Dynamic Modification of a Mutant Cytoplasmic Cysteine Residue Modulates the Conductance of the Human 5-HT$_{3A}$ Receptor*

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Structural models suggest that Arg$^{436}$ lies within five cytoplasmic portals of the 5-HT$_{3A}$ receptor. We tested both the accessibility of residue 436 and the influence of its charge on single channel conductance ($\gamma$) by substituting Arg$^{436}$ with Cys and examining the effect of methanethiosulfonate (MTS) reagents on $\gamma$. Inclusion of positively charged 2-aminoethyl-MTS (MTSEA) within the electrode solution reduced $\gamma$ of 5-HT$_{3A}$(R436C) receptors in outside-out patches from 7.8 $\pm$ 0.5 to 5.0 $\pm$ 0.5 picosiemens (pS). To increase $\gamma$, we substituted Arg$^{436}$ by Cys in the 5-HT$_{3A}$(R432Q,R440A) mutant, yielding the 5-HT$_{3A}$(QCA) construct with a $\gamma$ of 17.7 $\pm$ 0.4 pS. Modification of 5-HT$_{3A}$(QCA) receptors by MTSEA or 2-(trimethylammonium)ethyl-MTS reduced $\gamma$ to 8.7 $\pm$ 0.5 and 6.7 $\pm$ 0.4 pS, respectively, both significantly below that of channels exposed to nonpolar propyl-MTS. Extracellular MTSEA, but not 2-(trimethylammonium)ethyl-MTS, crossed the membrane and in so doing slowly ($\tau = 94$ s) reduced $\gamma$. MTSEA more rapidly ($\tau = 15$ s) reduced the $\gamma$ of 5-HT$_{3A}$(QCA) receptors in inside-out patches, an effect reversed by the reducing agent dithiothreitol. Cys$^{436}$ modification by negatively charged 2-carboxyethyl-MTS and 2-sulfonatoethyl-MTS increased $\gamma$ to 23 $\pm$ 1.0 and 26 $\pm$ 0.7 pS, respectively. MTS reagents did not affect $\gamma$ values for 5-HT$_{3A}$(QDA) constructs with Cys substituted for Lys$^{431}$ predicted to be outside the entrance to the portals. Collectively, the data demonstrate that the dynamic modification of the charge of a cytoplasmic residue regulates $\gamma$, consistent with the existence of cytoplasmic portals that impose a rate-limiting barrier to ion conduction in Cys loop receptors.

Cysteine loop (Cys loop) receptors encompass five families of gene products. Nicotinic-acetylcholine (nACh)$^{35}$ receptors, 5-hydroxytryptamine type-3 (5-HT$_3$) receptors, and Zn$^{2+}$-activated channels conduct cations, whereas $\gamma$-aminobutyric acid type A and glycine receptors conduct anions (1). Cys loop receptor subunits combine as pentamers forming a central ion pore that traverses the plasma membrane. Studies examining the role of residues comprising the pore-lining second transmembrane (TM2) helices of the nACh receptor led to the traditional view that this region of Cys loop receptors serves as the rate-limiting barrier to ion flux. Among the cation-selective Cys loop receptor subunits, conserved acidic residues influence unitary conductance ($\gamma$) by forming concentric rings of negative charge positioned along the vertical axis of the central pore (2).

Although the role of residues within the TM2 helix of Cys loop receptors in controlling $\gamma$ is well established, recent studies demonstrate a similar function for residues located in the large cytoplasmic TM3–4 loop (1, 3, 4). Based on a 4.6 Å resolution cryoelectron microscopy image of the Torpedo marmorata nACh receptor, Miyazawa et al. (5) first speculated that amino acids in the TM3–4 loop form “transverse tunnels” that may contribute to the ion conduction pathway. Subsequent electrophysiological studies demonstrated that cytoplasmic residues within membrane-associated (MA) helices of the 5-HT$_3$ and $\alpha_x\beta_2$ nACh receptors are determinants of $\gamma$ (3, 4). Arg$^{436}$ in the 5-HT$_{3A}$ subunit at a position termed MA 0’ is critical for maintaining the anomalously low $\gamma$ ($\sim$900 femtosiemens) that is a hallmark of the homomeric 5-HT$_{3A}$ receptor. Furthermore, introduction of MA 0’ Arg into $\alpha_x\beta_2$ nACh receptors halved $\gamma$ (4).

A 4 Å resolution model of the T. marmorata nACh receptor indicates that adjacent MA helices frame portals with apertures only slightly larger than partially hydrated permeant cations (6). Using this structure as a template, homology models of the 5-HT$_{3A}$ and $\alpha_x\beta_2$ nACh receptors indicate that charged amino acids line their cytoplasmic portals (4).

Although it is now clear that specific MA helix residues influence the $\gamma$ of nACh and 5-HT$_3$ receptors, it remains to be determined whether their charge plays a role. We used substituted cysteine modification to address this question, a method free transmembrane; WT, wild type; $\gamma$, single channel conductance; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl-MTS; PMTS, propyl-MTS; MTSEC, 2-carboxyethyl-MTS; MTSES, 2-sulfonatoethyl-MTS; MTSET, 2-(trimethylammonium)ethyl-MTS; DTT, dithiothreitol; pS, picosiemens.

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3 The abbreviations used are: nACh, nicotinic acetylcholine; nAChR, nACh receptor; 5-HT, 5-hydroxytryptamine; MA, membrane-associated; TM,
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quently used to determine whether residues are located within water-accessible regions of proteins (7). Methanethiosulfonate (MTS) reagents form disulfide bonds rapidly with accessible cysteine residues. Instead of examining accessibility per se, we used a series of MTS compounds with differing charges to examine how the properties of the modified cysteine residue, substituted at the 5-HT$_{3A}$ receptor MA 0’-position, influence $\gamma$. This approach is advantageous compared with systematic mutagenesis; basic amino acids are large compared with acidic residues; thus, charge and volume cannot be treated as separate variables. Cysteine modification by contrast enables alterations of charge while minimizing changes in side chain volume. Furthermore, the effect of sulfhydryl modification on $\gamma$ can be observed in real time and reversed by administration of the reducing agent, dithiothreitol, thereby diminishing the likelihood that altered function is caused by gross changes in assembly and/or tertiary structure.

Our results demonstrate that the dynamic modification of the charge of the MA 0’ Cys residue modulates the $\gamma$ of the 5-HT$_{3A}$ receptor.

**EXPERIMENTAL PROCEDURES**

5-HT$_{3A}$ Constructs and Transfection of Subunit cDNAs—cDNAs encoding human wild type 5-HT$_{3A}$ and mutant constructs were cloned into pGW1. Point mutations were introduced into the 5-HT$_{3A}$ construct using standard molecular biological techniques, and all constructs were sequenced to confirm fidelity. Transient transfection of HEK293 or tsA-201 cells with cDNA was performed using the calcium phosphate precipitation method or electroporation, respectively, as described previously (3, 8). Cells were subcultured twice weekly and incubated in a medium composed of Dulbecco’s modified Eagle’s medium and 10% calf serum, supplemented with 100 $\mu$g/ml streptomycin and 100 units/ml penicillin. Cells were maintained at 37 °C in an atmosphere of 5% CO$_2$ (100% relative humidity). HEK293 cells were incubated at 37 °C overnight during the transfection and were washed after 16 h with medium. Cell culture reagents were purchased from Invitrogen.

Electrophysiology—Outside-out and inside-out patch configurations were used to record single channel currents from patches excised from transfected cells. The bath solution contained 140 mM NaCl, 2.8 mM KCl, 2.0 mM MgCl$_2$, 1.0 mM CaCl$_2$, 10 mM glucose, and 10 mM HEPES (pH 7.2 adjusted with NaOH). Patch electrodes in the outside-out configuration were filled with a solution comprising 130 mM potassium gluconate, 5 mM NaCl, 2 mM MgCl$_2$, 5 mM EGTA, 0.1 mM ATP (Mg$_2^+$ salt), and 10 mM HEPES (pH 7.2 adjusted with KOH). The same bath solution was used for whole-cell current recordings, and electrodes contained 140 mM CsCl, 2.0 mM MgCl$_2$, 0.1 mM CaCl$_2$, 1.1 mM EGTA, 0.1 mM ATP (Mg$_2^+$ salt), 10 mM HEPES, pH 7.2.

5-HT was dissolved in the bath solution and applied locally by pressure ejection to whole cells and outside-out patches held at −60 and −74 mV, respectively (the latter includes correction for a 14-mV liquid junction potential associated with the use of potassium gluconate). Inside-out patch electrodes were filled with the bath solution containing 5-HT (1 $\mu$m), and the electrode potential was clamped at +80 mV, corresponding to a holding potential of −80 mV. Stock solutions of MTS reagents (200 mM) obtained from Toronto Research Chemicals (Ontario, Canada) were stored at −20 °C. Prior to each experiment, MTS reagents were diluted in the electrode solution (or in the extracellular solution in some experiments using MTSEA and 2-((trimethylammonium)ethyl-MTS (MTSET)) for outside-out patch experiments and in the bath solution for inside-out patch experiments to yield a final concentration of 200 $\mu$m.

Single channel currents were recorded using an Axopatch 200A (Axon Instruments) and low pass-filtered at 1 kHz. Data were digitized (Digidata 1322A; Axon Instruments) at 10 kHz onto the hard drive of a PC for subsequent offline analysis. Sections of data recorded from outside-out patches ~10 s in length, in which unitary events predominated were selected for analysis and leak-subtracted using Clampfit (pCLAMP 8.0; Axon Instruments) for the generation of all-points amplitude histograms using Fetchan (pCLAMP 8.0). Multiple Gaussian distributions were fitted (least squares minimization) to all-points amplitude histograms using the Simplex method within pSTAT (pCLAMP 8.0) and used to determine single channel current amplitude as described previously (4). Single channel conductance ($\gamma$) values are reported as the chord conductance determined from the relationship $\gamma = i/(V_m - E_{rev})$, in which $i$ is the current amplitude of single channel events, $V_m$ is the holding potential, and $E_{rev}$ is the experimentally determined reversal potential. The onset of reduced 5-HT–activated single channel amplitude following local pressure application of MTSEA (200 $\mu$m) to inside-out patches was monitored by measuring the amplitudes of all unitary events individually using cursors in Clampfit. The recovery of single channel amplitude following the local pressure application of 100 mM dithiothreitol (DTT) was similarly monitored. The time courses of channel amplitude attenuation by MTSEA and its reversal by DTT were determined by fitting each with an exponential function. To assess the influence of extracellularly applied MTSEA (200 $\mu$m) or MTSET (200 $\mu$m), single channel events recorded over 1-min epochs were used to construct amplitude histograms from which $\gamma$ was derived. The rates at which extracellularly applied MTSEA modified the amplitudes of single channel and macroscopic currents, recorded from outside-out patches, and whole cells were determined by fitting single exponential functions to plots of mean current amplitude (recorded every 60 and 30 s, respectively) versus time.

Statistics—Data are presented as the mean ± either S.E. or S.D., as indicated. Data sets were routinely compared using one-way analysis of variance with a post hoc Tukey’s test. However, the paired t test was used to compare $\gamma$ values obtained from inside-out and outside-out patch recordings before and after the addition of MTS reagents.

**Modeling the Structures of WT 5-HT$_{3A}$ and Mutant Receptors Using the 4 Å Resolution Model of the T. marmorata nACh Receptor**—Homology models were generated as previously described (4). Briefly, amino acid sequences for the human WT 5-HT$_{3A}$, the 5-HT$_{3A}$(R432Q,R436C,R440A), and the 5-HT$_{3A}$(K431C,R432Q,R436D,R440A) subunits were aligned against the T. marmorata $\alpha$, $\beta$, $\delta$, and $\gamma$ subunits using MultiAlign (available on the World Wide Web at prodes.toulouse.inra.fr/multalin/multalin.html). Gaps between the template and query sequences in the extracellular
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FIGURE 1. MTSEA reduces the γ of point mutant 5-HT₃A(R436C) receptors by modifying the substituted cysteine at the 0'-position within the MA helix. A, left, a homology model of the WT 5-HT₃A receptor using the T. marmorata model as a template. The functional domains are indicated thus: extracellular ligand binding domain (LBD), pore-forming transmembrane helices (TM), and the portal-framing cytoplasmic MA helices (MAA). Right, an alignment of amino acids within the MA helices of WT 5-HT₃A and 5-HT₃B subunits and the mutant 5-HT₃A constructs used in this study, indicating the positions of −4', 0', and 4' residues (boxed). 5-HT₃A(QCA) and 5-HT₃A(QDA) represent the triple mutant 5-HT₃A(R432Q,R436C,R440A) and 5-HT₃A(R432Q,R436D,R440A) receptors, respectively. Substituted residues within MA helices of mutant constructs are indicated in boldface type. Amino acids are numbered according to the human 5-HT₃A amino acid sequence (20). Note that the sequences shown do not illustrate the entire MA helices. B, a general scheme for an MTS compound reacting with the sulfhydryl group on the side chain of a cysteine residue. R represents the chemical group that varies among MTS compounds (see Table 1 for structures). C, 5-HT (10 μM)-activated current recorded at −74 mV from an excised outside-out patch containing the single point mutant 5-HT₃A(R436C) receptor under control conditions. The all points amplitude histogram derived from single channel events during the deactivation of the exemplar current was fitted with two Gaussian distributions representing the open and closed states. The single channel current amplitude was −0.72 pA, corresponding to a γ of 9.7 pS. D, 5-HT (10 μM)-activated current recorded with the addition of MTSEA (200 μM) to the electrode solution. Gaussian distributions fitted to the all points amplitude histogram provide an estimated single channel amplitude of −0.28 pA, corresponding to a γ in this case of 3.8 pS. In C and D, expanded sections of data are shown below each trace. In both cases, after acquisition, digitized currents were low pass-filtered at 500 Hz with a Gaussian filter to improve the signal/noise ratio. See Table 1 for mean γ values for control and MTSEA-modified receptors.

RESULTS

Substituted Cysteine Modification of Mutant 5-HT₃A Receptors by Methanethiosulfonate Reagents—

from the template subunits. The structure of the T. marmorata nACh receptor was downloaded from the RCSB Protein Data Bank (Protein Data Bank code 2BG9) and loaded into a Deepview Swiss-PdbViewer for imaging and modeling. Wild type 5-HT₃A and 5-HT₃A(QCA) query sequences were threaded onto the backbone of the nAChR model using the “Fit Raw Sequence” and “Alignment” features in Deepview. Models were then submitted to SwissModel (available on the World Wide Web at swissmodel.expasy.org) for optimization. Following energy minimization (Gromos96, SwissModel), structures were uploaded into Deepview for imaging. The subunit interface illustrated (Fig. 6) is equivalent to the T. marmorata 1α-5γ portal. The Protein Data Bank model of cysteine-MTSEA was imported from Spartan’04 (Wavefunction Inc., Irvine, CA) and for illustrative purposes was manually aligned and positioned onto the cysteine residues of the 5-HT₃A(QCA) and 5-HT₃A(K431C,QDA) homology models.

Amino Acid Volumes—We used Spartan’04 (Wavefunction Inc., Irvine, CA) to estimate all volumes to ensure consistency when comparing amino acids and cysteine residues modified by MTS reagents. Structures were energy-minimized in the extended chain conformation. The volumes of amino acids determined by Spartan’04 correlated well with those previously published by Zamyatnin (9). A linear regression fitted to a plot of the amino acid volumes determined by Spartan’04 versus those published previously (9) yielded a coefficient of determination (r²) of 0.97 (data not shown).

RESULTS

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domain were filled in with corresponding amino acids from the template sequence. The C termini of the query sequences were shorter than the templates and were filled in with amino acids
by variance analysis to be ~900 femtosiemens) derives from residues located in the MA stretch that form putative intracellular portals depicted in the ribbon rendering of the 5-HT$_{3A}$ receptor homology model (Fig. 1A). The γ of 5-HT$_{3}$ receptor channels is enhanced to the resolvable range either by incorporation of the 5-HT$_{3B}$ subunit into heteropentameric 5-HT$_{3A/B}$ receptors (8, 10) or by replacing the 5-HT$_{3A}$ subunit MA helix residues located in the MA stretch that form putative intracellular portals depicted in the receptor homology model (Fig. 1A). The estimated volume of cysteines modified by these reagents: 2-aminoethyl-MTS (MTSEA), 2-propyl-MTS (PMTS), 2-carboxyethyl-MTS (MTSCE), and 2-sulfonatoethyl-MTS (MTSES) with similar van der Waals volumes estimated in Spartan’04 (see “Experimental Procedures”).

Table 1: MTS reagents affect the γ of 5-HT$_{3A}$ mutants with MA 0’ cysteine residues

| Condition         | MTS reagent variable group: volume$^a$ | Outside-out γ | Inside-out QCA γ |
|-------------------|----------------------------------------|---------------|-----------------|
|                   |                                        | R436D | R436C | QDA | K431C,QDA | QCA | p$^a$ | p$^a$ |
| Control           | Unmodified Cys: 108 Å$^3$               | 9.1 ± 0.6$^a$ | 7.8 ± 0.5 | 34.9 ± 1.6 | 42.4 ± 1.6 | 17.7 ± 0.4 | 17.5 ± 1.1 |
| MTSEA (ic)        | -S-CH$_2$-CH$_2$-NH$_2$: 173 Å$^3$    | 9.8 ± 0.9   | 5.0 ± 0.5$^b$ | 34.5 ± 0.9 | 38.9 ± 0.4 | 8.7 ± 0.5$^d$ | 8.2 ± 0.4$^d$ |
| MTSET (ic)        | -S-CH$_2$-CH$_2$-N(NH$_2$): 231 Å$^3$ | NT        | NT   | 31.8 ± 1.2 | NT        | 5.8 ± 0.9 | NT |
| MTSET (ec)        | -S-CH$_2$-CH$_2$-N(NH$_2$): 231 Å$^3$ | NT | NT | 31.8 ± 1.2 | NT | 6.7 ± 0.9 | NT |
| PMTS (ic)         | -S-CH$_2$-CH$_2$: 178 Å$^3$           | NT | NT | 34.3 ± 1.3 | NT | 11.9 ± 0.4 | NT |
| MTSCS (ec)        | -S-CH$_2$-CH$_2$:COO$: 185 Å$^3$      | NT | NT | 34.4 ± 0.3 | NT | 22.7 ± 1.0 | NT |
| MTSES (ec)        | -S-CH$_2$-CH$_2$:SO$_2$: 196 Å$^3$    | NT | NT | 34.4 ± 0.3 | NT | 41.7 ± 0.8 | NT |

$^a$ Values are calculated (see “Experimental Procedures”) volumes of a cysteine residue either unmodified (control) or modified by the indicated MTS reagent.
$^b$ Includes values reported previously (4).
$^c$ Includes values reported previously (3).
$^d$ Statistical significance was determined by unpaired t-test, p < 0.01, compared with control.
$^e$ Statistical significance was determined by analysis of variance with post hoc Tukey’s test.
$^f$ Statistical significance was determined by analysis of variance with post hoc Tukey’s test, p < 0.001, compared with all other values in the MA column.

To address this issue, we used substituted cysteine modification to examine the γ of mutant receptors containing varying chemical entities at the MA 0’ position. We created 5-HT$_{3A}$ constructs in which Cys replaced Arg at the 436-position (Fig. 1A) and subsequently modified the physicochemical properties of the substituted residue with MTS compounds that react rapidly and specifically with thiolate groups (Fig. 1B). When examining the influence of charge, we utilized the MTS compounds: 2-aminoethyl-MTS (MTSEA), propyl-MTS (PMTS), 2-carboxyethyl-MTS (MTSCE), and 2-sulfonatoethyl-MTS (MTSES) with similar van der Waals volumes estimated in Spartan’04 (see “Experimental Procedures”). The estimated volumes of cysteines modified by these reagents were 173, 178, 185, and 196 Å$^3$, respectively (Table 1).

Thus, such agents differ predominantly in the substituent attached to an ethanethiol chain (i.e. positively charged ammonium, neutral methyl, and negative carboxylate and sulfonate groups) (Table 1). We also utilized the larger quaternary ammonium compound MTSET. The estimated volume of cysteine modified by MTSET was 231 Å$^3$ (Table 1).

MTSEA reduces the γ of the 5-HT$_{3A}$ (R436C) but not the 5-HT$_{3A}$ (R436D) mutant receptor—The single amino acid substitution of the MA 0’ Arg by Cys in the mutant homomeric 5-HT$_{3A}$ (R436C) receptor increased γ to a level that could be directly observed in recordings from outside-out patches. The transient application of 5-HT (10 μM) to outside-out membrane patches clamped at −74 mV elicited rapidly rising inward currents from which unitary events with a mean γ of 7.8 ± 0.5 pS (Table 1) were discernable during current deactivation (Fig. 1C). Inclusion of MTSEA (200 μM) within the electrode solution, thus exposing the intracellular face of the membrane to the reagent, reduced the γ of unitary events mediated by the 5-HT$_{3A}$ (R436C) receptor to 5.0 ± 0.5 pS, a value close to the limit of resolution (Fig. 1D and Table 1). Since the γ of WT 5-HT$_{3A}$ receptors is too low to enable direct observation of unitary events (3, 4, 8), we used homomeric mutant 5-HT$_{3A}$ (R436D) receptors as a control to exclude potential effects of MTSEA that may occur independently of the substituted cysteine residue. MTSEA had no significant effect on the γ of events mediated by homomeric 5-HT$_{3A}$ (R436D) receptors, which was 9.1 ± 0.6 and 9.8 ± 0.9 pS in the absence and presence of the reagent, respectively (Table 1). Therefore, the reduction in γ of the 5-HT$_{3A}$ (R436C) receptor was caused specifically by modification of the substituted MA 0’ Cys.

MTS Reagents Modulate the γ of the Mutant 5-HT$_{3A}$(R432Q,R436C,R440A) Receptor—In subsequent experiments, we employed a 5-HT$_{3A}$(R432Q,R436C,R440A) construct, hereafter termed 5-HT$_{3A}$ (QCA), which we anticipated from our previous studies (3, 4) would produce channels with a γ more amenable to the quantitative analysis of the modulatory effects of MTS reagents. In comparison with 5-HT$_{3A}$ (R436C), the γ of the 5-HT$_{3A}$ (QCA) construct determined from recordings performed on outside-out membrane patches was indeed substantially increased (17.7 ± 0.4 pS) (Fig. 2A and Table 1), although not to the extent found for the previously characterized 5-HT$_{3A}$ (QDA) receptor (4), which has a γ of 34.9 ± 1.6 pS (Fig. 2B and Table 1). MTSEA (200 μM) reacted with 5-HT$_{3A}$ (QCA) receptors, causing a substantial reduction in γ to 8.7 ± 0.5 pS (Fig. 2C and Table 1). The specificity of this effect was confirmed in parallel experiments performed on the 5-HT$_{3A}$ (QDA) receptor, the γ of which was unaffected by MTSEA (Fig. 2D and Table 1).

MTSEA is sufficiently membrane-permeant to access residues engineered within a cytoplasmic region of the Shaker B K$^+$ channel when applied to the extracellular aspect of the membrane (11). Therefore, we investigated whether extracellular MTSEA could access the MA 0’ residue of the 5-HT$_{3A}$ (QCA)
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**FIGURE 2.** MTSEA reduces γ by reacting with the substituted Cys at the MA 0<sup>+</sup>-position of the triple mutant 5-HT<sub>3A</sub>(QCA) receptor. Data were recorded from excised outside-out patches containing either 5-HT<sub>3A</sub>(QCA) or 5-HT<sub>3A</sub>(QDA) receptors as indicated (see Fig. 1A for position of mutations within the MA helix). 5-HT (10 μM) was applied by pressure ejection onto patches at a holding potential of −74 mV. All points amplitude histograms are shown below each trace. The 5-HT-activated patch currents mediated by 5-HT<sub>3A</sub>(QCA) receptors in the absence (A) and in the presence (C) of MTSEA (200 μM) in the electrode solution are shown. Also shown are the 5-HT-activated patch currents mediated by 5-HT<sub>3A</sub>(QDA) receptors in the absence (B) and in the presence (D) of MTSEA (200 μM). All points amplitude histograms were fitted with the sum of two Gaussians, and single channel current amplitudes were calculated to be as follows: 5-HT<sub>3A</sub>(QCA), −1.34 and −0.72 pA; 5-HT<sub>3A</sub>(QDA), −2.58 and −2.54 pA, in the absence and presence of MTSEA, respectively.

In extracellular application of MTSEA did not decrease the γ of 5-HT<sub>3A</sub>(QDA) receptors in outside-out patches (γ = 31.8 ± 1.2, n = 6), confirming that the inhibition of γ observed with 5-HT<sub>3A</sub>(QCA) receptors is due to the specific reaction of MTSEA with MA 0<sup>+</sup> Cys, rather than a rapid "flickery" nonselective pore block from the extracellular environment that could, in principle, yield the impression of a reduction in γ (Fig. 3, B and D).

In parallel experiments, bath application of the membrane-impermeant compound MTSET (200 μM) (11) to 5-HT<sub>3A</sub>(QCA) receptors in excised outside-out patches had no influence upon γ (17.2 ± 0.8 pS, n = 6). However, in common with MTSEA, MTSET was clearly effective when applied to the intracellular aspect of the membrane, reducing γ to 6.7 ± 0.4 pS (Fig. 3, C and D, and Table 1).

**Extracellular MTSEA Crosses the Cell Membrane to Reduce 5-HT<sub>3A</sub>(QCA)-mediated Currents—**MTSEA has a smaller volume than MTSET (Table 1); thus, it is possible that the former, but not the latter, is able to pass through the open channel of the 5-HT<sub>3A</sub>(QCA) receptor and modify Cys<sup>436</sup> when applied to the extracellular aspect of an outside-out patch. Alternatively, MTSEA, which exists in both charged and uncharged species (11), may access the cytoplasmic Cys<sup>436</sup> residue by permeating the membrane, a route denied to MTSET, which is permanently charged and therefore membrane-impermeant. We performed whole-cell experiments to determine whether the construct in excised outside-out patches. In such experiments, single channel events evoked by pressure-applied 5-HT (10 μM) were recorded prior to MTSEA application to obtain a control data set (Fig. 3, A and D). The subsequent addition of MTSEA (200 μM) to the perfusate caused an ~50% reduction in γ (to 8.5 ± 0.9 pS, n = 6) within 2 min (Fig. 3, A and D). A single exponential function fitted to the onset of the reduction of the mean conductance by MTSEA yielded a τ = 94 s (fit not shown). The extent of the depression of γ was essentially identical to that caused by intracellular application of MTSEA at the same concentration (Fig. 3A and Table 1). A washout period of up to 20 min did not reverse the inhibition of γ by extracellularly applied MTSEA (data not shown). Furthermore, extracellular application of MTSEA did not decrease the γ of 5-HT<sub>3A</sub>(QDA) receptors in outside-out patches (γ = 31.8 ± 1.2, n = 6), confirming that the inhibition of γ observed with 5-HT<sub>3A</sub>(QCA) receptors is due to the specific reaction of MTSEA with MA 0<sup>+</sup> Cys, rather than a rapid "flickery" nonselective pore block from the extracellular environment that could, in principle, yield the impression of a reduction in γ (Fig. 3, B and D).

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the recording chamber. After a 240-s exposure to MTSEA, in
the absence of channel activation, we resumed 5-HT applica-
tion every 30 s (Fig. 4B). Using this approach, MTSEA applied
in the absence of repetitive 5-HT3A(QCA) receptor activation
inhibited currents subsequently activated by 5-HT by
\(50\%\), similar to the level achieved over the same time course with
repetitive channel activation (Fig. 4C). Such data suggest that,
under the conditions of these experiments, MTSEA gained
access to Cys436 primarily by permeating the cell membrane.

Negative Charge of the MA 0
Residue Overcomes Steric
Hindrance to Increase
—Neutral PMTS also caused a sig-
nificant reduction in
(\(11.9\ ±\ 0.4\ pS\)) of 5-HT3(QCA)
receptors relative to control (Fig. 5A), but the effect was not
as pronounced as that found for the positively charged
MTSEA or for MTSET (\(p\ <\ 0.001\); Table 1). These data
suggest that for cysteines modified by MTS reagents of com-
parable volume (Table 1), the introduction of a positive
charge causes an additional attenuation of \(\gamma\). The impor-

**FIGURE 3. Extracellular application of MTSEA, but not MTSET, reduces the \(\gamma\) of mutant 5-HT3A(QCA) receptor.** Data were recorded from excised outside-out patches expressing either 5-HT3A(QCA) or 5-HT3A(QDA) receptors as indicated (see Fig. 1A for the positions of mutations within the MA helix). 5-HT (10 \(\mu M\)) was applied by pressure ejection onto patches at a holding potential of \(-74\ mV\). A, representative single channel events elicited by 5-HT acting at 5-HT3A(QCA) receptors in the absence (left) and in the presence of extracellular (ec; middle) or intracellular (ic; right) MTSEA (200 \(\mu M\)). Note that MTSEA is effective in reducing single channel current amplitude irrespective of the membrane aspect to which it was applied. B, exemplar single channel events evoked by 5-HT acting at 5-HT3A(QDA) receptors in the absence (left) and in the presence of extracellular (ec; middle), or intracellular (ic; right) MTSEA (200 \(\mu M\)). C, typical single channel events gated by 5-HT acting at 5-HT3A(QCA) receptors in the absence (left) and in the presence of extracellular (ec; middle) or intracellular (ic; right) MTSET (200 \(\mu M\)). Note that MTSET is only effective when applied to the inner aspect of the plasma membrane. D, graphical depiction of the time course of the action of MTSEA or MTSET on mutant 5-HT3A receptors. 5-HT (30 \(\mu M\)) was applied at 1-min intervals by local pressure ejection, and MTSEA (200 \(\mu M\)) was applied via the superfusate during the period indicated by the horizontal bar. 5-HT was applied during the course of the MTSEA application (closed circles) or after 240 s of exposure (open squares). Data points are the mean of four experiments, and vertical bars depict ± S.E.

**FIGURE 4. Extracellular MTSEA gains access to the MA 0’ Cys by crossing the cell membrane.** 5-HT (30 \(\mu M\))-activated currents recorded at a holding potential of \(-60\ mV\) from HEK293 cells expressing 5-HT3A(QCA) receptors. A, 5-HT-activated currents recorded at 30-s intervals (alternate currents illustrated) before and during the extracellular application of MTSEA (200 \(\mu M\)) illustrate the onset of inhibition due to Cys modification. B, currents were recorded before and following a 240-s exposure to MTSEA (200 \(\mu M\)). C, graphical depiction of the time course of the action of MTSEA on 5-HT3A(QCA) receptors. 5-HT (30 \(\mu M\)) was applied at 30-s intervals by local pressure ejection, and MTSEA (200 \(\mu M\)) was applied via the superfusate during the period indicated by the horizontal bar. 5-HT was applied during the course of the MTSEA application (closed circles) or after 240 s of exposure (open squares). Data points are the mean of four experiments, and vertical bars depict ± S.E.
The local potential was confirmed by the inclusion of the negatively charged MTSCE in the electrode solution, which caused a significant increase in $\gamma$ to 22.7 ± 1.0 pS (Fig. 5B and Table 1). Similarly, the introduction of negative charge by reaction of the 5-HT$_{3A}$(QCA) receptor with MTSES (Fig. 5C) also increased $\gamma$ to 26.2 ± 0.7 pS (Table 1).

Interestingly, the $\gamma$ determined in the presence of MTSES was statistically greater than that observed for MTSCE ($p < 0.001$; Table 1). The $\gamma$ values for the 5-HT$_{3A}$(QDA) receptor in the absence and presence of all MTS compounds are summarized in Table 1. Importantly, none of the MTS reagents caused a significant change in $\gamma$ of the 5-HT$_{3A}$(QDA) receptor. Collectively, these data demonstrate that MTS reagents modulate $\gamma$ of 5-HT$_{3A}$(QCA) receptors by reacting specifically with the thiolate group present on the cysteine residue substituted into the MA 0-position and emphasize the importance of charge at this position.

MTS reagents with different physicochemical properties differentially affect the $\gamma$ of mutant 5-HT$_{3A}$(QCA) receptors. Currents were activated by 5-HT (10 $\mu$M) applied to excised outside-out patches containing 5-HT$_{3A}$(QCA) receptors held at a holding potential of −74 mV. Patch electrodes were filled with recording solution containing the indicated MTS reagent (200 $\mu$M). Patch currents recorded with electrodes containing PMTS (A), MTSCE (B), or MTSES (C) are shown. All points amplitude histograms of unitary events during the deactivation of currents are shown below each trace. The Gaussian fits to the open and closed states revealed that the amplitudes of single channel events mediated by 5-HT$_{3A}$(QCA) receptors were 0.83, 1.50, 2.13 pA when modified by PMTS, MTSCE, or MTSES, respectively.

Control data obtained in parallel on the 5-HT$_{3A}$(QDA) receptor confirming that such MTS reagents do not affect $\gamma$ in constructs without a substituted cysteine are summarized in Table 1.
fied unitary events had a mean amplitude of 1.5 pA from the all points amplitude histogram. Mean values of single channel currents recorded from inside-out patches by inclusion within the recording electrode. The patch was held at +80 mV and bathed with a symmetrical NaCl-based solution. The unitary current amplitude of sporadic 5-HT-activated currents in control was determined to be 1.5 pA from the all points amplitude histogram fitted with the sum of two Gaussians. The effect of MTSEA (200 μM) applied by pressure ejection for 40 s reduced the amplitude of single channel currents recorded from inside-out patches containing 5-HT3A(QCA) mutant receptors before and after MTSEA application are provided in Table 1. Application of the reducing agent DTT (100 μM) caused an increase in the amplitude of single channel currents compared with those recorded from the same patch following modification by MTSEA (8). The corresponding all points amplitude histogram reveals that the amplitude of unitary currents after treatment with DTT was 1.3 pA. The time course of the effects of MTSEA and DTT on the amplitudes of single channels recorded from the inside-out patch from which data were obtained for A–C. The amplitudes of all unitary events were measured to construct the graph of current amplitude versus time illustrated in D. Data points before and after the abscissa break represent the average single channel current amplitudes for events occurring in the preceding 4 and 10 s, respectively. The vertical lines indicate ±S.D. and data points were fitted with single exponential functions, from which the time constants of onset and reversal of the actions of MTSEA were determined. Cysteine modification by MTS reagents can be reversed by high concentrations of reducing agents, such as DTT (11). Pressure application of DTT (100 nM) to an inside-out patch containing 5-HT3A(QCA) receptors modified by MTSEA caused a slow recovery of the amplitude of 5-HT-activated single channels toward values observed prior to the application of MTSEA (Fig. 6C). We investigated the time course of MTSEA-modification by measuring the amplitude of 5-HT-activated single channels before, during, and after MTSEA application. The successful completion of this experiment requires sustained channel opening events over a prolonged period of time despite the confounding influence of desensitization caused by constant exposure of the receptors to 5-HT within the electrode solution. Unfortunately, the majority of patches exhibited sporadic channel activity with insufficient events, and thus temporal resolution, to determine the rate at which MTSEA reduced γ. However, in the exemplar patch (Fig. 6D), channel activity was relatively frequent, and the reduction in γ had a τ = 15 s, determined by fitting an exponential function to the data points during the onset of the action of MTSEA. Reversal of the effect of MTSEA by DTT had a τ = 77 s (Fig. 6D). This experiment demonstrates that real time modification of the physicochemical properties of MA 0’ residue reduces γ, an effect that can be reversed by application of a reducing reagent. Modification of a Cysteine Residue at MA −5’ outside the Putative Portal Did Not Affect γ—We constructed homology models of wild-type and mutant 5-HT3A receptors based on the structural model of the T. marmorata (Fig. 7). One of the five putative cytoplasmic portals framed by adjacent MA helices is shown in each case. Three arginine residues lie at the entrance to portals of the wild-type 5-HT3A receptors (Fig. 7A), located at the MA −4’-, −0’-, and 4’-positions (Fig. 1A), each of which contribute to the receptor’s characteristically low γ (4). The portal of the 5-HT3A(QCA) receptor by comparison contains smaller uncharged residues (Fig. 7B). Modification of the MA 0’ Cys by MTSEA adds substantial bulk and a positive charge within the portal (Fig. 7C). Consistent with the model, this modification substantially reduces the γ of the 5-HT3A(QCA) receptor (Figs. 2, 3, and 6 and Table 1). To further test the validity of the structural model, we substituted a Cys for Lys431 in the 5-HT3A(QDA) mutant receptor background. The model (Fig. 7D) predicts that Cys431 at the MA −5’-position (Fig. 1A) resides outside the portal at a locus where modification of its charge and volume by MTS reagents would be anticipated to have a minimal effect upon γ. We specifically selected MA −5’ Lys for modification, because the replacement of this basic residue by Cys is broadly analogous to the Arg to Cys mutation at the MA 0’-position. Outside-out patch recordings revealed that 5-HT-activated single channels mediated by the 5-HT3A(K431C,QDA) receptor had a small but significantly (p < 0.01) increased γ compared with those mediated by the 5-HT3A(QDA) receptor (Table 1). However, consistent with the predicted location of Cys431 outside the portal (Fig. 7D),
modification of the residue by intracellular application of either MTSEA or MTSES to outside-out patches did not significantly affect γ (Table 1).

**DISCUSSION**

We used cysteine modification by a series of MTS reagents to investigate the mechanism by which the substituted 0′ residue within the cytoplasmic MA helix influences γ of the human 5-HT$_{3A}$ receptor. Substitution of the MA 0′ Arg by Cys within either the WT 5-HT$_{3A}$ receptor background or the 5-HT$_{3A}$(R432Q,R440A) construct in which 0′ and 4′ arginines were replaced by glutamine and alanine, respectively, substantially increased γ compared with equivalent values observed previously in the absence of the 0′ Cys residue (4). Furthermore, modification of substituted MA 0′ Cys residues by MTS reagents either further increased or reduced γ, depending on the physicochemical properties of the reagent. This study lends further support to the cryo-electron microscopy-derived atomic scale model of Cys loop receptors based on the T. marmorata nACh receptor in which adjacent MA helices frame cytoplasmic portals (Fig. 7), forming an obligate pathway through which ions pass to traverse the cell membrane (6).

The homology model of the human homomeric WT 5-HT$_{3A}$ receptor based on the 4 Å resolution structure of the T. marmorata nACh receptor (6) suggests that MA –4′, 0′, and 4′ arginine residues lie near the mouth of each putative intracellular portal (Fig. 7A). Indeed, there is a preponderance of basic residues in the MA helices of the 5-HT$_{3A}$ receptor (Fig. 1A), and it is likely that this concentration of positive charge contributes to the anomalously low γ of the channel (4, 8). Consistent with this hypothesis, structural models of the T. marmorata and α$_{4}$β$_{2}$ nACh receptors, which both have vastly higher γ values than that of the WT 5-HT$_{3A}$ receptor, indicate that acidic residues predominate within the portals and their immediate vicinity (4, 6).

Both the volume and charge of residues within the TM2 helices lining the central channel pore play a role in determining γ. The identity of the amino acid at the TM2 –1′ position near the cytoplasmic face of the membrane is a crucial determinant of γ. Substitution of the –1′ Glu by polar uncharged Gln in the α subunit reduced the γ of the Torpedo californica nACh receptor –4-fold (2, 12). Even more profound reductions in γ ensue from the mutation of the –1′ Glu, or –2′ Gly, of the β-subunit of adult muscle nACh receptor to lysine (13). Charge inversion substitutions at the extracellular 0′ and intracellular 4′ rings of the T. californica α-subunit resulted in a 50% reduction in γ. Introduction of the bulky Tyr residue in place of the smaller Thr or Ser at the 2′ central ring position also reduced γ by 50% (14), suggesting that the pore narrows considerably in this region. Recent data indicate the most constricted region of the adult muscle nACh receptor to be at positions –1′ and –2′ (13).

Consistent with the strategic location of the cytoplasmic MA 0′ arginine in the 5-HT$_{3A}$ subunit, wherein the side chain gua-
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nidium group is predicted to protrude into the mouth of the portal (Fig. 7A), its replacement by various other amino acids has a large impact on $\gamma$. Single point substitutions to Asp, Glu, Gln (4), or Cys cause increases in $\gamma$ values of mutant 5-HT$_{3A}$(R436X) receptors, such that single channels can be resolved in outside-out patch recordings. All of these residues differ from the native MA 0' arginine in both their smaller side chain volume and lack of positive charge. Either or both of these physicochemical properties may contribute to an increased $\gamma$.

The 4 Å model of the $T. marmorata$ nACh receptor reveals that cytoplasmic portals have a maximum width of only ~8 Å, which is approximately equal to the diameter of a sodium or potassium ion that retains its first hydration shell. A comparison of homology models of the $\alpha_4\beta_2$ nACh receptor and the 5-HT$_{3A}$ receptor suggests that the portals of the latter are further constricted (4). Consistent with the idea that portals of the 5-HT$_{3A}$ receptor hinder ion flow due to their extreme narrowness, substitution of the MA 0' arginine (173 Å$^3$) by the large aromatic residue phenylalanine (174 Å$^3$) yielded a 5-HT$_{3A}$(R436F) mutant receptor with a low $\gamma$, estimated by variance analysis to be ~400 femtosiemens (4).

The availability of a variety of MTS reagents that differ by charge and/or volume provides a useful tool for correlating modifications in the structure of the cysteine-modified MA 0' residue of the 5-HT$_{3A}$ subunit with function, in this case $\gamma$. Single channels recorded from outside-out patches containing mutant 5-HT$_{3A}$(R436C) receptors were readily resolvable, although the $\gamma$ of this mutant was smaller than that observed when the MA 0' Arg was replaced by either Glu or Asp (4). In bulk water, cysteine is largely uncharged at physiological pH. Therefore, the smaller $\gamma$ of mutant 5-HT$_{3A}$(R436C) receptors compared with either 5-HT$_{3A}$(R436E) or 5-HT$_{3A}$(R436D) receptors might be explained by a relative lack of a negative charge. However, it is not clear what the $pK_a$ of the cysteine side chain is within the environment of the MA helix of the 5-HT$_{3A}$ receptor. The recent demonstration that the $pK_a$ values of amino acid residues within the TM2 of the $\delta$-subunit of the adult muscle nAChR can deviate markedly from their bulk water values highlights the need for caution in this respect (13).

Modification of 5-HT$_{3A}$(R436C) receptors by inclusion of MTSEA in the electrode solution caused an ~50% reduction in $\gamma$ (to 5 pS), making single channel events difficult to resolve. By contrast, MTSEA had no effect on $\gamma$ of 5-HT$_{3A}$(R436D) receptors. We previously demonstrated that replacement of MA 0' arginine remained while the 4' and 4' positions of the 5-HT$_{3A}$(QDA) triple mutant increased $\gamma$ by ~4-fold in comparison with the $\gamma$ of the single mutant 5-HT$_{3A}$(R436D) receptor (4). Similarly, the triple mutant 5-HT$_{3A}$(QCA) receptor had a $\gamma$ more than double that of the single point mutant 5-HT$_{3A}$(R436C) receptor (Table 1). The homology model of the 5-HT$_{3A}$(QCA) construct predicts that the relatively small size of the amino acid side chains at MA 0', 0', and 4' increases the dimensions of the portals compared with the WT 5-HT$_{3A}$ receptor (Fig. 7). We took advantage of the increased signal/noise ratio of the 5-HT$_{3A}$(QCA) receptor in experiments investigating the modification of the MA 0' Cys.

Inclusion of the uncharged PMTS in the electrode solution significantly decreased the $\gamma$ of 5-HT$_{3A}$(QCA) receptors in outside-out patches. Such data support the hypothesis that increased volume at MA 0' hinders ion conduction (Table 1). However, whereas PMTS and the positively charged reagent MTSEA modify cysteines to achieve similar estimated volumes (Table 1), the latter caused a significantly greater reduction in $\gamma$ to less than half that recorded in the absence of the reagent.

MTSEA was equally effective as an inhibitor of $\gamma$ when applied to the intracellular or extracellular aspect of the membrane. When applied to whole cells, the degree of inhibition of 5-HT-activated macroscopic currents was similar to the reduction in $\gamma$ observed in excised patch recordings. Furthermore, the inhibition by MTSEA was independent of channel activation. Thus, under the conditions of these experiments, MTSEA primarily accesses the MA 0' residue by diffusion through the membrane as the uncharged species (11, 15). Consistent with this interpretation, MTSEA reduced $\gamma$ more rapidly when applied directly onto the intracellular aspect of an inside-out patch. Using this approach, the rate of reduction of the amplitude of unitary events was still substantially slower than the rate of MTS modification of simple thiol compounds and easily accessible substituted Cys residues in the outer mouth of the Shaker B K$^+$ channel (16, 17). Such data suggest that MTSEA encounters a rate-limiting barrier en route to the MA 0' residue within the portal. However, the estimated rate of accessibility derived from MTSEA application to the inside-out patch needs to be treated with caution due to the sporadic nature of channel gating with 5-HT included in the recording electrode.

The membrane-impermeant quaternary ammonium compound, MTSET, caused a reduction in $\gamma$ only when applied to the intracellular aspect of the membrane. MTSET caused a marginally larger reduction in $\gamma$ than was observed with MTSEA, in keeping with the larger estimated volume of the MTSET-modified cysteine (Table 1). Such observations are consistent with the hypothesis that the positive charge of the modified MA 0' Cys causes electrostatic repulsion of permeating cations. Interestingly the $\gamma$ of the 5-HT$_{3A}$(QCA) receptor modified by MTSEA (or MTSET) (Table 1) is comparable with that of the 5-HT$_{3A}$(QRA) construct (~6.5 pS), in which the MA 0' arginine remained while the 4' and 4' arginines were replaced by Gln and Ala, respectively (4). This is perhaps not surprising, given that both the estimated volume and charge of the MTSEA-modified Cys resemble those of arginine (Fig. 6).

Based on the effects of MTSEA, MTSET, and PMTS, it would have been unsurprising if additional volume contributed by any MTS reagent reduced $\gamma$ of modified 5-HT$_{3A}$(QCA) receptors. However, negatively charged MTSES and MTSCE reagents increased $\gamma$ to values greater than that of the unmodified 5-HT$_{3A}$(QCA) receptor (Table 1). Such data suggest that the electrostatic attraction exerted by the negatively charged reagents more than compensates for the steric hindrance imposed by the increased volume of the modified MA 0' Cys. MTSCE contains a carboxyethyl group that is identical in structure to a glutamic acid side chain, yet the $\gamma$ of 5-HT$_{3A}$(QCA)-MTSCE is less than that of the 5-HT$_{3A}$(QEA) receptor by >10
pS (4). This is consistent with greater steric hindrance of the MA 0’ Cys plus a carboxyethyl group compared with a MA 0’ Glu (volumes estimated to be 185 and 136 Å³, respectively). Interestingly, although MTSCE and MTSES both carry one net negative charge, the latter, despite its larger volume (Table 1), caused a greater increase in the γ of the 5-HT₃ₐ(QCA) receptor. This is potentially due to the more strongly acidic nature of the sulfonate group compared with the carboxylate group. However, the pKₐ values for the sulfonate and carboxylate side groups of MTSES and MTSCE, respectively, in the environment of the 5-HT₃ₐ portal are unknown.

The reducing agent DTT reversed the reduction in γ of the 5-HT₃ₐ(QCA) receptor observed in the inside-out patch configuration following exposure to MTSEA. These data demonstrate that the effects of MTS reagents were caused specifically by cysteine modification. Furthermore, MTS-dependent changes in γ were seen only in channels containing a substituted MA 0’ Cys. MTS reagents were without effect on the mutant 5-HT₃ₐ(QDA) receptor even upon substitution of Cys for the MA 0’ Lys. These data support the structural model of the 5-HT₃ₐ receptor, which predicts that the MA 0’ residue lies outside the portal (Fig. 7D). In future studies, it should be feasible to employ the substituted cysteine accessibility method to identify all residues within the MA stretch that impinge upon the permeation pathway in a manner analogous to that adopted to evaluate the channel-lining TM2 residues of, for example, the 5-HT₃ₐ receptor (18, 19).

Our results suggest that the MA 0’ residue affects the γ of the 5-HT₃ₐ receptor both by steric and electrostatic influences. Determination of whether the MA helix residues within the anionic subgroup of Cys loop receptors (i.e. γ-aminobutyric acid type A and glycine receptors) influence γ by similar mechanisms awaits additional studies. The existence of residues accessible to the cytoplasmic milieu that dictate γ raises the possibility of dynamic regulation of fundamental properties previously thought to be obdurate in Cys loop receptors.

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