Steroids differentially inhibit the nicotinic acetylcholine receptor

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The effect of various natural and synthetic steroids on the function of the nicotinic acetylcholine receptor (AChR) was studied at the single-channel level. AChR channel kinetics was affected by some substitutions in the cyclopentaneperhydrophenantrene ring. Functionally relevant substitutions shortened channel open state duration, an effect that varied for different steroids. The presence of a polar group at C11 contributed to the inhibitory potency of the steroid. Among mono-hydroxylated steroids such as 11- and 17-OH progesterone, the highest potency was displayed by the former showing a level similar to that of the reference compound, hydrocortisone. When the effects were analyzed in terms of the octanol–water partition coefficient, a linear relationship was unexpectedly found between the hydrophilicity of the steroids and their inhibitory potency. *NeuroReport* 12:227–231 © 2001 Lippincott Williams & Wilkins.

Key words: Channel blockade; Nicotinic receptor; Patch clamp; Steroids

INTRODUCTION

In addition to its genomic steroid-receptor related actions, steroids also display activity through non-genomic pathways. These rapid non-genomic effects may occur either by a direct interaction of steroids with membrane proteins or by disturbing the membrane lipid. Given their cell-surface location, ligand-gated ion channels (LGIC), which are integral membrane proteins, are logical targets for steroid modulation. The nicotinic acetylcholine receptor (AChR) has been the prototype member of the superfamily of LGIC (for review see [1]). Adult muscle AChR is composed of four homologous subunits assembled in an $\alpha_2\beta\varepsilon\delta$ stoichiometry. For each subunit, the occurrence of four transmembrane segments (M1–M4) has been proposed. It has been shown that the AChR protein associates preferentially with some sterols [2,3], and photoaffinity labeling established the close proximity of cholesterol to the AChR protein [4].

We have demonstrated that natural and synthetic glucocorticoids act as noncompetitive inhibitors of the muscle-type AChR (for review see [5]). At the single-channel level, the main effect of dexamethasone, hydrocortisone (HC) and 11-deoxycortisone on the AChR consists of a decrease in channel open duration, an observation that we have interpreted as an allosteric blockade [6–8].

The site of action of steroids on the AChR has not been identified as yet, as is the case with other non-competitive AChR inhibitors. Early experimental data [2] led us to suggest that the site of action of such compounds is located in receptor domains probably in contact with the membrane lipid. Electrophysiological data further reinforced this view [8]. This contention is strongly supported by recent photoaffinity labeling experiments of purified AChR with the progestin steroid, promegestone. The steroid strongly labeled residues located in the hydrophobic M4 transmembrane segment of the α, β and γ subunits [9]. The M4 segment is currently accepted to be in extensive contact with membrane lipid. In contrast to muscle or electroplax AChR, an extracellular location has been proposed for the progesterone binding site in the case of neuronal AChR [10].

In the present study, single-channel currents were recorded with the patch-clamp technique in adult muscle AChR in the presence of different steroids. The changes in AChR channel mean open time were used as a measure of their relative inhibitory potency. A correlation could be established between the extent of the observed decrease in AChR channel open time and the chemical structure and hydrophilicity of certain steroids.

MATERIALS AND METHODS

Expression of AChR in HEK-293 cells: Mouse cDNAs used were subcloned into the cytomegalovirus-based expression vector pRBG4 [11]. HEK-293 cells were transfected with the cDNAs corresponding to the α, β, ε, and δ subunits using calcium phosphate precipitation at a subunit ratio of 2:1:1:1, respectively, essentially as described previously [12,13]. For transfections, cells at 40–50% confluence were incubated for 8–12 h at 37°C with the calcium phosphate precipitate containing the cDNAs in DMEM supplemented with 10% fetal bovine serum. Cells were used for single-channel measurements 1–2 days after transfection. In some experiments, the wild-type ε, subunit was replaced by the mutant ε_{T264P} [14].

Patch clamp recordings: Recordings were obtained in the cell-attached configuration [15] at a membrane potential of −70 mV and at 20°C. The bath and pipette solutions contained 142 mM KCl, 5.4 mM NaCl, 1.8 mM $CaCl_2$,1.7 mM $MgCl_2$ and 10 mM HEPES, pH 7.4. Patch pipettes were pulled from 7052 capillary tubes (Garner Glass, Claremont CA) and coated with Sylgard (Dow Coming, Midland, MI). Pipette resistances ranged from 5 to 7 MΩ. Acetylcholine (ACh) at a final concentration of 1 μM was present in the pipette solution. Steroids were dissolved in ethanol and dried under a stream of N_2. Dried steroids were resuspended in pipette solution by alternating sonication–heating (45–50°C) cycles. The same procedure, but without addition of steroids, was used in control recordings.

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.), digitized at 94 kHz with a PCM adapter (VR-10B, Instrutech) and transferred to a computer using the programme Acquire (Bruxton Corporation). Detection of single-channel events followed the half-amplitude threshold criterion using the program TAC (Bruxton Corporation) at a bandwidth of 5 kHz. Open- and closed-time histograms were plotted using a logarithmic abscissa and a square root ordinate [16] 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 < 1 ms.

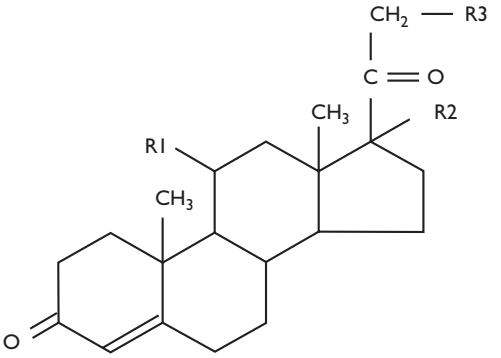
The partition coefficient of steroids was experimentally determined using the octanol-water partition method and related to literature data [17].

RESULTS

Extent of modification of the AChR mean open time by different steroids: The influence of the position and structure of the substituent groups in the cyclopentaneperhydrophenantrene ring of different steroids (Fig. 1) was studied using the patch-clamp technique in the cell-attached mode.

Qualitatively, all steroids affected the duration of the AChR channel open time in a similar manner, shortening the period that the channel spent in the open state, albeit to a different extent. In the presence of 1 μM ACh HEK-293, cells transfected with α, β, ε, and δ cDNAs exhibited channel openings typical of adult AChR (Fig. 2). The open time histograms (Fig. 2) showed a main component of 0.94 ± 0.12 ms ($n = 12$). When cell-attached patches were made in the presence of different steroids, the main effect observed was a decrease in the duration of the open state (Fig. 2).

All steroids tested affected AChR kinetics similarly: AChR channel open time distributions in the presence of steroids were displaced to briefer durations. They did not cause flickering nor changes in channel amplitude (Fig. 2, Table 1). In addition, the distribution of closed time duration in the presence of steroids did not show statistically significant differences with respect to control recordings at 1 μM ACh (Table 1). These effects were not time dependent, since longer exposure to steroid (up to several minutes) did not result in further modification of AChR channel kinetics. AChR channel mean open time was therefore adopted as a measure of steroid inhibitory potency.



Steroid	R1 (C11)	R2 (C17)	R3 (C21)
Hydrocortisone	OH	OH	OH
Cortisone	=O	OH	OH
Cortexolone	H	OH	OH
21Deoxycortisol	OH	OH	H
Corticosterone	OH	H	OH
Aldosterone	OH	H	OH
17OH Progesterone	H	OH	H
11OH Progesterone	OH	H	H
Progesterone	H	H	H
Pregnenolone	H	H	H
17OH Pregnenolone	H	OH	H

Fig. 1. Schematic representation of the steroids used in the present study. R1, R2 and R3 are the substituent groups at C11, C17 and C21 of the cyclopentaneperhydrophenantrene ring, respectively.

Channel mean open time in the presence of a given steroid with respect to that obtained under control conditions varied between 0.4 and 0.8 for all steroids tested at a final concentration of 200 μM (Fig. 3a). At this concentration, aldosterone produced the strongest reduction among all steroids tested (Table 1).

Because of their low solubility in aqueous media, progesterone and pregnenolone were used at a concentration of 100 μM, and their effects were compared to those of their hydroxylated forms. At 100 μM, progesterone, pregnenolone and 17OH progesterone did not significantly affect AChR open time (Fig. 3). In contrast, at this concentration the hydroxylated progesterone derivative, 11OH-progesterone, significantly modified channel open time ($p < 0.001$).

In order to discard the possibility that the lack of effect of progesterone was due to the insufficient solubilization of the steroid in the aqueous medium, we decided to test lower steroid concentrations. Though normal muscle-type AChR exhibits rather short open durations, further shortened in the presence of steroid, the AChR mutant ε_{T264P}

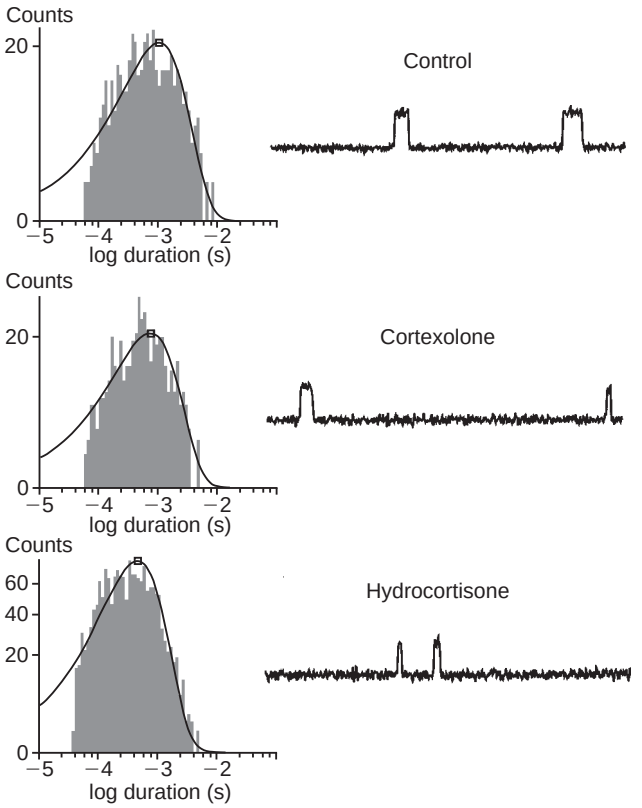


Fig. 2. Open time histograms (left) and single-channel recordings (right) obtained in the absence (top trace) and presence of 200 μM cortexolone (middle) or hydrocortisone (bottom) in the pipette solution. Recordings were obtained from HEK-293 cells expressing adult AChR Membrane potential: −70 mV. Filter: 5 kHz.

[14] dramatically prolongs the channel mean open time to several milliseconds, thus extending the sensitivity of the single-channel recordings to test lower steroid concentrations. At a concentration of 30 μM, HC caused a diminution of 65% in the main open time component of the ε_{T264P} AChR mutant ($\tau_o = 18.3 \pm 1.6$ and 5.31 ± 0.8 ms in the absence and presence of HC, respectively). In contrast, 30 μM progesterone, a concentration below its maximal water solubility (36 μM), did not affect AChR mean open time ($\tau_o = 17.9 \pm 2.2$ ms).

Relationship between inhibitory potency of steroids and their hydrophilicity: Initially, we set to investigate whether there was any relationship between the decrease in AChR channel mean open time and the hydrophilicity of steroids, as measured by their octanol-water partition coefficient. The latter is considered to be a faithful reflection of the partition behavior of compounds in a biological membrane–water system [18]. The ratio between AChR mean open time in the presence of a given steroid and the control condition for each experiment was plotted against the logarithm of the octanol–water partition coefficient for each compound (Fig. 4). Unexpectedly, an inverse linear relationship between the hydrophobicity of the steroid and the decrease in AChR mean open time was observed. From

Table 1. AChR channel properties in the presence of steroids. Currents were measured with the patch-clamp technique in the presence of a fixed concentration of steroid (200 μM). τ_{o1} and τ_{o2} are the AChR channel mean open times obtained from the corresponding histograms. τ_{C1} and τ_{C2} are the mean AChR channel closed times. τ_{burst1} and τ_{burst2} are mean burst durations, and A is the corresponding amplitude.

Steroid	n	τ_{o1} (μs)	τ_{o2} (μs)	τ_{burst1} (μs)	τ_{burst2} (μs)	τ_{c1} (μs)	τ_{c2} (ms)	A (pA)
Control	12	940 ± 120	–	1050 ± 120	–	–	–	4.9 ± 0.1
Corticosterone	5	552 ± 129	–	671 ± 160	–	71 ± 31	14 ± 6	4.7 ± 0.1
11OH-Progesterone	5	624 ± 98	–	675 ± 118	–	97 ± 47	54 ± 26	4.9 ± 0.3
17OH-Progesterone	4	690 ± 83	–	751 ± 105	–	78 ± 29	41 ± 16	5.2 ± 0.1
Aldosterone	5	437 ± 48	–	486 ± 75	–	64 ± 21	28 ± 5	4.5 ± 0.4
Cortexolone	5	735 ± 135	–	841 ± 134	–	–	–	5.1 ± 0.1
Hydrocortisone (HC)	14	547 ± 84	184 ± 46	638 ± 108	97 ± 32	85 ± 38	17.3 ± 8	5.0 ± 0.1
Dehydroepiandrosterone sulphate (DHEAS)	4	534 ± 69	–	568 ± 89	–	69 ± 30	370 ± 215	4.9 ± 0.2
21 Deoxycortisol	7	610 ± 56	198 ± 28	652 ± 70	194 ± 19	43 ± 29	129 ± 123	5.4
Cortisone	4	471 ± 75	153 ± 29	558 ± 140	148 ± 36	258 ± 59	419 ± 152	5

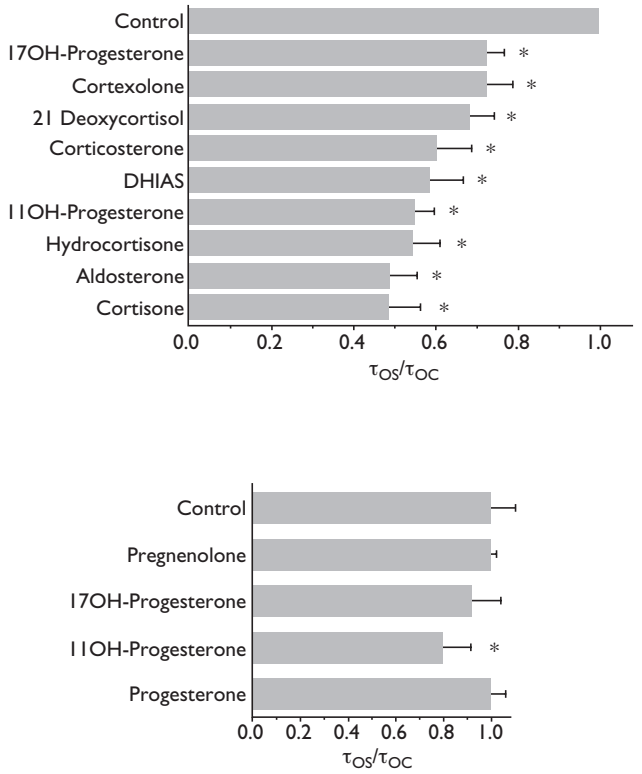


Fig. 3. Upper panel: Effect of steroids on AChR channel mean open time. Results are expressed as the ratio of the mean open time (τ_o) of channels recorded in the presence of a given steroid (200 μM) (τ_{os}) and that obtained under control conditions (τ_{oc}). The data are expressed as the mean ± s.d. of at least 3 recordings for each condition. * $p < 0.001$ (Student's *t*-test). Lower panel: Effect of less soluble steroids at a concentration of 100 μM on AChR mean open time. Data represent the mean ± s.d. of at least 3 independent determinations for each condition. * $p < 0.05$.

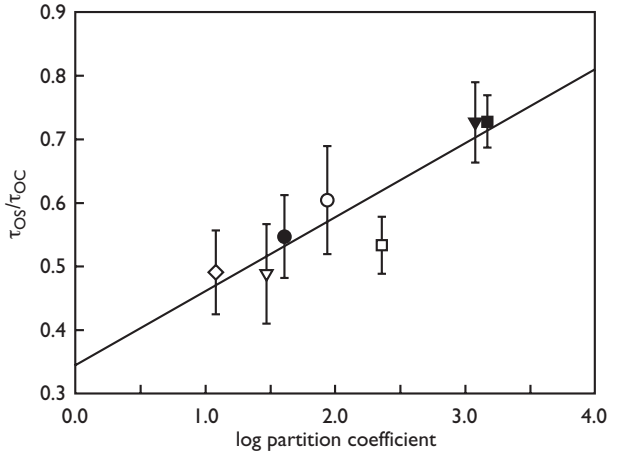


Fig. 4. Relationship between AChR channel open time and the octanol–water partition coefficient of hydrocortisone (closed circles), corticosterone (open circles), aldosterone (open diamonds), cortexolone (closed triangles), cortisone (open triangles), 17OH progesterone (closed squares) and 11OH progesterone (open squares). τ_{oc} is the mean open time in the absence of the steroid; τ_{os} corresponds to the mean open time in the presence of a given steroid at a concentration of 200 μM.

the slope of the relationship we could determine that a decrease of one unit in the octanol–water partition coefficient of the steroid leads to a 12% decrease in AChR mean open time; the inhibitory potency of the series of steroids studied therefore increases proportionally to their water solubility. In agreement with this observation, we found that steroids affected AChR channel mean open time in a manner inversely proportional to their retention time in a (hydrophobic) reverse-phase HPLC column (not shown).

DISCUSSION

We studied the relationship between the structure of a series of steroids and their inhibitory effects on AChR function. We could demonstrate that a series of steroids modify AChR channel kinetics in a qualitatively similar but quantitative different manner (Fig. 1, Fig. 2, Fig. 3). The main common effect consists of a diminution in AChR channel duration, reaching values of up to 60% in some cases.

We could establish the influence of different ring substituent groups on the potency of the steroid in diminishing AChR channel mean open time. The synthetic glucocorticoid HC was used as a reference compound. This steroid possesses three OH groups in positions 11, 17 and 21 and reduces AChR mean open time by 50% at a concentration of ~200 μM. The lack of a hydroxyl group in C11 and C21, as in cortexolone and 21 deoxycortisol, causes these two latter compounds to be less potent AChR inhibitors. In contrast, the absence of the OH group in C17, as in corticosterone, does not introduce any significant change in potency as compared to HC (Table 1). Cortisone possesses a keto group at position C11 and produces an effect of similar magnitude similar to that of HC. Thus, a conclusion from this series of experiments is that the presence of a polar group at position 11 contributes to the potency of a given steroid as an inhibitor of AChR function. Among mono-hydroxylated steroids such as 11 and 17OH progesterone, the former is the most potent, with a potency in the order of that of HC.

The importance of position 11 could be related to the lipo/hydrophilicity of the compound: the presence of an OH group at position 11 increases hydrophilicity, whereas the presence of OH groups in the other two positions has less influence on this physicochemical property of the steroid. Interestingly, the presence of the OH group in position 11 has been reported to be a determining factor in other important functions of steroids. Thus, the introduction of a hydroxyl group in C11 leads to an ~5-fold enhancement of binding affinity of steroids for the cytoplasmic steroid receptor [19]. Similar trends in steroid effects are observed here with the AChR, suggesting common structural characteristics between the two types of steroid-binding proteins.

Our results show that the mean open time of the adult muscle-type AChR is not modified by progesterone at a concentration of 100 μM (Fig. 3b). The data were confirmed in a mutated AChR that possesses a substitution in a single amino acid residue in the M2 transmembrane segment of the ε subunit (ε_{T264P}) [14]. Because this mutation dramatically prolongs the channel mean open time, it constitutes an excellent model to study the effect of drugs that shorten AChR open state. We first used this model system to study

the effect of HC [8]. In the present study we observed that progesterone did not modify the mean open time of ε_{T264P}AChR even at a concentration of 30 μM, whereas at the same concentration HC decreased AChR open time by 60%. In contrast to the lack of action of progesterone on a muscle-type AChR as observed here, progesterone has been shown to inhibit a neuronal AChR [10]. Differences are also apparent in the case of dexamethasone, a synthetic glucocorticoid that behaves as a potent inhibitor of muscle-type AChR [6] but is inactive on α4-containing neuronal AChR [20]. Thus our results demonstrate that neuronal and muscle AChRs differ in their selectivity for different steroids.

Cholesterol and cholesterol analogs locate in the membrane with their planar rigid hydrophobic ring oriented perpendicular to the membrane bilayer, with their polar moiety at the same depth as position 3 in the phospholipid polar head region, and the isooctyl side chain deeply buried in the center of the bilayer [21,22]. Cholesterol and cholesterol analogs exhibit transbilayer dimerization in multilamellar and large unilamellar vesicles, through the partial overlap of their aliphatic tails [23–25]. It is surmised that other sterols adopt a position in the membrane similar to that of cholesterol [23,24]. The presence of substituent OH groups may affect the extent of penetration in the membrane.

Here we show that steroid potency on AChR function is inversely correlated with their lipophilicity and thus does not follow a Meyer-Overton behavior, suggesting that their effect on AChR channel kinetics is not exerted through a simple perturbation of the membrane lipid, e.g. by altering membrane fluidity. Instead, the quantitatively different effects of different steroids on the AChR channel probably reflect their varying extent of penetration in the membrane, resulting in different accessibilities to their site(s) of action. Alternatively, differences in the inhibitory potency of different steroids could arise from subtle variations in structure brought about by different ring substituent groups.

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