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CAPACITATIVE Ca^{2+} ENTRY IN HUMAN PLATELETS IS RESISTANT TO NITRIC OXIDE

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SUMMARY: Both sodium nitroprusside (SNP) and a phospholipase C inhibitor U73122, when applied to human platelets after the stimulation with thrombin (0.2 U/ml), caused dose-dependent inhibition of Ca^{2+} influx. The inhibition, however, was not complete for either substance and the U73122-resistant Ca^{2+} influx was also resistant to SNP. Two lines of evidence suggested that the SNP/U73122-resistant Ca^{2+} influx was due to the capacitative Ca^{2+} entry. First, U73122-resistant fraction of Ca^{2+} influx induced by thapsigargin was also resistant to SNP. Second, both U73122 and SNP failed to inhibit the Ca^{2+} influx induced by an acid extract from thrombin-stimulated platelets that contained the Ca^{2+} influx factor activity. We suggest that the capacitative Ca^{2+} entry in human platelets, once triggered by inositol trisphosphate-induced store-depletion, is not affected by nitric oxide.

The anti-aggregatory effect of nitric oxide (NO)-generating agents such as sodium nitroprusside (SNP) is mainly attributed to the inhibitory effect of NO on agonist-induced increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) of platelets (1-3). A strong blood coagulant thrombin induces an increase in the $[Ca^{2+}]i$ of human platelets either by stimulating the Ca^{2+} release from intracellular stores or the Ca^{2+} influx from extracellular space. In contrast to the well-characterized, inositol trisphosphate (IP_3)-dependent signaling which underlines the Ca^{2+} release, the molecular mechanism(s) for the Ca^{2+} influx is still unclear (4). In nonexcitable cells, the presence of at least two biochemical mechanisms involved in the Ca^{2+} influx, however, has been suggested. One is the opening of plasma membrane Ca^{2+} channels by messenger molecules generated by receptor activation; the candidate molecules include IP_3 or its metabolites (5). This process is, at least in theory, independent of the filling state of the intracellular Ca^{2+} stores. The other is the capacitative Ca^{2+} entry (6) which is triggered by depletion of the intracellular Ca^{2+} stores; recent studies indicated the presence of a diffusible messenger molecule

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termed Ca^{2+} influx factor (CIF) that is either generated or released by store-depletion and opens the plasma membrane Ca^{2+} channels (7,8).

Previous studies described the inhibitory effect of NO on the agonist-induced generation of IP_3 (4), i.e., the activation of phospholipase C (PLC). Therefore, the question to be answered is whether the inhibitory effect of NO against the agonist-induced rise in $[Ca^{2+}]i$ is solely due to the inhibition of PLC or not. Thus the present study was designed to examine the effect of NO on the capacitative Ca^{2+} entry in human platelets.

MATERIALS AND METHODS

Materials: Thapsigargin, hexokinase and thrombin were from Sigma Chemical Co. (St. Louis, MO). Fura-2 acetoxymethyl ester (fura-2/AM) was from Dojin Chemicals (Tokyo, Japan). U73122 (1-(6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl)-1H-pyrrole-2,5-dione) was from Funakoshi (Tokyo, Japan). Calf intestinal alkaline phosphatase was from TOYOBO (Tokyo, Japan). All other reagents were of purist grade available and were obtained commercially.

Preparation of human platelets and measurement of $[Ca^{2+}]i$: Fresh human blood was anti-coagulated with ACD solution containing trisodium citrate (2.5g/dl), citric acid (1.5g/dl) and D-glucose (2g/dl) and was centrifuged at 200 g for 20 min to give platelet rich plasma (PRP). PRP was supplemented with aspirin (100 μM) and then incubated with 4 μM fura-2/AM at 20°C for 45 min. The fura-2-loaded platelets were recovered by centrifugation at 1,200 g for 20 min, washed twice and then resuspended in Ca^{2+}-free Hepes-buffered saline (HBS) (10 mM HEPES pH 7.4, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose) containing 0.1 mM EGTA at the cell density of ~10^8 cells/ml. The fluorescence of fura-2-loaded cells was measured with a CAF-100 spectrofluorometer (Japan Spectroscopy Inc., Tokyo, Japan) with excitations at 340 nm and 380 nm and emission at 500 nm. The $[Ca^{2+}]i$ was calculated from the ratio of fluorescence intensities as described (9).

Extraction of CIF: The acid extract from human platelets which contained the CIF activity was prepared according to the procedures described by Randriamampita and Tsien (6). In brief, platelets were suspended in Ca^{2+}-free HBS/0.1 mM EGTA at the density of ~10^9/ml and then stimulated with thrombin (0.5 U/ml) for 5 min followed by centrifugation at 1,200 g for 5 min. The cell pellet (~0.2 ml packed volume) was suspended in 0.7 ml of Ca^{2+}-free HBS plus 0.1 ml of 1 N HCl and incubated at 25°C for 20 min. Insoluble material was removed by centrifugation and the acid extract was neutralized with 1 N NaOH to give a final volume of ~0.9 ml. The extract was successively treated by hexokinase (2 U/ml at 37°C for 20 min) and then by heat (65°C for 30 min). After clarification by centrifugation, the extract was acidified by the addition of 1% trifluoroacetic acid (TFA) (1:10, v/v) and loaded onto SEP-PAK C18 column (Waters Associates, Milfold, MA). The column was washed with 0.1% TFA and the CIF activity was recovered in 2 ml of 0.1% TFA/50% methanol. The eluate was dried under vacuum, dissolved in 0.1 ml of Ca^{2+}-free HBS and was immediately used for the assay. Where indicated, the extract was treated with calf intestinal phosphatase (1 U/ml) at 37°C for 30 min before use. One hundred μl of the final, reconstituted sample was obtained from a starting cell mass of ~10^9 cells.

RESULTS

Effects of SNP and U73122 on thrombin-induced increase in $[Ca^{2+}]i$. To examine the Ca^{2+} release and influx separately, fura-2-loaded platelets were suspended in Ca^{2+}-free buffer and extracellular Ca^{2+} was replenished 5 min after the stimulation with thrombin. Thrombin caused

dose-dependent increases both in the Ca^{2+} release and influx and there were clear differences in the profiles of the dose-dependency; the concentrations to cause the maximum peak increments of Ca^{2+} release were ~0.5 U/ml and the EC_{50} values were ~0.1 U/ml while the concentrations to cause the maximum peak increments of Ca^{2+} influx were ~1 U/ml and the EC_{50} values were ~0.5 U/ml (Fig. 1). Similar values were obtained when extracellular Ca^{2+} was supplemented 3-10 min after the stimulation (data not shown). There was also a clear difference in the effects of SNP on the thrombin-induced Ca^{2+} release and influx. SNP, when applied after the stimulation with thrombin (0.2 U/ml), caused dose-dependent inhibition of Ca^{2+} influx. The inhibition, however, was not complete; there was always a significant influx even in the presence of extreme concentrations of SNP (up to 3 mM) (Fig. 2A). In contrast, when applied before the stimulation, SNP (100 μM) completely suppressed both the thrombin (0.2 U/ml)-induced Ca^{2+} release from intracellular stores and Ca^{2+} influx across the plasma membrane (data not shown). The same was the case for a PLC inhibitor U73122. The inhibition of Ca^{2+} influx by U73122 was not complete up to the concentration of 5 μM (Fig. 2B), although 5 μM U73122 completely suppressed both the Ca^{2+} release and Ca^{2+} influx (data not shown). The U73122-resistant Ca^{2+} influx was also resistant to SNP; co-addition of SNP (100 μM) with U73122 (5 μM) failed to further decrease the influx (Fig. 2C). These results suggested the presence of PLC-dependent and -independent components of thrombin-induced Ca^{2+} influx and the resistance of the PLC-independent influx against NO.

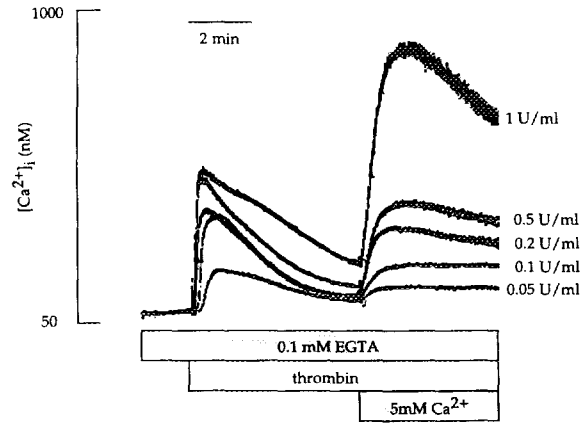

Fig. 1. Dose dependencies for the effects of thrombin on Ca^{2+} release and influx of human platelets. Fura-2-loaded platelets were suspended in Ca^{2+}-free HBS/0.1 mM EGTA and then stimulated with increasing concentrations of thrombin. After 5 min, extracellular Ca^{2+} was supplemented at the concentration of 5 mM. The $[Ca^{2+}]i$ was monitored as described in "Materials and Methods". Shown is the representative of at least three independent experiments.

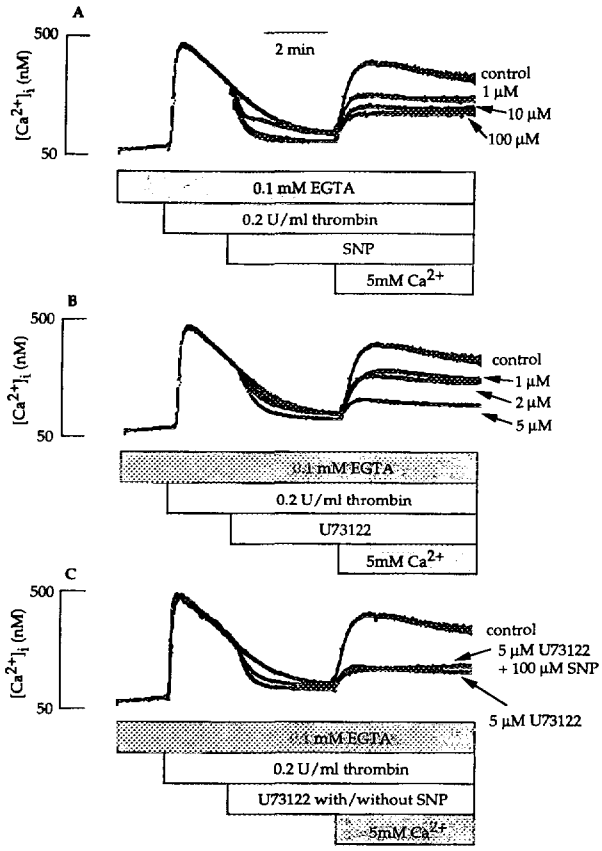

Fig. 2. Effects of SNP and/or U73122 on thrombin-induced Ca^{2+} influx of human platelets. Fura-2-loaded platelets were suspended in Ca^{2+}-free HBS/0.1 mM EGTA and then stimulated with thrombin (0.2 U/ml). At 2 min, increasing concentrations of either SNP (**A**) or U73122 (**B**) were added and, at 5 min, extracellular Ca^{2+} was supplemented at the concentration of 5 mM. SNP, at the concentrations higher than 100 μM up to 3 mM caused no further reduction in the $[Ca^{2+}]i$. In **C**, SNP and U73122 were added simultaneously at the concentrations high enough to cause the maximum inhibition of the Ca^{2+} influx. The $[Ca^{2+}]i$ was monitored as described in "Materials and Methods". Shown is the representative of at least three independent experiments.

Effects of SNP and U73122 on thapsigargin-induced increase in $[Ca^{2+}]i$. To test the hypothesis that the SNP-resistant, PLC-independent Ca^{2+} influx was due to the capacitative Ca^{2+} entry, we examined the effects of SNP and U73122 on the Ca^{2+} influx induced by a Ca^{2+} pump inhibitor thapsigargin. Thapsigargin caused a dose-dependent increase in $[Ca^{2+}]i$ which was totally dependent upon the extracellular Ca^{2+} (Fig. 3A). SNP caused only a marginal inhibition of the thapsigargin-induced Ca^{2+} influx while U73122 partially inhibited the influx (Fig. 3B,C). As in the case of thrombin-induced Ca^{2+} influx, the U73122-resistant fraction of the thapsigargin-induced Ca^{2+} influx was also resistant to SNP (Fig. 3C). These results suggested the presence of

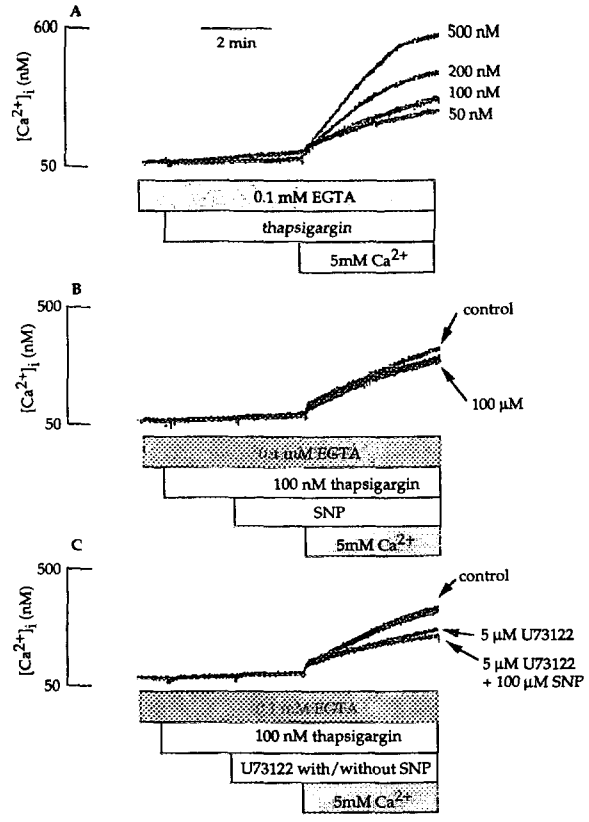

Fig. 3. Effects of SNP and/or U73122 on thapsigargin-induced Ca^{2+} influx of human platelets. **A.** Dose dependency for the effect of thapsigargin. Fura-2-loaded platelets were suspended in Ca^{2+}-free HBS/0.1 mM EGTA and then incubated with increasing concentrations of thapsigargin. At 4 min, extracellular Ca^{2+} was supplemented at the concentration of 5 mM. Either SNP (**B**) or U73122 (**C**) was added at 2 min after the incubation with thapsigargin at the concentrations indicated. The $[Ca^{2+}]i$ was monitored as described in "Materials and Methods". Shown is the representative of at least three independent experiments.

the capacitative Ca^{2+} entry pathway in human platelets and that the entry was independent of PLC activity and was resistant to NO.

Effects of SNP and U73122 on CIF-induced increase in [Ca^{2+}]i. To obtain more direct evidence for the hypothesis aforementioned, we examined that the effects of SNP and U73122 on the activity of CIF that is supposed to act as a diffusible messenger in the capacitative Ca^{2+} entry. The acid extract containing the CIF activity was prepared from the thrombin-stimulated platelets according to the procedures adopted for the extraction of CIF from Jurkat cells (6). The extract, when applied extracellularly, caused an increase in $[Ca^{2+}]i$ that was totally dependent upon the extracellular Ca^{2+} (Fig. 4A). The activity was completely abolished by prior treatment of the

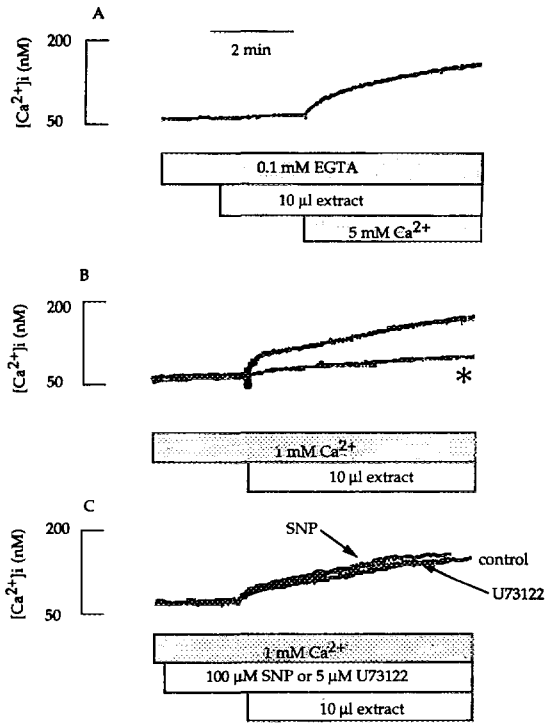

Fig. 4. Effects of SNP or U73122 on the Ca^{2+} influx of human platelets induced by an acid extract from thrombin-stimulated platelets. **A.** Dependency of the effect of the extract on extracellular Ca^{2+}. Ten μl of the acid extract prepared as described in "Materials and Methods" was applied to 0.5 ml (~10^8 cells/ml) of the fura-2-loaded platelets suspended in Ca^{2+}-free HBS/0.1 mM EGTA. At 2 min, extracellular Ca^{2+} was supplemented at the concentration of 5 mM. **B.** Ten μl of the acid extract, either treated (*) or untreated with calf intestinal phosphatase was applied to the fura-2-loaded platelets suspended in Ca^{2+} (1 mM)-containing HBS. **C.** The same volume of the extract as in B was applied to the the fura-2-loaded platelets in the absence or presence of either SNP or U73122 at the concentrations indicated. The $[Ca^{2+}]i$ was monitored as described in "Materials and Methods". Shown is the representative of at least three independent experiments.

extract with calf intestinal alkaline phosphatase (Fig. 4B) and the extract-induced response was not desensitized by prior stimulation with either ADP or thrombin (data not shown). These properties were exactly the same as described for the CIF activity extracted from Jurkat cells (6). In contrast to their effects on thrombin- or thapsigargin-induced Ca^{2+} influx, neither SNP nor U73122 caused any changes in the Ca^{2+} influx induced by the acid extract (Fig. 4C), indicating that the CIF-induced Ca^{2+} influx was independent of the PLC activity and was resistant to NO.

DISCUSSION

Following the report by Randriamampita and Tsien (6), there have been several studies describing the successful extraction of CIF activity from various cell lines including neutrophils, macrophages and lacrimal cells (10-14). The CIF is an yet unidentified molecule with a molecular

mass of less than 500 and with a phosphate group(s) that is essential for its activity (6). There is, however, still a controversy on the identity of CIF; Gilon et al. (14) reported that the observed Ca^{2+} influx induced by the extract was in fact caused by some contaminant(s) that acted as a muscarinic agonist(s) and suggested the influx was a receptor-dependent event. We could detect the similar activity in the acid extract from thrombin-activated human platelets and showed that the activity was independent of PLC activity and was resistant to NO. Because human platelets lack muscarinic receptors (15), the activity cannot be due to any muscarinic agonists contained in the extract. Furthermore, the PLC-independency on and resistance against NO exclude the possibility that the activity was due to any Ca^{2+} mobilizing agents such as thrombin or ADP. Thus the data obtained in the present study was in favor of the presence of an unidentified molecule that beared the activity.

In accordance with the notion for the presence of at least two biochemical mechanisms involved in the agonist-induced Ca^{2+} influx (5), the thrombin-induced Ca^{2+} influx was composed of at least two fractions; one which was sensitive to and the other which was resistant to NO (Fig. 2). The NO-resistant fraction was apparently identical with the U73122-resistant, hence PLC-independent influx. The same was the case for the thapsigargin-induced Ca^{2+} influx (Fig. 3). Together with the negative effects of SNP and U73122 on the CIF-induced Ca^{2+} influx (Fig. 4), we suggest that the capacitative Ca^{2+} entry in human platelets, once triggered by IP_3-induced store-depletion, is not affected by NO. The resistance against NO may be a useful pharmacological tool to distinguish the Ca^{2+} influx caused by the capacitative Ca^{2+} entry from that caused by any other mechanisms involved.

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