Modulation of active Ca^{2+} uptake by the islet-cell endoplasmic reticulum

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The possible effects of calmodulin and cyclic AMP on active Ca^{2+} uptake by the islet-cell endoplasmic reticulum were investigated. Neither calmodulin nor cyclic AMP affected the rate of active Ca^{2+} uptake, or the steady-state filling capacity of the endoplasmic reticulum when measured in the absence of oxalate. Consistent with these results, calmodulin did not activate the Ca^{2+} -stimulated ATPase activity associated with this cell fraction. During the course of these experiments, it was unexpectedly discovered that the rate of Ca^{2+} uptake, as well as the steady-state Ca^{2+} filling capacity of the endoplasmic reticulum, were markedly increased by unidentified factor(s) in the cytosol. This effect could be demonstrated by reconstitution of the membranes in cytosol, or by direct addition of fresh or dialysed cytosol to the Ca^{2+} uptake assays. The degree of activation by the cytosol indicates that the endoplasmic reticulum may play a prominent role in controlling β -cell Ca^{2+} concentrations and that the unidentified activator(s) present in the cytosol may be involved in regulation of this function.

Extracellular Ca^{2+} is a requirement for glucose-stimulated insulin secretion from the β -cell (Grodsky & Bennett, 1966). Furthermore, insulinogenic stimuli significantly alter Ca^{2+} fluxes in intact islets (Malaisse-Lagae & Malaisse, 1971; Hellman *et al.*, 1976, 1977; Naber *et al.*, 1977; Malaisse *et al.*, 1978), leading to the generally held view that the interaction of Ca^{2+} with specific intracellular sites initiates a sequence of events resulting in the secretion of insulin. In this sequence of events, critical concentrations of Ca^{2+} are believed to be regulated both at the cell surface as well as by intracellular organelles.

We have recently described a technique for obtaining an islet-cell fraction that is enriched in endoplasmic reticulum and have characterized a system for studying ATP-dependent Ca^{2+} uptake by this fraction (Colca *et al.*, 1982). Ca^{2+} uptake was ascribed to the endoplasmic reticulum by morphological and biochemical characterization, the use of selective inhibitors, and by simultaneous comparison of active Ca^{2+} uptake by various subcellular fractions of islet cells. The present paper seeks to identify physiological agents that modulate this active Ca^{2+} uptake.

In cardiac muscle, the rate of Ca^{2+} uptake by the sarcoplasmic reticulum appears to be regulated by the Ca^{2+} binding protein, calmodulin, secondary to the phosphorylation of phospholamban (Le Peuch *et al.*, 1979; Lopaschuck *et al.*, 1980; Kirchberger &

Antonetz, 1982), and also through a similar mechanism by cyclic AMP-dependent protein kinase (Kirchberger *et al.*, 1974; Kirchberger & Chu, 1976; Le Peuch *et al.*, 1979). Calmodulin is present in islet cells (Sugden *et al.*, 1979; Landt *et al.*, 1982) and has been indirectly implicated in glucose-induced insulin secretion by studies that have utilized calmodulin antagonists (Krausz *et al.*, 1980; Gagliardino *et al.*, 1980). Calmodulin has also been shown to stimulate (Ca^{2+} + Mg^{2+})-dependent ATPase activity (Kotagal *et al.*, 1982) and Ca^{2+} uptake by the islet-cell plasma membrane (Pershadsingh *et al.*, 1980a). The importance of cyclic AMP in potentiating glucose-induced insulin secretion is well known (Malaisse *et al.*, 1967; Lambert *et al.*, 1971). It has been postulated that the modification of insulin secretion by cyclic AMP occurs secondary to the liberation of intracellular Ca^{2+} (Siegel *et al.*, 1980).

The present paper reports on detailed investigations of the possible impact of calmodulin, cyclic AMP and cytosolic activators on active Ca^{2+} uptake and Ca^{2+} -stimulated ATPase activity in the islet-cell endoplasmic reticulum.

Experimental

Materials

Male Wistar rats (200–300 g) were purchased from Charles Rivers Laboratories (Wilmington,

MA, U.S.A.). Collagenase (CLS IV) was obtained from Worthington (Freehold, NJ, U.S.A.), $^{45}\text{CaCl}_2$ (20–30 Ci/g) from New England Nuclear (Boston, MA, U.S.A.), and calmodulin from Calbiochem (La Jolla, CA, U.S.A.). Vanadate-free ATP, cyclic AMP-dependent protein kinase (type I) and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Islet fractionation

Islets were obtained by collagenase digestion (Lacy & Kostianovsky, 1967) of pancreata dissected from 30–33 rats. The isolated islets were harvested and purified by separation on Ficoll gradients (Shibata *et al.*, 1976) followed by repeated washes in Hanks solution and selection under a dissecting microscope. The islets were cultured overnight in CMRL 1066 (GIBCO, Grand Island, NY, U.S.A.) medium containing 10% heat-inactivated foetal-calf serum, 1% penicillin, 1% streptomycin, 1% L-glutamine and 8 mM-glucose [24°C under CO_2/air (1:19)] before subcellular fractionation (Colca *et al.*, 1982).

The cultured islets (6000–10000) were pooled and fractionated by the technique of Naber *et al.* (1980) with modifications as described previously (Colca *et al.*, 1982). Briefly, the islets were homogenized in 1 ml of fractionation buffer [50 mM-MeS (4-morpholine-ethanesulphonic acid)/250 mM-sucrose/1 mM-EDTA, pH 7.2 at 4°C] in a no. 19 Kontes glass homogenizer with a Teflon pestle. The homogenate was centrifuged twice at 600 g for 5 min to remove nuclei and cell debris. The supernatants were pooled and centrifuged at 20000 g for 20 min, and the resulting post-mitochondrial supernatant was pelleted at 150000 g for 90 min to yield an endoplasmic-reticulum-enriched pellet. This fraction contained a 2–3-fold enrichment of endoplasmic-reticulum marker enzymes (NADH-cytochrome *c* reductase and glucose 6-phosphatase) relative to the homogenate and contained virtually no contamination with mitochondria or secretory granules (Colca *et al.*, 1982).

The supernatant resulting from the 150000 g centrifugation was considered to represent the cytosol and was itself devoid of membranes capable of accumulating Ca^{2+} . Protein was measured by a fluorimetric technique using fluorescamine (Orr *et al.*, 1976).

Ca^{2+} uptake

ATP-dependent or active Ca^{2+} uptake was measured by a technique described previously (Colca *et al.*, 1982) using oxalate-facilitated Ca^{2+} uptake with $^{45}\text{Ca}^{2+}$, followed by rapid membrane filtration. Briefly, incubations (0.1 ml) containing 50 mM-Tris, 10 mM-oxalate, 5 mM- MgCl_2 and 100 mM-KCl (pH 6.8 at 37°C), ± 1.25 mM-ATP,

were initiated by the addition of 1–5 μg of endoplasmic-reticulum protein, and terminated by filtration over Millipore filters (0.22 μm pore size). Addition of oxalate linearizes the rate of Ca^{2+} uptake for at least 30 min. In cases where the steady-state Ca^{2+} filling capacity was studied, oxalate was omitted from the incubation medium. Ca^{2+} (0.1–0.2 μCi of $^{45}\text{Ca}^{2+}$ and 2–20 nmol of total Ca^{2+} per tube) was buffered by the addition of 0.2 mM-EGTA. In some experiments, added Ca^{2+} was buffered only by oxalate and ATP present in the medium. Free Ca^{2+} levels were calculated as described previously (Colca *et al.*, 1982) by using the following association constants: Ca^{2+} –EGTA = $10^{5.94}$; Ca^{2+} –oxalate = $10^{2.3}$; Mg^{2+} –oxalate = $10^{2.53}$; Ca^{2+} –ATP = $10^{4.5}$; Mg^{2+} –ATP = $10^{4.94}$. Omission of EGTA from the incubation medium results in a higher specific radioactivity of $^{45}\text{Ca}^{2+}$, thus providing greater sensitivity, and also obviates the possible artifactual effect of EGTA (Kotagal & McDaniel, 1982).

The effect of cytosol on Ca^{2+} uptake

To evaluate the effect of cytosol on Ca^{2+} uptake by the endoplasmic reticulum, some experiments utilized the post-mitochondrial supernatant (S_2). S_2 contained the endoplasmic-reticulum fraction together with cytosol. The effect of cytosol on Ca^{2+} uptake was evaluated by: (1) rehomogenization of endoplasmic reticulum with cytosol before assay (reconstitution studies) and (2) direct addition of cytosol to assays employing the standard endoplasmic-reticulum preparation (direct addition assays). In reconstitution protocols S_2 was divided into several portions before centrifugation at 150000 g. The membrane pellets were then individually rehomogenized in the supernatant cytosol or in fractionation buffer. In these reconstitution studies, Ca^{2+} uptake assays were then initiated by the addition of 10 μl of the membrane suspension. Separate experiments involved direct addition of cytosol to the uptake assays. When cytosol was added directly to the uptake assays, 10 μl of cytosol (8–12 μg of protein) was added in addition to 10 μl of the standard endoplasmic-reticulum membrane suspension.

Ca^{2+} -stimulated ATPase

ATPase activity was measured by a modification of the technique of Seals *et al.* (1978). The isotopic assay measures the liberation of [^{32}P]P_i from [γ - ^{32}P]ATP in incubations (100 μl) consisting of 50 mM-Tris, 0.1 mM- MgCl_2 , 12.5 mM-KCl, 0.5 mM-ATP (0.1–0.2 μCi /tube) and 0.2 mM-EGTA $\pm$ 0.1–0.2 mM- CaCl_2 (pH 6.8 at 37°C). Assays were initiated by the addition of 0.5–1 μg of endoplasmic-reticulum protein and terminated after 1–30 min with addition of 50 μl of 3% sodium dodecyl sulphate.

ATPase assays utilized endoplasmic reticulum protein that had been sonicated (Branson sonifier; 5 s at 19 W) and rendered transport-incompetent. The rate of ATP hydrolysis was linear for at least 30 min, and in no case was more than 5% of the ATP hydrolysed. Ca²⁺ levels were varied by addition of CaCl₂, and free Ca²⁺ concentrations were calculated as described above. Ca²⁺-stimulated ATPase activity was determined by subtracting the ATPase activity obtained in the presence of 0.2 mM-EGTA alone from the activity obtained in parallel incubations that contained Ca²⁺. Endogenous Ca²⁺ levels were less than 5 μM as determined by atomic-absorption spectrophotometry.

Unless otherwise indicated *n* refers to the number of individual tissue preparations used for a particular experiment. Statistical evaluations were by analysis of variance or by paired *t* analysis, as appropriate.

Results

Effect of calmodulin

Initial studies showed that calmodulin at concentrations up to 30 μg/ml (1.8 μM) at subsaturating (1 μM) or saturating (15 μM) Ca²⁺ concentrations did not affect the rate of Ca²⁺ uptake by the islet-cell endoplasmic reticulum. These studies routinely employed 100 mM-K⁺, which maximally stimulates Ca²⁺ uptake by this system (Colca *et al.*, 1982). As the effect of K⁺ may preclude or obscure further stimulation by calmodulin (Lopaschuck *et al.*, 1980), calmodulin was added to calcium uptake assays, which were performed in the presence or absence of 100 mM-KCl. Again, calmodulin did not exert a detectable effect in the presence or absence of 100 mM-KCl and whether or not 200 mM-sucrose was added as an osmotic replacement for the omitted KCl. Fig. 1 shows the lack of an effect of calmodulin on Ca²⁺ uptake when tested in the presence or absence of 100 mM-KCl over a complete range of Ca²⁺ concentrations (0–30 μM). Although K⁺ stimulated Ca²⁺ uptake (*P* < 0.05), calmodulin did not exert a detectable effect on Ca²⁺ uptake in either case.

In many of these studies EGTA was used to buffer and maintain free Ca²⁺ concentrations. As several

reports from other laboratories (Sarkadi *et al.*, 1979; Al-Jabore & Roufogalis, 1981), as well as our own (Kotagal & McDaniel, 1982), have indicated that EGTA may itself alter Ca²⁺-sensitive enzyme systems, thus reducing or obscuring a response to calmodulin, these experiments were repeated with lower concentrations of total Ca²⁺ without added EGTA. In no case, with or without EGTA-buffered Ca²⁺ concentrations, did calmodulin stimulate the rate of Ca²⁺ uptake by the islet-cell endoplasmic reticulum (Table 1).

Effect of cyclic AMP

When ATP-dependent Ca²⁺ uptake was measured at 1 μM-free Ca²⁺ (140 μM-CaCl₂ and 200 μM-EGTA), 5–100 μM-cyclic AMP did not affect the rate of Ca²⁺ uptake by the islet-cell endoplasmic reticulum. Addition of 1–100 μM-cyclic AMP to standard assays employing 16–19 μM-free calcium (minus EGTA) also did not affect the rate of Ca²⁺ uptake during a 15 min incubation (Table 2).

Fig. 1. Effect of calmodulin on Ca²⁺ uptake by islet-cell endoplasmic reticulum (±K⁺)

Ca²⁺ uptake was measured under conditions described in the Experimental section; 15 min incubations ± 15 μg of calmodulin/ml were performed in the presence and absence of 100 mM-KCl at various Ca²⁺ concentrations. Results are means of four experiments; S.E.M. bars are omitted from the graph for clarity.

Table 1. Effect of calmodulin on active Ca²⁺ uptake by islet-cell endoplasmic reticulum

Ca²⁺ uptake was measured in 15 min incubations at 37°C in 50 mM-Tris/5 mM-MgCl₂/10 mM-oxalate/200 mM-sucrose (–KCl) ± 1.25 mM-ATP. [Ca²⁺] was maintained at 1 μM with 0.2 mM-EGTA or at 16–18 μM (44–48 μM total calcium) in the absence of EGTA. Results are means ± S.E.M. for the numbers of experiments indicated in parentheses.

Calmodulin (μg/ml) ...	Ca ²⁺ uptake (nmol/mg per min)				
	0	2.0	7.5	15	30
1.0 μM-Ca ²⁺ (+EGTA)	0.34 ± 0.05	0.38 ± 0.09	0.39 ± 0.11	0.49 ± 0.08	0.40 ± 0.09 (4)
16 μM-Ca ²⁺ (–EGTA)	0.72 ± 0.15	0.70 ± 0.08	0.73 ± 0.10	0.74 ± 0.08	0.82 ± 0.11 (5)

Table 2. *Effect of cyclic AMP on active Ca²⁺ uptake by islet-cell endoplasmic reticulum*

Ca²⁺ uptake was measured in 15 min incubations at 37°C in 50 mM-Tris/5 mM-MgCl₂/100 mM-KCl/10 mM-oxalate, $\pm$ 1.25 mM-ATP. [Ca²⁺] was maintained at 1 μ M with 0.2 mM-EGTA or at 16–18 μ M in the absence of EGTA. Results are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses.

[Cyclic AMP] (μ M) ...	Ca ²⁺ uptake (nmol/mg per min)				
	0	5	10	50	100
1.0 μ M-Ca ²⁺ (+EGTA)	0.54 $\pm$ 0.11	0.49 $\pm$ 0.13	0.53 $\pm$ 0.12	0.51 $\pm$ 0.12	0.55 $\pm$ 0.13 (4)
16 μ M-Ca ²⁺ (–EGTA)	0.89 $\pm$ 0.18	0.81 $\pm$ 0.13	0.82 $\pm$ 0.18	0.84 $\pm$ 0.16	0.85 $\pm$ 0.15 (3)

Table 3. *Effect of cyclic AMP and cyclic AMP-dependent protein kinase on active Ca²⁺ uptake in islet-cell endoplasmic reticulum*

Active Ca²⁺ uptake was measured in 15 min incubations by the standard technique as described in the Experimental section. [Ca²⁺] was maintained at 1 μ M with 0.2 mM-EGTA. Results are means $\pm$ S.E.M. for four experiments that contained the indicated concentrations of type I protein kinase with or without cyclic AMP.

Protein kinase (mg/ml) ...	Ca ²⁺ uptake (nmol/mg per min)				
	0	0.05	0.10	0.25	0.50
No Cyclic AMP	0.73 $\pm$ 0.12	0.73 $\pm$ 0.12	0.73 $\pm$ 0.12	0.72 $\pm$ 0.10	0.65 $\pm$ 0.08
10 μ M-Cyclic AMP	0.68 $\pm$ 0.09	0.69 $\pm$ 0.10	0.70 $\pm$ 0.10	0.74 $\pm$ 0.10	0.64 $\pm$ 0.09

Similarly, cyclic AMP did not affect Ca²⁺ uptake at any time period of incubation (5–45 min; results not shown).

As an effect of cyclic AMP on Ca²⁺ uptake would likely depend on the presence of a cyclic AMP-dependent protein kinase, which may be removed by the fractionation procedure, similar studies were repeated with addition of various concentrations of bovine heart cyclic AMP-dependent protein kinase (0–0.5 mg/ml). The addition of protein kinase with or without 10 μ M-cyclic AMP also did not affect the rate of Ca²⁺ uptake by the endoplasmic reticulum (Table 3). Similar results were obtained in an additional experiment, which employed a similar protocol, but where the endoplasmic reticulum was pre-incubated for 5 min with and without cyclic AMP and protein kinase before a 15 min assay.

To further test the possibility that cyclic AMP may affect Ca²⁺ uptake by an endogenous islet-cell protein kinase, a series of experiments were performed using the endoplasmic reticulum fraction which was suspended in cytosol, or using the post-mitochondrial supernatant before pelleting the endoplasmic-reticulum-enriched membranes. Under these conditions cyclic AMP (1–50 μ M $\pm$ 5 mM-theophylline) still did not detectably alter Ca²⁺ uptake by the islet-cell endoplasmic reticulum, whether these studies were performed at 16 μ M-Ca²⁺ in the absence of EGTA (10–90 min incubations; n = 3), or at 0.5–30 μ M-free Ca²⁺ (results not shown). Similarly, pretreatment of the post-mitochondrial supernatant

or endoplasmic-reticulum fraction (with or without reconstitution in cytosol) with 10 μ M-cyclic AMP (5 min at 37°C) did not affect the subsequent rate of Ca²⁺ uptake.

Reconstitution with cytosol

During the course of these studies it became apparent that the rate of Ca²⁺ uptake is much greater in the post-mitochondrial supernatant (S₂) than in the subsequently isolated endoplasmic reticulum (Fig. 2), although the cytosol removed by this fractionation did not itself accumulate Ca²⁺. The subsequent Ca²⁺ uptake activity in the endoplasmic-reticulum fraction seemed to depend on the concentration of cytosol present during the isolation of these membranes. Thus, dilution (2-fold) of the post-mitochondrial supernatant before separation of the endoplasmic reticulum reduced the subsequent rate of Ca²⁺ uptake by 38 $\pm$ 10% (P < 0.05, n = 6). Furthermore, reconstitution of the endoplasmic reticulum with cytosol produced a 2–3-fold increase in the rate of Ca²⁺ uptake compared with uptake of endoplasmic reticulum resuspended in fractionation buffer (Fig. 2). Heat treatment of cytosol for 5 min in a boiling-water bath reduced this activation of Ca²⁺ uptake by 25–30%. Although reconstitution of the endoplasmic reticulum with cytosol increased the velocity of uptake at all Ca²⁺ concentrations tested, the apparent K_m of 5.8 $\pm$ 3 μ M for free Ca²⁺ determined in the absence of EGTA was not changed by reconstitution with cytosol.

Fig. 2. Effect of reconstitution of the islet-cell endoplasmic reticulum with cytosol on Ca²⁺ uptake

Islet cells were fractionated by the technique described in the Experimental section. Ca²⁺ uptake was measured over a range of Ca²⁺ concentrations (–EGTA) using the post-mitochondrial supernatant (S₂) or the subsequently obtained endoplasmic-reticulum-enriched fraction (ER). The endoplasmic-reticulum-enriched fraction was reconstituted with cytosol from the same subcellular fractionation. A portion of the cytosol was heat-treated (5 min in a boiling-water bath) before reconstitution with the endoplasmic-reticulum-enriched fraction. Results were obtained from four experiments.

Direct addition of cytosol

Addition of cytosol to the Ca²⁺ uptake assay employing the endoplasmic reticulum resuspended in fractionation buffer (as shown in Fig. 3) increased the velocity of Ca²⁺ uptake by 24% ($V_{\max}$, 2.1 ± 0.1 compared with 1.7 ± 0.05 nmol/mg per min; $P < 0.05$). This treatment did not alter the apparent K_m for Ca²⁺ measured in the absence of EGTA (6.2 ± 0.13 compared with 6.4 ± 0.8 μM respectively) as determined by double-reciprocal analysis. The effect of directly added cytosol on Ca²⁺ uptake was concentration-dependent and was reduced by $65 \pm 11\%$ by prior heat treatment of cytosol (Fig. 4).

To further characterize the nature of the activation afforded by the added cytosol, cytosol from four separate preparations of islets (40000) was dialysed against distilled water using a 1000-mol.wt.-cut-off dialysis membrane and concentrated by freeze-drying. The addition of the freeze-dried and dialysed cytosol produced a concentration-dependent increase in the rate of active Ca²⁺ uptake ($n = 3$; 30% maximum; $P < 0.05$; results not shown).

Steady-state filling capacity

It is possible that the active steady-state filling capacity of the endoplasmic reticulum may be modified without alteration of the rate of Ca²⁺ uptake. Such an effect may be masked when the rate

Fig. 3. Effect of cytosol on Ca²⁺ uptake by the islet-cell endoplasmic reticulum

Ca²⁺ uptake was measured under standard conditions using the endoplasmic-reticulum-enriched fraction as described in the Experimental section. Fractionation buffer or cytosol obtained from the same subcellular fractionation was added directly to the Ca²⁺ uptake assays during a 15 min incubation at 37°C. Results are means $\pm$ S.E.M. (represented by the bars) of three experiments.

Fig. 4. Effect of dilutions of fresh or heat-treated cytosol on Ca²⁺ uptake by islet-cell endoplasmic reticulum

Ca²⁺ uptake was measured under the conditions described in the Experimental section. Total calcium (44–48 μM) was buffered only by ATP and oxalate resulting in [Ca²⁺] being 16–18 μM. A portion of cytosol obtained from the same subcellular fractionation as the endoplasmic reticulum was placed in a boiling-water bath for 5 min. Heat-treated or untreated cytosol was added directly to the uptake assay before a 15 min incubation. Results are means $\pm$ S.E.M. (represented by the bars) of five experiments.

of Ca²⁺ uptake is sustained by incubation with oxalate. Therefore, the effects of calmodulin, cyclic AMP and cytosol were also evaluated in the absence of oxalate. Under these conditions steady-state

Fig. 5. Steady-state Ca^{2+} uptake measured in the absence of oxalate

Steady-state active Ca^{2+} uptake was measured in 15 min incubations in medium containing 50 mM-Tris, 100 mM-KCl, 5 mM- MgCl_2 , 44–48 μM -total calcium, 1.25 mM-ATP and 2–4 μg of endoplasmic-reticulum protein. The assays employed the post-mitochondrial supernatant (S_2), endoplasmic reticulum (ER) fraction that was reconstituted with cytosol or untreated endoplasmic reticulum. In each case cyclic AMP (10 μM) or calmodulin (16 $\mu\text{g}/\text{ml}$) was added directly to the Ca^{2+} uptake assay. Results are means $\pm$ S.E.M. ($n = 3$).

uptake in the presence of ATP is achieved in 15 min (Colca *et al.*, 1982). The results of these studies are summarized in Fig. 5. The addition of cytosol by prior reconstitution with the endoplasmic-reticulum-enriched membranes or by direct addition to the Ca^{2+} uptake assays increased the steady-state ATP-dependent Ca^{2+} uptake. Although the addition of cyclic AMP under these conditions produced no change whatsoever, calmodulin (15 $\mu\text{g}/\text{ml}$) tended to increase Ca^{2+} uptake in two of the three experiments ($P > 0.1$).

Ca^{2+} -stimulated ATPase activity

Active Ca^{2+} transport by the endoplasmic reticulum, if analogous to other Ca^{2+} -transporting systems, is undoubtedly coupled to Ca^{2+} -stimulated ATP hydrolysis. The Ca^{2+} -stimulated ATPase of the islet endoplasmic reticulum has not previously been characterized. Initial experiments were therefore performed to define Ca^{2+} -stimulated ATPase activity in this fraction. ATPase activity of the endoplasmic-reticulum-enriched fraction measured at endogenous concentrations of Mg^{2+} (8–9 μM), i.e., without added Mg^{2+} , showed a similar dependence on Ca^{2+} as did active transport. The basal rate of ATP hydrolysis measured in the absence of Ca^{2+} was 0.42 ± 0.24 nmol/mg per min and increased by 21.4 ± 13.8 nmol/mg per min on maximal activation

Fig. 6. Effect of calmodulin on Ca^{2+} -stimulated ATPase activity associated with islet-cell endoplasmic reticulum. Ca^{2+} -stimulated ATPase activity was measured in 15 min incubations as described in the Experimental section. Results are means $\pm$ S.E.M. ($n = 5$).

by Ca^{2+} , with half-maximal activation occurring at 3.0 ± 1.1 μM ionized calcium ($n = 3$). As the concentration of Mg^{2+} in the assay was increased, it became progressively more difficult to measure the Ca^{2+} stimulation over the Mg^{2+} -stimulated ATPase activity. However, addition of 50 or 100 μM - Mg^{2+} , although reducing the apparent V_{max} of Ca^{2+} -stimulated ATPase activity (12.5 ± 4 and 11 ± 0.4 nmol/mg per min respectively), did not change the apparent K_m for Ca^{2+} (2.7 ± 1.1 and 3.6 ± 0.7 ; $n = 3$). Hence ATPase assays employed 100 μM - Mg^{2+} to approximate conditions where active Ca^{2+} uptake could also be measured.

Since K^+ stimulates Ca^{2+} uptake by the endoplasmic reticulum, the effect of K^+ on the ATPase activity was also evaluated. In three experiments, 6.25 and 12.5 mM- K^+ increased Ca^{2+} -stimulated ATPase activity by $20 \pm 11\%$, whereas at higher K^+ concentrations of 50 and 100 mM the rate of the Ca^{2+} -stimulated component of the ATPase activity was significantly reduced by $31 \pm 3\%$ and $61 \pm 11\%$ respectively. Subsequent assays therefore employed 12.5 mM- K^+ .

The addition of 8 μg of calmodulin/ml did not significantly alter the Ca^{2+} -stimulated ATPase activity at any of the Ca^{2+} concentrations tested (Fig. 6). Neither did calmodulin stimulate Ca^{2+} -stimulated ATPase activity in three other experiments that utilized higher concentrations of calmodulin (15–30 $\mu\text{g}/\text{ml}$; results not shown). The only effect of calmodulin in these experiments was to elevate the background Mg^{2+} -stimulated ATPase. This effect served to reduce the calculated Ca^{2+} -stimulated activity.

As the islet-cell cytosol contains significant ATPase activity, it was not possible to interpret the effect of native cytosol on ATPase activity of the endoplasmic reticulum fraction. However, the ad-

dition of heat-treated cytosol to the ATPase assays gave similar results to those for the addition of exogenous calmodulin. Namely, at higher concentrations of heat-treated cytosol Mg²⁺-stimulated ATPase activity increased ($33 \pm 7\%$, $P < 0.05$), whereas the rate of Ca²⁺-stimulated ATP hydrolysis was reduced (-14% ; $P > 0.05$).

Discussion

The addition of calmodulin to isolated islet-cell endoplasmic reticulum did not significantly increase the rate of active or ATP-dependent Ca²⁺ uptake. This result is consistent with the lack of effect of calmodulin on Ca²⁺ uptake by endoplasmic reticulum derived from adipocytes (Pershad Singh *et al.*, 1980b) and by sarcoplasmic reticulum derived from skeletal (Chiesi & Carafoli, 1982) and smooth muscle (Wuytack *et al.*, 1980; Wibo *et al.*, 1981). It would appear, therefore, that calmodulin activation of Ca²⁺ uptake in cardiac-muscle sarcoplasmic reticulum may represent a unique adaptation serving the peculiar function of that tissue.

As there is some controversy as to whether the presence of high concentrations of K⁺ may obscure or preclude a response of cardiac sarcoplasmic reticulum to calmodulin (Lopaschuck *et al.*, 1980; Kirchberger & Antonetz, 1982), the possible effects of calmodulin in the present study were evaluated with and without K⁺ in the incubation buffer. Although K⁺ stimulated the rate of Ca²⁺ uptake by the islet-cell endoplasmic reticulum, calmodulin was without effect.

The effect of calmodulin was also evaluated on Ca²⁺ uptake under conditions in which free Ca²⁺ concentrations were buffered with or without EGTA. It is possible that EGTA or Ca²⁺-EGTA may itself exert an effect on Ca²⁺-sensitive processes (Sarkadi *et al.*, 1979; Al-Jabore & Roufogalis, 1981). We have recently shown that high concentrations of Ca²⁺-EGTA obscure or preclude activation by calmodulin of the (Ca²⁺ + Mg²⁺)-dependent ATPase localized to the islet-cell plasma membrane (Kotagal & McDaniel, 1982). However, unlike the islet-cell plasma membrane (Pershad Singh *et al.*, 1980a; Kotagal *et al.*, 1982), the Ca²⁺ uptake system of the endoplasmic reticulum was not stimulated by calmodulin. In general, it would appear that the Ca²⁺-transporting systems of the plasma membrane and endoplasmic reticulum are regulated in a fundamentally different manner. Thus, although both Ca²⁺ uptake and Ca²⁺-stimulated ATPase activity in plasma membrane derived from a variety of tissues are stimulated by calmodulin (Larsen & Vincenzi, 1979; Sobue *et al.*, 1979; Pershad Singh *et al.*, 1980b; Caroni & Carafoli,

1981; Kotagal *et al.*, 1982), calmodulin does not appear to stimulate Ca²⁺ uptake in the endoplasmic reticulum. Consistent with the lack of effect on Ca²⁺ transport by the islet-cell endoplasmic reticulum, calmodulin also did not increase Ca²⁺-stimulated ATPase activity in this cell fraction.

Therefore, although calmodulin is certainly important in the regulation of β -cell function (Tomlinson *et al.*, 1982), and although we have previously shown that the addition of Ca²⁺ and calmodulin (but not Ca²⁺ alone) increases protein kinase activity in this islet-cell fraction (Landt *et al.*, 1982), calmodulin does not appear to regulate the rate of active Ca²⁺ transport by the islet-cell endoplasmic reticulum.

Cyclic AMP-dependent protein kinase has been reported to increase Ca²⁺ uptake in cardiac sarcoplasmic reticulum secondary to phosphorylation of an intrinsic protein, phospholamban (Kirchberger *et al.*, 1974; Kirchberger & Chu, 1976; Le Peuch *et al.*, 1979). Although the effect of cyclic AMP-dependent protein kinase on phosphorylation is a direct effect, it is not clear whether the cyclic AMP-mediated stimulation of Ca²⁺ uptake by the cardiac sarcoplasmic reticulum requires the concomitant presence of calmodulin [Le Peuch *et al.*, 1979; cf. the results of Kirchberger *et al.* (1974) and Kirchberger & Chu (1976)]. Cyclic AMP has also been reported to increase Ca²⁺ uptake by microsomes prepared from platelets (Kaser-Glanzmann *et al.*, 1977). The possible effects of cyclic AMP on the islet-cell endoplasmic reticulum were investigated by using direct addition of cyclic AMP with and without addition of exogenous cyclic AMP-dependent protein kinase. Cyclic AMP was also added to endoplasmic-reticulum fractions that had been resuspended in cytosol such that assay conditions would contain endogenous protein kinase (Harrison & Ashcroft, 1982) as well as calmodulin [$2-4 \mu\text{g/ml}$ (Landt *et al.*, 1982)]. In no case, with or without the addition of the phosphodiesterase inhibitor theophylline, did cyclic AMP exert an effect on Ca²⁺ uptake by the islet-cell endoplasmic reticulum. Sehlin (1976) demonstrated that cyclic AMP decreased steady-state Ca²⁺ uptake by a crude islet-cell microsomal preparation. However, this latter cell fraction was not completely characterized, and may not have been completely cleared of mitochondria since the Ca²⁺ uptake was sensitive to azide. Howell & Montague (1975) also reported that cyclic AMP reduced Ca²⁺ uptake by islet-cell homogenates, but that this effect could be localized to a subcellular fraction enriched in mitochondria and secretory granules. In our own studies (results not shown), 5 mM-theophylline produced a $22 \pm 6\%$ decrease ($P < 0.05$; $n = 3$) in Ca²⁺ uptake by a fraction enriched in mitochondria and secretory granules (20 000 g pellet), whereas cyclic AMP did

not exert an additional effect. This is consistent with the results of Sugden & Ashcroft (1978), who showed that theophylline, but not cyclic AMP, reduced Ca^{2+} uptake by a preparation of islet-cell mitochondria.

It is conceivable that Ca^{2+} accumulation by the endoplasmic reticulum may be regulated physiologically without detectably altering Ca^{2+} -stimulated ATPase activity or the rate of active Ca^{2+} uptake. This may be accomplished, for example, by altering the number of intravesicular binding sites. As such an effect may be obscured when the rate of Ca^{2+} uptake is maintained by the addition of a permeant anion, the steady-state filling capacity of the islet-cell endoplasmic reticulum was investigated in the absence of oxalate. Under these conditions neither cyclic AMP nor calmodulin exerted a significant effect on the amount of active Ca^{2+} uptake.

Studies that were designed to test the effect of cyclic AMP on endoplasmic reticulum suspended in cytosol demonstrated that the endoplasmic reticulum fragments that were suspended in cytosol exhibited a much higher rate of Ca^{2+} uptake than the subsequently isolated endoplasmic reticulum. Although part of the loss of activity may be explained by physical damage to the membranes during the subsequent centrifugation, the existence of a cytosolic activator(s) is supported by the following evidence: (1) dilution of the post-mitochondrial supernatant before centrifugation leads to a greater loss of uptake activity; (2) resuspension of the isolated endoplasmic reticulum in cytosol increases the rate of Ca^{2+} uptake; (3) direct addition of cytosol to Ca^{2+} uptake assays increases the rate of Ca^{2+} uptake in a concentration-dependent manner. The cytosolic activator is either highly charged and/or has a mol.wt. >1000 as it is retained by membrane dialysis. This cytosolic activator is not likely to be calmodulin, since calmodulin does not increase Ca^{2+} uptake under these conditions and the factor is at least partially heat-labile, whereas calmodulin is heat-stable under these conditions.

The activation of Ca^{2+} uptake by cytosol could not be correlated with a concomitant stimulation of Ca^{2+} -stimulated ATPase activity. The effect of fresh cytosol on ATPase activity could not be tested, since the cytosol itself contained ATPase activity. Heat-treatment (5 min in a boiling-water bath) destroyed the cytosol ATPase activity. Although the heat-treated cytosol still retained partial stimulatory activity with regard to Ca^{2+} uptake, it did not increase Ca^{2+} -stimulated ATPase activity. The interpretation of these studies concerning Ca^{2+} -stimulated ATPase activity is complicated as this is not the only ATPase activity present in this fraction. Further studies are required to convincingly demonstrate that this activity actually represents the Ca^{2+} transport enzyme in the endoplasmic reticulum.

Cytosol, either by prior reconstitution with the endoplasmic-reticulum membrane or by direct addition to the Ca^{2+} uptake assays, increased the steady-state accumulation of Ca^{2+} as well as the rate of oxalate-sustained active Ca^{2+} uptake. The effect of cytosol on steady state accumulation of Ca^{2+} may well be more important physiologically than the effect on the initial rate of transport that was measured in the presence of oxalate. The cytosol factor(s) may stimulate the Ca^{2+} uptake without directly affecting the transport enzyme, e.g. by increasing intravesicular binding sites. In any event, the activated steady-state accumulation of Ca^{2+} by the endoplasmic reticulum indicates that the endoplasmic reticulum has the capacity to play a significant role in controlling Ca^{2+} concentrations in the β -cell.

The nature of the cytosolic activator(s) remains to be elucidated. The possibility exists that β -cell lipids may be involved in this response. Lipids are known to be required for Ca^{2+} transport in the sarcoplasmic reticulum (de Meis, 1981). The importance of cellular phospholipids as possible physiological modulators has recently been emphasized (Berridge, 1981). Additionally, unidentified protein modulators of Ca^{2+} handling by the plasma membrane (McDonald *et al.*, 1981; Lotersztajn *et al.*, 1981), mitochondria (Fleschner *et al.*, 1982), as well as the sarcoplasmic reticulum (Chiesi & Carafoli, 1982), in other systems have been reported.

McDonald and colleagues (Bruns *et al.*, 1976; Black *et al.*, 1980) have also reported that the rate of Ca^{2+} uptake by the adipose endoplasmic reticulum is higher in the post-mitochondrial supernatant than in the subsequently isolated fraction. Although these investigators did not test the effectiveness of resuspension of the isolated endoplasmic reticulum in cytosol, they did report that addition of Mg^{2+} and ATP to the postmitochondrial supernatant, before centrifugation to yield the endoplasmic-reticulum fraction, preserved the rate of Ca^{2+} uptake in the isolated fraction (Black *et al.*, 1980). We have also observed that addition of 0.5 mM-ATP and 3 mM- MgCl_2 to the postmitochondrial supernatant produced a 55–60% increase in the rate of Ca^{2+} uptake by the subsequently isolated islet-cell endoplasmic reticulum (results not shown). It is not clear if this activation is by the same mechanism(s) as the activation of Ca^{2+} uptake in the isolated fraction by the addition of cytosol. However, it is interesting to speculate that the activation produced by the islet-cell cytosol is an active process resulting in either the phosphorylation of endoplasmic-reticulum components or involving the active transport of cytosolic activators into the endoplasmic reticulum. In any event, the magnitude of these effects of the cytosol on Ca^{2+} uptake indicates that these factors could play a significant role in modulating β -cell Ca^{2+}

concentrations, and that the nature of these effects should be further explored.

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