Effects of caffeine on cytoplasmic free Ca^{2+} concentration in pancreatic β-cells are mediated by interaction with ATP-sensitive K^+ channels and L-type voltage-gated Ca^{2+} channels but not the ryanodine receptor

Md. Shahidul ISLAM, Olof LARSSON, Thomas NILSSON and Per-Olof BERGGREN*

The Rolf Luft Center for Diabetes Research, Department of Molecular Medicine, Karolinska Institute, Karolinska Hospital, S-171 76 Stockholm, Sweden

In the pancreatic β-cell, an increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) by caffeine is believed to indicate mobilization of Ca^{2+} from intracellular stores, through activation of a ryanodine receptor-like channel. It is not known whether other mechanisms, as well, underlie caffeine-induced changes in $[Ca^{2+}]_i$. We studied the effects of caffeine on $[Ca^{2+}]_i$ by using dual-wavelength excitation microfluorimetry in fura-2-loaded β-cells. In the presence of a non-stimulatory concentration of glucose, caffeine (10–50 mM) consistently increased $[Ca^{2+}]_i$. The effect was completely blocked by omission of extracellular Ca^{2+} and by blockers of the L-type voltage-gated Ca^{2+} channel, such as D-600 or nifedipine. Depletion of agonist-sensitive intracellular Ca^{2+} pools by thapsigargin did not inhibit the stimulatory effect of caffeine on $[Ca^{2+}]_i$. Moreover, this effect of caffeine was not due to an increase in cyclic AMP, since forskolin and 3-isobutyl-1-methylxanthine (IBMX) failed to raise $[Ca^{2+}]_i$ in unstimulated β-cells. In β-cells, glucose and sulphonylureas increase $[Ca^{2+}]_i$ by causing closure of ATP-sensitive K^+ channels (K_{ATP} channels). Caffeine also caused inhibition of K_{ATP} channel activity, as measured in excised inside-out patches. Accordingly, caffeine (> 10 mM) induced insulin release from β-cells in the presence of a non-stimulatory concentration of glucose (3 mM). Hence, membrane depolarization and opening of voltage-gated L-type Ca^{2+} channels were the underlying mechanisms whereby the xanthine drug increased $[Ca^{2+}]_i$ and induced insulin release. Paradoxically, in glucose-stimulated β-cells, caffeine (> 10 mM) lowered $[Ca^{2+}]_i$. This effect was due to the fact that caffeine reduced depolarization-induced whole-cell Ca^{2+} current through the L-type voltage-gated Ca^{2+} channel in a dose-dependent manner. Lower concentrations of caffeine (2.5–5.0 mM), when added after glucose-stimulated increase in $[Ca^{2+}]_i$, induced fast oscillations in $[Ca^{2+}]_i$. The latter effect was likely to be attributable to the cyclic AMP-elevating action of caffeine, leading to phosphorylation of voltage-gated Ca^{2+} channels. Hence, in β-cells, caffeine-induced changes in $[Ca^{2+}]_i$ are not due to any interaction with intracellular Ca^{2+} pools. In these cells, a direct interference with K_{ATP} channel- and L-type voltage-gated Ca^{2+}-channel activity is the underlying mechanism by which caffeine increases or decreases $[Ca^{2+}]_i$.

INTRODUCTION

Cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) plays a key role in the stimulation of insulin secretion from the pancreatic β-cell [1]. In this cell, glucose metabolism is coupled to an increase in $[Ca^{2+}]_i$ through the participation of at least two types of ion channels: the ATP-sensitive K^+ channel (K_{ATP} channel) and the L-type voltage-gated Ca^{2+} channel [1,2]. According to the current model, metabolism of glucose increases the cytosolic ATP/ADP ratio leading to closure of the K_{ATP} channel with consequent depolarization of the cell, opening of voltage-gated L-type Ca^{2+} channels resulting in subsequent influx of Ca^{2+}, increase in $[Ca^{2+}]_i$ and finally exocytosis [2,3]. While Ca^{2+} influx through the voltage-gated Ca^{2+} channels is believed to be the dominant mechanism, mobilization of Ca^{2+} from intracellular Ca^{2+} pools also contributes to the increase in $[Ca^{2+}]_i$ in the β-cell [1,4].

Stimulation of receptors linked to the phospholipase C system induces formation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], which upon binding to its receptor (IP3R) triggers Ca^{2+} release from intracellular stores and Ca^{2+} influx through the plasma membrane [5]. Another major intracellular Ca^{2+} release channel, the ryanodine receptor, is also present in many cells. The ryanodine receptor was originally described in sarcoplasmic reticulum, where it mediates Ca^{2+}-induced Ca^{2+} release [6]. The endogenous ligand for the receptor is unknown, although cyclic adenosine diphosphate ribose (cyclic ADPR) is a candidate [7]. Experimentally, the receptor can be activated by nanomolar concentrations of the plant alkaloid ryanodine or millimolar concentrations of caffeine [6,8]. The caffeine–ryanodine-sensitive intracellular Ca^{2+} pool is typically present in excitable cells [9–11] and has also been described in non-excitable cells [12]. In some cells the ryanodine receptor co-exists with the IP3R, but the distribution of the former is much restricted compared with the ubiquitous IP3R [11,12]. Three ryanodine receptors have been cloned and at least one of them seems to be more widely distributed [13].

The most commonly used pharmacological tool for the study of the ryanodine receptor is caffeine [8]. This substance, notably, has other actions unrelated to its effect on the ryanodine receptor. These include elevation of cyclic AMP by inhibition of cyclic nucleotide phosphodiesterases and inhibition of plasma membrane Ca^{2+} channels [14,15]. To what extent such effects of

Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; K_{ATP}, ATP-sensitive K channel; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; IP3R, Ins(1,4,5)P_3 receptor; IBMX, 3-isobutyl-1-methylxanthine; NMDG, *N*-methyl-D-glucamine; PKA, protein kinase A; *R*pcAMPS, (*R*)-p-cyclic adenosine-3′,5′-monophosphorothioate.

* To whom correspondence should be addressed.

caffeine account for its effects on $[Ca^{2+}]_i$ under different experimental conditions remains unclear. The effects of caffeine on the ryanodine receptor have been reported to vary. In addition to the stimulatory effect, an inhibitory effect of caffeine on the ryanodine receptor has been described in non-muscle cells [16]. Furthermore, one type of ryanodine receptor is insensitive to the activation by caffeine [13]. There is no consensus about the presence of a caffeine-sensitive intracellular Ca^{2+} pool in insulin-secreting cells. Several studies showed that in intact β-cells and islets, caffeine markedly increased $[Ca^{2+}]_i$ [17,18]. On the other hand, in permeabilized insulin-secreting cells, Ca^{2+} release by caffeine is at best marginal [19,20]. Because of such discrepant results, we questioned whether caffeine, in the β-cell, might cause changes in $[Ca^{2+}]_i$ by mechanisms other than those affecting Ca^{2+} mobilization from intracellular stores. In the present study we re-examined the effect of caffeine on $[Ca^{2+}]_i$ and identified the molecular mechanisms by which this compound produced distinct changes in $[Ca^{2+}]_i$ in the pancreatic β-cell.

MATERIALS AND METHODS

Isolation of islets and preparation of β-cells

Pancreatic islets from obese (*ob/ob*) mice from a local non-inbred colony were isolated by collagenase digestion and dispersed into small cell clusters by shaking in a Ca^{2+}- and Mg^{2+}-deficient medium, as previously described [3]. Cells were cultured on glass coverslips or in plastic Petri dishes for 1–3 days, in RPMI 1640 medium containing 5.5 mM glucose, supplemented with fetal-calf serum (10%, v/v), penicillin (100 i.u./ml) and streptomycin (100 μg/ml).

Measurements of $[Ca^{2+}]_i$ by microfluorimetry

Cells attached to coverslips were loaded with fura-2 by incubating in basal medium (in mM): NaCl 125, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.28, Hepes 25, glucose 3, BSA 0.1% (pH 7.4 with NaOH) and fura-2 acetoxymethyl ester 2 μM, for 20 min at 37 °C. Coverslips were washed twice with the buffer and mounted as the bottom of an open chamber placed on the stage of an inverted epi-fluorescence microscope (Zeiss, Axiovert 35M). The perifusate volume in the chamber was 0.2 ml and the perifusion rate 0.3 ml/min. After switching the perifusion solutions, there was a lag period of about 30 s before the new solution reached the chamber. The stage of the microscope was thermostatically controlled, to maintain a temperature of 37 °C in the perifusate inside the chamber. The microscope was connected to a SPEX fluorolog-2 CM1T11I system, for dual-wavelength excitation fluorimetry. The excitation wavelengths generated by two monochromators were directed to the cell by a dichroic mirror. The emitted light, selected by a 510 nm filter, was monitored by a photomultiplier attached to the microscope. The excitation wavelengths were alternated at a frequency of 1 Hz and the length of time for data collection at each wavelength was 0.33 s. The emissions at the excitation wavelength of 340 nm (F_{340}) and that of 380 nm (F_{380}) were used to calculate the fluorescence ratio ($R_{340/380}$). Small clusters of cells (usually 3 or 4), isolated optically by means of the diaphragm of the microscope, were studied using a 100×, 1.3 NA oil-immersion objective (Zeiss, Plan Neofluar). Background fluorescence was measured after quenching of the fura-2 fluorescence with manganese and was subtracted from the traces before calculation of $[Ca^{2+}]_i$ according to the method of Grynkiewicz et al. [21]. Maximum and minimum fluorescence ratios were determined in separate experiments using 1 μl drops of an intracellular-like buffer, containing 10 μM fura-2 free acid and either 2 mM Ca^{2+} or no Ca^{2+} in the presence of 2 mM EGTA. The K_d for the Ca^{2+}–fura-2 complex was taken as 225 nM. In order to compensate for variations in output light intensity from the two monochromators, all experiments were corrected for by the inclusion of a fluorescence ratio where both monochromators were set at 360 nm. No correction was made for interference of fura-2 fluorescence by caffeine and, where $[Ca^{2+}]_i$ was measured in the presence of caffeine, the estimated $[Ca^{2+}]_i$ was probably lower than the actual $[Ca^{2+}]_i$.

Electrophysiological recordings

We used the inside-out and whole-cell configurations of the patch–clamp technique [22]. Pipettes were prepared from borosilicate glass capillary tubes, coated with Sylgard resin (Dow Corning) near the tips, fire-polished and had resistances of 2–6 MΩ. For the study of the K_{ATP} channel, single-channel currents were recorded from inside-out membrane patches at 0 mV membrane potential. Currents were recorded using an Axopatch 200 patch–clamp amplifier (Axon Instruments Inc., Foster City, CA, U.S.A.). During experiments the current signals were stored using a VR-100A digital recorder (Instrutech Corp., U.S.A.) and a high-resolution video-cassette recorder (JVC, Japan). Channel records are displayed according to the convention, with upward deflections denoting outward currents. K_{ATP}-channel activity was identified on the basis of sensitivity to ATP and unitary amplitude (1.5–2 pA). The extracellular solution contained (in mM): NaCl 138, KCl 5.6, $MgCl_2$ 1.2, $CaCl_2$ 2.6, Hepes 5 (pH 7.4 with NaOH). The intracellular-like solution consisted of (in mM): KCl 125, $MgCl_2$ 1, EGTA 10, KOH 30, Hepes 5 (pH 7.15 with KOH). Patches were excised into nucleotide-free solution and ATP was first added to test for channel inhibition. ATP was then removed and patches were exposed to solutions containing caffeine. Mg-ATP (0.1 mM) was present in the intracellular solution for most of the time to reduce run-down of K_{ATP}-channel activity. Each experimental condition was tested, with identical results, in three to six different patches. The current signal was filtered at 100 Hz (−3 dB value) by using an eight-pole Bessel filter (Frequency Devices, Haverhill, U.S.A.). Single-channel amplitude was measured directly from a digital oscilloscope.

For the study of the voltage-gated Ca^{2+} channels, cells were washed with a solution composed of (in mM): NaCl 138, KCl 5.6, $MgCl_2$ 1.2, $CaCl_2$ 10, tetraethylammonium chloride 10, Hepes 5 (pH 7.4 with NaOH). The pipette solution contained (in mM): *N*-methyl-D-glucamine (NMDG) 150, HCl 110, $MgCl_2$ 1, $CaCl_2$ 2, EGTA 10, Mg-ATP 3, Hepes 5 (pH 7.15). NMDG was substituted for K^+ in the pipette solution to block outwardly directed K^+ currents. Voltage-steps were generated, digitized and stored using the programs pClamp (Axon Instruments) and Labmaster ADC (Scientific Solutions, U.S.A.). The current responses were filtered at 2 kHz. The pulse protocol is given in the Figure legends. Figures were made by plotting segments of the records on a chart recorder, scanning the segments using a HP scanner and incorporating them into Corel Draw graphics software program.

Measurement of insulin release

β-cells obtained from *ob/ob* mice were cultured overnight in RPMI 1640 medium containing 11 mM glucose and otherwise with the same composition as described under 'Isolation of islets and preparation of β-cells'. Cells were washed twice in the basal medium containing 3 mM glucose and thereafter about 2×10^5 cells were mixed with Bio-Gel P4 polyacrylamide beads (Bio-Rad) in a 0.5 ml column and perifused with the same medium at 37 °C with a flow rate of 120 μl/min. The column was washed by

perifusing for 15 min and then fractions were collected at 1–2 min intervals. Insulin concentrations in the collected fractions were measured by radioimmunoassay with crystalline rat insulin as reference. Values are expressed as percentages of the average insulin release during the 5 min period preceding the addition of caffeine.

Materials

Forskolin was from Hoechst and D-600 from Knoll AG (Ludwigshaffen, Germany). (*R*)-p-cyclic adenosine-3′,5′-monophosphorothioate (*R*pcAMPS) was a gift from Biolog (Bremen, Germany). All other chemicals were from Sigma.

Statistical analysis

Statistical significance was judged by Student's *t* test for unpaired data.

RESULTS

Effect of caffeine on fura-2 fluorescence

In an intracellular-like solution caffeine was only weakly fluorescent (< 15% increase in signal), but it affected fura-2 fluorescence considerably. In a cell-free system, caffeine (40 mM) increased fura-2 fluorescence to a variable extent, depending on the wavelengths and ambient $[Ca^{2+}]$ (Figures 1a and 1b). At 100 nM Ca^{2+}, caffeine caused a large increase in F_{380} (190% of control) followed by an increase in F_{360} (175%) and F_{340} (160%), while net increase in fluorescence in arbitrary units was 284, 173, and 26 respectively. At a $[Ca^{2+}]$ of 1 μM, the increase in F_{380} was 132% (net increase in arbitrary units 61) and F_{360} 122% (40) with no change in F_{340}. Caffeine did not have significant effect on cellular autofluorescence.

Effects of caffeine on $[Ca^{2+}]_i$

In the presence of extracellular Ca^{2+} and a non-stimulatory glucose concentration (3 mM), caffeine increased $[Ca^{2+}]_i$ in a concentration-dependent manner. The minimal and maximal effective concentrations of caffeine, as added to the perifusion system, were 10 mM and 50 mM respectively. At 3 mM glucose,

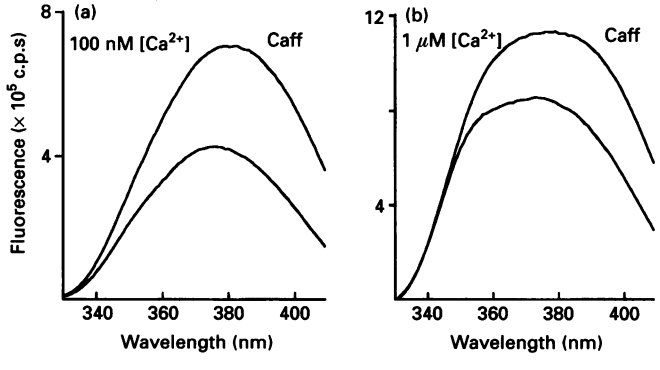


Figure 1 Effects of caffeine on fluorescence excitation spectra of fura-2

Spectra were obtained in a drop of intracellular-like solution containing 120 mM KCl, 1 mM EGTA, 25 mM Hepes (pH 7.2) and 10 μM fura-2 free acid. Fluorescence emission was measured at 510 nm. $[Ca^{2+}]$ was adjusted to 100 nM (**a**) or 1 μM (**b**), as verified by using a Ca^{2+}-sensitive mini-electrode. In caffeine-containing solutions caffeine (40 mM) was added with iso-osmotic replacement of KCl.

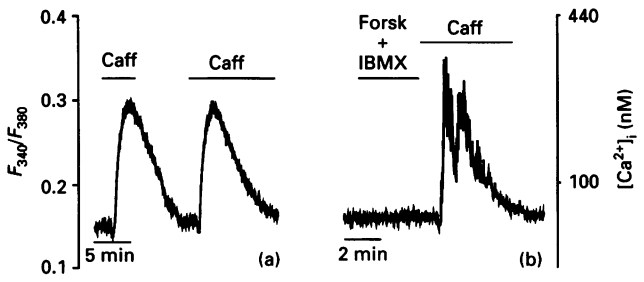


Figure 2 Caffeine increases $[Ca^{2+}]_i$ in pancreatic β-cells by a mechanism unrelated to cyclic AMP formation

$[Ca^{2+}]_i$ was measured in fura-2-loaded β-cells by dual-wavelength microfluorimetry in the presence of 1.28 mM extracellular Ca^{2+} and 3 mM glucose. Times indicated in the labels are times of switching to the new solutions. Caffeine was dissolved in buffer with iso-osmotic replacement of NaCl. (**a**) The effect of repeated application of caffeine (50 mM) is shown. Apparent initial lowering of $[Ca^{2+}]_i$ after addition of caffeine is an artifact due to interference of caffeine with fura-2 fluorescence. The trace is representative of six different experiments. (**b**) At points indicated, forskolin (30 μM) + IBMX (1 mM) and caffeine (50 mM) were added. The trace is representative of three different experiments.

lower concentrations of caffeine (1–5 mM) had no effect on $[Ca^{2+}]_i$. Entry of caffeine into the cytosol was signalled by an abrupt increase in F_{380} and a much smaller increase in F_{340}, giving an initial lowering of $R_{340/380}$. This was an artifact resulting from interference of caffeine with fura-2 fluorescence as mentioned above. Following this, there was a lag of 5–20 s after which $[Ca^{2+}]_i$ increased rapidly to a peak. The lag period was shorter and the rise in $[Ca^{2+}]_i$ faster, when basal glucose concentration was 5 mM instead of 3 mM, most likely reflecting that the β-cells are fuel-deprived at low glucose concentrations [23]. After the increase induced by caffeine, $[Ca^{2+}]_i$ returned to basal levels in the continued presence of the substance (Figure 2a). Repeated application of caffeine to the cells elicited similar responses, although some run-down was seen. The effect was not specific for caffeine, since the related methylxanthine aminophylline also increased $[Ca^{2+}]_i$, although the effect was less consistent (results not shown). Since caffeine increases intracellular cyclic AMP concentration by inhibiting phosphodiesterase, we tested whether the increase in $[Ca^{2+}]_i$ by caffeine was mediated by cyclic AMP. In the presence of 3 mM glucose, elevation of intracellular cyclic AMP by forskolin (30 μM) or by forskolin plus the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 1 mM) did not affect $[Ca^{2+}]_i$ (Figure 2b). Ryanodine (1 μM) did not affect $[Ca^{2+}]_i$ (results not shown).

Diazoxide, a hyperglycaemic sulphonamide, inhibits glucose- and tolbutamide-induced increase in $[Ca^{2+}]_i$ in β-cells by opening the K_{ATP} channel [3]. As shown in Figures 3(a) and 3(b), diazoxide (400 μM) completely blocked $[Ca^{2+}]_i$ increase induced by tolbutamide (100 μM) but reduced caffeine-induced increase in $[Ca^{2+}]_i$ to only 64% of that achieved in the absence of diazoxide, an effect that was not statistically significant ($P = 0.12$, $n = 7$) (cf. Figure 2a). We also examined whether depletion of agonist-sensitive intracellular Ca^{2+} pools affected caffeine-induced increase in $[Ca^{2+}]_i$. To deplete the agonist-sensitive intracellular Ca^{2+} pools, cells were incubated for 20 min with thapsigargin (2.5 μM), a potent inhibitor of sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [24]. As shown in Figure 3(c), such pretreatment by thapsigargin did not block the caffeine-induced increase in $[Ca^{2+}]_i$, although it did abolish $[Ca^{2+}]_i$ increase by the muscarinic agonist carbachol. In cells not treated with thapsigargin, carbachol always induced a rapid increase in $[Ca^{2+}]_i$

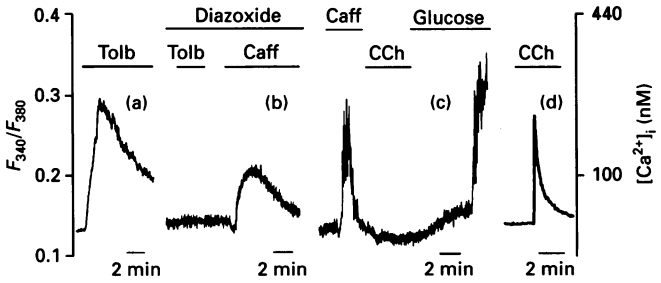


Figure 3 Effect of diazoxide and thapsigargin on caffeine-induced increase in $[Ca^{2+}]_i$

Experimental conditions were the same as mentioned in the legend to Figure 2. (**a**) Tolbutamide (100 μM) increased $[Ca^{2+}]_i$ in β-cells. In (**b**) cells were exposed to diazoxide (400 μM) for 5 min before addition of tolbutamide (100 μM) or caffeine (50 mM). In (**c**) cells were incubated for 20 min with thapsigargin (2.5 μM) before beginning the experiment. At points indicated, carbachol (CCh) (200 μM), caffeine (50 mM) and glucose (10 mM) were added. (**d**) Cells not treated with thapsigargin always responded to carbachol (200 μM) by an increase in $[Ca^{2+}]_i$. Figures are typical of at least three different experiments with similar results.

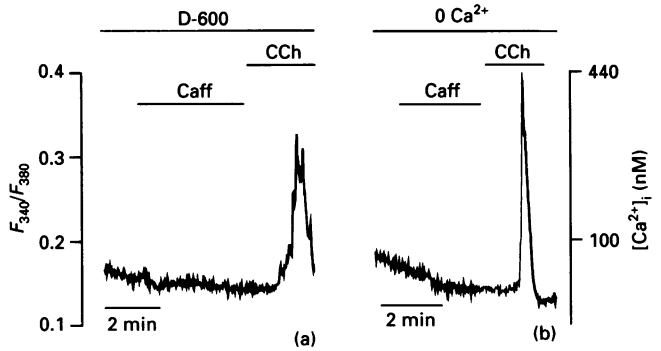


Figure 4 $[Ca^{2+}]_i$ increase by caffeine was blocked by L-type voltage-gated Ca^{2+}-channel blocker or by omission of extracellular Ca^{2+}

(**a**) In the presence of 1.28 mM extracellular Ca^{2+} and the L-type voltage-gated Ca^{2+}-channel blocker D-600 (50 μM), 50 mM caffeine did not increase $[Ca^{2+}]_i$. The Figure is representative of at least three different experiments. (**b**) Cells were superfused for 1–2 min with extracellular buffer containing EGTA (0.5–2 mM) and no added Ca^{2+}. The $[Ca^{2+}]$ of the buffer was then adjusted to 100 nM, as verified by a Ca^{2+}-selective mini-electrode. Caffeine (50 mM) did not release Ca^{2+}, while marked Ca^{2+} release was obtained by carbachol (200 μM). This effect of caffeine (5–50 mM) was seen in 18 out of 19 experiments (six preparations).

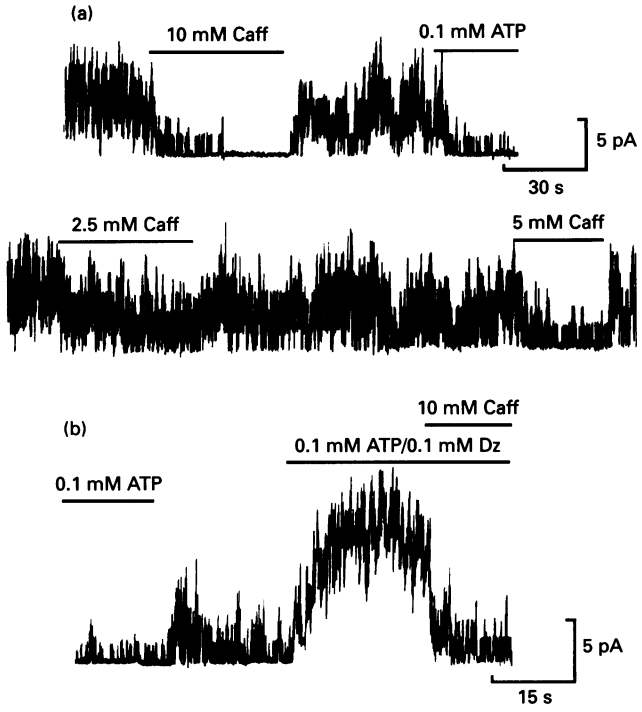


Figure 5 Effects of caffeine on K_{ATP}-channel activity

K_{ATP}-channel activity was measured in inside-out patches shortly after excision. (**a**) Caffeine (10 mM) completely blocked K_{ATP}-channel activity. Inhibition was fully reversible upon withdrawal of the compound. The inhibitory action of caffeine was dose-dependent, the minimal effective concentration being 2.5 mM. Addition of 5 mM caffeine to the same patch induced a further decrease in K_{ATP}-channel activity. In (**b**), K_{ATP}-channel activity stimulated by diazoxide (Dz) (100 μM) was almost completely blocked by caffeine (10 mM), even in the continued presence of diazoxide.

(Figure 3d). The lack of effect of carbachol in thapsigargin-treated cells was not due to prior exposure to caffeine, since in cells not treated with thapsigargin, application of carbachol after caffeine caused release of Ca^{2+} (Figure 4b). Dantrolene sodium (10–50 μM), which blocks Ca^{2+} release in skeletal muscle [6], also did not inhibit caffeine-induced increase in $[Ca^{2+}]_i$ (results not shown).

We tested whether Ca^{2+} entry through the voltage-gated Ca^{2+} channels was involved in the caffeine-induced increase in $[Ca^{2+}]_i$. As shown in Figure 4(a), the effect of caffeine was completely blocked by the L-type Ca^{2+}-channel blocker D-600 (methoxyverapamil) (50 μM). Similar inhibition was also observed with the dihydropyridine blocker nifedipine (10 μM) (results not shown). To see whether caffeine released Ca^{2+} from intracellular stores, its effect was tested in '$low\text{-}Ca^{2+}$' extracellular medium, in the presence of either 3 mM or 11 mM glucose. The '$low\text{-}Ca^{2+}$' solution was prepared by omitting $CaCl_2$ from the basal medium and adding 0.5–2.0 mM EGTA. By adding $CaCl_2$, the $[Ca^{2+}]$ of this medium was adjusted to 100 nM as measured by a Ca^{2+}-selective mini-electrode. To avoid significant depletion of intracellular Ca^{2+} stores, cells were superfused with the '$low\text{-}Ca^{2+}$' buffer for only 1–2 min. Under these conditions, no increase in $[Ca^{2+}]_i$ was induced by caffeine (5–50 mM) in 18 out of 19 experiments (six preparations). To verify that the intracellular Ca^{2+} pools were not depleted by pretreatment with '$low\text{-}Ca^{2+}$' medium, carbachol was added. This substance caused a marked increase in $[Ca^{2+}]_i$, indicating that these pools were indeed filled with Ca^{2+} (Figure 4b). In some experiments, $[Ca^{2+}]_i$ was first raised by depolarizing the cell with glucose, KCl or glipizide, in the presence of extracellular Ca^{2+}, in an attempt to load the intracellular Ca^{2+} pools further and thereby maximize the possibility of detecting intracellular Ca^{2+} release. Such pretreatment also failed to elicit Ca^{2+} release by caffeine in the presence of a '$low\text{-}Ca^{2+}$' buffer.

The main mechanism whereby glucose and antidiabetic sulphonylureas increase $[Ca^{2+}]_i$ in β-cells is closure of the K_{ATP} channel [3]. We therefore examined whether caffeine also acted by a similar mechanism. In excised inside-out patches, caffeine inhibited K_{ATP}-channel activity in a dose-dependent manner. The minimal concentration of caffeine for the inhibition of the K_{ATP} channel was 2.5 mM. The inhibitory effect of 10 mM caffeine was rapid and led to total block of K_{ATP}-channel activity (Figure 5a). Channel activity returned promptly when caffeine-containing solution was replaced by caffeine-free solution. Aminophylline (10 mM) also blocked K_{ATP}-channel activity in a reversible

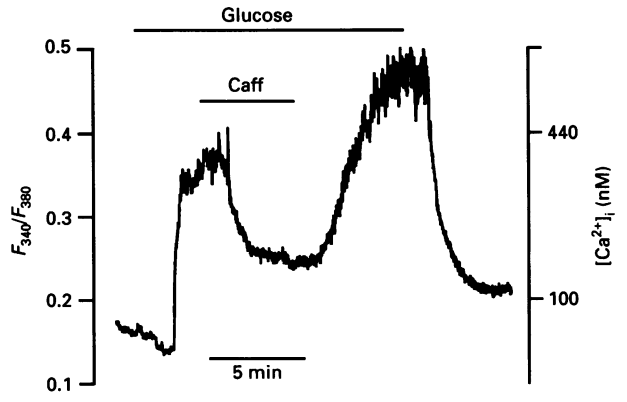
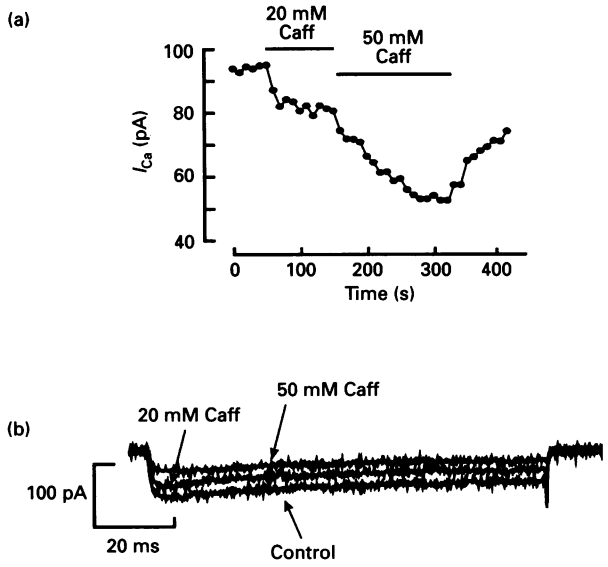


Figure 6 Lowering of $[Ca^{2+}]_i$ by caffeine in glucose-stimulated β-cells

Conditions of experiment were as described in legend to Figure 2. $[Ca^{2+}]_i$ was increased by stimulating the β-cells with glucose (8–11 mM). Addition of caffeine (10 mM) caused a reduction in $[Ca^{2+}]_i$. The trace is representative of six different experiments.



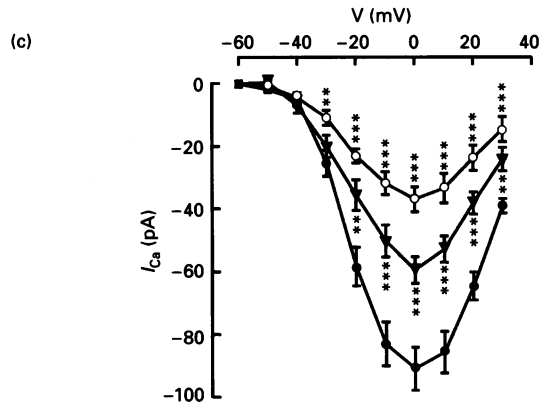


Figure 7 Effects of caffeine on whole-cell peak Ca^{2+} currents

(**a**) Depolarizing voltage steps (100 ms) to 0 mV, from a holding potential of −70 mV, were applied every 20 s. Caffeine inhibited depolarization-induced whole-cell Ca^{2+} currents in a dose-dependent manner. Caffeine was present in the chamber as indicated by the bars. Prior to addition of caffeine, peak current averaged 94 pA. This was reduced to about 80 pA after 20 mM caffeine and, following 50 mM of the compound, peak current further decreased to less than 50 pA. The Figure is representative of eight different experiments. (**b**) Examples of current traces, filtered at 2 kHz, prior to addition of caffeine, during exposure to 20 and 50 mM caffeine (**c**) Full current–voltage relationship in the absence (○) and presence of 20 (▼) or 50 mM (●) caffeine. The cells were depolarized from −60 mV to 30 mV, from a resting potential of −70 mV. Statistical significances were evaluated by comparing currents before and after addition of caffeine at each voltage step by using Student's *t* test for paired data. $^{**}P < 0.01$; $^{***}P < 0.001$.

manner. In experiments described in Figure 5(b), K_{ATP}-channel activity was increased by diazoxide (100 μM), and caffeine (10 mM) was added in the continued presence of diazoxide. Also, under these conditions, caffeine inhibited K_{ATP}-channel activity, which may suggest that caffeine interacts with a binding site separate from that for the hyperglycaemic sulphonamide.

When β-cells were stimulated by glucose (8–11 mM), $[Ca^{2+}]_i$ increased rapidly to a plateau. Addition of caffeine at this stage did not increase $[Ca^{2+}]_i$ further; rather it decreased $[Ca^{2+}]_i$, as indicated by a decrease in $R_{340/380}$ and notable anti-parallel change in the F_{340} and F_{380}. The effect was reversed completely upon switching back to caffeine-free solution (Figure 6). The lowering effect on $[Ca^{2+}]_i$ was always observed when caffeine was used at concentrations of 10 mM or higher. We further tested whether this reduction in $[Ca^{2+}]_i$ by caffeine could be explained by a reduction in Ca^{2+} entry through the L-type voltage-gated Ca^{2+} channels, by using the whole-cell mode of the patch–clamp technique. Figure 7 shows that caffeine reduced depolarization-evoked whole-cell Ca^{2+} current in β cells in a dose-dependent manner. In Figure 7(a), the cells were depolarized to 0 mV from a holding potential of −70 mV. The depolarizing voltage steps were given every 20 s. When 20 mM caffeine was added to the extracellular medium, there was 10–20% reduction in peak whole-cell Ca^{2+} currents. Increasing the concentration of caffeine to 50 mM further reduced the depolarization-evoked Ca^{2+} currents to about 50%. The inhibitory effect of caffeine on peak Ca^{2+} currents was fully reversible following wash-out and was reproducible in the same cell on subsequent re-exposure. An example of Ca^{2+} current traces from a cell exposed to 20 and 50 mM caffeine can be seen in Figure 7(b). The full current–voltage relationship is shown in Figure 7(c). The cells were depolarized from −60 mV to 30 mV, from a holding potential of −70 mV. Each cell was then exposed to 20 and 50 mM caffeine. The inhibitory effect of caffeine was most pronounced at around 0 mV and amounted to $34 \pm 3\%$ ($P < 0.001$, $n = 8$) and $62 \pm 3\%$ ($P < 0.001$, $n = 8$) inhibition in the presence of 20 and 50 mM caffeine respectively.

In experiments described in Figure 8(a), $[Ca^{2+}]_i$ was first raised by stimulating the cells with glucose (8–10 mM). This resulted in either a sustained elevation of $[Ca^{2+}]_i$ or slow oscillations in $[Ca^{2+}]_i$ (0.1–0.9/min). After the increase in $[Ca^{2+}]_i$ by glucose, caffeine was added at a low concentration (2.5–5.0 mM). This induced fast $[Ca^{2+}]_i$ oscillations (0.9–3.5/min). Characteristically, these oscillations were superimposed on an elevated level of $[Ca^{2+}]$, contained large-amplitude Ca^{2+} transients (6–12 s) and disappeared after withdrawal of caffeine. Caffeine-induced oscillations were seen in eight out of nine experiments. In one experiment, 5 mM caffeine did not induce $[Ca^{2+}]_i$ oscillations but instead caused a reduction in $[Ca^{2+}]_i$, as described above for higher concentrations of the compound.

The effect of *R*pcAMPS, an antagonist of protein kinase A (PKA) [25], on caffeine-induced $[Ca^{2+}]_i$ oscillations was tested in

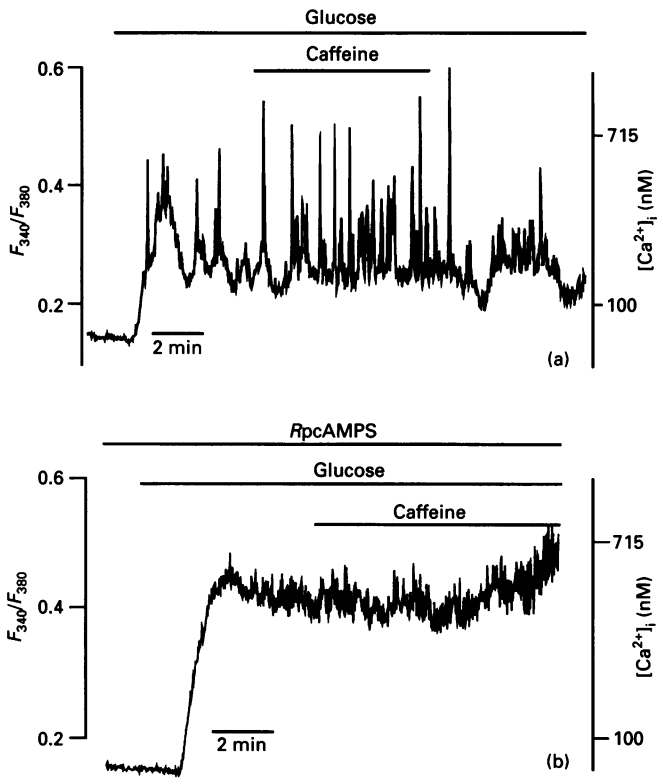


Figure 8 Caffeine-induced fast $[Ca^{2+}]_i$ oscillations in glucose-stimulated β-cells

Fura-2-loaded β-cell clusters (2–4 cells) were stimulated by glucose (8–10 mM). (**a**) Addition of caffeine (5 mM), at point indicated, induced fast $[Ca^{2+}]_i$ oscillations which reversed upon withdrawal of the compound. Similar results were observed in eight out of nine different experiments. In (**b**), *R*pcAMPS (50 μM) was present both during loading with fura-2 (for 20 min) and the rest of the experiment. The trace is representative of three different experiments. Control experiments using cells from the same cell preparation showed fast $[Ca^{2+}]_i$ oscillations.

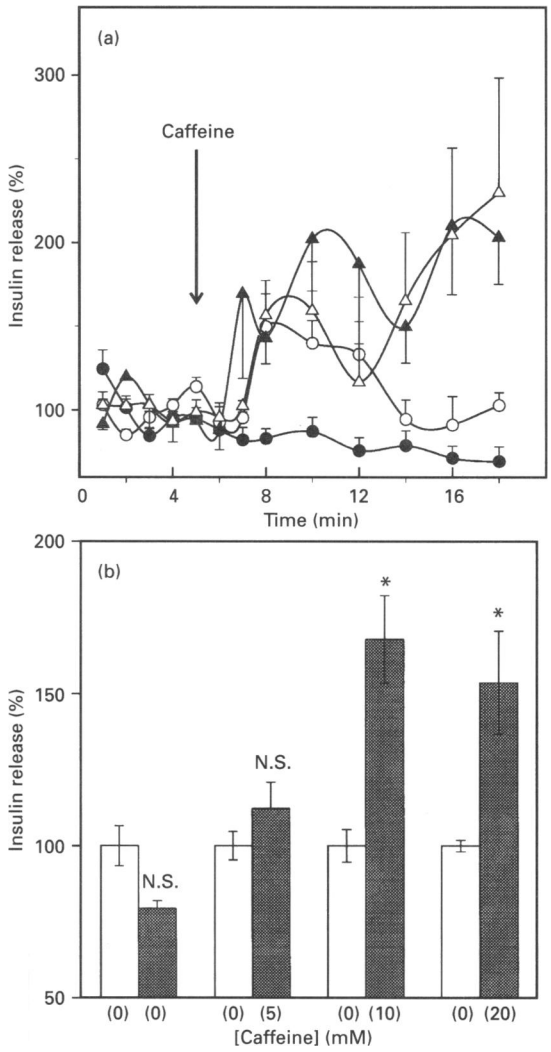


Figure 9 Effect of caffeine on insulin-release

Insulin release was studied by perifusing β-cells mixed with Bio-Gel P4 polyacrylamide beads as described in the Materials and methods section. Glucose concentration in the perifusion medium was 3 mM. Average insulin release during the first 5 min was taken as 100% and all values are given relative to this. (**a**) Dynamics of insulin release by caffeine. Cells were perifused with the basal medium containing 3 mM glucose. At the point indicated by the arrow, the perifusion medium contained caffeine at 0 mM (●), 5 mM (○), 10 mM (▲) or 20 mM (△). Each trace shows mean ± S.E.M. of three different experiments using cells from three cell preparations. (**b**) Mean ± S.E.M. of insulin release during the period before (empty bars) and after (shaded bars) the time point of switching to the caffeine-containing perifusion medium. Insulin release is estimated from the mean of concentrations of insulin in the perifusate obtained at time points shown in (**a**). N.S., not significant; $^*P < 0.05$.

Figure 8(b). In these experiments, cells were pretreated with *R*pcAMPS (50 μM) for 20 min, by adding the substance to the buffer during incubation with fura-2. *R*pcAMPS was continuously present in the perifusion during the rest of the experiment. With this treatment, cells responded to glucose with an increase in the $[Ca^{2+}]_i$, but $[Ca^{2+}]_i$ oscillations induced by 5 mM caffeine were largely prevented.

Insulin release by caffeine

Since inhibitors of the K_{ATP} channel usually induce insulin secretion from the β-cells, we investigated whether caffeine could do the same. When β-cells were perfused with a non-stimulatory concentration of glucose (3 mM), basal insulin secretion showed slow decline over the observation period (18 min) (Figure 9a, filled circles). Switching to perifusion medium containing 3 mM glucose and caffeine at different concentrations induced a rapid increase in insulin release (Figure 9a). At 5 mM caffeine (open circles) increase in insulin release was only transient but more sustained increase was seen at 10 (filled triangles) and 20 (open triangles) mM. In Figure 9(b) average insulin release after addition of caffeine is compared with that before addition of the substance. Insulin release is estimated from the mean of concentrations of insulin in the perifusate obtained at different time points. While all concentrations of caffeine used in the experiment increased insulin release, the increase was statistically significant for 10 and 20 mM caffeine ($P < 0.05$).

DISCUSSION

Because of the lack of more suitable pharmacological tools, caffeine is used for investigating problems related to Ca^{2+} signalling that involve Ca^{2+}-induced Ca^{2+} release mediated by the ryanodine receptor. In such studies, it is often necessary to use caffeine at concentrations as high as 40–50 mM to obtain maximal Ca^{2+} release [26,27]. The most well-known mechanism

by which caffeine increases $[Ca^{2+}]_i$ is through the activation of the ryanodine receptor. Hence, an increase in $[Ca^{2+}]_i$ by caffeine is generally taken as indicative of Ca^{2+} release from intracellular Ca^{2+} stores [16–18]. The results in the present study caution against such a simplistic view and demonstrate novel mechanisms by which caffeine can affect $[Ca^{2+}]_i$.

It was claimed that, depending on the concentration, caffeine can either stimulate or inhibit intracellular Ca^{2+} release [16,18]. In the present study, we therefore used caffeine over a wide range of concentrations. However, it is to be noted that in our microfluorimetric experiments, concentrations of caffeine refer to the concentrations as added to the perifusion medium. The concentration in the perifusion chamber, at the onset of a response, was probably lower. In most of the experiments where caffeine was used at the maximally effective concentration, increase in $[Ca^{2+}]_i$ occurred within 1–2 min of switching to the caffeine-containing solution, whereas an estimated time of about 3.45 min would be required before 90% of the intended concentration of the compound could be attained in the perifusion chamber.

Interference of caffeine with fluorescence of Ca^{2+} indicators has been appreciated in several studies [27]. In our study, caffeine increased the fluorescence of fura-2 in a $[Ca^{2+}]$- and wavelength-dependent manner. A much greater increase of F_{380} compared with F_{340}, after addition of caffeine, resulted in a reduction in basal $R_{340/380}$ in all experiments. This reduction in $R_{340/380}$ was not due to a decrease in $[Ca^{2+}]_i$, where F_{340} should decrease. The opposing effects of caffeine and Ca^{2+} on F_{380} may make it difficult to detect a small change in $[Ca^{2+}]_i$.

Our study showed that in unstimulated β-cells, in the presence of extracellular Ca^{2+}, caffeine consistently increased $[Ca^{2+}]_i$. This confirms earlier reports which attributed similar effects to the intracellular Ca^{2+}-mobilizing action of caffeine [17,18]. In the present study, however, three lines of evidence indicated that Ca^{2+} release from intracellular stores was not involved. First, in the presence of a 'low-Ca^{2+}' solution extracellularly there was no increase in $[Ca^{2+}]_i$, as tested with various concentrations of caffeine in a large number of experiments, despite the presence of releasable Ca^{2+} in the intracellular Ca^{2+} pools. Secondly, when cells were treated for a prolonged period of time with the potent SERCA inhibitor thapsigargin, caffeine still increased $[Ca^{2+}]_i$. Thirdly, the $[Ca^{2+}]_i$ increase by caffeine was completely blocked by the L-type voltage-gated Ca^{2+}-channel blockers D-600 and nifedipine, indicating that Ca^{2+} entry through this channel was involved. Moreover, dantrolene, a blocker of ryanodine receptor, did not block caffeine-induced increase in $[Ca^{2+}]_i$.

Since caffeine is a well-known inhibitor of phosphodiesterase, our first thought was that caffeine might increase $[Ca^{2+}]_i$ by elevating cyclic AMP and thereby phosphorylating L-type voltage-gated Ca^{2+} channels. However, this seemed unlikely since in the presence of a non-stimulatory concentration of glucose, forskolin and IBMX did not increase $[Ca^{2+}]_i$, whereas caffeine always did. It has been reported that, under resting conditions, cyclic AMP increases $[Ca^{2+}]_i$ by stimulating Ca^{2+} entry through voltage-gated Ca^{2+} channels in a hamster β-cell line (HIT T-15) [28], but not in normal β-cells [29] or insulin-secreting RINm5F cells [30]. Hence, our results indicated that caffeine affected Ca^{2+}-handling by mechanisms not dependent on cyclic AMP, resulting in opening of voltage-gated Ca^{2+} channels.

In β-cells, resting membrane potential is maintained by K_{ATP} channel conductance. Closure of this channel causes cell depolarization, opening of voltage-gated L-type Ca^{2+} channels and Ca^{2+} influx [3,31]. The $[Ca^{2+}]_i$ increase caused by glucose and antidiabetic sulphonylureas, both of which act by closing the K_{ATP} channel, is inhibited by diazoxide, an opener of this channel. In our experiments, diazoxide did not have a significant inhibitory effect on the caffeine-induced increase in $[Ca^{2+}]_i$. Single-channel recordings in excised inside-out patches confirmed that caffeine was an efficient blocker of the K_{ATP} channel. In spite of being highly membrane-permeable, the concentration of caffeine required for maximal $[Ca^{2+}]_i$ increase in intact cells was five times greater than that required for maximal inhibition of K_{ATP}-channel activity in excised patches. This may be due to the fact that in intact cells, the inhibitory effect is opposed by the stimulatory effect of intracellular ADP on K_{ATP} channel activity [32]. The inhibitory effect of caffeine on the K_{ATP} channel was unlikely to be mediated by cyclic AMP, since even a large increase in cyclic AMP does not significantly affect activity of the channel (M. S. Islam, O. Larsson and P.-O. Berggren, unpublished work). The lack of inhibition by diazoxide of the $[Ca^{2+}]_i$-increasing effect of caffeine was consistent with the finding that caffeine inhibited K_{ATP}-channel activity in excised patches, even under conditions of stimulation by diazoxide. Henquin used $^{86}Rb^+$ efflux as a measure of K_{ATP}-channel activity and reported a reduction in $^{86}Rb^+$ efflux from rat islet cells by another xanthine, theophylline [33]. Our present report is the first direct demonstration of an inhibitory effect of caffeine on K_{ATP}-channel activity, an effect that underlies the observed elevation in $[Ca^{2+}]_i$ subsequent to stimulation of intact β-cells with the compound. ATP and xanthine derivatives share the purine ring which may be a possible basis for the inhibitory action of caffeine on the K_{ATP} channel, since at a high concentration (5 mM) the purine base adenine also blocks channel activity [34]. An inhibitory effect of caffeine on K_{ATP}-channel activity is consistent with our finding that caffeine induced a modest increase in insulin release in spite of the presence of a non-stimulatory concentration of glucose, a finding that has been reported in at least one other study [35].

The effects of caffeine were complex in that it could not only increase but also decrease $[Ca^{2+}]_i$. The latter phenomenon was observed when caffeine was added subsequent to a glucose-induced increase in $[Ca^{2+}]_i$. This $[Ca^{2+}]_i$-lowering effect was unlikely to be due to interference of caffeine with glucose metabolism, since such reduction was seen even when $[Ca^{2+}]_i$ was raised by depolarization with a high concentration of KCl (results not shown). Moreover, at least one previous study has examined the effect of caffeine on glucose metabolism and excluded any significant effect [36]. A reduction of $[Ca^{2+}]_i$ in glucose-stimulated islets by caffeine was described by Roe et al. [18], an effect that could be interpreted as a reduction in Ca^{2+} release from intracellular stores. However, since mobilization of Ca^{2+} from intracellular stores is not a major mechanism of glucose-induced increase in $[Ca^{2+}]_i$ we needed to find an alternative mechanism for this phenomenon. In the β-cell, Ca^{2+} entry through the L-type voltage-gated Ca^{2+} channel is the major mechanism by which $[Ca^{2+}]_i$ is increased after glucose stimulation. We tested whether a reduction in Ca^{2+} entry through this channel might underlie the lowering effect of caffeine on $[Ca^{2+}]_i$. Patch–clamp studies on the effects of caffeine on the L-type voltage-gated Ca^{2+} channel in the β-cell confirmed that caffeine indeed reduced depolarization-induced whole-cell Ca^{2+} current. Inhibition of I_{Ca} by caffeine has also been reported in other cell types, e.g. smooth muscle cells and sympathetic neurons [10,37]. Thus caffeine, by closing the K_{ATP} channel, caused cell depolarization and opening of the L-type voltage-gated Ca^{2+} channel in unstimulated β-cells. On the other hand, caffeine inhibited influx through the same Ca^{2+} channel, when the latter was already activated by depolarization. This apparent paradox can be explained by the fact that the former action was due to depolarization brought about by inhibition of the K_{ATP} channel

by caffeine, while the latter was a direct inhibitory effect of caffeine on the L-type Ca^{2+} channel.

In β-cells stimulated by glucose, a low concentration of caffeine (2.5–5.0 mM) induced fast $[Ca^{2+}]_i$ oscillations and Ca^{2+} transients superimposed on an elevated $[Ca^{2+}]_i$. This is likely to be attributable to the cyclic AMP-elevating action of caffeine, since previous studies have demonstrated that administration of cyclic AMP analogues, or cyclic AMP-elevating agents like GLP-1-(7–36)-amide, to glucose-stimulated β-cells induces similar fast $[Ca^{2+}]_i$ oscillations ([38] and L. Juntti-Berggren and P.-O. Berggren, unpublished work). Here, we further show that this effect of caffeine is inhibited by blocking PKA with *R*pcAMPS. The precise mechanism underlying this phenomenon is unclear, but a possible explanation lies in the fact that cyclic AMP also increases electrical activity and Ca^{2+} action potentials in glucose-stimulated β-cells [39]. It is well-documented that a cyclic AMP-dependent phosphorylation of the L-type Ca^{2+} channel, which may be favoured by a voltage-dependent change in the conformation of the subunits, increases the number of active channels and their probability of opening [40,41]. In β-cells, PKA phosphorylation increases the depolarization-evoked Ca^{2+} current [42].

Our previous studies showed that in permeabilized insulin-secreting cells, Ca^{2+} release by caffeine was, if anything, very small [19,20]. In those studies, an optimal concentration of caffeine could not be used because of difficulty in obtaining a sufficiently concentrated stock solution of the substance. In the present study caffeine was used at concentrations as high as 50 mM, by dissolving it directly in the perifusion buffer. Even in this situation, a small Ca^{2+} release was observed only rarely. We can not exclude the possibility that under these conditions caffeine released a small amount of Ca^{2+}, which remained undetected because of uptake into other pools or was masked due to the action of the plasma membrane Ca^{2+} pump and/or interference of caffeine with fura-2 fluorescence. Herchuelz et al., by measuring $^{45}Ca^{2+}$ outflow, demonstrated a small Ca^{2+} release from rat pancreatic islets by caffeine [17]. However, a large proportion of cells in rat islets are non-β cells and might constitute the caffeine-sensitive pool in their experiments. In some cells, $[Ca^{2+}]_i$ oscillations have been demonstrated in the presence of high extracellular Ca^{2+}, even after depletion of intracellular Ca^{2+} stores by endoplasmic reticulum Ca^{2+}-ATPase inhibitors [43]. A caffeine-sensitive Ca^{2+} store is implicated, because the oscillations are stimulated by low concentrations of caffeine and inhibited by high concentrations of ryanodine [43]. The identity and mechanism of filling of the putative caffeine-sensitive pool in these cells are unresolved. From our study it appears that, as far as β-cells are concerned, the mechanisms responsible for caffeine sensitivity are located in the plasma membrane.

In summary, inhibition of K_{ATP}-channel activity was a distinct mechanism by which caffeine increased $[Ca^{2+}]_i$ in pancreatic β-cells. Caffeine-induced changes in $[Ca^{2+}]_i$ were not mediated by its action on the ryanodine receptor. In these cells a caffeine-sensitive intracellular Ca^{2+} pool was either absent or very small, compared with Ins(1,4,5)P_3-sensitive stores. The basis for the $[Ca^{2+}]_i$-lowering effect of caffeine in glucose-stimulated β-cells was inhibition of L-type voltage-gated Ca^{2+} channels. Furthermore, in glucose-stimulated β-cells, caffeine may induce fast Ca^{2+} oscillations, possibly by a cyclic AMP-dependent phosphorylation of voltage-gated Ca^{2+} channels.

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