A Role for the $\beta_1-\beta_2$ Loop in the Gating of 5-HT$_3$ Receptors

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aligned with AChR αV46, to cysteine. We monitored the functional effect of cysteine substitution on channel gating and conductance, the effects of covalent modification with thiol-reactive reagents, and disulfide cross-linking to engineered cysteines in the M2–M3 loop.

Reagents. (2-(Trimethylammonium)ethyl) methanethiosulfonate bromide (MTSET$^+$), (2-aminoethyl) methanethiosulfonate hydrobromide (MTSEA$^+$), sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES$^-$), and MTS 5(6)-carboxytetramethylrhodamine (MTS-TAMRA) were purchased from Biotium (Hayward, CA). Benzophenone-4-carboxamidocysteine methanethiosulfonate (BPMTS) and *p*-chloromercuribenzene sulfonate (pCMBS$^-$) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Methyl methanethiosulfonate (MMTS) was purchased from Sigma (St. Louis, MO). All other reagents, unless specified otherwise, were purchased from Sigma or Fisher Scientific (Hampton, NH).

Site-directed mutagenesis of ligand-gated ion channel subunits. Mutagenesis of full-length cDNAs encoding the mouse 5-HT$_{3A(s)}$ and 5-HT$_{3B}$ subunits, the rat GABA$_A$ α_1 and β_1 subunits in the pGEMHE vector, and the mouse AChR α_1 subunit in the pSP64T vector was performed using the Quikchange mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Mutagenic primers were obtained from Sigma, and sequences are available on request. All plasmid constructs were sequenced to confirm the presence of the desired mutations and to rule out a secondary mutation. Wild-type (WT) and mutant 5-HT$_3$R subunit cDNAs were subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) for expression in mammalian cells. An additional round of mutagenesis was performed on the 5-HT$_{3A}$ construct to introduce the set of mutations R437Q/R441D/R445A (Kelley et al., 2003), and the constructs were resequenced as above.

Expression and electrophysiological recording in Xenopus *oocytes. In vitro* RNA transcription, preparation, and injection of *Xenopus* oocytes were performed as described previously (Horenstein and Akabas, 1998). Currents were recorded under two-electrode voltage clamp from oocytes perfused continuously at 5 ml min^{-1} with Ca^{2+}-free Frog Ringer buffer (in mM: 115 NaCl, 2.5 KCl, 1.8 MgCl$_2$, and 10 HEPES, pH 7.5 with NaOH) using equipment and procedures described previously (Horenstein and Akabas, 1998). The perfusion chamber volume was 200 μl. Agarose cushion electrodes were filled with 3 M KCl and had a resistance of <2 M . The ground electrode was connected to the bath by a 3 M KCl/agar bridge. The holding potential was maintained at -80 mV, unless specified otherwise. EC$_{50}$ values were determined by nonlinear least-squares fits to the Hill equation as described previously (Reeves et al., 2001). The irreversible effects of reaction with thiol-reactive reagents were measured as described previously (Reeves et al., 2001). Briefly, pairs of test pulses at a saturating 5-HT concentration (50 μM) were applied, followed by washout for 3 min and reaction for 1 min with a saturating concentration of thiol-reactive reagent, as indicated. After a 3 min washout, an additional pair of test pulses of 50 μM 5-HT were applied, and the effect of reaction was quantitated as follows:

$$\%\text{effect} = ((I_{5\text{-HT, after}}/I_{5\text{-HT, before}}) - 1) \times 100,$$

where $I_{5\text{-HT, after}}$ is the mean peak current of the 5-HT test pulses after thiol-reactive reagent application and $I_{5\text{-HT, before}}$ is the mean peak current of the initial 5-HT applications. The data for the effect of each reagent on mutant receptors were compared with that obtained with WT receptors using one-way ANOVA (Prism 3.0; GraphPad Software, San Diego, CA). The effects of reaction of the thiol-reactive reagents with GABA$_A$ and AChR were determined using a similar protocol. We also used this procedure to characterize the effect of oxidation by copper phenanthroline (Cu:phen; 100:200 μM) and reduction with dithiothreitol (DTT; 10 mM) on the receptors.

The rates of reaction of thiol-reactive reagents with substituted-cysteine mutant receptors were determined as described previously (Reeves et al., 2001). Briefly, a test pulse of 5-HT (50 μM) was applied, followed by a 3 min washout period and five to eight short (10–20 s)
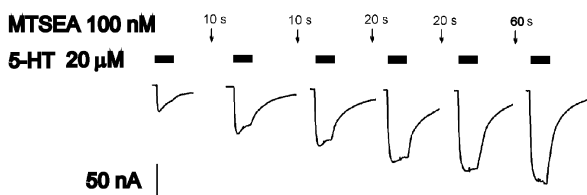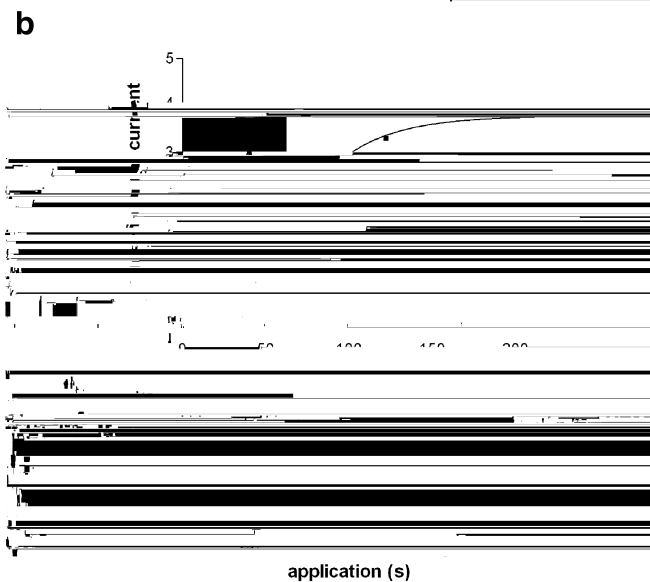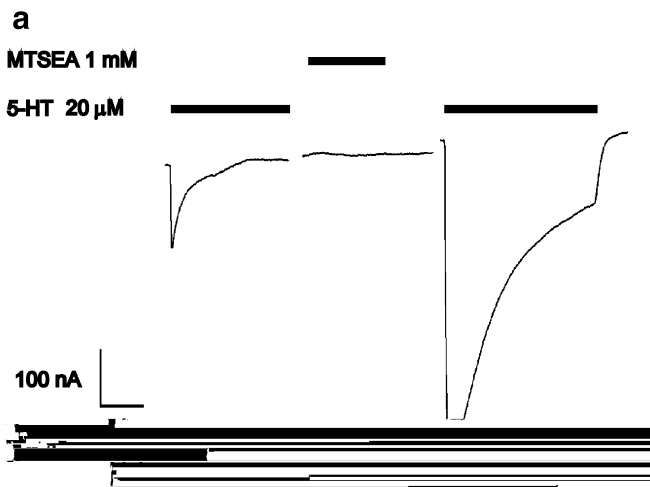
pulses of methanethiosulfonate (MTS) reagent (0.1–20 μM) in the absence or presence of 5-HT, alternating with test pulses of 5-HT. The peak currents induced by the 5-HT test pulses were normalized to the first test pulse, plotted as a function of the cumulative duration of MTS reagent application, and fitted with a single exponential function. The second-order rate constant was calculated by dividing the pseudo-first-order rate constant obtained from the exponential fit by the MTS reagent concentration. For a given mutant, second-order rate constants were independent of the MTS reagent concentration used.

Expression in human embryonic kidney 293 cells. Human embryonic kidney cells (HEK293T; American Tissue Culture Collection, Manassas, VA) were grown at 37°C in DMEM supplemented with 10% fetal calf serum, 2.5 mM L-glutamine, 100 IU of penicillin, and 170 μM streptomycin in an atmosphere of 5% CO$_2$/95% air. Cells were seeded in 100 mm plates at a density of 1.2–1.5 $\times$ 10^6 cells and transfected 24 h later for 12 h using the calcium phosphate precipitation technique (Chen and Okayama, 1988) with 5–7 μg of plasmid DNA. Constructs containing the mutant receptor subunits were cotransfected with empty pXOON plasmid (Jespersen et al., 2002), which encodes a neomycin–enhanced green fluorescent protein (GFP) fusion protein for the visual identification of expression in transfected tedtransf72arw12.2(trhpression12.2h-25wSn12.2h-29sm49

a potential energy function for rotation around the S-S bond created by the covalent reaction of each MTS reagent with cysteine using quantum chemical calculations (Dewar et al., 1985; Stewart, 1990). MTS-modified cysteines were energy minimized using semi-empirical PM3-Hamiltonian calculations (Spartan '04; Wavefunction, Irvine, CA). After rotation of a starting conformation through 180° in 10 steps, the free energy difference between the calculated conformational isomer with the lowest and highest energy was as follows: $MTSES^-$

ceptors, yielding a peak current level about half that of WT homomeric receptors. After modification, the apparent rate of desensitization was decreased by $367 \pm 18\%$ ($n = 3$).

We measured the rate of reaction of MTSEA

a

MTSEA 1 mM

5-HT 20 μM

100 nA

b

application (s)

MTSEA 100 nM

5-HT 20 μM

10 s   10 s   20 s   20 s   60 s

50 nA

parent rate of desensitization compared with WT 5-HT$_{3A}$ receptors. Fast time-scale changes in receptor kinetics cannot be resolved using whole-cell recordings from *Xenopus* oocytes, because their large size places a practical limit of a few seconds on the speed of solution exchange around the cell. Therefore, we performed similar experiments using whole-cell patch-clamp recording from HEK293 cells transiently transfected with the WT

5-HT$_{3B}$ subunit and either the WT 5-HT$_{3A}$ or the K81C mutant subunits (Fig. 3c). Mean peak currents were as follows: for WT 5-HT$_{3A}$/5-HT$_{3B}$ receptors, 3520 $\pm$ 560 pA ($n = 4$); for 5-HT$_{3A}$/K81C/5-HT$_{3B}$ receptors, 310 $\pm$ 63 pA ($n = 4$); for 5-HT$_{3A}$/K81C/5-HT$_{3B}$ receptors after treatment with MTSET$^+$, 3830 $\pm$ 460 pA

and K81C(QDA) treated with MTSET$^+$ than for K81C(QDA). We obtained enough events to measure a predominant single-channel conductance of 38 ± 7.7 pS for WT(QDA) receptors (Fig. 5a) and 40 ± 7.7 pS for K81C(QDA) receptors (Fig. 5b). Therefore, the reduction in the peak current observed with the K81C-mutant whole-cell currents was not caused by a decrease in single-channel conductance. Pretreatment of cells expressing 5-HT$_3$/K81C(QDA) for 3 min with 20 μM MTSET$^+$ or MTSEA$^+$ allowed recording from modified 5-HT$_3$/K81C receptors (Fig. 5c). Modification by either MTSET$^+$ or MTSEA$^+$ did not significantly alter single-channel conductance (41 ± 7.5 and 38 ± 7.3 pS, respectively) compared with WT(QDA) or untreated K81C(QDA) receptors. Of note, bursts containing multiple opening and closing events were observed in the records for WT(QDA) and MTS-modified K81C(QDA) (Fig. 5a,c, insets) In contrast, openings of K81C(QDA) channels had one or, at most, a few opening and closing events per burst (Fig. 5b, inset). This is consistent with a reduced opening rate for the K81C channels, such that the opening rate was comparable to or slower than the fast desensitization rate.

Disulfide cross-linking scan of the extracellular end of M2 and the M2–M3 loop

K81C, at the tip of the β_1–β_2 loop, should be in close proximity to the extracellular end of M2 and the M2–M3 loop, based on a 5-HT$_{3A}$ subunit homology model (Fig. 6a) constructed using the atomic coordinates from AChBP (PDB code I19B) and the *Torpedo* AChR TMD (PDB code 1OED). We tested this hypothesis using disulfide cross-linking experiments (Horenstein et al., 2005). We created eight double-cysteine mutants by mutating the residues T303 (25$'$) to I310 (32$'$), one at a time to cysteine in the presence of the K81C mutation (Fig. 6c). The residues T303 (25$'$) to G306 (28$'$) are part of the extracellular end of M2 that extends above the membrane surface (Bera et al., 2002) and T307 to I310 form the proximal portion of the M2–M3 loop (Miyazawa et al., 2003).

No 5-HT-induced currents were observed from oocytes injected with mRNA for any of the double mutant 5-HT$_{3A}$ subunits alone (data not shown). However, when coexpressed with the WT 5-HT$_{3B}$ subunit, small 5-HT-induced currents, ranging from 15 to 145 nA, were recorded from oocytes expressing either 5-HT$_{3A}$/K81C/A304C or 5-HT$_{3A}$/K81C/G306C. We tested the effects of oxidation and reduction on oocytes expressing these double mutant constructs. Oxidation by 100:200 μM Cu:phen in the absence of 5-HT, inhibited the current from 5-HT$_{3A}$/K81C/A304C/5-HT$_{3B}$-expressing oocytes. The 5-HT currents were restored after subsequent reduction with 10 mM DTT (Fig. 6b) ($n = 3$). This suggested that we could form and break a disulfide bond at this position. DTT reversal of Cu:phen-induced inhibition was not observed when the same protocol was applied to receptors containing either the K81C or A304C mutations alone (data not shown). Oxidation and reduction had no significant effects on currents from oocytes expressing 5-HT$_{3A}$/K81C/G306C/5-HT$_{3B}$.

For the other six double-cysteine mutants that did not give

currents, we assayed for the presence of spontaneously formed disulfide bonds by treating the oocytes with 10 mM DTT for 3 min. For one of these mutants, 5-HT$_{3A}$/K81C/I305C/5-HT$_{3B}$, after DTT treatment,-1.157e39Tc82.6(7e3922.6(of)urrents)-216(of)ere ofof iliection

5-HT activated K81C-containing channels, but the macroscopic currents were significantly reduced compared with WT (Figs. 2, 3). The reduction in current in K81C-expressing cells was not attributable to a change in single-channel conductance (Fig. 5) or to a decrease in expression level, because after modification by MTS reagents, currents were restored to WT levels (Fig. 3). Desensitization was also significantly different between WT and mutant currents. In HEK cells, WT currents desensitized with two time components, one fast (170 ms) and the one slow (4800 ms). This implies the existence of at least two desensitized states. Currents from K81C-expressing cells desensitized with only the fast time constant. To explain the effects of the K81C mutation on peak currents and desensitization, we infer that the mutation altered two aspects of channel kinetics. The change from two components of desensitization in WT to one in the mutant occurred either because mutant channels did not return from the fast desensitized state or because entry into the slow desensitized state, from which the rate of return is very low, was accelerated to a rate comparable with entry into the fast desensitized state. Although we favor the former explanation, we cannot distinguish these possibilities experimentally. However, neither of these changes can completely explain the magnitude of the decrease in peak current amplitudes. Thus, we infer that an additional aspect of channel gating was altered. The most parsimonious explanation would be that the entry rate into the open state, β, is reduced in K81C

surface appears to return to its resting state position before recovery from desensitization is complete (Dahan et al., 2004). In our mechanism, a certain amount of molecular bulk at K81 and other αV46-aligned residues could maintain the interaction between the β_1–β_2 loop and the M2–M3 loop, allowing rapid recovery from the fast desensitized state. However, rigid groups attached to K81C, such as pCMBS$^-$, would be more likely to inhibit channel opening, because they may hinder the movement of the β_1–β_2 loop relative to the M2–M3 loop required for channel opening.

Desensitization of 5-HT$_3$R has been shown previously to depend on the subunit composition, the binding site occupancy, and the primary structure of the M2 transmembrane segment (Yakel, 1996; Mott et al., 2001; Hapfelmeier et al., 2003). Because Cys-loop receptor desensitization can be important *in vivo* for the modulation of synaptic responses (Quick and Lester, 2002), the lack of conservation at αV46-aligning residues may indicate that this position is a candidate for evolutionary tuning of the divergent functional responses of different Cys loop receptors.

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