Mechanosensitivity of the Cardiac Muscarinic Potassium Channel

A NOVEL PROPERTY CONFERRED BY Kir3.4 SUBUNIT*

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Muscarnic potassium channels are heterotetramers of Kir3.1 and other Kir3 channel subunits and play major roles in regulating membrane excitability in cardiac atrial, neuronal, and neuroendocrine tissues. We report here that rabbit atrial muscarinic potassium channels are rapidly and reversibly inhibited by membrane stretch, possibly serving as a mechanoelectrical feedback pathway. To probe the molecular basis for this phenomenon, we heterologously expressed heteromeric Kir3.1/Kir3.4 channels in Xenopus oocytes and found that they possess similar mechanosensitivity in response to hypo-osmolar stress. This could be attributed in part, if not exclusively, to the Kir3.4 subunit, which reproduced the mechanosensitivity of the heteromeric channel when expressed as a homomeric channel in oocytes. Kir3.4 is the first stretch-inactivated potassium channel to be identified molecularly. Physiologically, this feature may be important in atrial volume-sensing and other responses to stretch.

Although excitation-contraction coupling is the major mechanism regulating cardiac function, mechanoelectrical feedback plays important modulatory roles (1). Mechanoelectrical feedback is particularly essential in the atria of the heart, which regulate vascular volume through secretion of atrial natriuretic peptides when atrial myocytes are stretched. A number of mechano-sensitive ion channels have been identified in atrial tissue, including stretch-activated potassium, chloride, and nonselective cation channels (2–6) and stretch-inactivated potassium channels (7). However, the molecular identities of these channels are currently unknown. Since cardiac muscarinic potassium channels (K_ACh)1 (8, 9) regulated by G proteins (10) are preferentially expressed in atrial tissues (11), they seemed likely candidates to examine for mechanosensitive properties. Moreover, they have been characterized at the molecular level as heterotetramers of Kir3.1 (GIRK1) and Kir3.4 (GIRK4) proteins (12).

MATERIALS AND METHODS

**Atrial Myocyte Isolation and Current Recording**—Rabbit atrial myocytes were isolated enzymatically and patch-clamped in the whole-cell recording configuration as described previously (13, 14). Calibrated positive pressure was applied to myocytes through a water-filled U-tube connected to the patch electrode. Application of positive pressure (10 cm of H2O) did not significantly change the series resistance (6.2 ± 0.9 to 5.9 ± 0.8 megohms, n = 9). Patch pipettes (resistance 0.2–0.5 megohms) contained 150 mM KCl, 1 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, pH 7.2, and the bath solution contained 150 mM NaCl + NaOH, 10 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 0.02 mM tetrodotoxin, 0.01 mM nifedipine, pH 7.4. The calculated potassium equilibrium potential was −65 mV.

**cRNA Synthesis and Current Recording from Oocytes**—Full-length cDNA encoding the Kir3.4 protein from a rat brain library (confirmed by sequencing) was subcloned into pBlueScript (Stratagene, San Diego, CA), and cRNA was made using standardized in vitro methods (Ambion, Austin, TX). The coding region was subcloned such that the 5’ end had a Kozak sequence and the 3’ end had a poly(A) tail. *Xenopus laevis* oocytes were isolated by enzymatic digestion (2 mg/ml collagenase). Stage IV-V oocytes were used for injection. Current usually became detectable 24 h after injection, and experiments were carried out between 24 and 96 h afterward. Whole-oocyte currents were recorded at room temperature with the two-electrode voltage clamp technique (15) using a Dagan (Minneapolis, MN) CA-1 oocyte clamp amplifier, a TL-1 DMA interface for data acquisition and pCLAMP software (Axon Instruments, Foster City, CA). Recording electrodes were pulled from borosilicate pipette glass (A-M Systems Inc., Seattle, WA) and filled with 3 M KCl. Capacitance and leak currents were subtracted after blocking K+ currents with 5 mM BaCl2. The standard bath solution contained 98 mM KCl, 2 mM KOH, 1.8 mM CaCl2, 1.0 mM MgCl2, and 5.0 mM HEPES, pH 7.2. For the experiments measuring reversal potentials, KCl was replaced isotonically by NaCl. Giant cell-attached patches were formed on de-vitellinized oocytes (16, 17), and currents were recorded under voltage clamp conditions as described previously (17). The bath and pipette solutions (room temperature) contained 98 mM KCl, 2 mM KOH, 1.8 mM CaCl2, 1.0 mM MgCl2, and 5.0 mM HEPES, pH 7.2.

**Hypo-osmotic Challenge**—Currents were first recorded using the two-electrode voltage clamp technique in oocytes superfused with bath solution containing 50 mM KCl and 100 mM sucrose. Hypo-osmotic challenge (50%) was induced by removal of sucrose for 15–30 min. Leak current was subtracted after blocking K+ current with 5 mM BaCl2.

**RESULTS**

Isolated rabbit atrial myocytes were patch-clamped in the whole-cell configuration, and whole-cell currents were either recorded at a steady holding potential of −100 mV (Fig. 1a) or during periodic voltage ramps from −100 to −20 mV (0.1 mV/ ms) with Na+ and Ca2+ currents blocked (Fig. 1b). After potentiating the inward potassium current by exposing the myocyte to 10 μM carbachol, 10 cm of H2O positive pressure was applied to the patch pipette to stretch the cell membrane. Positive pressure caused a rapid (within 500 msec) decrease in the carbachol-sensitive current, averaging 15 ± 3% at −100 mV in 5 myocytes (p < 0.05) (Fig. 1d). Upon withdrawal of positive pressure, the current recovered rapidly and completely. Both before and after the application of positive pressure, current was fully blocked by 5 mM external Ba2+. Addi-

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1 The abbreviation used is: K_ACh, muscarinic potassium channels.
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Mechanosensitivity of $I_{K,Ca}$ in isolated rabbit atrial myocytes. $a$, effects of applying 10 cm of H$_2$O positive pressure to the patch pipette on whole cell current recorded at −100 mV in an isolated rabbit atrial myocyte. Positive pressure had no effect on the holding current before exposure to carbachol (Carb) but rapidly and reversibly decreased the carbachol-potentiated current by 15%. Application of external 5 mM Ba$^{2+}$ completely blocked the current, after which reaplication of positive pressure had no effect. $b$, superimposed current (carbachol-sensitive)-voltage relations obtained from voltage ramps (0.1 mV/ms) from −100 to −20 mV before (Carb), during (Carb + 10-cm H$_2$O), and after (Rec) application of 10-cm H$_2$O positive pressure in another atrial myocyte after exposure to carbachol (10 μM). $c$ and $d$, summary of the effects of positive pressure on carbachol-stimulated $I_{K,Ca}$, in 5 atrial myocytes (mean ± S.E.) $p$ values were obtained by paired Student’s $t$ test.

Effect of hypo-osmotic challenge on heteromeric Kir currents. $a$, time course of changes in inward whole-oocyte currents at −100 mV in response to a 50% reduction in osmolality of the superfusate (Hyp), recorded during the two-electrode voltage clamp of an oocyte expressing heteromeric Kir3.1/3.4 and G$_{bg}$, in excess (23:1 excess of injected G$_{bg}$ cRNA). $b$, superimposed whole-oocyte currents during a voltage ramp from −100 to +40 mV before, during, and after hypo-osmolar challenge from the same oocyte in a. c and d, lack of effects of hypo-osmotic challenge on the I-V relationship from oocytes expressing either Kir1.1 (c) or Kir2.1 channels (d). Con, control. $e$, summary of effects of hypo-osmotic challenge on the amplitude of current at −100 mV (mean ± S.E.) in oocytes expressing Kir3.1/3.4 + G$_{bg}$ (n = 4), Kir1.1 (n = 3), and Kir2.1 (n = 3). * indicates $p < 0.02$ by paired Student’s $t$ test.
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Fig. 3. Characteristics of homomeric Kir3.4 currents. a, superimposed whole-oocyte currents recorded with the two-electrode voltage clamp technique from an oocyte expressing Kir3.4, with 100 mM extra-cellular [K⁺]. Voltage was clamped for 60 ms to membrane potentials from −100 to + 80 mV (20 mV increments) from a holding potential of −20 mV. The dotted line is zero current level. b, I-V relationship for a.

c, current amplitudes (at −100 mV) as a function of the amount of Kir3.4 cRNA injected into batches of oocytes obtained on the same day (n = 6–9 oocytes/point). d, single channel currents from a giant cell-attached patch at the voltages indicated after rundown left only a few active channels. The single channel conductance was 33 pA. e, stimulation of Kir3.4 current by the muscarinic agonist carbachol in an oocyte coexpressing Kir3.4 and the m₂ muscarinic receptor. The control current (Con) recorded during a voltage ramp (0.1 mV/ms) from −80 to +40 mV was increased by 158% when the oocyte was exposed to 100 mM potassium in the bath perfusate from of oocytes from the same frog on the same day (Fig. 3). f, the current magnitude to saturate as well as its large magnitude compared with small endogenous currents in un.injected oocytes (−0.13 ± 0.23 μA at −100 mV, n = 8) argues strongly against the possibility that Kir3.4 proteins were combining with an endogenous Xenopus protein such as Kir3.5 (XIR) (22) to form heteromeric channels. With 100 mM potassium in the bath solution, the large inward currents showed no or mild relaxation, and outward currents were minimal at potentials positive to the potassium equilibrium potential, typical for strong inward rectifier potassium channels (25) (Fig. 3, a and b). The current was highly selective for potassium over sodium and blocked in a voltage-dependent manner by extra-cellular cesium and barium, with Kᵦₛ values at −60 mV of 61 ± 3 μM (n = 4) and 92 ± 13 μM (n = 4) respectively (data not shown).

Unitary current amplitudes at different voltages obtained from all-points histogram analysis revealed a single channel conductance of 33.2 ± 0.3 pico Siemens (n = 4) with 100 mM K⁺ in the pipette solution (Fig. 3d), similar to an earlier estimate (12). In contrast to the previous studies in which single channel openings were flickery and more short-lived (11, 12), openings lasting 5–50 ms were commonly observed. Like native Kᵦₛ channels (26), currents through homomeric Kir3.4 channels increased by an average of 76 ± 17% at −100 mV (n = 7) in response to 10 μM carbachol when coexpressed with the m₂ muscarinic receptor (Fig. 3, e and f), as has been noted previously (12, 24). The effect was maximal within 1–2 min and then gradually lessened, probably due to desensitization.

Co-expression of Kir3.4 with Gₛ subunits (at 23:1 excess of injected Gₛ cRNA) also boosted Kir3.4 currents by an average of >100-fold compared with oocytes from the same batch injected with the same amount of Kir3.4 cRNA alone (n = 4) (Fig. 3f). These results confirm that homomeric Kir3.4 channels are also classic G-protein-regulated potassium channels.

In attempting to measure unitary currents through homomeric Kir3.4 channels, we noted that channels ran down very rapidly after formation of a cell-attached patch (>50 patches). This rapid rundown precluded single channel analysis from standard patches (electrode tip, 1–3 μm) but could be quantified in giant patches (electrode tip, 20–30 μm), with the mean time constant of rundown averaging 3.2 ± 0.6 min (n = 5 giant patches). Single channel events could often be resolved when only a few active channels were left in the giant patch (Fig. 3d).

Patch excision always led to an immediate disappearance of channel activity. Since the formation of a gigaseal (or patch excision) subjects the underlying membrane to considerable mechanical forces, these observations suggested that homomeric Kir3.4 channels might be sensitive to membrane stretch.

To test directly for mechanosensitivity, we examined the effects of cell swelling induced by hypo-osmotic challenge on whole-oocyte homomeric Kir3.4 currents measured with the two-electrode voltage clamp. A 50% reduction of osmotic strength of the bath solution reversibly caused a 27 ± 4% reduction in Kir3.4 current at −100 mV (n = 6) (Fig. 4). This finding indicates that the Kir3.4 subunit is responsible in part, if not exclusively, for conferring mechanosensitivity to heteromeric Kir3.1/3.4 channels and to the cardiac G-protein-regulated potassium channel.

Whether the Kir3.1 subunit shares similar mechanosensitive properties is uncertain at this point.

To examine the mechanism underlying the mechanosensitivity of Kir3.4 channels, we investigated whether membrane stretch inhibited Kir3.4 currents indirectly by an effect on G-protein signaling. First, we tested the effects of hypo-osmolar challenge on Kir3.4 currents that had been maximally stimulated with carbachol in oocytes coexpressing Kir3.4 and the m₂ receptor. The carbachol-stimulated Kir3.4 currents demonstrated a similar decrease in response to hypo-osmolar challenge as under basal conditions (averaging 31 ± 4%, n = 4)
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The mechanism responsible for mechanosensitivity in these channels is unclear at this point. Our findings argue against a mass action effect of stretch on Gbg subunits as the underlying mechanism, since the mechano-sensitive response remained intact and of comparable magnitude over a wide range of ambient Gbg levels (Fig. 4d). For the case in which oocytes were co-injected with Kir3.4 and Gbg, cRNA at a 1:23 ratio, we presume that this includes a saturating range of Gbg subunits relative to Kir3.4 molecules, although the ratio of protein molecules cannot be assumed to be the same as the ratio of cRNA injected. Even if a mass action effect is unlikely, G-protein signaling might be involved if membrane stretch inhibited the ability of Gbg subunits to activate the channels by an allosteric, rather than mass action, effect. By constructing chimeric proteins between Kir3.4 and non-G-protein-regulated Kir proteins, it may be possible to resolve this question. Alternatively, the mechanism of mechanosensitivity may not directly involve G-protein signaling. A recent report has demonstrated inhibition of Kir3 currents by protein kinase C (29). It is possible that membrane stretch-induced activation of phospholipase C (30, 31) could in turn activate protein kinase C to inhibit the channels. Finally, a direct interaction between Kir3.4 and cytoskeletal elements or direct sensitivity of the channel to membrane curvature are possible mechanisms for mechanosensitivity (32).

Actin has been implicated as a cytoskeletal transducer of mechanical force for other mechano-sensitive channels (33) and has been shown to regulate the function of a number of ion channels such as epithelial sodium channels (34). Also, Kir2 channels such as Kir2.1 have been shown to link to the actin cytoskeleton as a means of spatially localizing them at specific regions in the cell (35), although no similar consensus linkage sites have been identified in Kir3 channels. These actin-binding protein sites do not confer mechanosensitivity to Kir2.1 when expressed in oocytes, however, as shown in Fig. 2d. In preliminary experiments, we were also unable to restore Kir3.4 channel activity in excised giant inside-out patches by adding F-actin to the cytoplasmic surface of the patch. Further studies, perhaps involving chimeric constructs between Kir3.4 and other Kir family members, will be required to unravel the molecular basis for stretch-induced inactivation of these channels.

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FIG. 4. Effects of oocyte swelling induced by hypo-osmolar challenge on homomeric Kir3.4 currents. a, time course of changes in inward whole-oocyte currents at −100 mV in response to a 50% reduction in osmolarity of the superfusate (Hyp), recorded during the two-electrode voltage clamp of an oocyte expressing Kir3.4. b, superimposed whole-oocyte currents during a voltage clamp from −20 to −100 mV before (Con), during (Hyp), and after (Rec) hypo-osmolar challenge from the same oocyte in a. c, I-V relationship for the experiment in a, d, summary of effects of hypo-osmotic challenge on the amplitude of current at −100 mV in oocytes expressing Kir3.4 alone (n = 6), Kir3.4 + the m2 muscarinic receptor (n = 5) during maximal stimulation by 10 µM carbachol (Carb), and Kir3.4 + Gbg, in excess (23.1 mM of injected Gbg, cRNA) (n = 5). * indicates p < 0.01 by paired Student’s t test.

Discussion

Our results demonstrate for the first time that KACH channels in the atrium are mechano-sensitive, consistent with their participation in the volume-sensing role of this organ. Physiologically, stretch-induced inactivation of KACH channels during atrial distension would facilitate membrane depolarization and enhance excitability and could potentially contribute to a variety of stretch-induced responses, including contraction-excita
coupling, atrial natriuretic peptide release, stretch-induced arrhythmias, and/or hypertrophic gene programming.

By demonstrating that both heteromeric Kir3.1/3.4 channels and homomeric Kir3.4 channels exhibit similar mechanosensitivity as native rabbit atrial KACH channels, we provide the first molecular identity of a mammalian stretch-inactivated potassium channel, which will permit structure-function studies to characterize the molecular mechanisms involved. Interestingly, the predicted overall topological structure of Kir3 channels is similar to nonmammalian mechano-sensitive ion channels cloned from Escherichia coli and Caenorhabditis elegans (27, 28), suggesting a common structural motif for these mechano-sensitive ion channels.

(c) Kir3.4

(a) Kir3.4

(b) Kir3.4

(d) Kir3.4

Hyp

Hyp

Con

Rec

V (mV)

0

100

-100

-12

-6

-60

-120

20

10 µA

% of Control

Kir3.4

Kir3.4 + Gbg

Kir3.4 + Gbg + C821–C830

Kir3.4 + Gbg + Rec

Kir3.4 + Gbg + Hyp

Kir3.4 + C821–C830

Kir3.4 + C821–C830 + Hyp

Kir3.4 + Gbg + Rec

Kir3.4 + Gbg + Rec

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