The Roles of Intracellular and Extracellular Ca^{++} in Glucose-Stimulated Biphasic Insulin Release by Rat Islets

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ABSTRACT Verapamil, an agent known rapidly to block calcium uptake into islets of Langerhans, has been used to study the roles of intra- and extracellular calcium in the two phases of glucose-induced insulin release. Rates of calcium uptake and insulin release during the first phase were measured simultaneously over 5 min in rat islets after maintenance in tissue culture for 2 days. Rates of $^{45}Ca^{++}$ efflux and insulin release during the first and second phases were also measured simultaneously under perifusion conditions. For this, islets were loaded with $^{45}Ca^{++}$ during the entire maintenance period to complete isotopic equilibrium. Under static incubation conditions 5 μM Verapamil had no effect upon Ca^{++} uptake or insulin release in the presence of 2.8 mM glucose. By contrast, glucose-stimulated calcium influx was totally abolished without there being any significant effect upon first phase insulin release. Thus first phase insulin release is independent of increased uptake of extracellular calcium. The lack of effect of 5 μM Verapamil blockade on first phase insulin release was confirmed, under perifusion conditions, and was in marked contrast to the observed 55% inhibition of second phase release. $^{45}Ca^{++}$ efflux was inhibited during both phases of the insulin release response.

The results show that increased calcium uptake in response to glucose is not involved in the mechanism of first phase insulin release but is required for the full development and maintenance of the second phase release. It seems possible that intracellular calcium is the major regulatory control for first phase insulin release and that intracellular calcium and increased uptake of extracellular calcium contribute almost equally to the second phase of glucose-induced release.

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INTRODUCTION

The β-cells of islets of Langerhans secrete insulin in response to a constant glucose stimulus with a characteristic biphasic pattern (1–4). This consists of a rapid surge of insulin output which peaks after a few minutes and then declines (first phase release), followed by a sustained period of slowly increasing insulin output (second phase release). Several explanations for this pattern have been proposed and generally these are along four lines: (*a*) that the insulin-containing granules in the β-cells are not a homogeneous pool, but heterogeneous either in their sensitivity to the stimulus, or by fortuitous positioning close to the plasma membrane or other structures involved in the release mechanism (5–7); (*b*) that the β-cells themselves have different sensitivities (8) or release rates in response to glucose; (*c*) that the initial rapid release of insulin causes a feedback inhibition of subsequent release (9–10); (*d*) that the initiation and maintenance of the constant glucose stimulation sets up a chain of events that results in a biphasic change in the concentration of an intracellular mediator of insulin release (11). Along the lines of this last possibility, it seemed that the pattern of insulin release could be dictated by a biphasic pattern of the cytosolic Ca^{++} concentration. Calcium is known to be required for insulin release (12, 13), and calcium alone has been shown to trigger the release process. For example, when the extracellular Ca^{++} concentrations are raised (14, 15) or when the ionophore A23187 is used to raise intracellular Ca^{++} concentrations, a prompt and sustained release of insulin is achieved (16–19).

The effects of glucose on calcium handling have been described by several groups. It seems clear that glucose has at least two actions on the movement of calcium into and out of the islet cells. Thus, it has been shown that glucose has a rapid effect to decrease the rate of efflux of $^{45}Ca^{++}$ from preloaded isolated

islets (20–22), an effect that could raise the cytosol Ca^{++} concentrations. Glucose has also been shown to cause an increase in Ca^{++} uptake by the islet (23–31). This effect also could increase the cytosol concentration, an increase which would be reinforced by the effect of glucose to decrease the rate of efflux. It seemed possible therefore that the timing and confluence of these two effects of glucose on the handling of calcium, and thus on the cytosol Ca^{++} concentration, could be responsible for the biphasicity of insulin release. To investigate this possibility studies have been performed with Verapamil, an agent that rapidly blocks calcium uptake by islet cells, to assess the roles of the two effects of glucose on Ca^{++} efflux and influx on the generation of the biphasic insulin secretory response to glucose.

METHODS

Preparation and maintenance of islets. Pancreatic islets were isolated by the collagenase digestion technique (32) from male Wistar rats. 200–250 islets were maintained in 5 ml of medium 199 containing 10% heat-inactivated calf serum, 14 mM sodium bicarbonate, 8.3 mM glucose, 400 IU/ml sodium penicillin G, and 200 μg/ml streptomycin sulfate in 60-mm diameter plastic Petri dishes at 37°C and gassed with air and CO_2. The islets remained unattached as individual islets throughout the 45- to 47-h maintenance period.

$^{45}Ca^{++}$ uptake. After the maintenance period, the islets were washed twice at room temperature with a modified Krebs-Ringer bicarbonate buffer (KRB-Hepes)[1] containing 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 250 kallikrein inhibitory U/ml Trasylol, 0.5% dialyzed bovine serum albumin, 10 mM *N*-2-hydroxy-ethylpiperazine-*N'*-ethane sulfonic acid (Hepes), and 2.8 mM glucose, pH 7.4. The islets were distributed into 400-μl polyethylene tubes containing 200 μl of a mixture of dibutyl- and dinonylphthalate (10:3 vol/vol) layered on top of 20 μl of 6 M urea. 10 islets in 50 μl of KRB-Hepes buffer were carefully placed against the tube wall so as to leave an air-layer between the buffer and the oil mixture. The incubation was started by adding another 50 μl of warm (37°C) KRB-Hepes buffer containing glucose with or without Verapamil to yield appropriate final concentrations, 0.8 μCi of $^{45}CaCl_2$ and 1.4 μCi [6,6'(*n*)^3*H*]sucrose (4 μM), as a marker of the extracellular space (33, 34). The tubes were incubated at 37°C without shaking. At the end of 5 min the incubation was stopped and the islets were separated from the incubation buffer by centrifugation for 15 s at 8,000 × *g* in a Greiner microfuge (type ZF1, Greiner A. G., Lucerne, Switzerland). By this procedure (26, 28, 35, 36), the islets were separated from the buffer by passage through the phthalate mixture and into the urea layer. Insulin release was assayed on an aliquot of the supernatant buffer. The bottoms of the tubes were cut above the urea layer and placed in 5 ml Instagel for liquid scintillation spectrometry. In every experiment blanks containing cut microfuge tubes without islets, standards of the radioactive medium (20 μl), and samples containing $^{45}Ca^{++}$ only, for the estimation of spillover of $^{45}Ca^{++}$ counts into the ^{3}H channel, were added for spectrometry. Blanks without islets did not differ from background counts. Ca^{++} uptake was calculated from the $^{45}Ca^{++}$ space in excess of the [^{3}H]sucrose space. The sucrose space became maximal within 1 min of incubation and remained constant over 30 min. At 5 min the extracellular space was 1.22±0.10 nl/islet (n = 15) (mean±SEM) in the presence of 2.8 mM glucose and 1.25±0.11 nl/islet (n = 14) in the presence of 16.7 mM glucose. $^{45}Ca^{++}$ uptake, in both low and high glucose, was linear for 5 min (28).

The insulin release, determined on the same islets as the Ca^{++} uptake, was corrected to indicate the true release over the 5-min incubation period by subtraction of the values measured at zero-time. Immunoreactive insulin (IRI) was measured by the method of Herbert et al. (37) using rat insulin as standard.

Perifusion system and measurement of $^{45}Ca^{++}$ efflux. Islets used for $^{45}Ca^{++}$ efflux studies were labeled during the entire maintenance period with 100 μCi of $^{45}CaCl_2$/ml of culture medium. $CaCl_2$ in the culture medium was 1.8 mM and the final specific radioactivity was approximately 54 μCi/μmol. 40 islets were perifused per chamber as described in detail elsewhere (4, 38). The volume of the perifusion chamber was 70 μl, and two rotating oxygen distributors also serving as medium reservoirs were connected to each chamber. The dead space of the system was ≅1.4 ml and the flow rate was 1.4 ml/min. The perifusate consisted of KRB containing 1.0 mM $CaCl_2$, 0.5% dialyzed bovine serum albumin, and 2.8 mM glucose. The islets, after loading, were placed directly in the perifusion chamber without washing. From zero time to 46 min the islets were perifused with KRB-buffer containing 2.8 mM glucose. At 46 min, 1 min after the solution change, the glucose concentration in the chamber increased to 16.7 mM and the stimulation period continued for another 44 min. Verapamil was added during the stimulation period only. No sample collections were made during the first 40 min of the washing and equilibrium period. Fractions were then collected every minute between 41 and 65 min and thereafter every 5th min. An aliquot of the sample was assayed for IRI. To 0.8 ml of the samples, 8 ml of Instagel was added for assay of $^{45}Ca^{++}$ by liquid scintillation spectrometry. After background subtraction, the counts per minute (cpm) were normalized by setting the mean cpm of the five samples collected between 41 and 45 min to 100% and expressing the subsequent values as a percentage of this mean. The mean basal efflux ranged between 100 and 200 cpm. Statistical analysis was by Student's t test.

The materials employed and their sources were as follows: collagenase, 150 U/mg (Worthington Biochemical Corp., Freehold, N. J.), medium 199 and Hepes solution (Grand Island Biological Co., Grand Island, N. Y.), sodium penicillin G (Pfizer Inc., New York), streptomycin sulfate (Novo Research Institute, Copenhagen, Denmark), plastic Petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.), bovine serum albumin (Behring-Werke AG., Marburg Lahn, W. Germany), Trasylol (kindly provided by Dr. H. Rüf, Bayer Pharma A.G., Zurich, Switzerland), Verapamil (kindly provided by Professor Oberdorf, Knoll A.G., Ludwigshafen, W. Germany), guinea-pig anti-pork insulin serum (a generous gift from Dr. P. Wright, University of Indiana, Indianapolis, Ind.), rat insulin (provided by Dr. J. Schlichtkrull, Novo Research Institute), $^{45}CaCl_2$ and [6,6'(n)^{3}H]sucrose (the Radiochemical Centre, Amersham, Eng.), and Instagel (Packard Instrument Co., Inc., Downers Grove, Ill.).

[1] *Abbreviations used in this paper*: IRI, immunoreactive insulin; KRB-Hepes, Krebs-Ringer bicarbonate buffer containing 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 250 kallikrein inhibitory u/ml Trasylol, 0.5% dialyzed bovine serum albumin, 10 mM *N*-2-hydroxy-ethyl-piperazine-*N'* ethane sulfonic acid, and 2.8 mM glucose.

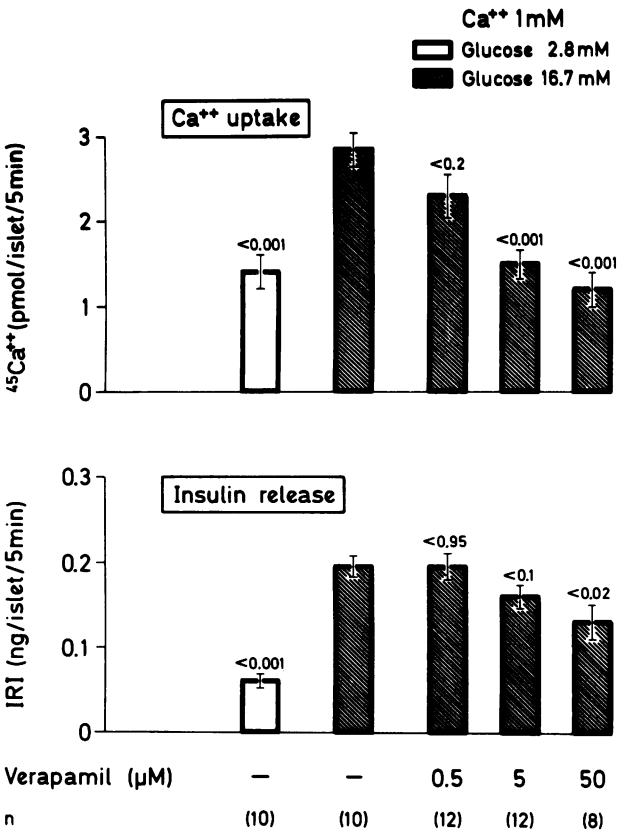

FIGURE 1 The effect of different concentrations of Verapamil on glucose-stimulated $^{45}Ca^{++}$ uptake and insulin release. The numbers of observations are in parentheses. Vertical lines represent ±SEM. The *P* values above the bars are relative to the high glucose control.

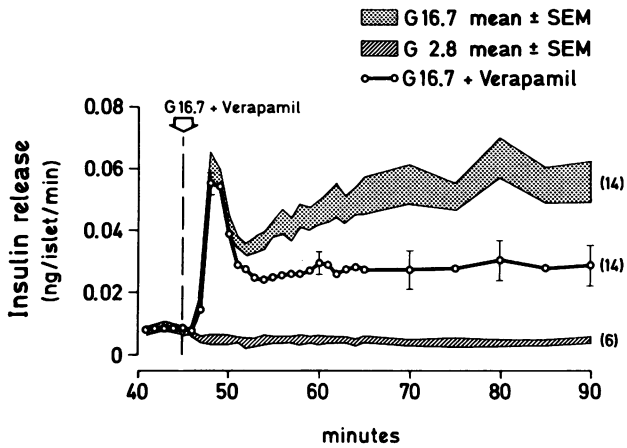

FIGURE 2 The effect of 5 μM Verapamil on glucose-stimulated insulin release from perifused islets. The numbers of observations are in parentheses. G, glucose.

RESULTS

Effect of Verapamil on first phase IRI release and Ca^{++} uptake. IRI release and $^{45}Ca^{++}$ uptake were studied simultaneously under control conditions with 2.8 mM glucose and under test conditions with 16.7 mM glucose, the latter in the absence or presence of 0.5, 5, and 50 μM Verapamil. With the method used here $^{45}Ca^{++}$ uptake is linear with time for 5 min in the presence of low or high glucose concentrations (28). All uptake studies were therefore performed over the first 5 min after addition of the test agents so that with respect to glucose stimulation, it is the period of first phase insulin release that is under examination. The results are shown in Fig. 1. 16.7 mM glucose caused a 225% increase in IRI release and a 100% increase in $^{45}Ca^{++}$ uptake. The presence of 0.5 μM Verapamil inhibited the stimulated $^{45}Ca^{++}$ uptake by 35%, although this was not statistically significant, without influencing the IRI release. 5 μM Verapamil completely abolished the glucose stimulation of $^{45}Ca^{++}$ uptake ($P < 0.001$) but did not significantly reduce the IRI release. 50 μM Verapamil reduced $^{45}Ca^{++}$ uptake further but to levels that were not significantly different from the control (2.8 mM glucose) series. In additional experiments the effect of 5 μM Verapamil was studied in the presence of 2.8 mM glucose. Calcium uptake in the control was 1.31±0.17 pmol/islet per 5 min compared with 1.37±0.13 pmol/islet per 5 min with Verapamil. Thus 5 μM Verapamil has no effect upon calcium uptake under basal conditions ($P < 0.6, n = 28$). It is concluded that first phase insulin release in response to 16.7 mM glucose occurs even in the absence of increased Ca^{++} uptake.

Effect of Verapamil on first and second phase IRI release and $^{45}Ca^{++}$ efflux. Under perifusion conditions, Verapamil was tested for its effect on IRI release in response to 16.7 mM glucose. From the results shown

TABLE I
$^{45}Ca^{++}$ Efflux and Insulin Release during the First and Second Phase Periods

	First phase		Second phase	
	$^{45}Ca^{++}$ efflux	Insulin release	$^{45}Ca^{++}$ efflux	Insulin release
	% basal	*ng/islet*	*% basal*	*ng/islet*
Control	391±21	0.16±0.02	2,685±256	1.67±0.22
Verapamil	254±19	0.14±0.01	1,395±67	0.75±0.13
Inhibition, %	35	12	48	55
P	<0.001	<0.4	<0.001	<0.001
	n = 6	*n* = 14	*n* = 6	*n* = 14

The data were obtained by integrating the stimulated $^{45}Ca^{++}$ efflux and insulin release in the perifusate samples collected at 47–51 min and at 52–90 min.

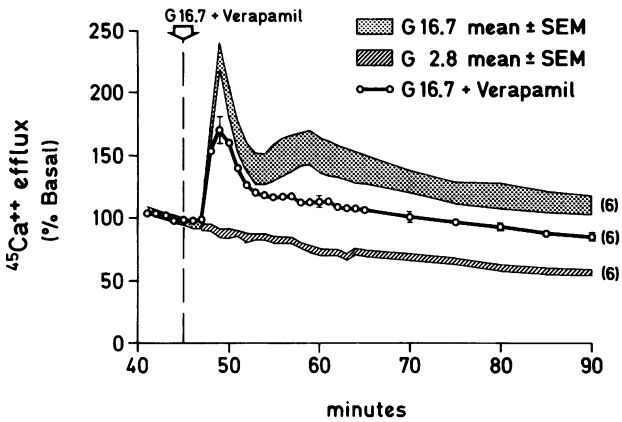

FIGURE 3 The effect of Verapamil on glucose-stimulated $^{45}Ca^{++}$ efflux from perifused $^{45}Ca^{++}$-loaded islets. After 45 min of perifusion in 2.8 mM glucose, the islets were subjected to 2.8 mM glucose or 16.7 mM glucose plus 5 μM Verapamil, as for the experiments described in Fig. 2. The numbers of observations are in parentheses. G, glucose. The 100% value for $^{45}Ca^{++}$ efflux was 137±10 cpm (n = 12).

in Fig. 2, it can be seen that 5 μM Verapamil had only a slight and nonsignificant inhibitory effect upon insulin release during the first phase period but had a marked effect upon the second phase release. The data presented in Table I show the stimulated insulin release in the two phases. This was obtained by integration of the incremental insulin release in the perifusate samples collected at 47–51 min (to correspond with first phase release and 5 min Ca^{++} uptake data) and at 52–90 min (second phase). The sample obtained at the 46th min was not included in the first phase period because this is the dead-space time of the perifusion apparatus before the islets are exposed to glucose. Insulin release during the first phase was not significantly inhibited by Verapamil and was decreased by only 12%. In contrast, the 55% inhibition of second phase release was highly significant.

The effect of 5 μM Verapamil on $^{45}Ca^{++}$ efflux in the presence of 16.7 mM glucose is shown in Fig. 3. With a change from 2.8 to 16.7 mM glucose, $^{45}Ca^{++}$ efflux increased rapidly after the 47th min, i.e., after 1 min of exposure to glucose. $^{45}Ca^{++}$ efflux then more than doubled in rate within 2 min. Thereafter, the rate declined rapidly for 3 min to a nadir, rose thereafter transiently, and remained well above control (2.8 mM glucose) levels for the duration of the test period. It should be noted that in these maintained islets the initial effect of glucose to reduce $^{45}Ca^{++}$ efflux is masked by the rapidity of onset of stimulated efflux and can be detected only by lowering the temperature or Ca^{++} concentration of the perifusate (22). Verapamil had a rapid effect on $^{45}Ca^{++}$ efflux so that 2 min from the onset of stimulated efflux it was significantly reduced relative to the control ($P < 0.01$). Subsequently, the efflux rate remained inhibited though still above basal (2.8 mM glucose). Integration of stimulated $^{45}Ca^{++}$ efflux during the two phases (Table I) showed that in the first phase $^{45}Ca^{++}$efflux was inhibited by 35% ($P < 0.001$) and in the second phase by 48% ($P < 0.001$).

Effect of Verapamil on insulin release and Ca^{++} uptake in the presence of a submaximal glucose concentration. If the rates of glucose-induced insulin release are maximal at 16.7 mM glucose, it is possible that under these conditions the cytosol Ca^{++} concentration rises to levels that saturate the release mechanism. If this were the case, an inhibition of Ca^{++} uptake would fail significantly to affect insulin release. To investigate this possibility experiments were carried out in the presence of a submaximal glucose concentration. As is shown in Table II, 8.3 mM glucose stimulated Ca^{++} uptake by 48% and first phase insulin release by 40% relative to 2.8 mM glucose. Although 5 μM Verapamil inhibited the stimulated Ca^{++} uptake, first phase insulin release was not significantly inhibited. It can also be seen in Table II that 16.7 mM glucose caused a further increase in both Ca^{++} uptake and insulin release compared to 8.3 mM glucose. Thus, using a weak glucose stimulus, a dissociation between the effects of Verapamil on Ca^{++} uptake and first phase insulin release was observed, similar to that seen when a strong glucose stimulus was employed.

TABLE II
Effect of Verapamil on $^{45}Ca^{++}$ Uptake and Insulin Release in the Presence of Glucose 8.3 mM

	Ca^{++} uptake	P	Insulin release	P	Number
	pmol/islet/5 min		*ng/islet/5 min*		
Glucose, 2.8 mM	0.73±0.06	<0.001	0.05±0.01	<0.05	16
Glucose, 8.3 mM	1.08±0.07	—	0.07±0.01	—	16
Glucose, 8.3 mM and Verapamil, 5 μM	0.81±0.07	<0.02	0.06±0.01	<0.3	18
Glucose, 16.7 mM	1.72±0.09	<0.001	0.18±0.02	<0.001	14

The P values are relative to glucose 8.3 mM.

TABLE III
Effect of Verapamil on K^+-Induced Insulin Release

	Insulin release, *ng/islet/5 min*			
	Control	Test 5 μM Verapamil	*P*	Number
Glucose, 2.8 mM	0.06±0.01	0.06±0.01		18
Glucose, 16.7 mM	0.28±0.04*	0.24±0.03	<0.5	19
Glucose, 2.8 mM, and 24 mM KCl	0.23±0.02*	0.12±0.01	<0.001	19

* Both significantly stimulated relative to glucose 2.8 mM ($P < 0.001$).

Effect of Verapamil on K^+-induced insulin release. In view of the failure of 5 μM Verapamil to inhibit first phase insulin release in response to glucose, it was thought worthwhile to study the effect of Verapamil on another stimulus to release. High K^+ was chosen because of its prompt action to depolarize the plasma membrane and stimulate insulin secretion in a calcium-dependent manner. The results are shown in Table III, and insulin release was measured over the 5-min period after addition of the stimulators. Basal insulin release in the presence of 2.8 mM glucose was the same in the presence and absence of Verapamil. Glucose-stimulated insulin release, as expected, was not significantly inhibited by Verapamil. In contrast, insulin release stimulated by 24 mM KCl was inhibited by 66%. Thus Verapamil is capable of causing a rapid inhibition of stimulated insulin release.

DISCUSSION

Verapamil inhibits calcium uptake in cardiac muscle (39, 40), uterus (41), and pituitary cells (42). It also inhibits $^{45}Ca^{++}$ net uptake by isolated islets incubated for 90 min in the presence of the label (43) and is a useful agent for the exploration of the role of calcium in cell function. In the results reported here it is apparent that the glucose-stimulated unidirectional influx of $^{45}Ca^{++}$ in isolated islets of Langerhans is rapidly inhibited by Verapamil. Also, Verapamil inhibits glucose-stimulated $^{45}Ca^{++}$ efflux from islets preloaded to isotopic equilibrium. This differs from previous observations with Verapamil (43) and D-600 (44), the methoxy derivative of Verapamil, in that these agents were not found to affect $^{45}Ca^{++}$ efflux. However, in the latter studies the imposed experimental conditions differed from the ones reported here as $^{45}Ca^{++}$ efflux was examined in Ca^{++}-deprived media containing ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetate. Under such conditions the stimulatory effect of glucose upon $^{45}Ca^{++}$ efflux is not seen. Although we have no explanation for the inhibition of $^{45}Ca^{++}$ efflux by Verapamil, it should be noted that a similar inhibitory effect was observed when D-600 was used to study $^{45}Ca^{++}$ efflux from pituitary cells (45).

When insulin release was measured over the first 5 min after exposure of the islets to high glucose, Verapamil failed to cause significant inhibition of release despite a total blockade of glucose-stimulated calcium uptake. Under perifusion conditions, the lack of effect of Verapamil on first phase insulin release was confirmed and a differential effect on the two phases of release observed. Thus even though Verapamil failed to inhibit the first phase, it caused a marked inhibition of the second. That Verapamil has a rapid onset of action is shown by the fact that 5 μM was sufficient to completely prevent glucose-stimulated Ca^{++} influx over the first 5 min and by rapid inhibition of insulin release due to high K^+ concentration. Thus, failure to observe significant inhibition of first phase release in response to glucose cannot be a result of a slow onset of action. Moreover, Verapamil failed to inhibit the insulin release over 5 min evoked by a submaximal glucose concentration (8.3 mM), which stimulates Ca^{++} uptake to a lesser extent than 16.7 mM glucose. It seems unlikely, therefore, that failure of Verapamil to inhibit first phase insulin release is owing to a saturation of the release mechanism by maximally raised cytosol Ca^{++}, which in turn would not be affected by inhibition of Ca^{++} uptake. An alternative explanation for the failure of Verapamil to inhibit first phase insulin release is that the stimulated Ca^{++} uptake is unrelated to insulin release. This alternative appears unlikely, especially in view of the dose-related effect of glucose on initial Ca^{++} uptake. The major conclusion from these results is that first phase insulin release is independent of glucose-stimulated uptake of extracellular calcium. Assuming that Ca^{++} is an essential trigger to insulin release, the first phase release must be the result of changes in the handling of intracellular calcium.

Previous studies on the effects of Verapamil and D-600 have shown inhibition of both phases of insulin release (43, 44, 46) but in these studies the β-cells were exposed to the agents by perifusion for lengthy periods before the glucose stimulus. These conditions allow the possibilities for Verapamil to gain access to intracellular sites of action or for calcium depletion of the islets before the glucose stimulus is applied. Ca^{++} depletion is suggested by the finding that under basal conditions net uptake of $^{45}Ca^{++}$ after 90 min is markedly reduced (43, 44). Similarly, in studies in which cobalt was used to block calcium uptake and both phases of insulin release were inhibited, lengthy preperifusion was also performed (26). That calcium depletion by blockade of uptake could be dramatic is illustrated by a comparison of the total calcium content of the islets—approximately 10–11 pmol/islet (22)—and the fluxes of Ca^{++} across the plasma membrane. Assuming equilibrium conditions in the presence of

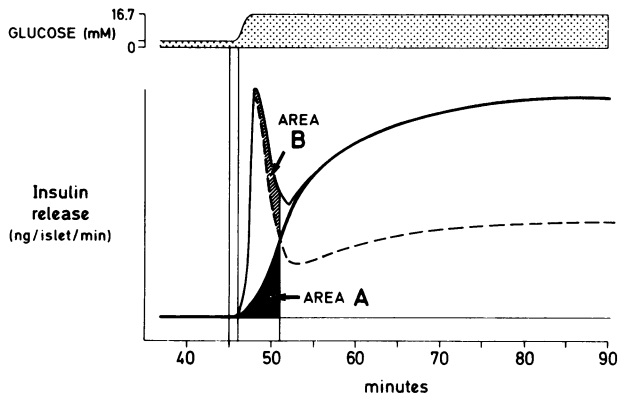

FIGURE 4 Schematic representation of the biphasic pattern of insulin release by perifused islets in response to 16.7 mM glucose (thin solid line) and the effect of 5 μM Verapamil (dashed line). In the data given in the text and Table I, first phase and second phase insulin release was assessed by measuring IRI in the effluent collected at 47–51 and 52–90 min, respectively. The pattern is drawn so that the second phase is a separate entity that starts immediately after exposure to high glucose and can be extrapolated back through the first phase period to the 46th min (heavy solid line). Thus area A represents the contribution to insulin release during the first phase period that is provided by the second phase process. Area B represents the inhibition of insulin release that was observed during the first phase period. As the second phase release was inhibited by 55% with 5 μM Verapamil, then 55% of area A should also be inhibited. This degree of inhibition is quantitatively similar to area B. This interpretation implies that the first phase insulin release process is totally unaffected by 5 μM Verapamil, a concentration that abolishes the glucose-induced stimulation of calcium uptake.

2.8 mM glucose and an influx of calcium across the membrane of 0.28 pmol/islet per min (Fig. 1) then influx (and efflux) rates for calcium in 35–40 min are equal to the total islet calcium content.

The effect of Verapamil on the two phases of insulin release can be expressed in a different fashion from that shown in Table I, when all insulin released during 46–51-min was compared in the presence or absence of the drug. Assuming that both phases of insulin release have independent components and start simultaneously, then by extrapolating the second phase to the start of the glucose stimulus (see Fig. 4), the component of the second phase contributing to the first phase can be calculated. By using the 16.7 mM glucose experiments of Fig. 2, for the extrapolation, this component amounts to 23% of the first phase. As second phase release is inhibited by 55%, then the second phase contribution to the inhibition observed during the time of first phase release should be 0.55 × 23%. This value, 13%, is essentially the same as that measured, 12%, and is in accord with the mechanism for first phase insulin release which is totally independent of the stimulation of Ca^{++} uptake. Thus, the present results show a striking difference in the dependency of the two phases of insulin release on Ca^{++} uptake, without disputing the hypothesis that Ca^{++} triggers insulin release. The β-cells contain Ca^{++} stores, as evidenced by histochemical localization (29, 30, 47, 48), which are thought to be under physiological regulation (48, 49). The results obtained with Verapamil show that glucose can cause first phase insulin release without recourse to increased Ca^{++} influx. This leads to the possibility that the inhibitory effect of glucose on Ca^{++} efflux (and perhaps a simultaneous mobilization of Ca^{++} from intracellular storage sites) is sufficient to cause a transitory rise in cytosol Ca^{++} and to trigger the first phase release. Subsequent increased Ca^{++} influx would then be responsible for the full development of the second phase, as demonstrated by the fact that 5 μM Verapamil blocked the second phase release by some 55%. Thus, in the second phase, the handling of intracellular Ca^{++} and the net uptake of extracellular Ca^{++} both contribute to the full display of insulin release.

In this delineation of the roles of intracellular and extracellular Ca^{++} in biphasic insulin release, it is assumed that glucose has two effects on Ca^{++} handling, namely, blockade of efflux (20–22) and stimulation of influx (23–28, 31). Both these effects could account for the raised cytosol Ca^{++} and net gain of intracellular Ca^{++} content. Under the conditions imposed for this study, the inhibition of efflux is obscured by the rapidity of onset of increased $^{45}Ca^{++}$ efflux (22). Thus the question arises as to the significance of increased $^{45}Ca^{++}$ efflux from preloaded islets. Under basal equilibrium conditions, the β-cell has to remove Ca^{++} from its cytosol because of the unfavorable inward electrochemical gradient. It is thought that major Ca^{++} extrusion against the concentration gradient is an energy-dependent process that may utilize energy directly, as in a calcium pump (50, 51) or indirectly as in the case of a Na^{+}/Ca^{++} exchange process (52–54). It follows that significant inhibition of Ca^{++} efflux by glucose must be exerted on an energy-dependent step. Removal of Ca^{++} from the cytosol is achieved by the plasma membrane and by the membranes of intracellular organelles which act as Ca^{++} stores and buffers. Glucose inhibition of outward fluxes could apply equally to these membranes. In consequence, glucose would cause a rise in cytosol Ca^{++} that would result in increased $^{45}Ca^{++}$ efflux. At present it is not possible to define the quantitative aspects of the $^{45}Ca^{++}$ efflux, nor do we know the extent to which the $^{45}Ca^{++}$ efflux represents Ca^{++} pumping, diffusion, Ca^{++}/Ca^{++} exchange, or Na^{+}/Ca^{++} exchange. It is known that only an insignificantly small amount of the $^{45}Ca^{++}$ is extruded via the granule secretion process per se (22, 28). Thus, the overall pattern of $^{45}Ca^{++}$ efflux could reflect the cytosol Ca^{++} concentration. Bearing in mind that the specific activity of the intracellular $^{45}Ca^{++}$ is continually being decreased

as unlabeled Ca^{++} enters the cells, it is noteworthy that the pattern of $^{45}Ca^{++}$ efflux resembles that of insulin release (Figs. 2 and 3). Thus, there is a first phase of $^{45}Ca^{++}$ efflux, a well-defined nadir, and subsequently a second rising phase of efflux. The lack of persistence of the second phase of $^{45}Ca^{++}$ efflux could be the result of the continually diminishing specific activity of the $^{45}Ca^{++}$ in the islets. Thus, if the pattern of $^{45}Ca^{++}$ efflux reflects the cytosol Ca^{++} concentration, then the biphasic insulin release is a reflection of a biphasic change in the cytosol Ca^{++} concentration. The reason for the transient nature of the first phase of insulin release, and presumably of the first peak in cytosol Ca^{++} concentration, is not known although an overshoot phenomenon would be a likely explanation.

The possibility that the two phases of insulin release could be regulated by separate mechanisms has been implicit in the observation that in "prediabetic" and mildly diabetic people, the sensitivity to glucose is less in the first phase than in the second (55–58). Thus Ca^{++} storage or ability to mobilize Ca^{++} stores in response to glucose could be involved in the impaired first phase insulin release in the diabetic state.

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