Caffeine inhibits cytosolic calcium oscillations induced by noradrenaline and vasopressin in rat hepatocytes

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The effects of caffeine on agonist-induced changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were studied in single fura 2-loaded cells and suspensions of rat hepatocytes. In single cells, caffeine (5–10 mM) inhibited $[\text{Ca}^{2+}]_i$ oscillations induced both by noradrenaline (0.1 μM) and by vasopressin (0.1 nM). Caffeine shifted the dose–response curves of the $[\text{Ca}^{2+}]_i$ rise induced by vasopressin (0.5 to 2 nM) and noradrenaline (from 80 to 580 nM) in suspensions of liver cells loaded with quin2. This inhibitory effect of caffeine was not due to inhibition of phosphodiesterase

enzymes and elevation of cyclic AMP levels, because application of 3-isobutyl-1-methylxanthine, forskolin or 8-bromo cyclic AMP had no inhibitory effect on the intracellular Ca^{2+} rise induced by inositol 1,4,5-trisphosphate (InsP_3)-dependent agonists. We demonstrate that the inhibitory effect of caffeine may result from at least three actions of caffeine: (1) inhibition of receptor-stimulated InsP_3 formation; (2) inhibition of agonist-stimulated Ca^{2+} influx; and (3) direct inhibition of the InsP_3 -sensitive Ca^{2+} -release channel.

INTRODUCTION

Inositol 1,4,5-trisphosphate (InsP_3) formed after activation of Ca^{2+} -mobilizing receptors binds to specific intracellular receptors and thereby stimulates Ca^{2+} release from intracellular stores and causes an increase in the cytoplasmic $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$) (Berridge and Irvine, 1989). A sustained increase in $[\text{Ca}^{2+}]_i$ depends on Ca^{2+} influx from the extracellular space (Mauger et al., 1985). Measurements on single cells reveal additional complexities; the intracellular Ca^{2+} signal appears to be precisely organized in both space and time, with different areas of the cell responding differently and with signals often taking the form of a series of spikes (Berridge and Galione, 1988; Jacob, 1990). InsP_3 appears to play a part, directly or indirectly, in regulating Ca^{2+} entry (Berridge and Irvine, 1989; Putney, 1990) and in controlling the more complex features of the Ca^{2+} signal (Berridge and Galione, 1988; Jacob, 1990; Rooney and Thomas, 1991). Models describing these actions of InsP_3 suggest that there is regulated communication between discrete intracellular Ca^{2+} stores, only some of which are directly sensitive to InsP_3 . The release of Ca^{2+} from the InsP_3 -insensitive stores may be Ca^{2+} -induced (Berridge and Galione, 1988).

Ca^{2+} -induced Ca^{2+} release ('CICR') has been studied extensively in muscle tissue (Endo, 1975) by methods using pharmacological tools such as caffeine. Caffeine has increased the open probability of the ryanodine-sensitive Ca^{2+} channels involved in Ca^{2+} -induced Ca^{2+} release via an increase in their sensitivity to Ca^{2+} (Martonosi, 1984; Sistsapesan and Williams, 1990). Thus caffeine can release Ca^{2+} from the sarcoplasmic reticulum of a wide variety of cellular systems, including both electrically excitable (skeletal, cardiac and smooth muscle, and neurons; for review, see Sawynok and Yaksh, 1993) and non-excitable cells such as exocrine acinar cells (Osipchuk et al., 1990).

Ryanodine receptors have been reported in liver (Shoshan-Barmatz et al., 1990). The isoform of the ryanodine receptor found in the liver is different from that in electrically excitable

cells (Otsu et al., 1990; Shoshan-Barmatz et al., 1991); it may, nevertheless, be implicated in Ca^{2+} mobilization in hepatocytes (Bazotte et al., 1991).

Caffeine has been reported to inhibit the Ca^{2+} oscillations induced by agonists and the mobilization of Ca^{2+} from the internal compartments of different cell types, including *Xenopus* oocytes (Berridge, 1991; Parker and Ivorra, 1991) and pancreatic acinar cells (Wakui et al., 1990). We investigated the effects of caffeine on the Ca^{2+} rise induced by the hormone vasopressin and the neurotransmitter noradrenaline in single cells and suspensions of isolated rat hepatocytes. Caffeine had inhibitory effects due to one or more of at least three activities: inhibition of receptor-stimulated InsP_3 formation, inhibition of agonist-stimulated Ca^{2+} influx and inhibition of the InsP_3 -sensitive Ca^{2+} release channel.

MATERIALS AND METHODS

Materials

Quin2, quin2/AM, fura2 and fura2/AM were obtained from Molecular Probes, Williams medium E was from GIBCO, ionomycin from Calbiochem, collagenase from Boehringer, and InsP_3 from Amersham Corp. All other chemicals were purchased from Sigma and were of the highest grade commercially available.

Preparation and incubation of hepatocytes

Isolated hepatocytes were prepared by collagenase perfusion of the livers of fed female Wistar rats as previously described (Combettes et al., 1986). Briefly, rat hepatocytes were maintained (2×10^6 cells/ml) in an Eagle's medium consisting of 116 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl_2 , 0.8 mM MgCl_2 , 0.96 mM NaH_2PO_4 , 25 mM NaHCO_3 , 15 mg/ml gelatin, vitamins and amino acids, and 1 g/l glucose, gassed with O_2/CO_2 (19:1) (pH 7.4) at 37 °C. Cell viability, estimated by Trypan Blue

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; InsP_3 , inositol 1,4,5-trisphosphate; cAMP, cyclic AMP; IBMX, 3-isobutyl-1-methylxanthine.

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exclusion, was always greater than 96 % and remained stable for 4–5 h.

Quin2 loading and measurement of $[Ca^{2+}]_i$ in cell suspensions

The incubation conditions used for loading cells with quin2 and measuring changes in $[Ca^{2+}]_i$ have been described previously (Combettes et al., 1986). Cells (2×10^6 cells/ml) were incubated with 50 μ M quin2/AM for 150 s, then washed twice and transferred to a continuously stirred spectrofluorimeter cuvette (0.5×10^6 cells/ml) at 37 °C and gassed with O_2/CO_2 (19:1). $[Ca^{2+}]_i$ was measured, calibrated and corrected for auto-fluorescence as reported elsewhere (Combettes et al., 1986).

Loading of hepatocytes with fura2

Cells were washed twice with Eagle's medium, then suspended (2.5×10^5 cells/ml) in 2 ml of Williams medium E supplemented with 10 % foetal-calf serum, penicillin (200 000 units/ml), streptomycin (100 mg/ml) and 3 μ M fura2/AM. Hepatocytes (5×10^5 cells/Petri dish; 90–95 % viability as assessed by cell exclusion of Trypan Blue) were plated on to glass coverslips, coated with collagen. The dishes were incubated for 45 min at 37 °C under an atmosphere of 5 % CO_2 in air. The coverslips were then washed twice with saline solution (10 mM Hepes, 116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.96 mM NaH_2PO_4 , 5 mM $NaHCO_3$, 1 g/l glucose, pH 7.4). No intracellular heterogeneity of fura2 distribution was observed by fluorescence microscopy.

Measurement of $[Ca^{2+}]_i$ in individual cells

Culture dishes were made by cutting a hole (12 mm diameter) in a small plastic Petri dish (35 mm diameter) and attaching a glass coverslip to the bottom of the dish with adhesive (Selgard) to cover the hole. Dish coverslips were put into a temperature-regulated holder on the stage of a Zeiss Axiovert 35 microscope set up for epifluorescence microscopy. The temperature was kept at 34 °C because, in agreement with the findings of Kawanishi et al. (1989), intracellular fura2 fluorescence decreased rapidly at 37 °C as dye was extruded from liver cells. The cells were continuously superfused (1 ml/min) in a stream of solution from any one of the seven inlet tubes containing control and test solutions converging on the chamber.

The excitation light was supplied by a high-pressure xenon arc lamp (75 W), and the excitation wavelengths were selected by using 340 and 380 nm filters (10 nm bandwidth) mounted in a processor-controlled rotating filter wheel (MSP 21; Zeiss) between the u.v. lamp and the microscope. A computer-controlled shutter (Zeiss) was used to limit the exposure of the cells to excitation light, to minimize photobleaching. Photons emitted by fura2 within the cells were collected by the objective (Zeiss Neophluar, $\times 40$) and counted by a photomultiplier tube through an interference filter (500 nm) and a circular diaphragm, focused on a single cell. The integration interval was generally 3 s for data collection, but in some longer experiments 10 s was employed, to decrease photobleaching. Fluorescence resulting from both excitation wavelengths and the ratio were displayed on-line on a computer monitor, offering immediate access to changes in $[Ca^{2+}]_i$ due to experimental manipulations.

Fura2 fluorescence was calibrated for $[Ca^{2+}]_i$ for individual cells and calculated from the ratio of 340/380 nm fluorescence values as described by Grynkiewicz et al. (1985). At the end of an experiment, R_{max} was determined by adding ionomycin (1 μ M) to the fura2-loaded cells to increase the $[Ca^{2+}]_i$ to give maximal Ca^{2+} -dependent fura2 fluorescence (Williams and Fay, 1990).

EGTA (3 mM) was then added to the bathing solution to give R_{min} . Typical values for R_{max} , R_{min} , and B (F_{380} at R_{min} / F_{380} at R_{max}) were 0.7, 0.04 and 7 respectively.

Cells were loaded with fura2 by micro-injection and tested similarly. The results obtained by the two techniques were similar.

Ca^{2+} release from saponin-treated cells

Hepatocytes (2.5×10^6 cells/ml) were incubated at 37 °C in a 'cytosol-like medium' and permeabilized with saponin (50 μ g/ml) as reported by Burgess et al. (1984). The medium consisted of 20 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 0.96 mM NaH_2PO_4 , 25 mM Hepes (pH 7.15, with KOH) containing 1.5 mM ATP, 5 mM phosphocreatine, 5 units of creatine kinase/ml and 20 μ M quin2. The concentration of free Ca^{2+} and the amount of Ca^{2+} released by the cells were calculated as reported in Combettes et al. (1993). In some experiments, we added $^{45}Ca^{2+}$ tracer (2 μ Ci/ml). External free Ca^{2+} was monitored continuously by quin2 fluorescence in the spectrofluorimeter cuvette. The $^{45}Ca^{2+}$ efflux, after steady-state loading of the cellular stores, was measured simultaneously by diluting portions of the cell suspension in the cytosol-like medium (which did not contain saponin, quin2, or the regenerating system, but which did contain $^{40}Ca^{2+}$ and EGTA to buffer the free Ca^{2+} concentration at pCa 7) in the presence or absence of caffeine. $InsP_3$ was added 10 s after dilution of $^{45}Ca^{2+}$ -loaded cells. At 15 s after cell dilution, 1 ml of the diluted cells was filtered through a Whatman GF/C glass-fibre filter, washed and counted for radioactivity in a scintillation counter.

Ca^{2+} contamination and optical interference by caffeine with quin2 and fura2

Contaminating Ca^{2+} was detected in caffeine by atomic-absorption spectroscopy and quin2 and fura2 fluorescence. A 10 mM solution of caffeine contained about 0.6 μ M Ca^{2+} . In accordance with the previous results of O'Neill et al. (1990), caffeine (2–10 mM) did not alter the binding properties (K_D and maximal binding to Ca^{2+}) of free quin2 or fura2 added to saline solutions. However, caffeine had a small effect (less than 3 %) on the fluorescence intensity of quin2 and fura2. This effect appeared to be unrelated to $[Ca^{2+}]_i$. These changes in fluorescence occurred as rapidly as cells could be exposed to caffeine, and were reversed when caffeine was removed (results not shown). This effect was also observed in the absence of cells. Caffeine increased fluorescence at both 340 and 380 nm, and decreased the ratio by increasing the latter more than the former. Similar effects of caffeine on fura2 fluorescence have been reported previously (Iino, 1989; Friel and Tsien, 1992).

Influx of $^{45}Ca^{2+}$

The influx of $^{45}Ca^{2+}$ was determined from the initial rate of $^{45}Ca^{2+}$ uptake by isolated rat hepatocytes as previously described (Mauger et al., 1985). Briefly, cells (2×10^6 cells/ml) were incubated in Eagle's medium with a trace amount of $^{45}Ca^{2+}$ (1–3 μ Ci/ml). Samples (100 μ l) were taken at 15, 45, 75 and 105 s after addition of $^{45}Ca^{2+}$. Each sample was rapidly diluted in 4 ml of ice-cold washing solution containing 144 mM NaCl, 5 mM $CaCl_2$ and 5 mM Tris/HCl (pH 7.4), filtered through Whatman GF/C glass-fibre filters, and washed with 3×4 ml of washing solution. The radioactivity bound to the filter was determined with a liquid-scintillation spectrometer and the $^{45}Ca^{2+}$ influx was calculated as previously described (Mauger et al., 1985).

Determination of total cell Ca^{2+} , K^{+} and Na^{+} contents

Total ion contents were measured as described previously (Combettes et al., 1988). Samples (5×10^6 cells/ml) were centrifuged in 10 ml of an ice-cold solution containing 150 mM choline, 0.5 mM EGTA (pH 7.4 with KOH) and 10% (w/v) sucrose, and the cell pellets dispersed in water. Cell proteins were precipitated and the concentrations of Ca^{2+} , K^{+} and Na^{+} in the medium were determined by atomic-absorption spectroscopy.

Measurement of InsP_3 fraction

Cells were incubated with *myo*-[2- ^3H]inositol for 90 min, washed and resuspended in a modified Eagle's medium containing 10 mM LiCl in place of 10 mM NaCl (106 mM NaCl only) and incubated for 10 min. The [^3H]Ins P_3 fraction was measured as described by Combettes et al. (1988). Briefly, cell samples were precipitated with 10% HClO_4 , the sample was neutralized with KHCO_3 and the supernatant was diluted in 0.5 mM EDTA and 5 mM tetraborate. The [^3H]inositol phosphates were separated by anion-exchange chromatography on a Dowex AG X8 column (0.5 cm $\times$ 3 cm) (formate form; 100–200 mesh; Bio-Rad).

Determination of protein content

Protein was determined by the method of Lowry et al. (1951), with BSA as a standard.

RESULTS

Effect of caffeine on Ca^{2+} oscillations induced by vasopressin and noradrenaline

Single fura2-loaded rat liver cells respond to application of hormones or neurotransmitters with a characteristic fluorescence response and an oscillatory $[\text{Ca}^{2+}]_i$ increase (Rooney and Thomas, 1991). As previously described by Woods et al. (1986) and by Rooney et al. (1989), using single aequorin- and fura2-loaded hepatocytes respectively, the frequency of $[\text{Ca}^{2+}]_i$ oscillations was dependent on the agonist concentration. The responses of single fura2-loaded hepatocytes to application of vasopressin and noradrenaline are shown in Figures 1 and 2. The frequency of the oscillations of the Ca^{2+} responses evoked by vasopressin (0.1–10 nM) or noradrenaline (0.1–5 μM) increased with increasing agonist concentration. High doses of these agonists caused sustained Ca^{2+} responses. Moreover, in accordance with previous reports (Rooney et al., 1989; Kawanishi et al., 1989), the peak Ca^{2+} level achieved in each oscillation was independent of the applied agonist concentration. The cells were then superfused with the same agonist solutions containing caffeine (5 or 10 mM). Caffeine rapidly abolished the $[\text{Ca}^{2+}]_i$ oscillations evoked by low concentrations of hormones (0.1 nM vasopressin, Figure 1a; 0.1 μM noradrenaline, Figure 2a) and slowed $[\text{Ca}^{2+}]_i$ oscillations evoked by higher agonist concentrations (Figures 1b and 2b). The inhibitory effect of caffeine was rapidly and completely reversible. Agonist-induced oscillations re-appeared after removal of caffeine. However, caffeine had no effect on the sustained maximal response induced by vasopressin (10 nM) and had either little (30% of cells tested, $n = 25$) or no effect on the response to noradrenaline (1–5 μM) (Figures 1c and 2c).

It is well known that caffeine is a phosphodiesterase inhibitor (Butcher and Sutherland, 1962; Sawynok and Yaksh, 1993) and can thus elevate the level of cyclic AMP (cAMP) in cells. To determine whether this effect was responsible for the inhibition of the agonist response by caffeine, we tested the effects of 3-isobutyl-1-methylxanthine (IBMX) (a more potent inhibitor than

caffeine of phosphodiesterase; Butcher and Sutherland, 1962), the adenylate cyclase activator forskolin and 8-bromo cAMP (8Br-cAMP), which is a cell-permeant analogue of cAMP (Figure 3). At 5 mM, caffeine decreased the frequency of $[\text{Ca}^{2+}]_i$ oscillations induced by noradrenaline (0.5 μM), whereas 500 μM IBMX, a concentration that maximally inhibits phosphodiesterase (Butcher and Sutherland, 1962) did not alter the response (Figure 3a). Similar results were obtained with vasopressin (results not shown: 18 cells with noradrenaline and 12 with vasopressin in two independent experiments).

Additional evidence against the involvement of cAMP in inhibition of the responses is that no inhibition of agonist-evoked responses was detected during bath application of either forskolin (10 μM) or 8Br-cAMP (100 μM) (Figures 3b and 3c). On the contrary, in accordance with the observation by Sanchez-Bueno et al. (1993), perfusion of these agents during $[\text{Ca}^{2+}]_i$ oscillations induced by vasopressin (0.1 nM) or noradrenaline (0.1 μM) increased the frequency, and in some cases the amplitude, of the response (Figures 3b and 3c). Moreover, in agreement with

Figure 1 Effect of caffeine on $[\text{Ca}^{2+}]_i$ oscillations induced by vasopressin

Hepatocytes loaded with fura2 were challenged with vasopressin (VP) for the time shown by the upper horizontal bars. Caffeine (5 mM) was applied as indicated by the lower horizontal bars. VP was applied at the following concentrations: 0.1 (a), 0.5 (b) and 10 nM (c). Each trace is representative of traces obtained from 30–40 cells in 6 independent experiments.

Figure 2 Effect of caffeine on $[Ca^{2+}]_i$ oscillations induced by noradrenaline

Hepatocytes loaded with fura2 were challenged with noradrenaline (NOR) as indicated by the upper horizontal bars. Caffeine (5 mM) was applied as indicated by the lower horizontal bars. Noradrenaline was applied at the following concentrations: 0.1 (a), 0.5 (b) and 1 μ M (c). Each trace is representative of traces obtained from 25–30 cells in 6 independent experiments.

previous results with single guinea-pig hepatocytes (Capiod et al., 1991), 8Br-cAMP (100 μ M) induced $[Ca^{2+}]_i$ oscillations in about 10% of the cells tested ($n = 44$) (Figure 3c). Thus the effects of caffeine on the agonist-induced $[Ca^{2+}]_i$ oscillations were not due to effects on phosphodiesterase or cAMP levels.

Finally, caffeine itself (2–20 mM) did not elicit any measurable Ca^{2+} release (results not shown).

Effect of caffeine on the dose–response curves for vasopressin- and noradrenaline-induced Ca^{2+} increases

These findings suggested that caffeine may shift the dose–response curve of these agonists. Because the frequency of agonist-induced Ca^{2+} spikes was very different in individual liver cells (Rooney et al., 1989; Kawanishi et al., 1989), it was not easy to quantify these responses in terms of the dose–response-curve relationship. We therefore measured $[Ca^{2+}]_i$ in suspensions of rat hepatocytes loaded with quin2. Isolated rat hepatocytes were incubated in Eagle's medium in the absence or presence of caffeine (10 mM) for 5 min. Agonist was then added and the fluorescence signal calibrated as usual (see the Materials and methods section). In

Figure 3 Effect of cAMP-dependent agonists on $[Ca^{2+}]_i$ oscillations induced by vasopressin (VP) and noradrenaline (NOR)

Hepatocytes loaded with fura2 were challenged with $InsR_3$ -dependent agonists as indicated by the upper horizontal bars. cAMP-dependent agonists or caffeine (5 mM) were applied as indicated by the lower horizontal bars. (a) Effects of caffeine (5 mM) and IBMX (0.5 μ M) on noradrenaline (0.5 μ M)-induced oscillations. This trace is representative of those obtained by using 18 cells in 3 independent experiments. Similar results were obtained with VP (1 nM; 12 cells in 3 independent experiments; results not shown). (b) Effect of forskolin (Fk, 10 μ M) on vasopressin (0.1 nM)-induced oscillations. This trace is representative of those obtained by using 22 cells in 3 independent experiments. Similar results were obtained with NOR (0.1 μ M; 7 cells in 3 independent experiments; results not shown). (c) 8Br-cAMP-induced oscillations and potentiated NOR-induced Ca^{2+} spikes (compare with a or Figure 2b). This trace is representative of those obtained by using 18 cells in 3 independent experiments. Similar results were obtained with VP (0.1 or 1 nM; 26 cells in 3 independent experiments; results not shown).

the presence of caffeine (10 mM), the noradrenaline- and vasopressin-induced $[Ca^{2+}]_i$ increase was smaller than in its absence (Figure 4). Caffeine shifted the EC_{50} value of vasopressin from 0.8 nM, a value similar to that obtained previously (Combettes et al., 1986), to 2 nM and shifted the EC_{50} of noradrenaline from about 80 nM to 580 nM.

Interestingly, addition of caffeine to quin2-loaded hepatocytes resulted in a significant fall in $[Ca^{2+}]_i$ from 170 ± 5 to 120 ± 6 nM ($n = 20$). This latter effect of caffeine did not seem to result from quenching of fluorescence, as has been described for indo-1 (O'Neill et al., 1990). On the contrary, in some cases caffeine increased the fluorescence intensity of quin2 (see the Materials and methods section). This fall in $[Ca^{2+}]_i$, although observed in some isolated fura2-loaded hepatocytes, was more difficult to

Figure 4 Dose/response curves for vasopressin- and noradrenaline-induced $[Ca^{2+}]_i$ responses in the absence (●) and presence (○) of caffeine

Cells were loaded with quin2 and incubated with or without caffeine (10 mM) for 5 min in Eagle's medium containing 1.8 mM Ca^{2+} . $[Ca^{2+}]_i$ was measured as described in the Materials and methods section. $[Ca^{2+}]_i$ increases are expressed as percentages of the resting $[Ca^{2+}]_i$ (before agonist), which was 170 ± 5 nM in the absence of caffeine and 120 ± 6 nM in the presence of caffeine. Data are means $\pm$ S.E.M. of 7–10 determinations.

detect, probably due to the lower affinity of fura2 for Ca^{2+} and the weaker fluorescence obtained at the resting $[Ca^{2+}]_i$.

Effect of caffeine on Ca^{2+} influx and intracellular Ca^{2+} mobilization

It has been reported previously in hepatocytes that removal of extracellular Ca^{2+} can decrease (Rooney et al., 1989) the frequency of agonist-induced $[Ca^{2+}]_i$ oscillations, or abolish them completely (Somogyi and Stucki, 1991). Thus the ability of caffeine to decrease both the basal $[Ca^{2+}]_i$ and the potency of $InsP_3$ -dependent agonists could result from inhibition of a component of the resting influx and from inhibition of stimulated Ca^{2+} influx normally induced by these agents. Indeed, it has been shown that caffeine can inhibit the entry of Ca^{2+} into a vascular-smooth-muscle cell line (Otun et al., 1991). Therefore we measured the $^{45}Ca^{2+}$ influx in hepatocytes stimulated by the $InsP_3$ -dependent agonists, vasopressin and noradrenaline, in the presence and absence of caffeine. In these experiments, cells were incubated in Eagle's medium containing a trace amount of $^{45}Ca^{2+}$ and agonist as appropriate. Results are shown in Table 1. The basal $^{45}Ca^{2+}$ influx was 0.21 nmol/min per mg, a value similar to that published previously (Mauger et al., 1985), and was not affected in the presence of caffeine (10 mM), as previously found by Kashiwagura et al. (1987). In contrast, the $^{45}Ca^{2+}$ influx stimulated by highest concentration of vasopressin (10 nM) was decreased by about 40 %, whereas the $^{45}Ca^{2+}$ influx stimulated by noradrenaline (1 μ M) was decreased by about 20 % (not statistically significant; Table 1). This inhibitory effect of caffeine does not result from an effect on cAMP. Indeed, as previously reported by Mauger et al. (1985), 8Br-cAMP (100 μ M) raised the initial $^{45}Ca^{2+}$ influx and potentiated the $^{45}Ca^{2+}$ influx stimulated by vasopressin (Table 1).

The mechanism of the Ca^{2+} influx stimulated by $InsP_3$ -dependent agonists remains unclear (Irvine, 1992; Putney, 1993). Receptor activation may activate Ca^{2+} entry either through $InsP_3$ production (Irvine, 1990) or emptying of an intracellular compartment (Putney, 1990). The inhibitory effects of caffeine may thus result from an effect on either of these mechanisms.

We measured, in absence of external Ca^{2+} , the $[Ca^{2+}]_i$ increase stimulated by the two agonists in the presence of caffeine.

Table 1 Effect of caffeine on agonist-induced $^{45}Ca^{2+}$ influx

Hepatocytes were preincubated for 3 min in Eagle's medium with or without caffeine (10 mM). Uptake was initiated by adding $^{45}Ca^{2+}$ 10 s after addition of noradrenaline (1 μ M) or vasopressin (10 nM). Results are means $\pm$ S.E.M. of 3 determinations in 4 experiments: ** indicates that the means are significantly different from paired controls ($P < 0.01$); N.D., not determined.

	$^{45}Ca^{2+}$ influx (nmol/min per mg)		
	H ₂ O	Noradrenaline (1 μ M)	Vasopressin (10 nM)
Control	0.21 $\pm$ 0.02	0.35 $\pm$ 0.03	0.39 $\pm$ 0.03
Caffeine (10 mM)	0.22 $\pm$ 0.01	0.27 $\pm$ 0.02	0.23 $\pm$ 0.02**
8Br-cAMP (100 μ M)	0.35 $\pm$ 0.01**	N.D.	0.78 $\pm$ 0.04**

Table 2 Effect of caffeine on agonist-induced $[Ca^{2+}]_i$ increase in the absence of external Ca^{2+}

Experimental conditions were identical with those described in Figure 4. EGTA (2 mM) was added 15 s before addition of agonist. $[Ca^{2+}]_i$ increases are expressed as percentages of the resting $[Ca^{2+}]_i$. Data are means $\pm$ S.E.M. of 5–12 determinations: ** indicates that the means are significantly different from paired controls ($P < 0.01$).

	$[Ca^{2+}]_i$ (% of resting value)			
	Noradrenaline		Vasopressin	
	0.1 μ M	5 μ M	1 nM	10 nM
Control	181 $\pm$ 13	251 $\pm$ 17	201 $\pm$ 16	318 $\pm$ 24
Caffeine (10 mM)	153 $\pm$ 17	253 $\pm$ 13	125 $\pm$ 8**	295 $\pm$ 35

Experiments were performed as in the presence of external Ca^{2+} . Quin2-loaded cells were resuspended in Eagle's medium with or without caffeine for 5 min, then EGTA (2 mM) was added 15 s before addition of agonists. At intermediate concentrations of vasopressin (1 nM) and noradrenaline (0.1 μ M), caffeine (10 mM) decreased the agonist-induced $[Ca^{2+}]_i$ increase, but had no effect on the response to a maximal concentration of these agonists (respectively 10 nM and 5 μ M) (Table 2). Because of the poor solubility of caffeine, it was not possible to add caffeine after EGTA to observe directly whether or not caffeine could induce a $[Ca^{2+}]_i$ decrease in these conditions. To overcome this problem, we attempted to measure the size of the intracellular Ca^{2+} pool in the presence and absence of caffeine. Addition of ionomycin (5 μ M), in the presence of EGTA (2.5 mM) to give an external Ca^{2+} concentration less than 1 μ M, induced a large rise in $[Ca^{2+}]_i$, and this effect was significantly more pronounced when cells were treated with caffeine (results not shown). To confirm these results, we measured the total cellular Ca^{2+} concentration. The results obtained (Table 3) show that, whereas the total cellular concentration of K^+ and Na^+ was not affected by caffeine, the total cellular Ca^{2+} concentration was significantly higher in the presence of caffeine. It therefore seems that caffeine can induce an increase in the total cellular Ca^{2+} , possibly indirectly by causing the overloading of the intracellular Ca^{2+} pool via the Ca^{2+} - Mg^{2+} -ATPase (see the Discussion below).

Table 3 Effects of caffeine on the total concentration of cellular ions

Hepatocytes were incubated for 3 min in Eagle's medium with or without caffeine (10 mM). Portions (1 ml) were then removed and cell ion concentrations determined (see the Materials and methods section). Data are means $\pm$ S.E.M. of 4 determinations in 6 separate experiments: * indicates that the means are significantly different from paired controls ($P < 0.05$).

	Concn. (nmol/mg of protein)		
	Ca ²⁺	K ⁺	Na ⁺
Control	3.56 $\pm$ 0.26	356 $\pm$ 17	46 $\pm$ 3
Caffeine (10 mM)	4.51 $\pm$ 0.30*	350 $\pm$ 20	55 $\pm$ 4

Effect of caffeine on agonist-induced InsP₃ formation

The results with InsP₃-dependent agonists suggest that caffeine inhibits the release of Ca²⁺ from intracellular stores. This effect of caffeine could arise from (1) a decrease in the ability of InsP₃ to release Ca²⁺ and/or a decrease in the amplification of the Ca²⁺-induced signal for Ca²⁺ release observed in other cells (Berridge, 1991; Parker and Ivorra, 1991; Wakui et al., 1990; Brown et al., 1992), or (2) inhibition of the production of InsP₃. To distinguish between these possibilities, we measured the agonist-evoked production of InsP₃. Intact hepatocytes were prelabelled with [³H]inositol (Berridge et al., 1983; Combettes et al., 1988), and then incubated with vasopressin (10 nM) and noradrenaline (1 μ M). In accordance with previous results (Tennes et al., 1987; Combettes et al., 1988), stimulation of the cells with vasopressin or noradrenaline caused a substantial increase in the level of the InsP₃ fraction (Table 4). In the presence of caffeine (10 mM), this increase was significantly inhibited. Moreover, when caffeine was present, the maximal InsP₃ production induced by each agonist was delayed by about 60 s (90 s, versus 30 s in the control experiment; results not shown). Finally, caffeine alone significantly decreased the basal InsP₃ content of cells (see Table 4).

Effect of caffeine on InsP₃-induced Ca²⁺ release from saponin-treated cells

To test whether the effects of caffeine could also result from direct action on the intracellular InsP₃-sensitive Ca²⁺ pool, as previously suggested in other cell types (Parker and Ivorra, 1991; Brown et al., 1992), caffeine was added to hepatocytes permeabilized with saponin (Burgess et al., 1984). Intact cells were incubated in the cytosol-like medium as described in the Materials and methods section. Under these conditions, the predominant compartment concentrating Ca²⁺ is the endoplasmic reticulum (Burgess et al., 1984). Loading of the Ca²⁺ stores was simultaneously monitored by using ⁴⁵Ca²⁺ and quin2 fluorescence (Combettes et al., 1993). When the fluorescence measurements indicated that the Ca²⁺ content of stores had reached steady state, portions of the cell suspension were diluted in an unlabelled efflux medium and various amounts of InsP₃ were added in the absence or presence of caffeine (10 mM). Samples were then filtered to evaluate the residual ⁴⁵Ca²⁺ content of the intracellular stores. Because caffeine was contaminated with Ca²⁺ (see the Materials and methods section), the efflux medium was buffered with a mixture of ⁴⁰Ca²⁺ and EGTA in amounts sufficient to buffer the free Ca²⁺ concentration at pCa 7 (Combettes et al., 1993). Under these conditions, the InsP₃ dose-response curve was shifted, the EC₅₀ value increasing from 0.5 μ M in control

Table 4 Effect of caffeine on the accumulation of [³H]InsP₃ evoked by noradrenaline and vasopressin

The InsP₃ content was measured in hepatocytes prelabelled with [³H]inositol, incubated with 1.24 mM EGTA for 15 s and with the agents for 20 s. For caffeine-treated cells, caffeine was added 3 min before EGTA. Reactions were terminated and levels of [³H]InsP₃ (c.p.m./10 mg of cell protein) were determined. Data are means $\pm$ S.E.M. of 3 determinations in 5 experiments: * and ** indicate that the means are significantly different from paired controls ($P < 0.05$ and $P < 0.01$ respectively).

	[³ H]InsP ₃ (c.p.m./10 mg of cell protein)		
	H ₂ O	Noradrenaline (1 μ M)	Vasopressin (10 nM)
Control	367 $\pm$ 45	615 $\pm$ 70	1444 $\pm$ 183
Caffeine	229 $\pm$ 31*	430 $\pm$ 30*	673 $\pm$ 102**

Figure 5 Dose/response curves for InsP₃-induced Ca²⁺ release in saponin-treated hepatocytes in the absence (●) and presence (○) of 10 mM caffeine

Cells were permeabilized and loaded with ⁴⁵Ca²⁺ in the spectrofluorimeter cuvette at 20 °C. After equilibration, samples were transferred and diluted in a cytosol-like efflux medium buffered at pCa 7 with or without caffeine (10 mM). Ca²⁺ release was measured after addition of various concentrations of InsP₃ to the efflux medium 5 s after cell dilution (see the Materials and methods section). Data are the means $\pm$ S.E.M. of 3 determinations in 5 experiments.

conditions to about 1 μ M (Figure 5). This effect and these values are identical with those described by Brown et al. (1992) in cerebellum microsomes. Similar results were obtained when caffeine was added before saponin permeabilization to ensure a longer time of effect (results not shown). Moreover, no caffeine-induced Ca²⁺ release was observed when caffeine (10 mM) was added alone. This is in accordance with previous results reported by McNulty and Taylor (1993), showing that caffeine induces a small release of Ca²⁺ at high (≥ 10 mM) concentrations.

DISCUSSION

The methylxanthine caffeine is known to have many different actions (for review, see Sawynok and Yaksh, 1993), including inhibition of adenosine receptors (Fredholm, 1980), phosphodiesterase inhibition (Butcher and Sutherland, 1962), and activation of Ca²⁺-induced Ca²⁺ release (Endo, 1975). In muscle and neuronal cells, caffeine has been shown to release intracellularly stored Ca²⁺ by activating a putative Ca²⁺-induced Ca²⁺-release channel distinct from the InsP₃ receptor (Saida and van Breemen, 1983; Thayer et al., 1988), and such Ca²⁺ release

has been suggested to contribute to Ca^{2+} oscillations and Ca^{2+} waves observed in these cells (Berridge and Galione, 1988). In other cell types, however, the action of caffeine may be more complicated. In exocrine pancreas, for example, low caffeine concentrations induced Ca^{2+} oscillations, whereas higher concentrations inhibited oscillations induced by acetylcholine (Osipchuk et al., 1990). A similar inhibitory effect of caffeine on hormone-induced Ca^{2+} oscillations was observed in the present study: caffeine completely suppressed Ca^{2+} oscillations induced by vasopressin and noradrenaline in single rat hepatocytes (Figures 1 and 2). This inhibitory effect of caffeine appeared to result from an inhibition of both basal and agonist-stimulated InsP_3 production (Table 4). These results are in agreement with the observations by Toescu et al. (1992), who reported caffeine-induced inhibition of InsP_3 production in exocrine pancreas. Caffeine also decreased the ability of InsP_3 to mobilize intracellular Ca^{2+} (Figure 5). This effect could result from inhibition of the opening of InsP_3 -sensitive channels, as described in cerebellar microsomes by Brown et al. (1992), rather than an effect on InsP_3 binding, since caffeine has been reported not to affect InsP_3 binding in hepatocytes (Feng et al., 1992).

Hormones that act via cell-surface receptors to generate InsP_3 promote a transient rise in $[\text{Ca}^{2+}]_i$ that is biphasic: Ca^{2+} release from the intracellular store is accompanied by some form of receptor-mediated Ca^{2+} entry at the plasma membrane (Berridge and Irvine, 1989; Irvine, 1992; Putney, 1993). The mechanism of the Ca^{2+} influx stimulated by InsP_3 -dependent agonists may be controlled by InsP_3 acting alone or together with InsP_4 (Kuno and Gardner, 1987) and may be regulated by the degree of filling of the intracellular Ca^{2+} store (Putney, 1990). In the present study, caffeine inhibited the receptor-activated Ca^{2+} influx (Table 1). Diminution of the receptor-activated InsP_3 production and lower efficiency of InsP_3 mobilization of intracellular Ca^{2+} in the presence of caffeine would clearly decrease Ca^{2+} influx controlled by both these mechanisms. Taken together, the inhibitory effects of caffeine on InsP_3 production, InsP_3 -mediated Ca^{2+} release and InsP_3 -associated Ca^{2+} influx, could therefore explain the inhibitory action of caffeine on InsP_3 -dependent-hormone Ca^{2+} signals in hepatocytes observed in this study.

The mechanisms underlying the inhibitory actions of caffeine on InsP_3 metabolism and InsP_3 -mediated Ca^{2+} release are not clear, but they appear distinct from the well-established ability of caffeine to elevate the concentration of cAMP by phosphodiesterase inhibition. This notion is supported by the observation that elevating cAMP by three independent methods, IBMX pretreatment (Figure 3a), forskolin (Figure 3b) and 8Br-cAMP addition (Figure 3c), all failed to inhibit hormone-induced Ca^{2+} signals in the present study. Moreover, these agents induced $[\text{Ca}^{2+}]_i$ oscillations and/or potentiated the Ca^{2+} response evoked by the InsP_3 -dependent agonists, as previously reported (Capiod et al., 1991; Sanchez-Bueno et al., 1993). This effect could result in part from an increase in the sensitivity of the InsP_3 receptor (Burgess et al., 1991; Joseph and Ryan, 1993).

These observations are in agreement with previous studies, obtained on hepatocytes suspensions, showing that elevation of cAMP induced a large increase in $[\text{Ca}^{2+}]_i$ both by mobilizing intracellular Ca^{2+} and by stimulating Ca^{2+} influx (Charest et al., 1983; Mauger et al., 1985; Combettes et al., 1986; for review, see Bygrave and Benedetti, 1993) and potentiated the effects of InsP_3 -dependent hormones on Ca^{2+} release and Ca^{2+} influx (Mauger et al., 1985; Combettes et al., 1986; Bánhegyi et al., 1991).

Although the elevation of cAMP is not responsible for the inhibitory actions of caffeine on InsP_3 -dependent hormone-induced $[\text{Ca}^{2+}]_i$ signals, it may be responsible for the observed

increase in total cell Ca^{2+} content (Table 3). cAMP has been shown to increase Ca^{2+} uptake by hepatocytes (Taylor et al., 1979; Bánhegyi et al., 1991) and subcellular fractions of rat liver (Bygrave and Tranter, 1978; Andia-Waltenbaugh et al., 1980). Increase in total cell Ca^{2+} in the presence of caffeine could therefore result from the overloading of the intracellular Ca^{2+} pool via the Ca^{2+} - Mg^{2+} -ATPase by elevating the cAMP level, as has been reported for the sarcoplasmic reticulum (Fabiato and Fabiato, 1975; Fredholm, 1980). Interestingly, activation of the Ca^{2+} - Mg^{2+} -ATPase has been reported to modulate $[\text{Ca}^{2+}]_i$ oscillations in some cells (Camacho and Lechleiter, 1993; Petersen et al., 1993). Such an activation may participate indirectly in the inhibitory effects of caffeine on $[\text{Ca}^{2+}]_i$ oscillation.

In summary, these multiple effects of caffeine on the two major intracellular signalling pathways for $[\text{Ca}^{2+}]_i$ regulation (Berridge and Irvine, 1989; Bygrave and Benedetti, 1993), i.e. increase in cAMP level, decrease in both basal InsP_3 level and hormone-induced InsP_3 synthesis and decrease in InsP_3 -mediated Ca^{2+} release, probably combine to underlie its complex actions on cellular $[\text{Ca}^{2+}]_i$ in hepatocytes. For example, such an explanation may apply to the biphasic effect of caffeine on gluconeogenesis and urea synthesis in hepatocytes (Kashiwagura et al., 1987). These synthetic pathways are Ca^{2+} - and cAMP-dependent (Kashiwagura et al., 1987), are stimulated by InsP_3 -mobilizing hormones, and are potentiated by low (0.1–1 mM), but inhibited by higher (> 1 mM), caffeine concentrations (Kashiwagura et al., 1987). One possibility is that, at low concentrations, caffeine has a greater influence on cAMP metabolism (i.e. phosphodiesterase inhibition) than on InsP_3 metabolism, and this may result, for example, in sensitization of the InsP_3 receptor. At higher caffeine concentrations, however, the direct inhibitory effects of caffeine on InsP_3 synthesis and InsP_3 -mediated Ca^{2+} release begin to predominate, and the response becomes attenuated. Clearly, caffeine induces a variety of effects in rat hepatocytes, and results obtained by its use should be interpreted with caution.

We thank Dr. T. R. Cheek, Dr. T. J. McNulty and Dr. C. W. Taylor for helpful discussion, R. Leuillet for preparing beautiful liver cells, and A. Edelman for his help in editing the manuscript. The work was supported in part by a grant from the Fondation pour la Recherche Medicale.

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