Bisandrographolide from Andrographis paniculata Activates TRPV4 Channels

Paula L. Smith 1§, Katherine N. Maloney 2§, Randy G. Pothen ‡, Jon Clardy ‡ and David E. Clapham 1‡

Many transient receptor potential (TRP) channels are activated by various compounds found in plants; two prominent examples include the activation of TRPV1 channels by capsaicin and the activation of TRPM8 channels by menthol. We sought to identify additional plant compounds that are active on other types of TRP channels. We screened a library of extracts from 50 Chinese herbal plants using a calcium-imaging assay to find compounds active on TRPV3 and TRPV4 channels. An extract from the plant Andrographis paniculata potently activated TRPV4 channels. The extract was fractionated further, and the active compound was identified as bisandrographolide A (BAA). We used purified compound to characterize the activity of BAA on certain TRPV channel subtypes. Although BAA activated TRPV4 channels with an EC50 of 790–950 nM, it did not activate or block activation of TRPV1, TRPV2, or TRPV3 channels. BAA activated a large TRPV4-like current in immortalized mouse keratinocytes (308 cells) that have been shown to express TRPV4 protein endogenously. This compound also activated TRPV4 currents in cell-free outside-out patches from HEK293T cells overexpressing TRPV4 cDNA, suggesting that BAA can activate the channel in a membrane-delimited manner. Another related compound, andrographolide, found in abundance in the plant Andrographis was unable to activate or block activation of TRPV4 channels. These experiments show that BAA activates TRPV4 channels, and we discuss the possibility that activation of TRPV4 by BAA could play a role in some of the effects of Andrographis extract described in traditional medicine.

TRPV4 4 is a member of the transient receptor potential superfamily of ion channels. Presumably, the assembly of four polypeptide subunits into a pore permeable to cations forms TRP channels. The pore opens and closes (gates) to allow cations to cross the membrane, and gating is usually controlled by one or more stimuli (e.g. ligand binding). Many TRP channels including TRPV4 are involved in sensing properties of the extracellular or intracellular environment (e.g. temperature, acidity, osmolarity, etc.). These characteristics provide a general description of TRP channels; however, a universal function for the TRP channel superfamily has yet to be found.

TRPV4 is one of six members of the vanilloid TRP subfamily; the first member of this group of channels is TRPV1, a channel gated by multiple stimuli including capsaicin, protons, heat, and anandamide (1–3). TRPV4 is most closely related to TRPV1, TRPV2, and TRPV3 as determined by sequence homology. In addition these four channels are functionally similar because they are all activated by increases in temperature. TRPV5 and TRPV6 are notably different from the other four TRPV channels as determined by sequence homology and channel function; both TRPV5 and TRPV6 are highly calcium-selective in contrast to most other TRP channels (4, 5).

Similar to TRPV1, TRPV4 can be activated by a wide range of stimuli including low osmolarity solutions; heating to warm temperatures; metabolites of arachidonic acid (epoxyeicosatrienoic acids) and 4-α-phorbol 12,13-didecanoate (4αPDD), a synthetic phorbol ester often used as a negative control for phorbol 12,13-didecanoate (6–10). The channel is widely expressed and can be found in kidney, skin, brain, lung, smooth muscle, vascular endothelium, liver, and a number of other areas (4). Functional studies, channel localization, and analysis of TRPV4 knock-out mice suggest that the channel may play a role in osmosensation, nociception, and heat sensation; however, the mechanistic details of these roles are still being investigated (11–15).

Plant compounds activate several TRP channels. For example, TRPV1 is activated by capsaicin, the “hot” ingredient in chili peppers; TRPV3 is activated by camphor from an evergreen tree, carvacrol in oregano, and eugenol in thyme (16, 17); TRPM8 is activated by menthol, the cooling compound in peppermint (18, 19), and TRPA1 is activated by allyl isothiocyanate, the pungent compound in mustard oil (20). These are several prominent examples, but other compounds with activity on TRP channels also have been described.

To search for novel compounds active on TRP channels, we used the Starr collection, a library of prefractionated extracts of 2-aminoethoxydiphenyl borate; eGFP, enhanced green fluorescent protein; BAA, bisandrographolide A.
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50 plants used in traditional Chinese medicine, which was assembled by the Osher Institute at Harvard Medical School. We used a calcium-imaging assay to screen the library for activity on TRPV3 and TRPV4 channels. Extracts active in the calcium-imaging screen were analyzed further in an electrophysiology assay. Extracts displaying activity in the electrophysiology assay were purified, chemically analyzed, and tested to identify the active compound in the extract. We show that a compound called bisandrographolide A (BAA) contained in extracts of the plant Andrographis paniculata activates TRPV4 channels. We speculate about the possibility that activation of TRPV4 by BAA might play a role in some of the reported effects of Andrographis extract in traditional medicine.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK293T cells were maintained at 37°C in media containing 90% Dulbecco’s modified Eagle’s medium/Ham’s F-12 (1:1), 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO2. 308 cells from a mouse keratinocyte cell line were maintained at 37°C in medium containing 90% Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO2. We received the 308 keratinocytes from S. H. Yuspa at the National Cancer Institute; this cell line is a papilloma-derived keratinocyte cell line from adult BALB/c mouse skin (21).

Calcium Imaging—35-mm dishes of HEK293T cells were transiently transfected with 3 μg of either human TRPV3 or mouse TRPV4 cDNA and 0.25 μg of dsRed2 cDNA (Clontech) using Lipofectamine 2000 (Invitrogen). After 24 h transfected cells were subcultured into 96-well plates. 48–72 h after transfection cells were subcultured onto glass coverslips, and recordings were made 24–72 h after transfection. We visualized eGFP- or dsRed2-positive cells with a fluorescence microscope (Olympus, ×40, N.A. 0.9) and recorded currents using an Axopatch 200B amplifier and pClamp8 software (Axon Instruments). During voltage ramps, currents were sampled at 10 kHz, and the recordings were filtered at 2 kHz. For all experiments the membrane potential was held at ~−60 mV. Borosilicate glass pipettes with resistances of ~2-4 megohm were used for recording. For recording HEK293T cells, the bath solution contained 135 mM NaCl, 5 mM CsCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM glucose, pH 7.4. For recording 308 cells we used a low chloride bath solution containing 135 mM sodium gluconate, 10 mM NaCl, 5 mM CsMES, 2 mM calcium gluconate, 1 mM MgSO4, 10 mM HEPES, and 10 mM glucose, pH 7.4. For recording cell-free patches we used a nominally Ca++-free external solution containing 135 mM NaCl, 5 mM CsCl, 1 mM MgCl2, 10 mM HEPES, and 10 mM glucose, pH 7.4. In all recordings measuring TRPV4 currents, the pipette solution contained 100 mM CsMES, 10 mM CsCl, 3 mM MgATP, 0.2 mM NaGTP, 10 mM CsH BAPTA, 80 μM CaCl2, 10 HEPES, and 20 mM mannitol, pH 7.3. Free [Ca++] in the pipette solution was ~1 nm as calculated by MaxChelator. For measurement of TRPV1, TRPV2, or TRPV3 currents the pipette solution contained 100 mM CsMES, 4 mM CsCl, 3 mM MgATP, 0.2 mM NaGTP, 10 mM CsH BAPTA, 3.2 mM CaCl2, 10 HEPES, and 20 mM mannitol, pH 7.3. Free [Ca++] in the pipette solution was ~100 nm as calculated by MaxChelator. Junction potentials were corrected in all experiments.

Reagents—fraction H11 (from the Starr collection), BAA, 4αPDD (Calbiochem), andrographolide (Calbiochem), capsaicin (Sigma), and 2-aminoethoxydiphenyl borate (2-APB, Calbiochem) were dissolved in Me2SO at high concentrations then diluted in the appropriate extracellular buffer before addition to the bath. Final Me2SO concentrations were 0.1–0.5%. Continuous perfusion during BAA experiments was not feasible because the quantity of BAA was limited. For these experiments we diluted BAA to the indicated concentration and added 500 μl of solution directly to the bath; bath volume was ~250 μl.

Plant Material—Fifty herbs that are used widely in traditional Chinese medicine were selected and obtained by Dr. David Eisenberg and his colleagues at the Osher Institute at Harvard Medical School.

Extraction and Prefractionation—Extraction and prefractionation steps were performed at the Instituto Nacional de Biodiversidad (INBio) in Costa Rica. Gram quantities of each of the Chinese medicinal plants were extracted using methanol with grinding. The extract from each plant was evaporated in a minimum volume of methanol and adsorbed onto Diaion HP20 (Mitsubishi Chemical). The resin was washed with deionized water and extracted with ethanol. This solution was adjusted to 20% aqueous ethanol and loaded onto a 500-ml RP-C18 column, where it was separated into 48 fractions. Fractions of sufficient weight, as judged by evaporative light-scattering detection, were evaporated, reconstituted in Me2SO, arrayed in 96-well plates, and shipped to the Institute of Chemistry and Cell Biology-Longwood at Harvard Medical School for integration into their screening platform.
Isolation and Characterization of BAA—The active fraction from Andrographis was separated by HPLC on an Agilent 1100 chromatograph (Agilent Technologies) using a semipreparative Discovery HS-C18 column (Supelco, 250 × 10 mm, 5-mm particle size) with an acetonitrile-water gradient to afford 0.4 mg of pure, active compound.
One-dimensional $^1$H and two-dimensional (double quantum filtered $^1$H-$^1$H COSY (dqfCOSY), $^1$H-$^{13}$C heteronuclear multiple quantum coherence (HMQC), $^1$H-$^{13}$C heteronuclear multiple bond correlation (HMBC), and nuclear Overhauser effect spectroscopy (NOESY)) NMR spectra were obtained on a Varian Oxford NMR AS500 spectrometer with standard pulse sequences operating at 500 MHz. CD$_3$OD was used as solvent. Offline processing was conducted using Mestre-C NMR software (Mestrelab Research). The NMR data revealed a diterpene dimer with the bisandrographolide carbon skeleton shown in Fig. 1E. $^1$H chemical shifts were identical to literature values for BAA (22) (supplemental Fig. 1 and supplemental Table 1). The liquid chromatography mass spectrum (LC/MS) of the active compound was obtained using a Micromass Platform LC-Z spectrometer equipped with a Waters 2690 LC system and Waters 2690 photodiode array detector, was processed using MassLynx software (Waters Corporation), and revealed a molecular ion [M + 1]$^+$ of 665.53, consistent with BAA.

**FIGURE 2.** BAA does not activate closely related TRPV channels. Experiments showing application of 2.6 µM BAA to HEK293T cells transiently overexpressing: A, TRPV1; B, TRPV2; C, TRPV3 cDNA. Current-voltage relationships (left panels) are in response to voltage ramps from -80 mV to +80 mV. Traces recorded under control bath conditions are shown in black; traces recorded after addition of 2.6 µM BAA to the bath are shown in red; and traces recorded after addition of a known agonist for the channel (A, 100 nM capsaicin; B, 500 µM 2-APB; C, 100 µM 2-APB) are shown in blue. The time course of the response in each experiment is shown in the right panels. Symbols are the same as in Fig. 1B. The red bars indicate the presence of BAA in the bath solution; the blue bars indicate the presence of the known agonist for each type of channel.
RESULTS

We screened a library of extracts from 50 plants used in traditional Chinese medicine using a Ca\(^{2+}\)-imaging assay. The assay could detect compounds that opened or blocked TRPV3 channels and those that opened TRPV4 channels. Each extract at \(\sim 10 \mu\text{g/ml}\) was added to HEK293T cells transfected with either TRPV3 or TRPV4 cDNA and loaded with the Ca\(^{2+}\) indicator Fluo-4. Extracts that specifically altered Ca\(^{2+}\) influx in the Ca\(^{2+}\)-imaging screen were tested further in an electrophysiological assay to verify activity. Although one extract displayed blocking activity for TRPV3 in the Ca\(^{2+}\)-imaging assay, the effect could not be repeated in the electrophysiology assay.

Several fractions stimulated Ca\(^{2+}\) influx in TRPV4-transfected cells (supplemental Fig. 2). In the electrophysiology assay 10 \(\mu\text{g/ml}\) fraction H11 from the Starr collection caused activation of a large current in HEK293T cells overexpressing TRPV4 channels (Fig. 1, A and B). NMR analysis showed that fraction H11 contained several compounds; H11 was separated further by reverse phase HPLC, and the fractions were tested in the electrophysiology assay to identify a single active compound. This compound was identified as bisandrographolide A by NMR and mass spectrometry (Fig. 1E). No other compounds in fraction H11 activated TRPV4 current. We applied purified BAA to TRPV4-expressing cells to confirm that the compound activated TRPV4 channels similar to fraction H11 (Fig. 1, C and D). We also obtained a dose-response curve for activation of TRPV4 channels by BAA. Fig. 1F shows the average maximum inward current density measured at \(-110\) mV plotted against concentration of BAA; data are fitted with a Hill equation (EC\(_{50}\) = 950 nM BAA, Hill coefficient = 1.4). A dose-response curve plotting average I/I\(_{\text{max}}\) (current normalized to the maximum inward current measured at \(-110\) mV in each experiment) against the concentration of BAA yielded a similar result: EC\(_{50}\) = 790 nM BAA, Hill coefficient = 1.8 (data not shown).

To determine whether the activity of BAA was specific for TRPV4, we applied BAA to other closely related vanilloid TRP channels. As shown in Fig. 2, BAA did not activate TRPV1, TRPV2, or TRPV3 channels overexpressed in HEK293T cells even though these channels were robustly activated by known agonists (100 nM capsaicin, 500 \(\mu\text{M}\) 2-APB, or 100 \(\mu\text{M}\) 2-APB, respectively; \(n = 5\) for each experiment). In addition, we tested whether the compound could block activation of TRPV1, TRPV2, or TRPV3 channels. However, we did not observe blocking after application of 5 \(\mu\text{M}\) BAA (data not shown). We did not test other TRP channel subtypes.

To test whether BAA activates endogenously expressed TRPV4 channels similar to heterologously expressed channels, we investigated currents in 308 cells, a mouse keratinocyte cell line previously shown to express both TRPV3 and TRPV4 channels (23). We could stimulate large TRPV4 currents in 308 cells (see also Ref. 23). Activation of a current in response to 4\(\alpha\)PDD occurred in 7 of 16 cells. Block trace, recorded under control conditions; red trace, recorded after addition of 2.6 \(\mu\text{M}\) BAA to the bath. Activation of a current in response to 4\(\alpha\)PDD to the bath. Activation of a current in response to BAA occurred in 6 of 11 cells. Lower panel, time course of activation by BAA. Symbols are same as in Fig. 1B; the red bar indicates presence of 2.6 \(\mu\text{M}\) BAA in the bath.
erologously expressed TRPV4 channels. We note that the reversal potential of the current activated by BAA is shifted slightly to the left of 0 mV. We believe this effect was due to activation of residual chloride current by Ca$^{2+}$/H$^{+}$ entering through TRPV4 channels during prolonged activation. These results support the hypothesis that BAA is an effective activator of endogenous TRPV4 channels.

To determine whether this compound activates TRPV4 in a membrane-delimited manner, we applied 5 μM BAA to cell-free outside-out patches excised from HEK293T cells overexpressing TRPV4 cDNA (Fig. 4). These experiments were performed in an external solution with no added Ca$^{2+}$ to slow inactivation of the current. 5 μM BAA can activate a substantial current in excised patches with properties similar to whole-cell currents under similar conditions. The reversal potential, rectification of the current through the channel, and the time course of activation are similar to TRPV4 current measured in whole cells bathed in nominally Ca$^{2+}$-free external solution. We note that the reversal potential of the current in Fig. 4A is also shifted to the left of 0 mV. In this instance we attribute the shift to the lack of external Ca$^{2+}$. The ability of BAA to activate TRPV4 in patches suggests that the action of BAA does not require signaling through soluble internal factors.

The most abundant compound isolated from A. paniculata is andrographolide (Fig. 5A), the monomer of BAA. Andrographolide has been shown previously to block NF-κB activation with an IC$_{50}$ of ~10 μM and to display anti-inflammatory properties in mouse models of inflammation (25). We applied high concentrations of andrographolide to TRPV4-expressing cells to determine whether andrographolide has activity similar to BAA. However, application of 0.1–1 mM andrographolide did not activate TRPV4 channels, although a large TRPV4 current could be elicited by the agonist 4αPDD (Fig. 5, B and C). High concentrations of andrographol-
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In this study we have identified a novel activator of TRPV4 channels from the plant *A. paniculata* used in traditional medicine in many regions of Asia. The compound, bisandrographolide A, has an EC50 of ~790–950 nM for TRPV4 activation and is not active on other closely related members of the vanilloid TRP subfamily (TRPV1, TRPV2, and TRPV3) at concentrations that fully activate TRPV4 channels. We have shown that BAA activated endogenous TRPV4 channels in keratinocytes. We also demonstrated that BAA activated TRPV4 channels in cell-free outside-out patches, suggesting that activation is membrane-delimited and not dependent on diffusible intercellular factors. Furthermore, we tested the activity of the monomer andrographolide on TRPV3 channels, and found that andrographolide neither activated nor blocked TRPV1, -V2, -V3, or -V4 channels.

TRPV4 is a nonselective cation channel that can be activated by a wide range of stimuli, like several other transient receptor potential potential channels. Activation causes opening of a pore that allows Na+, K+, and Ca2+ ions to cross the membrane. The resulting increase in internal free [Ca2+] can affect cell signaling. TRPV4 has been implicated in osmoregulation, nociception, regulation of vascular tone, and heat sensation.

The plant *A. paniculata* is used in traditional medicine in various parts of Asia for a wide array of ailments. Extracts are typically used as an anti-inflammatory agent or immunosuppressant. Indications include upper respiratory tract infections, diarrhea, fever, tonsillitis, snakebite, and many others (26). How the extract is used seems to vary widely depending on the region. Extracts of *Andrographis* are used as an herbal remedy for the common cold in Sweden where pills with a standardized amount of *Andrographis* extract and another herb (*Eleutherococcus senticosus*) are made and sold under the name Kan Jang.

Because of the widespread use of *Andrographis* extract in traditional medicine, there have been a relatively large number of studies investigating the effects of *Andrographis* extract as well as compounds purified from the extract. Many articles have focused on andrographolide, the most abundant compound in *Andrographis*. In one study, andrographolide was shown to inhibit NF-κB activation and reduce inflammatory response in a variety of mouse models (25). This mechanism may account for some of the anti-inflammatory effects of *Andrographis*.

In addition to andrographolide, many other compounds have been identified in *Andrographis* extract. BAA was first identified in 1994 in a screen of naturally occurring substances that induce differentiation of mouse myeloid leukemia (M1) cells (22). These authors identified 18 compounds found in methanol extracts of *Andrographis paniculata*, including BAA and three other andrographolide dimers; they also showed that BAA and two stereoisomers stimulated phagocytosis and inhibited growth in M1 cells, suggesting that these compounds can induce differentiation of these cells (22). Although several other compounds found in the *Andrographis* extract had similar effects on M1 cells, BAA and its stereoisomers had the strongest effects (22).

Many experiments on *Andrographis* have focused on its anti-inflammatory and immunosuppressant properties; however, there have been a small number of studies on the cardiovascular effects of *Andrographis*. These studies were prompted by the use of *Andrographis* extract for hypertension in Malaysian traditional medicine. The authors found that administration of *Andrographis* extract transiently reduced the blood pressure of rats. Furthermore, they found that administration of pure andrographolide did not produce a similar reduction in blood pressure, suggesting that other compounds were eliciting the effect (27, 28). Further studies by this group investigated how 14-deoxyandrographolide and 14-dideoxy-11,12-didehydroandrographolide, two compounds also found in *Andrographis* extract, might be responsible for the observation (29–31). Based on our results and several other studies on the role of TRPV4 in the vascular system, it is possible that activation of TRPV4 by BAA also might contribute to the observed reduction in blood pressure. A recent article suggests that TRPV4 activation may result in smooth muscle hyperpolarization and arterial dilation (32). The experiments from Earley et al. (32) support the hypothesis that Ca2+ influx through TRPV4 channels in arterial smooth muscle cells increases the number of Ca2+ sparks that activate large conductance, Ca2+-activated K+ channels, and the resulting increase in potassium current causes hyperpolarization and arterial dilation. *Andrographis* extract containing BAA presumably could act on vascular smooth muscle by the same mechanism to cause arterial dilation and a decrease in blood pressure.

According to a data base of information on compounds found in plants used in traditional Chinese medicine (26), *Andrographis* also is used to treat snake bite and eczema, two conditions relating to the skin. Unfortunately there is little research on the efficacy of *Andrographis* extracts for treating these conditions. There are, however, some hints that TRPV4 might play a role in skin function. TRPV4 is highly expressed in skin, particularly in keratinocytes (10, 33, 34). As shown previously (35) as well as in our experiments, very large currents can be elicited in keratinocytes by TRPV4 agonists. The Ca2+ influx through TRPV4 channels could play a role in skin function, because it is known that Ca2+ signaling plays an important role in the proliferation and terminal differentiation of keratinocytes (36). In addition, there is some evidence that epoxyeicosatrienoic acid signaling may be involved in keratinocyte cornification (37). Although there is evidence that TRPV4 is present in mouse and human skin, we do not know enough about its functional role to speculate further on how activation of the channel could affect injury to the skin.

Our experiments show that TRPV4 is activated by BAA, a compound contained in extracts of the herbal medicine *A. paniculata*, with an EC50 of 790–950 nM. *Andrographis* extract is used as a traditional medicine in various parts of Asia for a wide variety of illnesses. It is possible that activation of TRPV4 by BAA could play a role in some of the effects of *Andrographis* extract that have been reported previously.
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Acknowledgments—S. H. Yuspa from the National Cancer Institute kindly provided the 308 cells. Constructs for rat TRPV1 and rat TRPV2 were kindly provided by D. Julius. We thank D. Eisenberg and T. Kapchuk of the Osher Institute for information about the plants used to make the library of extracts.

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