0026-895X/01/360-541–552$3.00
MOLECULAR PHARMACOLOGY
Vol. 3, No. 60
753/923910
Mol Pharmacol 3:541–552, 2001
Printed in U.S.A.

2-Aminoethoxydiphenyl Borate Directly Inhibits Store-Operated Calcium Entry Channels in Human Platelets

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Received December 12, 2000; accepted May 21, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

In this study, we examined 2-aminoethoxydiphenyl borate (2APB) as an inhibitor of Ca^{2+} influx in human platelets. 2APB was found to inhibit thrombin-mediated intracellular Ca^{2+} mobilization rapidly in platelets incubated in the absence of extracellular Ca^{2+}. This result supports an intracellular action of 2APB on inositol 1,4,5-trisphosphate (IP_3)-receptor Ca^{2+} channels. 2APB was without effect on the ability of thapsigargin to mobilize intracellular Ca^{2+}. This result suggests that the efflux of Ca^{2+} from the endoplasmic reticulum mediated by thapsigargin is not via IP_3 Ca^{2+} channels. However, 2APB was able to prevent the entry of Ca^{2+} and Sr^{2+} through thapsigargin-activated, store-operated Ca^{2+} channels (SOCC). This result supports a direct inhibitory effect of 2APB on SOCC. 2APB was also able to block the entry of Sr^{2+}, Ba^{2+}, and Mn^{2+} entry into unstimulated platelets, which suggests that 2APB was inhibiting the Ca^{2+} influx channels directly. The capacity of 2APB to prevent Ca^{2+} influx and Sr^{2+} influx was rapid because it occurred immediately upon addition to the platelets. The inhibition of Ca^{2+} and Sr^{2+} influx by 2APB was similar to that seen with the cell-impermeable nonselective Ca^{2+}-channel blocker La^{3+} or the Ca^{2+} chelator EGTA. Diphenylboronic anhydride and 2,2-diphenyltetrahydrofuran, two compounds that are structurally similar to 2APB, also inhibited Ca^{2+} influx. It was concluded that 2APB was a rapid and effective direct inhibitor of SOCC in human platelets; as such, it cannot be used to support the involvement of IP_3 receptors in the activation of SOCC.

2-Aminoethoxydiphenyl borate (2APB) was originally characterized as a cell-permeable inhibitor of inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release (Maruyama et al., 1997). 2APB inhibited IP_3-induced Ca^{2+} release from cerebellar microsomes without affecting IP_3 binding. 2APB also inhibited agonist-induced increases in intracellular free calcium ($[Ca^{2+}]_i$) in platelets and neutrophils and blocked agonist-induced contractions in thoracic aorta, but it had no effect on KCl-induced contractions. 2APB has been used extensively to inhibit the release of intracellular Ca^{2+} (Cui and Kanno, 1997; Ascher-Landsberg et al., 1999; Gysembergh et al., 1999; Hamada et al., 1999; Ma et al., 2000; van Rossum et al., 2000).

2APB has the ability to form a five-membered boroxazolidine heterocyclic ring (Fig. 1) when an internal coordinate bond is formed between the nitrogen in the ethanolamine side chain and the tricoordinated boron (Strang et al., 1989). This heterocyclic form of 2APB (*B,B*-diphenylboroaxozolidine) forms crystals in staggered arrays of molecules. Each molecule links with two others through hydrogen bonds (Rettig and Trotter, 1976); this feature most probably accounts for the fact that 2APB is soluble in water (see below). This heterocyclic species of 2APB would be more hydrophobic than the compound without the heterocyclic ring and should enter cells more rapidly than the primary amine open-chain species that could be protonated. It is also known that boron-nitrogen coordination results in the formation of dimers (Nöth, 1970); van Rossum et al. (2000) suggested that 2APB also exists as a dimer (Fig. 1).

The recent study conducted by Ma et al. (2000) relied on the specificity of 2APB as a blocker of Ca^{2+} release via the IP_3 receptor in the endoplasmic reticulum (ER) of several different cell lines. The authors discounted a direct effect of 2APB on plasma membrane Ca^{2+} channels (see Fig. 4 in Ma et al., 2000). In another study, van Rossum et al. (2000) showed that 2APB, when added with thapsigargin to DDT_1-MF2 cells had no effect on the Ca^{2+} release component but inhibited the Ca^{2+} entry component (see Fig. 1 in van Rossum et al., 2000). An interpretation of this result, although not considered by the authors, was that 2APB was blocking SOCC directly.

The laboratory of P.F.B. was supported by grants from the Jeffress Memorial Trust; the American Heart Association, VA affiliate; and the Virginia Academy of Science. Some of the data presented in this article were presented at Experimental Biology; 2001 March 31 to April 4; in Orlando, FL (abstract 718.6).

ABBREVIATIONS: 2APB, 2-aminoethoxydiphenyl borate; IP_3, inositol 1,4,5-trisphosphate; SOCC, store-operated Ca^{2+} channels; hTrp, human transient receptor potential; fura-2, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid; ER, endoplasmic reticulum; AM, acetoxymethyl ester; SERCA, smooth endoplasmic reticulum Ca^{2+} ATPase; DPBA, diphenylborinic anhydride; IP_3R, inositol 1,4,5-trisphosphate receptor; DPTTF, 2,2-diphenyltetrahydrofuran.

We have been investigating the mechanism by which phytoestrogens were able to inhibit platelet aggregation and found that several phytoestrogens inhibited Ca^{2+} influx in platelets induced by thrombin (Dobrydneva et al., 1999). The phytoestrogens were inhibiting the entry of Ca^{2+} through SOCC, because the phytoestrogen *trans*-resveratrol was also able to inhibit thapsigargin-mediated Ca^{2+} influx and basal Ba^{2+} ion influx (Dobrydneva et al., 1999). Thapsigargin is believed to promote Ca^{2+} entry through SOCC by depleting the IP_3-sensitive Ca^{2+} stores in platelets (Sage, 1997). To further investigate the mechanism by which phytoestrogens inhibit Ca^{2+} influx, we sought to prevent thrombin-mediated Ca^{2+} mobilization from IP_3-sensitive Ca^{2+} stores by using 2APB.

The mechanism by which SOCC is activated in a variety of cells has received much attention recently (Putney, 1999a,b; Berridge et al., 2000). Current evidence supports a model in which there is reversible trafficking and coupling of the IP_3 receptor/channel with the plasma membrane SOCC, a process called conformational coupling (Kiselyov et al., 1998; Boulay et al., 1999; Patterson et al., 1999; Yao et al., 1999; Ma et al., 2000). In platelets, evidence supports this conformational coupling mechanism because coimmunoprecipitation experiments show a coupling of endogenously expressed hTrp1 with type II IP_3 receptors when intracellular Ca^{2+} stores are depleted (Rosado et al., 2000; Rosado and Sage, 2000a). There is some very strong evidence against the conformational coupling mechanism for SOCC activation. When the IP_3 receptor-deficient B-cell line DT40 was stimulated with anti-IgM or carbachol, there was no increase in $[Ca^{2+}]_i$; however, the ability of thapsigargin to increase $[Ca^{2+}]_i$ was unaffected (Sugawara et al., 1997). These data were interpreted to mean that IP_3 receptors were not the mediator between the endoplasmic reticulum and SOCC. Also, studies in T lymphocytes lacking type 1 IP_3 receptors showed that depletion of intracellular stores with thapsigargin resulted in stimulation of Ca^{2+} influx, whereas agonist-induced influx was inhibited (Jayaraman et al., 1995).

In the study by Maruyama et al. (1997), 2APB was shown to inhibit thrombin-mediated elevation in $[Ca^{2+}]_i$ when extracellular Ca^{2+} was present. However, the effect of 2APB to inhibit thrombin-mediated intracellular Ca^{2+} mobilization (platelets incubated without extracellular Ca^{2+}, which prevents Ca^{2+} influx) was not examined (Maruyama et al., 1997). Also, the effect of 2APB on SOCC activation by thapsigargin was not investigated in platelets (Maruyama et al., 1997). In the present study, 2APB inhibited thrombin-induced intracellular Ca^{2+} mobilization in platelets but not thapsigargin-mediated intracellular Ca^{2+} mobilization. However, thapsigargin-stimulated Ca^{2+} influx after store depletion was inhibited by 2APB. 2APB also rapidly inhibited basal uptake of Sr^{2+} and Mn^{2+}, which suggests a direct effect of 2APB on plasma membrane SOCC channels. Therefore, 2APB may not be a specific inhibitor of IP_3-mediated intracellular Ca^{2+} mobilization as currently believed. Nevertheless, 2APB rapidly inhibited thrombin- and thapsigargin-mediated increases in $[Ca^{2+}]_i$ in human platelets by at least two different mechanisms.

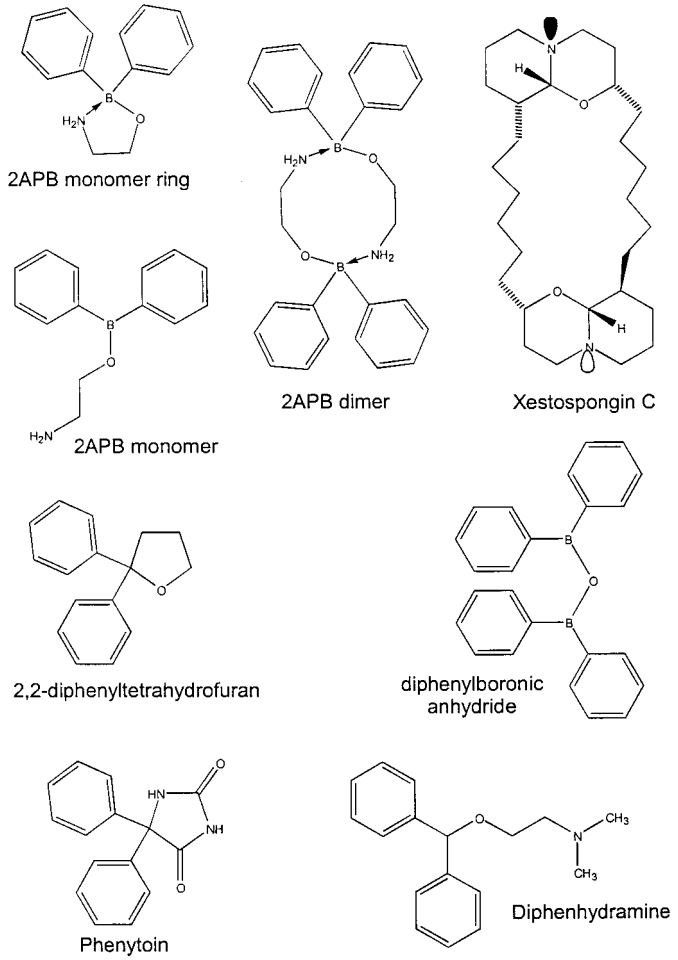

Fig. 1. Structures of 2APB dimer, 2APB monomer ring, 2APB monomer, xestospongin C, 2,2-diphenyltetrahydrofuran, diphenylboronic acid, phenytoin, and diphenhydramine. The 2APB monomer ring is formed when the boron forms a coordinate bond with the nitrogen, giving boron a full outer-shell octet of electrons, thus allowing for a tetragonal boron bond configuration (Strang et al., 1989). This compound will not carry a positive charge, whereas the 2APB monomer (no ring) will probably have a positive charge at physiological pH because it is a primary amine. Considering the ability of the boron in 2APB to form a coordinate bond with the ethanolamine nitrogen, neutral polymers of 2APB could theoretically exist, such as the 2APB dimer shown.

Materials and Methods

Reagents and Material Sources. Thrombin and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO). Thapsigargin, cyclopiazonic acid, and ionomycin were obtained from Calbiochem (San Diego, CA). Fura-2/AM was from Molecular Probes (Eugene, OR). Diphenylboronic acid, ethanolamine ester [also called 2-aminoethoxydiphenyl borate (CAS registry number [524–95-8])], diphenylboronic anhydride, and 2,2-diphenyltetrahydrofuran were from Aldrich Chemical (Milwaukee, WI). Other chemicals and reagents were from Fisher Scientific (Fair Lawn, NJ) and Sigma.

Blood Donors and Platelet Preparation. All donors were healthy volunteers (aged 20–40 years) who had not consumed any medication known to affect platelet function (e.g., calcium-channel blockers and aspirin) for at least 10 days before the study. Venous blood was collected into 1/10 volume of 74.8 mM sodium citrate, 38.1 mM citric acid, and 123 mM dextrose, pH 6.4 (Baxter, McGaw Park, IL). The blood was centrifuged at 250*g* for 10 min at room temperature to obtain platelet-rich plasma. The platelet-rich plasma was centrifuged at 550*g* for 12 min to sediment the platelets. The platelets were then suspended in a modified Tyrode's physiological salt solution (e.g., 145 mM NaCl, 4 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 10 mM Na/HEPES, and 6 mM glucose; pH 7.4) containing 1.0 mM EGTA, which acted to prevent spontaneous aggregation

during the various experimental manipulations by binding extracellular Ca^{2+} (Nolan and Lapetina, 1990). The platelets were washed once (at 500*g* for 15 min) and finally suspended Tyrode's solution, nominally Ca^{2+}-free (without EGTA), at a count of approximately 3×10^8 platelets/ml. In experiments involving the use of La^{3+}, the platelets were suspended in Tyrode's solution without Na_2HPO_4 to prevent the formation of insoluble lanthanum phosphate.

Drug Solution Preparations. Stock solutions of the drugs (thapsigargin, 2APB, diphenylboronic anhydride, and 2,2-diphenyltetrahydrofuran) in Me_2SO (10 mM) were prepared and stored at −20°C. Just before each experiment, aliquots were thawed and diluted to the desirable concentration with Me_2SO (see individual figure legends for concentrations used). In some experiments, we dissolved 2APB in water (10 mM), although vigorous shaking of the suspension was required for it to go into solution. This aqueous solution of 2APB produced the same results as 2APB dissolved in Me_2SO or ethanol (data not shown).

Platelet Loading with Fura-2 and Measurement of $[Ca^{2+}]_i$. Calcium measurements $[Ca^{2+}]_i$ were made using the fluorescent dye fura-2, which involved incubating the platelets with the cell-permeating acetoxymethyl ester (fura-2/AM) (Sargeant et al., 1992). A suspension of human platelets (isolated as described above) was incubated with 2 μM fura-2/AM for 1 h at room temperature on a rocking platform. Excess fura-2/AM was removed by centrifugation (500*g* for 10 min), and the platelets were suspended in Tyrode's solution without added Ca^{2+} or EGTA. Platelet suspensions (0.5 ml) were placed into 1.5-ml aggregometer tubes containing a magnetic stir bar (CHRONO-LOG, Havertown, PA). Just before $[Ca^{2+}]_i$ measurements were performed, Ca^{2+} was added back to the platelets to a final concentration of 1.0 mM 30 s before the commencement of the experiment, then 2APB (various concentrations), thapsigargin, or thrombin was added (see individual figure legends for concentrations used). The various agents were added to the cuvette as data was being collected; if the pipette tip was placed into the light path, a small transient decrease in fluorescence ratio was observed. The measurements of $[Ca^{2+}]_i$ was performed at room temperature in a SPEX ARCM spectrofluorometer (SPEX Industries, Edison, NJ) using excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. Calibration was performed as described previously for human sperm (Blackmore et al., 1990). $[Ca^{2+}]_i$ was calculated with the use of the SPEX dM3000 software package.

Measurement of $[Ba^{2+}]_i$ and $[Sr^{2+}]_i$. To assess basal Ca^{2+}-channel activity without agonist stimulation, Sr^{2+} or Ba^{2+} was added to platelets in Ca^{2+}-free medium to act as a Ca^{2+} surrogate. Sr^{2+} and Ba^{2+} enter the cell through Ca^{2+} channels; unlike Ca^{2+}, however, Ba^{2+} cannot be extruded from the cell by plasma membrane Ca^{2+}-ATPase pump, whereas Sr^{2+} can be extruded (Ozaki et al., 1992). Once inside the cell, Sr^{2+} and Ba^{2+} form fluorescent complexes with the dye fura-2 in a manner similar to that of Ca^{2+} but with a different affinity. The intensity of the fluorescence is directly proportional to the $[Sr^{2+}]_i$ or $[Ba^{2+}]_i$. $SrCl_2$ or $BaCl_2$ (10 mM) was added to the fura-2–loaded platelets in the absence of extracellular Ca^{2+} and in the absence of agonist. The fura-2/Ba^{2+} or fura-2/Sr^{2+} fluorescent complex was measured, and the results were expressed as 340:380-nm ratios. To show that Ba^{2+} and Sr^{2+} were entering through Ca^{2+} channels, Ca^{2+} (1.0, 2.0, 3.0, and 5.0 mM) was added to the platelets along with either Sr^{2+} or Ba^{2+}. The presence of Ca^{2+} acted as a competitor of Sr^{2+} and Ba^{2+} influx because Ca^{2+} produced a dose-dependent reduction of the 340/380-nm fluorescence signal that was increased by Sr^{2+} and Ba^{2+} influx (data not shown).

The influx of Sr^{2+} and Ba^{2+} into unstimulated platelets has not been fully characterized; therefore, our data must be interpreted cautiously. At present, we have no good explanation for the biphasic kinetics of cation uptake (e.g., Figs. 5 and 6). We may be observing multiple Ca^{2+} channels (Jenner and Sage, 2000; Sun and Kambayashi, 2000). Also, sequestration of the Sr^{2+} and Ba^{2+} by the endoplasmic reticulum may indirectly influence SOCC activity by altering the Ca^{2+} content of the endoplasmic reticulum; alternatively, these cations may also affect SOCC activity directly.

Thapsigargin-Induced $[Ca^{2+}]_i$ Entry. After Ca^{2+} was added back to the platelet suspensions, thapsigargin (dissolved in Me_2SO) was added to a final concentration of 100 nM, and the fluorescence was monitored as described previously (Dobrydneva et al., 1999). Alternatively, thapsigargin was added to platelets in the absence of extracellular Ca^{2+} after several minutes of incubation with EGTA (see specific experiments). Ca^{2+} was then added to activated platelets, and this resulted in a rapid increase in $[Ca^{2+}]_i$, which predominantly represented Ca^{2+} influx through SOCC. The same results were obtained if either 0.1 mM EGTA was added or no EGTA was used (Tyrode's solution used was nominally Ca^{2+}-free because no Ca^{2+} was added).

Molecular Modeling. Molecular modeling and energy minimization protocols were performed using CambridgeSoft Chem3D software (version 3.5.1; Cambridge, MA). Minimal energy conformations were obtained using the default settings provided in the MM2 (molecular mechanics) calculation package, part of the Chem3D software.

Statistical Analysis. Data are reported as mean ± S.E.M. for the number of individual experiments specified in each figure legend. Different platelet donors were used for each experiment.

Results and Discussion

Effect of 2APB on Thrombin and Thapsigargin to Increase $[Ca^{2+}]_i$ in the Presence and Absence of Extracellular Ca^{2+}. The data in Fig. 2A show the effect of three different concentrations of 2APB on the action of 0.05 U/ml thrombin to increase $[Ca^{2+}]_i$. The 2APB was added approximately 30 s before thrombin, and subsequent experiments (see below) show that this preincubation was not required to observe 2APB-mediated inhibition of agonist-induced elevation of $[Ca^{2+}]_i$. A concentration of 100 μM 2APB produced total inhibition of thrombin and 10 μM 2APB caused an approximately 50% inhibition, whereas 1 μM 2APB produced a small suppression in the peak effect and slowed the rate of $[Ca^{2+}]_i$ increase. The addition of 1.0 mM EGTA to the medium reduced the ability of thrombin to increase $[Ca^{2+}]_i$ substantially, the increase in $[Ca^{2+}]_i$ being approximately 30 nM (Fig. 2B), whereas when extracellular Ca^{2+} was present, the increase was approximately 240 nM (Fig. 2A). 2APB inhibited, in a dose-dependent manner, the ability of thrombin to increase $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} and, hence, in the absence of Ca^{2+} influx. These results therefore confirm the findings of Maruyama et al. (1997) by showing that 2APB was able to inhibit the ability of thrombin to increase $[Ca^{2+}]_i$ in human platelets when extracellular Ca^{2+} was present. In addition, we also demonstrate that 2APB inhibited thrombin-mediated mobilization of intracellular Ca^{2+} when extracellular Ca^{2+} was absent (Fig. 2B). Therefore, 2APB was able to inhibit both intracellular Ca^{2+} mobilization and Ca^{2+} influx, either directly on the Ca^{2+} influx channel or indirectly by its capacity to inhibit IP_3-mediated Ca^{2+} store depletion and, hence, prevent activation of SOCC by conformational coupling. The data in Fig. 3 summarize the dose-response data for 2APB to inhibit the ability of thrombin to increase $[Ca^{2+}]_i$ when extracellular Ca^{2+} was present (Fig. 2A) and absent (Fig. 2B). Although not shown, 1, 10, and 100 μM 2APB had no effect on basal $[Ca^{2+}]_i$. The addition of 500 μM of 2APB produced an elevation in $[Ca^{2+}]_i$ that was the same whether extracellular calcium was present or not. This suggests that the increase in $[Ca^{2+}]_i$ was caused by intracellular mobilization of Ca^{2+} by 2APB; this Ca^{2+}-mobilizing effect was not examined any further in the present study.

Most evidence supports the notion that thrombin increases $[Ca^{2+}]_i$ by mainly promoting the influx of Ca^{2+} through SOCC in platelets (Sargeant et al., 1992; Sage, 1997; Rosado and Sage, 2000c), although another Ca^{2+} channel may also be involved (Jenner and Sage, 2000; Sun and Kambayashi, 2000). We therefore used thapsigargin to activate SOCC and examined whether 2APB was able to inhibit Ca^{2+} influx in platelets. Thapsigargin activates SOCC by

inhibiting the smooth ER Ca^{2+} ATPase (SERCA) pump, thus promoting a loss of Ca^{2+} via a "leak" process in the ER (Pozzan et al., 1994; Treiman et al., 1998). This Ca^{2+}-depleted condition of the ER then causes an increase in Ca^{2+} influx through SOCC. The data in Fig. 4 show that 2APB elicited a dose-dependent inhibition of thapsigargin-mediated Ca^{2+} influx through SOCC. 2APB (100 μM) also completely inhibited the action of cyclopiazonic acid (another SERCA inhibitor) to increase $[Ca^{2+}]_i$ (data not shown). In Fig. 3, the dose-dependent data show that 2APB inhibits the effects of thapsigargin on $[Ca^{2+}]_i$. The dose response of 2APB to inhibit both thrombin- and thapsigargin-mediated increases in $[Ca^{2+}]_i$ were similar. One interpretation of this result, given the known action of 2APB, was that 2APB was inhibiting the thapsigargin-mediated release of Ca^{2+} via the ER IP_3-sensitive Ca^{2+} channel, thereby preventing the activation of plasma membrane SOCC by the conformational coupling mechanism. However, as will be shown later in this article, 2APB (Fig. 9, B and C) does not inhibit thapsigargin-mediated mobilization of intracellular Ca^{2+}; therefore, Ca^{2+} efflux from the ER in platelets seems to be independent of IP_3 receptors and probably occurs via a leak process (Pozzan et al., 1994). Thus, the inhibition of thapsigargin-mediated elevation of $[Ca^{2+}]_i$ by 2APB (Fig. 4) seems to be mediated by a more direct inhibitory effect on SOCC.

Measurement of Ca^{2+} Influx Using the Ca^{2+} Surrogates Sr^{2+}, Ba^{2+}, and Mn^{2+}. The data in Fig. 5 show the effect of adding Ba^{2+} directly to platelets in the absence of Ca^{2+} and without any agonist. When Ba^{2+} enters the fura-2–loaded platelet, it binds to fura-2 and causes an increase in the 340/380 nm fluorescence ratio (Dobrydneva et al., 1999). After a slight delay, Ba^{2+} caused an increase in the 340/380 nm fluorescence ratio, and 2APB displayed a dose-dependent inhibition of Ba^{2+} influx, consistent with 2APB directly inhibiting the influx of Ba^{2+} through plasma membrane Ca^{2+} channels. We showed previously that thapsigargin potentiated Ba^{2+} influx; therefore, Ba^{2+} influx represents, at least in part, the activity of SOCC (Dobrydneva et al., 1999). Likewise, when Sr^{2+} was added to fura-2–loaded platelets in the absence of extracellular Ca^{2+} and agonist, 2APB caused a dose-dependent inhibition of Sr^{2+} influx (Fig. 6A). The data showing the dose-dependent effect of 2APB to inhibit Sr^{2+} influx is summarized in Fig. 3. We believe that Sr^{2+} influx (Fig. 6A) occurs predominantly via SOCC because thapsigargin was able to stimulate Sr^{2+} influx further (Fig. 6B). When thapsigargin was added to platelets in the absence of extracellular Ca^{2+}, there was a very small increase in $[Ca^{2+}]_i$ (approximately 5% increase over basal within several minutes) (also see Figs. 9B and 10B). This result indicates that the increase in 340/380 nm fluorescence ratio (Figs. 5 and 6) was caused by an influx of Ba^{2+} or Sr^{2+}, not by Ca^{2+} release from the ER, because this could only represent a very small contribution to the overall fura-2 signal when extracellular Ca^{2+} was absent (Rosado and Sage, 2000c). The thapsigargin-stimulated Sr^{2+} influx was also inhibited by 2APB in a dose-dependent manner (Fig. 6B), with 100 μM 2APB causing complete inhibition of Sr^{2+} influx. These results are also compatible with the inhibition of SOCC activity by 2APB.

The data in Fig. 7 show that the ability of thrombin to stimulate Sr^{2+} influx through SOCC was also inhibited in a dose-dependent manner by 2APB. In this experiment, thrombin was added to the platelets in the absence of extracellular Ca^{2+} to mobilize intracellular Ca^{2+} and thereby activate SOCC. After 200 s of thrombin stimulation to allow the activation of SOCC, Sr^{2+} and different concentrations of 2APB were added simultaneously to the platelets. In the absence of 2APB, the addition of Sr^{2+} caused an immediate increase in 340/380 nm fluorescence ratio, consistent with SOCC being activated (Fig. 6A shows a comparison in the rate of 340/380 nm increase in the absence of thrombin). The presence of 2APB caused a dose-dependent inhibition of thrombin-mediated Sr^{2+} influx. Because 2APB and Sr^{2+} were added to the platelets simultaneously, the effect of 2APB to inhibit Sr^{2+} influx was essentially instantaneous. This rapid inhibitory effect would be consistent with 2APB having an action at the cell surface, possibly SOCC itself.

Another technique commonly used to measure Ca^{2+} influx is to monitor the quenching of fura-2 by Mn^{2+}, which enters cells, including platelets, via Ca^{2+} channels in the plasma membrane (Merritt and Hallam, 1988). The data in Fig. 8 show that treating platelets with 2APB reduced the rate at which Mn^{2+} quenched fura-2 in the absence of any agonist. There was a slight but rapid decrease in fura-2 fluorescence after Mn^{2+} was added to the platelet suspension. This was most probably caused by a small amount of fura-2 that had leaked out of the platelets into the medium and that would immediately bind Mn^{2+} when it was added. Incubating platelets with 2APB or dimethyl sulfoxide solvent (control solvent for 2APB) produced a slight decrease in fura-2 fluorescence over time. Although not shown, both thrombin and thapsigargin stimulate the rate of Mn^{2+}-induced fura-2 quenching; thus, Mn^{2+} entry represents SOCC activity, at least in part.

These results using Sr^{2+}, Ba^{2+} and Mn^{2+} were compatible with direct inhibition by 2APB of the Ca^{2+}-influx channel in the plasma membrane. This is because the entry of these cations could be ob-

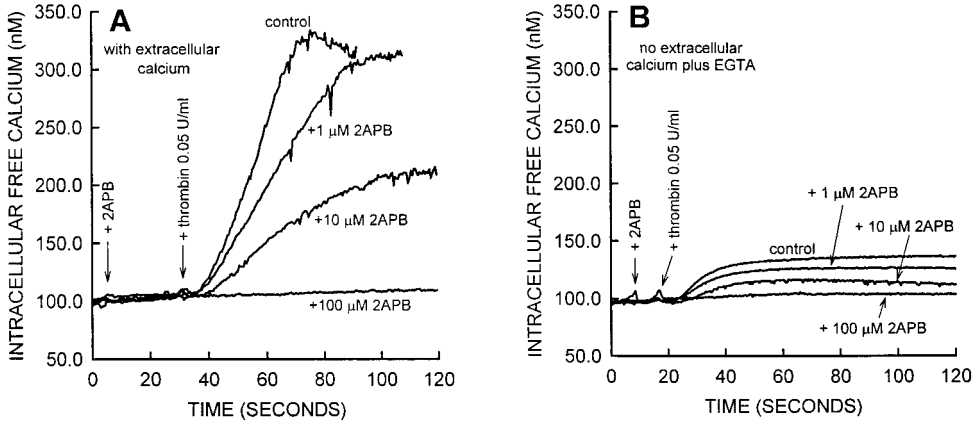

Fig. 2. Effect of 2APB on thrombin-mediated increase in $[Ca^{2+}]_i$ in the presence (A) and absence (B) of extracellular Ca^{2+}. Platelets were loaded with fura-2 as described under *Materials and Methods* and incubated in the absence of added extracellular Ca^{2+}, which was nominally Ca^{2+}-free. Calcium (1.0 mM) was added to 0.5 ml of platelets 30 s before data collection was started. Various concentrations of 2APB in Me_2SO were added to the platelets at 5 s, and thrombin was added at 30 s. A representative of five experiments is shown (A), and the averages of the inhibitory effects of 2APB from these experiments are shown in Fig. 3. B, platelets were incubated in the absence of added Ca^{2+}, and 1.0 mM EGTA was added to the platelets 30 s before data collection was started. After 5 s, 100 μM 2APB or Me_2SO (control) was added; 20 s later thrombin was added. The increase in $[Ca^{2+}]_i$ observed under these conditions was much smaller than the increase in $[Ca^{2+}]_i$ observed when Ca^{2+} was present in the medium (A). 2APB was able to produce a large inhibition of the increase in $[Ca^{2+}]_i$ elicited by thrombin. A representative of four experiments is shown, and the average of these experiments are shown in Fig. 3.

served in the absence of any agonist-induced depletion (thrombin or thapsigargin) of intracellular Ca^{2+} stores; hence, SOCC activity would be operating only at a basal level. However, 2APB (Figs. 5 and 6A) could inhibit this basal activity of SOCC. Also, thrombin- and thapsigargin-stimulated Sr^{2+} influx through SOCC was inhibited by 2APB (Figs. 7 and 6B, respectively).

Measurement of SOCC Activity after Depletion of Intracellular Ca^{2+} Stores with Thrombin, Thapsigargin, or Thrombin Plus Thapsigargin. Another common approach used to examine SOCC activity is to mobilize intracellular Ca^{2+} using an agonist (e.g., thrombin) or thapsigargin in the absence of extracellular Ca^{2+} plus EGTA in the medium (Fig. 7). In platelets, this results in a small increase in $[Ca^{2+}]_i$ caused by the mobilization of Ca^{2+} from intracellular pools when agonists are added (Rosado et al., 2000). To observe the SOCC activity, Ca^{2+} is then added back to the stimulated cells. A large and rapid influx of Ca^{2+} is then observed (Putney and McKay, 1999). The data in Fig. 9A show the effect of thrombin to mobilize intracellular Ca^{2+} in the absence of extracellular Ca^{2+}. A small increase in $[Ca^{2+}]_i$ was seen that declined gradually as intracellular Ca^{2+} pools were being depleted and Ca^{2+} was extruded from the cell by the plasma membrane Ca^{2+} ATPase (Paszty et al., 1998). At 150 s, 2.0 mM Ca^{2+} was added to the platelets in the presence and absence of 2APB (2APB and Ca^{2+} were added simultaneously). The presence of 2APB almost totally inhibited the increase in $[Ca^{2+}]_i$. The addition of 2APB alone to thrombin-treated platelets in the absence of extracellular Ca^{2+} caused a gradual decline in $[Ca^{2+}]_i$ to basal levels within approximately 2 min (data not shown). This decrease in $[Ca^{2+}]_i$, in the absence of extracellular Ca^{2+}, would be consistent with either Ca^{2+} being sequestered again into the ER by SERCA and/or being expelled from the cell by the Ca^{2+}-ATPase pump (Paszty et al., 1998) after 2APB inhibition of Ca^{2+} release from the ER by the IP_3 receptor (Maruyama et al., 1997).

The data in Fig. 9B show the effect of thapsigargin on intracellular Ca^{2+} mobilization, which was very small when Ca^{2+} was present in the extracellular medium (Fig. 4) (Rosado et al., 2000), followed by the addition of Ca^{2+} back to the platelets with or without 2APB. The small increase in $[Ca^{2+}]_i$ under these conditions possibly occurred because thapsigargin does not increase IP_3; therefore, IP_3 receptors are not activated. Hence, Ca^{2+} was effluxing from the ER by a leak process that seems to be not very active in the platelet (Pozzan et al., 1994). The ability of thapsigargin to increase $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} was not affected by 2APB (Fig. 9C). This result implies that Ca^{2+} was effluxing from the ER independent of IP_3 receptors and probably occurred via a leak process. The presence of 2APB totally prevented the influx of Ca^{2+} induced by thapsigargin, when Ca^{2+} was added back to thapsigargin treated platelets (Fig. 9B). When 100 μM 2APB was added to platelets treated with 100 nM thapsigargin for 15 min in the absence of extracellular Ca^{2+}, there was no effect on $[Ca^{2+}]_i$. This result, therefore, is consistent with the idea that thapsigargin-mediated mobilization of Ca^{2+} is not mediated by IP_3 receptors, because if it were an IP_3-mediated effect, then

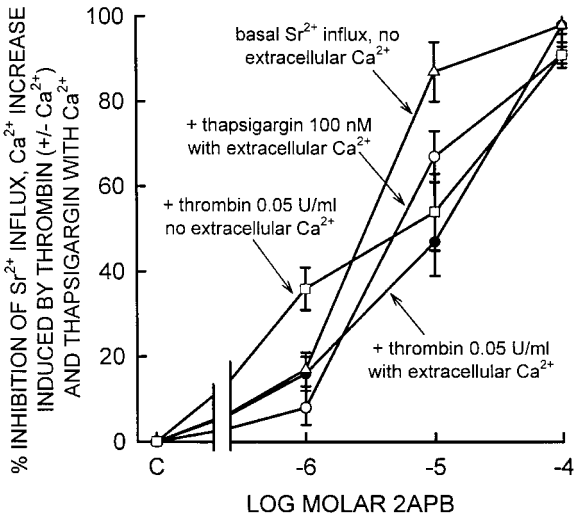

Fig. 3. Dose response of 2APB to inhibit 0.05 U/ml thrombin (with and without extracellular Ca^{2+}) and 0.1 μM thapsigargin-mediated increases in $[Ca^{2+}]_i$ (with extracellular Ca^{2+}). The inhibitory effect of 2APB on basal Sr^{2+} influx is also shown. The data presented are from experiments shown in Figs. 2, 4, and 6A. The values are means ± S.E.M. from four to five separate experiments.

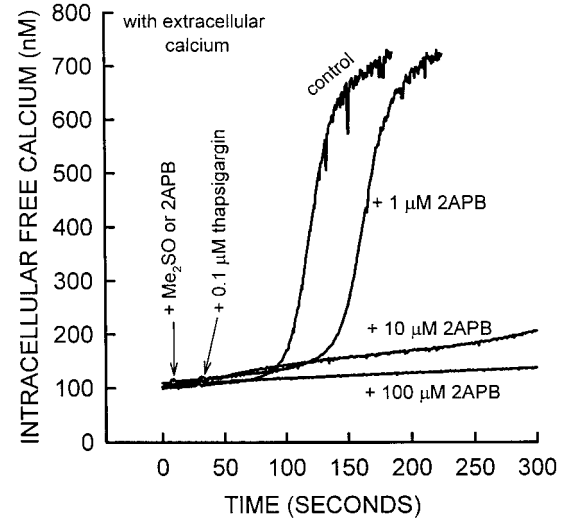

Fig. 4. Dose response of 2APB to inhibit thapsigargin-mediated (0.1 μM) increases in $[Ca^{2+}]_i$ when extracellular Ca^{2+} was present. Calcium (1.0 mM) was added to the platelets 30 s before data collection was started. 2APB was added at 10 s, and 0.1 μM thapsigargin was added at 30 s. A representative of five experiments is shown. The average of the five experiments is shown in Fig. 3.

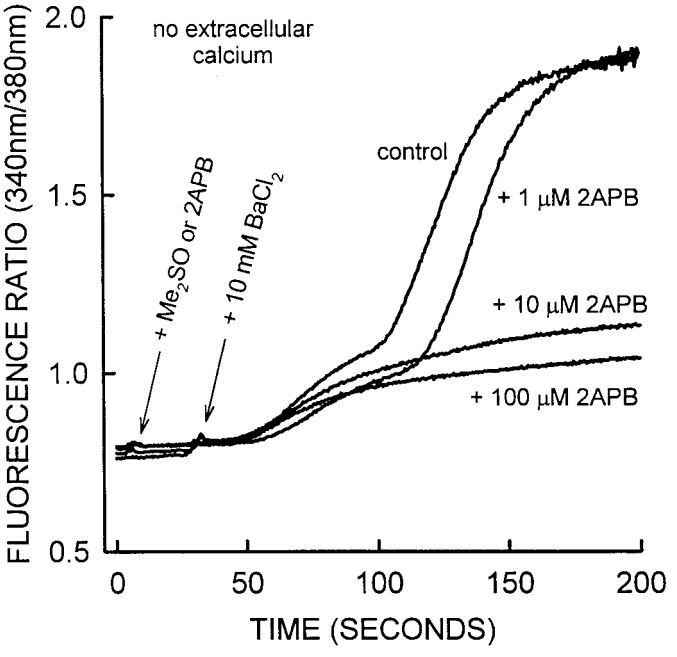

Fig. 5. Dose response of 2APB to inhibit Ba^{2+} influx in unstimulated platelets (no agonist) in the absence of extracellular Ca^{2+}. Platelets were incubated in the absence of added extracellular Ca^{2+}. At 5 s, 2APB or Me_2SO solvent control was added. At 30 s, 10 mM $BaCl_2$ was added, and the 340/380 nm fluorescence ratio was measured. 2APB produced a dose-dependent inhibition of Ba^{2+} influx as measured by a decrease in the fura-2 fluorescence ratio. This result implies that 2APB was blocking Ca^{2+} channels directly. A representative of four experiments is shown.

2APB should inhibit the thapsigargin-mediated increase in $[Ca^{2+}]_i$ as it does with thrombin (Fig. 10C).

The data in Fig. 9, A and B, show that thrombin and thapsigargin alone produced a small elevation in $[Ca^{2+}]_i$ when extracellular Ca^{2+} was absent. The data in Fig. 9D showed that combining thapsigargin and thrombin produced a larger increase in $[Ca^{2+}]_i$ than when either agent alone was added; in fact, the increase in $[Ca^{2+}]_i$ was synergistic (data not shown). This combination of agents would be more conducive to a greater elevation in $[Ca^{2+}]_i$ because the Ca^{2+} mobilized from the ER by thrombin would be prevented from being taken up again into the ER because SERCA would be inhibited by thapsigargin. The addition of Ca^{2+} at 250 s produced an immediate increase in $[Ca^{2+}]_i$ that was totally inhibited when 2APB was added simultaneously with Ca^{2+}. 2APB added alone caused a very small decrease in $[Ca^{2+}]_i$ when added to thrombin- and thapsigargin-treated platelets in the absence of extracellular Ca^{2+} at 250 s, probably because SERCA was inhibited by thapsigargin (thus, Ca^{2+} could not be sequestered by the ER) and because of a relatively low activity of the plasma membrane Ca^{2+}-ATPase pump, which acts to expel Ca^{2+} from the platelet (Paszty et al., 1998). This result implies that the ER Ca^{2+} pool was depleted substantially because of the prolonged action of thrombin-generated IP_3 and thapsigargin inhibition of SERCA. This Ca^{2+}-depleted condition of the ER would also ensure that the SOCC was maximally activated, which was evident by the large and immediate increase in $[Ca^{2+}]_i$ when Ca^{2+} was added to the Ca^{2+}-depleted platelets at 250 s (Fig. 9D). Because 2APB had no effect on intracellular mobilization under this thrombin-plus-thapsigargin condition (Fig. 9D), the effect of 2APB to totally inhibit the Ca^{2+} influx was most probably caused by a direct effect on SOCC itself and not by 2APB preventing the release of Ca^{2+} from the ER and uncoupling SOCC from the IP_3 receptor (because the ER would be substantially depleted of Ca^{2+}).

It is possible that 2APB binds to IP_3 receptors that are coupled to SOCC, causing a conformational change that uncouples IP_3 receptors from SOCC, thereby inhibiting Ca^{2+} influx through SOCC. No such effect of 2APB on IP_3 receptors has been characterized apart from the ability of 2APB to inhibit IP_3-mediated Ca^{2+} efflux from the endoplasmic reticulum.

Time Course of the 2APB to Inhibit Thrombin- and Thapsigargin-Stimulated Ca^{2+} Influx and Basal Sr^{2+} Influx. The experiments presented above predominantly involved incubating platelets with 2APB for short periods (10–30 s) or adding 2APB together with either Ca^{2+} or Sr^{2+} at the same time (Figs. 7 and 9, A–C). The following experiments were performed to investigate just how rapid the effect of 2APB was in inhibiting the actions of thrombin and thapsigargin. The data in Fig. 10A show that 2APB caused an immediate and large decrease in the thrombin-mediated increase in $[Ca^{2+}]_i$. The 2APB was added when the $[Ca^{2+}]_i$ had increased to its maximum level. For comparison purposes, 100 μM La^{3+} was also added to thrombin-stimulated platelets, and it also produced an immediate cessation in the elevation of $[Ca^{2+}]_i$. The comparison of the effects of La^{3+} was used recently to show that LY294002 and farnesylcysteine analogs were not direct Ca^{2+}-channel blockers in platelets (Rosado and Sage, 2000b,c). LY294002 had no effect on $[Ca^{2+}]_i$, although farnesylcysteine analogs inhibited $[Ca^{2+}]_i$ only after a delay of 30 s, whereas La^{3+} produced an immediate inhibitory effect. La^{3+}, a nonselective Ca^{2+}-channel blocker, is unlikely to enter the platelet because of its positive charge; therefore, its action would be confined to plasma membrane Ca^{2+} channels and not intracellular Ca^{2+} channels (Hoth and Penner, 1993). The combination of La^{3+} plus 2APB did not produce any greater decrease in Ca^{2+} influx than that observed with either La^{3+} or 2APB alone; all these treatments produced identical decreases in $[Ca^{2+}]_i$ (Fig. 10A). These results imply that 2APB and La^{3+} were blocking the same channel(s) in the plasma membrane.

In another experimental protocol, we used thapsigargin to promote Ca^{2+} entry through SOCC. In these experiments, we first mobilized intracellular Ca^{2+} with thapsigargin in the absence of extracellular Ca^{2+} (Fig. 10B). After 2 min, 1.0 mM Ca^{2+} was added to the stimulated platelets to promote entry through activated SOCC. The $[Ca^{2+}]_i$ began to increase immediately to a peak value approximately 2.0 min later (Fig. 10B). When the $[Ca^{2+}]_i$ neared the maximum level, either La^{3+}, 2APB, or both were added. Both La^{3+} and 2APB produced an immediate decline in $[Ca^{2+}]_i$, and the inhibitory effects of La^{3+} plus 2APB were not additive (Fig. 10B). This result is compatible with the idea that 2APB and La^{3+} block the same channel, which was likely to be SOCC.

Another approach to blocking Ca^{2+} influx into platelets is to add an amount of EGTA to the extracellular medium in excess of the total calcium in the medium. This procedure will reduce the extracellular free Ca^{2+} to less than micromolar levels. The data in Fig. 10C show that when Ca^{2+} was added to thrombin-stimulated platelets in the

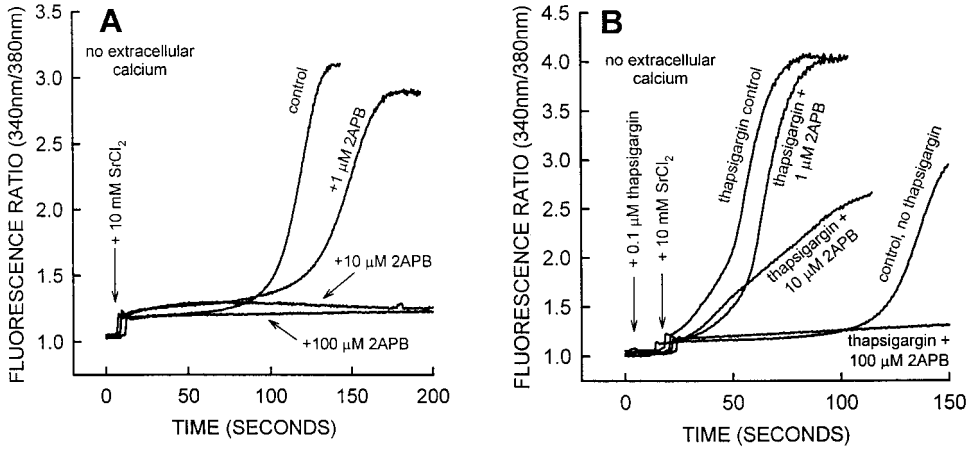

Fig. 6. Dose response of 2APB to inhibit Sr^{2+} influx into unstimulated platelets, without extracellular Ca^{2+} (A), and influence of thapsigargin on Sr^{2+} influx and the effect of 2APB on thapsigargin-stimulated Sr^{2+} influx (B). Platelets were incubated in the absence of added extracellular Ca^{2+}. Various concentrations of 2APB were added 10 s before data collection was started. At 5 s, 10 mM $SrCl_2$ was added. The increase in fura-2 340/380 nm fluorescence ratio indicates the entry of Sr^{2+} through Ca^{2+} channels. 2APB blocked the entry of Sr^{2+}, suggesting a direct effect on the plasma membrane Ca^{2+} channels (A). A representative of four experiments is shown. B, platelets were incubated in the absence of extracellular Ca^{2+}. Various concentrations of 2APB were added 10 s before data collection was initiated. Thapsigargin (100 nM) was added at 10 s, and 10 mM $SrCl_2$ was then added at 20 s. Thapsigargin treatment greatly potentiated the influx of Sr^{2+}, such that Sr^{2+} influx increased without delay. This result suggests that Sr^{2+} influx in platelets predominantly represents SOCC because thapsigargin was able to stimulate Sr^{2+} influx. The ability of thapsigargin to increase Sr^{2+} influx was inhibited by 2APB, with 100 μM 2APB causing an almost total inhibition of thapsigargin-stimulated Sr^{2+} influx. A representative of four experiments is shown.

absence of extracellular Ca^{2+}, there was a rapid increase in $[Ca^{2+}]_i$ similar to that shown in Fig. 9A. When EGTA was added 20 s after the addition of Ca^{2+}, there was an immediate and linear decline in $[Ca^{2+}]_i$ consistent with the prevention of Ca^{2+} influx through activated plasma membrane Ca^{2+} channels by EGTA and the continued extrusion of Ca^{2+} from the platelet by the plasma membrane Ca^{2+}-ATPase pump. When 2APB was added to the platelets 20 s after the addition of Ca^{2+}, there was also an immediate and rapid decline in $[Ca^{2+}]_i$ that was more rapid than that seen with EGTA. This could be attributed to 2APB action at two sites: the internal IP_3R and the plasma membrane Ca^{2+} influx channel. Blocking the efflux of Ca^{2+} from the endoplasmic reticulum via the IP_3R and blocking Ca^{2+} influx across the plasma membrane would produce a lower level of $[Ca^{2+}]_i$ than that seen by blocking Ca^{2+} influx alone. When 2APB was combined with EGTA, there was also a rapid and immediate decline in $[Ca^{2+}]$ that was the same as that observed with 2APB alone. Therefore, 2APB, like EGTA, was able to block influx, and it was also able to block internal release of Ca^{2+}.

We also examined the effect of 2APB and La^{3+} on basal Sr^{2+} influx. 2APB was added when the rate of Sr^{2+} influx was maximal. The data in Fig. 11 shows that 50 μM 2APB caused an immediate decline of Sr^{2+} influx. Because the Sr^{2+} fura-2 signal declined after 2APB addition, Sr^{2+} was being rapidly removed from the cytoplasm either by sequestration into the ER by SERCA or by the activity of the plasma membrane Ca^{2+}-ATPase pump (Ozaki et al., 1992). The addition of La^{3+} also caused an immediate decline in Sr^{2+} influx, and the effect of La^{3+} and 2APB together to inhibit Sr^{2+} influx was not additive. This supports the notion that 2APB and La^{3+} were inhibiting the same Ca^{2+} channel(s), which, given the data presented above, seem to be SOCC.

Lack of Effect of 2APB on the Ability of Ionomycin to Increase $[Ca^{2+}]_i$. The action of the Ca^{2+} ionophore ionomycin to increase $[Ca^{2+}]_i$ in platelets should not be influenced by 2APB if 2APB were blocking Ca^{2+} channels in either the plasma membrane or ER, because ionomycin merely moves Ca^{2+} ions across membranes independent of Ca^{2+} channels. The data in Fig. 12 support this hypothesis, because the capacity of 2APB to influence the increase in $[Ca^{2+}]_i$ induced by ionomycin was minimally affected. Two methods were used to study the ability of ionomycin to increase $[Ca^{2+}]_i$. In one approach, ionomycin was added to platelets in the presence of extracellular Ca^{2+}; in the other approach, ionomycin was added to platelets in the absence of extracellular Ca^{2+} so that the ionomycin could mobilize intracellular Ca^{2+}; then Ca^{2+} was added back to the platelets. The Ca^{2+} influx observed at this stage most probably represented the ability of ionomycin to translocate Ca^{2+} across the plasma membrane. The rate of increase in $[Ca^{2+}]_i$ was slightly inhibited, but the maximum effect on $[Ca^{2+}]_i$ was not affected using either protocol. This result also shows that 2APB does not influence the Ca^{2+}/fura-2 fluorescence signal in platelets and therefore cannot account for the inhibitory effects of 2APB on $[Ca^{2+}]_i$ observed in this study.

Pharmacophore Responsible for the Inhibition of Ca^{2+} Influx. We examined several compounds that are structurally related to 2APB for Ca^{2+} influx-blocking activity (Fig. 1). The first was diphenylboronic anhydride (DPBA), in which the two diphenylboronic groups are separated by an oxygen atom. The data in Fig. 13 show a dose response of DPBA to inhibit the ability of thrombin to increase $[Ca^{2+}]_i$ in the presence and absence of extracellular Ca^{2+}. The IC_{50} value for DPBA to inhibit thrombin-mediated elevation of $[Ca^{2+}]_i$ when extracellular Ca^{2+} was either present or absent was approximately 2 μM. A greater inhibition (90% at 100 μM) of the increase in $[Ca^{2+}]_i$ was observed when extracellular Ca^{2+} was present compared with when Ca^{2+} was absent (60% at 100 μM). The IC_{50} value for DPBA to inhibit $[Ca^{2+}]_i$ was approximately five times lower than the IC_{50} value for 2APB to inhibit the thrombin-induced $[Ca^{2+}]_i$ increase in the presence and absence of Ca^{2+}, which was 10 μM (Fig. 3). Therefore, it seems that the diphenylboronic moiety is the sole requirement for producing an inhibition of Ca^{2+} influx.

According to the crystal structure data (Rettig and Trotter, 1976) the ethanolamine chain of 2APB forms an internal coordinate N→B

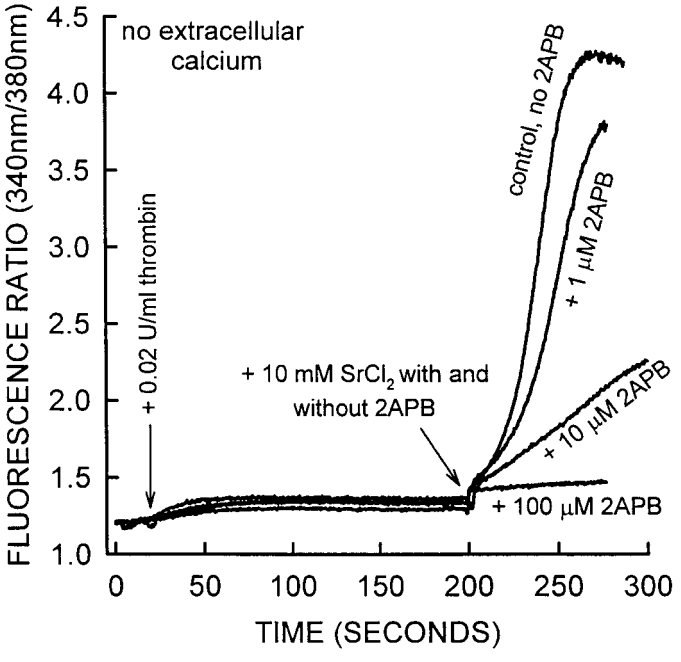

Fig. 7. Dose response of 2APB to inhibit thrombin-mediated Sr^{2+} influx into platelets. Platelets were incubated in the absence of added extracellular Ca^{2+}. Thrombin 0.02 U/ml was added at 15 s, and at 200 s, 10 mM $SrCl_2$ was added together with various concentrations of 2APB. 2APB produced an immediate dose-dependent inhibition of Sr^{2+} influx as measured by a decrease in the fura-2 fluorescence ratio compared with the control. A representative of four experiments is shown.

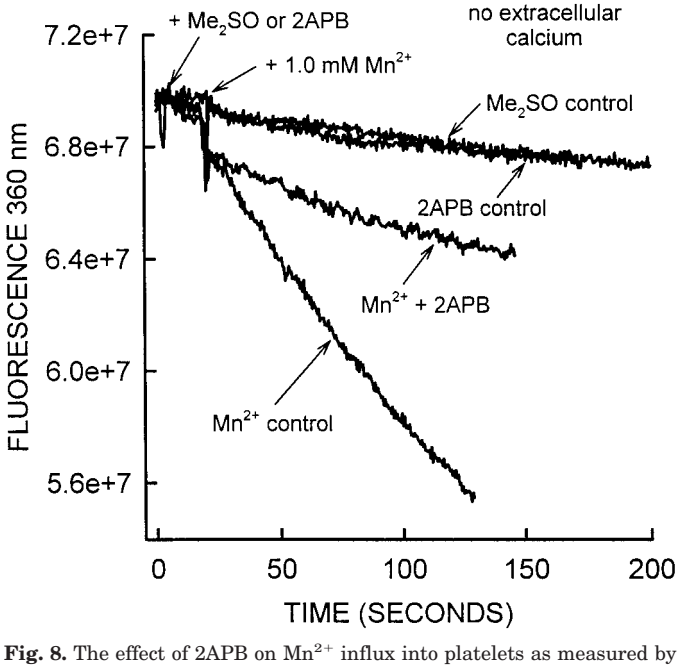

Fig. 8. The effect of 2APB on Mn^{2+} influx into platelets as measured by quenching of fura-2. Platelets were incubated in the absence of added extracellular Ca^{2+}. 2APB or Me_2SO was added at 5 s, and at 20 s, 1.0 mM Mn^{2+} was added. There was a slight decline in fura-2 fluorescence with 2APB or Me_2SO (without Mn^{2+}), and the addition of Mn^{2+} produced a rapid decline in fura-2 fluorescence measured at 360 nm. The presence of 2APB was able to almost totally prevent the Mn^{2+}-induced quenching of fura-2, such that the rate of fura-2 fluorescence decline was the same as that for the Me_2SO control. A representative of three experiments is shown.

bond with tetrahedral boron, which results in the formation of a boroaxozolidine ring (Fig. 1, 2APB monomer ring). Thus, as noticed by Niedenzu and Dawson (1967), such compounds have an unusual hydrolytic stability in water. The pK_b value we measured for an aqueous solution of 2APB was approximately 10.5, whereas the pK_b value for ethanolamine is 4.6. These data are further evidence that in solution, 2APB retains the internal coordinate N→B bond in a boroaxozolidine ring, which makes the free electron pair of nitrogen less available for protonation. Existence of the open-chain form (Fig. 1, 2APB monomer), although not supported by the crystallographic data, is also theoretically possible. Therefore, we sought a stable isoelectronic analog of the 2APB heterocycle in which the N→B coordinate bond was replaced by an isoelectronic C—C covalent bond, retaining the geometry of a 2APB molecule. One such compound, 2,2-diphenyltetrahydrofuran (DPTTF), was available commercially. This structure possesses a five-membered ring containing an oxygen atom but no nitrogen or boron atoms, and the two phenyl groups are attached to a tetrahedral carbon atom (Fig. 1). This compound displayed Ca^{2+}-blocking activity comparable with that seen with DPBA (Fig. 13). It seems, therefore, that the presence of the five-membered tetrahydrofuran ring attached to the diphenyl groups in DPTTF is not deleterious for the activity of this compound to inhibit Ca^{2+} influx. It also seems that the presence of the boron atom in 2APB is not an absolute requirement for the activity.

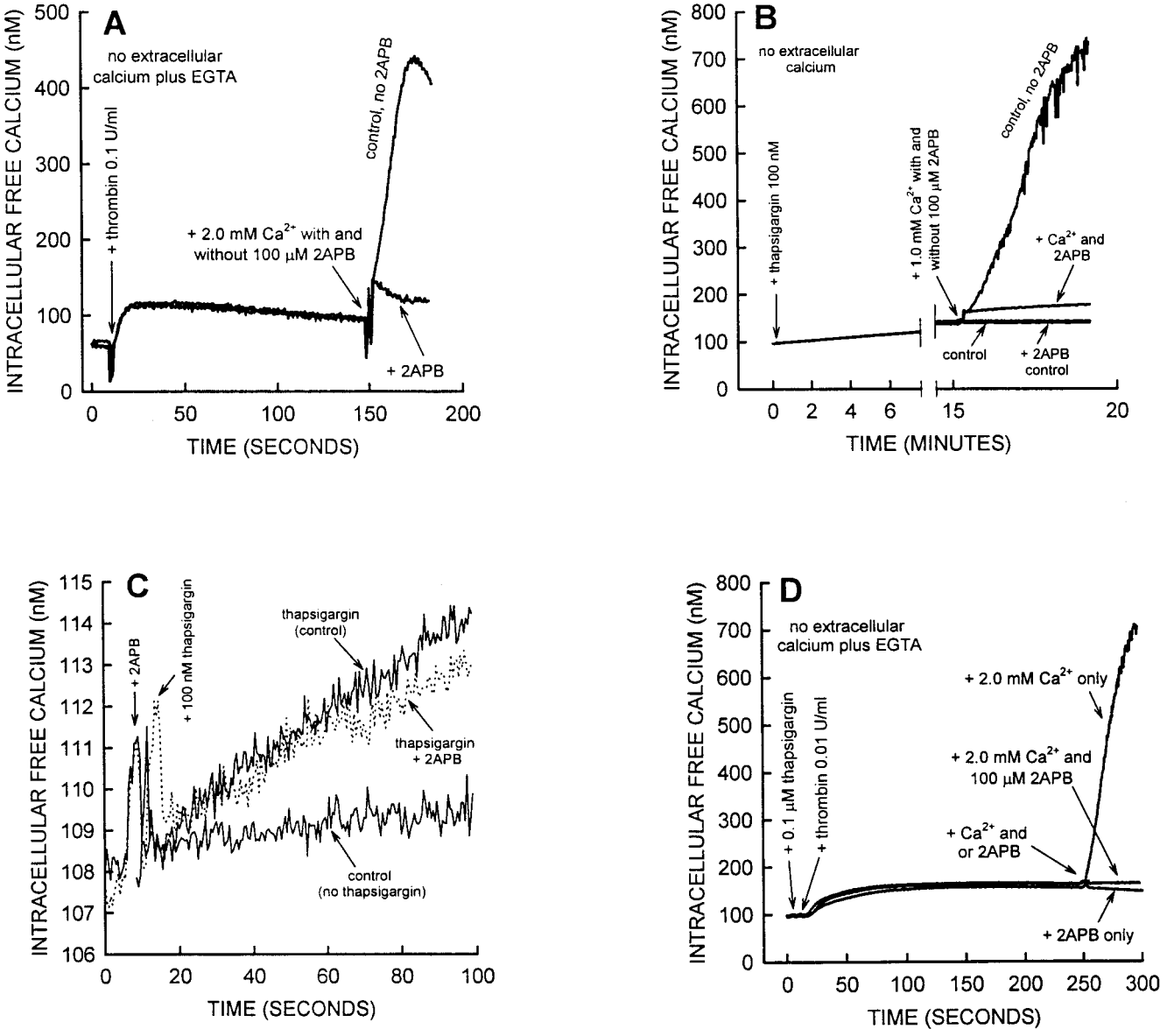

Fig. 9. The effect of 2APB on calcium influx initiated after mobilization of intracellular Ca^{2+} by thrombin (A), thapsigargin (B and C), and thrombin plus thapsigargin (D). A, platelets were incubated in the absence of extracellular Ca^{2+} and in the presence of 1.0 mM EGTA. Thrombin was added at 10 s. At 150 s, when $[Ca^{2+}]_i$ was declining because of depletion of intracellular stores, 2.0 mM Ca^{2+} with or without 2APB was added simultaneously to the platelets. In the presence of 100 μM 2APB, there was almost no increase in $[Ca^{2+}]_i$. A representative of three experiments is shown. B, platelets were incubated in the absence of extracellular Ca^{2+}. Thapsigargin (100 nM) was added at 20 s, and $[Ca^{2+}]_i$ began to increase gradually, leveling off between 10 and 15 min. At 15 min, 1.0 mM Ca^{2+} with or without 2APB was added simultaneously to the platelets. The presence of 100 μM 2APB totally prevented the increase in $[Ca^{2+}]_i$ that was observed in the absence of 2APB when Ca^{2+} was added. A representative of three experiments is shown. C, the effect of 2APB on the ability of thapsigargin to increase $[Ca^{2+}]_i$ was examined in the absence of extracellular Ca^{2+}. 2APB had no effect on thapsigargin to increase $[Ca^{2+}]_i$. A representative of three experiments is shown. D, platelets were incubated in the absence of Ca^{2+} and with 1.0 mM EGTA. Thapsigargin (0.1 μM) plus thrombin (0.01 U/ml) were added at 5.0 and 10.0 s, respectively. At 250 s, either 2APB alone, 2APB plus Ca^{2+}, or Ca^{2+} alone was added. 2APB completely prevented the increase in $[Ca^{2+}]_i$ induced by the addition of Ca^{2+} to the extracellular fluid. 2APB alone had a small effect on $[Ca^{2+}]_i$ stimulated by thapsigargin and thrombin. A representative of three experiments is shown.

Phenytoin has structural features similar to those of 2APB, such as two phenyl groups attached to the tetrahedral carbon of a five-membered ring. Phenytoin is much more polar than 2APB because of the nature of the heterocyclic imidazolidinedione moiety, which has acidic protons. Phenytoin was a very weak inhibitor of thrombin-mediated increases in $[Ca^{2+}]_i$ ($22 \pm 5\%$ inhibition at 100 μM). Therefore, extensive modification of the five-membered ring cannot be tolerated (two nitrogen atoms and two ketone groups in phenytoin compared with DPTTF).

An analog of the 2APB monomer that does not contain boron is diphenhydramine (Fig. 1). Two phenyl groups are attached to the tertiary carbon, and the secondary-amine nitrogen bears two methyl groups. There is no possibility for the internal coordinate-bond formation and the ring closure in this structure. Diphenhydramine was almost devoid of the inhibitory activity necessary to block thrombin-induced $[Ca^{2+}]_i$ elevation ($4 \pm 4\%$ inhibition at 100 μM). Therefore, the presence of the diphenyl groups attached to a tertiary carbon alone may not be sufficient for activity, and a moderately hydrophobic five-membered ring may also potentiate the activity. From this limited structure-activity relationship study, we see that two diphenyl groups attached to a tetrahedral atom of a five-membered ring seem to be structural requirements for calcium-blocking activity. This five-membered ring, however, cannot tolerate much modification (phenytoin is far less active at blocking thrombin-induced $[Ca^{2+}]_i$ elevation, whereas DPTTF is very active).

There have been some suggestions that 2APB and xestospongin C are acting in a similar manner to inhibit IP_3 channels because they "share some distant structural similarity" (van Rossum et al., 2000). Thus, it has been proposed that 2APB exists predominantly as a dimer in which a coordinate N→B bond forms between two open-chain molecules of 2APB (van Rossum et al., 2000). This is found when one looks at the structures of both compounds displayed side by side (Wilcox et al., 1998; van Rossum et al., 2000) (Fig. 1). The data in Fig. 14 show minimized energy structures of xestospongin C and 2APB dimer using the MM2 force field. It is clear that both structures have totally different space-filling orientations and charge distributions. In the 2APB dimer, the two aromatic rings at either end of the molecule are projecting away from the boron atom in a propeller-like arrangement at an angle of 119° with the planes of the rings at an angle of 48° to each other. The aromatic rings posses freedom of rotation around single bonds. Xestospongin C has no aromatic character for each one of two bis-1-oxaquinolizidine–saturated heterocyclic rings at either end of the molecule. The 2APB dimer has a 10-membered ring compared with a 20-membered saturated methylene ring in xestospongin C. Xestospongin C also has two tertiary amine groups in each of the rings that can be protonated at the physiological pH (Gafni et al., 1997). Because hypothetical 2APB dimer and xestospongin C are so structurally and spatially dissimilar (Fig. 14), we suggest that both compounds are probably binding to discrete sites on the IP_3R to inhibit Ca^{2+} release. Earlier studies showed that these sites were not the IP_3-binding pocket because these compounds did not prevent IP_3-binding (Gafni et al., 1997; Maruyama et al., 1997).

Concluding Comments. This study demonstrates a lack of specificity of 2APB as a unique inhibitor of IP_3-induced Ca^{2+} release in human platelets. We predict that 2APB would have similar effects on other cells because many other cell types have been shown to possess both SOCC- and IP_3-induced Ca^{2+}-release mechanisms. If 2APB had

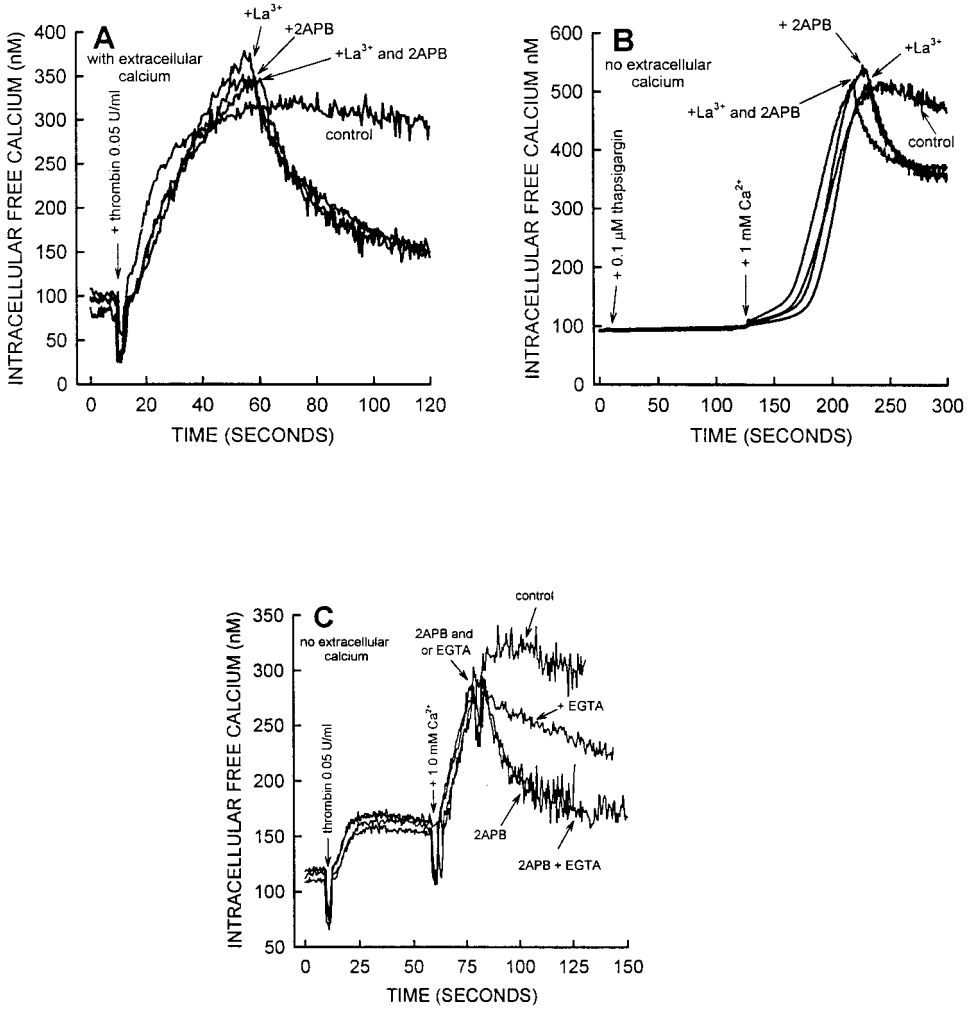

Fig. 10. Time course for the effect of La^{3+} and 2APB to inhibit the increase in $[Ca^{2+}]_i$ elicited by thrombin (A) and thapsigargin (B) in the presence of extracellular Ca^{2+}. C, the effect of EGTA, 2APB, and 2APB plus EGTA to inhibit thrombin-mediated Ca^{2+} influx is shown. A, thrombin was added to platelets at 10 s in the presence of 1.0 mM extracellular Ca^{2+}. At 60 s, either La^{3+}, 2APB, or both were added. The $[Ca^{2+}]_i$ began to decline immediately (A). The inhibitory effects of La^{3+} and 2APB were not additive, Also, the inhibitory effects of 2APB and La^{3+} were the same, which suggests that both agents were inhibiting the same Ca^{2+} channel(s) in platelets. A representative of three experiments is shown. B, thapsigargin (100 nM) was added to platelets at 10 s in the absence of added Ca^{2+}. The influx of Ca^{2+} was then initiated by adding 1.0 mM Ca^{2+} at 130 s. When the increase in $[Ca^{2+}]_i$ had reached a maximal level, either La^{3+}, 2APB, both were added. Both La^{3+} and 2APB caused an immediate decline in $[Ca^{2+}]_I$, and the inhibitory effect observed when both La^{3+} plus 2APB were added together was no greater than that seen with either La^{3+} or 2APB alone. A representative of three experiments is shown. C, thrombin was added at 10 s to platelets in Ca^{2+}-free buffer, and at 60 s, 1.0 mM Ca^{2+} was added to initiate Ca^{2+} influx. After 80 s, either 2APB, EGTA, or 2APB plus EGTA was added. A representative of four experiments is shown.

two sites of action, it would seem from this study to be an unusual Ca^{2+}-channel antagonist, because it reduces agonist-stimulated increases in $[Ca^{2+}]_i$ by inhibiting both internal release of Ca^{2+} (Fig. 2B) and Ca^{2+} influx across the plasma membrane via SOCC.

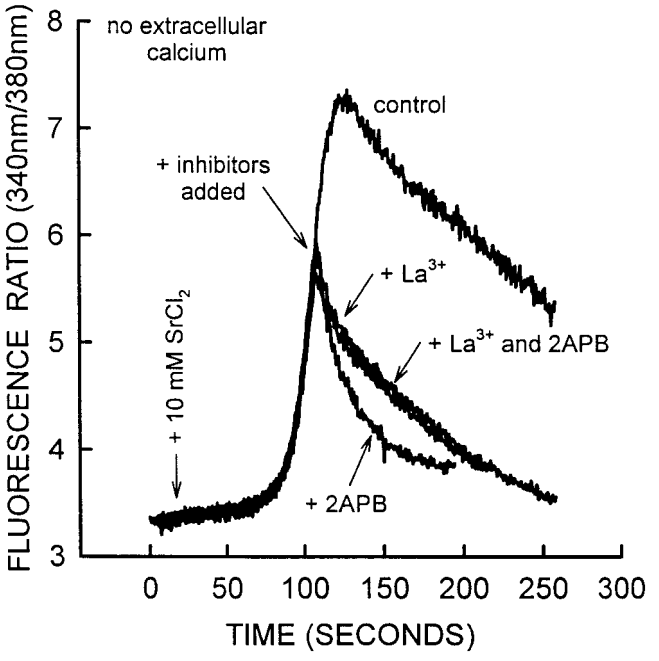

Fig. 11. Time course for the effects of La^{3+} and 2APB to inhibit Sr^{2+} influx in unstimulated platelets (no agonist). The influx of Sr^{2+} was initiated by adding 10 mM $SrCl_2$ at 10 s. When Sr^{2+} influx was at a maximum rate (110 s), either La^{3+}, 2APB, or La^{3+} plus 2APB was added. These treatments caused an immediate cessation in Sr^{2+} influx, consistent with La^{3+} and 2APB both blocking Ca^{2+} channels in the plasma membrane. A representative of four experiments is shown.

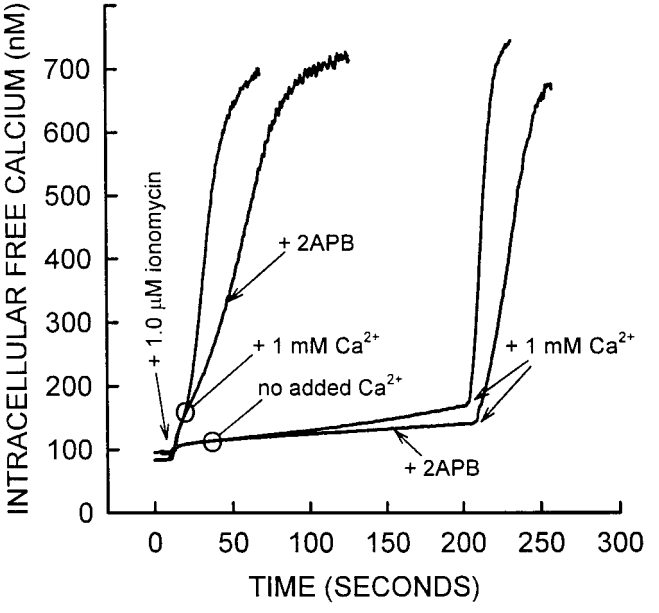

Fig. 12. The effect of 2APB on the ability of ionomycin to increase $[Ca^{2+}]_i$. Ionomycin (1.0 μM) was added to platelets in the presence of 1.0 mM extracellular Ca^{2+} in the presence or absence of 100 μM 2APB (added 10 s before data collection was started). 2APB reduced the rate of increase in $[Ca^{2+}]_i$ but had no effect on the maximum effect. In another experiment, 1.0 μM ionomycin was added to platelets in the absence of extracellular Ca^{2+} in the presence or absence of 100 μM 2APB. After 200 s, Ca^{2+} was added to the platelets, and the increase in $[Ca^{2+}]_i$ was measured. 2APB reduced the rate of increase in $[Ca^{2+}]_i$, but it had no effect on the maximum effect. A representative of three experiments is shown.

We believe that the most convincing evidence that 2APB has a direct effect on inhibition of SOCC is that 2APB was able to inhibit Ba^{2+}, Sr^{2+}, and Mn^{2+} influx into platelets that were not being stimulated by any agonist (Figs. 5, 6A, 8, and 11). In this situation, Ca^{2+} would permeate the SOCC at a basal rate. Recent studies show that SOCC was activated by direct coupling of the ER IP_3 receptors/

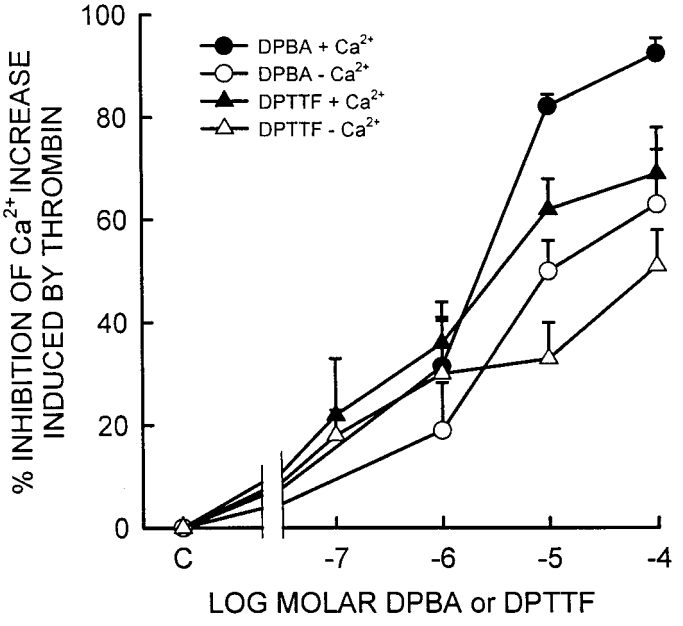

Fig. 13. Dose response of DPBA and DPTTF to inhibit thrombin-mediated elevation in $[Ca^{2+}]_i$ in the presence and absence of extracellular Ca^{2+}. The dose-response experiments were performed in a manner similar to that described for 2APB in the legends to Figs. 2 and 3. The values are means ± S.E.M. from four to five separate experiments.

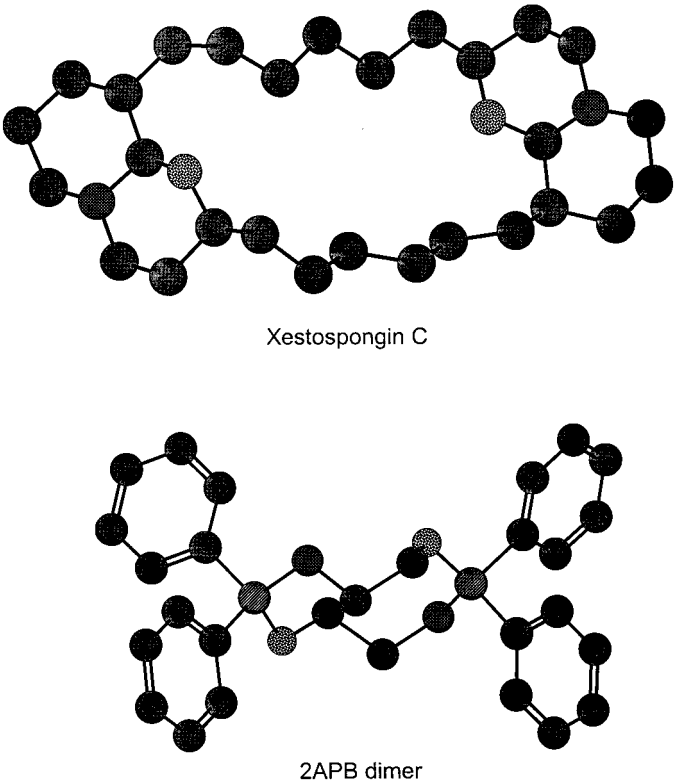

Fig. 14. Minimized structures of xestospongin C (top) and 2APB dimer (bottom) obtained by MM2 force field. Hydrogen atoms have been omitted for clarity.

channel to the plasma membrane SOCC (Kiselyov et al., 1998; Barritt, 1999; Boulay et al., 1999; Patterson et al., 1999; Yao et al., 1999; Ma et al., 2000). This coupling was believed to be initiated by the depletion of ER Ca^{2+} stores that produces a conformational change in the IP_3 receptor, which then caused it to interact and couple with SOCC. Therefore, from this model, there would be little or no coupling of plasma membrane SOCC to the ER in the basal state, and the Ca^{2+} stores would be filled with Ca^{2+}. Hence any basal SOCC activity would be acting independently of Ca^{2+} store-filling state. The action of 2APB on the IP_3-sensitive Ca^{2+} stores in the resting state would be to prevent Ca^{2+} release and might actually promote further filling of the stores because of the continued action of SERCA. This situation would also not contribute to the activation of SOCC by conformational coupling because the IP_3-sensitive Ca^{2+} stores would probably contain more Ca^{2+}. However, 2APB was able to inhibit basal cation (Sr^{2+}, Ba^{2+}, and Mn^{2+}) influx in this condition, which suggests an additional site of action for this compound, probably the SOCC itself. In the thrombin-activated state, 2APB would most probably block SOCC directly and prevent the conformational coupling by inhibiting the loss of Ca^{2+} from the ER Ca^{2+} stores via the IP_3 receptor.

When platelets were treated with thapsigargin, there was no involvement of IP_3-mediated Ca^{2+} mobilization (Fig. 9, B and C). The elevation of $[Ca^{2+}]_i$ by thapsigargin is mediated by SERCA inhibition following a Ca^{2+} "leak" from the ER (Pozzan et al., 1994). The fact that 2APB has no influence on thapsigargin-mediated intracellular Ca^{2+} mobilization in platelets (Fig. 9 B and C) confirms a lack of involvement of Ca^{2+} efflux via the ER IP_3 receptors in this process. However, when Ca^{2+} influx was initiated after thapsigargin treatment (Fig. 9B) through the addition of extracellular Ca^{2+}, this Ca^{2+} influx was totally abolished by 2APB. This result, therefore, is consistent with 2APB blocking SOCC directly. It is also possible that 2APB is interacting with some protein other than the IP_3 receptor that regulates SOCC.

Recently, the type III IP_3 receptors were identified in purified plasma membranes from human platelets (El-Daher et al., 2000). This finding indicates that Ca^{2+} may enter the platelet through these channels. Indeed, there is evidence for a direct role of IP_3 stimulating Ca^{2+} into platelets (Sage and Rink, 1987; Somasundaram and Mahaut-Smith, 1995; Lu et al., 1998). The effects of 2APB to inhibit Ca^{2+} influx, observed in the present study, may therefore be attributable to an effect on plasma membrane type III IP_3 receptor/channels. If SOCC is the type III IP_3 receptor in platelets, then the data reported here support this notion because Maruyama et al. (1997) claimed that 2APB acted on both type I and type III IP_3 receptors. Alternatively, if SOCC is hTrp1 or hTrp3 in platelets (El-Daher et al., 2000), then these trp channels may also be targets for 2APB and might suggest that both trp channels and IP_3 receptor/channels share some common characteristics such that they both bind 2APB. The studies of Rosado and Sage (2000a) support the concept that in platelets, Htrp1 couples with the type II IP_3R with depleted stores. If Htrp1 in platelets is the SOCC, then we have identified pharmacological agents that can inhibit this channel directly: 2APB, DPBA, and DPTTF. These inhibitors are in addition to the phytoestrogens, such as *trans*-resveratrol, that we have previously identified as inhibitors of SOCC in human platelets (Dobrydneva et al., 1999).

Previous studies using 2APB (Ma et al., 2000) may need to be reevaluated if 2APB produced effects in the cells used in those studies that were similar to those seen in platelets (this study). If 2APB was also blocking SOCC directly in the studies performed by Ma et al. (2000), then their data cannot be interpreted as evidence for the coupling of IP_3 receptors with SOCC. We would therefore express caution when using 2APB to investigate the release of Ca^{2+} from IP_3-sensitive Ca^{2+} stores. Along with the inhibition of internal release of Ca^{2+}, 2APB produces direct inhibition of SOCC. Despite the presence of at least two sites of action for 2APB, it should be an effective pharmacological tool for investigating the signal transduction pathways regulating $[Ca^{2+}]_i$, because it prevents the entry of Ca^{2+} into the cytoplasm by both blocking intracellular IP_3-receptor Ca^{2+} channels and SOCC directly. While our manuscript was being reviewed, a study was published by Braun et al. (2001) in which single-channel recordings were performed in RBL-2H3 m1 cells. The results of this study suggested that 2APB was a direct blocker of SOCCs; this finding therefore confirms our studies in platelets, which suggest that 2APB inhibits SOCCs in human platelets.

Acknowledgments

We thank Patricia G. Loose for expert technical assistance.

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