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

Ludwig MISSIAEN,* Jan B. PARYS, Humbert DE SMEDT, Bernard HIMPENS and Rik CASTEELS

Laboratorium voor Fysiologie, K. U. Leuven Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium

We have investigated the effect of various methylxanthines on the basal and InsP2-stimulated unidirectional \(^{45}\text{Ca}^{2+}\) efflux from permeabilized A7r5 cells under different experimental conditions. We report that caffeine and theophylline inhibit the InsP2-induced Ca\(^{2+}\) release, whereas the basal Ca\(^{2+}\) leak remained largely unaffected. The effect on InsP2-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 \(^{3}H\)InsP1 binding to the receptor. ATP and MgATP (5 mM) prevented the inhibition, suggesting that caffeine may interact with an ATP-binding site on the InsP2 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 (InsP2)-producing agonists [4–11]. It has been proposed that millimolar concentrations of caffeine inhibit the InsP2 receptor [8,12–14]. However, in other experimental systems, caffeine concentrations up to 20 mM [10] and 50 mM [15] did not affect the InsP2 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 InsP2-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 InsP2-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 InsP2-mediated Ca\(^{2+}\) release and that this inhibition must represent a specific interaction. The inhibition occurs independently of the cytosolic [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. \(10^5\) cells/cm\(^2\). Experiments were carried out with confluent monolayers of cells on day 7 after plating, at an average cell density of \(7.5 \times 10^4\) cells/cm\(^2\).

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 \(\mu\)g/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 Na\(_2\)ATP and 238 nM free Ca\(^{2+}\) (23 \(\mu\)Ci/ml) at 25 °C. The cells 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 Na\(_2\)ATP 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 [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. InsP2 at the indicated concentration or A23187 (10 \(\mu\)M) was added for 2 min between min 4 and 6 of efflux.

InsP2 and ATP were from Boehringer, Mannheim, Germany. Caffeine, saponin, Na\(_2\)ATP, 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 InsP2 (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 \(\mu\)M

* To whom correspondence and reprint requests should be addressed.

Abbreviation used: InsP2, inositol 1,4,5-trisphosphate.
Ruthenium Red, which blocks the ryanodine receptor (results not shown); (3) the extent of inhibition was independent of the cytosolic free [Ca$^{2+}$] in the range 10 nM–10 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$_2$-induced Ca$^{2+}$ release

InsP$_2$ 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$_2$ [16]. We investigated the effect of caffeine on the Ca$^{2+}$ release induced by 3.2 μM InsP$_2$ in efflux medium A, which contained 1 mM ATP, after loading the stores to steady state with $^{40}$Ca$^{2+}$ (Figure 2a). Ca$^{2+}$ release induced by this sub-maximal InsP$_2$ 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 μM cyclic AMP (results not shown). The inhibitory effect of caffeine was observed over a wide range of InsP$_2$ concentrations (Figure 2b). Caffeine had no significant effect on the Hill coefficient of the InsP$_2$ 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$_2$ 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$_2$-induced Ca$^{2+}$ release increased 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$_2$-sensitive than those of passage 15: the EC$_{50}$ shifted from 1.4 μM InsP$_2$ (passage 15) to 13.1 μM InsP$_2$ (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$_2$ receptor expressed in these cells. We have observed by reverse-transcriptase polymerase chain reaction a slight increase in the relative amount of the type 1 receptor as compared with the other isoforms (results not shown).

Caffeine-induced inhibition of the InsP$_2$-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-
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 InsP3-mediated Ca2+ release at two cytosolic [Ca2+]**

The InsP3 receptor is stimulated by cytosolic Ca2+ [19–22]. It has been suggested for guinea-pig portal-vein smooth muscle that caffeine will only inhibit that part of the Ca2+-release response that is activated by cytosolic Ca2+ [14]. Figure 3 shows the effect of caffeine on the Ca2+ release in response to 7 μM InsP3 at a free [Ca2+] of less than 10 mM and at a free [Ca2+] of 1 μM. Raising the [Ca2+] from 71.8 ± 2.2% to 83.6 ± 2.0% (n = 3) under these conditions, confirming that also the A7r5 InsP3 receptors are stimulated by cytosolic Ca2+ [23]. Caffeine inhibited the InsP3-induced Ca2+ release at both levels of cytosolic Ca2+. There was a tendency for a decreased effectiveness of caffeine at higher [Ca2+]. The action of caffeine in A7r5 cells was therefore not specific for the Ca2+-stimulated part of the release.

**ATP prevents the caffeine-induced inhibition of the InsP3 receptor**

Since caffeine bears a structural resemblance to ATP, and since ATP-binding sites were described on the InsP3 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 Ca2+ release induced by 4 μM InsP3 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 Ca2+ release. ATP (5 mM) increased the Ca2+ release in the presence of 4 μM InsP3 from 61.6 ± 2.2% to 81.5 ± 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 μM InsP3 still released 59.4 ± 1.1% of the ionophore-releasable Ca2+ in the presence of 50 mM caffeine (compared with 79.7 ± 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 InsP3 receptor. This effect seems to require a specific binding site on the InsP3 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 InsP3 receptor would have a similar binding domain. It is also possible that caffeine interacts with some associated protein, which then affects the InsP3-receptor function. Cyclic ADP-ribose, which is the endogenous ligand of some types of ryanodine receptor [17], was without effect on the InsP3-induced Ca2+ release in A7r5 cells under these conditions (results not shown). Recently, 9-methyl-7-bromo-9-deoxyadenosin-β-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$_2$-induced Ca$^{2+}$ release.

Caffeine does not affect the binding of InsP$_2$ to its receptor [10,13]. We have also tested the effect of 50 mM caffeine on the binding of 7 nM [H]InsP$_2$ to A7r5-cell microsomes and failed to observe an inhibitory effect (results not shown). The Hill coefficient 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$_2$ 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$_2$ 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$_2$ receptor have been described [24–26]. ATP, ADP and AMP competitively inhibited [α-32P]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$_2$ receptor expressed in A7r5 cells [30]. Moreover, each InsP$_2$ 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$_2$ 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$_2$ receptor [18] or the ryanodine receptor [31–35]. Ca$^{2+}$ spiking through the InsP$_2$ receptor may depend on a sensitization of the InsP$_2$ receptor to low doses of InsP$_2$ [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$_2$ 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$_2$ receptor.

J.B.P. is Senior Research Assistant of the Belgian National Fund for Scientific Research, N.F.W.O.

REFERENCES

Received 15 October 1993/31 December 1993; accepted 14 January 1994