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INOSITOL 1,4,5-TRISPHOSPHATE-INDUCED CALCIUM RELEASE IN PERMEABILIZED PLATELETS IS COUPLED TO HYDROLYSIS OF INOSITOL 1,4,5-TRISPHOSPHATE TO INOSITOL 1,4-BISPHOSPHATE

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SUMMARY: Metabolism of inositol 1,4,5-trisphosphate (IP_3) and IP_3-induced release of Ca^{2+} was analyzed in permeabilized human platelets. Both rapid Ca^{2+} release and hydrolysis of IP_3 to inositol 1,4-bisphosphate (IP_2) was observed after addition of IP_3 to permeabilized, Ca^{2+}-loaded platelets. In the absence of ATP or in the presence of inhibitors of the Ca^{2+}-ATPase of the endoplasmic reticulum, no release of Ca^{2+} and little hydrolysis of IP_3 occurs, indicating a coupling between the Ca^{2+} gradient across the membrane of the IP_3-sensitive Ca^{2+} store and conversion of IP_3 to IP_2. In addition, the rapid recovery of the sensitivity of the IP_3-sensitive Ca^{2+} store after successive additions of IP_3 (increment detection) appears to be associated with hydrolysis of IP_3.

Inositol 1,4,5-trisphosphate (IP_3) is an intracellular second messenger generated through receptor-mediated activation of phospholipase C (1). IP_3 induces a release of Ca^{2+} from intracellular stores into the cytosol upon binding to an IP_3-gated Ca^{2+} channel (2). In permeabilized cells addition of IP_3 at non-saturating concentrations leads to the release of a fraction of the IP_3-sensitive Ca^{2+} pool. The amount of Ca^{2+} released depends on the concentration of added IP_3, hence IP_3-induced Ca^{2+} release is termed 'quantal' (3). Moreover, IP_3-sensitive Ca^{2+} stores retain the ability of IP_3-induced Ca^{2+} release even after many successive additions of IP_3, a phenomenon denoted as 'increment detection' (4). Both quantal release and increment detection disappear at low temperature, suggesting the involvement of enzymatic steps (5). Quantal IP_3-induced Ca^{2+} release is also observed in vesicles containing purified IP_3 receptor (6) indicating that quantal release is a property of the IP_3 receptor. A variety of models have been proposed to explain quantal release and increment detection (3,5,7,8).

In the present study we analyzed IP_3-induced Ca^{2+} release and metabolism of IP_3 in permeabilized human platelets. The results demonstrate that hydrolysis of IP_3 to inositol 1,4-

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Abbreviations: DTE, 1,4-dithioerythritol; IP_3, D-myo-inositol-1,4,5-trisphosphate; IP_2, D-myo-inositol-1,4-bisphosphate; IP_1, D-myo-inositol-1-monophosphate. PMSF, phenylmethanesulfonyl fluoride.

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bisphosphate (IP_2) is activated by a Ca^{2+} gradient across the membrane of the IP_3-sensitive stores and indicate a relationship between hydrolysis of IP_3 and increment detection of the IP_3-gated Ca^{2+} channel.

MATERIALS AND METHODS

IP_3 was purchased from Boehringer, cAMP, ADP, ATP, GMP, GDP and GTP from Fluka. Pentosan polysulfate and saponin were obtained from Sigma. Fluo-3 pentapotassium salt was from Molecular Probes (Eugene, Oregon). Fluo-3 was stored in 50 µl aliquots at a concentration of 1 mM in dimethylsulfoxide at -70°C. Thapsigargin was from LC Services Corporation. Aminoethyl Biogel P-2 was purchased from Bio-Rad, and Sepharose CL-2B from Pharmacia. ^{3}H-labelled IP_1, IP_2 and IP_3 (1 Ci/mmol) was purchased as an 1:1:1 mixture from Amersham.

All experiments were performed in 20 mM HEPES buffer containing 155 mM KCl, 5 mM NaCl, 2 mM $MgCl_2$, 2 mM DTE and 0.3 mM PMSF at pH 7.2. Ca^{2+} was removed from solutions by means of chromatography on EDTA-polyacrylamide which was prepared as described previously (9) except using Aminoethyl Biogel P-2 instead of Affigel 102. Since $MgCl_2$ was found to substantially reduce the efficiency of the chelating chromatography material, $MgCl_2$ was added after passage through EDTA-polyacrylamide. Ca^{2+} was removed from cell suspensions by addition of 10 mg of EDTA-polyacrylamide per ml. The suspension was stirred briefly and the gel allowed to settle for 2 min.

Platelets were prepared from platelet-rich plasma by gel filtration (10) using Sepharose CL-2B equilibrated in the HEPES/KCl buffer. Platelets were diluted to 10^8 cells per ml and permeabilized by addition of 20 µg saponin per 10^8 cells.

Ca^{2+} concentrations were measured by means of the fluorescent Ca^{2+} indicator fluo-3. The indicator was added at a final concentration of 1 µM to 1 ml of a suspension of permeabilized platelets placed into a 1 cm quartz cuvette. The suspension was continuously stirred using a teflon coated magnetic stirrer. Fluorescence was measured by means of a Perkin-Elmer 650-10S fluorimeter, interfaced via an analog/digital converter (PC-28 from Instrumatic AG, Switzerland) to a microcomputer (Twix XT-88 personal computer). Fluorescence of fluo-3 was excited at 505 nm and observed at 530 nm using 2 nm slits for both excitation and emission.

Metabolism of inositol polyphosphates was analyzed as follows: IP_3 (200 pmol) supplemented with ^{3}H-labelled IP_1, IP_2 and IP_3 (10 nCi, 0.01 pmol each) was added to 1 ml of a suspension of permeabilized platelets. The reaction was stopped by addition of HCl at a final concentration of 20 mM. After centrifugation (Eppendorf centrifuge) the supernatant was stored frozen at -20°C. Analysis of pure ^{3}H-labelled IP_1, IP_2 and IP_3 revealed no chemical degradation by this treatment. The frozen samples were thawed on ice, neutralized with NaOH, supplemented with cAMP, ADP, ATP, GMP, GDP and GTP (50 µM each), and analyzed in 80 µl aliquots by HPLC (11) using an Alltech Partisil 10 SAX 10U (250 x 4.6 mm) column equipped with a Kontron SAX 10 µm guard column cartridge. Absorbance at 260 nm was recorded to assess the separation of nucleotides. Solvent A was plain water, solvent B 1 M ammonium phosphate pH 4.0. After injection of the sample the column was eluted at a constant flow rate of 1.25 ml/min according to the following protocol: 0% B for 5 min; linear gradient to 15% B within 15 min; linear gradient to 100% B within 15 min; 100% B for 15 min; linear gradient to 0% B within 5 min; 0% B for 15 min. Typical elution times of inositol polyphosphates and nucleotides were 14.3 min (cAMP), 15.5 min (IP_1), 18.2 min (GMP), 25.8 min (ADP), 27.0 min (IP_2), 28.7 min (GDP), 33.9 min (ATP), 35.0 min (IP_3), and 36.2 min

(GTP). 0.4 to 0.8 ml fractions were collected and analyzed by liquid scintillation counting after addition of 0.1 volumes of concentrated HCl and 5 volumes of scintillation fluid (Quickszint 212, Zinsser Analytic).

RESULTS AND DISCUSSION

In physiological conditions, permeabilized platelets exhibit increment detection, i.e. recovery of the sensitivity of the IP_3-sensitive Ca^{2+} stores after addition of IP_3. Figure 1A shows that increment detection disappears at low temperature, in agreement with other studies (4,12). In order to examine putative soluble factors mediating increment detection, the properties of the supernatant of permeabilized platelets were analyzed. In contrast to pure IP_3 the supernatant of permeabilized, IP_3-stimulated platelets is unable to induce Ca^{2+} release in permeabilized platelets loaded with Ca^{2+} by ATP-driven Ca^{2+} uptake (Ca^{2+}-loaded platelets), indicating that IP_3 disappears after Ca^{2+} release (Fig. 1B, 1C). In the absence of ATP or in the presence of thapsigargin, an inhibitor of the endoplasmic Ca^{2+} ATPase, no Ca^{2+} is released upon addition of IP_3. The supernatant of these platelets induces a Ca^{2+} release when added to freshly permeabilized, Ca^{2+}-loaded platelets (Fig. 1C). Thus, IP_3 persists in the absence of Ca^{2+} release.

At least two mechanisms may account for the observed disappearance of IP_3 upon Ca^{2+} release. IP_3 may be removed from the cytosol by translocation into the Ca^{2+} store. Alternatively, IP_3 may be converted to a compound unable to release Ca^{2+}. To distinguish between these possibilities, IP_3 was supplemented with a trace amount of ^3H-labelled IP_1, IP_2 and IP_3 prior to addition to permeabilized platelets. ^3H-labelled inositol polyphosphates present in the soluble and particulate fractions of permeabilized platelets were analyzed by HPLC. All of the ^3H label was found in the soluble fraction, indicating that IP_3 is not transported into membrane vesicles (not shown). Figure 2 shows that IP_3 is converted to IP_2 in permeabilized, Ca^{2+}-loaded platelets. Under the same conditions, Ca^{2+} release is observed. Apart from IP_1, IP_2 and IP_3 (see abbreviations) no other inositol polyphosphate isomers were discernible (Fig. 2). Only little hydrolysis is observed when cells were not allowed to fill their Ca^{2+} stores (Ca^{2+}-depleted platelets). Hydrolysis of IP_3 to IP_2 occurs also in the presence of pentosan polysulfate, where no IP_3-induced Ca^{2+} release is observed (Table 1). Therefore, hydrolysis of IP_3 is activated by a Ca^{2+} gradient across the membrane of the IP_3-sensitive Ca^{2+} store and is not inhibited by pentosan polysulfate.

In order to test whether hydrolysis of IP_3 accounts for quantal release and increment detection, the time course of conversion of to IP_3 to IP_2 was determined at 37°C in Ca^{2+}-loaded and Ca^{2+}-depleted platelets, and at 10°C in Ca^{2+}-loaded platelets. The half-life period of IP_3 in Ca^{2+}-loaded platelets was 270 s at 10°C and 20 s at 37°C. Thus, hydrolysis of IP_3 to IP_2 is substantially attenuated at low temperature, i.e. under conditions of non-incremental and non-quantal Ca^{2+} release (Fig. 1A). The half-life period of IP_3 at 37°C in a suspension of permeabilized platelets supplemented with thapsigargin (1 μM) was approximately 420 s, i.e. 20-fold larger than in Ca^{2+}-loaded platelets, confirming the data shown in Table 1.

IP_3-induced Ca^{2+} release is markedly biphasic at 37°C. About half of the Ca^{2+} is released within 1 s after addition of IP_3 (Fig. 1B), i.e. much faster than hydrolysis of IP_3.

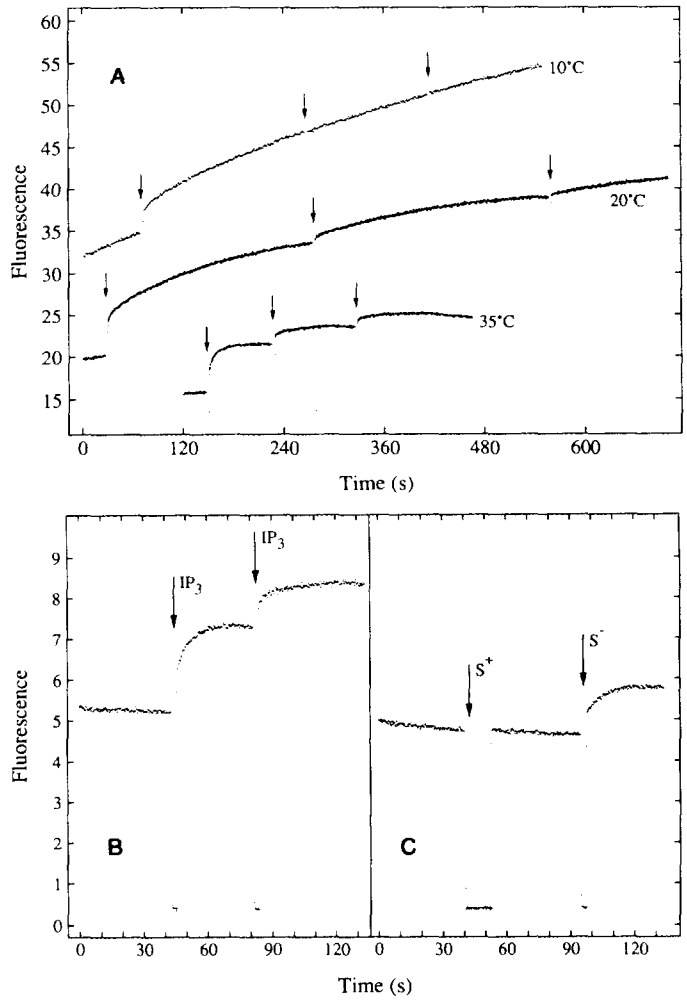

Figure 1. *Characteristics of IP_3-induced Ca^{2+} release in platelets.* **A.** Increment detection disappears at low temperature. A suspension of permeabilized, Ca^{2+}-loaded platelets was equilibrated at the indicated temperature and three aliquots of IP_3 (200 nM, arrows) were added. The slope of the transients increases at low temperature indicating that ATP-driven Ca^{2+} uptake slows down more profoundly than Ca^{2+} leak of the stores. **B.** Time course of the fluorescence signal of fluo-3 upon addition of two aliquots (200 nM, final concentration) of IP_3 to a suspension of permeabilized platelets. ATP (1 mM) was present to allow active transport of Ca^{2+} into the IP_3-sensitive stores. **C.** Supernatant (S^+) of the platelet suspension of experiment A does not induce Ca^{2+} release when added to permeabilized, Ca^{2+}-loaded platelets. In contrast, supernatant (S^-) from permeabilized platelets stimulated with IP_3 (400 nM) in the presence of thapsigargin (1 μM) induces a release of Ca^{2+}. 500 μl of each supernatant was added to 1 ml of cell suspension. The supernatants were adjusted to low Ca^{2+} concentrations by means of EDTA-polyacrylamide (see Materials and Methods).

Thus, a rapid and partial deactivation of the IP_3-sensitive Ca^{2+} channel may control the Ca^{2+} flux of the fast phase of Ca^{2+} release. The half-life of the slow phase of IP_3-induced Ca^{2+} release is similar to that of IP_3 hydrolysis (Fig. 1B). Since IP_3-induced Ca^{2+} release appears to be highly cooperative (4,5,13), only little Ca^{2+} is released at low concentration of IP_3. Therefore, Ca^{2+} release is expected to quickly cease after fall of the concentration of IP_3

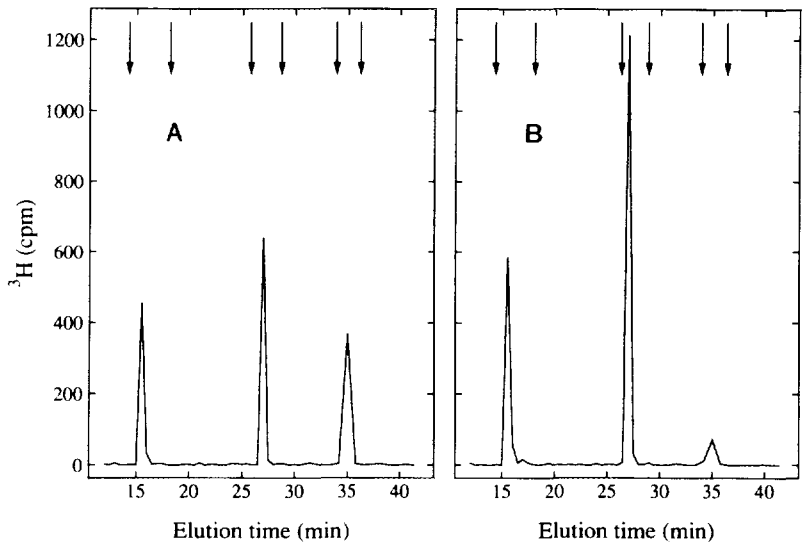

Figure 2. *Separation of inositol polyphosphates by HPLC.* **A.** Inositol polyphosphates after addition of IP_3 (200 nM) and a trace amount of ^{3}H-labelled IP_1, IP_2 and IP_3 to permeabilized platelets in the absence of ATP. No Ca^{2+} is released due to the lack of a Ca^{2+} gradient across the membrane of the store. Inositol phosphates elute in the order IP_1, IP_2 and IP_3 (see Materials and Methods). **B.** Inositol polyphosphates after addition of IP_3 to permeabilized platelets loaded with Ca^{2+} in the presence of ATP (1 mM), under conditions of Ca^{2+} release. In A and B the arrows indicate the elution times of the following nucleotides: cAMP (14.3 min), GMP (18.2 min), ADP (25.8 min), GDP (28.7 min), ATP (33.9 min), GTP (36.2 min).

below a critical value. Thus, hydrolysis of IP_3 may regulate the Ca^{2+} flux of the slow phase. After some time, the Ca^{2+} channel will return to its original, IP_3-sensitive state, due to depletion of IP_3. In this way, IP_3 hydrolysis may account for increment detection, at least in permeabilized platelets.

At low temperature, IP_3 hydrolysis in permeabilized, Ca^{2+}-loaded platelets is slow (half-life period around 240 s at 10°C). The slow phase of IP_3-induced Ca^{2+} release disappears and Ca^{2+} release becomes monoexponential (12). Therefore, and since ATP-driven Ca^{2+}

Table 1. *Coupling of IP_3 hydrolysis to the Ca^{2+} gradient of the IP_3-sensitive store*

conditions[a]			Relative amounts of inositol polyphosphates (%)		
ATP	TG	PP	IP_1	IP_2	IP_3
+	-	-	32.4±1.4	63.2±1.7	4.4±0.5
-	-	-	32.1±1.5	43.1±1.5	24.8±1.2
+	+	-	34.0±1.3	44.3±1.4	21.7±0.9
+	-	+	36.0±1.1	62.7±1.2	1.3±0.2

[a]The relative amounts of IP_1, IP_2 and IP_3 2 min after addition of IP_3 (200 nM) supplemented with a trace amount of ^{3}H-labelled IP_1, IP_2 and IP_3 are listed. Experiments were performed in the presence or absence of ATP (1 mM), thapsigargin (TG, 1 μM), and pentosan polysulfate (PP, 150 μg/ml). Ca^{2+} release is observed only in the presence of ATP and in the absence of both thapsigargin and pentosan polysulfate (+ATP -TG -PP).

uptake is also slowed down, the IP_3-sensitive stores are depleted upon addition of IP_3 before IP_3 is hydrolyzed to a major extent. Indeed, no further IP_3-induced Ca^{2+} release is observed 3 min following the first IP_3 aliquot at 10°C (Fig. 1A). Under these conditions the concentration of IP_3 drops to about 60% of its original value. The apparent lack of rapid deactivation of the IP_3-gated Ca^{2+} channel at low temperature may reflect either a change of the IP_3-sensitive Ca^{2+} store, e.g. a transition of the membrane from a liquid to a crystalline state, or a conformational change of the channel protein itself.

Hydrolysis of IP_3 to IP_2 under conditions of Ca^{2+} release has been found in rat cerebellum (13). In the cytosol of human platelets, 2 types of inositol polyphosphate 5-phosphatase have been identified and isolated (14,15). In rat liver and human placenta membrane-bound inositol polyphosphate 5-phosphatases have been characterized (16,17). Whether some of these enzymes are activated by a Ca^{2+} gradient of the IP_3-sensitive store, or whether the IP_3 receptor itself exhibits a 5-phosphatase activity has not yet been analyzed. In addition, it is unclear whether analogs of IP_3 capable of quantal Ca^{2+} release (5) are also subject to hydrolysis. However, the results of the present study suggest a close coupling between hydrolysis of IP_3 to IP_2 and IP_3-induced Ca^{2+} release. In addition, rapid inactivation of the IP_3-gated Ca^{2+} channel and the concomittant activation of hydrolysis of IP_3 may be responsible for quantal release and increment detection in human platelets.

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