

# Dependency of Cyclic AMP-induced Insulin Release on Intra- and Extracellular Calcium in Rat Islets of Langerhans

EBERHARD G. SIEGEL, CLAES B. WOLLHEIM, MASATOSHI KIKUCHI,  
ALBERT E. RENOLD, and GEOFFREY W. G. SHARP, *Institut de Biochimie Clinique,  
University of Geneva, 1211 Geneva 4, Switzerland*

**ABSTRACT** Calcium and cyclic AMP are important in the stimulation of insulin release. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) raises islet cAMP levels and causes insulin release at nonstimulatory glucose concentrations. In isolated rat pancreatic islets maintained for 2 d in tissue culture, the effects of IBMX on insulin release and  $^{45}\text{Ca}^{++}$  fluxes were compared with those of glucose. During perfusion at 1 mM  $\text{Ca}^{++}$ , 16.7 mM glucose elicited a biphasic insulin release, whereas 1 mM IBMX in the presence of 2.8 mM glucose caused a monophasic release. Decreasing extracellular  $\text{Ca}^{++}$  to 0.1 mM during stimulation reduced the glucose effect by 80% but did not alter IBMX-induced release. Both glucose and IBMX stimulated  $^{45}\text{Ca}^{++}$  uptake (5 min).  $^{45}\text{Ca}^{++}$  efflux from islets loaded to isotopic equilibrium (46 h) was increased by both substances. IBMX stimulation of insulin release, of  $^{45}\text{Ca}^{++}$  uptake, and of efflux were not inhibited by blockade of  $\text{Ca}^{++}$  uptake with verapamil, whereas glucose-induced changes are known to be inhibited. Because IBMX-induced insulin release remained unaltered at 0.1 mM calcium, it appears that cAMP-stimulated insulin release is controlled by intracellular calcium. This is supported by perfusion experiments at 0  $\text{Ca}^{++}$  when IBMX stimulated net  $\text{Ca}^{++}$  efflux. In addition, glucose-stimulated insulin release was potentiated by IBMX. These results suggest that cAMP-induced insulin release is mediated by increases in cytosolic  $\text{Ca}^{++}$  and that cAMP causes dislocation of  $\text{Ca}^{++}$  from intracellular stores.

## INTRODUCTION

Cyclic AMP (cAMP)<sup>1</sup> plays an important role in cell function in general and in hormone secretion in particular (1). With regard to insulin release from the pancreatic  $\beta$ -cell, it is well established that agents that increase cellular cAMP levels potentiate glucose-induced insulin release both in vivo (2–4) and in vitro (5–12). Whether increases in the concentration of cellular cAMP mediate glucose-induced insulin release is still unclear. On the one hand, stimulation of insulin release by glucose has been reported to be associated with increased cAMP levels in isolated islets (9–14). On the other hand, increasing cAMP levels with agents other than glucose does not necessarily stimulate insulin release (11–13). Furthermore, some authors failed to show increased cAMP in response to glucose (15, 16) and, in contrast to freshly isolated islets, Rabinovitch et al. (14) found that in islets maintained in tissue culture medium for periods from 4 h to 6 d, glucose stimulates insulin release without changing the concentrations of cAMP. Recently, no increases in cAMP levels were observed in the perfused rat pancreas in response to glucose (17). Current evidence favors the hypothesis that an increase in the concentration of ionized cytosolic  $\text{Ca}^{++}$  directly mediates the effect of glucose to stimulate insulin release (18–22). Thus, in the present study glucose-induced modifications in  $\text{Ca}^{++}$  movements accompanying insulin release were compared with the changes induced by cAMP. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine

Dr. Sharp's present address is Department of Physiology, Tufts University School of Medicine, Boston, Mass.

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<sup>1</sup>Abbreviations used in this paper: cAMP, cyclic AMP; IBMX, 3-isobutyl-1-methylxanthine; KRB, Krebs-Ringer bicarbonate.

(IBMX) which has been shown to stimulate insulin release at nonstimulatory glucose concentrations (8, 11, 14) was used to raise islet cAMP levels in islets maintained in tissue culture (14). In addition, the relative importance of intra- and extracellular calcium in insulin release stimulated by glucose or IBMX was assessed.

## METHODS

### *Isolation of the islets and maintenance in tissue culture.*

Pancreatic islets were isolated by the collagenase digestion technique (23) from male Wistar rats weighing 220–270 g. Batches of 250–500 islets were washed and maintained for 46 h in Petri dishes (6 cm Diam) containing 4 ml Dulbecco's modified Eagle's Medium (24) supplemented with 10% heat-inactivated newborn calf serum, 14 mM NaHCO<sub>3</sub>, 8.3 mM glucose, 400 IU/ml sodium penicillin G, 200 µg/ml streptomycin sulfate. For <sup>45</sup>Ca<sup>++</sup> efflux studies, the medium contained 100 µCi/ml of <sup>45</sup>Ca<sup>++</sup> at a final concentration of 1.05 mM CaCl<sub>2</sub>. The phosphate concentration was 1.0 mM (normal phosphate medium). For one experimental series islets were maintained in the presence of 5.0 mM phosphate for the purpose of increasing intracellular Ca<sup>++</sup> content (22). A 1-M solution of NaH<sub>2</sub>PO<sub>4</sub> was titrated with a 1-M solution of Na<sub>2</sub>HPO<sub>4</sub> to pH 7.40. To 50 ml of phosphate-free culture medium 50 or 250 µl of this phosphate solution was added for normal or high phosphate medium, respectively. Islets used for <sup>45</sup>Ca<sup>++</sup> uptake studies were maintained either in the normal phosphate medium or in medium 199 as described previously (22, 25). Basal and stimulated <sup>45</sup>Ca<sup>++</sup> uptake were not different after culture in the two types of media. The islets were maintained at 37°C, pH 7.4, in an atmosphere saturated with water and gassed with an air-CO<sub>2</sub> mixture.

*Static incubation for measurement of <sup>45</sup>Ca<sup>++</sup> uptake and insulin release.* After the maintenance period the islets were washed twice by centrifugation (150 g) at room temperature with a modified Krebs-Ringer bicarbonate (KRB) Hepes buffer containing 5 mM NaHCO<sub>3</sub>, 1 mM CaCl<sub>2</sub>, 250 kallikrein inhibitory U/ml Trasylol, 0.5% dialyzed bovine serum albumin, 10 mM Hepes, and 2.8 mM glucose, pH 7.4. <sup>45</sup>Ca<sup>++</sup> uptake and insulin release were measured as described (25). In brief, 10 islets were incubated for 5 min in microfuge tubes on top of an oil layer in KRB-Hepes at 37°C in the presence of the test substances. The incubation buffer also contained 0.8 µCi of <sup>45</sup>CaCl<sub>2</sub> and 1.4 µCi [6,6'-n<sup>3</sup>H] sucrose (4 µM) as a marker of the extracellular space (25). The islets were separated from the radioactive medium by centrifugation at 8,000 g through the oil layer into 6 M urea. Insulin release was measured in an aliquot of the supernatant buffer. The bottom of the tubes was cut above the urea layer and placed in 5 ml Ready-Solv HP for liquid scintillation spectrometry. <sup>45</sup>Ca<sup>++</sup> uptake was calculated from the <sup>45</sup>Ca<sup>++</sup> space in excess of the [<sup>3</sup>H]sucrose space. The sucrose space became maximal within 1 min of incubation and remained constant over 30 min (25). At 5 min the extracellular space was 0.67±0.06 nl/islet (n = 25) (mean±SEM) and unchanged by any of the test agents. <sup>45</sup>Ca<sup>++</sup> uptake was linear for 5 min in the presence of both 2.8 and 16.7 mM glucose (25). Immunoreactive insulin was measured by the method of Herbert et al. (26) using rat insulin as standard.

*Perfusion for measurement of <sup>45</sup>Ca<sup>++</sup> efflux and insulin release.* The islets were perfused using 40 islets per chamber as described in detail previously (25, 27, 28). The perfusate consisted of KRB buffer containing 1.0 mM CaCl<sub>2</sub> (except when stated), 0.5% dialyzed bovine serum albumin, and 2.8

mM glucose. The phosphate concentration was 1.0 mM (normal KRB buffer). Islets maintained in a medium containing 5 mM phosphate were perfused with a KRB buffer containing 5 mM phosphate. The phosphate salt used was H<sub>2</sub>PO<sub>4</sub><sup>-</sup> and the concentration of Cl<sup>-</sup> in the high phosphate KRB was reduced to maintain iso-osmolarity. The islets were placed directly in the perfusion chamber without washing. From 0 to 46 min the islets were perfused with the appropriate KRB buffer containing 2.8 mM glucose. After 46 min the perfusate was changed to a KRB buffer containing the respective stimulus and the stimulation period continued for another 44 min. Changes in CaCl<sub>2</sub> concentration are detailed in the text.

Samples were collected every minute between minutes 41 and 55 and thereafter every 5th min, whereas no samples were taken during the initial 40-min equilibration period. An aliquot was assayed for immunoreactive insulin (26). To 0.5 ml of the samples, 5 ml of Instagel was added for measurement of <sup>45</sup>Ca<sup>++</sup> by liquid scintillation spectrometry. After background subtraction, the counts per minute were normalized by setting the mean counts per minute of the six samples collected between minutes 41 and 46 to 100% and expressing the subsequent values as a percentage of this mean for every individual chamber. The mean basal efflux ranged between 70 and 150 cpm.

*Measurement of islet cAMP content.* Batches of 10 islets were incubated for 20 min in KRB-Hepes buffer at 37°C. The islets were then boiled in 0.05 M acetate buffer (pH 6.2). Islet cAMP levels were determined by radioimmunoassay using a commercially available kit (Becton Dickinson & Co., Basel, Switzerland) after succinylation of samples and standards according to the method of Cailla et al. (29).

Statistical analysis was by Student's *t* test for unpaired data.

The materials employed and their sources were as follows: collagenase (Serva GmbH, Heidelberg, West Germany); Dulbecco's modified Eagle's Medium, medium 199, and Hepes solution (Grand Island Biological Co., Grand Island, N. Y.); sodium penicillin G (Pfizer Chemicals, Div. Pfizer, Inc., New York, N. Y.); streptomycin sulfate (Novo Industri A.S., Copenhagen, Denmark); plastic Petri dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.); bovine serum albumin (Behring-Werke AG, Marburg/Lahn, West Germany), Trasylol (kindly provided by Professor Haberland, Bayer AG, Wuppertal, West Germany); verapamil (kindly provided by Professor Oberdorf, Knoll AG, Ludwigshafen, West Germany); 3-isobutyl-1-methyl-xanthine and EGTA (Sigma Chemical Co., St. Louis, Mo.); guinea pig anti-pork insulin serum was a generous gift from Dr. H. H. Schoene, Farbwerke Hoechst AG, Frankfurt, West Germany); rat insulin standard (Novo Research Institute, Copenhagen, Denmark); <sup>45</sup>CaCl<sub>2</sub> and [6,6'-n<sup>3</sup>H]sucrose (The Radiochemical Centre, Amersham, England); Instagel (Packard Instrument International S.A., Zurich, Switzerland); and Ready-Solv HP (Beckman Instruments International SA, Geneva, Switzerland).

## RESULTS

*Effects of lowering the extracellular Ca<sup>++</sup> on glucose- and IBMX-induced insulin release.* At normal extracellular Ca<sup>++</sup> 16.7 mM glucose elicited a biphasic insulin release (Fig. 1, left panel). The rise started 1 min after exposure; a peak at 3 min and a nadir at 6–7 min were followed by an increasing second phase. To limit the availability of extracellular Ca<sup>++</sup>, the perfusate Ca<sup>++</sup> concentration was lowered from

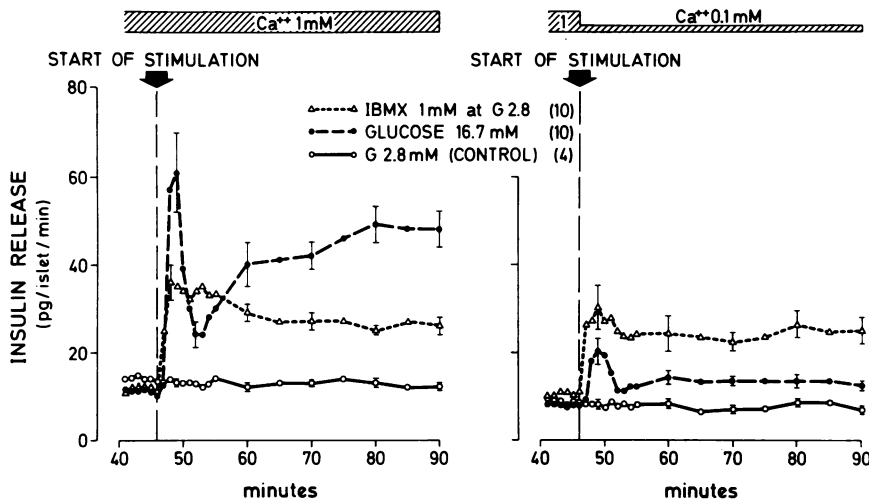


FIGURE 1 Comparison of the effects of glucose and IBMX on insulin release at normal and low extracellular calcium. Islets maintained in tissue culture for 46 h were perfused for 46 min with 2.8 mM glucose and then stimulated for 44 min with either 16.7 mM glucose or 1 mM IBMX in the continued presence of 2.8 mM glucose. In experiments presented on the right panel the calcium concentration was changed from 1 to 0.1 mM at the start of the stimulation. Numbers of observations are indicated in parentheses. G, glucose. All values are mean  $\pm$  SEM.

1 to 0.1 mM as the glucose was increased from 2.8 to 16.7 mM (Fig. 1, right panel). This caused a marked inhibition of both first and second phase insulin release. The total release above base line over the whole stimulation period was only 20% of the situation at normal extracellular  $\text{Ca}^{++}$  (see also Table IA). IBMX, in the presence of 2.8 mM glucose and 1 mM  $\text{Ca}^{++}$ , elicited a monophasic insulin release. The release was of rapid onset reaching a plateau level at 1 min. In contrast to the stimulation with 16.7 mM glucose, IBMX-induced insulin release was not affected by lowering the extracellular  $\text{Ca}^{++}$  (comparing the right with the left panel of Fig. 1). At no point in time was there a significant difference between the release rates at 1 and 0.1 mM  $\text{Ca}^{++}$ . Furthermore, there was no difference in the integrated insulin release above base line (Table IA). In control experiments, the shift from 1 to 0.1 mM  $\text{Ca}^{++}$  in the continued presence of 2.8 mM glucose alone had no effect on insulin release.

**Effects of glucose and IBMX on islet cAMP content.** The release data of Fig. 1 were compared with the effects of IBMX and glucose on islet cAMP levels after 20 min of static incubation (Table II). At 1 mM  $\text{Ca}^{++}$ , the islet cAMP content in the presence of 16.7 mM glucose was not significantly different from 2.8 mM glucose. By contrast, an almost fourfold increase was seen in response to 1 mM IBMX. Reduction of the  $\text{Ca}^{++}$  to 0.1 mM  $\text{Ca}^{++}$  did not significantly alter islet cAMP levels in the presence of 2.8 or 16.7 mM glucose. At 0.1 mM  $\text{Ca}^{++}$ , IBMX enhanced islet cAMP to a similar extent as that seen at 1 mM  $\text{Ca}^{++}$ .

**Insulin release,  $^{45}\text{Ca}^{++}$  efflux, and  $^{45}\text{Ca}^{++}$  uptake in the presence of IBMX at low glucose and the effects**

**of verapamil.** The monophasic insulin release induced by 1 mM IBMX at 1 mM  $\text{Ca}^{++}$  was paralleled by an increase in  $^{45}\text{Ca}^{++}$  efflux (Fig. 2).  $^{45}\text{Ca}^{++}$  efflux remained elevated during the entire stimulation period. 5  $\mu\text{M}$  verapamil, a dose that has been shown to completely block glucose-stimulated  $^{45}\text{Ca}^{++}$  uptake over 5 min (19) was without any significant effect on IBMX-induced insulin release (Table IB) and  $^{45}\text{Ca}^{++}$  efflux. Basal  $^{45}\text{Ca}^{++}$  efflux (2.8 mM glucose) decreased gradually, as anticipated, during the entire perfusion period.

When  $^{45}\text{Ca}^{++}$  uptake and insulin release were measured over 5 min, 1 mM IBMX stimulated both parameters significantly (Table IIIA). The addition of 5  $\mu\text{M}$  verapamil failed to inhibit significantly the stimulatory effects of IBMX on  $^{45}\text{Ca}^{++}$  uptake ( $P > 0.05$ ) and insulin release ( $P > 0.05$ ). To find out, therefore, whether these stimulatory effects of IBMX could be dissociated, a lower concentration was tested. As shown in Table IIIB, 0.1 mM IBMX did not affect  $^{45}\text{Ca}^{++}$  uptake, although it stimulated insulin release threefold. Verapamil did not inhibit basal  $^{45}\text{Ca}^{++}$  uptake and insulin release (Table IIIA).

**Effects of IBMX at high glucose.** The biphasic glucose-induced insulin release was clearly potentiated by the addition of 1 mM IBMX (Fig. 3). The total release was increased by 250% (Table IC). In the presence of 16.7 mM glucose, both phases of insulin release were associated with an increase in  $^{45}\text{Ca}^{++}$  efflux that was markedly enhanced by IBMX.  $^{45}\text{Ca}^{++}$  uptake and insulin release over 5 min were stimulated by 16.7 mM glucose (Table IIIC). IBMX potentiated the glucose-induced insulin release to an

**TABLE I**  
*Integrated Insulin Release above Base Line during 44 min of Stimulation*

		Insulin release	
		pg/44 min	
<b>A. Comparison of glucose and IBMX at normal and low extracellular calcium</b>			
G	16.7 mM at 1 mM Ca <sup>++</sup>	1,319±153 (10)	<0.001
G	16.7 mM at 0.1 mM Ca <sup>++</sup>	255±42 (10)	
G	2.8 mM + IBMX 1 mM at 1 mM Ca <sup>++</sup>	695±82 (10)	>0.20
G	2.8 mM + IBMX 1 mM at 0.1 mM Ca <sup>++</sup>	539±86 (10)	
<b>B. Effect of verapamil on IBMX-induced insulin release</b>			
G	2.8 mM + IBMX 1 mM	860±68 (5)	>0.40
G	2.8 mM + IBMX 1 mM + verapamil 5 μM	781±63 (5)	
<b>C. Effect of IBMX at high glucose</b>			
G	16.7 mM	1,609±263 (4)	<0.01
G	16.7 mM + IBMX 1 mM	5,870±1,000 (4)	
<b>D. Effect of IBMX at low glucose in normal and calcium-loaded islets at normal and low extracellular calcium</b>			
G 2.8 mM + IBMX 1 mM in			
	Normal islets at 1 mM Ca <sup>++</sup>	641±94 (6)	>0.70
	Normal islets at 0.1 mM Ca <sup>++</sup>	579±129 (5)	>0.30
	Calcium-loaded islets at 0.1 mM Ca <sup>++</sup>	429±72 (6)	>0.30
	Calcium-loaded islets at 1 mM Ca <sup>++</sup>	376±84 (5)*	>0.30

Insulin release values over 44 min were calculated by integrating the insulin release from 47–90 min of perfusion (see Figs. 1–3) after subtraction of the basal values in the presence of 2.8 mM glucose (mean of the insulin release between minutes 41 to 46) for every individual experiment. Values are presented as mean±SEM, number of experiments in parentheses; G, glucose.

\*  $P > 0.05$  vs. normal islets.

extent similar to that seen in the perfusion experiments, but had no significant further effect on glucose-stimulated <sup>45</sup>Ca<sup>++</sup> uptake.

*Effects of IBMX in calcium-deficient media.* In these experiments the islets were perfused from the start of the experiments with a buffer prepared without the addition of calcium. When 1 mM IBMX was added after 46 min, no increase of insulin release occurred (Fig. 4), but there was a small stimulation of <sup>45</sup>Ca<sup>++</sup> efflux.

Between 48 and 65 min all the values for <sup>45</sup>Ca<sup>++</sup> efflux were significantly higher in the presence of IBMX compared with control values obtained in islets from the same batches ( $P < 0.02$ ). A similar stimulation was also seen when a calcium chelator was added (1 mM EGTA); data not shown.

*Effects of IBMX on insulin release by islets with increased calcium stores.* Islets maintained in tissue culture for 46 h in the presence of 5 mM phosphate

**TABLE II**  
*Islet cAMP Content in the Presence of IBMX or Glucose at Different Ca<sup>++</sup> Concentrations*

	cAMP content (fmol/islet) at 1 mM Ca <sup>++</sup>	P vs. G 2.8	cAMP content (fmol/islet) at 0.1 mM Ca <sup>++</sup>	P vs. G 2.8
G 2.8 mM	11.2±1.5 (9)		10.3±1.7	
G 16.7 mM	10.3±1.2 (10)	>0.60	12.2±1.3	>0.30
G 2.8 mM + IBMX 1 mM	39.7±3.2 (10)	<0.001	35.0±3.5*	<0.001

The islet content of cAMP was determined after 20 min of incubation in KRB-Hepes buffer following 46 h maintenance in tissue culture (see Methods). The different Ca<sup>++</sup> concentrations were imposed from the beginning of the incubation. Values are presented as mean±SEM, numbers of experiments in parentheses; G, glucose.

\*  $P > 0.30$  vs. G 2.8 mM + IBMX 1 mM at 1 mM Ca<sup>++</sup>.

## DISCUSSION

The *in vitro* maintenance of isolated pancreatic islets used in this study has two main advantages compared with freshly isolated islets. First, islets used for  $^{45}\text{Ca}^{++}$  efflux studies have been loaded to isotopic equilibrium (28). Second, the pattern of glucose-induced insulin release is clearly biphasic with a spikelike first phase followed by a distinct nadir and a progressively increasing second phase (Figs. 1 and 3), a pattern similar to that observed in the perfused pancreas (17, 30) or the portal vein in humans (31). In these islets, glucose can cause insulin release without an increase in cAMP levels (14). This was confirmed in the present study using a more direct method (Table II). This is in contrast to freshly isolated islets (14) where several authors have reported glucose to raise cAMP levels (32). In the islets used in this study, IBMX raised islet cAMP fourfold (Table II) and stimulated insulin release at non-stimulatory glucose concentrations (Figs. 1 and 2) in a manner similar to that observed in the perfused pancreas (33).

Stimulation of  $^{45}\text{Ca}^{++}$  efflux from preloaded cells during cellular activation is generally thought to reflect increases in cytosolic  $\text{Ca}^{++}$  (1). This appears also to apply to isolated islets, particularly because stimulated  $^{45}\text{Ca}^{++}$  efflux can be dissociated from the event of insulin release *per se* (18, 20, 25, 28). It is therefore improbable that a major part of the  $^{45}\text{Ca}^{++}$  is released together with the content of the secretory granules. Increases in cytosolic  $\text{Ca}^{++}$  are now generally believed to trigger glucose-induced insulin release (18–22). The following findings suggest that increases in cytosolic  $\text{Ca}^{++}$  are also involved in cAMP-induced insulin release. First, the IBMX-induced insulin release at low glucose was accompanied by a parallel rise in  $^{45}\text{Ca}^{++}$  efflux (Fig. 2). Second, glucose-stimulated  $^{45}\text{Ca}^{++}$  efflux was further increased when insulin release was potentiated by IBMX (Fig. 3). Third, in islets of which the calcium content had been decreased by preperfusion with calcium-deficient media (21, 28, 34) IBMX failed to cause insulin release (Fig. 4). Because ~80% of the islet cells are  $\beta$ -cells (35), changes in  $^{45}\text{Ca}^{++}$  efflux can be assumed to reflect changes in these cells.

The increase in cytosolic  $\text{Ca}^{++}$  during glucose stimulation may be the result of an increased  $\text{Ca}^{++}$  uptake (18, 19, 25), an inhibition of  $\text{Ca}^{++}$  efflux (20, 21, 28, 36), and/or utilization of  $\text{Ca}^{++}$  from cellular stores (18, 19, 22, 37). Calcium has been localized mainly in mitochondria, secretory granules, and the plasma membrane (38–40). For IBMX-induced insulin release, the effect of cytosolic  $\text{Ca}^{++}$  does not appear to depend on increased  $\text{Ca}^{++}$  uptake. Although 1 mM IBMX at low glucose increased  $\text{Ca}^{++}$  uptake (Table III), it is unlikely that this contributes to a major extent to the increased cytosolic  $\text{Ca}^{++}$ , because the insulin release was not

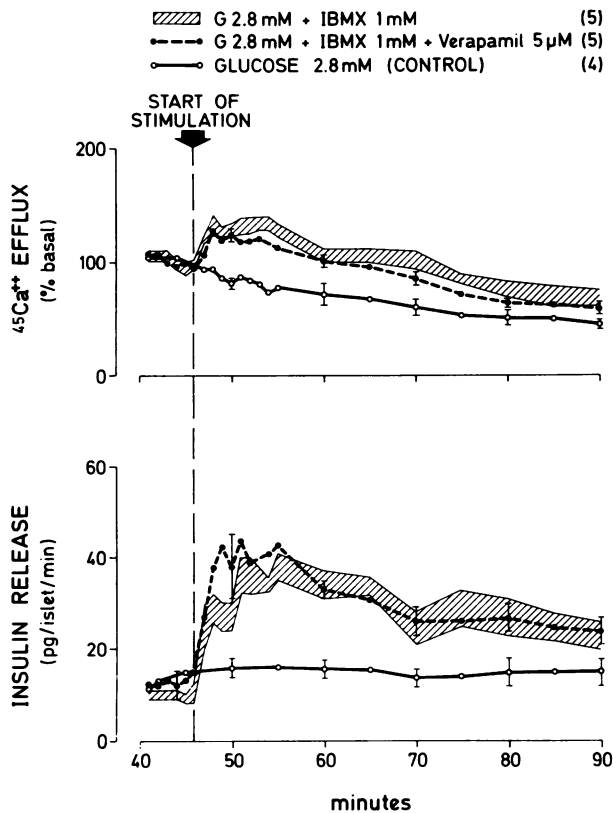


FIGURE 2 Effects of verapamil on IBMX-induced  $^{45}\text{Ca}^{++}$  efflux and insulin release. Islets preloaded with  $^{45}\text{CaCl}_2$  for 46 h were perfused at 1 mM calcium and 2.8 mM glucose throughout. At 46 min, the islets were stimulated with 1 mM IBMX. At the same time, 5  $\mu\text{M}$  verapamil was added to one group and the stimulation continued for another 44 min. For every individual experiment, mean  $^{45}\text{Ca}^{++}$  efflux during minutes 41–46 was set to 100% and the subsequent changes expressed as percentage of this mean. G, glucose. All values are mean  $\pm$  SEM.

have been shown to have a 50-fold increase in intracellular calcium content (22). To investigate whether this increased intracellular calcium could be readily utilized by IBMX to cause enhanced insulin release, these islets were stimulated with 1 mM IBMX at normal and low (0.1 mM) extracellular  $\text{Ca}^{++}$ . In these experiments, islets from the same initial pool were maintained at either 1 (controls) or 5 mM (calcium-loaded) phosphate. Because the release profile of the calcium-loaded islets was similar to that of the controls (cf. Fig. 1), the data are shown as the integrated insulin release above base line over 44 min of stimulation (Table ID). There was no significant difference in IBMX-stimulated release between calcium-loaded and control islets, whether the experiments were performed at normal or low extracellular  $\text{Ca}^{++}$ . IBMX thus failed to cause enhanced insulin release from islets with increased calcium stores.

TABLE III  
*IBMX-induced Insulin Release and Simultaneous Measurement of <sup>45</sup>Ca<sup>++</sup> Uptake*

	<sup>45</sup> Ca <sup>++</sup> uptake	P	Insulin release	P
	pmol/islet/5 min		pg/islet/5 min	
A. Effects of 1 mM IBMX and verapamil at low glucose				
G 2.8 mM	0.907±0.060 (15)		42.3±5.6 (15)	
G 2.8 mM + IBMX 1 mM	1.544±0.087 (25)	<0.001	252.3±23.4 (25)	<0.001
G 2.8 mM + IBMX 1 mM + verapamil 5 μM	1.282±0.133 (21)	>0.05	188.2±26.1 (21)	>0.05
G 2.8 mM + verapamil 5 μM	0.954±0.127 (10)*		62.1±9.1 (10)‡	
B. Effects of 0.1 mM IBMX at low glucose				
G 2.8 mM	0.846±0.101 (9)		34.8±7.7 (9)	
G 2.8 mM + IBMX 0.1 mM	0.743±0.078 (8)	>0.40	104.8±18.1 (8)	<0.001
C. Effects of 1 mM IBMX at high glucose				
G 2.8 mM	1.233±0.201 (9)		34.3±16.2 (9)	
G 16.7 mM	2.724±0.258 (15)	<0.001	148.5±24.6 (15)	<0.005
G 16.7 mM + IBMX 1 mM	3.395±0.330 (11)	>0.10	519.6±49.2 (11)	<0.001

Insulin release and <sup>45</sup>Ca<sup>++</sup> uptake were measured over 5 min, on the same batches of islets. Values are presented as mean±SEM, numbers of experiments in parentheses; G, glucose.

\* P > 0.70 vs. G 2.8 mM alone.

‡ P > 0.05 vs. G 2.8 mM alone.

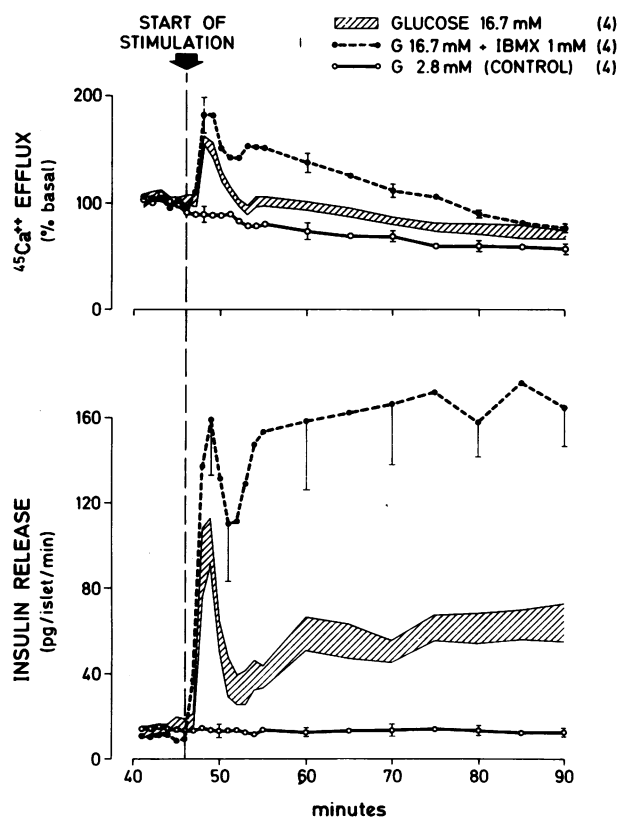


FIGURE 3 Effects of IBMX on glucose-stimulated <sup>45</sup>Ca<sup>++</sup> efflux and insulin release at 1 mM calcium. Islets preloaded with <sup>45</sup>CaCl<sub>2</sub> for 46 h were used. The perfusate glucose concentration was changed from 2.8 to 16.7 mM at 46 min and the glucose stimulation continued for another 44 min in the presence or absence of 1 mM IBMX. G, glucose. All values are mean±SEM.

affected when the extracellular Ca<sup>++</sup> was lowered to 0.1 mM (Fig. 1). More direct evidence, however, is that 0.1 mM IBMX stimulated insulin release without an increase in Ca<sup>++</sup> uptake, and at 16.7 mM glucose, a marked potentiation of insulin release occurred without a further increase of glucose-stimulated Ca<sup>++</sup> uptake (Table III). In an earlier report, no increase in calcium net uptake was observed either when glucose-induced insulin release was potentiated with theophylline (6).

Ca<sup>++</sup> uptake via the voltage-dependent Ca<sup>++</sup> channel which is opened as a consequence of depolarization of the cell membrane can be inhibited by verapamil in various tissues (41) including islets (19, 42, 43). Depolarization of the β-cell membrane by glucose (18, 44) is thought to be causally related to the stimulation of Ca<sup>++</sup> uptake. Inhibition of this Ca<sup>++</sup> uptake by verapamil has been shown not to affect the first phase of glucose-induced insulin release, whereas the second phase was markedly inhibited (19). In contrast, verapamil did not alter IBMX-stimulated insulin release or Ca<sup>++</sup> uptake (Fig. 2 and Table III). This finding is not so surprising in view of the fact that 10 mM theophylline at non-stimulatory glucose concentrations (2.8 mM) does not induce electrical activity in β-cells, but only causes a slight depolarization of 6–7 mV.<sup>2</sup> The mechanism by which Ca<sup>++</sup> enters the islet cells in response to IBMX is unknown. One possible explanation for the enhanced <sup>45</sup>Ca<sup>++</sup> uptake may be that an increase in cytosolic Ca<sup>++</sup> in turn triggers the exchange between radioactive and nonradioactive Ca<sup>++</sup> (21, 28).

<sup>2</sup> Meissner, H. P. Personal communication.

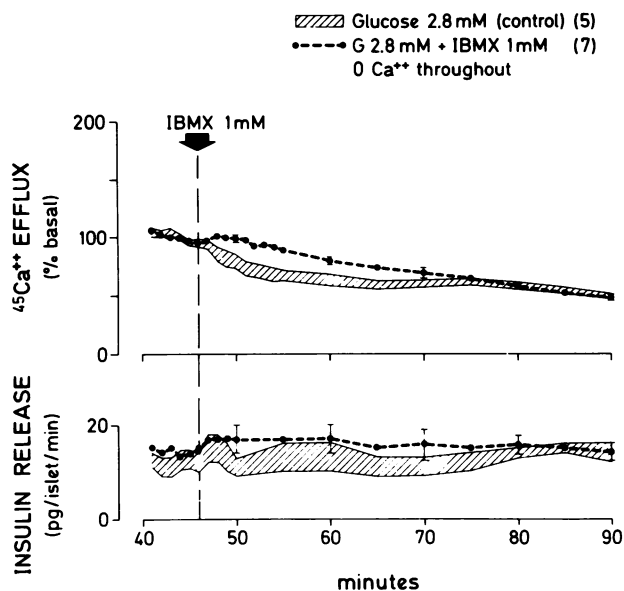


FIGURE 4 Effects of IBMX on  $^{45}\text{Ca}^{++}$  efflux and insulin release in the absence of extracellular calcium. Islets preloaded with  $^{45}\text{CaCl}_2$  for 46 h were perfused in a buffer prepared without the addition of calcium throughout the perfusion experiments. From 47 to 90 min 1 mM IBMX was added to the perfusate. G, glucose. All values are mean  $\pm$  SEM.

Because the IBMX action does not appear to be dependent on increased  $\text{Ca}^{++}$  uptake, the possibility that IBMX inhibits  $\text{Ca}^{++}$  efflux across the plasma membrane to raise cytosolic  $\text{Ca}^{++}$  was considered. Under conditions when glucose clearly inhibits  $^{45}\text{Ca}^{++}$  efflux, i.e., at zero  $\text{Ca}^{++}$  (20, 21, 28, 36), IBMX was found to stimulate it (Fig. 4). Indeed, the stimulation of  $^{45}\text{Ca}^{++}$  efflux from these islets that had attained isotopic equilibrium (28) and were then perfused in the absence of  $\text{Ca}^{++}$  to prevent isotopic dilution unequivocally permits the conclusion that IBMX causes a net increase of  $\text{Ca}^{++}$  efflux. It is concluded, therefore, that IBMX uses cellular  $\text{Ca}^{++}$  to stimulate insulin release. In an earlier study on 1-h preloaded islets a similar conclusion was drawn when theophylline was used (45). In an attempt to assess the effect of cAMP on intracellular calcium stores, IBMX-induced insulin release was measured in islets with increased calcium stores after culture in 5 mM phosphate (22). Insulin release was not increased compared with control islets (Table ID) thus indicating that the increased cellular calcium cannot be readily utilized by cAMP.

It is still unclear whether the presumed increase in cytosolic  $\text{Ca}^{++}$  is a result of mobilization of  $\text{Ca}^{++}$  from cellular stores or of inhibition of uptake into these stores. Howell et al. (39) reported cAMP to inhibit  $\text{Ca}^{++}$  uptake into rat islet homogenates, whereas IBMX and theophylline had no effect. In a mitochondria-rich fraction cAMP also inhibited  $\text{Ca}^{++}$  uptake. Using a

mitochondrial fraction from rat islets, Sugden and Ashcroft (37) showed an inhibition with IBMX and no effect of cAMP. Sehlin (46) using a microsomal fraction from ob/ob mouse islets found both cAMP and theophylline to inhibit  $\text{Ca}^{++}$  uptake. Thus, it is likely that cAMP or phosphodiesterase inhibitors interfere with the uptake of  $\text{Ca}^{++}$  by cellular organelles.

Two lines of evidence support the assumption that the changes induced by IBMX in this study are mediated by increased cellular cAMP levels. First, IBMX enhanced the islet content of cAMP at 1 mM  $\text{Ca}^{++}$  and its effects both on insulin release and cAMP remained unaltered at 0.1 mM  $\text{Ca}^{++}$  (Table II and Fig. 1). Second, when glucagon, a specific stimulator of adenylate cyclase, was used to potentiate insulin release, both glucose-stimulated insulin release and  $^{45}\text{Ca}^{++}$  efflux were enhanced to a similar extent as observed for IBMX in Fig. 3 (43). In addition, like IBMX, glucagon did not alter glucose-stimulated  $\text{Ca}^{++}$  uptake (43).

After perfusion for 45 min in the absence of  $\text{Ca}^{++}$  IBMX failed to stimulate insulin release. Under similar conditions both theophylline (13) and IBMX (47) have been shown to enhance islet cAMP levels. A possible explanation for failure of IBMX to stimulate insulin release may be the depletion of a labile  $\text{Ca}^{++}$  pool (34) necessary for insulin release. Although in the absence of extracellular  $\text{Ca}^{++}$  glucose does not elicit insulin release (6, 21, 28), the combination of glucose and agents that raise cAMP levels stimulates insulin release (6, 13, 47, 48). This may be the result of a combined effect of glucose or its metabolites (37) and cAMP (37, 39, 46) on cellular calcium stores and inhibition of  $\text{Ca}^{++}$  efflux across the plasma membrane by glucose (20, 21, 28, 36). Therefore, cytosolic  $\text{Ca}^{++}$  may rise high enough to cause insulin release. Phosphorylation of islet proteins with IBMX has been demonstrated (49), and this could be the manner by which cAMP utilizes cellular calcium. In addition to the above considerations, cAMP could also sensitize the release machinery to the effects of calcium ions (8, 47).

The results presented here may also have clinical implications. Patients with decreased serum calcium resulting from hypoparathyroidism show an impaired insulin response to an oral glucose load (3, 50). The infusion of theophylline was shown to overcome this impairment (3). Similarly, Cerasi and Luft (4) have reported that the defective early insulin response to a glucose infusion in prediabetic patients could be restored by aminophylline infusion. In rat islets, first phase insulin release has been shown to depend mainly on cellular  $\text{Ca}^{++}$  stores (19, 22) and a defective cellular  $\text{Ca}^{++}$  handling would thus mainly affect the first phase. The beneficial effect of aminophylline in the prediabetic subjects might therefore be a result of an action of cAMP on cellular  $\text{Ca}^{++}$  stores as shown for IBMX in this study.

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