Caffeine-stimulated Ca^{2+} release from the intracellular stores of hepatocytes is not mediated by ryanodine receptors

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Caffeine has been much used to examine the possibility that ryanodine receptors similar to those found in skeletal and cardiac muscle may be more widely distributed and perhaps contribute to regenerative Ca^{2+} signals in electrically inexcitable cells. In permeabilized hepatocytes loaded with $^{45}\text{Ca}^{2+}$, caffeine (≥ 5 mM) decreased the $^{45}\text{Ca}^{2+}$ content of the intracellular stores by up to 60%; the effect was substantially reversible and it was not mimicked by the closely related methylxanthine theophylline

(20 mM). Ryanodine ($5\text{ }\mu\text{M}$) stimulated a far smaller Ca^{2+} mobilization ($7 \pm 1\%$). Procaine (1 mM), Ruthenium Red ($10\text{ }\mu\text{M}$) and ryanodine ($5\text{ }\mu\text{M}$) did not affect the Ca^{2+} release evoked by InsP_3 ($3\text{ }\mu\text{M}$) or caffeine (30 mM). We conclude that caffeine can specifically cause Ca^{2+} release from the intracellular stores of hepatocytes, but the effect is unlikely to be mediated by ryanodine receptors.

INTRODUCTION

The intracellular Ca^{2+} signals that follow activation of receptors linked to formation of InsP_3 are complex and typically consist of repetitive transient increases in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (Woods et al., 1986; Berridge, 1990). The mechanisms that generate this Ca^{2+} spiking are unknown, but variations of two models have been proposed. The first suggests that oscillating levels of intracellular InsP_3 directly trigger periodic mobilization of Ca^{2+} from InsP_3 -sensitive stores (Meyer and Stryer, 1991). The alternative model suggests that intracellular stores periodically release their Ca^{2+} contents in the presence of a sustained increase in the intracellular InsP_3 concentration. One version of this second scheme, 'the two-pool model', proposes that Ca^{2+} initially released from InsP_3 -sensitive stores is re-sequestered into InsP_3 -insensitive stores from which it is suddenly released when either the luminal or the cytosolic free $[\text{Ca}^{2+}]$ reaches a threshold level that triggers Ca^{2+} -induced Ca^{2+} release (Berridge and Galione, 1988).

Ryanodine receptors are intracellular Ca^{2+} channels that open in response to increased $[\text{Ca}^{2+}]_i$, and they have therefore been proposed to be the channels responsible for propagating intracellular Ca^{2+} signals by Ca^{2+} -induced Ca^{2+} release. Although ryanodine receptors are probably expressed in several tissues (Ashley, 1989; Cheek et al., 1991; Whicher et al., 1992), the properties of the skeletal and cardiac muscle forms are best understood. These channels are activated by caffeine (≤ 10 mM), which may both directly open the channels and increase their sensitivity to $[\text{Ca}^{2+}]_i$ (Sitsapesan and Williams, 1990); they are locked in a sub-conductance state by low concentrations ($\leq 10\text{ }\mu\text{M}$) of ryanodine and are fully closed by higher ryanodine concentrations, and they are inhibited by elevated Mg^{2+} concentrations (millimolar), by local anaesthetics (e.g. procaine) and by Ruthenium Red ($< 1\text{ }\mu\text{M}$) (Palade, 1987; Lai et al., 1989). These pharmacological properties, most notably the ability of caffeine to stimulate Ca^{2+} mobilization through muscle ryanodine receptors, have been used to examine the possibility that similar Ca^{2+} channels may be present in other tissues (Osipchuk et al., 1990; Stauderman et al., 1991).

Earlier studies have suggested the existence of ryanodine receptors linked to Ca^{2+} mobilization in the liver (Bazotte et al., 1991), but this organ appears not to express mRNA encoding the cardiac or skeletal muscle isoforms of ryanodine receptors (Otsu et al., 1990; Shoshan-Barmatz et al., 1991). In the present study, we have examined the effects of caffeine on the intracellular Ca^{2+} stores of saponin-permeabilized hepatocytes and compared the effects of caffeine with the effects of other agents known to modulate ryanodine receptors in muscle.

MATERIALS AND METHODS

Hepatocytes were prepared from livers of male Wistar rats and stored in cold Eagle's medium as previously described (Nunn and Taylor, 1992). Cells were resuspended in cytosol-like medium (mM: KCl, 140; NaCl, 20; MgCl_2 , 2; EGTA, 1; Pipes, 20, pH 7) and permeabilized by incubation with saponin ($50\text{ }\mu\text{g/ml}$) for 10 min at 37°C . After permeabilization, cells ($> 98\%$ permeable) were washed twice and resuspended (4×10^6 cells/ml) in cytosol-like medium supplemented with carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) ($10\text{ }\mu\text{M}$), $^{45}\text{CaCl}_2$ ($2\text{ }\mu\text{Ci/ml}$), ATP (1.5 mM), phosphocreatine (5 mM), creatine kinase (5 units/ml) and with enough CaCl_2 ($390\text{ }\mu\text{M}$) to give a free $[\text{Ca}^{2+}]$ of 200 nM. After 10 min at 37°C the intracellular stores had loaded to steady-state with Ca^{2+} . In some experiments involving high concentrations of caffeine, some of the KCl (25 mM) was replaced by mannitol (50 mM) in the final control medium (see below). Caffeine is both sparingly soluble and effective only at relatively high concentrations; the effects of caffeine were therefore examined by diluting cells 2-fold into media containing caffeine at twice its final concentration. In most experiments, caffeine iso-osmotically replaced either KCl or mannitol in the control medium; similar results were obtained with either replacement and when no osmotic correction was made (results not shown). Media were prepared to ensure that, after dilution of the cells, the compositions of the incubation media, including the specific radioactivity of the $^{45}\text{Ca}^{2+}$, were otherwise unchanged.

Incubations were terminated by rapid filtration of cells through

Whatman GF/C filters, followed by two rapid washes with ice-cold sucrose (310 mM) and sodium citrate (1 mM) (Nunn and Taylor, 1992). The $^{45}\text{Ca}^{2+}$ contents of the intracellular stores were then calculated after subtraction of non-specific $^{45}\text{Ca}^{2+}$ binding determined in the presence of ionomycin (10 μM). Where appropriate, concentration-response curves were fitted to a logistic equation using GraphPad InPlot software as described earlier (Nunn and Taylor, 1992).

Caffeine, Ruthenium Red and procaine were from Sigma. Ryanodine was from Research Biochemicals Inc. All other materials were from suppliers listed previously (Nunn and Taylor, 1992).

RESULTS AND DISCUSSION

Caffeine, at relatively high concentrations (5–50 mM), caused a decrease in the $^{45}\text{Ca}^{2+}$ content of the non-mitochondrial stores in permeabilized hepatocytes. The effect was concentration-

Figure 1 Concentration-dependent effects of caffeine on intracellular Ca^{2+} stores

Permeabilized hepatocytes, loaded to steady-state with $^{45}\text{Ca}^{2+}$, were incubated with caffeine (■) or theophylline (□, 20 mM) for 5 min in media where the methylxanthines iso-osmotically replaced the mannitol present in control medium. Results, expressed relative to the $^{45}\text{Ca}^{2+}$ contents of control cells, are shown as means $\pm$ S.E.M. of six independent experiments; error bars are obscured by the symbols. C, control (no caffeine or theophylline).

Table 1 Effects of agents that modulate ryanodine receptors on intracellular Ca^{2+} stores

Permeabilized hepatocytes were loaded to steady-state with $^{45}\text{Ca}^{2+}$ and then incubated with Ruthenium Red or procaine for 1 min before addition of InsP_3 , caffeine or ryanodine. After 5 min, the incubations were stopped and the $^{45}\text{Ca}^{2+}$ contents of the cells were determined. Results, expressed as percentage of the control (i.e. no pre-treatment and no subsequent drug addition), are shown as means $\pm$ S.E.M. of 14 (top row) or 3 (lower rows) independent experiments.

Pre-incubation	$^{45}\text{Ca}^{2+}$ content (% of control)			
	Control	InsP_3 (3 μM)	Caffeine (30 mM)	Ryanodine (5 μM)
Control	100	61 $\pm$ 2	71 $\pm$ 3	93 $\pm$ 1
Ruthenium Red (1 μM)	96 $\pm$ 5	67 $\pm$ 3	75 $\pm$ 7	92 $\pm$ 13
Ruthenium Red (10 μM)	102 $\pm$ 10	63 $\pm$ 4	81 $\pm$ 8	101 $\pm$ 8
Procaine (1 mM)	107 $\pm$ 2	71 $\pm$ 2	75 $\pm$ 5	106 $\pm$ 6

dependent, but even when caffeine was present at 50 mM there was no evidence that the response was maximal (Figure 1). At the highest practicable concentration tested (50 mM), caffeine released $42 \pm 2\%$ ($n = 6$) of the intracellular Ca^{2+} stores. The effect of caffeine was specific, as the closely related methylxanthine theophylline (20 mM), which differs by only one methyl group from caffeine, had no effect (Figure 1).

Permeabilized cells did not survive the repeated centrifugations needed to completely remove caffeine, and we therefore examined the reversibility of the caffeine effect using a dilution protocol. Cells were incubated with 50 mM caffeine for 5 min and then diluted 10-fold to reduce the caffeine concentration to 5 mM and incubated for a further 10 min before assessing the $^{45}\text{Ca}^{2+}$ contents of the intracellular stores. With this protocol, we observed that dilution of the cells caused a modest stimulation ($38 \pm 10\%$, $n = 3$) of $^{45}\text{Ca}^{2+}$ uptake into the intracellular stores; this effect was corrected by expressing the Ca^{2+} contents of caffeine-treated cells relative to those of control cells that had undergone identical dilution steps. Treatment with 5 mM and 50 mM caffeine reduced the $^{45}\text{Ca}^{2+}$ content of the stores to $77 \pm 11\%$ and $38 \pm 3\%$ ($n = 3$) of their control values respectively (results not shown). When cells were first exposed to 50 mM caffeine and then diluted to bring the final caffeine concentration to 5 mM, the $^{45}\text{Ca}^{2+}$ content of the stores recovered to $74 \pm 6\%$ ($n = 3$) of the level in control cells, which is not significantly different from the $^{45}\text{Ca}^{2+}$ content of cells exposed to 5 mM caffeine throughout ($86 \pm 3\%$, $n = 3$). The effects of caffeine are therefore substantially reversible.

InsP_3 stimulated Ca^{2+} mobilization from the intracellular stores; the maximal effect (release of $38 \pm 5\%$ of the stores; $n = 3$) occurred with 3 μM InsP_3 , and the half-maximal effect occurred with 470 ± 110 nM ($n = 3$; $h = 2.68 \pm 0.07$). Caffeine (10 or 50 mM) had no significant effect on the responses to maximal (5 μM) or submaximal (500 nM) concentrations of InsP_3 ($n = 6$; results not shown). In our earlier studies with hepatocytes, we reported that caffeine antagonized the effects of the InsP_3 , but the inhibition occurred only at concentrations of InsP_3 that were substantially lower than those used in the present study (Missiaen et al., 1992). Others have also reported inhibitory effects of caffeine in *Xenopus* oocytes (Parker and Ivorra, 1991) and cerebellum (Brown et al., 1992), but the nature of the interaction is unclear because caffeine does not appear to affect the binding of InsP_3 to its receptor (Brown et al., 1992). We conclude that the effects of caffeine in permeabilized hepatocytes are unlikely to be mediated by an interaction with InsP_3 receptors.

Our results suggesting that caffeine specifically and reversibly decreases the Ca^{2+} content of the intracellular stores in hepatocytes prompted us to examine the possible involvement of ryanodine receptors. Our results from experiments using a variety of agents known to interact with muscle ryanodine receptors are shown in Table 1. Ryanodine (50 nM or 5 μM) caused only a modest decrease ($9 \pm 1\%$ and $7 \pm 1\%$ at 50 nM and 5 μM respectively; $n = 9$ and 14) in the Ca^{2+} content of the stores, an effect that was substantially smaller than that evoked by caffeine ($42 \pm 2\%$ loss with 50 mM caffeine; $n = 6$). Ruthenium Red (1 μM and 10 μM), procaine (1 mM) and ryanodine (5 μM) had no effect on the response to a maximal concentration of InsP_3 (3 μM), confirming that ryanodine receptors are not involved in the response to InsP_3 (see Taylor and Richardson, 1991). The effects of caffeine (30 mM) were also entirely insensitive to pre-incubation with procaine, Ruthenium Red (Table 1) or ryanodine ($31 \pm 3\%$ and $29 \pm 2\%$ Ca^{2+} release by 30 mM caffeine with and without 5 μM ryanodine respectively; $n = 9$ and 14). We conclude that, while caffeine specifically and reversibly decreases the Ca^{2+} content of the intracellular stores of

hepatocytes, the effect is unlikely to be mediated by a Ca^{2+} channel similar to the ryanodine receptors found in muscle.

Earlier studies have also identified atypical effects of agents known to modulate muscle ryanodine receptors. Ryanodine has been reported to decrease the Ca^{2+} content of permeabilized hepatocytes, but the effect is insensitive to concentrations of Ruthenium Red that inhibit muscle ryanodine receptors (Bazotte et al., 1991). In adrenal chromaffin cells also, caffeine-stimulated Ca^{2+} mobilization is only partially sensitive to a high concentration of Ruthenium Red (10 μM ; Cheek et al., 1991). Our own results suggest a modest decrease in the Ca^{2+} contents of the intracellular stores after incubation with high (5 μM) or low (50 nM) concentrations of ryanodine, but the effect is too small to reliably establish its sensitivity to potential inhibitors (Table 1). High-affinity binding of ryanodine to liver microsomes differs in many respects from that found in sarcoplasmic reticulum (SR): (1) the former is inhibited by caffeine, whereas the latter is stimulated; (2) Ruthenium Red inhibits ryanodine binding to SR but not to liver microsomes; (3) the binding kinetics are very different in liver and SR; and (4) Ca^{2+} modulates ryanodine binding to SR but not to liver microsomes (Shoshan-Barmatz et al., 1991). Furthermore, both immunological and molecular biological studies have failed to detect muscle-like ryanodine receptors in liver (Otsu et al., 1990; Shoshan-Barmatz et al., 1991).

Our results do not exclude the possibility that in hepatocytes caffeine and ryanodine decrease the Ca^{2+} content of the intracellular stores by activating specific channels which share pharmacological properties with muscle ryanodine receptors. Indeed, pancreatic acinar cells have Ca^{2+} channels that are activated by caffeine, inhibited by Ruthenium Red, and insensitive to ryanodine and Ca^{2+} (Schmid et al., 1990). It is, however, clear from both our present results and the results of earlier studies that neither caffeine nor ryanodine can be used to

unequivocally demonstrate the presence of muscle-like ryanodine receptors in non-muscle cells.

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