Phosphoinositide Hydrolysis and Insulin Release From Isolated Perifused Rat Islets

Studies With Glucose

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The ability of glucose to promote the hydrolysis of prelabeled [2-^{3}H]inositol-containing phosphoinositides (PI) was assessed by measuring the efflux of ^{3}H in response to glucose and the accumulation of labeled inositol phosphates. The inclusion of nonradioactive inositol (1 mM) in the perifusion medium dramatically improved our ability to monitor glucose-induced increases in ^{3}H efflux. Efflux studies with this method revealed the following. *1*) ^{3}H efflux is significantly greater at 7 than at 2.75 mM glucose, and this parallels a small but significant increase in insulin secretion. *2*) D-*manno*-Heptulose reduces ^{3}H efflux with 7 mM glucose to a level approximating that seen in the presence of 2.75 mM glucose and has no effect on ^{3}H efflux with 2.75 mM glucose. *3*) In the presence of 20 mM glucose plus 1 mM inositol, ^{3}H efflux is rapid and biphasic, a response that parallels the timing and amplitude of the biphasic pattern of insulin secretion. Direct measurements of labeled inositol and inositol phosphate levels in islets revealed the following. *4*) After 50 min of perifusion with 2.75 or 7 mM glucose, labeled inositol phosphates were significantly greater with 7 mM glucose. *5*) In response to 20 mM glucose alone, islet levels of free inositol, inositol monophosphate (IP_1), and inositol bisphosphate (IP_2) increased. *6*) In response to 20 mM glucose plus 1 mM cold inositol, islet levels of free inositol increased, whereas islet levels of IP_1, IP_2, and inositol trisphosphate (IP_3) were reduced compared with values obtained with 20 mM glucose alone. *7*) In response to perifusion with 20 mM glucose, IP_3 accumulation was biphasic in nature, and this response precedes the biphasic pattern of both insulin output and ^{3}H efflux by several minutes. These results suggest that PI hydrolysis in islets is tightly regulated by the ambient glucose level and that second-messenger signals generated by activation of this pathway may contribute to the biphasic pattern of glucose-induced insulin secretion. *Diabetes* 37:1294–1300, 1988

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Phosphoinositide (PI) hydrolysis in pancreatic islets participates in the insulin secretory response induced by various agonists (1–5). In a series of publications, we (5–8) and others (2,9–11) have monitored agonist-induced PI hydrolysis by measuring the efflux of ^{3}H from *myo*-[2-^{3}H]inositol–prelabeled islets. This label is exclusively incorporated into the family of phosphoinositides, including phosphatidylinositol, phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP_2) (2,11). Consequently, the appearance of increased amounts of perifusate ^{3}H reflects in a dynamic, albeit indirect, manner the hydrolysis of membrane PI. In addition, and most important considering the variability in collagenase-isolated islet response characteristics, both the functional integrity (insulin release) and PI hydrolysis can be dynamically monitored in the same groups of islets and in the same perifusate samples, thus strengthening interpretation of the data. One major problem with this approach, however, is that a significant delay in any increases in perifusate ^{3}H in response to high glucose has been consistently observed, whereas insulin secretion is rapid (5,9–11). However, because labilized inositol may be reincorporated back into PI, the possibility exsits that the hydrolysis of these lipids may be underestimated with this otherwise experimentally sound approach. In an attempt to circumvent this possible shortcoming, we have included nonradioactive inositol in the perifusion medium to compete with the reincorporation of labilized radioactive inositol back into membrane PI. Whereas this manipulation has no effect on glucose-induced insulin secretion, it dramatically improves our ability to kinetically monitor PI hydrolysis in isolated perifused islets. We also determined the chemical nature of the radioactive compounds appearing in the effluent and dynamic changes in

islet levels of labeled free inositol and inositol phosphates during glucose stimulation.

MATERIALS AND METHODS

Male Sprague-Dawley rats purchased from Charles River (Wilmington, MA) were used in all studies. The animals were fed ad libitum and weighed 300–400 g. After Nembutal-induced (50 mg/kg) anesthesia, islets were isolated by collagenase digestion (12). Batches of 40–80 islets were loaded onto nylon filters and placed in small glass vials. They were incubated for 2 h in 200 μl of a *myo*-[2-^{3}H]inositol–containing solution prepared by adding 10 μCi *myo*-[2-^{3}H]inositol (initial sp act 16.6–19.9 Ci/mmol) to 250 μl of incubation medium. The medium used for this incubation procedure was similar to that employed during the islet perifusion and consisted of 115 mM NaCl, 5 mM KCl, 2.2 mM $CaCl_2$, 1 mM $MgCl_2$, 24 mM $NaHCO_3$, and 0.17 g/dl bovine serum albumin. The solution was gassed with 95% O_2/5% CO_2. Glucose (2.75 mM) was also present during the incubation. It was not necessary to prelabel islets in high glucose to demonstrate glucose-induced changes in PI hydrolysis. After termination of the incubation, the islets, still attached to the nylon filters, were washed with 5 ml nonradioactive medium. These islets were directly perifused. The pH of the perifusion medium was maintained at 7.4, the temperature at 37°C, and the flow rate at 1 ml/min. Islets were usually perifused for 30 min to establish stable insulin secretory rates and then exposed to the various agonists indicated in the figure legends. Perifusate samples were collected at time intervals indicated in the figures, and 200-μl aliquots were analyzed for ^{3}H content as well as insulin, with rat insulin (615-D63-12-3, Lilly, Indianapolis, IN) as standard (13). In some experiments, aliquots of perifusate samples were directly analyzed for [^{3}H]inositol, glycerophosphoinositol (GPI), and inositol phosphates. The cellular contents of radioisotope after the perifusion were also determined. In other experiments, cellular levels of labeled inositol phosphates were measured at various times after exposure to 20 mM glucose (5,14). Briefly, after neutralization with 0.25–0.28 ml of 6 N KOH, addition of 5 ml water, and centrifugation, the supernatant was applied to columns. These columns were prepared by adding anion-exchange resin (AGI-X8 formate form, Bio-Rad, Richmond, CA) to Pasteur pipettes to achieve a length of 3 cm. Further additions to the column included 10 ml water (to elute free inositol) and 5 ml of 5 mM Borax/60 mM sodium formate (to elute glycerophosphoinositol). Elution of the inositol phosphates was accomplished by the sequential addition of 10 ml of 0.1 M formic acid/0.2 M ammonium formate (inositol monophosphate, IP_1), 0.1 M formic acid/0.4 M ammonium formate (inositol bisphosphate, IP_2), and 0.1 M formic acid/1 M ammonium formate (inositol trisphosphate, IP_3). This methodology does not distinguish between the inositol trisphosphate isomers, inositol 1,4,5-trisphosphate and inositol 1,3,4-trisphosphate. Aliquots (0.4 ml) of the eluate were then analyzed for radioactive contents. The radioisotope used to measure insulin release (^{125}I-labeled insulin) was purchased from New England Nuclear (Boston, MA) and the *myo*-[2-^{3}H]inositol from Amersham (Arlington Heights, IL). *myo*-Inositol, bovine serum albumin, D-*manno*-heptulose, formic acid, and the salts used to make the perifusion medium were purchased from Sigma (St. Louis, MO). Ammonium formate was purchased from Fisher (Fair Lawn, NJ). Where appropriate, statistical significance was determined via Student's *t* test for unpaired data, and *P* values <.05 were taken as significant. Values presented in the figures represent the means ± SE of the specified number of observations.

RESULTS

After a 2-h labeling period with [^{3}H]inositol, the efflux of ^{3}H in the presence of 2.75 mM glucose averages ~0.2%/min after 30 min of perifusion (Fig. 1, *top*). This rate continues

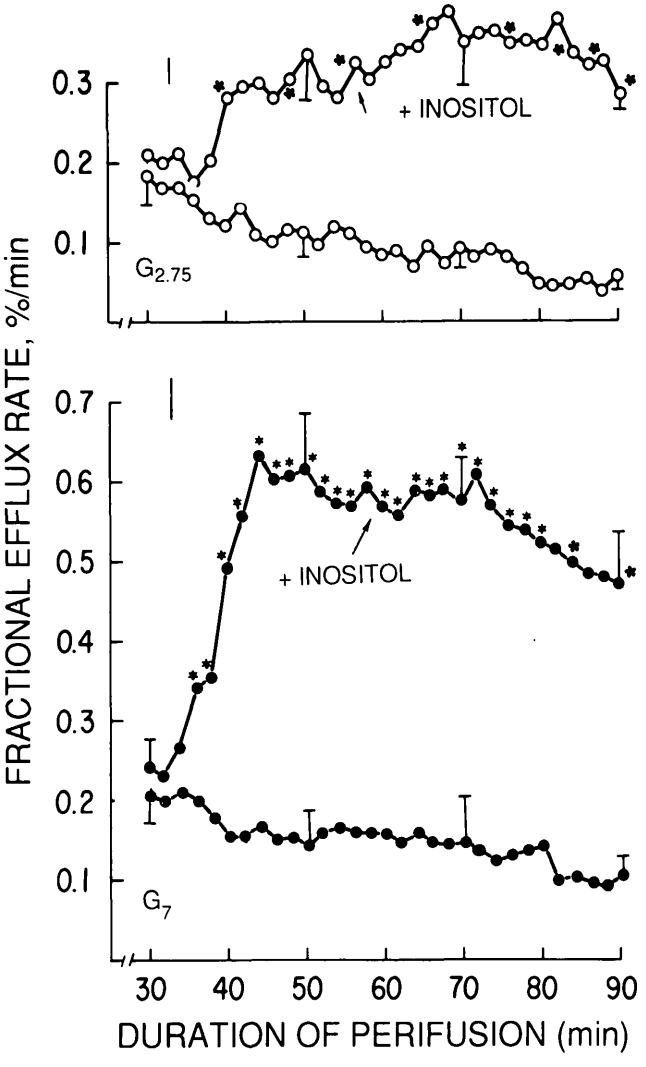

FIG. 1. Effects of glucose and inositol on ^{3}H efflux patterns from isolated perifused islets. *Top*: after 2-h prelabeling period with *myo*-[2-^{3}H]inositol, batches of islets were washed and then perifused. Fractional efflux rates of ^{3}H were calculated as previously described (25) in presence of 2.75 mM glucose plus or minus 1 mM inositol added 30 min after initiation of perifusion. $n \geq 4$ for each condition. *Bottom*: after labeling with *myo*-[2-^{3}H]inositol, islets were perifused for 30 min with 7 mM glucose. In some experiments, 1 mM nonradioactive *myo*-inositol was included in medium, and perifusion was continued for additional 60 min. This and all subsequent figures have been corrected for dead space (3 ml, ~3 min with flow rate of 1 ml/min) in perifusion system. After switching, new solution took ~1.5 min to reach islets and another 1.5 min to traverse remainder of perifusion apparatus. Means plus selected SEs of ≥4 experiments are given. *$P < .05$, statistically significant for selected points.

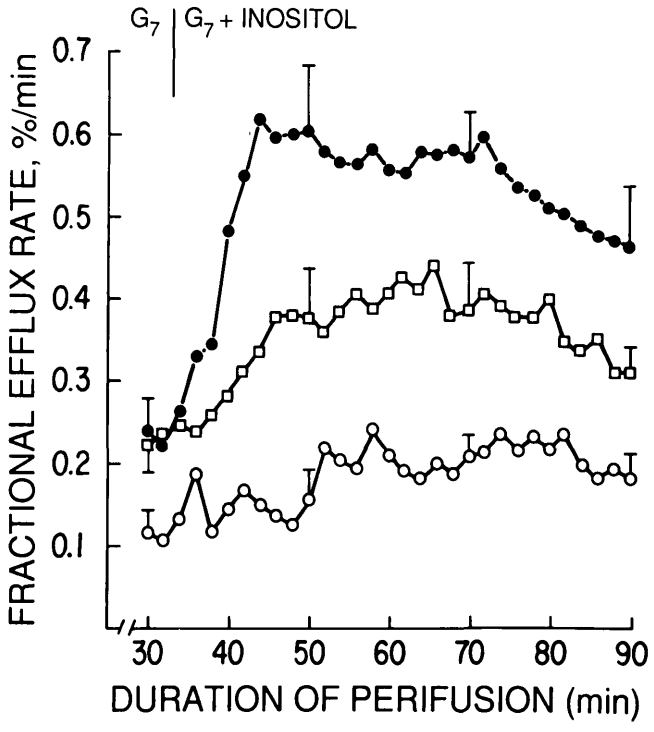

FIG. 2. Effects of D-*manno*-heptulose (□, 10 mM) and lithium chloride (○, 10 mM) on fractional efflux rates of ^{3}H. After prelabeling, islets were perifused for 30 min with 7 mM glucose. Control islets (●) were then exposed to 1 mM inositol for 60 min. In another group, 10 mM D-*manno*-heptulose was included in perifusate with 1 mM inositol for final 60 min. In third group, lithium chloride (10 mM) was added to medium 10 min before addition of 1 mM inositol and maintained at this level for duration of perifusion. $n \geq 4$ for each condition.

to fall during the perifusion, averaging ~0.05–0.08%/min after 90 min. If 1 mM inositol is added to the medium after 30 min with 2.75 mM glucose alone, the fractional efflux rate of ^{3}H rapidly increases, reaching a peak value of ~0.4%/min. For the duration of the perifusion, ^{3}H efflux rates in these nonradioactive inositol-treated islets are significantly greater than control efflux rates. Similar perifusion studies were conducted with 7 mM glucose (Fig. 1, *bottom*). Efflux rates with 7 mM glucose alone were slightly, but not significantly, higher than those noticed with 2.75 mM glucose alone. The addition of 1 mM inositol was again accompanied by a significant increase in ^{3}H efflux, a response that significantly exceeded that observed with 2.75 mM glucose plus 1 mM inositol.

TABLE 1
Labeled inositol phosphate levels after glucose perifusion

	Inositol phosphate levels (cpm/40 islets)		
Glucose (mM)	IP_1	IP_2	IP_3
2.75	388 ± 32	95 ± 11	72 ± 6
7.0	550 ± 37*	131 ± 8*	105 ± 14*

Groups of islets were perifused for 50 min with 2.75 or 7 mM glucose. Labeled inositol phosphate accumulation was determined after extraction with 10% perchloric acid. Values are means ± SE. IP_1, inositol 1-phosphate; IP_2, inositol 1,4-bisphosphate; IP_3, inositol 1,4,5-trisphosphate plus inositol 1,3,4-trisphosphate. $n \geq 8$ for each condition.
*Significant difference vs. 2.75 mM glucose.

Analysis of insulin release rates with 2.75 or 7 mM glucose revealed the following. After 30 min of perifusion with 2.75 mM glucose alone, release rates averaged 21 ± 3 pg · islet^{-1} · min^{-1} (mean ± SE). This value fell to 11 ± 2 pg · islet^{-1} · min^{-1} when measured after 90 min. The inclusion of 1 mM inositol did not affect this secretory pattern; e.g., insulin release measured 60 min after perifusion with 2.75 mM glucose plus 1 mM inositol averaged 10 ± 3 pg · islet^{-1} · min^{-1}. With 7 mM glucose, release rates averaged 49 ± 5 pg · islet^{-1} · min^{-1} after 30 min of perifusion, and this value fell to 28 ± 4 pg · islet^{-1} · min^{-1} after 90 min. Insulin secretory rates in response to 7 mM glucose, although small, were significantly greater than those observed with 2.75 mM glucose.

The seven-carbon sugar D-*manno*-heptulose (10 mM), a competitive inhibitor of glucose phosphorylation by islet glucokinase (15), reduced the fractional efflux of ^{3}H noted with 7 mM glucose plus 1 mM inositol (Fig. 2). Efflux rates now approximated those noted with 2.75 mM glucose plus 1 mM inositol. D-*manno*-Heptulose had no significant effect on the

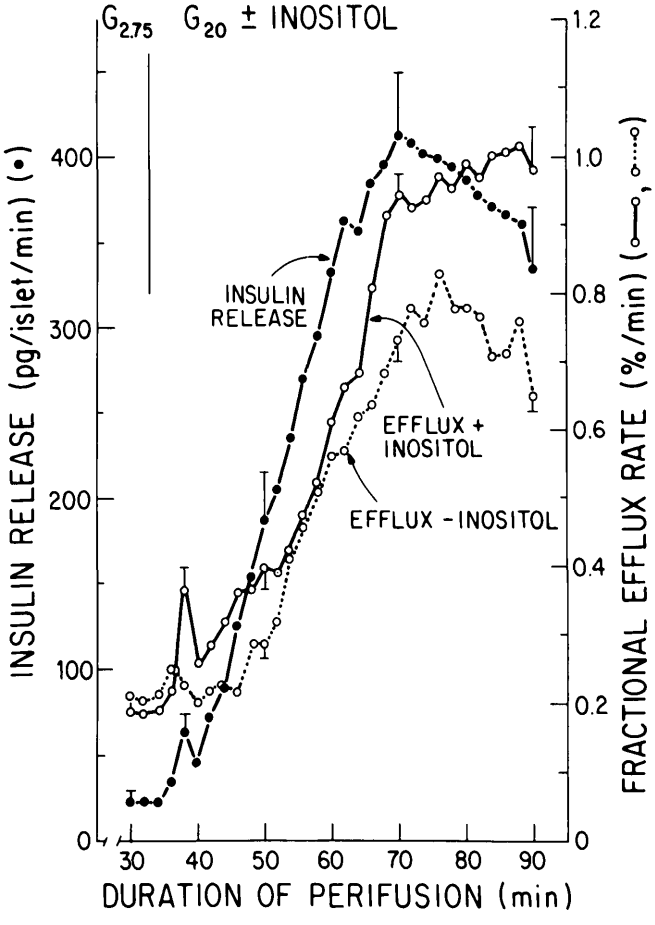

FIG. 3. Insulin output and fractional efflux rates of ^{3}H in response to glucose stimulation. After prelabeling, islets were perifused for 30 min with 2.75 mM glucose to establish stable basal insulin release and ^{3}H efflux rates. They were then stimulated for 60 min with 20 mM glucose plus or minus 1 mM *myo*-inositol. Insulin and ^{3}H contents were then measured in duplicate in same perifusate samples. Insulin secretory profile shown here was obtained from islets perifused with 20 mM glucose plus 1 mM inositol. Similar secretory results were noted in response to 20 mM glucose alone. Nine experiments were performed with each protocol.

^{3}H efflux patterns observed with 2.75 mM glucose plus 1 mM inositol. After 60 min with 10 mM D-*manno*-heptulose plus 1 mM inositol, ^{3}H efflux rates with 2.75 mM glucose averaged 0.30 ± 0.03%/min compared to 0.28 ± 0.02%/min in the absence of D-*manno*-heptulose. Lithium (10 mM), an inhibitor of the intracellular phosphates responsible for the degradation of inositol phosphates into more membrane-permeable species (16), also attenuated the increase in fractional efflux noticed with 7 mM glucose plus 1 mM inositol.

In other groups of islets, the levels of labeled inositol phosphates were monitored after a 50-min perifusion with 2.75 or 7 mM glucose alone. The results demonstrate that at the higher hexose level the amounts of labeled inositol phosphates are consistently increased above values noted with the lower hexose level (Table 1).

In response to 20 mM glucose alone, the insulin secretory response was biphasic (data not shown). Peak first-phase release, reached ~5 min after the onset of stimulation, averaged 71 ± 6 pg · islet^{-1} · min^{-1} (n = 9). Release after 60 min of stimulation with 20 mM glucose averaged 361 ± 23 pg · islet^{-1} · min^{-1} (n = 9). In response to 20 mM glucose plus 1 mM inositol, a similar biphasic insulin secretory response was evoked (Fig. 3). Peak first-phase release averaged 63 ± 10 pg · islet^{-1} · min^{-1} (n = 9) compared to the prestimulatory rate of 22 ± 6 pg · islet^{-1} · min^{-1} (n = 9). Release after 60 min of stimulation averaged 335 ± 36 pg · islet^{-1} · min^{-1}. In the presence of 20 mM glucose alone, the efflux of ^{3}H changed slightly, but not significantly, during the first few minutes of glucose stimulation. As the perifusion progressed, these rates increased dramatically, reaching an average value of 0.65%/min after 60 min of stimulation. Fractional efflux rates of ^{3}H measured in the presence of 20 mM glucose plus cold inositol were biphasic. In both timing and amplitude, ^{3}H efflux rates paralleled the biphasic insulin secretory response. Similar to insulin release rates, ^{3}H efflux rates quickly increased for the first several minutes after glucose stimulation, fell slightly for the next few minutes, and then slowly but inexorably increased to values of ~1.0%/min. When compared with efflux rates of islets maintained for 90 min with 2.75 mM glucose alone, these stimulated values are ~15-fold greater than the nonstimulatory efflux rates noted with 2.75 mM glucose (Fig. 1). Interestingly, insulin release rates are also ~15- to 20-fold greater in these stimulated islets.

In another group of studies, the accumulation of labeled inositol phosphates was monitored in islets after exposure to 20 mM glucose (Fig. 4). The levels of IP_1, IP_2, and IP_3 were similar in islets exposed to 2.75 mM glucose for 30–60 min. Levels of all these labeled phosphates changed dramatically in response to the addition of 20 mM glucose alone. IP_1 levels in islets rose within 2.5 min after 20 mM glucose stimulation and remained elevated for the entire 30-min period with 20 mM glucose. IP_2 levels in islets also changed rapidly, although the kinetics of the response were slightly different than that observed for IP_1; e.g., IP_2 levels in islets increased most dramatically 4.5 min after 20 mM glucose stimulation and then slowly thereafter. A biphasic pattern of IP_3 generation was observed after glucose stimulation. IP_3 content was characterized by an early peak 2.5 min after the addition of 20 mM glucose. Levels of IP_3 fell for the next 2 min and then increased slowly to values approximately threefold greater than those noted in control islets. The pattern of this compound's accumulation is similar to that noted for both ^{3}H efflux (in the presence of 1 mM inositol) and insulin output (Fig. 3). Furthermore, the biphasic accumulation of IP_3 precedes the biphasic pattern of ^{3}H efflux and insulin output by several minutes.

The nature of the ^{3}H-containing moieties in perifusate samples was analyzed during the final 10 min with 2.75 mM glucose alone, 2.75 mM glucose plus 1 mM cold inositol, 20 mM glucose alone, and 20 mM glucose plus 1 mM cold inositol (Table 2). In addition, the levels of free [^{3}H]inositol, GPI, and labeled inositol phosphates in islets were also measured. With 2.75 mM glucose alone, of the 214 ± 18 counts per minute (cpm)/40 islets collected during the final 10 min of the perifusion, 147 ± 16 (69%) appeared as free [^{3}H]inositol. The small remainder eluted with GPI (39 ± 7 cpm) and inositol phosphates (28 ± 6 cpm). With 2.75 mM

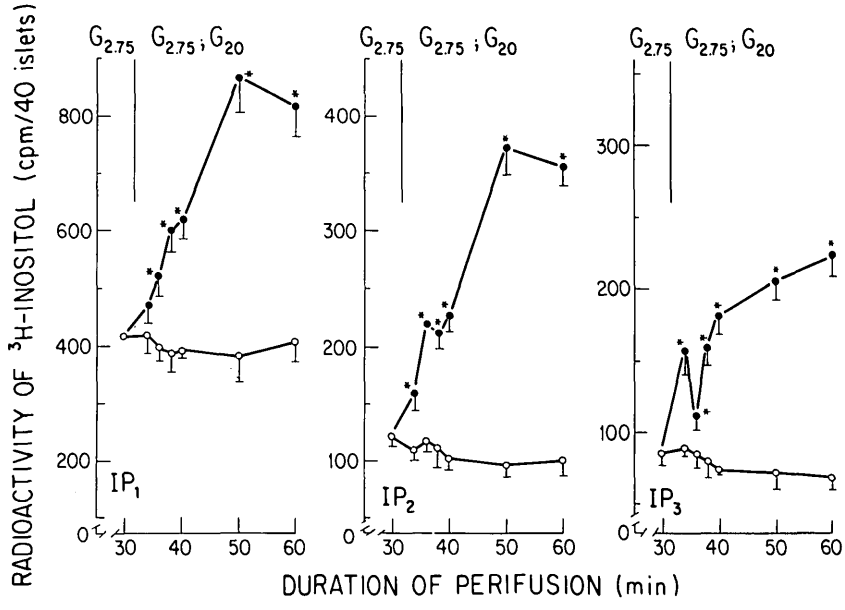

FIG. 4. Inositol 1-phosphate (IP_1), inositol 1,4-bisphosphate (IP_2), and inositol trisphosphate (IP_3) accumulation in islets after glucose stimulation. Batches of islets were incubated for 2 h in *myo*-[2-^{3}H]inositol and then perifused. Control islets (○) were maintained with 2.75 mM glucose for 30–60 min; other groups of islets (●) were perifused for various times with 20 mM glucose. This figure has been corrected for time (1.5 min) necessary for high glucose to reach islets; i.e., at 34 min of perifusion (1st time point analyzed after 20 mM glucose), islets had been exposed to glucose for 2.5 min. Similarly, 36 min of perifusion corresponds to 4.5 min of glucose (20 mM) stimulation. Means ± SE are given. At least 4 experiments with 20 mM glucose were performed at each time point. Lithium was not used in these measurements. *$P < .05$, statistically significant vs. control.

TABLE 2
Effect of glucose stimulation on islet and effluent levels of [^{3}H]inositol, glycerophosphoinositol, and inositol phosphates

Glucose (mM)	Inositol (mM)	[^{3}H]inositol	GPI	IP_1	IP_2	IP_3
Islets						
2.75 → 2.75		1422 ± 241*	97 ± 8	415 ± 23*	94 ± 10	69 ± 12
2.75 → 2.75	1	2084 ± 217	110 ± 13	340 ± 25	69 ± 18	57 ± 14
2.75 → 20		2642 ± 115*	81 ± 15	815 ± 62*	357 ± 26*	222 ± 14*
2.75 → 20	1	4698 ± 355	64 ± 19	609 ± 49	230 ± 31	122 ± 12
Effluent						
2.75		147 ± 16*	39 ± 7		28 ± 6†	
2.75	1	317 ± 47	41 ± 6		35 ± 8†	
20		661 ± 75*	52 ± 11		36 ± 8†	
20	1	958 ± 83	55 ± 10		39 ± 7†	

Islet levels expressed in cpm/40 islets; effluent levels in cpm · 40 islets^{-1} · 10 min^{-1}. After 2-h incubation with [2-^{3}H]inositol to label phosphoinositides, groups of 60–80 islets were washed and then perifused for 30 min with 2.75 mM glucose to establish basal stable ^{3}H efflux and insulin secretion rates. Islets were maintained with 2.75 mM glucose, 2.75 mM glucose plus 1 mM inositol, 20 mM glucose, or 20 mM glucose plus 1 mM inositol for additional 30 min. At termination of perifusion, islets were placed in 10% perchloric acid (PCA), and various compounds were measured as described in MATERIALS AND METHODS. Effluent samples were collected during final 10 min of perifusion with substances indicated and pooled 5-ml samples acidified with 500 μl PCA. After neutralization with 6 N KOH and centrifugation, samples were analyzed for contents. Values are means ± SE. $n \geq 4$ for each condition. GPI, glycerophosphoinositol; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate.
*$P < .05$ vs. treatment with inositol.
†Because levels of individual labeled inositol phosphates were so low in effluent, they were extracted together by addition of 10 ml of 0.1 M formic acid + 1 M ammonium formate.

glucose plus 1 mM inositol, 81% of the total effluent radioactivity (393 ± 28 cpm) eluted with [^{3}H]inositol and the remainder with GPI (41 ± 6 cpm) and inositol phosphates (35 ± 8 cpm). In response to 20 mM glucose alone, 88 ± 3% of the total effluent radioactivity (749 ± 62 cpm/40 islets) was identified as [^{3}H]inositol. With 20 mM glucose plus 1 mM cold inositol, 91 ± 4% of the 1052 ± 78 cpm · 40 islets^{-1} · 10 min^{-1} was free inositol. With 20 mM glucose or 20 mM glucose plus inositol, GPI and inositol phosphates accounted for the small remainder of effluent radioactivity. Under both stimulatory conditions, total labeled inositol phosphates accounted for 3–5% of effluent radioactivity. With 2.75 mM glucose alone, the content of free [^{3}H]inositol averaged 1422 ± 241 cpm/40 islets, and the addition of 1 mM inositol increased this value to 2084 ± 217 cpm/40 islets. After 30 min with 20 mM glucose alone, the total islet radioactivity recovered as free [^{3}H]inositol increased to 2642 ± 115 cpm/40 islets, and the presence of 1 mM cold inositol further increased this value to 4698 ± 355 cpm/40 islets. Interestingly, levels of radioactive IP_1, IP_2, and IP_3 were significantly ($P < .05$) reduced in islets treated with 1 mM inositol plus 20 mM glucose when compared with values obtained in the presence of 20 mM glucose alone. For example, IP_1, IP_2, and IP_3 levels after 30 min with 20 mM glucose alone averaged 815 ± 62, 357 ± 26, and 222 ± 14 cpm/40 islets, respectively. These values fell to 609 ± 49, 230 ± 31, and 122 ± 12 cpm/40 islets, respectively, in islets treated with 20 mM glucose plus 1 mM cold inositol.

DISCUSSION

A variety of different methodologies have been employed to monitor PI turnover in pancreatic islets (1–5). Attempts have been made from the data generated to assign second-messenger roles for various PI-derived molecules in the insulin secretory response. In many previous studies, results obtained during static incubation and in the presence of lithium [an inhibitor of stimulated insulin secretion (8,17)] have been extrapolated to the dynamic insulin secretory response. PI metabolism is usually monitored in one group of islets, and insulin secretion, if measured at all, is determined in other groups of islets. Because of the variability in collagenase-isolated islet response characteristics, we have analyzed insulin output and PI hydrolysis in the same groups of islets. We have taken advantage of the fact that radioactive inositol is exclusively incorporated into the family of membrane PIs (2,11). Because the only appreciable source of ^{3}H is assumed to be islet PI, any increase in perifusate ^{3}H above basal rates reflects an increase in PI hydrolysis. The studies of Axen et al. (11), Clements and Rhoten (2), and Mathias et al. (10) have established the validity of this concept. Several possible problems with this approach exist. For example, the possible contribution of free intracellular [^{3}H]inositol to efflux patterns noted in response to various agonists has not been thoroughly considered. Also, PI-derived [^{3}H]inositol phosphates, after partial or complete removal of the phosphate moieties by cellular phosphatases, may be reincorporated back into PI. To circumvent this problem, we included nonradioactive inositol in the perifusion medium. The results obtained in these efflux experiments were compared with those obtained by direct measurements of labeled inositol phosphates. The nature of the ^{3}H-containing molecules in both the islets and islet effluent was also determined. Several significant observations were made with this approach, and further comment is appropriate.

First, the hydrolysis of membrane PI in the presence of 1 mM inositol, monitored by measuring fractional efflux rates of ^{3}H (25), is ~2- to 2.5-fold greater at a glucose concentration of 7 mM than at 2.75 mM. This increase in PI hydrolysis is paralleled by small but significant differences in the levels of islet inositol phosphates and a slight elevation in insulin secretory rates (Table 1). The data presented here and elsewhere indicate that glucose metabolism by the islet determines to a large extent PI hydrolysis (8,18). The studies utilizing the metabolic inhibitor D-*manno*-heptulose perhaps best support this. This compound inhibits glucose phosphorylation and glucose-induced PI turnover and reduces

glucose-induced insulin output in a parallel fashion (8,15,19). In our study, 10 mM D-*manno*-heptulose reduces the increase in ^3H efflux usually noted in response to 7 mM glucose plus 1 mM inositol. In fact, the fractional efflux rates of ^3H under this condition (10 mM D-*manno*-heptulose, 7 mM glucose, and 1 mM inositol) resemble those obtained under similar conditions with 2.75 mM glucose plus 1 mM inositol. Note also that the efflux rates in the presence of 1 mM inositol were 2- to 2.5-fold greater with 7 mM glucose than with 2.75 mM glucose. The metabolic rate at the higher hexose level is also ~2- to 2.5-fold greater than at the lower hexose level (20). These results suggest that any increase in the metabolic rate of the islet (normally achieved by elevating the glucose level bathing them) is paralleled quantitatively by commensurate increases in PI hydrolysis.

Previous studies leave little doubt that the increase in ^3H efflux noted in these studies reflects the hydrolysis of islet PI (2,11). This is further supported herein, where 10 mM lithium blocks the increase in fractional efflux of ^3H by inhibiting the action of cellular phosphatases and trapping the inositol phosphates intracellularly. For this reason, lithium is commonly employed in many studies to facilitate measurements of intracellular inositol phosphates. Unfortunately, lithium reduces glucose-stimulated insulin output, thus somewhat limiting its usefulness in delineating the biochemical events responsible for stimulated insulin output (8,17).

Perhaps the most pertinent finding in these studies concerning how PI hydrolysis may contribute to stimulated insulin release was obtained when islets were stimulated with 20 mM glucose. Under this experimental condition, glucose evokes a kinetically similar biphasic pattern of IP_3 accumulation and insulin output. Moreover, the inclusion of cold inositol with 20 mM glucose, a maneuver without any effect on the insulin secretory response, converts a small and delayed ^3H-efflux pattern into a biphasic one. Furthermore, high glucose with or without cold inositol increases the islet content of free [^3H]inositol. The only significant source of [^3H]inositol is [^3H]inositol phosphates derived from [^3H]PI. Our results suggest that in the presence of 20 mM glucose, nonradioactive inositol competes with this expanding pool of free [^3H]inositol for reincorporation back into islet PI, and this manipulation allows more [^3H]inositol to escape into the effluent. This scenario is further supported by an analysis of [^3H]inositol phosphate levels in the presence of 20 mM glucose plus or minus 1 mM inositol. Levels of these phosphorylated derivatives are significantly reduced in the presence of cold inositol, indicating that reuptake of [^3H]inositol back into PI contributes significantly to the capacity of islets to maintain these sustained increases in labeled inositol phosphate levels. In the absence of nonradioactive inositol, reincorporation of [^3H]inositol into PI also reduces the amount of [^3H]inositol that effluxes from the cell. Cold inositol appears to compete with this rapidly expanding pool of free [^3H]inositol, derived from phosphatase action on [^3H]inositol phosphates, for reincorporation back into PI. This results in the significant increases in free perifusate [^3H]inositol noted in these experiments.

We did not measure the nature of all possible phosphorylated derivatives of inositol. Therefore, it might be argued that the biphasic pattern of IP_3 accumulation in 20-mM-glucose–stimulated islets does not really reflect a biphasic pattern of PI hydrolysis. For example, IP_3 may be rapidly converted into inositol 1,3,4,5-tetrakisphosphate (IP_4), thus accounting for the fall in its levels. If this were so, ^3H efflux would be reduced during the early minutes of glucose stimulation, an event reflecting sequestering of the label into IP_4. The fact that this did not occur suggests that the biphasic generation of IP_3 reflects a biphasic pattern of PI hydrolysis. Future experiments designed to address the nature of all inositol phosphates in stimulated islets seem appropriate.

Previous studies by various investigators have employed the same basic methodological approach to analyze PI turnover in islets, and discrepancies exist. For example, Clements and Rhoten (2) found that ^3H efflux from [^3H]inositol-prelabeled islets was characterized by a brisk initial burst of radioactivity when these islets were stimulated with high glucose. These results stand in contrast not only to the present ones but also to those of Axen et al. (11), Blachier et al. (9), and Mathias et al. (10). In all of these subsequent reports, increases in ^3H efflux were initially delayed in response to high glucose but then increased substantially as the perifusion continued. Perhaps the low rate of ^3H incorporation accounts, at least in part, for the results of Clements and Rhoten. The chemical nature of the ^3H-containing molecules present in the effluent after glucose stimulation has also been examined in several reports, and again, discrepancies exist. Although most reports agree that free [^3H]inositol is the major labeled moiety, Clements and Rhoten reported that with glucose stimulation, large increases in phosphorylated inositol derivatives were measurable. Axen et al. found that 53% of the ^3H-labeled material in the effluent from stimulated islets was free inositol with another 25% identified as GPI, formed by the removal of the two fatty acids from phosphatidylinositol. IP_1 accounted for another 6%. In the report of Mathias et al., most (70–80%) radioactivity effluent from unstimulated islets was free inositol, and in response to various agonists including glucose, only free [^3H]inositol increased in the effluent. In their study and in ours, the levels of labeled inositol phosphates in the effluent were low and did not increase with high glucose. Clearly, our results are in excellent agreement with this report. These results indicate that most of the radioactivity in the effluent from unstimulated islets perifused with a low nonstimulatory glucose level is free [^3H]inositol and, with the addition of a stimulatory glucose level, the amount of this label increases and accounts for virtually all the increase in perifusate ^3H.

Finally, a note of caution concerning the present efflux methology should be sounded. The ^3H efflux patterns observed in these studies are the net result of complex and incompletely understood interactions between various metabolic pathways (21). For example, let us assume that the only source of ^3H measured in the perifusate is derived from PIP_2, not an unreasonable assumption based on the comprehensive studies of Biden, Wollheim, and associates (22–24). First, PIP_2 must be hydrolyzed by phospholipase C. Second, the IP_3, apparently generated in a biphasic pattern, must be catabolized into more membrane-permeable species by islet phosphatases (Fig. 4; 24). Finally, the free inositol formed must escape biosynthetic pathways and avoid reincorporation back into PI. The situation becomes more complex if PIP and phosphatidylinositol hydrolysis participate in the genesis of inositol phosphates. In any event,

experimental manipulation of this distal metabolic step (blocking reincorporation of label by excess nonradioactive inositol) has revealed that ^3H is mobilized from glucose-stimulated islets in a biphasic pattern that, in both timing and amplitude, mirrors the biphasic pattern of insulin secretion. Moreover, the usefulness of this approach has been corroborated by direct measurements of labeled inositol phosphate accumulation.

In summary, the rate of PI hydrolysis in isolated perifused islets is tightly regulated by the ambient glucose level. This response is at least partly determined by the metabolism of the hexose. Finally, 20 mM glucose addition to [^3H]inositol-prelabeled islets results in a biphasic efflux of ^3H, a biphasic pattern of IP_3 generation, and a biphasic pattern of insulin output. Although we suggest that PI hydrolysis to a large extent regulates this biphasic insulin secretory response, further experiments to validate this important concept are required.

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