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Calcium-induced Insulin Release in Monolayer Culture of the Endocrine Pancreas

STUDIES WITH IONOPHORE A23187*

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SUMMARY

The role of Ca^{2+} on insulin release has been studied by the use of ionophore A23187. The ionophore complexes divalent cations and permits Ca^{2+} entry into cells by acting as a carrier in the plasma membranes. Cultured cells obtained by enzymatic digestion of pancreases from newborn rats were studied on the 3rd day of culture. With Ca^{2+} in the incubation medium the ionophore induced sustained insulin release even in the absence of glucose. Optimal effects of the ionophore were observed at 3 and 10 μg per ml in the presence of 0.3 to 1.0 mM Ca^{2+}. Under these conditions the insulin release was greater than that caused by 16.7 mM glucose. A graded response was observed to changes in Ca^{2+} concentration from 0.1 to 1.0 mM Ca^{2+}. Higher Ca^{2+} concentrations caused a large amount of insulin to be released promptly, but the release was not sustained. Mg^{2+} and Sr^{2+} were not found to substitute for Ca^{2+}. Ba^{2+} at 0.3 mM stimulated insulin release even in the absence of ionophore. Cyclic adenosine 3′:5′-monophosphate was able to increase ionophore-induced insulin release. The α-adrenergic effect of epinephrine to inhibit insulin release was not observed in the presence of Ca^{2+} and the ionophore, and a stimulatory effect of epinephrine was seen. This unusual stimulatory effect of epinephrine was blocked by propranolol indicating a β-adrenergic mechanism for epinephrine. It is concluded that Ca^{2+}, which plays an essential role in the stimulus-secretion coupling, can alone initiate and cause sustained insulin release.

Calcium is known to play an important role in hormone secretion of endocrine glands. In the case of insulin release from the pancreatic β-cell, glucose, the most important physiological stimulus to release, is dependent for its action on the presence of calcium in the extracellular medium (1–4). Malaisse-Lagae and Malaisse (5) found that glucose stimulated net uptake of radioactive calcium by isolated islets of Langerhans. The same workers later suggested that the mechanism by which the islets gained calcium in response to glucose was by a decrease in calcium efflux from the cells (6). Hellman *et al.* (7), using different techniques, reported that glucose stimulated the entry of calcium into islets but were not able to detect a change in steady state content. These studies suggested that the accumulation of intracellular calcium was an important component of the insulin release mechanism. The precise role played by calcium, however, remains to be determined.

With the description in recent years of the ionophore A23187, a carboxylic acid antibiotic that specifically transports divalent cations across biological membranes (8–11), it became possible to examine the effects of calcium on insulin release more directly and in the absence of normal stimulators of the release mechanism. The use of the ionophore in studying the biological effects of Ca^{2+} has already been exploited for studies of histamine release from mast cells (12, 13), fluid secretion by fly salivary gland cells (14), K^+ release in the parotid gland (15), contractility in frog eggs (16), and ATP secretion by platelets (17). In these studies it was shown that calcium in the presence of A23187 could mimic physiological responses by the tissues. The present investigation was undertaken to examine the hypothesis that an increased intracellular calcium concentration could elicit insulin release from pancreatic β-cells. Because of the need for optimal exposure of β-cell membranes to the ionophore, the monolayer culture preparation of newborn rat pancreas was chosen for the study.

EXPERIMENTAL PROCEDURES

The culture procedure for endocrine pancreas has been described previously (18, 19). In brief (for one culture preparation) 100 to 120 pancreases from 1- to 3-day-old Wistar rats were excised by sterile technique. The pooled pancreases were cut into pieces 1 to 2 mm in diameter. After one washing in calcium- and magnesium-free phosphate-buffered saline the pieces of pancreas were submitted to approximately 10 consecutive treatments with a mixture of trypsin and collagenase in calcium- and magnesium-free PBS[1] containing 2.8 mM glucose at 37°. The three to four

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[1] The abbreviations used are: PBS, phosphate-buffered saline; KRB, Krebs-Ringer bicarbonate buffer; cyclic AMP, cyclic adenosine 3′:5′-monophosphate.

initial supernatants were discarded. The subsequent supernatants containing isolated cells were kept and diluted with cold (+4°) culture medium (Medium 199) containing 10% calf serum, 14 mM sodium bicarbonate, 16.7 mM glucose, and sodium penicillin G (400 units per ml). The pooled supernatants were washed twice by spinning at 150 × *g* and resuspending the packed cells in Medium 199. The final solution was diluted to contain about 700,000 cells per ml and plated in 60-mm diameter plastic Petri dishes. By this procedure 30 to 50 culture dishes were obtained. The cells were cultured at 37° in an incubator with automatic pH regulation. About 14 hours after plating, at a time when a major portion of fibroblastoid cells but only a minor portion of the epithelioid cells had attached to the bottom of the culture dishes, the supernatants were decanted into new Petri dishes. (The residual dishes were discarded.) As reported previously (18, 19) this decantation yielded cultures which were rich in epithelioid cells. Forty-four hours after plating, when the epithelioid cells had attached to the bottom of the dish and rearranged in clusters of varying size, the cells were washed, and fresh culture medium containing 5.6 mM glucose was added. On the 3rd day of culture the cells were used for short incubations in Krebs-Ringer bicarbonate buffer. By this time amylase activity could not be detected in the culture medium, and exocrine cells did not contain zymogen granules when examined by electron microscopy (20).

The normal KRB buffer contained 0.5% dialyzed bovine serum albumin, 250 kallikrein inhibitory units per ml of Trasylol, and different concentrations of glucose and the agents under test. The ionic composition of the standard KRB buffer was: Na^+, 142.9 mM; K^+, 5.8 mM; Ca^{2+}, 2.5 mM; Mg^{2+}, 1.2 mM; Cl^-, 128.4 mM; SO_4^{2-}, 1.2 mM; $H_2PO_4^-$, 0.8 mM; and HCO_3^-, 24.4 mM. Magnesium-deficient medium was obtained by omission of $MgSO_4$ from the standard buffer; calcium-deficient buffer was obtained by omission of $CaCl_2$ and replacement of $MgSO_4$ by $MgCl_2$ where appropriate. To the calcium-depleted buffer varying concentrations of $CaCl_2$, $MgSO_4$, $SrCl_2$, or $BaCl_2$ were added. Ascorbic acid, at a concentration of 1.1 mM, was added to the KRB buffer in experiments with epinephrine. A23187 was dispersed in the incubation buffer in small glass tissue homogenizers to yield a fine concentrated suspension, since the ionophore does not dissolve in water. The suspension of A23187 was added to the culture dish immediately prior to the incubation.

Incubations were carried out over 2 hours at 37°, and pH was maintained at 7.40 ± 0.05. In most experiments an aliquot of the incubation medium was taken at 30 min and at 2 hours. Samples were immediately frozen and stored at −20° until assay. Insulin content of the cultured cells was determined by extracting the hormone with 0.15 N HCl in 75% ethanol and assaying at high dilution. Insulin was measured by radioimmunoassay with guinea pig antiporcine insulin serum and rat insulin as standard. Antibody-bound hormone was separated from free hormone with the use of dextran-coated charcoal as described by Herbert *et al.* (21). To avoid interference in the assay by A23187 two different methods were employed. The samples were either centrifuged 20 min at 3500 × *g* and the supernatant decanted or 200 µl of carbon tetrachloride were added to extract A23187 from the media. Both methods were equally effective in eliminating interference in the assay. Results are expressed as means of immunoreactive insulin ± S.E. Petri dishes were paired before the experiments into test and control dishes. Statistical analysis was by paired Student's *t* test. Where an occasional Petri dish was lost, its pair was deleted from the analysis.

The chemicals employed and their sources were as follows: trypsin (Difco Laboratories, Detroit, Mich.), collagenase (Worthington Biochemicals, Corp., Freehold, N. J.), Medium 199 (Grand Island Biological Co., Grand Island, N. Y.), sodium penicillin G (Charles Pfizer and Co., Inc., Brooklyn, N. Y.), Petri dishes (Falcon Plastics, Oxnard, Calif.), bovine serum albumin (Behringwerke A.G., Marburg, FRG), Trasylol (Bayer Pharma A.G., Zurich, Switzerland), A23187 (kindly provided by Dr. O. Behrens, Eli Lilly, Indianapolis, Ind.), anti-insulin serum (generously provided by Dr. P. Wright, University of Indiana, Indianapolis, Ind.), 10× recrystallized rat insulin (Novo Research Institute, Copenhagen, Denmark), carbon tetrachloride and ascorbic acid (Merck A.G., Darmstadt, FRG), epinephrine (Vifor S. A., Geneva, Switzerland), cyclic adenosine 3′:5′-monophosphate (Fluka AG, Buchs, Switzerland), sodium pyruvate (Boehringer Mannheim GmbH, Mannheim, FRG), propranolol (Imperial Chemical Industries, Alderley Park, United Kingdom).

RESULTS

When A23187 was added to the pancreatic monolayer culture in Krebs-Ringer bicarbonate medium containing 2.8 mM glucose and 2.5 mM $CaCl_2$, a marked stimulation of insulin release occurred. Increased release of insulin occurred in the first 30 min after addition of the ionophore, and the increase was sustained up to 2 hours, the longest time period studied (see Fig. 1, *left-hand panels*). Maximal stimulation under these conditions was achieved at an ionophore concentration of 10 µg per ml. Concentrations of the ionophore below 1 µg per ml had no effect upon insulin release.

In the presence of 16.7 mM glucose, which itself stimulated insulin release, a complex pattern of ionophore effects was observed (see Fig. 1, *right-hand panels*). During the first 30 min, glucose-induced insulin release was enhanced only by ionophore concentrations of 3 and 10 µg per ml. At 2 hours, glucose-induced insulin release was further stimulated only by the ionophore at 3 µg per ml, and a slight but nonsignificant decrease in release occurred with 10 µg per ml of ionophore.

In these experiments it was possible to compare the insulin-releasing ability of the ionophore in the presence of a low (non-stimulating) glucose concentration with the ability of a high glucose concentration to release insulin. Over 30 min the stimulation of insulin release by ionophore (10 µg per ml) in low glucose was similar to that achieved by high glucose alone. Over 2 hours, however, high glucose caused a significantly greater insulin release than the ionophore ($p < 0.01$).

At 3 µg per ml of ionophore in the presence of low glucose, little increase in insulin release occurred over the first 30 min while high release was observed over the next 90 min, higher in

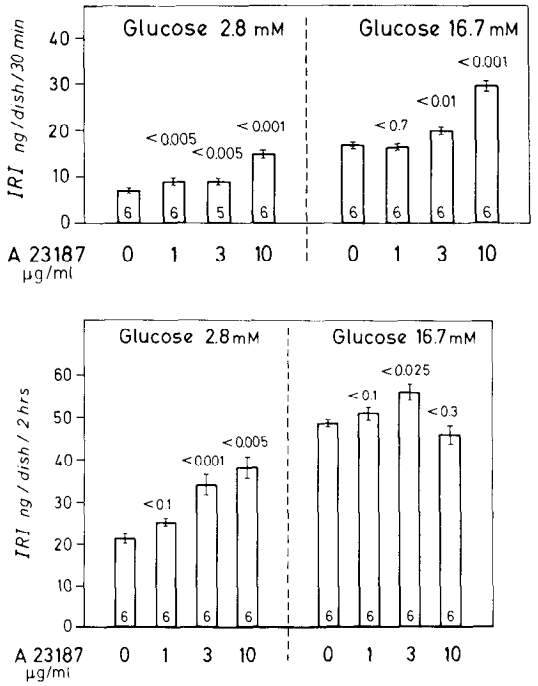

FIG. 1. Effect of different concentrations of ionophore A23187 on insulin release. Experiments performed in the presence of 2.5 mM Ca^{2+} and 2.8 mM glucose (*left hand panels*) or 16.7 mM glucose (*right hand panels*). Insulin release was measured after 30 min of incubation (*upper panels*) and 2 hours (*lower panels*). *Vertical lines* represent ±S.E. The number of experiments is indicated in each block. *IRI*, immunoreactive insulin.

TABLE I

Effect of ionophore A23187 at different concentrations on insulin release expressed as percentage of release relative to total insulin content of cultured cells (n = 6)

Glucose concentration	Ionophore	Release ± S.E.
mM	μg/ml	%
2.8	0	3.7 ± 0.2
2.8	10	8.2 ± 0.7
2.8	3	6.6 ± 0.4
2.8	1	5.1 ± 0.3
16.7	0	8.5 ± 0.2
16.7	10	10.4 ± 0.6
16.7	3	11.3 ± 0.3
16.7	1	10.3 ± 0.3

TABLE II

Effect of ionophore A23187 (10 μg per ml) on insulin release in presence of different concentrations of Ca^{2+} and 16.7 mM glucose

Ca^{2+} concentration	Insulin release[a]			p	n
	Control	Test	Δ		
mM					
30 Min					
0.3	5.5 ± 0.7	5.2 ± 0.6	−0.3 ± 0.5	<0.7	17
1.0	11.6 ± 0.7	14.2 ± 0.9	2.6 ± 0.8	<0.01	11
2.5	14.5 ± 0.9	23.5 ± 2.2	9.0 ± 1.5	<0.001	11
2 Hs					
0.3	15.3 ± 1.7	21.2 ± 1.7	5.9 ± 2.1	<0.02	16
1.0	30.2 ± 1.5	31.9 ± 1.5	1.7 ± 2.3	<0.5	11
2.5	41.7 ± 2.5	37.6 ± 3.5	−4.1 ± 2.3	<0.3	11

[a] Insulin release measured after 30 min and after 2 hours. Results expressed in nanograms per Petri dish ± S.E.

fact than with 10 μg per ml of ionophore, which gave a marked stimulation over the first 30 min. A likely explanation of this result is that the calcium entry at 3 μg per ml of ionophore was rate limiting over the early time period but became less so as the ionophore action developed. In the case of 10 μg per ml of ionophore, insulin release was rapid in the first 30 min and then decelerated during the next 90 min, perhaps by permitting excessive calcium entry and inhibition of calcium-sensitive enzyme systems. This is perhaps seen better in the case of high glucose and 10 μg per ml of ionophore where the intracellular calcium accumulation mechanisms of glucose and the ionophore are presumably reinforcing each other.

In these experiments the immunoreactive insulin release has also been expressed as a percentage of the total insulin content in the cultured cells. From the results shown in Table I, it can be seen that the release was lowest in 2.8 mM glucose (3.7%) and highest in the presence of high glucose + 3 μg per ml of ionophore (11.3%).

Further evidence along these lines was obtained from experiments in high glucose (16.7 mM) and different concentrations of Ca^{2+}. In these experiments (see results in Table II) Ca^{2+}, at 1.0 and 2.5 mM in the presence of 10 μg per ml of ionophore, stimulated insulin release at 30 min more than did high glucose

TABLE III

Effect of ionophore A23187 (10 μg per ml) on insulin release in presence of different concentrations of Ca^{2+} and 2.8 mM glucose

Ca^{2+} concentration	Insulin release[a]			p	n
	Control	Test	Δ		
mM					
30 Min					
0.1	7.9 ± 1.2	7.9 ± 1.3	0.0 ± 0.5		16
0.3	8.2 ± 1.8	8.2 ± 1.4	0.0 ± 0.8		14
1.0	6.5 ± 1.1	8.2 ± 1.1	1.7 ± 0.6	<0.02	16
2.5	7.0 ± 1.2	9.6 ± 1.1	2.6 ± 0.6	<0.001	16
2 Hs					
0.1	14.6 ± 2.3	22.7 ± 2.2	8.1 ± 1.4	<0.001	15
0.3	14.6 ± 2.4	24.5 ± 3.9	9.9 ± 2.5	<0.005	15
1.0	15.1 ± 2.5	24.1 ± 1.8	9.0 ± 2.0	<0.001	15
2.5	13.2 ± 2.0	17.6 ± 2.2	4.4 ± 1.6	<0.02	16

[a] Insulin release measured after 30 min and after 2 hours. Results expressed in nanograms per Petri dish ± S.E.

alone. Ca^{2+} (0.3 mM) did not increase insulin release over this time. At 2 hours, however, the situation reversed. Ca^{2+} at 1.0 and 2.5 mM did not stimulate more than high glucose whereas Ca^{2+} at 0.3 mM did. Thus we have an effect of high Ca^{2+} to cause marked insulin release rapidly, but not to sustain the release, and an effect of lower Ca^{2+} (0.3 mM) to induce insulin release after a longer time period.

In view of these data further experiments were designed to determine the optimum conditions for ionophore action and then to see whether the ionophore under low glucose conditions could elicit a greater insulin release than that achieved by the stimulus of high glucose concentration. Consequently, the effects of different calcium concentrations were studied with the use of Krebs-Ringer bicarbonate medium, 2.8 mM glucose, and 10 μg per ml of A23187 (see Table III). In the first 30 min of incubation, insulin release was significantly increased only by calcium concentrations of 1.0 and 2.5 mM. Calcium at 0.1 mM and 0.3 mM was without effect. Over the next 90 min, however, all four concentrations of calcium produced a significant increase in insulin release. Optimal effects at 2 hours were seen with calcium at 0.3 and 1.0 mM. With 2.5 mM calcium, insulin release was clearly less than that achieved by 0.3 and 1.0 mM over the longer incubation period ($p < 0.001$ compared with 1.0 mM Ca^{2+}). In the absence of Ca^{2+} in the medium no increase of insulin release was detected (Table IV, A). As optimum effects were seen at 0.3 mM and 1.0 mM, these concentrations were chosen for all subsequent studies with the ionophore. Thus a further comparison was made of the effects of ionophore under optimal conditions, relative to high glucose in the medium. From the results shown in Fig. 2 it can be seen that over 2 hours with 0.3 mM Ca^{2+} in the medium, the ionophore caused an increase in insulin release which was significantly greater than caused by 16.7 mM glucose. Thus in contrast to the results obtained in the presence of 2.5 mM Ca^{2+}, the ionophore was now more effective than high glucose in eliciting insulin release over 2 hours. The combination of ionophore and high glucose gave greater insulin release than high glucose and was not significantly different from that achieved with ionophore and low glucose.

To exclude the possibility that A23187 altered the sensitivity

TABLE IV

Effects of Sr^{2+}, Ba^{2+}, and Mg^{2+} on insulin release over 2 hours

Experiments performed with different concentrations of the divalent cations. Ionophore A23187 (10 μg per ml) was used in test dishes. Glucose (2.8 mM) present throughout. Results expressed in nanograms per Petri dish ± S.E.

Ca^{2+} concentration	Test ion	Insulation release			p	n
mM		Control	Test	Δ		
			A			
0	0	8.5 ± 0.6	9.0 ± 0.7	0.5 ± 0.5	<0.4	5
0.3	0	6.9 ± 0.6	14.1 ± 0.8	7.2 ± 1.0	<0.005	5
0	0.3 Sr^{2+}	7.9 ± 0.6	9.6 ± 0.5	1.7 ± 1.0	<0.2	5
0	3.0 Sr^{2+}	8.3 ± 0.7	10.2 ± 0.2	1.9 ± 0.8	<0.1	5
			B			
1.0	0	7.2 ± 0.5	13.9 ± 1.7	6.7 ± 1.8	<0.02	5
0	0.03 Ba^{2+}	6.0 ± 0.5	7.1 ± 0.6	1.1 ± 0.8	<0.3	5
0	0.1 Ba^{2+}	6.2 ± 0.6	6.7 ± 0.5	0.5 ± 0.4	<0.3	5
0	0.3 Ba^{2+}	22.3 ± 0.7	18.4 ± 0.4	−3.9 ± 0.8	<0.01	5
			C			
0.3	1.2 Mg^{2+}	6.6 ± 0.7	19.4 ± 1.4	12.8 ± 1.6	<0.005	5
0.3	0	7.4 ± 0.7	19.9 ± 1.4	12.5 ± 1.1	<0.001	5
0	12.0 Mg^{2+}	6.6 ± 0.6	5.9 ± −0.6	−0.7 ± 1.0	<0.6	5
0.03	1.0 Mg^{2+}	4.1 ± 0.4	6.7 ± 1.0	2.6 ± 0.7	<0.005	12
0.03	10.0 Mg^{2+}	3.7 ± 0.9	6.2 ± 0.8	2.5 ± 0.8	<0.01	12
0.03	20.0 Mg^{2+}	3.1 ± 0.4	6.2 ± 0.2	3.1 ± 0.5	<0.005	6

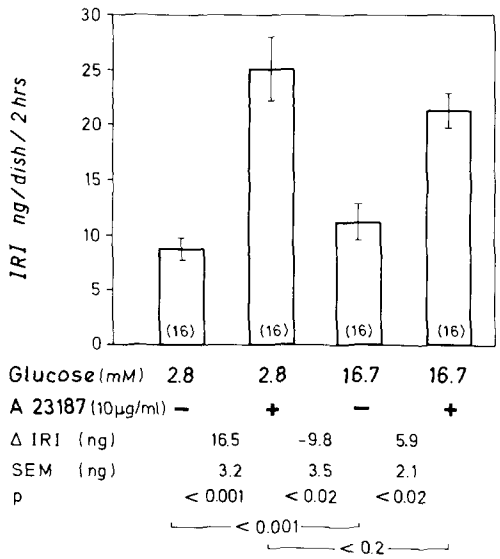

FIG. 2. Effects of ionophore A23187 on insulin release. Experiments performed with 2.8 mM or 16.7 mM glucose in the presence or absence of 10 μg per ml of ionophore. Ca^{2+} concentration was 0.3 mM throughout. *Vertical lines* represent ±S.E. The number of experiments is indicated in each block. *IRI*, immunoreactive insulin.

of the recognition of glucose by the β-cell and that 2.8 mM glucose thereby became a stimulating concentration, the effects of the ionophore were studied under conditions in which glucose was completely absent from the bathing medium, or in the absence of glucose but in the presence of pyruvate. Ca^{2+} (0.3 mM) was present throughout. In six paired experiments performed in the absence of glucose, insulin release was 15.6 ± 1.0 ng per Petri dish per 2 hours, and after addition of A23187 it was 24.8 ± 0.8 ng per Petri dish per 2 hours (Δ = 9.2 ± 1.4, $p < 0.005$). When 11 mM pyruvate was present and glucose absent the results were similar; it was found that the control insulin release in seven paired experiments was 14.0 ± 1.6 ng per Petri dish per 2 hours and after A23187 22.2 ± 2.0 ng per Petri dish per 2 hours (Δ = 8.2 ± 2.0, $p < 0.01$). Thus, the ionophore was capable of eliciting insulin release in the absence of glucose in the medium.

The specificity of the calcium-induced insulin release in the presence of ionophore was tested by replacement experiments using Sr^{2+}, Ba^{2+}, and Mg^{2+}. In Table IV (A) are shown the results obtained when experiments were performed in the absence of calcium or in the presence of 0.3 mM Ca^{2+} and when the calcium was replaced by 0.3 and 3.0 mM Sr^{2+}. Insulin release was elicited only when calcium was present. In the absence of calcium, or when Sr^{2+} replaced calcium in the medium, no significant increase in insulin release was detected. In the experiments in which Ba^{2+} replaced Ca^{2+}, Ba^{2+} concentrations of 0.03, 0.1, and 0.3 mM were tested. From the results in Table IV (B) it can be seen that no response was elicited by 0.03 or 0.1 mM Ba^{2+} in the presence of ionophore. Higher concentrations could not be tested because Ba^{2+} at 0.3 mM was stimulatory to insulin release even in the absence of ionophore. This stimulation was inhibited slightly by ionophore treatment. The role of Mg^{2+} in the Ca^{2+}-induced release reaction was studied in experiments in which Mg^{2+} was either removed from the media (its usual concentration being 1.2 mM) or its concentration increased to 12 mM (see Table IV, C). Removal of Mg^{2+} had no effect upon the insulin release due to A23187 and 0.3 mM Ca^{2+}. Furthermore, 12 mM Mg^{2+} in the absence of Ca^{2+} was incapable of eliciting insulin release in the presence of the ionophore. A second series of experiments tested the effect of 1, 10, and 20 mM Mg^{2+} on ionophore-induced insulin release in the presence of a low Ca^{2+} concentration (0.03 mM), to seek for evidence that Mg^{2+} competes

with Ca^{2+} for the ionophore and thus inhibits entry into the cell. Under all three conditions, the ionophore caused a significant increase in insulin release, and no effect of Mg^{2+} was detected.

In a further characterization of the insulin release mechanism evoked by A23187 and Ca^{2+}, the well documented inhibition of insulin release by epinephrine (22–24) was studied. Experiments were performed over 2 hours in the presence of 0.3 mM Ca^{2+} and high glucose concentration (16.7 mM). From the results seen in Fig. 3 it is clear that epinephrine exerted its usual strong inhibitory effect upon the insulin release due to high glucose. The combination of high glucose and ionophore gave a greater insulin release than high glucose alone, a result in accord with those shown in Fig. 2 and Table II. Furthermore, epinephrine had no inhibitory effect in the presence of the ionophore but had, in marked contrast, a significant stimulatory effect. Further experiments were carried out to determine whether this stimulatory effect of epinephrine was mediated by a β-adrenergic effect and whether cyclic AMP was capable of stimulating the rate of ionophore-induced insulin release. Thus experiments were performed using the β-adrenergic blocking agent propranolol. In these experiments the effect of 10 μg per ml of A23187 was tested in the presence of epinephrine, propranolol, and the combination of epinephrine and propranolol. The results are shown in Table V. Epinephrine (2.7×10^{-7} M) enhanced the ionophore-induced insulin release, and propranolol (7.7×10^{-6} M) virtually eliminated the effect of epinephrine. Propranolol alone, however, enhanced the effect of the ionophore. In a further experiment propranolol at 9.6×10^{-7} M also enhanced the ionophore response. The results of experiments with cyclic AMP performed in the presence of 2.8 mM glucose and 1.0 mM Ca^{2+} are shown in Fig. 4. Cyclic AMP (10 mM) stimulated the basal insulin release at 30 min ($p < 0.001$) and at 2 hours ($p < 0.001$). A23187 produced its stimulation of release, and this was significant at 30 min ($p < 0.005$) and at 2 hours ($p < 0.001$). When cyclic AMP and the ionophore were added in combination a further increase in insulin release relative to ionophore alone was seen ($p < 0.001$ at 30 min, $p < 0.05$ at 2 hours). Cyclic AMP was also tested under conditions of low and high glucose in the absence of Ca^{2+} from the media. The results are given in Table VI. While the cells responded to 16.7 mM glucose in the presence of 1 mM Ca^{2+}, no effect was elicited in the absence of Ca^{2+}.

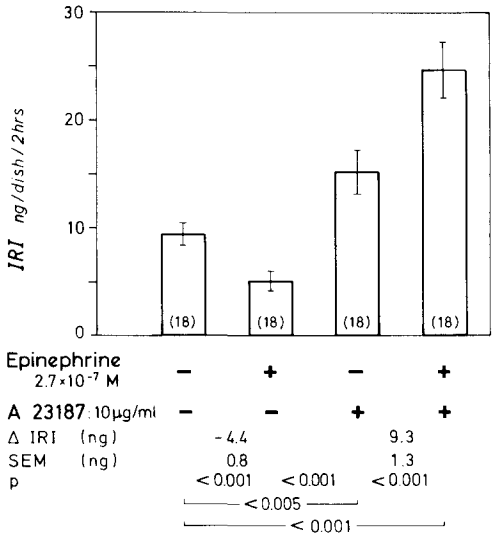

FIG. 3. Effects of epinephrine (2.7×10^{-7}M) on glucose-induced, and Ca^{2+}-and ionophore-induced insulin release. A23187 was used at a concentration of 10 μg per ml. All experiments were carried out in 0.3 mM Ca^{2+} and 16.7 mM glucose. *Vertical lines* represent ±S.E. The number of experiments is indicated in each block. *IRI*, immunoreactive insulin.

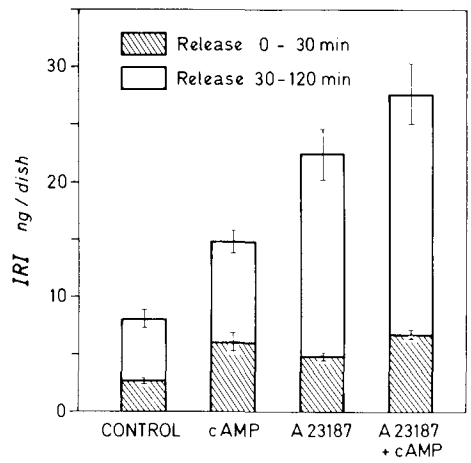

FIG. 4. Effect of cyclic AMP (10 mM) on Ca^{2+} and ionophore-induced insulin release. All experiments were carried out in 1 mM Ca^{2+} and 2.8 mM glucose. A23187 was used at a concentration of 10 μg per ml. ▨, release 0 to 30 min, □, release 30 to 120 min. *Vertical lines* represent ±S.E. $n = 11$; *IRI* = immunoreactive insulin.

TABLE VI

Effect of 10 mM cyclic AMP on insulin release in absence of Ca^{2+} from media and in presence of 2.8 mM or 16.7 mM glucose

Glucose concentration	Ca^{2+}	Insulin release[a]			p	n
		Control	+ Cyclic AMP	Δ ± S.E.		
mM						
2.8	1	6.5 ± 0.4				12
16.7	1	14.9 ± 1.8				12
2.8	0	7.5 ± 1.1	9.7 ± 1.7	2.2 ± 0.7	<0.02	12
16.7	0	6.4 ± 0.7	9.8 ± 1.1	3.4 ± 0.7	<0.001	12

[a] Insulin release measured after 2 hours. Results expressed in nanograms per Petri dish ± S.E.

TABLE V

Effect of propranolol (7.7×10^{-6} M) on insulin release induced by epinephrine (2.7×10^{-7} M) in presence of A23187

All experiments were carried out in the presence of 16.7 mM glucose, 0.3 mM Ca^{2+}, and 10 μg per ml of A23187. Insulin release was measured over 2 hours. Results expressed in nanograms per Petri dish ± S.E.

Control	Epinephrine	Propranolol	Epinephrine + propranolol	Δ	p	n
9.8 ± 1.1	15.2 ± 0.6			5.4 ± 1.4	<0.005	10
		15.7 ± 1.5		5.9 ± 1.4	<0.005	10
			10.4 ± 1.2	0.6 ± 1.4	<0.8	10

Cyclic AMP (10 mM) produced a significant increase in insulin release in the absence of Ca^{2+} in both low and high glucose. No significant increase in the response to cyclic AMP was observed in the high glucose incubations relative to the low glucose incubations.

DISCUSSION

The results of this work show that Ca^{2+}, with the aid of the ionophore A23187, can induce sustained insulin release from pancreatic β-cells. The ionophore is without effect in the absence of Ca^{2+}, and the latter has a concentration-graded effect upon insulin release. In these respects the results are analogous to those of Foreman *et al.* (12) and Cochrane and Douglas (13) who reported on histamine release from mast cells and of Prince *et al.* (14) who reported mimicry of physiological responses in fly salivary gland.

A23187 is an ionophore for divalent cations and not solely for Ca^{2+}. Thus the effects of Ca^{2+} which could not be reproduced by Sr^{2+} or Mg^{2+} suggest a tissue specificity for Ca^{2+} in the release mechanism. Ba^{2+} could not be tested in the presence of ionophore at concentrations as high as the other divalent cations, because at 0.3 mM Ba^{2+} alone has a stimulatory effect upon insulin release. However, at 0.03 and 0.1 mM it did not stimulate release in the presence of ionophore. Ba^{2+} has been reported previously to stimulate insulin release (25, 26) and to successfully replace Ca^{2+} in supporting insulin release due to glucose (27); thus failure in our experiments to demonstrate a Ba^{2+} effect at 0.03 and 0.1 mM could be due to either too low a Ba^{2+} concentration or a reduced transfer capacity of the ionophore for Ba^{2+} relative to Ca^{2+}. The transfer ability of ionophore A23187 with respect to different divalent cations is $Mn^{2+} > Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$ (28). Thus we do not conclude that Ba^{2+} cannot replace Ca^{2+} under optimal conditions but simply that under our conditions with the ionophore, Ba^{2+} was not shown to be effective. Similarly the low transfer capacity for Sr^{2+} could account for the lack of stimulatory effect of this ion. Given the similar affinities of Ca^{2+} and Mg^{2+} for the ionophore it is possible that competition for the ionophore could occur. That this was not apparent may suggest that at 10 μg per ml, the ionophore concentration in the membrane is in excess of that required for Ca^{2+} entry. Thus even in the face of 20 mM Mg^{2+} in the medium the rate of transfer of Ca^{2+} into the cell is still sufficient to elicit the characteristic response for a particular Ca^{2+} concentration.

It seems clear that at some point in the chain of events between recognition of glucose and the final release of insulin an increase in intracellular Ca^{2+} concentration is a crucial event. Whether this is early or late in this chain of events or even the final trigger to the secretion process, remains unknown. That the Ca^{2+} concentration has to be regulated precisely can be seen from the results of our experiments with different concentrations of Ca^{2+} and different concentrations of ionophore. For instance, at high Ca^{2+} and glucose concentrations, insulin release was stimulated rapidly, but the insulin release was not sustained. The inability of the cells to sustain insulin release under conditions of high Ca^{2+} and glucose concentrations was not due to depletion of the insulin content of the cells, because the highest release (at 3 μg per ml of ionophore and 16.7 mM glucose) amounted to only 11% of the content of the culture. Thus almost 90% of the insulin remained available for release under these conditions. In contrast, at lower Ca^{2+} concentrations (below 1 mM) insulin release was not stimulated rapidly, but large amounts of insulin were released between 30 and 120 min. Insulin release in these experiments will be determined by the amount of ionophore used, its rate of association with cell membranes, and the diffusion rate for the Ca^{2+}-inophore complex across the cell membranes. The latter, in turn, will be regulated by the extracellular Ca^{2+} concentration relative to the intracellular concentration. In seeking for optimal insulin release the duration of exposure to ionophore, the amount of ionophore, and the external Ca^{2+} concentration will all be independently variable factors. As the ionophore is taken out of the aqueous phase the amount of ionophore available, as well as its concentration in suspension, will affect the response of the β-cell. From the results it appears that high concentrations of ionophore coupled with high extracellular Ca^{2+} may permit the entry of excessive amounts of Ca^{2+} into the cells. Thus the deleterious effect noted in Fig. 1 and Tables II and III of 10 μg per ml of ionophore and 2.5 mM Ca^{2+} may be due to Ca^{2+}-induced inhibition of key enzyme systems such as adenylate cyclase or protein kinase. Optimal Ca^{2+} concentration for the ionophore effect was somewhat lower than that required for glucose effects. With 0.3 mM Ca^{2+} in the medium the effect of the ionophore on insulin release was greater than that of 16.7 mM glucose. The important features of this work, however, are not the relative release rates but the fact that sustained insulin release is possible with Ca^{2+} and the ionophore and that insulin release can be elicited in the absence of glucose in the medium.

The lack of inhibitory effect of epinephrine on the Ca^{2+}-induced release reported here is of considerable interest. Assuming that the ionophore does not destroy the α receptor it may be that the effect of Ca^{2+} to stimulate insulin release occurs at a point distal to the α-adrenergic inhibitory effect of epinephrine, *i.e.* that the site of inhibition is bypassed by the Ca^{2+} stimulus to release, or that the α-adrenergic effect of epinephrine is to block the accumulation of Ca^{2+} induced by glucose and other stimulators of insulin release. Indeed, Malaisse-Lagae and Malaisse (5) have reported epinephrine to inhibit glucose-stimulated net uptake of $^{45}Ca^{2+}$ in isolated islets of Langerhans. In addition, Brisson and Malaisse (29) showed that epinephrine, in the absence of Ca^{2+} in the medium, caused a transient increase in $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$ preloaded islets. Epinephrine also decreased the theophylline-induced efflux of $^{45}Ca^{2+}$. They suggested that the effect of epinephrine is to deplete the cell cytosol of Ca^{2+} by virtue of two effects, one on efflux across the plasma membrane and the other by uptake of Ca^{2+} into compartments such as mitochondria. An interesting observation is that another divalent cation ionophore, X537A, binds norepinephrine and epinephrine (10). This raises the speculation that natural ionophores could be involved in the increased concentration of intracellular Ca^{2+} in response to glucose. Furthermore, binding by epinephrine to such an ionophore in the β-cell could possibly block Ca^{2+} movement and inhibit insulin release. The existence of a Ca^{2+} ionophore in beef heart muscle mitochondria has recently been reported (30). In contrast to this line of reasoning is the report by Selinger *et al.* that A23187 and Ca^{2+} in the parotid gland mimicked the α-adrenergic effect of epinephrine to cause K^+ loss from the cells (15). It is not possible at the moment to put forward a common hypothesis for the action of Ca^{2+} in the parotid to produce an α-adrenergic effect compatible with our observations on the same ionophore which does not reproduce the α-adrenergic inhibitory effect of epinephrine on insulin release and which in fact eliminates the inhibitory effect of epinephrine. At the same time it appears possible that the β-adrenergic mechanism to stimulate adenylate cyclase, increase cyclic AMP levels, and stimulate release could be intact and functioning. This is suggested by the fact that cyclic AMP and epinephrine both enhanced insulin release in the presence of the ionophore and that

the epinephrine effect was blocked by propranolol. The effect of propranolol, however, to also enhance the ionophore response presents problems of interpretation. We are unable to explain how propranolol can both stimulate insulin release in the presence of ionophore and block the effect of epinephrine. As cyclic AMP can increase the Ca^{2+} and ionophore-induced insulin release, even at optimal Ca^{2+} concentrations for the ionophore effect, it becomes questionable whether cyclic AMP acts by increasing intracellular Ca^{2+} levels as has been suggested (31). Certainly it is difficult to imagine that the release of more Ca^{2+} from bound sites within the cell could enhance maximal ionophore-stimulated release, although this has not been proven. Precise measurement of intracellular-free Ca^{2+} will be required for definition of this question. From the results shown in Table VI it is clear that cyclic AMP can stimulate insulin release in the absence of Ca^{2+} from the medium as reported previously (31). Thus either cyclic AMP acts independently of Ca^{2+} or it can mobilize sufficient intracellular Ca^{2+} to increase even further the ionophore-stimulated increase in cytosol Ca^{2+}.

In conclusion, current theories for the essential role of Ca^{2+} in the mechanism of insulin release are supported by the direct demonstration of insulin release by Ca^{2+} reported here. The mechanism by which Ca^{2+} produces its effect remains to be elucidated, and the ionophore A23187 should prove a useful tool in these investigations.

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