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Progesterone modulates a neuronal nicotinic acetylcholine receptor

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ABSTRACT **The major brain nicotinic acetylcholine receptor is assembled from two subunits termed α4 and nα1. When expressed in *Xenopus* oocytes, these subunits reconstitute a functional acetylcholine receptor that is inhibited by progesterone levels similar to those found in serum. In this report, we show that the steroid interacts with a site located on the extracellular part of the protein, thus confirming that inhibition by progesterone is not due to a nonspecific perturbation of the membrane bilayer or to the activation of second messengers. Because inhibition by progesterone does not require the presence of agonist, is voltage-independent, and does not alter receptor desensitization, we conclude that the steroid is not an open channel blocker. In addition, we show that progesterone is not a competitive inhibitor but may interact with the acetylcholine binding site and that its effect is independent of the ionic permeability of the receptor.**

Steroid hormones are synthesized from cholesterol in the adrenal gland (the gluco- and mineralocorticoids) and in the gonads and placenta (androgens and estrogens). Their lipophilicity explains their passage of the blood–brain barrier. Furthermore, neurosteroids are synthesized by oligodendrocytes and released at concentrations of up to 0.1 μM (1). Steroids have also been demonstrated to reduce brain activity and alphaxalone is used clinically. The potency of anesthetics has been correlated with their liposolubility (2, 3), yet the molecular basis of these effects remains obscure.

Some steroids, including 5α-pregnan-3α-hydroxy-20-one, enhance chloride fluxes at the γ-aminobutyric acid (GABA) synapses of the type A GABA ($GABA_A$) receptors (4, 5) and in transfected cells expressing $GABA_A$ receptors (6). In addition, progesterone attenuates cation fluxes evoked by excitatory amino acids in cerebellar Purkinje cells (7) and current induced by acetylcholine (AcCho) in chromaffin cells (8) and in reconstituted brain nicotinic AcCho receptor (nAcChoR) (9). In contrast, pregnenolone inhibits $GABA_A$ receptors in rat cortex neurons (10) and a progesterone-induced reduction of glycine-evoked current has been reported for spinal cord neurons (11).

The aim of this work is to investigate the mode of action of progesterone on the major brain neuronal nAcChoR α4/nα1 reconstituted in *Xenopus* oocytes.

MATERIALS AND METHODS

Oocyte preparation and recording procedures were as described (12). Data acquisition, storage, and analysis were done on an IBM-PC/AT using the software DATAC (13). Inhibition curves were fitted to the empirical Hill equation. Inhibition by progesterone as a function of the AcCho concentration was fitted by using a Michaelis equation in the form:

$$y = (1 - a) + a/[(1 + x)/IC_{50}],$$

where y is the normalized current, a is a constant, x is the AcCho concentration, and IC_{50} is apparent inhibition constant. All steroids were purchased from Sigma. Stock solutions of steroids and progesterone-3-(*O*-carboxymethyl)oxime (P-3; Sigma P-3277) were at 0.01 M, in ethanol. Stock solutions were kept at −20°C and diluted in OR-2 (12) just before use. P-3-conjugated bovine serum albumin (P-3-BSA; Sigma P-4778, progesterone/BSA ratio = 38:1) and 11α-hydroxyprogesterone hemisuccinate (P-11)-conjugated BSA (P-11-BSA; Sigma H-4508, progesterone/BSA ratio = 19:1) were diluted directly in OR-2.

RESULTS

We reported (9) that neuronal nAcChoRs are inhibited by low concentrations of progesterone. This effect is fast and reversible upon removal of the steroid. Inhibition of AcCho responses depended upon steroid type and was not mediated by intracellular pathways. Thus it was proposed that steroids are acting directly on the AcChoRs protein. The experiments presented below attempt to discriminate between the various mechanisms shown in Fig. 1.

Steroid Selectivity of the α4/nα1 Receptor. We determined the sensitivity of reconstituted α4/nα1 receptors to cholesterol, progesterone, testosterone, and pregnenolone (Fig. 2*A*). Steroids were assayed using a combined application of AcCho and the drug (Fig. 2*B*). To avoid obscuring the inhibitory effects of steroids, care was taken to use low AcCho concentrations that produce no detectable desensitization. Inhibition by progesterone was tested on 15 batches of oocytes. Fig. 2*A* shows that the α4/nα1 receptor is sensitive to certain steroids (progesterone and testosterone) and unaffected by others (cholesterol and pregnenolone). Furthermore, inhibition by progesterone was not affected by the concomitant application of cholesterol (20 μM, 5 cells; data not shown). Pregnenolone, which induces oocyte maturation in the range 10 nM–1 μM (16), produces no effect on the AcCho responses (Fig. 2*A*). These results, and particularly the lack of action of cholesterol at concentrations as high as 20 μM, suggest that steroids inhibiting the AcChoR bind to a specific site or sites on the α4 or nα1 receptors. To verify that progesterone acts via the extracellular compartment, we used progesterone coupled to BSA, a water soluble compound that does not partition into the plasma membrane. Two forms of derivatized progesterone, P-3 and the corresponding BSA conjugate P-3-BSA, inhibited the currents evoked by AcCho (Fig. 2*B*). Another form of coupled progesterone, P-11-BSA, gave similar results. The P-3-BSA inhibition curve obtained on two batches of oocytes yields an IC_{50} value of 2.9 μM (Fig. 2*C*). BSA alone (10 cells) had no detectable effects

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Abbreviations: AcCho, acetylcholine; nAcChoR, nicotinic acetylcholine receptor; BSA, bovine serum albumin; OCB, open channel blocker; P-3, progesterone-3-(*O*-carboxymethyl)oxime; P-11, 11α-hydroxyprogesterone *n*-hemisuccinate; DHβE, dihydro-β-erythroidine.

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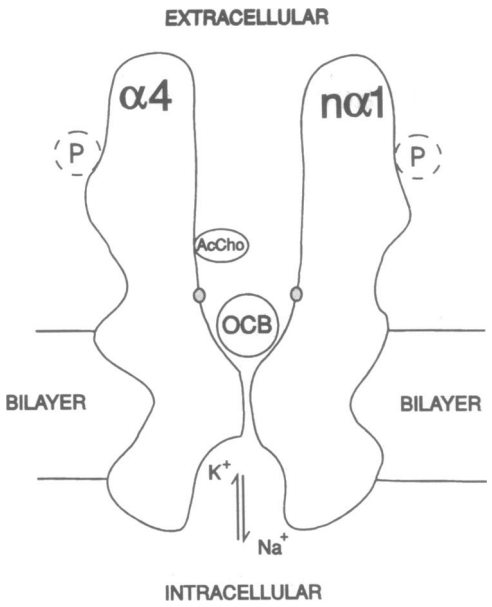

FIG. 1. Representation of the nicotinic $\alpha 4/n\alpha 1$ receptor and possible sites of action of progesterone, based on the model derived from the extensive structural data obtained from the *Torpedo* electroplax receptor (14, 15). AcCho binding site, the OCB site, and other possible sites of progesterone binding (P) are indicated. Shaded areas indicate the point mutations.

on the AcCho-evoked currents when applied before or during the AcCho test pulse. Ethanol, the solvent for steroid stock solutions, did not affect AcCho responses when added at up to 1% in OR-2, a concentration roughly twice as high as that resulting from the most concentrated steroid applications.

Is Inhibition by Steroid Caused by an Open-Channel Block? Progesterone could act as an open channel blocker (OCB) by entering the ion pore and occluding the channel, as demonstrated for hexamethonium or the local anaesthetic QX-222 (17, 18). In general, OCBs are strongly voltage-dependent, due to the charge they carry in the transmembrane field. To determine the influence of the membrane potential on the progesterone-induced inhibition of AcCho-evoked currents, the current–voltage relationship of responses to AcCho was recorded first in control conditions and then in presence of progesterone (Fig. 3*B*). As the conductance of both sets of data can be fitted by Boltzmann equations differing only by a scaling factor (Fig. 3*A*), these results demonstrate that progesterone inhibition of AcCho-evoked currents is voltage-independent. In addition OCBs lack effectiveness when applied before the agonist challenge, as demonstrated for hexamethonium on reconstituted $\alpha 4/n\alpha 1$ receptors (19). This property results from the difference in channel conformation in the absence or presence of agonist. As progesterone is an effective inhibitor of AcCho responses when applied in prepulses (Fig. 4*B*, trace d), we conclude that the steroid is not an OCB.

Inhibition by Steroid Is Not Competitive but Increases with AcCho Concentration. Inhibition by progesterone appears strongly dependent upon agonist concentration (9). To determine whether this reflects competition at the AcCho binding site, a protection experiment was done using the competitive inhibitor dihydro-β-erythroidine (DHβE). As demonstrated by the shift of the agonist dose–response curve determined in absence or presence of a fixed concentration of DHβE, this compound behaves as a typical competitive inhibitor (Fig. 4*A*). If progesterone and AcCho bind to the same site, simultaneous prepulse application of DHβE should compete with progesterone and block its effect. Prepulses of progesterone mixed with a DHβE concentration that abolishes AcCho-evoked currents (5 μM) resulted in the same degree of inhibition as progesterone alone (Fig. 4*B*).

However, measurements of currents evoked by several AcCho concentrations first in control conditions and then in presence of 2 μM and 5 μM of progesterone (Fig. 5*A*) demonstrate that inhibition by steroid increases with increasing agonist concentration (Fig. 5*B*).

Desensitization of $\alpha 4/n\alpha 1$ Receptor Is Not Modified by Progesterone. Reconstituted $\alpha 4/n\alpha 1$ receptor desensitizes noticeably during sustained agonist application. Effects of progesterone on desensitization were tested by progesterone application (*i*) during agonist challenge and (*ii*) as a 10-s prepulse followed by coapplication with AcCho (Fig. 6). Desensitization of the AcCho-evoked current is fitted by a dual exponential process (20). The ratios of the exponential time constants obtained during the progesterone tests and in the control conditions were computed to quantify a possible variation of desensitization induced by the steroid. For the steady-state conditions (protocol ii, Fig. 6, trace c, six cells from two ovaries), ratios of 1 ± 0.25 and 1.5 ± 1 were computed for the fast and slow time constants, respectively. The ratios obtained for concomitant AcCho and progesterone (Fig. 6, trace b, four cells, one batch) were 0.84 ± 0.31 and 1.3 ± 0.78, respectively. These results indicate that progesterone does not significantly affect the desensitization properties of the $\alpha 4/n\alpha 1$ receptor.

Inhibition by Steroid Is Not Affected by Point Mutations at the Channel Outer Mouth. Mutation of Glu-266 → Lys (E266K) of the $\alpha 4$ subunit and of Lys-260 → Glu (K260E) of the $n\alpha 1$ subunit has been shown to affect the single-channel conductance of reconstituted $\alpha 4/n\alpha 1$ receptors (21), as the corresponding mutations do in endplate receptors (22). By assuming that the structure of the $\alpha 4/n\alpha 1$ receptor closely resembles the structures of electroplax and muscle nAcChoRs, it follows that mutations E266K on $\alpha 4$ and K260E on $n\alpha 1$ must lie close to the AcCho binding site. To investigate whether charge differences or changes in channel permeability can affect the steroid-induced inhibition of AcCho-evoked currents, dose–response inhibition experiments were performed with these mutants. We found that progesterone had essentially the same effect on the mutated receptors $\alpha 4/n\alpha 1$ K260E and $\alpha 4$ E266K/$n\alpha 1$ K260E and on the wild-type $\alpha 4/n\alpha 1$ receptor (data not shown).

DISCUSSION

The steroid-induced inhibition of neuronal nAcChoRs reconstituted in *Xenopus* oocytes was examined by voltage clamp. All the active steroids tested inhibited the $\alpha 4/n\alpha 1$ receptor in a dose-dependent manner and the inhibition curves were described by the empirical Hill equation (Fig. 2). AcCho-evoked currents were reduced by progesterone, testosterone, P-3, P-3-BSA, P-11-BSA, and dexamethasone (9) at concentrations comparable to those measured in rat plasma (6–20 μM, ref. 23) but were unaffected by cholesterol, pregnenolone, and 5α-pregnan-3α-hydroxy-20-one (9). It is known that progesterone, testosterone, and pregnenolone can induce maturation of *Xenopus* oocytes *in vitro* (16). In our experiments, pregnenolone does not affect AcCho currents, thereby ruling out a possible involvement of the early events of meiotic reinitiation. Inhibition by progesterone thus seems independent of second messenger activation, in agreement with patch-clamp experiments (9) and, therefore, the known early effects of progesterone on adenylate cyclase (24, 25) and intracellular Ca^{2+} concentration (26) appear not to be implicated in AcChoR modulation. The water-soluble coupled progesterones P-3-BSA and P-11-BSA are effective in reducing the AcCho-evoked currents. Since the partition of these compounds in the plasma membrane is unlikely to occur, this result proves that progesterone acts via the

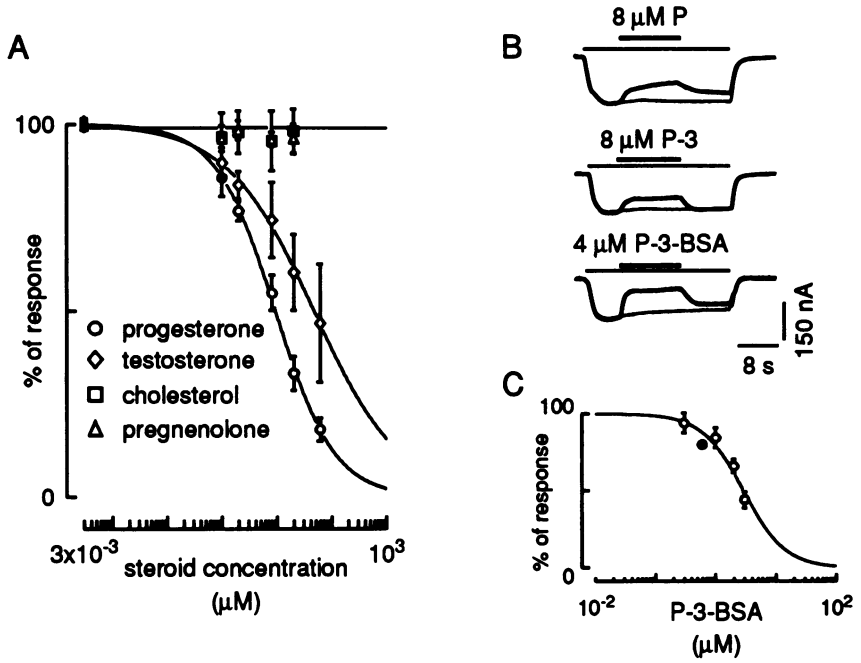

FIG. 2. Inhibition of AcCho-evoked currents is steroid-specific. (*A*) Steroid dose–response inhibition of nondesensitizing AcCho currents. Cells were held at −100 mV and currents were measured using the test protocol illustrated in *B* (AcCho = 50 nM). Normalized values obtained for cholesterol (□; 8 cells, three ovaries), pregnenolone (△; 5 cells, two ovaries), progesterone (○; 8 cells, two ovaries), and testosterone (◇; 10 cells, two ovaries) are superimposed. Progesterone and testosterone inhibitions were fitted with the empirical Hill equation with IC_{50} = 9 μM (n = 0.81) and IC_{50} = 46 μM (n = 0.55), respectively. (*B*) AcCho-evoked currents are inhibited by progesterone, P-3 (7 cells, two ovaries), and P-3-BSA (5 cells, two ovaries). Cells were held at −100 mV and a test pulse of steroid or conjugate was applied during exposure to 50 nM AcCho. (*C*) Dose–response inhibition curve of P-3-BSA. Inhibition was determined as in *A*. Measurements were obtained from two oocyte batches and from 3 to 5 cells for each point. Line is the best fit obtained with the Hill equation, IC_{50} = 2.9 μM (n = 1.3).

extracellular compartment. It has been demonstrated that 5–10 cholesterol molecules must be bound to the receptor proteins to form functional nAcChoR (27, 28). Moreover, the action of the general anaesthetic halothane is inversely correlated to the cholesterol concentration around nAcChoRs (29). This mode of action of cholesterol has been attributed to an alteration of the lipid composition of the plasma membrane surrounding the nAcChoRs. The lack of action of cholesterol on reconstituted α4/nα1 receptors indicates that progesterone inhibition is unlikely to occur via a membrane

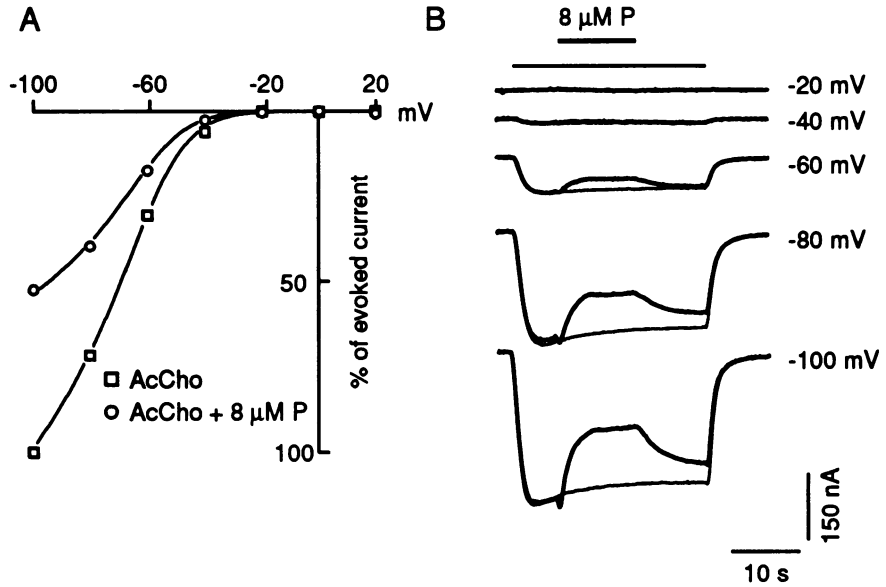

FIG. 3. Inhibition by progesterone is voltage-independent. (*A*) Currents evoked by a constant AcCho application (as in *B*) were measured at different potentials and plotted as a function of holding potential (□, mean of three cells). Currents were normalized with respect to the value recorded at −100 mV, in control conditions. Currents measured at the end of the steroid test pulse [8 μM progesterone (P)] were plotted on the same graph (○). Conductances from both sets of experiments were fitted using Boltzmann's equation, with a = 0.1 and b = −59 mV. The current–voltage relationship was reconstructed by multiplying the conductance by the driving force, assuming a reversal potential of −5 mV. The two curves differ by a scaling factor of 0.5. (*B*) Steroid-induced inhibition as a function of holding potential. A test pulse of 8 μM progesterone (P) was applied during a steady exposure to 50 nM AcCho (thick traces). The thin traces were obtained during the same AcCho exposure without steroid test pulse. The small spike observed at the most hyperpolarized potentials is an artefact produced by the valves.

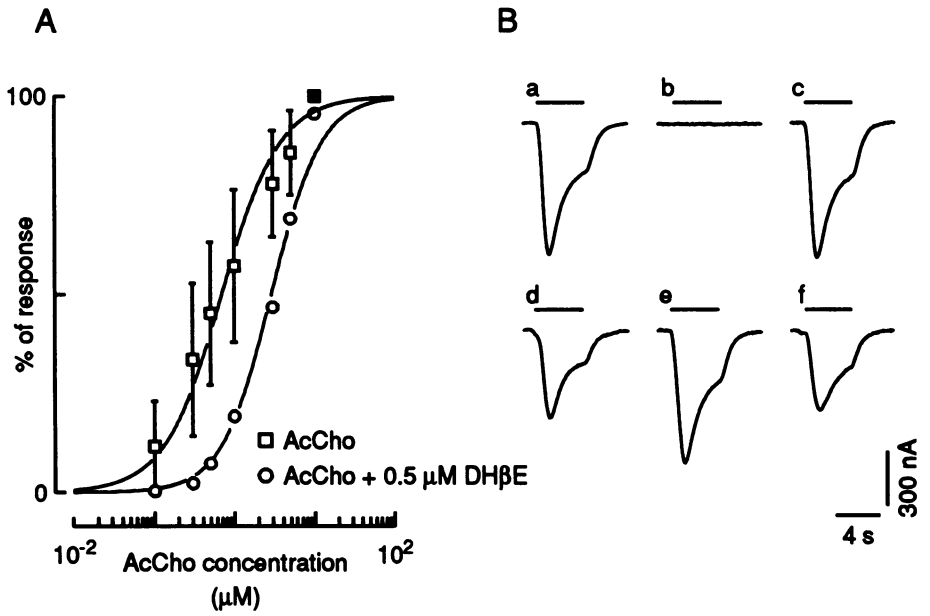

FIG. 4. Progesterone-induced inhibition is not masked by the competitive inhibitor DHβE. (*A*) DHβE is a competitive inhibitor of the neuronal α4/nα1 receptor. The AcCho dose–response curve obtained in control conditions (□, mean of eight cells) is compared to the dose–response curve obtained in the presence of 0.5 μM DHβE (○, mean of three cells). Curves are best fits obtained with the empirical Hill equation with EC_{50} = 0.7 μM (n = 1.2; for AcCho) and EC_{50} = 2.9 μM (n = 1.4; for AcCho plus DHβE). (*B*) DHβE does not protect from inhibition by steroid. Currents evoked by 0.5 μM AcCho (traces a and c) are reversibly abolished in the presence of 5 μM DHβE (trace b). When a 10-s prepulse of 40 μM progesterone and a 15-s wash with control solution are applied before the AcCho test pulse, the evoked currents are strongly reduced (trace d). Inhibition by steroid is fully reversible within 1 min (trace e). Exposure to a constant 5 μM DHβE during steroid application does not prevent the inhibition induced by the steroid pulse (trace f).

perturbation but rather reflects a direct action on the receptor.

Several experiments were attempted to determine whether steroids act as OCBs on the neuronal receptor. For OCBs, the presence of agonist is required to let the substance enter the channel after the receptor has undergone an agonist-induced conformational change (19, 30, 31). In contrast to OCBs, progesterone is equally effective when applied in prepulse or during agonist exposure (9), indicating that it can interact with the nAcChoR in its closed state. Moreover, most OCBs are sensitive to the transmembrane electrical field, yet inhibition by progesterone is voltage-insensitive (Fig. 3), which can be attributed either to the uncharged nature of steroids or to their mode of action. OCBs have been

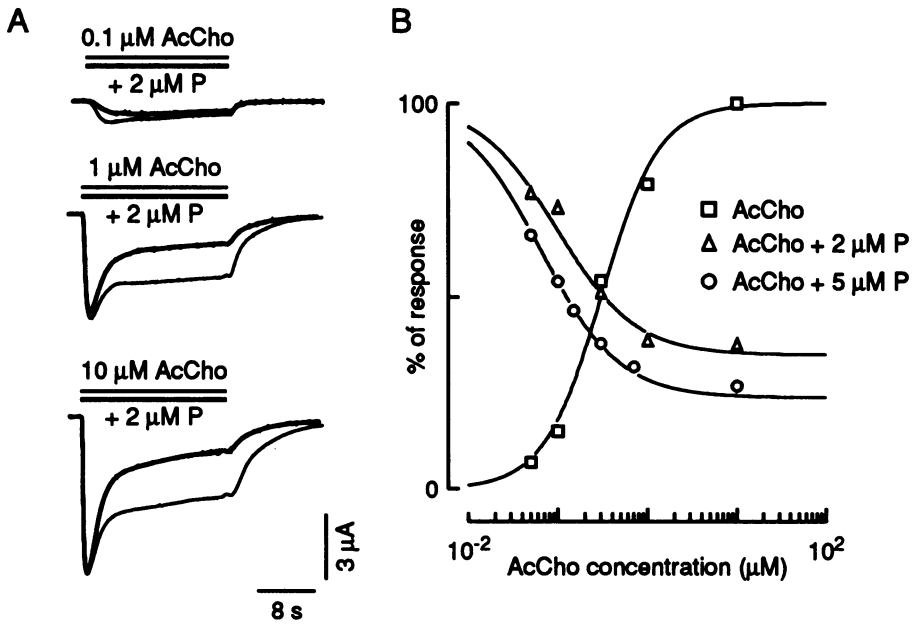

FIG. 5. Inhibition by progesterone depends upon the AcCho concentration. (*A*) Recordings evoked by three AcCho concentrations (0.1, 1, and 10 μM) are shown (thin traces). Applications of the same AcCho concentrations mixed with 2 μM progesterone (P) evoke identical peak currents but lower plateau currents (thick traces). (*B*) Steroid inhibition plotted as a function of the AcCho concentration. Percent inhibition obtained for 2 μM (△, 3 cells) and 5 μM progesterone (P; ○, 5–13 cells, two ovaries) are plotted as a function of the logarithm of the AcCho concentration. Lines are best fit obtained with a Michaelis equation (1) with IC_{50} = 0.065 μM, a = 0.76 for 5 μM progesterone and IC_{50} = 0.098 μM and a = 0.65 for 2 μM progesterone. Plateau currents of the AcCho dose–response curve from the same oocytes, obtained in control conditions, are indicated for comparison (□, n = 3). Data were fitted with the Hill equation, EC_{50} = 0.3 μM (n = 1.35).

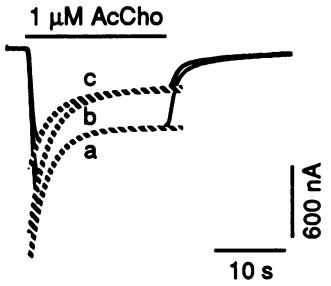

FIG. 6. Inhibition by progesterone and desensitization are independent processes. Currents evoked by test pulses with 1 μM AcCho were recorded at a holding potential of −100 mV. Trace a was obtained in control conditions. In trace b, 8 μM progesterone was added simultaneously with the AcCho test pulse. Trace c was obtained as in trace b but after progesterone prepulse application (10 s, 8 μM). Dashed lines are the best fits obtained with two exponentials (see text).

shown to alter the decay of the time course of muscle nAcChoR responses (32). Determination of the response decay time constant in the absence or presence of a steady-state concentration of progesterone (Fig. 6, traces a and c) revealed that inhibition by progesterone does not modify the decay time course. The slightly faster decay observed during the concomitant application of AcCho and progesterone cannot be attributed either to an increase in desensitization or to an open channel block but rather to a progressive block induced by progesterone that develops during the application. Comparison of the progesterone inhibition curves with the AcCho activation profile shows that inhibition is already near maximal at the EC_{50} for AcCho. This indicates that there is no correlation between the probability of channel opening of the α4/nα1 receptor and the amount of inhibition by steroid, another indication that steroids are unlikely to act as OCBs.

To determine the nature of the interaction between progesterone and receptor, we designed protection experiments using the competitive inhibitor DHβE. Since progesterone inhibition could not be masked by DHβE, we concluded that the sites recognized by competitive inhibitors and modulating steroids must differ.

The complex nature of progesterone action is also demonstrated by the unexpected dependence of inhibition upon the AcCho concentration (Fig. 5*B*). This interaction is reminiscent of the allosteric interaction observed between AcCho and barbiturate in *Torpedo* electroplax nAcChoR (33).

Point mutations (α4 E266K and nα1 K260E) that modify α4/nα1 channel permeability did not affect progesterone sensitivity, thereby indicating that inhibition by progesterone is not directly related to channel permeability.

In some respects, the effect of progesterone on nAcChoRs resembles the block induced by histrionicotoxin, meproadifen, or tricyclic antidepressants, which are voltage-insensitive, effective when applied in prepulse, and enhanced by increases in agonist concentration (34). In conclusion, we postulate that inhibition by progesterone results from a negative heterotopic interaction between the AcCho binding site and a specific progesterone binding site.

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