ELSEVIER

Biochimica et Biophysica Acta 1564 (2002) 14–20

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Modulation of acetylcholine receptor channel kinetics by hydrocortisone

Ewa Nurowska [a,b,*], Fabio Ruzzier [a]

[a] *Department of Physiology and Pathology and B.R.A.I.N. Centre for Neuroscience, University of Trieste, via Fleming 22, I-34127 Trieste, Italy*
[b] *Department of Biophysics, Agricultural University SGGW, ul. Rakowiecka 26/30, 02-528 Warsaw, Poland*

Received 16 August 2001; received in revised form 14 December 2001; accepted 17 January 2002

Abstract

The kinetics of the nicotinic acetylcholine receptor (AChR) channel were analysed in the presence of hydrocortisone (HC, 100–400 μM), an electrically neutral steroid. The channel open time decreased, and in contrast to control conditions did not show any voltage dependency. However, HC induced a new (blocked) component in the closed time distribution, with a time constant that decreased with membrane hyperpolarization. HC decreased also, in a concentration-dependent way, the open time per burst. After coupling HC to bovine serum albumin, to restrict the place of steroid action at the external surface of the membrane, a voltage dependency of steroid action persisted. The effects of HC on the open and blocked time constants did not depend on agonist concentration, but was dependent on the type of agonist used (acetylcholine or nicotine). These results support the hypothesis that HC molecules bind near the agonist binding site.

Keywords: Acetylcholine receptor; Hydrocortisone; Steroid; Voltage-dependent modulation; C2C12 cell

1. Introduction

Many steroids are known to alter the kinetics of ligand-activated receptors, showing, however, a large diversity in action. Some of them block [1–4], while others enhance [5,6] the activity induced by an agonist. Moreover, some steroids exert both activities, depending on concentration [1,7]. Hydrocortisone (HC) was shown to block the nicotinic acetylcholine receptor (AChR) channel activity inducing bursts of openings [8,9], an action that was voltage dependent [9].

Many charged or ionizable agents can modulate the burst activity of AChR channels in a voltage-dependent manner, while the action of electrically neutral agents usually does not depend on membrane potential [10,11]. An exception seems to be HC, an uncharged molecule that decreases the open time in a voltage-dependent way. It has been already suggested [12] that the voltage-dependent burst activity caused by charged agents could originate from their interaction with the transmembrane region of the receptor (i.e. well within the membrane potential field). Bouzat and Barrantes [9] also proposed such site of action for HC. However, experiments performed with other hormones suggest an extracellular localisation of the steroid binding site [4,13]. In this paper, we present results from experiments aiming at clarifying the HC binding site localisation. This could help us to understand better the mechanisms by which HC blocks AChR activity.

Some of these results have already been briefly presented [14].

2. Materials and methods

2.1. Cell culture

C2C12 cells were grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with foetal calf serum (20%), L-glutamine (4 mM), penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}). To induce cell fusion, 1 day after plating, the medium was shifted to DMEM supplemented with horse serum (2%) plus L-glutamine, penicillin and streptomycin as above. All chemicals were from ICN Biomedicals (Costa Mesa, CA, USA).

2.2. Single-channel measurements

Electrophysiological experiments were performed on myotubes 4–8 days after changing the culture medium. Experiments were performed at room temperature in the cell-attached configuration of the patch clamp technique.

* Corresponding author. Department of Biophysics, Agricultural University SGGW, ul Rakowiecka 26/30, 02-528 Warsaw, Poland. Fax: +48-22-849-16-76.
E-mail address: nurowska@delta.sggw.waw.pl (E. Nurowska).

0005-2736/02/$ - see front matter
PII: S0005-2736(02)00356-5

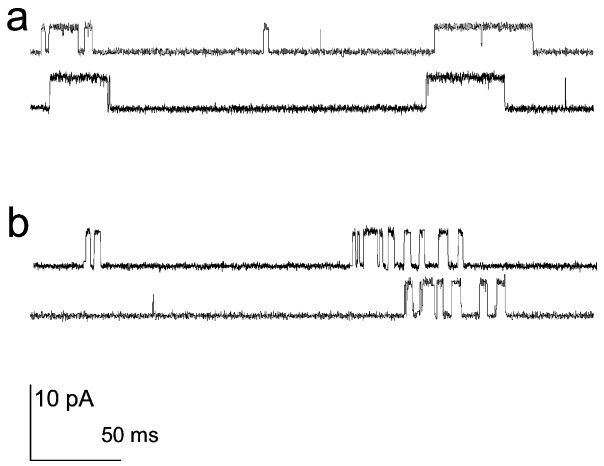


Fig. 1. HC affects the AChR channel activity. Recordings were made with 50 nM ACh in the pipette, from a cell-attached patch (a) in the absence and (b) in the presence of 100 μM HC in the pipette solution. The pipette potential was held at+60 mV. Inward currents are plotted as upward deflections.

Just before the experiments, the medium was substituted for a bath saline (NES) containing (mM): NaCl 140, KCl 2.8, $CaCl_2$ 2, $MgCl_2$ 2, glucose 10, HEPES buffer 10 (pH 7.4). The pipettes contained the same saline solution supplemented with acetylcholine (ACh, 50 nM; Sigma, St Louis, MO, USA), or nicotine (Nic, 1–2 μM; Sigma) if not otherwise stated. HC (up to 400 μM; Calbiochem, San Diego, CA, USA) and HC 21-hemisuccinate–bovine serum albumin (HC–BSA, 100 μM, 23 mol steroid/mol BSA; Sigma) was dissolved in NES on the same day of the experiment and added to the pipette solution.

2.3. Data analysis

Single AChR channel currents were recorded with the Axopatch 200 amplifier (Axon Instruments, Foster City, CA, USA). The signals were filtered (2–5 kHz, −3 dB), video-recorded with 14-bit resolution, and transferred at 50 kHz to a computer using a DigiData 1200 interface and the Fetchex program (pClamp 6.0.4, Axon Instruments). Channel events were detected using the Fetchan program (pClamp 6.0.4), with the threshold set at half of the unitary current. With 50 nM ACh in the pipette solution, overlapping openings were rarely observed. Only openings to the first level were included in the dwell time distributions, while all openings were detected for the P_{open} analysis. Open and closed time histograms were plotted using a logarithmic abscissa from more than 450 events (usually about 1000). The time constants τ_i of the open and the closed distributions were fitted by the method of maximum likelihood using PSTAT program (pClamp 6.0.4) with a probability density function being the sum of n exponential terms, i.e.

$$f(t) = \sum a_i(1/\tau_i)\exp(-t/\tau_i) \quad (1)$$

where a_i represents the area of the i-th component ($\Sigma a_i = 1$), $i = c_1$ corresponds to the brief, $i = c_2$ to the middle and $i = c_3$ to the long component of closures, while $i = o_1$ corresponds to the brief and $i = o_2$ to the long component of openings. Bursts of openings are defined [15] as groups of openings separated by gaps shorter than τ_c, including also isolated openings. τ_c was found by solving the following equation with numerical methods:

$$a_{c_3}(1 - \exp(-\tau_c/\tau_{c_3})) = a_{c_2}(\exp(-\tau_c/\tau_{c_2}))$$

In control recordings, i.e. without steroids, τ_c did not change significantly with pipette potential; thus, for simplicity, we used the τ_c calculated at 60 mV to analyse also the data

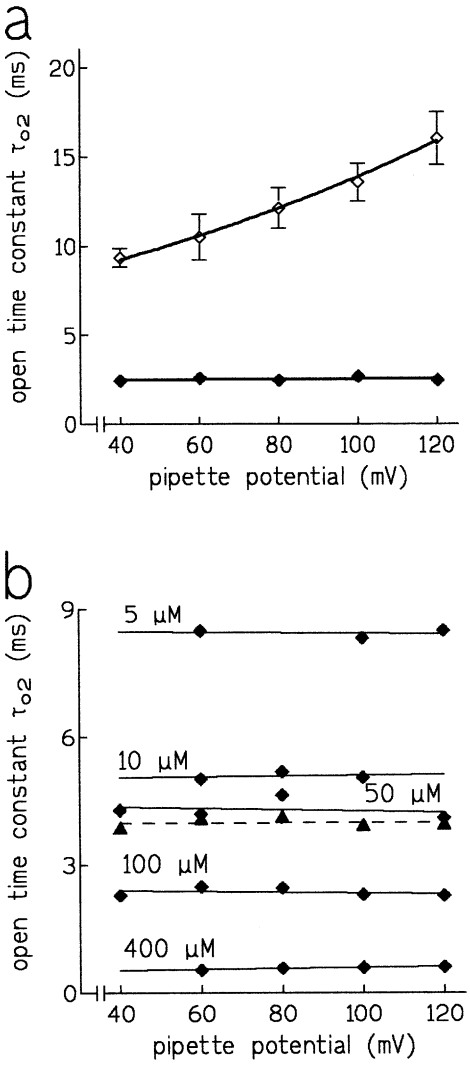


Fig. 2. HC affects the voltage dependency of the receptor open time duration. The open time constant τ_{o_2} at different pipette potentials: (a) in the absence (◇) and in the presence (◆) of 100 μM HC in the pipette solution; (◇) are fitted with the function $f = A \cdot \exp(V/b)$, where V is the pipette potential, $A = 7.0$, $b = 147$ mV; each point represents the mean ± S.E. from (◇) eight or (◆) six membrane patches; (b) at different HC concentrations (◆) and when 100 μM HC–BSA (▲) was present in the pipette solution; each line determined by a linear regression fits data from the same patch; numbers correspond to HC concentration. All recordings are from cell attached patches in the presence of 50 nM ACh.

recorded at other pipette potentials in the same patch. In all other experiments, τ_c was calculated separately for each pipette potential. To calculate the open time within a burst, τ_{ob}, firstly the time duration of all openings within one burst were added. Then, the distribution of such events were plotted and fitted with the Eq. (1) with two components only: a brief corresponding to brief isolated openings and a long corresponding to the τ_{ob}. Sequences of open and blocked events were defined as bursts without isolated openings. Distribution of open events within sequences was well fitted with the Eq. (1) with only one component corresponding to long openings.

Data are given as mean ± S.E. The Student's *t*-test was used to examine the statistical significance.

2.4. Calculation of the kinetic rate constants in the blocking scheme (2)

The probability $P(r)$ calculated for the blocking scheme (2) to have r openings per burst is:

$$P(r) = (\pi_{OB}\pi_{BO})^{r-1}(\pi_{OS} + \pi_{OB}\pi_{BS})$$

where $\pi_{OB}=[HC]k_+/(\alpha+[HC]k_+)$; $\pi_{OS}=\alpha/(\alpha+[HC]k_+)$; $\pi_{BO}=k_-/(k_-+k_c)$; $\pi_{BS}=k_c/(k_-+k_c)$ are the probabilities that the channel undergo the transition from state i to state j (i, j = O, B, S for open, blocked and shut states). $[HC]k_+$, k_- represent the transition rates from open to blocked and from blocked to open states, k_c is the rate constant for transition from blocked to shut state, β and α are rate constants for opening and closing the receptor. The mean number of openings per burst m is given by the expression

$$m = \sum_{r=1}^{\infty} rP(r) / \sum_{r=1}^{\infty} P(r) = \sum_{r=1}^{\infty} rP(r)$$

and is related to the mean open time per burst by $\tau_{ob}=m\tau_{o_2}$. Short algebra shows that the reciprocal of the mean open time per burst ($1/\tau_{ob}$) is:

$$1/\tau_{ob} = \alpha + ((k_c k_+)/(k_c + k_-))[HC]$$

The rate k_c was found by identification of the coefficient $(k_c k_+)/(k_c + k_-)$ with the slope of the reciprocal $1/\tau_{ob}$ versus HC concentration, the rate k_+ with the slope of the reciprocal $1/\tau_{o_2}$ versus HC concentration and the coefficient $1/(k_c + k_-)$ with τ_{c_2}.

3. Results

3.1. Control experiments

After seal formation, with 50 nM ACh in the pipette, AChR channel activity was easily observed in almost every patch (e.g. Fig. 1a). The *I–V* relation was linear in the range from 40 to 100 mV pipette potential, giving a slope conductance of 34 ± 1.0 pS (n = 9).

The channel open time distribution was best fitted with a sum of two exponential functions, the first, with time constant τ_{o_1}, corresponding to brief openings, the second, with time constant τ_{o_2}, corresponding to long openings. The time constant τ_{o_2} of the long component increased exponentially with hyperpolarization (Fig. 2a), but there was no evidence of such dependence for the brief time constant.

The closed time distributions were well fitted with the sum of two or three exponential functions, with the brief and long components present in all recordings, while the middle component was absent in about 50% of patches (Table 1).

About 40% of all openings occurred in bursts, defined as sequences of open states divided by closures with brief (0.10 ms) or middle (0.72 ms) time constants, and the open time within bursts was ca. 15 ms (Table 1).

3.2. The effects of HC

When HC was added to the ACh pipette solution, significant changes in the channel gating kinetics were observed, without any statistically significant effect on the mean channel conductance (32 ± 1.0 pS, n = 7). The most evident effect of HC was a decrease in the channel open time (Figs. 1b and 2). More precisely, HC (100 μM) reduced only the time constant of the long openings τ_{o_2} ($P < 0.0001$, Table 1), while the brief opening duration was unaffected (Table 1). Interestingly, in the presence of HC, the brief openings were observed less frequently, being about 30% of

Table 1
Open, closed and open time per burst distributions

	n	Open time		Closed time			Open time per burst τ_{ob} (ms)
		τ_{o_1} (ms)	τ_{o_2} (ms)	τ_{c_1} (ms)	τ_{c_2} (ms)	τ_{c_3} (ms)	
		%	%	%	%	%	
ACh 50 nM	20	0.191 ± 0.010	10.2 ± 0.7	0.188 ± 0.03	1.08 ± 0.03	124 ± 23	15.2 ± 0.8
		56 ± 2	44 ± 2	33 ± 6	10[a] ± 2	65 ± 6	
ACh 50 nM + HC 100 μM	10	0.177 ± 0.013	2.32 ± 0.09	0.253 ± 0.072	4.48 ± 0.27	323 ± 82	9.54 ± 0.85
		29 ± 2	71 ± 2	11[b] ± 3	53 ± 4	39 ± 3	

[a] Calculated from 10 patches; in remaining 10 patches, the component was absent.
[b] Calculated from seven patches; in remaining three patches, the component was absent.

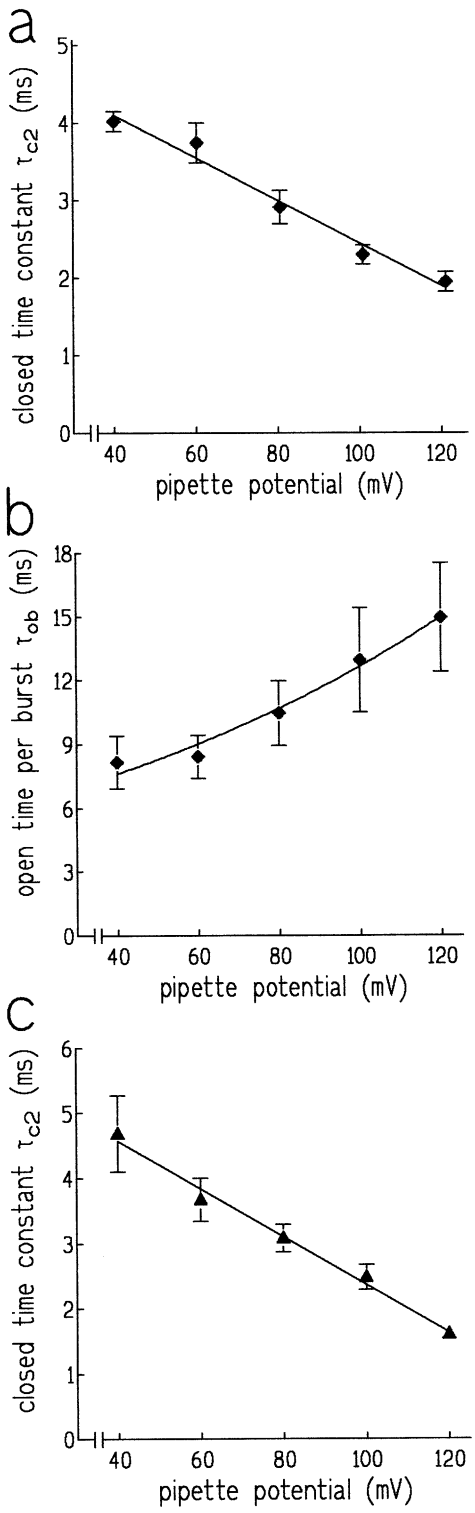


Fig. 3. HC effects are voltage dependent. Effect of pipette potential on (a,c) the closed time constant τ_{c_2} and (b) open time per burst τ_{ob} at different pipette potentials when (a,b) 100 μM HC and when (c) 100 μM HC–BSA is added to the pipette solution. Data points represent the mean ± S.E. (a) from six membrane patches or (b,c) from three to four membrane patches. In (a,c), the continuous line is determined by a linear regression; both slopes are significantly different than zero. In (b), data points are fitted with the function $f = A \cdot \exp(V/b)$, where V is the pipette potential, $A = 5.4$, $b = 118$ mV.

all openings, in comparison to 56% in control conditions ($P < 0.0001$, Table 1). The time constant of the long openings did not change when the membrane potential changed, and this effect was independent on HC concentration (Fig. 2b).

Moreover, in the presence of HC, a new component in the closed time distribution appeared representing 50% of all closures (Table 1). For sake of simplicity, we will term these events "blocked events". The time constant τ_{c_2} of this HC-induced component was dependent on membrane potential, decreasing with hyperpolarization (Fig. 3a). On the other hand, we did not observe any statistically significant change in τ_{c_2} while changing HC concentration (Fig. 4a).

HC increased the time constant of the long component in the closed time distribution ($P = 0.005$, Table 1). In recordings performed for 15–20 min (400 μM HC, $n = 2$), we did not observe any progressive decrease of the probability of channel openings.

In the presence of HC 100 μM in the pipette, the bursts appeared as groups of openings divided by closures with a brief component and a new middle component with τ_{c_2} of 4.5

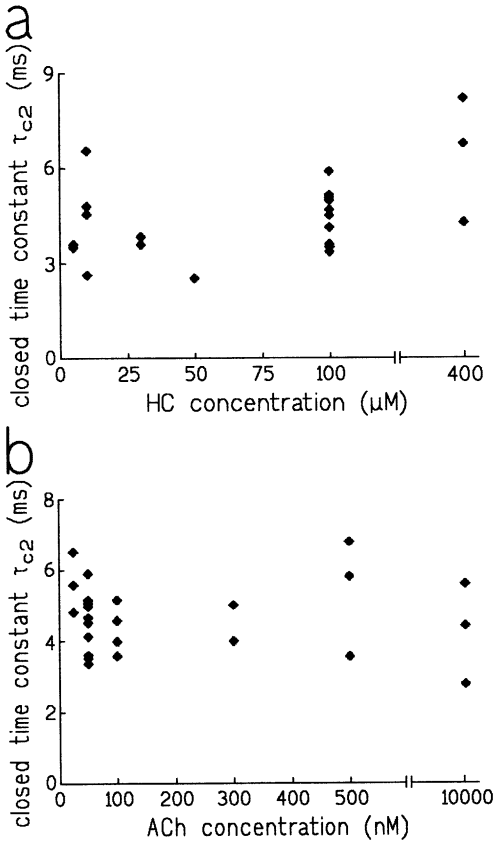


Fig. 4. Closed time constants τ_{c_2} do not depend on HC and ACh concentrations. Closed time constants τ_{c_2} (a) at different HC concentrations when 50 nM ACh was present in the pipette solution, and (b) at different ACh concentration when 100 μM HC was present in the pipette solution. Data points represent values from different membrane patches. Pipette potential was +60 mV.

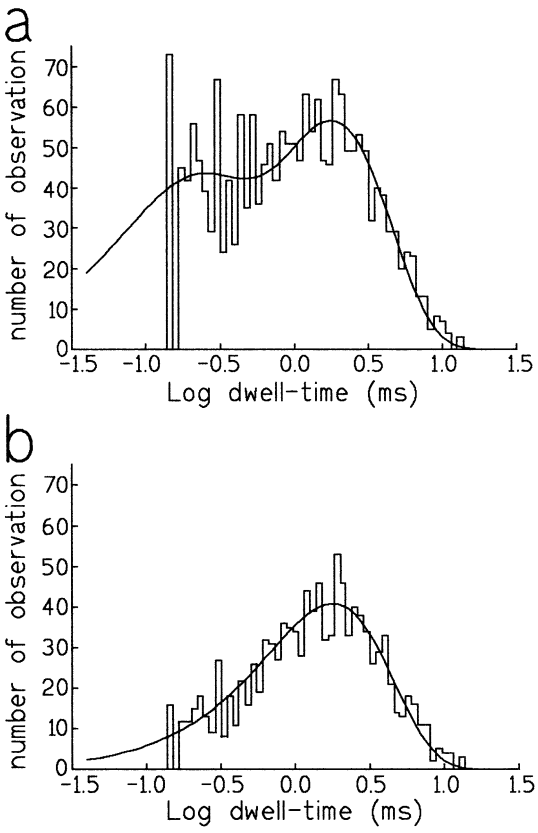


Fig. 5. Brief events are absent within the sequences of open and blocked events. Open time distribution in the presence of 100 μM HC from the same membrane patch when (a) all open events are included and when (b) only events within sequences (i.e. excluding isolated openings) are included. The histogram of all openings is best fitted by the sum of two exponentials, with time constants $\tau_{o_1} = 0.153$ ms and $\tau_{o_2} = 1.78$ ms. In the histogram of openings within sequences, only one component is present, with a time constant $\tau_{o_2} = 1.78$ ms.

ms. During the sequences of open and blocked events, only one open time component was observed (Fig. 5), and open time per burst increased with membrane hyperpolarization (Fig. 3b). The effects of HC were concentration dependent, both on the open time constant and on the open time per burst (Fig. 6). For example, 100 μM HC significantly decreased the open time per burst from 15 to 9.5 ms (Table 1, $P = 0.0002$), and 400 μM HC to 3.85 ± 0.63 ms ($n = 3$, $P < 0.0001$, Fig. 6). However, the burst duration increased, in the presence of 100 μM HC, from 15.89 ± 1.01 to 21.98 ± 2.60 ms ($P = 0.0125$).

To exclude the possibility that HC exerts these effects acting within the cell membrane, we performed a series of experiments ($n = 5$) using HC coupled to BSA, a very hydrophilic molecule. In this way, the coupled hormone has to be restricted to the external surface of the membrane. The effects caused by HC–BSA were similar to those observed with the uncoupled hormone. We noticed a decrease in the open time constant τ_{o_2} (3.18 ± 0.33 ms), and the appearance of a new component of 3.85 ± 0.31 ms in the closed time distribution. Similarly to what was observed when using HC alone, the effects of the coupled hormone depended on the membrane potential (Figs. 2b and 3c).

In three experiments in which we used ACh (50 nM) and BSA alone, at the same concentration as the one used in experiments with the HC–BSA conjugate, we did not observe significant changes in the open or the closed time constants compared to recordings without BSA.

Since it was already reported [4] that the inhibition of ACh-induced currents caused by another steroid hormone, progesterone, was dependent on the agonist concentration, we studied the effect of HC at different ACh concentrations (25 nM–10 μM). We did not observe any obvious trend in the time constants of the closed τ_{c_2} (Fig. 4b) or open τ_{o_1}, τ_{o_2} constants (data not shown) after changing the concentration of ACh.

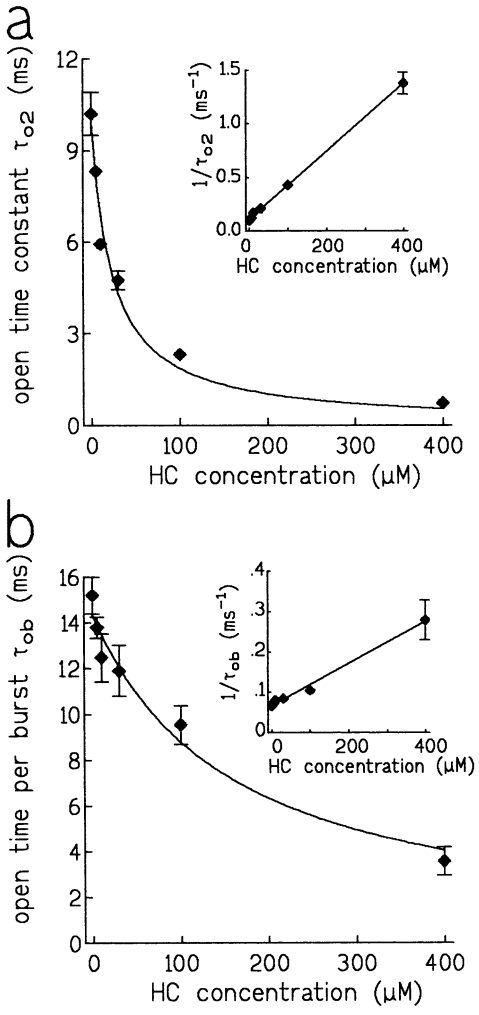


Fig. 6. HC effects depend on HC concentrations. Effects of different HC concentrations on (a) the open time constant τ_{o_2}, and on (b) the open time per burst τ_{ob}. Data points representing the mean ± S.E. from 2 to 10 membrane patches are fitted with the function $f = 1/(\alpha + k[\mathrm{HC}])$; α and k values are: in (a) 0.11 ms^{-1} and 3.16 mM^{-1} ms^{-1}, in (b) 0.07 ms^{-1} and 0.44 mM^{-1} ms^{-1}. Insets: reciprocal of the (a) open time constant τ_{o_2} and (b) open time per burst τ_{ob}; lines are determined by a linear regression.

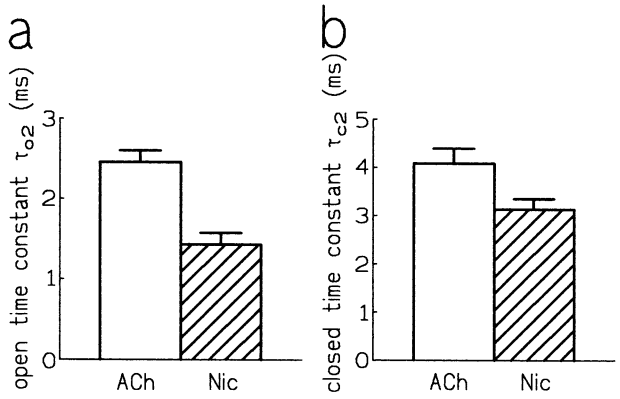


Fig. 7. HC effects depend on the nature of the agonist used to activate the receptor. Comparison of (a) the mean open time constant $\tau_{o_2} \pm$ S.E. and (b) the mean closed time constant $\tau_{c_2} \pm$ S.E. measured from cell-attached patches in the presence of 100 μM HC, when receptors are activated either by ACh (50 nM, $n=8$) or Nic (1–2 μM, $n=8$). Pipette potential was +60 mV.

To test for possible interactions between the hormone and the agonist molecule at the binding site, we performed experiments in which ACh was replaced by nicotine (Nic, 1–2 μM). When HC was absent ($n=8$), we observed channel openings with an open time τ_{o_2} of about 6.05 ± 0.23 ms, and with only two components in the closed time distribution. HC added to the pipette solution induced a burst activity and decreased the long open time τ_{o2}, as in the presence of ACh. However, in comparison to experiments with ACh and HC ($n=8$), the open time constant τ_{o_2} decreased to 1.43 ± 0.12 ms ($P<0.0001$, Fig. 7) and the closed time constant τ_{c_2} decreased to 3.14 ± 0.22 ms (unpaired test $P=0.018$, paired test $P=0.06$, Fig. 7). The current amplitudes in experiments using either Nic or ACh on the same cells, were similar, thus suggesting that in such conditions, also the real membrane potential (influencing the τ_{c_2}) was similar.

4. Discussion

Our experiments show that HC (100–400 μM) is a potent modulator of the AChR channel kinetics. HC induces a new component in the closed time distribution (Table 1), and reduces the time constant τ_{o_2} of the long open events (Fig. 2). Some HC effects are voltage-dependent: increasing the pipette potential, we observed a decrease of the time constant of the newly appeared component in the closed time distribution. Such voltage dependency has not been reported before. Moreover, HC eliminates voltage dependency of the receptor open time: in the presence of HC, contrary to experiments in control conditions, the time duration of the open events did not vary with pipette potential changes. This only partially agrees with previous results [9], in which a weak voltage dependency of the open time in the presence of HC was reported.

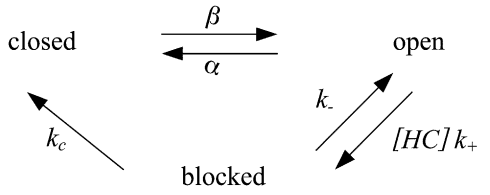
In our experiments, we have seen that a voltage-dependent modulation (i) can be present when an uncharged molecule like HC, is used, and (ii) that such effects are seen also when the place of its action is restricted to the external surface of the membrane, using HC–BSA. This suggests that HC does not bind to the transmembrane part of the receptor (contrary to what was previously proposed [9]), but acts on its extracellular part. Moreover, the voltage-dependent decrease of the blocked time suggests that the membrane potential affects the structure of such extracellular site of HC action.

A similar extracellular site of action was already proposed for progesterone on the neuronal type of nicotinic AChRs [4], for dexamethasone on chromaffin cells [16] and for corticosterone on C2C12 cells [13].

We show also that HC effects do not depend on agonist concentration, while they depend on the nature of the agonist molecule. When ACh is replaced by nicotine, the duration of open and blocked events is reduced (see Fig. 7). We explain this in terms of close localisation of HC and agonist binding sites.

The nature of action of HC seems to be complex. In fact, we have seen that HC decreases the number of brief openings, thus suggesting that HC is a noncompetitive blocker. On the other hand, the observation that HC cause a decrease of the open time per burst suggests that HC does not act as a classical open channel blocker (the so-called 'total open time per burst paradox' [17]).

When analysed in the terms of del Castillo and Katz's model [18], such decrease in the open time per burst suggests that the receptor undergoes the transition from the blocked to its closed conformation according to the blocking scheme:



(2)

without reentering the open state with the rate $k_c=31\ s^{-1}$. Kinetics rates in the above model calculated for 60 mV of pipette potentials are: $\alpha=110\ s^{-1}$, $[HC]k_+=3.16\times10^6\ M^{-1}\ s^{-1}$, $k_-=192\ s^{-1}$. This model is the simplest following from the del Castillo and Katz's model, explaining the decrease in the open time per burst. However, further analysis on mutated receptors is needed to be able to confirm or not this model.

Concluding, we proposed that the closing of the channel can occur independently of whether or not the blocker, a HC molecule, is bound. We demonstrated that HC acts on the extracellular part of the receptor possibly near the agonist binding site. HC could modulate the channel conformation by allosteric mechanism. We suggest that membrane potential directly or indirectly affects the HC binding site. It could be that charged residues in the transmembrane region cause

long-range distortions in the protein structure at HC binding site.

Acknowledgements

We wish to thank Drs. A. Constanti, F. Grassi and P. Lorenzon for critical reading of a preliminary version of the manuscript, and Prof. R. Miledi for the final revision. We also thank Dr. A. Bernareggi for her help in cell cultures. The financial support of Telethon-Italy (grant 785 to F.R.) is gratefully acknowledged. This work was also supported by grants from MURST-Italy and University of Trieste to F.R., and from SGGW to E.N.

References

[1] F.S. Wu, T.T. Gibbs, D.H. Farb, Inverse modulation of γ-aminobutyric and glycine-induced currents by progesterone, Mol. Pharmacol. 40 (1990) 333–336.
[2] L. Ke, R.J. Lukas, Effects of steroid exposure on ligand binding and functional activities of diverse nicotinic acetylcholine receptor subtypes, J. Neurochem. 67 (1996) 1100–1112.
[3] D.J. Calvo, R. Miledi, Antagonism of human homomeric $GABA_{\rho 1}$ receptors by steroids, Soc. Neurosci. Abstr. 23 (1997) 952abs. 378.1.
[4] S. Valera, M. Ballivet, D. Bertrand, Progesterone modulates a neuronal nicotinic acetylcholine receptor, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 9949–9953.
[5] S.D. Erulkar, D.M. Wetzel, 5α-dihydrotestosterone has nonspecific effects on membrane channels and possible genomic effects on ACh-activated channels, J. Neurophysiol. 61 (1989) 1036–1052.
[6] R.M. Woodward, L. Polenzani, R. Miledi, Effects of steroids on gamma-aminobutyric acid receptors expressed in *Xenopus* oocytes by poly(A)+RNA from mammalian brain and retina, Mol. Pharmacol. 41 (1992) 89–103.
[7] M.D. Majewska, Neurosteroids: endogenous bimodal modulators of GABAa receptor: mechanism of action and physiological significance, Prog. Neurobiol. 38 (1992) 379–395.
[8] C. Bouzat, F.J. Barrantes, Hydrocortisone and 11-desoxycortisone modify acetylcholine receptor channel gating, NeuroReport 4 (1993) 143–146.
[9] C. Bouzat, F.J. Barrantes, Modulation of muscle acetylcholine receptors by the glucocorticoid hydrocortisone, J. Biol. Chem. 271 (1996) 25835–25841.
[10] J.W. Karpen, G.P. Hess, Cocaine, phencyclidine, and procaine inhibition of the acetylcholine receptor: characterization of the binding site by stopped-flow measurements of receptor-controlled ion flux in membrane vesicles, Biochemistry 25 (1986) 1777–1785.
[11] H.R. Arias, Topology of ligand binding sites on the nicotinic acetylcholine receptor, Brain Res. Rev. 25 (1997) 133–191.
[12] E. Neher, J.H. Steinbach, Local anaesthetics transiently block currents through single acetylcholine-receptor channels, J. Physiol. 277 (1978) 153–176.
[13] E. Nurowska, F. Ruzzier, Corticosterone modifies the murine acetylcholine receptor channel kinetics, NeuroReport 8 (1996) 77–80.
[14] E. Nurowska, F. Ruzzier, Voltage dependent effects of hydrocortisone on acetylcholine receptor channel kinetics in C2C12 myotubes, Pflügers Arch. 439 (2000) R262.
[15] K.L. Magleby, B.S. Pallotta, Burst kinetics of single calcium-activated potassium channels in cultured rat muscle, J. Physiol. 344 (1983) 605–623.
[16] M. Inoue, H. Kuriyama, Glucocorticoids inhibit acetylcholine-induced currents in chromaffin cells, Am. J. Physiol. 257 (1989) C906–C912.
[17] D. Colquhoun, A.G. Hawkes, The principles of the stochastic interpretation of ion-channel mechanism, in: B. Sakmann, E. Neher (Eds.), Single-Channel Recordings, Plenum, New York, 1995, pp. 397–482.
[18] J. del Castillo, B. Katz, Interaction at endplate receptors between different choline derivatives, Proc. R. Soc. Lond., B 149 (1957) 369–381.