Mechanism of Inhibition of TRPC Cation Channels by 2-Aminoethoxydiphenylborane

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ABSTRACT

We investigated the actions of the organoborane, 2-aminoethoxydiphenylborane (2APB), on Ca$^{2+}$ signaling in wild-type human embryonic kidney (HEK) 293 cells and in HEK293 cells stably expressing canonical transient receptor potential (TRPC) channels. Previous reports have suggested that 2APB inhibits agonist activation of TRPC channels because of its ability to act as a membrane-permeant inhibitor of inositol 1,4,5-trisphosphate (IP$_3$) receptors. 2APB was specifically said to inhibit TRPC3 channels when activated through a phospholipase C-linked receptor but not when activated more directly by a synthetic diacylglycerol, oleyl-acetyl-glycerol (OAG) [Science (Wash DC) 287:1647–1651, 2000]. However, we subsequently reported that IP$_3$ does not activate TRPC3; rather the mechanism of activation by phospholipase C-linked receptors seemed to result from diacylglycerol [J Biol Chem 278:16244–16252, 2003]. Thus, the current study was carried out to address the mechanism of action of 2APB in inhibiting TRPC channels. We found that, although the release of Ca$^{2+}$ by a muscarinic agonist was reduced by high concentrations of 2APB, this effect was indistinguishable from that seen when stores were discharged by thapsigargin, which does not involve IP$_3$ receptors. This indicates that 2APB is incapable of significant inhibition of IP$_3$ receptors when applied to intact cells. We found that 2APB partially inhibits divalent cation entry in cells expressing TRPC3, TRPC6, or TRPC7 and that this partial inhibition was observed whether the channels were activated by a muscarinic agonist or by OAG. Thus, as concluded for store-operated channels, 2APB seems to inhibit TRPC channels by a direct mechanism not involving IP$_3$ receptors.

TRPC channels are believed to function as multifunctional calcium-permeable cation channels (Putney, 2004). Depending on cell type, expression level, or expression environment, the channels can be activated through the phospholipase C pathway or, in some instances, by depletion of intracellular Ca$^{2+}$ stores (Venkatachalam et al., 2002; Vazquez et al., 2004). A subgroup of TRPCs, TRPC3, TRPC6, and TRPC7, can be activated by synthetic diacylglycerols, such as oleyl-acetyl-glycerol (OAG) (Hofmann et al., 1999; Okada et al., 1999), leading to the suggestion that the mechanism by which phospholipase C-linked receptors activate these channels is through diacylglycerol formation (Trebak et al., 2003a,b). However, some investigators have suggested that activation of TRPCs, especially TRPC3, involves interaction with inositol 1,4,5-trisphosphate (IP$_3$) and the IP$_3$ receptor (Kiselyov et al., 1998; Ma et al., 2000). One of the arguments for this idea was the inhibition of TRPC3 by the organoborane 2-aminoethoxydiphenylborane (2APB) (Ma et al., 2000).

2APB was originally described as a membrane-permeant inhibitor of IP$_3$ receptors (Maruyama et al., 1997). Thereafter, the ability of 2APB to inhibit endogenous store-operated channels as well as TRPC3 channels was taken as evidence for a role of the IP$_3$ receptor in the mechanism of activation of both of these channel types (Ma et al., 2000). A key observation was that 2APB blocked the activation of TRPC3 channels by a muscarinic agonist (which would activate phospholipase C) but did not block more direct activation with OAG. The proposed role of the IP$_3$ receptor was consistent with the conformational coupling hypothesis for store-operated channels (Irvine, 1990; Berridge, 1995), whereby underlying endoplasmic reticulum IP$_3$ receptors interact with and signal to plasma membrane store-operated channels. In addition, this interpretation was consistent with previous published findings indicating that the activation of expressed TRPC3 channels involved interaction with endoplasmic reticulum IP$_3$ receptors (Kiselyov et al., 1998). However, other studies cast

ABBREVIATIONS: TRPC, canonical transient receptor potential; 2APB, 2-aminoethoxydiphenylborane; ANOVA, analysis of variance; HEK, human embryonic kidney; OAG, oleyl-acetyl-glycerol; IP$_3$, inositol 1,4,5-trisphosphate; DMEM, Dulbecco’s modified Eagle’s medium; FLIPR, fluorometric imaging plate reader.

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doubt on a role of IP₃ receptors in the mechanism of store-operated entry (Sugawara et al., 1997; Broad et al., 2001). In the case of TRPC3 channels, more recent studies found no evidence of IP₃ or IP₃ receptor involvement and concluded that stimulation of TRPC3 through the phospholipase C pathway involved the formation of and activation by diacylglycerol, a process mimicked by OAG activation (Venkat-achalam et al., 2001; Trebak et al., 2003a). In addition, other studies from a number of laboratories presented evidence of IP3 or IP3 receptor involvement and concluded that stimulation of TRPC3 through the phospholipase C pathway involved the formation of and activation by diacylglycerol, a process mimicked by OAG activation (Venkat-achalam et al., 2001; Trebak et al., 2003a). In addition, other studies from a number of laboratories presented evidence that 2APB blocked capacitative calcium entry directly, rather than as a result of action on IP₃ receptors (Bakowski et al., 2001; Braun et al., 2001; Dobrydneva and Blackmore, 2001; Iwasaki et al., 2001; Prakriya and Lewis, 2001). In light of this conclusion, the original report that 2APB blocks TRPC3 when activated by a muscarinic agonist but not when activated by OAG or OAG presents something of a conundrum. Therefore, in the current work, we have re-examined the effect of 2APB on agonist- and OAG-activated TRPC3, as well as the close structural relatives, TRPC6 and TRPC7. Our findings indicate that 2APB does not in fact act as a membrane-permeable inhibitor of IP₃ receptors, at least in HEK293 cells. In addition, we find that 2APB induces partial inhibition of all three channels, whether activated by agonist or by OAG. The reasons for the differences from previously published results are discussed.

Materials and Methods

Reagents. Thapsigargin, methacholine, and OAG were purchased from Sigma Chemical (St. Louis, MO). Cell Culture and Transfection. HEK293 cells stably expressing TRPC3-green fluorescent protein fusion protein were described previously (McKay et al., 2000). Stable transfections of wild-type HEK293 cells obtained from American Type Culture Collection (Manassas, VA) were carried out using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Wild-type HEK293 cells transfected with either pCDNA3 vector containing the coding sequence for human TRPC6 (kindly provided by Dr. T. Gudermann (Institut für Pharmakologie, Berlin, Germany) (Hofmann et al., 1999) or pCDNA3.1(−) expression vector encoding for wild-type human TRPC7 [WT TRPC7 (leucine at position 111)] (jointly supplied by Christine Murphy and Adrian Wolstenholme (University of Bath, Bath, United Kingdom) and John Westwick (Novartis, Horsham, United Kingdom)] were grown for 4 weeks under continuous selection with 500 μg/ml Geneticin (Invitrogen) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine (complete DMEM) in a humidified 95% O2/5% CO2 incubator. TRPC6- and TRPC7-transfected cell populations were then tested for the ability of a phospholipase C-linked agonist (methacholine) to activate the channels in the presence of 5 μM Gd³⁺ by calcium measurements performed in single cells (see results below). TRPC6- and TRPC7-transfected cells (80–90%) showed a receptor-mediated Ca²⁺ entry insensitive to Gd³⁺, a percentage of responding cells not significantly different from for the TRPC3-green fluorescent protein-expressing cell population (data not shown; McKay et al., 2000).

Measurement of Intracellular Calcium. For wild-type and stably TRPC3-, TRPC6-, and TRPC7-expressing HEK293 cells, calcium measurements were performed on Fluo-4-loaded cells [4 μM Fluo-4 AM (Invitrogen) in minimal essential medium for 45 min at 37°C] with a fluorometric imaging plate reader (FLIPR™; Molecular Devices, Sunnyvale, CA) as described previously (Trebak et al., 2002). Because FLIPR experiments use a single wavelength dye, fluorescence intensities for each well are normalized by proportion to a single, average initial value, thus compensating for small variances in the number of cells scanned in each well. Barium entry measurements were performed with single cells attached to glass coverslips mounted in a Teflon chamber and incubated at 37°C for 30 min in complete DMEM containing 2 μM Fura-2/AM (Molecular Probes, Inc. Eugene, OR) and 1 μM barium chloride.

![Figure 1](https://example.com/fig1.png)  
**Fig. 1.** Effects of 2APB on Ca²⁺ release and Ca²⁺ entry in methacholine (MCh)- and thapsigargin (TG)-stimulated wild-type and TRPC3-expressing HEK293 cells. Ca²⁺ mobilization was assessed in Fluo-4 loaded wild-type or TRPC3-expressing HEK293 with a FLIPR™ plate reader system. The cells were initially incubated in medium lacking extracellular Ca²⁺. Varying concentrations of 2APB were added as indicated followed by either 300 μM methacholine or 2 μM thapsigargin and, subsequently, 1.8 mM Ca²⁺. The traces shown are averages from four wells on a 96-well plate.
Eugene, OR. Cells were then washed and bathed in a HEPES-buffered saline solution for at least 10 min before fluorescence measurements were made. Measurements of intracellular Ca\(^{2+}\) and Ba\(^{2+}\) changes with Fura-2 were recorded and analyzed with an InCyt Im2 digital fluorescence imaging system (Intracellular Imaging Inc., Cincinnati, OH) as described previously (Trebak et al., 2003a). All experiments were conducted at room temperature, and data are reported as fluorescence intensity for calcium measurements in FLIPR\(^{384}\) or as the initial rate of rise of the ratio of fluorescence due to excitation at 340 and 380 nm for Ba\(^{2+}\) entry. For Fluo-4, peak fluorescence intensities (i.e., release with methacholine) were at most 50 to 60% of those for dye saturation.

**Statistics.** Statistical analyses (two-way ANOVA and post hoc tests) were carried out with JMP statistical software, version 5.0.1.2, produced by SAS Institute (Cary, NC).

**Results and Discussion**

We first examined the concentration-effect relationship for the inhibitory actions of 2APB on agonist-induced Ca\(^{2+}\) release, which reflects IP\(_3\) receptor function, and on Ca\(^{2+}\) entry due to agonist and thapsigargin in wild-type and TRPC3-expressing cells. In wild-type cells, the entry in response to agonist and thapsigargin involves capacitative calcium entry channels (Luo et al., 2001), whereas in TRPC3-expressing cells, a component of the agonist-induced entry reflects activity of TRPC3 channels (Zhu et al., 1998). These experiments were carried out using an automated high-throughput system, FLIPR\(^{384}\), that uses robotic solution pipetting and yields [Ca\(^{2+}\)]

![Fig. 2. Statistical summary of Ca\(^{2+}\) release and Ca\(^{2+}\) entry data from the experiments illustrated in Fig. 1. •, experiments with methacholine; ○, experiments with thapsigargin. Means ± S.E.M. from three experiments for wild-type cells and four experiments for TRPC3-expressing cells with four wells per plate (experiment) for each condition. Where mean ± S.E.M. bars are not visible, they are smaller than the symbol size. Each data set was analyzed by two-way ANOVA. For both release and entry data, the peak increase in Fluo-4 fluorescence after agonist addition or Ca\(^{2+}\) restoration, respectively, was taken. For release data, the responses varied somewhat from plate to plate, and for this reason and to facilitate comparison between methacholine- and thapsigargin-induced release, the data were normalized to the maximal release seen in the absence of 2APB. For release data in both wild-type and TRPC3-expressing cells, the effects of agonist were not significant, unlike the effects of 2APB concentration. Interaction was not significant. For entry data for both wild-type and TRPC3-expressing cells, the effects of agonist, 2APB concentration, and interaction were all significant. Post hoc analyses by Tukey-Kramer Highest Significant Difference revealed that, for the TRPC3-expressing cells, the values for entry in the presence of 30 and 100 μM 2APB were significantly higher with methacholine than with thapsigargin. No such significant difference was detected in wild-type cells.](https://example.com/figure2.png)
tantly, there was no significant difference in the peak release of Ca\(^{2+}\) in the methacholine-stimulated cells compared with the thapsigargin group. This indicates that, despite the documented ability of 2APB to block IP\(_3\) receptors in broken or permeable cell preparations, the drug seems unable to block IP\(_3\) receptors to a significant extent when applied to intact cells, at least in this cell line and in this concentration range.

Examination of the effects of 2APB on Ca\(^{2+}\) entry reveals that entry in both wild-type and TRPC3 cells is inhibited in a concentration-dependent manner. In addition, in both cell types, entry is inhibited whether activated by methacholine or thapsigargin. Entry due to thapsigargin occurs through endogenous store-operated channels in both the wild-type and TRPC3-expressing cells, because TRPC3 does not form store-operated channels under these expression conditions. However, as shown previously (Trebak et al., 2002), methacholine-activated entry is not completely blocked in the TRPC3-expressing cells, even at the highest concentration used (100 \(\mu\)M). These findings indicate that 2APB is capable of completely blocking entry through store-operated channels but induces only a partial block of entry through agonist-activated TRPC3 channels.

We next examined the action of the maximal inhibitory concentration of 2APB (30 \(\mu\)M) on Ca\(^{2+}\) entry activated by either methacholine or the diacylglycerol analog OAG. We carried out these experiments with HEK293 cells stably expressing each of the three OAG-sensitive TRPCs, TRPC3, TRPC6, and TRPC7. We used Ba\(^{2+}\) as a surrogate for Ca\(^{2+}\); Ba\(^{2+}\) is a sensitive indicator of divalent cation entry and is not subject to a number of potential artifacts that affect Ca\(^{2+}\) movements, such as altered rates of Ca\(^{2+}\) transport (Ba\(^{2+}\) is not a substrate for Ca\(^{2+}\) transporters) or secondary effects on Ca\(^{2+}\)-activated channels (Vanderkooi and Martonosi, 1971; Uvelius et al., 1974; Schilling et al., 1989; Kwan and Putney, 1990; Broad et al., 1996; McKay et al., 2000; Trebak et al., 2002; Vazquez et al., 2002; Lievremont et al., 2004). In addition, 5 \(\mu\)M Gd\(^{3+}\) was included to insure that endogenous capacitative calcium entry was blocked and that only entry through TRPC channels could be observed. Figure 3, top, shows two selected traces demonstrating inhibition by 30 \(\mu\)M 2APB of methacholine- and OAG-induced Ba\(^{2+}\) entry in TRPC3-expressing cells (discussed below). The entry is significantly reduced in both cases, although the effect seems somewhat less for OAG. Figure 3, bottom, summarizes the effects of 30 \(\mu\)M 2APB on methacholine- and OAG-induced entry in TRPC3, TRPC6, and TRPC7 cells. For all three cell types, entry was significantly yet only partially inhibited by 2APB whether activated by methacholine or OAG. In general, the entry induced by OAG was greater than that by methacholine, perhaps because added OAG causes maximal or near maximal channel activation, whereas the amount of endogenous DAG formed may not be sufficient for maximal activation. We considered that this difference might explain the apparent lesser sensitivity of the OAG response to 2APB inhibition. Thus, for TRPC3, we repeated this experiment using a 3 \(\mu\)M concentration of OAG, a concentration producing just less than a half-maximal activation of TRPC3. With this concentration of OAG, the control rate of Ba\(^{2+}\) entry was 0.29 \(\pm\) 0.08, and in the presence of 30 \(\mu\)M 2APB, the entry was 0.11 \(\pm\) 0.03 (F340/F380 s/100; \(n = 4\)) (i.e., a reduction of more than 50%). This may explain why OAG activation of TRPC3 seemed insensitive to 2APB in previous studies (Ma et al., 2000). In addition, in the latter study, entry was determined using Sr\(^{2+}\) or Ca\(^{2+}\), cations that are substrates for Ca\(^{2+}\) pumps and buffers, which might make it more difficult to see partial inhibitory effects.

In conclusion, we have found that the actions of 2APB on TRPC channels probably do not involve IP\(_3\) receptors. First, our results indicate that, despite claims from previous reports, 2APB seems incapable of inhibiting IP\(_3\) receptors in intact cells. Second, we find that, in contrast to previously published reports, 2APB inhibits TRPC channels similarly.

Fig. 3. 2APB partially inhibits methacholine-induced Ba\(^{2+}\) entry in TRPC3-, TRPC6-, and TRPC7-transfected HEK293 cells. Cells were incubated in the absence of added Ca\(^{2+}\) and the continual presence of 5 \(\mu\)M Gd\(^{3+}\). Top, 300 \(\mu\)M methacholine (MeCh) or 100 \(\mu\)M OAG in the absence (solid line) or in the presence (dotted line) of 30 \(\mu\)M 2APB followed by 2 mM Ba\(^{2+}\) was added where indicated. Ba\(^{2+}\) entry measurements were performed with single cells attached to coverslips as described under Materials and Methods. The trace presented is the average of three coverslips with at least 50 cells attached per coverslip from one experiment performed in triplicate. Bottom, initial rates of Ba\(^{2+}\) entry (fluorescence increase upon addition of Ba\(^{2+}\) to the external medium) in TRPC3, TRPC6, and TRPC7 cells were calculated. Presented are the means \(\pm\) S.E. from three to six independent experiments performed with TRPC3, TRPC6, and TRPC7-expressing cells. Under conditions for measuring Ba\(^{2+}\) entry by single cell digital imaging, basal rates of Ba\(^{2+}\) entry were negligible. The data were analyzed by two-way ANOVA, which revealed a weak interaction (\(p < 0.05\)) for both the methacholine and OAG data sets. In both cases, the differences among TRPCs and differences due to 2APB were significant at \(p < 0.0001\). Because of the significance of the interaction, individual means were compared by Tukey-Kramer Highest Significant Difference, and in all cases the effect of 2APB was significant at \(p < 0.05\).
whether activated through a phospholipase C-coupled receptor or more directly by the DAG analog, OAG. The reason that 2APB induces only a partial block of the channels is not known, but this finding suggests that the drug does not act by simply occluding the channel pore. In this regard, the inhibition is clearly distinct from that seen with native store-operated channels that are completely inhibited in this same concentration range.

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References


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