MOLECULAR PHARMACOLOGY, 8, 759–769

The Pancreatic β-Cell Recognition of Insulin Secretagogues

V. Binding and Stimulatory Action of Phlorizin

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(Received June 23, 1972)

SUMMARY

Phlorizin (10 mM) inhibited glucose-stimulated insulin release from microdissected pancreatic islets of obese-hyperglycemic mice. In the absence of glucose, phlorizin (5–15 mM) as well as phloretin (10 mM) stimulated insulin release. These stimulatory effects were inhibited by mannoheptulose, suggesting that phlorizin and phloretin were sensed by the system which recognizes glucose as an insulin secretagogue. However, the mechanism sensitive to phlorizin does not seem to possess the full competence of the glucose-recognizing system, since phlorizin did not potentiate the insulin-releasing actions of arginine or theophylline. Leucine, but not pyruvate or succinate, enhanced the stimulatory effect of phlorizin. Radioactive phlorizin rapidly accumulated in amounts far exceeding the urea space of the islets. This uptake was concentration-dependent up into the millimolar concentration range. It was not significantly influenced by glucose. Antimycin A, *p*-chloromercuriphenylsulfonic acid, and chlorpromazine, which increase the uptake of extracellular space markers, stimulated the uptake of phlorizin in whole islets but not in islet homogenates. It is suggested that phlorizin binds predominantly to the plasma membranes of intact β-cells. Although the binding may not be specific for glucose sites, reaction with such a site could be responsible for the phlorizin-induced insulin release.

INTRODUCTION

Phlorizin has several interesting effects on the pancreatic β-cells. At a concentration of 1 mM it strongly inhibits glucose transport but has no effect on insulin release (1). A 10-fold higher concentration of phlorizin inhibits glucose-stimulated insulin release but stimulates insulin release in the absence of glucose (1). These observations raise fundamental questions as to the site and nature of the recognition system which enables β-cells to sense glucose as an insulin secretagogue. Taken together, the ability of phlorizin to stimulate insulin release, its ability to interact with glucose-sensing systems, and its poor ability to penetrate epithelial cells (2) suggest that insulin release may be triggered at the β-cell plasma membrane. Although this chain of arguments is loose, its validity deserves experimental testing because little is known about the mechanisms of glucose-stimulated insulin release. On the one hand there are data suggesting that glucose stimulates insulin release through its metabolism (3–9). On the other, some authors feel that insulin release can be stimulated by glucose analogues which are not, or only poorly, metabolized (10, 11). The relevance of phlorizin-induced insulin release to this problem depends primarily on whether or not phlori-

This work was supported by Grants 12x-562 and 12x-2288 from the Swedish Medical Research Council.

zin utilizes the physiological recognition system. It is also important to know whether phlorizin can readily penetrate β-cells or is essentially confined to the outside of the plasma membrane. With these questions in mind we have investigated how the insulin secretory responses to phlorizin and its aglucone analogue, phloretin, may be modified by some compounds which are known to affect glucose-stimulated insulin release. In addition, the uptake of radioactively labeled phlorizin by isolated islets was studied. Islets containing more than 90% β-cells were obtained by freehand microdissection of the pancreatic glands from obese-hyperglycemic mice.

MATERIALS AND METHODS

Chemicals. Nonradioactive phlorizin was obtained from Fluka AG, Buchs, Switzerland, and phloretin was purchased from Pfaltz and Bauer, New York. The purity of these preparations with regard to free D-glucose was determined by gas chromatography. Phlorizin was found to contain 0.1% (w/w) D-glucose, whereas no D-glucose was detected in the phloretin preparation. In some experiments the phlorizin was used after treatment with activated charcoal and recrystallization from hot water. There were no detectable differences between results obtained with the commercial and the recrystallized phlorizin. [^{3}H]Phlorizin was prepared by Farbwerke Hoechst AG, Frankfurt am Main, Germany. [^{125}I]Insulin was purchased from Farbwerke Hoechst AG and from the Radiochemical Centre, Amersham, England. The Radiochemical Centre also supplied [^{14}C]urea, [U-^{14}C]sucrose, 3-*O*-methyl-D-[U-^{14}C]glucose, [6,6′-^{3}H]sucrose, and insulin antibodies. Nonradioactive D-glucose, sucrose, pyruvate, and succinate were obtained from British Drug Houses, Ltd., and 3-*O*-methyl-D-glucose urea, L-leucine, L-arginine, D-mannoheptulose, theophylline, antimycin A, and *p*-chloromercuriphenylsulfonic acid, from Sigma Chemical Company. Chlorpromazine chloride was a gift from AB Leo, Helsingborg. Crystalline mouse insulin was prepared by Novo A/S, Copenhagen, and human serum albumin was obtained from AB Kabi, Stockholm.

Animals and isolation of islets. Adult obese-hyperglycemic mice (gene symbol, *obob*) of either sex were taken from a local colony (12). Unless otherwise stated, the animals were fasted overnight before being killed. With the excised pancreas suspended in Krebs-Ringer-bicarbonate buffer equilibrated with O_2–CO_2 (95:5), fresh islets were isolated by freehand microdissection (13). When the subsequent incubations were performed in the presence of serum albumin, the dissection medium also contained albumin at a concentration of 0.3–0.5%.

General aspects of incubations. Krebs-Ringer-bicarbonate buffer equilibrated with O_2–CO_2 (95:5) was used as the basal medium in all experiments. Unless otherwise stated, the media contained 0.3–0.5% human serum albumin. The presence of various test substances is specified in the legends to the figures and tables. All experiments were started by preliminary incubation of the islets at 37° for 30–70 min. This temperature was also used in all further incubations except when studying the uptake of 3-*O*-methyl-D-glucose, which was done at 8°.

Insulin release. The techniques for measuring insulin release have been dealt with in detail elsewhere (14). After preliminary incubation, either the islets were incubated in 115 μl of medium for 5 min (early phase) followed by incubation in 315 μl of medium for 60 min (late phase), or they were merely incubated in 315 μl of medium for 60 min. Samples of insulin were taken at the end of each incubation period and were assayed radioimmunologically. Ethanol was used to separate free and antibody-bound insulin (15). Crystalline mouse insulin was used as a reference. It was ascertained that phlorizin did not interfere with the insulin assay (Fig. 1).

Uptake of 3-O-methyl-D-glucose. We used the kind of double-label procedure that was previously employed in studies on D-glucose transport in pancreatic islets (1, 16). Batches of three islets were incubated at 8° for 3 min in media containing 2.0 mM 3-*O*-methyl-D-[U-^{14}C]glucose (5.0 mCi/mmole) and 0.1 mM [6,6′-^{3}H]sucrose (150 mCi/mmole). The islets were then freeze-dried, weighed, and analyzed for ^{14}C and ^{3}H. The uptake of 3-*O*-methyl-D-glucose in excess of the sucrose space was taken as an indication of transport

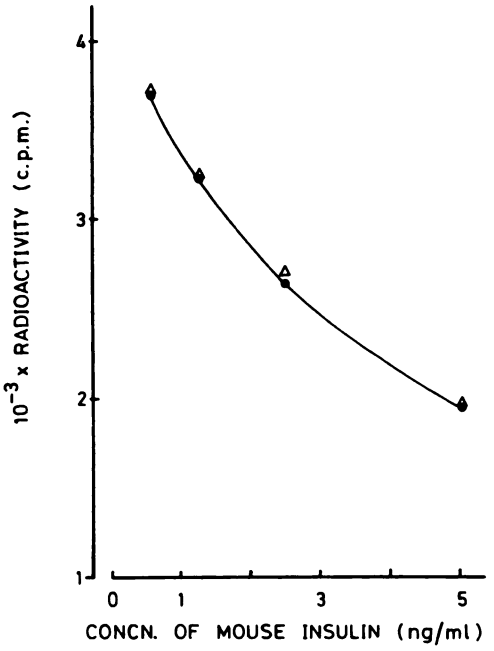

FIG. 1. *Mouse insulin standard curve*
Crystalline mouse insulin was dissolved in 0.1 M phosphate buffer (pH 7.4) containing (△) or lacking (●) 10 mM phlorizin. After incubation at 37° for 15 min, samples were taken for radioimmunological assay. The points show the antibody-bound radioactivity as a function of mouse insulin concentration.

across the β-cell membrane, since sucrose is an extracellular space marker in the present kind of islets (16, 17).

Uptake of phlorizin. Batches of three islets were incubated in media containing [^{3}H]-phlorizin and either [U-^{14}C]sucrose or [^{14}C]-urea. Urea is assumed to be distributed in the total tissue water of pancreatic islets (17). The concentration of radioactive compounds and other test substances as well as the incubation time are indicated in the legends to the figures and tables. After freeze-drying and weighing, the islets were analyzed for ^{3}H and ^{14}C. Results are expressed as the islet uptake of phlorizin in excess of the sucrose or urea spaces.

Experiments were also performed to study the uptake of phlorizin by subcellular islet particles. In one type of experiment 25–30 islets were homogenized in 75 μl of basal medium, using a microscale pestle homogenizer. After centrifugation at about 400 × *g* for 10 min, 10-μl samples of the supernatant fraction were assayed for protein (18) or were incubated for 90 min with 90 μl of medium containing [^{3}H]phlorizin, [^{14}C]urea, and test substances as indicated in the legend to Table 4. The mixtures of homogenate and medium were then filtered on membrane filters with a pore size of 0.5 μ (Oxoid, Ltd., England). After drying, the filters were analyzed for ^{3}H and ^{14}C, and the uptake of phlorizin in excess of the urea space was calculated per unit of protein of the incubated homogenate.

Weighing of islets and counting of radioactivity. Incubated islets were placed on pieces of aluminum foil, and with the aid of a micropipette they were freed of as much contaminating fluid as possible. This procedure was standardized to take only a few seconds, after which the islets were plunged into isopentane cooled to its melting point. The islets were then freeze-dried (−40°, 0.001 mm Hg) overnight and were weighed on a quartz fiber balance. The weighed islets were dissolved by incubation for 45 min at room temperature in 100 μl of Hyamine. Ten milliliters of scintillation liquid (5 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[5-phenyloxazol-2-yl]benzene in 1 liter of toluene) were added, and the radioactivity was counted in a liquid scintillation spectrometer. The same scintillation liquid was used when counting the radioactivity of subcellular particles. Particles attached to Oxoid filters were counted after dissolution in Hyamine as above. The setting of the discriminators was such that 25% of the counts in the ^{14}C channel were also counted in the ^{3}H channel. Spillover of ^{3}H counts to the ^{14}C channel was less than 1%. After correction for spillover and blanks, the observed counts per minute were translated into millimoles by comparison with external standards. These standards consisted of 5-μl samples of medium counted in parallel with the samples.

RESULTS

Effects of Phlorizin and Phloretin on Insulin Release

Dose-response relationships. Figure 2 shows the effects of different concentrations of phlorizin on insulin release in the absence of glucose. A dose-dependent stimulation was

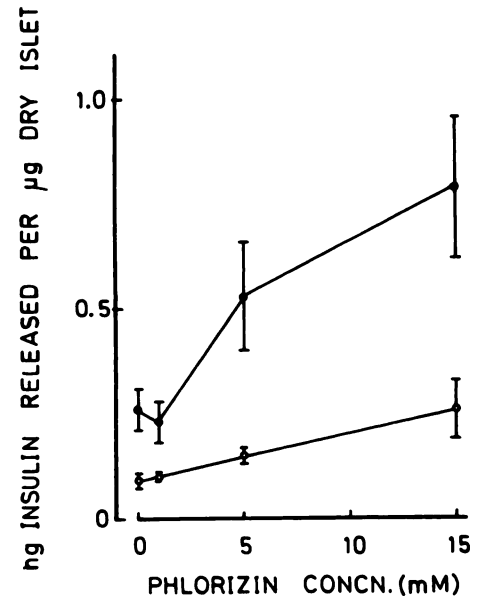

FIG. 2. *Effect of phlorizin on insulin release in the absence of glucose*

Islets from fed mice were incubated in basal medium supplemented with different concentrations of phlorizin. The points show the amounts of insulin released during the first 5 min (○) and subsequent 60 min (●) of incubation. Mean values ± standard errors for seven different experiments are given.

observed up to 15 mM phlorizin during both the early and late phases of incubation. Higher concentrations of phlorizin could not be tested because of its limited solubility at 37°. No significant effect was obtained with 1 mM phlorizin.

The inhibitory action of 10 mM phlorizin on insulin release at various glucose concentrations is shown in Fig. 3. The data are compatible with a noncompetitive mechanism of inhibition: the effect of phlorizin was not overcome by a glucose concentration as high as 40 mM.

Table 1 summarizes some experiments with phloretin, the aglucone analogue of phlorizin. In contrast to phlorizin, phloretin did not significantly inhibit glucose-stimulated insulin release. However, when tested in the absence of glucose, 10 mM phloretin stimulated insulin release.

Effects of metabolic substrates on phlorizin-stimulated insulin release. It is theoretically possible that glucose stimulates insulin release by a twofold mechanism involving both a direct receptor and intermediary metabolism (19). It was therefore of interest to determine whether the stimulatory effect of phlorizin could be enhanced by metabolizable substrates. As shown in Table 2, neither pyruvate nor succinate had any effect on phlorizin-stimulated insulin release. There was, however, a greater secretory response to 10 mM phlorizin in the presence of 10 mM leucine than to 10 mM phlorizin alone. This effect was significantly larger than the sum of effects caused by phlorizin and leucine.

Effect of mannoheptulose on insulin release in response to phlorizin or phloretin. In the construction of hypotheses about the molecular mechanisms of glucose-stimulated insulin release, the inhibitory effect of mannoheptulose stands out as a key observation (4–6, 20, 21). Since mannoheptulose does not inhibit the insulin-releasing action of leucine (22), it probably acts on the glucose-recognizing system. The possibility that this system is sensitive to phlorizin and phloretin

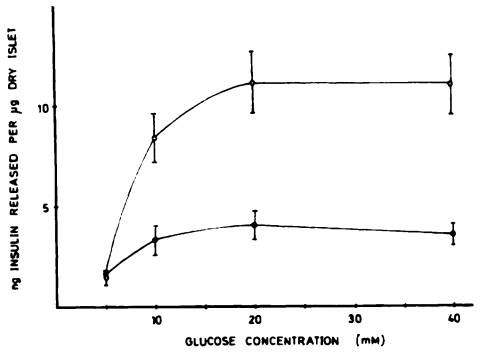

FIG. 3. *Effect of phlorizin on glucose-stimulated insulin release*

Islets from fed mice were incubated for consecutive periods of 5 and 60 min in basal medium supplemented with different concentrations of glucose. Test media also contained 10 mM phlorizin (●), whereas control incubations were performed in the absence of phlorizin (○). Amounts of insulin released during the 60-min period are presented as mean values ± standard errors for six different experiments. Similar inhibition by phlorizin was apparent also during the initial 5 min, although the differences from controls were not statistically significant.

TABLE 1

Effect of phloretin on insulin release

After preliminary incubation in basal medium, incubations were carried out in the presence of phloretin and glucose at the concentrations listed. Values represent amounts of insulin released during the initial 5 min (early phase) and subsequent 60 min (late phase) of incubation. Mean values ± standard errors are given for the numbers of experiments indicated within parentheses.

Phase	Phloretin	Insulin released	
		0 mM glucose	10 mM glucose
	mM	*ng/μg dry islet*	
Early	0	0.18 ± 0.03 (6)	0.82 ± 0.17 (6)
	1		0.77 ± 0.09 (6)
	5		0.65 ± 0.14 (5)
	10	0.30 ± 0.08 (6)	0.83 ± 0.18 (6)
Late	0	0.63 ± 0.22 (6)	8.67 ± 1.05 (6)
	1		8.73 ± 0.72 (6)
	5		8.15 ± 2.31 (5)
	10	1.19 ± 0.26[a] (6)	7.72 ± 1.67 (6)

[a] $p < 0.025$.

would be strengthened if mannoheptulose were found to inhibit insulin release in response to these substances. Table 2 shows that this is the case. Mannoheptulose at a concentration of 10 mM significantly inhibited the stimulatory effects of 10 mM phlorizin or 10 mM phloretin.

Effects of arginine and theophylline on phlorizin-induced insulin release. In the kind of islets used, 10 mM arginine has no effect on insulin release in a medium containing 3 mM glucose (23). However, in the presence of 10 mM glucose, graded secretory responses are obtained with 5–20 mM arginine (23). Similarly, stimulation of insulin release by theophylline (24) or caffeine (21) has been found to depend on the glucose concentration in the medium. That glucose potentiates the stimulatory action of theophylline on our islets is shown in Table 2. As is also shown in Table 2, phlorizin could not replace glucose in the role of potentiator of insulin release in response to arginine or theophylline.

Effect of Phloretin on Glucose Transport

It was previously shown that phlorizin inhibits glucose transport in the pancreatic β-cells (1, 16). One series of experiments was performed to test whether phloretin has a similar effect. Islets were incubated at 8° in basal medium containing 2 mM 3-*O*-methyl-D-[U-^{14}C]glucose (5.0 mCi/mmole), 0.1 mM [6,6′-^{3}H]sucrose (150 mCi/mmole), and 10 mM phloretin. Control islets were incubated without phloretin in the medium. After 3 min of incubation the uptake of 3-*O*-methyl-D-glucose in excess of the sucrose space was 2.12 ± 0.25 mmoles/kg (dry weight) of control islets (mean ± standard error for five experiments). The corresponding figures for islets incubated with 10 mM phloretin were 0.61 ± 0.17 mmole/kg (dry weight) ($n = 5$), which means significant ($p < 0.01$) inhibition of 3-*O*-methyl-D-glucose uptake. Since 3-*O*-methyl-D-glucose utilizes the D-glucose transport system in the β-cells,[1] the results indicate that phloretin is an inhibitor of this system.

Uptake of Phlorizin by Islets

Time course of phlorizin uptake and release. Figure 4 shows the islet uptake of phlorizin with time. Phlorizin was taken up in considerable excess of the sucrose and urea spaces. The uptake did not increase significantly after 7 min, the shortest period of incubation tested. This means that phlorizin was equilibrated much more rapidly than any of the smaller organic molecules which have previously been found to be concentrated in the islet cells (17, 25, 26).

Effect of glucose on phlorizin uptake. Binding of radioactive phlorizin has been fruitfully employed as a tool in the analysis of glucose receptors in the kidney (27, 28). Clear-cut effects of glucose on phlorizin binding are an obvious condition for such an

[1] B. Hellman, J. Sehlin, and I.-B. Täljedal, manuscript in preparation.

TABLE 2

Effects of various compounds on insulin release in response to phloretin or phlorizin

After preliminary incubation in basal medium, islets were incubated for 60 min in media supplemented with phloretin or phlorizin as well as test substances as indicated. The amounts of insulin released are given as mean values ± standard errors for the numbers of experiments stated within parentheses. In each experiment parallel control and test incubations were performed with islets from a single animal. The statistical significances of effects were judged from the mean differences between parallel test and control incubations over a series of repeated experiments.

Test substance	Insulin released	
	0 mM phlorizin or phloretin	10 mM phlorizin or phloretin
	ng/μg dry islet	
Experiments with phloretin		
None	0.19 ± 0.06 (8)	1.16 ± 0.27[a] (8)
Mannoheptulose, 10 mM	0.25 ± 0.10 (8)	0.69 ± 0.17[b] (8)
Experiments with phlorizin		
Experimental series I		
None	0.61 ± 0.12 (10)	1.23 ± 0.37[c] (10)
L-Leucine, 10 mM	1.93 ± 0.36 (10)	3.51 ± 0.57[d] (9)
Pyruvate, 20 mM	0.80 ± 0.21 (10)	1.38 ± 0.43 (9)
Succinate, 20 mM	0.60 ± 0.14 (10)	1.38 ± 0.22 (9)
Experimental series II		
None	0.79 ± 0.19 (10)	3.23 ± 0.59[e] (10)
Arginine, 10 mM	0.67 ± 0.16 (10)	2.38 ± 0.61 (10)
Mannoheptulose, 10 mM	0.87 ± 0.21 (10)	1.84 ± 0.43[f] (10)
Experimental series III		
None	0.48 ± 0.15 (7)	2.28 ± 0.48[g] (7)
Theophylline, 5 mM	1.54 ± 0.59 (6)	2.85 ± 0.83 (6)
Check of potentiation: theophylline-glucose		
None	0.19 ± 0.06 (8)	
Glucose, 10 mM	2.17 ± 0.53 (8)	
Theophylline, 5 mM, + glucose, 10 mM	7.80 ± 1.90 (8)	

[a] $p < 0.005$ (10 mM vs. 0 mM phloretin).

[b] $p < 0.02$ (effect of mannoheptulose on phloretin-stimulated release).

[c] $p < 0.1$ (10 mM phlorizin vs. 0 mM phlorizin).

[d] In experimental series I the same statistical principle was used for testing the possible interaction between leucine and phlorizin. Within each of nine experiments the sum of effects caused by phlorizin and leucine alone was subtracted from the effect produced by both compounds together. The mean ± standard error of these differences over the whole experimental series was 1.40 ± 0.34 ng of insulin per microgram of dry islet. This means that interaction between phlorizin and leucine was significant at $p < 0.005$.

[e] $p < 0.005$ (10 mM vs. 0 mM phlorizin).

[f] $p < 0.02$ (effect of mannoheptulose on phlorizin-stimulated release.

[g] $p < 0.025$ (10 mM phlorizin vs. 0 mM phlorizin).

experimental approach. Table 3 shows that 20 mM glucose did not measurably inhibit the islet uptake of phlorizin. Nor did 20 mM glucose have any effect on the loss of radioactivity from islets loaded with 0.1 mM radioactive phlorizin. These results do not rule out the possibility that phlorizin may interact with glucose-binding sites in the β-cells. They suggest, however, that the uptake of phlorizin in whole islets is not specific for such sites.

Effects of antimycin A, p-chloromercuri-

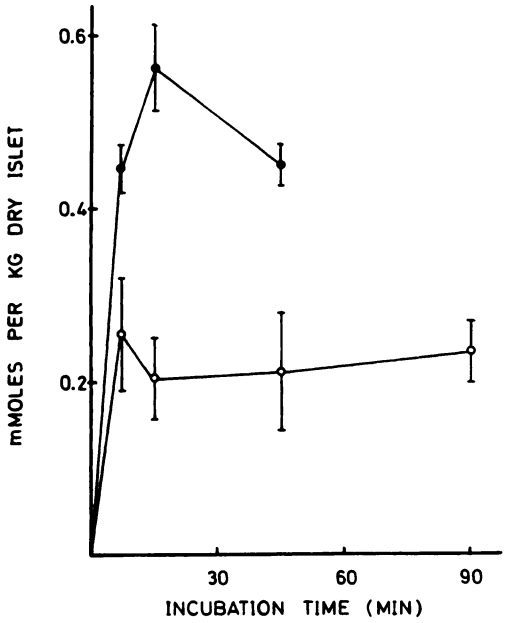

FIG. 4. *Islet uptake of phlorizin with time*
Islets were incubated with 0.2 mM [^{3}H]phlorizin (50 mCi/mmole) for different periods of time. The incubation media also contained either 0.1 mM [U-^{14}C]sucrose (100 mCi/mmole) or 0.5 mM [^{14}C] urea (20 mCi/mmole). The points denote islet uptake of phlorizin in excess of the spaces occupied by sucrose (●) or urea (○). Mean values ± standard errors are presented for six or seven (●) or 10 (○) experiments.

phenylsulfonate, and chlorpromazine on phlorizin uptake. Although uptake of phlorizin in excess of the urea space (Fig. 4) suggested that phlorizin was bound to intact islet cells, it was not known whether binding occurred exclusively on the β-cell surface or whether phlorizin entered these cells. An experimental approach to this problem was suggested by our observation that phlorizin can bind to several subcellular fractions of homogenized islets. If phlorizin is normally restricted to the outside of the β-cells, increased membrane permeability may result in a demonstrable enhancement of phlorizin uptake. We have previously observed that islets exposed to antimycin A, CMPS,[2] or chlorpromazine have an enlarged sucrose space, indicative of an increased permeability of the β-cell plasma membranes[1] (29). Orci *et al.* (30) reported that millimolar concentrations of chlorpromazine exert a lytic effect on rat islet cells. Table 4 shows that antimycin A, CMPS, and chlorpromazine markedly increased the islet uptake of phlorizin. In contrast, none of these compounds had significant effect on the uptake of phlorizin by the particles in islet homogenates. These results are compatible with the idea that phlorizin does not readily enter β-cells with intact plasma membranes.

[2] The abbreviation used is: CMPS, *p*-chloromercuriphenylsulfonic acid.

TABLE 3
Effects of glucose on phlorizin uptake and release

In uptake studies islets were first incubated for 30 min with 0 or 20 mM glucose. They were then incubated for 60 min with the same concentrations of glucose as well as 0.1 mM [^{3}H]phlorizin (50 mCi/mmole) and 0.5 mM [^{14}C]urea (20 mCi/mmole). No albumin was present in the incubation media. In studies of phlorizin release the islets were first incubated for 45 min with radioactive phlorizin and urea as above. They were then incubated for 5 or 15 min in media lacking phlorizin and urea but containing 0 or 10 mM glucose. In both types of experiments the islet contents of phlorizin, corrected for label in the urea space, are given as mean values ± standard errors for the numbers of experiments stated within parentheses.

Measurement	Incubation time	Phlorizin	
		0 mM glucose	20 mM glucose
	min	*mmole/kg dry islet*	
Uptake	60	0.20 ± 0.05 (8)	0.30 ± 0.09 (8)
Release	5	0.27 ± 0.03 (9)	0.23 ± 0.03 (9)
	15	0.15 ± 0.02 (9)	0.15 ± 0.01 (9)

Concentration dependence of phlorizin uptake. In spite of the apparently nonspecific binding of phlorizin to β-cell membranes, it was of interest to know if the uptake is concentration-dependent up into the millimolar range, where the effects on insulin release are seen. Figure 5 shows that this is the case.

DISCUSSION

The results reported here extend our previous observation that phlorizin stimulates

TABLE 4

Effects of antimycin A, CMPS, and chlorpromazine on uptake of phlorizin by intact and homogenized islets

In experiments with intact islets ("islets"), these were incubated for 90 min in albumin-free basal medium supplemented with test substance as listed. During the last 60 min the incubation medium also contained 0.1 mM [^{3}H]phlorizin (50 mCi/mmole) and 0.5 mM [^{14}C]urea (20 mCi/mmole). The islet contents of phlorizin are given after correction for label in the urea space. In another series of experiments ("homogenates") 25 islets were first homogenized in 75 µl of basal medium. After centrifugation at 400 × *g* for 10 min, 10-µl samples of the supernatant fluid were assayed for protein or were incubated for 90 min with 90 µl of medium containing test substance, phlorizin, and urea as above. The mixture of homogenate and medium was then filtered, and the retained particles were analyzed for ^{3}H and ^{14}C. After correction for label in the urea space, the amounts of particle-bound phlorizin were calculated per unit of protein of the incubated homogenate. All results are given as mean values ± standard errors for the numbers of experiments stated within parentheses.

Test substance	Islets	Homogenates
	mmoles/kg dry islet	*mmoles/kg protein*
Experiments with antimycin A and CMPS		
None (control)	0.18 ± 0.07 (5)	1.54 ± 0.26 (11)
Antimycin A, 10 µM	0.72 ± 0.06[a] (5)	1.57 ± 0.47 (6)
CMPS, 0.5 mM	1.15 ± 0.02[b] (5)	0.96 ± 0.45 (5)
Experiments with chlorpromazine		
None (control)	0.20 ± 0.07 (6)	1.91 ± 0.41 (6)
Chlorpromazine, 0.5 mM	1.58 ± 0.23[a] (6)	1.73 ± 0.04 (6)

[a] $p < 0.005$.
[b] $p < 0.001$.

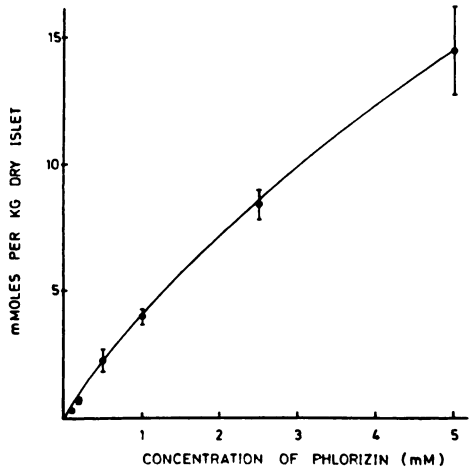

FIG. 5. *Concentration dependence of phlorizin uptake*

Islets were incubated for 45 min in albumin-free media supplemented with different concentrations of [^{3}H]phlorizin (2–50 mCi/mmole) and 0.1 mM [U-^{14}C]sucrose (100 mCi/mmole). The points denote uptake of phlorizin in excess of the sucrose space and represent the mean values of two to six experiments. Bars indicate the standard errors in the cases of five or six experiments.

insulin release in the absence of glucose (1). The purpose of the present study was to shed some light on the mechanism of this stimulation. Two questions appear to be particularly important in relation to the problem of physiological stimulus recognition: (a) Is there any similarity between phlorizin and glucose in their actions as insulin secretagogues? (b) Can the effect of phlorizin be attributed to a direct action on the β-cell plasma membrane?

The major reason for raising the first of these questions is, of course, that phlorizin has long been known to interact with glucose-binding transport systems of different cells (31). We have shown that phlorizin inhibits mediated glucose transport in the type of islet preparation used (1, 16). Although the glucose transport system in the β-cells does not seem to be identical with the recognition system responsible for insulin release (1, 20), there could well be other glucose-binding sites with a similar ability to react with phlorizin. It does not follow, however, that the insulin-releasing effect of phlorizin must be mediated by a glucose-

binding site, since phlorizin has been reported to inhibit other systems as well, *e.g.*, ($Na^+ + K^+$)-dependent adenosine triphosphatase in brain (32). Furthermore, since phlorizin is a polyphenol compound, it could perhaps induce rather nonspecific alterations of the tertiary structure of the β-cell plasma membrane. The possibility that the insulin-releasing action of phlorizin may not involve glucose-binding sites is obvious from the fact that 5 mM phlorizin is required for this effect. Inhibition of sugar transport in other cells has been reported to require micromolar concentrations of phlorizin (2, 31). In the β-cells 1 mM phlorizin inhibited glucose transport by at least 50% at a glucose concentration of 10 mM (1).

In an attempt to answer the first question we investigated how the effect of phlorizin is modified by some substances known to affect glucose-stimulated insulin release. These substances were chosen so as to cover different molecular aspects of glucose-stimulated insulin release. Since mannoheptulose inhibits the stimulatory action of glucose (4, 5, 20) but not that of leucine (22), it probably reacts with the glucose recognition system. The present observation that mannoheptulose significantly inhibited insulin release in response to phlorizin and phloretin therefore suggests that these compounds are sensed by the glucose recognition system. This conclusion can be evaded by postulating the existence of a separate insulinogenic mechanism sensitive to phlorizin and mannoheptulose, but there is no support for such a concept. The stimulatory action of phloretin and the fact that this action was also inhibited by mannoheptulose indicate that phlorizin does not stimulate insulin release by virtue of its glucose residue. This does not invalidate the idea that phlorizin is sensed by a glucose recognition system. Both phlorizin and phloretin inhibit glucose transport in the pancreatic islets as well as in other tissues (31).

The fact that mannoheptulose inhibits insulin release in response to phlorizin or phloretin has some implications for the interpretation of how mannoheptulose inhibits secretion in response to glucose. Since mannoheptulose is an inhibitor of islet hexokinases, it has been natural to assume that this may represent the mechanism by which glucose-stimulated insulin release is inhibited (5, 33). This explanation is consonant with the hypothesis that glucose stimulates insulin release through its metabolism (3–9). Since the β-cells are permeable to mannoheptulose (20), it would be rather surprising if mannoheptulose did not affect the enzymes of intact islets in a manner similar to that seen with islet homogenates. However, it is difficult to visualize phlorizin and phloretin as metabolic substrates for the hexokinases of intact β-cells. Furthermore, phlorizin was found not to affect the contents of glucose 6-phosphate and fructose 1,6-diphosphate in islets incubated without glucose (1). It therefore seems reasonable to assume that mannoheptulose can inhibit insulin release in other ways than through blockade of glucose degradation. Although these deliberations undoubtedly converge to the hypothesis that at least part of the glucose recognition may not involve metabolism, we wish to emphasize the tentative nature of this idea. The literature contains examples of speculations about a direct receptor mechanism (1, 11, 16, 19, 34–36), but the available experimental support either is fragmentary or has not yet been confirmed.

If one accepts the hypothesis that phlorizin stimulates insulin release by acting on a glucose-sensitive direct receptor mechanism, the results obtained with arginine and theophylline may indicate that this mechanism does not possess the full competence of the glucose-recognizing system. Unlike glucose, phlorizin did not permit arginine to act as an insulin secretagogue. The mechanism of the permissive action of glucose is not known. However, bearing in mind that it can apparently be prevented with mannoheptulose (22), it is best visualized as an integral part of the full glucose-recognizing system. This part of the system appears not to be altogether specific for glucose, since the stimulatory effect of arginine can also be seen in the presence of leucine (37). One property that leucine shares with glucose is the ability to undergo fairly rapid oxidation in the β-cells (38), and it is conceivable

that arginine requires the production of some intermediate or cofactor of glucose or leucine metabolism in order to be fully potent as an insulin secretagogue. This interpretation fits the observation that arginine is in itself poorly oxidized in the β-cells (38). Randle *et al.* (19) suggested that the glucose recognition system might well be composed of two parts, a direct receptor for the glucose molecule as well as glucose metabolism. Matschinsky *et al.* (36) and Landgraf *et al.* (11) interpreted some of their recent data as supporting this view. In the same vein, the fact that phlorizin stimulated insulin release but failed to potentiate the effect of arginine may be explained by assuming that phlorizin stimulates only the direct receptor.

The ability of methylxanthines to potentiate glucose-stimulated insulin release has been well established (21, 24) and is documented here with microdissected islets from obese-hyperglycemic mice. Brisson *et al.* (39) explained this synergism by postulating that theophylline causes a translocation of calcium within the β-cell, which leads to insulin release only if glucose or some other agent prevents excessive calcium efflux from the cell. If this idea is correct, the failure of theophylline to potentiate the secretory response to phlorizin might suggest that calcium efflux is controlled predominantly by the metabolic component of the glucose recognition system.

The above bimodal concept of glucose recognition would seem to predict that the stimulatory action of phlorizin should be subject to regulation by metabolism of glucose or leucine. We have previously shown that 5–10 mM phlorizin inhibits glucose oxidation (1), which may contribute to the inhibitory action of phlorizin on glucose-stimulated insulin release. In the present study, leucine appeared to potentiate the insulin-releasing action of phlorizin. The picture is somewhat blurred by the failure of pyruvate or succinate to enhance the phlorizin-stimulated insulin release. It is possible that improved insight into the β-cell metabolism of these compounds may remove this apparent contradiction to the bimodal concept of stimulus recognition.

Electron microscopic radioautography has shown that phlorizin accumulates on the surface of gut epithelium but does not readily penetrate into the cells (2). These observations were made with micromolar concentrations of phlorizin. It is thus far from certain that phlorizin in millimolar concentrations would affect the pancreatic β-cells predominantly at the plasma membrane. However, this idea is supported by the present data on the uptake of radioactively labeled phlorizin by intact islets. Binding to the islets was apparent from the much greater uptake of phlorizin than of urea. The accumulation of phlorizin was markedly increased by exposing the islets to CMPS, antimycin A, or chlorpromazine, three compounds which also increase the islet uptake of sucrose, an extracellular space marker[1] (29). The interpretation that CMPS and antimycin A opened the β-cells to phlorizin is supported by the failure of these compounds to increase the binding of phlorizin to particles of homogenized islets.

It was not possible to demonstrate any effect of glucose on the binding of phlorizin to the β-cells. This failure indicates that under the present conditions phlorizin does not react specifically with glucose-binding sites. If nonspecific binding can be reduced by altering the experimental conditions, this would greatly increase the value of phlorizin as a tool for the identification of the hypothetical insulinogenic glucose receptor in the β-cell membrane.

ACKNOWLEDGMENTS

Radioactive phlorizin was donated by the Swedish Branch of Farbwerke Hoechst through the courtesy of Dr. H. Heise. We also thank AB Leo, Helsingborg, for a gift of chlorpromazine, and Novo A/S, Copenhagen, for preparing crystalline mouse insulin. Dr. L. Höglund, Department of Zoology, University of Umeå, kindly performed the gas chromatographic analyses of phloretin and phlorizin.

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