References and Notes

1. P. Narahari, C. R. Bhatia, T. Gopalakrishna, R. K. Mitra, in *Evaluation of Seed Protein Alterations by Mutation Breeding* (STI/PUB/426, International Atomic Energy Agency, Vienna, 1976), pp. 119–127; C. W. Stuber, V. A. Johnson, J. W. Schmidt, *Crop Sci.* **2**, 506 (1962).
2. B. O. Eggum, in *Genetic Control of Diversity of Plants*, A. Muhammed, R. Aksel, R. C. von Borstel, Eds. (Plenum, New York, in press).
3. T. R. Sinclair and C. T. de Wit, *Science* **189**, 565 (1975).
4. F. W. T. Penning de Vries, A. H. M. Brunsting, H. H. van Laar, *J. Theor. Biol.* **45**, 339 (1974).
5. F. W. T. Penning de Vries, in *Photosynthesis and Productivity in Different Environments, International Biological Programme* (Cambridge Univ. Press, Cambridge, England, 1975), vol. 3, pp. 459–480.
6. B. J. Miflin, in *Proceedings of the 11th Colloquium of the International Potash Institute* (International Potash Institute, Bern, 1975), pp. 53–74.
7. L. T. Evans, in *Crop Physiology*, L. T. Evans, Ed. (Cambridge Univ. Press, Cambridge, England, 1975), pp. 327–355.
8. W. S. Spector, Ed., *Handbook of Biological Data* (Saunders, Philadelphia, 1956), p. 87.
9. R. B. Austin and H. G. Jones, in *Annual Report* (Plant Breeding Institute, Cambridge, England, 1974), pp. 20–73.
10. C. M. Perez, G. B. Cagampang, B. V. Esmama, U. Monserrate, B. O. Juliano, *Plant Physiol.* **51**, 537 (1972).
11. V. R. Johnson and P. J. Mattern, *Report of Research Findings—Improvement of Nutritional Quality of Wheat Through Increased Protein Content and Improved Amino Acid Balance. January 1973–March 1975* (Agency for International Development, Washington, D.C., 1975), p. 186.
12. A. P. Rhodes and G. Jenkins, *J. Agric. Sci.* **86**, 57 (1976).
13. D. T. Canvin, in *Proceedings of an International Workshop on Genetic Improvement of Seed Protein, March 1974* (National Academy of Sciences–National Research Council, Washington, D.C., 1976), pp. 172–195.
14. C. R. Bhatia, *Euphytica* **24**, 789 (1975).
15. F. H. McNeal, M. A. Berg, C. F. McGuire, V. R. Stewart, D. E. Baldridge, *Crop Sci.* **12**, 599 (1972).
16. H. J. Evans, Ed., *Enhancing Biological Nitrogen Fixation, Proceedings of a Workshop, June 6, 1974* (Division of Biological and Medical Sciences, National Science Foundation, Washington, D.C., 1975).
17. B. O. Eggum, in *Nuclear Techniques for Seed Protein Improvement* (STI/PUB/320, International Atomic Energy Agency, Vienna, 1973), pp. 391–408.
18. V. A. Johnson, P. J. Mattern, J. E. Stroike, K. D. Wilhelmi, *in Proceedings of the 2nd International Winter Wheat Conference* (Univ. of Nebraska, Lincoln, 1975), pp. 316–333.
19. The International Winter Wheat Performance Nursery is a cooperative program of the U.S. Department of Agriculture's Agricultural Research Service and the University of Nebraska, supported by funds from the U.S. Agency for International Development. Its purpose is to evaluate the relative agronomic performance of selected winter wheat genotypes under a wide range of environmental conditions in wheat-growing regions around the world.
20. O. E. Nelson, in *New Approaches to Breeding for Improved Plant Protein* (STI/PUB/212, International Atomic Energy Agency, Vienna, 1969), pp. 41–54.
21. L. Munck, *Hereditas* **72**, 1 (1972).
22. We are grateful to F. W. T. Penning de Vries and H. H. van Laar for the computer analysis of comparative energy requirements for the different maize and barley genotypes. Reprint requests should be sent to R.R.

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Calcium and Secretion: Distinction Between Two Pools of Glucose-Sensitive Calcium in Pancreatic Islets

Abstract. *D-Glucose, but not L-glucose or 3-O-methyl-D-glucose, stimulates $^{45}Ca^{2+}$ uptake by both lanthanum-displaceable and lanthanum-nondisplaceable pools in pancreatic islets. The nondisplaceable pool probably represents secretory granules, while the displaceable pool may be located in the β-cell membrane. Kinetic studies with isotopically labeled islets suggest that only the displaceable pool participates in the short-term coupling of the glucose stimulus with secretion.*

Glucose causes a net movement of Ca^{2+} from the extracellular to the intracellular space of pancreatic islets containing more than 90 percent of β-cells (*1*). On fractionation of ^{45}Ca-labeled islets, the isotope taken up in response to glucose was recovered with the insulin secretory granules (*2*). In intact islets the glucose-sensitive calcium pool displayed a marked inertia to provocations aimed at initiating its mobilization (*1*, *2*). Therefore, it is doubtful whether the short-term insulin-releasing action of glucose is mediated by mechanisms for intracellular Ca^{2+} uptake. The rapidity with which changes of the extracellular calcium influence insulin release (*3*) suggests that secretion may depend on a labile pool that is perhaps located in the periphery of the β-cells.

To measure specifically the intracellular calcium, in previous experiments we used lanthanum ions to wash the islets free of extracellular calcium and to prevent losses of intracellular ion (*1*). Lanthanum displaces calcium from cell surfaces. Therefore, if the β-cells contain a membrane-located calcium pool that is important for secretion, it is possible that the lanthanum wash technique prevented the detection of such a pool. In the present study we labeled the islets with ^{45}Ca and studied the rate of disappearance of isotope from both lanthanum-displaceable and lanthanum-nondisplaceable pools. The results indicated that the β-cells contain a glucose-sensitive calcium pool that is displaceable with lanthanum and shows the mobility required of a plausible mechanism for coupling the glucose stimulus with secretion.

Fresh islets containing more than 90 percent β-cells were microdissected from the pancreatic glands of noninbred *ob/ob* mice; the islets were not exposed to collagenase during the isolation procedure. They were incubated at 37°C in a salt-balanced tris or bicarbonate buffer (*1*) supplemented with sugars (see Fig. 1 and Tables 1 and 2). The cells were incubated for 10 or 120 minutes in buffer containing trace amounts of $^{45}CaCl_2$; the calcium concentration was 2.56 m*M* in all media. The ^{45}Ca-labeled islets were incubated for various periods of time in nonradioactive medium, and the radioactivity retained by the islets was measured by liquid-scintillation counting after they were freeze-dried (−40°C, 0.1 pascal) overnight, weighed on a quartz-fiber balance, and dissolved in hyamine. Before being freeze-dried, some islets were washed for 60 minutes with 2 m*M* $LaCl_3$ (*1*). The radioactivity of islets not washed with lanthanum will be referred to as "total" calcium, that of lanthanum-washed islets as "lanthanum-nondisplaceable" calcium, and the difference between the two groups as "lanthanum-displaceable" calcium. Samples of the $^{45}CaCl_2$-containing medium used for labeling the islets were used as external standards in the counting procedure. Islet radioactivities are expressed in terms of millimoles of calcium with same specific radioactivity as the medium used for labeling the islets.

When $^{45}Ca^{2+}$-labeled islets are placed in a nonradioactive buffer, the spontaneous disappearance of isotope is much slower from lanthanum-nondisplaceable than from lanthanum-displaceable pools; virtually all of the $^{45}Ca^{2+}$ retained by islets after 90 minutes of efflux is nondisplaceable with lanthanum (*1*). Therefore, in the present experiments we subjected the ^{45}Ca-labeled islets to very brief periods of incubation (0 to 5 minutes) in nonradiactive buffer. Figure 1 shows the retention of $^{45}Ca^{2+}$ by islets incubated with $^{45}CaCl_2$ for 120 minutes in the presence of 3 m*M* D-glucose, 20 m*M* D-glucose, or 3 m*M* D-glucose plus 17 m*M* L-glucose. D-glucose, but not L-glucose, stimulated the $^{45}Ca^{2+}$ uptake to the lanthanum-nondisplaceable pool, and there was only a negligible loss of isotope from this pool during efflux in nonradioactive buffer. After 5 minutes of efflux, the effect of 20 m*M* D-glucose on the lanthanum-nondisplaceable pool fully accounts for the increase of total islet $^{45}Ca^{2+}$ at this time point. However, after 1 or 2 minutes of efflux, the labeling of the total islet calcium appeared to be greater than that of the lanthanum-nondisplaceable pool. This difference sug-

gests that 20 m*M* D-glucose had also stimulated the incorporation of $^{45}Ca^{2+}$ into a lanthanum-displaceable pool of such mobility as to permit complete release of the label in only 5 minutes.

After incubation of the islets with $^{45}CaCl_2$ for 10 minutes, the radioactive labeling of the lanthanum-nondisplaceable pool was about one-third of the labeling achieved by incubating the islets for 120 minutes (compare Fig. 1 and Table 1). This is in contrast to the lanthanum-displaceable pool, the rather similar labeling or which after incubating the islets for 10 minutes (Table 1) or 120 minutes (Fig. 1) is consistent with it being more mobile than the lanthanum-nondisplaceable calcium. In the experiments shown in Table 1, islets were incubated with $^{45}CaCl_2$ together with 3 or 20 m*M* D-glucose, the effect of glucose being computed in each individual experiment. The presence of 20 m*M* D-glucose significantly increased the labeling of the lanthanum-nondisplaceable pool at all time points studied. Moreover, after 1 minute of efflux, the effect of 20 m*M* D-glucose on the total islet calcium was larger than that on the lanthanum-nondisplaceable pool ($P < .025$). This statistically significant difference strongly suggests that D-glucose stimulated the incorporation of $^{45}Ca^{2+}$ into both lanthanum-nondisplaceable and lanthanum-displaceable pools.

Table 1. Retention of $^{45}Ca^{2+}$ by different calcium pools in islets during efflux. Islets were first incubated for 10 minutes in ^{45}Ca-labeled tris buffer containing 3 or 20 m*M* D-glucose; they were then incubated for various periods of time in nonradioactive medium containing 3 m*M* glucose. The retention of label in each group is expressed as millimoles of Ca^{2+} with the same specific activities as the medium used for labeling the islets. The differences between parallel incubations in ^{45}Ca-labeled medium containing 20 or 3 m*M* glucose are also given; the significance of these differences were examined by Student's *t*-test (see footnotes to table). The values are means ± S.E. for the numbers of experiments stated in parentheses.

Efflux (time (minutes)	Glucose during labeling (m*M*)	Islet content of labeled calcium (mmole/kg dry weight)		
		Total	Nondisplaceable with lanthanum	Displaceable with lanthanum
0	3	24.75 ± 2.06 (11)	2.69 ± 0.23 (8)	22.06
0	20	26.32 ± 1.40 (11)	3.52 ± 0.24 (8)	22.80
0	Difference	1.57 ± 2.30 (11)	0.82 ± 0.23* (8)	
1	3	9.72 ± 0.76 (19)	2.18 ± 0.16 (16)	7.54
1	20	12.83 ± 0.66 (19)	3.36 ± 0.20 (16)	9.47
1	Difference	3.09 ± 0.60† (19)	1.15 ± 0.34‡ (16)	
2	3	7.31 ± 0.66 (11)	2.22 ± 0.20 (8)	5.09
2	20	8.93 ± 0.41 (11)	2.98 ± 0.27 (8)	5.95
2	Difference	1.62 ± 0.85 (11)	0.77 ± 0.17‡ (8)	
5	3	3.29 ± 0.20 (11)	1.54 ± 0.12 (7)	1.75
5	20	4.66 ± 0.21 (11)	2.42 ± 0.30 (7)	2.24
5	Difference	1.37 ± 0.27† (11)	0.87 ± 0.22‡ (7)	

*$P < .02$. †$P < .001$. ‡$P < .01$.

Table 2. Effects of various conditions during labeling on the subsequent retention of $^{45}Ca^{2+}$ during efflux for 1 minute. The efflux medium contained 3 m*M* D-glucose in all experiments. Differences from 3 m*M* D-glucose were evaluated by Student's *t*-test (see footnotes to table). Values are means ± S.E.

Compounds present during loading	Experiments (No.)	Islet content of labeled calcium (mmole/kg dry weights)		
		Total	Nondisplaceable with lanthanum	Displaceable with lanthanum
Labeling in tris buffer for 10 minutes				
3 m*M* D-glucose	8	10.32 ± 1.21	1.99 ± 0.17	8.33 ± 1.12
20 m*M* D-glucose	8	15.02 ± 1.69*	2.40 ± 0.14*	12.62 ± 1.67†
3 m*M* D-glucose plus 17 m*M* L-glucose	8	11.60 ± 1.13	2.01 ± 0.16	9.60 ± 1.16
3 m*M* D-glucose plus 17 m*M* 3-*O*-methyl--D-glucose	8	9.20 ± 1.24	1.70 ± 0.16	7.50 ± 1.25
Labeling in tris buffer for 10 minutes				
3 m*M* D-glucose	8	7.53 ± 1.35	1.74 ± 0.12	5.79 ± 1.30
20 m*M* D-glucose	8	11.26 ± 0.83*	2.49 ± 0.21*	8.77 ± 0.79†
20 m*M* D-glucose plus 125 μg/ml diazoxide	8	10.54 ± 0.85	1.74 ± 0.10	8.80 ± 0.88
Labeling in tris buffer for 120 minutes				
3 m*M* D-glucose	17	13.18 ± 0.87	6.43 ± 0.48	6.74 ± 0.90
20 m*M* D-glucose	10	25.67 ± 2.83‡	11.84 ± 1.04‡	13.83 ± 2.22*
3 m*M* D-glucose plus 17 m*M* L-glucose	7	14.76 ± 2.23	6.94 ± 0.65	7.82 ± 1.99
Labeling in bicarbonate buffer for 10 minutes				
3 m*M* D-glucose	8	15.57 ± 2.69	3.66 ± 0.30	11.90 ± 2.74
20 m*M* D-glucose	8	23.01 ± 3.14†	5.06 ± 0.49†	17.95 ± 2.81†

*$P < .01$. †$P < .05$. ‡$P < .001$.

To show even more convincingly that D-glucose affects a pool of very mobile calcium in the islets, we designed some experiments that would permit a strict comparison of the lanthanum-nondisplaceable and lanthanum-displaceable calcium pools in parallel incubations of islets from the same animals. Islets were incubated with $^{45}CaCl_2$ for 10 or 120 minutes and subsequently incubated for 1 minute in nonradioactive buffer. In each experiment half of the islets were then freeze-dried and their radioactivity counted, while the other half was first washed with lanthanum. The mean value and its error could thus be estimated for the lanthanum-displaceable calcium in a series of repeated experiments. In complete corroboration of the conclusions drawn from Fig. 1 and Table 1, incubation with $^{45}CaCl_2$ in the presence of 20 m*M* D-glucose resulted in a significantly greater labeling of the lanthanum-displaceable calcium pool than did loading in 3 m*M* D-glucose (Table 2). Neither L-glucose nor 3-*O*-methyl-D-glucose had such an effect. The effect of D-glucose was significant in both tris and bicarbonate buffer, although the magnitude of the $^{45}Ca^{2+}$ incorporation appeared greater in the bicarbonate buffer. The retention of lanthanum-displaceable $^{45}Ca^{2+}$ was not significantly influenced by the glucose concentration in the nonradioactive efflux medium (not shown). Thus, the effect of 20 m*M* D-glucose on the $^{45}Ca^{2+}$ uptake is predominantly to enhance the rate constant for association between Ca^{2+} and islets.

It is generally believed that Ca^{2+} participates in stimulus-secretion coupling. Several studies have shown that glucose influences the handling of $^{45}Ca^{2+}$ in pancreatic islets (*1, 2, 4*), but the exact role of the ion in insulin secretion is unclear. As previously reported (*1*) and confirmed here, D-glucose increases the incorporation of $^{45}Ca^{2+}$ into a lanthanum-nondisplaceable pool. This lanthanum-

nondisplaceable calcium appears to be associated with the insulin secretory granules (*2*).

The lanthanum-nondisplaceable $^{45}Ca^{2+}$ uptake in β-cells resembles the enhanced labeling of granule-derived vesicles in leucocidin-stimulated leucocytes (*5*). In either case it is unlikely that this $^{45}Ca^{2+}$ incorporation results from a general permeability increase of the plasma membranes to calcium (*1*, *5*). In leucocidin-induced exocytosis, the site of contact between the granule and plasma membranes is thought to be permeable to the secretory proteins as well as to $^{45}Ca^{2+}$, although a visible aperture need not appear; when the granule withdraws as a partly emptied vesicle, it will be labeled with the isotope (*5*). Exocytosis from β-cells is usually considered to proceed through a secretory aperture, the inward return of membrane material occurring in the form of endocytotic vesicles (*6*). Some of the lanthanum-nondisplaceable $^{45}Ca^{2+}$ in the β-cells is perhaps due to endocytosis, but endocytosis can hardly explain the microscopically demonstrable calcium uptake in true granule vesicles (*7*). The information available about exocytosis in the β-cells does not seem to rule out that in these cells, too, the granules are able to make brief contacts with the plasma membranes to release some insulin and take up calcium. That the lanthanum-nondisplaceable $^{45}Ca^{2+}$ uptake may depend on secretion is suggested by experiments with diazoxide, a potent inhibitor of glucose-induced insulin release (*8*). This drug completely abolished the stimulatory effect of glucose on the lanthanum-nondisplaceable pool, apparently without affecting the lanthanum-displaceable $^{45}Ca^{2+}$ uptake (Table 2).

Whatever the mechanism of granular uptake of $^{45}Ca^{2+}$, the lanthanum-nondisplaceable calcium has such a low mobility that it does not seem to mediate the initial signal for secretion (*1*). The glucose-sensitive calcium pool that is displaceable with lanthanum is likely to occur in the β-cells, since the islets under study contain more than 90 percent β-cells and exhibit insulin secretory responses to D-glucose in both the tris and bicarbonate buffers employed (*1*). The displacement with lanthanum indicates that the calcium is located superficially in the cells, perhaps in the β-cell plasma membranes. Although we cannot be certain that this calcium pool plays a causative role in secretion, it is notable that it responded to D-glucose but not to L-glucose or 3-*O*-methyl-D-glucose. Among these closely related sugars only D-glucose is an insulin secretagogue.

The data on the lanthanum-displaceable calcium pool are also in good agreement with the situation in leucocytes. Having found that leucocidin does not cause a general influx of $^{45}Ca^{2+}$ into those cells, Woodin and Wieneke (*5*) concluded that secretion is triggered by enhanced calcium binding to the inner cell membrane surface. That glucose-stimulated insulin release is associated with a deposition of calcium in the β-cell plasma membrane has been reported on the basis of studies of pyroantimonate precipitation and electron microscopy (*7*). Our experiments suggest that a glucose-sensitive calcium pool is localized in the β-cell plasma membrane, and that the membrane-located calcium has sufficient mobility to meet the kinetic demands requisite for a particular calcium pool to serve in the process of stimulus-secretion coupling.

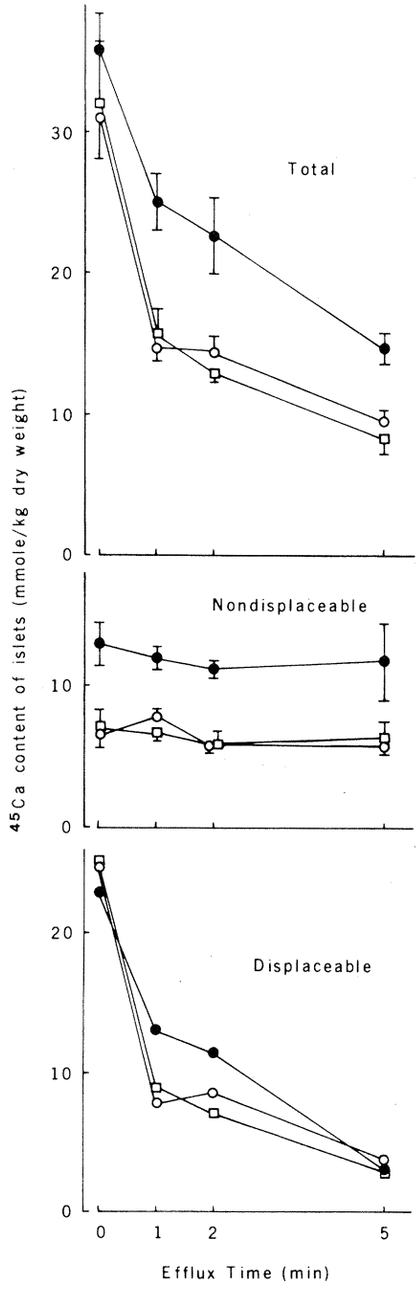

Fig. 1. Content of labeled calcium in microdissected islets that were incubated for 120 minutes in tris buffer containing $^{45}CaCl_2$ and then for 0 to 5 minutes in nonradioactive medium. The radioactive medium used for labeling contained 3 m*M* D-glucose (○), 20 m*M* D-glucose (●), or 3 m*M* D-glucose plus 17 m*M* L-glucose (□), while the efflux medium contained 3 m*M* D-glucose in all cases. Mean values ± standard error of 5 to 29 experiments are given for total islet calcium (top panel) and for calcium remaining after washing with lanthanum (middle panel); the bottom panel shows the difference between total and lanthanum-nondisplaceable calcium pools. Results are expressed as millimoles of labeled calcium with the same specific radioactivity as the medium used for labeling the islets.

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References and Notes

1. B. Hellman, J. Sehlin, I.-B. Täljedal, *J. Physiol. (London)* **254**, 639 (1976).
2. Fractions were isolated by differential centrifugation at 400*g*, 5,000*g*, 28,000*g*, and 110,000*g*. The 28,000*g* fraction showed the highest radioactivity per unit of protein and a significant increase of labeling after the islets were incubated in 20 m*M* glucose as compared with 3 m*M*. The retention of $^{45}Ca^{2+}$ by islets incubated in nonradioactive medium after incubation with $^{45}CaCl_2$ was unaffected by glucose, dibutyryl adenosine 3′,5′-monophosphate, theophylline, and pentobarbital.
3. G. M. Grodsky, *Diabetes* **21** (Suppl. 2), 584 (1972); B. Hellman, L.-Å. Idahl, Å. Lernmark, J. Sehlin, I.-B. Täljedal, *Biochem. J.* **132**, 775 (1973).
4. F. Malaisse-Lagae and W. J. Malaisse, *Endocrinology* **88**, 72 (1971); B. Hellman, J. Sehlin, I.-B. Täljedal, *Am. J. Physiol.* **221**, 1795 (1971); W. J. Malaisse and D. G. Pipeleers, in *Diabetes. Proceedings of the 8th Congress of the International Diabetes Federation*, W. J. Malaisse and J. Pirart, Eds. (Excerpta Medica, Amsterdam, 1974), pp. 95–103.
5. A. M. Woodin and A. A. Wieneke, in *Calcium and Cellular Function*, A. Cuthbert, Ed. (Macmillan, London, 1970), pp. 183–197.
6. L. Orci, F. Malaisse-Lagae, M. Ravazzola, M. Amherdt, A. E. Renold, *Science* **181**, 561 (1973).
7. L. Herman, T. Sato, C. N. Hales, *J. Ultra struct. Res.* **42**, 298 (1973); M. Ravazzola, F. Malaisse-Lagae, M. Amherdt, A. Perrelet, W. J. Malaisse, L. Orci, *J. Cell Sci.*, **21**, 107 (1976).
8. That diazoxide inhibits $^{45}Ca^{2+}$ accumulation in islets has been shown in a study in which islets were washed without lanthanum to free them of extracellular label (*4*). The radioactivity retained after such washing is likely to represent a fraction of the lanthanum-nondisplaceable pool (*1*). Whether the effect on $^{45}Ca^{2+}$ uptake is the cause of, or is caused by, the concomitant inhibition of insulin secretion is unknown because the two processes are difficult to dissociate experimentally. An apparent dissociation of $^{45}Ca^{2+}$ uptake from insulin release has been observed in islets incubated in a virtually calcium-free medium containing only the radioactive tracer, $^{45}CaCl_2$ (*4*). However, at very low calcium concentrations, the specific activity of the tracer may be so high that a negligible and barely detectable change of the secretory rate is sufficient to cause an appreciable change in the isotope uptake by a secretion-dependent mechanism.
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