

Tarasov et al

SUPPLEMENTARY INFORMATION

I. Use of recombinant K_{ATP} channels expressed in HEK cells

For expression in HEK cells, Kir6.2 was tagged at the C-terminus with EYFP to enable visualization of transfected cells by epifluorescence (1). Attachment of EGFP to the C-terminus of Kir6.2 did not markedly alter K_{ATP} channel ATP sensitivity (Table1, main text), as previously reported by (2). Previous studies have shown that addition of GFP or an HA tag to the C terminus of Kir6.2 allows some expression of Kir6.2 at the membrane surface independently of SUR1 (2). Because Kir6.2 has a lower ATP sensitivity than Kir6.2/SUR1 (IC_{50} of 100 μ M and 10 μ M, respectively), and because unlike Kir6.2/SUR1, Kir6.2 is not activated by MgADP, this could potentially result in a small population of K_{ATP} channels with altered ATP sensitivity. This may, in part, explain why the ATP sensitivity in the inside-out patch measured in the absence of Mg^{2+} is somewhat lower for recombinant K_{ATP} channels expressed in HEK cells than it is for native β -cell channels. It is also possible that differences in endogenous channel modulators (e.g. lipids) vary between β -cells and HEK cells. When Kir6.2/SUR1 and Kir6.2-GFP/SUR1 were expressed in the same cells there was no difference in ATP sensitivity (2).

In the SUR1-KA/KM construct lysine residues in NBD1 (K719) and NBD2 (K1384), which are critical for ATP binding and/or hydrolysis, have been mutated to alanine and methionine, respectively. Cytosolic ATP levels in HEK cells were 3.3 ± 0.5 mM (n=6) and 2.5 ± 0.7 mM (n=4) when estimated from cell-attached channel activity and

the ATP-sensitivity of wild-type and Kir6.2/SUR1-KAKM channels, respectively, in open-cell patches.

II. Whole-cell recordings

For whole-cell experiments (perforated patch and standard whole-cell), the pipette solution contained (mM): 76 K₂SO₄, 10 NaCl, 10 KCl, 1 MgCl₂, 5 HEPES (pH 7.35 with KOH) plus 0.24 mg/ml amphotericin. No ATP was added. The bath solution contained (mM): 137 NaCl, 5.6 KCl, 10 HEPES (pH 7.4 with NaOH), 2.6 CaCl₂, 1.1 MgCl₂ and glucose as indicated. Recordings were initiated 30 min after exposure to glucose-free solution. All experiments were conducted at 21-23°C and the bath solution was perfused continuously.

Whole-cell currents were recorded at a holding potential of -70mV in response to hyperpolarizing 20mV voltage steps. Currents and conductances were normalized to cell capacitance to correct for differences in cell size.

To measure the relationship between the extracellular glucose concentration and whole-cell K_{ATP} conductance, we used the perforated-patch configuration, in which the patch membrane is perforated with amphotericin thereby giving access to the cytoplasm without destroying cell metabolism. Glucose was added in increasing concentrations. Once the concentration-response curve had been measured, the standard whole-cell configuration was formed by applying negative pressure to the patch. This led to destruction of the patch membrane allowing ATP to dialyze out of the cell. The loss of ATP led to a large increase in the K_{ATP} current. The peak whole-cell K_{ATP} current (referred to as the washout current) was measured once the current

had reached its maximal value. The conductance was then calculated from the current response to the 20-mV voltage step.

Because K_{ATP} currents sometimes run down with time in standard whole-cell recordings when $[ATP]_i$ is absent (3), we estimated the maximal K_{ATP} current by extrapolating the peak washout current to time zero by fitting a polynomial to the time course of rundown.

III. Advantages of cell permeabilization with α -toxin

Cell permeabilization with α -toxin offers several advantages over other methods of permeabilization. First, the pores produced by the toxin are small (1.5nm) and exclude substances with molecular masses of $>3kDa$, which includes almost all proteins. Second, permeabilization produces pores over the whole of the cell surface, so that the membrane behaves as a molecular sieve: as some of these pores may be adjacent to the patch membrane rapid exchange of solutions and a good concentration clamp is possible. Third, because α -toxin does not penetrate the plasma membrane, intracellular organelles are not permeabilized. Finally, the cytoskeleton of the cell remains intact, as evidenced by the fact that insulin secretion can be elicited from isolated beta-cells or beta-cell lines permeabilized with α -toxin (5,6). A previous study has shown that α -toxin permeabilized beta-cells are permeable to ATP (500 Da), and trypan blue (950 Da) (4). The pores formed by the toxin are known not to seal with time (5).

The favoured method of cell permeabilization for patch-clamp studies has been the use of the detergents saponin and digitonin. These have the disadvantage that they

cannot be applied close to the patch or they destroy it. Cardiac cells are large enough that the detergent can be applied to one end of the cell without damaging a cell-attached patch at the other end; however, the technique is much less successful in small cells like β -cells. Furthermore, the distance between the patch and the ruptured membrane means applied solutions must dialyse through much of the cell cytoplasm (which means that concentrations of applied nucleotides may be modified). A third disadvantage is that the membrane is destroyed enabling leakage of large cytosolic proteins in an uncontrolled fashion. Finally, in β -cells, disruption of the membrane of the secretory granules might alter [ATP] in the vicinity of the K_{ATP} channel. A few studies have used streptolysin O (SLO) to permeabilize β -cells (7), but the pores produced are much larger than those of α -toxin (30nm; cut-off MW 150 kDa). Furthermore, like digitonin, SLO can affect the membrane of intracellular organelles.

An alternative strategy for measuring the ATP sensitivity of K_{ATP} channel in the 'intact' cell has been to dialyse β -cells with different concentrations of ATP using the standard whole-cell recording configuration (8). A difficulty with this method is that it is only possible to test one ATP concentration per cell, rather than construct the whole ATP concentration-inhibition curve for a single cell (as with our method). Secondly, the rate of diffusion of ATP into or out of the cell is slow. In whole-cell recordings of recombinant K_{ATP} channels expressed in HEK cells the time-to-peak current when dialysed with nucleotide free solution was 167s (8). In contrast, the block by 1 mM ATP reversed within 60s in our experiments, a time that also includes the perfusion rate of the chamber. The rapidity with which ATP inhibition can be washed out in α -toxin permeabilized cells, and the fact that the ATP concentration-

inhibition curves in Mg^{2+} -free solution are identical in open-cell and inside-out patches, suggest that the inside of the cell is efficiently perfused in our experiments.

Finally, the ATP sensitivity has been estimated by measuring glibenclamide-sensitive Rb^+ efflux in intact RINm5F cells and depleting cellular ATP with metabolic poisons (9). This experiment suffered from the problem that the contribution of ATP within the secretory granules was not taken into account, so that $[ATP]_i$ may be overestimated. Furthermore, metabolic poisoning may elevate $[MgADP]$ above that found in the absence of glucose (or in dialysed cells). Finally, Rb^+ efflux may not be a good measure of K_{ATP} channel activity, as the cation causes a voltage-dependent block of the channel (10). This may explain why the estimated IC_{50} for ATP block was higher than other estimates (0.8 mM).

Cell permeabilization with SLO

In some experiments, streptolysin O (SLO, Sigma-Aldrich Co Ltd, Poole, England) was used to permeabilize the cells. It was activated with dithiothreitol (5.6 mM per 1,000 HU of SLO), aliquoted at 6,250 HU/ml and frozen at $-20^{\circ}C$. A separate aliquot was thawed, kept on ice, and used during that day only.

When cells were permeabilized with SLO, the IC_{50} for ATP inhibition in the open-cell patch was not significantly different from that found for cells permeabilized with α -toxin. However, the rate of ATP block was slower for cells permeabilized with SLO, K_{ATP} channels ran down rapidly, and the cells were more fragile. Thus, for all subsequent experiments we used α -toxin to permeabilize cells. However, the fact that the IC_{50} for ATP inhibition was not significantly different between cells

permeabilized with SLO or with α -toxin suggests that a non-diffusible agent, or one with a molecular weight >150 kDa is responsible for the difference in the ATP sensitivity of the excised patch and intact cell.

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