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Extracellular calcium concentration controls the frequency of intracellular calcium spiking independently of inositol 1,4,5-trisphosphate production in HeLa cells

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Stimulation of single HeLa cells with histamine evoked repetitive increases of the intracellular calcium ion concentration (Ca^{2+} spikes). The frequency of Ca^{2+} spiking increased as the extracellular hormone concentration was elevated. In addition, the frequency of Ca^{2+} spiking could be accelerated by increasing the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) in the presence of a constant hormone concentration. The range of $[Ca^{2+}]_o$ over which the spiking frequency could be titrated was nominally-zero to 10 mM, being half-maximally effective at approx. 1 and 2.5 mM for 37 and 22 °C respectively. The effect of $[Ca^{2+}]_o$ on inositol phosphates production was also examined. Changes of $[Ca^{2+}]_o$ over a range which had been found to affect the frequency of Ca^{2+} spiking did not have any effect on the rate of *myo*-inositol 1,4,5-trisphosphate (InsP_3) production, although an increase in inositol phosphates production was observed as $[Ca^{2+}]_o$ was increased from zero to values giving less than half-maximal Ca^{2+} spike frequency. These data suggest that at low Ca^{2+} spike frequency, Ca^{2+}-stimulated activation of phospholipase C may contribute to Ca^{2+} spiking in HeLa cells, but under some conditions the availability of Ca^{2+} to the intracellular stores, rather than changes in the rate of InsP_3 production, determines the Ca^{2+} spike frequency.

INTRODUCTION

Hormones that activate phospholipase C (PLC) cause an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in many cell types [1,2]. Hormone-stimulated signals often have a complex temporal arrangement, consisting of a series of repetitive Ca^{2+} spikes [3,4]. Ca^{2+} spikes are often initiated by a slowly-elevating 'pacemaker' $[Ca^{2+}]_i$ rise, that eventually leads to a more rapid upstroke. The spatial correlate of a Ca^{2+} spike is a Ca^{2+} wave, where an initial localized $[Ca^{2+}]_i$ elevation leads to the propagation of a regenerative $[Ca^{2+}]_i$ rise throughout the cytoplasm [5,6]. The link between activation of PLC and $[Ca^{2+}]_i$ elevation is the intracellular messenger *myo*-inositol 1,4,5-trisphosphate (InsP_3). InsP_3 mobilizes Ca^{2+} from intracellular stores by binding to specific receptors located on intracellular stores; these receptors also form the channels through which stored Ca^{2+} is released [1].

Although many of the biochemical events leading from PLC activation to InsP_3-mediated Ca^{2+} release have been characterized, the mechanism underlying Ca^{2+} spiking is not fully resolved. The regenerative nature of agonist-stimulated Ca^{2+} spikes and waves makes it very likely that the underlying process displays strong positive feedback. Several alternative models have been proposed to explain the generation of Ca^{2+} spiking, which place emphasis on different mechanisms to generate this positive feedback and fall broadly into two categories: the first group suggests that Ca^{2+} spiking is driven by cyclical changes in the rate of InsP_3 production, whilst other models favour schemes where the repetitive Ca^{2+} release occurs independently of changes in InsP_3 concentration, due to a Ca^{2+}-induced Ca^{2+} release (CICR) process [7–12].

Meyer and Stryer [10] suggested that cyclical increases in InsP_3 could be produced by the activation of a Ca^{2+}-sensitive PLC isoenzyme during each Ca^{2+} spike. This InsP_3–Ca^{2+} cross-coupling (ICC) scheme is supported by several lines of evidence. Firstly, the activity of PLC can be regulated by Ca^{2+} [13–15], and Ca^{2+}-stimulated inositol phosphate production has been demonstrated in several cell types following permeabilization [16–18]. Second, experimental manipulations which alter the amplitude of hormone-mediated Ca^{2+} signals in intact cells can affect inositol phosphate production in some cell types [17,19–21], although not in all [22]. In addition, mathematical modelling of the ICC model has suggested that it can plausibly account for Ca^{2+} spiking [10,23,24].

Despite the evidence supporting ICC, models for Ca^{2+} spike generation involving CICR have become widely accepted and have generally discounted the contribution of Ca^{2+}-dependent PLC activation [25] or have relegated it to a secondary process [26]. Several experimental observations have increased the prominence of CICR models. Firstly, in many cell types Ca^{2+} spiking can be stimulated by introduction of InsP_3 (or non-metabolizable InsP_3 analogues) into intact cells [6,27,28], suggesting that a constant level of intracellular messenger can lead to Ca^{2+} spiking. Second, several studies have suggested that Ca^{2+} spiking can be induced by agents that do not trigger InsP_3 production [29–32]. Most importantly, InsP_3-induced Ca^{2+} release has a biphasic dependence on the cytosolic Ca^{2+} concentration [33–37]. The positive feedback required for Ca^{2+} spiking can therefore be due to a regenerative autocatalytic Ca^{2+} release from InsP_3 receptors, whilst the negative feedback component may contribute to the termination of each spike.

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; $Ca^{2+}{}_o$, extracellular Ca^{2+}; PLC, phospholipase C; InsP_3, *myo*-inositol 1,4,5-trisphosphate; DMEM, Dulbecco's modified Eagle's medium; CICR, Ca^{2+}-induced Ca^{2+} release; ICC, InsP_3–Ca^{2+} cross-coupling; EM, extracellular medium.

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However, these data do not completely exclude a role for ICC in the Ca^{2+} spiking mechanism. For example, Ca^{2+} spiking following injection of InsP_3 into intact cells could be due to Ca^{2+}-dependent InsP_3 production, if the injected InsP_3 triggered a slow release of Ca^{2+} from the intracellular stores that was amplified by ICC. In this scheme, oscillations of the InsP_3 concentration could occur on top of the constant injected InsP_3 level. Interestingly, in rat megakaryocytes, injection of various InsP_3 concentrations did not mimic thrombin-stimulated Ca^{2+} spiking, whereas slow infusion of Ca^{2+} gave similar responses [38]. These data suggest that tonically elevated InsP_3 concentrations may not support Ca^{2+} spiking in some cell types. Furthermore, even though cells may rely on CICR, ICC could act as an additional 'booster' helping to cause regenerative Ca^{2+} release.

The aim of the present study was to examine how HeLa cells utilize the initial pacemaker release of Ca^{2+} from InsP_3-sensitive stores to produce the rapid, regenerative $[Ca^{2+}]_i$ elevation observed during a Ca^{2+} spike. The possible contribution of Ca^{2+}-dependent PLC activation to hormone-stimulated Ca^{2+} spiking in HeLa cells was examined using variations in the extracellular Ca^{2+} ($Ca^{2+}{}_o$) concentration ($[Ca^{2+}]_o$) as a method of altering Ca^{2+} spike frequency, without needing to change hormone concentration. The corresponding effect of changing $[Ca^{2+}]_o$ on inositol phosphates formation suggests that ICC may partly contribute to the generation of Ca^{2+} spikes in HeLa cells, although under certain conditions ICC does not underlie Ca^{2+} spiking.

EXPERIMENTAL

Cell culture

For fluorescence measurements, HeLa cells were grown in minimal essential medium supplemented with 5% mixed serum (50% newborn calf/50% fetal calf), 2 mM glutamine, 60 units/ml penicillin and 50 μg/ml streptomycin, in a humidified atmosphere (5% CO_2). For analysis of [^{3}H]inositol phosphates production, the cells were grown under similar conditions, except that the minimal essential medium was replaced with Dulbecco's modified Eagle's medium (DMEM; Gibco).

Single-cell imaging

To allow firm attachment, HeLa cells were transferred from plastic culture dishes to glass coverslips (22 mm diameter; Chance Propper Ltd, Smethick, Warley, U.K.) 48 h before use. The culture medium was replaced with an extracellular medium (EM) containing (mM): NaCl, 121; KCl, 5.4; $MgCl_2$, 1.6; $CaCl_2$, 1.8; $NaHCO_3$, 6; glucose, 9; Hepes, 25, at pH 7.4. Cells were loaded with fura 2 by incubation with 1 μM fura 2 acetoxymethyl ester (Molecular Probes Inc.) for 30 min at room temperature (22 °C), followed by an EM wash and a further 30 min incubation to allow de-esterification of the loaded dye. A single glass coverslip was mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope. Fluorescent images were obtained by alternate excitation at 340 and 380 nm (40 ms at each wavelength) using twin xenon arc lamps (Spex Industries Inc.). The emission signal at 510 nm was collected using a charge-coupled device video camera (Photonic Science) and the digitized signals were stored and processed as previously described [39]. When cells were imaged at 37 °C, they were loaded with fura 2 at room temperature as described above and then slowly warmed to 37 °C on the stage of the microscope.

Population fluorescence measurements

Cells were removed from their culture dishes by brief enzymic dissociation, pelleted by centrifugation (1000 ***g*** for 5 min) and resuspended at 6×10^6 cells/ml in EM. The cells were loaded with fura 2 as described above for single cells. Fura 2 fluorescence was measured using a Perkin-Elmer LS-50B, as previously described [40].

Measurement of [^{3}H]inositol phosphates accumulation in HeLa cells

HeLa cells were grown to near-confluence in DMEM. The culture medium was removed and the cells were washed with 10 ml of PBS before the addition of inositol-free DMEM supplemented with 10% dialysed calf serum (Gibco), 10 μM *myo*-inositol and 0.16 μM *myo*-[2-^{3}H]inositol (18 Ci/mmol; NEN). The cells were further incubated for 20–24 h at 37 °C in a CO_2 incubator. The [^{3}H]inositol-labelled cells were washed once with PBS and dissociated by treatment with trypsin (500–700 BAEE units/ml; Sigma) and EDTA (0.6 mM). The cells were pelleted by centrifugation (220 ***g*** for 5 min) and resuspended in EM and 50 μl of cells (approx. 1×10^5 cells) were then added to 190 μl of EM containing LiCl (final concentration 30 mM). The cells were incubated for a further 15 min at 37 °C, whilst being constantly shaken, before addition of histamine or H_2O. After 30 min, the stimulation was terminated by addition of 250 μl of ice-cold 10% perchloric acid solution containing 1 mg/ml phytic acid and 1 mM EDTA. [^{3}H]Inositol phosphates were extracted by addition of tri-n-octylamine/1,1,2-trichlorofluoroethane (1:1 v/v; 0.4 ml), vortexing and centrifugation (950 ***g*** for 5 min). A portion (0.38 ml) of the upper aqueous phase was transferred to a vial and 3 ml of 20 mM Hepes solution (pH 7.6) was added.

[^{3}H]Inositol phosphates were separated by anion-exchange chromatography using Bio-Rad Poly-prep chromatography columns containing 2 ml of Dowex AG 1-X8 resin (100–200 mesh; Bio-Rad). The extracted [^{3}H]inositol phosphates were applied to the columns and free [^{3}H]inositol was washed out using 10 ml of distilled H_2O. [^{3}H]Glycerophosphoinositol was eluted by addition of 10 ml of 5 mM disodium tetraborate/60 mM ammonium formate. [^{3}H]Inositol phosphates were collected into scintillation vials by stepwise elution with 10 ml formate buffers of increasing ionic strength: InsP_1, 0.2 M ammonium formate/0.1 M formic acid; InsP_2, 0.4 M ammonium formate/0.1 M formic acid; InsP_3, 0.8 M ammonium formate/0.1 M formic acid. The [^{3}H] content from each elution was determined by scintillation counting.

In control experiments, preincubation of cells with LiCl, under the conditions used for [^{3}H]inositol phosphates measurements, did not affect the duration or amplitude of histamine-stimulated Ca^{2+} signals (results not shown). In addition, loading cells with fura 2, under the conditions described above, did not alter the effects of $[Ca^{2+}]_o$ on [^{3}H]inositol phosphates measurement described in the Results section (results not shown).

RESULTS

Ca^{2+}-dependent and Ca^{2+}-independent [^{3}H]inositol phosphate production in HeLa cells

Stimulation of HeLa cells with histamine leads to H1-receptor-mediated production of InsP_3 and mobilization of Ca^{2+} from stores within HeLa cells (half-maximally effective histamine concentration is 2.3 μM [40,41]). In populations of cells the histamine-stimulated $[Ca^{2+}]_i$ elevation is biphasic (Figure 1a), with a large initial spike reflecting Ca^{2+} mobilization and a sustained plateau dependent upon $[Ca^{2+}]_o$. To investigate whether the histamine-stimulated PLC activity in HeLa cells is sensitive

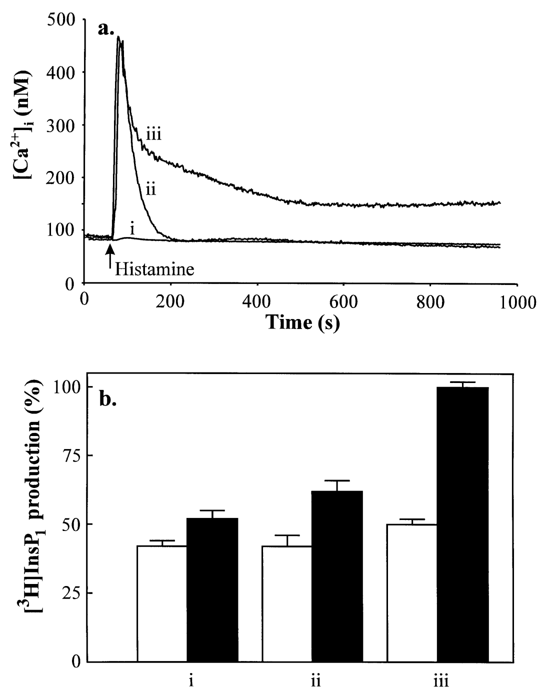

Figure 1 Reduction of intracellular Ca^{2+} signals decreases histamine-stimulated $[^3H]$inositol phosphates formation in HeLa cells

A population of $[^3H]$inositol-labelled HeLa cells was split into two equivalent portions for fluorimetry (**a**) or $[^3H]$inositol phosphates analysis (**b**). Before obtaining the traces shown in (**a**), the cells were treated with 100 μM histamine in Ca^{2+}-free EM for 10 min to deplete the intracellular Ca^{2+} stores, followed by resuspension in Ca^{2+}-containing EM (**iii**), brief (5 min) resuspension in Ca^{2+}-containing EM (**ii**) or resuspension in Ca^{2+}-free EM without any reloading period (**i**). Ten minutes after the initial histamine application, the cells were restimulated with histamine (100 μM). The responses obtained during this second histamine application are shown. For (**b**), the cells were treated with histamine as described above, so that the Ca^{2+} responses of the cells in (**b**) matched those in (**a**). (**b**) shows the accumulation of $[^3H]InsP_1$ over 10 min following the second histamine application (filled bars) or without histamine addition (open bars). The experiments described in (**a**) and (**b**) were repeated three times [with duplicate samples in (**b**)]. The traces in (**a**) are typical of the three experiments; the results in (**b**) represent the means $\pm$ S.E.M. of the three experiments. The data in (**b**) are plotted as a percentage of the response of the cells in Ca^{2+}-containing EM. The data from $[^3H]InsP_1$ accumulation are shown since this was the largest $[^3H]$inositol phosphate fraction obtained, however, similar data were obtained for $[^3H]InsP_2$ and $[^3H]InsP_3$ fractions. The average basal and 100 μM histamine-stimulated $[^3H]$ (d.p.m.) for (**b**, iii) were 1164 $\pm$ 61 and 2328 $\pm$ 49 respectively (mean $\pm$ S.E.M.). For (**b**, i–iii), the histamine-stimulated $InsP_1$ accumulations were significantly different from their respective controls ($P < 0.05$; calculated using Student's *t*-test).

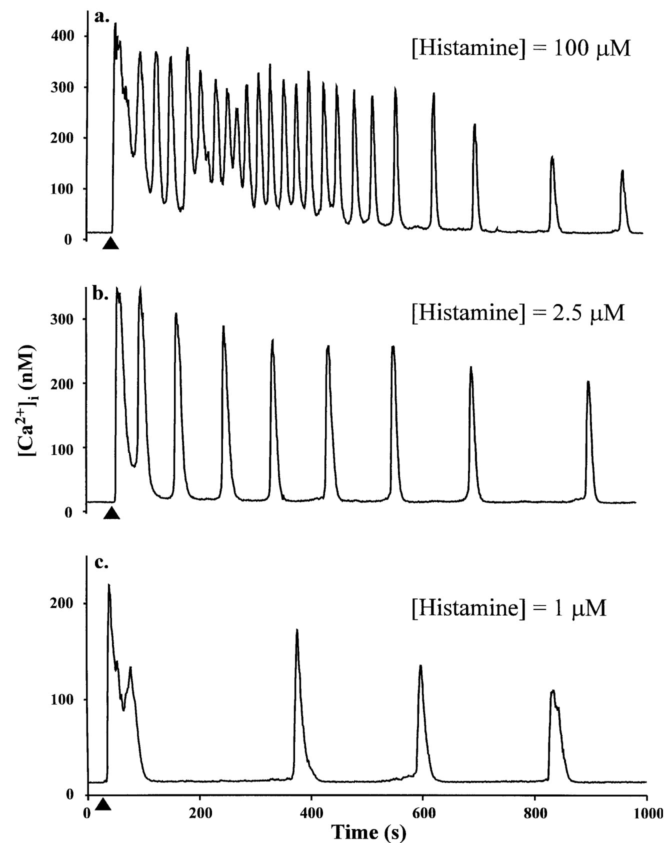

Figure 2 Histamine-stimulated Ca^{2+} spikes in single fura 2-loaded HeLa cells

The arrowheads denote the start of perfusion of the cells with EM containing 10 mM Ca^{2+} and supplemented with various histamine concentrations: (**a**) 100 μM; (**b**) 2.5 μM; and (**c**) 1 μM. The responses shown are typical of at least 90 cells for each concentration.

to the intracellular Ca^{2+} signal, we manipulated the Ca^{2+} response of HeLa cell populations and monitored the effect on the production of $[^3H]$inositol phosphates (Figure 1b). Briefly, three equivalent cell populations were treated with a maximal histamine concentration (100 μM) in Ca^{2+}-free EM for 10 min to deplete the intracellular Ca^{2+} stores, after which the histamine was removed. Following the histamine stimulation, one population was resuspended and maintained in Ca^{2+}-containing EM (Figure 1a, iii), another was briefly (5 min) resuspended in Ca^{2+}-containing EM and then transferred back to Ca^{2+}-free EM (Figure 1a, ii) and the remaining population was maintained in Ca^{2+}-free EM (Figure 1a, i). Ten minutes after the removal of histamine, the individual cell populations were again stimulated with histamine (100 μM) to determine the status of their intracellular Ca^{2+} signals. The cells that were resuspended and maintained in Ca^{2+}-containing EM gave a typical biphasic response to histamine (Figure 1a, iii). The cells that were briefly incubated in Ca^{2+}-containing EM displayed a transient $[Ca^{2+}]_i$ signal, reflecting Ca^{2+} mobilization from intracellular stores (Figure 1a, ii). The histamine-stimulated Ca^{2+} signal was abolished in the cells that were incubated solely in Ca^{2+}-free EM (Figure 1a, i).

The effect of these manipulations of the intracellular Ca^{2+} signal on the production of $[^3H]$inositol phosphates is shown in Figure 1b. The cells that were resuspended in Ca^{2+}-containing EM (Figure 1a, iii) and were able to display a typical biphasic response to histamine, showed the highest level of $[^3H]$inositol phosphates accumulation (Figure 1b, iii). Manipulations which reduced the Ca^{2+} signal to a mobilization phase (Figure 1a, ii) or abolished the intracellular response (Figure 1a, i) also reduced the accumulation of $[^3H]$inositol phosphates (Figure 1b, i and ii).

The data presented in Figure 1 suggest that the production of $[^3H]$inositol phosphates in HeLa cells is partly independent of $[Ca^{2+}]_i$ elevation and partly $[Ca^{2+}]_i$ dependent, since in the absence of an intracellular Ca^{2+} signal, histamine stimulated a small increase in $[^3H]$inositol phosphates, which was amplified in cells with typical Ca^{2+} signals. These data raise the possibility that a positive feedback loop exists between the release of Ca^{2+} from intracellular stores and the activity of PLC, which could be involved in the generation of Ca^{2+} spikes. However, the manipu-

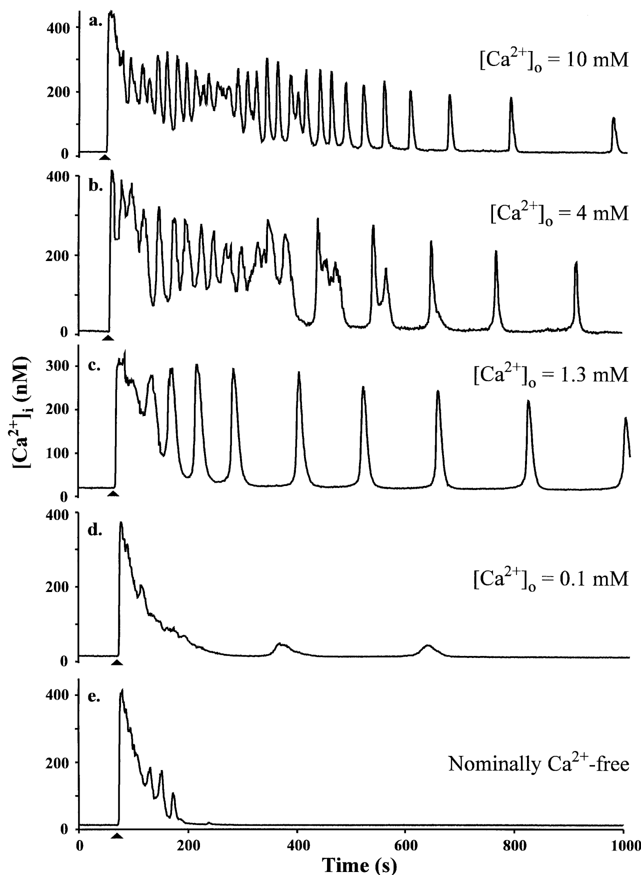

Figure 3 Control of Ca^{2+} spike frequency by $[Ca^{2+}]_o$

The arrowheads denote the start of perfusion of the cells with EM containing 100 μM histamine and supplemented with various Ca^{2+} concentrations: (**a**) 10 mM; (**b**), 4 mM; (**c**) 1.3 mM; (**d**) 0.1 mM; and (**e**) nominally zero. Histamine-stimulated Ca^{2+} spiking persisted in most cells for at least 30 min, however only the first 1000 s of the response is shown in the Figure to allow the initial rapid spikes to be distinguished.

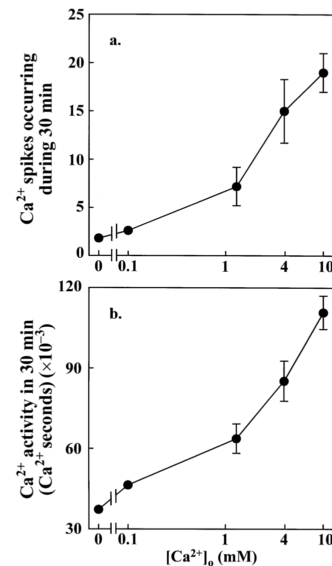

Figure 4 Averaged responses to histamine-stimulation in various $Ca^{2+}{}_o$ concentrations

The Figure shows averaged responses from experiments such as that shown in Figure 2. (**a**) The number of Ca^{2+} spikes occurring over a 30 min stimulation period. (**b**) The integrated Ca^{2+} signal occurring during the 30 min stimulation. A Ca^{2+} spike was distinguished as a discrete $[Ca^{2+}]_i$ increase of $\geqslant$ 25 nM, which was easily distinguished from noise and resting $[Ca^{2+}]_i$. However, at high $[Ca^{2+}]_o$ concentrations (4 and 10 mM), the Ca^{2+} spikes were so rapid that they fused together and were frequently difficult to distinguish. In these cases, the train of fused Ca^{2+} spikes was counted as a single spike. This means that the data presented in (**a**) slightly underestimates the Ca^{2+} spiking frequency at 4 and 10 mM $Ca^{2+}{}_o$. The integrated data presented in (**b**) (calculated from the area under the Ca^{2+} spiking traces) does not suffer from this problem and may present a more accurate representation of the Ca^{2+} activity in the cells during the experiment.

lations of the histamine-stimulated Ca^{2+} signals shown in Figure 1 were far from a steady-state situation and quite extreme, i.e. Ca^{2+} signals were normal or abolished. We therefore attempted to investigate the possible feedback of Ca^{2+} on PLC under conditions approaching a steady-state.

Effect of $[Ca^{2+}]_o$ on histamine-stimulated Ca^{2+} signals

Single fura 2-loaded HeLa cells responded to stimulation by histamine with a series of repetitive Ca^{2+} spikes (Figures 2 and 3). The response of single HeLa cells to histamine parallels the response of cell populations: initially rapid Ca^{2+} spikes reflecting mobilization of Ca^{2+} from intracellular stores (Figure 3e), followed by a period of less frequent Ca^{2+} spiking which was dependent upon Ca^{2+} entry (e.g. Figure 3c). The frequency of Ca^{2+} spiking gradually declined during perfusion with histamine over a period of 30 min, reflecting the desensitization of the signalling machinery [42].

Consistent with observations from many other tissues [43–45], the frequency of Ca^{2+} spiking was dependent upon the histamine concentration perfusing the cells (Figure 2). In addition, at a constant histamine concentration (100 μM), the frequency of Ca^{2+} spiking (Figure 3 and Figure 4a) and integrated histamine-stimulated Ca^{2+} activity (Figure 4b) was modulated by $[Ca^{2+}]_o$ within the range 0–10 mM. Above 0.1 mM $Ca^{2+}{}_o$, there was no change in the shape of the histamine-induced Ca^{2+} spikes, despite the large changes in frequency. At 0.1 mM $Ca^{2+}{}_o$, all the cells which exhibited Ca^{2+} spiking activity after the initial Ca^{2+} mobilization phase (75%, $n = 144$), showed infrequent, low-amplitude Ca^{2+} spikes (Figure 3d).

Stimulation of HeLa cell populations with histamine in various $[Ca^{2+}]_o$ produced results consistent with the effects on single cells described above. Increasing $[Ca^{2+}]_o$ from nominally zero to 10 mM had no effect on the resting $[Ca^{2+}]_i$ (Figure 5d), or on the initial peak $[Ca^{2+}]_i$ response to 1, 2.5 or 100 μM histamine (Figure 5c), but it did enhance $[Ca^{2+}]_i$ during the period dependent upon Ca^{2+} entry (Figures 5a, 5b and 6).

Although the frequency of Ca^{2+} spiking in HeLa cells can be modulated by $[Ca^{2+}]_o$, the extent of the frequency increase appears to be limited by the concentration of histamine (Figure 5b). With each histamine concentration (1, 2.5 and 100 μM) the effective $[Ca^{2+}]_o$ range was similar; the minimum $[Ca^{2+}]_o$ that gave a detectable $[Ca^{2+}]_i$ increase was 0.1 mM and the responses saturated at approx. 10 mM (Figure 5b and results not shown). Furthermore, although the magnitude of the $[Ca^{2+}]_i$ response could be augmented by increasing $[Ca^{2+}]_o$ either before (e.g. Figure 5a) or following histamine addition (Figure 6), the maximal amplitude obtained with saturating $[Ca^{2+}]_o$ was dependent upon the histamine concentration (Figures 5b and 6).

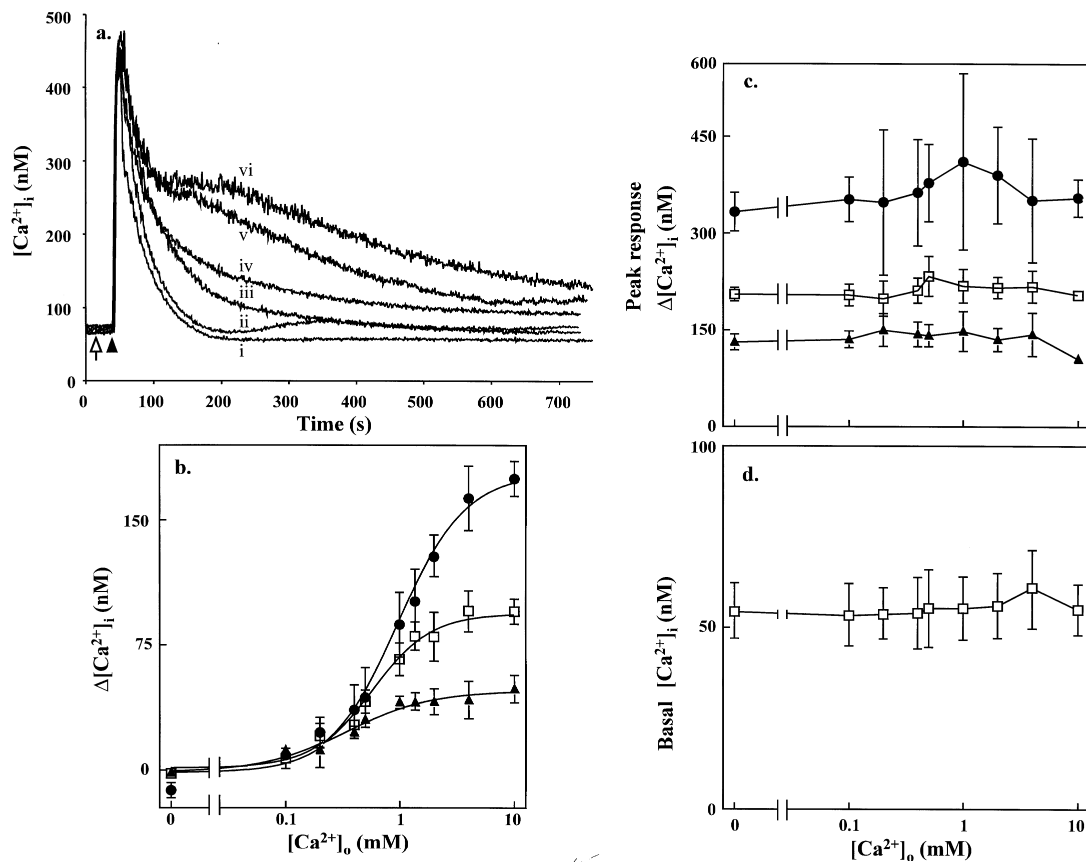

Figure 5 Response of HeLa cell populations to histamine-stimulation in various $Ca^{2+}{}_o$ concentrations

(**a**) The open arrow denotes the addition of EM containing various $Ca^{2+}{}_o$ concentrations to resting cells. The final $Ca^{2+}{}_o$ concentrations were: (i) nominally zero; (ii) 0.1 mM; (iii) 0.3 mM; (iv) 1.3 mM; (v) 4 mM; and (vi) 10 mM. The black arrowhead denotes the subsequent addition of histamine (100 μM) to the cells. (**b**) Averaged data showing the effect of $[Ca^{2+}]_o$ on the plateau phase of the response to three different histamine concentrations. The graph shows the average $[Ca^{2+}]_i$ rise observed 180 s after histamine addition. At this time the response of the cells is maintained by $Ca^{2+}{}_o$. (**c**) The effect of $[Ca^{2+}]_o$ on the peak response to histamine. The data represent the peak $[Ca^{2+}]_i$ elevation obtained directly after histamine addition. This phase of the response is due almost entirely to Ca^{2+} release from intracellular stores. (**d**) Effect of $[Ca^{2+}]_o$ on resting $[Ca^{2+}]_i$. The data show that addition of various $Ca^{2+}{}_o$ concentrations to the cells before stimulation with histamine had no significant effect on basal $[Ca^{2+}]_i$. Symbols in (**b**) and (**c**) represent: ●, 100 μM histamine; □, 2.5 μM histamine; ▲, 1 μM histamine. The data are means ± S.E.M. from 3–5 independent experiments for each histamine concentration.

The rate of entry of Ca^{2+} is therefore partly determined by $[Ca^{2+}]_o$ and is also partly dependent on the level of cell stimulation.

Effect of $[Ca^{2+}]_o$ on [³H]inositol phosphates production

We examined the effect of $[Ca^{2+}]_o$, over the range which affected intracellular Ca^{2+} signals, on the histamine-stimulated production of [³H]inositol phosphates. Stimulation of [³H]inositol-labelled HeLa cells with 100 μM histamine for 30 min in various concentrations of $Ca^{2+}{}_o$ caused an increase in the production of [³H]inositol phosphates (Figure 7). The majority of [³H]inositol phosphates production was detected as an increase in InsP_1 levels (Figure 7a), but smaller increases in InsP_2 (Figure 7b) and InsP_3 (Figure 7c) fractions were also found. Histamine stimulated [³H]inositol phosphates production at all $[Ca^{2+}]_o$ tested, although the production of [³H]inositol phosphates increased as $[Ca^{2+}]_o$ was elevated from zero to 1.3 mM. At $[Ca^{2+}]_o$ greater than 1.3 mM there was no additional increase in histamine-stimulated [³H]inositol phosphates accumulation, even though the intracellular Ca^{2+} signals stimulated by 100 μM histamine increased in both single cells (Figures 3 and 4) and cell populations (Figure 5) if $[Ca^{2+}]_o$ was elevated above 1.3 mM.

Effect of $[Ca^{2+}]_o$ on histamine-stimulated Ca^{2+} signals and [³H]inositol phosphates production at 37 °C

The experiments presented in Figures 1–7 above were conducted at room temperature (22 °C). Some of these experiments were repeated at 37 °C. These two temperatures conveyed different advantages for the present study. The lower temperature facilitates the measurement of Ca^{2+} signals in HeLa cells using fura 2, since there is virtually no leakage of fura 2 from the cells at 22 °C. At 37 °C, fura 2 leaks rapidly from HeLa cells to the extracellular medium. The fura 2 leakage can be attenuated, but not prevented, by addition of the anion transport inhibitor sulphinpyrazone. The loss of fura 2 from cells incubated at 37 °C prevented the measurement of histamine-stimulated Ca^{2+} signals of HeLa cell populations at this temperature, since the leakage of fura 2 into the extracellular medium caused a continuous drift in the baseline fluorescence. However, by continuously perfusing attached cells to remove extracellular fura 2, we were able to measure histamine-stimulated Ca^{2+} signals in single cells. The advantage gained by using cells at 37 °C is that the extent of [³H]inositol phosphates production is greatly increased compared with the response at 22 °C, thus providing a more substantial measure of the effect of $[Ca^{2+}]_o$ on this response.

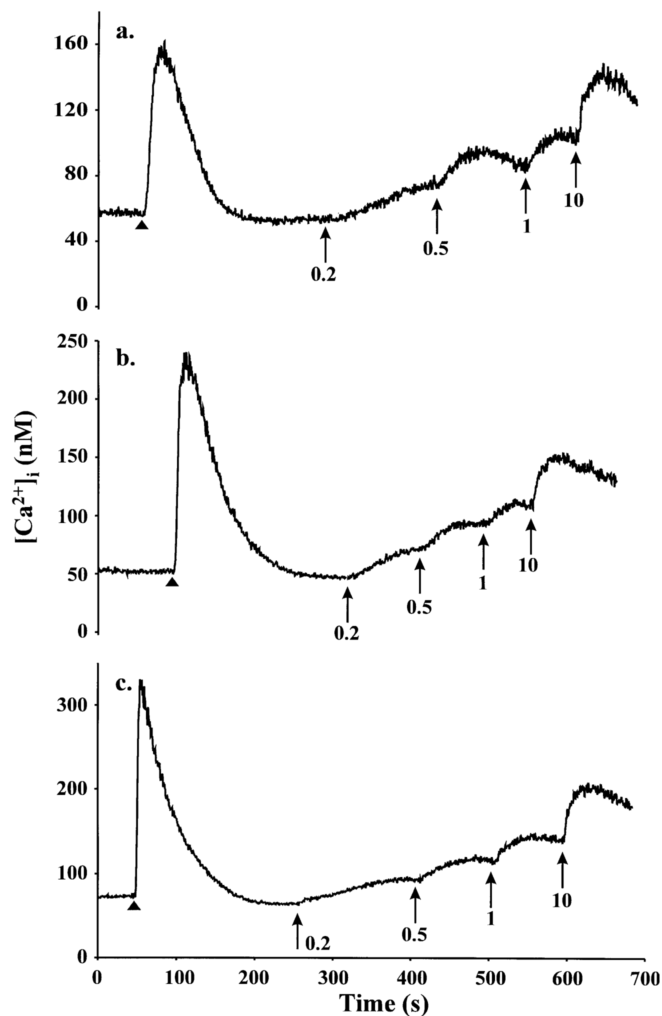

Figure 6 Response of HeLa cell populations to various $Ca^{2+}{}_o$ concentrations following histamine stimulation

The arrowheads denote addition of histamine to the cells in nominally Ca^{2+}-free EM. Final histamine concentrations were: (**a**) 1 μM; (**b**) 2.5 μM; (**c**) 100 μM. Subsequent increases of $Ca^{2+}{}_o$ are denoted by the arrows, at the concentrations shown (mM).

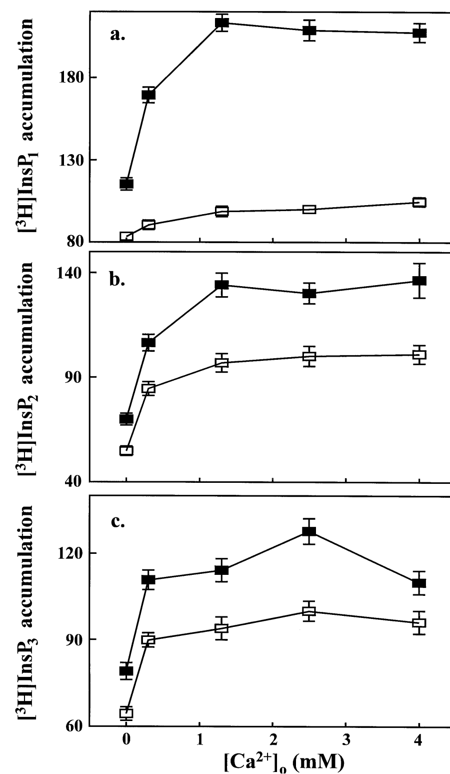

Figure 7 Effect of $[Ca^{2+}]_o$ on histamine-stimulated [^{3}H]inositol phosphates accumulation

The graphs represent the accumulation of [^{3}H]inositol phosphates during a 30 min incubation without histamine (□) or with 100 μM histamine (■), at the $[Ca^{2+}]_o$ shown. The results are presented as a percentage of the unstimulated [^{3}H]inositol phosphates accumulation at 2.5 mM $Ca^{2+}{}_o$: (**a**) InsP_1; (**b**) InsP_2; and (**c**) InsP_3. The data are means ± S.E.M. of three independent experiments, each with quadruplicate samples. The average basal and 100 μM histamine-stimulated [^{3}H]InsP_1 (d.p.m.) for cells in 2.5 mM $Ca^{2+}{}_o$ were 1641 ± 30 and 3359 ± 73 respectively (means ± S.E.M.).

The effect of changing $[Ca^{2+}]_o$ on histamine-stimulated Ca^{2+} signals in single HeLa cells incubated at 37 °C is shown in Figures 8 and 9. Similar to the effects at 22 °C, increasing $[Ca^{2+}]_o$ from nominally zero to 10 mM increased the frequency of Ca^{2+} spiking (Figure 8 and Figure 9a) and the integrated Ca^{2+} activity (Figure 9b). The cells appeared to be more sensitive to $[Ca^{2+}]_o$ at 37 °C compared with 22 °C. For the single-cell responses shown in Figures 3 and 8, the $[Ca^{2+}]_o$ giving half-maximal effects were 1 and 2.5 mM for 37 and 22 °C respectively (assuming the responses were saturated at 10 mM $Ca^{2+}{}_o$).

Histamine-stimulated [^{3}H]inositol phosphates accumulation was also sensitive to $[Ca^{2+}]_o$ at 37 °C (Figure 10). Similar to the effect on Ca^{2+} signalling, the accumulation of [^{3}H]inositol phosphates appeared to be more sensitive to $[Ca^{2+}]_o$ at 37 than at 22 °C, since at 37 °C the response to histamine (100 μM) was saturated at 0.3 mM $Ca^{2+}{}_o$ (Figure 10).

The data obtained using cells at 22 and 37 °C were similar: at both temperatures the histamine-stimulated production of [^{3}H]inositol phosphates was sensitive to $[Ca^{2+}]_o$, however, this response was saturated by $Ca^{2+}{}_o$ at lower concentrations than the histamine-stimulated Ca^{2+} signals.

DISCUSSION

The aim of this study was to examine the possible contribution of Ca^{2+}-dependent InsP_3 production during hormone-stimulated Ca^{2+} spiking. Our approach to this question was to investigate the relationship between Ca^{2+} spiking frequency and the production of InsP_3. Since techniques for measuring InsP_3 at the single-cell level are not yet available, we reverted to using HeLa cell populations, so that the accumulation of [^{3}H]inositol phosphates could be measured over long periods.

Initial experiments were designed to find experimental conditions that reproducibly altered Ca^{2+} spiking frequency. In common with many cell types, the Ca^{2+} spiking frequency displayed by single HeLa cells could be elevated by increasing the concentration of extracellular agonist (Figure 2). Changing the agonist concentration also alters the production of [^{3}H]inositol phosphates in HeLa cells [31,41,46]. The Ca^{2+} spiking frequency could also be reproducibly adjusted by titrating $Ca^{2+}{}_o$ (Figure 3). Similar effects of $[Ca^{2+}]_o$ on Ca^{2+} spiking frequency have been

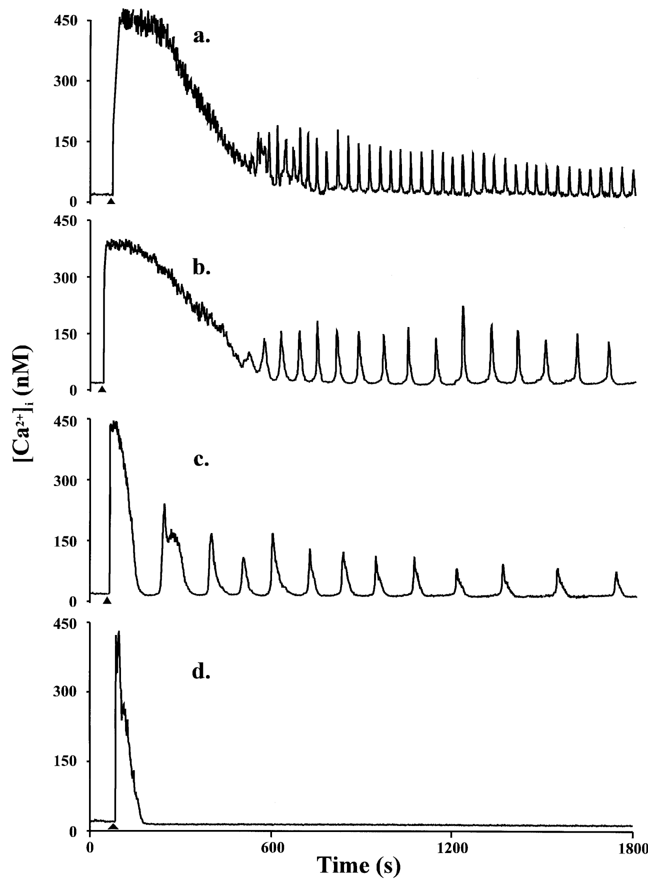

Figure 8 Control of Ca^{2+} spike frequency by $Ca^{2+}{}_o$ at 37 °C

The arrowheads denote the start of perfusion of the cells with EM containing 100 μM histamine and supplemented with various Ca^{2+} concentrations: (**a**) 10 mM; (**b**) 4 mM; (**c**) 0.3 mM; and (**d**) nominally zero.

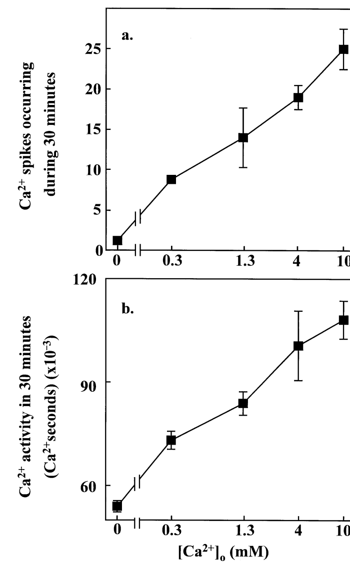

Figure 9 Averaged responses to histamine-stimulation in various $Ca^{2+}{}_o$ concentrations at 37 °C

(**a**) The number of Ca^{2+} spikes occurring over a 30 min period following stimulation with histamine (100 μM). (**b**) The integrated Ca^{2+} signal occurring during the 30 min stimulation. The initial high-amplitude sustained signal seen with 4 and 10 mM $Ca^{2+}{}_o$ was counted as a single spike, which means that the data presented in (**a**) slightly underestimates the Ca^{2+} spiking frequency at 4 and 10 mM $Ca^{2+}{}_o$.

seen in several other cell types, including REF52 fibroblasts [47], hepatocytes [48], AR42J pancreatoma cells [49] and MDCK cells [50].

The relationship between $[Ca^{2+}]_o$ and [^{3}H]inositol phosphate accumulation was complex for HeLa cells. At 22 and 37 °C, the accumulation of [^{3}H]inositol phosphates increased as $[Ca^{2+}]_o$ was elevated from nominally zero to 1.3 and 0.3 mM respectively (Figures 7 and 10). Above these concentrations, $Ca^{2+}{}_o$ had no effect on [^{3}H]inositol phosphates accumulation, even though further increases in Ca^{2+} spiking frequency could be evoked (Figures 3, 4 and 8).

The data obtained from HeLa cells suggest that PLC activity is sensitive to elevated $[Ca^{2+}]_i$, although some PLC activity occurs in the absence of an intracellular Ca^{2+} signal (Figure 1). The Ca^{2+} signal-independent PLC activation may reflect a low rate of PLC activity which is responsible for initiating a response. The ensuing $[Ca^{2+}]_i$ elevation could lead to enhanced PLC activity, causing progresssively more Ca^{2+} release and eventually culminating in a Ca^{2+} spike.

Interestingly, the PLC response saturates at frequencies of Ca^{2+}-spiking below the maximum. A possible explanation for these data is that Ca^{2+} binds to PLC during a Ca^{2+} spike and enhances its activity, but the off-rate of Ca^{2+} unbinding from the enzyme is relatively slow. In this scheme, Ca^{2+} can bind to, and unbind from, PLC at low spiking frequency, but will effectively remain bound to PLC at high spiking frequencies. The observation that the Ca^{2+} spiking frequency can be increased by $Ca^{2+}{}_o$ when the PLC response in HeLa cells is saturated is particularly important, since it suggests that the frequency of Ca^{2+} spiking can also be controlled independently of the production of InsP_3 in these cells. The mechanism underlying the ability of $Ca^{2+}{}_o$ to influence spiking frequency is unclear. However, $Ca^{2+}{}_o$ could control the release of Ca^{2+} from intracellular stores by modulating the rate of store refilling between each Ca^{2+} spike. In this situation, the positive feedback responsible for Ca^{2+} spiking must be generated by CICR, since PLC activity is essentially saturated. A similar acceleration of Ca^{2+} spiking was observed in *Xenopus* oocytes following a hyperpolarization-induced increase in Ca^{2+} entry [51]. The acceleration of spiking was explained on the basis of an elevated baseline $[Ca^{2+}]_i$, promoting the co-agonist action of Ca^{2+}. However, in the present study, increasing $[Ca^{2+}]_o$ did not cause any detectable increase in baseline $[Ca^{2+}]_i$.

The data presented in this study suggest that Ca^{2+}-stimulated activation of PLC may contribute to Ca^{2+} spiking in HeLa cells, as predicted by the ICC model [10]. The exact contribution of ICC to HeLa cell Ca^{2+} spikes is difficult to assess, since the population measurements described in this study do not distinguish between Ca^{2+}-activated [^{3}H]inositol phosphates production being a cause or consequence of each Ca^{2+} spike. However, if the feedback of Ca^{2+} onto PLC is sufficiently rapid, the increased rate of InsP_3 production could contribute to the rapid $[Ca^{2+}]_i$ elevation at the onset of a Ca^{2+} spike.

This scheme may have widespread significance, since experimental manipulations which alter the magnitude or duration of intracellular Ca^{2+} signals have been found to affect the production of [^{3}H]inositol phosphates in many cell types [17,19–21,52]. Comprehensive models for Ca^{2+} spiking therefore

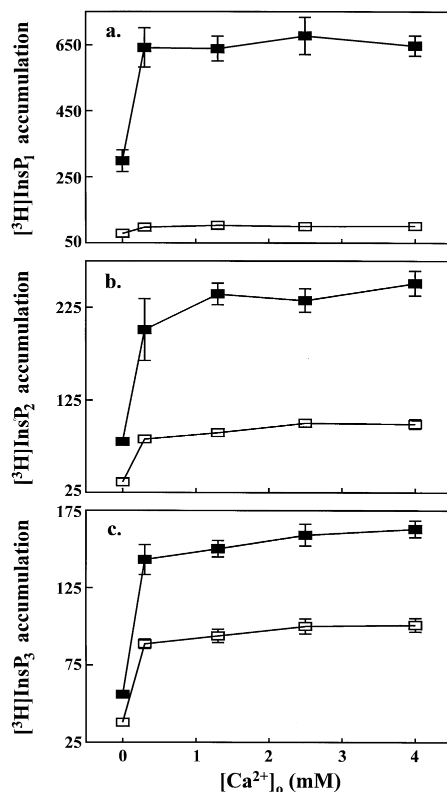

Figure 10 Effect of $[Ca^{2+}]_o$ on histamine-stimulated [^{3}H]inositol phosphates accumulation at 37 °C

The graphs represent the effect of $[Ca^{2+}]_o$ on the accumulation of [^{3}H]inositol phosphates during a 30 min incubation at 37 °C without histamine (□) or with 100 μM histamine (■). The results are presented as a percentage of the unstimulated [^{3}H]inositol phosphates accumulation at 2.5 mM $Ca^{2+}{}_o$: (**a**) $InsP_1$; (**b**) $InsP_2$; and (**c**) $InsP_3$. The data are means ± S.E.M. of three independent experiments.

need to account for the potential contribution from ICC. However, ICC cannot fully underlie Ca^{2+} spike generation in HeLa cells, since alteration of the Ca^{2+} spiking frequency can be achieved when PLC activity is saturated. The Ca^{2+} spiking mechanism may therefore involve contributions from both ICC and CICR. Models with both CICR and ICC operating have been proposed and suggest that such dual-feedback systems may allow cells to generate particularly complex Ca^{2+} spiking patterns [26,53].

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