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Identification of threonine 422 in transmembrane domain αM4 of the nicotinic acetylcholine receptor as a possible site of interaction with hydrocortisone

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Abstract

The modulatory effects exerted by the glucocorticoid hydrocortisone (HC) on the nicotinic acetylcholine receptor (AChR) were studied in mutants of the α subunit M4 transmembrane region. Based on the photoaffinity labeling of αM4 412 with the steroid promegestone this position was mutated to different residues to explore the properties of side-chain volume, hydrophobicity, and charge on AChR–steroid interactions. All mutants showed channel kinetics indistinguishable from those of the wild-type AChR, both in the absence and presence of HC (200 and 400 μM), in single-channel recordings at different acetylcholine (ACh) concentrations. An alanine-substituted quadruple mutant of four putative lipid-exposed residues in αM4 (L411, M415, C418 and T422) exhibited less inhibition by HC than that observed in wild-type AChR. When we dissected the quadruple mutant into four individual alanine-substituted receptors, we found that the T422 mutant AChR behaved like the quadruple mutant, whereas the other three were indistinguishable from the wild-type. We conclude that T422, a residue close to the extracellular-facing membrane hemilayer in αM4, has direct bearing on the changes in HC sensitivity and propose its involvement in the steroid-AChR interaction site.

Keywords: Cholinergic receptor; Mutations; Steroid; Glucocorticoid; Patch-clamp

1. Introduction

The nicotinic acetylcholine receptor (AChR) is the prototype member of the superfamily of ligand gated ion channels, which also comprises the 5-HT_3, $GABA_A$ and glycine receptors (for a review see Barrantes, 1998). The embryonic muscle AChR is a transmembrane pentameric protein composed of four different homologous subunits arranged in stoichiometry $\alpha_2\beta\gamma\delta$, whereas in the adult the γ subunit is replaced by ε. Each subunit is composed of an extracellular N-terminal domain, four helical transmembrane segments (called M1–M4) and a short extracellular C-terminal tail. The agonist binding sites, two per receptor molecule, are formed at the interfaces between the extracellular domains of the α–ε and α–δ subunits. There is consensus on the view that the M2 segment of all subunits delimits the pore region of the cation-selective channel (Giraudat et al., 1986, 1987, 1989). The function of the other transmembrane segments is not so well understood. M1 and M3 are partially in contact with the membrane and are most likely involved in ion conduction. M4 has always been considered the firmest candidate to be in contact with the lipid bilayer since it is the most hydrophobic segment and is extensively labeled by photoaffinity reagents such as TID, phosphatidylserine derivatives and other hydrophobic probes (Blanton and Wang, 1991; Blanton and Cohen, 1992, 1994). These characteristics make this segment the most likely one through which hydrophobic molecules modify channel kinetics.

Lipids and other hydrophobic molecules modulate AChR activity (for reviews, see Barrantes, 1993; Barrantes et al., 2000a). Among lipids, sterols are of particular physiological significance. Their presence in the AChR microenvironment was demonstrated in early

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experiments (Marsh and Barrantes, 1978; Marsh et al., 1981). Sterols were also shown to modulate the agonist-induced receptor state transitions and the kinetics of the agonist-induced permeability changes in vitro (Criado et al., 1982, 1984). Subsequent work from our laboratory demonstrated that synthetic glucocorticoids exert non-competitive inhibitory effects on the muscle-type AChR (Bouzat et al., 1993a,b, 2000). Thus, dexamethasone produces a diminution in the AChR channel mean open time without modifying its ion conductance, through an alteration in channel kinetics that was interpreted as resulting from channel blockage in the closed state. Similar results were obtained with hydrocortisone (HC) or 11-deoxycortisone (Bouzat and Barrantes, 1993b). We further suggested that HC interacted with transmembrane regions of the AChR protein different from those that form the walls of the channel proper (Bouzat and Barrantes, 1996). More recently, we carried out a more systematic study of the steroid effect and found that different steroids affect AChR channel kinetics in a qualitatively similar manner, albeit to different extents (Garbus et al., 2001). We described an inverse relationship between the lipophilicity of some steroids and their inhibitory effect (Garbus et al., 2001). One general conclusion from our studies on steroids is that these compounds do not affect the membrane in a global and unspecific manner, but rather that the effect exerted by each depends on the degree of access to sites at the AchR–lipid interface (Barrantes et al., 2000a).

Photoaffinity labeling experiments with hydrophobic probes such as, TID (Blanton and Cohen, 1994), the cholesterol analog azido-cholesterol (Corbin et al., 1998) the steroid derivative promegestone (Blanton et al., 1999) and a phosphatidylserine arylazido derivative (Blanton and Wang, 1991), all support the view that the transmembrane segment M4 is in contact with membrane lipids. In *Torpedo* AChR all these probes labeled amino acid residues in all four subunits, principally in transmembrane domains M1, M3 and M4 (Blanton and Cohen, 1994; Blanton et al., 1999; Corbin et al., 1998). The periodicity of the labeling in αM4 suggests an α-helical secondary structure (Blanton and Cohen, 1992, 1994). More recent fluorescent spectroscopy studies from our laboratory are compatible with an α-helical structure in αM4 and γM4, whereas αM1 and possibly γM1 possess non-helical structure (Barrantes et al., 2000b).

In a photoaffinity study with a synthetic progestin, 17,21-dimethyl-19-nor-pregn-4,9-diene-3,20-dione (Promegestone) probe, labeling was observed in some of the *Torpedo* AChR residues labeled with TID, predominantly in C412 (Blanton et al., 1999). Given the steroidal nature of the probe, these results suggested that C412 could be involved in the steroid–AChR interaction in general. Following this reasoning, in the recent study we focused our attention on the αM4 412 position, which in murine AChR corresponds to a glycine residue. We set out to substitute G412 for tyrosine, tryptophan, cysteine, serine and asparagine, to explore the effects of side chain volume, hydrophobicity, and charge on AChR–steroid interactions.

We first studied the effect of various mutations in the αM4 transmembrane regions of the AChR on the pharmacological response to steroids. We subsequently measured single-channel activity in wild-type and mutated AChRs in the presence of low (1 μM) and high (30 μM) concentrations of ACh, in the absence and presence of a high dose of HC (200 μM). Finally, we undertook an alanine scanning mutagenesis study of the other relevant residues in αM4, namely L411, M415, C418, and T422 and found that the sensitivity of the corticoid effect changed upon mutation of T422 only.

2. Methods

2.1. Construction of mutant α subunits

Point mutations were carried out on the cDNA of the mouse AChR α subunit subcloned into the pBRG4 cytomegalovirus-based expression vector (Sine, 1993). Basically, we used the naturally occurring restriction sites for *Bsp*M I, *Bst*X I and *Spe* I to obtain two fragments of 1900 and 3600 bp. The plasmid was reconstructed by ligating these two fragments with a synthetic double-stranded oligonucleotide carrying the desired mutation through the action of the enzyme T4 ligase. The *E. coli* strain DH5αF′ was transformed with the reconstituted plasmid. Plasmid DNA was isolated from bacteria and sequenced by the dideoxynucleotide technique using polyacrylamide gels in order to confirm the correctness of the constructs. Those strains carrying the correct plasmid were amplified and the cDNA purified on a large scale.

2.2. Expression of mutated AChR in HEK-293 cells

Mouse AChR cDNAs corresponding to the α (mutated or wild-type), β, ε and δ subunits, subcloned into the cytomegalovirus-based expression vector pRBG4, were transfected into HEK-293 cells using the calcium phosphate precipitation technique at a subunit ratio of 2:1:1:1, respectively. Cells at 40–50% confluence were incubated for 8–12 h at 37 °C with calcium phosphate containing the cDNAs in DMEM supplemented with 10% fetal bovine serum. Cells were used for single-channel measurements 1–2 days after transfection.

2.3. Patch-clamp recording

Recordings were obtained in the cell-attached configuration (Hamill et al., 1981) at a membrane potential

of −70 mV and at 20 °C. The bath and pipette solutions contained 142 mM KCI, 5.4 mM NaCI, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$ and 10 mM HEPES (pH 7.4). Patch pipettes were pulled from Kimax-51 capillary tubes (Kimble Products, Vineland, NJ), coated with Coat D (M-Line accessories, Measurements Group, Raleigh, NC), and fire-polished. Pipette resistances ranged from 5 to 7 MΩ. Acetylcholine at a final concentration of 1 or 30 μM was present in the pipette solution. Hydrocortisone (200 or 400 μM) was incorporated in the pipette solution by dissolving it in the bath solution.

Single-channel currents were recorded at a membrane potential of −70 mV and 20 °C using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Inc., Union City, CA), digitized at 94 kHz with an ITC-16 interface (Instrutech Corporation, Long Island, NY) and transferred to a computer using the program Acquire (Bruxton Corporation, Seattle, WA). Detection of single-channel events using the program TAC followed the half-amplitude threshold criterion (Bruxton Corporation) at a bandwidth of 5 kHz. Open- and closed-time histograms were plotted using a logarithmic abscissa and a square root ordinate (Sigworth and Sine, 1987) and fitted to the sum of exponential functions by the maximum likelihood criterion using the program TACFit (Bruxton Corporation). Bursts were defined as a group of opening events separated by closed times briefer than a specified time given by the intersection between the first component and the following component of the closed-time histograms. In recordings at 1 μM agonist, the time was set as 1 ms; in the recordings at 30 μM ACh the value was obtained each time from the histograms. With values always close to 5 pA, none of the mutants showed differences in channel amplitude with respect to wild-type AChR either in the absence or presence of the steroid.

2.4. Statistical analysis

Differences in mean open time were analyzed using the Student's *t*-test. When results were expressed as the ratio of mean open time in the presence and the absence of the steroid HC, the following algorithm was used to obtain the standard deviation (SD) of the above quotient:

$$SD\ (X1/X2) = (X1/X2) * [(SD1/X1)^2 + (SD2/X2)^2]^{1/2}$$

(Johnson and Kotz, 1969). The Student's *t*-test was not applicable in such cases because each value was obtained as the ratio between numerical media of a different number of observations. We thus analyzed the significance of the differences in terms of superposition of confidence intervals.

3. Results

We employed two different strategies to identify the site of action of the steroid hydrocortisone (HC) on the AChR: (a) Given the predominant labeling of C412 with the synthetic progestin, promegestone in *Torpedo* AChR (Blanton et al., 1999), the candidate residue was explored initially in more detail, and replaced by amino acid residues of different physicochemical characteristics; (b) several other positions in M4 were submitted to alanine scanning mutagenesis. In both cases the effects of the mutations were examined by single-channel patch-clamp recording.

3.1. Effect of mutations of αG412 by W, Y, S and C on HC–AChR interactions at low agonist concentration

The amino acid C412, possibly located in the inner leaflet of the membrane in αM4 (Ortells et al., 1998) has been labeled with the progestin derivative promegestone (Blanton et al., 1999). In mouse AChR, position 412 corresponds to a glycine residue. To evaluate the influence of the amino acid side chain substitution at this position on the well-known inhibitory effect of the steroid HC on the AChR (Uki et al., 1999; Bouzat and Barrantes, 1993, 1996; Kindler et al., 2000), electrophysiological recordings of wild-type and mutant AChRs activated by low (1 μM) ACh were made in the absence and presence of HC at two different steroid concentrations. The analysis of the results showed that in normal AChRs the presence of HC (200 and 400 μM) causes a diminution in the channel mean open time (τ_{open}) of about 50 and 70%, respectively. Analysis of the HC effect on AChR channel mean burst time (τ_{burst}) yielded similar results (Table 1). Recordings done on mutated AChRs showed neither quantitative nor qualitative differences in single-channel kinetics with respect to the wild-type AChR, as measured by τ_{open} or τ_{burst} in G412 mutated to tryptophan, serine, tyrosine and cysteine (Table 1).

3.2. Effect of αG412 by W, Y, N and C mutations on HC–AChR interactions at high agonist concentration

At this point, we can already surmise that the effect of HC on mutant AChR does not differ from that on willd-type AChR. Before definitely discarding the possibility that αG412 is involved in HC–AChR interactions, we performed further experiments using high (30 μM) ACh doses, because this concentration regime has desensitizing effects on the AChR, and under such conditions bursts of single-channel events group into clusters. Clusters, in turn, reflect the opening and closing events of a single channel (Sakmann et al., 1980). Clusters are separated by a minimum closed interval equivalent to the intersection between the main component of the closed time distribution (c1) and the following component. Cluster analysis was used in order to gain more insight into the behavior of the mutant AChRs, given the fact that the high agonist concentration regime permits one

Table 1
Channel mean open time[a,b] at two HC concentrations in wild-type and G412 mutated AChRs at low (1 μM) agonist concentration

	$\tau_{open\ mut}/\tau_{open\ c}$	$\tau_{open\ mut}$ HC 200/$\tau_{open\ c}$	$\tau_{open\ mut}$ HC 400/$\tau_{open\ c}$
control	1.00±0.11 (*n*=14)[c]	0.48±0.03 (*n*=19)	0.30±0.02 (*n*=12)
G412S	1.23±0.12 (*n*=5)	0.42±0.07 (*n*=5)	0.27±0.08 (*n*=3)
G412C	1.08±0.22 (*n*=3)	0.41±0.11 (*n*=5)	0.26±0.01 (*n*=2)
G412W	1.09±0.09 (*n*=11)	0.55±0.03 (*n*=7)	0.28±0.03 (*n*=4)
G412Y	0.92±0.19 (*n*=3)	0.64±0.06 (*n*=4)	0.38±0.01 (*n*=4)

[a] The mean open time was normalized in all cases with respect to that of the control, wild-type AchR ($\tau_{open\ c}$) and expressed as the ratio between the dwell time of the mutated AChR in the absence ($\tau_{open\ mut}/\tau_{open\ c}$, left column) and presence of 200 (middle column) and 400 μM (right column) HC, respectively.
[b] Recordings were obtained at 1 μM ACh, at a temperature of 20–22 °C and at a membrane potential of −70 mV.
[c] The number of individual recordings is indicated in parenthesis.

to explore whether mutations affected mean burst time (τ_{burst}) and number of events per burst.

As observed at low agonist concentration, τ_{open} diminished in wild-type receptors opened by high ACh concentrations by about 50% in the presence of 200 μM HC (1.03±0.15 and 0.53±0.07 ms in the absence and presence of the steroid, respectively). The inhibitory effect of HC over τ_{burst} and the number of events per burst reached ~90% (see Table 2). In wild-type channels, closed time histograms are well fitted by the sum of three components with durations of 2±0.8 (*n*=15, area=0.83) 1522±672 (0.07) and 27±16 ms (0.07), whereas in the presence of HC, a new component of 167±118 ms (*n*=8, area=0.14) in the closed time distribution is observed (Fig. 1). This observation is in agreement with previous data from our laboratory (Bouzat and Barrantes, 1996) and is likely due to the transition of the AChR to the blocked state induced by HC, since the steroid inhibits the AChR from switching between closed and open states.

When we analyzed the HC effect in G412 mutants, we found no significant differences in any of the parameters previously analyzed for wild-type AChRs, either in the absence or presence of HC (Table 2).

3.3. Effect of other M4 mutants

Once it was established that position αM4412 appeared to have no influence on the steroid–AChR interaction, we studied the effect of HC on AChRs mutated in other residues along the αM4 segment. In view of the labeling pattern with lipophilic photoaffinity reagents, it has been proposed that M4 is an α-helix in which residues C412, M415, C418, T422 and V425 all lie on the same, lipid-exposed face of the helix (Blanton and Cohen, 1992, 1994). We therefore considered the possibility that these lipid-exposed residues could be involved in the steroid binding site, and consequently single-channel recordings were obtained using AChRs mutated in these residues. Previous work from our laboratory demonstrated that residues C418 and T422 significantly affect the AChR channel gating kinetics when replaced by alanine (Bouzat et al., 1998), whereas C412 and M415 mutants did not. Results from position C412 mutants were described in a previous section (Tables 1 and 2).

We first evaluated the kinetics of a quadruple mutant AChR in which residues L411, M415, C418 and T422 were all substituted by alanine. We resorted to this

Table 2
Normalized mean open time, mean burst time and number of events per burst for G412 mutant AChRs at high (30 μM) agonist concentration[a]

	τ_{open} HC/τ_{open}	τ_{burst} HC/τ_{burst}	Events per burst HC/events per burst in control	c1 HC/c1
WT	0.49±0.07	0.12±0.07	0.15±0.07	1.50±0.53
G412W	0.43±0.06	0.14±0.07	0.14±0.03	1.10±0.52
G412N	0.54±0.06	0.08±0.03	0.17±0.08	1.03±0.10
G412C	0.43±0.05	0.11±0.04	0.14±0.07	1.28±0.17
G412Y	0.51±0.08	0.20±0.08	0.21±0.07	1.05±0.17

[a] Results show the ratio of the mean open time (τ_{open}), mean burst time (τ_{burst}) and number of events per burst in the presence and the absence of HC (200 μM), either in wild type (first row) or mutant (following rows) AChRs. Each ratio was obtained as the quotient between media of at least three independent observations for each condition. SDs were obtained as described in Section 2. The analysis in terms of confidence intervals showed no significant differences in the normalized data for mutant and wild-type receptors.

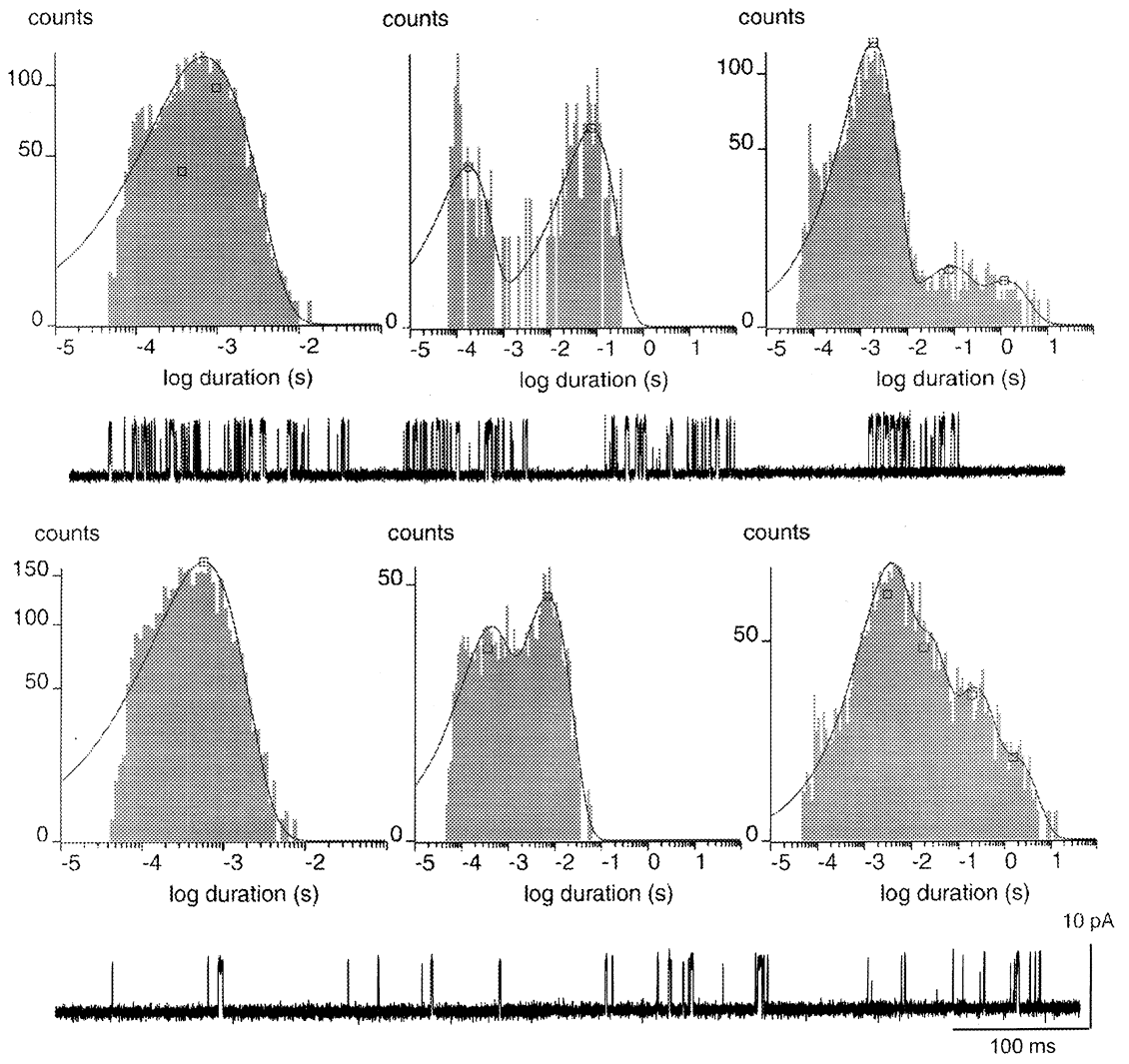

Fig. 1. Effect of HC on wild-type AChR. Open (left), burst (middle) and closed (right) time histograms obtained from single-channel recordings in the presence of 30 μM ACh in the absence (top row) or presence (bottom row) of 200 μM HC. Crude traces are shown below the histograms.

mutant AChR previously obtained in our laboratory (Bouzat et al., 1998) because it constitutes a useful model of lipid-exposed residues in αM4 (Blanton and Wang, 1991; Blanton and Cohen, 1992, 1994). Furthermore, as demonstrated in the previous section, neither L411 nor the αG412 mutant has any effect on steroid modulation. When activated by 30 μM ACh, the quadruple mutant channel opened in clusters with durations (395±93 ms, n=4) longer than those observed with the wild-type AChR (66.7±18 ms, n=9) (Fig. 2). The intra-cluster closed time interval was noticeably different: 1.95±0.05 and 18±1 ms for wild-type and mutant channel, respectively (Fig. 2).

In the presence of HC, cluster duration diminished by ~90% for wild-type AChR. In the mutant channel this diminution was about fivefold (Fig. 2). The intra-cluster duration was not affected by the presence of steroid in neither wild-type nor mutant channels. The intra-cluster open probability showed no variation in the presence of the steroid. When activated by 30 μM ACh, the mean open time of the mutant channel was 0.26±0.01 ms (n=3) whereas in the presence of 200 μM HC this parameter diminished to 0.19±0.02 ms (n=3, $p<0.01$) (Fig. 3 and Table 3). In order to compare these results with data obtained with wild-type AChR, the variation was normalized by expressing it as the ratio between the mean open time in the absence and presence of HC. The ratios obtained were 0.50±0.07 and 0.73±0.08 for wild-type and mutant AChRs, respectively, thus indicating that the inhibition produced by HC is lower in the quadruple mutant AChR (Fig. 4).

In order to further refine the search for the residue(s) responsible for the differences in corticoid action, we dissected the quadruple mutation into four different mutant AChRs. Two of them, L411A and M415A, with a mean open time similar to that of the wild-type AChR, were evaluated at 1 μM ACh. C418A and T422A, with open time durations, varying considerably from that of

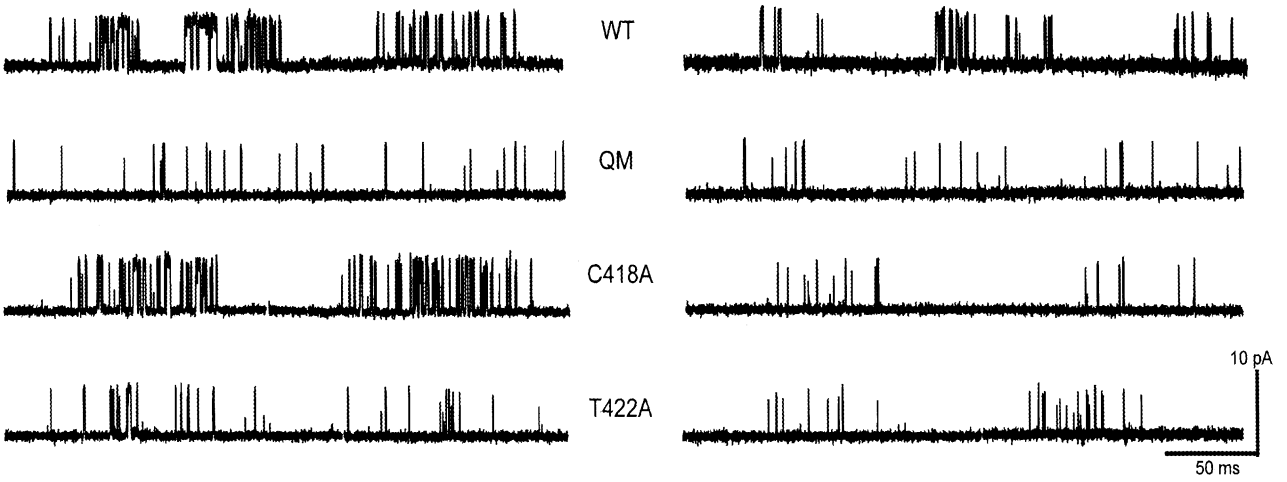

Fig. 2. Effect of mutations on HC response. Representative crude single-channel recordings of wild-type and quadruple (QM), C418A and T422A mutant AChRs at high (30 μM) ACh (left column). The right column shows the effect of 200 μM HC. The time scale was chosen to optimize observation of differences in burst duration rather than single-channel events.

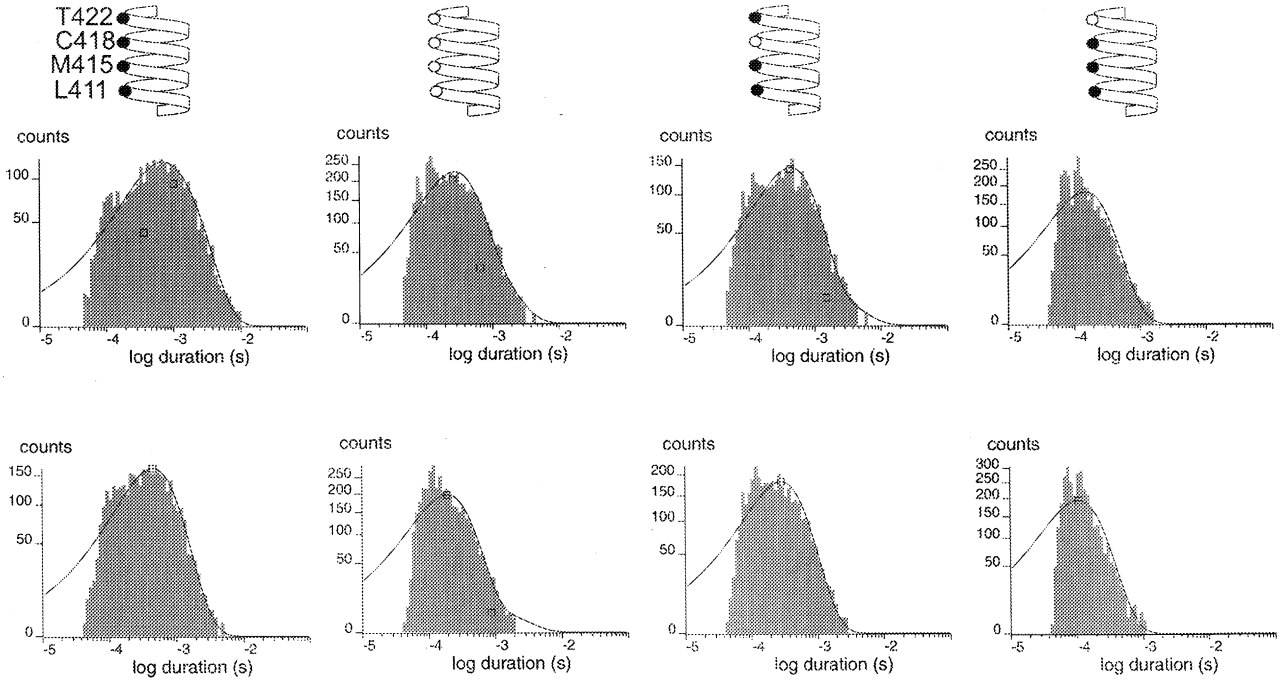

Fig. 3. Open time histograms of wild-type (left column) and mutant AChR channels opened by 30 μM ACh in the absence (upper row) and presence (lower row) of 200 μM HC. The schematic helices above the columns depict the lipid-exposed residues (black circles); mutations to alanine are shown as empty circles, e.g. four alanine substitutions are shown in the quadruple mutant (second column). Membrane potential: −70 mV.

the wild-type AChR, were evaluated at 30 μM ACh. In the L411A mutant AChR the mean open time did not differ from that of the wild-type AChR in the absence (1.08±0.013 ms, n=3) or presence (0.49±0.09 ms, n=3) of HC.

The main component in channel mean open time for M415A mutant AChR was 1.08±0.12 ms (n=4) as against 1.07±0.12 ms (n=4) for the wild-type with a mean burst time of 1.24 and 1.23±0.015 ms, respectively at 1 μM ACh. In the presence of 200 μM HC, τ_{open} for wild-type and M415A AChRs yielded values of 0.47±0.08 ms and 0.49±0.09 ms (n=3), respectively (data not shown). There were no significant differences in the effect caused by HC on this mutant and on the wild-type receptor (Fig. 4).

Fig. 2 further shows that the C418A mutant AChR channel opens in clusters longer than wild-type ones. Wild-type AChR and the C418A mutant AChR have similar intra-cluster closed durations, thus differing from that of the quadruple mutant channel. The burst duration

Table 3
τ_{open}, τ_{burst} and number of events per burst for wild-type and mutant AChRs at high (30 μM) agonist concentration[a]

	τ_{open} (ms)		τ_{burst} (ms)		Number of events per burst	
	−HC	+HC	−HC	+HC	−HC	+HC
WT	1.03±0.15 (*n*=4)	0.53±0.07 (*n*=12)	67±18	6.6±2.0	24±5	3.7±0.4
Quadruple	0.26±0.01 (*n*=3)	0.19±0.02 (*n*=3)	395±92	77±16	36±11	6.4±0.7
C418A	0.45±0.03 (*n*=5)	0.25±0.03 (*n*=4)	162±64	16±4.6	55±20	6±1
T422A	0.15±0.02 (*n*=4)	0.11±0.01 (*n*=3)	82±11	76±12	11.4±1.9	8.8±0.6

[a] τ_{open}, τ_{burst} and number of events per burst at 30 μM ACh for wild-type and mutant channels having mean open statistically different from those of wild-type AChR. The first column indicate the type of channel studied, and the next three columns list τ_{open}, τ_{burst} and number of events per burst, in the absence (left) and presence (right) of HC (200 μM).

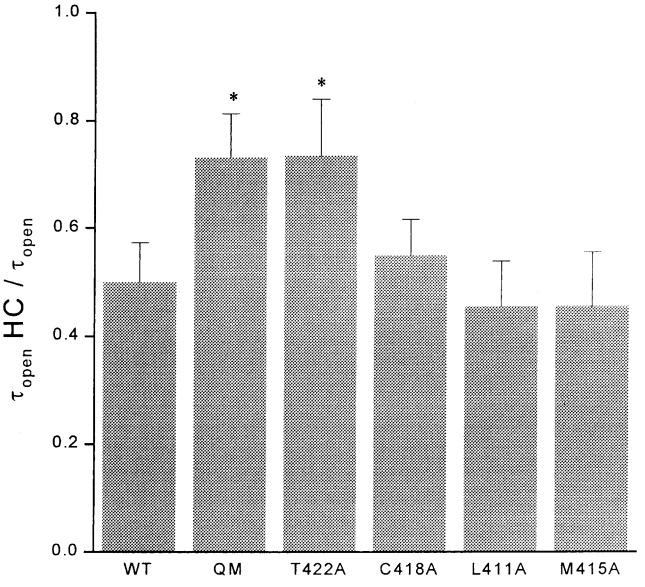

Fig. 4. Normalized effect of HC on the mean open time of wild-type and mutant AChR channels activated by 30 μM ACh. Values are the ratio between the mean open time in the presence and absence of HC. The symbol "∗" above the bar indicates statistically significant differences in the confidence interval of the quadruple (QM) and T422A mutants with respect to wild-type (WT) AChR.

in the C418A mutant diminished by 90% in the presence of HC (Table 3). The mean open time of the C418A mutation yielded a value of 0.45±0.03 ms (*n*=5) and 0.25±0.03 ms (*n*=4) in the absence and presence of HC, respectively (see histograms in Fig. 3). Here again, we expressed the mean net effect of HC as the ratio between the mean open time obtained in the presence and the absence of the drug. The ratio 0.55±0.07 was not different from that of the wild-type AChR (Fig. 4).

The T422A mutant exhibited clusters with mean durations shorter than those of the other mutants (82±11 ms) and intra-cluster closed durations remarkably similar to those of the quadruple mutant (Table 3). The mean burst duration and the number of events per burst were both found to decrease only slightly as a consequence of HC treatment. The mean open time obtained at 30 μM ACh was 0.15±0.02 ms (*n*=4) and 0.11±0.01 ms in the absence and the presence of 200 μM HC, respectively. The ratio between the τ_{open} in the absence and presence of HC was 0.76±0.11, which differs from that obtained for wild-type AChR, but is indistinguishable from that of the quadruple mutant (Fig. 4).

In order to normalize the results obtained with the five mutants, we further recorded the activity of the L411A and M415A mutants at 30 μM ACh and observed that the magnitude of the effect caused by HC on these mutants does not differ significantly from the effect on the wild-type receptor (Fig. 4).

4. Discussion

The fact that the M4 segment is the most likely domain of the AChR to be in contact with the lipid also makes it the strongest candidate for modulation by the membrane microenvironment and hydrophobic molecules (see review in Barrantes et al., 2000a). Our laboratory has a long-standing interest in elucidating the possible site and mechanism through which the steroids interact with, and modulate, the AChR protein. The process by which such modulation occurs is unlikely to result from a mere disturbance of the bulk membrane bilayer, since the effect can be reproduced in different cell lines having different membrane lipid compositions (see review in Barrantes, 2001). In the case of glucocorticoids, the alteration in AChR channel kinetics can be interpreted in terms of a blocking mechanism, probably allosteric in nature.

Work from our laboratory established that acute exposure of the AChR to HC induced a dose-dependent reduction in the single-channel mean open time and burst duration, and an increase in the closed time, with no changes in channel amplitude (Bouzat and Barrantes,

1993b). Subsequently we determined (Bouzat and Barrantes, 1996) that HC acts as a noncompetitive inhibitor of adult and embryonic AChRs. Other authors also studied the effect of this steroid on macroscopic currents. Kindler et al. (2000) for instance, expressed functional fetal and adult AChRs in *Xenopus laevis* oocytes and studied them with the two-electrode voltage-clamp technique. They found that both forms of the muscle-type AChR were potently inhibited by HC; 50% inhibition was found in the 1–2 mM range. In acutely dissociated superior cervical ganglion neurons, HC reversibly suppressed both the peak and steady-state nicotine-induced currents at a concentration of >10 μM (Uki et al., 1999).

Experimental evidence supports the notion that residues located in M4 play a key role in channel gating kinetics (Li et al., 1990; Bouzat et al., 1994; Lasalde et al., 1996). In the light of our current results, and in agreement with electrophysiological experiments using macroscopic current recordings of *Torpedo* AChR channels heterologously expressed in Xenopus laevis oocytes (Lasalde et al., 1996) position 412 in αM4 does not appear to be involved in AChR channel gating. When αG412 mutant AChRs were exposed to HC (Tables 1 and 2) we found no significant differences in the extent of steroid inhibition as compared with that seen in wild-type AChR. Initially, we also planned to evaluate the influence of changes in αC412 side-chain physical properties on HC–AChR interactions, given the predominant photoaffinity labeling of this residue with the synthetic progestin, promegestone in *Torpedo* AChR (Blanton et al., 1999). The lack of effect of varying side-chain at this position reinforces the view that αG412 is not involved in HC–receptor interactions in the case of murine skeletal muscle AChR.

We studied next a quadruple mutant in which those residues putatively in contact with membrane lipid (Blanton and Cohen, 1994) were all substituted by alanine. At high agonist concentration this quadruple mutant channel opened in longer clusters, with remarkably longer intra-cluster closed time intervals than those of the wild-type AChR, whereas the mean open time was about four times shorter than that of the wild-type AChR (Figs. 2 and 3; Table 3). In the presence of HC the kinetics of the quadruple mutant channel changed, but the extent of the decrease did not reach the level observed with wild-type AChR. The ratios between mean open time in the presence and absence of HC differed in terms of confidence intervals for wild-type AChR and the quadruple mutant (0.50±0.07 and 0.73±0.08, respectively).

When we dissected the quadruple mutant into four single mutant AChRs we observed that in three of them, L411A, M415A and C418A, the magnitude of the HC effect was equal to that of the wild-type AChR. The ratio between the mean open time in the presence and absence of HC was 0.45±0.08, 0.45±0.01 and 0.55±0.07, respectively (Fig. 4). The mutant T422A showed briefer single-channel openings in the presence of the steroid, but the magnitude of the diminution was ~30% instead of the ~50% typically observed in the presence of this glucocorticoid with the wild-type AChR (here the ratio was 0.73±0.10). Thus the alanine substitution in this residue appears to mimic the effect observed in the quadruple mutant, providing evidence to account for the steroid sensitivity so far found only with this residue. Such outcome is not totally unexpected, in the light of previous findings from our laboratory showing that T422 plays a key role in channel gating kinetics, probably due to its ability to form hydrogen bonds (Bouzat et al., 2000).

Cholesterol and its analogues locate in the membrane with their hydrophobic tail deeply buried in the bilayer, and their head at the level of the phospholipid polar head region (Franks, 1981; Villalain, 1996). Steroids adopt a similar location in the membrane, but differences in ring substituents lead to different degrees of penetration in the bilayer (Garbus et al., 2001). It will be of interest to investigate whether T422 is also important for the interaction of other steroids with the AChR, and whether other residues play a role in the interaction with further steroids, given their different penetration in the membrane.

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References

Barrantes, F.J., 1993. Structural-functional correlates of the nicotinic acetylcholine receptor and its lipid microenvironment. FASEB Journal 7, 1460–1467.

Barrantes, F.J., 1998. The Nicotinic Acetylcholine Receptor: Current Views and Future Trends. Springer Verlag, Berlin/Heidelberg and Landes Publishing Co, Georgetown, TX.

Barrantes, F.J., 2001. Fluorescence studies of the acetylcholine receptor: Structure and dynamics in the membrane environment. Journal of Fluorescence 11, 273–285.

Barrantes, F.J., Antollini, S.S., Bouzat, C., Garbus, I., Massol, R.H., 2000a. Nongenomic effects of steroids on the nicotinic acetylcholine receptor. Kidney International 57, 1382–1389.

Barrantes, F.J., Antollini, S.S., Blanton, M.P., Prieto, M., 2000b. Topography of nicotinic acetylcholine receptor membrane-embedded domains. Journal of Biological Chemistry 275, 37333–37339.

Blanton, M.P., Wang, H.H., 1991. Localization of regions of the *Torpedo* californica nicotinic acetylcholine receptor labeled with an aryl azide derivative of phosphatidylserine. Biochemica Biophysica Acta 1067, 1–8.

Blanton, M.P., Cohen, J.B., 1992. Mapping the lipid-exposed regions in the *Torpedo californica* nicotinic acetylcholine receptor. Biochemistry 31, 3738–3750.

Blanton, M.P., Cohen, J.B., 1994. Identifying the lipid-protein interface of the *Torpedo* nicotinic acetylcholine receptor: secondary structure implications. Biochemistry 33, 2859–2872.

Blanton, M.P., Xie, Y., Dangott, L.J., Cohen, J.B., 1999. The steroid promegestone, is a noncompetitive antagonist of the *Torpedo* nicotinic acetylcholine receptor that interacts with the lipid-protein interface. Molecular Pharmacology 55, 269–278.

Bouzat, C., Barrantes, F.J., 1993a. Acute exposure of nicotinic acetylcholine receptor to the synthetic glucocorticoid dexamethasone alters single channel gating properties. Molecular Neuropharmacology 3, 109–116.

Bouzat, C., Barrantes, F.J., 1993b. Hydrocortisone and 11-desoxycortisone modify acetylcholine receptor channel gating. NeuroReport 4, 143–146.

Bouzat, C., Bren, N., Sine, S., 1994. Structural basis of the different gating kinetics of fetal and adult acetylcholine receptors. Neuron 13, 1395–1402.

Bouzat, C., Barrantes, F.J., 1996. Modulation of muscle nicotinic acetylcholine receptors by the glucocorticoid hydrocortisone. Possible allosteric mechanism of channel blockade. Journal of Biological Chemistry 271, 25835–25841.

Bouzat, C., Roccamo, A.M., Garbus, I., Barrantes, F.J., 1998. Mutations at lipid-exposed residues of the acetylcholine receptor affect its gating kinetics. Molecular Pharmacology 54, 146–153.

Bouzat, C., Barrantes, F.J., Sine, S., 2000. Nicotinic receptor fourth transmembrane domain: hydrogen bonding by conserved threonine contributes to channel gating kinetics. Journal of General Physiology 115, 663–671.

Corbin, J., Wang, H.H., Blanton, M.P., 1998. Identifying the cholesterol binding domain in the nicotinic acetylcholine receptor with [125l] azido-cholesterol. Biochimica Biophysica Acta 1414, 65–74.

Criado, M., Eibl, H., Barrantes, F.J., 1982. Effects of lipids on acetylcholine receptor. Essential need of cholesterol for maintenance of agonist-induced state transitions in lipid vesicles. Biochemistry 21, 3622–3629.

Criado, M., Eibl, H., Barrantes, F.J., 1984. Functional properties of the acetylcholine receptor incorporated in model lipid membranes. Differential effects of chain length and head group of phospholipids on receptor affinity states and receptor-mediated ion translocation. Journal of Biological Chemistry 259, 9188–9198.

Franks, N.P., 1981. Membrane spectroscopy. In: Grell, E. (Ed.), Molecular Biology Biochemistry and Biophysics, vol 31. Springer-Verlag, Berlin.

Garbus, I., Bouzat, C.B., Barrantes, F.J., 2001. Steroids differentially inhibit the nicotinic acetylcholine receptor. Neuroreport 12, 227–231.

Giraudat, J., Dennis, M., Heidmann, T., Chang, J.-Y., Changeux, J.P., 1986. Structure for the high-affinity binding site for noncompetitive blockers of the acetylcholine receptor: serine 262 of the delta subunit is labeled by [3H] chlorpromazine. Proceedings of the National Academy of Science 83, 22719–22723.

Giraudat, J., Dennis, M., Heidmann, T., Haumont, P.Y., Lederer, F., Changeux, J.P., 1987. Structure for the high-affinity binding site for noncompetitive blockers of the acetylcholine receptor: [3H] chlorpromazine labels homologous residues in the beta and delta chains. Biochemistry 26, 2410–2418.

Giraudat, J., Galzi, J.L., Revah, F., Changeux, J.P., Haumont, P.Y., Lederer, F., 1989. The noncompetitive blocker [3H] chlorpromazine labels segment M2 but not segment M1 of the nicotinic acetylcholine receptor α-subunit. FEBS Letters 253, 190–198.

Hamill, O.P., Marty, A., Neher, E., Sakmann, E., Sigworth, F.J., 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391, 85–100.

Johnson, N.L., Kotz, S., 1969. Distribution in Statistics. Houghton-Mifflin, Boston.

Kindler, C.H., Verotta, D., Gray, A.T., Gropper, M.A., Yost, C.S., 2000. Additive inhibition of nicotinic acetylcholine receptors by corticosteroids and the neuromuscular blocking drug vecuronium. Anesthesiology 92, 821–832.

Lasalde, J.A., Tamamizu, S., Butler, D.H., Vibat, C.R.T., Hung, B., McNamee, M.G., 1996. Tryptophan substitutions at the lipid-exposed transmembrane segment M4 of *Torpedo californica* acetylcholine receptor govern channel gating. Biochemistry 35, 14139–14148.

Li, L., Schuchard, M., Palma, A., Pradier, L., McNamee, M.G., 1990. Functional role of the cysteine 451 thiol group in the M4 helix of the gamma subunit of *Torpedo californica* acetylcholine receptor. Biochemistry 29, 5428–5436.

Marsh, D., Barrantes, F.J., 1978. Immobilized lipid in acetylcholine receptor-rich membranes from *Torpedo marmorata*. Proceedings of the National Academy of Science USA 75, 4329–4333.

Marsh, D., Watts, A., Barrantes, F.J., 1981. Phospholipid chain immobilization and steroid rotational immobilization in acetylcholine receptor-rich membranes from *Torpedo marmorata*. Biochemica Biophysica Acta 645, 97–101.

Ortells, M.O., Barrantes, G.E., Barrantes, F.J., 1998. Molecular modeling of the nicotinic acetylcholine receptor. In: Barrantes, F.J. (Ed.), The Nicotinic Acetylcholine Receptor: Current View and Future Trends, Neuroscience Intelligence Unit. Springer Verlag and Landes Publishing, Berlin and Austin, TX.

Sakmann, B., Patlak, J., Neher, E., 1980. Single acetylcholine-activated channels show burst-kinetics in presence of desensitizing concentrations of agonist. Nature 286, 71–73.

Sigworth, F., Sine, S.M., 1987. Data transformations for improved display and fitting of single-channel dwell time histograms. Biophysical Journal 52, 1047–1052.

Sine, S.M., 1993. Molecular dissection of subunit interfaces in the acetylcholine receptor: identification of residues that determine curare selectivity. Proceedings of the National Academy of Science USA 90, 9436–9440.

Uki, M., Nabekura, J., Akaike, N., 1999. Suppression of the nicotinic acetylcholine response in rat superior cervical ganglionic neurons by steroids. Journal of Neurochemistry 72, 808–814.

Villalain, J., 1996. Location of cholesterol in model membranes by magic-angle-sample-spinning NMR. European Journal of Biochemistry 241, 586–593.