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AMP-Activated Protein Kinase Connects Cellular Energy Metabolism to K_{ATP} Channel Function

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Abstract

AMPK is an important sensor of cellular energy levels.

Objective—The aim of these studies was to investigate whether cardiac K_{ATP} channels, which couple cellular energy metabolism to membrane excitability, are regulated by AMPK activity.

Research Design and Methods—We investigated effects of AMPK on rat ventricular K_{ATP} channels using electrophysiological and biochemical approaches

Results—Whole-cell K_{ATP} channel current was activated by metabolic inhibition; this occurred more rapidly in the presence of AICAR (an AMPK activator). AICAR had no effects on K_{ATP} channel activity recorded in the inside-out patch clamp configuration, but ZMP (the intracellular intermediate of AICAR) strongly activated K_{ATP} channels. An AMPK-mediated effect is demonstrated by the finding that ZMP had no effect on K_{ATP} channels in the presence of Compound C (an AMPK inhibitor). Recombinant AMPK activated Kir6.2/SUR2A channels in a manner that was dependent on the AMP concentration, whereas heat-inactivated AMPK was without effect. Using mass-spectrometry and co-immunoprecipitation approaches, we demonstrate that the AMPK α-subunit physically associates with K_{ATP} channel subunits.

Conclusions—Our data demonstrate that the cardiac K_{ATP} channel function is directly regulated by AMPK activation. During metabolic stress, a small change in cellular AMP that activates AMPK can be a potential trigger for K_{ATP} channel opening.

Keywords

ATP-sensitive K^+ channels; AMP-activated protein kinase; Potassium channels

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INTRODUCTION

ATP-sensitive K^+ (K_{ATP}) channels are found in most tissue beds, including the heart, skeletal and smooth muscle, brain, kidney and pancreatic β-cells [1]. Although the various subtypes of K_{ATP} channels differ from each other in terms of their gating properties and pharmacological sensitivities, a common property is their regulation by intracellular nucleotides. The cellular ATP:ADP ratio is considered to be the prime regulator of K_{ATP} channels in cardiac muscle and the pancreatic β-cell [2]. As such, K_{ATP} channels function to couple intracellular metabolic events to membrane excitability and cellular effector responses. The cloning of the molecular subunits of K_{ATP} channels have revealed them to be hetero-octameric complexes, consisting of four pore-forming inward rectifier subunits (Kir6.1 or Kir6.2) and four regulatory subunits (SUR1 or SUR2) [2]. Since the discovery of K_{ATP} channels over two decades ago, much work has gone into the delineation of mechanisms that regulate their activity, including their modulation by nucleotides, cellular metabolic events, pharmacological agents and regulation by signaling pathways. Despite these advances, the triggers for K_{ATP} channel opening during myocardial ischemia and metabolic impairment are not fully understood.

AMP-activated protein kinase (AMPK) represents the mammalian form of the core component of a kinase cascade that is conserved between fungi, plants, and animals [3]. When activated, AMPK switches off ATP consuming pathways (e.g. biosynthetic pathways) while switching on a variety of pathways to enhance ATP production and cell survival. The latter includes increased β-oxidation of free fatty acids, increased formation of creatine phosphate, and enhanced membrane glucose transport by membrane translocation of GLUT-4 [3]. AMPK is a heterotrimeric protein composed of a catalytic α-subunit and regulatory β and γ subunits, which are important for protein stability and substrate specificity. Each subunit has two or more different isoforms [3]. The α1 subunit is widely expressed, while the α2 subunit is predominantly found in liver, heart and skeletal muscle [4]. AMPK is activated through Thr^{172} phosphorylation by one or more upstream kinases (AMPKK) and allosterically by increases in the ATP:AMP and creatine:phosphocreatine ratios [3, 5]. AMPK has previously been proposed to regulate K_{ATP} channels trafficking in a cellular model of hypoxia-induced “ischemic preconditioning” [6], but there are no reports that AMPK directly affects cardiac K_{ATP} channel function. Our data demonstrate that rat ventricular K_{ATP} channel activity is regulated by AMPK, and that this kinase therefore directly connects alterations in cellular energy metabolism (i.e. the ATP:AMP ratio) with the cardiac K_{ATP} channel function. Furthermore, the AMPK α-subunit associates with K_{ATP} channel subunits, suggesting that AMPK may be a local signalling component of the K_{ATP} channel macromolecular complex.

METHODS

Preparation of single ventricular myocytes

Ventricular myocytes were enzymatically isolated from male Sprague-Dawley rats (~200 g). Hearts were rapidly excised after pentobarbital overdose (60 mg/kg), rinsed with ice-cold Tyrode’s solution (in mM: NaCl 137, KCl 5.4, HEPES 10, $MgCl_2$ 1, NaH_2PO_4 0.33, $CaCl_2$ 1.8; glucose 10; pH 7.4), cannulated and retrogradedly perfused with oxygenated Tyrode’s solution for 3–5 min at 37°C. The perfusate was switched to nominally Ca^{2+}-free Tyrode’s buffer for 5 min, followed by perfusion for 11–13 min with the same solution containing collagenase (type I, 3 mg/mL; Sigma) and protease (type XIV, 0.44 U/mL; Sigma). The enzyme was washed out by 5 min perfusion with KB solution (in mM: taurine 20, L-glutamic acid 50, HEPES 10, EGTA 0.5, $MgSO_4$ 3, KH_2PO_4 30, KCl 30, KOH 78; pH 7.2 adjusted with KOH). The heart was removed from the cannula, and cells were isolated by

gentle titration, filtered (150 μm mesh) and kept in KB buffer for at least 30 min at room temperature before experimentation. All data were collected within 8 hours of cell isolation.

Whole-cell patch clamping

Whole-cell recordings were performed as previously described [7]. Pipettes (3–4 MΩ) were filled with (in mM): L-Aspartic acid (K^+ salt) 115, KCl 20, EGTA 5, HEPES 10, Na_2ATP 5, $MgCl_2$ 1, pH 7.2. Cardiomyocytes were superfused with Tyrode's solution. The following compounds were added to Tyrode's solution as needed: NaCN, 2-deoxy-glucose, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and/or 8-p-sulfophenyl-theophyline (all from Sigma). Experiments were conducted at 37°C.

Excised patch recordings

K_{ATP} channels were recorded in excised patches using the inside-out configuration as described [8]. Pipettes (2–4 MΩ) were filled with (in mM): KCl, 140; $MgCl_2$, 1; HEPES, 10; EGTA, 1; $CaCl_2$, 2; pH 7.4 adjusted with KOH. The bath solution contained (in mM) KCl, 140; EGTA, 1; HEPES, 10; $MgCl_2$, 1.2; pH 7.2 adjusted with KOH. Experiments were performed at room temperature at a holding potential of −60 or −80 mV. Solution changes were performed using a multi-barrel rapid solution changer (RSC-200; Molecular kinetics, Inc., IN). Recombinant AMPK (Upstate Biotechnology, Lake Placid, NY) was used at a final concentration of 1 U/mL.

Solutions containing ATP, AMP or ZMP were freshly prepared from frozen stocks (100 mM). Compound C (10 μM) was prepared from a 10 mM stock solution made in DMSO. Channel open probability (N.Po) was calculated using all points histograms (using 15s of continuous recording) or by dividing the mean patch current by the unitary current amplitude.

Heterologous Expression of K_{ATP} channel subunits

For electrophysiological studies, COS7L cells (Invitrogen, Carlsbad CA) were transfected (Fugene 6, Roche Applied Science) with mouse Kir6.2 and SUR2A cDNA (a gift from Dr. S. Seino, Kobe University) as well as a GFP reporter vector. For biochemical experiments, we used Kir6.2-HA cDNA, which has four C-terminal HA epitopes [9].

Antibodies

Rabbit polyclonal anti-Kir6.2 antibodies (W62 or W62b) were raised against the N terminal region of human Kir6.2 [10]. We also used goat anti-Kir6.2 (G-16; Santa Cruz, CA) and anti-SUR2A antibodies (M-19; Santa Cruz, CA), rabbit polyclonal anti-AMPKα (U; α-pan; Upstate Biotechnology, Billerica, MA), rabbit polyclonal anti-AMPKα (C; α-pan; Cell Signaling Technology, Beverly, MA), mouse monoclonal anti-GFP antibodies (BD Biosciences, San Jose, CA), mouse monoclonal anti-HA (12CA5) and rat monoclonal anti-HA-peroxidase (3F10; both from Roche Applied Science, IN).

Immunoprecipitation

Forty-eight hour post-transfection, each 100 mm dish was lysed in ice-cold immunoprecipitation lysis (IP) buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100, plus protease inhibitors [1 mM phenylmethanesulfonyl fluoride and 10 μl/ml protease inhibitor cocktail (Sigma-Aldrich)]. Immunoprecipitation was performed as described [10]. Briefly, anti-Kir6.2 (W62), anti-Kir6.2 (G-16), anti-SUR2A (M-19), anti-AMPKα (U), anti-AMPKα (C), anti-HA, or anti-GFP antibodies were added to 400μg of the cell lysates and incubated overnight at 4°C. Protein G beads (25 μl; Amersham Biosciences, NJ) was added and incubated at 4°C with rotation for 3 hours. As negative

controls we used unrelated antibodies (e.g. anti-GFP). The immunoprecipitates were washed (4x) with the IP buffer, modified to contain 0.1% Triton X-100. Washed beads were resuspended in 40 μl of 2× Laemmli sample buffer.

For native immunoprecipitations, enzymatically isolated ventricular myocytes from rat heart were lysed in ice cold buffer containing 250 mM sucrose, 10 mM HEPES, 1 mM EDTA, 1 mM DTT, pH 7.4, supplemented with protease inhibitors (Roche Complete cocktail). Membranes were prepared essentially as described [11]. Anti-Kir6.2 (W62b) antibody or unrelated IgG (as control) were bound to protein A/G beads (Invitrogen). Membrane proteins (120μg) were added to the antibody-bead complexes and incubated overnight at 4°C with rotation. Beads were washed, proteins were eluted and subjected to SDS-PAGE using AMPK antibodies.

Western blotting

Samples were fractionated by 12% SDS-PAGE, transferred to PVDF membranes (Bio-Rad) and incubated overnight at 4°C in blocking solution, consisting of 5% non-fat milk in TN-buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 0.1% Tween-20). Membranes were incubated (1 h) with primary antibodies at room temperature, washed with TN-buffer (3 times), and incubated (1 h) at room temperature in peroxidase-linked donkey anti-rabbit IgG (Amersham Biosciences) or monoclonal anti-goat IgG-peroxidase (Sigma-Aldrich) in TN-buffer. For HA tagged proteins, we used anti-HA peroxidase (1:800 dilution). Detection was with chemiluminesence (Supersignal West Pico, Pierce Biotechnology, Rockford, IL). A minimum of three independent experiments were performed.

RESULTS

We recently characterized the cardiac K_{ATP} channel macromolecular complex using proteomic approaches and found the enzymes of the glycolytic pathway to be important associated proteins [8]. These mass spectrometry experiments also identified 5'-AMP-activated protein kinase (AMPK) catalytic α subunit in an immunoprecipitate obtained with an anti-Kir6.2 antibody (data not shown). Experiments were therefore performed to investigate the functional relevance of this observation.

AMPK activation predisposes to K_{ATP} channel opening during metabolic stress

We used the membrane-permeable 5-aminoimidazole-4-carboxyamide (AICAR) to activate AMPK [12] in initial experiments with intact cells. After being taken up into cells by a nucleoside transporter, AICAR is phosphorylated to the monophosphate form, 5-amino-4-imidazolecarboxamide (also known as AICA ribotide or ZMP), which is a normal intermediate in the purine nucleotide synthesis pathway. ZMP accumulates inside the cell because of the slow rate of purine metabolism and, as an AMP analog, activates AMPK signalling [13–14].

Isolated rat ventricular myocytes were subjected to whole-cell voltage clamping and K_{ATP} channel opening was induced by metabolic inhibition with 2-deoxyglucose plus cyanide (Fig. 1). The time to half-maximal activation was 76±9.17s (n=5). Another group of cells were pre-treated for 10 minutes with 100 μM AICAR before the onset of the experiment. Upon metabolic inhibition the K_{ATP} channel current activated more rapidly in the AICAR-treated cells, with maximal activation occurring at 24±3.42 s ($p<0.05$; n=9). The time required for the K_{ATP} channel to activate during metabolic inhibition dose-dependently decreased with increasing AICAR concentrations (Fig. 2).

To exclude the possibility that AICAR directly activated K_{ATP} channels (instead of acting though ZMP formation), we examined its effect in the inside-out patch clamp configuration.

Robust K_{ATP} channel activity was observed following patch excision with typical dose-dependent inhibition by ATP (Fig. 3). AICAR (1 mM) was applied in the presence of an ATP concentration (100 M) that produces sub-maximal channel inhibition, which allows us to observe either stimulatory or inhibitory effects. However, AICAR had no effect on the mean patch current (Fig. 3A), demonstrating that neither the ATP-sensitivity nor the channel open probability was affected. This result is supportive of a secondary action of AICAR on K_{ATP} currents, possibly mediated by intracellular formation of the ZMP metabolite.

We next investigated the effects of ‚intracellular' monophosphate nucleosides (AMP and ZMP) on K_{ATP} channel activity. Experiments were performed as described above (i.e. nucleotides were applied in the presence of ATP at a concentration that produces sub-maximal block). First, we examined effects of AMP. We consistently saw that K_{ATP} channel activity recorded in inside-out patches of rat ventricular myocytes was significantly increased by AMP (100 M; data not shown; see also [15–7]). This activation may either be the result of a phosphotransfer reaction mediated by adenylate kinase [17] and/or AMP-induced activation of AMPK. We therefore used ZMP (the AICAR metabolite), which is not an adenylate kinase substrate [18–19]. ZMP (100 M) led to a significant activation of K_{ATP} channel activity within ~40s (Fig. 3B). This effect was reversible upon washout. Overall, ZMP increased channel open probability ($N.P_o$) from 2.3±0.61 to 4.8±1.68 ($p<0.01$; n=10; paired t-test). An AMPK-mediated effect is demonstrated by the finding that ZMP was without effect on K_{ATP} channel activity in the presence of the specific AMPK inhibitor, Compound C (10 M; Fig 3C; the $N.P_o$ respectively was 3.1±0.93 and 3.4±1.06; n=10; $p>0.05$ before and after application of ZMP). Compound C itself was without effect on K_{ATP} channel activity (Fig 3; also see Supplemental Figure S1). This result is consistent with our whole-cell experiments, demonstrating that AMPK regulates K_{ATP} channel function.

To further investigate the effect of this kinase, we used recombinantly purified AMPK. Experiments were performed using COS-7 cells transfected with mouse Kir6.2 and rat SUR2A cDNAs and K_{ATP} channels were recorded in the inside-out patch configuration. In the absence of "cytosolic" ATP, high levels of channel activity occurred, which were completely blocked by ATP (Fig. 4), characteristic of Kir6.2/SUR2A channels. Channels were then partially blocked by application of 300 μM ATP. In most patches, further application of AMP (100–300 μM) had little effect on channel activity. This is in contrast to patches obtained from cardiac myocytes, where AMP stimulates K_{ATP} channel activity under similar conditions [our observations; also see [16]]. The reasons for the different AMP response between K_{ATP} channels in heterologous expression systems and ventricular myocytes are not obvious, but may involve differences in the regulation by AMP-activated processes in the two cell types. To investigate the direct effect of AMPK on Kir6.2/SUR2A channels, we applied purified AMPK protein (Upstate Biotechnology) together with AMP to the cytosolic face of the same patch. A significant stimulation of K_{ATP} channel activity was observed (Fig. 4). The degree of stimulation of Kir6.2/SUR2A channels by AMPK was dependent on the AMP concentration. With 100 μM AMP, recombinant AMPK stimulated K_{ATP} channel mean patch current only mildly (by about 20%; Fig. 5), whereas AMPK enhanced the mean patch current several fold in the presence of 300 μM AMP (Figs. 4 and 5). The unusual AMP dose-response might reflect the possibility that AMPK becomes dephosphorylated in our in-vitro system [20]. Heat-inactivated AMPK did not stimulate K_{ATP} channel activity (Figs. 4 and 5).

To determine whether AMPK and K_{ATP} channel subunits form protein complexes in mammalian cells, COS7L cells were transiently transfected with Kir6.2-HA and SUR2A cDNAs. Whole cell extracts were immunoprecipitated with two separate anti-AMPK antibodies, with anti-Kir6.2 or anti-SUR2A antibodies (anti-GFP antibodies were used as a

negative control). The immunoprecipitates were assayed in immunoblotting using a peroxidase-conjugated anti-HA antibody and AMPK antibody (Fig. 6). As expected, Kir6.2-HA subunits were detected as a ~44 kDa band in the positive controls, but not in the negative control (Fig 6B). Kir6.2-HA was detected in the immunoprecipitate obtained with two separate antibodies against the AMPK α-subunit (Fig 6 B and C). Reciprocally, AMPK α-subunits are detected in immunoprecipitates containing SUR2 subunits. These data demonstrate that AMPK α-subunits physically interact with K_{ATP} channel subunits.

To investigate the physiological relevance of this interaction, the co-immunoprecipitation experiment was repeated using membranes prepared from enzymatically isolated rat ventricular myocytes. Immunoprecipitation was performed using an anti-Kir6.2 antibody (or unrelated IgG as a control). Immunoblotting of the immunoprecipitates confirmed detection of AMPK α-subunits in the experimental (but not negative control) lane (Fig 6D). These data confirm the mass-spectrometry data (see earlier) and demonstrate that AMPK and K_{ATP} channel subunits physically interact.

DISCUSSION

Physiological actions of AMPK and its effect on K_{ATP} channels

Biochemically, AMPK has been identified over 2 decades ago as an ultra-sensitive sensor of cellular stress. Even small rises in cellular AMP levels activate AMPK, which results in ATP consumption pathways to be inhibited and ATP synthesis pathways to be activated [3]. The activity of AMPK is increased during muscle contraction, hypoxia, ischemia, heat shock, a decrease in pH, inhibition of glycolysis and by uncouplers of oxidative phosphorylation [21–22]. In addition to acting as a fuel gauge under conditions of stress, AMPK has been assigned other roles, including stimulation of glucose-stimulated insulin secretion from pancreatic islets [23], stimulation of vascular endothelial growth factor (VGEF) expression and angiogenesis in skeletal muscle [24] and stimulation of nitric-oxide synthesis in human aortic endothelial cells [25]. Some ion channels have also been described to be targets of AMPK. Epithelial sodium channels, for example, are inhibited by AMPK [26] and AMPK plays a physiological role in modulating CFTR activity [27–29]. Until now, however, there have been few reports of AMPK affecting the expression or function of cardiac K_{ATP} channels. An inhibitory role for AMPK on K_{ATP} channel activity has been suggested by the finding that AICAR stimulates insulin release from pancreatic β-cells [23]. Furthermore, anti-diabetic drugs (such as rosiglitazone and phenformin) also activate AMPK, and stimulate insulin release [30–31]. The picture is clouded by the observation that some of these anti-diabetic drugs have direct inhibitory effects on the pancreatic β-cell subtype of the K_{ATP} channel independent of AMPK activation [32] and that AMPK stimulation actually *increases* β-cell K_{ATP} channel activity, in part by enhanced surface trafficking [33]. Effects of AMPK on the cardiac ventricular K_{ATP} channel are less well documented. One report demonstrated that whole-cell K_{ATP} channel density in isolated mouse ventricular myocytes is increased by repeated hypoxic episodes, and that this increase does not occur in myocytes isolated from a transgenic mouse with cardiac-specific expression of a dominant negative AMPK α2 subunit [6]. The same study demonstrated that repeated hypoxia leads to elevated K_{ATP} channel subunit levels in sarcolemmal membrane fractions and that this increase does not occur in the transgenic mouse hearts. These studies support the concept that hypoxia-induced activation of AMPK leads to increased surface expression of K_{ATP} channel subunits, but do not address the question whether K_{ATP} channels are direct targets of the AMPK signalling cascade. Our data demonstrate that AMPK does have a direct activating role in the rat ventricular K_{ATP} channel function. In whole-cell patch clamp conditions, we found that the K_{ATP} channel current was activated more readily during metabolic inhibition in the presence of AMPK activation by AICAR. This compound had no effect on K_{ATP} channels in inside-out membrane patches, whereas

ZMP (the downstream metabolite of AICAR) activated K_{ATP} channels. Moreover, the stimulatory effect of ZMP was prevented by the AMPK inhibitor, Compound C. Heterologously expressed Kir6.2/SUR2A channels were activated by recombinant AMPK in an AMP-dependent manner, but not by heat-inactivated AMPK. Our co-immunoprecipitation data demonstrate that the AMPK α-subunit associates with K_{ATP} channel subunits, which raises the possibility of localized signalling of the K_{ATP} channel subunits and/or proteins with which they associate.

Kir6 and SUR K^+ channel subunits as possible substrates for AMP-activated protein kinase

AMPK is a serine /threonine kinase. In a recent study, the Kir6.2 subunit of the pancreatic β-cell K_{ATP} channel was shown to be phosphorylated at Ser-385 [31], which does not conform to a typical AMPK consensus sequence [34]. It is likely that this Ser residue is also AMPK phosphorylated in the cardiac K_{ATP} channel, which is thought to be composed of a Kir6.2/SUR2A subunit combination [35]. It is interesting that the SUR2A subunit also contains several consensus sequences for AMPK phosphorylation, including Ser-401 (located within the intracellular linker between TM7 and TM8), Ser-1468 and Ser-1508 (both contained within the intracellular distal C-terminus). Our ongoing experiments are directed to map the K_{ATP} channel subunit phosphorylation sites and whether K_{ATP} channel associated subunits are also substrates for AMPK.

Dual regulation of K_{ATP} channels by AMP

AMP inhibits K_{ATP} channels at high millimolar concentrations [36]. This inhibition is likely to be due to interaction of AMP with the Kir6.2 ATP-binding site and is different from the stimulation of K_{ATP} channel activity observed at low AMP concentrations [15–17]. An interesting possibility was recently reported to explain the mechanism by which AMP activates K_{ATP} channels. Since AMP was found to stimulate K_{ATP} channel activity only in the presence of Mg^{2+} and a hydrolysable analog of ATP [16], a kinase-mediated phosphorylation event was suggested. Further, P^1,P^5-di-adenosine-5'-pentaphosphate (Ap_5A), which blocks adenylate kinase activity (albeit rather non-specifically [37]), prevented AMP-dependent stimulation of K_{ATP} channel activity. This led to the suggestion that AMP effects occur through the action of phosphotransfer reactions that are mediated in part by adenylate kinase [16]. This represents an important manner by which metabolism can be coupled to K_{ATP} channel activity. Our data demonstrate that AMP might have dual regulatory role in that it may additionally activate K_{ATP} channels by acting through AMPK.

Role of AMPK in ischemia – effects on K_{ATP} channels?

The concept that AMP at low micromolar levels may provoke K_{ATP} channel opening during ischemia is interesting and important, since AMP levels are almost undetectably low in normal, well-oxygenated heart tissue (due to the action of adenylate kinase). However, AMP levels rise rapidly early during ischemia [38–39]. In dog heart, for example, the ATP:AMP ratio decreases from 78 to 46 within just 1 minute of regional ischemia [38] – a change mediated predominantly by changes in the AMP levels. Since the ATP:AMP ratio is approximately the square of the ATP:ADP ratio [40], it is a far more sensitive indicator of alterations in the cellular metabolic status. An increase in cytosolic AMP is therefore an ideal candidate as a signaling molecule to indicate cellular stress. Stimulation of K_{ATP} channel activity by AMP could thus represent one of the earliest events in the ischemic heart. It is unlikely for AMP to act via adenylate kinase under ischemic conditions for the following reasons. First, the balance of the adenylate kinase reaction may not be favorable to produce AMP from ADP under ischemic conditions. Second, adenylate kinase activity is inhibited during myocardial ischemia [41]. Third, unlike the protective effect of K_{ATP} channel openers [42], inhibitors of adenylate kinase do not affect functional recovery of the

ischemic heart [43]. Other potential mechanisms should therefore be considered by which AMP might signal metabolic stress and regulate the activity of key proteins, such as K_{ATP} channels.

AMPK is emerging as an important signaling protein during myocardial ischemia. In the isolated heart, ischemia strongly activates AMPK [44]. Low-flow or regional ischemia also rapidly activates the activity of the upstream kinase, AMPKK, which further promotes phosphorylation of AMPK [45–46]. Support for a protective role of AMPK during ischemia comes from transgenic mice expressing a kinase dead AMPK subunit, which demonstrated significantly impaired recovery of post-ischemic contractile function in the setting of ischemia and reperfusion [47]. In rabbit heart, ischemic preconditioning also activates AMPK [48]. Historically, the literature provides ample support for the concept that AMPK activation may be protecting the heart from ischemic episodes. The widely used AMPK activator, AICAR (also known as acadesine), has been known for more than a decade to be cardioprotective by improving post-ischemic heart recovery and preconditioning [49–51]. Initially it was used in an attempt to enhance the rate of repletion of the nucleotide pool in post-ischemic myocardium [49], but has since been found to be without major effects on the cellular nucleotide complement [50]. Given our preliminary data that AMPK activates K_{ATP} channels and the known protective role of K_{ATP} channels in ischemic damage, we hypothesize that the protective effect of AICAR may be mediated (at least in part) through activation of AMPK and stimulation of K_{ATP} channel activity. Thus, AMP may act as a signaling molecule early during ischemia to stimulate K_{ATP} channel opening through activation of AMPK. There are two catalytic AMPK α-subunits, both of which are expressed in the heart. The AMPK α1-subunit is expressed widely, whereas the α2 subunit is expressed predominantly in the heart, liver and skeletal muscle [4, 40, 52]. It is likely that, similar to the regulation of CFTR channels [27], cardiac K_{ATP} channels may be regulated by both α1- and α2-subunits.

AMPK as a membrane-associated protein

There are reports to indicate that membrane proteins or ion channels are affected by AMPK [23, 26–27, 53]. Our co-immunoprecipitation show that the AMPK α-subunit physically interacts with K_{ATP} channel subunits. The AMPK α-subunit has also been described to interact with the Cl^- channel subunit CFTR [27], which is part of the same ABC cassette family of proteins to which SUR subunits belong. In the case of CFTR, the interaction domain has been mapped to residues 1420–1457 [27], which corresponds to the distal C-terminus of SUR (amino acids 1523–1549 of SUR2 and 1555–1581 or SUR1). This corresponds to the alternatively spliced region in SUR2 and it would be extremely interesting if the AMPK α-subunit also interacts with SUR in this region, since this raises the possibility of isoform-specific interaction. Of course, it remains possible that interaction is not direct and that unidentified adaptor proteins are involved, akin to the regulation of PKA that interacts with membrane channels through specific anchoring proteins (e.g. AKAPs) to target kinases close to their effector sites [54–55].

Significance of these studies

K_{ATP} channels are strongly regulated by the ATP:ADP ratio: the channel is activated both by decreasing ATP levels and increasing ADP levels during metabolic demand. Our data show that elevated AMP levels positively regulate K_{ATP} channels, hence adding another layer of regulation during metabolic stress (in addition to the well described changes in ATP:ADP ratio). In the cardiovascular system, the coupling between AMPK activation and K_{ATP} channel opening (due to alterations in the ATP:AMP ratio) may be particularly relevant during increased metabolic demand (such as intense exercise) and under pathophysiological conditions, such as hypoxia and ischemia.

HIGHLIGHTS

- During metabolic impairment, a change in the ATP:ADP ratio stimulates K_{ATP} channels opening.
- The ATP:AMP ratio is an acquisitively sensitive indicator of alterations in the metabolic status.
- We show that AMP-activated protein kinase (AMPK) activity promotes K_{ATP} channel opening.
- AMPK physically interacts with K_{ATP} channel subunits, suggestive of local signalling.
- Thus, small changes in AMP may trigger K_{ATP} channel availability under ischemic conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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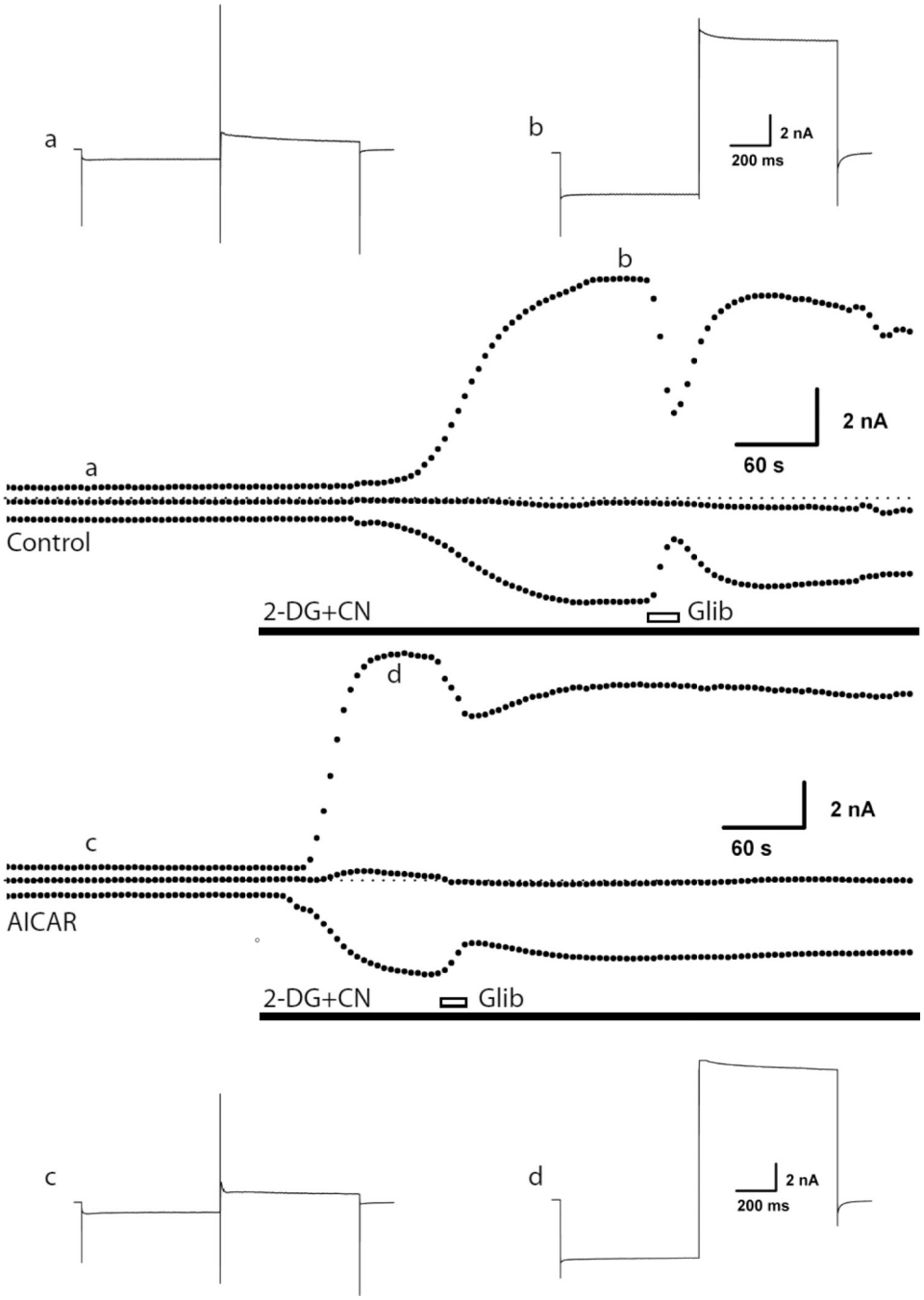

Fig 1.

Pretreatment of rat ventricular myocytes with AICAR shortened the time required for K_{ATP} channels to be activated by metabolic inhibition. Ventricular myocytes were voltage-clamped in whole-cell mode. From a holding potential of −70 mV, a dual voltage step was applied repeatedly (every 5s), consisting of a 700 ms hyperpolarization step to −100 followed by a 700 ms depolarization step to 0 mV. Two examples of currents recorded are shown at a collapsed time scale and each point represents a voltage clamp episode. For each recording, the top points are current at 0 mV, the middle are the holding current and the bottom points represent the current at −100 mV. The dotted lines represent the zero current level. The insets depict currents of individual voltage clamp episodes recorded at the points

indicated. K_{ATP} channel current was activated with metabolic inhibition (2-deoxyglycose plus cyanide; 2-DG+CN) and blocked by glibenclamide (10μM; Glib). One of the cells was pre-incubated with AICAR (100 μM) for 10 min, whereas the control cell was perfused with Tyrode's solution for an identical time. The adenosine receptor antagonist [8 (*p*-sulfophenyl) theophyline); 100μM] was included in the bath solution since AICAR may be a weak adenosine receptor agonist. Consistent with a previous report of incomplete sulfonylurea block of K_{ATP} channels during metabolic impairment [56], the current was only partially blocked by glibenclamide.

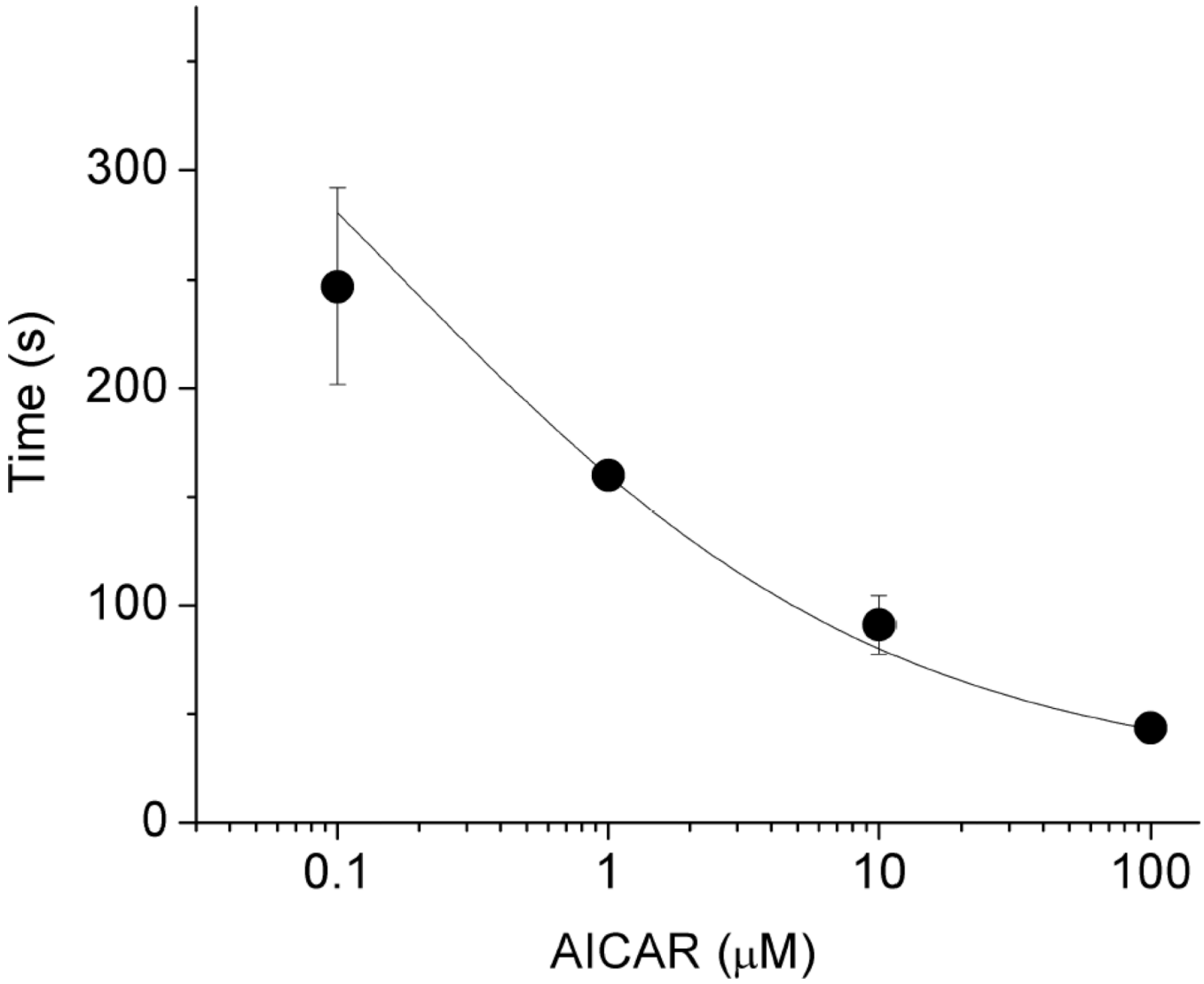

Fig 2.

AMPK dose-dependently shortened the time to maximal activation of K_{ATP} channels by metabolic inhibition. Cells (n=5–9) were incubated with various concentrations of AICAR before metabolic inhibition (as in the previous figure). The time to maximal activation of K_{ATP} channels is plotted as a function of the AMPK concentration. The line was drawn by fitting data points to a Boltzman distribution.

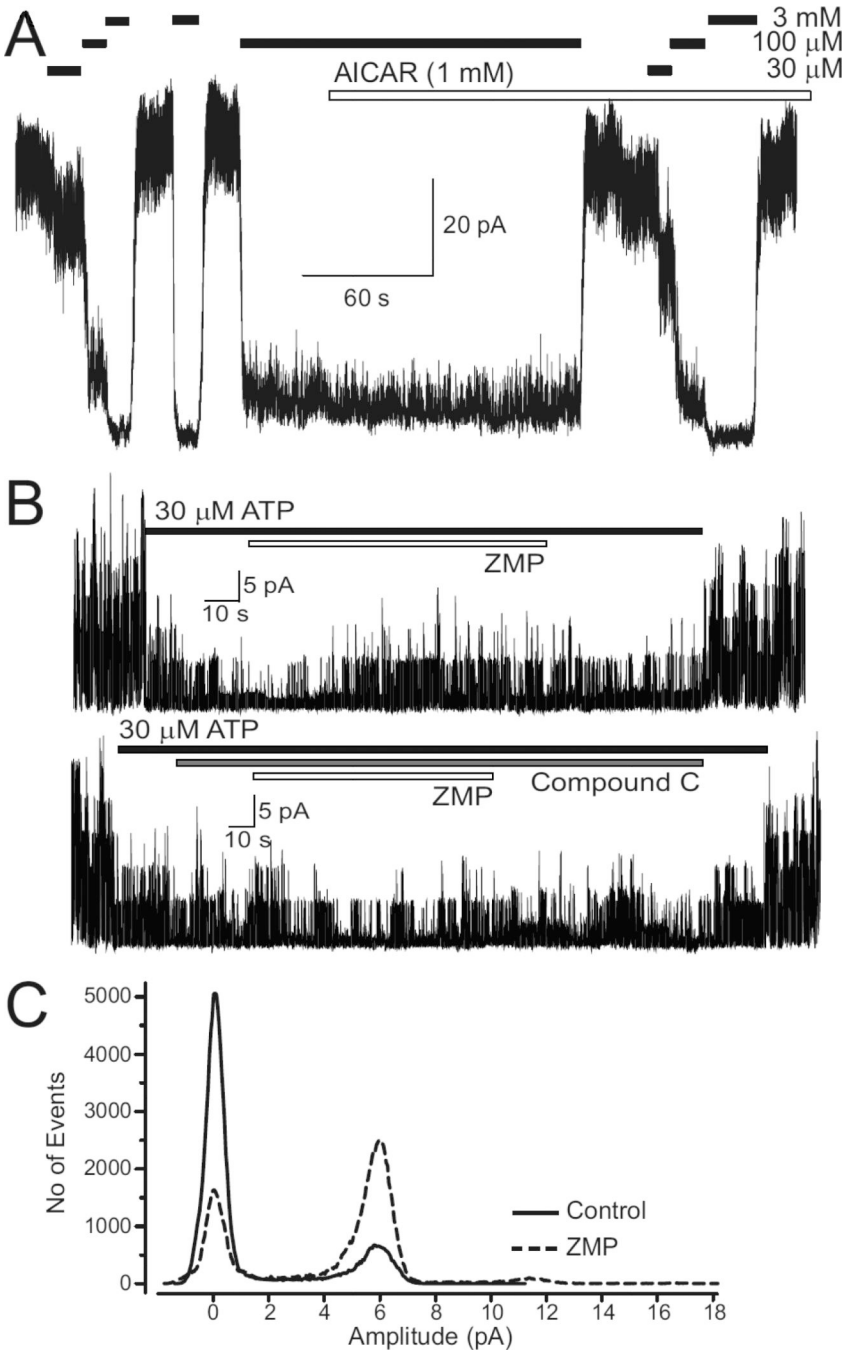

Fig 3.

ZMP (the AICAR metabolite) activates rat ventricular K_{ATP} channels without an effect on the unitary conductance. A, Inside-out recordings demonstrating that K_{ATP} channels are dose-dependently blocked by cytosolic ATP. There was no effect of 1 mM AICAR when applied in the presence of 100 μM ATP. Similar results were observed in 4 other patches. B, Upper panel: K_{ATP} channel activity was partially blocked by 30 μM ATP; subsequent addition of ZMP (100 μM) led to a progressive and reversible increase in K_{ATP} channel activity. Lower panel: pre-application of Compound C (10 μM) prevented the ZMP-induced activation of K_{ATP} channel activity. C, All-points histograms of K_{ATP} channel activity before (control) and after application of ZMP (100 μM) demonstrates that there was no

effect on the unitary current amplitude (~5.9pA in this patch). The patch potential was −80mV. The result depicted is representative of 10 similar recordings.

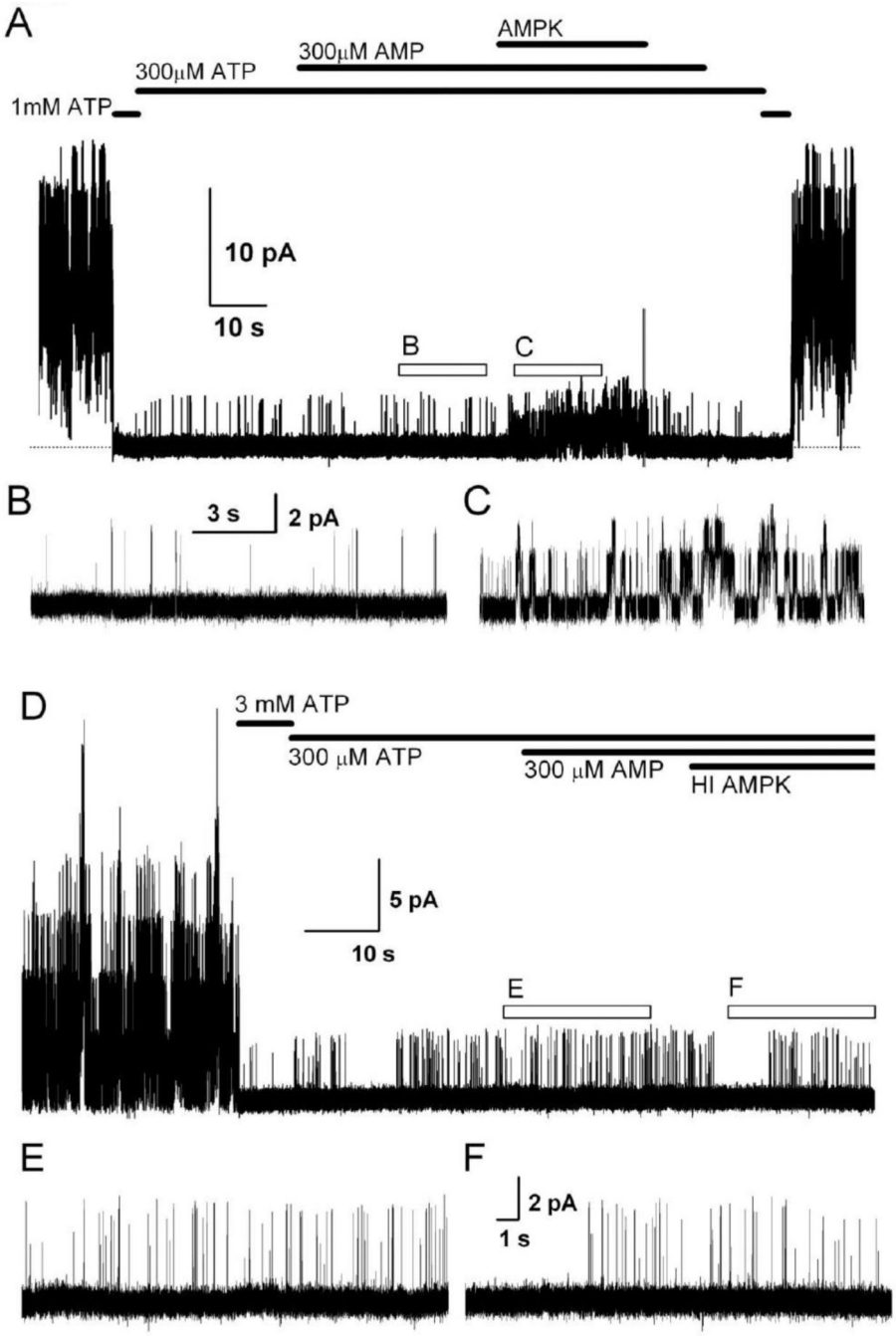

Fig 4.

The effects of AMPK on recombinant K_{ATP} (Kir6.2/SUR2A) channels. *A*, Representative recordings of Kir6.2/SUR2A currents from excised inside-out membrane patches from transiently transfected COS7L cells. Substances were applied as indicated. The functional expression of K_{ATP} channels was confirmed by the presence of large currents that were inhibited by 1 mM ATP. In the presence of 300 μM ATP and 300 μM AMP, recombinant AMPK (1U/ml), applied to the cytosolic face of the membrane, significantly increased channel activity. The holding potential was −60mV. B. and C. are expanded current sections from the regions in the upper panel as marked by the open bars. D. Similar experiment as in

the upper panel, but here we used heat-inactivated AMPK (HI AMPK). E, F: Expanded sections of the recording depicted in panel D, as indicated.

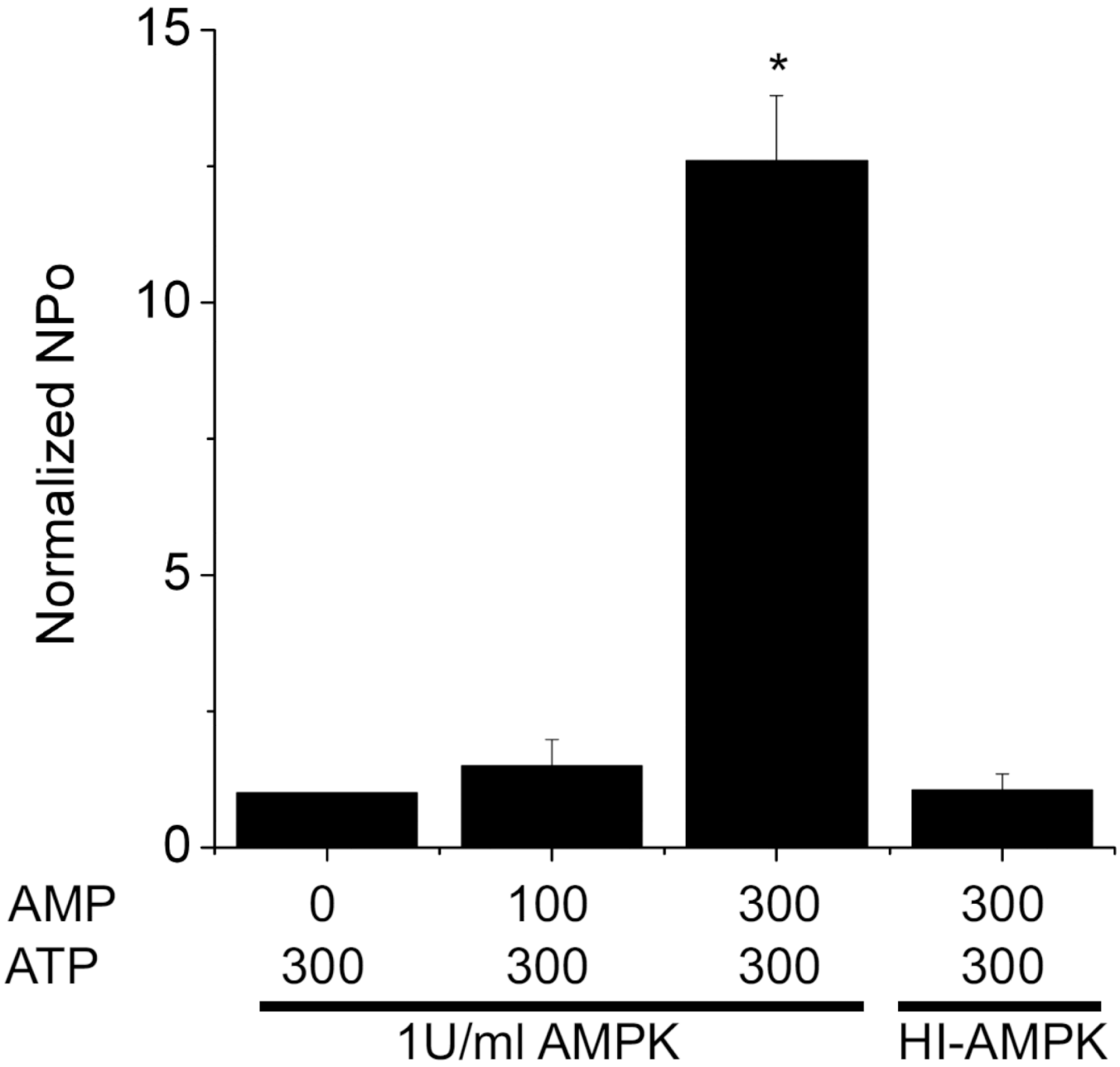

Fig 5.

The effect of AMPK is dependent on the AMP concentration. The bar graph depicts K_{ATP} channel current recorded at −60 mV in the presence of 300 μM ATP and 1U/ml AMPK. In control, no AMP was present. Increasing the AMP concentration dose-dependently increased K_{ATP} channel activity. The right-most bar represents recordings made with 300 μM AMP and heat-inactivated AMPK. In all cases, NPo was normalized to recordings made in the presence of 300 μM ATP and absence of AMP.

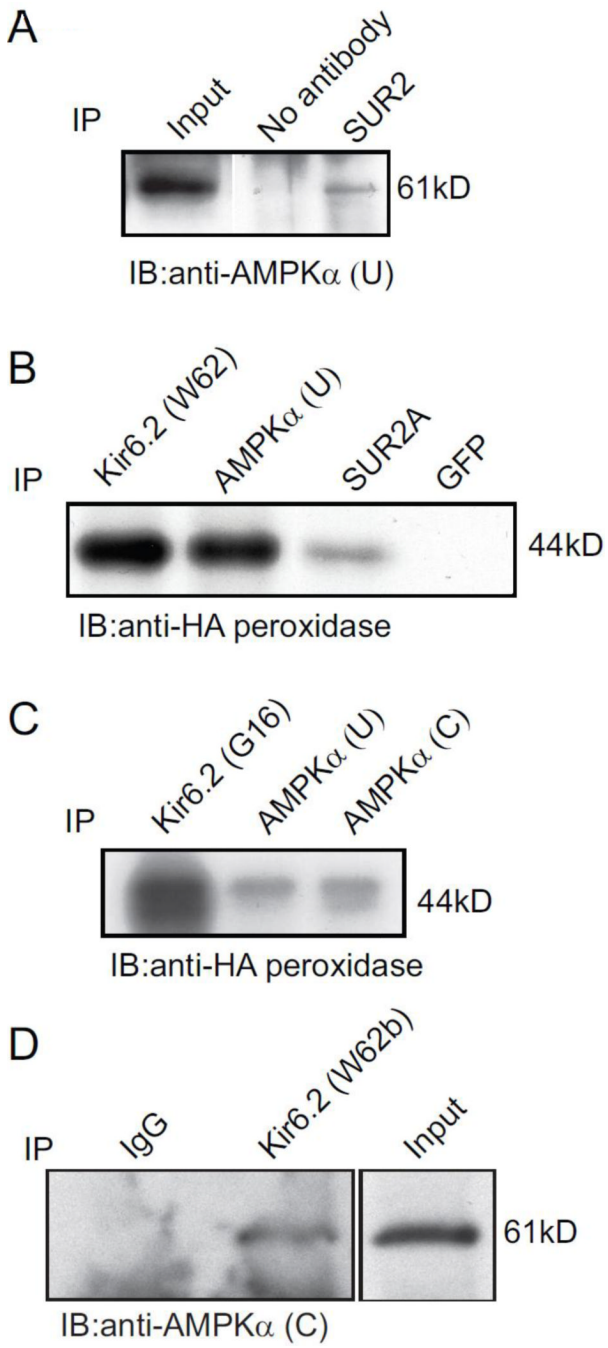

Fig 6.

Co-immunoprecipitation of AMPK α-subunits and K_{ATP} channel subunits. Co-immunoprecipitation of cell lysates from cells transfected with Kir6.2-HA and SUR2A cDNAs and rat ventricular tissue. Detection of AMPKα in the immunoprecipitate obtained with anti-SUR2A antibodies. B, Kir6.2-HA protein was detected in the immunoprecipitate obtained with the anti-Kir6.2 (W62), anti-AMPKα (U) and anti-SUR2A antibodies, but not with anti-GFP antibodies that were used as a negative control. C, Detection of Kir6.2-HA protein in immunoprecipitates obtained with a different anti-Kir6.2 (G16) antibody, as well as the immunoprecipitate obtained with two separate anti-AMPKα antibodies. D, Detection of AMPKα subunits in immunoprecipitates obtained with an anti-Kir6.2 (W62b) antibody.

An unrelated antibody (rabbit IgG) was used as a negative control. The results shown are representative of 2–3 separate experiments, which showed similar results.