Inositol trisphosphate activates a voltage-dependent calcium influx in *Xenopus* oocytes

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Injection of inositol trisphosphate (IP_3) into oocytes of *Xenopus laevis* induces the appearance of a transient inward (T_{in}) current on hyperpolarization of the membrane. This current is carried largely by chloride ions, but is shown to depend on extracellular calcium, because it is abolished by removal of calcium in the bathing fluid or by addition of manganese. Recordings with aequorin as an intracellular calcium indicator show that a calcium influx is activated by hyperpolarization after intracellular injection of IP_3 as well as after activation of neurotransmitter receptors thought to mediate a rise in IP_3. Furthermore, by substituting barium for calcium in the bathing solution, inward barium currents can be recorded during hyperpolarization. We conclude that intracellular IP_3 modulates the activity of a class of calcium channels, so as to allow an influx of calcium on hyperpolarization. In normal Ringer solution this then leads to the generation of a chloride current, because of the large numbers of calcium-dependent chloride channels in the oocyte membrane.

INTRODUCTION

Many neurotransmitters and hormones appear to exert their action through a system in which receptor binding leads to the breakdown of a membrane inositol phospholipid to yield inositol trisphosphate (IP_3) and diacylglycerol (Berridge & Irvine 1984; Hirasawa & Nishizuka 1985). Both of these substances then act as intracellular messengers. The IP_3 is thought to function by causing the release of calcium from intracellular stores, and the resulting rise in intracellular free calcium in turn activates the cellular response (Berridge & Irvine 1984). Release of calcium from intracellular organelles probably occurs through IP_3-activated calcium channels in the internal membranes. It seems reasonable to wonder, therefore, whether similar channels might exist also in the surface membrane (Putney 1986a) where they would, of course, be much more amenable to experimental study.

There is already evidence suggesting that this might be the case in oocytes. *Xenopus* oocytes are known to possess calcium channels, activated by depolarization, which give rise to a transient outward current (Miledi 1982; Barish 1983) because the resulting rise in intracellular calcium then activates calcium-dependent chloride channels in the membrane (Miledi & Parker 1984). Moreover, in addition to these calcium channels activated by depolarization, receptors to neurotransmitters, such as serotonin and acetylcholine, which are thought to act via phospho-

inositide metabolism, induce the appearance of a calcium-dependent chloride current activated by *hyper*polarization (Parker *et al.* 1985). This current is abolished by intracellular injection of the calcium-chelating agent EGTA (Parker *et al.* 1985); a likely explanation is that the agonists cause the modification of a class of calcium channels in the membrane so that they allow a calcium influx on hyperpolarization. Furthermore, injection of IP_3 into *Xenopus* oocytes is able to mimic the appearance of the calcium-dependent chloride current in the absence of agonists (Miledi *et al.* 1987), and in sea-urchin eggs injection of IP_3 produces changes in potential which are partly dependent on external calcium (Slack *et al.* 1986). In this paper we describe in more detail the induction of the calcium-dependent current by IP_3 and demonstrate, by using aequorin as an intracellular calcium monitor and by recording inward barium currents, that IP_3 modulates a calcium influx into the oocyte.

METHODS

Oocytes of *Xenopus laevis* were penetrated with two KCl-filled microelectrodes for voltage clamping, and with two additional micropipettes to allow intracellular injection of aequorin and IP_3 by means of pneumatic pressure pulses. Procedures for electrophysiological recording, pressure injection and monitoring of light emission by aequorin were as described previously (Parker & Miledi 1986). Aequorin (supplied by Dr J. R. Blinks) was injected at a concentration of 1 mg ml^{-1}. IP_3 (Sigma Chemical Co.; D-*myo* inositol trisphosphate) was injected as a 1 mM solution, together with 50 µM EDTA and 5 mM HEPES. During recording, oocytes were continuously superfused with frog Ringer solution at room temperature (*ca.* 24 °C).

RESULTS

Development of T_{in} current after IP_3 injection

Xenopus oocytes usually gave only comparatively passive 'ohmic' increases in clamp current in response to hyperpolarizing voltage pulses (figure 1*b*, frame 1; see also Kusano *et al.* (1982)). However, intracellular injection of IP_3 elicited an oscillatory membrane current (which is small at the gain of figure 1), and accompanying this a transient inward (T_{in}) current developed on hyperpolarization (figures 1–3). The properties of this current, including its time course, voltage dependence and ionic dependence, are similar to those of the T_{in} current induced after serotonergic or muscarinic activation in the oocyte (Parker *et al.* 1985). In particular, the current appears to be largely carried by chloride ions. The tail currents recorded on stepping the membrane potential to different levels just after the peak of the T_{in} current inverted direction at about -20 mV; this potential is close to the chloride equilibrium potential in the oocyte (Kusano *et al.* 1982; Barish 1983), and shifted to more positive potentials with reduction of extracellular chloride. Furthermore, the current must depend on extracellular calcium, because it was reduced when the calcium concentration in the Ringer solution was decreased from 12 to 1.8 mM (figure 1), and was abolished by addition of 5 mM manganese (figure 3) or by substitution of barium for calcium (figure 5*a*).
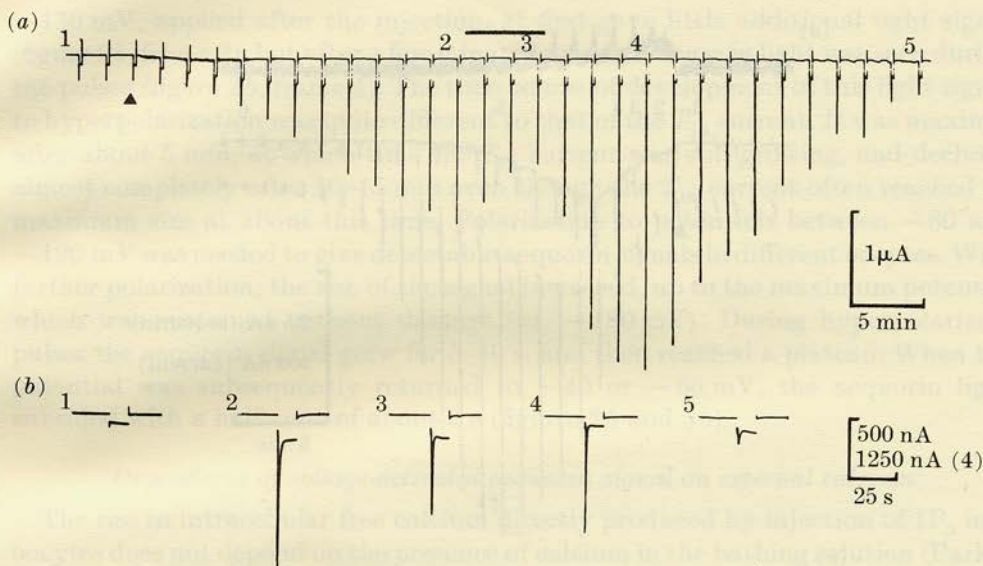
(a)

(b)

FIGURE 1. Development of T_{in} current after intracellular injection of IP_3. The oocyte was voltage-clamped at -40 mV, and hyperpolarized to -140 mV by pulses of 10 s duration at intervals of 2 min. Injection of IP_3 was made as indicated by the arrowhead, by means of a single pressure-pulse, of 100 kPa and 200 ms duration, applied to a pipette containing 1 mM IP_3. (In this, and other figures, downward deflections correspond to inward membrane currents.) (a) Continuous record of clamp current at a slow speed. The oocyte was bathed with Ringer solution containing 12 mM calcium except where shown by the bar, when the calcium concentration was reduced to 1.8 mM. (b) Selected records from (a), at a faster sweep. Note that the recording gain was reduced for frame 4.

The development and decay of the T_{in} current occurred slowly after injection of a single pulse of IP_3 (figures 1 and 2). The maximum current was not reached until 10–30 min after injection; and the response then declined over many minutes. This time course was slower than that of the current directly induced by IP_3 (figures 1 and 2) (see also Parker & Miledi 1986; Dascal *et al.* 1985), and large T_{in} currents could be obtained at times when the direct current was undetectable. The peak T_{in} current often grew to be as large as several microamps, even in oocytes where the direct current response to IP_3 was small; for example, the oocyte in figure 1 gave a peak T_{in} current of 3 µA, although the IP_3-induced current was only about 50 nA.

Aequorin signals after IP_3 injection

The dependence on extracellular calcium of the T_{in} current suggests that this current arises because of a calcium influx on hyperpolarization, which then activates a chloride conductance. To test this hypothesis, we used aequorin to monitor the intracellular calcium level. Experiments were usually made in a high-calcium (12 mM) Ringer solution, so as to increase any calcium influx and thus improve detection.

Shortly after loading oocytes with aequorin, a resting light emission was often seen, which increased with membrane hyperpolarization and with increases in
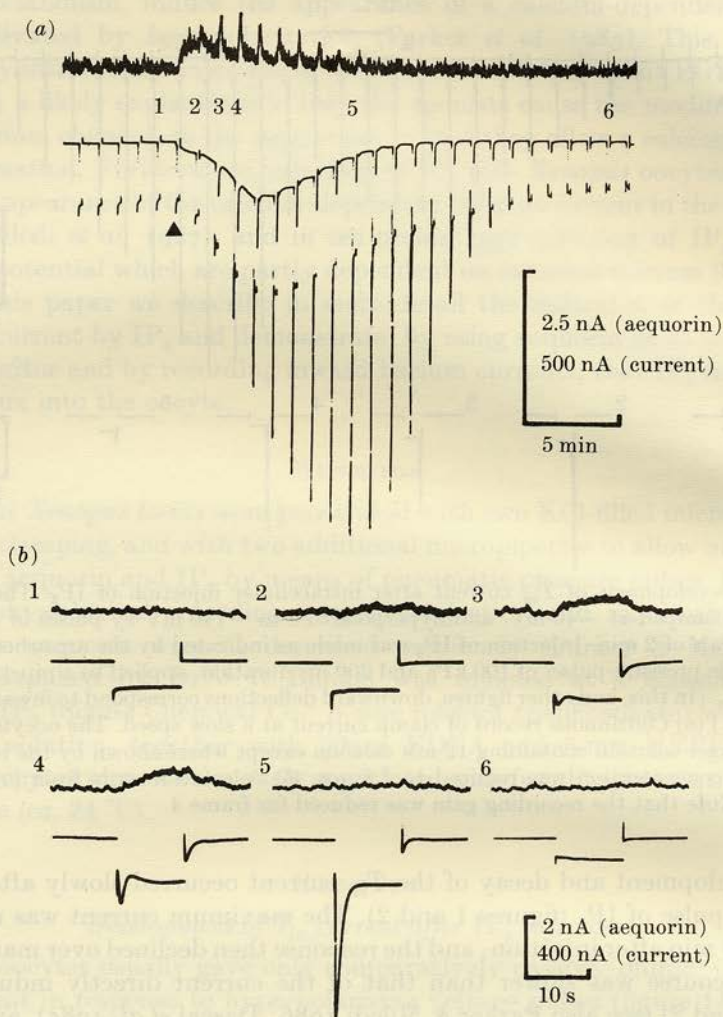
FIGURE 2. Intracellular calcium signals and membrane currents evoked by hyperpolarizing
pulses after intracellular injection of IP$_3$ into an aequorin-loaded oocyte. In (a) and (b), the
upper traces show aequorin light emission measured as photomultiplier current, and the
lower traces show membrane current. IP$_3$ was injected at the arrowhead (100 kPa, 3 s pulse),
and the oocyte was continuously superfused with 12 mM calcium Ringer. (a) Continuous
record at a slow sweep; (b) selected responses from (a) at a faster sweep.

extracellular calcium. This may have arisen because of calcium influx around the
sites of electrode impalement, or because of increased membrane permeability due
to stretch (Miledi & Parker 1984). Therefore, oocytes were allowed to settle for at
least 30 min after loading, at which time hyperpolarizing pulses usually elicited
almost no detectable light signal (figure 2b, frame 1; figure 3a).

Injection of IP$_3$ into aequorin-loaded oocytes elicited a light emission, which
peaked within one or two minutes of the injection, and then declined over several
minutes (figure 2) (see also Parker et al. 1986). Hyperpolarizing pulses to

-130 mV, applied after the injection, at first gave little additional light signal (figure 2b, frame 2), but after a few minutes a clear increase in light was seen during the pulses (figure 2b, frame 4). The time course of development of this light signal to hyperpolarization was quite different to that of the T_{in} current. It was maximal after about 5 min, at which time the T_{in} current was still growing, and declined almost completely after 10–15 min even though the T_{in} current often reached its maximum size at about this time. Polarization to potentials between -80 and -120 mV was needed to give detectable aequorin signals in different oocytes. With further polarization, the size of the signal increased, up to the maximum potential which was sustained without damage (*ca.* -180 mV). During hyperpolarizing pulses the aequorin signal grew for 5–10 s, and then reached a plateau. When the potential was subsequently returned to -40 or -60 mV, the aequorin light subsided with a half-time of about 2 s (figures 2b and 3b).

Dependence of voltage-activated aequorin signal on external calcium

The rise in intracellular free calcium directly produced by injection of IP₃ into oocytes does not depend on the presence of calcium in the bathing solution (Parker & Miledi 1986) and probably arises because of release of calcium from intracellular stores. In contrast, the additional calcium signal evoked by hyperpolarization showed a marked dependence upon extracellular calcium. The size of this signal was reduced when the calcium concentration was lowered from 12 to 1.8 mM and, as shown in figure 3, it was abolished by addition of manganese to the bathing solution.
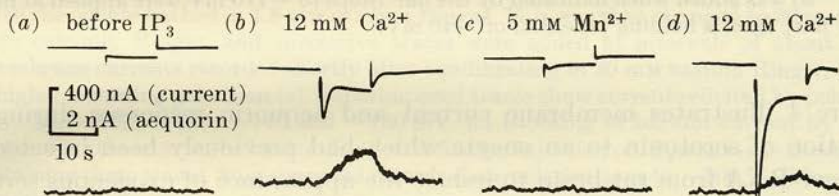

(a) before IP₃   (b) 12 mM Ca²⁺   (c) 5 mM Mn²⁺   (d) 12 mM Ca²⁺

400 nA (current)
2 nA (aequorin)
10 s

FIGURE 3. Blocking of IP₃-induced aequorin and membrane current responses to hyperpolarization by manganese. In each frame the upper trace shows membrane current in response to hyperpolarization from -40 to -140 mV, and the lower trace the corresponding aequorin light response. The oocyte was bathed in Ringer solution containing 12 mM calcium for all records except (c). (a) Control records before injection of IP₃. (b) Responses 5 min after injection of IP₃ (120 kPa, 4 s pulse). (c) Records obtained 4 min after (b), in Ringer solution containing 5 mM manganese and 1.8 mM calcium. (d) Records after changing back to 12 mM calcium Ringer, obtained 4 min after those in (c). The peak T_{in} current was 2.4 µA, and was off-scale on the trace shown.

Aequorin signals during serotonergic activation

One explanation for the increase in aequorin light on hyperpolarization might be that a calcium influx occurs after some mechanical disturbance associated with the injection of IP₃ solution. For example, movement of the injection pipette could disrupt the membrane around the site of impalement and allow a calcium influx, which would be increased by hyperpolarization because of the increased

driving force for calcium entry. However, this explanation seems unlikely, since the aequorin signal did not reach maximum size until a few minutes after the injection, whereas any mechanical effects would be expected to be greatest immediately after the pressure pulse. A stronger argument is that voltage-dependent aequorin signals also could be elicited during activation of muscarinic and other neurotransmitter receptors in the oocyte membrane, which are thought to cause an increase in intracellular IP_3 (Oron *et al.* 1985; Miledi *et al.* 1986).
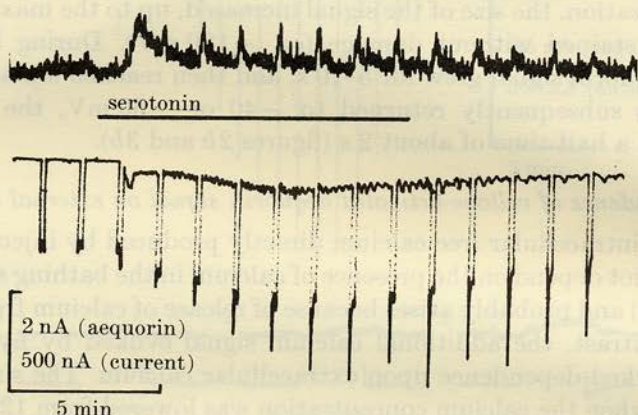

FIGURE 4. Aequorin signals (upper trace) and membrane currents (lower trace) during application of serotonin to an oocyte injected with rat brain messenger RNA. The oocyte was continuously perfused with Ringer solution including 12 mM calcium, to which serotonin (10^{-5} M) was added when indicated by the bar. Steps to -110 mV were applied at intervals of 1 min, from a holding potential of -40 mV.

Figure 4 illustrates membrane current and aequorin responses during bath application of serotonin to an oocyte which had previously been injected with messenger RNA from rat brain to induce the appearance of exogenous serotonin receptors (Gundersen *et al.*, 1983). Serotonin (10^{-5} M) elicited an oscillatory membrane current, and a T_{in} current developed with pulses from -40 to -140 mV. The aequorin light showed a transient increase coincident with the onset of the membrane-current response to serotonin, and then subsided within about òne minute to a level which was maintained slightly above the resting baseline. Hyperpolarizing pulses gave no increase in aequorin light before the serotonin was applied but, as with IP_3 injections, a signal developed within about 3 min of the onset of serotonin activation. This then remained nearly constant throughout the remainder of a 10 min application of serotonin, and declined after washing.

Barium currents after IP_3 injections

An influx of calcium ions into the oocyte through membrane channels is expected to generate an inward membrane current, but this would normally be obscured by the much larger chloride current produced by the activation of calcium-dependent chloride channels. To try to record the current through the calcium

channels in isolation, we replaced the calcium in the Ringer solution by a higher concentration (30 mM) of barium. Barium ions are generally permeant through calcium channels, but are relatively ineffective in activating the calcium-dependent chloride channels (Miledi & Parker 1984). In this way, it had previously been possible to record currents through the calcium channels in the oocyte activated by depolarization, with little contamination from chloride currents (Dascal *et al.* 1986; Miledi *et al.* 1986).

Perfusion with Ringer solution containing 30 mM barium and no added calcium rapidly and reversibly abolished the T_{in} current activated by hyperpolarization in oocytes injected with IP_3 (figure 5*a*). However, when examined at a higher recording gain, a much smaller, slowly rising current could be seen (figure 5*b*),
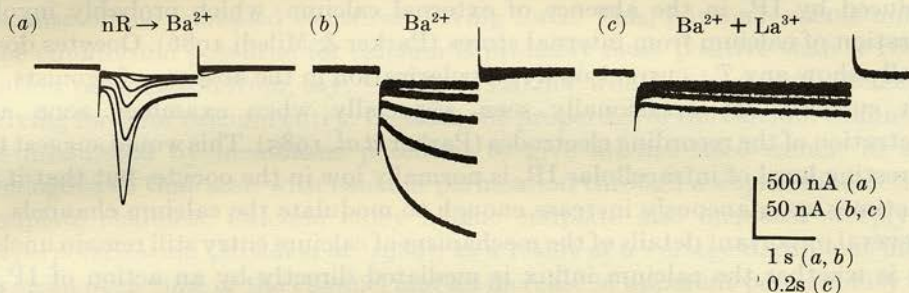

FIGURE 5. Barium currents elicited during hyperpolarization in a native oocyte injected with IP_3. The holding potential in all frames was -40 mV. (*a*) superimposed records of T_{in} currents elicited by polarization to -140 mV. The first trace (largest current) was recorded with the oocyte bathed in 1.8 mM calcium Ringer. This was then changed to 30 mM barium, zero calcium, Ringer, and successive traces were added at intervals of about 10 s. (*b*) Membrane currents recorded shortly after equilibrating in 30 mM barium Ringer, shown at a higher recording gain than (*a*). Superimposed traces show currents elicited by polarization to -80, -100, -120, -140 and -160 mV. (*c*) Blocking of barium current by addition of 1 mM lanthanum to the barium Ringer. A further injection of IP_3 was made shortly before obtaining these traces. Superimposed records show polarizations to -100, -120, -140 and -160 mV.

which was not evident in the barium solution before injection of IP_3. This current was strongly reduced by addition of 1 mM lanthanum (figure 5*c*); this result suggests that it arose largely because of a voltage- and IP_3-dependent influx of barium into the oocyte. However, at present it is difficult to exclude the possibility of some contribution by an inward chloride current, activated by intracellular barium or by entry of contaminating calcium in the bathing solution.

The barium current activated after IP_3 injection became detectable with hyperpolarization to about -100 mV, and increased steeply with further polarizations to beyond -160 mV (figure 5*b*). At the onset of polarization the current showed an initial rapid increase, followed by a slower rise with a half-time of a few hundred milliseconds. The maximal current was reached after about 2 s, and thereafter a slight decline was evident during hyperpolarizing pulses lasting 10–20 s.

DISCUSSION

The main conclusion from this work is that intracellular IP_3 induces the appearance of a voltage-dependent calcium influx in oocytes, presumably by modulating the activity of calcium channels in the cell membrane. Evidence supporting this view is that intracellular injection of IP_3 induces the following responses during hyperpolarization: (i), a calcium-dependent chloride current; (ii), an increase in intracellular free calcium, which depends upon extracellular calcium; and (iii), an inward barium current, recorded after substituting barium for calcium in the bathing fluid so as to avoid complications from the calcium-activated chloride current. The rise in intracellular free calcium resulting from the IP_3- and hyperpolarization-dependent calcium influx should be distinguished from the rise produced by IP_3 in the absence of external calcium, which probably involves liberation of calcium from internal stores (Parker & Miledi 1986). Oocytes do not usually show any T_{in} current on hyperpolarization in the absence of agonists, but such currents are occasionally seen, especially when examined soon after penetration of the recording electrodes (Parker et al. 1985). This would suggest that the resting level of intracellular IP_3 is normally low in the oocyte, but that it can sometimes spontaneously increase enough to modulate the calcium channels.

Several important details of the mechanism of calcium entry still remain unclear. One is whether the calcium influx is mediated directly by an action of IP_3 on calcium channels, or whether some additional messenger molecule is involved. This might be released by the oocyte in response to IP_3, or be formed as a metabolic product from IP_3 (cf. Irvine et al. 1986). Another question is whether the entering calcium passes directly into the cytoplasm or, as proposed by Putney (1986a), enters first into an intracellular pool (such as the endoplasmic reticulum) from where it is subsequently liberated into the cytoplasm.

There are also some puzzling discrepancies between the properties of the T_{in} current and the properties of the calcium entry which we suppose to be responsible for its activation. A striking difference is that the calcium influx appears to be well maintained throughout hyperpolarizations lasting many seconds, whereas the T_{in} current declines within a few hundred milliseconds. Rectification of the current through the calcium-activated chloride channels may contribute to the transient nature of the chloride current (Miledi & Parker 1984; Miledi et al. 1987), but it could also be that the activity of the chloride channels is affected by some other, as yet unknown, mechanism. Another discrepancy is that the aequorin signal elicited by hyperpolarization appears and decays following IP_3 injection more rapidly than the T_{in} current. Thus, the largest T_{in} current is often recorded after the aequorin signal has almost completely declined (figure 2). This lack of correspondence might be attributable to geometrical considerations and the characteristics of aequorin as a calcium indicator. Shortly after injection, the IP_3 will be localized at relatively high concentration in a small area. Thus, a large aequorin signal might be expected because of the resulting high resting free-calcium concentration in this region. On the other hand, the T_{in} current would be small, because only a small area of membrane would be involved. As the IP_3 diffused throughout the large oocyte cell (diameter ca. 1 mm) increasing areas of membrane would become activated, giving

a larger T_{in} current, but the free-calcium level at each point would be lower, and possibly fall below the concentration needed to give a detectable aequorin signal. The power-law relation that is observed under some conditions between the free-calcium concentration and light emission by aequorin (Blinks *et al.* 1981) might further accentuate this effect.

A curious feature of the calcium influx mediated by IP$_3$ is that it becomes prominent only at strongly negative membrane potentials. This voltage sensitivity is in the opposite sense to the calcium channels in the oocyte, which underlie the transient outward current activated by depolarization (Miledi 1982; Barish 1983), and also opposite to calcium channels in many other cell types (Reuter 1983). One possibility is that the activity of the calcium channels is relatively independent of potential, but that a greater calcium influx occurs with hyperpolarization because of the increased electrical driving force. This, however, seems unlikely. The equilibrium potential for calcium is probably more positive than $+100$ mV, so that polarization from, say, -40 to -120 mV would only slightly increase the driving force for calcium entry. Instead, the properties of the calcium channel may be modulated by membrane potential to give an increased influx, in a way analogous to that seen with calcium permeation through acetylcholine-activated endplate channels. Calcium entry at the endplate also increases steeply with hyperpolarization (Miledi *et al.* 1980), as a result of a voltage-dependent increase in mean open time of the channel and an increase in apparent permeability of the channel to calcium (Bregestovski *et al.* 1979).

It has been suspected for many years that calcium entry into cells may be associated with receptors which stimulate phospholipid turnover (Michell 1975; Fain & Berridge 1979; for review see Putney 1986b). Our results support this view, and suggest that the *Xenopus* oocyte may be a convenient preparation in which to study the gating of calcium influx by phosphoinositides. In particular, the ability to record inward barium currents through the calcium channels should facilitate the detailed characterization of these channels, by means of voltage- and patch-clamp techniques.

REFERENCES

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

Berridge, M. J. & Irvine, R. F. 1984 Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature, Lond.* **312**, 315–321.

Blinks, J. R., Wier, W. G., Hess, P. & Prendergast, F. G. 1981 Measurement of calcium concentration in living cells. *Prog. Biophys. molec. Biol.* **40**, 1–114.

Bregestovski, P. D., Miledi, R. & Parker, I. 1979 Calcium conductance of acetylcholine-induced endplate channels. *Nature, Lond.* **279**, 638–639.

Dascal, N., Gillo, B. & Lass, Y. 1985 Role of calcium mobilization in mediation of acetylcholine-evoked chloride currents in *Xenopus laevis* oocytes. *J. Physiol., Lond.* **366**, 299–313.

Dascal, N., Snutch, T. P., Lubbert, H., Davidson, N. & Lester, H. 1986 Expression and

modulation of voltage-gated calcium channels after RNA injection in *Xenopus* oocytes. *Science, Wash.* **231**, 1147–1150.

Fain, J. N. & Berridge, M. J. 1979 Relationship between hormonal activation of phosphatidylinositol hydrolysis, fluid secretion and calcium flux in the blowfly salivary gland. *Biochem. J.* **178**, 45–58.

Gundersen, C. B., Miledi, R. & Parker, I. 1983 Serotonin receptors induced by exogenous messenger RNA in *Xenopus* oocytes. *Proc. R. Soc. Lond.* B **219**, 103–109.

Hirasawa, K. & Nishizuka, Y. 1985 Phosphatidylinositol turnover in receptor mechanism and signal transduction. *A. Rev. Pharmac. Toxicol.* **25**, 147–170.

Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. 1986 The inositol tris/tetrakis phosphate pathway – demonstration of Ins(1,4,5)P$_3$-kinase activity in animal tissues. *Nature, Lond.* **320**, 631–634.

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

Michell, R. H. 1975 Inositol phospholipids and cell surface receptor function. *Biochim. biophys. Acta* **415**, 81–147.

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.

Miledi, R., Parker, I. & Schalow, G. 1980 Transmitter induced calcium entry across the post-synaptic membrane at frog end-plates measured using arsenazo III. *J. Physiol., Lond.* **300**, 197–212.

Miledi, R., Parker, I. & Sumikawa, K. 1986 Calcium channels expressed in *Xenopus* oocytes by mRNA from rat brain. *J. Physiol., Lond.* **374**, 38P.

Miledi, R., Parker, I. & Sumikawa, K. 1987 Oscillatory chloride current evoked by temperature jumps during muscarinic and serotonergic activation in *Xenopus* oocyte. *J. Physiol., Lond.* **383**, 213–229.

Oron, Y., Dascal, N., Nadler, E. & Lupu, M. 1985 Inositol 1,4,5-trisphosphate mimics muscarinic response in *Xenopus* oocytes. *Nature, Lond.* **313**, 141–143.

Parker, I., Gundersen, C. B. & Miledi, R. 1985 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. 1986 Changes in intracellular calcium and in membrane currents evoked by injection of inositol trisphosphate into *Xenopus* oocytes. *Proc. R. Soc. Lond.* B **228**, 307–315.

Petersen, O. H. & Maruyama, Y. 1984 Calcium activated potassium channels and their role in secretion. *Nature, Lond.* **307**, 693–696.

Putney, J. W., Jr 1986a A model for receptor-regulated calcium entry. *Cell Calcium* **7**, 1–12.

Putney, J. W., Jr 1986b Identification of cellular activation mechanisms associated with salivary secretion. *A. Rev. Physiol.* **48**, 75–88.

Reuter, H. 1983 Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature, Lond.* **301**, 569–574.

Slack, B. E., Bell, J. E. & Benos, D. J. 1986 Inositol 1,4,5-trisphosphate injection mimics fertilization potentials in sea urchin eggs. *Am. J. Physiol.* **250**, C340–344.