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Inhibition of TRPC5 channels by intracellular ATP

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Abbreviations: TRPC1, 3, 4, or 5, canonical transient receptor potential channel, subfamily 1, 3, 4, or 5, respectively; PLC, phospholipase C; TRPM4b or 7, melastatin transient receptor potential, subfamily 4b or 7, respectively; Kir, inward rectifier potassium channel; eGFP, enhanced green fluorescent protein; HEK, human embryonic kidney; DMEM, dulbecco's modified eagle's medium; AMP-PNP, adenosine 5'-(β,γ -imido)triphosphate; BAPTA, 1,2-bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate; PKC, protein kinase C; K_{ATP} , ATP-sensitive potassium channel; GPCR, G-protein coupled receptor; FBS, fetal bovine serum; PS, penicillin-streptomycin; PMT, *Pasteurella multocida* toxin; PIP_2 , phosphatidylinositol 4,5-bisphosphate

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Abstract

TRPC5 channels are Ca^{2+} -permeable non-selective 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 HEK-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 (PMT), 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 non-hydrolysable 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.

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TRPC5 channels are non-selective cation channels composed of subunits that likely 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 (Goel et al., 2002; Strubing et al., 2001; Strubing et al., 2003). 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 (Plant and Schaefer, 2003; Riccio et al., 2002). 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).

Since 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* photoreceptors cause cell death (Yoon et al., 2000). In addition, TRPM7, a non-selective Ca^{2+} -permeable channel that is distantly related to the TRPC subfamily, has been shown to be involved in cell death following ischemia (Aarts et al., 2003).

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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. TRPC5 channels can be activated by at least two signaling pathways. In the mouse, the channels appear to be activated only by G-protein coupled receptors (GPCRs) (Strubing et al., 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 (Plant and Schaefer, 2005; Schaefer et al., 2000).

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 (Plant and Schaefer, 2005; Schaefer et al., 2000). We have found that intracellular ATP profoundly inhibits TRPC5 channels and suggest that ATP may be a key endogenous regulator of channel activity.

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Materials and Methods

Plasmid Preparation. Plasmids were maintained in vectors suitable for mammalian cell expression (pcDNA3 or pcDNA3.1) and propagated in *E. coli* DH5 α . The mouse TRPC5 clone was a gift from Dr. Michael Schaefer (Freie Universität, Berlin); 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 DMEM/Ham's F12 (1:1) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) in 5% CO₂ at 37 °C in a humidified incubator. Two to three days before transfection, cells were plated in 35 mm Nunclon dishes (Fisher Scientific Company, Suwanee, GA). Cells were transfected with 1.5 μ g each of the TRPC5 and/or M1 plasmids and 0.4 μ g 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 re-plated onto poly-L-lysine coated glass coverslips in 35 mm Nunclon dishes. In some experiments, the selective G_{oq} inhibitor, *Pasteurella multocida* toxin (PMT, 1 μ g/mL), was added to the growth medium 24 hours before whole-cell recordings. Currents were recorded 48-72 hours after transfection. Transfection efficiency was about 40%. Chemicals and culture media were purchased from Sigma-Aldrich Corporation (St. Louis, MO).

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Electrophysiology: Solutions: The standard external buffer contained (in mM): NaCl 140, KCl 5.4, CaCl₂ 2, MgCl₂ 1, glucose 10, HEPES 10, and pH adjusted to 7.4 with NaOH. The standard pipette solution contained (in mM): CsOH 120, gluconic acid 120, MgCl₂ 2, CaCl₂ 3, Cs₄-BAPTA 5, HEPES 10, and pH adjusted to 7.3 with gluconic acid. For Mg²⁺-free pipette solution, 2 mM MgCl₂ was substituted with 3 mM NaCl. Free Ca²⁺ for the standard pipette solution or Mg²⁺-free pipette solution was calculated to be ~100 nM using the CaBuf program (<ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip>). Mg²⁺-free pipette solution was used in studies where NaATP, MgATP, NaADP, LiAMP-PNP, NaGTP, MgCl₂ and LiCl were added to the pipette solution or intracellular perfusion solution. In experiments where 4 mM NaATP or 4 mM MgATP was added to Mg²⁺-free pipette solution, free ATP⁴⁻ was calculated to be ~150 nM or ~40 nM, respectively (Cockcroft and Gomperts, 1979) and total free ATP in various forms including ATP³⁻ was calculated to be ~4 mM or ~0.5 mM, respectively (WebMax C, available at <http://www.stanford.edu/~cpatton/downloads.htm>). MgATP, NaATP, NaADP, LiAMP-PNP, MgCl₂, NaGTP and LiCl were prepared as 250 mM stock solutions, aliquotted, 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 corrected. Cs₄-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

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were recorded for 300 ms out of every 4 seconds 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 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 prior to 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 figures 1A and B, 3A-D, and supplementary figure 2A were not leak-subtracted since the currents prior to 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 to 20 μ M) 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. Since 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

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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 (SPSS Inc., Chicago, IL) or Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA). Statistical analyses were carried out using KaledaGraph 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. ANOVA with a post-hoc Dunnett test ($\alpha = 0.05$) was used to compare control carbachol-induced TRPC5 currents to carbachol-induced TRPC5 currents in the presence of ATP, ADP, AMP-PNP, or $MgCl_2$.

Results

Nucleotides in the patch pipette inhibit 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. Since the M1 receptor is a GPCR and stimulation of the M1 receptor has been reported to gate TRPC5 channels (Strubing 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 about 500 μ M (Silver et al., 1997; Traut, 1994). 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. 1A, 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. 1B, C), whereas TRPC5 currents were large and robust in the absence of MgATP (971 ± 177 pA, $n = 9$; Fig. 1A, C). Similarly, with 500 μ M 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, $n =$

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17) compared to 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 ($2 \mu\text{M}$) produced currents that developed maximally over 25-40 seconds and then decayed slowly, whereas higher concentrations of carbachol ($10\text{-}20 \mu\text{M}$) 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; Strubing et al., 2001). Carbachol did not induce any current in cells transfected with the M1 receptor and eGFP in the absence of TRPC5 (data not shown), indicating that the ATP-sensitive current is indeed mediated through TRPC5.

Carbachol-induced activation of TRPC5 currents requires a G-protein. Stimulation of GPCRs, such as the M1 receptor, can activate TRPC5 channels (Strubing 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. Since 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.

Since 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_{α_q} inhibitor (Wilson and Ho, 2004) on M1 receptor-activated TRPC5 currents to determine if G_q is involved. No selective inhibitor of G_{11} is

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currently available. In these experiments, currents were recorded in control cells and in cells incubated with PMT (1 $\mu\text{g/ml}$) for 24 hours prior to 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; Strubing et al., 2001). In cells treated with PMT, (Fig. 2A 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 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. 2A, C and E). This indicates that activation by Gd^{3+} does not require G_q and that PMT does not have a general, non-specific effect on TRPC5 currents.

Intracellular perfusion of ATP inhibits 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 second 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

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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) nor did it 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 although 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 inhibition was observed at very depolarized potentials (Fig. 3B, *inset*). Similarly, steady-state currents were markedly inhibited by NaATP at $+80$ and -60 mV (Figs. 3B & 4). The effect of ATP was concentration-dependent; $400 \mu M$ NaATP reduced TRPC5 currents by $40 \pm 21\%$, whereas 4 mM NaATP reduced currents by $82 \pm 6\%$. In some cells Gd^{3+} ($100 \mu M$) was also applied extracellularly. In these cells, TRPC5 currents were measured in the presence of carbachol and subsequently in the presence of carbachol plus $100 \mu M Gd^{3+}$. 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 \pm 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-

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down ($8 \pm 11\%$) over 80 seconds 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). Prior to perfusion of 4 mM $MgCl_2$ currents were -256 ± 98 pA at -60 mV and 1239 ± 260 pA at $+80$ mV, and at 80 seconds 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 about $+10$ and $+40$ mV (Obukhov and Nowycky, 2005) was already maximal with the residual endogenous intracellular Mg^{2+} .

AMP-PNP, but not ADP, inhibits 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 PKC-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 & 4). These results indicate that the γ -phosphate of ATP is necessary for inhibition of TRPC5. Therefore, a non-hydrolysable 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

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phosphorylation reactions. Since AMP-PNP is a Li^+ salt, the effect of LiCl was also tested. LiCl had no effect on TRPC5 currents ($89 \pm 7\%$ of control at +80 mV and $84 \pm 19\%$ of control at -60 mV, measured 80 seconds after the onset of intracellular perfusion) compared with pipette solution ($86 \pm 10\%$ of control at +80 mV and $83 \pm 10\%$ of control at -60 mV, measured 80 seconds after the onset of intracellular perfusion). AMP-PNP, like ATP, inhibited TRPC5 currents (Fig. 3D & 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.

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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 (Plant and Schaefer, 2003; Plant and Schaefer, 2005; Schaefer et al., 2000; Strubing et al., 2001). 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 PLC β (Hubbard and Hepler, 2006). Recently, PMT has been reported to selectively inhibit G_q , but to have no effect on G_{11} , making it a 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 , since PMT inactivates the α subunit of G_q .

Normally, 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., the

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β_2 -adrenoreceptor-Gs system was activated in the absence of GTP but in the presence of GDP or GDP β s, a phosphorylation resistant analog of GDP (Ugur et al., 2005).

The major finding of the present work was that intracellular ATP profoundly inhibits TRPC5 currents. Since 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 (Silver et al., 1997; Traut, 1994), although the free cytosolic concentration is difficult to assess and is likely 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 due to 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 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 due to 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 Ca²⁺ ions and subsequent cell death.

In a survey of the literature, we found twenty four papers from thirteen different research groups reporting whole-cell patch clamp recordings of recombinant TRPC5 channels

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(Supplementary Table 1). Of those thirteen 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 about 80%. Thus, it is conceivable that in studies where ATP and/or GTP is commonly included in the pipette solution (e.g., nos. 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 appears to require a MgATP complex rather than free ATP (Demeuse et al., 2006; Nadler et al., 2001). 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

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report clearly have structural specificity, with ATP and AMP-PNP but not ADP inhibiting TRPC5 currents.

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 (PIP₂). PIP₂ 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 PIP₂ in a cell is related to the level of intracellular ATP, and ATP is necessary to maintain kinase activity and thus PIP₂ levels (Rohacs, 2007; Sun et al., 1995). Conceivably, inclusion of ATP in the patch pipette may be sufficient to maintain intracellular PIP₂ levels and, if PIP₂ 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 PIP₂ levels (Xie et al., 1999), also markedly inhibits TRPC5 currents suggesting that the effects of ATP on TRPC5 channels do not involve modulation of PIP₂ 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

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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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References

- Aarts M, Iihara K, Wei WL, Xiong ZG, Arundine M, Cerwinski W, MacDonald JF and Tymianski M (2003) A key role for TRPM7 channels in anoxic neuronal death. *Cell* **115**:863-877.
- Ashcroft FM (1988) Adenosine 5'-triphosphate-sensitive potassium channels. *Annu Rev Neurosci* **11**:97-118.
- Bush EW, Hood DB, Papst PJ, Chapo JA, Minobe W, Bristow MR, Olson EN and McKinsey TA (2006) Canonical transient receptor potential channels promote cardiomyocyte hypertrophy through activation of calcineurin signaling. *J Biol Chem* **281**:33487-33496.
- Cockcroft S and Gomperts BD (1979) Activation and inhibition of calcium-dependent histamine secretion by ATP ions applied to rat mast cells. *J Physiol* **296**:229-243.
- Demeuse P, Penner R and Fleig A (2006) TRPM7 channel is regulated by magnesium nucleotides via its kinase domain. *J Gen Physiol* **127**:421-434.
- Ferrari R, Pedersini P, Bongrazio M, Gaia G, Bernocchi P, Di Lisa F and Visioli O (1993) Mitochondrial energy production and cation control in myocardial ischaemia and reperfusion. *Basic Res Cardiol* **88**:495-512.
- Goel M, Sinkins WG and Schilling WP (2002) Selective association of TRPC channel subunits in rat brain synaptosomes. *J Biol Chem* **277**:48303-48310.
- Greka A, Navarro B, Oancea E, Duggan A and Clapham DE (2003) TRPC5 is a regulator of hippocampal neurite length and growth cone morphology. *Nat Neurosci* **6**:837-845.
- Hardie RC (2007) TRP channels and lipids: from Drosophila to mammalian physiology. *J Physiol* **578**:9-24.

MOL #40899

- Harkness RA (1997) Is post-hypoxic-ischemic cell damage associated with excessive ATP consumption rather than a failure of ATP production? *Acta Paediatr* **86**:1-5.
- Hubbard KB and Hepler JR (2006) Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins. *Cell Signal* **18**:135-150.
- Jung S, Muhle A, Schaefer M, Strotmann R, Schultz G and Plant TD (2003) Lanthanides potentiate TRPC5 currents by an action at extracellular sites close to the pore mouth. *J Biol Chem* **278**:3562-3571.
- Kennedy HJ, Pouli AE, Ainscow EK, Jouaville LS, Rizzuto R and Rutter GA (1999) Glucose generates sub-plasma membrane ATP microdomains in single islet beta-cells. Potential role for strategically located mitochondria. *J Biol Chem* **274**:13281-13291.
- Liman ER (2003) Regulation by voltage and adenine nucleotides of a Ca²⁺-activated cation channel from hamster vomeronasal sensory neurons. *J Physiol* **548**:777-787.
- Liu D, Scholze A, Zhu Z, Krueger K, Thilo F, Burkert A, Streffer K, Holz S, Harteneck C, Zidek W and Tepel M (2006) Transient receptor potential channels in essential hypertension. *J Hypertens* **24**:1105-1114.
- Nadler MJ, Hermosura MC, Inabe K, Perraud AL, Zhu Q, Stokes AJ, Kurotaki T, Kinet JP, Penner R, Scharenberg AM and Fleig A (2001) LTRPC7 is a Mg.ATP-regulated divalent cation channel required for cell viability. *Nature* **411**:590-595.
- Nichols CG (2006) KATP channels as molecular sensors of cellular metabolism. *Nature* **440**:470-476.
- Nichols CG, Shyng SL, Nestorowicz A, Glaser B, Clement JPt, Gonzalez G, Aguilar-Bryan L, Permutt MA and Bryan J (1996) Adenosine diphosphate as an intracellular regulator of insulin secretion. *Science* **272**:1785-1787.

MOL #40899

- Nilius B, Prenen J, Voets T and Droogmans G (2004) Intracellular nucleotides and polyamines inhibit the Ca²⁺-activated cation channel TRPM4b. *Pflugers Arch* **448**:70-75.
- Obukhov AG and Nowycky MC (2005) A cytosolic residue mediates Mg²⁺ block and regulates inward current amplitude of a transient receptor potential channel. *J Neurosci* **25**:1234-1239.
- Philipp S, Hambrecht J, Braslavski L, Schroth G, Freichel M, Murakami M, Cavalie A and Flockerzi V (1998) A novel capacitative calcium entry channel expressed in excitable cells. *Embo J* **17**:4274-4282.
- Plant TD and Schaefer M (2003) TRPC4 and TRPC5: receptor-operated Ca²⁺-permeable nonselective cation channels. *Cell Calcium* **33**:441-450.
- Plant TD and Schaefer M (2005) Receptor-operated cation channels formed by TRPC4 and TRPC5. *Naunyn Schmiedebergs Arch Pharmacol* **371**:266-276.
- Riccio A, Medhurst AD, Mattei C, Kelsell RE, Calver AR, Randall AD, Benham CD and Pangalos MN (2002) mRNA distribution analysis of human TRPC family in CNS and peripheral tissues. *Brain Res Mol Brain Res* **109**:95-104.
- Rohacs T (2007) Regulation of TRP channels by PIP(2). *Pflugers Arch* **453**:753-762.
- Schaefer M, Plant TD, Obukhov AG, Hofmann T, Gudermann T and Schultz G (2000) Receptor-mediated regulation of the nonselective cation channels TRPC4 and TRPC5. *J Biol Chem* **275**:17517-17526.
- Silver IA, Deas J and Erecinska M (1997) Ion homeostasis in brain cells: differences in intracellular ion responses to energy limitation between cultured neurons and glial cells. *Neuroscience* **78**:589-601.

MOL #40899

Strubing C, Krapivinsky G, Krapivinsky L and Clapham DE (2001) TRPC1 and TRPC5 form a novel cation channel in mammalian brain. *Neuron* **29**:645-655.

Strubing C, Krapivinsky G, Krapivinsky L and Clapham DE (2003) Formation of novel TRPC channels by complex subunit interactions in embryonic brain. *J Biol Chem* **278**:39014-39019.

Sun GY, Zhang JP, Lin TA, Lin TN, He YY and Hsu CY (1995) Inositol trisphosphate, polyphosphoinositide turnover, and high-energy metabolites in focal cerebral ischemia and reperfusion. *Stroke* **26**:1893-1900.

Thompson RJ, Zhou N and MacVicar BA (2006) Ischemia opens neuronal gap junction hemichannels. *Science* **312**:924-927.

Topolnik L, Azzi M, Morin F, Kougioumoutzakakis A and Lacaille JC (2006) mGluR1/5 subtype-specific calcium signalling and induction of long-term potentiation in rat hippocampal oriens/alveus interneurons. *J Physiol* **575**:115-131.

Trapp S, Tucker SJ and Ashcroft FM (1997) Activation and inhibition of K-ATP currents by guanine nucleotides is mediated by different channel subunits. *Proc Natl Acad Sci U S A* **94**:8872-8877.

Traut TW (1994) Physiological concentrations of purines and pyrimidines. *Mol Cell Biochem* **140**:1-22.

Ugur O, Oner SS, Molinari P, Ambrosio C, Sayar K and Onaran HO (2005) Guanine nucleotide exchange-independent activation of Gs protein by beta2-adrenoceptor. *Mol Pharmacol* **68**:720-728.

Wilson BA and Ho M (2004) Pasteurella multocida toxin as a tool for studying Gq signal transduction. *Rev Physiol Biochem Pharmacol* **152**:93-109.

MOL #40899

Xie LH, Horie M and Takano M (1999) Phospholipase C-linked receptors regulate the ATP-sensitive potassium channel by means of phosphatidylinositol 4,5-bisphosphate metabolism. *Proc Natl Acad Sci U S A* **96**:15292-15297.

Yoon J, Ben-Ami HC, Hong YS, Park S, Strong LL, Bowman J, Geng C, Baek K, Minke B and Pak WL (2000) Novel mechanism of massive photoreceptor degeneration caused by mutations in the trp gene of *Drosophila*. *J Neurosci* **20**:649-659.

Zeng F, Xu SZ, Jackson PK, McHugh D, Kumar B, Fountain SJ and Beech DJ (2004) Human TRPC5 channel activated by a multiplicity of signals in a single cell. *J Physiol* **559**:739-750.

Zhu MH, Chae M, Kim HJ, Lee YM, Kim MJ, Jin NG, Yang DK, So I and Kim KW (2005) Desensitization of canonical transient receptor potential channel 5 by protein kinase C. *Am J Physiol Cell Physiol* **289**:C591-600.

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Footnotes

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FIGURE LEGENDS

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 μ M) 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 μ M carbachol with and without 4 mM MgATP in the pipette (mean \pm s.e.m., $n = 9$, *** $p < 0.0005$).

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 Figure 1. Carbachol (10 μ M) or Gd^{3+} (100 μ M) 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 ($n = 25$), while 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 hours in PMT (1 μ g/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 \pm s.e.m., $n = 15-30$ cells, * $p < 0.05$ compared to 10 μ M carbachol control. B – E, Representative I-V relationships of TRPC5 currents induced by carbachol (B, D) and Gd^{3+} (C, E) in control cells (B,C) and PMT treated cells (D, E).

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Fig. 3. Effects of intracellular perfusion of nucleotides on TRPC5 currents. In all panels, currents were measured every 4 seconds at -60 mV (\bullet) and after stepping transiently to $+80$ mV (\circ); 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*).

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 sec intervals in cells voltage-clamped at -60 mV. *A* & *C*, Values were obtained by subtracting the leak current (measured immediately prior to application of carbachol) from the carbachol-induced current. Carbachol-induced currents were then normalized to the current at the onset of intracellular perfusion (I_0). 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* & *D*, The fractional TRPC5 current measured at 80 seconds ($I_{80 \text{ sec}}$) after the onset of perfusion for each group. Values are mean \pm s.e.m., $n = 5-9$ cells; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0005$ compared to pipette solution.

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Fig. 5. Intracellular perfusion of MgCl₂ does not alter TRPC5 currents. TRPC5 currents were recorded at 4 sec 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 prior to 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 (I₀). 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 (MgCl₂) at +80 mV and -712 ± 182 pA (pipette solution) and -256 ± 98 pA (MgCl₂) at -60 mV. *B*, Representative I-V relationship of carbachol-induced TRPC5 current prior to the onset of intracellular perfusion (Carb) and 80 seconds after the onset of intracellular perfusion with 4 mM MgCl₂ (Carb + MgCl₂).

Figure 1

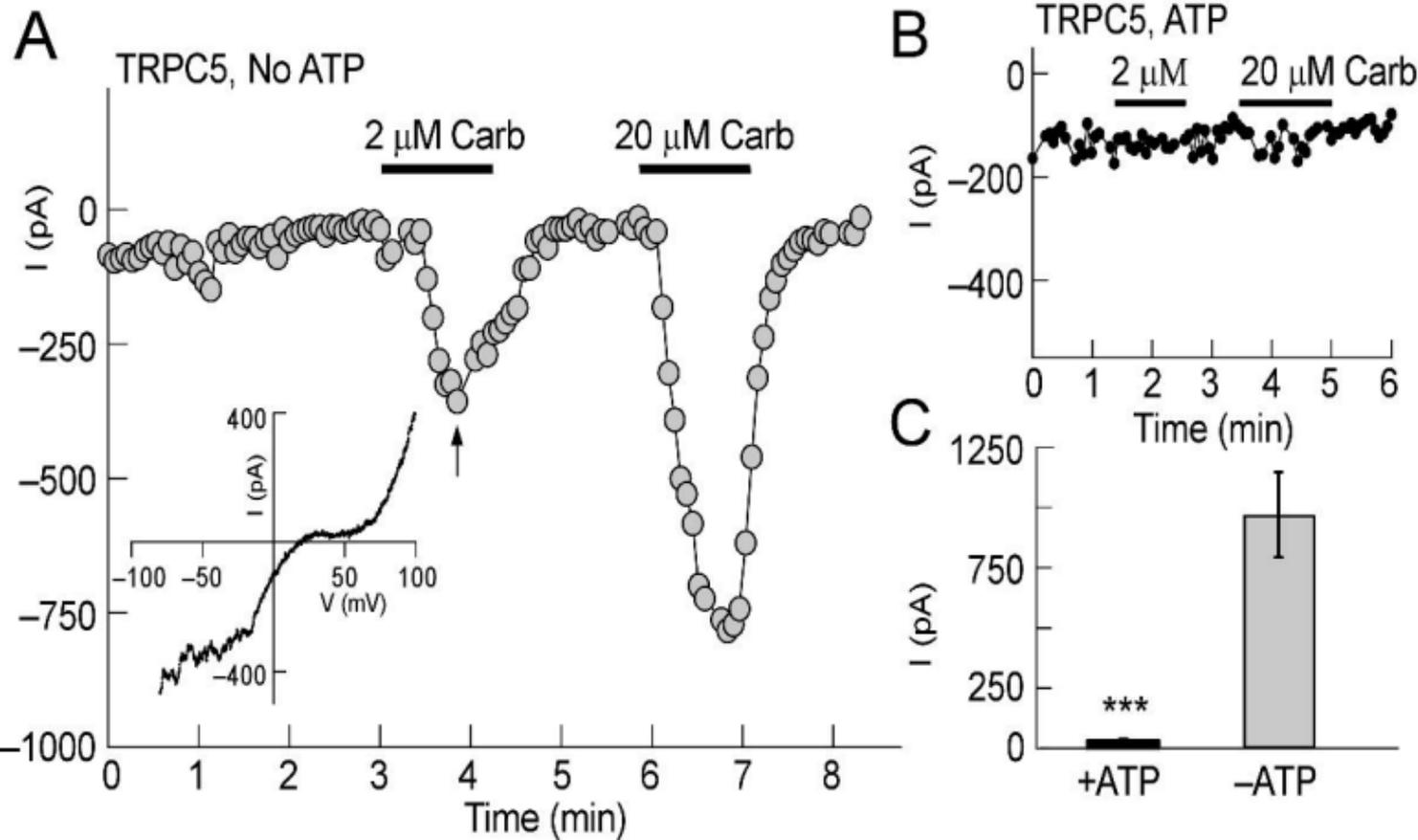


Figure 2

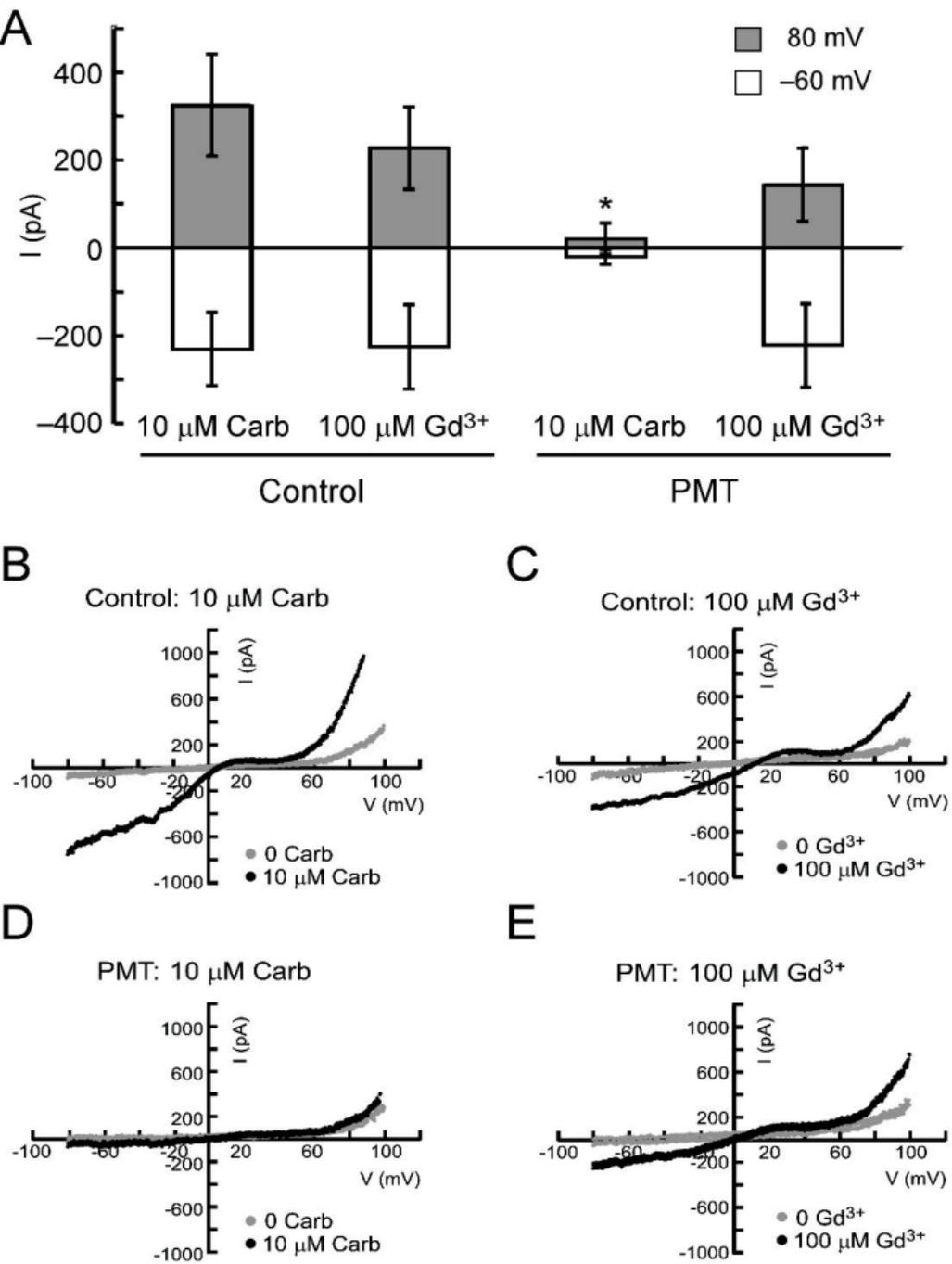
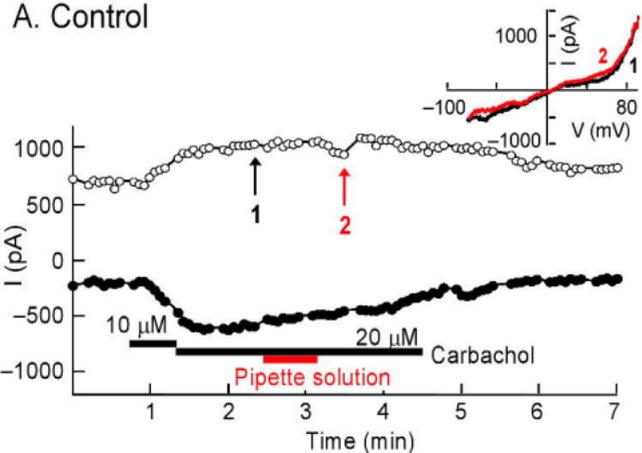
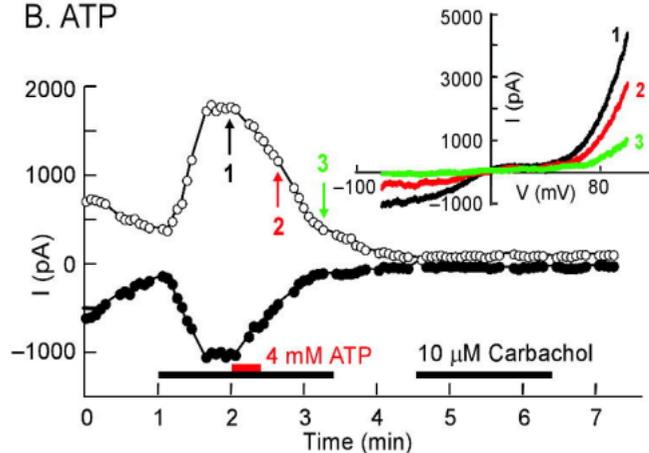


Figure 3

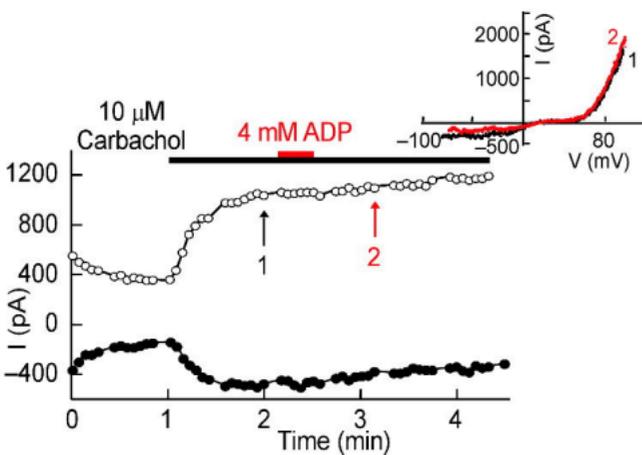
A. Control



B. ATP



C. ADP



D. AMP-PNP

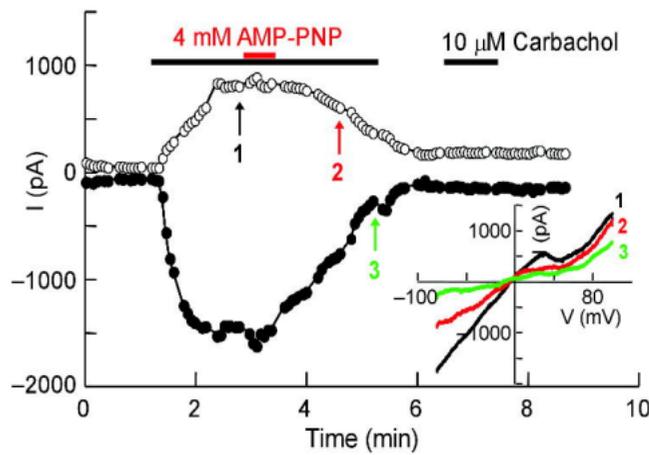
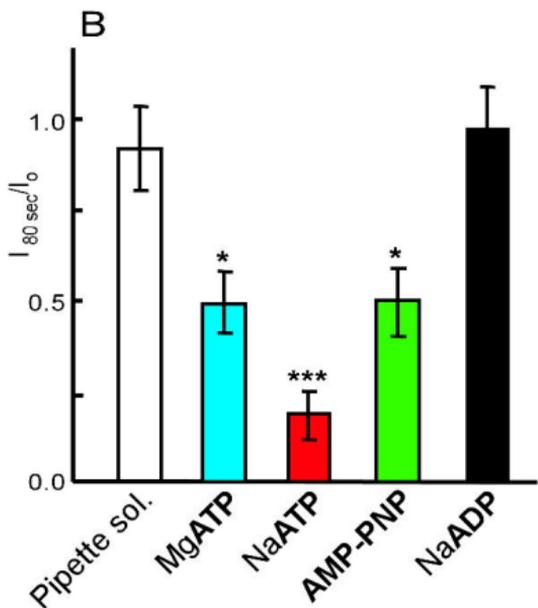
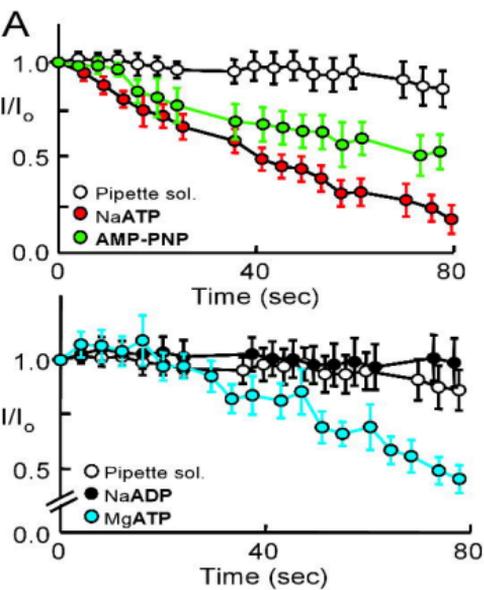


Figure 4

+80 mV



-60 mV

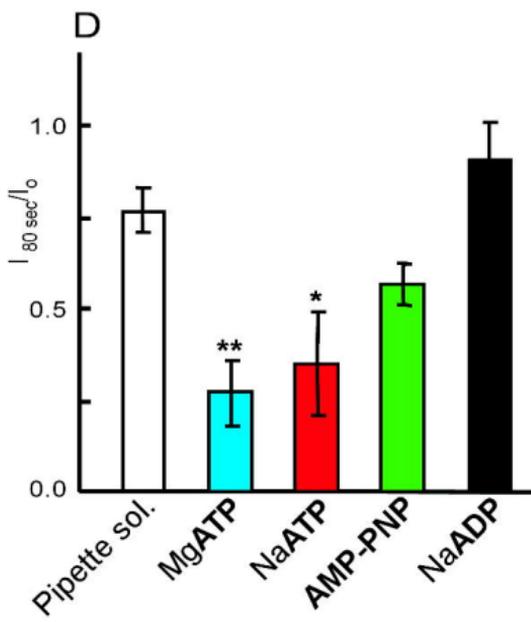
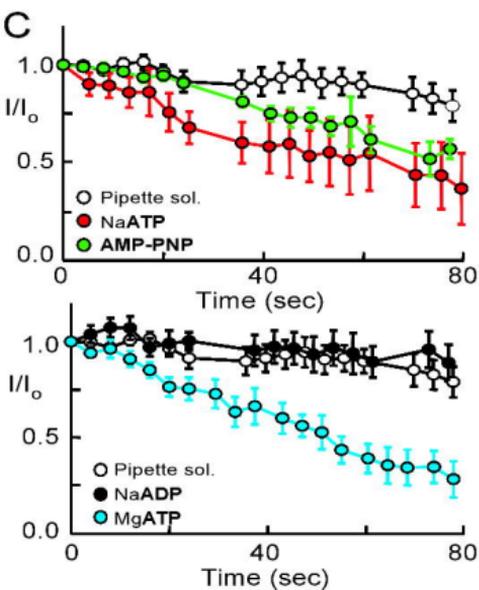


Figure 5

