

# Latency, Threshold and Facilitation in Phosphoinositide Signalling

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## INTRODUCTION

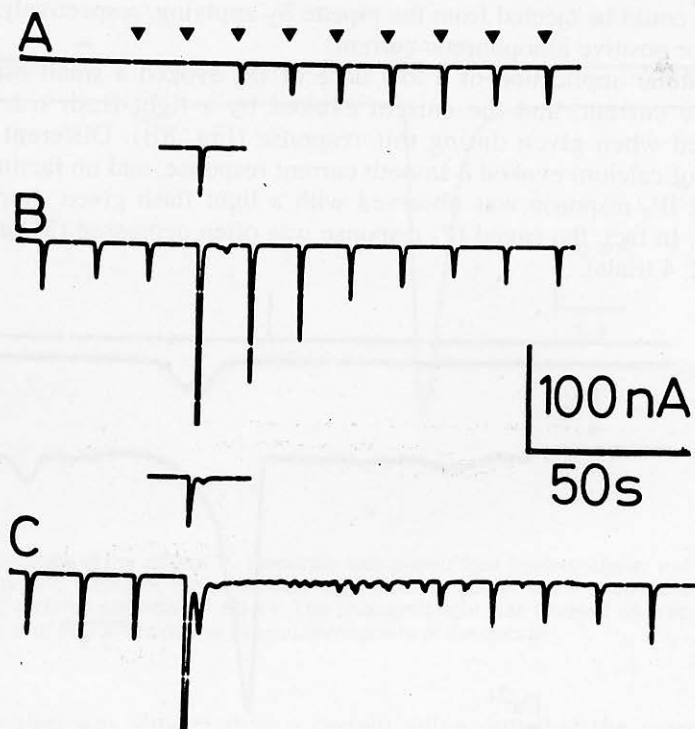
### **Intrinsic and second messenger-linked receptor-channel coupling**

The nicotinic acetylcholine receptor (nAChR) is a classical example of a neurotransmitter receptor in which the receptor sites are a part of the same molecule as the channel which they operate. The relatively simple architecture of this 'intrinsic' receptor/channel molecule endows it with relatively straightforward functional properties. For example, membrane responses generated by the opening of nAChR channels begin within microseconds of the application of agonist, vary as about the second power of agonist concentration, and cease within a few milliseconds following the removal of agonist (19,37). In marked contrast to this, many other neurotransmitters and hormones act on receptors which are quite distinct from the channels which they operate. Here, the functional link between receptors and channels is provided by intracellular second messengers, and this more elaborate system introduces greater complexity in the responses mediated via second messengers. Most strikingly, responses begin following a long (hundreds of ms or several s) latency after application of agonist, they may vary in a highly non-linear way with agonist concentration, and when the agonist is removed the currents may persist for a considerable time (14,16,17,20,29).

The characteristics imposed by the second messenger pathways linking receptors to their respective ion channels undoubtedly have important consequences for the transmission of information at synapses, and for communication between cells in general. I discuss here the application of oocytes from *Xenopus laevis* as a model system in which to study the functional characteristics of the phosphoinositide messenger pathway,

### Repetitive stimulation of the caged $\text{IP}_3$ response

Fig. 9 illustrates some other aspects of the facilitation of the caged  $\text{IP}_3$  response seen with repetitive stimulation. A train of light flashes at 15 s intervals was applied to the oocyte, with the flash duration (70 ms) set so that a single flash was sub-threshold. The first two flashes in the train evoked no response, but a small current was evoked by the third, and this grew with successive stimuli to reach a maximal level by about the fifth stimulus (Fig. 9A).



**Fig. 9.** Facilitation and depression of caged  $\text{IP}_3$  responses during repetitive stimulation. **A**, membrane currents evoked by a train of light flashes (duration 70 ms) given at intervals of 15 s, as indicated by the arrowheads. These test flashes continued at the same rate throughout the records in (B) and (C). Note that the first two flashes in the train failed to evoke any response. **B**, the duration of the fourth light flash in this record was increased to 300 ms; all other flashes remained of 70 ms duration. The current evoked by the long flash was off-scale on the main recording trace, and is shown at lower gain in the inset record, for which the calibration bar corresponds to 1  $\mu\text{A}$ . **C**, same as (B), except that the duration of the fourth flash in the train was further increased to 3 s. Records are from a single oocyte, which was illuminated by a light spot of 250  $\mu\text{m}$  diameter positioned on the vegetal hemisphere close to the equator.

concentrating on two particular aspects; (i) the latency of the response and, (ii) non-linearity and facilitation of the evoked currents.

### Oocytes as a model cell system

The study of intracellular signalling pathways in neurons is greatly complicated by their small size, complex geometry and relative inaccessibility.

Instead, we sought a preparation which would be technically easier to approach, and selected the *Xenopus* oocyte as a suitable model cell (28). The oocytes are large (diameter 1 mm or more) spherical cells, and are readily penetrated by micropipettes which can be used to voltage clamp the cell, and for intracellular microinjection (7,39).

Oocytes as they are obtained from the frog already possess muscarinic receptors to ACh (refs. 31,32), and receptors to a component of blood serum (G. Tigyi, C.M. Matute & R. Miledi; unpublished data), both of which mediate a chloride membrane current through activation of a phosphoinositide signalling pathway. Furthermore, we are not restricted only to the study of these native receptors. A great advantage of the oocyte is that it can be 'engineered' to express many other types of receptors, by injection of foreign mRNA (1,7,11,28).

For example, mRNA can be extracted from the brain and injected into oocytes, where it is translated to express many types of functional receptors in the oocyte membrane (12,28). It is then possible to record electrophysiological responses mediated by these neurotransmitter receptors after they have been transplanted from the brain to the more amenable oocyte.

## RESULTS

### Responses mediated by intrinsic and second-messenger systems in the oocyte

The ability to transplant foreign receptors to the oocyte facilitates a comparison in the same cell of responses involving intrinsic receptor-channel complexes (eg. the nAChR), and those involving second-messenger linked pathways.

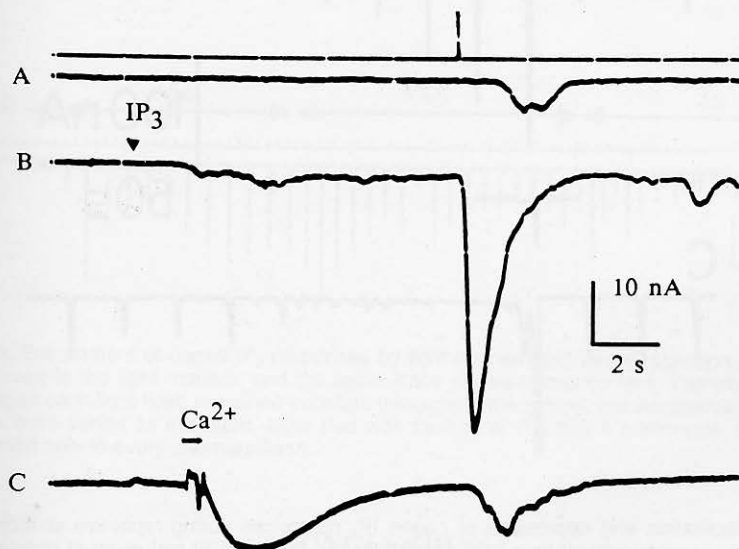
This is illustrated in Fig. 1. The upper trace (A) shows the membrane current evoked in a voltage-clamped oocyte by local ionophoretic application of ACh.

Native muscarinic receptors were present in this oocyte, and they mediated a response which began after a delay of several seconds, and showed a

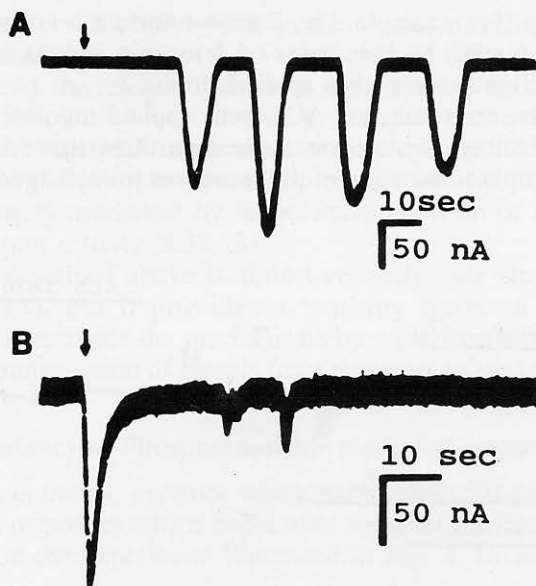
### Interactions between intracellular $IP_3$ or calcium and caged $IP_3$ response

The threshold and facilitation described above might occur if a threshold level of  $IP_3$  is needed to evoke calcium liberation from intracellular stores, or if a threshold level of calcium is needed to activate the membrane chloride conductance. To discriminate between these possibilities, we looked for interactions between responses to photolysis of caged  $IP_3$  and responses evoked by injection free  $IP_3$  or calcium into the oocyte (32,35). Fig. 8 illustrates results from an experiment where either  $IP_3$  or calcium were injected from a single micropipette into an oocyte previously loaded with caged  $IP_3$ . Because  $IP_3$  and calcium bear opposite electrical charges, either substance could be ejected from the pipette by applying, respectively, pipette negative or positive ionophoretic current.

Intracellular application of a low dose of  $IP_3$  evoked a small oscillatory membrane current, and the current evoked by a light flash was greatly potentiated when given during this response (Fig. 8B). Different to this, injection of calcium evoked a smooth current response, and no facilitation of the caged  $IP_3$  response was observed with a light flash given shortly after (Fig. 8C). In fact, the caged  $IP_3$  response was often depressed (Mean = 54% of control, 4 trials).



**Fig. 8.** Facilitation of the caged  $IP_3$  response by intracellular injection of  $IP_3$ , but not by injection of calcium. The three records show membrane currents elicited by an identical light flash given at the time indicated by the marker in the top trace. **A**, the light flash was applied alone. **B**, the light flash was preceded by an ionophoretic injection of  $IP_3$  into the oocyte, made at the time indicated by the arrowhead. **C**, the light flash was preceded by an injection of calcium, at the time indicated by the bar. The light was focused as a 200  $\mu m$  spot on the vegetal hemisphere, centered on the tip of the ionophoretic pipette used to inject  $IP_3$  and calcium.

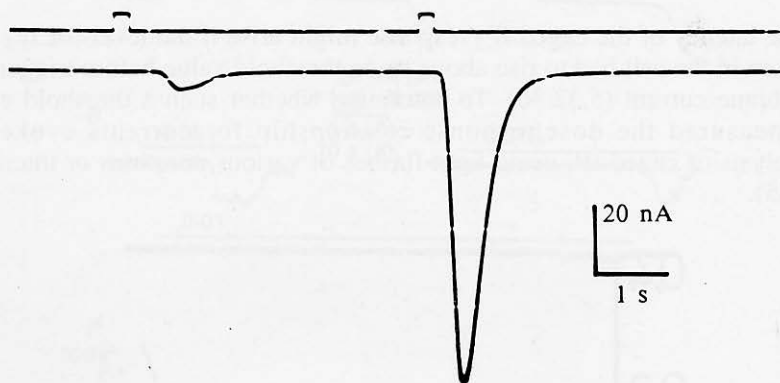


**Fig. 1.** Muscarinic and nicotinic response to ACh applied ionophoretically to *Xenopus* oocytes. Each record shows the change in clamp current elicited by an ionophoretic pulse of ACh of 1 s duration, given at the times indicated by the arrows. In this, and all other figures, downward deflections correspond to inward membrane currents. **A**, muscarinic response to ACh in a native (non mRNA-injected) oocyte. Clamp potential was -60 mV. **B**, predominantly nicotinic response to ACh in an oocyte injected with mRNA from denervated cat muscle. Clamp potential was -100 mV. A small muscarinic component is also present 20-30 s after the ACh pulse. (From ref 27).

prolonged oscillatory nature. In contrast, a very different response was obtained by application of ACh to a different oocyte, which had been induced to express nicotinic receptors by injecting it with mRNA from denervated cat muscle (27). The current mediated by nicotinic receptors increased almost immediately following ACh application, and showed a simple rise and fall (Fig. 1B), like that seen when ACh is applied to muscle endplates.

In addition to the native muscarinic receptors, several types of receptor expressed in the oocyte by brain mRNA (eg. receptors to serotonin and glutamate) also give rise to oscillatory membrane currents like those elicited by ACh (11,12). Although these receptors can be distinguished pharmacologically (12), and are encoded by different mRNAs (38), it is likely that they all link in to a common second messenger pathway to cause the generation of the membrane current (30,36,40). The ability to express a variety of receptors allows a further property of the second messenger pathway to be demonstrated; that of facilitation between responses to

Brief (less than about 1 s) light flashes evoked only a single 'spike' of current (eg. Fig. 7), rather than a prolonged oscillatory response. Measurements of the peak amplitude of the current are shown in Fig. 6, where an oocyte was illuminated by flashes of fixed intensity and varying duration. A striking result was that no response could be detected when the



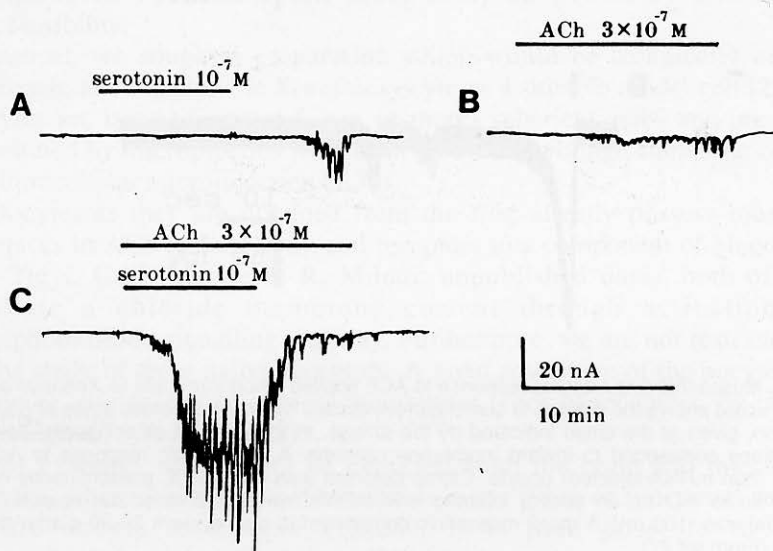
**Fig. 7.** Facilitation of the caged  $IP_3$  response with paired light flashes. Upper trace monitors the intensity and timing of the photolysis light flashes. Lower trace shows clamp current recorded at a clamp potential of  $-60 \mu V$ . The photolysis light was focused as a spot of about  $200 \mu m$  diameter positioned on the vegetal hemisphere of the oocyte.

flash duration was shorter than a certain value, but that the current grew sharply when the duration was increased above the threshold. Results like that illustrated were obtained in many other experiments, in which either the flash duration or intensity were altered.

Associated with this threshold phenomenon was a marked facilitation in responses evoked by paired light flashes (35). For example, in Fig. 7 a light flash which by itself was just supra-threshold, greatly potentiated the response to an identical flash repeated after an interval of 3 s. Potentiation was still evident even when the conditioning flash was reduced below threshold, and the facilitatory effect of a conditioning flash could be detected after intervals as long as one minute.

different agonists. For example, Fig. 2 shows currents evoked in a rat brain mRNA-injected oocyte by low doses of serotonin and ACh. Each agonist evoked only small responses when applied alone.

However, when serotonin and ACh were applied together a much larger response was obtained, which was more than three times larger than that expected by a simple summation of the responses to each agonist alone.



**Fig. 2.** Facilitation between responses to low doses of ACh and serotonin. Traces show clamp currents recorded at a potential of -60 mV in an oocyte injected with brain mRNA. Drugs were applied in the bathing fluid at the concentrations indicated and for the times marked by the bars. (From ref. 36).

### Phosphoinositide signalling in the oocyte

Because of the long delay of the muscarinic response in the oocyte, Kusano et al. (31,32) postulated that the current evoked by ACh does not arise because the agonist directly opens membrane channels, but instead arises through the mediation of a substance that has to accumulate inside the oocyte. Subsequently, it has become clear that the responses are generated through an intracellular messenger pathway involving inositol phosphates and calcium (4,6,25,31,33,34,40), similar to that described in many other cell types (for reviews see refs. 2,3,10). Briefly, this cascade is thought to be initiated when the binding of agonist to receptors activates a class of G-proteins coupled to the enzyme phospholipase C. This enzyme hydrolyses

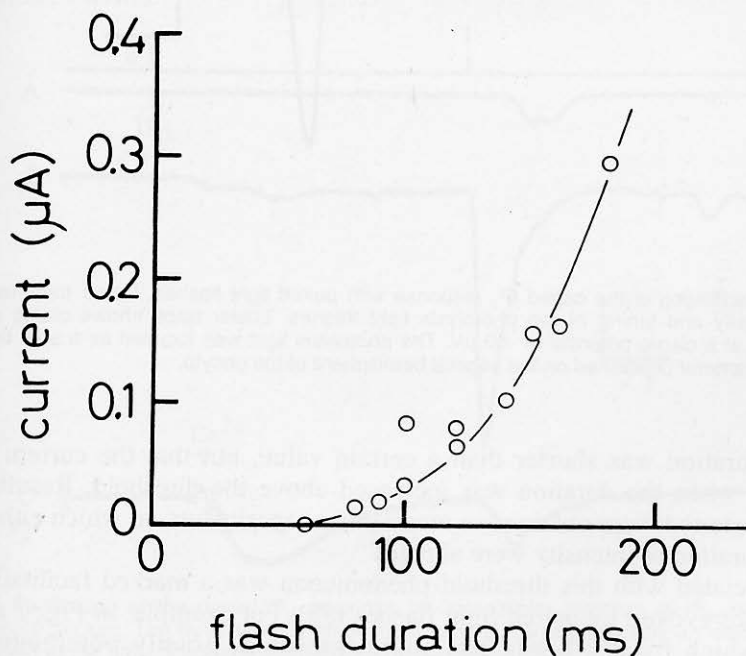


that the latency to onset of the current became shorter when more caged  $\text{IP}_3$  was loaded into the oocyte and when the intensity of the photolysis light was increased (26). For example, in the oocyte illustrated in Fig. 5 the current began after about 150 ms at the highest light intensity, and after about 75 s at the lowest intensity.

This experiment demonstrates, therefore, that very long latencies may arise when there is a slow formation of  $\text{IP}_3$ , as would be expected when low doses of agonist are applied to the oocyte.

### Threshold and facilitation of caged $\text{IP}_3$ responses

The latency of the caged  $\text{IP}_3$  response might arise if the levels of  $\text{IP}_3$  or of calcium in the cell had to rise above some threshold value before triggering a membrane current (5,32,36). To determine whether such a threshold exists, we measured the dose/response relationship for currents evoked by photolysis of caged  $\text{IP}_3$  using light flashes of various durations or intensities (32,35).



**Fig. 6.** Variation in peak size of the caged  $\text{IP}_3$  response with different durations of light flash of constant intensity. Records were obtained from an oocyte loaded with about 4 pmol of caged  $\text{IP}_3$ . The light was focused as a spot of about  $200\mu\text{m}$  diameter, positioned on the vegetal hemisphere of the oocyte and close to the equator.

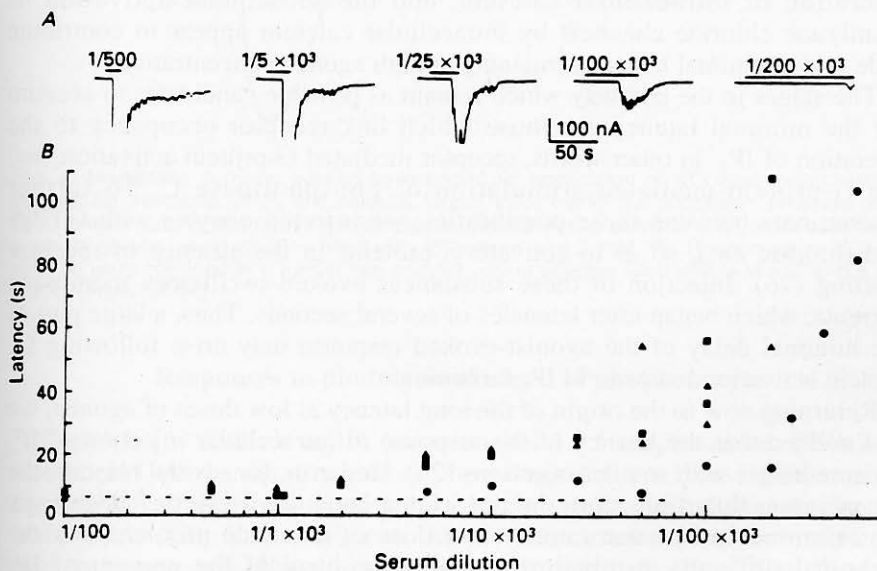


phosphatidylinositol 4,5-bisphosphate in the plasma membrane, forming the intracellular messengers inositol 1,4,5-trisphosphate ( $IP_3$ ) and diacylglycerol (DAG).  $IP_3$  causes the release of calcium from intracellular stores which, in turn, activates calcium-gated chloride membrane channels in the oocyte membrane to give the final membrane current response. The role of DAG in the generation of the chloride current is presently unclear, since responses to agonists are closely mimicked by intracellular injection of  $IP_3$  alone, in the absence of receptor activity (9,31,33).

The scheme described above is almost certainly over simplified (see, for example ref. 15), but it provides a working basis on which to base experiments to investigate the mechanisms by which the messenger pathway influences the transmission of signals from receptors to ion channels.

### Latency of Phosphoinositide mediated response

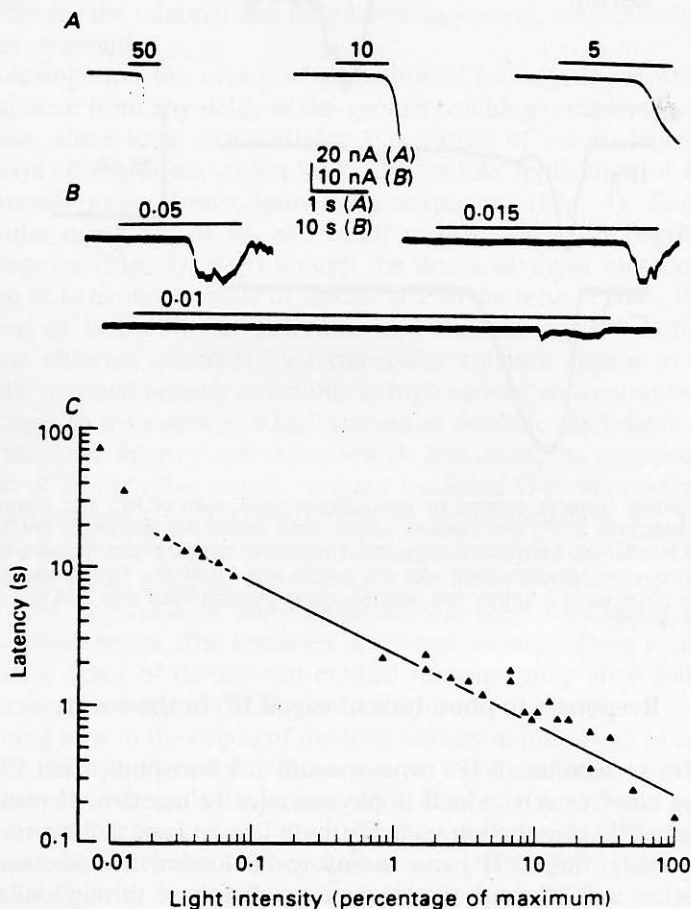
As already mentioned, agonists which act through the phosphoinositide pathway evoke responses which begin after long delays. This was examined in more detail in the experiment illustrated in Fig. 3. Different dilutions of



**Fig. 3.** Latencies of responses evoked by bath application of various dilutions of serum to *Xenopus* oocytes. **A**, sample traces showing currents in response to serum applications given as marked by the bars. Times of application were monitored by including a fluorescent marker dye in the solution, to avoid errors due to dead space in the perfusion system. Currents at high serum doses are off-scale. **B**, variation in latency to onset of the current response with increasing dilution of serum. Data are from three oocytes. The dashed line indicates the minimal latency. (From ref. 26).

over the illuminated region of the cell, (ii) the intensity and duration of the photolysis light can be varied to give a precise control of the amount of  $\text{IP}_3$  liberated and (iii) because only a small fraction of the caged  $\text{IP}_3$  is consumed during each flash, many reproducible responses can be recorded from each oocyte.

Prolonged irradiation of oocytes loaded with caged  $\text{IP}_3$  evoked oscillatory membrane currents, resembling those evoked by bath application of serum and other agonists (compare Fig. 5A,B and Fig. 3). A striking feature was



**Fig. 5.** Latencies of currents evoked by photolysis of caged  $\text{IP}_3$ . **A** and **B**, traces show currents evoked by exposure to light of various intensities (indicated next to each trace as a percentage of the maximum). The light was extinguished shortly after the onset of the membrane current, and the bars indicate the durations of light exposure. The oocyte was loaded with about 10 pmol caged  $\text{IP}_3$ , and the clamp potential was -60 mV. **C**, double-logarithmic plot showing the relation between intensity of photolysis light and the latency to onset of the membrane current (From ref. 26).

serum were bath applied to a voltage-clamped oocyte, and the latencies to onset of the membrane current were measured after correcting for dead time in the perfusion system (26). Low doses of serum, close to the threshold to elicit any response, gave currents beginning after delays of a minute or more. As the dose was increased, the currents grew larger and began after shorter latencies. However, although the latency varied steeply with dose at low doses of serum, at higher concentrations it approached a minimal value of about 8 s (Fig. 3B; and see also refs. 5 23,41).

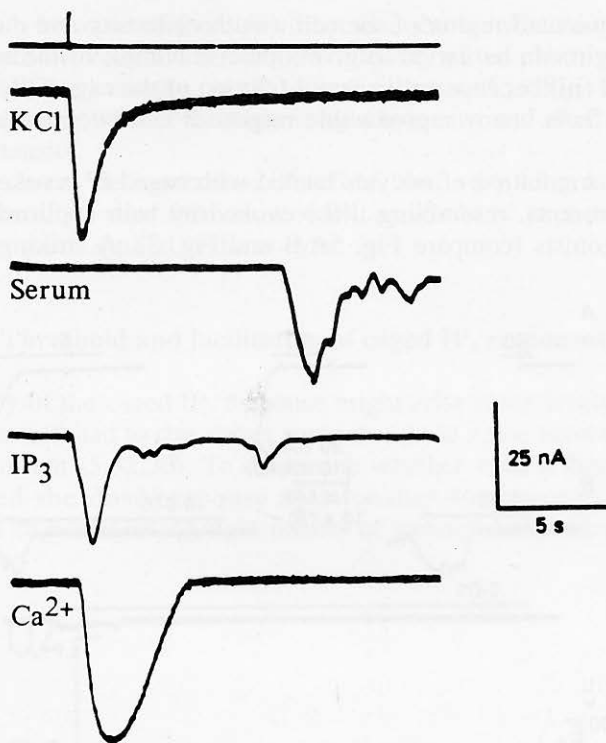
These observations suggested that two different mechanisms might be responsible for the minimal and long latencies seen at, respectively, high and low doses of serum.

Considering first the origin of the minimal latency, it seemed that this could not arise from any delay in the agonist reaching receptors in the oocyte membrane, since local extracellular application of serum from a 'puffer' pipette evoked responses with a long delay, while application of KCl in the same manner gave almost immediate responses (Fig. 4). Furthermore, intracellular injections of  $IP_3$  and calcium gave responses beginning after short latencies (Fig. 4), even though the doses of these messengers were chosen so as to evoke currents of similar size to the serum. Thus,  $IP_3$ -induced liberation of intracellular calcium, and the subsequent activation of membrane chloride channels by intracellular calcium appear to contribute little to 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  $IP_3$ : in other words, receptor mediated G-protein activation, and the G-protein-mediated stimulation of phospholipase C. To further discriminate between these possibilities, we injected oocytes with GTP $\gamma$ S and fluoride ions, so as to activate G-proteins in the absence of receptor binding (26). Injection of these substances evoked oscillatory membrane currents, which began after latencies of several seconds. Thus, a large part of the minimal delay of the agonist-evoked response may arise following G-protein activation but prior to  $IP_3$  formation.

Returning now to the origin of the long latency at low doses of agonist, we had noticed that the latency of the response to intracellular injection of  $IP_3$  became longer with smaller injections (26). However, for several reasons, the use of intracellular injections did not seem a good way to further investigate the relationship between latency and dose of  $IP_3$ . One problem was the technical difficulty in obtaining accurate control of the amount of  $IP_3$  injected.

Secondly, injection of  $IP_3$  through a micropipette is expected to result in a point source of high concentration around the pipette tip, unlike the more diffuse formation of  $IP_3$  near the inner surface of the membrane which presumably occurs following bath application of agonists. To better study this problem we therefore employed a novel technique involving caged  $IP_3$ .



**Fig. 4.** Membrane currents evoked by extracellular application of KCl and serum, and by intracellular injections of  $\text{IP}_3$  and calcium. Upper trace marks the pneumatic pressure pulse used to eject substances from the micropipette. Responses to serum and  $\text{IP}_3$  were obtained in a single oocyte, using pipettes filled with 4% serum and 2 mM  $\text{IP}_3$ . Responses to KCl and calcium were obtained in a further two oocytes, using pipettes filled with 3 M KCl and 5 mM  $\text{CaCl}_2$ . (From ref. 26).

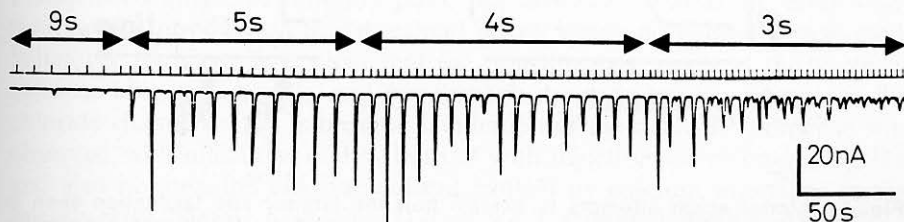
### Responses to photolysis of caged $\text{IP}_3$ in the oocyte

Caged  $\text{IP}_3$  is an ester of  $\text{IP}_3$  (myo-inositol 1,4,5-trisphosphate,  $\text{P}^{4(5)}\text{-1-(2-nitrophenyl ethyl ester)}$ ), which is physiologically inactive. However, it is photolabile, so that irradiation with UV light can be used to liberate free  $\text{IP}_3$  (refs. 13,18,42). Caged  $\text{IP}_3$  can therefore be loaded into the oocyte by microinjection and allowed, in the dark, to distribute throughout the cell. Appropriate illumination is then used to photolyse a small fraction of the caged  $\text{IP}_3$ , causing the liberation of free  $\text{IP}_3$  and the subsequent generation of a chloride membrane current (32, 35). Because the animal hemisphere of the oocyte is pigmented, all experiments described here were done by illuminating the vegetal (non-pigmented) hemisphere.

Important advantages of this technique are; (i) the  $\text{IP}_3$  is liberated diffusely

After the response had attained a stable level, a single flash of longer (300 ms) duration was interposed. This elicited a large current, and responses to subsequent test stimuli were facilitated for about 1 min afterwards. However, when a still longer (3 s) conditioning stimulus was interposed, this depressed, rather than facilitated, responses to subsequent test pulses. The reason for this depression is not yet clear, but it may arise through the inhibition by calcium of the  $IP_3$  response as mentioned previously.

Another interesting phenomenon which occurred during stimulation with repetitive light flashes is shown in Fig. 10. With the light flash intensity and duration used in this experiment, stimulation at intervals of 9 s or longer failed to evoke any appreciable response. Currents were, however, obtained when the interval was shortened to 5 s but, surprisingly, they occurred only with every alternate light flash. This phenomenon was still present when the interval was shortened to 4 s, but a further reduction to 3 s gave only small and irregular currents. A 'frequency division' effect like that illustrated was seen in several other oocytes and in all cases it was critically dependent upon the intensity and duration of the light flash.



**Fig. 10.** Entrainment of caged  $IP_3$  responses by light flashes at different repetition rates. The upper trace is the light monitor, and the lower trace shows clamp current. The intensity and duration of each light flash remained constant throughout the record, but the intervals between flashes were varied as indicated. Note that with flashes at 5 s and 4 s intervals, the oocyte responded only to every alternate flash.

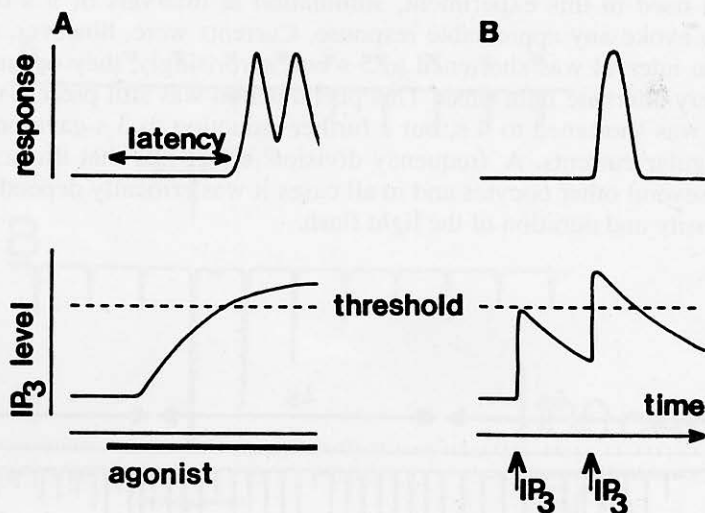
## CONCLUSIONS

### Threshold and facilitation in $IP_3$ action

The major finding described here is the existence of an apparent threshold in the level of intracellular  $IP_3$  needed to trigger release of intracellular calcium. Observations of an almost 'all or nothing' dose/response relationship for activation by low doses of agonists (11,36), and by

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intracellular  $IP_3$  and  $IP_4$  (ref. 34) had previously indicated that the phosphoinositide signalling pathway in the oocyte operates in a highly non-linear manner. The use of caged  $IP_3$  provided a more precise approach to study these phenomena and facilitated the identification of the stage in the signalling pathway responsible for the non-linearity. As illustrated diagrammatically in Fig. 11, the finding of a threshold in the phosphoinositide pathway may provide an explanation for several of its functional characteristics.



**Fig. 11.** Model which attempts to explain how the latency and facilitation seen in phosphoinositide-mediated responses may arise from the existence of a threshold level of  $IP_3$  necessary to trigger calcium release. See text for explanations.

Firstly, the threshold is expected to introduce a variable latency to onset of membrane responses elicited by agonist activation (Fig. 11A). For simplicity, we presume that  $IP_3$  is formed at a steady rate, while its subsequent metabolism proceeds at a rate proportional to its intracellular concentration. Thus, when the agonist is applied, 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  $IP_3$  level rises above the threshold, and will thus become shorter at higher agonist doses, since  $IP_3$  will be formed more rapidly (5,26). In addition to this dose-dependent latency, there is also a fixed minimal latency, which arises from the earlier stages in the signalling pathway linking receptor occupancy to formation of  $IP_3$ .

A second feature of the phosphoinositide response is the facilitation seen



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with paired stimuli. A possible basis for this is illustrated in Fig. 11B for an experiment where  $IP_3$  is liberated from caged  $IP_3$  by paired light flashes (cf. Fig. 7), but the same idea would apply also to the activation of an oocyte by simultaneous application of low doses of two agonists (cf. Fig. 2). The first stimulus raises the intracellular level of  $IP_3$  but, because this does not exceed the threshold, no response is seen. However, the  $IP_3$  produced by a second stimulus summates with that remaining from the first and, because the threshold is now exceeded, evokes a response. In this model the decay of facilitation with increasing pulse interval should reflect the decline in intracellular  $IP_3$ , and measurements in the oocyte indicate that this occurs over several tens of seconds (35).

Measurements of  $IP_3$ -induced calcium release in permeabilized cells indicate that the rate of calcium liberation varies as about the third power of  $IP_3$  concentration (24). The currents evoked by photolysis of caged  $IP_3$  in the oocyte also follow a similar relation, and double-logarithmic plots of the current versus light flash duration or intensity give a limiting slope of about 3.7 (ref. 35). Thus, the opening of the intracellular release channel by  $IP_3$  may require the cooperative binding of at least three or four molecules of  $IP_3$ . This steep power function for  $IP_3$  action might account for the apparent threshold, because currents would reduce very sharply with reducing stimulus strength and could become too small to be detected in voltage-clamp recordings. It remains possible, however, that some additional mechanism introduces a true threshold below which no current is activated. Whatever the case, it is clear that the non-linearity arises at the stage of calcium liberation by  $IP_3$ , and not through the calcium-dependence of the chloride channels. This is because facilitation of the caged  $IP_3$  response was observed with injections of  $IP_3$ , but not with injections of calcium (Fig. 8); and also because the chloride current evoked by calcium injections varies linearly with the dose of calcium (I. Ivorra & I. Parker, unpublished data).

### Relevance to synaptic functioning

The mechanisms of phosphoinositide signalling in neurons are probably very similar to those in the oocyte (2,3), and this view is strengthened by the ability of brain neurotransmitter receptors, transplanted to the oocyte by injection of brain mRNA, to link in to the oocyte's endogenous signalling pathway (30, 36, 40).

Thus, it is interesting to speculate how the characteristics of the phosphoinositide pathway described here might influence the transmission of information at 'slow' synapses in the brain which utilize phosphoinositide signalling in the postsynaptic cells.

Perhaps the most important point is that the finding of a threshold for  $IP_3$  action suggests the basis for a novel form of synaptic integration, where

summation in the post-synaptic cell arises from a summation of  $IP_3$  levels.

Because of its 'chemical' basis, this integration is expected to show properties very different to the classical interaction of electrical signals across the post-synaptic membrane. The summation of  $IP_3$  signals will be spatially restricted since it depends upon the diffusion of an intracellular messenger, while electrical signals will spread more widely, limited only by the membrane space constant. Conversely, phosphoinositide signalling is likely to show a long lasting temporal facilitation, dependent upon the metabolism of  $IP_3$ , whereas the decay of postsynaptic potentials is set by the more rapid electrical time constant of the membrane. Finally, the inherent oscillatory nature of the  $IP_3$ -mediated response may be important for the generation and transmission of rhythmic activity in neurons (5,35). A nice example is illustrated by the oocyte in Fig. 10. If a neuron were to behave in a similar way, it would follow rhythmic input signals over only a narrow frequency range and, by means of chemical not electrical mechanisms, respond at a rate one half that of the input signal.

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