2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of $InsP_3$-induced Ca^{2+} release

MARTIN D. BOOTMAN, TONY J. COLLINS, LAUREN MACKENZIE,
H. LLEWELYN RODERICK, MICHAEL J. BERRIDGE, AND CLAIRE M. PEPPIATT
Laboratory of Molecular Signalling, The Babraham Institute, Babraham, Cambridge CB2 4AT, UK

ABSTRACT **Since its introduction to Ca^{2+} signaling in 1997, 2-aminoethoxydiphenyl borate (2-APB) has been used in many studies to probe for the involvement of inositol 1,4,5-trisphosphate receptors in the generation of Ca^{2+} signals. Due to reports of some nonspecific actions of 2-APB, and the fact that its principal antagonistic effect is on Ca^{2+} entry rather than Ca^{2+} release, this compound may not have the utility first suggested. However, 2-APB has thrown up some interesting results, particularly with respect to store-operated Ca^{2+} entry in nonexcitable cells. These data indicate that although it must be used with caution, 2-APB can be useful in probing certain aspects of Ca^{2+} signaling.—Bootman, M. D., Collins, T. J., Mackenzie, L., Roderick, H. L., Berridge, M. J., Peppiatt, C. M. 2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of $InsP_3$-induced Ca^{2+} release. *FASEB J.* 16, 1145–1150 (2002)**

Of the multiple presently recognized mechanisms of mobilizing intracellular Ca^{2+} stores (1), the pathway involving release of Ca^{2+} by inositol 1,4,5-trisphosphate ($InsP_3$) has possibly the least useful membrane-permeant pharmacology. Ryanodine receptors in intact cells, for example, can be rapidly activated by caffeine and specifically inhibited in a use-dependent manner by ryanodine (for reviews, see refs 2, 3). $InsP_3$ receptors ($InsP_3Rs$) can be specifically activated using a membrane-permeant $InsP_3$ ester (4; for example, see ref 5), but the choice of antagonists of $InsP_3$ action is limited. One of the most commonly used $InsP_3R$ antagonists is heparin. This compound is limited because it has multiple actions, including uncoupling G-protein signaling and activating ryanodine receptors (for reviews, see refs 2, 6). Furthermore, heparin has to be either injected or infused into cells, although it has been suggested that low molecular weight heparin species may cross the plasma membrane and thus inhibit $InsP_3Rs$ within intact cells (7). Xestospongins, which were first described as specific membrane-permeant $InsP_3R$ antagonists by Pessah and colleagues (8), have been used many times to prove whether $InsP_3Rs$ are involved in particular responses. Although there are many examples of xestospongin inhibiting Ca^{2+} signaling, its mechanism of action has not been fully elucidated. Xestospongin is expensive, often slow to act, and has not been successful in all labs. It was against this backdrop of a shortage of rapidly working, low-cost membrane-permeant antagonists of phosphoinositide signaling that 2-APB appeared to have great potential.

Although there are numerous examples of 2-APB inhibiting Ca^{2+} signaling in intact cells, its mechanism of action is unclear. In addition to attenuating the release of internal Ca^{2+} stores, 2-APB can also inhibit the store-operated channels (SOCs) that replenish the Ca^{2+} pool, suggesting that the inhibition of SOC may be a primary target for 2-APB.

SPECIFIC ACTIONS OF 2-APB ON Ca^{2+} RELEASE BY $InsP_3$ RECEPTORS

The initial report by Mikoshiba and colleagues demonstrated that 2-APB gave a concentration-dependent inhibition of $InsP_3$-induced Ca^{2+} release from mouse cerebellar membranes without affecting $InsP_3$ binding (9). It did not modulate $InsP_3$ production in platelets nor did it affect Ca^{2+} release from striated muscle vesicles activated by modest concentrations of caffeine, signifying that it did not interact with ryanodine receptors. Smooth muscle cell contraction in response to an $InsP_3$-generating stimulus was inhibited by 2-APB, whereas that triggered by KCl-induced depolarization was unaffected, suggesting there was an effect on neither voltage-operated Ca^{2+} entry nor the contractile machinery. 2-APB appeared to be rapidly cell permeant. Most of the claims made in the study by Maruyama et al. (9) have been substantiated by later studies, although 2-APB does not appear to be equally potent at inhibiting $InsP_3$-induced Ca^{2+} release in all cell types.

Ironically, the inhibitory effect of 2-APB on Ca^{2+} release via $InsP_3Rs$ is perhaps the most controversial of its actions. The cell types where 2-APB has been shown to have effects consistent with inhibition of $InsP_3$-

[1] Correspondence: Laboratory of Molecular Signalling, The Babraham Institute, Babraham, Cambridge CB2 4AT, UK. E-mail: martin.bootman@bbsrc.ac.uk

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induced Ca^{2+} release include platelets (9, 10), suprachiasmatic nucleus (11), ventricular cardiomyocytes (12), various types of smooth muscle (9, 13–18), toad sinus venosus (19), pancreatic β cells (20), fungal growth tips (21), hippocampal neurons (22), skeletal myotubes (23), neutrophils (24), and endothelial cells (25). Generally, these studies found that over the range 1–100 μM, 2-APB inhibited Ca^{2+} signals due to the release of intracellular stores and subsequent Ca^{2+} entry. However, in many of these studies, 2-APB has been used to demonstrate the involvement of $InsP_3Rs$ without consideration of additional effects.

Results obtained using permeabilized cells, cell membranes, and intact cells suggest that 2-APB may not be a consistent blocker of $InsP_3Rs$. Whereas Maruyama et al. (9) and others (e.g., refs 26, 27) found that $InsP_3$-induced Ca^{2+} release in permeabilized cells was inhibited in a concentration-dependent manner by 2-APB, different studies have reported either weak inhibition or no effect on $InsP_3$-induced Ca^{2+} release (e.g., 28).

Why 2-APB has such a variable effect on $InsP_3$-induced Ca^{2+} release in intact or permeabilized cells is unclear. One possibility, suggested by Kukkonen et al. (29), is that 2-APB shows selectivity for different $InsP_3R$ isoforms. Consistent with this notion, some of the most prominent effects of 2-APB have been seen in cell types that largely express types 1 and 3 $InsP_3Rs$, whereas cells that express largely type 2 $InsP_3Rs$ seem to be insensitive. However, there are exceptions to this rule, which suggests it is perhaps only a partial explanation. An alternative possibility derives from the mixed mode of inhibition of $InsP_3$-induced Ca^{2+} release by 2-APB. The inhibitory effect of 2-APB can be partially out-competed by increasing $InsP_3$ concentration. The IC_{50} for inhibition of $InsP_3$-evoked Ca^{2+} release is therefore dependent on the ambient $InsP_3$ concentration. In experiments by Maruyama et al. (9), Ca^{2+} released from cerebellar membranes by 100 nM $InsP_3$ was inhibited by 2-APB with an IC_{50} of ~10–20 μM. The maximal response of cerebellar microsomes to $InsP_3$ is achieved with 10 μM $InsP_3$; at this concentration of $InsP_3$, the IC_{50} for 2-APB is ~1 mM (J. Bilmen and F. Michelangeli, personal communication). The discrepant effects of 2-APB in blocking agonist-induced Ca^{2+} release in intact cells may therefore indicate that different cell types generate substantially varying levels of $InsP_3$. However, this is also likely to be only a partial explanation, since the same concentrations of 2-APB and $InsP_3$ have been shown to have contrasting effects in different cell types (e.g., compare refs 27, 28). Although there is no apparent resolution to the divergent effects of 2-APB on $InsP_3$-induced Ca^{2+} release in different studies, these data do highlight the need for caution in simply using 2-APB as an $InsP_3R$ antagonist. In some situations, 2-APB attenuates Ca^{2+} signaling without modulating Ca^{2+} flux through $InsP_3Rs$. In these cases, the action of 2-APB can be explained by inhibition of Ca^{2+} entry.

SPECIFIC ACTIONS OF 2-APB ON Ca^{2+} ENTRY THROUGH SOCs

Even though the data presented by Maruyama et al. (9) made a reasonable case for 2-APB acting as a specific membrane-permeable $InsP_3R$ antagonist, emerging data show that its primary action on cells is not to block Ca^{2+} release, but rather to inhibit SOC influx. The study that really brought 2-APB into the limelight—demonstrating the involvement of $InsP_3Rs$ in coupling to SOC channels (30)—must be interpreted in the light that 2-APB can inhibit SOC directly without involvement of $InsP_3Rs$ (27, 28, 31–34).

Despite its widespread use, there is presently no clear-cut evidence for 2-APB inhibiting Ca^{2+} signaling by solely targeting $InsP_3Rs$. At best, it seems that in some cells 2-APB can inhibit both agonist-induced Ca^{2+} release and the concomitant SOC with the same efficacy (e.g., refs 16, 27). In contrast, there are several studies of intact cells where 2-APB was found not to inhibit $InsP_3$-induced Ca^{2+} release, but it could still block Ca^{2+} entry. For example, Barritt and colleagues (28) found that concentrations of 2-APB of up to 100 μM had no effect on Ca^{2+} release during vasopressin stimulation of primary hepatocytes, but it did cause a concentration-dependent inhibition of Ca^{2+} influx with an IC_{50} of ~10 μM. Thapsigargin-induced Ca^{2+} influx was inhibited with a comparable concentration dependence. A similar effect of 2-APB inhibiting SOC without affecting Ca^{2+} release has been demonstrated for CHO cells (29).

The best evidence for 2-APB inhibiting SOC through a mechanism not involving $InsP_3Rs$ is that it is still effective in cells that do not express $InsP_3Rs$ at all (27, 31, 34, 35). Activation of *Drosophila* photoreceptors relies on the gating of light-activated cation channels in the plasma membrane of these cells. It is well established that the $InsP_3Rs$ expressed in *Drosophila* photoreceptors are not necessary for vision, yet 2-APB still blocked light-induced cation entry (27, 36).

2-APB does not inhibit SOC when internally perfused within cells (31, 32) or applied to the cytosolic surface of excised membrane patches (33). Extracellularly applied 2-APB inhibits SOC in platelets with no measurable delay, similar to inorganic ions such as lanthanum (37). These data indicate that 2-APB blocks SOC by interacting at the outside of cells. The manner in which 2-APB prevents SOC is unclear. The agonist-induced activation of the putative SOC channel TRP3 heterologously expressed in HEK cells was inhibited by 2-APB whereas activation of the same channel by diacylglycerol analogs was not (30). Similarly, in *Drosophila* photoreceptors, 2-APB blocked light-induced cation entry but not that evoked by metabolic stress (36). If 2-APB simply occluded these channels, it should prevent their activation by any means. These data therefore suggest that 2-APB probably does not bind directly to the channels, but rather interacts upstream in their activation mechanism (see also ref 38).

If 2-APB is to be used as a SOC inhibitor, it is essential

that it has good selectivity for those channels. The original study demonstrated that it had no effect on voltage-operated Ca^{2+} channels (9), a finding supported by several other studies (11, 13, 16, 19, 23). Several other non-voltage-activated Ca^{2+} entry pathways that are distinct from SOC are not inhibited by 2-APB, including S-nitrosylation-induced influx in a smooth muscle cell line (39), maitotoxin-evoked Ca^{2+} entry in hepatocytes (28), Ca^{2+} influx caused by diacylglycerol analogs in PC12 cells (40), muscarinic activation of nonselective cation channels in smooth muscle (41), and arachidonate-stimulated Ca^{2+} channels in HEK293 cells (38) and HeLa cells (authors' unpublished observations).

Despite its lack of action on the non-SOC Ca^{2+} entry pathways listed above, 2-APB is not completely specific for SOC channels, as it has been shown to inhibit MagNuM channels (also known as LTRPC7 and TRP-PLIK; ref 42). MagNuM is a widely expressed ion channel that conducts Ca^{2+} and Mg^{2+} at negative membrane potentials. This channel is not regulated by store depletion, but instead is activated by a decrease in intracellular levels of magnesium nucleotides (43). Although SOC and MagNuM may be difficult to discriminate if they are simultaneously switched on, since the latter is not activated by store depletion it is likely that the flux of Ca^{2+} through this channel will not be appreciably altered by mild treatments that activate SOC. Under many experimental conditions, therefore, it is likely that a block of Ca^{2+} entry by 2-APB would reflect and affect SOC channels rather than MagNuM. In rat basophilic leukemia cells, the effect of 2-APB on MagNuM was rapidly reversible whereas its effects on SOC were not (42). The reversibility of 2-APB may therefore provide a means of discriminating between different targets.

2-APB also modifies the conductance through CaT1 channels (44). These channels were suggested to underlie I_{CRAC}, a well-characterized form of SOC (45). However, whereas 2-APB irreversibly inhibits I_{CRAC}, it reversibly potentiates the conductance of CaT1 channels, suggesting they are not the same (44).

At present, the bulk of data supports the notion that 2-APB is an almost universal blocker of SOC and some TRP isoforms (35) while being a rather variable inhibitor of $InsP_3$-induced Ca^{2+} release. Whereas substantially different concentrations of 2-APB are needed to inhibit $InsP_3$-induced Ca^{2+} release in different cell types, SOC is generally fully inhibited by 50–100 μM 2-APB. The present exceptions to this are SH-SY5Y cells, which appear to be completely insensitive to 2-APB (29), and neutrophils where Ca^{2+} signals in response to platelet-activating factor were totally blocked by 100 nM 2-APB (46).

POTENTIALLY UNHELPFUL ACTIONS OF 2-APB

In addition to its effects on $InsP_3$-induced Ca^{2+} release and SOC, 2-APB has been shown to enhance the nonspecific leak of Ca^{2+} from the intracellular Ca^{2+} pool (26) and to inhibit the SERCA pumps responsible for loading intracellular Ca^{2+} stores (9, 26). Consistent with the enhancement of a Ca^{2+} leak, in some intact cell studies 2-APB caused a rise in basal Ca^{2+} levels (9, 10, 12, 28). When applied to electrically paced cardiac myocytes at concentrations ≥ 10 μM, 2-APB caused a transient increase in the amplitude of systolic Ca^{2+} rises, followed by a progressive diminution of the electrically evoked responses (authors' unpublished observations). This effect is consistent with an inhibition of SERCA activity leading initially to an enhanced response due to less Ca^{2+} sequestration, but the lack of Ca^{2+} store refilling eventually leads to a failure of excitation-contraction coupling.

2-APB prevents mitochondria from releasing Ca^{2+} they have sequestered (31), possibly by an inhibitory action on the sodium/Ca^{2+} exchanger. We have found that 2-APB at 100 μM causes mitochondria to swell and change shape, but without causing them to depolarize (authors' unpublished observations).

These actions of 2-APB may render it useless in some situations. However, in experimental protocols investigating SOC activity, the intracellular Ca^{2+} pool is often completely discharged by application of substances such as thapsigargin. Under those conditions, any effect of 2-APB on the Ca^{2+} leak or SERCAs would be irrelevant. Although mitochondria and intracellular Ca^{2+} signaling systems communicate bidirectionally, the effect of inhibiting mitochondrial Ca^{2+} release on a particular response can be directly tested by using the sodium/Ca^{2+} exchange blocker GCP37157 (47).

Another potentially limiting feature of 2-APB is that it is poorly reversible in some cell types. In toad sinus venosus, for example, it took > 1 h for the inhibitory effects of 60 μM 2-APB to be reversed (19, see also refs 27, 31, 32). These data contrast with other studies where the inhibitory effects 2-APB were reversed within a few minutes (28) or even immediately (36). In studies of cardiac myocytes, we have found that the concentration of 2-APB applied to cells determined its reversibility. 2-APB inhibited spontaneous diastolic Ca^{2+} transients evoked by endothelin 1. At concentrations of < 5 μM, this effect was readily reversible, whereas higher concentrations gave a prolonged inhibition (unpublished results).

Some of the discrepant effects of 2-APB may be due to its ability to exist as different species. Besides the open-chain monomeric form, it has been suggested to dimerize (39) and form a monomeric heterocyclic compound with an internal coordinate nitrogen-boron bond (10). It was proposed that 2-APB dimers resemble Xestospongins, indicating a common mechanism of action (39), but this seems unlikely (10). A comparison of structural analogs of 2-APB that inhibit Ca^{2+} signaling in platelets has suggested that the diphenyl moiety is important for its activity (10).

The pK_b of 2-APB is ~ 10, which means that in

physiological pH its amine group will be protonated, giving the molecule a net positive charge (J. Bilmen and F. Michelangeli, personal communication; ref 10). We have found that 2-APB causes a rapid cytoplasmic acidification that is slowly reversible, suggesting that it either carries protons across the membrane or displaces them from internal sites (authors' unpublished observations).

SIMILARITY BETWEEN THE ACTIONS OF 2-APB AND XESTOSPONGIN

The observations of multiple conflicting effects of 2-APB may also sound a note of caution for users of Xestospongins. Ironically, Xestospongins have been used in more studies than 2-APB, yet we probably know more about the latter compound. In several studies, the results of using 2-APB and Xestospongins have been the same, and their common effect interpreted as clear evidence for involvement of $InsP_3Rs$ (e.g., refs 25, 30). However, since the interpretation of data obtained using 2-APB has to be reevaluated in the light of its action on SOC, so Xestospongin cannot simply be regarded as an $InsP_3R$ antagonist. Indeed, Xestospongin is capable of inhibiting SOC in DT40 cells where $InsP_3Rs$ are absent (D. L. Gill, personal communication). Just like 2-APB, Xestospongin can inhibit SERCAs (48) and cause release of Ca^{2+} from stores in intact cells (e.g., 49). There is a striking similarity between Xestospongin and 2-APB in the range of targets (SOC, $InsP_3Rs$, SERCA) and in their rather variable action on different cell types.

POTENTIATION OF Ca^{2+} SIGNALING BY 2-APB

Although much of the data obtained using 2-APB have suggested that it blocks Ca^{2+} signaling in intact cells, there are several reports of 2-APB enhancing Ca^{2+} release and Ca^{2+} entry. In rat basophilic leukemia cells and Jurkat cells, I_{CRAC} had a biphasic dependence on 2-APB concentration; it was potentiated by low micromolar concentrations of 2-APB and inhibited by higher levels of 2-APB (31). This was independent of any effects of 2-APB on $InsP_3Rs$, since I_{CRAC} in DT40 cells not expressing $InsP_3Rs$ showed a similar biphasic dependence on 2-APB concentration (31; but see ref 44). A transient facilitation of the light-induced current by 2-APB was observed in *Drosophila* photoreceptors (36). 2-APB potentiated Ca^{2+} signals in mouse pancreatic cells during agonist stimulation or during internal perfusion with $InsP_3$ (50).

The enhanced leak of Ca^{2+} from the endoplasmic reticulum and inhibition of SERCA activity by 2-APB may underlie some of the observed potentiation of Ca^{2+} signaling and the suggestion that 2-APB may act as an agonist of $InsP_3Rs$ (51). Ca^{2+} that is nonspecifically released by stores can positively stimulate further $InsP_3$ production or Ca^{2+} release (e.g., 52). Removal of Ca^{2+} sequestration by SERCA inhibition will remove a negative feedback component from the responses. Therefore, whereas potentiatory effects of 2-APB on Ca^{2+} entry might reflect modulation of channels (31), enhancement of Ca^{2+} release probably reflects actions of 2-APB on the Ca^{2+} leak and SERCAs.

SUMMARY

2-APB has been rapidly adopted as an $InsP_3R$ antagonist by the Ca^{2+} signaling community. However, 2-APB cannot simply be used as an $InsP_3R$ antagonist in most situations, since it has multiple targets and can potentiate some Ca^{2+} responses. Inhibition of $InsP_3$-mediated Ca^{2+} release by 2-APB is extremely variable between studies. An almost universal observation is that 2-APB inhibits SOC. However, this is not via an effect on $InsP_3Rs$, nor is it due to direct blocking of the Ca^{2+} entry channels. The multiple actions of 2-APB are strikingly similar to those of Xestospongin, implying that the data obtained with both compounds must be interpreted cautiously. Despite the many effects of 2-APB, it does have uses, for example, in investigating the activation of SOC and in discriminating between different forms of Ca^{2+} entry. Careful titration of 2-APB concentration may allow inhibition of $InsP_3$-induced Ca^{2+} release and SOC without significant SERCA inhibition or nonspecific Ca^{2+} release. In many situations 2-APB cannot be regarded as a marker for the involvement of $InsP_3Rs$ in the generation of Ca^{2+} signals. FJ

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