Inhibition of inositol trisphosphate-induced calcium release by caffeine is prevented by ATP

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We have investigated the effect of various methylxanthines on the basal and InsP_3 -stimulated unidirectional $^{45}\text{Ca}^{2+}$ efflux from permeabilized A7r5 cells under different experimental conditions. We report that caffeine and theophylline inhibit the InsP_3 -induced Ca^{2+} release, whereas the basal Ca^{2+} leak remained largely unaffected. The effect on InsP_3 -induced Ca^{2+} release was

specific, since isocaffeine was not effective. The inhibition occurred similarly in the absence or presence of extravesicular Ca^{2+} and was not associated with a decrease in the $[\text{H}^3]\text{InsP}_3$ binding to the receptor. ATP and MgATP (5 mM) prevented the inhibition, suggesting that caffeine may interact with an ATP-binding site on the InsP_3 receptor or some associated protein.

INTRODUCTION

Caffeine stimulates Ca^{2+} release through some, but not all, types of ryanodine receptors [1–3] and has been widely used as a tool for demonstrating the presence of a ryanodine receptor in a variety of cells. In these studies, it became apparent that caffeine concentrations in the range 10–25 mM inhibited Ca^{2+} responses induced by inositol 1,4,5-trisphosphate (InsP_3)-producing agonists [4–11]. It has been proposed that millimolar concentrations of caffeine inhibit the InsP_3 receptor [8,12–14]. However, in other experimental systems, caffeine concentrations up to 20 mM [10] and 50 mM [15] did not affect the InsP_3 receptor. This discrepancy indicates that the inhibitory effect of caffeine must be strongly dependent on experimental conditions and/or on cell type.

We have investigated the effect of caffeine on the basal and InsP_3 -stimulated unidirectional $^{45}\text{Ca}^{2+}$ efflux from the non-mitochondrial Ca^{2+} stores of permeabilized A7r5 smooth-muscle cells. The following two questions were specifically addressed: (1) does caffeine inhibit the InsP_3 -induced Ca^{2+} release and, if so, does this inhibition represent a specific action of caffeine? (2) is the extent of inhibition of the release dependent on experimental conditions?

We report that caffeine does inhibit InsP_3 -mediated Ca^{2+} release and that this inhibition must represent a specific interaction. The inhibition occurs independently of the cytosolic $[\text{Ca}^{2+}]$, and the presence of ATP or MgATP in the assay medium prevents this inhibitory effect of caffeine.

MATERIALS AND METHODS

A7r5 aortic smooth-muscle cells were cultured as described previously [16]. The cells were used between passages 10 and 20 after receipt from the American Type Culture Collection (Bethesda, MD, U.S.A.), unless otherwise indicated. They were seeded in 12-well clusters at a density of approx. 10^4 cells/cm². Experiments were carried out with confluent monolayers of cells on day 7 after plating, at an average cell density of 7.5×10^4 cells/cm².

Permeabilization with saponin was performed by incubating the cells for 10 min in a solution containing 120 mM KCl,

30 mM imidazole/HCl (pH 6.8), 2 mM MgCl_2 , 1 mM ATP, 1 mM EGTA and 20 $\mu\text{g}/\text{ml}$ saponin at 25 °C. The non-mitochondrial Ca^{2+} stores were then loaded with $^{45}\text{Ca}^{2+}$ for 40 min in a medium containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 5 mM MgCl_2 , 5 mM ATP, 0.44 mM EGTA, 10 mM NaN_3 and 238 nM free Ca^{2+} (23 $\mu\text{Ci}/\text{ml}$) at 25 °C. The wells were then washed twice in efflux medium A containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 2 mM MgCl_2 , 1 mM ATP, 1 mM EGTA and 5 mM NaN_3 at 25 °C. In those experiments where the effect of changing the ATP concentration was tested, the efflux medium only contained 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 1 mM EGTA and the indicated ATP concentration (efflux medium B). The efflux media A and B had a calculated free $[\text{Ca}^{2+}]$ of less than 10 nM. The medium was replaced every 2 min for a 16 min period. Methylxanthines were added to the efflux medium for 4 min from min 2 to 6 of efflux. InsP_3 at the indicated concentration or A23187 (10 μM) was added for 2 min between min 4 and 6 of efflux.

InsP_3 and ATP were from Boehringer, Mannheim, Germany. Caffeine, saponin, NaN_3 , thapsigargin, A23187 and the various methylxanthines were from Sigma Chemical Co., St. Louis, MO, U.S.A. $^{45}\text{Ca}^{2+}$ was from Amersham International, Amersham, Bucks, U.K. All other reagents were of the highest purity commercially available.

RESULTS

Effect of caffeine on the passive Ca^{2+} leak

Permeabilized A7r5 cells were loaded to steady state with $^{45}\text{Ca}^{2+}$ and then exposed for 4 min to caffeine (1,3,7-trimethylxanthine), isocaffeine (1,3,9-trimethylxanthine) or theophylline (1,3-dimethylxanthine) in the absence of added InsP_3 (Figure 1). The highest concentrations tested (50 mM for caffeine and isocaffeine and 25 mM for the less soluble theophylline) induced a slight decrease in the Ca^{2+} content of the stores. This represented a non-specific effect on the passive Ca^{2+} leak and not the activation of a possible ryanodine receptor, for the following reasons: (1) isocaffeine, which is about 5 times less effective than caffeine in activating the ryanodine receptor [17], was equally potent as caffeine in decreasing the Ca^{2+} content of the store; (2) the effect of the methylxanthines also occurred in the presence of 10 μM

Abbreviation used: InsP_3 , inositol 1,4,5-trisphosphate.

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Figure 1 Effect of methylxanthines on the passive Ca^{2+} leak in permeabilized A7r5 cells

The non-mitochondrial Ca^{2+} stores were first loaded to steady state with $^{45}\text{Ca}^{2+}$ and then exposed to efflux medium A, to which the indicated concentration of caffeine (●), isocaffeine (■) or theophylline (○) was added for 4 min. The Ca^{2+} content of the stores after this application was expressed as a percentage of the Ca^{2+} content of the stores in the absence of these methylxanthines. The Ca^{2+} contents were corrected for non-specific binding, which was taken as the residual Ca^{2+} after addition of $10 \mu\text{M}$ A23187. Non-specific binding amounted on average to 3% of total Ca^{2+} uptake. For clarity, the S.E.M. ($n = 4$) is only shown for the data at 50 mM methylxanthine. The S.E.M. was always lower than 5%.

Ruthenium Red, which blocks the ryanodine receptor (results not shown); (3) the extent of inhibition was independent of the cytosolic free $[\text{Ca}^{2+}]$ in the range 10 nM – $1 \mu\text{M}$ (results not shown); (4) no evidence for a ryanodine receptor in A7r5 cells was found [9,16]. A similar non-specific but more pronounced increase in the passive Ca^{2+} leak has also been observed in permeabilized hepatocytes [15].

Effect of caffeine on the InsP_3 -induced Ca^{2+} release

InsP_3 is an ubiquitous messenger that releases Ca^{2+} from internal stores [18]. In A7r5 cells, more than 95% of the releasable Ca^{2+} is mobilized by InsP_3 [16]. We investigated the effect of caffeine on the Ca^{2+} release induced by $3.2 \mu\text{M}$ InsP_3 in efflux medium A, which contained 1 mM ATP, after loading the stores to steady state with $^{45}\text{Ca}^{2+}$ (Figure 2a). Ca^{2+} release induced by this sub-maximal InsP_3 concentration, expressed as a percentage of the ionophore-releasable Ca^{2+} , was decreased by caffeine. It is noteworthy that a significant inhibition of the release occurred with caffeine concentrations that are usually applied to intact cells (up to 25 mM) to activate pharmacologically a possible ryanodine receptor [4–11]. Inhibition of phosphodiesterases was not responsible for these effects, since Ca^{2+} release could not be inhibited by $10 \mu\text{M}$ cyclic AMP (results not shown). The inhibitory effect of caffeine was observed over a whole range of InsP_3 concentrations (Figure 2b). Caffeine had no significant effect on the Hill coefficient of the InsP_3 dose–response relationship (0.9 ± 0.2 in the absence of caffeine and 1.1 ± 0.1 in the presence of 50 mM caffeine).

The effects of caffeine on the InsP_3 dose–response relationship were tested on cells from the same passage, which was always between 10 and 20 after receipt of the cells. This precaution was necessary, since the EC_{50} for InsP_3 -induced Ca^{2+} release increased

Figure 2 Effect of methylxanthines on the InsP_3 -induced Ca^{2+} release

(a) Effect of caffeine (●), isocaffeine (■) and theophylline (○) on the Ca^{2+} release induced by a sub-maximal dose of InsP_3 ($3.2 \mu\text{M}$). The methylxanthines were added to the efflux medium A 2 min before the InsP_3 challenge as described in the Materials and methods section. The release induced by $3.2 \mu\text{M}$ InsP_3 is expressed as a percentage of the release induced by $10 \mu\text{M}$ A23187. (b) Stores were challenged with the indicated InsP_3 concentration in efflux medium A in the absence (■) or in the presence (●) of 50 mM caffeine. The results shown are typical for three independent experiments.

with passage number. This effect was particularly evident at passage numbers higher than 30. For example, the cells of passage 35 were much less InsP_3 -sensitive than those of passage 15: the EC_{50} shifted from $1.4 \mu\text{M}$ InsP_3 (passage 15) to $13.1 \mu\text{M}$ InsP_3 (passage 35) (results not shown). No significant difference in the amount of Ca^{2+} accumulated in the stores was observed between these two groups of cells. The change in EC_{50} in high-passage-number cells may be related to the type of InsP_3 receptor expressed in these cells. We have observed by reverse-transcriptase polymerase chain reaction a slight increase in the relative amount of the type I receptor as compared with the other isoforms (results not shown).

Caffeine-induced inhibition of the InsP_3 -mediated Ca^{2+} release is a specific effect

The approach used to investigate whether the observed inhibition by caffeine was specific was to study the effect of closely related methylxanthines (Figure 2a). The effect of xanthine, 3,7-dimethylxanthine (theobromine) and 3-isobutyl-1-methyl-

Figure 3 Effect of cytosolic Ca^{2+} on the caffeine-induced inhibition of the InsP_3 response

The release induced by $7 \mu\text{M}$ InsP_3 in efflux medium A containing either no added Ca^{2+} (■) or $1 \mu\text{M}$ cytosolic Ca^{2+} (●) was measured as a function of the caffeine concentration. The indicated $[\text{Ca}^{2+}]$ was added at the time of InsP_3 addition. Means $\pm$ S.E.M. of three experiments are shown.

Figure 4 Effect of ATP on the caffeine-induced inhibition of the InsP_3 response

The stores were challenged with $4 \mu\text{M}$ InsP_3 in efflux medium B containing 120 mM KCl, 30 mM imidazole/HCl, 1 mM EGTA, the indicated caffeine concentration and either no ATP (●) or 5 mM ATP (■). The InsP_3 -induced Ca^{2+} release was expressed as a percentage of the Ca^{2+} release induced by $10 \mu\text{M}$ A23187. Means $\pm$ S.E.M. are shown ($n = 4$).

xanthine could not be tested, because of their poor solubility in water at 25°C . Theophylline seemed to be as efficient as caffeine in inhibiting InsP_3 -mediated Ca^{2+} release. Isocaffeine, which only differs from caffeine in the position of one of the three methyl

groups on the xanthine ring, did not inhibit the release, even at a concentration of 50 mM. This finding indicates that the inhibitory effect of caffeine was not the result of a non-specific inhibition induced by the presence of a high concentration of the methylxanthine.

Caffeine-induced inhibition of the InsP_3 -mediated Ca^{2+} release at two cytosolic $[\text{Ca}^{2+}]$

The InsP_3 receptor is stimulated by cytosolic Ca^{2+} [19–22]. It has been suggested for guinea-pig portal-vein smooth muscle that caffeine will only inhibit that part of the Ca^{2+} -release response that is activated by cytosolic Ca^{2+} [14]. Figure 3 shows the effect of caffeine on the Ca^{2+} release in response to $7 \mu\text{M}$ InsP_3 at a free $[\text{Ca}^{2+}]$ of less than 10 nM and at a free $[\text{Ca}^{2+}]$ of $1 \mu\text{M}$. Raising the $[\text{Ca}^{2+}]$ to $1 \mu\text{M}$ increased the Ca^{2+} release in the absence of caffeine from $71.8 \pm 2.2\%$ to $83.6 \pm 2.0\%$ ($n = 3$) under these conditions, confirming that also the A7r5 InsP_3 receptors are stimulated by cytosolic Ca^{2+} [23]. Caffeine inhibited the InsP_3 -induced Ca^{2+} release at both levels of cytosolic Ca^{2+} . There was a tendency for a decreased effectiveness of caffeine at higher $[\text{Ca}^{2+}]$. The action of caffeine in A7r5 cells was therefore not specific for the Ca^{2+} -stimulated part of the release.

ATP prevents the caffeine-induced inhibition of the InsP_3 receptor

Since caffeine bears a structural resemblance to ATP, and since ATP-binding sites were described on the InsP_3 receptor [24–27], we investigated the effects of ATP on the caffeine-induced inhibition. Figure 4 compares the effect of increasing concentrations of caffeine on the Ca^{2+} release induced by $4 \mu\text{M}$ InsP_3 in efflux medium B containing either no ATP or 5 mM ATP. Raising the caffeine concentration to 50 mM in the absence of ATP resulted in an almost complete inhibition of the Ca^{2+} release. ATP (5 mM) increased the Ca^{2+} release in the presence of $4 \mu\text{M}$ InsP_3 from $61.6 \pm 2.2\%$ to $81.5 \pm 0.8\%$ ($n = 4$). The inhibitory effect of caffeine was largely prevented by 5 mM ATP (Figure 4). Similar results were obtained with 5 mM MgATP, because $4 \mu\text{M}$ InsP_3 still released $59.4 \pm 1.1\%$ of the ionophore-releasable Ca^{2+} in the presence of 50 mM caffeine (compared with $79.7 \pm 0.7\%$ in the absence of caffeine; $n = 3$), indicating that MgATP was also able to decrease the inhibitory effect of caffeine.

DISCUSSION

The major conclusion from this work is that caffeine, either directly or indirectly, inhibits the InsP_3 receptor. This effect seems to require a specific binding site on the InsP_3 receptor or some associated protein, since isocaffeine, which differs from caffeine by only the position of one methyl group, was ineffective. A similar specificity has been found for activation of the ryanodine receptor [17]. The site of interaction of caffeine with the ryanodine receptor has not been resolved as yet [28], and it is therefore impossible to say whether the InsP_3 receptor would have a similar binding domain. It is also possible that caffeine interacts with some associated protein, which then affects the InsP_3 -receptor function. Cyclic ADP-ribose, which is the endogenous ligand of some types of ryanodine receptor [17], was without effect on the InsP_3 -induced Ca^{2+} release in A7r5 cells under these conditions (results not shown). Recently, 9-methyl-7-bromoeudistomin-D has been reported to bind with high affinity to the ryanodine receptor of skeletal-muscle sarcoplasmic reticulum, and this inhibition was competitively antagonized by caffeine [29]. It will be interesting to investigate whether 9-

methyl-7-bromoeudistomin-D would also interfere with InsP_3 -induced Ca^{2+} release.

Caffeine does not affect the binding of InsP_3 to its receptor [10,13]. We have also tested the effect of 50 mM caffeine on the binding of 7 nM [^3H] InsP_3 to A7r5-cell microsomes and failed to observe an inhibitory effect (results not shown). The Hill co-efficient of the dose-response relationship was not affected by caffeine in our A7r5 aortic smooth-muscle cells, confirming the findings of Hirose et al. [14] on portal-vein smooth muscle. In contrast, a caffeine-induced decrease in co-operativity was observed for the rat cerebellar receptor [13].

The InsP_3 receptor is stimulated by cytosolic Ca^{2+} [19–22]. It has been suggested that caffeine will only inhibit that part of the Ca^{2+} -release response that is activated by cytosolic Ca^{2+} [14]. Caffeine did not affect the InsP_3 receptor in A7r5 cells in this way, because caffeine inhibited the release both in the absence of added Ca^{2+} , i.e. at a calculated free [Ca^{2+}] of less than 10 nM, and in the presence of 1 μM Ca^{2+} (Figure 3). We actually observed that the inhibition by caffeine was somewhat less pronounced at higher free [Ca^{2+}]. The reason for this discrepancy is unknown.

ATP-binding sites on the InsP_3 receptor have been described [24–26]. ATP, ADP and AMP competitively inhibited [α - ^{32}P]ATP binding to these sites, whereas GTP was not effective, indicating that ATP probably interacts with the receptor through its adenine moiety [27]. Our finding that both ATP and MgATP prevented the inhibitory effect of caffeine indicates that probably the adenine part of the caffeine molecule will allow caffeine to interact with the ATP-binding site. Since isocaffeine is not effective, we can suppose that a methyl group on N-9 prevents interaction with this site on the receptor. A precise kinetic analysis is difficult, since there are several isoforms of the InsP_3 receptor expressed in A7r5 cells [30]. Moreover, each InsP_3 receptor monomer seems to have more than one ATP-binding site [24–26]. Our data suggest that caffeine may interact with any of these ATP-binding sites. It is conceivable that such interaction could induce a conformational change of the receptor, which then affects the gating properties, resulting in an inhibition of the Ca^{2+} release. An alternative possibility is that caffeine would interact with some associated protein. Our finding that ATP counteracted the inhibitory effect of caffeine might explain why already very low concentrations of caffeine (2 mM) inhibited the InsP_3 receptor in the absence of ATP [14], whereas very high caffeine concentrations (50 mM) did not inhibit [15] or only partially inhibited [13] the release in the presence of 1.5 mM ATP.

Ca^{2+} spiking in non-excitable cells is due to a periodic release of Ca^{2+} from internal stores. The oscillatory release can occur through the InsP_3 receptor [18] or the ryanodine receptor [31–35]. Ca^{2+} spiking through the InsP_3 receptor may depend on a sensitization of the InsP_3 receptor to low doses of InsP_3 [12,18,36,37]. High concentrations of caffeine can inhibit Ca^{2+} spiking also in cells that do not express a ryanodine receptor. Our findings and those of others [8,12–14] indicate that caffeine inhibits the InsP_3 receptor. The relatively high intracellular MgATP concentration (in the millimolar range) would limit the inhibitory effect of caffeine, but some inhibition remains even in

the presence of 5 mM MgATP. We therefore propose that the inhibitory effect of caffeine on intracellular Ca^{2+} signalling may, at least partly, be explained by an inhibitory effect of this methylxanthine on the InsP_3 receptor.

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