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Block of Kcnk3 by Protons

EVIDENCE THAT 2-P-DOMAIN POTASSIUM CHANNEL SUBUNITS FUNCTION AS HOMODIMERS*

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KCNK subunits have two pore-forming P domains and four predicted transmembrane segments. To assess the number of subunits in each pore, we studied external proton block of Kcnk3, a subunit prominent in rodent heart and brain. Consistent with a pore-blocking mechanism, inhibition was dependent on voltage, potassium concentration, and a histidine in the first P domain (P1H). Thus, at pH 6.8 with 20 mM potassium half the current passed by P1H channels was blocked (apparently via two sites ~10% into the electrical field) whereas channels with an asparagine substitution (P1N) were fully active. Furthermore, pore blockade by barium was sensitive to pH in P1H but not P1N channels. Although linking two Kcnk3 subunits in tandem to produce P1H-P1N and P1N-P1H channels bearing four P domains did not alter these attributes, the mixed tandems P1H-P1N and P1N-P1H were half-blocked at pH ~6.4, apparently via a single site. This implicates a dimeric structure for Kcnk3 channels with two (and only two) P1 domains in each pore and argues that P2 domains also contribute to pore formation.

Leak currents are key to normal electrical activity of sympathetic ganglia, myelinated axons, carotid body type 1 cells, and cardiac myocytes (1). 50 years after their description (2, 3) leak currents have been revealed to pass via potassium channels formed by subunits with two P domains (1). Preceded by TOK1, a non-voltage-gated outward rectifier from Saccharomyces cerevisiae with eight predicted membrane-spanning segments (a 2P/8TM topology) (4), the first canonical leak channel, KCNK0, was isolated from Drosophila melanogaster and has a predicted 2P/4TM topology (5–7). To date, 15 mammalian genes (KCNK1–15) have been identified to encode subunits with a 2P/4TM topology (1). Family members that show function (KCNK0, -2, -3, -4, -5, -6, -9, -10, -13, and -15) are potassium-selective leak channels; open at rest, they pass potassium without apparent delay in response to changes in membrane potential across the physiological voltage range. The functional repertoire of KCNK channels is, however, more varied, as phosphorylation can reversibly transform cloned and hippocampal KCNK2 between an open and voltage-dependent phenotype (8). Other KCNK subunits have yet to reveal their biophysical attributes (KCNK1, -7, -8, and -12).

Previously, we studied the genomic structure, cardiac localization, and biophysical properties of murine Kcnk3 (9). Thus, at steady-state, Kcnk3 channels behave like potassium-selective, transmembrane holes inhibited by physiological levels of protons. With voltage steps, Kcnk3 currents activate (or decay) in two phases; one phase appears to be instantaneous, and another appears to be time-dependent (τ ~ 5 ms). Both proton block and time-dependent gating are potassium-sensitive, producing an anomalous increase in outward flux as external potassium levels rise because of decreased proton block. At physiological levels of potassium, proton inhibition at ~120 mV is half-maximal at ~pH 7.6, indicating that Kcnk3 is poised to sense and respond to changes in pH value. Moreover, Kcnk3-like channels have been recorded in rat central nervous system nuclei, where they were shown to influence spike frequency in response to volatile anesthetics and neurotransmitters (10–14), and in murine cardiac myocytes, where they appear to contribute to the action potential plateau current called IkP (9, 15). These findings suggest a potential role for Kcnk3 in the physiology of cerebral and cardiac acidosis.

Four KCNK channels are notably sensitive to extracellular pH in the physiological range, (KCNK3, -5, -9, and -15) (1, 16). Kcnk3 and -9 appear to be closely related sharing 54% amino acid identity and a histidine at position 98 in the first P domain (P1) in the midst of signature sequence residues that form the potassium selectivity filter (GYGH); KCNK5 and -15 do not possess this histidine. Kim et al. (17) have demonstrated that histidine 98 in KCNK9 confers sensitivity to external pH. In this study, we demonstrate a similar role for histidine 98 in Kcnk3 and employ this finding to evaluate subunit stoichiometry. Our findings support the conclusions that there are two P1 domains present in the channel that contribute equivalently to pore formation and, thus, that two Kcnk3 subunits assemble to form each channel.

MATERIALS AND METHODS

Molecular Biology—Cloning of Kcnk3 (GenBank® accession number AB008537) and the pbP2 vector have been described (9). Point mutants were produced by Pfu mutagensis with a QuikchangeTM kit (Stratagene, Inc., La Jolla, CA) followed by insertion of mutant gene fragments into translationally silent restriction sites. Sequences were confirmed by DNA sequencing. To produce dimer constructs, a cDNA fragment encoding all but the first 6 amino acids of Kcnk3 was excised from its full-length EcoRI fragment (GenBankTM accession number X58175). The result was further digested with I and EcoRI and ligated behind another copy of the gene resulting in a fragment encoding both P1H and P1N. The fragment was then ligated into pcDNA3 vector, resulting in the P1H-P1N construct. Using these constructs, we have produced transgenic mice expressing either Kcnk3 or Kcnk5.

Electrophysiology—Oocytes were isolated from Xenopus laevis frogs (Nasco, Atkinson, WI), treated with collagenase to ease follicle removal, and injected with 0.1–0.4 ng of Kcnk3 cRNA in 46 nl of sterile water. Currents were measured 1–4 days after injection by two-electrode voltage clamp using a Geneclamp 500 amplifier (Axon Instrumets, Foster City, CA). Data were sampled at 4 kHz, filtered at 1 kHz, and analyzed using Pulse (Instrutech, Great Neck, NY) and Sigmplot

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Kenk3 Subunits Function as Dimers

Native Kenk3 and a Mutant—Native Kenk3 channels expressed in oocytes are notable for at least four reasons. First, the currents activate and deactivate in response to voltage changes in two phases, one instantaneous, another time-dependent (Fig. 1A). A non-zero holding current at voltages from −120 to 60 mV (Fig. 1A and B) indicates that the channels are open across the physiological voltage range, as has been observed directly at the single channel level (9). Second, Kenk3 currents are non-inactivating, remaining at a stable level until voltage is again altered. Third, Kenk3 currents show open rectification; as expected for an open, potassium-selective portal, the relationship of current and voltage becomes linear as potassium is raised to nearly equal levels across the membrane (Fig. 1B). Conversely, at physiological levels of bath potassium (−5 mM), Kenk3 passes large outward currents and small inward currents, because permeant ions flow more readily from a side of high concentration. Fourth, Kenk3 channels are inhibited by protons in the physiological pH range. Currents are close to maximal at pH 8.0, partially suppressed at pH 7.0, and essentially absent at pH 6.0 (Fig. 1C).

Mutation of Kenk3 histidine 98 (P1H) to asparagine (P1N) markedly diminishes pH sensitivity of Kenk3 so that little block is now seen at neutral pH (Fig. 1D). Moreover, wild type channels show anomalously low outward current in 5 mM potassium compared with 100 mM at pH 7.4 because of increased proton blockade (Fig. 1B) (9) whereas this effect is absent in the mutant (Fig. 1E). Otherwise, the P1N mutation did not appear to significantly change channel-gating kinetics, rectification properties, or ion selectivity (Fig. 1, D and E, and data not shown). These findings suggested that pH inhibition of Kenk3 resulted from protonation of histidine 98 in the selectivity filter.

Potassium and Voltage Affect Proton Block—The influence of external potassium concentration on inhibition of Kenk3 by proton was next studied (Fig. 2). At −120 mV, with 5 mM potassium, Kenk3 current was half-maximal at pH 7.6 ± 0.6 (n = 16 cells) whereas with 20 mM and 100 mM potassium, half-block required lowering the pH to 6.83 ± 0.01 and 6.35 ± 0.02, respectively (n = 10 and 20 cells). Moreover, the current-pH relationship with 5 mM potassium showed a Hill coefficient of 0.96 ± 0.06 whereas with 20 and 100 mM potassium, Hill coefficients of 2.02 ± 0.14 and 1.86 ± 0.21 were determined. This suggested that protonation of a single pore site was sufficient to inhibit at low bath potassium whereas protonation
of more than one site was required at higher potassium levels.

Consistent with the idea that proton enters the superficial portion of the Kcnk3 pore to block, inhibition was sensitive to transmembrane voltage. In the presence of 20 mM potassium the apparent fraction of the electric field traversed by proton to reach its site ($\delta$) was estimated to be 0.08 ± 0.01 ($n = 6$; see “Materials and Methods”).

pH Alters Pore Block by Barium—To assess the effect of proton blockade on ions entering and leaving the Kcnk3 pore, blockade by external barium was studied. Inward Kcnk3 currents are inhibited by barium in a voltage-dependent manner ($z = 0.45 ± 0.01$; $n = 5$ cells) (9), apparently via occupancy of a potassium site in the pore as in 1-P-domain (19, 20) and another 2-P-domain channel (7). Thus, with 20 mM potassium at pH 8.0, Kcnk3 was half-blocked by 0.40 ± 0.02 mM barium with a Hill coefficient of 1.1.1 ± 0.1 (−120 mV; $n = 7$ cells). When the pH value was lowered from 8.0 to 6.5, inhibition by 1 mM barium decreased from 66 ± 3% to 37 ± 4% ($n = 8$ cells; see Fig. 3A). Although the onset of barium block was not resolved from the capacitance transient associated with a voltage step to −150 mV, release from steady-state barium inhibition at −150 mV could be assessed by a step to 60 mV; $\tau_{off}$ was slowed −2-fold (from 53 ± 8 and 100 ± 10 ms) when the pH value was lowered from 8.0 to 6.5 ($n = 7$ cells) (Fig. 3B).

Studies with the proton-insensitive Kcnk3 channel mutant (P1N) supported the conclusion that the proton(s) that modify barium blockade reside on histidine 98. Thus, P1N channels were inhibited by barium like wild type ($K_{1/2}$ was 0.42 ± 0.06 with a Hill coefficient of 0.9 ± 0.1; $n = 8$ cells). However, blockade of P1N channels by 1 mM barium was insensitive to pH, showing 77 ± 2% and 75 ± 3% inhibition at pH 8.0 and 6.5, respectively ($n = 5$ cells; see Fig. 3A). Moreover, unblock kinetics for P1N channels were less sensitive to pH than wild type channels, showing $\tau_{off}$ of 59 ± 3 and 67 ± 5 ms at pH 8.0 and 6.5, respectively ($n = 5$ cells; see Fig. 3B).

Proton Block of Tandem Dimers—When two subunits were linked in tandem to produce constructs with four P domains, those with two native subunits (P1H-P1H) or two mutant subunits (P1N-P1N) showed the same sensitivity to proton as channels formed by the related monomer subunits, P1H or P1N (Fig. 4A). Thus, with 20 mM potassium at −120 mV the $K_{1/2}$ and Hill coefficient for wild type tandems was 6.75 ± 0.04 and 1.84 ± 0.11, respectively, similar to wild type monomer, suggesting protonation at more than one site. In contrast, tandem constructs with a single histidine in the first or second P1 position (P1H-P1N and P1N-P1H, respectively) showed decreased pH sensitivity and evidence for a single proton blocking site. Values for P1H-P1N channels were 6.28 ± 0.05 and 1.10 ± 0.14 whereas those for P1N-P1H channels were 6.34 ± 0.04 and 1.18 ± 0.21. These results support the conclusion that two P1H sites are protonated in channels formed by wild type subunits, and one P1H is protonated in mixed tandems whether it resides in the first or downstream position.

**DISCUSSION**

The First P Domain of Kcnk3 Contributes to the Pore—Here, we demonstrate that Kcnk3 senses pH value changes in the physiological range via protonation of histidine 98 leading to channel blockade. We employ proton blockade to demonstrate that the first P domain in Kcnk3 subunits contributes to pore function and then provide evidence that the pore contains two P1 domains. Four experiments support the conclusions that inhibition results from protonation of sites in the pore and that P1 domains are pore-forming. First, block is mediated by histidine 98, which is located in P1 adjacent to residues that coordinate potassium ions in the ion-conduction pathway (21). Second, block depends on voltage as if the protons enter the transmembrane electric field to bind. Third, protons alter steady-state affinity of barium on its pore site (as well as barium block kinetics). Fourth, increasing external potassium decreases the sensitivity of the channel to proton (perhaps because of increased occupancy of the pore by potassium). Finally, evaluation of linked subunits bearing four P domains (P1-P2-P1-P2) demonstrate that P1 mediates proton blockade in the first or third position arguing for their functional equivalence.
Kcnk3 Subunits Function as Dimers

Kcnk3 Subunit Number—Because four P domains are necessary and sufficient to form the pore in voltage-gated and inwardly rectifying potassium channels we expect the same number of P domains in each Kcnk3 pore. The arrangement and number of Kcnk3 subunits should account for the following four findings in this report: first, wild type channels are expected to have more than one proton binding site (based on a Hill coefficient of -2 in 20 mM potassium); second, occupancy of one of the sites is sufficient to block (based on a Hill coefficient of -1 in 5 mM potassium); third, the site is formed with histidine residues on the external side of the pore (as the P1N mutant is insensitive); and fourth, only one site is available in channels formed by mixed tandem constructs with a single P1H domain (based on a Hill coefficient of -1 in 20 mM potassium).

The most likely model for pore formation is a dimer with two P1 and two P2 domain as this provides two histidine residues to bind protons in P1H (Fig. 4B, upper) and P1H-P1H channels (Fig. 4B, lower), no histidine sites in P1N (Fig. 4C, upper) and P1N-P1N channels (Fig. 4C, lower), and one histidine in P1H-P1N (Fig. 4D, upper) and P1N-P1H channels (Fig. 4D, lower). It is appropriate to rule out both channels formed by four P2 domains, as P1 domains contribute to pore function (Fig. 4E, left), and channels formed only by the central P domains in linked tandems, because P1H-P1N channels (Fig. 4E, right) show proton blockade. Finally, it seems extremely unlikely but remains possible that four P1 domains form the pore (Fig. 4F) if KCNK P2 domains are non-functional (more on this below).

Indeed, four glutamate residues (on four 1P6TM subunits) form only two proton binding sites in a cyclic nucleotide-gated channel pore (22), and a single proton site is formed by a cluster of four glutamate residues in an L-type cardiac voltage-dependent calcium channel (23). However, to account for our results with mixed tandem constructs, P1 domains would either need to function non-equivalently (which is not what we observed), and/or single protons must block via imidazole/imidazolate pairs (an arrangement that has not been described in other proteins), and/or single protons must block across the mouth of the pore (Fig. 4E, right). Thus, it is reasonable to argue that Kcnk3 channels are homodimeric (Fig. 4B, upper).

Functional evidence that 2-P domain channels are dimeric has been expected since the isolation of the first channel clones (1). Indeed, an early report argued that a cysteine in the first external loop of KCNK1 was required for both dimer formation and function (24). However, that channel (TWIK1) has subsequently been demonstrated to be non-functional in oocyte plasma membranes, and the cysteine residue revealed to be absent from other KCNK channels (1).

How Protons Block—Protonation of Kcnk3 histidine 98 is likely to alter channel gating rather than permeation. Thus, Kim et al. (15, 17) found that proton blockade decreased open probability of Kcnk3 and KCNK9 whereas single channel conductance was not significantly altered. Moreover, increasing external permeant ion concentration destabilizes the long-lasting closed state of single KCNK0 channels whereas changing the external permeant ion from potassium to rubidium increases the open probability of single KCNK2 channels (8). These findings suggest that protonation favors Kcnk3 channel closure whereas permeant ions in the pore favor the open state.

The position homologous to P1H in Kcnk3 is also a histidine in KCNK1, -3, -7/8, and -9 but is occupied by aspartate (GYGD) in TOK1 and asparagine (GYGN) in KCNK0. The analogous position in the second P domain of all KCNK channels is aspartate (GYGD) as found in voltage-gated potassium channels; when this P2D was mutated to histidine there was no evidence for Kcnk3 channel function whether a histidine, asparagine, or aspartate was in position 98 (not shown) demonstrating that homologous P1 and P2 residues are not functionally equivalent.

Kir2.1 inward rectifier potassium channels are formed by 1P2TM subunits and have an arginine residue in the P domain (GYFR) that has been argued to form an electrostatic barrier to divalent cation permeation, because a histidine at the site (GYFH) yields channels with increased sensitivity to magnesium until proton levels increase and the site is thought to bear a proton (26); a similar mechanism may explain the effects of pH on barium blockade via P1H in Kcnk3 (Fig. 3).

Little Is Known about P2 Domains—Remarkably, a P2 domain has yet to be shown to contribute to pore formation. Thus far, it has been demonstrated that mutations in the TOK1 P2 domain (and flanking transmembrane segments) alter potassium-sensitive gating kinetics without affecting its single channel current (25, 27, 28). Thus, external potassium concentration influences both the magnitude and rate of TOK1 current development, and mutations in some P2 sites modify this effect. As homologous sites in Shaker channels are known to affect the potassium sensitivity of C-type inactivation, these P2 residues have been suggested to contribute to channel regulation. A homodimeric stoichiometry for Kcnk3 demands that P2 domains are, indeed, pore-forming.

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REFERENCES
18. Woodhull, A. M. (1973) J. Gen. Physiol. 61, 687–708