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INHIBITORS OF INOSITOL TRISPHOSPHATE-INDUCED Ca^{2+} RELEASE FROM ISOLATED PLATELET MEMBRANE VESICLES

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Abstract—Platelet membrane vesicles accumulated Ca^{2+} in an ATP-dependent fashion, and 25–50% of the accumulated Ca^{2+} was released by the addition of 10 μM inositol 1,4,5-trisphosphate (IP_3). The concentration of IP_3 required for half-maximal Ca^{2+} release was approximately 0.5 μM. The inhibition of IP_3-induced Ca^{2+} release from these membrane vesicles by various agents was examined. Of the plasma membrane Ca^{2+} channel blockers, cinnarizine and flunarizine were found to be potent inhibitors of IP_3-induced Ca^{2+} release while having no effect on ATP-dependent Ca^{2+} uptake. The IC_{50} value for both cinnarizine and flunarizine as inhibitors of IP_3-induced Ca^{2+} release was below 10^{-6} M. Nifedipine, verapamil, bepridil, and diltiazem did not significantly inhibit IP_3-induced Ca^{2+} release at the highest concentration tested (50 μM). The "intracellular Ca^{2+} antagonists" ryanodine, TMB-8 (8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate), dantroline, trifluoperazine and chlorpromazine were not inhibitors of IP_3-induced Ca^{2+} release at 50 μM. The local anesthetics benzocaine and lidocaine weakly inhibited the IP_3-induced Ca^{2+} release with IC_{50} values of approximately 5 and 50 μM, respectively, whereas other local anesthetics tested were less potent inhibitors. The potent inhibitors described may prove useful as probes of the IP_3-induced Ca^{2+} release channels.

Increases in the platelet intracellular Ca^{2+} concentration are important in controlling shape change, aggregation and secretion [1–5], and in activating a number of platelet enzymes [6–8]. The increases in cytoplasmic Ca^{2+} observed during platelet activation result from both increased permeability of the plasma membrane to Ca^{2+} and mobilization of Ca^{2+} from the cytoplasmic dense tubule system [3–5]. The dense tubule system functions in a manner similar to the sarcoplasmic reticulum in muscle. It contains a Ca^{2+} ATPase that actively accumulates cytosolic Ca^{2+} and releases the stored Ca^{2+} during platelet activation.

Inositol 1,4,5-trisphosphate (IP_3)† has been identified recently as a signal for intracellular Ca^{2+} mobilization [9]. Receptor-activated phospholipase C in the platelet plasma membrane hydrolyzes phosphatidylinositol diphosphate to generate diacylglycerol and IP_3. IP_3 activates Ca^{2+} release from the cytoplasmic dense tubule system [9], while diacylglycerol activates protein kinase C [8].

The importance of IP_3-induced intracellular Ca^{2+} release is emphasized by observations that thrombin-induced platelet shape change and secretion [10] can occur in the absence of extracellular Ca^{2+}, and that the addition of IP_3 alone to saponin-permeabilized platelets stimulates protein phosphorylation, aggregation and secretion, which can be inhibited by Ca^{2+} chelation [11–13].

To better understand the role of IP_3-induced Ca^{2+} release in platelet function we have studied the effect of several agents on this process. We also wanted to determine if compounds reported to block Ca^{2+} channels in other membranes had any effects on the intracellular IP_3-regulated Ca^{2+} channel.

IP_3-induced Ca^{2+} release from isolated platelet membrane vesicles (presumed to be derived from the platelet dense tubule system) has been demonstrated recently [14–16]. We have used this preparation to study IP_3-induced Ca^{2+} release, and this report describes some inhibitors of that process.

MATERIALS AND METHODS

Materials. Outdated human platelet-rich plasma was obtained from the American Red Cross (Evansville, Indiana Office). The platelet-rich plasma had "expired" no more than 10 hr prior to use and was maintained under agitation until used for membrane preparations. Bovine brain inositol 1,4,5-trisphosphate (IP_3) (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled H_2O to make a stock solution of 2 mM. Prostaglandin I_2 (Sigma Chemical Co.) was freshly prepared prior to use. Protease inhibitors were made as a stock solution consisting of 40 mM phenylmethylsulfonylfluoride, 10 mM pepstatin A and 10 mM leupeptin in dimethyl sulfoxide and diluted 1:1000 into the appropriate media prior to usage. $^{45}Ca^{2+}$ was obtained from DuPont NEN Research Products (Boston, MA), and glass fiber filters GF/C were obtained from Whatman Ltd. (Hillsboro, OR).

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† Abbreviations used: IP_3, myo-inositol 1,4,5-trisphosphate; TMB-8, 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; and EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate.

Platelet membrane preparation. The membrane vesicles were prepared from 16 to 24 units (800–1200 ml) of platelet rich plasma to which 0.01 vol. of 0.25 M Na_2EDTA (pH 6.8) was added following previously described procedures [15]. PGI_2 was also added to the platelet-rich plasma to a final concentration of 125 ng/ml to prevent platelet aggreation. Contaminating red blood cells were removed by centrifugation at 365 *g* for 5 min [1500 rpm in a Sorvall (Dupont Co., Wilmington, DE) GSA rotor in an RC2-B centrifuge for 5 min]. All centrifugations of intact platelets were performed at room temperature. The platelets were recovered by sedimentation at 2000 *g* for 10 min (3500 rpm in the Sorvall GSA rotor for 10 min). The platelets were resuspended in original volume of 0.15 M NaCl, 2.7 mM Na_2EDTA, pH 6.8, using a large plastic pipet and bulb. The platelets were again sedimented at 2000 *g* for 10 min and resuspended in original volume of 0.15 M NaCl, 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid), pH 6.8. The platelets were again sedimented at 2000 *g* for 10 min and then resuspended in 0.05 original volume of ice-cold homogenization medium containing 100 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM HEPES/KOH (pH 7.0), 40 μM phenylmethylsulfonylfluoride, 10 μM pepstatin A, 10 μM leupeptin, 0.01 mg/ml aprotinin and 2 mM ATP. All remaining procedures were performed at 0–4°. The platelets were sonicated 3 × 15 sec at power setting No. 6 using a Biosonik III sonicator (Bronwill Scientific, Rochester, NY).

The platelet homogenate was then sedimented at 12,000 *g* for 20 min (10,000 rpm in the Sorvall SS-34 rotor in the RC2-B centrifuge for 20 min). The supernatant fractions were removed and immediately centrifuged at 193,000 *g* for 45 min [40,000 rpm in the Beckman 50.2 ti rotor in the L2-65B or L8-70M ultra centrifuge (Beckman Instruments, Palo Alto, CA)]. The platelet membrane vesicles were then resuspended to 0.005 original volume in 100 mM KCl, 0.3 M sucrose, 20 mM HEPES/KOH (pH 7.0), 100 μM EDTA, 40 μM phenylmethylsulfonylfluoride, 10 μM pepstatin A, 10 μM leupeptin, and 0.01 mg/ml aprotinin. The membrane vesicles (10–15 mg protein/ml) were aliquoted into small tubes, rapidly frozen in liquid nitrogen, and stored frozen at −70° until used.

Ca^{2+} *uptake and release.* The platelet membrane preparations were thawed by hand-warming and immediately loaded into an ice-cold 50 μl Hamilton syringe for dispensing. Ca^{2+} uptake and subsequent IP_3-induced release were assayed at room temperature.

The Ca^{2+} uptake reaction was started by addition of 2 μl of microsomal membrane vesicles into 200 ml of Ca^{2+} uptake medium containing 100 mM KCl, 2 mM MgATP, 50 mM NaN_3, 10^5–10^6 cpm/ml of $^{45}CaCl_2$, 20 mM HEPES/KOH, pH 7.4, with either 5 μM or no added $CaCl_2$. The starting free Ca^{2+} concentration in the uptake medium was determined using a Ca^{2+}-sensitive electrode (Orion Research Inc.) standardized with Ca^{2+}/EGTA buffers. The EGTA–Ca^{2+} dissociation constants used to make the free Ca^{2+} concentration standards were those given in Ref. 17. Unless otherwise indicated, the starting free Ca^{2+} concentration in the assay medium used where no Ca^{2+} was added was between 2 and 5×10^{-7} M. At the indicated time points during the Ca^{2+} uptake reaction, the reaction was stopped by

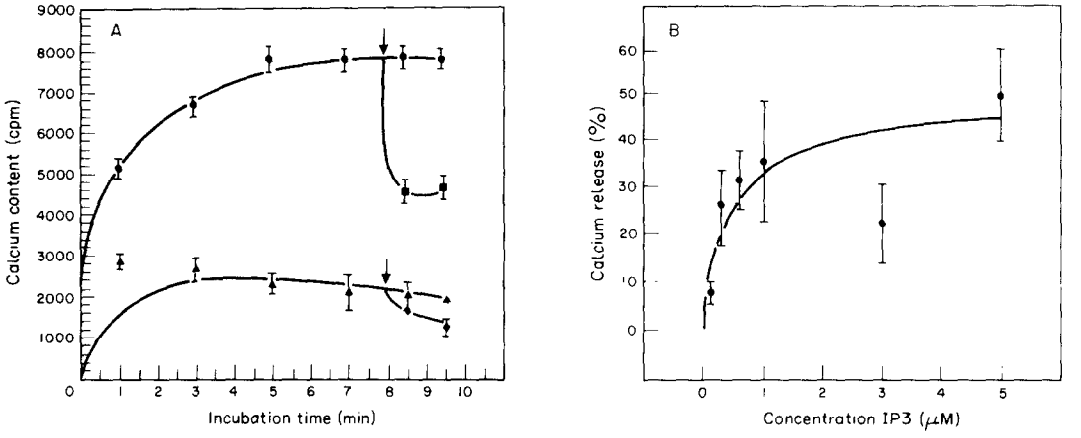

Fig. 1. (A) Ca^{2+} uptake and release from isolated platelet membrane vesicles. Ca^{2+} uptake was measured as described in Materials and Methods in the presence (●) or absence (▲) of 2 mM MgATP in a medium containing 100 mM KCl, 50 mM NaN_3, 20 mM HEPES, pH 7.4, 1.02×10^5 cpm/ml $^{45}Ca^{2+}$, and no added Ca^{2+} (starting free Ca^{2+} concentration was 3×10^{-7} M; the total Ca^{2+} concentration was 9×10^{-7} M). The reaction was started by the addition of 2 μl of platelet membrane vesicles (10.4 mg/ml protein) to 200 ml of uptake medium. At the arrow, 10 μM IP_3 was added to release the IP_3-sensitive Ca^{2+} pool. This panel represents a typical experiment (the bars indicate the standard deviation of triplicate determinations). At the 8.5 min time point, typically used for 100% determinations in subsequent figures, the amount of Ca^{2+} accumulated was 3 nmol/mg. (B) Dependence of Ca^{2+} release on IP_3 concentration. ATP-dependent Ca^{2+} uptake (in the presence of 5 μM added $CaCl_2$) was allowed for 8 min, after which the indicated concentration of IP_3 was added to the membrane vesicles (100% = 3 nmol/mg). This panel represents the average of two platelet membrane preparations, triplicate determinations on each preparation (the bars indicate the standard error of the mean).

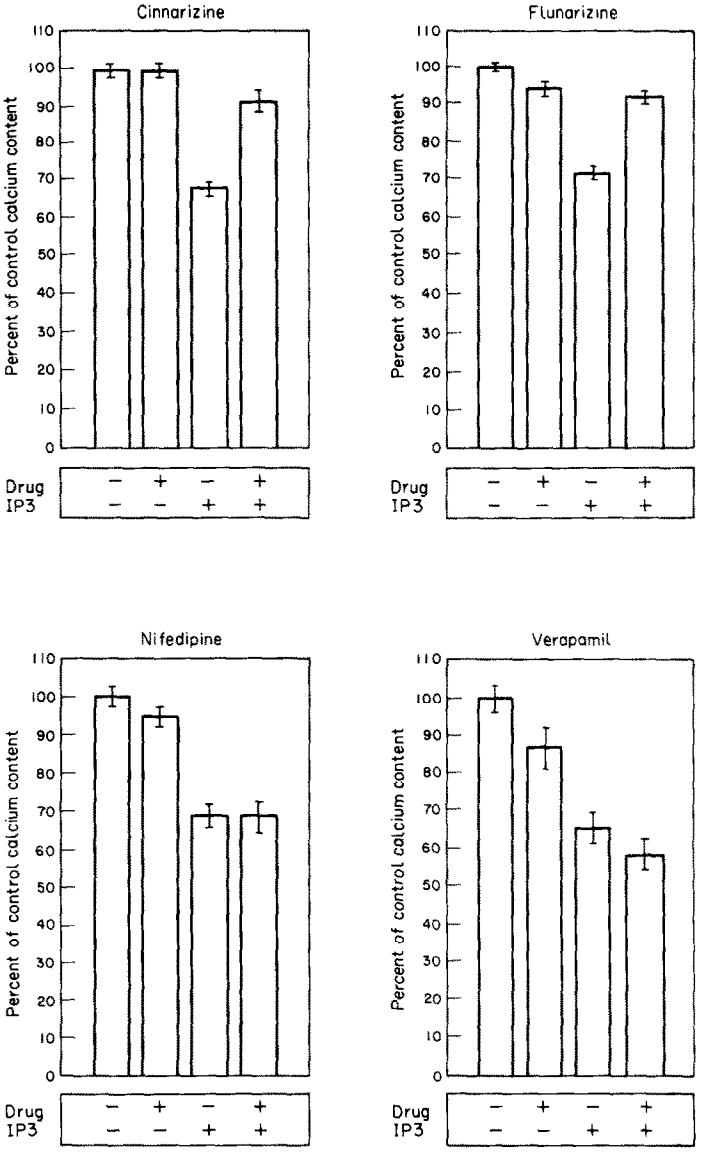

Fig. 2. Effects of plasma membrane Ca^{2+}-channel blocking agents on IP_3-induced Ca^{2+} release from isolated platelet membranes. The platelet membranes were loaded with $^{45}Ca^{2+}$ in the presence of ATP (in the presence or absence of drug) until steady-state Ca^{2+} accumulation conditions were reached (8 min). Ten micromolar IP_3 (final concentration) or the equivalent amount of water was added, and 30 sec later the vesicles were diluted, filtered, and counted to determine the amount of $^{45}Ca^{2+}$ remaining. The compound tested is given above the appropriate panel. The concentration of compounds tested was 50 μM. (For the cinnarizine and flumarizine experiments, 100% = 9 nmol/mg; for the nifedipine and verapamil experiments, 100% = 4 nmol/mg.) In this and subsequent figures, the bars represent standard deviation of triplicate determinations on a single (representative) preparation.

the addition of 5 ml of ice-cold wash medium containing 100 mM KCl, 20 mM HEPES, 5 mM NaN_3, 5 mM $LaCl_3$ and 0.1 mg/ml bovine serum albumin (25-fold dilution). The sample was vortex-mixed and rapidly filtered through Whatman GF/C filters. The filtration time was less than 10 sec. The filters were removed and blotted dry, and the trapped $^{45}Ca^{2+}$ was measured by scintillation counting.

For compound testing Ca^{2+} uptake was allowed to proceed for 8 min (in the presence and absence of compound to determine the direct effect of compound on the Ca^{2+}-accumulating ability of the membrane vesicles), after which time either H_2O (control) or 10 μM IP_3 was added to the membrane vesicles. After the IP_3 or water additions, the vesicles were further incubated for 30 sec before being diluted and filtered. Blank values where Ca^{2+} uptake medium was diluted and filtered in the absence of added membrane vesicles were subtracted.

RESULTS

Ca^{2+} uptake and IP_3-dependent release. Isolated platelet membrane vesicles were used to study Ca^{2+}

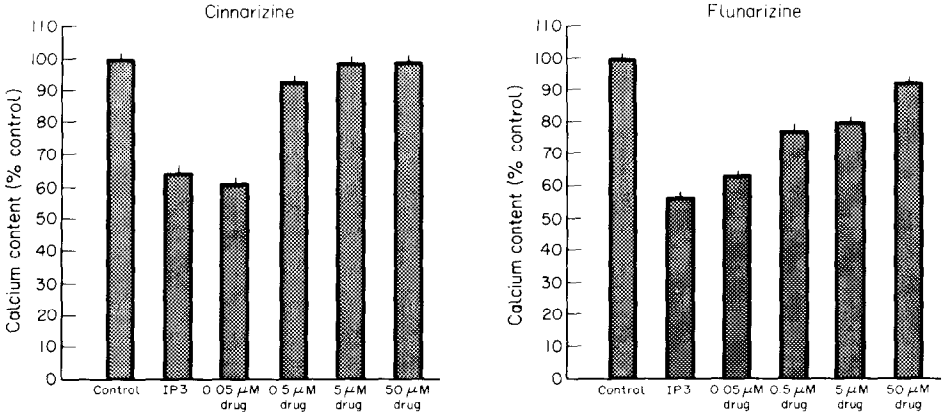

Fig. 3. Concentration dependence of drug inhibition of IP_3-induced Ca^{2+} release from isolated platelet membrane vesicles. ATP-dependent Ca^{2+} uptake and IP_3-induced release were performed as described in Materials and Methods and in the legend to Fig. 2. The column labeled "control" represents the ATP-dependent Ca^{2+} uptake where distilled water (vehicle for the IP_3) was added (100% = 2 nmol/mg). The columns labeled "IP_3" (and the remaining bars) indicate the amount of Ca^{2+} left in the vesicles 30 sec after the addition of 10 μM IP_3. The remaining columns indicate the effects of the various drugs (at the indicated concentrations) on the IP_3-induced Ca^{2+} release.

accumulation and release by platelet intracellular membranes. The translocation of calcium was followed using $^{45}Ca^{2+}$ as a tracer. The platelet membranes rapidly accumulated $^{45}Ca^{2+}$ in the presence of ATP (Fig. 1A). In preliminary experiments, A23187 prevented the ATP-dependent Ca^{2+} accumulation. The Ca^{2+} accumulation could be stimulated by the presence of a Ca^{2+}-precipitating agent such as potassium oxalate (data not shown). In the absence of a Ca^{2+}-precipitating agent, the ATP-dependent Ca^{2+} uptake reached steady state by 5 min. The addition of 10 μM inositol 1,4,5-trisphosphate (IP_3) caused a rapid release of between 25 and 50% of the Ca^{2+} accumulated within 30 sec of the IP_3 addition (Fig. 1A). The percentage of Ca^{2+} released was somewhat preparation dependent and decreased with prolonged storage (greater than a month) of the preparation at −70°.

The amount of the Ca^{2+} released in the first 30 sec after IP_3 addition depended on the concentration of IP_3 added (Fig. 1B). The half-maximal Ca^{2+} release occurred at approximately 0.5 μM IP_3. The concentration dependence of IP_3 required for Ca^{2+} release is very similar to that reported by others for Ca^{2+} release from isolated platelet membrane vesicles [14–16] and from saponin-permeabilized platelets [11–13].

Inhibition of IP_3*-induced* Ca^{2+} *release.* The influence of potential inhibitors of IP_3-induced Ca^{2+} release was tested. The platelet membrane vesicles were allowed to accumulate Ca^{2+} in the presence of ATP until steady-state conditions were reached. At the 8 min time point, 10 μM (final concentration) IP_3 or an equivalent volume of vehicle (distilled H_2O) was added to the Ca^{2+}-loaded membrane vesicles. After the addition of IP_3 or H_2O, the vesicles were further incubated 30 sec to allow for Ca^{2+} to exit the vesicles before dilution with wash medium and filtration. The compound to be tested was incubated with the membrane vesicles during the course of ATP-dependent Ca^{2+} uptake and subsequent IP_3-induced release. (The possible inhibition of ATP-dependent Ca^{2+} uptake by the compound required a set of controls where the Ca^{2+} uptake and release occurred in the absence of test compound. Compounds that inhibited the ATP-dependent Ca^{2+} uptake greater than 50% of control did not permit accurate analysis of the influence of the compounds on IP_3-induced Ca^{2+} release.)

A number of compounds were tested as possible inhibitors of IP_3-induced Ca^{2+} release in platelets. Plasma membrane voltage-dependent and receptor-operated Ca^{2+} channel blockers were tested for their ability to block IP_3-induced Ca^{2+} release, and sample results are shown in Fig. 2. Cinnarizine and flunarizine inhibited IP_3-induced Ca^{2+} release, whereas the others, nifedipine, diltiazem and verapamil, were not effective inhibitors at the concentrations tested (see Figs. 2 and 4). The dose–effect relations were determined (Fig. 3), and the IC_{50} values were less than 10^{-6} M for both compounds (Fig. 3).

Dantroline sodium has been shown to inhibit both the Ca^{2+} pumping activity and IP_3-induced Ca^{2+} release in adrenal glomerulosa cells [18]. Dantroline did not inhibit the platelet IP_3-induced Ca^{2+} release at 50 μM (Fig. 4). Ryanodine has been shown to modulate Ca^{2+} channels in sarcoplasmic reticulum in cardiac and skeletal muscle. At concentrations of up to 200 μM, ryanodine did not influence the IP_3-induced Ca^{2+} release from platelet vesicles (Fig. 4). Methylenedioxyindene, proposed to be an intracellular Ca^{2+} antagonist in a number of cell systems [19], also did not inhibit IP_3-induced Ca^{2+} release (Fig. 4).

In experiments not shown, we tested the effects of a number of other reportedly "intracellular Ca^{2+} antagonists". Bepridil, TMB-8, W-7, doxorubicin, sodium nitroprusside, imidazole and caffeine did not inhibit platelet IP_3-induced Ca^{2+} release when tested at 50 μM. We also found that quercetin, tri-

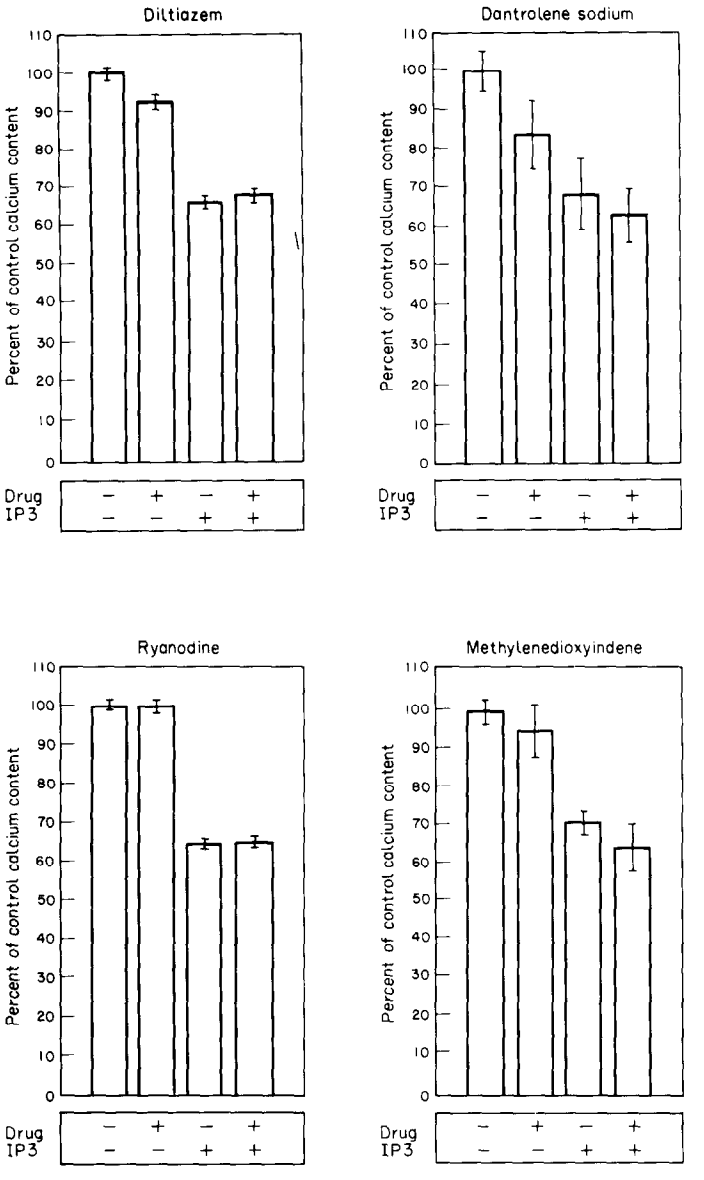

Fig. 4. Effects of selected compounds on IP_3-induced Ca^{2+} release from isolated platelet membranes. The effect of compounds on ATP-dependent Ca^{2+} uptake and IP_3-induced Ca^{2+} release was determined as described in the legend to Fig. 2. The concentration of compound tested was 50 μM, except for ryanodine, where 100 μM was used. (One hundred percent = 4 nmol/mg for the diltiazem and ryanodine experiments and 9 nmol/mg for the dantroline and methylenedioxyindene experiments.)

fluoperazine and chloropromazine did not inhibit the IP_3-induced Ca^{2+} release at 25 μM. Higher concentrations of these agents inhibited the ATP-dependent Ca^{2+} uptake, which made assessment of IP_3-induced Ca^{2+} release difficult.

Since local anesthetics have been shown to inhibit intracellular Ca^{2+} mobilization in muscle [20], we evaluated their effects on IP_3-induced Ca^{2+} release in platelet membranes. Benzocaine and lidocaine were found to inhibit IP_3-induced Ca^{2+} release (Fig. 5), with IC_{50} values of approximately 5 μM for benzocaine (Fig. 6) and approximately 50 μM for lidocaine (data not shown). Other local anesthetics tested (procaine, procainamide, dibucaine, tetracaine and SKF-525A) had IC_{50} values much greater than 50 μM (data not shown).

DISCUSSION

This study describes the effects of selected compounds on the isolated platelet membrane IP_3-induced Ca^{2+} release and identifies several inhibitors of this process. Cinnarizine and flunarizine were potent inhibitors of platelet IP_3-induced Ca^{2+} release (IC_{50} less than 10^{-6} M), while other known plasma membrane Ca^{2+} channel blocking agents (including nifedipine, verapamil and diltiazem) did not inhibit this intracellular Ca^{2+} channel. The local anesthetics

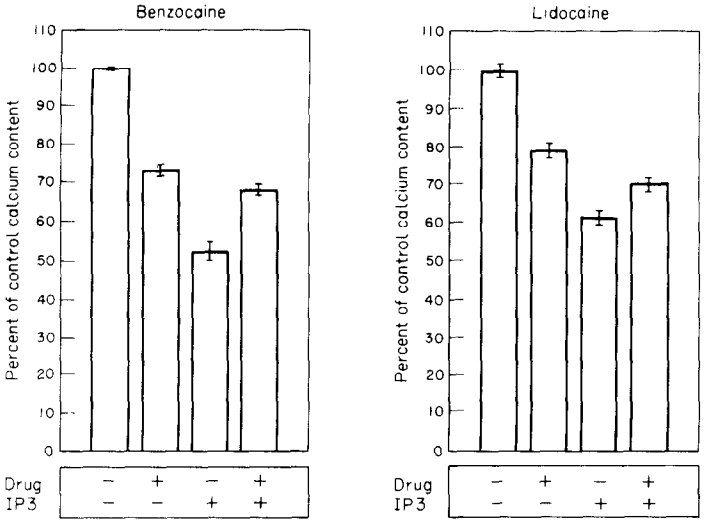

Fig. 5. Effects of benzocaine and lidocaine on IP_3-induced Ca^{2+} release. The effect of 50 μM benzocaine or lidocaine on IP_3-induced Ca^{2+} release was determined as described in the legend to Fig. 2. (One hundred percent = 2 nmol/mg.)

benzocaine and lidocaine inhibited IP_3-induced Ca^{2+} release, although they were less potent than cinnarizine or flunarizine.

Cinnarizine and flunarizine were the most potent inhibitors of platelet membrane IP_3-induced Ca^{2+} release tested in this study. These drugs have been shown to prevent Ca^{2+} influx into smooth muscle cells via both voltage-sensitive and norepinephrine-activated Ca^{2+} channels [21, 22]. However, there is little indication that either compound blocks intracellular Ca^{2+} mobilization in intact vascular smooth muscle cells [21–24], nor is it known if these compounds block IP_3-induced Ca^{2+} release in smooth muscle. Flunarizine has little influence on platelet aggregation [23], but it is not known if either compound is capable of inhibiting intracellular Ca^{2+} mobilization within intact platelets. In preliminary experiments, we found that cinnarizine at concentrations which block Ca^{2+} release using isolated membranes only slightly inhibited thrombin-induced intracellular Ca^{2+} mobilization within intact platelets. Since these compounds are charged molecules, they may not reach high enough intracellular concentrations to inhibit intracellular Ca^{2+} mobilization, or there may be other intracellular signal mechanisms (in addition to IP_3) that activate intracellular Ca^{2+} mobilization.

It is interesting that the "intracellular Ca^{2+} antagonists" dantroline, ryanodine and methylenedioxyindene did not inhibit IP_3-induced Ca^{2+} release in these isolated platelet membrane vesicles. Dantroline had been shown by Kojima *et al.* [18] to inhibit IP_3-induced Ca^{2+} release from (as well as Ca^{2+} uptake into) permeabilized bovine adrenal glomerulosa cells. The reasons for the observed differences are not clear, but they may reflect tissue specificity. Ryanodine has been shown by others to modulate Ca^{2+} release from intracellular membrane storage sites in both cardiac and skeletal muscle [25–30], but it had no effect on IP_3-induced Ca^{2+} release in this study, which suggests pharmacological differences between these intracellular Ca^{2+} channels.

Several local anesthetics were found to inhibit IP_3-induced Ca^{2+} release from the platelet vesicles. Others have shown that local anesthetics inhibit Ca^{2+} release from intracellular storage sites of a number of cell systems, including skeletal muscle [31–35], smooth muscle [36] and liver [37], as well as platelets [38]. Local anesthetics also inhibit Ca^{2+} release from isolated skeletal muscle sarcoplasmic reticulum caused by several different mechanisms, including "spontaneous" Ca^{2+} release [39], Ca^{2+}-induced Ca^{2+}

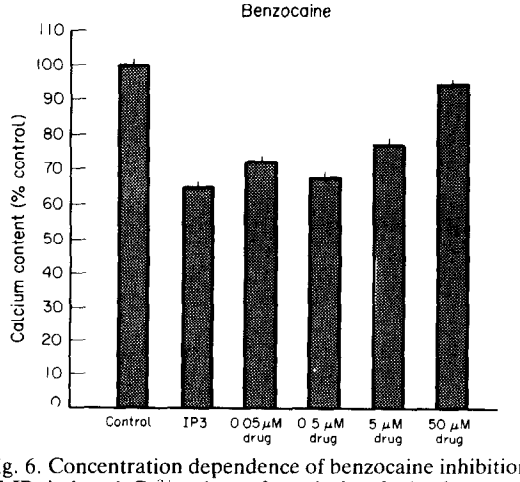

Fig. 6. Concentration dependence of benzocaine inhibition of IP_3-induced Ca^{2+} release from isolated platelet membranes. The concentration dependence was determined as described in the legend to Fig. 3. (One hundred percent = 4 nmol/mg.)

release [40–42], and caffeine-induced Ca^{2+} release [32, 43]. Our findings are consistent with suggestions by others that local anesthetics could inhibit Ca^{2+} mobilization in whole platelets [38].

This report describes several inhibitors of IP_3-induced Ca^{2+} release from isolated platelet membrane vesicles. In isolated platelet membrane vesicles, cinnarizine and flunarizine are the most potent inhibitors of IP_3-induced Ca^{2+} release thus far reported. At concentrations that completely block IP_3-induced Ca^{2+} release, these compounds have little influence on ATP-dependent Ca^{2+} uptake. Therefore, these compounds may be useful as pharmacological probes of IP_3-induced Ca^{2+} release phenomenon in isolated membranes and permeabilized cell systems and may contribute to biochemical characterization and understanding of the IP_3-induced Ca^{2+} release channel in platelets, as well as other cell types.

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