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A calcium-independent chloride current activated by hyperpolarization in *Xenopus* oocytes

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Hyperpolarization of oocytes of *Xenopus laevis* usually elicits mainly passive currents. However, when polarized to potentials more negative than about −100 mV, oocytes obtained from some donors show a relatively well maintained current that is carried mainly by chloride ions. This response does not depend upon external calcium, and is thus clearly different from the calcium-dependent transient chloride current previously described.

Introduction

Considering that they are electrically passive cells, the immature oocytes of *Xenopus laevis* display a surprising diversity of ionic currents. At present, we know they possess calcium-activated chloride channels (Miledi & Parker 1984), calcium channels activated by depolarization (Miledi 1982; Barish 1983; Leonard *et al.* 1987) and by hyperpolarization (Parker *et al.* 1984; Parker & Miledi 1987*a*), at least two types of sodium channel (Baud *et al.* 1982; Parker & Miledi 1987*b*), and two types of potassium channel (Peres *et al.* 1985; R. Miledi, unpublished data). When the object is to examine exogenous membrane channels, expressed in the oocyte after injection of foreign messenger RNAs, the presence of these endogenous channels can sometimes be a nuisance (cf. Parker & Miledi 1987*b*). On the other hand, the membrane channels in native oocytes provide a fruitful field of study in their own right, particularly as they are located in a large cell, which greatly facilitates electrophysiological and biochemical analysis.

Some of the above-mentioned membrane currents are almost invariably present, and probably serve a function in the development of the oocyte or the embryo. An example is the calcium-activated chloride current, which appears to be involved in the generation of the fertilization potential (Whitaker & Steinhardt 1982). However, other currents, such as the transient tetrodotoxin-sensitive sodium current (Parker & Miledi 1987*b*), are prominent in oocytes from only a small proportion of donors. In this case it seems unlikely that the current would have any function in the oocyte and, instead, it may arise from the apparently random expression of a gene coding for channels which are important in some later stage of development of the frog. In the present paper we add to the list of infrequently observed currents in the *Xenopus* oocyte, by describing a chloride current activated by hyperpolarization, which we first noticed while studying other currents (Miledi 1982). This chloride current is independent of external calcium, and is thus different from the calcium-dependent transient chloride current previously described (Peres & Bernardini 1983; Parker *et al.* 1984).

Methods

Experiments were made on oocytes of *Xenopus laevis*, which, except where otherwise noted, were treated with collagenase to remove enveloping cells (Miledi & Parker 1984). Oocytes were voltage clamped, by using a two electrode system (Kusano *et al.* 1982; Miledi 1982), and membrane currents were recorded on stepping the potential to different levels from a holding potential of −50 mV. During recording, the oocytes were continuously superfused with Ringer solution (composition in mM: NaCl, 120; KCl, 2; $CaCl_2$, 1.8; HEPES, 5; pH about 7.0) at room temperature (22–24 °C). Calcium-free Ringer contained no added calcium, and additionally 0.5 mM EGTA and 2 mM $MgCl_2$. Solutions containing low sodium or low chloride were made by replacing NaCl by, respectively, tetraethylammonium (TEA) chloride or sodium methylsulphate. High potassium and high calcium solutions were made by adding KCl or $CaCl_2$ to normal Ringer, without compensating for the small increase in osmotic and ionic strength.

Results

Inward current on hyperpolarization

Currents recorded from 'native' *Xenopus* oocytes (i.e. without injection of exogenous messenger RNA) usually show comparatively passive increases in clamp current in response to hyperpolarizing steps up to about −140 mV, although a small fraction of oocytes give a transient inward chloride current (Peres & Bernardini 1983; Parker *et al.* 1984). This was not evident in the oocytes described here, which instead showed a quite different type of current activated on hyperpolarization. The results are based on recordings in oocytes obtained from a single donor, and we have occasionally seen similar current in oocytes from other donors examined over several years.

The current activated on hyperpolarization is illustrated in figure 1*a*, *b*, which shows membrane currents elicited by stepping an oocyte to various potentials between +10 and −190 mV, from a holding potential of −50 mV. Between about 0 mV and −100 mV, changes in potential elicited ohmic changes in current, following a close-to-linear current–voltage relation (figure 1*c*). However, stepping the potential to −110 mV induced a more-than-linear increase in inward current, and this grew steeply with further polarization. During steps to moderately hyperpolarized potentials (*ca.* −110 to −140 mV) the current rose slowly, reaching a maximum after about 1 s, and then remained fairly steady. As the potential was made more negative than this, the activation of the current after a voltage step became progressively more rapid, but it also began to decline during maintained polarization (figure 1*b*).

The voltage-dependence of activation of the hyperpolarization-activated current was estimated in figure 1*c* (●) by extrapolating the passive electrical properties of the oocyte between 0 and −90 mV to more negative potentials, and by subtracting this linear current–voltage relation from the observed currents (○). In this way, it is seen that the current begins to activate at potentials between about −100 and −110 mV, and that beyond −130 mV it increases about linearly with

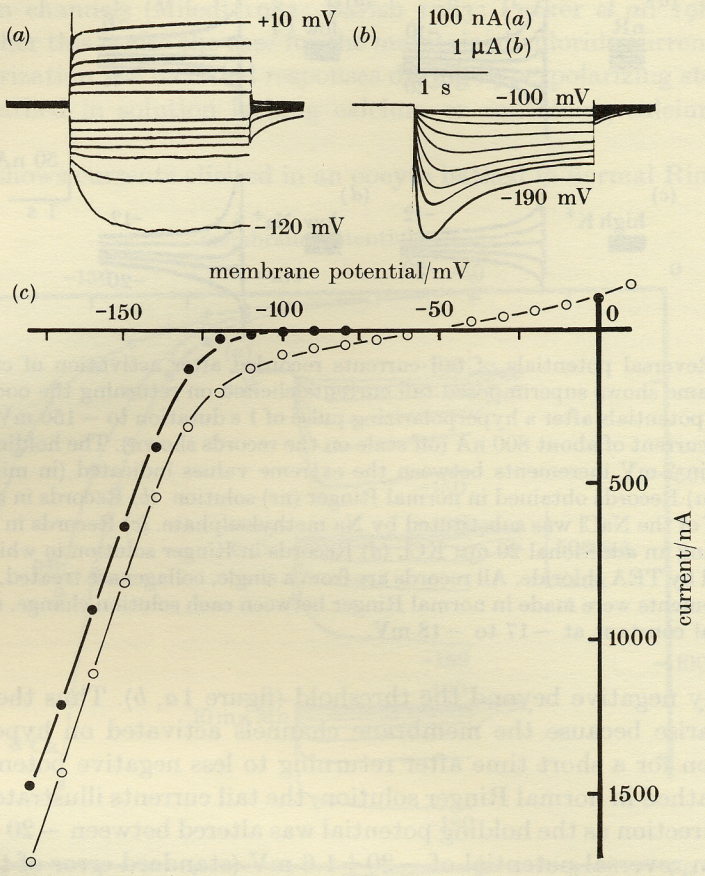

FIGURE 1. Membrane currents and current–voltage relation in an oocyte showing a pronounced chloride current on hyperpolarization. (*a*, *b*) Superimposed traces showing membrane currents evoked by briefly stepping the clamp potential to different voltages from a holding potential of −50 mV. In (*a*) the potential during the step was altered in 10 mV increments from +10 mV (upper trace) to −120 mV (lower trace). Records in (*b*) were obtained at a lower recording gain, and show potential steps in 10 mV increments from −100 to −190 mV. (*c*) Current–voltage relation measured from the records in (*a*, *b*). Open circles show the maximal current recorded at each potential, measured with respect to the holding current at −50 mV. Filled circles show the current remaining after subtraction of passive leakage currents, estimated by extrapolation of the linear current–voltage relation between −100 and 0 mV (see text). Collagenase treated oocyte, at 23 °C.

hyperpolarization up to the maximum potential (−190 mV) that the oocyte would withstand.

Ionic basis of the hyperpolarization-activated current

To derive information about the ions that carry this current, we measured the reversal potential of the tail currents recorded on returning the membrane potential to different levels following hyperpolarizing steps (figure 2). Such tail currents were not seen when the hyperpolarizing step was below the threshold for eliciting the inward current, but they grew in size progressively as the test pulse was made

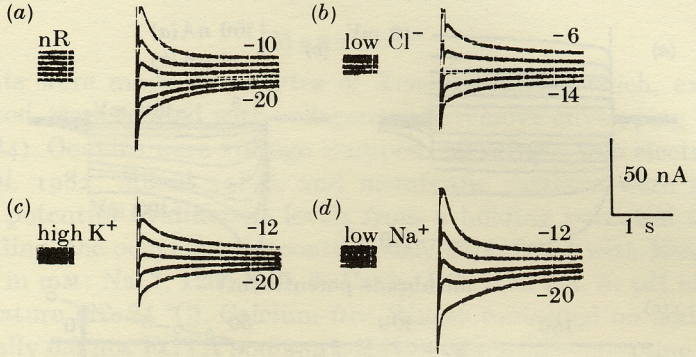

FIGURE 2. Reversal potentials of tail currents recorded after activation of chloride current. Each frame shows superimposed tail currents elicited on returning the oocyte to different holding potentials after a hyperpolarizing pulse of 1 s duration to −150 mV, which gave an inward current of about 800 nA (off scale on the records shown). The holding potential was altered in 2 mV increments between the extreme values indicated (in millivolts) in each frame. (*a*) Records obtained in normal Ringer (nr) solution. (*b*) Records in a solution where one half of the NaCl was substituted by Na methylsulphate. (*c*) Records in Ringer solution containing an additional 20 mM KCl. (*d*) Records in Ringer solution in which all NaCl was replaced by TEA chloride. All records are from a single, collagenase treated, oocyte. Repeat measurements were made in normal Ringer between each solution change, and the reversal potential constant at −17 to −18 mV.

increasingly negative beyond the threshold (figure 1*a*, *b*). Thus the tail currents probably arise because the membrane channels activated on hyperpolarization remain open for a short time after returning to less negative potentials.

When bathed in normal Ringer solution, the tail currents illustrated in figure 2*a* inverted direction as the holding potential was altered between −20 and −16 mV, and a mean reversal potential of -20 ± 1.6 mV (standard error of the mean) was obtained in four oocytes. This corresponds to the chloride equilibrium potential in the oocyte (Kusano *et al.* 1982; Barish 1983), suggesting that the current is carried largely by a flux of chloride ions. To further test this conclusion, the effects of changes in ionic composition of the bathing fluid were examined on the reversal potential of the tail currents. Increasing the concentration of potassium ions tenfold, from 2 to 20 mM, gave virtually no change in reversal potential (figure 2*c*), as did the complete substitution of sodium by TEA (figure 2*d*). Thus it seems that sodium and potassium ions cannot contribute appreciably to the current, both because of the lack of effect of ion substitutions on the reversal potential, and because the high concentration of TEA would be expected to block some potassium channels. Different to this, the partial replacement of chloride in the bathing solution by relatively impermeant methylsulphate ions shifted the reversal potential to more positive values (figure 2*b*), as is expected if the current is carried by chloride ions.

The chloride current does not depend upon extracellular calcium

The surface membrane of *Xenopus* oocytes contains many chloride channels which are activated by intracellular calcium (Miledi & Parker 1984), and which give rise to chloride currents subsequent to the entry of calcium through voltage-

gated calcium channels (Miledi 1982; Barish 1983; Parker *et al.* 1984). To determine whether this is also the case for the maintained chloride current activated on hyperpolarization, we recorded responses during hyperpolarizing steps applied to oocytes bathed in solution lacking calcium or containing calcium blocking agents.

Figure 3*a* shows currents elicited in an oocyte bathed in normal Ringer, and in

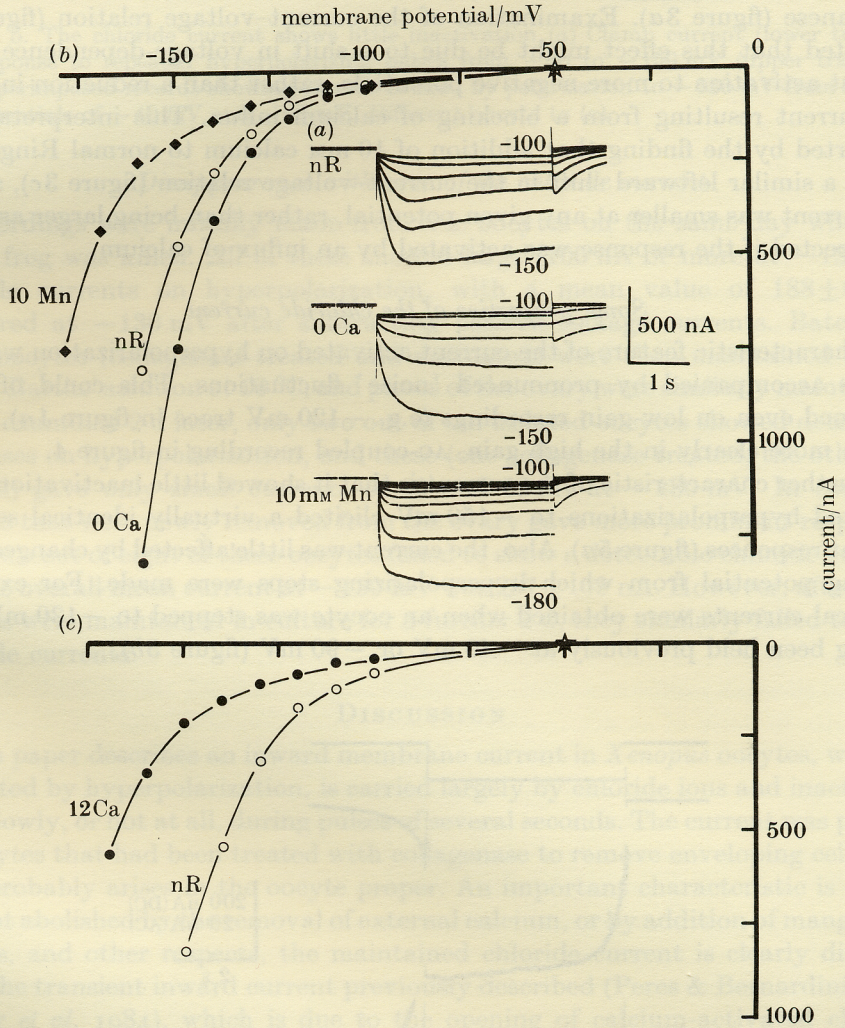

FIGURE 3. The chloride current activated on hyperpolarization does not depend on calcium. (*a*) Superimposed traces showing membrane currents evoked by hyperpolarization to different potentials from a holding potential of −50 mV. In each frame, the voltage during the pulse was raised in 10 mV increments between the minimum and maximum values indicated (in millivolts). Recordings were made in a single oocyte during superfusion with normal Ringer (nR, upper frame), calcium-free Ringer (middle frame) and Ringer solution containing 10 mM $MnCl_2$ (lower frame). (*b*) Current–voltage relations measured in a different oocyte, bathed in normal Ringer (nR) (○), calcium free Ringer (●), and Ringer containing 10 mM Mn^{2+} (◆). (*c*) Current–voltage relation in a third oocyte, measured in normal Ringer (○), and in Ringer containing an additional 10 mM Ca^{2+} (●).

a Ringer solution in which the free calcium concentration was reduced to very low levels with EGTA. Large chloride currents were still seen on hyperpolarization in the calcium-free solution, and indeed these were slightly larger than the control responses in normal Ringer at the corresponding potentials (figure 3*b*). Responses also remained after addition of 10 mM manganese to normal Ringer solution, even though this blocks calcium entry in the oocyte (Miledi 1982; Parker *et al.* 1984). However, the currents elicited at any given potential were appreciably reduced by manganese (figure 3*a*). Examination of the current–voltage relation (figure 3*b*) indicated that this effect might be due to a shift in voltage-dependence of the current activation to more negative potentials, rather than a reduction in size of the current resulting from a blocking of calcium influx. This interpretation is supported by the finding that addition of 10 mM calcium to normal Ringer produced a similar leftward shift in the current–voltage relation (figure 3*c*), so that the current was smaller at any given potential, rather than being larger as would be expected if the response was activated by an influx of calcium.

Some properties of the chloride current

A characteristic feature of the current activated on hyperpolarization was that it was accompanied by pronounced 'noise' fluctuations. This could often be discerned even on low-gain recordings (e.g. −120 mV trace in figure 1*a*), and is shown more clearly in the high gain, AC-coupled recording in figure 4.

A further characteristic of the current is that it showed little inactivation. Thus repeated hyperpolarizations to −150 mV elicited a virtually identical series of current responses (figure 5*a*). Also, the current was little affected by changes in the holding potential from which hyperpolarizing steps were made. For example, identical currents were obtained when an oocyte was stepped to −130 mV after having been held previously at −40 mV or −90 mV (figure 5*b*).

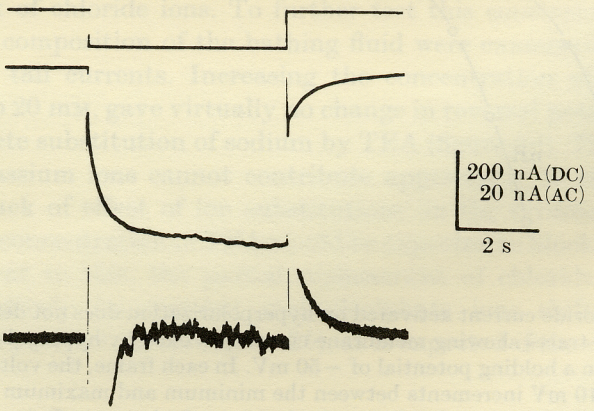

FIGURE 4. Noise fluctuations during activation of the chloride current. The oocyte was polarized from −50 to −150 mV during a 3 s duration pulse. Traces show (from top to bottom) clamp potential, DC record of clamp current, and a high-gain record of clamp current, bandpass filtered at 2–200 Hz.

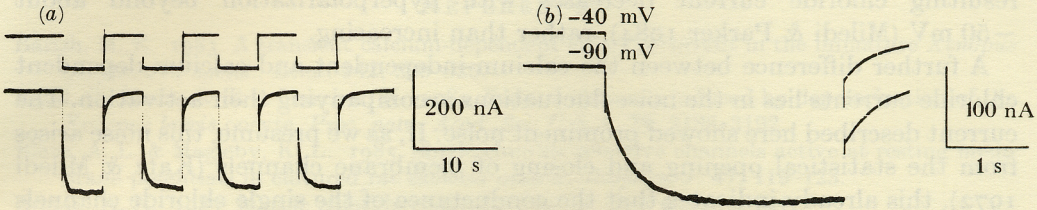

FIGURE 5. The chloride current shows little inactivation (*a*) Clamp current (lower trace) in response to repeated hyperpolarizing pulses from −50 to −150 mV (upper trace). (*b*) Superimposed records showing current evoked by polarization to −130 mV from holding potentials of −40 mV and −90 mV. Different oocyte to (*a*).

Disappearance with time of the chloride current

Recordings were initially made from five oocytes on the same day when the donor frog was killed. All of these showed large (100 nA or more at −130 mV) chloride currents on hyperpolarization, with a mean value of 188±69 nA, measured at −130 mV after subtracting passive leakage currents. Batches of these oocytes (collagenase-treated and untreated) were then maintained in culture in Barths' solution at 16 °C, and pieces of the ovary were similarly maintained. When examined 2 d later, only two out of ten isolated oocytes showed detectable responses on hyperpolarization, and these (one collagenase treated, the other untreated) gave only small currents of 40 and 20 nA at −130 mV. In contrast, oocytes that were newly removed from the ovary gave more prominent responses. Only two out of eight of these oocytes failed to show a detectable chloride current, and the overall mean current at −130 mV was 207±109 nA. However, after these oocytes were maintained in culture for a further 2 d, they similarly failed to show chloride currents.

DISCUSSION

This paper describes an inward membrane current in *Xenopus* oocytes, which is activated by hyperpolarization, is carried largely by chloride ions and inactivates only slowly, or not at all, during pulses of several seconds. The current was present in oocytes that had been treated with collagenase to remove enveloping cells, and thus probably arises in the oocyte proper. An important characteristic is that it was not abolished by the removal of external calcium, or by addition of manganese. In this, and other respects, the maintained chloride current is clearly different from the transient inward current previously described (Peres & Bernardini 1983; Parker *et al.* 1984), which is due to the opening of calcium-activated chloride channels subsequent to the entry of calcium through channels modulated by hyperpolarization and by inositol phosphates (Parker & Miledi 1987*a*). Thus it seems that oocytes from some donors express a voltage-activated chloride channel, in addition to the calcium-activated chloride channels that they normally possess (Miledi & Parker 1984). An alternative possibility is that the maintained current could arise through the calcium-activated chloride channels, if the resting level of intracellular free calcium is sufficiently high to cause channel activation. This, however, seems unlikely, because when calcium is injected into an oocyte the

resulting chloride current decreases with hyperpolarization beyond about −50 mV (Miledi & Parker 1984), rather than increasing.

A further difference between the calcium-independent and calcium-dependent chloride currents lies in the noise fluctuations accompanying their activation. The current described here showed prominent noise. If, as we presume, this noise arises from the statistical opening and closing of membrane channels (Katz & Miledi 1972), this already indicates that the conductance of the single chloride channels is large and their lifetime long. In contrast, the calcium-activated chloride channel has a low unitary conductance (Methfessel *et al.* 1986; Takahashi *et al.* 1987), and little noise can be discerned in voltage-clamp recordings of calcium-activated chloride currents (cf. Miledi & Parker 1984). Glycine-activated chloride channels, expressed in the oocyte by messenger RNA from brain, have properties intermediate between these two. The single-channel conductance is about 20 pS (Gundersen *et al.* 1986) and, although noise fluctuations are apparent on current records, they are smaller than those accompanying the hyperpolarization-activated chloride current.

Voltage-activated, calcium-independent chloride currents have previously been described in a variety of preparations (for review see Bretag (1987)), but many of these, including chloride channels in muscle (Blatz & Magleby 1985), glia (Gray *et al.* 1984) and *Torpedo* electric organ (White & Miller 1979; Sumikawa *et al.* 1984) show a voltage dependence in the opposite sense to the oocyte chloride channel; that is to say, they open with depolarization and close with hyperpolarization. The native chloride channel in the oocyte does, however, more closely resemble hyperpolarization-activated chloride currents described in *Aplysia* neurons (Chesnoy-Marchais 1983; Chesnoy-Marchais & Evans 1986) and in rat sympathetic neurons (Selyanko 1984). Because of the infrequency with which the calcium-independent chloride current is found in oocytes from different donors, it seems unlikely that it would serve any important function in the oocyte. Instead, the chloride channels may arise because of an apparently random expression of a gene that becomes of importance at a later stage in the development of the frog. Thus it will be interesting to see if channels similar to those in the oocyte are expressed in any tissues of the adult animal.

An interesting feature of the chloride current is that it was greatest in oocytes that had been freshly removed from the ovary, and disappeared almost completely after oocytes were maintained in culture for a few days. A similar phenomenon is seen with potassium currents evoked by certain neurotransmitter and hormone receptors in the oocyte (Woodward & Miledi 1987); but in those cases it is thought that the current is generated in the follicular cells, which gradually lose their electrical coupling to the oocyte. This cannot be the explanation for the disappearance of the hyperpolarization-activated chloride current, because this is present in defolliculated oocytes. Instead, it may be that the ovary produces some factor that serves to regulate the functional activity of the chloride channels, or their expression in the membrane.

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References

Barish, M. E. 1983 A transient calcium-dependent chloride current in the immature *Xenopus* oocyte. *J. Physiol., Lond.* **342**, 309–325.

Baud, C., Kado, R. T. & Marcher, K. 1982 Sodium channels induced by depolarization of the *Xenopus laevis* oocyte. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3188–3192.

Blatz, A. L. & Magleby, K. L. 1985 Single chloride-selective channels active at resting membrane potentials in cultured rat skeletal muscle. *Biophys. J.* **47**, 119–123.

Bretag, A. H. 1987 Muscle chloride channels. *Physiol. Rev.* **67**, 618–724.

Chesnoy-Marchais, D. 1983 Characterization of a chloride conductance activated by hyperpolarization in *Aplysia* neurons. *J. Physiol., Lond.* **342**, 277–308.

Chesnoy-Marchais, D. & Evans, M. G. 1986 Chloride channels activated by hyperpolarization in *Aplysia* neurons. *Pflügers Arch. Eur. J. Physiol.* **407**, 694–696.

Gray, P. T. A., Bevan, S. & Ritchie, J. M. 1984 High conductance anion-selective channels in rat cultured Schwann cells. *Proc. R. Soc. Lond.* B **221**, 395–409.

Gundersen, C. B., Miledi, R. & Parker, I. 1986 Voltage dependence of human brain glycine receptor-channels in *Xenopus* oocytes. *J. Physiol., Lond.* **377**, 40*P*.

Katz, B. & Miledi, R. 1972 The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol., Lond.* **224**, 665–699.

Kusano, K., Miledi, R. & Stinnakre, J. 1982 Cholinergic and catecholaminergic receptors in the *Xenopus* oocyte membrane. *J. Physiol., Lond.* **328**, 143–170.

Leonard, J. P., Nargeot, J., Snutch, T. P., Davidson, N. & Lester, H. A. 1987 Ca channels induced in *Xenopus* oocytes by rat brain mRNA. *J. Neurosci.* **7**, 875–881.

Methfessel, C., Witzemann, V., Sakmann, B., Takahashi, T., Mishina, M. & Numa, S. 1986 Patch clamp measurements on *Xenopus laevis* oocytes: currents through endogenous channels and implanted acetylcholine receptors and Na channels. *Pflügers Arch. Eur. J. Physiol.* **407**, 577–588.

Miledi, R. 1982 A calcium-dependent transient outward current in *Xenopus laevis* oocytes. *Proc. R. Soc. Lond.* B **215**, 491–497.

Miledi, R. & Parker, I. 1984 Chloride current induced by injection of calcium into *Xenopus* oocytes. *J. Physiol., Lond.* **357**, 173–183.

Parker, I., Gundersen, C. B. & Miledi, R. 1984 A transient inward current elicited by hyperpolarization during serotonin activation in *Xenopus* oocytes. *Proc. R. Soc. Lond.* B **223**, 279–292.

Parker, I. & Miledi, R. 1987*a* Inositol trisphosphate activates a voltage-dependent calcium influx in *Xenopus* oocytes. *Proc. R. Soc. Lond.* B **231**, 27–36.

Parker, I. & Miledi, R. 1987*b* Tetrodotoxin-sensitive sodium current in native *Xenopus* oocytes. *Proc. R. Soc. Lond.* B**232**, 289–296.

Peres, A. & Bernardini, G. 1983 A hyperpolarization-activated chloride current in *Xenopus laevis* oocytes under voltage clamp. *Pflügers Arch. Eur. J. Physiol.* **399**, 157–159.

Peres, A., Bernardini, G., Mancinelli, E. & Ferroni, A. 1985 A voltage-dependent K^+ channel controlling the membrane potential in frog oocytes. *Pflügers. Arch. Eur. J. Physiol.* **403**, 41–46.

Selyanko, A. A. 1984 Cd^{2+} suppresses a time dependent Cl^- current in rat sympathetic neuron. *J. Physiol., Lond.* **350**, 49*P*.

Sumikawa, K., Parker I., Amano, T. & Miledi, R. 1984 Separate fractions of mRNA from *Torpedo* electric organ induce chloride channels and acetylcholine receptors in *Xenopus* oocytes. *EMBO J.* **3**, 2291–2294.

Takahashi, T., Neher, E. & Sakmann, B. 1987 Rat brain serotonin receptors in *Xenopus* oocytes are coupled to endogenous channels. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5063–5067.

Whitaker, M. J. & Steinhardt, R. A. 1982 Ionic regulation of egg activation. *Q. Rev. Biophys.* **15**, 593–666.

White, M. M. & Miller, C. 1979 A voltage-gated anion channel from the electric organ of *Torpedo californica*. *J. biol. Chem.* **254**, 10161–10166.

Woodward, R. M. & Miledi, R. 1987 Hormonal activation of membrane currents in follicle-enclosed *Xenopus* oocytes. *Proc. natn. Acad. Sci. U.S.A.* **84**, 4135–4139.