Single Amino Acid Residue in the Extracellular Portion of Transmembrane Segment 2 in the Nicotinic α7 Acetylcholine Receptor Modulates Sensitivity to Ketamine

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Background: Ketamine inhibits the activation of both heteromeric and homomeric nicotinic acetylcholine receptors. The site of molecular interaction is unknown.

Methods: The inhibition of α7 nicotinic acetylcholine receptors by ketamine was compared to that of 5-hydroxytryptamine-3A (5HT3A) receptors that are resistant to ketamine inhibition in Xenopus laevis oocytes. To determine whether the region of transmembrane segments 2 and 3 is relevant for ketamine inhibition of nicotinic receptors, the authors identified single amino acid residues that differ in the sequence alignment of the two proteins. They created 22 mutant α7 nicotinic receptors that contain the single homologous amino acid residue in the 5HT3A sequence.

Results: Of the 22 mutant α7 nicotinic receptors tested, only one (α7 A258S) was significantly resistant to 20 μM ketamine. The ketamine concentration response relationship for the α7 A258S mutant was shifted to the right with the IC50 for ketamine increased from 17 ± 2 for wild type to 30 ± 3 μM in the mutant (P < 0.001). Agonist activation was unchanged by the mutation. The homologous amino acid residue in the 5HT3A receptor was mutated to the alanine that occurs in the wild-type nicotinic receptor. This mutation made the previously insensitive 5HT3A receptor sensitive to ketamine (P < 0.001).

Conclusions: Conservative mutation of a single amino acid in the extracellular transmembrane segment 2 domain induces resistance to ketamine inhibition in the α7 nicotinic receptor and sensitivity to inhibition in the 5HT3A receptor. This region may represent a ketamine binding site in the α7 nicotinic receptor, or it may be an important transduction site for ketamine action.

WE have previously demonstrated that ketamine inhibits neuronal nicotinic acetylcholine receptors in a clinically relevant concentration range.1,2 Ketamine also inhibits N-methyl-D-aspartate receptor activation in a similar concentration range.3 Unlike many other anesthetics, ketamine has little effect on the inhibitory γ-aminobutyric acid (GABA) type A (GABA A) response.1,4,5 The molecular nature of ketamine’s action as an analgescic and anesthetic drug are unknown.

Individual amino acid residues have recently been identified in GABA A and glycine receptors that, when mutated, cause the receptor to be insensitive to anesthetics.6–9 The identified residues for these chemically diverse anesthetic drugs have been in homologous positions within the extracellular portions of transmembrane segments (TM) 2 and 3. It is unknown whether this region is also important for ketamine inhibition in neuronal nicotinic acetylcholine receptors.

To determine the potential relevance of the TM2/TM3 region for ketamine inhibition of neuronal nicotinic acetylcholine receptors, we studied the effect of molecular changes in this region. We aligned the protein sequence for the α7 nicotinic subunit that forms homooligomeric receptors sensitive to ketamine inhibition with the sequence for the 5-hydroxytryptamine-3A (5HT3A) subunit that forms ketamine insensitive receptors. We constructed 22 individual mutant α7 subunits, corresponding to the 22 amino acid residues that differed between the α7 nicotinic and the 5HT3A sequences. Only a single mutation in this region induced significant ketamine resistance.

Materials and Methods

Molecular Biology

The protein sequence containing the predicted TM2 and TM3 regions of the α7 (accession no. NP038589) and 5HT3A (accession no. NM013561) proteins were aligned using the Clustal W algorithm in Align X (Vector MI, Informax, Bethesda, Maryland). The numbers used to identify the amino acid residues were based on the human nicotinic α7 numbering starting after the leader sequence. The location of the transmembrane segments is based on that published using predictions from several algorithms.10 Twenty-two amino acid residues were found to differ between the two sequences within the identified region. In each case in which an amino acid residue differed between the two sequences, a mutant α7 nicotinic subunit was constructed to contain the amino acid residue naturally occurring in that position in the 5HT3A sequence. The single amino acid residue identified with this strategy that resulted in an α7 nicotinic receptor resistant to ketamine was also studied in the homologous position in the 5HT3A receptor. In the background of the 5HT3A subunit, the serine residue that aligns with A258 in the α7 nicotinic receptor was mutated to alanine.

Single Site Mutant Production

The wild-type human α7 nicotinic acetylcholine receptor was subcloned in a pMXT expression vector, and the mouse 5HT3A gene was subcloned in PCDM8. Single amino acid mutation was performed using a polymerase...
chain reaction-based method with a Quick Change kit (Stratagene, La Jolla, CA). Briefly, mutagenic primers were made to contain the desired mutation, and the rest of the clone and vector were copied using the polymerase chain reaction. The sequence of all mutant constructs was confirmed by commercial sequencing (Gene Wiz, North Brunswick, NJ). All α7 nicotinic-based constructs were linearized with XbaI. Using the SP6 RNA polymerase, cRNA was made using a standard protocol. 5HT3A-based constructs were injected directly into the nucleus of the Xenopus oocyte.

**Oocyte Extraction and Injection**

*Xenopus laevis* oocytes were extracted from anesthetized females and placed in ND-96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 2 mM HEPES, 2.5 mM Na-pyruvate, 0.5 mM theophylline, and 10 mg/1 gentamicin; adjusted to pH 7.5). The oocyte clusters were incubated in 0.2% collagenase type IA (Sigma-Aldrich, St. Louis, MO) in ND-96 medium for defolliculation. Oocytes were agitated at 18.5°C for 4 h and afterward were rinsed with Barth’s medium (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 15 mM HEPES; pH 7.6). The oocytes were left to recover for 24 h in L-15 oocyte medium (Specialty Media, Phillipsburg, NJ) before injection of cRNA or cDNA. Approximately 10 ng of α7 cRNA or 10 ng 5HT3 cDNA was injected into individual oocytes in volumes of approximately 100 nl using a manual injector (Nanoject; Drummond Scientific, Broomall, PA). The oocytes were incubated at 17°C for 5 to 7 days in the ND-96 medium prior to electrophysiologic recording.

**Electrophysiology**

Current recordings were made from whole oocytes at 19–22°C using a Gene-Clamp 500 two-microelectrode voltage-clamp amplifier (Axon Instruments, Inc., Foster City, CA). The recording electrodes were pulled from glass capillary tubing (Drummond Scientific) to obtain a resistance of between 1 and 5 MΩ and filled with 3 mM KCl.

The buffer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM BaCl2, 10 mM HEPES, and 1 mM atropine; pH 7.5) used for recordings contained atropine to prevent muscarinic receptor stimulation, and barium in place of calcium to avoid current amplification by a calcium-activated chloride current. Oocytes were maintained at a holding potential of −60 mV and were held in a 125-ml cylindrical channel. Acetylcholine was applied at a flow rate of 4 ml/min for 5 s, and serotonin was applied at the same flow rate for 30 s. Activation was complete within these time periods. Prior to coapplication with an agonist, the oocytes were preequilibrated with ketamine for 2 min. To minimize the contribution of desensitization, at least 4 min passed between agonist applications. This protocol resulted in reproducible results.

**Analysis**

A baseline response to an agonist was measured before each agonist-antagonist coapplication. The response to an agonist was measured again after each agonist and antagonist application. The current response in the presence of an antagonist is taken as a percentage of the average current measured in response to an agonist alone. Ketamine and serotonin were made as stock solutions and were serially diluted to the appropriate concentrations on the day of the experiment.

Mutant α7 nicotinic receptors were screened for inhibition by 20 μM ketamine (of activation by 1 mM acetylcholine). This ketamine concentration was chosen because there was over 60% inhibition of α7 nicotinic receptor current and no effect on 5HT3A receptors. The response of each mutant α7 nicotinic receptor to 20 μM ketamine was compared to that in the wild-type α7 receptor. The difference was compared to the wild-type response with a t test corrected for multiple observations. The single amino acid change that resulted in this mutant was compared to the wild-type receptor.

**Statistics**

Clampex 7 (Axon Instruments, Inc., Foster City, CA) was used for data acquisition, and Microcal Origin 7.0 (Microcal, Northampton, MA) was used for graphics. A Student t test, corrected for multiple comparisons, was used to compare the response of each receptor to 20 μM ketamine. A mixed-effects repeated measures, and one-way ANOVA using an Excel (Microsoft Corporation, Redmond, WA) macro was used to compare the ketamine concentration response curves for wild-type and mutant receptors. P < 0.05 was considered significant, and the data were expressed as mean ± SE.

**Results**

As we have previously shown, the α7 nicotinic receptor is inhibited by ketamine (fig. 1A). The IC50 is 17 ± 2


MOLECULAR DETERMINANTS OF KETAMINE SENSITIVITY

659

Fig. 1. Nicotinic α7 receptor is sensitive to inhibition by ketamine and the 5-hydroxytryptamine-3A (5HT3A) receptor is resistant. (A) A control current activated by 1 mM acetylcholine from an α7 nicotinic acetylcholine receptor before (left) and during a 5 s acetylcholine application with 20 μM ketamine (middle), and after ketamine washout (right). (B) A control current activated by 2 μM serotonin from a 5HT3A receptor before (left) and during a second serotonin application with 20 μM ketamine (middle), and after ketamine washout (right). (C) Concentration response relationship for ketamine inhibition of the activation of the α7 nicotinic receptor (○) and the 5HT3A serotonin receptor (●). The IC50 for ketamine inhibition of the α7 nicotinic receptor was 17.3 ± 2.0 μM ketamine (Hill coefficient, 1.1 ± 0.2). Ketamine causes slight but statistically significant inhibition of the 5HT3A serotonin receptor at concentrations greater than 100 μM (15 ± 2%; t test, P < 0.001). Number of oocytes for each data point = 5–7. Values are expressed as mean ± SE.

μM ketamine. Although the 5HT3A receptor is insensitive to clinical ketamine concentrations (fig. 1B), it is not completely insensitive across a full concentration range because it is inhibited by 15 ± 2% by 100 μM ketamine (fig. 1C).

The protein sequences of the α7 nicotinic and 5HT3A subunits in the TM2/TM3 region align with 49% percent homology. Twenty-two amino acid residues differ between the two sequences within the selected region (fig. 2A). We screened the individual mutant α7 nicotinic receptor constructs with 20 μM ketamine. There was good separation of effect at this concentration, with ketamine having no significant effect on the 5HT3A receptor. The wild-type α7 nicotinic receptor was inhibited by 61 ± 3%. Of the 22 mutant α7 nicotinic constructs for which RNA was created, four could not be detected in our oocyte expression system. Of the remaining 18, only mutation of a single residue, A258S, had statistically significant resistance to the inhibition by 20 μM ketamine (35 ± 1% inhibition; t test, P < 0.001; fig. 2B). In contrast, there was no difference in inhibition by 5 nM α-bungarotoxin between A258S and the wild-type receptor (40 ± 9 and 38 ± 8%, respectively).

When the A258S mutant α7 receptor is tested with a full range of ketamine concentrations, the response relationship is shifted to the right with the ketamine IC50 increased to 30 ± 3 μM ketamine (ANOVA, P < 0.001) and the Hill slope unchanged (fig. 3). When mutated to alanine, the homologous amino acid residue in the 5HT3A subunit (5HT3A S258A, using nicotinic numbering) causes the serotonin receptor to become sensitive to inhibition by 20 μM ketamine (fig. 4A). Although the inhibitory response to 20 μM ketamine is significantly greater than that of the wild-type 5HT3A receptor (t test, P < 0.001), it is not as great as that of the wild-type α7 nicotinic receptor (fig. 4B). Ketamine is a fully efficacious inhibitor of the 5HT3A S258A mutant, with an IC50 of 158 ± 42 μM ketamine and a Hill coefficient of 0.6 ± 0.1 (fig. 4C). The inhibitory response to ketamine in the S258A mutant is greater than for wild-type (ANOVA, P < 0.001), but the Hill coefficient is unchanged. There is no difference between S258A and the wild-type 5HT3 re-
A receptor for inhibition by 1 nM ondansetron (45 ± 1 and 49 ± 1%, respectively).

The agonist response of the wild-type α7 nicotinic receptor does not form a perfect sigmoid response, potentially suggesting more than one activating conformation. However, when A258 is mutated to serine there is little difference on agonist activation, and the resulting concentration response curves are not different by ANOVA (fig. 5A). Mutation of serine 258 to alanine does not change the activation response relationship in the 5HT3A receptor (ANOVA, P < 0.05; fig. 5B).

Discussion

We and others have previously demonstrated that both homomeric α7 and heteromeric α4β2 and α4β4 nicotinic acetylcholine receptors are inhibited by clinically relevant concentrations of ketamine.1,2,5 In these experiments, we have taken advantage of the relative insensitivity of the evolutionarily related 5HT3A receptor to ketamine. Some studies have reported potentiation of...
Molecular determinants of ketamine sensitivity

5H1T3 responses in a nodose ganglion preparation by low ketamine concentrations.11,12 We detected no potentiation of current response in our oocyte preparation, perhaps because the native receptors are likely to be composed of 5HT3A and 5HT3B heteromeric receptors. For simplicity of interpretation, we expressed homomeric receptors composed only of the 5HT3A subunit in our Xenopus oocyte preparation. Alternatively, the difference could be due to the difference between mammalian cells and Xenopus oocytes.

We targeted the TM2 pore-forming domain and the extracellular loop connecting TM2 and TM3 that has been implicated in the mechanisms of other anesthetic drugs. We expected that if an individual amino acid residue were critical for the ability of ketamine to inhibit the a7 nicotinic receptor, mutation of that residue to the homologous residue in the 5HT3A sequence would induce ketamine resistance. Of the 22 amino acid residues that differed between the two protein sequences in this region, only the mutation of A258 to serine induced significant resistance to ketamine. This mutation decreased the potency of ketamine, but ketamine remained an efficacious antagonist. Similarly, we anticipated that if this amino acid residue were important to ketamine action in the a7 nicotinic receptor, the mutation of this residue in the background of the 5HT3A receptor might increase sensitivity to inhibition by ketamine. Figure 4 demonstrates that mutation of S258 to alanine in the 5HT3A receptor significantly increases ketamine sensitivity. However, the mutation of a single amino acid does not completely recapitulate the behavior of the other wild-type receptor to ketamine in either case. This is not unexpected, because other aspects of the molecular environment remain unchanged. What is unexpected is that the relatively conservative exchange between alanine and a serine residue would make any difference at all. Although the molecular volume of the side chains is nearly identical (88.6 Å3 for alanine and 89 Å3 for serine), the difference may lie in the possibility for hydrogen bonding. When A258S is depicted in a model of the nicotinic a7 subunit developed by Trudell and Bertaccini,13 it appears that the side chain of the A258 faces the interior of a 4-helix bundle formed by the membrane-spanning segments of a single a7 subunit. The substitution of a serine may allow for hydrogen bonding with reducing residues within the bundle.

It is interesting that the amino acid residue identified in our analysis for ketamine inhibition of the nicotinic a7 receptor is predicted to be at the most extracellular portion of the TM2 segment (the 19′ position, according to numbering used in torpedo). This residue is homologous to the hyperekplexia mutant described in the glycine receptor.14 The residue that we have identified is approximately one helical turn extracellular to the position of the amino acid residues thought to be important in the action of volatile anesthetics in the GABAA α-subunit and the glycine α1-subunit, as well as those identified in the GABAA β-subunit as modulating propofol and etomidate sensitivity.6,7,15,16 Both of these residues are predicted to project internally to the 4-helical bundle predicted to be formed by the transmembrane domains of a single subunit. The interior of this 4-helical bundle in the GABAA receptor is predicted to contain a water-filled crevice that is larger in the presence of agonist.17

Our earlier studies suggested that the pharmacologic mechanism was different even though both heteromeric and homomeric a7 nicotinic receptors were inhibited by ketamine. Ketamine inhibition of the heteromeric nicotinic receptors was strongly activation- and voltage-dependent, although inhibition was more potent with activation at lower agonist concentrations, suggesting a mixed mechanism of inhibition.2 Similarly, in both muscle type nicotinic receptors and N-methyl-D-aspartate receptors, ketamine is thought to act as both an open channel blocker and an allosteric inhibitor.18-21 In the homomeric a7 nicotinic receptors, the extent of inhibition by ketamine was neither use-dependent nor voltage-dependent, suggesting that the drug acts at a more superficial, exposed portion of the channel that may be accessible when the channel is in a closed state. The residue that we have identified, A258, is well positioned to form part of such a ketamine binding site, as it is predicted to be located at the extreme extracellular position in the TM2 domain and, thus, would not experience significant electrostatic potential from the membrane field.

Fig. 5. Agonist concentration response curves. (A) Concentration response relationship for acetylcholine activation of the wild-type a7 nicotinic receptor (-) and a7 A258S receptor (■). The EC50 activation of the a7 nicotinic receptor was 349 ± 52 nM acetylcholine (Hill coefficient, 1.2 ± 0.2). The EC50 activation of a7 A258S nicotinic receptor was 355 ± 84 nM acetylcholine (Hill coefficient, 1.0 ± 0.2). The curves were not significantly different by ANOVA analysis. (B) Concentration response relationship for serotonin activation of the wild-type 5HT3A receptor (○) and the 5HT3A S258A receptor (■). The EC50 activation of the 5HT3A receptor was 2.0 ± 0.1 μM serotonin (Hill coefficient, 3.7 ± 0.4). The EC50 activation of 5HT3A S258A receptor was 1.9 ± 0.1 μM serotonin (Hill coefficient, 2.9 ± 0.2). The curves were not significantly different by ANOVA analysis. Number of oocytes for each data point = 5-12. Values are mean ± SE.
Although our experiments suggest that the A258 residue in the nicotinic α7 receptor is important in the action of ketamine, they provide no direct information as to whether this amino acid represents part of a binding site for ketamine or simply a region important in the transduction of the effect of ketamine from a remote binding site. Because we have not studied the importance of other regions of the α7 nicotinic receptor, we have not ruled out the importance of other areas. In fact, a traditional binding site cannot be formed with a single amino acid, and we would predict that others could be identified by other means guided by molecular modeling.

In support of this region being part of a potential binding site for ketamine, a recent biochemical analysis of ketamine binding to the muscle type nicotinic receptor from the torpedo electroplax organ has shown that the ketamine binding site overlaps with the binding sites for tetracaine but not TID, suggesting by independent analysis the existence of a binding site accessible in the closed state that lies in the superficial extracellular portion of the channel pore.22

References
5. Yamakura T, Chavez-Noriega LE, Harris RA: Subunit-dependent inhibition of human neuronal nicotinic acetylcholine receptors and other ligand-gated ion channels by dissociative anesthetics ketamine and dizocilpine. ANESTHESIOLOGY 2000; 92:1144–53
19. Scheller M, Butler J, Hertle I, Schnick H, Franke C, Koch E: Ketamine blocks currents through mammalian nicotinic acetylcholine receptor channels by interaction with both the open and the closed state. Anesth Analg 1996; 83:830–6
20. Orser BA, Pennefather PS, MacDonald JF: Multiple mechanisms of ketamine blockade of N-methyl-D-aspartate receptors. ANESTHESIOLOGY 1997; 86:903–17