The M1P1 Loop of TASK3 K2P Channels Apposes the Selectivity Filter and Influences Channel Function*

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Channels of the two-pore domain potassium (K2P) family contain two pore domains rather than one and an unusually long pre-pore extracellular linker called the M1P1 loop. The TASK (TASK1, TASK3, and TASK5) subfamily of K2P channels is regulated by a number of different pharmacological and physiological mediators. At pH 7.4, TASK3 channels are selectively blocked by a number of different pharmacological and physiological mediators. At pH 7.4, TASK3 channels are selectively blocked by zinc in a manner that is both pH2+- and [K+]o-dependent. Mutation of both the Glu-70 residue in the M1P1 loop and the His-98 residue in the pore region abolished block, suggesting the two residues may contribute to a zinc binding site. Mutation of one Glu-70 residue and one His-98 residue to cysteine in TASK3 fixed concatamer channels gave currents that were enhanced by dithiothreitol and then potently blocked by cadmium, suggesting that spontaneous disulfide bridges could be formed between these two residues. Swapping the M1P1 loops of TASK1 and TASK3 channels showed that the M1P1 loop is also involved in channel regulation by pH. Therefore, the TASK3 M1P1 loop lies close to the pore, regulating TASK3 channel activity.

Background, or leak, potassium currents play an important role in the regulation of the resting membrane potential and excitability of mammalian neurons. The two-pore domain potassium (K2P) channel family is open across the physiological voltage range and is believed to underlie many of these leak currents (1–5). So named as each α-subunit of K2P channels contains two pore domains, or P-domains, the channels also contain an unusually large extracellular pre-pore linker called the M1P1 loop. This linker is believed to form a self-interacting domain that is essential for channel dimerization (6) and may play a role in channel regulation (7–9).

There are currently 15 members of this family, which can be divided into six subfamilies on the basis of structural and functional properties (4, 10, 11). Among these subfamilies is the TWIK-related acid-sensitive potassium subfamily (TASK1 (Ktpr 3.1), TASK3 (Ktpr 9.1), and TASK5 (Ktpr 15.1)). TASK1 and TASK3 K2P channels are regulated by a wide variety of chemical stimuli (12, 13) and are responsible for leak potassium currents in many neurons, including cerebellar granule neurons (e.g. 14–20).

TASK channels are sensitive to extracellular acidification, with a histidine at position 98 shown to be crucial for the pH sensitivity of TASK1 and TASK3 channels (7, 21–23), although the mechanism behind TASK1 pH sensitivity is not yet fully understood as mutation of this histidine in TASK1 merely shifts pH sensitivity rather than abolishes it (7). We have shown previously that zinc is a selective blocker of TASK3 channels with little effect on TASK1 in physiological conditions (24). This selective block involves both His-98 and a glutamate residue (Glu-70) within the M1P1 loop, suggesting that the M1P1 loop plays an important role in channel regulation (24). Indeed, Glu-70 on the M1P1 loop is also essential for the block of TASK3 current by ruthenium red (25) and other divalent cations (26).

The long extracellular M1P1 loop is not conserved in potassium channels with known crystal structures. However, a structural homology model of TASK1 based on known potassium channel structures shows the M1P1 loops lying in close association at position Asn-53 (27), a position homologous to TWIK1 C69, an M1P1 loop cysteine residue responsible for channel dimerization. However, the engineered disulfide approach, involving introduction of cysteine residues and looking for disulfide bond formation, is a useful tool that has been widely used to determine regions of close proximity for a number of membrane proteins, including potassium channels (29).

In this study we describe in more detail the mechanism of TASK3 zinc block. We confirm that the zinc block involves a glutamate residue in the M1P1 loop and His-98 in the pore region, and we demonstrate that engineered cysteine residues at positions 70 and 98 are able to form both inter- and intrasubunit spontaneous disulfide bonds. The M1P1 loops of TASK3 channels therefore lie in close apposition to the pore, and through exchange of the M1P1 loops between TASK3 and TASK1 channels we demonstrate that this unusual linker plays a role in the TASK3 channel pH-sensing mechanism.

EXPERIMENTAL PROCEDURES

Mutations and Truncations—Point mutations were introduced by site-directed mutagenesis into the TASK3 channel
clones using the QuickChange kit (Stratagene). A pair of short (25–35 bases) complementary oligonucleotide primers incorporating the intended mutation were synthesized (MWG-Biotech, Ebersberg, Germany). TASK3 concatamers channels were created with a 6-amino acid linker between the two subunits. In each case, point mutations were made to individual subunits before the final concatamers were formed. Chimeric TASK channels were constructed by swapping M1P1 loops between family members. This was achieved by the introduction of silent mutations to create unique restriction sites, XhoI and BamH1, either side of the M1P1 region using standard PCR techniques described above. Chimeras were then formed by “cutting and pasting” of this region between the TASK family members. For TASK2 channels a non-silent BamH1 restriction site was created for cutting and pasting and further point mutations undertaken to remove the non-silent mutations. Mutant DNA and chimeric constructs were sequenced (MWG-Biotech, UK/SUPAMAC, Sydney) to confirm the introduction of the correct mutated bases.

**tsA-201 Cell Culture Preparation**—Modified human embryonic kidney 293 cells (tsA-201) were maintained in 5% CO2 in a humidified incubator at 37 °C in growth medium (89% Dulbecco’s modified Eagle’s medium, 10% heat-inactivated fetal bovine serum, 1% penicillin (10,000 units ml⁻¹) and streptomycin (10 mg ml⁻¹)). When the cells were 80% confluent, they were split and plated for transfection onto glass coverslips coated with poly-d-lysine (1 mg ml⁻¹) to ensure good cell adhesion. The cells were transiently transfected using the calcium phosphate method. 0.3–1 μg of cDNA expression vector encoding a mouse or human TASK3 subunit was added to each 15-mm well, and 0.3–1 μg of a plasmid encoding the cDNA of green fluorescent protein was included to identify cells expressing TASK3 (H98A) reduced the effect of zinc on TASK3 channels by 87% (mean S.E., 8) (see Fig. 1, A and B, also Refs. 19, 24). Mutation of the histidine residue adjacent to the selectivity filter in the first pore domain of TASK3 (H98A) reduced the effect of zinc on TASK3 chan-

**RESULTS**

Zinc Is a Relatively Selective Blocker of TASK3 Channels Compared with TASK1 Channels—In control solutions (pHo 7.4, [K], 2.5 mM), zinc (100 μM) substantially inhibited TASK3 currents by 87 ± 2% (mean ± S.E., n = 12) but inhibited TASK1 currents by only 11 ± 4% (n = 8) (see Fig. 1, A and B, also Refs. 19, 24). Mutation of the histidine residue adjacent to the selectivity filter in the first pore domain of TASK3 (H98A) reduced the effect of zinc on TASK3 chan-

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4 The abbreviations used are: MES, 4-morpholineethanesulfonic acid; DTT, dithiothreitol; WT, wild type.
Zinc (100 µM) and zinc (namely the His at position 98) suggests that the commonality in an identified site of action of pH (see Ref. 22) and zinc become a significantly more effective blocker of TASK1 channels when pH was raised to 8.4 (inhibition of 55 ± 4%, n = 8).

The interaction between zinc and hydrogen ions could also be demonstrated by considering recovery of current amplitude following block by zinc. In the example shown, TASK3 currents were potently blocked at pH 8.4 by 100 µM zinc (Fig. 1E). Full recovery from block took a considerable time (~700 s for the example shown); however, this recovery was much faster (>3-fold faster in this cell) if the channels were exposed briefly to pH 6.4 external solution during wash out of zinc. Because mutation of histidine at position 98 interferes with both zinc and hydrogen ion block, the most parsimonious explanation for these data is that zinc and hydrogen ions compete for an overlapping binding site.

Hydrogen ions are less effective blockers of TASK channels in the presence of high extracellular [K] (31), so it was of interest to determine whether changing the extracellular [K] also interfered with block by zinc of TASK3 channels. Fig. 1E shows the effect of 100 µM zinc on TASK3 channels at pH 7.4.
sensitivity of TASK3 channels (Fig. 2, A–D). Thus, the block of TASK3 channels by zinc is only recapitulated in TASK2 channels when both the M1P1 loop and the pore histidine residue from TASK3 are present.

The Effect of DTT on TASK3 Channel Cysteine Mutations—
The importance of both Glu-70 and His-98 in block by zinc suggests that these amino acids may be located close to each other in the channel to form a binding site for zinc (see also Ref. 24). Mutation of both Glu-70 and His-98 to Cys gave channels that were virtually non-functional (2 ± 24). Mutation of both Glu-70 and His-98 to Cys gave channels when both the M1P1 loop and the pore histidine residue from TASK3 are present.

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FIGURE 2. The M1P1 loop of TASK3 and a histidine at position 103 confer zinc sensitivity to TASK2 channels. A–D, current-voltage relationships showing the effect of zinc (100 μM) on WT TASK2 channels (A), TASK2_E70C channels (B), TASK2_H98C channels (C), and TASK2_H98C chimeric channels (D). In each panel (A–D) = a schematic of the TASK2 channel construct is shown. Mutations and chimeric alterations are shown in white. E, histogram showing block of WT and mutated TASK2 channels by zinc (100 μM). F, concentration-response curves for zinc block of TASK3 channels and mutated TASK2 channels. The filled triangles are TASK3, the filled circles are TASK2, and the open squares are TASK2/M1P1. Error bars in E and F are S.E. of the mean.

The amplitude of current through constructs where one Glu-70 residue and one His-98 residue were mutated to cysteine on one or the other of the two subunits of the tandem construct was significantly enhanced by DTT treatment (Fig. 3, E and F). Currents through the TASK3<sub>E70C,H98C</sub> construct were enhanced by 43 ± 9% (n = 6) whereas DTT enhanced currents through the TASK3<sub>E70C</sub> construct by 39 ± 7% (n = 12), the latter construct consisting of channels with one mutation in each subunit.
Our data therefore suggest that some degree of cross-linking can occur, spontaneously, between the Cys-70 and Cys-98 residues in these mutants, even when the cysteine residues are on different subunits of the dimer. The M1P1 loop therefore must lie close to the pore. Because the enhancement by DTT is similar in magnitude whether the cysteine residues at positions Cys-70 and Cys-98 are on the same subunit or opposite subunits, this suggests that inter- and intrasubunit interactions are equally strong. To test this idea further, we used cadmium ions that can bind strongly to two closely apposed cysteine residues (e.g. Ref. 34). Cadmium (at 10 μM) had no significant effect on WT TASK3 channels (2 ± 2% inhibition, n = 4, Fig. A4A); however, it caused a small, reversible inhibition of the mutated concatamers (Fig. 4, B–D). For the TASK3<sub>E70C,H98C</sub>/TASK3 construct, cadmium produced a 13 ± 6% inhibition (n = 4) whereas the TASK3<sub>E70C</sub>/TASK3<sub>H98C</sub> construct was inhibited by 18 ± 2% (n = 10). This is comparable with the block seen for the single mutant concatamer channel (TASK3/TASK3<sub>H98C</sub>) that was blocked by 10 μM cadmium by 25 ± 3% (n = 6) before DTT and 27 ± 2% (n = 6) following DTT and suggests reversible cadmium binding to single free cysteine residues (see also Ref. 34). Following treatment with DTT, cadmium was significantly (p < 0.05) more effective at blocking current through these concatamer channels (Fig. 4, B and C). The TASK3<sub>E70C,H98C</sub>/TASK3 construct was inhibited by cadmium (10 μM) by 57 ± 5% (n = 5) after DTT, whereas the TASK3<sub>E70C</sub>/TASK3<sub>H98C</sub> construct was inhibited by 63 ± 4% (n = 3). Furthermore, following DTT treatment, block by cadmium was difficult to reverse unless DTT was reapplied to the cells (Fig. 4, B and D), again supporting the hypothesis that these residues are in close apposition.

The TASK3<sub>N53C</sub> Channel Construct Is Insensitive to DTT and Cadmium—in contrast to this, M1P1 residues that have previously been assumed to be closely associated in TASK1 (35) that are also conserved in TASK3 may not, in fact, be in close apposition. We created a TASK3<sub>N53C</sub> mutant and looked for evidence of disulfide bridge formation. Expression of the mutant in Xenopus oocytes gave currents that were WT-like (8.5 ± 2 μA (n = 8) and 7.0 ± 1 μA (n = 13) for mutant and WT TASK3 channels, respectively). Full pH response curves showed no change in pH sensitivity of the mutant channel with a pK<sub>a</sub> of 6.4 ± 0.1 (n = 4), compared to 6.6 ± 0.03 (n = 13) for WT TASK3 (Fig. 4F). Application of 5 mM DTT or 10 μM cadmium had no significant effect on current size at 30 mV, with a 6.9 ± 4% (n = 8) increase and a 3.7 ± 1% (n = 5) decrease in current recorded, respectively. A similar lack of effect of DTT and cadmium on this mutant was seen when these channels were expressed in tSA-201 cells (Fig. 4F).

The M1P1 Loop Influences the pH Sensitivity of TASK1 Channels—TASK1 channels are more sensitive to extracellular pH than TASK3 (7, 21–23). The close proximity of residues on the M1P1 loop to the apparent pH sensor in TASK3 suggests that the M1P1 loop could play a role in determining the pK<sub>a</sub> of channel conductance. To test this hypothesis we generated chimeric channels in which the M1P1 loop was swapped between TASK1 and TASK3 and vice versa.

Expression of the chimeras formed from TASK1 with the TASK3 M1P1 loop (TASK1<sub>TASK3</sub>M1P1) and TASK3 with the TASK1 M1P1 loop (TASK3<sub>TASK1</sub>M1P1) in Xenopus oocytes...
gave functional currents that were still potassium-selective. Similarly to WT TASK channels, the chimeras were sensitive to extracellular pH changes (Fig. 5, A–D). Full pH response curves (Fig. 5E) showed a pK_a for inhibition of TASK3 TASK1M1P1 that was significantly shifted by 0.3 of a pH unit compared with WT TASK3 (TASK3 TASK1M1P1, pK_a = 6.7 ± 0.02 (n = 11), TASK3 pK_a = 6.4 ± 0.09 (n = 10), p < 0.005). The pK_a of the TASK1 TASK1M1P1 was significantly shifted by 0.5 of a pH unit compared with WT TASK1 (TASK1 TASK1M1P1, pK_a = 7.0 ± 0.06, (n = 15), TASK1, pK_a = 7.5 ± 0.03 (n = 11), p < 0.005). Furthermore, zinc sensitivity could be imparted to TASK1 channels by inserting the TASK3 M1P1 loop, and, conversely, zinc sensitivity was lost in TASK3 channels when the M1P1 loop was replaced with that from TASK1 (Fig. 5F). Overall, these data demonstrate that the M1P1 loop can regulate TASK channel function by influencing sensitivity to both zinc and pH.

**DISCUSSION**

**Zinc Block of TASK3 Channels**—We have shown that zinc block of TASK3 channels depends on both [H] and [K], with zinc being less effective in acidic pH or high extracellular potassium. Thus, zinc is a clear discriminator between TASK3 and TASK1 channels only under physiological recording conditions. The interaction between zinc and hydrogen ion block of TASK channels may suggest that these ions compete for an overlapping binding site on the channel. This idea is supported by the observation that, as seen for zinc ions in this study, hydrogen ions are less effective blockers of TASK channels in the presence of high extracellular [K] (31). Indeed, an identified amino acid, His-98, is involved in both zinc block (24) and in the pH-sensing mechanism of TASK3 channels (22, 23). Although we cannot completely rule out the possibility that it is the reduction of [Na] rather than the increase in [K], that modifies zinc and pH sensitivity, we think it more likely that the increase in [K], is responsible (see also Ref. 31) because replacement of [Na] with choline or N-methyl-D-glucamine, although reducing current amplitude considerably, does not alter the sensitivity of TASK3 channels to changes in pH.

In terms of the mechanism of zinc block of TASK channels, our original explanation of the results we obtained (24) was that zinc was at its most potent when a four-coordinate binding site (of 2 × Glu-70 and 2 × His-98) is present, such as in the TASK3 WT homodimer channel, because the action of zinc was reduced either when His-98 or Glu-70 was mutated or, in this study, when His-98 was protonated. These findings are supported by our additional data showing that a TASK3 TASK1 chimera (TASK3 TASK1M1P1) was also not sensitive to zinc and, importantly, that TASK2 channels can acquire zinc sensitivity only when both the TASK3 M1P1 loop and His-103 are present (TASK2 TASK1M1P1 N103).

**Glu-70 in the M1P1 Loop Is Closely Apposed to His-98 in the Pore Region**—Because Glu-70 and His-98 are suggested to form a binding site for zinc, this implies that these amino acids are located close to each other in the tertiary TASK3 channel structure. Our cysteine mutation studies and experiments on
The M1P1 loop influences the pH sensitivity of TASK1 and TASK3. Example currents demonstrate pH sensitivity in oocytes expressing WT TASK1 (A), WT TASK3 (B), TASK1\_TASK3\_M1P1 chimera (C), and TASK3\_TASK1\_M1P1 chimera (D), respectively. In each panel (A–D), a schematic of the chimeric channel construct is shown with TASK1 components in white and TASK3 in black. E, full pH response curves showed a pKₐ for inhibition of TASK3\_TASK1\_M1P1 (closed circle) that was slightly but significantly shifted compared with WT TASK3 (dashed line, open circle). The pKₐ of the TASK1\_TASK3\_M1P1 chimera (closed triangle) was also significantly shifted by 0.5 of a pH unit compared with WT TASK1 (dashed line, open triangle). F, zinc concentration response curves demonstrate that WT TASK1 (dashed line, open triangle) and TASK3\_TASK1\_M1P1 (closed circle) are essentially zinc-insensitive, whereas the zinc sensitivity of the TASK1\_TASK3\_M1P1 chimera (closed triangle) overlies that of WT TASK3 (dashed line, open circle).

TASK3 channel concatamers add support to this suggestion. A significant enhancement of current through mutated TASK3 channel concatamers by DTT was observed, whether the E70C and H98C are on the same subunit in the concatamer or on opposite subunits, whereas a concatamer with only H98C on the second subunit was unaffected by DTT.

Cadmium can bind strongly to two closely apposed cysteine residues to form long-lasting bonds, providing they are separated by 5 Å or less (34). At a concentration that had no effect on WT TASK3 channels, we observed a small block of mutated TASK3 channel concatamers, suggesting that some reversible binding to free, introduced cysteine residues occurs. However, following treatment of the cells with DTT, cadmium became a powerful blocker of the mutated concatamers with an effect that was hard to reverse unless we re-applied DTT. This suggests that the disulfide bonds broken by DTT release closely apposed cysteine residues that were now available to bind strongly to cadmium ions and alter channel conformation to block current flow. Taken together, these data provide strong evidence that Glu-70 and His-98 are in close apposition (within 4–5 Å of each other) (28, 34) in TASK3 channels.

A recent structural homology model of TASK1 (27) proposed that the M1P1 loops are forced in close association at position Asn-53, a position homologous to the TWIK1 M1P1 cysteine responsible for channel dimerization (6). In this study, TASK3 N53C mutant channels were no different to wild type both in terms of current size and pH sensitivity. While our data do not rule out the possibility that these amino acids are in close apposition and that disulfide bonds may be still be formed between the M1P1 loops at this position, if they do occur these must either be inaccessible or simply do not affect channel function, because application of DTT or cadmium had no effect on currents through the channel.

The pH-sensing Mechanism of TASK Channels—His-98 acts as the pH sensor of both TASK3 and TASK1 channels (7, 21–23). However, although mutation of His-98 abolishes TASK3 pH sensitivity, it only reduces the pH sensitivity of TASK1, implying an additional pH-sensing mechanism in TASK1 channels (7, 23). An aspartate (Asp-204) within the second pore region optimizes pH sensitivity in TASK1 (35). However, this aspartate is conserved throughout the entire K2P family, and it may be that it serves an important role in structuring the selectivity pathway rather than being a pH sensor per se. Indeed, recent data from Yuill et al. (27) show that mutations throughout the pore region of TASK1 that affect the selectivity of the channel also affect the pH sensitivity.

Intriguingly, the recently discovered Drosophila TASK channels, named dTASK6 and dTASK7, have pH sensitivity that is independent of His-98 (9). In these channels a region including the first 20 of the 48 amino acids that form the M1P1 loop of dTASK6 was determined to play a role in proton sensing; however, full M1P1 chimeras were non-functional. Our own functional and potassium-selective chimeras now show that the differential pH sensitivity of TASK1 and TASK3 is due, in part, to the M1P1 loop. The presence of the TASK1 M1P1 loop (and hence a positively charged lysine at position 70) increases the pH sensitivity of TASK3 whereas the presence of the TASK3 M1P1 loop (and therefore introduction of a negatively charged
TASK3 Channel Pore Region Structure

glutamate at position 70) decreases the pH sensitivity of the TASK1 channel.

TASK2 channels are highly sensitive to extracellular alkalinization (36), and an arginine at position 224 was recently reported to be the pH sensor of TASK2 channels (37). Mutation of charged residues within the M1P1 loop of TASK2 previously thought to form the pH sensor of these channels (8) leads to a shift of the pKa by 0.6 pH units from a pKa of 8 to ~7.4 (37). Niemeyer et al. (37) hypothesized that this anomalous shift may be due to structural changes or perhaps a partial collapse of the M1P1 loop. Our data, however, show similar shifts in pH sensitivity with M1P1 exchange between TASK1 and TASK3 channels, despite zinc sensitivity data demonstrating that there must be correct outer mouth structure in these chimeras. As such, our data suggest that the M1P1 loop of TASK channels has a more complex role to play in channel regulation than mere electrostatic effects at the outer mouth.

The only potassium channel family with a similarly long extracellular pre-pore loop as K2P channels is the ether-a-go-go (EAG) family. The best characterized member of this family is the human ether-a-go-go gene (HERG) potassium channel, which has a 42-amino acid pre-pore extracellular loop called the SSP linker. We and others have shown this linker forms an amphipathic α-helix that is critical for HERG channel regulation and likely to interact with the mouth of the pore, playing a central role in HERG rapid C-type-like inactivation (30, 38, 39). However, the positioning of this helical linker and the mechanism of this C-type-like inactivation are still unknown. K2P channels are also suggested to undergo C-type-like inactivation (40); indeed, it has been recently hypothesized that closure of both TASK2 and TASK1 channels upon extracellular pH changes is analogous to C-type-like inactivation (27, 37). Thus, in addition to influencing external regulation of these channels, the M1P1 loop may play a role in intrinsic K2P channel gating.

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