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2APB, 2-Aminoethoxydiphenyl Borate, a Membrane-Penetrable Modulator of Ins(1,4,5)P_3-Induced Ca^{2+} Release

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The effects of a novel membrane-penetrable modulator, 2APB (2-aminoethoxy diphenyl borate), on Ins(1,4,5)P_3-induced Ca^{2+} release were examined. 2APB inhibited Ins(1,4,5)-P_3-induced Ca^{2+} release from rat cerebellar microsomal preparations without affecting [^{3}H]Ins(1,4,5)P_3 binding to its receptor. The IC_{50} value (concentration producing 50% inhibition) of 2APB for inhibition of Ins(1,4,5)P_3 (100 nM) induced Ca^{2+} release was 42 μM. Further increase in the concentration of 2APB (more than 90 μM) caused a gradual release of Ca^{2+} from cerebellar microsomal preparations. Addition of 2APB to the extracellular environment inhibited the cytosolic Ca^{2+} ($[Ca^{2+}]_c$) rise in intact cells such as human platelets and neutrophils stimulated by thromboxane-mimetic STA_2 or thrombin, and leukotriene B_4 (LTB_4) or formyl-methionine-leucine-phenylalanine (FMLP), respectively. 2APB inhibited the contraction of thoracic aorta isolated from rabbits induced by angiotensin II (AII), STA_2, and norepinephrine in a non-competitive manner, but showed no effect on the contraction of potassium-depolarized muscle. 2APB had no effect on the Ca^{2+} release from the ryanodine-sensitive Ca^{2+} store prepared from rat leg skeletal muscle and heart. Although the specificity of 2APB with respect to the intracellular signaling system was not fully established, 2APB is the first candidate for a membrane-penetrable modulator of Ins(1,4,5)P_3 receptor, and it should be a useful tool to investigate the physiological role of the Ins(1,4,5)P_3 receptor in various cells.

Key words: 2APB, calcium release, cytosolic calcium concentration, inositol 1,4,5-trisphosphate, membrane-penetrable modulator.

The intracellular Ca^{2+} concentration is controlled by both Ca^{2+} influx from the outside of the cell and Ca^{2+} release from the intracellular Ca^{2+} store sites (*1, 2*). The voltage-sensitive Ca^{2+} channels (*3*) and ligand-gated cation channels (*4, 5*) located in the plasma membrane play roles in the Ca^{2+} influx from outside the cell. The internal mobilization of Ca^{2+} is mediated either by *myo*-Ins(1,4,5)P_3 receptors (IP_3R) or by ryanodine receptors (RyR) (*6*). IP_3R is involved in various intracellular phenomena such as Ca^{2+} oscillations and Ca^{2+} waves (*7, 8*). We have shown that the monoclonal antibody (mAb) 18A10 raised against IP_3R (*9, 10*) inhibited Ins(1,4,5)P_3-induced Ca^{2+} release (*11*). Microinjection of mAb18A10 into cells demonstrated the importance of Ins(1,4,5)P_3 in fertilization of hamster oocytes (*12*). Because technical problems with microinjection limit the investigation of many other cells, we have been searching for a plasma membrane-penetrable inhibitor of Ins(1,4,5)P_3, and we found a novel Ins(1,4,5)P_3 modulator, 2APB, which is active when added to the extracellular space. TMB-8, PCMB, and cinnarizine inhibit Ins(1,4,5)-P_3-induced Ca^{2+} release (*13*) *via* blockade of Ca^{2+} channel opening or by inhibiting Ins(1,4,5)P_3 binding, but they also act as general Ca^{2+} antagonists on the Ca^{2+} channel located in the plasmamembrane or intracellular vesicles. 2APB (Fig. 1) is the first Ins(1,4,5)P_3 modulator which does not affect the Ca^{2+} influx from outside of the cell and that is active even in intact cells, and it should provide a basis to develop more potent and selective inhibitors for Ins(1,4,5)P_3 receptor subtypes.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources: Fura 2, Fura 2-AM, EGTA, and Hepes from Dojindo Laboratories (Kumamoto); [^{3}H]Ins(1,4,5)P_3 from NEN; creatine kinase, phosphocreatine, oligomycin and disodium ATP, thrombin, FMLP from Sigma; Ins(1,4,5)P_3 from Funakoshi (Tokyo). All of the other chemicals were of the highest purity commercially available. STA_2 and ONO-NT-126 were synthesized at Ono Pharmaceutical.

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Abbreviations: 2APB, 2-aminoethoxydiphenyl borate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; STA_2, 9,11-epithio-11,12-methanothromboxane A_2; LTB_4, leukotriene B_4; FMLP, formyl-methionine-leucine-phenylalanine; AII, angiotensin II; CCK-8, cholecystokinin octapeptide; mAb, monoclonal antibody; $[Ca^{2+}]_c$, cytosolic calcium concentration.

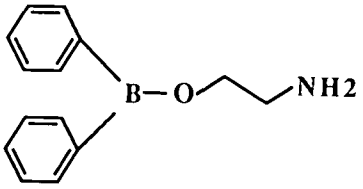

Fig. 1. **Chemical structure of 2APB, 2-aminoethoxydiphenyl borate.**

Synthesis of 2APB—Bis phenyl(2-aminoethoxy)borate, (2APB) was synthesized by the procedure reported elsewhere (*14*). 2APB was synthesized by the esterification of diphenylboronic acid with aminoethanol in ethanol. Diphenylboronic acid was obtained by the reaction of methyl borate with phenylmagnesium bromide at low temperature (*14*).

Measurement of Ins(1,4,5)P_3-Induced Ca^{2+} Release—Rat cerebellar microsomal fraction was prepared according to the method reported by Nakade *et al.* (*11*). Briefly the cerebella isolated from Sprague Dawley rats (male, 200-250 g body weight) were homogenized in 9 volumes of buffer containing 0.32 M sucrose, 1 mM EGTA, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μM leupeptin, 10 μM pepstatin A, 1 mM dithiothreitol (DTT), and 5 mM Tris-HCl (pH 7.4) using Teflon-glass Potter homogenizer. After centrifugation at 10,500$\times g$ for 15 min at 4°C, the supernatant was collected and then centrifuged at 105,000$\times g$ for 60 min at 4°C. The resultant precipitate was resuspended in a solution containing 0.1 M sucrose, 0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A, 1 mM DTT, and 10 mM Tris-HCl, pH 7.4, washed three times by centrifugation at 105,000$\times g$ for 15 min at 4°C and resuspended in the same buffer at a protein concentration of 3 mg/ml. This microsomal fraction could be stored in liquid N_2 for more than 3 months without significant loss of Ins(1,4,5)P_3-induced Ca^{2+}-releasing activity. Ca^{2+} uptake was initiated by adding 40 μl of microsomal fraction into a glass cuvette containing 360 μl of a medium composed of (final concentration, mM): KCl 110, Hepes 10, $MgCl_2$ 1, DTT 1, NaCl 10, KH_2PO_4 5, disodium ATP 1.25, and phosphocreatine 1.25 plus creatine kinase (10 units/ml), oligomycin (2.5 μg/ml) and Fura 2 free acid (2 μM), pH 7.2, with continuous stirring at 600 rpm. After 8 min of incubation, various concentrations of Ins(1,4,5)P_3 were tested in the presence or absence of 2APB. 2APB was added 1-2 min prior to Ins(1,4,5)P_3 administration to test the inhibitory activity of Ins(1,4,5)P_3-induced Ca^{2+} release. When we tested the effect of 2APB on Ca^{2+} uptake induced by ATP, 2APB was administered with ATP. The free Ca^{2+} concentration of the solution was monitored with Fura 2 under constant agitation at 37°C in a dual-excitation-wavelength CAM 220 spectrofluorometer (JASCO, Tokyo). Changes in fluorescence were calculated as described elsewhere (*11*).

Measurement of [^3H] Ins(1,4,5)P_3 Binding—[^{3}H] Ins(1,4,5)P_3 binding was measured as described previously (*11*).

Measurement of Ca^{2+} Release from Cardiac and Skeletal Sarcoplasmic Reticulum Vesicles—Cardiac sarcoplasmic reticulum vesicles were prepared by a slight modification of the method reported by Meissner *et al.* (*15*), which is almost the same as that for rat cerebellum described above. Briefly, excised hearts from anesthetized rats were minced and homogenized in 7 volumes of the same buffer as in the case of cerebellum for 60 s at 18,000 rpm in a Hiscotron. The 105,000$\times g$ precipitate of the 10,500$\times g$ supernatant was obtained as a crude microsomal fraction for measurement of caffeine-induced Ca^{2+} release. A crude membrane fraction of sarcoplasmic reticulum was prepared as reported by Meissner (*16*). Briefly, 10 g of back and leg muscles of rats was minced and homogenized in 4 volumes of buffer containing 0.1 M NaCl, 2.5 mM EGTA, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μM leupeptin, 10 μM pepstatin A, and 5 mM Tris-Maleate, pH 7.0, at 4°C for 60 s in a Hiscotron at 18,000 rpm. The homogenate was centrifuged for 30 min at 2,600$\times g$. A crude microsomal fraction was obtained from the supernatant by centrifugation for 30 min at 10,000$\times g$. The pellets were resuspended in buffer containing 0.6 M KCl, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μM leupeptin, 10 μM pepstatin A, 2.5 mM EGTA, and 5 mM Tris-Maleate, pH 7.0, and washed twice by centrifugation for 30 min at 10,000$\times g$. The resultant pellet was resuspended in buffer containing 0.1 M KCl, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μM leupeptin, 10 μM pepstatin A, 2.5 mM EGTA, and 5 mM Tris-Maleate, pH 7.0, at a protein concentration of 3 mg/ml, and used as a heavy fraction of sarcoplasmic reticulum for measurement of caffeine-induced Ca^{2+} release. ATP-induced Ca^{2+} uptake and caffeine-induced Ca^{2+} release in cardiac and skeletal microsomal fraction was determined by measuring the changes of Fura 2 fluorescence intensity as above.

Experiments with Human Platelets—Platelets were prepared from freshly drawn human blood as previously described (*17*). According to the method of Merrit *et al.* (*18*), the platelet-rich plasma was incubated with 2 μM Fura 2-AM, 20 μM indomethacin, and 2.5 mM probenecid at 37°C for 30 min. The platelets were then resuspended in medium containing NaCl (145 mM), KCl (5 mM), $MgCl_2$ (1 mM), Hepes (10 mM, pH 7.2 at 37°C), and $CaCl_2$ (0.2 mM). For measurement of $[Ca^{2+}]_c$, Fura 2 fluorescence was monitored as described above. Aggregation of platelets was monitored in a 6-Channel aggregometer (NBS HEMA tracer 601 Niko Biochemical) using cells prepared as above without Fura 2-AM treatment. The extent of aggregation was assessed 3 min after addition of the stimulus.

Experiments with Human Neutrophils—The preparation of human neutrophils and measurement of $[Ca^{2+}]_c$ change by LTB_4 and FMLP were performed by the method of Seya *et al.* (*19*).

Measurement of Contraction of Rabbit Aorta—Rabbits were anesthetized and killed, and the thoracic aorta was isolated. The contraction of helically cut strips of thoracic aorta was measured by the classical Magnus method as described elsewhere (*20*). Isotonic contraction was recorded on a Recticorder (RJG4128 Nihon Kohden).

RESULTS

Effects of 2APB on Ca^{2+} Release in Cerebellar Microsome—It was previously reported that Ins(1,4,5)P_3 could release Ca^{2+} from the mouse microsomal fraction after Ca^{2+} was incorporated into the luminal side in the presence of the ATP and an ATP-regenerating system (*11, 13, 21-23*). The Ins(1,4,5)P_3-induced Ca^{2+} release is rapidly restored to the

resting level due to rapid Ca^{2+} re-uptake and degradation of $Ins(1,4,5)P_3$. These observations were further confirmed in rat microsomes. Figure 2a shows the inhibitory effect of 2APB (30 μM×2) on the 100 nM $Ins(1,4,5)P_3$-induced Ca^{2+} efflux. Increase in the concentration of 2APB caused proportional inhibition of $Ins(1,4,5)P_3$-induced Ca^{2+} release (Fig. 2c). In the present study, the response to various doses of $Ins(1,4,5)P_3$ (10–1,000 nM) was recorded in the presence or absence of 2APB. At 20 μM, 2APB shifted the dose-dependent curve of $Ins(1,4,5)P_3$ to the right. This inhibitory effect was stronger at 60 μM 2APB. Interestingly, the ability of 2APB was decreased by increasing the concentration of $Ins(1,4,5)P_3$ and Ca^{2+} efflux was not inhibited in the presence of a maximal concentration of $Ins(1,4,5)P_3$. We also tested the effects of 2APB on $Ins(1,4,5)P_3$ binding to rat cerebellar microsomes. However, at 1 mM, 2APB had no effect on [^{3}H]$Ins(1,4,5)P_3$ binding (data not shown). These findings suggest that 2APB inhibits Ca^{2+} channel opening by $Ins(1,4,5)P_3$ without affecting $Ins(1,4,5)P_3$ binding to the receptor.

We found that 2APB induced Ca^{2+} release from microsomal preparation at a relatively high concentration (Fig. 2b). Such a stimulative effect could be due to inhibition of Ca^{2+}-ATPase activity, which sequestrates Ca^{2+} into endoplasmic reticulum as in the case of thapsigazine (*24, 25*) or direct action of IP_3R molecule that induces Ca^{2+} channel opening. To explore these possibilities, we examined the effect of 2APB on ATP-dependent Ca^{2+} uptake in rat cerebellar microsomal preparations (Fig. 3). After addition of various doses of 2APB before initiating Ca^{2+} uptake by ATP, Ca^{2+} sequestration was apparently inhibited by 2APB in a dose-dependent manner. After Ca^{2+} uptake reached a plateau in the presence and absence of 2APB, we measured the Ca^{2+} content in the $Ins(1,4,5)P_3$-sensitive store by

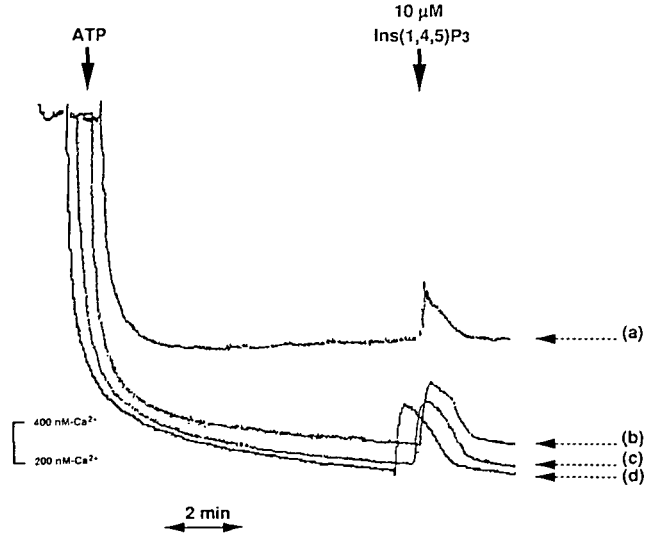

Fig. 3. **The effect of 2APB on ATP-dependent Ca^{2+} uptake into rat cerebellar microsome.** ATP-dependent Ca^{2+} uptake and $Ins(1,4,5)P_3$-induced Ca^{2+} release were measured as described in the experimental procedure section and the legend to Fig. 2. 2APB was added in combination with ATP. After Ca^{2+} uptake reached a plateau, 10 μM $Ins(1,4,5)P_3$ was added to determine the amount of Ca^{2+} in the $Ins(1,4,5)P_3$-sensitive Ca^{2+} store. (a) 100 μM 2APB, (b) 30 μM 2APB, (c) 10 μM 2APB, (d) no 2APB.

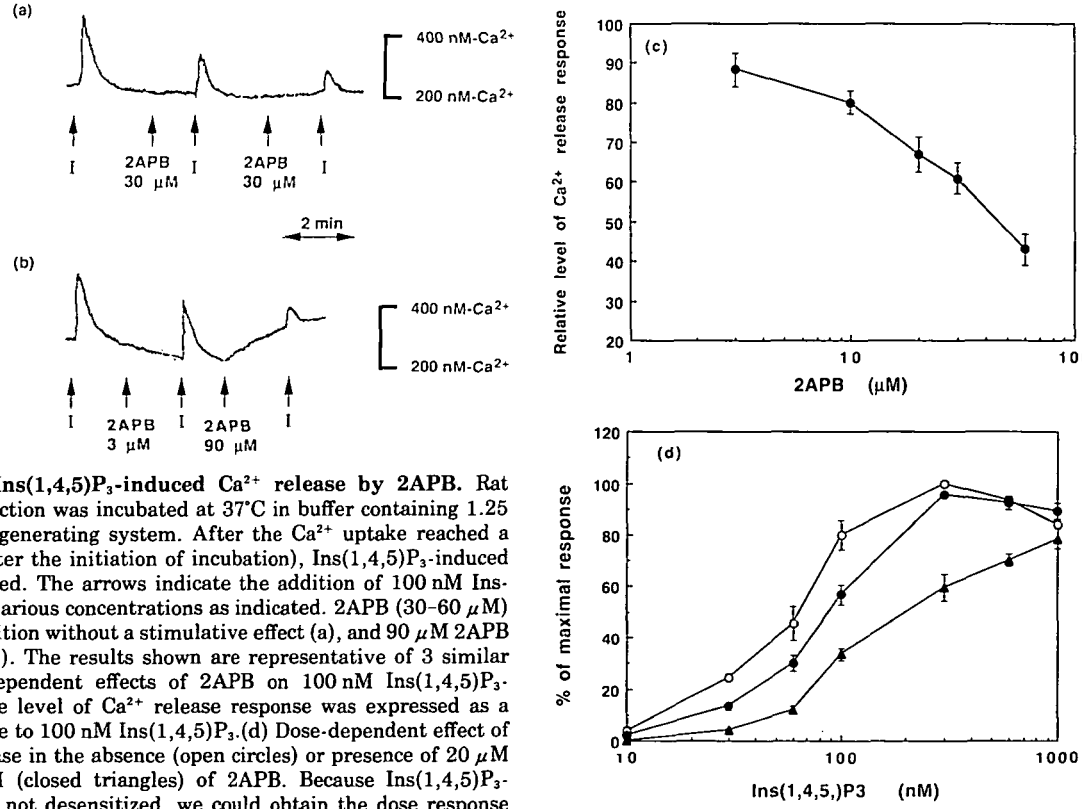

Fig. 2. **Inhibition of $Ins(1,4,5)P_3$-induced Ca^{2+} release by 2APB.** Rat cerebellar microsomal fraction was incubated at 37°C in buffer containing 1.25 mM ATP and an ATP-regenerating system. After the Ca^{2+} uptake reached a plateau (usually 8 min after the initiation of incubation), $Ins(1,4,5)P_3$-induced Ca^{2+} release was monitored. The arrows indicate the addition of 100 nM $Ins(1,4,5)P_3$ (I) or 2APB at various concentrations as indicated. 2APB (30–60 μM) showed an apparent inhibition without a stimulative effect (a), and 90 μM 2APB released Ca^{2+} by itself (b). The results shown are representative of 3 similar experiments. (c) Dose-dependent effects of 2APB on 100 nM $Ins(1,4,5)P_3$-induced Ca^{2+} release. The level of Ca^{2+} release response was expressed as a percentage of the response to 100 nM $Ins(1,4,5)P_3$. (d) Dose-dependent effect of $Ins(1,4,5)P_3$ on Ca^{2+} release in the absence (open circles) or presence of 20 μM (closed circles) or 60 μM (closed triangles) of 2APB. Because $Ins(1,4,5)P_3$-induced Ca^{2+} release was not desensitized, we could obtain the dose response curve by the addition of increasing amounts of 2APB to one microsomal preparation in a cuvette. The amount of Ca^{2+} release is expressed as a percent of the maximal response. Each plotted value is the mean ± SE of 3–4 independent experiments.

addition of 10 μM Ins(1,4,5)P_3. Interestingly, the plateau level of ATP-dependent Ca^{2+} uptake in the presence of 100 μM 2APB (a) was above the peak level of maximum Ca^{2+} release induced by 10 μM Ins(1,4,5)P_3 in the absence of 2APB (d), and 10 μM Ins(1,4,5)P_3 was still effective to induce Ca^{2+} release in the presence of 100 μM 2APB (a); this corresponded to about 80% of that obtained in the absence of 2APB (d). These findings suggest that 2APB can partly inhibit ATP-dependent Ca^{2+} uptake into Ins-(1,4,5)P_3-sensitive stores.

Effects of 2APB on Caffeine-Sensitive Ca^{2+} Store—2APB (100 μM) did not inhibit 2 mM caffeine-induced Ca^{2+} release from the vesicular preparations of sarcoplasmic reticulum of cardiac and skeletal muscle of rats (Fig. 4, a and b).

Effects of 2APB on Agonist-Induced $[Ca^{2+}]_c$ Release in Fura 2-Loaded Human Platelets—The effects of extracellularly added 2APB were further examined in intact cells such as platelets, neutrophils and aorta strips treated with various agonists coupled to the phospholipase C-Ins-(1,4,5)P_3 generation system.

Figure 5 shows typical fluorescence traces of Fura 2-loaded human platelets stimulated by 100 nM STA_2 (a) or 0.1 U/ml of thrombin (b). 2APB (10-100 μM) caused dose-dependent inhibition of agonist-stimulated $[Ca^{2+}]_c$ increase, with almost complete inhibition at 100 μM. Figure 5c shows the relationship between 2APB concentration and inhibition of STA_2- and thrombin-induced $[Ca^{2+}]_c$ increase. Degree of inhibition was expressed as percent inhibition of fluorescence rise (measured in arbitrary fluorescence units) with respect to the level of agonist-stimulated $[Ca^{2+}]_c$ increase. The IC_{50} for inhibition of STA_2- and thrombin-stimulated $[Ca^{2+}]_c$ increase by 2APB was 13±5 and 32±6 μM, respectively (mean±SE, n=3 experiments). We next examined whether the inhibition of $[Ca^{2+}]_c$ increase by 2APB might influence platelet function. Figure 5d shows the dose-response curve for inhibition of platelet aggregation by 2APB. The IC_{50} values of 2APB for STA_2- and thrombin-stimulated platelet aggregation were 8±4 and 25±8 μM, respectively (mean±SE, n=3 experiments). These values are similar to those for inhibition of

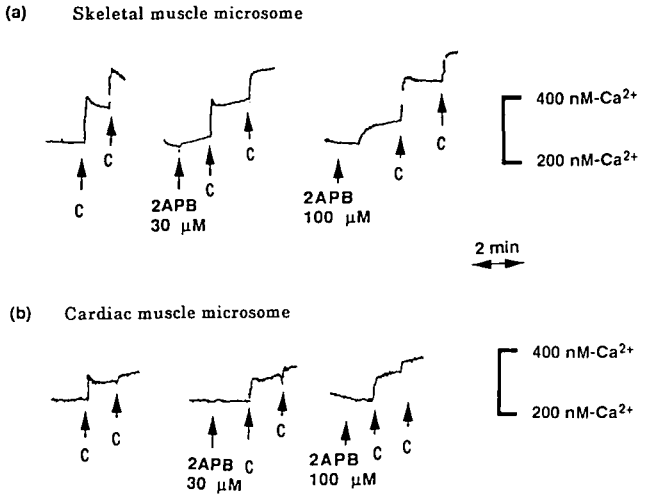

Fig. 4. **Effect of 2APB on caffeine-induced Ca^{2+} release in microsomal fraction of rat cardiac and skeletal muscle.** Ca^{2+} release from the microsomal fraction of rat skeletal muscle (a) and heart (b) was measured by the method described under experimental procedures and in the legend to Fig. 2. After ATP-dependent Ca^{2+} uptake reached a plateau, 2 mM caffeine-induced Ca^{2+} release was monitored. The arrow indicates the addition of 2 mM caffeine (C) or 2APB at various concentrations as indicated.

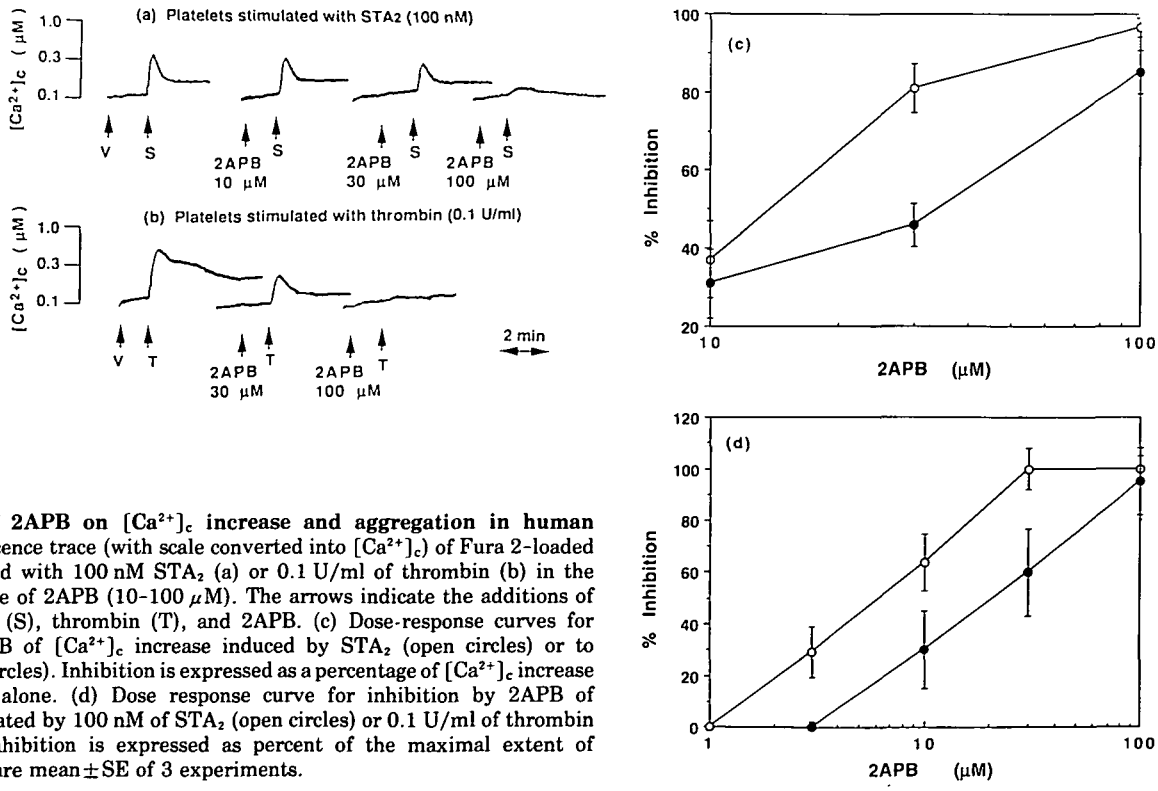

Fig. 5. **Effect of 2APB on $[Ca^{2+}]_c$ increase and aggregation in human platelets.** Fluorescence trace (with scale converted into $[Ca^{2+}]_c$) of Fura 2-loaded platelets stimulated with 100 nM STA_2 (a) or 0.1 U/ml of thrombin (b) in the presence or absence of 2APB (10-100 μM). The arrows indicate the additions of vehicle (V), STA_2 (S), thrombin (T), and 2APB. (c) Dose-response curves for inhibition by 2APB of $[Ca^{2+}]_c$ increase induced by STA_2 (open circles) or to thrombin (closed circles). Inhibition is expressed as a percentage of $[Ca^{2+}]_c$ increase by the stimulant alone. (d) Dose response curve for inhibition by 2APB of aggregation stimulated by 100 nM of STA_2 (open circles) or 0.1 U/ml of thrombin (closed circles). Inhibition is expressed as percent of the maximal extent of aggregation. Data are mean±SE of 3 experiments.

the $[Ca^{2+}]_c$ increase.

Since 2APB has a stimulatory effect at a high concentration as mentioned above, we examined this in platelets. In platelets, 2APB up to 100 μM showed no stimulative effect on $[Ca^{2+}]_c$ measurement, but markedly increased intracellular Ca^{2+} concentration by itself at a concentration higher than 200 μM (Fig. 6). This stimulative effect was not influenced by addition of EGTA to the assay medium. PGE_1, which inhibits agonist-stimulated $[Ca^{2+}]_c$ increase and aggregation *via* cAMP accumulation (*26*), and ONO-NT-126, a thromboxane receptor antagonist (*27*), showed no effect on 2APB (200 μM)-induced $[Ca^{2+}]_c$ increase. These observations indicate that the stimulative effect of 2APB is due to neither Ca^{2+} entry nor cAMP, nor a process *via* the activation of thromboxane A_2 receptor. Surprisingly, 2APB up to 1 mM showed no stimulative effect on human platelet aggregation (data not shown). The mechanism of the stimulative effect of 2APB remains unclear. Increase in intracellular Ca^{2+} by a high concentration of 2APB may not influence the platelet function. A higher concentration of 2APB may inhibit some platelet-aggregatory process such as calmodulin activation.

Effect of 2APB on Ins(1,4,5)P_3 and cAMP Production in Human Platelets—Because the cAMP-generating stimulant, PGE_1, decreases the $[Ca^{2+}]_c$ increase induced by STA_2, we examined the possibility that 2APB stimulates cAMP formation and decreases the $[Ca^{2+}]_c$ increase caused by STA_2 or thrombin. Human platelets were prepared as above (without Fura 2 loading), and incubated with 2APB or PGE_1, at 37°C for 2 min. The reaction was stopped by the addition of ice-cold 6% trichloroacetic acid. The cAMP content in the cells was determined by radioimmunoassay with an Amersham cyclic AMP [^{125}I] assay system. PGE_1 produced a significant amount of cAMP, 180±11 pmol/10^9

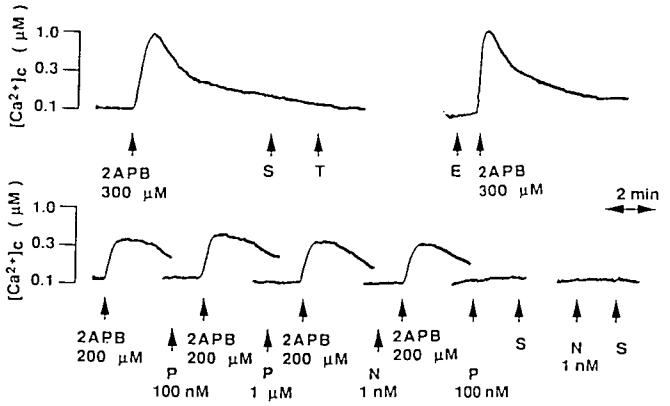

Fig. 6. **Stimulative effect of 2APB on $[Ca^{2+}]_c$ in Fura 2-loaded human platelets.** Typical fluorescence traces (with scale converted into $[Ca^{2+}]_c$) of Fura 2-loaded platelets. The arrows indicate the addition of: E (1 mM EGTA), S (100 nM STA_2), T (0.1 U/ml thrombin) and the indicated dose of PGE_1 (P), 2APB or ONO-NT-126 (N).

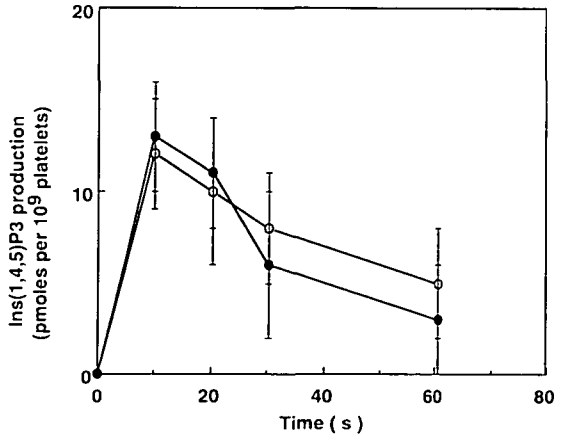

Fig. 7. **Effect of 2APB on STA_2-induced elevation of Ins-(1,4,5)P_3 in human platelets.** Human platelets were stimulated with 100 nM STA_2 in the presence (open circles) or absence (closed circles) of 2APB (100 μM). The reaction was stopped at the times indicated, and Ins(1,4,5)P_3 levels were analyzed. Agonist-induced responses was determined by subtracting the response seen in unstimulated platelets from that in the corresponding STA_2-treated samples. Values presented are the means±SE of four experiments.

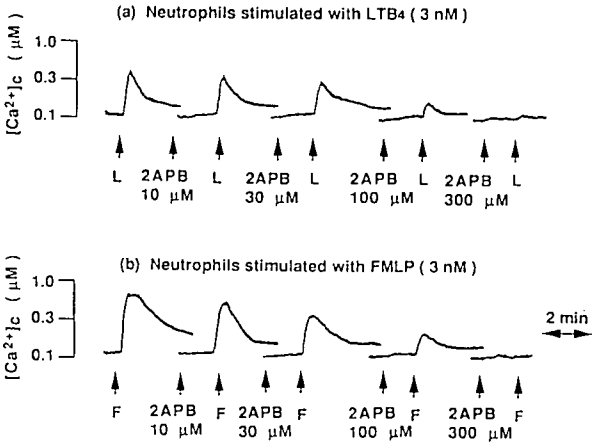

Fig. 8. **Effect of 2APB on $[Ca^{2+}]_c$ increase in human neutrophils.** Typical fluorescence traces, with scale converted into $[Ca^{2+}]_c$, of Fura 2-loaded neutrophils stimulated with 3 nM LTB_4 (a) and 3 nM FMLP (b) in the presence or absence of 2APB. The arrows indicate the addition of LTB_4 (L), FMLP (F), or 2APB.

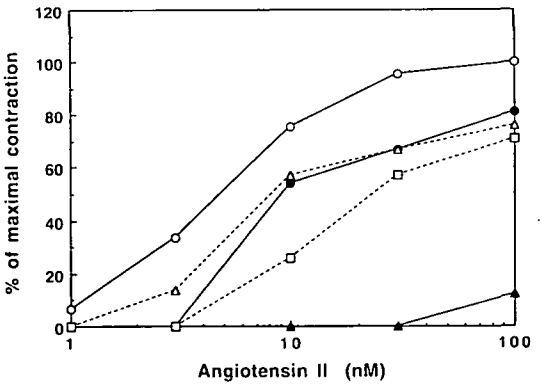

Fig. 9. **Effect of 2APB and nifedipine on contraction of aortic strips in response to cumulative increases of AII.** Strips were preincubated with 2APB (solid line of closed symbols, 10 μM: circles, 100 μM: triangles), nifedipine (dashed line of open symbols; 10 μM: triangles, 100 μM: squares), none (open circles) for 10 min and, without washout, were tested with cumulatively increasing concentrations of AII. The results shown are the means of five independent experiments.

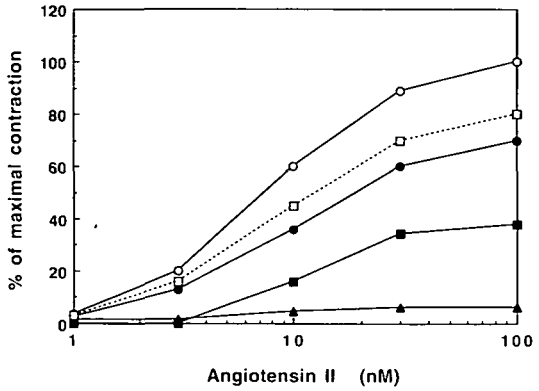

Fig. 10. **Effect of 2APB on responses of aortic strips to AII in the Ca^{2+}-free medium.** Strips were incubated with 2APB (10 μM: closed circles, 30 μM: closed squares, 100 μM: closed triangles), nifedipine (100 μM: dashed line of open squares) or none (open circles) for 10 min in Ca^{2+}-deficient Krebs-Henseleit solution and, without washout, were exposed to cumulatively increasing concentrations of AII. The results shown are the means of five independent experiments.

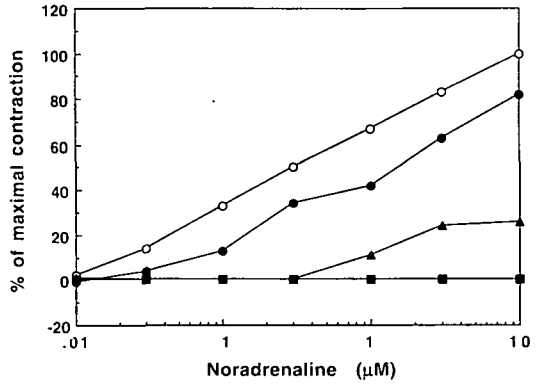

Fig. 11. **Effect of 2APB on responses of aortic strips to cumulatively increasing concentrations of noradrenaline.** Strips were incubated with 2APB (10 μM: closed circles, 100 μM: closed triangles, 1 mM: closed squares) or none (open circles) for 10 min and, without washout, were exposed to cumulatively increasing concentrations of noradrenaline. The results shown are the means of five independent experiments.

platelets at 0.1 μM. However, the amount of cAMP produced by 2APB (100 μM) was 40±6 pmol/10^9 platelets, which was similar to the control level (35±3 pmol/10^9 platelets). Next, we tested the effect of 2APB on the Ins(1,4,5)P_3 production induced by STA_2, using the method reported by Yamaja Setty *et al.* (*28*). Human platelets prepared as above were incubated at 37°C with 100 nM STA_2 in the presence or absence of 2APB (100 μM). The reaction was terminated by mixing with an equal volume of 6% TCA before and at various time intervals after the addition of STA_2. Ins(1,4,5)P_3 in the TCA extract was assayed using an Amersham Ins(1,4,5)P_3 radioreceptor assay kit. As shown in Fig. 7, 2APB (100 μM) did not affect the Ins(1,4,5)P_3 production induced by STA_2 in human platelets.

Effects of 2APB on $[Ca^{2+}]_c$ *Responses in Fura 2-Loaded Human Neutrophils*—Figure 8 shows typical traces of Fura 2-loaded neutrophils stimulated by 3 nM LTB_4 (a) and 3 nM FMLP (b). As with platelets, 10-300 μM 2APB caused dose-dependent inhibition of both stimulant-evoked responses. The IC_{50} value of 2APB for inhibition of LTB_4- and FMLP-induced $[Ca^{2+}]_c$ increases were 60±5.0 and 62±8.2 μM, respectively (mean±SE, n=3 experiment). These values were higher than those in platelets, suggesting that penetration of 2APB into the cells is lower in neutrophils than in platelets. 2APB showed no stimulative effect up to 300 μM in neutrophils.

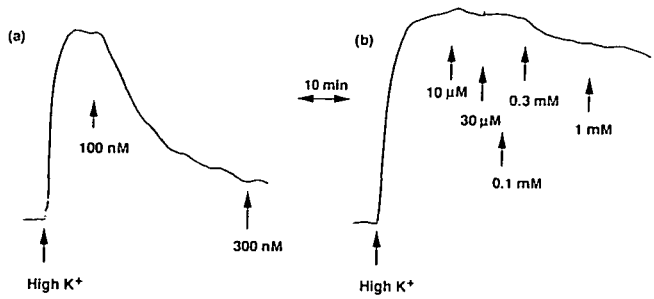

Fig. 12. **Effect of nifedipine and 2APB on high K^+ (40 mM)-induced contraction of aortic strip.** (a) Effect of nifedipine (10^{-7} M) added after the response to potassium had reached a plateau. (b) Effect of 2APB (10^{-5}-10^{-3} M) added after the response to potassium had reached a plateau. The results shown are typical traces.

Effects of 2APB on the Contraction of Rabbit Thoracic Aorta—The effect of 2APB on contraction by angiotensin II (AII) was examined. Figure 9 shows the effects of 2APB and the L type Ca^{2+} channel blocker nifedipine on the dose-response curve of AII-induced rabbit thoracic aorta contraction. 2APB inhibited the contraction in a non-competitive manner. 2APB at 100 μM shows almost complete inhibition of the maximum contraction of AII. The partial inhibition by 100 μM nifedipine indicates that the contribution of voltage-dependent L-type Ca^{2+} influx to contraction by AII is relatively small. Figure 10 shows the effects of 2APB on contracture of AII in the absence of Ca^{2+} in the medium. Under this condition, 2APB still showed similar inhibition, but nifedipine had no effect. Similar effects of 2APB were also seen in rabbit aorta contracted by noradrenaline (Fig. 11) and STA_2 (data not shown). Figure 12 shows the effects of nifedipine and 2APB on the contraction of K^+-depolarized aorta. As expected for a voltage-dependent Ca^{2+} channel antagonist, nifedipine at 10^{-7} M relaxed the muscle contraction. However, 2APB showed no effect even at 1 mM. These findings indicate that 2APB has no antagonistic function on the voltage-dependent Ca^{2+} channels. 2APB (up to 1 mM) caused no contraction by itself.

DISCUSSION

The $[Ca^{2+}]_c$ level can be regulated by a number of mechanisms, for example Ca^{2+} entry into cells through channels operated by voltage or by receptors and Ca^{2+} release from an intracellular Ca^{2+} store *via* the Ins(1,4,5)-P_3-induced or Ca^{2+}-induced mechanism, which seems to be mediated by cyclic adenosine diphosphate-ribose (*29, 30*). Is the 2APB function specific to the Ins(1,4,5)P_3-induced Ca^{2+} release? Inhibition of agonist-stimulated $[Ca^{2+}]_c$ increase may occur by decreasing Ca^{2+} entry. However, it is unlikely that 2APB inhibits voltage-dependent Ca^{2+} entry (at least *via* the L-type Ca^{2+} channel) considering that

2APB has no effect on the high potassium induced contraction of rabbit aorta as shown in Fig. 12. SK&F96365, a receptor mediated Ca^{2+} entry blocker, is effective in inhibiting the Ca^{2+} entry by certain agonists in various non excitable cells (*31-33*). We also demonstrated the inhibitory effects of SK&F96365 on Ca^{2+} entry by CCK-8 and carbacol in rat pancreatic acinar cells (*34*). Interestingly, SK&F96365 and 2APB show an additive effect on the inhibition of $[Ca^{2+}]_c$ increase by CCK-8 and carbacol (data in preparation). To study the effect on Ca^{2+} release from the intracellular store, we first examined the effect of 2APB on Ca^{2+}-induced Ca^{2+} release which is sensitive to ryanodine and is potentiated by caffeine. We prepared the heavy sarcoplasmic reticulum from rat heart and leg skeletal muscle as described by Meissner *et al.* (*15, 16*). However, 2APB did not inhibit Ca^{2+} release induced by caffeine in either preparation (Fig. 4, a and b).

2APB (100 μM) showed almost complete inhibition of agonist-induced increase in $[Ca^{2+}]_c$ in platelets (Fig. 5), neutrophils (Fig. 8), and aorta (Figs. 9-11). It is therefore clear that 2APB can penetrable through the plasma membrane. This is one of the great advantages of 2APB compared to the newly developed agonist adenophostine (*35*), which is not membrane-penetrable. It remains to be elucidated how 2APB penetrates the plasma membrane and how it works inside the cell.

2APB will give us more information on the physiological role of the Ins(1,4,5)P_3 receptor in hormonal regulation of $[Ca^{2+}]_c$ and function. Recent studies have identified 3 or 4 subtypes and several isomers of the Ins(1,4,5)P_3 receptor (*36-46*). We have no direct evidence to show which type among these Ins(1,4,5)P_3 receptors is affected by 2APB. However, 2APB may work on type 1 Ins(1,4,5)P_3 receptor, since the Ins(1,4,5)P_3 receptor in the cerebellum is mainly type 1. Furthermore, 2APB may also act on type 3 Ins(1,4,5)P_3 receptor, because our recent study (*Biomed. Res.*, in press) showed that 2APB abolished the recurrent $[Ca^{2+}]_c$ spikes induced by a physiological concentration of CCK-8, 2 pM, in isolated rat pancreatic acini, which contain type 3 Ins(1,4,5)P_3 receptor (*46*).

The findings presented here show that 2APB can inhibit the Ca^{2+} release from the cerebellar microsomes. Since 2APB does not inhibit Ins(1,4,5)P_3 binding to its receptor, its site of action on the receptor is different from the Ins(1,4,5)P_3 recognition site. We have previously reported that mAb18A10 inhibited Ca^{2+} release by Ins(1,4,5)P_3 (*11*). mAb18A10 does not inhibit [^{3}H]Ins(1,4,5)P_3 binding to mouse cerebellar microsomes, but enhances it, presumably by modifying the conformation (*11*). The dose-response relation for Ins(1,4,5)P_3-induced Ca^{2+} release in the presence of 2APB is similar to that with mAb18A10. mAb18A10 binds to the C-terminal region of the Ins(1,4,5)P_3 receptor and may suppress channel activity by allosteric modulation of the channel formation or by causing steric hindrance to Ca^{2+} flow. 2APB may inhibit Ca^{2+} release by modulating the structure of IP_3R or changing the membrane environment. Relatively high concentrations of 2APB showed stimulative effects such as Ca^{2+} release from microsomal preparation (Fig. 2) and $[Ca^{2+}]_c$ increase in platelets (Fig. 6). 2APB could partly inhibit ATP-dependent Ca^{2+} uptake into Ins(1,4,5)P_3-sensitive stores at 100 μM (Fig. 3). These stimulative effects of 2APB might be due to partial inhibition of Ca^{2+}-ATPase activity, which sequestrates Ca^{2+} into endoplasmic reticulum, similar to the effects of thapsigazine (*24, 25*). These possibilities remain to be examined.

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