Role for SUR2A ED Domain in Allosteric Coupling within the $K_{\text{ATP}}$ Channel Complex

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Allosteric regulation of heteromultimeric ATP-sensitive potassium ($K_{\text{ATP}}$) channels is unique among protein systems as it implies transmission of ligand-induced structural adaptation at the regulatory SUR subunit, a member of ATP-binding cassette ABCC family, to the distinct pore-forming $K^+$ (Kir6.x) channel module. Cooperative interaction between nucleotide binding domains (NBDs) of SUR is a prerequisite for $K_{\text{ATP}}$ channel gating, yet pathways of allosteric intersubunit communication remain uncertain. Here, we analyzed the role of the ED domain, a stretch of 15 negatively charged aspartate/glutamate amino acid residues (948–962) of the SUR2A isoform, in the regulation of cardiac $K_{\text{ATP}}$ channels. Disruption of the ED domain impeded cooperative NBDs interaction and interrupted the regulation of $K_{\text{ATP}}$ channel complexes by MgADP, potassium channel openers, and sulfonylurea drugs. Thus, the ED domain is a structural component of the allosteric pathway within the $K_{\text{ATP}}$ channel complex integrating transduction of diverse nucleotide-dependent states in the regulatory SUR subunit to the open/closed states of the $K^+$-conducting channel pore.

INTRODUCTION

Adoption of different functional states in response to effector binding at a site distal from the catalytic site within homomeric systems, with one or more axes of molecular symmetry, defines the allosteric regulation of enzymes (Monod et al., 1965; Monod, 1966). This is the case with ATP-binding cassette (ABC) transporters where adenine nucleotide interactions with nucleotide binding domains (NBDs) drive structural rearrangement necessary for cargo translocation across the cell membrane (Linton and Higgins, 2007). Within the ABC superfamily, regulation of the ATP-sensitive $K^+$ ($K_{\text{ATP}}$) channel complex is unique as it relies not only on structural coupling and cooperativity, induced by nucleotide interaction within the ATPase-harboring regulatory sulfonylurea receptors (SUR), but also on transmission of such interaction to the associated pore-forming Kir6.x channel (Alekseev et al., 2005).

The $K_{\text{ATP}}$ channel heteromer is comprised of four SUR modules, members of the ABCC subfamily, and four $K^+$-conducting pores (Aguilar-Bryan et al., 1995; Inagaki et al., 1995; 1996; Tucker et al., 1997; Lorenz and Terzic, 1999; Nichols, 2006). SUR, like other ABCC proteins, consists of two bundles of six hydrophobic transmembrane-spanning domains (TMDs) fused to hydrophilic nucleotide-binding domains (NBDs) (TMD$_{\sigma}$-NBD1-TMD$_{\sigma'}$-NBD2) (Lorkowski and Cullen, 2002). An additional TMD$_{\sigma}$ module with five transmembrane domains (TMD$_{\sigma}$-TMD$_{\sigma'}$-NBD1-TMD$_{\sigma'}$-NBD2) anchors the channel pore to the SUR module (Babenko and Bryan, 2003; Fang et al., 2006; Hosy et al., 2007). While SUR shares the properties of ATP interaction and hydrolysis with ABC proteins (Bienengraeber et al., 2000; Matsuo et al., 2000), a catalysis-coupled transport function has not been identified for $K_{\text{ATP}}$ channels. Catalysis in SUR, while not required for ion permeation down the electrochemical gradient, has been implicated in allosteric regulation of the heteromeric $K_{\text{ATP}}$ channel complex (Zingman et al., 2001). In this way, the concept of allosteric regulation, developed for homomers, could be expanded to heteromers, where ligand-induced conformation adaptation within the regulatory module is translated to the functional state of the distal subunit.

While the allosteric hierarchy controlling $K_{\text{ATP}}$ channel operation has not been dissected, the proposed model of nucleotide-dependent channel gating (Abraham et al., 2002; Selivanov et al., 2004) implies that four identical binding sites for ATP and ATP/ADP coexist on the pore-forming and regulatory subunits, respectively, within the octameric complex (Clement et al., 1997). Binding of ATP to the Kir6.x pore inhibits channel opening (Tucker et al., 1997; Drain et al., 1998), whereas MgADP at the corresponding regulatory SUR subunit antagonizes ATP-induced pore closure (Shyng et al., 1997; Matsuo et al., 2000). Cooperative rather than individual contribution of SUR NBDs gate channel operation as

Abbreviations used in this paper: ABC, ATP-binding cassette; CL, cytoplasmic loop; HRP, horseradish peroxidase; KCO, potassium channel opener; NBD, nucleotide binding domain; SUR, sulfonylurea receptor; TMD, transmembrane-spanning domain.
ATP binding to NBD1, supported by hydrolysis at NBD2, is a necessary step in securing structural arrangement of both NBDs (Ueda et al., 1999; Matsuo et al., 2000, 2002; Zingman et al., 2002). The outcome on KATP channel pore state is not ultimately determined by the rate of NBD2-mediated catalysis at SUR (Zingman et al., 2001; 2002), but relies on the probability that NBD2 will adopt post-versus prehydrolytic conformations reflecting cooperative NBD interaction (Bienengraeber et al., 2004; Alekseev et al., 2005). Although this model is in agreement with experimentally defined nucleotide-dependent gating (Alekseev et al., 1998; Abraham et al., 2002), it does not elucidate the structural determinants underlying intersubunit communication.

Pharmacological KATP channel regulators, potassium channel openers and sulfonylurea drugs, albeit with distinct binding topologies within SUR also rely on nucleotide-dependent NBD states. Nucleotides and drugs induce an identical fingerprint in channel behavior, modulating termination of burst openings while maintaining an intact pattern of interburst kinetics (Alekseev et al., 1998; Bienengraeber et al., 2000). Potassium channel openers require cooperative NBD interaction, since drug effects are dependent on intracellular nucleotides and Mg2+, intact NBDs, and post-hydrolytic SUR conformations (Gribble et al., 1997; Schwanstecher et al., 1998; Bienengraeber et al., 2000; Ashcroft and Gribble, 2000; Zingman et al., 2001; Moreau et al., 2005). Biochemical studies further demonstrate that binding of sulfonylureas to SUR modulates cooperative interaction between NBDs, inducing ATP dissociation from NBD1 (Ueda et al., 1999). Further, neutralization of positively charged arginine moieties in Kir6.x produces a KATP channel phenotype insensitive to MgADP-induced activation and sulfonylurea-induced inhibition (John et al., 2001). These features of KATP channel regulation collectively indicate that conformational rearrangements of NBDs induced by exogenous (openers and sulfonylurea) or endogenous (MgADP) ligands are coupled with the open or closed state of the channel pore through a common allosteric pathway.

To serve as transducer of diverse signals to the channel pore, components of such a pathway should not be conserved among ABC proteins, which, unlike SUR, function as complete enzymatic modules and do not require communication with a functionally distinct protein. This domain should not interfere with critical binding sites for drugs or nucleotides, yet to facilitate readout of nucleotide binding it may be located in the vicinity of NBDs. Lastly, it may comprise a significant negative charge recognized in protein–protein interactions (Shin et al., 2000; Ma et al., 2001).

The present study analyzes an aspartate/glutamate-rich domain as a candidate common transducer securing allosteric regulation in the cardiac KATP channel complex, comprised of Kir6.2 and SUR2A channel isoforms. SUR2A contains a stretch of 15 negatively charged aspartate and glutamate residues, designated the ED domain adjacent to NBD1 within the sixth cytoplasmic loop (Fig. 1A). The ED domain does not overlap with known binding sites for KATP channel ligands (Aguilar-Bryan et al., 1998; Uhde et al., 1999; Moreau et al., 2000; Mikhailov et al., 2001; Gribble and Reimann, 2003). Sequence alignment revealed that the ED domain is not conserved among ABC subfamily members (Fig. 1B), yet it matches the location of the R domain in ABCC7 encoding the CFTR channel (Fig. 1A), which when phosphorylated permits nucleotide-dependent gating of Cl– conduction (Aguilar-Bryan et al., 1998; Ostgaard et al., 2001). Thus, the ED domain emerges as a candidate allosteric transducer within KATP channel complexes.

**MATERIALS AND METHODS**

**Recombinant KATP Channels**

SUR2A and Kir6.2 were subcloned into the pcDNA3.1 vector. Overlap PCR mutagenesis was used to create deletions and mutations within the acidic domain (Ho et al., 1989). KATP channel
respectively; tated SUR2A (Schwappach et al., 2000). Tagged SUR2A were co-

between transmembrane helices 16 and 17 of wild-type or mu-

ted by using HA tag incorporated in the extracellular loop [54x130]

and 40 mM Tris-HCl pH 7.5 at 37 °C for 3 min. Unbound 8-azido-

A surface expression of K ATP channel constructs were deter-

mined by using FuGene (Roche). Experiments were performed 48 – 72 h

Labeling protocols using 8-azido-[γ-32P]ATP followed established

post-transfection. (A) Amino acid composition of the wild-type (WT) 

Channel complexes measured as chemiluminescence from extracellu-

lar HA epitope–labeled WT versus ED domain–mutated SUR2A coex-

pressed with Kir6.2. For each construct, a p-value is provided indicating no significant

reduction in surface expression, except Kir6.2 alone, which was used as a nega-

tive control. Sample number is indicated in parentheses. (C) ATP concentration–

response curves for each of the ED mutants measured in the presence of Mg 2+

manifested by a rightward shift relative to wild type (WT). Curves are Hill plots constructed based on fitting results revealing the following

I C50 values: WT, 28 ± 3.3 μM (n = 8); entire deletion, 51 ± 4.6 μM (n = 6); first half deletion, 73 ± 3.7 μM (n = 3); second half deletion, 35 ± 4.1 μM (n = 5); entire Q/N mutant, 64 ± 4.9 μM (n = 6). (D) ATP concentration–response curves for WT, entire deletion, and entire Q/N mutant measured in the absence of Mg 2+, and characterized by indistinguishable IC50 values: 14 ± 3.7, 16 ± 3.3, and 15 ± 2.6 μM, respectively; n = 3 for each construct.

subunits were expressed in HEK293 cells, cultured in Dulbecco’s Modified Eagle’s Medium with 10% FBS and 2 mM glutamate at 5% CO 2 . Subunits were transiently transfected (5 μg SUR2A:1 μg Kir6.2) with 0.5 μg of the reporter gene GFP us-

ing FuGene (Roche). Experiments were performed 48–72 h post-transfection.

Photoaffinity Labeling
Labeling protocols using 8-azido-[γ-32P]ATP followed established

post-incubation procedures (Ueda et al., 1999). Membrane samples (40 μg) isolated from HEK293 cells cotransfected with wild-
type or mutated SUR2A and Kir6.2 were incubated with 100 μM 8-azido-[γ-32P]ATP, 3 mM MgCl 2 , 0.1 mM EGTA, 2 mM ouabain, and 40 mM Tris-HCl pH 7.5 at 37°C for 3 min. Unbound 8-azido-

-[γ-32P]ATP was removed after centrifugation (13,000 g for 5 min at 4°C). Pellets were resuspended with the same buffer and then

mixed with the indicated concentration of ADP. The mixture

solutions were incubated for 15 min at 37°C and then irradiated on ice at maximal output (∼1 J/cm²) for 8 min in a UVP CL1000 ultraviolet cross-linker. Samples were electrophoresed on 12% SDS-

PAGE and autoradiographed.

Membrane Expression of K ATP Channel Constructs
A surface expression of K ATP channel constructs were deter-

mined by using HA tag incorporated in the extracellular loop

between transmembrane helices 16 and 17 of wild-type or mu-
ted SUR2A (Schwappach et al., 2000). Tagged SUR2A were co-

transfected with Kir6.2. After 48 h, transfected cells were fixed with 4% formaldehyde for 15 min and washed twice with PBS. After 30 min of blocking (1% FBS in PBS), cells were incubated with monoclonal HA antibody 3F10 (Roche) for 1 h, washed, and incubated with goat anti-mouse horseradish peroxidase (HRP)–conjugated secondary antibody (20 min). After washing, power signal ELISA solution (Pierce Chemical Co.) was added and chemiluminescence read after 15 s in a plate reader (Molecular Devices). Chemiluminescence signal was normalized to the protein content.

Immunoblotting
Proteins were separated in 3–8% Tris-Acetate gel (Invitrogen)

from membrane samples isolated from HEK293 cells cotrans-

fected with Kir6.2 and wild-type or ED-mutated SUR2A. Res-

olved proteins were transferred by electrophoresis onto PVDF

membrane. The presence of SUR2A was probed by incubation

with SUR2A-specific antibody (Santa Cruz Biotechnology, Inc.)
counterstained by anti-goat IgG HRP conjugate. Membranes were

incubated in Super Signal West Pico Substrate (Pierce Chemical Co.), and signals captured on CL-XPosure film (Pierce Chemical Co.).

Electrophysiology
Recombinant K ATP channel activity was recorded using the inside-

cout configuration of the patch-clamp methodology at a membrane

potential of −60 mV. Patch pipettes contained (in mM) KCl 140,

CaCl 2 1, MgCl 2 1, HEPES-KOH 5 (pH 7.3). Cells were bathed in internal solution containing (in mM) KCl 140, MgCl 2 1, EGTA 5,

HEPES-KOH 5 (pH 7.3). After patch excision, K ATP channel activity

was measured in the same internal solution, supplemented by
drugs and/or nucleotides. Mg-free internal solution was supple-

mented by 5 mM EDTA with omitted MgCl 2 . Recordings were

performed at 32 ± 1°C using the HI-25x heating element and the TC2

temperature controller (Cell Micro Controls). Determination of the open state probability (P o ), through calculation of NP o values, was performed in channel recordings when a clear determination of the number of simultaneously operating K ATP channels (n < 10) was possible (Jovanovic et al., 1996). P o were calculated with known

N using obtained NP o values averaged for a 30–40-s-long duration of channel activity.
The ED domain mutants exhibited a moderate reduction in ATP sensitivity manifested by a rightward shift of MgATP concentration-dependent curves with the observed IC$_{50}$ ranging from 35 to 73 nM, relative to 28 nM measured for the wild type (Fig. 2 C). However, in the absence of Mg$^{2+}$, the ATP concentration-dependent relationships constructed for wild-type, entire deletion, and entire Q/N mutant K$_{ATP}$ channels revealed identical IC$_{50}$ values (Fig. 2 D), indicating that deletion or neutralization of negative charges of the ED domain did not affect apparent ATP binding to the channel pore. Inhibition of wild-type K$_{ATP}$ channels by 300 μM ATP was readily reversed by 300 μM MgADP (Fig. 3 A). While 300 μM ATP inhibited the ED mutant SUR2A/Kir6.2 channels similarly to the wild type, MgADP was unable to reverse such inhibition in ED mutants (Fig. 3, A and B). Thus, while the ED domain of SUR2A is not directly involved in ATP-induced pore inhibition, it is essential in mediating MgADP-dependent channel activation.

ED Domain Critical for Cooperative Interaction of NBDs

MgADP, through binding or as a product of MgATP hydrolysis at NBD2, stabilizes nucleotide binding to NBD1 of SUR, suggesting that cooperative interaction between NBDs is a critical conformational rearrangement for ADP-induced K$_{ATP}$ channel activation (Ueda et al., 1999; Matsuo et al., 2000; Zingman et al., 2002). To test the role of the ED domain in cooperative NBD interaction, the effect of ADP on photoaffinity labeling of membrane proteins by 8-azido-[$\gamma^{32}$P]ATP in cells coexpressing Kir6.2 with wild-type or ED-mutated SUR2A was assessed using a post-incubation procedure established to evaluate cooperative binding (Ueda et al., 1999). Untransfected cells, used as control, were photolabeled in the

**Figure 3.** Intact ED domain required for MgADP-dependent channel activation. (A) Mg-nucleotide-dependent K$_{ATP}$ channel gating was probed as the channel response to 0.3 mM MgADP in WT and ED mutants inhibited by 0.3 mM ATP. Disruption of the ED domain prevented MgADP-dependent channel activation in inside-out patches. Dotted lines represent zero-current levels. (B) Activation of ED domain mutants by MgADP is significantly blunted when compared with WT ($P < 0.05$) in inside-out patches. Channel activity expressed relative to that measured in the absence of nucleotides. Sample size is indicated in parentheses.

**RESULTS**

Role of the ED Domain in Nucleotide-dependent Channel Gating

To examine the role of the ED domain in K$_{ATP}$ channel regulation, deletion and mutations were made within this unique region of SUR2A. Specifically, three deletion mutants were constructed: first half deletion (Δ948–954), second half deletion (Δ955–962), and entire deletion (Δ948–962) (Fig. 2 A). In addition, to ensure that the effects with deletion mutants are not due to nonspecific shortening of the cytoplasmic loop, the entire Q/N mutant was made replacing negatively charged aspartates and glutamates with neutrally charged asparagines and glutamines (Fig. 2 A). Expression of Kir6.2 with the ED domain mutants generated channels that retained basic properties of K$_{ATP}$ channels. These included an identical single channel conductance (∼85 pS at 32°C) and intrinsic probability of the open state ($P_o$) measured based on $NP_o$ values in the absence of drug or nucleotides ($P_o = 0.76$-0.8), indicating unimpaired pore gating in ED mutants versus wild-type K$_{ATP}$ channel constructs (Fig. 2 A). All SUR2A ED domain mutants coexpressed with Kir6.2 produced vigorous K$_{ATP}$ channel activity, detected via patch-clamp, indicating unaltered surface expression compared with wild-type K$_{ATP}$ channels, further confirmed by chemiluminescence measured from extracellular HA epitope–labeled channel constructs (Fig. 2 B).

The ED domain mutants exhibited a moderate reduction in ATP sensitivity manifested by a rightward shift of MgATP concentration–dependent curves with the observed IC$_{50}$ ranging from 35 to 73 μM, relative to 28 μM measured for the wild type (Fig. 2 C). However, in the absence of Mg$^{2+}$, the ATP concentration–dependent relationships constructed for wild-type, entire deletion, and entire Q/N mutant K$_{ATP}$ channels revealed identical IC$_{50}$ values (Fig. 2 D), indicating that deletion or neutralization of negative charges of the ED domain did not affect apparent ATP binding to the channel pore. Inhibition of wild-type K$_{ATP}$ channels by 300 μM ATP was readily reversed by 300 μM MgADP (Fig. 3 A). While 300 μM ATP inhibited the ED mutant SUR2A/Kir6.2 channels similarly to the wild type, MgADP was unable to reverse such inhibition in ED mutants (Fig. 3, A and B). Thus, while the ED domain of SUR2A is not directly involved in ATP-induced pore inhibition, it is essential in mediating MgADP-dependent channel activation.

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the cytoplasmic loop 7 (CL7) between TM helices 13 and 14, as well as TM helices 16 and 17 of SUR2 (Uhde et al., 1999; Moreau et al., 2000). Specifically, 50 μM pinacidil antagonized the inhibition of wild-type K<sub>ATP</sub> channels by 300 μM ATP, reversing channel activity close to the maximum level measured in the absence of nucleotides (Fig. 5). In contrast, channel activity induced by the same concentration of pinacidil, in the presence of 300 μM ATP, was 30–50% lower for the ED domain mutants when compared with the wild-type counterpart (Fig. 5). Thus, pinacidil-induced antagonism of ATP-induced Kir6.2 pore was impaired, albeit not abolished, in ED domain mutants.

Role of ED Domain in Sulfonylurea-induced K<sub>ATP</sub> Channel Inhibition

On wild-type K<sub>ATP</sub> channels, the sulfonylurea glyburide (10 μM) exhibited a dual time course of inhibition, with fast and slow rates manifested by instantaneous blockade of only a fraction (59 ± 11%; n = 9) of the channel population (Fig. 6, A and B) followed by gradual dissipation of channel activity (93 ± 3%; n = 9) in the remaining subpopulation within 2–3 min (Fig. 6 A). Deletion of the entire ED domain generated a monophasic response to glyburide, with loss of the slow phase of inhibition and a reduced fast phase so that glyburide-induced reduction in overall channel activity was only 21 ± 5%; n = 3 (Fig. 6, A and B). Similarly, first half deletion, second half deletion, and the entire Q/N mutant produced channels with reduced glyburide responsiveness manifested absence or presence of MgADP in the range from 0.05 to 1.0 mM. Nonspecific labeling in untransfected samples was negligible and unaffected by ADP titration (Fig. 4 A). Due to inherent catalytic activity centered at NBD2, leading to hydrolysis of the ATP γ-phosphate, 8-azido-[γ-<sup>32</sup>P]ATP labeling reflects the presence of nucleotides at NBD1 that harbors low or no ATPase activity (Ueda et al., 1999; Bienengaerger et al., 2000; Matsuo et al., 2000; Fig. 4 B). Immunoblotting with a SUR2A-specific antibody validated that photolabeled bands in wild-type or ED domain mutants corresponded to SUR2A (Fig. 4 C). In the absence of MgADP, the entire deletion and Q/N mutants demonstrated reduced 8-azido-[γ-<sup>32</sup>P]ATP labeling compared with wild type (Fig. 4, B and D). After titration with MgADP, wild-type constructs exhibited increased 8-azido-[γ-<sup>32</sup>P]ATP labeling, reaching a maximum at 500 μM, consistent with previous findings (Ueda et al., 1999). In contrast, MgADP within the applied concentration range did not affect labeling of the ED-mutated constructs (Fig. 4, B and E). Thus, an intact ED domain is critical for cooperative NBD interaction and may therefore be essential for readout and transmission of nucleotide interactions to the channel pore.

Role of ED Domain in Opener-induced K<sub>ATP</sub> Channel Activation

ED disruption blunted the activating effect of the potassium channel opener (KCO) pinacidil (Fig. 5), although the ED domain does not overlap with binding sites for pinacidil and related cyanoguanidines that interact with the cytoplasmic loop 7 (CL7) between TM helices 13 and 14, as well as TM helices 16 and 17 of SUR2 (Uhde et al., 1999; Moreau et al., 2000). Specifically, 50 μM pinacidil antagonized the inhibition of wild-type K<sub>ATP</sub> channels by 300 μM ATP, reversing channel activity close to the maximum level measured in the absence of nucleotides (Fig. 5). In contrast, channel activity induced by the same concentration of pinacidil, in the presence of 300 μM ATP, was 30–50% lower for the ED domain mutants when compared with the wild-type counterpart (Fig. 5). Thus, pinacidil-induced antagonism of ATP-induced Kir6.2 pore was impaired, albeit not abolished, in ED domain mutants.

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primarily by a modest slow phase of glyburide-induced inhibition compared with wild type (Fig. 6, A and B). Thus, the ED domain contributes to sulfonylurea-induced K<sub>ATP</sub> channel inhibition affecting to various degrees both phases of glyburide-mediated inhibition.

To assess the mechanism underlying the dual phase of the K<sub>ATP</sub> channel response to glyburide, the effect of the sulfonylurea was tested on wild-type channels. Removal of glyburide did not reverse current inhibition, which required reapplication of ATP (Fig. 6 A), suggesting that at least the slow phase of inhibition is pertinent to the nucleotide-dependent state of the channel complex and resembles the described phenomenon of K<sub>ATP</sub> channel rundown (Trube and Hescheler, 1984; Findlay and

Figure 5. Intact ED domain required for potassium channel opener–induced channel activation. (A) Disruption of ED domain blunts the activating effect of pinacidil (50 μM) on recombinant K<sub>ATP</sub> channels in the presence of inhibitory concentration of ATP (300 μM). (B) In all mutant constructs, the effect of pinacidil was significantly blunted when compared with WT channels (P < 0.05). Number of values averaged for each experimental condition is indicated in parentheses. Channel activity expressed relative to that measured in the absence of nucleotides and drugs.

Figure 6. Role of ED domain in glyburide-induced K<sub>ATP</sub> channel inhibition. (A) All ED domain mutants, in comparison with WT, exhibited an essentially monophasic response toward glyburide (10 μM), with little to no slow phase. (B) K<sub>ATP</sub> channel activity was measured at the end of the fast phase and at the end of 3 min after glyburide application (slow phase) and expressed relative to the control activity in the absence of drugs and nucleotide. The effect of the drug on ED-mutated constructs was significantly different from WT (P < 0.05) except for the fast phase measured in the second half deletion mutant. Number of values averaged for each experimental condition is indicated in parentheses.
At a membrane potential of $-60$ mV and a single channel conductance of 85 pS, the single channel current amplitude was estimated at $\sim 5$ pA and at a given level of protein expression and open state probability of $\sim 0.8$, patches with estimated 100–200 operating recombinant channels were selected to ensure discrimination of the probabilistic properties of the time course of K$_{ATP}$ channel closure (Fig. 7 A). In the presence of 50 $\mu$M MgATP, glyburide induced only a fast phase of K$_{ATP}$ channel inhibition. In the presence of 300 $\mu$M MgADP, glyburide maintained the dual pattern of K$_{ATP}$ channel blockade as measured without nucleotides, but both phases of inhibition were blunted. In the presence of AMP-PNP, a nonhydrolyzable ATP analogue, the profile of glyburide inhibition was virtually indistinguishable from the effect of glyburide in the absence of nucleotides. (B) Number of values averaged for each experimental condition is indicated in parentheses. Fast and slow phases of glyburide inhibition were quantified as in Fig. 6 B. (C) In the presence of hydrolyzable ATP (50 $\mu$M), glyburide-induced channel inhibition was reversed after washout of glyburide. (D) AMP-PNP (50 $\mu$M) did not prevent channel rundown initiated by the sulfonylurea and did not support restoration of channel activity after removal of glyburide.

Dunne, 1986; Nichols and Lederer, 1991; Tung and Kurachi, 1991; Findlay, 1994; Terzic et al., 1994). One of the contributing conditions of K$_{ATP}$ channel rundown is a fall in PIP$_2$ levels, which however is a slow process lasting $>1$ h following nucleotide removal (Ribalet et al., 2000), and therefore per se cannot explain the slow phase of glyburide-induced channel inhibition that occurs with a significantly faster time course. To test whether the slow phase of glyburide-induced channel inhibition could be accounted based on nucleotide-dependent states of SUR2A, glyburide action was further probed in the presence of adenine nucleotides or the nonhydrolyzable AMP-PNP analogue known to induce distinct NBD formations (Gribble et al., 1998b; Matsuo et al., 2000; Zingman et al., 2001; Alekseev et al., 2005; Nichols, 2006).

Figure 7. Biphasic glyburide-induced channel inhibition of wild-type K$_{ATP}$ channels. (A) In the absence of nucleotide only a fraction of the channel population present in the patch (100–200 channels) was instantaneously blocked by the sulfonylurea glyburide (10 $\mu$M). The rest manifested gradual and eventually complete dissipation of activity after glyburide application. Reapplication and removal of ATP (300 $\mu$M) restored K$_{ATP}$ channel activity. In the presence of 50 $\mu$M MgATP, glyburide induced only a fast phase of K$_{ATP}$ channel inhibition. In the presence of 300 $\mu$M MgADP, glyburide maintained the dual pattern of K$_{ATP}$ channel blockade as measured without nucleotides, but both phases of inhibition were blunted. In the presence of AMP-PNP, a nonhydrolyzable ATP analogue, the profile of glyburide inhibition was virtually indistinguishable from the effect of glyburide in the absence of nucleotides. (B) Number of values averaged for each experimental condition is indicated in parentheses. Fast and slow phases of glyburide inhibition were quantified as in Fig. 6 B. (C) In the presence of hydrolyzable ATP (50 $\mu$M), glyburide-induced channel inhibition was reversed after washout of glyburide. (D) AMP-PNP (50 $\mu$M) did not prevent channel rundown initiated by the sulfonylurea and did not support restoration of channel activity after removal of glyburide.
accomplished by dissociation of ATP from NBD1. Such NBDs/ED domain conformational rearrangement is critical for transduction of inhibitory channel gating originating from SUR2A to pore closure. (C) Disruption of the ED domain stabilizes the NBDs of SUR2A in a suboptimal conformation characterized by residual communication between constitutive channel subunits. Hypothetic occlusion of MgADP within such suboptimal NBD conformation endows moderate antagonism of ATP-induced pore inhibition (right shift of ATP concentration responses), partial channel activation by KCO, and impaired MgADP-induced channel opening. The inflexibility of this NBD formation in the absence of the ED domain precludes glyburide-induced K$_{ATP}$ channel inhibition, yet prevents the rundown process associated with nucleotide-dependent states of SUR2A.

glyburide maintained the dual pattern of K$_{ATP}$ channel blockade as measured without nucleotides, but both phases were blunted (Fig. 7 A). Although it partially antagonized sulfonylurea-induced channel inhibition as previously reported (Gribble et al., 1998a; Reimann et al., 2003), MgADP apparently could not equivalently replace MgATP in supporting nucleotide-driven NBD formation. This is further supported by the observation that in the presence of AMP-PNP, a nonhydrolyzable analogue of ATP, the profile of glyburide inhibition was indistinguishable from the effect of glyburide in the absence of nucleotides (Fig. 7, A and B). While in the presence of hydrolyzable ATP, channel inhibition could be reversed (Takano et al., 1990; Furukawa et al., 1994), following washout of glyburide, the nonhydrolyzable ATP analogue did not prevent channel rundown initiated by the sulfonylurea and did not support restoration of channel activity after removal of glyburide (Fig. 7, C and D). Restoration of channel activity after glyburide treatment in the presence of AMP-PNP required MgATP (Fig. 7 D). Taken together, these observations suggest that the effect of the sulfonylurea glyburide relies upon nucleotide-dependent conformations of SUR2A associated with its catalytic function involving cooperative interaction between NBDs (Ueda et al., 1999; Matsuo et al., 2000; Zingman et al., 2001, 2002). Thus, disruption or neutralization of the ED domain interrupted allosteric communication of channel inhibition induced by a sulfonylurea at SUR2A to the K$_{ATP}$ channel pore.

**DISCUSSION**

Gating of the cardiac K$_{ATP}$ channel complex implies transduction of ligand-induced conformational transitions in the regulatory SUR2A module to the Kir6.2 pore. In the present study, the negatively charged ED domain was identified as a structural component within SUR2A contributing to allosteric signal communication between constitutive K$_{ATP}$ channel subunits. Specifically, K$_{ATP}$ channel regulation by MgADP, potassium channel openers, and sulfonylurea drugs, which relies on nucleotide-dependent NBD states, was dependent on the intactness of the ED domain. Indeed, while the ED domain does not overlap with known ligand binding sites, deletion or charge neutralization of this acidic domain impeded K$_{ATP}$ channel pore function in response to either activating or inhibitory SUR2A ligands. The strategic location of the ED domain downstream of NBD1, in the cytoplasmic loop 6 (CL6) between TMD1 and TMD2, facilitated readout of cooperative NBD formation communicated to the channel pore. Whether communication of SUR2A and Kir6.2 involves direct electrostatic interaction via the ED domain or implies additional intermediate structural components remains to be determined and will require more detailed elaboration of the K$_{ATP}$ channel architecture.

**ED Domain Equivalents in ABC Proteins**

Although the ED domain is not conserved among other ABCC proteins, structural alignment indicated topological equivalence of the ED domain in SUR with the R domain in CFTR channels, a related family member. In fact, PKA-mediated phosphorylation of serine residues within the R domain negatively charges this region, permitting Cl$^-$ conductance by the CFTR protein (Rich et al., 1993; Seibert et al., 1999; Ostedgaard et al., 2001) presumably after cooperative NBD interaction (Ostedgaard et al., 1997; Aleksandrov et al., 2001, 2002; Vergani et al., 2003; Howell et al., 2004). This effect is mimicked by mutating serines to negatively charged aspartate residues, allowing CFTR operation in the absence of PKA phosphorylation.
Both NBDs as well as the engagement of NBD2 into post-hydrolytic conformations are prerequisites for $K_{\text{ATP}}$ channel activation by MgADP and potassium channel openers (Gribble et al., 1997; Schwanstecher et al., 1998; Bienengraeber et al., 2000; Zingman et al., 2001). Disruption of the ED domain antagonized $K_{\text{ATP}}$ channel opening induced by MgADP and the potassium channel opener pinacidil. This could be explained by destabilization of NBD dimer formation required for channel activation (Gribble et al., 1998b; Zingman et al., 2002; Alekseev et al., 2005). Moreover, the observed minor rightward shift of MgATP (but not Mg-free ATP)-induced $K_{\text{ATP}}$ channel inhibition in the ED mutants despite loss of MgADP efficacy could result from stabilization of the NBD2 ATPase cycle in a post-hydrolytic conformation (Bienengraeber et al., 2004). Although an alternative pathway for potassium channel openers cannot be excluded (Russ et al., 2003), the residual effectiveness of potassium channel openers measured in the ED domain mutants could also be related to the stabilization of such post-hydrolytic conformation (Schwanstecher et al., 1998; Zingman et al., 2001). Thus, the intact ED domain secures optimal cooperativity of NBDs and proper $K_{\text{ATP}}$ channel activation, whereas disruption in the ED domain locks NBDs in a suboptimal state, allowing only residual communication between SUR2A and the channel pore (Fig. 8).

**ED Domain Controls Cooperative NBD Interaction**

Nucleotide-dependent gating of $K_{\text{ATP}}$ channels is coupled to the catalytic function of NBD2 and requires nucleotide coordination at NBD1 (Gribble et al., 1997, 1998b; Zingman et al., 2001, 2002; Matsuo et al., 2002), implying determinate linkage between catalysis-driven SUR conformations and pore gating (Alekseev et al., 2005). Evidence for conformational association of SUR NBDs stems from the dimeric structures of distinct ABC proteins (Hung et al., 1998; Diederichs et al., 2000; Hopfner et al., 2000; Campbell et al., 2003) and is supported by direct labeling studies (Ueda et al., 1999; Matsuo et al., 2000). Mutations in the ED domain were here found to disrupt cooperative nucleotide binding at NBD. Thus, the ED domain is integral to the allosteric machinery controlling the readout of NBD states essential for optimal channel regulation (Fig. 8).

**ED Domain Mediates $K_{\text{ATP}}$ Channel Activation**

Both NBDs as well as the engagement of NBD2 into post-hydrolytic conformations are prerequisites for $K_{\text{ATP}}$ channel activation by MgADP and potassium channel openers (Gribble et al., 1997; Schwanstecher et al., 1998; Bienengraeber et al., 2000; Zingman et al., 2001). Disruption of the ED domain antagonized $K_{\text{ATP}}$ channel opening induced by MgADP and the potassium channel opener pinacidil. This could be explained by destabilization of NBD dimer formation required for channel activation (Gribble et al., 1998b; Zingman et al., 2002; Alekseev et al., 2005). Moreover, the observed minor rightward shift of MgATP (but not Mg-free ATP)-induced $K_{\text{ATP}}$ channel inhibition in the ED mutants despite loss of MgADP efficacy could result from stabilization of the NBD2 ATPase cycle in a post-hydrolytic conformation (Bienengraeber et al., 2004). Although an alternative pathway for potassium channel openers cannot be excluded (Russ et al., 2003), the residual effectiveness of potassium channel openers measured in the ED domain mutants could also be related to the stabilization of such post-hydrolytic conformation (Schwanstecher et al., 1998; Zingman et al., 2001). Thus, the intact ED domain secures optimal cooperativity of NBDs and proper $K_{\text{ATP}}$ channel activation, whereas disruption in the ED domain locks NBDs in a suboptimal state, allowing only residual communication between SUR2A and the channel pore (Fig. 8).

**ED Domain and Inhibitory Channel Gating**

The reduced sensitivity of ED-mutated $K_{\text{ATP}}$ channel constructs toward the inhibitory effect of glyburide, which interacts by its benzamido moiety with the cytoplasmic loop 3 (CL3) of SUR2 between TMD0 and TMD1 (Mikhailov et al., 2001), may also be explained by a suboptimal NBD
state induced by disruption of the ED domain. The present findings provide a framework for a mechanistic model of channel regulation to interpret the nucleotide dependence of glyburide inhibition of K\textsubscript{ATP} channels (Fig. 9). Upon removal of ATP, a population of K\textsubscript{ATP} channels remains in a nucleotide-bound state in line with cooperative dimer conformation of SUR2A NBDs. This is in accord with SUR properties to stabilize bound ATP and MgADP at NBD1 and NBD2, respectively (Ueda et al., 1999; Matsuo et al., 2000). When glyburide is added, two phases of inhibition are observed. Binding of the sulfonylurea to dimerized SUR disrupts cooperative interaction of NBDs and promotes ATP dissociation from NBD1 (Ueda et al., 1999) allosterically associated with immediate pore closure (Fig. 9A). The K\textsubscript{ATP} channel subpopulation that at glyburide application is outside of cooperative NBD interaction can resist sulfonylurea-induced channel closure, and contributes only to the slower phase of channel inhibition characterized as sulfonylurea-accelerated rundown. This is supported by glyburide-induced K\textsubscript{ATP} channel inhibition in the presence of 50 \mu M MgATP (Fig. 9B), an ATP concentration that prevents rundown and, although insufficient per se to keep channel closed, can apparently support cooperative interaction of NBDs. Under this condition, virtually the whole K\textsubscript{ATP} channel population is primed for sulfonylurea-induced channel block manifested by exclusively fast inhibition (Fig. 9B). MgADP blunted both phases of glyburide-induced K\textsubscript{ATP} channel inhibition in line with the known property of nucleotide diphosphates to prevent rundown, and the inability to mimic ATP-bound SUR conformations that are most sensitive to sulfonylurea (Nichols and Lederer, 1991; Tung and Kurachi, 1991; Aleksseev et al., 1998; Reimann et al., 2003). Thus, the attenuated sensitivity of ED domain mutants to glyburide, which revealed essentially a monophasic profile of channel inhibition, could indicate that deletion or neutralization of negative charges in the ED domain stabilizes the NBDs in suboptimal conformation, rendering K\textsubscript{ATP} channels less sensitive to rundown. Collectively, sulfonylurea, which binds to SUR2A, induced pore inhibition through a common NBD-dependent mechanism involving the ED domain–mediated pathway (Fig. 8).

In conclusion, transmission of ligand-induced structural adaptation of NBDs at the SUR2A module to the channel pore defines the operation of cardiac K\textsubscript{ATP} channels. The present study identifies the acidic ED domain of SUR2A as a structural component of allosteric K\textsubscript{ATP} channel gating integrating transduction of diverse nucleotide-dependent states in the regulatory module to the K\textsuperscript{+}-conducting channel pore. In this way, the identified role for the ED domain in allosteric coupling within the K\textsubscript{ATP} channel complex could provide a foundation for the further understanding of structure–function relationships among ABC proteins.

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