ATP inhibition of K_{ATP} channels: control of nucleotide sensitivity by the N-terminal domain of the Kir6.2 subunit

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1. To gain insight into the role of the cytoplasmic regions of the Kir6.2 subunit in regulating channel activity, we have expressed the sulphonylurea receptor SUR1 with Kir6.2 subunits containing systematic truncations of the N- and C-termini. Up to 30 amino acids could be truncated from the N-terminus, and up to 36 amino acids from the C-terminus without loss of functional channels in co-expression with SUR1. Furthermore, Kir6.2ΔC25 and Kir6.2ΔC36 subunits expressed functional channels in the absence of SUR1.

2. In co-expression with SUR1, N-terminal truncations increased $K_{i,ATP}$ ([ATP] causing half-maximal inhibition of channel activity) by as much as 10-fold, accompanied by an increase in the ATP-insensitive open probability, whereas the C-terminal truncations did not affect the ATP sensitivity of co-expressed channels.

3. A mutation in the near C-terminal region, K185Q, reduced ATP sensitivity of co-expressed channels by approximately 30-fold, and on the Kir6.2ΔN2–30 background, this mutation decreased ATP sensitivity of co-expressed channels by approximately 400-fold.

4. Each of these mutations also reduced the sensitivity to inhibition by ADP, AMP and adenosine tetraphosphate.

5. The results can be quantitatively explained by assuming that the N-terminal deletions stabilize the ATP-independent open state, whereas the Kir6.2K185Q mutation may alter the stability of ATP binding. These two effects are energetically additive, causing the large reduction of ATP sensitivity in the double mutant channels.

ATP-sensitive potassium channels (K_{ATP}) are unique among K^+ channels in being rapidly and reversibly inhibited by the non-hydrolytic binding of cytoplasmic adenine nucleotides (Noma, 1983; Ashcroft, 1988; Nichols & Lederer, 1991). The realization that these channels are encoded by an ATP binding cassette (ABC) protein, the sulphonylurea receptor (SUR1 or SUR2), in addition to an inward rectifier K^+ channel subunit (Kir6.1 or Kir6.2; Aguilar-Bryan *et al.* 1995; Inagaki *et al.* 1995, 1996) led to the natural presumption that inhibitory ATP binding should occur at the nucleotide binding folds (NBFs) of the SUR subunit. Mutagenic studies have now demonstrated that the NBFs of SUR1 control activation of the channel by Mg^+-bound nucleotides and diazoxide, probably as a consequence of these agents mimicking, or enhancing, an effect of nucleotide hydrolysis (Nichols *et al.* 1996; Gribble *et al.* 1997; Shyng *et al.* 1997*b*). However, none of these studies has demonstrated an effect of SUR1 mutations on the intrinsic sensitivity of expressed channels to ATP inhibition. On the other hand, tandem dimeric constructs of SUR1 and Kir6.2 express channels with reduced ATP sensitivity (Clement *et al.* 1997; Shyng & Nichols, 1997), and, moreover, mutations of Kir6.2

can alter the inhibitory effect of ATP on channels formed by co-expression with SUR1 (Shyng *et al.* 1997*a*; Tucker *et al.* 1997, 1998). In the study of Shyng *et al.* (1997*a*), mutations of asparagine 160 in the second transmembrane segment alter sensitivity to polyamine-induced rectification, as well as decreasing the apparent ATP sensitivity approximately 5-fold. Tucker *et al.* (1997) reported that a C-terminally truncated Kir6.2 subunit (Kir6.2ΔC36) could express ATP-sensitive channels in the absence of SUR1, and reported a significant reduction of ATP sensitivity for Kir6.2ΔC36 channels with an additional mutation of lysine 185 in the truncated C-terminus. Lorenz *et al.* (1998) have confirmed these findings, and John *et al.* (1998), as well as Mikhailov *et al.* (1998), have most recently demonstrated that even full length Kir6.2 can form ATP-sensitive channels in the absence of SUR1. These results are fully consistent with the alternative hypothesis that ATP inhibits the channel through a direct interaction with the Kir6.2 subunit.

Shyng *et al.* (1997*a*) demonstrated a correlation between the shift in apparent K_i for ATP inhibition that results from mutations of amino acid 160 and changes in the channel open probability in the absence of ATP. This suggested that

the K_i shift might result from a change in the energetics of the ATP-independent states (i.e. altering the coupling between ATP binding and channel closure), rather than from a change in ATP binding itself. In the present study we report experiments designed to systematically examine the role of the N- and C-terminal regions of the Kir6.2 subunit in regulating channel activity and ATP sensitivity, by making serial truncations at each terminus. A preliminary report of these findings has been presented to the Biophysical Society (Koster *et al.* 1998).

METHODS

Construction of deletion mutants

Kir6.2 constructs containing truncations in either the C- or N-terminus were prepared by deletional mutagenesis using polymerase chain reaction (PCR). The Kir6.2K185Q point mutant was prepared by overlap extension at residue 185 by sequential PCR. Resulting PCR products were subcloned into the *Eco*RI–*Cla*I sites of pCMV6b and the encoded protein expressed under control of the cytomegalovirus (CMV) promoter. SUR1 was cloned into the pECE expression vector. The nucleotide sequences of all mutant Kir6.2 constructs were verified by fluorescence-based cycle sequencing using AmpliTaq DNA polymerase, FS (Perkin-Elmer, Foster City, CA, USA) and an ABI PRISM DNA sequencer (Perkin-Elmer).

Expression of K_{ATP} channels in COSm6 cells

COSm6 cells were plated at a density of approximately 2.5×10^5 cells per well (30 mm 6-well dishes) and cultured in Dulbecco's modified Eagle's medium plus 10 mM glucose (DMEM-HG), supplemented with fetal calf serum (FCS, 10%), penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}). The following day, cells were transfected by incubation for 4 h at 37 °C in DMEM-HG medium containing 10% Nu-Serum I (Collaborative Biomedical Products, Bedford, MA, USA), 0.4 μg ml^{-1} diethylaminoethyl-dextran, 100 μM chloroquine, and 5 μg each of pCMV6b-Kir6.2, pECE-SUR1 and pECE-GFP (green fluorescent protein) cDNA. Cells were subsequently incubated for 2 min in Hepes-buffered salt solution containing DMSO (10%), and returned to DMEM-HG plus 10% FCS and penicillin–streptomycin.

Rubidium flux experiments

^{86}RbCl (1 mCi ml^{-1}) was added in fresh growth medium 48 h after transfection. Cells were incubated for 24 h before assaying Rb$^+$ flux. For efflux measurements, cells were incubated for 30 min at 25 °C in Krebs–Ringer solution with metabolic inhibitors (2.5 μg ml^{-1} oligomycin plus 1 mM 2-deoxy-D-glucose). At selected time points, the solution was aspirated from the cells and replaced with fresh solution. The ^{86}Rb$^+$ in the aspirated solution was counted in a scintillation counter.

Patch-clamp measurements

Patch-clamp experiments were made at room temperature in an oil-gate chamber which allowed the solution bathing the exposed surface of the isolated patch to be changed in less than 50 ms (Lederer & Nichols, 1989). Micropipettes were pulled from thin-walled glass (WPI Inc., New Haven, CT, USA) on a horizontal puller (Sutter Instrument, Co., Novato, CA, USA). Electrode resistance was typically 0.5–1 MΩ when filled with K$_{int}$ solution (140 mM KCl, 10 mM K-Hepes, 1 mM K-EGTA, pH 7.3). Micro-electrodes were sealed onto cells that fluoresced green under UV illumination, by applying light suction to the rear of the pipette.

Membrane patches were voltage clamped with an Axopatch 1B patch-clamp (Axon Instruments). All currents were measured at a membrane potential of −50 mV (pipette voltage, +50 mV). Inward currents at this voltage are shown as positive-going signals. Data were normally filtered at 0.5–3 kHz, signals were digitized at 22 kHz (Neurocorder, Neurodata, NY, NY, USA) and stored on video tape. Experiments were replayed onto a chart recorder, or digitized into a microcomputer using Axotape software (Axon Instruments). The standard bath (intracellular) and pipette (extra-cellular) solution contained K$_{int}$, with the addition of [ATP] in the bath as indicated.

Data analysis

Off-line analysis was performed using Axotape and Microsoft Excel programs. Stationary fluctuation analysis of macroscopic currents (Neher & Stevens, 1977; Sigworth, 1980) was performed on short (< 1 s) recordings of currents at +50 mV following a step to zero [ATP] (over which time mean channel activity rundown, or inactivation, was negligible) and in 5–10 mM [ATP]. Currents (400 pA < mean current > 7 nA, corresponding to ~100–2000 channels) were filtered at f_c (cut-off frequency) of 3 kHz and digitized at 10 kHz with 16-bit amplitude resolution. Mean patch current (I) and variance (σ^2) in the absence of ATP were obtained by subtraction of mean patch current and variance of patch current in 5 or 10 mM ATP (all channels closed) from mean current and variance estimated in zero ATP. For the ATP-insensitive dual mutant channel, Kir6.2ΔN2–30/K185Q + SUR1, the baseline current was determined by irreversibly inhibiting channel activity with K$_{int}$ solution supplemented with 10 mM MgCl$_2$ and 2 mM CaCl$_2$. Single channel current (i) was assumed to be constant at 3.75 pA, corresponding to single channel conductance of 75 pS for Kir6.2wt, Kir6.2ΔN2–30, Kir6.2K185Q and Kir6.2ΔN2–30/K185Q mutant channels (data not shown) The mean open probability (P_o) was then calculated by fitting the following:

$$P_o = 1 - (\sigma^2/(iI)). \tag{1}$$

Model simulations of steady-state [ATP], [AMP], [ADP] and [adenosine tetraphosphate] dose–response relationships were generated using Microsoft Excel. Wherever possible, data are presented as the mean ± S.E.M. (standard error of the mean). Microsoft Solver was used to fit data by a least-squares algorithm. For calculation of standard free energy change the following equation was used:

$$\Delta G^{\circ\prime} = -2.303\, RT \log_{10} K'_{eq}, \tag{2}$$

where R represents the gas constant (1.98×10^{-3} kcal mol^{-1} deg^{-1}), T is absolute temperature (298 °K at 25 °C), and K'_{eq} the [ATP] of half-maximal inhibition.

RESULTS

Kir6.2 N-terminal deletions decrease ATP sensitivity of SUR1 co-expressed channels

In order to determine the role of different structural regions of Kir6.2 in inhibition of the K_{ATP} channel by ATP, the Kir6.2 subunit was systematically truncated at the N- and C-termini (Fig. 1*A*) and the effect of these truncations on the expressed channels was examined. Up to 30 amino acids could be truncated from the N-terminus without loss of functional channels when co-expressed with SUR1. However, truncations of 40 amino acids or beyond (Kir6.2ΔN40, Kir6.2ΔN50) did not generate functional channels. Figure 1*B* shows representative Rb$^+$ flux assays of

K_{ATP} channel activity from various N-terminal deletion constructs, co-expressed with SUR1. In all cases, metabolic inhibition activated a flux comparable to that seen with expression of wild-type K_{ATP} currents. Only a small Rb^{+} efflux was observed in untransfected cells under the same experimental conditions ($> 10\%$). The Kir6.2ΔN2–10, Kir6.2ΔN2–20 and Kir6.2ΔN2–30 mutants were functional when co-expressed with SUR1, although currents above background were not observed with Kir6.2ΔN2–40 (data not shown). As with wild-type Kir6.2, active channels were not observed from any of the N-terminal deletions when expressed alone, either by inside-out patch-clamp (data not shown, $n = 2$–4 patches) or by Rb^{+}-efflux analysis (Fig. 1B; for Kir6.2ΔN2–30). This is in contrast to C-terminal truncations (Kir6.2ΔC25 and Kir6.2ΔC36), which displayed K_{ATP} channel activity when expressed in the absence of SUR1 (data not shown) (cf. Tucker *et al.* 1997). These data indicate that the N-terminal 30 amino acids, unlike the C-terminus, are not involved in prohibiting functional expression of the wild-type Kir6.2 in the absence of SUR1. Interestingly, recent reports (John *et al.* 1998; Mikhailov *et al.* 1998) describe functional expression of full-length Kir6.2 in the absence of SUR1, with properties similar to Kir6.2ΔC25 channels alone. These observations may indicate that a population of K_{ATP} channels can normally form without SUR1 or simply that suppression of a normal

endoplasmic reticulum retention mechanism may result from overexpression of the Kir6.2 subunit under non-physiological conditions.

The response of N-terminal deletion constructs, Kir6.2-ΔN2–10 and Kir6.2ΔN2–30, to the application of cyto-plasmic ATP, in the absence of Mg^{2+} (see Lederer & Nichols, 1989) is shown for representative inside-out patches in Fig. 2. It is clear that the sensitivity to inhibition by ATP was decreased by N-terminal deletions. Mean data from all patches ($n = 5$–10 for each construct) are shown as dose–response relationships in Fig. 3.

Previous detailed analyses of ATP sensitivity indicate that multiple ATP molecules are likely to bind sequentially to the channel (Nichols *et al.* 1991). In this case the Hill equation does not describe such a kinetic scheme, and is thus useful only empirically. In this paper we are primarily concerned with relative sensitivities of different constructs and nucleotides. For ATP inhibition of wild-type channels, the Hill coefficient, n_H, and K_i were free to vary, and the obtained best fit ($n_H = 1 \cdot 3$) was then used to constrain subsequent fits to other constructs and nucleotides.

The Kir6.2ΔN2–30 channels were approximately 10-fold less sensitive to inhibition by ATP ($K_i = 124\ \mu$M) than wild-type channels ($K_i = 12\ \mu$M). Similarly, Kir6.2ΔN2–10 and Kir6.2ΔN2–20 patches displayed a 3-fold decrease in steady-state [ATP] dependence for both mutant channels ($K_i = 44$ and $36\ \mu$M, respectively), consistent with residues
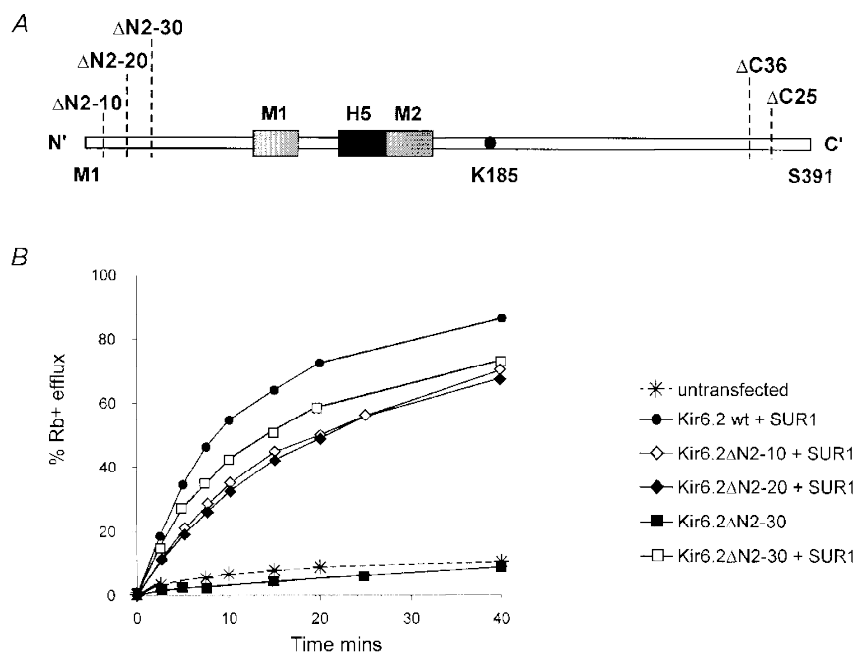

Figure 1. $^{86}Rb^{+}$ **efflux from untransfected COSm6 cells and cells expressing truncated Kir6.2 subunits alone or with SUR1**

A, schematic diagram of the Kir6.2 subunit illustrating the regions in the cytoplasmic C- and N-termini that were deleted or mutated. The Kir6.2 subunit contains two transmembrane segments, M1 and M2, a pore-forming H5 segment, and cytoplasmic N- and C-termini. A lysine residue at position 185 has previously been implicated in inhibition of the K_{ATP} channel by ATP (Tucker *et al.* 1997). *B*, percentage efflux of $^{86}Rb^{+}$ as a function of time in the presence of metabolic inhibitors (see Methods) for wild-type Kir6.2 and N-terminal deletion constructs, with or without SUR1 as indicated. All transfections were done in parallel. Graph shows results from a typical experiment (repeated 2–3 times for each construct).
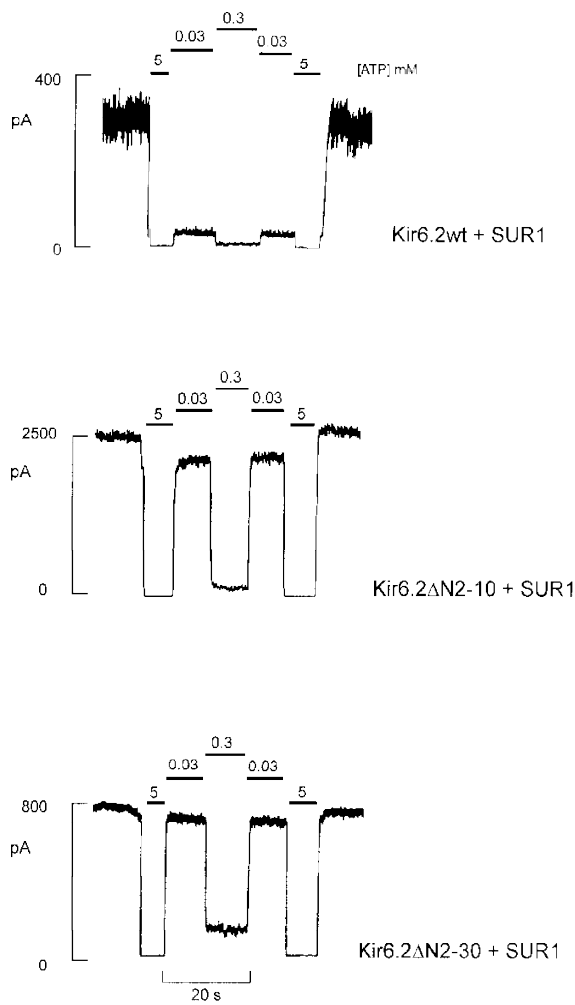
Figure 2. Currents from cells co-expressing N-terminal truncated Kir6.2 subunits and SUR1

Representative currents recorded from inside-out membrane patches containing wild-type or mutant K_{ATP} channels at -50 mV in K_{int} solution (see Methods). Patches were exposed to differing [ATP] as indicated.

2–10 and 20–30 being major determinants of the N-terminal regulation of ATP sensitivity.

Kir6.2 C-terminal deletions do not affect ATP sensitivity of SUR1 co-expressed channels

Systematic C-terminal deletions in Kir6.2 were generated in order to examine control of ATP sensitivity by this region of the channel. In co-expression with SUR1, deletions of up to 36 amino acids were tolerated, consistent with the previous report of Tucker *et al.* (1997). Figure 4*B* shows ATP sensitivity of currents through Kir6.2ΔC25 channels expressed alone or co-expressed with SUR1. Similar to a previous report (Tucker *et al.* 1997), the apparent ATP sensitivity of Kir6.2ΔC25 channels alone was decreased approximately 10-fold, as compared with the wild-type
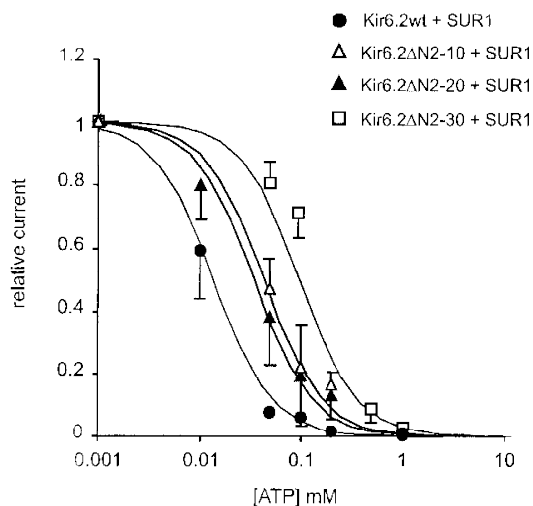

Figure 3. [ATP] dependence of N-terminal truncated K_{ATP} mutant channels

Steady-state dependence of membrane current (mean ± S.E.M. relative to current in zero ATP) on [ATP] for wild-type and mutant channels (from records such as those shown in Fig. 2). Data points represent the mean ± S.E.M. ($n = 3$–8 patches). The fitted lines correspond to least-squares fit of the Hill equation (relative current = $100/(1 + ([ATP]/K_i)^{n_H})$, with n_H fixed at 1·3, and $K_i = 12$ μM (wild-type), 44 μM (Kir6.2ΔN2–10), 36 μM (Kir6.2ΔN2–20 + SUR1), and 124 μM (Kir6.2ΔN2–30 + SUR1).

channel ($K_i = 12$ μM for wild-type, 125 μM for Kir6.2ΔC25 alone). However, ATP sensitivity could be restored to the wild-type level by co-expressing Kir6.2ΔC25 with SUR1 ($K_i = 14$ μM, Fig. 4B). The Kir6.2ΔC25 truncation was also introduced on the Kir6.2ΔN2–30 background. Figure 4A and B shows current densities and ATP sensitivity of currents through Kir6.2ΔN2–30ΔC25 channels expressed alone or co-expressed with SUR1. Consistent with a role for the N-terminus, but not the C-terminus, in controlling ATP sensitivity, the C-terminal truncation did not decrease ATP sensitivity further over the level observed for Kir6.2-ΔN2–30 + SUR1 channels (Kir6.2ΔC25ΔN2–30 + SUR1, $K_i = 94$ μM). Moreover, when expressed in the absence of

SUR1, the double deletion also did not decrease ATP sensitivity further over the level observed for Kir6.2ΔC25 alone (Kir6.2ΔC25ΔN2–30, $K_i = 117$ μM, Fig. 4).

The N-terminus and lysine 185 of Kir6.2 make energetically additive contributions to ATP sensitivity

Tucker *et al.* (1997) demonstrated that a neutralization mutation in the near C-terminal region of Kir6.2 (K185Q) significantly reduced the ATP sensitivity of Kir6.2ΔC26 channels expressed in the absence of SUR1. It was suggested that this residue may represent a binding site for inhibitory ATP by co-ordinating and neutralizing the negatively charged phosphate group(s) of the inhibitory molecule. We
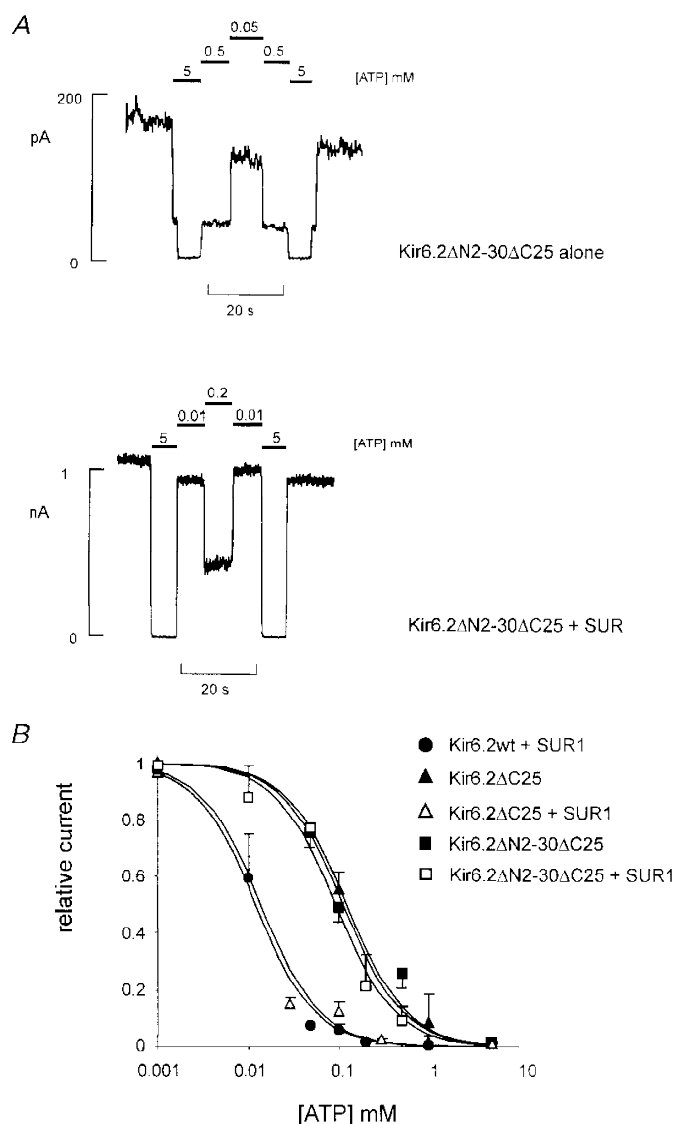

Figure 4. [ATP] dependence of Kir6.2ΔN2–30ΔC25 channel expressed alone or with SUR1

A, representative currents (from $n = 3$–5 patches) recorded from inside-out membrane patches containing Kir6.2ΔN2–30ΔC25 channels alone (above), or with SUR1 (below) at -50 mV in K_{int} solution (see Methods). Patches were exposed to differing [ATP] as shown. *B*, steady-state dependence of membrane current relative to current in zero ATP (mean $\pm$ s.e.m., $n = 3$–5 patches for each construct) on [ATP] for wild-type and Kir6.2ΔC25 mutant channels (as indicated). The data were fitted as described in Fig. 3. $K_i = 12$ μM (wild-type), 128 μM (Kir6.2ΔC25 alone), $14{\cdot}0$ μM (Kir6.2ΔC25 + SUR1), 117 μM (Kir6.2-ΔN2–30ΔC25 alone) and 94 μM (Kir6.2ΔN2–30ΔC25 + SUR1).

introduced the K185Q mutation into the wild-type Kir6.2 and C- and N-terminal truncations and co-expressed each mutant with SUR1. As shown in Fig. 5, Kir6.2K185Q + SUR1 channels in inside-out patches are considerably less sensitive to inhibition by ATP than wild-type channels. The [ATP] dose–response relationship compiled from all patches was fitted by a K_i of 308 μM, representing an ~30-fold decrease of ATP sensitivity compared with wild-type Kir6.2 + SUR1 channels (Fig. 6A). As with the N-terminal truncations, introduction of the C-terminal truncation on the K185Q background did not cause any further reduction of the ATP sensitivity, with Kir6.2ΔC25/K185Q + SUR1 channels exhibiting a K_i of 304 μM (Fig. 6A). Thus alteration of ATP sensitivity by both N-terminal and K185Q mutations is unaffected by introduction of these mutations onto the ΔC25 background. These results strengthen the conclusion that, while C-terminal truncation permits or enhances expression of K_{ATP} channels in the absence of SUR1, it does not alter the ATP sensitivity of expressed channels.

As shown in Figs 5 and 6, however, combining the N-terminal deletion with the K185Q mutation has a significant, and additive, effect on apparent ATP sensitivity. The combination mutant channel Kir6.2ΔN2–30/K185Q +

SUR1 is highly insensitive to inhibition by ATP with an estimated K_i of 4·9 mM (compared with a K_i of 124 and 308 μM for ΔN2–30 and K185Q mutants, respectively; Fig. 6A). This represents an ~400-fold reduction in apparent ATP sensitivity over wild-type channels and indicates that the N-terminal deletion and the K185Q mutation are energetically additive in terms of their effect on ATP sensitivity. Each mutation alone corresponds to a reduction of 5·3 and 7·4 kJ mol^{-1} in apparent association energy for ATP (Kir6.2ΔN2–30 and Kir6.2K185Q, respectively), whereas the paired mutant is reduced by 13·6 kJ mol^{-1} compared with wild-type channels (see eqn (2) in Methods).

The open state is stabilized in the Kir6.2ΔN2–30 mutant

The data presented thus far are consistent with lysine 185 in the near C-terminus comprising part of the binding site for inhibitory ATP. However, an alternative explanation seems to underlie the decrease in sensitivity of Kir6.2-ΔN2–30 channels. As previously reported, mutations of asparagine 160 in the second transmembrane segment of Kir6.2 (Shyng et al. 1997a) change the open probability of the channel in the absence of ATP, and stabilization of an open state, away from ATP-bound closed states, was postulated to account for the decreased apparent ATP
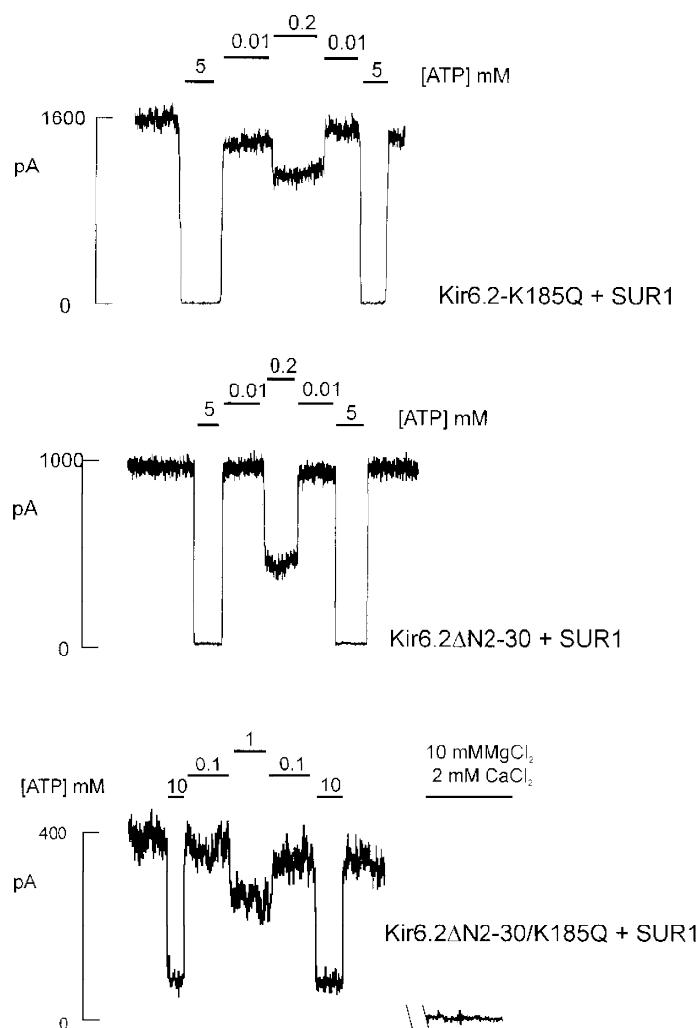

Figure 5. Representative current traces from cells co-expressing Kir6.2ΔN2–30, Kir6.2K185Q or Kir6.2ΔN2–30/K185Q mutant subunits and SUR1

Currents recorded from inside-out membrane patches containing wild-type or mutant K_{ATP} channels at −50 mV in K_{int} solution (see Methods). Patches were exposed to differing [ATP] as shown. For the ATP-insensitive dual mutant channel Kir6.2ΔN2–30/K185Q + SUR1 the baseline current was determined by subsequent irreversible inhibition of channel activity with 10 mM MgCl$_2$ and 2 mM CaCl$_2$ (see Methods).

sensitivity of the mutant channels. The single channel conductance of Kir6.2ΔN2–30 channels is the same as wild-type (75 pS, data not shown), but as shown in Fig. 7, the channel open probability in the absence of ATP ($P_{o,max}$) is considerably higher for Kir6.2ΔN2–30 channels than for wild-type channels. Whereas, in the two records shown, wild-type channels show brief bursts of openings separated by longer closed events ($\tau = 72$ ms, Fig. 7B), ΔN2–30 channels are well described by continuous bursting, without the longer interburst closed times. Similar results were seen in two other wild-type single channel patches, and one other ΔN2–30 channel patch. In order to obtain statistical significance, we estimated $P_{o,max}$ using noise analysis. In good agreement with single channel patches, $P_{o,max} \approx 0\cdot8$ ($n = 5$, Fig. 6B) for Kir6.2ΔN2–30 channels is considerably higher than for wild-type channels ($P_{o,max} \approx 0\cdot5$, $n = 7$; Fig. 6B). In contrast, the open probability of the

Kir6.2K185Q mutant channel, in the absence of ATP, is not significantly different from the parental wild-type channel (Fig. 6B). Additionally, the double mutant channel, Kir6.2ΔN2–30/K185Q, exhibited an open probability similar to Kir6.2ΔN2–30 channels. As discussed in detail below, stabilization of the open state, relative to a closed state, in Kir6.2ΔN2–30 channels can account for the difference in ATP sensitivity of the deletion mutants relative to the wild-type channel as well as the energetically additive effects of the N-terminal deletion and the K185Q point mutation.

Sensitivity to other adenosine nucleotides parallels alteration of ATP sensitivity

To define the altered nucleotide sensitivity of the mutant K_{ATP} channels further, the effect of additional adenosine nucleotides on channel activity was examined. Reducing the
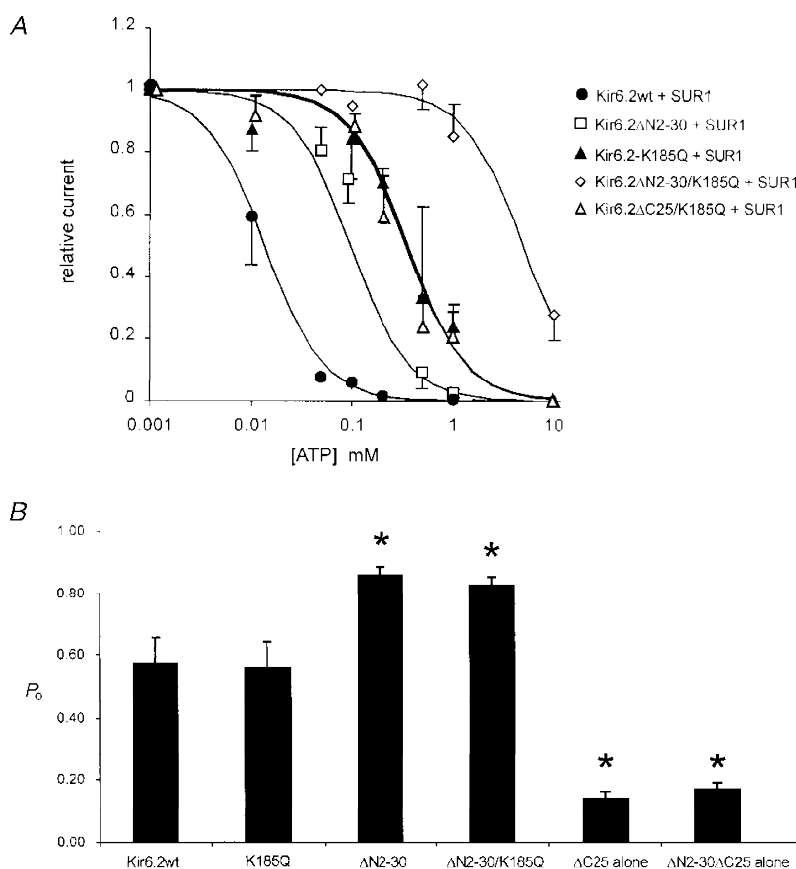

Figure 6. ATP dose–response relationship and mean peak open probability of N-terminal deletion and K185Q mutant channels

A, steady-state dependence of membrane current (relative to current in zero ATP) on [ATP] for wild-type and mutant channels (as indicated) co-expressed with SUR1. Data points represent the mean $\pm$ s.e.m. ($n = 3$–8 patches). The data were fitted as described in Fig. 3. $K_i = 12$ μM (wild-type), 124 μM (Kir6.2ΔN2–30 + SUR1), 308 μM (Kir6.2K185Q + SUR1), 304 μM (Kir6.2ΔC25/K185Q + SUR1), and 4·9 mM (Kir6.2ΔN2–30/K185Q + SUR1). B, mean peak open probability (P_o) of mutant Kir6.2 subunits was measured in the absence of inhibitory ATP by steady-state noise analysis using eqn (1) (see Methods). Asterisks denote statistical difference between wild-type $P_{o,max}$ and mutant channel $P_{o,max}$ using Student's unpaired t test (*$P < 0\cdot05$). Peak open probabilities (P_o) were as follows ($n = 4$–7 patches; $\pm$ s.e.m.): Kir6.2wt $= 0\cdot57 \pm 0\cdot08$; K185Q $= 0\cdot56 \pm 0\cdot08$; ΔN2–30 $= 0\cdot86 \pm 0\cdot02$; ΔN2–30/K185Q $= 0\cdot83 \pm 0\cdot03$; ΔC25 alone $= 0\cdot14 \pm 0\cdot02$; ΔN2–30/ΔC25 alone $= 0\cdot17 \pm 0\cdot02$.

number of phosphate groups from three to two (ADP) or one (AMP), markedly reduces the inhibitory effect on native K_{ATP} channels (Noma, 1983; Ashcroft, 1988; Nichols & Lederer, 1991), and this is true for reconstituted and mutant K_{ATP} channels (Fig. 8). Wild-type channels are ~10-fold less sensitive to ADP than ATP ($K_i = 144$ and $12\,\mu M$, respectively) and ~140-fold less sensitive to AMP ($K_i = 1\cdot8$ mM). The data are summarized in Fig. 9 for inhibition of mutant channels by ATP, ADP and adenosine tetra-phosphate (AtetP). As can be seen, qualitatively similar differences are observed for the sensitivity of the mutant K_{ATP} channels, although the sensitivities to each nucleotide begin to converge for the K185Q and double-mutant channels, consistent with the K185Q mutation reducing the discrimination between adenosine nucleotides. Wild-type and mutant channels are almost insensitive to GTP (data not shown), and sensitivities to AtetP are similar to ATP sensitivities for each channel.

DISCUSSION

Control of nucleotide sensitivity by the Kir6.2 subunit

The pore-forming Kir6.2 subunit of the K_{ATP} channel consists of two transmembrane segments, M1 and M2, with an extracellular P-loop, and cytoplasmic N- and C-termini.

While previous studies have implicated the pore-lining M2 segment in controlling the gating and rectification properties of the channel (Shyng et al. 1997a), little is known of the role of the cytoplasmic domains, particularly the N-terminus, in channel activity. The present study demonstrates that Kir6.2 subunits with deletions of up to 30 to 40 amino acids from either the cytoplasmic N- or C-termini can be co-expressed with SUR1 to generate functional ATP-sensitive K^+ channels. Moreover, deletions of 25 or 36 amino acids from the C-terminus (cf. Tucker et al. 1997), but not the N-terminus, lead to the generation of significant ATP-sensitive K^+ channel current in the absence of SUR1. These data confirm a role of the C-terminus, and not the N-terminus, in preventing expression of channel activity from Kir6.2 subunits in the absence of co-expressed SUR1. Deletions of the N-terminus result in up to a 10-fold reduction in ATP sensitivity, the critical amino acids being in residues 2–10 and 20–30, associated with a significant increase in open probability in the absence of ATP. In contrast, even though deletions of the C-terminus permit expression of functional channels without SUR1, the ATP sensitivity of co-expressed channels is indistinguishable from the sensitivity of wild-type channels, suggesting that the C-terminal 36 amino acids are not involved in controlling intrinsic ATP sensitivity. Consistent with a previous report

Figure 7. Kir6.2ΔN2–30 channels have a higher open probability than wild-type channels

A, representative single channel currents recorded from cells expressing wild-type (Kir6.2 + SUR1) or Kir6.2ΔN2–30 + SUR1 channels, $f_c = 1$ kHz. c and o indicate the closed and open channel levels. B, unconditional open and closed time distributions from the records shown in A, using log binning. Open times were well fitted by single exponential distributions for both wild-type ($T_O = 9\cdot5$ ms) and ΔN2–30 ($T_O = 11\cdot9$ ms). Two time constants ($T_{C1} = 5\cdot9$ ms, relative fraction = 0·6; $T_{C2} = 71\cdot9$ ms, relative fraction = 0·4) were necessary to describe wild-type closed distributions, but a single time constant ($T_{C1} = 4\cdot0$ ms) was sufficient to describe ΔN2–30 channel closed time distribution.
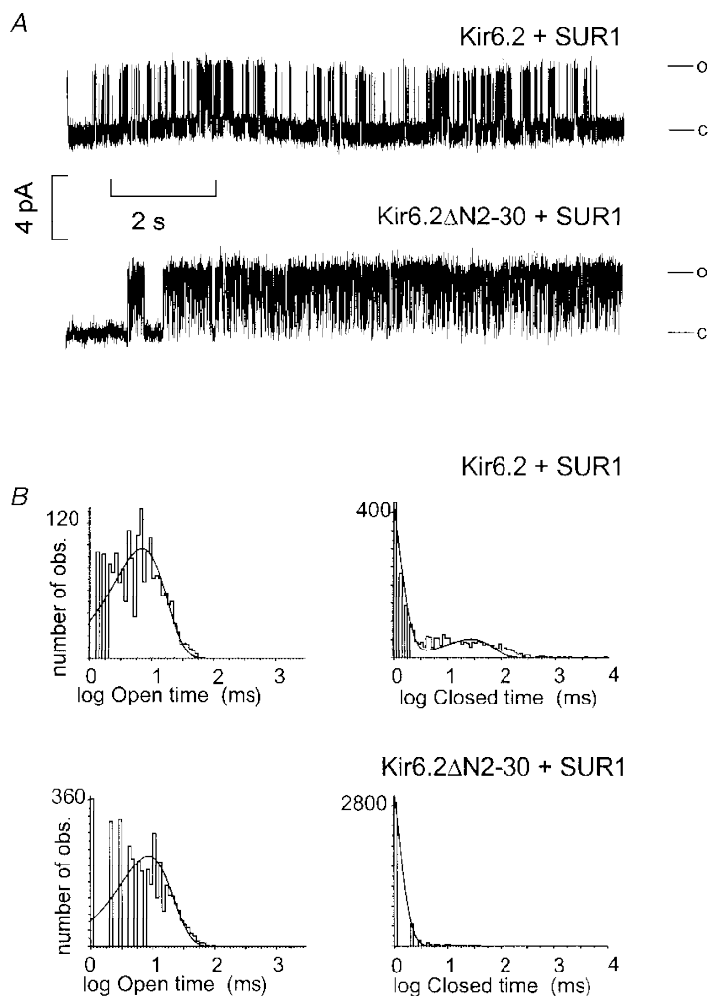
(Tucker *et al.* 1997), lysine 185 in the proximal C-terminus beyond the second transmembrane region (M2), when mutated to a glutamine, causes an ~30-fold reduction in ATP sensitivity, though, unlike the Kir6.2ΔN2–30 channels, this mutation is not associated with a change in open probability (see below).

The cloning of the SUR1 subunit (Aguilar-Bryan *et al.* 1995), and demonstration that it represents a requisite component of the functional K_{ATP} channel complex (Inagaki *et al.* 1995) suggested the obvious possibility that ATP inhibition, the defining property of these channels, results from an interaction of Mg^+-free ATP with the nucleotide

binding folds (NBF) of the SUR1 subunit. Consistent with this possibility was the further demonstration that co-expression of Kir6.2 with SUR2A generated channels with a slightly lower K_i for ATP than co-expression with SUR1 (Inagaki *et al.* 1996). However, several studies have now demonstrated that mutations of the nucleotide binding folds, while changing or abolishing channel activation by Mg-ADP, MgATP and potassium channel openers (KCO), do not alter the intrinsic sensitivity to inhibition by ATP (Nichols *et al.* 1996; Gribble *et al.* 1997, 1998; Shyng *et al.* 1997a). In contrast, mutations of both pore-forming, and cytoplasmic residues of the Kir6.2 subunit have been shown
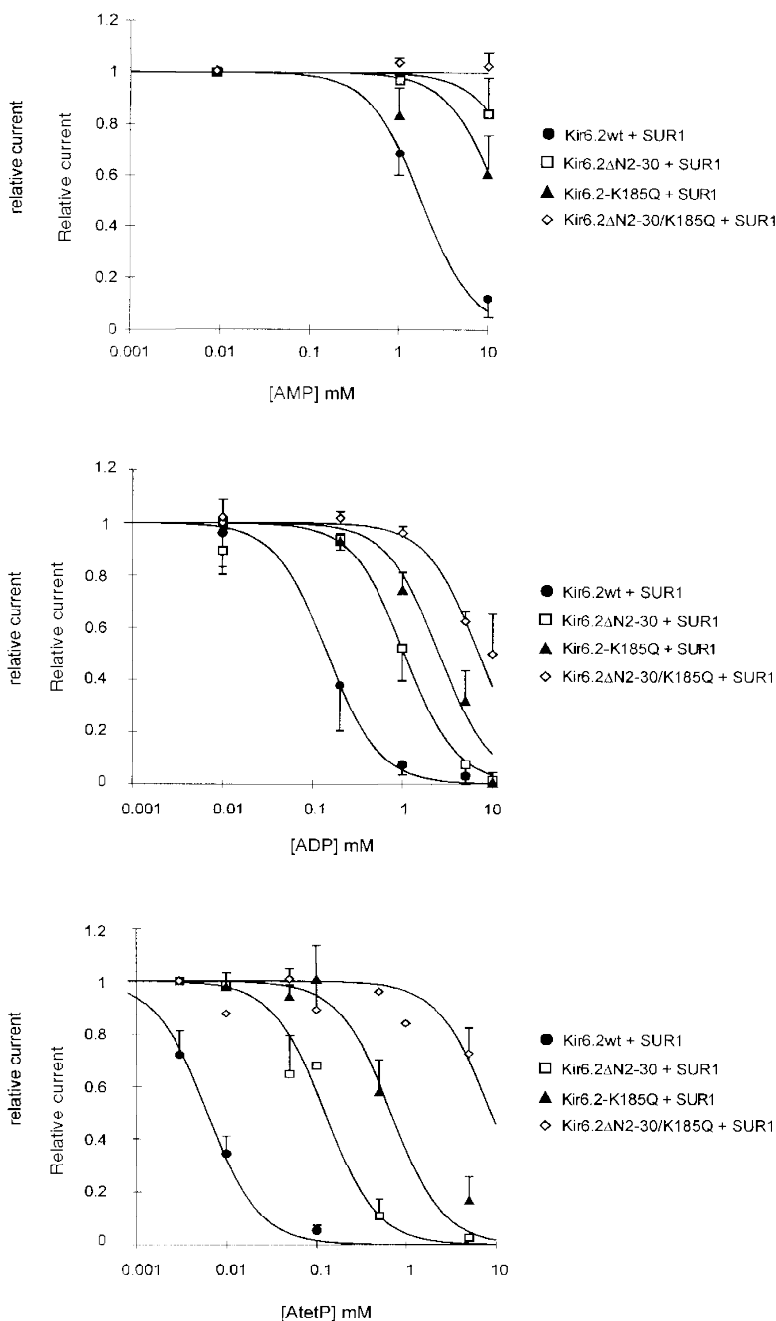

Figure 8. Steady-state dependence of mutant K_{ATP} channel activities on [AMP], [ADP] and [adenosine tetra-phosphate]

Dependence of membrane current (relative to current in zero adenosine nucleotide) on [adenosine nucleotides] for wild-type and mutant channels (as indicated). Data points represent the mean ± S.E.M. ($n = 3$–10 patches). The data were fitted as described in Fig. 3. K_i for AMP = 1·8 mM (wild-type), 31·9 mM (Kir6.2ΔN2–30 + SUR1), and 13·6 mM (Kir6.2K185Q + SUR1). Not determined for Kir6.2ΔN2–30/K185Q + SUR1. K_i for ADP = 144 μM (wild-type), 1·1 mM (Kir6.2ΔN2–30 + SUR1), 2·5 mM (Kir6.2K185Q + SUR1), and 7·1 mM (Kir6.2ΔN2–30/K185Q + SUR1). K_i for adenosine tetraphosphate = 6 μM (wild-type), 124 μM (Kir6.2ΔN2–30 + SUR1), 680 μM (Kir6.2K185Q + SUR1), and 8·8 mM (Kir6.2ΔN2–30/K185Q + SUR1).

to significantly alter the intrinsic ATP sensitivity of co-expressed channels (Shyng *et al.* 1997*b*; Tucker *et al.* 1997, 1998).

We have demonstrated previously that mutations of residue 160, within the pore-forming M2 segment, alter the ATP sensitivity of expressed mutant channels, by altering the relative stability of the open channel (Shyng *et al.* 1997*b*). According to the proposed model, Kir6.2N160X mutant channels with increased peak open probabilities would spend less time in distant ATP-bound closed states, resulting in an apparent decrease in ATP affinity. A similar mechanism seems to be responsible for the decrease in the K_i for ATP that results from N-terminal truncation. Figure 10*A* shows a simplified version of a kinetic model initially proposed by Nichols *et al.* (1991) and modified to explain the N160 mutational effects (for details see Shyng *et al.* 1997*b*). This model satisfactorily accounts for the following, generally observed, kinetic features of K_{ATP} channels: (1) increase of inter-burst closed times with increasing [ATP]; (2) lack of dependence of intra-burst closed and open times on [ATP]; and (3) steady-state dependence of channel on [ATP] that is steeper than a Hill coefficient of 1. The model does not explicitly provide an explanation for the stimulatory actions of diazoxide or Mg-ADP, acting through interactions with SUR1, and at this juncture there is no unifying kinetic model to explain all such data. For Kir6.2ΔN2–30 + SUR1 mutant channels, both the decreased steady-state [ATP] dependence and increased peak open probability can be explained by a shift in the coupling transition (K_{CO}) between the ATP-unbound closed state (C) and the open state (O) (Fig. 10*C*). In this case the ATP binding constant (K_A) remains unaltered. In contrast, for Kir6.2K185Q + SUR1 channels a shift in the ATP-binding constant alone (K_A) can explain the decrease in apparent ATP affinity, with unmodified peak open probability. Finally, the striking effect of the double mutant Kir6.2ΔN2–30/K185Q on ATP sensitivity of co-expressed channels, in which the K_i for ATP is reduced 400-fold, is then explained by the additive effect of the two mutations, acting at different steps in the linearly coupled kinetic process (K_{CO} and K_A). As shown in

Fig. 10*B*, the proposed model accurately simulates the kinetic behaviour of both the wild-type and mutant K_{ATP} channels with respect to observed peak open probability and ATP sensitivity.

One goal of these studies is to understand the physical basis of gating, and the question arises as to how the N-terminus, which is not directly connected to the pore of the channel, is able to control open probability. Analysis of control of channel activity by the SUR1 subunit (Shyng *et al.* 1997*b*), led us to hypothesize that SUR1 exerts a hypersensitizing effect on the ATP inhibition of Kir6.2 channel activity that reduces the effective K_i from around 100 μM (as seen with Kir6.2ΔC25 expressed without SUR1) to around 10 μM. This 10-fold increase of ATP sensitivity could result from a shift in the coupling between ATP binding and the open state, in which the interaction of SUR1 destabilizes the open state relative to the ATP accessible closed state (i.e. a reduction in K_{CO} in the model of Fig. 10).

In light of the realization that the expression of Kir6.2 in the absence of SUR1 results in channels which actually have a considerably lower $P_{o,max}$ (Trapp *et al.* 1998; John *et al.* 1998), we are forced to suggest that expression of Kir6 in the absence of SUR has an additional effect that results in the lowering of P_o to much lower values (~0·1), by an effect besides that of a simple shift in the equilibrium between the open state and ATP accessible states, i.e. there is an additional lowering of the open-state stability that may be unrelated to transitions to ATP-accessible closed states. Given the clearly intimate physical interaction between SUR and Kir6 subunits (Clement *et al.* 1997), such additional kinetic effects may not be surprising.

That the N-terminus of Kir6.2 might be involved in transducing this effect is suggested by the subsequent observation that tethering the N-terminus of Kir6.2 to the C-terminus of SUR1 may also serve to uncouple the hypersensitizing action, the ATP sensitivity of channels formed from SUR1-Kir6.2 dimers being reduced 2- to 8-fold (Shyng & Nichols, 1997; Clement *et al.* 1997; Inagaki *et al.* 1997). Thus we might envisage that the normal 'role' of the N-terminus is to destabilize the open state under the influence of SUR1, and that this action is blocked by physical
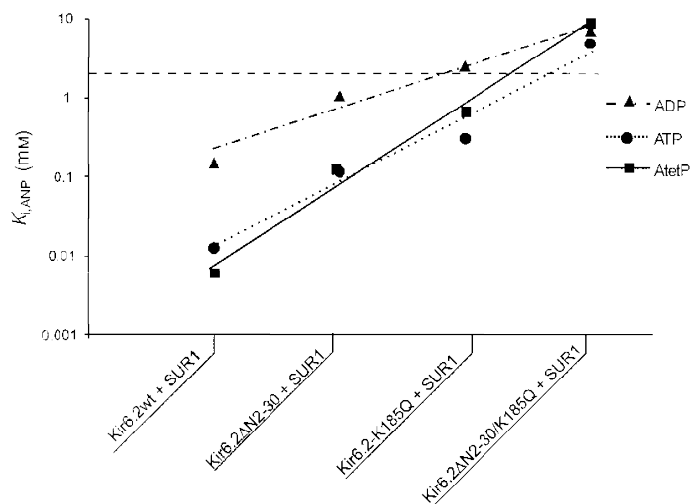

Figure 9. Relative sensitivity of mutant K_{ATP} channels to inhibition by adenosine nucleotides
Calculated K_i for ATP, ADP and AtetP from Fig. 8 for mutant K_{ATP} channels. The dashed line above 3 mM indicates unreliability of estimated parameters. The lines connect values predicted by adjustments to the model of Fig. 10 (see text for details).

tethering of the N-terminus to SUR1, and is therefore absent both after deletion of the Kir6.2 N-terminus, or upon expression of Kir6.2 without SUR1. This would explain the lack of additivity of the effect of N- and C-terminal deletions expressed without SUR1 (Figs 4 and 6*B*).

Reduced sensitivity to other adenosine nucleotides

As shown in Fig. 8, qualitative discrimination between adenosine nucleotides is maintained for both N-terminal truncations and for the K185Q mutation. Given the inability to examine the effects of nucleotide concentrations greater than about 10 mM (due to effects on free [K$^+$]), estimated K_i becomes unreliable above $\sim$3 mM. If conclusions above are justified ((1) that N-terminal deletions affect open probability; (2) that N-terminal deletions and K185Q mutant effects are independent and additive) then appropriate decreases of the binding affinity of the wild-type channel for ADP (by a factor of 1/12), and AMP (by a factor of $1/12^2 = 1/144$) should also predict the affinity of ΔN2–30 channels for these additional nucleotides. Figure 9 shows that appropriate changes of K_A (the nucleotide binding constant) lead to reasonable predictions of the observed sensitivity (K_i) of wild-type and mutant channels to different nucleotides.

Consistent with the K185Q mutation acting by reducing the intrinsic binding affinity for nucleotide phosphates, similar, although quantitatively smaller, reductions of binding affinity for ADP (by a factor of 1/5), and for AMP (by a factor of $1/5^2$, i.e. 1/25) reasonably predict not only the sensitivity of K185Q mutant channels to these nucleotides, but also the double mutant channels (Fig. 9).

The data are consistent with K185 forming part of the inhibitory ATP binding site, but this remains a point of speculation. The Kir6.2 subunit does not contain a consensus ATP-binding motif (Inagaki *et al.* 1995), and there is thus far no evidence for ATP binding to Kir6.2. In agreement with a preliminary study by Babenko & Bryan, (1998), we observe strong labelling of SUR1, but not Kir6.2, with a photo-affinity derivative of ATP, 8-azido-ATP (J. C. Koster & C. G. Nichols, unpublished data).

In conclusion, the present results demonstrate that truncations of the N- and C-termini of the Kir6.2 subunits can generate K_{ATP} channels with novel properties. Deletions of the C-terminus, while not affecting the intrinsic channel ATP sensitivity, allow the generation of functional channels in the absence of SUR1, confirming the results of Tucker *et al.* (1997). Deletions of the N-terminus, though not generating functional channels without SUR1, nevertheless

Figure 10. Model simulation of the observed kinetic behaviour of Kir6.2wt, Kir6.2ΔN2–30, Kir6.2K185Q, and Kir6.2ΔN2–30/K185Q mutant channels

A, model scheme for simulation of K_{ATP} channel activity (see Shyng *et al.* 1997*a*). Only two sequential ATP binding steps are necessary to approximate the steepness of steady-state dependence of channel activity on [ATP]. C$'$ refers to the rapid closed state observed within a burst. Equilibrium constants for each transition are indicated. *B*, model predicted K_i [ATP] and $P_{o,max}$ plotted against measured K_i [ATP] and $P_{o,max}$, respectively (see Fig. 6*A* and *B*). *C*, table showing the model parameters for equilibrium constants (K_{CO} and K_f) and the ATP binding constant (K_A). Only the boxed constants were altered to produce the necessary simulations of mutant channel activity.

	K_A	K_{CO}	K_f
wt	10	2	0.15
ΔN2-30	10	150	0.15
K185Q	240	2	0.15
ΔN30/ K185Q	240	150	0.15
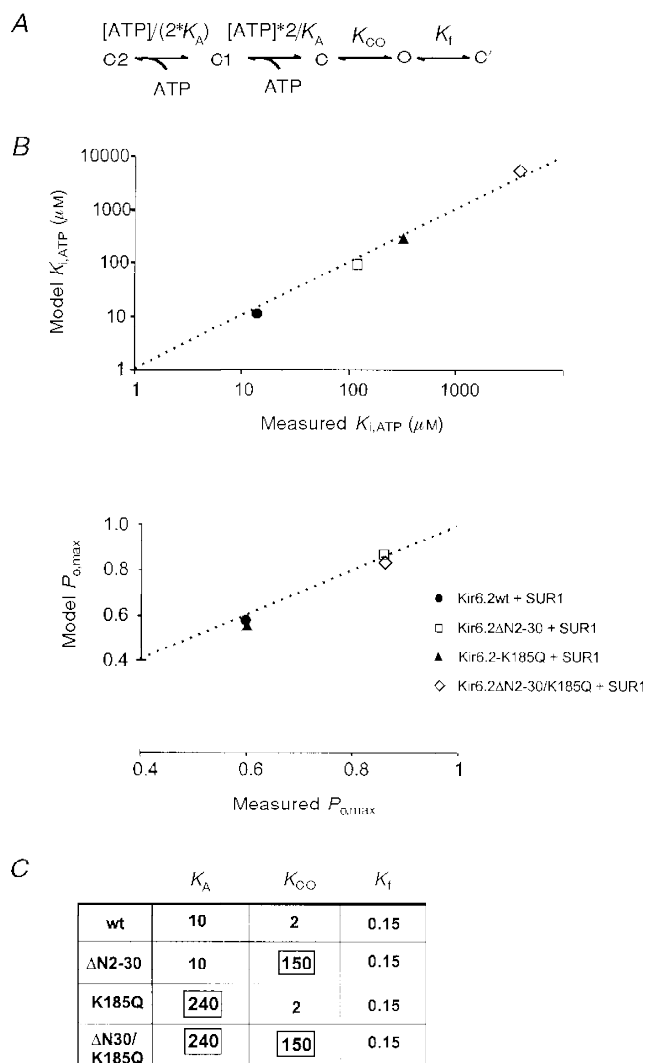
stabilize the open channel, leading to a reduction of apparent ATP sensitivity in the presence of SUR1. This leads us to suggest that the N-terminus may function to transduce a destabilizing effect of SUR1 on the open state. A mutation in the near C-terminal region (K185Q) reduced the ATP sensitivity of SUR1-co-expressed channels without changing peak open probability, consistent with a reduction of ATP binding affinity. Finally, a dual mutant channel containing both the N-terminal truncation and lysine 185 mutation has very low ATP sensitivity, resulting from the energetically additive effects of open-state stabilization and reduction of apparent ATP affinity.

AGUILAR BRYAN, L., NICHOLS, C. G., WECHSLER, S. W., CLEMENT, J. P. IV, BOYD, A. E. III, GONZALEZ, G., HERRERA SOSA, H., NGUY, K., BRYAN, J. & NELSON, D. A. (1995). The β cell high affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* **268**, 423–426.

ASHCROFT, F. M. (1988). Adenosine 5′-triphosphate-sensitive potassium channels. *Annual Review of Neuroscience* **11**, 97–118.

BABENKO, A. P. & BRYAN, J. (1998). What have we learned from C-terminus truncated Kir6.2 channels? *Biophysical Journal* **74**, A114.

CLEMENT, J. P. IV, KUNJILWAR, K., GONZALEZ, G., SCHWANSTECHER, M., PANTEN, U., AGUILAR-BRYAN, L. & BRYAN, J. (1997). Association and stoichiometry of K_{ATP} channel subunits. *Neuron* **18**, 827–838.

GRIBBLE, F. M., TUCKER, S. J. & ASHCROFT, F. M. (1997). The essential role of the Walker A motifs of SUR1 in K-ATP channel activation by Mg-ADP and diazoxide. *EMBO Journal* **16**, 1145–1152.

GRIBBLE, F. M., TUCKER, S. J., HAUG, T. & ASHCROFT, F. M. (1998). MgATP activates the beta cell K-ATP channel by interaction with its SUR1 subunit. *Proceedings of the National Academy of Sciences of the USA* **95**, 7185–7190.

INAGAKI, N., GONOI, T., CLEMENT, J. P. IV, NAMBA, N., INAZAWA, J., GONZALES, G., AGUILAR-BRYAN, L., SEINO, S. & BRYAN, J. (1995). Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor. *Science* **270**, 1166–1170.

INAGAKI, N., GONOI, T., CLEMENT, J. P. IV, WANG, C. Z., AGUILAR-BRYAN, L., BRYAN, J. & SEINO, S. (1996). A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K^+ channels. *Neuron* **16**, 1011–1017.

INAGAKI, N., GONOI, T. & SEINO, S. (1997). Subunit stoichiometry of the pancreatic β-cell ATP-sensitive K^+ channel. *FEBS Letters* **409**, 232–236.

JOHN, S. A., MONCK, J. R., WEISS, J. N. & RIBALET, B. (1998). The sulfonylurea receptor SUR1 regulates ATP-sensitive mouse Kir6.2 K^+ channels linked to green fluorescent protein in human embryonic kidney cells (HEK 293). *Journal of Physiology* **510**, 333–345.

KOSTER, J. C., SHYNG, S.-L., SHA, Q. & NICHOLS, C. G. (1998). Involvement of the N-terminus of Kir6.2 in regulating ATP-sensitivity of K_{ATP} channels. *Biophysical Journal* **74**, A230.

LEDERER, W. J. & NICHOLS, C. G. (1989). Nucleotide modulation of the activity of rat heart K_{ATP} channels in isolated membrane patches. *Journal of Physiology* **419**, 193–211.

LORENZ, E., ALEKSEEV, A. E., KRAPIVINSKY, G. B., CARRASCO, A., CLAPHAM, D. E. & TERZIC, A. (1998). Evidence for direct physical association between a K^+ channel (kir6.2) and an ATP-binding cassette protein (SUR1) which affects cellular distribution and kinetic behavior of an ATP-sensitive K^+ channel. *Molecular and Cellular Biology* **18**, 1652–1659.

MIKHAILOV, M. V., PROKS, P., ASHCROFT, F. M. & ASHCROFT, S. J. (1998). Expression of functionally active ATP-sensitive K-channels in insect cells using baculovirus. *FEBS Letters* **429**, 390–394.

NEHER, E. & STEVENS, C. (1977). Conductance fluctuations and ionic pores in membranes. *Annual Review of Biophysics and Bioengineering* **6**, 345–381.

NICHOLS, C. G. & LEDERER, W. J. (1991). ATP-sensitive potassium channels in the cardiovascular system. *American Journal of Physiology* **261**, H1675–1686.

NICHOLS, C. G., LEDERER, W. J. & CANNELL, M. B. (1991). The ATP-dependence of K_{ATP} channel kinetics in isolated membrane patches from rat ventricle. *Biophysical Journal* **60**, 1164–1177.

NICHOLS, C. G., SHYNG, S.-L., NESTOROWICZ, A., GLASER, B., CLEMENT, J. P. IV, GONZALEZ, G., AGUILAR-BRYAN, L., PERMUTT, A. M. & BRYAN, J. P. (1996). Adenosine diphosphate as an intra-cellular regulator of insulin secretion. *Science* **272**, 1785–1787.

NOMA, A. (1983). ATP-regulated K^+ channels in cardiac muscle. *Nature* **305**, 147–148.

SHYNG, S.-L., FERRIGNI, T. & NICHOLS, C. G. (1997a). Control of rectification and gating of cloned K_{ATP} channels by the Kir6.2 subunit. *Journal of General Physiology* **110**, 141–153.

SHYNG, S.-L., FERRIGNI, T. & NICHOLS, C. G. (1997b). Regulation of KATP channel activity by diazoxide and MgADP: distinct functions of the two nucleotide binding folds of the sulfonylurea receptor. *Journal of General Physiology* **110**, 643–654.

SHYNG, S.-L. & NICHOLS, C. G. (1997). Octameric stoichiometry of the K_{ATP} channel. *Journal of General Physiology* **110**, 655–664.

SIGWORTH, R. J. (1980). The conductance of sodium channels under conditions of reduced current at the node of Ranvier. *Journal of Physiology* **307**, 131–142.

TRAPP, S., PROKS, P., TUCKER, S. J. & ASHCROFT, F. M. (1998). Molecular analysis of ATP-sensitive K channel gating and implications for channel inhibition by ATP. *Journal of General Physiology* **112**, 333–349.

TUCKER, S. J., GRIBBLE, F. M., PROKS, P., TRAPP, S., RYDER, T. J., HAUG, T., REIMANN, F. & ASHCROFT, F. M. (1998). Molecular determinants of K-ATP channel inhibition by ATP. *EMBO Journal* **17**, 3290–3296.

TUCKER, S. J., GRIBBLE, F. M., ZHAO, C., TRAPP, S. & ASHCROFT, F. M. (1997). Truncation of Kir6.2 produces ATP-sensitive K^+ channels in the absence of the sulphonylurea receptor. *Nature* **387**, 179–183.

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