Modulation of inwardly rectifying potassium channels in cultured bovine pulmonary artery endothelial cells

M. Kamouchi, K. Van Den Bremt, J. Eggermont, G. Droogmans and B. Nilius*

Laboratorium voor Fysiologie, Campus Gasthuisberg, KU Leuven, B-3000 Leuven, Belgium

1. We have used the patch-clamp technique to study modulation of the inwardly rectifying K^+ current ($I_{K(IR)}$) in cultured bovine pulmonary artery endothelial cells (CPAE cells). In whole-cell mode, $I_{K(IR)}$ was defined as the Ba^{2+}-sensitive current. In single channel recordings, we observed a strongly inwardly rectifying and K^+-selective channel with a conductance of 31 ± 3 pS.

2. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis and functional data suggest that the endothelial IRK is most probably Kir2.1.

3. Intracellular ATP is required to prevent run-down of IRK in whole-cell mode. Single channel activity disappeared in inside-out patches exposed to ATP-free solution and in cell-attached patches on cells exposed to metabolic inhibition (KCN, 2-deoxyglucose).

4. The non-hydrolysable ATP analogues, ATPγS and adenylyl imidodiphosphate (AMP-PNP), did not prevent run-down. Run-down did not occur in the presence of okadaic acid, a phosphatase inhibitor, but was enhanced in the presence of protamine, an activator of phosphatase 2A (PP2A).

5. GTPγS and AlF_4^- inhibited IRK, also in the presence of ATP. GTPβS antagonized the GTPγS effect. Pretreatment of the cells with PTX did not affect the GTPγS-induced inhibition. Okadaic acid, however, slowed this inhibition.

6. Neither activation of protein kinase A (PKA) nor activation of protein kinase C (PKC) affected IRK. Additionally, neither cytochalasin B nor a high concentration of intracellular Ca^{2+} affected the time course of IRK run-down.

7. We conclude that run-down of IRK is probably due to dephosphorylation by PP2A. Activation of a PTX-insensitive G protein inhibits this current by a mechanism that is neither mediated via the PKA and PKC pathways nor by intracellular Ca^{2+}, but supposedly by a G protein-dependent activation of a phosphatase.

Inwardly rectifying K^+ channels play an important role in the electrogenesis of the resting potential in both excitable and non-excitable cells. Two types of inwardly rectifying K^+ channels were initially cloned from a rat kidney and mouse macrophage cell line and were termed ROMK1 and IRK1, respectively (Ho *et al.* 1993; Kubo, Baldwin, Jan & Jan, 1993*a*). Screening of homologous sequences with these channels led to isolation of a G protein-coupled inwardly rectifying K^+ channel in rat heart (GIRK1; Kubo, Reuveny, Slesinger, Jan & Jan, 1993*b*). The pharmacological fingerprint of IRK1 channels is a high affinity inhibition by external Cs^+ and Ba^{2+}. The subfamily Kir2 of IRKs has three members, referred to as Kir2.1, Kir2.2 and Kir2.3, with single channel conductances of 23, 34 and 10 pS, respectively (Kubo *et al.* 1993*a*; Makhina, Kelly, Lopatin, Mercer & Nichols, 1994; Takahashi *et al.* 1994). Among these members, Kir2.1 is insensitive to changes in the pH, whereas Kir2.3 is inhibited at acidic extracellular pH (Coulter, Perier, Radeke & Vandenberg, 1995; Fakler & Ruppersberg, 1996).

The modulation of these channels under normal and pathophysiological conditions is of special interest, but so far little is known about the modulation of each of these cloned Kir2 channels. In addition, evidence for the involvement of protein kinases is contradictory, since experimental data in favour of as well as against a role of protein kinase A (PKA) and/or protein kinase C (PKC) in the regulation of Kir2.1 and/or Kir2.3 have been reported (Fakler, Brandle, Glowatzki, Zenner & Ruppersberg, 1994; Henry, Pearson & Nichols, 1996; Wischmeyer & Karschin, 1996).

Inwardly rectifying K^+ channels also contribute to the resting potential of vascular endothelial cells and therefore to the driving force for Ca^{2+} ions (Voets, Droogmans & Nilius, 1996). Production and release of vasoactive agents are mainly initiated by Ca^{2+}-dependent mechanisms, and

* To whom correspondence should be addressed.

the inwardly rectifying K^+ channel may therefore be an important regulator of signal transduction in various vascular functions (Nilius, Viana & Droogmans, 1997). It has been reported that G proteins might modulate these endothelial inwardly rectifying K^+ channels (Nilius, Schwarz & Droogmans, 1993; Pasyk, Cipris & Daniel, 1996), but a detailed analysis of this channel modulation is lacking. In addition, the molecular identity of the endothelial IRK has not yet been elucidated.

We show here that the inwardly rectifying K^+ channel in macrovascular endothelial cells is probably Kir2.1, and these channels are modulated by endogenous G protein-linked phosphatases.

METHODS

Cells

We have used cells from a cultured bovine pulmonary artery endothelial cell line (CPAE, ATCC CCL 209) grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% (v/v) human serum, 2 mmol l^{-1} L-glutamine, 2 units ml^{-1} penicillin and 2 mg ml^{-1} streptomycin. Cultures were maintained at 37 °C in a fully humidified atmosphere of 10% CO_2 in air.

Electrophysiology

The conventional patch-clamp technique in the whole-cell, cell-attached and inside-out configuration was used. In whole-cell mode, we applied a ramp protocol every 5 s consisting of a voltage step to −80 mV for 300 ms, followed by a step to −150 mV for 100 ms and a 1300 ms linear ramp to +100 mV. The amplitude of the membrane current at −80 or −150 mV was measured at the end of the corresponding voltage step. Currents were monitored with a EPC-9 (sampling rate 1 ms, 8-Pole Bessel filter 3 kHz).

For single channels measurements, 2 s sweeps were recorded with a sampling interval of 1 ms, 2048 points per sweep, and 5 s interval between the sweeps (filter settings 500 Hz).

Solutions

The standard extracellular solution was a Krebs solution containing (mM): 150 NaCl, 6 KCl, 1·5 $CaCl_2$, 1 $MgCl_2$, 10 Hepes and 10 glucose. The osmolality of this solution was 320 ± 5 mosmol kg^{-1} as measured with an osmometer (Wescor 5500 osmometer, Schlag Instruments, Gladbach, Germany). The standard internal pipette solution contained (mM): 140 KCl, 2 $MgCl_2$, 0·1 EGTA, 10 Hepes and 4 mM Na_2ATP. In the ATP-free pipette solution that was used to induce run-down of the inwardly rectifying K^+ channels Na_2ATP was replaced by 8 mM NaCl. The pH of the extracellular solutions was adjusted to 7·4 with NaOH, and that of the internal solutions to 7·2 with KOH.

Cells were depleted of ATP by applying 2 mM KCN and 5 mM 2-deoxyglucose (2-DG, Sigma) to the bath solution. For some experiments cells were pretreated with 500 ng ml^{-1} pertussis toxin (PTX, Sigma) for 4–12 h at 37 °C (Braun, Fedida & Giles, 1992; Inoue & Imanaga, 1993) to test for a possible involvement of PTX-sensitive G proteins. PKA phosphorylation was traced by application of 2 or 10 μM forskolin and 20 or 40 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma). For PKC modulation, 100 nM or 1 μM phorbol 12-myristate 14-acetate (PMA, Sigma) was used. Forskolin, IBMX and PMA were dissolved in dimethyl sulphoxide (DMSO) as stock solutions.

ATPγS (lithium salt, Sigma), AMP-PNP (lithium salt, Sigma), okadaic acid (Calbiochem), protamine (Sigma), GTPγS (lithium salt, Sigma), GDPβS (lithium salt, Boehringer), KF, $AlCl_3$ and cytochalasin B (Sigma) were added to the internal solution. When KF was added to the internal solution, KCl was reduced by an equimolar amount. Cytochalasin B and okadaic acid were dissolved in DMSO. The final concentration of DMSO was less than 0·2% (v/v).

For single channel recordings in cell-attached mode, we used a high K^+ solution for both the extracellular and pipette solution. This solution contained (mM): 150 KCl, 10 Hepes, 1 $MgCl_2$, 1·5 $CaCl_2$ and 10 glucose. Inside-out excised patches were exposed to a bathing solution that contained (mM): 150 KCl, 10 Hepes, 2 $MgCl_2$ and 0·1 EGTA. The solutions were buffered at pH 7·40 with KOH.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Reverse transcriptase reactions were carried out on total RNA from CPAE cells with You-Prime First-Strand Beads (Ready-To-Go, Pharmacia Biotech) using a random primer. RT reactions were quality controlled with a PCR for actine, which yielded the expected 144 nucleotide fragment. PCR reactions for Kir channels were carried out with Goldstar DNA polymerase (Eurogentec, Seraing, Belgium) in the supplied reaction buffer with the addition of 1·5 mM $MgCl_2$. After 2 min at 94 °C, Goldstar was added to the PCR reaction mixture, performing a hot start, followed by 40 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The following primers were used for PCR:

forward IRKF01 primer:

5′TCAGGATCC I AC I ATHGGNTAYGG3′

(with H = A + T + C, N = A + G + T + C and Y = C + T)

and reverse IRKR01 primer:

5′ACTAAGCTTCRTANGTYTTRTG RAA3′

(with R = A + G, N = A + G + T + C and Y = C + T).

IRKF01 contained a *Bam*HI restriction site, and IRKR01 a *Hind*III restriction site for cloning purposes. PCR products were separated on a 1% (w/v) agarose gel. The amplified PCR fragment (621 nucleotides) was recovered from the agarose gel with a QIAEX kit (QIAGEN, Hilden, Germany). This fragment was digested with *Bam*HI and *Hind*III and subcloned into a pBluescript vector. Subcloned fragments were sequenced using an ALF sequencer (Pharmacia). Sequences were analysed with the Genetics Computer Group Wisconsin Package (version 8).

All experiments were performed at room temperature (20–22 °C). Significance was tested at the $P = 0.05$ level using Student's t test. Pooled data are given as the mean ± S.E.M.

RESULTS

Functional characterization of the inwardly rectifying K^+ current

We have recently shown that basically three conductances determine the resting potential of non-stimulated CPAE cells (Voets *et al.* 1996). The inwardly rectifying K^+ current component ($I_{K(IR)}$) can be blocked by extracellular Ba^{2+}, which does not significantly affect the other current components. This could be inferred from the pronounced inhibition by 1 mM Ba^{2+} of the current at −150 mV, an effect that did not occur at a potential of −80 mV, close to the K^+ equilibrium potential (Fig. 1*A*). We therefore define

$I_{K(IR)}$ functionally as the Ba^{2+}-sensitive whole-cell membrane current. This is illustrated in Fig. 1*B* for a voltage ramp protocol, which was used to measure the instantaneous current–voltage relationship. It shows current traces in the absence (*a*) and presence (*b*) of 1 mM Ba^{2+} as well as the difference current (*a*–*b*). The difference current shows strong inward rectification and reverses at −77 mV, close to the K^+ equilibrium potential of −80 mV. It also shows that Ba^{2+} does not significantly affect the outward current component, which mainly occurs through Cl^- and non-selective cation channels (Voets *et al.* 1996). The inwardly rectifying component is strongly potentiated if the extracellular K^+ concentration is increased from 6 to 140 mM (Fig. 1*C*). The Ba^{2+}-sensitive component is, under these conditions, also inwardly rectifying and reverses at the equilibrium potential for K^+ (Fig. 1*D*).

Voltage steps to various potentials evoked large inward but only small outward currents. However, the currents at potentials more negative than −100 mV inactivate rapidly (Fig. 2*A*). Again, 1 mM Ba^{2+} blocked the inward currents efficiently without affecting outward currents (Fig. 2*B*). Figure 2*C* illustrates the steady-state *I–V* curves of the currents in the absence and in the presence of Ba^{2+}, and that of the difference current. These results show that the Ba^{2+}-sensitive current in CPAE cells has the characteristics of inwardly rectifying K^+ currents. The current density of the Ba^{2+}-sensitive component at −150 mV was 15·5 ± 1·6 pA pF^{-1} in the standard internal solution ($n = 58$ cells).

We have also recorded unitary currents in symmetrical high K^+ solutions in the cell-attached configuration (Fig. 2*D*). Single channel activity was observed at negative potentials, but never occurred at positive potentials. The current amplitude at each potential was determined from amplitude histograms and the pooled data from ten cells were plotted as a function of voltage (Fig. 2*E*). From the linear regression through the experimental data, we calculated a single channel slope conductance of 31·0 ± 3·1 pS.

It has been reported that HIR, a member of the Kir2 family (Kir2.3), is extremely sensitive to extracellular pH in the physiological range. On the other hand, IRK1 is insensitive to intra- and extracellular pH in that range (Coulter *et al.* 1995; Fakler & Ruppersberg, 1996). We therefore checked the sensitivity of IRK in CPAE cells to intra- and extracellular pH. Neither changes in intracellular pH in the range 5·4–8·4 ($n = 4$–7, data not shown), nor changes in

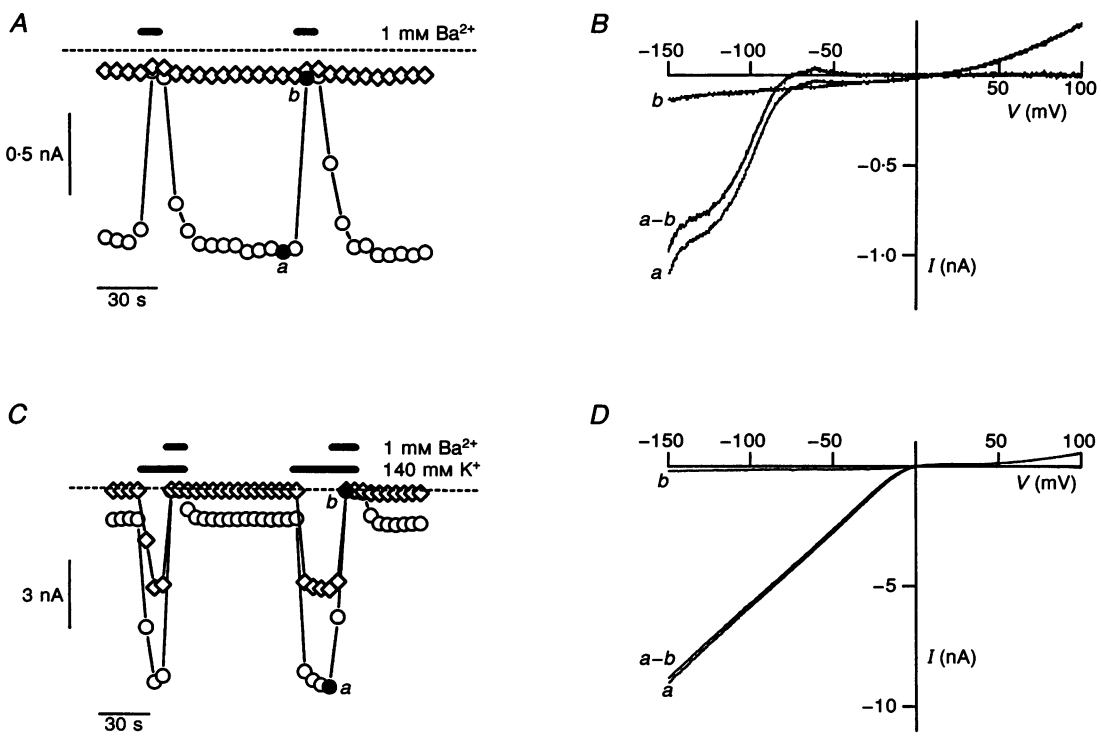

Figure 1. Dissection of $I_{K(IR)}$ from whole-cell currents in cultured bovine pulmonary artery endothelial cells (CPAE cells)

A and *C*, the amplitude of the membrane currents at −80 mV (◇) and −150 mV (○) was measured every 5 s and plotted against time. In Fig. 1*A*, the external solution contained 6 mM K^+, whereas in Fig. 1*C* the extracellular K^+ concentration was increased from 6 to 140 mM (indicated by bars). Ba^{2+} (1 mM) was added to the external solution as indicated by bars. Dashed lines always mark the zero current. *B* and *D*, current–voltage relationships obtained from the ramp protocol in normal Krebs solution (*B*) or high K^+ solution (*D*). The difference current was obtained by the subtraction of the current in the presence of 1 mM Ba^{2+} (filled circles at point *b* in panel *A* and *C*) from controls (filled circles at point *a* in *A* and *C*).

extracellular pH in the range 6·4–8·4 ($n = 6$, data not shown) affected IRK in CPAE cells.

The endothelial IRK is a member of the Kir2 family

The strong inward rectification of IRK in CPAE cells suggests that it might belong to the Kir2.0 subfamily. In order to check the molecular identity of the CPAE K^+ channel, we performed a RT-PCR using degenerated primers that were designed against conserved regions in the Kir2.0 family. The forward primer IRKF01 corresponded to the conserved T I GYG sequence (position 142 to 146 of Kir2.1; AC P48049) in the H5 pore region of Kir channels. The reverse primer IRKR01 was designed against a conserved region of the Kir2.0 channels FHKTYE (position 344 to 349 of Kir2.1; AC P48049) (Doupnik, Davidson & Lester, 1995). PCR with this primer set is predicted to amplify a 621 nucleotide fragment. RT-PCR in CPAE RNA with these primers resulted in a single band of the expected length (Fig. 3). Seven clones were sequenced and were found to be 100% identical to each other.

Basic local alignment search tool (BLASTX) analysis (Altschul, Gish, Miller, Meyers & Lipman, 1990; Gish & States, 1993) of the CPAE sequence confirmed that it belonged to the Kir2.0 family. Pairwise alignment (BESTFIT, Genetic Computer Group, Wisconsin University) of the CPAE amino acid sequence with different mouse Kir2.0 channel proteins revealed a very high identity (95·2%) with Kir2.1(AC P35561). In contrast, the identity score at the amino acid level with mouse Kir2.2 (AC P52187) and Kir2.3 (AC P52189) dropped to 78·8 and 74·9%, respectively. Comparison of the CPAE sequence with Kir2.1 K^+ channels from different species (mouse: AC P35561; human: AC P48049; rabbit: AC P49656; rat: AC L48490;

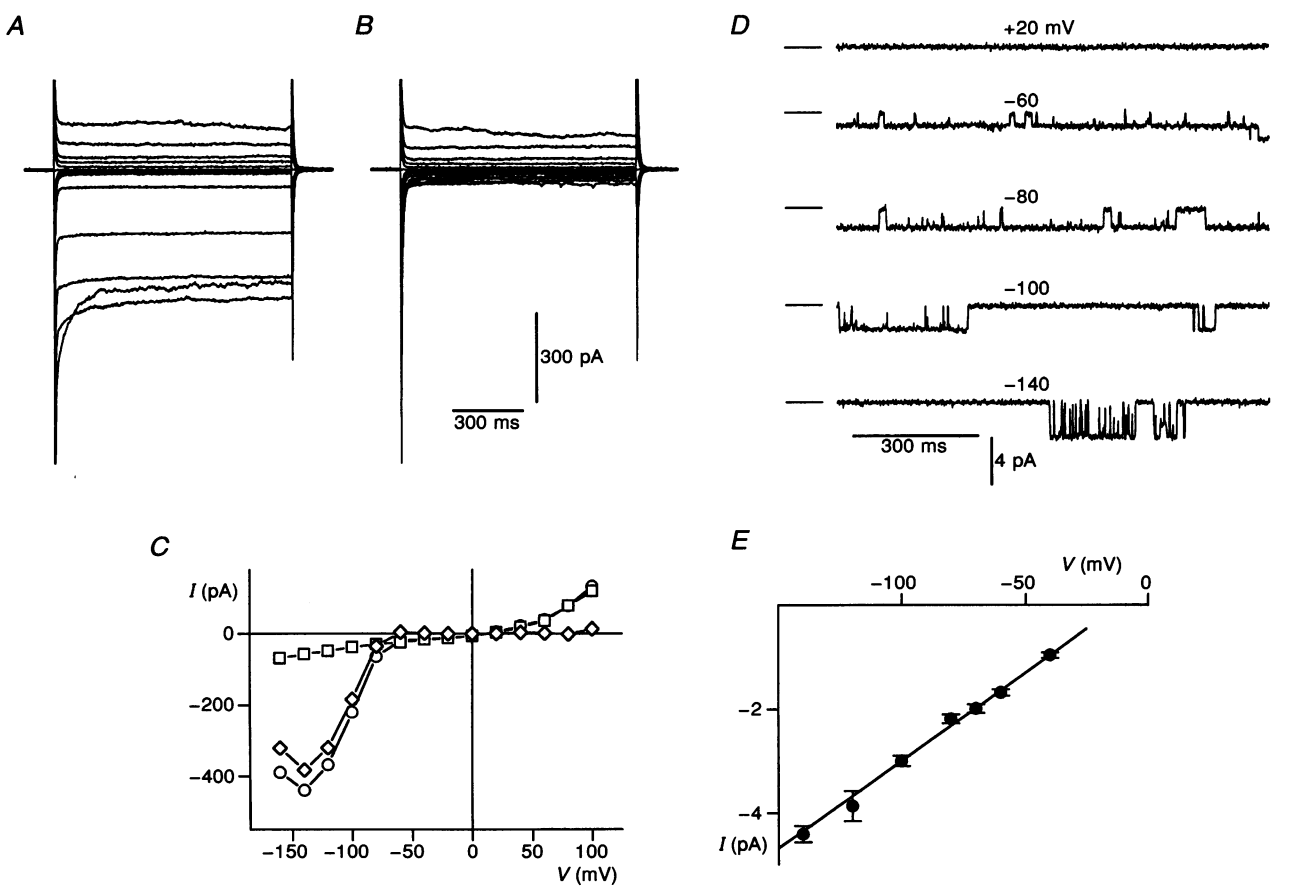

Figure 2. Features of the inwardly rectifying K^+ current, $I_{K(IR)}$, in CPAE cells

A and *B*, membrane currents evoked by the 1 s step voltage pulse in the absence (*A*) and presence (*B*) of 1 mM Ba^{2+}. Voltage steps were applied from −160 to +100 mV with increments of 20 mV. The external solution contained 6 mM K^+ (normal Krebs solution). *C*, current–voltage relationship obtained from the protocols shown in *A* and *B* at the end of each step. The current–voltage relationship for the difference current (◇) was obtained by subtraction of the current in the presence of 1 mM Ba^{2+} (□) from the controls (○). *D*, single channel currents at different membrane potentials recorded in the cell-attached configuration. Bath and pipette solutions contained 150 mM K^+. The currents were recorded at 0·5 ms sample intervals and filtered at 500 Hz. The closed level is indicated by the bars at the left. *E*, current–voltage relationship for the unitary currents in the cell-attached patches in symmetrical 150 mM K^+ solutions. The amplitude of the currents was measured from amplitude histograms. Data were obtained from 3 to 15 different patches and plotted as means ± S.E.M. against voltage. Single channel conductance (γ), 31 pS.

chicken: AC P52186) showed an identity between 95·2 and 93·2% at the amino acid level. These data clearly show that the endothelial inwardly rectifying K^+ channel is a member of the Kir2 family.

Run-down of IRK in ATP-depleted cells

The amplitude of IRK was remarkably constant after breaking the cell membrane and did not significantly run-down over time periods of 25 min or longer, provided that the pipette solution contained 4 mM ATP. This is illustrated in Fig. 4*A*, which shows *I–V* curves of the Ba^{2+}-sensitive current obtained from voltage ramp protocols at different times after breaking the patch membrane. In contrast, this current gradually declines with time if the cells are patched with an ATP-free pipette solution. To compare the rate and extent of run-down under various experimental conditions, we measured the amplitude of the Ba^{2+}-sensitive current at −150 mV at different times after breaking into a cell, and normalized these values to the current amplitude in the same cell 1 or 5 min after breaking in ($I_{-150,rel}$). The pooled data, as summarized in Fig. 4*C*, show that the current does not significantly decrease with time if the cells are dialysed with 4 mM ATP ($n = 7$), but slowly declines if ATP is omitted from the pipette solution ($n = 10$). This run-down is much faster if the ATP depletion is accelerated by applying 2 mM KCN, an inhibitor of oxidative phosphorylation (cytochrome oxidase), and 5 mM 2-deoxyglucose (2-DG), an inhibitor of glycolysis, to the external solution, as illustrated in Fig. 4*B* (typical *I–V* curves from 7 cells). If these metabolic inhibitors were applied separately, we could, however, not observe any significant change in the rate of run-down (1 mM KCN, $n = 5$ and 5 mM 2-DG, $n = 9$; Fig. 4*C*).

We have also investigated how intracellular ATP affects IRK at the single channel level. In the cell-attached configuration, the activity of IRK channels recorded at −80 mV did not change significantly with time. To quantify these data, we have calculated NP_o (where N is the number of channels in the patch and P_o is the probability of the channel being open), i.e. the average current divided by the single channel current amplitude, in successive current traces of 2048 data points sampled at 1 kHz. These values of NP_o are represented as a function of time for a cell-attached patch in Fig. 5*A* ($n = 15$ patches). If the membrane patch was excised and activity was monitored in the inside-out configuration in an ATP-free internal solution, channel activity disappeared rapidly ($n = 5/6$ patches; Fig. 5*B*). If 2 mM KCN and 5 mM 2-DG were added to the external solution, the channel activity also disappeared in the cell-attached configuration, but resumed after washing out the metabolic inhibitors ($n = 3/3$ patches; Fig. 5*C*).

These results, together with those of the whole-cell experiments, clearly demonstrate that intracellular ATP is necessary to maintain the activity of IRK in CPAE cells. It is not clear whether ATP is hydrolysed and whether phosphorylation of the channel is essential for its normal functioning, as has been reported for the cloned Kir2.1 and

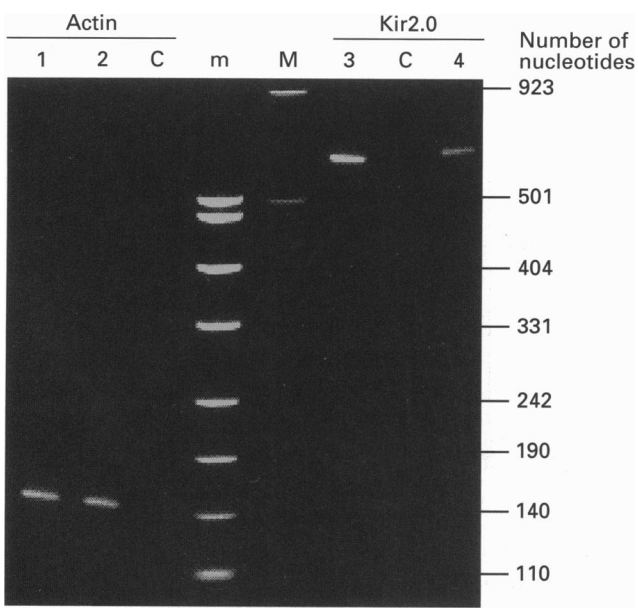

Figure 3. RT-PCR analysis of Kir2.0 expression in CPAE cells

Total RNA of CPAE cells (lanes 1 and 3) and polyA^+-enriched RNA of human brain (lanes 2 and 4) were reverse transcribed with random primers. PCR was performed with a primer set specific for actin (lanes 1 and 2) or for Kir2.0 K^+ channels (lanes 3 and 4). Lanes marked with C correspond to control PCR reactions in which the RT mix was replaced by H_2O. Lanes m and M contain marker fragments (corresponding lengths are indicated on the right). The ±600 nucleotide Kir2.0 fragment of CPAE cells (lane 3) was subcloned and sequenced (see text).

Kir2.3 channels (Fakler *et al.* 1994; Henry *et al.* 1996; Wischmeyer & Karschin, 1996). We have therefore tested whether channel activity is maintained if ATP is substituted with non-hydrolysable analogues. However, run-down was even faster if the pipette solution contained either AMP-PNP or ATPγS (Fig. 6*A*). In the presence of 4 mM ATP, 4 mM AMP-PNP and 4 mM ATPγS, $I_{-150,rel}$ at 25 min was 0·95 ± 0·11 ($n = 7$), 0·24 ± 0·10 ($n = 8$) and 0·06 ± 0·04 ($n = 12$), respectively (here and in the following results, the relative amplitude ($I_{-150,rel}$) at 25 min is mentioned and compared). These results suggest that hydrolysis of ATP rather than its mere binding is necessary to maintain channel activity. This finding is also consistent with the observation that the phosphatase inhibitor okadaic acid at a concentration of 1 μM prevents run-down in ATP-free solution (with and without okadaic acid: 1·02 ± 0·04, $n = 7$ and 0·57 ± 0·07, $n = 10$, respectively; Fig. 6*B*) and decelerates it in the presence of metabolic inhibitors (with and without okadaic acid: 0·45 ± 0·11, $n = 5$ and 0·08 ± 0·04, $n = 7$, respectively; Fig. 6*C*). It is therefore likely that run-down in ATP-free solution is due to channel dephosphorylation by a phosphatase. Okadaic acid interacts specifically with phosphatase 1 (PP1) and phosphatase 2A (PP2A). To differentiate between these phosphatases we have used protamine, a polycation that is known to activate PP2A (Cohen, 1989). If added at a concentration of

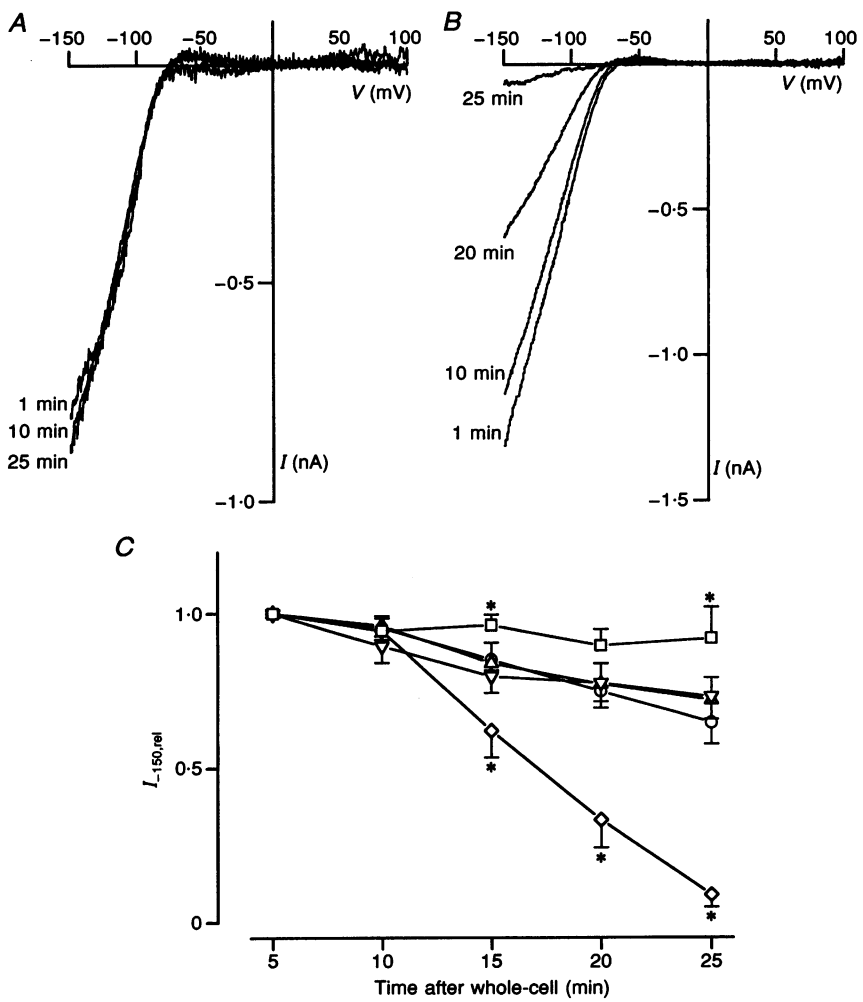

Figure 4. Changes in the amplitude of $I_{K(IR)}$ in the absence or presence of ATP

A and *B*, representative current–voltage relationships for $I_{K(IR)}$ measured from voltage ramps in the presence (*A*) or absence (*B*) of ATP. At the left-hand side, the times which elapsed from getting whole-cell access to the depicted *I–V* trace are indicated. In *B*, 2 mM KCN and 5 mM 2-DG were additionally applied to the external solution immediately after the establishment of whole-cell configuration. *C*, average time course of $I_{K(IR)}$ measured at −150 mV in the absence (○, $n = 10$) or presence (□, $n = 7$) of 4 mM ATP in the internal solution. 1 mM KCN (△, $n = 5$), 5 mM 2-DG (▽, $n = 9$) or 2 mM KCN plus 5 mM 2-DG (◇, $n = 7$) were additionally applied immediately after establishment of whole-cell configuration. Metabolic inhibitors were administered to external solution. No ATP was applied to the pipette solution. The time course of the amplitude of $I_{K(IR)}$ was expressed relative to that observed at 5 min after break-in (* $P < 0·05$, significant differences as compared with the current in the ATP-free pipette solution (○)).

10 μg ml^{-1} to the ATP-free pipette solution, it significantly accelerated the run-down of IRK (0·15 ± 0·10, $n = 7$; Fig. 6*B*). In contrast, it did not cause any run-down if the pipette solution contained 4 mM ATP (1·02 ± 0·02, $n = 5$, data not shown), suggesting that its effect is rather specific. These findings therefore indicate that dephosphorylation of the channel by PP2A is the most likely mechanism of run-down.

G protein-mediated modulation of IRK

It is well established that G proteins inhibit IRK in many cell types. In endothelial cells, it has been reported that histamine inhibits IRK, presumably by a G protein-dependent mechanism (Nilius *et al.* 1993). To investigate whether G proteins really affect the channel activity of IRK, we added 100 μM of the non-hydrolysable GTP analogue GTPγS to the internal pipette solution. Under these conditions the current amplitude gradually decreased even in the presence of 4 mM ATP (0·16 ± 0·08, $n = 11$; Fig. 7*A*). This effect of GTPγS could be partially reversed if 3 mM GDPβS, which binds to G proteins with a similar affinity as GTPγS but without stimulating them, was added to the pipette solution (0·55 ± 0·17, $n = 6$; Fig. 7*A*). These results suggest that activation of a G protein inhibits IRK at least partially.

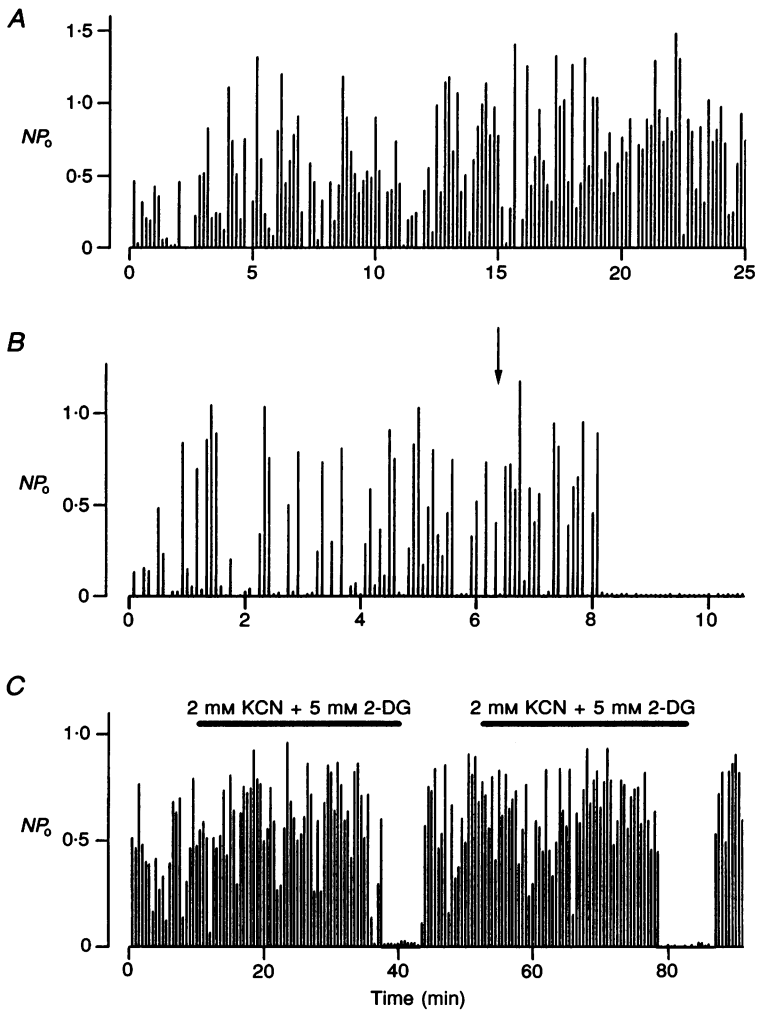

Figure 5. Changes in the open probability of single IRK channels in different patch configurations

A, time course of NP_o (N, number of channels in the patch; P_o, probability of the channel being open) of inwardly rectifying K^+ channels measured in the cell-attached configuration. NP_o was calculated from averaged current during each 2 s sweep divided by the single channel current and is plotted against the time after seal forming. *B*, the change in open probability of inwardly rectifying K^+ channels after patch excision at time zero. The inside-out patch was then exposed to an ATP-free solution at the point shown by the arrow. *C*, the change in open probability of inwardly rectifying K^+ channels evoked by addition of metabolic inhibitors. The current was recorded in cell-attached configuration. KCN (2 mM) and 2-DG (5 mM) were added to the external solution where indicated by bars. In all patches, the membrane was held at −80 mV. Open probability was calculated from each sweep (*B*), from two sweeps (*A*) or six sweeps (*C*) (sampling interval for all measurements 1 ms, 2048 points per sweep and 5 s intervals between the sweeps).

An alternative method to activate G proteins is by using fluoride. Figure 7*B* shows $I_{-150,rel}$ at 25 min in the presence of the agents related to G protein modulation. F^- (10 mM) added to the internal solution inhibited IRK, even in the presence of 4 mM ATP (0·30 ± 0·15, $n = 6$). If 100 μM Al^{3+} was applied together with 10 mM fluoride to the internal solution, run-down developed more rapidly (0·07 ± 0·06, $n = 6$). These results support the idea that inhibition of IRK is to some extent caused by activation of a G protein.

Activation of a pertussis toxin (PTX)-sensitive G protein is rather unlikely, because preincubation of CPAE cells with 500 ng ml^{-1} PTX for 4–12 h at 37 °C (Braun *et al.* 1992; Inoue & Imanaga, 1993) does not affect the current inhibition induced by GTPγS. As shown in Fig. 7*B*, there was no significant difference between untreated cells and cells treated with PTX (0·23 ± 0·08, $n = 12$). This implies that G proteins other than G_i or G_o may mediate the GTPγS-induced inhibition of IRK.

The next problem we addressed was the mechanism by which G proteins might inhibit IRK. We first investigated whether the effect of G proteins was mediated via PKA or PKC. PKA was activated by applying 2 or 10 μM forskolin,

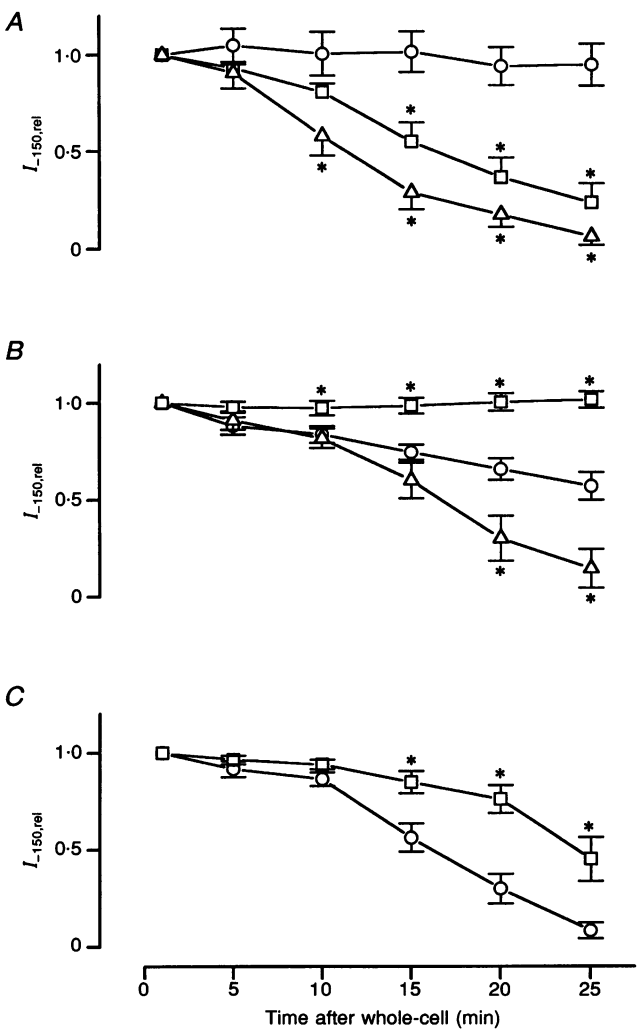

Figure 6. Effects of ATP analogues, the phosphatase inhibitor okadaic acid and the phosphatase activator protamine on $I_{K(IR)}$ inhibition

A, changes in the amplitude of $I_{K(IR)}$ at −150 mV in the presence of 4 mM ATP (○, $n = 7$), 4 mM AMP-PNP (□, $n = 8$) and 4 mM ATPγS (△, $n = 12$). In the presence of AMP-PNP or ATPγS no ATP was administered (*$P < 0·05$, significant differences as compared with the control currents in the presence of 4 mM ATP). *B*, time course of $I_{K(IR)}$ in the absence (○, $n = 10$) and presence of 1 μM okadaic acid (□, $n = 7$) and 10 μg ml^{-1} protamine (△, $n = 7$). Okadaic acid and protamine were included in the internal solution without ATP (*$P < 0·05$, significant differences as compared with currents measured in the absence of ATP). *C*, the effect of 1 μM okadaic acid on the time course of the inhibition of $I_{K(IR)}$ induced by 2 mM KCN and 5 mM 2-DG (○, $n = 7$). Okadaic acid was included in the internal solution (□, $n = 5$). KCN and 2-DG were applied immediately after break-in (*$P < 0·05$, significantly different as compared with the current in the presence of the metabolic inhibitors).

an activator of adenylyl cyclase, and 20 or 40 μM IBMX, a phosphodiesterase inhibitor, to the bath solution. This PKA-activating cocktail did not, however, induce any run-down (lower concentrations, $1{\cdot}00 \pm 0{\cdot}09$, $n = 4$, data not shown; and higher concentrations, $1{\cdot}02 \pm 0{\cdot}04$, $n = 4$; Fig. 7*B*). Also activation of PKC by 100 nM or 1 μM of the phorbol ester analogue PMA did not induce any run-down of IRK (100 nM, $1{\cdot}05 \pm 0{\cdot}09$, $n = 6$, data not shown and 1 μM, $0{\cdot}90 \pm 0{\cdot}11$, $n = 4$; Fig. 7*B*). We therefore conclude that PKA and PKC are not involved in the G protein-induced inhibition of IRK.

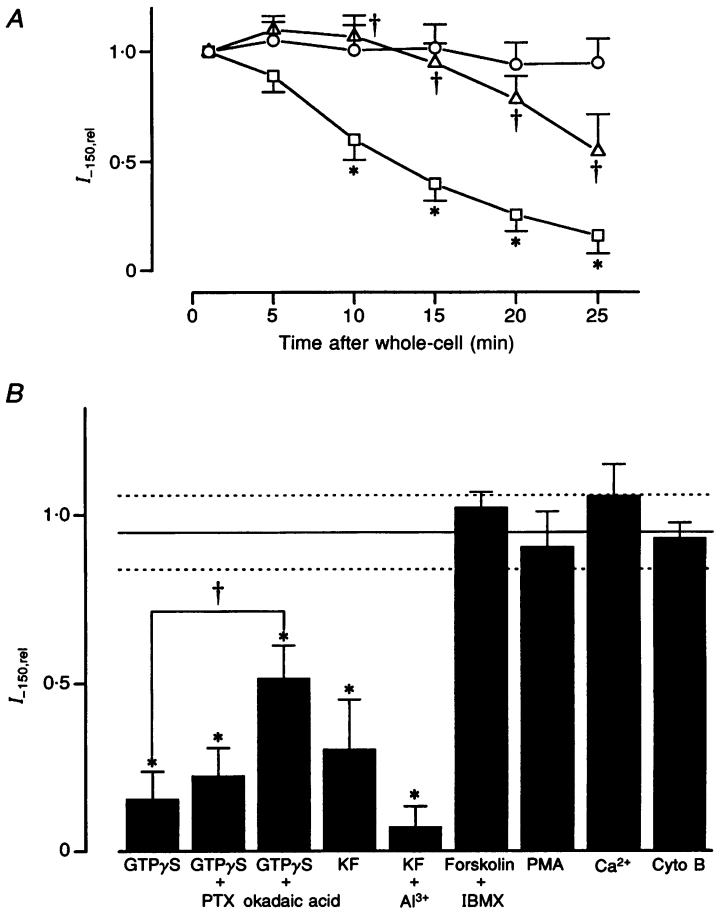

Figure 7. G protein-mediated modulation of $I_{K(IR)}$

A, the effect of GTPγS and GDPβS on the time course of the inhibition of $I_{K(IR)}$ is shown. The internal solution contained 100 μM GTPγS in the absence (□, $n = 11$) and the presence of 3 mM (△, $n = 6$) GDPβS. ATP (4 mM) was present in the internal solution (*$P < 0{\cdot}05$, significantly different compared with the control currents measured with standard internal solution (○); †$P < 0{\cdot}05$, significant differences compared with currents in the presence of 100 μM GTPγS (□)). *B*, comparison of $I_{-150,rel}$ at 25 min in the presence of the G protein activators: GTPγS (100 μM); GTPγS plus pertussis toxin (a G protein inhibitor; see Methods); GTPγS plus okadaic acid; the PKA activater forskolin plus a phosphodiesterase inhibitor, IBMX; the PKC activator PMA; as well as a high concentration of intracellular Ca^{2+} and application of cytochalasin B (Cyto B). The horizontal lines indicate the mean (continuous line) ± S.E.M. (dotted lines) of $I_{-150,rel}$ at 25 min in the standard internal solution ($0{\cdot}95 \pm 0{\cdot}11$, $n = 7$). KCl (10 mM) was replaced by equimolar KF in the internal solution without caring about the trace concentration of Al^{3+} ($n = 6$). $AlCl_3$ (100 μM) was added to the internal solution together with KF ($n = 8$). Okadaic acid (1 μM) was applied through the pipette ($n = 7$). An activator cocktail of adenylyl cyclase, forskolin (10 μM) and phosphodiesterase inhibitor IBMX (40 μM), and a PKC activator, phorbol ester analogue, PMA (1 μM) were added to the bath 5 min after breaking the cell membrane ($n = 4$ for both experiments). In these experiments, the amplitude of the current was normalized to that at 5 min, which was recorded just before application of the agents. Concentration of free Ca^{2+} was calculated in the presence of 4 mM ATP and adjusted to 10 μM ($n = 9$). Cytochalasin B (50 μM) was included in the internal solution and perfused into the cells via the patch pipette ($n = 6$, *$P < 0{\cdot}05$, significant differences as compared with the control current measured with standard internal solution; †$P < 0{\cdot}05$, significantly different compared with the current in the presence of 100 μM GTPγS).

It has been reported recently that the GTPγS-induced inhibition of Ca^{2+}-activated K^+ channels and IRK can be prevented by phosphatase inhibitors, implying that the G protein-induced inhibition is mediated by activation of a phosphatase (Bielefeldt & Jackson, 1994; Inoue & Imanaga, 1995). Also in CPAE cells, 1 μM okadaic acid caused a small but significant reduction of the GTPγS-induced inhibition of IRK (0·52 ± 0·10, $n = 7$; Fig. 7*B*). This indicates that the G protein-mediated inhibition in these cells is also at least partially mediated by the activation of a phosphatase.

To investigate whether G protein-mediated inhibition is caused by the elevation of intracellular Ca^{2+}, the effect of a high concentration of intracellular Ca^{2+} was tested. When 10 μM of intracellular free Ca^{2+} was included in the internal solution, the amplitude of IRK did not change (1·06 ± 0·09, $n = 9$; Fig. 7*B*). This result indicates that intracellular Ca^{2+} did not affect the activity of IRK.

Effect of cytochalasin B

It has been shown that polymerization of actin might be essential for protection of the availability of inwardly rectifying K^+ channels (Mazzanti, Assandri, Ferroni & DiFrancesco, 1996). Since ATP hydrolysis occurs during actin assembly (Stossel, 1993), it may be possible that run-down in ATP-free solution is due to depolymerization of actin. However, the actin depolymerizing agent cytochalasin B (50 μM) did not induce run-down in the presence of internal ATP (0·93 ± 0·05, $n = 6$; Fig. 7*B*). Also it did not accelerate the run-down observed in ATP-free internal medium (5 μM, 0·71 ± 0·10, $n = 6$ and 50 μM cytochalasin B, 0·68 ± 0·13, $n = 6$, data not shown). It is therefore unlikely that actin depolymerization is responsible for run-down.

DISCUSSION

Modulation of inwardly rectifying K^+ channels is an important mechanism because of the role of these ion channels in the control of the resting potential that regulates various cell functions (for a review see Nilius *et al.* 1997). We describe here the observation that endothelial IRK can be modulated by two mechanisms, the one depending on channel phosphorylation, the other via a G protein-mediated mechanism, probably by activation of a phosphatase.

We have previously demonstrated the existence of an IRK channel in CPAE cells which could be dissected from other current components based on its rapid and complete inhibition by Ba^{2+}. The present experiments confirm these findings, while our RT-PCR data strongly indicate that these channels belong to the Kir2 family. The weak sensitivity of the current for changes in intra- and extracellular pH makes it unlikely that these channels belong to the Kir2.3 subfamily (Coulter *et al.* 1995; Fakler & Ruppersberg, 1996) but rather that they belong to the Kir2.1 subfamily.

Our data from whole-cell and single channel experiments clearly show that the activity of IRK depends not only on the presence of ATP but also on its hydrolysis. Dialysis of the cell with an ATP-free pipette solution or application of metabolic inhibitors that rapidly deplete intracellular ATP (Kuhne, Besselmann, Noll, Muhs, Watanabe & Piper, 1993) induces a gradual decrease of the current amplitude. Channel activity also disappeared after patch excision and exposure to ATP-free solution. Similar observations have also been reported for other tissues (Mccloskey & Cahalan, 1990; Olesen & Bundgaard, 1993; Inoue & Imanaga, 1995; Wischmeyer, Lentes & Karschin, 1995), although the run-down in the present experiments is much slower. Since the non-hydrolysable ATP analogues AMP-PNP and ATPγS cannot substitute for ATP, it is obvious that ATP hydrolysis is necessary. Also the observation that okadaic acid prevents run-down is consistent with a modulation of IRK via phosphorylation and dephosphorylation. At the concentration of 1 μM used in the present experiments, okadaic acid completely inhibits PP1 and PP2A phosphatases (Cohen, Holmes & Tsukitani, 1990). The run-down of IRK induced by the basic polypeptide protamine, which activates PP2A and inhibits PP1 (Cohen, 1989), suggests that dephosphorylation of serine/threonine via PP2A is involved.

The kinase responsible for the phosphorylation of the target protein that modulates IRK and is dephosphorylated by PP2A is unknown. It is also unclear whether modulation occurs by a direct phosphorylation/dephosphorylation of the channels. A Walker type-A motif, representing a single putative ATP-binding site, has been identified in ROMK1 (Ho *et al.* 1993). More detailed information about the sequence of the endothelial IRK1 is required to find out whether it contains a similar site.

It is well known that ATP hydrolysis occurs during actin assembly (Stossel, 1993), and that compounds which interfere with actin assembly and disassembly modulate the activity of K^+ channels (Wang, Cassola & Giebisch, 1994; Furukawa, Yamane, Terai, Katayama & Hiraoka, 1996). However, cytochalasin B at concentrations that have been reported to disintegrate F-actin in endothelial cells (Oike *et al.* 1994) did not affect IRK. It is therefore unlikely that channel activity is modulated by the cytoskeleton.

It is well known that non-hydrolysable GTP analogues inhibit IRK in various tissues (Mccloskey & Cahalan, 1990; Braun *et al.* 1992; Inoue & Imanaga, 1993, 1995; Wischmeyer *et al.* 1995; Pasyk *et al.* 1996). In the present experiments we show that GTPγS, which persistently activates G proteins by binding to the Gα subunit, inhibits IRK in CPAE cells, also in the presence of ATP. Activation of G proteins by F^-, alone or in combination with Al^{3+} (Gilman, 1987), has a similar effect on IRK. In addition, GDPβS, which binds to G proteins with a similar affinity as GTPγS but does not stimulate them, dose dependently antagonizes the GTPγS-induced inhibition.

These results therefore support the idea that endothelial IRK is inhibited by activation of G proteins. PTX, an inhibitor of G_i or G_o, did not affect the GTPγS-induced inhibition, suggesting that G proteins other than G_i or G_o are involved. It has been shown (Kahn, 1991) that AlF_4^- complexes fail to activate monomeric G proteins with molecular mass of 20–25 kDa, suggesting that small G proteins are also not involved.

Activation of PKA did not affect IRK in CPAE cells, and it is therefore unlikely that the G protein-induced inhibition is mediated by a G_s-coupled activation of adenylyl cyclase. These findings are consistent with those observed in cloned Kir2.1 or Kir2.3 channels expressed in *Xenopus* oocytes (Henry *et al.* 1996), but at variance with the inhibitory effects in other cell types (Koumi, Wasserstrom & Ten Eick, 1995; Pasyk *et al.* 1996). They also do not fit the contradictory observations that PKA activation is necessary for Kir2.1 channel activity (Fakler *et al.* 1994) or that it inhibits this channel (Wischmeyer & Karschin, 1996).

Activation of phospholipase C by G_q produces inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, and subsequently activates PKC. The phorbol ester PMA, which activates PKC, does not, however, affect IRK in CPAE cells. A similar finding was observed in atrial myocytes (Braun *et al.* 1992), but inhibition of IRK has also been reported in many cell types (Sato & Koumi, 1995; Takano, Stanfield, Nakajima & Nakajima, 1995). In addition Kir2.1 (Fakler *et al.* 1994) and IRK expressed by a polyA$^+$ brain mRNA injection into *Xenopus laevis* oocytes (DiMagno, Dascal, Davidson, Lester & Schreibmayer, 1996) were inhibited by PKC. In a recent report, it was shown that Kir2.3 but not Kir2.1 was regulated by PKC (Henry *et al.* 1996).

The observation that okadaic acid partially prevents the inhibition by GTPγS may suggest that it is linked to a G protein-regulated phosphatase. These results are consistent with those of Ruppersberg & Fakler (1996), which showed that the phosphatase inhibitor microcystin inhibited the effect of GTPγS on cloned Kir2.1 channels expressed in *Xenopus* oocytes. Based on similar evidence, Bielefeldt & Jackson (1994) and Inoue & Imanaga (1995) proposed a direct coupling between the G proteins and the phosphatases involved in dephosphorylation of Ca^{2+}-activated K^+ channels or inwardly rectifying K^+ channels. From our experimental data obtained in the whole-cell configuration, it is not possible to discriminate between a soluble and membrane-bound phosphatase. The slow rate of inhibition of IRK during dialysis of the cell with a GTPγS-containing pipette solution is in marked contrast to the fast inhibition during agonist stimulation of human endothelial (histamine, Nilius *et al.* 1993) and CPAE cells (ATP, B. Nilius, unpublished observation). This finding, which is indicative of the slow diffusion of GTPγS from the pipette to the target G protein that regulates IRK, is therefore consistent with the model of a membrane-bound G protein and phosphatase. It is interesting to note that recently a membrane-associated G protein-coupled receptor phosphatase (GRP), which is a member of the PP2A family, has been described (Pitcher, Payne, Csortos, DePaoli Roach & Lefkowitz, 1995). It is therefore tempting to speculate that this GRP may be involved in regulation of IRK. It is also not clear from our experiments how the G protein interacts with the phosphatase, but it has recently been suggested that the $G_{\beta\gamma}$ subunit may regulate the phosphatase (Ruppersberg & Fakler, 1996).

Our experiments show that neither PKA nor PKC activation is involved in the regulation of IRK, although several potential PKA and PKC phosphorylation sites have been demonstrated in the amino acid sequence of the Kir2 family (Kubo *et al.* 1993*a*; Makhina *et al.* 1994; Takahashi *et al.* 1994). They also show a marked inhibition of endothelial IRK mediated by a G protein-coupled activation of a phosphatase belonging to the PP2A family. This phosphatase may also be responsible for current run-down when the supply of intracellular ATP is compromised. This mechanism might be of importance for the control of the endothelial resting potential and the driving force for Ca^{2+} entry during cell stimulation under physiological and pathophysiological conditions.

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Author's email address

B. Nilius: bernd.nilius@med.kuleuven.ac.be

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