REGULATION BY NUCLEOTIDES OF ^{45}CALCIUM UPTAKE IN HOMOGENATES OF RAT ISLETS OF LANGERHANS

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1. Introduction

The presence of an adequate extracellular concentration of calcium is essential for the maintenance of insulin secretory responses to a variety of stimuli [1,2], and it has been postulated that in the pancreatic B-cell as in other cell types this ion might provide the trigger for the initiation of secretion. Some insight into the nature of the calcium requirements for the insulin secretory process has been provided by Malaisse and his colleagues in a comprehensive series of studies of rates of influx and efflux of ^{45}calcium in isolated islets of Langerhans, both in static incubations and in perifusion systems, as reviewed by Malaisse [3]. Briefly, these workers concluded that neither the patterns of influx of calcium into the B-cell nor its efflux could by themselves qualify them as initiators of secretion, but rather that the variations in the concentration of free ionised calcium in the cytosol might regulate the rate of release [3]. Glucose and amino acids were considered to increase the concentration of free calcium by preventing its efflux from the B-cell whereas cyclic AMP was suggested to raise the level by stimulating calcium release from an organelle-bound pool.

We have now attempted to clarify this role of calcium in the B-cell by studying some factors which alter the rates of uptake and storage of ^{45}calcium in homogenates and subcellular fractions of isolated islets of Langerhans. Calcium accumulation by particulate fractions during incubation in the presence of inorganic phosphate is strongly stimulated by ATP, ADP or the ATP analogue AMP–PNP but not by UTP, GTP, AMP or adenosine. Accumulation is diminished in the presence of cyclic AMP.

2. Methods

2.1. *Tissue preparation*

Islets of Langerhans were isolated from rat pancreas by collagenase digestion [4] using a bicarbonate buffered medium [5] containing 5.5 mM glucose and 2 mM $CaCl_2$. Final rinsing and separation of the islets was performed in medium from which calcium was omitted.

After removal of the medium, islets were homogenised in 0.25 M sucrose buffered with 10 mM Tris–HCl (pH 7.2). In some experiments subcellular fractions were obtained from the homogenate by centrifugation at 4°C as follows: 600 *g* for 5 min to give a nuclei + debris pellet; the supernatant was centrifuged at 24 000 *g* for 10 min to give a mitochondrial + storage granule pellet and this supernatant was in turn centrifuged at 105 000 *g* for 60 min to give a microsomal pellet and final supernatant. All pellets were resuspended in 0.25 M sucrose, 10 mM Tris–HCl (pH 7.2) and assayed simultaneously at the end of the centrifugation.

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2.2. *Assay of ^{45}Ca accumulation*

Accumulation of ^{45}Ca by homogenates or isolated subcellular fractions was estimated by incubation of 50 μl aliquots of homogenate or subcellular fraction resuspended in sucrose with 50 μl of a solution (pH 7.2) which was prepared to give final concentrations of: 0.125 M sucrose, 35 mM KCl, 10 mM Tris–HCl, 4 mM $MgCl_2$, 4 mM K_2HPO_4, 10 mM sodium succinate, 20 μM $CaCl_2$ and $^{45}CaCl_2$ (5 μC/ml obtained from the Radiochemical Centre, Amersham, Bucks.). Nucleotides and other agents were added at the concentrations indicated in individual experiments.

After 20 min incubation at 23°C the particulate-bound calcium was separated by filtration through Millipore membranes (0.45 μ, HAWP), each membrane being rinsed three times with 0.25 M sucrose in 10 mM Tris–HCl (pH 7.2) to remove unbound radioactivity. Radioactivity remaining on the filters was determined in a liquid scintillation spectrometer (Beckman LS 233) using Instagel (Packard Instruments) as scintillant. Blank values obtained in the absence of tissue never exceeded 10% of the counts obtained in the presence of homogenate. Protein concentrations were determined by the Lowry procedure [6] using crystalline bovine albumin as standard, and calcium accumulation was expressed as nmoles Ca accumulated per mg protein per 20-min incubation period.

3. Results

The system used in this study of calcium uptake by particulate components of islet homogenates involved the rapid separation of particulate bound from free ^{45}calcium on Millipore membranes, a method which has been successfully used in studies of calcium accumulation by mitochondria [7], plasma membranes from muscle [8] or liver [9], and sarcoplasmic reticulum fragments [10]. Preliminary experiments to investigate the characteristics of ^{45}calcium uptake were performed with freshly prepared homogenates in the presence of 1.25 mM ATP and showed that optimal binding occurred at an initial incubation medium pH of 7.2 (fig.1). Accumulation was a very rapid process, reaching equilibrium in less than 2.5 min (the shortest period tested) at 23°C and being maintained for at least 30 min; the extent of uptake

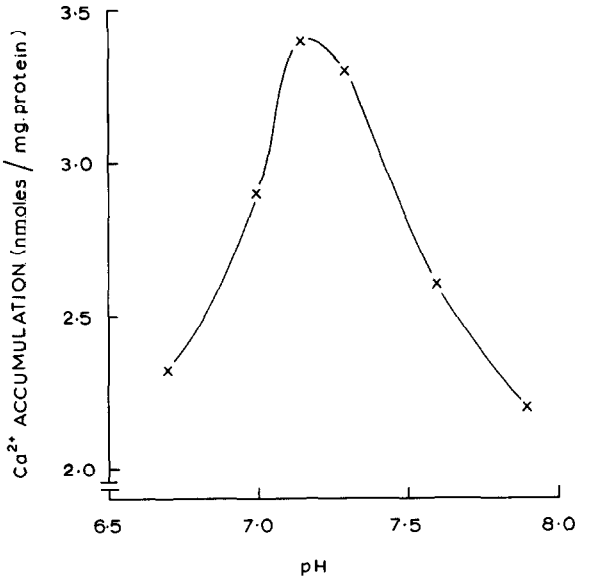

Fig.1. Effect of pH on the accumulation of ^{45}calcium by islet homogenates during a 20-min period of incubation at 23°C in the presence of 1.25 mM ATP.

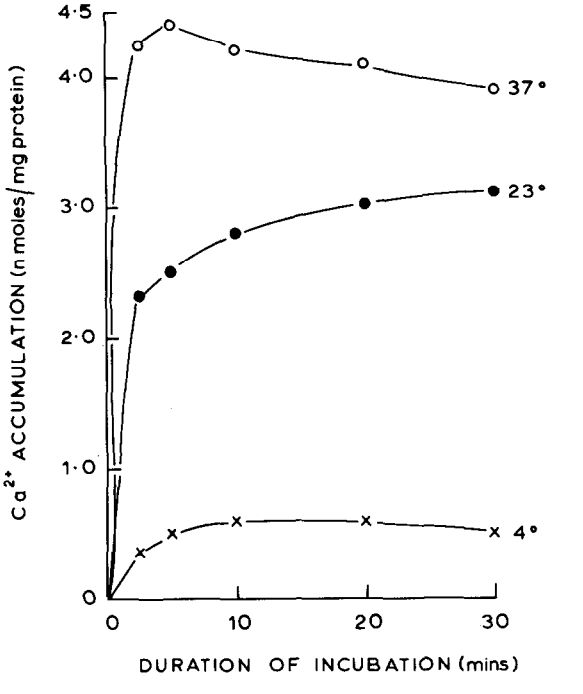

Fig.2. Effect of temperature of incubation on accumulation of ^{45}calcium by islet homogenates in the presence of 1.25 mM ATP. Incubations were performed at 4°C (X–X–X), 23°C (•–•–•), 37°C (o–o–o).

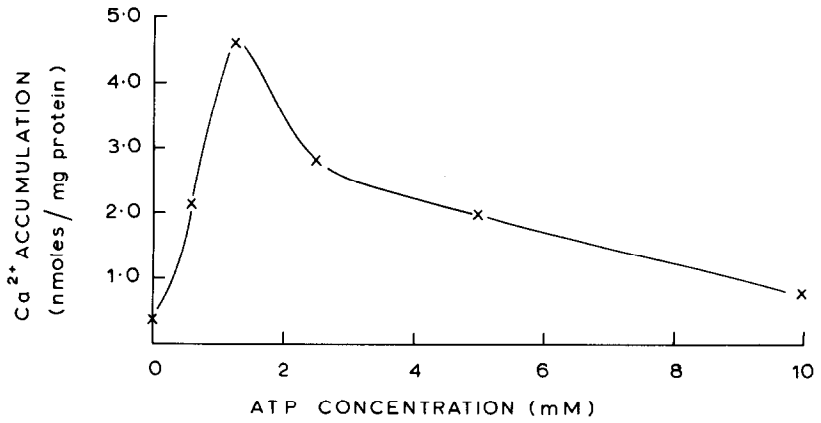

Fig.3. Effect of ATP concentration on the accumulation of ^{45}calcium by islet homogenates. Incubations were performed for 20 min at 23°C.

was dependent on the temperature of incubation (fig.2). Calcium uptake was dramatically increased by addition of ATP to the incubation medium, the maximum effect being obtained at a concentration of 1.25 mM (fig.3). Accumulation was stimulated to a lesser extent by addition of ADP or the ATP analogue adenylyl imidodiphosphate (AMP–PNP) but not by GTP, UTP, AMP or adenosine (table 1). Addition of potassium oxalate or an increased concentration of potassium phosphate during incubations performed in the presence of 1.25 mM ATP resulted in a proportional increase in the amount of ^{45}Ca accumulation, whereas omission of a permeant anion (phosphate or oxalate) resulted in a reduction of binding by homogenates to very low levels, either in

Table 1
Effects of nucleotides on ^{45}calcium accumulation by homogenates of islets of Langerhans

Additions	% of accumulation observed in control incubations
None	100
ATP	842 ± 58*
UTP	121 ± 12
GTP	108 ± 7
ADP	388 ± 20*
AMP–PNP	435 ± 35*
AMP	87 ± 8
Adenosine	104 ± 7

Each nucleotide was added at a concentration of 1 mM, and incubation was continued at 23°C for 20 min. Results are shown as mean and standard error of the mean of six observations;
* indicates result which is significantly different from the control level ($p < 0.05$).

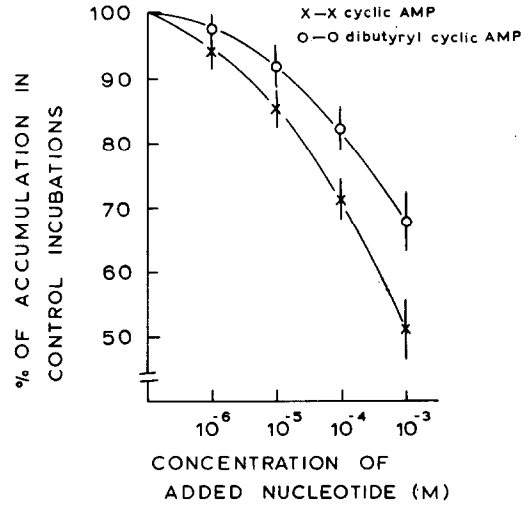

Fig.4. Effect of cyclic AMP (X–X–X) or dibutyryl cyclic AMP (o–o–o) on ^{45}calcium accumulation by islet homogenates during incubation at 23°C for 20 min. The results shown are the mean and the standard error of the mean (9 observations) percentage of the accumulation which was obtained in the absence of either agent.

the absence or the presence of ATP. Heating of the homogenate to 80°C for 3 min before incubation abolished calcium accumulation.

The effects of cyclic nucleotides on calcium uptake were initially studied on freshly prepared islet homogenates. The nucleotides were added to the basal incubation medium containing 1.25 mM ATP, 20 μM $CaCl_2$ and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.5 mM); addition of this agent by itself had no effect on calcium uptake. The effects of addition of various concentrations (10^{-6}–10^{-3}M) of cyclic AMP or dibutyryl cyclic AMP are shown in fig.4. It is clear that cyclic AMP and to a lesser extent dibutyryl cyclic AMP are capable of reducing the net accumulation of ^{45}Ca by the homogenates; smaller effects were obtained with cyclic GMP or dibutyryl cyclic GMP. These effects could be demonstrated when the nucleotide was added after 5, 10 or 15 min of a 20-min incubation period. Similar alterations of calcium uptake in the presence of 1 mM cyclic AMP could be demonstrated during incubation at 37° or 4°C, or in the presence of medium calcium concentrations in a range 5 to 100 μM.

Further studies concentrated on attempts to identify the sites of ATP dependent binding of calcium in B-cells and of the sites of effect of cyclic AMP on binding, using impure fractions prepared by differential centrifugation. The results indicated that the highest specific activity of basal and ATP stimulated calcium uptake was obtained in the mitochondrial + storage granule fraction, and that cyclic AMP was effective principally on this fraction (results not shown).

4. Discussion

Studies of calcium distribution in islet cells performed after precipitation of calcium with pyroantimonate [11,12] have suggested that insulin storage granules and plasma membranes might represent sites of calcium binding, while Schäfer and Klöppel [12] also indicated that endoplasmic reticulum and mitochondria may also be important storage sites for calcium, particularly in B-cells which were not in a state of active secretion. Furthermore, direct determinations performed by X-ray microanalysis of the calcium distribution in frozen sections of unfixed islet tissue prepared by cryo-ultramicrotomy [13] have shown that B-cell mitochondria may contain concentrations of calcium while other organelles, including storage granules, also contain significant quantities of this ion [14].

The purpose of the present study was to investigate some of the characteristics of calcium accumulation by components of the A and B cells of the islets of Langerhans, and to determine whether this binding might be subject to regulation by nucleotides and, in particular, by ATP or cyclic AMP. The possibility that cyclic AMP might alter intracellular calcium distribution in this way, suggested by Rasmussen [15], was further indicated by Malaisse [3] in studies of influx and efflux of ^{45}Ca from intact islet cells, and by Borle [16] who showed directly that cyclic AMP could promote efflux from mitochondria isolated from kidney, heart or liver. In view of the likelihood that there might be several organalles which could play an important role in the regulation of calcium binding in islet cells, the major part of the study was performed using homogenates; only in the later stages have preliminary attempts been made to identify and separate the organelles which may be involved.

Optimal accumulation of calcium by islet homogenates could be obtained using the buffer and incubation procedure described by Borle [16]. Negligible uptake was seen in the absence of phosphate or oxalate, either in the presence or absence of ATP, and uptake was markedly stimulated by ATP, by ADP or by AMP–PNP. This nucleotide specificity appears comparable to that for ^{45}calcium uptake by mitochondria [17], by plasma membranes [8,9] or by sarcoplasmic reticulum [10]. Only in the case of mitochondria, however, has an effect of cyclic nucleotides in diminishing net accumulation been observed [16]. Indeed, calcium binding to liver plasma membranes [9] or to cardiac sarcoplasmic reticulum fragments [19] appears to be stimulated by high concentrations of cyclic AMP.

The observed effect of cyclic AMP in diminishing calcium accumulation by islet particulate fractions is consistent with the interpretation of Malaisse [3] of patterns of calcium influx and efflux in B-cells during incubation in the presence of agents which raise cyclic AMP levels, and again suggests that one

mechanism of the action of cyclic AMP in B-cells may be to increase levels of free intracellular calcium. Studies are at present in progress to determine the relative importance of the various sites of calcium accumulation in islet cells, and how uptake at these sites is regulated by nucleotides including ATP and cyclic AMP.

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