CALMODULIN-SENSITIVE ATP-DEPENDENT CALCIUM TRANSPORT BY THE RAT PAROTID ENDOPLASMIC RETICULUM

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1. Introduction

ATP-dependent Ca^{2+} pumps have been identified in a variety of tissues [1–4]. These Ca^{2+} transport systems are associated with either the plasma membrane [1,2,4] or intracellular membranes such as the sarcoplasmic reticulum [1], endoplasmic reticulum [1,3] or Golgi vesicles [5]. In view of their high affinity for Ca^{2+}, it is generally accepted that the function of these Ca^{2+} transport systems is the maintenance of low levels of intracellular free Ca^{2+}.

In the rat parotid gland many aspects of metabolism including K^+ and enzyme secretion are influenced by changes in intracellular Ca^{2+} [6,7]. Since the Ca^{2+} transport system identified by Selinger et al. [8] in rat parotid microsomes may be important in regulating intracellular Ca^{2+} levels, it was of interest to us to study the mechanism by which Ca^{2+} accumulation by this system is controlled. Recently, it has been shown that many Ca^{2+} transport systems are activated by the calcium binding protein, calmodulin. These include ATP-dependent Ca^{2+} transport by sarcoplasmic reticulum [9], heart sarcolemma [2], erythrocyte plasma membrane [10] and adipocyte plasma membrane [4]. By activating Ca^{2+} uptake, calmodulin is believed to mediate a self-regulating negative feedback system that enables cells to maintain Ca^{2+} homeostasis following stimulation with hormones and metabolic signals that increase intracellular free Ca^{2+}. We report here evidence suggesting that calmodulin may play a similar function in parotid gland metabolism by activating Ca^{2+} uptake by the endoplasmic reticulum.

2. Materials and methods

2.1. *Isolation of membrane*

A membrane fraction enriched in endoplasmic reticulum was obtained from parotid glands by differential centrifugation. The glands were excised from overnight starved, male Wistar rats, cleared of extraneous tissue and homogenised in 0.25 M sucrose, 0.1 mM dithiothreitol, 5 mM Tris–HCl (pH 7.4), to give a 10% (w/v) homogenate. Homogenising was carried out in a glass homogeniser with a teflon pestle (Thomas, size A) driven at 500 rev./min. Nuclei and cell debris were removed by centrifugation at 1000 × *g* for 10 min and the supernatant then centrifuged at 10 000 × *g* for 10 min. The pellet consisting mainly of mitochondria was discarded and the supernatant subjected to further centrifugation at 15 000 × *g* for 20 min. The resulting 'heavy microsomal' pellet was washed and resuspended in a small volume of the same buffer. This fraction, which showed the highest enrichment of NADPH-cytochrome *c* reductase activity, was used for determination of ^{45}Ca uptake. All steps were carried out at 0–4°C.

2.2. *^{45}Ca uptake*

Calcium uptake was measured at 30°C in 1 ml of medium having the following composition: 100 mM KCl, 5 mM Tris–oxalate, 10 μM $CaCl_2$ containing 0.2 μCi/ml of ^{45}Ca, 10 mM Tris–HCl (pH 7.4), 2.5 mM Mg–ATP and ~100 μg of membrane protein. Uptake was initiated by the addition of ATP. Aliquots were removed at 30 s and 5.5 min after addition of ATP, filtered through millipore filters (pore diameter 0.45 μm) and washed with 5 ml of ice-cold 100 mM KCl, 1 mM EGTA and 10 mM Tris–HCl (pH 7.4). The filters were dried and radioactivity determined in toluene based scintillation cocktail. Under the assay conditions, ^{45}Ca uptake proceeded at a linear rate for at least 10 min.

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2.3. Enzyme and other assays

Protein was determined by the method of Lowry et al. [11]. Calmodulin-sensitive cyclic AMP phosphodiesterase was prepared from beef heart as described in [12] and cyclic AMP phosphodiesterase activity determined by the method of Loten et al. [13]. NADPH-cytochrome *c* reductase activity was measured as in [14]. One unit is the amount of enzyme that reduces 1 μmol of cytochrome *c*/min at 30°C.

All biochemicals were obtained from Sigma, USA. ^{45}Ca was from the Radiochemical Centre, UK. Trifluoperazine was a gift from Smith, Klyne and French Laboratories. Purified beef brain calmodulin was donated by Dr J. H. Wang of the University of Manitoba, Canada.

3. Results

In preliminary studies, we observed that ATP-dependent ^{45}Ca uptake by subcellular fractions of rat parotid glands co-purified with the endoplasmic reticulum marker enzyme NADPH-cytochrome *c* reductase. Moreover, this Ca^{2+} uptake was insensitive to inhibition by ruthenium red, an inhibitor of mitochondrial Ca^{2+} uptake, suggesting that under the conditions used for fractionation, there is little interference from the mitochondrial Ca^{2+} transport system. This may be ascribed to the omission of EGTA from the preparative medium which results in uncoupling of parotid mitochondria and consequent loss of Ca^{2+} transport capacity [15]. The heavy microsomal fraction used in the present study showed the highest enrichment of NADPH-cytochrome *c* reductase activity (18.4 ± 0.3 munits/mg membrane protein, mean ± SEM, $n = 4$), being ~6.5-fold greater than in tissue homogenate.

Fig.1 shows the effect of trifluoperazine, a calmodulin antagonist, on the rate of ^{45}Ca accumulation by the endoplasmic reticulum. Increasing the concentration of trifluoperazine from 10–40 μM resulted in progressively greater inhibition of ^{45}Ca uptake. 50% inhibition of ^{45}Ca uptake required 17 μM trifluoperazine.

Since the effects of trifluoperazine on ^{45}Ca uptake are likely to arise from antagonism of calmodulin function, membrane associated calmodulin was estimated using calmodulin-free, cyclic AMP phosphodiesterase. The endoplasmic reticulum fraction was heated at 90°C for 1.5 min and denatured proteins

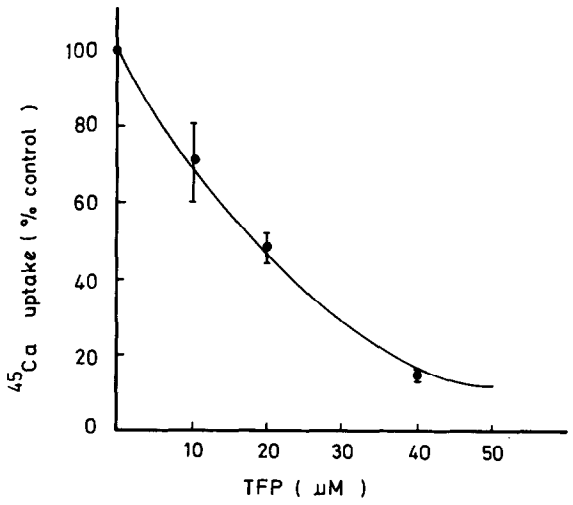

Fig.1. Effect of trifluoperazine on ^{45}Ca uptake by parotid endoplasmic reticulum. ^{45}Ca uptake by endoplasmic reticulum vesicles was determined as described in section 2. Trifluoperazine (TFP) was added to the assay mixture 5 min before initiation of ^{45}Ca uptake with ATP. Results expressed as a % of the rate of ^{45}Ca uptake in the absence of trifluoperazine (28.7 ± 2.1 nmol . 5 min^{-1} . mg $protein^{-1}$, $n = 6$) are the mean of 6 determinations.

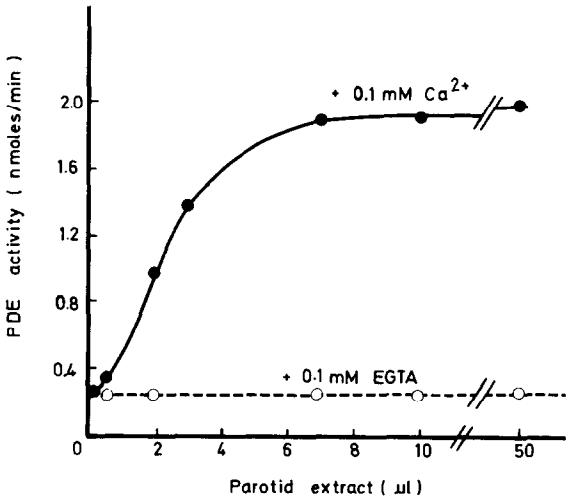

Fig.2. Activation of phosphodiesterase by endoplasmic reticulum-associated calmodulin. Parotid endoplasmic reticulum fraction containing 4.2 mg of protein/ml of 0.25 M sucrose, 5 mM Tris–HCl (pH 7.4), was heated at 90°C for 1.5 min. The suspension was centrifuged and the supernatant (parotid extract) assayed for calmodulin activity. 0.002 units cyclic AMP phosphodiesterase activity of calmodulin-deficient beef heart phosphodiesterase (PDE) was determined in the presence of varying concentrations of parotid extract and 0.1 mM Ca^{2+} (–●–●–) or 0.1 mM EGTA (–○––○–). Results shown are representative of 3 experiments.

removed by centrifugation. Addition of the heat-treated supernatant to cyclic AMP phosphodiesterase increased phosphodiesterase activity in a concentration dependent manner (fig.2). Activation required Ca^{2+} and was abolished in the presence of 0.1 mM EGTA. A similar activation profile was obtained when phosphodiesterase activity was determined in the presence of varying concentrations (0–50 ng) of pure beef brain calmodulin. By comparison, membrane associated calmodulin was estimated to be 4.4 μg/mg membrane protein. Stimulation of phosphodiesterase activity by the heat-treated parotid supernatant was reversed by trifluoperazine. At 20 μM and 40 μM trifluoperazine, calmodulin activated phosphodiesterase activity was inhibited by 72 and 92%, respectively.

Purified calmodulin at concentrations ranging from 1–10 μg/ml had no effect on the rate of ^{45}Ca uptake by the parotid endoplasmic reticulum. Some other Ca^{2+} transport systems containing endogenously bound calmodulin also show a similar lack of sensitivity to calmodulin unless previously depleted of calmodulin [2,5]. Since hypotonic washing in the presence of a Ca^{2+} chelator has been successfully used to deplete erythrocyte membranes of calmodulin [16], the parotid endoplasmic reticulum fraction was washed twice with 0.1 mM EGTA, 10 mM Tris–HCl. As shown in table 1, although the hypotonic treatment resulted in marked loss of Ca^{2+} uptake capacity, it was now possible to demonstrate calmodulin sensitivity. Calmodulin (5 μg/ml) activated Ca^{2+} uptake by the washed membranes by almost 50%.

Table 1
Effect of calmodulin on ^{45}Ca uptake by endoplasmic reticulum before and after hypotonic treatment

Condition	^{45}Ca uptake (nmol . 5 min^{-1} . mg $protein^{-1}$)
Endoplasmic reticulum	22.49 ± 1.56
Endoplasmic reticulum + 5 μg/ml calmodulin	21.05 ± 1.29
Washed endoplasmic reticulum	5.29 ± 0.05
Washed endoplasmic reticulum + 5 μg/ml calmodulin	8.13 ± 0.04

The endoplasmic reticulum fraction was depleted of calmodulin by washing twice with 10 mM Tris–HCl pH 7.4 containing 1 mM EGTA. Ca^{2+} uptake by the membrane vesicles before and after washing was determined as described in section 2 in the presence and absence of added calmodulin. Results are the mean ± SEM of three determinations

4. Discussion

The Ca^{2+}-dependent regulatory protein calmodulin has been shown to activate a variety of Ca^{2+} transport systems [2,4,5,9,10]. We suggest here that it plays a similar regulatory function in controlling Ca^{2+} uptake by the endoplasmic reticulum of rat parotid glands. Like most calmodulin-sensitive systems, Ca^{2+} uptake by the parotid endoplasmic reticulum was inhibited by trifluoperazine. The sensitivity to inhibition was comparable to that shown by beef heart cyclic AMP phosphodiesterase activated with calmodulin. Thus, it is likely that the inhibition of Ca^{2+} uptake arises from specific antagonism of calmodulin function rather than other non-specific effects of the phenothiazine.

For many Ca^{2+} transport systems, activation by calmodulin may be readily demonstrated by addition of exogenous calmodulin [4,17,18]. In this study however, it was necessary to first deplete membranes of calmodulin before sensitivity to this protein could be demonstrated. This suggests that the parotid endoplasmic reticulum contains sufficient calmodulin tightly associated with it for maximal activation of the Ca^{2+} transport system. Such activation would enable the endoplasmic reticulum of parotid acinar cells to counter any increase in intracellular Ca^{2+} that may arise from stimulation of acinar cells with hormones or neurotransmitters, and thereby help to maintain intracellular Ca^{2+} homeostasis.

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