Glucose-Stimulated $^{45}$Ca Calcium Efflux from
Isolated Rat Pancreatic Islets

BARBARA J. FRANKEL, WALTER T. IMAGAWA, MICHAEL D. L. O’CONNOR,
INGMAR LUNDIQUIST, JOHN A. KROMHOUT, RUDOLPH E. FANSKA, and
GEROLD M. GRODSKY, Metabolic Research Unit and the Department of
Biochemistry and Biophysics, University of California, San Francisco,
California 94143

A B S T R A C T  Kinetics of $^{45}$Ca efflux and insulin release were studied in collagenase-isolated rat islets
during 2-h perfusions with calcium-depleted (0.05 mM) bicarbonate-phosphate buffer containing 2.2 mM
glucose. Addition of glucose (16.7 mM) suppressed $^{45}$Ca efflux by 30%. Removal of glucose caused an “off
response” of insulin release. The perfusion of a normal concentration of Ca (2.3 mM) greatly stimulated
$^{45}$Ca efflux, indicating Ca ↔ $^{45}$Ca exchange. When Ca and glucose were superimposed, the effects on
$^{45}$Ca efflux and insulin release depended upon the order of presentation of the stimuli: when Ca was added
to an ongoing 16.7-M glucose perfusion, biphasic patterns of $^{45}$Ca and insulin release were seen; when
the glucose was superimposed on a Ca perfusion, an inhibition of the Ca-stimulated $^{45}$Ca efflux occurred, and
a reduced but clearly biphasic insulin response was seen. The subsequent insulin off response after with-
drawal of the glucose was also reduced.

Mathematical “peeling” of $^{45}$Ca efflux curves from unstimulated islets suggests that there are at least two,
and probably three, different intracellular Ca compartments (not including the extracellular sucrose space).
At the beginning of perifusion, these three compartments (I, II, III) contain 25, 56, and 19% of the intra-
cellular $^{45}$Ca, and their rates of efflux are 6.7, 1.2, and 0.1 %/min, respectively. Glucose appears to suppress
efflux from the largest compartment (II); Ca appears to exchange with $^{45}$Ca from a more inert compart-
ment (III). The relationship between insulin and $^{45}$Ca release is not stoichiometric.

INTRODUCTION

The role of calcium (Ca) ion movements during glu-
cose-stimulated insulin release from the pancreatic $\beta$-
cell is unclear. Several studies (1–4) have shown that
Ca is necessary for insulin secretion. In studies of $^{45}$Ca
efflux, Malaisse et al. (5), and later Bukowiecki and
Freinkel (6), showed that an increased glucose load
(with approximately 1 mM Ca), caused a short depres-
sion followed immediately by an impressive spike of
$^{45}$Ca efflux. This spike occurred about the time that the
first phase of insulin release is usually seen (7). At a
very low external Ca level with EGTA in the medium,
glucose only depressed $^{45}$Ca efflux (5). Glucose, in the
presence of external Ca, causes an increased net up-
take of $^{45}$Ca (8–10). It is the free cytosolic Ca, or Ca
in a small compartment, which is believed to be di-
rectly related to biphasic insulin release and the elec-
trical phenomena which accompany it (11). Both de-
creased efflux and increased influx across the plasma
membrane could lead to increasing this Ca. It, and
insulin secretion, may also be increased by solubiliza-
tion of plasma membrane-bound Ca or release from an
intracellular organelle. Histochemical studies indi-
cate that Ca deposits are found within the “halo” of the
beta granule, near the plasma membrane, and in the
mitochondrial matrix and endoplasmic reticulum of the
$\beta$-cell (12–14). In the presence of a high level of
glucose, the granules’ stores and those on the inner
side of the plasma membrane appear to increase while
the mitochondrial and endoplasmic reticulum ones
tend to dissipate. Thus, glucose-stimulated movements
of Ca are complex and probably the summation of many
different processes.

In the present studies with isolated perifused islets,
the effect of glucose and Ca on the kinetics of $^{45}$Ca
efflux and insulin release were studied. Efflux during
the absence of stimulation was also studied to attempt
mathematical estimation of the number, size, and efflux
rates of the intracellular Ca pools within the islets.

METHODS

Islets were isolated by the collagenase method of Lacy and
Kostianovsky (15) from 350- to 375-g, fed, Long-Evans rats


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and incubated at 37°C for 60 min in the presence of 44Ca (100–150 μCi) in 0.5 ml of medium. The incubation medium was a bicarbonate-phosphate buffer (pH 7.4) from which all Ca except radioactive Ca was omitted; the total Ca concentration was approximately 0.3 mM. The buffer was otherwise composed of 29 mM bicarbonate, 1.5 mM phosphate, 140 mM sodium, 111 mM chloride, 58 mM potassium, 1.2 mM magnesium, 0.3% human serum albumin, and 16.7 mM glucose. Preliminary uptake experiments showed that islets reached 50%–70% isotopic equilibrium after 60 min incubation with 44Ca. Saturation occurred at 2.5–3 h. After the incubation, 150–200 islets were washed once and perfused for 120 min at a flow rate of 1.0 ml/min, during which 44Ca efflux and insulin release were measured. The medium was used for washing and perfusion of the islets identical to the incubation medium, except that the isotope was omitted and the glucose concentration was lowered to 2.2 mM. The Ca concentration, as determined by atomic absorption spectrophotometry, was 0.05 mM (“calcium-depleted medium”). Most of this Ca was probably not free, but rather bound to the albumin in the medium. The perfusion technique used was that described by Lacy et al. (16). In some experiments after perfusion for 60 min, the glucose and Ca concentrations in the perfusate were brought to 16.7 and 2.3 mM, respectively; these reagents were dissolved in 0.9% saline solution and added by a Harvard infusion pump (Harvard Apparatus Co., Inc., Millis, Mass.) at a rate of 0.05 ml/min. These stimuli were added in saline solution because it was not possible to inject the 20-fold concentration of Ca in a phosphate-containing buffer. The stimulation period was 45 min. In other experiments, glucose and Ca were infused concomitantly during the middle one-third of the stimulation period. From each collected sample, 0.4 ml was removed for liquid scintillation counting in Aquasol (New England Nuclear, Boston, Mass.). The remainder of the sample was saved for insulin determination by solid-phase, single-antibody radioimmunoassay (17). At the end of the perfusion, the filter that contained the islets and 0.4 ml perfusate was counted in Aquasol. All counts and insulin values were expressed per 200 islets. To establish the time required for extracellular washout of isotope, the extracellular space marker [6,6’(n)-3H]sucrose (2 mCi) (ICN Pharmaceuticals, Inc., Cleveland, Ohio) was added to the incubation medium after 50 min (n = three experiments), and [3H]sucrose washout was monitored. The [3H]sucrose counts decreased to near background levels by 20 min (see Fig. 1). Therefore, in all the subsequent 44Ca efflux perifusions, collections were begun after 20 min.

Analysis of the perifusion control curves was as follows. Each of six experiments, in which the effluent was collected from 1 to 120 min, was normalized to 100% at 20 min. The resulting values were then multiplied by the average counts per minute of all the curves at 20 min, giving a set of individual curves, each normalized to the average 20-min value. The mean of these normalized curves was then analyzed by simple peeling, with the BMD-X nonlinear, least-squares-fitting routine of the University of California (San Francisco) IBM 360-50 computer (IBM Corp., White Plains, N. Y.). First, a slow compartment (II) was fitted with the later points in the curve. After subtraction of this compartment, the fast compartment (I) was fitted. A third, much slower compartment (III) was calculated by subtracting the calculated number of counts remaining in compartments I and II from the average number of counts measured in the islets at the end of the experiments. The release rate from this third compartment was not resolvable within the practical perifusion period, and release was assumed to be approximately zero.

In the top portion of Figs. 1 through 5, 44Ca efflux (counts per minute/200 islets) is presented as a semilogarithmic plot. Displayed in this manner, efflux at a constant rate from a single compartment would appear as a straight line. To reduce the statistical variation among experiments, these data are also presented as the fractional efflux rate1 (percent of islet content effluxed per minute, where islet content at time t is the sum of the counts remaining in the islets at the end of the 120-min perfusion plus the counts not yet effluxed during the period from t to 120 min). Estimated counts remaining in the islets at each time period are also shown. Data shown as mean±SEM.

Differences in specific activity of the incubation medium (100–150 μCi/0.5 ml) and/or actual differences in uptake by different batches of islets led to variations in the absolute counts effluxed during individual experiments. This difference is most notable in the top of Fig. 5, where the counts per minute effluxed at any one time for the two experimental curves differs by about twofold. However, the fact that the efflux curves are parallel on a log scale indicates that their rates of efflux are identical. This is seen when the efflux was recalculated as fractional efflux (percent counts per minute; middle of Fig. 5). Therefore, differences in labeling do not invalidate the comparison of the rates of efflux and acute changes in the rate of efflux in response to a stimulus.

RESULTS

Fig. 1 shows the efflux of 44Ca and [3H]sucrose from perifused islets incubated for 60 and 10 min, respectively, with the isotopes. Counts per minute are reported on a logarithmic scale to show the kinetics of 44Ca and [3H]sucrose washout. The extracellular marker, [3H]sucrose, was eliminated within 25 min. Calculated fractional efflux rate of 44Ca was not constant throughout the 120-min experiment, but declined from 8 to 0.5%/min. Throughout most of the efflux period, therefore, the total islet content of 44Ca fell very slowly; release during any 1 min was only a fraction of the total 44Ca in the islets. As expected, almost no insulin release occurred in the calcium-depleted medium.

Control for the subsequent experiments (Fig. 2) showed no effect of saline solution infusion on 44Ca efflux or insulin release.

As seen in Fig. 3, and again in Fig. 4, by increasing the glucose concentration after 60 min from 2.2 to 16.7 mM in calcium-depleted medium we suppressed the efflux of 44Ca by about 30% within 5 min. This effect was maintained throughout the 45-min period of glucose perfusion. When the glucose perfusion was stopped, efflux returned to normal (Fig. 3) and a marked overshoot of insulin release occurred (Fig. 4). The addition of 2.3 mM Ca during the middle one-third of the glucose perfusion caused biphasic insulin release and biphasic Ca efflux and potentiated the insulin overshoot when the glucose was removed.

Fig. 5 shows that, during the slow perifusion of 2.3 mM Ca, the rate of 44Ca efflux increased for approximately 25 min and then remained constant at about twice the control level. When Ca perfusion was

1 Essentially the same as Bolle’s Efflux Rate Coefficient (20).
stopped, $^{45}\text{Ca}$ efflux promptly fell toward the control level without overshoot. By raising the glucose concentration from 2.2 to 16.7 mM in the middle of the Ca perifusion we suppressed the ongoing $^{45}\text{Ca}$ efflux and caused biphasic insulin release. Changes in $^{45}\text{Ca}$ efflux and insulin release were opposite to each other at this time. The amount of insulin released was somewhat less than in those experiments in which the order of addition of Ca and glucose was reversed (Fig. 4). Removal of glucose in experiments of either design was usually associated with an overshoot of insulin release and $^{45}\text{Ca}$ efflux (Figs. 4 and 5).

In both types of experiments, the observed efflux reflected only slight changes in the curves showing $^{45}\text{Ca}$ remaining in the islets. This indicated that islet

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\textsuperscript{2} Although mean release was reduced by 60\%, this difference was not significant because of the large variation: 145±60 vs. 59±15 ng/15 min (SEM).

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**FIGURE 1** Efflux of $^{45}\text{Ca}$ from prelabeled rat islets. [$^{3}\text{H}$]Sucrose was used as a marker for the extracellular space. Basal perifusate contained 2.2 mM glucose and 0.05 mM Ca\textsuperscript{2+}. Fractional $^{45}\text{Ca}$ efflux was calculated at 2-min intervals as explained in Methods. Data shown as mean±SEM.

**FIGURE 2** Lack of effect of saline solution infusion on efflux of $^{45}\text{Ca}$ from prelabeled rat islets. Basal perifusate contained 2.2 mM glucose and 0.05 mM Ca\textsuperscript{2+}. Data shown as mean±SEM. Control experiments ($n=8$) shown by dotted line.

$^{45}\text{Ca}$ content was an insensitive measure of the effect of glucose on Ca efflux.

Analysis of the kinetics of $^{45}\text{Ca}$ efflux during the control (unstimulated) experiments suggested that the efflux was from at least two (I and II), and probably a third (III), separate Ca compartments within the islets. These compartments and their rates of efflux are shown in Figs. 6 and 7. At the beginning of efflux, in medium containing 0.05 mM Ca and 2.2 mM glucose, the approximate distribution of $^{45}\text{Ca}$ in the compartments was: 25\%(I), 56\%(II), and 19\%(III). After 60 min perifusion, compartment I was nearly empty (emptying rate 6.7 \%/min); II was one-half empty (emptying rate 1.2 \%/min); and compartment III was nearly unchanged (emptying rate 0.1 \%/min). By increasing the glucose concentration to 16.7 mM we reduced efflux (Fig. 3)

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**FIGURE 3** Effect of glucose (16.7 mM) on efflux of $^{45}\text{Ca}$ from prelabeled rat islets. Basal perifusate contained 2.2 mM glucose and 0.05 mM Ca. Data shown as mean±SEM. Control experiments ($n=8$) shown by dotted line. (These data taken from Fig. 4 and presented here on an expanded scale.)

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in a pattern that could be approximated by decreasing the release rate from compartment II by about 30%. By increasing the Ca concentration to 2.3 mM we increased total efflux about twofold (Fig. 5).

DISCUSSION

The function of Ca ion movement during glucose-stimulated insulin release is unclear. The present studies attempted to clarify the relationship between Ca + glucose-stimulated insulin release and concomitant changes in 45Ca efflux.

Computer analysis of the mean 45Ca efflux curve from unstimulated islets (Fig. 1) revealed the existence of at least two intracellular compartments (I and II) exclusive of the extracellular (sucrose) space. A third intracellular Ca compartment (III) was assumed to exist in order to better fit the experimental efflux curve and the large number of counts remaining in the islets after 120 min. 45Ca in the extracellular space, which bound very loosely to the outside of the islets and which was released from a possibly very labile compartment (18), was presumably washed away during the first 20–25 min of perfusion (19) ("[3H]sucrose space"). This first 20-min period of 45Ca efflux was not shown in subsequent Figs. 2–5. Compartment sizes were determined by extrapolation of the efflux from each of the three compartments back to zero time (beginning of perfusion) while assuming a constant rate of emptying throughout the experimental period. It was further assumed that the three compartments were parallel (i.e., that one compartment did not release into another compartment before release to the exterior of the cell). Also, efflux from each compartment was assumed inde-
dependent of Ca movements in the others. However, the data are not incompatible with the possibility that the compartments are in series (e.g., Ca release from a "slow" compartment into a rapidly emptying compartment and then to the exterior) (20).

From the studies of isolated kidney cells (21) Borle found three phases of Ca-efflux which he tentatively ascribed to extracellular, cytoplasmic, and mitochondrial Ca compartments. He further suggested that the mitochondrial pool may be inhomogeneous, consisting of bound and free components. By comparison with these (21) and other (22) studies it is possible that the islets' most rapidly emptying intracellular compartment (I) may correspond to the cytosol. Compartment II, which is larger and empties more slowly, may correspond to the Ca-storing mitochondria (23), insulin granules (12-14), plasma membrane (13), or other intracellular Ca-storing sites (24). The nature of compartment III is more difficult to define. In the absence of the stimulators, glucose and Ca, compartment III is relatively inert and may correspond to the more inert (bound Ca?) portions of the mitochondria, granules, plasma membrane, etc. However, there is no assurance that Ca compartments in pancreatic islet cells resemble those in kidney and liver.

Malaisse et al. (5) have previously shown inhibition of Ca efflux from islets by glucose (16.7 mM) in medium without added Ca and containing EGTA. Fig. 3 shows that this suppressive effect is fairly constant throughout a 45-min glucose infusion. Based on the theoretical efflux from each compartment (Fig. 6), it can be seen that compartments I and III contribute only about 10% of the total Ca efflux during this period (60-105 min) in unstimulated islets. Therefore, at the time of glucose infusion, efflux was primarily from compartment II, and the suppressive effect of glucose was probably directed toward this compartment. However, the present data cannot distinguish between an inhibi-

### Figure 6
Hypothetical division of observed Ca efflux (dotted line) into efflux from three parallel, intra-islet, calcium compartments, not including the extracellular sucrose space (broken line). Basal perfusate contained 2.2 mM glucose and 0.05 mM Ca. Observed efflux was the mean of six experiments, each normalized with reference to the initial 20-min point. (Relative size of compartments from which efflux occurs shown in Fig. 7.)

### Figure 7
Theoretical calcium compartments within the β-cell. Relative size of the Ca-filled compartments is calculated for the beginning of perfusion, time 0 (solid line), and at 60 min (broken line). (Efflux from these compartments shown in Fig. 6.)
tion of efflux at the plasma membrane and an inhibition at some intracellular organelle or site. Inhibition of the normal movement of Ca out through the plasma membrane might cause a temporary accumulation of Ca within the cytosol. However, because there was not a significant overshoot of 45Ca efflux after we stopped the glucose infusion (Fig. 4), the excess 45Ca from compartment II may have been redeposited into a more inaccessible site.

In contrast to the effect of glucose, perfusion of 2.3 mM Ca in glucose-depleted medium caused a marked 45Ca efflux which was sustained at about 200% of the control level (Fig. 5). At the end of the Ca infusion (105 min), if the excess 45Ca had come from compartment II, and if that compartment had gone unplenished, it would have been about one-half as full, and efflux would have fallen to about 50% of the control level. However, because efflux returned to near the control level, the external Ca appears to have mobilized or exchanged with 45Ca from a more inert compartment (III?). Studies with isolated heart mitochondria have shown an approximate 1:1 exchange of unlabeled Ca for labeled 45Ca (25). From studies of isolated adipocyte plasma membranes (22), it seems unlikely that there would have been enough labeled 45Ca bound to the plasma membrane to have sustained the high level of 45Ca efflux for the entire 45-min period. The Ca-induced 45Ca efflux probably was not a manifestation of islet damage, because Ca-stimulated 45Ca efflux was immediate and biphasic when Ca was superimposed on glucose infusion, and because the biphasic insulin release promptly ceased after termination of the Ca perfusion. Raising the Ca concentration from 2.3 to 4.6 mM in similar studies produced a small but definite increase in 45Ca efflux (data not shown). Thus, these results suggest that islets, like other tissues (25, 26), possess a Ca ↔ 45Ca exchange system which may occur in the mitochondria (25), or other β-cell locations which accumulate Ca (granules, plasma membrane, endoplasmic reticulum, Golgi apparatus) (12–14).

A further complication to the precise interpretation of these compartmental results is that the isolated rat islet only consists of about 60–70% β-cells—the remainder being α, δ, pancreatic polypeptide, other?, and endothelial cells (27–29). Efflux from the non-β-cells of the islet may complicate the compartmental analysis. The development of a pure β-cell technique would help solve this question.

The order in which glucose and Ca were presented to the islets influenced insulin secretion. When glucose was perfused first, a larger biphasic insulin release occurred. Thus, a potentiating effect of glucose can occur in a Ca-depleted medium. As seen in the perfused pancreas (30), prior exposure to glucose can potentiate the insulin response, possibly by increasing proinsulin, metabolic intermediates, ATP, cyclic AMP, or microtubule protein (31–34).

In experiments in which glucose was superimposed 15 min after addition of Ca (Fig. 5), glucose suppressed 45Ca efflux even though it stimulated insulin release. These experiments differ in design but are consistent with recent reports (35, 36) in suggesting that calcium efflux does not occur stoichiometrically with insulin release, as was originally suggested (5).

It is interesting to note that, in all instances in which glucose was stopped during perfusion, an overshoot or "off response" of insulin release and a possible 45Ca off response occurred. The relationship between 45Ca and insulin was not stoichiometric, and the cause of this off response is not clear. However, studies by Blackard et al. (37) suggest that the abrupt decrease in osmolarity on removal of glucose may have caused at least the insulin off response.

Although the various changes in efflux were easily discernible when the minute-by-minute efflux of 45Ca was measured, the rates of efflux were small. Therefore, as illustrated in Figs. 1, 4, and 5, the attempt to detect changes in efflux by measuring the small depletion of islet content may not always be sufficient for monitoring acute changes in efflux (9).

The use of 45Ca to analyze storage and efflux of Ca could depend upon the conditions of prior 45Ca loading. We used stimulatory levels of glucose and low levels of unlabeled Ca because, as noted by others (8), this caused enhanced loading of the isotope and more sensitive measurement of efflux. Loading was for 60 min, corresponding to 50–70% of isotopic equilibrium. In comparative studies (data not shown), in which loading occurred at normal Ca levels over a 20-h period in culture to ensure isotopic equilibrium within the islets, efflux curves were in qualitative agreement with the results shown in Figs. 1, 3, 4, and 5.

In conclusion, the present study suggests that 45Ca efflux was derived from at least three compartments within the unstimulated islets. Glucose decreased efflux from the largest compartment in the presence or absence of previously added Ca. Calcium caused a marked 45Ca efflux from a relatively stable compartment, indicating the presence of a Ca pump or a rapidly exchangeable Ca-binding site. 45Ca efflux and insulin release were dependent upon the order of addition of glucose and Ca consistent with the possibility that glucose can affect intracellular Ca distribution and potentiate subsequent insulin release even in low extracellular Ca. During stimulation with glucose, Ca efflux does not occur stoichiometrically with insulin release.

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