Molecular modelling of the nicotinic acetylcholine receptor transmembrane region in the open state

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Abstract
A model of the nicotinic acetylcholine receptor transmembrane region has been constructed which may represent the channel in its open-state. The positions of helices flanking the ion channel match those observed by electron microscopy and previously reported by others. Residues labelled, mutated or by other means known to have a strong influence on ion flux are each accessible from the lumen of the modelled channel. The model provides new insights into our current understanding of the ion channel structure, and suggests some novel explanations for the results of labelling and mutation studies such as those involving ion channel blockers and residue-dependent changes in ion selectivity.

Keywords: molecular modelling/nicotinic acetylcholine receptor/pore/ion channel/structure–function relationships

Introduction
The nicotinic acetylcholine receptor (AChR) is the best understood member of the Ligand-Gated-Ion Channels (LGIC) superfamily. The members of this important group of signalling proteins, which also comprises the 5HT₃, GABAₐ and glycine receptors (Ortells and Lunt, 1995), are assumed to share common secondary, tertiary and quaternary structures on the basis of a very high sequence similarity. All attempts, however, to obtain high resolution structures by crystallization and spectroscopic techniques have failed and hence, most of the structural information is inferred from biochemical and mutational data. The notable exception is the work of Unwin (1993, 1995), who has progressively increased the resolution of his electron microscope images of the AChR from Torpedo electroplax down to 9 Å, at which point it is possible to observe individual helices.

LGIC receptors are believed to be composed of five homologous subunits, and this is certainly the case for the AChR (Unwin, 1993). Until very recently, it was assumed that each subunit was constituted by a large extracellular N-terminal domain (encompassing the ligand binding site), four putative helical transmembrane (TM) regions, M1–M4, and a short extracellular C-terminal domain (Karlin, 1993). This view of the transmembrane region is now seriously challenged. Structurally, the best understood part of the transmembrane region (and actually of the whole AChR) is that corresponding to the ion channel (Bertrand et al., 1993). This is composed mainly of M2, and biochemical, mutational [see reviews by Bertrand et al. (1993) and Karlin and Akabas (1996)] and electron microscopy (Unwin, 1993, 1995) data have confirmed its α-helical nature. The secondary structure of the remaining TM segments (M1, M3 and M4) is, however, uncertain. Unwin (1993, 1995) could resolve only five helical structures in the TM region and these were interpreted as the M2 segments of each subunit. Gorne-Tschelnokow et al. (1994) detected 40% of β-sheet plus turn structure in the TM region using Fourier transform infrared spectroscopy, a value which argues strongly against an all-helical structure. Ortells and Lunt (1994), using molecular modelling, concluded that an all-helix bundle was an unlikely structure for the TM. Blanton and Cohen (1994), on the basis of a series of affinity labelling studies using lipophilic photoaffinity probes, have repeatedly produced experimental evidence which they interpret as supporting the orthodox view that the four transmembrane segments of each subunit are helical. In their experiments M1, M3 and M4 were labelled and they concluded that for M3 and M4, the labelling pattern was compatible with a helical conformation. The pattern obtained for M1, however, could not be assigned unambiguously to either a helix or a β-sheet; moreover, the loop connecting M2 and M3, a region which the classical helical model does not place in the membrane, was also labelled. In a detailed molecular model of the α7 AChR, Ortells and Lunt (1996) were able to accommodate all the available information on the transmembrane region. In their model, M1 was built as a three-strand β-sheet, M3 as a half β-sheet and half helix and M4 was modelled as a full α-helix. The model was concordant in both overall size and shape with Unwin’s (1993) EM images of the AChR. In addition, the secondary structure deduced from the model agreed quite well with that measured for the TM region by Gorne-Tschelnokow et al. (1994). Interestingly, the loop connecting M2 and M3 was actually modelled inside the membrane, thereby providing an acceptable structural explanation for the lipophilic labelling observed by Blanton and Cohen (1994). The core of the model was based on the structure of the B5 pentamer of the heat-labile enterotoxin (Sixma et al., 1991). This template included five α-helices, one from each subunit, forming a central channel in the toxin and matched to the M2s of the AChR. In the final minimized configuration of the model, these helices had a configuration very similar to the closed-state AChR as described in Unwin’s work (1993, 1995), and to another model of the ion-channel in the closed state (Sansom et al., 1995). We describe here a further refinement of the AChR model which we believe may represent the open state of the channel.

Materials and methods
Basic strategy
The model was constructed on the basis of the coordinates of our previous model (Ortells and Lunt, 1996). Unwin (1995) proposed that the main structural changes in the transmembrane region involved in the opening of the ion channel entail only the M2 helices, which he reported as having a marked kink whereas the external rim has the same shape in both structures (open and closed states). We therefore speculated that the open-state could be modelled by substituting the M2s of the closed-state AChR model with appropriately kinked M2...
structures, and searched the Protein Data Bank (Bernstein et al., 1977) for helices with a kink around their middle point and having a length of ≈22 residues. We considered the possibility that the kink seen by Unwin in the M2s could be due to the presence of a short stretch (two or three residues) in an extended conformation as recently suggested by Akabas et al. (1994). Hence, pairs of helices of appropriate size connected by two or three extended residues were sought, using the programme Iditis (Oxford Molecular). The constraints in this search were that the N-terminal helix should have a length of between nine and 12 residues and that the overall length (both helices plus the extended stretch) be between 22 and 24 residues (the actual length of M2 is 22 residues). All candidate helices (a total of 30) were compared in terms of shape with the images reported by Unwin (1995) for M2 and those that fitted best (eight) were chosen for model building.

As a first step, the 22mer old (closed-state) M2s of the five subunits were replaced by a copy of each of the eight candidates for the new M2, in such a way that they approximately matched the position of the M2s in Unwin’s images (1995). Each M2 candidate was transformed into an AChR α7 M2 by means of an iterative mutational procedure consisting of replacing the original side chains of the candidate helices, with those corresponding to the AChR α7 M2 (keeping, when possible, shared torsion angles between the old and new side-chains), with three constraints: the first constraint was to maximize the number of side chains accessible from the lumen of the channel of those residues labelled, or by other means (e.g. mutational or electrophysiological studies) known to have strong influence on the AChR ion flux in the open-state. Residues in homologous positions 4, 7, 11, 14, 15, 18, 21 and 25 in Table I are the most probable candidates for lining the lumen of the ion channel. Thus these residues were those that constrained the orientation of the M2s (see Results and Discussion for references on these residues); the second constraint was that the leucine believed to be part of the M2 kink (the conserved Leu14 in Table I) and to be involved in the closing of the channel (Unwin 1993, 1995) had to be close to the kink; and the third constraint was that the smallest diameter of the ion channel had to be at the level of between positions 2 and 7 of Table I, whereas the upper region (above Leu14) had to be much wider (Villarroel et al., 1991; Unwin, 1995). In addition, slight modifications to the original position (small rotations and translations) were made manually to better satisfy these constraints.

With all such constraints in place the best candidate was a fully helical region found in aconitase (Brookhaven code 6ACN), extending from residues 110 to 134 and having a kink from residues Asp114 to Val123. This helix was therefore used for the construction of the new M2s. The loops connecting the M2s to M1 and to M3 in the closed-state were not altered since their extremities were adjacent to the new M2s.

Minimization procedures

(i) The first regions to be minimized were the loops between M1s and M2s, and those connecting M2s with M3s; the remaining TM region was left fixed. The loops were initially energy minimized by means of the steepest descents algorithm and using a harmonic bond stretch function, no charges and no cross-terms until a maximum first-derivative of 2.0 kcal mol⁻¹ Å⁻¹ was reached. This procedure relaxed the loops to their new, slightly different positions.

(ii) The whole TM was fixed, except the M2s. The latter were then minimized with steepest descents as in (i), but the main chain atoms were forced to remain in their original positions using a forcing constant of 350 kcal Å⁻¹. As a result, M2 side chains were relaxed, without significantly altering the positions of the helices. In addition, OG and HG atoms of the serines at position 9 (Table I) were forced (with a forcing constant of 500 kcal Å⁻¹) to remain in place in such a way as to shape a channel with a diameter of around 7 Å at that level (Zhorov, 1991; Villarroel et al., 1991).

(iii) The whole TM was allowed to move and minimize as above, but the main chain atoms were forced to their original positions with a forcing constant of 700 kcal Å⁻¹. OG and HG atoms of serines at position 9 were also forced as above. Further steps minimized the TM as above, but with decreasing forcing constants for the main chain atoms of 350 and 10 kcal Å⁻¹. The forcing constant for the OG and HG serine atoms was lowered to 5 kcal Å⁻¹.

(iv) For the whole TM, minimization was carried out by using conjugate gradients with a Morse bond potential, charges and cross-terms in a maximum first-derivative of 0.5 kcal mol⁻¹ Å⁻¹. However, a forcing constant of 500 kcal Å⁻¹ was applied to main chain atoms.

(v) Finally, M2 regions were allowed to minimize as in (iv)—though without any template forcing—until a maximum first-derivative of 0.05 kcal mol⁻¹ Å⁻¹ was reached.

Computational procedures

Insight and Discover programmes from Biosym (San Diego, CA) were used to display and minimize the models using a Silicon Graphics Onyx computer at the Modelling Unit of the Institute of Biochemistry, Bahía Blanca, Argentina. The programme Iditis (Oxford Molecular) was run at Daresbury Laboratory, Warrington, UK.

Results and Discussion

Overall structure of the open-channel AChR model and differences with the closed-channel state model

Table I shows a sequence alignment of the four different subunits of the Torpedo AChR, and the α7 subunit. The table introduces the numbering that is used throughout the text and compares it with the conventional numbering used in sequencing and related studies.

A general schematic view of the TM region of the AChR as seen in the open-channel model from the synaptic side is shown in Figure 1a. One of the most striking differences between the new open-channel model and the closed-channel model (Ortells and Lunt, 1996) is that modifications introduced in M2 lead to a slight protrusion of M1 and M3 towards the membrane lipid.

Another important difference is that the final disposition of the five M2s in the open-channel model is asymmetrical. Even though the M2s are identical in sequence and initial conformation, the energy minimization resulted in their backbones occupying similar but not identical positions; furthermore, some of the side chains at homologous positions have different accessibilities from the lumen of the channel.

Figure 1b shows the estimated molecular surface of the modelled AChR TM region, coloured by the electrostatic surface potential calculated by the programme Delphi
Table I. Sequence alignment between the neuronal AChR α7 and Torpedo (α1, β1, γ1 and δ1) subunits

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Solid black background indicates that residues at that position are exposed to the channel lumen in the model. The light grey background indicates partial exposure and white background non-exposure. The 'conventional' sequence numbering of Torpedo α1 and chick α7 are also given to facilitate the identification of the residues listed under n, an arbitrary numbering adopted for homologous positions along the M2s (residues 4–25) and the adjacent N-terminal loop.

Fig. 1. (a) Schematic synaptic view of the whole transmembrane region of the AChR. Each of the five subunits is coloured differently. Cylinders are α-helices; flat ribbons are β-strands; and ropes are loops. Generated with the programme SETOR (Evans, 1993). (b) Molecular surface generated by the programme GRASP (Nicholl, 1991), and coloured by the electrostatic potential calculated by the programme Delphi. Left, synaptic view; right, lateral (membrane) view.
Fig. 2. CPK representation of the ion channel region. Residue numbering 1–25 corresponds to that in Table 1. Numbers 26, 27 and 28 correspond to the α7 subunit residues Tyr 209 from M1, Met 278 and Ile 279 from M3, respectively. The colours represent the following numbers: red, 1, 7, 13, 19 and 25; green, 2, 8, 14, 20 and 26; blue, 3, 9, 15, 21 and 27; magenta, 4, 10, 16, 22 and 28; yellow, 6, 12, 18 and 24. (a) Synaptic view. (b) Cytoplasmic view. Generated with the programme GRASP (Nicholl, 1991).

Fig. 3. Molecular surface of the ion channel lumen, as viewed from outside the channel, in three successive rotations of about 72° each. Numbering and colouring as in Figure 2.

(Nicholl et al., 1991). It can be seen that within the ion channel the electrostatic potential is slightly more negative, as might be anticipated for a cation-selective channel.

Figures 2a and b show general views of the open-channel model from the synaptic and cytoplasmic sides, respectively. Table I lists those residues accessible from the lumen of the ion channel. Figure 3 shows 'external' views of the surface of the ion channel with individual side-chain contributions coloured differently.

The synaptic region of the ion channel

In this region there is one ring of residues (position 25 in Table I) that has influence on the channel conductance (Imoto et al., 1988) and is labelled by the non-competitive inhibitor mepropradifen mustard (MPM) in the presence of carbamoylcholine (Pedersen et al., 1992). The ring was used as a constraint in the search for appropriate helices in the process of building the open-channel model (see Materials and Methods) and is consequently accessible from the lumen.
An important feature of the model is that residues at position 23 (see Table I) are facing the channel, albeit with different degrees of exposure due to the asymmetry of the channel model. At this position, the threonine found in the Torpedo AChR δ subunit was labelled by the photoaffinity label 3-(trifluoromethyl)-3-m-([I125]iodophenyl)diazirine (TID) in the presence of agonist (White and Cohen, 1992). These authors proposed that threonine 23 was the labelled residue, and was therefore probably pointing towards the lipid–protein interface. They also indicated that this residue ‘lies on the opposite side of the M2 α-helix from the residues presumed to form the ion channel’. As shown, such an explanation is redundant in our model, because the residue is readily accessible from the lumen of the channel.

There are two leucines in the α7 receptor (positions 21 and 22 in Table I) involved in the permeability of divalent cations (Bertrand et al., 1993). One was used as a constraint (position 21) and its accessibility from the lumen was maintained. This residue, corresponding to Leu258 position of mouse α subunit was labelled when mutated to cysteine, by the positively charged, hydrophilic, sulphhydryl-specific reagent methane-thiosulphonate ethylammonium (MTSEA) in the presence of agonist (Akabas et al., 1994). In this region the M2s are quite separated from each other as in Unwin’s images (1995), and therefore most of the residues of M2 are ‘visible’ to some degree from the lumen of the channel, with the exception of residues at positions 16, 20 and 24 (Table I). Unwin (1995) stated that in the upper (synaptic) leaflet of the channel, the M2 helical segments are sufficiently far apart to permit access to side chains of residues not in M2. In our model, the α7 M1 residue Tyr209 (residue 26 in Figures 2 and 3), and M3 residues Met278 and Ile279 (residues 27 and 28, respectively in Figures 2 and 3) are accessible from the ion channel. In the model, Tyr209 is at the beginning of M1 and Met278 and Ile279 are in the second β strand of M3.

Three residues at consecutive positions (17, 18 and 19 in Table I) are also accessible from the lumen in the model. Residue 18, used as a constraint during model building, was labelled by TID in the closed state, though with less intensity, in the presence of agonist (White and Cohen, 1992). When mutated to cysteine, this position was also labelled by MTSEA (Akabas et al., 1994).

The residue corresponding to position 17 (corresponding to the mouse α subunit) was claimed to be labelled in the absence of agonist by Akabas et al. (1992). In a subsequent paper, however, they retracted this observation. The side chains of these residues only partially face the channel lumen, and after mutation to cysteine, the reactive sulphhydryl moiety is probably hidden. This might explain their lack of, or erratic reactivity when mutated. Residues at position 19 are also poorly exposed.

Middle and lower (cytoplasmic) regions of the open-state AChR channel

Residues at positions 7, 11, 14 and 15 in Table I were used as constraints in the construction of the model and are quite exposed. In all cases, however, and because of the asymmetry of the channel, the five homologous side chains are not equally exposed, and are at slightly different levels along the main axis of the channel. It is important to note the relationship between this differential exposure and the different degree of labelling of the four types of subunits (α, β, γ and δ) in the muscle-type and Torpedo electroplax AChR, respectively, an observation in agreement with the evidence showing that these residues face the ion channel in the open-state or in the presence of agonist (see references below).

Mutations of residues 11, 14 and 15 alter the binding of the open channel blocker QX-222 (Charnet et al., 1990; Revah et al., 1991). Furthermore, MTSEA labels residues of the mouse α-subunit at these positions and at position 7, in the presence of agonist (Akabas et al., 1994). In addition, positions
7, 11 and 14 were labelled by TID in the presence of agonist (White and Cohen, 1992) and by chlorpromazine in the open-state (Revah et al., 1990). Position 11 was also labelled by the non-competitive blocker triphenyl-methyl-phosphonium (Hucho et al., 1986).

The structure of the open-channel model in this region is worthy of particular comment. The M2s here are at an angle of around 45° with respect to the plane of the membrane. Following minimization, this places labelled residues at positions 7, 11 and 14 at the same level. As a consequence of the asymmetry of the model, residues in two non-consecutive helices are at a slightly ‘higher’ level towards the synaptic, extracellular side. Thus, residues at position 14 of these ‘higher’ levels fall in the same plane as those of position 15 in the ‘lower’ ones. Residues at position 15 of the ‘higher’ level are above the other residues of positions 7, 11 and 14. This region is the narrowest part of the channel and the locus where the closely clustered side-chains are labelled by the channel blockers chlorpromazine, QX-222 and TPP. This cluster of residues constitutes an annular constriction about 8 Å in depth, i.e. about the same dimension as the narrowest part of the ion channel as estimated in electrophysiological studies (Zhorov et al., 1991). Figure 4 shows a schematic view of the pore region of the AChR channel depicting its overall profile and its asymmetric shape.

Other residues accessible from the lumen in the model are those at positions 6 and 10, which have not been reported to be labelled. The residue at position 8 is partially accessible and has been labelled in the absence of agonist (Akabas et al., 1994).

Finally, a negatively charged ring of residues at position 4 faces the channel. These residues have been shown to have a very important influence on channel conductance and cation selectivity (Imoto et al., 1988; Galzi et al., 1992) and were also used as constraints in the modelling.

The M1–M2 loop region

Surprisingly, some residues in the loop between M1 and M2 (positions 1, 2 and 3 in Table I) adopt conformations that constitute a continuation of the orthodox ion channel. Even though they are not closely packed, these residues shape a well-defined path for ion flow. Of particular note in this context are the glycines at position 3 (Figure 3). It has been shown by Galzi et al. (1992) that mutations at this region can change AChR ion selectivity from cationic to anionic. These authors speculated that this conversion was not directly related to a change in the channel lumen, but rather to indirect changes in the geometry of the M2s. It is not possible, on the basis of our model, to give a complete explanation for this phenomenon, but the model unquestionably shows that, although not located on the M2, the changes may well directly affect the properties of the channel lumen.

Comparison between the open and closed states

Comparison between the closed- and open-channel models is important to gain further insight into the function of the AChR molecule. The issue can be separated into two topics: the M2 region and the external rim, respectively. The comparative topology of the M2 region poses some difficulties. In the closed-state model this region corresponds to the heat-labile enterotoxin B subunit structure, and hence it is not optimized to match Unwin’s images of the M2s in the closed state, as it is in the present work in the case of the open-state model. However, the M2 region in the final closed-state model of Ortells and Lunt (1996) does indeed resemble the electron density interpreted to correspond to this region in Unwin’s (1993) images of the closed-state channel and thus enables a valid comparison between the two channel states.

Our first comparison involved superimposing all atoms (except hydrogens) of the TM regions of the two channel forms. The minimized open-state model exhibited disruptions in the M1 and M3 regions with respect to the closed-state model. The overall r.m.s. value between the open and closed states was 20. Excluding the M2s atoms to guide the superimposition of the whole structure, the r.m.s. between the outer rims (corresponding to open and closed states) is 22, whilst the value between the M2s only is 6.9. The interesting conclusion follows that the modelled M2 region is significantly less disturbed than the whole structure. Figure 5 shows the superimposed M2 structures in the open- and closed-channel states, as well as the relative positions of the leucines (position 14 of Table I) that are believed to play a key role in the closure of the channel. However, according to Unwin (1993, 1995) the only differences between the closed and open states in the transmembrane region are at the level of the M2s. The displacement of the open M2s is not the same for the five subunits, as seems to be the case in the images published by Unwin (1993, 1995). This is not unexpected given the fivefold rotational averaging applied by this author. Our current model suggests the existence of structural changes in the external rim during AChR channel opening, a feature that may be subjected to experimental test as improvements in the attainable resolution become available.

Acknowledgements

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References

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