

Expression of functionally active ATP-sensitive K-channels in insect cells using baculovirus

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Received 18 May 1998

Abstract We have expressed active ATP-sensitive K-channels (K_{ATP} channels) in *Spodoptera frugiperda* (Sf9) cells using a baculovirus vector. A high yield of active channels was obtained on co-infection with SUR1 and Kir6.2 engineered to contain N- and/or C-terminal tags to permit detection by Western blotting. Channel activity was sensitive to ATP, glibenclamide and diazoxide. Channel activity was also obtained on expression of a C-terminally truncated Kir6.2 (Kir6.2ΔC26): these channels were blocked by ATP but were insensitive to sulphonylureas. In contrast to *Xenopus* oocytes and mammalian cells the full length Kir6.2 also gave rise to active channels in Sf9 cells when expressed alone. The highest yield of active K_{ATP} channels was obtained on infection with a fusion protein containing SUR1 linked to Kir6.2ΔC26 via a 6-amino acid linker.

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Key words: Potassium channel; ATP; Sulphonylurea receptor; Baculovirus; Gene expression

1. Introduction

ATP-sensitive K-channels (K_{ATP} channels) play a central role in the control of insulin secretion from pancreatic β -cells [1–3]. Their closure in response to changes in intracellular adenine nucleotide concentrations couples changes in β -cell glucose metabolism to membrane depolarisation and hence to Ca^{2+} -influx and insulin release. They are also closed by sulphonylureas such as tolbutamide, which are used in the treatment of non-insulin-dependent diabetes mellitus (NIDDM) [4], and they are opened by diazoxide, a sulfonamide drug used to treat the excessive insulin secretion found in insulinoma or persistent hypoglycaemia and hyperinsulinaemia of infants [5]. K_{ATP} channels are made up of two components, a channel-forming subunit, Kir6.2, and a larger regulatory subunit, SUR1, which mediates the effects of sulphonylureas and diazoxide on channel activity [6,7]. SUR1 belongs to the ATP-binding cassette (ABC) family of proteins and contains two putative nucleotide-binding folds which mediate the activation of the channel by MgADP; current evidence suggests, however, that the major inhibitory effect of ATP is mediated by Kir6.2 [8]. Studies of the stoichiometry of K_{ATP} channels indicate that the active channel is an octomer consisting of 4 molecules each of Kir6.2 and SUR1 [9,10]. Transient transfection of mammalian cells with SUR1 leads to the expression of high-affinity sulphonylurea binding sites. However, both subunits are required for expression of active

K_{ATP} channels in *Xenopus* oocytes or mammalian cells [7,8,11–15]. A truncated mutant of Kir6.2 lacking 26 amino acid residues at the carboxyl terminal (Kir6.2ΔC26) did, however, give rise to active channels in oocytes and mammalian cells [8].

Detailed functional and structural analysis of Kir6.2 and SUR1 and of how they interact to form an active K_{ATP} channel requires an expression system capable of providing substantial amounts of the active proteins. In this study we show that *Spodoptera frugiperda* (Sf9) insect cells simultaneously infected with recombinant baculoviruses containing the genes coding for Kir6.2 and SUR1 under the control of the polyhedrin promoter produce functionally active K_{ATP} channels in large amounts. We show that at the high level of expression attained in this system Kir6.2 alone gives rise to active channels. We also demonstrate high channel activity in Sf9 cells infected with Kir6.2ΔC26. The highest levels of sulphonylurea-sensitive K_{ATP} channel activity were obtained in Sf9 cells infected with baculovirus encoding a fusion protein of SUR1-Kir6.2ΔC26.

2. Materials and methods

2.1. Cells and viruses

Sf9 cells were propagated at 28°C in TC100 medium containing 10% fetal calf serum. Cells were infected by each recombinant baculovirus at a multiplicity of infection (moi) of 10 and expressed products were analyzed at 2 days post-infection (pi). For virus stocks, Sf9 cells were infected by each recombinant baculovirus at a moi of 0.1 and the supernatant collected 5 days later.

2.2. Construction of plasmid DNAs and recombinant baculoviruses

We constructed transfer vectors containing DNA fragments encoding rat SUR1 [6] and mouse Kir6.2 [15] under control of the polyhedrin promoter in the pAcYM1 vector. Sequences encoding His⁶ and FLAG (NYKNNNNK) tags (indicated by H and F, respectively, in the DNA sequences described below) were introduced by PCR onto the 3' ends of SUR1 cDNA. As a result transfer vectors pAcSUR1H and pAcSUR1F were obtained. The FLAG tag was introduced on the N-terminal of Kir6.2 and the His⁶ tag on the C-terminal; pAcKir6.2H, pAcKir6.2F were obtained by cloning the corresponding PCR products in pAcYM1. Truncated sequences of Kir6.2 without the 26 carboxyterminal amino acids were obtained by PCR and inserted in the *Bam*HI site of pAcYM1. His⁶ and FLAG tags were inserted as described above. Transfer vectors pAcKir6.2ΔH and pAcKir6.2ΔF were created. To link SUR1 and truncated Kir6.2 in a single polypeptide in head-to-tail fashion the nucleotide sequence TCTGCTTCTGCCTCTGCA, coding for a spacer (SerAla)³, was introduced by fusion PCR between SUR1 and Kir6.2. DNA coding for the fusion protein with a His⁶ tag at the C-terminus was cloned in pAcYM1. Transfer vector pAcFusΔH was obtained. Transfer vectors were used for co-transfection of Sf9 cells together with *Autographa californica* nuclear polyhedrosis virus (AcNPV PAK6) [16]. Recombinant baculoviruses AcSUR1H, AcSUR1F, AcKir6.2H, AcKir6.2F, AcKir6.2ΔH, AcKir6.2ΔF, AcFusΔH were obtained by using the corresponding transfer vectors, three times plaque purified and used for infection of Sf9 cells.

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2.3. Electrophysiology

Whole-cell and single-channel currents were recorded using an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany) and stored on videotape for later analysis. Whole-cell currents flowing through K_{ATP} channels were monitored using alternate ± 20 -mV pulses of 250 ms duration which were applied at a frequency of 0.5 Hz from a holding potential of -70 mV. For all experiments, the internal solution contained (mM): 107 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 EGTA, 10 HEPES (pH 7.2 with KOH), plus 0.3 mM or 1 mM MgATP as indicated. For whole-cell recordings, the external solution contained (mM): 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$ and 10 HEPES (pH 7.4 with NaOH). For single-channel recordings, the external (pipette) solution contained (mM): 140 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES (pH 7.4 with KOH). All experiments were carried out at room temperature (22 – $24^\circ C$).

Whole-cell currents were filtered at 2 kHz, digitised at 1 kHz using a Digidata 1200 Interface and subsequently analysed using pClamp software (Axon Instruments, Burlingame, USA). Single-channel currents were filtered at 5 kHz, digitised at 10 kHz and analysed using a combination of pClamp and in-house software written by Dr. P.A. Smith (Oxford University).

2.4. Rubidium efflux assay

Sf9 cells (5×10^5 cells; 24 h pi) were incubated for 16–20 h in TC100 medium with 10% fetal calf serum and $^{86}RbCl$ (0.4 mCi/ml). Cells were then incubated for 30 min in solution A (120 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM KCl, 20 mM HEPES, pH 7.5) containing $^{86}RbCl$ (0.4 mCi/ml) under one of four conditions: no additions (basal); with 2.5 mg/ml oligomycin (metabolically inhibited); with oligomycin and 1 μM glibenclamide (to inhibit K_{ATP} channels); with 400 μM diazoxide (to activate K_{ATP} channels). Subsequently cells were washed once in $^{86}RbCl$ -free solution A with or without additives and then exposed to the same solution for 2 min. The ^{86}Rb released to the medium was measured by liquid scintillation spectrometry. Rubidium efflux was expressed as a percentage of the total rubidium uptake (the sum of ^{86}Rb -efflux and the ^{86}Rb remaining in the cells and released by adding 50 mM Tris-HCl, pH 8.0, 2% SDS, 150 mM NaCl).

2.5. SDS-PAGE and immunoblotting analysis

Protein dissociation buffer ($2 \times$) (4% (v/v) β -mercaptoethanol, 4% (w/v) SDS, 25% (v/v) glycerol, 10 mM Tris (pH 6.8), 0.02% (w/v) bromophenol blue) was added in equal volume to each sample, and mixtures were heated to $55^\circ C$ for 15 min. Proteins were resolved by SDS-PAGE (10%) and stained with Coomassie blue. Proteins were then electroblotted onto an Immobilon membrane (Millipore International). The membrane was incubated for 1 h at room temperature in blocking buffer (5% (w/v) skimmed milk in Tris buffer saline). Anti-His⁶ tag antibody (Penta-His, Qiagen) or anti-FLAG antibody (Anti-FLAG M2 Antibody, Eastman Kodak Company) was added and the membrane was incubated for 1 h at room temperature. After three 5-min washes in blocking buffer, the bound antibody was detected by alkaline phosphatase conjugated with anti-mouse IgG.

2.6. Measurement of ATP

Sf9 cells (10^6 cells/ml) were incubated in 100 μl TC100 medium in the absence or presence of 2.5 $\mu g/ml$ oligomycin. After 20 min at room temperature, 50 μl ice-cold 10% PCA was added. Aliquots of the extracts (10 μl) were assayed for ATP by addition to 1 ml of a solution containing 100 mM Tris-HCl, pH 7.8, 5 mM $MgSO_4$, 0.5 mM EDTA, 0.5 mM dithiothreitol and 0.1 mg/ml BSA. Following addition of 40 μl firefly lantern extract, luminescence was measured on a BioOrbit 1253 Luminometer. Standard ATP samples (1–100 pmol) were used to calibrate the luminescence.

2.7. [3H]Glibenclamide binding

Sf9 cells resuspended at a density of 2×10^6 cells/ml in TC100 were incubated at room temperature for 90 min with [3H]glibenclamide (20 nM) and test substances in a final volume of 400 μl . The incubation was stopped by rapid separation on Whatman GF/C filters soaked in TC100 for 30 min beforehand. Filters were washed and specific binding determined as previously described [17].

2.8. Expression of data

Data are shown as mean $\pm$ S.E.M. and the significance of differences was assessed by Student's *t*-test.

3. Results and discussion

Fig. 1 shows the expression of SUR1, Kir6.2, Kir6.2 $\Delta C26$ and the fusion protein SUR1-Kir6.2 $\Delta C26$ in Sf9 cells infected with the corresponding recombinant baculoviruses. Proteins were detected by Coomassie staining (Fig. 1A) or using antibodies directed against the N-terminal or C-terminal tags incorporated into the recombinant proteins (Fig. 1B,C). There are strong single bands corresponding to SUR1 and SUR1-Kir6.2 (Fig. 1A,B for His⁶ tagged proteins, Fig. 1C for FLAG tagged), in contrast to expression in heterologous mammalian cells where two differently glycosylated forms of SUR1 were observed [9,18]. This finding confirms a difference in glycosylation pattern in insect and mammalian cells. For expression of Kir6.2 and Kir6.2 $\Delta C26$ (Fig. 1B,C) strong monomeric bands corresponding to protein with the appropriate molecular weight can be seen together with dimeric and tetrameric forms reflecting strong protein-protein interaction and the two-fold symmetry of the complex. Fig. 1A,D demonstrate expression of SUR1H and Kir6.2F after co-infection of insect cells with the corresponding viruses. Using different tags and antibodies allowed separate determination of the expression of each recombinant protein.

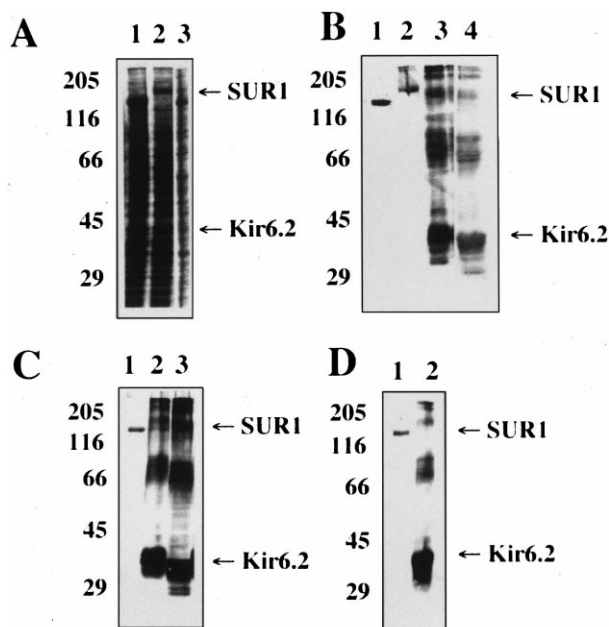


Fig. 1. Expression in Sf9 cells of recombinant SUR1 and Kir6.2 containing His⁶ (H) or FLAG (F) tags. A: Coomassie staining. Lane 1: SUR1H; lane 2: SUR1-Kir6.2 $\Delta C26$ H; lane 3: SUR1H+Kir6.2F co-expression. B–D: Western blots. B: With anti-His⁶ antisera; lane 1: SUR1; lane 2: SUR1-Kir6.2 $\Delta C26$ H; lane 3: Kir6.2H; lane 4: Kir6.2 $\Delta C26$ H. C: With anti-FLAG antisera; lane 1: SUR1F; lane 2: Kir6.2F; lane 3: Kir6.2 $\Delta C26$ F. D: SUR1H and Kir6.2F co-expression; lane 1: with anti-His⁶ antisera; lane 2: with anti-FLAG antisera.

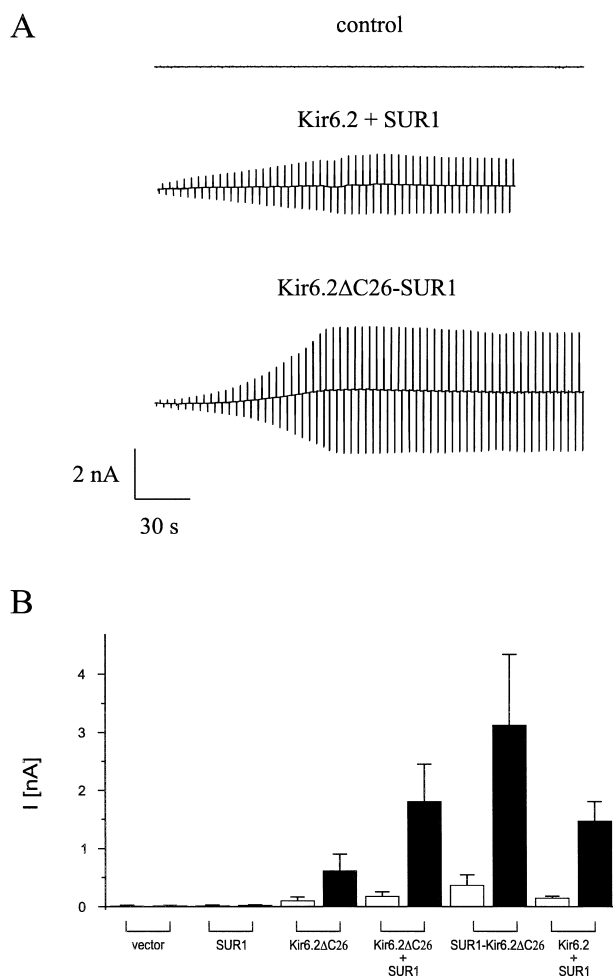


Fig. 2. A: Whole-cell K_{ATP} currents recorded from a control Sf9 cell (top) and from Sf9 cells expressing Kir6.2+SUR1 (middle) or the SUR1-Kir6.2ΔC26 fusion protein (bottom). Currents were recorded in response to alternate ± 20 -mV voltage steps from a holding potential of -70 mV. B: Mean current amplitudes ($n=6$) recorded from Sf9 cells expressing the indicated proteins immediately after obtaining the whole-cell configuration (white bars) and when the current was maximal (black bars). In those cases where no washout current was evident, the current was measured after 5 min of dialysis.

Sf9 cells infected with SUR1 expressed [3 H]glibenclamide binding activity. The specific binding activity at 20 nM [3 H]glibenclamide amounted to 2.5 ± 10^6 binding sites per cell. In comparison, the density of sulphonylurea binding sites in pancreatic β -cells is approximately 1.9×10^3 sites per cell [17]. A high density of [3 H]glibenclamide sites was also obtained on infection of Sf9 cells with the fusion protein SUR1-Kir6.2ΔC26.

The whole-cell patch-clamp technique was used to study the expression of K_{ATP} channel activity (Fig. 2). Sf9 cells infected with the viral vector AcNPV PAK6 alone expressed very little endogenous K^+ current: the mean current amplitude evoked by a 20-mV step from -70 mV was 22 ± 5 pA ($n=6$) (Fig. 2B). The magnitude of this current did not change significantly during 5 min of infusion with an intracellular solution containing 0.3 mM ATP (Fig. 2A). Mean current amplitudes were also unchanged by infection with SUR1. When Sf9 cells were co-infected with Kir6.2 and SUR1, however, whole-cell currents were initially larger than in cells infected with the

vector alone and further increased to -1480 ± 330 pA ($n=6$, $P<0.001$), within the next 2–3 min (Fig. 2A,B). A similar effect is found in pancreatic β -cells where it has been attributed to the washout of ATP from the cell following dialysis with pipette solution [19]. Single-channel currents were observed in the cell-attached mode in most cells, and exhibited properties similar to those of native K_{ATP} channels. These results confirm that Kir6.2/SUR1 can be expressed in insect cells.

A similar increase in current was observed with the truncated form of Kir6.2 (Kir6.2ΔC26) that is capable of independent expression in *Xenopus* oocytes and mammalian cells [8] (Fig. 2B). This suggests that the resting level of ATP in Sf9 cells is sufficient to cause marked inhibition of Kir6.2ΔC26. Since this construct is inhibited, but not activated, by adenine nucleotides an estimate of the resting intracellular ATP concentration can be obtained from the increase in current that follows replacement of the endogenous ATP concentration with a solution containing 300 μ M ATP. The measured increase in current was ~ 6 -fold, and we took the K_i to be 150 μ M and the Hill coefficient as 1 [8]. This gives a lower estimate of 2.5 mM for the endogenous level of submembrane ATP in Sf9 cells. By direct measurement of ATP in Sf9 cells we obtained a value of 50.5 fmol/cell. Assuming a cellular volume of 1 pL, the total ATP content amounts to 5 mM, in good agreement with the electrophysiological estimate.

Co-infection with SUR1 plus Kir6.2ΔC26 enhanced the current amplitude, as previously reported [8]: when the cDNA encoding Kir6.2ΔC26 was linked to that of SUR1, the current amplitudes were even greater (Fig. 2B). Since SUR1 enhances functional expression of Kir6.2, it seems probable that this result reflects the fact that not all Kir6.2 subunits are coupled to SUR1 when the cDNAs are cotransfected (in contrast to the fusion construct). There was no significant difference in current amplitude when Kir6.2, rather than Kir6.2ΔC26, was coinfecting with SUR1 (Fig. 2B). This provides additional support for the idea that truncation of the last 26 amino acids of Kir6.2 is without functional effect [8].

Although the full length form of Kir6.2 did not express measurable currents in *Xenopus* oocytes [8,14], COS cells

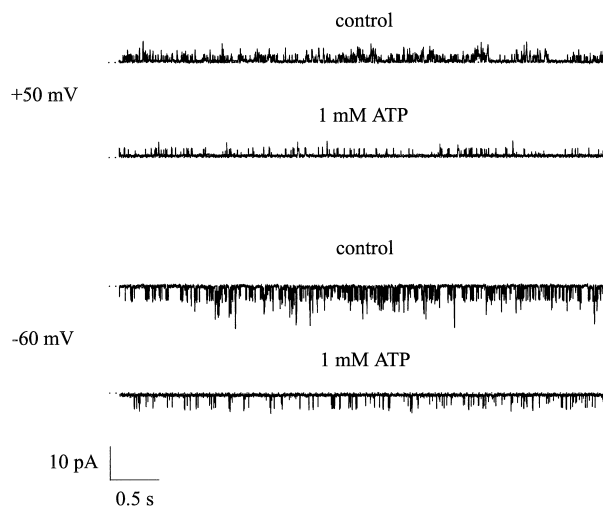


Fig. 3. Single channel currents recorded at +50 mV or -60 mV, as indicated, from inside-out patches excised from Sf9 cells expressing Kir6.2 alone. ATP (1 mM) was present in the internal solution as indicated.

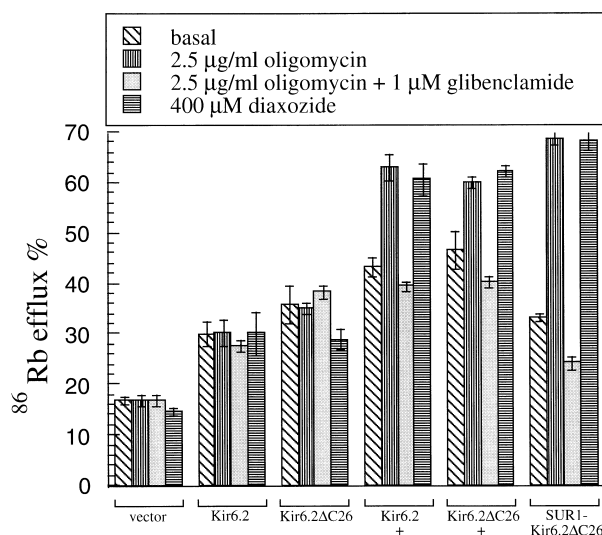


Fig. 4. ⁸⁶Rb-efflux from Sf9 cells expressing the indicated proteins. Values are given as mean $\pm$ S.E.M. ($n=6$) for ⁸⁶Rb-efflux during 2 min expressed as a percentage of the initial ⁸⁶Rb content of the cells.

[7,11,13] and HEK cells [12,15], this was not the case in insect cells. Fig. 3 shows single channel currents recorded from inside-out patches excised from Sf9 cells infected with Kir6.2 alone. The amplitude of these currents was 3.9 ± 0.1 pA ($n=3$) at -60 mV, similar to that of wild-type K_{ATP} , of Kir6.2/SUR1 and of Kir6.2ΔC26 channels [7,8,15]. The mean open probability was 0.06 ± 0.01 ($n=3$) and the mean open time was 0.81 ± 0.13 ms ($n=3$); these values are similar to those observed for Kir6.2ΔC26 channels [20]. Furthermore, Kir6.2 currents were blocked by ATP (Fig. 3) with a sensitivity similar to that found for Kir6.2ΔC26: assuming a Hill coefficient of unity, as is the case for Kir6.2ΔC26 [8], the estimated K_i for Kir6.2 was 194 μ M, compared with a value of 106–175 μ M for Kir6.2ΔC26 [8]. Thus it appears that Kir6.2 is capable of independent expression in Sf9 cells and exhibits properties similar to those of Kir6.2ΔC26. This may be due to the much higher levels of expression that can be achieved using the baculovirus system; alternatively, it may mean that Sf9 cells, but not mammalian cells or *Xenopus* oocytes are capable of functional expression of Kir6.2. We were unable to examine the whole-cell currents in cells infected with Kir6.2 alone because of the presence of large leakage currents.

To study metabolic activation of expressed K_{ATP} channels, which requires intact cells, we used ⁸⁶Rb-efflux assays. Fig. 4 shows ⁸⁶Rb-efflux from Sf9 cells infected with recombinant baculoviruses. The efflux from Sf9 cells was the same (17%) for uninfected cells as for cells infected with the vector AcNPV PAK6 alone or expressing SUR1 alone (not all data shown). A significant increase in ⁸⁶Rb-efflux was observed when cells expressed Kir6.2 (30%) or Kir6.2ΔC26 (36%) and the greatest increase was found when SUR1 and Kir6.2 were expressed together (44–47%). Native K_{ATP} channels are blocked by ATP and glibenclamide and activated by diazoxide [1]. We first established that addition of 2.5 μ g/ml oligomycin elicited a significant reduction in Sf9 cell ATP content. In the absence of oligomycin, the mean ATP of cells was 5 ± 0.5 fmol/cell and was reduced to 2.35 ± 0.01 fmol/cell

on incubation for 20 min with oligomycin. Decreasing $[ATP]_i$ in this way produced a significant stimulation of ⁸⁶Rb-efflux (to 61–69%) in Sf9 cells co-expressing SUR1 together with Kir6.2 or Kir6.2ΔC26, or the fusion construct SUR1-Kir6.2ΔC26, but not in cells expressing Kir6.2 or Kir6.2ΔC26 alone. Glibenclamide (1 μ M) blocked this increase in ⁸⁶Rb-efflux suggesting it flows through K_{ATP} channels. In support of this view, diazoxide also increased ⁸⁶Rb-efflux in these cells. By contrast, in cells expressing Kir6.2 or Kir6.2ΔC26 alone, neither diazoxide nor glibenclamide was effective; this is expected since both agents mediate their action via SUR1. Reducing $[ATP]_i$ did not enhance ⁸⁶Rb-efflux in cells infected with Kir6.2 or Kir6.2ΔC26. Although Kir6.2ΔC26 is affected by metabolic inhibition in oocytes [8], the effects are small. Taken together, these data support the view that metabolic inhibition of the K_{ATP} channel is primarily mediated by the SUR1 subunit. These experiments demonstrate that ⁸⁶Rb-efflux is effectively regulated in insect cells expressing SUR1 and Kir6.2 together. In cells expressing fusion protein, regulation was more marked than in the case of co-expression. This may reflect more uniform channel formation in the fusion protein since after co-expression SUR1 and Kir6.2 may not be expressed equally, leading to unregulated Kir6.2 [9]. Alternatively there may exist a slightly different conformation of molecules in the channel in the fusion protein.

In summary, our data demonstrate that expression of functional K_{ATP} channels and independent expression of their individual protein components can be achieved in high yield using the baculovirus-insect cell system. This system provides sufficient material to allow future detailed biochemical and structural analysis.

Acknowledgements: These studies were supported by grants from the Wellcome Trust, and the British Diabetic Association.

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