Biochimica et Biophysica Acta 902 (1987) 87–92
Elsevier

BBA 73636

The role of a (Ca^{2+} + Mg^{2+})-ATPase of the rough endoplasmic reticulum in regulating intracellular Ca^{2+} during cholinergic stimulation of rat pancreatic acini

Graham R. Brown, Alun E. Richardson and Robert L. Dormer

Department of Medical Biochemistry, University of Wales College of Medicine, Cardiff (U.K.)

(Received 6 February 1987)

Key words: ATPase, (Ca^{2+} + Mg^{2+})-; Intracellular calcium; Cholinergic stimulation; (Rat acini)

Rough endoplasmic reticulum membranes, purified from isolated rat pancreatic acini stimulated by carbachol, had a decreased Ca^{2+} content and increased (Ca^{2+} + Mg^{2+})-ATPase activity. Ca^{2+} was regained and ATPase activity reduced to control levels only after blockade by atropine. The (Ca^{2+} + Mg^{2+})-ATPase was activated by free Ca^{2+} (half-maximal at 0.17 μM; maximal at 0.7 μM) over the concentration range which occurs in the cell cytoplasm. Pretreatment with EGTA, at a high concentration (5 mM), inhibited ATPase activity which, our results suggest, was due to removal of a bound activator such as calmodulin. The rate of (Ca^{2+} + Mg^{2+})-ATPase actively declined during the 10-min period over which maximal active accumulation of Ca^{2+} by membrane vesicles occurs. In the presence of ionophore A23187, which released actively accumulated Ca^{2+} and stimulated the (Ca^{2+} + Mg^{2+})-ATPase, this time-dependent decline in activity was not observed. Our data provide evidence that the activity of the Ca^{2+}-transporting ATPase of the rough endoplasmic reticulum is regulated by both extra and intravesicular Ca^{2+} and is consistent with a direct role of this enzyme in the release and uptake of Ca^{2+} during cholinergic stimulation of pancreatic acinar cells.

Introduction

Observations of a rapid rise in cytoplasmic free Ca^{2+} concentration [1–3], a positive correlation between increased Ca^{2+} concentration and the degree of enzyme release [1,4,5] and inhibition of stimulated enzyme release by intracellular EGTA [6] have provided strong evidence that Ca^{2+} is a primary trigger for the stimulation of pancreatic enzyme secretion by cholinergic agonists. Experiments in which extracellular Ca^{2+} was omitted immediately prior to stimulation [7–9] and measurements of $^{45}Ca^{2+}$ and total calcium fluxes [10–13] have supported the hypothesis that enzyme release is initially triggered by the release of intracellular Ca^{2+} into the cytoplasm, but that the stimulated rate of secretion is maintained by an increased influx of extracellular calcium.

We have previously investigated the nature of the site of intracellular Ca^{2+} release using subcellular fractionation techniques [14,15] and suggested that it was the rough endoplasmic reticulum. Ca^{2+} release from the inner surface of the plasma membrane has also been suggested to be involved [16,17]. However, the discovery of inositol 1,4,5-trisphosphate as a putative Ca^{2+}-mobilizing intracellular messenger [18,19], and the demonstration that it is produced rapidly in response to cholinergic agents in pancreatic acinar cells [20] and acts directly to release calcium from

Correspondence: R.L. Dormer, Department of Medical Biochemistry, University of Wales College of Medicine, Heath Park, Cardiff, U.K.

isolated rough endoplasmic reticulum membranes [20,21] has lent further support for the rough endoplasmic reticulum as the intracellular site of Ca^{2+} release.

In the present study, we have demonstrated that cholinergic stimulation of isolated pancreatic acini causes net release of Ca^{2+} from the rough endoplasmic reticulum, which is not regained during sustained stimulation. A $(Ca^{2+} + Mg^{2+})$-ATPase linked to Ca^{2+} transport has been demonstrated, which is regulated by the cytoplasmic free Ca^{2+} concentration, and by intraluminal Ca^{2+}. We suggest that this plays an important role in buffering entry of extracellular Ca^{2+} induced by cholinergic stimulation. Part of this work has been published in preliminary form [22,33].

Materials and Methods

Materials

$^{45}CaCl_2$ (10–40 mCi/mg Ca) was obtained from the Radiochemical Centre, Amersham, U.K. The following were purchased from Sigma, Poole, U.K: soybean trypsin inhibitor (type 1-S), collagenase (type III, fraction A), hyaluronidase (type 1), ATP (disodium from equine muscle, vanadium-free), benzamidine, carbamylcholine chloride, (carbachol) DL-dithiothreitol, EGTA and L-glutamine. Bovine serum albumin, fraction V, was from Miles Laboratories, Slough, U.K., minimal Eagle's medium amino acid supplement, minus glutamine, from Gibco Bio-Cult, Paisley, U.K., and *p*-methylaminophenol sulphate from Kodak Limited, Liverpool, U.K. All other reagents were AnalaR Grade from BDH Chemicals, U.K.

Isolation of rat pancreatic acini

Pancreatic acini were isolated from overnight-fasted Wistar rats (200–250 g) as described previously [15] and preincubated for at least 30 min at 37 °C in Krebs-Henseleit bicarbonate medium (see Ref. 1) containing 1% (w/v) bovine serum albumin.

Isolation of rough endoplasmic reticulum

Rough endoplasmic reticulum membranes were purified from isolated rat pancreatic acini as previously described [15] with some minor modifications. Briefly, acini were homogenised in 0.3 M sucrose buffered with 10 mM Hepes, pH 7.4, containing 1 mM benzamidine, 2 mM sodium azide and 5 mM dithiothreitol. A post-mitochondrial supernatant was prepared and centrifuged (90 min at $100\,000 \times g$) on a sucrose layer consisting of 40% (w/w) sucrose in imidazole buffer (100 mM imidazole, adjusted to pH 6.8 at 4 °C, containing 5 mM $MgCl_2$, 2 mM sodium azide, 100 mM KCl). The purified rough endoplasmic reticulum membranes were recovered as a pellet and resuspended in 0.25 M sucrose in the imidazole buffer, adjusted to pH 6.8 at 37 °C. We have demonstrated [15] that these membranes are virtually free of plasma and Golgi membranes, as assessed by enzyme markers.

Measurement of $(Ca^{2+} + Mg^{2+})$-ATPase activity

Rough endoplasmic reticulum membranes (approximately 50 μg protein) were mixed with 1 mM EGTA alone or with Ca-EGTA (1 mM EGTA plus various amounts of $CaCl_2$) at a range of free Ca^{2+} concentrations, calculated using an iterative program for interacting ligands (EGTA, ATP, azide and imidazole) and ions (Ca^{2+}, Mg^{2+}, H^+, Na^+, K^+) with association constants taken from Sillen and Martell [24]. 5 mM ATP was added and incubation continued at 37 °C for the times denoted in the figure and table legends. The reaction was stopped by addition of 0.2 M perchloric acid and, after 10 min on ice, the precipitate was centrifuged and the supernatant assayed for P_i by the method of Lebel et al. [25]. The results are expressed as the difference in ATPase activity in the presence and absence of Ca^{2+} in nmol P_i produced.

Measurement of $^{45}Ca^{2+}$ uptake

This was carried out using the Millipore filtration technique described previously [15] at pH 6.8 and in the presence of 1 mM EGTA plus 0.5 mM $^{45}CaCl_2$ of specific radioactivity 3.1 mCi/nmol (free Ca^{2+}, 0.7 μM).

Measurement of total Ca^{2+} content

Rough endoplasmic reticulum membranes suspended in either buffer or distilled water were diluted in 1% $LaCl_3$ and assayed for calcium by atomic absorption spectroscopy.

Protein was estimated by the method of Lowry et al. [26] using bovine serum albumin as standard.

Statistical significance of results was assessed using Student's *t*-test for unpaired samples.

Results

Effect of cholinergic stimulation on rough endoplasmic reticulum calcium handling

Fig. 1 shows that, following 15 min stimulation with a concentration of carbachol (3 μM) which causes a maximal secretory response [6], the (Ca^{2+} + Mg^{2+})-ATPase activity of the rough endoplasmic reticulum membranes isolated from stimulated acini was significantly greater than that in membranes isolated from control acini. This effect was observed at times from 2 min to 40 min following stimulation in the continual presence of carbachol (data not shown). Concurrently with the increased ATPase activity, the total Ca^{2+} content of membranes from stimulated cells was significantly decreased (Fig. 1). When acini were stimulated with carbachol for 15 min and then atropine (10 μM) added for 10 min, as shown in Fig. 1, both the ATPase activity and total Ca^{2+} content of isolated membranes had returned to the same level as controls.

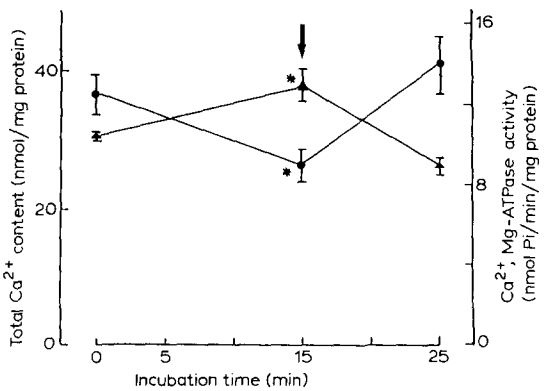

Fig. 1. Effect of cholinergic stimulation followed by atropine inhibition of isolated acini on (Ca^{2+} + Mg^{2+})-ATPase activity and total Ca^{2+} content of rough endoplasmic reticulum membranes. Isolated rat pancreatic acini were preincubated for 30 min at 37 °C in Krebs-Henseleit bicarbonate medium and then resuspended in fresh Krebs-Henseleit bicarbonate medium, an aliquot was removed, and 3 μM carbachol added. Another aliquot was removed after 15 min incubation at 37 °C, 10 μM atropine added (↓) and a final aliquot taken 10 min later. Rough endoplasmic reticulum membranes were isolated from each aliquot and (Ca^{2+} + Mg^{2+})-ATPase assayed immediately, at a free Ca^{2+} concentration of 0.7 μM. Total Ca^{2+} content of the membranes was measured by atomic absorption spectroscopy. Results are means ± S.E. for four observations (ATPase) or three observations (total Ca^{2+}). *P less than 0.01 for difference from control and atropine-inhibited acini.

Regulation of the rough endoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase

Fig. 2 shows that the (Ca^{2+} + Mg^{2+})-ATPase is sensitive to free Ca^{2+} in the medium over the range of concentrations found in the cell cytoplasm. Activity was maximal at 0.7 μM and half-maximal at 0.17 μM, which is the same as that previously shown for ATP-dependent $^{45}Ca^{2+}$ uptake by these membrane [15].

One explanation for the observed increases in (Ca^{2+}-Mg^{2+})-ATPase (Fig. 1) and $^{45}Ca^{2+}$ uptake activity [15] following cholinergic stimulation is that it is the direct result of the release of Ca^{2+} from the endoplasmic reticulum lumen. We have tested this hypothesis by the use of agents which can remove Ca^{2+} from isolated membranes.

Effects of EGTA

The Ca^{2+} chelator EGTA promotes a slow release of actively accumulated $^{45}Ca^{2+}$ from isolated pancreatic microsomal vesicles [27] and sig-

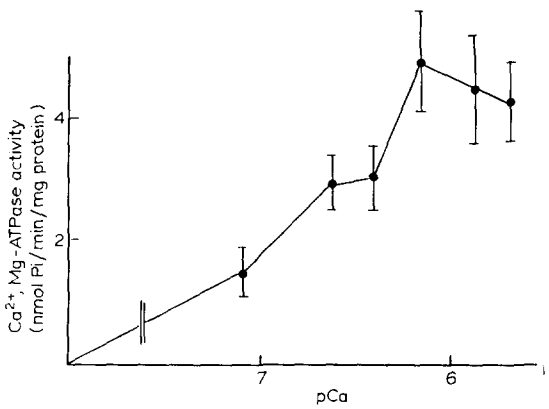

Fig. 2. Dependence on free Ca^{2+} concentration of (Ca^{2+} + Mg^{2+})-ATPase activity of rough endoplasmic reticulum membranes. Isolated rough endoplasmic reticulum membranes were assayed for (Ca^{2+} + Mg^{2+})-ATPase activity at a range of free Ca^{2+} concentrations as described in Materials and Methods. Points are means ± S.E. for six determinations.

nificantly reduces their total Ca^{2+} content if included in the buffers used for homogenisation and purification [14]. We tested the effect of adding different concentrations of EGTA to the homogenisation and gradient media, on $^{45}Ca^{2+}$ uptake and $(Ca^{2+} + Mg^{2+})$-ATPase activity by purified rough endoplasmic reticulum vesicles. Inclusion of 3 mM EGTA during purification of rough endoplasmic reticulum vesicles reduced the total Ca^{2+} content by $36.4 \pm 8.4\%$ (mean $\pm$ S.E. ($n = 4$ experiments)). EGTA at 1 or 3 mM did not stimulate $^{45}Ca^{2+}$ uptake either in the absence (passive binding) or presence (active uptake) of ATP (data not shown). Treatment with 3 mM EGTA also did not stimulate $(Ca^{2+} + Mg^{2+})$-ATPase activity (50.4 ± 9.2 ($n = 4$) nmol P_i/mg protein per 10 min compared to 58.0 ± 16.4 ($n = 7$) for controls). However, a higher concentration of EGTA (5 mM) decreased $(Ca^{2+} + Mg^{2+})$-ATPase in five out of the seven preparations tested (29.6 ± 4.5 nmol), suggesting removal of a bound activator such as calmodulin, as is observed in isolated plasma membranes [28]. We have previously shown a small activation by calmodulin of $^{45}Ca^{2+}$ uptake by a rat pancreatic microsomal fraction [29]. In three experiments, the direct effect of calmodulin (59 nM) on $(Ca^{2+} + Mg^{2+})$-ATPase of rough endoplasmic reticulum membranes, isolated in the presence or absence of 5 mM EGTA, was tested. No significant effect of calmodulin was observed on membranes isolated in the absence of EGTA, but in EGTA-treated membranes the activity was significantly ($P < 0.05$) increased from 2.58 ± 0.30 nmol P_i/min per mg protein to 8.88 ± 1.65 nmol P_i/min per mg protein.

Effect of ionophore A23187

The bivalent cation ionophore A23187 significantly stimulated the $(Ca^{2+} + Mg^{2+}$-ATPase activity at a concentration which we have previously shown [15] to release all $^{45}Ca^{2+}$ which is actively accumulated by the vesicles (176.3 ± 18.8 ($n = 4$) nmol P_i/mg protein per 10 min in 5 μM A23187-treated membranes compared to 48.5 ± 19.1 ($n = 4$) for controls; $P < 0.01$). The solvent, dimethyl sulphoxide, also stimulated ATPase activity in some experiments but the effect was not significant (83.8 ± 16.9 ($n = 4$) nmol P_i/mg protein per 10 min). Similarly, in some experiments, dimethyl sulphoxide caused more release of accumulated $^{45}Ca^{2+}$ (data not shown), suggesting that non-specific release of calcium by this agent may have a similar effect to that of the ionophore. The effect of A23187 suggested that accumulation of Ca^{2+} inside the membrane vesicles was inhibitory to the ATPase. If this were true, it would be expected that the initial rate of ATPase activity stimulated by Ca^{2+} would decline over the 10-min period during which the maximum Ca^{2+} gradient is formed [15]. Table I shows that the rate of ATPase activity, measured over 5 min, was greater than that measured over 10 min, such that the rate for the second 5-min period was significantly decreased (77%) compared to the rate for the first 5 min. Table I also shows that in the presence of A23187, when the vesicles are unable to accumulate Ca^{2+}, the rate of $(Ca^{2+} + Mg^{2+})$-ATPase activity was not significantly reduced between 5 and 20 min.

TABLE I

RATE OF $(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITY OF ISOLATED ROUGH ENDOPLASMIC RETICULUM MEMBRANES: EFFECT OF IONOPHORE A23187

Rough endoplasmic reticulum membranes were isolated and $(Ca^{2+} + Mg^{2+})$-ATPase activity assayed as described, in the presence or absence of 0.7 μM free Ca^{2+} for either 5 min or 10 min at 37°C. Results are means $\pm$ S.E. for the number of experiments denoted in parentheses.

Period of assay (min)	$(Ca^{2+} + Mg^{2+})$-ATPase activity (nmol P_i/min per mg protein)	
Addition:	None	5 μM A23187
0– 5	5.35 ± 0.94 (6)	7.23 ± 1.56 (3)
0–10	3.27 ± 0.63 (6)	6.46 ± 0.98 (3) $^{+}$
5–10	1.18 ± 0.40 (6) *	5.68 ± 0.53 (3) $^{++}$

* P less than 0.05 for difference from 0–5 min period.
$^{+}$ P less than 0.05.
$^{++}$ P less than 0.001 for difference from control (no addition).

Discussion

We have previously reported [15] that Ca^{2+} transport by purified rough endoplasmic reticulum membrane vesicles has properties consistent with its proposed role as a releasable Ca^{2+} store in pancreatic acinar cells. The present report pro-

vides direct evidence that the rough endoplasmic reticulum is involved in the release and reuptake of Ca^{2+} during a cycle of cholinergic stimulation and recovery of isolated rat pancreatic acini. Thus, following stimulation of isolated acini by carbachol, a sustained decrease in the total Ca^{2+} content of rough endoplasmic reticulum membranes was observed (Fig. 1). The Ca^{2+} was regained when receptor occupancy was blocked by atropine. Studies of whole cell $^{45}Ca^{2+}$ fluxes [12] also suggested that the secretagogue-sensitive Ca^{2+} store was not refilled during sustained stimulation of enzyme release.

The sensitivity of the $(Ca^{2+} + Mg^{2+})$-ATPase to free Ca^{2+} (half-maximal at 0.17 μM; maximal at 0.7 μM) is the same as that previously determined for $^{45}Ca^{2+}$ uptake [15], suggesting that the two processes are linked. Rough endoplasmic reticulum membranes purified from single rat pancreatic acinar cells by Percoll density-gradient centrifugation [30] showed $^{45}Ca^{2+}$ uptake and $(Ca^{2+} + Mg^{2+})$-ATPase activities in the same range (half-maximal at 0.5–0.6 μM and maximal at 2 μM Ca^{2+}).

In stimulated acini, the rise in cytoplasmic free Ca^{2+} is transient, returning to a concentration close to the resting level within 5 min [1–3,31]. Thus, our observation (Fig. 1) of increased $(Ca^{2+} + Mg^{2+})$-ATPase activity in membranes isolated from acini stimulated by carbachol for 15 min suggested that it is regulated by factors other than the cytoplasmic (extravesicular) free Ca^{2+} concentration. Since, during stimulation and recovery of the cell, the $(Ca^{2+} + Mg^{2+})$-ATPase activity appeared to be inversely related to the Ca^{2+} content of the membranes, we investigated the hypothesis that intravesicular Ca^{2+} also regulates $(Ca^{2+} + Mg^{2+})$-ATPase activity.

Reducing the Ca^{2+} content of isolated membranes by EGTA did not produce consistent stimulation of either $^{45}Ca^{2+}$ uptake or $(Ca^{2+} + Mg^{2+})$-ATPase activities. However, inhibition of $(Ca^{2+} + Mg^{2+})$-ATPase activity by the highest EGTA concentration (5 mM) implied that the Ca^{2+} removed was from a different site to that released during stimulation. Our data suggest that a high concentration of EGTA acts by removing a bound activator such as calmodulin.

The finding that ionophore A23187 stimulated $(Ca^{2+} + Mg^{2+})$-ATPase activity and sustained a linear rate of activity when Ca^{2+} accumulation was prevented suggests that there is an inhibitory effect of intravesicular Ca^{2+}. Similar effects have been reported for rat liver microsomal fractions [32,33]. This would in part explain the plateau of Ca^{2+} accumulation seen in isolated rough endoplasmic reticulum membranes at 5–10 min in the absence of precipitating anions [15], as was proposed for sarcoplasmic reticulum [34,35]. At steady state, the ATPase continues to turn over, in order to balance outward leakage of Ca^{2+} [36]. $(Ca^{2+} + Mg^{2+})$-ATPase activity in pancreatic rough endoplasmic reticulum membranes [30], measured in the presence of A23187, showed complete inhibition at 10–100 μM free Ca^{2+}. This is likely to be of physiological relevance only if Ca^{2+} acts at an intravesicular site, since cytoplasmic free Ca^{2+} only reaches these concentrations in a damaged cell [37], but, by analogy to the sarcoplasmic reticulum [38], such a high Ca^{2+} concentration may be achieved in the endoplasmic reticulum lumen.

In the intact cell the free Ca^{2+} concentration in the endoplasmic reticulum lumen may be determined by the rate of Ca^{2+} leakage, by the presence of specific Ca^{2+}-binding proteins analogous to calsequestrin in the sarcoplasmic reticulum [39] and whether the Ca^{2+} taken up is transported through the secretory pathway to the zymogen granules. Our observation that the endoplasmic reticulum Ca^{2+} was not regained during continual stimulation could be due to Ca^{2+} removal by transport through the secretory pathway or because the 'Ca^{2+}-release channel' remains open. The possibility that endoplasmic reticulum Ca^{2+} is directed through the secretory pathway requires further investigation. During continual cholinergic stimulation of isolated mouse pancreatic acini we demonstrated [14] an increased uptake of $^{45}Ca^{2+}$ into zymogen granules without a net gain in total Ca^{2+}, suggesting a flux of Ca^{2+} through the secretory pathway. Kinetic experiments using intact pancreas in vivo [40] and in vitro [41] suggested that $^{45}Ca^{2+}$ enters the secretory pathway beyond the level of the endoplasmic reticulum, whereas studies by X-ray microanalysis [42] indicated net flux of Ca^{2+} from the endoplasmic reticulum to the apical end of the cell, following cholinergic stimulation. The findings

[20,21] that inositol 1,4,5-trisphosphate release actively accumulated Ca^{2+} from isolated rough endoplasmic reticulum membranes and has a time-course of formation [20] consistent with a role as second messenger linking receptor occupancy to Ca^{2+} release and the stimulation of enzyme secretion provide an alternative mechanism. Thus, as recently discussed by Putney [43], the continued formation of inositol 1,4,5-trisphosphate, or another product of inositol phospholipid breakdown, might maintain the permeability of the rough endoplasmic reticulum to Ca^{2+} and prevents its reaccumulation.

In conclusion, therefore, we have presented direct evidence that cholinergic stimulation of rat pancreatic acini results in release of Ca^{2+} from the rough endoplasmic reticulum which releases an inhibition of the Ca^{2+}-transporting ATPase allowing rapid restoration of the Ca^{2+} store when receptor occupancy is terminated.

Acknowledgements

We are grateful to the Welsh Scheme for the Development of Health and Social Research and the Medical Research Council for financial support. We wish to thank Dr. M.A. McPherson for much helpful discussion, and Mr. S. Hann for carrying out the atomic absorption measurements.

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