

LATENCIES OF MEMBRANE CURRENTS EVOKED IN *XENOPUS* OOCYTES BY RECEPTOR ACTIVATION, INOSITOL TRISPHOSPHATE AND CALCIUM

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SUMMARY

1. Application of serum to *Xenopus* oocytes elicits an oscillatory chloride membrane current, which begins after a latency of several seconds or minutes, and is mediated through a phosphoinositide–calcium signalling pathway. We studied the characteristics and origin of this latency in voltage-clamped oocytes.

2. Bath application of low doses of serum evoked responses beginning after latencies of 1 min or more. The latency decreased with increasing dose and reached a minimal value of several seconds that did not decrease with further increases in serum concentration. Experiments to study this minimal latency were done by applying brief ‘puffs’ of serum and other agonists at high concentrations from a local extracellular pipette.

3. The mean latency of the response evoked by local serum application was about 7 s (at 22–24 °C), but individual responses showed a wide variation, from 2 s to over 20 s. Diffusion of serum from the pipette tip to the membrane did not contribute appreciably to this delay, since short (< 100 ms) delays were obtained when KCl was applied in the same way.

4. Currents evoked by acetylcholine and serotonin, in oocytes induced to acquire muscarinic and serotonergic receptors following injection of brain messenger RNA, began following latencies similar to that of the serum response.

5. The response latency was shorter when serum was applied to the vegetal rather than the animal hemisphere of the oocyte, even though smaller currents were obtained.

6. The latency showed a slight dependence upon membrane potential, becoming shorter with depolarization.

7. Cooling to temperatures below about 22 °C produced a striking lengthening of the delay, corresponding to a Q_{10} of about 5. In contrast, above 22 °C the temperature dependence was slight, with a Q_{10} of about 1.25.

8. Intracellular injections of calcium and inositol 1,4,5-trisphosphate (IP_3) evoked chloride currents with short (a few tens of milliseconds) latency. Short (100 ms) latency responses were also evoked when intracellularly loaded caged IP_3 was photolysed by strong illumination, but weak illumination gave responses with latencies of over 1 min.

9. Measurements of intracellular free calcium, made with Fura-2 and Indo-1, showed an increase following serum application beginning coincident with the onset of the membrane current response.

10. Generation of the chloride current responses to serum, acetylcholine and serotonin is thought to involve the following sequential stages: receptor binding, activation of a G protein, activation of a phosphoinositidase enzyme which produces IP_3 , liberation of calcium from intracellular stores by IP_3 and, finally, the opening of calcium-dependent chloride membrane channels. Our results indicate that, at high agonist concentrations, the stages between receptor binding and IP_3 liberation are rate-limiting and account for almost all of the response latency. However, at low agonist concentrations the latency becomes much longer, probably because an additional delay is introduced because intracellular levels of IP_3 rise slowly towards a threshold required before calcium release is triggered.

INTRODUCTION

The receptor sites for some neurotransmitters are part of the same molecule as the membrane channel which they regulate, and these receptor-channel complexes are able to mediate very rapid synaptic responses. An example is the nicotinic acetylcholine (ACh) receptor, which begins to respond within 20 μ s or less after application of ACh (Katz & Miledi, 1965, and unpublished data). In contrast, many other neurotransmitter and hormone receptors are physically separated in the membrane from their respective ion channels, but are functionally linked via intracellular messenger pathways. These receptors typically mediate responses which are slow in both their onset and duration (Kehoe & Marty, 1980; Hartzell, 1981; Iversen, 1984; Iversen & Goodman, 1986). For example, response latencies of tens of milliseconds to several seconds have been observed for muscarinic activation in sympathetic neurones (Dodd & Horn, 1983), in the heart (del Castillo & Katz, 1955; Hill-Smith & Purves, 1978; Pott, 1979) and in *Xenopus* oocytes (Kusano, Miledi & Stinnakre, 1976, 1982; Miledi, Parker & Sumikawa, 1982). The existence of an appreciable delay between receptor binding and the final membrane response will clearly have important consequences for the synaptic activation of cells. We describe here a study of the characteristics, and possible origin, of the latency of responses mediated by a phosphoinositide signalling system in *Xenopus* oocytes.

These oocytes possess native muscarinic receptors (Kusano *et al.* 1982) and receptors to an as yet unidentified component of blood serum (G. Tigyi, C. S. Matute & R. Miledi, unpublished data), both of which mediate an oscillatory chloride membrane current. Furthermore, exogenous receptors to serotonin (Gundersen, Miledi & Parker, 1983), ACh (Sumikawa, Parker & Miledi, 1984) and glutamate (Gundersen, Miledi & Parker, 1984), induced in the oocyte following injection of messenger RNA from brain, also give rise to similar chloride currents. The currents elicited by the agonists all show appreciable (several seconds) latencies to onset, and the responses probably arise because the different endogenous and exogenous receptors 'link in' to a common, endogenous, signalling pathway in the oocyte (Parker, Sumikawa & Miledi, 1987; Takahashi, Neher & Sakmann, 1987). Recent experiments indicate that this signalling pathway operates through phosphoinositide

and calcium metabolism (Miledi & Parker, 1984; Dascal, Gillo & Lass, 1985; Oron, Dascal, Nadler & Lupu, 1985; Parker, Gundersen & Miledi, 1985; Dascal, Ifune, Hopkins, Snutch, Lubbert, Davidson, Simon & Lester, 1986; Parker & Miledi, 1986; Nomura, Kaneko, Kato, Yamagishi & Sugiyama, 1987; Berridge, 1988) in a similar manner to that described in other cell types (Berridge & Irvine, 1984; Hokin, 1985; Berridge, 1986, 1987). Briefly, receptor binding causes a membrane G protein (G_p) to bind GTP and subsequently dissociate. The free α_{GTP} subunit then interacts with a phosphoinositidase enzyme (phospholipase C), causing it to cleave membrane phosphatidylinositol 4,5-bisphosphate to yield inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. Inositol 1,4,5-trisphosphate acts as a messenger to release calcium from intracellular stores, and the resulting rise in free calcium level in turn activates calcium-dependent chloride channels present in the plasma membrane (Miledi, 1982; Miledi & Parker, 1984) to give the final current response.

The large size (> 1 mm diameter) and simple geometry of the oocyte greatly facilitate study of the processes which lead to the delay in activation of the agonist responses. In particular, membrane currents can be recorded under voltage clamp in response to the rapid extracellular application of agonists or to intracellular injections of messengers. Our main findings are that the limiting factors determining the response latency at high agonist concentrations arise from stages in the messenger pathway between receptor binding and IP_3 liberation, while at low agonist levels an additional and longer latency is introduced, probably because of a slow build-up of IP_3 towards a threshold level required before calcium release is activated.

METHODS

Experiments were done on immature oocytes of *Xenopus laevis*, obtained after killing the donor frogs. The oocytes were treated with collagenase to remove enveloping cells (Miledi & Parker, 1984) and, in some cases, the vitelline envelope was also removed, using sharpened forceps with the oocytes bathed in normal Ringer solution or solution made slightly hypertonic with NaCl. For electrophysiological study, oocytes were penetrated by two KCl-filled microelectrodes, to allow voltage clamp using a conventional two-electrode system (Kusano *et al.* 1982; Miledi, 1982). Except where otherwise noted, the membrane potential was held at -50 mV. During recording, the oocyte was continually superfused with Ringer solution (composition (in mM): NaCl, 120; KCl, 2; $CaCl_2$, 1.8 and HEPES, 5, at pH about 7.0) which unless otherwise noted was at room temperature (22–24 °C). The temperature of the solution was monitored by a thermocouple placed just downstream of the oocyte and was altered, when required, by immersing the inlet tube to the recording chamber in a dish containing ice or hot water. Serum was bath applied to the oocyte by switching the superfusate to Ringer solution including various dilutions of rabbit serum (type 100: Diagnostic Biochemistry Inc., San Diego, CA, USA). To avoid uncertainties in timing due to dead space in the tubing, a small amount of Lucifer Yellow was added to the serum solutions to mark when this reached the oocyte. Fluorescence of the dye was monitored from the solution directly above the oocyte, using the microscope system described below.

Local extracellular applications of serum and other agonists were made by pneumatic pressure pulses (50–500 ms duration, 10–200 kPa) applied with blunt (tip diameter *ca* 5 μ m) micropipettes, positioned so as to just touch the oocyte surface. The pneumatic pulses were controlled through a solenoid-operated valve, and the operating voltage for this was displayed on one trace of the recording oscilloscope to provide a pulse marker. Mechanical inertia in the valve introduced a lag of about 50 ms, but this was usually unimportant at the time scale used in the present experiments. Serum pipettes were filled with type 100 rabbit serum, filtered through a 0.2 μ m filter and diluted with Ringer solution to concentrations between 2 and 10%. Serotonin and ACh were similarly

applied at pipette concentrations of, respectively 1 and 10 mM. Potassium pipettes were filled with 3 M-KCl. Intracellular injections were also made with the same system (Parker & Miledi, 1987), though using sharper micropipettes. With the exception of calcium, all compounds for intracellular injection were dissolved in an aqueous solution including 50 μ M-EDTA and 5 mM-HEPES at pH 7. Inositol 1,4,5-trisphosphate (*D-myo*-inositol 1,4,5-trisphosphate, trilithium salt) was obtained from Calbiochem (La Jolla, CA, USA) and injected at concentrations between 10 and 2000 μ M. GTP γ S was obtained from Sigma and injected as a 10 mM solution. Sodium fluoride was made up as a 100 mM solution, and calcium as 5 or 50 mM solutions of CaCl₂. Estimates of the amounts of compounds ejected with each pressure pulse were made by measuring the diameter of the fluid droplets expelled with the pipette tip in the air. In other experiments, IP₃ and calcium were injected by iontophoresis from pipettes filled with 2 mM-IP₃ or 50 mM-CaCl₂.

In some experiments, a compound microscope fitted with an epifluorescence system (Zeiss) was used to monitor the fluorescence of marker dyes and fluorescent calcium indicators. For use with the calcium indicators Fura-2 and Indo-1 (Grynkiewicz, Poenie & Tsien, 1985), light from a 75 W Xenon lamp (Photon Technology) was used for fluorescence excitation, passed through a narrow band interference filter (373 nm, half bandwidth 10 nm; Oriol Corp) and focused onto the oocyte through a 6 \times Neofluor objective (Zeiss). Emitted light was measured by a photomultiplier through a filter transmitting wavelengths longer than 500 nm. An adjustable diaphragm in the excitation light path allowed signals to be recorded from the whole oocyte or from a restricted area. Fura-2 and Indo-1 (Molecular Probes Inc.) were injected into oocytes as 1 mM aqueous solutions. Fluorescence of Lucifer Yellow was monitored using a fluorescein filter set (Zeiss).

The same epi-illumination system also was employed as a source of UV light to photolyse caged IP₃ loaded into oocytes (Walker, Somlyo, Goldman, Somlyo & Trentham, 1987; Parker, 1988). For these experiments, no interference filters were placed in the light path, but an electronically controlled shutter was used to control the duration of light exposure. The light intensity was controlled by an iris diaphragm in combination with neutral density filters, and the photomultiplier was used to monitor the light incident on the oocyte. Caged IP₃ (*myo*-inositol 1,4,5)trisphosphate, P⁴(⁶)-1-(2-nitrophenyl ethyl ester)) was obtained from Calbiochem and oocytes were injected with about 10 pmol from a 10 mM aqueous solution. After injection, oocytes were left for at least 1 h before recording. To avoid light absorption by pigment in the animal hemisphere, oocytes were positioned so that the vegetal (white) hemisphere faced the light source.

RESULTS

Oscillatory chloride currents induced by serum

It was found previously (G. Tigyi, C. S. Matute & R. Miledi, unpublished data) that bath application of serum to voltage-clamped oocytes elicited a characteristic oscillatory membrane current (Fig. 1A). This current inverted direction at about the chloride equilibrium potential in the oocyte (*ca* -20 mV) and arises through calcium-activated chloride membrane channels, via a second messenger system like that which mediates native muscarinic responses in the oocyte (Kusano *et al.* 1982) as well as responses to induced serotonin and other brain neurotransmitter receptors (Gundersen *et al.* 1983; Parker *et al.* 1985).

In the present experiments the parameter of interest was the latency between application of serum and the onset of the membrane current response. This is illustrated in Fig. 1 for currents evoked by bath application of different concentrations of serum. Low doses of serum, close to the threshold to elicit any response, gave small currents beginning after delays of a minute or more. As the dose of serum was increased, the currents grew progressively larger and began after shorter latencies. However, although the latency varied steeply with dose at low concentrations of serum, at higher concentrations it appeared to approach a minimal value of about 8 s and was relatively independent of dose (Fig. 1B).

A major object of this study was to examine the minimal latency approached at high agonist doses. Bath superfusion of agonists was not well suited to this purpose, because with our present system of perfusion it was difficult to obtain rapid and precisely timed applications. Also, the responses to high doses of serum showed desensitization, so that intervals of 10 min or more were needed between each trial. To circumvent these difficulties, we used a 'puffer' pipette to locally apply high concentrations of serum and other agonists onto the oocyte surface. Electronic

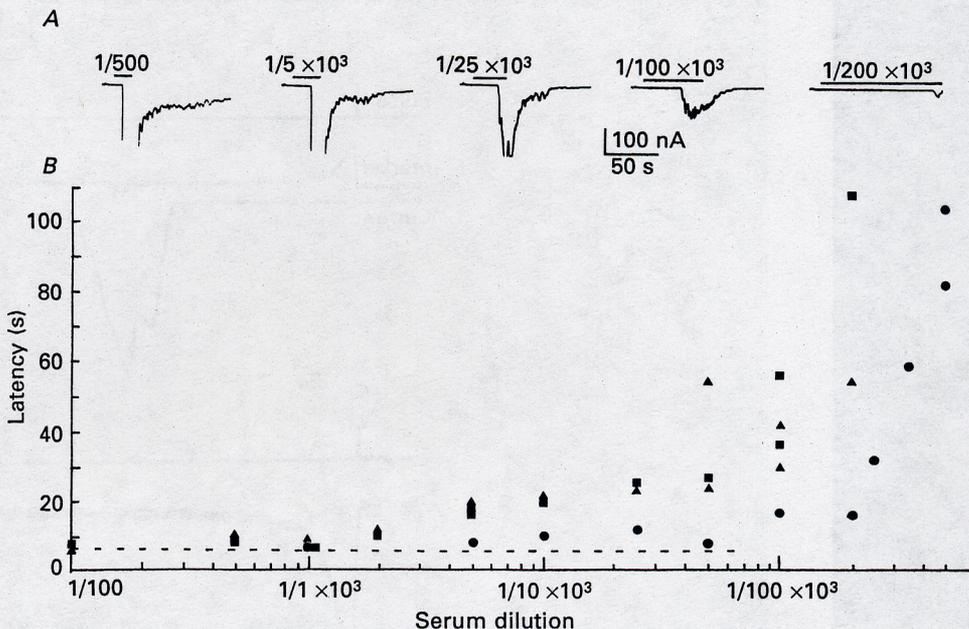


Fig. 1. Latencies of responses evoked by bath application of various dilutions of serum to *Xenopus* oocytes. *A*, sample traces showing currents in an oocyte voltage-clamped to a potential of -60 mV. Bars indicate the times of serum application, monitored by the use of a fluorescent marker dye (see Methods). The final dilutions of serum in the Ringer solution are given next to each trace. Intervals of 10 min or more were allowed following each exposure to high doses (dilutions $< 1/10 \times 10^3$) of serum. In this and other figures downward deflections correspond to inward membrane currents. The maximal peak currents at high serum doses reached values of several microamps and are off-scale on the records shown. *B*, variation in latency to onset of the current response with increasing dilutions of bath-applied serum. Each point is a single measurement, from records like those in *A*. Data from three oocytes, obtained from a single donor. The dashed line indicates the limiting minimal latency. Temperature was 22–24 °C.

control of the pneumatic pressure pulses used to eject agonists gave precise timing. Furthermore, the pipette tip could be moved to a new area of the oocyte between each trial to avoid complications from desensitization.

Figure 2*A* shows a typical response evoked in this way. At room temperature the latency to onset of the current was about 6 s (see later), and in any individual trial was very clearly defined, since the onset of the current was abrupt. For example, in the record in Fig. 2*A*, there was no detectable change in current (< 1 nA) following the serum pulse until, after about 9 s, the current rose abruptly to reach a peak of nearly 200 nA.

The latency does not arise from diffusion of agonist

The serum pipettes were positioned so their tips just dimpled the oocyte surface. Thus, it is unlikely that the distance the serum had to diffuse to reach receptors on the membrane would have been sufficient to introduce a delay of several seconds. Furthermore, the epithelial and follicular cell layers were removed from the oocytes, and although the vitelline envelope usually remained, this is a thin and loose network of collagen-like fibres (Dumont & Brummett, 1978) which would not be expected to

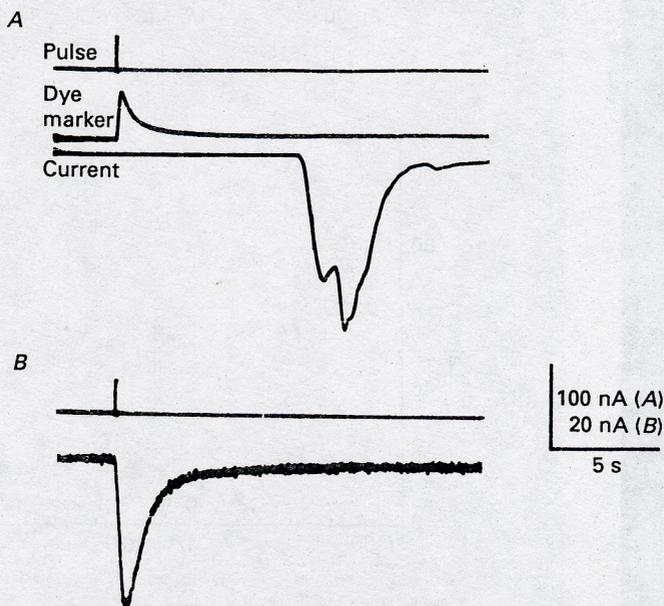


Fig. 2. Membrane currents evoked by local extracellular application of serum (*A*) and KCl (*B*). In each frame, the upper trace is a pulse marker and the lower trace monitors clamp current at a membrane potential of -50 mV. The serum and KCl pipettes were positioned just touching the animal hemisphere of the oocytes. *A*, pipette contained 2% serum together with 1 mM-Fura-2. The lower trace shows the oscillatory membrane current elicited following a pulse of serum. The middle trace monitors Fura-2 fluorescence from an area including all of the oocyte; thus it indicates the time course of ejection of serum from the pipette and its subsequent removal from the vicinity of the oocyte. *B*, membrane current evoked by a pulse of KCl applied from a pipette containing 3 M-KCl. Records are from different oocytes, at temperatures of 22–23.5 °C.

hinder diffusion. In agreement with this, similar latencies of several seconds were recorded to the onset of serum responses in oocytes from which the vitelline envelope was mechanically stripped.

Other evidence also indicates that a time lag in serum reaching the membrane receptors does not contribute importantly to the response latency. For example, when KCl was substituted for serum in the pipette, an inward membrane current began almost coincident with the onset of the ejection pulse (Fig. 2*B*), and a mean latency of 90 ms was measured in ten trials. Also, Fig. 2*A* shows the results of an experiment in which the fluorescent dye Fura-2 was added to serum in the pipette

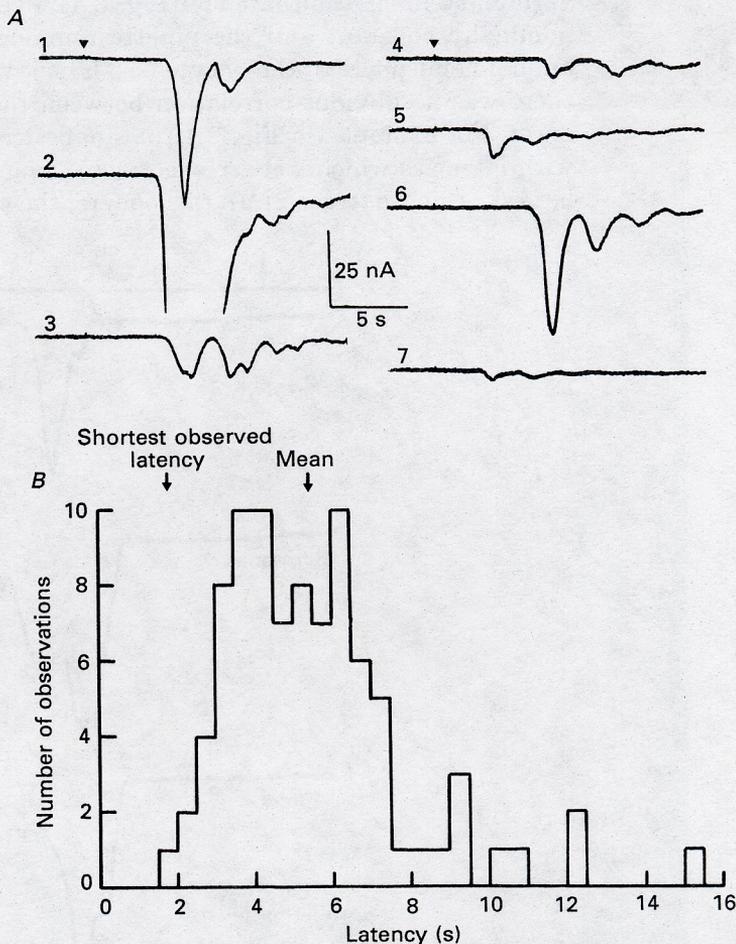


Fig. 3. Variability in the latency to onset of the serum response. *A*, sample records in one oocyte, showing membrane currents evoked by pulses of serum applied as marked by the arrow-heads. The serum pipette was moved slightly between each trace, but remained between latitudes of 20–40 deg in the animal hemisphere. Clamp potential was -50 mV and temperature 23 °C. *B*, distribution of latencies to serum response collected in eighty-nine trials on seven oocytes from one donor. Arrows mark the mean latency (5.5 ± 0.25 s; s.e. of the mean) and the shortest observed latency (1.9 s). Temperature was 22–23.5 °C.

to give an indication of the time course of ejection of serum and its subsequent removal from the vicinity of the oocyte by the continuous flow of Ringer solution. The fluorescence signal rose rapidly during the pressure pulse and then declined with a half-time of about 0.5 s. By the time the membrane current response began, the fluorescence signal, and hence presumably the level of serum, had fallen almost to zero.

Variability in latency

As mentioned before, experiments were usually done by moving the serum pipette a small distance between each trial. Responses elicited in this way showed great

variability in size and latency (Fig. 3A), even though the dose of serum remained nominally constant and the pipette remained at about the same latitude on the animal hemisphere of the oocyte (see later). With this method of serum application, there was no obvious correlation between the size of a response and its latency to onset. For example, in Fig. 3A the smallest responses shown (traces 4 and 7) began with latencies which were, respectively, longer and shorter than the latency to the largest response (trace 2). In this oocyte, the correlation coefficient between response

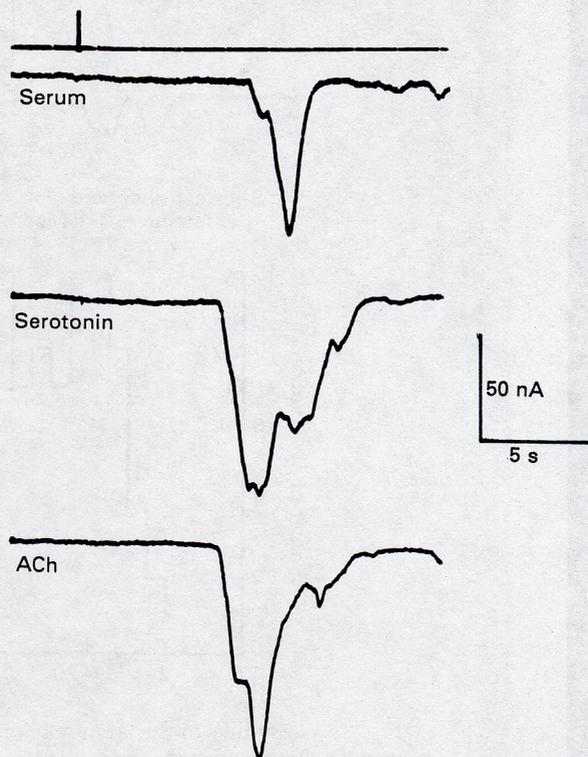


Fig. 4. Serum, serotonin and ACh all evoke similar responses in an oocyte previously injected with mRNA from rat brain. Pulses of agonists were applied from extracellular pipettes, placed at similar positions on the animal hemisphere. Upper trace marks the time of application. Pipettes contained 4% serum, 1 mM-serotonin or 10 mM-ACh. Temperature was 23.5 °C.

latency and size was 0.05 (twenty-one trials), and a second oocyte gave a still lower correlation (0.03, sixteen trials). Furthermore, increasing the dose of serum did not give responses with shorter latency, even though larger currents were evoked. One oocyte was stimulated alternately with brief (20 ms) and long (100 ms) 'puffs' of serum. The long 'puffs' evoked much larger responses than the short (mean sizes respectively 118 ± 7 and 18.6 ± 0.3 nA; s.e. of the mean, ten trials each), but these were of slightly longer latency (respective means 10.1 ± 0.3 and 8.4 ± 0.6 s).

Figure 3B shows a histogram of response latencies, collected in eighty-nine trials in seven oocytes from a single donor. This followed a skewed distribution, with a mean of 5.5 s. The shortest latency recorded was 1.9 s and the longest 15.5 s. As well

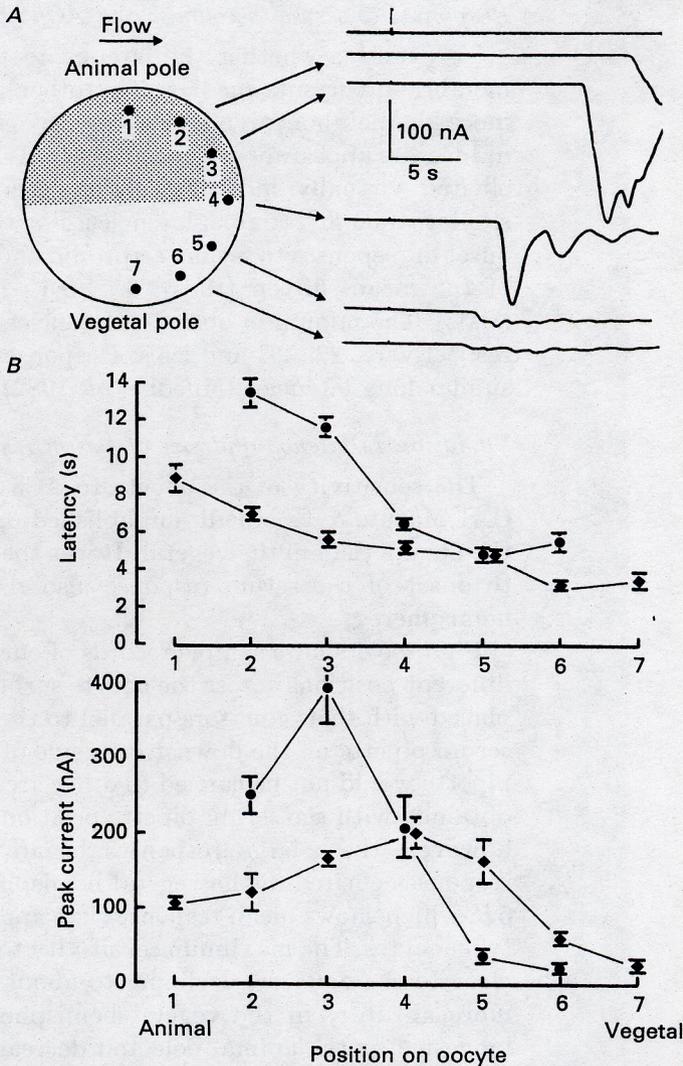


Fig. 5. *A*, membrane currents in response to pulses of serum applied at different positions across the oocyte surface. The upper trace marks the time of the pressure pulse, which remained constant at all positions. The direction of flow of Ringer solution across the oocyte is indicated. Temperature was 23 °C. *B*, variations in size (lower graph) and latency to onset (upper graph) of serum responses evoked by local application at different points across the oocyte surface. Numbers on the abscissae correspond to the positions marked in *A*. Data are shown from two oocytes (different symbols). Each point is a mean value (± 1 s.e. of the mean) from five to eight trials. Temperature was 22–23.5 °C.

as the variability of latency within measurements from an individual oocyte, there also appeared to be differences in the mean latencies between different oocytes. Measurements from sixteen oocytes (three donors) gave mean latencies varying between 4.6 and 14.7 s in individual oocytes, with an overall mean of 6.95 s.

Responses to serum, serotonin and ACh have similar latencies

To examine whether the latency to the serum response was similar to that of membrane currents mediated by other receptors thought to operate via phosphoinositide metabolism, oocytes were injected with messenger RNA from rat brain to induce the appearance of receptors to ACh and serotonin. All three of these agonists elicited virtually indistinguishable oscillatory membrane currents (Fig. 4) with similar, though variable, latencies. For the oocyte illustrated, the mean latencies to onset of responses to serum, serotonin and ACh were, respectively, 7.4 ± 0.7 s (± 1 s.e. of the mean, fifteen trials), 6.7 ± 0.5 s (twenty-one trials) and 6.5 ± 0.5 s (twenty trials). The minimum observed latencies were also similar for these agonists, being, respectively, 2.6, 2.3 and 2.5 s. Responses to ionophoretically applied agonists have similar long latencies (Miledi *et al.* 1982).

Variation in latency and size of serum response across the oocyte

The sensitivity of the oocyte to ACh (Kusano *et al.* 1982) and serum (G. Tigyi, C. S. Matute & R. Miledi, unpublished data) is greater in the animal hemisphere of the oocyte than in the vegetal. It was therefore of interest to see whether the latency to onset of the serum response also showed a consistent difference between the hemispheres.

Figure 5A shows sample records of currents in response to application of serum at different positions across the oocyte surface. For these experiments, the oocytes were placed with their equators parallel to the direction of flow of bathing fluid, with the serum pipette on the downstream side of the oocyte, so that serum ejected from the pipette would not be carried to other areas of the oocyte. The largest responses were obtained with the serum pipette positioned on the animal hemisphere. Surprisingly, however, these large responses began after a longer latency than the smaller responses elicited in the vegetal hemisphere. These findings are summarized in Fig. 5B, which shows mean response sizes and latencies obtained at different positions on two oocytes. The maximum sensitivity to serum was found in the animal hemisphere close to the equator, declining to about one-half near the animal pole, and falling more abruptly in the vegetal hemisphere. In contrast, the response latency was longest near the animal pole and decreased progressively towards the vegetal pole. Differences in latencies between the poles were observed in four oocytes examined, and the latency near the animal pole was on average 2.1 times longer than near the vegetal pole (range 1.4–2.5).

Because of this variation in response latency, except where otherwise noted, all results described in this paper were obtained with agonist and intracellular injection pipettes positioned at latitudes between about 10 and 40 deg in the animal hemisphere.

Voltage dependence of the latency

To determine whether the latency to onset of the serum response was dependent upon membrane potential, applications were made while the oocyte was alternately clamped at -10 or -100 mV. The membrane current responses were inverted at -10 mV and were also generally larger than at -100 mV, probably because of the

rectification of the calcium-activated chloride current at negative potentials (Miledi & Parker, 1984). Differences in latency between the two potentials were not obvious, but comparison of mean latencies averaged over many trials at each potential indicated that the response began slightly later at the more negative voltage. For example, in one oocyte, the latency to the serum response was 10.0 ± 0.7 s at -100 mV, as compared to 7.45 ± 0.6 s at -10 mV (s.e. of the mean, thirty-two trials at each potential), and similar results were obtained in another oocyte to which serotonin was applied ionophoretically (latencies 7.14 ± 0.24 s at -90 mV and 6.26 ± 0.3 s at -10 mV; ten trials at each potential). Two further oocytes also showed a longer latency response to serum at more negative potentials, but here the differences were not statistically significant: for the first oocyte the latencies were 8.0 ± 1.1 s at -100 mV and 6.8 ± 1.2 s at -10 mV; for the second, 6.5 ± 0.3 s at -100 mV and 6.3 ± 0.4 s at -10 mV (eleven trials at each potential for both oocytes).

Temperature dependence

The latency to onset of the serum-evoked current became strikingly longer as the temperature of the bathing fluid was lowered (Fig. 6A), increasing in the oocyte illustrated by a factor of sixteen on cooling from 23 to 11 °C. The duration of the oscillatory current response was also greatly prolonged. On the other hand, warming above room temperature produced a much smaller shortening in latency (Fig. 6B). Measurements of these effects were made by initially equilibrating the oocyte at a low temperature, since cooling elicited large 'temperature jump' fluctuations in current (Miledi, Parker & Sumikawa, 1987) which subsided only after several minutes. The oocyte was then gradually re-warmed and maintained at selected temperatures while readings were obtained. At temperatures above about 35 °C the response to serum was abolished and it recovered only slowly, or not at all, when the oocyte was returned to room temperature.

An Arrhenius plot of the serum response latency is shown in Fig. 6C, measured in six oocytes from two donors, over a temperature range from 11 to 35 °C. This showed a clear transition point. At temperatures below about 22 °C the latency varied very steeply with temperature, and the line drawn through the data corresponds to a Q_{10} of about 5. Very different to this, above 22 °C the temperature dependence was slight, corresponding to a Q_{10} of only about 1.25.

Intracellular injections of IP_3 and Ca^{2+}

As described in the Introduction, the chloride membrane currents evoked by serum and serotonin are thought to arise because receptor occupancy causes liberation of IP_3 , which then releases calcium from intracellular stores; this calcium in turn finally activates the chloride channels. Thus, by injecting IP_3 and calcium into oocytes, we sought to identify those stages in the messenger pathway which might be primarily responsible for the latency in the agonist-evoked response.

It already appeared that intracellular injections of calcium (Miledi & Parker, 1984) and IP_3 (Oron *et al.* 1985; Parker & Miledi, 1986) elicit membrane current responses with quite short delay. Figure 7 shows that the latencies of responses to both of these messengers were negligible as compared to the response to serum, even when IP_3 and

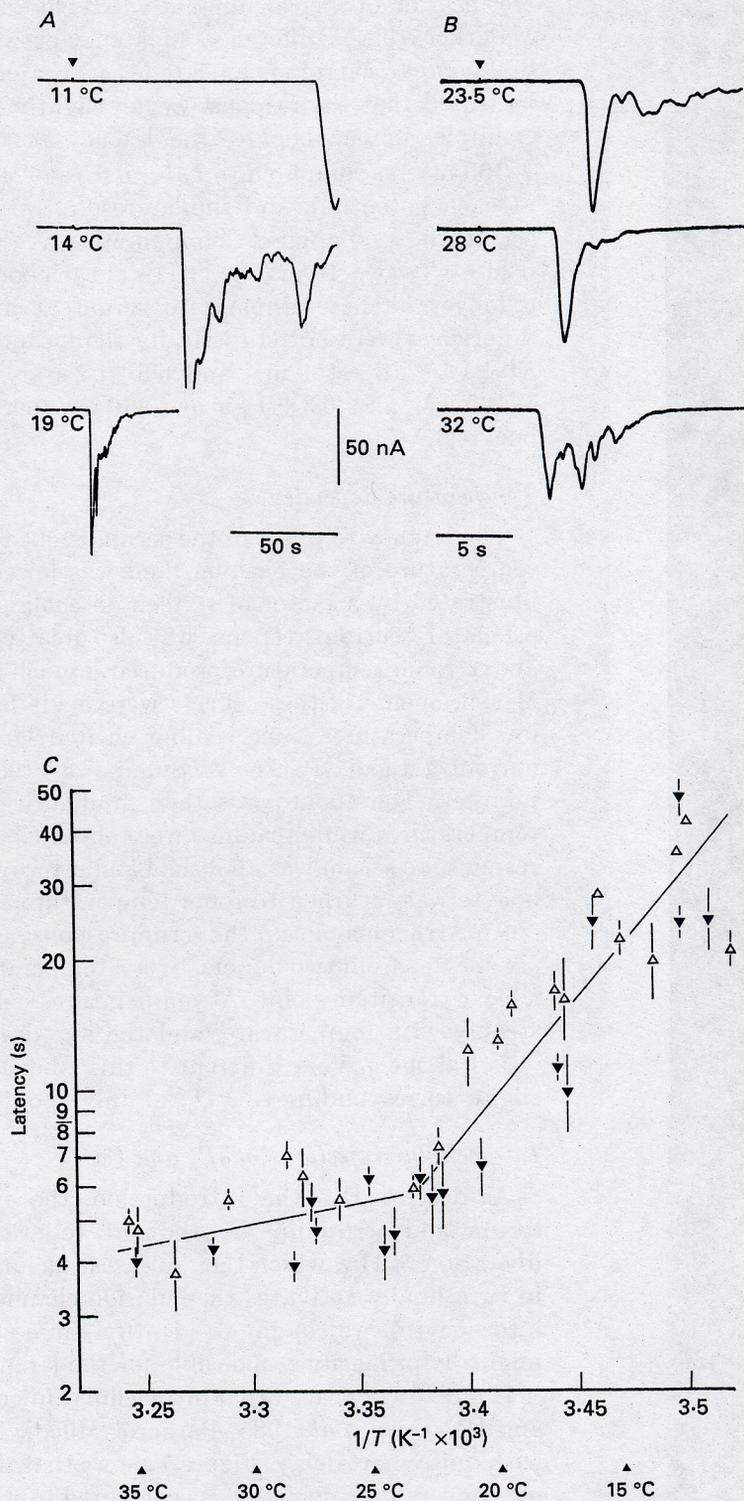


Fig. 6. For legend see facing page.

serum were applied to the same oocyte, at doses selected to give responses of similar size. Intracellular injection of IP_3 evoked an initial spike of current which, like the response to serum, was usually followed by a series of oscillations (cf. Oron *et al.* 1985; Parker & Miledi, 1986). Calcium, at the doses used here, gave only a smooth rise and fall in current (cf. Miledi & Parker, 1984). The possibility that the initial response to

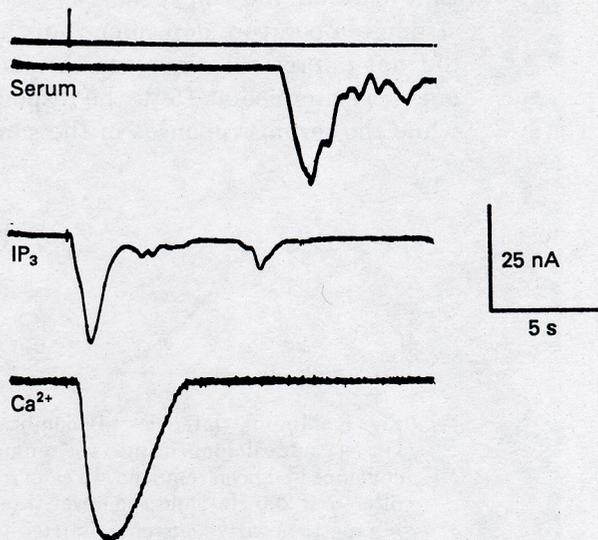


Fig. 7. Membrane currents evoked by extracellular application of serum and by intracellular injections of IP_3 and calcium. Upper trace is a pulse marker. The responses to serum and IP_3 were obtained in the same oocyte, using pipettes filled with 4% serum or 2 mM- IP_3 . The tip of the serum pipette was positioned close to the point of insertion of the IP_3 pipette, which remained in the oocyte. The response to intracellular injection of calcium was recorded in a second oocyte, using a pipette filled with 5 mM- $CaCl_2$. Temperature was 23 °C for all records.

IP_3 arose because of contaminating calcium in the injection solution is unlikely, since EDTA (50 μ M) was present in this solution to chelate any calcium, and testing of the fluid in the pipettes with Arsenazo III indicated a low (micromolar or less) free calcium level. Furthermore, short-latency responses to IP_3 were observed when IP_3 was injected by ionophoresis, even though the pipette negative ionophoretic current used to expel IP_3 would be expected to retain calcium ions within the pipette.

Fig. 6. Effect of temperature on responses to serum. *A*, and *B* currents evoked by 'puffs' of serum, applied as marked by the arrow-heads. Traces on the left and right were obtained in two different oocytes; note the difference in recording speed. The serum pipettes were moved slightly between each trace, but remained at about the same latitude on the animal hemisphere of the oocytes. *C*, Arrhenius plot, showing temperature dependence of the mean latency to onset of the serum response, measured from records like those in *A*. Data are from six oocytes from two donors (Δ , \blacktriangledown). Each point shown with error bars is a mean of four to sixteen trials, and the bars indicate ± 1 s.e. of the mean. Points without bars are means from one to three measurements. Lines were fitted by eye.

The latencies of responses to IP_3 and calcium, injected by pneumatic pressure, varied between about 50 and 900 ms in different insertions in several oocytes. Much of this latency, and its variability, probably arose because the tip of the injection pipette penetrated to different depths in the cytoplasm, introducing a variable delay in diffusion from the ejection site to the inner surface of the cell membrane. In agreement with this, sharper injection pipettes, which dimpled the oocyte less before they entered the cell, generally gave responses with shorter latency. Unlike the strong temperature dependence of the serum response latency, cooling of the oocyte did not appreciably increase the latency of the response to IP_3 injection. In two oocytes examined at 15 °C the response to injections of IP_3 began within 50–130 ms, while the serum responses in the same oocytes showed latencies of more than 20 s.

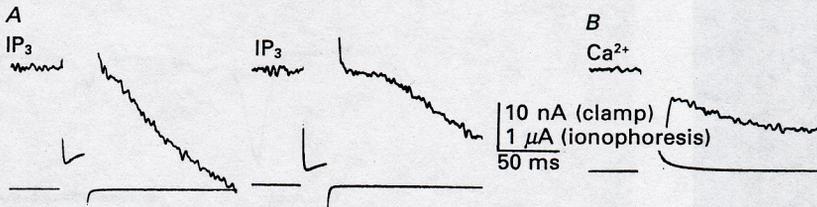


Fig. 8. Minimal latencies of membrane currents evoked by ionophoretic injections of IP_3 (A) and calcium (B) into the animal hemisphere of an oocyte from which the vitelline envelope had been removed. In each frame, the upper trace shows clamp current (low-pass filtered at 200 Hz), and the lower trace monitors ionophoretic current (upward deflection = pipette negative current). Capacitative artifacts obscure the clamp current during and shortly after the ionophoretic pulses. The two traces in A show responses to ionophoretic applications of IP_3 of different sizes.

To obtain a better estimate of the minimal response latency to intracellular IP_3 and calcium, injections were made after removal of the vitelline membrane from the oocyte. Injection pipettes would then enter the oocyte with very little deformation of the membrane, and could be withdrawn a few micrometres at a time to position the pipette tip close to the inner membrane surface. Also, injections were made by ionophoresis, since this allowed better control of the pulse timing, without the mechanical lag of the pneumatic system. Figure 8 illustrates responses evoked in this way. Capacitative currents from the ionophoretic pulse obscured the clamp record for about 25 ms. Nevertheless, it can be seen that the current evoked by a large dose of IP_3 began within less than 30 ms following the onset of the ionophoretic pulse, although smaller doses gave responses with a longer latency. The onset of the response to calcium was even shorter, but it is difficult to know whether this arose from differences in position of the pipette tips.

Caged IP_3

As mentioned above, measurements of the latency of responses evoked by intracellular injections of IP_3 are complicated by uncertainties regarding the position of the pipette tip, and also by the difficulty in controlling the amounts injected. To avoid these problems, we used an alternative technique employing caged IP_3 (Walker *et al.* 1987). Oocytes were injected with caged IP_3 , which was allowed to distribute

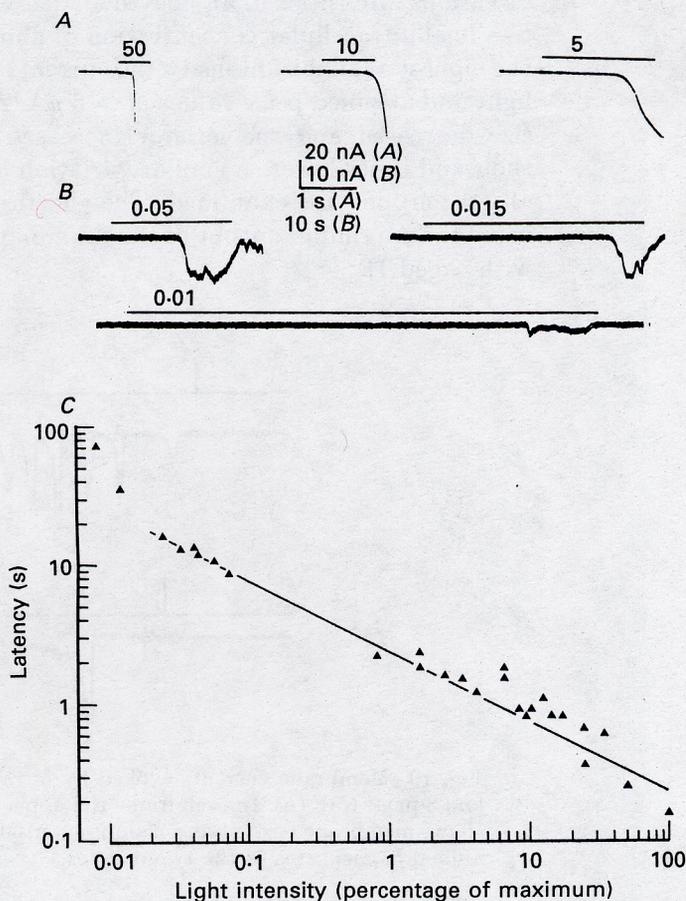


Fig. 9. Latency of membrane currents evoked by photolysis of intracellularly loaded caged IP_3 . *A* and *B*, traces show membrane currents evoked by exposures to light of various intensities, indicated next to each trace as a percentage of the maximum intensity. The light was extinguished shortly after the onset of the membrane current response and the bars indicate the durations of light exposure. The oocyte was loaded with about 10 pmol caged IP_3 and the clamp potential was -60 mV. Note the differences in sweep speed and recording gain between *A* and *B*. The peak currents evoked by the maximal light intensity were $> 5 \mu\text{A}$, and the currents are off-scale in *A*. *C*, double logarithmic plot showing the relation between intensity of photolysis light and latency to onset of the membrane current. Each point is a single measurement, from the same oocyte as in *A* and *B*.

throughout the cell for 1 h or more. Irradiation with UV light was then used to photolyse the compound, causing the liberation of free IP_3 (Parker, 1988).

Light flashes applied to oocytes loaded with caged IP_3 evoked membrane currents (Fig. 9*A* and *B*) which, like those evoked by intracellular injection of IP_3 , were carried largely by chloride ions and depended upon intracellular, though not extracellular, calcium (Parker, 1988). A striking feature was that the latency to onset of the responses became shorter when more caged IP_3 was loaded into an oocyte and when the intensity of the light was increased. Figure 9 illustrates responses to light

of various intensities in an oocyte loaded with about 10 pmol caged IP₃ (equivalent to a final intracellular concentration of about 10 μM assuming even distribution). At the highest available intensity the currents began about 150 ms after the onset of the light and attained peak values of > 5 μA. As the intensity was reduced the response size decreased and the latency increased to as long as 75 s at an intensity ten thousand times lower. A similar variation in latency with light intensity was seen in all (> ten) oocytes examined. The shortest latencies observed were about 100 ms, using the maximum output of the illuminator in oocytes heavily loaded (> 30 pmol) with caged IP₃.

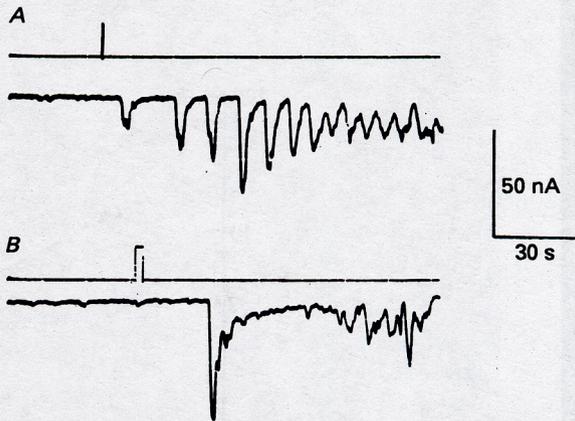


Fig. 10. Membrane currents evoked by intracellular injections of 0.15 pmol GTP γ S (A) and 3 pmol NaF (B). In each frame the upper trace is a pulse marker, and the lower trace shows membrane current at a clamp potential of -60 mV. Injections were made into the animal hemispheres of the two oocytes.

Injections of GTP γ S and fluoride

The G_p protein which mediates phosphoinositide signalling can be activated, in the absence of receptor binding, by intracellular GTP γ S (a non-hydrolysable analogue of GTP) and by fluoride ions (Berridge, 1987). We therefore injected these substances into the oocyte to determine the latency of responses evoked when G_p was activated by a route bypassing the surface membrane receptors.

Injection of GTP γ S evoked a gradually increasing series of oscillations in membrane current, which persisted for 30 min or more (Fig. 10A, and see Dascal *et al.* 1986). The mean latency to onset of the first oscillation was 10.6 ± 3.3 s, measured in five trials in four oocytes.

Injections of NaF also evoked oscillatory currents (Fig. 10B) which were more transient than those generated by GTP γ S and began with a 'spike' of current resembling that seen with injection of IP₃ (Parker & Miledi, 1986). The mean latency from injection to the onset of the current spike was 20 ± 6 s, measured in four trials in two oocytes.

Changes in intracellular calcium

If the membrane current evoked by serum does indeed arise as a consequence of a rise in intracellular calcium, the above results suggest that the intracellular calcium

level should rise after a latency almost identical to that of the corresponding current response. To see if this was the case, the fluorescent probes Fura-2 and Indo-1 (Grynkiewicz *et al.* 1985) were used to monitor the intracellular free calcium level.

Figure 11 shows simultaneous records of membrane current and Fura-2 fluorescence, obtained following application of serum to an oocyte. Neither the calcium signal nor the current showed any change for about 11 s after the serum pulse, but the calcium signal then abruptly rose at about the same time as the membrane current response began. This record was obtained with 375 nm excitation, a wavelength at which Fura-2 shows a decrease in fluorescence with increasing calcium. Control records were also obtained with 350 nm excitation, where the fluorescence change with calcium is in the opposite direction (Grynkiewicz *et al.*

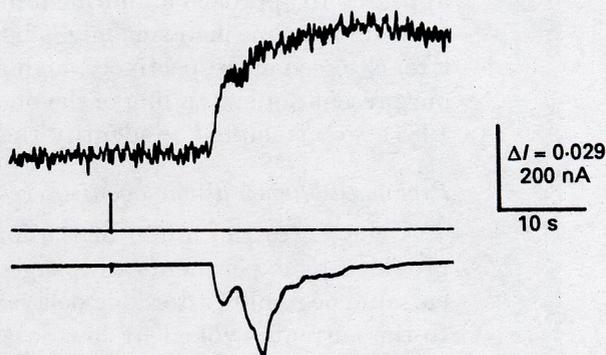


Fig. 11. Simultaneous recording of changes in intracellular free calcium level and in membrane current evoked by a pulse of serum. Traces show, from top to bottom, Fura-2 fluorescence with 375 nm excitation, time of pressure pulse applied to serum pipette and membrane current. A decrease in fluorescence is displayed as an upward deflection, so as to correspond to increasing free calcium. The calibration bar corresponds to a change in fluorescence intensity of 2.9%. The serum pipette was positioned just on the vegetal side of the equator, and fluorescence signals were monitored from a spot of about $50\ \mu\text{m}$ diameter centred on the tip of the serum pipette. See text for further details. Temperature was $23\ ^\circ\text{C}$.

1985). These showed a delayed increase in fluorescence after application of serum, consistent with an increase in intracellular free calcium, but the records were very noisy, since the optical system employed transmitted little light at 350 nm. Results like those illustrated were obtained in a further three oocytes loaded with Indo-1, monitoring the decrease in fluorescence emission at 500 nm as an indicator of increasing intracellular free calcium. The calcium signals with both indicators declined slowly after the peak and returned to the baseline only after a few minutes, even though the current responses ceased much sooner.

DISCUSSION

The results are concerned mainly with the latency to onset of currents evoked by a component of serum in *Xenopus* oocytes. This agonist was chosen largely for convenience, because almost all oocytes show a high sensitivity to serum (G. Tigyi, C. S. Matute & R. Miledi, unpublished data), while responses to neurotransmitters

such as ACh are less common (Kusano *et al.* 1982). However, responses mediated by muscarinic and serotonergic receptors showed an almost identical latency and probably arise through the activation of a common signalling pathway (see Introduction). Thus, the mechanism responsible for the latency in the oocyte is likely to be of widespread importance in the generation of responses to many neurotransmitters and hormones. In particular, it may be relevant to slow synaptic transmission in neurones, since brain serotonin and ACh receptors, 'transplanted' to the oocyte by injection of messenger RNA, are able to link into the already existing messenger pathway.

Application of low concentrations of serum by bath superfusion evoked responses with very long (tens of seconds) and variable latencies. Progressive increases in the dose of serum at first caused a marked shortening of the latency, but the latency then appeared to approach a limiting minimal value of 6–10 s. This observation suggested that different mechanisms might be primarily responsible for the minimal and long latencies seen at, respectively, high and low concentrations of serum. On the basis of our present understanding of the phosphoinositide signalling system (Berridge, 1986, 1987), we attempted to identify these mechanisms.

Prolonged latency at low agonist levels

A clue as to the origin of the long latencies seen with low levels of serum was provided by experiments with caged IP_3 , where dim photolysis lights evoked small currents beginning after long delays. These responses were similar in size and latency to the currents evoked by low doses of bath-applied serum (compare Figs 1A and 9B). Thus, it seems that long latencies are associated with conditions where IP_3 is expected to be formed at a slow rate in the oocyte. A possible explanation for this latency is provided by recent experiments which indicate that intracellular IP_3 must rise above a threshold level before release of intracellular calcium occurs (Parker, 1988). On this basis we propose a model which is closely analogous to the scheme we had previously suggested to account for the latency of E–C coupling in muscle (Miledi, Parker & Zhu, 1983). Inositol 1,4,5-trisphosphate is presumed to be formed at a steady, slow rate during continued application of low doses of serum, while its subsequent metabolism is presumed to proceed at a rate proportional to its intracellular concentration. Thus, when serum application begins, the intracellular level of IP_3 will rise towards a steady-state value following an exponential time course. The latency arises from the time taken before the concentration of IP_3 rises above the threshold.

This model predicts that the latency should decrease as the rate of formation of IP_3 increases, and that it should approach zero for very high rates of formation. In experiments where IP_3 was liberated directly in the oocyte this was approximately true, since intracellular injections of IP_3 and massive photolysis of caged IP_3 gave response latencies as short as 30–100 ms. However, the latency of currents evoked by serum did not reduce to shorter than several seconds, even with very high doses. One possibility for this is that the response to serum becomes saturated, so that high doses cause only a modest rate of formation of IP_3 . Several observations indicate that this is not so. (1) The size of the membrane current and (hence presumably the rate of IP_3 formation) continues to rise steeply as the concentration of serum is raised

to as high as 1% (G. Tigyí, C. S. Matute & R. Miledi, unpublished data), yet the latency fails to shorten appreciably as the concentration is raised above 0.1% (Fig. 1*B*). (2) Increasing the dose of serum applied locally from an extracellular pipette gave larger current responses, but did not shorten the latency. (3) Intracellular injections of IP_3 gave responses with short (*ca* 100 ms) latency, even when the response sizes matched those of the long-latency responses evoked by local serum application (Fig. 7).

The minimal latency at high serum doses appears, therefore, not to arise from a slow build-up of IP_3 , and other mechanisms for its origin are discussed below.

Origin of the minimal latency at high agonist levels

Experiments to study the minimal latency were done by locally applying high concentrations of agonist from a 'puffer' or ionophoretic pipette. With these techniques, it seems that events prior to receptor activation could not have introduced any appreciable delay. The distance from the tip of the agonist pipette to the oocyte membrane was small and, as expected from this, responses to KCl (Fig. 2) and to activation of induced nicotinic receptors (Miledi *et al.* 1982) were of short latency. Furthermore, the possibility that serum (as well as serotonin and muscarinic ACh) receptors might be located exclusively within deep infoldings of the plasma membrane seems unlikely, and in any case the high temperature dependence of the latency indicates that it does not arise from a diffusion delay. A slow binding of the agonist to the receptor is also improbable, since brief 'puffs' of serum evoked responses which began only after the concentration near the oocyte had fallen nearly to zero.

Considering next the final stages in the signalling pathway, we have already mentioned that high levels of IP_3 evoke membrane currents with short (< 100 ms) delay. Furthermore, injections of calcium into the oocyte evoked responses with latencies which were negligible (a few tens of milliseconds) as compared to the agonist-evoked responses, and intracellular free calcium levels (monitored with Fura-2 and Indo-1) did not rise following application of serum until after a latency almost identical to that of the membrane current response. All this indicates that the IP_3 -induced liberation of intracellular calcium, and the subsequent activation of membrane chloride channels by intracellular calcium, appear to contribute little towards the minimal latency remaining at high agonist concentrations.

The stages in the pathway which remain as possible candidates to account for the minimal latency are those which link receptor occupancy to the liberation of IP_3 ; i.e. the receptor-mediated activation of G_p , the binding of GTP to G_p , and the activation of phosphoinositidase by G_p . To try to further discriminate between these possibilities we injected oocytes with GTP γ S and fluoride ions, so as to activate G_p in the absence of receptor binding. Injections of these substances evoked oscillatory currents beginning after latencies of several seconds. This result would seem to indicate that a large part of the latency of the agonist-evoked response arises subsequent to the activation of G_p , but the interpretation is difficult, because we do not know whether GTP γ S and fluoride activate G_p as rapidly as does the normal physiological pathway.

Attempts have previously been made to model slow electrical responses in terms

of a sequence of stages of exponential delay (e.g. Fuortes & Hodgkin, 1964; Hartzell, Kuffler, Sickgold & Yoshikami, 1977). The abrupt onset of the response in the oocyte, following several seconds or even minutes of total quiescence, suggests that such a model is not appropriate here, since an unrealistic number of cascaded stages would be needed to reproduce the observed behaviour. An alternative explanation is provided by the concept of a threshold, above which the level of an intermediate in the signalling pathway must rise before subsequent stages are activated. As described above, we believe that one such threshold arises at the stage of IP_3 -induced calcium liberation. In addition, another threshold may exist at earlier stages in the pathway linking receptor activation to IP_3 formation, which is responsible for the minimal latency at high agonist levels.

An interesting feature of the minimal serum response latency was its dramatic dependence upon temperature. Above about 22 °C the latency shortened only slightly with increasing temperature, with a Q_{10} (1.25) no more than would be expected for a diffusion-limited process. In contrast, at lower temperatures the latency increased greatly, corresponding to a Q_{10} of about 5. The origin of this steep temperature dependence is not yet clear, but it seems that it must arise prior to the liberation of IP_3 , since injections of IP_3 still elicited short (< 100 ms) latency responses at 15 °C. Possible causes for the transition point at 22 °C include a membrane-lipid phase transition (Chapman, 1975), a conformational change in some protein (cf. Levy, Sharon & Koshland, 1959), or that different steps in the signalling pathway are rate-limiting in different parts of the temperature range (Dixon & Webb, 1964). It is difficult to discriminate between these alternatives at present, but a lipid phase transition would offer an attractive explanation, as the movement of a G protein coupling receptors to phosphoinositidase activation might be expected to be strongly influenced by the fluidity of membrane lipids.

Application of serum near the vegetal pole of the oocyte gave responses which were smaller, but of shorter latency, than those evoked from the animal hemisphere. This variation in response size resembles that observed for currents mediated by native muscarinic receptors (Kusano *et al.* 1982) and those evoked by intracellular injections of IP_3 (Berridge, 1988). The asymmetrical distribution of calcium-activated chloride channels across the oocyte surface (Miledi & Parker, 1984) probably accounts for much of these variations in response size between the two hemispheres, but a lower density of channels in the vegetal hemisphere would not be expected to result in a shorter latency. Instead, other components of the signalling pathway might also show regional differences; for example, there may be more receptors or G proteins in the vegetal hemisphere, or the lipid composition of the membrane could differ. Whatever the explanation, it seems that the mean latency is not an invariant parameter defined (at any given temperature) by the molecular properties of the signalling pathway. Furthermore, the observation that the latency differs systematically across the oocyte surface raises the possibility that the phosphoinositide signalling pathway may be 'tailored' in different cells to give responses with specific delays.

Note added in proof. Horn & Marty (1988) have recently described that the delay of muscarinic responses in lacrymal cells shows a similar dose dependence and approaches a minimal value at high agonist concentrations.

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