Stimulation of Insulin Secretion from Isolated Rat Islets by SaRI 59-801

Relation to cAMP Concentration and Ca^{2+} Uptake

RONALD L. HANSON AND CRAIG M. ISAACSON

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/34/7/691/354154/34-7-691.pdf by guest on 30 March 2023

SUMMARY

The mechanism of stimulation of insulin release from isolated rat islets by 0.3 mM SaRI 59-801 (DL-α-dimethylaminomethyl-2-[3-ethyl-5-methyl-4-isoxazoyl]-1H-indole-3-methanol) was investigated, considering cAMP concentration and Ca^{2+} uptake. Ten millimolar theophylline or 1 mM 1-methyl-3-isobutylxanthine, which inhibit cAMP phosphodiesterase, each greatly increased the stimulation of insulin release by 59-801. Forskolin (0.1 mM), an activator of adenylate cyclase, or 1 mM dibutyryl cAMP also potentiated 59-801, suggesting that 59-801 does not elevate islet cAMP but is potentiated by other compounds that do. Measurement of cAMP in islets by radioimmunoassay confirmed that it was not significantly elevated by 59-801 but was increased sevenfold by forskolin or 1-methyl-3-isobutylxanthine. SaRI 59-801 was not effective in the absence of Ca^{2+} and presence of 1 mM EGTA. Agents that block entry of Ca^{2+} into β-cells, verapamil, nifedipine, or $CoCl_2$, inhibited the release of insulin in response to 59-801. Studies of $^{45}Ca^{2+}$ uptake by isolated islets revealed an increased uptake in the presence of 59-801 and blockage of this effect by 50 μM verapamil. Thus, the stimulation of insulin secretion by 59-801 appears to involve a stimulation of Ca^{2+} uptake rather than an increase of cAMP concentration. The mechanism of stimulation of Ca^{2+} uptake by 59-801 requires further investigation.
DIABETES 1985; 34:691–95.

SaRI 59-801 is an oral hypoglycemic agent, effective in several species, which has been found to elevate serum insulin in vivo[1] and to stimulate insulin secretion by isolated rat pancreatic islets in the presence of 1-methyl-3-isobutylxanthine.[2] Insulin release from islets was stimulated by 59-801 at 0, 3, or 5 mM but not at 10 or 20 mM glucose and the effect of 59-801 at 3 mM glucose was not inhibited by mannoheptulose, suggesting that stimulation of insulin release by 59-801 is independent of glucose metabolism.[2] In addition, the rate of conversion of 5 mM [5-^{3}H]-glucose to ^3H_2O by islets, a measure of the rate of glycolysis, was not affected by 59-801.[2]

Insulin release can occur in response to increased metabolism of glucose or certain other substrates, which somehow is coupled to increased Ca^{2+} uptake by islets and may also increase islet cAMP.[3] Efflux of Ca^{2+} from β-cells and release of Ca^{2+} from intracellular organelles may also be altered.[4] The signal for insulin release is generally considered to be a rise in the concentration of Ca^{2+} in the cytoplasm of the β-cell. Elevated cAMP is not considered to be a primary stimulus for insulin secretion but, rather, amplifies the amount of insulin release in response to a primary stimulus.[5]

The mechanism of stimulation of insulin release by 59-801 has been further investigated. This report describes studies of possible effects of 59-801 on cAMP concentration and Ca^{2+} uptake by islets. For comparison, results with tolbutamide are also presented.

MATERIALS AND METHODS

Insulin secretion by islets. Pancreatic islets were isolated from fed, male, 320–350-g Sprague-Dawley rats by the collagenase digestion technique[6] and purified on a gradient of Ficoll 400.[7] The procedures for incubation of islets and measurement of insulin secretion were described previously.[2] Groups of 5 or 10 islets per beaker were incubated for 1 h in medium containing Krebs bicarbonate buffer (122 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, and 15.5 mM $NaHCO_3$), 20 mg/ml bovine serum albumin, 1 mM 1-methyl-3-isobutylxanthine, and 3 mM glucose, with additions or deletions as noted in the table and figure legends.

cAMP content of islets. Three groups of five islets were incubated for 45 min at 37°C in conical, 12-ml test tubes containing 0.5 ml Krebs bicarbonate buffer with 3 mM glucose, 2 mg/ml bovine serum albumin, and additions shown in Table 2. These experiments were performed separately

From the Sandoz Research Institute, Route 10, East Hanover, New Jersey 07936.
Address reprint requests to Dr. R. L. Hanson at the above address.
Received for publication 10 October 1984 and in revised form 15 December 1984.

from the secretion experiments. Incubations were stopped by placing the tubes in boiling water for 5 min, then islets were dispersed by sonication for 10 s. The tubes were centrifuged for 10 min at 1700 × *g*, and the supernatants were assayed for cAMP by radioimmunoassay, using the more sensitive procedure given in the kit from New England Nuclear (Boston, Massachusetts) in which the samples and standards are acetylated.

$^{45}Ca^{2+}$ uptake by islets. $^{45}Ca^{2+}$ uptake was measured by a modification of the method of Henquin and Lambert.[8] Incubations were carried out in 0.4-ml polyethylene tubes containing 25 μl of 6 M urea beneath 150 μl of a mixture of Versilube F50 silicone fluid and Thomas silicone fluid 6428-R15 (4:1 vol/vol). Batches of 10 islets in 50 μl of modified Krebs bicarbonate buffer (containing 1 mM instead of 2.5 mM $CaCl_2$, 5 mg/ml bovine serum albumin, 2.8 mM glucose, and 1 mM 1-methyl-3-isobutylxanthine) were carefully layered over the silicone fluid and allowed to preincubate for 5 min under 95% O_2/5% CO_2. Incubation was started by adding another 50 μl of modified Krebs bicarbonate buffer containing test substances, 1 μCi $^{45}CaCl_2$, and 1 μCi [6,6′(n)-^{3}H]-sucrose (9.8 Ci/mmol). The tubes were incubated in a Dubnoff shaker at 37°C under water-saturated 95% O_2/5% CO_2 at 40,rpm for 20 min. The incubation was stopped by adding 10 μl of 25 mM EGTA, and the islets were immediately separated from the incubation buffer by centrifugation for 20 s at maximum speed in a Beckman Microfuge B. The bottoms of the tubes were cut off at the junction of the 6 M urea and silicone fluid layers and placed in a scintillation vial to which 10 ml of Aquasol was added. Tubes without islets were run as blanks. ^{45}Ca and ^{3}H were counted with a Beckman LS7500 scintillation counter equipped with a data reduction accessory for dual label counting. $^{45}Ca^{2+}$ uptake per islet was calculated after subtraction of radioactivity in blanks and correction for extracellular $^{45}Ca^{2+}$ based on [^{3}H]-sucrose present.

Statistical analysis. Results are reported as mean ± SEM, and significance of difference was calculated by Student's *t*-test. Outliers were eliminated by the Q-test.

Materials. SaRI 59-801 was synthesized by Dr. Jeffrey Nadelson and Leonard J. Brand, Sandoz Research Institute. Tolbutamide, nifedipine, and verapamil were gifts from the Upjohn Co., Kalamazoo, Michigan; Bayer AG, Alemania, FRG; and Knoll Pharmaceutical Co., Whippany, New Jersey, respectively. Commercial sources of materials were: Aquasol, $^{45}CaCl_2$, and cAMP radioimmunoassay kits, New England Nuclear; [6,6′(n)-^{3}H]-sucrose and insulin radioimmunoassay kits, Amersham, Arlington Heights, Illinois; forskolin, Calbiochem, La Jolla, California; Versilube F50 silicone oil, General Electric, Waterford, New York; silicone fluid 6428-R15, Thomas Scientific, Philadelphia, Pennsylvania; and type IV collagenase, Worthington, Freehold, New Jersey.

TABLE 1
Effect of 1-methyl-3-isobutylxanthine, theophylline, dibutyryl cAMP, or forskolin on stimulation of insulin release by SaRI 59-801 or tolbutamide

Addition	Insulin* (μU/islet/h)	P†	P‡
A. None	38.2 ± 4.2		
1 mM 1-Methyl-3-isobutylxanthine	55.5 ± 4.4	<0.05	<0.05
0.3 mM 59-801	39.0 ± 2.1		>0.1
0.3 mM 59-801 + 1 mM 1-methyl-3-isobutylxanthine	178 ± 11.8	<0.001	<0.001
0.37 mM Tolbutamide	56.6 ± 7.4		<0.1
0.37 mM Tolbutamide + 1 mM 1-methyl-3-isobutylxanthine	172 ± 4.3	<0.001	<0.001
B. None	19.8 ± 3.9		
10 mM Theophylline	39.9 ± 3.6	<0.02	<0.02
0.3 mM 59-801	31.5 ± 12.3		>0.1
0.3 mM 59-801 + 10 mM theophylline	119 ± 10	<0.01	<0.001
0.37 mM Tolbutamide	19.2 ± 6.3		>0.1
0.37 mM Tolbutamide + 10 mM theophylline	79.5 ± 6.3	<0.005	<0.002
C. None	27.6 ± 6.6		
1 mM Dibutyryl cAMP	77.4 ± 26.7	>0.1	>0.1
0.3 mM 59-801	57.3 ± 18		>0.1
0.3 mM 59-801 + 1 mM dibutyryl cAMP	169 ± 37	<0.1	<0.05
0.37 mM Tolbutamide	48.0 ± 10.8		>0.1
0.37 mM Tolbutamide + 1 mM dibutyryl cAMP	198 ± 8	<0.001	<0.001
D. None	12.6 ± 3.6		
0.1 mM Forskolin	32.4 ± 12	>0.1	>0.1
0.3 mM 59-801	45.3 ± 7.8		<0.02
0.3 mM 59-801 + 0.1 mM forskolin	105 ± 17.1	<0.05	<0.01
0.37 mM Tolbutamide	27.6 ± 6.9		>0.1
0.37 mM Tolbutamide + 0.1 mM forskolin	95.7 ± 15.9	<0.02	<0.01

*Mean ± SEM for 3 groups of 10 islets in A and 3 groups of 5 islets in B, C, and D. Islets were incubated as described in MATERIALS AND METHODS, except 1-methyl-3-isobutylxanthine was omitted unless noted.
†Plus or minus 1-methyl-3-isobutylxanthine in A, ±theophylline in B, ±dibutyryl cAMP in C, and ±forskolin in D.
‡Versus no addition.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/34/7/691/354154/34-7-691.pdf by guest on 30 March 2023

TABLE 2
cAMP content of islets treated with SaRI 59-801, tolbutamide, and/or 1-methyl-3-isobutylxanthine

Addition	cAMP* (fmol/islet)	P†	P‡
None	20.8 ± 4.3		
1 mM 1-Methyl-3-isobutylxanthine	147 ± 15	<0.001	
0.3 mM 59-801	23.5 ± 4.3	>0.1	
0.3 mM 59-801 + 1 mM 1-methyl-3-isobutylxanthine	141 ± 13	<0.001	>0.1
0.37 mM Tolbutamide	24.6 ± 6.1	>0.1	
0.37 mM Tolbutamide + 1 mM 1-methyl-3-isobutylxanthine	267 ± 57	<0.001	<0.1

*Results from three separate experiments were combined, N = 9.
†Versus no addition.
‡Versus 1 mM 1-methyl-3-isobutylxanthine.

RESULTS

Islet cAMP. When islets were incubated with 3 mM glucose, 0.3 mM 59-801 or 0.37 mM tolbutamide alone produced little stimulation of insulin secretion, but addition of any of several agents that can elevate cAMP allowed these compounds to stimulate insulin release (Table 1). Addition of the cAMP phosphodiesterase inhibitors, 1 mM 1-methyl-3-isobutylxanthine or 10 mM theophylline, allowed a four- to sixfold stimulation of insulin release by 59-801 or tolbutamide. The phosphodiesterase inhibitors alone produced only a relatively small stimulation of insulin release from islets incubated with 3 mM glucose. Addition of 1 mM dibutyryl cAMP also was effective at stimulating insulin release in combination with tolbutamide or 59-801, but was relatively ineffective alone.

Forskolin (0.1 mM), an activator of adenylate cyclase in many tissues,[9] also greatly elevated cAMP in islets (from 18.7 ± 2.0 to 131 ± 9 fmol/islet, $P < 0.002$). Forskolin alone produced a small elevation of insulin release that was not statistically significant (Table 1). However, forskolin potentiated the action of 59-801 or tolbutamide to allow an eightfold stimulation of insulin release from islets incubated with 3 mM glucose.

These results suggested that 59-801 or tolbutamide do not elevate islet cAMP, but require elevation of cAMP by some other means to be able to effectively stimulate insulin release. To obtain more direct evidence for this, islet cAMP concentrations were measured by radioimmunoassay (Table 2). In medium containing 3 mM glucose, basal cAMP was 20.8 ± 4.3 fmol/islet. One millimolar 1-methyl-3-isobutylxanthine elevated cAMP by 604%, whereas 0.3 mM 59-801 or 0.37 mM tolbutamide did not significantly affect islet cAMP. With 59-801 plus 1-methyl-3-isobutylxanthine, cAMP levels were also elevated but were not significantly different from that with the diesterase inhibitor alone. The addition of tolbutamide increased cAMP by 82% over the value with 1-methyl-3-isobutylxanthine alone, but this effect was of borderline statistical significance. Because stimulation of insulin release by 59-801 or tolbutamide was potentiated by cAMP, 1 mM 1-methyl-3-isobutylxanthine was added to the incubation medium to elevate cAMP in all other experiments reported.

Requirement for Ca^{2+} uptake. The requirement for Ca^{2+} uptake for stimulation of insulin release by 59-801 was explored because of its importance for many other insulin secretagogues. Ca^{2+} channel blockers have been shown to inhibit uptake of Ca^{2+} and release of insulin by islets.[4] As shown in Figure 1, verapamil, $CoCl_2$, or nifedipine each decreased the stimulation of insulin release by 59-801 or tolbutamide. When Ca^{2+} was omitted from the incubation medium, and 1 mM EGTA was added to remove residual Ca^{2+}, 59-801 and tolbutamide did not stimulate insulin release (control, 43.2 ± 4.8 μU/islet/h; 0.3 mM 59-801, 53.4 ± 10.2, $P > 0.1$; 0.37 mM tolbutamide, 39.3 ± 5.7, $P > 0.1$, N = 7 in each group).

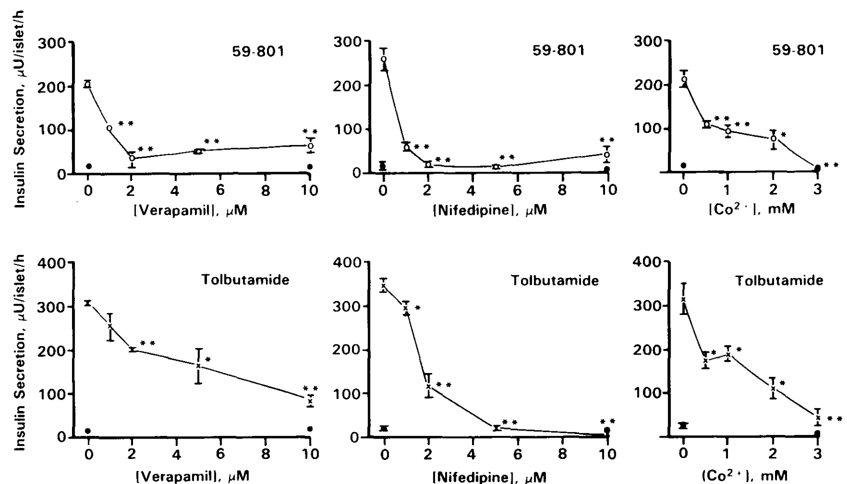

FIGURE 1. Inhibition of 59-801- or tolbutamide-stimulated insulin secretion by verapamil, nifedipine, or $CoCl_2$. The glucose concentration was 3 mM. Each point is a mean value for three groups of five islets with SEM shown as a vertical bar. ●, No secretagogue; ○, 59-801; and x, tolbutamide. *$P < 0.05$; **$P < 0.005$ versus no inhibitor.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/34/7/691/354154/34-7-691.pdf by guest on 30 March 2023

TABLE 3
Effect of SaRI 59-801, tolbutamide, or glucose on $^{45}Ca^{2+}$ uptake by isolated rat islets

Addition	$^{45}Ca^{2+}$ uptake (pmol/islet/20 min)	N	P*	P†
A. None	2.11 ± 0.32	15		
50 μM Verapamil	1.64 ± 0.19	15	>0.1	
0.3 mM 59-801	4.64 ± 0.46	15	<0.001	
0.3 mM 59-801 + 50 μM verapamil	2.93 ± 0.50	16	>0.1	<0.02
B. None	2.22 ± 0.32	14		
50 μM Verapamil	1.92 ± 0.57	12	>0.1	
0.37 mM Tolbutamide	6.06 ± 0.55	14	<0.001	
0.37 mM Tolbutamide + 50 μM verapamil	2.12 ± 0.33	14	>0.1	<0.001
C. None	3.04 ± 0.25	7		
20 mM Glucose	5.66 ± 0.61	6	<0.005	
20 mM Glucose + 50 μM verapamil	3.90 ± 0.55	7	>0.1	<0.1

*Versus no addition.
†Plus or minus verapamil.

The experiments with inhibitors of Ca^{2+} uptake or omission of Ca^{2+} showed that Ca^{2+} uptake is necessary for stimulation of insulin release by 59-801. To more directly measure an effect of 59-801 on Ca^{2+} uptake by islets, uptake of $^{45}Ca^{2+}$ was studied (Table 3). During a 20-min incubation period with 2.8 mM glucose, uptake of $^{45}Ca^{2+}$ was stimulated 120% by 0.3 mM 59-801. The increment of uptake stimulated by 59-801 was inhibited 68% by 50 μM verapamil, but basal uptake was not significantly inhibited by the Ca^{2+} channel blocker. In agreement with the results of Malaisse et al.,[10] 0.37 mM tolbutamide at 2.8 mM glucose, or 20 mM glucose also increased $^{45}Ca^{2+}$ uptake during a 20-min period by 173% and 86%, respectively. In both cases, $^{45}Ca^{2+}$ uptake was reduced by verapamil.

DISCUSSION

Several different methods were used to investigate the relationship of islet cAMP to stimulation of insulin release by 59-801. Both 59-801 and tolbutamide were potentiated by dibutyryl cAMP, forskolin, or by the phosphodiesterase inhibitors, theophylline and 1-methyl-3-isobutylxanthine. We conclude, therefore, that the major effect of these hypoglycemic agents is not due to elevation of islet cAMP. Tolbutamide has been reported to activate adenylate cyclase,[5,11] inhibit phosphodiesterase,[5,11] and transiently elevate islet cAMP.[12,13] However, more recent reports have cast doubts on the significance of these results to the mechanism of stimulation of insulin release by sulfonylureas.[14,15] In agreement with our results, 1-methyl-3-isobutylxanthine[13] and theophylline[14] have been shown to increase insulin release in response to tolbutamide.

Direct measurement of islet cAMP by radioimmunoassay supported the indirect studies. SaRI 59-801 or tolbutamide did not significantly affect islet cAMP levels, although 1-methyl-3-isobutylxanthine greatly increased cAMP concentrations both in the presence or absence of the hypoglycemic agents. Reports of elevated cAMP in response to tolbutamide indicated a rapid increase followed by a decline in 10 or 20 min.[12,13] The lack of effect of tolbutamide on islet cAMP in our experiments may be because the level at 45 min after addition of drug was measured. Islet perifusion experiments will be necessary to establish the time course of insulin release stimulated by 59-801 and to determine whether there is more than one phase of release, as with some other secretagogues. When this information is available, the cAMP levels at other time points would be of interest.

Extracellular Ca^{2+} is required for stimulation of insulin release from rat pancreas by tolbutamide and glucose.[16] Co^{2+8} and verapamil[10] are known to inhibit glucose-stimulated Ca^{2+} uptake and insulin release by islets. However, Co^{2+} has recently been reported to inhibit glucose-stimulated insulin release from rat islets by a mechanism independent of inhibition of Ca^{2+} uptake.[17] Another Ca^{2+} channel blocker, nifedipine, has also been reported to be a potent inhibitor of glucose-stimulated insulin release by rat islets.[18] Verapamil has been shown to block stimulation of insulin release by a sulfonylurea[19] and to inhibit tolbutamide-stimulated Ca^{2+} uptake by islets.[10] In agreement with these reports, we found that stimulation of insulin release by tolbutamide was blocked by the absence of Ca^{2+} or by the addition of the Ca^{2+} antagonists, verapamil, nifedipine, or Co^{2+}. The action of 59-801 also required external Ca^{2+} and was inhibited by Co^{2+}, nifedipine, or verapamil. This indirect evidence indicated that 59-801 stimulates insulin release by causing uptake of Ca^{2+} into islet cells. Further evidence was obtained by measurement of $^{45}Ca^{2+}$ accumulation by islets, which was stimulated by 59-801, tolbutamide, or 20 mM glucose and inhibited by verapamil in each case. Of course, this technique does not discriminate between net uptake and $^{45}Ca^{2+}$–$^{40}Ca^{2+}$ exchange.

Increased Ca^{2+} uptake can occur in several ways. Some possibilities are: ionophorous activity, which has been found for tolbutamide[15] but not 59-801 (R. L. Hanson, unpublished results); activation of the Ca^{2+} channel by depolarization of the cell membrane, in response to elevated K^+ or ouabain, for example; or by a more direct effect on the Ca^{2+} channel. Further studies are necessary to more precisely determine the mechanism by which 59-801 stimulates Ca^{2+} uptake and thereby stimulates insulin release.

ACKNOWLEDGMENTS

We thank Dr. Ali Surve and his associates for performance of the insulin radioimmunoassays.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/34/7/691/354154/34-7-691.pdf by guest on 30 March 2023

This manuscript is dedicated to Eugene P. Kennedy on the occasion of his 65th birthday.

REFERENCES

[1] Ho, R. S., Wiseberg, J. J., Brand, L. J., Nadelson, J., and Boyajy, L. D.: A novel, orally effective hypoglycemic agent, SaRI 59-801, in laboratory animals. In press. Drug Research Development, 1985.

[2] Hanson, R. L., Isaacson, C. M., and Boyajy, L. D.: Stimulation of insulin secretion from isolated rat islets by SaRI 59-801. Diabetes 1985; 34:548–52.

[3] Hedeskov, C. J.: *Mechanism of glucose-induced insulin secretion.* Physiol. Rev. 1980; 60:442–509.

[4] Wollheim, C. B., and Sharp, G. W. G.: Regulation of insulin release by calcium. Physiol. Rev. 1981; 61:914–73.

[5] Sharp, G. W. G.: The adenylate cyclase-cyclic AMP system in islets of Langerhans and its role in the control of insulin release. Diabetologia 1979; 16:287–96.

[6] Lacy, P. E., and Kostianovsky, M.: Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 1967; 16:35–39.

[7] Lernmark, Å., Nathans, A., and Steiner, D. F.: Preparation and characterization of plasma membrane-enriched fractions from rat pancreatic islets. J. Cell Biol. 1976; 71:606–23.

[8] Henquin, J. C., and Lambert, A. E.: Cobalt inhibition of insulin secretion and calcium uptake by isolated rat islets. Am. J. Physiol. 1975; 228:1669–77.

[9] Seamon, K., Padgett, W., and Daly, J. W.: Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc. Natl. Acad. Sci. USA 1981; 78:3363–67.

[10] Malaisse, W. J., *Herchuelz, A., Levy, J., and Sener, A.: Calcium antagonists* and islet function. III. The possible site of action of verapamil. Biochem. Pharmacol. 1977; *26*:735–40.

[11] Montague, W., and Howell, S. L.: Cyclic AMP and the physiology of the islets of Langerhans. *In* Advances in Cyclic Nucleotide Research, Vol. 6. Greengard, P., and Robison, G. A., Eds. New York, Raven Press, 1975:201–43.

[12] Charles, M. A., Lawecki, I., Steiner, A. D., and Grodsky, G. M.: Cyclic nucleotides in pancreatic islets. Tolbutamide- and arginine-induced insulin release. Diabetes 1976; 25:256–59.

[13] Grill, V., and Cerasi, E.: Interacting effects of sulfonylureas and glucose on cyclic AMP metabolism and insulin release in pancreatic islets of the rat. J. Clin. Invest. 1978; 61:1346–54.

[14] Henquin, J. C.: Tolbutamide stimulation and inhibition of insulin release: studies of the underlying ionic mechanisms in isolated rat islets. Diabetologia 1980; 18:151–60.

[15] Couturier, E., and Malaisse, W. J.: Insulinotropic effects of hypoglycaemic and hyperglycaemic sulphonamides: the ionophoretic hypothesis. Diabetologia 1980; 19:335–40.

[16] Curry, D. L., Bennett, L. L., and Grodsky, G. M.: Requirement for calcium ion in insulin secretion by the perfused rat pancreas. Am. J. Physiol. 1968; 214:174–78.

[17] Wollheim, C. B., and Janjic, D.: Cobalt inhibition of insulin release: evidence for an action not related to Ca^{2+} uptake. Am. J. Physiol. 1984; 246:C57–62.

[18] Malaisse, W. J., and Sener, A.: Calcium-antagonists and islet function. XII. Comparison between nifedipine and chemically related drugs. Biochem. Pharmacol. 1981; 30:1039–41.

[19] Devis, G., Somers, G., Van Obberghen, E., and Malaisse, W. J.: Calcium antagonists and islet function. I. Inhibition of insulin release by verapamil. Diabetes 1975; 24:547–51.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/34/7/691/354154/34-7-691.pdf by guest on 30 March 2023