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EFFECTS OF ALCOHOLS ON RESPONSES EVOKED BY INOSITOL TRISPHOSPHATE IN *XENOPUS* OOCYTES

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SUMMARY

1. The effects of ethanol and other alcohols on inositol 1,4,5-trisphosphate ($InsP_3$) signalling were studied in *Xenopus* oocytes by the use of flash photolysis of caged $InsP_3$. Calcium liberation induced by $InsP_3$ was monitored by voltage-clamp recording of Ca^{2+}-activated membrane currents, and by fluorescence of the Ca^{2+} indicator Fluo-3.

2. Membrane current and fluorescence Ca^{2+} signals evoked by light flashes giving small responses were initially potentiated by bath application of ethanol (80–400 mM). However, the responses subsequently declined while ethanol was present and were strongly reduced or suppressed when it was removed.

3. These effects did not arise artifactually from changes in photolysis of caged $InsP_3$, as similar results were seen with responses evoked by intracellular injections of $InsP_3$. Also, the effects on the membrane current did not arise primarily through actions on the Ca^{2+}-dependent Cl^- channels, since currents evoked by intracellular injections of Ca^{2+} were little changed by ethanol

4. Ethanol reduced the threshold level of $InsP_3$ required to cause Ca^{2+} liberation. Thus, potentiation was most prominent with small responses evoked by brief light flashes, whereas the predominant effect on larger responses was inhibitory.

5. The facilitatory and inhibitory actions of ethanol persisted after removing extracellular Ca^{2+}.

6. Intracellular injections of ethanol produced an initial inhibition of $InsP_3$ responses, followed, in some oocytes, by a potentiation.

7. Methanol had little effect on $InsP_3$ responses, whereas butanol and other long-chain alcohols produced strong inhibition, but little or no potentiation.

8. We conclude that extracellular application of ethanol produces a rapid potentiation of $InsP_3$-mediated Ca^{2+} liberation, and a more slowly developing inhibition. The potentiation may arise through stimulation of $InsP_3$ formation at the plasma mebrane, whereas the inhibition occurs more deeply in the cell. Both actions were evident at relatively low concentrations (a few tens of millimoles per litre), and might thus be important in the behavioural effects of ethanol intoxication.

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INTRODUCTION

Inositol 1,4,5-trisphosphate ($InsP_3$) functions as an intracellular messenger molecule in virtually every cell in the body, where it acts by liberating Ca^{2+} from intracellular stores (for recent reviews see: Berridge & Irvine, 1989; Rana & Hokin, 1990). Because of this ubiquitous role, drugs acting on the intracellular $InsP_3$ receptor and other parts of the messenger pathway are expected to have widespread and varying effects on cellular function. We were thus motivated to look for pharmacological agents affecting the $InsP_3$ system. For this purpose, oocytes of *Xenopus laevis* offer a convenient experimental system, since they possess a messenger pathway whereby $InsP_3$-mediated Ca^{2+} liberation leads to the generation of a Cl^- membrane current (Miledi & Parker, 1984; Oron, Dascal, Nadler & Lupu, 1985; Parker & Miledi, 1986), and their large size greatly facilitates procedures including voltage-clamp recording and intracellular microinjection. Furthermore, flash photolysis of caged $InsP_3$ (McCray & Trentham, 1989) provides an elegant means by which to evoke repetitive, reproducible elevations of cytosolic $InsP_3$ concentration (Parker & Miledi, 1989). We had previously used these techniques to examine the influence of caffeine on $InsP_3$-evoked responses (Parker & Ivorra, 1991), and now use similar approaches to study the actions of alcohols.

The work presented here stems from an observation, made several years ago, that bath application of ethanol to oocytes could generate oscillatory currents (R. Miledi, unpublished data). This effect was seen also by Watford, Dunwiddie & Harris (1989), who further reported that oscillatory currents were induced by injection of ethanol into oocytes. These currents are carried by Cl^- ions (Wafford *et al.* 1989) and closely resemble those evoked by intracellular injections of $InsP_3$, suggesting that they arise through intracellular Ca^{2+} mobilization. However, high concentrations (several hundred millimoles per litre) of ethanol are required to evoke appreciable oscillatory currents (Wafford *et al.* 1989; the present paper), thus raising doubts as to whether actions on the $InsP_3$–Ca^{2+} signalling pathway could be important in the behavioural effects of ethanol that become apparent at concentrations of a few tens of millimoles per litre. Nevertheless, we surmised that ethanol might modulate $InsP_3$-mediated signalling at concentrations lower than those which directly evoked responses, and looked for such effects on responses evoked by photorelease of $InsP_3$ in the oocyte. We describe here how ethanol (40–400 mM) causes rapid potentiation of $InsP_3$-evoked responses, together with a slowly developing depression. Because of this latter phenomenon, strong effects on $InsP_3$ signalling are apparent at concentrations of ethanol well below that required to directly evoke responses.

METHODS

Procedures for obtaining oocytes of *Xenopus laevis* for voltage-clamp recording and for light flash photolysis of caged $InsP_3$ were as previously described (Parker & Miledi, 1989; Parker 1991). Briefly, defolliculated oocytes were voltage clamped at a potential of -60 or -50 mV, and membrane currents were recorded while the oocytes were continuously superfused with frog Ringer solution at room temperature. Normal Ringer solution had the composition (in mM): NaCl, 120; KCl, 2; $CaCl_2$, 1·8; HEPES, 5; at about pH 7·0. Calcium-free Ringer was made by omitting $CaCl_2$ and adding 1 mM-EGTA and 5 mM-$MgCl_2$. In most experiments, oocytes were each loaded with 1–10 pmol of caged $InsP_3$ (*myo*-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester), and

were stimulated by brief flashes of near UV light to cause intracellular release of free $InsP_3$. The photolysis light was normally arranged as a square with sides of about 100 μm, positioned on the vegetal hemisphere close to the equator. Bath applications of alcohols were accomplished by switching the intake of the superfusion system. The bars marked on the figures indicate the time at which the solution was changed, and do not correct for the dead time of 20–60 s (depending on flow rate) of the perfusion system. Solutions of alcohols were prepared by volumetric dilution, and the resulting molar concentrations calculated from their densities at room temperature. As a guide, a dilution of 0·1 % ethanol corresponds to about 16 mM. Intracellular injections were made by applying pneumatic pressure pulses to glass micropipettes broken to a tip diameter of a few micrometres. $InsP_3$, caged $InsP_3$ and ethanol were injected at respective concentrations of 100 μM, 1 mM and 1 M, in aqueous solutions including 50 μM-EDTA and 5 mM-HEPES at pH 7·0. Recordings of intracellular Ca^{2+} transients were made as described previously (Parker & Ivorra, 1990*a*, *b*; Parker, 1991), by use of the fluorescent indicator Fluo-3 (Minta, Kao & Tsien, 1989). $InsP_3$ and caged $InsP_3$ were obtained from Calbiochem (La Jolla, CA, USA), Fluo-3 from Molecular Probes Inc. (Eugene, OR, USA) and alcohols were obtained from Sigma Chemical Co. (St Louis, MO, USA).

RESULTS

Ethanol potentiates and inhibits caged $InsP_3$ responses

Figure 1 illustrates the basic phenomena with which this paper is concerned. An oocyte that had previously been loaded with caged $InsP_3$ was stimulated by a train of ultra-violet (UV) light flashes at 30 s intervals, so as to evoke a series of transient membrane current responses. These currents arose because the free $InsP_3$ that was formed by photolysis caused liberation of Ca^{2+} from intracellular stores and that, in turn, activated Ca^{2+}-dependent Cl^- membrane currents (Parker & Miledi, 1989). Furthermore, because each oocyte was loaded with a large excess of caged $InsP_3$, it was possible to evoke hundreds of responses without depleting the reserve. The flash duration in this experiment was set slightly above the threshold necessary to evoke any response, so that a series of small and roughly constant currents were obtained, and ethanol was bath applied at a concentration of 400 mM for the time indicated by the bar. Initially, the light flash responses were greatly potentiated, but during continued application of ethanol they declined progressively. On washing out the ethanol, the currents were at first almost completely suppressed, but subsequently showed a partial recovery over several minutes.

Results like those illustrated were consistently observed in more than forty other oocytes examined although, as discussed later, the relative magnitude of the potentiation and inhibition varied with the concentration of ethanol and the duration of the stimulus light flash.

Facilitation and depression of responses to microinjected $InsP_3$

The phenomena illustrated in Fig. 1 were observed with responses generated by photorelease of $InsP_3$. Before progressing further to investigate their mechanisms, it was first necessary to exclude the possibility that the effects arose artifactually from actions of ethanol upon the photolysis of caged $InsP_3$. We thus examined the action of ethanol on responses evoked by microinjection of free $InsP_3$ into oocytes.

Figure 2*A* shows responses evoked by injection of a low dose of $InsP_3$. With the oocyte bathed in normal Ringer solution this gave only a single small spike of current. However, after addition of 450 mM-ethanol the same amount of $InsP_3$ produced a much larger and more prolonged response. Results obtained in ten similar

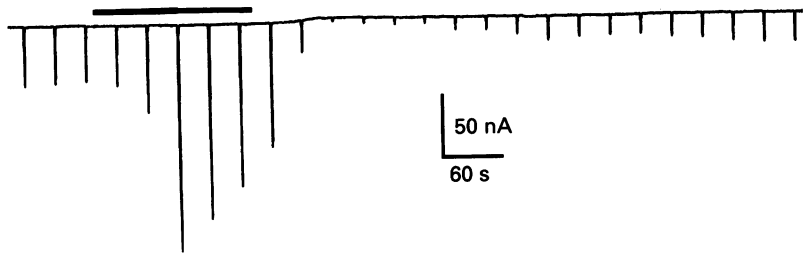

Fig. 1. Potentiation and depression of $InsP_3$-evoked currents by bath application of ethanol. In this and other figures, traces show membrane currents recorded at a clamp potential of −60 mV; downward deflections correspond to inward membrane currents. The oocyte was loaded with about 0·8 pmol caged $InsP_3$ and was stimulated by repetitive light flasher (duration 24 ms) at 30 s intervals. During the time marked by the bar, the superfusate was switched to a solution including 400 mM-ethanol.

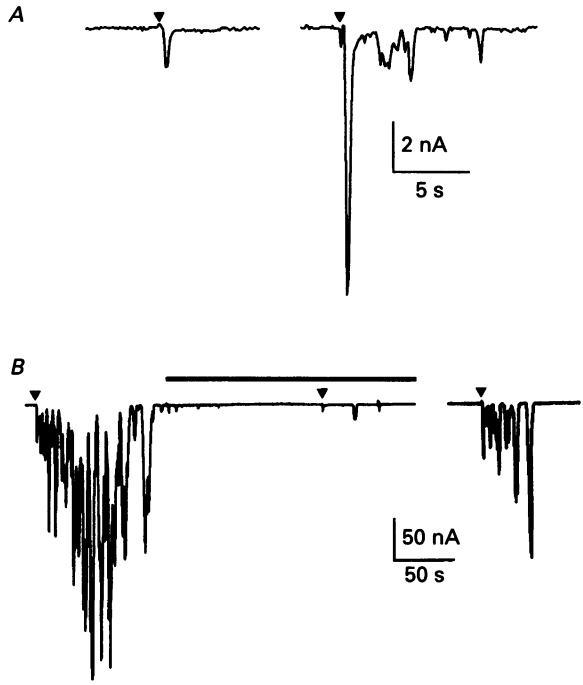

Fig. 2. *A*, potentiation of response to intracellular injections of low doses of $InsP_3$ by ethanol. Traces show successive responses to injections of about 0·2 fmol $InsP_3$, given at times indicated by the arrow-heads. The record on the left is a control response. That on the right was obtained a few minutes later, beginning about 70 s after bath applying 450 mM-ethanol. *B*, inhibition by 1-hexanol of responses evoked by greater amounts of $InsP_3$. Arrow-heads indicate when intracellular injections of roughly 40 fmol $InsP_3$ were made. The superfusion solution included 0·05 % 1-hexanol when indicated by the bar. The third injection was made 5 min after washing out hexanol. Records in *A* and *B* are from different oocytes.

experiments were quantified by measuring the charge displacements (i.e. area under the current traces) induced by $InsP_3$. The mean control response evoked by a near-threshold dose of $InsP_3$ was $3{\cdot}4 \pm 1{\cdot}1$ nC (± 1 S.E.M.), whereas corresponding injections

of $InsP_3$ gave a mean response of $15{\cdot}3 \pm 3{\cdot}8$ nC in the presence of ethanol (160–450 mM).

To investigate whether alcohol depressed – as well as facilitated – responses to injected $InsP_3$, larger doses of $InsP_3$ were used. Furthermore, we used hexanol in

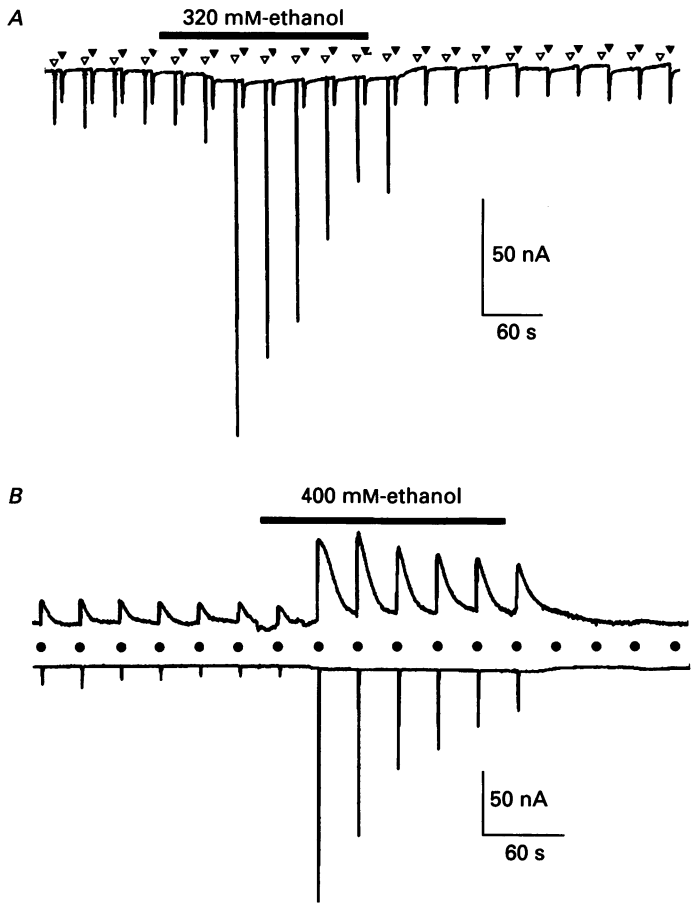

Fig. 3. The effects of ethanol arise primarily from actions on intracellular Ca^{2+} release, and not on the Ca^{2+}-activated chloride currents. *A*, trace shows membrane currents evoked by alternate stimulation by light flashes (open arrow-heads) and by intracellular injections of about 7 fmol Ca^{2+} (filled arrow-heads). Ethanol (320 mM) was bath applied when indicated by the bar. *B*, simultaneous records of intracellular Ca^{2+} transients and membrane currents evoked by repetitive light flashes (marked by ●). The upper trace monitors fluorescence of the Ca^{2+} indicator dye Fluo-3 previously loaded into the oocytes together with caged $InsP_3$. Upward deflections correspond to increasing fluorescence and increasing free Ca^{2+} concentration; the magnitude of the trace is uncalibrated. Ethanol (400 nmM) was bath applied during the time indicated by the bar. The unsteady baseline of the fluorescence following the solution change was the result of bubbles in the perfusion system.

place of ethanol because, as described later, the actions of the higher alcohols were almost entirely inhibitory and thus simplified interpretation of the results. The oocyte in Fig. 2*B* was stimulated by repeated injections of about 40 fmol $InsP_3$. In

control solution this evoked a large oscillatory current persisting for over 1 min. After addition of 0·05% 1-hexanol the response was greatly reduced and $InsP_3$ evoked only a single small spike of current, beginning after a latency of about 20 s. This inhibition was at least partly reversible, since a larger response was obtained to an injection of $InsP_3$ made 5 min after washing out the hexanol.

Intracellular Ca^{2+} liberation is facilitated and inhibited by ethanol

The $InsP_3$-evoked membrane currents arise because $InsP_3$ liberates Ca^{2+} from intracellular stores, and the resulting rise in cytoplasmic Ca^{2+} then activates Ca^{2+}-dependent chloride channels in the plasma membrane (Miledi & Parker, 1984; Parker & Miledi, 1989). The effects of ethanol might thus arise through actions on $InsP_3$-mediated Ca^{2+} liberation, or through actions on the Ca^{2+}-activated chloride channels. We used two approaches to discriminate between these possibilities.

In the first, ethanol was applied while currents were evoked alternately by light flashes (to release $InsP_3$) and by injections of Ca^{2+} (Fig. 3*A*). The light flash response was at first greatly (600%) facilitated by ethanol, but then declined during the application and was completely suppressed on washing. In contrast, the currents evoked by Ca^{2+} injection showed no facilitation. Instead they were reduced by about 15% while ethanol was present, but recovered rapidly to about the control level on washing. Results like those illustrated were obtained in six other experiments.

The second approach to determine whether ethanol affects $InsP_3$-mediated Ca^{2+} liberation utilized a fluorescent Ca^{2+} indicator dye (Minta *et al.* 1989) to more directly monitor intracellular free Ca^{2+} levels. Figure 3*B* illustrates an experiment in which Fluo-3 was used to monitor Ca^{2+} simultaneously with measurements of membrane currents. The oocyte was injected with a mixture of caged $InsP_3$ and Fluo-3 (Parker & Ivorra, 1990*a*), and repetitive flashes of UV light were used to evoke small current responses. Application of ethanol produced a large initial facilitation of the currents, but these subsequently declined during continued perfusion and were suppressed after washing. Similar to this, the fluorescent Ca^{2+} signals also showed a clear initial facilitation, with the responses subsequently declining during application of ethanol and being suppressed after washing. Some quantitative differences were, however, apparent between the fluorescence and current recording. Most notably, the maximal facilitation of the Fluo-3 signal was smaller (300%, as compared to 1900% for the current) and the decline during ethanol application was less (45% decline from peak response, compared to 84% for the current).

Eight trials in three oocytes gave similar results and, in all cases, the Ca^{2+} signal was potentiated during ethanol application and depressed after washing. Differences between the fluorescent and current signals were, however, sometimes more marked than in the record illustrated. For example, small fluorescent signals often remained after washing ethanol, even though the corresponding currents were undetectable.

Potentiation is most evident with small $InsP_3$ responses

Figure 4 shows the effects of various concentrations of ethanol on responses evoked by just-suprathreshold light flashes that evoked small (1–2 nA) currents, and on larger (about 100 nA) currents evoked by longer flashes. With the brief flashes, currents were potentiated during application of ethanol, and the extent of the

facilitation increased as the ethanol concentration was raised from 80 to 400 mM (Fig. 4*A*). Very different to this, the larger currents evoked by long flashes showed almost no facilitation at any concentration of ethanol tested (Fig. 4*B*). Instead, the currents were reduced by proportionally great extents with increasing concentrations

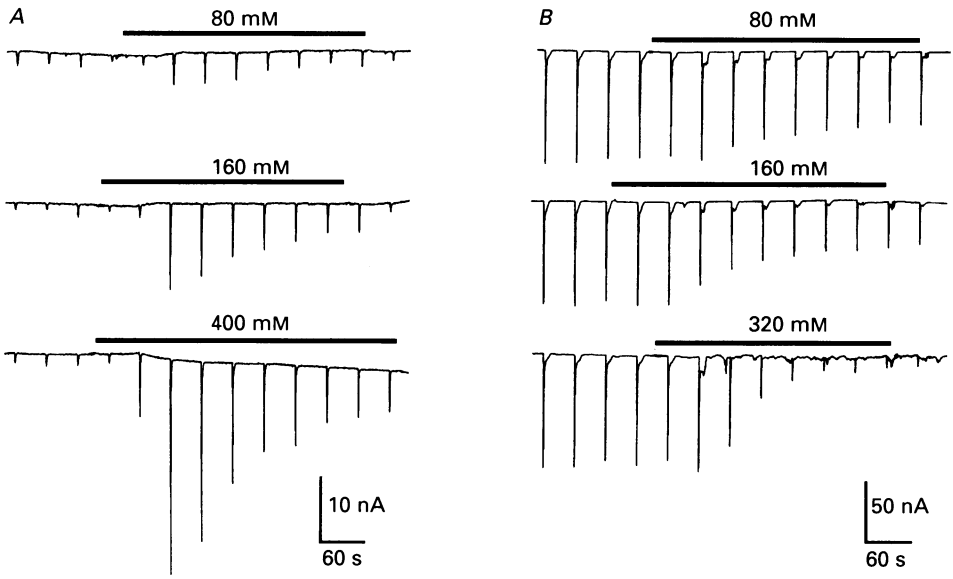

Fig. 4. Ethanol potentiates responses to brief light flashes but inhibits response to larger flashes. All traces were obtained from a single oocyte which was stimulated by repetitive light flashes at 40 s intervals. Records in *A* were obtained with flash durations (25–33 ms) that were just suprathreshold, whereas those in *B* were obtained with a flash duration of 220 ms. Ethanol was bath applied at the time and concentration indicated by the bars.

of ethanol. A dramatic comparison of the differing effects of ethanol on small and large $InsP_3$-evoked currents is provided by the recordings at high (400 or 320 mM) concentrations of ethanol. The small response was potentiated by 1800%, whereas the larger response was reduced to 10%.

Ethanol decreases the threshold amount of InsP$_3$ required to evoke a current

We have previously shown (Parker & Miledi, 1989; Parker & Ivorra, 1990*a*, *b*) that a certain threshold level of $InsP_3$ is required in the oocyte before Ca^{2+} begins to be released from intracellular stores. It seemed, therefore, that the greater facilitation seen with small $InsP_3$-evoked responses might arise if ethanol reduced the threshold for $InsP_3$ action. This idea was examined more fully by measuring currents evoked by flashes of various durations, so as to obtain dose–response curves for $InsP_3$ in the absence and presence of ethanol. Figure 5 shows the results from one oocyte. In normal Ringer solution, a threshold flash duration of about 20 ms was required to evoke any detectable response, and the current then grew as the flash duration was lengthened until it reached a maximum with flashes longer than about 70 ms. A difficulty arose in obtaining similar data in the presence of ethanol, because of the progressive decline of the response during maintained application. Measurements

were made, therefore, by obtaining control responses to flashes of a particular duration, and then measuring the peak size of currents evoked by the same flash shortly after adding ethanol. To minimize errors resulting from slow recovery from the depressant effect of ethanol, applications were kept short (1 min or less) and the

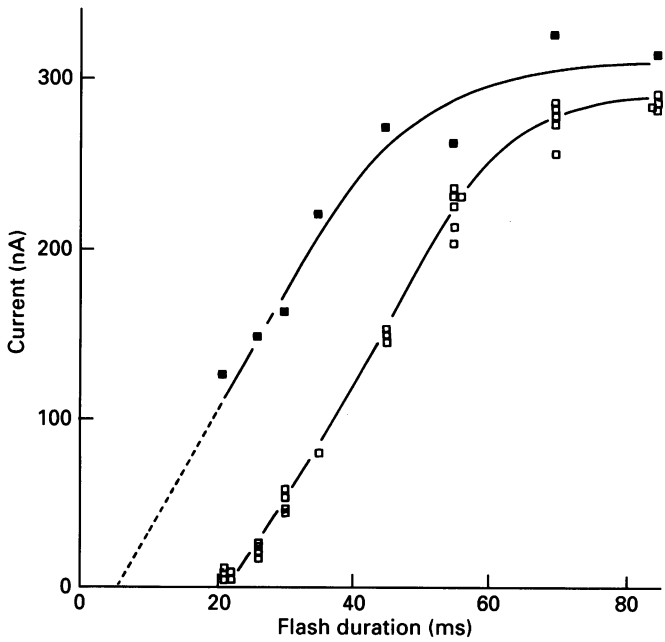

Fig. 5. Ethanol reduces the threshold amount of $InsP_3$ required to evoke a response. Points show measurements of peak currents evoked in a single oocyte to light flashes of various durations. These were obtained by repetitively stimulating the oocyte by flashes of a particular duration at 30 s intervals. □, control measurements; ■, the peak response obtained during bath application of 250 mM-ethanol.

oocyte was washed for about 10 min between trials. The filled symbols in Fig. 5 show measurements in the presence of 250 mM-ethanol, and indicate that the predominant effect was a leftward shift of the $InsP_3$ dose–response relationship. Maximal responses evoked by longer flashes were increased little, whereas responses evoked by brief flashes were greatly potentiated. Unfortunately, measurements were not made at flash durations shorter than 20 ms, but extrapolation of the curve indicated that the threshold flash duration would have been reduced to about 5 ms in the presence of ethanol, as compared to the control value of about 20 ms.

Dose dependence of ethanol actions

The facilitatory effects of different concentrations of ethanol were estimated in experiments like that in Fig. 1*A*, in which ethanol was bath applied during repetitive stimulation by light flashes with durations that were set to evoke small (*ca* 5 nA) control responses. Peak currents were then measured following ethanol applications, and are expressed in Fig. 6*A* as the percentage potentiation of the corresponding control values. At a concentration of 40 mM the responses were potentiated by an

average of about 70%, and potentiation increased progressively to about 1500% as the concentration of ethanol was raised to 400 mM. On double-logarithmic co-ordinates the data fitted to a line with a slope of about 1·5, indicating that the facilitation varied as a steeper than linear function of ethanol concentration.

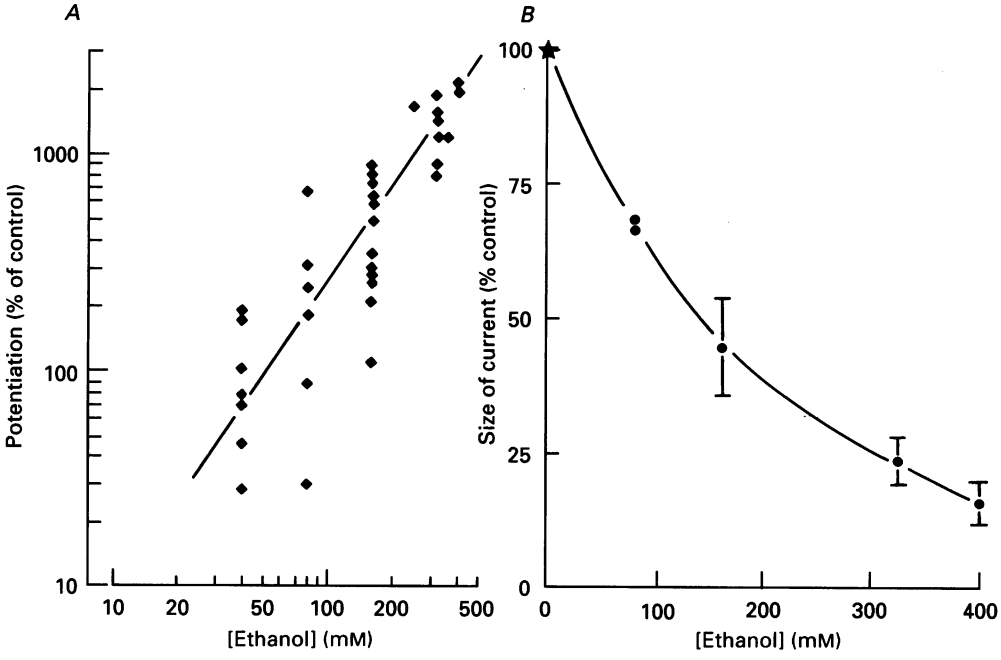

Fig. 6. Dose dependence of ethanol-induced facilitation and depression. *A*, dose dependence of facilitation, determined in fifteen oocytes that were stimulated by light flashes with durations set to give small (5–10 nA) control current responses. The points show the maximal facilitation induced by various concentrations of ethanol as a percentage of the control response. The line is drawn by eye. *B*, dose dependence of depression estimated using longer light flashes that evoked control currents of about 100 nA. Data are from four oocytes, and show the size of the currents after 3–4 min exposure to ethanol, expressed as a percentage of the control currents (★). Error bars indicate ±1 S.E.M. from four to eight trials, except for the points at 80 mM-ethanol which are individual measurements. The curve was drawn by eye.

To examine the dose dependence of the ethanol-induced depression, ethanol was applied during repetitive stimulation by light flashes that evoked relatively large (*ca* 100 nA) currents. Measurements were made of responses recorded 3–4 min after applying ethanol, at which time the currents had fallen to a roughly steady level. Figure 6*B* shows these data, scaled as a percentage of the corresponding control responses. Half-maximal inhibition occurred at a concentration of about 150 mM-ethanol, and the responses were reduced to about 20% by 400 mM-ethanol.

Effects of ethanol do not depend upon extracellular Ca^{2+}

Chloride current responses to photoreleased $InsP_3$ arise through liberation of intracellular Ca^{2+}, and do not require the presence of extracellular Ca^{2+} (Parker & Miledi, 1989). Nevertheless, it remained possible that the facilitatory or inhibitory

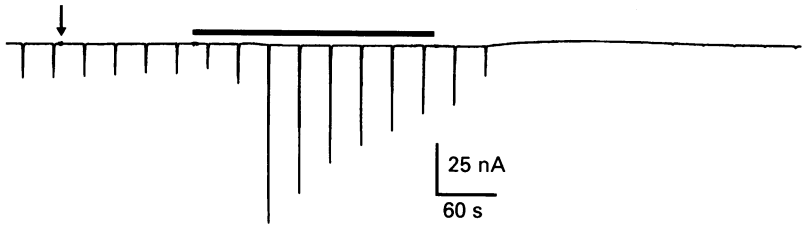

Fig. 7. Potentiation and depression do not depend upon extracellular Ca^{2+}. Trace shows currents evoked by repetitive light flashes, and 400 mM-ethanol was bath applied during period indicated by bar. The superfusate was switched from normal (1·8 mM-Ca^{2+}) Ringer solution to Ca^{2+}-free Ringer solution at time indicated by arrow.

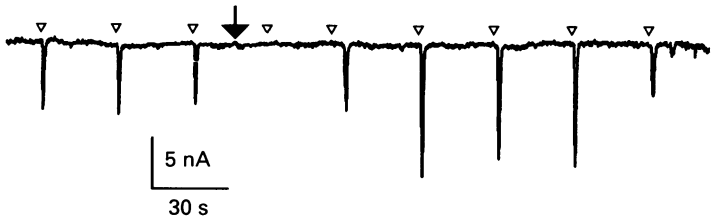

Fig. 8. Intracellular injection of ethanol first inhibits and then potentiates responses to $InsP_3$. The traces show currents evoked by repetitive light flashes at 30 s intervals, as marked by the arrow-heads. At the arrow, about 900 pmol ethanol was injected into the oocyte through a micropipette centred in the photolysis light spot.

effects of ethanol might require extracellular Ca^{2+}. To test this, we repeated the experiment of Fig. 1 in an oocyte bathed in a solution in which the free Ca^{2+} concentration was reduced to very low levels by chelation with EGTA. As can be seen in Fig. 7, ethanol gave strong facilitatory and inhibitory effects, like those in normal (1·8 mM-Ca^{2+}) Ringer solution. Recovery of the responses after washing out ethanol was less obvious in this experiment as compared to Fig. 1, proably as a result of the smaller size of the control response and longer duration of ethanol application, rather than because of the absence of external Ca^{2+}.

Intracellular injections of ethanol

To determine whether ethanol acts at intracellular sites, we injected ethanol into the oocyte whilst evoking repetitive light flash responses. The amounts of ethanol injected were smaller, by factors of 10–100, than the doses used by Wafford *et al.* (1989) and usually did not themselves evoke oscillatory current responses. Nevertheless, as illustrated in Fig. 8, responses to the light flashes were strongly depressed shortly after injection of ethanol but subsequently became facilitated – the reverse sequence of events to that seen following bath application of ethanol. In a total of eleven trials in three oocytes, injections of ethanol always caused a rapid depression of the $InsP_3$ responses. The subsequent facilitation was more variable, and was clearly seen in only four trials.

Because intracellular injections of Ca^{2+} also inhibit caged $InsP_3$ responses (Parker & Ivorra, 1990*a*), we were concerned that the depression seen with intracellular

ethanol might have arisen artifactually because of contaminating Ca^{2+} in the injection solution. This, however, was unlikely for several reasons. Firstly, care was taken to avoid contamination by Ca^{2+}. The fluid near the pipette tip (which becomes contaminated by Ca^{2+} from the bathing fluid) was expelled immediately before

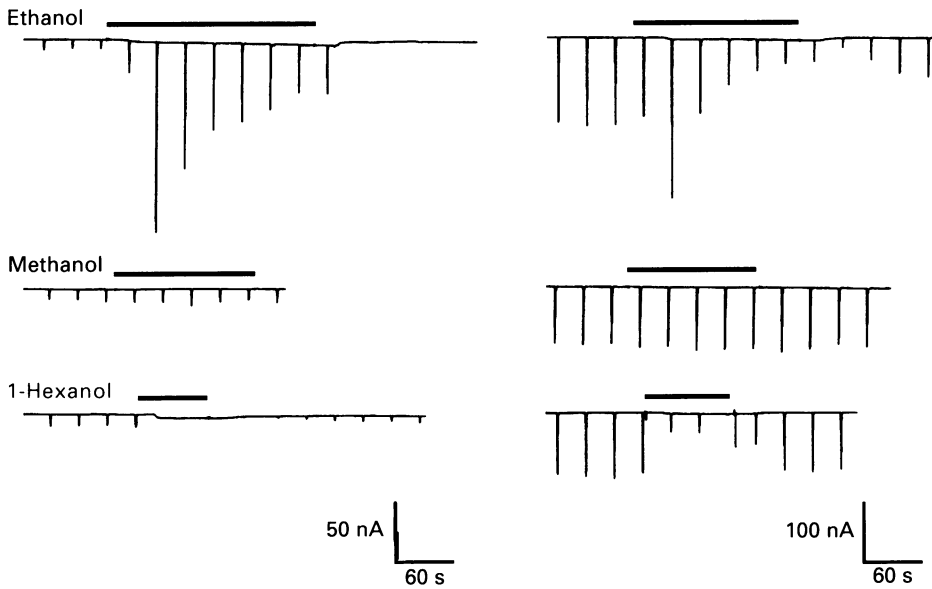

Fig. 9. Effects of various alcohols on caged $InsP_3$ responses. Traces on the left show responses to just-suprathreshold light flashes that evoked control currents at about 10 nA; those on the right were obtained with longer light flashes that evoked control currents of 100–150 nA. Ethanol, methanol and 1-hexanol were bath applied at respective dilutions of 2, 2 and 0·05 % during periods indicated by the bars. All data are from a single oocyte.

penetration of the oocyte, the injection solution included 50 μM-EDTA to chelate contaminating Ca^{2+}, and injections of a control solution lacking ethanol failed to produce depression. Secondly, depression was still evident in experiments in which the injection itself evoked little or no current response (e.g. Fig. 8), whereas a Ca^{2+}-activated Cl^- current should have been evident if any appreciable Ca^{2+} were present in the injection solution (Parker & Ivorra, 1990*a*).

Other alcohols

Various alcohols were tested for actions on $InsP_3$ signalling in the same way as described above for ethanol; that is to say, they were bath applied during stimulation by brief light flashes to look for potentiation and during stimulation by longer flashes to look for inhibition.

Figure 9 shows representative records obtained in a single oocyte that was exposed to methanol, ethanol and 1-hexanol. Ethanol produced the usual potentiation and depression. On the other hand, methanol was virtually without effect, even at a concentration of 500 mM. There was no detectable depression of responses to the longer flash and responses to the brief flash showed only a slight (about 50 %)

potentiation, in marked contrast to the potentiation of 2100% seen with 400 mM-ethanol. This relative ineffectiveness of methanol on caged $InsP_3$ responses was confirmed in four other experiments. Furthermore, methanol (500 mM) produced no obvious changes in the Fluo-3 signals evoked by light flashes, or in the currents evoked by intracellular injections of Ca^{2+} (data not shown).

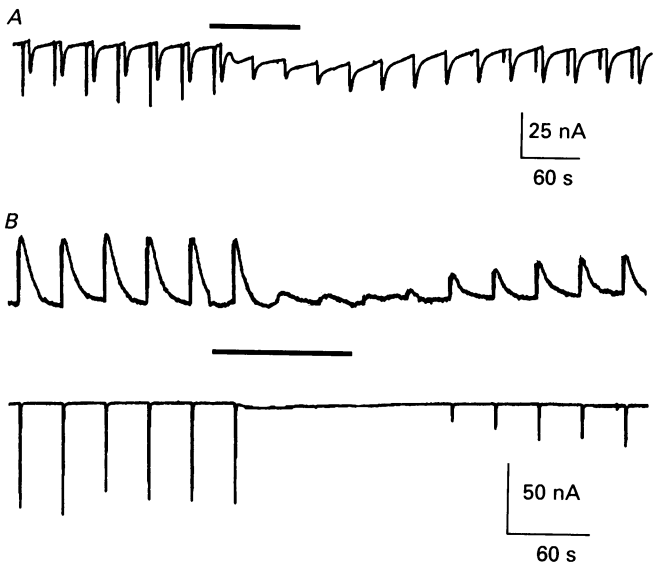

Fig. 10. *A*, trace shows membrane currents evoked by alternate stimulation by light flashes (brief responses) and by intracellular injections of Ca^{2+} (longer responses). During the time indicated by the bar, 0·025% 1-heptanol was added to the superfusate. *B*, simultaneous records of Fluo-3 Ca^{2+} transients and membrane currents evoked by repetitive light flashes at 30 s intervals. Details are as in Fig. 3*B*. The superfusate included 0·025% 1-heptanol during the time marked by the bar.

Different again to ethanol, application of 1-hexanol produced a marked depression of responses to both the brief and long light flashes, without any sign of potentiation. Similar results were obtained with other long-chain alcohols tested, including iso-butanol, heptanol and pentanol. Each of these was tested in at least three oocytes, and produced strong inhibition but no detectable potentiation. We did not examine the dose effectiveness of these alcohols in detail, but all depressed responses to 20% or less of the control value at dilutions of 0·2%. The most potent appeared to be 1-heptanol, which almost completely suppressed large (100 nA) current responses at a dilution of 0·025%. For comparison, the same dilution of ethanol (4 mM) would not cause any measurable depression (Fig. 6*B*).

To examine whether the depressant effects of the longer chain alcohols arose in a similar way to the ethanol-induced depression, we repeated the experiments of Fig. 3 using 0·025% 1-heptanol. The trace in Fig. 10*A* shows membrane currents evoked by alternate light flashes and intracellular Ca^{2+} injections. During application of heptanol the light flash responses were abolished, while the currents evoked by Ca^{2+} injections were reduced to about 36% of the control. Figure 10*B* shows simultaneous

records of Fluo-3 and membrane current signals evoked by light flashes during application of heptanol. The currents were suppressed by heptanol, and the Fluo-3 signals reduced to about 10% of the control. Both showed a partial recovery after washing for 3 min. Thus, it is clear that heptanol reduced $InsP_3$-mediated Ca^{2+} liberation. However, the appreciable reduction of the Ca^{2+}-activated Cl^- current indicates that this mechanism may also play an important part in the depression of $InsP_3$-mediated currents by heptanol.

DISCUSSION

The major finding is that ethanol both potentiates and depresses membrane current responses evoked by $InsP_3$ in *Xenopus* oocytes. Among the alcohols, this combination of effects appears unique to ethanol; methanol produced no inhibition and only slight potentiation, whereas various longer chain alcohols produced strong depression but no detectable potentiation. Both the facilitatory and depressant effects appear to arise primarily through actions on $InsP_3$-mediated liberation of sequestered Ca^{2+}, because they were evident in recordings of intracellular free Ca^{2+} made using the indicator dye Fluo-3, as well as in records where membrane Cl^- currents were used as a monitor of intracellular free Ca^{2+}. However, a part of the depressant effects on the current may also arise from inhibitory effects on the Ca^{2+}-activated Cl^- channels, as the Fluo-3 signals were reduced to a lesser extent than the current, and because currents evoked by intracellular injections of Ca^{2+} were depressed by ethanol and higher alcohols.

When ethanol was applied by bath superfusion, responses were first potentiated and then declined in the maintained presence of ethanol. In contrast, intracellular injection of ethanol produced an immediate depression followed, in some oocytes, by a potentiation. A possible explanation for this is that the potentiation arises at, or close to the cell membrane, whereas the depression arises more deeply into the cell. The slow time course of onset and recovery of the depression with bath applied ethanol may, therefore, reflect the time required for ethanol to equilibrate between the bathing solution and the cell interior. On this basis, the time course of records like that in Fig. 1 can be explained by a summation of facilitatory and inhibitory affects. Thus, the initial response to bath application of ethanol is a marked potentiation, because of the rapid onset of facilitation. During maintained application of ethanol the response then declines as the depressant effect grows and, after washing, the response is greatly reduced becuse the facilitation rapidly disappears whereas recovery from depression is slow.

Regarding the mechanism underlying the facilitation, a strong clue is provided by the observation that it arises through a reduction in the amount of $InsP_3$ required to evoke a threshold response. We have previously described a similar facilitation arising from procedures such as agonist activation and intracellular injection of low doses of $InsP_3$, that elevate the background level of $InsP_3$ in the oocyte (Parker & Miledi, 1989). Thus, the facilitatory effect of ethanol may arise if it elevates the intracellular level of $InsP_3$, possibly by stimulating its formation by the break-down of phosphatidylinositol bisphosphate in the plasma membrane. Such a mechanism is consistent with the localization of the facilitatory effect at the cell membrane

discussed above. It is also consistent with findings that ethanol can evoke oscillatory Cl^- currents in the oocyte (Wafford *et al.* 1989), which almost certainly arise as a result of $InsP_3$ formation, and that ethanol mobilizes Ca^{2+} in hepatocytes by activation of phospholipase C (Hoek, Thomas, Rubin & Rubin, 1987). However, the contrary effect has been reported in platelets, where ethanol inhibits thrombin-stimulated $InsP_3$ formation (Rand, Vickers, Kinlough-Rathbone, Packham & Mustard, 1988).

The depressant effects of ethanol and higher alcohols on $InsP_3$-mediated Ca^{2+} release presumably arise within the cell at, or close to, the sites of Ca^{2+} release. At present the mechanisms remain unclear, but we can consider two possibilities. One is that the alcohols act directly to inhibit the binding of $InsP_3$ to its intracellular receptors, or to modulate the gating of the Ca^{2+} release channels. Against this, ethanol at concentrations up to 500 mM has been found to have no effect on binding to cerebellar $InsP_3$ receptors (Smith, 1987). The second possibility is that alcohols may raise the cytoplasmic free Ca^{2+} concentration, either through an $InsP_3$-independent mechanism or by increasing the efficacy of endogenous $InsP_3$, and thus cause a Ca^{2+}-dependent inhibition of $InsP_3$-mediated Ca^{2+} liberation (Parker & Ivorra, 1990*a*; Payne, Flores & Fein, 1990). Since the depressant effect of ethanol remains when oocytes are bathed in a Ca^{2+}-free medium, the source of this calcium would have to be intracellular. Evidence in support of this hypothesis is that ethanol increases intracellular Ca^{2+} levels in various cells (Daniell, Brass & Harris, 1987; Rabe & Weight, 1988; Davidson, Wilce & Shanley, 1988), including oocytes (Cuthbertson, Whittingham & Cobbold, 1981), and that it is able to release Ca^{2+} from $InsP_3$-insensitive intracellular compartments (Machu, Woodward & Leslie, 1989). We were therefore interested to determine whether the intracellular free Ca^{2+} concentration was raised during depression of the $InsP_3$-mediated responses. The results, however, were inconclusive. Recordings of Fluo-3 fluorescence failed to show any clear rise during or after application of ethanol at concentrations sufficient to almost completely suppress the light flash responses, although increases were sometimes seen during superfusion with longer chain alcohols. Furthermore, the small membrane currents evoked by ethanol application did not appear to arise primarily through activation of the Ca^{2+}-dependent Cl^- conductance. The reversal potential for Cl^- in the oocyte is about -25 mV (Kusano, Miledi & Stinnakre, 1982), whereas reversal potentials of the ethanol-induced currents varied widely in different oocytes between about -10 mV to more negative than -60 mV. Also, the ethanol currents subsided rapidly on washing, in contrast to the slow recovery of the $InsP_3$-mediated responses. However, it remains possible that if ethanol were to release sequestered Ca^{2+} at sites close to the $InsP_3$ receptors, the localized Ca^{2+} concentration could be sufficient to inhibit $InsP_3$ action without giving rise to detectable fluorescence or membrane current signals.

Acute behavioural effects of ethanol are apparent at concentrations of a few tens of millimoles per litre. For example, the drunk driving limit in many States in the USA (0·1%) corresponds to a blood alcohol concentration of about 16 mM, and ataxia is produced in mice at concentrations between 40 and 75 mM (Gallagher, Parsons & Goldstein, 1982). The mechanisms underlying these effects are not understood, but actions of ethanol on many different receptors, channels and

enzymes have been implicated (for reviews see: Gandhi & Ross, 1989; Ticku, 1989). Although the facilitatory and depressant effects that we describe were most prominent at high ethanol concentrations (a few hundred millimoles per litre), clear actions were apparent at physiologically relevant levels. They might, therefore, be important in the generation of some symptoms of ethanol intoxication, resulting from modulation of phosphoinositide-mediated synaptic transmission. In this respect it is interesting that the most pronounced effect during mild alcohol intoxication is ataxia, and that the cerebellum contains by far the highest density of $InsP_3$ receptors of any brain region (Worley, Baraban & Snyder, 1989).

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REFERENCES

BERRIDGE, M. J. & IRVINE, R. F. (1989). Inositol phosphates and cell signalling. *Nature* **341**, 197–205.

CUTHBERTSON, K. S. R., WHITTINGHAM, D. G. & COBBOLD, P. H. (1981). Free Ca^{2+} increases in exponential phases during mouse oocyte activation. *Nature* **294**, 754–757.

DANIELL, L. C., BRASS, E. P. & HARRIS, R. A. (1987). Effect of ethanol on intracellular ionized calcium concentration in synaptosomes and hepatocytes. *Molecular Pharmacology* **32**, 831–837.

DAVIDSON, M., WILCE, P. & SHANLEY, B. (1988). Ethanol increases synaptosomal free calcium concentration. *Neuroscience Letters* **89**, 165–169.

GALLAGHER, E. J., PARSONS, L. M. & GOLDSTEIN, D. B. (1982). The rapid onset of tolerance to ataxic effects of ethanol in mice. *Psychopharmacology* **78**, 67–70.

GANDHI, C. R. & ROSS, D. H. (1989). Influence of ethanol on calcium, inositol phospholipids and intracellular signalling mechanisms. *Experientia* **45**, 407–413.

HOEK, J. B., THOMAS, A. P., RUBIN, R. & RUBIN, E. (1987). Ethanol-induced mobilization of calcium by activation of phosphoinositidase specific phospholipase C in intact hepatocytes. *Journal of Biological Chemistry* **262**, 682–691.

KUSANO, K., MILEDI, R. & STINNAKRE, J. (1982). Cholinergic and catecholaminergic receptors in the *Xenopus* oocyte membrane. *Journal of Physiology* **328**, 143–170.

MCCRAY, J. A. & TRENTHAM, D. R. (1989). Properties and uses of photoreactive caged compounds. *Annual Reviews of Biophysics and Biophysical Chemistry* **18**, 239–270.

MACHU, T., WOODWARD, J. J. & LESLIE, S. W. (1989). Ethanol and inositol 1,4,5-trisphosphate mobilize calcium from rat brain microsomes. *Alcohol* **6**, 431–436.

MILEDI, R. & PARKER, I. (1984). Chloride current evoked by injection of calcium into *Xenopus* oocytes. *Journal of Physiology* **357**, 173–183.

MINTA, A., KAO, J. P. Y. & TSIEN, R. Y. (1989). Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *Journal of Biological Chemistry* **264**, 8171–8178.

ORON, Y., DASCAL, N., NADLER, E. & LUPU, M. (1985). Inositol 1,4,5-trisphosphate mimics muscarinic response in *Xenopus* oocytes. *Nature* **313**, 141–143.

PARKER, I. (1991). Use of caged intracellular messengers for studying the inositol phosphate pathway. In *Neuromethods*, vol. 19, *Intracellular Messengers*, ed. BOULTON, A. A., BAKER, G. B. & TAYLOR, C. W. Humana Press, New Jersey (in the Press).

PARKER, I. & IVORRA, I. (1990*a*). Inhibition by Ca^{2+} of inositol trisphosphate-mediated Ca^{2+} liberation: A possible mechanism for oscillatory release of Ca^{2+}. *Proceedings of the National Academy of Sciences of the USA* **87**, 260–264.

PARKER, I. & IVORRA, I. (1990*b*). Localized all-or-none calcium liberation by inositol trisphosphate. *Science* **250**, 977–979.

PARKER, I. & IVORRA, I. (1991). Caffeine inhibits inositol trisphosphate-mediated liberation of intracellular calcium in *Xenopus* oocytes. *Journal of Physiology* **433**, 229–240.

PARKER, I. & MILEDI, R. (1986). Changes in intracellular calcium and in membrane currents evoked by injection of inositol trisphosphate in *Xenopus* oocytes. *Proceedings of the Royal Society* B **228**, 307–315.

PARKER, I. & MILEDI, R. (1989). Non-linearity and facilitation in phosphoinositide signalling studied by the use of caged inositol trisphosphate in *Xenopus* oocytes. *Journal of Neuroscience* **9**, 4068–4077.

PAYNE, R., FLORES, T. M. & FEIN, A. (1990). Feedback inhibition by calcium limits the release of calcium by inositol trisphosphate in *Limulus* ventral photoreceptors. *Neuron* **4**, 547–555.

RABE, C. S. & WEIGHT, F. F. (1988). Effects of ethanol on neurotransmitter release and intracellular free calcium in PC12 cells. *Journal of Pharmacology and Experimental Therapeutics* **244**, 417–422.

RANA, R. S. & HOKIN, L. E. (1990). Role of phosphoinositides in transmembrane signalling. *Physiological Reviews* **70**, 115–164.

RAND, M. L., VICKERS, J. D., KINLOUGH-RATHBONE, R. L., PACKHAM, M. A. & MUSTARD, J. F. (1988). Thrombin-induced inositol trisphosphate production by rabbit platelets is inhibited by ethanol. *Biochemical Journal* **251**, 279–284.

SMITH, T. L. (1987). Chronic ethanol consumption reduces [^{3}H]inositol(1,4,5)trisphosphate specific binding in mouse cerebellar membrane fragments. *Life Sciences* **41**, 2863–2868.

TICKU, M. K. (1989). Ethanol and the benzodiazepine–GABA receptor–ionophore complex. *Experientia* **45**, 413–418.

WAFFORD, K. A., DUNWIDDIE, T. V. & HARRIS, R. A. (1989). Calcium-dependent chloride currents elicited by injection of ethanol into *Xenopus* oocytes. *Brain Research* **505**, 215–219.

WORLEY, P. F., BARABAN, J. M. & SNYDER, S. H. (1989). Inositol 1,4,5-trisphosphate receptor binding: autoradiographic localization in rat brain. *Journal of Neuroscience* **9**, 339–343.