Two distinct modes of Ca^{2+} signalling by ACh in rat pancreatic β-cells: concentration, glucose dependence and Ca^{2+} origin

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1. Calcium signalling by acetylcholine (ACh) in single rat pancreatic β-cells was studied. The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by dual-wavelength fura-2 microfluorometry.

2. In the presence of basal glucose (2·8 mM), 10^{-6} to 10^{-4} M ACh (high ACh) transiently increased $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ response to 10^{-5} M ACh was little altered under Ca^{2+}-free conditions. Brief pulses of 10^{-5} M ACh evoked successive $[Ca^{2+}]_i$ responses, which were progressively inhibited by 0·2–0·5 μM thapsigargin, a specific inhibitor of the endoplasmic reticulum (ER) Ca^{2+} pump.

3. Elevation of glucose to 8·3 mM, a concentration which stimulates insulin release, increased $[Ca^{2+}]_i$ to an initial peak followed by a sustained, moderate elevation. Addition of 10^{-8} to 10^{-7} M ACh (low ACh) evoked a further increase in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ response to 10^{-7} M ACh was completely inhibited under Ca^{2+}-free conditions by 1 μM nitrendipine, a blocker of L-type Ca^{2+} channels, and by 100 μM diazoxide, an opener of ATP-sensitive K^+ channels.

4. In the presence of 8·3 mM glucose, $[Ca^{2+}]_i$ responses to 10^{-5} M ACh were reduced but not abolished by Ca^{2+}-free conditions, nitrendipine and diazoxide. Successive $[Ca^{2+}]_i$ transients induced by 10^{-5} M ACh pulses in the presence of nitrendipine were progressively inhibited by thapsigargin.

5. The results revealed two distinct modes of Ca^{2+} signalling: low ACh increases $[Ca^{2+}]_i$ by stimulating Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels only in the β-cells in which glucose has already elevated $[Ca^{2+}]_i$, while high ACh increases $[Ca^{2+}]_i$ at basal as well as stimulatory glucose concentrations by releasing Ca^{2+} from the ER. The former mechanism is likely to relate to the potentiator action and the latter to the initiator action of ACh on insulin release. High ACh and elevated glucose provoke both modes of Ca^{2+} signalling.

The autonomic nervous system plays an important role in the regulation of insulin release, and the neurotransmitter acetylcholine (ACh) mediates insulin release during vagal stimulation (Wollheim & Sharp, 1981; Ahren, Taborsky & Porte, 1986). ACh stimulates insulin release both *in vivo* and *in vitro*, and the effect is largely due to its direct action on pancreatic β-cells via muscarinic receptors (Iversen, 1972; Bergman & Miller, 1973; Wollheim & Sharp, 1981; Ahren *et al.* 1986). The muscarinic activation is coupled to an activation of phospholipase C (Prentki & Matschinsky, 1987) leading to a liberation of inositol 1,4,5-trisphosphate (IP_3) (Wollheim & Biden, 1986; Nilsson, Arkhammer, Hallberg, Hellman & Berggren, 1987) and an activation of protein kinase C (Peter-Reisch, Fathi, Schlegel & Wollheim, 1988; Persaud, Jones, Sugden & Howell, 1989), and also involves a membrane depolarization with bursts of action potentials (Gagerman, Idahl, Meissner & Taljedal, 1978; Cook, Crill & Porte, 1981; Hermans, Schmeer & Henquin, 1987), increases in the plasma membrane Na^+ permeability (Henquin, Garcia, Bozem, Hermans & Nenquin, 1988) and the cytosolic Na^+ concentration (Gilon & Henquin, 1993). All these activities are known to increase the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). It is well established that the insulin exocytosis is regulated by the level of $[Ca^{2+}]_i$ (Penner & Neher, 1988; Ämmälä, Eliasson, Bokvist, Larsson, Ashcroft & Rorsman, 1993). An increase in $[Ca^{2+}]_i$ by muscarinic activation has been demonstrated in RINm5F (Wollheim & Biden, 1986) and HIT insulinoma cells (Regazzi, Li, Deshusses & Wollheim, 1990; Hughes, Chalk & Ashcroft, 1990), *ob,ob* mouse islet cells (Nilsson *et al.* 1987) and β-cells (Gylfe, 1991), rat pancreatic β-cells (Yada & Sorimachi, 1989; Theler *et al.* 1992; Wang, Vaimbridge & Brown, 1992), and mouse whole islets (Gao, Gilon & Henquin, 1994). However, the nature of the $[Ca^{2+}]_i$ activity in

response to ACh in normal pancreatic β-cells is as yet largely undetermined. ACh increases insulin release mildly in the presence of basal glucose concentrations and extensively in the presence of glucose concentrations which are stimulatory for insulin release (Iversen, 1972; Bergman & Miller, 1973; Gagerman *et al.* 1978; Garcia, Hermans & Henquin, 1988; Regazzi *et al.* 1990). Hence ACh can be considered as a mild initiator as well as a strong potentiator of insulin secretion. Therefore it is of particular importance to disclose the β-cell $[Ca^{2+}]_i$ activities which specifically relate to the initiator and potentiator actions of ACh on insulin release.

In the present study, we attempted was made to characterize precisely the β-cell $[Ca^{2+}]_i$ activities in response to ACh and to determine their concentration and glucose dependence. $[Ca^{2+}]_i$ was measured in normal pancreatic β-cells isolated from Wistar rats by dual-wavelength fura-2 microfluorometry. We found two distinct actions of ACh on $[Ca^{2+}]_i$, one to release Ca^{2+} from the endoplasmic reticulum (ER), a mechanism which operates only at high ACh concentrations and at both basal and stimulatory glucose concentrations, and the other to stimulate Ca^{2+} influx through the L-type Ca^{2+} channel in the β-cell plasma membrane, a mechanism which operates only in the presence of stimulatory glucose concentrations and which has a lower threshold for ACh.

METHODS

Preparation and selection of islet β-cells

Islets of Langerhans were isolated from Wistar rats aged 8–12 weeks by collagenase digestion. Animals were anaesthetized by intraperitoneal injection of pentobarbitone at 80 mg kg^{-1}. The abdomen was opened, and collagenase (3 mg ml^{-1}) dissolved in 5 ml of 5 mM Ca^{2+}-containing Krebs–Ringer bicarbonate buffer (KRB) solution was injected into the common bile duct at the distal end after ligation of the duct proximal to the pancreas. The pancreas was dissected out and incubated at 37 °C for 17 min. Islets were collected and immediately dispersed into single cells in Ca^{2+}-free KRB solution. The single cells were plated on coverslips and maintained in short-term culture for up to 3 days in Eagle's MEM containing 5·6 mM glucose supplemented with 10% fetal bovine serum, 100 mg ml^{-1} streptomycin and 100 U ml^{-1} penicillin at 37 °C in a 95% air–5% CO_2 atmosphere. The cells during this period responded to glucose, ACh and tolbutamide (TB) in a consistent manner.

β-Cells were selected according to the previously reported procedure (Yada, Itoh & Nakata, 1993). Briefly, when the cells with a diameter of 12·5–17·5 μm were examined, the majority of the cells responded to glucose (8·3 and 16·7 mM) and all the cells to tolbutamide (300 μM) with increases in $[Ca^{2+}]_i$. These responses are typical of β-cells that are identified by immunocytochemistry as positive for insulin (Yada *et al.* 1994). Only the data from glucose- and TB-responsive cells were analysed.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured according to a method previously described (Yada, Kakei & Tanaka, 1992; Yada *et al.* 1994) with slight modification. In brief, the single cells on coverslips were incubated with 1 μM fura-2 acetoxymethyl ester (Grynkiewicz, Poenie & Tsien, 1985) for 30 min at 37 °C in KRB solution containing 2·8 mM glucose. The cells were then mounted in a chamber, placed on the stage of TMD inverted microscope (Nikon), and superfused using a peristaltic pump (Watson Marlow) at 1 ml min^{-1} at 37 °C with KRB solution with basal (1·0 and 2·8 mM) or elevated glucose concentrations (5·0, 8·3 and 16·7 mM). The fura-2 fluorescence from the cells due to excitation at 340 nm (F_{340}) and that at 380 nm (F_{380}) were detected every 2·5 s by an intensified charge-coupled device (ICCD) camera, and the ratio (F_{340}/F_{380}) was produced by an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan). Ratio values were converted to $[Ca^{2+}]_i$ according to the calibration curves obtained from the relationship between free Ca^{2+}

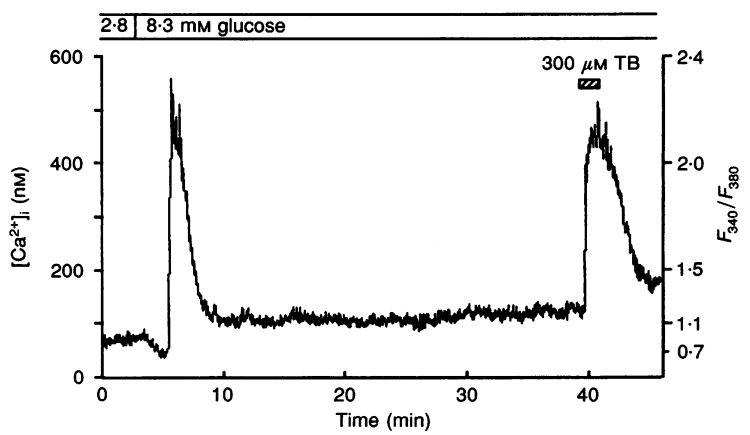


Figure 1. Typical $[Ca^{2+}]_i$ responses to a rise in glucose concentration and to tolbutamide in a single rat pancreatic β-cell

A rise in glucose concentration evoked an initial small decrease and a subsequent large increase in $[Ca^{2+}]_i$ in a single rat pancreatic β-cell. The increase in $[Ca^{2+}]_i$ was characterized by an initial peak followed by a sustained, moderate elevation of $[Ca^{2+}]_i$. The cells also responded to 300 μM tolbutamide (TB) with a monophasic increase in $[Ca^{2+}]_i$. The result is expressed as both the ratio of fura-2 fluorescence signals (F_{340}/F_{380}) and the calculated $[Ca^{2+}]_i$. The tracing is representative of 30 similar experiments. Glucose concentrations are indicated above the trace and the hatched bar specifies the period of exposure to TB.

concentration and the ratio determined in a cytosol-mimicking solution using Ca-EGTA buffer and fura-2 free acid.

Solutions and chemicals

Measurements were carried out in KRB solution composed of (mM): 129 NaCl, 5·0 $NaHCO_3$, 4·7 KCl, 1·2 KH_2PO_4, 1·0 $CaCl_2$, 1·2 $MgSO_4$ and 10 Hepes at pH 7·4 supplemented with 0·1% bovine serum albumin. Ca^{2+}-free KRB solution was made with 0·1 mM EGTA and no added Ca^{2+}. Fetal bovine serum was from Flow Laboratories. Fura-2 free acid and fura-2 acetoxymethyl-ester were from Molecular Probes and Dojin Chemical (Kumamoto, Japan). EGTA was from Dojin Chemical. Acetylcholine buromide and tolbutamide were from Nacalai Tesque (Kyoto, Japan). Thapsigargin and diazoxide were from Sigma. Nitrendipine was a gift from Yoshitomi Pharmaceutical (Osaka, Japan).

RESULTS

Effects of high glucose concentration on $[Ca^{2+}]_i$ in single rat pancreatic β-cells

$[Ca^{2+}]_i$ in single β-cells at a basal glucose concentration (2·8 mM) was around 50–110 nM. Elevation of the glucose concentration from 2·8 to 8·3 or 16·7 mM elicited an initial decrease and subsequent increase in $[Ca^{2+}]_i$ (Figs 1 and 2), as previously demonstrated in mouse and rat β-cells (Grapengiesser, Gylfe & Hellman, 1988; Hellman, Gylfe, Grapengiesser, Lund & Marcström, 1992; Yada *et al.* 1992), as well as in mouse whole islets (Valdeolmillos, Santos, Contreras, Soria & Rosario, 1989; Valdeolmillos, Nadal, Contreras & Soria, 1992). The increase in $[Ca^{2+}]_i$ was characterized by an initial transient peak to 300–800 nM followed by a maintained elevation at a lower level. The maintained elevation of $[Ca^{2+}]_i$ was stable in the majority of cells stimulated with 8·3 mM glucose (Fig. 1), while it was accompanied by oscillations of $[Ca^{2+}]_i$ in a minor proportion of cells (11 of 58 cells, 19%). The number of cells showing oscillations increased as the glucose concentration was raised further to 16·7 mM. In the present study, ACh was administered once the $[Ca^{2+}]_i$ increase had stabilized at a mildly elevated level after the initial transient.

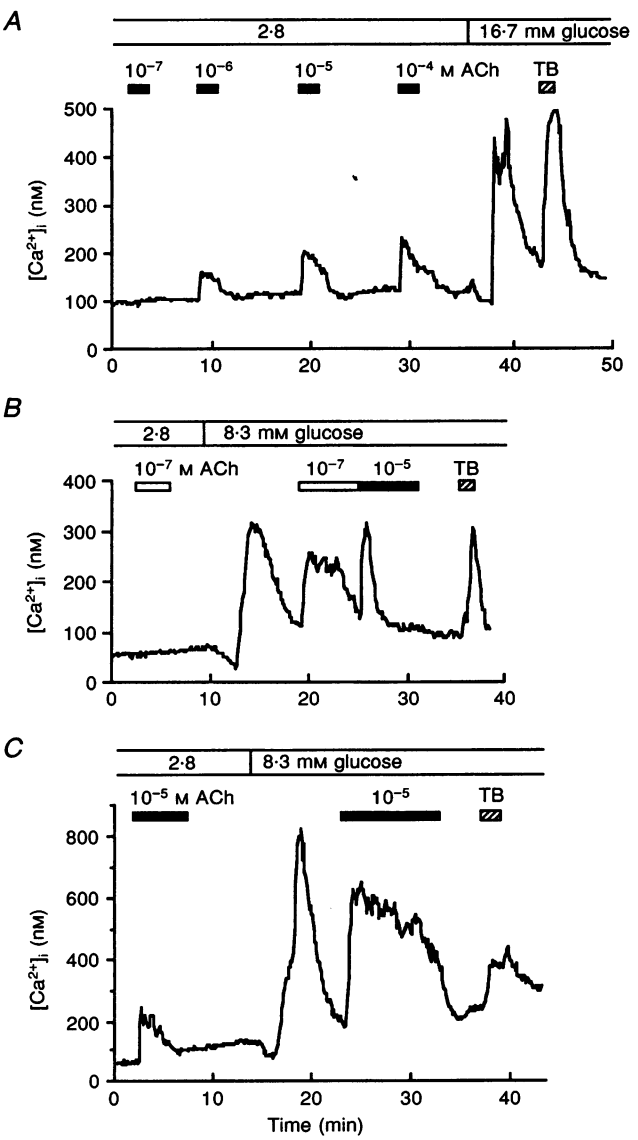


Figure 2. Concentration- and glucose-dependent ACh-evoked increase in $[Ca^{2+}]_i$ in single β-cells

A, in the presence of 2·8 mM glucose, a basal concentration, ACh at 10^{-6}, 10^{-5} and 10^{-4} M evoked an increase in $[Ca^{2+}]_i$, while ACh at 10^{-7} M was without effect. This cell subsequently responded to 16·7 mM glucose and 300 μM TB. *B*, ACh at 10^{-7} M was without effect in the presence of 2·8 mM glucose but subsequently evoked an increase in $[Ca^{2+}]_i$ after the glucose concentration was elevated to 8·3 mM, a concentration at which insulin release is stimulated. A further rise in ACh to 10^{-5} M produced an additional increase in $[Ca^{2+}]_i$. *C*, ACh at 10^{-5} M evoked a rather transient increase in $[Ca^{2+}]_i$ in the presence of 2·8 mM glucose, whereas it produced an increase in $[Ca^{2+}]_i$ of larger amplitude and longer duration in 8·3 mM glucose. Glucose concentrations are indicated above the traces and the bars specify the periods of exposure to ACh (open and filled bars) and TB (hatched bars). The results shown are representative of 4 similar experiments in *A*, 20 in *B*, and 7 in *C*.

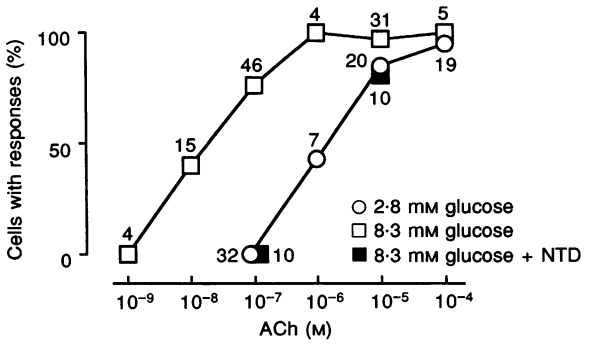


Figure 3. Concentration–response relationship for ACh-induced increase in $[Ca^{2+}]_i$ in single β-cells

Percentage of cells responding to ACh in the presence of 2·8 (○) and 8·3 mM glucose without (□) and with 1 μM nitrendipine (NTD) (■), plotted against ACh concentration. The number near each point indicates the number of cells examined.

Concentration dependence of ACh-induced increase in $[Ca^{2+}]_i$ in basal and elevated glucose

In the presence of 2·8 mM glucose, ACh $\geqslant 10^{-6}$ M evoked an increase in $[Ca^{2+}]_i$ in the cells in which high glucose (8·3 or 16·7 mM) and tolbutamide (TB, 300 μM) induced increases in $[Ca^{2+}]_i$ (Fig. 2). ACh $\leqslant 10^{-7}$ M was without effect. ACh at 10^{-6} M evoked a $[Ca^{2+}]_i$ response in about half of the cells, and at 10^{-5} and 10^{-4} M in 85% or more of the cells examined, showing a concentration-dependent effect of ACh (Figs 2*A* and 3). Upon stimulation with ACh at 10^{-5} M, a maximal concentration, $[Ca^{2+}]_i$ rose rapidly without an appreciable lag before onset, peaked at around 0·5–1 min, and declined toward the pre-stimulatory level (Fig. 2*A*). The rather transient characteristic of the $[Ca^{2+}]_i$ response was observed also in the continuous presence of ACh (Fig. 2*C*).

In the presence of 8·3 mM glucose, ACh at 10^{-7} M, a concentration which was ineffective at 2·8 mM glucose, evoked a rapid and large increase in $[Ca^{2+}]_i$ (Fig. 2*B*). This demonstrates that the effect of 10^{-7} M ACh is glucose dependent. ACh at 10^{-5} M produced an increase in $[Ca^{2+}]_i$ with a greater amplitude in a longer-lasting manner than that elicited by 10^{-5} M ACh at 2·8 mM glucose (Fig. 2*C*). When the ACh concentration was elevated further from 10^{-7} to 10^{-5} M, an additional transient increase in $[Ca^{2+}]_i$ was induced (Fig. 2*B*). ACh at 10^{-8} M evoked an increase in $[Ca^{2+}]_i$ in six out of fifteen cells, at 10^{-7} M in thirty-five out of forty-six cells, and at 10^{-6} to 10^{-4} M in almost all β-cells examined (Fig. 3).

Glucose dependence of ACh-induced increase in $[Ca^{2+}]_i$

It appeared that the effect of ACh at low concentrations (10^{-8} to 10^{-7} M) required an elevated glucose concentration, whereas that at higher concentrations (10^{-5} to 10^{-4} M) was independent of glucose (Fig. 3). We then carefully examined the glucose dependence of the effect of low (10^{-7} M) and high (10^{-5} M) ACh concentrations on $[Ca^{2+}]_i$.

At 10^{-5} M, ACh evoked a $[Ca^{2+}]_i$ response in 85–100% of individual β-cells, regardless of the concentration of glucose (1, 2·8, 5 and 8·3 mM; Fig. 4). On the other hand, 10^{-7} M ACh had no effect in the presence of 2·8 mM glucose, a concentration at which insulin release is not stimulated, whereas it elicited a close to maximal $[Ca^{2+}]_i$ response in the presence of 8·3 and 16·7 mM glucose (Figs 3 and 4), concentrations at which insulin release is stimulated and $[Ca^{2+}]_i$ increased (Hellman *et al.* 1992; Yada *et al.* 1992), in about 70% of individual β-cells. In the presence of 5 mM glucose, a concentration near the threshold for insulin release (Hellman *et al.* 1992), an intermediate fraction of β-cells (6 out of 18 cells, 33%) responded to 10^{-7} M ACh.

The question next arises as to the mechanism underlying the glucose dependence of the effect of low ACh concentration. One possibility is that glucose-induced depolarization and Ca^{2+} influx are required. We therefore investigated whether there is a correlation between the occurrence of a $[Ca^{2+}]_i$ rise evoked by 5 mM glucose and the subsequent effect of ACh on $[Ca^{2+}]_i$ in individual β-cells. No effect of 10^{-7} M ACh on $[Ca^{2+}]_i$ was observed in nine cells

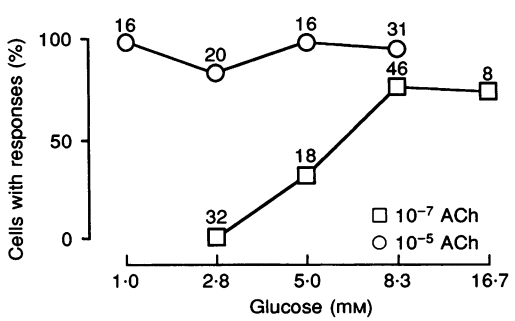


Figure 4. Glucose dependence of ACh-induced increase in $[Ca^{2+}]_i$ in single β-cells

Percentage of cells responding to 10^{-7} M (□) and 10^{-5} M ACh (○). plotted against glucose concentration. The number near each point indicates the number of cells examined.

which had failed to respond to 5 mM glucose but subsequently responded to 16·7 mM glucose (Fig. 5*A* and *C*). In contrast, 10^{-7} M ACh evoked an increase in $[Ca^{2+}]_i$ in six out of nine cells which had already responded to 5 mM glucose (Fig. 5*B* and *C*), and this frequency (67%) of response was close to that observed in 8·3 or 16·7 mM glucose (Fig. 4). Thus, low ACh (10^{-7} M) was ineffective in all β-cells in which 5 mM glucose did not raise $[Ca^{2+}]_i$, whereas it evoked $[Ca^{2+}]_i$ responses in cells in which glucose had initiated an increase in $[Ca^{2+}]_i$. Once the cells exhibited $[Ca^{2+}]_i$ increases in response to glucose, the frequency of the subsequent ACh responses was maximal (around 70%) irrespective of the glucose concentrations used (5, 8·3 and 16·7 mM). In comparison, high ACh (10^{-5} M) evoked $[Ca^{2+}]_i$ increases in all cells irrespective of whether a $[Ca^{2+}]_i$ response to 5 mM glucose was observed (Fig. 5*C*).

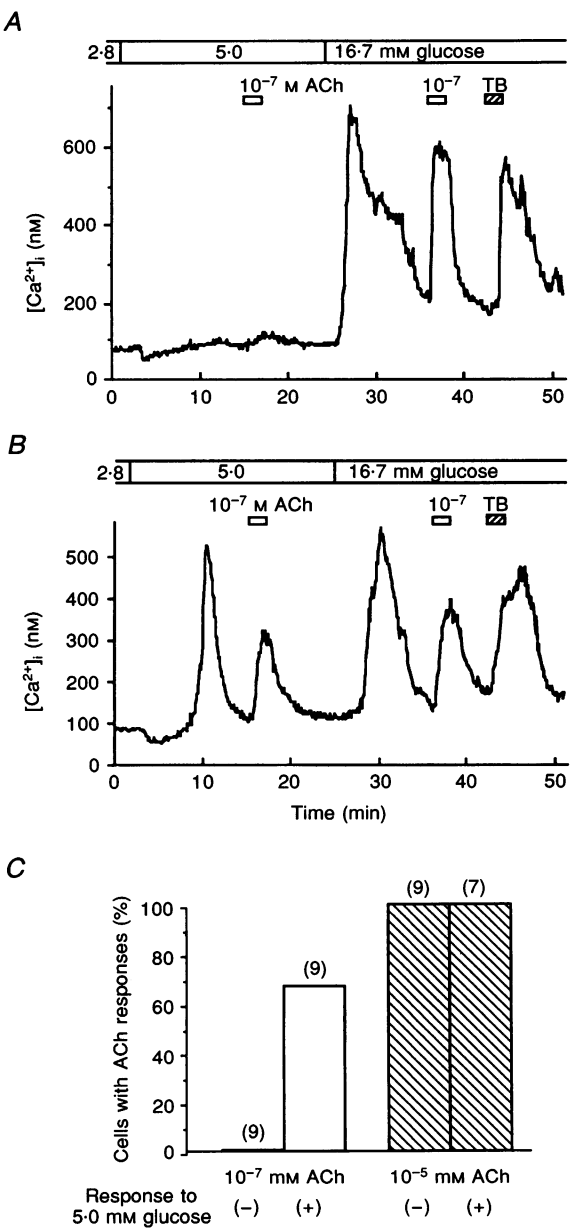
Figure 5. Correlation between 5 mM glucose-induced $[Ca^{2+}]_i$ activity and effectiveness of 10^{-7} M ACh in increasing $[Ca^{2+}]_i$ in single β-cells

A, 10^{-7} M ACh failed to evoke an increase in $[Ca^{2+}]_i$ in a cell in which 5 mM glucose did not raise $[Ca^{2+}]_i$. Subsequently, a $[Ca^{2+}]_i$ response to 10^{-7} M ACh was produced after a rise in $[Ca^{2+}]_i$ induced by 16·7 mM glucose. *B*, 10^{-7} M ACh evoked an increase in $[Ca^{2+}]_i$ in a cell in which 5 mM glucose raised $[Ca^{2+}]_i$. The traces in *A* and *B* were obtained from the same recording. *C*, left-hand columns show the relationship between the $[Ca^{2+}]_i$ response to 5 mM glucose and that to 10^{-7} M ACh administered subsequently in the presence of 5 mM glucose. Right-hand columns show the relationship between the $[Ca^{2+}]_i$ response to 5 mM glucose and that to 10^{-5} M ACh in the presence of 5 mM glucose. The number above each column indicates the number of cells examined.

Effects of Ca^{2+}-free conditions, a Ca^{2+} channel blocker, and an ATP-sensitive K^+ channel opener on the ACh-induced increase in $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ response to 10^{-5} M ACh observed in the presence of 2·8 mM glucose was not affected under Ca^{2+}-free conditions, which were obtained by substituting 0·1 mM EGTA for Ca^{2+} in the KRB solution (Fig. 6*A*). In contrast, the $[Ca^{2+}]_i$ response to 10^{-7} M ACh, observed only in the presence of elevated glucose, was completely inhibited under Ca^{2+}-free conditions in a reversible manner (Fig. 6*B*). The $[Ca^{2+}]_i$ response to 10^{-5} M ACh at 8·3 mM glucose was reduced under Ca^{2+}-free conditions (Fig. 6*C*).

In the presence of 8·3 mM glucose, the $[Ca^{2+}]_i$ response to 10^{-7} M ACh was also completely inhibited by 1 μM nitrendipine, a specific blocker of the L-type Ca^{2+} channel,



or by 100 μM diazoxide, an agent which opens the ATP-sensitive K^+ channel and consequently hyperpolarizes the plasma membrane (Fig. 7). Upon washing out nitrendipine or diazoxide, and after a rebound of $[Ca^{2+}]_i$ which occasionally took place, the $[Ca^{2+}]_i$ response to 10^{-7} M ACh was restored (Fig. 7). The $[Ca^{2+}]_i$ response to 10^{-5} M ACh, in contrast, was only partly reduced by nitrendipine and diazoxide (Fig. 7). These results suggest that in the presence of elevated glucose the $[Ca^{2+}]_i$ response to 10^{-7} M ACh is triggered by stimulation of Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels, whereas that to 10^{-5} M ACh involves a mechanism independent of extracellular Ca^{2+}.

Effect of an inhibitor of the endoplasmic reticulum Ca^{2+} pump on the ACh-induced increase in $[Ca^{2+}]_i$

In the presence of 2·8 mM glucose, when 10^{-5} M ACh was administered by successive brief pulses of 1–2 min with intervals of 10 min or longer, the cells responded with a train of $[Ca^{2+}]_i$ responses and the amplitude was unaltered at least for the first three responses (Fig. 8*A*). Upon administration of 0·2–0·5 μM thapsigargin, an inhibitor of the ER Ca^{2+} pump (Thastrup, Cullen, Drobak, Hanley & Dawson, 1990; Lytton, Westlin & Hanley, 1991), $[Ca^{2+}]_i$ gradually rose, peaked at around 2 min, and then slowly declined towards a level somewhat elevated above basal levels (Fig. 8*B*). In the presence of thapsigargin, $[Ca^{2+}]_i$ responses to ACh pulses were initially reduced and then inhibited (Fig. 8*B*). Thapsigargin at concentrations of 0·2–1 μM affected neither the increase in $[Ca^{2+}]_i$ induced by tolbutamide nor that induced by a high glucose concentration. The elevation of the basal $[Ca^{2+}]_i$ level confirms that thapsigargin blocks ER Ca^{2+} uptake in the resting state. The inhibition of the successive $[Ca^{2+}]_i$ responses to ACh pulses suggests that the ER Ca^{2+} uptake after ACh responses is also inhibited by thapsigargin,

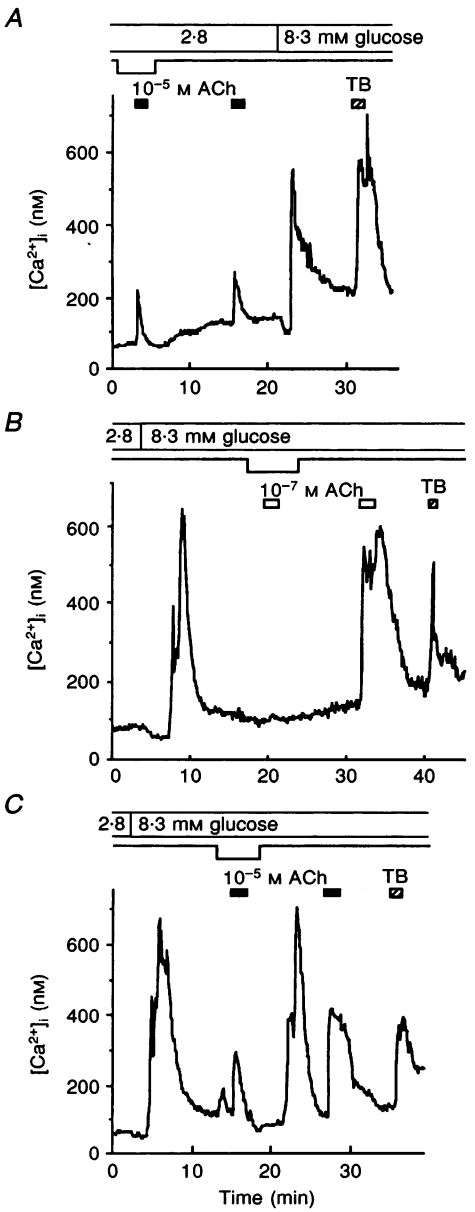


Figure 6. Effects of Ca^{2+}-free conditions on $[Ca^{2+}]_i$ responses to 10^{-7} M and 10^{-5} M ACh in basal and elevated glucose in single β-cells

A, in the presence of 2·8 mM glucose, 10^{-5} M ACh evoked an almost identical increase in $[Ca^{2+}]_i$ in the absence and presence of extracellular Ca^{2+}. *B*, in the presence of 8·3 mM glucose, the 10^{-7} M ACh-evoked increase in $[Ca^{2+}]_i$ was abolished in the absence of extracellular Ca^{2+} in a reversible manner. *C*, in the presence of 8·3 mM glucose, the 10^{-5} M ACh-evoked increase in $[Ca^{2+}]_i$ was reduced in the absence of extracellular Ca^{2+}. Ca^{2+}-free conditions were achieved by substituting 0·1 mM EGTA for Ca^{2+} in the KRB solution. The results shown are representative of 18 similar experiments in *A*, 12 in *B*, and 13 in *C*.

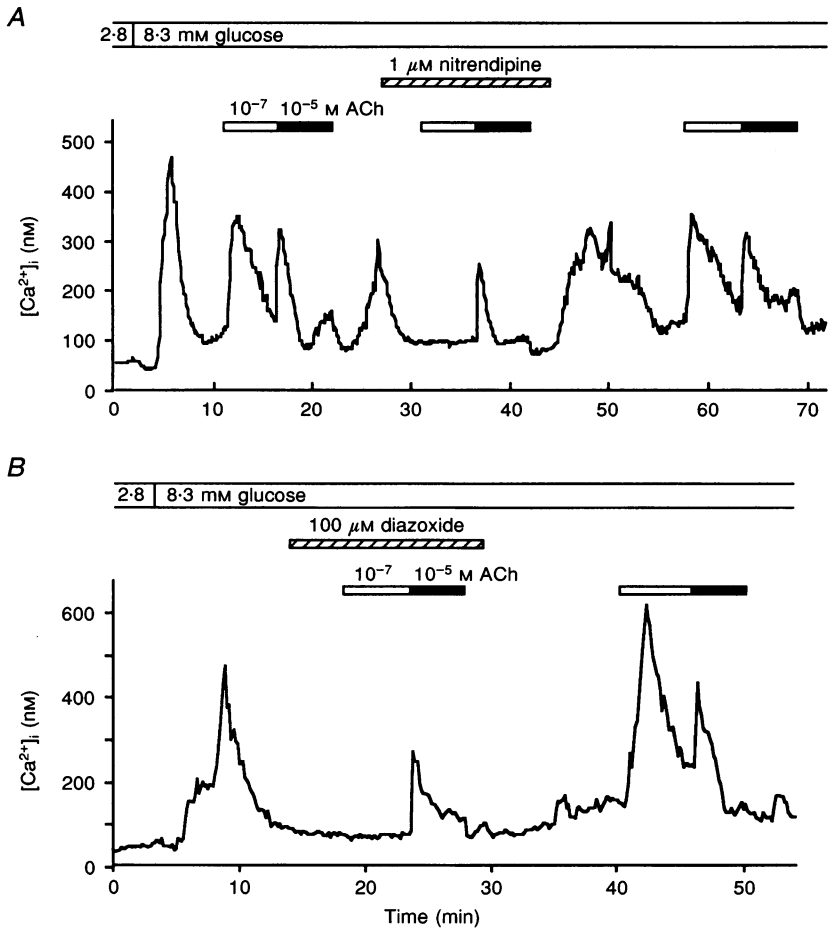


Figure 7. Inhibition of 10^{-7} M ACh-induced increase in $[Ca^{2+}]_i$ at elevated glucose by a Ca^{2+} channel blocker and an ATP-sensitive K^+ channel opener in single β-cells

In the presence of 1 μM nitrendipine (*A*) and 100 μM diazoxide (*B*), the $[Ca^{2+}]_i$ response to 10^{-7} M ACh in the presence of 8·3 mM glucose was completely and reversibly inhibited, whereas the $[Ca^{2+}]_i$ response to 10^{-5} M ACh was only slightly attenuated. The results shown are representative of 7 similar experiments in *A* and 6 in *B*. An additional oscillatory increase in $[Ca^{2+}]_i$ occasionally observed during and after exposure to ACh (*A*) was also inhibited by nitrendipine.

Table 1. Concentration, glucose and Ca^{2+} dependencies and pharmacological properties of $[Ca^{2+}]_i$ responses to ACh in rat pancreatic β-cells

Glucose	ACh (M)	$[Ca^{2+}]_i$ response				Mechanism
		Control	EGTA	Nitrendipine	Thapsigargin	
Basal	10^{-8} to 10^{-7}	—				
	10^{-6} to 10^{-4}	++	++		—	ER
Stimulatory	10^{-8} to 10^{-7}	++		—	++*	LCC
	10^{-6} to 10^{-4}	++	+	+		LCC,ER

$[Ca^{2+}]_i$ responses were classified into three types: ++, intact response; +, suppressed response; —, no or inhibited response. ER, Ca^{2+} release from endoplasmic reticulum. LCC, Ca^{2+} influx through L-type Ca^{2+} channels. * Data were obtained after washing out thapsigargin when the effect of 10^{-5} M ACh at basal glucose was completely inhibited.

thereby reducing the amount of Ca^{2+} stored in ER. In many cases, the $[Ca^{2+}]_i$ response to 10^{-5} M ACh was still inhibited for a long period after washing out the drug, during which low ACh (10^{-7} M) in the presence of 8·3 mM glucose evoked a $[Ca^{2+}]_i$ response similar to that observed in control conditions (Fig. 8*B*). The results suggest that ER Ca^{2+} release plays a key role in the action of a high level of ACh (10^{-5} M) in increasing $[Ca^{2+}]_i$, but not in the action of a low level of ACh (10^{-7} M) in the presence of elevated glucose. The long-lasting inhibitory effect of thapsigargin observed in our study is in agreement with the report that the interaction between thapsigargin and the ER and sarcoplasmic reticulum Ca^{2+}-ATPase is irreversible (Lytton *et al.* 1991).

In the presence of elevated glucose, the effect of 10^{-5} M ACh was examined in the continuous presence of nitrendipine which eliminates the component of the Ca^{2+} influx that passes through L-type channels. In the presence of nitrendipine, repeated brief pulses of 10^{-5} M ACh produced a train of $[Ca^{2+}]_i$ responses, and the amplitude of the response was only slightly attenuated by time or by repetitive stimulation (Fig. 9*A*). In the presence of 0·5 μM thapsigargin, $[Ca^{2+}]_i$ responses to ACh pulses were initially reduced and then inhibited (Fig. 9*B*). Thapsigargin by itself induced a rise in $[Ca^{2+}]_i$, which was smaller at its peak but longer lasting than that evoked by 10^{-5} M ACh. Thus, in the presence of elevated glucose and nitrendipine, the effect of 10^{-5} M ACh on $[Ca^{2+}]_i$ and its inhibition by thapsigargin were similar to those observed at basal glucose levels.

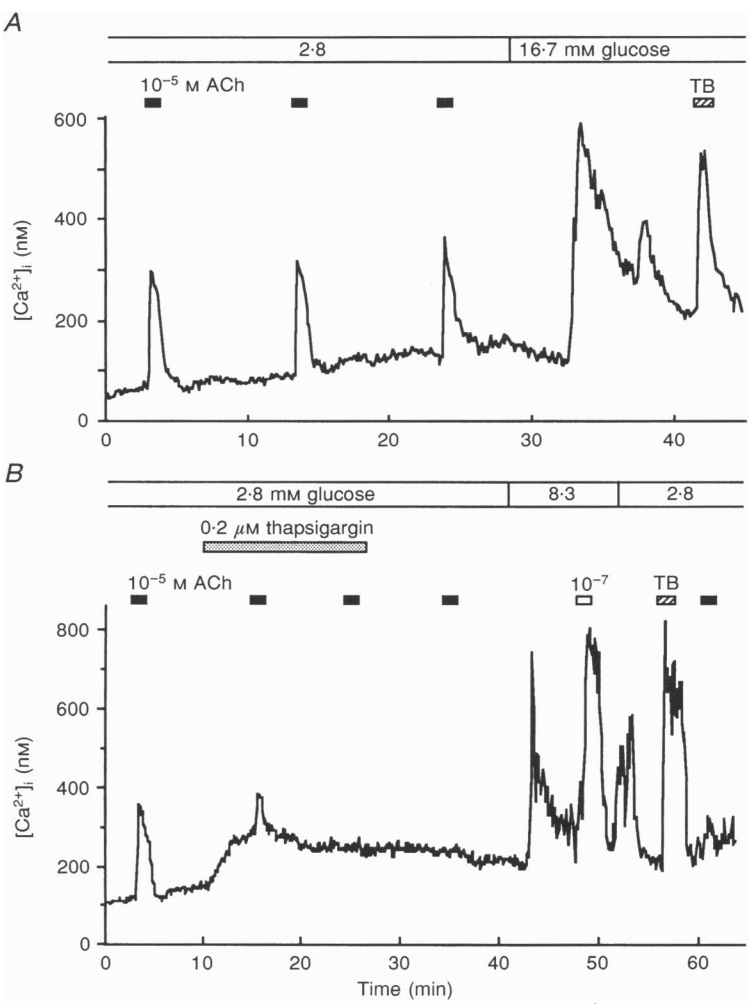


Figure 8. Effects of an ER Ca^{2+} pump blocker on successive $[Ca^{2+}]_i$ responses to repetitive administration of 10^{-5} M ACh at basal glucose in single β-cells

A, in the presence of 2·8 mM glucose, repeated ACh administration in brief pulses produced successive $[Ca^{2+}]_i$ increases the amplitude of which was not significantly altered for the first three responses. *B*, thapsigargin (0·2 μM) increased basal $[Ca^{2+}]_i$ and progressively inhibited the $[Ca^{2+}]_i$ responses to repeated pulses of 10^{-5} M ACh. Note also that at around 30 min after washing out thapsigargin, the response to 10^{-5} M ACh was still inhibited, but a $[Ca^{2+}]_i$ response to 10^{-7} M ACh in the presence of 8·3 mM glucose was obtained. The results shown are representative of 15 similar experiments in *A* and 12 in *B*.

DISCUSSION

In the presence of glucose concentrations which did not stimulate insulin release (1 and 2·8 mM), relatively high concentrations of ACh ($\geqslant 10^{-6}$ M) evoked a rather transient increase in $[Ca^{2+}]_i$ both in the presence and absence of extracellular Ca^{2+}, indicating that ACh releases Ca^{2+} from intracellular stores (Table 1). This is in accord with previous reports that muscarinic agonists induce Ca^{2+} release from internal stores, transient increases in $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ efflux in the absence of extracellular Ca^{2+} in rat (Yada & Sorimachi, 1989; Theler *et al.* 1992) and mouse β-cells (Nilsson *et al.* 1987; Gylfe, 1991), mouse islets (Hellman & Gylfe, 1986; Gao *et al.* 1994), and RINm5F cells (Wollheim & Biden, 1986). Repeated pulses of 10^{-5} M ACh evoked successive $[Ca^{2+}]_i$ responses, which were progressively inhibited in the presence of thapsigargin. Thapsigargin by itself moderately elevated $[Ca^{2+}]_i$, as has previously been reported in rat β-cells (Herchuelz & Lebrun, 1994). The inhibition by thapsigargin was specific to the ACh-evoked release of Ca^{2+}, because the $[Ca^{2+}]_i$ increase in response to glucose was not affected by the drug (Hamakawa & Yada, 1995). It has been shown that thapsigargin specifically

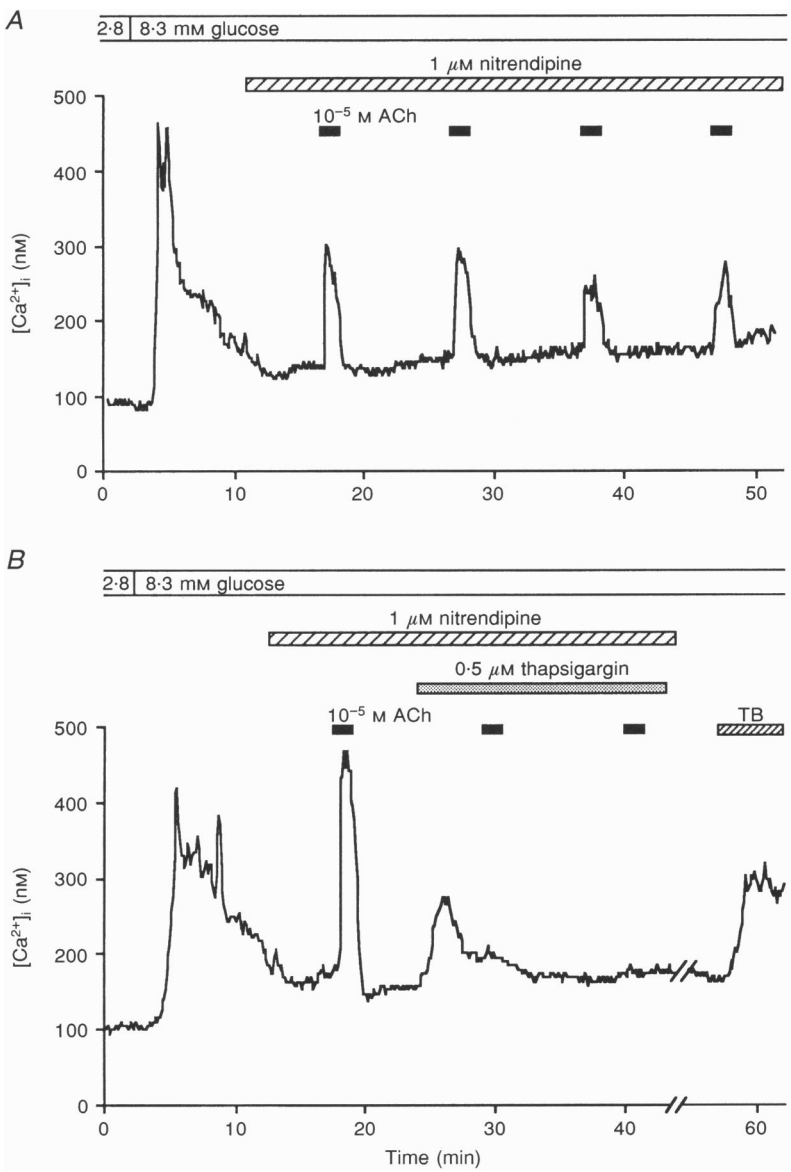


Figure 9. Effects of a Ca^{2+} channel blocker plus an ER Ca^{2+} pump blocker on successive $[Ca^{2+}]_i$ responses to repetitive administration of 10^{-5} M ACh in elevated glucose in single β-cells

A, in the presence of 8·3 mM glucose and 1 μM nitrendipine, repetitive 10^{-5}M ACh administration in brief pulses produced successive $[Ca^{2+}]_i$ increases. *B*, thapsigargin (0·5 μM) increased $[Ca^{2+}]_i$ and inhibited subsequent $[Ca^{2+}]_i$ responses to repeated pulses of 10^{-5} M ACh. The results shown are representative of 7 similar experiments in both *A* and *B*.

inhibits the ER and sarcoplasmic reticulum Ca^{2+}-ATPase (Ca^{2+} pump) without influencing the plasma membrane Ca^{2+}-ATPase and Na^+–K^+-ATPase in a variety of preparations including hepatocytes, cardiac and fast twitch muscles (Thastrup *et al.* 1990; Lytton *et al.* 1991). Thapsigargin-induced depletion of the ACh-sensitive intracellular Ca^{2+} pool in exocrine pancreatic AR4-2J cells has also been reported (Bird, Takemura, Thastrup, Putney & Menniti, 1992). The present results indicate, therefore, that ACh releases the ER Ca^{2+} that is sequestered by the thapsigargin-sensitive Ca^{2+} pump. This action of ACh in β-cells may be mediated by IP_3 in view of the following reports: (1) muscarinic activation stimulates formation of IP_3 in mouse islets (Nilsson *et al.* 1987; Gao *et al.* 1994) and RINm5F cells (Wollheim & Biden, 1986), (2) IP_3 mobilizes Ca^{2+} in permeabilized mouse islet cells (Nilsson *et al.* 1987; Hellman *et al.* 1992), RINm5F cells (Prentki & Matschinsky, 1987) and mouse islet cells in which caged IP_3 is injected and liberated (Ämmälä *et al.* 1993), and (3) an IP_3 receptor subtype is expressed in rat islets and RINm5F cells (Blondel, Takeda, Janssen, Seino & Bell, 1993).

The present results demonstrate that in the presence of glucose concentrations which stimulate insulin release (8·3 and 16·7 mM), ACh at low concentrations (10^{-8} to 10^{-7} M) produced a rise in $[Ca^{2+}]_i$ which was longer lasting than that evoked by high ACh (10^{-5} M) in the presence of basal glucose levels. In the presence of 5 mM glucose, a concentration near the threshold for insulin release, 10^{-7} M ACh evoked $[Ca^{2+}]_i$ responses in about 70% of the cells in which 5 mM glucose had previously increased $[Ca^{2+}]_i$, and this frequency was similar to that of the responses observed in the presence of 8·3 and 16·7 mM glucose. In contrast, 10^{-7} M ACh never evoked a $[Ca^{2+}]_i$ response in the cells in which 5 mM glucose had not been adequate to raise $[Ca^{2+}]_i$. Thus, it appears that the effect of low ACh in increasing $[Ca^{2+}]_i$ is dependent upon the glucose-induced rise in $[Ca^{2+}]_i$, irrespective of the concentration of glucose used (5·0, 8·3 or 16·7 mM). The glucose-induced rise in $[Ca^{2+}]_i$ has been shown to result from membrane depolarization and consequent activation of voltage-dependent Ca^{2+} channels (Valdeolmillos *et al.* 1992). The $[Ca^{2+}]_i$ response to 10^{-7} M ACh was completely inhibited by nitrendipine. Therefore it is likely that low ACh acts on the β-cells in which glucose previously induced sufficient depolarization and subsequent activation of L-type Ca^{2+} channels to potentiate Ca^{2+} channel activity either directly or via further depolarization of the membrane, leading to the increase in $[Ca^{2+}]_i$ (Table 1). This is in accord with the observation that ACh stimulation of insulin release requires that the β-cell membrane be sufficiently depolarized to reach the threshold potential at which Ca^{2+} channels are activated (Hermans *et al.* 1987). Alternatively, the increase in $[Ca^{2+}]_i$ caused by glucose may condition the cells to allow subsequent responses to low ACh.

In the presence of elevated glucose concentrations, a rise in ACh from 10^{-7} to 10^{-5} M provoked an additional increase in $[Ca^{2+}]_i$, which was only slightly reduced by nitrendipine, indicating that only a minor portion of the additional $[Ca^{2+}]_i$ response was due to a further stimulation of Ca^{2+} influx (Fig. 7). The $[Ca^{2+}]_i$ increase in response to a fresh administration of 10^{-5} M ACh was partly reduced under Ca^{2+}-free conditions and by nitrendipine, leaving a substantial, transient component. The component that remained in the presence of nitrendipine was completely inhibited by thapsigargin. Thus, the $[Ca^{2+}]_i$ response to high ACh (10^{-5} M) in the presence of elevated glucose appeared to comprise two modes: a nitrendipine-sensitive influx of extracellular Ca^{2+} and a Ca^{2+} release from thapsigargin-sensitive ER (Table 1). It is likely that ACh, when raised to 10^{-6} to 10^{-4} M, becomes capable of mobilizing ER Ca^{2+}, in addition to its role in augmenting Ca^{2+} influx at lower concentrations (10^{-8} to 10^{-7} M). In support of this idea, it has previously been reported that 10^{-6} to 10^{-4} M ACh, in the presence of glucose concentrations which stimulate insulin release, induced a sustained increase in $^{45}Ca^{2+}$ efflux from mouse islets, the pattern of which was transformed to a transient response by blockers of voltage-dependent Ca^{2+} channels (Hermans & Henquin, 1989), and that only a transient increase in $[Ca^{2+}]_i$ was evoked by high ACh (10^{-4} M) in the presence of elevated glucose under Ca^{2+}-free conditions (Gao *et al.* 1994) or in the presence of diazoxide which hyperpolarizes the β-cell membrane (Gilon, Shepherd & Henquin, 1993).

The present study revealed two distinct modes of $[Ca^{2+}]_i$ signalling by ACh in pancreatic β-cells (Table 1). The first is a transient increase in $[Ca^{2+}]_i$ due to the Ca^{2+} release from ER. This mechanism requires high concentrations of ACh (10^{-6} to 10^{-4} M) but can operate at basal glucose concentrations and without extracellular Ca^{2+}. The second is a longer-lasting increase in $[Ca^{2+}]_i$ due to an augmented Ca^{2+} influx through the voltage-dependent L-type Ca^{2+} channels of the β-cell plasma membrane. This mechanism already operates at low concentrations of ACh (10^{-8} to 10^{-7} M) but only when glucose is raised to concentrations which stimulate insulin release (5 mM or higher). High concentrations of ACh in the presence of stimulatory glucose levels can evoke the two mechanisms simultaneously. It has been demonstrated that insulin release from mouse islets is stimulated by ACh at concentrations as low as 10^{-7} M in the presence of 11 mM glucose whereas high concentrations of ACh (10^{-5} to 10^{-4} M) are required in the presence of 3 mM glucose (Garcia *et al.* 1988). Furthermore, ACh at high concentrations in the presence of elevated glucose stimulates insulin release via two mechanisms, a sustained mechanism that is inhibited under Ca^{2+}-free conditions and by Ca^{2+} channel blockers (Hermans & Henquin, 1989) and a transient mechanism that occurs under Ca^{2+}-free conditions

(Garcia *et al.* 1988). The two modes of the ACh action on $[Ca^{2+}]_i$ in β-cells demonstrated in the present study appear to fit well with these two components of ACh-stimulated insulin release. It is therefore suggested that Ca^{2+} release from ER relates to the initiation of insulin release by high ACh, while augmented Ca^{2+} influx through L-type Ca^{2+} channels relates to the potentiation of glucose-induced insulin release by low and high ACh. It should be kept in mind, however, that the insulinotropic action of ACh could also involve β-cell activities other than Ca^{2+}, such as an activation of protein kinase C, which is also likely to contribute to the potentiating effect (Prentki & Matschinsky, 1987; Peter-Reisch *et al.* 1988; Persaud *et al.* 1989) and increases in the plasma membrane Na^+ permeability (Henquin *et al.* 1988) and the cytosolic Na^+ concentration (Gilon & Henquin, 1993).

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