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2-Aminoethoxydiphenyl Borate Modulates Kinetics of Intracellular Ca^{2+} Signals Mediated by Inositol 1,4,5-Trisphosphate-Sensitive Ca^{2+} Stores in Single Pancreatic Acinar Cells of Mouse

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ABSTRACT

Regulation of the kinetics of intracellular Ca^{2+} signals with a novel, membrane-penetrable, inositol 1,4,5-trisphosphate (InsP$_3$) receptor/Ca^{2+} channel modulator, 2-amino-ethoxydiphenyl borate (2APB), has been investigated using patch-clamp, whole-cell recording to monitor Ca^{2+}-activated Cl$^-$ currents in single isolated pancreatic acinar cells. 2APB itself fails to evoke a detectable current response but it dramatically changes the kinetics of agonist-induced Ca^{2+} release from pulsatile spikes to long-lasting, huge Ca^{2+} waves, suggesting that 2APB coordinates local Ca^{2+} release to generate global Ca^{2+} signals. The regulation by 2APB can be elicited by internal perfusion of InsP$_3$ in a concentration-dependent manner, indicating that this regulation is not mediated through membrane receptors or G protein signal transduction. The InsP$_3$ receptor blocker heparin, but not the ryanodine-sensitive receptor blockers ruthenium red or ryanodine, abolishes 2APB-mediated regulation of Ca^{2+} release. This results also suggest that 2APB effects are mediated through InsP$_3$ receptors. 2APB substantially modifies single inward Cl$^-$ current pulse evoked by the photolytic release of caged InsP$_3$ but not by caged Ca^{2+}. These data indicate that 2APB-induced regulation is mediated neither by Ca^{2+}-induced Ca^{2+} release nor by affecting Cl$^-$ channel activity directly. We conclude that 2APB regulates the kinetics of intracellular Ca^{2+} signals, represented as the change in the Ca^{2+} oscillation patterns from brief pulsatile spikes to huge, long-lasting Ca^{2+} waves. Moreover, this regulation seems to be mediated through InsP$_3$-sensitive Ca^{2+} pools. 2APB may act as a novel, useful pharmacological tool to study the genesis of intracellular Ca^{2+} signals.

Intracellular Ca^{2+} signals play an important role in regulating cell functions, including secretion, contraction, differentiation, and proliferation. In a variety of nonexcitable cells, biostimulators induce Ca^{2+} release from intracellular pools in an oscillatory rather than sustained manner (Petersen, 1992; Berridge, 1993). Although the precise mechanism for these effects is unclear, accumulating data indicate that the cooperation of two types of intracellular Ca^{2+} pools plays an important role in generating such oscillatory signals (Petersen and Wakui, 1990; Wakui et al., 1990; Bootman and Berridge, 1995). One type of intracellular Ca^{2+} pool is sensitized by inositol 1,4,5-trisphosphate (InsP$_3$), which activates InsP$_3$-induced Ca^{2+} release (IICR). Another is sensi-

tized by ryanodine, which activates Ca^{2+}-induced Ca^{2+} release (CICR) process (Petersen and Wakui, 1990). Caffeine, ryanodine, and ATP are membrane-penetrable modulators for ryanodine-sensitive Ca^{2+} pools (Petersen and Wakui, 1990), whereas there is still no ideal membrane-penetrable, specific modulator for InsP$_3$-sensitive Ca^{2+} pools. We recently developed a novel, membrane-penetrable modulator, 2APB, which is a specific modulator for InsP$_3$-sensitive Ca^{2+} pools. Our previous data indicated that 2APB inhibited InsP$_3$-induced Ca^{2+} release in cerebellar microsomes (Maruyama et al., 1997b) and inhibited cholecystokinin (CCK)-8-induced intracellular Ca^{2+} release in isolated acini of rat pancreas (Maruyama et al., 1997a). Our previous data also showed that the modulation by 2APB of Ca^{2+} release was concentration- and preparation-dependent. For instance, 2APB at low concentration (IC$_{50}$ of 42 μM) inhibited InsP$_3$-induced Ca^{2+}

ABBREVIATIONS: InsP$_3$, inositol 1,4,5-trisphosphate; IICR, inositol 1,4,5-trisphosphate-induced Ca^{2+} release; CICR, Ca^{2+}-induced Ca^{2+} release; 2APB, 2-aminoethoxydiphenyl borate; CCK, cholecystokinin; Ach, acetylcholine; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; InsP$_3$R, inositol 1,4,5-trisphosphate receptor; DTT, dithiothreitol; RyR, ryanodine receptor; DMSO, dimethyl sulfoxide; SH, sulfhydryl.

release, whereas 2APB at high concentrations (more than 90 μM) increased Ca²⁺ release from cerebellar microsomal preparations (Maruyama et al., 1997b). In the present investigation, we further study the regulation of 2APB on the kinetics of intracellular Ca²⁺ signals using patch-clamp, whole-cell recording to monitor the Ca²⁺-activated Cl⁻ currents in single isolated pancreatic acinar cells. We focused in particular on regulation by 2APB at low concentrations (3–30 μM), because the patch-clamp technique is suitable for monitoring local Ca²⁺ release events from stores closed to the cell membrane (Osipchuk et al., 1990).

Materials and Methods

Single Pancreatic Acinar Cell Isolation. The preparation for single dissociated acinar cells has been described previously (Wu et al., 1996). Briefly, pancreatic glands were taken from ether-anesthetized mice, and fragments of the tissue were minced and digested by collagenase (200 U/ml, 25–30 min, 37°C; Wako Pure Chemicals, Osaka, Japan) in the presence of 1 mM Ca²⁺. At the end of collagenase digestion, the cell suspension was gently pipetted to obtain further separation of the cells and then washed with physiological saline containing 0.2% bovine serum albumin. A 100-μl volume of the suspension was then poured into the extracellular solution in a 2-ml experimental bath. Only single cells were used in the experiments.

Patch-Clamp, Whole-Cell Recording. The conventional whole-cell, patch-clamp method was used to record the Ca²⁺-activated Cl⁻ currents for monitoring intracellular Ca²⁺ signal oscillations as reported previously (Wakui et al., 1989, 1990). The recording pipettes, made of borosilicate glass capillaries, had a resistance of 2 to 4 MΩ when filled with a standard pipette solution. After a GΩ seal was established between the cell membrane and pipette, the whole-cell configuration was achieved by brief negative suction. Transmembrane currents were recorded with a patch-clamp amplifier (EPC-7; List Electronik, Darmstadt, Germany) at a holding potential (V$_H$) of −30 mV. All experiments were performed at room temperature (22 ± 1°C).

Light-Flash Photolysis of Caged Compounds. When caged InsP₃ was tested, D-myo-inositol trisphosphate, P4(5)-1(2-nitrophenyl)-ethyl ester (Calibiochem, La Jolla, CA) was dissolved in the pipette solution at a concentration of 20 μM. Establishment of whole-cell recording allowed caged InsP₃ to diffuse into the recorded cell. When caged Ca²⁺ was tested, the pipette solution contained 2 mM DM-nitrophen (Calbiochem, La Jolla, CA) and 1 mM Ca²⁺. Photolysis of caged compounds was accomplished with a xenon arc flash system (SA-200E; Nissin Electronic Co., Tokyo, Japan). The maximum intensity of the flash was 200 J (Takeo et al., 1997).

Drug Application. The recorded cell in the experimental bath was continuously perfused with a stream of standard extracellular solution. For extracellular drug application, a multibarrel (five barrels) system was employed. For intracellular drug application, agents were dissolved in the pipette solution. Establishment of the whole-cell configuration allowed the drug to diffuse into the cell.

Solutions and Chemicals. The standard extracellular solution contained 140 mM NaCl, 4.7 mM KCl, 1.0 mM CaCl₂, 1.13 mM MgCl₂, 10 mM HEPES, and 10 mM glucose. The solution was adjusted to pH 7.2 with NaOH. A Ca²⁺-free solution was prepared by replacing Ca²⁺ with Na⁺ (142 mM NaCl and 0 mM CaCl₂) and adding 1 mM EGTA. The pipette solution contained 140 mM KCl, 0.24 mM EGTA, 1.13 mM MgCl₂, 5 mM Na₂ATP, 10 mM glucose, and 10 mM HEPES, pH 7.2. The drugs used in this study were myo-inositol trisphosphate, acetylcholine (ACh), cholecystokinin, GTPγS, heparin, ruthenium red, ryanodine, dithiothreitol, D-myo-inositol-triphosphate, P4(5)-1(2-nitrophenyl)-ethyl ester and DM-nitrophen. All drugs were purchased from Sigma (St. Louis, MO) except D-myo-inositol-triphosphate, P4(5)-1(2-nitrophenyl)-ethyl ester and DM-ni-

trophen (Calbiochem). 2-Aminoethoxydiphenyl borate (Fig. 1) was synthesized by the esterification of diphenylboronic acid with aminoethanol in ethanol. Diphenylboronic acid was obtained by the reaction of methylborate with phenylmagnesium bromide at low temperature (Povlock and Lippincott, 1958).

Results

2APB Regulates Agonist-Induced Ca²⁺ Oscillations. The first experiment was performed to determine whether 2APB regulates the kinetics of Ca²⁺ oscillations evoked by various agonists at low concentrations. Figure 2A shows that 2APB alone failed to elicit any detectable current response even at a high concentration (100 μM). However, with continuous perfusion of low concentrations of ACh (10 nM), cholecystokinin (4 pM), or GTPγS (20 μM), 2APB (30 μM) clearly changed the kinetics of Ca²⁺ oscillations from very brief pulsatile spikes to long-lasting, huge Ca²⁺ waves (Fig. 2B-D). Therefore, 2APB seems to coordinate local pulsatile Ca²⁺ release events to generate global Ca²⁺ waves.

2APB Regulates InsP₃-Induced Ca²⁺ Oscillations. Because 2APB is designed as a specific InsP₃ receptor (InsP₃R) modulator (Maruyama et al., 1997a,b), its ability to regulate Ca²⁺ oscillations might be mediated directly through intracellular InsP₃R rather than through cell-surface membrane receptors (ACh or CCK receptor) or G protein signal transduction. To test this hypothesis, the effect of 2APB on Ca²⁺ oscillations evoked by direct internal perfusion of InsP₃ was examined. After breaking into the whole-cell configuration, InsP₃ (20 μM) was continuously diffused from the recording pipette into the cell to produce repetitive inward, pulsatile Cl⁻ current spikes corresponding to spikes of intracellular Ca²⁺ release (Fig. 3 A-C). The duration of spikes at 50% amplitude was 0.9 ± 0.09 s ($n = 9$, mean ± S.E.). Effect of 3 μM 2APB on Ca²⁺ spikes appeared as a potentiation of spike amplitude (123.8 ± 3.7%, $n = 4$, $P < .05$) and an increase in spike frequency (238.5 ± 16.3%, $n = 4$, $P < .01$) without a clear change in spike duration (Fig. 3A). Increasing 2APB to 10 μM increased spike duration dramatically (400 ± 22.3%, $n = 4$, $P < .01$), and increasing 2APB to 30 μM induced the typical global Ca²⁺ waves (Fig. 3B, C). To determine whether the regulation by 2APB was mediated by Ca²⁺ influx pathway or by a change in SH group function, we removed extracellular Ca²⁺, or we bath-applied the SH group-reducing agent dithiothreitol (DTT). As shown in
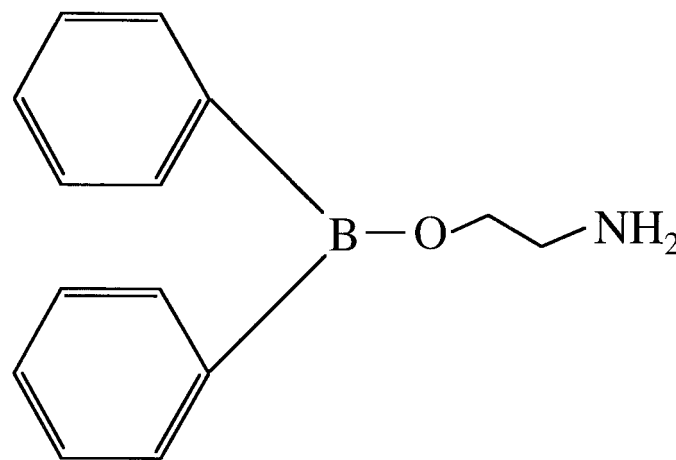

Fig. 1. Chemical structure of 2-aminoethoxydiphenyl borate.

Fig. 3D, neither a Ca^{2+}-free bath solution nor DTT (200 μM) prevented 2APB-induced regulation. These results support the idea that 2APB regulates the kinetics of Ca^{2+} release by directly acting on intracellular Ca^{2+} pools, rather than on SH group function or Ca^{2+} influx pathway.

2APB Regulates Ca^{2+} Oscillations Mediated by InsP$_3$-Sensitive Ca^{2+} Pools. The ryanodine receptor

(RyR)-mediated CICR process plays a role in the generation of repetitive Ca^{2+} oscillations (Petersen and Wakui, 1990; Berridge, 1993). Therefore, it is useful to consider whether 2APB regulates local pulsatile Ca^{2+} release events mediated by CICR. The most straightforward way to answer this question is to block RyR or InsP$_3$R pharmacologically. Figure 4 shows the results obtained when the InsP$_3$R blocker, heparin
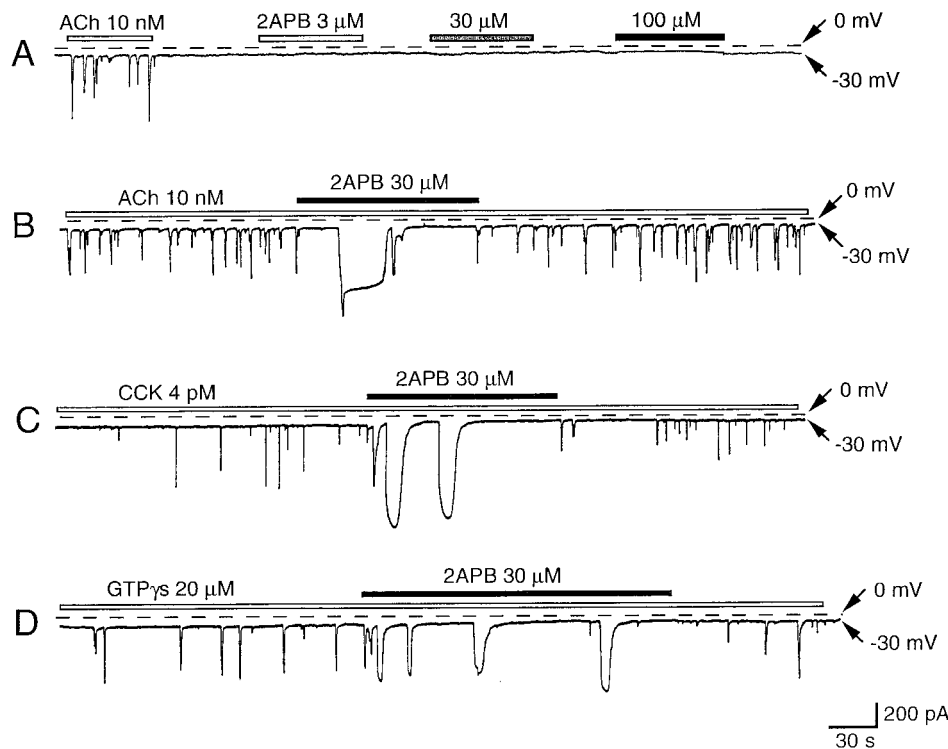

Fig. 2. 2APB regulates local pulsatile Ca^{2+} spikes. A, 2APB itself fails to evoke Ca^{2+} spikes. B to D, 2APB changes the kinetics of local Ca^{2+} release elicited by ACh (10 nM), CCK (4 pM), and GTPγS (20 μM) from brief spikes to long-lasting, huge Ca^{2+} waves. The open bar above each trace indicates the application of an agonist; black bars indicate the application of 30 μM 2APB. Traces (A-D) are from different cells, and each trace is a typical case from four to seven cells.
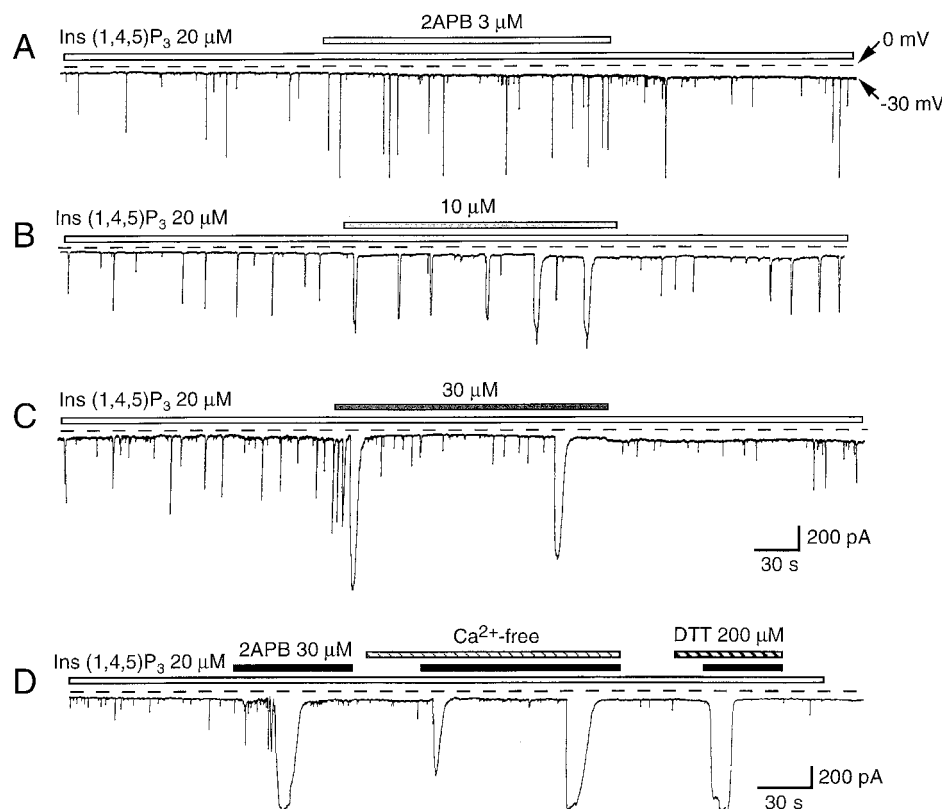

Fig. 3. Concentration-dependent magnification of 2APB on InsP$_3$-induced Ca^{2+} oscillation. A, 3 μM 2APB increases amplitude and frequency of brief spikes of Ca^{2+} oscillations evoked by internal perfusion of 20 μM InsP$_3$. B, 10 μM 2APB increases both amplitude and duration of InsP$_3$-induced pulsatile Ca^{2+} oscillations. C, 30 μM 2APB induces long-lasting and large-amplitude global Ca^{2+} waves accompanied by a decrease in spike frequency and amplitude. D, 2APB-induced regulation is not prevented either by free Ca^{2+} in a bath or DTT (200 μM). Traces (A-C) from the same cell are typical of cases from four cells.

(1 mg/ml), was present with $InsP_3$ (20 μM) in the pipette solution. After the patch-clamp, whole-cell recording was established, $InsP_3$ was infused into the cell and evoked spike responses. Under this condition, the first application of 2APB (30 μM) quickly magnified pulsatile Ca^{2+} spikes into Ca^{2+} waves. However, after heparin blocked $InsP_3R$ as shown by the gradual reduction in the ACh-induced spikes (the sign that $InsP_3R$ was blocked), 2APB lost its effect (Fig. 4A). This result further supports our early data (Fig. 2A) suggesting that the activation of $InsP_3R$ is necessary for 2APB to play its regulation in intracellular Ca^{2+} release events. Using the same procedure, the RyR blocker ruthenium red (100 μM) reduced $InsP_3$-induced Ca^{2+} spikes and blocked RyR (as measured by reduction of the ACh-induced Ca^{2+} spike responses) but did not prevent 2APB (30 μM)-induced regulation (Fig. 4B). This result suggests that 2APB regulates kinetics of local pulsatile Ca^{2+} release through $InsP_3$-sensitive Ca^{2+} pools rather than through RyR-sensitive Ca^{2+} pools. In addition, bath-applied ryanodine (10 μM) increased $InsP_3$-induced Ca^{2+} spikes; the 2APB-mediated effect was potentiated instead of blocked (Fig. 4C). The duration (50% amplitude) of Ca^{2+} spikes in the presence of 30 μM 2APB or in the presence of 10 μM ryanodine plus 30 μM 2APB were 5.9 $\pm$ 0.4 s and 15.2 $\pm$ 1.2 s ($n = 4$, $P < .01$), respectively. Because 10 μM ryanodine completely blocked RyR-mediated Ca^{2+} oscillations evoked by agonists and cyclic ADP-ribose in pancreatic acinar cells (Thorn et al., 1994), this data further supports lack of the RyR-mediated CICR processes in 2APB-induced regulation.

The results presented thus far indicate that 2APB magnifies local pulsatile Ca^{2+} release events to produce global Ca^{2+} waves through the IICR process. This inference, however, could be disputed because $InsP_3$-induced spike responses activated by internal perfusion of $InsP_3$ are involved

in the CICR process (Thorn et al., 1994). To distinguish the effect of 2APB on IICR or CICR, we examined the effect of 2APB on photolytic release of caged $InsP_3$ or caged Ca^{2+} (Fig. 5A and B). Like internal perfusion of $InsP_3$ (Fig. 5A), 200 J of flash-release of caged $InsP_3$ evoked a single, brief, inward Ca^{2+}-activated Cl^- current. The spike duration at 50% amplitude was 0.85 $\pm$ 0.16 s ($n = 6$). 2APB coordinated this short-duration spike into a huge, long-lasting wave, and the effect was reversible (Fig. 5B). Figure 5, C and D, compares the regulation effects of 2APB and the SH group reagent thimerosal. Results show that kinetics of 2APB regulation were different from that for thimerosal. This suggests that 2APB and thimerosal regulated local pulsatile Ca^{2+} release events by different mechanisms. The flash-released caged Ca^{2+} (200 Joules) induced an inward Ca^{2+}-activated Cl^- current spike that failed to be affected by 2APB (30 μM; Fig. 5D). Therefore, 2APB seems to act neither on the CICR process nor on cytoplasmic Cl^- channels to induce this regulation.

Discussion

The major and important findings of this study are that at low concentrations, 2APB regulates the kinetics of the agonist-induced intracellular Ca^{2+} oscillations from brief, pulsatile spikes to long-lasting, huge Ca^{2+} waves. This regulation is mediated through intracellular $InsP_3$-sensitive pools.

Previously, we showed that 2APB inhibited $InsP_3$-induced Ca^{2+} release in cerebellar microsomal preparations (Maruyama et al., 1997b) and that 2APB inhibited CCK-induced Ca^{2+} release in rat pancreatic acini (Maruyama et al., 1997a). There are three differences between this study and our previous studies.

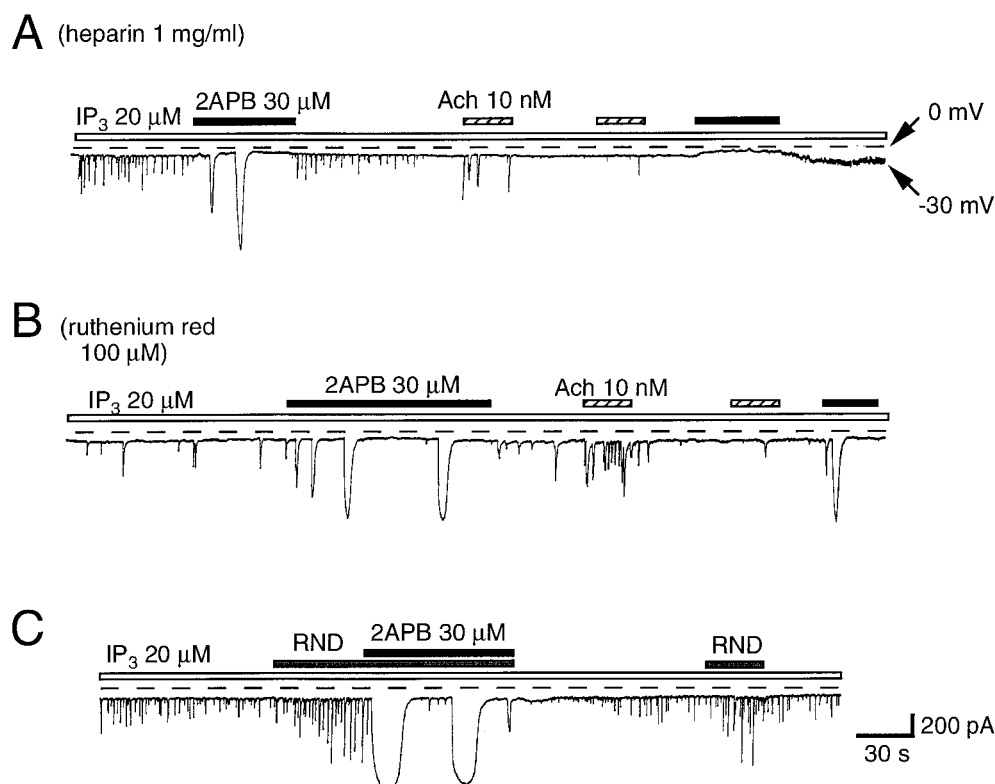First, the experimental preparation is different. Rat cere-

A (heparin 1 mg/ml)

B (ruthenium red 100 μM)

C

Fig. 4. Effects of heparin, ruthenium red, and ryanodine on 2APB-induced regulation. A, preloading heparin (1 mg/ml) gradually blocks ACh-induced spikes and prevents the regulation mediated by 2APB. B, preloading RR (100 μM) blocks ACh-induced spikes but does not prevent the regulation mediated by 2APB. C, bath-applied ryanodine (10 μM) enhances $InsP_3$-induced Ca^{2+} spikes and potentiates the regulation mediated by 2APB. A to C are from different cells; each is a typical trace from three to four cells.

bellar microsomes, rat cardiac and skeletal, human platelet, and rabbit aorta preparations were used in our first study (Maruyama et al., 1997b). A difference in InsP$_3$ receptor subtype expressed cerebellar microsomes (InsP$_3$R-1; Furuichi et al., 1989) and pancreatic acinar cells (InsP$_3$R-3; Nathanson et al., 1994) has been reported. Interestingly, cytosolic Ca^{2+} regulates the binding of InsP$_3$ to InsP$_3$ receptor subtypes 1 and 3 in opposite ways (Yoneshima et al., 1997), implying that 2APB may regulate InsP$_3$R-mediated Ca^{2+} releases in a different manner in cerebellar microsomes and pancreatic acinar cells. In our second study (Maruyama et al., 1997a), rat pancreatic acini (rather than single acinar cells) were used. The sensitization of whole pancreatic acini of rat to agonist and 2APB may be different from that of

mouse single acinar cells. In whole acini, 2APB (10, 30, and 100 μM) did not significantly inhibit the physiological concentrations (1–10 pM) of CCK-induced amylase release (Maruyama et al., 1997a; Fig. 1). CCK (2 pM)-induced recurrent spikes were inhibited by 2APB, but only at high concentrations of 2APB (100 μM, Maruyama et al., 1997a; Fig. 2). In contrast, in mouse single acinar cells, 30 μM 2APB clearly increased pulsatile Ca^{2+} release evoked by 4 pM CCK (Fig. 2C).

Second, the way to evoke InsP$_3$ receptor-mediated Ca^{2+} release is different between the present study and our second study. In the present studies, the major data were collected by examination of 2APB effects on pulsatile Ca^{2+} spikes elicited by internal perfusion of InsP$_3$. This way ruled out any
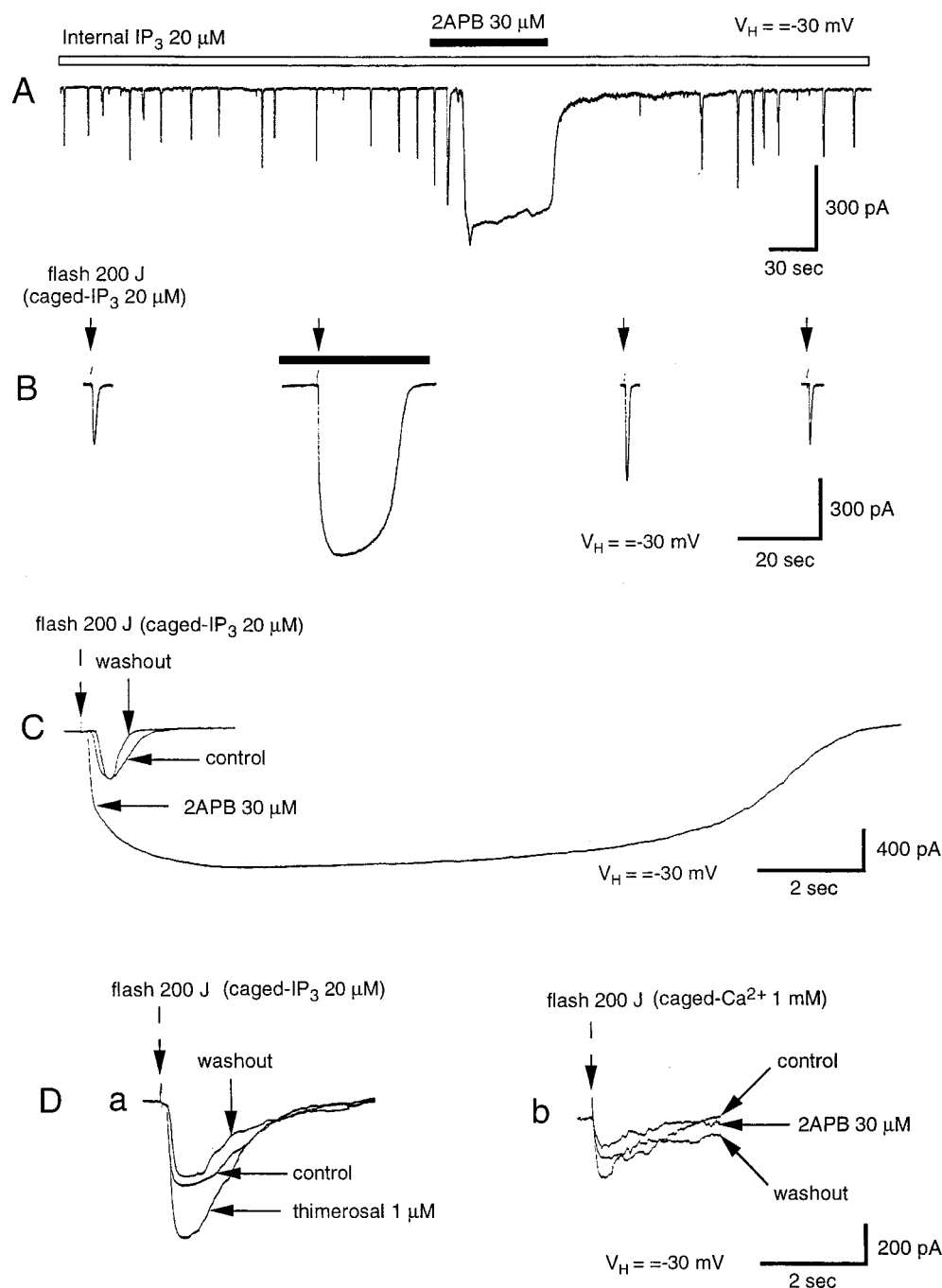

Fig. 5. Effects of 2APB on a single inward Cl$^-$ pulse evoked by the photolytic release of caged InsP$_3$ or caged Ca^{2+}. A and B, comparison of 2APB-induced regulation on pulsatile Ca^{2+} spikes elicited by internal perfusion of InsP$_3$ (A) or a single inward Cl$^-$ pulse evoked by photolytic release of caged InsP$_3$ (B). In the caged InsP$_3$ experiments, four flash stimulations were applied at an interval of 2 min. C and D, a, comparison of effect of 2APB and thimerosal on a single inward Cl$^-$ pulse evoked by photolytic release of the caged InsP$_3$. Thimerosal (1 μM) was pretreated for 2 min and 2APB (30 μM) was pretreated for 1 min. D, b, 2APB (30 μM) does not show a detectable effect on the photolytic release of caged Ca^{2+}. The dashed arrow above each response indicates the flash stimulation. Traces (A-D) from different cells are typical of cases from three to five cells.

effect of 2APB on membrane receptors or G protein signal transduction. In fact, our preliminary experiments indicated an inhibitory effect of 2APB (60 μM) on ACh-induced pulsatile Ca²⁺ spikes in single acinar cells (data not shown). In control experiments with DMSO however, DMSO itself ($\geq$0.03%) suppressed ACh-induced pulsatile Ca²⁺ spikes (data not shown). Figure 2B showed that 30 μM 2APB initially suppressed and then potentiated ACh-induced Ca²⁺ spikes. This initial inhibition may be caused by effects of DMSO. Because 2APB was dissolved as a 100 mM stock solution, the inhibitory effect by high concentrations ($>$30 μM) of 2APB should involve DMSO-induced inhibition in single mouse acinar cells. Consequently, the pure inhibitory effect of 2APB seems not to be suitable to investigate at relatively high concentrations in single pancreatic acinar cells of mouse. Therefore, we designed our experiment to examine the effect of 2APB at low concentrations (3–30 μM).

Third, the measurement of InsP₃ receptor-mediated Ca²⁺ release is different. Here, for the first time, we employ patch-clamp, whole-cell recording to examine effects of 2APB on agonist-induced Ca²⁺ release. Our previous experiments have showed that the measurement of whole-cell current by patch clamp is more sensitive for monitoring local Ca²⁺ release events than the measurement of single-cell microfluorometry (Osipchuk et al., 1990). This technique allows us to investigate the effect of low concentrations (3–30 μM) of 2APB and allow us to find this interesting potentiation of local Ca²⁺ release.

Under our experimental conditions, the low concentrations of agonists induced brief and repetitive Ca²⁺ spikes (Figs. 2 and 3). These pulsatile Ca²⁺ spikes can be defined as local Ca²⁺ release from intracellular Ca²⁺ stores near the plasma membrane (Osipchuk et al., 1990; Thorn et al., 1993). The present results indicate that 2APB regulates the kinetics of agonist-induced brief Ca²⁺ oscillations represented as the increase in spike frequency and amplitude at a low concentrations (Fig. 3A) and as a dramatic increase in spike duration at relatively high concentrations (Fig. 3, B and C). The 2APB-induced regulation does not require stimulation of cell-surface membrane receptors (ACh or CCK) or G protein signal transduction because brief, pulsatile Ca²⁺ spikes elicited by direct application of InsP₃ into the recorded cells were regulated by 2APB (Fig. 3). The regulation is not mediated through extracellular Ca²⁺ influx or by modification of SH group function because experimental removal of extracellular Ca²⁺ or bath-application of the SH group-reducing agent DTT failed to abolish 2APB-induced regulation (Fig. 3D). The regulation is not mediated through ryanodine-sensitive CICR process because pharmacological blockade of ryanodine-sensitive Ca²⁺ release channels did not prevent 2APB regulation (Fig. 4, B and C). Moreover, this regulation is not mediated through membrane Cl⁻ channel activities because 2APB failed to regulate Cl⁻ current pulse evoked by photolytic release of caged Ca²⁺, which does not involve any InsP₃R activation (Fig. 5D, b).

Therefore, we hypothesize that 2APB itself does not mobilize Ca²⁺ from intracellular stores; rather, it modulates the kinetics of agonist-induced Ca²⁺ release by acting specifically on the InsP₃R/Ca²⁺ release channels. This hypothesis is based on the following evidence.

First, InsP₃R activation is necessary for 2APB to regulate intracellular Ca²⁺ release. Under resting conditions (neither activation nor blockade of InsP₃R), 2APB fails to release Ca²⁺ from stores, indicating that 2APB cannot exert its effects without activation of InsP₃R (Fig. 2A). This idea was further confirmed by studies shown in Fig. 4A, in which the specific InsP₃R antagonist heparin was infused into the recorded cell with InsP₃. This procedure allows us to sequentially examine the effect of 2APB in two conditions (activation and blockade) of InsP₃R in the same recorded cell. As shown in Fig. 4A, the formation of whole-cell configuration led to infusion of InsP₃ and heparin from the pipette to the cell. Before heparin started its effects, infused InsP₃ quickly activates InsP₃Rs, and 2APB potentiated the pulsatile Ca²⁺ spikes, indicating that under activation of InsP₃Rs, 2APB plays a role in regulation of the kinetics of local Ca²⁺ release. However, after heparin gradually blocked InsP₃Rs, 2APB lost its effect, demonstrating that 2APB regulates Ca²⁺ release only under condition of InsP₃R activation.

Second, RyR seemed not to be involved in the 2APB regulation. Figure 4B clearly shows that pharmacological blockade of RyR by internal perfusion of ruthenium red cannot block 2APB-induced regulation. Traditionally, one believes that ruthenium red is a selective RyR blocker that has no effect on the InsP₃-activated Ca²⁺ channels (Ehrlich and Watras, 1988). However, accumulating data indicate an interaction between two types of intracellular Ca²⁺ stores (Thorn et al., 1994). As shown in Fig. 4B, ruthenium red not only blocked ACh-induced Ca²⁺ spikes but also obviously reduced spontaneous Ca²⁺ spikes evoked by continuously internal perfusion of InsP₃, as reported previously (Thorn et al., 1994). The mechanism of this effect is unclear. It may imply that the leakage a small amount of local Ca²⁺ through RyR may sensitize InsP₃ receptors (Bezprozvanny et al., 1991). Therefore, complete blockade of RyR by ruthenium red (100 μM) abolishes this sensitization effect, then reduces InsP₃-mediated Ca²⁺. This explanation is further supported by the finding that ryanodine (10 μM) increased InsP₃ -induced Ca²⁺ spikes (Fig. 4C; Thorn et al., 1994). Ryanodine blocks RyR by stabilizing an open-channel configuration at a low conductance, which serves to enhance Ca²⁺ leak through RyR and further sensitizes InsP₃ receptors, already stimulated by InsP₃, leading to an enhancement of InsP₃-induced Ca²⁺ spikes (Thorn et al., 1994). A previous report (Thorn et al., 1994) indicated that 10 μM ryanodine completely blocked ACh (30 nM)- and CCK (2 pM)-induced Ca²⁺ spikes. In particular, 10 μM ryanodine completely blocked RyR-mediated Ca²⁺ spikes stimulated by 1 μM cADP-ribose. Therefore, we believe that 10 μM ryanodine is concentrated enough to completely block RyR-mediated Ca²⁺ spikes in single pancreatic cells.

The mechanism that 2APB accesses to coordinate local Ca²⁺ release events to generate global Ca²⁺ waves is unclear. One possible explanation is that 2APB, at low concentrations, may increase sensitivity of InsP₃R/Ca²⁺ release channels to InsP₃. First, the present results clearly show that the regulation of the pulsatile Ca²⁺ spikes by 2APB depends on the presence of threshold concentrations of InsP₃ because 2APB, even at a high concentration (100 μM, Fig. 2A), never released Ca²⁺ at the resting level of InsP₃. Other evidence to support our explanation is shown in Fig. 4C, in which InsP₃ receptors have been sensitized first by InsP₃, then by Ca²⁺ (leaks through low conductance blockade of RyR). Under this condition, the potentiating effect of 2APB was increased sig-

nificantly (Fig. 4C). Second, 2APB enhanced local Ca^{2+} release events in a concentration-dependent manner. At a low concentration (3 μM), 2APB increased the frequency and amplitude of local Ca^{2+} release events without affecting spike duration (Fig. 3A). This excitatory effect is probably an increase in the sensitivity of $InsP_3$/Ca^{2+} release channels to $InsP_3$, which leads to activation of more Ca^{2+} release units during the continuous perfusion of $InsP_3$. At a relatively high concentration (30 μM), 2APB dramatically increased spike duration, perhaps reflecting the summation of the activated Ca^{2+} release units. This increase in spike duration may also reflect the fact that 2APB inhibits, at least in part, the ATP-dependent Ca^{2+} uptake system (Maruyama et al., 1997b). Pharmacological blockade of the ATP-dependent Ca^{2+} uptake system by thapsigargin induced an elevation of intracellular Ca^{2+} concentration, which involves an $InsP_3$-dependent Ca^{2+} release process in pancreatic acinar cells (Toescu and Petersen, 1994). During the activation of $InsP_3$/Ca^{2+} release channels, the appropriate blockade of the ATP-dependent Ca^{2+} uptake system (Ca^{2+}-ATPase activity) may delay the removal of cytosolic Ca^{2+}, leading to the long-lasting Ca^{2+} waves.

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