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Tunicamycin Increases Desensitization of Junctional and Extrajunctional Acetylcholine Receptors Expressed in *Xenopus* Oocytes by a Mechanism Independent of *N*-Glycosylation Blocking

TOMOYUKI NISHIZAKI and KATUMI SUMIKAWA

Department of Psychobiology, University of California, Irvine, California 92717-4550

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SUMMARY

Extrajunctional and junctional mouse muscle acetylcholine receptors (AChRs) expressed in *Xenopus* oocytes in the presence of tunicamycin desensitized more rapidly than the corresponding AChRs synthesized in the absence of tunicamycin. The two types of AChR expressed in non-tunicamycin-treated oocytes could be distinguished by their different rates of desensitization, but tunicamycin diminished this difference. The effect of tunicamycin on the AChR desensitization appeared to be reversible, and coapplication of tunicamycin with acetylcholine (ACh) also caused a similar effect on desensitization of these AChRs, suggesting that the effect of tunicamycin was mediated by a mechanism independent of *N*-glycosylation blocking. In addition, tunicamycin increased the amplitude of membrane current elicited by application of lower doses of ACh and accelerated the rate of desensitization, which suggests that tunicamycin favors an open channel state and, therefore, accelerates transition towards a desensitized state. Tunicamycin also increased the membrane current decay elicited by ACh in oocytes expressing incomplete AChRs, missing the β, γ, ϵ, or δ subunits.

The role of *N*-glycosylation in the expression of functional nicotinic AChRs has been studied (1–4) by the use of tunicamycin, an inhibitor of protein *N*-glycosylation (5), and by the expression in *Xenopus* oocytes of mutant AChRs lacking potential *N*-glycosylation sites (6, 7). In muscle cells, it was shown that tunicamycin reduced the appearance of functional AChRs in the plasma membrane by either blocking the assembly of AChR subunits (3) or increasing the rate of degradation of AChR molecules (2). In oocytes microinjected with *Torpedo* mRNAs and treated with tunicamycin, the expression of functional AChRs in the plasma membrane was reduced. This appeared to be due to retention of non-*N*-glycosylated oligomeric AChR complexes in intracellular compartments (4). A study using mutant subunits, lacking the conserved *N*-glycosylation site, also found that *N*-glycosylation appeared to contribute to stability of the subunits and/or efficient insertion of assembled subunits into the plasma membrane (7).

In oocytes injected with *Torpedo* AChR mRNAs and treated with external tunicamycin alone, *N*-glycosylation was only partially blocked (4). Therefore, in these oocytes relatively large membrane current responses to ACh could still be observed. This was presumably due to the expression of *N*-glycosylated AChRs in the plasma membrane. Interestingly, the membrane currents elicited in theses oocytes appeared to decay more rapidly than those in non-tunicamycin-treated oocytes.[1] Recently, however, it has been reported that tunicamycin increases desensitization of AChRs in cultured mouse muscle cells by blocking *N*-glycosylation (8), suggesting insertion of functional non-*N*-glycosylated AChRs in the plasma membrane. Thus, we were prompted to assess the effect of tunicamycin on the membrane currents elicited by ACh in oocytes expressing mouse muscle AChRs. These studies culminated in the finding that tunicamycin accelerated desensitization of both junctional and extrajunctional muscle AChRs, expressed in oocytes, by a mechanism independent of blocking of *N*-glycosylation.

Materials and Methods

***In vitro* transcription.** Full-length cDNA coding for the ϵ subunit of mouse AChRs was isolated by the polymerase chain reaction, as described elsewhere,[2] and was subcloned into the transcription vector

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[1] K. Sumikawa, R. Woodward, and R. Miledi, unpublished observations.
[2] A. Morales and K. Sumikawa. Desensitization of junctional and extrajunctional nicotinic ACh receptors expressed in *Xenopus* oocytes. Manuscript submitted for publication.

ABBREVIATIONS: AChR, acetylcholine receptor; ACh, acetylcholine.

pGEM4Z. The resulting plasmid DNA was used to synthesize capped mouse ϵ subunit mRNA (4, 9–11). Plasmids BMA 407, BMB 49, BMG 419, and BMD 451 (kindly provided by J. Boulter and S. Heinemann, Salk Institute) were also used as templates for the synthesis of the mouse α, β, γ, and δ subunit mRNAs, respectively.

Translation of mRNA in *Xenopus* oocytes. Collagenase treatment of oocytes was carried out, to remove the follicular cell layer, 1 day before microinjection (12). Oocytes were injected (about 40 nl) with various combinations of AChR subunit mRNAs (12), as indicated. Injected oocytes were incubated at 16–18° in Barth's solution without any antibiotics (12). In some experiments, injected oocytes were incubated in the presence of tunicamycin (2 μg/ml) for about 48 hr. In the present study, oocytes were not pretreated with external tunicamycin (2 μg/ml) for 24 hr before injection, unlike in previous experiments (4, 13).

Electrophysiology. Two days after mRNA injection, oocytes were transferred to a recording chamber, and electrophysiological recordings were made using a conventional two-electrode voltage/clamp technique, in a bath continuously superfused with frog Ringer's solution (12). ACh, with or without tunicamycin, was applied by bath perfusion, at a flow rate of 18 ml/min. Oocytes were clamped at −60 mV. The membrane currents evoked by ACh in the presence or absence of tunicamycin were digitized and stored on hard disk for subsequent analysis. The decay of ACh-evoked current consists of the sum of two exponential curves, the fast component (nonlinear regression) and the slow component (linear regression). Relative fast (τ_f) and slow (τ_s) time constants of the current decay were measured by using a laboratory computer. The statistical analyses of the data were performed by using Student's t test.

Results and Discussion

All of our previous observations with tunicamycin (4, 13) were made using oocytes cultured in medium with gentamicin, which was later found to accelerate the membrane current decay elicited by ACh (14). Thus, in the present study all experiments were carried out using oocytes cultured in the absence of gentamicin. Initially, oocytes were injected with a mixture of all four mouse junctional (α, β, ϵ, and δ) or extrajunctional (α, β, γ, and δ) AChR mRNAs, cultured either in the presence or in the absence of tunicamycin (2 μg/ml) for 48 hr, and then assayed electrophysiologically for membrane current responses elicited by ACh. Because the external application of tunicamycin alone was only partially effective in blocking N-glycosylation in oocytes (4) (also see Ref. 15), we expected to see relatively large (but smaller than in control oocytes) ACh-evoked membrane currents in tunicamycin-treated oocytes. However, the amplitudes of the currents elicited with 10^{-4} M ACh in tunicamycin-treated oocytes were unexpectedly large and similar to those produced in control oocytes, regardless of injected mRNAs (Fig. 1, A and B). Because N-glycosylation was required for the expression of functional AChRs in the plasma membranes of muscle cells and oocytes (1–4), these results indicate that tunicamycin failed to block N-glycosylation of either junctional or extrajunctional AChRs under the conditions used (see Materials and Methods). Interestingly, although membrane currents elicited by extended exposure to ACh desensitized with at least two components in both control and tunicamycin-treated oocytes, ACh responses in tunicamycin-treated oocytes desensitized more rapidly than those in control oocytes, regardless of expressed receptor types (Fig. 1, A and B; Table 1). Because tunicamycin-treated oocytes were kept in a recording chamber continuously superfused with frog Ringer's solution, without tunicamycin, for a maximum of

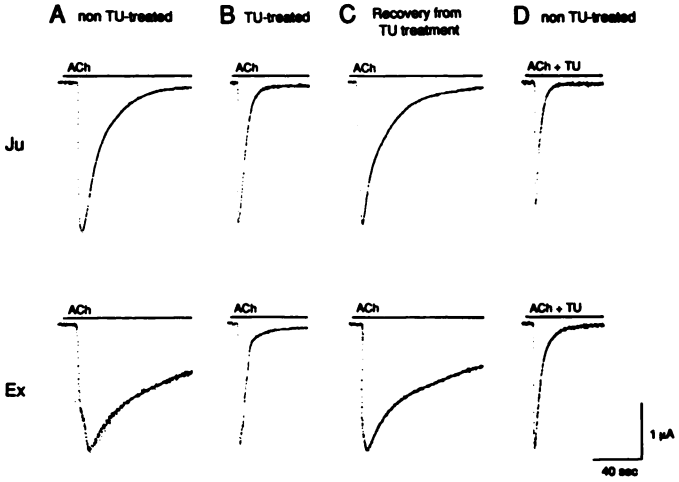

Fig. 1. Effects of chronic and acute treatment with tunicamycin (*TU*) on desensitization of junctional and extrajunctional AChRs. *Upper*, oocytes injected with junctional AChR mRNAs (a mixture of α, β, ϵ, and δ subunit mRNAs); *lower*, oocytes injected with extrajunctional AChR mRNAs (a mixture of α, β, γ, and δ subunit mRNAs). Microinjected oocytes were incubated in the absence (A and D) or presence (B) of tunicamycin (2 μg/ml) for 48 hr. C, Injected oocytes were incubated with tunicamycin (2 μg/ml) for 48 hr and then washed in frog Ringer's solution for 1 hr before electrophysiological recordings. ACh (10^{-4} M), with (D) or without (A, B, and C) tunicamycin (2 μg/ml), was applied by bath perfusion for the times indicated by the *bars*. In this and all following figures, inward membrane currents correspond to downward deflections.

TABLE 1

Effects of tunicamycin on the time constants of the current decay elicited by high doses of ACh in oocytes expressing junctional and extrajunctional AChRs

The time constants were measured from current responses similar to those in Fig. 1. Each value represents the mean ± standard deviation (n = 5).

	Junctional	Extrajunctional
τ_f (sec)		
Control	24.33 ± 4.01	56.53 ± 6.32
Rapid tunicamycin	6.82 ± 1.68	6.26 ± 1.79
Prolonged tunicamycin	8.76 ± 0.70	7.01 ± 0.48
Recovery	20.50 ± 2.92	55.63 ± 13.48
τ_s (sec)		
Control	48.37 ± 4.28	90.53 ± 12.65
Rapid tunicamycin	28.50 ± 3.80	33.51 ± 10.98
Prolonged tunicamycin	22.30 ± 9.66	85.44 ± 18.18
Recovery	43.21 ± 3.10	82.17 ± 18.52

5 min before ACh applications, the effect of tunicamycin appeared not to be rapidly reversible. However, when tunicamycin-treated oocytes were washed for about 1 hr before electrophysiological recording, the membrane currents elicited in these oocytes decayed like those in control oocytes (Fig. 1, A and C; Table 1). Thus, the effect of tunicamycin on the current decay appeared to be reversible, and this also suggests that the observed effect was not due to lack of N-glycosylation. Further evidence supporting this conclusion was obtained by acute treatment with tunicamycin. As shown in Fig. 1, A and D, the membrane currents elicited by coapplication of tunicamycin with ACh desensitized more rapidly than those produced by ACh alone. No significant differences in the time constants of the current decay between acute and chronic treatments of tunicamycin were observed, except for the slow time constant, with extrajunctional AChRs (Table 1). In addition, the effect caused by acute treatment of tunicamycin was rapidly reversible (data not shown), unlike chronic treatment with tunicamycin.

To investigate the dose-dependent effect of tunicamycin on AChR desensitization, different concentrations of tunicamycin were applied together with 10^{-4} M ACh, and the membrane currents elicited were recorded (data not shown). In both junctional and extrajunctional AChRs, the fast time constant of the current decay was dose-dependently reduced (Fig. 2A), whereas for the slow time constant such dose dependency was not found (Fig. 2B).

We have previously found that junctional AChRs expressed in oocytes in the presence of gentamicin appeared to desensitize more rapidly than extrajunctional AChRs synthesized in the presence of gentamicin.[2] The difference in desensitization between the two types of AChR still persisted in the absence of gentamicin (Fig. 1A; Table 1), although the current decay elicited in non-gentamicin-treated oocytes was slower than that in gentamicin-treated oocytes. However, in tunicamycin-treated oocytes, junctional AChRs desensitized like extrajunctional AChRs (Fig. 1B). There was also no significant difference in fast and slow time constants between junctional and extrajunctional AChRs expressed in oocytes acutely treated with tunicamycin, at any concentration used (Figs. 1D and 2; Table 1), although the slow time constant for extrajunctional AChRs expressed in oocytes chronically treated with tunicamycin was slower than that for junctional AChRs treated in a similar manner. It has been established that the two types of AChR differ in their subunit structures. The junctional AChR consists of α, β, ϵ, and δ subunits, whereas the extrajunctional AChR is composed of α, β, γ, and δ subunits (16–18). Thus, it appears that structural differences between the ϵ and γ subunits were responsible for the different rates of desensitization and that tunicamycin diminished the differences in desensitization of the two AChR types by accelerating the rates of desensitization of both AChR types to similar levels.

Subsequently, we examined the effect of acute tunicamycin treatment on the current decay evoked by lower doses (10^{-6} M) of ACh. When lower ACh doses alone were applied to oocytes expressing either junctional or extrajunctional AChRs, the ACh-activated membrane currents were well maintained (Fig. 3). However, coapplication of ACh with tunicamycin induced the fast component of the current decay in these oocytes (Fig. 3; Table 2), and junctional AChRs desensitized more rapidly than extrajunctional AChR ($p<0.001$). In addition, the amplitudes of the membrane currents elicited in oocytes expressing junctional or extrajunctional AChRs increased to 133% ($p<0.01$) or 164% ($p<0.001$) of control, respectively (Fig. 3; Table 2). The enhancement in desensitization rates and current amplitudes was also observed when tunicamycin was applied together with ACh to AChRs that had previously been activated by ACh alone (Fig. 3C). These results suggest that tunicamycin favors an open channel state and accelerates a transition towards a desensitized state.

In an attempt to determine the tunicamycin binding site on AChRs, various incomplete AChR molecules, missing one subunit, were expressed in oocytes, and the rates of desensitization

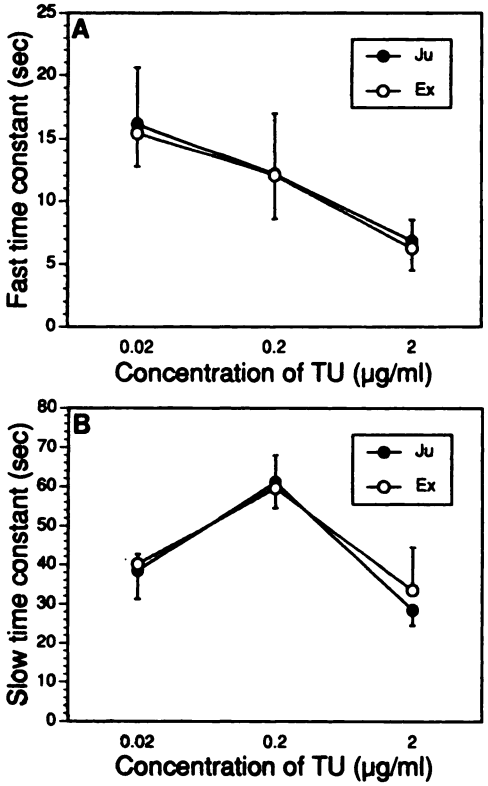

Fig. 2. Dose-dependent effects of tunicamycin (*TU*) on the time constant of the membrane current decay elicited by ACh in oocytes expressing either junctional or extrajunctional AChRs, showing the effects of tunicamycin on the fast (A) and slow (B) time constants. Oocytes were injected with either junctional (*Ju*) or extrajunctional (*Ex*) AChR mRNAs and cultured in the absence of tunicamycin for 48 hr. Responses elicited by ACh (10^{-4} M) with different concentrations of tunicamycin were measured from records similar to those in Fig. 1. Each *point* represents the average from five oocytes, and standard deviation is shown by the *bar*.

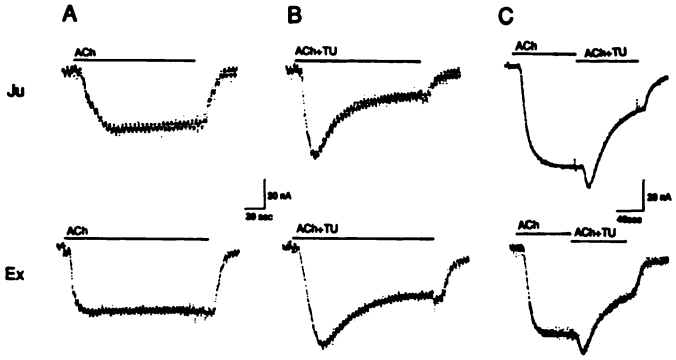

Fig. 3. Effects of tunicamycin (*TU*) on the current response elicited by lower doses (10^{-6} M) of ACh. *Upper*, oocytes injected with junctional AChR mRNAs; *lower*, oocytes injected with extrajunctional AChR mRNAs. Microinjected oocytes were incubated in the absence of tunicamycin for 48 hr before electrophysiological recordings. ACh (10^{-6} M), with or without tunicamycin (2 μg/ml), was applied as indicated. The current responses in A and B were obtained from the same oocytes by repeated exposures to Ach without (A) or with (B) tunicamycin at 5-min intervals of wash.

TABLE 2

Effects of tunicamycin on desensitization of junctional and extrajunctional AChRs induced by 10^{-6} M ACh

The time constants were determined from current responses similar to those in Fig. 3. Each value represents the mean ± standard deviation (n = 5).

	Junctional	Extrajunctional
τ_f (sec)		
Control	—	—
Tunicamycin	28 ± 6.5	115 ± 24
I_{peak} (nA)		
Control	63 ± 7.9	71 ± 8.6
Tunicamycin	83 ± 7.7	116 ± 13

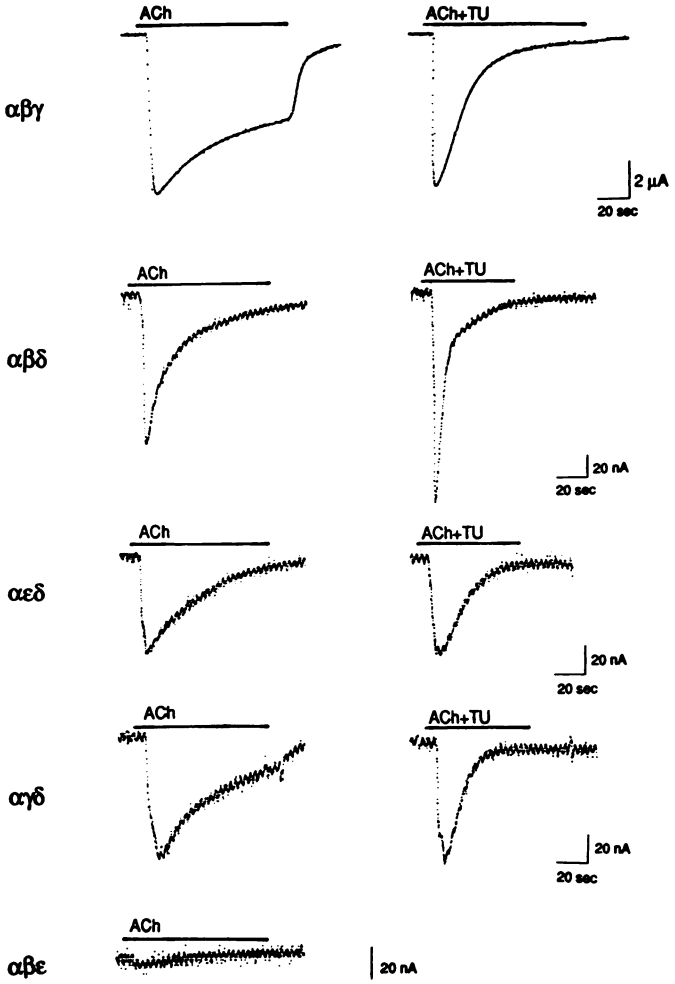

Fig. 4. Desensitization of incomplete AChR molecules expressed in oocytes. Oocytes were injected with mRNAs as indicated and were incubated as in Fig. 3. ACh (10^{-4} M), with or without tunicamycin (*TU*) (2 μg/ml), was applied as indicated. Responses on the *left* and *right* were obtained from the same oocytes.

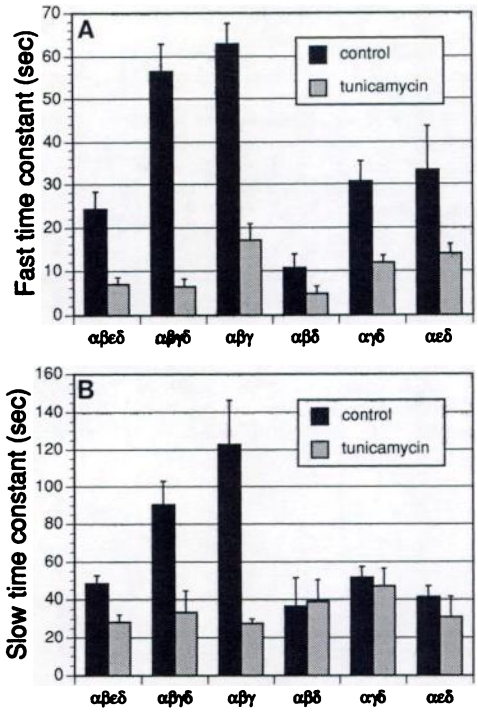

Fig. 5. Effects of tunicamycin on the time constant of the current decay elicited in oocytes expressing complete or incomplete AChR molecules. Various different AChR molecules were expressed in oocytes, as indicated. Responses elicited by ACh (10^{-4} M), with or without tunicamycin (2 μg/ml), were measured from records similar to those in Figs. 1 and 4, and the effects of tunicamycin on fast (A) and slow (B) time constants are presented as the mean ± standard deviation from five oocytes.

of these AChR molecules, in the absence and presence of tunicamycin, were measured. The amplitudes of membrane currents elicited by ACh in oocytes expressing these incomplete AChR molecules varied. The largest responses were obtained from oocytes injected with α, β, and γ subunit mRNAs, whereas oocytes expressing α, β, and ε subunits gave almost no responses (Fig. 4). The ACh-evoked membrane current decay also varied among incomplete AChR molecules. The fast phase of the current decay was decreased in the order of αβγ (the greatest decrease), αβγδ, αεδ, αγδ, αβεδ, and αβδ (Figs. 4 and 5). The slow phase of the current decay was also reduced, in similar order (Figs. 4 and 5). The coapplication of tunicamycin accelerated desensitization of all incomplete AChRs to different degrees (Figs. 4 and 5), which may suggest that the primary binding site of tunicamycin is on the α subunit. However, we are unable to exclude the possibility that tunicamycin alters desensitization by interacting with membrane components surrounding AChRs. It is notable that the fast component of the current decay elicited in oocytes expressing αβεδ, αβγδ, or αβδ was more rapid than that in oocytes synthesizing αβγ, αγδ, or αεδ ($p<0.001$) (Fig. 5A). The presence of the β and δ subunits appeared to be required for a maximum effect of tunicamycin on AChR desensitization. In contrast to the fast component, there was no significant difference, among these AChR molecules, in the slow time constant of the current decay elicited by ACh together with tunicamycin (Fig. 5B).

The results presented here clearly demonstrate that tunicamycin enhances the rates of desensitization of junctional and extrajunctional AChRs without blocking *N*-glycosylation, which is the most widely known action of tunicamycin. One possible explanation for the effect could be that tunicamycin alters channel-gating kinetics, such as the channel mean open time and/or the frequency of channel opening. We are currently testing this possibility with single-AChR channel recordings, using the patch-clamp technique.

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References

1. Fambrough D. M. Control of acetylcholine receptors in skeletal muscle. *Physiol. Rev.* **59**:165–227 (1979).
2. Prives, J. M., and K. Olden. Carbohydrate requirement for expression and stability of acetylcholine receptor on the surface of embryonic muscle cells in culture. *Proc. Natl. Acad. Sci. USA* **77**:5263–5267 (1980).
3. Merlie, J. P., R. Sebbane, S. Tzartos, and J. Lindstrom. Inhibition of glycosylation with tunicamycin blocks assembly of newly synthesized acetylcholine receptor subunits in muscle cells. *J. Biol. Chem.* **257**:2694–2701 (1982).
4. Sumikawa, K., and R. Miledi. Assembly and *N*-glycosylation of all ACh receptor subunits are required for their efficient insertion into plasma membranes. *Mol. Brain Res.* **5**:183–192 (1989).
5. Tkacz, J. S., and J. O. Lampen. Tunicamycin inhibition of polyisoprenyl *N*-acetylglucosaminyl pyrophosphate formation in calf-liver microsomes. *Biochem. Biophys. Res. Commun.* **65**:248–257 (1975).
6. Mishina, M., T. Tobimatsu, K. Imoto, K. Tanaka, Y. Fujita, K. Fukuda, M. Kurasaki, H. Takahashi, Y. Morimoto, T. Hirose, S. Inayama, T. Takahashi, M. Kuno, and S. Numa. Location of functional regions of acetylcholine

receptor α-subunit by site-directed mutagenesis. *Nature (Lond.)* **313**:364–369 (1985).
7. Gehle, V. M., and K. Sumikawa. Site-directed mutagenesis of the conserved *N*-glycosylation site on the nicotinic acetylcholine receptor subunits. *Mol. Brain Res.* **11**:17–25 (1991).
8. Giovannelli, A., F. Grassi, F. Eusebi, and R. Miledi. Tunicamycin increases desensitization of acetylcholine receptors in cultured mouse muscle cells. *Proc. Natl. Acad. Sci. USA* **88**:1808–1811 (1991).
9. Melton, D. Injected anti-sense RNAs specifically block messenger RNA translation *in vitro*. *Proc. Natl Acad. Sci. USA* **82**:144–148 (1985).
10. White, M. M., K. M. Mayne, H. A. Lester, and N. Davidson. Mouse-*Torpedo* hybrid acetylcholine receptors: functional homology does not equal sequence homology. *Proc. Natl. Acad. Sci. USA* **82**:4852–4856 (1985).
11. Kurosaki, T., K. Fukuda, T. Konno, Y. Mori, K. Tanaka, M. Mishina, and S. Numa. Functional properties of nicotinic acetylcholine receptor subunits expressed in various combinations. *FEBS Lett.* **214**:253–258 (1987).
12. Sumikawa, K., I. Parker, and R. Miledi. Expression of neurotransmitter receptors and voltage-activated channels from brain mRNA in *Xenopus* oocytes. *Methods Neurosci.* **1**:30–45 (1989).
13. Sumikawa, K., I. Parker, and R. Miledi. Effect of tunicamycin on the expression of functional brain neurotransmitter receptors and voltage-operated channels in *Xenopus* oocytes. *Mol. Brain Res.* **4**:191–199 (1988).
14. Okamoto, T., and K. Sumikawa. Antibiotics cause changes in the desensitization of ACh receptors expressed in *Xenopus* oocytes. *Mol. Brain Res.* **9**:165–168 (1991).
15. Colman, A. Translation of eukaryotic messenger RNA in *Xenopus* oocytes, in *Transcription & Translation, A Practical Approach* (B. D. Hames and S. J. Higgins, eds.). IRL Press, Oxford, UK, 271–302 (1984).
16. Mishina, M., T. Takai, K. Imoto, M. Noda, T. Takahashi, S. Numa, C. Methfessel, and B. Sakmann. Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. *Nature (Lond.)* **321**:406–411 (1986).
17. Gu, Y., and Z. W. Hall. Immunological evidence for a change in subunits of the acetylcholine receptor in developing and denervated rat muscle. *Neuron* **1**:117–125 (1988).
18. Witzemann, V., B. Barg, M. Criado, E. Stein, and B. Sakmann. Developmental regulation of five subunit specific mRNAs encoding acetylcholine receptor subtypes in rat muscle. *FEBS Lett.* **242**:419–424 (1989).

Send reprint requests to: Dr. K. Sumikawa, Department of Psychobiology, University of California, Irvine, CA 92727-4550.