Pflügers Arch – Eur J Physiol (1996) 431 : 371–378

ORIGINAL ARTICLE

Carlos Villalobos · Javier García-Sancho

Caffeine-induced oscillations of cytosolic Ca^{2+} in GH_3 pituitary cells are not due to Ca^{2+} release from intracellular stores but to enhanced Ca^{2+} influx through voltage-gated Ca^{2+} channels

Received: 31 July 1995/Received after revision: 1 September 1995/Accepted: 4 September 1995

Abstract Caffeine, a well known facilitator of Ca^{2+}-induced Ca^{2+} release, induced oscillations of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in GH_3 pituitary cells. These oscillations were dependent on the presence of extracellular Ca^{2+} and blocked by dihydropyridines, suggesting that they are due to Ca^{2+} entry through L-type Ca^{2+} channels, rather than to Ca^{2+} release from the intracellular Ca^{2+} stores. Emptying the stores by treatment with ionomycin or thapsigargin did not prevent the caffeine-induced $[Ca^{2+}]_i$ oscillations. Treatment with caffeine occluded phase 2 ($[Ca^{2+}]_i$ oscillations) of the action of thyrotropin-releasing hormone (TRH) without modifying phase 1 (Ca^{2+} release from the intracellular stores). Caffeine also inhibited the $[Ca^{2+}]_i$ increase induced by depolarization with high-K^+ solutions (56% at 20 mM), suggesting direct inhibition of the Ca^{2+} entry through voltage-gated Ca^{2+} channels. We propose that the $[Ca^{2+}]_i$ increase induced by caffeine in GH_3 cells takes place by a mechanism similar to that of TRH, i.e. membrane depolarization that increases the firing frequency of action potentials. The increase of the electrical activity overcomes the direct inhibitory effect on voltage-gated Ca^{2+} channels with the result of increased Ca^{2+} entry and a rise in $[Ca^{2+}]_i$. Consideration of this action cautions interpretation of previous experiments in which caffeine was assumed to increase $[Ca^{2+}]_i$ only by facilitating the release of Ca^{2+} from intracellular Ca^{2+} stores.

Key words Ca^{2+} influx · Ca^{2+}-induced Ca^{2+} release · Caffeine · Ryanodine · Intracellular Ca^{2+} stores · GH_3 pituitary cells · Thapsigargin

C. Villalobos · J. García-Sancho (✉)
Instituto de Biología y Genética Molecular, Universidad de Valladolid and Consejo Superior de Investigaciones Científicas, Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, E-47005 Valladolid, Spain

Introduction

GH_3 pituitary cells display oscillations of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) that are driven by electrical activity and related to the control of prolactin secretion [5, 18, 26, 36]. Physiological secretagogues such as thyrotropin-releasing hormone (TRH) or vasoactive intestinal peptide (VIP) increase both electrical activity and $[Ca^{2+}]_i$ oscillations [5, 13, 29, 36]. The effect of TRH on electrical activity is thought to be mediated by a TRH-induced inhibition of an inwardly rectifying K^+ current [2, 3]. This results in depolarization of the plasma membrane potential, an increase of the frequency of action potential firing and the ensuing $[Ca^{2+}]_i$ oscillations.

In cardiac muscle and some neuron types $[Ca^{2+}]_i$ oscillations are started by Ca^{2+} entry and amplified by Ca^{2+}-induced Ca^{2+} release (CICR) from the intracellular stores that takes place through ryanodine receptors [11, 12, 22, 24, 31]. This mechanism has been proposed to apply to other cell systems, often on the basis of the effects of caffeine, a drug that is able to activate the ryanodine receptor Ca^{2+}-release pathway [10, 25, 27]. However, caffeine may also affect other cell parameters related to Ca^{2+} homeostasis, including adenosine 3′,5′-cyclic monophosphate (cAMP) levels [7], Ca^{2+} release mediated via inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] [10] and voltage-sensitive Ca^{2+} [19, 20, 32] and K^+ [17, 32] channels of the plasma membrane.

Using permeabilized GH_4C_1 cells Tanaka and Tashjian [28] have identified three different pools of stored Ca^{2+}: (1) an Ins(1,4,5)P_3-sensitive pool which is also emptied by treatment with the endomembrane Ca^{2+}-ATPase inhibitor thapsigargin [30]; (2) a pool which is sensitive to thapsigargin but not emptied by Ins(1,4,5)P_3; (3) a residual pool emptied by caffeine. All the three pools were emptied by treatment with the Ca^{2+} ionophore ionomycin. The same group proposed recently that an intracellular Ca^{2+} pool located near

the plasma membrane is essential for spontaneous $[Ca^{2+}]_i$ oscillations, which are favoured by caffeine. According to this view, CICR would amplify $[Ca^{2+}]_i$ oscillations in GH_4C_1 cells [6, 35].

Here we have studied the effects of caffeine on the homeostasis of Ca^{2+} in GH_3 pituitary cells. Caffeine and theophylline induced $[Ca^{2+}]_i$ oscillations, but the mechanism did not involve a major contribution of CICR, as they were not substantially modified by emptying the Ca^{2+} stores using ionomycin or thapsigargin. Instead, $[Ca^{2+}]_i$ oscillations were due to increased Ca^{2+} entry through L-type Ca^{2+} channels. This seems to be secondary to an increase of electrical activity, probably caused by a caffeine-induced membrane depolarization, perhaps by inhibition of a K^+ conductance pathway. Caffeine and theophylline also had a partial inhibitory action on voltage-dependent Ca^{2+} channels, which was overcome, in GH_3 cells, by the other effects of these drugs.

Materials and methods

GH_3 pituitary cells were kindly provided by Dr. F. Barros (Universidad de Oviedo, Spain). They were grown in RPMI 1640 medium supplemented with 15% horse serum and 2.5% fetal calf serum at 37° C and in an atmosphere of 95% air and 5% CO_2.

For fluorescence measurements, GH_3 cells were allowed to attach to poly-L-lysine-coated (0.01 mg/ml, 5 min) glass coverslips and grown for 2–3 days. The cell-coated coverslips were washed with standard medium containing (in mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; glucose, 10; 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, Na salt (sodium-HEPES),10; pH, 7.4. and loaded with fura-2 by incubation with 5 μM fura-2/AM (i.e. the acetoxymethyl ester) at room temperature for about 1 h. $[Ca^{2+}]_i$ measurements were performed either on cell populations or at the single-cell level, as described below.

For $[Ca^{2+}]_i$ measurements and Mn^{2+} entry assays using cell populations, glass coverslips were introduced at a fixed angle (45°) into quartz cuvettes placed in the sample compartment of a spectrophotometer that allowed rapid (30–300 Hz) alternation of up to six different excitation wavelengths (Cairn Research, Newnhan, Sittingbourne, Kent, UK). Temperature was 30° C. Fluorescence emitted above 510 nm was measured and integrated every second. $[Ca^{2+}]_i$ was estimated from the ratio of the fluorescence values excited at 340 nm and at 380 nm [14]. Calibration was performed by comparison with fura-2 standards. Mn^{2+} entry was evidenced by the quenching of the fura-2 fluorescence excited at 360 nm, a wavelength which is not sensitive to changes in Ca^{2+} concentration [15]. This procedure has been described in detail elsewhere [1]. Perfusion with different media allowed the repeated stimulation and washing of the same cells, in order to compare inhibition within the same cell lot and to document reversibility.

For single-cell measurements, the coverslips coated with fura-2-loaded cells were mounted under the microscope (Nikon Diaphot) in a temperature-controlled (36° C) chamber and epi-illuminated alternately at 340 and 380 nm. Light emitted above 510 nm was recorded by an extended ISIS-M camera (Photonic Science, Robertsbridge, East Sussex, UK) and analysed using an Applied Imaging Magical image processor (Sunderland, Tyne and Wear, UK). Four video frames at each wavelength were averaged by hardware, with an overall time resolution of about 3 s for each pair of images at alternate wavelengths. Consecutive frames obtained at 340 and 380 nm excitation were ratioed pixel by pixel and $[Ca^{2+}]_i$ was estimated by comparison with fura-2 standards [23, 34].

Quantification of $[Ca^{2+}]_i$ changes of single cells were performed using two parameters, the mean $[Ca^{2+}]_i$ increase and the oscillation index [23]. The mean $[Ca^{2+}]_i$ increase was computed as the average of all the $[Ca^{2+}]_i$ values during the test period (usually 3 min) minus the average of all the $[Ca^{2+}]_i$ values during the control period. This parameter reflects increases of $[Ca^{2+}]_i$, whether or not they are oscillatory. The oscillation index was computed as the average of all the differences (in absolute values) between each $[Ca^{2+}]_i$ value and the next, throughout the whole integration period; units are nM/3 s. The increase of this parameter reflects an increase of oscillations (either amplitude or frequency) and it is largely independent of the actual $[Ca^{2+}]_i$ values [23, 33].

Fura-2/AM was obtained from Molecular Probes, Eugene, Ore., USA. Caffeine and theophylline were obtained from Sigma, Madrid, Spain. Nisoldipine was a generous gift from Bayer, Germany. Thapsigargin was obtained from Alomon Laboratories, Jerusalem, Israel. Griseolic acid and its dipivaloyloxymethyl (diPOM) ester derivative were generous gifts from Dr. Masakatsu Kaneko, Sankyo, Tokyo, Japan. Other chemicals were obtained from Sigma, or from E. Merck, Darmstadt, Germany.

Results

Caffeine induces a $[Ca^{2+}]_i$ increase due to Ca^{2+} entry through L-type Ca^{2+} channels

Addition of caffeine (1–20 mM) to GH_3 cells perfused with Ca^{2+}-containing medium induced an increase in $[Ca^{2+}]_i$, which started within a few seconds after adding caffeine and persisted for several minutes. The action of caffeine was quickly reversed upon washing out the drug. Figure 1 shows typical results from three representative single cells (A–C) and the average of all the cells (D). In the single-cell traces it seems clear that caffeine increases $[Ca^{2+}]_i$ oscillations. In the average trace, the effect of caffeine is evidenced by an increase of $[Ca^{2+}]_i$ which declines with time. Figure 2 shows the average of the responses to caffeine from 388 single cells, analysed in terms of the mean $[Ca^{2+}]_i$ increase and oscillation index, and compares it to the responses to ionomycin, thapsigargin or TRH. Results are expressed as a percentage of the control activity, before addition of the drugs. As explained in Materials and methods, the mean $[Ca^{2+}]_i$ increase is sensitive to both static and dynamic changes of $[Ca^{2+}]_i$, whereas the oscillation index is sensitive only to dynamic changes. Ionomycin and thapsigargin affected only the static component, detected by the increase of the mean $[Ca^{2+}]_i$, but not the dynamic one, as the oscillation index was not significantly modified. We have shown before that the increase of $[Ca^{2+}]_i$ is due, in these cases, to increased Ca^{2+} entry through a capacitative mechanism which is activated on emptying the intracellular Ca^{2+} stores [33]. TRH increased both the mean $[Ca^{2+}]_i$ increase and the oscillation index. The second effect is due to stimulation of $[Ca^{2+}]_i$ oscillations during phase 2 of the action of TRH [33]. Caffeine also increased both parameters suggesting that the rise of $[Ca^{2+}]_i$ is due to the increase of $[Ca^{2+}]_i$ oscillations. The effect of caffeine was even larger than that of TRH (compare

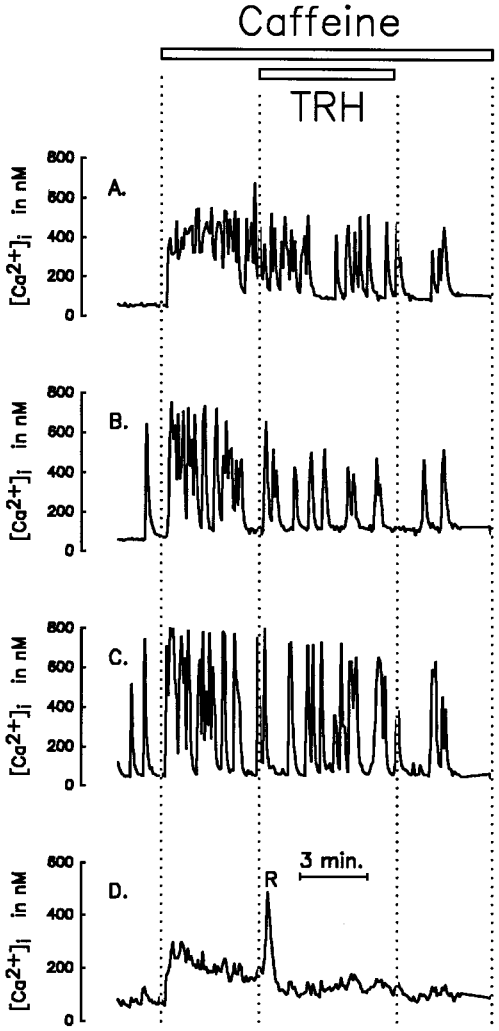

Fig. 1A–D Caffeine increases $[Ca^{2+}]_i$ oscillations of GH_3 cells. **A–C** The effects on three representative single cells and, **D** the average of 47 cells present in the same microscope field are shown. The synchronous release of Ca^{2+} from the intracellular Ca^{2+} stores of all the cells induced by thyrotropin-releasing hormone (TRH) is evidenced as a neat $[Ca^{2+}]_i$ peak (labelled *R*) in the averaged trace (**D**). The concentrations of caffeine and TRH were 10 mM and 100 nM, respectively

the last four bars in Fig. 2). When TRH was added after caffeine, it produced an early $[Ca^{2+}]_i$ peak, synchronous in all the cells, due to Ca^{2+} release from the intracellular stores (phase 1, labelled "R" in Fig. 1D; see also [19]). However, the increase of $[Ca^{2+}]_i$ oscillations characteristic of phase 2 of the action of TRH was usually not observed in these caffeine-treated cells (Fig. 1A–C). This is also evidenced in the average trace (Fig. 1D) as there is not an increase in the mean $[Ca^{2+}]_i$ signal during phase 2 as compared to the level observed before TRH addition. Therefore, the treatment with caffeine seemed to occlude the effect of TRH on $[Ca^{2+}]_i$.

The increase of $[Ca^{2+}]_i$ produced by caffeine was due to Ca^{2+} entry from the extracellular medium, since it was completely blocked by Ca^{2+} removal (Fig. 3A), by 5 mM Ni^{2+} (not shown) or by dihydropyridines

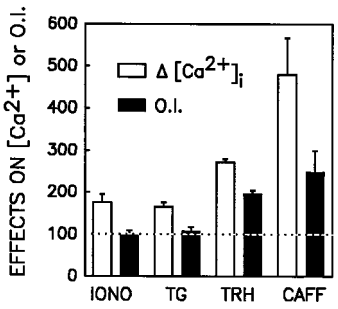

Fig. 2 Comparison of the effects of ionomycin (*IONO*, 100 nM), thapsigargin (*TG*, 500 nM), TRH (100 nM) and caffeine (*CAFF*, 20 mM) on the mean $[Ca^{2+}]_i$ level ($\Delta[Ca^{2+}]_i$) and on the oscillation index (*O.I.*). The values represent means ± SEM of 68, 100, 281, and 388 single cells analysed, respectively. Data are expressed as the percentage of the values just before the treatment; thus a value of 100 (*dotted line*) means no effect. See Materials and methods for more details on computation of mean $[Ca^{2+}]_i$ and O.I.. The two integration periods used for calculations were of 3 min, one just before treatment and the other starting 1 min (CAFF), 2 min (IONO and TRH), or 5 min (TG) after treatment with the drug

(Fig. 3B). We have shown before that Mn^{2+} is able to enter through voltage-operated Ca^{2+} channels in GH_3 cells [34]. Figure 3C shows that caffeine also accelerated the entry of Mn^{2+}, estimated from the quenching of the fura-2 fluorescence excited at 360 nm, a wavelength that is insensitive to Ca^{2+} (see Materials and methods). The entry of Mn^{2+} induced by caffeine was fully blocked by Ni^{2+} or by dihydropyridines (results not shown).

Theophylline but not other phosphodiesterase inhibitors reproduces the effects of caffeine

Theophylline, another methyl-xanthine derivative, had the same effects as caffeine on $[Ca^{2+}]_i$ of GH_3 cells. As a matter of fact, theophylline was about twice as potent as caffeine (half-maximal increases were obtained at about 2 and 1 mM, respectively; results not shown). Figure 4A–C shows the effects of caffeine and theophylline in three representative single cells. A final pulse application of TRH is also shown for comparison. It is apparent that the cells responding better to caffeine responded also better to theophylline. Figure 4D shows the correlation between the increases of $[Ca^{2+}]_i$ produced by both xanthine derivatives in 99 single cells. There was a good correlation between the responses to the two xanthine derivatives (correlation coefficient, $r = 0.85$), suggesting that both drugs were acting by the same mechanism. Theophylline was also able to induce entry of Mn^{2+} and this effect was blocked by dihydropyridines (results not shown).

Since both theophylline and caffeine are well known phosphodiesterase inhibitors [7], the observed effects on $[Ca^{2+}]_i$ could be attributed to an increase of cAMP levels. To investigate this point we studied the effects of other cAMP-increasing agents. Two other phospho-

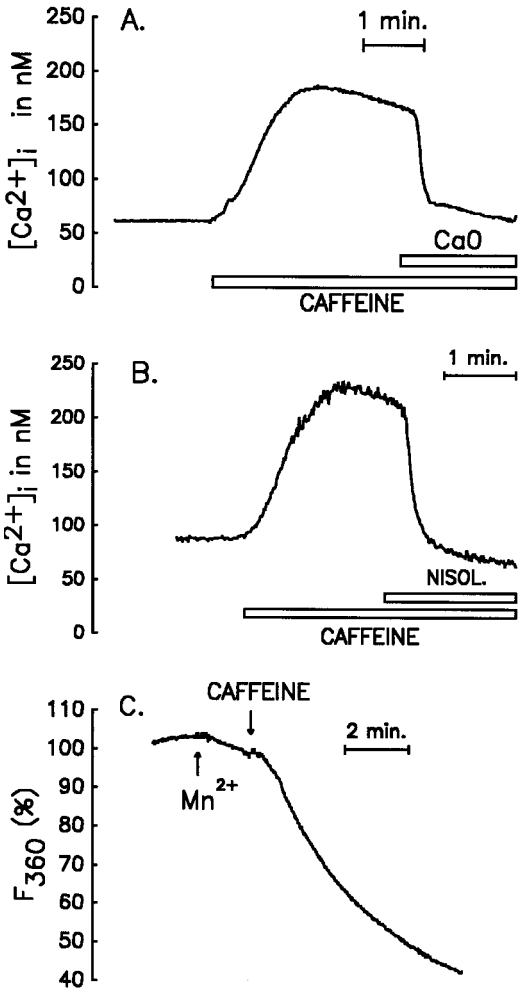

Fig. 3A–C Caffeine induces entry of Ca^{2+} and Mn^{2+}. The averaged signal obtained from a cell population is shown. The increase of $[Ca^{2+}]_i$ is prevented by Ca^{2+} removal (*Ca0*, containing 0.5 mM EGTA; **A**) and blocked by nisoldipine (*NISOL*; **B**). **C** The acceleration of Mn^{2+} entry evidenced by the quenching of the fura-2 fluorescence excited at 360 nm (see Materials and methods) is shown. The concentration of caffeine was 20 mM in the three cases. The concentrations of nisoldipine and Mn^{2+} were 500 nM and 200 μM, respectively. Each trace is representative of 3–7 similar experiments

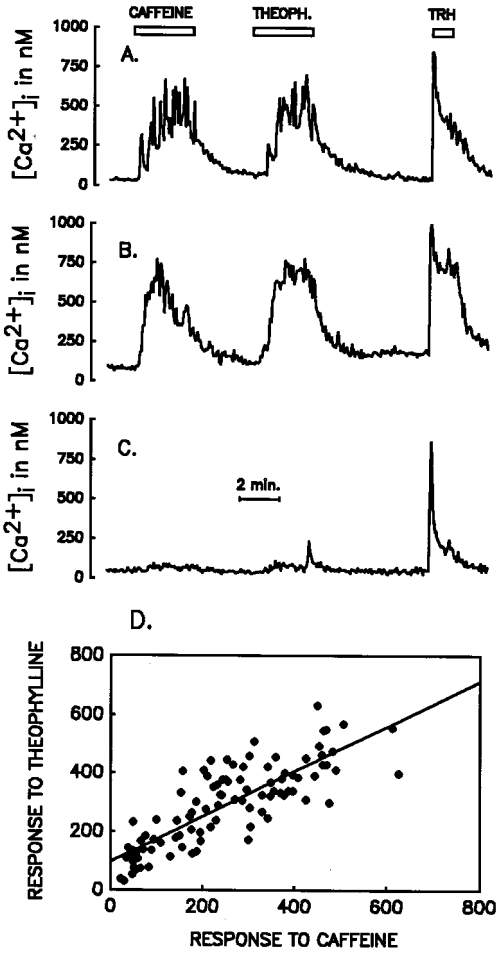

Fig. 4A–D Comparison of the effects of caffeine and theophylline in single GH_3 cells. **A–C** The effects of sequential perfusion with 10 mM caffeine and 5 mM theophylline (*THEOPH.*) during 3-min periods in three representative single cells is illustrated. The effect of TRH (100 nM) is also shown. **D** The correlation between the responses to caffeine and theophylline in 99 single cells. Responses were quantified as the mean $[Ca^{2+}]_i$ increase during the last 2 min of the perfusion period with the drug. The line was adjusted by the least-squares procedure. The correlation coefficient (r) was 0.85

diesterase inhibitors, 3-isobutyl-1-methylxanthine (IBMX; [7]) and the structurally unrelated and highly specific compound griseolic acid [37], were tested. The effects of the adenylate cyclase activator forskolin [8] and the membrane-permeable cAMP analogue dibutyryl-cAMP were also studied. All the cAMP-increasing agents produced an increase of $[Ca^{2+}]_i$ which was blocked by dihydropyridines, as illustrated for forskolin in Fig. 5A. However, the increase of $[Ca^{2+}]_i$ was smaller than that produced by caffeine or theophylline and developed more slowly (compare to Figs. 1, 3 and 4). In addition, caffeine or theophylline added as well as forskolin (Fig. 5B), griseolic acid (Fig. 5C), IBMX (100 μM) or dibutyryl-cAMP (300 μM, not shown) produced an additional increase of $[Ca^{2+}]_i$, whereas the reverse did not apply (not shown). We conclude that the effects of caffeine and theophylline on $[Ca^{2+}]_i$ of GH_3 cells cannot be explained by the increase of cAMP levels.

Does release of Ca^{2+} from the intracellular Ca^{2+} stores contribute to the $[Ca^{2+}]_i$ increase induced by caffeine in GH_3 cells?

Caffeine is able to release Ca^{2+} from the intracellular Ca^{2+} stores (ICS) by facilitating the opening of the Ca^{2+} channels associated with ryanodine receptors, which are responsible for CICR [27]. Caffeine has been reported to release Ca^{2+} from the ICS of GH_3 cells on

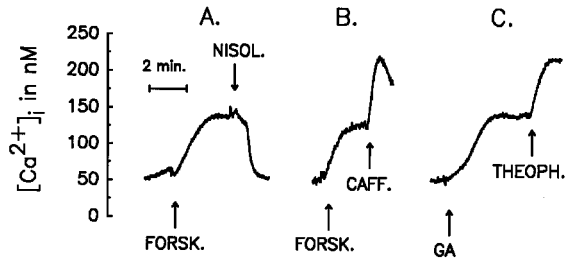

Fig. 5A–C The effects of caffeine and theophylline are additive with those of other agents that increase adenosine 3′,5′-cyclic monophosphate (cAMP) levels. **A** Forskolin (*FORSK.*; 5 μM) produced an increase of $[Ca^{2+}]_i$ that was prevented by nisoldipine (*NISOL.*; 500 nM). **B** Caffeine (*CAFF.*; 20 mM), added in conjunction with forskolin (5 μM) produced an additional increase of $[Ca^{2+}]_i$. **C** Theophylline (*THEOPH.*; 10 mM) added in conjunction with the dipiraloyloxymethyl ester of griseolic acid (*GA*; 10 μM) produced an additional effect on $[Ca^{2+}]_i$. Other cAMP-increasing agents tested (3-isobutyl-1-methylxanthine IBMX, 100 μM; dibutyryl-cAMP; 300 μM) had the same effects as those illustrated here for forskolin and griseolic acid (not shown)

the basis of experiments performed with both intact and digitonin-permeabilized cells [28, 35]. We studied the effects of caffeine in GH_3 cells whose bathing medium was switched to Ca^{2+}-free solution 15–30 s before caffeine addition, under the rationale that caffeine-induced Ca^{2+} release should produce a transient increase of $[Ca^{2+}]_i$. In none of six experiments performed with different batches of GH_3 cells (measurements of average $[Ca^{2+}]_i$ in the whole population) were we able to observe a $[Ca^{2+}]_i$ increase after addition of caffeine (results not shown). In three similar experiments performed using the fluorescence microscope, we found a caffeine-induced $[Ca^{2+}]_i$ peak in none out of 112 single cells analysed (92 of them responding to caffeine in Ca^{2+}-containing medium; results not shown). In parallel experiments with isolated bovine chromaffin cells (experimental conditions as described in [34]) we found clear effects of caffeine using the same protocol (L. Nuñez, M.T. de la Fuente, unpublished results). In an additional experiment with GH_3 cells we tried to overload the ICS by increasing $[Ca^{2+}]_i$ by depolarization with high-K^+ (75 mM) solution for 90 s. Then perfusion was switched to standard (5 mM K^+) medium and 20 mM caffeine was added 1 min later, when $[Ca^{2+}]_i$ was still high (average about 500 nM). Under these conditions we found a weak response (transient $[Ca^{2+}]_i$ increase of 20–100 nM) in only 6 out of 57 cells analysed. Therefore, in our hands, there was no indication of caffeine-facilitated release of Ca^{2+} from the ICS in GH_3 cells.

Another series of experiments was designed to test whether CICR could contribute to the increase of $[Ca^{2+}]_i$ induced by caffeine in GH_3 cells incubated in Ca^{2+}-containing medium. In these experiments the ICS were emptied of Ca^{2+} by treatment with either the endomembrane ATPase inhibitor thapsigargin or with the Ca^{2+} ionophore ionomycin before caffeine treatment. These treatments produce > 95% emptying of

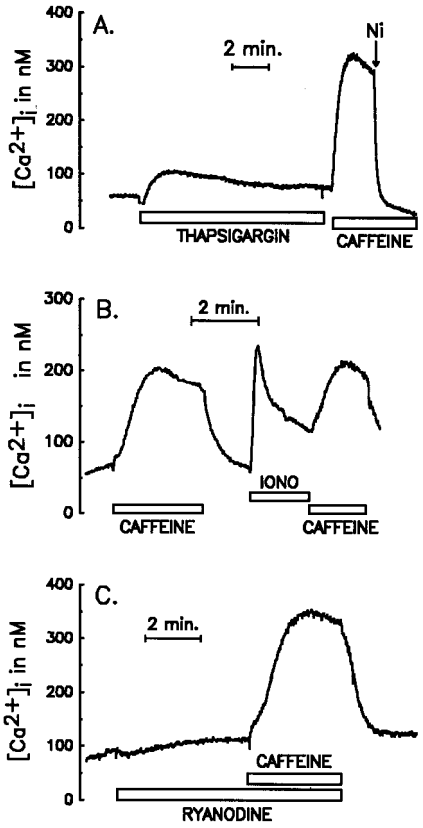

Fig. 6 The effect of caffeine is not prevented by emptying the Ca^{2+} stores with thapsigargin (**A**) or ionomycin (**B**) nor by treatment with ryanodine (**C**). Averaged $[Ca^{2+}]_i$ traces from cell populations are shown. The concentrations of the drugs were: caffeine, 20 mM; thapsigargin, 500 nM; ionomycin, 100 nM; ryanodine, 10 μM. Experiments representative of 3–5 similar ones

the ICS [33]. Pretreatment with either thapsigargin (Fig. 6A) or ionomycin (Fig. 6B) did not substantially modify the effect of caffeine, suggesting that stored Ca^{2+} does not play a prominent role in the increase of $[Ca^{2+}]_i$ induced by caffeine in GH_3 cells. On the other hand, the effect of caffeine was not modified by ryanodine (Fig. 6C), an inhibitor of CICR [10, 27].

Caffeine and theophylline also inhibit Ca^{2+} entry through voltage-operated Ca^{2+} channels

Caffeine has been recently reported to inhibit Ca^{2+} entry through L-type Ca^{2+} channels in several tissues, including GH_3 cells [19, 32, 38]. We have tested the effects of caffeine and theophylline on the increase of $[Ca^{2+}]_i$ induced by depolarization of GH_3 cells with high-K^+ solutions (Fig. 7). These $[Ca^{2+}]_i$ peaks were inhibited by more than 90% by 1 μM nisoldipine (results not shown; see also [34]), suggesting that most of the Ca^{2+} entry takes place through L-type Ca^{2+} channels. Both caffeine (Fig. 7A) and theophylline (Fig. 7B) inhibited the high-K^+-induced $[Ca^{2+}]_i$ increase. Theophylline was more potent than caffeine

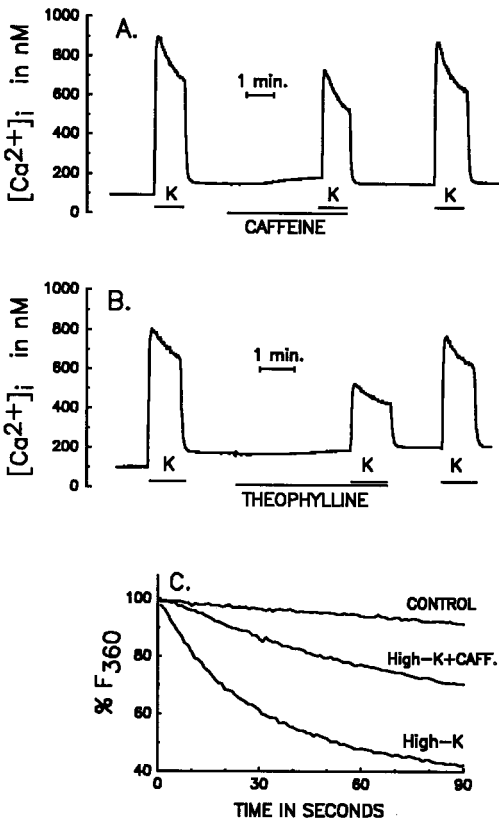

Fig. 7A–C Effects of caffeine and theophylline on the entry of Ca^{2+} and Mn^{2+} induced by depolarization with high-K^+ solutions. Averaged traces from cell populations are shown. **A**,**B** The cells were depolarized by perfusion with standard medium containing 75 mm KCl (replacing an equivalent amount of NaCl, labelled "*K*"). The protocol was as shown in the figure. The concentrations of caffeine and theophylline were both 5 mM. **C** Perfusion was switched at $t = 0$ from standard medium to medium containing 0.2 mM Mn^{2+}, either standard (*CONTROL*), high-K^+ (75 mM, *High-K*) or high-K^+ also containing 20 mM caffeine (*High-K+CAFF.*). The rate of quenching was the same in High-K+CAFF solution containing no Ca^{2+} (not shown). The fluorescence of fura-2 excited at 360 nm, normalized to 100% with regard to the initial value, is shown on the *ordinate* (see Materials and methods for more details)

(40% versus 20% inhibition at 5 mM and 66% versus 56% inhibition at 20 mM). The inhibition of the $[Ca^{2+}]_i$ increases induced by caffeine or theophylline was reversed by washing out the drugs (Fig. 7). Figure 7C documents that caffeine was also able to inhibit Mn^{2+} entry, estimated from the quenching of fura-2 fluorescence, induced by depolarization with high-K^+ solutions. The experiments were performed with similar results in both Ca^{2+}-free and in Ca^{2+}-containing media, suggesting that inhibition of the entry pathway by caffeine is not secondary to the increase of $[Ca^{2+}]_i$ it may induce (results not shown).

Discussion

The operation of CICR mechanisms has been well documented in heart muscle and some nerve cells [11, 12, 22, 24, 31]. In these tissues, entry of Ca^{2+} through the plasma membrane triggers massive Ca^{2+} release from the ICS by activation of type-2 ryanodine receptors [27], this resulting in amplification of the $[Ca^{2+}]_i$ signal. Caffeine increases the sensitivity to Ca^{2+} of CICR, thus facilitating the $[Ca^{2+}]_i$ changes involving the cooperation of this mechanism [24, 25]. $[Ca^{2+}]_i$ oscillations in GH_3 cells have, for a long time, been attributed to the entry of Ca^{2+} associated with electrical activity [26]. Recently, it has been suggested that CICR could contribute to the amplification of $[Ca^{2+}]_i$ oscillations in GH_4C_1 cells [35], a GH clone closely related to the GH_3 strain. This proposal was based on the kinetics of the oscillations, their modification by Ca^{2+} removal and readdition and the pro-oscillatory effects of caffeine [35].

In our hands caffeine was not able to release Ca^{2+} from the ICS in GH_3 cells, since it did not produce an increase of $[Ca^{2+}]_i$ in cells incubated in Ca^{2+}-free medium. In standard Ca^{2+}-containing medium, caffeine and theophylline increased $[Ca^{2+}]_i$, but by stimulating Ca^{2+} entry through L-type Ca^{2+} channels. The effect was entirely dependent on the presence of external Ca^{2+} and it was blocked by Ni^{2+} and by dihydropyridines (Fig. 3). In addition, caffeine and theophylline were also shown to induce entry of Mn^{2+} (Fig. 3), which is able to permeate L-type Ca^{2+} channels in GH_3 cells [34].

Caffeine-induced Ca^{2+} release from the ICS did not seem to play a major role in the increase of $[Ca^{2+}]_i$ produced by caffeine since complete emptying of the stores by treatment with either thapsigargin or ionomycin did not modify the effect of caffeine (Fig. 6). On the other hand, treatment with caffeine did not prevent the increase of $[Ca^{2+}]_i$ induced during phase 1 of TRH action, which is due to mobilization of stored Ca^{2+} (Fig. 1; see also [20]). Finally, the effect of caffeine was not substantially modified by ryanodine, a blocker or CICR (Fig. 6).

The lack of contribution of release of stored Ca^{2+} to the action of caffeine may seem rather surprising since the ability of caffeine to mobilize Ca^{2+} has been documented in permeabilized GH_4C_1 cells [28]. This discrepancy may depend on the experimental conditions, such as differences in $[Ca^{2+}]_i$ and loading state of the stores. Tanaka and Tashjian [28] showed that Ca^{2+} redistribution among different stored Ca^{2+} pools can take place depending on the experimental conditions, so that the caffeine-sensitive stores might refill more in permeabilized cells than they do in intact cells. In any case, we were unable to induce Ca^{2+} mobilization from the ICS by caffeine in intact GH_3 cells.

Since caffeine and theophylline are well known phosphodiesterase inhibitors [7], their effects on $[Ca^{2+}]_i$ could be secondary to the increase of cAMP levels.

Phosphorylation of L-type Ca^{2+} channels by protein kinase A has been reported to be essential for function of these channels in several cell types [21]. In GH_3 cells cAMP induces prolactin secretion which is associated with a dihydropyridine-sensitive Ca^{2+} influx [16]. We find that either inhibition of phosphodiesterase by IBMX [7] or griseolic acid [37], addition of membrane-permeable cAMP analogues or activation of adenylate cyclase by forskolin [8] produced an increase of $[Ca^{2+}]_i$ which was sensitive to dihydropyridines. However, the effect was smaller than that with caffeine or theophylline and developed more slowly. In addition, cells whose cAMP levels had been increased by any of the above-described treatments, or by a combination of them (for example, forskolin + IBMX) continued to respond to caffeine with a similar increase of $[Ca^{2+}]_i$. We conclude that, even though the increase of cAMP is able to produce a small increase of $[Ca^{2+}]_i$, the effects of caffeine and theophylline cannot be explained only by the rise of cAMP levels.

Caffeine and theophylline inhibited the increase of $[Ca^{2+}]_i$ induced by depolarization in high-K^+ solutions, which is due to Ca^{2+} entry through L-type Ca^{2+} channels. It should be noted that these blocking effects of caffeine and theophylline, even at the highest concentrations tested (20 mM), were always incomplete. The inhibitory effects on Ca^{2+} channels shown here are consistent with recent reports on the action of caffeine on Ca^{2+} currents in several cell types, including GH_3 [19, 32, 38].

How could caffeine and theophylline antagonize L-type Ca^{2+} channels and, at the same time, stimulate Ca^{2+} entry through them? A possibility would be that caffeine increased the rate of action potential firing, as does TRH [26, 29, 36]. The effects of caffeine on $[Ca^{2+}]_i$ were reminiscent of those observed during phase 2 of the action of TRH, with an increase of both the mean $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$ oscillations (Fig. 2). In addition, caffeine occluded the action of TRH during phase 2 (Fig. 1). In the case of TRH, the stimulation of electrical activity is due to increased excitability caused by membrane depolarization by inhibition of an inwardly rectifying K^+ current [2, 3, 9]. Caffeine has been reported to inhibit an inwardly rectifying K^+ current in heart cells [32]. We propose that caffeine and theophylline may act in GH_3 cells by inhibiting the inwardly rectifying K^+ current, this causing membrane depolarization, increased firing frequency and elevation of $[Ca^{2+}]_i$. The partial block of Ca^{2+} entry by a direct effect on Ca^{2+} channels would be overcome by the increase of electrical activity. The inhibition of the inwardly rectifying K^+ current and the increase of firing frequency we propose here have been confirmed by direct electrophysiological measurements [4].

We have reported recently that caffeine has a dual effect on bovine adrenal chromaffin cells showing veratridine-induced $[Ca^{2+}]_i$ oscillations. These oscillations were inhibited by caffeine in cells showing high $[Ca^{2+}]_i$ activity, whereas the reverse was observed in cells with low $[Ca^{2+}]_i$ activity [23]. This outcome could reflect the effects on the Ca^{2+} and on the K^+ currents, respectively. Our results on the effects of caffeine in GH_3 cells are very close to those reported recently in pancreatic B cells [17]. In these cells caffeine increased $[Ca^{2+}]_i$, but the effects were not due to Ca^{2+} release from the ICS nor to changes in cAMP levels. Instead, caffeine caused closure of ATP-sensitive K^+ channels with the ensuing membrane depolarization and Ca^{2+} entry through L-type Ca^{2+} channels [17].

In summary, to the widely acknowledged actions of caffeine, i.e. facilitating Ca^{2+} release from the ICS, inhibiting phosphodiesterase activity or blocking voltage-operated Ca^{2+} channels, inhibition of K^+ channels leading to increased Ca^{2+} entry through voltage-gated Ca^{2+} channels should be added. Therefore, extreme caution should be used when interpreting the effects of this drug.

Acknowledgements Financial support from the spanish Dirección General de Investigación Científica y Técnica (DGICYT, project grant PB92-0268 and fellowship to C.V.) is gratefully acknowledged.

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