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Inhibition of TRPC5 Channels by Intracellular ATP[S]

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ABSTRACT

TRPC5 channels are Ca^{2+}-permeable nonselective cation channels activated by G-protein-coupled receptors, although the mechanisms responsible for channel activation and regulation are poorly understood. Carbachol-activated TRPC5 currents were recorded by the whole-cell patch clamp technique from human embryonic kidney 293 cells transiently transfected with TRPC5 and the M1 muscarinic receptor. Some published studies of TRPC5 currents have included ATP and/or GTP in the patch pipette, whereas others used an ATP- and GTP-free pipette solution. We initially included these two nucleotides in the patch pipette but found that TRPC5 currents were absent or were very small. Recordings made with an ATP- and GTP-free pipette solution produced large and robust TRPC5 currents. Under these conditions, treatment of cells with *Pasteurella multocida* toxin, a selective inhibitor of $G\alpha_q$, almost abolished TRPC5 currents indicating that $G\alpha_q$ is necessary for activation of TRPC5 by the M1 receptor. To study the effect of intracellular ATP on TRPC5 channels, an intracellular perfusion system was used. Perfusion of ADP or control pipette solution had no effect, whereas perfusion of ATP or AMP-PNP, a nonhydrolyzable analog of ATP, significantly inhibited TRPC5 currents. Thus, the effects of ATP have structural specificity and probably involve a direct effect on the channel rather than a phosphorylation-mediated effect. The activity of TRPC5 channels may be linked to cellular metabolism via changes in ATP levels and could be involved in Ca^{2+} overload occurring after ischemia when ATP is depleted.

TRPC5 channels are nonselective cation channels composed of subunits that probably have intracellular N and C termini, six transmembrane domains, and a pore-forming re-entrant loop between the fifth and sixth transmembrane domains. The subunits can assemble as homomeric channels or as heteromers with the related TRPC1, -3, or -4 subunits (Strübing et al., 2001, 2003; Goel et al., 2002). TRPC5 channels are expressed in many tissues including the brain where high levels are found in CA1 pyramidal cells, the olfactory bulb, amygdala, cingulate gyrus, and cerebellar nuclei (Riccio et al., 2002; Plant and Schaefer, 2003). The channels can regulate neurite extension and may play a role in the development of the nervous system (Greka et al., 2003) and in the induction of some forms of long-term potentiation (Topolnik et al., 2006). In peripheral tissues, high levels of TRPC5 have been detected in the heart and lungs (Riccio et al., 2002). In the heart, increased expression of TRPC5 promotes the induction of cardiac hypertrophic factors (Bush et al., 2006), presumably contributing to hypertrophy seen in heart failure. In addition, increased expression of TRPC5 and increased TRPC5 currents have been reported in patients with essential hypertension (Liu et al., 2006).

Because TRPC5 channels are highly permeable to Ca^{2+} (Plant and Schaefer, 2003), excessive or prolonged activation of these channels could lead to Ca^{2+} overload and Ca^{2+}-mediated cell death. In this regard, it has been reported that mutations that produce constitutively active TRP channels in *Drosophila melanogaster* photoreceptors cause cell death (Yoon et al., 2000). In addition, TRPM7, a nonselective Ca^{2+}-permeable channel that is distantly related to the TRPC subfamily, has been shown to be involved in cell death after ischemia (Aarts et al., 2003).

Although a physiological role for TRPC5 channels in the heart or mature nervous system has not yet been documented, understanding the mechanisms involved in activation and regulation of these channels will be necessary to understand their roles in physiological and pathological processes. At least two signaling pathways can activate TRPC5 channels. In the mouse, the channels seem to be activated only by G-protein-coupled receptors (GPCRs) (Strübing et al.,

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ABBREVIATIONS: TRPC1, -3, -4, or -5, canonical transient receptor potential channel, subfamily 1, 3, 4, or 5, respectively; TRPM4b or 7, melastatin transient receptor potential, subfamily 4b or 7, respectively; GPCR, G-protein-coupled receptor; PLC, phospholipase C; eGFP, enhanced green fluorescent protein; HEK, human embryonic kidney; PMT, *Pasteurella multocida* toxin; I-V, current-voltage; AMP-PNP, adenyl-5′-yl imidodiphosphate; BAPTA, 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate; PIP_2, phosphatidylinositol 4,5-bisphosphate; Kir, inward rectifier potassium channel; K_{ATP}, ATP-sensitive potassium channel.

2001), but in some species, including rabbit and human, TRPC5 channels can also be store-operated, being activated by depletion of intracellular Ca^{2+} stores (Philipp et al., 1998; Zeng et al., 2004). GPCR activation of TRPC5 channels is mediated by phospholipase C (PLC), although the exact mechanisms downstream of PLC that are involved in channel activation are unclear (Schaefer et al., 2000; Plant and Schaefer, 2005).

The activity of many types of ion channels can be modulated by extracellular or intracellular factors. Extracellular modulation of TRPC5 channels by the trivalent cations La^{3+} and Gd^{3+} has been documented—these ions increase macroscopic TRPC5 currents (Jung et al., 2003). However, there is little information regarding intracellular modulators of TRPC5 channels beyond the factors thought to be responsible for gating these channels (Schaefer et al., 2000; Plant and Schaefer, 2005). We have found that intracellular ATP profoundly inhibits TRPC5 channels and suggest that ATP may be a key endogenous regulator of channel activity.

Materials and Methods

Plasmid Preparation. Plasmids were maintained in vectors suitable for mammalian cell expression (pcDNA3 or pcDNA3.1) and propagated in *Escherichia coli* DH5α. The mouse TRPC5 clone was a gift from Dr. Michael Schaefer (Freie Universität, Berlin, Germany); the muscarinic receptor M1 clone was from the Guthrie cDNA Resource Center (Rolla, MO); eGFP was from Clontech (Mountain View, CA). Plasmids for transfection were prepared using a QIAGEN HiSpeed Maxi Kit (QIAGEN Corporation, Valencia, CA).

Cell Culture. HEK-293 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in 5% CO_2 at 37°C in a humidified incubator. Two to three days before transfection, cells were plated in 35-mm Nunclon dishes (Thermo Fisher Scientific, Waltham, MA). Cells were transfected with 1.5 μg each of the TRPC5 and/or M1 plasmids and 0.4 μg of eGFP plasmid by the calcium phosphate method or the Lipofectamine method using kits from Invitrogen (Carlsbad, CA). The eGFP serves as a marker to identify transfected cells by fluorescence microscopy. Six hours after transfection, the medium was replaced with fresh medium. Twenty-four hours after transfection, cells were replated onto poly-L-lysine–coated glass coverslips in 35-mm Nunclon dishes. In some experiments, the selective $G\alpha_q$ inhibitor, *Pasteurella multocida* toxin (PMT, 1 μg/ml), was added to the growth medium 24 h before whole-cell recordings. Currents were recorded 48 to 72 h after transfection. Transfection efficiency was approximately 40%. Chemicals and culture media were purchased from Sigma-Aldrich Corporation (St. Louis, MO).

Electrophysiology: Solutions. The standard external buffer contained 140 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES; pH adjusted to 7.4 with NaOH. The standard pipette solution contained 120 mM CsOH, 120 mM gluconic acid, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 5 mM Cs_4-BAPTA, and 10 mM HEPES; pH adjusted to 7.3 with gluconic acid. For Mg^{2+}-free pipette solution, 2 mM $MgCl_2$ was substituted with 3 mM NaCl. Free Ca^{2+} for the standard pipette solution or Mg^{2+}-free pipette solution was calculated to be ~100 nM using the CaBuf program (ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip). Mg^{2+}-free pipette solution was used in studies where NaATP, MgATP, NaADP, LiAMP-PNP, NaGTP, $MgCl_2$ and LiCl were added to the pipette solution or intracellular perfusion solution. In experiments in which 4 mM NaATP or 4 mM MgATP was added to Mg^{2+}-free pipette solution, free ATP^{4-} was calculated to be ~150 or ~40 nM, respectively (Cockcroft and Gomperts, 1979) and total free ATP in various forms, including ATP^{3-} was calculated to be ~4 or ~0.5 mM, respectively (WebMax C, available at http://www.stanford.edu/~cpatton/downloads.htm). MgATP, NaATP, NaADP, LiAMP-PNP, $MgCl_2$, NaGTP and LiCl were prepared as 250 mM stock solutions, separated into aliquots, and stored at −80°C until use. On the day of recording, aliquots were thawed and added to the pipette solution or intracellular perfusion solution, and the pH was corrected. Cs_4-BAPTA was purchased from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Whole Cell Recording. Currents were recorded from HEK-293 cells using the patch-clamp technique in the whole-cell mode at room temperature. Glass coverslips with transfected cells were placed in a recording chamber on the stage of an inverted Nikon TS-100 microscope equipped with fluorescence capabilities. Cells were voltage clamped at −60 mV and currents were recorded for 300 ms of every 4 s at −60 mV and by stepping to +80 mV for 150 ms (Supplementary Fig. 1). Steady-state currents were measured by averaging the amplitude of the current recorded over ~100 ms at each holding potential (Supplementary Fig. 1). An AxoPatch 200B amplifier (Molecular Devices, Sunnyvale, CA) was used to amplify whole-cell currents. The series resistance circuit of the amplifier was used to compensate 80% of the apparent series resistance. Clamp settling time was typically less than a millisecond. Leak-subtraction was performed off-line by subtracting the current immediately before carbachol application from the maximal current in the presence of carbachol to determine the carbachol-induced current. The data shown in the current versus time traces in Fig. 1, A and B, 3, A–D, and Supplementary Fig. 2A were not leak-subtracted because the currents before application of carbachol consisted of leak current and any constitutive TRPC5 current. A fast-flow extracellular perfusion system located near the tip of the recording electrode superperfused cells with varying concentrations of carbachol (2–20 μM) to activate TRPC5 currents. Although the size of the average response varied from day to day, experimental and control conditions were studied in the same batches of transfected cells, and recordings from each group were carried out on the same day. Because HEK-293 cells have been reported to express endogenous M1 receptors, initial whole-cell recordings were done on cells transfected with TRPC5 and eGFP DNA, with and without the M1 receptor. Only 4 of 14 cells (28%) not transfected with the M1 receptor responded to carbachol, whereas 7 of 8 cells (88%) transfected with the M1 receptor responded to carbachol; thus, we routinely transfected cells with the M1 receptor together with TRPC5 and eGFP.

After stable TRPC5 currents were obtained, intracellular perfusion, via a perfusion system within the recording electrode (ALA Scientific Instruments, Inc., Westbury, NY), was used to introduce either control solutions or nucleotides into the patch pipette and thus into the cell (Supplementary Fig. 2). Using this system, nucleotides such as ATP or AMP-PNP were introduced into the pipette and the cell, but could not be subsequently washed out. The final free concentration is unknown because of dilution of the nucleotides with the pipette solution and cell cytosol. Voltage ramps from −80 mV to +100 mV over 100 ms were applied periodically to assess current-voltage (I-V) relationships. Control voltage ramps were measured before application of carbachol and were subtracted from I-V relationships measured in the presence of carbachol or carbachol plus nucleotides to obtain leak-subtracted carbachol-induced currents.

Data Analysis. Data were analyzed with SigmaPlot version 8.02 (Systat Software, Inc., Point Richmond, CA) or Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA). Statistical analyses were carried out using KaleidaGraph version 4.0 (Synergy Software, Inc., Reading, PA). An unpaired t test ($\alpha = 0.05$) was used to compare carbachol- or gadolinium-induced TRPC5 currents in the presence or absence of PMT. Analysis of variance with a post hoc Dunnett test ($\alpha = 0.05$) was used to compare control carbachol-induced TRPC5 currents with carbachol-induced TRPC5 currents in the presence of ATP, ADP, AMP-PNP, or $MgCl_2$.

Results

Nucleotides in the Patch Pipette Inhibited TRPC5 Currents. In initial experiments designed to study the pharmacology of TRPC5, we used whole-cell patch-clamp recording of HEK-293 cells expressing TRPC5 and the M1 muscarinic receptor. Because the M1 receptor is a GPCR and stimulation of the M1 receptor has been reported to gate TRPC5 channels (Strübing et al., 2001), we initially included ATP and GTP in the patch pipette because we assumed these nucleotides would be necessary to maintain G-protein-mediated activity. Under these conditions, TRPC5 currents were undetectable or very small. Some studies of TRPC5 currents have used a pipette solution without added GTP and ATP (Supplementary Table 1). In intact cells under normal conditions, the intracellular concentration of ATP is maintained in the millimolar range, and the intracellular concentration of GTP is approximately 500 μM (Traut, 1994; Silver et al., 1997). However, during whole-cell recording using a pipette solution that lacks ATP and GTP, rapid dialysis between the pipette solution and the inside of the cell substantially lowers the intracellular concentration of ATP and GTP. Using a patch pipette solution that lacks GTP and ATP, we were subsequently able to record robust carbachol-induced TRPC5 currents (Fig. 1, A and C). To determine the effect of ATP and GTP on TRPC5 currents, we then recorded currents using pipette solutions that contained either ATP or GTP and made the surprising discovery that inclusion of MgATP in the patch pipette almost abolished TRPC5 currents (Fig. 1). With 4 mM MgATP in the pipette, carbachol activated only very small currents (34 ± 8 pA, $n = 9$; Fig. 1, B and C), whereas TRPC5 currents were large and robust in the absence of MgATP (971 ± 177 pA, $n = 9$; Fig. 1, A and C). Likewise, with 500 μM GTP but no ATP in the patch pipette, carbachol-induced TRPC5 currents were very small (−49.6 ± 43.8 pA at −60 mV and 62.7 ± 60.7 pA at +80 mV, $n = 17$) compared with currents recorded in the absence of GTP (−588 ± 192 pA at −60 mV and 494 ± 151 pA at +80 mV, $n = 15$).

Without ATP or GTP in the pipette, a low concentration of carbachol (2 μM) produced currents that developed maximally over 25 to 40 s and then decayed slowly, whereas higher concentrations of carbachol (10–20 μM) produced more stable currents (Fig. 1A). The I-V relationship (Fig. 1A, inset) showed a double rectification characteristic of TRPC5 channels (Schaefer et al., 2000; Strübing et al., 2001). Carbachol induced no current in cells transfected with the M1 receptor and eGFP in the absence of TRPC5 (data not shown), indicating that the ATP-sensitive current was indeed mediated through TRPC5.

Carbachol-Induced Activation of TRPC5 Currents Required a G-Protein. Stimulation of GPCRs, such as the M1 receptor, can activate TRPC5 channels (Strübing et al., 2001). However, in many reported studies of whole-cell patch-clamp recordings of TRPC5 channels, GTP was not included in the patch pipette (Supplementary Table 1) and, as described above, inclusion of GTP in the patch pipette actually inhibits TRPC5 currents. Because GTP is required for G-protein-mediated signaling and therefore for the maintenance of GPCR responses, this raises the question of whether G-proteins really are involved in the activation of TRPC5 channels by GPCRs. We therefore carried out experiments to address this issue directly.

Because the M1 receptor can activate signaling cascades via the G-proteins G_q and G_{11}, we studied the effects of PMT, a selective $G\alpha_q$ inhibitor (Wilson and Ho, 2004) on M1 receptor-activated TRPC5 currents to determine whether G_q is involved. No selective inhibitor of G_{11} is currently available. In these experiments, currents were recorded in control cells and in cells incubated with PMT (1 μg/ml) for 24 h before whole-cell recording. This treatment has been shown to reduce or abolish active $G\alpha_q$ (Wilson and Ho, 2004). Currents through TRPC5 channels were obtained by bath application of either 10 μM carbachol, which activates the M1 receptor, or 100 μM Gd^{3+}, which may potentiate spontaneous channel activity or may activate the channel by a mechanism that does not require G-proteins (Jung et al., 2003; Zeng et al., 2004). All recordings were done without ATP and GTP in the pipette solution. As shown in Fig. 2A and B, in control cells, 10 μM carbachol produced robust TRPC5 currents (−231 ± 84 pA at −60 mV and 324 ± 115 pA at +80 mV, $n = 25$) with a doubly rectifying I-V relationship characteristic of TRPC5 currents (Schaefer et al., 2000; Strübing et al., 2001). In cells treated with PMT (Fig. 2, A and D) carbachol produced only very small currents (−21 ± 16 pA at −60 mV and 21 ± 35 pA at +80 mV, $n = 30$), indicating that M1 receptor-induced

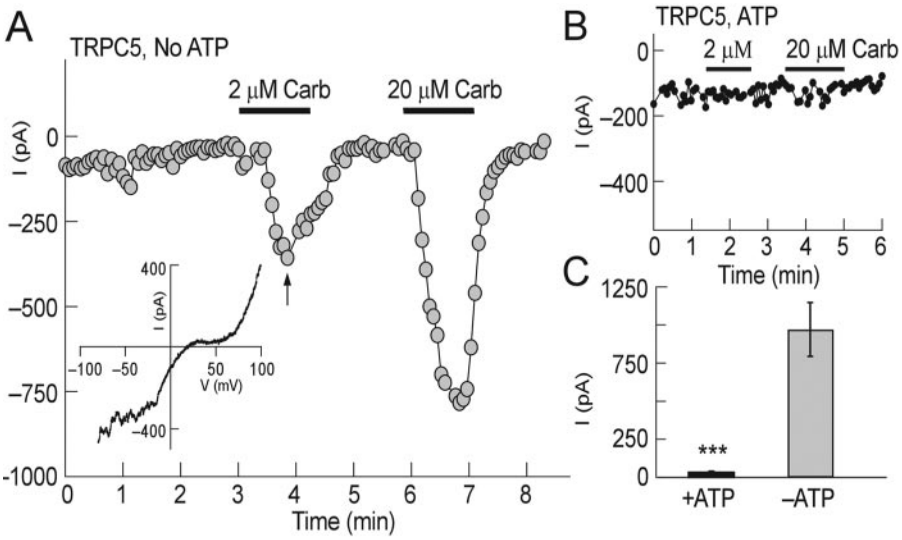

Fig. 1. Intracellular ATP inhibits TRPC5 currents. A and B, whole-cell currents at −60 mV were recorded from individual HEK-293 cells expressing M1 receptors and TRPC5, without (A) or with (B) 4 mM MgATP in the patch pipette. Carbachol (Carb; 2 or 20 μM) was applied during the times shown by the horizontal bars. A, inset, I-V relationship of the carbachol-induced current measured at the time shown by the arrow. C, maximum TRPC5 currents activated by 20 μM carbachol with and without 4 mM MgATP in the pipette (mean ± S.E.M., $n = 9$; ***, $p < 0.0005$).

activation of TRPC5 channels is indeed mediated via the G_q protein. In contrast, currents induced by Gd^{3+} in the absence of carbachol were similar in control cells (−226 ± 96 pA at −60 mV and 227 ± 93 pA at +80 mV, $n = 25$) and in cells treated with PMT (−222 ± 96 pA at −60 mV and 143 ± 83 pA at +80 mV, $n = 20$), (Fig. 2, A, C, and E). This indicates that activation by Gd^{3+} does not require G_q and that PMT does not have a general, nonspecific effect on TRPC5 currents.

Intracellular Perfusion of ATP Inhibited TRPC5 Currents. To study the effect of ATP on TRPC5 currents within the same cell, an intracellular perfusion system was used. Whole-cell currents were initially recorded without ATP in the pipette, and subsequently after introduction of ATP or other nucleotides via the perfusion system (Supplementary Fig. 2). Currents were recorded at 4-s intervals at −60 mV and after briefly stepping to +80 mV, and I-V relationships were monitored by using voltage ramps. In some experiments, spontaneous TRPC5 currents were seen in the absence of carbachol as determined by the characteristic TRPC5 I-V relationship (Supplementary Fig. 3). In control experiments, perfusion of pipette solution did not alter spontaneous TRPC5 currents (data not shown) or affect carbachol-induced TRPC5 currents (Fig. 3A).

Because intracellular Mg^{2+} can affect TRPC5 currents (Obukhov and Nowycky, 2005) and can reduce the level of free ATP^{4-} (Cockcroft and Gomperts, 1979), we also studied the effects of NaATP. Intracellular perfusion of 4 mM NaATP inhibited spontaneous and carbachol-induced TRPC5 currents (Fig. 3B). After perfusion of ATP, subsequent applications of carbachol did not elicit a current, suggesting that the effect is sustained because ATP cannot be washed out using the pipette perfusion system. In the absence of ATP, repeated applications of carbachol still induced TRPC5 currents, albeit of smaller magnitude than the initial current (data not shown). NaATP inhibited TRPC5 currents across a voltage range of −80 to +100 mV, with the effect showing little or no voltage dependence, although in some cells, a slight relief of

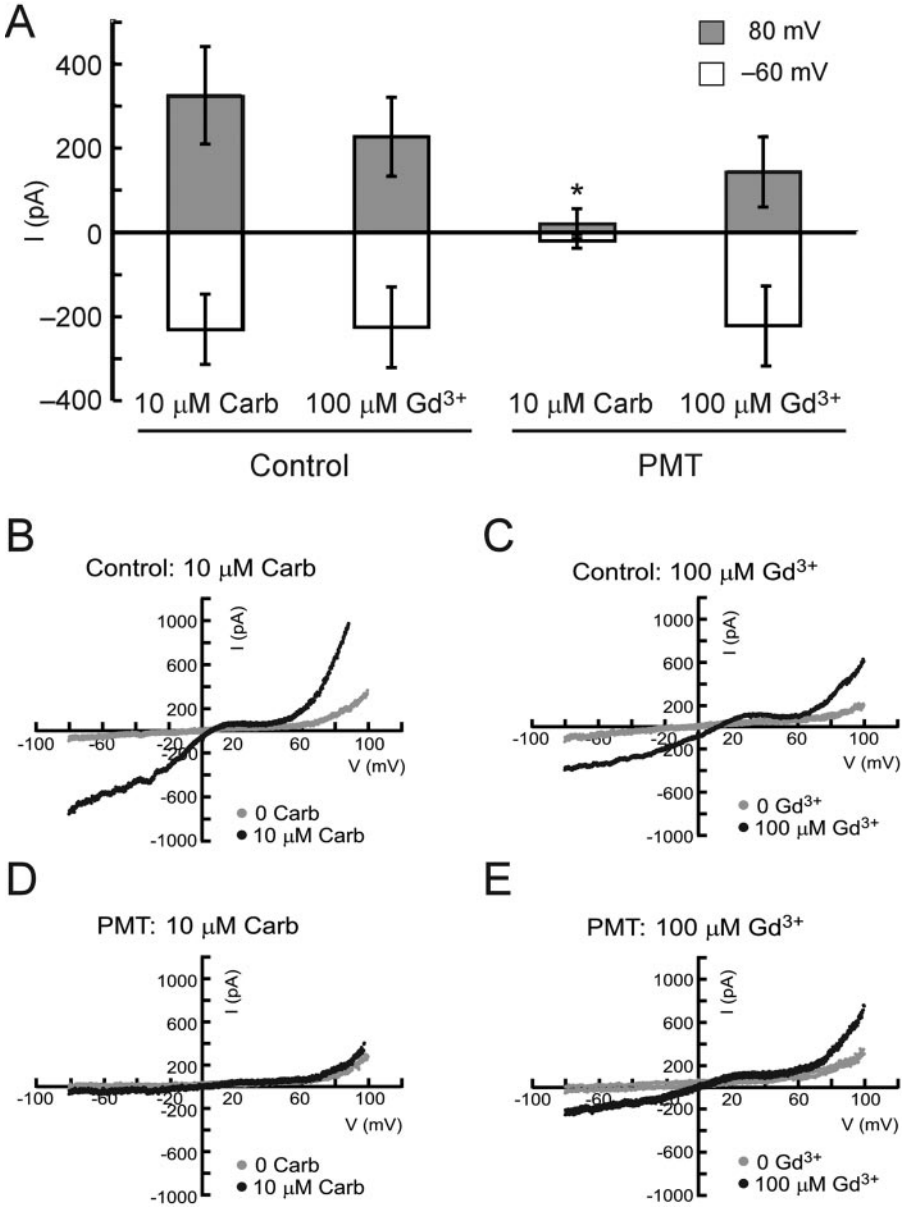

Fig. 2. PMT inhibits M1-induced activation of TRPC5 channels. A, whole-cell currents at −60 mV and +80 mV were recorded from control and PMT-treated HEK-293 cells expressing M1 receptors and TRPC5 channels. Peak currents were measured as shown in Supplementary Fig. 1. Carbachol (10 μM) or Gd^{3+} (100 μM) was applied extracellularly to activate TRPC5 channels. In control cells, TRPC5 currents activated by carbachol were 324 ± 115 pA at +80 mV and −231 ± 84 pA at −60 mV ($n = 25$), whereas Gd^{3+}-induced TRPC5 currents were 227 ± 93 pA at +80 mV and −226 ± 96 pA at −60 mV ($n = 15$). In cells incubated for 24 h in PMT (1 μg/ml), carbachol-activated TRPC5 currents were 21 ± 35 pA at +80 mV and −21 ± 16 pA at −60 mV ($n = 30$), whereas Gd^{3+}-induced TRPC5 currents were 143 ± 83 pA at +80 mV and −222 ± 96 pA at −60 mV ($n = 20$). Values are mean ± S.E.M., $n = 15$–30 cells; *, $p < 0.05$ compared with 10 μM carbachol control. B–E, representative I-V relationships of TRPC5 currents induced by carbachol (B and D) and Gd^{3+} (C and E) in control cells (B and C) and PMT-treated cells (D and E).

inhibition was observed at very depolarized potentials (Fig. 3B, inset). Likewise, steady-state currents were markedly inhibited by NaATP at +80 and −60 mV (Figs. 3B and 4). The effect of ATP was concentration-dependent; 400 μM NaATP reduced TRPC5 currents by 40 ± 21%, whereas 4 mM NaATP reduced currents by 82 ± 6%. In some cells, Gd^{3+} (100 μM) was also applied extracellularly. In these cells, TRPC5 currents were measured in the presence of carbachol and subsequently in the presence of carbachol plus 100 μM Gd^{3+}. Gd^{3+} increased the currents from −127 ± 27 pA to −642 ± 99 pA at −60 mV and from 205 ± 61 pA to 519 ± 135 pA at +80 mV (mean ± S.E.M., n = 11 cells). Gd^{3+} did not alter the effects of ATP or other nucleotides on TRPC5 currents. Control carbachol-induced TRPC5 currents showed little run-down (8 ± 11%) over 80 s when perfused with pipette solution (Fig. 4). We also studied intracellular perfusion of MgATP and found that its effects were similar to those of NaATP (Fig. 4).

To further rule out effects of Mg^{2+} on the ATP-mediated inhibition of TRPC5 currents, we recorded currents before and after intracellular perfusion of 4 mM $MgCl_2$, which had no effect on carbachol-induced TRPC5 currents (Fig. 5). Before perfusion of 4 mM $MgCl_2$, currents were −256 ± 98 pA at −60 mV and 1239 ± 260 pA at +80 mV; 80 s after the onset of perfusion of $MgCl_2$, currents were −240 ± 69 pA at −60 mV and 1062 ± 227 pA at +80 mV. Intracellular perfusion of $MgCl_2$ had no effect on steady-state TRPC5 currents or on the I-V relationship (Fig. 5) suggesting that the block between approximately +10 and +40 mV (Obukhov and Nowycky, 2005) was already maximal with the residual endogenous intracellular Mg^{2+}.

AMP-PNP, but Not ADP, Inhibited TRPC5 Currents. Intracellular ATP could have a direct effect on the TRPC5 channel or a closely associated protein or it could have an indirect effect by altering the activity of a protein kinase, for example by altering protein kinase C-mediated desensitization of TRPC5 currents (Zhu et al., 2005). To distinguish between these possibilities, and to determine whether there is structural specificity to the effects of ATP, we studied two related nucleotides, ADP and AMP-PNP. In contrast to ATP, ADP had no effect on steady-state TRPC5 currents (Figs. 3C and 4). These results indicate that the γ-phosphate of ATP is necessary for inhibition of TRPC5. Therefore, a nonhydrolyzable analog of ATP, AMP-PNP, was also studied to determine the role of the γ-phosphate. AMP-PNP cannot donate its γ-phosphate; therefore, it is unable to serve as a substrate for kinase activation or to participate directly in phosphorylation reactions. Because AMP-PNP is a Li^+ salt, the effect of LiCl was also tested. LiCl had no effect on TRPC5 currents (89 ± 7% of control at +80 mV and 84 ± 19% of control at −60 mV, measured 80 s after the onset of intracellular perfusion) compared with pipette solution (86 ± 10% of control at +80 mV and 83 ± 10% of control at −60 mV, measured 80 s after the onset of intracellular perfusion). AMP-PNP, like ATP, inhibited TRPC5 currents (Figs. 3D and 4), indicating that ATP does not act as a phosphorylation substrate. Thus, the effects of AMP-PNP and ATP on TRPC5 currents are not likely to be due to changes in phosphorylation, and may involve a direct effect on the channel.

Discussion

Previous reports have shown that TRPC5 channels can be activated by a number of different GPCRs, and this activation was presumed to be mediated by G_q or G_{11}, although there was no direct evidence for the involvement of a particular G-protein (Schaefer et al., 2000; Strübing et al., 2001; Plant and Schaefer, 2003, 2005). Stimulation of the M1 receptor leads to activation of PLC by G-proteins of the G_q family, including G_q and G_{11}, which are ubiquitously expressed, have a very high degree of sequence homology, and are indistinguishable with regard to activation of PLCβ (Hubbard and Hepler, 2006). PMT has been reported to selectively inhibit G_q but to have no effect on G_{11}, making it a

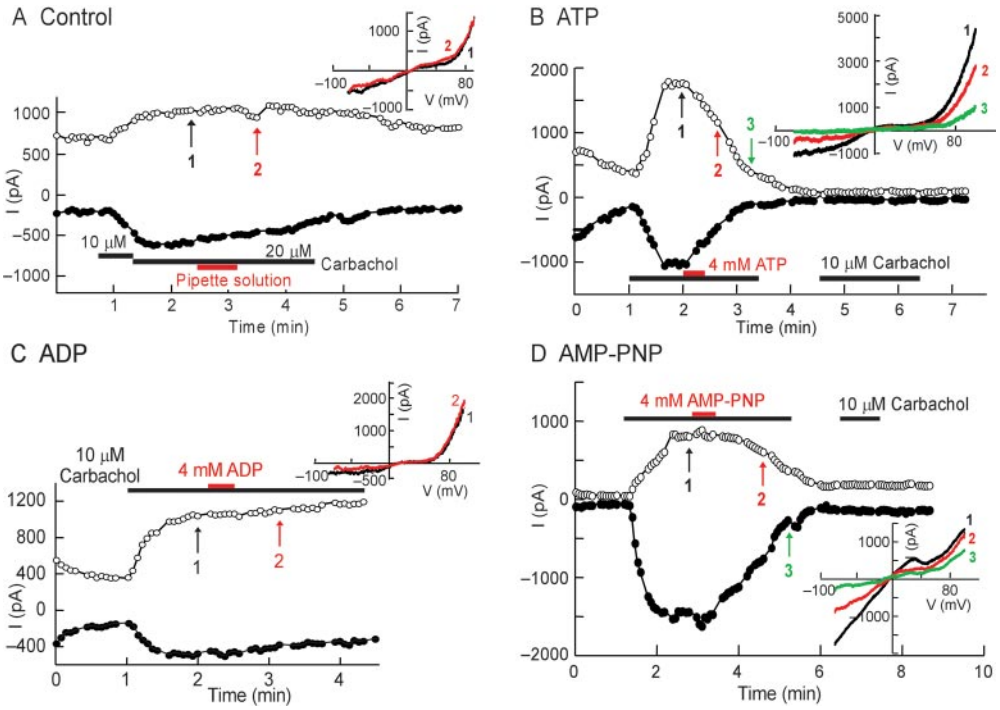

Fig. 3. Effects of intracellular perfusion of nucleotides on TRPC5 currents. In all panels, currents were measured every 4 s at −60 mV (●) and after stepping transiently to +80 mV (○). Application of carbachol is shown by black horizontal bars; intracellular perfusion of pipette solution or nucleotides (4 mM) by red horizontal bars; arrows and numbers indicate where voltage ramps were applied (see insets with corresponding numbers). Intracellular perfusion of pipette solution did not alter TRPC5 currents (A), whereas currents were inhibited by NaATP (B) and AMP-PNP (D) but not by NaADP (C).

valuable tool to distinguish between these two G-proteins (Wilson and Ho, 2004). We found that treatment of cells with PMT almost abolished the M1-mediated activation of TRPC5 currents, thus demonstrating that activation of TRPC5 channels by the M1 receptor is indeed mediated by G_q, because PMT inactivates the α subunit of G_q.

In general, the presence of GTP is required for the activation of G-proteins and the maintenance of GPCR responses; however, inclusion of GTP in the patch pipette significantly reduced whole-cell TRPC5 currents. Although exogenous GTP is not required for activation of TRPC5 channels, and the intracellular concentration of GTP is lower after dialysis with the GTP-free pipette solution, there is presumably still GTP remaining inside the cell. It is likely that the lowered concentration of GTP is still sufficient to support the G_q-mediated activation of TRPC5 channels. Alternatively, it may be that GTP is not required for G_q to activate and maintain TRPC5 currents. In this regard, it has been suggested that G-proteins can be activated independent of guanine nucleotide exchange (Ugur et al., 2005). In the study by Ugur et al. (2005), the β_2-adrenoreceptor-Gs system was activated in the absence of GTP and in the presence of GDP or GDPβs, a phosphorylation resistant analog of GDP (Ugur et al., 2005).

The major finding of the present work was that intracellular ATP profoundly inhibited TRPC5 currents. Because ATP is vital for the maintenance of cellular energy processes and ultimately for the viability of cells, intracellular levels of ATP are tightly regulated. Total ATP levels have been reported to be around 6 mM in glia and neurons (Traut, 1994; Silver et al., 1997), although the free cytosolic concentration is difficult to assess and is probably lower than 6 mM because of compartmentalization and binding of ATP to intracellular cations. If the free ATP concentration in the local microenvironment of TRPC5 channels is sufficiently low because of compartmentalization or buffering by intracellular cations, then TRPC5 channels will be activated under normal physiological conditions and could act as sensors of cellular metabolism and viability, responding to subtle changes in ATP levels analogous to the role of K^+-ATP channels in pancreatic

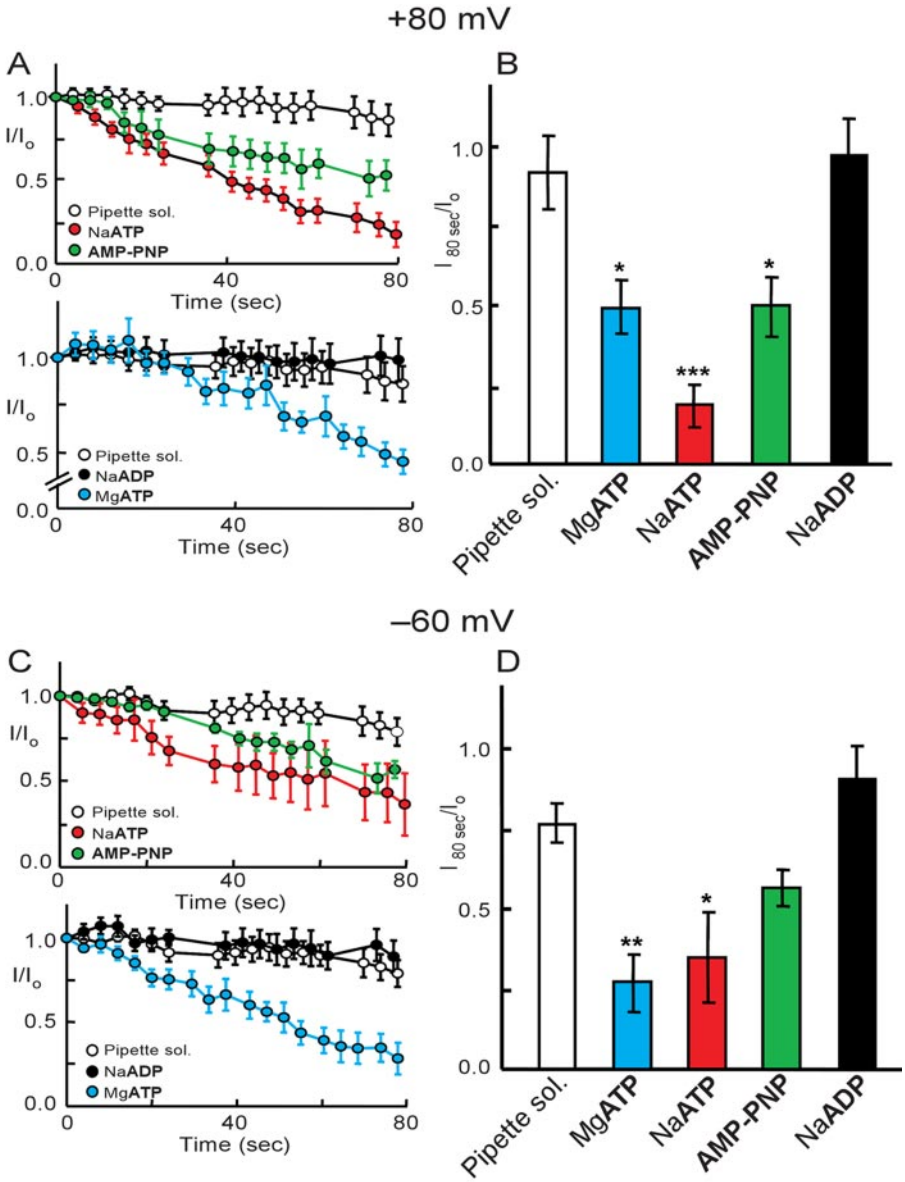

Fig. 4. Effects of nucleotides on TRPC5 currents recorded at +80 mV (A and B) and −60 mV (C and D). TRPC5 currents were recorded at 4-s intervals in cells voltage-clamped at −60 mV. A and C, values were obtained by subtracting the leak current (measured immediately before application of carbachol) from the carbachol-induced current. Carbachol-induced currents were then normalized to the current at the onset of intracellular perfusion (I_o). Carbachol-induced currents just before onset of intracellular perfusion were not significantly different in each of the groups. Currents measured 437 ± 142 pA (pipette solution, $n = 7$), 671 ± 155 pA (MgATP, $n = 5$), 645 ± 166 pA (NaATP, $n = 6$), 543 ± 101 pA (AMP-PNP, $n = 5$), and 614 ± 114 pA (NaADP, $n = 9$) at +80 mV and −627 ± 187 pA (pipette solution, $n = 7$), −659 ± 239 pA (MgATP, $n = 5$), −466 ± 165 pA (NaATP, $n = 6$), −624 ± 188 pA (AMP-PNP, $n = 5$), and −482 ± 111 pA (NaADP, $n = 9$) at −60 mV. B and D, the fractional TRPC5 current measured at 80 s (I_{80sec}) after the onset of perfusion for each group. Values are mean ± S.E.M., $n = 5$–9 cells; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0005$ compared with pipette solution.

islet β-cells (Kennedy et al., 1999). In this regard, it has been reported that under conditions of metabolic stress, such as oxygen-glucose deprivation, and in some pathological conditions, such as epilepsy and stroke, levels of ATP decline acutely as a result of increased consumption and/or decreased production of ATP (Ferrari et al., 1993; Harkness, 1997). An acute fall in ATP levels under conditions of increased metabolic stress may cause relief of the ATP-mediated inhibition of TRPC5 channels leading to a large influx of Ca^{2+} ions and subsequent cell death.

In a survey of the literature, we found 24 papers from 13 different research groups reporting whole-cell patch-clamp recordings of recombinant TRPC5 channels (Supplementary Table 1). Of those 13 research groups, seven used recording conditions with no ATP and no GTP in the patch pipette, and the other six groups used 1 to 5 mM ATP, in some cases together with GTP, in the patch pipette. In our studies, inclusion of 4 mM ATP in the patch pipette produced an average reduction of TRPC5 currents of approximately 80%. Thus, it is conceivable that in studies where ATP and/or GTP is commonly included in the pipette solution (e.g., numbers 8–13 in Supplementary Table 1), the TRPC5 current measured in the presence of ATP might be markedly smaller than it would otherwise be without ATP.

Intracellular ATP is known to affect several classes of ion channels including some Ca^{2+}-activated cation channels (Liman, 2003), K^+ channels (Ashcroft, 1988; Nichols, 2006), and some TRP channels (Nilius et al., 2004). A direct effect of ATP on TRPC5 channels may be analogous to effects on the Kir6.2 subunit of K_{ATP} channels, which are strongly inhibited by intracellular ATP (Ashcroft, 1988; Nichols, 2006). Unlike K_{ATP} channels, which are sensitive to both ATP and ADP (Ashcroft, 1988; Nichols et al., 1996), TRPC5 channels were unaffected by ADP. The TRPM4b (Nilius et al., 2004) and TRPM7 channels are also inhibited by intracellular ATP, although inhibition of TRPM7 seems to require a MgATP complex rather than free ATP (Nadler et al., 2001; Demeuse et al., 2006). Like TRPM4b and TRPM7, TRPC5 channels may monitor changes in intracellular ATP levels, with enhanced opening under conditions where ATP levels are reduced, thereby serving as a link between cellular metabolism, cellular excitability, and Ca^{2+} influx. If the ATP binding site is located on the TRPC5 channel, it is possible that GTP also acts at this site to reduce channel activity, similar to effects of GTP on Kir 6.2 channels (Trapp et al., 1997). In any event, the effects of the nucleotides studied in this report clearly have structural specificity, with ATP and AMP-PNP but not ADP inhibiting TRPC5 currents.

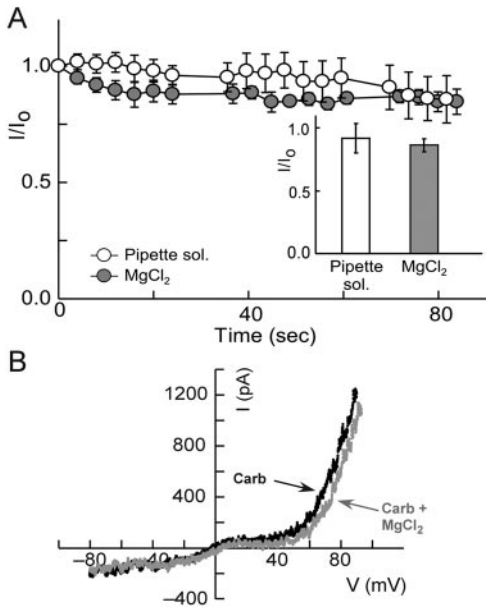

Fig. 5. Intracellular perfusion of $MgCl_2$ does not alter TRPC5 currents. TRPC5 currents were recorded at 4-s intervals in cells voltage-clamped at −60 mV and briefly stepped to +80 mV. A and inset, values were obtained by subtracting the leak current (measured immediately before application of carbachol) from the carbachol-induced current at +80 mV. Carbachol-induced currents were then normalized to the current at the onset of intracellular perfusion (I_o). Carbachol-induced currents immediately before onset of intracellular perfusion were not significantly different between the two groups of cells: 608 ± 166 pA (pipette solution) and 1239 ± 260 pA ($MgCl_2$) at +80 mV and −712 ± 182 pA (pipette solution) and −256 ± 98 pA ($MgCl_2$) at −60 mV. B, representative I-V relationship of carbachol-induced TRPC5 current before the onset of intracellular perfusion (Carb) and 80 s after the onset of intracellular perfusion with 4 mM $MgCl_2$ (Carb + $MgCl_2$).

If ATP does not act directly on the TRPC5 channel, it may act indirectly to inhibit TRPC5 currents by altering the levels of phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_2 has been shown to regulate a number of ion channels, including TRPV, TRPM, and TRPP channels, distant relatives of the TRPC channels (Hardie, 2007; Rohacs, 2007). The level of PIP_2 in a cell is related to the level of intracellular ATP, and ATP is necessary to maintain kinase activity and thus PIP_2 levels (Sun et al., 1995; Rohacs, 2007). It is conceivable that inclusion of ATP in the patch pipette may be sufficient to maintain intracellular PIP_2 levels and, if PIP_2 normally inhibits TRPC5 channels, to dampen TRPC5 currents accounting for the effects of ATP seen in this study. However, this scheme is not supported by our finding that AMP-PNP, which is unable to maintain PIP_2 levels (Xie et al., 1999), also markedly inhibits TRPC5 currents, suggesting that the effects of ATP on TRPC5 channels do not involve modulation of PIP_2 levels.

In the context of ATP regulation of TRPC5 channels, oxygen-glucose deprivation, which leads to reduced levels of ATP, has been reported to open large-conductance hemichannels in hippocampal pyramidal cells leading to neuronal death. Blockade of these channels uncovered a smaller current having characteristics similar to TRPC5 that was also induced by oxygen-glucose deprivation (Thompson et al., 2006). TRPC5 channels may play a role in cell damage and cell death during conditions in which intracellular ATP levels are reduced (for example, in ischemia). An understanding of the regulation of TRPC5 channels by ATP should be important for unraveling the signaling pathways that activate these channels as well as understanding their physiological roles, including their documented effects on neurite extension (Greka et al., 2003) and their reported roles in hypertension (Liu et al., 2006) and cardiomyocyte hypertrophy (Bush et al., 2006).

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