# Cloning and functional expression of the cDNA encoding an inwardly-rectifying potassium channel expressed in pancreatic β-cells and in the brain

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**Abstract** **A cDNA clone encoding an inwardly-rectifying K-channel (BIR1) was isolated from insulinoma cells. The predicted amino acid sequence shares 72% identity with the cardiac ATP-sensitive K-channel rc$K_{ATP}$ ($K_{ATP}$-1; [6]). The mRNA is expressed in the brain and insulinoma cells. Heterologous expression in *Xenopus* oocytes produced currents which were $K^+$-selective, time-independent and showed inward rectification. The currents were blocked by external barium and caesium, but insensitive to tolbutamide and diazoxide. In inside-out patches, channel activity was not blocked by 1 mM internal ATP. The sequence homology with $K_{ATP}$-1 suggests that BIR1 is a subunit of a brain and β-cell $K_{ATP}$ channel. However, pharmacological differences and the lack of ATP-sensitivity, suggest that if, this is the case, heterologous subunits must exert strong modulatory influences on the native channel.**

*Key words:* K-channel; Inward rectifyer; ATP-sensitive K-channel; Pancreatic β-cell; BIR1

## 1. Introduction

The β-cell ATP-sensitive potassium channel ($K_{ATP}$ channel) plays a central role in glucose-stimulated insulin secretion by linking metabolic and membrane events involved in stimulus-secretion coupling [3,5]. At the resting potential of the unstimulated β-cell, the $K_{ATP}$ channel is spontaneously active. Glucose metabolism produces a concentration-dependent inhibition of channel activity which is believed to be mediated by associated changes in the intracellular concentrations of ATP and ADP. The activity of the $K_{ATP}$ channel is responsible for the maintenance of a negative membrane potential, so that channel closure results in membrane depolarization and subsequent activation of voltage-gated calcium channels, calcium influx, and insulin secretion. The β-cell $K_{ATP}$ channel is also inhibited by sulfonylureas, drugs used in the treatment of non-insulin-dependent diabetes mellitus, resulting in the stimulation of insulin secretion [4,20]. Thus, the $K_{ATP}$ channel is the target for the most important physiological (glucose) and pharmacological (sulfonylureas) stimuli for insulin release. In addition, the channel is activated by the potassium channel opener diazoxide, which inhibits insulin secretion [20].

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The GenBank accession number of the rat BIR1 nucleotide sequence is X83583.

Recently, a $K_{ATP}$ channel has been cloned from cardiac muscle [6]. This channel (rc$K_{ATP}$; $K_{ATP}$-1) belongs to a family of inwardly rectifying potassium channels [7,12–16]. All members of this family show inward rectification which is at least in part induced by intracellular cations which act as voltage-dependent blocking particles and inhibit outward $K^+$-fluxes [8,17]. The molecular architecture of the inward rectifier channels is thought to consist of two transmembrane domains (TMs) with both N- and C-termini residing within the cell. These TMs are linked by a highly conserved segment, the P-region, which is believed to line the channel pore and within which resides the $K^+$-selectivity filter [10,11]; only the P-region shows high homology with the voltage-gated $K^+$ channels. Tissue distribution studies showed that $K_{ATP}$-1 mRNA is not expressed in β-cells [6], suggesting that the β-cell $K_{ATP}$ channel is a different protein. An additional K-ATP channel, u$K_{ATP}$-1, has also been cloned and although widely expressed is not found in insulin-secreting cell lines [13]. We now report the cloning and functional expression of an inwardly-rectifying K-channel with homology to $K_{ATP}$-1 which is found in β-cells and brain.

## 2. Materials and methods

### 2.1. Cloning of BIR1

Poly(A)$^+$ RNA was isolated from RINm5F cells using a Fast Track RNA isolation kit (Invitrogen), and converted to cDNA by reverse transcription with random primers. Oligonucleotides directed to bases 471–491 and 632–652 of $K_{ATP}$-1 were employed in the polymerase chain reaction [7] (94°C, 30 s; 53°C, 30 s; 72°C, 30 s for 40 cycles). A single band was visible by ethidium bromide staining. This reaction product was subcloned into M13mp19 and the nucleotide sequence of the insert was determined (Sequenase, USB). The sequence predicted a protein related to, but distinct from $K_{ATP}$-1. From this sequence, new oligonucleotides were synthesized and employed in 5′ and 3′ RACE using RINm5F cDNA [7,9]. In addition, a rat brain cDNA library (Stratagene) was screened using a radiolabeled oligonucleotide directed to the amplified sequence, bases 516–549 (5′CCTCTTAATCCAGTCCGTGTTGGGGTCCATTGTC3′; hybridization at 37°C, 50% formamide, and washed at 0.1× SSC, 0.1% SDS, 55°C) and 24 positively hybridizing clones were examined. All clones predicted the same protein sequence and 4 clones contained full length open reading frames: the nucleotide sequences of the PCR products from RINmF5 cells and the clones isolated from the brain cDNA library were identical. Oligonucleotides were synthesized on an Applied Biosystems model 391 DNA synthesizer, and PCRs were performed on a Perkin-Elmer 9600 thermocycler using Taq polymerase (Perkin-Elmer). All enzymes were purchased from BRL, and radionucleotides from NEN. Sequence analyses were performed using the Genetics Computer Group (GCG) suite of programs.


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*2.2. RT-PCR*

1 μg of total RNA from the indicated tissues was reverse-transcribed using random primers. Reactions were heated to 100°C for 5 min and quenched on ice prior to the PCR. Amplification was performed using unique oligonucleotides directed against 5′ untranslated and N-terminal coding sequences, bases 9–27 (sense) and 349–365 (antisense) respectively (94°C, 30 s; 59°C, 30 s; 72°C, 20 s; for 40 cycles).

*2.3. Northern blotting*

PolyA$^+$ RNA was isolated from rat heart, cerebellum, hindbrain, cortex and RINmF cells using TRI-REAGENT (Molecular Research Centre, Inc.) and polyAT tract mRNA isolation kit (Promega). 2–3 μg of each polyA$^+$ RNA, separated on a 1% agarose-formaldehyde gel, were transferred to nylon membrane (Hybond-N, Amersham) and probed with random primed BIR1 cDNA (entire coding region). The blots were washed at high stringency (0.1× SSC, 65°C for 1–2 h).

*2.4. Oocyte expression*

In vitro synthesis of mRNA and oocyte injection and handling were carried out as previously described [1]. Whole-cell currents were measured 48–96 h after injection using a 2-electrode voltage-clamp (Geneclamp 500, Axon Instruments, Foster City, USA). Microelectrodes were filled with 3 M KCl and had resistances of 0.7–2 MΩ. Data acquisition and analysis were carried out using a Labmaster AD converter (Axon Instruments, Foster City, CA) with a 486 PC computer and pClamp software. Control oocytes were injected with diethylpyrocarbonate treated water. The high potassium (90 K$^+$) bath solution contained (mM): 90 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES (pH 7.4 with KOH). The zero potassium solution contained (mM): 90 NaCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES (pH 7.4 with NaOH). Different K$^+$ concentrations were made by mixing the appropriate amounts of these two solutions. Tolbutamide was prepared as a 0.5 M stock in DMSO. $BaCl_2$ or CsCl were added directly to the solution. Oocytes were continuously perfused with solution and the experiments carried out at room temperature (18–24°C).

Single channel currents were recorded from cell-attached or excised membrane patches using an EPC 7 amplifier (List Electronik, Darmstadt, Germany) at 22–25°C. Patch electrodes were prepared from aluminosilicate glass and had resistances of 2–5 MΩ when filled with pipette solution. The pipette solution contained (mM): 140 KCl, 1 $MgCl_2$, 0.1 $GdCl_3$ (to suppress stretch-activated channels), 10 HEPES (pH 7.4). The bath solution was either (mM): 110 KCl, 10 EDTA, 30 KOH, 10 HEPES (pH 7.2) or 140 KCl, 1 $MgCl_2$ (free $Mg^{2+}$ 0.96 mM), 1 EGTA and 10 HEPES (pH 7.2). This solution also comprised the intracellular solution in inside-out patch recordings. Inside-out patches were removed from the vicinity of the oocyte (0.5 to 1 cm) and the bath solution exchanged to avoid any influence of the loss of oocyte contents on channel activity: in some experiments the bath was continuously

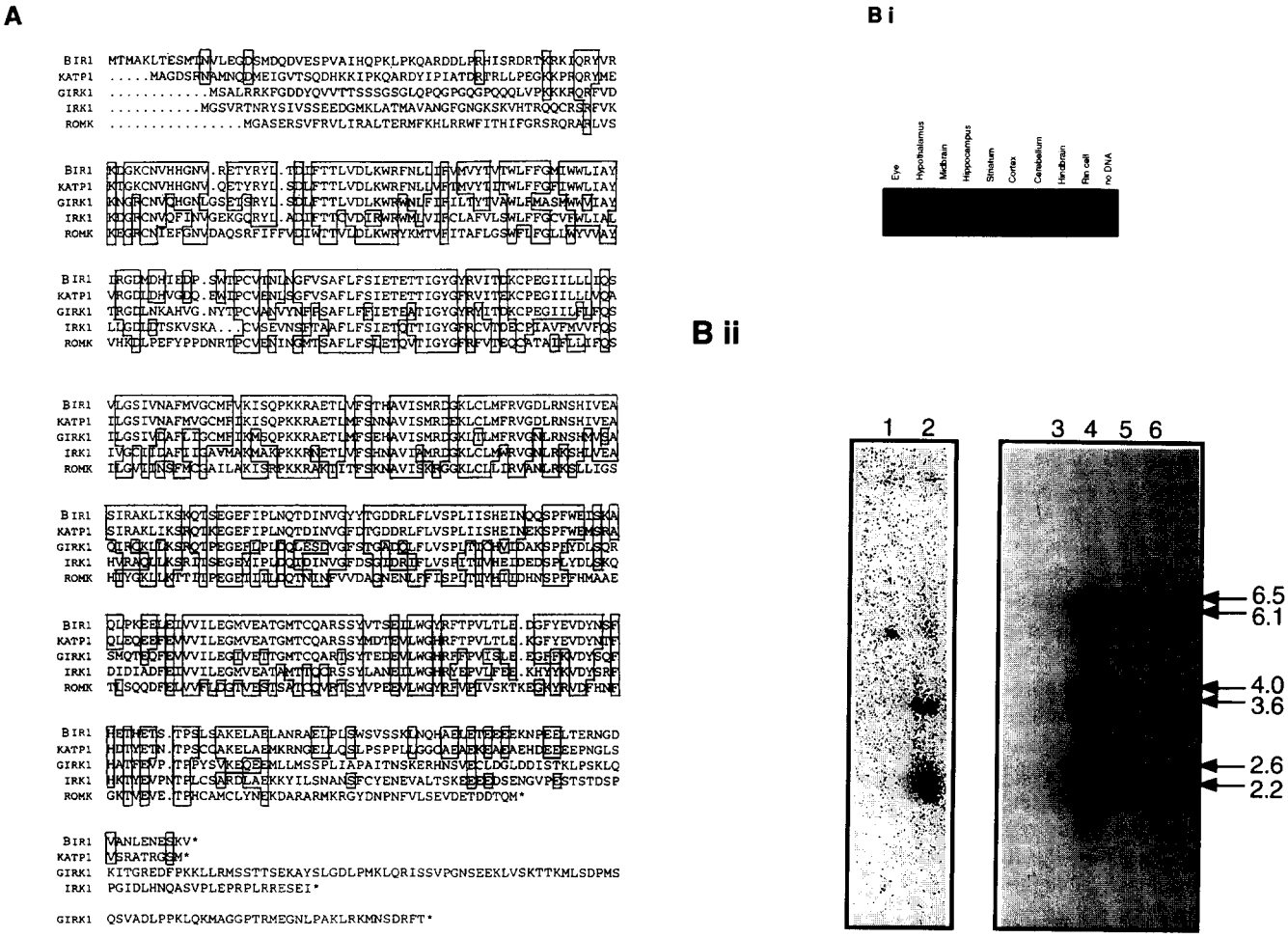


Fig. 1. (A) Predicted amino acid sequence of BIR1 and comparison with other members of the inward rectifier family. Residues shared by at least $K_{ATP}$-1 and BIR1 are boxed; putative transmembrane domains are overlined. Dots represent minimal gaps introduced to maximize the comparison. (B) Tissue distribution of BIR1. (i) *RT-PCR.* Total RNA was extracted from the indicated tissues. 1 μg was reverse-transcribed and subjected to PCR using BIR1-specific oligonucleotides. Following amplification, reaction products were separated on an agarose gel. (ii) *Northern blots.* PolyA$^+$ RNA was extracted from tissues and probed with random-primed BIR1. Lane 1, cardiac; 2, RINm5F cells; 3, hindbrain; 4, cerebellum; 5, cortex; 6, RINm5F cells. The sizes of bands are indicated in kb. Lanes 1 and 2 were exposed in a PhosphorImager cassette, and represent a much longer exposure than lanes 3–6 (autoradiography, 48 h).

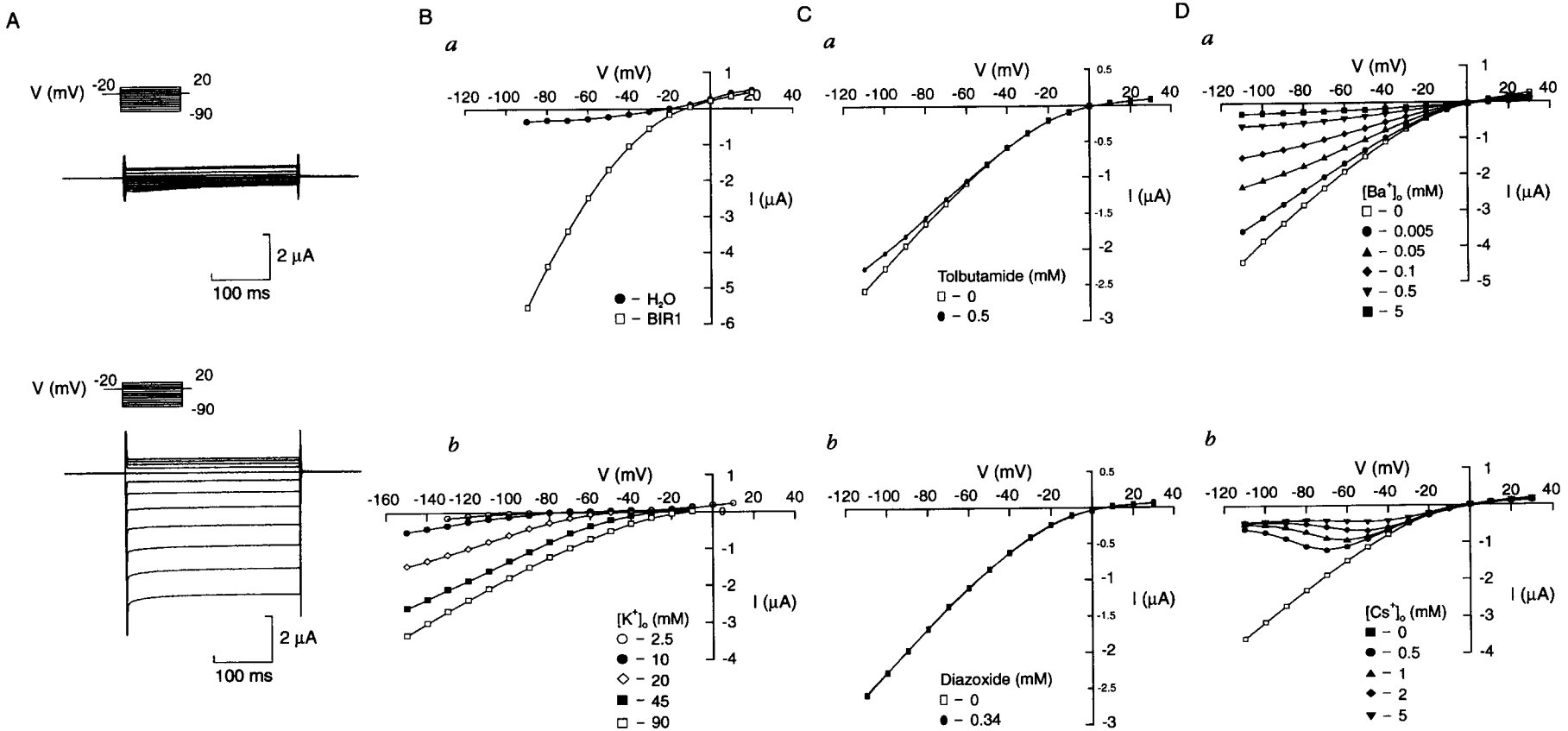


Fig. 2. (A) Whole-cell currents recorded in 90 mM $[K^+]_o$ from a *Xenopus* oocyte injected with water (above) or BIR1 mRNA (below). The holding potential ($V_h$) was −20 mV and the voltage protocol is shown above the current traces (on a different time base). (B) (*a*) Current–voltage (*I–V*) relationship of the data shown in A: water-injected (●), BIR1 mRNA injected (□). (*b*) *I–V* relationship recorded from an oocyte injected with BIR1 mRNA and recorded in: 90 mM $[K^+]_o$ (□, $V_h$ = −10 mV), 45 mM $[K^+]_o$ (■, $V_h$ = −30 mV), 20 mM $[K^+]_o$ (◇, $V_h$ = −50 mV), 10 mM $[K^+]_o$ (●, $V_h$ = −70 mV), 2.5 mM $[K^+]_o$ (○, $V_h$ = −80 mV). (C) *I–V* relationships recorded from 2 different oocytes in control solution (90 mM $[K^+]_o$; open symbols) and 0.5 mM tolbutamide (*a*, ●) or 340 μM diazoxide (*b*, ●: the data overlie one another). $V_h$ = −10 mV. (D) *I–V* relationships recorded from 2 different oocytes in control solution (90 mM $[K^+]_o$; open symbols) and the presence of barium (*a*, filled symbols) or Cs (*b*, filled symbols) at the concentrations indicated. $V_h$ = −10 mV.

perfused, but no differences were observed. Continuous recordings were stored on VCR and for subsequent analysis the data were filtered at 2 or 10 kHz by an 8-pole Bessel filter, sampled at 5 or 25 kHz using a Digidata A/D converter (Axon Instruments) and stored on a computer. The distribution of open and closed times was obtained by measurement of the lifetime of open and closed events and the distributions fitted using a Simplex maximum likelihood method.

## 3. Results

When oligonucleotides directed to $K_{ATP}$-1 were employed in RT-PCR under reduced stringency conditions, a product was detected from RINm5F cells and brain. The nucleotide sequence of this product predicted a protein related to, but distinct from, $K_{ATP}$-1. The full length coding sequence of this product was isolated (BIR1; Fig. 1A). The nucleotide sequence of BIR1 predicts a protein of 425 amino acids, sharing 72% identity with $K_{ATP}$-1 [6], 53% with GIRK-1 [15], 48% with IRK-1 [14] and 41% with ROMK-1 [12].

The tissue distribution of BIR1 mRNA was investigated and compared to that of $K_{ATP}$-1. RT-PCR revealed BIR1 expression throughout the brain and in insulinoma cells (Fig. 1Bi), but no expression was detected in thymus, adrenal gland, small intestine, lung, submaxillary gland, spleen, liver, kidney, skeletal muscle, heart ventricle, atrium and spetum, testis, uterus or ovary (not shown). In contrast, $K_{ATP}$-1 mRNA was detected in heart, some brain regions and multiple peripheral tissues, but was not detected in RINm5F cells or brain cortex [6]. Northern blot analysis detected six transcripts of 2.2, 2.6, 3.6, 4.0, 6.1 and 6.5 kilobases in cerebellum. Three of these were also detected in cortex (2.6, 4.0 and 6.5 kb) and two in RINm5F cells (2.2 and 3.6 kb); no hybridization was detected from hindbrain or heart (Fig. 1Bii).

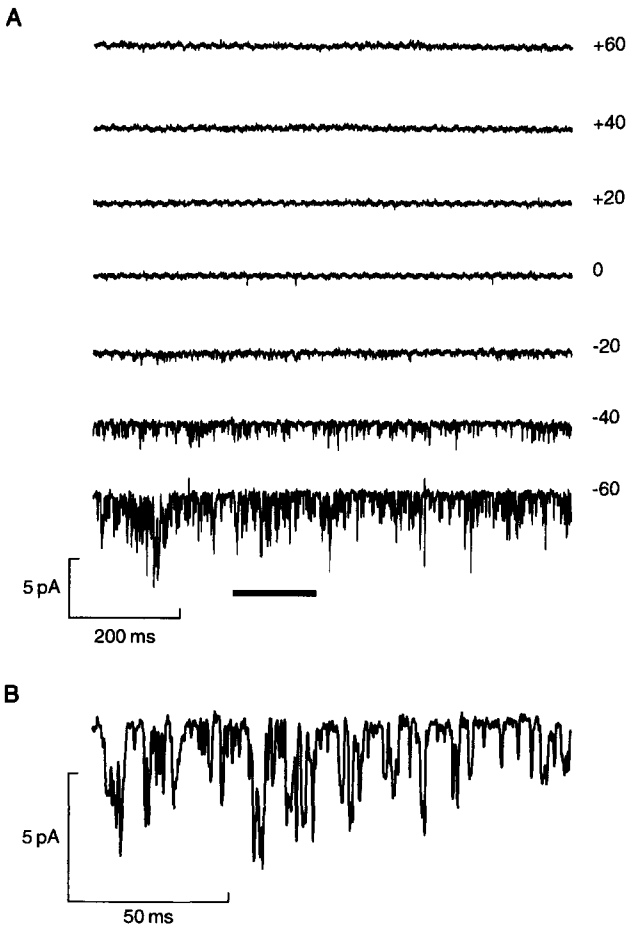


Fig. 3. (A) Single channel currents exhibiting mode 1 activity recorded from a cell-attached patch on an oocyte injected with BIR1 mRNA. The numbers to the right of each trace indicate the membrane potential. (B) Expanded section of the trace shown in A (indicated by the bar).

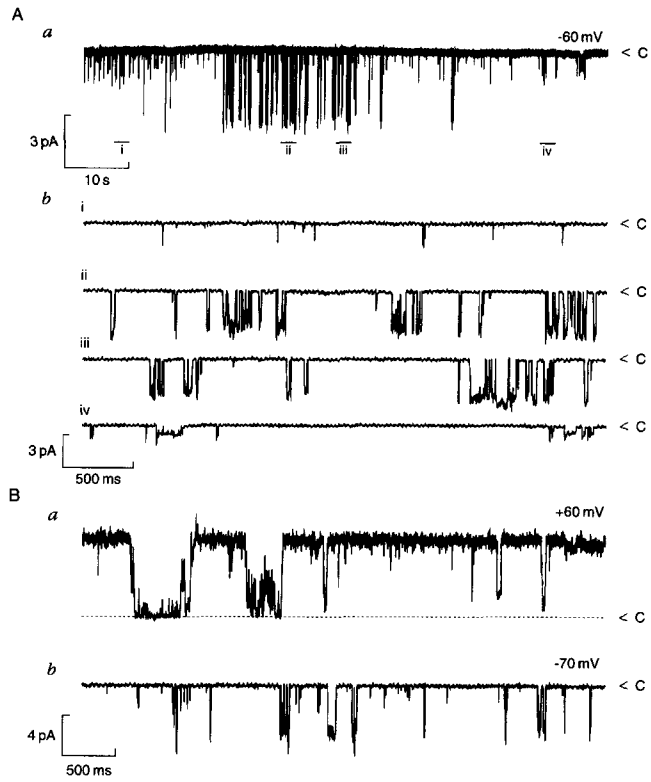


Fig. 4. (A) (*a*) Single channel recordings at −60 mV from an inside-out patch on an oocyte injected with BIR1 mRNA displaying the change between mode 1 and mode 2 activity. (*b*) Expanded sections of the trace shown in A. C = closed state. The current amplitude in mode 2 was i.e. −3.6 pA. (B) Mode 2 single channel recordings at +60 mV and −70 mV from a cell-attached patch on an oocyte injected with BIR1 mRNA. C = closed state. The current amplitude was −4.7 pA at −70 mV and 5.9 pA at +60 mV.

*Xenopus* oocytes were injected with BIR1 mRNA and whole-cell currents examined using the 2-electrode voltage clamp. Inwardly-rectifying currents were detected which activated rapidly upon hyperpolarization and which were 10- to 100-fold larger than those seen in water-injected control oocytes (Fig. 2A,B). To determine whether the expressed current was $K^+$-selective, the current-voltage relationship was measured at different external potassium concentrations (Fig. 2Bb). The shift in reversal potential was 54 mV/10-fold change in $[K^+]_o$, confirming that the channel is highly K-selective ($n$ = 5). The slope conductance also increased when external potassium was increased. The pharmacology of BIR1 expressed in oocytes is shown in Fig. 2C,D. The channel was almost completely inhibited by 5 mM external barium ($n$ = 40) and 1 mM caesium ($n$ = 7). The block by $Cs^+$ was strongly voltage-dependent, whereas that produced by $Ba^{2+}$ showed little voltage-dependence (Fig. 2D). The native $\beta$-cell $K_{ATP}$ channel is inhibited by sulfonylureas, but application of a maximally effective concen-

tration of tolbutamide (0.5 mM) was without effect on BIR1 currents (Fig. 2C, $n = 9$). Likewise, the sulfonamide diazoxide (340 $\mu$M), which potentiates the native $\beta$-cell $K_{ATP}$ current, was without effect on BIR1 expressed in oocytes ($n = 7$).

The sequence of BIR1 is almost identical (98%) with GIRK-2 [16], a brain inward rectifier K-channel which is modulated by G-protein activation. However, addition of dopamine to oocytes coinjected with BIR1 and the D2 receptor did not result in an increase in the whole-cell K-current (data not shown) suggesting that BIR1 is not activated by G-proteins.

Single BIR1 channel currents were studied in cell-attached and excised patches from oocytes. No endogenous currents were detected in water-injected oocytes (25 patches on 4 oocytes) with the exception of stretch-activated currents which were not fully blocked by gadolinium. However, the latter were not active in the absence of suction. A novel channel was detected in oocytes injected with BIR1 mRNA. Single BIR1 channel currents recorded from cell-attached patches with 140 mM external potassium exhibited two distinct gating modes. Mode 1 exhibited kinetics and conductance properties shown in Fig. 3. At negative membrane potentials, these currents showed rapid flickers with no clearly resolved open state, making it impossible to construct a reliable current-voltage relationship. At positive membrane potentials no outward currents were observed. Similar results were observed in inside-out patches, with no outward currents being observed at positive membrane potentials even in the absence of blocking ions ($Mg^{2+}$, $Na^+$, polyamines) in the intracellular solution.

Channel activity ran down very quickly (< 1 min) following formation of the inside-out configuration. This made testing the effect of ATP on channel activity difficult, but in 4 patches where this was possible 1 mM ATP was without effect on mode 1 activity.

In 2 out of 19 patches, one cell-attached and one inside-out, the channel briefly shifted into a different mode of gating (mode 2: Fig. 4). Thus, at negative membrane potentials inward currents consisted of clearly resolved channel openings which were clustered into bursts separated by long closed periods. In both configurations, the estimated single channel conductance at negative membrane potentials was 63 pS. In the cell-attached patch, large outward currents with several subconductance states were found: at +60 mV, the largest conductance state had an amplitude of 5.9 pA (100 pS). As intracellular blocking ions will be present in the cell-attached configuration, this suggests that BIR1 lacks rectification when operating in mode 2. We were unable to examine the mode 2 properties at positive potentials in the case of the inside-out patch, as the channel rapidly switched back to mode 1 behaviour. The single channel kinetics were analysed for mode 2 openings at −60 mV in the inside-out patch. The open time distribution was best fit by a single exponential with time constants of 5.4 ms and the closed time distribution was best fit by the sum of 2 exponentials with time constants of 0.6 ms and 148 ms.

## 4. Discussion

The results presented here demonstrate that BIR1 encodes an inwardly-rectifying K-channel expressed in both pancreatic $\beta$-cells and in brain.

Two pieces of evidence suggest that BIR1 may represent a pore-forming subunit of the $\beta$-cell $K_{ATP}$ channel. First, the predicted amino acid sequence shares 72% identity with $K_{ATP}$-1. Secondly, when expressed in oocytes, the whole-cell BIR1 currents are $K^+$-selective, time-independent and blocked by external application of caesium and barium, properties which are shared with native $K_{ATP}$ channels [6,18,19].

In most respects, however, the heterologously expressed BIR1 channel differs from the native $\beta$-cell $K_{ATP}$ channel. Firstly, BIR1 currents are insensitive to intracellular ATP, to sulfonylureas and to potassium channel openers. Secondly, the whole-cell currents showed strong inward rectification, whereas that of the native $\beta$-cell $K_{ATP}$ channel is weak. Thirdly, the $Ba^{2+}$ block of whole-cell currents is far less voltage-dependent than that of the native channel (unpublished observations). Fourthly, the kinetics, conductance and rectification properties of the most common mode of behaviour (mode 1) at the single channel level are very different from those of the native $\beta$-cell $K_{ATP}$ channel. Furthermore, although the single channel conductance in mode 2 at −60 mV resembles that of native $\beta$-cell $K_{ATP}$ channel, the kinetics and rectification properties are not similar.

Heterologously expressed BIR1 channels normally gate in mode 1 in which events appear flickery and unresolved. This behaviour is reminiscent of the rapid block of the conduction pathway of voltage-gated K-channels by external TEA. For BIR1 channels, mode 1 activity persists even in excised patches and with solutions which do not contain potential blocking cations such as $Mg^{2+}$ and polyamines. One explanation for the two different modes of gating is that in mode 1 part of the intracellular domain of the channel may act to block the permeation pathway. Inward currents flowing through the pore may transiently relieve the block by briefly forcing the blocking particle out of the permeation pathway. If this blocking and unblocking occur very fast, open events will not be fully resolved and single channel currents will appear as rapid flickers of reduced conductance. Outward currents may enhance the block by forcing the blocking particle further into the pore. Mode 2 behaviour may result if the blocking particle is removed completely from the vicinity of the pore, unmasking the fully opened properties of the channel. If this is the case, then the native channel may normally show mode 2 behaviour if additional subunits which form part of the channel complex alter the conformation of the blocking particle so that it is no longer free to enter the permeation pathway.

In conclusion, we have identified an inward rectifier K-channel subunit which is expressed in brain and $\beta$-cells. BIR1 has significant homology to $K_{ATP}$-1. However, if BIR1 represents a pore-forming subunit of the $\beta$-cell $K_{ATP}$ channel then associated subunits have profound effects on the channel properties, including modulation of gating and of sensitivity to ATP.

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