Korean J Biol Sci 3: 181-185, 1999

Improved Method for Heterologous Expression of Ion Channels in *Xenopus* Oocyte: a PCR Shortcut to Oocyte Expression

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Key Words:
Xenopus oocyte
Heterologous expression
cRNA
Nuronal nicotinic receptor
α_7 subtype

***Xenopus* oocyte is one of the widely used heterologous expression systems of ion channels for electrophysiological studies. Here we describe a new method in which cRNA produced by polymerase chain reaction (PCR) and *in vitro* transcription is injected to express ion channels in oocytes. This method enables us (1) to eliminate all or a part of the untranslated region of the cDNA and to replace it with a known sequence which helps increase the expression level in oocytes, and (2) to use the PCR product for *in vitro* transcription without subcloning. Using this method, the expression level of one of the neuronal nicotinic acetylcholine receptors (nAChRs) α_7 subtype in oocytes was systematically increased by more than 100-fold, which was confirmed both by the α-Bungarotoxin (αBgt) binding assay and the current measurement.**

Xenopus oocyte is a convenient expression system of the cDNA encoding an ion channel or a receptor for electrophysiological studies (Quick and Lester, 1994). The most common method of introducing cDNAs of ion channels is the cytoplasmic injection of RNA transcripts of cDNAs, so called cRNAs. *In vitro* synthesis of cRNA is carried out using a viral polymerase, so it requires cloning or subcloning a cDNA into a vector containing a viral promoter such as the T7 or SP6 promoter. Occasionally, a high level expression of an ion channel is not achieved although the cRNA is properly sized and faithfully translated in oocytes. This may result (1) from the instability of the transcripts, (2) from the presence of an incorrect sequence context surrounding the transcript initiation site or (3) from unfavorable 5′- and/or 3′-untranslated regions. Polymerase chain reaction (PCR) has been demonstrated to be useful to remove and/or modify the unfavorable untranslated sequences, and be able to provide *in vitro* transcript templates without further subcloning (Jobling and Gehrke, 1987; Figl et al., 1992).

We examined a new method, *in vitro* transcription of the PCR-amplified cDNA, to improve the expression level of an ion channel in oocytes, using the neuronal α_7 nicotinic acetylcholine receptor (nAChR) as a model since the expression level of the avian α_7 nAChR was low in many heterologous expression systems (Bertrand et al., 1990). The avian α_7 nAChR has some unique features: (1) the α subunit forms a homo-oligomeric channel and (2) the channel is blocked by α-Bungarotoxin (αBgt), a toxin shown to block muscle-type nAChRs. The homo-oligomeric channel formation eliminates the need for coinjection of non-α subunit transcripts to form a functional channel. The high affinity αBgt binding site present on the α_7 receptor was utilized for a binding assay. In this study, special attention was given to the substitution of 5′ and 3′ untranslated sequences with the alfalfa mosaic virus RNA 4 5′-leader sequence (AMV sequence) and with the poly(A) sequence, respectively.

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Materials and Methods

Materials and chemicals

The avian clone of the nAChR α_7 subtype was provided by Dr. Lester of the California Institute of Technology (Couturier et al., 1990), and subcloned into the *Eco*RI site of the pBSII-SK vector (Stratagen). The primers used in PCR were custom-synthesized and purified (Korea Biotech). The sequence of a typical primer used to replace the 5′-untranslated region of the α_7 cDNA was, 5′-GAGCTCGAGCTCATTTAGGTGACACTATATTTCAAATACTTCCGCCATGGGCCTCCGGGCG-3′, which was composed of two consecutive *Sac*I recognition sequences for subcloning if necessary, SP6 promoter, a various length of the 5′-untranslated sequence of the AMV sequence plus the 9-base "Kozak" sequence (CCGCCATGG) (Kozak, 1991), followed by the remaining 11-base long coding sequence of the cDNA. A typical primer used to replace the 3′-untran-

slated region was 5′-GAGCTCGAGCTCTTT----TTTCC-CCTTCTGAAAGGGTGCTCC-3′, which was composed of two *Sac*I sites, vargying lengths of the poly(T) tail, followed by the 21-base long coding sequence including the stop codon of the cDNA. Acetylcholine chloride, (-)nicotine, flufenamic acid, and $BaCl_2$ were purchased from Sigma. [^{125}I]Tyr^{54}-αBgt was purchased from NEN.

PCR and in vitro transcription

PCR was conducted in a DNA thermal cycler (model 480, Perkin Elmer) according to the manufacturer's manual. The annealing temperature was 60℃, and the polymerization was repeated for 25 cycles. The resulting PCR product was gel-purified with the Qiaex extraction kit (Qiagen) and then used to synthesize cRNAs using the Ambion *in vitro* transcription kit with SP6 RNA polymerase (Ambion).

Electrophysiology

Stage IV-V of *Xenopus* oocytes were prepared as previously described (Quick and Lester, 1994). An oocyte was injected with 5-20 ng cRNAs, and maintained at 20℃ in an incubation medium of ND96^{++} (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 1.8 mM $CaCl_2$, 2.5 mM Na-pyruvate, 50 μg/ml gentamicin, pH 7.4-7.6). The cationic inward current elicited by a nicotinic agonist was measured by a standard two electrode voltage clamp method, as previously described (Smith et al., 1980). The drugs were applied to the bathing solution (ND96^{++} without gentamicin and HEPES) by perfusion. In order to eliminate the secondary Ca^{2+}-activated Cl^- current, the oocyte was preincubated in 500 μM flufenamic acid, a general Cl^- channel blocker, for at least 1 h and the recording was made by substituting Ba^{2+} for Ca^{2+} in the bathing solution.

Toxin-binding assay

The number of the α_7 receptors expressed on the surface of an oocyte was measured by a binding assay with [^{125}I] αBgt, a specific antagonist to the α_7 receptor, according to the method described previously (Yoshii et al., 1987). In short, oocytes were prewashed in a solution of 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6, and then placed in the same solution supplemented with 1 mg/ml bovine serum albumin and 5 nM [^{125}I] αBgt for 1 h. Oocytes were then washed four times and counted individually in a gamma counter (total binding). Nonspecific binding was determined from the mock-injected or uninjected oocytes (Yoshii et al., 1987).

Results and Discussion

Experimental designs of a PCR shortcut to oocyte expression

The experimental outline of modifying sequences of the nAChR α_7 subtype using PCR and their expression in *Xenopus* oocytes is depicted in Fig. 1. The α_7 cDNA in the pBSII-SK vector was primed with two primers, the 5′ primer composed of two *Sac*I restriction sites, SP6 promoter, the AMV sequence, Kozak sequence, and 11-base long coding sequence following ATGG at the second codon of the cDNA, and the 3′ primer composed of two *Sac*I sites, the poly(A) sequence, the coding sequence starting from the stop codon in an antisense orientation. The PCR-produced, modified cDNA was then *in vitro* transcribed by SP6 RNA polymerase, and the resulting cRNA was injected into oocytes. About 5-20 ng of cRNA was injected per oocyte. This procedure has many features: (1) eliminating or substituting a portion of the α7 sequence is easily carried out with PCR; (2) subcloning prior to *in vitro* transcription is not necessary. The PCR product can be directly used as a template of *in vitro* transcription by virtue of its phage RNA polymerase promoter generated during PCR.

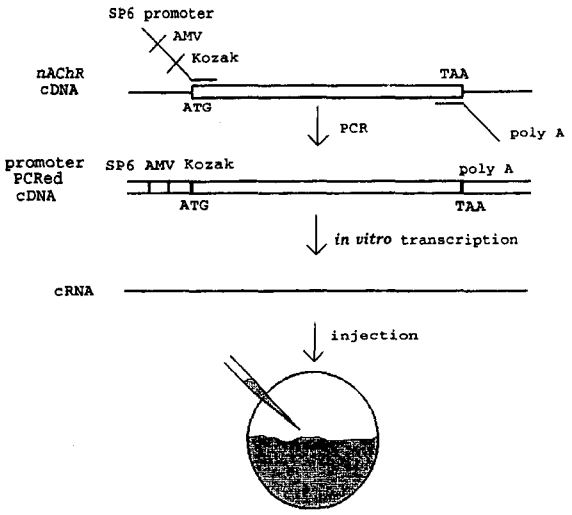

Fig. 1. General scheme of modifying sequences of an ion channel using PCR and its expression in *Xenopus* oocytes. The cDNA of the nicotinic receptor α_7 is modified and amplified by a 5′-primer containing the SP6 RNA polymerase promoter and a consensus ribosomal binding site and a 3′-primer containing the poly(A) tail. This is followed by a direct *in vitro* transcription of the PCR product and injection of the resulting mRNAs into oocytes. This method was proven to increase the expression level of the α_7 receptor by more than 100-fold.

Correlation between the number and the current of nAChR α_7

Three to four days after the cRNA injection, the number of the nAChR α_7 subtype expressed on the surface of an oocyte was determined by binding to 5 nM [^{125}I] α Bgt. The binding of αBgt to the nAChR is known to be almost irreversible with high affnity in the order of pM units. Thus, we chose one point binding in a saturable concentration, and the nonspecific binding was obtained from the mock-injected or uninjected oocyte (Yoshii et

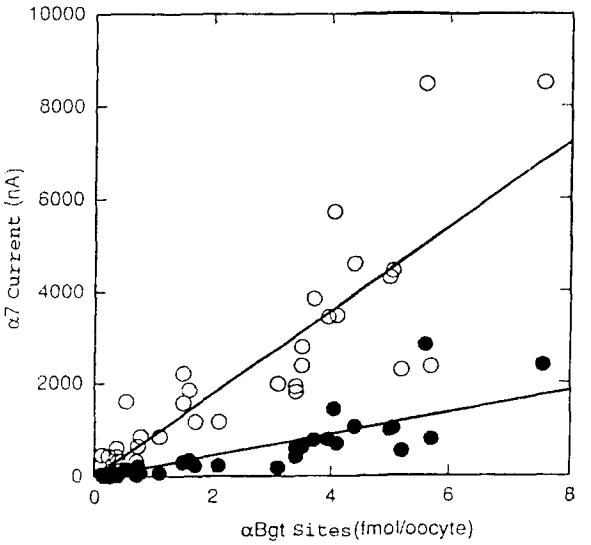

Fig. 2. Correlation between the number of the α_7 receptors expressed and the α_7 current. Agonist-induced responses of oocytes expressing the α_7 receptors were recorded under two electrode voltage clamps at a holding potential of -80 mV. Currents were elicited either by application of 10 μM ACh (●) or by 100 μM nicotine (○). The number of the α_7 receptors expressed on the surface of an oocyte was determined by binding with [^{125}I] αBgt.

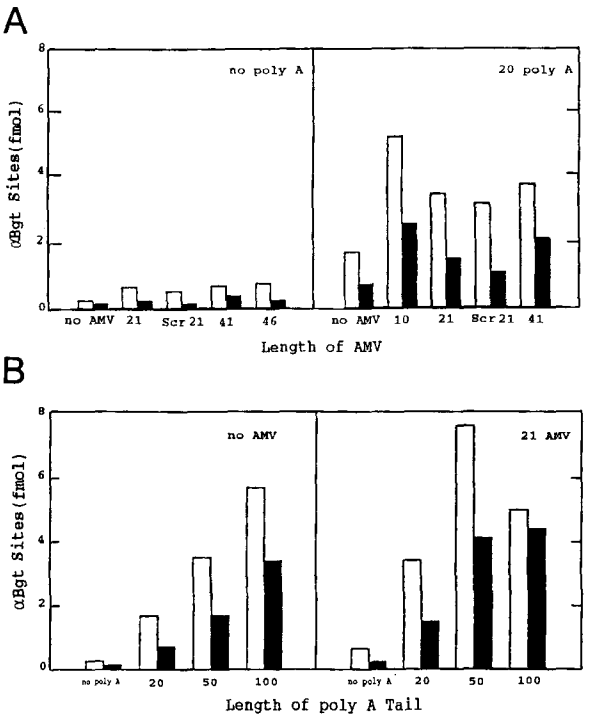

Fig. 3. Profile of the translational efficiency of the α_7 receptors in *Xenopus* oocytes. The numbers of the α_7 receptors expressed in oocytes were plotted as a function of the length of the alfalfa mosaic virus RNA 4 leader sequence (AMV sequence) of panel A and as a function of the length of the poly(A) tail of panel B. Twenty ng (open bars) and 5 ng (closed bars) mRNAs were injected pairwise. Scrambled 21 base AMV sequences (scr 21) represented the 21-base AMV sequence that is randomly mixed except for the first 5 bases (CCGCC) to preserve the Kozak sequence. Note also that for pair comparison, no AMV already contains the 9-base Kozak sequence (CCGCCATGG).

al., 1987). The number of the α_7 receptor shown in Fig. 2 was nonspecific binding-subtracted. As shown in Fig. 2, the number of the α_7 receptor per oocyte varied from <0.1 fmole to 7.8 fmoles depending upon the expression level. The α_7 current was elicited either by 10 μM acetylcholine (ACh) or 100 μM (-) nicotine at a holding potential of -80 mV. ACh and (-) nicotine showed similar potency of activating the α_7 receptor since ACh and (-) nicotine gave rise to the same amount of current at the same concentration (data not shown). Among oocytes from the same ovary receiving identical injection, there was a >10-fold range in the conductance induced by a given concentration of an agonist. This variation among oocytes is a general phenomenon in many laboratories that study channels and receptors induced by foreign RNA. This is seen, for instance, with *Torpedo* nAChR (Yoshii et al., 1987). Therefore, we attempted to minimize the cell-to-cell variation in order to draw conclusions. An oocyte was impaled first for the current measurement and then subject to the binding assay. This method may ensure that the number of the α_7 receptor reflects the functional receptors. For instance, an oocyte having 7.8 fmoles of the receptor sites gave rise to 8,200 nA current induced by 100 μM (-) nicotine at -80 mV holding potential, whereas 2.1 moles corresponded to 1,800 nA. The mock-injected or uninjected oocyte gave no significant current (<10 nA).

Translational efficiency of the α_7 receptor in Xenopus oocytes

One way to increase the expression level of a protein in a cell is to introduce a sequence which is known to enhance the translational efficiency. The sequences we chose were the AMV sequence, Kozak sequence, and the poly(A) sequence. The length of the AMV sequence and the poly(A) sequence attached to the α_7 receptor cDNA were systematically varied and the number of the receptor expressed in *Xenopus* oocytes was determined correspondingly. To reduce the artifact due to variation of the amount of cRNA injected, either 20 ng and 5 ng cRNA was injected pairwise. In the upper panel of Fig. 3, the length of the poly(A) sequence was fixed at either 0 or 20 bases, while that of AMV sequence was increased systematically. For fair comparison, it should be noticed that all the AMV sequences in the x-axis already contains a 9-base Kozak sequence. Introducing the Kozak sequence alone significantly increased the translational efficiency compared to the one without the Kozak sequence (<0.1 fmoles *vs.* 0.27 fmoles, data not shown). However, increasing the length of the AMV sequence reflected a 2-3 fold increase in the translational efficiency, and the maximal enhancement was achieved with the AMV sequence as short as 10-20 bases. In contrast, the lower panel (Fig. 3B), where the AMV sequence is fixed either at 0 or 21 bases while the poly (A) sequence was increased in length, showed the linear

dependence of the translational efficiency on the length of the poly(A) tail. Without any modification in either of the 5′- and 3′-untranslated sequence of the α_7 cDNA, the expression level was negligible with a maximum of 5-10 nA current and <0.1 fmole of receptors per oocyte. By replacing the 5′- and 3′-untranslated sequences with varying lengths of the AMV/Kozak and the poly(A) tail, respectively, we were able to increase the expression level of the α_7 receptor by more than 100-fold in *Xenopus* oocytes (from <0.1 fmole up to 7.8 fmoles α Bgt sites or from 5-10 nA to 8,500 nA current). The AMV sequence has been shown to enhance translation of a chimeric mRNA containing it by 35-fold *in vitro* (Jobling and Gehrke, 1987). In this study, a maximum of 10-fold increase in the translation efficiency *in vivo* was demonstrated by introducing the AMV and the Kozak sequences at a fixed poly(A) tail. The other point we should mention here is that the scrambled AMV sequence (*scr* AMV in Fig. 3A) was equally potent for enhancing the translational efficiency. So the order of bases of the AMV sequence did not seem to be important for the proper function of the sequence. On the 3′-side of the transcript, it has been shown that *Xenopus* uses the length of the poly(A) tail as a trigger for mRNA translation during the egg maturation. Cytoplasmic polyadenylation of the message accompanies an increase in its translation, and depolyadenylation stops the translation (Bachvarova, 1992). Thus, introducing the poly(A) tail by PCR will enhance the rate of translation or increase the stability of the message. Poly(A) tails of 50-100 bases in length, for instance, increased the translational efficiency by about 20-fold (Fig. 3B). Thus, this method will be especially helpful when expressing a cDNA which contains a number of initiation codons (pseudo initiation codons) in the 5′-side and no polyadenylation consensus sequence in the 3′-side of the cDNA.

Characterization of the α_7 current

The α_7 channel has a greater Ca^{2+} permeability than any other nAChRs. The relative permeability ratio of Ca^{2+} to Na^+ (P_{Ca}/P_{Na}) has been shown to be about 20 (Segual et al., 1993). *Xenopus* oocytes are known to have endogenous Ca^{2+}-activated Cl^- channels (Uezono et al, 1993). To block the secondary Ca^{2+}-activated Cl^- current, a recording was made in the absence of external Ca^{2+} (substituted by Ba^{2+}), and oocytes were preincubated in 500 µM flufenamic acid, a general Cl^- channel blocker. The α7 current was elicited by 30 µM ACh at -40 mV. Fig. 4A showed that flufenamic acid eliminated the secondary Cl^- current without reducing the peak current (145 nA *vs.* 130 nA). If the blockers were completely washed out, the current recovered to nearly the same amplitude seen in the absence of the blocker. Under this condition, the current-voltage relation was linear and the reversal potential was about 10 mV, indicating that there was very little Cl^- current

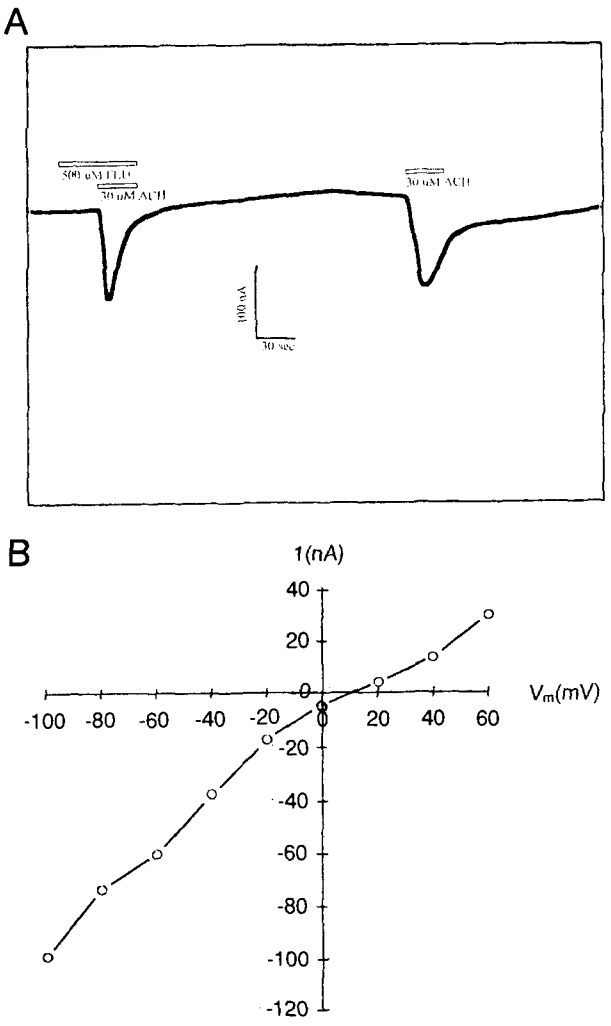

Fig. 4. ACh-induced current in the absence or presence of Ca^{2+}-activated chloride channel blocker flufenamic acid. A, Current induced by 30 µM ACh at a holding potential of -40 mV were shown from an oocyte expressing the α_7 receptors bathed in Ca^{2+}-free $ND96^{++}$ solution where 3 mM Ba^{2+} was substituted for Ca^{2+}. A set of oocytes were preincubated in 500 µM flufenamic acid for at least 1 h before a recording was made. B, The peak current elicited by 10 µM ACh was measured at different holding potentials and plotted as a function of the membrane potential in Ca^{2+}-free solution containing 3 mM Ba^{2+}.

contamination (Fig. 4B). One obvious feature of the α7 response shown in Fig. 4 is their fast and thorough desensitization at high agonist concentrations. In all cells we recorded from (n=10), the time course of desensitization was similar and completed within 30 s, which is in sharp contrast to the incomplete desensitization of other neuronal nAChRs (Bertrand et al., 1990). The other feature is the lack of rectification. Most neuronal nAChRs have shown inward rectification at depolarizing membrane potentials (Bertrand et al., 1990). The third feature is the ligand sensitivity. ACh and (-) nicotine showed similar sensitivity and amplitude while αBgt blocked the channel completely at nM concentrations (data not shown). In other neuronal nAChRs, the response to (-) nicotine was always greater (>5 times)

than ACh (Bertrand et al., 1990; Couturier et al., 1990).

In conclusion, we have developed a new method which utilizes PCR and *in vitro* transcription to produce chimeric cRNAs. The chimeric cRNA was constructed by attaching the AMV/Kozak sequence to the 5′-side of the initiation codon and the poly(A) tail to the 3′-side of the stop codon. Its newly introduced sequences increased the expression level of the α7 nAChR in *Xenopus* oocytes by as much as 100-fold. The α7 nAChR expressed on the surface of oocytes reflected functional channels since there was a close correlation between the number of the receptor and the size of the current. By blocking and eliminating the secondary Ca^{2+}-activated Cl^- current, the α7 current was isolated and verified pharmacologically as well as electrophysiologically.

Acknowledgements

This work was supported by a Genetic Engineering Research grant given by MOE to C. K. Min in 1996 and by the Korea Science and Engineering Foundation through the Brain Disease Research Center at Ajou University.

References

Bachvarova RF (1992) A maternal tail of poly(A): the long and the short of it. *Cell* 69: 895-897.

Bertrand D, Ballivet M, and Rungger D (1990) Activation and blocking of neuronal nicotinic acetylcholine receptor reconstituted in *Xenopus* oocytes. *Proc Natl Acad Sci USA* 87: 1993-1997.

Couturier SD, Bertrand D, Matter J-M, Hernandez M-C, Bertrand S, Miller N, Valera S, Barkas T, and Ballivet M (1990) A neuronal nicotinic acetylcholine receptor subunit (α_7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-BTX. *Neuron* 5: 847-856.

Figl A, Cohen BN, Quick MW, Davidson N, and Lester HA (1992) Regions of β4 · β2 subunit chimeras that contribute to the agonist selectivity of neuronal nicotinic receptors. *FEBS Lett* 308: 245-248.

Jobling SA and Gehrke L (1987) Enhanced translation of chimaeric messenger RNAs containing a plant viral untranslated leader sequence. *Nature* 325: 622-625.

Kozak M (1991) Effects of long 5′ leader sequences on initiation by eukaryotic ribosomes *in vitro*. *Gene Exp* 1: 117-125.

Quick MW and Lester HA (1994) Methods for expression of excitability proteins in *Xenopus* oocytes. In: Conn PM (ed), Ion Channels of Excitable Cells. Academic Press, San Diego, pp 261-279.

Seguela P, Wadiche J, Dineley-Miller K, Dani JA, and Patrick JW (1993) Molecular cloning, functional properties, and distribution of rat brain α_7: a nicotinic cationic channel highly permeable to calcium. *J Neurosci* 13: 596-604.

Smith TG, Barker JL, Smith BM, and Colburn TL (1980) Voltage clamping with microelectrodes. *J Neurosci Meth* 3: 105-128.

Uezono Y, Bradley J, Min C, McCarty NA, Quick M, Riordan JR, Chavkin C, Zinn K, Lester HA, and Davidson N (1993) Receptors that couple to 2 classes of G proteins increase cAMP and activate CFTR expressed in *Xenopus* oocytes. *Recep Chan* 1: 233-241.

Yoshii K, Yu L, Mayne KM, Davidson N, and Lester HA (1987) Equilibrium properties of mouse-*Torpedo* acetylcholine receptor hybrids expressed in *Xenopus* oocytes. *J Gen Physiol* 90: 553-573.

[Received March 4, 1999; accepted April 16, 1999]