2-Aminoethyl diphenyl borinate (2-APB) inhibits TRPM7 channels through an intracellular acidification mechanism

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2-APB is a widely used compound in ion channel research. It affects numerous channels including inositol 1,4,5-trisphosphate receptors, store-operated calcium channels and TRP channels, TRPV3 and TRPM7 among them. A characteristic property of TRPM7 channels is their sensitivity to intracellular Mg²⁺ and pH. Using patch clamp electrophysiology we find that in Jurkat T lymphocytes, 100–300 μM extracellular 2-APB reversibly inhibits TRPM7 channels when internal HEPES concentration is low (1 mM). Increasing the concentration to 140 mM abolishes the 2-APB effect. Using single-cell fluorescence pH video imaging, we show that at concentrations of 100 μM and higher, 2-APB potently acidifies the cytoplasm. We conclude that TRPM7 sensitivity to 2-APB is not direct but rather, can be explained by cytoplasmic acidification and a resulting channel inhibition.

Introduction

2-aminoethyl diphenyl borinate (2-APB) is a membrane-permeant, lipophilic compound that was originally described as a blocker of intracellular inositol 1,4,5 trisphosphate [Ins(1,4,5)P₃] receptors.¹² Soon afterwards, it was discovered that this compound can block store-operated calcium channels in various cell types without engaging the Ins(1,4,5)P₃ receptors.³⁻⁸ During the last decade, effects of 2-APB on numerous other ion transport proteins have been described. For example, 2-APB is an inhibitor of sarcoplasmic-endoplasmic reticulum Ca²⁺-ATPase (SERCA) pumps,⁹ gap junction connexins,¹⁰ potassium channels¹¹ and volume-regulated anion channels¹² at micromolar concentrations. Members of the Transient Receptor Potential (TRP) family of cation channels were also shown to be sensitive to this compound. Thus, TRPC3, TRPC5, TRPC6, TRPM2, TRPM3 and TRPM7 are blocked by 2-APB at micromolar concentrations.¹,¹³⁻²⁰

2-APB inhibits native and overexpressed TRPM7 channels in a voltage-independent manner, with IC₅₀ values in the 70–170 μM range.¹⁵⁻²⁴ Jurkat T-cell TRPM7 channels are sensitive to cytoplasmic Mg²⁺, described by a biphasic inhibition dose-response curve with IC₅₀ values of -10 μM and -165 μM in whole-cell recordings.²⁶ These channels are also sensitive to intracellular pH with a single IC₅₀ of pH 6.3.²⁶,²⁷

We recently found that several non-steroidal anti-inflammatory drugs (NSAIDs) inhibit TRPM7 channels in Jurkat T lymphocytes by a mechanism of cytosolic acidification.²⁸ We therefore hypothesized that 2-APB may also exert its inhibitory action on this channel through a similar mechanism. In order to test this hypothesis, we compared the effectiveness of channel blockade by extracellular 2-APB for Jurkat T cells internally perfused with 1 mM and 140 mM HEPES-containing solutions in whole-cell patch clamp. In agreement with previous reports, we found that 100 and 300 μM 2-APB potently and reversibly inhibited TRPM7 channels when the internal solution contained low concentration of pH buffer. Inhibition was abolished, however, when the internal solution included a high concentration of HEPES buffer. Addition of 2-APB at the same concentrations to the pipette solution did not inhibit TRPM7 channels, suggesting that 2-APB binds on the cell surface. Using ratiometric pH imaging of BCECF-loaded Jurkat T cells, we found that 2-APB at concentrations effective for channel inhibition, reversibly acidified the cell cytoplasm. Based on these findings we conclude that 2-APB inhibits TRPM7 channels indirectly through a cytoplasmic acidification mechanism. This finding will help to evaluate some of the previously described effects of 2-APB on other ion channels and ion pumps.

Results

2-APB was previously reported to inhibit TRPM7 channel monovalent current of Jurkat T cells at 50 μM with a slow onset and recovery.¹⁵ We used internal solutions containing a low concentration of HEPES, 1 mM, to record TRPM7 channels. 2-APB, at 10, 100 and 300 μM concentrations, was applied to the

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cells after TRPM7 current had fully developed in response to a gradual depletion of cellular Mg²⁺ (Fig. 1). Ten micromolars of 2-APB did not produce a noticeable inhibition of the current in any of the cells tested. In agreement with previous studies, application of 100 and 300 μM 2-APB resulted in a slow decline of current amplitude, which was reversible upon washout with DMSO-containing control solutions (Fig. 1A and B). This inhibition was not voltage-dependent (Fig. 1A).

We repeated these experiments in recordings with internal solutions containing 140 mM HEPES buffer. Elevated intracellular HEPES prevented any significant inhibition by 100 or 300 μM 2-APB, concentrations effective for low HEPES internal solutions. Figure 1C shows the time course of development of TRPM7 current in a Jurkat T cell, recorded with a high HEPES pipette solution. Application of 300 μM 2-APB elicited only a small reduction in the current magnitude. Addition of 10 μM spermine in the presence of 2-APB resulted in a fast and reversible channel block, as previously reported for TRPM7 channels. These findings suggest that 2-APB inhibits TRPM7 channels by acidifying the cytosol, which would be expected to reduce TRPM7 channel activity. We therefore tested if external ammonium would relieve channel inhibition by 2-APB. In Figure 1D, we applied 100 μM 2-APB after TRPM7 channels were fully activated. As expected, 2-APB significantly reduced the current. When, however, 50 mM NH₄⁺ was applied in the presence of 2-APB, the current inhibition was reversed. Most likely the NH₄⁺-mediated relief of block reflects cytoplasmic alkalization counteracting the 2-APB-induced acidification. Figure 1E depicts a summary bar graph comparing the degree of TRPM7 current inhibition by 10, 100, and 300 μM 2-APB for low and high HEPES-containing internal solutions and in the presence of NH₄⁺. We conclude from these experiments that extracellular 2-APB likely alters the pH inside the Jurkat T cell and this effect is counteracted by the inclusion of high concentration of pH buffer or by extracellular NH₄⁺. Previously we showed that increased internal HEPES reduced the effect of extracellular propionate, which inhibits TRPM7 channels by cytoplasmic acidification. Elevated intracellular HEPES concentration did not affect the potency of channel block by spermine, which is voltage-dependent and involves the channel pore.

It is worth noting that we did not observe activation of other TRP channels by 2-APB, such as TRPV1 and TRPV2, reportedly expressed in lymphocytes (reviewed in ref. 30). Since 2-APB is a cell-permeant compound due to its lipophilic nature, it can potentially block TRPM7 channels from the external or internal faces of the membrane. Access of channels from the inside could explain the slow onset of channel inhibition compared with spermine block, which acts from outside (Fig. 1B and C). In order to determine which side of the membrane 2-APB acts on, we included 100 μM 2-APB, a concentration sufficient to inhibit TRPM7 channels from outside, to the pipette solution. Figure 2 shows that TRPM7 channel current developed normally, when exposed to internal 2-APB, showing no apparent reduction in expected maximum amplitude. After the current had developed fully, we added the same concentration of 2-APB in the bath and this caused a significant inhibition of the current with a slow onset (n = 5 cells). From this experiment we conclude that 2-APB most likely inhibits TRPM7 by interacting with a target located on the exterior of the cell, as was found for TRPM2 channels.

In order to directly test whether 2-APB is capable of changing cytosolic pH from the outside, we loaded Jurkat T cells with the pH-sensitive fluorescent dye BCECF-AM. Figure 3A shows a time course of ratiometric measurements of BCECF fluorescence at two excitation wavelengths, 440 and 490 nm. Application of 10 μM did not alter the cell pH noticeably. Increasing 2-APB concentration to 100 μM, however, resulted in a significant drop in the ratiometric signal, corresponding to cytoplasmic acidification. This effect was partially reversible upon washout of the drug (Fig. 3A). Calibrations conducted with nigericin and high K⁺ solutions in the group of cells exposed to 2-APB (Fig. 3A), showed that the pH achieved in presence of 10 and 100 μM 2-APB was 7.25 and 6.91 respectively (Fig. 3B and C). The pH value of 6.91 is expected to be sufficient to reduce TRPM7 channel current magnitude, since its IC₅₀ of proton inhibition is pH 6.3. It should be noted however that measurements shown in Figure 3 describe an average value of pH over the whole cytoplasm, whereas TRPM7 channels likely sense the pH near the plasma membrane. In Figure 3D application of 300 μM and 900 μM 2-APB to Jurkat T cells produced increased acidification with increased concentration of the compound. Removal of 2-APB resulted in partial reversal of acidification. We also performed nigericin calibrations in the presence of 100 μM 2-APB and found no evidence of quenching of the BCECF signal by this compound (n = 45 cells, data not shown). In summary, 2-APB at concentrations of 100–900 μM acidifies the cytoplasm of Jurkat T cells.

**Discussion**

In the present report we demonstrate that in Jurkat T lymphocytes, TRPM7 channels are dose-dependently inhibited by 2-APB at concentrations of 10–300 μM through a cytoplasmic acidification mechanism. The extent of inhibition did not depend on voltage, as previously reported by others, and displayed a relatively slow onset. Inhibition was observed when the internal solution contained 1 mM HEPES (Fig. 1A and B). Increasing HEPES concentration to 140 mM greatly diminished 2-APB-induced inhibition at all tested concentrations, suggesting that cytoplasmic pH change is responsible for the 2-APB effect (Fig. 1C and E). In agreement with this, application of NH₄⁺ in the presence of 2-APB relieved channel inhibition (Fig. 1D). Simply raising the pH of the internal low HEPES solution to 7.6 was sufficient to substantially diminish the inhibitory effect of 100 μM 2-APB (n = 3 cells, data not shown). Intracellular pH measurements in BCECF-loaded Jurkat T lymphocytes showed that at 100–900 μM, 2-APB potently and reversibly acidifies the cytoplasm (Fig. 3). Ten micromolars of 2-APB, which did not inhibit TRPM7 channel currents measurably (Fig. 1), was also ineffective in lowering cellular pH (Fig. 3A). We therefore conclude that 2-APB inhibits TRPM7 channels indirectly, by acidifying TRP channels by 2-APB, such as TRPV1 and TRPV2, report other channels. It is worth noting that we did not observe activation of other TRP channels by 2-APB, such as TRPV1 and TRPV2, reportedly expressed in lymphocytes (reviewed in ref. 30). Since 2-APB is a cell-permeant compound due to its lipophilic nature, it can potentially block TRPM7 channels from the external or internal faces of the membrane. Access of channels from the inside could explain the slow onset of channel inhibition compared with spermine block, which acts from outside (Fig. 1B and C). In order to determine which side of the membrane 2-APB acts on, we included 100 μM 2-APB, a concentration sufficient to inhibit TRPM7 channels from outside, to the pipette solution. Figure 2 shows that TRPM7 channel current developed normally, when exposed to internal 2-APB, showing no apparent reduction in expected maximum amplitude. After the current had developed fully, we added the same concentration of 2-APB in the bath and this caused a significant inhibition of the current with a slow onset (n = 5 cells). From this experiment we conclude that 2-APB most likely inhibits TRPM7 by interacting with a target located on the exterior of the cell, as was found for TRPM2 channels.

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Figure 1. The extent of inhibition of TRPM7 channels by extracellular 2-APB depends on cytosolic pH buffering. Jurkat T cells were dialyzed with Mg²⁺-free internal solution containing 1 mM HEPES which led to activation of TRPM7 channels. (A) Monovalent TRPM7 current-voltage relations obtained from voltage ramps in the absence and presence of 100 μM 2-APB (100th voltage ramp after drug application). Reduction in current magnitude is voltage-independent. (B) Time course of development and inhibition of TRPM7 current in the same cell as in A. Zero indicates the time of break-in. 2-APB was applied after ~10 min of dialysis with the Mg²⁺-free pipette solution containing 1 mM HEPES. Symbols correspond to measurements of TRPM7 current amplitude taken at -100 mV every 2.5 sec. 2-APB induced a slow reduction in current magnitude which was almost fully reversed upon washout of the compound. (C) Time course of TRPM7 current development with Mg²⁺-free internal solution containing 140 mM HEPES. 300 μM 2-APB was applied for the duration indicated by the horizontal bar on top, followed by addition of TRPM7 channel blocker spermine (10 μM) in presence of 2-APB. Note that in this cell TRPM7 current was pre-activated at break-in. (D) Inhibition of TRPM7 channels by 100 μM 2-APB is relieved by ammonium. One hundred micromolars of 2-APB was applied as indicated by the horizontal bar and elicited pronounced inhibition of TRPM7 current. Subsequent perfusion of 100 μM 2-APB + 50 mM NH₄⁺ resulted in recovery from inhibition. (E) Summary of experiments similar to those shown in (A–D) from multiple cells. Percent inhibition was calculated by dividing the current amplitude measured at ramp number 100 after the start of 2-APB application by the current amplitude measured immediately before. Each of bars in light color shows the extent of current inhibition with 1 mM HEPES in the pipette and the indicated extracellular concentration of 2-APB and NH₄⁺. Inclusion of 140 mM HEPES in the recording pipette (dark gray bars) prevented significant reductions of TRPM7 current amplitude. Numbers above the bars indicate the number of cells tested. Cells exposed to spermine were not included in the analysis. For cells treated with 2-APB+NH₄⁺, the current amplitude at 70th ramp after NH₄⁺ application was divided by current amplitude measured immediately before 2-APB application. Data represent mean ± SEM. Relevant significantly different pairs are shown by asterisk. *p < 0.001.
In the plasma membrane. Normally, NHE1 extrudes protons sensitive sodium-proton exchanger isoform 1 (NHE1), expressed molecules responsible for T-cell pH homeostasis is the amiloride-govern the steady-state pH in the cell. One of the most studied reported and may involve proteins in the plasma membrane that TRPM7 channels.

It is unlikely that 2-APB acidifies the cytoplasm by blocking NHE1, however, because this Na+-H+ antiporter is thought to require the presence of Na+ on an extracellular site for its function, whereas our recordings were done after extracellular Na+ was replaced with Cs+. It is thought that NHE1 is unable to utilize extracellular Cs+ in place of Na+ for pumping out H+ ions but can use Li+. Accordingly, application of 10 μM ethyl isopropyl amiloride (EIPA), an NHE blocker, did not inhibit TRPM7 currents (n = 3 cells, not shown). It is probable then that other ion transporters in the plasma membrane respond to 2-APB and acidify the cytoplasm. We did not investigate such possibilities in the current study. We did not see any evidence for activation by 2-APB of an electrogenic ion transport pathway or any additional ion conductances when TRPM7 was blocked (see Fig. 1C). Therefore, the ion transport mechanism affected by 2-APB is likely to be electroneutral. Future investigations involving high-throughput genome-wide screens will allow the identification of the molecule(s) responsible for 2-APB-induced pH change.

2-APB has been shown to activate several TRP channels, and in view of their intracellular pH sensitivity, it is possible that acidification effects participate in 2-APB actions. Caution should be exercised when interpreting 2-APB blocking and activating effect on native Orai1 and Orai3 store-operated Ca2+ channels since other pH-sensitive conductances in cellular membrane may also be modified by this compound. For example, Kv1.3 potassium channels, which are highly expressed in T lymphocytes, are known to be inhibited by intracellular acidification. Thus, inhibition of store-operated Ca2+ entry by 2-APB in these cells may also be accompanied by inhibition of Kv1.3 and this would result in a diminished driving force for Ca2+ entry into the cell. In such cases 2-APB would reduce the cytoplasmic Ca2+ levels more drastically than would be expected if it only affected Orai channels.

In studies where 2-APB blockade of TRP channels has been investigated in detail, it has been reported to involve direct interaction with the channel protein. Thus, for TRPC5 channels 2-APB acts from the extracellular but not intracellular face of the membrane. The block showed voltage-dependence, indicating that the drug may directly bind to the channel protein and affect the ion conduction pathway. For connexin channels, 2-APB blocks various subtypes (Cx36, Cx43, Cx50) with differential affinities. The primary effect at the single-channel level is a reduced probability of opening without significant reduction in unitary conductance. Importantly, the differential sensitivity of various connexins to 2-APB was shown to be unrelated to their sensitivity to pH. As for TRPM7 and TRPC5, 2-APB blocks connexins only from the extracellular side of the membrane.

2-APB block of SERCA pumps is isoform-specific, as it is for connexins, and involves decreased affinity for Ca2+ and diminished phosphoryl transfer from ATP. For Ins(1,4,5)P3 receptors, 2-APB acts as an allosteric inhibitor at high micromolar concentrations. In other cases, 2-APB also acts from the extracellular side of the membrane, e.g., ref. 40. For store-operated Ca2+ channels in platelets and overexpressed Orai channels, 2-APB effect appears to be direct and involves specific transmembrane segments. Modifications of this compound in the pursuit of highly specific agonists for Orai channels need to also be screened against their effects on intracellular pH since many diverse ion transport proteins are known to be pH-dependent.

Physiologic studies of ion channel function routinely use 2-APB concentrations that fall in the range where it acidifies the cytoplasm. Even though we have not conducted detailed studies in other cell types, it is expected that 2-APB-induced acidification mechanism is general and not restricted to Jurkat T cells, since we observed a similar effect of 2-APB in HEK293 cells (data not shown). It is likely, however, that the extent of acidification induced by 2-APB will depend on the particular cell type. Thus, in vascular smooth muscle cells, TRPM7-mediated
Mg\(^{2+}\) influx was reduced by 10 μM 2-APB,\(^{20}\) a concentration that neither blocked TRPM7 channels nor measurably acidified Jurkat T cells (see Figs. 1 and 3).

In order to separate the specific effects of 2-APB on ion channels from the drug's global cellular acidification effect, we suggest to use the simple maneuver of including high concentrations of pH buffers in internal solutions as we have done in Figure 1. We note that the choice of the particular pH buffer will depend on the properties of the ion channel studied; thus, anion channels may not be permeable to HEPES etc. Obviously, this approach will only be feasible in patch-clamped but not intact cells. In essence, the present study adds a new twist to the decade-long interpretation of results from studies involving this fascinating compound.

**Materials and Methods**

**Cell culture.** Human leukemic Jurkat T lymphocytes were the kind gift of Dr T. L. Brown, Wright State University. Cells were
maintained at 37°C in a CO₂ incubator (NAPCO 8000, Thermo Scientific) and grown in RPMI-1640 medium (Lonza) and 10% fetal bovine serum (BioWest). Cells were passaged twice a week by diluting the cell suspension in the culture medium.

**Patch-clamp electrophysiology.** Whole-cell patch clamp was used for recording Jurkat T-cell TRPM7 channels as previously described. Briefly, TRPM7 (Mg²⁺-inhibited cation) currents were recorded with Mg²⁺-free pipette solution which contained (in mM): 112 Cs glutamate, 8 NaCl, 0.09 CaCl₂, 12 EGTA, 1 HEPES, pH 7.3. The high-HEPES internal solution contained (in mM) 140 HEPES, 8 NaCl, 0.09 CaCl₂, 12 EGTA, pH 7.3 with CsOH. The extracellular (bath) solution was composed of (in mM) 136 Cs aspartate, 7.5 CsCl, 10 HEDTA, 10 HEPES, pH 7.3. In divalent cation-free external solutions the current-voltage relation of TRPM7 channels is semi-linear, reversing at around 0 mV (Figs. 1A and 2A). Cs⁺, L-glutamate and L-aspartate were used to minimize currents through potassium and chloride channels expressed in Jurkat T cells. The Cs⁺-based extracellular solution also prevented contamination by calcium-release activated Ca²⁺ currents, which are almost impermeable to Cs⁺.

Whole-cell recordings were performed using the computer-driven EPC-10 patch-clamp amplifier (HEKA Elektronik) and PatchMaster (v. 2.6) software. Instantaneous current-voltage relations were obtained from 10 ms duration command voltage steps to -100 mV followed by ramps of 200 ms duration spanning -100 mV to +85 mV, applied every 2.5 sec. Currents were sampled at 5 kHz and low-pass filtered at 2.9 kHz. Monovalent TRPM7 current amplitudes were measured at -100 mV to +85 mV, applied every 2.5 sec. Currents were measured at -100 mV and usually reached a maximum 3–6 min after break-in.

Two hundred millimolar stock solution of 2-Aminoethyl diphenyl borinate or 2-Aminoethoxy diphenyl borate (2-APB) (Sigma-Aldrich, D9754) was prepared in dimethyl sulfoxide (DMSO) (Fisher Scientific, BP231-100), and aliquots kept frozen. 2-APB was diluted to the desired concentration in the extracellular solutions on the day of the experiment. Ten millimolar L-aspartate were used to minimize currents through potassium channels expressed in Jurkat T cells. The Cs⁺-based extracellular solution also prevented contamination by calcium-release activated Ca²⁺ currents, which are almost impermeable to Cs⁺.

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**Single-cell pH measurement.** For intracellular pH imaging experiments, 35 mm glass-bottom imaging chambers were used with solution volume of ~1 ml. Jurkat T cells were seeded in these chambers after coating with poly-L-lysine and incubated in presence of 4 μM BCECF-AM ester (Invitrogen, B3051) and 0.02% pluronic F-127 (Sigma-Aldrich, 2443) for ~45 min at room temperature. The fluorescent dye-containing solution was then aspirated and replaced with normal external solution composed of (in mM): 2 CaCl₂, 4 KCl, 160 CsCl, 10 HEPES-Na⁺, 10 glucose, pH 7.3. The imaging chambers with attached cells were then mounted on the movable stage of an inverted microscope (Olympus). pH in individual cells was ratiometrically measured by illuminating cells in a selected field every 5 sec at 490 and 440 nm wavelengths using a Lambda 10B shutter and filter wheel (Sutter Instrument). Fluorescence was measured at 535 nm. The light source was a 175 W Xenon lamp (QED). Images were taken with PixelFly CCD camera (PCO Imaging) and InCyt1M 2 software (Intracellular Imaging). Emitted light intensities averaged for individual cells in the imaging field were plotted against time using Origin. Solutions in the imaging chamber were exchanged with a syringe-driven perfusion system. The high K⁺ pH calibration solution contained 130 mM KCl, 20 mM NaCl, 1 mM CaCl₂, 0.5 mM MgSO₄, 1 mM NaH₂PO₄, 10 mM HEPES, 5 mM dextrose. The ionophore nigericin (sodium salt, Calbiochem, 481990) was added to this solution at 10 μM final concentration and pH adjusted to values indicated in Figure 3. The ratio value was taken when pH achieved a steady-state in presence of nigericin. pH calibrations were performed in the same groups of cells after experiments with 2-APB applications were completed.

In one experiment, 2-APB effect was studied using a bathing solution containing NaCl instead of CsCl and yielded identical results (31 cells, data not shown).

All imaging and electrophysiology experiments were performed at room temperature. Salts were purchased from Sigma-Aldrich or Acros.

**Statistical analysis.** Results are presented as mean ± SEM. Significant differences were assessed by non-parametric independent samples Kruskal-Wallis test performed among all six groups in Figure 1E and Tamhane’s T2 test for post-hoc pairwise comparisons. Only cells exposed to 10, 100 and 300 μM 2-APB for durations longer than 100 voltage ramps, corresponding to approximately 4.4 min, were included in the analysis shown in Figure 1E.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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