The Role of Ca\(^{2+}\) and Cyclic Adenosine 3':5'-Monophosphate in Insulin Release Induced in Vitro by the Divalent Cation Ionophore A23187*

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SUMMARY

Insulin release from isolated perfused pancreatic islets was stimulated by the divalent ionophore A23187 in the absence of exogenous glucose. In addition, A23187 produced a 2-fold elevation of cyclic adenosine 3':5'-monophosphate (cAMP) levels in isolated perfused islets. The elevation of cAMP levels coincided with peak insulin release. Ionophore-induced insulin release was unaffected by pretreatment of the islets with theophylline (5 mM). Stimulation of insulin release produced by the ionophore occurred either in the presence or absence of extracellular Ca\(^{2+}\); however, cAMP accumulation required the presence of extracellular Ca\(^{2+}\). The ionophore (10 \(\mu\)M) had no effect on adenylate cyclase activity of homogenates of isolated islets. The results of this study are interpreted as indicating that intracellular Ca\(^{2+}\) has an essential role in the insulin releasing mechanism, whereas the cAMP system has a modulatory effect on this process.

Cyclic adenosine 3':5'-monophosphate and calcium (Ca\(^{2+}\)) have been implicated as intracellular mediators of glucose-induced insulin release from pancreatic \(\beta\) cells (1). Several observations support this hypothesis: (a) hormones and transmitters which are known to alter adenylate cyclase activity in homogenates of islet cells or to alter cAMP levels in isolated islets are potent modulators of insulin release (2, 3); (b) phosphodiesterase inhibitors, such as theophylline, potentiate insulin release stimulated by various agents (e.g. glucose or tolbutamide) (4); (c) cAMP-sensitive protein kinases have been described in pancreatic islets (5); and (d) extracellular Ca\(^{2+}\) is absolutely necessary for insulin release stimulated by glucose and other agents, a requirement shared by some other cAMP-dependent processes (6).

Nonetheless, the respective roles of Ca\(^{2+}\) and cAMP or possible interrelationships between the two substances in the process of insulin release, particularly in release induced by the major physiological stimulant glucose, are not clear. For example, 10 \(\mu\)M theophylline alone is capable of augmenting islet cAMP levels 4-fold yet, in the absence of glucose, does not result in an increased rate of insulin secretion (4, 7).

We have sought new approaches to examine more closely the possible interrelated roles of Ca\(^{2+}\) and cAMP in insulin release. In the present study, the effects of a divalent cation ionophore, A23187, on insulin release and the behavior of cAMP levels in isolated perfused rat islets were examined. This ionophore, obtained from streptomycin, stimulates secretory responses in a number of tissues and it is thought that this occurs as a result of altered transmembranous Ca\(^{2+}\) fluxes (8-10), leading to elevation of intracellular Ca\(^{2+}\) levels.

EXPERIMENTAL PROCEDURE

Perfusion Studies—Islets from fed adult male Sprague-Dawley rats (Holtzman, Madison, Wis.) were isolated according to the method of Lacy and Kostianovsky (11). For studying the kinetics of insulin release and of cAMP level changes in islets, a perfusion system similar to that first employed by Burr (12) and modified by Lacy et al. (13) was used. Batches of 100 to 200 islets were used in each experiment. A 30-min preperfusion period was followed by stimulation with test substances for various time periods, as evident from the tables and figures under "Results." For tissue analysis, islets, still attached to the Millipore filter, were removed quickly from the chamber, submerged for 30 s in Freon-12, which had been cooled to its freezing point (-150°), and then placed in cooled glass jars for storage at -80°. cAMP 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 hydrated ethyl ether, and the acid-free solution then was dried with a stream of nitrogen. The solid residue was dissolved in 125 \(\mu\)l of 0.1M sodium acetate buffer, pH 6.0. The tissue extracts were assayed (in triplicate) for cAMP using a modification of the radioimmunoassay of Steiner et al. (14).

The perfusate was composed of 0.5% crystalline bovine serum albumin in a salt solution buffered with bicarbonate (pH 7.4) and continuously gassed with a mixture of 95% \(\text{O}_2\) and 5% \(\text{CO}_2\). The salt composition was: NaCl, 115 mM; KCl, 5 mM; NaHCO\(_3\), 24 mM; CaCl\(_2\), 2.1 mM; and MgCl\(_2\), 1.0 mM. The flow rate was maintained close (±10%) to 1 ml/min.
**Insulin Assay**—Immunoreactive insulin was measured according to the method of Hales and Randle (15) using porcine insulin as a standard.

**Adenylate Cyclase Assay**—Adenylate cyclase was assayed using a modification of the method previously reported by Steiner et al. (10). Two hundred freshly isolated islets were homogenized in 200 μl of 50 mM Tris-HCl buffer, pH 7.4, in a ground glass homogenizer with a tight fitting pestle. Twenty microliters of this homogenate were added to 20 μl of reagent containing: 50 mM Tris-HCl buffer, pH 7.4; 12 mM theophylline; 60 mM phosphocreatine; 1.25 mM ATP; 4.5 mM MgCl₂; 0.05% bovine serum albumin; 0.01 mM EGTA; and 0.8 mg/ml of creatine kinase. After mixing, the tubes were incubated for 45 min at 30°C, and the reaction was stopped by placing the assay tube in boiling water for 3 min. Control tubes were placed into boiling water without incubation at 30°C. The resulting mixture was centrifuged at low speed, and the supernatant fluid was assayed for CAMP using the radioimmunoassay of Steiner et al. (14).

**Materials**—The ionophore, A23187, was a generous gift of Dr. Robert Hammill, Eli Lilly. Crystallized bovine serum albumin was obtained from Armour Pharmaceuticals. Enzymes and other biochemicals were obtained from Sigma Chemical Co. (St. Louis) or from Boehringer-Mannheim.

**RESULTS**

**Effects of A23187 on Insulin Release**

A23187, at a concentration of 10 μM in the presence of 2.1 mM Ca²⁺ and in the absence of glucose, caused a significant but transient release of insulin from perfused islets (Fig. 1). It is worth emphasizing here that in most experiments performed in this study there was no glucose added to the perfusion fluid. In one set of experiments, with 2.75 mM glucose present throughout, exposure to 10 μM ionophore yielded insulin release of similar magnitude as observed when glucose was absent (95 ± 10 versus 40 ± 8 microunits/100 islet × min in experiments (n = 3) versus controls (n = 3)). The onset of release occurred as quickly as that observed when islets were challenged with high concentrations of glucose. The peak rate of insulin release produced by 10 μM of the ionophore was twice the basal rate. In comparison, first phase insulin release produced by 27.5 mM glucose was 3 times the basal rate.

After the ionophore (or the solvent in the controls) had been removed from the perfusion medium, the islets retained their full capacity to respond in characteristic biphasic fashion to high glucose concentrations. Thus, it is apparent that the ionophore did not produce irreversible damage to the insulin releasing mechanism.

Dose-response studies with the ionophore revealed a steep curve (Figs. 2 and 3). The threshold level was 1 μM and maximal insulin release was achieved by 10 μM. After exposure to a higher concentration of the ionophore (25 μM), the first phase of the insulin release in response to the subsequent addition of 27.5 mM glucose was blunted, suggesting that this high concentration of ionophore may be deleterious.

The role of extracellular Ca²⁺ on ionophore-stimulated insulin release was examined. Preliminary experiments (not shown) indicated that the endocrine response to ionophore was equal with and without added calcium in the perfusate. More convincingly, experiments carried out in EGTA-Ca²⁺ buffers clearly demonstrated that the response to the ionophore was elicited in the complete absence of Ca²⁺ in the perfusate (Fig. 4). However, the peak of insulin release was delayed by 3 to 4 min and was more pronounced. When 27.5 mM glucose and 2.1 mM Ca²⁺ were then reintroduced, both experimentals and controls responded. However, the islets previously perfused by Ca²⁺-free medium lacked the first phase response.

Since other studies have revealed significant intracellular calcium stores (17), the response to the ionophore was ascribed to a failure to deplete these stores. Since theophylline has been shown to increase Ca²⁺ flux from pancreatic islets (18), we used pretreatment with this agent in an attempt to deplete intracellular Ca²⁺ stores and to demonstrate a calcium dependency for the action of the ionophore. Therefore, islets were preperfused in the absence of Ca²⁺ for 30 min in the presence of EGTA and 5 mM theophylline. This pretreatment did not eliminate the secretory response to the ionophore (also not shown).

**Effect of A23187 on cAMP Levels of Islet Tissue**

Cytosolic calcium has been thought of as an inhibitor of adenylate cyclase (1). As predicted, A23187 has been reported to decrease intracellular cAMP levels in the presence of extracellular Ca²⁺ in other tissues (8). Since the precise roles of cAMP and Ca²⁺ in insulin release are not clear and since the roles of the two factors might be interdependent, we examined the effect of the ionophore on islet cAMP levels as a function of the extracellular Ca²⁺ concentration. A nearly 2-fold increase in cAMP levels of islets was found 3 min after exposure to the ionophore in the presence of 2.1 mM Ca²⁺ (Table 1). This interval was chosen for tissue sampling since it coincided with maximal insulin release following introduction of the ionophore (Fig. 1).

In agreement with others, we previously have described elevation of cAMP levels in islets stimulated by a high concentration of glucose (20, 21). In addition, we have demonstrated that such an effect is dependent upon extracellular Ca²⁺, as indeed is insulin release itself in response to high concentrations of glucose (21). Since the ionophore is capable of stimulating insulin release in the absence of extracellular Ca²⁺, we have examined its effects upon cAMP levels in the absence of Ca²⁺. We found that cAMP levels were unaltered in response to the ionophore in the absence of

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**FIG. 1. Temporal insulin release profile in response to 10 μM ionophore A23187.** Islets were preperfused in the absence of extracellular glucose for 30 min and then exposed to the ionophore. Control islets were exposed to the solvent (0.25% acetone plus 1% ethanol). Both groups subsequently were exposed to high glucose. The numbers of experiments are given in parentheses. The means of the results are recorded and the standard errors (± S.E.) are indicated at strategic points of the release profiles.
Fig. 2. Insulin release in response to varying ionophore concentrations. The protocol was as described in the legend to Fig. 1 and under "Experimental Procedure." The micromolar concentrations of A23187 are indicated. The means of the results from at least three perfusion experiments are recorded for each level of the ionophore. The standard errors were as recorded in Fig. 1 but are not shown for reasons of clarity of the graphs.

Fig. 3. Rate of insulin release 3 min after exposure to varying concentrations of ionophore. The means + S.E. values of the results contained in Fig. 2 are recorded.

exocellular Ca\(^{2+}\), although release of insulin still occurred and was even more pronounced than with Ca\(^{2+}\) present (Table I and Fig. 4).

As previous studies have demonstrated that ethanol is capable of stimulating adenylate cyclase activity (19), the effect of the solvent used here for the ionophore (0.25% acetone plus 1% ethanol) upon cAMP levels in islets was examined. The solvent

Fig. 4. Insulin release in response to ionophore in various Ca\(^{2+}\)-EGTA buffers. Islets were preperfused in presence or absence of Ca\(^{2+}\) in the presence of 2 mM EGTA for 30 min prior to and during exposure to 10 \(\mu M\) ionophore. The profile designated zero Ca\(^{2+}\) had no added Ca\(^{2+}\) and MgCl\(_2\) was increased to 3 mM. The profile designated 2.1 mM Ca\(^{2+}\) had 1 mM Mg\(^{2+}\), 27.5 mM glucose, 4.1 Ca\(^{2+}\) and EGTA. The means of an indicated number of experiments are recorded and the standard errors are shown at essential points of the release profiles (± S.E.).
The islets were preperfused for 30 min with Krebs-Ringer buffer (KRB) containing 0.5% bovine serum albumin but in the absence of glucose and were sampled 3 to 4 min after exposure to the ionophore as indicated. The cAMP level of control islets perfused for 33 min in the absence of both glucose and solvent was 4.72 ± 0.67 pmol/100 islets (n = 4). Results in the presence of 10 µM ionophore (Experimental) are compared with the corresponding solvent controls (0.25% acetone plus 1% ethanol). The mean ± S.E. values of an indicated number of experiments are recorded.

| Conditions, sampling time | cAMP pmol/100 islets | Insulin release microunits/min x 100 islets |
|--------------------------|----------------------|------------------------------------------|
|                          | Solvent controls     | Experiments                              |
| a. A23187 (10 µM), normal KRB (3 min) | 4.01 ± 0.33 (9) | 7.54 ± 0.6 (5) |
| b. A23187 (10 µM), zero Ca++, 3 mM MgCl₂, 2 mM EGTA (4 min) | 2.54 ± 0.26 (6) | 2.53 ± 0.36 (6) |
| c. A23187 (10 µM), normal KRB, 5 mM theophylline (3 min) | 7.19 ± 0.59 (7) | 6.13 ± 0.45 (6) |

* The values for insulin recorded here were obtained 1 min prior to sampling the islets and therefore do not represent the peak rate of hormone release but indicate that the islets responded to the ionophore as predicted (compare the results in the corresponding figures).

b Significantly different from corresponding solvent controls, p < 0.002.

**TABLE II**

**Lack of effect of A23187 on adenylate cyclase activity of islet tissue homogenate**

The means ± S.E.M. values of an indicated number of experiments are recorded.

| Conditions | Adenylate cyclase activity pmol cAMP formed/µg protein/45 min |
|-----------|-------------------------------------------------------------|
| No additions | 1.50 ± 0.43 (6) |
| Solvent control (0.25% acetone plus 1% ethanol) | 1.20 ± 0.14 (6) |
| A23187 (10 µM) plus above solvent | 1.34 ± 0.22 (6) |

alone had no effect on cAMP levels, as indicated in the legend to Table I.

Lack of glucose in the perfusate did cause an almost 3-fold elevation of the base-line levels of cAMP, as is apparent when the present data are compared with previously published values from this laboratory (20). Average control values in our hands in the presence of 2.75 mM glucose has been 1.6 pmol/100 islets. There was a decrease in the base-line levels of cAMP (−36%) in the solvent controls during perfusion in Ca⁺⁺-free medium.

The mechanism of ionophore-stimulated increases in cAMP in the presence of Ca⁺⁺ is unclear. A priori, one might postulate that the ionophore itself, or changes in Ca⁺⁺ fluxes caused by the ionophore might either stimulate adenylate cyclase or inhibit phosphodiesterase. To investigate those possibilities, the following experiments were designed.

**Experiment 1**—The possible effect of the ionophore and the solvent on adenylate cyclase activity was explored in the absence of added Ca⁺⁺ in rat islet homogenates. Neither the ionophore nor the solvent (0.25% acetone plus 1% alcohol) significantly altered adenylate cyclase activity in rat islet homogenates (Table II). These experiments were carried out in the absence of added Ca⁺⁺ and the presence of 1 mM EGTA. It would be desirable to perform similar studies as a function of the Ca⁺⁺ concentration in the assay media. Since, however, physiological levels (i.e. extracellular Ca⁺⁺ concentration) of Ca⁺⁺ (2.1 mM) greatly suppress adenylate cyclase activity (more than 80%) and since we were unable to perform activity measurements at a fixed low Ca⁺⁺ level (e.g. 10⁻⁵ M) because of the substantial but variable Ca⁺⁺ contamination from the islet tissue homogenates themselves, such studies have not been performed to date.

**Experiment 2**—The possible involvement of phosphodiesterase was examined with the aid of theophylline by measuring the effect of this agent in the perfusion system upon insulin release and cAMP levels in response to the ionophore. If elevations of cAMP and insulin release were due to an action of the ionophore on phosphodiesterase, blocking that enzyme with theophylline should obviate these responses to the ionophore. It was shown that the insulin release stimulated by the ionophore is not modified in the presence of 5 mM theophylline (Fig. 5). The levels of cAMP were elevated in theophylline-treated control islets, indicating that phosphodiesterase activity was inhibited and the ionophore caused no further increase of cAMP levels in these tissues (Table I). Furthermore, it was noted that theophylline exposure in the solvent controls increased cAMP levels without a
significant increase in insulin secretion (Table I, compare a and c). In the course of these studies it was also observed that the increase of cAMP elicited by 5 mM theophylline in the absence of extracellular glucose occurred independent of extracellular Ca2+ (6.42 ± 0.25 and 6.30 ± 0.40 pmol of cAMP/100 islets following theophylline in the presence and absence, respectively, of Ca2+ as compared to 4.01 ± 0.33 pmol/100 islets in controls).

DISCUSSION

The divalent cation ionophore A23187 proved to be an effective stimulator of insulin release in the absence of extracellular glucose. Islets exposed to the ionophore exhibited a brisk monophasic release of insulin, and the same islets subsequently challenged by high glucose responded with typical biphasic insulin release. While it has been postulated that the ionophore's action on many systems is mediated through augmented Ca2+ influx across the cell membranes (8), we were unable to show a dependency on extracellular calcium in perfused islets. In the absence of extracellular calcium and in the presence of 10 μM of the ionophore, insulin release occurred, although somewhat delayed and magnified as compared to the experiments with Ca2+ present. Three possible explanations for this Ca2+-independent effect deserve to be considered. First, intracellular stores of calcium (e.g. mitochondria), which almost certainly are mobilized by the agent (10), are sufficiently high to allow insulin release in the absence of extracellular calcium. The delay of the response may be an expression of the involvement of such an intracellular Ca2+ pool. Second, since the ionophore binds and transports magnesium as well as calcium (10), the observed effects may be due to altered magnesium distribution and due to secondary effects on the numerous Mg2+-dependent processes of the cell. A possible involvement of magnesium cannot be ruled out currently. Third, the ionophore's action may be independent of the translocation of divalent cations. Detailed studies on fluxes of Ca2+ and Mg2+ might clarify this point.

cAMP plays a prominent role as modulator of insulin discharge, perhaps mediating the effects of various hormones and transmitters. Several groups of investigators recently have demonstrated a rapid rise of tissue levels of this nucleotide after the exposure of islets to high concentrations of glucose (1, 20–23). These observations may imply that cAMP is also the second messenger for glucose-stimulated insulin release. Zawalich et al. (21) and Charles et al. (24) have documented the calcium dependency of this cAMP rise. The present studies have shown that the ionophore also elevates cAMP levels in isolated islets in a Ca2+-dependent manner. This contrasts with the findings of Prince et al. (8) who reported that cAMP levels decreased in fly salivary gland on stimulation of secretion by the ionophore. In the presence of theophylline, cAMP levels of islets were elevated as predicted, but when the ionophore was superimposed, no further rise of the cyclic nucleotide occurred. Nevertheless, neither the kinetics nor the magnitude of ionophore-induced hormone release were modified. This lack of potentiation of the insulin response argues against a primary involvement of the cAMP system in the ionophore induced insulin secretion. Indeed, islets perfused without extracellular calcium showed no rise of cAMP due to the ionophore, while stimulation of insulin secretion was preserved.

Based on experimental evidence presented, it is suggested that insulin release due to the ionophore results from mobilization of calcium from either extracellular or intracellular sources. Mobilization of Ca2+ by the ionophore has two effects: first, insulin release occurs, and second, the cAMP level of islets increases. However, the two events may not be coupled since, in islets exposed to the ionophore in the absence of extracellular calcium, insulin release occurred but the cAMP level (as measured in these experiments) remained constant.

It is tempting to speculate that altered Ca2+ conductivity or binding induced either by glucose (25) or by the ionophore specifically at the site of the plasma membrane might lead to an activation of adenylate cyclase in the plasma membrane. Mobilization of Ca2+ from intracellular stores (e.g. mitochondria), although capable of causing insulin release, lacks this effect on adenylate cyclase. Thus, enhanced insulin release is a result of elevated intracellular Ca2+ levels whatever the source of Ca2+ might be, whereas adenylate cyclase activation depends on a circumscribed change of Ca2+ distribution within or across the cell membrane. Such a concept would explain why to date all attempts to demonstrate an effect of glucose on adenylate cyclase in cell free systems have been unsuccessful.

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