

Synergistic Insulin Release Induced by Glucose and Carbachol is not associated with an Increase in Cytoplasmic Free Calcium Levels

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Abstract. Synergistic insulin release is observed in combination with muscarinic agonist and glucose. In order to define the role of changes in the cytoplasmic free Ca^{2+} level ($[\text{Ca}^{2+}]_i$), the effect of combination with carbachol and glucose on both phosphoinositide breakdown in rat pancreatic islets and $[\text{Ca}^{2+}]_i$ dynamics in single fura 2-loaded B-cells was investigated. When the islets were prelabeled with myo-[2- ^3H]inositol, phosphoinositide breakdown was obtained and inositol trisphosphate levels were increased in the presence of carbachol (0.5 mM). Inositol trisphosphate levels induced by carbachol were further increased by co-stimulation with 16.7 mM glucose. $[\text{Ca}^{2+}]_i$ in single-B-cells was increased by 16.7 mM glucose. A prompt, transient rise in $[\text{Ca}^{2+}]_i$ was induced by carbachol. However, carbachol failed to augment the $[\text{Ca}^{2+}]_i$ response in the presence of 16.7 mM glucose. These data suggest that a synergistic release of insulin occurs without an amplification of $[\text{Ca}^{2+}]_i$ regardless of an increase in inositol trisphosphate.

Key words: Insulin release, Ca^{2+} , Fura-2, Phosphoinositide breakdown

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IT IS WELL known that the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is involved in insulin secretion induced by a variety of secretagogues [1]. Glucose promotes closure of ATP-sensitive K^{+} -channels, resulting in depolarization and opening of voltage-dependent Ca^{2+} channels [2–4]. Glucose promptly increases $[\text{Ca}^{2+}]_i$ in pancreatic B-cells [5–9]. A receptor-mediated increase in $[\text{Ca}^{2+}]_i$ was proposed from an experiment with carbachol, a muscarinic agonist, which is known to activate phospholipase C, leading to the generation of inositol 1,4,5-trisphosphate (IP_3) and diglyceride [10]. It has been suggested that IP_3 acts as the second messenger by which carbachol mobilizes intracel-

lular Ca^{2+} [11]. Synergistic insulin secretion occurs in the presence of glucose and muscarinic agonist [12, 13], probably resulting from amplification of the Ca^{2+} signaling machinery. However, direct measurement of $[\text{Ca}^{2+}]_i$ remains to be investigated. In the present study, we examined the combined effects of glucose and carbachol on insulin release and inositol trisphosphate production in rat pancreatic islet. We then determined $[\text{Ca}^{2+}]_i$ levels in single B-cells. The data presented here suggest that the potentiation of insulin release can be promoted without an increase in $[\text{Ca}^{2+}]_i$ in the B-cells.

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Materials and Methods

Insulin secretion

Wistar strain male rats of 8–10 weeks old were

used throughout the experiments. After decapitation, pancreatic islets of Langerhans were isolated by collagenase digestion [14]. After preincubation at 37°C in 2 ml Krebs-Ringer bicarbonate buffer (KRBB) containing 2.8 mM glucose and 0.5 mg/ml BSA, batches of 10 islets were incubated with 1 ml KRBB containing glucose (2.8 mM or 16.7 mM) with or without carbachol (0.5 mM) for 20 min. Then the media were collected and kept at -20°C for measurement of the insulin concentration by specific radioimmunoassay as previously described [15].

Phosphoinositide breakdown

Islets were labeled with 10 μ Ci myo-[2-³H]inositol (18.9 Ci/mmol, Amersham, Buckinghamshire, UK) in 0.2 ml KRBB containing 2.8 mM glucose for 2 h under 95% O₂-5% CO₂ at 37°C. The ³H-labeled islets were washed five times with 1 ml KRBB. Forty islets were then incubated with 10 mM LiCl and 1 mM inositol. An appropriate test medium was also added. Incubation was terminated by the addition of 10% perchloric acid. The inositol phosphates of the islets were separated by anion exchange chromatography as described by Zawulich *et al.* [16]. This method does not distinguish between the inositol trisphosphate isomers, inositol, 1,4,5-trisphosphate and inositol 1,3,4-trisphosphate. Samples of the eluate were then counted in a liquid scintillation spectrometer.

Measurement of cytoplasmic free calcium concentration

Single islet cells were further prepared from the islets by pipetting in a Ca²⁺-deficient medium, suspended in RPMI 1640 medium (Flow Lab., Irvine, UK) containing 100 IU/ml penicillin and 100 μ g/ml streptomycin, and allowed to attach to circular coverglasses coated with poly-L-lysine (Sigma, St. Louis, MO) after incubation for 3-4 days at 37°C under an atmosphere of humidified 5% CO₂-95% air. Islet cell viability, assessed by trypan blue exclusion, was 95% in each experimental group. All experiments were performed on individual cells >10 μ m in diameter and not in contact with other cells, because non-insulin producing cells reportedly are characterized by their small size [17]. In addition, an increase in [Ca²⁺]_i due to glucose confirms the identity of the B-cells, since A-cells do

not respond [18].

Single islet cells attached on the cover glass were loaded with 1 μ M fura-2/AM (Molecular probes, Eugene, OR) in 2 ml of culture medium for 20-30 min at room temperature. The cells were rinsed and plated on the bottoms of open chambers designed for microscopic work. The chamber was placed on the stage of an inverted microscope (Olympus, Tokyo) equipped for epifluorescence measurements with a 40 \times UV-fluorite oil immersion objective. The cells were perfused with KRBB containing 2.8 mM glucose for 20 min and successively challenged with test medium at a flow rate of 3 ml/min at 34-37°C by heating the stage of the microscope and the medium. The intracellular Ca²⁺ concentration was estimated from fura-2 fluorescence by a ratiometric method using dual wavelength excitation (340 and 380 nm) and single emission (500 nm). [Ca²⁺]_i was calculated according to the method of Kudo and Ogura [19]. The calibration curve for [Ca²⁺]_i was obtained with calibration medium containing 145 mM KCl, 20 mM Tris/HCl (pH 6.8), 20 μ M fura-2 pentapotassium salt, EGTA and CaCl₂.

Statistical analysis

Results are expressed as the means \pm SEM. Statistical differences were evaluated by Student's *t* test or by analysis of variance followed by Scheffe's multiple-comparison test. *P* < 0.05 was considered significant.

Results

Insulin release from the isolated islets as a functional marker was assessed by batch incubation (Table 1). Basal secretion of insulin from the islets was slightly but not significantly increased by 0.5 mM carbachol. When the glucose concentration was raised from 2.8 mM to 16.7 mM, insulin release was increased to five times the basal level. Glucose-induced insulin release was synergistically potentiated by the addition of carbachol.

Carbachol stimulated phosphoinositide breakdown in the presence of 2.8 mM glucose, and inositol monophosphate (IP₁) and inositol trisphosphate (IP₃) levels were increased (Table 2). Higher concentrations of glucose (16.7 mM) stimulated greater phosphoinositide breakdown. The com-

bined addition of glucose and carbachol enhanced IP_1 and IP_3 production much more than glucose alone. An increase in IP_3 was also found in the islets after 5 min incubation.

The mean (\pm SE) level of basal $[\text{Ca}^{2+}]_i$ was 27.0 ± 1.7 nM ($n=24$) in the presence of 2.8 mM glucose. When the glucose concentration was raised to 16.7 mM, $[\text{Ca}^{2+}]_i$ was markedly increased but considerable cellular heterogeneity was obtained (Fig. 1). $[\text{Ca}^{2+}]_i$ showed a monophasic or biphasic pattern and a spike-shaped increase with various oscillations in most B-cells. As shown in Fig. 1b, a small decrease in $[\text{Ca}^{2+}]_i$ preceded a subsequent rise in

$[\text{Ca}^{2+}]_i$ in some but not all cells. The mean (\pm SE) peak value for $[\text{Ca}^{2+}]_i$ was 221.1 ± 28.5 nM ($n=12$). The increase in $[\text{Ca}^{2+}]_i$ remained even though the glucose level was lowered from 16.7 mM to 2.8 mM. An increase in $[\text{Ca}^{2+}]_i$ induced by 16.7 mM glucose was completely abolished when extracellular Ca^{2+} was absent (Fig. 1c). This reduction of $[\text{Ca}^{2+}]_i$ response had already been observed at a concentration of 1 mM Ca^{2+} (data not shown).

In the presence of 2.8 mM glucose, 0.5 mM carbachol caused a prompt and transient increase in $[\text{Ca}^{2+}]_i$, which was obtained with a mean (\pm SEM) lag of 28.0 ± 2.4 sec ($n=7$) (Fig. 2a). The mean

Table 1. Effect of carbachol on insulin release induced by glucose from rat pancreatic islets *in vitro*

Line	Glucose (mM)	Carbachol (mM)	Insulin release ($\mu\text{U}/\text{islet}/20$ min)
1	2.8	0	8.6 ± 1.0 (13)
2	2.8	0.5	15.6 ± 2.1 (12)
3	16.7	0	40.4 ± 4.2 (15)
4	16.7	0.5	97.7 ± 10.2 (15)
<i>P</i> values			
1) vs. 2)			ns
1) vs. 3)			0.001
2) vs. 4)			0.001
3) vs. 4)			0.005

Islets were preincubated for 30 min at 37°C . Then the batches of 10 islets were further incubated for 20 min in 1.0 ml test medium. The numbers of experiments are shown in parentheses. Statistical significance was determined by ANOVA. ns, not significant.

Table 2. Effect of carbachol on glucose-induced phosphoinositide breakdown in rat pancreatic islets

Contents of Incubation Medium		Time (min)	Radioactivity (cpm/40 islets)		
Glucose (mM)	Carbachol (mM)		IP_1	IP_2	IP_3
2.8	0	5	2934 ± 148	347 ± 20	179 ± 36
		20	2733 ± 250	237 ± 35	152 ± 22
2.8	0.5	5	3054 ± 469	413 ± 64	191 ± 50
		20	$5289 \pm 549^*$	$468 \pm 60^{**}$	$275 \pm 30^*$
16.7	0	5	2949 ± 468	422 ± 61	223 ± 33
		20	$5726 \pm 907^\#$	$520 \pm 66^\#$	$277 \pm 29^{##}$
16.7	0.5	5	3322 ± 362	$616 \pm 49^*$	$432 \pm 77^*$
		20	$8779 \pm 1055^*$	$592 \pm 83^*$	$352 \pm 33^*$

Values are shown as the mean \pm SE for 3–5 different experiments. IP_1 , inositol monophosphate; IP_2 , inositol bisphosphate; IP_3 , inositol trisphosphate. Statistical significance was determined with ANOVA for the group of 5 min (A) and 20 min (B). A: $*P < 0.05$ vs. 16.7 mM glucose without carbachol. B: $*P < 0.05$, $**P < 0.025$ vs. 2.8 mM or 16.7 mM glucose without carbachol. $^\#P < 0.025$, $^{##}P < 0.01$ vs. 2.8 mM glucose without carbachol.

(\pm SEM) peak value for $[Ca^{2+}]_i$ from seven different experiments was 163 ± 53 nM. There was little heterogeneity in $[Ca^{2+}]_i$ changes induced by carbachol. In the absence of extracellular Ca^{2+} , 0.5 mM carbachol also induced a steep, transient rise in

$[Ca^{2+}]_i$ (Fig. 2b). The mean (\pm SE) peak value (174 ± 22 nM) and the lag time (35 ± 7 sec) were not different from those in the presence of extracellular Ca^{2+} . However, $[Ca^{2+}]_i$ decreased rapidly to the basal level without a long shoulder in the absence of extracellular Ca^{2+} .

When the B-cells were perfused with 0.5 mM carbachol in the presence of 16.7 mM glucose, biphasic changes in $[Ca^{2+}]_i$ were always obtained (Fig. 3a). The first phase was transient and spiked in shape. The second phase was late and showed a gradual increase in $[Ca^{2+}]_i$ response, which was characterized by a heterogeneous increase in $[Ca^{2+}]_i$ with multiple oscillations. The mean (\pm SE) $[Ca^{2+}]_i$ in the first phase was 110.5 ± 20.1 nM, while that in the second phase was 140.9 ± 17.5 nM ($n=9$).

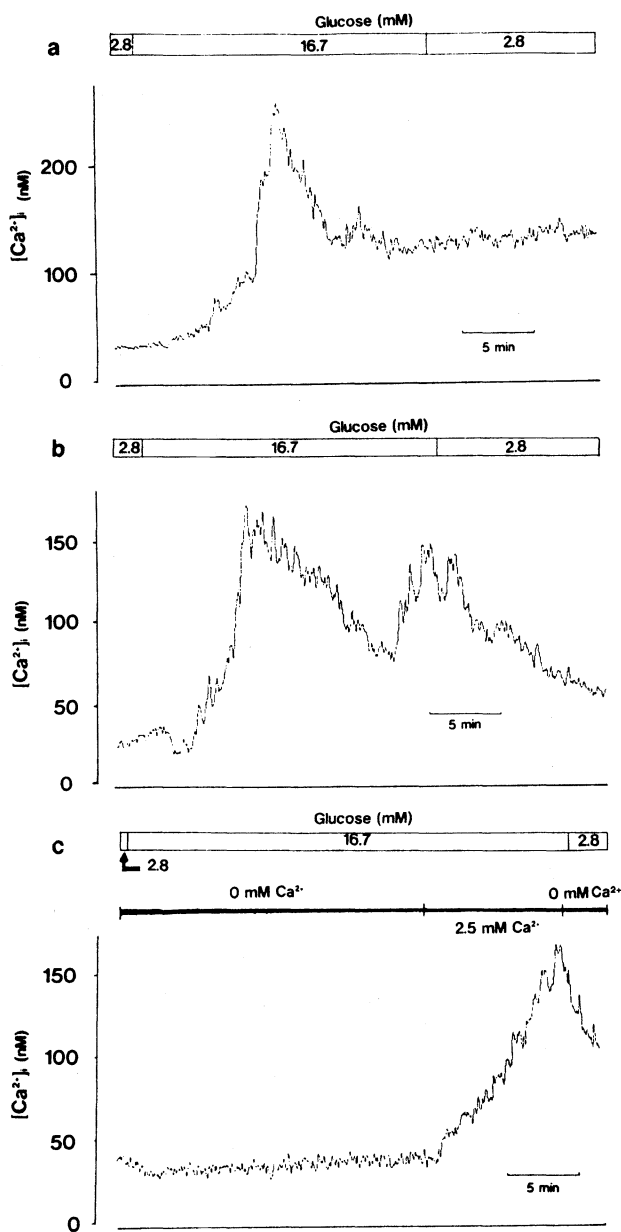


Fig. 1. Heterogeneous response of $[Ca^{2+}]_i$ induced by 16.7 mM glucose in single fura-2-loaded B-cells in the presence of 2.5 mM Ca^{2+} (Figs. 1a and 1b). Effect of the absence of extracellular Ca^{2+} on $[Ca^{2+}]_i$ response to glucose is shown in Fig. 1c. The glucose concentration in the perfusate is indicated by horizontal bars.

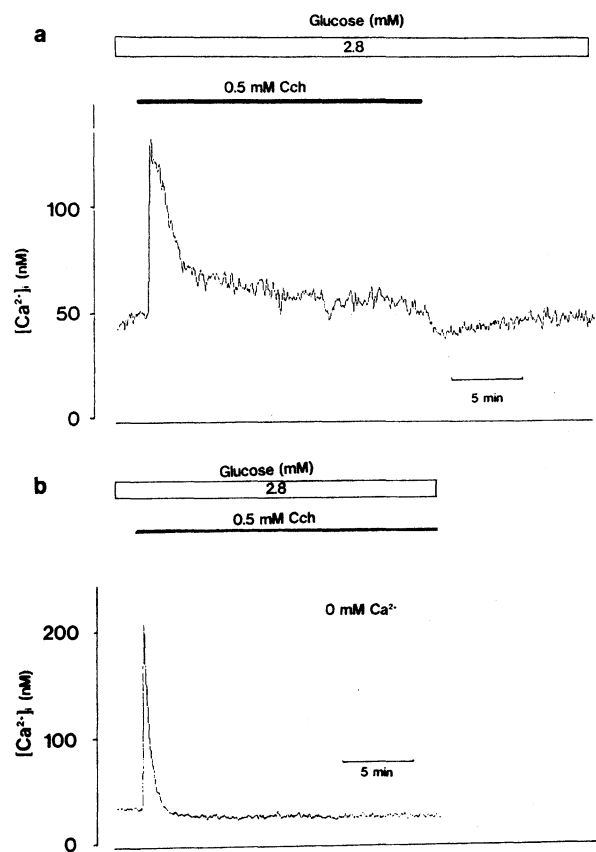


Fig. 2. Effect of 0.5 mM carbachol (Cch) on changes in $[Ca^{2+}]_i$ in a single fura-2-loaded B-cell. The glucose concentration in the perfusate was 2.8 mM. The solid line indicates the perfusion period of carbachol in the presence (a) or absence (b) of 2.5 mM Ca^{2+} . Figures are representative of 7 and 4 observations, respectively.

The $[\text{Ca}^{2+}]_i$ responses induced by the combination of 16.7 mM glucose and 0.5 mM carbachol were significantly ($P < 0.05$) reduced compared with 16.7 mM glucose alone. The mean (\pm SE) value for the lag period in the first phase was 36.3 ± 3.1 sec. When the combined effect of 16.7 mM glucose and 0.5 mM carbachol on changes in $[\text{Ca}^{2+}]_i$ was examined in the absence of extracellular Ca^{2+} , the first phase of $[\text{Ca}^{2+}]_i$ response was restored, while the second phase was completely abolished (Fig. 3b). After the addition of 2.5 mM Ca^{2+} to the perfusate, a rebound of $[\text{Ca}^{2+}]_i$ response occurred.

Discussion

Insulin secretion from pancreatic B-cells is influenced by a large number of modulators. Although an increase in $[\text{Ca}^{2+}]_i$ is functionally coupled with the initiation of insulin secretion [20, 21], it remains to be fully elucidated whether an increase in $[\text{Ca}^{2+}]_i$ is associated with the potentiation of insulin secretion.

Carbachol synergistically augmented insulin release from pancreatic islets incubated with high concentrations of glucose. These findings are in good agreement with previous reports [12, 13]. The Ca^{2+} -mobilizing agonists are poor secretagogues when added alone, but potentiate insulin secretion in the presence of stimulatory glucose concentrations.

Although carbachol failed to stimulate significant insulin release from pancreatic islets in the presence of low concentrations of glucose, phosphoinositide breakdown was augmented. High concentrations of glucose stimulated both insulin release and inositol phosphate production. It is interesting that the magnitude of inositol-lipid hydrolysis induced by 16.7 mM glucose was almost the same as that induced by 0.5 mM carbachol. These findings suggest that the magnitude of phosphoinositide breakdown is not well correlated to the amount of insulin secretion induced by each secretagogue. Phosphoinositide breakdown induced by 16.7 mM glucose was more pronounced in the presence of 0.5 mM carbachol. The IP_3 level was greater under this condition than with each stimulant alone. We therefore expected that combined stimulation could cause pronounced $[\text{Ca}^{2+}]_i$ response in B-cells.

We confirmed the profile and magnitude of $[\text{Ca}^{2+}]_i$ response induced by glucose and also by carbachol, as described before [5–9]. The $[\text{Ca}^{2+}]_i$ increase stimulated by glucose appears to be due to Ca^{2+} influx via voltage-sensitive Ca^{2+} channels, since it was completely inhibited in the absence of extracellular Ca^{2+} . On the other hand, a transient and spike shaped increase in $[\text{Ca}^{2+}]_i$ was obtained in the cells stimulated by carbachol in the absence of extracellular Ca^{2+} . However, the finding that $[\text{Ca}^{2+}]_i$ rapidly returned to basal level suggests that the carbachol-induced increase in $[\text{Ca}^{2+}]_i$ partly depends on a Ca^{2+} influx.

In the presence of stimulatory concentrations of

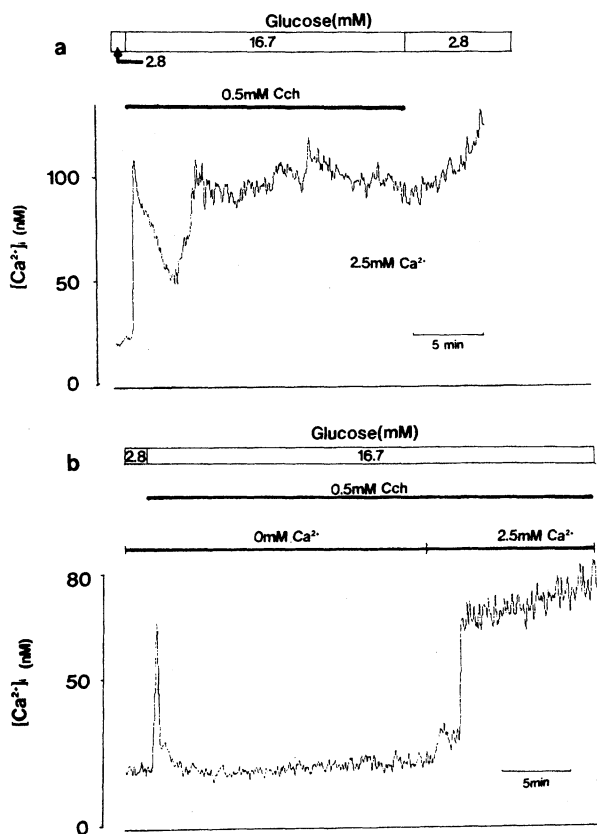


Fig. 3. Combined effect of 16.7 mM glucose and 0.5 mM carbachol (Cch) on changes in $[\text{Ca}^{2+}]_i$ in a single pancreatic B-cell. The glucose concentration in the perfusate was raised from 2.8 mM to 16.7 mM. The solid line indicates the perfusion period of carbachol in the presence of 2.5 mM Ca^{2+} (a) and in the presence of either 0 mM or 2.5 mM Ca^{2+} (b). Figures are representative of 9 and 5 observations.

glucose, carbachol caused biphasic changes in $[Ca^{2+}]_i$ in single B-cells. The first phase of $[Ca^{2+}]_i$ response was consistently obtained in the experiment in which extracellular Ca^{2+} was eliminated. The second phase of $[Ca^{2+}]_i$ response varied widely among the B-cells examined and was completely abolished in the absence of external Ca^{2+} . The combined addition of glucose and carbachol therefore only showed the summation of the $[Ca^{2+}]_i$ profile induced by each secretagogue. Our data on islet B-cells are consistent with results recently reported by Baffy *et al.* [22]. Sluggish insulin release induced by glucose was potentiated by co-stimulation with carbachol from a B-cell line derived from transgenic mice (beta-TG3). The $[Ca^{2+}]_i$ response evoked by combination with glucose and carbachol in these cells was very similar to that obtained in the islet B-cells. Taken together, these observations suggest that the potentiation of carbachol on glucose-induced insulin secretion may occur without amplification of $[Ca^{2+}]_i$. Tamagawa *et al.* [23] demonstrated that forskolin and phorbol ester stimulate insulin release without a rise in $[Ca^{2+}]_i$. This suggests that cyclic AMP and C-Kinase sensitized the secretory machinery of the B-cells to Ca^{2+} .

Although IP_3 was significantly increased by glu-

cose and carbachol, the finding that the combination rather lowered $[Ca^{2+}]_i$ suggests that the IP_3 levels do not directly correlate with an increase in $[Ca^{2+}]_i$. Biden *et al.* [24] suggested that the generation of IP_3 induced by glucose might be a secondary event following Ca^{2+} influx. They also found that IP_3 levels stimulated by carbachol were not affected either in the presence or absence of extracellular Ca^{2+} . Since both the first and second phases of $[Ca^{2+}]_i$ were decreased by the combination with glucose and carbachol, it is suggested that Ca^{2+} influx was inhibited. Garcia *et al.* [12] found that Ca^{2+} influx was markedly stimulated by acetylcholine at 10 mM glucose, but not at 3 mM- or 30 mM-glucose, when radioactive Ca^{2+} was monitored. Although further study is required, our data seem to indicate that a synergistic release of insulin occurs without a pronounced increase in $[Ca^{2+}]_i$ in pancreatic B-cells.

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