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Caffeine inhibits Ca^{2+} uptake by subplasmalemmal calcium stores ('alveolar sacs') isolated from *Paramecium* cells

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Abstract

Caffeine inhibits $^{45}Ca^{2+}$ sequestration by subplasmalemmal calcium stores ('alveolar sacs') of low thapsigargicin sensitivity which we have isolated from the ciliated protozoan, *Paramecium tetraurelia*. Inhibition depends on caffeine concentration, with an IC_{50} of 31.8 mM. According to kinetic evaluation this is compatible with non-competitive inhibition of Ca^{2+} uptake, rather than with superimposed $^{45}Ca^{2+}$ release during sequestration. It remains to be analysed whether this mechanism might be of possible relevance also for Ca^{2+}-mediated activation in vivo in this or in any other secretory system. Such an effect could also operate indirectly, e.g., by Ca^{2+}-release induction via sequestration inhibition. This is the first description of caffeine-mediated inhibition of Ca^{2+} uptake by calcium stores from a secretory system. Our data are compatible with some observations with sarcoplasmic reticulum from striated muscle fibers.

Keywords: ATPase, Ca^{2+}-; Caffeine; Calcium ion uptake; Calcium store; Secretion; SERCA; (*Paramecium*)

1. Introduction

Caffeine (trimethylxanthine) is frequently used to induce exocytosis in a variety of cell types by mobilizing Ca^{2+} from intracellular stores [1–3]. Caffeine activated stores are frequently, though not always sensitive also to ryanodine or vice versa [3–5], but almost always they are insensitive to inositol 1,4,5-trisphosphate ($InsP_3$) [1,3]. The activation mechanism of caffeine is known to mimic Ca^{2+}-induced Ca^{2+} release, CICR. This mechanism normally involves a precedent increase of free intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, by mobilization from other sources [3]. Similarly caffeine is known to activate Ca^{2+} release from skeletal muscle SR [2,6] where it operates via the ryanodine receptor coupled to the voltage sensor in the cell membrane [7]. While a $[Ca^{2+}]_i$ increase does not precede activation of SR in skeletal muscle in vivo [6], CICR or caffeine can trigger Ca^{2+} release from isolated SR in vitro [6]. In secretory cells, CICR or caffeine can trigger exocytosis, even when a voltage sensor-coupled ryanodine receptor does not occur.

Altogether the mechanism of caffeine-induced Ca^{2+} release, therefore, is not well understood, particularly in secretory cells. Also quite remarkable are the high concentrations of caffeine required, up to 50 mM or even more, to achieve full activation of Ca^{2+} release and of secretion [8]. Intriguingly the Ca^{2+} release mechanism may depend on the concentration of Ca^{2+} and caffeine may exert different effects depending on the concentration applied [9]. Beyond this, caffeine is known to exert some other, widely different effects unrelated to Ca^{2+} release (see Section 4).

We have isolated 'alveolar sacs', the vast subplasmalemmal Ca^{2+} stores from *Paramecium* cells [10,11] for functional characterization [12]. We have evidence that alveolar sacs provide Ca^{2+} for exocytosis of trichocysts in *Paramecium* [11–13]. We have also previously found that Ca^{2+} release cannot be induced by any of the established second messengers, including $InsP_3$ and cADPR [12]. Alveolar sacs possess a non-calmodulin stimulated Ca^{2+}-pump [10]. Inhibitors of SR-ER-type Ca^{2+}-ATPases (SERCA) of intracellular calcium stores [14] cause reduced uptake of $^{45}Ca^{2+}$ by isolated alveolar sacs in vitro and they trigger some trichocyst exocytosis in vivo, as does caffeine [12]. We now show that $^{45}Ca^{2+}$ uptake is inhib-

Abbreviations: ATP, adenosine trisphosphate; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CICR, Ca^{2+}-induced Ca^{2+}-release; $InsP_3$, inositol 1,4,5-trisphosphate; SERCA, sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TG, thapsigargicin.

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ited in vitro not only by the SERCA inhibitor thapsigargicin (the less hydrophilic analogue of thapsigargin used in this study), though at relatively high concentrations, but also by caffeine – an aspect not previously recognized with any secretory system. This may be of possible importance also for other secretory cells.

2. Materials and methods

We have isolated alveolar sac fractions from *P. tetraurelia* wild-type (7S) cells according to Stelly et al. [10], in presence of protease inhibitors, as modified by Länge et al. [12]. (See this reference also for detailed indications on methods, including protein and $[Ca^{2+}]$ determinations, and for sources of chemicals.) All chemicals were of the highest purity available. Thapsigargicin was from Calbiochem (San Diego, CA), caffeine from Sigma (Deisenhofen, Germany). $^{45}CaCl_2$ (Amersham Buchler, Braunschweig, Germany) was used as an aqueous solution (38.9 MBq/μmol).

Alveolar sac fractions were usually analysed in 20 mM Tris/maleate buffer pH 7.2, 250 mM sucrose, 5 mM $MgCl_2$, 5 U creatine phosphokinase (EC 2.7.3.2), 5 mM phosphocreatine and 1 mM Na_2ATP. After 10 min preincubation at 27°C we added 20 μM $^{45}CaCl_2$ ($2 \cdot 10^5$ Bq/μmol) to start $^{45}Ca^{2+}$ uptake measurements over 10 min. $[Ca^{2+}]$ was kept at 20 μM by control with a Ca^{2+}-selective electrode [12]. In some experiments (Fig. 1) the sequence of adding ATP and Ca^{2+} was reversed and occasionally ATP was omitted. ATP concentrations were eventually modified in experiments for Michaelis-Menten kinetics. Usually experiments were carried out with freshly isolated fractions (~ 100 μg of protein in an assay volume of 500 μl). An exception were experiments on 'ageing' of these fractions. $^{45}Ca^{2+}$ retained in isolated fractions was determined, after washing with an excess of ice-cold washing solution (20 mM Tris/maleate pH 7.2, 250 mM sucrose, 40 mM NaCl) on Whatman GF/C glass microfibre filters, in a liquid scintillation counter. With some aliquots $^{45}Ca^{2+}$ sequestration was ascertained by adding ionophore A23187. Alternatively adsorbed, i.e., non-sequestered $^{45}Ca^{2+}$ was determined by experiments without ATP supply. The background values thus determined were below 10% of the actual total $^{45}Ca^{2+}$ content of samples after washing. This background was subtracted. For determination of K_M and V_{max} values we used the Enzfitter computer program (Elsevier Biosoft). For analysis of statistical significance we performed a Student's *t*-test.

3. Results

We found that $^{45}Ca^{2+}$ uptake by isolated alveolar sac fractions is reduced by ~60% within 24 h (0°C) after isolation (Table 1). Therefore, freshly isolated fractions were used throughout this work. We tried to rule out any influence of variable purity of fractions or of partial ageing on our kinetic analyses by running in parallel both, experiments and controls.

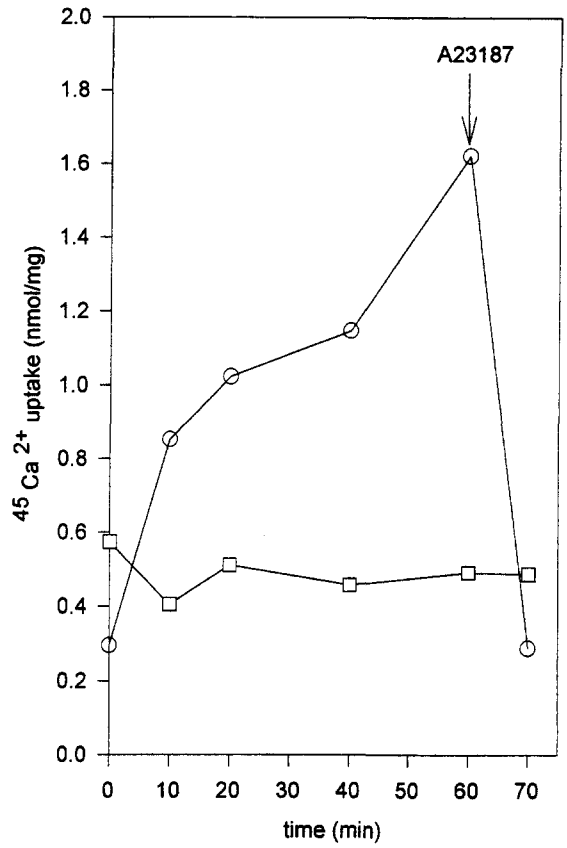

Fig. 1. ATP-dependent $^{45}Ca^{2+}$ sequestration shown in a typical experiment. Alveolar sac fractions were supplied with the ATP regenerating system 10 min after adding $^{45}Ca^{2+}$ (upper curve, circles). Ionophore A23187 added after 60 min releases $^{45}Ca^{2+}$ after sequestration. [ATP] = 1.5 mM, free $[Ca^{2+}] = 10$ μM. The lower curve (squares) was obtained in presence of $^{45}Ca^{2+}$ but in absence of ATP.

To guarantee that isolated alveolar sacs sequester $^{45}Ca^{2+}$ by an ATP-dependent mechanism, i.e., by a Ca^{2+}-ATPase type pump, we recorded in Fig. 1 the absence of $^{45}Ca^{2+}$ uptake in the absence of ATP (lower curve), the immediate beginning of $^{45}Ca^{2+}$ uptake upon addition of ATP (upper

Table 1
Some characteristics of $^{45}Ca^{2+}$ uptake by alveolar sacs

Ca^{2+}	
K_M	5.8 μM
V_{max}	0.33 nmol/min/mg protein
ATP	
K_M	13.2 μM
V_{max}	0.15 nmol/min/mg protein
Caffeine	
IC_{50}	31.8 mM
'Ageing'	
Residual activity after 24 h (0°C)	40.5%

curve) and $^{45}Ca^{2+}$ release by ionophore A23187. In this experiment fractions were preincubated with $^{45}Ca^{2+}$ before activating the ATP regenerating system, opposite to experiments described below (see also Section 2). Nevertheless, the time course of $^{45}Ca^{2+}$ sequestration was quite similar when Fig. 1 is compared, e.g., with Figs. 2 and 3 (10 min ATP preincubation → $^{45}Ca^{2+}$ supply).

From Lineweaver-Burk diagrams we calculated for Ca^{2+} a $K_M = 5.8$ μM and a $V_{max} = 0.33$ nmol/min/mg protein (data not shown). [Ca^{2+}] was kept constant at 20 μM (see Section 2). Figs. 2 and 3 are typical experiments documenting increasing inhibition of $^{45}Ca^{2+}$ uptake by increasing concentrations of either thapsigargicin or caffeine. Subsequent release by the Ca^{2+}-ionophore A23187 makes $^{45}Ca^{2+}$ leakage during uptake an unlikely explanation. Fig. 4 represents the dependency of $^{45}Ca^{2+}$ uptake on ATP concentrations. Kinetic values for ATP dependency, as derived from Lineweaver-Burk plots, are $K_M = 13.2$ μM and $V_{max} = 0.15$ nmol/min/mg protein for controls without caffeine (Fig. 4). According to Figs. 3 and 4, $^{45}Ca^{2+}$ sequestration is increasingly reduced by 10 mM and by 20 mM caffeine. In Fig. 4 values of V_{max} are

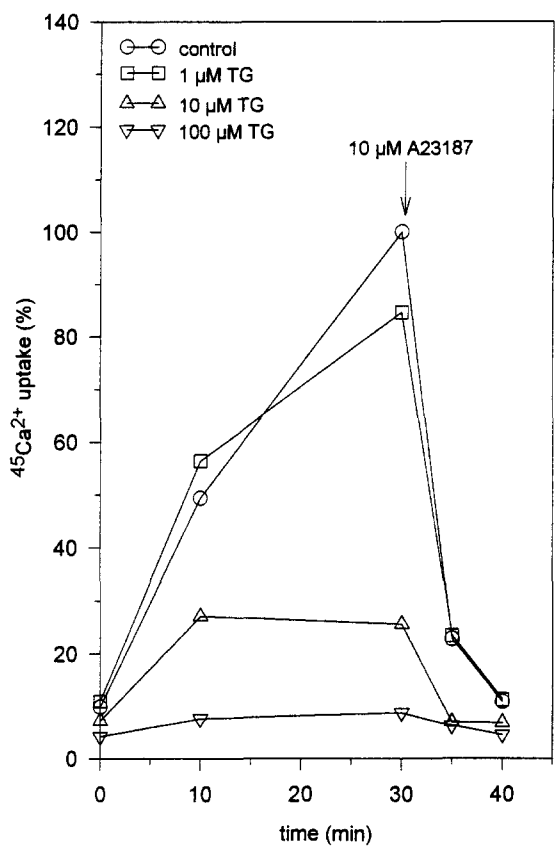

Fig. 2. Typical experiment showing inhibition of $^{45}Ca^{2+}$ uptake by thapsigargicin (TG). [ATP] = 1.5 mM, free [Ca^{2+}] = 10 μM. Alveolar sacs were preincubated for 10 min with the ATP regenerating system before adding $^{45}Ca^{2+}$. Data are normalized to 100% for maximal sequestration; for absolute values, see Fig. 4. Sequestered $^{45}Ca^{2+}$ is released by the Ca^{2+}-ionophore A23187.

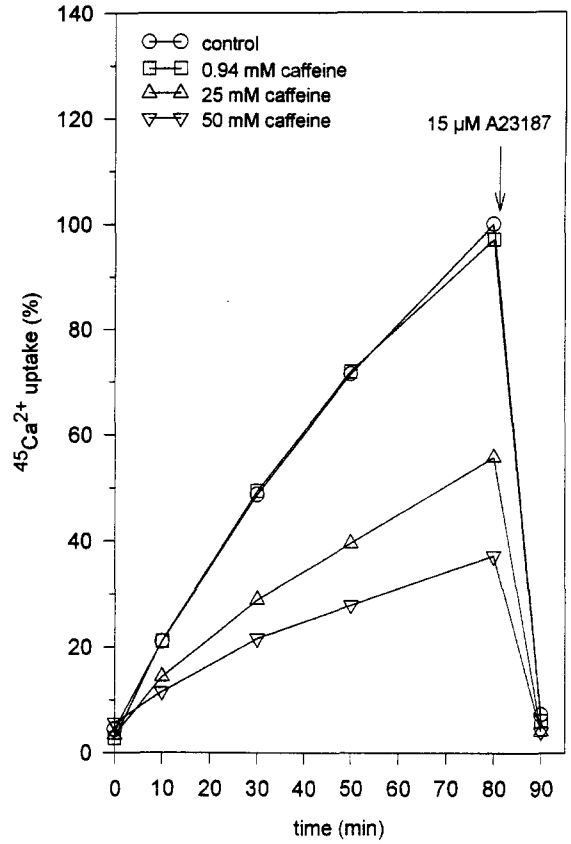

Fig. 3. Typical experiment showing inhibition of $^{45}Ca^{2+}$ uptake by caffeine. [ATP] = 1.5 mM, free [Ca^{2+}] = 10 μM. Alveolar sacs were preincubated for 10 min with the ATP regenerating system before adding $^{45}Ca^{2+}$. Data are normalized to 100% for maximal sequestration; for absolute values, see Fig. 4. Sequestered $^{45}Ca^{2+}$ is released by the Ca^{2+}-ionophore A23187.

reduced from 0.15 nmol/min/mg protein (controls) to 0.11 (10 mM caffeine) and 0.10 nmol/min/mg protein (20 mM caffeine). Statistical analysis of Fig. 4 revealed that these values are significantly different from controls on a level of $P < 0.05$. K_M values for ATP derived from Lineweaver-Burk diagrams are 13.2 μM for samples without caffeine, 13.0 with 10 mM caffeine and 12.1 μM for 20 mM caffeine. Statistical analysis showed these values of K_M not to be significantly different ($P > 0.5$). This formally indicates non-competitive inhibition of Ca^{2+} uptake. Essential data are summarized in Table 1.

We also varied caffeine concentrations in presence of two widely different ATP concentrations, 0.1 and 1.5 mM (Fig. 5). With both ATP concentrations tested, $^{45}Ca^{2+}$ uptake without caffeine was normalized to 100% in Fig. 5. Absolute values for Ca^{2+} sequestered were 0.13 and 0.80 nMol Ca^{2+}/min/mg protein, using 0.1 and 1.5 mM ATP, respectively. The curves in Fig. 5 decline by identical coefficients (without any statistically significant difference) as caffeine concentrations are increased. This again supports non-competitive inhibition of $^{45}Ca^{2+}$ uptake by caffeine. It also makes it unlikely that the effect of caf-

feine, in our case, would be due to latent $^{45}Ca^{2+}$ release (diffusion) from the store during uptake since release would depend on the filling state, i.e., on ATP-concentration dependent pump activation. This conclusion is supported by the following data. In the experiments presented in Fig. 5 $^{45}Ca^{2+}$ uptake occurred for 10 min in presence of caffeine. The amount of $^{45}Ca^{2+}$ sequestration achieved in Fig. 5 is about 5-times less than the amount we could release in previous work [12] by adding caffeine only after $^{45}Ca^{2+}$ sequestration was completed (also within 10 min and also using 1.5 mM ATP). For all these reasons we conclude that, with isolated alveolar sacs, caffeine causes inhibition of $^{45}Ca^{2+}$ uptake, probably in a non-competitive mode, rather than any substantial release of $^{45}Ca^{2+}$. Nevertheless, in situ, the releasing effect may prevail (see Section 4).

We determined the concentration of caffeine required for 50% inhibition of $^{45}Ca^{2+}$ uptake as $IC_{50} = 31.8$ mM (Table 1). For TG, an $IC_{50} \sim 5$ μM and an $IC_{100} \sim 20$ μM can be extrapolated from experiments of the type shown in Fig. 2. Extrapolation to full inhibition would result in values well beyond 50 μM, as required for full activation of calcium stores in some other secretory systems [8].

4. Discussion

We interpret the data obtained with our in vitro system, the subplasmalemmal calcium stores isolated from *Paramecium* cells, as inhibition of Ca^{2+} uptake by caffeine. Our data are formally compatible with non-competitive inhibition of Ca^{2+} uptake. It has previously been shown that a SERCA-type Ca^{2+}-pump occurs in alveolar sacs [10,12]. We also have shown that, in vitro, the release of sequestered $^{45}Ca^{2+}$ achieved with caffeine is as slow as with SERCA inhibitors, whereas in vivo caffeine provokes rapid trichocyst exocytosis [12] and rapid $[Ca^{2+}]_i$ increase (not shown). It remains to be seen whether Ca^{2+}-pump inhibition by caffeine plays a role in vivo or whether in this case a genuine Ca^{2+} releasing effect may prevail. Interestingly not only caffeine (see Section 1) but also SERCA inhibitors can cause a simultaneous induction of a $[Ca^{2+}]_i$ increase and of secretory activity with a variety of electrically excitable or non-excitable cells [15–17].

Besides activation of CICR in some systems (see Section 1), several other unrelated effects have been observed with caffeine [2,3,18]. Among them are the inhibition of voltage-gated Ca channels and of K channels in pancreatic

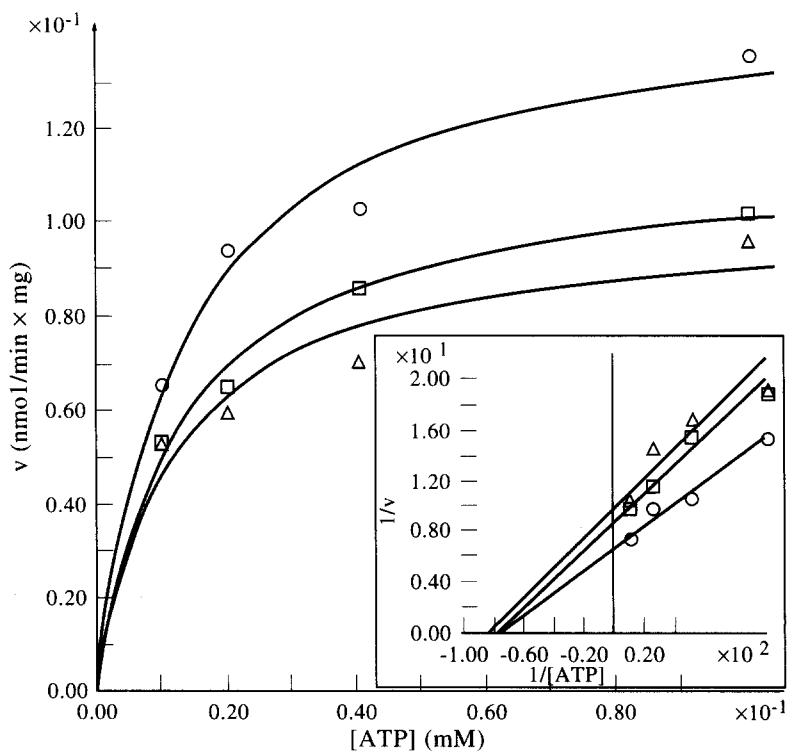

Fig. 4. [ATP] dependent $^{45}Ca^{2+}$ uptake (10 min, 27°C) by alveolar sacs and its inhibition by caffeine. Each data point is the mean of four independent experiments. (Ordinate: $\times 10^{-1}$ (inset $\times 10^{1}$), abscissa: $\times 10^{-1}$ (inset $\times 10^{2}$)). Values for K_M and V_{max} were determined by the Enzfitter computer program. Circles = no caffeine ($K_M = 13.2$ μM, $V_{max} = 0.15$ nmol/min/mg protein); squares = 10 mM caffeine ($K_M = 13.0$ μM, $V_{max} = 0.11$ nmol/min/mg protein); triangles = 20 mM caffeine ($K_M = 12.1$, $V_{max} = 0.10$ nmol/min/mg protein). Whereas K_M values obtained with the two caffeine concentrations tested are not significantly different from controls ($P > 0.5$), values calculated for V_{max} are significantly different on a $P < 0.05$ level.

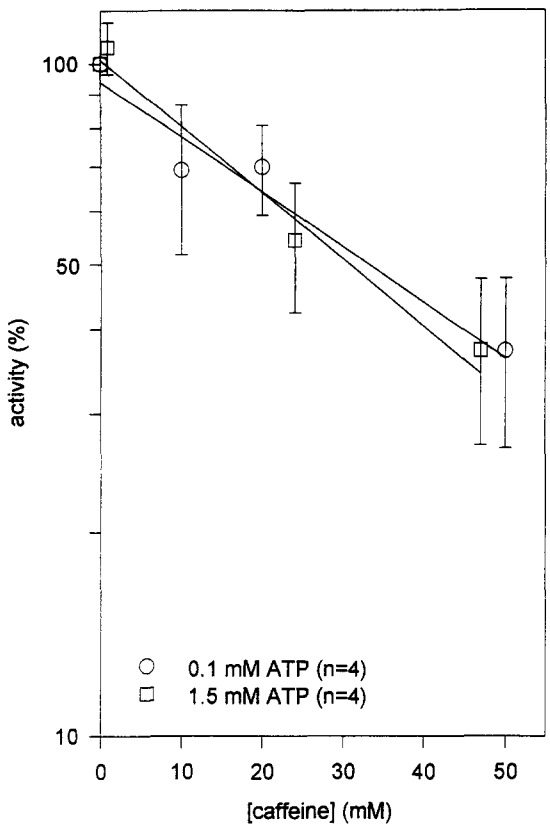

Fig. 5. Inhibition of $^{45}Ca^{2+}$ uptake by caffeine does not depend on ATP concentration. For one series we used 1.5 mM ATP (standard conditions), for another series only 0.1 mM ATP. Values without caffeine are normalized to 100%; for absolute values, see text. Caffeine inhibited $^{45}Ca^{2+}$ sequestration equally under both conditions. An IC_{50} = 31.8 mM was calculated.

β-cells [19], activation of non-selective cation channels (carrying also Ca^{2+}) in gastric smooth muscle cells [20], as well as inhibition of phosphodiesterase activity in some cells [2,18]. Furthermore, in pancreatic acinar cells caffeine activates (at low concentration) or blocks (at higher concentrations) agonist induced $[Ca^{2+}]_i$ oscillations [21], as it does in oocytes [22,23] and in hepatocytes [24]. In the latter case suppression of $InsP_3$-mediated Ca^{2+} release by caffeine has been proposed [23,25]. Thus, caffeine may not simply be regarded as a CICR activator, but one also has to consider some 'unorthodox' effects. Among them the Ca^{2+} transport inhibition described here is novel for secretory cells and may be relevant also for other systems.

So far to our knowledge the effect of caffeine on Ca^{2+} sequestration by isolated calcium stores has been analysed only with SR, with the following intriguing results. Although caffeine reduced Ca^{2+} uptake [26], this effect was believed to be due to superimposed Ca^{2+} release, rather than to inhibition of the Ca^{2+} pump [27,28]. A more scrutinized analysis revealed either activation or inhibition of Ca^{2+} uptake by caffeine-related drugs, depending on conditions used [29]. More recent analyses with SR fractions more clearly showed the inhibition of Ca^{2+} uptake or of Ca^{2+}-ATPase activity, respectively [30,31].

The following aspects have to be kept in mind with secretory systems. In widely different cells SERCA-type pumps may be sensitive to caffeine or to established inhibitors to a different extent. As with our system, caffeine has to be applied at relatively high concentrations to achieve full activation of Ca^{2+} release even with some mammalian secretory cells [8]. Some populations of mast cells from rat also require 20 μM thapsigargin to trigger maximal histamine release [15] and even higher concentrations are required to reduce the peak amplitude of caffeine-induced contracture in skinned fibres from frog skeletal muscle [32]. Finally, latent thapsigargin incubation causes expression of a SERCA-type pump of low thapsigargin sensitivity in some mammalian cells [33,34], with an $IC_{50} \sim 4$ μM [34]. Similarly we found alveolar sacs to be little sensitive to TG ($IC_{50} \sim 5$ μM), and possibly this SERCA-type may be selectively inhibited by caffeine. It also has to be kept in mind that effects achieved in vitro may not strictly reflect the situation in vivo and our failure to induce CICR in vitro [12] might be due to loss of regulatory components in these studies. Nevertheless, secretory systems may be activated by a small amount of $[Ca^{2+}]_i$ increase which can be caused by Ca^{2+}-pump inhibition and which secondarily may induce an extensive response via CICR. Clearly more detailed analyses are required to settle these aspects in our as well as in other secretory systems.

5. Conclusions

It cannot be taken for granted that caffeine activates secretory systems only by the mechanisms commonly assumed, i.e., by direct activation of Ca^{2+} release channels in subcellular stores. Even small inhibition of Ca^{2+} sequestration could be amplified by CICR and, thus, has to be envisaged as an additional or alternative mechanism of the Ca^{2+}-dependent activation phenomena observed with caffeine in secretory systems.

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