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Incorporation of reconstituted acetylcholine receptors from *Torpedo* into the *Xenopus* oocyte membrane

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ABSTRACT ***Xenopus* oocytes are a valuable aid for studying the molecular structure and function of ionic channels and neurotransmitter receptors. Their use has recently been extended by the demonstration that oocytes can incorporate foreign membranes carrying preassembled receptors and channels. Here we show that when reconstituted in an artificial lipid matrix and injected into *Xenopus* oocytes, purified nicotinic acetylcholine receptors are efficiently inserted into the plasma membrane, where they form "clusters" of receptors that retain their native properties. This constitutes an innovative approach that, besides allowing the analyses of membrane fusion processes, is also a powerful technique for studying the characteristics and regulation of many membrane proteins (with their native stoichiometry and configuration) upon reinsertion into the membrane of a very convenient host cell system.**

The functional properties of nicotinic acetylcholine receptors (nAcChoRs) have been elucidated by many approaches: for example, electrophysiological recordings from muscle (1), measurements of ion fluxes in membrane vesicles containing nAcChoRs (2), expression of mRNAs encoding nAcChoRs in *Xenopus* oocytes (3–5), and, more recently, by injecting oocytes with membranes from *Torpedo* electroplaques (6). The latter approach results in the incorporation of native *Torpedo* nAcChoRs and other proteins into the oocyte's plasma membrane (6). This procedure has some important advantages over the usual oocyte expression system, since it allows the study of receptors that have been fully processed and assembled, with their natural subunit stoichiometry, in the original cell membrane. Nevertheless, there are many reasons that make the incorporation of well-defined purified proteins into the host cell membranes highly desirable. Therefore, we set to find out whether purified nAcChoRs reconstituted in a lipid matrix could be incorporated in a host cellular system such as the *Xenopus* oocyte, in which the function and cellular regulation of the protein can be examined in detail. Preliminary results have been presented elsewhere (7, 8).

METHODS

Solubilization and Reconstitution of nAcChoRs. nAcChoR-rich membranes from the electric organ of *Torpedo marmorata* were used to purify nAcChoRs by affinity chromatography in the presence of asolectin lipids and with cholate as detergent (9–11) (Fig. 1). The specific activity of the purified nAcChoRs was ≈8 nmol of α-bungarotoxin bound per mg of protein (10).

Reconstitution of nAcChoRs in asolectin lipid vesicles was accomplished by a detergent dialysis method (10). Final concentrations in the reconstitution mixtures were as follows: nAcChoRs, 0.3–1.2 mg of protein per ml; asolectin lipids, ≈5 mg/ml; and sodium cholate, 1% (wt/vol). After dialysis, reconstituted nAcChoR samples were aliquoted and injected immediately into oocytes or stored in liquid nitrogen, either alone or in the presence of trehalose (5 mg/mg of protein) to prevent protein denaturation (10).

Oocyte Preparation and Microinjection. *Xenopus laevis* oocytes at stages V and VI (12) were isolated from segments of ovary under aseptic conditions. The oocytes were kept at 15–16°C in a Barth's solution supplemented either with nystatin (50 units/ml) and gentamicin (0.1 mg/ml) or with penicillin (100 units/ml) and streptomycin (0.1 mg/ml), until used for electrophysiological recordings. One day before injection the oocytes were treated with collagenase (0.5 mg/ml) for ≈1 hr to remove the surrounding layers (13).

Oocytes were microinjected with 50 or 100 nl of fresh or thawed reconstituted nAcChoRs by use of an electronic nanoliter injector. Frozen–thawed samples were rehomogenized prior to injection.

Electrophysiological Recordings and Data Analysis. Membrane current recordings were performed at room temperature (21–25°C) 4–60 hr after injection. Oocytes were placed in a 150-μl chamber that was continuously perfused with a Ringer's solution to which 0.5 μM atropine sulfate was added to block any muscarinic responses (14). During recordings the flow rate was 3 ml/min.

Oocytes were voltage-clamped with a two-electrode voltage-clamp system and exposed to acetylcholine (AcCho) with the membrane potential held at −60 mV, unless otherwise noted. Membrane currents were usually low-pass filtered at 30–500 Hz and simultaneously recorded on a chart recorder and on a digital oscilloscope (Nicolet model 310) with disc storage for subsequent analyses. In some experiments the membrane potential was briefly stepped to different levels before and during AcCho application to obtain a current/voltage (*I/V*) relationship. Localized extracellular or intracellular AcCho applications were made through glass micropipettes filled with 1 mM AcCho, using 40-ms pneumatic pressure pulses of around 200 kPa (Picospritzer, General Valve, Fairfield, NJ).

Unless otherwise specified, values given in the text correspond to the mean ± standard deviation. Among-group differences were determined by analysis of variance, and the group means were compared by the Student–Newman–Keuls test. When two group means were compared, the Student *t* test was used.

RESULTS

Functional Incorporation of Reconstituted nAcChoRs. Purified nAcChoRs, reconstituted into asolectin lipid vesicles, underwent agonist-induced affinity transitions between sensitized and desensitized states and exhibited their characteristic ion-flux activity in response to cholinergic agonists (9, 11). Microinjection of up to 100 nl of these reconstituted nAc-

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Abbreviations: AcCho, acetylcholine; nAcChoR, nicotinic acetylcholine receptor.

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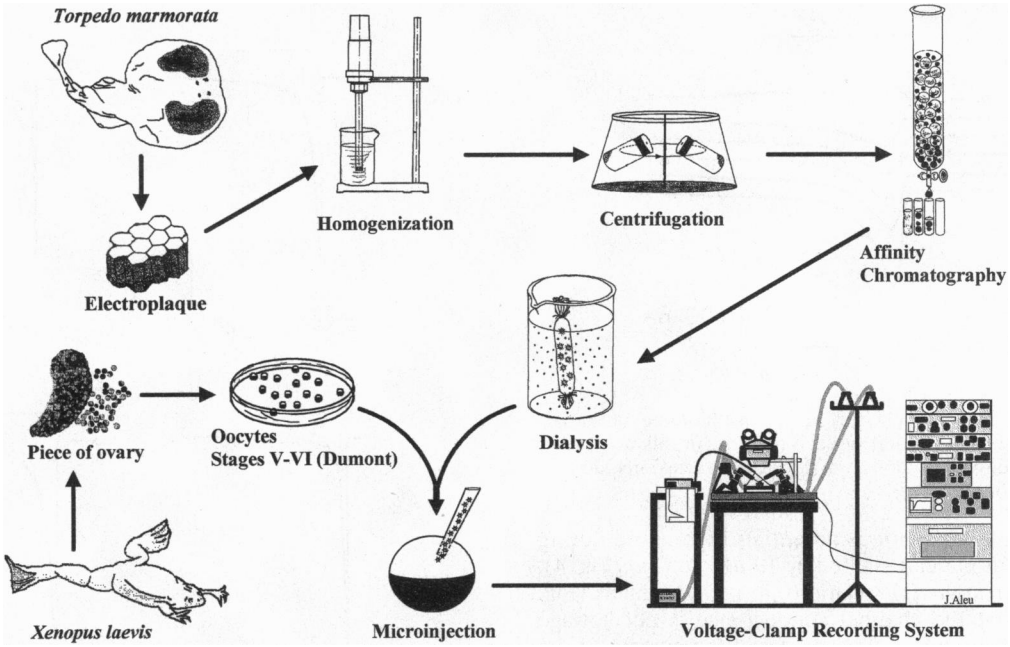

FIG. 1. Steps followed for purification, reconstitution, and incorporation of *Torpedo* nAcChoRs into *Xenopus* oocytes, and their subsequent functional analyses.

ChoRs into oocytes was well tolerated. The reconstituted nAcChoRs were rapidly incorporated into the oocyte's plasma membrane; AcCho currents could already be elicited 4 hr after injection and persisted for over 60 hr. In 148 out of 163 injected oocytes, bath application of AcCho (100 μM) elicited membrane currents similar to those previously described for *Torpedo* nAcChoRs expressed in oocytes after injection of their coding mRNA (3, 4). The AcCho-current amplitude and rate of desensitization were concentration dependent (Fig. 2 *Inset*). The amplitude of the AcCho current varied greatly (5 to 1600 nA for 100 μM AcCho, n = 148), probably depending on variations in the donor frogs as well as on the different samples of reconstituted nAcChoRs. Application of AcCho (100 μM) to oocytes of one donor injected with 50 nl or 100 nl of the same reconstituted nAcChoR sample elicited currents of 79 ± 85 nA (n = 13) and 457 ± 412 nA (n = 9), respectively ($P < 0.01$; Student t test). This clearly indicates that, in addition to the observed experimental variability, the current amplitude depended on the amount of nAcChoRs injected. Fig. 2 shows a dose–response curve obtained from four oocytes injected with two different samples of nAcChoRs. The Hill coefficient, estimated from the fitted dose–response curve, was 2.08 and the EC_{50} was 72 μM, values which are in good agreement with previously published data (1, 5, 15).

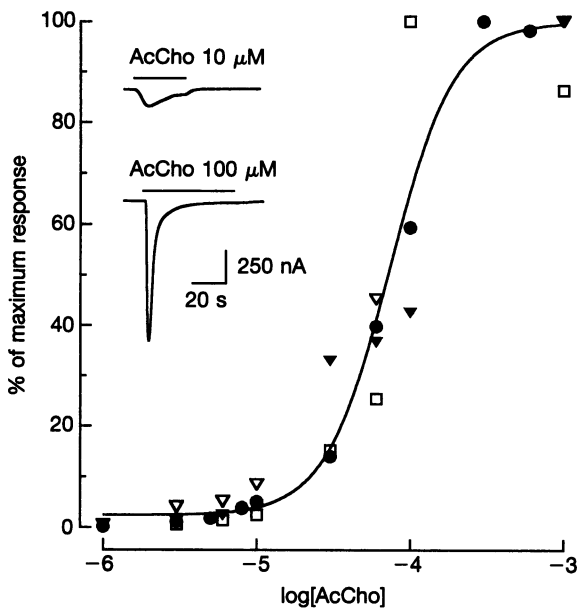

FIG. 2. Dose–response relationship in four oocytes (each represented by a symbol) injected with reconstituted *Torpedo* nAcChoRs. (*Inset*) Examples of currents elicited by two different AcCho concentrations. In this and following figures AcCho application is indicated by bars, downward deflections denote inward currents, and the holding potential was −60 mV, unless otherwise specified.

If we disregard desensitization, from the maximum AcCho current elicited (2.19 μA with 1 mM AcCho) it is possible to estimate roughly the number of functional nAcChoR channels incorporated into the oocyte membrane. For a single-channel conductance of about 40 pS (16, 17), a peak conductance change (peak AcCho current)/(membrane potential − reversal potential) of 40 μS would mean that at least 10^6 functional channels were incorporated at a given time into the oocyte membrane. As we did not usually test oocytes with such high AcCho concentrations, the maximum response value is probably underestimated. Thus, in one oocyte 10 μM AcCho elicited a current of 480 nA, which, from the obtained dose–response curve, would correspond to the presence of $>5 \times 10^6$ functional channels.

Electrophysiological Characteristics of Reconstituted and Incorporated nAcChoRs. It was important to determine whether the properties of the incorporated nAcChoRs were similar to those previously described after oocyte injection of electroplaque membranes (6) or of native or cloned mRNAs (5, 17), because the purification procedure or the lipid environment can modify nAcChoR channel properties (18, 19). Therefore, we determined the reversal potential of AcCho currents by applying brief pulses of AcCho (10 μM) while holding the membrane potential at various levels (Fig. 3). The average reversal potential obtained in five oocytes was −5 mV (range, −17 to +4 mV) which is similar to the values obtained for nAcChoRs expressed by native or cloned mRNA in oocytes (3, 20). In a few oocytes we obtained the I/V relationship by

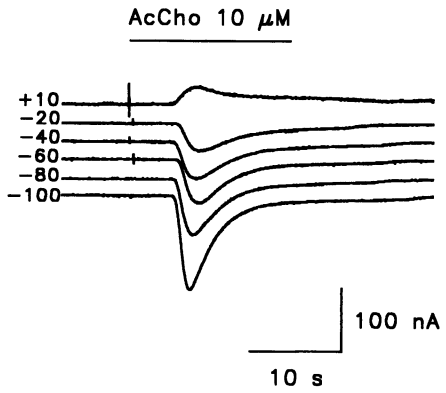

FIG. 3. AcCho currents elicited at various holding potentials (indicated in millivolts at the left of each record). In this oocyte the current reversed direction at −2 mV. Baseline currents do not correspond to real levels.

applying short pulses to various potentials before and during the AcCho-current plateau elicited by 10 μM AcCho (Fig. 4). Since the instantaneous I/V relationship is fairly linear (Fig. 4*B*), it follows that the channel conductance is not voltage dependent. However, when we considered the current at the end of 800-ms pulses, a marked inward rectification was observed. This effect was seen in all the oocytes tested, although its magnitude was quite variable.

The well-known desensitization of *Torpedo* nAcChoRs (21) was also preserved when nAcChoRs were purified, reconstituted, and incorporated into the *Xenopus* oocyte membrane

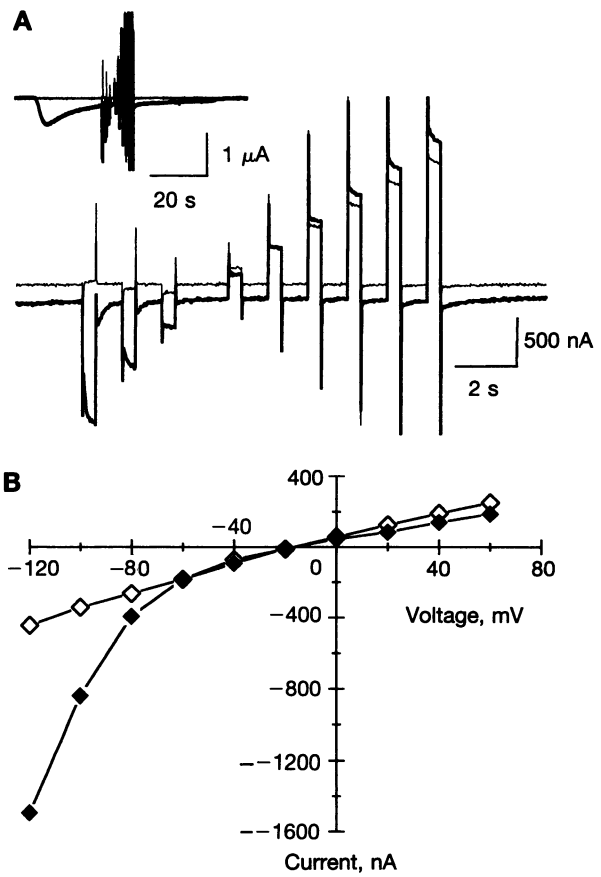

FIG. 4. Voltage dependence of currents elicited by AcCho (10 μM). (*A*) Responses to voltage pulses (800 ms) before AcCho (thin trace) and during the current plateau induced by 10 μM AcCho (thick trace). (*Inset*) Whole AcCho current. (*B*) Plot of instantaneous (◇) and end-of-pulse (◆) I/V relationships for the same oocyte as in *A*.

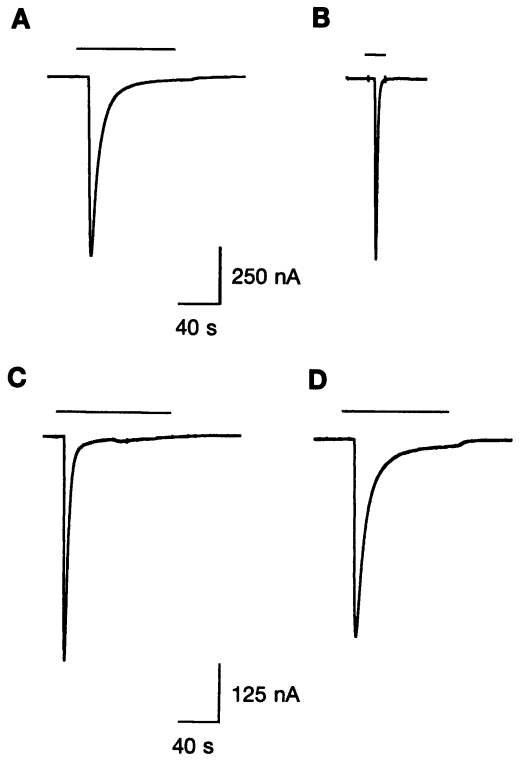

FIG. 5. Effects of antibiotics on desensitization of currents elicited by AcCho (100 μM). (*A*) Record from an oocyte not exposed to antibiotics. (*B*) Oocyte incubated with gentamicin (0.1 mg/ml). (*C*) Oocyte incubated with penicillin (100 units/ml) and streptomycin (0.1 mg/ml). (*D*) Oocyte kept as in *C* but with trehalose (5 mg/mg of protein) in the sample of reconstituted receptors. *C* and *D* are from the same donor.

(Fig. 5). Desensitization of nAcChoRs induced by prolonged application of AcCho (100 μM) showed at least two components and was similar in magnitude to that of nAcChoRs induced by mRNA injection (22, 23). Gentamicin, an antibiotic frequently used for oocyte culture, is known to increase the rate of desensitization of *Torpedo* nAcChoRs expressed from cDNA clones (24) or native mRNA (R.M., unpublished results). Similarly, gentamicin increased the rate of desensitization of reconstituted nAcChoRs (Fig. 5 *A* and *B*; Table 1). A similar result was obtained for nAcChoRs expressed following the injection of *Torpedo* electroplaque membranes (R.M., G. Tigyi, and J. Marsal, unpublished results). Because of this effect most of the recordings were done in oocytes maintained without antibiotics or in the presence of penicillin and streptomycin, which have only a small effect on desensitization (Table 1). Unexpectedly, oocytes incubated with penicillin and streptomycin and injected with nAcChoRs that had been frozen in the presence of trehalose showed a significantly slower desensitization, similar to that of oocytes not treated with antibiotics (see Fig. 5*D* and Table 1).

Table 1. nAcChoR desensitization induced by 100 μM AcCho

Incubation medium	% desensitization 10 s	% desensitization 40 s	No. of oocytes
No antibiotics	78 ± 17	94 ± 4	12
Gentamicin	96 ± 6*	—	19
Penicillin/streptomycin			
Frozen without trehalose	83 ± 9	97 ± 3	28
Frozen with trehalose	75 ± 13†	92 ± 7†	20

*$P < 0.05$ between no-antibiotics group and any other.
†$P < 0.05$ between penicillin/streptomycin groups.

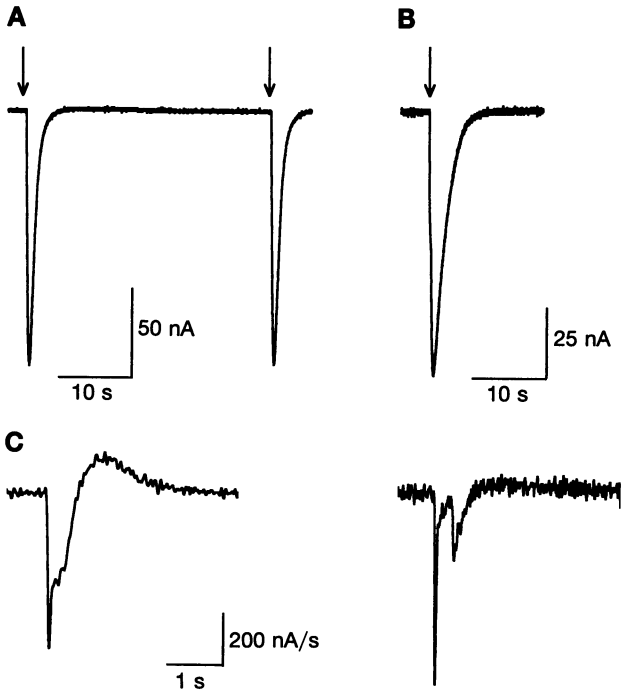

FIG. 6. (*A* and *B*) AcCho currents elicited in an oocyte by focal pulses of AcCho (arrows) applied in the animal (*A*) or vegetal (*B*) hemisphere. (*C*) Derivatives of AcCho currents on an expanded time scale. nAcChoRs were injected in the vegetal hemisphere.

Localization of Incorporated nAcChoRs. The distribution of nAcChoRs incorporated in the plasma membrane was examined to determine whether the incorporation was restricted to the site of injection. In three oocytes in which nAcChoRs were injected near the vegetal pole, AcCho elicited currents in both the animal and vegetal hemispheres (Fig. 6 *A* and *B*), with responses ranging from 7 to 205 nA and from 10 to 150 nA, respectively (10–20 trials per oocyte). Frequently, AcCho elicited a current at one spot but not a few hundred micrometers away. This suggests that nAcChoRs are incorporated in "clusters," consistent with the observation that sometimes the responses were wider in places near the injection point and showed an inflection in their rising phase (Fig. 6*B*). This inflection, presumably caused by AcCho reaching two different clusters of nAcChoRs, is seen better in the differentiated AcCho-current records (Fig. 6*C*).

Since externally applied AcCho elicited membrane currents, it was clear that nAcChoRs had been incorporated with their agonist binding sites on the extracellular side of the oocyte membrane. To see whether receptors were also incorporated with the opposite orientation, AcCho was applied intracellularly in those areas that showed responses to extracellular pulses of AcCho. As in the example shown in Fig. 7, intracellular pulses of AcCho applied just inside three such areas failed to elicit AcCho currents.

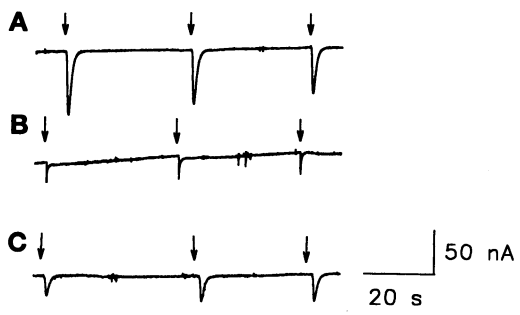

FIG. 7. Orientation of nAcChoRs incorporated into the membrane. (*A*) Local extracellular application of pulses of AcCho in the vegetal hemisphere induced AcCho currents. (*B*) Lack of AcCho current when the injection micropipette was just intracellular. The sharp transients are mechanical artifacts. (*C*) AcCho currents were again evoked when the pipette was withdrawn.

DISCUSSION

nAcChoRs from *Torpedo* electroplaques can be isolated and reconstituted in artificial membranes and still retain their functional properties (25–27). Here we have demonstrated that reconstituted nAcChoRs are incorporated into the *Xenopus* oocyte membrane and retain their functional characteristics. Although the amplitudes of the AcCho currents were sometimes as high as several microamperes, this accounted for only a small fraction of the receptors injected. The number of functional receptors at a given time, estimated from the largest AcCho currents, was a few million when about 6×10^{10} receptors were injected (estimated from the number of α-bungarotoxin binding sites). That is, only about 1 in 10^5 of the receptors injected appeared as a functional receptor in the plasma membrane. Several factors may contribute to this difference. (*i*) The maximal current is clearly underestimated because of receptor desensitization; and the single-channel conductance of the reconstituted receptor could be smaller than assumed. (*ii*) Some of the injected receptors may be sequestered or incorporated into intracellular organelles or may be rapidly degraded after injection. (*iii*) Some of the injected receptors may also be silent—i.e., nonfunctional, as has been proposed for muscle nAcChoRs expressed after mRNA injection (5). (*iv*) Receptors incorporated into the plasma membrane could have a fast turnover, even though AcCho currents could still be elicited 2–3 days after the injection. More work is required to examine these and other possibilities, but it is already evident that sufficient receptors are incorporated to allow their detailed functional study.

The properties of the reconstituted and incorporated nAcChoRs were similar to those previously described for *Torpedo* nAcChoRs (15). Thus, the Hill coefficient suggests that two molecules of AcCho need to bind to a receptor to open the channel, and the reversal potential of −5 mV indicates that the channel permeability is also similar to that previously described for *Torpedo* nAcChoRs in oocytes (3, 20). That is, the AcCho current seems to be carried mostly by Na^+ and K^+ ions, although some Ca^{2+} also permeates the channel. Further, the *I/V* relation obtained during the maintained AcCho current elicited by a low dose of AcCho shows a reversal potential similar to that of the peak current, indicating a similar ionic selectivity in both phases of the response. On the other hand, the nonlinearity of nAcChoR currents observed in some oocytes at potentials more negative than −60 mV was more evident than previously reported for *Torpedo* cDNA-derived receptors (17). A similar nonlinear *I/V* relation observed in muscle nAcChoR channels expressed in oocytes has been ascribed to a voltage dependency of the channel mean open time (5, 17). The reason underlying the high variability in the *I/V* relation still remains to be determined.

Like the native *Torpedo* nAcChoRs or those expressed in *Xenopus* from mRNA, the reconstituted receptors also showed desensitization. Interestingly, the effects of antibiotics such as gentamicin or penicillin/streptomycin on AcCho currents (24) were also reproduced by the reconstituted receptors. Therefore, the antibiotic effect appears to be due to a direct or mediated action on the receptor itself, rather than to any alteration in the receptor processing by the cell. Since trehalose by itself did not appreciably alter nAcChoR desensitization, it appears that this freezing-protection agent may be acting on the same mechanism affected by the antibiotics.

nAcChoRs expressed in oocytes from their mRNA are oriented correctly in the oocyte membrane and are not randomly distributed at the cell surface (4). Similarly, the reconstituted nAcChoRs incorporated into the oocyte plasma membrane appeared in small patches (clusters) distributed in both hemispheres. Nevertheless, more clusters appeared near the site of injection, probably due to the higher concentration of receptor-containing vesicles in this zone. Although most of the receptors seemed to incorporate with the right-side-out orientation, at present we cannot completely exclude the possibility of a "wrong" orientation of a small number of receptors.

The results presented here confirm previous data on *Torpedo* nAcChoRs and suggest the use of oocytes for studying the function and regulation of single membrane proteins (enzymes, channels, or receptors) in a highly amenable host cell system. In addition, injection of reconstituted proteins instead of their coding cDNA or mRNA will be very useful in cases where changes in the posttranslational processing due to the oocyte may result in an absence or alteration of function (28). Finally, oocytes arise as a suitable system for studying the mechanisms involved in membrane fusion processes within a live cell through the use of specific lipid vehicles containing an easily detectable protein.

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