets. We prevented thapsigargin-induced tyrosine phosphorylation by buffering cytosolic calcium rise with the calcium chelator 1,2-bis(o-aminophenoxo)ethane-N,N,N',N'-tetraacetic acid-acetomethoxyester (BAPTA-AM). Calcium influx, induced by thapsigargin and measured by 45Ca2+ accumulation, persisted in BAPTA-loaded platelets in the absence of tyrosine phosphorylation. This calcium influx was blocked by the SOC influx inhibitor SKF-96365. Tyrosine kinase inhibitors have been used to demonstrate a role for tyrosine phosphorylation in SOC influx. We compared the effects of four tyrosine kinase inhibitors genistein, methyl-2,5-dihydroxycinnamate (erbstatin analog), tyrphostin A47, and lavendustin A, on thapsigargin-induced tyrosine phosphorylation in control platelets and on thapsigargin-induced SOC influx into BAPTA-loaded platelets in absence of tyrosine phosphorylation. Tyrphostin A47 prevented all measurable tyrosine phosphorylation in control platelets, but did not decrease calcium influx into BAPTA-loaded platelets. Genistein and the erbstatin analog were poor inhibitors of tyrosine phosphorylation but decreased SOC influx into BAPTA-loaded platelets to 55.8 ± 3% and 51.9 ± 7.5% of control, respectively. Lavendustin A did not decrease tyrosine phosphorylation or calcium influx. Thus, thapsigargin-induced SOC influx can occur without detectable tyrosine phosphorylation and the inhibition of SOC influx by tyrosine kinase inhibitors does not correlate with their ability to prevent tyrosine phosphorylation.

Prolonged calcium influx in nonexcitable cells, such as platelets, is regulated by levels of calcium remaining in calcium stores (1). This calcium influx is referred to as store-operated calcium (SOC) influx (2). The mechanisms facilitating the connection between calcium stores and changes in calcium permeability to plasma membrane are unknown. A variety of second messengers have been suggested to mediate this inter-

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‡ The abbreviations used are: SOC, store-operated calcium; BAPTA-AM, 1,2-bis(o-aminophenoxo)ethane-N,N,N',N'-tetraacetic acid-acetomethoxyester; MeSO, dimethyl sulfoxide.

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healthy human volunteers and stored as platelet-rich plasma overnight at room temperature in PL 146 plastic storage bags (Baxter Healthcare Corp., Deerfield, IL) with constant rotation. Ten ml of platelet-rich plasma were removed from the bags, treated with prostaglandin E₁ (1 µM) and centrifuged at 750 × g for 3 min to remove red and white blood cells. The platelet-containing supernatant was immediately gel-filtered on Sepharose 2B (Pharmacia LKB, Uppsala, Sweden) equilibrated with Tyrode’s balanced salt solution without calcium and magnesium (in mM: NaCl, 138; KCl, 2.9; NaH₂CO₃, 12; NaH₂PO₄, 0.4; dextrose, 5.0; Heps, 5.0; BSA, Fraction V, fatty acid-free, 0.35%, pH 7.4). After filtration platelets were diluted to a count of 400,000/µl and allowed to rest for 30 min at room temperature prior to initiation of studies. Experiments were also carried out on fresh platelets collected by venipuncture into 3.8% sodium citrate (9 parts blood:1 part citrate). Platelet-rich plasma was obtained by centrifugation of whole blood at 750 × g for 3 min, and platelets were gel-filtered as described above. Freshly collected platelets produced the same results on calcium influx and tyrosine phosphorylation as pheresed platelets.

### Calcium Influx into Platelets—Apyrase (2 units/ml) and indomethacin (1 µM) were added to gel-filtered platelets for 15 min at room temperature. The cells were then divided, and one-half was treated with BAPTA-AM (30 µM) in dimethyl sulfoxide (Me₂SO) or another calcium chelator for 30 min. These cells were then divided, and thapsigargin (1 µM) was added to one-half, while Me₂SO was added to the other half. Platelets not treated with calcium chelators received equal volume of Me₂SO (final concentration of all Me₂SO was 0.02%) and were otherwise treated the same. After 10 min, calcium 45 (45Ca²⁺) (50 µM, 71 Cl⁻, Amersham Corp.) and MgCl₂ (0.5 mM) were added to all platelets, and immediately aliquots were collected at time 0 and the time at indicated time points for determination of internalized 45Ca²⁺. For experiments with 2 mM extracellular calcium, additional nonradioactive calcium was added to make up the final concentration.

Calcium accumulation inside cells was assessed by collecting 30 µl of platelet suspension and mixing it with an equal volume of EGTA (2 mM, pH 7.4) to strip off calcium bound to the outside of cells. Forty-five µl of this mixture was then filtered on 0.65 µm filters (Multiscreen Durapore, Millipore, Bedford, MA) prewetted with Tyrode’s balanced salt solution. The filters were washed four times with 200 µl of Tyrode’s solution, air-dried, cut out, mixed with scintillation fluid, and their radioactivity was determined in a scintillation counter (Beckman LS 3801, Beckman Instruments, Fullerton, CA). Each time point was collected and processed in duplicate.

### Detection of Tyrosine Phosphorylation—Samples of platelet suspension (75 µl) for determination of tyrosine phosphorylation were collected at indicated time points, mixed with equal volume of preheated (95 °C) SDS-polyacrylamide gel electrophoresis sample buffer (20% glycerol, 0.125 M Tris-HCl, pH 6.8, 0.4% SDS), and boiled for 3 min. Proteins were separated on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with a monoclonal anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology, Inc., Lake Placid, NY) as described previously (9). For experiments testing the efficacy of tyrosine kinase inhibitors on thapsigargin-induced tyrosine phosphorylation, the gel-filtered platelets with either no calcium added or with 2 mM calcium were mixed with the inhibitors and allowed to sit for 10 or 30 min. Thapsigargin (1 µM) was then added, and samples were collected 10 min later. Tyrosine phosphorylation in the presence of tyrosine kinase inhibitors was quantitated by digitizing the autoradiographs with a FluoroSlide system (Fotodyne, Hartland, WI) and analyzing the images with NIH Image software.

### RESULTS AND DISCUSSION

Adding thapsigargin to gel-filtered platelets induces pronounced tyrosine phosphorylation of proteins with molecular masses of 120, 80, and 65 kDa, along with less pronounced tyrosine phosphorylation of 117-, 95-, and 85-kDa proteins (Fig. 1). A constitutively tyrosine phosphorylated protein at 60 kDa most likely is p60⁵⁶k, a tyrosine kinase (20). The new onset of tyrosine phosphorylation is due to elevation of cytosolic calcium by thapsigargin (9, 21, 22) secondary to inhibition of the sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA) (23). It is not due to release of endogenous platelet agonists, ADP and thromboxane (24), since inhibitors for these agonists, apyrase and indomethacin, are present in the medium. To prevent tyrosine phosphorylation dependent on elevation of cytosolic calcium, we used three intracellular calcium chelators, EGTA-AM, BAPTA-AM, and difluoro-BAPTA-AM and compared their ability to prevent thapsigargin-induced tyrosine phosphorylation. These calcium buffers differ in their affinity for calcium with a Kᵦ of 67 nM for EGTA (25), 192 nM for BAPTA (26), and 708 nM for difluoro-BAPTA (27). Platelets were incubated with 30 µM concentrations of each chelator for 30 min followed by addition of thapsigargin and determination of tyrosine phosphorylation 45 min later. The effect of 50 µM extracellular calcium on thapsigargin-induced tyrosine phosphorylation in control cells and chelator-treated cells was also tested, since SOC influx was measured at these calcium concentrations. BAPTA-AM prevented thapsigargin-induced tyrosine phosphorylation in platelets with no extracellular calcium added, indicating that it successfully buffered the release of stored calcium by thapsigargin. Moreover, BAPTA-AM also prevented thapsigargin-induced tyrosine phosphorylation in presence of 50 µM calcium, thus demonstrating sufficient buffering capacity to buffer the influx of this concentration of extracellular calcium for 45 min. In comparison, EGTA-AM was not effective at preventing tyrosine phosphorylation, while difluoro-BAPTA-AM was only slightly less effective than BAPTA-AM. The reason that EGTA-AM was not able to inhibit tyrosine phosphorylation may be because it did not accumulate intracellularly to the same extent as BAPTA-AM.
To mimic the effects of physiological levels of extracellular calcium, we added 2 mM calcium to the BAPTA-loaded platelets either with or without prior activation with thapsigargin. Within 5 min, tyrosine phosphorylation of proteins with molecular masses of 120, 80, and 65 kDa reached levels found in control platelets activated with thapsigargin (Fig. 1B). This observation indicates that calcium influx from 2 mM extracellular calcium can easily saturate 30 μM concentration of BAPTA and further demonstrates that tyrosine phosphorylation of this set of proteins depends on elevation of cytosolic calcium regardless of whether it is released from internal stores or occurs through extracellular influx.

Since BAPTA inhibited thapsigargin-induced tyrosine phosphorylation, even in the presence of 50 μM extracellular calcium, we investigated the effect of BAPTA on thapsigargin-induced calcium influx. Platelets were loaded with BAPTA-AM and then treated with thapsigargin for 10 min prior to addition of 50 μM extracellular 45Ca2+. Influx was measured as accumulation of calcium over 45 min and is expressed in nanomoles/10^10 cells (Fig. 2). Without BAPTA, platelets accumulated 3.44 ± 0.37 nmol/10^10 cells and this was increased to 7.79 ± 0.82 nmol/10^10 cells by thapsigargin. This calcium accumulation underestimates the extent of calcium influx, since plasma membrane calcium ATPases can remove cytosolic calcium back into the medium (28). With BAPTA present, internalized calcium is chelated and trapped inside the cells thus giving a better estimate of calcium influx. The extent of 45Ca2+ accumulation in BAPTA-treated cells is 8.85 ± 1.16 nmol/10^10 cells and 18.3 ± 3.1 nmol/10^10 cells in BAPTA- and thapsigargin-treated cells (Fig. 2).

In platelets, calcium influx mediated by the SOC mechanism was fully inhibited by the compound SKF-96365 (29). Influx of calcium into BAPTA-loaded platelets was also inhibited by SKF-96365, indicating that calcium crosses the plasma membrane by the same pharmacologically defined mechanism. The initial incubation of platelets with BAPTA without extracellular calcium partially depletes internal calcium stores (30), which increases plasma membrane permeability to calcium by a SOC mechanism. Adding thapsigargin to BAPTA-loaded cells further potentiated calcium influx by additional depletion of internal calcium stores through inhibition of calcium pumps.
and was also fully inhibited by SKF-96365 (Fig. 2). Thus, calcium influx mediated by the SOC mechanism occurred in BAPTA-loaded platelets where there was no detectable increase in tyrosine phosphorylation of platelet proteins, suggesting that a tyrosine phosphorylation step is not required for influx.

Tyrosine phosphorylation in platelets has been reported to occur in periodic waves with certain proteins being rapidly phosphorylated and dephosphorylated (31). To determine whether there was any tyrosine phosphorylation during the course of calcium influx, we measured calcium accumulation and tyrosine phosphorylation simultaneously in the same set of platelets. In BAPTA-loaded cells no increase in protein tyrosine phosphorylation was evident at 10, 20, 30, and 45 min (Fig. 3A). At the same time, thapsigargin-induced accumulation of intracellular calcium rose above control levels from 5 to 45 min in 50 μM extracellular calcium (Fig. 3B). With 2 mM calcium, thapsigargin-induced accumulation of calcium above control levels was already evident at 2 min (Fig. 3C). At both calcium concentrations, the thapsigargin-induced accumulation was linear over time with a slight upward curve, indicating that it has not been saturated in the time frame tested.

The hypothesis that tyrosine phosphorylation directly regulates SOC influx has been supported by the observation that tyrosine kinase inhibitors decrease SOC influx (10, 11, 14–17). We wanted to test whether the tyrosine kinase inhibitors commonly used in these studies would also inhibit SOC influx in absence of a detectable increase in tyrosine phosphorylation. First we tested the effect of structurally distinct tyrosine kinase inhibitors, genistein, tyrphostin A47, lavendustin A, and 2,5-dihydroxycinnamate (erbstatin), and their respective analogs inactive against tyrosine kinases, daidzen, tyrphostin A1, and lavendustin B, on thapsigargin-induced platelet tyrosine phosphorylation. Genistein, at 100 μM, did not inhibit tyrosine phosphorylation without extracellular calcium present but did slightly decrease tyrosine phosphorylation when extracellular calcium was available for influx (Fig. 4A). Extending the incubation of platelets with genistein to 30 min did not increase its effectiveness. Both lavendustin A and the erbstatin analog decreased thapsigargin-induced tyrosine phosphorylation at 100 and 200 μM in the absence of extracellular calcium (Fig. 4B) but were not effective inhibitors at the same concentrations when 2 mM calcium was present (Fig. 4C). In comparison, tyrphostin A47 had the best efficacy as an inhibitor of thapsigargin-induced increase in tyrosine phosphorylation at both 50 and 100 μM concentrations (Fig. 4D). Presence of 2 mM extracellular calcium did not change the effect of this tyrphostin.

The inhibitory effects of the tyrosine kinase inhibitors on thapsigargin-induced tyrosine phosphorylation in the presence of 2 mM calcium were quantitated from three separate blots by

Fig. 4. Effect of tyrosine kinase inhibitors on thapsigargin-induced tyrosine phosphorylation in platelets. Inhibitors, at indicated concentrations, were incubated with platelets for 10 min prior to addition of thapsigargin (TG) (1 μM) and determination of tyrosine phosphorylation. Thirty-minute incubations with tyrosine kinase inhibitors produced the same results. A, genistein, 25, 50, and 100 μM. B, lavendustin A (Laven A) 100 and 200 μM; lavendustin B (Laven B), 100 and 200 μM; erbstatin analog (erbstat) 100 and 200 μM, with no added extracellular calcium (C). D, tyrphostins A47, 50 and 100 μM (Tyrph 47, 50, and 100); and Tyrphostin A1, 100 μM (TyrphA1 100).
Phosphate metabolism by inhibiting phosphatidylinositol kinase inhibitors. The most frequently used tyrosine kinase inhibitors have a number of actions that apparently are not related to tyrosine phosphorylation (32–35). Finally, the effects of genistein and erbstatin analog both, of which had not detectable (Fig. 6). With 50 μM extracellular calcium, genistein (100 μM) and erbstatin (200 μM) inhibited thapsigargin-induced 45Ca2⁺ influx to 55.8 ± 3.0% and 51.9 ± 7.5% (mean ± S.E., n = 3) of control influx, respectively, while tyrophostin A47 (100 μM) and lavendustin A (200 μM) were not inhibitory. When extracellular calcium was increased to 2 mM, only genistein remained inhibitory, although its effect was decreased to 79.5 ± 19.5% (mean ± S.E., n = 3) of control. Both erbstatin and lavendustin A slightly potentiated SOC influx to 126.4 ± 26.4% and 126.3 ± 22.3% of control at 2 mM calcium. The observations on decreased inhibition by genistein and erbstatin analog at high calcium suggest that either these compounds compete with calcium at the channel site or a separate additional calcium influx mechanism induced by thapsigargin and not blocked by these compounds is present at 2 mM calcium. The latter possibility is more likely, since even SKF-96365 was less effective at 2 mM calcium (59.8 ± 9.9% of control) than at 50 μM calcium (0% of control), and SKF-96365 has been shown not to compete with calcium (29).

The arguments for tyrosine phosphorylation mediating capacitative calcium influx have been based on observations that 1) thapsigargin induces tyrosine phosphorylation of specific proteins and that 2) some tyrosine kinase inhibitors inhibit SOC influx in a variety of cells. The weakness of this argument lies in the relative nonspecificity of some of these tyrosine kinase inhibitors. The most frequently used tyrosine kinase inhibitor in SOC influx experiments has been genistein, which is not a particularly good inhibitor of tyrosine phosphorylation in platelets, as demonstrated in Fig. 4A and as reported by others (32). Genistein has also been found to alter inositol phosphate metabolism by inhibiting phosphatidylinositol 4-monophosphate 5-kinase (33), to block the thromboxane receptor (32) and to inhibit voltage-gated calcium channels (34, 35).

In experiments that did not rely on tyrosine kinase inhibitors, we have now demonstrated that SOC influx into platelets can occur without evidence of increased tyrosine phosphorylation. This was achieved by preventing the rise in cytosolic calcium associated with depletion of calcium stores and suggests that initiation of SOC influx does not require either a rise in cytosolic calcium or an increase in tyrosine phosphorylation. The caveat to this argument is that tyrosine phosphorylation of a key protein could still occur, although at levels too low to detect by our methodology. To deal with this possibility we resorted to the use of tyrosine kinase inhibitors. Of the four that we tested, tyrophostin A47 was by far the best inhibitor of thapsigargin-induced tyrosine phosphorylation in control platelets. It uniformly decreased tyrosine phosphorylation of all proteins to levels less than found in unactivated platelets and was effective regardless of whether extracellular calcium was present or not. However, tyrophostin A47 did not inhibit SOC influx in BAPTA-loaded platelets, suggesting that even additional tyrosine phosphorylation was not involved. On the other hand, genistein and erbstatin analog both, of which had low efficacy against tyrosine phosphorylation, did decrease SOC influx into BAPTA-loaded platelets. Thus, it could be argued that these two drugs are specific inhibitors of a tyrosine kinase, which phosphorylates unidentified protein(s) involved in SOC influx, but we do not consider this likely for several reasons. First, we did not find any evidence (Figs. 4, A–C) that either genistein or erbstatin analog selectively inhibited tyrosine phosphorylation of a subgroup of platelet proteins. Rather, the inhibition produced by these drugs seemed general, like that of tyrophostin A47. Second, the pharmacological properties of these tyrosine kinase inhibitors have not been studied sufficiently to be certain that they do not have other effects that could affect SOC influx. In fact, genistein has been shown to have a number of actions that apparently are not related to tyrosine phosphorylation (32–35). Finally, the effects of genistein and erbstatin analog on the SOC influx were diminished by higher extracellular concentrations of calcium, suggesting that the inhibition produced by these drugs maybe similar to other SOC inhibitors such as econazole (36) and SKF-96365.
In conclusion, we have demonstrated that SOC influx in platelets can occur without detectable tyrosine phosphorylation, which accompanies thapsigargin-induced depletion of the calcium stores. Although we cannot rigorously exclude that SOC influx is dependent on tyrosine phosphorylation of a class of proteins not measurable by current methods, our experiments raise enough doubt about the relationship between tyrosine phosphorylation and SOC influx to consider other potential mechanisms.

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