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RESEARCH PAPER

Probing the mechanisms underlying modulation of quinidine sensitivity to cardiac I_{Ks} block by protein kinase A-mediated I_{Ks} phosphorylation

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Background and purpose: Cardiac I_{Ks} is enhanced by protein kinase A (PKA) stimulation. And PKA-stimulated I_{Ks} is about threefold less sensitive to quinidine block than basal current. In this study, we further tested two competing hypotheses: I_{Ks} phosphorylation either (i) modulates access of blocking drugs to a binding site; or (ii) destabilizes the drug–channel interaction.
Experimental approach: To distinguish between these hypotheses, we studied quinidine block of I_{Ks} channels in which three PKA site residues of the α-subunit KCNQ1 were mutated with a bulky negative charged aspartic acid (D). To study alleviation of I_{Ks} block by quinidine, we compared activating current at +60 mV, either with or without 5 s hyperpolarizing prepulses to –120 mV.
Key results: Without PKA stimulation, quinidine (100 μM) blocked wild-type current to a similar extent with and without the prepulse (93 ± 2% of pre-drug current at +60 mV vs. 95 ± 1%). With PKA-stimulated wild-type channels, however, there was less block with the hyperpolarization to –120 mV: at +60 mV, block was 71 ± 2% (–prepulse) versus 58 ± 3% (+prepulse). Individual D-mutations and the triple-D mutant were resistant to quinidine block similar to that seen with PKA-stimulated wild-type I_{Ks}.
Conclusions and implications: We conclude that phosphorylation-induced insertion of bulky negative charges alleviates quinidine block and that PKA-induced stimulation, by conferring negative charges to the channels, blunts I_{Ks} block as the interaction between the channels and blockers becomes destabilized. These effects would be of clinical significance in providing protective mechanisms against pro-arrhythmias caused by drug-induced inhibition of I_{Ks} and I_{Kr}.
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Keywords: KCNQ1; I_{Ks}; protein kinase A (PKA) sites; PKA stimulation; negative charged residue; quinidine block; whole-cell voltage clamp

Abbreviations: CHO, Chinese hamster ovary; GFP, green fluorescent protein; I_{KCNQ1}, the KCNQ1 current; I_{Ks}, the slowly activating delayed rectifier potassium current; KCNE1, the gene that encodes the β-subunit of cardiac I_{Ks} channel complex; KCNQ1, the gene that encodes the α-subunit of cardiac I_{Ks} channel complex; PKA, protein kinase A

Introduction

Cardiac repolarization involves multiple outward K^+ currents. Among these, the slowly activating delayed rectifier (I_{Ks}) is an important repolarizing component that underlies cardiac repolarization reserve (Roden, 1998). The I_{Ks} channel is mainly formed by KCNQ1 and KCNE1 (Barhanin *et al.*, 1996; Sanguinetti *et al.*, 1996): KCNQ1 is the pore-forming α-subunit of the channel; whereas KCNE1 acts predominantly as a regulatory β-subunit of the channel complex, although other KCNE subunits (e.g. KCNE2-5) may interact with KCNQ1 to enable variable modulation of I_{Ks} (Lundquist *et al.*, 2005). I_{Ks} acts as a major source in maintaining cardiac repolarization reserve (Roden, 1998; Roden and Yang, 2005), especially during β-adrenoceptor stimulation that markedly increases I_{Ks} (Sanguinetti *et al.*, 1991), an effect that shortens cardiac action potential duration through acceleration of cardiac repolarization. It is speculated that an increase in I_{Ks} would strengthen repolarization reserve against the

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pro-arrhythmic risk under conditions where other K^+ channels (especially HERG) are suppressed by drugs, or when these channels are down-regulated in some diseases or suffer loss of function in congenital long QT syndromes (Jost *et al.*, 2007).

Protein kinase A (PKA) is a cAMP-dependent serine/threonine kinase. During activation of β-adrenoceptors, intracellular cAMP binds to PKA and initiates phosphorylation of serines or threonines on target proteins, including ion channels. Analysis of the KCNQ1 sequence reveals one 'typical' PKA consensus site in the N-terminal residue 27 (RRG**S**, Ser27) and multiple 'atypical' PKA sites (such as Ser468/Thr470) in the C-terminus (Figure 1). We have previously shown that stimulation of PKA directly increases the KCNQ1 channel current, a PKA-mediated phosphorylation effect, as ablation of PKA target sites on the ion channel protein altered the response to PKA stimulation (Yang *et al.*, 2003). And the PKA-stimulated increase in I_{Ks} is blunted but not eliminated when the 'typical' PKA site mutant S27A is expressed with or without KCNE1 co-expression; others have also reported similar findings (Lo and Numann, 1998; Boucherot *et al.*, 2001), although another group has suggested that the S27A mutant channel is totally unaffected by PKA (Marx *et al.*, 2002).

Cardiac I_{Ks} channels are drug targets. The β-adrenoceptor agonist isoprenaline increases I_{Ks} and accelerates cardiac repolarization, presumably through activation of the PKA-signalling pathway discussed above. Small molecule I_{Ks} activators (e.g. stilbenes, fenamates and L-364,373) have been described (Salata *et al.*, 1998; Abitbol *et al.*, 1999). In addition, blockers of both I_{KCNQ1} (the KCNQ1 current) and I_{Ks} have been also reported. Chromanol 293B (Bosch *et al.*, 1998), L-735,821 (Seebohm *et al.*, 2003) and HMR-1556 (Thomas *et al.*, 2003) are selective I_{Ks} blockers that have the ability to prolong action potentials. Many anti-arrhythmics are also non-selective I_{Ks} blockers: quinidine (Balser *et al.*, 1991; Yang *et al.*, 2003), amiodarone (Balser *et al.*, 1991), azimilide (Busch *et al.*, 1994) and clofilium (Yang *et al.*, 1997).

In a previous study we found that PKA-stimulated I_{Ks} and I_{KCNQ1} are about threefold less sensitive to block by drugs (e.g. quinidine) than basal currents, and the onset of drug block is markedly slowed by PKA stimulation (Yang *et al.*, 2003). These findings let us to propose two competing hypotheses: channel phosphorylation either (i) limits access of blockers to a binding site; and/or (ii) destabilizes the drug–channel interaction. To distinguish between these two hypotheses, therefore, we further studied quinidine (un)trapping and (un)blocking in wild-type I_{Ks} and channels in which three KCNQ1 cytoplasmic serine/threonine residues (S27, S468 and T470) targeted by the PKA-mediated phosphorylation were mutated to a bulky permanent negative charge (aspartic acid, D). We found that insertion of bulky negative charges by phosphorylation or by aspartate mutations alleviated drug block, supporting the concept that PKA stimulation destabilizes the interaction between KCNQ1 channels and blocking drugs.

Methods

Site-directed mutagenesis

The nomenclature for the ion channels used in this work conforms to 'Guide to receptors and channels' by *British Journal of Pharmacology* (Alexander *et al.*, 2008). Typical and atypical PKA sites of the KCNQ1 were identified by sequence analysis (Figure 1), with a broadly used online prediction programme (http://www.cbs.dtu.dk/services/NetPhos/). Multiple PKA site mutations were individually made by substitution with alanine (A) or a bulky permanent negative charge (aspartic acid, D) by recombinant PCR mutagenesis.

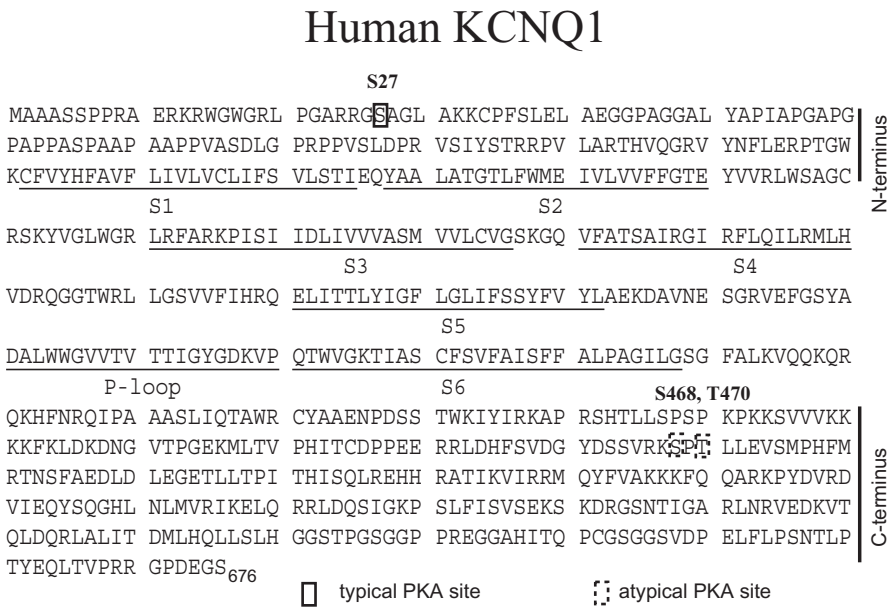

Figure 1 Human cardiac KCNQ1 sequence and protein kinase A (PKA) sites. Residue S27 (solid box) in the N-terminus is considered a typical PKA phosphorylation site, while residues S478 and T470 (dash box) in the C-terminus are atypical PKA sites. These PKA sites were predicted by a commonly used online programme (http://www.cbs.dtu.dk/services/NetPhos/).

The A-substituted mutation was a triple construct (S27A + S468A + T470A). The D-substituted mutations included individual S27D, S468D and S27D + S468D + T470D. For each mutation, four primers were used: two were mutagenic primers, and two were flanking primers. The mutagenic primers contained the desired point mutation at their centre and were complementary to each other. The flanking primers were complementary to target sequences 5′ and 3′ of the desired mutation. They were chosen to abut convenient restriction sites to allow those sites to be used for re-insertion into the target sequence. The target sequence was denatured, and the mutagenic primers were annealed and extended by using Taq polymerase. The two extension products carrying the point mutations close to their 5′ ends were then allowed to anneal and were then extended, creating a DNA duplex with the mutation roughly at the centre. The mutagenic primers were then removed by using a QIAquick PCR purification kit following the manufacturer's instructions. The two flanking primers were then used in a standard PCR reaction by using the mutated DNA as template. The resulting mutated PCR fragment was then inserted into the target sequence via the restriction sites within the flanking primers. Standard PCR conditions used to accomplish these reactions included 10–100 ng of template DNA, 40 pmol each primer, 10 mM Tris (pH 9.0, room temperature), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM each dATP, dCTP, dGTP, dTTP and 5 units of Taq DNA polymerase. The temperature profiles used typically were: 95°C for 5 min at which point the polymerase was added. This was followed by 30 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min. A final extension step for 5 min at 72°C was then added. To optimize the PCR conditions, Mg^{2+} concentration ranged from 1 to 3 mM, and the annealing temperatures ranged from 53 to 62°C. Duplicate mutated cDNA fragments were sequenced to ensure that polymerase or other errors had not been introduced.

Electrophysiology, FuGENE6-mediated channel expression and cell transfection

Wild-type or mutant KCNQ1 was cloned into pCI/CMV-vector, while KCNE1 was cloned into a bicistronic vector that also included green fluorescent protein (GFP).

Chinese hamster ovary (CHO) cells were used for transient transfections. The cells lack endogenous outward currents and are thus suitable for K^+ current studies. The cells were grown to confluence in F-12 nutrient mixture (HAM) medium supplemented with 10% horse serum at 37°C. To generate I_{Ks}, the cells were transiently transfected with KCNQ1 and the bicistronic KCNE1-IRES-eGFP (pCI/CMV) plasmids by using 2 μg of ion channel plasmid and 12 μL of FuGENE6 in 0.5 mL serum-free medium for 30 min, after which standard medium was restored for 48 h in culture. In experiments using KCNQ1 alone to generate the current (I_{KCNQ1}), a second plasmid encoding only GFP was co-transfected.

Cells showing green fluorescence were chosen to identify successfully transfected cells for the voltage–clamp analysis. The cells were removed from the dish by brief trypsinization and stored in standard medium for the experiments within the next 12 h.

Whole-cell voltage clamp

The voltage–clamp studies were performed by using the same methods as previously reported (Balser *et al.*, 1991; Yang *et al.*, 2003; Kanki *et al.*, 2004). To determine the percentage of I_{Ks} reduction by a blocking drug (quinidine), activating current was elicited with 5 s single repetitively depolarizing pulse from the holding potential of –80 to +60 mV, and tail current was recorded upon return to –40 mV. Pulses were delivered every 30 s, and drug block was measured at the end of activating I_{Ks}. Whole-cell voltage–clamp experiments were conducted to assess drug block kinetics at 22–23°C.

To study whether quinidine is trapped in the channel or relieves from block, we compared the unblocked portion of the activating current at the end of a 5 s pulse to +60 mV, either with or without a 5 s prepulse to a hyperpolarizing potential of –120 mV. Quinidine was used at two concentrations (10 and 100 μM). The rationale for using this hyperpolarization protocol is described below (see *Results*). Two protocols with and without this hyperpolarization to –120 mV were applied in order at each experiment. First, after basal steady-state current had been recorded with 5 s repetitive pulses to +60 mV from a holding potential of –80 mV, cells were subjected to 5 min of drug exposure without pulsing, and then pulses to +60 mV were delivered to establish steady-state block. Second, when block reached a stable level, 5 s hyperpolarizing pulses to –120 mV were then applied at 30 s intervals for another 5 min to allow channels to re-enter closed states, and thus untrapped drug to leave the channel (Mitcheson *et al.*, 2000). To detect the unblocked current (as alleviation of block), depolarizing pulses to +60 mV were given again from –120 mV. The percentage of the unblocked current was compared in the absence and presence of PKA stimulation. D-substituted mutants were also examined in the same fashion. These two voltage–clamp protocols were used in order in the same cell, shown in Figure 2.

Minimizing I_{Ks} run-down

I_{Ks} run-down with time after rupture of the cell membrane has been a concern when studying the current in isolated cardiac myocytes (Harvey and Hume, 1989; Balser *et al.*, 1991) and heterologously expressed systems, such as CHO and COS cells (Yang *et al.*, 2000; Caballero *et al.*, 2001; Loussouarn *et al.*, 2003). Such run-down is variably seen with the corresponding recombinant KCNQ1 construct (±KCNE1). The run-down is associated with loss or dilution of some intracellular compositions, which help stabilize the ion channel activity by exchanging the pipette-filling solution with the intracellular milieu in the course of cell dialysis (Harvey and Hume, 1989; Balser *et al.*, 1990).

In the present study, therefore, four conditions were used to minimize I_{Ks} run-down according to literature (Giles and Shibata, 1985; Balser *et al.*, 1990; Sanguinetti and Jurkiewicz, 1990; Balser *et al.*, 1991): use of short-shanked small tip (3–5 mΩ) electrodes, elevation of $[K^+]_i$ to ~235 mM, maintenance of negative pressure inside the electrode and of bath temperature at 22–23°C. By using these methods, cell dialysis was minimized, and changes of driving force and osmolarity due to possible K^+ leakage from the pipette into the cell were negligible. In experiments with the two $[K^+]_i$, we did not see

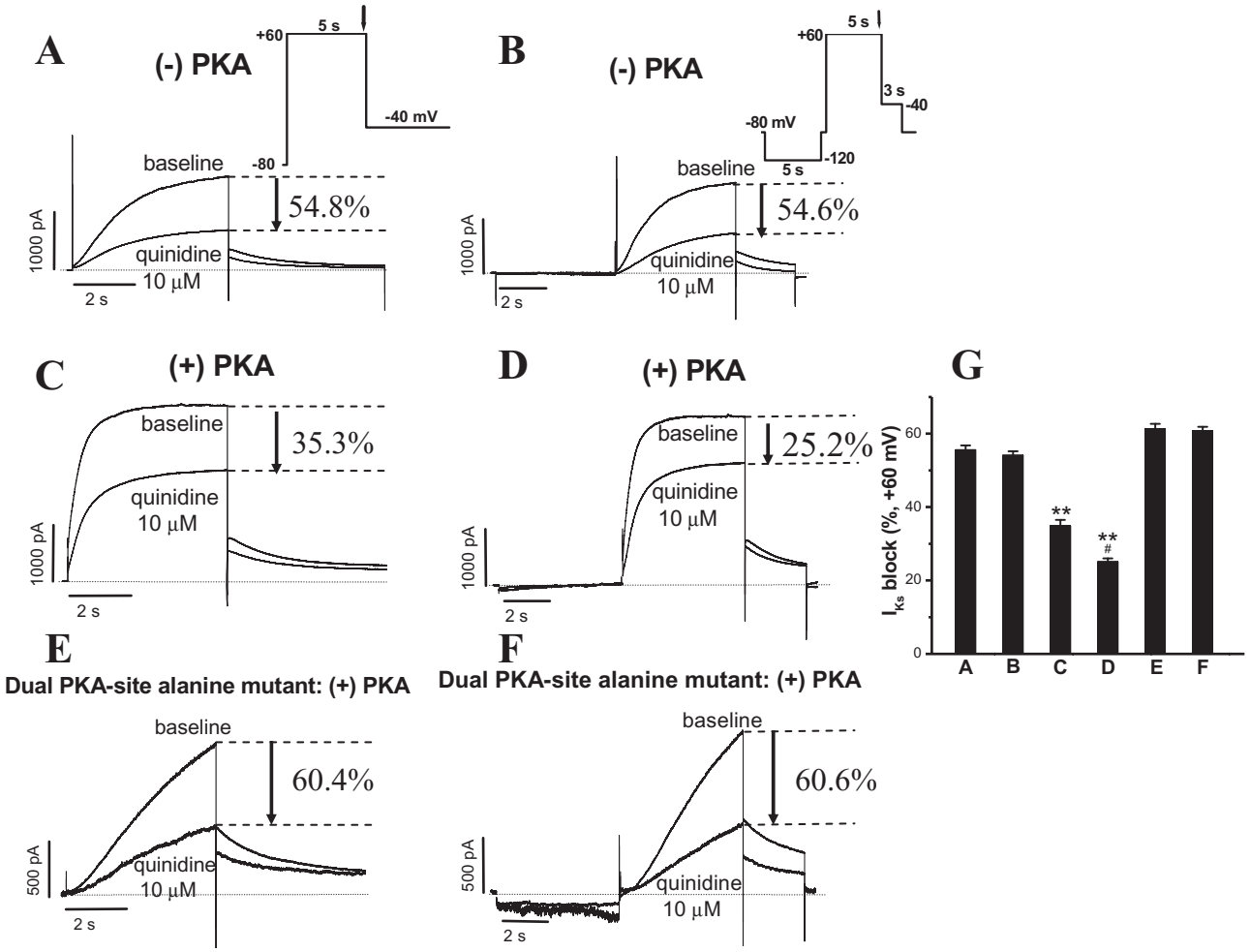

Figure 2 Block induced by quinidine and its alleviation in wild-type and PKA site mutated channels with or without PKA stimulation. After stable current had been established, two voltage–clamp protocols shown were applied in order to the same cell to determine block, and the reduction of this block, induced by quinidine at a concentration of 10 μM (~IC_{50}) in the absence and presence of PKA stimulation. In (A), (C) and (E), a regular 5 s single repetitive pulse protocol without a hyperpolarizing prepulse to –120 mV was used to elicit the current to compare I_{Ks} magnitudes prior to and after drug in the absence and presence of the PKA stimulating cocktail. In (B), (D) and (F), a 5 s hyperpolarizing repetitive pulse to –120 mV was applied prior to pulsing to +60 mV. The current traces in (A,B), (C,D) and (E,F) were obtained from three different cells respectively. (G) A summary of data from different groups of cells (n = 7 each) shown in (A–F) with the two voltage–clamp protocols. $^{**}P < 0.01$ versus four other groups; $^{\#}P < 0.05$ versus the group for (C). I_{Ks}, the slowly activating delayed rectifier potassium current; PKA, protein kinase A.

any differences in the properties of I_{Ks} (e.g. magnitude and gating), except for the fact that the current run-down was minimized. However, we do recognize that even under these conditions, I_{Ks} run-down cannot be completely prevented. To ensure the results obtained were accurate, cells with >50% run-down were still excluded. For experiments in which the effects of drugs on I_{KCNQ1} or I_{Ks} with and without PKA stimulation were determined, data were obtained after the current had stabilized in the absence and presence of the PKA cocktail or drug; as PKA stimulation increased the current, there was no ambiguity about the effect during the studies.

Solutions and drugs

The internal pipette filling solution contained (in mM): KCl 200, K_4BAPTA 5, K_2ATP 5, $MgCl_2$ 1 and HEPES 10. The solution was adjusted to pH 7.2 with KOH. To assess the stability of currents over time and the drug effect, repetitive pulses to +60 mV from –80 mV are used pre- and post-drugs. The external solution was normal Tyrode's, containing (in mM) NaCl 130, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10 and glucose 10, and was adjusted to pH 7.35 with NaOH. Quinidine was used as an I_{Ks} blocker at a concentration of 10 μM (~IC_{50}) or 100 μM. To observe the inhibitory effect, cells were exposed to quinidine for 20 min before any recordings were performed. The PKA stimulating 'cocktail' consisted of forskolin (10 μM) and 8'-Br-cAMP (200 μM) and was incubated with the cells for 30 min, as necessary. The drugs were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). HAM medium, Invitrogen (Carlsbad, CA, USA); FuGENE6, Roche (Indianapolis, IN, USA).

Statistical analysis

Data are expressed as mean ± s.e.mean. For comparisons among means of more than two groups, ANOVA was used, with

post hoc pair-wise comparisons by Duncan's test if significant differences among means were detected. If only two groups were being compared, Student's *t*-test was used. A *P*-value <0.05 was considered statistically significant.

Materials

cDNAs for wild-type human KCNQ1 and KCNE1 were provided by Dr Michael Sanguinetti (University of Utah) and Dr Mark Keating (currently at Novartis Institute for Biomedical Research, Cambridge, MA, USA). QIAquick PCR purification kit was obtained from QIAGEN Inc. (Chatsworth, CA, USA).

Results

PKA-mediated I_{Ks} phosphorylation in wild-type and PKA site mutated channels

Figure 3 shows I_{Ks} or I_{KCNQ1} run-down phenomenon after the cell membrane had been ruptured in this study. When the current stabilized after the run-down in the absence and presence of the β-subunit KCNE1, addition of a PKA-stimulating 'cocktail' then resulted in an increase in current. Previously, we have shown that WT-I_{Ks} increase by the PKA stimulation was reduced (but not eliminated) in a S27A or S468A/T470A mutation with or without KCNE1 co-expression. In the dual mutant (containing S27A and S468A/T470A), however, the effect of PKA stimulation was completely abolished. These results strongly indicate that I_{Ks} phosphorylation requires an integration of both typical and atypical PKA sites in the KCNQ1 channel. In good agreement with other studies (Lo and Numann, 1998; Boucherot *et al*., 2001), these results were obtained in the absence of co-expression with an exogenous PKA anchoring protein (AKAP) yotiao (Marx *et al*., 2002), which is believed to act as a partner of the I_{Ks} channel complex to permit PKA to have an effect. Even so, the possibility that some endogenous AKAP(s) may mediate a PKA-stimulated increase in the current cannot be excluded, because a cell line that does not express AKAPs has not yet been described.

Quinidine trapping in the I_{Ks} channel as a potential blocking mechanism

During cell membrane depolarization, it is thought that the pore region of S6 segment in K^+ channels can open wide enough to allow blocking drugs to enter from the cytoplasm and bind to the sites in the inner pore (Yellen, 2002). When the channels deactivate at closure, movement of the pore region may limit drug dissociation and exit from the channel vestibule. This effect can prolong blockade through trapping of drug molecules inside the channels. This trapping mechanism has been described for the K^+ channel blockers TEA (Armstrong, 1971) and MK-499 (Mitcheson *et al*., 2000), the

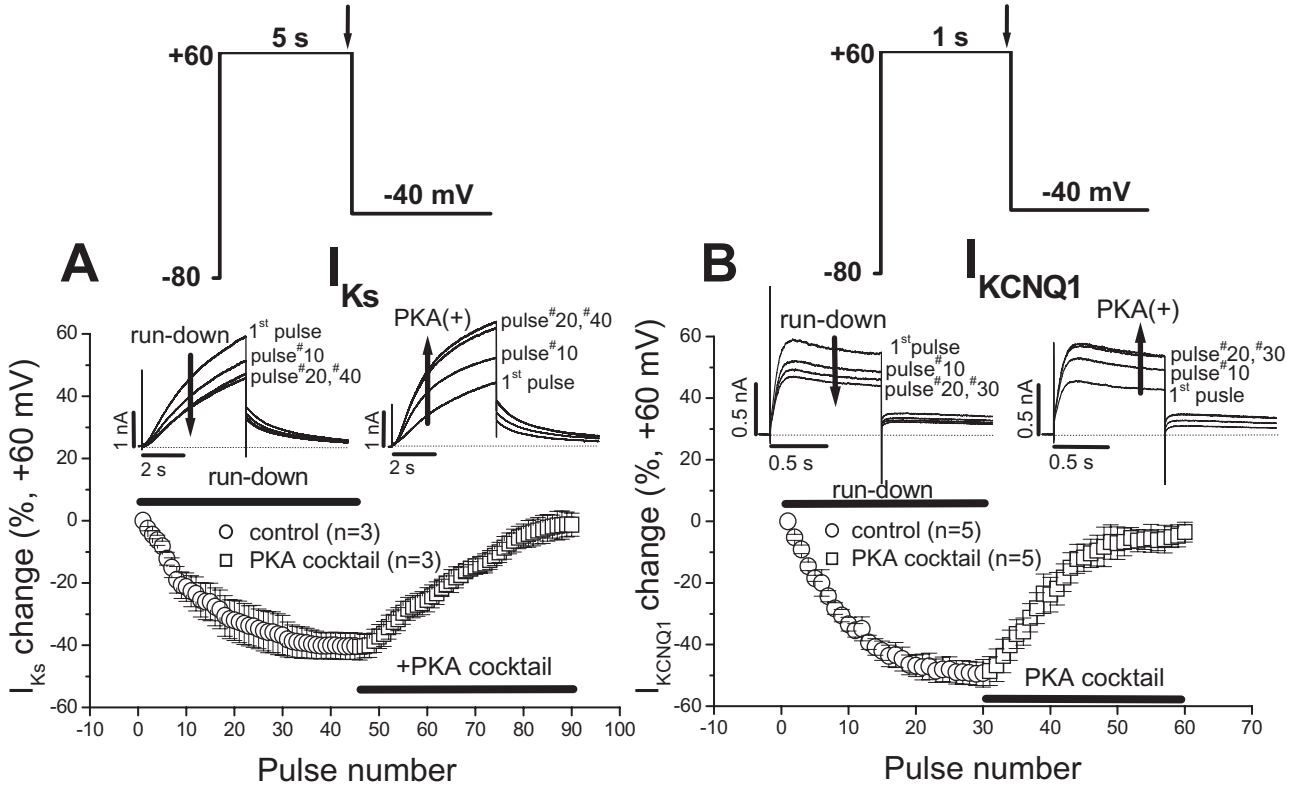

Figure 3 Changes of I_{KCNQ1} and I_{Ks} prior to and after PKA stimulation. As the voltage–clamp protocols shown, activating currents were elicited with repetitive pulses to +60 mV from the holding potential of −80 mV for 1 (I_{KCNQ1}) or 5 s (I_{Ks}), and tail currents were recorded upon repolarization to −40 mV at 22–23°C. Pulses were delivered at intervals of 15 s (for I_{KCNQ1}) or 30 s (for I_{Ks}). Representative traces are shown as insets. I_{KCNQ1}, the KCNQ1 current; I_{Ks}, the slowly activating delayed rectifier potassium current; KCNQ1, the gene that encodes the α-subunit of cardiac I_{Ks} channel complex; PKA, protein kinase A.

Na^+ channel blocker flecainide (Ramos and O'leary, 2004) and the pacemaker blocker ZD7288 (Shin *et al.*, 2001). Therefore, we hypothesized that quinidine trapped in the I_{Ks} channel could be a potential blocking mechanism.

To test this hypothesis, we first examined the effect of quinidine on I_{Ks} in the absence and presence of PKA stimulation using two voltage–clamp protocols (Figure 2). The sequence of the two protocols is described above (see *Methods*). When the cell is hyperpolarized during exposure to a blocking drug, three potential effects can be generated (Carmeliet, 1992; 1993; Mitcheson *et al.*, 2000): (i) minimizing drug binding to the closed-state channel at a very negative membrane potential, (ii) favouring unbinding of a positively charged drug in the channel; and (iii) facilitating drug trapping by rapid closure of the activation gate in the channel .

Accordingly, we hypothesized that in PKA-phosphorylated or D-mutant I_{Ks} channels, a hyperpolarizing pulse would either (i) enhance block if drug access is modulated; or (ii) leave the partial current unblocked if the drug–channel interaction is destabilized. Figure 2 shows the experimental results to test this hypothesis. In Figure 2A, C and E from three individual cells, I_{Ks} block of quinidine (10 μM, ~IC_{50}) was tested in the wild-type and dual PKA site alanine mutant channels in the absence and presence of PKA stimulation when a regular 5 s single pulsing protocol was repetitively applied. Both wild-type channel (Figure 2A without the PKA cocktail) and dual alanine mutant channel (Figure 2E with the PKA cocktail) responded to quinidine block to a similar extent (55–60% reduction in these examples), indicating that while the dual alanine mutant channel lost responses to PKA stimulation, it retained wild-type drug sensitivity. In the presence of PKA cocktail, however, block of wild-type I_{Ks} by quinidine was blunted with only ~35% reduction (Figure 2C), further supporting our previous results (Yang *et al.*, 2003) Why did quinidine strongly suppress the current in the wild-type channel without PKA stimulation or in dual PKA site alanine mutant with PKA stimulation? One possible explanation is that the drug may be trapped in the channel under these two conditions.

To determine whether such drug trapping mechanism occurs under these conditions, we examined I_{Ks} alleviation from block by quinidine. In these particular experiments, basal steady-state current was first recorded with regular 5 s repetitive pulses to +60 mV from a holding potential of –80 mV at 30 s intervals (the protocol in Figure 2A). Cells were then hyperpolarized to –120 mV and depolarized to +60 mV again to establish a new stable current (Figure 2B). During exposure to 10 μM quinidine, the two protocols were repeated in order. Delivery of 5 s repetitive hyperpolarizing pulses to –120 mV would allow channels to re-enter closed states and thus untrapped drug to leave the channels, because membrane hyperpolarization favours unbinding of a positively charged drug (such as quinidine) in the channel (Mitcheson *et al.*, 2000). Then, depolarizing pulses to +60 mV were again used to record whether the unblocked current exists.

These experiments were conducted in the absence and presence of PKA stimulation. We predicted that PKA-mediated phosphorylation promotes channel unblocking and limits drug trapping inside the channels. Should phosphorylation-induced drug untrapping or alleviation from block be observed, a logical next step will be to repeat these protocols in PKA site alanine mutated phosphorylation-resistant and D-substituted channel mutants. To further confirm this possibility, we used a hyperpolarizing pulsing protocol to determine whether or not the unblocked current was recorded under the three conditions shown in Figure 2. In the absence of PKA stimulation, obviously, the hyperpolarizing protocol did not reveal any unblocked component of the wild-type channel (Figure 2B) by 10 μM quinidine, compared with Figure 2A. This effect was similar in the dual PKA site alanine mutant channel (Figure 2F). In the presence of PKA stimulation, surprisingly, the hyperpolarizing protocol revealed only ~25% of current block (Figure 2D) in this experiment, with a 10% reduction in block compared with that shown in Figure 2C. A summary of these results is presented in Figure 2G. These findings were further confirmed by using a 10-time higher concentration of quinidine (100 μM). In the absence of PKA stimulation, 100 μM quinidine suppressed wild-type current to a similar extent with and without the hyperpolarizing prepulse to –120 mV (93 ± 2% vs. 95 ± 1%, $P > 0.05$, $n = 4$ each). With PKA-stimulated wild-type channels, however, a reduced block was seen with the hyperpolarizing prepulses to –120 mV: block was 71 ± 2% without prepulse versus 58 ± 3% with prepulse ($P < 0.05$, $n = 4$ each). This finding strongly indicates that PKA-mediated phosphorylation results in less sensitivity of I_{Ks} channel to quinidine block by promoting drug untrapping in the channel.

The triple-D-substituted mutant restores resistance to quinidine block seen with PKA-stimulated wild-type channels

Next, we tested whether substitution of serine and/or threonine in the PKA sites with a bulky negatively charged residue, aspartic acid (D), altered quinidine block in a manner similar to the PKA-stimulated wild-type channel. In this series of experiments with the use of same two protocols shown in Figure 2, multiple D-substituted PKA site mutant channels were tested for I_{Ks} block by a high concentration of quinidine (100 μM), and compared with the wild-type channel with and without PKA stimulation. Without PKA stimulation, 100 μM quinidine suppressed the wild-type current by 95 ± 1% (Figure 4A,D), whereas such a high concentration of drug only reduced the current by 71 ± 2% ($P < 0.01$) in the presence of PKA stimulation (Figure 4B,D). This blunted block was mimicked with the triple-D-substituted PKA site mutant (Figure 4C,D). The effects of quinidine on individual wild-type and mutant channels are summarized in Figure 4D. These results indicate that substitution of typical and/or atypical PKA sites in the KCNQ1 channel with a bulky negatively charged aspartic acid (D) significantly reduces the sensitivity of the channel to quinidine block, as seen in the wild-type channel after application of PKA stimulators.

Another piece of evidence supporting this argument is presented in Figure 5, which compares the effects of a repetitively applied depolarizing pulse with the use of a hyperpolarizing prepulse to –120 mV (Figure 5B). While a high concentration of quinidine (100 μM) blocked non-PKA-stimulated wild-type current by ~100% (Figure 4A), the triple-D mutant displayed only a 50% reduction (Figure 5A) by quinidine. When the

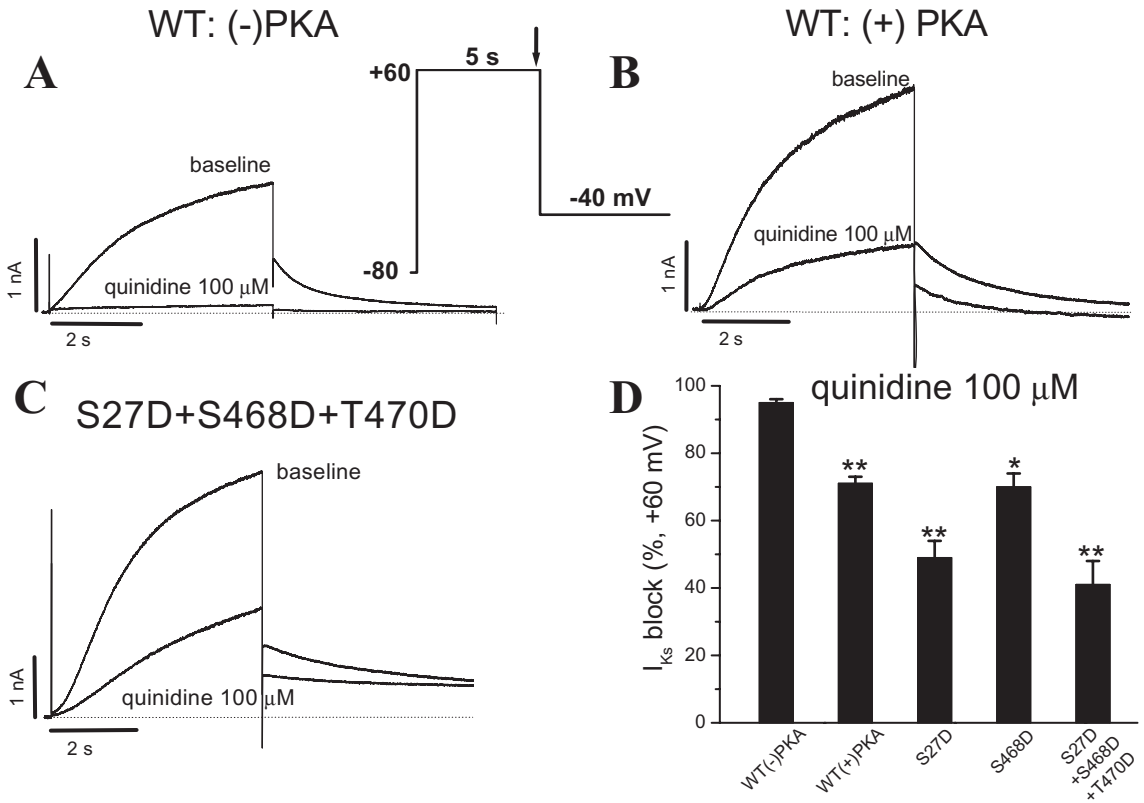

Figure 4 Blunted block of I_{Ks} (the slowly activating delayed rectifier potassium current) by quinidine. (A) and (B) Block of wild-type (WT) I_{Ks} by 100 μM quinidine in the absence and presence of PKA stimulation. The mutant channels were those in which the PKA sites of KCNQ1 channel were substituted with a negatively charged amino acid (aspartic acid, D). The blunting of quinidine block by PKA stimulation was restored in the triple-D protein kinase A (PKA) site substituted mutant (C). (D) A summary of the block of I_{Ks} by quinidine in wild-type and individual D-mutant channels. The number of cells was four to six in each group: $*P < 0.05$ or $**P < 0.01$ versus WT(–)PKA.

hyperpolarizing prepulse was used, further alleviation of drug block was observed, with only 35% block (Figure 5B). In the triple-D PKA site mutation, I_{Ks} block over time with these two protocols is compared in Figure 5C. With the prepulse protocol, the percent of the block alleviated in four groups of experiments is summarized in Figure 5D. These results suggest that promotion of drug untrapping is related to enhanced exit of drug from the channel. This effect nearly exactly restores the result we observed in the wild-type channel under PKA stimulation.

Discussion

In this study, we have further explored the mechanism(s) underlying PKA-mediated I_{Ks} phosphorylation and modulation of quinidine sensitivity to I_{Ks} block by using aspartic acid (D)-substituted PKA site mutants in the KCNQ1 channel and specifically designed protocols. Taken together with results from our previous study (Yang *et al.*, 2003), these data indicate that PKA-mediated phosphorylation of cardiac I_{Ks} channel reduces quinidine block by (i) limiting drug access to a binding site; and (ii) decreasing quinidine block by preventing drug from trapping in the channel.

We have previously demonstrated that PKA stimulation eliminates first pulse-induced quinidine block of I_{Ks} and dramatically slows the onset of block (Yang *et al.*, 2003), suggesting that PKA stimulation limits drug access to a binding site in the channel. To further test this concept, a logical next step was to determine whether the effects occur with phosphorylation-deficient mutants, and with mutants in which a permanent negative charge (D) was introduced at phosphorylation sites, as described above. Most drugs access channels that they block via intracellular routes (Mitcheson *et al.*, 2000; Perry *et al.*, 2004; Mitcheson *et al.*, 2005; Perry *et al.*, 2007). Because quinidine and most other blockers are neutral or positively charged at physiological pH, addition of a negative charge in the N- and/or C-terminus of the KCNQ1 channel is unlikely to explain blunted drug block. However, it is possible that negative charges in the N- or C-termini interact with positive charges on the intracellular face of the protein to limit drug access to a binding site, or even to interact with the binding site itself. We found that in the triple-D-substituted PKA site mutant, an increase in the current amplitude was similar to that seen in the PKA-stimulated WT-I_{Ks} (Figure 4B,C, baseline) and PKA-stimulation did not further increased the triple-D mutant generated current. In addition, all individual D-substituted mutants were resistant to quinidine block (Figure 4D). This finding strongly suggests that a negative charge at these PKA sites is a critical modulator of drug block.

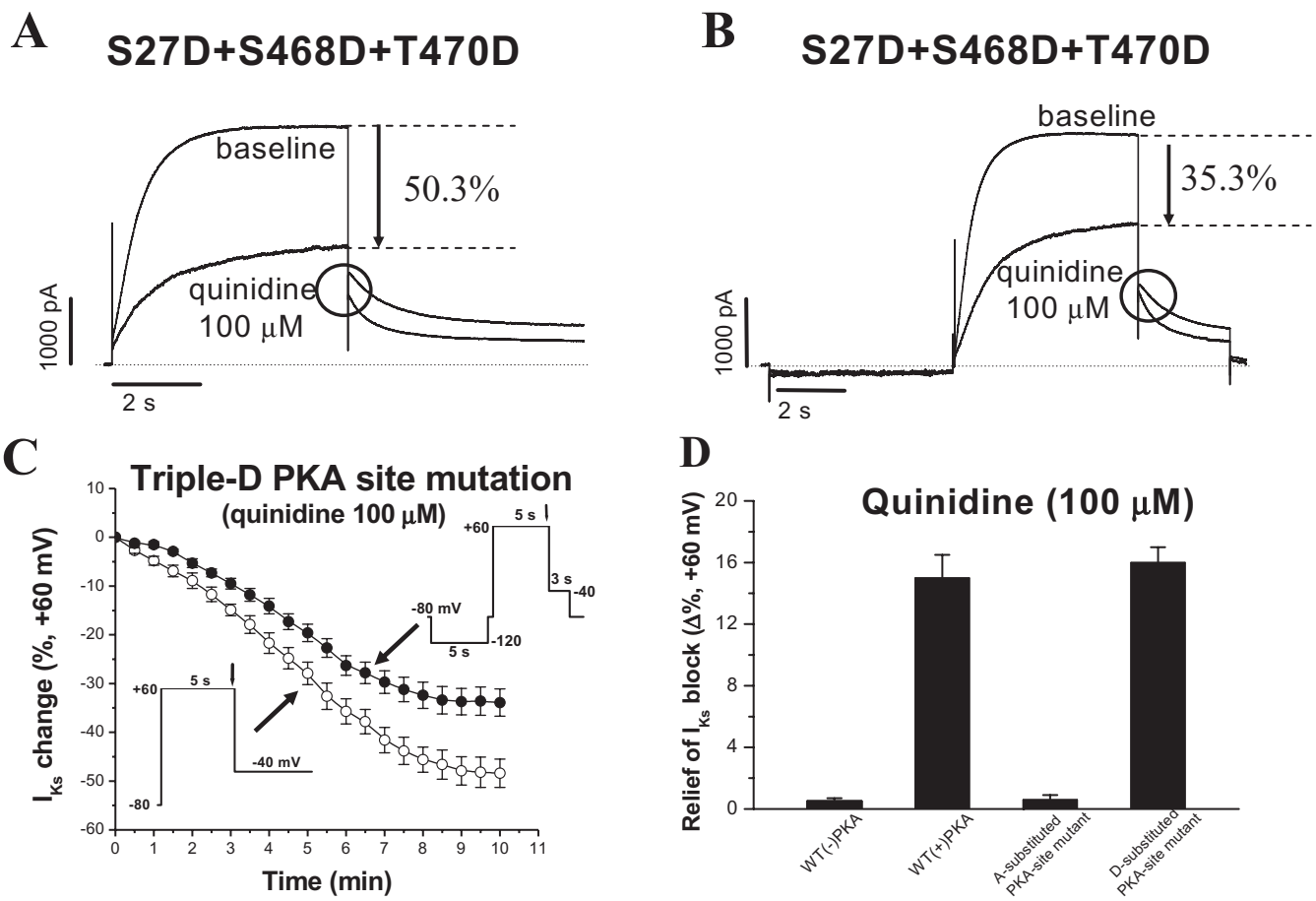

Figure 5 Alleviation of the I_{Ks} block by quinidine in the triple-D-substituted PKA site mutant channel. A hyperpolarizing prepulse to –120 mV provided even further relief of block in the Triple-D PKA site mutant channel (B), compared with the block in (A). The changes in tail currents are highlighted. (C) Shows that steady-state activating I_{Ks} changes over time during applications of the two voltage–clamp protocols to the same cell in two groups ($n = 4$ each). In the triple-D mutant channel, the reduction of the blocked current induced by quinidine was virtually identical to that seen in wild-type channel with PKA stimulation (D; cell numbers were four in each group). I_{Ks}, the slowly activating delayed rectifier potassium current; PKA, protein kinase A.

KCNQ1, along with most K^+ channels (except HERG), is thought to have a short and narrow inner pore cavity, which may act to limit drug trapping inside the channels when the channel closes (Yellen, 2002). However, the idea that phosphorylation or insertion of a negative charge decreases the sensitivity of ion channels to blocking drugs has not been tested. To probe this issue, we further determined whether (i) block of I_{Ks} by quinidine results partially from a trapping mechanism; and (ii) PKA stimulation prevents drug from being trapped in the KCNQ1 channel. Using the protocols specifically designed to address these issues, it becomes obvious that quinidine can be trapped in the channel upon channel closure following the channel opening. The drug trapping and block were partially alleviated either by the PKA-mediated phosphorylation of I_{Ks} channel, or by insertion of bulky negative charged residue (aspartic acid, D) in the KCNQ1 channel.

It is generally believed that I_{Ks} plays little role in cardiac action potential repolarization in the absence of increased sympathetic tone (Varro *et al.*, 2000; Han *et al.*, 2001; Volders *et al.*, 2003; Jost *et al.*, 2005). During sympathetic stimulation, however, an increase in I_{Ks} may provide an important 'braking' function to limit cardiac action potential prolongation (Han *et al.*, 2001). Recent studies provide further evidence that the I_{Ks} acts as a major source of repolarization reserve that protects against pro-arrhythmias during I_{Kr} inhibition or down-regulation (Jost *et al.*, 2005; Roden and Yang, 2005; Silva and Rudy, 2005; Roden, 2006; Roden, 2008). Our study found that block of I_{Ks} by quinidine was attenuated or alleviated during the PKA stimulated I_{Ks} increase that thereby strengthens repolarization reserve. In clinical therapeutics, these effects would be of great significance in preventing the pro-arrhythmic risk from excessive prolongation of cardiac repolarization during drug blockade of I_{Ks} and other K^+ currents (such as I_{Kr} and I_{To}).

In conclusion, therefore, our studies have demonstrated that insertion of bulky negative charges in the KCNQ1 channel alleviates block by promoting quinidine untrapping in the channel, and therefore strongly supports this as a mechanism for reduced drug block in PKA-stimulated channels. This suggests that PKA-mediated phosphorylation of KCNQ1 channels blunts quinidine block of I_{Ks} by destabilizing the interaction between the channels and the blocking drugs through conferring negative charges to the channels.

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Conflict of interests

The authors state no conflict of interest.

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