MOLECULAR PHARMACOLOGY, 15, 287-293

2,4-Diamino-5-cyano-6-halopyridines and Analogues. A New Family of Insulin Secretogogues That Resemble Glucose in Hydrogen Bonding Possibilities

DAVID G. JOHNSON AND CHRISTOPH DE HAËN

Division of Clinical Pharmacology and Division of Metabolism, Endocrinology and Gerontology, Department of Medicine, University of Washington, Seattle, Washington 98195

(Received April 10, 1978)
(Accepted October 5, 1978)

SUMMARY

DAVID G. JOHNSON & DE HAËN, CHRISTOPH. (1979) 2,4-Diamino-5-cyano-6-halopyridines and analogues. A new family of insulin secretogogues that resemble glucose in hydrogen bonding possibilities. *Mol. Pharmacol.* **15**, 287-293.

Rat pancreas was perfused *in situ* with medium containing 300 mg/dl glucose and 2,4-diamino-5-cyano-6-bromopyridine (Compound I) or several closely related analogues. Addition of 0.1 mM Compound I caused a 3 to 4-fold increase in insulin release. At 1.0 mM concentration Compound I increased insulin release 7 to 20-fold greater than that caused by glucose alone. The augmented release of insulin was biphasic, with a brief initial spike followed by a secondary rise that lasted at least 60 min. Similar results were obtained with 2,4-diamino-5-cyano-6-iodopyridine and 2,4-diamino-3,5-dicyano-6-bromopyridine. Twenty minutes after giving Compound I by gavage to anesthetized rats an intravenous bolus of glucose (0.625 g/kg) was given. During the 60 min interval following glucose, drug-treated animals had almost 3 times higher serum insulin concentrations compared to control animals. The glucose disappearance curves were similar in both groups. These results indicate that Compound I and its analogues are potent insulin secretogogues *in vivo* and *in vitro*. The similarity of the hydrogen bonding possibilities of these compounds to glucose suggests that their ability to release insulin may be due to interaction with a glucoreceptor in the pancreatic β-cell.

INTRODUCTION

In most mammals glucose is the primary physiologic regulator of pancreatic insulin secretion. With initial glucose concentrations below 115 mg/dl, sudden increases in glucose concentrations result in a biphasic insulin secretory response, i.e., a first phase insulin spike followed by a slower increase of insulin output until a new steady state is reached. A variety of hormones, such as glucagon (1), gastrin (2, 3), pancreozymin (4), secretin (2, 5), gastric inhibitory polypeptide (6), and caerulein (7) also stimulate insulin release. In most cases elevated glucose concentrations are required for these hormones to be effective. Two models have been suggested to explain how glucose regulates insulin secretion (8). Either glucose needs to be metabolized, and a metabolite triggers insulin secretion, or glucose binds to a receptor and induces an altered function without necessarily being metabolized (for review see [9]). These two models are not mutually exclusive.

To investigate the nature of the secretory stimulus produced by glucose we have studied the effects on insulin release of a series

This work was supported by USPHS Grants AM-02456, AM-17698, and through the Diabetes Center (AM-17047).

0026-895X/79/020287-07$02.00/0

of compounds that were designed to mimic glucose in the configuration of hydrogen bonding possibilities, but which could not share a metabolic pathway with glucose. Hershfield and Richards (10) described glucose transport inhibitory properties of a series of substituted pyridines and attributed the properties of these compounds to similarities in hydrogen bonding possibilities with glucose. This paper reports the insulinotropic action of one of these substituted pyridines, 2,4-diamino-5-cyano-6-bromopyridine, as well as other analogues.

MATERIALS AND METHODS

Materials. 2,4-Diamino-5-cyano-6-bromopyridine (Compound I) was synthesized according to Carboni *et al.* (11). The procedure was slightly modified and also used to synthesize 2,4-diamino-5-cyano-6-iodopyridine (Compound II). 2,4-Diamino-3,5-dicyano-6-bromopyridine (Compound III) and 2-amino-3,5-dicyano-4,6-dibromopyridine (Compound IV) were synthesized by the method of Little *et al.* (12), and samples of these compounds were the generous gifts of Dr. V. A. Engelhardt, E. I. du Pont de Nemours and Co. Pentachloropyridine (Compound V) was obtained from Aldrich Chemical Comp., Milwaukee, Wisc. Other drugs used were 1-butyl-3-(p-tolylsulfonyl) urea (tolbutamide) from Upjohn, Kalamazoo, Michigan and sodium pentobarbital from Med. Tech. Inc., Elkwood, Kansas. Male Wistar rats were purchased from Simonsen Laboratories, Gilroy, California, and fed *ad libitum* with Purina rat chow.

In situ pancreas perfusion. The method was that of Penhos *et al.* (13) as modified by Johnson *et al.* (14). Drugs were dissolved in perfusion medium in 1 mM concentration by stirring at 40° for ½ hour.

In vivo insulinotropic effects. Crystalline drug (10 mg) was pressed into the end of 10 cm of teflon tubing (i.d. 0.042 mm, o.d. 0.062 mm, #18, Bolab Inc., Derry, N. H.) and plugged with 1 mm of 1% agar.

Weight-matched pairs of rats (400 to 700 g) were anesthetized with intraperitoneal pentobarbital (50 mg/kg). To facilitate obtaining blood samples the rats were kept warm with an Air curtain incubator (Sage-Orion, Model 279, Cambridge, Mass.) at 35°. Drug was given by gavage using the teflon tubing and a syringe containing 0.2 ml of water. The control animal received water only. The first blood sample (0.22 ml) was taken from the amputated tail immediately before drug administration. A 100 µl aliquot of each blood sample was injected into 1 ml of 3% trichloroacetic acid for subsequent glucose assay. The remainder was put into a 0.4 ml microfuge tube, centrifuged in a Microfuge B (Beckman, Palo Alto, Calif.) for 30 sec, and the serum separated and kept frozen until assayed for insulin. An incision was made in the skin overlapping the left femoral vein. Twenty minutes after giving the drug, a bolus of glucose (0.625 g/kg as a 12.5% solution) was injected through a 25 gauge needle over a 30 sec interval. Blood specimens were taken from the tail at 0, 5, 10, 20, 30 and 60 min.

Radioimmunoassays. Insulin was studied by radioimmunoassay, using cellulose to adsorb the free insulin (15). Glucagon was studied by radioimmunoassay according to Rocha *et al.* (16), using antiserum 30K. Gastric inhibitory polypeptide assays were courtesy of Dr. Robert H. Williams and were performed using antiserum (Gö AIS No. 5, 7/5/75) provided by Drs. John C. Brown and W. Creutzfeldt.

Glucose assay. The aldosaccharide assay of Hultman (17) was used.

RESULTS

Perfused pancreas studies. Rat pancreas preparations were perfused with 0.1 mM and 1 mM of Compound I for periods of 10 min, separated by 10 min of perfusion medium alone, in medium containing either 100 or 300 mg/dl glucose. At 100 mg/dl glucose essentially no effects of the drug on insulin release were observed. At 300 mg/dl glucose, 0.1 mM Compound I caused a 3 to 4-fold stimulation of insulin release over that caused by glucose alone, and 1 mM Compound I resulted in 7 to 20-fold stimulation (Fig. 1A). Removal of the compound resulted in a rapid return of insulin release to basal levels. At 1 mM concentrations of Compound I the response was biphasic, with a transient peak of insulin release followed by a secondary rise in insulin concentration. The biphasic pattern of the insulin

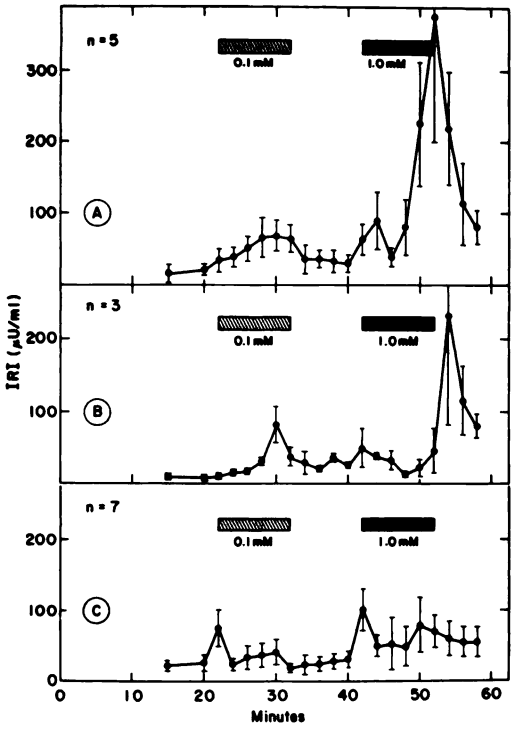

FIG. 1. *Insulin release by the perfused rat pancreas in the presence of 300 mg/dl glucose as stimulated by Compound I (A); Compound II (B), and tolbutamide (C)*

The drug concentrations are given below the bars which indicate the periods of drug perfusion. The results represent the mean ± standard error of the mean of n experiments.

secretory response resembled the well known biphasic release of insulin in response to glucose. Only a slight biphasic response was discernable at 0.1 mM drug. Perfusion pressure remained constant at approximately 80 mm Hg throughout the experiment, indicating that there was no significant effect of the compounds on the vasculature of the pancreas. No effect on glucagon release was observed at 300 mg/dl glucose. In one animal an attempt was made to measure gastric inhibitory peptide release. All levels were below the detection limit of 125 pg/ml, although the assay was capable of detecting the hormone in rat plasma. Compound II had a similar effect on insulin release as Compound I, although the first phase was blunted (Fig. 1B). For comparison, perfusion was performed with tolbutamide (Fig. 1C) at 300 mg/dl glucose, using concentrations equimolar with those of Compounds I and II. Tolbutamide appeared to affect primarily first phase insulin release, and was less potent than Compound I at 300 mg/dl glucose concentration.

To investigate structural requirements for the insulinotropic pyridines and to test whether the same structure-function relationships applied as those for the glucose transport inhibitory function described by Hershfield and Richards (10), a number of other analogues were tested in the same system (Table 1). The observations are consistent with the hypothesis of Hershfield and Richards that hydrogen binding possibilities, in particular in positions 1, 2, 4 and 5 are essential for the drug to mimic glucose. The lack of activity of Compound IV indicated that the substituent in position 4 was of particular importance for biologic activity. The additional cyano group in position 3 of Compound III did not interfere with its activity. Interestingly, pentachloropyridine (Compound V) was devoid of activity, in marked contrast to its potent glucose transport inhibitory function in the erythrocyte (10).

During prolonged perfusions of the pancreas with 1 mM Compound I in the presence of 300 mg/dl glucose, a biphasic response was again observed (Fig. 2). In one of the three experiments (the average insulin release of which is shown), the second phase response reached a plateau. In the other two experiments secretion was still increasing at 60 min.

In vivo studies. Twenty minutes after giving 10 mg of Compound I by gavage there was no change in either basal blood glucose or insulin levels. The intravenous glucose bolus (0.625 g/kg) caused an almost three-fold greater insulin response in drug-treated animals compared with controls (Fig. 3B). This difference was significant from 10 to 60 min. The increased insulin secretion was not reflected in a difference in the glucose disappearance curve (Fig. 3A). In one animal gastric inhibitory polypeptide levels were measured for 1 hour following oral administration of 10 mg of Compound I. No significant change from the basal level of 765 pg/ml was observed.

TABLE 1

Structure-function relationships of insulinotropic pyridine derivatives and the role of hydrogen bonds

	Compound	Insulinotropic response in pancreas	Hydrogen Bonding of Atoms or Substituent					
			Pos. 1	Pos. 2	Pos. 3	Pos. 4	Pos. 5	Pos. 6
2,4-diamino-5-cyano-6-bromopyridine	I	+	A	A/D	0	A/D	A	0
2,4-diamino-5-cyano-6-iodopyridine	II	+	A	A/D	0	A/D	A	0
2,4-diamino-3,5-dicyano-6-bromopyridine	III	+	A	A/D	A	A/D	A	0
2-amino-3,5-dicyano-4,6-dibromopyridine	IV	−	A	A/D	A	0	A	0
pentachloropyridine	V	−	A	0	0	0	0	0

A., Acceptor; D, Donor; 0, neither A or D.

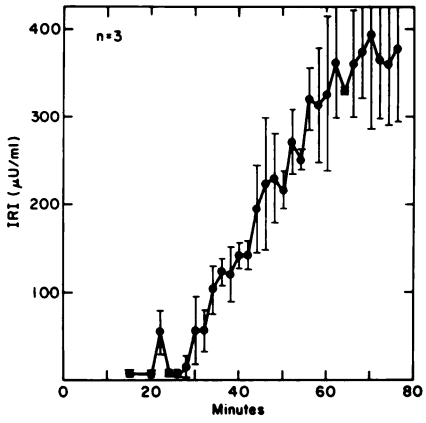

FIG. 2. *Insulin release during prolonged perfusion of rat pancreas with 1 mM Compound I and 300 mg/dl glucose*

Drug perfusion was initiated at 20 min. The results represent the mean values ± standard error of the mean from three experiments.

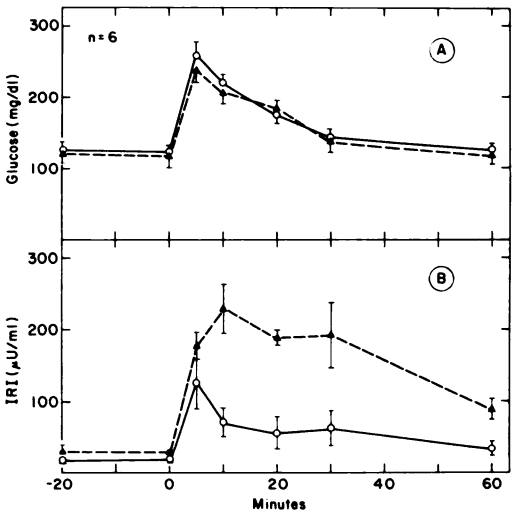

FIG. 3. *Effect of oral Compound I on insulin release in an IV glucose tolerance test in the rat. (A) glucose levels; (B) immunoreactive insulin levels.*

Details of the experiment are given in the text. ——○—— control; - - -▲- - - plus Compound I. The results represent the mean values ± standard errors of the mean from six experiments.

Compound I (50 mg) administered to 200 g rats by stomach tube did not produce signs of acute toxicity. Four weeks later the rats showed no noticeable adverse effects and autopsy revealed no abnormalities.[1]

DISCUSSION

A group of substituted pyridines that resemble glucose with respect to hydrogen bonding acceptor capacities in position 1 and the substituents in positions 2, 4 and 5 have been found to stimulate pancreatic insulin secretion. Previously some of these compounds, which conform to the hydrogen bonding requirements for optimal binding of sugars to the erythrocyte glucose transport system (18–21), had been found to be potent transport inhibitors (10). However, one potent transport inhibitor, pentachloropyridine, constituted a disturbing exception to the rules. Since pentachloropyridine was devoid of activity in the pancreas system, the above rules concerning hydrogen bonding requirements appear to apply more strictly in determining whether a compound will elicit insulin release.

Since iodoacetamide, a sulfhydryl alkylating agent, is known to promote prolonged insulin secretion, or transient stimulation followed by inhibition depending on the concentrations of reagent and glucose (22),

[1] Compound I dissolved in 1 mM concentration in growth medium did not disturb noticeably the development of fruitfly larvae (Drosophila melanogaster) to adult fly.

it was important to note that the effects of the 6-bromo- and 6-iodopyridine derivatives were rapidly reversible (Fig. 1). Therefore it is unlikely that the effect of the substituted pyridine compounds is mediated by covalent modification, arylation in this case, of sulfhydryl groups on or in the β-cell. The chemical reactivity of the compounds with sulfhydryl groups is currently being investigated.

The fact that the pyridine derivatives resemble glucose in the configuration of hydrogen bonding possibilities suggests that the secretory mechanism of the pancreatic β-cell can be triggered without the triggering substance being metabolized by a common metabolic pathway with glucose. However, the lack of effect of Compound I and its analogues at 100 mg/dl glucose concentration shows that these agents cannot substitute entirely for glucose in eliciting an insulin response. They require even higher than normal fasting glucose concentrations to exert their effects. In this respect these compounds resemble the hormonal potentiators of insulin release (23, 24). The same mechanism that protects animals against excessive insulin release stimulated by endogenous hormones may also serve to protect animals given the synthetic compounds *in vivo* from releasing too much insulin at normal fasting glucose levels and thus developing hypoglycemia. In this respect the pyridine derivatives also differ from the sulfonylureas in their requirement for elevated glucose levels. Whereas acutely the sulfonylureas can cause hypoglycemia, it appears that, at least in rats, the pyridine derivatives cannot. The requirement for elevated glucose concentrations in stimulation of insulin secretion is also a characteristic of agents that elevate β-cell cyclic AMP (25–27). Although we cannot exclude the possibility that Compound I acts by elevating cyclic AMP, previous studies with phosphodiesterase inhibitors and prostaglandins (27) showed that agents promoting insulin release by that mechanism seldom give increases of more than 3-fold, while Compound I reached 7- to 20-fold stimulation.

As outlined in the introduction, two alternative models of how glucose increases insulin secretion have been proposed (8, 9). One model is based on a glucoreceptor on the β-cell membrane that recognizes increasing ambient glucose concentrations. In the second model, one or several of the intracellular metabolites of glucose regulate insulin release. The results obtained with Compound I suggest that perhaps both models are partially correct, a suggestion made previously (28, 29). Although the pyridine compounds may interact with glucoreceptors because of their resemblance to glucose (illustrated in reference 10), it is very unlikely that they can be metabolized to compounds that resemble glucose metabolites. The elevated glucose concentration necessary to detect stimulation of insulin release with the pyridine compounds suggests that higher levels of glucose metabolites are required intracellularly before activation of the glucoreceptor on the cell membrane can affect insulin release.

The insulin secretogogue 2,4-diamino-5-cyano-6-bromopyridine was first described as reversible glucose transport inhibitor in erythrocytes with an apparent inhibition constant of 80 μM (10). Strong stimulation of insulin secretion occurred at slightly higher concentrations (0.1–1 mM). It is noteworthy that in the case of other glucose transport inhibitors, like phlorizin and phloretin, the half-maximal dose for glucose transport inhibition is also different from that for insulin release and depends on the concentration of glucose present (30–32).

While Compound I produced increased insulin release in an intravenous glucose tolerance test in rats, glucose levels were unchanged compared to that in control animals. It is conceivable that the normal amounts of insulin released by glucose were so effective that the additional insulin released by drug-treated animals led to no increase in glucose disappearance rate. In view of the glucose transport inhibitory activity of Compound I in erythrocytes (10), it is also possible that the lack of a difference in the glucose disappearance curve was the result of competition between glucose and Compound I for the glucose transport system in peripheral tissues. In the latter case the increased concentrations of insulin would have no net effect on glucose

transport, but insulin could still direct whatever amounts of glucose enter the cell to appropriate metabolic pathways. Presumably the increased insulin levels produced by Compound I can also affect other cellular processes regulated by insulin such as the uptake of amino acids. Finally, it is conceivable that the additional insulin secreted under the influence of Compound I did increase glucose disposal in peripheral tissues, but the drug stimulated compensatory glucose production by the liver. A distinction between these possibilities will require additional studies.

This new family of insulinotropic agents is interesting for several reasons. First, use of these agents may help clarify the mechanism of glucose-induced insulin release in the pancreatic β-cell. Second, the oral insulinotropic activity deserves testing in both normal and diabetic animals to determine whether these or related compounds have therapeutic potential in the treatment of diabetes mellitus. Finally, these compounds are an example of rational drug design, still an ususual way of finding a new class of specific bioactive agents.

ACKNOWLEDGMENTS

We thank Mrs. Glenda Schneider for excellent technical assistance, Dr. Frederic M. Richards for a sample of Compound I and Dr. V. A. Engelhardt for samples of Compounds III and IV.

REFERENCES

1. Patton, G. S., E. Ipp, R. E. Dobbs, L. Orci, W. Vale, & R. H. Unger. Pancreatic immunoreactive somatostatin release. *Proc. Nat. Acad. Sci. USA* **74**, 2140–2143, 1977.
2. Unger, R. H., H. Ketterer, J. Dupré, & A. M. Eisentraut. The effects of secretin, pancreozymin, and gastrin on insulin and glucagon secretion in anesthetized dogs. *J. Clin. Invest.* **46**, 630–645, 1967.
3. Kaneto, A., Y. Tasaka, K. Kosaka, & K. Nakao. Stimulation of insulin secretion by the C-terminal tetrapeptide amide of gastrin. *Endocrinology* **84**, 1098–1106, 1969.
4. Frame, C. M., M. B. Davidson, & R. A. L. Sturdevant. Effects of the Octapeptide of cholecystokinin on insulin and glucagon secretion in the dog. *Endocrinology* **97**, 549–553, 1975.
5. Pfeiffer, E. F., M. Telib, J. Ammon, F. Melani, & H. Ditschuneit. Direkte Stimulierung der Insulin-sekretion *in vitro* durch Sekretin. *Dtsch. med. Wochenschr.* **90**, 1663–1669, 1965.
6. Dupré, J., S. A. Ross, D. Watson, & J. C. Brown. Stimulation of insulin secretion by gastric inhibitory polypeptide in man. *J. Clin. Endocrinol. Metab.* **37**, 826–828, 1973.
7. Bertaccini, G., G. DeCaro, & P. Melchiorri. The effects of caerulein on insulin secretion in anaesthetized dogs. *Brit. J. Pharmacol.* **40**, 78–85, 1970.
8. Randle, P. J., S. J. H. Ashcroft, & J. F. Gill. Carbohydrate metabolism and release of hormones, in *Carbohydrate Metabolism and its Disorders* (Dickens, F., P. J. Randle, and W. J. Whelan, eds.), Vol. 1. Academic Press, London, 1968, 427–447.
9. Ashcroft, S. J. H. The control of insulin release by sugars, in *Polypeptide Hormones; Molecular and Cellular Aspects* (Porter, R., and D. W. Fitzsimons, eds.). Elsevier Excerpta Medica, North Holland, Amsterdam, 1976, 117–139.
10. Hershfield, R., & F. M. Richards. Reversible inhibition of glucose transport in human erythrocytes by a series of pyridine derivatives. *J. Biol. Chem.* **251**, 5141–5148, 1976.
11. Carboni, R. A., D. D. Coffman, & E. G. Howard. Cyanocarbon Chemistry. XI. Malononitrile dimer. *J. Amer. Chem. Soc.* **80**, 2838–2840, 1958.
12. Little, E. L., Jr., W. J. Middleton, D. D. Coffman, V. A. Engelhardt, & G. W. Sausen. Cyanocarbon chemistry. X. Pyridines from tetracyanopropenes. *J. Amer. Chem. Soc.* **80**, 2832–2838, 1958.
13. Penhos, J. C., C. H. Wu, J. C. Basabe, N. Lopez, & F. W. Wolff. A rat pancreas-small gut preparation for the study of intestinal factor(s) and insulin release. *Diabetes* **18**, 733–738, 1969.
14. Johnson, D. G., J. W. Ensinck, D. Koerker, J. Palmer, & C. J. Goodner. Inhibition of glucagon and insulin secretion by somatostatin in the rat pancreas perfused *in situ*. *Endocrinology* **96**, 370–374, 1975.
15. Zaharko, D. S., & L. V. Beck. Studies of a simplified plasma insulin immunoassay using cellulose powder. *Diabetes* **17**, 444–457, 1968.
16. Rocha, D. M., G. R. Faloona, & R. H. Unger. Glucagon-stimulating activity of 20 amino acids in dogs. *J. Clin. Invest.* **51**, 2346–2351, 1972.
17. Hultman, E. Rapid specific method for determination of aldosaccharides in body fluids. *Nature* (London) **183**, 108–109, 1959.
18. Le Fevre, P. G. Sugar transport in the red blood cell: structure-activity relationships in substrates and antagonists. *Pharmacol. Rev.* **13**, 39–70, 1961.
19. Kahlenberg, A., B. Urman, & D. Dolansky. Preferential uptake of D-glucose by isolated human erythrocyte membranes. *Biochemistry* **10**, 3154–3162, 1971.
20. Kahlenberg, A., & D. Dolansky. Structural requirements of D-glucose for its binding to iso-

lated human erythrocyte membranes. *Can. J. Biochem.* **50,** 638–643, 1972.

21. Barnett, J. E. G., G. D. Holman, & K. A. Munday. Structural requirements for binding to the sugar-transport system of the human erythrocyte. *Biochem. J.* **131,** 211–221, 1973.
22. Hellman, B., L.-Å. Idahl, Å. Lernmark, J. Sehlin, & I.-B. Täljedal. Iodocetamide-induced sensitization of the pancreatic β-cells to glucose stimulation. *Biochem. J.* **132,** 775–789, 1973.
23. Pederson, R. A., & J C. Brown. The insulinotropic action of gastric inhibitory polypeptide in the perfused isolated rat pancreas. *Endocrinology* **99,** 780–785, 1976.
24. Schauder, P., J. Arends, B. Schindler, R. Ebert, & H. Frerichs. Permissive effect of glucose on the glucagon-induced accumulation of cAMP in isolated rat pancreatic islets. *Diabetologia* **13,** 171–175, 1977.
25. Malaisse, W. J., F. Malaisse-Lagae, & D. Mayhew. A possible role for the adenylcyclase system in insulin secretion. *J. Clin. Invest.* **46,** 1724–1734, 1967.
26. Ammon, H. P. T., & J. Steinke. Effect of 6-aminonicotinamide on insulin release and C-14 glucose oxidation by isolated pancreatic rat islets: difference between glucose, tolbutamide, and aminophylline. *Endocrinology* **91,** 33–38, 1972.
27. Johnson, D. G., W. Y. Fujimoto, & R. H. Williams. Enhanced release of insulin by prostaglandins in isolated pancreatic islets. *Diabetes* **22,** 658–663, 1973.
28. Ashcroft, S. J. H., L. C. C. Weeransinghe, & P. J. Randle. Interrelationship of islet metabolism, adenosine triphosphate content and insulin release. *Biochem. J.* **132,** 223–231, 1973.
29. Davis, B., & N. R. Lazarus. An *in vitro* system for studying insulin release caused by secretory granules-plasma membrane interactions: Definition of the system. *J. Physiol.* (Lond.) **256,** 709–729, 1976.
30. Hellman, B., Å. Lernmark, J. Sehlin, & I.-B. Täljedal. Effects of phlorizin on metabolism and function of pancreatic β-cell. *Metabolism* **21,** 60–66, 1972.
31. Hellman, B., Å. Lernmark, J. Sehlin., & I.-B. Täljedal. The pancreatic β-cell recognition of insulin secretagogues V. Binding and stimulatory action of phlorizin. *Mol. Pharmacol.* **8,** 759–769, 1972.
32. Ashcroft, S. J. H., & S. Nino. Effects of phloretin and dextran-linked phloretin on pancreatic islet metabolism and insulin release. *Biochim. Biophys. Acta* **538,** 334–342, 1978.