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GLYBURIDE PRIMING OF BETA CELLS

POSSIBLE INVOLVEMENT OF PHOSPHOINOSITIDE HYDROLYSIS

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Abstract—In the simultaneous presence of 5.5 mM glucose, exposure of isolated perifused islets to the sulfonylurea glyburide (500 nM) acutely stimulated insulin release and amplified the subsequent insulin secretory responses to 10 mM glucose or 10 mM arginine. This sensitizing effect of glyburide developed within 10 min, was maintained for at least 40 min after glyburide removal from the perifusion medium, and was attenuated by the calcium channel blocker nitrendipine. In islets whose inositol-containing lipids were prelabeled during a 2-hr incubation period with *myo*[2-^{3}H]inositol, glyburide induced a concentration-dependent increase in labeled inositol phosphate accumulation. Nitrendipine abolished this stimulatory effect of glyburide. In perifused islets, the stimulatory effect of glyburide on phosphoinositide (PI) hydrolysis persisted after its removal from the medium and the duration of this effect paralleled the duration of sensitization. These findings suggest that glyburide-induced increases in PI hydrolysis account, at least in part, for its acute stimulatory effect on insulin output and its ability to sensitize islets to subsequent stimulation.

Commonly employed in the therapy of non-insulin-dependent diabetes, the orally effective sulfonylureas amplify the insulin secretory response of pancreatic beta cells to a variety of agonists including glucose [1–4]. Their efficacy in activating the secretory apparatus depends to a large extent on the ambient glucose level; they are most effective at postprandial (5–8 mM) glucose levels and least effective at low (less than 3–4 mM) glucose levels. Despite their increasing popularity as a therapeutic modality, their biochemical mechanism of action remains enigmatic. It has been demonstrated that they influence ATP-dependent potassium channels [3, 5], an action thought to result in beta cell membrane depolarization and the opening of voltage-dependent calcium channels. The resulting influx of extracellular calcium participates in the insulinotropic effect of the sulfonylurea. We recently reported that the first generation sulfonylurea tolbutamide induces a glucose- and calcium-dependent increase in beta cell phosphoinositide (PI) hydrolysis [4]. This action appeared not only to participate in the acute insulin stimulatory effect of the compound but also to influence the sensitivity of the beta cell to other insulinotropic agonists as well. Like a wide variety of compounds which activate PI hydrolysis in islets [6–9], tolbutamide is capable of inducing time-dependent potentiation (TDP [10]) or memory in this tissue. In the present report we determined if the second generation sulfonylurea glyburide increased PI hydrolysis in islets, the duration of this effect, and whether glyburide also induces TDP. These findings further emphasize the importance of PI hydrolysis in beta cell stimulus–response coupling and the induction of TDP.

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METHODS

Male Sprague–Dawley rats purchased from Charles River were used in all studies. The animals were fed *ad lib.* and weighed 300–400 g. After nembutal (50 mg/kg)-induced anesthesia, islets were isolated by collagenase digestion and hand-picked under a stereomicroscope using a glass loop pipette. Then some groups of islets were directly perifused to establish secretory responsiveness to various agonists. In other experiments, batches of 22–35 islets were loaded onto nylon filters and placed in small glass vials. They were incubated for 2 hr 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 to 19.0 Ci/mmol) to 250 μL of incubation medium to label their inositol-containing phospholipids. 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. We did not find it necessary to incubate islets in a higher glucose level to demonstrate agonist-induced PI hydrolysis. After termination of the incubation, the islets, still attached to the nylon filters, were washed with 5 mL nonradioactive medium. Some of these islets were then perifused to assess fractional rates of [^{3}H]inositol efflux [11], inositol phosphate accumulation [7–9, 12], and insulin secretion in response to various treatments. Others were statically-incubated and subsequently analyzed for the accumulation of labeled inositol phosphates under various conditions. Lithium chloride (10 mM) was used in these batch-incubated islets to facilitate these measurements (see Table 1). Briefly, after neutralization with 0.25 to 0.28 mL of 6 N KOH, the further addition of 5 mL water, and centrifu-

gation, the supernatant was applied to columns. These columns were prepared by adding anion-exchange resin (AG1-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 of water and 5 mL of 5 mM Borax/60 mM sodium formate. 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 1-phosphate, IP_1) 10 mL of 0.1 M formic acid/0.4 M ammonium formate (inositol 1,4-bisphosphate, IP_2), and 10 mL of 0.1 M formic acid/1 M ammonium formate (primarily inositol 1,4,5-trisphosphate plus inositol 1,3,4-trisphosphate, IP_3). Aliquots (0.4 mL) of the eluate were then analyzed for radioactive content.

For the perfusion studies, the pH of the medium was maintained at 7.4, the temperature at 37°, and the flow at 1 mL/min. Islets were usually perifused for 30–40 min to establish stable insulin secretory rates and then treated as 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]inositol content, when appropriate, and insulin using rat insulin (615-D6312-3, Lilly, Indianapolis, IN) as standard. In the [^{3}H]inositol experiments, the cellular content of radioisotope after the perifusion was also determined. 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). Bovine serum albumin, lithium chloride, arginine, and the salts used to make the perifusion medium were purchased from Sigma (St. Louis, MO). Potassium glyburide was the gift of the Upjohn Co. (Kalamazoo, MI). Nitrendipine was the gift of Dr. A. Scriabine of the Miles Institute for Preclinical Pharmacology.

Statistics. Where appropriate, statistical significance was determined using analysis of variance in conjunction with the Newman–Keuls multiple comparison test or the Student's *t*-test for unpaired data. A P value of <0.05 was taken as significant. Values presented in the figures are means ± SEM of at least three experiments.

RESULTS

In the simultaneous presence of 5.5 mM glucose, a brief 10-min exposure to 500 nM potassium glyburide resulted in a significant insulin secretory response (Fig. 1). Removal of the sulfonylurea, along with a reduction of the glucose level to 2.75 mM, was accompanied by a fall in insulin release rates. After 20 min of perifusion with 2.75 mM glucose, these islets were subsequently stimulated with 10 mM glucose. When compared to the insulin secretory responses noted from naive islets, prior exposure to glyburide significantly amplified the subsequent insulin response to 10 mM glucose (Fig. 1). Particularly dramatic was the increase in first phase release. While peak first phase release from control, unprimed islets averaged 163 ± 18 pg/islet/min (mean ± SEM), this value increased to 501 ± 33 pg/islet/min after prior glyburide exposure. Second phase release was also amplified by prior glyburide exposure, although this effect waned as the perifusion progressed. As shown in Fig. 2, the sensitizing effect of glyburide was maintained for at least 40 min after its removal from the medium. Again, particularly dramatic was the initial response to 10 mM glucose. In addition, the sensitizing action of glyburide was not confined to glucose. As shown in Fig. 3, a 10-min exposure to glyburide (500 nM) also sensitized islets to arginine (10 mM) stimulation.

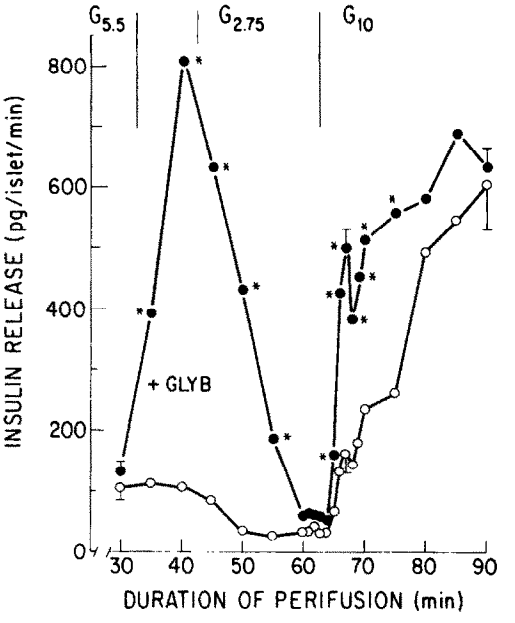

Fig. 1. Induction of time-dependent potentiation by glyburide. Groups of 12–16 islets were collagenase isolated and perifused with 5.5 mM glucose (G) for 30 min. One group (●) was stimulated with 500 nM potassium glyburide (GLYB) for 10 min. Both groups were then perifused for 20 min with 2.75 mM glucose prior to a 30-min stimulatory period with 10 mM glucose. Mean values of four experiments are given ± selected SEMs. The asterisks indicate significant differences ($P < 0.05$) between the two groups. This and all subsequent figures have been corrected for the dead space in the perifusion system, 2.5 mL or 2.5 min with a flow rate of 1 mL/min.

In the next series of experiments, we determined if glyburide influences phosphoinositide hydrolysis in islets. As a barometer of this effect, the accumulation of labeled inositol phosphates was determined in islets whose inositol-containing lipids were prelabeled with *myo*[2-^{3}H]inositol. The results are given in Table 1. Potassium glyburide induced a concentration-dependent increase in all of the labeled inositol phosphates measured (Table 1, lines 2 and 3 and lines 7 and 8), an effect that was effectively attenuated by a 5 μM concentration of the calcium influx inhibitor nitrendipine (Table 1, lines 5 and 10). Nitrendipine (5 μM) also abolished the acute stimulatory effect of 500 nM glyburide (in the presence of 5.5 mM glucose) on insulin release and abolished the sensitizing effect of glyburide on

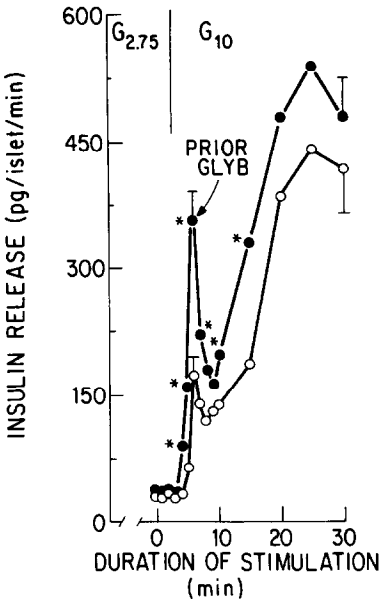

Fig. 2. Duration of glyburide-induced time-dependent potentiation. Groups of islets were treated as in Fig. 1 except that the period of perifusion with 2.75 mM glucose was extended at 40 min. A 30-min stimulatory period with 10 mM glucose followed and this period is shown here. Mean values of four experiments are given ± selected SEMs. Asterisks indicate significant differences ($P < 0.05$) between groups.

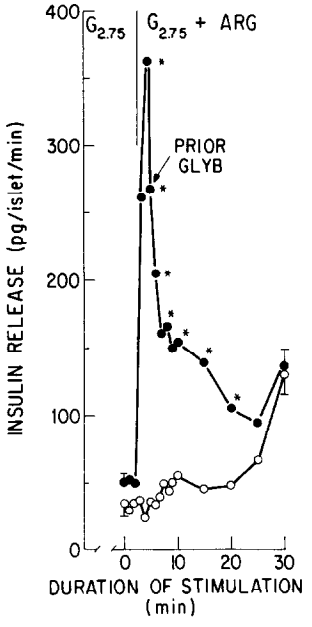

Fig. 3. Glyburide-induced time-dependent potentiation to arginine (ARG). Two groups of islets were perifused for 40 min with 5.5 mM glucose. During the final 10 min of this period 500 nM glyburide was included in one group (●). After 20 min with 2.75 mM glucose alone, both groups were stimulated with 10 mM arginine. Mean values of at least three experiments are given ± selected SEMs. The asterisks indicate significant ($P < 0.05$) differences between the two groups at the indicated time of perifusion.

first phase insulin release (compare Figs. 1 and 4). A significant residual stimulatory effect of glyburide on second phase release was noted, however. In perifused islets at least, the impact of this level of nitrendipine was not readily reversible since its inclusion in the medium attenuated control responses as well (compare control responses in Figs. 1 and 4, open circles).

We previously reported that the protein hormone cholecystokinin [6], high glucose [7], interleukin-1 [9], and the sulfonylurea tolbutamide [4] evoke sustained increases in phosphoinositide hydrolysis in islets. Whether a similar phenomenon occurs after glyburide exposure was assessed next. After labeling in *myo*[2-^{3}H]inositol, islets were perifused with 5.5 mM glucose. After an appropriate stabilization period, some islets were stimulated with 500 nM glyburide for 10 min. Marked increases in both insulin release and [^{3}H]inositol efflux occurred, although the increase in [^{3}H]inositol efflux lagged behind the insulin response (Fig. 5). After removal of the sulfonylurea, the increases in both insulin release and [^{3}H]inositol efflux declined slowly. However, this response as well as the sustained increase in [^{3}H]inositol efflux were effectively attenuated by 10 mM lithium chloride (Fig. 5).

It might be reasonably argued that the persistent increase in [^{3}H]inositol efflux (Fig. 5) represents the hydrolysis of preformed (during stimulatory period with glyburide) inositol phosphates into membrane permeable [^{3}H]inositol and is not the result of a sustained hydrolysis of [^{3}H]inositol-containing phosphoinositides. To address this important issue, newly formed inositol phosphates were trapped intracellularly during the perifusion with 10 mM lithium chloride. The results of these studies are given in Table 2. First, glyburide, even in the absence of lithium chloride, significantly increased inositol phosphate levels in perifused islets (Table 2, compare lines 1, 2, and 3). Removal of glyburide and further perifusion for 20 min with 5.5 mM glucose alone was, surprisingly, accompanied by a marked fall only in the IP_3 level (line 4). Thus, at least part of the sustained [^{3}H]inositol efflux response may be the result of the hydrolysis of these inositol phosphates. However, an even more important component of this [^{3}H]inositol efflux response is the sustained hydrolysis of membrane PI. This is demonstrated by the results given in Table 2, lines 5 and 6. In these experiments, the inclusion of lithium chloride had only a small effect on total inositol phosphates in naive, unprimed islets (Table 2, line 5). In islets previously exposed to glyburide and perifused for 20 min with 5.5 mM glucose alone, the subsequent addition of 10 mM lithium chloride was accompanied by significant increase in inositol phosphate levels, particularly IP_1 (Table 2, line 6). A reasonable explanation for this significant increase in islet inositol phosphates noted with lithium chloride is the sustained hydrolysis of labeled islet phosphoinositides.

DISCUSSION

Sulfonylureas evoke an insulin stimulatory response from pancreatic beta cells, a response that

Table 1. Influence of glyburide (GLYB) on labeled inositol phosphate accumulation in isolated, batch-incubated islets

Protocol (min)	Labeled inositol phosphates (cpm/40 islets)		
	IP_1	IP_2	IP_3
1. $G_{2.75}$ (40)	2351 ± 361	242 ± 47	126 ± 35
2. $G_{2.75}$ (10) → $G_{2.75}$ + GLYB, 100 nM (30)	4954 ± 349	503 ± 40	210 ± 13
3. $G_{2.75}$ (10) → $G_{2.75}$ + GLYB, 500 nM (30)	5776 ± 432	533 ± 35	252 ± 28
4. $G_{2.75}$ + Nitr (40)	1451 ± 185	213 ± 53	135 ± 9
5. $G_{2.75}$ + Nitr (10) → $G_{2.75}$ + Nitr + GLYB, 500 nM (30)	1616 ± 199	252 ± 29	102 ± 14
6. $G_{5.5}$ (40)	3996 ± 346	370 ± 31	179 ± 22
7. $G_{5.5}$ (10) → $G_{5.5}$ + GLYB, 100 nM (30)	4879 ± 148	591 ± 69	287 ± 18
8. $G_{5.5}$ (10) → $G_{5.5}$ + GLYB, 500 nM (30)	6748 ± 704	634 ± 82	338 ± 49
9. $G_{5.5}$ + Nitr (40)	3005 ± 168	283 ± 25	99 ± 21
10. $G_{5.5}$ + Nitr (10) → $G_{5.5}$ + Nitr + GLYB, 500 nM (30)	3443 ± 335	343 ± 41	108 ± 8

Groups of 22–27 islets were labeled for 2 hr in a *myo*[2-^{3}H]inositol-containing solution. After washing to remove unincorporated label, they were incubated for 10 min in 200 μL of a glucose (2.75 or 5.5 mM)-containing solution supplemented with 10 mM lithium chloride. After this, 200 μL of a glyburide-containing solution was added to achieve the concentrations indicated. Lithium was maintained at 10 mM. In the nitrendipine (Nitr) experiments, this compound at 5 μM was present during the entire 40-min incubation period. The glucose (G) level, in millimolarity, is given in subscript. Values are means of at least three experiments ± SEM.

Statistical analysis:
Line 1 vs line 2—$P < 0.05$ for all inositol phosphates.
Line 1 vs line 3—$P < 0.05$ for all inositol phosphates.
Line 1 vs line 4—$P < 0.05$ for IP_1.
Line 3 vs line 5—$P < 0.05$ for all inositol phosphates.
Line 6 vs line 7—$P < 0.05$ for all inositol phosphates.
Line 6 vs line 8—$P < 0.05$ for all inositol phosphates.
Line 8 vs line 9—$P < 0.05$ for all inositol phosphates.
Line 9 vs line 10—no significant differences.

depends to a large extent on both the glucose level bathing the islet and the influx of extracellular calcium [1–4]. While data have accumulated suggesting that this insulinotropic effect depends on their ability to influence ATP-dependent potassium channels [3, 5], the possibility that this insulin stimulatory action depends on PI hydrolysis has also been put forth [4, 13]. Indeed, it has been demonstrated that tolbutamide increases PI hydrolysis in islets and that this action, like its insulinotropic effect, is dependent on both glucose and calcium [4]. Of course, both actions of the sulfonylurea—its ability to influence ATP-regulated potassium channels and to increase PI hydrolysis—may be related cellular phenomena mediated through increases in intracellular calcium [14]. In any event, we examined the effects of the second generation sulfonylurea glyburide on PI hydrolysis in islets. Several findings were recorded and comment on them seems appropriate.

At therapeutic levels [15], glyburide induced a concentration-dependent increase in islet PI hydrolysis (Table 1). This effect, like that previously observed with the first generation sulfonylurea tolbutamide, depends on the influx of extracellular calcium. Thus, the calcium channel blocker nitrendipine effectively attenuated the ability of glyburide to increase PI hydrolysis (Table 1) and insulin release (Fig. 4) and it also attenuated sensitization (Fig. 4). These findings suggest that an increase in the intracellular calcium level, induced perhaps by the ability of glyburide to influence ATP-dependent potassium channels [3, 5], is sufficient to increase the activity of phospholipase C. This conclusion, arrived at previously by others [13, 14] as well, appears to explain the extracellular calcium dependency of sulfonylurea-induced insulin secretion. In the absence of calcium influx (achieved with nitrendipine), the effect of glyburide on potassium channels cannot be converted into an insulin secretory response. In the absence of calcium influx, there is no activation of phospholipase C, no increase in PI hydrolysis, no generation of multiple second messenger molecules [16], and no insulin release.

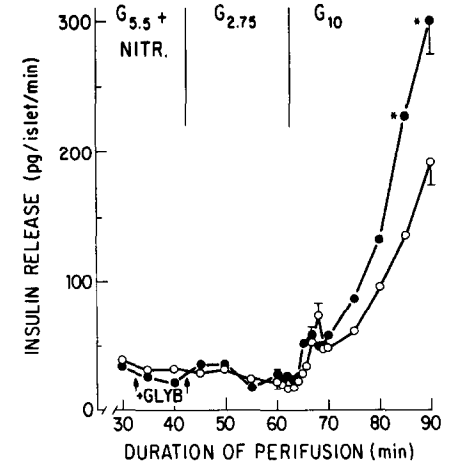

Fig. 4. Effect of nitrendipine on glyburide-induced time-dependent potentiation. Two groups of islets were perifused for 20 min with 5.5 mM glucose alone and for an additional 20 min with a 5 μM concentration of the calcium channel blocker nitrendipine (Nitr). In one group (●), 500 nM glyburide was included during the final 10 min with nitrendipine. After a 20-min period with 2.75 mM glucose, both groups were stimulated with 10 mM glucose. Four experiments were performed under each condition; values are means ± selected SEMs. The asterisks indicate significant ($P < 0.05$) differences between the two groups at the indicated time points.

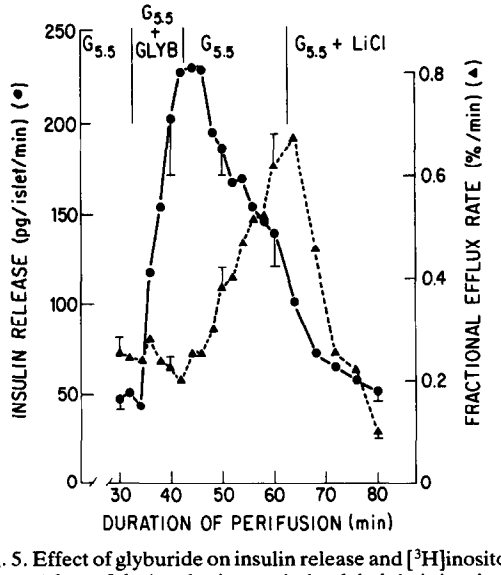

Fig. 5. Effect of glyburide on insulin release and [^{3}H]inositol efflux. After a 2-hr incubation period to label their inositol-containing phospholipids, groups of islets were perifused for a total of 80 min. After an initial 30-min period with 5.5 mM glucose alone, 500 nM glyburide was included in the medium for 10 min. The perifusion was continued for 20 min in 5.5 mM glucose alone and for an additional 20 min with 5.5 mM glucose plus 10 mM lithium chloride (LiCl). Insulin release rates (●) and fractional efflux rates of [^{3}H]inositol (▲) were monitored. Four experiments were performed; values are means ± selected SEMs.

One other aspect of glyburide action was also probed in the present work. We previously documented that several structurally distinct molecules possess the ability to sensitize islets to various agonists. Termed time-dependent potentiation (TDP [10]) or memory, the common denominator in this phenomenon appears to be the capacity of these memory-inducing compounds to increase PI hydrolysis in islets. Furthermore, this stimulatory effect on these strategically-located phospholipids appears to persist despite agonist removal. A sustained supply of PI-derived second messenger molecules, particularly perhaps diacylglycerol (DG), may serve a crucial messenger function here. For example, DG may serve to keep the enzyme protein kinase C in its membrane-associated, calcium-sensitive form [17]. Since the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an established activator of protein kinase C, also induces memory in islets [18, 19], it is difficult to dismiss the idea that protein kinase C occupies a pivotal position in both the induction and expression of TDP.

Herein we demonstrate that glyburide increases PI hydrolysis in prelabeled islets and, furthermore, this exposure sensitizes islets to other stimulants as well. These findings are certainly compatible with the idea expressed above indicating a primary role for protein kinase C in glyburide-induced sensitization. The persistent increase in PI hydrolysis, monitored by a sustained effect on inositol phosphate accumulation, would be accompanied by the provision of diacylglycerol, a compound thought to be generated stoichiometrically with the inositol phosphates. A persistent insulin stimulatory effect of glyburide, a result perhaps of its sustained effect on PI hydrolysis, has been noted previously [20]. The data in Figs. 1 and 4 support the idea that the glucose level is a key determinant of this persistent insulin stimulatory effect. If the glucose level was lowered to 2.75 mM, insulin release subsided quickly after glyburide removal (Fig. 1). However, if the level of the glucose was maintained at 5.5 mM, the decline in release was less precipitous (Fig. 5). It appeared that glyburide had sensitized islets to 5.5 mM glucose as well.

Before concluding, a few comments concerning our approach to monitoring PI hydrolysis in islets seem appropriate. In the present report, two distinct but complementary methods were used to assess changing rates of PI hydrolysis—the efflux of [^{3}H]inositol and the cellular accumulation of [^{3}H]inositol phosphates from [^{3}H]inositol-prelabeled islets. The data presented in Fig. 5 demonstrate that glyburide-induced increases in [^{3}H]inositol efflux lagged significantly behind glyburide-induced increases in insulin secretion. This does not imply that increases in PI hydrolysis display a similar latency and thus may not contribute to the insulin secretory response. The delay in [^{3}H]inositol efflux most likely represents the interaction between various metabolic pathways—the dephosphorylation of labeled inositol phosphates and the reincorporation of [^{3}H]inositol back into islet phosphoinositides. Only when the pool of [^{3}H]inositol exceeds the PI biosynthetic capacity of the beta cell will appreciable amounts of [^{3}H]inositol efflux from the cell. Our

Table 2. Influence of glyburide (GLYB) on labeled inositol phosphate levels in perifused islets

Perifusion protocol (min)	Labeled inositol phosphates (cpm/40 islets)		
	IP_1	IP_2	IP_3
1. $G_{5.5}$ (50)	516 ± 36	119 ± 17	92 ± 12
2. $G_{5.5} \rightarrow G_{5.5}$ + GLYB (30) (10)	682 ± 31	168 ± 14	129 ± 10
3. $G_{5.5} \rightarrow G_{5.5}$ + GLYB (30) (20)	803 ± 62	369 ± 21	148 ± 14
4. $G_{5.5} \rightarrow G_{5.5}$ + GLYB $\rightarrow G_{5.5}$ (30) (10) (20)	1061 ± 140	366 ± 23	106 ± 16
5. $G_{5.5} \rightarrow G_{5.5}$ + $LiCL_{10}$ (60) (20)	675 ± 63	172 ± 26	95 ± 18
6. $G_{5.5} \rightarrow G_{5.5}$ + GLYB $\rightarrow G_{5.5} \rightarrow G_{5.5}$ + $LiCL_{10}$ (30) (10) (20) (20)	3111 ± 341	480 ± 63	245 ± 42

Batches of 27–35 islets were labeled for 2 hr with [^{3}H]inositol as discussed in Methods. After washing, they were perifused using the protocols indicated. The glyburide level used was 500 nM. At least four experiments were performed under each experimental setting. Values are means ± SEM. Lithium chloride (10 mM) was used only in lines 5 and 6.

Statistical analysis:
Line 1 vs 2—$P < 0.05$ for all inositol phosphates.
Line 1 vs 3—$P < 0.05$ for all inositol phosphates.
Line 1 vs 4—$P < 0.05$ for IP_1 and IP_2.
Line 5 vs 6—$P < 0.05$ for all inositol phosphates.

previous studies with several islet stimulants which activate PI hydrolysis in islets are compatible with this interpretation [8, 21]. A similar latency in [^{3}H]inositol efflux has also been noted with glucose [21–23], even though labeled inositol phosphates accumulate rapidly in response to glucose stimulation [21]. Finally, significant increases in labeled inositol phosphates were observed at the earliest time points measured, suggesting that increases in PI hydrolysis occurred with sufficient rapidity to contribute to insulin secretion.

In summary, the findings reported indicate that glyburide-induced increases in PI hydrolysis participate not only in its acute insulin stimulatory effect but also in its ability to induce memory. Whether the maintenance of this increase in beta cell sensitivity to stimulation depends on protein kinase C activation and/or translocation to the cell membrane is an important clinical issue and one that deserves critical scrutiny. Finally, since failure to respond to their hypoglycemic action is a major therapeutic problem, studies designed to assess the involvement of diacylglycerol and protein kinase C in islets refractory to the sulfonylureas appear warranted. These issues require further experimental analysis.

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