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### Research Article

Breakdown of phosphatidylinositol (PI) has been shown to be increased during Ca<sup>2+</sup>-mediated stimulation of cellular responses in many systems and has been proposed to be involved in stimulus-secretion coupling. The effects on PI breakdown of insulin secretagogues that alter cellular Ca<sup>2+</sup> or cyclic (c)AMP levels were investigated in perfused rat islets of Langerhans. Isolated islets were labeled with myo-[2-3H(N)]inositol and the efflux of 3H-labeled metabolites was monitored. Glucose (16.7 mM) greatly increased 3H release in a manner that paralleled the second phase of the insulin secretory response; by 60 min, the amount of [3H]PI in the islet decreased by 50%. Removal of Ca<sup>2+</sup> from the perfusate or blockade of Ca<sup>2+</sup> entry through the voltage-dependent channels by D600 (20 microM) abolished the glucose-induced increase in 3H efflux. Depolarization with 47 mM K<sup>+</sup>, which increases Ca<sup>2+</sup> entry, stimulated protracted 3H and insulin release. Glucose-stimulated output of 3H was not prevented by epinephrine (1 microM) even though the insulin response was abolished. In contrast, 3H output was not affected by isobutylmethylxanthine (1 mM), known to raise cellular levels of cAMP, although insulin release was stimulated. These findings indicate that PI breakdown is not related to the exocytotic process since stimulation of insulin release and PI breakdown could be uncoupled, and that it is not associated with cAMP-mediated regulation of insulin release. PI breakdown [...]

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# Role of $\text{Ca}^{2+}$ in Secretagogue-stimulated Breakdown of Phosphatidylinositol in Rat Pancreatic Islets

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**ABSTRACT** Breakdown of phosphatidylinositol (PI) has been shown to be increased during  $\text{Ca}^{2+}$ -mediated stimulation of cellular responses in many systems and has been proposed to be involved in stimulus-secretion coupling. The effects on PI breakdown of insulin secretagogues that alter cellular  $\text{Ca}^{2+}$  or cyclic (c)AMP levels were investigated in perfused rat islets of Langerhans. Isolated islets were labeled with myo-[2- $^3\text{H}$ (N)]inositol and the efflux of  $^3\text{H}$ -labeled metabolites was monitored. Glucose (16.7 mM) greatly increased  $^3\text{H}$  release in a manner that paralleled the second phase of the insulin secretory response; by 60 min, the amount of [ $^3\text{H}$ ]PI in the islet decreased by 50%. Removal of  $\text{Ca}^{2+}$  from the perfusate or blockade of  $\text{Ca}^{2+}$  entry through the voltage-dependent channels by D600 (20  $\mu\text{M}$ ) abolished the glucose-induced increase in  $^3\text{H}$  efflux. Depolarization with 47 mM  $\text{K}^+$ , which increases  $\text{Ca}^{2+}$  entry, stimulated protracted  $^3\text{H}$  and insulin release. Glucose-stimulated output of  $^3\text{H}$  was not prevented by epinephrine (1  $\mu\text{M}$ ) even though the insulin response was abolished. In contrast,  $^3\text{H}$  output was not affected by isobutylmethylxanthine (1 mM), known to raise cellular levels of cAMP, although insulin release was stimulated. These findings indicate that PI breakdown is not related to the exocytotic process since stimulation of insulin release and PI breakdown could be uncoupled, and that it is not associated with cAMP-mediated regulation of insulin release. PI breakdown in islets differs from the immediate, transient phenomenon reported in other systems in both its timing and requirement for  $\text{Ca}^{2+}$ . It appears to result from the entry of  $\text{Ca}^{2+}$  and not to be the mechanism by which glucose initiates  $\text{Ca}^{2+}$  influx.

## INTRODUCTION

Calcium plays a crucial role in stimulus-secretion coupling in the pancreatic  $\beta$ -cell and it is generally agreed that glucose promotes insulin release by raising the level of cytosolic free  $\text{Ca}^{2+}$  (1). There is evidence to indicate that glucose raises intracellular  $\text{Ca}^{2+}$  levels by stimulating  $\text{Ca}^{2+}$  influx (2), inhibiting cellular  $\text{Ca}^{2+}$  efflux (3), and mobilizing  $\text{Ca}^{2+}$  from intracellular storage sites (4). Although the role of  $\text{Ca}^{2+}$  is not as firmly established for secretagogues whose effects are mediated by cyclic (c) $^1\text{AMP}$ , findings suggest that cAMP may cause a redistribution of intracellular  $\text{Ca}^{2+}$  (5).

Although stimulation of insulin release by glucose requires cellular uptake and metabolism of glucose (6), it also has characteristics that resemble those of systems in which interaction of a ligand with a plasma membrane receptor is sufficient to initiate a response (1). It has been reported that glucose increases phosphatidylinositol (PI) turnover in pancreatic islets (7), possibly in a manner similar to the enhancement of PI turnover seen when a ligand-receptor interaction triggers a  $\text{Ca}^{2+}$ -mediated secretory response (8). The relationship between alterations in  $\text{Ca}^{2+}$  fluxes and phosphatidylinositol turnover is controversial, since it is unclear whether metabolites generated upon breakdown of PI are instrumental in bringing about changes in cellular  $\text{Ca}^{2+}$  fluxes or whether stimulation of PI metabolism is itself induced by  $\text{Ca}^{2+}$ .

The study reported herein was designed to investigate the role of  $\text{Ca}^{2+}$  in stimulated PI breakdown in islets, the relationship between PI breakdown and the insulin secretory response, and the effects of agents that modulate insulin release through changes in cellular

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<sup>1</sup> *Abbreviations used in this paper:* cAMP, cyclic AMP; D600,  $\alpha$ -isopropyl- $\alpha$ -p[(*N*-methyl-*N*-homoveratryl)- $\delta$ -aminopropyl]3,4,5-trimethoxyphenylacetone nitrile hydrochloride; IBMX, 3-isobutyl-1-methylxanthine; PI, phosphatidylinositol.

levels of cAMP. Breakdown of PI was assessed by monitoring the efflux of water-soluble metabolites of PI from perfused islets labeled with [<sup>3</sup>H]inositol. The results indicate that, in the pancreatic  $\beta$ -cell, stimulation of PI breakdown depends on cellular uptake of Ca<sup>2+</sup> and can be uncoupled from insulin secretion. Cyclic AMP-mediated insulin release does not appear to be associated with enhanced PI breakdown.

## METHODS

**Labeling of islets with [<sup>3</sup>H]inositol** Islets of Langerhans were obtained from fed male Wistar rats (~200 g body wt) by digestion of pancreatic fragments with collagenase (type IV, Worthington Biochemical Corp., Freehold, NJ) as described by Lacy and Kostianovsky (9). Islets (100/tube) were placed immediately in 100  $\mu$ l of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 2.5 mM Ca<sup>2+</sup>, 4.7 mM K<sup>+</sup>, 3 mg/ml bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, MO), 2.8 mM glucose, and 0.06 mM myo-inositol (Sigma Chemical Co.). 25  $\mu$ Ci of [<sup>3</sup>H]inositol (myo-[2-<sup>3</sup>H(N)]inositol, 15.8 Ci/mmol, New England Nuclear, Boston, MA) in distilled water were dried under a stream of nitrogen, redissolved in 100  $\mu$ l incubation buffer and added to the tube containing the islets. Islets were incubated at 37°C under 95% O<sub>2</sub> 5% CO<sub>2</sub> for 2 h.

**Perfusion of islets.** At the completion of the radiolabeling period, each batch of 100 islets was transferred by pipette to a Swinnex 13 chamber (Millipore Continental Water Systems, Bedford, MA) and placed atop two layers of Nitex HD3-80 nylon mesh (TETKO Inc., Elmsford, NY). The chamber was filled with buffer, connected to the perfusion line and immersed in a 37°C water bath. The islets were perfused using a peristaltic pump (Manostat Corp., New York) at a rate of 1 ml/min with Krebs-Ringer bicarbonate buffer of identical composition to that of the labeling period unless otherwise stated. When the K<sup>+</sup> or Ca<sup>2+</sup> compositions of the buffer were altered, the osmolarity was maintained by adjusting the concentration of NaCl. The pH of the buffer was adjusted after additions were made. Epinephrine bitartrate, 3-isobutyl-1-methylxanthine (IBMX), and EGTA were purchased from Sigma Chemical Co.;  $\alpha$ -isopropyl- $\alpha$ -(*N*-methyl-*N*-homoveratryl)- $\delta$ -amino-propyl] 3,4,5-trimethoxyphenylacetone nitrile hydrochloride (D600) was obtained from Knoll A.G. (Ludwigshafen, West Germany).

Islets were perfused for 30 min before any experimental alterations were made in the buffer and then for as long as 90 min thereafter with 1-min fractions of the perfusate collected automatically. Solutions flowing through the perfusion chamber were changed by a turn of the stopcock. All perfusions were performed in pairs using islets from the same rat or islets picked from the pooled products of two digestions. One of the paired chambers was always exposed to an agent known to have a stimulatory effect on insulin and/or <sup>3</sup>H release so that the responsiveness of the islets could be determined. The insulin concentration of effluent from the perfusion was measured by radioimmunoassay using <sup>125</sup>I-labeled bovine insulin, bovine insulin standard (Eli Lilly & Co., Indianapolis, IN), guinea pig anti-bovine insulin serum (Dr. P. Wright, University of Indiana, Indianapolis, IN) and dextran-coated charcoal to separate antibody-bound from free insulin (10). Results are expressed as nanograms of rat insulin/100 islets per min based on the cross-reactivity determined in this laboratory between bovine insulin and rat insulin standards (kindly supplied by Dr. M. Root, Eli Lilly & Co.). Radio-

activity in the perfusion effluent was measured by liquid scintillation counting of 500  $\mu$ l aliquots in 10 ml Aquasol (New England Nuclear).

**Lipid extraction.** At the completion of each experiment islets, still on nylon mesh disks, were extracted with 3 ml chloroform/methanol 1:2 (vol/vol), sonicated, and stored below 4°C. Following centrifugation to remove debris, which contained negligible radioactivity, a sample was removed to measure <sup>3</sup>H (total islet <sup>3</sup>H). The lipid phase was washed twice with 1 ml chloroform and 1 ml 1 M MgCl<sub>2</sub> with removal of the upper aqueous phase. An aliquot of the lipid phase was taken and all samples were dried at 50°C to eliminate quenching and were counted in 10 ml Aquasol.

Two kinds of control were used to monitor recovery. [<sup>14</sup>C]Phosphatidylcholine (Amersham Corp., Arlington Heights, IL) was added to each tube of the original extraction mixture to measure recovery of phospholipid in each particular tube; the extraction of tritiated phospholipid was corrected on the basis of recovery of <sup>14</sup>C for that sample. The calculated recovery averaged 107 $\pm$ 5% (mean $\pm$ SEM, *n* = 59). In separate experiments, the same extraction mixture, with or without unlabeled islets, was prepared and authentic [<sup>3</sup>H]PI, synthesized by hamster insulinoma cells incubated with [<sup>3</sup>H]inositol and identified by thin-layer chromatography (see below), was added. Recovery of [<sup>3</sup>H]PI was 74 $\pm$ 7% (mean $\pm$ SEM, *n* = 13). The data presented in Table I have been corrected for this degree of recovery of PI.

**Identification of metabolites.** To verify that [<sup>3</sup>H]inositol was incorporated into PI, lipid extracts were dried under nitrogen, dissolved in a small volume of chloroform/methanol/water = 60:29:4 (vol/vol), spotted on silica gel-coated plastic plates (Polygram silG, Brinkmann Instruments, Inc., Westbury, NY) and developed in two dimensions (chloroform/methanol/acetic acid/water = 52:20:7:3 and chloroform/methanol/40% aqueous methylamine/water = 13:7:1:1, both containing 0.05% butylated hydroxytoluene (BHT, wt/vol) (11). Identification of labeled phospholipids was made by liquid scintillation counting of spots migrating with authentic PI (Sigma Chemical Co.) as visualized by staining for phosphate (12).

Perfusate containing <sup>3</sup>H-labeled material was lyophilized and then desalted and deproteinized on a Biogel P-2 column (Bio-Rad Laboratories, Richmond, CA), and each fraction containing radioactivity was spotted in the center of the paper for separation by high-voltage electrophoresis (40 V/cm for 60 min) (13) under coolant. The identities of tritiated substances in each column fraction with detectable radioactivity were determined by comparison of their migrations with those of standard PI, inositol, or inositol 2-monophosphate (Sigma Chemical Co.). Because inositol 2-monophosphate and inositol 1-monophosphate (not commercially available) are not readily distinguishable in this solvent system (14) compounds migrating similarly to the inositol 2-monophosphate have been identified simply as inositol monophosphate. Inositol was detected with a silver stain (15) and inositol monophosphate with a ferrithiocyanate staining technique (16). Presence of <sup>3</sup>H-labeled compounds was ascertained by counting radioactivity in each square-inch section of the lane. Sections of paper were eluted with 1 ml deionized water, the paper was placed at the bottom of the vial, and the radioactivity of the total sample was counted in 10 ml Aquasol. Comparison of the sum of the counts per minute in the sections of a lane with counts per minute in a corresponding unseparated spot showed that no radioactivity was lost from the paper during electrophoresis.

**Statistics.** Statistical analyses were done by Student's *t* tests for paired and unpaired data, as noted (17).

## RESULTS

Islets incubated with myo-[2-<sup>3</sup>H(N)]-inositol incorporated  $1,147 \pm 147$  cpm/islet (mean  $\pm$  SEM), with most radioactivity ( $94 \pm 5\%$  in control islets) present in the lipid fraction. Thin-layer chromatography of lipid extracts of these islets showed that the majority of lipid-associated <sup>3</sup>H was present in PI; no radioactivity was detectable at the origin but a minor amount was present in the region corresponding to di- or triphosphoinositides.

Perfusion of islets, labeled with [<sup>3</sup>H]inositol, with 2.8 mM glucose resulted in an efflux of <sup>3</sup>H which remained low throughout the period of observation (Fig. 1). When the glucose concentration was increased to

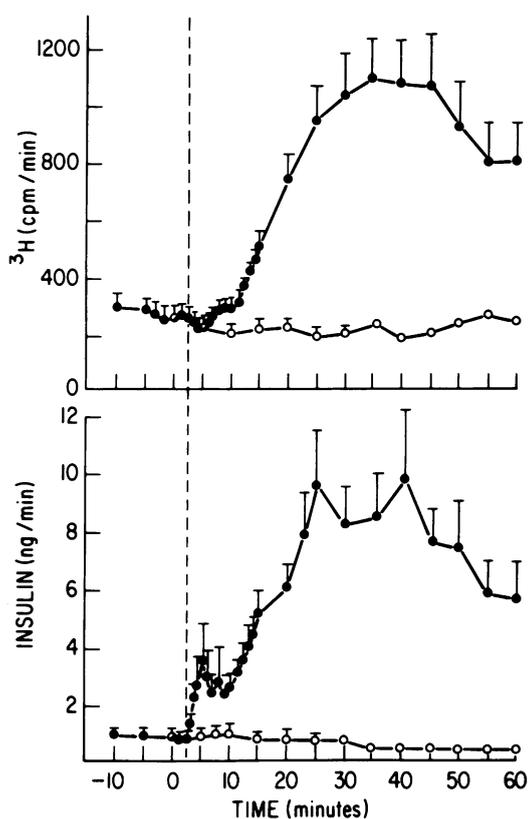


FIGURE 1 Effect of 16.7 mM glucose on release of <sup>3</sup>H-labeled compounds and insulin from [<sup>3</sup>H]inositol labeled, isolated rat islets. After the 2-h labeling period, each batch of 100 islets was perfused with buffer containing 2.8 mM glucose for 30 min (-30 to 0 min). At time 0, the perfusate was changed to one with either 16.7 mM glucose (filled circles,  $n = 12$ ) or 2.8 mM glucose (open circles,  $n = 5$ ). Efflux of <sup>3</sup>H (upper panel) and insulin (lower panel) are shown as means  $\pm$  SEM per 100 islets per minute. The vertical interrupted line at 2.5 min represents the approximate time at which perfusate from the buffer change at time 0 traversed the dead space of the system; this dead space applies to all subsequent figures.

2.8mM GLUCOSE	16.7mM GLUCOSE	
2.5mM Ca <sup>2+</sup>	0 Ca <sup>2+</sup>	0.5mM EGTA

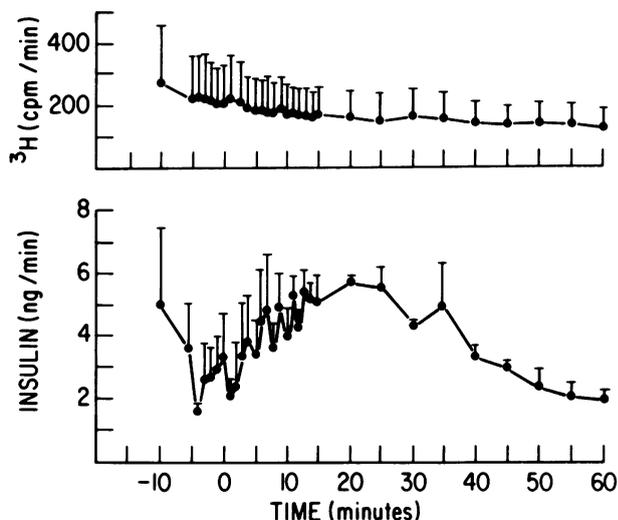


FIGURE 2 Effect of the removal of calcium on glucose-induced <sup>3</sup>H efflux and insulin release. Islets were labeled with [<sup>3</sup>H]inositol and perfused as described for Fig. 1 until time 0 when the perfusate was changed to a Ca<sup>2+</sup>-free buffer containing 16.7 mM glucose plus 0.5 mM EGTA. Release of <sup>3</sup>H (upper panel) and insulin (lower panel) per 100 islets per minute are shown as means  $\pm$  SEM for three experiments.

16.7 mM, a dramatic and sustained rise in both <sup>3</sup>H and insulin output occurred. Insulin release increased significantly above base line at 3 min ( $P < 0.05$ , paired  $t$  test); this represents a virtually immediate response because it required 2.5–3 min for the buffer to traverse the dead space of the system. A first phase secretory burst lasting 4 min was followed by a larger second phase that reached its peak at 35 min. Release of <sup>3</sup>H was delayed with respect to the onset of insulin release, rising at  $\sim 5$  min but only reaching a level that was significantly higher than control ( $P < 0.025$ ; paired  $t$  test) at 12 min (9 min if corrected for the dead space) and remaining elevated throughout the perfusion ( $P < 0.005$ ). The timing and amplitude of <sup>3</sup>H output and of second-phase insulin release in response to 16.7 mM glucose were similar.

Two types of experiments were performed to determine whether the observed <sup>3</sup>H efflux was due to the higher glucose concentration per se or to effects of glucose on Ca<sup>2+</sup> fluxes in the islet. In the first experiment, the perfusion buffer was switched from one containing 2.8 mM glucose and 2.5 mM Ca<sup>2+</sup> to one containing 16.7 mM glucose and 0.5 mM EGTA but no added Ca<sup>2+</sup> (Fig. 2). This completely inhibited glucose stimulated <sup>3</sup>H efflux but, as has been reported by others (18), showed a partially preserved insulin secretory re-

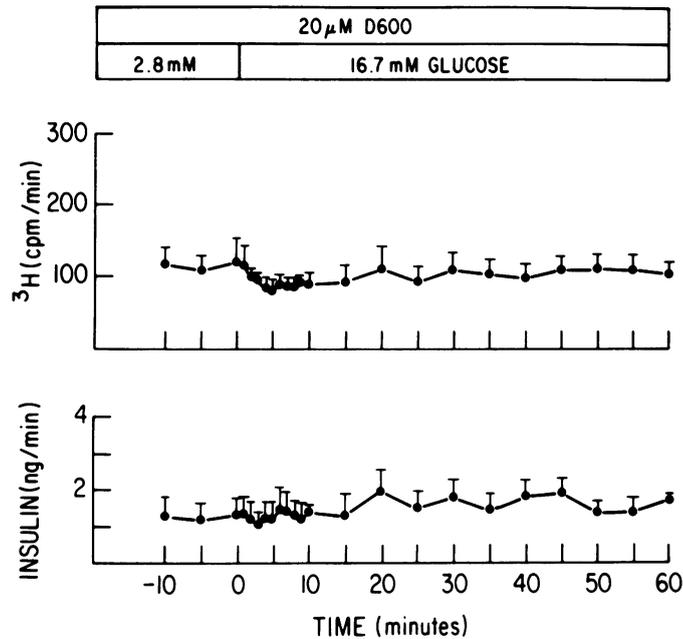
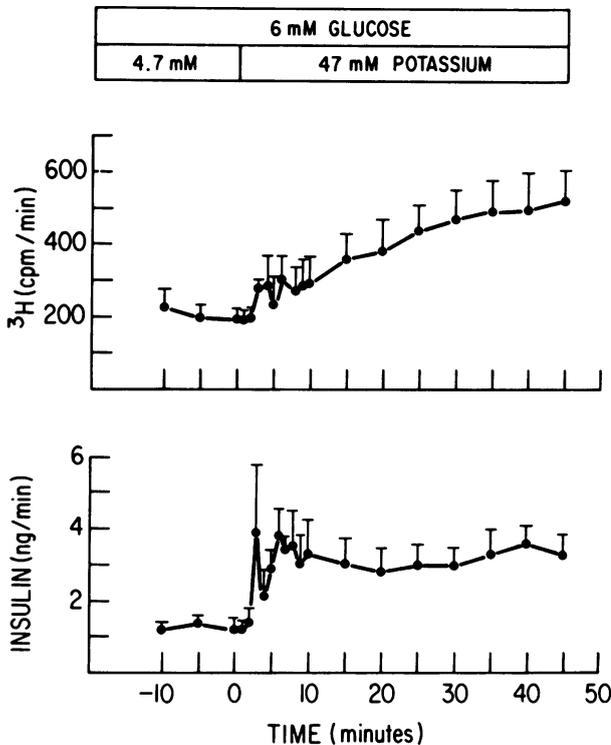


FIGURE 3 Effect of D600 on glucose-induced  $^3\text{H}$  efflux and insulin release. Islets were labeled with [ $^3\text{H}$ ]inositol and perfused as described for Fig. 1 until  $t = -15$  min when the buffer was changed to one containing 2.8 mM glucose plus 20  $\mu\text{M}$  D600. At time 0 the perfusate was switched to one containing 16.7 mM glucose plus 20  $\mu\text{M}$  D600. Release of  $^3\text{H}$  (upper panel) and insulin (lower panel) per 100 islets per minute are shown as means + SEM for four experiments.



response. In the second experiment, islets were exposed to D600, a blocker of the voltage-dependent  $\text{Ca}^{2+}$  channel for 15 min before and throughout perfusion with 16.7 mM glucose. Under these conditions glucose failed to stimulate either  $^3\text{H}$  or insulin release (Fig. 3). Collectively, these results indicate that glucose does not stimulate the efflux of  $^3\text{H}$  from [ $^3\text{H}$ ]inositol-labeled islets in the absence of an inward flux of  $\text{Ca}^{2+}$ .

The ability of  $\text{Ca}^{2+}$  influx to stimulate PI breakdown in the absence of a high concentration of glucose was examined by perfusing islets with 47 mM  $\text{K}^+$ . High concentrations of  $\text{K}^+$  are known to depolarize the islet and provoke influx of  $\text{Ca}^{2+}$  through the voltage-dependent channel (19). As shown in Fig. 4, 47 mM  $\text{K}^+$  in-

FIGURE 4 Effect of depolarization by 47 mM  $\text{K}^+$  on  $^3\text{H}$  efflux and insulin release. Islets were labeled for 2 h with [ $^3\text{H}$ ]inositol, perfused with buffer containing 2.8 mM glucose for 30 min ( $t = -60$  to  $-30$ ) and then with buffer containing 6 mM glucose for 30 min ( $t = -30$  to 0 min). This increase in glucose concentration did not stimulate either  $^3\text{H}$  efflux or insulin release (data not shown). At time 0 the perfusate was changed to one with 6 mM glucose and 47 mM potassium. Release of  $^3\text{H}$  (upper panel) and insulin (lower panel) per 100 islets per minute are shown as means + SEM for four experiments.

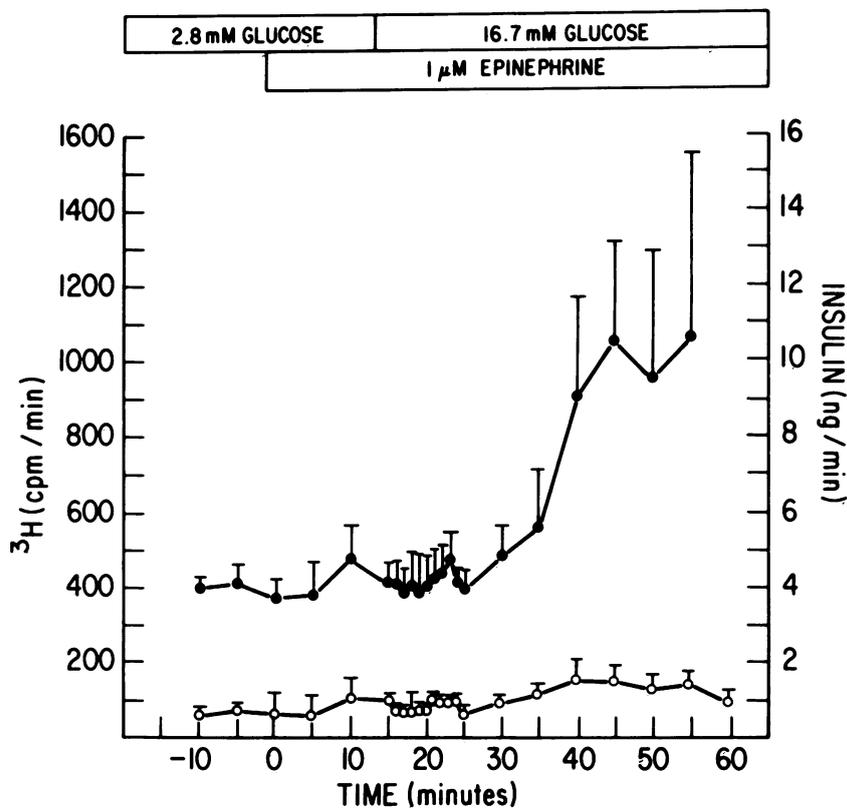


FIGURE 5 Effect of epinephrine on glucose-induced release of  $^3\text{H}$  and insulin. Islets were labeled with [ $^3\text{H}$ ]inositol and perfused as described for Fig. 1 until time 0 when the perfusate was changed to one containing 2.8 mM glucose plus 1  $\mu\text{M}$  epinephrine bitartrate. At  $t = 15$  min, the buffer was changed to one with 16.7 mM glucose plus 1  $\mu\text{M}$  epinephrine bitartrate. Release of  $^3\text{H}$  (filled circles) and insulin (open circles) per 100 islets per minute are shown as means + SEM for four experiments.

creased the release of both insulin and  $^3\text{H}$ . Increased output of  $^3\text{H}$  was immediate in three of four cases, in contrast with the consistently delayed output observed during stimulation with 16.7 mM glucose (Fig. 1).

Because epinephrine has been reported to prevent glucose-induced insulin secretion at concentrations that do not abolish the actions of glucose on  $\text{Ca}^{2+}$  influx or efflux (20), its effect on glucose-stimulated  $^3\text{H}$  efflux was examined. Epinephrine (1  $\mu\text{M}$ ) completely inhibited the insulin secretory response to 16.7 mM glucose (Fig. 5). However,  $^3\text{H}$  efflux was significantly increased ( $P < 0.05$ , paired  $t$  test) by  $t = 30$ , which is 15 min after the glucose concentration was raised (12 min if corrected for the dead space).

In contrast, perfusion of islets with IBMX (1 mM), an inhibitor of phosphodiesterase, which elevates cAMP levels, had no effect on  $^3\text{H}$  efflux despite stimulation of a rapid and sustained rise in insulin secretion (Fig. 6).

Table I shows the distribution of  $^3\text{H}$  in islets that had been perfused with buffer containing various secretory

agents (Figs. 1–6) and whose lipids were then extracted. Total  $^3\text{H}$  refers to all  $^3\text{H}$  that was in the labeled, washed islets at time 0 of the perfusion. The total  $^3\text{H}$  varied among preparations; the reason for this is not known but could be due to differences in the size of the islets. In the next two columns,  $^3\text{H}$  released from time 0 to the end of the perfusion and  $^3\text{H}$  remaining in the islets after perfusion, the two values that together comprise total  $^3\text{H}$ , are expressed as percentages of the total  $^3\text{H}$ . The lipid-soluble (PI) radioactivity within the islets at the end of the perfusion is listed in the next column. The last two columns show the [ $^3\text{H}$ ]PI remaining in the islets as a percentage of all  $^3\text{H}$  in the islets at the end and as a percentage of the total  $^3\text{H}$  at time 0.

[ $^3\text{H}$ ]PI, as a percentage of the  $^3\text{H}$  remaining in the islet, was reduced significantly from control in  $\text{K}^+$ -stimulated islets and those perfused with both 16.7 mM glucose and epinephrine. This suggests that [ $^3\text{H}$ ]PI was proportionately more depleted than any nonlipid forms of [ $^3\text{H}$ ]inositol in the islets. When [ $^3\text{H}$ ]PI remaining in

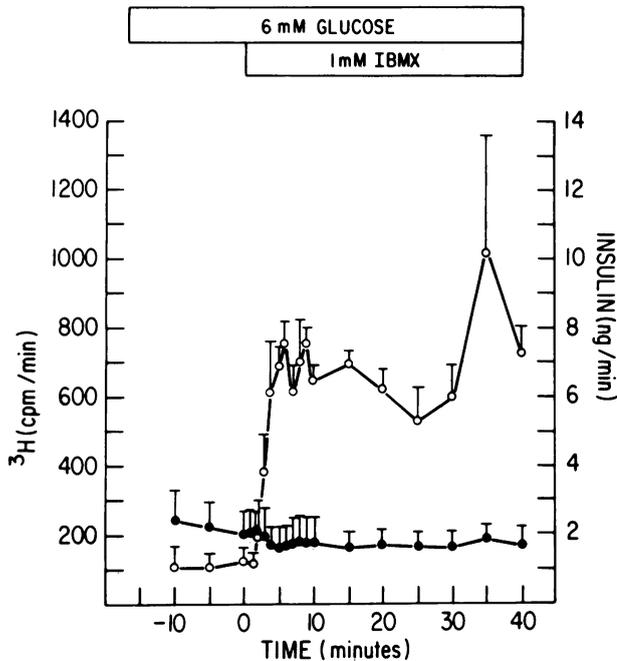


FIGURE 6 Effect of IBMX on  $^3\text{H}$  efflux and insulin release. Islets were labeled for 2 h with [ $^3\text{H}$ ]inositol, perfused with buffer containing 2.8 mM glucose for 30 min ( $t = -60$  to  $-30$  min) and then with buffer containing 6 mM glucose for 30 min ( $t = -30$  to 0 min). At time 0, the perfusate was changed to one with 6 mM glucose and 1 mM IBMX. Release of  $^3\text{H}$  (filled circles) and insulin (open circles) per 100 islets per minute are shown as means + SEM for five experiments.

the islet at the end of the perfusion is expressed as a percentage of the total  $^3\text{H}$  that was in the islet at the beginning (time 0) of the perfusion (final column), it can be seen that [ $^3\text{H}$ ]PI decreased significantly only in conditions in which  $^3\text{H}$  efflux was stimulated: 16.7 mM glucose, 47 mM  $\text{K}^+$  and 16.7 mM glucose plus 1  $\mu\text{M}$  epinephrine.

Myo-inositol comprised 53% of the  $^3\text{H}$ -labeled material in the perfusate emanating from stimulated islets. 6% of the total  $^3\text{H}$  was identified as an inositol monophosphate; 25% was tentatively identified as glycerylphosphorylinositol (a product of the removal of both fatty acyl groups from PI) by comparison with its relative migration in the same solvent system (21). The remaining radioactivity was not associated with any identified compounds and was evenly distributed.

## DISCUSSION

In this study the efflux of  $^3\text{H}$  from islets incubated with [ $^3\text{H}$ ]inositol was used as an index of PI breakdown during stimulation by various secretagogues. PI can be hydrolyzed by several types of enzymes including phospholipase  $\text{A}_2$ , which removes the fatty acid at the 2-position (usually arachidonic acid) either in an exchange reaction involving other acyl transferases or in conjunction with lysophospholipases, thereby producing glycerylphosphorylinositol, phospholipase C, which yields 1,2-diacylglycerol (rapidly metabolized to phosphatidic acid) and inositol 1:2-cyclic phosphate (con-

TABLE I  
Distribution of  $^3\text{H}$  from [ $^3\text{H}$ ]inositol-labeled Perfused Islets

Perfusion buffer	n	Total $^3\text{H}^*$ cpm $\times 10^{-3}$ per 100 islets	$^3\text{H}$ released, percentage of total	$^3\text{H}$ remaining in islet, percentage of total	[ $^3\text{H}$ ]PI remaining in islet†		
					cpm $\times 10^{-3}$ per 100 islets	Percentage of $^3\text{H}$ in islet	Percentage of total $^3\text{H}$
Glucose, 2.8 mM	5	108.7 $\pm$ 27.4	22 $\pm$ 7	78 $\pm$ 7	84.1 $\pm$ 24.3	94 $\pm$ 5	73 $\pm$ 6
Glucose, 16.7 mM	12	118.3 $\pm$ 20.5	49 $\pm$ 5	52 $\pm$ 5§	47.0 $\pm$ 10.7	76 $\pm$ 7	37 $\pm$ 4 <sup>  </sup>
Glucose, 16.7 mM D600, 20 $\mu\text{M}$	4	73.3 $\pm$ 14.1	18 $\pm$ 1	82 $\pm$ 1	49.7 $\pm$ 10.7	82 $\pm$ 5	67 $\pm$ 4
Glucose, 16.7 mM Calcium-free EGTA, 0.5 mM	3	104.4 $\pm$ 56.4	14 $\pm$ 1	86 $\pm$ 1	67.7 $\pm$ 32.0	81 $\pm$ 6	69 $\pm$ 6
Glucose, 16.7 mM Epinephrine, 1 $\mu\text{M}$	4	118.5 $\pm$ 27.4	38 $\pm$ 3	62 $\pm$ 3	49.4 $\pm$ 7.4	70 $\pm$ 5 <sup>  </sup>	44 $\pm$ 5 <sup>  </sup>
Potassium, 40 mM	4	65.1 $\pm$ 14.7	42 $\pm$ 7	58 $\pm$ 7	22.8 $\pm$ 7.4§	62 $\pm$ 12§	36 $\pm$ 8 <sup>  </sup>
IBMX, 1 mM	5	93.6 $\pm$ 21.8	16 $\pm$ 2	84 $\pm$ 2	74.2 $\pm$ 27.8	91 $\pm$ 10	77 $\pm$ 9

Values are expressed as means $\pm$ SEM and compared with responses to 2.8 mM glucose by unpaired  $t$  test.

\* Sum of  $^3\text{H}$  released from perfused islets, beginning at  $t = 0$ , plus total  $^3\text{H}$  remaining in the islets after perfusion.

† Corrected for 75 $\pm$ 6% recovery of PI.

§  $P < 0.05$ .

<sup>||</sup>  $P < 0.01$ .

verted to inositol monophosphate and then inositol plus phosphate (14, 22)), and phospholipase D, which yields inositol plus phosphatidic acid. The activities of phospholipases A<sub>2</sub> and C appear to predominate in the systems showing enhancement of PI turnover by Ca<sup>2+</sup>-mediated stimulants (8, 23).

Four lines of evidence indicate that the enhanced <sup>3</sup>H efflux observed in this study indeed reflected PI breakdown. Firstly, after incubation of islets with [<sup>3</sup>H]inositol, <sup>3</sup>H was assimilated into lipid, with most detectable radioactivity associated with PI, as shown by two-dimensional thin-layer chromatography. Secondly, when the output of <sup>3</sup>H increased, the amount of [<sup>3</sup>H]PI remaining in the islet decreased, particularly when viewed as a percentage of total <sup>3</sup>H (Table I). Thirdly, other metabolites of PI besides inositol—i.e., inositol monophosphate and glycerylphosphorylinositol—were identified among the radiolabeled substances emanating from the islet. Fourthly, the amount of <sup>3</sup>H released by glucose-stimulated islets (Table I, line 2, column 1 × column 2) was greater than that possibly available from aqueous pools in the islet, even assuming control <sup>3</sup>H efflux to be totally derived from the aqueous pools (Table I, line 1, column 1 minus column 4). In summary, these data show that the stimulated efflux of <sup>3</sup>H does not merely represent an exchange of extracellular inositol with unincorporated [<sup>3</sup>H]inositol within the islet. The only source that could provide enough <sup>3</sup>H for the large efflux observed in glucose-stimulated islets was [<sup>3</sup>H]PI.

This conclusion is supported by several related findings. High concentrations of glucose decrease the net uptake of inositol by islets (data not shown) and hepatocytes (24). Therefore, an increased glucose concentration would be expected to cause less replacement of unincorporated intracellular [<sup>3</sup>H]inositol by extracellular inositol and hence less and not more efflux of <sup>3</sup>H. Another relevant observation is the inhibition by Ca<sup>2+</sup> of an enzyme in *vas deferens* that exchanges free inositol with that incorporated into PI (25). Since the conditions in which <sup>3</sup>H efflux was increased in the present study were all associated with increased intracellular Ca<sup>2+</sup> levels, it is unlikely that the enhancement of <sup>3</sup>H efflux was due to increased exchange of nonlabeled inositol with [<sup>3</sup>H]inositol already in PI.

The combined evidence shows, therefore, that <sup>3</sup>H efflux is an index of PI turnover in the islet under these conditions. The actual reactions involved in PI breakdown were not determined. The potential for further metabolism of products (e.g., inositol monophosphates to inositol plus phosphate) precludes specification of the reactions responsible within the protocol of this study. The identities of the tritiated metabolites, however, are consistent with the actions of phospholipases

C and A. Activity of phospholipase A<sub>2</sub> in islets has been reported recently (26).

This study examined the effects of secretagogues on PI metabolism in islets. The effect of glucose on PI turnover in islets has been investigated previously. Fex and Lernmark (27) found that 20 mM glucose increased labeling of all phospholipids with <sup>32</sup>P; the fraction including PI appeared to be the most affected. Freinkel et al. (11) also showed a glucose-stimulated uptake of <sup>32</sup>P by phospholipids; the increased labeling of PI could be selectively inhibited by the anesthetic tetracaine. The first evidence in islets for stimulation by glucose of the actual breakdown of PI was reported by Clements and Rhoten (7) who monitored the release of <sup>3</sup>H from perfused islets labeled with [<sup>3</sup>H]inositol. The response to 28 mM glucose was rapid and transient, completely disappearing before the second phase of insulin release began. Clements et al. (28) later screened a variety of agents for their ability to alter the amount of lipid-bound <sup>3</sup>H (shown to be mainly [<sup>3</sup>H]PI) in [<sup>3</sup>H]inositol-labeled islets. After 10-min incubation with glucose, tolbutamide, or ouabain, both insulin release and loss of [<sup>3</sup>H]PI were stimulated. Agents known to alter cellular levels of cAMP—*isoproterenol*, *theophylline* and *dibutyryl-cAMP*—showed inconsistent effects both on insulin release and [<sup>3</sup>H]PI levels. Removal of Ca<sup>2+</sup> from the medium prevented the loss of [<sup>3</sup>H]PI associated with stimulation by 16.7 mM glucose. These results suggest that Ca<sup>2+</sup> and perhaps cAMP affect PI breakdown in the islet.

In the present study, perfusion with 16.7 mM glucose greatly stimulated PI breakdown in [<sup>3</sup>H]inositol-labeled islets (Fig. 1) but the response differed from that described by Clements and Rhoten (7) in its later onset, greater magnitude, and longer duration. Duplication of their protocol (using 0.5 mM Ca<sup>2+</sup> and 28 mM glucose) still yielded a response indistinguishable from that depicted in Fig. 1 (data not shown). Although the reason for the dissimilarity between the responses in the two studies is unknown, it may have been due to the nearly 50-fold greater incorporation of [<sup>3</sup>H]inositol into lipid as compared with that reported (7). However, [<sup>3</sup>H]inositol-labeled islets incubated with stimulatory concentrations of glucose (7, 28) contained less PI-associated radioactivity than control islets only after 10 min, a finding consistent with the timing of increased <sup>3</sup>H efflux in the present study.

Several conclusions can be made regarding the nature of the stimulation of PI turnover in the present study. The effect was not merely due to the presence of glucose per se but instead appeared to require the movement of Ca<sup>2+</sup> into the cell. In addition, the stimulated breakdown of PI was not merely the result of the fusion of insulin secretory granules with the plasma

membrane, since insulin release and  $^3\text{H}$  efflux were uncoupled under three different experimental conditions: exposure to 16.7 mM glucose in the absence of  $\text{Ca}^{2+}$ , to glucose plus epinephrine and to IBMX (Figs. 2, 5, 6).

The results also indicate that modulation of insulin secretion by changes in cAMP levels was not associated with altered PI breakdown (Figs. 5 and 6). If cAMP-mediated insulin release is, as has been proposed (1, 5, 31), due to a redistribution of intracellular  $\text{Ca}^{2+}$  that raises the concentration of cytosolic free  $\text{Ca}^{2+}$ , the cytosolic free  $\text{Ca}^{2+}$  either would not be the crucial pool or its level would not be altered enough to affect the breakdown of PI. A related deduction can be made from data obtained in experiments in which the removal of available extracellular  $\text{Ca}^{2+}$  coincided with the addition of 16.7 mM glucose (Fig. 2). Indeed, our experiments suggest the necessity for a  $\text{Ca}^{2+}$  influx from the extracellular space to stimulate the metabolism of PI. These findings support the hypothesis that  $\text{Ca}^{2+}$  influx causes PI turnover as opposed to the idea that PI breakdown causes the increased  $\text{Ca}^{2+}$  flux.

Recent investigations on stimulated lymphocytes (32), neutrophils (33), hepatocytes (34), and pancreas (35, 36) have also demonstrated that removal of  $\text{Ca}^{2+}$  from the extracellular environment decreases the stimulation of PI metabolism and suggest (34) a distinction between changes in the availability of  $\text{Ca}^{2+}$  within the cell and the actual entry of  $\text{Ca}^{2+}$  through specific channels in the plasma membrane. Thus, the conclusions of several other studies support a model in which PI turnover is itself mediated by  $\text{Ca}^{2+}$ .

The timing of PI breakdown observed in this study differs from that generally reported (8, 23) in its later onset and persistence throughout the cell's secretory response. A similar temporal pattern has been described for hepatocytes in which activation of PI breakdown is  $\text{Ca}^{2+}$  dependent (34). Furthermore, glucose-induced insulin release probably does not involve binding of a ligand to its receptor (6) whereas systems in which stimulated PI turnover is independent of  $\text{Ca}^{2+}$  influx do involve ligand-receptor interactions (8). The observed effects then may represent a distinct phenomenon from the immediate, transient and  $\text{Ca}^{2+}$ -independent breakdown of PI that has been postulated to initiate  $\text{Ca}^{2+}$  fluxes (8, 23).

Breakdown of PI in islets appears to be dependent on the movement of  $\text{Ca}^{2+}$  through the voltage-dependent  $\text{Ca}^{2+}$ -channel and may reflect biochemical events that occur during its activation. This breakdown or the resultant generation of metabolites of PI may be involved in stimulus-secretion coupling at a step distal to  $\text{Ca}^{2+}$  influx, mediating the effects of  $\text{Ca}^{2+}$  in some way. A possible role is the activation of a  $\text{Ca}^{2+}$ - and

phospholipid-dependent protein kinase, which has been shown to be stimulated by 1,2-diacylglycerol, a product of the hydrolysis of phospholipids (including PI) by a phospholipase C (37).

The results of the present study indicate that PI breakdown in glucose-stimulated insulin secretion occurs after the entry of  $\text{Ca}^{2+}$ . This represents a departure from the prevalent model, which proposes that  $\text{Ca}^{2+}$  influx is induced by PI turnover (8).

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