Incorporation of acetylcholine receptors and Cl⁻ channels in *Xenopus* oocytes injected with *Torpedo* electroplaque membranes

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ABSTRACT A method was developed to transplant assembled nicotinic acetylcholine receptors (AcChoRs) and Cl⁻ channels from the electric organ of *Torpedo* to the membrane of *Xenopus* oocytes. Membrane vesicles from *Torpedo* electroplaques were injected into the oocytes and, within a few hours, the oocyte membrane acquired AcChoRs and Cl⁻ channels. The mechanism of expression of these receptors and channels is very different from that which follows the injection of mRNA, since the appearance of receptors after membrane injection does not require *de novo* protein synthesis or N-glycosylation. This, and other controls, indicate that the foreign receptor-bearing membranes fuse with the oocyte membrane and cause the appearance of functional receptors and channels. All this makes the *Xenopus* oocyte an even more powerful tool for studies of the structure and function of membrane proteins.

Xenopus oocytes are a useful system to study the structure and function of many neurotransmitter receptors, ion channels, and other membrane proteins. In the first place, *Xenopus* oocytes and their enveloping follicular cells, which are electrically coupled to the oocyte, are endowed with a variety of native neurotransmitter and hormone receptors and voltage-operated channels (1–4). Moreover, after injection of appropriate mRNAs, *Xenopus* oocytes will express a large variety of foreign receptors and channels. The oocytes translate the heterologous mRNA, process the products, and incorporate them into their plasma membrane where they form functional receptors and channels (5).

The present experiments were done to see if the oocyte membrane could be made to acquire foreign receptors, already assembled in other cell membranes, without injecting the nucleic acids. If this could be achieved, the oocytes would become even more useful for studies of many receptors, channels, and signaling events. Our experiments, carried out early in 1990, showed that acetylcholine receptors (AcChoRs) and Cl⁻ channels from *Torpedo* can be transplanted directly from the *Torpedo* electroplaques to the oocyte membrane. Some of the results have been presented in brief (6).

MATERIALS AND METHODS

To determine whether foreign membranes carrying neurotransmitter receptors could be incorporated directly into the oocyte plasma membrane, *Xenopus* oocytes were injected with membranes isolated from the electric organ of *Torpedo*, because the electroplaque cells are very rich in AcChoRs and Cl⁻ channels (7–11).

Membrane Preparation. Membranes were prepared as described (10, 11) with slight modifications. About 300 g of frozen electric organ from *Torpedo californica* was thawed in 1–2 liters of a hypoosmotic solution (1 mM EDTA, 10 mM Hepes buffer, pH 7.2). This solution was discarded and fresh solution was added to make 1 liter. The thawed, or sometimes fresh, tissue was homogenized first in a Waring blender (high speed for 1 min), completed in a Teflon glass homogenizer, and filtered under vacuum through 100-mm nylon gauze. The filtrate was centrifuged for 1 hr at 25,000 × g. The pellet was suspended in EH buffer (0.1 mM EDTA, 10 mM Hepes, pH 7.2), gently placed on a 75-ml layer of 1.05 M sucrose in EH buffer, and centrifuged for 1 hr at 10,000 rpm. The viscous supernatant–sucrose interface was resuspended in EH buffer and centrifuged for 3 hr at 10,000 rpm. The pellet was resuspended in the same buffer and 6 ml was overlaid on a four-step sucrose density gradient consisting of 30–35–37.5–40% (wt/wt; 8 ml each) sucrose in EH buffer. The gradients were centrifuged for 16 hr at 25,000 rpm with an SW 28 rotor. The interface materials were collected, diluted with 5 mM glycine buffer (pH 7.0), and centrifuged at 60,000 rpm for 1 hr in a 60Ti rotor. Pellets were suspended in glycine buffer and used directly or kept at −70°C for later use. Sometimes the samples were thawed and sonicated for 10 s in a Branson B12 ultrasound bath in iced water before injection. Electron microscope inspection revealed that the fractions were made up of bi- and multilamellar membrane vesicles and cisternae of different sizes (0.05–4 μm), were devoid of cell nuclei, and were essentially free of rough endoplasmic reticulum.

Injection of Membrane Vesicles and of Electroplaque mRNA. The procedure used to transplant membranes from the electric organ of *Torpedo* into *Xenopus* oocytes is illustrated in Fig. 1. Oocytes were injected with membrane fractions (50–100 nl; 1–2 mg of protein per ml) and maintained in modified Barth's solution at 15–17°C (12), usually without antibiotics, until electrophysiological recording was performed, typically within 7 days after injection.

For comparative purposes, oocytes were injected with 5–50 ng of *Torpedo* electric organ mRNA, alone or together with an oligonucleotide antisense to the α subunit of the AcChoR. To block protein synthesis, or glycosylation, cycloheximide (25 μg/ml) or tunicamycin (2 μg/ml), respectively, was added to the Barth's solution immediately after isolating the oocytes. One day later, the oocytes were injected with membranes, or mRNA, and kept in Barth's solution still containing the blockers. To ensure maximal suppression of N-glycosylation, some oocytes were also injected with 0.2 ng of tunicamycin together with membranes or mRNA. For other experiments α-bungarotoxin (BTX) was dissolved in distilled water and added to the membranes or to the mRNA at a 5:1 or 2:1 (wt/wt) ratio, respectively, and incubated on ice for 3 hr before injection. In other experiments, membranes were incubated for 3 hr with trypsin (120 μg/ml) or 1 hr in RNase (100 μg/ml) (DNase and proteinase free) at room temperature. Trypsin activity was terminated by the addition of an equal amount of
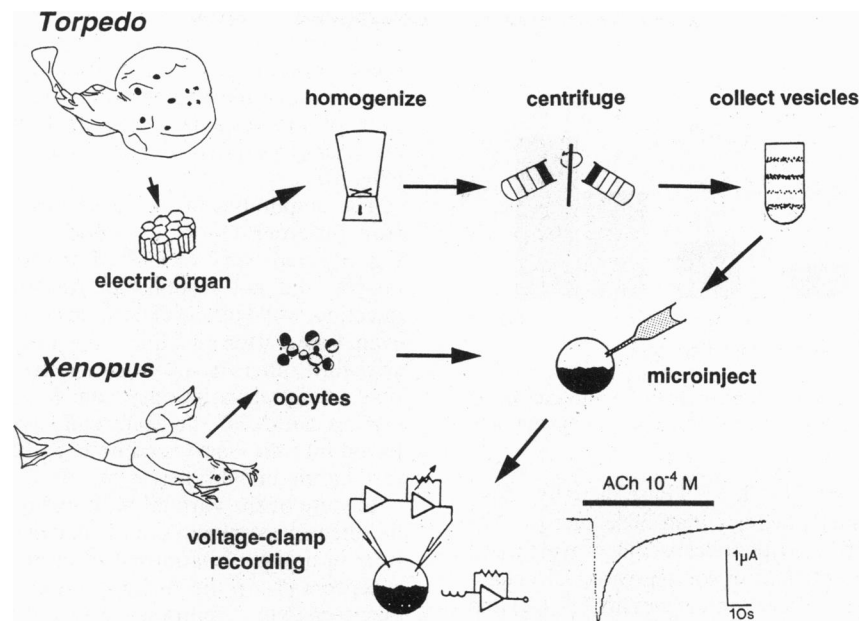
FIG. 1.　Method used to transplant assembled AcChoRs and Cl⁻ channels from the electric organ of *Torpedo* to the plasma membrane of *Xenopus* oocytes.

soybean trypsin inhibitor 30 min before injection of the membranes into oocytes. For electrophysiological recordings, oocytes were freed of epithelial and follicular cell envelopes, by collagenase treatment, 1–2 days after injection (13).

Preparation of mRNA and Synthesis of Antisense Oligonucleotides. Poly(A)⁺ RNA was isolated from fresh or frozen electric organ of *T. californica* as described (9). For antisense experiments, an oligonucleotide complementary to amino acid residues 4–10 of the α subunit of the *Torpedo* AcChoR was synthesized and coinjected with the membranes or mRNA (14).

Electrophysiological Recording and Other Methods. Membrane currents elicited by AcCho were monitored by a two-electrode voltage-clamp system as described (1, 2). The presence of voltage-gated Cl⁻ channels was assessed by the resting membrane conductance (at −20 mV) and the hyperpolarization induced rectification of the current–voltage relationship of the oocyte membrane (cf. refs. 9 and 14).

BTX was fluorescently labeled with tetramethylrhodamine isothiocyanate (15). Rhodaminated BTX (Rho-BTX) was incubated with the membrane fraction resuspended in 14 mM phosphate-buffered saline (PBS) solution (pH 7.2) for 2 hr in the dark at 4°C. The membrane fraction was pelleted by centrifuging for 1 hr (60,000 rpm) and washed repeatedly in PBS. The pellet was resuspended in 5 mM glycine buffer (pH 7.0) at 2 mg of protein per ml (final concentration) and injected into the oocytes. A control solution devoid of membranes was similarly processed. For subsequent microscopic observation, the oocytes were washed in glycine buffer and mounted in *p*-phenylendiamine (10 mg/ml) in 1:9 (vol/vol) PBS, pH 9/glycerol. In some experiments, oocytes injected with membranes were incubated for 15 hr to allow the *Torpedo* membranes to be incorporated into the oocyte plasma membrane and then treated with Rho-BTX, washed, and mounted as described above.

Protein determinations were carried out by the bicinchoninic acid assay. Binding of ¹²⁵I-labeled BTX to Triton X-100-solubilized membrane supernatants, and binding of ³⁵S-labeled butylbicyclophosphorothioate (TBPS), a putative marker for Cl⁻ channels, to the membrane fractions was done as described (16, 17). Na⁺,K⁺-activated ouabain-sensitive ATPase was measured at room temperature (18).

RESULTS

Partial Characterization of the Membrane Fractions. Postsynaptic membrane fragments, containing most of the AcChoR, and fragments of the dorsal noninnervated face of
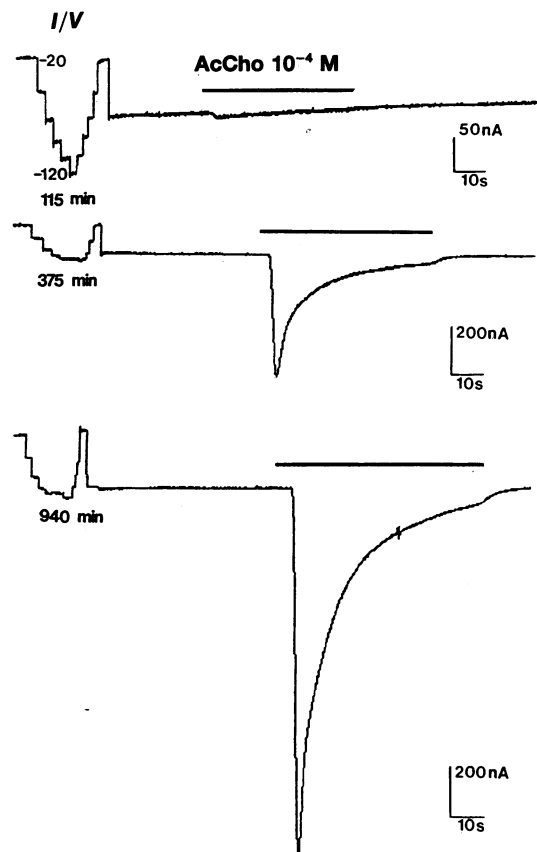

FIG. 2.　Current/voltage (*I/V*) relation and membrane current responses to AcCho in an oocyte injected with *Torpedo* electroplaque membranes. For this and subsequent figures, AcCho was applied, with the membrane potential held at −60 mV, during the time indicated by horizontal bars.
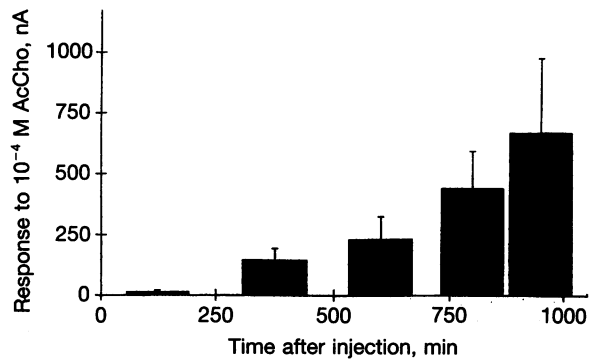
FIG. 3. Time course of development of responses to AcCho in oocytes (one frog) injected with *Torpedo* electroplaque membranes. Bars show means + SEM.

the electroplaques, rich in Na^+,K^+-ATPase activity, have similar equilibrium densities, heavier than 30% sucrose. We attempted to separate both activities because the dorsal face of the electroplaque is also enriched in voltage-gated Cl^- channels. For that purpose, we collected interfaces at 35% (A) and 37.5% (B) sucrose to be injected into oocytes. The amounts of AcChoR in A and B were 19.7 ± 9.6 and 46.65 ± 16 pmol per mg of protein, respectively (mean ± SEM; n = 5), and the difference was significant ($P < 0.013$). Na^+,K^+-ATPase activities in A and B were 1.69 ± 0.43 and 3.32 ± 0.432 mmol per mg of protein per hr and the difference was just significant (n = 3; $P < 0.0563$). TBPS binding to Cl^- channels in A was 15.05 ± 2.7 and in B was 9.64 ± 2.42 pmol per mg of protein and the difference was not significant (n = 8; $P < 0.16$). Subsequently, we used the fractions separately, or pooled, for injection into oocytes.

Expression of AcChoRs After Injection of Electroplaque Membranes. *Xenopus* oocytes do not have nicotinic AcChoRs but sometimes possess native muscarinic AcChoRs that mediate membrane current responses with an oscillatory time course characteristic of receptors coupled to the phosphatidylinositol pathway (1, 5). In contrast, the oocytes injected with

Torpedo electroplaque membranes acquired AcChoRs that gated smooth inward membrane currents (Fig. 1). These receptors could be detected within 1–2 hr after injection of the electroplaque membranes (Fig. 2), and thereafter the amplitude of the responses, and by implication the number of receptors incorporated, continued to increase for a few days (Fig. 3).

The amplitudes of the responses elicited in oocytes taken from different donors, or in oocytes from the same frog (e.g., Fig. 3), were very variable. For instance, in one frog some oocytes did not respond to AcCho (10^{-4} M) 10 hr after injection, while others generated currents as large as 3 μA, and even 4 days after injection a few oocytes remained unresponsive while others gave >5 μA. At present, we do not know the reason for such variation but one factor may be that, due to the viscous nature of the membrane preparations, the fluid injected into the oocytes contained variable amounts of receptor-bearing membranes.

In spite of the variability, it was quite clear that AcChoRs had been incorporated and that these receptors were nicotinic in nature. In contrast to native or induced muscarinic receptors (1, 5), the AcChoRs that appear after injection of electroplaque membranes were not blocked by atropine but were blocked by (+)-tubocurarine or BTX, two highly specific blockers of nicotinic receptors. Moreover, the currents elicited by AcCho in oocytes injected with electroplaque membranes were similar to currents generated in oocytes injected with mRNA isolated from the electric organ. For example, in both cases the equilibrium potential for the AcCho current elicited by AcCho was close to 0 mV, and the current desensitized rapidly during maintained application of AcCho (Figs. 1, 2, and 4) (19).

Cl^- Channels Expressed After Injection of Electroplaque Membranes. It has been shown previously that oocytes injected with electroplaque mRNA (9), or cloned RNA encoding the *Torpedo marmorata* Cl^- channel (20), acquire voltage-dependent Cl^- channels that resemble those seen when electroplaque membranes are incorporated into lipid bilayers (8). Similarly, injection of electroplaque membranes into oocytes caused them to acquire the Cl^- channels. A simple way to
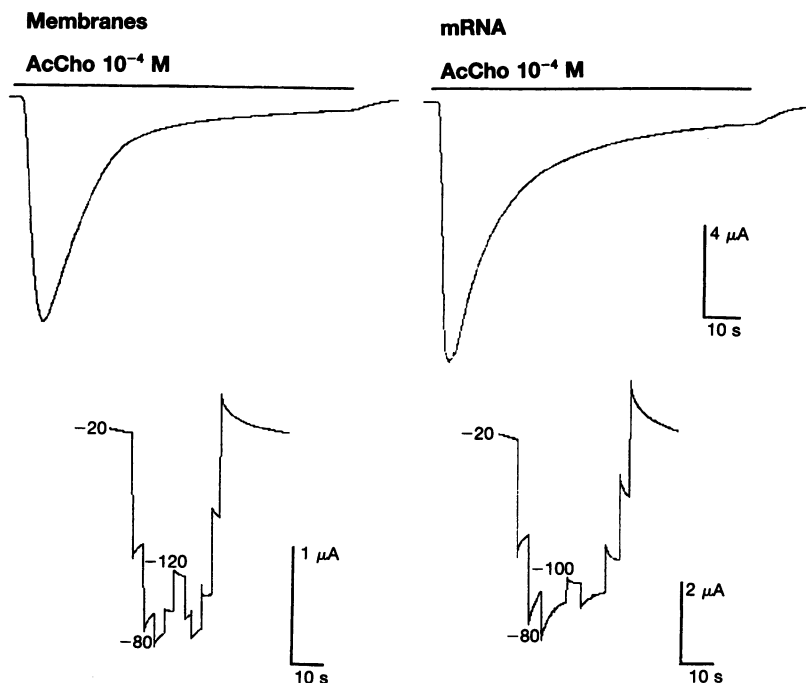

FIG. 4. (*Upper*) Similarity in responses to AcCho and current/voltage relations in oocytes injected with *Torpedo* electroplaque membranes or *Torpedo* mRNA. (*Lower*) Currents for 20-mV steps in the range indicated.

assess the presence of these Cl⁻ channels is to hold the membrane potential at -20 mV, at which potential many channels are open, and then step the potential progressively to -120 mV, at which potential many channels close. Instead of the fairly linear current/voltage relation of native oocytes (1, 2), the current for each voltage step decreased and even changed direction as channels closed during the hyperpolarizing steps (Figs. 2 and 4). Like the channels expressed by *Torpedo* electric organ mRNA, the Cl⁻ channels induced by injection of electroplaque membranes were blocked by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid, Zn^{2+}, and Cu^{2+} (R.M., unpublished data).

Transplantation of Assembled AcChoRs or *de Novo* Expression from mRNA? Since the electric organ is very rich in mRNAs coding for AcChoRs and Cl⁻ channels (9), it was necessary to consider the possibility that, after injecting the membranes, the appearance of AcChoRs and Cl⁻ channels was due not to a direct insertion of membranes containing receptors and channels but to translation of contaminating mRNA. This is unlikely because, while preparing the membranes, no precautions were taken to prevent degradation of mRNA. Furthermore, incorporation of receptors was sometimes evident 1 hr after membrane injection, and this rapidity of expression is seen only after injecting substantial amounts of AcChoR mRNA. Nonetheless, to ascertain that assembled receptors were being incorporated together with the membranes, four types of control experiments were performed.

(*i*) One way of assessing whether AcChoRs were newly synthesized or whether preassembled membrane-associated receptors were incorporated is to inactivate the receptors before injecting the membranes. For that purpose, membranes were preincubated with BTX and injected into the oocytes, and almost no functional receptors appeared in the oocyte membrane. In contrast, AcChoRs were still strongly expressed when *Torpedo* electroplaque mRNA was similarly mixed with BTX and injected into the oocytes (Fig. 5).

(*ii*) Further evidence that after injection of the membranes the receptors were not synthesized *de novo* comes from experiments designed to inhibit the synthesis or incorporation of receptors. For example, tunicamycin strongly inhibits N-glycosylation and incorporation of AcChoRs in oocytes injected with AcChoR mRNA (21), but it failed to inhibit the appearance of functional receptors in oocytes injected with *Torpedo* electroplaque membranes (Fig. 6).

(*iii*) Similarly, coinjection of AcChoR mRNA with an antisense oligonucleotide, complementary to a segment of the AcChoR α subunit, abolished almost completely the appearance of AcChoRs in oocytes injected with mRNA, but receptors were still incorporated in oocytes injected with the membranes plus the antisense oligonucleotide. Moreover, similar results were obtained by using cycloheximide to block protein synthesis (Fig. 6).

(*iv*) Finally, to destroy any possible contaminating mRNA, the injected membranes were pretreated with large amounts of RNase, and AcChoRs still appeared in the oocyte membrane. Conversely, when membranes were digested with trypsin before injection, the number of functional AcChoRs in the oocyte was greatly reduced.

These experiments indicate that, after injection of electroplaque membranes, the receptor-bearing membranes were incorporated into the oocyte plasma membrane and show further that, after coinjection of mRNA and BTX, the AcChoRs synthesized were not accessible to BTX during their path from their site of synthesis to the plasma membrane.

Incorporation of Fluorescently Labeled AcChoRs. The incorporation of membrane vesicles was also followed by fluorescence microscopy. No fluorescence was detected on the surface of control oocytes injected with Rho-BTX alone. In contrast, after injection of labeled membranes there were large fluorescent areas that were made up of smaller patches.

In some experiments, the incorporated membranes were labeled by applying Rho-BTX extracellularly, and again fluorescent patches were observed. All this confirms the electrophysiological finding of AcChoRs on the oocyte membrane.

DISCUSSION

The main conclusion to be drawn from our experiments is that AcChoRs and Cl⁻ channels, already assembled in the *Torpedo* electroplaque membrane, can rapidly and efficiently be transplanted to the oocyte membrane simply by injecting the oocyte with the membranes. This method combines the advantages of techniques using liposomes with those provided by a host cell such as the *Xenopus* oocyte and seems more efficient than the more difficult incorporation of AcChoRs and Cl⁻ channels into lipid bilayers (8, 22).

This comparatively simple procedure of expressing receptors in the plasma membrane of *Xenopus* oocytes lends itself
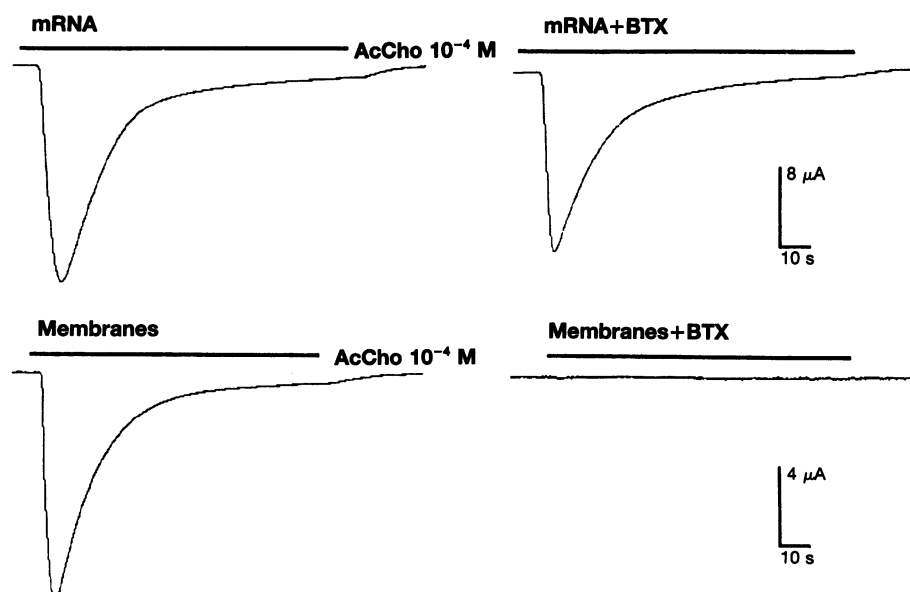
FIG. 5. Responses to AcCho in oocytes injected with *Torpedo* mRNA or membranes alone (*Left*) or preincubated and coinjected with BTX (*Right*).
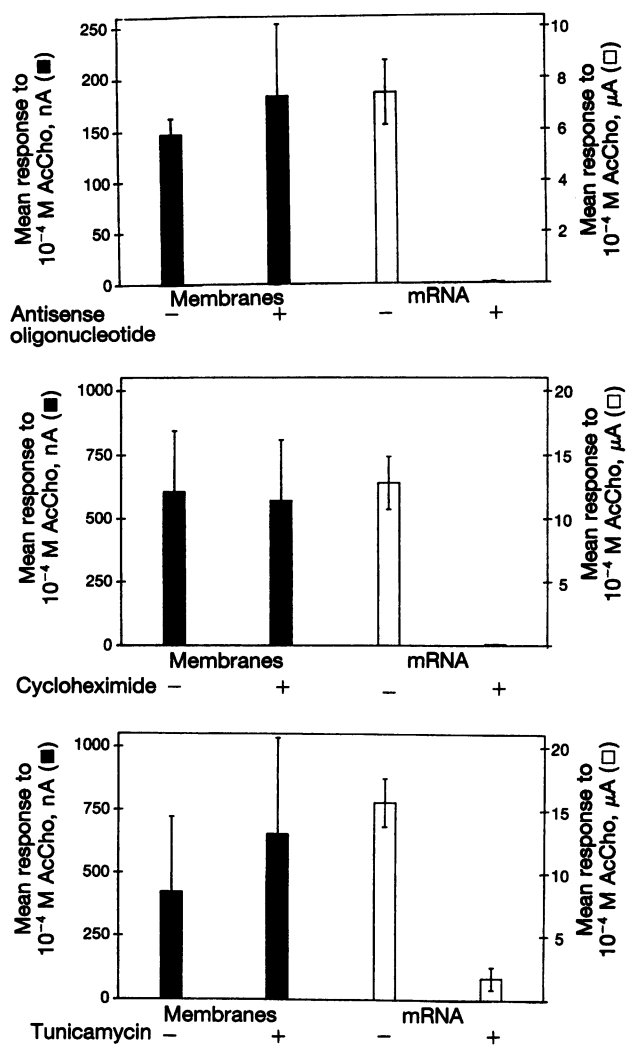
FIG. 6.   Responses to AcCho in oocytes injected with membranes or mRNA alone or together with an antisense oligonucleotide (α subunit), cycloheximide, or tunicamycin. Bars represent means $\pm$ SEM of 5–11 oocytes.

channel-bearing membranes from rat and fish brains can also be incorporated in *Xenopus* oocytes (R.M. and G.T., unpublished data). We are only at the beginning, but it is already clear that the usefulness of the *Xenopus* oocyte is greatly extended by this method.

very well to studying many processes, such as the mechanism of incorporation of receptors, channels, and other membrane proteins, in addition to studies of second messengers, receptor/channel modulation, secretion, etc. In fact since our work was completed, similar procedures have been used to express rhodopsin (23, 24) and chromaffin granule exocytosis (25). Moreover, preliminary experiments indicate that receptor/

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