2-Aminoethoxydiphenyl borate is a common activator of TRPV1, TRPV2, and TRPV3

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Running title: Activation of TRPV1, V2, and V3 by 2APB

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SUMMARY:

The transient receptor potential (TRP) superfamily contains a large number of proteins encoding cation permeable channels that are further divided into TRPC (canonical), TRPM (melastatin) and TRPV (vanilloid) subfamilies. Among the six TRPV members, TRPV1, V2, V3, and V4 form heat-activated cation channels, which serve diverse functions ranging from nociception to osmolality regulation. Although chemical activators for TRPV1 and V4 are well documented, those for TRPV2 and V3 are lacking. Here we show that in the absence of other stimuli, 2-aminoethoxydiphenyl borate (2APB) activates TRPV1, V2, and V3, but not TRPV4, V5, and V6 expressed in HEK293 cells. In contrast, 2APB inhibits the activity of TRPC6 and TRPM8 evoked by 1-oleoyl-2-acetyl-sn-glycerol and menthol, respectively. In addition, low levels of 2APB strongly potentiate the effect of capsaicin, protons, and heat on TRPV1 as well as that of heat on TRPV3 expressed in *Xenopus* oocytes. In dorsal root ganglia neurons, supra-additive stimulations were evoked by 2APB and capsaicin or 2APB and acid. Our data suggest the existence of a common activation mechanism for TRPV1, V2, and V3, which may serve as a therapeutic target for pain management and treatment for diseases caused by hypersensitivity and temperature misregulation.
INTRODUCTION:

The transient receptor potential (TRP) superfamily of cation channels consists of a large number of recently identified molecules that share sequence homology with the *Drosophila* protein named after a phototransduction mutant called *trp*. According to sequence similarities, the TRP channels are further divided into subfamilies, such as TRPC (canonical), TRPM (melastatin), and TRPV (vanilloid) [see reviews in ref. 1,2]. These channels are involved in diverse cellular functions including receptor and store-operated Ca\(^{2+}\) entry [3], Ca\(^{2+}\) transport [4,5], trace metal detection [6], and temperature [7-9] and osmolality [10,11] sensations. The activation mechanisms for most of the TRP channels remain to be elucidated. Specific ligands have been found for TRPC3, C6, C7, V1, V4, M2, M4, M5, M7, and M8. These include endogenous substances, such as lipids (diacylglycerol [12], anandamide [13,14], and phosphatidylinositol 4,5-bisphosphate [15]), nucleotides (ADP-ribose [16]), and calcium ions [17,18], as well as exogenous materials either synthesized or extracted from plants, e.g. capsaicin [7], menthol [19], and 4 α-phorbol 12,13-didecanoate (4αPDD) [20]. Although several blockers are also available, except for a limited number of TRPV1 antagonists [7,21], other TRP inhibitors are nonspecific. Ruthenium red blocks all TRPV channels [1,22]. 2-Aminoethoxydiphenyl borate (2APB) appears to block a number of TRPC and TRPM channels [1,22].

First reported as a membrane permeable inhibitor of inositol 1,4,5-trisphosphate receptors [23,24], 2APB was soon found to directly block native store-operated channels [25-27], SERCA pumps [28], mitochondrial permeability transition pore [29] and a few other ion channels [30]. The mechanism of action for 2APB is likely to be complex. In
addition to inhibition, low concentrations of 2APB enhanced the activity of store-operated channels [26]. At greater than 50 μM, 2APB activated a Ca^{2+} permeable non-selective cation channel with a 50 pS single channel conductance and very low open probability in rat basophilic leukemia (RBL) cells [31].

2APB has been perceived as a general inhibitor of TRP channels [1]. However, except for TRPC3 [24,32], the effects of this drug were often examined on presumptive endogenous TRP channels [33-35], of which the molecular compositions are uncertain. It is possible that an unknown subunit confers the 2APB sensitivity of the native channels. Therefore, it is important to confirm the effects of 2APB on heterologously expressed TRP channels. Here, we used TRPC6, TRPM8 and TRPV1 to represent each of the three major TRP subfamilies and examined the effects of 2APB on their activities. We confirmed that TRPC6 and TRPM8 were inhibited by 2APB. However, to our surprise, 2APB activated TRPV1 expressed in HEK293 cells and in *Xenopus* oocytes. In rat dorsal root ganglion (DRG) neurons, 2APB elicited currents that were potentiated by capsaicin or low pH. Finally, we showed that 2APB also activated TRPV2 and V3 and therefore is a common activator of three TRPV channels.

**EXPERIMENTAL PROCEDURES:**

DNA constructs, cell culture, and transfections: cDNA for murine TRPC6 was cloned as previously described [36]. cDNAs for murine TRPV1 (GenBank Acc# AY452083) and TRPM8, murine TRPV3, and human TRPV4 were isolated from total RNA prepared from mouse DRG, mouse skin, and human endothelial cells, respectively, by RT-PCR using oligonucleotide primers designed based on published sequences. The correctness
of the cDNAs was confirmed by DNA sequencing. cDNAs for rat TRPV1, murine
TRPV2, rat TRPV5 and TRPV6 were kindly provided by Drs. M. Caterina, M. Kanzaki,
and J. Peng. For expression in HEK293 cells and intracellular Ca\(^{2+}\) measurements, the
cDNAs were subcloned in pcDNA3.

HEK293 cells were grown in Dulbecco’s minimal essential medium (DMEM)
containing 4.5 mg/ml glucose, 10% heat-inactivated fetal bovine serum, 50 units/ml
penicillin, and 50 µg/ml streptomycin. For intracellular Ca\(^{2+}\) measurements, cells were
transfected with the desired DNA constructs in the wells of 96-well plates without pre-
seeding using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the
protocol provided by the manufacturer. To prevent cell loss from subsequent washing,
the wells were treated with 20 µg/ml polyornithine (MW >30,000, Sigma, St Louis, MO,
USA) for >15 min and rinsed once with Hank’s balanced salt solution (HBSS) without
Mg\(^{2+}\) and Ca\(^{2+}\). For each well, the plasmid DNA (25 ng) and Lipofectamine 2000 (0.4
µl) were mixed in 50 µl OptiMEM (Invitrogen) and added to the well before the addition
of 120,000 cells suspended in 100 µl of the medium without antibiotics. The cells were
incubated for 24-28 hrs without medium change. The transfection efficiency was about
70% as determined using an enhanced green fluorescence protein (EGFP) expression
vector. For whole-cell recordings, murine TRPV1, V3, and M8 were subcloned in the
bicistronic expression vector, pIRES2-EGFP (Clontech, Palo Alto, CA, USA). TRPC6
was subcloned in pEGFP-N1 (Clontech) and expressed as a GFP-fusion protein. The
transfections were performed in 35 mm dishes using Lipofectamine 2000. For expression
in *Xenopus* oocytes, murine TRPV1 and V3 were subcloned into the pAGA3 vector
(GenBank Acc# AY452085).
Whole cell recordings of HEK293 cells: Transfected HEK293 cells were reseeded in 35 mm dishes one day after the transfection. Whole cell recordings were performed in the following day. Recording pipettes were pulled from micropipette glass (World Precision Instruments Inc, Sarasota, FL, USA) to 2-4 MΩ when filled with a pipette solution containing (in mM): 140 CsCl, 0.6 MgCl₂, 1 EGTA, 10 Hepes, pH 7.20 and placed in the bath solution containing (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 Hepes, pH 7.40. Isolated cells were voltage-clamped in the whole-cell mode using an EPC9 (HEKA Instruments Inc, Southboro, MA, USA) amplifier. For TRPC6, the bath solution was changed to an external solution containing (in mM): 160 NaCl, 7.2 N-methyl-D-glucamine, 10 Hepes, pH 7.4, after the establishment of the whole-cell configuration to facilitate the detection of the TRPC6 currents. Voltage commands were made from the Pulse+Pulse Fit program (version 8.53, HEKA) and currents were recorded at 5 kHz. Voltage ramps of 100 ms to +100 mV after a brief (20 ms) step to –100 mV from holding potential of 0 mV were applied every 0.5 s. Cells were continuously perfused with the bath solution through a gravity-driven multi-outlet device with the desired outlet placed about 50 µm away from the cell being recorded. Stock solutions of 2APB, N-arachidonyl dopamine, capsaicin, capsazepine, menthol, 1-oleoyl-2-acetyl-sn-glycerol (OAG), and resiniferatoxin were made in dimethyl sulfoxide (DMSO). Ruthenium red was dissolved in water. Drugs were diluted in the appropriate external solutions to the desired final concentrations and applied to the cell through perfusion. The acidic solution used for TRPV1 contained (in mM): 140 NaCl, 5 KCl, 2
CaCl$_2$, 1 MgCl$_2$, 10 Glucose, 10 MES, pH 6.50. All whole-cell experiments were performed at the room temperature (20-24°C).

**Intracellular Ca$^{2+}$ measurements:** Transiently transfected HEK 293 cells in 96-well plates were washed once with an extracellular solution (ECS) containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$, 10 mM glucose, 0.1% bovine serum albumin, and 15 mM Hepes, pH 7.4 and then incubated in 50 µl ECS supplemented with 2 µM fluo4/AM and 0.05% Pluronic F-127 (both were from Molecular Probes, Eugene, OR, USA) at 37°C for 60 min. Probenecid (2 mM) was included in all solutions to prevent the leakage of fluo4 from the cells. At the end of the incubation, cells were washed three times with ECS and placed in 80 µl of the same solution. Intracellular Ca$^{2+}$ was measured using a fluid handling integrated fluorescence plate reader, Flex Station (Molecular Devices, Sunnyvale, CA, USA). 2APB and other drugs were diluted into ECS at 3x the desired final concentrations and delivered to the sample plate by the integrated robotic 8-channel pipettor at the preprogrammed time points. The fluo4 fluorescence was read at excitation of 494 nm and emission of 525 nm from the bottom of the plate at 0.67 Hz. All experiments were performed at 32°C unless indicated otherwise.

cRNA synthesis and expression in *Xenopus* oocytes: TRPV1 and V3 in the pAGA3 vector were linearized using HindIII. cRNAs were synthesized using mMessage mM Machine reagents and protocols obtained from Ambion (Austin, TX, USA). The resulting cRNAs were dissolved in diethylpyrocarbonate-treated H$_2$O. Sexually mature female *Xenopus* laevis of older than 2.5 years of age were purchased from Xenopus I,
Inc. (Dexter, MI, USA). The frogs were quarantined for at least two weeks before being used. For oocyte isolation, small pieces of ovarian lobe were dissected out from anesthetized frogs and shaken gently at 19°C for 90 min in a solution containing (in mM) 82.5 NaCl, 2 KCl, 1 MgCl₂, 5 Hepes, pH 7.4, and supplemented with 1 mg/ml collagenase (Worthington Biochem, Lakewood, NJ, USA). Denuded, healthy-looking oocytes of more than 1 mm in diameter were selected and injected in a volume of 50 nl/cell with a total of 5 ng cRNA. The injected oocytes were incubated at 19°C for 2-5 days in sterile Barth's saline (in mM, 88 NaCl, 1 KCl, 0.41 CaCl₂, 0.33 Ca(NO₃)₂, 0.82 MgSO₄, 2.4 NaHCO₃, 7.5 Tris-HCl, pH 7.6, supplemented with 20 U/ml penicillin and 20 mg/ml streptomycin). The solution was changed daily.

Two-electrode voltage clamp: Oocytes were placed in a 50 µl chamber, which was perfused with a bath solution containing (in mM): 100 NaCl, 2.5 KCl, 1 MgCl₂, 1.5 EGTA, 5 Hepes, pH 7.4. The cell was impaled with two intracellular glass electrodes filled with 3 M KCl connected to a TEV-700 Two Electrode Voltage Clamp Workstation (Warner Instruments, Hamden, CT, USA). The oocytes were clamped at –40 mV and currents were continuously recorded using a chart recorder (Astro Med, Inc., West Warwick, RI, USA) and at the same time digitized at 100 Hz using a PowerLab Data Acquisition System (ADInstruments, Colorado Springs, CO, USA). The low pH solutions were made of (in mM) 100 NaCl, 2.5 KCl, 1 MgCl₂, 1.5 EGTA, 5 MES, adjusted to the desired pH with NaOH. 2APB, capsaicin, and other drugs were dissolved in the bath solution at the desired final concentrations and applied to the cells by perfusion. Temperature changes were made using a CL-100 Bipolar Temperature
Controller connected to a SC-20 Dual In-line Solution Heater/Cooler (Warner Instruments).

Isolation and culture of rat DRG neurons: Male Sprague-Dawley rats (150–220 g) were anaesthetized with 4% halothane in air and decapitated. DRG (T1-T10) were extracted under a dissecting microscope and placed in ice-cold DMEM/F12 solution. Each ganglion was desheathed, cut into ∼10 pieces, placed in 0.125% type IV collagenase, and incubated in a humidified chamber for 1 hour in 5% CO2 in air at 37°C. The ganglion suspension was centrifuged (150 × g, 5 min) and supernatant aspirated. The ganglion pellet was resuspended in 0.05 % trypsin and 0.53 mM EDTA in HBSS, incubated for 5 min and centrifuged (150 × g, 5 min). The ganglion pellet was then resuspended in a modified DMEM/F12 (DMEM/F12 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µM MEM non-essential amino acids) and gently triturated with a small-bore fire-polished Pasteur pipette. The dispersed cell suspension was centrifuged (500 × g, 8 min) through a layer of 15% bovine serum albumin to separate the cells from the myelin debris. The pellets were resuspended in the modified DMEM/F12 solution supplemented with 50 ng/ml 2.5S-nerve growth factor and plated onto poly-L-lysine-coated glass coverslips, and then incubated overnight (5% CO2 in air at 37°C).

Whole-cell perforated patch-clamp recording of DRG neurons: The coverslip containing the attached cells were centered in a small-volume (0.2 ml) perfusion chamber, which was perfused by gravity feed with 2APB, capsazepine, ruthenium red, or vehicle (extracellular...
solution) at 2 ml/min, while the chemical stimulants (2APB, capsaicin, and acid) were delivered by a pressure-driven perfusion system (ALA-VM8, ALA Scientific Instruments, Westbury, NY, USA), with its tip positioned to ensure that the cell was fully within the stream of the perfusate. The extracellular solution consisted of (in mM): 136 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 0.33 NaH₂PO₄, 10 glucose, 10 Hepes, pH 7.4. The intracellular solution contained (in mM): 92 potassium gluconate, 40 KCl, 1 CaCl₂, 0.5 MgCl₂, 10 EGTA, 10 Hepes, pH 7.2. Recordings were made in the whole cell perforated patch configuration (gramicidin 100 µg/ml) using Axopatch 200B/pCLAMP9 (Axon Instruments, Union City, CA, USA). Experiments were performed at room temperature (20-24°C). Data were acquired at 5 kHz and filtered at 2 kHz. Series resistance was compensated at ~80%. Membrane potential was held at -70 mV. Data were collected from only one cell per dish to avoid possible drug contamination of the cells.

RESULTS:

Differential effects of 2APB on TRP channels

In transiently transfected HEK293 cells, the OAG (10 µM)-evoked TRPC6 currents and the menthol (100 µM)-elicited TRPM8 currents were inhibited by 2APB in a dose-dependent manner (Fig. 1A&B). When activated, both channels gave rise to outwardly rectifying currents, which reversed near 0 mV. 2APB blocked both the inward and the outward currents with the blockage of the inward current being slightly more effective. The TRPC6 currents were incompletely blocked by 300 µM 2APB to 47.6 ± 6.7% at -100 mV and 35.4 ± 4.3% at +100 mV of the control response (n = 5). The IC₅₀ values were 10.4 ± 2.5 µM at -100 mV (Hill coefficient, n₀ = 1.0 ± 0.3, n = 5) and 13.9 ± 2.3 µM at
+100 mV (nᵦ = 1.1 ± 0.2). The TRPM8 currents were abolished by 300 µM 2APB. The IC₅₀ values were 7.7 ± 2.2 µM (nᵦ = 0.9 ± 0.2, n = 5) and 11.6 ± 1.2 µM (nᵦ = 1.2 ± 0.2) at −100 mV and +100 mV, respectively. In contrast to TRPC6 and TRPM8, TRPV1 was dose-dependently activated by 2APB in the absence of any TRPV1 agonists (Fig. 1C). At 0.3-1.0 mM, 2APB evoked a current that was comparable to that elicited by 1 µM capsaicin, both in current amplitudes and the shape of the current-voltage (I/V) curves. The EC₅₀ values of 2APB for TRPV1 activation were 197 ± 13 µM at −100 mV (nᵦ = 1.8 ± 0.1, n = 6) and 130 ± 17 µM at +100 mV (nᵦ = 1.5 ± 0.2).

The response to 2APB was also detected in *Xenopus* oocytes injected with the cRNA for mouse TRPV1 (Fig. 2). Defolliculated *Xenopus* oocytes were injected with TRPV1 cRNA and TRPV1 activity was measured 2-4 days later using two-electrode voltage-clamp techniques. Cells were held at −40 mV and placed in a nominally Ca²⁺-free external solution to minimize the Ca²⁺-activated Cl⁻ conductance and the inactivation of TRPV1 channels. Bath application of 2APB elicited inward currents only in oocytes injected with the cRNA for TRPV1 but not in uninjected oocytes or oocytes injected with the cRNA for an inactive form of TRPV1 that lacks 10 amino acids at the N-terminus (GenBank Acc# AY452084). The 2APB-evoked currents were abolished by 3 µM ruthenium red (Fig. 2A&C). However, the competitive capsaicin antagonist, capsazepine, at 30 µM only blocked ~30% of the response elicited by 2APB, although it almost completely inhibited the response activated by 1 µM capsaicin (Fig. 2A-D). The weak effect of capsazepine on 2APB-induced currents was not unexpected because for the mouse TRPV1, the antagonist was also very weak at inhibiting the currents elicited by acid or heat (data not shown). Similarly poor inhibitions of acid- and heat-induced
responses by capsazepine were found for rat but not human TRPV1 channels [37].

Together, the above data demonstrate that 2APB is an activator of TRPV1.

**Potentiation of TRPV1 responses by 2APB**

TRPV1 is a polymodal sensor responsive to multiple stimuli such as, heat, protons, and the principal pungent ingredient of chilli peppers, capsaicin [7,21]. When expressed in HEK293 cells, TRPV1 is partially activated by low concentrations of 2APB (100 µM) or capsaicin (0.3 µM). When added together, these drugs caused a large increase in TRPV1 activity (Fig. 3A). The average increase was 9.0 ± 2.0 fold at +100 mV and 32 ± 11 fold at –100 mV (n = 7) as compared to the currents elicited by 2APB alone. Similarly, the response to 2APB was strongly potentiated by weak extracellular acidification to pH 6.5, which by itself had a very small effect on TRPV1. The average increases evoked by 2APB at pH 6.5 were 25.0 ± 6.0 and 21.0 ± 8.0 fold (n = 6) at +100 and –100 mV, respectively, as compared to the current elicited by 2APB at pH 7.4 (Fig. 3B). For the transiently transfected HEK293 cells, the response to the low concentration of 2APB as well as to that of capsaicin or protons was quite variable, presumably because of differences in the levels of channel expression. Noticeably, there was a negative association between the initial response to 2APB and the increase that was elicited by the co-application with the low level of capsaicin or weak acid. Cells that responded weakly to 2APB, which also had weak responses to capsaicin or acid, typically displayed more potentiation than those that responded strongly (Fig. 3C).

The effect of 2APB on capsaicin- and acid-induced activation and that of capsaicin and acid on 2APB-evoked activation of TRPV1 were studied in more detail in *Xenopus*...
oocytes. As shown in Fig. 4A, 100 µM 2APB strongly potentiated the response induced by 0.1 and 0.3 µM capsaicin. At this 2APB concentration, the dose response curve for capsaicin was shifted to the left with the EC$_{50}$ value changed from 1.34 ± 0.12 µM ($n_h = 2.0 ± 0.3$) in the absence of 2APB to 0.35 ± 0.08 µM ($n_h = 1.2 ± 0.3$) in the presence of 2APB. Inversely, 0.3 µM capsaicin caused a left shift of the dose response curve for 2APB (Fig. 4B) with a decrease in the EC$_{50}$ value from 315 ± 13 µM ($n_h = 2.27 ± 0.24$) to 34 ± 2 µM ($n_h = 0.92 ± 0.05$). Likewise, the EC$_{50}$ value for protons to activate TRPV1 changed from pH 5.220 ± 0.001 ($n_h = 1.37 ± 0.02$) in the absence of 2APB to pH 6.036 ± 0.005 ($n_h = 1.00 ± 0.01$) in the presence of 100 µM 2APB (Fig. 4C). The EC$_{50}$ value of 2APB also decreased from 322.0 ± 0.8 µM ($n_h = 3.10 ± 0.04$) at pH 7.5 to 159.1 ± 5.9 µM ($n_h = 1.60 ± 0.10$) at pH 6.5 (Fig. 4D). These results indicate that 2APB and the other two activators of TRPV1 (capsaicin and acid) sensitized effect of each other. Interestingly, with the presence of another stimulus, the Hill coefficient of the dose response curve to a given ligand was typically decreased to half of the original value when it was applied alone, suggesting that sensitization is accompanied with a decrease in cooperativity. In addition to the chemical activators, the response to 2APB was also enhanced by heat. At 40°C, the TRPV1 current evoked by 100 µM 2APB was about 9 times larger than that obtained at 22°C (Fig. 4E). Therefore, 2APB acts synergistically with other known activating factors of TRPV1 channels.

**Effect of 2APB on DRG neurons**

In order to examine its effects on native capsaicin receptors, we applied 2APB to acutely cultured rat DRG neurons. In capsaicin-sensitive cells held at –70 mV with
perforated patches, 2APB dose-dependently evoked an inward current (Fig. 5A), which was inhibited by 3 µM ruthenium red (Fig. 5B) and, to a lesser extent, 10 µM capsazepine (Fig. 5C). Although 30 µM 2APB alone did not activate any current, it significantly increased the current induced by 0.3 µM capsaicin (Fig. 6A). Similarly, the response to weak acid (pH 6.5) was also strongly increased in the presence of 30 and 100 µM 2APB (Fig. 6B). The potentiated responses were inhibited by 10 µM capsazepine (Fig. 6C&D). Therefore, like the heterologously expressed TRPV1, native channels in DRG neurons were activated by 2APB and this effect was augmented by capsaicin and protons.

Notably, the inhibition by capsazepine showed a clear difference depending on whether or not the channels were activated by capsaicin. The current elicited by 0.3 µM capsaicin plus 30 µM 2APB was totally inhibited whereas that activated by 300 µM 2APB alone or 100 µM 2APB in pH 6.5 was only blocked less than 60% by the antagonist (Fig. 5C and Fig. 6C&D). This, together with the result shown in Fig. 2, suggests that 2APB and capsaicin activate the TRPV1 channels using different mechanisms.

**Activation of TRPV1-3 by 2APB**

To determine the specificity of 2APB in the activation of TRPV channels, we examined its effect on the six known TRPV channels in transiently transfected HEK293 cells by measuring intracellular Ca\(^{2+}\) concentrations after loading the cells with fluo4/AM. As shown in Fig. 7A, 0.5 mM 2APB induced increases in fluo4 fluorescence in cells that expressed rat TRPV1, mouse TRPV1, V2, and V3. Small increases detected in cells expressing human TRPV4, rat TRPV5 and V6 were similar to that obtained in control cells transfected with the vector plasmid and thus represented the endogenous response to
2APB. Fig. 7B shows the specificity of cDNAs used in these experiments. For the same sets of transfections, both mouse and rat TRPV1, but not other TRPVs, were activated by resiniferatoxin and N-arachidonyl dopamine, two known activators of TRPV1 [7,38]. Only TRPV4 was activated by its selective activator, 4αPDD [20]. Cells transfected with TRPV5 and V6 had elevated basal fluorescence levels (not shown), consistent with them being constitutively active [4,5]. Therefore, among all TRPV channels, TRPV1-3, but not TRPV4-6, were activated by 2APB. Concentration response curves showed that in the Ca\textsuperscript{2+} assay performed at 32°C, the EC\textsubscript{50} values of 2APB were 114 ± 8, 129 ± 13, and 34 ± 12 µM (n = 3) for TRPV1, V2, and V3, respectively (Fig. 7C). These results demonstrated that 2APB is a common activator of TRPV1, V2, and V3. At 32°C, 2APB is more effective at stimulating TRPV3 than TRPV1 and V2.

2APB-induced TRPV2 and TRPV3 currents

2APB-evoked whole-cell currents were recorded in HEK293 cells that expressed TRPV2 and V3 at the room temperature. At 1 and 3 mM, 2APB evoked weakly rectifying currents that reversed at 0 mV in cells that expressed TRPV2 (Fig. 7D). Both the inward and outward currents were inhibited by 3 µM ruthenium red, but the inward current was blocked more strongly. For cells that expressed TRPV3, significant currents were elicited at 30-300 µM 2APB (Fig. 8E). The I/V curves revealed stronger outward and inward rectifications than TRPV2 and the reversal potential was also at 0 mV. These data established 2APB as the first known chemical activator of TRPV2 and TRPV3. The activation by 2APB appeared to require an extracellular binding site because infusion of up to 1 mM 2APB through the patch pipette for > 6 min failed to elicit any current (Fig.
8A, n = 5) in cells that expressed TRPV3. However, subsequent application of 2APB in the bathing solution elicited TRPV3 currents. Ruthenium red (3 µM) abolished the inward current evoked by 2APB and also caused a large increase in the outward current at potentials higher than 40 mV (Fig. 8A, I/V curves, trace d, gray). Similarly, intracellular injection of 40 mM 2APB did not activate TRPV1 expressed in *Xenopus* oocytes. Nor did it affect the response to the extracellular application of 2APB. The EC$_{50}$ values for the extracellularly applied 2APB were 249 ± 19 µM (n$_h$ = 1.9 ± 0.3, n = 7) and 216 ± 10 µM (n$_h$ = 2.4 ± 0.2 n = 6) for the uninjected cells and cells injected with 2APB, respectively. Note the oocytes used for these experiments were 7 days, instead of 4 days, after the cRNA injection. Therefore, the EC$_{50}$ values for 2APB are lower than those in Fig. 4 because of higher expression levels of the channels.

2APB (300 µM) activated TRPV3 expressed in *Xenopus* oocytes and the response was blocked by ruthenium red but not by capsazepine (Fig. 8B). Since TRPV3 is a heat-activated channel, we examined whether there was a synergistic effect between 2APB and heat on TRPV3 activity. Fig. 8C shows that although application of 100 µM 2APB at 22°C or increasing the bath temperature from 22 to 40°C (within 15 sec) evoked only small increases in the inward current, the same concentration of 2APB at 40°C evoked a large current increase, which was 35 ± 6 times (n = 14) that induced by 2APB at 22°C. The dose response curves for 2APB, as determined by the fluorescence measurements in fluo4-loaded cells, were also shifted to the left as the temperature increased from 24 to 32 and then to 37°C (Fig. 8D). Therefore, like the behavior of TRPV1, the 2APB-induced response of TRPV3 was potentiated by another activating factor, heat.
DISCUSSION:

TRPV1 is the founding member for the TRPV family, among which, TRPV1, V2, V3, and V4 form heat-activated channels responding to temperatures higher than 43, 52, 31, and 25 °C, respectively. TRPV1 is well-known for its contributions to acute thermal nociception and injury-elicited hyperalgesia [39,40]. TRPV1 channels are activated by heat, acid, and a large number of chemical stimuli including the vanilloid compounds and endogenous substances, such as anandamide, N-arachidonyl dopamine, and several lipoxygenase products [7,13,21,38,41]. TRPV4 is activated by 4αPDD and several epoxygenase products derived from arachidonic acid [14,20]. The use of chemical ligands has greatly facilitated the understanding of the structural and functional relationships as well as the physiological roles of TRPV1 and V4. Until recently, a similar tool had been lacking for TRPV2 and V3, although these channels are expected to share equal importance with TRPV1 in sensing warm to noxious heat and other functions related or unrelated to TRPV1. While the expression of TRPV2 in DRG and trigeminal ganglia is distinct from that of TRPV1 [8], TRPV3 was found to co-express and form heteromultimers with TRPV1 [9]. Our finding that 2APB is an activator of TRPV2 and TRPV3 should significantly help in the study of these channels.

To the best of our knowledge, other than heat, there is no common stimulus for TRPV channels. 2APB is the first chemical ligand now shown to activate TRPV1, V2 and V3. This suggests a common mechanism for the activation of these channels. Identification of the critical sequence region(s) involved in the 2APB-evoked responses will be necessary for understanding the mechanism. Our finding that intracellular application of 2APB in the HEK293 cells and the oocytes did not activate TRPV1 and
TRPV3 suggests that one or more extracellular sites must be important for activation by the drug. The quick washout of the 2APB effect in TRPV1, as compared to the slow washout of the capsaicin response, is consistent with the above hypothesis, which also agrees with the proposal that the 2APB binding site is located extracellularly for the inhibition of TRPC3 channels [32]. Notably, 2APB displayed selectivity for specific members of the TRPV subfamily. TRPV4, V5, and V6 are not activated by 2APB. Others found that 2APB slightly increased the constitutive activity of TRPV6 when it was overexpressed in HEK293 or RBL cells [42, 43]. However, at low expression levels, the store-operated channels formed by TRPV6 in the RBL cells were inhibited by 2APB [43].

While this article was under revision, another report appeared showing that 2APB not only activated TRPV3 heterologously expressed in HEK293 cells at the room temperature but also endogenous channels in mouse primary keratinocytes when it was combined with heating [44]. This is in agreement with our data on TRPV3. However, in contrast to our data on TRPV1 and V2, 2APB was reported to be ineffective on TRPV2 and very weak on activating TRPV1 [44]. The discrepancy may be explained by the different concentration ranges of 2APB used between the two studies. Our results clearly demonstrate that rather than being a specific agonist of TRPV3, 2APB is a common and strong activator of these TRPV channels.

The sensitivity to 2APB was greatly enhanced when it was combined with other stimuli for the TRPV channels. Inversely, the presence of 2APB also increased the sensitivity of the TRPV channels to other stimuli. This shift in sensitivity may explain the so-called “supra-additive effect” found for the different cues that activate TRPV1 and V4 [7, 45, 46]. Apparently, the presence of one kind of ligand reduced the cooperativity of the
channel to another stimulus, suggesting that the channel could be simultaneously occupied by two different ligands. However, direct competition between these ligands for binding to the same site(s) on the channel is unlikely since different mechanisms are involved in the activation of TRPV1 by vanilloids, protons, and heat [47-49] and the stimulation of TRPV4 by 4αPDD, heat, and cell swelling [50]. It is likely that the mechanism of channel activation by 2APB differs from that for capsaicin or protons because 2APB activates not only TRPV1, but V2 and V3 as well. On the other hand, the fact that heat and 2APB have the same potency order (V3>V1>V2) suggests that a similar mechanism may be involved for the activation by these two stimuli.

Our data show that in HEK293 cells, the diacylglycerol-activated TRPC6 currents were partially inhibited by 2APB. The effect of 2APB on TRPC3, a closely related channel, has been controversial with reports describing either a complete [24] or a partial [32] block of receptor-activated, but not diacylglycerol-stimulated TRPC3 activity [24]. In chicken B lymphocytes, TRPC3 was activated by 2APB, presumably through activation of a phospholipase C γ complex [51]. The inhibition of TRPM8 by 2APB is consistent with the blockade of menthol-elicited currents in DRG neurons [35]. Most importantly, we show that 2APB is not only an inhibitor of TRPC and TRPM channels but also an activator of TRPV1, V2, and V3. Whether or not 2APB has a common mechanism of action on each channel remains to be elucidated. Nevertheless, analogs of 2APB are attractive candidates for development of more selective agonists and/or antagonists for TRP channels, some of which may prove to be valuable in future studies for therapeutic use in pain management and treatments of disorders that might be related to dysfunctions of heat-activated TRPV channels.
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FOOTNOTE:

Abbreviations used: 2APB, 2-aminoethoxydiphenyl borate; 4αPDD; 4 α-phorbol 12,13-didecanoate; DMEM, Dulbecco’s minimal essential medium; DRG, dorsal root ganglia; ECS, extracellular solution; EGFP, enhanced green fluorescence protein; HBSS, Hank’s balanced salt solution; OAG, 1-oleoyl-2-acetyl-sn-glycerol; RBL, rat basophilic leukemia; TRP, transient receptor potential; TRPV, vanilloid subfamily of TRP.

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FIGURE LEGENDS:

Figure 1. Differential effects of 2APB on TRPC6, TRPM8 and TRPV1. HEK293 cells were transfected with murine TRPC6 (A), TRPM8 (B) or TRPV1 (C). Cells were held at 0 mV in whole-cell mode and currents were recorded using voltage ramps from –100 to 100 mV at 2 Hz. Drugs were applied as indicated. A. Inhibition of OAG-activated TRPC6 current by different concentrations of 2APB. Left, membrane current at –100 mV (open circles) and +100 mV (filled circles) for a representative cell. The dashed line indicates zero current. Middle, I/V curves obtained by the voltage ramps at the indicated time points in the left. Right, a summary of dose-dependent inhibition of OAG response by 2APB at –100 (open) and +100 mV (filled). Data are averages ± SEM for 5 cells. B. Inhibition of menthol-evoked TRPM8 current by 2APB. Similar to A. Data at the right are from 5 cells. C. Dose-dependent activation of TRPV1 by 2APB. Data at the right show dose-dependent activation by 2APB normalized to the response obtained by 1 µM capsaicin (Cap) for 6 cells.

Figure 2. 2APB-evoked currents in Xenopus oocytes that expressed mouse TRPV1. Oocytes-injected with TRPV1 cRNA were voltage-clamped at –40 mV and currents recorded continuously at 22°C. Drugs were applied as indicted. A. Representative current record for a cell sequentially stimulated with 300 µM 2APB in the absence or presence of capsazepine (CPZ) or ruthenium red (RR). B. Current record for another cell sequentially stimulated with 2APB or capsaicin (Cap) in the absence or presence of CPZ. C & D.
Averages ± SEM of currents normalized to the response to 1 mM 2APB under conditions indicated. The concentrations were 2APB, 300 µM; Cap, 1 µM; CPZ, 30 µM; RR, 3 µM.

Figure 3. Potentiation of TRPV1 response to capsaicin (A) and protons (B) by 2APB in HEK293 cells. Drugs were applied either separately or in combinations as indicated. Left panels show currents at –100 (open) and +100 mV (filled). Middle panels show leak-subtracted I/V curves at the time points indicated in the left. Note trace b (gray) in B represents a transient hASIC1a current found in all HEK293 cells [50]. It does not interfere with the TRPV1 current obtained at the later phase of the acid stimulation (trace c). Right panels show averages ± SEM of responses at –100 and +100 mV normalized to that obtained by 100 µM 2APB alone. n = 7 for A; n = 6 for B. C. Negative association of the initial 2APB response to that potentiated by 0.3 µM capsaicin or pH 6.5. Individual data points used for A and B (right panels) are plotted as the response to 100 µM 2APB vs the fold increase due to the addition of capsaicin or protons. The relationship is not linear. The gray line represents the least square fit of the equation: $y = P_{\text{max}}/(1 + x/I_{50})$, where $P_{\text{max}} = 43.3$ is the maximal fold increase of the potentiated current and $I_{50} = 112.4$ pA is the initial 2APB-evoked current corresponding to 50% of the $P_{\text{max}}$.

Figure 4. 2APB-induced sensitization of TRPV1 response to capsaicin, protons, and heat in Xenopus oocytes. Oocytes expressing TRPV1 were clamped at –40 mV and currents recorded continuously at 22°C unless indicated otherwise. Drugs were applied as indicted. A. 100 µM 2APB increased the sensitivity of TRPV1 to capsaicin. Left shows a representative current trace from one oocyte consecutively stimulated with capsaicin from
the low to high concentrations in the absence or presence of 2APB. Sufficient time was allowed for the cell to recover from the prior stimulation. Right shows dose response curves for capsaicin with and without 2APB. Data are averages ± SEM for 4-11 cells of currents normalized to the response to 1 mM 2APB and fitted to Hill equation. B-D. Similar to A, showing the sensitization of 2APB response by 0.3 µM capsaicin (B) and weak acid (pH 6.5, D) and the sensitization of TRPV1 to protons by 100 µM 2APB (C). E. Heating enhanced 2APB-evoked TRPV1 current. Left shows a representative current trace for an oocyte stimulated by heating to 40°C or by 100 µM 2APB at either 22 or 40°C. Right shows averages ± SEM for 13 oocytes of the currents normalized to that obtained from 100 µM 2APB at 22°C.

**Figure 5. Effect of 2APB on rat DRG neurons.** Acutely cultured DRG neurons were voltage-clamped at –70 mV and membrane currents recorded at 22-24°C. Drugs were applied as indicated. A. Dose-dependent activation of DRG neurons by 2APB. B & C. Inhibition of 2APB-evoked currents in DRG neurons by 3 µM ruthenium red (RR, B) and 10 µM capsazepine (CPZ, C). Left panels show representative traces. Right panels show averages ± SEM for the number of cells as indicated. *p<0.01, significantly different from corresponding control response, by one-way ANOVA with post hoc of Neuman-Keul test.

**Figure 6. Potentiation of capsaincin- and proton-response of DRG neurons by 2APB.** Similar to Fig. 5, acutely cultured DRG neurons were voltage-clamped at –70 mV and membrane currents recorded at 22-24°C. Drugs were applied as indicated. The
response to capsaicin (A) and acid (B) was enhanced by the low concentrations of 2APB and the overall response was inhibited by capsazepine (CPZ, C & D). Left panels show representative traces. Right panels show averages ± SEM for the number of cells as indicated. * p<0.01, significantly different from corresponding control response, † p<0.01, significantly different from response to 30 µM 2APB, by one-way ANOVA with post hoc of Neuman-Keul test.

Figure 7. Activation of TRPV1-3 by 2APB. A. 2APB-evoked changes in intracellular Ca<sup>2+</sup> in HEK293 cells expressing TRPV1-6. Cells transiently transfected with cDNAs for murine TRPV1 (mV1), TRPV2 (V2), and TRPV3 (V3), human TRPV4 (V4), rat TRPV1 (rV1), TRPV5 (V5), and TRPV6 (V6), or pcDNA3 vector (cont) in a 96-well plate were loaded with fluo4 and fluorescence was read at 32°C. 2APB was added at 20 sec. Shown are baseline subtracted fluorescence values. B. The same series of transiently transfected cells as shown in A were stimulated with resiniferatoxin (upper), N-arachidonyl dopamine (middle), or 4αPDD (lower) as indicated. Baseline subtracted fluorescence values are shown. The color codes for the traces are the same as in A. C. Dose response curves derived from Ca<sup>2+</sup> measurement for mV1 (open circles), V2 (filled circles), and V3 (filled triangles). Data for V4 (open triangles) were normalized to the maximal response of V3. Endogenous responses to 2APB were subtracted using values obtained from control vector-transfected cells. Data are averages ± SEM for three measurements. D&E. 2APB-activated whole-cell currents in HEK293 cells that expressed TRPV2 (D) or TRPV3 (E). Membrane currents at indicated potentials (left) and I/V curves (right) are shown as in Fig. 1C. RR, ruthenium red.
Figure 8. 2APB-evoked TRPV3 activity and its sensitization by heat.  

A. Extracellular application is required for 2APB to activate TRPV3. For the TRPV3-transfected cell, 2APB (1 mM) was included in the pipette solution and recording began right after the establishment of the whole-cell configuration. No current was elicited for >6 min. 2APB and ruthenium red (RR) were applied extracellularly later to the same cell as indicated. Open and closed circles represent membrane currents at –100 and +100 mV, respectively. The insert above shows the I/V curves at the indicated time points.  

B. Activation of TRPV3 by 2APB at 22°C and the inhibition by RR in *Xenopus* oocytes. *Left*, a representative trace. *Right*, averages ± SEM of the effects of capsazepine (CPZ, n = 5) and RR (n = 7) on 2APB-evoked response.  


D. Sensitization of TRPV3 response to 2APB by rising temperatures. Dose response curves to 2APB of HEK293 cells expressing TRPV3 were generated at 24 (filled circles), 32 (filled triangles), and 37 (open circles) °C using the fluorescence Ca^{2+} assay as described for Fig. 7C. Data are averages ± ranges of duplicated measurements of representative experiments.
Hu et al., 2004, Fig. 2
**A**  
2APB (μM)  30  100  300  
0.2 nA  4 sec  

**B**  
300 μM 2APB  3 μM RR  
0.5 nA  4 sec  

**C**  
300 μM 2APB  10 μM CPZ  
0.2 nA  4 sec  

Hu et al., 2004, Fig. 5
Hu et al., 2004, Fig. 6
A}

B

C

D

Hu et al., 2004, Fig. 8
2-Aminoethoxydiphenyl borate is a common activator of TRPV1, TRPV2, and TRPV3
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