Pinacidil, a K_{atp} channel opener, identified as a novel agonist for TRPA1

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Received October 13, 2011; accepted December 28, 2011; published online March 22, 2012

The transient receptor potential Ankyrin 1 (TRPA1) cation channel is activated by various pungent and irritant compounds, and it also mediates the perception of noxious cold. Identification of different agonists for this channel is important for understanding its activation mechanism. Therefore, a screen for novel TRPA1 agonists was performed using an agonist-induced calcium influx assay. Out of 90 compounds screened, pinacidil was identified as a novel agonist for this channel. Pinacidil is a known opener of the K_{atp} channel, for which it has an EC₅₀ value of 1–3 μmol/L. In comparison, the EC₅₀ value of pinacidil for TRPA1 is relatively high (260 μmol/L). Recombinant HEK-TRPA1 cells did not respond to P1075, another K_{atp} channel opener, suggesting that the effect of pinacidil on TRPA1 was highly specific. Further studies revealed that the agonist activity of pinacidil could be blocked by the TRP channel inhibitors, ruthenium red and HC-030031. Using glutathione (GSH) and site-specific mutagenesis, we demonstrated that pinacidil could activate TRPA1 by covalent modification of the critical amino acids C619, C639 and C663 in the N-terminus of TRPA1.

TRPA1, pinacidil, covalent modification

Citation: Ma L H, Deng Y, Zhang B, et al. Pinacidil, a K_{atp} channel opener, identified as a novel agonist for TRPA1. Chin Sci Bull, 2012, 57: 1810–1817, doi: 10.1007/s11434-012-5035-0

TRPA1 (ANKTM1, p120) belongs to the transient receptor potential (TRP) superfamily of cation channels and is present in all species from yeast to mammals. It is critical for animal survival by influencing their ability to sense noxious environments, such as high or low temperatures [1], toxic chemicals [2] and strong mechanical stress [3]. Mammalian TRP channels, which are composed of six-transmembrane (6-TM) domains with a pore region between TM5 and TM6, can be divided into six subfamilies based on their sequence homology. These subfamilies are: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin) [4–6]. TRPA1 is the only member of the TRPA sub-family, and it is distin-

guished from other TRP channels by the presence of 14 repeated ankyrin domains within its N-terminus [7].

TRPA1 was originally identified in cultured human lung fibroblasts (IMR-90 cell line) [8]. Subsequent studies found that TRPA1 is highly expressed in dorsal root, trigeminal and nodose ganglion sensory neurons, and in cells of the inner ear [9,10]. Furthermore, some studies reported that the human TRPA1 protein is widely expressed outside of the CNS (e.g. intestine, lung, pancreas), and dysregulation of TRPA1 during oncogenic transformation has been postulated [11,12]. Researchers have found that TRPA1 is sensitive to cold temperatures of less than 17°C, and that it can be activated by a host of exogenous or endogenously-produced compounds including cinnamaldehyde [13,14], diallyl disulfide [15], 2-pentenal [16], hydroxyl fatty acid

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[17], and morphanthridine analogue [18]. In addition, bradykinin, a potent algogenic peptide released from sites of injury and inflammation, can also activate TRPA1 via G-protein coupled bradykinin B2 receptors [13]. Studies on TRPA1 deficient mice supported the hypothesis that the channel plays a role in sensation of noxious cold, inflammatory pain and cough [16]. Therefore, TRPA1 is a promising target not only for the development of novel pain medications, but also for the development of potential new anti-tussive treatments for smoking-related diseases and environmental irritation [19].

TRP channels belong to the group of non-voltage gated, cation-permeable ion channels [20]. TRPA1 is a calcium-permeable, non-selective cation channel. Based on its channel characteristics, the activation of TRPA1 can be monitored by calcium assay and electrophysiology. In this study, we established an integrated assay system that used a Fluo3-based calcium influx assay together with whole cell patch-clamp analysis to identify novel TRPA1 channel agonists. Using this system, we demonstrated that pinacidil could activate recombinant human TRPA1 channels in HEK293 cells.

1 Materials and methods

1.1 Reagents

The HDB WASH Free Calcium Assay Kit was supplied by HD Biosciences (Shanghai, China). The culture medium, pcDNA3.1-hygro(+) vector, hygromycin B and Lipofectamine 2000 were purchased from Invitrogen (USA). Cinnamaldehyde, *trans*-2-pentenal, tetraethylthiuram disulfide, ruthenium red, pinacidil and P1075 were purchased from Sigma (USA).

1.2 Cloning and expression of human TRPA1

The human TRPA1 cDNA in the pCMV6-XL4 vector was purchased from ORIGENE (USA), and the sequence was verified by restriction mapping and DNA sequencing. The pCMV6-XL4-TRPA1 plasmid was digested with the restriction enzyme *Not* I to isolate the human TRPA1 fragment. The obtained fragment was inserted into the mammalian expression vector, pcDNA3.1-hygro(+), at the *Not* I site to generate the pcDNA3.1-hygro(+)-TRPA1 recombinant plasmid. The pcDNA3.1-hygro(+)-TRPA1 plasmid was then transfected into HEK293 cells using Lipofectamine 2000. Stable HEK293 cells expressing full length human TRPA1 were selected in the presence of 1 mg/mL hygromycin B and cultured in a humidified atmosphere of 5% CO₂ at 37°C in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 2 mmol/L *L*-glutamine.

A series of cysteine to serine TRPA1 mutants at C619, C639 and C663 were created using the Stratagene (USA) site-directed mutagenesis kit according to manufacturer's

instructions. Mutations were confirmed by DNA sequencing. The mutant and wild-type TRPA1 constructs were then transiently transfected into HEK293 cells for functional detection by calcium influx assay.

1.3 Calcium influx assay

A HDB WASH Free Calcium Assay Kit (HD Biosciences, Shanghai, China) was used to measure intracellular calcium concentration on a FlexStation 384^{II} plate reader according to the manufacturer's instructions. Cells were seeded at a density of 4×10⁴ cells/well in a Matrigel-coated, 96-well black plate and incubated in a CO₂ incubator overnight before running the assay. The growth medium was replaced next day with calcium assay buffer containing 4 μmol/L Fluo-3/AM, 1 mmol/L probenecid and 0.4 g/L pluronic F127. The assay was performed by incubating the plate at 37°C for 1 h on the FlexStation plate reader, with an excitation wavelength of 485 nm and an emission wavelength of 525 nm. In the antagonist test experiment, following preincubation of the antagonist on the cells for 10 min, agonist was applied to elicit a residual fluorescence signal that was recorded on the FlexStation plate reader.

1.4 Electrophysiology

HEK293-TRPA1 cells were transferred to a continuously-perfused recording chamber mounted on the stage of an inverted microscope. For whole-cell patch clamp experiments, the standard bath solution consisted of: 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 10 mmol/L HEPES, 10 mmol/L *D*-glucose, pH 7.4 (NaOH). The pipette solution for whole-cell experiments contained 140 mmol/L KCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 10 mmol/L EGTA, 10 mmol/L HEPES, 10 mmol/L *D*-glucose, pH 7.3 (KOH). All experiments were performed at room temperature using a HEKA EPC-10 amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany). Patch pipettes of 3–5 megaohms were fabricated from borosilicate glass capillaries. Whole cell access was obtained by using negative pressure to rupture the cell membrane, and series resistance compensated for optimal voltage control of the cell membrane. A holding potential of –60 mV was applied from an HEKA EPC-10 amplifier controlled by Patchmaster™ software. Experiments were carried out and analyzed under the control of the Patchmaster™ software (HEKA Electronics). Whole-cell currents were filtered at 3 kHz (four-pole Bessel filter) and sampled continuously at 5 kHz.

1.5 Glutathione adduct reaction

Electrospray ionization mass spectrometry (ESI-MS) was used to determine that reaction of TRPA1 agonists and glutathione in water at room temperature produced glutathione adducts.

1.6 Data analysis

Calcium assay data were recorded as RFU values (relative fluorescence units) using SoftMax Pro 4.8 software. The concentration dose responses were analyzed using Graph-Pad Prism 4 software. Data are reported as mean $\pm$ SEM (n indicates the number of experiments). Student's t -test was used for all statistical calculations.

2 Results

2.1 Primary screen of TRPA1 agonists by calcium influx assay

Based on the calcium-permeable property of the TRPA1

channels, a high-throughput screen based on calcium influx in response to TRPA1 activation was established. The assay was optimized for cell density, loading time and DMSO tolerance. Cinnamaldehyde (CA), *trans*-2-pentenal (2-TP) and tetraethylthiuram disulfide (TED), which are known agonists of TRPA1, were used to test the assay system. As expected, all three compounds induced a large and sustained increase in fluorescence in TRPA1 cells, while generating no response in the non-transfected HEK293 cells (data not shown). Around 90 compounds were tested by this screening system, and four hits, including pinacidil, were identified as potential agonists of TRPA1 (Figure 1(a)). Comparable calcium increases were observed with 200 μ mol/L pinacidil, while no response to pinacidil, even at high concentration, was observed in non-transfected HEK293 cells

Figure 1 Activation of the TRPA1 channel by pinacidil in recombinant HEK293 cells. (a) Primary screen of 90 compounds assayed by the change in Relative Fluorescence Units (RFU). Four hits were identified by the screen. (b) Pinacidil induced Ca^{2+} influx in a dose-dependent manner in HEK293-hsTRPA1 cells, while it failed to evoke Ca^{2+} influx in non-recombinant HEK293 host cells. (c) Chemical structures of cinnamaldehyde and pinacidil. (d) P1075 does not activate TRPA1. (e) The TRP channel antagonists, ruthenium red and HC-030031, inhibit the calcium response induced by pinacidil in TRPA1 cells. The curve is plotted as the change in RFU in response to 300 μ mol/L pinacidil for 10 min after the addition of antagonist. RFU were calculated by normalizing each data point to the maximal response. Data points represent means $\pm$ SEM ($n = 4-5$).

(Figure 1(b)). EC_{50} values for the known TRPA1 agonists cinnamaldehyde, *trans*-2-pentenal, tetraethylthiuram disulfide were also measured using the calcium influx assay (Table 1), and the data obtained were similar to other published data.

Pinacidil is an ATP-dependent K^+ channel activator (its structure is shown in Figure 1(c)). The application of pinacidil on human coronary artery vascular smooth muscle cells can cause an outward current [21]. Although it has been reported that HEK293 cells do not express any endogenous ATP-dependent K^+ channels [22–24], to rule out the possibility that pinacidil-induced TRPA1 activation was not caused by activation of K_{ATP} channels, another K_{ATP} opener, P1075 [25], with a similar structure to pinacidil, was tested in the calcium influx assay. As shown in Figure 1(d), P1075 had no effect on TRPA1 activation.

The nonselective TRP channel antagonist, ruthenium red, and the TRPA1-specific antagonist, HC-030031, were used to test if they could block pinacidil-induced calcium influx in HEK293-TRPA1 cells. The results demonstrated a concentration-dependent inhibition with IC_{50} values ranging from 80–800 nmol/L for ruthenium red (RR) and 5–25 μ mol/L for HC-030031 (HC) (Figure 1(e)).

2.2 Confirmation of TRPA1 agonists by electrophysiology

In electrophysiological studies, the characteristic inward current and desensitization with repeated stimulation was observed when applying cinnamaldehyde (1 mmol/L) to TRPA1-expressing cells (data not shown). These cinnamaldehyde-induced currents were inhibited by the universal antagonist, RR, and the TRPA1-specific antagonist, HC-030031 (Figure S1(A,B)). This system was further applied to confirm the activation effect of pinacidil on TRPA1. We found that large membrane currents were elicited by pinacidil (1 mmol/L) at -60 mV in HEK293 cells that expressed human TRPA1 (Figure S1(E)) but not in non-transfected HEK293 cells (data not shown). Moreover, these agonist-induced current responses were inhibited by RR (30 μ mol/L) and HC-030031 (50 μ mol/L) (Figure S1(C,D)).

2.3 Selectivity study

To test whether pinacidil acts specifically on TRPA1, the

effect of pinacidil on two related cation channels, human TRPV3 and TRPM8, was tested. Pinacidil exerted no effect on HEK293-hsTRPV3 cells and evoked a very weak fluorescence increase in HEK293-hsTRPM8 cells at high concentration (100–1000 μ mol/L) (Figure 2(a),(b)). Considering these data together with our other results, we therefore suggest that pinacidil acts as a specific agonist for TRPA1.

2.4 ER calcium stores are not responsible for pinacidil-induced calcium release

The increased calcium levels detected in the HEK293-TRPA1 cells by the calcium influx assay may be due to release from intracellular Ca^{2+} stores (the endoplasmic reticulum) or influx from the extracellular solution. The calcium contained in the ER can be depleted by blocking ATP-dependent Ca^{2+} uptake with thapsigargin. To determine the source of the pinacidil induced calcium, the TRPA1 cells were pre-incubated with or without thapsigargin (10 μ mol/L) and then stimulated with TRPA1 agonists. No significant difference was observed following stimulation with cinnamaldehyde (30 μ mol/L) or pinacidil (300 μ mol/L) (Figure 2(c)). These observations suggested that the calcium signal increase mainly resulted from the opening of cell membrane TRPA1 channels, rather than release from the ER.

2.5 *In vitro* glutathione adduct formation

Covalent modification of cysteines in TRPA1 is regarded as an important activation mechanism mediated by most TRPA1 agonists. A glutathione (GSH) adduction test was performed to confirm the covalent modification of cysteine residues by TRPA1 agonists *in vitro*. To investigate the activation mechanism of the TRPA1 agonists, GSH was combined with cinnamaldehyde, pinacidil or P1075 and allowed to react at room temperature. The samples were then measured by electrospray ionization mass spectrometry (ESI-MS). GSH alone existed as both a monomer and a dimer in solution, MH^+ , m/z (307, 613) (Figure S2(A)). When reacted with cinnamaldehyde and pinacidil, glutathione adducts of the predicted mass were detected by ESI-MS (Figures 3(a), S2(B)). However, no adducts were observed from the reaction of P1075 and GSH sample (data not shown). These data suggest that pinacidil can covalently modify cysteine residues in TRPA1.

Table 1 Summary of the activity of TRPA1 agonists in HEK293-hsTRPA1 cells^{a)}

Agonist	Cinnamaldehyde	<i>Trans</i> -2-pentenal	Tetraethylthiuram	Pinacidil
EC_{50} (μ mol/L)	12.96 $\pm$ 2	25.57 $\pm$ 3.4	0.10 $\pm$ 0.1	260 $\pm$ 18
HILLSLOPE	2.393	2.147	1.308	3.21
Maximum response (%)	113.3 $\pm$ 6.1	125.9 $\pm$ 9.3	153 $\pm$ 17	119.5 $\pm$ 6
RR IC_{50} (μ mol/L)	0.83 $\pm$ 0.07	0.75 $\pm$ 0.05	0.23 $\pm$ 0.04	0.08 $\pm$ 0.01
HC-030031 IC_{50} (μ mol/L)	21.94 $\pm$ 7.5	18.75 $\pm$ 5.6	6.26 $\pm$ 2.8	6.6 $\pm$ 2.3

a) Maximum response is the ratio of the high response relative to the response of HEK293-hsTRPA1 cells to 40 μ mol/L cinnamaldehyde. Values are the mean and standard deviations for $n=4$ –5 concentration-response experiments.

Figure 2 Selectivity of pinacidil on different HEK293-hsTRP cell lines, and the effect of thapsigargin on the HEK-TRPA1 cell line. (a) Dose-dependent changes on calcium influx induced by DPBA (TRPV3 agonist) and pinacidil in HEK293-hsTRPV3 cells. (b) Dose-dependent calcium influx responses induced by Menthol (TRPM8 agonist) and pinacidil in HEK293-hsTRPM8 cells. The y-axis represents the fluorescence change mediated by Fluo-3 in RFU. (c) Induction of calcium responses in HEK293-hsTRPA1 cells induced by the test compounds with or without 10 μmol/L thapsigargin pre-incubation. Data points represent means $\pm$ SEM ($n = 4-5$).

2.6 Competitive inhibition of TRPA1 agonists by GSH

To further verify the activation mechanism, cinnamaldehyde (1 mmol/L) or pinacidil (1 mmol/L) were reacted with different concentrations of GSH and applied to TRPA1 cells. A significant reduction in calcium influx was observed with the addition of higher concentrations of GSH (Figures 3(b), S2(C)). Together with the *in vitro* glutathione adduct formation data, it is clear that TRPA1 and GSH are both capable of reaction with pinacidil.

2.7 Three cysteine residues on TRPA1 are critical for pinacidil activation

Covalent modification of cysteine residues in the N-terminus of TRPA1 is thought to underlie the agonist activity of chemically reactive compounds such as cinnamaldehyde. Furthermore, three cysteine residues (C619, C639 and C663) were identified as the specific requirement site for agonists that activate the TRPA1 channel by covalent modification [26]. To determine whether a similar mechanism underlies the activity of the novel TRPA1 agonist, pinacidil, a series of single point-mutations and multiple point-mutations in TRPA1 were generated. We studied the effects of cinnamaldehyde and pinacidil on calcium fluorescence in HEK293 cells transiently transfected with WT TRPA1 or cysteine mutants of TRPA1 (Figures 4(b), S2(D)). As illustrated in Figure 4(b), single point-mutations of C619 or C639, and double point-mutation of C639/663, significantly changed the pinacidil-induced Ca^{2+} influx compared to wide-type TRPA1. The observation that double point mutation of C639/663S resulted in a greater reduction of Ca^{2+} influx compared to the C639 single point mutation suggested that C663 also contributes to the sensitivity of TRPA1 to pinacidil. All of these results indicated that the three cysteine residues in the intracellular N-terminus of TRPA1 confer sensitivity to pinacidil.

3 Discussion

In this study, we established an integrated assay system to screen and verify TRPA1 agonists. This system included a calcium influx assay and patch-clamp whole cell electrophysiology. Through this approach, the previously reported K_{atp} channel opener, pinacidil, was identified as a novel agonist for TRPA1.

TRPA1 is a non-selective cation channel that is permeable to calcium, and its activation leads to an elevation of intracellular calcium. Therefore, TRPA1-mediated intracellular calcium influx can be monitored by the use of the fluorescent calcium indicator, Fluo-3. Because cinnamaldehyde, tetraethylthiuram disulfide and *trans*-2-pentenal have been reported as agonists of the TRPA1, we used these compounds to characterize and validate our TRPA1 screening system. The known agonist activity of cinnamaldehyde was observed by calcium influx in TRPA1-expressing cells, but not in wild-type cells, indicating that the calcium influx was mediated by TRPA1 activation. Also, our dose response curve data and EC_{50} values for the agonists were consistent with those reported in the literature [9,13,27]. The TRP channel antagonists, ruthenium red and HC-030031, have been used to test the specificity of potential TRPA1 agonists. Ruthenium red is a universal antagonist for the TRP channels, and it displayed a potent, membrane-impermeable antagonism effect on TRPA1 currents. HC-

Figure 3 GSH forms adducts with pinacidil and competitively inhibits TRPA1 agonists applied to HEK293-hsTRPA1 cells. (a) Pinacidil and glutathione were mixed in water at room temperature to produce glutathione adducts. Adduct formation was determined by electrospray ionization mass spectrometry (ESI-MS). (b) Calcium responses induced by pinacidil in HEK293-hsTRPA1 cells in the presence of 50 or 15 mmol/L GSH. * $P < 0.05$, ** $P < 0.01$ versus pinacidil group.

030031, a newly-reported, selective antagonist of TRPA1 channels [28,29], showed an effect similar to that of ruthenium red. The antagonist effects of ruthenium red and HC-030031 against TRPA1 agonists were confirmed in our assays, with IC_{50} values similar published data being observed [10,28]. Together, these data validated our assay system for the screening of TRPA1 agonists. For the screening assay, a series of compounds was tested on the HEK293 TRPA1-expressing cells. To identify false positives and exclude non-specific compounds, the hits were screened in a secondary assay using cells not expressing recombinant TRPA1. As a result, pinacidil was identified as able to evoke a calcium response that could be blocked by the TRPA1

blockers, ruthenium red and HC-030031. All of these data suggest that pinacidil is an agonist of the TRPA1 channel. Moreover, in contrast to TRPA1, pinacidil did not stimulate calcium influx via TRPV3 and TRPM8. These results suggest that pinacidil may be a specific agonist of TRPA1, rather than a general agonist of TRP family channels.

To investigate ion channel function, electrophysiological measurements, in particular patch clamp, have long been considered as the "Gold Standard". To confirm the TRPA1 agonistic activity of pinacidil by a relatively direct approach, we used the whole-cell patch clamp technique. It has been reported that TRPA1 is often desensitized in the presence of extracellular Ca^{2+} [10,30]. It has even been reported that

Figure 4 Three cysteine residues in the intracellular N-terminus of TRPA1 confer sensitivity to pinacidil. (a) Structure of one TRPA1 subunit; the dark dots downstream of the ankyrin repeats represent the target Cysteine C663, C639 and C619 (Adapted from Michael J. Caterina, 2007). (b) Calcium influx mediated by WT and cysteine mutant TRPA1 in response to 300 μmol/L pinacidil. * $P < 0.05$, ** $P < 0.01$ versus wtTRPA1 group.

calcium ions alone can directly gate heterologously expressed TRPA1 [31,32]. Therefore, many researchers choose to use a calcium-free solution in the patch clamp experiment to avoid desensitization. However, some reported TRPA1 agonists such as icilin, which uses a different activation mechanism to other TRPA1 agonists, display a requirement for calcium ions to elicit an agonist response [31,33]. Calcium ions were shown to potentiate the agonist-mediated activation of TRPA1 without changing the property of the agonists. To avoid the misjudgment of compound activity, we chose to use a solution containing calcium ions in our electrophysiology experiments. Under these conditions, pinacidil and cinnamaldehyde evoked a slowly developing current, and desensitization was observed with repeated stimulation. These currents were impeded by the TRP channel non-specific antagonist, ruthenium red, and the TRPA1-specific antagonist, HC-030031. All of the results showed remarkable consistency with previously reported TRPA1 channel properties, and strongly suggested that pinacidil is a novel agonist for TRPA1.

Although TRPA1 can be activated by various stimuli in-

cluding noxious cold, irritant substances, activation of some PLC-related GPCRs, and even mechanical stimulation, the best characterized activation mechanism is via covalent modification of intracellular cysteines [34]. The *in vitro* formation of adducts between GSH and TRPA1 agonists reported by Macpherson was investigated to determine the mechanism of action of pinacidil. Pinacidil weakly formed adducts with the cysteine-containing tripeptide glutathione (Glu-Cys-Gly) compared to cinnamaldehyde, while P1075 did not form adducts with GSH. When pinacidil was applied to TRPA1-expressing cells together with different concentrations of GSH, a significant reduction in the calcium signal was observed at higher concentrations of GSH. GSH has been shown to be a competitive inhibitor of TRPA1 agonists. Our results also suggested that pinacidil activated TRPA1 by covalent modification of intracellular cysteines. Comparing the structure of pinacidil and P1075, a conjugated system of double and triple bonds is observed in pinacidil, while this conjugation is broken by one more single bond in P1075. This structural difference may explain the different reactivity of TRPA1 with pinacidil and P1075.

Pinacidil is an ATP-sensitive K channel opener and has been approved for the clinical treatment of mild to moderate hypertension [35]. Here, we present for the first time its off-target activity on TRPA1. A recent review summarized that a number of marketed drugs have been reported to activate the TRPA1 channel in addition to their intended targets, suggesting that their side effects may be partially explained by interaction of these drugs with TRP channels [14]. Headache is the main side effect of pinacidil treatment in antihypertension [36]. Over the past decade, several studies have revealed that members of the TRP ion channel family, including TRPA1, are fundamental molecules for the sensation of pain [37,38]. Our data suggest that activation of the TRPA1 receptor by pinacidil is a possible explanation for this drug-induced headache side effect. Therefore, structural modification of these compounds to remove their activity at TRPA1 may alleviate their side effects of pain.

The authors thank greatly Dr Fan LIU of HD Biosciences for her helpful assistance during the patch clamp experiment. J. Liu was supported by grants from the Ministry of Science and Technology (2007CB914200 and 2010DFA32140), the National Natural Science Foundation of China (31130028 and 30973514) and Program of Introducing Talents of Discipline to Universities of Ministry of Education (B08029).

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Supporting Information

Figure S1 Electrophysiology data proved that pinacidil activates TRPA1.

Figure S2 GSH adducts and GSH competitively reacted with agonists on HEK293-hsTRPA1 cells.

The supporting information is available online at csb.scichina.com and www.springerlink.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.