[2] Expression of Neurotransmitter Receptors and Voltage-Activated Channels from Brain mRNA in *Xenopus* Oocytes

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The small size and inaccessibility of neurons in the brain greatly complicate the study of the neurotransmitter receptors and voltage-activated channels of the brain. A way around many of the difficulties encountered when studying these molecules in the brain cells has recently become available, with the finding that functional neurotransmitter receptors can be "transplanted" from the brain into the membrane of frog oocytes. This is done by injecting *Xenopus* oocytes with mRNA extracted from the brain. The mRNA is translated by the oocyte, and functional receptors and channels are inserted in the plasma membrane. Because of the large size of the oocyte (over 1 mm diameter), the receptor and channel molecules can then be studied by electrophysiological and biochemical techniques with much greater ease than is possible when they are present in their "native" neuronal cells (for reviews, see Refs. 1 and 2). Using this method, we have transplanted into oocytes most of the known receptors to neurotransmitters [acetylcholine (ACh), serotonin, γ-aminobutyric acid (GABA), glycine, glutamate, *N*-methyl-D-aspartate (NMDA), aspartate, noradrenaline, dopamine, β-alanine, etc.), as well as the voltage-operated channels (Na^+, K^+, Ca^{2+}, Cl^-). This chapter describes the use of *Xenopus* oocytes for the study of brain neurotransmitter receptors and ion channels.

Procedures used for the transplantation of neurontransmitter receptors and voltage-activated ion channels are shown diagrammatically in Fig. 1 and each stage is described in more detail below.

Extraction and Purification of mRNA

Extraction of Total RNA from the Brain

Extraction of total RNA is the most important step in the transplantation, because the quality of the mRNA dictates the type and number of neurotransmitter receptors and voltage-operated ion channels which are expressed in the oocyte membrane. Several techniques are available for

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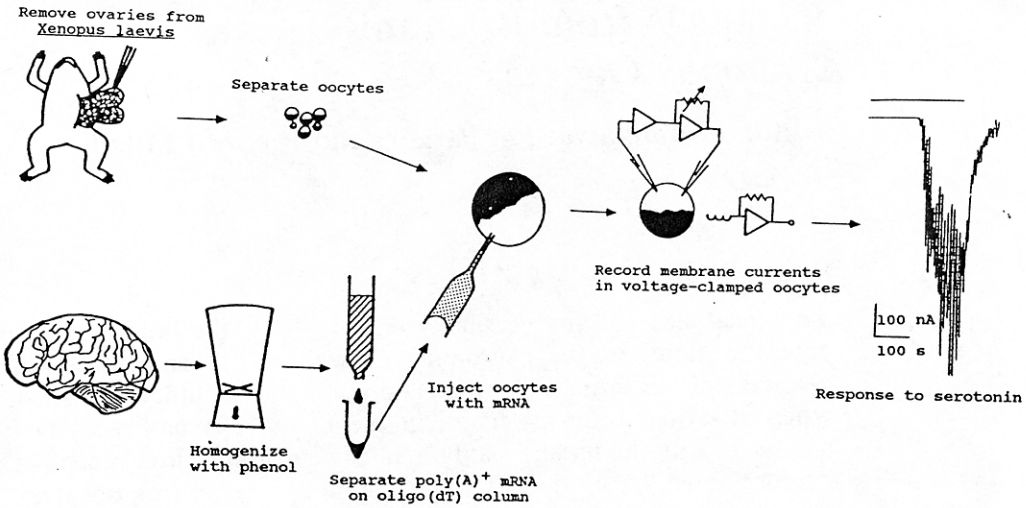

FIG. 1 Stages in the transplantation of receptors and channels from brain to oocyte.

isolating intact RNA from fresh and frozen tissues (3, 4). Among them, the guanidinium thiocyanate method (5) is the most widespread, because it is a more efficient protein denaturant (i.e., more efficient ribonuclease inactivator) than other denaturants. However, in our hands the RNA extracted from the brain with this method is generally less effective in expressing functional receptors and ion channels in the oocyte as compared with RNA extracted by the phenol–chloroform method. Therefore, we describe here a protocol for the extraction of RNA using phenol–chloroform as a protein denaturant and a means of separating the proteins from the nucleic acids, which consistently provides us with active mRNA coding for many brain neurotransmitter receptors and voltage-activated ion channels.

For safety, goggles and disposable plastic gloves should be worn during the following procedure. Disposable plastic gloves also protect RNA solutions from ribonucleases present on the skin. Always use sterile solutions, glassware, and plasticware. Water and all buffer solutions are sterilized by autoclaving for 1 hr. All glassware is baked for at least 4 hr at 250°C (or for at least 8 hr at 180°C). Plastic centrifuge tubes [Sepcor polypropylene centrifuge tubes with fluorocarbon O ring (Fisher, Pittsburgh, PA) or its equivalent] are placed in 1 *N* HCl overnight and rinsed thoroughly with distilled water and autoclaved for 0.5 hr. Individually wrapped, sterile disposable glass pipettes and sterile disposable tips for pipettors are used for handling solutions.

1. Place homogenizer probe (Polytron or its equivalent) in 0.1% (v/v)

diethyl pyrocarbonate (DPC; Sigma, St. Louis, MO) for 0.5 hr at room temperature and rinse thoroughly with autoclaved water.

2. Dissolve 100 g phenol [white loose crystals (Fisher, Mallinckrodt, Paris, KY; Baker, Phillipsburg, NJ)] in 11 ml of homogenization buffer [200 m*M* Tris-HCl, pH 9.0/50 m*M* NaCl/10 m*M* EDTA/0.5% (w/v) SDS] at 37°C just before use and add 0.11 g 8-hydroxyquinoline.

3. Pipette 10 ml of homogenization buffer into a sterile 50-ml cylinder (Pyrex). Add 0.01 g heparin (Sigma) and 10 ml phenol presaturated with homogenization buffer.

4. Place homogenizer probe into the 50-ml cylinder and start mixing the solution. Drop 1 g of brain tissue into the mixing solution and homogenize for 2–3 min at a setting of 5–6 at room temperature (for more RNA, 10 g of brain tissue can be processed with a 10-fold scale up).

5. Transfer homogenate into a 50-ml Sepcor centrifuge tube and shake vigorously for 5 min at room temperature.

6. Centrifuge in a swinging bucket rotor (Beckman JS 13.1 rotor or its equivalent) at 11,000 rpm for 10 min at about 15°C.

7. Use a glass pipette to remove the bottom phenol layer and discard this. Save the top layer and fluffy white material.

8. Add 10 ml chloroform and shake vigorously for 5 min at room temperature. Centrifuge as before.

9. Remove and discard the bottom chloroform layer. Save the top layer including the fluffy white material.

10. Repeat step 8.

11. If the fluffy white material is packed into a narrow band forming a small interface layer, collect upper aqueous layer (avoiding taking the interface layer) into a fresh 50-ml centrifuge tube (Sepcor) containing 10 ml phenol/chloroform (1:1). Shake vigorously for 5 min at room temperature.

If there is a large interface layer, remove the chloroform layer, and repeat step 8 before collecting the aqueous layer.

12. Centrifuge as before.

13. Remove upper aqueous layer, avoiding white interface material, and collect into a fresh 50-ml centrifuge tube (Sepcor) containing 10 ml phenol/chloroform (1:1). Shake hard for 5 min at room temperature and centrifuge as before.

14. Repeat step 13 until the interface is gone (generally 2–3 extractions).

15. Then collect the upper aqueous layer into a fresh 50-ml centrifuge tube (Sepcor) containing 10 ml chloroform. Shake hard and centrifuge as before.

16. Collect the upper aqueous layer, measuring its volume with a pipette, and transfer into a fresh 50-ml centrifuge tube (Sepcor) kept on an ice. Add 4 *M* NaCl solution to make a final concentration of 0.2 *M* (i.e., add 50 μl NaCl solution per milliliter collected), and then add 2.5 vol of cold (−20°C)

absolute ethanol. Allow the DNA and RNA to precipitate at −20°C for at least 2 hr or at −80°C for at least 0.5 hr (it is possible to leave the preparation indefinitely at this stage).

17. Centrifuge to collect DNA and RNA in a swinging bucket rotor (Beckman JS 13.1 or its equivalent) at 11,000 rpm for 30 min at 4°C.

18. Remove the ethanol supernatant as much as possible and discard. Dissolve the DNA and RNA pellet (it is not necessary to first dry the pellet) in 5 ml of cold 20 m*M* HEPES–NaOH, pH 7.5. With the solution on ice, slowly add 0.9 g of NaCl (use a baked spatula) to bring solution to 3 *M*. Leave overnight at −15°C or on ice. This step precipitates RNA, leaving most of the DNA in solution.

19. Centrifuge as in step 17 and discard the supernatant, saving the pellet. Wash the pellet with 10 ml of cold 3 *M* sodium acetate, pH 6.0. For this purpose, the pellet is first ground well with a baked glass rod, and then sodium acetate is added and vortexed.

20. Centrifuge as in step 17 for 5 min and discard the supernatant. Wash the pellet as in step 19.

21. Repeat step 20.

22. Centrifuge as in step 17 for 5 min and remove most of the supernatant. Wash the pellet with cold (−20°C) 75% ethanol as in step 19.

23. Repeat step 22.

24. Centrifuge as in step 17 for 5 min and discard supernatant. (If the RNA is not used immediately, store the RNA in 75% ethanol at −80°C.)

25. Dry the pellet in vacuum and dissolve the pellet (i.e., RNA) in 400 μl cold 20 m*M* HEPES–NaOH, pH 7.5. The concentration of RNA can be determined by measuring the OD_{260} of a 10 μl RNA solution. An OD_{260} of 1 corresponds to approximately 40 μg/ml RNA. About 1 mg of total RNA should be recovered. The OD_{260}/OD_{280} ratio, which reflects the degree of contamination by protein and phenol, should be 2.0. The RNA solution can be stored in sterile microcentrifuge tubes at −80°C or used for isolation of poly(A)$^+$ mRNA as described below.

Purification of Poly(A)$^+$ mRNA by Chromatography on Oligo(dT)-Cellulose

To date, the great variety of receptors and channels expressed by brain mRNA in oocytes have all been induced by poly(A)$^+$ mRNA. A few preliminary experiments showed that oocytes injected with poly(A)$^-$ mRNA did not respond to the various neurotransmitters tested. Therefore, the next stage is the removal of unwanted RNA species from the poly(A)$^+$ mRNA.

All these manipulations are carried out at room temperature.

1. Autoclave a column (Econo-column 0.7 × 10 cm; Bio-Rad or its equivalent) and connect it to a UV monitor (Pharmacia or its equivalent) via autoclaved silicone tubing.

2. Suspend 0.2 g oligo(dT)-cellulose (Sigma or its equivalent) in autoclaved water and pour into the column.

3. Wash the column and all connecting system successively with
(a) 20 ml of 0.1 *N* NaOH,
(b) 20 ml of autoclaved water,
(c) 20 ml of 0.5 *M* KCl/5 m*M* HEPES–NaOH, pH 7.5.

4. Add an equal volume of 1 *M* KCl to the RNA solution to bring to 0.5 *M* KCl and slowly apply the RNA solution onto the column.

5. Collect the unbound RNA, which elutes, and reapply it to the column.

6. Wash the column with 0.5 *M* KCl/5 m*M* HEPES–NaOH, pH 7.5, until the OD_{260} returns to close to zero. The flow rate can be increased to save time.

7. Wash the column with 0.1 *M* KCl/5 m*M* HEPES–NaOH, pH 7.5, until the OD_{260} returns to close to zero.

8. Elute poly(A)$^+$ mRNA with warm (30–40°C) 5 m*M* HEPES–NaOH, pH 7.5, and collect the poly(A)$^+$ mRNA peak into a 15-ml sterile (baked), siliconized Corex tube on ice. The volume of the mRNA solution should be minimized to ensure good recovery of the mRNA in step 9.

9. Add 4 *M* NaCl to the mRNA solution to make to 0.2 *M* final concentration and precipitate the mRNA with 2.5 vol of absolute ethanol at −20°C overnight.

10. Collect the mRNA by centrifugation in a swinging bucket rotor (Beckman JS 13.1 or its equivalent) at 10,000 rpm for at least 0.5 hr at 4°C. Discard the supernatant and rinse the pellet (mRNA) carefully with 5 ml of cold (−20°C) 75% ethanol. Discard the ethanol. The mRNA can be stored in 75% ethanol at −80°C indefinitely, or it can be dried and dissolved in 50 μl of sterile water. The OD_{260} of a small sample should be measured to determine recovery. The yield of mRNA is normally 2–5% of the RNA applied to the column. The mRNA solution should be divided into small aliquots in sterile microcentrifuge tubes and stored at −80°C. Avoid repeated freezing and thawing of the mRNA solution.

Preparation and Injection of Oocytes

Preparation of Xenopus Oocytes for Microinjection

Female *Xenopus laevis* are obtained from commercial suppliers (e.g., Nasco Inc, Fort Atkinson, WI; *Xenopus* I, Ann Arbor, MI), and sacrificed by decapitation. Pieces of ovary are dissected out, and individual oocytes at

Dumont stages V and VI (6) are manually stripped from the ovary using watchmaker's forceps under a dissecting microscope. These oocytes are maintained in culture at 16°C, in Barth's solution [88 m*M* NaCl, 1 m*M* KCl, 2.4 m*M* $NaHCO_3$, 0.82 m*M* $MgSO_4$, 0.33 m*M* $Ca(NO_3)_2$, 0.41 m*M* $CaCl_2$, 7.5 m*M* Tris-HCl at pH 7.6] containing 50 units (U)/ml nystatin (Sigma) and 0.1 mg/ml gentamycin (Sigma). The dissected oocytes are cultured overnight and healthy oocytes are selected for injection. It is not advisable to use oocytes immediately after manual dissection, because some oocytes may be damaged. After incubation overnight the damaged oocytes can be easily identified. For more details, see Ref. 6.

Injection of mRNA into Xenopus Oocytes

This subject has been described in detail (6) and is mentioned only briefly here. *Xenopus* oocytes are injected with about 50 nl of poly(A)$^+$ mRNA solution (routinely 1 mg/ml), using hydraulic pressure ejection from a calibrated glass micropipette. Avoid injection into the pigmented region and do not allow the oocytes to dry out during injection. The RNA solution should be centrifuged briefly before use for injection. This helps remove particles which may otherwise be present in the RNA solution and might block the injection micropipette. The number of functional receptors or ion channels expressed in the plasma membrane of the oocyte varies with the amount of mRNA injected and, in the cases of voltage-activated sodium channel and kainate receptor (7), is directly proportional to the amount of mRNA injected over a large range. However, the expression of some other types of receptors varies nonlinearly with the amount of mRNA injected (M. Carpenter, unpublished observations), so it is advisable to determine empirically the optimal amount of mRNA to be injected for any particular receptor or ion channel of interest.

After injection, the oocytes are cultured at 16°C in sterile Barth's solution. Culturing at 16°C, instead of 18–21°C (normal incubation temperature), the oocytes survive for longer periods (some oocytes survived for >2 months). Before doing detailed electrophysiological experiments on oocytes injected with mRNA, it is necessary to assess the time course of expression of receptor interested in oocyte's membrane. This is because different receptor proteins and/or the corresponding mRNAs have different stabilities in the oocytes (Table I). We usually examine the oocytes 2 to 7 days after injection.

Preparation of Oocytes for Recording

Electrophysiological recordings may be made from intact follicular oocytes as they are obtained from the ovary, i.e., the oocyte proper, surrounded by

TABLE I Time-Dependent Expression of GABA and Kainate Receptors in Oocytes[a]

Receptor	Current (nA)	
	3–5 days after injection	11–15 days after injection
GABA	634 ± 136 (n = 8)	192 ± 112 (n = 4)
Kainate	284 ± 58 (n = 8)	853 ± 165 (n = 4)

[a] Data are given as means ± SEM. Oocytes were injected with chick brain mRNA and were tested with GABA (1 m*M*) and kainate (0.1 m*M*).

enveloping follicular, epithelial, and other layers. However, the presence of additional layers makes the oocytes more difficult to penetrate with microelectrodes. Furthermore, the follicular cells are electrically coupled to the oocyte, and possess a variety of neurotransmitter receptors (8, 9), which may complicate recordings. It is often helpful, therefore, to treat oocytes with collagenase so as to remove the follicular and other cell layers before recording (10).

A few tens of oocytes are placed in small vials containing 0.5–1 mg collagenase (type 1, Sigma) dissolved in 1 ml of Ringer's solution (composition in m*M*; NaCl, 120; KCl, 2; $CaCl_2$, 1.8; HEPES, 5, at pH about 7.0). The vials are gently rotated for about 1 hr at room temperature, and the oocytes are then washed with several changes of Ringer's solution. Gentle shaking will often detach the enveloping cell layers, producing ghosts, which can readily be seen by eye. If the envelopes do not detach with shaking, they can be peeled off using fine forceps under a dissecting microscope. Prolonged treatment with collagenase at higher concentrations removes or destroys some of the induced channels and receptors in the oocyte membrane.

Shortly after collagenase treatment, the oocytes have low resting potential and input resistance. Thus, treatment is best done a day or more before recording starts, and the oocytes are returned to culture in Barth's medium in the meantime. We normally treat oocytes with collagenase about 2 days after they have been injected with mRNA, but it is also possible to treat the oocytes prior to injection. After they recover from the collagenase treatment, the oocytes have a higher resting potential (around −100 mV), a higher input resistance (1 MΩ or more), and a reduced capacitance.

It is also possible, though more laborious, to remove the follicular cells mechanically without the use of collagenase. The first step is to "pop out" the oocytes from their epithelia when removing them from the ovary. The oocytes are then rolled over the surface of a polylysine-coated microscope slide, in the bottom of a dish filled with Ringer's solution (9). The follicular

cells stick tightly to the slide, and detach, forming a ribbon which is visible by eye.

Electrophysiological Recording from mRNA-Injected Oocytes

Recordings of membrane currents in mRNA-injected oocytes provide a highly sensitive and selective assay for the expression of ion channels gated by voltage or ligands, and allow the functional properties of the channels to be explored. Voltage clamping of the oocyte using a two-electrode clamp is the most simple and straightforward technique, and the following sections describe our methods in detail. The sensitivity of the oocyte expression and recording system is so high that it is possible to detect the translation of just a few copies of a particular message. For example, the limit of resolution with the voltage clamp is about 1 nA, so that it is possible to detect the opening of less than 1000 channel molecules, if the current passing through each is 1 pA or more.

An alternative approach is to use patch-clamp techniques to record from single channels. This allows a more detailed characterization of single-channel properties, but is not so useful for assaying the ability of mRNA preparations to induce particular receptors of channels. The application of patch-clamp recording to the oocyte is described in Ref. 11.

Several recent reviews (1, 2) deal with the types of electrical responses which may be induced in the oocyte by foreign mRNA, and with those receptors and channels which are native to the oocyte.

Voltage-Clamp System

Oocytes are clamped using a conventional two-electrode clamp, in which one electrode serves to monitor the membrane potential, while the other is used to inject current so as to maintain the potential at any desired value.

Both electrodes are made from standard glass micropipettes, pulled from glass capillary tubing including an internal filament (World Precision Instruments, New Haven, CT; A-M Systems, Everett, WA; and many other suppliers) for ease of filling. The voltage recording pipette is filled with 3 *M* KCl, and the current electrode with 3 *M* potassium acetate or KCl (potassium acetate is better if large outward currents are to be clamped). An important point is that both electrodes must be of low resistance, a few megaohms or less. This can be achieved by gently breaking the tips of the pipettes against the base of the recording chamber, or against each other,

while monitoring the resistance. In the case of the recording pipette, a low resistance minimizes the noise introduced into the clamp. For the current electrode, a low resistance is needed so that large currents may be injected into the oocyte. Currents of tens of microamperes are sometimes required to charge rapidly the membrane capacitance when stepping the membrane potential to activate voltage-gated channels, and similarly large ligand-gated currents may be induced by potent mRNA preparations. In practice, the tip size of the pipettes is a matter of compromise; the electrical properties of the pipettes improve with increasing size, but more damage is done to the oocyte on impalement. Some donors yield oocytes which can withstand the use of large pipettes, while other oocytes may be very fragile.

The voltage-clamp circuit must be able to produce quite large (>10 μA) output currents. Some commercial designs use a series resistor in the current path, which may produce an unacceptable limitation in maximum current. We use a homemade design (Fig. 2), in which the output is driven by a high-voltage op-amp, to allow greater currents to be injected through an electrode of given resistance.

The clamp current is monitored through a virtual ground circuit, connected to the fluid in the recording chamber. It is helpful to use two switched feedback resistors in this circuit, with values of 100 KΩ and 1MΩ. The higher value gives a higher recording gain (1 mV per nanoampere) for use when examining small currents, while the lower value avoids overload during large responses or capacitative spikes. A range of switched capacitors across the feedback resistors provide a simple low pass filter, with cut-off frequencies between 100 Hz and 5 KHz.

Chlorided silver wires establish electrical contact with the micropipettes, and with the fluid in the recording chamber. The bath electrodes are connected via glass tubes filled with agar in Ringer solution, so that the ionic composition of the bathing solution may be changed without altering the junction potential of the electrodes. Separate electrodes, and agar brides, are used to connect the current monitor and the indifferent side of the voltage recording amplifier to the bath, since a common electrode may introduce errors in measurement of the potential when large currents are clamped.

The function of the current-passing electrode is controlled by a reed switch, mounted in a head-stage close to the electrode. This allows the electrode to be connected either to the clamp output, or to a voltage-recording amplifier. It is convenient to monitor the potential while inserting the electrode into the oocyte, and after insertion of both electrodes the clamp can be activated by switching the electrode to the current-passing mode.

Immediately after inserting the microelectrodes, the resting potential and

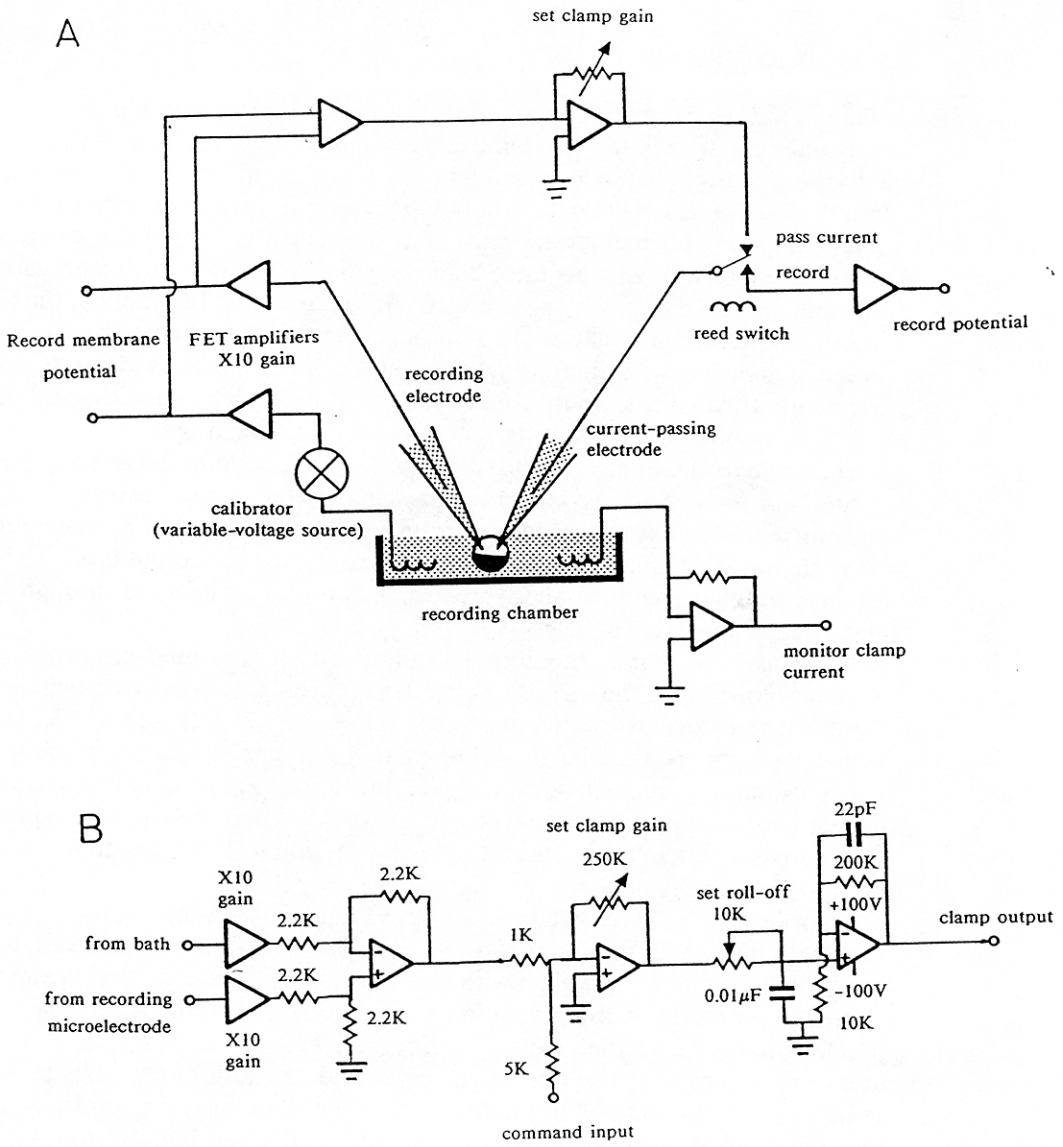

FIG. 2 Two-electrode voltage clamp for recording membrane currents in oocytes. (A) Schematic of complete clamp system. (B) Circuit diagram of high-voltage clamp. The output op-amp is type 3582J (Burr-Brown), and other op-amps are type OF 720 (Computing Techniques), or equivalent high-quality FET op-amps.

input resistance of the oocyte are usually low, and spontaneous oscillations in membrane current may be evoked. Allowing the oocyte to recover for a few minutes will often give an improvement in potential and a more stable baseline.

Recording Chamber

Recordings are made while the oocyte is continuously superfused with frog Ringer's solution in a small (0.5 ml or less) plexiglass chamber. Solution flows into the chamber by gravity from a reservoir placed on a shelf about 50 cm above the chamber. An inverted U tube of glass is connected to the inflow of the chamber by a length of silicone rubber tubing containing the perfusate, and the end of the U tube dipped into a beaker or flask. Air is first removed from the tube, and the solution then flows continuously by siphon action, at a rate which is adjusted by a clamp on the rubber tube. Changes in the perfusate are made by pinching the rubber tube (to stop the introduction of air bubbles), and transferring the U tube to a different flask. For most purposes, this extremely simple system works well, and its only major disadvantage is the dead time of several seconds introduced by the column of fluid in the tubing.

Solution is removed from the chamber through an outflow pipe, which is connected to a vacuum line (or water pump) via a series of two side-arm flasks. The flasks serve as a reservoir to collect the fluid, and as an air gap to electrically isolate the fluid in the recording chamber from ground. The second flask serves as a backup in case the first accidentally overflows, and increases the electrical isolation. A flask with a capacity of 2 liters is sufficient to allow several hours recording without emptying. Some care is needed in selecting the type of tubing used to connect the chamber outflow to the side-arm flask, since many tubes (especially silicone rubber) produce electrical artifacts in the current monitor circuit when fluid and air bubbles pass along the tube. The length of both the inflow and exit tubes should be kept as short as is convenient, and they should be arranged to minimize vibration or movement, as this can introduce electrical artifacts.

Since the oocyte is spherical, it must be held in position in the chamber. We use either of two methods to do this. In the first, the oocyte is placed in a conical hole (~1 mm diameter) in a small stainless steel disk (the egg cup) placed on the floor of the chamber. The second method involves forming a ring of petroleum jelly (Vaseline) on the chamber floor, to make a central depression into which the oocyte fits. This is done by extruding Vaseline through a hypodermic syringe using a blunted needle. In both cases, the oocyte is dropped into place in the chamber, after transfering it with a Pasteur pipette.

A dissecting microscope with a magnification of ×10 to ×40 gives adequate visibility to position and insert the microelctrodes. The oocyte is illuminated from above by a microscope lamp or fiber optic illuminator. Because of the large size of the oocyte, almost any type micromanipulator is adequate to position the electrodes, and it is unnecessary to take great precautions to isolate the recording system from vibrations.

Microinjection into Oocyte While Recording

In some experiments it is convenient to be able to inject substances into the oocyte while recording; for example, EGTA may be injected to block the oscillatory chloride currents mediated by several neurotransmitters (12), or calcium may be injected to activate chloride channels (10). Electrically charged molecules and ions may be injected by iontophoresis, but it is generally preferable to use pressure ejection. A micropipette is filled with the solution to be injected and, if sufficient quantities are available, this should be passed through a 0.22-μm Millipore filter to remove particles which might block the pipette. The micropipette is then mounted in the recording setup, and connected to a device which can apply brief (10–1000 msec) pneumatic pulses of variable (0–200 KPa) pressure. Commercial instruments are available for this purpose (e.g., Picopump, World Precision Instruments, New Haven, CT), or they can be easily made from a solenoid valve and a pressure regulator. As pulled, the micropipettes are too fine to eject fluid, and the tip should be gently broken to a diameter of a few micrometers. The ability of the pipette to eject fluid can be monitored by applying pneumatic pulses with the tip raised into the air. With newly made pipettes the expelled fluid usually runs up the side of the pipette, making quantitation difficult. However, after a pipette has been inserted in an oocyte, pneumatic pulses applied with the pipette in the air usually produce a discrete droplet of fluid at the tip. The diameter of the droplet can be measured by a calibrated micrometer in the microscope eyepiece, and used to estimate the volume. It is convenient, for this purpose, to draw a calibration curve on double-log axes, of droplet diameter versus volume.

Insertion of the injection pipette into an oocyte can be monitored by recording membrane current while clamping at a potential of −60 mV. The injection pipette is gradually advanced toward the oocyte, and its penetration is signaled by a rapid, transient inward deflection at the current trace. We sometimes also monitor insertion of the recording pipette by recording the potential from this pipette.

Volumes of a few nanoliters may be safely injected without causing damage. However, it is important to avoid contamination by calcium in the

injection solution, since concentrations of only a few picomolar are sufficient to activate chloride currents. Thus, the solution for injection should usually include about 50 μM EDTA, to chelate any contaminating calcium. Furthermore, a few pressure pulses are applied just before inserting the pipette, to flush out fluid in the tip of the pipette which becomes contaminated by calcium from the bathing solution.

Other Applications

Use of Size-Fractionated mRNA

The use of fractionated rather than whole poly(A)$^+$ mRNA offers many important advantages for the study of receptors and ion channels expressed in the oocyte (13). For instance, specific mRNAs are more concentrated, so that larger responses are obtained. More importantly, by injecting the appropriate fraction, it becomes possible to incorporate only a desired receptor or ion channel type. For example, if one wants to study the characteristics of Na^+ and K^+ channels in the brain, it is normally necessary to use pharmacological agents to block one type of channel. However, this is not necessary in oocytes because the Na^+ and K^+ channel mRNAs can be separated, and injected into the oocytes to express only one type of channel (13). Another example is that the two mRNA species coding for glutamate receptors can be separated and used to characterize the two glutamate receptor subtypes encoded (13). A further advantage of fractionation is that it may disclose heterogeneity among mRNAs encoding a particular receptor (14). The mRNA size fractionation is also useful to examine the presence of other mRNA species with different sizes encoding a second subunit or a factor, such as an enzymatic activity that modulates the properties of the channels (15, 16).

Partial purification of mRNA can be achieved either by sucrose density gradient centrifugation (13) or by gel electrophoresis (4, 17). However, we usually found that the mRNAs fractionated by agarose gel electrophoresis do not translate well in *Xenopus* oocyte. An important advantage of sucrose gradient centrifugation is that fractionated mRNA can be recovered easily and efficiently and is still translationally active. However, the resolving power of this technique is much less than that of gel electrophoresis. We describe here the procedure for partial purification of mRNA by sucrose gradient centrifugation. For further purification of mRNA by gel electrophoresis; see Ref. 17.

1. Treat centrifuge tubes for the Beckman SW 40 Ti (or its equivalent) and

gradient maker with 0.2% (v/v) DPC overnight, and rinse thoroughly with autoclaved water before use.

2. Prepare 10% (w/w) and 31% (w/w) sucrose solutions in 10 m*M* HEPES–NaOH, pH 7.5/1 m*M* EDTA/0.1% (w/v) lithium dodecyl sulfate and treat with 0.05% (v/v) DPC overnight. Autoclave the DPC-treated sucrose solutions for 15 min. Autoclaving can ensure complete removal of DPC.

3. Make 10–31% linear sucrose gradients in centrifuge tubes and keep on ice for 30 min. The gradients may be prepared with a gradient maker or using frozen step gradients by the method of Luthe (18). [The latter method is easier, reproducible, and provides equally good fractionation (M. M. Panicker and A. Morales, unpublished results).]

4. Heat the poly(A)$^+$ mRNA solution (100–200 μg in 100–200 μl of 5 m*M* HEPES-NaOH, pH 7.5) at 65°C for 5 min, cool on ice, and load onto the sucrose gradient.

5. Centrifuge immediately for 19 hr at 2°C on a Beckman SW 40 Ti rotor at 39,000 rpm.

6. Collect fractions (about 0.4 ml) in sterile microcentrifuge tubes. Add 20 μl of 4 *M* NaCl and 2.5 vol of cold (−20°C) absolute ethanol. Allow the mRNA to precipitate at −20°C overnight.

7. Centrifuge in a swinging bucket rotor (Beckman JS 13.1 or its equivalent) at 11,000 rpm for 1 hr at 2°C. Discard supernatant and rinse the RNA pellets twice with 1 ml of cold (−20°C) 75% ethanol.

8. Dry the RNA pellets in vacuum and dissolve in 10–20 μl of autoclaved water. Store at −80°C until used for injection.

Use of Antisense RNAs and Oligonucleotides

Gene expression in various cells, including *Xenopus* oocytes and mammalian cells, can be selectively inhibited by antisense RNA (RNA that is complementary to mRNA) (for a review, see Ref. 19). This inhibition sometimes involves hybridization between an antisense RNA and its counterpart mRNA, which results in an inhibition of mRNA translation (20). Synthetic oligonucleotides, which are complementary to mRNA, can also be used to inhibit translation of corresponding mRNA. Complementary oligonucleotides appears to promote target mRNA degradation by an RNase H-like activity (21, 22). Since many receptor and ion-channel cDNAs have been isolated and their mRNA sequences are available, antisense RNA and complementary oligonucleotides to a particular receptor or ion-channel mRNA can be synthesized. These molecules can be injected into oocytes together with total brain mRNA to block the translation of complementary

mRNA. This approach is useful to examine receptor or ion-channel heterogeneity, blocking expression of one receptor (or ion channel) subtype with a specific oligonucleotide and studying another subtype encoded by a similar, but not identical, mRNA. Use of antisense RNA and complementary oligonucleotides to inhibit the expression of functional receptors in *Xenopus* oocytes has been described (23) and, therefore, will be only briefly summarized here. For more details, see Refs. 23 and 24.

1. Synthesize antisense RNA or complementary oligonucleotide (20-mers work well). For a detailed procedure for the synthesis of antisense RNA, see Refs. 23 and 25. Custom syntheses of oligonucleotides of specific sequences are now readily available from commercial sources.
2. To ensure absence of ribonuclease activity, extract the antisense RNA or complementary oligonucleotide solution with an equal volume of phenol/chloroform (1:1) in a microcentrifuge tube. Centrifuge briefly and collect the upper aqueous solution into a siliconized, sterile microcentrifuge tube. Add 4 *M* NaCl solution to a final concentration of 0.2 *M* and precipitate the antisense RNA, or complementary oligonucleotide, with 2.5 vol of absolute ethanol overnight at −20°C.
3. Centrifuge in a swinging bucket rotor (Beckman JS 13.1 or its equivalent) at 11,000 rpm for 1 hr at 4°C. Discard supernatant and rinse the pellet carefully with 1 ml of cold (−20°C) 75% ethanol. Dry the pellet under vacuum and dissolve in a small volume of sterile water.
4. Measure the absorbance of a diluted sample at 260 nm to estimate the concentration of antisense RNA, or complementary oligonucleotide. An OD_{260} of 1 corresponds to approximately 20 μg oligonucleotide/ml. Adjust concentration of 1 μg/μl and store at −80°C until use.
5. For injection into oocytes, mix 1 μl of mRNA solution (2 mg/ml) with 0.1–1 μg of antisense RNA, or complementary oligonucleotide, in 1 μl of water on a sterile tissue culture dish (35 × 10 mm; Falcon) on ice. Since overinjection of these molecules results in nonspecific inhibition of expression of all receptors and ion channels, an optimal concentration for each antisense molecule must be determined. Five to fifty nanograms per oocyte is a good concentration to try first.
6. The above mixture can then be injected into oocytes to study the effect of antisense RNA or complementary oligonucleotide on the expression of functional receptors or ion channels.

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