

Microtransplantation of membranes from cultured cells to *Xenopus* oocytes: A method to study neurotransmitter receptors embedded in native lipids

Eleonora Palma^{*†}, Flavia Trettel^{*†}, Sergio Fucile^{*†}, Massimiliano Renzi^{*}, Ricardo Miledi[‡], and Fabrizio Eusebi^{*†§}

^{*}Istituto Pasteur-Fondazione Cenci Bolognietti and Dipartimento di Fisiologia Umana e Farmacologia, Università di Roma "La Sapienza," Piazzale Aldo Moro 5, I-00185 Rome, Italy; [†]Dipartimento di Scienze Internistiche, San Raffaele Pisana, Tosinvest-Sanità, Via della Pisana 235, I-00163 Rome, Italy; and [‡]Laboratory of Cellular and Molecular Neurobiology, Department Neurobiology and Behavior, University of California, Irvine, CA 92697-4550

Contributed by Ricardo Miledi, December 27, 2002

The *Xenopus* oocyte is used as a convenient cell expression system to study the structure and function of heterogenic transmitter receptors and ion channels. Recently, we introduced a method to microtransplant already assembled neurotransmitter receptors from the human brain to the plasma membrane of *Xenopus* oocytes. The same approach was used here to transplant neurotransmitter receptors expressed from cultured cells to the oocytes. Membrane vesicles prepared from a human embryonic kidney cell line (HEK293) stably expressing the rat glutamate receptor 1 were injected into oocytes, and, within a few hours, the oocyte plasma membrane acquired α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors, which had the same properties as those expressed in the original HEK cells. Analogously, oocytes injected with membranes prepared from rat pituitary GH(4)C1 cells, stably expressing homomeric human neuronal α 7 nicotinic acetylcholine receptors (α 7-AcChoRs), incorporated in their plasma membrane AcChoRs that behaved as those expressed in GH(4)C1 cells. Similar results were obtained with HEK cells stably expressing heteromeric human neuronal α 4 β 2-AcChoRs. All this makes the *Xenopus* oocyte a powerful tool for detailed investigations of receptors and other proteins expressed in the membrane of cultured cells.

The original approach (1) of injecting heterogenic mRNAs, isolated from brains and other tissues, into *Xenopus* oocytes to express functional neurotransmitter receptors in the oocyte membrane (1) has been used extensively in the last 20 yr to study the molecular structure and function of many receptors and voltage-operated channels (2–4). A method was subsequently developed to incorporate in the oocyte membrane foreign receptors that had already been assembled in their native cells. Essentially, this method consists in injecting the oocytes with cell membranes prepared from foreign tissues. The oocyte plasma membrane incorporates the foreign membranes and efficiently acquires functional neurotransmitter receptors and voltage-operated channels (5–7). Those experiments showed that the incorporated receptors and channels behaved very much like the ones in the original cells. However, we deemed it important to determine in more detail whether the properties of the receptors incorporated in the oocyte membrane are the same as those of the receptors while still in the “donor” cells. For that purpose, we microtransplanted three types of neurotransmitter receptors assembled in the membranes of transfected cells to the oocytes and found that the properties of the receptors in their native membrane are retained after transplantation to the oocytes. Therefore, this method may be useful to study many of the proteins present in the membranes of cultured cells.

Materials and Methods

Cell Lines. The cell lines used for electrophysiological recordings and membrane isolation were as follows: (i) HEK 293 cells stably transfected with the rat flip variant of glutamate receptor 1 (HEK-GluR1, a gift from Dr. P. Bregestovski, Centre National

de la Recherche Scientifique, Marseille, France); (ii) HEK293 cells stably transfected with the human α 4 and β 2 nicotinic receptor subunits (HEK- α 4 β 2 indicated elsewhere also as A4B2.2 cells; cf. ref. 20; a gift from from Merck, Whitehouse Station, NJ); and (iii) the rat pituitary GH(4)C1 cells stably transfected with the human α 7 nicotinic receptor [GH(4)C1- α 7; ref. 8; also a gift from Merck]. HEK cells were grown in DMEM and the GH(4)C1 in Ham's F10 media; and both were supplemented with 10% FBS, penicillin, streptomycin, and geneticin (G418 sulfate, 500 μ g/ml). All culture media were purchased from Invitrogen.

Membrane Preparation. Membranes were prepared as described (7). Briefly, about 10^7 cells were collected, rinsed in PBS and stored frozen at -80°C . Cells were then homogenized by using a Teflon glass homogenizer with 2 ml of glycine buffer of the following composition (in mM): 200 glycine, 150 NaCl, 50 EGTA, 50 EDTA, and 300 sucrose; plus 20 μ l of protease inhibitors (P2714; Sigma); pH 9 was adjusted with NaOH. The homogenate was centrifuged for 15 min at $9,500 \times g$ with a Beckman centrifuge (C1015 rotor). The supernatant was collected and centrifuged for 2 h at $10^5 \times g$ with a TL-100 rotor at 4°C . The pellet was washed, resuspended in assay buffer (glycine 5 mM), and used directly, or aliquotted and stored at -80°C for later use.

Oocyte Injection of Membrane Vesicles. Preparation of *Xenopus laevis* oocytes and injection procedures were detailed elsewhere (9). Oocytes were injected with membranes (≈ 100 nl; 9–14 mg of protein per ml) dissolved in 5 mM glycine, and maintained in modified Barth's solution plus antibiotics (kanamycin, 10^4 units $\cdot\text{ml}^{-1}$; penicillin, 10^4 units $\cdot\text{ml}^{-1}$; streptomycin, 10 mg $\cdot\text{ml}^{-1}$) at 16°C until electrophysiological recordings were performed. As controls, oocytes were injected with 100 nl of water or 5 mM glycine alone.

Patch-Clamp Recordings. Whole-cell current recordings were performed from HEK-GluR1, HEK- α 4 β 2 cells, or GH(4)C1- α 7 cells 2–3 days after plating. Recordings and data analysis were performed as detailed (10) by using borosilicate glass patch pipette (3- to 6-M Ω tip resistance) connected to an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Data were stored on a PC computer by using PCLAMP8 software (Axon Instruments). Whole cell capacitance and patch series resistances (5–15 M Ω) were estimated from slow transient compensations, with a series resistance compensation of 75–90%. Unless otherwise indicated, the cells were voltage-clamped at a holding

Abbreviations: $T_{0.1}$, the time for the current to decay by 10%; n_H , Hill coefficient; AcCho, acetylcholine; AcChoR, AcChol receptor; GluR1, glutamate receptor 1; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, cyano-7-nitroquinoxaline-2,3-dione; MLA, methyllycaconitine; 5OHind , 5-hydroxyindole; DH β E, dihydro- β -erythroidine.

[§]To whom correspondence should be addressed. E-mail: fabrizio.eusebi@uniroma1.it.

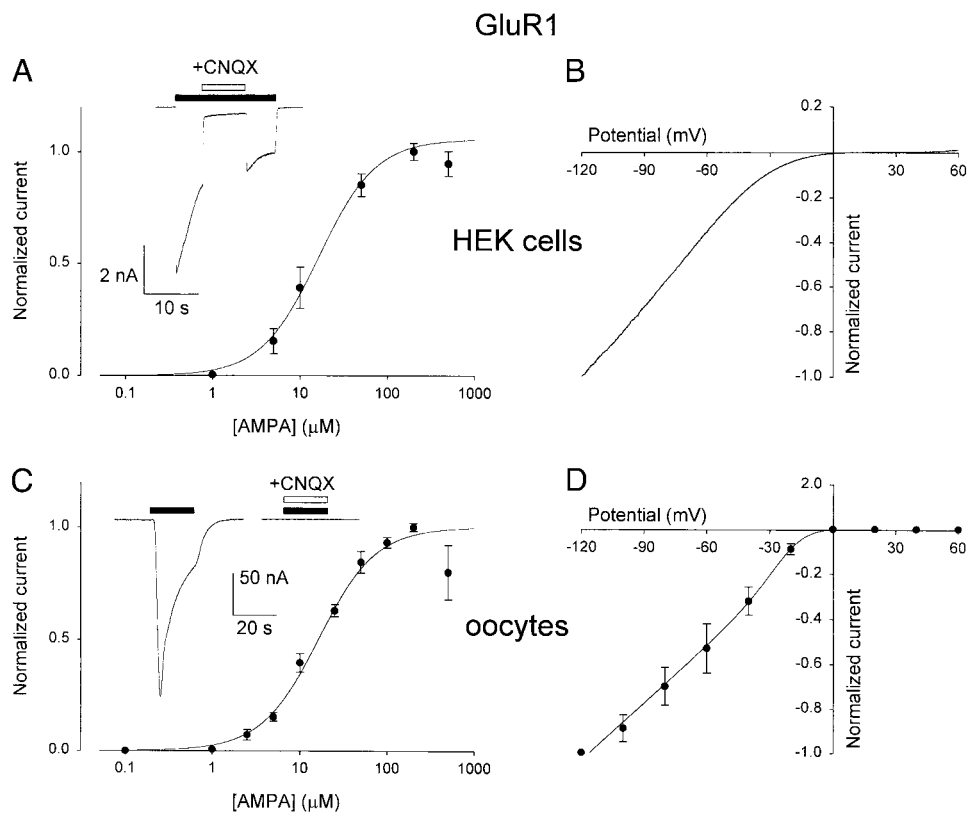


Fig. 1. Properties of AMPA currents in HEK-GluR1 cells and in oocytes microtransplanted with HEK-GluR1 cell membranes. (A) AMPA-dose/current relation from six HEK-GluR1 cells. Peak currents normalized to I_{\max} of each cell (mean $I_{\max} = -5.3 \pm 0.8$ nA; AMPA, 200 μM). Data represent mean \pm SEM. (Inset) Sample current elicited by AMPA (100 μM) and blocked by CNQX (20 μM). In all figures, the solid bars show the timing of drug applications. (B) Current-voltage relation, from potential ramps, -120 mV to $+60$ mV, applied during the steady-state current induced by AMPA (50 μM). Data normalized to the current at -120 mV (-7.7 ± 1.6 nA; average of four HEK-GluR1 cells). (C) AMPA-dose/current response relation from 10 oocytes injected with HEK-GluR1 cell membranes. Peak currents normalized to I_{\max} of each oocyte (mean $I_{\max} = -187 \pm 92$ nA; AMPA, 200 μM). (Inset) Drug concentrations and horizontal bars are as in A. (D) AMPA-current/voltage relation, from four oocytes injected as in C, fitted to a Boltzmann equation (see *Materials and Methods*). Data normalized to the currents, -149 ± 50 nA amplitude, elicited by AMPA (50 μM) at -120 mV.

potential of -70 mV and continuously superfused by using a gravity-driven perfusion system consisting of independent tubes for standard and agonist-containing solutions (positioned 50–100 μm from the patched cell). To reduce the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) current desensitization, all measurements of AMPA currents in cultured cells were made in the presence of 50 μM cyclothiazide (stock was 50 mM in DMSO; ref. 11). Potential ramps (-120 mV to $+60$ mV, 1.5 s duration) were applied during the steady-state current induced by neurotransmitters. Dose–response relationships were constructed as detailed in ref. 12 and below.

Oocyte Recordings. A few hours (from 5 to 48) after cell membrane injection, membrane currents were recorded from voltage-clamped oocytes by using two microelectrodes filled with 3 M KCl (13). The oocytes were placed in a recording chamber (volume, 0.1 ml) and perfused continuously, 10–11 ml/min, with oocyte Ringer’s solution at room temperature (20 – 22°C). To obtain agonist-dose/current response relations, the substances were applied to the oocytes at 4-min intervals, holding the oocyte membrane potential at -60 mV. The current-voltage relations were fitted with the Boltzmann equation as below. The half maximal concentration (EC_{50}) was estimated by fitting the data to Hill equations, using least-square routines:

$$I/I_{\max} = [A]^{n_H}/([A]^{n_H} + EC_{50}^{n_H}) \quad [1]$$

where $[A]$ are the doses of neurotransmitter, n_H is the Hill coefficient, and I_{\max} is the maximum current response. Solution exchange was achieved by using electromagnetic valves and a computer controlled perfusion system (Biologique, Claix, France). Current-voltage relationships were constructed holding the cells at -60 mV and stepping the membrane potential for 2–4 min at the desired value before applying the neurotransmitter.

The current-voltage relations were fitted to the Boltzmann equation:

$$I = \gamma \frac{V}{1 + e^{-\frac{V-V_0}{k}}} + c \quad [2]$$

where I is the current, V is the membrane potential, γ is the conductance, V_0 is the threshold potential, and c and k are constant values. To reduce AMPA current desensitization, all measurements of AMPA currents were again made in the presence of 50 μM cyclothiazide.

Chemicals and Solutions. Oocyte Ringer’s solution had the following composition (in mM): NaCl 82.5, KCl 2.5, CaCl_2 2.5, MgCl_2 1, and HEPES-NaOH 5, adjusted to pH 7.4 with NaOH. The HEK or GH(4)C1 cells were superfused with (in mM): NaCl 140, KCl 2.5, CaCl_2 2, MgCl_2 2, HEPES-NaOH 10, and glucose 10 (pH 7.3). Whole-cell patch-clamp recording pipettes were filled with (in

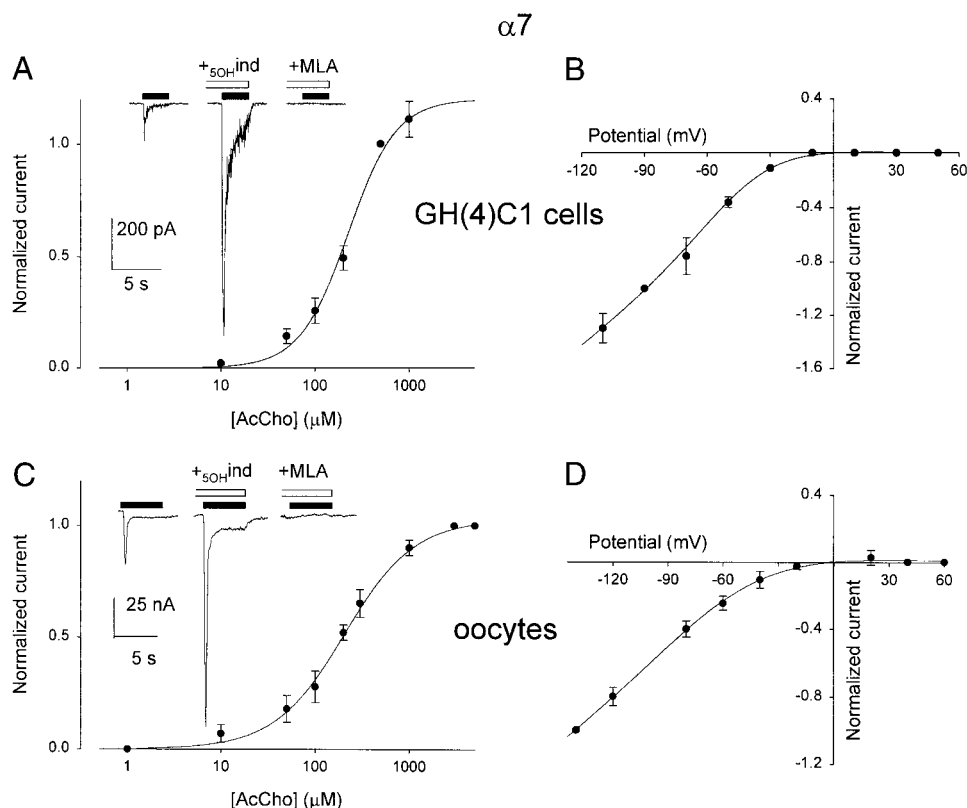


Fig. 2. AcChO currents from GH(4)C1- α 7 cells compared with those of oocytes injected with GH(4)C1- α 7 cell membranes. (A) Dose-current response relation from six GH(4)C1- α 7 cells. Peak currents normalized to I_{\max} of each cell (mean $I_{\max} = -167 \pm 52$ pA; $n = 6$; AcChO, 200 μ M). (Inset Left) Current elicited by 100 μ M AcChO; Center, plus 50Hind (1 mM; horizontal empty bar); Right, plus MLA (50 nM). (B) AcChO-current-voltage relation obtained with AcChO (100 μ M) at different membrane potentials. Data normalized to the AcChO-current at -90 mV (-45 ± 14 pA), and averaged from three cells. (C) AcChO-dose/current response relation from six oocytes injected with GH(4)C1- α 7 cell membranes. Peak currents normalized to normalized to I_{\max} of each oocyte (mean $I_{\max} = -30.2 \pm 5.3$ nA; AcChO, 3 mM). (Inset) As in A. (D) AcChO-current/voltage relation, fitted as in Fig. 1, from four oocytes injected as in C. Data normalized to AcChO currents (-40 ± 7 nA; 1 mM AcChO), at -120 mV. MLA and 50Hind were preapplied for 4 s in A and 20 s in C.

mM): CsCl 140, MgCl₂ 2, Hepes 10, Na-ATP 4, and EGTA 0.25, equilibrated at pH 7.3 with CsOH. All drugs were purchased from Sigma, with the exception of AMPA, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and cyclothiazide from Tocris Neuramin (Bristol, U.K.); and of methyllycaonitine (MLA) from Research Biochemicals (Natick, MA).

Results

GluR1 Receptors. Application of AMPA (200 μ M) to the HEK-GluR1 cells elicited inward currents, -5.3 ± 0.8 nA amplitude (mean \pm SEM; $n = 6$), that decayed with a $T_{0.1}$ value (the time for the current to decay by 10%) of 1.1 ± 0.2 s, and were rapidly and reversibly inhibited by the classical competitive blocker CNQX (Fig. 1A Inset). The amplitude of these currents increased with AMPA concentration, and the AMPA-dose/current-response curves, fitted to the Hill equation, gave n_H and EC_{50} values of 1.3 and 16 μ M (Fig. 1A). These values are similar to those reported for cerebellar granule cells (10). Moreover, the AMPA current was nonlinear, with membrane voltage showing a clear inward rectification beyond -40 mV (Fig. 1B), as described elsewhere using a variety of experimental approaches (14, 15).

Within 2 hr postinjection, the oocytes injected with HEK-GluR1 membranes acquired AMPA-type receptors that gated inward membrane currents, which varied in amplitude among different oocytes and also between donor frogs. For instance, 24 h after cell membrane injection, oocytes from one donor AMPA (200 μ M) elicited currents of -179 ± 25 nA ($n = 10$;

range, -62 to -307 nA). The AMPA currents in the oocytes desensitized fairly rapidly, showing a $T_{0.1}$ value (0.49 ± 0.05 s; Fig. 1C Inset) significantly different compared with $T_{0.1}$ value determined in HEK cells (Student's t test, $P < 0.05$). Furthermore, CNQX again blocked the currents elicited by AMPA in oocytes injected with HEK-GluR1 membranes. The AMPA-dose/current-response relation of the oocytes (Fig. 1C) yielded n_H and EC_{50} values of 1.32 and 16.2 μ M, respectively, matching the values reported here for HEK-GluR1 cells (see Fig. 1A), and elsewhere for *Xenopus* oocytes expressing GluR1 receptors, after the injection of GluR1 cRNA (16). Furthermore, the oocyte's AMPA currents increased fairly linearly, with membrane potential between -120 and -20 mV, and showed a considerable rectification beyond -20 mV (Fig. 1D). Fitting the I - V relationships to a Boltzmann equation (see Materials and Methods), we found similar slopes for both *Xenopus* oocytes and HEK cells ($\gamma_{\text{HEK}} = 8.5 \times 10^{-3}$ mV⁻¹; $\gamma_{\text{oocytes}} = 8.6 \times 10^{-3}$ mV⁻¹).

α 7 Nicotinic Acetylcholine Receptors (AcChOR). Patch-clamped GH(4)C1- α 7 cells responded to AcChO application with an inward current that decayed very rapidly. Specifically, AcChO (200 μ M) elicited a mean current of -167 ± 52 pA ($n = 6$), with a $T_{0.1}$ value of 4.5 ± 1.6 ms, and the rate of decay became faster as the neurotransmitter concentration was raised (not shown). The AcChO currents were blocked by the α 7 nicotinic antagonist MLA (e.g., Fig. 2A Inset), and were not affected by atropine (not shown). On the other hand, it is known that AcChO responses elicited by activation of α 7 receptors, both in native nerve cells

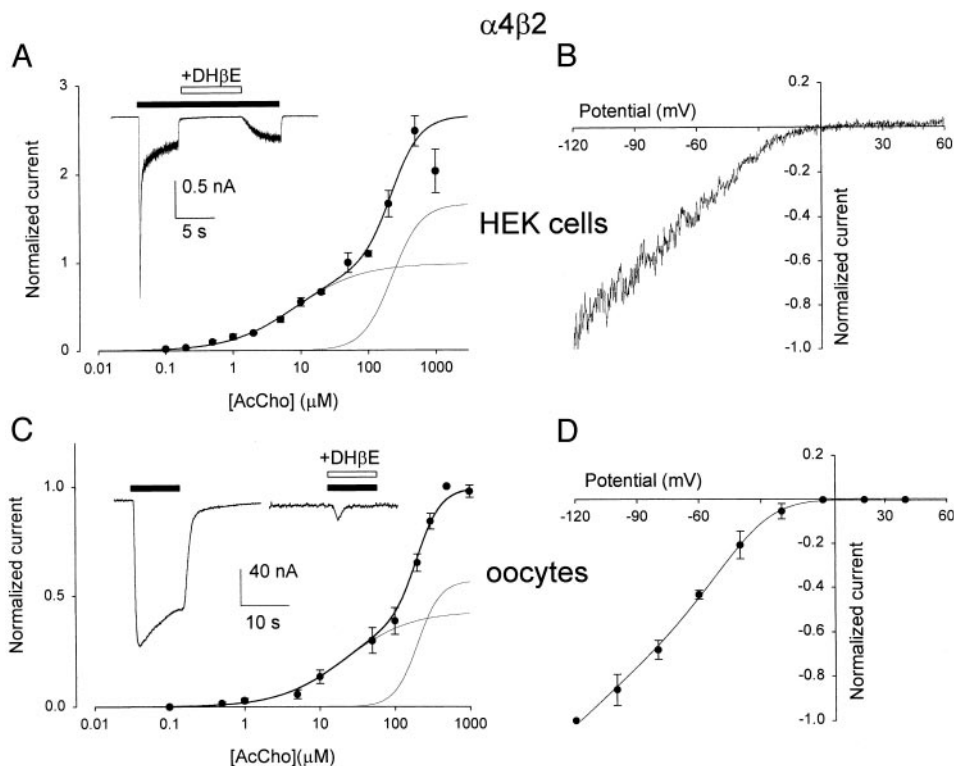


Fig. 3. AcChO currents of HEK- $\alpha 4\beta 2$ cells compared with those of oocytes injected with HEK- $\alpha 4\beta 2$ cell membranes. (A) Bimodal AcChO-dose/current relation ($n = 6$ for each point), best fitted to the sum of two Hill equations (superimposed lines) with the following best fitting parameters: $n_H = 0.90$, $EC_{50} = 8.3 \mu M$; and $n_H = 2.0$, $EC_{50} = 221 \mu M$. Data normalized to the I_{max} of each cell (mean peak current -536 ± 60 pA; $n = 18$; AcChO, $50 \mu M$). (Inset) Current induced by AcChO ($30 \mu M$, horizontal filled bar) and reversibly blocked by DH β E ($5 \mu M$). (B) Current-voltage relation. Potential ramp, -120 mV to $+60$ mV, on the steady-state current induced by AcChO ($50 \mu M$). Data normalized to the current at -120 mV (-218 ± 52 pA; four cells). (C) AcChO-dose/current response relation from six oocytes injected with HEK- $\alpha 4\beta 2$ cell membranes. Peak currents, normalized to I_{max} of each oocyte (mean $I_{max} -405 \pm 7$ nA; AcChO, $500 \mu M$) and best-fitted with a sum of two Hill equations (superimposed lines), gave: $n_H = 1$ and $EC_{50} = 21.4 \mu M$; $n_H = 2.8$ and $EC_{50} = 200 \mu M$, respectively. (Inset) As in A. (D) Current-voltage relation, fitted as in Fig. 2, from four oocytes injected as in C. Data normalized to currents (-331 ± 107 nA) elicited by AcChO ($50 \mu M$) at -120 mV.

and in cell expression systems, are positively modulated by 5-hydroxyindole ($5OHind$; ref. 17). Accordingly, we found that the AcChO currents were greatly potentiated by $5OHind$ applied 4 s before the AcChO (e.g., Fig. 2A Inset). The AcChO concentration/current response relationship (Fig. 2A) gave EC_{50} and n_H values of $239 \mu M$ and 1.9, similar to values reported elsewhere (18). Furthermore, the AcChO current was nonlinear, with membrane voltage showing marked rectification beyond -30 mV (Fig. 2B), as described (12).

As expected, injection of GH(4)C1- $\alpha 7$ cell membranes into the oocytes could lead to the incorporation of $\alpha 7$ receptors; and, 24 h postinjection, AcChO ($200 \mu M$) generated inward currents with a mean of 22 ± 3 nA (range, 15 to 35 nA, $n = 6$), and which decayed unlike those of the native cells ($T_{0.1} = 220 \pm 25$ ms; $P < 0.005$). In addition, the AcChO currents were again potentiated by $5OHind$, and blocked by MLA (Fig. 2C Inset); and the dose/current relation gave EC_{50} and n_H values of $206 \mu M$ and 1.2 that matched those obtained for the GH(4)C1- $\alpha 7$ cells (Fig. 2C). Furthermore, the $I-V$ relation was linear at hyperpolarized membrane potentials, while it was bent at -30 to $+30$ mV (e.g., Fig. 2D), with a pattern similar to that of native GH(4)C1- $\alpha 7$ cells. Furthermore, the $I-V$ relationships fitted to a Boltzmann equation showed similar slopes ($\gamma_{HEK} = 9.2 \times 10^{-3} \text{ mV}^{-1}$; $\gamma_{oocytes} = 9.4 \times 10^{-3} \text{ mV}^{-1}$).

$\alpha 4\beta 2$ Nicotinic AcChRs. Application of AcChO ($50 \mu M$) to whole-cell patch-clamped HEK- $\alpha 4\beta 2$ cells elicited inward currents, -536 ± 60 pA amplitude, that decayed with a $T_{0.1}$ of 25 ± 3 ms ($n = 18$). The AcChO currents were inhibited by the competitive

nicotinic blocker dihydro- β -erythroidine (DH β E; Fig. 3A Inset), and, as reported (19), the AcChO-dose/current-response curve was bimodal and was best fitted to the sum of two Hill equations (Fig. 3A). The AcChO current was nonlinear, rectifying strongly beyond -20 mV (Fig. 3B).

Oocytes injected with HEK- $\alpha 4\beta 2$ cell membranes rapidly acquired AcChRs that gated inward membrane currents of variable amplitude. Specifically, 24 h after membrane injection, AcChO at $50 \mu M$ elicited currents that ranged from -42 and -220 nA (Fig. 3C Inset) and had a mean of -116 ± 27 nA ($n = 9$). The currents decayed with a $T_{0.1}$ of 2.6 ± 0.65 s and, as illustrated in Fig. 3C Inset, DH β E again blocked the AcChO currents. The AcChO-dose/current relation of the oocytes was again bimodal (Fig. 3C), and the EC_{50} and n_H values resembled those of the donor HEK- $\alpha 4\beta 2$ cells (see also refs. 19 and 20). Furthermore, the AcChO currents increased with the membrane potential in the range -120 to -20 mV and showed a considerable rectification similarly to the currents elicited in the HEK- $\alpha 4\beta 2$ (e.g., Fig. 3D). The $I-V$ relationships fitted to the Boltzmann equation again showed similar slopes ($\gamma_{HEK} = 7.6 \times 10^{-3} \text{ mV}^{-1}$; $\gamma_{oocytes} = 8.4 \times 10^{-3} \text{ mV}^{-1}$).

Discussion

In this work, we report an efficient method to easily study transmitter receptors originally assembled in cultured cell lines and then microtransplanted to a sturdy and convenient host cell system, the *Xenopus* oocyte. This method has been recently used to transplant assembled transmitter receptors from human brain to *Xenopus* oocyte (7), following a method developed a few years

ago to microtransplant AcChoRs and chloride channels from the electric organ of *Torpedo* to the *Xenopus* oocyte membrane (5, 6). Here, we injected membrane vesicles prepared from cultured cell lines, and this approach led to a rapid incorporation of neurotransmitter receptors in the oocyte plasma membrane. In this way, functional AMPA-type GluR1, α 7-AcChoRs, and α 4 β 2-AcChoRs from cultured cells were microtransplanted to the oocytes, and their respective transmitter-activated currents were analyzed.

We report here that the gross electrophysiological and pharmacological characteristics of the transmitter-gated receptors constitutively assembled in the cultured cells are, for the most part, retained after transplantation to the oocytes. Specifically, we found that (i) the receptor apparent transmitter affinity, (ii) the transmitter binding site cooperativity, (iii) the receptor sensitivity to the antagonists, and (iv) the *I-V* pattern observed in the native donor cells are substantially equivalent to those detected from membrane-injected oocytes, with the notable exception of the kinetic behavior of the current responses: α 4 β 2- and α 7-AcCho currents desensitized more rapidly when the receptors were in the native cells than when they were transplanted to the oocytes whereas GluR1-AMPA currents decayed faster in oocytes vs. HEK cells. The reasons for these differences are not known, and several possibilities need to be examined. To mention just two, it may be that the α 4 β 2 receptors internalize after AcChoR activation due to internal signaling (21) and this process may be usually more marked in the cells than in the oocytes. Another possibility is raised by the fact that the

intracellular medium and the cellular signaling machinery are different in the amphibian oocytes (22) vs. the mammalian cells (23). Whatever the correct explanation for the different rates of desensitization turns out to be, our results already show that these factors are not relevant to many other receptor properties and point to the molecular structure of the receptor as the main determining factor.

Finally, we also further report that the α 7-AcChoRs are positively modulated by the allosteric modulator 5OHind , both in the donor cells and after receptor transplantation to the oocytes. Therefore, our approach may be useful to investigate also membrane receptor modulation due to the action of allosteric effectors and possible intracellular cascade signals, thus addressing many questions related to biochemical processes linking the cell membrane and its receptors to the whole cell.

A large body of evidence indicates that the functional properties of transmitter-gated receptors may be influenced by the host heterologous cell expression system (24, 25); but the roles played by the membrane, the intracellular ionic composition, the vast array of intracellular cell proteins, etc. are still not clear. One outcome of the present work is that our approach to microtransplant proteins, with their natural structure and still embedded in their natural lipid membrane, from donor cells to the host oocyte opens the way to many pathophysiological investigations.

We thank Drs. Francesca Grassi and Fabio Ruzzier for critical reading of the manuscript and Gregorio Torchia for help. This work had the financial support of Ministero Istruzione Università Ricerca (to F.E.) and the National Science Foundation (to R.M.).

- Barnard, E. A., Miledi, R. & Sumikawa, K. (1982) *Proc. R. Soc. London Ser. B Biol. Sci.* **215**, 241–246.
- Gundersen, C. B., Miledi, R. & Parker, I. (1984) *Nature* **308**, 421–424.
- Miledi, R., Parker, I. & Sumikawa, K. (1989) in *Fidia Award Lecture Series*, ed. Smith, J. (Raven, New York), Vol. 3, pp. 57–90.
- Valera, S., Hussy, N., Evans, R. J., Adams, N., North, R. A., Suprenant, A. & Buell, G. (1994) *Nature* **371**, 516–519.
- Marsal, J., Tigy, G. & Miledi, R. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 5224–5228.
- Morales, A., Aleu, J., Ivorra, I., Ferragut, J. A., Gonzalez-Ros, J. M. & Miledi, R. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 8468–8472.
- Miledi, R., Eusebi, F., Martinez-Torres, A., Palma, E. & Trettel, F. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 13238–13242.
- Virginio, C., Giacometti, A., Aldegheri, L., Rimland, J. M. & Terstappen, G. C. (2002) *Eur. J. Pharmacol.* **445**, 153–161.
- Palma, E., Mileo, A. M., Eusebi, F. & Miledi, R. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 11231–11235.
- Lax, P., Limatola, C., Fucile, S., Trettel, F., Di Bartolomeo, S., Renzi, M., Ragozzino, D. & Eusebi, F. (2002) *J. Neuroimmunol.* **129**, 66–73.
- Patneau, D. K., Vyklicky, L., Jr., & Mayer, M. L. (1993) *J. Neurosci.* **13**, 3496–3509.
- Fucile, S., Palma, E., Mileo, A. M., Eusebi, F. & Miledi, R. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 3643–3648.
- Miledi, R. (1982) *Proc. R. Soc. London Ser. B Biol. Sci.* **215**, 491–497.
- Derkach, V., Barria, A. & Soderling, T. R. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 3269–3274.
- Palma, E., Esposito, V., Mileo, A. M., Di Gennaro, G., Quarato, P., Giangaspero, F., Scoppetta, C., Onorati, P., Trettel, F., Miledi, R. & Eusebi, F. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 15078–15083.
- Wahl, P., Madsen, U., Banke, T., Krogsgaard-Larsen, P. & Schousboe, A. (1996) *Eur. J. Pharmacol.* **308**, 211–218.
- Zwart, R., De Filippi, G., Broad, L., McPhie, G., Pearson, K., Baldwinson, T. & Sher, E. (2002) *Neuropharmacology* **43**, 374–384.
- Gopalakrishnan, M., Buisson, B., Touma, E., Giordano, T., Campbell, J. E., Hu, I. C., Donnelly-Roberts, D., Arneric, S. P., Bertrand, D. & Sullivan, J. P. (1995) *Eur. J. Pharmacol.* **290**, 237–246.
- Buisson, B. & Bertrand, D. (2001) *J. Neurosci.* **21**, 1819–1829.
- Chavez-Noriega, L. E., Gillespie, A., Stauderman, K. A., Crona, H. J., Claeps, B. O. N., Elliot, K. J., Reid, R. T., Rao, T. S., Velicelebi, G., Harpold, M. M., et al. (2000) *Neuropharmacology* **39**, 2543–2560.
- Bhattacharyya S., Puri, S., Miledi, R. & Panicker, M. M. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 14470–14475.
- Kusano, K., Miledi, R. & Stinnakre, J. (1982) *J. Physiol. (London)* **328**, 143–170.
- Burton, R. F. (1983) *Comp. Biochem. Physiol. B* **76**, 663–671.
- Lewis, T. M., Harkness, P. C., Sivilotti, L. G., Colquhoun, D. & Millar, N. S. (1997) *J. Physiol.* **505**, 299–306.
- Fucile, S., Barabino, B., Palma, E., Grassi, F., Limatola, C., Mileo, A. M., Alemà, S., Ballivet, M. & Eusebi, F. (1997) *NeuroReport* **8**, 2433–2436.