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A Strongly Inwardly Rectifying K^+ Channel that Is Sensitive to ATP

Anthony Collins,[1] Michael S. German,[2] Yuh Nung Jan,[1] Lily Y. Jan,[1] and Biao Zhao[1]

[1]Howard Hughes Medical Institute, Departments of Physiology and Biochemistry, and [2]Hormone Research Institute, University of California, San Francisco, California 94143-0724

We have cloned an inwardly rectifying K^+ channel from the hamster insulinoma cDNA library and shown that it is inhibited by cytoplasmic ATP. The channel is 90–97% identical to the IRK3 channels cloned from other species, and its mRNA is found primarily in the brain. When expressed in *Xenopus* oocytes, the channel displays strong inward rectification typical of inward rectifiers. The channel is inhibited reversibly by physiological concentrations of ATP via a mechanism that does not appear to involve ATP hydrolysis, as shown by studies of channels in excised inside-out membrane patches. This effect is antagonized by ADP, again in the physiological range, implying that this channel is sensitive to the index of metabolic state, i.e., the intracellular [ATP]/[ADP] ratio. This channel is different from previously known ATP-sensitive K^+ channels, although it may also be stimulated by MgATP, as are other ATP-sensitive K^+ channels. The potential physiological significance of these ATP-dependent regulations will be discussed.

Key words: potassium channel; inward rectifier; ATP; ADP; Xenopus oocyte; molecular cloning; hamster insulinoma

Regulation of channel activities by ATP allows the excitability of a cell to vary with the metabolic state of the cell. A well known example of this is the inhibition of K^+ channels by ATP in the pancreas, which allows insulin release to be controlled by sugar level (Ashcroft et al., 1984; Henquin and Meissner, 1984; Rorsman and Trube, 1985, 1988; Misler et al., 1986; Ribalet et al., 1988). ATP-sensitive K^+ channels have also been found in neurons and muscles and have been suggested to provide protection against injury resulting from ischemia or anoxia (Grigg and Anderson, 1989; Nichols and Lederer, 1990; Jiang et al., 1992, 1994).

Of the five known types of ATP-sensitive channels (reviewed by Ashcroft and Ashcroft, 1990), Types 1–3 are K^+-selective. Type 1 ATP-sensitive K^+ channels (Noma, 1983), such as the ATP-sensitive K^+ channels found in pancreas, heart, and skeletal muscle, appear to be members of the family of inwardly rectifying K^+ channels. Inwardly rectifying K^+ channels pass more inward than outward current and have been found to be important for controlling the resting potential and excitability of the cell (Katz, 1949; Hille, 1992). The degree of rectification varies among different channels. Type 1 ATP-sensitive K^+ channels are weakly rectifying and pass outward current even at potentials 60 mV or more above the K^+ equilibrium potential, in contrast to "strong" inward rectifiers, which pass little or no outward current at these potentials and consequently have a current–voltage relationship with a range of negative slope conductance. To date, none of the strong inward rectifiers has been reported to be inhibited by ATP and nonhydrolyzable ATP analogs.

The recent cloning of inwardly rectifying K^+ channels (Dascal et al., 1993; Ho et al., 1993; Kubo et al., 1993a,b; Ashford et al., 1994; Ishii et al., 1994; Makhina et al., 1994; Morishige et al., 1994; Périer et al., 1994; Stanfield et al., 1994; Takahashi et al., 1994; Tang and Yang, 1994; Bredt et al., 1995; Inagaki et al., 1995) has opened the possibility of cloning ATP-sensitive inwardly rectifying K^+ channels, and two cDNA clones have been reported to give rise to weakly rectifying K^+ channels sensitive to ATP (Ashford et al., 1994; Inagaki et al., 1995). Here we report the cloning and expression of a strongly rectifying K^+ channel from a hamster insulinoma (HIT-T15) cDNA library, as well as inhibition of this channel by ATP in the physiological range. The predicted amino acid sequence is 90–97% identical to that of IRK3 from mouse (Morishige et al., 1994), rat (Bredt et al., 1995), and human (Makhina et al., 1994; Périer et al., 1994; Tang and Yang, 1994) and, therefore, is referred to as IRK3(HIT). The functional characteristics of this channel expressed in *Xenopus* oocytes indicate that it is not equivalent to any previously characterized ATP-sensitive channel, because it is a strongly rectifying inward rectifier that is inhibited by ATP and nonhydrolyzable ATP analogs.

MATERIALS AND METHODS

Molecular cloning of IRK3(HIT) and cRNA transcription. We screened 1.4×10^6 independent recombinants from a random primed cDNA library constructed in the λgt11 vector from HIT-T15 cells. After lifting from bacterial plates, nylon filters were hybridized to degenerate oligonucleotide probes H5S2 {5′-ACC,AT(ATC),CG(TC),TAT,GG(ATC), T(TA)C,(CA)G}, which encode the consensus amino acid sequence in the H5 region: TIGYG(FY)R. The hybridization was done at 43°C (prehybridization and hybridization solutions consisted of 5× SSPE, 5× Denhardt's solution, 100 μg/ml single-stranded salmon sperm DNA, and 0.2% SDS), and final wash was done in 0.2× SSPE, 0.5% SDS at room temperature. The cDNA inserts were then subcloned into the pBluescript SK vector (Stratagene, La Jolla, CA) at the *Eco*RI site for sequencing with the Sequenase kit (United States Biochemical, Cleveland, OH). To boost the expression efficiency of the channel in *Xenopus* oocytes, the insert of the H5 clone was excised at the *Sal*I/*Not*I sites and subcloned into pSD64TR (a gift of Dr. Terry Snutch, University of British Columbia, Vancouver, British Columbia, Canada) at the *Xho*I/*Not*I sites. Com-

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Correspondence should be addressed to Dr. Lily Y. Jan, HHMI/UCSF, 3rd & Parnassus Ave., San Francisco, CA 94143-0724.

plementary RNA was transcribed from the clone by using the mMessage mMachine kit (Ambion, Austin, TX).

Northern analysis. Total RNA was prepared with Tri-Reagent (Molecular Research Center, Cincinnati, OH), and polyA$^+$ RNA was isolated with Dynabeads (DYNAL, Great Neck, NY), all according to the manufacturer's instructions. For Northern analysis, 10 μg of polyA$^+$ RNA was resolved by electrophoresis on formamide agarose gel and blotted onto nylon filter. The filter was then hybridized to randomly primed DNA probes using the IRK3(HIT) cDNA clone (H3) as a template, and the final wash was done at 65°C with 0.1× SSC, 0.2% SDS.

Oocyte preparation. Stages V–VI *Xenopus* oocytes were prepared by digestion with 2 mg/ml collagenase (Worthington, Freehold, NJ, type CLS3, or Boehringer Mannheim, Indianapolis, IN, type A) for 2 hr with agitation in 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4. Separated oocytes were then rinsed and stored in 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.4 (ND-96), at 18°C with 50 μg/ml gentamycin, 60 μg/ml ampicillin, and 2.5 mM pyruvate added. Up to 24 hr after digestion, oocytes were injected with 46–50 nl of 20 ng/μl cRNA (Nanoject, Drummond, Broomall, PA). Two-electrode voltage-clamp or patch-clamp recording was performed at 21–23°C 3–14 d after injection.

Electrophysiology. Two-electrode voltage-clamp (Axoclamp 2A, Axon Instruments, Foster City, CA) recordings were low pass-filtered at 1 kHz, digitized at 2 kHz, and stored on disk using pClamp software (Axon Instruments). Electrodes were filled with 3 M KCl and had resistances of ~1 MΩ. The recording bath contained 3 mM $MgCl_2$ and 5 mM HEPES with either 90 mM KCl (90K) or 19 mM KCl plus 75 mM Tris (19K), pH 7.4.

Patch-clamp recording was performed using a List-Medical (Darmstadt, Germany) EPC 7 amplifier. Data from giant patches were filtered at a corner frequency of 20 KHz, acquired at 50 KHz, and stored on disk using Axobasic software (Axon Instruments). Capacitance transients were canceled as far as possible by analog compensation. Pipettes were either 7052 (Garner, Claremont, CA) or Pyrex (Corning, Newark, CA) glass with tips of 30–40 μm inner diameter, dipped in a Parafilm-mineral oil mixture to aid sealing (modified from Hilgemann, 1989; Collins et al., 1992), and filled with 140 mM KCl, 2 mM $MgSO_4$, 5 mM $CaCl_2$, and 5 mM HEPES, pH 7.4. For seal formation, oocytes were placed in a recording dish containing 140 mM KCl, 10 mM EDTA, 5.6 mM $CaCl_2$ (50 nM free Ca^{2+}), 10 mM HEPES, pH 7.2 (Int_{0Mg}). Inside-out patches were excised into this solution. Patches were perfused using a perfusion chamber similar to one previously described (Collins et al., 1992). Perfusion solutions used were as follows: (1) Int_{0Mg}; (2) 140 mM KCl, 10 mM EDTA, 9 mM $MgCl_2$ (100 μM free Mg^{2+}), 0.6 mM $CaCl_2$ (50 nM free Ca^{2+}), and 10 mM HEPES, pH 7.2 ($Int_{0.1Mg}$); and (3) 140 mM KCl, 10 mM EGTA, 2 mM $MgCl_2$, and 10 mM HEPES, pH 7.3 (Int_{2Mg}). Int_{0Mg} and $Int_{0.1Mg}$ were prepared with and without added nucleotides from 2× or 5× stock solutions containing the 1× equivalent of 100 mM K^+. In the case of $Int_{0.1Mg}$, [total $MgCl_2$] was adjusted to maintain [free Mg^{2+}] at 100 μM, depending on the concentration and type of nucleotide added. The pH was adjusted with KOH after addition of nucleotides, then [K^+] was made up to 140 mM with KCl and, finally, the pH was checked carefully again. The [Cl^-], therefore, was actually <140 mM and varied depending on the amount of nucleotide added. Control experiments were performed to confirm that the 50 nM Ca^{2+} in these solutions was too low to activate the endogenous Ca-activated Cl^- conductance. Concentrations of free and bound metals and nucleotides were calculated using the "Bound and Determined" computer program (Brooks and Storey, 1992).

Single-channel recordings were digitized at 94.4 kHz and stored on videotape (VR10B, Instrutech, Great Neck, NY) and then transferred to disk at 5.9 kHz for further analysis. Pipettes were of borosilicate glass (VWR, West Chester, PA) and contained 140 mM KCl, 5 mM $CaCl_2$, 2 mM $MgSO_4$, and 5 mM HEPES, pH 7.4. The bath solution was 140 mM KCl, 10 mM EGTA, 2 mM $MgCl_2$, and 10 mM HEPES, pH 7.1.

Salts and nucleotides were purchased from Sigma (St. Louis, MO), except adenylyl-imidodiphosphate (AMP-PNP) and adenylyl (β,γ-methylene)-diphosphate (AMP-PCP) (lithium salts), which were purchased from Boehringer Mannheim, and EDTA and KOH, which were purchased from Aldrich (Milwaukee, WI).

RESULTS

IRK3(HIT) is expressed primarily in the brain

To look for cDNA clones that encode inwardly rectifying K^+ channels, we screened a hamster insulinoma cell line (HIT-T15) cDNA library under low stringency with degenerate oligonucleotide probes encoding the H5 region, and we isolated two independent, full-length cDNA clones that encode the same channel. The deduced amino acid sequence is ~60% identical to that of IRK1 (Kubo et al., 1993a) and ~40% identical to those of ROMK1 (Ho et al., 1993) or GIRK1 (Dascal et al., 1993; Kubo et al., 1993b). During preparation of this paper, several IRK3 (homologs of IRK1) clones have been reported from human, mouse, and rat (Makhina et al., 1994; Morishige et al., 1994; Périer et al., 1994; Tang and Yang, 1994; Bredt et al., 1995). Because our cDNA encodes a protein that is 90–97% identical to the amino acid sequence of these IRK3s, we named our clone IRK3(HIT).

IRK3(HIT) has an open reading frame of 444 amino acid residues and two putative transmembrane segments (M1, M2) based on hydropathy analysis (Fig. 1*A*). The sequence reveals a richness in putative sites of phosphorylation by protein kinases. In addition, there is a proline-rich region (amino acid residues 361–367) that bears resemblance to the SH3-binding domain of other proteins (Ren et al., 1993).

To examine the tissue distribution of IRK3(HIT), we performed Northern analysis. As evident from Figure 1*B*, IRK3(HIT) is expressed mainly in the brain (a strong signal at 2.4 kb and a weak signal at ~6 kb). The BHK cell line (a fibroblast cell line derived from hamster kidney) also displays a weak signal at ~1.6 kb. To our surprise, IRK3(HIT) could not be detected in the HIT cells on our Northern blot. This suggests that IRK3(HIT) is expressed at a much lower level in the HIT cells than in the brain.

Functional expression of IRK3(HIT)

Injection of IRK3(HIT) cRNA into *Xenopus* oocytes resulted in the expression of strongly inwardly rectifying K^+ currents, as recorded by two-electrode voltage-clamp (Fig. 2*a*) and in cell-attached patches (Fig. 2*b*, *trace 1*). External Ba^{2+} blocked currents in a voltage-dependent manner (data not shown), and outward currents showed a range of negative slope conductance at voltages positive to the K^+ equilibrium potential (Fig. 2*a*). The macroscopic IRK3(HIT) currents resemble those of classical native inward rectifiers (Hagiwara et al., 1976; Standen and Stanfield, 1978; Hagiwara and Yoshii, 1979; Sakmann and Trube, 1984) and other cloned "strong" inward rectifiers (Kubo et al., 1993a; Takahashi et al., 1994; Stanfield et al., 1994; Ishii et al., 1994). In cell-attached patches, single-channel conductance was ~10 pS (Fig. 2*c–f*). These results are consistent with those reported for other cloned channels with almost identical primary structure: MB-IRK3 (Morishige et al., 1994), HRK1 (Makhina et al., 1994), HIR (Périer et al., 1994), and hIRK2 (Tang and Yang, 1994). External application of 300 μM tolbutamide (a sulfonylurea antagonist of Type 1 ATP-sensitive K^+ channels) had little or no effect on the IRK3(HIT) currents. In 10 oocytes, the current was inhibited by ≤7%. In one oocyte, inhibition was 24% (data not shown).

Because IRK3(HIT) was cloned from the HIT cell, which expresses ATP-sensitive K^+ channels, we considered the possibility that IRK3(HIT) was inhibited by ATP. To determine the dependence of IRK3 current on cytoplasmic ATP and related compounds, it was necessary to expose the cytoplasmic side of the membrane to solutions of various compositions. Excision of inside-out patches into Mg^{2+}-containing solutions resulted in a rapid, irreversible decrease ("rundown") of IRK3(HIT) currents. However, rundown could be slowed greatly by excising into Mg^{2+}-free solution (Int_{0Mg}), followed

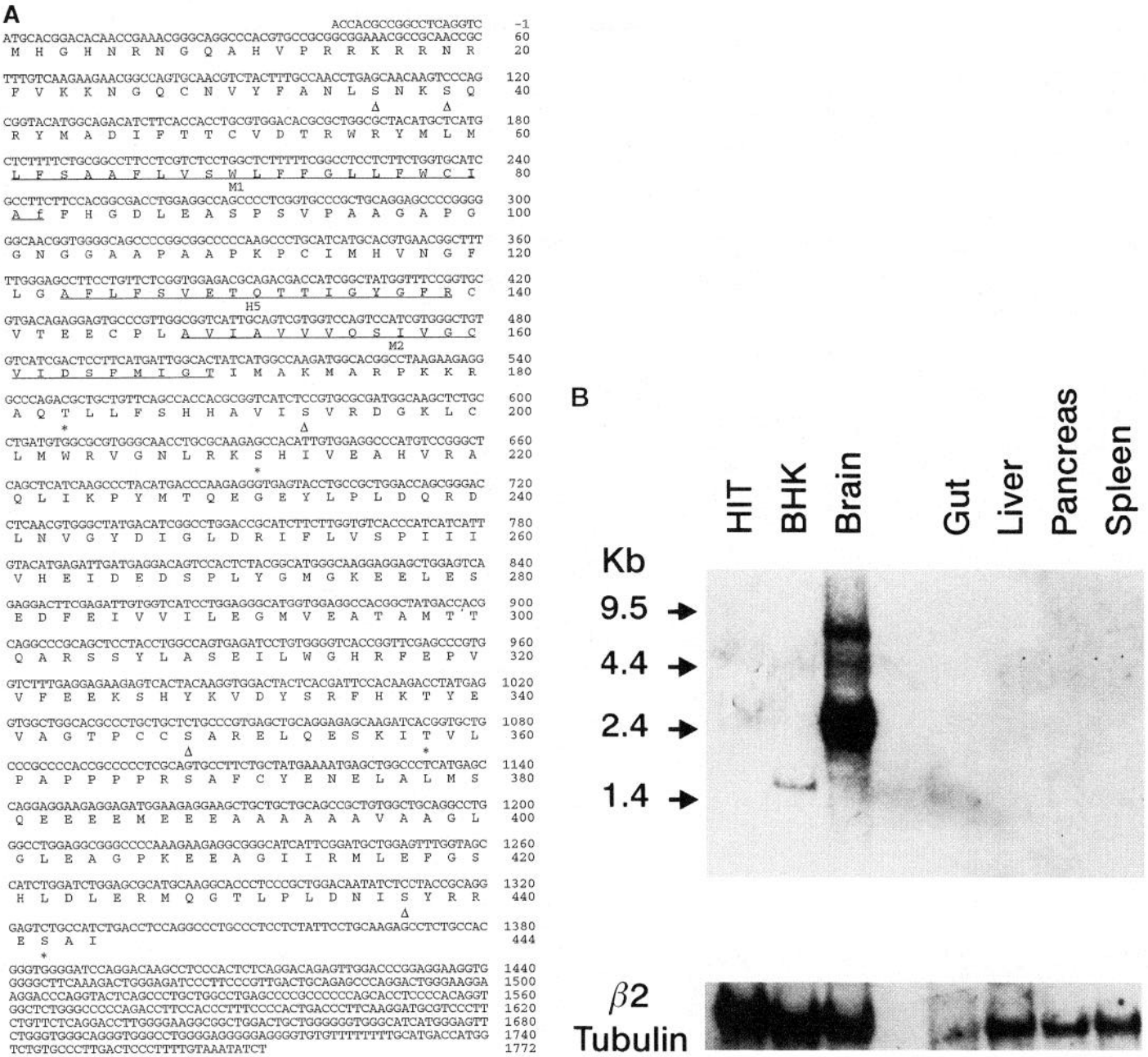

Figure 1. *A*, The nucleotide and predicted amino acid sequence of IRK3(HIT). The sequence shown here is that of the cDNA clone used for expression in the oocytes. The other clone is ~280 nucleotides longer in the 5′-untranslated region and ~50 nucleotides longer in the 3′-untranslated region. Proposed transmembrane segments (*M1*, *M2*) are assigned by hydropathy analysis. Putative phosphorylation sites are underlined by *triangles* for protein kinase C and *asterisks* for protein kinase A. *B*, Northern analysis of IRK3(HIT). Each lane contains 10 μg of polyA$^+$ RNA. The IRK3(HIT) cDNA was radioactively labeled and used for hybridization. The film was exposed for 120 hr. β2 tubulin was used as control for the quality and quantity of RNA preparations, and an exposure time of 4 hr was used for this control.

by perfusion with the same solution. This procedure resulted in loss of rectification and loss of slow activation kinetics at negative potentials (Fig. 2*b*, *traces 2* and *3*), probably caused by washout of blocking agents such as Mg^{2+} and polyamines (Matsuda et al., 1987; Vandenberg, 1987; Ficker et al., 1994; Lopatin et al., 1994; Fakler et al., 1995). With 140 mM K^+ on both sides of the membrane and in the absence of rectification, the IRK3(HIT) current is indistinguishable, in principle, from a linear leak conductance with a reversal potential of 0 mV. However, the identity of this current as IRK3(HIT) could be confirmed by the initial presence of rectification in the cell-attached mode and by the re-establishment of rectification after application of 2 mM Mg^{2+} (Int_{2Mg}) to the cytoplasmic surface of the patch (Fig. 2*b*, *trace 4*) (Matsuda et al., 1987; Vandenberg, 1987). Continued perfusion with Int_{2Mg} caused the IRK3(HIT) current to run down completely, providing an opportunity to reapply experimental solutions as a negative control, if needed.

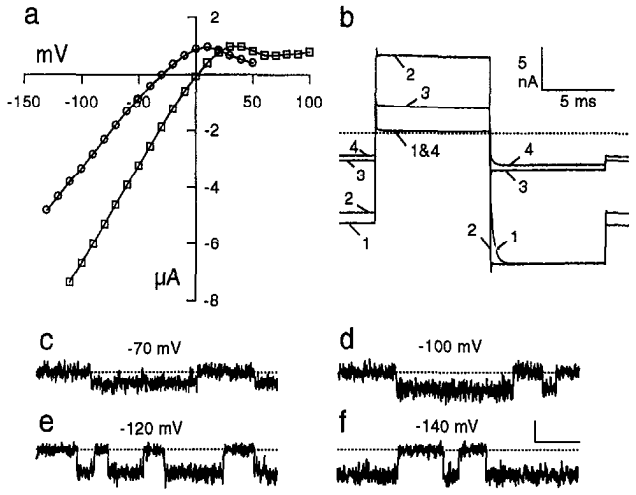

Figure 2. IRK3(HIT) channels expressed in *Xenopus* oocytes. Horizontal *dotted lines* in this and subsequent figures indicate zero current level. *a*, Current–voltage relationship for inwardly rectifying currents obtained by two-electrode voltage-clamp from an oocyte injected with IRK3(HIT) cRNA. External $[K^+]$ was 90 mM (*open squares*) or 19 mM (*open circles*). Membrane potential was held at 0 mV and stepped to potentials between +100 and −130 mV for 400 msec. Current at the end of the test pulse is shown. The increase in conductance between +60 and +100 mV was attributable to activation of an endogenous voltage-activated current. *b*, Macroscopic currents in a giant patch. Membrane potential was held at −60 mV and stepped to +60 mV, then to −80 mV (except *trace 2*: holding potential −30 mV; test potentials +30 and −50 mV). *Trace 1*, Cell-attached. *Trace 2*, After excision into and perfusion with Int_{0Mg}. *Trace 3*, In Int_{0Mg}; 20 min, 16 sec after *trace 2*. *Trace 4*, In Int_{2Mg}; 4 sec after *trace 3*. *c–f*, Single-channel records in cell-attached patches; digitally low pass-filtered at 500 Hz. Bath and pipette solutions both contained 140 mM K^+. All records except the −120 mV record were obtained from the same oocyte. A single-channel conductance of 9.8 pS was obtained from the relationship of mean single-channel current amplitude versus membrane potential. Calibration bar: 1 pA, 100 msec.

Inhibition of IRK3(HIT) by ATP and nonhydrolyzable ATP analogs

ATP applied to inside-out patches in the absence of free Mg^{2+} inhibited the current reversibly in a concentration-dependent manner (31/36 patches). ATP also had an inhibitory effect in the presence of 0.1 mM Mg^{2+} ($Int_{0.1Mg}$; 22/27 patches); this inhibitory effect was superimposed on the stimulatory effect of MgATP, which presumably involves ATP hydrolysis (see Fig. 7 and final section in Results). Although the inhibitory effect of ATP was evident in the presence or absence of Mg^{2+}, this effect could be quantitated more readily when Mg^{2+} was absent, thereby eliminating the stimulatory effects induced by MgATP and reducing the rate of channel rundown. In most cases, the time course of rundown could be approximated closely by a single exponential or linear decay (Fig. 3*a*), which made it possible to correct for rundown and normalize current records (Fig. 3*b*). Rundown-compensated and normalized data were pooled from several patches to obtain a dose–response curve; assuming a single ATP-binding site, ATP inhibited IRK3(HIT) current with an apparent K_d of 1.47 mM (Fig. 3*d*, *circles*). The fraction of current sensitive to ATP was variable, with a mean of 0.49. As a control, currents in patches from oocytes expressing IRK1 (Kubo et al., 1993a) were not inhibited by up to 10 mM ATP, both in the absence of Mg^{2+} (Fig. 3*d*, *triangles*) and in the presence of 30 μM free Mg^{2+} (data not shown). Therefore, IRK3(HIT), but not IRK1, is inhibited by physiological concentrations of ATP.

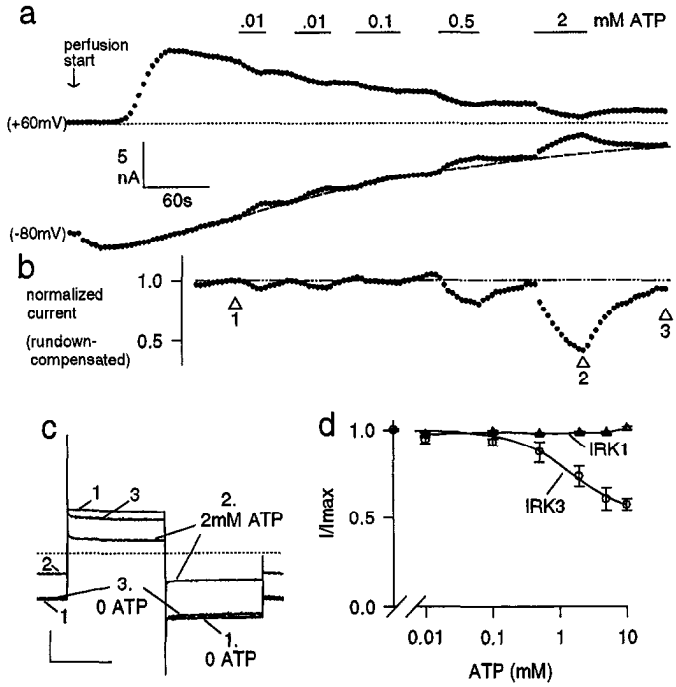

Figure 3. Inhibition of IRK3(HIT) current by ATP. Membrane potential was held at −60 mV and stepped every 4 sec to +60 mV for 8 msec, then to −80 mV for 8 msec. *a*, The *arrow* at the beginning of the trace indicates the start of perfusion with Int_{0Mg}. *Filled circles* represent current amplitude at the end of the 8 msec pulses to +60 and −80 mV. *Bars* above the record indicate perfusion with 0.01, 0.1, 0.5, and 2 mM ATP. At the end of each of these perfusions, ATP was removed quickly by perfusion with Int_{0Mg}. The *dashed line* is a single exponential fitted to the current in 0 ATP at −80 mV (time constant = 256.6 sec), to indicate the time course of channel rundown. *b*, Current at −80 mV compensated for rundown and normalized to the predicted current at the beginning of the trace. The time axis is aligned with *a*. *c*, Individual traces from the experiment in *a*, recorded at the times indicated by the *open triangles* in *b*. *Traces 2* and *3* are rundown-compensated and scaled to *trace 1*. Calibration bars: 5 msec, 5 nA. *d*, Dose response for ATP in Int_{0Mg}. (*open circles*) IRK3(HIT); data from 10 patches were rundown-compensated and normalized as in *b*. The line represents a fit to the data according to $I/I_{max} = -f/((K_d/[ATP]) + 1) + 1$. $K_d = 1.47$ mM; $f = 0.49$. (*filled triangles*) IRK1; data from two patches. Data points are mean ± SEM.

The observation that ATP inhibited IRK3(HIT) current in the absence of Mg^{2+} suggested that hydrolysis of ATP is not required. Indeed, the nonhydrolyzable ATP analogs AMP-PNP and AMP-PCP both inhibited the current (Fig. 4). Inhibition by AMP-PCP was similar to the effect of ATP (5/5 patches), whereas inhibition by AMP-PNP (3/6 patches) was irreversible. No consistent inhibitory effects were observed for CTP and GTP (5 mM in Int_{0Mg}; 5 patches, data not shown). AMP had little or no effect at concentrations of up to 10 mM (2 patches, data not shown). These findings indicate that ATP inhibits IRK3(HIT) without the involvement of kinases or other enzymes that require ATP hydrolysis.

The inhibitory effect of ATP is antagonized by ADP at physiological concentrations

Binding of ADP may lead to a reduction of the effect of ATP on some ATP-sensitive channels (Dunne and Petersen, 1986; Kakei et al., 1986; Misler et al., 1986; Findlay, 1988) and ATP-utilizing enzymes (Morrison and O'Sullivan, 1965). We therefore asked whether the inhibition of IRK3(HIT) currents by ATP was reduced in the presence of ADP. Application of ATP in Int_{0Mg} to the cytoplasmic side reduced the amplitude of both inward and

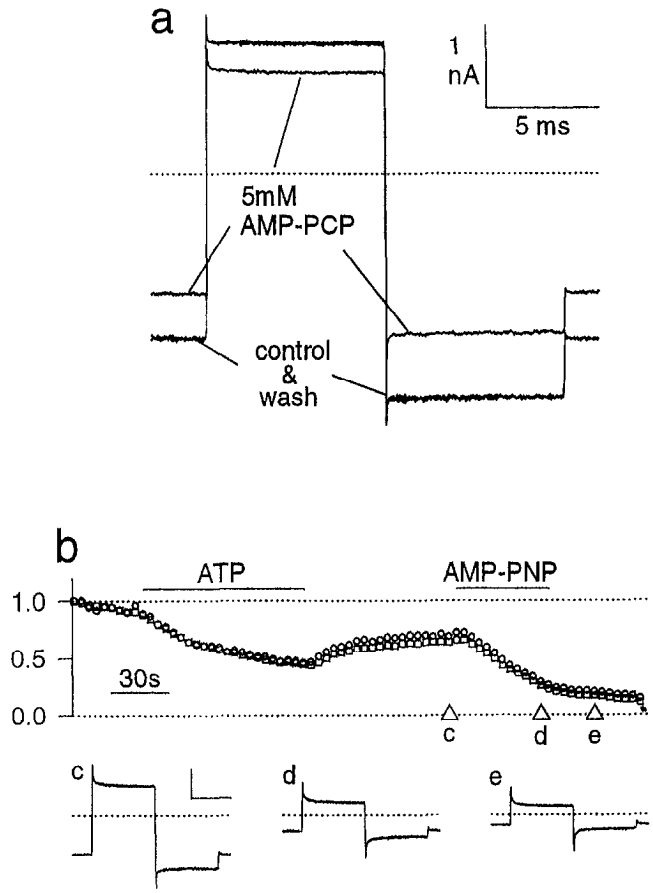

Figure 4. Inhibition of IRK3(HIT) by nonhydrolyzable ATP analogs. These analogs were in the form of lithium salts, and for this reason the perfusion solutions (Int_{0Mg}) all contained 20 mM Li^+, which partially blocked outward IRK3(HIT) current. *a*, AMP-PCP. Superimposed traces from a single patch with unusually stable current. Membrane potential was held at −60 mV and stepped to +60 mV, then to −80 mV. *Control* and *wash* indicate perfusion with Int_{0Mg} + 20 mM Li^+ before and after 5 mM AMP-PCP, respectively. *b*, AMP-PNP. Normalized current amplitudes at +60 mV (*open circles*) and −80 mV (*open squares*); no rundown compensation. *Bars* indicate perfusion with 5 mM ATP and 5 mM AMP-PNP, followed in each case with perfusion with ATP-free solution. *c–e*, Individual traces recorded at the times indicated by the *open triangles* in *b*. Calibration bars: 500 pA, 5 msec.

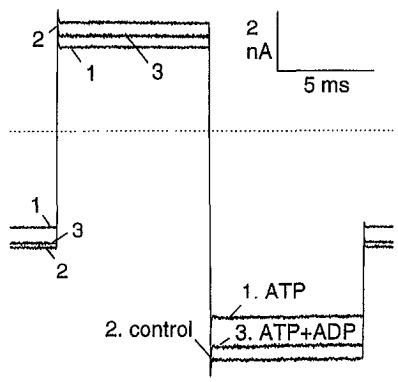

Figure 5. Antagonism of ATP by ADP. Superimposed traces from a single patch perfused with Int_{0Mg}. Membrane potential was held at −60 mV and stepped to +60 mV, then to −80 mV; no rundown compensation. *Trace 1*, Recorded during perfusion with 2 mM ATP. *Trace 2*, Recorded 64 sec after *trace 1*, in Int_{0Mg}. *Trace 3*, 20 sec after *trace 2*, in the presence of 2 mM ATP and 10 μM ADP.

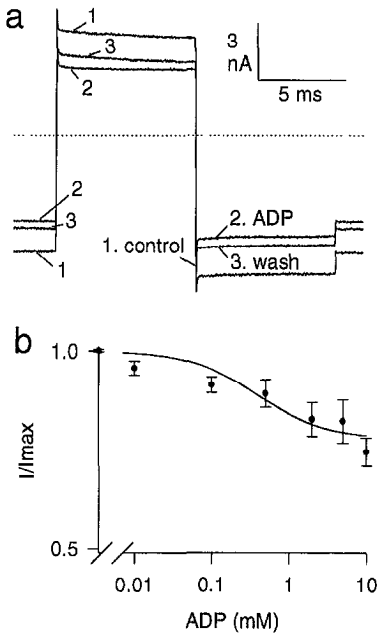

Figure 6. Partial inhibition of IRK3(HIT) by high [ADP]. *a*, Superimposed traces from a single patch perfused with Int_{0Mg}; no rundown compensation. Membrane potential was held at −60 mV and stepped to +60 mV, then to −80 mV. *Trace 1*, Recorded during perfusion with Int_{0Mg}. *Trace 2*, 144 sec after *trace 1*, in the presence of 5 mM ADP. *Trace 3*, 72 sec after *trace 2*, after washout of ADP. *b*, Dose response for ADP in Int_{0Mg}. Data from seven patches are rundown-compensated and normalized as in Figure 3. Data points are mean ± SEM. The *line* represents a fit to the data according to $I/I_{max} = -f/((K_d/[ADP]) + 1) + 1$. K_d = 0.40 mM; f = 0.22.

outward currents. Addition of ADP in the continued presence of ATP caused an increase in the current (9/11 patches). The effect of 2 mM ATP with and without 10 μM ADP is shown in Figure 5. Note that the traces in this figure are not corrected for rundown and are labeled in chronological order, so that if rundown influenced the apparent effect of ADP, the effect was actually larger than it appears to be in the figure. The concentration at which the ADP effect was the largest was generally 10–50 μM (in one patch, this optimal concentration was ~500 μM), within the range of physiological concentrations of free cytosolic ADP (Veech et al., 1979; Meyer et al., 1985).

Inhibitory effect of ADP at millimolar concentrations

Although physiological concentrations of ADP were effective in antagonizing the inhibitory effect of ATP, this effect of ADP was reduced further, by raising ADP concentration to the millimolar range. This biphasic dose response suggests that, in addition to antagonizing the inhibitory effect of ATP, ADP exerts other effects on IRK3(HIT). Indeed, IRK3(HIT) current was inhibited by ADP alone, with an apparent K_d of 0.40 mM. The mean fraction of current inhibitable by ADP was 0.22 (Fig. 6). Thus, in addition to the site(s) for the antagonistic effects of ATP and ADP at physiological concentrations, this channel appears to contain another site with lower affinity for ADP.

MgATP can stimulate IRK3(HIT)

In addition to the inhibitory effect of ATP, observed either in the presence or the absence of Mg^{2+}, stimulatory effects of MgATP were evident in some of the experiments. Figure 7 shows the effect of ATP in 100 μM free Mg^{2+}. Switching from 0 to 100 μM Mg^{2+} decreased the outward current with little

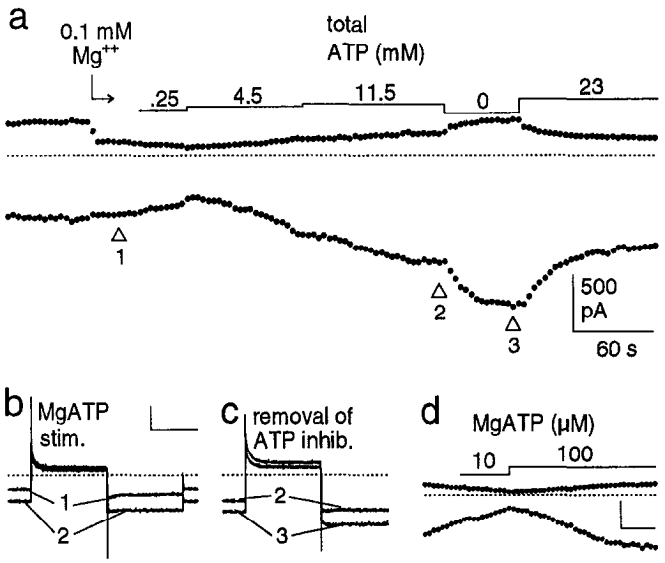

Figure 7. Stimulation of IRK3(HIT) current by MgATP. This stimulatory effect lasts for at least 1 min after ATP is removed and does not preclude the inhibitory effect of ATP. Membrane potential was held at −60 mV and stepped every 4 sec to +60 mV for 8 msec, then to −80 mV for 8 msec. *a*, The *arrow* at the beginning of the trace indicates the switch from Int_{0Mg} to $Int_{0.1Mg}$. *Filled circles* represent current amplitude at the end of the pulses to +60 and −80 mV. *Bars* above the record indicate perfusion with 0.25, 0.45, 11.5, and 23 mM total ATP (0.1, 2, 5, and 10 mM MgATP, respectively). *Triangles* labeled 1–3 indicate times corresponding to current traces shown in *b* and *c*. *b*, Individual traces recorded (*1*) before and (*2*) after stimulation by MgATP, as indicated by the *open triangles* in *a*. Calibration bars: 500 pA, 5 msec. *c*, Individual traces recorded after stimulation by MgATP and (*2*) before and (*3*) after ATP removal. Calibration as in *b*. *d*, MgATP (10 and 100 μM) was applied to an inside-out patch (40 and 130 μM total ATP, respectively) as indicated above the current record. Current stimulation was evident in the presence of 100 μM MgATP. The perfusion solution contained 30 μM free Mg^{2+} and (in mM): 140 K^+, 140 (2-[*N*-morpholino]ethanesulfonic acid), 20 Cl^-, 1 EGTA, and 20 HEPES, pH 7.2. Calibration bars: 200 pA, 30 sec.

effect on the inward current, as would be expected of the inward rectification attributable to Mg^{2+} block of the channel pore (Vandenberg, 1987; Matsuda et al., 1987). Application of MgATP resulted in a marked increase of the inward current (Fig. 7*b*,*d*). As shown in Figure 7, *a* and *c*, after removal of ATP (total concentration 11.5 mM; calculated [MgATP] = 5 mM), the current was increased further because of the removal of the inhibitory effect of ATP; addition of 23 mM total ATP (10 mM MgATP) restored inhibition. The stimulatory effect of MgATP lasted for at least one min while ATP was withdrawn, so that the same current level was obtained after reintroduction of ATP. This stimulatory effect of ATP in the presence of Mg^{2+} was variable in excised patches, being seen in 19/34 cases where MgATP was applied. Because the stimulatory effect of ATP was induced by MgATP but not by ATP in the absence of Mg^{2+}, it was probably caused by kinases and/or other enzymes that require ATP hydrolysis, analogous to the stimulation by ATP of other inwardly rectifying K^+ channels (Takano et al., 1990; Fakler et al., 1994). The stimulatory effect of MgATP did not preclude the inhibitory effect of ATP, similar to what has been shown for Type 1 ATP-sensitive K^+ channels (Ashcroft and Ashcroft, 1990). Thus, these channels may respond both to modulatory processes that require ATP hydrolysis and to more direct actions of ATP without its hydrolysis.

DISCUSSION

The main finding of this study is that a strongly rectifying inward rectifier potassium channel, IRK3(HIT), is inhibited reversibly by physiological concentrations of ATP via a mechanism that does not appear to involve ATP hydrolysis. This effect is antagonized by ADP, again in the physiological range. Here we discuss mechanistic aspects of IRK3(HIT) regulation and its possible physiological role in terms of tissue distribution and responses to changes in cellular metabolism.

IRK3(HIT) is regulated by concentrations of ATP and ADP in the physiological range

Estimates of cytoplasmic ATP concentration range from 2 to 8 mM (Ashcroft et al., 1973; Veech et al., 1979; Kakei et al., 1986). During periods of metabolic stress, such as ischemia or anoxia, the ATP concentration is reduced only mildly when cellular function or viability becomes seriously compromised (Gudbjarnason et al., 1970; Dhalla et al., 1972; Neeley et al., 1973). Therefore, for an ATP-sensitive channel to provide protection from damage caused by metabolic stress, its affinity for ATP would have to be in the millimolar range. Because the apparent K_d of IRK3(HIT) for ATP inhibition is on the order of 1.5 mM (Fig. 3), the channel activity is likely to increase during early stages of metabolic stress, thereby reducing membrane depolarization and toxicity caused by overexcitation.

Free cytosolic [ADP] in brain and muscle is estimated to be ~30 μM based on substrate levels of cytosolic kinases at equilibrium (Veech et al., 1979), or up to 14 μM based on studies combining nuclear magnetic resonance and biochemical data (Meyer et al., 1985). We have found that ADP antagonizes the inhibitory effect of ATP on IRK3(HIT) current at these physiological concentrations (Fig. 5). The antagonizing effect increases with ADP concentration until a low affinity site for ADP (K_d = 0.4 mM) is activated to cause moderate inhibition of the channel (Fig. 6). A rise in tissue ADP levels in the early stages of ischemia (Neeley et al., 1973), therefore, may increase the antagonizing effect of ATP inhibition. Thus, an increase of ADP concentration and a decrease of ATP concentration could act in concert to increase IRK3(HIT) channel activity during metabolic stress.

Inwardly rectifying potassium channels control the resting potential of central neurons, e.g., nucleus accumbens neurons (Uchimura et al., 1989) and neostriatal spiny projection neurons (Nisenbaum and Wilson, 1995). If IRK3(HIT) channels constitute a significant fraction of the inward rectifiers of some central neurons, the expected increase in IRK3(HIT) channel activity caused by a decrease in the cytosolic [ATP]/[ADP] ratio during ischemia/hypoxia would cause significant hyperpolarization of the plasma membrane, analogous to the reported involvement of ATP-sensitive K^+ channels in anoxic responses (Grigg and Anderson, 1989; Schaeffer and Lazdunski, 1991; Luhmann and Heinemann, 1992; Godfraind and Krnjević, 1993; Zini et al., 1993). Interestingly, neuronal hyperpolarization shortly after the onset of cerebral anoxia has been demonstrated in the hippocampus (Hansen et al., 1982; Fujiwara et al., 1987; Ben-Ari, 1989) and the neocortex (Luhmann and Heinemann, 1992), brain regions in which *in situ* hybridization studies indicate expression of the rat IRK3 homolog (Bredt et al., 1995).

Inhibition of IRK3(HIT) by ATP does not require hydrolysis

Inhibition of IRK3(HIT) currents by ATP does not require Mg^{2+} and is mimicked by nonhydrolyzable ATP analogs, as is

the case for other ATP-sensitive K^+ channels (Spruce et al., 1987; Ashcroft and Kakei, 1989; Lederer and Nichols, 1989), indicating that hydrolysis of ATP is not a requisite step. The mechanism of ATP action, therefore, is more likely to involve direct binding of ATP to the channel. So far, we have not been able to identify potential structural elements involved in ATP/ADP sensitivity by examining the amino acid sequence of IRK3(HIT) (Saraste et al., 1990). It is possible that such structures can be revealed by examining chimeras of IRK1 and IRK3(HIT); although IRK3(HIT) shares 60% identity with IRK1 in amino acid sequence, IRK1 is not sensitive to ATP inhibition. Alternatively, the ATP-binding site may not reside in IRK3(HIT) but, rather, in other proteins associated with the channel. Possible candidates for such proteins include members of the ATP-binding cassette family of proteins, because they have been suspected recently of interacting with inwardly rectifying K^+ channels (Fakler et al., 1994; Aguilar-Bryan et al., 1995).

ATP inhibition of IRK3(HIT) is antagonized by ADP, similar to what has been reported previously for ATP-sensitive K^+ channels (Dunne and Petersen, 1986; Kakei et al., 1986; Misler et al., 1986; Findlay, 1988). This could arise from competition of ATP and ADP for a common nucleotide-binding site, a mechanism that has been suggested for an ATP-sensitive K^+ channel in the rat renal cortical collecting duct (Wang and Giebisch, 1991). Alternatively, ADP could bind to a site distinct from the inhibitory ATP-binding site and stimulate channel activity. This latter possibility is analogous to the model proposed for the cardiac Type 1 ATP-sensitive K^+ channel (Tung and Kurachi, 1991; Hopkins et al., 1992; Terzic et al., 1994), although Mg^{2+} appears to be necessary for the ADP stimulation of the cardiac channel. In the case of IRK3(HIT), Mg^{2+} is not necessary either for the high affinity site for ADP to antagonize ATP inhibition or for the low affinity site for ADP to inhibit the channel.

IRK3(HIT) is different from known ATP-sensitive channels

Evidently, IRK3(HIT) expressed heterologously in *Xenopus* oocytes is not equivalent to any known native ATP-sensitive channel. The IRK3(HIT) currents are not blocked by sulfonylureas. The apparent ATP affinity of IRK3(HIT) is similar to that of Types 2 and 3, but these channels do not inwardly rectify (Ashford et al., 1988, 1990). The rectification of Type 1 channels is weaker than that of IRK3(HIT), and their sensitivity to ATP is much higher than that of IRK3(HIT) (Cook and Hales, 1984; Trube and Heschler, 1984; Findlay et al., 1985; Kakei et al., 1985; Misler et al., 1986; Sturgess et al., 1986; Ribalet and Ciani, 1987; Spruce et al., 1987; Ashcroft and Kakei, 1989; Ashcroft et al., 1989; Lederer and Nichols, 1989; Niki et al., 1989). This difference in ATP sensitivity appears to correlate with the different physiological requirements for ATP-sensitive K^+ channels in pancreatic β cells and central neurons. The high ATP sensitivity of Type 1 ATP-sensitive K^+ channels in β cells may allow a substantial decrease in channel activities to be induced by elevating sugar level, leading to sufficient depolarization and triggering insulin release. By contrast, the IRK3(HIT) channel activities may increase by up to twofold after relief of ATP inhibition. This extent of change of inward rectifier activities has been shown to cause significant changes in membrane excitability (Nisenbaum and Wilson, 1995) and could afford protection during metabolic stress.

IRK3 is expressed primarily in the brain, especially in the neocortex and hippocampus; IRK3 expression in the heart and skeletal muscle was found in the human but not in rodents (Morishige et al., 1994; Périer et al., 1994; Tang and Yang, 1994; Bredt et al., 1995). Because most work on native ATP-sensitive K^+ channels has been performed in rodent muscle, heart, and pancreatic cells, perhaps it is not surprising that there have been no previous reports of ATP-sensitive K^+ channels similar to IRK3(HIT). Moreover, because central neurons usually contain multiple channel types, the small conductance of IRK3 may be obscured by subconductance states of other channels (Penington et al., 1993), as has been pointed out recently by Périer et al. (1994). The susceptibility of this channel to rundown in excised patches also may have rendered it difficult to demonstrate the ATP sensitivity. Thus, characterization of the functional properties and expression patterns of IRK3 may facilitate studies of such channels *in vivo*. It is also possible that IRK3 coassembles with other channel subunits in central neurons. Heteromeric channel formation has been reported for other members of the inwardly rectifying K^+ channel family, and it has been suggested that a recently cloned ATP-sensitive potassium channel (Ashford et al., 1994) and its homologs represent native subunits of G-protein-gated heteromeric channels (Duprat et al., 1995; Kofuji et al., 1995; Krapivinsky et al., 1995). Coexpression of IRK3(HIT) with other cloned channel subunits may reveal whether they form channels closely resembling those known to exist in central neurons.

Stimulation of IRK3(HIT) by MgATP

The stimulatory effect of MgATP on IRK3(HIT) (Fig. 7) may be analogous to that reported for native inward rectifiers (Takano et al., 1990) and/or IRK1 (Fakler et al., 1994). In the case of IRK1, MgATP has a dual action, involving phosphorylation by protein kinase A and a separate requirement for ATP hydrolysis. Examination of the amino acid sequence of IRK3(HIT) reveals the presence of multiple putative sites for protein kinases. It remains to be determined whether this channel could be regulated by signaling processes that alter the phosphorylation state of the channel and whether the activities of this channel in central neurons represent an integration of such signaling events and regulation by the internal metabolic state of the neuron.

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