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Lack of Ca^{2+} Ionophoretic Activity of Hypoglycemic Sulfonylureas in Excitable Cells and Isolated Secretory Granules

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SUMMARY

β-Cell-rich pancreatic islets, neurohypophyses, and adrenal medullae were used for exploring whether hypoglycemic sulfonylureas exhibit Ca^{2+} ionophoretic properties. Exposure of these excitable organs to depolarizing concentrations of K^+ resulted in stimulation both of ^{45}Ca uptake and efflux. Although tolbutamide does not bind preferentially to pancreatic islets, this sulfonylurea was specific in stimulating the fluxes of ^{45}Ca in these endocrine specimens. A chromaffin granule preparation was used for studies of both the net transport of Ca^{2+} with the metallochromic indicator arsenazo III and the proton concentration gradient (ΔpH) with the fluorescent probe 9-aminoacridine. Even at high concentrations, tolbutamide and glibenclamide did not mediate Ca^{2+}-H^+ exchange diffusion whether or not the granules were made permeable to protons by the addition of the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, nor did the sulfonylureas affect Ca^{2+}-H^+ exchange diffusion induced by the addition of A-23187. The data indicate that the Ca^{2+} fluxes associated with sulfonylurea-stimulated insulin secretion do not result from the Ca^{2+}-ionophoretic properties of the drugs but rather reflect depolarization of the β-cells.

INTRODUCTION

The mechanisms by which hypoglycemic sulfonylureas stimulate insulin secretion are still incompletely understood. Considerable attention has been paid recently to the idea that they function as Ca^{2+} ionophores and/or can interact directly with native Ca^{2+} ionophores in the β-cell plasma membrane (1–7). This concept is based essentially on studies in which the translocation of ^{45}Ca has been measured into or across a hydrophobic immiscible domain containing sulfonylurea, antibiotic ionophores, or pancreatic islet extracts. The physiological relevance of these observations has been a matter of controversy (8–11). In the present study the effects of sulfonylureas on ^{45}Ca fluxes in pancreatic islets were compared with those in other excitable endocrine organs. Tests were also conducted to determine whether the drugs affect net fluxes of Ca^{2+} and proton distribution in a chromaffin granule preparation. The results suggest that sulfonylureas interact specifically with the β-cell plasma membrane. The drugs neither acted as fully competent Ca^{2+} ionophores nor influenced the activity of native or antibiotic ionophores.

MATERIALS AND METHODS

β-Cell-rich pancreatic islets were microdissected from non-inbred *ob/ob* (12) mice that had been fasted overnight. Adrenal medullae and neurohypophyses were taken from the normal littermates of these mice. A local slaughterhouse supplied the bovine adrenals used for preparation of chromaffin granules on isotonic Percoll density gradients (13). The basal medium used for the isolation of the mouse tissues and the subsequent studies of ^{45}Ca uptake and efflux was a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4) physiologically balanced with cations; Cl^- was the sole anion (14). For efflux studies the organs were loaded with 1.28 mM ^{45}Ca (390 Ci/mole) during 90 min of incubation in medium supplemented with 3 mM glucose and 25 mM KCl (equimolar substitution for NaCl). The procedures for measuring the uptake of La^{3+}-nondisplaceable ^{45}Ca (15) and ^{45}Ca efflux (16) have been described previously. The ^{45}Ca uptake and efflux experiments were terminated by freeze drying the organ specimens and weighing them on a quartz fiber balance. The data were expressed as millimoles of ^{45}Ca per kilogram of dry weight, assuming the same specific radioactivity as in the loading medium.

Chromaffin granules were suspended in 0.27 M sucrose buffered at pH 7.0 with 30 mM Tris-maleate. Protein was measured by the method of Lowry et *al.* (17) with bovine serum albumin as reference. Variations in the transmembrane proton concentration gradient (ΔpH) across the chromaffin granule membrane were monitored with the membrane-permeable fluorescent dye 9-aminoacridine (18). Net fluxes of Ca^{2+} were measured with the metallochromic indicator arsenazo III by dual wavelength

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spectrophotometry (19) using a time-sharing multichannel spectrophotometer (20). Details about the experiments are given in the legends to the figures and the table.

Sulfonylureas and ionophores were added as 1000-fold concentrated stock solutions in dimethyl sulfoxide and other additives as 100- to 1000-fold concentrated aqueous solutions. Statistical significances were calculated from mean values ± standard error of the mean of differences between paired test and control experiments.

RESULTS

Table 1 shows the effect of 1 mM tolbutamide and 25 mM K^+ on the uptake of La^{3+}-nondisplaceable ^{45}Ca in the different endocrine organs. K^+ depolarization was associated with an increased ^{45}Ca uptake in pancreatic islets, neurohypophyses, and adrenal medullae, whereas tolbutamide stimulated the uptake only into the islets. The specificity of tolbutamide for the pancreatic islets was apparent also when studying the efflux of ^{45}Ca taken up in response to K^+ depolarization. In contrast to the stimulatory effect of K^+ on all of the organs, tolbutamide stimulated the radioactive efflux only from the islets (Fig. 1).

The possibility that sulfonylureas possess Ca^{2+} ionophoretic properties was also tested more directly in a chromaffin granule preparation by measurements of the proton gradient (Figs. 2 and 3) and net Ca^{2+} transport (Figs. 4 and 5). In the presence of Ca^{2+} the antibiotic Ca^{2+} ionophore A-23187 induced an H^+-Ca^{2+} counter-transport indicated by both the collapsing proton gradient (Fig. 2) and the concomitant net uptake of Ca^{2+} (Fig. 4). Tolbutamide (100 μM or 1 mM) or glibenclamide (10 μM or 100 μM) did not mimic these effects of A-23187 even in the presence of the proton ionophore FCCP[1] (Figs. 2–5), nor did the sulfonylureas affect the rates of proton gradient collapse (Fig. 3) or Ca^{2+} uptake at low concentrations of A-23187 (Fig. 5). The small decrease in Δ absorbance observed upon the addition of 1 mM tolbutamide (Fig. 5) was not due to Ca^{2+} uptake by the chromaffin granules, since an identical effect was observed when the sulfonylurea was added to a medium lacking granules.

TABLE 1

Effects of tolbutamide and K^+ on the uptake of La^{3+}-nondisplaceable ^{45}Ca by different endocrine organs

Pancreatic islets were microdissected from *ob/ob* mice, and neurohypophyses and adrenal medullae were taken from their normal littermates. Groups of three islets or two pieces of the neurohypophysis and the adrenal medulla (approximately one-half of the gland) were incubated for 90 min at 37° in 200 μl (islets) or 400 μl of basal medium containing 3 mM glucose, 1.28 mM ^{45}Ca (15.6 or 7.8 Ci/mole), and the additives stated. When KCl was used as the test substance, osmotic compensation was achieved by equimolar reduction of the concentration of NaCl. La^{3+}-nondisplaceable uptake of ^{45}Ca is expressed in terms of labeled Ca^{2+} with the same specific activity as in the medium. Values are means ± standard error of the mean for 9 or 10 experiments.

Organ and additive	^{45}Ca uptake	
	Primary data	Test minus control
	mmoles/kg protein	
Neurohypophysis		
None (control)	4.98 ± 0.36	
K^+ (25 mM)	7.90 ± 0.33	2.91 ± 0.53[a]
Tolbutamide (1 mM)	4.84 ± 0.96	−0.14 ± 0.48
Adrenal medulla		
None (control)	9.85 ± 1.25	
K^+ (25 mM)	12.61 ± 0.97	2.76 ± 0.73[b]
Tolbutamide (1 mM)	10.57 ± 1.27	0.72 ± 1.43
Islets		
None (control)	4.72 ± 0.41	
K^+ (25 mM)	8.53 ± 0.75	3.81 ± 0.48[a]
Tolbutamide (1 mM)	6.42 ± 0.54	1.70 ± 0.50[b]

[a] $p < 0.001$.
[b] $p < 0.01$.

DISCUSSION

The direct stimulatory action of hypoglycemic sulfonylureas on the pancreatic β-cell secretion of insulin is well established (21). The effect is associated with a sustained depolarization of the β-cell (22). There is a requirement for extracellular Ca^{2+} (23), and blocking of the voltage-dependent Ca^{2+} channels in the plasma membrane results in secretory inhibition (24). Sulfonylurea stimulation of the entry of Ca^{2+} is evident from an increased uptake of ^{45}Ca (10). Furthermore, the efflux of the isotope from preloaded islets is increased, presumably due to a Ca-Ca exchange resulting from enhanced entry of nonradioactive Ca^{2+} (10, 11). In analogy with the effects of sulfonylureas, exposure of pancreatic β-cells to A-23187 or high K^+ concentrations leads to stimulation of insulin release by increased entry of calcium. The sulfonylureas consequently may act as classical Ca^{2+} ionophores and/or increase the uptake of Ca^{2+} by opening voltage-dependent channels after β-cell depolarization.

The effects of hypoglycemic sulfonylureas are not restricted to stimulation of the pancreatic β-cells. The drugs also affect hormone release from other endocrine cells (25, 26). However, this influence is not always stimulatory, as indicated by the diminished secretion of pancreatic glucagon (25) and adrenal catecholamines (26). The evidence that sulfonylureas act as Ca^{2+} ionophores has been obtained exclusively from experiments with artificial systems (1–7). Sulfonylureas induced translocation of ^{45}Ca from an aqueous buffer into or across a hydrophobic region consisting of toluene/butanol (2–5). The drugs have been reported to mediate exchange diffusion in a manner like that of A-23187, and their potency even increased in the presence of this antibiotic ionophore (5). Most important, the sulfonylureas potentiated the activity of an islet extract in facilitating exchange diffusion (1), and the drugs also enhanced the efflux of ^{45}Ca from liposomes (6). All of these observations were made after the addition of the sulfonylureas to the organic phase at concentrations much higher than those required for maximal stimulation of insulin release.

There are several lines of evidence suggesting that sulfonylureas do not act as ionophores in the pancreatic β-cells. First, no correlation has been found between the ionophoretic capacity and the insulinotropic potency of

[1] The abbreviation used is: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

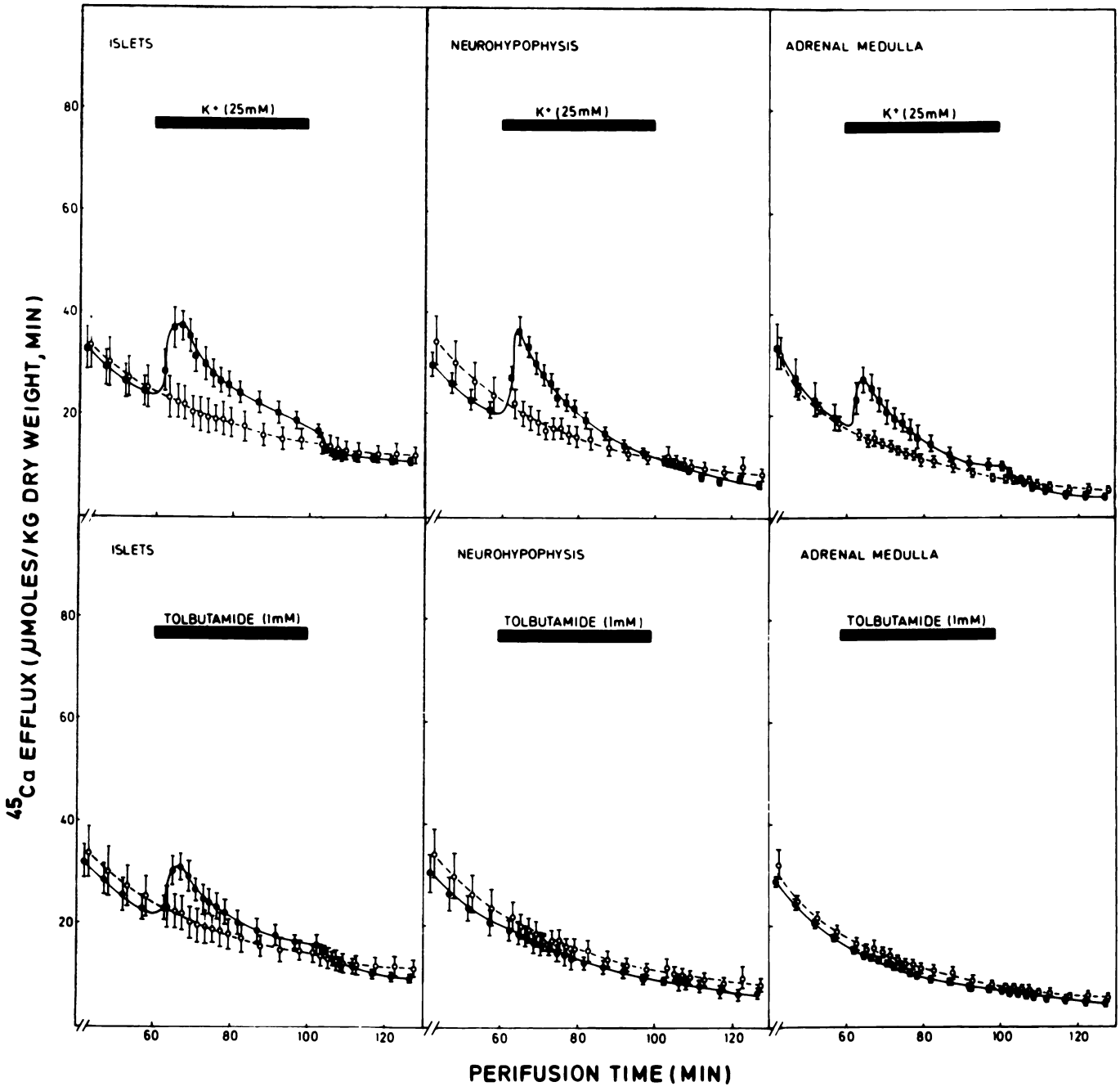

FIG. 1. *Effects of K^+ and tolbutamide on ^{45}Ca efflux*

Ten pancreatic islets, one or two neurohypophyses, and one adrenal medulla (three or four pieces) were loaded with ^{45}Ca in the presence of 3 mM glucose and a depolarizing concentration of K^+. The glucose-deficient perifusion medium was replaced either with an identical medium (○) or a medium containing the test substance (●) during the period indicated by the *horizontal bar*. In the *upper panels* the effect of K^+ depolarization was tested by replacing 25 mM NaCl with equimolar amounts of KCl; in the *lower panels* 1 mM tolbutamide was added. The results indicate mean values ± standard error of the mean for four experiments.

the drugs (4). Second, unlike genuine ionophores, sulfonylureas do not appear to penetrate the β-cell (21). Third, whereas the ionophore-stimulated efflux of ^{45}Ca from preloaded islets reaches particularly high levels in the absence of extracellular Ca^{2+} (27), sulfonylureas stimulate the efflux only when the ion is present in the medium (10). The present measurements of ^{45}Ca fluxes add the specificity of sulfonylureas for pancreatic islets to the arguments against an ionophoretic action of these drugs. Although the concentration of tolbutamide was about 10 times higher than that required for maximal insulin release, the drug did not significantly affect the Ca^{2+} fluxes in the neurohypophysis or adrenal medulla. Other studies have indicated that there is no preferential binding of sulfonylureas to the β-cells (28). In representing excitable tissues, all of the endocrine specimens reacted to K^+ depolarization with increased uptake and efflux of ^{45}Ca. The stimulated ^{45}Ca efflux can be explained by the

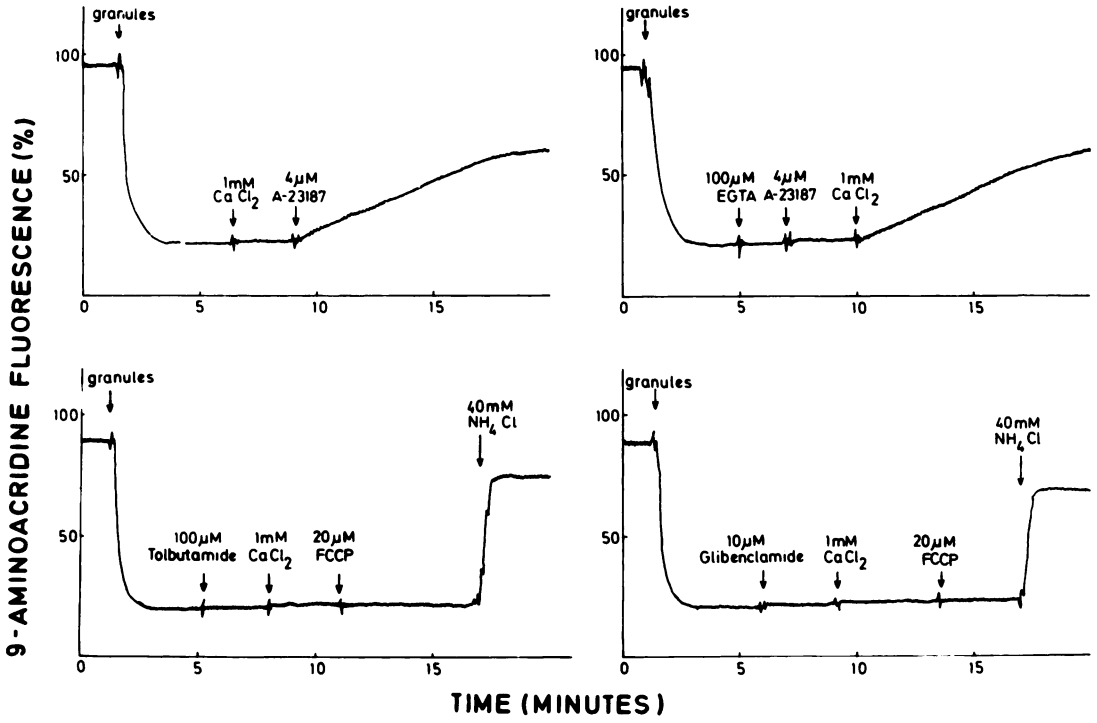

FIG. 2. *Effects of sulfonylureas on ΔpH in chromaffin granules*

The cuvette contained 90 μl of 0.27 M sucrose, 30 mM Tris-maleate (pH 7.0), and 11.1 μM 9-aminoacridine. After the addition of 10 μl of a chromaffin granule suspension (final protein concentration 5.1 mg/ml), the fluorescence of 9-aminoacridine (400/435 nm) was quenched by the ΔpH-dependent uptake of the dye. The *upper panels* show ΔpH after the addition of Ca^{2+} and A-23187. The *lower panels* indicate the corresponding data in the presence of Ca^{2+} and sulfonylureas alone or in combination with FCCP.

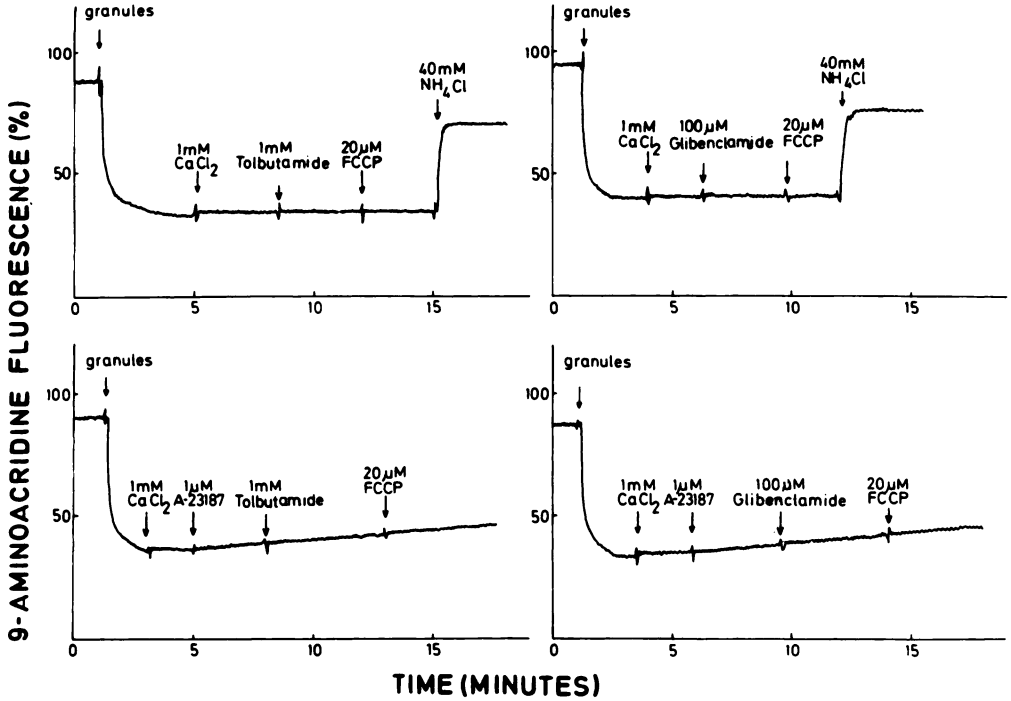

FIG. 3. *Effects of high concentrations of sulfonylureas on ΔpH in chromaffin granules*

The cuvette contained 90 μl of 0.27 M sucrose, 30 mM Tris-maleate (pH 7.0), and 11.1 μM 9-aminoacridine. After the addition of 10 μl of a chromaffin granule suspension (final protein concentration 7.1 mg/ml), the fluorescence of 9-aminoacridine (400/435 nm) was quenched by the ΔpH-dependent uptake of the dye. The *upper panels* show ΔpH after the addition of Ca^{2+} and sulfonylureas alone or in combination with FCCP. The *lower panels* indicate the corresponding data in medium supplemented with A-23187.

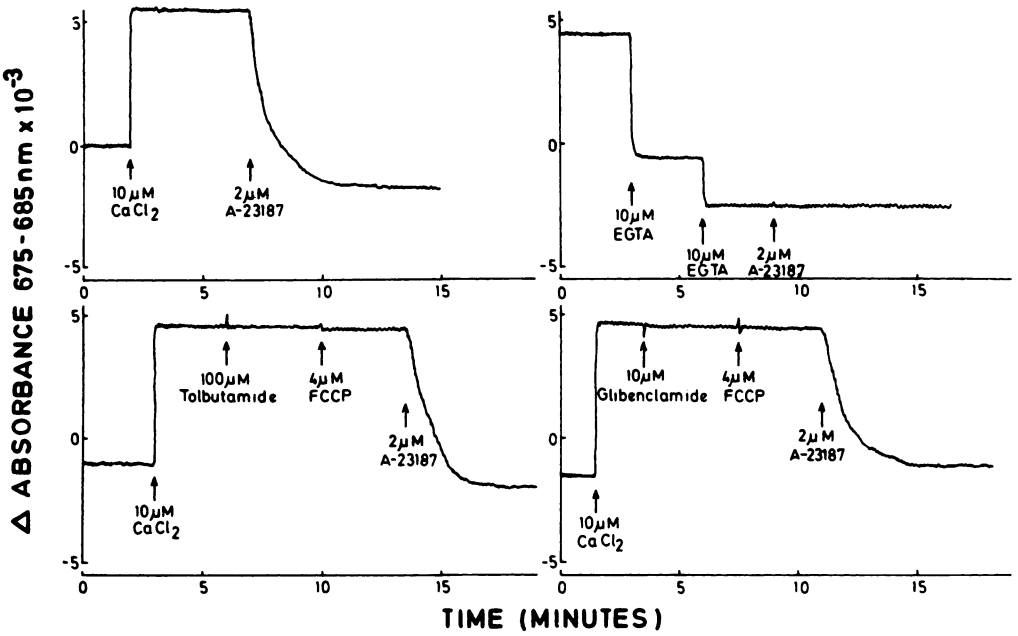

FIG. 4. *Effects of sulfonylureas on net Ca^{2+} fluxes in chromaffin granules*

The cuvette contained 250 μl of 0.27 M sucrose, 30 mM Tris-maleate (pH 7.0), 20 μM arsenazo III, and chromaffin granules (5.1 mg/ml). The absorbance difference at 675–685 nm of arsenazo III was used to indicate variations in the Ca^{2+} concentration of the medium. The *upper panels* show the concentration of Ca^{2+} in the medium after the addition of A-23187. The *lower panels* indicate the corresponding data in the presence of sulfonylureas and FCCP.

increased uptake of nonradioactive Ca^{2+} which subsequently exchanges with sequestered ^{45}Ca. The process of Ca-Ca exchange is consequently sufficient to overcome the competitive inhibition of the ^{45}Ca outward transport exerted by the entering Ca^{2+}.

The chromaffin granules are known to be essentially impermeable to H^+ and Ca^{2+} under the present experimental conditions (29). This impermeability makes the granules ideal for studies of ionophoretic actions. The interior of the granules is maintained at a relatively low

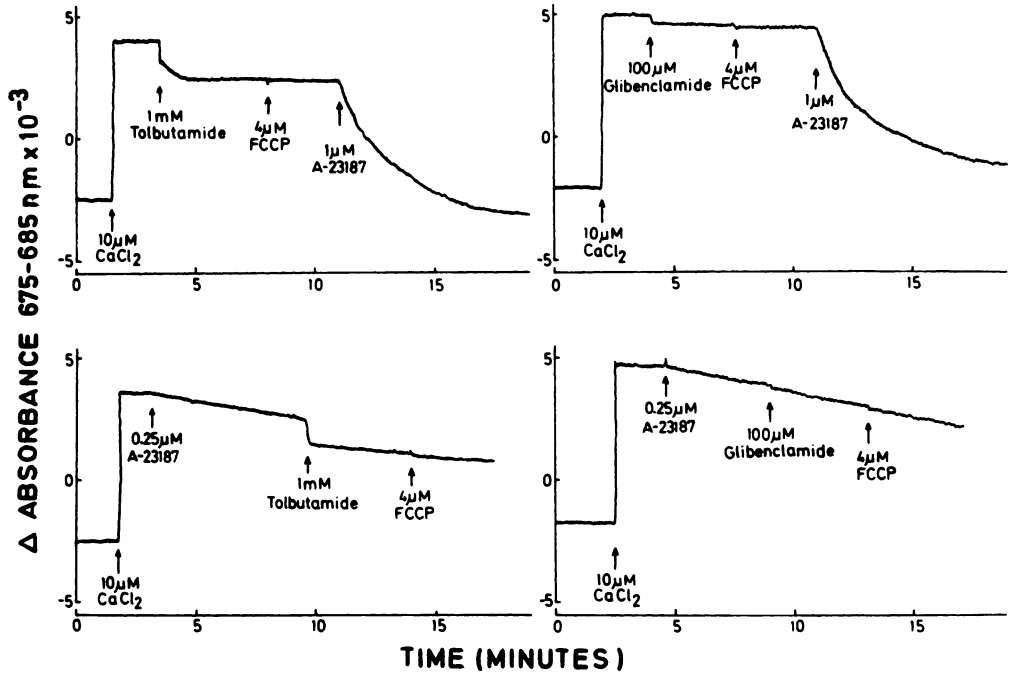

FIG. 5. *Effects of high concentrations of sulfonylureas on net Ca^{2+} fluxes in chromaffin granules*

The cuvette contained 250 μl of 0.27 M sucrose, 30 mM Tris-maleate (pH 7.0), 20 μM arsenazo III, and chromaffin granules (7.1 mg/ml). The absorbance difference at 675–685 nm of arsenazo III was used to indicate variations in the Ca^{2+} concentration of the medium. The *upper panels* show the concentration of Ca^{2+} in the medium after the addition of sulfonylureas alone or in combination with FCCP. The *lower panels* indicate the corresponding data in medium supplemented with A-23187.

pH (18, 29). Provided that Ca^{2+} is present, the addition of the Ca^{2+} ionophore A-23187 results in accumulation of Ca^{2+} at the expense of the proton gradient (29). This phenomenon can be explained as exchange diffusion with a Ca^{2+}/H^+ ratio of 1:2. The present data on proton distribution and net transport of Ca^{2+} confirm the observation that A-23187 mediates exchange of Ca^{2+} for H^+. However, concentrations of tolbutamide and glibenclamide up to 100 times higher than those necessary for maximal stimulation of insulin release did not promote Ca^{2+}-H^+ exchange even after the addition of FCCP to make the granule membrane permeable to protons. Furthermore, the sulfonylureas were unable to modify the rates of proton gradient collapse and the net uptake of Ca^{2+} induced by low concentrations of A-23187.

It can be concluded that tolbutamide and glibenclamide lack detectable Ca^{2+} ionophoretic or ionophore-regulating effects when tested in biological systems. Although sulfonylureas do not bind preferentially to the pancreatic β-cells (28), they exhibit a great degree of specificity in stimulating the entry of Ca^{2+} into these cells. It is likely that the observed ^{45}Ca fluxes results from opening of voltage-dependent Ca^{2+} channels subsequent to the sulfonylurea depolarization of the β-cells (22). The mechanism for this depolarization is unknown. A decreased K^+ conductance (8, 9) following the interaction of sulfonylureas with sulfhydryl groups in the β-cell plasma membrane (30) might be considered.

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REFERENCES

1. Anjaneyulu, K., R. Anjaneyulu, and W. J. Malaisse. The stimulus-secretion coupling of glucose-induced insulin release. XLIII. Na-Ca countertransport mediated by pancreatic islet native ionophores. *J. Inorg. Biochem.* **13**:179–188 (1980).
2. Anjaneyulu, R., K. Anjaneyulu, E. Couturier, and W. J. Malaisse. Opposite effects of hypoglycemic and hyperglycemic sulfonamides upon ionophore-mediated calcium transport. *Biochem. Pharmacol.* **29**:1879–1882 (1980).
3. Couturier, E., and W. J. Malaisse. Insulinotropic effects of hypoglycaemic and hyperglycaemic sulphonamides: the ionophoretic hypothesis. *Diabetologia* **19**:335–340 (1980).
4. Couturier, E., and W. J. Malaisse. Ionophoretic activity of hypoglycemic sulfonylureas. *Arch. Int. Pharmacodyn. Ther.* **245**:323–334 (1980).
5. Couturier, E., and W. J. Malaisse. Synergistic effects of hypoglycaemic sulphonylureas and antibiotic ionophores upon calcium translocation. *Br. J. Pharmacol.* **71**:315–320 (1980).
6. Deleers, M., E. Couturier, M. Mahy, and W. J. Malaisse. Calcium transport in liposomes containing hypoglycemic and hyperglycemic sulfonamides. *Arch. Int. Pharmacodyn. Ther.* **246**:170–172 (1980).
7. Malaisse, W. J., A. R. Carpinelli, and A. Herchuelz. Tolbutamide stimulates Ca^{2+} influx in islet cells without reducing K^+ conductance. *Diabetologia* **19**:85 (1980).
8. Henquin, J.-C. Tolbutamide stimulation and inhibition of insulin release: studies of underlying ionic mechanisms in isolated rat islets. *Diabetologia* **18**:151–160 (1980).
9. Henquin, J.-C. Mechanisms of tolbutamide-stimulation of pancreatic β-cells—a reply. *Diabetologia* **19**:162–163 (1980).
10. Hellman, B. Tolbutamide stimulation of ^{45}Ca fluxes in microdissected pancreatic islets rich in β-cells. *Mol. Pharmacol.* **20**:83–88 (1981).
11. Hellman, B. Lack of ionophoretic effect in tolbutamide-induced translocation of Ca^{2+} in pancreatic β-cells. *Diabetologia* **21**:281 (1981).
12. Hellman, B. Studies in obese-hyperglycemic mice. *Ann. N. Y. Acad. Sci.* **131**:541–558 (1965).
13. Carty, S. E., R. G. Johnson, and A. Scarpa. The isolation of intact adrenal chromaffin granules using isotonic Percoll density gradients. *Anal. Biochem.* **106**:438–445 (1980).
14. Hellman, B. The significance of calcium for glucose stimulation of insulin release. *Endocrinology* **97**:392–398 (1975).
15. Hellman, B. Calcium and pancreatic β-cell function. 3. Validity of the La^{3+}-wash technique for discriminating between superficial and intracellular ^{45}Ca. *Biochim. Biophys. Acta* **540**:534–542 (1978).
16. Gylfe, E., and B. Hellman. Calcium and pancreatic β-cell function: mobilization of glucose-sensitive ^{45}Ca from perifused islets rich in β-cells. *Biochim. Biophys. Acta* **538**:249–257 (1978).
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
18. Salama, G., R. G. Johnson, and A. Scarpa. Spectrophotometric measurements of transmembrane potential and pH gradients in chromaffin granules. *J. Gen. Physiol.* **75**:109–140 (1980).
19. Scarpa, A., F. J. Brinley, T. Tiffert, and G. R. Dubyak. Metallochromic indicators of ionized calcium. *Ann. N. Y. Acad. Sci.* **307**:86–112 (1978).
20. Chance, B., V. Legallais, J. Sorge, and N. Graham. A versatile time-sharing multichannel spectrophotometer, reflectometer, and fluorometer. *Anal. Biochem.* **66**:498–514 (1975).
21. Hellman, B. and I. B. Täljedal. Effects of sulfonylurea derivatives on pancreatic β-cells, in *Insulin II* (A. Hasselblatt, and F. von Bruchhausen, eds.). Springer-Verlag, Berlin, 175–194 (1975).
22. Meissner, H. P., and I. J. Atwater. The kinetics of electrical activity of β-cells in response to a "square wave" stimulation with glucose or glibenclamide. *Horm. Metab. Res.* **8**:11–16 (1976).
23. Curry, D. L., L. L. Bennett, and G. M. Grodsky. Requirement for calcium ion in insulin secretion by the perfused rat pancreas. *Am. J. Physiol.* **214**:174–178 (1968).
24. Malaisse, W. J., G. Devis, D. G. Pipeleers, and G. Somers. Calcium-antagonists and islet function. IV. Effect of D600. *Diabetologia* **12**:77–81 (1976).
25. Efendic, S., F. Enzmann, A. Nylen, K. Uvnäs-Wallensten, and R. Luft. Effect of glucose/sulfonylurea interaction on release of insulin, glucagon, and somatostatin from isolated perfused rat pancreas. *Proc. Natl. Acad. Sci. U. S. A.* **76**:5901–5904 (1979).
26. Hsu, C.-Y., G. Brooker, M. J. Peach, and T. C. Westfall. Inhibition of catecholamine release by tolbutamide and other sulfonylureas. *Science (Wash. D. C.)* **187**:1086–1087 (1975).
27. Hellman, B. Calcium and pancreatic β-cell function. 5. Mobilisation of a glucose-stimulated pool of intracellular ^{45}Ca by metabolic inhibitors and the ionophore A-23187. *Acta Endocrinol.* **90**:624–636 (1979).
28. Hellman, B., L.-Å. Idahl, H. Tjälve, Å. Danielsson, and Å. Lernmark. Beobachtungen zum wirkungsmechanismus des hypoglykämisch wirksammen sulfonylharnstoff-präparates HB 419. *Drug Res.* **19**:1472–1476 (1969).
29. Johnson, R. G., and A. Scarpa. Ion permeability of isolated chromaffin granules. *J. Gen. Physiol.* **68**:601–631 (1976).
30. Hellman, B., A. Lernmark, J. Sehlin, M. Söderberg, and I. B. Täljedal. On the possible role of thiol groups in the insulin-releasing action of mercurials, organic disulphides, alkylating agents and sulfonylureas. *Endocrinology* **99**:1398–1406 (1976).

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