Channel activators regulate ATP-sensitive potassium channel (KIR6.1) expression in chick cardiomyocytes

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Abstract ATP-sensitive potassium channels (K_{ATP}) are widely expressed and yet little is known about the mechanisms regulating their expression. Here we report that expression of chick heart Kir6.1 is regulated by channel activators. Activation of K_{ATP} with either ATP depletion or pinacidil, up-regulated Kir6.1 mRNA 1.8- to 2.4-fold in cultured ventricular myocytes as measured by competitive PCR. Pinacidil treatment also increased Kir6.1 protein as detected using an antibody to Kir6.1. Glibenclamide, a K_{ATP} inhibitor, completely blocked the pinacidil-induced increase in Kir6.1 levels. It appears that Kir6.1 is up-regulated by an unknown signal transduction pathway initiated by K_{ATP} opening.

Key words: Potassium channel; Sulfonylurea; mRNA regulation; Cardiac ion channel; Pinacidil; ATP sensitive

1. Introduction

ATP-sensitive K^+ channels (K_{ATP}, e.g. Kir6.n) are inwardly rectifying two-transmembrane domain channels first described in heart but also found in other tissues including pancreas, brain, smooth muscle and skeletal muscle [1–4]. A distinguishing feature of K_{ATP} channels is that their activity is inhibited by typical intracellular ATP concentrations, but stimulated when ATP levels diminish [1]. Rat Kir6.1 (uK_{ATP}-1) is a candidate cardiac K_{ATP} that is highly expressed in heart [3], while two other cloned K_{ATP} candidates, Kir6.2 and IRK3(HIT), are not [5,6]. K_{ATP} is postulated to be an important coupler between energy state and electrical excitability in cells [7]. The physiological roles of K_{ATP} channels in most tissues are not understood. In beta cells of the pancreas this pathway couples glucose metabolism with insulin secretion [8]. In heart, decreasing ATP levels (e.g. via hypoxia) opens K_{ATP} causing K^+ efflux, thus stabilizing membrane potential and perhaps protecting cells against further damage from voltage-activated Ca^{2+} entry [9,10].

Little is known about the mechanisms that regulate the expression of inwardly rectifying potassium channels (Kir). We have previously shown that K_{ATP}-associated sulfonylurea receptors are up-regulated in chick heart cells both by receptor ligands and by channel openers such as pinacidil [11]. In addition, the G-protein-coupled inwardly rectifying K^+ channels, GIRK1 and GIRK4, are down-regulated in ovo following muscarinic acetylcholine receptor activation [12]. Although the mechanisms underlying these events are not yet known, the results suggest that channel activity can regulate channel and channel-associated protein expression. These observations led us to ask whether channel activity mediated by chemical modulators also affects channel expression. These questions have special significance since potassium channel modulators are used to treat various disorders including diabetes, hypertension and asthma. For example, glibenclamide, a specific K_{ATP} inhibitor, is used to treat non-insulin-dependent diabetes mellitus [8] and pinacidil, a K_{ATP} opener, is used to treat hypertension [13]. Here we report the partial cloning of an avian ATP-sensitive potassium channel, Kir6.1, and the regulation of its expression by channel activators. These studies represent some of the first aimed at defining these mechanisms at the molecular level.

2. Materials and methods

2.1. Cloning of chick Kir6.1

Total RNA isolated from 9-day embryonic chick heart by a one-step method [14] was subjected to reverse transcription according to the manufacturer's protocol (Life Technologies). Polymerase chain reaction (PCR) was performed using degenerate primers matching the N-terminal (7-IIPEEYV-13: AT(ACT)AT(ACT)CCIGA(AG)-GA(AG)TA(CT)GT) and the putative pore-forming domain (138-QVTIGFG-144: CC(AG)AAICC (AGT)AT(ACGT)GTIAC(CT)TG) of the rat Kir6.1 [3] at a 55°C annealing temperature for 30 cycles. The major amplified product was 400 bp in agreement with the expected size based on the rat sequence and was subcloned into Bluescript II SK(−) (Stratagene) at the *Eco*RV site. The 363 bp DNA sequence (Applied Biosystems 373 DNA Sequencer) with the primer sequences removed for fidelity was deposited into the GenBank with the accession number U92977.

2.2. Northern blot analysis

Total RNA (25 μg/lane) from chick tissues was subjected to Northern blot analysis with 5×10^6 cpm/ml [α-^{32}P]dATP-labeled chick Kir6.1 [15,16]. After hybridization at 42° for 16 h and high-stringency washing at 65°, the blots were exposed to Kodak X-omat film. As an internal control, blots were stripped and hybridized with a ribosomal L-27 probe following the same procedure [17].

2.3. Cell culture

Homogeneous cultures of myocytes were prepared from 8-day chick embryo ventricles and cultured essentially as described except the medium was supplemented with 10% horse serum [11,18]. Cells were maintained for 2 days before the medium was changed and the cells were maintained for 1 more day before drug treatments were initiated. Cromakalim, diazoxide, pinacidil and glibenclamide were prepared as stock solutions in 60% DMSO. Control cells were treated with equal final concentrations of DMSO. The ATP level of cultured cells was measured using an ATP assay kit based on the luciferin-luciferase reaction (Calbiochem, La Jolla, CA) using the manufacturer's protocol. Reaction products were quantitated by liquid scintillation.

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Abbreviations: K_{ATP}, ATP-sensitive potassium channel; GIRK, G-protein-coupled inwardly rectifying potassium channel; Kir, inwardly rectifying potassium channel; RT-PCR, reverse transcription-polymerase chain reaction

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2.4. Competitive RT-PCR

Competitive reverse transcription-PCR (RT-PCR) was used for mRNA quantification based on the linear relationship of Log(Pt/Pc) vs. $LogNc^0$ [19]. Pt and Pc are the amounts of the final products amplified from the target and the competitor DNAs, and Nt^0 and Nc^0 are their starting molecule numbers, respectively. Relative $LogNt^0$ is determined from the shift in x-intercept of the plots between control and treatment groups. The competitors for Kir6.1 and L27 were constructed by inserting a PCR-amplified 220 bp chick GIRK4 fragment (70–295 nt, Genbank #U71060) into the *Sty*I site of Kir6.1 and L27 cloned in Bluescript. The RT-PCR primers for Kir6.1 were designed from 17-FAAENL-22 and 118-VTCVRS-121, a region which, in rat, distinguishes Kir6.1 from Kir6.2 (see Fig. 1). Single-stranded cDNA competitors were added with the reverse-transcribed native templates for competitive RT-PCR. The products were subjected to Southern analysis using [α-^{32}P]dATP-labeled chick Kir6.1 as described above and quantitated by scanning densitometry (Ultroscan XL, Pharmacia). To normalize for differences both in RNA isolation from cultures and in the efficiency of reverse transcription, levels of ribosomal L27 mRNA were determined similarly as an internal control.

2.5. Immunoblot detection of proteins

Cultured cardiomyocytes were homogenized in PBS (mM: NaCl 13.7, KCl 0.27, Na_2HPO_4 0.81 and KH_2PO_4 0.15) containing 1 mM EDTA-Na_2, 1 mM iodoacetamide, 200 μM PMSF, 5 μg/ml trypsin inhibitor, 5 μg/ml pepstatin A and 1 μg/ml leupeptin. Membranes were collected by centrifugation and resuspended in the homogenization buffer and an aliquot from each sample was used for protein determination by a modified Lowry method [11]. Protein (30 μg/lane) was subjected to SDS-PAGE and transferred to nitrocellulose membranes as previously described [20]. The membranes were reversibly stained by Ponceau S to ensure equivalent protein transfer among samples. The membranes were blocked with 5% BSA and incubated overnight at 4°C with a polyclonal rabbit anti-rat Kir6.1 IgG (1:6000). This antibody was raised against a peptide (a.a. 397–406) of rat Kir6.1 [3] and its binding to rat Kir6.1 on immunoblots is competed for by the immunizing peptide (Inagaki and Seino, manuscript in preparation). Antibody–antigen complexes were visualized using enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Arlington Heights, IL) and quantitated by scanning densitometry. Data were analyzed using a one-sided Student's t-test.

2.6. Materials

Embryonated chick eggs (White Leghorn) were obtained from Gawlak Farms (Lawton, NY). The polyclonal anti-Kir6.1 antibody was generously provided by Dr. S. Seino (Chiba University School of Medicine, Japan). Cell culture reagents, molecular weight standards, M-MLV reverse transcriptase, restriction enzymes and *Taq* DNA polymerase were from Life Technologies Inc. (Grand Island, NY). Cromakalim, 2-deoxy-D-glucose, leupeptin, iodoacetamide and oligomycin were from Sigma Chemical Co. (St. Louis, MO). Diazoxide and pinacidil were from RBI (Natick, MA) and glibenclamide was from Aldrich (Milwaukee, WI). Trypsin inhibitor and pepstatin A were from Boehringer Mannheim (Indianapolis, IN). Sources of other reagents were as previously described [20,21].

3. Results

3.1. Cloning and expression of chick Kir6.1

In order to obtain the chick Kir6.1 homologue, RT-PCR was used to obtain a clone which was sequenced from both strands and found to contain an open reading frame that encoded a protein fragment of 135 amino acids which was 82% identical to rat Kir6.1 and 68% identical to rat Kir6.2 [22] and covered one-third of the total coding region (Fig. 1A). The chick Kir6.1 had just 41 and 46% identity with chick GIRK4 (Lu and Halvorsen, unpublished) and GIRK1 [23], respectively, over this same region. The most diverse region between avian and mammalian Kir6.1 was the extracellular loop between the first transmembrane domain (M1) and the pore-forming region (H5). Northern analysis revealed a single 3.6 kb (± 0.1, $n = 3$) species in chick heart, skeletal muscle and brain (Fig. 1B).

3.2. Regulation of Kir6.1 mRNA by channel modulators

We previously showed that chronic treatment with K_{ATP} openers (e.g. pinacidil) or the inhibitor, glibenclamide, up-regulates glibenclamide receptors in cultured chick heart cells [11]. Therefore, we tested if channel activity also regulates the expression of K_{ATP}. In order to enhance the detection sensitivity in cultured cells, Kir6.1 mRNA was quantitated by competitive RT-PCR (Fig. 2). Treatment of ventricular myocytes with pinacidil (50 μM, 12 h) caused a nearly 2.5-fold up-regulation of Kir6.1 mRNA (Fig. 3A). This is a concentration of pinacidil that is known from electrophysiological studies to activate K_{ATP} [24,25]. The K_{ATP} inhibitor glibenclamide (30 nM) prevented the up-regulation of Kir6.1 mRNA by pinacidil (Fig. 3A). This glibenclamide concentration was about 30-fold higher than its K_d for receptor binding as previously determined [11]. Glibenclamide alone induced a 40% decrease of Kir6.1 mRNA (Fig. 3A). Therefore, activation of K_{ATP} was accompanied by an increase in Kir6.1 mRNA and blockade of channel function was followed by a decrease in mRNA.

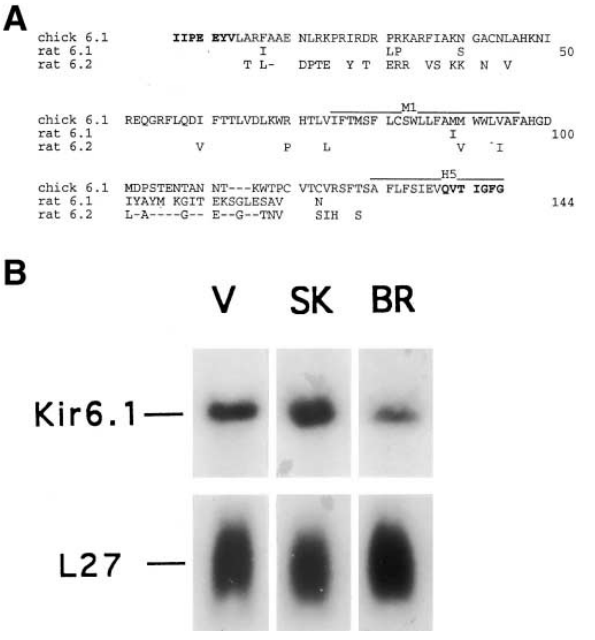

Fig. 1. Partial amino acid sequences of chick Kir6.1 with rat Kir6.1 and Kir6.2 and tissue distribution of chick Kir6.1. A: Alignment of deduced amino acid sequences in single-letter code. Locations of RT-PCR primers are indicated by bold typeface. The putative first transmembrane domain (M1) and the pore-forming region (H5) are indicated by lines above the residues. Residues that are non-identical with chick Kir6.1 are shown below the chick Kir6.1 sequence and gaps are indicated by dashes. The numbers at the right indicate the location of the last residue of rat Kir6.1 in each row. B: Northern blot of total RNA (25 μg) from 9-day chick embryonic tissues was hybridized with a ^{32}P-labeled chick Kir6.1 cDNA probe as described in Section 2. Upper panel: A 3.6 kb signal was detected in RNA from ventricles (V), skeletal muscle (SK) and brain (BR). Lower panel: A cDNA probe for L27 ribosomal RNA was used as a control for RNA loading.

3.3. Regulation of Kir6.1 mRNA by ATP depletion

One explanation for the up-regulation of Kir6.1 mRNA by pinacidil and its blockade by glibenclamide was that K_{ATP} opening initiated the regulation. To further test this we determined if activating K_{ATP} through lowering cellular ATP [26] also regulates Kir6.1. Ventricular myocytes were treated for 3 h with the metabolic inhibitors oligomycin (0.2 μg/ml) and 2-deoxy-D-glucose (5 mM), which decreased intracellular ATP concentrations to 0.2 mM from 1 mM (data not shown). Kir6.1 mRNA was up-regulated about 1.8-fold under these conditions of metabolic stress (Fig. 3B). Thus, two different treatments that activate K_{ATP} channels, either pinacidil or lowered ATP levels, each resulted in higher channel mRNA levels.

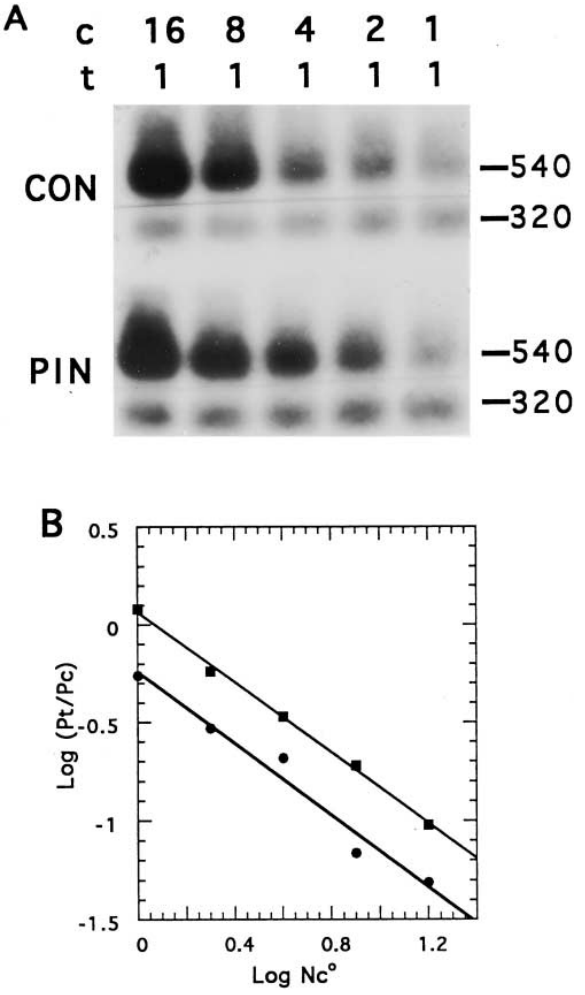

Fig. 2. Competitive RT-PCR analysis of Kir6.1 mRNA. A: Total RNA from control (CON) and pinacidil (PIN) treated (50 μM for 12 h) chick ventricle cultures was subjected to reverse transcription followed by competitive RT-PCR with increasing amounts from right to left of a competitor (c, in arbitrary units, 540 bp) and a constant amount of target Kir6.1 (t, 320 bp). Shown is a Southern blot of the PCR products. Typically, the amount of 'c' ranged from 1 to 16 fg per reaction. B: The PCR products from control (●) and pinacidil (■) treated ventricle cells from the experiment shown in (A) were quantitated by densitometry and plotted as described in Section 2. In this experiment a 1.8-fold increase was detected in samples from PIN-treated cells.

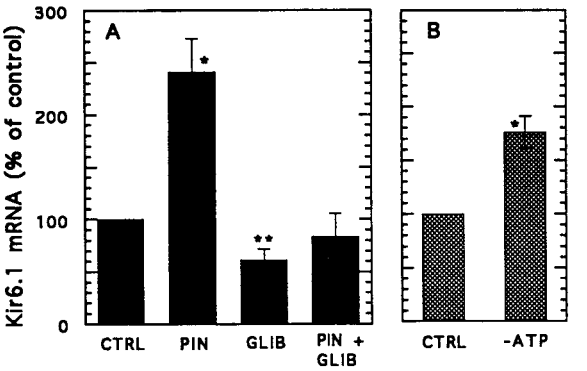

Fig. 3. Regulation of Kir6.1 mRNA by channel activity. A: Chick ventricle cells were cultured for 3 days and treated with the indicated modulators for the final 12 h. Total RNA was isolated for competitive RT-PCR and mRNA levels were determined as in Fig. 2. Shown are the means ± SEM of n determinations expressed as a percent of the value from vehicle-treated cells (CTRL) after normalization to L27. PIN: pinacidil 50 μM, $n=6$; GLIB: glibenclamide, 30 nM, $n=3$; PIN+GLIB: $n=3$ ($p<0.25$). (B) Cultured cells as in (A) were treated with oligomycin (0.2 μg/ml) plus 2-deoxy-D-glucose (5 mM) for 3 h for ATP depletion (-ATP, $n=3$) and competitive RT-PCR was performed to determine Kir6.1 mRNA levels as in (A). $^{*}P<0.005$ compared to control; $^{**}P<0.025$ compared to control.

3.4. Regulation of Kir6.1 protein by K_{ATP} channel modulators

In order to determine if the changes in Kir6.1 mRNA induced by channel modulators also resulted in changed protein expression, we used immunoblotting with an anti-rat Kir6.1 antibody. A single 47 ± 1 kDa ($n=3$) signal was detected in chick heart cells (Fig. 4A) and no proteins were detected when non-immune rabbit serum was used as a negative control (data not shown). A single 47 kDa protein was also detected in samples prepared from neonatal rat heart (data not shown). Treatment of ventricular myocytes with pinacidil induced an increase of nearly 2-fold in Kir6.1 protein which was completely inhibited by co-administration with glibenclamide (Fig. 4B). Glibenclamide treatment alone for 24 h appeared to cause a small decrease in Kir6.1 but the result was not statistically significant (Fig. 4B). An increase in Kir6.1 protein similar to that seen with pinacidil was also observed after treatment of cells for 24 h with two other K_{ATP} channel activators, diazoxide and cromakalim (Fig. 4A). Therefore, the increase in Kir6.1 mRNA seen following treatment with channel openers was accompanied by a similar increase in Kir6.1 protein.

4. Discussion

We have shown here that the expression of a cardiac K_{ATP} channel is up-regulated after channel activation. Kir6.1 mRNA and/or protein was up-regulated by several different channel openers, pinacidil, cromakalim and diazoxide. All three openers share a similar function in opening K_{ATP}, but they have different target sites and probably trigger different transducers in activating K_{ATP} [3,24,27,28]. Both pinacidil and diazoxide have been shown to activate exogenously expressed Kir6.1 [29,30]. Further, activation of K_{ATP} by ATP depletion from metabolic inhibitors also up-regulated Kir6.1 mRNA. The effects of pinacidil were blocked in the presence of glibenclamide which, at the concentration used, is a selective K_{ATP} inhibitor [2]. Together, these results support a hy-

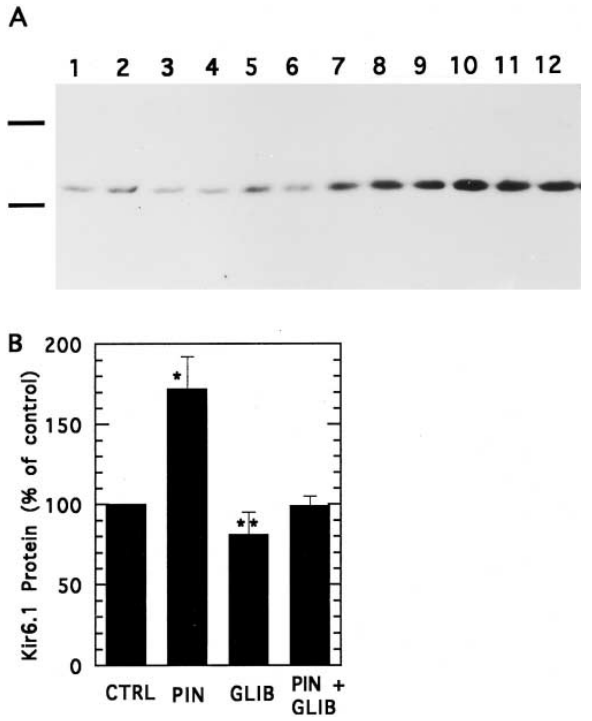

Fig. 4. Regulation of Kir6.1 protein by channel activity. A: Cultured cardiomyocytes were treated in duplicate with the indicated reagents for 24 h. Membrane proteins were analyzed by immunoblotting with anti-Kir6.1 antibody and visualized as described in Section 2. Lanes 1–2, control; lanes 3–4, glibenclamide, 30 nM; lanes 5–6, pinacidil, 50 μM+glibenclamide, 30 nM; lanes 7–8, pinacidil, 50 μM; lanes 9–10, cromakalim, 50 μM; lanes 11–12, diazoxide, 50 μM. The bars indicate the location of 43 and 68 kDa protein standards. B: Immunoblot results of Kir6.1 protein in control and treated cardiomyocytes as in (A) were quantitated and the mean ± SEM ($n = 3$) as a percent of control levels were plotted. CTRL, vehicle treated; PIN: pinacidil 50 μM; GLIB: glibenclamide 30 nM; PIN+GLIB: pinacidil 50 μM+glibenclamide 30 nM ($p < 0.25$). $^{*}P < 0.025$ compared to control; $^{**}P < 0.1$ compared to control.

pothesis that opening of K_{ATP}, including Kir6.1, is a common event in triggering an as yet unknown pathway to increase Kir6.1 expression.

Recent reports indicate that a K_{ATP} complex can be composed of either Kir6.1 or 6.2 and a sulfonylurea receptor (SUR1 or 2). The sensitivity to ATP and channel modulators appears to be primarily determined by the SUR component [24,29–31]. We previously showed that cardiac sulfonylurea receptors, measured by [^{3}H]glibenclamide binding, are up-regulated by both pinacidil and glibenclamide [11]. While here we found that Kir6.1 was up-regulated by pinacidil, glibenclamide had only modest effects on Kir6.1 expression. Therefore, the two components of the complex can be regulated either coordinately or independently. The effect of pinacidil on both sulfonylurea receptors and Kir6.1 might be through regulation of K_{ATP} activity, while the effect of glibenclamide on sulfonylurea receptors might be through a ligand-sulfonylurea receptor interaction.

This is the first report showing potassium channel up-regulation by channel activators. An increased expression of K_{ATP} would result in an increased channel density which may enhance its protective effects during hypoxia or ischemia. Therefore, it is important to understand the mechanisms that regulate expression of these channels. Down-regulation of sodium channels by neurotoxin activation and G-protein-coupled potassium channels by acetylcholine receptor activation has been reported [12,32]. In the latter case, the down-regulation of potassium channels could result from desensitization of channels after chronic exposure to muscarinic agonists. This would correlate with our treatment of cells with glibenclamide to inhibit K_{ATP} activity that appears to result in slight down regulation of Kir6.1. However, K_{ATP} activation by chemical activators or by lowered ATP may initiate pathways not leading to desensitization and thus resulting in the up-regulation of the Kir6.1 channel. The pathway(s) leading from K_{ATP} activity to the regulation of Kir6.1 expression is not yet defined, but may represent a mechanism also important for regulating other channels.

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