# Oscillations of cytosolic free calcium in bombesin-stimulated HIT-T15 cells

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# Oscillations of cytosolic free calcium in bombesin-stimulated HIT-T15 cells

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**Summary** The mechanism underlying the generation of cytosolic free $Ca^{2+}$ ($[Ca^{2+}]_i$) oscillations by bombesin, a receptor agonist activating phospholipase C, in insulin secreting HIT-T15 cells was investigated. At 25 μM, 61% of cells displayed $[Ca^{2+}]_i$ oscillations with variable patterns. The bombesin-induced $[Ca^{2+}]_i$ oscillations could last more than 1 h and glucose was required for maintaining these $[Ca^{2+}]_i$ fluctuations. Bombesin-evoked $[Ca^{2+}]_i$ oscillations were dependent on extracellular $Ca^{2+}$ entry and were attenuated by membrane hyperpolarization or by L-type $Ca^{2+}$ channel blockers. These $[Ca^{2+}]_i$ oscillations were apparently not associated with fluctuations in plasma membrane $Ca^{2+}$ permeability as monitored by the $Mn^{2+}$ quenching technique. 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ) and 4-chloro-*m*-cresol, which interfere with intracellular $Ca^{2+}$ stores, respectively, by inhibiting $Ca^{2+}$-ATPase of endoplasmic reticulum and by affecting $Ca^{2+}$-induced $Ca^{2+}$ release, disrupted bombesin-induced $[Ca^{2+}]_i$ oscillations. 4-chloro-*m*-cresol raised $[Ca^{2+}]_i$ by mobilizing an intracellular $Ca^{2+}$ pool, an effect not altered by ryanodine. Caffeine exerted complex actions on $[Ca^{2+}]_i$. It raised $[Ca^{2+}]_i$ by promoting $Ca^{2+}$ entry while inhibiting bombesin-elicited $[Ca^{2+}]_i$ oscillations. Our results suggest that in bombesin-elicited $[Ca^{2+}]_i$ oscillations in HIT-T15 cells: (i) the oscillations originate primarily from intracellular $Ca^{2+}$ stores; and (ii) the $Ca^{2+}$ influx required for maintaining the oscillations is in part membrane potential-sensitive and not coordinated with $[Ca^{2+}]_i$ oscillations. The interplay between intracellular $Ca^{2+}$ stores and voltage-sensitive and voltage-insensitive extracellular $Ca^{2+}$ entry determines the $[Ca^{2+}]_i$ oscillations evoked by bombesin.

## INTRODUCTION

It is well established that $Ca^{2+}$ is a universal intracellular signalling messenger during cell activation such as contraction, aggregation, secretion and fertilization. $Ca^{2+}$ plays a crucial role in the regulation of insulin secretion from pancreatic islets [1–3]. Glucose and other nutrients enter the β-cell and are metabolized, generating coupling factors such as ATP and NAD(P)H, leading to membrane depolarization [4]. Subsequently, $Ca^{2+}$ enters β-cells via voltage-gated channels and cytosolic free $Ca^{2+}$ ($[Ca^{2+}]_i$) is increased [5–8]. Many hormones and neurotransmitters modulate nutrient-induced insulin secretion and cause rapid changes in $[Ca^{2+}]_i$. These receptor agonists either stimulate adenylyl cyclase or phospholipase C via heterotrimeric G-proteins [9]. Activation of phospholipase C results in hydrolysis of phosphatidylinositol 4,5-bisphosphate, producing two messengers: inositol-1,4,5-trisphosphate [$Ins(1,4,5)P_3$] and diacylglycerol. The former mobilizes calcium from intracellular stores and the latter stimulates protein kinase C [10,11]. Receptor activation also directly or indirectly provokes the flux of calcium across the plasma membrane [10,12,13].

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*Abbreviations*: $[Ca^{2+}]_i$, cytosolic free $Ca^{2+}$ concentration; tBuBHQ, 2,5-di-(tert-butyl)-1,4-benzohydroquinone; CICR, $Ca^{2+}$-induced $Ca^{2+}$ release.

Both glucose and $Ca^{2+}$-mobilizing agonists evoke $[Ca^{2+}]_i$ oscillations in insulin secreting cells [7,14–22]. The $[Ca^{2+}]_i$ oscillations elicited by glucose seem to depend essentially on $Ca^{2+}$ influx. This influx is due to a rhythmical electrical activity which causes a corresponding periodical gating of L-type $Ca^{2+}$ channels [16,23]. Although the mechanism underlying the electrical activity is not completely understood, glucose-induced $[Ca^{2+}]_i$ oscillations probably result from the oscillatory generation of metabolic coupling factors [22,24–26].

Similarly, the mechanism of $[Ca^{2+}]_i$ oscillations induced by phospholipase C activators is not clearly defined, particularly in insulin secreting cells. These receptor agonists not only modulate glucose-evoked $[Ca^{2+}]_i$ oscillations but are also able to elicit the oscillations *per se* [14,18–20,27–30]. The agonist-induced $[Ca^{2+}]_i$ fluctuation depends on receptor occupancy and $Ca^{2+}$ influx mainly via non-L-channels [18,19]. Several models have been proposed for the generation of $[Ca^{2+}]_i$ oscillations by receptor agonists in non excitable cells. In one model, receptor activation may induce a pulsatile production of $Ins(1,4,5)P_3$ which causes oscillatory mobilization of $Ca^{2+}$ from $Ins(1,4,5)P_3$-sensitive pools [31]. In another model [10], the $[Ca^{2+}]_i$ oscillations are generated by $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) [32] triggered by the mobilization of $Ca^{2+}$ by $Ins(1,4,5)P_3$. This two-pool model has been revised recently, in that both $Ca^{2+}$ pools are $Ins(1,4,5)P_3$ sensitive [33]. The role of extracellular $Ca^{2+}$ in $[Ca^{2+}]_i$ oscillations is still unsettled and may vary from cell type to cell type. For instance, $[Ca^{2+}]_i$ oscillations are preserved or undergo gradual fading in the absence of extracellular $Ca^{2+}$ in some cells [10,34], while in other cells, including β-cells and RINm5F cells, $[Ca^{2+}]_i$ oscillations cease immediately following $Ca^{2+}$ removal [10,19,34–36]. Furthermore, it has also been reported that carbachol could induce fluctuations in $Ca^{2+}$ entry associated with $[Ca^{2+}]_i$ oscillations in carbachol-stimulated AR42J cells [36] but this phenomenon was not observed in histamine-stimulated endothelial cells [37]. In insulin secreting cells, it is not known whether there is a change in the rate of $Ca^{2+}$ influx associated with agonist-evoked $[Ca^{2+}]_i$ oscillations. In addition, the relative roles of $Ca^{2+}$ influx and $Ca^{2+}$ mobilization under such conditions have not been defined in these cells. The elucidation of these questions is the main goal of the present study.

## MATERIALS AND METHODS

### Materials

Krebs-Ringer-bicarbonate-HEPES buffer (KRBH) was used to superfuse the cells and its composition was (in mM): 136 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgSO_4$, 1 $KH_2PO_4$, 5 $NaHCO_3$, 20 HEPES, pH 7.4, 0.1% bovine serum albumin. Unless specified, the superfusion medium contained 0.5 mM glucose. Bombesin was from Bachem Bioscience, Bubendorf, Switzerland; Fura–2 acetoxymethyl ester from Molecular Probes, Eugene, OR, USA; ryanodine, 4-chloro-*m*-cresol and caffeine were from Fluka, Buchs, Switzerland.

### Cell culture

HIT-T15 cells (passages between 73–79) were cultured in RPMI 1640 medium in flasks (Falcon) as previously described [38]. For $[Ca^{2+}]_i$ measurement in single cells [6], the cells were detached by gentle trypsinization and approximately $1 \times 10^5$ cells were seeded on a bare glass coverslip (2 × 1.8 cm) and cultured for 2 days in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics.

### Fura–2 loading

Fura–2 acetoxymethyl ester was dissolved in dimethylsulphoxide (DMSO) in aliquots of 1 mM and kept at –20°C. The monolayer cultures were exposed to 1–2 μM of the Fura–2 ester for about 30 min at 37°C.

### $[Ca^{2+}]_i$ measurement by microfluorimetry

Ratiometric measurement [39] was used in a new system developed by W-F.P. and the Electronic Workshop of Geneva University Medical Centre. A xenon lamp coupled to quartz light guides provides the excitation light source. Pairs of synchronous spinning wheels, holding the required interference filters, are used to change the excitation and emission wavelengths and up to 5 excitation/emission pairs can be used simultaneously. This allows ratiometric measurement of fluorescence of Fura–2 or Indo–1 in combination with other fluorescence signals, such as NAD(P)H, with appropriate filter pairs. For Fura–2 assessment, two pairs of excitation/emission wavelengths, 340/510 nm and 380/510 nm, were chosen. An additional pair of filters (360/510 nm) was selected to monitor the total dye change when experiments of Fura–2 fluorescence quenching by $Mn^{2+}$ were performed [40]. The coverslip with the cells was sealed with silicon grease over the central hole of a Petri dish which served as a superfusion chamber installed on an inverted microscope. A stainless steel ring was placed on top of the coverslip to attenuate the mechanical impact of the superfusion and to prevent abrupt alterations in temperature. Cells were observed and monitored in the epifluorescence mode. The objective was a Nikon F-100 numerical aperture 1.3, with oil immersion. The emitted light was cut off at ~400 nm and directed

into either the eyepiece or a high sensitivity photomultiplier tube kept refrigerated at 9°C. In this system, the UV intensity of the excitation light can be adjusted by a specific diaphragm located optically before the objective. Another diaphragm is placed between the microscope and the photomultiplier, which allows the monitoring of selected areas or single cells in the microscopic field. The signals from the photomultiplier were digitized and computer processed. A specific Software, Specsys® working in the Windows® environment has been developed to control the sequence and integration time of each filter pair and the frequency of data acquisition and to process and calibrate the recordings.

The microscope was contained in a thermostatted box at 37°C, and the superfusion medium KRBH was equilibrated to this temperature by passage through a coil in the chamber. The cells were superfused with KRBH at a constant rate (main flow). Stimulating solutions were introduced by side-infusion through a pipette (internal diameter ~50 μm) placed in the vicinity of the cell under study as already described (6).

### Calibration of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ values were calculated from Fura-2 ratios (R) at dual excitation wavelengths of 340/380 nm by the equation [39]:

$$[Ca^{2+}]_i = K_d [(R - R_{min})/(R_{max} - R)] \beta \qquad \text{Eq. 1}$$

where $K_d$ is the dissociation constant of Fura-2 (225 nM); R is the fluorescence ratio at any experimental point during the recording ($F_{340}/F_{380}$). $R_{min}$ and $R_{max}$ are the ratios at the two excitation wavelengths obtained in zero (< 10 nM) or saturating calcium concentrations (> 1 mM), respectively. β is the fluorescence at 380 nm excitation under $Ca^{2+}$ free conditions divided by the fluorescence at the same wavelength under $Ca^{2+}$ saturating conditions. In practice, we obtained the constants by exposing HIT-T15 cells to the calcium ionophore ionomycin under ambient $Ca^{2+}$ kept either below 10 nM ($R_{min}$) or at 5 mM ($R_{max}$). $R_{min}$, $R_{max}$ and β determined in our system were 0.3, 4.5, and 4.1, respectively.

## RESULTS

### Glucose requirement for the bombesin-elicited $[Ca^{2+}]_i$ transients

The tetradecapeptide bombesin, a homologue of gastrin releasing factor, activates phospholipase C and raises $[Ca^{2+}]_i$ [41]. In HIT-T15 cell populations, the $[Ca^{2+}]_i$ response profile consists of a large initial $[Ca^{2+}]_i$ rise followed by a smaller sustained plateau [38]. As the main

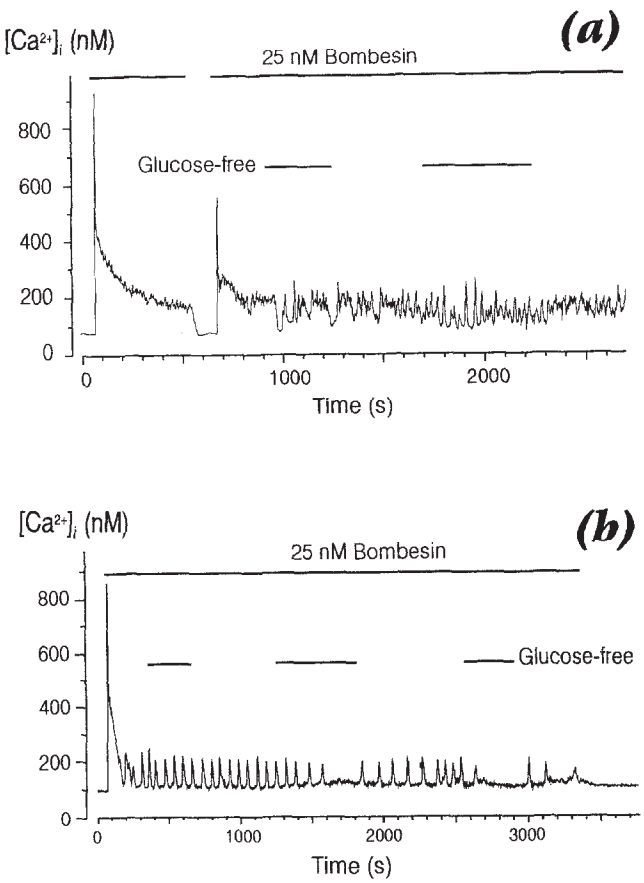


**Fig. 1** Effect of glucose withdrawal on bombesin-induced $[Ca^{2+}]_i$ oscillations in HIT-T15 cells. When present, the glucose concentration of the buffer was 0.5 mM. Each trace is representative of at least 3 experiments.

purpose of the present study was to elucidate the mechanisms by which bombesin induces $[Ca^{2+}]_i$ oscillations in HIT-T15 cells, we first established the concentration of glucose, itself a $[Ca^{2+}]_i$ raising agent, that had minimal effects on $[Ca^{2+}]_i$. This was necessary because bombesin was shown to synergize with nutrients in promoting membrane depolarization and $[Ca^{2+}]_i$ elevation in these cells [38]. Therefore, we selected a concentration of 0.5 mM glucose which, although slightly depolarizing the cell membrane potential, did not *per se* elicit a $[Ca^{2+}]_i$ rise in cell suspensions. In addition, this glucose concentration permitted stable $[Ca^{2+}]_i$ levels over long periods of recording (not shown). The effect of complete glucose removal was also tested on the bombesin-induced $[Ca^{2+}]_i$ responses (Fig. 1a,b). In cells displaying sustained plateau phase at 0.5 mM glucose, hexose deprivation changed the steady-state $Ca^{2+}$ elevations into irregular oscillations (Fig. 1a). In other cells, regular $[Ca^{2+}]_i$ oscillations were not affected by a short deprivation of glucose. However, more prolonged or repeated glucose deprivation resulted

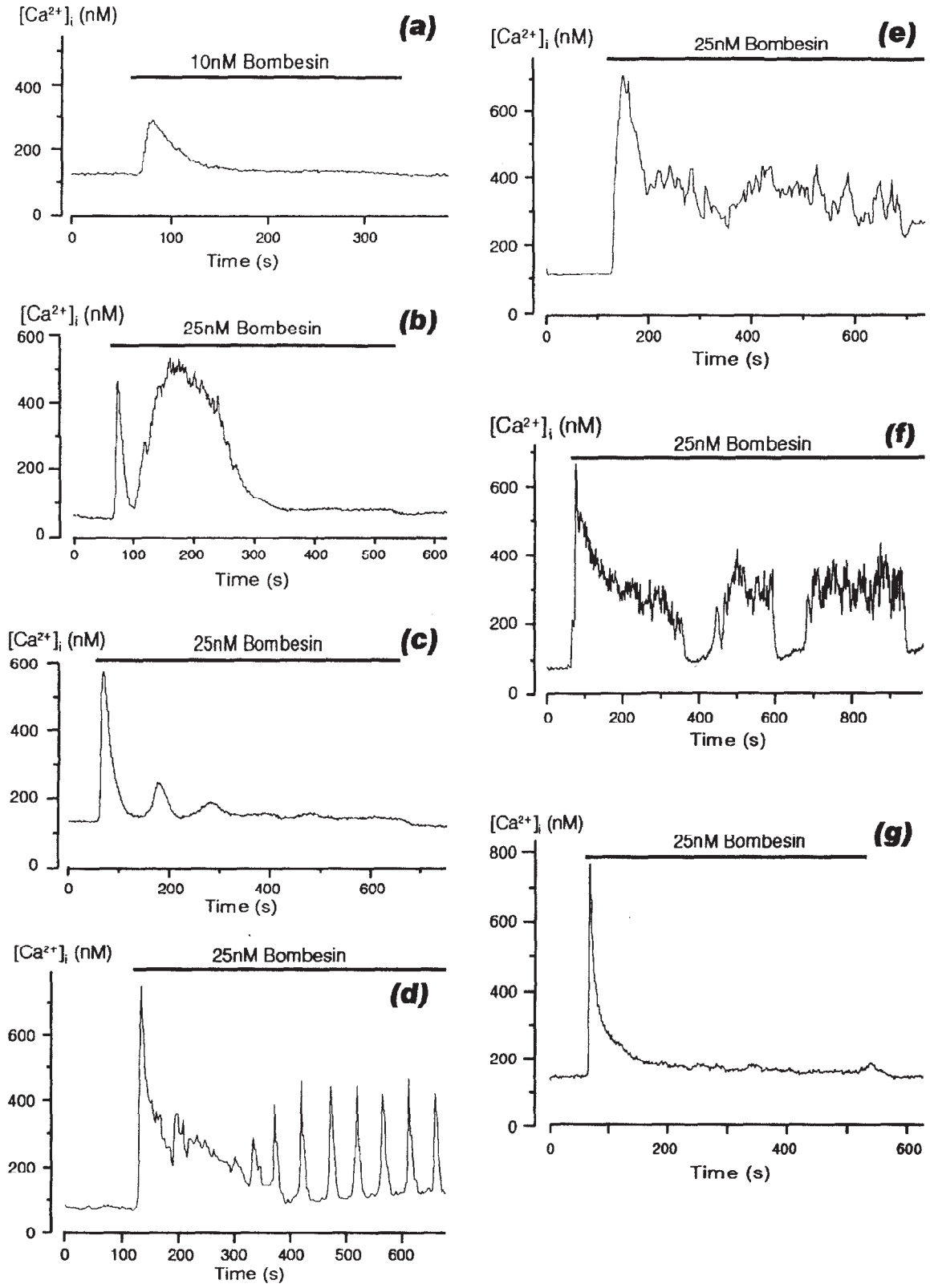


**Fig. 2** Heterogeneity of $[Ca^{2+}]_i$ responses to bombesin stimulation in single HIT-T15 cells. The cells were superfused with Krebs-Ringer-bicarbonate-HEPES buffer containing 0.5 mM glucose for about 30 min before and during bombesin stimulation.

in a gradual fading and faster disappearance of the oscillations. Reintroduction of glucose restored the bombesin-evoked $[Ca^{2+}]_i$ oscillations (Fig. 1b). From these observations it was concluded that minimal glucose was necessary to sustain the bombesin response and that a concentration of 0.5 mM was adequate for the purpose of this study. Therefore, HIT-T15 cells were always pre-equilibrated at this glucose concentration for about 30 min before being challenged by bombesin.

#### Heterogeneity of bombesin-induced $[Ca^{2+}]_i$ response

Bombesin (5–100 nM) increased $[Ca^{2+}]_i$ in the large majority of cells (> 90%). There was a marked heterogeneity in the $[Ca^{2+}]_i$ response profiles which also depended on the agonist concentrations (Fig. 2). This was particularly notable in the second phase period. The patterns of $[Ca^{2+}]_i$ rise can be grouped into three types of response. Type I exhibited only one or two transients (Fig. 2a–c), often seen at lower hormone concentrations (10 nM). Type II, seen preferentially at concentrations around 25 nM, displayed continuous $[Ca^{2+}]_i$ oscillations following a large initial peak (Fig. 2d–f). $[Ca^{2+}]_i$ oscillations could last as long as 50 min (Fig. 1). The frequency and profile of $[Ca^{2+}]_i$ oscillations varied. In some cells, these oscillations were regular with a frequency of around 1 transient every 30–100 s ($77 \pm 5$ s, $n = 33$); each cycle was started by a gradual increase of $[Ca^{2+}]_i$ over the basal level to a threshold from which a quick spike was triggered, rapidly returning thereafter to basal level (Fig. 2d). In some cells, rapid irregular oscillations were superimposed on an elevated $[Ca^{2+}]_i$ plateau (Fig. 2e). Combinations of Types I and II were also encountered (Fig. 2f). A third type of response was frequently noticed at high doses of the agonist, similar to the $[Ca^{2+}]_i$ response observed in cell populations, i.e. a large initial transient followed by a sustained elevation (Fig. 2g). At 25 nM bombesin, the percentage of Types I, II, and III of $[Ca^{2+}]_i$ responses in a total of 106 HIT-T15 cells examined was 15, 61 and 19%, respectively. The Type II pattern was regular in 55%, irregular in 29% and mixed in 12% of the cases. Since the majority of cells responded to 25 nM bombesin with oscillations in $[Ca^{2+}]_i$, this concentration was used in subsequent experiments.

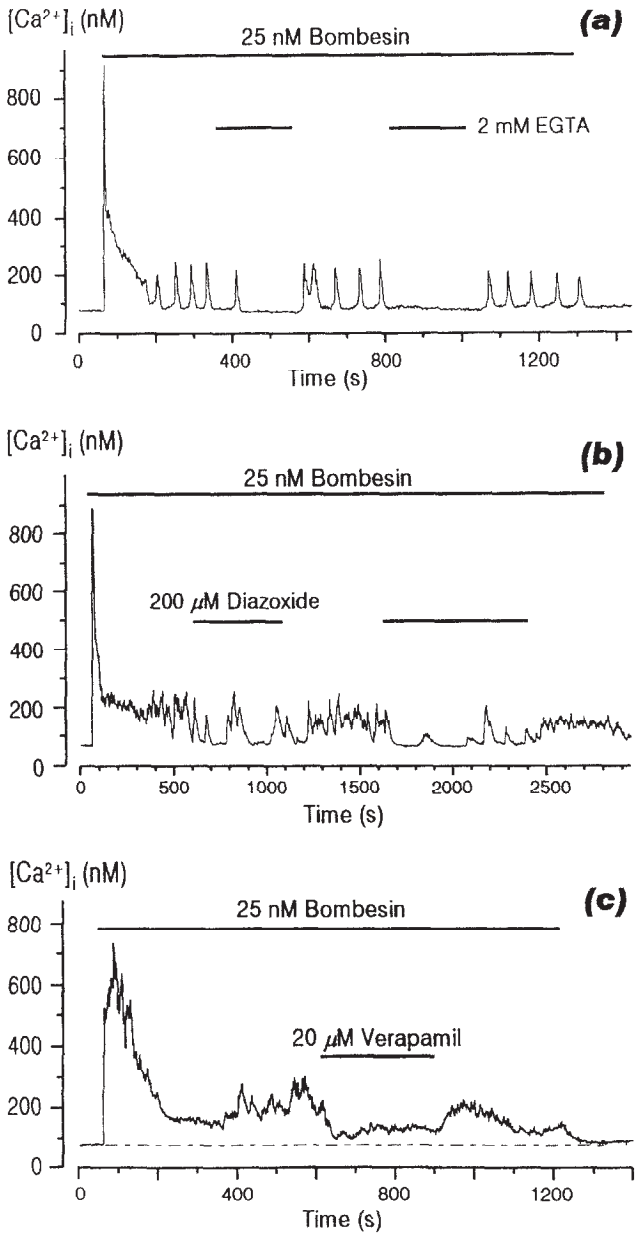


**Fig. 3** Effect of EGTA, diazoxide and verapamil on bombesin-induced $[Ca^{2+}]_i$ oscillations in HIT-T15 cells. The medium contained 0.5 mM glucose. Each trace is representative of at least 3 experiments.

#### Dependence of bombesin-induced $[Ca^{2+}]_i$ oscillations on extracellular $Ca^{2+}$ and voltage sensitive $Ca^{2+}$ permeability

In order to ascertain the contribution of $Ca^{2+}$ influx to the bombesin-induced $[Ca^{2+}]_i$ oscillations, extracellular $Ca^{2+}$ was first depleted by the application of the $Ca^{2+}$ chelator EGTA. 2 mM EGTA, which would lower the extracellular free $Ca^{2+}$ level to < 100 nM in the presence of 1 mM calcium in the medium, rapidly abolished bombesin-elicited $[Ca^{2+}]_i$ oscillations which reappeared when the chelator was withdrawn (Fig. 3a). During the first addition of EGTA, a single delayed $Ca^{2+}$ spike was still seen, indicating that bombesin can mobilize $Ca^{2+}$ from intracellular stores in $Ca^{2+}$ depleted medium.

In HIT-T15 cell suspensions, both voltage-dependent and independent $Ca^{2+}$ entry have been implicated in

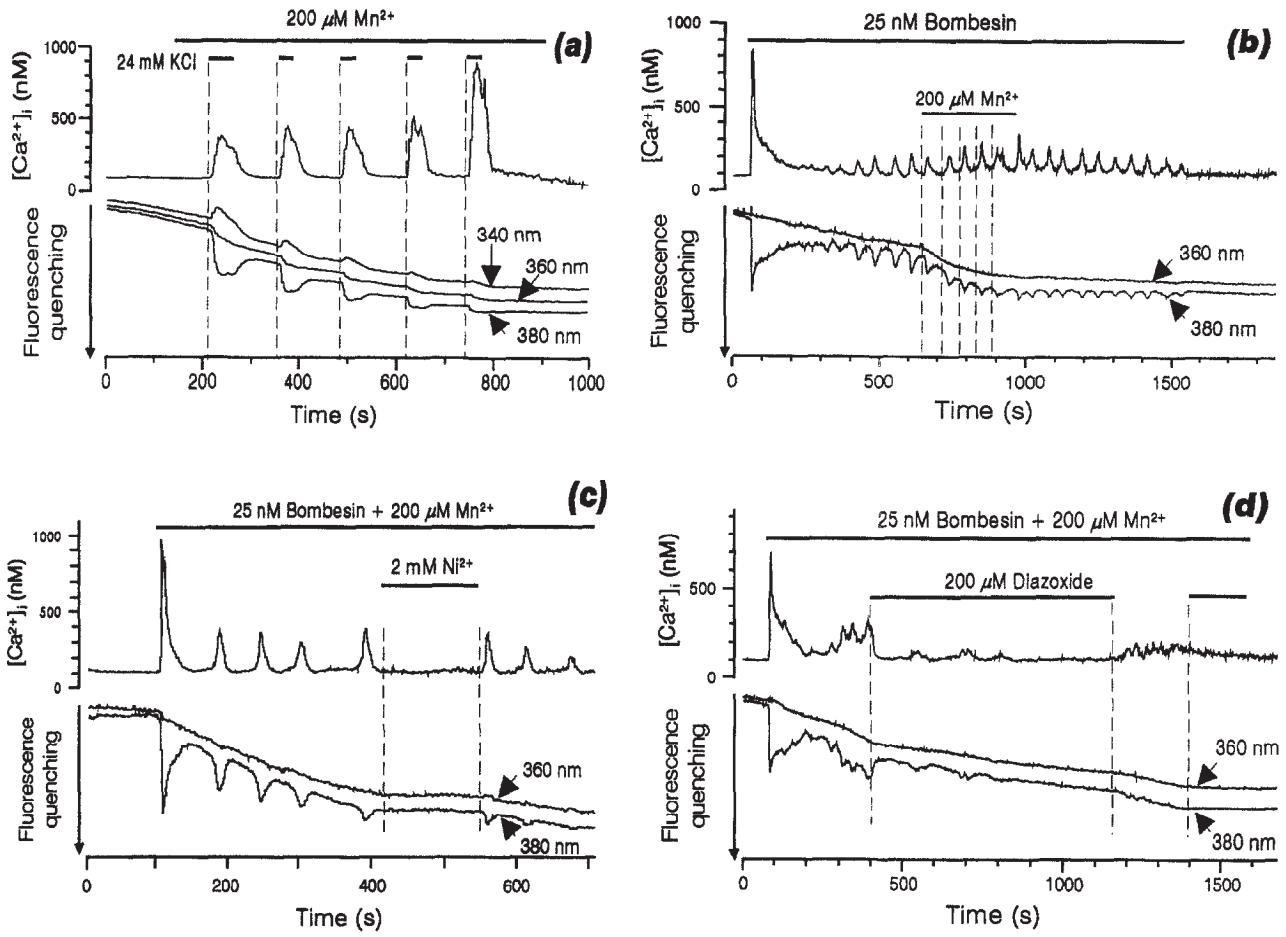


**Fig. 4** Effect of $Ni^{2+}$ and diazoxide on $[Ca^{2+}]_i$ and $Mn^{2+}$ influx in HIT-T15 cells stimulated with pulses of $K^+$ or continuous application of bombesin. (**a**) pulses of 24 mM KCl; (**b**) 25 nM bombesin; (**c**) blockade of the bombesin effect by 2 mM $Ni^{2+}$; (**d**) inhibition of the bombesin-induced $[Ca^{2+}]_i$ rise by 200 μM diazoxide. (For explanation *see* text.) Each trace is representative of at least 3 experiments.

bombesin-induced second phase $Ca^{2+}$ rise [38]. At the single cell level, hyperpolarization of cells with 200 μM diazoxide, which opens ATP-sensitive $K^+$ channels, attenuated bombesin-evoked $[Ca^{2+}]_i$ oscillations (Fig. 3b), suggesting that $[Ca^{2+}]_i$ fluctuations are partially dependent on membrane potential. Verapamil (20 μM), an L-type $Ca^{2+}$ channel blocker, also diminished these $[Ca^{2+}]_i$ oscillations (Fig. 3c). Similar results (not shown) were obtained with another L-type $Ca^{2+}$ channel blocker SR7037 at 1 μM [19,38], ω-conotoxin (3 μM) which blocks N-type $Ca^{2+}$ channels [12], or low concentrations of $Ni^{2+}$ (100 μM) inhibiting T-type channels [12], did not affect the bombesin-elicited $[Ca^{2+}]_i$ oscillations, indicating that neither N- nor T-channels are involved in the generation of the $[Ca^{2+}]_i$ spiking (not shown). In contrast, $Ni^{2+}$ at 2 mM, which obliterates receptor-mediated as well as voltage-gated $Ca^{2+}$ channels, also abrogated bombesin-induced $[Ca^{2+}]_i$ oscillations (Fig. 4c). These observations indicate that bombesin-elicited $[Ca^{2+}]_i$ oscillations strongly depend on extracellular $Ca^{2+}$ and that voltage sensitive $Ca^{2+}$ channels participate, at least in part, in the influx component.

### Absence of coordinate fluctuation in $Ca^{2+}$ influx during bombesin-induced $[Ca^{2+}]_i$ oscillations

The above results clearly demonstrated the dependency of bombesin-induced $[Ca^{2+}]_i$ oscillations on extracellular $Ca^{2+}$ influx. This raises the important question as to whether $[Ca^{2+}]_i$ oscillations are due to, or are accompanied by, correlated fluctuations of $Ca^{2+}$ permeability at the plasma membrane. To elucidate this question we applied extracellular $Mn^{2+}$, a $Ca^{2+}$ surrogate which quenches the Fura–2 fluorescence when entering the cell. Consequently, any change in the rate of fluorescence quenching should reflect an increased $Ca^{2+}$ influx

(Fig. 4). To monitor simultaneously changes in both $[Ca^{2+}]_i$ and $Mn^{2+}$-induced fluorescence quenching, three fluorescence signals were recorded simultaneously with appropriate filter pairs (Fig. 4a). The changes in fluorescence at 340 and 380 nm indicate the alterations in $[Ca^{2+}]_i$, while the $Ca^{2+}$-insensitive signal recorded at 360 nm (the isosbestic point of Fura–2) only reflects Fura–2 quenching by $Mn^{2+}$ influx [40]. Under resting conditions, the addition of 200 μM $Mn^{2+}$ scarcely enhanced the fluorescence decay due to Fura–2 leakage and photobleaching. In order to test whether transient signals of quenching could be monitored, short depolarizing $K^+$ pulses (24 mM) were applied in the presence of $Mn^{2+}$. A series of $Ca^{2+}$ rises ensued which were accompanied by a coordinate increase in fluorescence quenching. Therefore, a similar fluctuating quenching by $Mn^{2+}$ should be observed, if such a phenomenon occurs in bombesin-elicited $[Ca^{2+}]_i$ oscillations. The results shown in Figure 4b demonstrate that this was not the case. Bombesin evoked a series of $[Ca^{2+}]_i$ spikes whose height was similar to those caused by $K^+$. In contrast to the observation made with pulsatile $K^+$, an immediate and regular rate of fluorescence quenching was detected from the time of application of the agonist. Accordingly, when $Mn^{2+}$ was applied during the oscillation period, there was a rapid decrease in the 360 nm signal without coordinate quenching associated with the $[Ca^{2+}]_i$ spikes. These findings suggest that during bombesin-elicited $[Ca^{2+}]_i$ oscillations, cyclic changes in plasma membrane $Ca^{2+}$ permeability do not occur in HIT-T15 cells.

In agreement with the results presented in Figure 3, bombesin-induced $Mn^{2+}$ fluorescence quenching was abrogated by EGTA (not shown), by diazoxide (Fig. 4d) and by verapamil (not shown), indicating that $Mn^{2+}$ quenching indeed reflects bombesin-evoked $Ca^{2+}$ influx. Similar results were obtained with high $Ni^{2+}$ (2 mM: Fig. 4c).

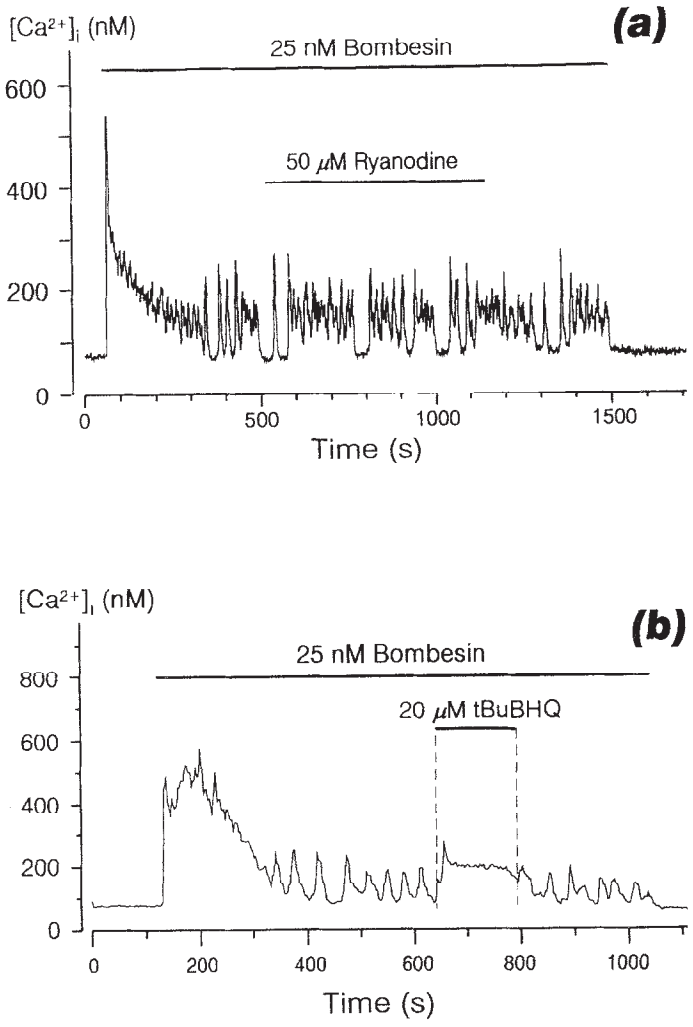


**Fig. 5** Effect of ryanodine and 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ) on bombesin-induced $[Ca^{2+}]_i$ oscillations in HIT-T15 cells. Each trace is representative of at least 3 experiments.

### Disruption of bombesin-induced $[Ca^{2+}]_i$ oscillations by agents affecting intracellular $Ca^{2+}$ stores

Bombesin is a phospholipase C activator which produces $Ins(1,4,5)P_3$-mediated mobilization of $Ca^{2+}$ from intracellular stores. Two compounds, ryanodine and 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ), were used to examine the role of intracellular $Ca^{2+}$ stores in the generation of $[Ca^{2+}]_i$ oscillations. Ryanodine interferes with CICR from intracellular stores [42], while tBuBHQ depletes intracellular $Ca^{2+}$ pools by inhibiting the $Ca^{2+}$-ATPase in the endoplasmic reticulum (ER) [43]. Ryanodine (50 μM) did not affect the $[Ca^{2+}]_i$ oscillations (Fig. 5a) and did not raise $[Ca^{2+}]_i$ when added (2–50 μM) alone (not shown). tBuBHQ (20 μM) disrupted bombesin-elicited $[Ca^{2+}]_i$ oscillations and caused a sustained $[Ca^{2+}]_i$ plateau (Fig. 5b). The tBuBHQ effect was reversible since the bombesin-induced $[Ca^{2+}]_i$ oscillations resumed after withdrawal of the drug.

The effect of another putative CICR activator, caffeine [44], was also investigated. Caffeine by itself (10 mM) was able to cause a moderate increase in $[Ca^{2+}]_i$ (Figs 6 & 7b). Ryanodine did not alter caffeine-induced $[Ca^{2+}]_i$ rise (Fig. 6a). The caffeine-evoked $[Ca^{2+}]_i$ increase, however, was rapidly abolished or completely prevented by the $Ca^{2+}$ chelator EGTA (Fig. 6b), indicating an extracellular source of $Ca^{2+}$. When caffeine was applied during bombesin stimulation, the hormone-elicited $[Ca^{2+}]_i$ oscillations were inhibited (Fig. 6c). This caffeine effect was reversible. However, when caffeine was added before bombesin, bombesin was still able to cause $[Ca^{2+}]_i$ fluctuations

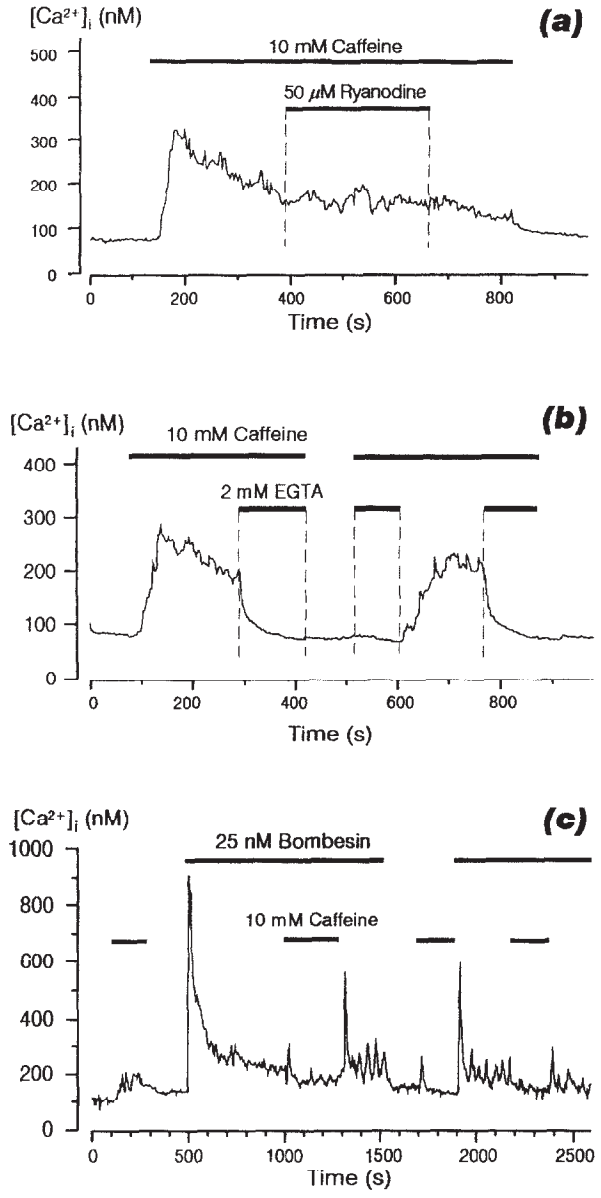


**Fig. 6** Effect of caffeine on basal and bombesin-elicited $[Ca^{2+}]_i$ rises in HIT-T15 cells. Action of ryanodine (**a**) and EGTA (**b**) on caffeine-raised $[Ca^{2+}]_i$; additions of caffeine during bombesin stimulation (**c**). Each trace is representative of at least 3 experiments.

which disappeared upon removal of caffeine (Fig. 7b). These results suggest that caffeine exerts complex effects on $Ca^{2+}$ handling in HIT-T15 cells.

Chlorocresol has been reported to mobilize $Ca^{2+}$ by acting on the CICR store [45]. In a set of experiments, we examined the action of 4-chloro-*m*-cresol on bombesin-induced $[Ca^{2+}]_i$ oscillations. Like tBuBHQ (*see* Fig. 5b), chlorocresol application resulted in a sustained $[Ca^{2+}]_i$ rise disrupting in a reversible manner the bombesin-elicited $[Ca^{2+}]_i$ fluctuations (Fig. 7a). This agent alone was capable of raising $[Ca^{2+}]_i$ to an extent larger than that due to caffeine as demonstrated in the same cell (Fig. 7b) and its action was not affected by ryanodine (Fig. 7c). The main effect of the drug appeared to be mediated through the mobilization of intracellular $Ca^{2+}$ stores, since EGTA only decreased the 4-chloro-*m*-cresol-induced $Ca^{2+}$ rise when added after it, and failed to block its effect when added before the cresol compound (Fig. 7c).

## DISCUSSION

$Ca^{2+}$-sensitive fluorescent probes made possible the demonstration that $[Ca^{2+}]_i$ oscillations play an essential role in the stimulation of insulin secretion induced by both glucose [7,8,15,20,22] and receptor agonists [14,16,18,21]. Glucose-elicited $[Ca^{2+}]_i$ oscillations correlated well with the electrical activity in the islet [23] and, as demonstrated more recently, with pulsatile insulin secretion [21,30,46]. Glucose induced $[Ca^{2+}]_i$ oscillations depend on extracellular $Ca^{2+}$ entry, but a recent study shows that intracellular $Ca^{2+}$ mobilization might possibly be implicated [47]. The presence of an oscillatory change in metabolism has been suggested [22,24–26]. This drives $[Ca^{2+}]_i$ oscillations, possibly via ATP/ADP ratio changes, leading to the closing/opening of ATP-sensitive $K^+$ channels. It has also been proposed that this may be due to the interaction between nutrients and intra islet hormone and neurotransmitters on the β-cell [48]. Since the glucose-elicited $[Ca^{2+}]_i$ oscillations were also observed in single β-cells, the fundamental device for the oscillation generation by the hexose does not appear to involve necessarily receptor agonists which, however, probably modulate the glucose action [27,28].

The main aim of the present study was to investigate the mechanisms of generation of $[Ca^{2+}]_i$ oscillations caused by bombesin, a phospholipase C activator that promotes insulin secretion from HIT-T15 cells [38,41]. This receptor agonist produced heterogeneous $[Ca^{2+}]_i$ responses in HIT-T15 cells, although most of the cells displayed a dose-dependent oscillatory pattern. The bombesin-elicited $[Ca^{2+}]_i$ oscillations required a non-stimulatory level of glucose. This glucose dependency was also seen in carbachol-evoked $[Ca^{2+}]_i$ oscillations in islet β-cells [7,18,20]. In HIT-T15 cell suspensions, bombesin caused a larger membrane depolarization and more marked $[Ca^{2+}]_i$ rises in the presence of nutrients [38]. Furthermore, glucose metabolism may also participate indirectly in the mechanisms of $[Ca^{2+}]_i$ entry via the generation of critical ATP and GTP concentrations necessary for the maintaining of receptor agonist-induced $Ca^{2+}$ entry [49]. Finally, reduced frequency of $[Ca^{2+}]_i$ oscillations seen in the absence of glucose could also result from the depletion of intracellular stores due to a deficient

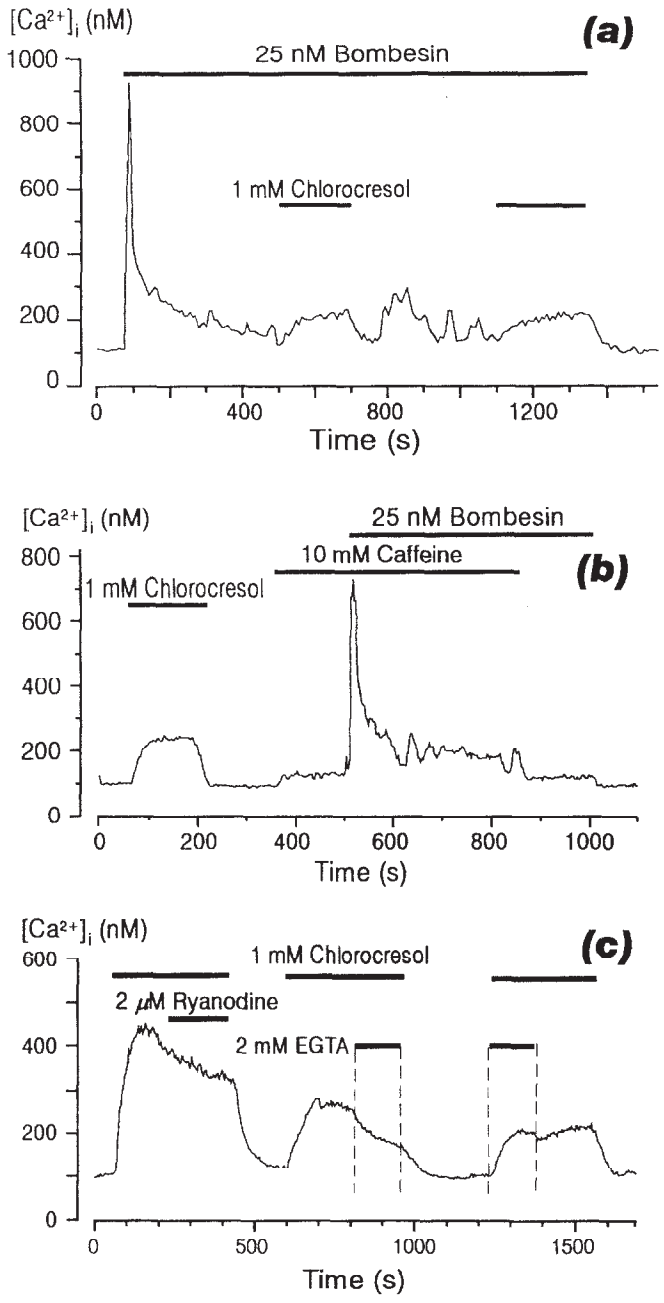


**Fig. 7** Comparison of 4-chloro-*m*-cresol (chlorocresol) and caffeine effects on basal and bombesin-elicited $[Ca^{2+}]_i$ rises (**a,b**). Note the failure of ryanodine to affect the chlorocresol-induced $[Ca^{2+}]_i$ rise and the only partial inhibition of EGTA on the latter (**c**). Each trace is representative of at least 3 experiments.

ATP-dependent $Ca^{2+}$ pumping from the cytosol into the stores [7].

Bombesin-elicited $[Ca^{2+}]_i$ oscillations not only depended on the presence of glucose but also required extracellular $Ca^{2+}$ influx. This was demonstrated by the removal of extracellular $Ca^{2+}$ by the application of $Ni^{2+}$, a non-selective cation blocker, and by the $Mn^{2+}$ quenching technique. This $Ca^{2+}$ influx was partially membrane potential-sensitive and occurred via L-type but apparently not via N- or T-type channels. This is reminiscent of the vasopressin-evoked $[Ca^{2+}]_i$ oscillations which were also affected by hyperpolarization with diazoxide [19]. Diazoxide did not completely abolish bombesin-elicited $[Ca^{2+}]_i$ oscillations, indicating that both voltage-sensitive and voltage-insensitive $Ca^{2+}$ permeability contribute to this $Ca^{2+}$ entry.

The key question, therefore, arises as to whether receptor agonist-induced $[Ca^{2+}]_i$ fluctuations in HIT-T15 are governed by an oscillating $Ca^{2+}$ conductance at the plasma membrane coordinated with the release of intracellular $Ca^{2+}$ stores. In AR42J cells, Loessberg et al. found [36], using the $Mn^{2+}$ quenching technique [40], that carbachol induced a repetitive opening of $Ca^{2+}$ channels which followed the $[Ca^{2+}]_i$ spikes. However, this could not be observed in endothelial cells [37], probably because of the omission of extracellular $Ca^{2+}$. Although we were able to generate synchronous oscillatory patterns of $[Ca^{2+}]_i$ and $Mn^{2+}$ quenching with $K^+$ pulses, similar $[Ca^{2+}]_i$ rises evoked by bombesin failed to do so. The reason for the discrepancy between our results and those of Loessberg et al. [36] is not clear, in particular since both studies employed physiological extracellular $Ca^{2+}$ concentrations. This may be due to inherent differences between the two cell types such as the expression of distinct $Ca^{2+}$ channel subtypes. Indeed, evidence for the presence of $Mn^{2+}$ impermeable $Ca^{2+}$ channels has been documented and cannot be totally discarded [50,51].

The following conclusions can therefore be drawn at this stage. Bombesin-elicited $[Ca^{2+}]_i$ oscillations do not involve fluctuations in plasma membrane permeability and a steadily increased $Ca^{2+}$ influx is sufficient for the preservation of these oscillations. Since bombesin-induced $[Ca^{2+}]_i$ oscillations are in part sensitive to membrane hyperpolarization and L-channel blockers, it is probable that one component may still result from a synergistic action of bombesin and glucose on membrane potential, causing a partial gating of voltage-sensitive $Ca^{2+}$ permeabilities.

Phospholipase C activators produce $Ins(1,4,5)P_3$ which mobilizes $Ca^{2+}$ from intracellular stores. The integrity of the intracellular $Ca^{2+}$ pools is essential for the generation of $[Ca^{2+}]_i$ oscillations [52]. At least two intracellular $Ca^{2+}$ pools have been described [10,33]: one $Ins(1,4,5)P_3$-sensitive and another sensitive to ryanodine or caffeine which is activated by $Ca^{2+}$ (CICR) [32,42,44]. There is evidence for the presence of CICR in insulin-secreting cells [53], based on the potentiation by caffeine of $Ca^{2+}$ release from an $Ins(1,4,5)P_3$-insensitive pool. In the present study, depletion of intracellular stores with tBuBHQ inhibited the generation of $[Ca^{2+}]_i$ oscillations. In contrast, ryanodine

did not alter bombesin-elicited $[Ca^{2+}]_i$ oscillations. Similarly, this alkaloid did not affect glucose-induced $[Ca^{2+}]_i$ oscillations in β-cells, although it blocked the potentiating effect of caffeine on glucose-evoked $[Ca^{2+}]_i$ rise [7]. We employed another potent CICR activator, 4-chloro-*m*-cresol, known to promote $Ca^{2+}$ release from an $Ins(1,4,5)P_3$-insensitive store via the activation of a ruthenium red and caffeine-sensitive channel [45]. This compound disrupted the bombesin-elicited $[Ca^{2+}]_i$ oscillations, suggesting the involvement of CICR. However, this is in discrepancy with our results obtained with ryanodine. This may be due to an insensitivity to ryanodine peculiar to the HIT-T15 cells, or to the action of 4-chloro-*m*-cresol on a $Ca^{2+}$ pool not participating in CICR. Further studies are required to define the precise involvement of CICR in $[Ca^{2+}]_i$ oscillations elicited by bombesin in the HIT-T15 cell line.

In HIT-T15 cells, intracellular $Ca^{2+}$ pools probably display fast turnover since $[Ca^{2+}]_i$ oscillations are quickly attenuated (*see* Fig. 3a) upon removal of extracellular $Ca^{2+}$ or blockade of $Ca^{2+}$ entry. This contrasts with many other cells in which agonist-induced $[Ca^{2+}]_i$ oscillations are preserved for long periods in $Ca^{2+}$-free medium [10,33]. It could be speculated that in the absence of $Ca^{2+}$ influx, the $Ca^{2+}$-ATPase of the plasma membrane in HIT-T15 cells outweighs that of $Ca^{2+}$ stores, thus explaining the strict dependency on $Ca^{2+}$ influx.

In addition to the voltage sensitive $Ca^{2+}$ entry [38], bombesin may promote $Ca^{2+}$ influx via another receptor-mediated effect. The underlying mechanism governing this $Ca^{2+}$ entry pathway is still under debate [10,19,54]. One model involves the activation of a $Ca^{2+}$ release-activated $Ca^{2+}$ current ($I_{CRAC}$) via a capacitative pathway [13]. A putative signalling molecule (oscillatory?) linking $Ca^{2+}$ stores emptying to $I_{CRAC}$ activation was isolated but still remains elusive [54]. It is noteworthy that in HIT-T15 cells stimulated with bombesin, the depletion of intracellular stores did not enhance such a $Ca^{2+}$ conductance at the plasma membrane. This is at variance with what we observed previously in vasopressin-stimulated RINm5F cells [19].

Caffeine had surprising effects on $[Ca^{2+}]_i$ in HIT-T15 cells. On the one hand, it raised $[Ca^{2+}]_i$ apparently by promoting $Ca^{2+}$ influx rather than by mobilizing it from intracellular stores. On the other hand, caffeine also inhibited bombesin-elicited $[Ca^{2+}]_i$ oscillations. This effect could be due to inhibition of phospholipase C since caffeine blocked agonist-stimulated $Ins(1,4,5)P_3$ generation in pancreatic acinar cells [55]. In our study, the reason for the suppressing effect of caffeine pretreatment on bombesin-elicited $[Ca^{2+}]_i$ oscillations may be similar to the observation reported in hyperpolarized β-cells stimulated with carbachol [29]. Indeed, the off responses seen upon removal of caffeine indicate that caffeine probably interferes within the signalling to $IP_3$ sensitive stores. This is also suggested from its inhibition of ATP-induced $[Ca^{2+}]_i$ oscillations in islet β-cells [56]. In studies on isolated islet β-cells, Islam et al. [57] recently found that caffeine caused $Ca^{2+}$ influx via L-channels at low glucose and decreased or potentiated the $[Ca^{2+}]_i$ rises induced by high glucose. Taken together, the results of this study and those of others [57] indicate that the multiple sites of action of caffeine render results with this pharmacological tool difficult to interpret, at least for the study of CICR in insulin secreting cells.

In conclusion, our observations indicate that $[Ca^{2+}]_i$ oscillations caused by bombesin in the insulin-secreting cell HIT-T15 result mainly from the interplay of intracellular $Ca^{2+}$ mobilization and extracellular $Ca^{2+}$ entry. Furthermore, we found no indication for the presence of coordinate fluctuating changes in the plasma membrane $Ca^{2+}$ permeability accompanying the $[Ca^{2+}]_i$ oscillations induced by bombesin. The extension of these results to normal β-cells remains to be established.

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