Opposite Effects of KCTD Subunit Domains on \( \text{GABA}_B \) Receptor-mediated Desensitization

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Background: \( \text{GABA}_B \) receptors in the brain assemble with auxiliary KCTD8, 12, 12b, and 16 subunits that influence the receptor response in distinct ways.

Results: Distinct KCTD domains exert opposing effects on desensitization of the receptor response.

Conclusion: KCTD12 and -12b acquired desensitizing properties by disposing of a C-terminal inhibitory domain.

Significance: This study defines the domains and motifs in KCTD proteins that generate functionally distinct \( \text{GABA}_B \) receptor subtypes.

\( \text{GABA}_B \) receptors assemble from principle and auxiliary subunits. The principle subunits \( \text{GABA}_B_{1,1,2} \) and \( \text{GABA}_B_{2,2} \) form functional heteromeric \( \text{GABA}_B(1,2) \) receptors that associate with homotetramers of auxiliary KCTD8, -12, -12b, or -16 (named after their K\(^+\) channel tetramerization domain) subunits. These auxiliary subunits constitute receptor subtypes with distinct functional properties. KCTD12 and -12b generate desensitizing receptor responses while KCTD8 and -16 generate largely non-desensitizing receptor responses. The structural elements of the KCTDs underlying these differences in desensitization are unknown. KCTDs are modular proteins comprising a T1 tetramerization domain, which binds to \( \text{GABA}_B \), and a H1 homology domain. KCTD8 and -16 contain an additional C-terminal H2 homology domain that is not sequence-related to the H1 domains. No functions are known for the H1 and H2 domains. Here we addressed which domains and sequence motifs in KCTD proteins regulate desensitization of the receptor response. We found that the H1 domains in KCTD12 and -12b mediate desensitization through a particular sequence motif, T/NFLEQ, which is not present in the H1 domains of KCTD8 and -16. In addition, the H2 domains in KCTD8 and -16 inhibit desensitization when expressed C-terminal to the H1 domains but not when expressed as a separate protein in trans. Intriguingly, the inhibitory effect of the H2 domain is sequence-independent, suggesting that the H2 domain sterically hinders desensitization by the H1 domain. Evolutionary analysis supports that KCTD12 and -12b evolved desensitizing properties by liberating their H1 domains from antagonistic H2 domains and acquisition of the T/NFLEQ motif.

\( \text{GABA}_B \) receptors are the G-protein-coupled receptors (GPCRs)\(^{5}\) for \( \text{GABA} \), the main inhibitory neurotransmitter in the central nervous system. They are widely distributed throughout the brain and have been implicated in a variety of disorders including cognitive impairments, addiction, anxiety, depression, and epilepsy (1, 2). \( \text{GABA}_B \) receptors activate \( \text{G}_i/o \)-type G-proteins that inhibit adenylyl cyclase and efficiently gate ion channels (3–5). Native \( \text{GABA}_B \) receptors are known to comprise auxiliary subunits that influence receptor properties in distinct ways (5, 6). The principal subunits form two core receptors, \( \text{GABA}_B_{(1,1,2)} \) and \( \text{GABA}_B_{(1,1,2)} \), that bind all \( \text{GABA}_B \) ligands, couple to G-proteins and regulate \( \text{GABA}_B \) receptor effectors, including G-protein-coupled inwardly rectifying K\(^+\) channels (GIRK channels, also known as Kir3 channels) and voltage-gated Ca\(^{2+}\) channels. The auxiliary subunits KCTD8, -12, -12b, and -16 are cytosolic proteins that modulate agonist potency and kinetic properties of the receptor response in distinct ways (6). In particular, KCTD12 or -12b produce fast desensitizing \( \text{GABA}_B \) receptor-mediated Kir3 currents characterized by time constants of seconds while KCTD8 and -16 produce currents with little desensitization. The molecular determinants in the \( \text{GABA}_B \) proteins and the mechanism underlying these kinetic differences in \( \text{GABA}_B \) responses

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\(^{5}\) The abbreviations used are: GPCR, G-protein-coupled receptor; KCTD, K\(^\pm\) channel tetramerization domain; GIRK, G-protein activated inwardly rectifying K\(^+\); Kir, K\(^+\) inwardly rectifying; GRK, G-protein-coupled receptor kinase; RGS, regulator of G-protein signaling; BTB, bric-a-brac, tramtrak, and broad complex; Luc, Luciferase.
are unknown. Importantly, fast desensitization of GABA<sub>B</sub> receptor-mediated K<sup>+</sup> currents is also observed with neurons (7, 8) expressing KCTD12 (9). However, it has not been specifically addressed whether this fast desensitization is due to the presence of KCTD12 in the receptor. In addition, desensitization of GABA<sub>B</sub> responses was shown to be regulated by GRK4 (10), RGS proteins (11–14) or phosphorylation of the GABAB2 (MEQKLISEEDLGEQKLISEEDLLEQKLISEEDLAAEF) into the cloned in-frame with the N-terminal tag of three c-Myc epitopes /H9004.113, a stop codon was inserted after residue Pro<sup>325</sup> in KCTD8. To generate 8AH2, a stop codon was inserted after residue Pro<sup>325</sup> in KCTD8. To generate 8AH2, Tyr<sup>279</sup> in the H1 domain of 8AH2 was mutated to Phe. To generate 16ΔH2F, His<sup>232</sup> in the H1 domain of 16ΔH2 was mutated to Phe. To generate 16ΔH2F, Lys<sup>231</sup>, His<sup>232</sup>, and Arg<sup>235</sup> in the H1 domain of 16ΔH2 were mutated to Asn, Phe, and Gln, respectively. To generate 12H, Phe<sup>278</sup> in the H1 domain of KCTD12 was mutated to His. To generate 12H, Lys<sup>231</sup>, His<sup>232</sup>, and Arg<sup>235</sup> in the H1 domain of 16ΔH2 were mutated to Asn, Phe, and Gln, respectively.

**Cell Culture**—CHO-K1 cells stably expressing human GABA<sub>B1b</sub> and rat GABA<sub>B2</sub> were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 500 μg/ml l-glutamine, 40 μg/ml l-proline, 0.5 mg/ml G418, 0.25 mg/ml zeocine, and 10% FCS in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C (21). Cells were transfected in 24-well plates at 80–90% confluency using 3 μl of Lipofectamine 2000 (Invitrogen) and 1.2 μg of pcDNA3.1 (22), 2–μg KCTD constructs in pCI and 0.3 μg of pEGFP-N1 (Clontech) to visualize transfected cells. 6 h after transfection the cells were plated onto plastic coverslips (Thermoax, Nalge Nunc International) at a dilution of 1:5 in 35 mm dishes and used for electrophysiological recordings 24–48 h later.

**EXPERIMENTAL PROCEDURES**

**Generation of Expression Plasmids**—All KCTD cDNAs were cloned in-frame with the N-terminal tag of three c-Myc epitopes (MEQKLISEEDLGEQKLISEEDLLEQKLISEEDLAAEF) into the cytomegalovirus-based expression vector pCI (Promega). Mutant constructs were generated using overlap extension PCR (20). To generate 12T1–16H1, residues Arg<sup>207</sup> to Glu<sup>279</sup> in KCTD12 were replaced by residues Arg<sup>207</sup> to Glu<sup>279</sup> of KCTD16. To generate 16T1–16H1H2, residues Arg<sup>207</sup> to Glu<sup>279</sup> in KCTD16 were added in-frame at the C terminus of KCTD12. To generate 16H2, a stop codon was inserted after residue Glu<sup>279</sup> in KCTD16. To generate 12–16H2, residues Pro<sup>280</sup> to Leu<sup>427</sup> of KCTD16 were exchanged by residues Arg<sup>207</sup> to Glu<sup>279</sup> of KCTD12. To generate 12–16H2, residues Pro<sup>280</sup> to Leu<sup>427</sup> of KCTD16 were added in-frame at the C terminus of KCTD12. To generate 12–16H2, residues Pro<sup>280</sup> to Leu<sup>427</sup> of KCTD16 were added in-frame at the C terminus of KCTD12. To generate 12–16H2, residues Pro<sup>280</sup> to Leu<sup>427</sup> of KCTD16 were added in-frame at the C terminus of KCTD12. To generate 12–16H2, residues Pro<sup>280</sup> to Leu<sup>427</sup> of KCTD16 were added in-frame at the C terminus of KCTD12.
Electrophysiology—EGFP-expressing CHO cells were identified via epifluorescence using a FITC filter set and patched under oblique illumination optics (BX51WI; Olympus). Kir3 currents were recorded at 30–32 °C in artificial cerebrospinal fluid containing (in mM): 119 NaCl, 2.7 KCl, 1.3 MgCl₂, 2.5 CaCl₂, 1 NaH₂PO₄, 26.2 NaHCO₃, 11 glucose, pH 7.3, equilibrated with 95% O₂/5% CO₂. Patch pipettes were pulled from borosilicate glass capillaries (resistance of 3–5 MΩ) and filled with a solution containing (in mM): 140 K-gluconate, 4 NaCl, 5 GABA, 1 Na₃-GTP, at pH 7.25 (adjusted with KOH). GABAB responses were evoked at 50 mV by fast application of 100 μM GABA (Sigma) for 40 s with a pressure pipette (standard patch pipette; 3 PSI, Picospritzer III, Intracell).

Data were acquired with a MultiClamp 700B (Molecular Devices), low-pass filtered at 2 kHz and digitized at 10 kHz using Clampfit 10.2 software (Molecular Devices). Whole-cell currents were analyzed using Clampfit 10.2 software (Molecular Devices). All values are expressed as mean ± S.D. Data were analyzed by one-way ANOVA followed by Dunnett’s test for pairwise comparison with the control group (Igor Pro software). p values < 0.05 were considered as statistically significant.

Protein Alignments and Phylogenetic Trees—For the tree of all KCTD proteins, all annotated human KCTD protein sequences were used to query the NCBI nr protein database with blastp and all sequences with an e-value less than 1e-10 were retrieved and aligned using MUSCLE. GBlocks (25) was used to retain only well-aligned blocks. This alignment was used as input into MrBayes. The tree shown in Fig. 5B has been pruned to retain only the KCTDs of M. musculus, Danio rerio, Branchiostoma floridae, Drosophila melanogaster, and Caenorhabditis elegans.

For the alignment of GABAB2 C termini, the human GABAβ2 was used to query the NCBI nr protein database with blastp, and sequences from several species were aligned using MUSCLE. The C termini of non-vertebrates do not align with vertebrates and are not shown in the alignment.

The alignment of vertebrate KCTD H2 domain sequences was performed on the most recent ENSEMBL draft genomes of the species indicated. These sequences were blasted with the KCTD12b protein sequence from Oryzias latipes using tblastn. All hits which had H2-like sequences within the same contig were retained. All potential H2-like open reading frames were separated by 9.4 (Tetraodon nigroviridis) to 103 kb (Ornithorhynchus anatinus) from the open reading frame containing the T1 and H1 domains. The retained sequences grouped with KCTD12b and not KCTD8 or -16, suggesting that they are indeed KCTD12b sequences.

RESULTS

Distinct KCTD Protein Domains Influence Desensitization of the GABAβ Receptor Response—KCTD subunits are built from T1, H1, and H2 domains, whereby only KCTD8 and 16 contain a H2 domain. The domain organization of KCTD12 and KCTD16 is illustrated in Fig. 1A. Previous work indicated that the lack of the H2 domain in KCTD12 and -12b correlates with strong
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desensitization of the receptor response (6). We therefore hypothesized that H1 domains facilitate and H2 domains inhibit desensitization. We tested this hypothesis using patch-clamp electrophysiology and CHO cells expressing GABA<sub>B<sub>(1b,2) receptors and effector Kir3 channels in the presence and absence of wild-type and mutant KCTD proteins. In the absence of KCTD proteins, activation of GABA<sub>B<sub> receptors for 40 s by GABA (0.1 mM) elicited Kir3 currents that slightly decreased in amplitude over time (Fig. 1A). The relative desensitization of GABA-activated Kir3 currents was calculated as the reduction in amplitude measured at the end of the GABA application normalized to the peak amplitude (Fig. 1C). In agreement with reported results (6), co-expression of KCTD12 significantly increased the desensitization of GABA-activated Kir3 currents, while KCTD16 had no significant effect on desensitization (Fig. 1, A and C; p < 0.001, compared with cells without KCTD). To identify the KCTD12 domain(s) responsible for desensitization we generated two chimeric proteins, 16T1–12H1 and 12T1–16H1H2, in which the H1 domain of KCTD12 and the H1/H2 domains of KCTD16 are swapped. The desensitization of GABA-activated Kir3 currents was significantly increased in cells expressing the chimeric protein 16T1–12H1 but not in cells expressing 12T1–16H1H2 (Fig. 1, B and C). Of note, both chimeric proteins contained the linker region of KCTD16. Western blot analysis confirmed that the chimeric KCTD proteins were expressed (Fig. 1D). In summary, these data show that the desensitizing properties of KCTD12 segregate with its H1 domain.

We next tested whether the H2 domain exerts an inhibitory influence on desensitization. We generated the mutant 12–16H2 with the H2 domain of KCTD16 attached to the C terminus of KCTD12. 12–16H2 lacks desensitizing properties, in line with a dominant inhibitory effect of the H2 domain on KCTD12-mediated desensitization (Fig. 2, A and D). However, removal of the H2 domain from KCTD16 in the mutant 16A2H2 does not produce a desensitizing KCTD protein (Fig. 2, A and D). Therefore, the H1 domain of KCTD16 is not sufficient for desensitization and differs in its functional properties from the H1 domain of KCTD12.

We next addressed whether the H2 domain of KCTD16 not only prevents KCTD12-mediated desensitization <em>in cis</em> but also <em>in trans</em>. When the H2 domain of KCTD16 is co-expressed with KCTD12 as an independent 16H2 protein GABA<sub>B<sub> receptors were activated by KCTD12-activated Kir3 currents still desensitize (KCTD12 + 16H2, Fig. 2, A and D; p < 0.001, compared with cells without KCTD). Expression of the 16H2 protein was confirmed by Western blot analysis (Fig. 2E). Therefore, the H2 domain only prevents KCTD12-mediated desensitization <em>in cis</em> but not <em>in trans</em>. In order to map the minimal size of the H2 domain preventing desensitization <em>in cis</em>, we generated C-terminal truncations of the chimeric 12–16H2 protein. Truncation of 60 of the 148 amino acid residues of the H2 domain in the 12–16H2Δ60 protein is insufficient to restore desensitization (Fig. 2, B and D). However, truncation of 113 amino acid residues in the 12–16H2Δ113 protein fully restored desensitization (Fig. 2, B and D). This shows that the size of the H2 domain is critical for inhibition of KCTD12-mediated desensitization. We tested whether addition of KCTD-unrelated protein domains to the C terminus of KCTD12 also prevents desensitization. Adding Luciferase (Luc) or the GFP variant Venus to the C terminus of KCTD12 in the 12-Luc and 12-Venus proteins completely prevented desensitization, suggestive of a sequence-unrelated steric hindrance of desensitization (Fig. 2, C and D). The 12–16H2, 12-Luc and 12-Venus proteins co-immunoprecipitate with GABA<sub>B<sub> (Fig. 2F). This demonstrates that the lack of desensitization of these proteins is not due to a lack of association with the receptor. Adding the Luc or Venus domains to the N terminus of KCTD12 in the Luc-12 and Venus-12 proteins did not prevent desensitization, showing that only protein domains C-terminal of the H1 domain are inhibitory (Fig. 2, C and D).

The Desensitization Motif of H1 Domains—We next determined the amino acid residues in the H1 domain of KCTD12 that differ from KCTD16 and mediate the desensitization. Sequence alignment revealed significant differences between KCTD12 and KCTD16 within the C-terminal half of their H1 domains (Fig. 3A). We therefore tested whether desensitizing properties can be transferred from KCTD12 to KCTD16 by replacing the C-terminal half of the H1 domain of KCTD16 with the corresponding sequence of KCTD12. We generated the two chimeric proteins 16T1–16/12H1G and 16T1–16/12H1N in which the 36 and 49 C-terminal residues, respectively, in the H1 domain of KCTD16 were replaced with those of KCTD12. In addition, we omitted in these chimeric proteins the H2 domain of KCTD16 to avoid its inhibitory effect on desensitization. The desensitization of GABA<sub>B<sub> receptor-activated Kir3 currents was small in CHO cell expressing 16T1–16/12H1G protein or the KCTD16. In contrast, cells expressing the 16T1–16/12H1N protein exhibited significantly more desensitization (Fig. 3, B and C; p < 0.001, compared with KCTD16). This result shows that the ability to desensitize GABA<sub>B<sub>-activated Kir3 currents can be transferred from the KCTD12 to KCTD16 H1 domain by exchanging the 49 C-terminal residues of the H1 domains. In addition, the result points at the 13 amino acid residues between Asn<sup>277</sup> and Ser<sup>289</sup> in the H1 domain of KCTD12 as being critical for desensitization. Sequence alignment of these 13 amino acid residues in KCTD8, 12, 12b, and 16 reveals that only Tyr<sup>278</sup> in KCTD8 is not identical or highly conserved with either KCTD12 or KCTD12b, which harbor an Phe residue at this position (Fig. 4A). We therefore addressed whether substitution of Tyr<sup>278</sup> in KCTD8 with Phe renders the H1 domain in KCTD8 desensitizing. We first established that deletion of the H2 domain of KCTD8 in the 8A2H2 protein was insufficient to convert KCTD8 into a desensitizing subunit (Fig. 4, B and D), similar as already observed with KCTD16 (Fig. 2). Strikingly, substitution of Tyr<sup>278</sup> with Phe in the 8A2H2 protein generated receptor responses with increased desensitization (Fig. 4, B and D; p < 0.05, compared with cells without KCTD). KCTD16 exhibits additional sequence divergence with KCTD12 and KCTD12b in the 13 amino acid residues under scrutiny (Fig. 4A). Accordingly, single amino acid substitution of His<sup>232</sup>, the residue homologous to Tyr<sup>278</sup> in KCTD8, with Phe in the 16A2H2 protein was insufficient to render the H1 domain of KCTD16 desensitizing (Fig. 4, C and D). However, additional substitution of two neighboring non-conserved residues (Asn for Lys<sup>311</sup> and Gln for Arg<sup>235</sup>) in 16A2H2NFQ also rendered the H1 domain of KCTD16 desensitizing (Fig. 4, C
and D; \( p < 0.01 \), compared with cells without KCTD). Finally, the converse substitution of these three residues in KCTD12 with the ones of KCTD16, but not the single amino acid substitution of Phe by His, rendered the H1 domain of KCTD12 non-desensitizing (Fig. 4, E and F; \( p < 0.001 \), compared with KCTD12). These experiments identify the motif T/NFLEQ in the H1 domain as a critical sequence element for KCTD-mediated desensitization.

Molecular Evolution of the KCTD Subunits—Our experiments show that the H1 domain is the functional unit responsible for desensitization of the receptor response. The H1 domains of KCTD8 and 16 lack desensitizing properties due to one or three amino acid substitutions, respectively, in the T/NFLEQ motif. The H2 domains in KCTD8 and -16 have antagonistic effects and inhibit desensitization by the H1 domains. To understand how the KCTD proteins acquired these regulatory domains we investigated their evolutionary history.

Analysis of the human and zebrafish KCTD proteins revealed that they are distinct from voltage-gated \( K^+ \) channels, due to...
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A sequence alignment of the C-terminal half of the H1 domains of KCTD12 and KCTD16. Identical and similar amino acids are marked with stars and dots, respectively. Arrows indicate the KCTD16/KCTD12 boundaries in the chimeric proteins 16T1–16/12H1N and 16T1–16/12H1G, which both lack the H2 domain of KCTD16. B, representative traces of GABA<sub>B</sub>-activated Kir3 currents recorded at −50 mV from CHO cells expressing GABA<sub>B</sub>(1b,2), Kir3 channels, and KCTD proteins. Kir3 currents exhibit strong desensitization in the presence of 16T1–16/12H1N but not 16T1–16/12H1G. C, bar graph summarizing the desensitization of Kir3 currents in the presence of KCTD proteins. Data are expressed as mean ± S.D.; **, p < 0.01 compared with cells transfected with KCTD16 (Dunnett's multiple comparison test).

Differences in their T1 domains and the absence of transmembrane domains (17, 26, 27). Our phylogenetic analysis based on the amino acid alignment of the T1 domains of all annotated human KCTD proteins and their orthologues revealed that they diverged deeply in time, preceding the split of animals from plants. However, some KCTD proteins, including the subfamily formed by KCTD8, -12, -12b, and -16, diverged more recently (Fig. 5A). An ancestral KCTD protein with T1 and H1 domains having homology to this subfamily of KCTD proteins is found in nematodes, insects as well as invertebrate chordates (Fig. 5B; C. elegans, D. melanogaster, and B. floridae, a lancelet). However, the C-terminal GABA<sub>B</sub> domain mediating the interaction with the T1 domain (6, 28) is absent in invertebrate GABA<sub>B</sub> receptors. Of note, the Tyr<sup>902</sup> residue in GABA<sub>B</sub> is critical for binding to the KCTD proteins (6) is conserved in all vertebrates. In addition, the H2 domain was added to the ancestral KCTD protein and GABA<sub>B</sub> receptors. Of note, the Tyr<sup>902</sup> residue in GABA<sub>B</sub> receptors is critical for binding to the KCTD proteins (6) is conserved in all vertebrates. In addition, the H2 domain was added to the ancestral KCTD protein and GABA<sub>B</sub> receptors.

Our phylogenetic analysis shows that soon after the emergence of vertebrates a number of events, occurring almost simultaneously in evolutionary terms, changed the structure of GABA<sub>B</sub> receptors. The C-terminal domain was added to the GABA<sub>B2</sub> subunit, thus enabling interaction between the ancestral KCTD protein and GABA<sub>B</sub> receptors. Of note, the Tyr<sup>902</sup> residue in GABA<sub>B2</sub> is critical for binding to the KCTD proteins (6). An ancestral KCTD protein then diversified into the KCTD16 and -12 lineages (Fig. 5A). A suite of amino acid changes occurred in the H1 domain of two of these lineages: in the KCTD16 lineage, the TYLEQ motif changed to NFLEQ (KCTD12) or TF/SLEQ (KCTD16). With the exception of placental mammals, most vertebrates retained a small part of the H2 domain in both sub-lineages. With the exception of placental mammals, most vertebrates retained a small part of the H2 domain in both sub-lineages. The C-terminal GABA<sub>B2</sub> domain mediating the interaction with the T1 domain (6, 28) is absent in invertebrate GABA<sub>B2</sub> receptors (Fig. 5C). Thus, it appears that the ancestral KCTD protein is not part of the invertebrate GABA<sub>B</sub> receptor complex.

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in the KCTD12 lineage. It remains to be addressed whether the residual H2 domain sequences in the KCTD12b genes of vertebrates are transcribed and translated. In this respect, it is interesting to note that all H2 domain sequences analyzed, including those of KCTD8 and -16, are encoded by a separate exon downstream of the exon encoding the T1 and H1 domains. Therefore it is possible that multiple KCTD8, -16, or -12b variants are generated by alternative splicing. In conclusion, our phylogenetic analysis shows that desensitizing KCTD12 and -12b proteins evolved from non-desensitizing KCTD proteins by disposal of the inhibitory H2 domains and acquisition of the T/NFLEQ motif in their desensitizing H1 domains.

FIGURE 5. KCTD8, -12, -12b, and -16 recently diversified from a single common ancestor. A, phylogenetic tree based on an amino acid alignment of the T1 domains of all KCTD proteins. Most KCTD proteins diverged deeply in time; however, a few groups have diverged more recently, including the group formed by KCTD8, -12, -12b, and -16 (box). TNFAIP1 and SHKBPI are grouped with the KCTD proteins due to T1 domain-related sequences. B, phylogenetic tree based on a nucleotide alignment of KCTD8, -12, -12b, and -16. For clarity, this tree is midpoint rooted. KCTD8, -12, and -16 evolved after the origin of chordates and rapidly diversified into the KCTD8, -12, and -16 lineages, with the H2 domain being lost in KCTD12b and -12b. Of note, D. rerio, but not M. musculus, retained part of the H2 domain at the genomic level. In both A and B, the circles at each node indicate the posterior probabilities (the probability that the descendent proteins are more closely related to each other than to other proteins in the tree): black, greater than 95%; gray, between 75 and 95%; white, between 50 and 75%. Two copies of KCTD12 and 16 are present in D. rerio. These duplicate copies are also present in the genomes of other fish, due to an ancient genome duplication (not shown).

FIGURE 6. Alignment of vertebrate KCTD H2 domain amino acid sequences. The C-terminal part of the H2 domain is retained in KCTD12b in most vertebrates, excluding placental mammals (e.g. M. musculus). Residues are numbered according to the H2 domain of D. rerio KCTD8. Conserved residues are highlighted: dark gray, present in more than 80% of the sequences; light gray, present in more than 50% of the sequences. The letters X in KCTD12b of Gadus morhua and Takifugu rubripes result from unspecified nucleotides in the genomic sequences. M. musculus, mouse; D. rerio, zebrafish; G. morhua, cod; Oreochromis niloticus, tilapia; T. rubripes, tiger pufferfish; T. nigroviridis, green-spotted pufferfish; O. latipes, medaka; Xenopus tropicalis, clawed frog; Pelodiscus sinensis, soft-shelled turtle; Gallus gallus, chicken; Taeniopygia guttata, zebra finch; G. anatinus, duck-billed platypus; Sarcophilus harrisii, tasmanian devil.
DISCUSSION

Association of auxiliary KCTD8, -12, -12b, and -16 subunits with principal GABA_B receptor subunits was recently shown to generate molecularly and functionally distinct receptor subtypes (5, 6, 9, 28). The four KCTD proteins are built from obligatory T1 and H1 domains and optional H2 domains. The T1 domains bind as tetramers to the GABA_B2 subunit and thus are crucial for formation of the receptor complex. No functional roles have been assigned to the H1 and H2 domains of the KCTD subunits yet. In this study we show that the H1 and H2 domains have opposite effects on fast desensitization of the receptor response. H1 domains containing the T/NFLEQ motif mediate the desensitization while the H2 domains antagonize this desensitization. The antagonistic effect of the H2 domain is only observed when the domain is expressed in cis with the H1 domain but not when the H2 domain is expressed as a separate protein in trans together with KCTD12. This suggests that the H2 domain does not inhibit desensitization through the binding to a specific site. More likely, the H2 domain acts by sterically hindering the binding of the H1 domain to a downstream effector responsible for fast desensitization such as, for example, proteins involved in G-protein signaling. In agreement with a steric hindrance of desensitization by the H2 domain KCTD-unrelated protein domains can substitute for the H2 domain and prevent KCTD12-mediated desensitization when tethered to the C terminus of KCTD12. It is unclear whether desensitizing and non-desensitizing KCTD proteins can simultaneously bind to the same receptor complex (6). If this is the case, our results suggest that the H2 domains of KCTD8 or -16 will be unable to inhibit desensitization by KCTD12 or -12b in trans. From an evolutionary perspective, it appears that the H2 domain was first acquired in an ancestral KCTD protein with non-desensitizing properties and subsequently lost in KCTD12 and -12b. It is therefore unlikely that the prime function of the H2 domain in KCTD8 and -16 is to antagonize desensitization.

KCTD12 and -12b evolved the T/NFLEQ motif within their H1 domain, which is necessary for KCTD-mediated desensitization of the receptor response. One to three amino acid substitutions within this motif can turn a desensitizing into a non-desensitizing H1 domain and vice versa. Secondary structure analysis predicts that the motif is part of a helix with amphipathic characteristics. It appears that an excess of positively charged amino acids within this helix, such as Arg, His, and Lys in KCTD16, is not permissive for desensitization. It is possible that these positively charged amino acids interact with negatively charged phospholipids of the plasma membrane and reduce the mobility of the helix. In addition, it appears that specific residues at the interface between the hydrophilic and the hydrophobic side of the helix are crucial for desensitization. Thus, substitution of Tyr276 with Phe was sufficient to render the H1 domain of KCTD8 desensitizing. Interestingly, amphipathic helices are widely found in proteins participating in membrane-associated biological processes. In particular, amphipathic helices within GPCRs, G-protein subunits or naturally occurring peptides were shown to regulate the G-protein activation-deactivation cycle (29–34). It is thus possible that the amphipathic helix in the H1 domain of KCTD12 and -12b directly regulates the G-protein that binds in its proximity to GABA_B2 (35–37). However, no binding partners for the H1 domain have yet been identified.

Our evolutionary analysis shows that receptor subtypes owing to auxiliary KCTD subunits emerged with the appearance of vertebrates. In this respect it is interesting to note that GABA_B1 subunit isoforms regulating axonal versus dendritic distribution of GABA_B receptors (38) also first evolved in vertebrates. This suggests that it became essential to control GABA_B receptor desensitization with the emergence of localized signaling. KCTD12 and -16 proteins appear to be present in pre- and postsynaptic GABA_B receptors (6) albeit to differing degrees (9). Biochemical data support that certain GABA_B receptors in the brain contain KCTD12 and others KCTD16 (6). However, whether association with specific KCTDs is responsible for the differences in desensitization between pre- and postsynaptic GABA_B receptors remains to be addressed (39–41). In conclusion, whereas the heteromeric nature and the activation mechanism of the GABA_B core receptor are conserved in evolution (42–46), only the vertebrate GABA_B receptors recruit functionally distinct auxiliary KCTD subunits and generate receptor subtypes.

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