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Transplanting Receptors from Brains into Oocytes

Ricardo Miledi, Ian Parker, and Katumi Sumikawa
Laboratory of Cellular and Molecular Neurobiology, Department of Psychobiology, University of California, Irvine, California 92717

The brain is the most complex and enigmatic organization in existence. It is made up of myriads of nerve cells that are interconnected by an incalculable number of synapses, and it is safe to say that those features that really make an individual unique—our thoughts, sensations, love, hate—all depend on the function of these synapses. Because of this, the brain has captivated human endeavor for thousands of years and, in attempting to understand how it works, humans have used many different methods. Today, we see one approach that was developed only recently.

The first recorded studies of the brain were carried out in Alexandria, some 5,000 years ago. In those studies, the Alexandrian doctors noted some correlations between skull and brain damage and impairment of movement and speech. This method of studying the brain by simple observation remained in operation for many centuries. It seems that Aristotle and Galen, about 2,000 years ago, were the first to use animals and experimental techniques to study the nervous system. For many experiments Galen used pigs, and his many important discoveries and interpretations were accepted without question until the Renaissance.

THE BIRTH OF ELECTROPHYSIOLOGY

Although less complicated than humans, Galen's apes and pigs still proved to be difficult subjects for studying the structure and function of the nervous system, so other animals were chosen. I could not find who was the first to use frogs as an experimental subject for studying the nervous system, but Leonardo da Vinci, in the late 15th century, used frogs to show that mechanical stimulation of the spinal cord caused muscles to contract.

A frog preparation similar to the one used by da Vinci was used by the great Italian scientist that we commemorate today. It is now about 200 years since the days when Luigi Galvani spent his afternoons on the roof of his house, waiting for a storm to break out. Figure 1, taken from Galvani's commentarius (*De Viribus*

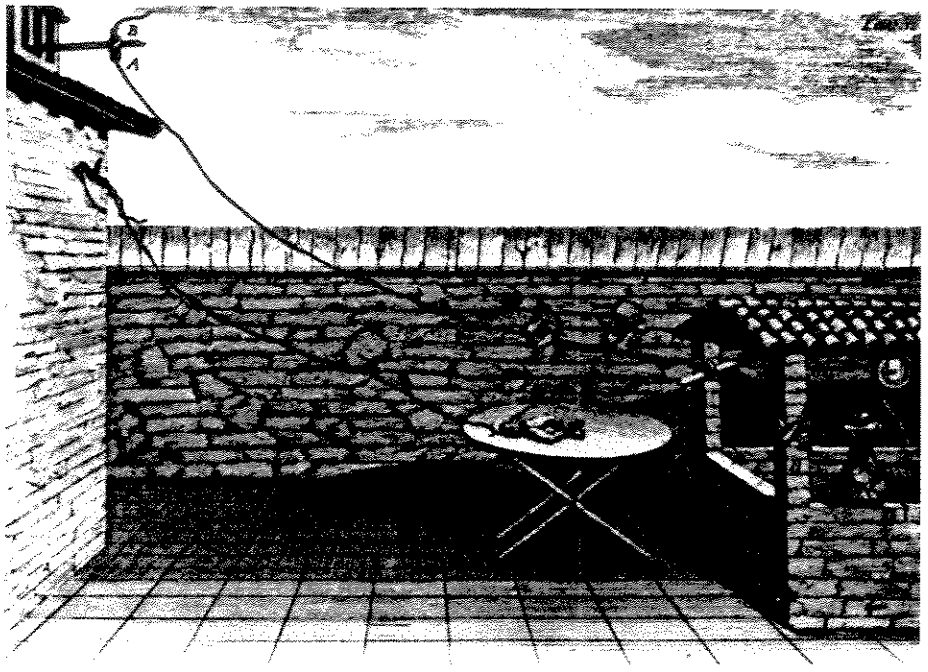

FIG. 1. Galvani's experiments on "atmospheric electricity" conducted during a thunderstorm. Whenever lightning struck the rod, the frog muscles contracted. (From ref. 8.)

Electricitatis in Motu Musculari, 1791) shows the hind quarters of a frog, attached by a wire to a lightning rod (8). Every time lightning struck the rod, the frog muscles contracted: Galvani was indeed lucky that he did not contract himself! This was really the birth of electrophysiology, and I like to think that it was born out of a thunder, whose reverberations are still felt today.

I have been following Galvani's trail and for many years have worked with frog nerves and muscles. So why now oocytes? The reason is quite simple. About 30 years ago, Diamond and I had mapped out the distribution of acetylcholine (ACh) sensitivity along fetal muscle fibers of the rat and found that after 17 days of gestation, at a time when nerve–muscle synapses had just been formed, the entire muscle fiber surface had ACh receptors (12,13). I then wondered when ACh receptors first appeared on the muscle fiber membrane. To examine this question I studied muscles earlier and earlier during development and found that even younger muscle fibers were sensitive to ACh. As you may imagine, the experiments became increasingly difficult to execute, as I used more and more immature animals, until finally I decided to approach the problem from the other end and asked myself the question: Are oocytes sensitive to ACh? At that time I did not fully anticipate all the consequences that would follow from such a simple question.

To explore this problem it was necessary to choose a suitable system. I was aware that *Xenopus* oocytes were very convenient, that they were easily available all through the year, and that embryologists had already accumulated a great deal of information on their structure, development, and changes after fertilization. So, about 20 years ago I performed a few unsuccessful experiments; but it was not until 1976 when Kiyoshi Kusano, from Chicago, and Jacques Stinnakre, from Paris, joined me in London that we really began to study *Xenopus* oocytes in earnest.

FROGS, OOCYTES, AND RECEPTORS

Presumably most of you have seen a *Xenopus*, but for those who have not, Fig. 2 shows two females. It can be seen that *Xenopus* are quite massive frogs—not unlike Sumo wrestlers in appearance. Comparing *Xenopus* with the more slender English *Rana temporaria* frogs, which I was accustomed to working with in London, is like comparing a beauty painted by Rubens with one painted by Modigliani.

The ovary of the adult *Xenopus* female contains thousands of oocytes, and if we remove a piece of ovary and look at it through a microscope it appears as in Fig. 3, which shows oocytes in different stages of development—from the very young and transparent ones to the fully grown oocytes which are about 1.5 mm in diameter. We can easily remove the oocytes from the ovary, place them in a chamber, insert two or more microelectrodes, and proceed to apply ACh to see if they are

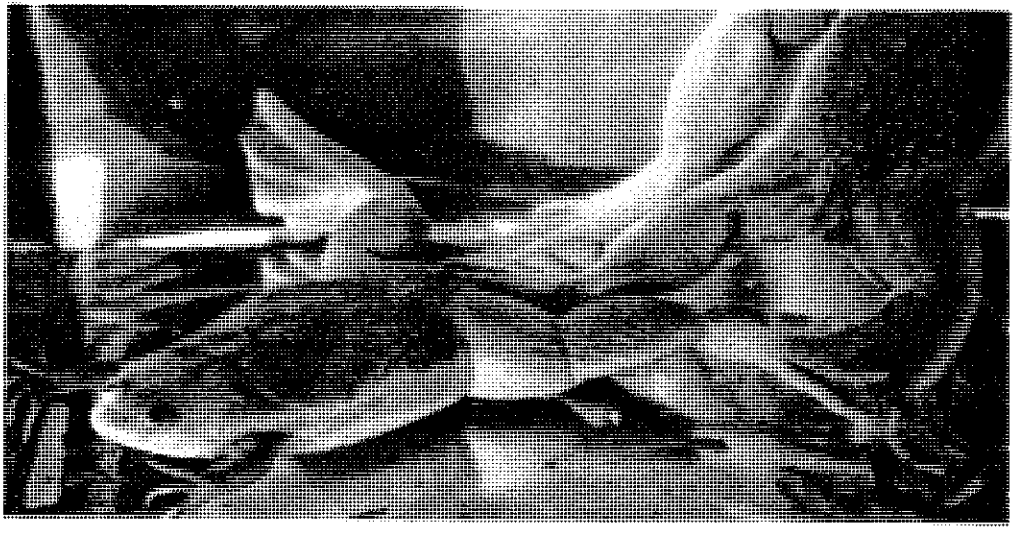

FIG. 2. *Xenopus laevis*: two female frogs. The length of each animal is about 8 inches from head to foot.

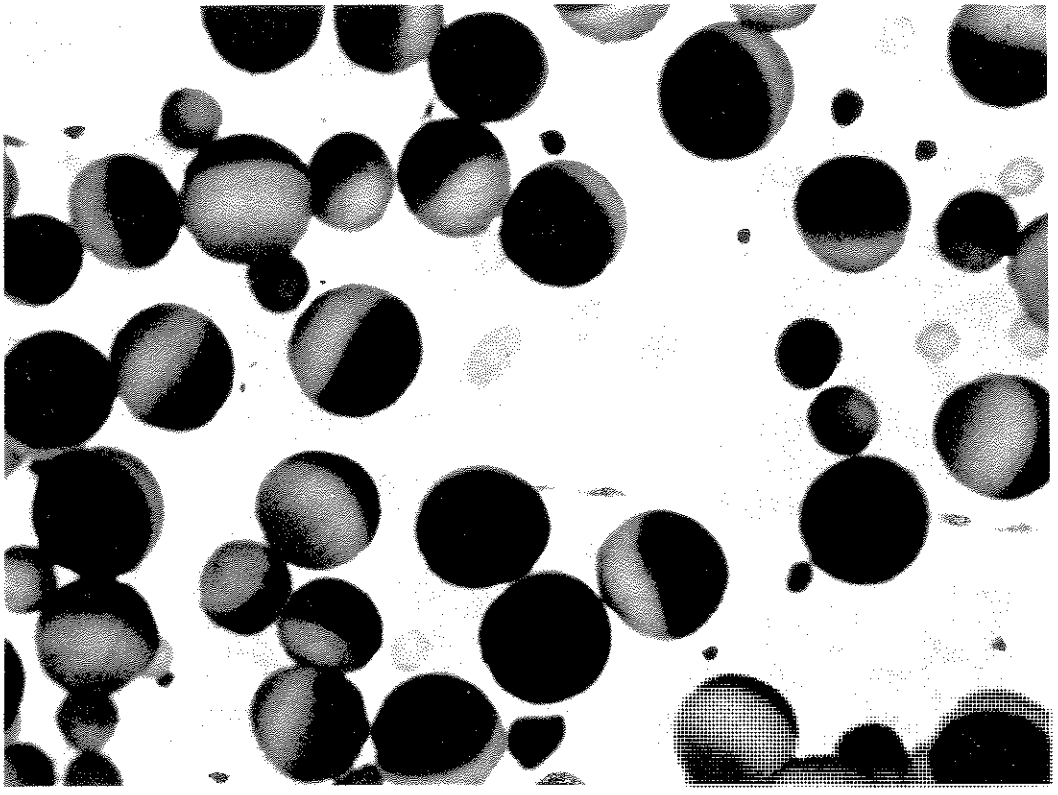

FIG. 3. Part of the ovary from a *Xenopus* showing oocytes at different stages of development.

sensitive to it. This is exactly what we did, and to our delight we found that many oocytes responded to ACh (29,30).

The response was an oscillatory current, and Fig. 4 shows one striking example. Without going into details, let me mention briefly some of the main features of these responses. First, they are blocked by atropine and not by curare or α-bungarotoxin, both of which are highly specific antagonists of nicotinic ACh receptors in muscle fibers. Therefore, the responses in the oocytes result from activation of muscarinic, and not nicotinic, ACh receptors. Second, the oscillatory current is carried mainly by chloride ions, and the onset of the response does not occur until after a very long delay has elapsed. In this particular case the first oscillation began only some 20 seconds after the pulse of ACh was applied. In other cases the delay could be minutes. This is in sharp contrast with similar experiments done earlier by Bernard Katz and myself on muscle fibers. There the stimulus–response delay was measured in microseconds—about a million times shorter! Because of the very long delay, Kusano, Stinnakre, and I (29,30) postulated that in the oocyte ACh

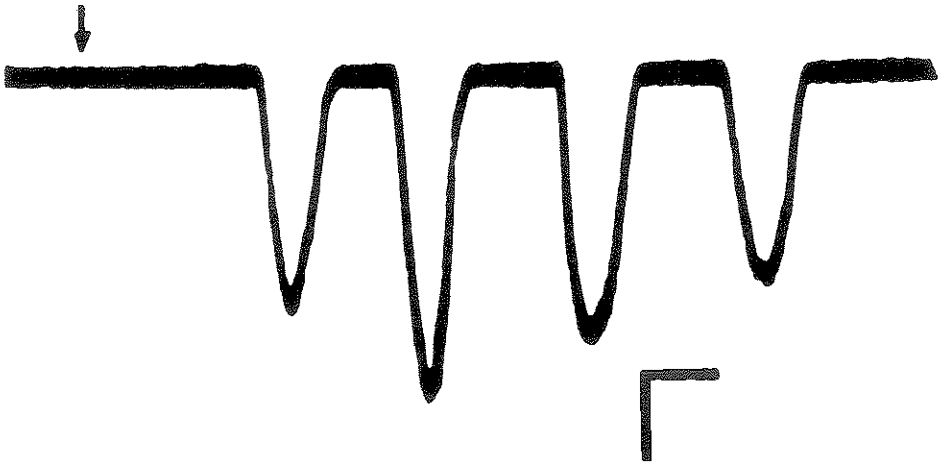

FIG. 4. Muscarinic response to ACh in a *Xenopus* oocyte. In this and subsequent figures the traces show membrane current recorded at a clamp potential of −60 mV (except where indicated); downward deflections correspond to inward currents. At the *arrow* a pulse of ACh was applied by ionophoresis to the oocyte surface. *Calibration bars* are 10 sec and 50 nA. (From ref. 37.)

did not open chloride channels directly but through the mediation of a substance that had to accumulate inside the oocyte.

All this was very encouraging, because it showed without question that *Xenopus* oocytes responded to ACh. But before we could definitely conclude that the oocyte membrane itself had ACh receptors, we had to consider other possibilities.

FOLLICLES AND OOCYTES

An oocyte simply plucked from the ovary as is commonly done is a follicle: it is not a single cell. It is actually a veritable cellular complex, formed by the oocyte proper, which is surrounded by thousands of follicular cells. This aggregate is in turn surrounded by thousands of epithelial cells. In view of the very long delay between the application of ACh and the onset of the response, it was possible that ACh was not acting directly on the oocyte but on the surrounding cells instead. The possibility of an indirect action was even more pertinent in the case of the follicular cells, because they make close contacts with the oocyte. As shown in Fig. 5A, the surface of the oocyte has many processes, some of which form gap junctions with other processes that arise from the follicular cells (10,62; A. R. Limbrick and R. Miledi, unpublished results). The follicular cells and the oocyte

FIG. 5. Electron micrographs of *Xenopus* oocytes. (A) Section of a follicle showing a follicular cell process meeting one from the oocyte. (Figure continues on *facing page*.)

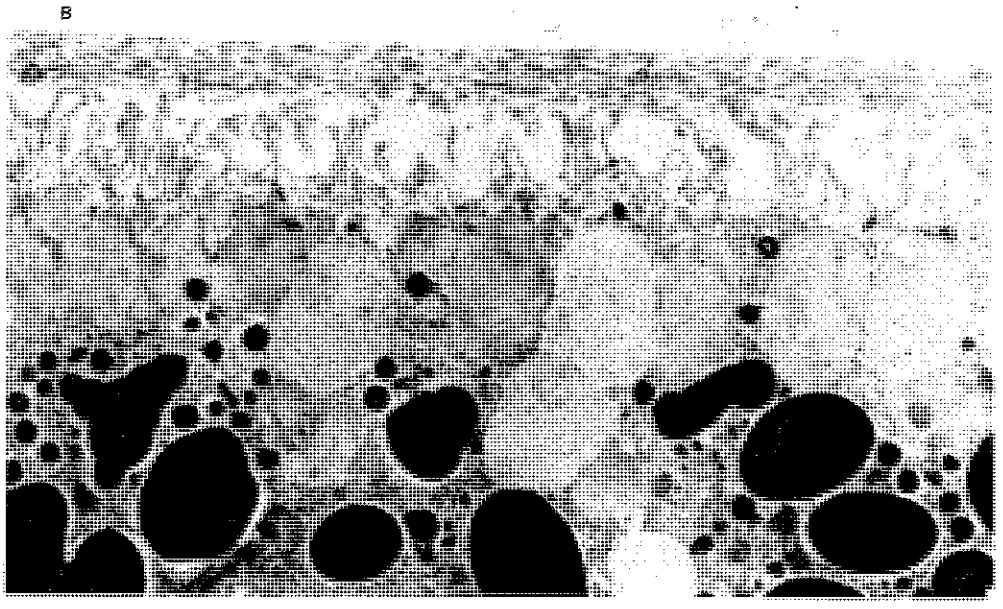

FIG. 5. *Continued.* (A) See *previous page*. (B) Section of a follicle that had been treated with collagenase to remove the cells enveloping the oocyte. (A. R. Limbrick and R. Miledi, unpublished results.)

are electrically coupled through these junctions, and it could be that ACh was combining with receptors situated on the follicular cells and that we were simply recording the ensuing response at a distance, from within the oocyte.

Fortunately, it is possible to remove the enveloping cells either manually or enzymatically, as in the case illustrated in Fig. 5B. After treatment with collagenase, the processes of the oocyte were still covered by the fibrous vitelline layer, but nothing else remained. Both follicular and epithelial cells had been removed. Nonetheless, oocytes like the one illustrated gave responses to ACh similar to those obtained with the follicles. Only then could we definitely conclude that the oocyte membrane itself has muscarinic ACh receptors which open chloride channels.

RESPONSE OF FOLLICLES TO MANY NEUROTRANSMITTERS AND HORMONES

Kusano, Stinnakre, and I also found that follicles respond to norepinephrine, dopamine, and serotonin, and later Richard Woodward and I (65,66) found that some follicles respond to other neurotransmitters and hormones. Table 1 shows a list of some of the agonists that elicit responses in follicles. However, it should be

TABLE 1. *Some agonists and hormones that evoke membrane current responses in native Xenopus follicles*

Acetylcholine	Gonadotropins
Norepinephrine	VIP
Dopamine	Prostaglandins
Adenosine	ANF
ATP	Oxytocin
GABA	Serotonin
Glutamate	Substance P
Glycine	

made clear that not every follicle responds to all these substances. Some follicles respond to only a few; and some neurotransmitters like glutamate, GABA, and glycine evoke responses that are only a few (<10) nanoamperes in amplitude. The most commonly effective agonists are the top three or four in each column. Moreover, it is interesting that all the follicles from a given donor tend to be selective for the same agonists. This suggests that the types of receptor expressed in the oocytes are under genetic control, which varies among different individuals. One wonders if this control is permanent or whether oocytes from the same frog manifest different receptors at different times.

Although a particular follicle does not necessarily have receptors to all the agonists listed in Table 1, it is abundantly clear that in some follicles several receptors can coexist. For example, Fig. 6 shows results from a follicle that responded to adenosine, follicle stimulating hormone, norepinephrine, and forskolin (an activator of adenylate cyclase). Note that all these substances elicited outward currents, except for ACh which evoked its usual oscillatory inward current. The gonadotropins, VIP, prostaglandins, and atrial natriuretic factor (ANF) also produced outward currents, all carried mainly by potassium ions. Furthermore, their actions are all mimicked by forskolin (Fig. 6) and by intra-oocyte injection of cyclic AMP. So we think that all these agonists do not open the K^+ channels directly but via a receptor–channel coupling mechanism that involves cAMP (31,63,65,66; R. M. Woodward and R. Miledi, unpublished results).

The available evidence indicates that the various outward current responses are mediated by specific receptors and, as in the case of the muscarinic ACh receptor, the question again arises as to where in the follicle those receptors are and where are the K^+ channels they operate?

DIFFERENT LOCATION OF THE TWO MAIN RECEPTOR–CHANNEL COUPLING SYSTEMS IN FOLLICLES

To determine where the receptors and channels are located, Woodward and I studied follicle-enclosed oocytes and oocytes from which the enveloping cells had

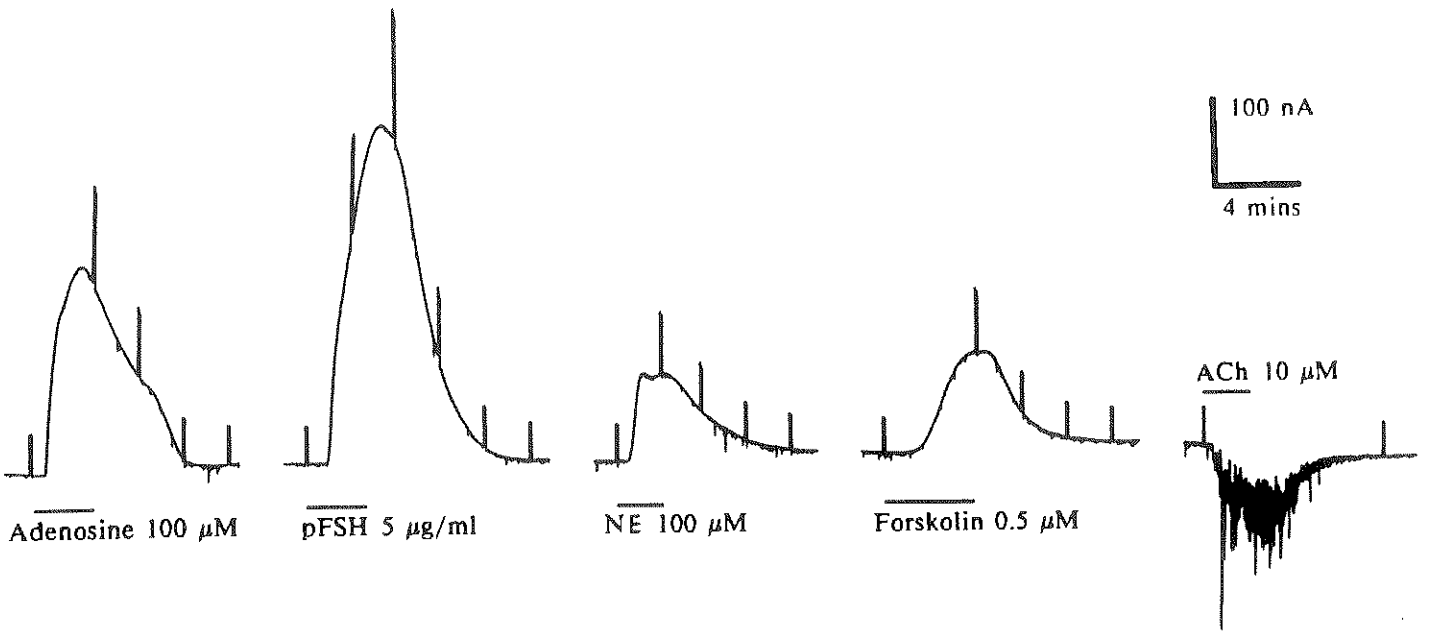

FIG. 6. Membrane currents recorded from a follicle-enclosed oocyte in response to adenosine, porcine follicle stimulating hormone (pFSH), norepinephrine (NE), forskolin, and ACh. Drugs were applied by bath superfusion for the times indicated by the *bars*. The periodic steps on the trace are currents resulting from voltage pulses (10 mV depolarizing) applied to monitor the oocyte's resistance. (From ref. 65.)

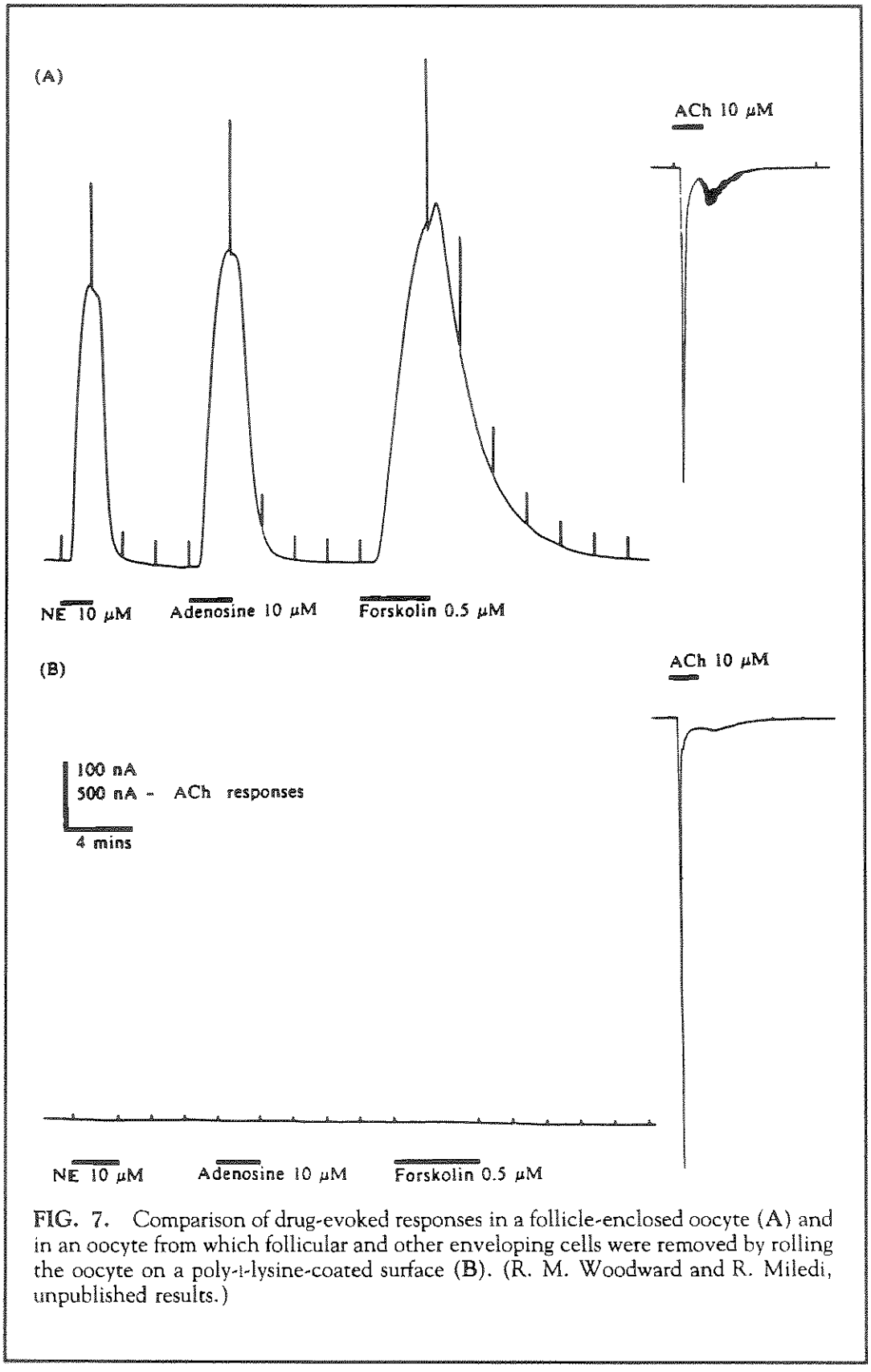

FIG. 7. Comparison of drug-evoked responses in a follicle-enclosed oocyte (A) and in an oocyte from which follicular and other enveloping cells were removed by rolling the oocyte on a poly-L-lysine-coated surface (B). (R. M. Woodward and R. Miledi, unpublished results.)

been removed. In Fig. 7, the top traces show the responses obtained in a follicle-enclosed oocyte exposed successively to norepinephrine, adenosine, forskolin, and ACh; the lower records show the effect of these same substances applied to a manually defolliculated oocyte obtained from the same donor. Note that the responses to norepinephrine, adenosine, and forskolin have vanished, while the response to ACh is still elicited after removing the follicular cells. Incidentally, the responses to gonadotropins, VIP, and prostaglandins are also abolished by defolliculation.

All these results may be explained in several ways. However, Woodward and I prefer the scheme illustrated in Fig. 8, which shows the follicular cells electrically coupled to each other as well as to the oocyte proper. We think that the receptors to catecholamines and hormones, which trigger the outward currents, are in the membrane of the follicular cells (65,66). When these receptors are activated, for instance, by norepinephrine as in the drawing, cyclic AMP is produced inside the follicular cells and this directly, or more probably through the action of protein

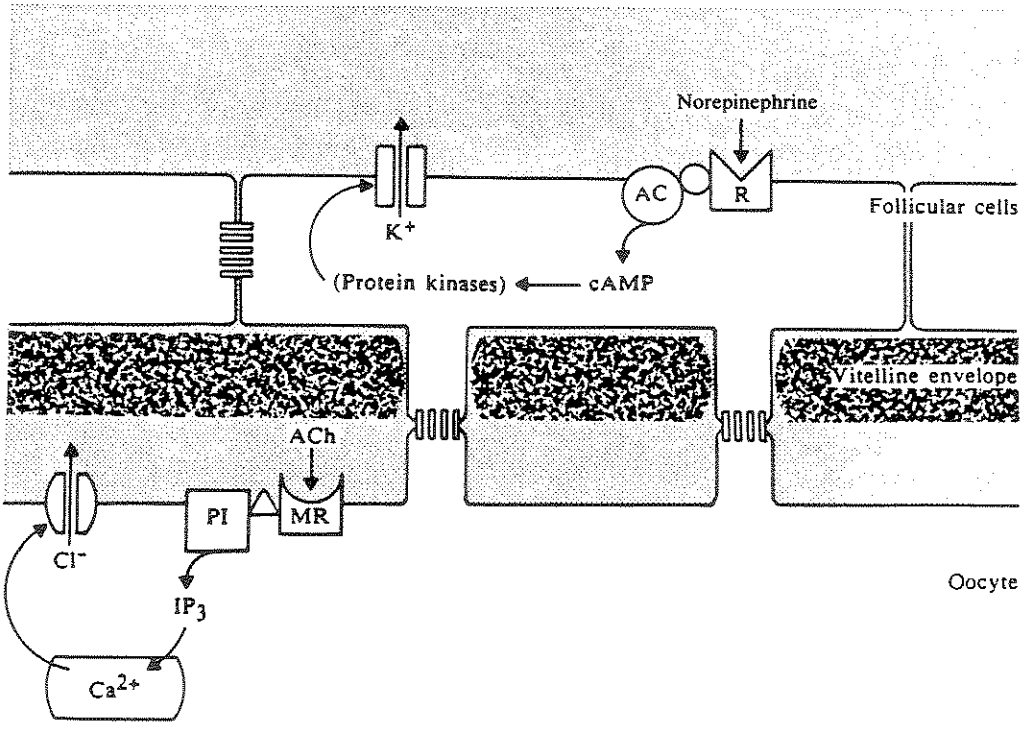

FIG. 8. Scheme illustrating the electrical coupling of follicular cells to the oocyte and the putative locations of neurotransmitter and hormone receptors and membrane channels. AC, adenylate cyclase; PI, phosphoinositidase; R, norepinephrine receptor; MR, muscarinic receptor. (R. M. Woodward and R. Miledi, unpublished results.)

kinases, opens the K^+ channels also located in the membrane of the follicular cells. When the follicular cells are removed, both the hormone receptors and the K^+ channels are eliminated, leaving muscarinic ACh receptors in the oocyte membrane coupled to chloride channels through a phosphatidyl–inositol system (see below).

Thus, it appears that, as far as plasma membrane channels are concerned, the two main receptor–channel coupling systems—cyclic AMP and phosphoinositides—have different intracellular locations in the follicle. The cyclic AMP system is preponderantly located in the follicular cells, while the phosphoinositide system is more abundant in the oocyte.

VOLTAGE-OPERATED MEMBRANE CHANNELS

As well as possessing a number of drug-activated currents, oocytes also display a surprising diversity of voltage-activated membrane currents. One of the largest and most common of these is a transient outward current seen on depolarization to potentials around 0 mV (3,37). This current arises because the oocyte membrane contains calcium channels that are opened when the membrane is depolarized, causing an influx of calcium into the oocyte. The ensuing rise in intracellular free calcium leads to the activation of calcium-dependent chloride channels (probably the same channels that mediate the ACh response) to give the final outward current response. More recently, Ian Parker and I (52–54) reexamined a number of other voltage-activated currents observed since we first started working with oocytes. Some of the currents are illustrated in Fig. 9. The first two of these are transient sodium and potassium currents, which are activated on depolarization to potentials around 0 mV and which have properties resembling the sodium and potassium currents of nerve cells. The third response is more unusual, being a chloride current that is activated by hyperpolarization to potentials beyond about -100 mV. It is not yet clear what role, if any, these currents play in the oocyte. Since some of these currents are not consistently present in the oocytes, it may be that the corresponding membrane channel proteins arise from an apparently random expression of some genes which become important at some later stage in the development of the frog.

TRANSPLANTING RECEPTORS FROM BRAIN CELLS INTO OOCYTES

With the variety of receptors and channels that they already have, *Xenopus* oocytes and follicles are very suitable subjects for study. However, I thought that their usefulness, as a model system for studies of the nervous system, would be

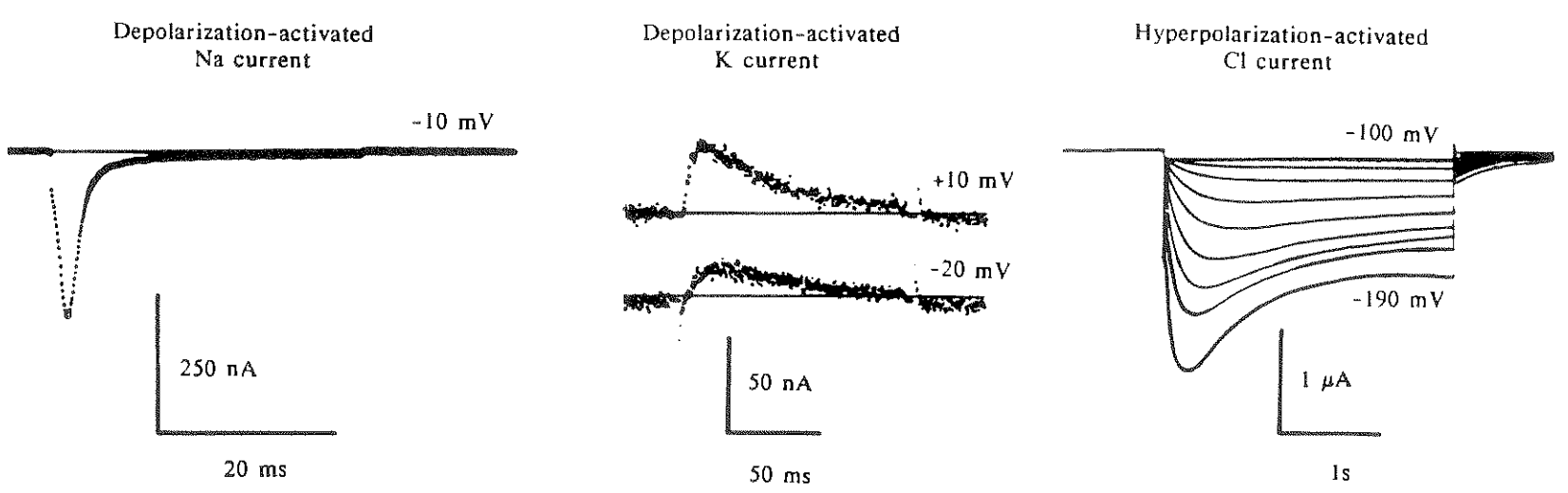

FIG. 9. Examples of membrane currents evoked by polarization in "native" *Xenopus* oocytes. All three traces show clamp currents in response to step changes to the voltages indicated, from holding potentials of −100 mV (first two figures on left) or −60 mV (last figure on right). Passive membrane currents were computer subtracted in the two figures on left. (From ref. 52–54.)

greatly increased if it were possible to induce the oocytes to acquire the neurotransmitter receptors and channels that are so crucial to the functions of the brain.

One obvious advantage in being able to do that is shown in Fig. 10. On the left is an oocyte and the little speck by its side shows a neuron at the same scale. On the right the same two cells are shown at higher magnification. Because the oocyte is so large and accessible, it allows us to do a variety of electrophysiological and biochemical experiments that would be very difficult, or nearly impossible, to carry out on the much smaller neuron. Clearly, it would be nice if we could transplant the receptors from the small nerve cells to the oocytes.

During the last years my colleagues, Katumi Sumikawa, Ian Parker, and Cameron Gundersen, and I have been doing just that. We are now able to transplant ACh receptors (both nicotinic and muscarinic) to the oocyte, as well as receptors to serotonin, dopamine, norepinephrine, glutamate, kainate, aspartate, NMDA, GABA, glycine, substance P, and neurotensin—in effect most of the known receptors (15–18,39,40,55,60). Other researchers are now adding to this list (9,11,21,47).

This so-called transplantation of receptors is not a surgical transplantation but a biochemical one; it is carried out as shown diagrammatically in Fig. 11. For example, we take the cerebral cortex from the human brain, homogenize it, and isolate the poly(A)$^+$ messenger RNA. It should be borne in mind that this mRNA

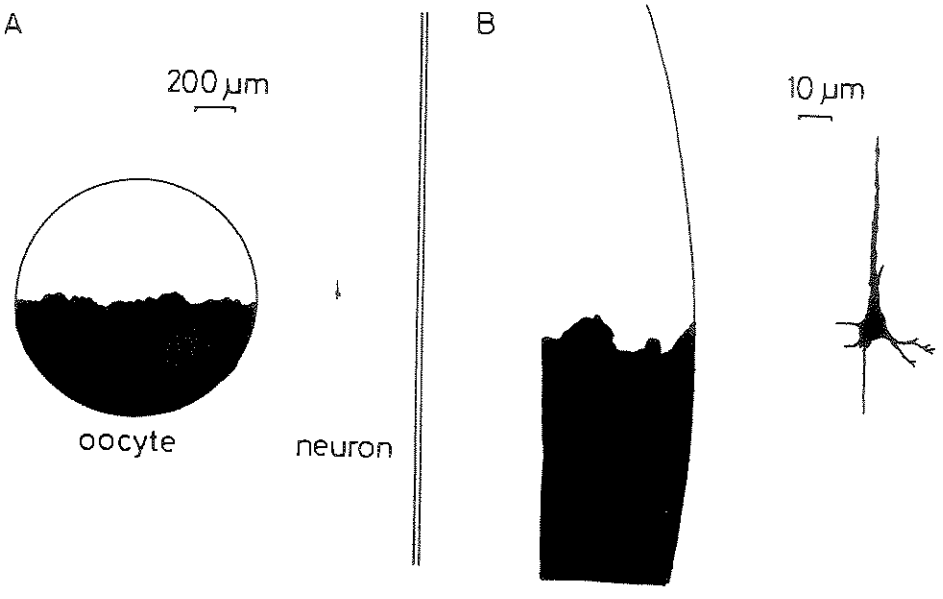

FIG. 10. Sketches illustrating the difference in size and shape between the frog oocyte and the cell body of a typical brain neuron (pyramidal cell) at lower (A) and higher (B) magnifications. The width of the neuron is less than one-hundredth of that of the oocyte.

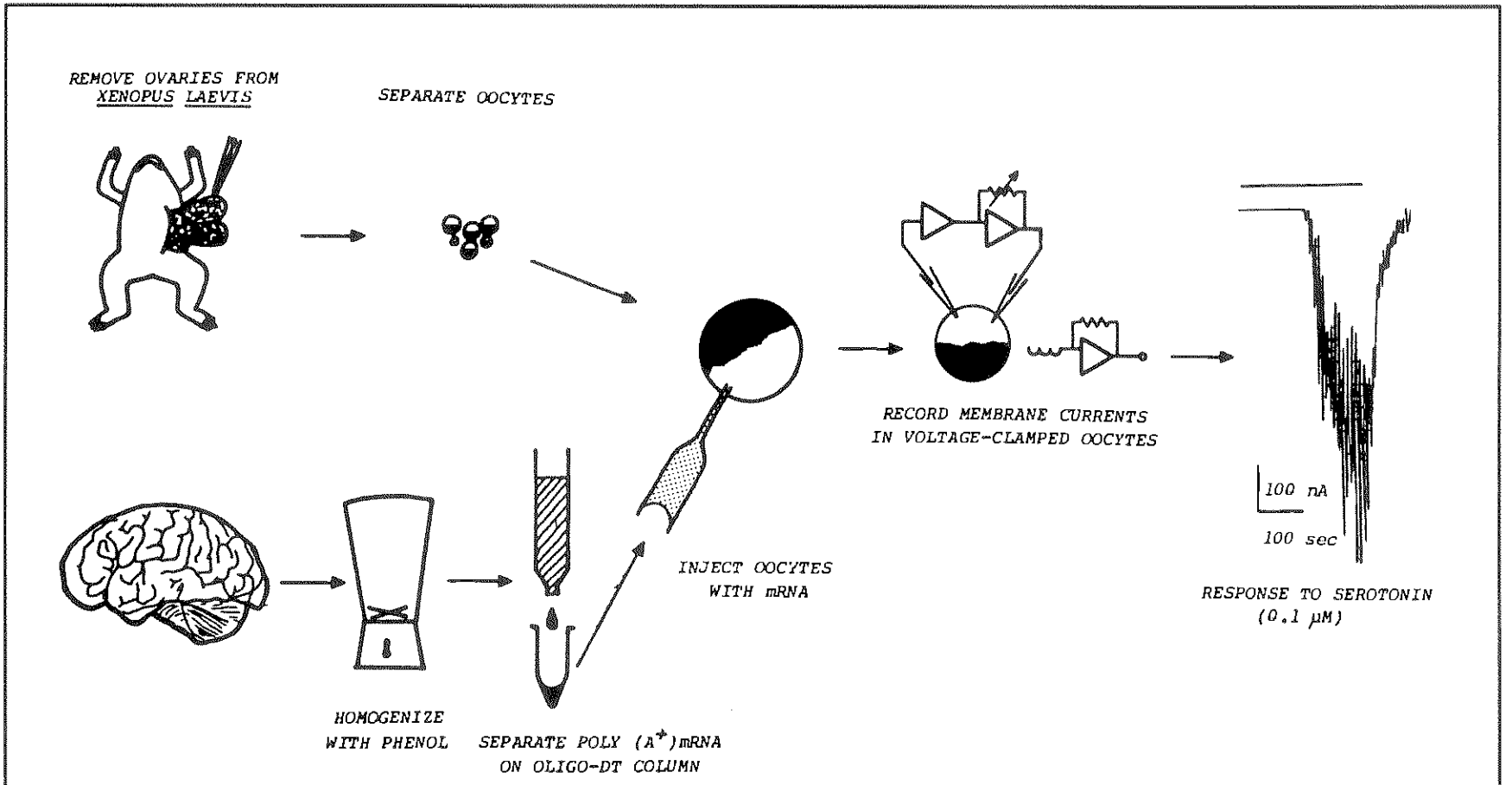

FIG. 11. Stages in the "transplantation" of neurotransmitter receptors into oocytes by the isolation and injection of mRNA from the brain.

is a mixture of many thousands of different messages, some of which carry the instructions for making neurotransmitter receptors. The next step is to inject the foreign mRNA into the oocytes. As was shown first by Gurdon and his colleagues (20), the foreign mRNA is translated by the oocytes' own protein-synthesizing machinery. We showed further that not only was the brain mRNA efficiently translated but the products of the translation were also processed, assembled, and inserted into the oocyte membrane, where they formed functional receptors. In the example illustrated here, activation of serotonin receptors from the human brain transplanted to the oocyte caused an oscillatory chloride current similar to that produced by ACh in some native oocytes. This was a pleasant finding. Quite frankly, when the first experiments were being done I thought that after the injection of such crude messengers the oocyte might soon die due to the production of proteases and other unwanted proteins. This was not so, and the oocytes acquired the foreign neurotransmitter receptors and retained them sometimes for more than 2 months.

CALCIUM, PHOSPHOINOSITIDES, AND OSCILLATORY CURRENTS IN OOCYTES

The transplantation of receptors and channels into the oocyte membrane is a very powerful and useful technique. Let me just mention briefly some of its potential.

For instance, once we have the receptors in the oocyte membrane we can study the way in which the receptors operate. Take, for example, the Cl^- current caused by activation of the serotonin receptors. Earlier, we had shown that the native oocyte membrane has Cl^- channels that open whenever the intracellular concentration of calcium ions is increased (37,38). Therefore, we wondered if the Cl^- channels opened by serotonin were Ca^{2+} dependent and, if so, from where the Ca^{2+} came.

As shown in Fig. 12, the response to serotonin in an oocyte injected with rat brain mRNA still occurred when all the Ca^{2+} was withdrawn from the external medium. Moreover, the oscillatory response to serotonin was abolished if the oocyte was pre-injected with EGTA to chelate calcium ions and prevent their intracellular level from rising (Fig. 13). These results indicated clearly that the Cl^- channels are operated by Ca^{2+} ions and that the Ca^{2+} derives from intracellular stores.

The Hokins, Michell, and especially Berridge and their colleagues have shown that some receptors activate phosphoinositide metabolism, leading to the production of inositol trisphosphate (IP_3), which then releases calcium (6,24,36). To see if a similar process occurred in the oocytes, Ian Parker and I injected IP_3 into oocytes. As shown in Fig. 14, we found that the IP_3 caused an increase in the intracellular level of Ca^{2+} ions (monitored here by the photoprotein aequorin)

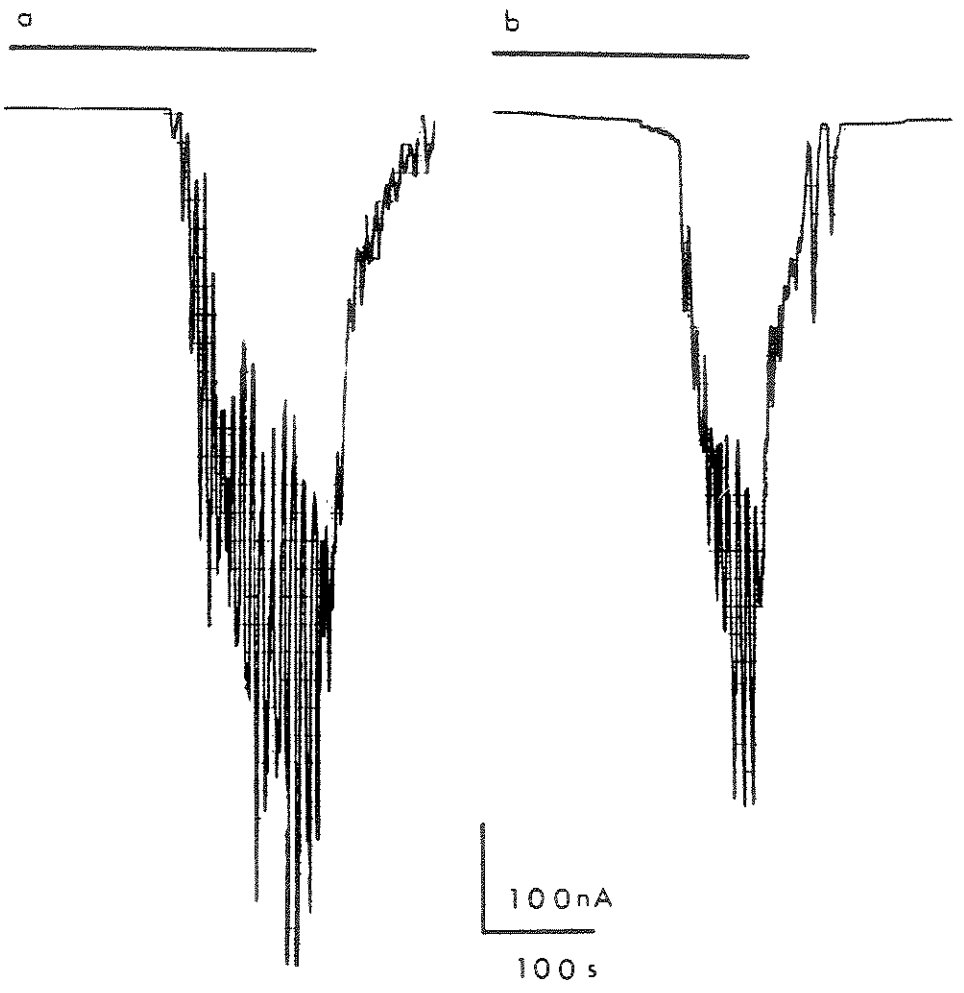

FIG. 12. The oscillatory response to serotonin is not abolished by removal of calcium from the external medium. Both traces show responses to serotonin (5×10^{-7} M):(a) in normal Ringer and (b) in Ringer with no added calcium and containing 2 mM EGTA and 5 mM $MgCl_2$. Perfusion with the zero calcium Ringer began several minutes before record (b). (From ref. 49.)

and also produced an oscillatory Cl^- current like that produced by serotonin or ACh (50). Furthermore, Nomura and his colleagues recently found that both serotonin and ACh cause an increased production of IP_3 in oocytes injected with rat brain mRNA (45). Thus, we believe that serotonin receptors, through the mediation of a GTP-binding protein, cause the production of IP_3, which then releases calcium from internal stores, the calcium finally opening the Cl^- channels.

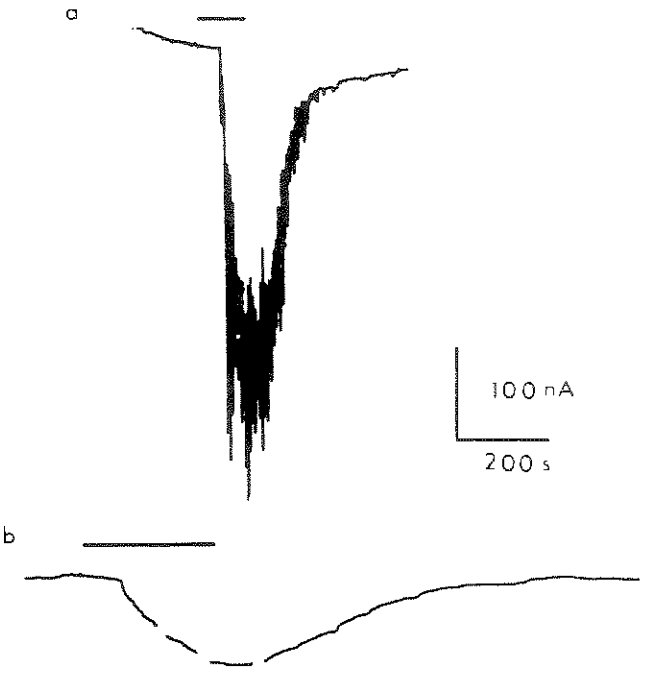

FIG. 13. Abolition of serotonin-induced oscillatory currents, by intracellular loading with EGTA. (a) Control record from an oocyte injected with rat brain mRNA. Serotonin (3×10^{-8} M) was applied by bath perfusion for the time indicated by the *bar*. (b) Record from the same oocyte but after intracellular loading with EGTA, using ionophoretic pulses of approximately 50 nA for about 1 hr. (From ref. 49.)

The messenger pathway described above already appears rather complicated, but recent experiments show that it may still be an oversimplified view of the events that mediate the serotoninergic response. For example, biochemical evidence indicates that IP_3 is further phosphorylated inside cells to inositol tetrakisphosphate (IP_4) (5). We therefore tried injecting IP_4 into oocytes to see if this compound acts as a second messenger and found that, like IP_3, it was able to evoke oscillatory chloride currents resulting from a rise in intracellular calcium (Fig. 15) (51). Interestingly, the characteristics of the currents evoked by IP_3 and IP_4 are different, suggesting that they might serve different roles as second messengers. IP_4 gave almost purely oscillatory responses, while IP_3 gave responses comprised of both smooth and oscillatory components (Fig. 15).

The characteristics of the phosphoinositide messenger pathway appear to determine many of the properties of the responses to serotonin and other agonists. A striking example of this is seen in the highly nonlinear dose–response relationship

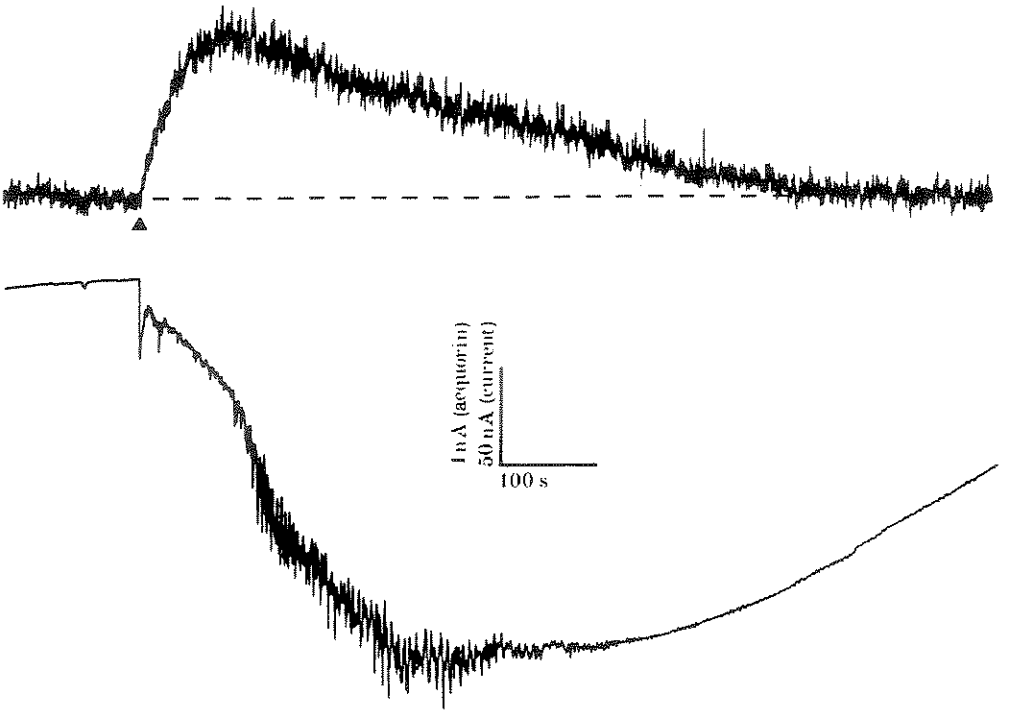

FIG. 14. Aequorin light signal and membrane current in response to injection of IP_3 into an aequorin-loaded oocyte. The upper trace monitors light emission and the lower trace shows clamp current. Upward deflections correspond to increasing light (i.e., increasing intracellular calcium) and outward membrane current. Injection of IP_3 was made at the time indicated by the *arrowhead*. (From ref. 50.)

for serotonin, which leads to an almost all-or-nothing membrane current response for doses close to threshold (15). Recent experiments, using the photolysis of caged IP_3 loaded into oocytes to provide a precisely controlled liberation of intracellular IP_3, indicate that this nonlinearity probably arises because a threshold level of IP_3 is required to trigger the liberation of intracellular calcium (48). The existence of the nonlinearity is likely to have important consequences for the functioning of synapses, which employ phosphoinositide signaling, since it may form the basis for a novel form of heterosynaptic facilitation. Thus, we were able to demonstrate in the oocyte (56) that large responses were obtained to the simultaneous application of low doses of ACh and serotonin, while the same dose of either agonist alone gave virtually no response (Fig. 16). Another interesting feature of this intracellular signaling system is that, once activated, it leaves membrane changes that greatly outlast the initial surge of current.

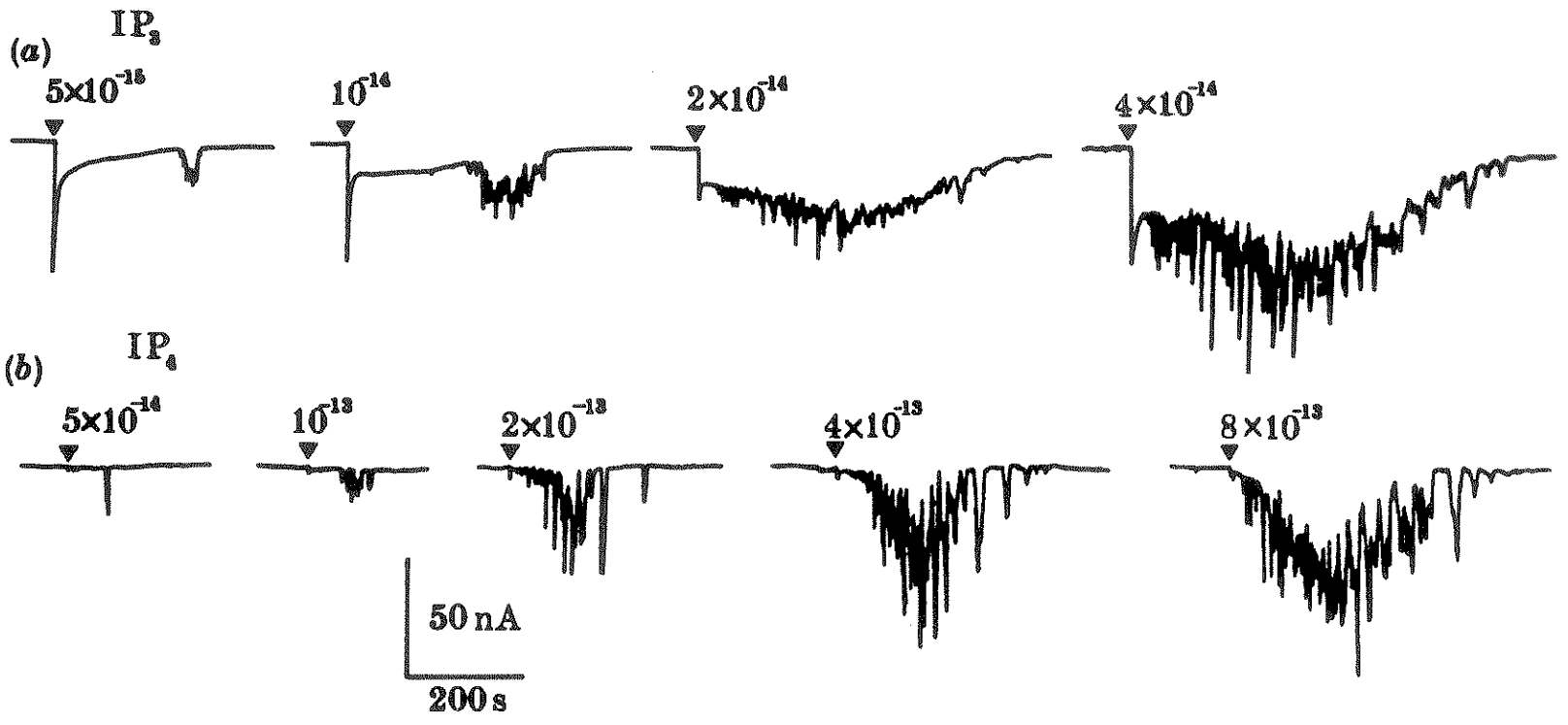

FIG. 15. Membrane currents recorded in a single oocyte in response to intracellular injections of different amounts of IP_3 (a) and IP_4 (b) (indicated in moles next to each trace). Clamp potential was -50 mV, and *arrowheads* mark the times of injection. (From ref. 51.)

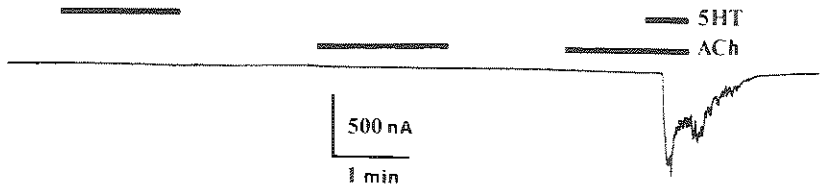

FIG. 16. Potentiation of membrane current response by simultaneous application of ACh (3×10^{-7} M) and 5-HT (serotonin, 3×10^{-8} M) to an oocyte injected with rat brain mRNA. The trace shows membrane currents, and the *bars* indicate the durations of drug application. ACh gave an inward current of about 5 nA and 5-HT (3×10^{-8} M), previously applied alone, gave 10 nA. Thus, the combined application of ACh and 5-HT elicited a response that was more than 50 times larger. (R. Miledi, unpublished results.)

NONOSCILLATORY CURRENT RESPONSES

As far as I know, nobody has yet described a serotonin receptor in the human or rat brain that functions in the same oscillatory way as the serotonin receptors transplanted to the oocyte membrane. Of course, it may be that the oscillatory behavior is peculiar to the oocyte. However, I am inclined to think that sooner or later someone will discover similar oscillatory currents in nerve cells.

Be that as it may, other receptors transplanted to the oocyte membrane behave very much like the receptors in the membrane of the cells from which the mRNA originated. For example, the properties of receptors to GABA, glycine, and kainate, as well as the nicotinic acetylcholine receptors transplanted to the oocyte, are similar in most respects to the properties of the original receptors in brain and muscle cells. Another recent example is the excitatory amino acid receptor activated by *N*-methyl-D-aspartate (NMDA).

A few years ago, we showed that rat brain mRNA induced the appearance of NMDA receptors in *Xenopus* oocytes (16). More recently, Philip Ascher and his colleagues showed that glycine potentiates the action of NMDA on cerebral neurons (2, 26). As shown in Fig. 17, rat NMDA receptors from brain transplanted to the oocyte are also greatly potentiated by glycine (see also ref. 64). Note that application of glycine alone produced a very small response—which in this experiment was undetectable at the amplification used to obtain the record. However, when NMDA was applied together with glycine, a large current was generated. Conversely, the right part of the figure shows that NMDA alone produced no detectable current, but again a large current was generated when both substances were applied together. Furthermore, Mayer, Westbrook, Ascher, and their colleagues have shown that Mg^{2+} ions block the responses to NMDA in nerve cells

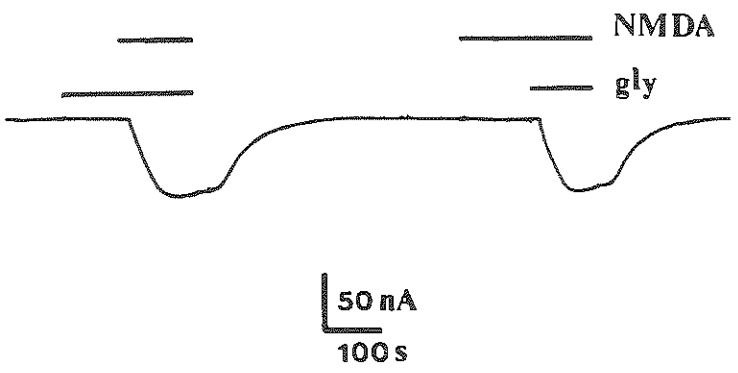

FIG. 17. Membrane currents in an oocyte injected with rat brain mRNA evoked by the concerted action of NMDA and glycine. NMDA (10^{-4} M) and glycine (10^{-5} M) were applied by bath perfusion for the times indicated by the *bars*. Ringer solution contained no magnesium. (R. Miledi, unpublished results.)

(34,35,46), and we observed that rat brain NMDA receptors in the oocyte are also inhibited in the presence of Mg^{2+} ions.

As well as responding to NMDA, oocytes injected with rat brain mRNA additionally show small currents in response to aspartate. These responses are also potentiated by glycine and are blocked by Mg^{2+} (Fig. 18). This suggests that

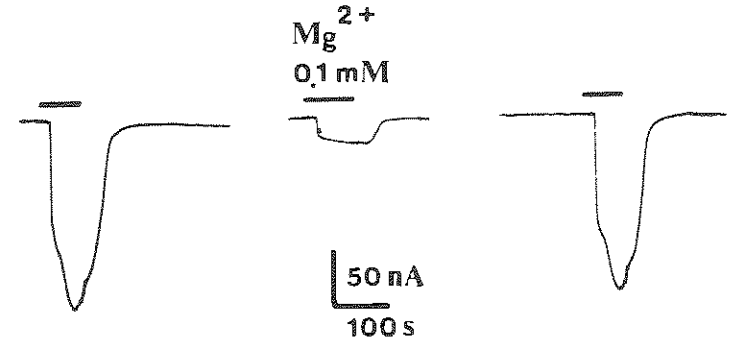

FIG. 18. Blocking action of Mg^{2+} ions on the current evoked by aspartate plus glycine. Each trace shows currents evoked by aspartate (10^{-4} M) plus glycine (10^{-5} M). The middle trace was obtained during perfusion with Ringer solution including 0.1 mM Mg^{2+}. Control records (*left* and *right*) were obtained in normal Ringer solution containing no Mg^{2+}. (R. Miledi, unpublished results.)

aspartate may act on the same receptors as NMDA, although we shall not be certain of this until the various receptors to excitatory amino acids are cloned.

MOLECULAR CHARACTERIZATION OF CHANNELS EXPRESSED IN OOCYTES

Xenopus oocytes also allow us to study the action of neurotransmitter receptors in molecular detail, which is sometimes difficult to do in nerve cells (consider, for instance, all the problems encountered if one wishes to study nerve cells of the human brain). Figure 19 shows results obtained from an oocyte that had been injected with human brain mRNA. Thus, this oocyte had been induced to acquire human glycine receptors and, when glycine was applied, it generated a Cl^- current. Note that as the current increased the membrane current noise, shown in the middle trace, also increased. This noise can be analyzed to derive single-channel characteristics (1,27,28), and in this way we found in this case the human brain glycine receptors transplanted to *Xenopus* oocytes open Cl^- channels that have a mean single-channel conductance of about 30 pS and remain open, on the average, for about 200 msec (cf. 19).

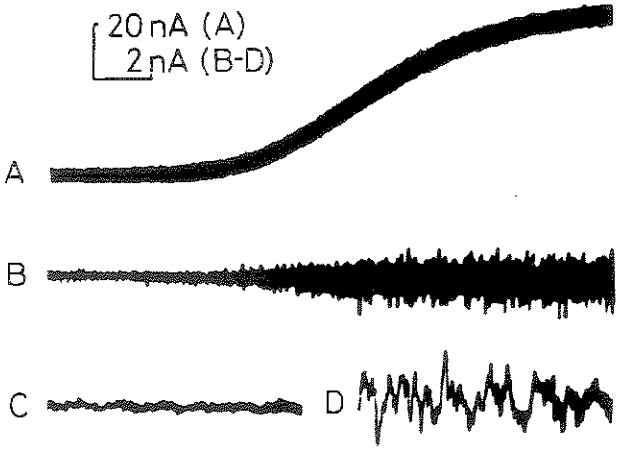

FIG. 19. Membrane current and accompanying current fluctuations ("noise") evoked by glycine in an oocyte injected with mRNA from fetal human brain. The upper trace (**A**) shows the rise in inward current evoked by glycine, monitored on a low recording gain. The same response is shown in (**B**), recorded at a higher gain and AC coupled, so as to demonstrate the increase in noise during glycine action. Traces in (**C**) and (**D**) were recorded at faster sweep speed and compare the noise levels before (**C**) and during (**D**) glycine activation. Horizontal bar, 15s (**A,B**) and 2s (**C,D**). (C. B. Gundersen, I. Parker, and R. Miledi, unpublished results.)

Rat brain mRNA also induces glycine receptors, together with many other types of receptors, including those to kainate. When we set out to analyze the properties of single channels activated by kainate, we were surprised to find that kainate currents were accompanied by almost no increase in noise fluctuations, despite the fact that glycine currents in the same oocytes showed clear noise (Fig. 20). This suggested that the elementary conductance of the kainate channel must be very small, and indeed noise analysis gave an estimate of 0.3 pS for single-channel

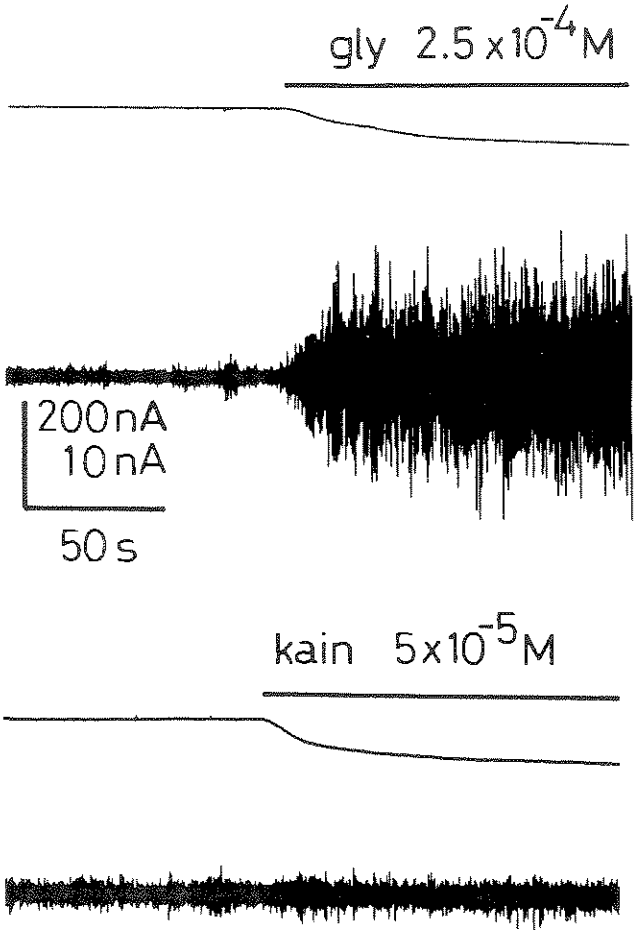

FIG. 20. Membrane current noise produced by glycine and kainate in oocytes injected with rat brain mRNA. In each frame the upper trace shows a low gain, DC-coupled record of clamp current, and the lower trace shows the current at high gain and band pass filtered between 1 and 50 Hz. Clamp potential was −60 mV. (I. Parker, C. B. Gundersen, and R. Miledi, unpublished results.)

conductance—a value 100 times smaller than that for the glycine channel (42). Thus, it is clear that the channel properties are determined largely by the properties of the proteins which form them and not by the lipid environment in which they reside. Furthermore, the rat brain mRNA appears to express very large numbers of kainate channels because large total currents (a few microamperes) could be obtained despite the small single-channel current.

Xenopus oocytes can also be used to record single-channel currents directly. For example, Fig. 21 shows results obtained from an oocyte injected with mRNA from both chick brain and denervated cat muscle which induced the oocyte membrane to acquire chick brain GABA receptors and cat muscle nicotinic ACh receptors. Single-channel currents were then recorded from the same oocyte with either GABA or ACh present in the patch pipette. Simple comparison of these records shows that the channels activated by GABA and ACh had similar conductances. In contrast, the average lifetime of the GABA-induced channels was 16 msec, while that of the channels induced by ACh was 3 msec (41). In these and other characteristics, the GABA and glycine receptors in the oocyte behave like those studied in neurons (4,7). All this shows that oocytes express faithfully some neurotransmitter receptors and channels and that the properties of the receptor–channel complexes depend mainly on the nature of the proteins encoded by the mRNA and not so much on the cell membrane in which the receptors are embedded.

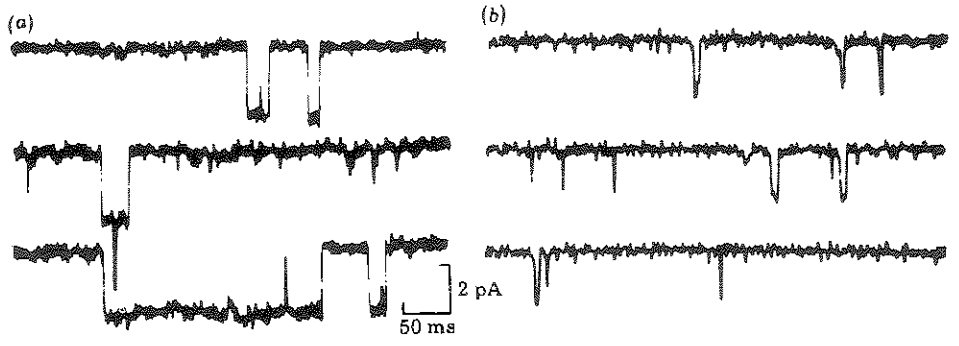

FIG. 21. Single-channel currents induced by GABA (a) and ACh (b) acting on receptors in one oocyte injected with mRNA derived from both chick optic lobe and denervated cat muscle. The records were obtained from membrane patches with seals of more than 10 GΩ. Patch pipettes contained 10^{-4} M GABA in (a) and 2×10^{-7} M ACh plus 5×10^{-7} M atropine in (b). Downward deflections of the traces correspond to inward currents and indicate channel openings. *Calibration bars* apply to all records. The membrane potential across the patch was about -110 mV in (a) and -90 mV in (b). Records were filtered at 1 kHz (a) and 500 Hz (b). Temperature range was 18 to 20°C. (From ref. 41.)

mRNA FRACTIONATION

Most of the experiments considered so far were done with whole mRNA preparations that gave rise to many neurotransmitter receptors and voltage-operated channels. Therefore, to reduce the number of different species of messages con-

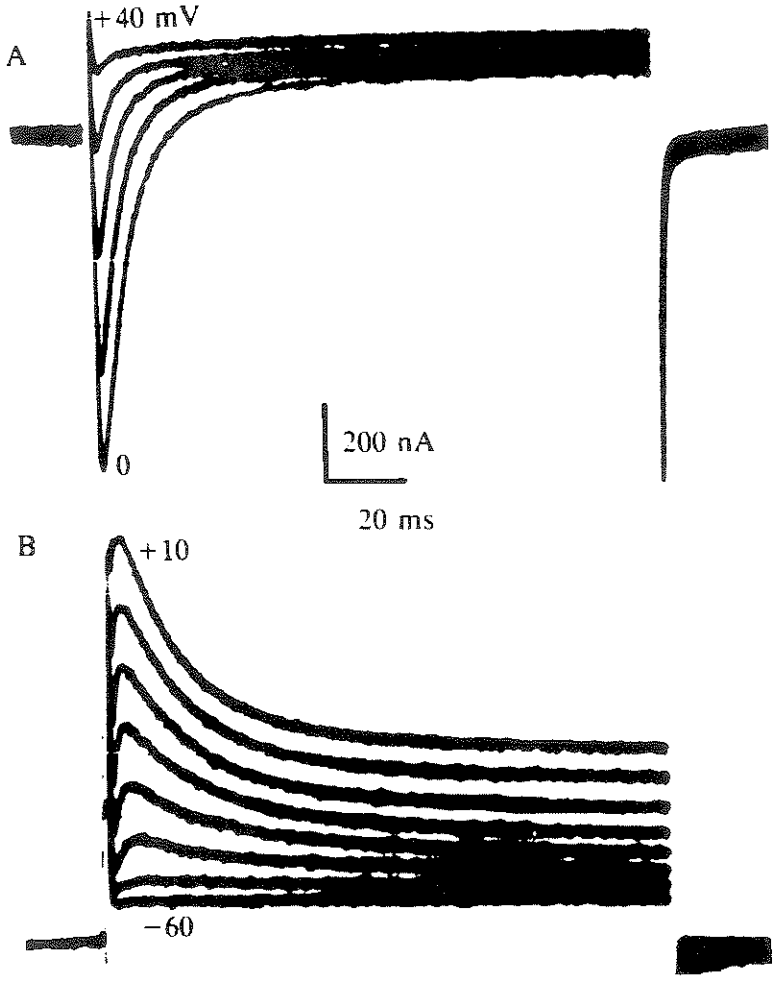

FIG. 22. Voltage-activated sodium (A) and potassium (B) currents recorded from two oocytes injected with different fractions of mRNA from chicken optic lobe. (A) Records of Na^+ currents in an oocyte injected with fraction 7 mRNA. The membrane potential was held at −100 mV and stepped to potentials of (from *bottom* to *top*) 0, +10, +20, +30, and +40 mV. (B) Records of K^+ currents in an oocyte from the same donor injected with mRNA fraction 11. The potential was held at −100 mV and stepped to (from *bottom* to *top*) −60, −50, −40, −30, −20, −10, 0, and +10 mV. (From ref. 59.)

tained in the mRNA, the whole mRNA preparations were fractionated by density gradient centrifugation or by gel electrophoresis. When the individual mRNA fractions were injected into the oocytes, some fractions were found to express only one type of receptor or voltage-operated channel. The fractionation of mRNA offers several advantages. For example, if one wants to study the characteristics of Na^+ and K^+ channels in nerve or muscle cells, it is normally necessary to use pharmacological agents to block one type of channel (cf. ref. 23). However, this is not necessary in oocytes because the mRNAs coding for different channels can be separated and injected into the oocytes to induce them to acquire one or the other type of channel (Fig. 22). For instance, although whole mRNA from the brain expressed both Na^+ and K^+ channels, oocytes injected with fraction 7 gave only inward Na^+ currents when depolarized to various levels. In contrast, depolarization of oocytes injected with fraction 11 did not elicit Na^+ currents but instead generated K^+ currents (59). When first done, these experiments indicated that Na^+ channels were encoded by a very large mRNA species, a fact that was later shown more conclusively when the Na^+ channel message was cloned and expressed by Numa and his colleagues (44).

OOCYTES AS A TOOL FOR CLONING RECEPTORS AND CHANNELS

Even though the mRNA fractions still contain many different messages, the partial purification of mRNAs is of great help in trying to understand how the brain works. First, the fractions of mRNA allow us to have oocytes tailor-made to contain a given receptor or channel. Perhaps more important, however, is the fact that the fractions are enriched for particular messages, and these fractions, combined with the use of oocytes as a translation system, provide us with a very powerful method for cloning any of the genes encoding the receptors.

The most straightforward way of cloning a receptor involves purifying the receptor protein and determining part of its primary amino acid sequence so as to be able to produce suitable oligonucleotide probes. Alternatively, one needs to purify, at least partially, the receptor protein and use it to make specific antibodies, which can then be used as probes. However, purifying receptors is frequently a very difficult and laborious task because some receptors are present in the cells only in comparatively small quantities and because the methods of purification have many pitfalls.

From the moment we succeeded in expressing neurotransmitter receptors in the oocytes we realized that these could be used to clone receptors and channels, without having first to purify them. Fractionation of mRNA was a further step toward that goal (59,61). For instance, one may take a fraction enriched for the message coding for a receptor or channel, construct a cDNA library, make mRNA from the cloned cDNA, and then screen the library, using functional assays in

Xenopus oocytes, for the gene encoding the receptor. The oocytes are very useful for these assays because they are extremely sensitive detectors of mRNA. I believe that it may even be possible to detect the presence in the oocyte of just one or a few transcripts coding for a receptor. For the initial screening, one may pool thousands of clones until a pool is identified that expresses the desired receptor, or voltage-gated membrane channel, in the oocytes. Then that pool is subdivided and tested again until the relevant clone is isolated.

Using *Xenopus* oocytes in essentially this way, Davidson, Lester, Nakanishi, and their colleagues have recently cloned the receptors for serotonin and substance K without having first to purify the receptors (32,33). Furthermore, this approach is not limited only to receptor proteins but can be applied to any protein whose expression in the oocyte can readily be screened. For example, the *Xenopus* expression system was recently employed to obtain a clone for the Na^+/glucose transporter from mammalian intestine (22).

ANTISENSE RNAs AND OOCYTES

I should mention that the positive assay—that is, expression of functional receptors by synthetic sense mRNA made from the clones—demands practically "full-length" cDNAs. Moreover, if the receptor or channel under investigation requires the presence of two or more different subunits in order to be functional, then selecting the clones by positive assays, although feasible, becomes more complicated and laborious. For these multiheterosubunit receptors, a better approach may be to use the cDNA library to produce antisense RNA and then inject the antisense RNA together with the whole or fractionated brain mRNA. In this case, hybridization of the antisense RNA with the corresponding sense target should inhibit expression of the receptor (reviewed in ref. 14; see ref. 57).

That this is indeed what happens is shown in Fig. 23. The record on the left shows the large response to ACh evoked in an oocyte injected with whole *Torpedo* electroplaque mRNA, which contained the mRNAs coding for the α, β, γ, and δ subunits of the nicotinic ACh receptor. The expression of functional nicotinic receptors was greatly inhibited when the same whole *Torpedo* mRNA was coinjected with synthetic α-subunit antisense RNA (Fig. 23B). The average response to ACh was reduced to much less than 1%. This inhibition was specific for the ACh receptors because the antisense RNA did not prevent the expression of the Cl^- channels (Fig. 23C), which are also encoded by mRNA from the electric organ of *Torpedo* (58). Furthermore, the different antisense RNAs specifically inhibited the translation of the corresponding target subunit RNAs synthesized from cloned cDNAs (Fig. 23D). Thus, by using the antisense approach, it should be possible to identify the clones encoding the subunits of a heteromeric receptor.

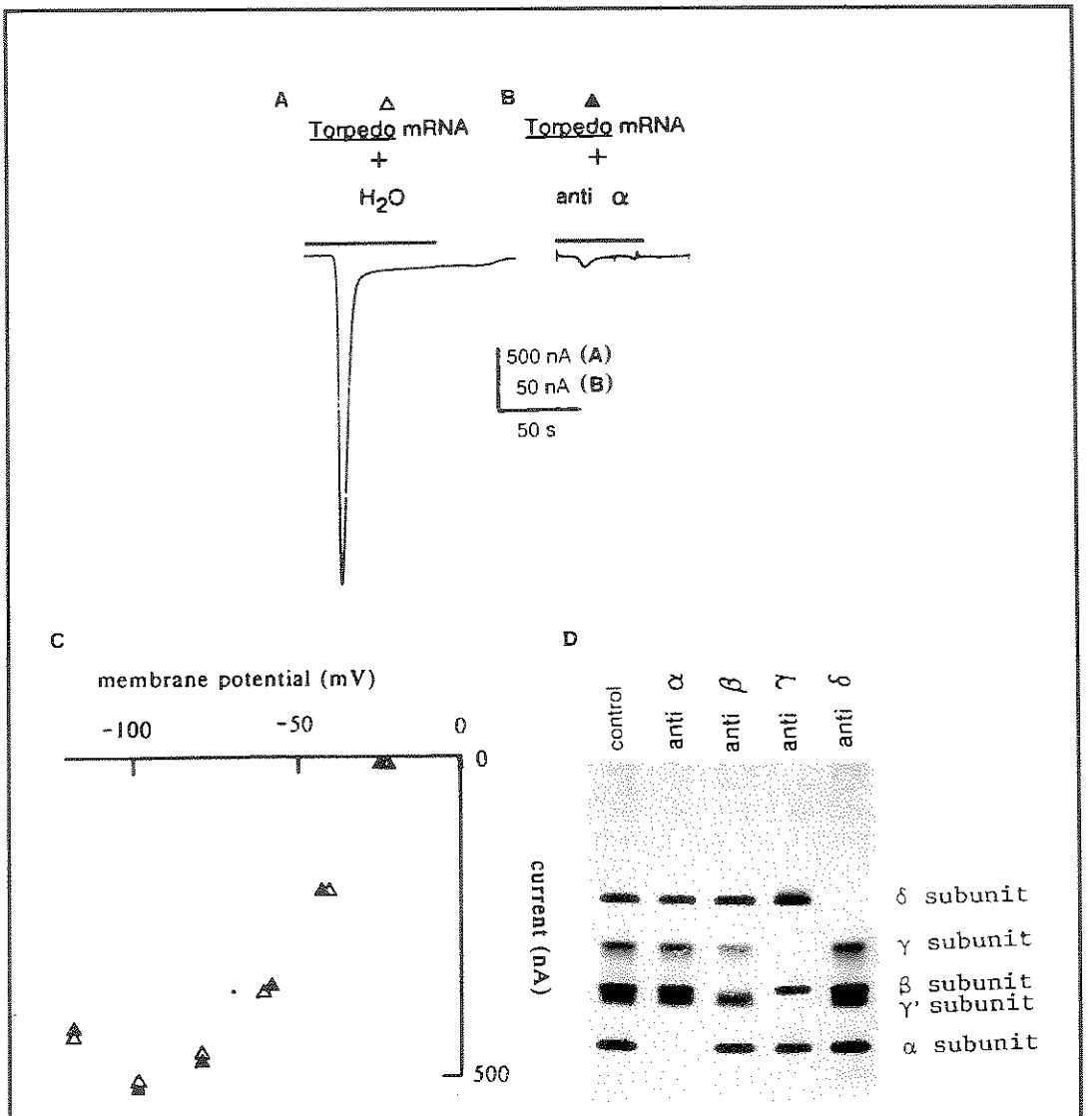

FIG. 23. ACh-activated currents (A,B) and current–voltage relationship of chloride current (C) induced in oocytes by *Torpedo* mRNA in the presence or absence of antisense RNA from the α subunit of the AChR. The antisense RNA almost abolished the responses to ACh (A,B) but had no effect on the expression of chloride channels (C). (D) SDS gel electrophoresis of AChR molecules synthesized in oocytes. A mixture of the four subunit mRNAs was injected into 10 oocytes with (1) H_2O, (2) antisense αRNA, (3) antisense βRNA, (4) antisense γRNA, or (5) antisense δRNA for immunoprecipitation assay. (K. Sumikawa and R. Miledi, unpublished results.)

DOING NEUROBIOLOGY IN OOCYTES

Once a receptor mRNA is cloned, be it using oocytes or otherwise, there are a million things that can be done, and here again the oocytes are very useful. For instance, one can use the oocytes to study the functional characteristics of selectively mutated receptors, as has been done by Numa, Sakmann, and their colleagues (25,43). Or they can be used to study the processes that control the synthesis, assembly, and membrane insertion of receptors and channels. Another very important practical application of the *Xenopus* oocyte system will be in the development and testing of new receptor-active drugs that can be used in medicine—and much more.

Actually, there is so much that can be done to study the brain with oocytes that they are becoming increasingly popular in neurobiology. A few years ago many journals did not even list *Xenopus* oocytes in their subject index. They do now and, as shown in Fig. 24, the number of papers describing research on receptors and channels in *Xenopus* oocytes is increasing rapidly (in the 5 years before 1977 there were only two or three papers), and it would be safe to predict that the numbers will grow even more rapidly in the ensuing years.

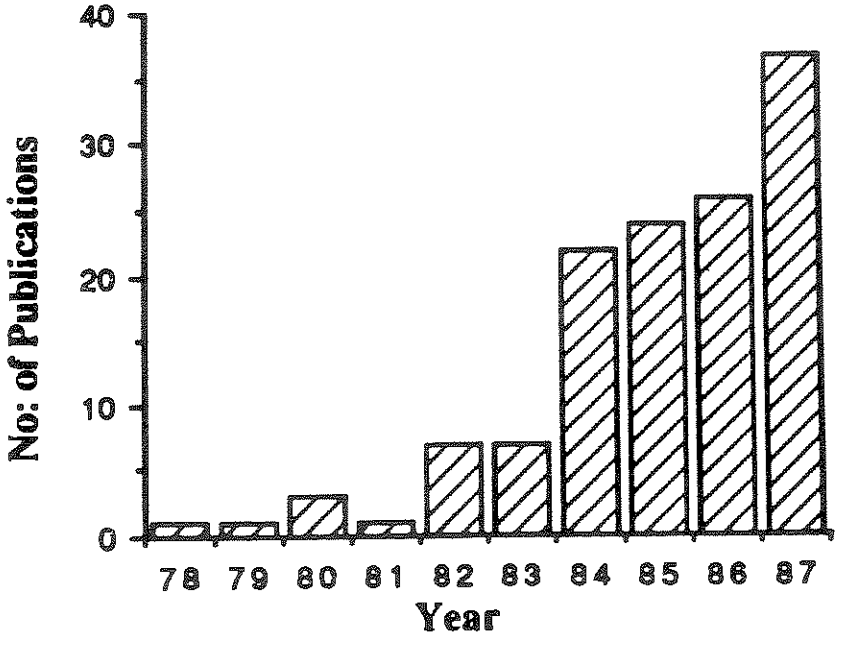

FIG. 24. Numbers of papers concerning membrane receptors and channels in *Xenopus* oocytes published per year in neurobiological journals.

I hope I have been able to convey to you a small portion of the excitement and potential of doing neurobiology in oocytes. However, we must not forget that *Xenopus* oocytes are only a model system to help us along while we find better ways to tackle the much more complex and intricate human brain. At some stage we must abandon Galvani's trail and return to that of the Alexandrian doctors to see in which way the information gained from oocytes can help us prevent or alleviate some of the many diseases that affect the human brain.

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