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Factors Governing Glucose Induced Elevation of Cyclic 3′5′ AMP Levels in Pancreatic Islets

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Summary. Adenosine 3′,5′-cyclic monophosphate (cAMP) levels of isolated perifused pancreatic islets were elevated by high levels of glucose concomitantly with initiation of enhanced insulin secretion. The rise of cAMP was biphasic and seemed to be related to the temporal biphasic kinetics of insulin release. However, the temporal profiles of cAMP level changes and of insulin release differed; the major rise of the cAMP levels was seen during the initial phase, whereas insulin secretion was more pronounced during the second phase of release. Glucose-induced cAMP elevation required the presence of extracellular Ca^{++}. Mannoheptulose completely blocked cAMP elevation due to high glucose. Exogenous insulin which has been shown by others to inhibit insulin secretion in vitro, blunted the glucose-induced cAMP rise. These observations and data in the literature are compatible with the concept that under physiological conditions glucose governs the intracellular cAMP levels in a Ca^{++} dependent manner – either directly or indirectly through metabolic effects.

Key words: Glucose, cyclic AMP, calcium, insulin, insulin secretion, receptor mechanism, second messenger.

The precise role of 3′,5′-cyclic adenosine monophosphate (cAMP) in the complex process of insulin release from pancreatic islets is poorly defined. There is no question that cAMP can influence profoundly B-cell function, as demonstrated by the fact that methylxanthines [1], cAMP [2] and dibutyryl-cAMP [1] all greatly potentiate glucose provoked insulin release (although they are very inefficient in causing hormone release in the absence of glucose). Furthermore, enteric peptide hormones [4] glucagon [2, 5], and the β-adrenergic agonist isoproterenol [3], substances which activate islet adenylate cyclase, also potentiate glucose-provoked insulin release, whereas the α-adrenergic agonist, epinephrine, which lowers cAMP in isolated pancreatic islets [7] blocks glucose-stimulated insulin release [6]. It remains an open question whether insulin release resulting from glucose, the major physiological B-cell stimulus, involves elevation of cAMP levels as an obligatory step. In several studies it was found that cAMP was elevated in isolated pancreatic islets exposed to high glucose [8–14]; however in an equally large number of papers it was reported that glucose alone did not alter the cAMP levels of islet tissue [15, 16] or had only a small transient effect [17]. Some have found that high glucose elevated cAMP when a phosphodiesterase inhibitor was present [8, 9, 17] others, however, did not confirm this phenomenon [15, 16].

This is a report of some of the results we have obtained in an effort to help clarify the issue.

The time courses of glucose-induced insulin release and possible related fluctuations in cAMP concentrations in islet tissue were investigated. Since glucose-induced insulin release requires extracellular Ca^{++} [18] and since this cation modifies adenylate cyclase activity in intact cells and in cell-free systems from many tissues [19], experiments were designed to delineate any role Ca^{++} might have in regulating cAMP levels in islets. In addition, since mannoheptulose and insulin have been used to modulate the endocrine response of the B-cells [6, 20], the possible influence of these agents on the cAMP system was investigated.

Materials and Methods

Islets from fed male Sprague-Dawley rats (Holtzman, Madison, Wisconsin) were isolated according to the method of Lacy and Kostianovsky [21]. For studying the kinetics of insulin release and of cAMP in islets, a perifusion system similar to that first employed by Burr *et al.* [22] and modified by Lacy, Walker, and Fink [23] was used. Batches of 100 to 200 islets were used in each experiment. A 45 min preperifusion period was followed by stimulation with test substances for various time periods as indicated in the Results section. The 'dead space' of the perifusion system was 2.5 ml. In presenting the results an appropriate correction for the time delay in delivering the stimulus to the islet chamber, i.e. 2.5 min was introduced. Islets, still attached to the millipore filter, were then quickly removed from the chamber, submerged for 30 sec in Freon-12 cooled to its freezing point (−150°) and then placed in glass jars for storage at −80° C. Cyclic AMP was extracted in 15% trichloroacetic acid (0.5 ml/batch of 100 to 200 islets).

The acid extract was washed four times with 6 volumes of ethyl ether, and the acid-free solution was then dried with a stream of nitrogen. The solid residue was dissolved in 0.125 ml 50 mM sodium acetate buffer. With the extraction method used recovery of exogenous cyclic AMP was greater than 90%.

The tissue extracts were assayed (in triplicate) for cyclic AMP using the radioimmunoassay of Steiner *et al.* [24, 25]. Because of the small amount of tissue available it was necessary to modify the assay technique originally described. Only one tenth of the assay volumes recommended by Steiner *et al.* [25] were used. Therefore, each assay tube contained 30 μl of standard or sample diluted in acetate buffer, 10 μl of diluted rabbit anti-cyclic AMP antibody, 10 μl of a solution of ^{125}I labeled succinyl-cyclic AMP tyrosine methyl ester (1200—1500 cpm per 10 μl) and 2 μl of goat anti-rabbit IgG. After all additions were made the tubes were incubated at 4° for 18—24 hours. To each assay tube 0.8 ml water was then added and the tubes were centrifuged at 1250 × g for 30 min. The supernatant fluid was decanted and discarded and the precipitate was counted in a gamma spectrometer. As a result of this modification, it was possible to measure as little as 7×10^{-14} moles of cyclic AMP (Fig. 1).

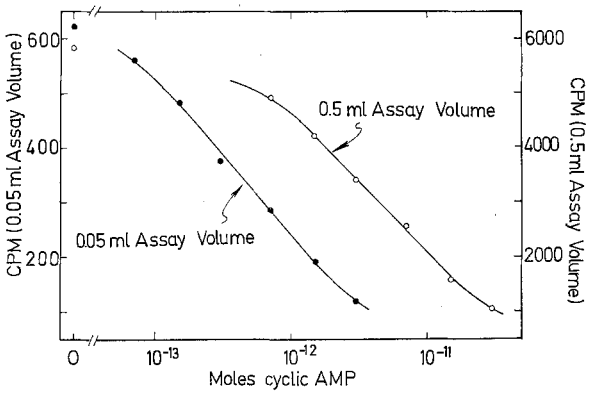

Fig. 1. *Standard curve of the micro modification of the radioimmunoassay for cAMP.* Each point represents the mean of three determinations

This is the amount of cyclic AMP present in 5—10 isolated islets under control conditions in the perifusion system. Assay variability with the modified method was less than 10%; this is not different from the results with the originally described procedure (Fig. 1).

Treatment of tissue extracts of control and stimulated islets with phosphodiesterase resulted in a greater than 95% decrease in cyclic AMP concentration indicating that essentially all of the measured substance was, in fact, cyclic AMP.

The medium used to perifuse the islets was composed of 0.5% crystalline bovine serum albumin (Armour Pharmaceuticals) in a salt solution buffered with bicarbonate (pH 7.4) and continuously gassed with a mixture of 95% O_2 and 5% CO_2. The salt composition was NaCl, 115 mM; KCl, 5 mM; $NaHCO_3$, 24 mM; $CaCl_2$, 2.1 mM; $MgCl_2$, 1 mM. The flow rate was maintained close (± 10%) to 1 ml/min. Immunoreactive insulin was measured according to the method of Hales and Randle [28] using porcine insulin as a standard.

Results

Glucose stimulated insulin release was biphasic, displaying a first peak at 2 min and a delayed second phase (Fig. 2). The cAMP levels in the islets also changed in a biphasic fashion: at 2 min cAMP was increased 3.1 fold; at 5 min, less dramatically, 1.4 fold and at 20 min, 2.8 fold, whereas at 1 min the levels were not significantly different from the controls. In the controls the cAMP levels declined by 50% in the course of the time period analyzed (20 min).

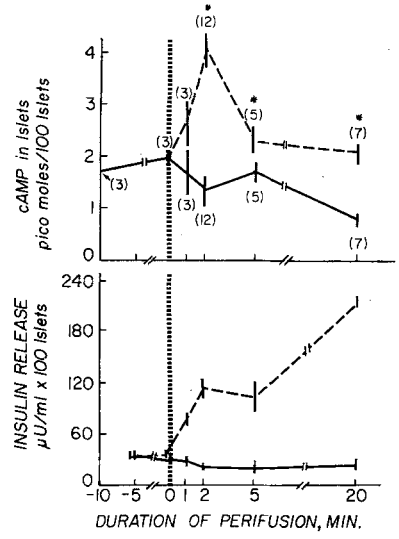

Fig. 2. *Kinetics of insulin release and cAMP level changes in isolated perifused rat islets.* In each experiment 200 islets were used. After establishing constant baselines of insulin release during a 45 min period using a medium containing basal glucose (2.75 mM) the stimulatory medium containing high glucose (27.5 mM) was fed into the system at t_0. The controls were maintained on the basal glucose level after a sham switch at t_0. The values recorded represent the means ± SEM of an indicated number of experiments. The cAMP points marked with a star differ statistically from the controls, ($p \leq 0.05$). All the differences of the insulin values after the switch to high glucose are statistically significant, ($p < 0.01$)

When Ca^{++} was deleted from the perifusion medium, the increases induced by high glucose in

Table 1. *The influence of Ca^{++}, mannoheptulose, and insulin on the glucose induced elevation of cAMP levels in islets of Langerhans and on insulin release*

Conditions		Cyclic 3′5′-AMP in islets (picomoles/100 islets, ± SEM (n))	Insulin release (uUnits/ml × min × 100 islets)
A.	$G_{2.75} \longrightarrow G_{2.75}$	1.33 ± 0.22 (6)	29 ± 2 (6)
	$G_{2.75} \longrightarrow G_{27.5}$	4.04 ± 0.33 (12)[a]	77 ± 5 (12)[a]
B.	$G_{2.75}, Ca_0^{++} \longrightarrow G_{2.75}, Ca_0^{++}$	1.52 ± 0.19 (3)	51 ± 6 (3)
	$G_{2.75}, Ca_0^{++} \longrightarrow G_{27.5}, Ca_0^{++}$	1.41 ± 0.08 (5)	45 ± 6 (5)
C.	$G_0, Ca_0^{++} \longrightarrow G_0, Ca_0^{++}$	2.36 ± 0.37 (4)[b]	34 ± 8 (4)
	$G_0, Ca_0^{++} \longrightarrow G_0, Ca_5^{++}$	2.07 ± 0.22 (6)	42 ± 6 (6)
D.	$G_{2.75} \longrightarrow G_{2.75} + MH_{27.5}$	1.50 ± 0.18 (3)	27 ± 8 (3)
	$G_{2.75} \longrightarrow G_{27.5} + MH_{27.5}$	1.69 ± 0.33 (4)[c]	23 ± 6 (4)[c]
E.	$G_{2.75}, INS_1 \longrightarrow G_{2.75}, INS_1$	1.42 ± 0,23 (4)	not measurable
	$G_{2.75}, INS_1 \longrightarrow G_{27.5}, INS_1$	2.54 ± 0.43 (8)[a, c]	not measurable

[a] $p \leq 0.05$ as compared to the corresponding controls
[b] $p \leq 0.05$ as compared to the controls of experiment A
[c] $p \leq 0.01$ as compared to the experimental group of experiment A

Batches of 100 to 200 islets were used. After 45 min of preperifusion the medium was changed as indicated under "Conditions", with a sham switch when the composition of the medium was not changed. Samples for cAMP and insulin measurements were taken 2 min after the switch. The concentrations of glucose (G), mannoheptulose (MH) and Ca^{++} are mM (as subscripts), those of insulin (INS), are μg/ml. Ca^{++} concentration unless noted was 2.1 mM

cAMP and insulin release were both blocked (Table 1, Experiment B). Basal release of insulin was however slightly increased under these conditions (compare B with controls of A). In the absence of both Ca^{++} and glucose, islet cAMP levels were 75% higher than in the presence of basal glucose and normal Ca^{++} (compare controls of A and C); readdition of 5 mM Ca^{++} alone was unable to change cAMP significantly or to elicit hormone release, at least not within the time interval studied here (2 min), indicating that insulin release as well as cAMP elevation requires the simultaneous presence of Ca^{++} and glucose (Experiment C). Equimolar mannoheptulose blocked completely the increase in cAMP and insulin release due to high glucose (Experiment D).

Porcine insulin at 1 μg/ml significantly inhibited the rise of cAMP due to high glucose (Experiment E). The use of such levels of extracellular insulin seemed justified because it was calculated that during maximal stimulation, the insulin level in the center of a pancreatic islet may amount to more than one μg/ml of water (Table 2).

Table 2. *Theoretical diffusion profile of insulin in isolated islets*

Distance from center (u)	Concentration of free insulin (μg/ml)	
	Nonstimulated islet	Stimulated islet
0	0.342	1.68
50	0.249	1.25
75	0.147	0.745
90	0.066	0.328
95	0.036	0.177

The insulin diffusion profiles of an average spherical islet with a diameter of 200 μ were calculated according to M. H. Jacobs [26] using the following formula:

$$u = c + \frac{\alpha}{6D}(R^2 - r^2)$$

where u = concentration of insulin at distance r from center [μg/ml]; c = concentration of insulin at the surface [μg/ml]; α = rate of insulin secretion in steady state [μg/sec × ml tissue]. D = diffusion constant of insulin [cm²/sec]; R = radius of sphere [cm]; r = distance from center of sphere [cm]. In the example given c is assumed to be 0.01 μg/ml and 0.002 μg/ml and α is calculated to be 0.16 and 0.032 μg/ml × sec for the stimulated and the non stimulated islet, respectively. D for the monomeric insulin is 1.6×10^{-6} cm²/sec [27]

Discussion

The present experiments demonstrated that glucose stimulation of islets produced marked elevation of cAMP levels, a result also reported by Charles *et al.* [8], by Selawry *et al.* [10] by Grill and Cerasi [9, 12] as well as by Capito and Hedeskov [14] but not confirmed by Cooper, Ashcroft and Randle [16] and Hellman *et al.* [17]. Reasons for these discrepancies are not readily apparent, but subtle differences in the procedures for isolating pancreatic islets with crude collagenase used by the several laboratories, species differences, or the nutritional state of the animal [14] seem to be the most likely causes. In the present study,

the rise of the nucleotide level exhibited an early peak (300% as compared to the appropriate control with low glucose). The relative elevation after 20 min with high glucose was equally pronounced (2.1 ± 0.15 vs 0.8 ± 0.1 picomoles/100 islets) but the absolute level at that time was only one-half of the transient peak level of the nucleotide. Charles *et al.* also reported that cAMP levels were elevated 2 min after exposure to high glucose (17 mM) but found that cAMP was as high or even higher after 20 min perifusion with high glucose [8]. Again, we have no sure explanation for the difference in the time course of cAMP response.

The rise of cyclic AMP might well be due to glucose activating adenylate cyclase. Although studies on islet homogenates have not revealed any direct effect of glucose on activation of adenylate cyclase it remains a distinct possibility that cellular integrity is a necessary prerequisite for this response. It seems less likely that inhibition of the Ca^{++} and glucose insensitive phosphodiesterase is involved [29, 30].

or incapable of inducing secretion [1, 2], it seems very probably that initiation of the release process by high glucose is accompanied by the simultaneous increase both of cAMP and of the islet Ca^{++} content.

Mannoheptulose, as previously reported by Capito and Hedeskov [14] for mouse islets, blocked the accumulation of cAMP due to high glucose.

The preceding considerations lead to the conclusion that the elevation of cyclic AMP levels may be a requirement for glucose-induced insulin release. One might also speculate that the increase of cyclic AMP levels caused by high glucose levels might augment the responsiveness of B-cells to other stimuli that are present in the physiological setting, i.e., amino acids, ketone bodies, fatty acids. Finally, there is little doubt that the cyclic AMP system can serve to transmit the modulatory function of many hormones and neurotransmitters that are known to influence insulin release. A schematic representation of this concept is proposed in Fig. 3. The inhibition of the cAMP rise by high

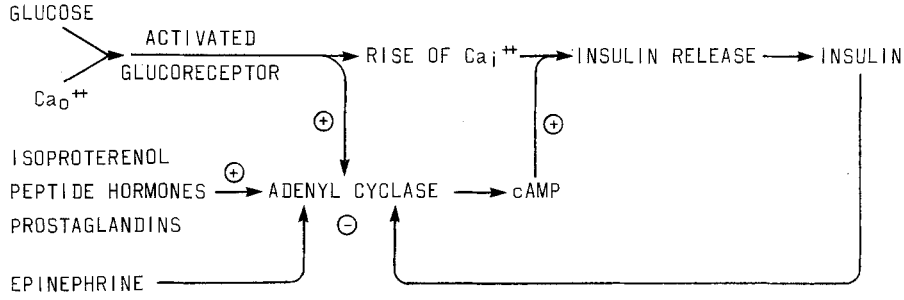

Fig. 3. *The role of cAMP in glucose induced insulin release.* Based on the present data and related information in the literature a scheme is presented describing the possible roles of glucose, Ca^{++} and cAMP in insulin release. Under physiological conditions glucose might activate a putative glucoreceptor [32] located in the cell membrane causing increased Ca^{++} entry into the cell [18, 19]. Concomitantly adenylate cyclase might be stimulated in a Ca^{++}-dependent manner. (It seems less likely that inhibition of the Ca^{++}- and glucose insensitive phosphodiesterase is involved [29, 30]. It remains, however undecided whether cAMP plays an obligatory role in glucose induced insulin discharge, or whether its role, although physiological, can be by-passed. Catecholamines, peptide hormones, prostaglandins and insulin are able to modulate the rate of release by altering the activity of adenylate cyclase most likely in a Ca^{++}- and glucose-independent manner [1–7, 18, 19]

It is assumed that the changes of cyclic AMP levels observed here occur in B-cells. However, one must not forget that islet tissue is heterogeneous (i.e. only 60–75% of the cells are B-cells), containing among others the functionally different but nevertheless glucose sensitive α-cells (i.e. 10–25%).

The calcium dependency of glucose-induced elevation of cAMP is remarkable since in many other systems with intact cells and in cell-free systems [19], Ca^{++} is not required for a specific stimulus to produce an accumulation of cAMP. Since the stimulation of insulin release by glucose demands that extracellular Ca^{++} be present [18] and since elevation of cAMP levels alone either by adding the nucleotide or by phosphodiesterase inhibitors is rather ineffective exogenous insulin may be compatible with the view that insulin functions as a feedback inhibitor of its release [20, 31, see also 30]. Although the existence of this feedback has been recently questioned, [33] it may only become apparent when higher amounts of exogenous insulin are added. Fig. 3 also offers a hypothesis for the mechanism of this inhibition.

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