PRESENCE AND POSSIBLE ROLE OF CALCIUM-DEPENDENT REGULATOR (CALMODULIN) IN RAT ISLETS OF LANGERHANS

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Received 29 June 1979

1. Introduction

A rise in the intracellular concentration of free Ca^{2+} has been implicated as a key event in the initiation of insulin secretion [1,2] but the mechanism by which Ca^{2+} triggers exocytosis is not known. In other Ca^{2+}-regulated processes, Ca^{2+} exerts a controlling influence by binding to specific proteins, e.g., troponin C in the initiation of muscle contraction, and phosphorylase kinase in the activation of glycogenolysis [3,4]. On the basis of amino acid sequence and X-ray crystallographic data a family of 5 homologous Ca^{2+}-modulated proteins: calcium dependent regulator (calmodulin); troponin C; myosin light chains; muscle pervalbumin; and intestinal calcium binding protein, has been described [5]. Binding of Ca^{2+} is suggested to depend on the presence of homologous α-helical domains referred to as EF-hands [5]. Calmodulin may be of special significance as a general intracellular Ca^{2+}-receptor protein in view both of its ubiquitous tissue distribution in eukaryotes and of the variety of proteins to which it confers sensitivity to Ca^{2+}. Originally discovered [6] as a heat-stable protein activator of brain cyclic nucleotide phosphodiesterase (PDE), calmodulin has been shown inter alia to activate a detergent-solubilized preparation of brain adenylyl cyclase [7] and a Ca^{2+}-ATPase in erythrocyte membranes [8] and to be a component of phosphorylase kinase [9] and of myosin light chain kinase [10] in skeletal muscle. In these studies we have investigated the hypothesis that calmodulin may play a role in Ca^{2+}-dependent insulin secretion. Extracts of rat islets of Langerhans were shown to contain a heat-stable activator of brain PDE. Activation was dependent on the presence of Ca^{2+} and was abolished by trifluoperazine, a specific inhibitor of calmodulin [11]. Trifluoperazine also inhibited glucose-stimulated insulin release but not islet glucose oxidation. These findings demonstrate the presence of calmodulin in islets of Langerhans and suggest its possible involvement in stimulus–secretion coupling in the β-cell.

Abbreviations: PDE, cyclic nucleotide phosphodiesterase (EC 3.1.4.1); cyclic AMP, cyclic adenosine-3′,5′-monophosphate; EGTA, ethylene glycol bis (β-aminoethyl)tetraacetic acid

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2. Materials and methods

2.1. *Reagents*

Bovine albumin (fraction V), 5′-nucleotidase and collagenase (type I) were obtained from Sigma (London) Chemical Co. Cyclic AMP was from the Boehringer Corp. (London) Ltd. (^{125}I)Insulin, cyclic[8-^{3}H]AMP and [U-^{14}C]glucose were from the Radiochemical Centre, Amersham. QAE–Sephadex was from Pharmacia (GB) Ltd. Guinea pig anti-porcine insulin serum was obtained from Wellcome Reagents Ltd. All other chemicals were from BDH Chemicals and were of the highest purity available. Trifluoperazine (Stelazine) was a gift from Smith, Kline and French Labs.

Calmodulin prepared from phosphorylase kinase [9,12] was a generous gift from Dr P. Cohen (Department of Biochemistry, University of Dundee). Cal-

modulin-deficient phosphodiesterase, prepared from bovine brain cortices by the method in [13], was a gift from Dr P. H. Sugden (Department of Clinical Biochemistry, University of Oxford).

2.2. *Preparation of islets*

Islets were prepared using collagenase digestion [14] from the pancreases of 200–300 g male albino Wistar rats fed ad libitum on a standard laboratory diet.

2.3. *Output of $^{14}CO_2$*

The rate of formation of $^{14}CO_2$ from [U-^{14}C]glucose was measured as in [15].

2.4. *Insulin release*

Batches of islets were incubated in vitro for 2 h and insulin release measured as in [16,17]. Radioimmunoassay of insulin was performed as in [17] except that Wellcome guinea pig anti-insulin serum was used as antibody.

2.5. *Assay of phosphodiesterase*

PDE assays were performed in 100 μl final vol. containing 50 mM Tris–HCl, (pH 8.0), 3 mM $MgCl_2$, 0.1 mg bovine albumin/ml, cyclic [^{3}H]AMP (30 Ci/mmol; ~1 × 10^6 dpm) and unlabelled cyclic AMP at 0.1 mM final conc. Activator-dependent activity was measured in the presence of 0.05 mM $CaCl_2$ and appropriate amounts of calmodulin or islet extracts. Tubes were incubated for 15 min at 30°C and the reaction terminated by placing the tubes in a boiling water-bath for 2 min. In all experiments the amount of enzyme caused hydrolysis of < 35% of the substrate. 2 μl snake venom 5′-nucleotidase (50 units/ml) were added to each tube. The tubes were then incubated for 15 min at 30°C and the reaction terminated by boiling as above. 0.4 ml 0.1 mM adenosine in 20 mM ammonium formate (pH 7.5) were added to each tube. Tritiated adenosine was separated from unreacted cyclic AMP by passing the contents of each tube over a column (0.5 × 1.0 cm) of quaternary aminoethyl (QAE)–Sephadex (A-25) in the formate form. The tubes were rinsed with 1.5 ml 20 mM ammonium formate (pH 7.5) which was also passed over the column. The effluent (2.0 ml) was collected and 0.8 ml aliquots counted in 18 ml methoxyethanol scintillation fluid [18].

2.6. *Preparation of islet extracts for assay of calmodulin*

Islets were placed into 10 mM Tris–HCl, (pH 8.0) and disrupted by sonication with three intervals of 5 s at position 2 on a Dawe Soniprobe. Islet sonicates were diluted with 10 mM Tris–HCl (pH 8.0) to a concentration equivalent to ~0.25 islets/μl and placed in a boiling water bath for 3 min. This procedure was found to completely inactivate islet phosphodiesterase. The boiled samples were centrifuged for 15 s in an Eppendorf 3200 centrifuge and 5–50 μl aliquots of the supernatants assayed for calmodulin activity by activation of PDE.

3. Results

3.1. *Assay of calmodulin*

In these experiments PDE activity was measured as the formation of [^{3}H]adenosine from cyclic 3′,5′-[^{3}H]AMP according to the equation:

$$\text{Cyclic } 3',5'\text{-AMP} \xrightarrow{\text{PDE}} 5'\text{-AMP}$$

$$5'\text{-AMP} \xrightarrow{5'\text{-nucleotidase}} \text{adenosine}$$

Conditions were chosen such that the time course of [^{3}H]adenosine formation was linear and the rate was proportional to the concentration of PDE. The activity of a fixed amount of calmodulin-free PDE (4×10^{-5} units) was increased by the addition of 1–100 ng calmodulin; a typical activation curve is shown in fig.1. Half-maximal activation required 10 ng calmodulin. The mean maximal activation was ~6-fold. Activation of PDE by calmodulin required the presence of Ca^{2+}; in the absence of added Ca^{2+} and with EGTA present to chelate traces of Ca^{2+} in the reagents, calmodulin did not stimulate PDE (fig.1). Trifluoperazine has been shown to bind to brain calmodulin [11]. The effects of increasing concentrations of trifluoperazine on the activation of PDE by 20 ng calmodulin are shown in fig.2. 60 μM trifluoperazine lowered PDE activity to values seen in the absence of calmodulin. Half-maximal inhibition under these conditions required 12 μM trifluoperazine.

These observations form the basis for the criteria

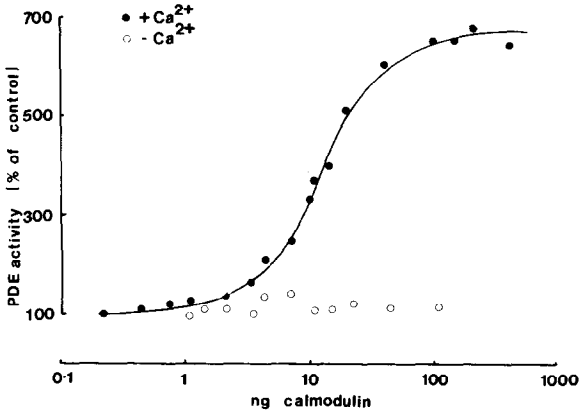

Fig.1. The Ca^{2+}-dependent activation of PDE by calmodulin. PDE activity was measured in the presence of varying amounts of calmodulin. Results are expressed as a percentage of the PDE activity in controls with no added calmodulin (37 pmol/min). The closed circles refer to assays conducted in the presence of Ca^{2+} (50 μM) and the open circles are values obtained in the absence of added Ca^{2+} and the presence of EGTA (1 mM).

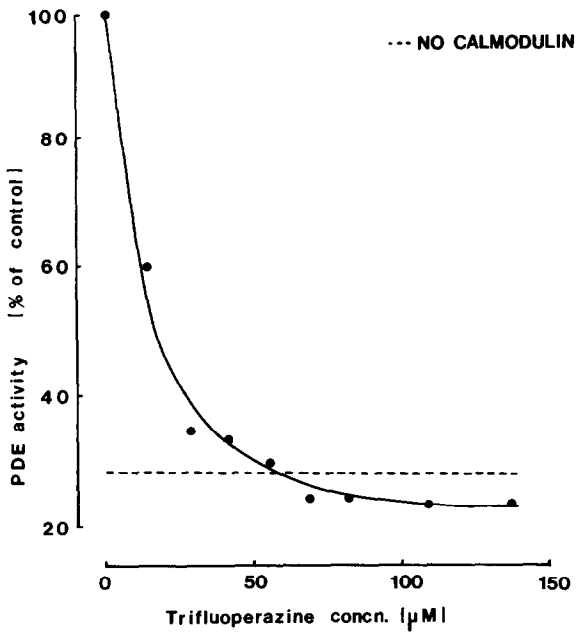

Fig.2. Effect of trifluoperazine on calmodulin-activated PDE. The activity of PDE stimulated by 20 ng calmodulin was measured in the presence of varying concentrations of trifluoperazine. The PDE activity is expressed as a percentage of its value in the absence of trifluoperazine (147 pmol/min). The dotted line indicates the activity of PDE in the absence of calmodulin.

used to demonstrate the presence of calmodulin in rat islets of Langerhans, viz.:

(i) The activation of PDE by extracts of rat islets;
(ii) The stability of the activating factor to boiling;
(iii) The dependence of activation on the presence of Ca^{2+};
(iv) Abolition of activation by trifluoperazine.

3.2. *Calmodulin in extracts of rat islets of Langerhans*

Extracts of rat islets prepared by sonication were boiled for 3 min and tested for their ability to activate brain PDE. Activation occurred (fig.3) to a maximum extent of 6-fold, i.e., equivalent to the maximum activation achieved with authentic calmodulin. Islet calmodulin was measured by using amounts of islet extract that gave sub-maximal activation of PDE. Islet calmodulin content could thus be quantitated by comparison with standard curves for PDE activation constructed using authentic calmodulin which were included in each experiment. The calculated amounts of calmodulin measured in this way were linearly related to the amount of islet extract assayed (fig.4). A mean value for 9 separate preparations of islets of 1.8 ± 0.2 ng calmodulin/islet was found. Assuming a mean intracellular volume of 3 nl/islet [19] and calmodulin mol. wt 16 700 [9], this corresponds to a mean of 36 μM calmodulin.

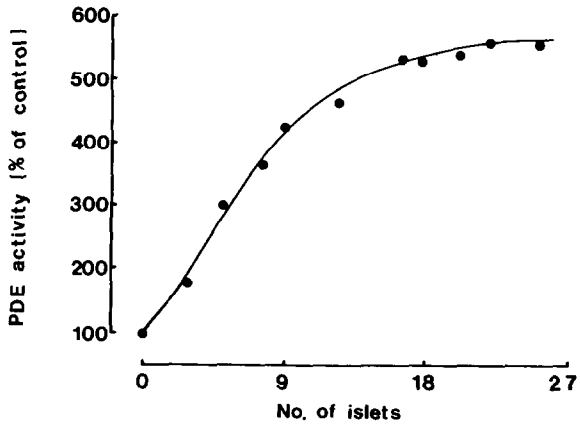

Fig.3. Activation of PDE by islet extracts. The effect of varying amounts of islet extract equivalent to the number of islets shown was tested on the activity of PDE as described in section 2. The PDE activity is expressed as a percentage of its value in the absence of added extract (42 pmol/min). The results are the mean values for 3 separate preparations of islet extract.

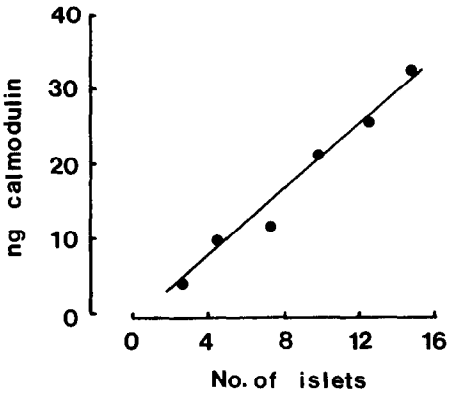

Fig.4. Islet content of calmodulin. Aliquots of islet extract corresponding to 2.5–15 islets were assayed for their ability to activate PDE. The islet content of calmodulin was calculated from PDE-activation curves using authentic calmodulin. The results are the mean values for 3 separate preparations of islet extract.

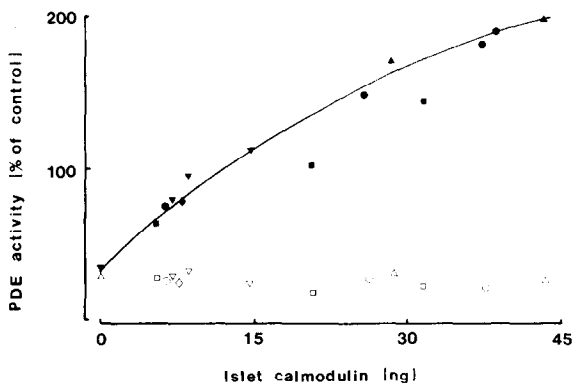

Fig.5. Inhibition by trifluoperazine of PDE activated by islet extract. Varying amounts of islet extract were assayed for their ability to activate PDE. The calmodulin in the extracts was quantified using PDE-activation curves with authentic calmodulin. The closed symbols refer to assays conducted in the absence of trifluoperazine and the open symbols to assays in which 60 μM trifluoperazine was included. Each symbol-type refers to a separate experiment with one islet extract and standard curve.

Activation of PDE by islet extract was totally dependent on addition of Ca^{2+}: three separate preparations of islet extract producing a mean activation of PDE of 2.4-fold in the presence of Ca^{2+} elicited no significant stimulation in the absence of Ca^{2+} and in the presence of EGTA (93 ± 4% of control activity in the absence of extract).

The lowest concentration of trifluoperazine that completely prevented activation of PDE by authentic calmodulin was tested on the activation of PDE by varying amounts of islet extract containing ⩽ 25 ng calmodulin. Figure 5 shows that 60 μM trifluoperazine abolished the activation of PDE by varying amounts of islet extract containing ⩽ 25 ng calmodulin. Trifluoperazine did not reduce the basal PDE activity observed in the absence of Ca^{2+}.

3.3. *Effects of trifluoperazine on intact islets*

Results are given in table 1.

Insulin release stimulated by 20 mM glucose was ~50% inhibited by 25 μM trifluoperazine but basal

Table 1
Effects of trifluoperazine on glucose-stimulated insulin release and [U-^{14}C]glucose oxidation by isolated islets

Parameter	Units	Glucose 20 mM	Trifluoperazine (μM)		
			0	25	50
Insulin release	μU .islet^{-1} .h^{-1}	–	31 ± 3 (44)	28 ± 4 (10)	108 ± 16 (9)[a]
		+	222 ± 17 (45)	132 ± 25 (15)[a]	125 ± 14 (44)[b]
[U-^{14}C]Glucose	$^{14}CO_2$ pmol .islet^{-1} .h^{-1}	+	40 ± 3 (8)	36 ± 3 (8)	44 ± 4 (7)

[a] $P \leqslant 0.01$
[b] $P \leqslant 0.001$

Batches of 5–10 islets were incubated at 37°C for 2 h in Krebs bicarbonate medium containing albumin (2 mg/ml) and the additions shown. Insulin release was determined by radioimmunoassay and the formation of $^{14}CO_2$ from [U-^{14}C]glucose (3 mCi/mmol) by liquid scintillation spectrometry. Results are given as mean ± SEM for the number of batches of islets stated in parenthesis. The significance of differences from the controls (no trifluoperazine) was assessed by Student's *t*-test

release was not affected. In the presence of 50 μM trifluoperazine basal release was enhanced but no stimulation by 20 mM glucose was observed. Since glucose-stimulated release is dependent on the ability of the islets to metabolize the sugar [20], the effect of trifluoperazine on the rate of oxidation of [U-^{14}C]-glucose by isolated islets was also tested. Neither 25 μM nor 50 μM trifluoperazine affected the rate of oxidation of 20 mM glucose.

4. Discussion

These studies demonstrate that rat pancreatic islets contain a heat-stable activator of brain cyclic nucleotide phosphodiesterase. Activation is dependent on the presence of Ca^{2+} and is abolished by trifluoperazine. The maximum activation is similar to that observed with muscle calmodulin. The findings constitute strong evidence for the existence of calmodulin in rat pancreatic islets. The amount of calmodulin measured in islet extracts is 300–450 mg/kg wet tissue (assuming 5–6 μg mean islet wet wt). This is close to the value reported for rabbit skeletal muscle [21] but considerably higher than that found in several other tissues tested [21].

Glucose-stimulated insulin release was impaired by a low concentration (25 μM) of trifluoperazine that did not affect islet glucose oxidation. This finding is consistant with the hypothesis that calmodulin may play a role in stimulus–section coupling in the β-cell. Interpretation of the results obtained with 50 μM trifluoperazine is more difficult since although the response to glucose was abolished, basal release was elevated. The possible nature of the involvement of calmodulin in insulin release is not known but consideration of the function of calmodulin in other systems raises several possibilities. For example the activation of both adenylyl cyclase and PDE by calmodulin might implicate the participation of calmodulin in the Ca^{2+}-dependent regulation of islet cyclic nucleotide concentrations: activation of adenylyl cyclase at the cell membrane by Ca^{2+}–calmodulin followed by activation of PDE as Ca^{2+} diffuses into the cytosol could underlie the transient, Ca^{2+}-dependent increase in islet cyclic AMP observed in islets exposed to glucose [22]. However evidence has been presented that cyclic AMP acts as a potentiator rather than a direct trigger for glucose-induced insulin release [23] and thus alternative or additional roles of calmodulin are likely.

Several studies have implicated a role for microtubules in the exocytotic discharge of insulin [24–26]; it is therefore relevant to note that, in rat brain, evidence has been presented that calcium regulates the assembly–disassembly of microtubules through the action of calmodulin [27]. Regulation of membrane ion fluxes is a further plausible function in exocytosis. Calmodulin has been shown to stimulate a Ca^{2+}-ATPase in erythrocyte membranes [8] and ATP-dependent Ca^{2+}-transport in dog cardiac sarcoplasmic reticulum [28]. Furthermore, calmodulin influences Ca^{2+}-dependent anion flux across rabbit ileum [29]. Finally it has been demonstrated that calmodulin mediates Ca^{2+}-dependent protein phosphorylation in membranes from various tissues [30]. Since Ca^{2+}-dependent protein phosphorylation has been demonstrated in mast cells stimulated with the Ca^{2+}-ionophore A23187 [31] it is tempting to speculate that phosphorylation events may participate in the membrane–membrane interactions involved in exocytosis. The cyclic AMP-independent protein phosphorylating activity recently demonstrated in rat pancreatic islets [32] may constitute a possible locus of action for islet calmodulin. Investigation of the effects of Ca^{2+} and calmodulin on this activity could substantiate such a hypothesis.

Acknowledgements

These studies were supported by grants from the Medical Research Council and the British Diabetic Association.

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