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Trek-like Potassium Channels in Rat Cardiac Ventricular Myocytes Are Activated by Intracellular ATP

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Abstract. Large (111 ± 3.0 pS) K^+ channels were recorded in membrane patches from adult rat ventricular myocytes using patch-clamp techniques. The channels were not blocked by 4-AP (5 mM), intracellular TEA (5 mM) or glybenclamide (100 mM). Applying stretch to the membrane (as pipette suction) increased channel open probability (P_o) in both cell-attached and isolated patches (typically, $P_o \approx 0.005$ with no pressure; ≈0.328 with 90 cm H_2O: $V_m = 40$ mV, $pH_i = 7.2$). The channels were activated by a decrease in intracellular pH; decreasing pH_i to 5.5 from 7.2 increased P_o to 0.16 from approx. 0.005 (no suction, V_m held at 40 mV). These properties are consistent with those demonstrated for TREK-1, a member of the recently cloned tandem pore family. We confirmed, using RT-PCR, that TREK-1 is expressed in rat ventricle, suggesting that the channel being recorded is indeed TREK-1. However, we show also that the channels are activated by millimolar concentrations of intracellular ATP. At a pH of 6 with no ATP at the intracellular membrane face, P_o was 0.048 ± 0.023, whereas P_o increased to 0.22 ± 0.1 with 1 mM ATP, and to 0.348 ± 0.13 with 3 mM ($n = 5$; no membrane stretch applied). The rapid time course of the response and the fact that we see the effect in isolated patches appear to preclude phosphorylation. We conclude that intracellular ATP directly activates TREK-like channels, a property not previously described.

Key words: TREK-1 — Stretch activated — Mechanosensitive — Acidosis — ATP activated — Potassium channel — Cardiac myocyte

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Introduction

Potassium-selective ion channels that are sensitive to stretch, and which therefore may contribute to mechano-electric responses, have been described in many tissues (e.g., astrocytes: Islas, Pasantes-Morales & Sanchez, 1993; epithelia: Kim, Dirksen, & Sanderson, 1993; smooth muscle: Ordway et al., 1995). Although they have been the subject of intense investigation for some time, the molecular identity of these channels was until recently unclear. With the recent cloning of a family of two-pore, four-membrane segment (2P4M), or tandem-pore channels (Fink et al., 1996), it has become apparent that the properties of some members of this family, when expressed in heterologous systems, are consistent with the properties of stretch-activated potassium channels previously described in a variety of tissues; in particular, TREK-1 appears to be a likely candidate for many of these channels, since it demonstrates stretch activation and a pH dependence (Maingret et al., 1999). Of particular interest is the role of stretch-activated channels in the heart. However, while there have been reports of stretch-activated potassium channels in atria, there are very few reports of such a channel in ventricular cells (for review *see* Hu & Sachs, 1997). Here we show that a stretch-activated potassium channel with properties consistent with TREK-1 is present at a relatively high density in rat ventricular cells. We also confirm that TREK-1 mRNA is expressed in the heart, in both atria and ventricles, strongly suggesting that this channel is indeed TREK-1. More importantly, we find that the channel is also activated by intracellular ATP, a property that has not previously been ascribed to TREK. The response to ATP is very rapid (in the order of seconds), and occurs in isolated patches, which appears to preclude the action of a kinase using ATP as a phosphorylation substrate. The response to ATP is also very dependent on the

pH, which may also indicate a direct action. The mechanism of the response to ATP, and the physiological role of this channel in the heart remain to be elucidated.

Materials and Methods

Animals used in these studies were cared for according to the National Health and Medical Council *Guidelines for the Care and Use of Animals*. The experimental procedures were subject to the approval of the University of Adelaide Animal Ethics Committee.

Isolation of Cardiac Myocytes

Enzymatic isolation of cardiac myocytes was performed according to the method of Farmer et al., (1983) and has been documented elsewhere (Saint, Ju & Gage, 1992). Briefly, 25 min after receiving heparin (2000 units i.p.), adult male Sprague-Dawley rats were killed by exsanguination under CO_2 anesthesia and the heart removed and retrogradely perfused through the aorta. After 5 min of wash in calcium-free Tyrode's solution, the heart was subjected to enzymatic dissociation using the same solution containing collagenase (1mg ml^{-1} Worthington CLS II) and fetal calf serum (1 μg ml^{-1}). After approximately 35–40 minutes the ventricles were removed, cut into small pieces in fresh 25 μM calcium-Tyrode's solution and triturated to dissociate myocytes. The resultant cell suspension was sieved through an 80-micron nylon mesh and layered onto a solution of bovine serum albumin (BSA, 1.29 g fraction V in 20 ml) for 15 min. The supernatant was removed and the cells were resuspended in 200 μM calcium-Tyrode's solution. Finally the cells were resuspended in Tyrode's solution containing 1 mM calcium and plated onto glass coverslips.

Patch-Clamp Recording

For most of the single-channel recordings, the cells were superfused with a bath solution containing (mM): K-aspartate, 150; EGTA, 10; no added Ca^{++}; TES, 10; pH adjusted to 7.2 ± 0.5 with KOH. Bath solutions of different pH were made by titrating this solution with HCl. The pipette solution contained (in mM) 150 KCl (NaCl in some experiments); EGTA 5; Mg^{++} 2; TES 10; pH adjusted to 7.2 ± 0.05 with KOH or NaOH as appropriate. Electrodes were prepared from borosilicate glass using a two-stage puller (Narishige Scientific Instruments, Tokyo, Japan) and resistances were typically between 1–5 MΩ when containing the pipette solution. Current-recording was performed using an Axopatch 200A amplifier (Axon Instruments, Union City, CA). Channel currents were filtered at 2 KHz and recorded via a 12-bit analogue-to-digital converter operating at 5 KHz using Axotape software (Axon Instruments).

The response of the channel to membrane stretch was investigated by applying suction to the patch pipette using a glass syringe. The pressure difference was recorded with an electronic pressure gauge (WPI model PM015D, Sarasota, FL), calibrated against a water manometer.

Where rapid switching of the solution bathing the membrane patch was required (for instance, in the investigation of the time course of the effect of ATP), a rapid-switching perfusion apparatus was used. In this design, the solutions converge in a manifold machined into a plexiglass rod, with the outflow being through a stainless steel needle placed within a few mm of the patch. The deadspace of this apparatus is of the order of 100 μl, and other experiments have shown solution-switching times at the membrane of 100 to 200 msec. Open probability was calculated from the channel records using a threshold-crossing algorithm (threshold generally at 50% of channel amplitude) and summing open (above threshold) and closed (below threshold) times.

Molecular Biology

Rats were killed by exsanguination under CO_2 anesthesia (the method approved by the NHMRC ethics committee). Hearts were removed and snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNA was isolated from atria and ventricles using TRIzol Reagent (Life Technologies, Frederick, MD), 2 μg of total RNA was treated with DNase (Life Technologies) and reverse-transcribed using random hexamer primer (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. PCR was performed for *TREK-1* (genebank accession number U73488) and *GAPDH* (genebank accession number M32599) using cDNAs corresponding to 200 ng total RNA synthesized from left and right atrium, and left and right ventricle. The gene-specific primers were: *TREK-1*, forward 5′ TTTGCTTTCTACTGGCTGGGG 3′; reverse 5′ TCGTCTTCTTAGAGATCACCG 3′; *GAPDH* forward 5′ ATGTTCCAGTATGACTCCACTCACG 3′; reverse 5′ GAAGACACCAGTAGACTCCACGACA 3′. These primers have been used by other groups to amplify these genes (Schoenfeld et al, 1998; Aimond et al., 2000). All primers were purchased from GeneSet (GenSet Pacific Pty., Australia). A touch-down PCR protocol was applied: 94°C, 45 sec, 20 cycles; 65 to 55°C (−0.5°C per cycle) for 45 sec; and 72°C for 90 sec followed by 20 cycles at annealing temperature of 55°C, and a final extension step for 8 min at 72°C.

Results

Basic Observations

Figure 1 shows a typical recording obtained in a cell-attached patch (panel *A*) with a pipette containing 150 mM NaCl, in a bath solution as detailed in the Methods. Note that, in this solution, the membrane potential of the cells is essentially zero. In such patches, occasional large channel openings could be seen at positive membrane potentials (pipette negative), but no channels could be recorded at negative membrane potentials (pipette positive). The channel activity was increased by application of suction to the patch pipette. When cell-attached recordings were made with KCl in the pipette, channels could be recorded at both negative and positive membrane potentials (panel *B*). The channel under these conditions had a slope conductance (at positive membrane potentials) of 111 ± 3 pS. Application of suction to the pipette markedly increased the channel activity in isolated patches as well as in cell-attached patches. Panel *C* shows the effect of application of 90 cm·H_2O suction to the pipette on the channel activity in a cell-attached patch. The segments of the record have been selected to show channel openings; in this patch, the channel-open probability (P_o) was 0.01 at +40 mV with no suction and 0.3 with a suction of 90 cm·H_2O applied. The current-voltage relation for the chan-

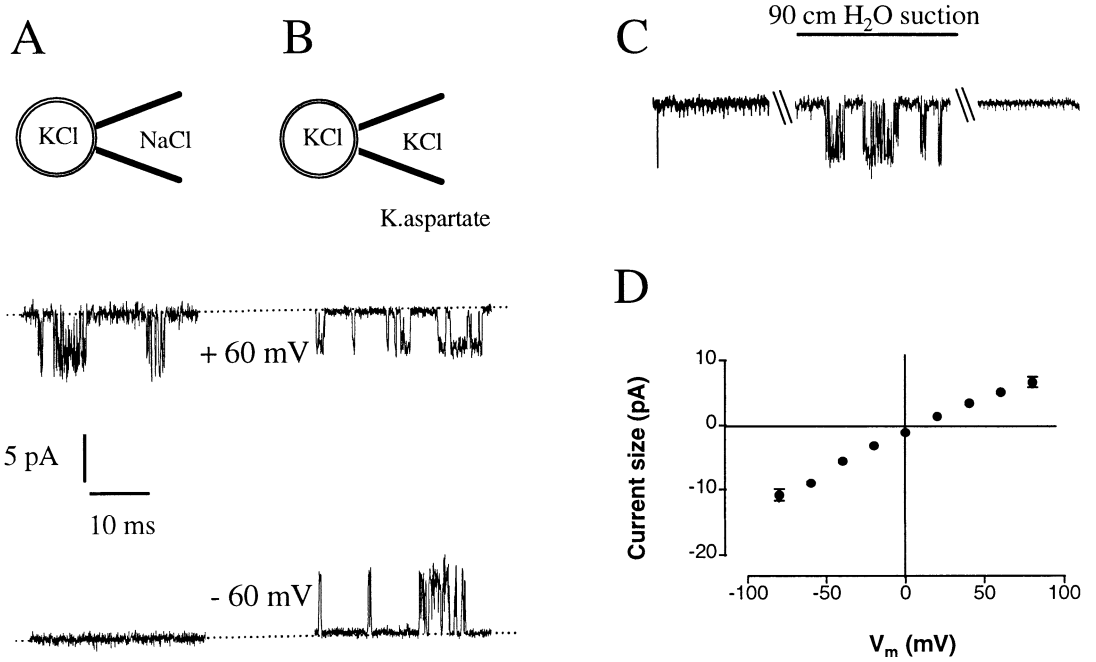

Fig. 1. The basic observations. Panel *A*: Examples of channel currents recorded in a cell-attached patch with 150 mM NaCl in the recording pipette. At a membrane potential of +60 mV (pipette −60 mV), when slight suction was applied to the membrane, large flickery channel openings could be seen. At a membrane potential of −60 mV, no channel openings could be recorded. When the recording pipette contained 150 mM KCl, channel openings activated by application of suction could be recorded at both positive and negative membrane potentials (panel *B*). The bath solution contained 150 mM K-aspartate, with other components as detailed in the methods. Panel *C*: An example of the response of the channel to applied suction (90 cm H_2O), recorded in a cell-attached patch at a constant membrane potential of 60 mV. Panel *D* : Current-voltage relation for the stretch-activated channel recorded in cell-attached patches, with 150 mM KCl in the pipette and the bath solution as given in the Methods (membrane potential of the cells should be close to zero). Data points show mean ± SEM ($n = 7$).

nels, with 150 mM KCl in the pipette and K-aspartate in the bath, is shown in panel *D*. This current-voltage relation is consistent with the permeant ion being K^+. Experiments in which K^+ was replaced with other ions showed that the channel had a selectivity (pK/pNa) of about 100, with no apparent permeability to anions or divalent cations. The channel was only weakly voltage dependent (open probability increased e-fold for 97 mV depolarization) (*data not shown*) and pharmacological experiments showed that the channel was not blocked by 4-AP (5 mM), intracellular TEA (5 mM) or glybenclamide (100 μM) (*data not shown*).

As noted in Fig. 1 , the channel was strongly activated by suction applied to the patch pipette in cell-attached patches. The response in isolated membrane patches was similar, and is shown in the form of a "dose response" curve for one patch in Fig. 2. The increase in P_o induced by suction was due primarily to an increase in frequency of opening, since mean open time did not change with the application of suction. The response to suction was very rapid—within the limits of our capability to measure it (i.e., less than one second). Channel size did not change with suction, as shown by the amplitude histograms produced at different suctions (panel 2*C*).

pH Sensitivity

Apart from this dramatic activation by suction applied across the patch, decreasing the pH at the intracellular face of the membrane also increased P_o. Figure 3*B* shows open probability plotted against pH at the intracellular membrane face in 7 patches with no suction applied to the patch and with the membrane potential held at 40 mV. Near physiological pH the open probability was very low, but rose linearly with decreasing pH (i.e., exponentially with increasing H^+ concentration). The response to pH was similar at a membrane potential of −40 mV, with P_o shifted down at all pH values. The interaction between pH and suction is shown as a "dose response" curve in panels *C* (membrane potential held at 40 mV) and *D* (membrane potential held at −40 mV). When suction was applied at the lower pH, the response was enhanced.

Stretch-activated channels with this suite of properties were seen in about 20% of patches where a giga-Ohm seal was successfully obtained (in 186 patches, 41 recordings containing these channels could be made). These channel properties are consistent with those reported for TREK-1. Using RT-

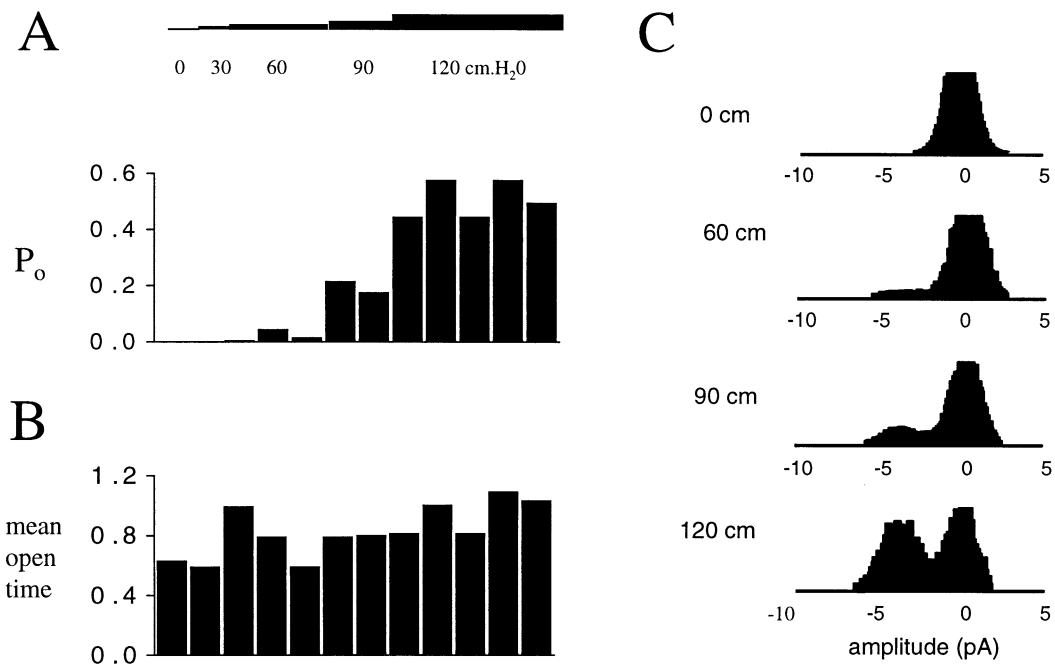

Fig. 2. Response of the channel to hydrostatic pressure. Channels were recorded at a constant membrane potential of 60 mV in an isolated membrane patch with 150 mM KCl in the pipette and 150 mM K-aspartate in the bath (pH 7.2). Suction was applied to the pipette side of the membrane and the open probability was measured. As the suction was increased from 0 to 120 cm H_2O, as depicted in the top bar, open probability increased from near zero to almost 0.6. The time course over which the suction was increased was about 2 minutes, so that each pause at the different suction levels lasts 20 to 30 seconds. The suction at 120 cm H_2O was held longer, to see if channel activity would "accommodate" to the suction, (i.e., fall with time). Such accomodation, or inactivation, was not seen in any patch, even for quite lengthy applications of suction. The increase in open probability was primarily due to an increase in frequency of opening, since mean channel-open time did not change appreciably (panel *B*). The amplitude of the channel current did not change appreciably as the suction was increased, as shown by the all-points amplitude histograms constructed from the recordings (panel *C*).

PCR techniques, we confirmed that the mRNA transcript for TREK-1 is indeed expressed in rat ventricles (Fig. 4), strongly suggesting that the channel being recorded was TREK-1. However, as a serendipitous finding in the patch-clamp experiments, we also noted that the channels were strongly activated by ATP at millimolar concentrations applied to the intracellular membrane face, a property not previously reported for TREK-1.

Activation by Intracellular ATP

Figure 5*A* shows examples of channel recordings in a patch with either zero, 1 mM or 3 mM ATP at the intracellular membrane face, with the membrane potential held constant at 40 mV, and no suction applied to the patch. This effect is plotted for 5 patches in panel *B*, which also illustrates the interaction of changing ATP concentration and pH, and in panel *C*, which illustrates the interaction of changing ATP concentration and membrane potential. ATP was almost ineffective at increasing P_o at a pH of 7, but was far more effective when pH was lowered slightly. Changing membrane potential had little effect on the response to ATP.

Only one other channel is widely recognized as being activated by intracellular ATP, the muscarinic potassium channel. In this case, activation involves a phosphorylation of the channel (Heidbuchel, Vereeke & Carmeliet, 1990) and, as a consequence, is comparatively slow, taking on the order of minutes (e.g., Shui & Boyett, 2000). Therefore, in order to investigate whether the response to ATP that we see might involve phosphorylation of the channel, and to differentiate this channel from $I_{K,Ach}$ we examined the time course of the response. A rapid-switching perfusion apparatus was used, in which the solution at the face of the membrane patch could be changed within times on the order of 100 msec. When the intracellular ATP concentration was raised rapidly with this technique, the channel-open probability rose essentially immediately, or at least within 5 seconds, the limit of resolution of the technique (the limit of resolution being that enough channel openings must be recorded to provide a reasonable estimate of P_o—this limits the minimum "time bin" that one can use) (Fig. 6). This time course is much faster than one would expect from a process involving a phosphorylation step (*see* Discussion).

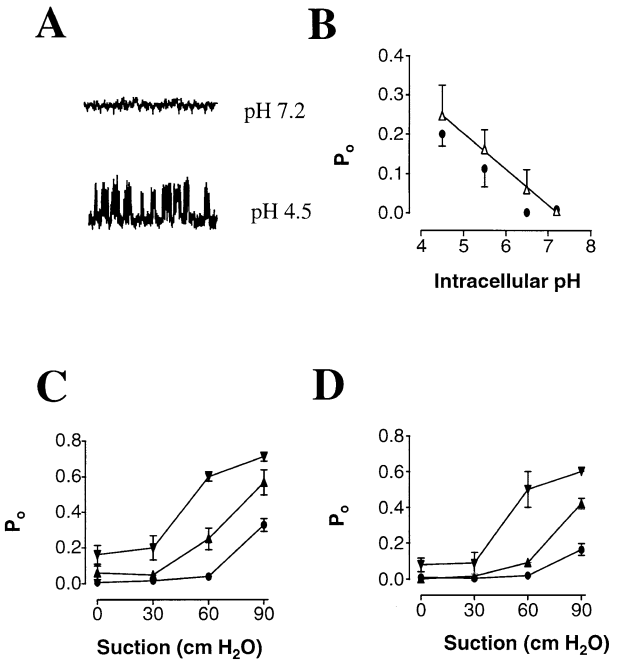

Fig. 3. Effect of pH. Panel *A* : Sections of a current record from an isolated membrane patch at a pH of 7.2 and at a pH of 4.5. In 7 isolated membrane patches, the pH in the bath was varied between 7.2 and 5.5 and the channel-open probability measured. The pipette contained 150 mM KCl and bath solution 150 mM K-aspartate. The mean open probability is shown plotted against pH in panel *B* (mean ± SEM). The data were recorded at constant membrane potential of either 40 mV (triangles) or −40 mV (filled circles). Panels *C* and *D*: The interaction of applied suction with pH. In 5 patches, suction of 0, 30, 60 and 90 cm H_2O was applied to the membrane at a bath pH of 7.2 (filled circles), 6.5 (triangles) and 5.5 (inverted triangles). The data points show P_0 (mean ± SEM) plotted against suction. The membrane potential was held constant at 40 mV (panel *C*) or −40 mV (panel *D*).

Discussion

We report here a potassium channel in adult rat ventricular myocytes with the following suite of properties: 1) it is highly potassium-selective, with a conductance of about 111 pS in symmetrical 150 mM K^+; 2) it is sensitive to membrane stretch, applied as hydrostatic pressure across the membrane patch; 3) it is activated by intracellular acidification; 4) the *I-V* relation is essentially linear in symmetrical potassium concentrations. Other experiments, not reported here, have shown that the channel is only slightly voltage dependent and is not blocked by TEA or 4-AP. At least some (or a combination of several) of these properties have been reported in various instances for stretch-activated potassium channels in several tissues, e.g., the heart (Hu & Sachs, 1996, 1997), and smooth muscle (Dopico et al., 1994).

However, a novel property of the channels we report here, in addition to the response to membrane stretch, is its activation by intracellular ATP. Although *inhibition* of potassium channels by intracellular ATP is of course well known (i.e., block of K_{ATP} channels (Noma, 1983) and BK channels (Groschner et al., 1991)) the only potassium channel widely reported to be activated by intracellular ATP is the muscarinic K_{Ach} channel (e.g., Sorota *et al.* 1998). Reports of other potassium channels being activated by ATP are very sparse, although a few instances can be found (e.g., Friel & Bean, 1990; Rowe, Treherne & Ashford, 1996), and reports of activation of potassium currents by, or at least in the presence of, ATP can also be found (Baron et al., 1999). It also appears that a channel related to the large calcium-activated channel ($K_{Ca,ATP}$), can be activated by intracellular ATP and may be present in some tissues (Albarwani et al., 1994; Kawakubo et al., 1999). (These latter channels generally have much higher conductances (> 200 pS) than that we report here and may represent a different class of channels).

Importantly, the combination of activation by stretch, intracellular acidification and intracellular ATP has not been reported for any channel, although we note that Kim (1992) showed that the mechanosensitive potassium channel from atrial cells could be activated by suction in the presence of ATP, although not that it was activated by ATP per se. A more recent report has also demonstrated a potassium channel in atrial cells activated by intracellular ATP

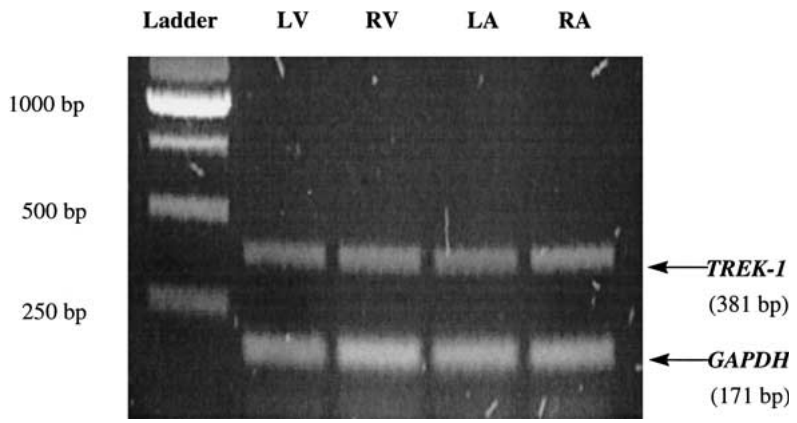

Fig. 4. Gene expression of TREK-1 in the heart. RT-PCR products of both *TREK-1* and *GAPDH* were analyzed in 1.2% agarose gel electrophoresis and visualized with ethidium bromide. For each lane, 5 μl of each post-PCR mixture for *TREK-1* and *GAPDH* were added. Given the published gene sequence of TREK-1 and the primers noted in the text, the expected fragment size for TREK-1 is 381 bp and 171 bp for GAPDH.

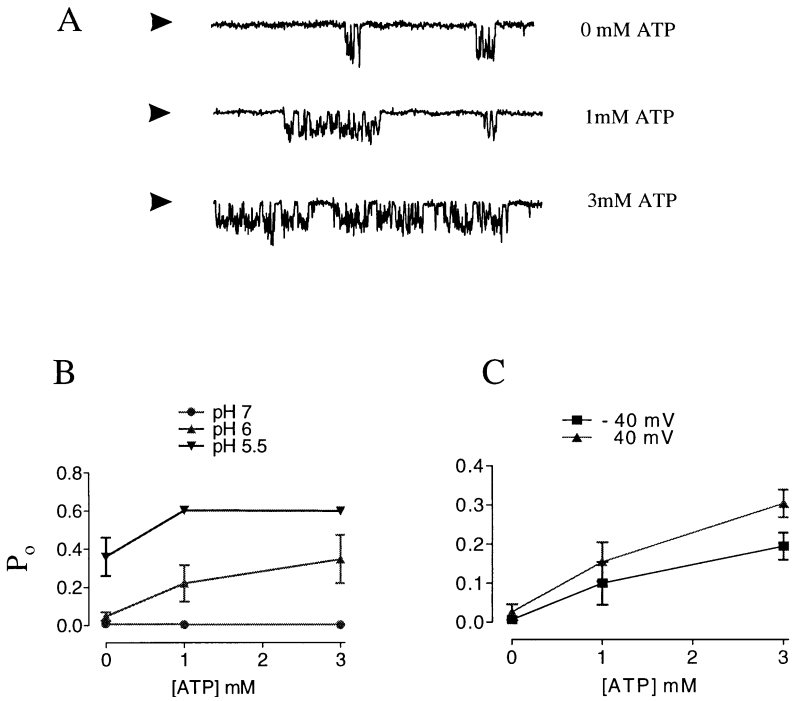

Fig. 5. Effect of intracellular ATP: Interaction with pH and membrane potential. Panel *A* shows selected segments of current records from a patch held at a membrane potential of +40 mV, with pipette and bath solutions as described in the Methods. With no hydrostatic pressure applied and a pH of 7.2 in the bath, P_o was very low (upper trace—selected to show channel openings). As ATP was added to the bath solution, P_o increased (middle trace at 1 mM ATP and lower trace at 3 mM). Panel *B*: The interaction of intracellular ATP and pH. Recordings were made from 5 patches with bath ATP concentrations of 0, 1 and 3 mM and different bath pH. The points show the increase in P_o produced by ATP at a pH of 7 (circles), 6 (triangles) and 5.5 (inverted triangles). Error bars show ± SEM. Panel *C*: Interaction of ATP and membrane potential. The increase in P_o produced by raising bath ATP concentration from 0 to 1 and then 3 mM with the membrane potential held at either −40 mV (squares) or +40 mV (triangles) is shown for 5 different patches. Error bars show ± SEM.

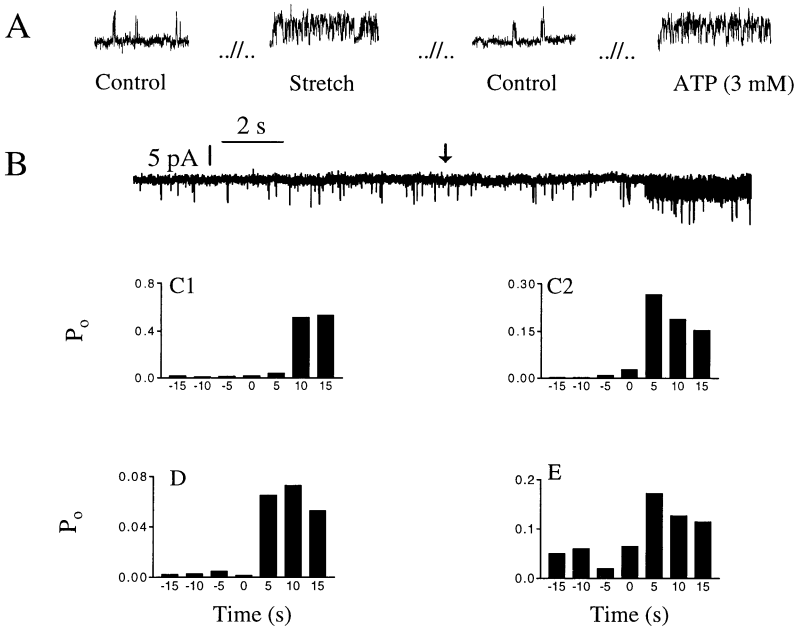

Fig. 6. Time course of the response to ATP. (*A*) Upper traces show current records from an inside-out membrane patch with a stretch-activated channel in the patch. The potential across the membrane was held constant at –40 mV and pH was 6.5. Applying 60 cm H_2O suction to the patch increased open probability dramatically. With no suction across the same patch, applying 3 mM ATP to the intracellular face of the patch also increased channel activity. (*B*) A section of a recording from a membrane patch, held at + 40 mV. 3 mM ATP was applied to the intracellular face of the patch at the arrow. *Lower panels* : The time course of the response to ATP shown as histograms in the panels below. Channel-open probability was measured in 5-second bins. ATP was applied at time zero (beginning of bin 0). Graphs *C*1 and *C*2 are repeats of the experiment in the same patch, panels *D* and *E* are different patches.

(Shui & Boyett, 2000), and these authors went to some lengths to differentiate this channel from the K_{Ach} channel, because of its different conductance, current-voltage relation and time course of activation by ATP. However, Shui and Boyett (2000) did not demonstrate sensitivity to stretch or pH of the ATP-activated channel. Nevertheless, it seems likely that their recordings are of the same type of channel shown here.

The identity (i.e., the gene product) of this channel is still uncertain. On the balance of evidence, we propose that the channel we report here is TREK-1. This channel is a member of the recently cloned 2P4T family of potassium channels, exemplified by TWIK (Lesage et al., 1996). TREK is widely expressed (Lesage & Lazdunski, 2000) and it has been shown that the mRNA coding for a TREK-like channel is expressed in rat cardiomyocytes (Aimond et al., 2000). In this study, we have also confirmed the expression of TREK-1 in rat atria and ventricles using RT-PCR (Fig. 4). TREK-1 has a conductance around 100 pS, is sensitive to intracellular pH, and is

activated by membrane stretch (Maingret et al., 1999), all properties consistent with the channel we report. It should be noted, however, that although TREK-1 has recently been shown to be activated by extracellular ATP, by a signalling cascade that appears to involve PLA_2 (Aimond et al 2000), activation by *intracellular* ATP of TREK has not yet been demonstrated.

The physiological role of this channel in the heart is as yet speculative. Shui and Boyett (2000) presented a rough calculation of the current likely to be carried by the ATP-activated channel in atrial cells. We record the channel somewhat more frequently than they report (in about 20 to 25% of patches), and so would expect a correspondingly larger whole-cell current. Ventricular cells have a larger capacitance than atrial cells, so the effect of activation of the channel may be similar. However, a caveat must be borne in mind; given the range of interacting factors that activate the channel, it is difficult to predict what the P_o for the channel would be *in vivo* any given time. Such an estimate would need accurate information on the membrane potential, intracellular pH, ATP concentration and membrane tension. Nevertheless, it does seem likely that the channel may underlie the hyperpolarization seen when the ventricles are subjected to stretch (Franz et al., 1992; Zabel et al., 1996), and hence may play a role in mechano-electric feedback in the heart.

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