Chimeric Mutations in the M2 Segment of the 5-Hydroxytryptamine-gated Chloride Channel MOD-1 Define a Minimal Determinant of Anion/Cation Permeability*

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The ionotropic receptor MOD-1 (for modulation of locomotion defective 1) has been cloned in the nematode Caenorhabditis elegans (1). This homeric assembly has been characterized as a 5-hydroxytryptamine (5-HT)\(^3\) ligand-gated chloride channel with a predicted protein structure similar to that of the members of the nicotinic acetylcholine receptor family of ligand-gated ion channels (LGICs). These receptors, which also include \(\gamma\)-aminobutyric acid (GABA\(_\)A\) receptors, glycine, and cationic 5-HT3 channels, are structurally similar allostERIC membrane proteins (2, 3) that mediate fast synaptic transmission (4, 5). They are believed to assemble as pentamers, allowing formation of a central water-filled pore (6) and are differentially selective; the 5-HT\(_3\) receptor and the nicotinic acetylcholine receptor (nAChR) are cation-selective, whereas the glycine receptor (GlyR) and the GABA\(_A\) receptor are anion selective. Individual subunits are predicted to contain a large N-terminal domain and four transmembrane-spanning domains (M1–M4), with the M2 domain forming the walls of the pore (7, 8). Agonist binding to the N-terminal domain of the LGICs promotes a rearrangement that converts the conformation of the M2 domains from a non-conducting to a conducting open state (9–11). Identification of the molecular determinants of ion selectivity (12) has been based in part on a comparison between anionic and cationic selective ionotropic receptors in such a closely related family (for review, see Ref. 13). Site-directed mutagenesis studies performed on nAChRs have identified rings of residues that alter channel gating (14), conductance (15), or the selectivity for monovalent (15–17) or divalent cations (18, 19). Studies of chimeric substitutions between the cationic M2 regions allowed the identification of three essential residues for determining selectivity of the pore (19–21). Subsequently, in the \(\alpha1\) GlyR a single mutation has also been identified that converts the selectivity of this channel (19).

MOD-1 offered an advantage to test the basis for anion selectivity over previously studied LGICs because it is a homeric receptor of identical subunits (1). We analyzed the basis for ionotropic selectivity of MOD-1 using different mutants that explored the roles of the extra- and intra-cellular rings of charge. A minimal substitution sufficient to convert the selectivity from anionic to cationic was identified at A270E. In addition, we also demonstrated that in MOD-1 the triplet of mutations not only induced a conversion in charge selectivity but also gave rise to a highly \(K^+\)-selective channel (Fig. 1).

**EXPERIMENTAL PROCEDURES**

*Site-directed Mutagenesis—*Complementary DNA encoding the wild type (WT) MOD-1 was subcloned into the pGEMHE expression vector (22). Site-directed point mutations were constructed by oligonucleotide-directed PCR mutagenesis using *Pfu* Turbo polymerase (Stratagene). The mutations were confirmed by sequencing with at least two different primers.

*Expression in Vitro—*For transcription, the expression construct was first linearized by PSTI. RNA transcripts encoding MOD-1 and mutants were synthesized from linearized constructs using the T7 mMessage mMachine kit (Ambion, Austin, TX). The quality of RNA was confirmed by agarose gel analysis. Stage V oocytes were removed from adult female *Xenopus laevis* frogs and prepared as described previously (23). Oocytes were incubated in ND-96 medium that consisted of 96 mm NaCl, 2 mm KCl, 1.8 mm CaCl\(_2\), 1 mmMgCl\(_2\), and 5 mM HEPES, pH 7.6, supplemented with 0.1 mg/ml gentamycin and 0.55 mg/ml pyruvate. RNA encoding WT or mutant versions of the MOD-1 channel was injected at ~50 ng/oocyte. Oocytes were incubated at 18 °C for ~24 h in ND-96 medium before voltage clamp experiments.

*Two-electrode Recordings—*Oocytes were placed in the recess of a small chamber and continuously superfused by gravity-driven flow (10 ml/min) with control or test solutions selected by computer-controlled...
solenoid valves (ALA-VM8; ALA Instruments). Currents activated by 5-HT were recorded from oocytes using a two-electrode voltage clamp (Warner OC-725C). Electrodes were made from borosilicate glass, pulled with a Sutter P-87 puller, and filled with a filtered 3 M KCl solution. All experiments were performed at room temperature.

The reversal potential ($E_{\text{rev}}$) was measured by continuously recording the membrane current as the voltage was ramped from $-70$ mV to $+50$ mV over 50 ms and then back again. This triangular voltage stimulus was applied repeatedly as the agonist (1 μM 5-HT) was washed over the oocytes. Ligand-activated channel opening results in more current flow whenever the membrane potential is away from its reversal value. Thus the $E_{\text{rev}}$ of 5-HT-activated channels was readily apparent from a superposition of current responses before and after the application of 5-HT. The data in Figs. 2 and 3 show MOD-1-specific currents, computed by subtracting the total current recorded before application of 5-HT. Ionic selectivity was determined by measuring shifts in $E_{\text{rev}}$ as the anionic composition of the bath was altered. Bath solutions contained 100 mM of the test ions (NaCl, KCl, choline chloride, or sodium gluconate) plus 0.2 mM calcium gluconate and 5 mM HEPES. The relative permeability of two cations, $a$ and $b$ (or two anions), was estimated from the shift in the reversal potential according to the Goldman-Hodgkin-Katz equation shown here as Equation 1,

$$P_a / P_b = [a] / [b] \exp \left( \frac{F}{RT} (E_{\text{rev}} - E_{\text{rev}, a}) \right)$$  \hspace{1cm} (Eq. 1)$$

where $R$, $T$, and $F$ have their usual meaning, $E_{\text{rev}, a}$ is the reversal potential obtained with external solution $a$, and $E_{\text{rev}, b}$ is obtained with solution $b$. This computation assumes that the permeability of the counter ion to $a$ or $b$ is negligible.

For the determination of agonist dose-response relationships (Fig. 5), an amplitude normalization was required because channels did not completely recover from the desensitization induced by high concentrations (>3 μM) of 5-HT. The peak current was measured in response to brief (5 s) applications of 5-HT beginning at 0.03 μM, followed by a 5-min wash and then subsequent test concentrations up to 1 μM. Complete recovery from desensitization was verified by repeating the measurement at 1 μM after a 20-min wash. Finally, a single response at 3, 10, or 30 μM 5-HT was measured, but no additional concentrations could be tested because of desensitization. The responses for each egg were normalized to an amplitude of 1 at a 5-HT concentration of 1 μM. Equation 2, shown here,

$$I = I_{\text{max}} (15\text{-HT}/([5\text{-HT}] + EC_50))$$  \hspace{1cm} (Eq. 2)$$

was fitted with normalized data pooled from multiple eggs. Finally, the data were renormalized by dividing by $I_{\text{max}}$ to generate the amplitude-normalized curves shown in Fig. 5. Data were analyzed using Clampfit (Axon Instruments, Foster City, CA) and Origin (MicroCal, Northampton, MA), and all parameter estimates are given as the mean ± S.E. for n independent experiments.

RESULTS

Determinants of Ionic Selectivity for MOD-1 Are Conserved Among the LGIC Family Members—WT MOD-1 and the mutants were expressed in Xenopus oocytes and characterized by recording 5-HT-gated whole-cell current in a two-electrode voltage clamp. To compare the ionic selectivity of the WT and mutant receptors, we measured current-voltage relationships in a number of different extracellular solutions, namely 100 mM NaCl, KCl, sodium gluconate, choline chloride, and, for mutant 3, N-methyl-D-glucamine. Sample traces illustrating current-voltage relations for WT MOD-1 channels in 100 mM external NaCl, KCl, choline chloride, and sodium gluconate are shown in Fig. 2. The mean and S.E. of the $E_{\text{rev}}$ values measured in these four different external solutions are given in Table II. The changes in $E_{\text{rev}}$ demonstrate that MOD-1 is a chloride-permeant anion channel. The reversal potential of WT MOD-1 was relatively insensitive to the external cation; $E_{\text{rev}}$ was $-27.5 ± 1.1$ mV (n = 11) for NaCl, $-31.9 ± 2.1$ mV (n = 11) for KCl, and $-24.3 ± 2.1$ mV (n = 11) for choline chloride. All of these values nearly matched the typical equilibrium potential for Cl in oocytes of about $-25$ mV in 100 mM external Cl⁻. In contrast, switching from NaCl to sodium gluconate shifted $E_{\text{rev}}$ by $+66$ mV. The interpretation is that MOD-1 is much more permeable to Cl⁻ than gluconate (20).

To test whether MOD-1 shares structural determinants of ion selectivity in common with other LGICs, several sets of mutations were introduced in the M2 segment based on conserved motifs in the pore region (Table I). Galzi et al. (19) showed that a triplet of mutations (Fig. 1) is required to change the selectivity of the $\alpha 7$ nAChR channel from cationic to anionic. We tested the hypothesis that the inverse mutation in the anion-selective channel MOD-1 would convert this channel into a cation-selective one. Fig. 3 shows I-V relationships measured for the selectivity triple mutant (mutant 1) using different external solutions, and the mean $E_{\text{rev}}$ values are listed in Table II. As expected, mutation 1 converted the selectivity from an anionic to a cationic channel. Quite unexpectedly, however, mutant 1 was highly selective for K⁺ over other cations (Fig. 3). The reversal potential was insensitive to an extracellular anion ($E_{\text{rev}}$ for NaCl was $-68.8 ± 1.8$ mV, n = 8; for sodium gluconate $E_{\text{rev}}$ was $-70.4 ± 1.4$ mV, n = 8) and remained near $-70$ mV for sodium or choline used as the cation, but it shifted to $-6.0 ± 1.1$ mV (n = 8) for KCl. Based on this 70-mV shift in $E_{\text{rev}}$, the relative permeability for $P_{K}/P_{Na}$ or $P_{K}/P_{choline}$ is 12.1. This observation was surprising, because the comparable mutation in GlyR channels results in a cation non-selective channel (25).

As has been observed in other LGICs (15, 26), the internal and external rings of charge were not primary determinants of cation versus anion permeability. Mutant MOD-1 receptors in which the positively charged lysine in the inner ring was replaced by negatively charged aspartate remained anion-per-
meant and impermeant to cations (Fig. 3, Mutant 6). One difference from WT channels was that the $E_{\text{rev}}$ in sodium gluconate was more positive by 15 mV (Table II), which implies that the selectivity for chlorine over gluconate is increased in mutant 6. The external ring of charge in MOD-1 is positive because of Lys-292, whereas in cation-selective channels this residue is negative (Asp or Glu; Fig. 1). The introduction of a negative charge in the external ring of MOD-1 (K292D, mutant 7) did not change the selectivity from anion to cation. The reversal potentials remained relatively insensitive to external cations, whereas for anions switching from NaCl to sodium gluconate the $E_{\text{rev}}$ shifted by +90 mV (Table II). Thus, in comparison to WT, replacing the positive charge in either the inner (mutant 6) or the outer (mutant 7) ring by inserting a negative aspartate residue increases the selectivity for chlorine over gluconate. The permeability ratio for $P_{\text{Cl}}/P_{\text{Gluc}}$ is 12 for WT, 30 for mutant 6, and 34 for mutant 7.

A Minimal Set of Mutations for Determining Anion/Cation Selectivity—Mutational studies of nAChR channels identified a tripeptidic subset of substitutions required to convert selectivity from cationic to anionic (19). More recently, Keramidas et al. have shown in the GlyR that part of this tripeptidic subset, a single Ala-to-Glu mutation in the intermediate ring of charge in M2, is sufficient to change selectivity from anionic to cationic (20). We constructed subsets of the selectivity tripeptide mutant (mutants 2, 3, 4, and 5 in Table I) to look for the minimal set of residues in MOD-1 required to determine anion/cation selectivity. First, we eliminated the polar to hydrophobic change of the tripeptide mutant. The resulting double mutant at intermediate ring (APro-269/A270E, mutant 2 in Table I) was cation-permeant but, unlike the tripeptide selectivity mutant, was not K+ -selective (Fig. 3). The modest 7-mV shift of $E_{\text{rev}}$ in switching from NaCl to sodium gluconate shows that the permeability to chlorine is relatively low. Conversely, the −36 mV shift of $E_{\text{rev}}$ from NaCl to choline chloride demonstrates substantial sodium permeability. Mutant 2 does not strongly discriminate cations tested in these experiments, and the $E_{\text{rev}}$ values show a permeability sequence of potassium > sodium > choline. We interpret these data as evidence that mutant 2 is a non-selective cation channel. The complementary mutation, wherein Pro-269 and Ala-270 remained unchanged but the third element of the triple selectivity mutant (T284V) was studied in isolation, did not alter the ion selectivity from that of WT MOD-1 channels (mutant 5; Table II).

Differences in the intermediate ring of charge between anion-permeant and cation-permeant LGICs are totally conserved. In MOD-1 and all other anion permeant channels, a PA occurs at this ring (Fig. 1). For cation-permeant LGICs the proline is absent and Ala is replaced by Gla. Deletion of this proline alone in MOD-1 (mutant 4) resulted in nonfunctional channels with no detectable 5-HT-activated current. The charge substitution alone, A270E (mutant 3), produced a cat-

### Table I

| M2 sequencea | EC50 | Selectivityb |
|--------------|------|--------------|
| nAChR        | ADEG-ETISLQTVLLLIVFVMLLVAIMPA   | 1.0 | C            |
| MOD-1 WT     | PKALPAETTVGILALLITIPQQNLKILPKV | 2.3 | C            |
| Mutant 1     | -E V                                     | 2.4 | C            |
| Mutant 2     | -E V                                     | 0.61| C            |
| Mutant 3     | - V                                      | ND | ND           |
| Mutant 4     | - V                                      | 0.59| A            |
| Mutant 5     | D                                         | 0.63| A            |
| Mutant 6     | D                                         | 0.63| A            |
| Mutant 7     | D                                         | 0.86| A            |

a Boldfaced type indicates mutation.
b A, anionic; C, cationic; ND, not determined.

Many experiments made on nAChRs have demonstrated that the channel pore of the LGICs was mainly localized in the putative M2 intermediate ring of charge, with the most profound effects on conductance and selectivity being obtained with mutations done in this region (15, 16). The organic cation permeability has been demonstrated to be dependent on the size and charge of these residues (29) and, furthermore, an adjacent ring of polar amino acids affected the monovalent cation permeation (30, 31). The relation between gating and accessibility to M2 residues in the aqueous inner vestibule of the pore has been demonstrated by cysteine-scanning mutagenesis coupled with cross-linking by thiol reagents (27, 28).

There is more recent work on the focus of cation/anion selectivity, Keramidas et al. (20) demonstrated that introducing both a negative charge (mutation A251E) and a conformational change to displace the positively charged residue Arg-252 caused primarily by the Pro-250 deletion in this constricted region was required to induce a selectivity change in the GlyR and that even the T265V mutation may not be required. The A270E mutation was enough to convert the selectivity of the
GlyR, suggesting that the introduced negatively charged glutamic acid residue overrode the adjacent positively charged arginine and confirming the relevance of the electrostatic effect in charge selectivity. In our experiments we showed that, like the GlyR, the charge selectivity conversion was achieved by mutations made in the intermediate ring of the MOD-1R. Our results demonstrated that two adjacent point mutations (ΔPro-269 and A270E) produced a channel selective for cations. The proline residue had been shown to be implicated in local geometrical change to the pore constriction in the α7 nAChR triple mutant (19, 24) or in WT GlyR (32). We thus can hypothesize that the deletion of this residue has induced modifications in the pore region at the conducting state level of the channel. Cation permeation should be provided by a suitable electrical potential and geometric environment, allowing the substituted glutamate (Ala-270) to be exposed to cationic ions (19). Because the single mutation A270E produced a cation selective channel but the P269D failed to form functional channels, we can conclude that electrostatic properties and pore geometry are mainly responsible for the conversion of selectivity. The introduction of a negatively charged residue (A270E) is also necessary for obtaining a functional cationic channel.

The mutant 3 was a cationic non-selective channel for small cation ions. More precisely, we noticed that the selectivity was higher for $K^+$ ions with a rightward shift in $E_{\text{rev}}$ values than that for the $Na^+$ ions. $Na^+$ ions were the most heavily hydrated in contrast with $Cl^-$ ions, which were the most easily dehydrated and require closer interactions with the charged residues of the selectivity filter. In both cases, the diameter of the selectivity filter sets constraints on how easily the charged residues of this filter can compensate adequately for the hydration energy of the ions. A similar precise fit hypothesis has been put forward to explain the high selectivity for $K^+$ ions in the KscA $K^+$ channel (33). Dutzler et al. (2002) (34) studied the CIC $Cl^-$ channel and, as for the KscA $K^+$ channel, α-helix dipoles in both cases contribute significantly to the charge selectivity filters. For the LGIC family, it is strongly suggested that the pore helix does not have a great effect on the charge selectivity. The changes in charge of the intracellular and extracellular rings from positive to anionic charge reduced anion selectivity to render the channel only mildly anion-selective. This suggested that both extracellular and intracellular rings have an effect on the selectivity of the channel. Taken separately, however, no conversion in selectivity was observed (mutants 6 and 7). Modifications in the intermediate ring were required to convert the selectivity of the channel. Here we showed the dramatic effect of the mutation T284V in mutant 1 induced selectivity for $K^+$ ions. At that point, we can argue that the geometrical changes in the pore induced by the introduction of a valine residue known to affect the geometry of the protein gave rise to a conformational change of the pore sufficient to enhance the selectivity for $K^+$ ions.

For mutants 2 and 3 $K^+$ was more permeant than $Na^+$, even though it is a larger ion. These results imply that the channel walls were lined with polar residues (threonine and serine), which provide a hydrogen bond acceptor. For the mutants 8, 9, and 10, the changes of charge in extracellular or intracellular rings had an influence on the selectivity, and we hypothesized that the changes in charge have modulated the diameter of the pore.

In addition to the change in selectivity, MOD-1 mutants presented changes in their gating behavior, with mutants activating more slowly than the WT. Presumably, changes in the slower component of desensitization were responsible for the different gating compared with WT.

Keramidas et al. (2000) (35) suggested that desensitization was not involved in the reduced rate of current decay in the triple mutant compared with WT. We showed here that a single exchange of one amino acid (mutant 3) prevented the desensitization of the MOD-1 receptor even if the process of LGIC desensitization was thought to be coupled to binding of the agonist. However, little is known about the structure and the mechanisms underlying the gating process.

A loss of function has also been described by Keramidas et al. (35). It indicated that the threonine-to-valine mutation would impair the gating of the open or activate state. We did not observe spontaneous opening, in contrast to Corringer et al. (36) who showed a gain of function with an effect on the open-closed equilibrium by the threonine-to-valine mutation. For all the mutants except mutant 3, we noticed that these mutations resulted in a “loss of function.” This property was characterized by an increase of affinity in response to 5-HT compared with WT, as shown by the sustained electrophysiological response following a 10-s application of 5-HT (Fig. 2, A and B), and an increase in EC$_{50}$ for these properties has led to the suggestion that the mutant receptors may attain a desensitized but conducting state in the presence of an agonist (19, 37, 38).
FIG. 4. Alterations in gating produced by mutations in the M2 segment. A, traces show individual currents elicited by 1 μM 5-HT applied under continuous flow for several minutes to oocytes clamped at −70 mV. WT MOD-1 channels have biphasic rapid and slowly desensitizing components. The triple selectivity mutant (mutant 1) lacks a rapidly desensitizing component. The minimal mutation to convert MOD-1 to cation-selective, A270E (mutant 3), does not desensitize in 1 μM 5-HT, and mutant 7 shows a slow desensitizing component. B, superposition of traces of MOD-1 WT and mutant 3. C, superposition of the traces of the three mutants.

Mutant 3 did not seem to be a conducting state. The channel closed after the application of the ligand and displayed a loss of function with an increase in the affinity for the ligand, (EC50 of 0.75 as compared with 1.23 for WT). Hille (39) has demonstrated that the ligand-gated ion channels induced a rapid open state in response to an application of an agonist. Also, in the continuous presence of the agonist these channels may enter an agonist-bound but non-conducting “desensitized” state with a higher affinity for agonist than the closed resting or open states of the channel. The marked reduction in the rate of activation and removal of desensitization as shown by the double electrode recordings could therefore be due to a similar phenomenon. If one or more of the desensitized states of the receptor has been changed into a conducting one by the mutation, then the reduced rate of current decay may reflect passage from the open state to a conducting desensitized state before subsequent entry into a further (non-conducting) desensitized state. Such a model may explain the long duration open times observed even in responses to short applications of agonist, where the receptor may remain in a high affinity desensitized (conducting) state until agonist unbinding occurs.

CONCLUSION

The present study elucidates several key determinants of ion-charge selectivity in the MOD-1 receptor, a 5-HT ligand-gated chloride channel. We demonstrate that a single point mutation, A270E, was required for the change in the selectivity (19) that also dramatically affected the desensitization of this receptor. The ability to convert an ionic channel into a potassium-conducting channel (triplet of mutations P269D, A270E, and T284V) implies that homologies in the selectivity features and T284V) implies that homologies in the selectivity features among these channels.

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