Proc. R. Soc. Lond. B **226**, 263–269 (1985)
Printed in Great Britain

On the orientation of foreign neurotransmitter receptors in *Xenopus* oocytes

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(*Received* 19 *July* 1985)

Xenopus oocytes can be made to incorporate into their membrane foreign neurotransmitter receptors and voltage-activated sodium channels. In their original location the receptors are normally activated by the extracellular action of transmitter substances. Tests were made to see if some of the newly synthesized foreign receptors were inserted in the oocyte membrane with their active site facing inwards. Since intracellular injections of acetylcholine, γ-aminobutyric acid, serotonin and kainic acid and tetrodotoxin into the oocyte failed to elicit a response, we conclude that very few, or none, of the receptor molecules expressed in the oocyte by the exogenous mRNA are inserted with the wrong orientation in the membrane.

Introduction

It has been known for some time that intracellularly applied acetylcholine does not activate the acetylcholine receptors of skeletal muscle (del Castillo & Katz 1955; Birks *et al.* 1960). This indicates that the acetylcholine binding site of the receptors is external to the muscle membrane; and the same is probably true for the receptors that mediate chemical synaptic transmission across nerve cells of the brain. However, it could be that some newly synthesized receptors are inserted in the cell membrane with their 'active' site facing inward. To test this possibility we have induced *Xenopus* oocytes to synthesize and incorporate into their membrane foreign neurotransmitter receptors (Barnard *et al.* 1982; Miledi *et al.* 1982; Gundersen *et al.* 1983*a*, 1984*a–c*), and examined the effects of extracellular and intracellular application of the transmitters.

Methods

The experiments were made on oocytes of *Xenopus laevis*, which had been injected one to several days previously with mRNA from one of various sources (*Torpedo* electric organ, rat brain or chick optic lobe) to induce the appearance of drug- and voltage-activated membrane channels. Procedures for isolation of

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mRNA and its injection into oocytes were as already described (Barnard *et al.* 1982; Miledi *et al.* 1982; Miledi & Sumikawa 1982; Gundersen *et al.* 1983*a*, 1984*a–c*). Membrane currents were recorded from voltage clamped oocytes, continually perfused with normal Ringer solution at room temperature (Kusano *et al.* 1982; Miledi 1982). Usually the oocytes were previously treated with collagenase to remove follicular and other enveloping cells (Miledi & Parker 1984).

Extracellular and intracellular applications of agonist drugs and blocking agents were made by pneumatic pressure injection from micropipettes, filled with 0.5 M acetylcholine (ACh), 100 mM kainate, 1 M γ-aminobutyric acid (GABA), 10 mM serotonin or 0.1 mM tetrodotoxin (TTX). In addition, 2 mM EGTA (ethylene glycol-bis(β-aminoethyl ether) *N,N*-tetra-acetic acid) was added to all solutions to chelate contaminating calcium, and prevent the activation of calcium-activated chloride channels in the oocyte membrane (Miledi & Parker 1984). In addition to the voltage recording electrode the membrane potential was also recorded through the drug micropipette, to monitor the penetration of the pipette into the oocyte. Results similar to those described here were obtained in earlier experiments in which ACh and other agonists were applied by iontophoresis (see also Miledi & Sumikawa 1982). Pressure injection was later preferred, because large amounts could be delivered quickly.

Results

Extracellular and intracellular application of agonists

Oocytes were voltage clamped at a potential of −60 mV, and membrane currents were recorded first in response to pulses of agonist applied with the tip of the pressure ejection pipette close to the outside of the oocyte. The presence of responses confirmed both that the pipette was functioning satisfactorily, and that the oocyte had acquired functional receptors. Next, the pipette was inserted into the oocyte, so that its tip lay close to the inner surface of the membrane, and the clamp current was recorded while applying pulses of agonist. Finally, the pipette was withdrawn and tested to ensure that it had not become blocked during penetration, by delivering further agonist pulses to the outside of the oocyte.

ACh receptors from electric organ or cat muscle

Nicotinic ACh receptors were induced in oocytes by injecting them with mRNAs derived from the electric organ of *Torpedo* or cat muscle (Barnard *et al.* 1982; Miledi & Sumikawa 1982; Sumikawa *et al.* 1984).

Puffs of ACh, applied with the tip of the injecting pipette placed a few tens of micrometres from the outer surface of the oocyte, evoked large inward currents due to activation of exogenous ACh receptors (figure 1*a*, *f*). In contrast, similar pulses of ACh applied with the tip of the pipette just inside the oocyte, evoked no detectable response, or only a small current like that illustrated in figure 1*b*. This response is seen more clearly in figure 1*c*, obtained at a higher gain and after applying a longer pulse of ACh. The presence of this response to intracellular ACh might be taken as an indication that some of the new receptors, synthesized from the foreign mRNA, were inserted with their active site facing inwards. However,

it could also be that some of the injected ACh leaked through the membrane, at the site of impalement, and activated external receptors. To test this latter possibility, we blocked the external receptors by adding curare or α-bungarotoxin (Miledi & Sumikawa 1982), to the solution flowing past the oocyte. A few seconds after adding curare, the response to the long intracellular pulse of ACh was abolished (figure 1*d*), but the sensitivity to ACh recovered rapidly after washing out the curare (figure 1*e*). While it might be argued that sufficient curare entered the oocyte to block ACh acting on the internal face of the membrane, this seems unlikely in view of the rapid onset and recovery from the blocking actions of curare.

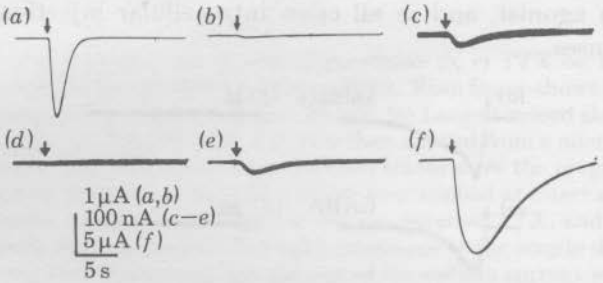

FIGURE 1. Extracellular (*a*, *f*) and intracellular (*b*–*e*) applications of ACh to an oocyte injected with mRNA from *Torpedo* electroplaques. The ACh was delivered by pressure pulses of 40 kPa and 200 ms (*a*, *b*, *f*) or 1 s (*c*–*e*) duration. (*a*) Membrane current elicited by an extracellular pulse of ACh with the pipette tip close to the pole in the vegetal (white) hemisphere of the oocyte. (*b*) Response to the same pulse of ACh after advancing the pipette into the oocyte. (*c*) Response to a longer intracellular pulse of ACh, recorded at higher gain. (*d*) Same as (*c*), but recorded a few seconds after adding curare (2×10^{-5} M) to the bathing solution. (*e*) Recovery of response after washing out curare. (*f*) Brief extracellular pulse of ACh applied immediately after withdrawing pipette. The oocyte was clamped at −60 mV in all records, and in this and other figures, downward deflections correspond to inward membrane currents.

Further evidence that the small responses seen after intracellular applications of ACh were due to ACh leaking and acting on the outside of the membrane, is that sometimes these responses were reduced as the membrane sealed around the injecting pipette; and that no responses to intracellular injections of ACh were observed in oocytes whose ACh receptors had been previously blocked irreversibly with α-bungarotoxin (R. Miledi & K. Sumikawa, unpublished). From all this we conclude that the small responses observed sometimes after intracellular injection of ACh are almost certainly due to leakage around the pipette, and that intracellular ACh does not activate the foreign nicotinic receptors. The native muscarinic ACh receptors are similarly unresponsive to intracellular ACh (Kusano *et al.* 1982).

Neurotransmitter receptors from the brain

For these experiments oocytes were injected with mRNA from chick optic lobe (to induce GABA and kainate receptors) or with mRNA from rat brain (to induce also serotonin and muscarinic ACh receptors). Oocytes not injected with mRNA show either no responses, or occasionally very small responses to GABA, kainate

and serotonin, so that the large currents elicited by externally applied puffs of these agonists (figure 2) arose because of the expression of exogenous receptors in the oocyte membrane (Gundersen *et al.* 1983*a*, 1984*a*; Miledi *et al.* 1982).

In contrast to the high extracellular transmitter sensitivity of these oocytes, we failed to detect any responses to intracellular injections of GABA, kainate, serotonin and ACh. This was the case even when several pulses were injected into oocytes which gave good responses to single extracellular pulses (figure 2). Large responses were, however, obtained after withdrawal of the pipettes, suggesting that the lack of intracellular responses was not due to blocking of the pipette. Experiments similar to those in figure 2 were repeated in between two and four oocytes for each agonist, and in all cases intracellular injections failed to give detectable responses.

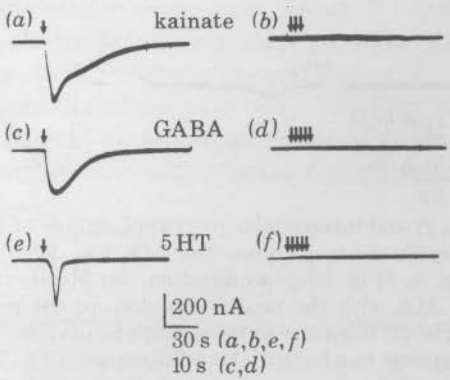

FIGURE 2. Extracellular (left) and intracellular (right) applications of agonists in oocytes injected with mRNA from chick optic lobe (*a–d*) or rat brain (*e*, *f*). Pressure pulses were 40–50 kPa and 1 s duration, applied when indicated by the arrows. Clamp potential was −60 mV.

Voltage-activated sodium currents

If voltage-activated channels were inserted the wrong way round in the oocyte membrane, we would expect their voltage sensitivity to be reversed. Thus, for example, the sodium current might be activated by depolarization from +100 mV to 0 mV, rather than from −100 to 0 mV as is usual. At present, we have not looked for channels with reversed voltage dependence, because of the difficulties in clamping the oocyte at high positive potentials for long periods. Instead, we investigated whether oocytes express any exogenous sodium channels that can be blocked by intracellular injection of tetrodotoxin (TTX); which would be the reverse of the normal situation where the TTX binding site is located on the extracellular side of the channel molecule (Armstrong 1981).

Depolarization of oocytes injected with rat brain mRNA from −100 to −10 mV elicited a transient inward membrane current (lower trace, figure 3*a*), resulting from the activation of voltage-dependent sodium channels (Gundersen *et al.* 1983*b*). This current was greatly reduced when TTX was applied externally to the oocyte by pressure ejection from a micropipette (figure 3*a*). In contrast to this,

several pulses of TTX applied after the pipette had been inserted into the oocyte failed to reduce appreciably the sodium current activated by the same depolarizing step (figure 3*b*, *c*).

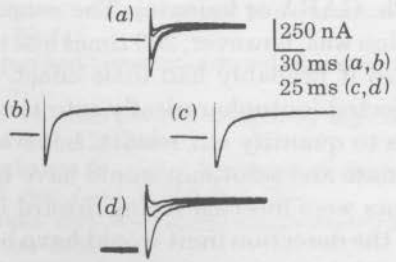

FIGURE 3. Action of extracellular (*a*, *d*) and intracellular (*b*, *c*) TTX on the sodium current recorded in an oocyte injected with rat brain mRNA. Each frame shows membrane currents elicited by depolarization from −100 to −10 mV. (*a*) Largest record shows control sodium current. Two pulses of TTX (40 kPA, 1 s) were then applied from a micropipette positioned outside the oocyte, and subsequent superimposed traces show the progressive reduction in the sodium current elicited by the same voltage step applied at intervals of a few seconds. (*b*) Sodium current recorded after washing out the external TTX, and inserting the TTX filled micropipette into the oocyte. The input resistance of the oocyte decreased as a result of damage during the impalement, but the size of the sodium current was unchanged from the control record in (*a*).(*c*) Same as (*b*), but recorded a few minutes after five pulses of TTX were injected into the oocyte. (*d*) Reduction of sodium current by extracellular application of TTX (four pulses) immediately after withdrawal of the TTX pipette to outside the oocyte.

DISCUSSION

Our main finding was that intracellular injections of various agonists (ACh, GABA, kainate and serotonin) into mRNA-injected oocytes failed to evoke detectable membrane currents, even though much smaller applications of these drugs gave large responses when applied to the outer face of the membrane. This indicates that although many functional receptor–channel molecules were translated from the exogenous mRNA and inserted into the oocyte membrane with the correct external orientation, very few, or none, were inserted with their transmitter binding site facing inwards.

Although intracellular injection experiments have some technical problems, we feel it unlikely that these could account for the lack of intracellular responses. For example, the injection pipettes all released agonist after withdrawal from the oocytes, indicating that they had not become plugged. Furthermore, the ejection of agonists from the micropipette and their subsequent diffusion to the membrane surface was probably not importantly hindered by oocyte cytoplasm, since calcium, EGTA and organic dyes diffuse readily throughout the oocyte after injection (Miledi & Parker 1984 and unpublished data). Perhaps the best evidence comes from experiments such as that in figure 1, where intracellular injection of ACh gave a small response, which almost certainly arose because of leakage of ACh at the impalement site. When the external receptors were blocked by curare the response was completely abolished, even though the concentration of ACh at the inside of the membrane must have been much higher than that resulting from leakage outside into the continuously flowing bathing medium. The experiment

with serotonin presents a special problem, since EGTA was included in the injection solution and we have recently found (Parker *et al.* 1985) that intracellular injection of EGTA abolishes the oscillatory response to external serotonin (though not the responses to ACh, GABA or kainate). The concentration of EGTA in the serotonin injection solution was, however, 200 times less than that injected to block the serotonin response, so it probably had little effect. Moreover, serotonin was also ineffective when injected iontophoretically into the oocytes.

We made no attempts to quantify our results, but we expect that responses to intracellular GABA, kainate and serotonin would have been detectable if 1 or 2% of the functional receptors were inserted facing inward in the membrane. For the ACh injection in figure 1 the detection limit would have been much lower than this. Of course, receptors could have been inserted wrongly, but if they were not functional we would not have detected them.

In addition to all the receptors incorporated in the surface membrane of the oocyte, many other receptors are being synthesized and are on their way to the surface. It could be postulated that transmitter substances might act intracellularly on these receptors and cause the release of ions, in particular calcium, into the cytoplasm. If that had been the case we would probably have detected a chloride current, because the oocytes are a very sensitive monitor of ionized intracellular calcium, which opens chloride channels in the surface membrane (Miledi 1982; Miledi & Parker 1984).

Our results are consistent with other reports (for review, see Lane 1983) which have examined the post-translational processing and subcellular disposition of foreign proteins expressed in oocytes following injection of heterologous mRNA. In general, it has been found that the oocytes faithfully perform a variety of covalent modifications of newly synthesized polypeptides and then route them to the appropriate subcellular compartment. This is the case for many cytoplasmic, membrane-associated and secreted enzymes and proteins (Lane 1983), and from our results it appears that the oocyte also inserts drug- and voltage-activated channels into the plasma membrane with the correct orientation.

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