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Research Article

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ABSTRACT Glucose-induced inhibition of Ca^{++} extrusion from the β-cell may contribute to the rise in cytosol Ca^{++} that leads to insulin release. To study whether interference with Na/Ca exchange is involved in this inhibition the effects of glucose were compared to those of ouabain. This substance inhibits Na/K ATPase, decreases the transmembrane Na^+ gradient in islets, and thus interferes with Na/Ca exchange. Collagenase isolated rat islets were maintained for 2 d in tissue culture with a trace amount of $^{45}Ca^{++}$. Insulin release and $^{45}Ca^{++}$ efflux were then measured during perifusion. In Ca^{++}-deprived medium (to avoid changes in tissue specific radioactivity) 16.7 mM glucose inhibited $^{45}Ca^{++}$ efflux. Initially 1 mM ouabain inhibited $^{45}Ca^{++}$ efflux in a similar fashion, the onset being even faster than that of glucose. The effects of 16.7 mM glucose and ouabain were not additive, indicating that both substances may interfere with Na/Ca exchange. In the presence of Ca^{++}, 16.7 mM glucose induced biphasic insulin release. Ouabain alone caused a gradual increase of insulin release. Again, the effects of ouabain and 16.7 mM glucose were not additive. In contrast, at a submaximal glucose concentration (7 mM) ouabain enhanced both phases of release. An important role for Na/Ca exchange is suggested from experiments in which Ca^{++} was removed at the time of glucose-stimulation (16.7 mM). The resulting marked inhibition of insulin release was completely overcome during first phase by ouabain added at the time of Ca^{++} removal; second phase was restored to 60%. This could be due to the rapid inhibitory action of ouabain on Ca^{++} efflux thereby preventing loss of cellular calcium critical for glucose to induce insulin release. It appears, therefore, that interference with Na/Ca exchange is an important event in the stimulation of insulin release by glucose.

INTRODUCTION

An increase in the concentration of ionized Ca^{++} in the cytosol of the pancreatic β-cell is generally believed to be one of the critical events in glucose-stimulated insulin release (1–6). The following effects of glucose on islet cell Ca^{++} handling could all contribute to an increase in cytosol Ca^{++}: (*a*) Glucose enhances Ca^{++} influx (1, 2, 7). (*b*) Glucose inhibits $^{45}Ca^{++}$ efflux from islets preloaded with the isotope (2–4, 8). (*c*) Glucose, possibly via cyclic AMP (9) or a metabolite (10), affects cellular Ca^{++} stores (4, 11). It is of interest that in two diabetic animal syndromes defects in Ca^{++} handling appear to be involved in the impaired insulin response to glucose (12, 13).

In view of the finding that the increase in Ca^{++} influx is not necessary for the early insulin response to glucose (1, 14, 15), as will also be demonstrated in this study, the other detectable early event, the inhibition of Ca^{++} efflux may be of particular importance in the initiation of insulin release. This inhibition can precede the increase in the rate of insulin release (2, 8) and is likely to occur throughout the stimulation, even when $^{45}Ca^{++}$ efflux is stimulated by glucose (2, 8). The mechanism by which glucose causes inhibition of Ca^{++} efflux is unknown. In other tissues, the extrusion of Ca^{++} against its electrochemical gradient occurs mainly via a Ca pump (16) and a Na/Ca exchange system (17, 18). The latter depends on an inward Na^+ gradient, which in turn is maintained by the activity of the Na pump (Na/K ATPase). Therefore, the effects of ouabain, an inhibitor of the Na pump (18), were studied and compared to those of glucose, to assess the involvement of a Na/Ca exchange in glucose-stimulated insulin release. The results suggest that inhibition of a Na/Ca

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exchange mechanism is important for the stimulation of insulin release by glucose.

METHODS

Isolation of islets and maintenance in tissue culture. Pancreatic islets were isolated by the collagenase digestion technique (19) from male Wistar rats weighing 200–270 g. Batches of 250–500 islets were washed and maintained for 46 h in petri dishes (6 cm Diam) containing 4 ml tissue culture medium 199, supplemented with 10% heat-inactivated newborn calf serum, 14 mM $NaHCO_3$, 8.3 mM glucose, 400 IU/ml sodium penicillin G, 200 μg/ml streptomycin sulfate. For $^{45}Ca^{++}$ efflux studies, the medium also contained 100 μCi/ml of $^{45}Ca^{++}$ at a final concentration of 1.8 mM. The islets were maintained at 37°C, pH 7.4 in an atmosphere saturated with water and gassed with an air-CO_2 mixture.

Static incubation for measurement of $^{45}Ca^{++}$ uptake and insulin release. After the maintenance period the islets were washed twice by centrifugation (150 *g*) at room temperature with a modified 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 Hepes, and 2.8 mM glucose, pH 7.4 (KRB-Hepes). $^{45}Ca^{++}$ uptake and insulin release were measured as described (1, 7). In brief, 10 islets were incubated for 5 min in microfuge tubes on top of an oil layer in KRB-Hepes at 37°C. The incubation buffer contained 0.8 μCi of $^{45}Ca^{++}$ and 1.4 μCi [6,6′-*N*-^{3}H]sucrose (4 μM) as a marker of the extracellular space (7). The islets were separated from the radioactive medium by centrifugation at 8,000 *g* through the oil layer into 6 M urea. Insulin release was measured in an aliquot of the supernatant buffer. The bottoms of the tubes were cut above the urea layer and placed in 5 ml Ready-Solv HP for liquid scintillation spectrometry. $^{45}Ca^{++}$ 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 (7). At 5 min the extracellular space was 0.67±0.06 nl/islet (n = 25) (mean±SEM) and not changed by any of the test agents. $^{45}Ca^{++}$ uptake was linear for 5 min in the presence of both 2.8 and 16.7 mM glucose (7). Immunoreactive insulin was measured by the method of Herbert et al. using rat insulin as standard (20).

Perifusion for measurement of $^{45}Ca^{++}$ efflux and insulin release. The islets were perifused using 40 islets per chamber as described in detail previously (7, 8). The chamber volume was 70 μl and the flow rate 1.2 ml/min, which results in a turnover of 17 chamber vol/min. The standard perifusion medium consisted of Krebs-Ringer bicarbonate buffer containing 1.0 mM $CaCl_2$, 0.5% dialysed bovine serum albumin and 2.8 mM glucose. After 46 min perifusion with this standard medium the islets were exposed to the test agents for another 44 min. In some experiments the islets were exposed to a Krebs-Ringer bicarbonate buffer prepared without the addition of $CaCl_2$ (Ca^{++}-deprived medium) simultaneously with the application of the test agents. The total Ca^{++} concentration of this medium was 14.0± 1.1 μM (n = 3) as determined by atomic absorption spectrophotometry. When Ca^{++} was removed the measured Ca^{++} concentration decreased from 1 mM to basal values (14.6±2.0 μM, n = 5) in the sample collected during the 2nd min. Assuming an exponential decay curve for the decrease it can be calculated from the measured Ca^{++} concentration over the 1st min (63.8±2.0 μM, n = 5) that the concentration surrounding the islets was <25 μM only 15 s after the change. Potassium-deprived medium was prepared by replacing KCl and KH_2PO_4 by the corresponding sodium salt. Samples were collected every minute between minutes 41 and 55 and thereafter every 5th min. An aliquot was assayed for immunoreactive insulin (20). To 0.5 ml of the samples 5 ml of Biofluor were added for measurement of $^{45}Ca^{++}$ by liquid scintillation spectrometry. After background subtraction, the counts per minute were normalized by setting the mean counts per minute of the six samples collected from each chamber between minutes 41 and 46 to 100%, and expressing the subsequent values as a percentage of this mean. The mean basal efflux ranged between 70 and 150 cpm.

Statistical analysis was by Student's t test for unpaired data.

The materials employed and their sources were as follows: collagenase, Serva GmbH, Heidelberg, West Germany; medium 199 and Hepes solution, Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.; sodium penicillin G, Pfizer Chemicals, Div. Pfizer Inc., New York; streptomycin sulfate, Novo Industri A.S., Copenhagen, Denmark; plastic petri dishes, Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.; bovine serum albumin, Behringwerke A.G., Marburg/Lahn, West Germany; Trasylol, kindly provided by Professor Haberland (Bayer AG, Wuppertal, West Germany); EGTA, Sigma Chemical Co., St. Louis, Mo.; guinea-pig anti-pork insulin serum was a generous gift from Dr. H. H. Schoene, (Farbwerke Hoechst AG, Frankfurt, West Germany), rat insulin standard, Novo Research Institute, Copenhagen, Denmark; $^{45}CaCl_2$ and [6,6′-*N*-^{3}H]sucrose, The Radiochemical Center, Amersham, England; Biofluor, New England Nuclear, Boston, Mass.; Ready-Solv HP, Beckman Instruments International SA, Geneva, Switzerland; and ouabain, Merck AG, Darmstadt, West Germany.

RESULTS

Effects of ouabain at a nonstimulatory glucose concentration. In Fig. 1 the effects of two concentrations of ouabain on $^{45}Ca^{++}$ efflux and insulin release in a medium containing 1 mM Ca^{++} and 2.8 mM glucose are shown. Under basal conditions (2.8 mM glucose) no significant changes in insulin secretion were observed. $^{45}Ca^{++}$ efflux decreased gradually with time, which can be explained by the decrease of specific radioactivity in the islets (8). A ouabain concentration of 0.1 mM, which has been shown to inhibit Na/K ATPase in rat islet homogenates (21, 22), failed to affect insulin secretion and $^{45}Ca^{++}$ efflux significantly. In contrast, 1 mM ouabain caused a significant increase of insulin secretion, which was most pronounced in the later part of the perifusion. A small effect, however, could also be detected within the first 5 min (Table I). The rise in insulin release was accompanied by a late and marked increase in the rate of $^{45}Ca^{++}$ efflux. In the following experiments, therefore, 1 mM ouabain was further tested.

Effects of ouabain at a submaximal glucose concentration. A concentration of 7 mM glucose caused a biphasic release of insulin (Fig. 2), with a first peak 4 min after exposure to glucose. When 1 mM ouabain was added together with 7 mM glucose insulin release rose faster and was higher during first phase (Table IC). No significant effects were detected between 50 and 55 min, whereas thereafter, insulin release rates were again increased. As expected, 7 mM glucose caused a small increase of $^{45}Ca^{++}$ efflux. As in the case of insulin secretion, addition of ouabain stimulated $^{45}Ca^{++}$ efflux

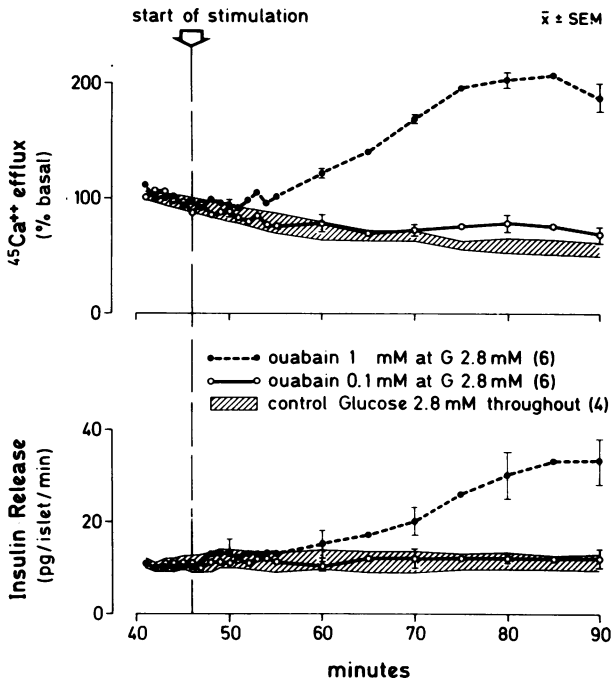


FIGURE 1 Effect of ouabain on $^{45}Ca^{++}$ efflux and insulin release at 2.8 mM glucose. After 2-d maintenance in the presence of $^{45}Ca^{++}$, islets were perifused for 46 min with a medium containing 2.8 mM glucose and then stimulated for 44 min with 0.1 or 1 mM ouabain. Numbers of observations in parentheses. G, glucose. For every individual experiment, mean $^{45}Ca^{++}$ efflux during the 6 min before stimulation was set to 100% and the subsequent changes expressed as a percentage of this mean.

early during first phase, and again from 60 min onwards, whereas no effect was seen from 52–55 min.

Effects of ouabain at a high glucose concentration. 16.7 mM glucose also caused a biphasic pattern of insulin release (Fig. 3). The rise started 1 min after exposure; a peak at 2–3 min was followed by a nadir at 5–7 min and an increasing second phase. Insulin release was accompanied by a peak of $^{45}Ca^{++}$ efflux during first phase and an elevated rate above basal during second phase. When 1 mM ouabain was added together with 16.7 mM glucose, a significant increase of insulin release was observed already in the 1st min of exposure (Fig. 3, arrow). This increase was evident in every individual experiment and was highly significant ($P < 0.001$ vs. 16.7 mM glucose alone). Otherwise no significant effect of 1 mM ouabain was detected above 16.7 mM glucose alone. Due to the rapid onset, ouabain enhanced overall insulin release during first phase (Table IB). No significant effect of ouabain on $^{45}Ca^{++}$ efflux was observed during first-phase period, whereas from 52 min onwards ouabain caused a significant rise of $^{45}Ca^{++}$ efflux until the end of the experiment.

Effects of ouabain on Ca^{++} uptake at different glucose concentrations. In Table II are shown results of measurement of Ca^{++} uptake in the presence of 2.8, 7, and 16.7 mM glucose. 7 mM glucose stimulated both insulin release ($P < 0.02$) and $^{45}Ca^{++}$ uptake over 5 min ($P < 0.001$) compared to 2.8 mM glucose. A further increase was seen in response to 16.7 mM glucose ($P < 0.001$ for insulin release and $P < 0.005$ for $^{45}Ca^{++}$ uptake vs. 7 mM glucose). 1 mM ouabain stimulated insulin release and $^{45}Ca^{++}$ uptake both at 2.8 and 7 mM glucose, whereas no significant stimulatory effect was seen above 16.7 mM glucose alone.

Effects of ouabain and glucose on $^{45}Ca^{++}$ efflux in Ca^{++}-deprived medium. In these experiments islets were perifused using a buffer prepared without the addition of $CaCl_2$ from the start of the perifusion. When 1 mM ouabain was added after 46 min (Fig. 4A) an inhibition of $^{45}Ca^{++}$ efflux was observed already in the 1st min ($P < 0.005$). $^{45}Ca^{++}$ efflux remained inhibited for 4 min ($P < 0.05$). Thereafter, it started to rise gradually and from the 60th min on, $^{45}Ca^{++}$ efflux was significantly increased compared to the control situation of 2.8 mM glucose throughout.

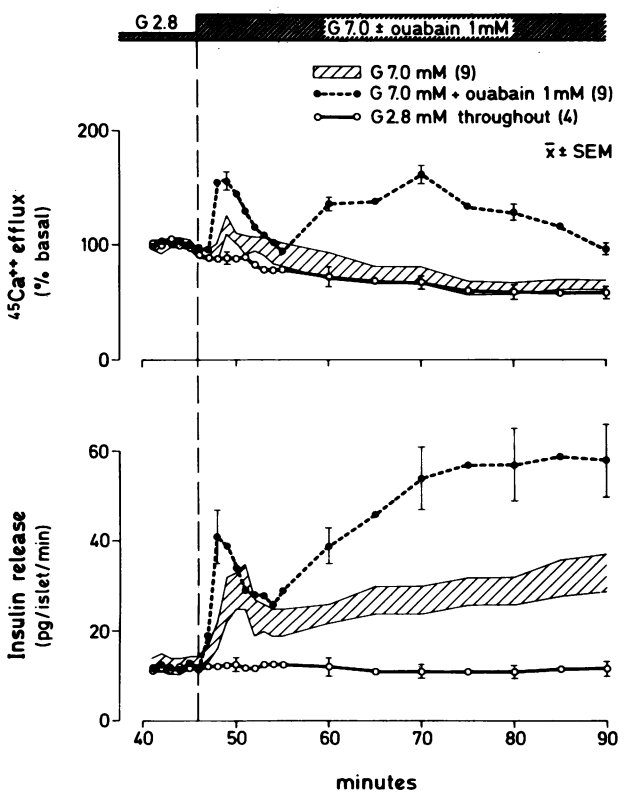
When 16.7 mM glucose was added during perifusion in the absence of Ca^{++} (Fig. 4B) an inhibition of $^{45}Ca^{++}$ efflux was also observed. This inhibition was significant from the 2nd min onward, which corresponds with the 1 min lag that glucose needed to induce insulin secretion at normal Ca^{++} (Fig. 3). When 1 mM ouabain was added with 16.7 mM glucose, the inhibition of $^{45}Ca^{++}$ efflux was significant already in the 1st min after exposure ($P < 0.05$), whereas no further effect was seen until 55 min compared to 16.7 mM glucose alone. From 55 min on $^{45}Ca^{++}$ efflux started to rise in a similar way as in response to ouabain at 2.8 mM glucose. No increase in the rate of insulin release was observed under the conditions described in Fig. 4.

Effects of ouabain and glucose addition at the time of Ca^{++} removal. Exposure to Ca^{++}-deprived medium decreases the calcium content of islets (3, 4). This makes it difficult to distinguish between the role of extra- and intracellular calcium (23). Therefore, the effects of ouabain were also investigated when Ca^{++} was removed (Fig. 5). Omission at the time of glucose stimulation caused a marked reduction of both phases of insulin release. The remaining insulin release was 26% for first and 12% for second phase when compared to the situation at 1 mM Ca^{++} (Table ID). When 1 mM ouabain was added at the time of glucose-stimulation insulin release, in spite of Ca^{++} removal, was greatly enhanced during both phases, resulting in a normal first phase (116 pg/islet) and an approximately half-normal second phase (60% of the control situation at 1 mM Ca^{++}). To exclude that the low Ca^{++} concentration (Methods) contributed to this restoration of insulin release experiments were also carried out with 100 μM

TABLE I
Integrated Insulin Release above Base Line

		Glucose		First-phase period	*P* value	Second-phase period	*P* value
		mM		*pg/islet/5 min*		*pg/islet/39 min*	
A	1	2.8	ouabain 0.1 mM	1.6±1.5 (6)		25±20	
	2	2.8	ouabain 1 mM	13±3 (6)		477±80	
B	1	16.7		117±16 (9)		1,494±177	
	2	16.7	ouabain 1 mM	173±17 (9)	<0.02 vs. B1	1,816±185	>0.20 vs. B1
C	1	7.0		49±10 (7)		639±73	
	2	7.0	ouabain 1M	99±16 (7)	<0.025 vs. C1	1,591±210	<0.005 vs. C1
D	1	16.7	Ca 1 mM	117±16 (9)		1,494±177	
	2	16.7	CaO	30±5 (8)	<0.001 vs. D1	176±34	<0.001 vs. D1
	3	16.7	CaO, ouabain 1 mM	116±11 (11)	<0.001 vs. D2	893±94	<0.001 vs. D2 <0.01 vs. D1
	4	16.7	CaO, KO	97±9 (9)	<0.001 vs. D2 >0.30 vs. D1	543±54	<0.001 vs. D2 and D1
	5	16.7	CaO, EGTA 0.1 mM ouabain 1 mM	110±10 (4)		498±128	
E	1	2.8	CaO, ouabain 1 mM	12±3 (7)		273±40	

Insulin release values were calculated by integrating the insulin release of the first 5 min or the subsequent 39 min of stimulation for first- and second-phase periods, respectively, after individual base-line subtraction (mean of the 6-min pre-stimulation). A, B, C, D1 perifused at 1 mM Ca^{++} throughout; D2–5, E Ca^{++} removal at the time of stimulation (text). Values are presented as mean±SEM, numbers of observations in parentheses. CaO and KO refer to media prepared without the addition of Ca and K, respectively.



EGTA present in the Ca^{++}-deprived medium. As shown in Table ID, the results were comparable whether EGTA was present or not.

To test whether this striking effect of ouabain is induced due to its inhibitory effect on the Na pump (20, 21), another condition known to inhibit the pump was chosen, exposure to K^+-deprived medium. This condition was again imposed at the time of glucose stimulation and Ca^{++} removal. Because the results were similar to those of ouabain they are only shown as the integrated areas in Table ID. The pronounced inhibition of glucose-induced insulin release in the absence of Ca^{++} was completely overcome during first phase and partially during second phase. Minor differences between the results obtained with ouabain or with K^+-deprived medium can be explained by a lower degree of inhibition of the Na pump achieved in the latter condition (24). In control experiments, it was found that 1 mM ouabain alone added at the time of Ca^{++} removal caused a slow and small increase of insulin release that was, however, reduced compared to the effects of ouabain

FIGURE 2 Effect of ouabain on $^{45}Ca^{++}$ efflux and insulin release at 7.0 mM glucose. Islets were perifused for 46 min with a medium containing 2.8 mM glucose and then exposed for 44 min to 7 mM glucose with or without 1 mM ouabain. Numbers of observations in parentheses. G, glucose.

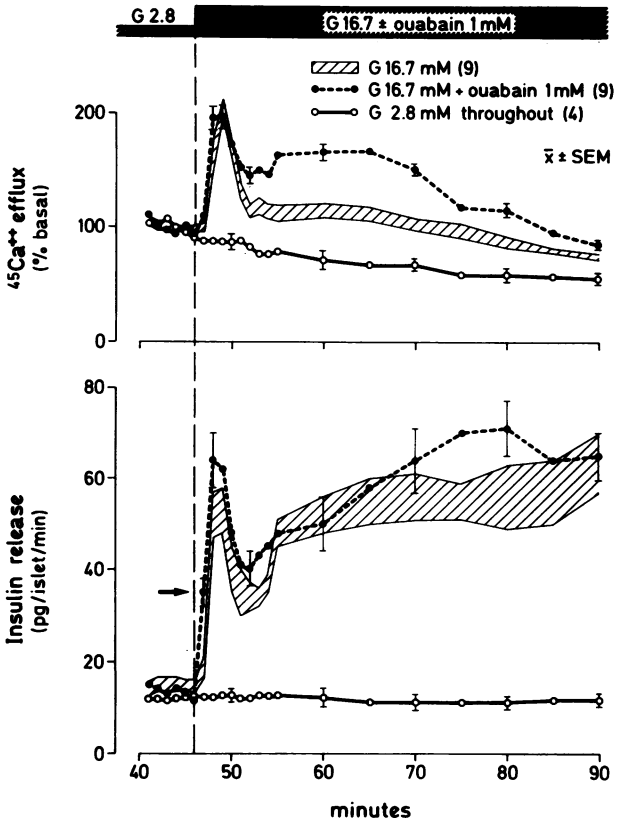


FIGURE 3 Effect of ouabain on $^{45}Ca^{++}$ efflux and insulin release at 16.7 mM glucose. Islets were perifused for 46 min with a medium containing 2.8 mM glucose and then stimulated for 44 min with 16.7 mM glucose with or without 1 mM ouabain. Numbers of observations in parentheses. G, glucose.

at normal Ca^{++}. (Results are included only as integrated areas in Table ID because the release pattern was similar to the effect of ouabain at normal Ca^{++}, Fig. 1 and Table IA.)

DISCUSSION

The mechanisms by which glucose stimulates insulin release are unknown. An early effect of glucose that may contribute to an increase in cytosol Ca^{++} is the inhibition of Ca^{++} efflux and it is this aspect of the action of glucose that is primarily considered in this paper. In other cells, Ca^{++} extrusion occurs, at least in part, via a Na/Ca exchange system (17, 18). Na^{+} entering the cell down its electrochemical gradient exchanges with, and extrudes, Ca^{++}. The energy applied to the outward movement of Ca^{++} against its electrochemical gradient is ultimately derived from ATP used by the Na pump, which maintains the Na^{+} gradient. Therefore, ouabain was used to interfere with the Na/Ca exchange.

In rat islet homogenates, inhibition of Na/K ATPase activity has been reported for ouabain concentrations between 0.05 and 1.0 mM (21, 22). Furthermore, ouabain increases the islet content of ^{22}Na (25, 26). From the results presented it is clear that ouabain has both early and late effects upon insulin release. The early stimulatory effect of ouabain, although detectable at low and high glucose, was most pronounced at an intermediate glucose concentration (Fig. 2) and in Ca^{++}-deprived medium at high glucose (Fig. 5). The late stimulatory effect was prominent at low and intermediate glucose, but did not occur at high glucose concentrations. Others have found that at nonstimulatory glucose concentrations ouabain either stimulated insulin release (27–31) or did not affect it (32, 33) and no effects were seen with high glucose concentrations (27, 29–31).

Concerning the early effect ouabain like glucose had a clear inhibitory effect on Ca^{++} efflux in Ca^{++} deprived medium (Fig. 4). As with glucose, this effect was not seen in the presence of Ca^{++}. At normal Ca^{++}, however, the insulin release induced by glucose or ouabain is most likely associated with an increase in cytosol Ca^{++},

TABLE II
Effects of Ouabain on Ca^{++} Uptake and Insulin Release

Glucose	$^{45}Ca^{++}$ uptake	*P* value	Insulin release	*P* value
mM	*pmol/islet/5 min*		*pg/islet/5 min*	
2.8	0.78±0.06 (21)	<0.001	41±5	<0.005
2.8 + ouabain 1 mM	1.51±0.16 (15)		72±9	
7.0	1.46±0.15 (14)	<0.02	67±11	<0.005
7.0 + ouabain 1 mM	2.28±0.26 (14)		154±21	
16.7	2.32±0.23 (12)	>0.40	207±18	>0.20
16.7 + ouabain 1 mM	2.65±0.37 (12)		235±19	

Insulin release and $^{45}Ca^{++}$ uptake were measured over 5 min on the same batches of islets. Values are presented as mean±SEM, numbers of experiments in parentheses. *P* values compare the effects of ouabain to the respective control without ouabain. For further statistical comparison, see text.

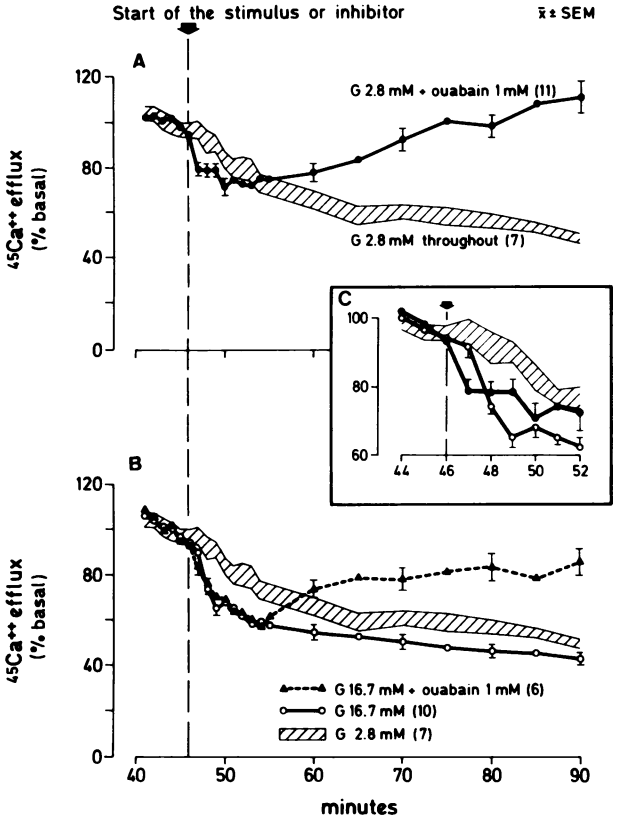


FIGURE 4 Effect of ouabain and glucose on $^{45}Ca^{++}$ efflux after exposure to Ca^{++}-deprived medium. (A) Islets were perifused throughout with 2.8 mM glucose and .Ca deprived medium (Methods). After 46 min, 1 mM ouabain was added. (B) Ca^{++} deprived medium throughout. After 46 min the glucose concentration was raised from 2.8 to 16.7 mM. To one group 1 mM ouabain was added at the same time. Numbers of observations in parentheses. G, glucose. (C) The early effects of ouabain (closed circles) and 16.7 mM glucose (open circles) are compared again on an expanded scale.

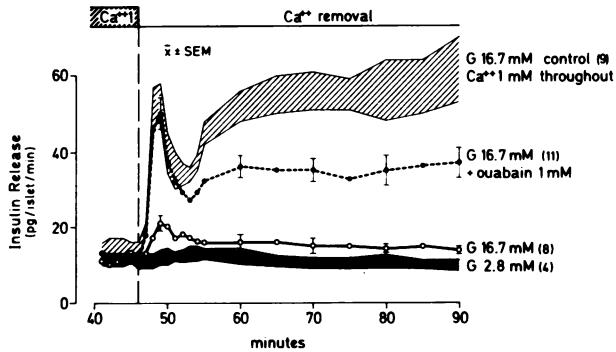


FIGURE 5 Effect of ouabain on glucose-induced insulin release when Ca^{++} is acutely removed. Islets were perifused for 46 min with 2.8 mM glucose and 1 mM Ca^{++}. Ca^{++}-deprived medium was introduced after 46 min containing either 16.7 mM glucose + 1 mM ouabain, 16.7 mM glucose alone or 2.8 mM glucose. For comparison, glucose-induced insulin release in the presence of 1 mM Ca^{++} is shown by the upper shaded area. Results obtained with ouabain addition were similar when 100 μM EGTA was present in the Ca^{++}-deprived medium (comparison; Table ID5); data not included. G, glucose.

which in turn is thought to be reflected by an increase in $^{45}Ca^{++}$ efflux (1, 5, 7, 8) as in other cells (34). Therefore, the underlying inhibitory effect is probably present, but not detectable (8). Four ouabain, this inhibition is probably due to interference with Na/Ca exchange such that increased intracellular Na^+ (Na^+_i) would compete with Ca^{++} for extrusion by a common carrier. Thus, the increase of Na^+_i by ouabain (25, 26) and the resulting decrease of the Na^+ gradient could explain the observed inhibition of $^{45}Ca^{++}$ efflux, as in the squid axon (18). This, of course, could explain the early effects of ouabain on insulin release at normal Ca^{++}. It seems likely that the inhibitory effect of ouabain on Ca^{++} efflux, although not detected, is also present at normal Ca^{++}, since ouabain induces the same change in the Na^+ gradient in the presence or absence of Ca^{++} (26). That a decrease of the Na^+ gradient indeed results in inhibition of Ca^{++} efflux can be inferred from the finding that in islets, decreasing extracellular Na^+ caused an increased Ca content (35) without an apparent effect on Ca^{++} influx. Strikingly, the inhibition of $^{45}Ca^{++}$ efflux by ouabain is detectable already within the 1st min of exposure. This is in accord with electrophysiological evidence that demonstrates a depolarizing effect of ouabain in <1 min (24). Further, the transmembrane flux of Na^+ has been estimated to be 25–35% of the total islet cell Na per min (26). Therefore, ouabain could rapidly affect the Na^+ gradient and in turn the Na/Ca exchange.

The major question is whether glucose also inhibits Ca^{++} efflux via the Na/Ca exchange. The similarity of the effects of ouabain and glucose on Ca^{++} efflux and the lack of additivity (Fig. 4), the potentiation of insulin release by ouabain at intermediate glucose concentrations, and the failure of ouabain and high glucose to exert additive effects on insulin release, suggest that both ouabain and glucose affect the same mechanism; the Na/Ca exchange. Further evidence is the finding that in Ca^{++}-depleted medium a decrease of extracellular Na^+ resulted in a decrease of $^{45}Ca^{++}$ efflux (36). Since this decrease was not observed at a high glucose concentration, it was concluded that glucose inhibits Ca^{++} efflux by interfering with Na/Ca exchange (36).

The mechanism by which glucose inhibits the Na/Ca exchange is unknown. Although in islet homogenates, glucose was found to inhibit Na/K ATPase activity in one report (21), but not in another (22), it is unlikely that glucose acts on Na/Ca exchange by inhibiting the Na pump like ouabain, since measurements of Na^+ and K^+ fluxes in islets have shown opposite effects of glucose and ouabain (25, 26, 37–39). The observation that

glucose did not increase the steady-state ^{22}Na content of islets (25, 26) is difficult to reconcile with the decrease of the Na^+ gradient that occurs with ouabain. One possibility of a common mechanism by which glucose and ouabain interfere with Na/Ca exchange could be that both agents depolarize the β-cell membrane (24, 40) and, at least in the squid axon, depolarization has been shown to inhibit Na-dependent Ca^{++} efflux (18).

Ouabain could also contribute to increase cytosol Ca^{++} by depolarization of the β-cell membrane (24, 40), which would permit Ca^{++} influx through voltage-dependent Ca^{++} channels (1, 24, 41). Such an effect was seen both at low and intermediate glucose concentrations but not at high (Table II), presumably since depolarization was maximal with high glucose alone (24, 41). Although this Ca^{++} influx may well contribute to the stimulation of insulin release it is not the major mode of action of ouabain, since insulin release due to ouabain was less affected than that due to glucose when extracellular Ca^{++} was removed (Fig. 5 and Table IA and E).

Late effects of ouabain were prominent on both $^{45}Ca^{++}$ efflux and on insulin release, the latter effect being similar to a previous report (27). Since ouabain increases Na_i^+ (25, 26), the slow increase of $^{45}Ca^{++}$ efflux is probably due to a continued gradual increase of cytosol Ca^{++} in turn due to the combined effects of inhibited Na/Ca exchange, increased Ca^{++} influx and possibly mobilization of Ca^{++} from stores by the increased Na_i^+. This latter effect has been demonstrated in isolated heart mitochondria (42) and Na^+ has been shown to inhibit Ca^{++}-ATPase in subcellular fractions of islets (43). Mobilization of stored Ca^{++} by the accumulation of intracellular Na^+ has also been proposed as the mechanism underlying veratridin-stimulation of insulin release (26, 27, 33). As mobilization of Ca^{++} by ouabain appears to be only a late effect it is possible that the Na/Ca exchange at the plasma membrane is more sensitive to Na_i^+ than is the mobilization of stored calcium.

One consequence of the early effect of ouabain to block Na/Ca exchange rapidly, can be seen in Fig. 5. Ca^{++} removal at the time of glucose addition resulted in a prompt reduction of insulin release. However, when ouabain was added with glucose, a striking restoration of first-phase release was seen. Since Ca^{++} efflux was inhibited more rapidly by ouabain than by glucose (Fig. 4), a likely explanation is that during the 1-min period before glucose stimulates insulin release, ouabain, by decreasing the Na^+ gradient, prevents loss of cellular Ca^{++}. This would in turn allow glucose to raise cytosol Ca^{++} high enough by the use of cellular calcium for a normal first phase of insulin release. This is further substantiated by the finding that when Ca^{++} was removed only 1 min after glucose application (i.e., before the increase of insulin release) a preserved first phase was again seen in Ca^{++}-deprived medium without ouabain (15). That the restoration of insulin release by ouabain is due to action on the Na pump is clear, since inhibition of the pump by K^+ removal gave an almost identical result (Table ID).

The only partial restoration of second-phase insulin release by ouabain in the absence of added Ca^{++} points to a more pronounced dependency of this phase on extracellular Ca^{++}. This agrees with studies using verapamil to block Ca^{++} uptake (1, 41) or islets with increased calcium stores (14) and demonstrates differences in the regulation of the two phases. The increase in cytosol Ca^{++} during the first phase of glucose-stimulated insulin release does therefore not appear to be due to increased Ca^{++} influx, but rather to be generated by cellular calcium. Interference with the Na/Ca exchange at the plasma membrane leading to inhibition of Ca^{++} efflux by itself is not sufficient to cause first-phase release because ouabain, causing similar inhibition of Ca^{++} efflux during this time period, failed to elicit first-phase release. Therefore, an additional effect of glucose on cellular Ca stores must be operative, possibly an interference with Ca^{++} sequestration into subcellular organelles (10, 44, 45). For the full development of second phase, however, both increased Ca^{++} uptake and use of cellular Ca^{++} are required (1, 41).

The most important conclusions from this work are that glucose in its action to stimulate insulin release raises cytosol Ca^{++} in part by inhibition of Ca^{++} efflux, which may be achieved by interference with Na/Ca exchange at the plasma membrane, and that glucose must have an effect on intracellular calcium stores.

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