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CALCIUM RELEASE INDUCED BY INOSITOL 1,4,5-TRISPHOSPHATE IN SINGLE RABBIT INTESTINAL SMOOTH MUSCLE CELLS

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SUMMARY

1. Single smooth muscle cells were isolated by enzymic digestion from the longitudinal muscle layer of rabbit jejunum, and the response of the cells to calcium (Ca^{2+}) release by $InsP_3$ (D-*myo*-inositol 1,4,5-trisphosphate) was studied. Changes in internal Ca^{2+} concentration were monitored by measuring Ca^{2+}-activated K^+ currents (outward currents) using the whole-cell voltage-clamp technique.

2. At break-through from cell-attached patch to whole-cell recording mode using a 100 μM-$InsP_3$-filled pipette, cells exhibited a brief outward current which reached its peak in 1·1 s and terminated within 10 s. Following this the generation of spontaneous transient outward currents (STOCs) was inhibited. (STOCs are considered to represent bursts of openings of Ca^{2+}-activated K^+ channels in response to spontaneous discharges of Ca^{2+} from the stores.) When a pipette filled with 20 μM-$InsP_3$ was used, similar current responses were also evoked, but some cells failed to respond.

3. The $InsP_3$-induced outward current at membrane break-through was similar in size and time course to the outward current response of normal cells to bath-applied carbachol (CCh, 100 μM) or caffeine (20 mM).

4. Dialysis with $InsP_3$-containing solution inhibited the caffeine-induced outward current, depending on the pipette $InsP_3$ concentration. Inclusion of heparin (5 mg/ml) in the pipette completely prevented inhibition by $InsP_3$ of the caffeine response and of STOC discharge. However, the $InsP_3$-induced current at break-through remained unchanged, probably because of the slower rate of diffusion of heparin.

5. In cells dialysed with pipette solution containing 30 or 100 μM-caged $InsP_3$, flash photolysis (producing up to 1·5 μM-$InsP_3$) induced an outward current response after a latency of 31·0 $\pm$ 1·8 ms (n = 15), which was followed by inhibition of STOCs. The reversal potential of the current to flash-release of $InsP_3$ followed closely the Nernst potential for K^+ ions (E_K), suggesting negligible contributions from channels other than Ca^{2+}-activated K^+ channels.

6. Photolysis of caged $InsP_3$ (30 or 100 μM) still produced a current response after 3–6 min in Ca^{2+}-free (3 mM-EGTA added) bathing solution, but no response occurred if the cell was exposed to either caffeine (20 mM) or CCh (100 μM) to deplete Ca stores. Inclusion of heparin (0·5 or 5 mg/ml) in the pipette resulted in blockade of the

current response to photolysis of caged $InsP_3$ but no inhibition of the caffeine (20 mM) response.

7. The results show that $InsP_3$ can extremely rapidly induce Ca^{2+} release from storage sites from which Ca^{2+} can also be released by CCh or caffeine. Heparin blocks the Ca^{2+}-releasing action of $InsP_3$. The similarity in the Ca^{2+}-releasing actions of $InsP_3$ and CCh provides evidence for the previous view (Komori & Bolton, 1990) that $InsP_3$ production is essential for Ca^{2+} release following activation of the muscarinic receptor.

INTRODUCTION

D-*Myo*-inositol 1,4,5-trisphosphate ($InsP_3$) is considered to be a second messenger substance that is liberated from the plasma membrane in response to receptor activation and which can release calcium (Ca^{2+}) from intracellular stores in smooth muscles and other cell types (reviewed by Berridge & Irvine, 1984). In our recent study (Komori & Bolton, 1990), where agonist-induced Ca^{2+} release was monitored by measuring the Ca^{2+}-activated K^+ current in isolated smooth muscle cells from the rabbit jejunum, it was suggested that Ca^{2+} release by activation of muscarinic receptors involved production of $InsP_3$, possibly in part mediated by a G-protein coupling the receptor to the enzyme phospholipase C (PLC). This was based on the following evidence: (1) bath application of either carbachol (CCh, a muscarinic agonist) or caffeine induced Ca^{2+} release from a common store, but only the effect of the former was reduced by GDPβS, which is a slowly hydrolysed analogue of GDP (Stryer & Bourne, 1986; Gilman, 1987); (2) CCh-induced, but not caffeine-induced, Ca^{2+} release was also blocked by heparin which competes with $InsP_3$ for binding to its receptors in cell membrane fragments of muscle and non-muscle cells (Hill, Berggren & Boynton, 1987; Worley, Baraban, Supattapone, Wilson & Snyder, 1987; Ghosh, Eis, Mullaney, Ebert & Gill, 1988; Kobayashi, Somlyo & Somlyo, 1988); (3) cell dialysis with GTPγS, a slowly hydrolysed analogue of GTP, resulted in the depletion of caffeine (or CCh)-releasable Ca stores, and this effect was also prevented by heparin. In addition, it was shown in vascular smooth muscle cells that the effect of GTPγS was competitively reduced by GDPβS (Komori & Bolton, 1989). It would be useful support for our scheme (Komori & Bolton, 1990) if it could be shown in single smooth muscle cells that $InsP_3$ caused Ca^{2+} release from the CCh (or caffeine)-releasable store and whether $InsP_3$ could mimic CCh-induced Ca^{2+} release. However, it has already been reported in patch-clamped smooth muscle cells of the rabbit portal vein, that $InsP_3$ (10 μM) did not produce outward current due to Ca store release although it was considered to facilitate STOC discharge (Ohya, Terada, Yamaguchi, Inoue, Okabe, Kitamura, Hirata & Kuriyama, 1988). An aim of the present study is to investigate these points.

The kinetic properties of the Ca^{2+} release process triggered by $InsP_3$ in smooth muscle cells are little known. In small, chemically 'skinned' strips, which contain many hundreds of smooth muscle cells and which were loaded with a photolabile, inactive precursor of $InsP_3$ (caged $InsP_3$), production of $InsP_3$ by a light flash resulted after a delay of about 0·5 s in tension development (Walker, Somlyo, Goldman, Somlyo & Trentham, 1987). In single isolated smooth muscle cells, the Ca^{2+}-activated K^+ current response to ionophoretic application of CCh began around

the end of the 0·5 s releasing pulse (Lim & Bolton, 1988; Bolton & Lim, 1989). These latter results indicate that the latency for $InsP_3$-induced Ca^{2+} release must be less than 0·5 s. It is desirable to determine more precisely how rapidly $InsP_3$ is capable of releasing stored Ca^{2+}.

In the present experiments, intracellular application of $InsP_3$ was performed in two different ways. One was to allow $InsP_3$ itself to diffuse from the pipette into the cell; a relatively high concentration (20 or 100 μM) was included in the pipette allowing for a high activity of metabolizing enzymes in smooth muscle (Walker *et al.* 1987; Somlyo, Walker, Goldman, Trentham, Kobayashi, Kitazawa & Somlyo, 1988). The other was to photochemically produce $InsP_3$ from a caged $InsP_3$ precursor previously allowed to diffuse into the cell. These two methods should allow, on the one hand, a comparison of the effects of a prolonged application of $InsP_3$ with those of bath application of CCh or caffeine and, on the other hand, to resolve the rapid kinetics of cellular events without the limitations imposed by diffusion.

The results show that $InsP_3$ can release most of the releasable Ca from the CCh (or caffeine)-releasable store after a latency of 30 ms, and that various features except for latency of the Ca^{2+}-releasing action of $InsP_3$ are similar to those of CCh.

METHODS

Preparation of cells

Rabbits of either sex, weighing 2–2·5 kg, were killed by injecting an overdose of sodium pentobarbitone into the ear vein. Lengths of longitudinal muscle from the jejunum were peeled from the underlying circular muscle and placed in physiological salt solution (composition given below).

Jejunal smooth muscle cells were dispersed as previously described (Komori & Bolton, 1990) using a combination of collagenase (0·5 mg/ml) and papain (0·5–1·0 mg/ml). The cells were suspended in 0·8 mM-Ca^{2+}-containing physiological salt solution, placed on cover-slips in a small aliquot and kept in a moist atmosphere at 4 °C until use on the same day.

Whole-cell recording

Dispersed cells were transferred to a small chamber (0·6 ml in volume) on a microscope stage and immersed in physiological salt solution. Whole-cell membrane-current recordings were made at room temperature (21–23 °C) using standard patch-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Experiments in which caged substances were used (see below) were performed under very low illumination. Patch pipettes had a resistance of 3–6 MΩ when filled with a pipette solution. The current amplifier used was a Bio-Logic RK-300 and current records were stored on FM tape and replayed onto a Gould brush recorder for illustration and analysis.

Smooth muscle cells from the rabbit jejunum possess abundant Ca^{2+}-activated K^+ channels (Benham, Bolton, Lang & Takewaki, 1986). Changes in free ionized Ca^{2+} concentration should thus be reflected in the activity of the K^+ channels, and the channel activity (outward membrane current positive to E_K) can be employed as a probe for monitoring rises in Ca^{2+} concentration. The holding potential in the whole-cell recording mode was usually set at 0 mV, at which agonist-induced current was outward under the ionic conditions used. This holding potential is close to the Nernst potential for chloride and to the reversal potential for non-selective cationic channels which are activated by muscarinic agonists (Benham, Bolton & Lang, 1985), but provides a large driving force for outward K^+ current (with a potassium Nernst potential of −78 mV). Thus, choosing a depolarized potential helps to detect Ca^{2+}-activated K^+ current virtually without interference from non-selective cationic current or from any Ca^{2+}-activated Cl^- current possibly present.

The amount of transferred charge (size of current × time = coulombs) in response to an agonist was estimated by measuring the area of the agonist-induced current response. The values in the text are the means ± S.E.M. Statistical significance was tested using an unpaired t test and differences were considered significant when $P < 0·05$.

Application of agonists

Carbachol or caffeine was introduced to the recording chamber by replacing the bathing solution with agonist-containing solution several times. Intracellular application of drugs such as $InsP_3$, $InsP_3S$, caged $InsP_3$, caged ATP and heparin was performed as follows: after obtaining a tight seal between the cell and a drug-filled pipette (on-cell patch), the patch membrane was ruptured by applying suction pulses to the pipette interior. The drug was allowed to diffuse into the cell under voltage clamp in the whole-cell recording mode.

Fluorescence measurements

In order to obtain some idea of the time course of diffusion into the cell of $InsP_3$ (molecular weight of trilithium salt 437) the fluorescent dye Indo-1 (molecular weight of pentasodium salt 759) was instead allowed to diffuse from the pipette. The influence of the difference in molecular weight on diffusion rate would be negligible, because the diffusion rate is affected by a factor of 1/cube root of molecular weight (Pusch & Neher, 1988). Single cells were centred under a circle of light created by a pinhole placed in the emission beam, and thus light collection was usually limited to an area including about 50–100% of the cell. Excitation was provided at 360 nm and emitted light was detected continuously after break-through of the membrane by two parallel photomultipliers at 420 and 480 nm.

Flash photolysis of caged substance

Photolysis of the caged substance was effected by a Model JML xenon arc flash lamp apparatus (240 J maximum output). The flash pulses (1 ms duration) were transmitted through a UG-11 filter (300–380 nm) which was positioned about 1·8 cm obliquely above the recording chamber, and the light intensity was adjusted via the capacitor charge voltage.

The efficiency of flash-induced hydrolysis in this experimental system was estimated by placing a 30 μl droplet containing 100 μM-caged $InsP_3$ or 600 μM-caged ATP on a cover-slip in place of the cells. A single flash (at a capacitor charge voltage of 300 V) photolysed approximately 1·5% of the caged $InsP_3$ (producing about 1·5 μM-$InsP_3$), as estimated by a bioluminescent method (Prestwich & Bolton, 1990), and a flash pulse (at 300 V and 385 V of an available maximum) converted 1·2 and 2·4% of the caged ATP, respectively, as estimated by high-performance liquid chromatography. The amount of released $InsP_3$ (up to 1·5 μM) was controlled by adjusting the light intensity. A small percentage conversion of caged compound at flash was desirable if repetitive pulses are intended to produce the same responses so an optical system giving a low percentage conversion was used.

Solutions and drugs

The physiological salt solution used in the experiments had the following composition (mM): NaCl, 126; KCl, 6; $CaCl_2$, 1·7; $MgCl_2$, 1·2; glucose, 14; HEPES, 10·5 (titrated to pH 7·2 with NaOH). Bathing solution without calcium had 3 mM-EGTA added, and a high-potassium solution had 25 mM-KCl substituted for NaCl. The patch-pipette solution had the following composition (mM); KCl, 134; $MgCl_2$, 1·2; MgATP, 1; EGTA, 0·05; glucose, 14; HEPES, 10·5 (titrated to pH 7·2 with NaOH). Pipette solutions containing $InsP_3$, caged $InsP_3$, caged ATP or a mixture of one of the first two and heparin were prepared by adding the highly concentrated substance (dissolved in distilled water or in the original pipette solution) in less than 5% of its total volume, to give the desired concentration.

In an attempt to protect $InsP_3$ from its metabolic inactivation in the cell, diphosphoglyceric acid (DGA, 2·5 mM) was added in the pipette solution which contained $InsP_3$. It had no effect in the present experiments and no effects on caffeine- or CCh-induced current responses.

Drugs used were ethyleneglycol-bis-(2-aminoethyl)tetraacetic acid (EGTA), adenosine 5′-triphosphate magnesium (MgATP), caffeine, carbachol chloride, heparin (from porcine intestinal mucosa; molecular weight 4000–6000), 2,3-diphospho-*d*-glyceric acid (DGA) (all from Sigma), D-*myo*-inositol 1,4,5-trisphosphate $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester trisodium salt (caged $InsP_3$), adenosine 5′-triphosphate P^3-1-(2-nitrophenyl)ethyl ester disodium salt (caged ATP), D-*myo*-inositol 1,4,5-trisphosphate trilithium salt ($InsP_3$) and Indo-1 pentasodium salt (Indo-1) (from

Calbiochem). $InsP_3$ was kindly donated by Calbiochem corporation. DL-*myo*-Inositol 1,4,5-trisphosphorothioate ($InsP_3S$) was synthesized by Dr B. V. L. Potter (Cooke, Gigg & Potter, 1987) and was kindly given to us by him.

RESULTS

Effects of $InsP_3$ entering the cell on the cell membrane current

To measure whole-cell currents at the instant that $InsP_3$ entered the cell, the holding potential was set at 0 mV in the cell-attached patch configuration and then the whole-cell recording mode was established by breaking through the membrane patch by pulses of suction. During this procedure, repetitive voltage pulses (10 mV, 200 ms) were usually applied at intervals of 0·8 s, so that establishment of the whole-cell mode could be judged by an increase of capacitative current. On some occasions this voltage protocol was interrupted during rupture of the membrane.

Control experiments

Without $InsP_3$ in the pipette, there was either no appreciable change in basal current upon break-through or sometimes outward current occurred. In the latter case, outward current reached a peak (up to 0·3 nA) 1 s or so after break-through, then decayed slowly in a roughly exponential manner and disappeared in 20–60 s depending on the size of the outward current. The average size of current deflection was $0{\cdot}15 \pm 0{\cdot}03$ nA ($n = 6$, range 0·05–0·30 nA). This outward current where it occurred may represent temporary damage to the pipette–cell seal and/or Ca^{2+} entry into the cell evoking outward current. Regardless of whether outward current occurred, spontaneous transient outward currents (STOCs; Benham & Bolton, 1986) appeared generally within a few seconds after break-through and then increased progressively in their size and/or discharge rate over 0·5–3 min to reach a stable level (Fig. 1*A*). STOCs are believed to represent the spontaneous, sporadic release of Ca^{2+} from storage sites in the cell in relation to Ca^{2+}-activated K^+ channels (Benham & Bolton, 1986). If caffeine (20 mM) was applied in the bathing solution to the cells after 3–6·5 min recording in whole-cell mode, then an outward current of 0·5–3·2 nA in size which continued for 5–15 s was elicited and it was followed by abolition of STOCs (Fig. 1*A*). Similar effects were obtained with bath application of carbachol (CCh, 100 μM; see Table 1). The resulting outward charge movement (current size × time; nC) was $3{\cdot}72 \pm 0{\cdot}68$ nC ($n = 11$) for caffeine responses and $3{\cdot}50 \pm 0{\cdot}64$ nC ($n = 7$) for the CCh responses. Caffeine (20 mM) elicited almost no response in the presence of CCh (100 μM), and vice versa. The above results suggested that both agonists act with a similar effectiveness on a common store to release Ca^{2+} (Bolton & Lim, 1989; Komori & Bolton, 1990).

Inclusion of 100 μM-$InsP_3$ in the pipette

With inclusion of $InsP_3$ (100 μM) in the pipette, a marked outward current was briefly evoked just following break-through, and then no STOCs were generally discharged for a time (1·5–7·5 min; Figs 1*B*, *C* and 3*A*). The time between the break-through artifact and the peak of the initial current was usually 0·7–1·5 s (eight out of nine recordings), and the current size varied from 0·4 to 3·5 nA, giving an average

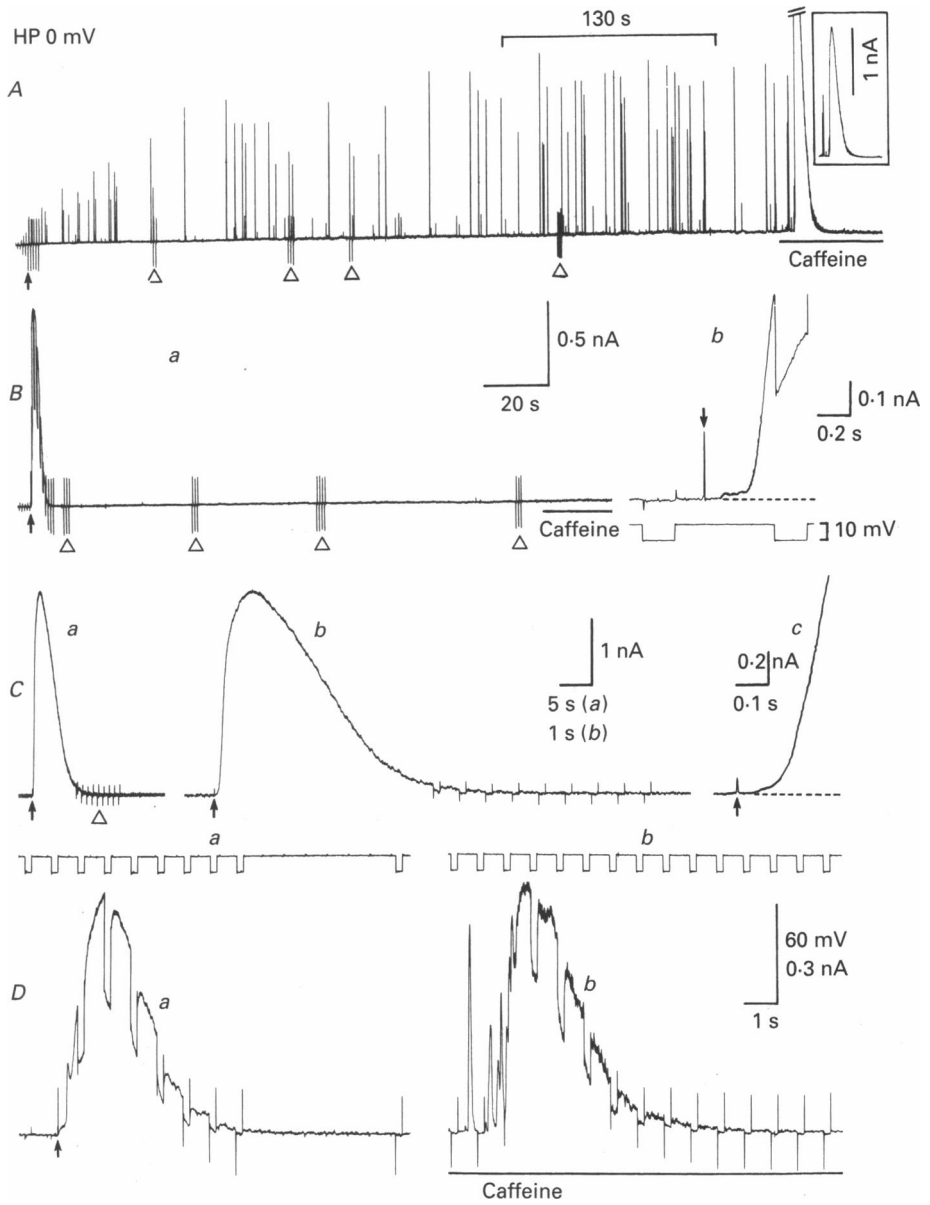

Fig. 1. Current responses to 100 μM-$InsP_3$ in the pipette at membrane break-through. Cells were voltage clamped at 0 mV, and the current was recorded. The transition to the whole-cell recording mode (↑) was obtained by breaking through the patch membrane with pulses of suction. Repetitive 10 mV, 200 ms hyperpolarizing pulses were usually applied at intervals of 0·8 s for a time before and after the break-through. Transition to the whole-cell mode resulted in an increase in the capacitative current. This voltage protocol was also used to check the access resistance during recording, and check points are indicated by open triangles (△) in this and subsequent figures. *A*, a typical current response of a control cell dialysed with control pipette solution without $InsP_3$. Note no notable change in the basal current just upon break-through (↑), a progressive development of spontaneous transient outward currents (STOCs) shortly after break-through, and a large current followed by abolition of STOCs in response to bath application of caffeine (20 mM). Time and size calibrations as for *Ba*. Inset shows caffeine

of 1·48 ± 0·36 nA (n = 9). (Table 1 includes results of these experiments and others in which heparin was present in the pipette; heparin did not affect the characteristics of the outward current so results with and without heparin were combined.) After reaching a peak, all the initial currents, except for one, declined to a stable level close to the pre-break-through level in 5–11 s. The large and transient current seen with

TABLE 1. Characteristics of $InsP_3$-, carbachol (CCh)- and caffeine-induced outward currents under voltage clamp

Agonist	Peak size (nA)	Time-to-peak (s)	50% decay (s)	n
100 μM-$InsP_3$	1·62 ± 0·26	1·10 ± 0·10	1·65 ± 0·13	15
100 μM-CCh	1·34 ± 0·20	1·34 ± 0·20	1·49 ± 0·34	7
20 mM-caffeine	1·60 ± 0·25	1·29 ± 0·12	1·35 ± 0·17	11

Figures represent means ± S.E.M. and number of experiments. $InsP_3$ with or without heparin was introduced into the cell by breaking through the patch membrane. Carbachol and caffeine were applied in the bathing solution. The agonist-induced outward currents were recorded at 0 mV. The characteristics for $InsP_3$-induced current were measured ignoring deflections produced by repetitive voltage pulses applied during the response (e.g. see Fig. 1*D*). The time-to-peak for CCh- or caffeine-induced current was counted from its onset and the corresponding time for $InsP_3$-induced current from the break-through artifact. The 50% decay time was the time taken for the response to decline to 50% of its peak size.

$InsP_3$ in the pipette compared to control cells where no outward current (or a smaller and longer lasting one) was seen, suggests that $InsP_3$ entering the cell at break-through discharges stored Ca.

Current recordings with an $InsP_3$-filled pipette were relatively less successful than control experiments without $InsP_3$ in the pipette, because in many cases a tight seal was lost during the transient current response at break-through. This happened especially during its decay phase, and input resistance decreased greatly, the holding current failing to settle down at a stable level near the pre-injection level before break-through.

As seen in the time-expanded traces of Fig. 1*Bb* and *Cc*, a delay between the break-through artifact and the onset of the initial current could be clearly seen in some recordings (40–90 ms; 66 ± 9 ms, n = 5). Also it can be seen in Fig. 1*Da* that the initial current response upon break-through was accompanied by a decrease in the

response at ×0·4 gain. *B*, current trace from a cell dialysed with a pipette solution containing $InsP_3$ (100 μM). *a*, note the large transient outward current response just following break-through. Subsequently bath application of caffeine (20 mM) elicited no response. *b*, current trace (upper) and voltage command (bottom) just before and after break-through. *C*, current response from another cell to $InsP_3$ (100 μM) at membrane break-through. Here, repetitive voltage pulses were not applied during breaking through the membrane. *a*, *b* and *c*, same record on different time scales and gains. Dashed line in *Bb* and *Cc* indicates current level before break-through. Note a delay between the break-through artifact and the onset of the response. *D*, comparison of the time course of the response of $InsP_3$ (100 μM) at break-through and that to bath application of caffeine (20 mM). *a*, current response from other cell to $InsP_3$ (100 μM) at membrane break-through. *b*, the caffeine-induced current recorded from a cell dialysed with normal pipette solution and held at 0 mV. During the recording, repetitive voltage pulses (10 mV, 200 ms; upper trace) were applied at intervals of 0·8 s. Note similar decreases in input resistance for the $InsP_3$- and caffeine-induced responses.

input resistance. However, the decreased input resistance seemed unlikely to result from a loss of tight seal between the cell and the pipette, since similar decreases of input resistance were observed during caffeine (20 mM)- or CCh (100 μM)-induced outward current in control cells (Fig. 1*Db*). This indicates that the decrease in input

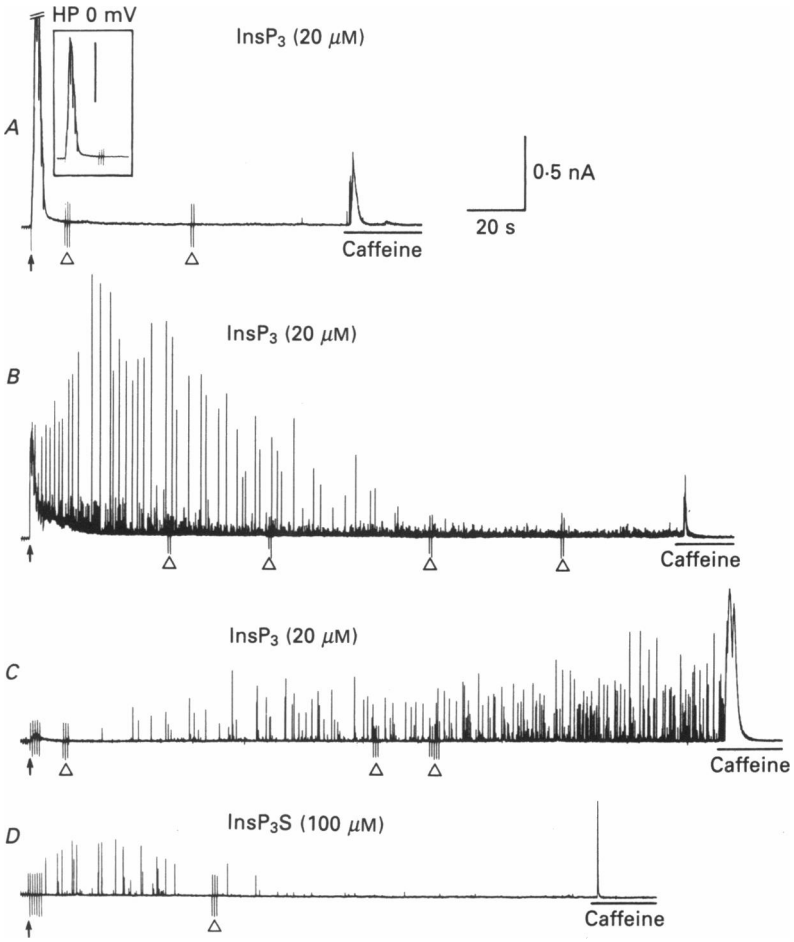

Fig. 2. Current response induced by cell dialysis at 0 mV with 20 μM-$InsP_3$ or 100 μM-$InsP_3S$-containing pipette solution. *A*, *B* and *C*, current traces from three different cells dialysed with $InsP_3$ (20 μM). In *A*, a marked outward current was evoked just following break-through (↑), no STOCs were seen, and a small response was induced by caffeine (20 mM). The inset shows $InsP_3$ response at ×0·4 gain. In *B* STOCs appeared immediately after break-through (↑), as in control (see Fig. 1*A*), but then declined in size, and the response to caffeine (20 mM) was very small. In *C* the current trace did not differ in any important respect from the control (see Fig. 1*A*). *D*, current trace from a cell dialysed with $InsP_3S$ (100 μM). Injection of $InsP_3S$ produced no outward current upon break-through in all tested cells ($n = 6$). See text for details.

resistance may represent an increase of membrane conductance (channel openings) associated with the current response. Thus, it was considered that the initial currents recorded with a pipette containing 100 μM-$InsP_3$ do not result from any artifact or damage to the cell associated with suction. Recently, using the same technique,

Ca^{2+}-activated current responses to $InsP_3$ have been obtained just after rupturing the membrane in acinar cells of rat pancreas (Maruyama, 1989) and lacrimal glands (Marty & Tan, 1989).

If caffeine (20 mM) was applied after 1·5–7·5 min (average 3·8 ± 0·7 min, $n = 8$) dialysis with the $InsP_3$ (100 μM) pipette solution, there was no response to caffeine recorded at 0 mV ($n = 6$) or only a small outward current ($n = 2$). The average outward charge transfer was only 0·10 ± 0·07 nC ($n = 8$), 2·6% of the control value (Fig. 4). In experiments in which a pipette containing 100 μM-$InsP_3$ produced similar initial currents (0·4–2·2 nA, $n = 3$), CCh (100 μM) application elicited no response 2–3 min after break-through. These results suggest that inclusion of 100 μM-$InsP_3$ in the pipette causes prolonged and lasting depletion of caffeine- or CCh-releasable Ca stores.

Inclusion of 20 μM-$InsP_3$ in the pipette

With inclusion of $InsP_3$ (20 μM) in the pipette, the current response on break-through was variable. Three types of response were commonly observed. In one type, STOCs were not or were infrequently seen for a time (1·5–5 min) after break-through as with 100 μM-$InsP_3$ ($n = 7$), and application of caffeine (20 mM) produced a relatively small outward current (Fig. 2*A*) or occasionally no response. The initial current just following break-through varied in size from 0·15 to 2·0 nA, and in four cells it was similar in time course to those commonly seen with 100 μM-$InsP_3$ (range 0·4–2·9 nA, mean 1·20 ± 0·42 nA, $n = 4$) (Fig. 2*A*). In a second type of cell, initial current responses ($n = 3$) were small in size and contained a slowly subsiding current component. STOCs were discharged soon after break-through and accelerated in size to reach a maximal level within 20–40 s, but then STOCs progressively disappeared or decreased in size within 1·5–3·5 min (2·8 ± 0·4 min, $n = 5$). Application of caffeine (20 mM) elicited no response or a small outward current followed by abolition of the residual STOCs (Fig. 2*B*). Such depression of STOCs was seen with, and also almost without, an initial current deflection upon break-through which was usually of the slowly subsiding type seen in the control ($n = 3$). There was no correlation between the time taken for STOCs to be inhibited (1·5–3·5 min) and the size of initial current deflection upon break-through (0–0·66 nA) (correlation coefficient $r = -0·256$, $n = 5$). The effects of $InsP_3$ on STOC discharge and on the caffeine response were reminiscent of those of GTPγS injection (Komori & Bolton, 1990). In a third type of cell the response did not differ appreciably from the control responses: no current or a slowly subsiding current deflection (up to 0·3 nA) upon break-through, the usual pattern of STOC discharge, and an outward current response to caffeine (20 mM) followed by abolition of STOCs were seen ($n = 6$) (Fig. 2*C*). In one cell, however, a sustained discharge of relatively large and high-frequency STOCs was seen. Such ineffectiveness of $InsP_3$ cannot be due to a predominant population of $InsP_3$-insensitive Ca stores in these cells nor to desensitization to $InsP_3$, since if CCh (100 μM) was applied to such cells than an outward current followed by abolition of STOCs was elicited. CCh is believed to act on Ca stores through the production of $InsP_3$ (Komori & Bolton, 1990).

If all the initial current deflections (0·04–2·0 nA) observed upon break-through were averaged, the mean was 0·050 ± 0·16 nA ($n = 14$). When caffeine (20 mM) was

applied after 1·5–7 min (average 3·9 ± 0·6 min, $n = 18$) dialysis with the 20 μM-$InsP_3$ pipette solution, the outward charge transfer was 1·51 ± 0·32 nC at 0 mV; this was significantly ($P < 0·01$) smaller than the control (3·72 ± 0·68 nC, $n = 11$) (Fig. 4).

The effects of the $InsP_3$ analogue DL-*myo*-inositol 1,4,5-trisphosphorothioate ($InsP_3S$) were also examined. The D-isomer of this compound acts as an agonist on

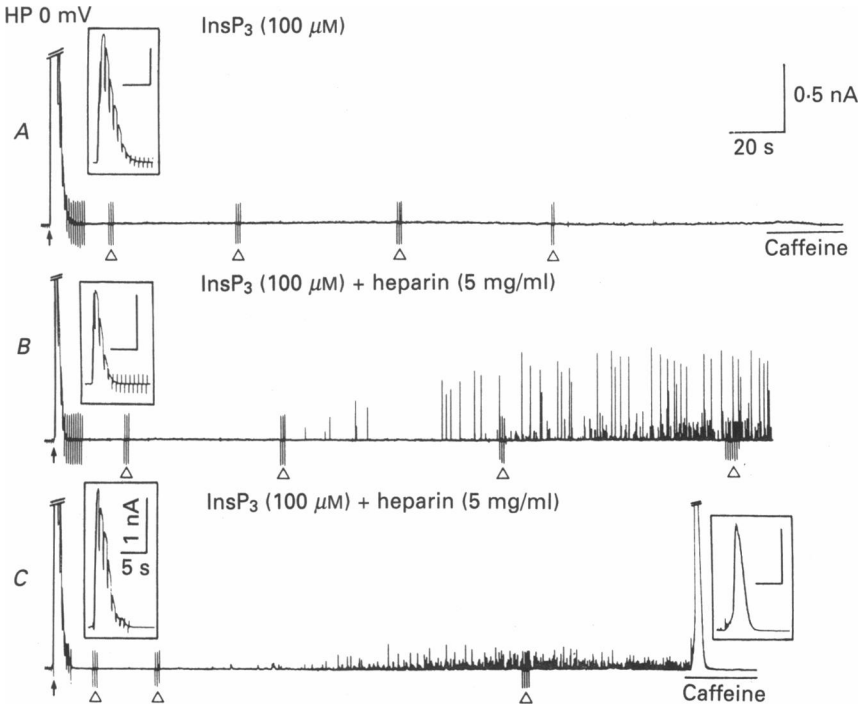

Fig. 3. Effects of inclusion of both $InsP_3$ and heparin in the pipette solution on the membrane current at 0 mV. *A*, control current trace of a cell of this series dialysed with $InsP_3$ (100 μM). The effects of $InsP_3$ are substantially similar to those shown in Fig. 1*B*. *B* and *C*, current traces from different cells dialysed with both $InsP_3$ (100 μM) and heparin (5 mg/ml). In *C*, caffeine (20 mM) was applied externally. Note a large initial current upon break-through, a progressive appearance of STOCs and a large current upon caffeine (20 mM) application. Each inset in *A*, *B* and *C* shows the full-sized response. Calibrations are 1 nA and 5 s (insets) and 0·5 nA and 20 s.

$InsP_3$ receptors in other systems and is resistant to enzymic attack by the 5-phosphomonoesterase and the 3-kinase (Nahorski & Potter, 1989). In four of five cells where 100 μM-$InsP_3S$ was present in the pipette solution, caffeine or carbachol produced no, or little, outward current; STOCs were absent or soon disappeared after achieving whole-cell recording mode in four of six cells. Outward current at break-through was not seen (Fig. 2*D*), however. In four cells where 20 μM-$InsP_3S$ was used, STOCs were normal (two cells) or small (two cells) and caffeine outward current was small in one of two cells where it was applied. If both 100 μM-$InsP_3S$ and 100 μM-$InsP_3$ were present in the pipette solution (two cells), the effects were indistinguishable from 100 μM-$InsP_3$ alone i.e. outward current at break-through was seen.

Inclusion of both heparin and $InsP_3$ in the pipette

Heparin has been shown to be a selective antagonist of $InsP_3$ binding to its receptor (Worley *et al.* 1987; Hill *et al.* 1987; Ghosh *et al.* 1988; Kobayashi *et al.* 1988), and it can also block the CCh-induced, but not the caffeine-induced, outward current in single smooth muscle cells of the rabbit jejunum (Komori & Bolton, 1990). Therefore, the effects of heparin on the actions of 100 μM-$InsP_3$ were investigated by including both heparin (5 mg/ml) and $InsP_3$ (100 μM) in the pipette.

Just following break-through of the patch membrane, the cell exhibited usually an initial current similar to those seen in the case where the pipette contained $InsP_3$ alone (0·8–2·7 nA, $n = 7$; Fig. 3*B* and *C*). However, in two other cells the initial current was small (0·1–0·3 nA) and slower in its decay. The average current in all experiments was $1{\cdot}40 \pm 0{\cdot}32$ nA ($n = 9$), not significantly different from the value for 100 μM-$InsP_3$ alone ($1{\cdot}48 \pm 0{\cdot}36$ nA; $n = 9$). After a 1–2 min dialysis, STOCs usually began and then increased progressively in their size and frequency to reach a stable level 3·5–6 min after break-through (Fig. 3*B* and *C*). This is in contrast to cells dialysed with 100 μM-$InsP_3$ alone where STOCs did not appear (Fig. 3*A*); the observation implies that with time, dialysis with heparin blocks the Ca-depleting action of $InsP_3$ on stores. This suggestion was supported by the observation that if caffeine (20 mM) was applied $4{\cdot}8 \pm 0{\cdot}3$ min ($n = 6$) after starting dialysis, an outward current followed by abolition of STOCs was elicited (Fig. 3*C*). The average charge movement during outward caffeine current was $4{\cdot}19 \pm 0{\cdot}98$ nC ($n = 6$), similar to the control value (Fig. 4).

The results suggest that at break-through $InsP_3$ can induce a rapid and significant Ca^{2+} release of caffeine (or CCh)-releasable Ca stores and heparin may allow the stores to be refilled in time with Ca by its interference with the action of $InsP_3$. The failure of heparin to block the transient response to $InsP_3$ just after break-through can be explained probably by the difference in diffusion rate resulting from their different molecular weights (Pusch & Neher, 1988). Blockade of the direct action of $InsP_3$ on Ca stores by heparin will be demonstrated below.

Characteristics of the current response to $InsP_3$ at break-through

The amplitude, time-to-peak and 50% decay time measured for the current response to $InsP_3$ (100 μM) observed at break-through into the whole-cell mode are presented in Table 1. Heparin was without apparent effect on the response so results were combined. The parameters were similar to those for the outward currents which were obtained by bath application of caffeine (20 mM) or CCh (100 μM) on cells dialysed with normal pipette solution and held at 0 mV. On the basis of these similarities (especially the same peak size), and that the application of any one of the three agents abolished the response to either of the other two subsequently applied, it can be suggested that the break-through current to $InsP_3$ may reflect the complete release of all releasable Ca stores. Despite metabolism of $InsP_3$, 100 μM in the pipette is sufficient to maintain Ca-store depletion for a prolonged period.

Diffusion profile of Indo-1

The current response to $InsP_3$ upon break-through seemed to involve all releasable Ca stores, as it was as large as the response to caffeine or CCh applied to the whole cell in the bathing solution. An attempt was made to follow the diffusion process

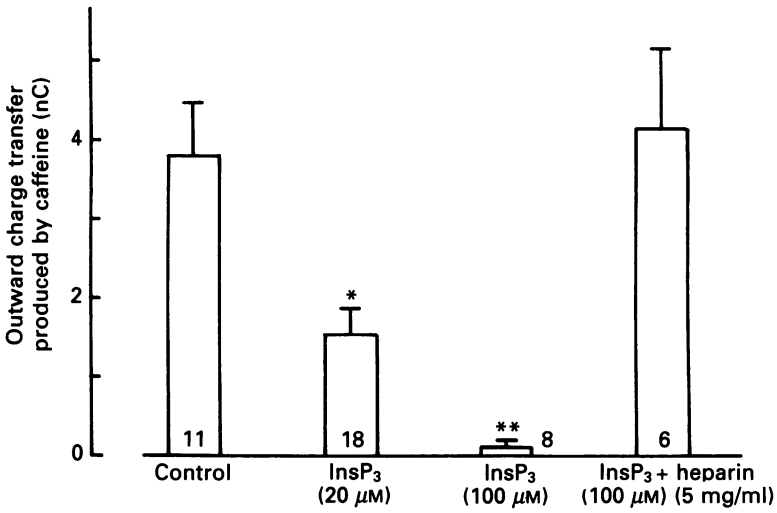

Fig. 4. Summary of the effects of $InsP_3$ alone, and of both $InsP_3$ and heparin, on the caffeine-induced outward current. Cells were dialysed with control solution, $InsP_3$ (20 or 100 μM), or both $InsP_3$ (100 μM) and heparin (5 mg/ml)-containing pipette solution, and the response to caffeine (20 mM) was recorded at 0 mV. Dialysis was for an average period of $4{\cdot}9 \pm 0{\cdot}4$ min for control, $3{\cdot}9 \pm 0{\cdot}6$ min for 20 μM-$InsP_3$, $3{\cdot}8 \pm 0{\cdot}7$ min for 100 μM-$InsP_3$ and $4{\cdot}8 \pm 0{\cdot}3$ min for both 100 μM-$InsP_3$ and 5 mg/ml heparin. Ordinate scale: outward charge movement (current size × time, nC), which was estimated by measuring the area of caffeine-induced response. Each column represents the mean obtained on the indicated number of cells; vertical line indicates one S.E.M. $*P < 0{\cdot}01$, $**P < 0{\cdot}001$.

using, instead of $InsP_3$, the fluorescent dye Indo-1 (60 μM, a concentration intermediate between the two $InsP_3$ concentrations used). The method for Indo-1 was similar to that for $InsP_3$ injection, except for the holding potential which was set at -40 to -60 mV. This favours the diffusion into the cell of molecules with negative charge such as $InsP_3$ or Indo-1.

Figure 5 shows fluorescence changes after break-through. In the graph, the fluorescence intensity is expressed relative to the intensity of peak fluorescence finally attained in the cell (at 420 nm) and is plotted against the time after break-through. The fluorescence started to increase in a roughly exponential manner immediately after break-through. Peak fluorescence was achieved in 90–180 s (132 ± 13 s, $n = 7$). Assuming a single-exponential fit, a normalized diffusion curve was obtained from the data, which was fitted by the equation $y = 1 - \exp(-0{\cdot}019t)$ where t is the time (s) after break-through and y is the relative value of fluorescence in the cell. Fluorescence measurements made simultaneously at 480 nm gave the same result. For comparison with the profile of Indo-1 diffusion into the cell, an averaged

$InsP_3$ current upon break-through is shown on the same graph in Fig. 5. It can be seen that the time course of the $InsP_3$ current was much faster than the diffusion profile of Indo-1 entering the cell and that peak calcium release corresponded to a time when the concentration of Indo-1 was about 2% of its steady-state value

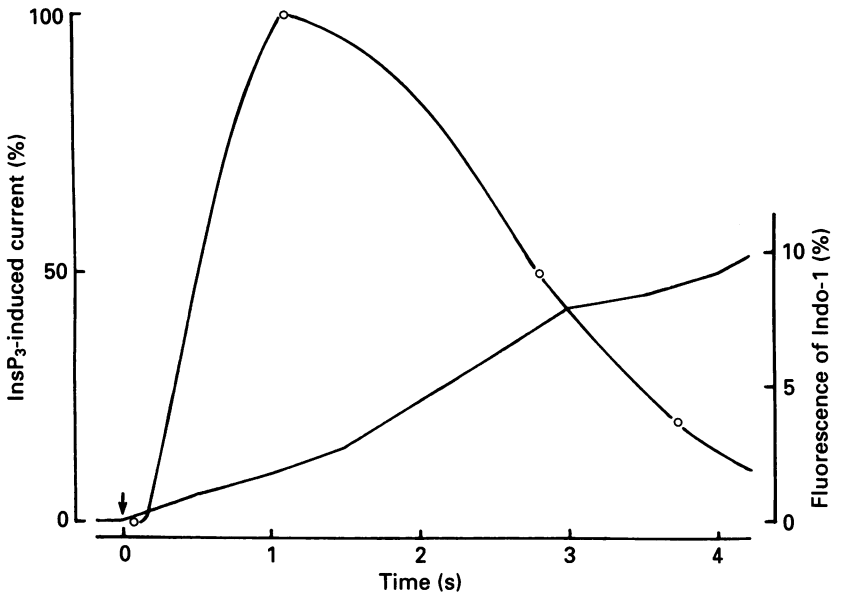

Fig. 5. Comparison of time course of the $InsP_3$-induced current with the fluorescence signal recorded in a cell after break-through using a pipette solution containing 60 μM-Indo-1. Fluorescence (at 420 nm) was continuously monitored under voltage clamp (see Methods). The $InsP_3$-induced currents were from those in Table 1. Ordinate, relative value of fluorescence or current; abscissa, time (s) after break-through (↓). The open circles represent data from the current responses in twelve to fifteen cells (not all points shown for clarity) and the other line is the fluorescence signal from a typical recording in a single cell. The current response shown represents a normalized time course of the $InsP_3$-induced current (from Table 1), which was constructed by connecting the time of break-through ($t = 0$) and the average values for the time-to-peak and to 50 and 80% decay times.

(60 μM). Allowing for enzymic destruction, this indicates that the effective concentration of $InsP_3$ which enters the cell to release stored Ca is less than 2 μM, since 100 μM- and sometimes 20 μM-$InsP_3$ in the pipette could evoke outward current at break-through.

Effects of flash photolysis of caged $InsP_3$ on membrane currents

Single jejunal cells were held under voltage clamp at 0 mV (as usual) using pipettes filled with solution containing caged $InsP_3$ (30 or 100 μM). Holding current and STOC discharge normally remained stable unless a flash pulse was applied.

Application of a single flash pulse elicited a rapid outward current lasting 1–8 s and up to about 4 nA in size; this was followed by abolition or depression of STOC discharge for a period (Fig. 6*B*, *C* and also see Fig. 10*A*, *B*). When pipettes filled with

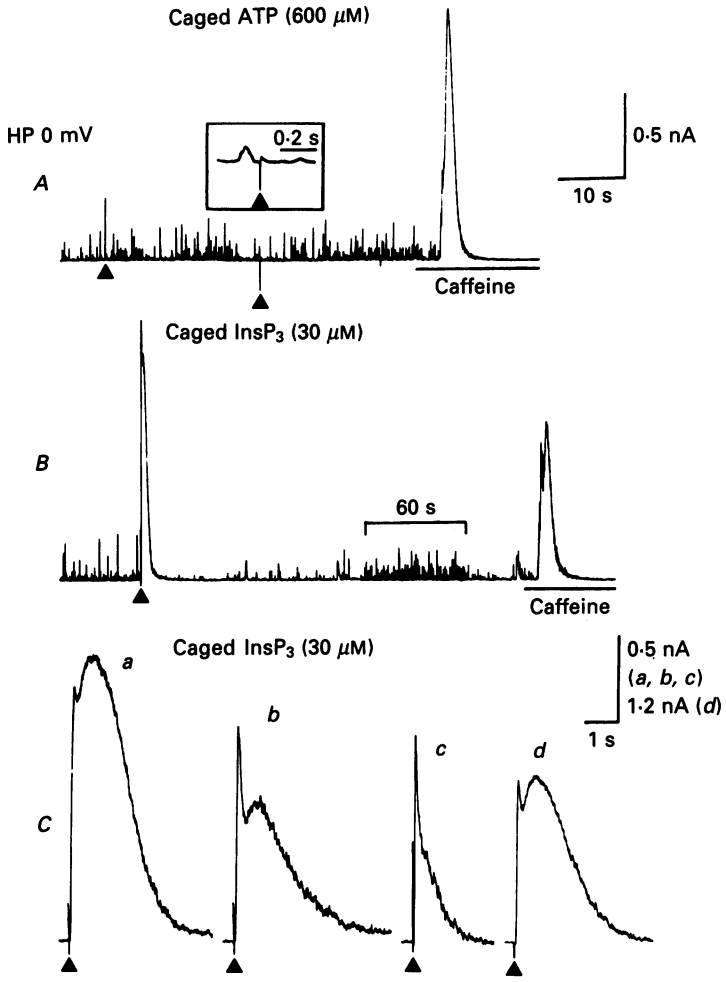

Fig. 6. Current responses to flash photolysis of caged compounds. Cells were dialysed with pipette solution containing the caged substance. Light-induced photolysis was induced using a xenon arc flash apparatus, and the current response of the cell was recorded at 0 mV. Application of a single flash pulse is marked with the closed triangle on each current trace in this and in subsequent figures. *A*, current trace from a cell dialysed with caged ATP (600 μM). No change in basal current or STOC activity is seen in response to a flash pulse (also see the inset of a time-expanded trace) and this is followed by a normal response to caffeine (20 mM). Note there is no effect of the flash itself or of flash-released by-product, 2-nitrosoacetophenone. Here, the effects of flash-released ATP, if any, may not show themselves because of the presence of 1 mM-ATP in the pipette solution. *B* and *C*, current traces from cells dialysed with caged $InsP_3$ (30 μM). In *B*, application of a flash pulse evoked an outward current followed by inhibition of STOCs similar to the response to caffeine (20 mM). In *C*, several responses to flash-released $InsP_3$ are shown to illustrate the variation seen. The response basically consisted of two components, an initial rapid and a subsequent slower component. *a* and *b*, from two different cells; *c* and *d*, from one other. The amount of $InsP_3$ released is more in *d* than in *c*. Note calibration size in *C*. Calibration in *A* also applies to *B*.

normal solution or solution containing caged ATP (600 μM) were used instead (six and four cells, respectively), no changes in basal current or STOC discharge occurred even upon application of a light flash. In these cells bath application of caffeine (20 mM) elicited a normal outward current in all cells (Fig. 6*A*). In the experiments

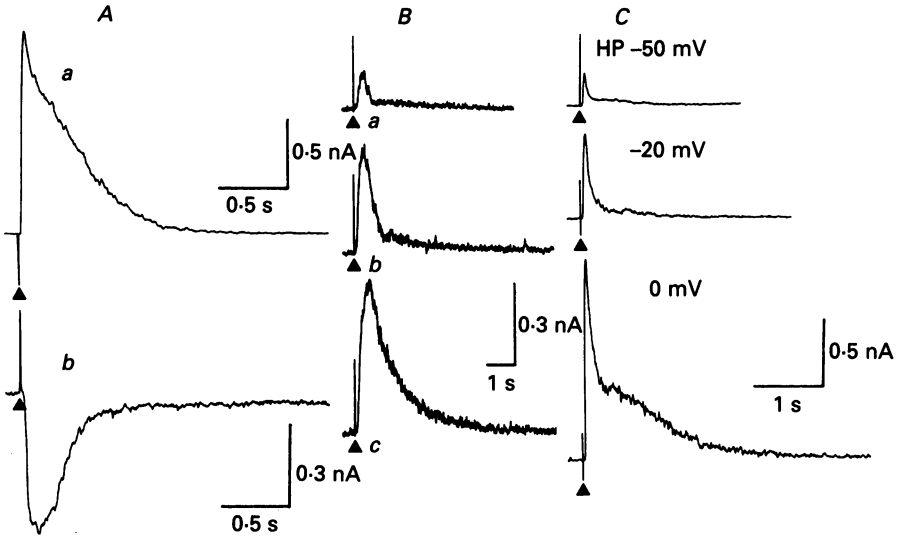

Fig. 7. Current responses to flash-released $InsP_3$. Cells were dialysed under voltage clamp with a solution containing caged $InsP_3$ (100 μM). *A*, high-speed current traces. Response to a flash pulse: from a cell held at −20 mV in 6 mM-external KCl (*a*); from another cell held at −60 mV in 25 mM-external KCl (*b*) (see also Fig. 8*B*). A sharp deflection, the artifact associated with application of flash pulse, precedes the onset of the current responses by 20–30 ms. *B*, effects of increasing the amount of released $InsP_3$. The amount was increased from *a* to *c* by increasing the intensity of the light flash. The current responses were recorded at 0 mV. *C*, effects of changing the holding potential (HP) as indicated. Note that peak size and response duration, but not latency and time-to-peak, of the $InsP_3$-induced current are noticeably dependent on the amount of released $InsP_3$ and on the holding potential.

with pipettes filled with caged ATP, any effects of flash-released ATP do not show themselves because the pipette solution also contained 1 mM-ATP (see Methods). The flash-induced release of the cage, 2-nitrosoacetophenone, was also without effect. Thus, the current response to flash photolysis of caged $InsP_3$ is very likely to be due to $InsP_3$ produced photochemically.

Temporal parameters of the $InsP_3$-induced current

The outward current responses to flash-released $InsP_3$ occurred with a delay after the flash pulse (duration about 1 ms) which was detected as an artifact on the pen recorder (Fig. 7*Aa*). This delay, the latency for the $InsP_3$-induced response, was not noticeably dependent on the amount of $InsP_3$ released (Fig. 7*B*) nor on the holding potential (at least from −60 to 20 mV; Fig. 7*C*). From fifteen measurements in

thirteen cells held at −20 or 0 mV and dialysed with 100 μM-caged $InsP_3$, a mean latency of 31·0 ± 1·8 ms was estimated with a range of 25–50 ms. As the latency was counted from the beginning of the artifact, which had a duration of 10 ms or so, then the values for the latency shown here might be somewhat overestimated. When the

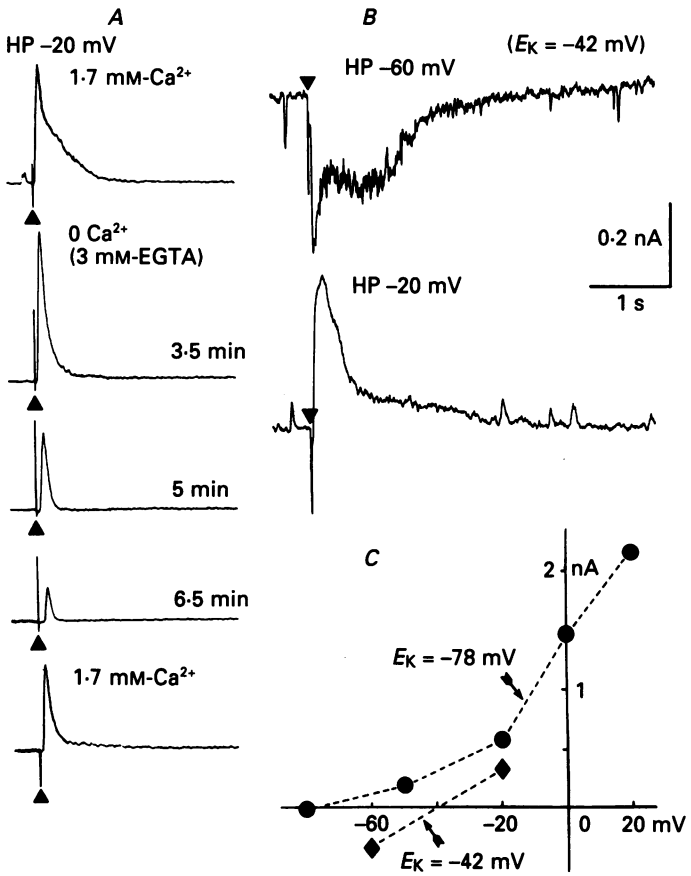

Fig. 8. Influence of the removal of external Ca^{2+} on the current response to flash-released $InsP_3$. Cells were dialysed with solution containing caged $InsP_3$ (100 μM), and the current response was recorded at the indicated holding potentials (HP). *A*, current responses to a constant flash pulse (▲) before (top) and 3·5 min, 5 min and 6·5 min after removal of external Ca^{2+} from bathing solution (3 mM-EGTA added), and (bottom) 2 min after re-admission of calcium (1·7 mM) to the bathing solution. *B*, reversal potential of the current response to release of $InsP_3$ by flash. The Nernst potential of K ions (E_K) was −42 mV (external and internal KCl, 25 and 134 mM, respectively). The current was inward at −60 mV HP (top trace) and outward at −20 mV HP (bottom trace). Records from the same cell. Calibration also applies to *A*. *C*, relationship between peak size of current response to flash-released $InsP_3$ and HP. ●, an experiment using a standard external solution with 6 mM-KCl (E_K was −78 mV, the same cell as in Fig. 7*C*); ◆, another experiment using an external solution with 25 mM-KCl (E_K was −42 mV, the same cell as *B*). Note that the reversal potential of the current closely follows E_K.

current response to flash-released $InsP_3$ was inward (see below), the latency was also between 25 and 50 ms ($n = 5$ from four cells) (Fig. 7*Ab*).

The current response rose rapidly to a peak 40–150 ms (76 ± 10 ms, $n = 15$) after

its onset, and its decay phase had diverse shapes. In some cells a change of slope or shoulder appeared on the decay phase. In other cells a second peak was visible (see Fig. 6*C*). The time-to-peak for the latter component was 534 ± 49 ms ($n = 7$), counted from the onset of the response, and the 50% decay time was at 761 ± 88 ms ($n = 7$). Its duration, as well its amplitude, was dependent on the amount of $InsP_3$ released (Figs 6*C* and 7*B*), and the latter component contributed particularly to the increase in duration.

Influence of Ca^{2+}-free solution

After 3–5 min in Ca^{2+}-free (3 mM-EGTA added) bathing solution, flash-released $InsP_3$ still evoked a substantial response, and this could still be elicited after 6·5 min in Ca^{2+}-free conditions (Fig. 8*A*). Latency increased in Ca^{2+}-free conditions and the duration of the $InsP_3$-induced current became briefer as under these conditions the stores probably begin to lose some of their Ca content. The result indicates that $InsP_3$-induced currents involve Ca^{2+} release from internal stores rather than Ca^{2+} entry into the cell although a contribution of Ca^{2+} entry to the shoulder or second phase of the response cannot be excluded.

Reversal potential for $InsP_3$-induced current

In normal bathing solution with 6 mM-KCl ($E_K = -78$ mV), the current response to flash-released $InsP_3$ was almost undetectable at -80 mV and displacement of the holding potential in a depolarizing direction resulted in a graded increase of outward current response to $InsP_3$ (Fig. 8*C*). In 25 mM-KCl-containing solution ($E_K = -42$ mV) $InsP_3$-induced current was inward at -60 mV and outward at -20 mV (Fig. 8*B*), such that the interpolated reversal potential of the current was close to E_K (Fig. 8*C*). Therefore, $InsP_3$-induced currents result from an increased K^+ conductance (Ca^{2+}-activated K^+ channels), and contributions from other ionic conductances were negligible in these cells.

Effects of caffeine and CCh on $InsP_3$-induced current

The current response to flash-released $InsP_3$ was absent after bath application of caffeine (20 mM; Fig. 9*A*) or CCh (100 μM; Fig. 9*B* and *C*) to deplete releasable Ca stores. Return to drug-free bathing solution allowed recovery and an outward current could be elicited in response to flash-released $InsP_3$ after 1 min or more. The results suggest again that $InsP_3$ acts on substantially the same Ca store as caffeine and CCh.

Effect of heparin on $InsP_3$-induced current

Heparin failed to block the transient current elicited by $InsP_3$ at break-through (see above), probably because of its slower diffusion rate. Therefore, the effect of heparin on $InsP_3$-induced current was investigated using caged $InsP_3$, where heparin could be allowed to diffuse first into the cell to reach an effective blocking concentration. Experiments were made alternatively on cells dialysed with caged $InsP_3$ (30 μM) alone (control) and with both caged $InsP_3$ (30 μM) and heparin (0·5 or

5 mg/ml). Current responses to flash-released $InsP_3$ were recorded at 0 mV after 4–8 min dialysis. In the control experiments, all the cells tested ($n = 13$) responded to flash-released $InsP_3$ with an outward current followed by suppression or abolition of STOC discharge for a time although the peak size of current was variable from cell

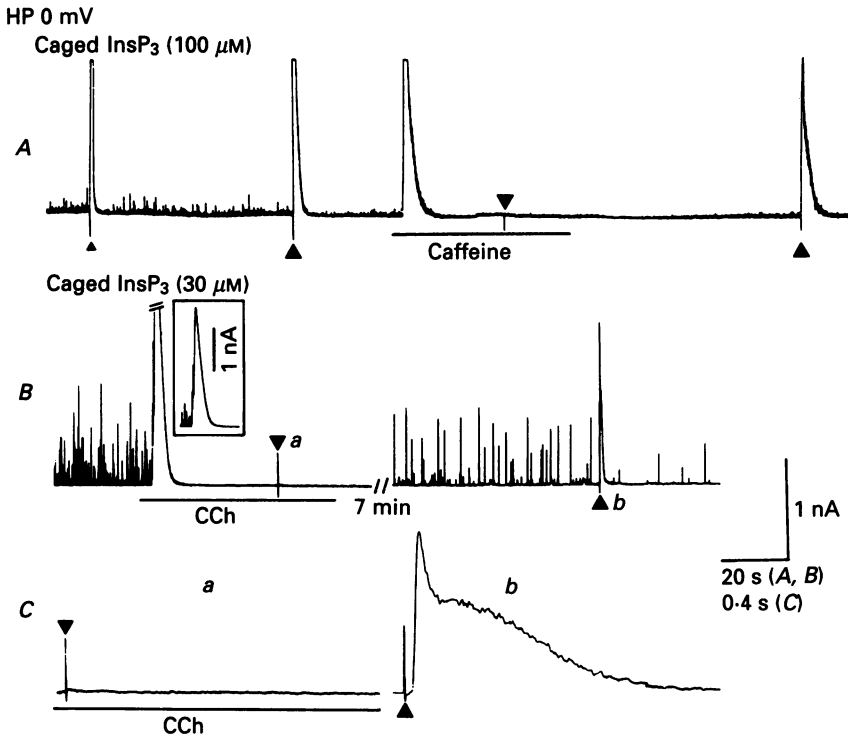

Fig. 9. Effects of caffeine and carbachol (CCh) on the current response to flash-released $InsP_3$. *A*, current trace from a cell dialysed with caged $InsP_3$ (100 μM). The response to flash-released $InsP_3$ (the first response marked by a smaller triangle (▲) was to a weaker intensity of flash than the others) was absent after application of caffeine (20 mM) which released Ca stores but reappeared after removal of caffeine and washing. The outward current responses exceeded the pen excursion of the recorder used here. *B*, current trace of another cell dialysed with caged $InsP_3$ (30 μM). Carbachol (100 μM) application elicited an outward current (see inset for its full size) followed by abolition of STOCs, and in the presence of CCh when Ca stores were depleted a flash pulse was without effect. After removal of CCh and washing a flash pulse now elicited an outward current followed by abolition of STOCs. The current trace was interrupted for 7 min as indicated. *C*, time-expanded traces from *B* as marked. Current calibration is the same for *A*, *B* and *C*.

to cell (range 0·5–3 nA, mean $1·66 \pm 0·24$ nA; $n = 13$) (Fig. 10*A* and *B*). In contrast, when both caged $InsP_3$ and heparin were included in the pipette, no current response was elicited in response to flash pulses, even at maximum available intensity ($n = 5$ and $n = 6$ for 0·5 and 5 mg/ml of heparin, respectively) (Fig. 10*C*). In these cells a normal current response was evoked by application of caffeine (20 mM), as would be expected.

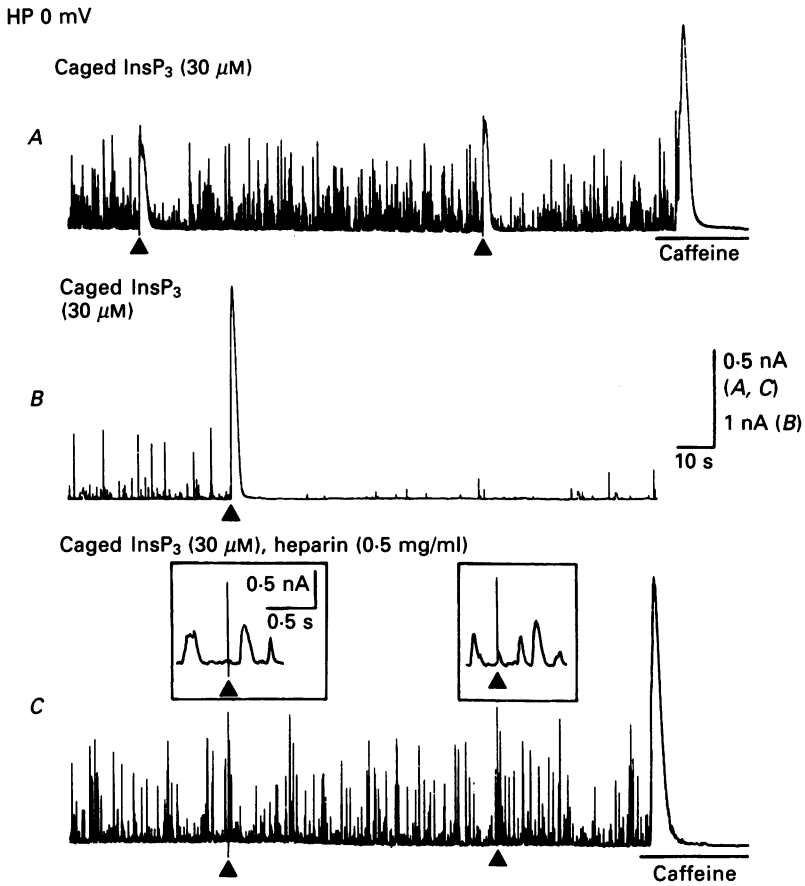

Fig. 10. Effects of heparin on the current response to flash-released $InsP_3$. Cells were dialysed with caged $InsP_3$ (30 μM) or both caged $InsP_3$ (30 μM) and heparin (0·5 mg/ml) pipette solution and the outward current response was recorded at 0 mV. *A* and *B*, control responses to flash-released $InsP_3$ of two different cells. In *A*, after application of the flash pulses, caffeine (20 mM) was applied externally. *C*, with both caged $InsP_3$ (30 μM) and heparin (0·5 mg/ml) in the pipette, flash pulses were without effect (see also time-expanded traces in insets) but a normal response to caffeine (20 mM) was elicited.

DISCUSSION

These results show that $InsP_3$ causes a rapid release of Ca^{2+} from store sites in single jejunal smooth muscle cells and that $InsP_3$ can mimic the Ca^{2+}-releasing action of CCh. The application of $InsP_3$ in the pipette solution to the interior of the cell in high concentrations depletes Ca stores which are also releasable by caffeine or CCh externally applied. The failure of Ohya *et al.* (1988) to observe such depletion was mainly because they used lower concentrations than those used in these experiments. The outward current response to $InsP_3$ entering the cell at break-through was similar to the CCh-induced response in time and size (see Table 1). Carbachol outward current was blocked by heparin introduced into the cell (see also Komori & Bolton,

1990), as was the response to $InsP_3$ photolytically released within the cell. Release of Ca^{2+} by caffeine was, however, not blocked by heparin. These results strongly support the idea that in these cells production of $InsP_3$ as a result of muscarinic receptor activation is essential and sufficient for Ca store release (Komori & Bolton, 1990).

There was a considerable difference in latency, however, between the onset of actions of $InsP_3$ and CCh. Outward current due to Ca-store release upon ionophoretic application of CCh did not begin until the end of the 0·5 s releasing pulse (Lim & Bolton, 1988; Bolton & Lim, 1989) suggesting a latency of around 0·5 s or more. In contrast, the latency for the current upon flash-release of $InsP_3$ was 31 ms on average, with a small variation (25–50 ms). Since $InsP_3$ is produced with a half-time of 3 ms by flash photolysis of caged $InsP_3$ (Walker *et al.* 1987), and an increase in Ca^{2+}-activated K^+ channel opening by Ca^{2+} entry upon depolarization is detectable within 2 ms (see Horn & Marty, 1988), the latency we see here may represent mainly the time taken for the steps: $InsP_3$ binding, $InsP_3$-receptor activation, opening of sarcoplasmic reticulum Ca^{2+} channels (Ehrlich & Watras, 1988) and Ca^{2+} release into the cytoplasm close to the cell membrane. Thus, the extra time for the CCh-induced response presumably reflects the time taken for the following steps: the binding of CCh to the muscarinic receptor, its activation, exchange of GTP for GDP on the α-subunit of the G-protein, dissociation of the α-subunit and its binding to the phospholipase C (if indeed there is a G-protein intermediate step, Komori & Bolton, 1990), activation of the enzyme and cleavage of $InsP_3$ from membrane phosphatidylinositol 4,5-bisphosphate.

In the present experiments, flash-released $InsP_3$ produced no detectable inward currents under conditions in which inward currents are elicited in response to CCh (Benham *et al.* 1985; Bolton & Lim, 1989; Komori & Bolton, 1990). Furthermore the fact that the reversal potential of the current upon a flash pulse closely followed E_K, indicates no contribution from the non-selective cationic current which is responsible for the inward CCh current and which has a reversal potential of around 0 mV (Benham *et al.* 1985). Thus, the results support the view that following stimulation of the muscarinic receptors, the non-selective cationic channels are induced to open by a system which involves neither $InsP_3$ action nor Ca store release (Bolton & Lim, 1989; Komori & Bolton, 1990).

$InsP_3$ produced two types of effect on Ca stores: rapid depletion leading to a Ca^{2+}-activated outward K^+ current response and a slower action leading to store depletion without evoking outward current. The latter effect was associated with a depression or loss of STOC discharge and of outward current responses to caffeine or CCH. The slow depletion is compatible with a diffusion-limited process (cf. the rate of rise of the Indo-1 signal) and this effect was sometimes seen alone with 20 μM-$InsP_3$. It is very similar to the effects of GTPγS or ryanodine when these are added to the pipette solution (Bolton & Lim, 1989; Komori & Bolton, 1989, 1990).

The brief outward current usually seen upon membrane break-through when 100 μM-$InsP_3$ was present in the pipette had the following features: (1) it started to rise after a latency of 66 ms, reached a peak in 1·1 s and lasted several seconds or so; (2) it was similar in size and time course to the responses to bath-applied caffeine and to carbachol; (3) lowering the concentration of $InsP_3$ in the pipette reduced the

fraction of cells which exhibited this brief current upon break-through; (4) it was monophasic. In contrast, flash-released $InsP_3$ elicited a biphasic response and the difference presumably arises because it is uniformly distributed throughout the cell whereas $InsP_3$ from the pipette at break-through enters at a single, localized point. However, the duration of the flash-released $InsP_3$ response was reduced and the second phase lost in Ca^{2+}-free conditions. It also tended to be lost if the cell was held at negative potentials perhaps because the Ca stores contain less Ca.

However, it is curious how the transient current resulting from the release of the Ca store was elicited immediately upon membrane break-through. Experiments with flash photolysis of caged $InsP_3$ revealed that $InsP_3$ could elicit a large current response at submicromolar concentrations (see Methods). Assuming that the $InsP_3$ concentration in the bulk of the cell after membrane break-through is similar to the diffusion curve for Indo-1, and that the maximum fluorescence achieved indicates a concentration equal to its pipette concentration, experiments suggest a cytoplasmic $InsP_3$ concentration 1·1 s after break-through of less than 2 μM with a 100 μM-$InsP_3$-filled pipette. This concentration seems to be enough for the release of all releasable Ca^{2+} in the cell to be induced by $InsP_3$ as the outward current was as large as that evoked by CCh or caffeine applied to the whole cell.

Flash-released $InsP_3$ elicited basically a biphasic response at 0 mV which consisted of an initial brief component (time-to-peak 76 ms) and a subsequent slow component (time-to-peak 534 ms). The causes of these two components are not obvious; the second was somewhat faster than the response evoked by $InsP_3$ at break-through. The second component disappeared in Ca^{2+}-free solution (Fig. 8*A*) suggesting that Ca^{2+} entry made a contribution (but this contribution may be simply that it maintains the level of Ca in the store). The caged form of $InsP_3$ we used may also inhibit metabolism of $InsP_3$ (Walker, Feeney & Trentham, 1989) and this may contribute in some way to the second phase. When the amounts of $InsP_3$ released are large, the two components may represent different $InsP_3$-sensitive Ca stores not distinguishable when $InsP_3$ enters the cell at break-through. If so, the Ca store responsible for the later component is relatively sensitive to external Ca^{2+}, since Ca^{2+}-free conditions reduced preferentially this phase of the $InsP_3$-induced response (see Fig. 8*A*). Another possibility is that the second component involves a different type of Ca store, from which Ca^{2+} is released more slowly following binding of $InsP_3$; perhaps a component of the Ca store is in close proximity to the K^+ channels in the cell membrane while another is deeper within the cell.

The effectiveness of flash-released $InsP_3$, at concentrations of up to 1·5 μM (see Methods), in eliciting Ca^{2+} release is consistent with the results obtained on cultured or freshly isolated, permeabilized smooth muscle cells, where the half-maximally effective concentrations of $InsP_3$ in releasing Ca^{2+} was approximately 1 μM (Suematsu, Hirata, Hashimoto & Kuriyama, 1984; Yamamoto & van Breemen, 1985). In contrast, the concentrations of $InsP_3$ in the pipette needed to evoke current responses were much higher (20–100 μM), and a 20 μM-$InsP_3$-filled pipette even had no effect sometimes, which indicates the existence of some factors which attenuate the actions of $InsP_3$. The most likely factor is the activity of enzymes such as $InsP_3$ 5-phosphomonoesterase, an enzyme which is known to be very active in $InsP_3$-producing cells (Somlyo *et al.* 1988). Such activity might cause a concentration

gradient between the cell and pipette which could result in reduction of the effects of $InsP_3$. However, $InsP_3S$, which is resistant to enzymic destruction, was not greatly different in potency. Another possible factor is a reduction in the diffusion rate (noticed in experiments with Indo-1), possibly coming from various sources including the access resistance to the cytoplasm, cell geography just beneath the pipette tip, cytoplasm viscosity and cell shape. The consistent effects of the 100 μM-$InsP_3$-filled pipette implies that an increase in the pipette $InsP_3$ concentration can somehow overcome the various impeding factors and bring about a sufficient cytoplasmic concentration of $InsP_3$ for the stimulation necessary for the rapid release of all releasable cell calcium.

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