

# Activation and blocking of neuronal nicotinic acetylcholine receptor reconstituted in *Xenopus* oocytes

(cDNA/channel/neurotransmitter/pharmacology/electrophysiology)

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Communicated by Gerald D. Fischbach, December 26, 1989

**ABSTRACT** Neuronal nicotinic acetylcholine receptor of the  $\alpha 4$ /non- $\alpha$  ( $\alpha 4/\alpha$ ) type was reconstituted in *Xenopus* oocytes after nuclear injection of cDNA expression vectors. Functional neuronal receptor was only formed when the two subunits  $\alpha 4$  and  $\alpha$  were coinjected, neither  $\alpha 4$  nor  $\alpha$  alone being effective. Responses to bath application of acetylcholine (AcCho) have been measured in voltage clamp. AcCho doses as low as 10 nM induce currents of up to 50 nA. Dose–response studies indicate a  $K_d$  of about  $0.77 \times 10^{-6}$  M and a Hill coefficient of 1.5, thus predicting more than one AcCho binding site per receptor molecule. The current–voltage relationship of AcCho-induced currents presents a strong inward rectification. Responses to AcCho were compared to those of three other agonists: L-nicotine, carbachol, and 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP). Sensitivities to AcCho, nicotine, and DMPP are quite similar. Sensitivity to carbachol is much lower, but the currents are otherwise indistinguishable from those induced by AcCho. Five AcCho antagonists—neuronal bungarotoxin ( $\kappa$ -bungarotoxin), tubocurarine (TC), hexamethonium bromide (Hex), decamethonium bromide (Dec), and mecamlamine (Mec)—have been tested. Neuronal bungarotoxin has no effect on the  $\alpha 4/\alpha$  channel, whereas 2.5  $\mu$ M TC reduces by half the current peak evoked by 1  $\mu$ M AcCho. The block by TC is independent of membrane voltage. By contrast, the block of AcCho-induced currents by Hex or Dec is strongly voltage dependent, suggesting that these substances enter the channel. The block by Mec is detectable at concentrations as low as 100 nM when applied together with 1  $\mu$ M AcCho and is voltage independent. Hex, Dec, and Mec are effective only when AcCho is present. While the effects of all other agents are fully reversible, the Mec block is persistent.

The nicotinic acetylcholine (AcCho) receptor (nAcChoR) mediating synaptic transmission at the neuromuscular junction of vertebrates has long been a favored model system in the study of ligand-gated ion channels. Because of the efforts of many, much is now known about its biochemistry, electrophysiology, and molecular biology (1). In contrast, little is known about the different types of neuronal nAcChoRs that recently have been identified in the central and peripheral nervous system by using antibody and cDNA probes derived from muscle receptor (2–8). At least six neuronal genes have been cloned, sequenced, and found to encode proteins homologous to the four related subunits assembled in the mature muscle receptor (2, 4–8). As functional nAcChoRs can be reconstituted in *Xenopus* oocytes by cytoplasmic injection of complementary RNA (2, 7, 8) or by nuclear injection of cDNA expression vectors (9), it is now possible to characterize the physiology and pharmacology of the numerous receptor types resulting from the assembly of cloned subunits. Such studies have revealed that functional

neuronal nAcChoRs assemble from two subunits only (an  $\alpha$  subunit and a non- $\alpha$ -subunit in our nomenclature), whereas four different subunits make up muscle receptor (1). Meanwhile, it was demonstrated biochemically (3) that the most abundant nAcChoR in chicken brain is indeed assembled from two subunits,  $\alpha 4$  and non- $\alpha$  ( $\alpha$ ) (5), and *in situ* hybridization has shown a widespread and complex pattern of differential expression of the various nAcChoR subunit mRNAs in neural tissue (8, 10, 11).

In this paper, we characterize some physiological and pharmacological properties of the avian nAcChoR composed of  $\alpha 4$  and  $\alpha$  subunits ( $\alpha 4/\alpha$  nAcChoR) expressed in *Xenopus* oocytes and compare them to those of other known nAcChoRs.

## MATERIALS AND METHODS

**DNA Expression Vectors and Injection into *Xenopus* Oocytes.** Preparation of oocytes and injection procedures are detailed elsewhere (12). Briefly, female *Xenopus laevis* were sacrificed, and pieces of ovary were dissected out. Oocytes were isolated by collagenase treatment [0.2% in OR-2 medium without  $\text{Ca}^{2+}$  (13)] and centrifuged at about  $400 \times g$ , and healthy stage 6 oocytes with a clear area indicating the position of the nucleus were selected under the binocular. Oocyte nuclei were injected with about 10 nl of injection buffer (88 mM NaCl/1 mM KCl/15 mM Hepes, pH 7) containing 1 ng each of  $\alpha 4$  and  $\alpha$  cDNA plasmid constructs. In these experiments, cDNAs were under the control of the simian virus 40 (SV40) early promoter because the heat treatment needed to induce transcription of heat shock-promoted expression vectors (9) resulted in high mortality of oocytes in some batches, particularly during the summer months. Plasmids p17neo- $\alpha 4$  and p17neo- $\alpha$  (9) were digested with restriction enzymes *Hind*III and *Sma* I, and the appropriate fragments were purified and ligated to place the  $\alpha 4$  and  $\alpha$  cDNAs in the correct orientation downstream of the SV40 promoter, which, in the original plasmids, controlled the neomycin-resistance gene. The resulting plasmids are flip  $\alpha 4$  and flip  $\alpha$ . We obtained high levels of expression and good oocyte survival over several days when oocytes were injected with 1–2 ng each of these constructs.

After injection, oocytes were kept at 19°C in complete OR-2 medium (13) supplemented with 100 units of penicillin, 100  $\mu$ g of streptomycin, and 20  $\mu$ g of kanamycin per ml. Each oocyte was kept in a separate well (8  $\times$  12 clusters; Nunc), and medium was changed daily.

Abbreviations: AcCho, acetylcholine; AcChoR, AcCho receptor; nAcChoR, nicotinic AcChoR; BTX, bungarotoxin;  $\alpha 4/\alpha$ ,  $\alpha 4$ /non- $\alpha$  subunits; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; TC, tubocurarine; Hex, hexamethonium bromide; Dec, decamethonium bromide; Mec, mecamlamine.

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**Electrophysiology.** AcCho-induced currents were measured by using a conventional two-electrode voltage clamp (9). Electrodes were made on a BB-CH-PC (Mecanex, Geneva) puller with quick-fill pyrex glass. The current-injecting electrode was shielded by aluminum foil wrapped around the barrel.

Oocytes were superfused during the entire experiment with OR-2 solution at a rate of  $\approx 12$  ml/min at room temperature. All solutions contained  $0.5 \mu\text{M}$  atropine to block muscarinic receptors. Drugs were dissolved in perfusion medium. Switching between control and test solution was achieved manually with a homemade pinch-tube valve.

Data acquisition, storage, and analysis were done on an IBM-PC AT using the program DATAC (14). Results were plotted on a flat bed plotter (Hewlett-Packard type 7550A).

**Normalization and Fitting Procedure.** Dose-response curves were normalized on the largest induced current. Equation curves were fitted with DATAC by using the minimization algorithm SIMPLEX.

**Drugs.** Drugs used were AcChoCl (Fluka), L-nicotine (Sigma), atropine sulfate (Fluka), carbamylcholine chloride (carbachol; Fluka), hexamethonium bromide (Hex; Fluka), decamethonium bromide (Dec; Fluka), (+)-tubocurarine chloride pentahydrate (TC; Fluka), 1,1-dimethyl-4-phenylpiperazine iodide (DMPP; Sigma), mecamlamine (Mec; Sigma), and neuronal bungarotoxin (also referred to as bungarotoxin 3.1, toxin F, and  $\kappa$ -bungarotoxin). The drugs were dissolved in the test solution just before use.

## RESULTS

**AcCho-Evoked Currents.** About 90% of the oocytes coinjected with  $\alpha 4$  and  $\alpha \alpha$  cDNA expression vectors presented AcCho-induced currents within 24 hr (60 cells). Currents could be as large as  $25 \mu\text{A}$  when a saturating AcCho dose was applied on cells held at  $-100$  mV [mean  $2.6 \mu\text{A} \pm 2.8$  (SD),  $n = 60$ ; tested with  $0.5 \mu\text{M}$  AcCho]. On the other hand, we never observed any response to AcCho in oocytes injected with only one subunit cDNA, even when testing was 3 or more days after the injection (15 cells were tested with  $\alpha 4$  and 5 cells with  $\alpha \alpha$  subunit cDNA).

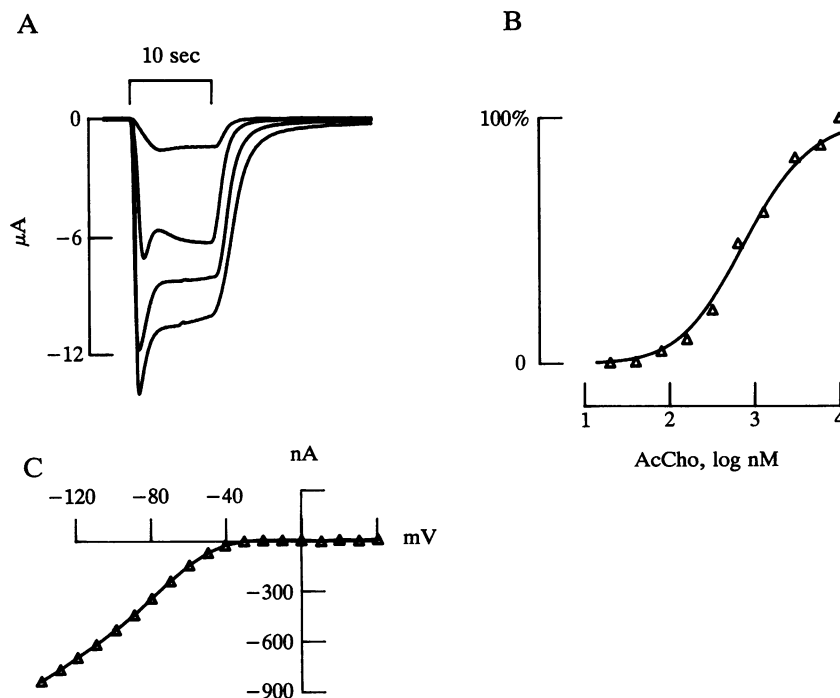
Currents evoked by four different AcCho concentrations are shown in Fig. 1A. The four superimposed recordings were

obtained successively from the same cell held at  $-100$  mV. AcCho was applied every minute for 10 sec. For low doses of AcCho, the induced current showed little desensitization. At high doses, the current first increased rapidly and then settled down to a plateau value. This decrease probably corresponds to a desensitization of the receptors (15, 16) or to a block induced by AcCho (17, 18). The relationship between a broad range of AcCho concentrations and the peak value of the evoked currents was recorded in one cell at a holding voltage of  $-100$  mV (Fig. 1B). The responses to 10 different concentrations were plotted on a semilogarithmic scale. A good fit was obtained by using the nonlinear Hill equation (continuous line in Fig. 1B), with a  $K_d$  of  $0.77 \times 10^{-6}$  M and a Hill coefficient of 1.5.

The current-voltage relationship was measured either by applying a constant AcCho dose at different voltages or by changing the holding voltage stepwise during a sustained response to a low concentration of AcCho (both methods yield exactly the same results, but the stepwise method is considerably faster). A typical current-voltage curve of the AcCho-induced responses is shown in Fig. 1C. We measured the current required for holding the cell at different voltages, first under control conditions and then during application of AcCho. The values plotted are the differences between these two measurements and represent the current flowing through the AcCho channels. The current-voltage relationship is roughly linear below  $-50$  mV but presents a strong rectification at more depolarized voltages. Since the current at all voltages above  $-40$  mV is close to zero, it was not possible to determine the reversal potential of these channels.

**Responses to Nicotine.** Application of nicotine evoked currents in all AcCho-responsive oocytes tested (see also ref. 9). In 82% of the cells studied (47 cells), currents induced by nicotine could not be distinguished from those evoked by AcCho (see Fig. 1A).

Fig. 2 shows the dose-response curves to AcCho (continuous line) and nicotine (triangles) over a broad concentration range. The peak of the response was expressed in percent of maximum as a function of the logarithm of agonist concentration. Since the two curves overlap, we conclude that the affinities of nicotine and AcCho for the  $\alpha 4/\alpha \alpha$  channel are approximately the same.



**FIG. 1.** AcCho-induced currents in *Xenopus* oocytes after nuclear injection with cDNAs derived from the neuronal  $\alpha 4$  and  $\alpha \alpha$  subunit genes. (A) Currents evoked by four different doses of AcCho ( $0.1$ ,  $0.3$ ,  $1$ , and  $5 \mu\text{M}$ ) were superimposed. The cell was clamped at  $-100$  mV, and AcCho was applied once every minute for about 10 sec. (B) Dose-response curve for AcCho in the range  $20 \text{ nM}$ – $4 \mu\text{M}$ . Triangles are the peaks of the AcCho-induced currents after normalization to the maximum value, plotted as a function of the logarithm of the AcCho concentration in nM. The cell was held at  $-100$  mV. The data are fitted to the nonlinear Hill equation  $y = 1/[1 + (K/A)^n]$ , in which  $K$  is the empirical dissociation constant ( $0.77 \times 10^{-6}$  M),  $A$  is the AcCho concentration, and  $n$  is the Hill coefficient (1.5). (C) Current-voltage relationship of AcCho-induced currents;  $0.5 \mu\text{M}$  AcCho was applied constantly, and the voltage was stepped up every 2 sec.

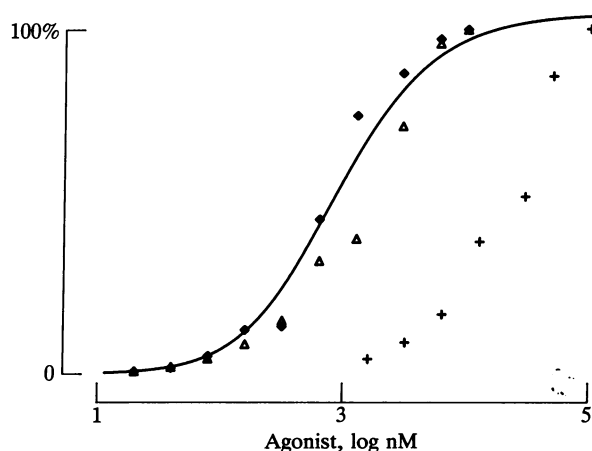


FIG. 2. Dose-response curves of  $\alpha 4/\alpha$  nAChR for four agonists. The cells were held at  $-100$  mV, and the current peaks were measured for different agonist concentrations. Data are normalized on the largest induced currents and are plotted as a function of the logarithm of the agonist concentration in nM. The continuous line is the dose-response for AcCho (same values in Fig. 1B).  $\blacklozenge$ , DMPP;  $\blacktriangle$ , L-nicotine; and  $+$ , carbachol.

**Currents Evoked by Carbachol and DMPP.** Carbachol induced responses in all AcCho-responding oocytes we tested (11 cells). These currents could not be distinguished by time course or saturating amplitude from those evoked by AcCho (Fig. 1A). However, sensitivity to carbachol is low, as shown in Fig. 2, which demonstrates that 25 times more carbachol than AcCho is needed to evoke half-saturating responses (4 cells).

DMPP, a rather specific agonist for ganglionic AcCho receptors, also induced inward currents in oocytes expressing the  $\alpha 4/\alpha$  channel. These currents were similar to those evoked by AcCho application. Moreover, receptor affinities for DMPP and AcCho appeared to be identical (Fig. 2).

**Lack of Response to Neuronal Bungarotoxin.** Neuronal bungarotoxin has been shown (2) to block rat  $\alpha 4/\beta 2$  channel ( $\beta 2$  subunit is the rat analogue of avian  $\alpha$ ) reconstituted in *Xenopus* oocytes. We found, however, that two different preparations of neuronal bungarotoxin failed to block the avian  $\alpha 4/\alpha$  receptor even when applied at 500 nM for 30 min (four cells). Stripped oocytes (three cells) also failed to

respond to neuronal bungarotoxin. The same toxin was subsequently tested for activity on quail ciliary ganglion neurons, where it rapidly blocked AcCho-induced currents.

**TC Block.** TC, a potent antagonist of AcCho-induced currents at the neuromuscular junction (19) and in several ganglia (20–22), also blocks the  $\alpha 4/\alpha$  receptor. Fig. 3A shows that its effect on AcCho-induced currents is fast and quickly reversible (within seconds). A block was observed only when TC and AcCho were applied together. TC alone did not affect the holding current or the following response to AcCho.

Fig. 3B shows the shift of the AcCho dose-response curve induced by  $0.5 \mu\text{M}$  TC. At the lowest AcCho concentrations, addition of TC decreased the amplitude of AcCho-induced currents, and the dose-response curve was shifted from 0.3 to  $0.6 \mu\text{M}$  at half maximal response. At high AcCho concentrations, however, the AcCho-induced currents were identical with or without the drug, indicating that TC is a competitive inhibitor.

In contrast to what has been observed in *Aplysia* (23), rat submandibular ganglion (20, 24), or at the frog neuromuscular junction (19, 25), the inhibition of the AcCho response induced by TC is not voltage dependent (even at membrane potentials as high as  $-140$  mV; Fig. 3C). In this respect, the  $\alpha 4/\alpha$  channel resembles the receptor in frog ganglia, which has also been shown to display little voltage sensitivity in its response to TC (21).

**Voltage-Dependent Block of  $\alpha 4/\alpha$  Channel by Methonium Salts.** Fig. 4A shows the action of Hex on AcCho-induced currents and its removal by inversion of the holding voltage in the presence of AcCho (we shall refer to this as “pop-out”). To quantify the block by Hex, we computed  $Z$ , the ratio of the current recorded at  $-100$  mV in the absence of antagonist, to the current recorded 1 min after application of the drug. When there is no inhibition,  $Z = 1$ , and  $Z - 1$  (an index of antagonistic effect) = 0. To measure the voltage dependence of Hex, we determined the index at different holding voltages. The holding voltage was stepped up to the test value a few seconds before application of Hex and stepped back to  $-100$  mV at the end of the AcCho pulse. Fig. 4B plots the value of  $Z - 1$  as a function of the holding voltage and clearly shows that the blocking action of Hex is markedly voltage dependent.

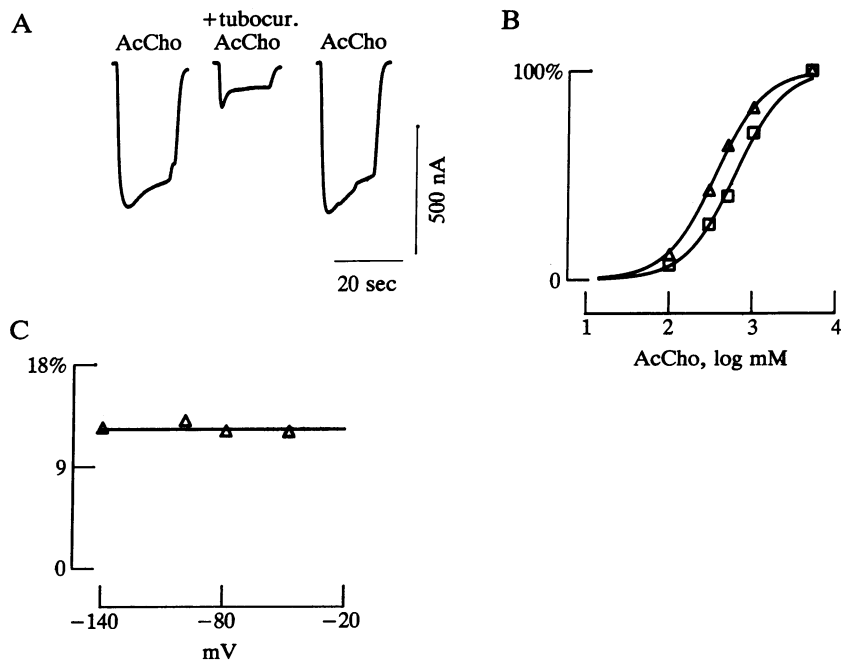


FIG. 3. Effect of TC on  $\alpha 4/\alpha$  nAChR. (A) AcCho-induced currents before, during, and after application of  $2.5 \mu\text{M}$  TC. The cell was held at  $-100$  mV, and AcCho was applied for 10 sec every minute. (B) Shift of the dose-response curve induced by constant application of  $0.5 \mu\text{M}$  TC (100% corresponds to  $4.8 \mu\text{A}$ ).  $\blacktriangle$ , Control condition;  $\square$ , TC added. (C) Residual conductance in the presence of  $2.5 \mu\text{M}$  TC, plotted as a function of holding voltage.

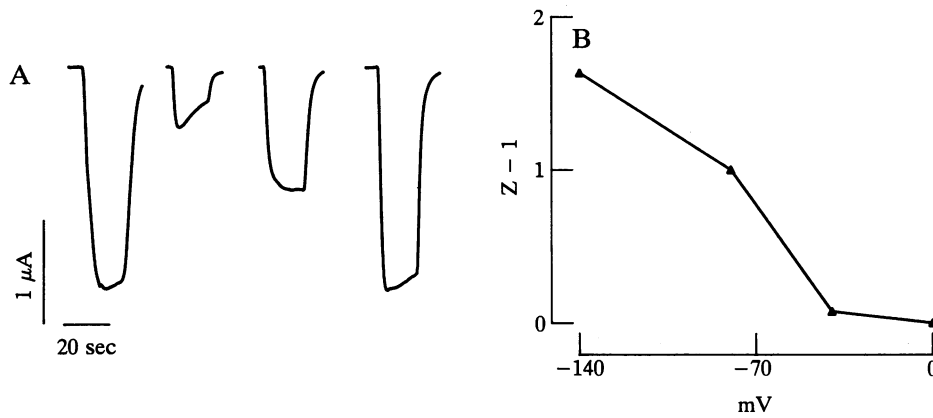


FIG. 4. Hex block of  $\alpha 4/\alpha n$  nAChR is voltage dependent. (A) Currents were evoked by application for 15 sec of 0.5  $\mu$ M AcCho before, during, and after application of 2.5  $\mu$ M Hex. The holding potential,  $-100$  mV, was constant during agonist application. The third trace was recorded 60 sec after the Hex trace. Later recordings did not show further recovery. To remove Hex completely, we used a "pop-out" protocol consisting of a 40-sec depolarization at  $+40$  mV, with application of 0.5  $\mu$ M AcCho. The AcCho-induced current after the "pop-out," fourth trace, is similar to the control. (B) Blocking effect of 2.5  $\mu$ M Hex, expressed as  $Z - 1$  (see text), as a function of membrane potential.

The  $\alpha 4/\alpha n$  channel was also blocked by Dec. As in the case of the rat submandibular ganglion (20, 26), Dec blocked less efficiently than Hex and about four times more Dec than Hex must be applied to observe comparable effects. Dec alone had no effects, and it did not wash out easily once applied together with AcCho. The "pop-out" protocol completely reversed the effects of Dec. The antagonist action of Dec was characterized by computing the  $Z$  ratio as for Hex: the plot of  $Z - 1$  as a function of holding voltage (Fig. 5A) reveals a marked voltage dependence, which differs only slightly from that of Hex.

**Mec, a Voltage-Independent Antagonist.** Mec applied at very low doses (10–100 nM) has been reported to block AcCho-induced currents in the rat submandibular ganglion in a voltage-independent manner (20). In the oocytes, application of 100 nM Mec alone had no effects on nAChR. When the antagonist was applied together with AcCho, the agonist-induced currents slowly decreased (over seconds). Mec could not be washed out even after several minutes, and no voltage protocol could reverse its effect. Thus, the effects of Mec, once established, are stable for long periods of time. Fig. 5B shows that the Mec block is voltage independent: the ratio of the currents measured in the presence and absence of the antagonist remains constant over the entire voltage range tested.

## DISCUSSION

Four agonists and five antagonists were used to characterize the  $\alpha 4/\alpha n$  nAChR reconstituted in *Xenopus* oocytes. We had shown previously (9) that this receptor yields a supra-linear response to AcCho in doses ranging from 10 to 200 nM. The dose-response curves measured here over a broader range of AcCho concentrations present roughly the same properties. They can be fitted with a Hill equation by using

a coefficient of 1.5 and an empirical  $K_d$  of  $0.77 \times 10^{-6}$  M. These values are quite different from those reported for BC3H-1, a muscle-like cell line (27) and from those observed in the chicken ciliary ganglion (28).

At high AcCho concentrations, the dose-response curve is probably not determined by ligand-binding alone but also by desensitization (17, 29–31). Moreover, because of saturation of the available channels, the current induced by high AcCho concentrations cannot be taken as a direct measure of the ligand-binding reaction.

The current-voltage relationship of the response to AcCho rectifies strongly (Fig. 1C), and the current induced by AcCho falls close to zero at  $-40$  mV. Therefore, it is indispensable to test the oocytes in voltage clamp at hyperpolarized potentials, as no current can be observed at potentials above  $-40$  mV. This in fact could explain the small responses observed in published current clamp experiments (2).

Since AcCho-activated  $\alpha 4/\alpha n$  single channels have a voltage-independent conductance of about 20 pS (9), the rectification of AcCho-induced currents observed in the whole cell must result from a change in single-channel kinetics. Thus, we predict that  $\alpha 4/\alpha n$  channel open time or close time will be found to be voltage dependent, as was observed for muscle nicotinic receptors (17, 32, 33) or in *Aplysia* and rat ganglia (20, 34). Recent studies of AcCho receptors in rat sympathetic ganglia have shown a similar voltage-dependence of whole-cell AcCho-induced currents, while the conductance of single channels follows Ohm's law. Measurements of single-channel kinetics revealed that channels simply open less frequently at positive potentials (35, 36).

The different agonists tested induce currents that could not be distinguished from those activated by AcCho on the basis of time course or amplitude. However, the  $\alpha 4/\alpha n$  channel is much less sensitive to carbachol than to AcCho: half-maximum responses are evoked by a concentration of car-

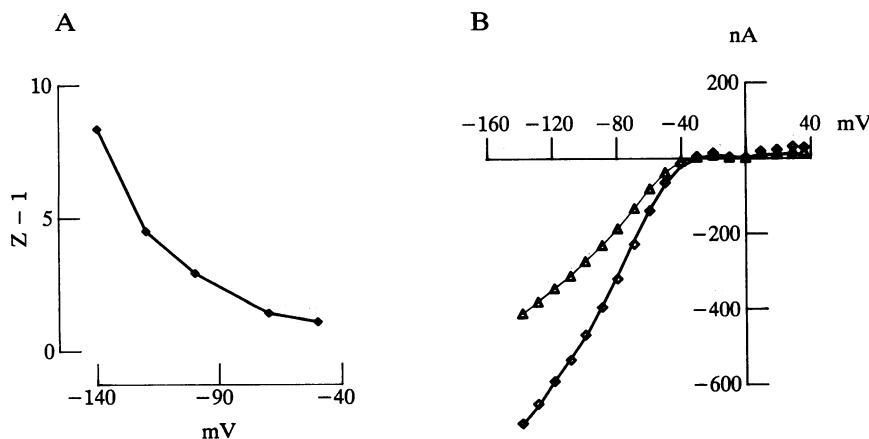


FIG. 5. Effect of Dec and Mec on  $\alpha 4/\alpha n$  nAChR currents. (A) Blocking effect of 10  $\mu$ M Dec, expressed as  $Z - 1$  (see text), as a function of membrane potential. (B) Current-voltage curves, obtained in the absence or presence of Mec. Thick line was recorded with 0.5  $\mu$ M AcCho (same procedure as Fig. 1C).  $\Delta$ , Measurements after exposition to 100 nM Mec together with 0.5  $\mu$ M AcCho for 20 sec at  $-100$  mV;  $\square$ , same values normalized for the AcCho current measured at  $-140$  mV.

bachol that is about 25 times greater than for AcCho. This ratio is much larger than those reported previously for the rat submandibular ganglion and the frog end plate, which are respectively 1/5th and 1/10th as sensitive to carbachol as to AcCho (17, 20). Recent studies on frog sympathetic ganglia (37) have shown that AcCho and nicotine evoked similar responses and had the same dose-response curve, whereas currents evoked by carbachol at saturation had an amplitude about 100 times smaller.

The  $\alpha 4/\alpha$  channel was not blocked by neuronal bungarotoxin even when applied at high concentration (500 nM) for 30 min. This was not due to toxin quality, since the same toxin batch and dilution were effective in blocking AcCho-induced currents in quail ciliary ganglion neurons. This result differs from those obtained on reconstituted rat  $\alpha 4/\beta 2$  receptor (2), where neuronal bungarotoxin applied at 100 nM for 30 min abolished AcCho-induced currents.

Another difference between the chicken and rat neuronal nAcChoR is that injection of the avian  $\alpha 4$  or  $\alpha$  subunit alone never produced a functional receptor, whereas rat  $\alpha 4$  has been reported to form functional channels (2).

TC is a competitive antagonist of the  $\alpha 4/\alpha$  nAcChoR (Fig. 3B), and the induced block is voltage independent (Fig. 3C) as in the frog ganglion (19, 21). In contrast, TC acts on the permeation of the ion channel in mammalian ganglia (20, 22, 24). TC has been shown to be a partial agonist at the neuromuscular junction (38), but it had no effect on the  $\alpha 4/\alpha$  receptor when applied alone.

Hex and Dec are also antagonists of AcCho-induced currents. The blocks induced by these two substances are voltage dependent, as reported for the rat submandibular ganglion (20, 24, 39). Both substances only block AcCho-induced currents when added with the agonist and when the cell is hyperpolarized. These antagonists appear to be trapped inside the channel and cannot be washed out unless one applies AcCho while depolarizing the cell. This "pop-out" procedure has previously been applied on receptors of the rat submandibular ganglion (26). It is most effective at very depolarized voltages (+40 mV), where the I-V relationship is flat (Fig. 1C). Therefore, AcCho must induce a modification of channel conformation at depolarized voltages, even though no net currents are detected. No significant differences were observed between trapping of Hex and Dec. This is another difference with the rat sympathetic ganglion neurons, where Dec is not trapped in the channel and can be washed away (26). Agonist trapping inside open nAcChoR channels is not peculiar to ganglionic receptors but also was observed at the neuromuscular junction with chlorisondamine (40).

Mec alone has no effects on the resting condition. However, low doses (100 nM) added together with the agonist are enough to reduce AcCho-induced currents significantly. The effect of Mec is voltage independent as reported for the rat submandibular ganglion (20).

In conclusion, we find that the  $\alpha 4/\alpha$  nAcChoR, as expressed in oocytes, differs in many significant ways from muscle receptors and also in more subtle ways from previously characterized neuronal receptors. Direct comparison between nAcChoR types from the same species and in the same environment will be possible in the future when  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ , and  $\alpha 3$  neuronal subunits (ref. 6; M.B., unpublished data) are expressed in *Xenopus* oocytes.

We are grateful to Philippe Ascher and to Ellis Cooper for critical comments on the manuscript. We also wish to thank Sonia Auderset for excellent technical assistance and Philippe Cand for help with the

electronics. We thank Alain Barth and Adrian Gross for participating in some of the experiments. Neuronal bungarotoxin was kindly provided by Darwin Berg and by Vincent Chiappinelli. This work was supported by Grants 3.594-0.87 (to D.B.), 3.586-0.87 (to D.R.), and 3.169-0.88 (to M.B.) from the Swiss National Foundation.

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