CAFFEINE INHIBITS INOSITOL TRISPHOSPHATE-MEDIATED LIBERATION OF INTRACELLULAR CALCIUM IN XENOPUS OOCYTES

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SUMMARY

1. Voltage-clamp recording of Ca\(^{2+}\)-activated chloride currents in Xenopus oocytes was used to study the effects of caffeine on the liberation of intracellular Ca\(^{2+}\) induced by photo-release of inositol 1,4,5-trisphosphate (InsP\(_3\)) from caged InsP\(_3\). Bath application of caffeine, at concentrations between 0·1 and 10 mM, reduced or abolished the current evoked by photo-release of InsP\(_3\) and by microinjection of InsP\(_3\).

2. Caffeine did not appreciably reduce currents evoked by injection of Ca\(^{2+}\) into oocytes, whereas measurements using the Ca\(^{2+}\) indicator Rhod-2 showed that it instead inhibited the liberation of Ca\(^{2+}\) by InsP\(_3\).

3. Caffeine increased the threshold amount of InsP\(_3\) required to evoke a current response and proportionally reduced the currents evoked by suprathreshold levels of InsP\(_3\).

4. Theophylline and 3-isobutyl-1-methylxanthine (IBMX) were much less potent than caffeine, and few changes were seen in the InsP\(_3\) responses following application of forskolin or intracellular injection of cyclic AMP. Thus, inhibition of InsP\(_3\) responses by caffeine does not arise through inhibition of phosphodiesterase enzymes.

5. Even at high (10 mM) concentrations, caffeine did not itself elicit any clear Ca\(^{2+}\)-activated current. It is therefore unlikely that inhibition of the InsP\(_3\) responses arise because caffeine itself liberates Ca\(^{2+}\) from intracellular stores.

6. The site of action of caffeine is intracellular, because injections of caffeine into the oocyte strongly inhibited responses to InsP\(_3\), whereas local extracellular applications of similar amounts were almost without effect.

INTRODUCTION

Inositol 1,4,5-trisphosphate (InsP\(_3\)) functions as part of a ubiquitous second messenger signalling system, in which it liberates Ca\(^{2+}\) from intracellular stores (for recent reviews see: Berridge & Irvine, 1989; Rana & Hokin, 1990). Drugs that act on InsP\(_3\)-mediated Ca\(^{2+}\) release would be of great utility as research tools and as potential therapeutic agents. However, relatively few drugs are as yet known to act on InsP\(_3\) signalling. Those that are available — for example, heparin (Hill, Berggren & Boynton, 1987; Ghosh, Eis, Mullaney, Ebert & Gill, 1988), tetraethylammonium (Shah & Pant, 1988), cinnarizine and flunarizine (Seiler, Arnold & Stanton, 1987) and
calmodulin antagonists (Hill, Campos-Gonzalez, Kindmark & Boynton, 1988) – are restricted in their use because of limited specificity or by their inability to cross the plasma membrane of intact cells. We report here that extracellular or intracellular application of caffeine to *Xenopus* oocytes inhibits InsP$_3$-mediated responses. This effect appears to arise by a mechanism different from the well-known actions of caffeine as a phosphodiesterase inhibitor (Butcher & Sutherland, 1962) and as an activator of intracellular Ca$^{2+}$ release (Endo, 1977; Neering & McBurney, 1984).

**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; Sumikawa, Parker & Miledi, 1989; Parker & Ivorra, 1990). Briefly, defolliculated oocytes were voltage clamped at a potential of $-60$ mV and membrane currents were recorded while the oocyte was continually superfused with frog Ringer solution at room temperature. In most experiments each oocyte was loaded with 1–10 pmol of caged InsP$_3$ *(myo-inositol 1,4,5-trisphosphate P$_4^{4,5,1}$-2-nitrophenyl)* ethyl ester, and was stimulated by brief flashes of UV light to cause intracellular release of free InsP$_3$. The photolysis light was focused on the oocyte as a square of about 100 $\mu$m sides, positioned on the vegetal hemisphere close to the equator. Bath application of caffeine and other compounds was accomplished by switching the intake of the superfusion system. The bars marked on the figures indicate the times 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. Intracellular injections were made by applying pneumatic pressure pulses to glass micropipettes broken to a tip diameter of a few micrometres. Caffeine, InsP$_3$ and caged InsP$_3$ were injected at respective concentrations of 5 $\mu$M, 100 $\mu$M and 1 mM, in aqueous solutions including 50 $\mu$M-EDTA and 5 mM-HEPES at pH 7.0. Calcium was injected as a 5 mM solution of CaCl$_2$. The volumes of solutions injected were estimated by measuring the sizes of fluid droplets expelled by the pressure pulses with the pipette tip in air. Injections of caffeine and Ca$^{2+}$ were made with the injection pipette inserted into the oocyte near the centre of the photolysis light spot. InsP$_3$ was injected into the animal hemisphere. Pipettes were tested before and after recording to ensure that they had not become plugged during the experiment. Recordings of intracellular Ca$^{2+}$ transients using fluorescent indicators (Minta, Kao & Tsien, 1989) were made as described previously (Parker & Ivorra, 1990), except that Rhod-2 was used as the indicator instead of Fluo-3. A rhodamine filter set was used to select the appropriate excitation and emission wavelengths. InsP$_3$ and caged InsP$_3$ were obtained from Calbiochem (La Jolla, CA, USA), Rhod-2 from Molecular Probes Inc. (Eugene, OR, USA), and all other reagents from Sigma Chemical Co. (St Louis, MO, USA).

**RESULTS**

*Caffeine inhibits InsP$_3$-mediated membrane currents*

Injection of InsP$_3$ into *Xenopus* oocytes evokes an oscillatory chloride membrane current, that arises because Ca$^{2+}$ is liberated from intracellular stores and subsequently activates Ca$^{2+}$-dependent chloride channels (Miledi & Parker, 1984; Oron, Dascal, Nadler & Lupu, 1985; Parker & Miledi, 1986, 1989). Figure 1A illustrates a response evoked by injection of InsP$_3$, during which the superfusate was briefly switched to a solution including 5 mM-caffeine. Application of caffeine rapidly abolished the current fluctuations evoked by the InsP$_3$, but these recovered after returning to normal Ringer solution.

A similar blocking action was seen also when caffeine (5 mM) was applied during bath application of acetylcholine (100 nM) to evoke oscillatory currents through activation of native muscarinic receptors in the oocyte membrane (Kusano, Miledi & Stinnakre, 1982).
Inhibition of responses evoked by photo-release of InsP$_3$

The responses to injections of InsP$_3$ are prolonged, and can vary appreciably from one injection to the next. To study better the effects of caffeine, the remaining experiments in this paper were done by the use of flash photolysis of caged InsP$_3$

![Graph A](image)

![Graph B](image)

Fig. 1. A, inhibition of InsP$_3$-evoked membrane currents by bath application of caffeine. In this and other figures, traces show membrane currents recorded at a clamp potential of $-60$ mV; downward deflections correspond to inward membrane currents. At the arrow a single injection of about 3.5 fmol InsP$_3$ was made into the oocyte. Caffeine (5 mM) was added to the bathing solution for the time indicated by the bar. B, inhibition by caffeine of responses evoked by photo-release of InsP$_3$ in oocytes loaded with caged InsP$_3$. Membrane currents were recorded in response to repetitive light flashes, delivered at 30 s intervals as indicated by the arrow-heads. The bathing solution included 5 mM-caffeine for the time marked by the bar.

(Mccray & Trentham, 1989), to liberate precisely controlled amounts of InsP$_3$ in the oocyte. Because each oocyte was loaded with a large excess of caged InsP$_3$ it was possible to evoke many tens or hundreds of responses (Parker & Miledi, 1989; Parker & Ivorra, 1990). The currents evoked by photo-release of InsP$_3$ were more transient than those evoked by injection of free InsP$_3$ (compare Figs. 1A and B) but arise similarly through the liberation of Ca$^{2+}$ from intracellular stores (Parker & Miledi, 1989).

Figure 1B shows membrane currents in an oocyte loaded with a caged InsP$_3$ in response to a train of light flashes at 30 s intervals. The duration of the flashes was set slightly above the threshold required to evoke a current (Parker & Miledi, 1989), so that a series of small and roughly constant responses were obtained. Caffeine was
bath-applied at a concentration of 5 mM for the time indicated by the bar. At first caffeine almost abolished the light-flash response but, during continued application of caffeine, the response recovered slightly (to about 7% of the control after 3 min). Upon returning to normal Ringer solution the response recovered rapidly and

\[ \text{Fig. 2. Caffeine inhibits the release of intracellular Ca}^{2+}, \text{but not the activation of the Ca}^{2+}-\text{dependent chloride current. A, trace shows membrane currents evoked by alternate stimulation by light flashes (filled arrow-heads) and by intracellular injections of about 50 fmol Ca}^{2+} (\text{open arrow-heads}). Caffeine (5 mM) was bath-applied when indicated by the bar. B, simultaneous records of intracellular Ca}^{2+}\text{transients and membrane currents evoked by repetitive light flashes at 40 s intervals (marked by arrow-heads). The lower trace shows membrane current. The upper trace monitors fluorescence of the Ca}^{2+}\text{indicator dye Rhod-2, previously loaded into the oocyte. Upward deflections correspond to increasing fluorescence and increasing free Ca}^{2+}\text{concentration; the magnitude of this trace is uncalibrated. Caffeine (5 mM) was bath-applied for the time indicated by the bar. The slight increase in baseline fluorescence during application of caffeine was not consistently seen in other experiments and may be artifactual.} \]

'overshot' the control level, transiently reaching a peak of about 170% of the control before declining back to the original level over a few minutes.

Inhibition of small light-flash responses, like those in Fig. 1B, was clearly seen with concentrations of caffeine as low as 100 μM; the current shortly after application of caffeine to the oocyte being reduced to a mean of 48±9% of the control (s.e. of mean, six trials in four oocytes). At a concentration of 1 mM the current was reduced to 17±7% of the control (eight trials in five oocytes).

Despite its marked effect on the InsP₃-evoked responses, caffeine usually produced little direct change in membrane current, even when applied at high (5 or 10 mM)
concentrations. Most oocytes (e.g., Fig. 1B) showed only very slight (1 nA or less) outward or inward currents, but in one oocyte (from a total of fifteen) a larger (5 nA) inward current was seen with 5 mm-caffeine.

**Caffeine inhibits Ca\(^{2+}\) liberation by InsP\(_3\)**

The inhibitory effect of caffeine described above might arise either because it suppresses the InsP\(_3\)-mediated release of intracellular Ca\(^{2+}\), or because it inhibits the opening of Ca\(^{2+}\)-dependent Cl\(^-\) channels. Two different approaches were used to distinguish between these possibilities.

The first is illustrated in Fig. 2A, which shows records from an oocyte that was loaded with caged InsP\(_3\) and stimulated repetitively at 30 s intervals by light flashes followed by injections of Ca\(^{2+}\). Bath application of 5 mm-caffeine completely abolished the responses to the light flashes. In contrast, the currents evoked by the Ca\(^{2+}\) injections remained virtually unchanged, indicating that caffeine does not act on the Ca\(^{2+}\)-activated Cl\(^-\) channels.

The oocyte in Fig. 2A showed spontaneous oscillations in membrane current (Kusano et al. 1982), which are apparent between stimuli near the beginning of the record. This spontaneous activity was reversibly abolished by the exposure to caffeine, and we observed a similar reversible reduction or abolition of spontaneous oscillations in several other oocytes.

A second, more direct method to test for inhibition by caffeine of InsP\(_3\)-induced Ca\(^{2+}\) release employed the fluorescent Ca\(^{2+}\) indicator Rhod-2 (Minta et al. 1989; Parker & Ivorra, 1990) as an intracellular Ca\(^{2+}\) monitor. Figure 2B shows simultaneous records of Rhod-2 fluorescence and membrane current signals evoked by repetitive light flashes in an oocyte loaded with caged InsP\(_3\) and Rhod-2. Bath application of caffeine (5 mm) reversibly abolished both signals, and a similar abolition or strong reduction of the Ca\(^{2+}\) signals was seen in a further ten trials in four oocytes. However, the membrane current response appeared to be more sensitive to caffeine than did the fluorescence Ca\(^{2+}\) signal. For example, during the onset of caffeine action in Fig. 2B (fourth flash) the current was reduced to about one-half, whereas the fluorescence signal was little altered. In some experiments, the currents were completely suppressed during application of 5 mm-caffeine, even though small (ca. 10%) fluorescence signals remained. This difference in sensitivities may arise because a certain elevation of intracellular free Ca\(^{2+}\) is required before any Cl\(^-\) current is detected (Ivorra & Parker, 1990), or because caffeine acts more potently on Ca\(^{2+}\) liberation close to the membrane than at greater depths into the cytoplasm.

**Caffeine increases the threshold for InsP\(_3\) action**

Dose–response relationships for the InsP\(_3\)-activated current were obtained by varying the duration of the light flash so as to release varying amounts of InsP\(_3\) (Parker & Miledi, 1989). As we have described previously (Parker & Miledi, 1989), there is a threshold flash duration below which no response is seen, whereas the current increases progressively with increasing flash duration above threshold. Caffeine altered this dose–response relationship in two ways: the threshold became longer, and the currents evoked by suprathreshold flashes were proportionally reduced in size. In the oocyte illustrated in Fig. 3, addition of 2.5 mm-caffeine roughly
Fig. 3. Caffeine raises the threshold amount of InsP₃ required to evoke a response. A, sample records of currents evoked by light flashes of different durations (indicated in milliseconds next to each trace). Control records in normal Ringer solution are shown on the left, and those on the right were obtained after addition of 2.5 mm-caffeine to the bathing solution. B, measurements of peak current amplitudes obtained from records like those in A are plotted as a function of flash duration. Open symbols are control measurements, filled symbols are measurements in the presence of 2.5 mm-caffeine. Two sets of measurements were alternately obtained in normal Ringer solution and in caffeine. For each solution, points marked by squares show the first set of measurements, and diamonds indicate the second set.

doubled the threshold flash duration from 95 to 210 ms, and the slope of the relationship with suprathreshold flashes was reduced to about one-half.

Because of the shift in threshold produced by caffeine, most of the other experiments described here were done using light flashes that were only slightly (about 20–50%) above threshold, so as to maximize the inhibition of the InsP₃ responses.
Inhibition does not arise through action on phosphodiesterase

Caffeine is a phosphodiesterase inhibitor (Butcher & Sutherland, 1962) and might thus elevate the level of cyclic AMP in the oocyte (Miledi & Woodward, 1989). To see if this was responsible for the inhibition of the InsP$_3$ response, we tested the effects of IBMX (3-isobutyl-1-methylxanthine) and theophylline, which are more potent inhibitors of phosphodiesterase than is caffeine (Butcher & Sutherland, 1962; Rall, 1980). Figure 4 illustrates results from one oocyte. At a concentration of 1 mM, caffeine strongly depressed the response to the light flash whereas at the same concentrations, theophylline gave only a slight reduction and IBMX failed to appreciably alter the response. Experiments like that shown were repeated in a total of three oocytes. The mean response sizes, expressed as a percentage of the control value before drug application (all concentrations 1 mM) were: caffeine, 19.7 ± 7.1 (S.E. of mean, seven trials); theophylline, 86.2 ± 7.9 (five trials); IBMX, 95.0 ± 1.7 (five trials).

Additional evidence against the involvement of cyclic AMP in depression of the InsP$_3$-evoked responses is that almost no changes were seen during bath application of 10 µM-forskolin, an activator of adenylate cyclase, nor following intracellular injections of 1.5–12 pmol cyclic AMP (mean response 107% of control; four trials in three oocytes).

Another established action of caffeine is to inhibit the binding of adenosine to cell surface purinergic receptors (Snyder, 1984). We therefore bath applied adenosine
(1 mM) to test for effects on the caged InsP₃ response, in the same way as illustrated in Fig. 4. The mean response during adenosine application was 91.2 ± 1.6% of the control (s.e. of mean, four trials in two oocytes).

**Intracellular injection of caffeine**

Inhibition of the InsP₃ responses might arise because caffeine binds to receptors on the outer surface of the cell membrane, or because it crosses the membrane and directly acts within the cytoplasm. Experiments in which caffeine was injected into the oocyte support the latter idea. For example, in Fig. 5A intracellular injection of 400 fmol caffeine greatly reduced the response to a light flash delivered 5 s later, and the current was almost completely suppressed following injection of 800 fmol caffeine. These injections themselves evoked little or no current response; in the oocyte illustrated, injection of 800 fmol caffeine evoked only a slight (about 1 nA) outward current.

Although the inhibition of the InsP₃ responses by caffeine most likely resulted through an action at intracellular sites, it remained possible that the caffeine injected into the cytoplasm might cross the plasma membrane to act on the extracellular membrane surface. To exclude this, we examined the effects of locally applying caffeine to the outside of the oocyte. Figure 5B shows records from the same oocyte as Fig. 5A, after withdrawing the caffeine pipette. Local extracellular application of 800 fmol caffeine produced almost no inhibition, even though intracellular injection of the same amount almost completely inhibited the InsP₃ response. Further increasing the amount of caffeine applied to 8 pmol caused the response to reduce by about one-half.
As shown in Fig. 5A, the InsP₃ response was strongly depressed within a few seconds of injection of caffeine, but recovered almost completely after about 30 s. The time course of this process was explored more fully in the experiment shown in Fig. 6, in which light flashes were delivered at various intervals after injecting caffeine. Responses were maximally inhibited when flashes were given 1–2 s after injection. At shorter intervals the inhibition was less pronounced, possibly because there was insufficient time for the caffeine to spread from the point of injection throughout that part of the cell illuminated by the photolysis light (100 μm-sided square). As the interval was further lengthened, the response recovered back to the control value after about 20 s, with a half-recovery time of about 9 s.
DISCUSSION

The results demonstrate that membrane currents evoked in *Xenopus* oocytes by InsP$_3$ are depressed in a dose-dependent manner by bath application of caffeine at concentrations between 0.1 and 10 mM. InsP$_3$ evokes currents because it liberates Ca$^{2+}$ from intracellular stores (Oron et al. 1985; Parker & Miledi, 1986, 1989) and that, in turn, opens Ca$^{2+}$-dependent chloride channels in the plasma membrane (Miledi & Parker, 1984). Caffeine did not reduce the currents evoked by intracellular injections of Ca$^{2+}$, but reduced or abolished InsP$_3$-evoked Ca$^{2+}$ transients as monitored by an intracellular fluorescent indicator. Thus, the inhibition of membrane current responses by caffeine almost certainly arises through a reduction in the ability of InsP$_3$ to liberate Ca$^{2+}$ from intracellular stores, and not through an action on the Ca$^{2+}$-activated chloride channels.

A well-established pharmacological action of caffeine is its ability to inhibit phosphodiesterase enzymes (Butcher & Sutherland, 1962). However, it seems that the effect of caffeine on InsP$_3$-evoked responses cannot arise through changes in cyclic nucleotide metabolism because applications of IBMX (a considerably more potent phosphodiesterase inhibitor) were almost without effect, and inhibition was not observed with the adenylate cyclase activator forskolin, nor with direct injection of cyclic AMP into the oocyte (see also Miledi & Woodward, 1989).

Another well-known action of caffeine is to release Ca$^{2+}$ from intracellular stores in muscle (Endo, 1977) and nerve (Neering & McBurney, 1984). If caffeine were to liberate Ca$^{2+}$ from stores in oocyte, it might inhibit responses to InsP$_3$ either by depleting the amount of Ca$^{2+}$ remaining available for release, or because the resulting rise in cytoplasmic Ca$^{2+}$ inhibits the InsP$_3$-mediated release of Ca$^{2+}$ (Parker & Ivorra, 1990). However, a strong argument against these possibilities can be made, because doses of caffeine which were sufficient to almost abolish the InsP$_3$ response themselves activated no detectable Ca$^{2+}$-dependent Cl$^-$ current (e.g. Figs 1B and 5A). If the depression were due to depletion of Ca$^{2+}$ from stores, caffeine might be expected to evoke a Cl$^-$ current at least as large as that elicited by InsP$_3$. Similarly, although injections of Ca$^{2+}$ into the oocyte inhibit subsequent responses to InsP$_3$, amounts sufficient to produce substantial inhibition themselves activate a large Cl$^-$ current (Parker & Ivorra, 1990). Furthermore, unlike the effect of caffeine on the InsP$_3$ dose–response relationship, the Ca$^{2+}$-inhibition of InsP$_3$-mediated Ca$^{2+}$ release does not shift the threshold for InsP$_3$ action (Parker & Ivorra, 1990).

Injections of caffeine into the oocyte were highly effective in reducing InsP$_3$-evoked responses, whereas local applications of similar amounts to the outside of the oocyte were almost ineffective. Thus, caffeine must act at an intracellular site, rather than on cell surface receptors. The ability of caffeine to bind with high affinity to cell surface adenosine receptors (Snyder, 1984) is therefore unlikely to be important for the results described here, and this conclusion is strengthened by the finding that bath application of adenosine produced only a slight reduction of the InsP$_3$-evoked responses. Following injection of caffeine into the oocyte, inhibition of the InsP$_3$-mediated response was maximal after about 1 s, and responses subsequently recovered over several seconds. Diffusion of caffeine from the injection site may be a major factor determining the onset of the inhibition, whereas diffusion into remote
regions of the cell and into the bathing fluid may explain the subsequent recovery of
the responses. Hence, inhibition of InsP3-mediated Ca2+ liberation is likely to be even
faster than indicated by the data in Fig. 6.

We do not yet know the mechanism by which caffeine inhibits InsP3-mediated
Ca2+ liberation although, as discussed above, several possibilities can be eliminated.
A simple explanation may be that caffeine antagonizes the binding of InsP3 to its
intracellular receptor. In support of this idea, intracellular injection of heparin, a
known inhibitor of InsP3 binding (Supattapone, Worley, Baraban & Snyder, 1988),
also inhibits InsP3-mediated responses in the oocyte and produces changes in the
dose–response relationship like those seen with caffeine (I. Parker and I. Ivorra,
unpublished data). However, some features of the results are not easily explained
on the basis of an inhibition of InsP3 binding. These include the partial recovery from
inhibition during maintained application of caffeine and the overshoot of the current
response after removing caffeine. Also, it has recently been reported that in
pancreatic acinar cells, caffeine potentiates InsP3-mediated responses following an
initial transient depression (Osipchuk, Wakui, Yule, Gallacher & Petersen, 1990).
Thus, caffeine may exert multiple actions on InsP3 signalling, and the relative
prominence of each may vary between different cell types.

Caffeine is widely used as a stimulant. Ingestion of a few cups of coffee (each
containing about 100 mg caffeine) results in plasma concentrations of few tens of
micromoles per litre and therapeutic doses of caffeine can produce plasma levels as
high as 100 μM (Rall, 1980). Because the effects of caffeine on phosphodiesterase
enzymes and on the direct release of Ca2+ from intracellular stores require
concentrations of several millimolar, it is thought that the stimulant actions of
caffeine arise largely through binding to adenosine receptors, which occurs with
micromolar affinity (Snyder, 1984). The finding of appreciable inhibition of InsP3-
mediated responses by 100 μM-caffeine raises the possibility that actions on
phosphoinositide signalling in neurons may also contribute to the behavioural effects
of caffeine. Finally, we suggest that caffeine might provide a useful starting point for
the development of new drugs which act with greater affinity and specificity on the
inositol phosphate signalling pathway.

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