

RESEARCH COMMUNICATION

The opening of the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} channel in rat cerebellum is inhibited by caffeine

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Ins(1,4,5) P_3 (Ins P_3)-induced Ca^{2+} release and [^3H]Ins P_3 binding were measured in rat cerebellar microsomes in the presence or absence of caffeine. The quantal Ca^{2+} release was shown to occur in an apparently co-operative fashion with a Hill coefficient (h) of 2.2. Half-maximal Ca^{2+} release was observed at 900 nM-Ins P_3 . Addition of caffeine caused changes both to the concentration of Ins P_3 required to cause half-maximal Ca^{2+} release (3.9 μM at 50 mM-caffeine) and to the apparent co-operativity ($h = 1.0$ at 50 mM-caffeine). Under standard conditions for [^3H]Ins P_3 binding, caffeine had no effect, and it had no effect on Ins P_3 metabolism. Cyclic AMP also had no effect on the quantal release induced by Ins P_3 . These results are consistent with the view that caffeine affects the opening (Ca^{2+} release) events rather than the ligand-binding events in the operation of the Ins P_3 -sensitive Ca^{2+} channel.

INTRODUCTION

Ins(1,4,5) P_3 (Ins P_3) is a second messenger responsible for the release of Ca^{2+} from intracellular Ca^{2+} stores after receptor activation [1]. The Ca^{2+} channel, which is sensitive to Ins P_3 , releases Ca^{2+} in a 'quantal' manner, with the amount of Ca^{2+} released dependent on Ins P_3 concentration [2]. This phenomenon may be due to regulation of the gating of this channel by changes in the intraluminal Ca^{2+} concentration [3] or due to heterogeneous populations of microsomes having different sensitivities to Ins P_3 and releasing their Ca^{2+} in an 'all-or-none' fashion [4].

Muscle contains a caffeine-sensitive Ca^{2+} channel that is distinct from the Ins P_3 -sensitive Ca^{2+} channel, which binds ryanodine with high affinity [5] and opens in the presence of millimolar concentrations of caffeine and micromolar concentrations of Ca^{2+} [6]. The use of antibodies raised against this caffeine-sensitive Ca^{2+} channel had led to the identification of a similar protein in non-muscle cells [7]. Caffeine also mobilizes Ca^{2+} in a variety of other non-muscle cells, including permeabilized and intact adrenal chromaffin cells [8,9]. This had led to the conclusion that a caffeine-sensitive Ca^{2+} channel exists in these cells, and recently these channels have been implicated in the propagation of Ca^{2+} waves observed in single cells that are stimulated by agonists which are coupled to Ins P_3 production [10].

A recent observation by Parker & Ivorra [11] has shown caffeine to be an inhibitor of Ins P_3 -mediated Ca^{2+} release in *Xenopus* oocytes. Here we report the effects of caffeine on Ins P_3 -induced Ca^{2+} release and [^3H]Ins P_3 binding using rat cerebellar microsomes.

METHODS

Fluo-3 was obtained from Molecular Probes, Ins P_3 from Calbiochem, [^3H]Ins P_3 from Amersham International, and caffeine from Sigma; all other reagents were of analytical grade. The rat cerebellar microsomes were prepared as described in [12].

 Ca^{2+} uptake and release

Ca^{2+} uptake and release were measured using fluo-3 as described elsewhere [13,14]. Rat cerebellar microsomes were suspended in a buffer containing 10 mM-potassium phosphate, 3.5 mM-potassium pyrophosphate, 250 nM-fluo-3, creatine kinase (10 $\mu\text{g}/\text{ml}$) and 10 mM-phosphocreatine, pH 7.2, and measurements were carried out at 37 °C. Typically, 0.2–0.3 mg of rat cerebellar microsomes/ml were added to a continuously stirred cuvette. Ca^{2+} uptake was initiated by the addition of 1.5 mM-MgATP, and the fluorescence change of fluo-3 was monitored in a Perkin-Elmer LS-50 fluorimeter, excitation being at 506 nm and detection of the fluorescence emission being at 526 nm. After ATP-dependent Ca^{2+} loading, further Ca^{2+} uptake was inhibited by the addition of 0.5 mM-sodium orthovanadate, which was prepared in alkaline solution and thus in an oligovanadate form that would not block Ins P_3 binding to the Ins P_3 -sensitive Ca^{2+} channel [27,28]. The absence of decavanadate in the orthovanadate solution was confirmed by the lack of any effect on [^3H]Ins P_3 binding, in either the presence or absence of caffeine. Ins P_3 was added, and the maximum amount of Ca^{2+} release for each [Ins P_3] monitored. Total Ca^{2+} accumulated within the microsomes was measured by permeabilization with 12.5 μg of ionophore A23187/ml.

Fluorescence intensity was related to [Ca^{2+}] by using the following equation [15]:

$$[\text{Ca}^{2+}] = K_d \times (F - F_{\text{min.}}) / (F_{\text{max.}} - F)$$

where K_d is the dissociation constant for Ca^{2+} binding to fluo-3 (900 nM at 37 °C, pH 7.2 [14]), F is the fluorescence intensity of the sample and $F_{\text{min.}}$ and $F_{\text{max.}}$ are the fluorescence intensities of the sample in 1 mM-EGTA and 2.5 mM- CaCl_2 , respectively. Caffeine was preincubated with the microsomes for several minutes before ATP-dependent Ca^{2+} uptake was measured.

[^3H]Ins P_3 binding

The effects of caffeine on Ins P_3 binding were assessed by displacement of [^3H]Ins P_3 with between 40 nM and 3 μM non-

Abbreviations used: Ins P_3 , Ins(1,4,5) P_3 ; Ins P_2 , Ins(1,4) P_2 .

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radioactive InsP_3 . The binding procedure was similar to that described by Alderson & Volpe [16]. Briefly, 0.2 mg of rat cerebellum microsomes were suspended in a medium containing 50 mM-Tris/HCl, pH 8.3, 1 mM-EDTA and 100 mM-KCl in a final volume of 0.5 ml. Total binding was measured at 40 nM- $[\text{H}]\text{InsP}_3$, and non-specific binding was measured in the presence of 10 μM non-radioactive InsP_3 . After a 10 min incubation on ice, the samples were centrifuged for 15 min at 15000 g and the pellet washed in distilled water. After drying, the pellets were solubilized in 0.25 ml of 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS and 62.5 mM-Tris/HCl, pH 6.8, and sonicated. The radioactivity of the samples was determined by liquid-scintillation spectrometry.

Metabolism of InsP_3

In membrane preparations, in the absence of cytosol, the most likely route of metabolism of InsP_3 is to $\text{Ins}(1,4)\text{P}_2$ (InsP_2) by the InsP_3 5-phosphatase. In order to assess the metabolism of InsP_3 by cerebellar microsomes, samples were preincubated for 20 min at 37 °C in conditions identical with those used for Ca^{2+} uptake and release, including the addition of 0.5 mM-sodium orthovanadate. Either radiochemical amounts of $[\text{H}]\text{InsP}_3$ or concentrations of up to 10 μM - InsP_3 doped with 0.003 μCi of $[\text{H}]\text{InsP}_3$ were added 2 min after the vanadate. The reaction was terminated after 30 s with 200 μl of ice-cold 10% (w/v) HClO_4 , neutralized with KOH, and products were separated on gravity-fed anion-exchange columns as described in [17]. The amounts of $[\text{H}]\text{InsP}_3$ metabolized and $[\text{H}]\text{InsP}_2$ formed were determined. To assess 5-phosphatase activity under simulated 'intracellular' conditions, cerebellar microsomes were similarly incubated in 102 mM-KCl, 10 mM-NaCl, 0.1 mM-EGTA, 5 mM-MgSO₄, 50 mM-Hepes, pH 7.2, and saponin (0.2 mg/ml) as described in [18].

RESULTS

Fig. 1 shows ATP-dependent Ca^{2+} uptake into, and InsP_3 -induced Ca^{2+} release from, rat cerebellar microsomes. Upon addition of ATP, a rapid decrease in $[\text{Ca}^{2+}]$ is observed as Ca^{2+} becomes complexed with ATP. This was confirmed through experiments where ATP was added to samples containing fluo-3 but devoid of microsomes (see [14]). The Ca^{2+} -uptake rate was 1.6 nmol/min per mg of cerebellar microsomal protein. Addition of 0.5 mM-sodium orthovanadate, which causes > 80% inhi-

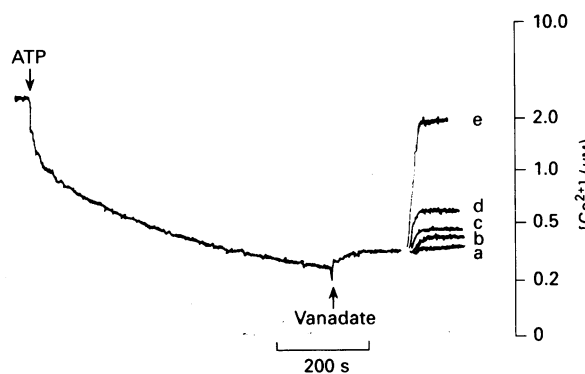


Fig. 1. ATP-dependent Ca^{2+} uptake and InsP_3 -induced Ca^{2+} release from rat cerebellar microsomes monitored using fluo-3

MgATP (1.5 mM) was added to initiate Ca^{2+} uptake, which was inhibited by the addition of 0.5 mM-sodium vanadate. The Ca^{2+} release was measured at different InsP_3 concentrations: 0.1 (trace a), 0.3 (trace b), 0.7 (trace c) or 10 (trace d) μM . Total Ca^{2+} release occurred with the addition of A23187 ionophore (12.5 $\mu\text{g}/\text{ml}$) (trace e).

tion of the Ca^{2+} pump [12], caused some release of Ca^{2+} due to passive leakage of the microsomes. Once a new steady state was established, where passive Ca^{2+} efflux was equal to Ca^{2+} influx, the monitored external $[\text{Ca}^{2+}]$ remained steady throughout the remainder of the experiment. Orthovanadate was added so that Ca^{2+} uptake did not occur after InsP_3 -induced Ca^{2+} release. Addition of InsP_3 caused Ca^{2+} release from the cerebellar microsomes, the amount of release being dependent on the concentration of InsP_3 added. The addition of ionophore A23187 caused complete release of the accumulated Ca^{2+} . At maximally effective InsP_3 concentrations ($\geq 20 \mu\text{M}$), only about 20% of the total accumulated Ca^{2+} was released. This was deemed to be due to release of Ca^{2+} through the InsP_3 -sensitive Ca^{2+} channel, as it was inhibited by heparin (results not shown).

Half-maximal InsP_3 -induced Ca^{2+} release occurred at 900 nM- InsP_3 (Fig. 2a). When these data are re-expressed as a Hill plot (Fig. 2b), they give an apparent h value of 2.2, similar to values previously measured in rat and canine cerebellar microsomes [14,19].

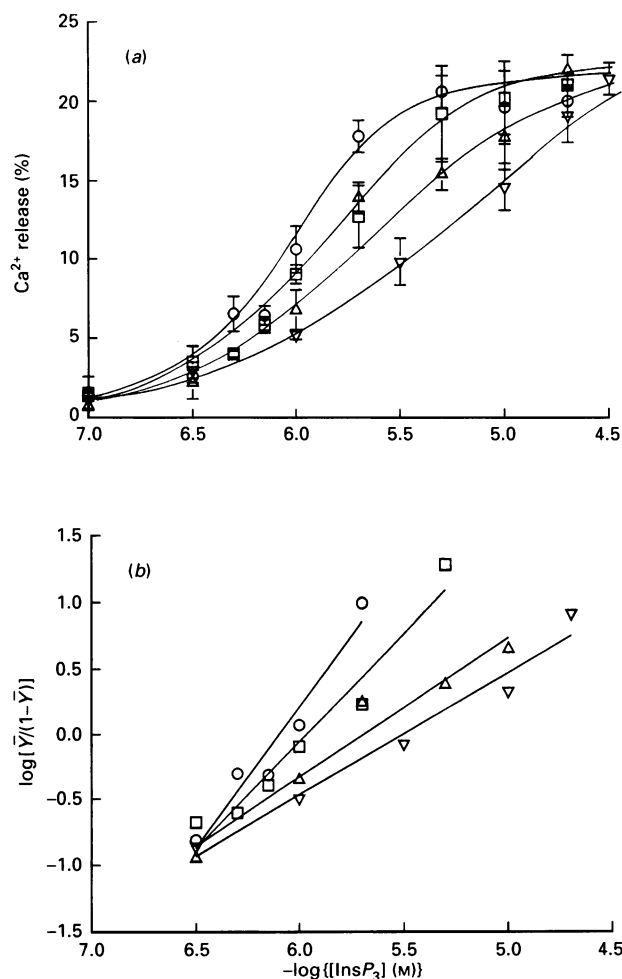


Fig. 2. Inhibition by caffeine of InsP_3 -induced quantal Ca^{2+} release from cerebellar microsomes

(a) The amounts of Ca^{2+} released as a function of InsP_3 concentrations expressed as a percentage of the Ca^{2+} released by the addition of ionophore A23187. Samples were either preincubated with no caffeine (○) or in the presence of 10 mM-caffeine (□), 25 mM-caffeine (△) or 50 mM-caffeine (▽). Each data point is the mean \pm S.D. of at least three determinations. (b) The data from (a) re-expressed as a Hill plot, where \bar{Y} is the fractional response (amount of Ca^{2+} released/maximal amount of Ca^{2+} released by 20 μM - InsP_3).

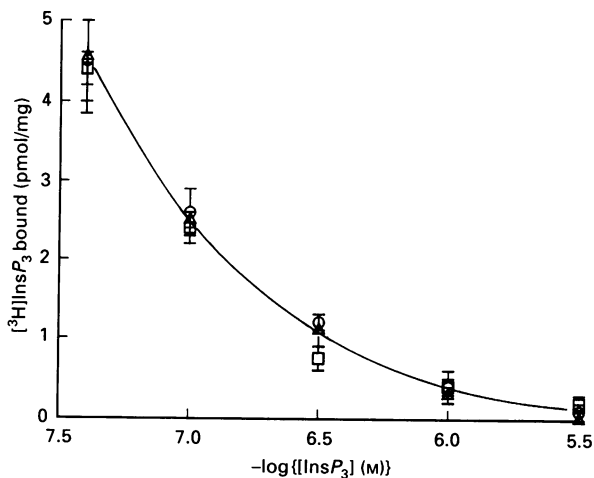


Fig. 3. Lack of any effect of caffeine on $[^3\text{H}]\text{InsP}_3$ binding

Displacement of $[^3\text{H}]\text{InsP}_3$ was achieved with between 40 nM and 3 μM non-radioactive InsP_3 . This was performed in the absence of caffeine (○) or in the presence of 10 mM-caffeine (□) and 25 mM-caffeine (△). Each data point is the mean \pm S.D. of at least three determinations.

Caffeine caused some quenching of the fluo-3 fluorescence, but did not affect the binding of Ca^{2+} to fluo-3. No caffeine-induced Ca^{2+} release was observed when caffeine was added to microsomes which had previously been loaded with Ca^{2+} .

Fig 2(a) shows the effect of caffeine on InsP_3 -induced Ca^{2+} release. It can be seen that addition of increasing amounts of caffeine reduced the amount of Ca^{2+} released by submaximal concentrations of InsP_3 . However, this effect was overcome by raising the concentration of InsP_3 . Half-maximal release of Ca^{2+} occurred at 900 nM- InsP_3 in the absence of caffeine, but at 3.9 μM - InsP_3 in the presence of 50 mM-caffeine. At concentrations of caffeine commonly used to elicit caffeine-induced Ca^{2+} release in muscle and non-muscle cells (i.e. 10 and 25 mM), half-maximal Ca^{2+} release occurred at 1.4 and 1.9 μM - InsP_3 respectively. When the Ca^{2+} -release curves are expressed as Hill plots (Fig. 2b), it can be seen that the h value for Ca^{2+} release also changes from 2.2 in the absence of caffeine to 1.0 in the presence of 50 mM-caffeine. The inhibition by caffeine of InsP_3 -induced Ca^{2+} release was also observed when Ca^{2+} uptake was partially inhibited by 50 nM-thapsigargin rather than orthovanadate, demonstrating that it was an effect of caffeine on the InsP_3 -sensitive Ca^{2+} channel rather than secondary to an effect of orthovanadate.

Fig. 3 shows that concentrations of up to 25 mM-caffeine had no effect on InsP_3 binding to cerebellar microsomes. Higher concentrations of caffeine precipitated out of the sample under our experimental conditions, and therefore binding studies could not be carried out at 50 mM-caffeine.

Under the experimental conditions used for measuring Ca^{2+} uptake and release, between $6 \pm 2\%$ of the InsP_3 was metabolized during 30 s, the period needed for the amount of Ca^{2+} released by InsP_3 to approach its maximum level. This metabolism was unaffected by the presence of caffeine. However, under simulated 'intracellular' conditions, 73% of the InsP_3 was metabolized by the same microsome preparations over this time period (results not shown). It has previously been reported that ATP, pyrophosphate and vanadate, all of which are present in the medium during Ca^{2+} flux measurements, inhibit the metabolism of the InsP_3 by the 5-phosphatase [18,20] (P. Hughes, personal communication).

DISCUSSION

Fluo-3 is an ideal monitor for measuring Ca^{2+} fluxes in muscle and non-muscle microsomes [13,14]. This method is also ideal for measuring both the rate and amount of Ca^{2+} released from microsomes after the addition of InsP_3 . Because the maximal amount of Ca^{2+} release is dependent on the added InsP_3 concentration, this process is referred to as quantal calcium release [2]. Here we have shown that InsP_3 -stimulated quantal Ca^{2+} release is co-operative in nature ($h = 2.2$). It is not known why InsP_3 -induced Ca^{2+} release is both quantal and co-operative, particularly as the kinetics of $[^3\text{H}]\text{InsP}_3$ binding do not show similar co-operativity [21]. Since the InsP_3 -sensitive Ca^{2+} channel is believed to exist as a tetramer in the membrane [22], one possible explanation is that the opening of one channel in the tetramer may somehow facilitate the opening of adjacent channels.

The quantal release of Ca^{2+} induced by InsP_3 is inhibited by caffeine, such that higher concentrations of InsP_3 become necessary in order to cause the release of comparable amounts of Ca^{2+} . The maximal amount of Ca^{2+} accessible to release by InsP_3 remains unchanged (about 22% of that sequestered by cerebellar microsomes). This might initially suggest competitive binding of caffeine at the InsP_3 -binding site. However, the apparent co-operativity of Ca^{2+} release is dramatically changed by caffeine, whereas $[^3\text{H}]\text{InsP}_3$ binding is unaffected, so such an explanation is extremely unlikely. Moreover, the effect of caffeine on InsP_3 -induced Ca^{2+} release is not due to changes in InsP_3 metabolism. It is also unlikely to be due to caffeine-mediated changes in cyclic AMP concentrations, since, in agreement with other studies [11,23], we found that addition of 10 μM cyclic AMP had no effect on the InsP_3 -induced quantal Ca^{2+} release (results not shown). Whatever the mechanism of the inhibition of InsP_3 -induced Ca^{2+} release by caffeine, its occurrence means that extra caution must be exercised in the interpretation of the effects of caffeine on intracellular Ca^{2+} .

At present, two models have been proposed to account for the phenomenon of quantal Ca^{2+} release. One model postulates the existence of an intraluminal Ca^{2+} -binding site on the receptor, which regulates the opening of the InsP_3 -sensitive Ca^{2+} channel [3]. The other model suggests that Ca^{2+} is released in an 'all-or-nothing' manner, with different InsP_3 -sensitive Ca^{2+} stores having channels with different sensitivities to InsP_3 [4]. In the present study we have shown that quantal Ca^{2+} release is normally co-operative, becoming non-co-operative upon the addition of caffeine. It is difficult to explain such changes in co-operativity solely in terms of distinct Ca^{2+} stores having different sensitivities to InsP_3 . These results can be explained more readily by the intraluminal Ca^{2+} -regulation model, with caffeine possibly affecting the regulation by intraluminal Ca^{2+} . However, the two models are not mutually exclusive, since it may be envisaged that a heterogeneous population of Ca^{2+} stores may exist with different sensitivities to InsP_3 , but which are also regulated by intraluminal Ca^{2+} concentrations. An analogy can be made to the ryanodine receptor in muscle, which shows a high sequence similarity to the InsP_3 -sensitive Ca^{2+} channel [24]. This Ca^{2+} channel is also controlled by the intra- and extra-luminal Ca^{2+} concentrations [25], with caffeine modulating the effects of Ca^{2+} ; in this case, it enhances Ca^{2+} release [26].

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