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The I182 Region of K_{ir}6.2 Is Closely Associated with Ligand Binding in K_{ATP} Channel Inhibition by ATP

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ABSTRACT The ATP-inhibited potassium (K_{ATP}) channel is assembled from four inward rectifier potassium (K_{ir}6.x) subunits and four sulfonylurea receptor (SURx) subunits. The inhibitory action of ATP is mediated by at least two distinct functional domains within the C-terminal cytoplasmic tail of K_{ir}6.2. The G334D mutation of K_{ir}6.2 virtually eliminates ATP-dependent gating with no effect on ligand-independent gating, suggesting a role in linkage of the site to the gate or in the ATP binding site, itself. The T171A mutation of K_{ir}6.2 strongly disrupts both ATP-dependent and ligand-independent gating, suggesting a role for T171 in the gating step. A neighboring mutation, I182Q, virtually eliminates ATP inhibition, but its effect on ligand-independent gating remained unknown. We have now characterized both the K_i values for inhibition by ATP and the ligand-independent gating kinetics of 15 substitutions at position 182. All substitutions decreased ATP-dependent inhibition gating as measured by the K_i, many profoundly so, yet had little or no effect on ligand-independent gating kinetics. Thus, substitutions at position 182 are unlikely to act by disrupting inhibition gate movement. Our results indicate an indispensable role for I182 in a step of the ATP binding mechanism, the linkage mechanism coupling the ATP binding site to the inhibition gate, or both.

INTRODUCTION

The ATP-inhibited potassium (K_{ATP}) channel couples energy metabolism to membrane electrical activity in a variety of cells and is important in several physiological systems (Aguilar-Bryan and Bryan, 1999; Ashcroft et al., 1984; Cook and Hales, 1984; Jovanovic et al., 1998; Noma, 1983). It is assembled from two distinct subunit types. A potassium pore-forming subunit, K_{ir}6.x (Inagaki et al., 1995), appears to be the primary seat of ATP-dependent inhibition gating (Drain et al., 1998; John et al., 1998; Mikhailov et al., 1998; Tucker et al., 1997, 1998). A sulfonylurea receptor subunit, SURx (Aguilar-Bryan et al., 1995), mediates inhibition by sulfonylureas and activation by MgADP and potassium channel openers (Babenko et al., 2000; Gribble et al., 1997, 1998; Nichols et al., 1996; Schwanstecher et al., 1998; Shyng et al., 1997a; Tucker et al., 1997). Although the channel's name reflects its characteristic inhibition by ATP, little is understood about the molecular and kinetic mechanisms underlying this property.

K_{ATP} channel activity occurs in bursts of brief openings that alternate with briefer closings, and these active burst episodes are separated by long-lived inactive interburst intervals (Alekseev et al., 1997; Ashcroft et al., 1984; Babenko et al., 1999a,b; Cook and Hales, 1984; Drain et al., 1998; Gillis et al., 1989; Nichols et al., 1991; Qin et al., 1989; Trapp et al., 1998). In the context of ATP inhibition gating, the K_{ATP} channel may be viewed simply as having two *major* functional conformations, an active burst state (conducting potassium ion flow) and an inactive interburst state (nonconducting). The briefer closings within the active burst state in this view may be thought to be due to an additional independent gate, and the simplification is to temporarily ignore these closings.

The transition from the active burst state to the inactive interburst state occurs at a relatively slow rate in the absence of ligand (ligand-independent gating) or at a greatly accelerated rate in the presence of ATP (ligand-dependent gating). When ATP binds to an active channel, it can be said to *cause* inhibition gate closure. ATP also binds to the inactive interburst state. When ATP binds to the inactive interburst state, bound ATP further stabilizes the already shut gate of the interburst state, prolonging its life. In this model, ATP may be acting as an allosteric effector biasing the conformational equilibrium toward the inactive interburst state. At very low ATP concentrations, most K_{ATP} channels are likely to first undergo the spontaneous transition to the inactive interburst state, and then bind ATP, which further stabilizes the inactive interburst state. When exposed to high ATP concentrations, most K_{ATP} channels are likely to first bind ATP, and then, at an accelerated rate, undergo transition to the inhibited state. Although ligand-independent and ATP-dependent gating to the interburst state differ in rate and by the presence of bound ATP, the two processes likely share the same mechanisms of pore occlusion (Drain et al., 1998; Loussouarn et al., 2000; Trapp et al., 1998; Tucker et al., 1998).

We recently showed that point mutation I182Q of the proximal C-terminal cytoplasmic tail of K_{ir}6.2 nearly eliminates ATP inhibition of the K_{ATP} channel (Drain et al., 1998), which raises the question of the precise role of this residue. Other previously characterized mutations, such as the neighboring T171A, disrupt both ligand-independent

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and ligand-dependent gating, suggesting a primary role in the gating step per se and not in an ATP binding step (Drain et al., 1998; Tucker et al., 1998). The N-terminal cytoplasmic segment of $K_{ir}6.2$ also is clearly involved (Babenko et al., 1999b; Koster et al., 1999; Proks et al., 1999) and can interact with the C-terminal cytoplasmic domain of $K_{ir}6.2$ (Tucker and Ashcroft, 1999). Here we tested for a role of I182 in the gating step by characterizing I182Q and all other substitutions at this position that express. We determined the effect of each of these substitutions both on ATP-dependent inhibition gating as measured by K_i and on ligand-independent gating as measured by appropriate single-channel kinetic parameters. Our results support models in which substitutions at I182 disrupt ATP inhibition, not by disrupting the gating step that controls burst-interburst transitions, but rather by acting primarily in an ATP binding step, a step that links it to the inhibition gate, or both. One possibility supported by the results presented here is that isoleucine at position 182 plays an indispensable role in a linkage mechanism between ATP binding and gating that is strongly engaged in the presence, but not in the absence, of bound ATP.

MATERIALS AND METHODS

Mutagenesis

Mouse $K_{ir}6.2$ and SUR1 cloned from the βHC9 cell line as previously reported (Drain et al., 1998) were used in this study. The truncated ΔC26 channel of Tucker et al. (1997) and the substitutions at position 182 in this background were constructed by a variation of the polymerase chain reaction (PCR) overlap extension technique. For the substitutions at position 182, we synthesized a single pool of mutagenic primers containing XXG/C at the 182 codon together with a silent restriction reporter site, where X represents any of the four nucleotides, which were present in equimolar concentrations (Reidharr-Olson et al., 1991). Thirty random, independent mutagenic primer-containing clones were selected as those containing the restriction reporter site, and sequenced. Fourteen different substitutions at 182 were obtained in this way. The remaining five substitutions at 182 were constructed separately using similar methods and confirmed by sequencing.

Expression in oocytes and electrophysiology

Preparation and injection of *Xenopus* oocytes, patch pipette fabrication, and recording techniques were as described (Drain et al., 1994, 1998). Briefly, macroscopic and single-channel currents were recorded with the inside-out patch configuration at −80 mV with the following pipette and bath solutions, unless indicated otherwise. Pipette solution (in mM): 150 KCl, 10 NaCl, 1 $CaCl_2$, 10 EGTA, and 10 HEPES, pH 7.4 ± 0.05. Bath solution: same as pipette solution but with 1.3 MgATP. Constant perfusion of the cytoplasmic face of patches was performed using a custom-made eight-sewer pipe syringe-pressurized system. Recordings were always begun within one minute after excision with the patch pipette partially inserted into one of the sewer pipes. ATP was added as the magnesium salt to minimize rundown (Trube and Hescheler, 1984) and no other ligands were added until after the ATP dose response data were obtained. Experiments showing rundown, characterized by a sudden significant decrease in channel open probability, P_O, were discarded. Patch clamp currents were amplified with Axopatch 200A (Axon Instruments, Inc., Foster City, CA) or EPC-9 (HEKA Elektronik, Lambrecht/Pfalz, Germany) instruments, low-pass filtered with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) at a corner frequency of 2 or 4 kHz, and sampled at 20 kHz using HEKA PULSE v.8.0 (HEKA Elektronik, Lambrecht/Pfalz, Germany). We did not correct for leak currents (as determined after complete rundown or inhibition with saturating tolbutamide or ATP), which were typically ≤10 pA, always ≤5% of the macroscopic K_{ATP} currents.

Data analysis

Analysis and display were done using TAC v.4.0 (Bruxton, Inc., Seattle, WA), IGOR Pro v.3.1 (WaveMetrics, Inc., Lake Oswego, OR), and PageMaker v.6.5 (Adobe Systems, Inc., San Jose, CA). Dose-response measurements were fit to the Hill equation, $I/I_{max} = 1/\{1 + ([ATP]/K_i)^{\alpha_H}\}$, where [ATP] is the concentration of ATP, I/I_{max} the fractional current at the indicated [ATP] relative to that in the same solution in the absence of added ATP, (I_{max} was defined as the average of measurements taken before and after current measurements in the presence of [ATP]), K_i the [ATP] at which inhibition is half-maximal, and α_H the slope factor, or Hill coefficient. Data are presented as mean ± SEM. For Hill plots of data from the mutant K_{ATP} channels with very large K_i values, we set $\alpha_H = 1.0$, a reasonable constraint given that when α_H was treated as a free variable, we found $\alpha_H = 1.0 \pm 0.1$ for the wild-type channel and $\alpha_H = 1.0 \pm 0.3$ for all our less severely affected K_i mutant channels. Single-channel current events were detected using the time of the half-amplitude of transitions between current levels with TAC v.4.0 (Bruxton, Inc., Seattle, WA). Durations were corrected for missed events during construction of duration histograms based on the filter corner frequency of the recording by the method of Colquhoun and Sigworth (1995). Duration analysis was done with TAC-FIT v4.0 (Bruxton, Inc., Seattle, WA), which uses the transformations of Sigworth and Sine (1987) to construct and fit duration histograms. Boxplots were constructed using IgorPro v3.14.

RESULTS

In the absence of ATP and at −80 mV, the wild-type K_{ATP} channel alternates between an active bursting state and an inactive interburst state, a behavior we refer to as ligand-independent gating. For the example shown in Fig. 1, the mean burst time was 28 ± 5 ms and the mean interburst closed time was 15 ± 4 ms. Within each burst the channel rapidly interconverts between its single open state and a brief closed state. The mean open time was 1.5 ± 0.1 ms and the mean brief closed time was 0.4 ± 0.04 ms. Thus, in the absence of ligand, when rundown is minimal, the fraction of time the K_{ATP} channel spends in the burst is ~0.65. Within a burst, the fraction of time the channel spends in the open state is ~0.8. Overall, the open probability of the wild-type channel in the absence of ligand typically ranges between 0.5 and 0.8, the variability due at least in part to rundown (Trube and Hescheler, 1984).

The ΔC26 truncated channel (Tucker et al., 1997) when expressed in the absence of SUR exhibits long interburst and short intraburst closed times similar to those of the wild-type channel. However, its burst and open times are significantly shorter (Drain et al., 1998; Tucker et al., 1998) not due to the truncation of ΔC26, but due to the absence of the SUR (Babenko et al., 1999a; Lorenz et al., 1998). For the truncated ΔC26 channel (without SUR), the mean burst

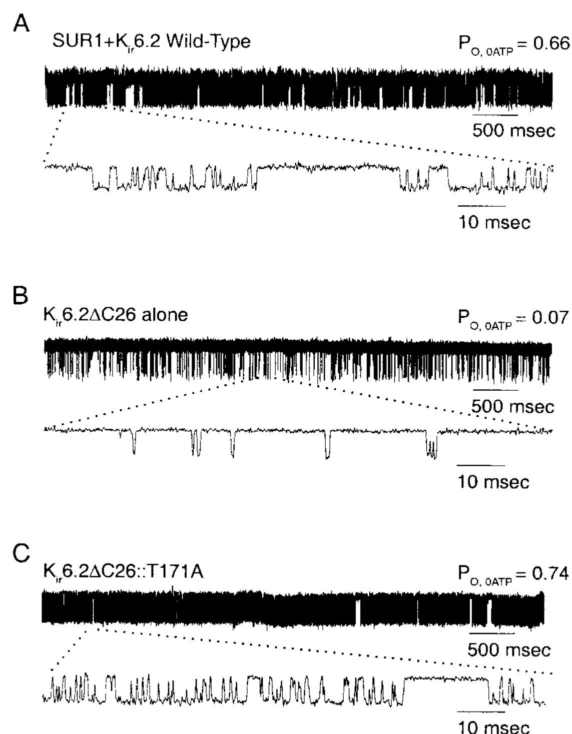

FIGURE 1 Comparison of the gating kinetics in the absence of ATP of three classes of K_{ATP} channels. (*A*) Wild-type channel exhibits a relatively high open probability (P_O) in the absence of ATP, characterized by long bursts, comprising many brief openings, separated by long interburst intervals. (*B*) Truncated ΔC26 channel without SUR1 exhibits a relatively low P_O in the absence of ATP, characterized by short bursts of one to a few openings, separated by long interburst intervals. (*C*) Truncated ΔC26 channel with the T171A substitution without SUR1 exhibits very high P_O in the absence of ATP. The bursts of the T171A/ΔC26 channel without SUR1 were longer than those of wild-type with SUR. All recordings in this and subsequent figures were made at −80 mV in symmetrical 150 mM KCl as permeant ion. For details see Materials and Methods.

duration was only 1.70 ± 0.06 ms and the mean open time was only 0.83 ± 0.05 ms. Thus, in the absence of ligand, the fraction of time the truncated ΔC26 channel spends in the burst state was ~0.10. The truncated ΔC26 channel typically opens only once, twice, or three times before exiting the burst. The dramatic reduction of mean burst duration from 28 to 1.7 ms suggests that the absence of SUR shifts the ligand-independent gating equilibrium in favor of the inactive interburst state through a greatly enhanced rate of spontaneous, ligand-independent transition to the inactive interburst state (Drain et al., 1998).

As previously shown, the shift in the ligand-independent gating equilibrium of $K_{ir}6.2$ΔC26 can be dramatically reversed by a point mutation at position 171, as well as at neighboring positions in the C-terminal third of the M2 transmembrane segment of $K_{ir}6.2$ (Drain et al., 1998; Trapp et al., 1998; Tucker et al., 1998). In the example revisited here, T171A in the truncated ΔC26 channel increased the fraction of time in the burst state by nearly an order of magnitude from ~0.10 to 0.90. Accordingly, the interburst intervals became infrequent, but remained comparable in duration to those of wild-type. Evidently, bursts that were dramatically abbreviated by the absence of SUR can be restored to very long duration by the T171A mutation. These results indicate that the T171A mutation, which decreases the ATP-dependent gating of the wild-type channel by nearly 40-fold as assessed by K_i, also greatly slows ligand-independent gating, suggesting a role for T171 in the gating step leading to the inactive interburst state. The results demonstrate that gating in the absence of ATP can be strongly altered by mutations originally selected for their ability to alter ATP-dependent inhibition gating, suggesting the two gating processes share a common mechanism.

We previously reported that a point mutation in the vicinity of T171, I182Q, when expressed in the wild-type $K_{ir}6.2$ background together with SUR1, virtually eliminated ATP-dependent inhibition gating of the channel as measured by K_i (Drain et al., 1998). Here, we expand on that finding by characterizing the K_i for ATP inhibition and the ligand-independent single-channel gating properties of the 15 substitutions at I182 in the truncated ΔC26 channel that expressed channel activity in the absence of SUR. Unless otherwise indicated, we describe here mutations at 182 studied in this truncated ΔC26 background expressed without SUR, which we refer to as I182/ΔC26 mutant channels, and compare them to the otherwise wild-type truncated ΔC26 control channel expressed without SUR.

Substitutions at position 182 in truncated ΔC26 channels strongly disrupt ATP-dependent inhibition

Fig. 2 shows macroscopic current responses of four different 182/ΔC26 mutant channels to the indicated doses of intracellular ATP. The ΔC26 control channels, which have the wild-type isoleucine at position 182, were strongly inhibited by 0.2 mM ATP and almost completely by 2 mM. The I182Q/ΔC26 channels, representative of the class of mutants whose ATP-dependent gating is most disrupted, were only weakly inhibited by 5 mM and moderately inhibited by 10 mM. The I182L/ΔC26 channels, representative of the less disrupted class of mutants, were weakly inhibited by 2 mM and moderately by 5 mM. The I182A/ΔC26 channels, representative of the least disrupted class of mutants, were moderately inhibited by 2 mM and strongly by 5 mM.

Fig. 3 shows fits of the Hill equation to dose-response data from ΔC26 control channels as well as from several representative 182/ΔC26 mutant channels. For the ΔC26 control channel wild-type at this position, the K_i was 0.19 ± 0.02 mM ATP. Even for the least effective mutation at this position, I182A, the K_i was 2.21 ± 0.12 mM, an 11.6-fold increase. More dramatic shifts in the K_i were found for all 14 other substitutions at position 182. For I182L (where a

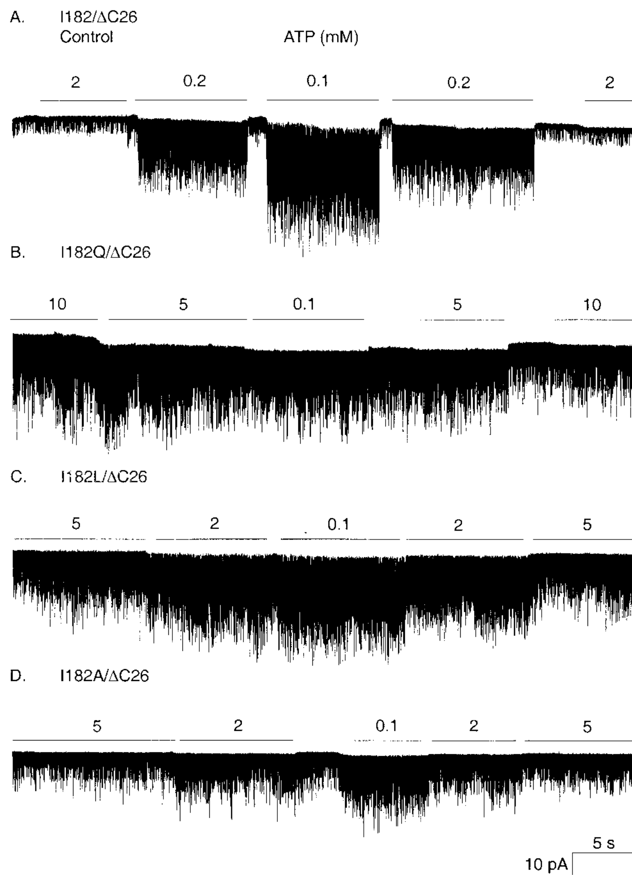

FIGURE 2 Effect of substitutions at position 182 on channel inhibition by intracellular ATP. (*A*) The truncated ΔC26 channel without SUR1 with the wild-type isoleucine (*I*) residue at position 182, which serves as the control for most of the experiments here, shows half-maximal inhibition by 0.2 mM ATP, and nearly complete inhibition by 2 mM. (*B*) The I182Q/ΔC26 channel, which shows little if any inhibition by 5 mM, and minor inhibition by 10 mM, represents the class of 182 substitutions with ATP inhibition most disrupted. Notice that channels with this substitution run down slightly, despite the best precautions. (*C*) The I182L/ΔC26 channel, which shows minor inhibition by 2 mM and nearly half-inhibition by 5mM, represents the class of 182 substitutions with ATP inhibition less disrupted. (*D*) The I182A/ΔC26 channel, which shows nearly half-maximal inhibition by 2 mM, and more than half-maximal inhibition by 5 mM, represents the class of 182 substitutions with ATP inhibition least disrupted.

hydrogen and a methyl group are swapped between adjacent carbons), the K_i was 6.22 ± 0.37, a 32.7-fold increase. For I182Q, the K_i was 12.25 ± 0.90 mM, a 65-fold increase. I182Q was originally selected in full-length $K_{ir}6.2$ expressed with SUR1 for its ability to increase K_i from 0.12 to ≥ 10 mM (Drain et al., 1998). Thus, the strong effect of this mutation on K_i was maintained in the truncated ΔC26 channel expressed without SUR1. For I182W/ΔC26, the K_i determinations were difficult at least in part because the channels are largely refractory to ATP up to the maximum tested 10 mM. Although variable, the K_i values for 182W were always ≥15 mM ATP, representing an increase in K_i of over 80-fold.

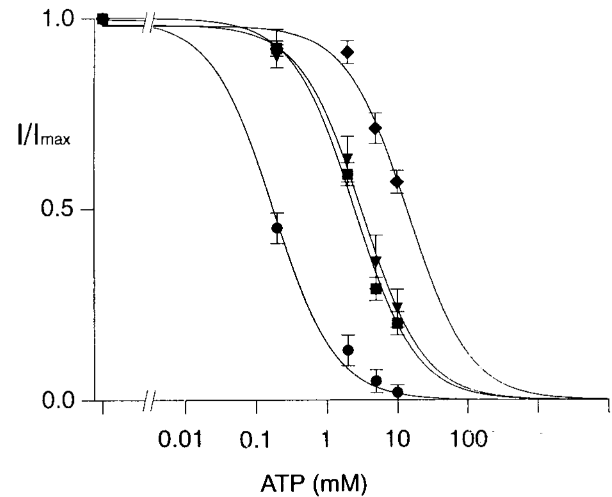

FIGURE 3 Substitutions at position 182 of truncated ΔC26 channels result in rightward shifts of the dose-response curve for ATP inhibition. Current in the presence of the ATP concentration indicated on the *x* axis relative to that in the absence of ATP was plotted for the 182 substituted ΔC26 channels. Data points were fit to the Hill plot to obtain the half-maximal ATP concentration in mM for the inhibition, or K_i. Slope factors for the fits ranged from 1.0 ± 0.1 to 1.3 ± 0.1. The fractional inhibition at a given concentration is indicated in the plot as follows: ●, ΔC26 control channels (n = 6); ■, I182A/ΔC26 channels (n = 5); ▼, I182L/ΔC26 channels (n = 6); and ♦, I182Q/ΔC26 channels (n = 5). Mean ± SEM.

Single-channel behavior, in the absence of ATP, of truncated ΔC26 channels with substitutions at position 182

The truncated ΔC26 control channel exhibits dramatically abbreviated burst durations, reflecting accelerated (compared to wild-type) transition to the inactive interburst state. However, ligand-independent gating typical of $K_{ir}6.2$ channels with SUR (i.e., longer bursts) can be restored to the ΔC26 control channels by certain substitutions at position 171. T171A, for example, slows down transition to the inactive interburst to rates comparable to those of $K_{ir}6.2$ channels with SUR. To explain these results we have proposed that the gate controlling the burst-interburst transition operates in both the absence and presence of bound ATP (Drain et al., 1998). In the absence of bound ATP, the gate closes relatively infrequently, as reflected in the relatively rare transitions from the active burst to the inactive interburst intervals. Bound ATP has the effect of greatly accelerating this pre-existing gating mechanism. The T171A mutation has the opposite effect, greatly slowing gating to the inactive interburst state, with or without ATP present, as reflected in the dramatically prolonged burst durations of the truncated ΔC26 channels. Because the mutation's effect is observed independent of the presence of ATP, it likely disrupts primarily the gating step per se, and not other steps such as ligand binding.

In contrast, we found that, of the 15 substitutions for I182 in the truncated ΔC26 background that expressed functional

channels, all greatly impaired ATP inhibition, yet none restored the long bursts typical of wild-type gating. Rather, all substitutions at position 182 in truncated ΔC26 channels preserved the short-burst gating characteristic of the otherwise wild-type truncated ΔC26 channel. Fig. 4 shows representative single-channel records in the absence of ATP for all substitutions at 182 of ΔC26 that expressed channel activity. Each record is labeled with the amino acid residue at the 182 position, the K_i for ATP inhibition, and the open probability (P_O) in the absence of ATP. The latter are convenient indicators of ligand-dependent and ligand-independent gating, respectively. The top record illustrates the brief bursts and frequent interbursts characteristic of the truncated ΔC26 control channel. For comparison, the bottom record demonstrates that T171A greatly lengthens the burst times of the truncated ΔC26 channel, to approximate

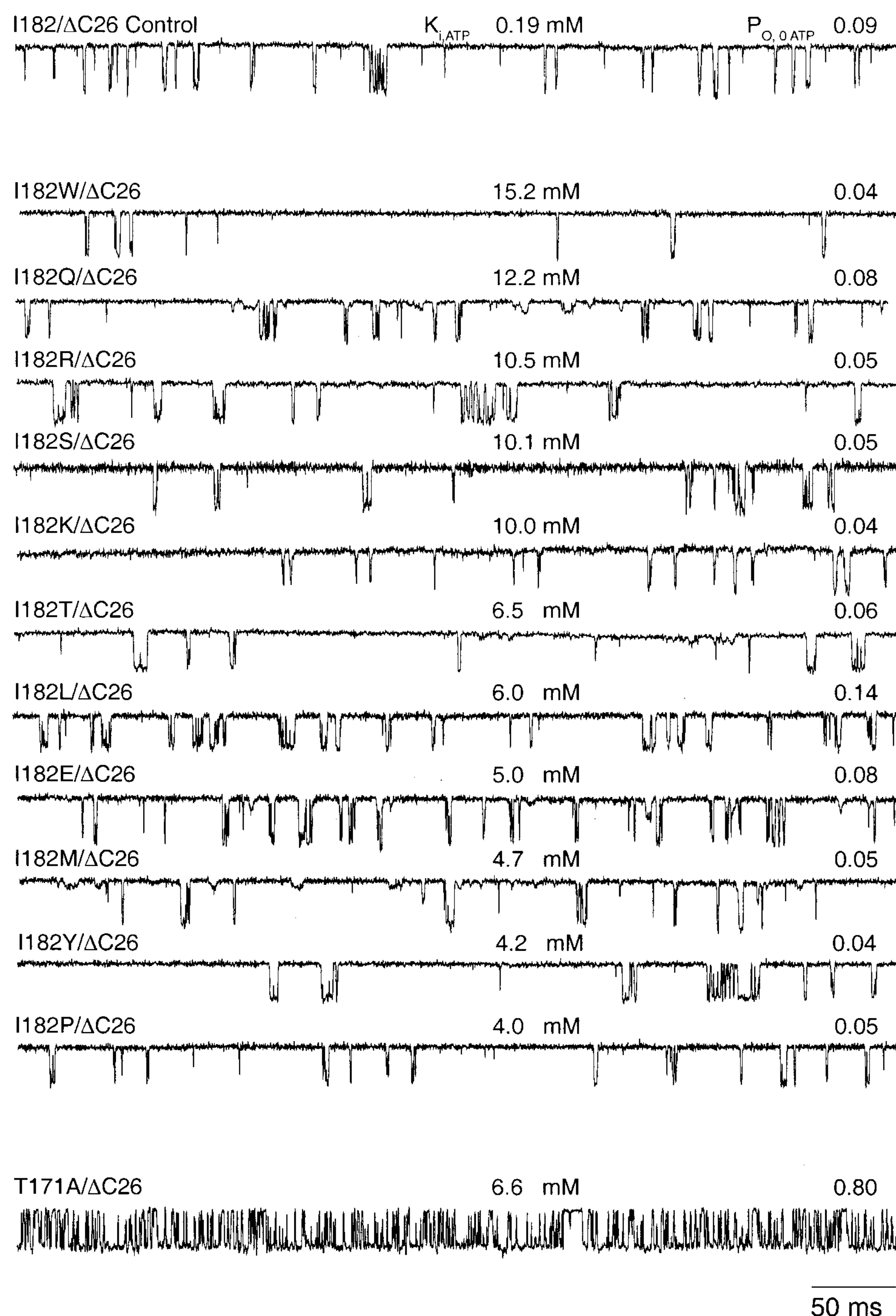

FIGURE 4 Substitutions at position 182 generally fail to restore long-duration bursts of activity to the truncated ΔC26 channel. Representative single-channel current records are shown with the single-letter abbreviation for the amino acid residue substituted in ΔC26 indicated, as well as the resulting K_i and open probability (P_O) in the absence of ATP. The top trace is from a channel with the wild-type residue I at position 182. These control channels had a relatively low K_i of 0.2 mM and a low P_O in the absence of ATP of 0.09. For comparison, the channel yielding the bottom trace also has the wild-type I residue at 182, but has in addition the T171A mutation, the latter of which disrupts both ATP-dependent inhibition gating (K_i of 6.6 mM, a 35-fold increase) and ligand-independent gating (P_O in the absence of ATP of 0.80, an 11-fold increase). Notice that, while the substitutions at position 182 greatly increase the K_i for ATP inhibition—in five cases more so than T171A does—they, unlike T171A, do not increase the ligand-independent open probability of the channel (P_O in the absence of ATP $\leq$ 0.14, comparable to the ΔC26 rather than to the ΔC26/T171A control). K_i values were determined as in Fig. 3. For both K_i and P_O values, $n \geq 4$ for each determination.

those of the full-length, wild-type K_{ATP} channel (compare with Fig. 1). The other records are for the 182/ΔC26 mutant channels, all in the absence of ATP. They clearly show that substitutions at 182 preserve the frequent gating to the inactive interbursts characteristic of the ΔC26 control channel. This holds true always for 13 substitutions at 182, and almost always for the remaining two. (Exceptions are described below.) Thus, 15 substitutions at position 182 increased K_i ≥10-fold, ten substitutions ≥25-fold, five substitutions ≥50-fold, yet all caused ≤1.13-fold increases in mean burst time, open time, or open probability.

Positively charged substitutions at position 182 can alter ligand-independent gating

For two of the substitutions at 182, we found rare exceptions to the general statement above. The two positively charged substitutions, 182K and 182R, each increased K_i by >50-fold and usually had little if any effect on ligand-independent gating parameters. Occasional patches, however, exhibited the long bursts typical of $K_{ir}6.2$ channels with SUR (Fig. 5). In 3 of 20 patches of 182K/ΔC26 channels and in 2 of 14 patches of 182R/ΔC26 channels, long bursts of openings were observed, comparable in duration to those of the 171A/ΔC26 and wild-type K_{ATP} channels in the absence of ATP. This rare behavior was not analyzed further.

Single-channel kinetics of the I182Q/ΔC26 channel in the absence of ATP

To quantify the effects of substitutions at position 182 on ligand-independent gating, in particular whether they had any effect on the transition rate from the active burst state to the inactive interburst state, we compare (Fig. 6) histograms of open, both intraburst and interburst closed, and burst dwell times of an I182Q/ΔC26 channel and a ΔC26 control channel. We found no significant differences in the means of these four parameters. This is in contrast to the dramatic difference in burst duration and nearly nonexistent inactive interburst times found for the T171A/ΔC26 channel. The results indicate that, in contrast to the T171A substitution, the I182Q substitution had little effect on the ligand-independent single-channel gating of the truncated ΔC26 channel, despite the 65-fold effect of I182Q on the K_i for ligand-dependent inhibition.

Comparison of gating parameters for 182/ΔC26 and T171A/ΔC26 channels

In Fig. 7, we compare six characteristics (the K_i for ATP inhibition and five single-channel gating parameters measured in the absence of ATP) of the I182X/ΔC26 mutant channels with those of the truncated I182/ΔC26 control and T171A/ΔC26 channels. For each characteristic, the panel

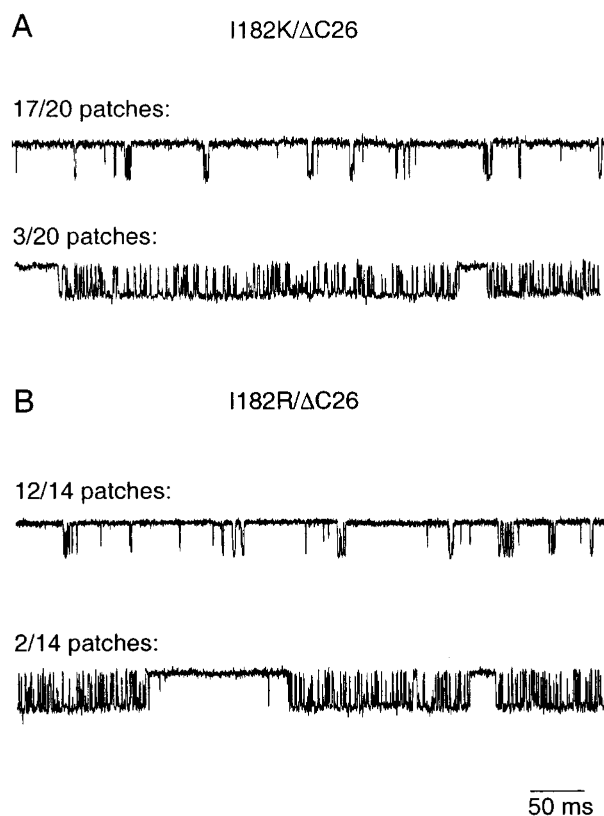

FIGURE 5 Exceptional restoration of long bursts of channel activity in the absence of ATP was observed only in positively charged substitutions at 182. (*A*) Of 20 single-channel I182K/ΔC26 patches, 17 exhibited the low P_O in the absence of ATP and short-duration bursts of one to few openings characteristic of the ΔC26 control; however, three patches exhibited the high P_O in the absence of ATP and long-duration bursts of openings characteristic of the T171A/ΔC26 channel. A representative segment from one of these rare, long-duration burst patches for the I182K/ΔC26 channel is shown. (*B*) Of 14 single-channel I182R/ΔC26 patches, 12 exhibited the low P_O in the absence of ATP and short-duration bursts characteristic of ΔC26 control; however, two exhibited the high P_O in the absence of ATP and long-duration bursts of openings characteristic of the T171A/ΔC26 channel. A representative segment from one of these two rare, long-duration burst patches for the I182R/ΔC26 channel is shown. For each panel, numbers indicate the fraction of oocytes having single-channel patches which in the absence of ATP exhibited bursts of the short-duration or long-duration class, respectively.

compares the range of mean values for all 15 of the I182X/ΔC26 mutant channels with the range of individual measurements for the I182/ΔC26 control and T171A/ΔC26 channels. Clearly, substitutions at 182 greatly increased the K_i over a wide range, with a median value similar to that for the T171A mutation (Fig. 7 *A*). Yet, substitutions at 182 exhibited no significant differences from I182/ΔC26 control in gating properties in the absence of ATP. For all 15 substitutions at position 182, the range of open probabilities, openings per burst, burst times, open times, and closed times (Fig. 7, *B–F*, respectively) were not significantly

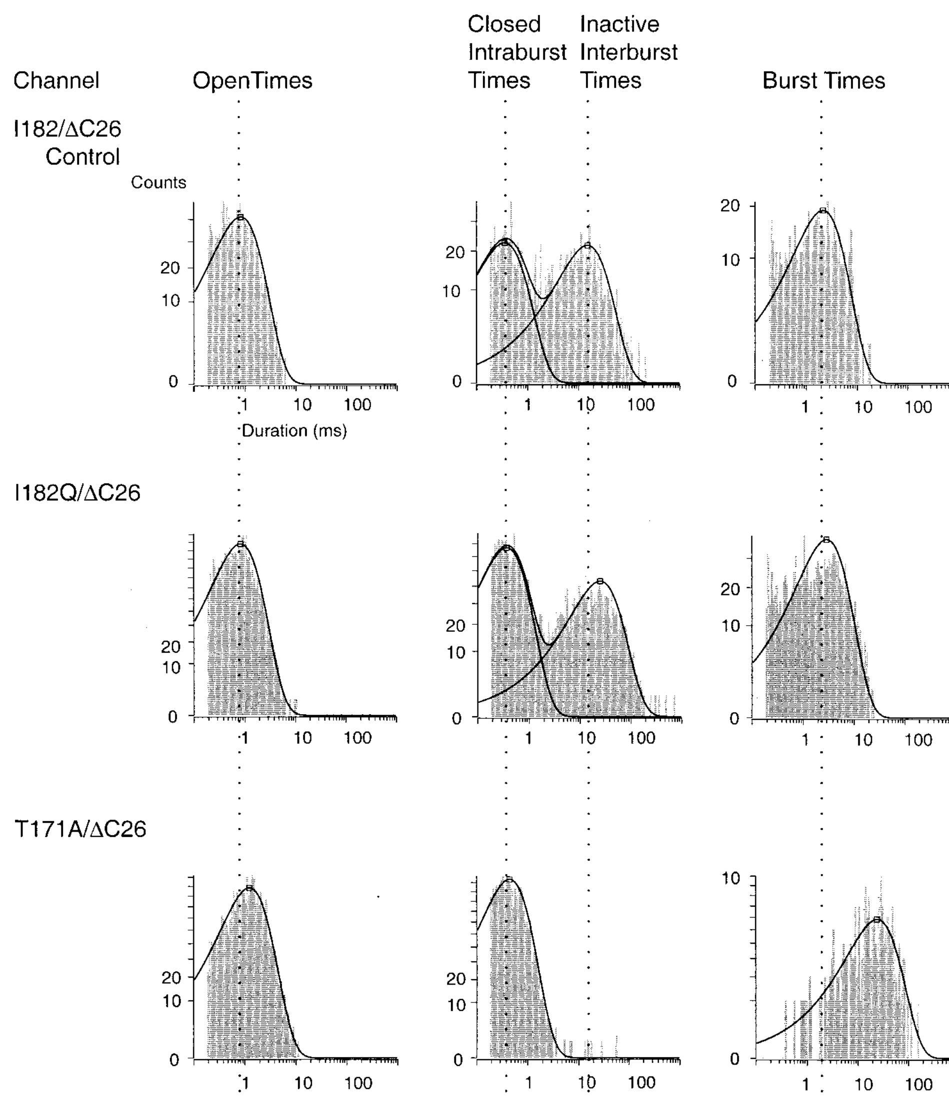

FIGURE 6 Comparison of ligand-independent single-channel kinetic properties of the I182Q/ΔC26 and T171A/ΔC26 channels, with those of ΔC26 control. Histograms of open times, intraburst and interburst closed times and burst times are shown for each channel. The logarithmic time-axis used for the histograms yields a fitted probability density function with peaked components. The position of the peak along the *x* axis of the peak corresponds to the component's time constant. To facilitate comparisons, vertical lines indicate the characteristic time constant for the exponential terms of the truncated ΔC26 control channel. The mean burst times for the truncated ΔC26 control, the I182Q/ΔC26, and T171A/ΔC26 channels were 1.70 ± 0.06, 1.58 ± 0.02, and 19.5 ± 0.5 ms, respectively. The mean open times were 0.83 ± 0.06, 0.73 ± 0.04, and 1.38 ± 0.02 ms, respectively. The mean brief, intraburst closed times were 0.42 ± 0.02, 0.40 ± 0.02, and 0.42 ± 0.02 ms, respectively. Moreover, whereas the long inactive interburst times of the I182Q/ΔC26 channel (mean duration 14.5 ± 2.0 ms) and ΔC26 control channel (14.1 ms ± 0.7 ms) contributed approximately half the closed events, the long inactive interburst events were nearly nonexistent in the T171A/ΔC26 channel. The duration histograms shown are representative of five similar experiments for each type of channel.

different from those for the truncated I182/ΔC26 control. This is in sharp contrast to the T171A/ΔC26 channel, whose open probability, openings per burst, burst times, and open times increased dramatically (Fig. 7, *B–F*), reflecting striking effects on ligand-independent gating by the T171A mutation, consistent with previous conclusions (Drain et al., 1998; Tucker et al., 1998).

Comparison of changes in K_i and burst duration in the absence of ATP for 182 substitutions in the truncated $K_{ir}6.2$ΔC26

The essential effects of 182 substitutions on the truncated ΔC26 channel are visually summarized in Fig. 8, where the mean burst time in the absence of ATP is plotted against the K_i for each of the 182/ΔC26 mutants. Each mutant is represented by the single-letter symbol for the amino acid residue at position 182. The ΔC26 control channel, represented by "I" in the plot, combines a relatively low K_i value with a low mean burst duration. All 15 of the I182/ΔC26 mutant channels combine a range of high K_i values with short and largely uniform mean burst times. The T171A/ΔC26 channel, represented by "T171A," combines a high K_i value with a high mean burst duration. The visual display emphasizes the lack of effect of substitutions at position 182 on ligand-independent burst times, compared to the T171A mutation.

182 substitution in the wild-type $K_{ir}6.2$/SUR1 channel also strongly increases K_i with little effect on ligand-independent gating

Initially, we were surprised by the general lack of experimental support for tight linkage between the gating mech-

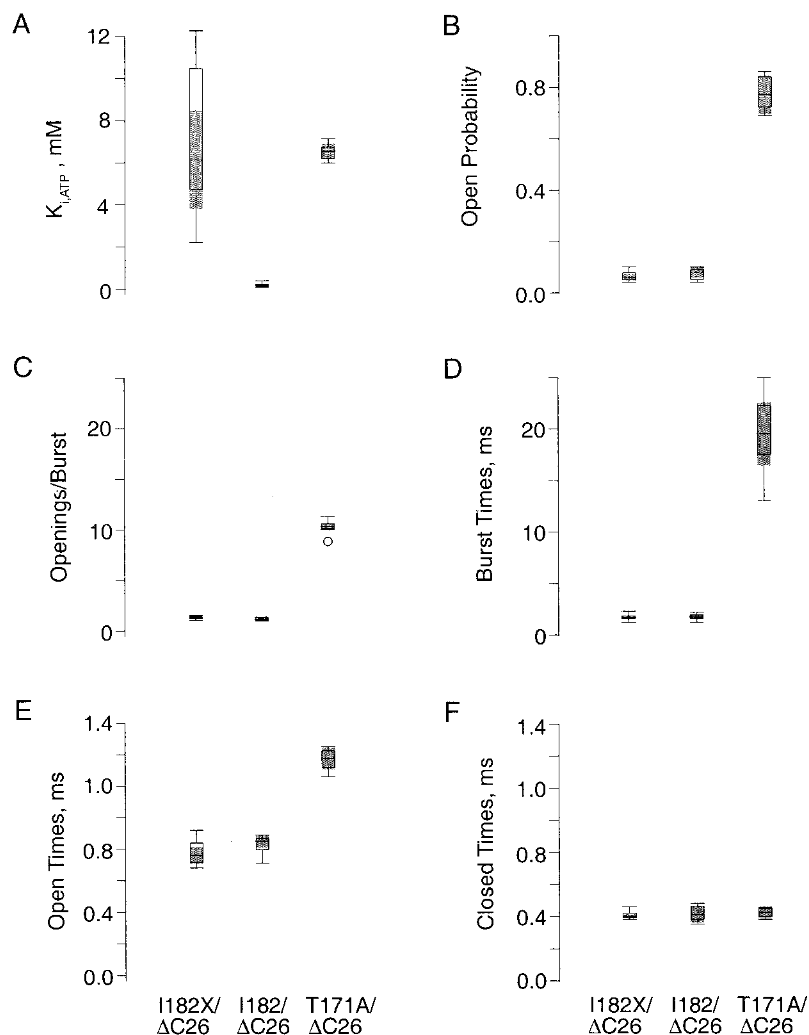

FIGURE 7 Ranges of K_i and ligand-independent single-channel parameter values for the 182 substitutions. Ranges of each parameter are shown by box plots. Measurements from the 15 substitutions at 182 studied here are pooled together and designated I182X/ΔC26, and compared with those of the I182/ΔC26 control and the T171A/ΔC26 channel. (*A*) K_i for ATP inhibition. The mean K_i values of the 15 mutants at position 182 covered a wide distribution with a median similar to that for T171A, and over 30-fold higher than that for the I182/ΔC26 control. The K_i of 15 mM for the I182W substitution (an 80-fold increase) is an outlier off the top of the plot, and not shown. (*B–F*) Distributions of various ligand-independent single-channel parameters. For every parameter, the I182X/ΔC26 mutant values were not significantly different from the corresponding I182/ΔC26 control values. For every parameter except closed times, the I182X/ΔC26 mutant values are significantly less than the corresponding T171A/ΔC26 values, except for the mean intraburst closed times (*panel F*). Each box covers the central half of the data with a line through the box at the median value. The single lines that extend vertically up and down from the box cover all but outliers of the data. The shaded region associated with each box approximates the 95% confidence interval of the median value.

anism and other components underlying K_{ATP} channel inhibition by ATP. We had reasoned that even substitutions of residues that are not directly part of the gating mechanism would nevertheless frequently alter gating via such tight linkage. In a strictly linked system, changes in the equilibrium position of the gate might be expected to follow changes at the site, including those arising from mutations isolated by their increase in K_i for ATP inhibition, regardless of the absence or presence of ligand. So far, we find little evidence for this not only from results of substitutions at 182, but also from similar results of 22 substitutions in the 334 region of $K_{ir}6.2\Delta C26$ without SUR (unpublished results, Li, Geng, and Drain). However, common to all the substitution experiments in the 182 and 334 regions is the ΔC26 mutation and the absence of SUR. Perhaps the lack of linkage to the gating mechanism in the absence of ligand is not a general property of substitutions at I182 or G334, but rather an artifact due to the truncation of $K_{ir}6.2$ or to the absence of SUR.

We tested whether linkage from I182 to the gating mechanism might depend on full-length $K_{ir}6.2$ or SUR1 by studying the I182Q substitution in a wild-type background. Fig. 9 shows that I182Q in the full-length $K_{ir}6.2$ with SUR1, as in the truncated subunit without SUR1, has little or no effect on ligand independent gating. I182Q, which results in virtual elimination of ATP inhibition (K_i >10 mM; Drain et al., 1998), did not affect the ligand-independent activity of the wild-type channel with SUR1 as measured by P_O in the absence of ATP. Thus, I182Q in both the $K_{ir}6.2\Delta C26$ channel and the full-length $K_{ir}6.2$ + SUR1 channel dramatically disrupts ATP inhibition without altering ligand-independent gating. The results are inconsistent with a linkage mechanism that always couples the ATP binding site to the inhibition gate in the presence of SUR, but not in its absence. Rather, the results suggest a ligand-dependent linkage mechanism that likely operates in the wild-type as well as the truncated channel (see below).

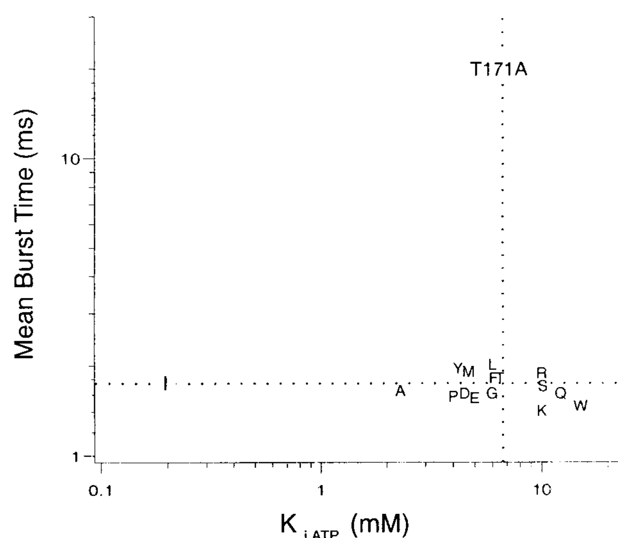

FIGURE 8 Ligand-independent burst times of I182/ΔC26 channels are independent of ligand-dependent gating as measured by K_i for ATP. The letters are the single-letter abbreviations for the amino acid substitutions at 182 studied in the ΔC26 channel and are plotted with coordinates representing the values for mean burst time and K_i. The corresponding values for T171A are also plotted for reference. The K_i values for I182/ΔC26 control and its substitutions at 182 cover a wide range from 0.19 to over 15 mM ATP, whereas the average mean burst times cover a narrow range from 1.46 to 1.92 ms. For T171A, the K_i is 6.6 mM and the average mean burst time is dramatically longer at 19.5 ms.

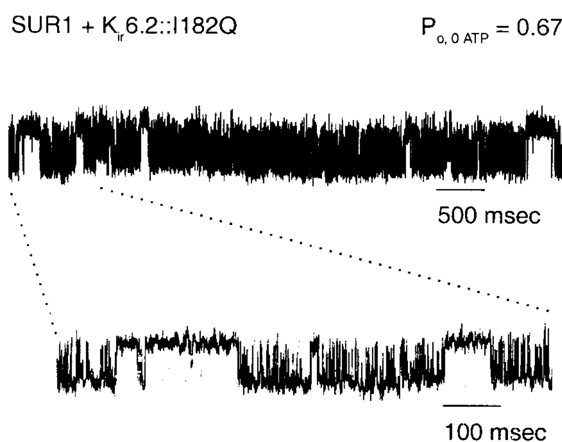

FIGURE 9 Mutation of I182 in the wild-type $K_{ir}6.2$/SUR1 background strongly disrupts ATP-dependent gating as measured by K_i with little effect on ligand-independent gating. Single-channel record segment of the channel formed by co-expression of SUR1 and $K_{ir}6.2$::I182Q. Five seconds of recording are shown together with the first segment greatly expanded below. The data are from a 138-s record taken immediately after patch excision into 0 ATP during which the channel exhibited no rundown, measured as time-dependent loss of activity. The open probability of the channel in this recording was 0.67, which is indistinguishable from that of the wild-type K_{ATP} channel. The I182Q mutation in the wild-type $K_{ir}6.2$ background with SUR1 results in over a 1000-fold loss in ATP inhibition, compared to wild-type control (Drain et al., 1998). Thus, mutation of I182 in the wild-type $K_{ir}6.2$ background co-expressed with SUR1 virtually eliminates ligand-dependent gating with little or no effect on ligand-independent gating. The results are representative of experiments of all 11 single-channel patches studied from six oocytes expressing $K_{ir}6.2$::I182Q + SUR1.

DISCUSSION

Isoleucine at 182 of $K_{ir}6.2$ is indispensable for wild-type ATP-inhibition

All 15 substitutions that express at position 182 of ΔC26 decreased ATP-dependent gating with no effect on ligand-independent gating. The alanine substitution, which had the least effect on ATP-dependent gating, nevertheless decreased it by over 10-fold. At the other extreme arginine, lysine, serine, and tryptophan each decreased ATP-dependent inhibition by 50-fold or more. Each of 15 substitutions at position I182 therefore increased the K_i by 10-fold or more, but none slowed ligand-independent gating of the channel to the interburst states, with rare exceptions discussed below. The increased K_i for ATP inhibition for all 15 of the I182 substitutions taken together indicates no other residue, including leucine, substituted well, and we conclude that isoleucine is indispensable for wild-type inhibition.

The effect on ligand independent gating of substitutions at 182 sharply contrasts that at 171

The lack of effect of I182 K_i mutations on ligand-independent gating is in sharp contrast to the effect of the T171A mutation, which increases both K_i and mean ligand-independent burst and open durations. In the full-length wild-type $K_{ir}6.2$ background expressed with SUR1, T171A increased K_i by 40-fold (Drain et al., 1998). In the truncated ΔC26 expressed without SUR1, T171A had the additional striking effect of restoring long bursts, increasing burst duration 10-fold or more compared to T171/ΔC26 (Drain et al., 1998; Tucker et al., 1998). This change in both K_i and the ligand-independent gating equilibrium of the ΔC26 channel is explained in part if T171A slows the transition rate from the active burst to the inactive interburst state regardless of the presence of ATP. In the wild-type channel, ATP at low concentration likely predominantly stabilizes channels that have already entered the inactive interburst state. At higher concentration, ATP also likely first binds to the burst state and greatly accelerates the transition from the active burst to the inactive interburst state.

Positively charged residues at 182 can alter ligand-independent gating

Whereas the indispensable role for I182 in ATP inhibition as part of the gating mechanism is excluded, the exceptional behavior of the I182K and I182R substitutions indicates residues at 182 can alter, and therefore must provide linkage

to, the gating mechanism. In 3 of 20 patches with I182K/ΔC26 channels and in 2 of 14 patches with I182R/ΔC26 channels, long-bursting gating kinetics dominated the current records. It may be of significance that altered ligand-independent gating was limited to channels with positively charged substitutions at position 182. Plausibly, the 182 region moves into proximity of charged or polar environments during the burst-interburst gating transitions. There is evidence for such pore domains contributed by residues from the second transmembrane domain M2 to T171A of $K_{ir}6.2$ (Drain et al., 1998; Loussouarn et al., 2000; Shyng et al., 1997b; Trapp et al., 1998; Tucker et al., 1998). The communication between charged residues at 182 and gating in the absence of ATP may reflect a tight linkage interaction between I182 and the gating mechanism that is engaged only during occupancy of the binding site by ATP in the wild-type channel.

The effect of charged substitutions on ligand-independent gating distinguishes I182 from other positions implicated in ATP inhibition that are distal to T171 in the C-terminal cytoplasmic tail of $K_{ir}6.2$. Although mutational analysis is not as complete at these distal positions, all such mutations that increased the K_i for ATP inhibition do so with ligand-independent gating unaltered, without exception. For example, K185Q in ΔC26 without SUR1 increased K_i by 40-fold (Tucker et al., 1997, 1998), without a ligand-independent gating change (Tucker et al., 1998). Other neighboring mutations E179Q and Q173A in the truncated ΔC26 channel without SUR increased the K_i more modestly, almost threefold, with little or no change in ligand-independent gating. Position 182 therefore is unique so far in not being part of the gating mechanism, but being able to provide a linkage to it when positively charged side chains occupy the position.

I182 neighbors a residue implicated in phosphoinositide-stimulated K_{ATP} channel activity

Fan and Makielski (1997) demonstrated that phosphoinositide stimulation was less effective when $K_{ir}6.2$ contained the R176A/R177A double mutation compared to wild-type. Subsequently, the application of phosphoinositides was shown to attenuate K_{ATP} channel inhibition by ATP, demonstrating functional antagonism between stimulation by phosphoinositides and inhibition by ATP (Baukrowitz et al., 1998; Shyng and Nichols, 1998; Fan and Makielski, 1999). Fan and Makielski (1999) proposed a speculative mechanism in which the phosphates of phosphoinositides and ATP compete at the inhibitory ATP binding site. They suggested that “the region T171-I182,” which includes the putative phosphoinositide binding site associated with increasing ligand-independent gating to the burst state, might also include the inhibitory ATP binding site. The ATP site, however, is unlikely to include position 176 because the substitution R176A had little or no effect on ATP inhibition (Baukrowitz et al., 1998). A recent report on the role of the neighboring residue K185 in channel inhibition by ATP concluded that ATP does not interact with the side chain of K185, but an interaction with the backbone or an indirect interaction of this residue with ATP remains possible (Reimann et al., 1999). An alternative hypothesis that combines all the results is that PIP_2 and the residues of this region all together do not directly contact ATP in the binding reaction but rather, as shown here for positively charged residues at 182, mediate the linkage between ATP binding and the gating mechanism.

Thus far our results do not distinguish between a role for I182 in the linkage of the ATP binding site to the gating mechanism or the ATP binding mechanism itself. On one hand, if the K_{ATP} channel tightly links the conformations of its gating states and ATP binding site (“ligand-independent linkage”), then either I182 substitutions disrupt the initial binding reaction with ATP or cleanly sever this linkage to explain the general lack of effect on gating. On the other hand, if linkage is engaged only when ATP binds (“ligand-dependent linkage”), then a linkage role fits well with the rare effect of charged substitutions on ligand-independent gating, and a role in the binding mechanism could not be discerned from these data. The latter ligand-dependent linkage mechanism is favored, as it explains the general lack of effect by 182 and distal mutations on gating in the absence of ligand as well as the rare effects of the positively charged substitutions at 182. In support of a ligand-dependent linkage mechanism underlying K_{ATP} channel inhibition by ATP, one of the strongest $K_{i,\ ATP}$ mutations, I182Q, was studied in the wild-type $K_{ir}6.2$ background co-expressed with SUR1. As is the case in the truncated $K_{ir}6.2$ΔC26 background expressed alone, the I182Q mutation strongly disrupted ATP inhibition but left ligand-independent gating indistinguishable from its wild-type counterpart. The results importantly indicate that the lack of effect of I182 substitution on linkage and gating mechanisms is not somehow due to the absence of SUR. We conclude that the lack of effect of I182 substitutions on ligand-independent gating likely reflects the nature of the mechanism underlying ATP inhibition of the K_{ATP} channel, and not the absence of an essential component of the linkage mechanism conferred by SUR.

Other regions of the K_{ATP} channel are likely to play an essential role in the ATP binding site. Mutations in the 334 region of $K_{ir}6.2$ can dramatically disrupt ATP-dependent gating with little or no effect on ligand-independent gating (Drain et al., 1998). The 334 region of the C-terminus includes an ATP-binding motif FX_4K that is highly conserved in the intracellular ATP-binding loop of ion-motive ATPases (McIntosh et al., 1996). The presence of this motif together with the lack of effect on ligand-independent gating make the 334 region a candidate for being part of the inhibitory ATP binding site.

In summary, the proposed ligand-dependent linkage model explains two remarkable features of the $K_{ir}6.2$ subunit of K_{ATP} channels. First, mutations including those in the 182–185 region shown here and by others (Tucker et al., 1998; Reimann et al., 1999) and the 334 region (Drain et al., 1998) can strongly disrupt ligand-dependent gating as measured by the K_i for ATP inhibition, but consistently with little or no effect on ligand-independent gating kinetics. This is generally unexpected of tightly coupled mechanisms (Monod et al., 1965) and suggests that the gating between burst and interburst can operate largely independently of changes at the ATP binding site in the absence of ligand, but the two become tightly coupled upon actual binding by ATP. Such ligand-induced linkage between occupied site and gate suggests induced-fit mechanisms (Koshland et al., 1966). Second, $K_{ir}6.2$ expression in the absence of the SUR subunit (Tucker et al., 1997; Drain et al., 1998; Babenko et al., 1999a; Alekseev et al., 1997; John et al., 1998) shows that the ligand-independent gating equilibrium can be changed to favor the interburst with a decrease—not an increase—in the apparent affinity for ATP binding. The ligand-dependent linkage model loosens the constraint that changes at the gate, and changes at the site always exhibit tight reciprocity. Thus the ligand-dependent linkage model better accommodates the opposite effects on ligand-independent gating and apparent ATP binding affinity observed in SUR-less K_{ATP} channels.

Overall, our results on the K_{ATP} channel are consistent with the proposal that channels gated by intracellular nucleotides share a common layout of C-terminal functional domains, with a proximal linking domain and a distal intracellular nucleotide binding site. The well-studied cyclic nucleotide-gated channel subunit contains a linking domain, the C-linker, in a proximal position and a separate cyclic nucleotide binding site in a distal position of the C-terminal cytoplasmic tail (Zagotta and Siegelbaum, 1996). Although mutations of both the C-linker of cyclic nucleotide-gated channels (Paoletti et al., 1999) and the I182 region of the K_{ATP} channels shown here profoundly affect ligand-dependent gating, these mutations do not affect the spontaneous, ligand-independent gating of their respective channels. Additional mutational analysis in the C-linker region of cyclic nucleotide-gated channels is consistent with the proposed role in linking the gating step with changes of the affinity of the channel for cyclic nucleotide (Gordon and Zagotta, 1995; Zhong et al., 1998). For the K_{ATP} channel, more detailed analysis of K_i mutations of $K_{ir}6.2$ combining both gating kinetics and physical measurements of ATP binding underlying channel inhibition will be important.

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