The methyl ester of pyruvic acid (methyl pyruvate) stimulated a dose-dependent increase in insulin secretion from isolated perfused rat islets. The threshold level for release was about 10 mM, and at 20 mM the addition of MP to perfused islets resulted in a large first phase of secretion followed by an insulin-secretory response that was sustained for at least 40 min. When compared to the effects of 20 mM glucose, peak first-phase release rates in response to 20 mM methyl pyruvate were comparable, but the second phase of release was only about 10–15% of that observed with an equimolar level of the hexose. The stimulatory effects of 20 mM methyl pyruvate on secretion were abolished by the K⁺-ATP channel blocker diazoxide (200 μM) and by the calcium channel antagonist nitrrendipine (500 nM). The glucokinase inhibitor mannose (20 mM) had no adverse effect on the secretory response to 20 mM methyl pyruvate, whereas 10 μM forskolin amplified the insulinotropic action of MP. Sodium pyruvate alone or in combination with 10 μM forskolin had no insulinotropic effect. In additional experiments islet phosphoinositide pools were labeled with myo-2-[3H]inositol, and the subsequent accumulation of labeled inositol phosphates was used to monitor the activation of phospholipase C. Methyl pyruvate stimulated a dose-dependent increase in inositol phosphate levels when measured after a 30-min incubation period with a maximal increase of about 300% at 20 mM methyl pyruvate. The increase in phosphoinositide hydrolysis caused by methyl pyruvate (20 mM) was, like insulin secretion, reduced by both diazoxide and nitrrendipine but was immune to inhibition by mannheptulose. Pyruvate (20 mM) had no effect on inositol phosphate accumulation. Prior short-term exposure to methyl pyruvate sensitized islets to subsequent stimulation with 15 mM glucose. Sodium pyruvate did not sensitize islets. These findings support the concept that the mitochondrial metabolism of nutrient molecules is an event sufficient to acutely augment insulin release from the beta cell, to increase phospholipase C-mediated phosphoinositide hydrolysis, and to induce time-dependent potentiation of insulin secretion.

The regulation of fuel-induced insulin secretion from pancreatic beta cells depends upon the intermediary metabolism of these compounds via several established metabolic pathways (1). In addition to glucose, mannose, glyceraldehyde, and dihydroxyacetone, which are metabolized initially by cytosolic glycolytic enzymes (2–5), a variety of insulinotropic nutrient molecules are metabolized solely within the mitochondria. These include among others leucine, monomethylsuccinate, and α-ketoisocaproate (6–10). Because these latter stimulants for insulin secretion share in common many of the same stimulatory features of glucose on the beta cell, the concept that mitochondrial-derived signals mediate, at least in part, the stimulatory actions of the hexose has been proposed (11, 12). For example, both glucose and α-ketoisocaproate stimulate similar changes in insulin secretion and the calcium-dependent hydrolysis of islet phosphoinositide (PI) pools and sensitize the beta cells to subsequent restimulation, a phenomenon also referred to as priming or time-dependent potentiation (TDP) (13–15).

Standing at odds with this unifying concept, implicating the importance of mitochondrial signals in the regulation of secretion regardless of the nutrient molecule used, are studies with pyruvate. Although it seems to be well metabolized, pyruvate alone has no insulinotropic effect, although in combination with stimulatory glucose, a small stimulatory action has been described (16). Most recently, the methylated derivative of pyruvate has been reported to augment the release of insulin from the beta cells of cultured mouse islets (17). Thus, methylation seems to confer upon pyruvate stimulatory actions not inherent in the unaltered pyruvate molecule. This situation is similar to the effects of methylation on the succinate molecule, which alone has no insulinotropic effect but when methylated now exhibits many of the same stimulatory characteristics as glucose (7, 10, 18). Presumably methylation renders succinate, and perhaps pyruvate as well, more membrane-permeable and allows higher levels of the compound to be achieved and acted upon by mitochondrial enzymes. In the present series of studies we explored in more detail the effects of methyl pyruvate (MP) on the beta cell. The dose dependence and calcium dependence of the insulinotropic effects of MP, the kinetics of insulin secretion from freshly isolated and perfused rat islets, as well as the ability of MP to influence PI hydrolysis in [3H]inositol-prelabeled islets were determined. The findings support the view that mitochondrial metabolism of methyl pyruvate is an event sufficient to generate the necessary metabolic and second messenger molecules to activate the beta cell insulin-secretory apparatus in a manner comparable in some regards to that seen with glucose. From a quantitative perspective, however, the maximal effects of MP on second-phase release from rat islets are significantly less than those observed with equimolar glucose.

The abbreviations used are: PI, phosphoinositide; MP, methyl pyruvate; KRB, Krebs-Ringer bicarbonate; PLC, phospholipase C; IP, inositol phosphate; TDP, time-dependent potentiation.

This paper is available online at http://www-jbc.stanford.edu/jbc/
**MATERIALS AND METHODS**

The detailed methodologies used to assess insulin output from collagenase-isolated islets have been described previously (19, 20). Male Sprague-Dawley rats were used in all studies. All animals were treated in a manner that complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The animals were fed *ad libitum* and weighed 300–450 g. After Nembutal (pentobarbital sodium) anesthesia, Abbott-induced islets were isolated by collagenase digestion and handpicked using a glass loop pipette under a stereo microscope. They were free of exocrine contamination.

After isolation some groups of islets were directly perfused in a Krebs-Ringer bicarbonate (KRB) buffer at a flow rate of 1 ml/min. Perifusate solutions were gassed with 95% O2, 5% CO2 and maintained at 37 °C. Perifusate samples were collected at appropriate times and analyzed for insulin content. To label PI pools, other groups of 18–22 freshly isolated islets were placed in small glass vials and incubated for 3 h in a *myo-[2-3H]inositol-containing KRB* solution made up as follows. 10 µCi of *myo-[2-3H]inositol* (specific activity, 16–23 Ci/mmol) were placed in a 10 mm × 75-mm culture tube. To this aliquot of label 250 µl of warmed (to 37 °C) and oxygenated KRB medium supplemented with 5 mM glucose were added. After mixing, 240 µl of this were gently added to the vial with islets. The vial was capped with a rubber stopper, gassed for 10 s with 95% O2, 5% CO2, and incubated at 37 °C. After 90 min the vials were again gently oxygenated. After the labeling period, the islets were washed with 5 ml of fresh KRB.

**Inositol Phosphate (IP) Measurements**—After washing, the islets on nylon filters were placed in small glass vials. Added gently to the vial were 400 µl of KRB supplemented with 10 mM LiCl to prevent IP degradation and the appropriate agonists as indicated. The vials were capped and gently gassed for 5 s with 95% O2, 5% CO2. After 30 min the generation of IPs was stopped by adding 400 µl of 20% perchloric acid. Total IPs formed were then measured using Dowex columns as described previously (21, 22).

**Reagents**—Hanks’ solution was used for the islet isolation. The perfusion medium consisted of 115 mM NaCl, 5 mM KCl, 2.2 mM CaCl2, 1 mM MgCl2, 24 mM NaHCO3, and 0.17 g/dl bovine serum albumin. Other compounds were added as indicated, and the solution was gassed with a mixture of 95% O2, 5% CO2. The [2-3H]labeled insulin used for the insulin assay was purchased from DuPont NEN. The *myo-[2-3H]inositol* was purchased from Amersham Corp. Bovine serum albumin (*radioimmunoassay grade*), glucose, mannose*trolulose, dioxazine, the sodium salt of pyruvate*, and pyruvic acid methyl ester as well as the salts used to make the Hanks’ solution and perfusion medium were purchased from Sigma. Forskolin was purchased from Calbiochem. Nitrendipine was a gift from Dr. A. Scriabine (Miles Institute for Preclinical Pharmacology, West Haven, CT). Rat insulin standard (lot 615-15-157) was the generous gift of Dr. Gerald Gold (Lilly). Collagenase (type I) was obtained from Boehringer Mannheim Biochemicals.

**Statististics**—Statistical significance was determined using Student’s *t*-test for unpaired data or analysis of variance in conjunction with the Newman-Keuls test for unpaired data. A *p* < 0.05 was taken as significant. Values presented in the figures and under "Results" represent the mean ± S.E. of at least three observations.

**RESULTS**

**Dose-Response Studies with Methyl Pyruvate**—In the initial series of experiments the dose dependence and the kinetics of the insulin-secretory responses to MP were determined. In these studies islets were perfused for 30 min in the absence of any added metabolic fuel and were perfused for an additional 40 min with varying concentrations of MP. As shown in Fig. 1, left, 5 mM MP had no insulinotropic effect, whereas 10 mM MP stimulated a pronounced first phase of release but no sustained insulin-secretory response. At a concentration of 20 mM MP, MP evoked a large first phase of insulin secretion and a sustained second-phase response (Fig. 1, right). A higher level (40 mM) of MP reduced the sustained release of the hormone when compared with 20 mM MP (Fig. 1, right).

**Comparison of the Insulinotropic Effects of MP with Glucose and the Sodium Salt of Pyruvate**—When compared to islets stimulated with 20 mM glucose (653 ± 198 pg/islet/min), peak first-phase insulin-secretory responses to 20 mM MP (728 ± 241 pg/islet/min) were comparable, but the second-phase insulin-secretory responses to 20 mM glucose measured 35–40 min after the onset of stimulation (1592 ± 92 pg/islet/min) were about 6 times greater than those observed with 20 mM MP (253 ± 71 pg/islet/min; see Fig. 2). At a level of 20 mM and in agreement with previous results obtained with freshly studied rat islets (16) or cultured mouse islets (17), the sodium salt of pyruvate at a level of 20 mM has no insulinotropic action (Fig. 2).

**Modulation of the Insulinotropic Action of MP**—Mannose*trolulose (20 mM)*, which blocks glucokinase and abolishes glucose-induced insulin secretion (4, 23, 24), had no inhibitory effect on insulin release in response to 20 mM MP (Fig. 3, middle panel). Similar to its potentiating effect on glucose-induced insulin secretion (25), the insulinotropic effects of MP (20 mM) on the beta cell were amplified by the addition of 10 µM forskolin (Fig. 3, lower panel). Peak first-phase secretion to 20 mM MP in the additional presence of forskolin averaged 1048 ± 186 pg/islet/min, whereas release rates measured 35–40 min after the onset of stimulation increased to 436 ± 91 pg/islet/min. The combination of 10 µM forskolin + 20 mM of the sodium salt of pyruvate exerted no insulinotropic effect on the beta cell (results not shown).

The stimulatory effect of 20 mM MP on insulin secretion was blocked by 500 nM of the calcium channel antagonist nitrendipine or by dioxazine (200 µM), which prevents closure of the ATP-dependent potassium channel (26). In the presence of nitrendipine or dioxazine, which were present during the entire 70-min perfusion period, peak first-phase secretion was reduced from 728 ± 241 pg/islet/min (control response in the absence of inhibitor) to 93 ± 17 pg/islet/min (in the presence of nitrendipine) or 107 ± 28 pg/islet/min (in the presence of dioxazine). When compared with sustained release measured 35–40 min after the onset of stimulation with MP alone (253 ±
71 pg/islet/min), release rates measured in the additional presence of nitrendipine (17 ± 7 pg/islet/min) or diazoxide (17 ± 4 pg/islet/min) were also decreased.

Effects of MP on Phospholipase C (PLC)-mediated \[^{3}H\]Inositol Phosphate Accumulation—In the next series of experiments we examined the effects of MP on the PLC-mediated hydrolysis of islet phosphoinositide (PI) pools. In the absence of any added agonist, basal IP levels averaged approximately 2700 cpm/40 islets/30 min (Table I, line 1). Basal levels were not significantly influenced by the addition of either nitrendipine or diazoxide (Table I, lines 2 and 3). As shown in Table I, the addition of MP to \[^{3}H\]inositol-prelabeled islets resulted in a significant increase in labeled IP accumulation, a sensitive index of PLC activation (27–30). This response was dose-dependent, with the maximal effect observed at 20 mM (Table I). Significant effects on IP accumulation were evident with low levels (2.5–5.0 mM) of MP (Table I, lines 4 and 5) devoid of any sustained insulinotropic action (see Fig. 1). Like insulin secretion, 20 mM MP-induced IP accumulation was inhabitable by diazoxide or nitrendipine. However, whereas insulin release was abolished by either inhibitor, IP accumulation, although reduced, was still significantly elevated above control levels (Table I, compare lines 2 and 3 with lines 7 and 8). Mannoheptulose (20 mM) had no deleterious effect on the capacity of 20 mM MP to increase IP accumulation (Table I, line 9). The maximal stimulatory effect of 20 mM MP on PLC-mediated PI hydrolysis was significantly less than that seen with 20 mM glucose (Table I, compare lines 6 and 11). Consistent with its the lack of any insulinotropic action, 20 mM of the sodium salt of pyruvate had no effect on IP accumulation under these conditions (Table I, line 12).

We considered the possibility that the stimulatory effects of MP on IP accumulation were solely dependent on the capacity of MP to increase Ca\(^{2+}\) influx into the beta cell. To address this issue additional studies were conducted with depolarizing levels (30 mM) of potassium and the calcium antagonist nitrendipine (Table I, lines 13 and 14). From a quantitative perspective the magnitude of the effect of 20 mM MP on IP accumulation could not be duplicated by potassium-induced beta cell depolarization (Table I, compare lines 6 and 13). Most importantly, nitrendipine completely abolished potassium-induced IP accumulation but only partially reduced the impact of 30 mM potassium (Table I, compare lines 7 and 14).

**FIG. 2. Comparison of the insulinotropic actions of 20 mM methyl pyruvate, 20 mM glucose, and 20 mM sodium pyruvate.** Groups of 14–18 freshly isolated islets were perfused for 30 min in the absence of any exogenous fuel and perfused for an additional 40 min (indicated by the vertical line in the figure) with 20 mM methyl pyruvate (○), 20 mM glucose (●), or 20 mM of the sodium salt of pyruvate (▲). At least three experiments were performed under each condition.

MP Induces Time-dependent Potentiation of Insulin Release—Many structurally diverse molecules sensitize the beta cell to subsequent restimulation. A common characteristic that links these agonists is their ability to increase PLC-mediated PI hydrolysis or to activate directly the enzyme protein kinase C. Because MP increases the activation of PLC, in the final set of experiments we determined whether MP induces time-dependent potentiation of insulin release. Similar to the effects of many compounds that increase PI hydrolysis in the beta cell, prior exposure to and subsequent removal of 20 mM MP primed or sensitized the islet to subsequent 15 mM glucose stimulation. As shown in Fig. 4, stimulation of control islets with 15 mM glucose after 60 min in 3 mM glucose resulted in a peak first-phase insulin response of 169 ± 36 pg/islet/min and a dramatic rising second phase of secretion. From a quantitative perspective, when compared with prestimulatory secretion rates of 28 ± 4 pg/islet/min, release rates measured 25–30 min after the onset of 15 mM glucose stimulation increased about 35-fold to 1102 ± 103 pg/islet/min. Prior exposure to 20 mM MP significantly amplified the first phase of release. For example, peak first-phase release averaged 169 ± 36 pg/islet/min from naive islets, but this response increased to 734 ± 188 pg/islet/min from MP-pretreated islets. Second-phase release rates measured 25–30 min after the onset of 15 mM glucose stimulation were comparable in naive and MP-primed islets. Consistent with the inability of the sodium salt of pyruvate to activate PLC and with the concept that PLC activation plays an important role in the induction of time-dependent potentiation, the sodium salt of pyruvate did not induce TDP. Peak first-phase
Regulation of Insulin Secretion by MP

TABLE I
Effects of various agonists on IP accumulation in isolated islets

| Stimulation condition | IP accumulation (cpm/40 islets/30 min) |
|-----------------------|-----------------------------------------|
| 1. No added agonist    | 2,694 ± 331                             |
| 2. No added agonist + nitrendipine | 2,241 ± 240                          |
| 3. No added agonist + diazoxide  | 2,728 ± 582                             |
| 4. 5 mM MP             | 5,194 ± 618                             |
| 5. 10 mM MP            | 6,392 ± 267                             |
| 6. 20 mM MP            | 8,255 ± 842                             |
| 7. 20 mM MP + nitrendipine | 5,295 ± 787                          |
| 8. 20 mM MP + diazoxide | 5,102 ± 494                             |
| 9. 20 mM MP + MH       | 10,064 ± 2,388                          |
| 10. 40 mM MP           | 6,319 ± 598                             |
| 11. 20 mM glucose      | 14,550 ± 1,783                          |
| 12. 20 mM pyruvate (sodium salt) | 2,276 ± 170                          |
| 13. 30 mM KCl         | 5,614 ± 877                             |
| 14. 30 mM KCl + nitrendipine | 2,412 ± 269                          |

Prior short-term exposure to 20 mM MP induces time-dependent potentiation of insulin release. Two groups of islets were studied. One group (●) was perifused for 60 min with 3 mM glucose followed by a 30-min stimulatory period with 15 mM glucose. The second group of islets (○) was perifused for 30 min with 3 mM glucose followed by 15 min with 3 mM glucose + 20 mM MP. After a 15-min washout in the presence of 3 mM glucose alone, these islets were restimulated with 15 mM glucose, and this is the period shown here. At least five experiments were conducted under each condition.

FIG. 4. Prior short-term exposure of perifused islets to 20 mM MP induces time-dependent potentiation of insulin release. Two groups of islets were studied. One group (●) was perifused for 60 min with 3 mM glucose followed by a 30-min stimulatory period with 15 mM glucose. The second group of islets (○) was perifused for 30 min with 3 mM glucose followed by 15 min with 3 mM glucose + 20 mM MP. After a 15-min washout in the presence of 3 mM glucose alone, these islets were restimulated with 15 mM glucose, and this is the period shown here. At least five experiments were conducted under each condition.

DISCUSSION

Nutrient-stimulated insulin secretion depends on the metabolic transformation of fuel and the generation of appropriate intracellular signals. Recent studies have supported an important role for the ATP level or the ADP/ATP ratio as an indispensable component of the signaling apparatus (31). In this view, increasing levels of ATP, a result of substrate interconversion, activate proteinkinase C (33–35). Membrane depolarization results in the subsequent opening of voltage-regulated Ca\(^{2+}\) channels, influx of the divalent cation, activation of PLC, and insulin secretion. The susceptibility of the insulinotropic actions of MP to diazoxide and nitrendipine and the immunity of MP to mannoheptulose are consistent with this proposed mechanism of action. At a higher level (40 mM), MP addition reduced the secretory response of the islet. The basis for this inhibitory effect was not explored further, but the results suggest caution when exposing islets to high levels of MP.

Similar to the actions of other nutrient molecules, MP activated beta cell PLC in a dose-dependent manner. The stimulatory effect of MP on IP accumulation was, like insulin release, immune to inhibition by mannoheptulose. Inositol phosphate accumulation in response to MP was reduced but not abolished by either diazoxide or nitrendipine. From a quantitative perspective the stimulatory effect of 20 mM MP on PLC activation was greater than that observed with depolarizing levels of potassium, an agonist whose stimulatory actions are dependent on calcium influx via voltage-regulated Ca\(^{2+}\) channels (26). These results suggest that more than just Ca\(^{2+}\) influx is involved in the activation of PLC by nutrient agonists. Moreover, nitrendipine totally abolished the stimulatory action of potassium on IP accumulation, whereas it only partially reduced the effect of MP or glucose (36). These results lead to the conclusion that both calcium and a metabolic signal participate in the regulation of nutrient-activated, PLC-mediated PI hydrolysis and that these agonists (glucose and MP) but not high potassium alone are capable of generating both of these stimulatory signals.

We also performed a series of limited studies with the sodium salt of pyruvate. In agreement with previous reports (16, 17) it was without any insulinotropic effect on the beta cell even at a level of 20 mM. Inclusion of forskolin together with pyruvate, which theoretically at least should amplify even a weak pyruvate-generated stimulatory signal for secretion, was also without any positive effect. Furthermore, pyruvate had no effect on IP accumulation in islets.

Prior short-term exposure to 20 mM MP enhanced the sensitivity of the islet to glucose stimulation. This amplified response, referred to TDP or priming (37), is reproduced by a large number of compounds that share in common the ability to...
activate one of the several isozymes of PLC identified in islets (13, 15, 19, 38). Thus, MP shares this property with such diverse agonists as carbachol, acetycholine, cholecystokinin, leucine, monomethylsuccinate, glucose, α-ketosocoprate, and tolbutamide. As a working hypothesis we have proposed that the induction of TDP is a result of an increase in information flow in the PLC/protein kinase C signaling cascade (29, 30, 39). Because TDP is also produced by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (40, 41), a unifying concept incorporates the activation of this enzyme and/or processes distal to it in its generation and maintenance. Not surprisingly, sodium pyruvate was impotent with regard to the induction of time-dependent potentiation of insulin release.

From a quantitative perspective, these results differ from a previous study examining the insulinotropic effects of MP on the islet. For example, Mertz et al. (17) reported that 20 mM MP was a 6-fold greater stimulus for insulin secretion than was 12 mM glucose. With rat islets, although the first-phase secretory response to 20 mM MP was comparable to 20 mM glucose, sustained second-phase release rates to MP were only 10–20% of those seen with 20 mM glucose. Several obvious points of departure exist between this and the previous report, however. We used freshly isolated rat islets, and insulin secretion was monitored during a dynamic perfusion. The report by Mertz et al. (17) used cultured mouse islets, and release was measured during static incubations. It is not known which of these or other methodological differences contribute to the quantitative disparity between the actions of MP and glucose on the beta cell. However, it is becoming increasingly apparent that the insulin-secretory responses and biochemical findings made with one rodent species may not necessarily be extrapolated to other rodent species or human beta cells (22, 42–47).

The precise nature of the metabolic signal or signals generated by MP that seem to interact with Ca²⁺ in the regulation of beta cell secretion and PLC activation is not known. For heuristic purposes at least, a common metabolic signal generated by all nutrient secretagogues seems to be a reasonable possibility. Based on our studies with nutrients metabolized exclusively within the mitochondria, we initially proposed that the signal was generated within this organelle. However, if the mitochondrial metabolism of glucose-derived pyruvate is exclusively responsible for the generation of this metabolic signal, then the results with sodium pyruvate remain problematic and difficult to interpret. This has to do with the inability of exogenously added pyruvate to activate either PLC or insulin secretion under conditions in which any small insulinotropic action of this compound should be manifest. A more global view might incorporate the concept that the metabolic signal may be generated by either cytosolic or mitochondrial pathways. For example, a recent report consistent with many other studies has suggested that ATP of either glycolytic or mitochondrial origin may be, along with Ca²⁺, the common signal involved in nutrient activation of the beta cell (31). In addition to regulating membrane polarization, ATP may serve as an important signaling molecule in other transduction systems as well, and the regulation of information flow in the PLC pathway may be one of them. This action of ATP may be exerted at multiple sites in the PLC/protein kinase C signaling cascade, including, but not confined to, the effect of ATP as a cofactor in protein kinase C phosphorylation events, the production of the variably phosphorylated phosphoinositides, or the transfer of phospholipids to the cell membrane (48). Other ATP-dependent steps have to be considered as well.

In conclusion, the findings presented demonstrate that the methylated and presumably more mitochondrial-permeable derivative of pyruvate stimulates the calcium-dependent activation of islet PLC and insulin release. Whereas the stimulatory actions of 20 mM MP on first-phase insulin secretion were comparable to 20 mM glucose, the effects of MP on sustained secretion were significantly less. MP induces TDP of release, a characteristic it shares in common with a variety of structurally distinct molecules linked by their ability to activate PLC directly or the enzyme protein kinase C (13, 15, 18, 19, 37, 38, 40, 41, 49). The judicious use of MP may prove to be a valuable tool in defining the nature of the biochemical factors and transduction pathways that interact so elegantly to regulate insulin secretion from the pancreatic beta cell.