The role of cytosolic free Ca^{2+} and protein kinase C in acetylcholine-induced insulin release in the clonal β -cell line, HIT-T15

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We examined the contribution of signal-transduction pathways to acetylcholine-induced insulin release in the clonal β -cell line HIT-T15. To assess the importance of changes in cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$), we studied time courses of the effects of glucose and acetylcholine on $[\text{Ca}^{2+}]_i$ and insulin release in quin 2-loaded HIT cells. Incubation in the presence of glucose (2 mM) resulted in a sustained increase in $[\text{Ca}^{2+}]_i$ in HIT cells from 98 ± 7 nM to 195 ± 12 nM measured after 9 min, whereas subsequent addition of acetylcholine (50 μM) produced a transient increase in $[\text{Ca}^{2+}]_i$ which reached a peak after 30 s (at 274 ± 10 nM), returning to pre-stimulus levels after 3 min. In contrast, incubation of HIT cells with acetylcholine in the presence of glucose produced a sustained increase in insulin release over and above that stimulated by glucose alone; after 10 min acetylcholine had potentiated glucose-stimulated insulin release by an additional increment of 135 %. The transient increase in $[\text{Ca}^{2+}]_i$ induced by acetylcholine was dose-dependent, and was prevented by omission of glucose or extracellular Ca^{2+} from the incubation medium. It was also inhibited by inclusion of 50 μM -verapamil in the incubation medium (by 87 ± 3 %) or by decreasing the Na^+ concentration in the medium (by 73 ± 6 %). To evaluate the role of the protein kinase C pathway, we have pretreated HIT cells with the phorbol ester 12-*O*-tetradecanoylphorbol acetate (TPA), to deplete the protein kinase C activity, and have compared their secretory activity with that of control cells. Protein kinase C activity was decreased by 73 % in HIT cells cultured in the presence of 200 nM-TPA for 22–24 h. TPA pre-treatment also significantly decreased the insulin content of HIT cells, but had no effect on cell number or the increases in $[\text{Ca}^{2+}]_i$ induced by glucose or acetylcholine. TPA-pre-treated cells responded comparatively less well to secretagogues than did control cells: glucose-stimulated insulin release was decreased by 40 %, whereas potentiation by TPA was significantly decreased by 50 % in comparison with control cells ($P < 0.05$, $n = 24$). Acetylcholine (50 μM) potentiated glucose-stimulated insulin release by 61 % in control cells. This effect was abolished in HIT cells pre-treated with TPA, whereas these cells still retained their normal secretory response to stimulation by forskolin. These data suggest that an early increase in $[\text{Ca}^{2+}]_i$ may be important for the initial increase in insulin release induced by acetylcholine in HIT cells. However, protein kinase C activation is necessary for sustained cholinergic-induced insulin release. This may involve sensitization of the secretory apparatus to intracellular Ca^{2+} , since insulin release takes place in the absence of any additional increase in $[\text{Ca}^{2+}]_i$.

INTRODUCTION

Several signal-transduction systems may be involved in the cholinergic stimulation of insulin release. It has been reported widely that insulin release induced by the neurotransmitter acetylcholine is dependent on the presence of extracellular Ca^{2+} (Griffey *et al.*, 1974; Burr *et al.*, 1976; Gagerman *et al.*, 1980; Wollheim *et al.*, 1980; Nenquin *et al.*, 1984; Mathias *et al.*, 1985; Hermans *et al.*, 1987; Hughes *et al.*, 1987; Garcia *et al.*, 1988) and that cholinergic agents stimulate $^{45}\text{Ca}^{2+}$ influx into the β -cell (Wollheim *et al.*, 1980; Hermans *et al.*, 1987; Garcia *et al.*, 1988). In addition, cholinergic agents have been shown to promote a rapid turnover of membrane inositol phospholipids (Best & Malaisse, 1984; Wollheim & Biden, 1986), releasing two potential intracellular mediators, inositol trisphosphate and diacylglycerol. The former regulates Ca^{2+} mobilization from the endoplasmic reticulum, which may augment the increasing intracellular Ca^{2+} resulting from Ca^{2+} influx, whereas the latter activates protein kinase C. Activation of protein kinase C may stimulate insulin release from the β -cell by sensitizing the secretory apparatus to Ca^{2+} (Tamagawa *et al.*, 1985; Jones *et al.*, 1985; Hughes *et al.*, 1987; Henquin *et al.*, 1987).

In the present work we have investigated cholinergic stimulation of insulin release in the clonal β -cell line HIT-T15 (Santerre *et al.*, 1981). This cell line essentially retains the secretory properties of normal islets, being responsive to nutrient secretagogues, including glucose, activators of cyclic-AMP-dep-

endent protein kinases or protein kinase C, and sulphonylureas (Hill & Boyd, 1985; Ashcroft *et al.*, 1986; Nelson *et al.*, 1987). In addition, both the ATP-sensitive K^+ channel and the voltage-sensitive Ca^{2+} channel have been characterized in HIT cells (Niki *et al.*, 1989; Keahey *et al.*, 1989), which suggests that the initial ionic events underlying the glucose-stimulated increase in $[\text{Ca}^{2+}]_i$ in this cell line (Hughes & Ashcroft, 1988a; Hughes *et al.*, 1989) are comparable with those in pancreatic β -cells.

We have attempted in the present study to determine the contributions of the signal-transduction systems to acetylcholine-induced insulin release in HIT cells. To do this, we have studied the effects of glucose and acetylcholine on changes in $[\text{Ca}^{2+}]_i$ and insulin release. To evaluate the importance of protein kinase C activation, we have examined the effects of acetylcholine and secretagogues on insulin release in HIT cells depleted of protein kinase C activity by prolonged incubation with the phorbol ester 12-*O*-tetradecanoylphorbol acetate (TPA).

MATERIALS AND METHODS

Materials

Tissue-culture materials were from Gibco Europe, Paisley, Strathclyde, U.K. TPA and 4 α -phorbol 12,13-didecanoate were from Pharmacia, Milton Keynes, Bucks., U.K. Guinea-pig anti-insulin serum was a gift from Dr. R. Turner, Diabetes Research Laboratory, Radcliffe Infirmary, Oxford, U.K. Rat insulin

standard was a gift from Dr. A. J. Moody, Novo Research Laboratories, Copenhagen, Denmark. [32 P]ATP and 125 I-labelled insulin were from Amersham International, Amersham, Bucks., U.K. Quin 2 and quin 2/AM were from Aldrich Chemical Co., Gillingham, Dorset, U.K. Forskolin was from Calbiochem, CP Laboratories, Bishops Cleeve, Herts., U.K. Triton X-100 was from BDH, Atherstone, Warwicks., U.K., and BSA was from BCL, Lewes, East Sussex, U.K. Acetylcholine (in 10 mg vials), verapamil, *N*-methyl-D-glucamine, phosphatidylserine, phenylmethanesulphonyl fluoride and benzamidine were all supplied by Sigma Chemical Co., Poole, Dorset, U.K.

Cell culture

HIT cells were cultured at 37 °C in RPMI 1640 supplemented with glucose (11 mM), antibiotics and foetal-calf serum (10%, v/v) as described previously (Ashcroft *et al.*, 1986). HIT cells were depleted of protein kinase C by culture for 22–24 h in medium containing 200 nM-TPA. Control cells were cultured in medium containing either carrier (dimethyl sulphoxide) or phorbol didecanoate. The cells used in the present studies were harvested between passages 74 and 83.

Loading with quin 2 and fluorescence measurements

HIT cells were loaded with quin 2 as described previously (Hughes & Ashcroft, 1988a) and were stored on ice at a density of 25×10^6 cells/ml before use. For some experiments, the cells were stored in a medium with a lower Na^+ content, which was prepared by substituting *N*-methyl-D-glucamine for NaCl (Henquin *et al.*, 1988). This buffer was used in subsequent incubations.

Fluorescence measurements were carried out in a Perkin-Elmer LS5 luminescence spectrometer essentially as described previously (Hughes & Ashcroft, 1988a). HIT cells (5×10^6) were preincubated at 37 °C for 5–7 min with continuous stirring in glucose-free Hepes-buffered Krebs medium (Christie & Ashcroft, 1985) before addition of glucose and test agents. Fluorescence measurements were recorded at 30 s or 1 min intervals. At the end of each experiment quin 2 fluorescence was calibrated as described previously (Hughes & Ashcroft, 1988a), and $[\text{Ca}^{2+}]_i$ was calculated as described by Rorsman & Abrahamsson (1985). The mean intracellular quin 2 concentration for HIT-cells suspension used in this study was calculated to be 1.1 ± 0.1 mM (mean $\pm$ S.E.M., $n = 9$).

Subcellular fractionation and protein kinase C assay

HIT cells $[(80-90) \times 10^6$ cells/flask] were detached with trypsin/EDTA, washed in 15 ml of culture medium and then in 20 ml of phosphate-buffered saline (Dulbecco's). The cells were resuspended in 1.0 ml of 5 mM-Tris buffer, pH 8.0, containing 1 mM-phenylmethanesulphonyl fluoride and 5 mM-benzamidine and left on ice for 40 min before homogenization with a hand-held glass homogenizer (20 passes) (Gaines *et al.*, 1988). The homogenate was centrifuged at 900 *g* for 10 min at 4 °C, and the resulting supernatant was centrifuged at 24000 *g* for 20 min at 4 °C, to yield membrane-particulate and supernatant fractions. The protein content of the fractions was measured as described by Bradford (1976), with BSA as standard. These fractions were assayed for protein kinase C activity as described previously (Hughes & Ashcroft, 1988b).

Insulin release

HIT-cell insulin release was measured as described previously (Ashcroft *et al.*, 1986). Multiwells were seeded with 5×10^5 cells and insulin release was measured after 4–5 days as follows. HIT

cells were preincubated for 1 h at 37 °C in glucose-free Hepes-buffered Krebs medium alone before incubation at 37 °C in the absence or presence of glucose and other additions. After 30 min, samples (0.2 ml) of incubation medium were collected and centrifuged at 190 *g* for 5 min at 4 °C to pellet any cells. In addition, insulin release was also measured in quin 2-loaded HIT-cell suspensions. At the times indicated, portions of cell suspension (0.1 ml) were collected and centrifuged as above. Insulin released into the supernatant was measured by radioimmunoassay as described previously (Ashcroft & Crossley, 1974).

Expression of data

Data are presented as means $\pm$ S.E.M. for the numbers of observations indicated. The significance of difference between mean values was assessed by Student's *t* test.

RESULTS

Effect of glucose and acetylcholine on $[\text{Ca}^{2+}]_i$ in HIT cells

Glucose (2 mM) and acetylcholine (50 μM) increased $[\text{Ca}^{2+}]_i$ in HIT cells (Fig. 1), although the characteristics of the increases induced by these two secretagogues were markedly different. As previously documented (Hughes & Ashcroft, 1988a), glucose stimulation resulted in a sustained rise in $[\text{Ca}^{2+}]_i$, which was characterized by a lag period of 1–2 min, followed by a rapid increase reaching a plateau (or rising slowly) 4–5 min after addition of glucose. In contrast, acetylcholine stimulation resulted in a transient increase in $[\text{Ca}^{2+}]_i$. This increase was immediate and had attained its maximal value 30–60 s after addition of acetylcholine. Thereafter, $[\text{Ca}^{2+}]_i$ declined, returning to pre-stimulus levels after a further 2 min (Fig. 1, control data). Despite the differences in $[\text{Ca}^{2+}]_i$ characteristics, the absolute magnitude of the effects of acetylcholine and glucose were similar, increasing $[\text{Ca}^{2+}]_i$ by increments of 94 ± 8 nM and 79 ± 10 nM respectively. The concentration-dependence of the effect of acetylcholine on $[\text{Ca}^{2+}]_i$ in glucose-stimulated HIT cells is shown in Fig. 2(a). At 50 nM, acetylcholine had no effect on $[\text{Ca}^{2+}]_i$ in HIT cells, although a 10-fold increase in the neurotransmitter concentration provoked a small transient increase in $[\text{Ca}^{2+}]_i$. The effect of acetylcholine was effectively maximal at between concentrations of 5 and 50 μM . Fig. 2(b) also shows that, in the absence of glucose, acetylcholine (50 μM) had no effect on $[\text{Ca}^{2+}]_i$ in HIT cells. In the absence of extracellular Ca^{2+} , neither glucose (2 mM) nor subsequent addition of acetylcholine (50 μM) had any significant effect on $[\text{Ca}^{2+}]_i$ in HIT cells.

The effect of verapamil (50 μM) on $[\text{Ca}^{2+}]_i$ in HIT cells stimulated by glucose (2 mM) is shown in Fig. 1(a). Addition of verapamil to the incubation medium resulted in a significant decrease ($P < 0.01$, $n = 3$) in $[\text{Ca}^{2+}]_i$ from 170 ± 4 to 122 ± 4 nM and largely inhibited the transient increase induced by the subsequent addition of acetylcholine. In comparison with cells not exposed to verapamil, the acetylcholine-induced $[\text{Ca}^{2+}]_i$ transient was inhibited by $87 \pm 3\%$ ($P < 0.01$, $n = 3$). The large increase in $[\text{Ca}^{2+}]_i$ induced by 40 mM- K^+ was also blocked by the presence of verapamil. Lowering the Na^+ content of the incubation medium to (approx.) 10 mM also inhibited the acetylcholine-induced $[\text{Ca}^{2+}]_i$ transient by $73 \pm 6\%$ ($P < 0.05$, $n = 4$) (Fig. 1b). However, the characteristics of the glucose-stimulated rise in $[\text{Ca}^{2+}]_i$ were also markedly altered in HIT cells suspended in lower- Na^+ medium; here, the increase in $[\text{Ca}^{2+}]_i$ was more rapid and of greater magnitude. Furthermore, the $[\text{Ca}^{2+}]_i$ increase was not sustained, falling below the level of $[\text{Ca}^{2+}]_i$ of the control cells 7 min after addition of glucose.

Fig. 1. Effect of glucose and acetylcholine on $[Ca^{2+}]_i$ in HIT cells

Quin 2-loaded HIT cells (5×10^6) were preincubated in glucose-free HEPES-buffered Krebs medium (G 0) for 6–7 min at 37 °C with continuous stirring, before addition (at final concentrations) of 2 mM-glucose (G 2 mM) followed by 50 μ M-acetylcholine (ACh) and 40 mM- K^+ (K^+) at the times indicated. In (a), verapamil (final concn. 50 μ M) was added to cells in parallel incubations 3.5 min before addition of acetylcholine (●). In (b), HIT cells were also incubated in medium with a decreased Na^+ concentration (●). In (c), HIT cells were pre-treated with TPA for 22–24 h in culture (●; PKC-depleted). Fluorescence was measured at 30 s intervals, and $[Ca^{2+}]_i$ was calculated as described by Rorsman & Abrahamsson (1985). Data are given as means $\pm$ S.E.M. for 3–4 observations.

Time course of the effect of acetylcholine on insulin release and $[Ca^{2+}]_i$ in glucose-stimulated HIT cells

Fig. 3 shows the effect of acetylcholine (50 μ M) on $[Ca^{2+}]_i$ and insulin release in quin 2-loaded HIT cell suspensions in parallel incubations. Fig. 3(b) shows the characteristic transient increase in $[Ca^{2+}]_i$ induced by acetylcholine followed by the return to pre-stimulus levels occurring over 3 min. In Fig. 3(a) the increase in amount of insulin released into the medium by glucose-stimulated HIT cells is presented. In glucose-stimulated cells, insulin release increased by 100% over a 10 min period. Addition of acetylcholine augmented this insulin release, resulting in a sustained increase in insulin release over and above that

Fig. 2. Effect of acetylcholine on $[Ca^{2+}]_i$ in HIT cells (a) at different concentrations and (b) in the absence of glucose or extracellular Ca^{2+}

In (a), quin 2-loaded HIT cells (5×10^6) were preincubated in HEPES-buffered Krebs medium for 6 min before addition of glucose [final concn. 2 mM (G 2 mM)]. After a further 9 min, acetylcholine (ACh) at concentrations of 50 nM (○), 0.5 μ M (●), 5.0 μ M (□), or 50 μ M (■) was added. Incubations in the absence of acetylcholine are also shown (continuous line). Data are presented as the percentage response relative to the effect of 50 μ M-acetylcholine (means $\pm$ S.E.M. for four observations). In (b), acetylcholine (ACh; final concn. 50 μ M) was added to HIT cells in the absence of glucose (○) or in the presence of 2 mM-glucose (G 2 mM) but in the absence of extracellular Ca^{2+} (●).

stimulated by glucose alone. After 2 min, insulin release was potentiated by an additional increment of 40%, whereas 10 min after addition of acetylcholine insulin release was potentiated by an additional increment of 135%.

Effect of TPA pre-treatment on protein kinase C activity and insulin release in HIT cells

The effect of TPA pre-treatment of HIT-cell protein kinase C activity is shown in Table 1. Under the conditions of preparation and assay, more than 90% of protein kinase C activity was found to reside in the supernatant fraction. This fraction also contained substantial amounts of protein kinase activity (0.44 pmol/5 min per μ g) that was both Ca^{2+} - and phospholipid-independent. Culture of HIT cells in the presence of 200 nM-TPA depleted protein kinase C activity in the supernatant fraction by 73%, but had no effect on Ca^{2+} -phospholipid-insensitive protein kinase activity. The pre-treatment had no significant effect on cell growth, as indicated by cell number [$69.4 (\pm 5.7) \times 10^6$ versus $69.2 (\pm 4) \times 10^6$ cells/flask in control and TPA-pre-treated cells respectively], although insulin content was significantly decreased ($P < 0.01$, $n = 4$, paired t test) in pre-treated cells (0.74 ± 0.17 munit/well) in comparison with control cells (1.07 ± 0.17 munits/well).

Fig. 3. Time course of the effect of acetylcholine on insulin release and [Ca²⁺]_i in glucose-stimulated HIT cells

Quin 2-loaded HIT cells (5×10^6) were preincubated in HEPES-buffered Krebs medium for 6 min at 37 °C with continuous stirring, before addition of glucose (G 2 mM). Fluorescence measurements (at 1 min intervals) commenced after a further 9 min, and 50 μ M-acetylcholine (ACh) was added at the time indicated. In parallel incubations, samples of cell suspension were collected at 2 min intervals, centrifuged, and supernatants were assayed for insulin release by radioimmunoassay. Insulin release is expressed as a percentage of the amount released at the first time point ($t = 14$ min). Data are presented as means $\pm$ S.E.M. of eight observations ([Ca²⁺]_i measurements) or of ten observations (insulin release).

Fig. 4. Time course of the effect of acetylcholine on insulin release and [Ca²⁺]_i in glucose-stimulated HIT cells pre-treated with TPA

Quin 2-loaded HIT cells (5×10^6) which had previously been cultured in the presence of 200 nM-TPA for 22–24 h were preincubated in HEPES-buffered Krebs medium for 6 min at 37 °C with continuous stirring, before addition of glucose (G 2 mM). Fluorescence measurements (at 1 min intervals) commenced after a further 9 min and 50 μ M-acetylcholine (ACh) was added at the time indicated. In parallel incubations, samples of cell suspension were collected at 2 min intervals, centrifuged, and supernatants were assayed for insulin release by radioimmunoassay. Insulin release is expressed as a percentage of the amount released at the first time point ($t = 14$ min). Data are presented as means $\pm$ S.E.M. of five observations ([Ca²⁺]_i measurements) or of six observations (insulin release).

Table 1. Effect of TPA pre-treatment of protein kinase C activity in HIT cells

HIT cells were cultured in the absence or presence of 200 nM-TPA for 22–24 h, detached with trypsin/EDTA, and subcellular fractions were prepared. Protein kinase C activity was assayed as follows. Protein (10–15 μ g) was incubated in the presence of 5 μ M-Ca²⁺, 0.8 μ g of phosphatidylserine and 100 nM-TPA. Data are presented as means $\pm$ S.E.M. for four preparations: ^a $P < 0.05$ compared with protein kinase C activity in the supernatant fraction from control cells (Student's t test).

	Enzyme activity (pmol/5 min per μ g)	
	Control	TPA-pre-treated
Protein kinase C		
Supernatant fraction	0.11 $\pm$ 0.03	0.03 $\pm$ 0.01 ^a
Membrane fraction	0.02 $\pm$ 0.01	0.01 $\pm$ 0.01
Ca ²⁺ -phospholipid-independent		
Supernatant fraction	0.44 $\pm$ 0.13	0.42 $\pm$ 0.07
Membrane fraction	0.15 $\pm$ 0.03	0.14 $\pm$ 0.03

Table 2. Effect of TPA pre-treatment on insulin release in HIT cells

HIT cells in multiwells were cultured in the absence or presence of 200 nM-TPA for 22–24 h. At the end of this period, they were preincubated in HEPES-buffered Krebs medium containing no glucose for 1 h at 37 °C, before incubation in medium containing zero, 2 mM- or 10 mM-glucose and secretagogues as indicated. After 30 min, samples were removed and centrifuged, and supernatants were assayed for insulin release by radioimmunoassay. Data are presented as means $\pm$ S.E.M. for 24 observations (6 experiments): ^a $P < 0.05$, ^b $P < 0.001$, NS not significant, compared with control cells under the same incubation conditions; ^c $P < 0.05$ compared with 2 mM-glucose, control cells (Student's t test).

Incubation condition	Insulin release (μ units/30 min per well)	
	Control	TPA-pre-treated
Zero glucose	3 $\pm$ 1	4 $\pm$ 1
2 mM-glucose (2G)	33 $\pm$ 6	20 $\pm$ 2 NS
10 mM-glucose	52 $\pm$ 9	29 $\pm$ 5 ^a
2G + acetylcholine	53 $\pm$ 7 ^c	19 $\pm$ 2 ^b
2G + TPA	124 $\pm$ 15	69 $\pm$ 14 ^a
2G + forskolin	195 $\pm$ 26	163 $\pm$ 22 NS

The effect of TPA pre-treatment on $[Ca^{2+}]_i$ in quin 2-loaded HIT cells is shown in Figs. 1(c) and 4. Pre-treatment of HIT cells with TPA had no significant effect on either the sustained rise induced by glucose or the transient increase in $[Ca^{2+}]_i$ induced by acetylcholine in HIT-cell suspensions. In contrast, TPA pre-treatment had a marked effect on the insulin-secretory properties of HIT cells (Table 2 and Fig. 4). In quin 2-loaded HIT-cell suspensions, the increase in insulin release induced by acetylcholine was abolished by prior TPA pre-treatment of the cells (compare Figs. 3a and 4a). In general, TPA-pre-treated cells responded comparatively less well to secretagogues than did control cells (Table 2). Glucose-stimulated insulin release was depressed in TPA-pre-treated cells by 39% and 44% in comparison with control cells when the incubation medium contained 2 mM- and 10 mM-glucose respectively. However, this decrease in insulin release achieved statistical significance only when the cells were stimulated by 10 mM-glucose. Acetylcholine (50 μ M) significantly potentiated glucose-stimulated insulin release in the control cells by 61%, an effect which was completely abolished in the TPA-pre-treated cells. TPA induced a 3.4-fold increase in insulin release in TPA-pre-treated HIT cells stimulated by glucose, although the absolute secretory response was significantly lower (by 50%) than the potentiation of insulin release induced by TPA in the control cells. In contrast, the effect of forskolin on glucose-stimulated insulin release was not significantly different in control (6-fold increase) and TPA-pre-treated (8-fold increase) cells.

DISCUSSION

In the present work we have investigated the second-messenger systems involved in the cholinergic stimulation of insulin release in the glucose-responsive clonal β -cell HIT-T15. The role of Ca^{2+} in the cholinergic stimulation of insulin release has long been recognized (Griffey *et al.*, 1974). Many studies have confirmed that acetylcholine-induced insulin release is dependent on the presence of extracellular Ca^{2+} (Burr *et al.*, 1976; Gagerman *et al.*, 1980; Wollheim *et al.*, 1980; Nenquin *et al.*, 1984; Mathias *et al.*, 1985; Hermans *et al.*, 1987; Hughes *et al.*, 1987; Garcia *et al.*, 1988) and that Ca^{2+} influx may play an important role in the secretory process. In the present study, we have characterized the effect of acetylcholine on $[Ca^{2+}]_i$ in HIT cells. Our data show that, although acetylcholine stimulated an increase in $[Ca^{2+}]_i$ in HIT cells, this increase was only transient in nature, lasting for approx. 2–3 min. This was in contrast with the effect of acetylcholine on insulin secretion, where the neurotransmitter induced a sustained increase in release from HIT-cell suspensions. Wollheim & Biden (1986) also showed that carbamoylcholine stimulated a transient increase in $[Ca^{2+}]_i$ and a sustained increase in insulin release in RIN m5F cells. Those authors obtained qualitatively similar results using quin 2 and the more sensitive fura 2, which suggested that the transient nature of the $[Ca^{2+}]_i$ signal did not reflect the ability of the fluorescent probe to buffer intracellular Ca^{2+} . Measurements of $[Ca^{2+}]_i$ have also been made in single HIT cells loaded with fura 2 (Prentki *et al.*, 1988). Here, carbamoylcholine induced oscillations and transients in $[Ca^{2+}]_i$ in individual cells, which were described as characteristic $[Ca^{2+}]_i$ patterns or ' Ca^{2+} fingerprints'.

The data from the present study are also in agreement with those of Sanchez-Andres *et al.* (1988), who showed that carbamoylcholine produced a transient increase in Ca^{2+} channel activity (lasting up to 1 min) in isolated mouse pancreatic islets. Those authors further suggested that extracellular Ca^{2+} was only necessary for the first 1 min to induce cholinergic-stimulated insulin release.

At variance with these data are studies in which Ca^{2+} fluxes

were measured in pancreatic islets in perfusion, using $^{45}Ca^{2+}$ (Nenquin *et al.*, 1984; Hermans *et al.*, 1987; Garcia *et al.*, 1988). Here the effects of cholinergic agents were sustained, producing biphasic efflux of $^{45}Ca^{2+}$. However, in one report carbamoylcholine stimulated $^{45}Ca^{2+}$ efflux from rat pancreatic islets, producing essentially a peak-shaped phenomenon in the early stages of perfusion (Mathias *et al.*, 1985).

Our data suggest that the cholinergic-induced $[Ca^{2+}]_i$ transient in HIT cells results essentially from Ca^{2+} influx, since it was abolished in the absence of extracellular Ca^{2+} or by inclusion of verapamil in the incubation medium. This is in contrast with the $[Ca^{2+}]_i$ transient induced by carbamoylcholine in RIN cells, which it was suggested resulted from inositol trisphosphate-induced mobilization of intracellular Ca^{2+} (Wollheim & Biden, 1986). The situation in pancreatic islets is again apparently more complex than in clonal β -cell lines. Although it has been reported that acetylcholine stimulates $^{45}Ca^{2+}$ uptake into islets (Wollheim *et al.*, 1980; Hermans *et al.*, 1987; Garcia *et al.*, 1988), Nilsson *et al.* (1987) have shown that carbamoylcholine stimulated a transient rise in $[Ca^{2+}]_i$ in dispersed pancreatic β -cells incubated in Ca^{2+} -free medium, which was coincident with a transient increase in insulin release. Studies in which islets were perfused in the absence of extracellular Ca^{2+} showed that cholinergic agents stimulated $^{45}Ca^{2+}$ efflux biphasically (Hellman & Gylfe, 1986; Henquin *et al.*, 1988). To explain the Ca^{2+} efflux pattern it was suggested that two distinct cholinergic-sensitive intracellular Ca^{2+} pools existed: one located in the endoplasmic reticulum, which was regulated by inositol trisphosphate, and the second sensitive to entry of Na^+ into the β -cell (Hellman & Gylfe, 1986). It has long been recognized that Na^+ fluxes play an important role in cholinergic stimulation of pancreatic islets (Gagerman *et al.*, 1980), and it has been suggested more recently that acetylcholine stimulates insulin release, at least in part by depolarizing the β -cell membrane via increased Na^+ permeability (Henquin *et al.*, 1988). The observation in the present study that decreasing Na^+ concentration in the medium abolished, at least in part, the transient increase in $[Ca^{2+}]_i$ induced by acetylcholine in HIT cells supports this hypothesis.

Our study also shows that, in the absence of glucose, acetylcholine is unable to induce the transient increase in $[Ca^{2+}]_i$. These data support the suggestion that acetylcholine stimulation of insulin release requires that the β -cell membrane be sufficiently depolarized to reach the threshold potential where Ca^{2+} channels are activated (Hermans *et al.*, 1987). The observation that cholinergic agents are effective at low glucose concentration (2 mM) reflects the finding that the rate-limiting step for glucose metabolism is transport rather than phosphorylation in HIT cells, the former process having a much lower K_m for glucose than the latter (Ashcroft & Stubbs, 1987).

Wollheim & Biden (1986) concluded that $[Ca^{2+}]_i$ is not a 'moment to moment' regulator of cholinergic-stimulated insulin release, and many authors have speculated about other possible modes of action of neurotransmitters in the β -cell (Mathias *et al.*, 1985; Garcia *et al.*, 1988). The transient nature of the $[Ca^{2+}]_i$ signal induced by acetylcholine in the present study led us to consider other possible second-messenger systems, particularly the protein kinase C pathway. Protein kinase C activation has been implicated in muscarinic-induced insulin release in the RINr cell line (Yamatani *et al.*, 1988). The approach that we adopted to investigate the role of protein kinase C in cholinergic-induced insulin release involved pretreating HIT cells in culture with the phorbol ester TPA. The procedure has been shown to deplete (selectively) cells, including β -cells, of protein kinase C activity (Hii *et al.*, 1987; Klip & Ramlal, 1987; Metz, 1988; Adams & Gullick, 1989). Studies using pancreatic islets depleted of protein kinase C have suggested that the enzyme does not play

an essential role in glucose-stimulated insulin release (Hii *et al.*, 1987; Metz, 1988).

In the present study, HIT cells cultured in the presence of TPA for 22–24 h still retained some protein kinase C activity. This residual activity probably enabled TPA-pre-treated cells to secrete insulin in response to TPA stimulation, albeit at a lower rate than control cells. It has been previously shown that pretreatment with the phorbol ester results in differential effects on cell lines, and that some cell lines still retain protein kinase C activity even after 30 h pre-treatment (Adams & Gullick, 1989). Nevertheless, a major finding of the present study was that HIT cells depleted of protein kinase C lost their secretory response to acetylcholine. This is in agreement with one other report in which pretreatment of pancreatic islets with TPA impaired their secretory response to carbamoylcholine (Persaud *et al.*, 1988). This suggests that protein kinase C plays a major role in cholinergic-induced insulin release in HIT cells. Data from the present study suggest that pretreatment with TPA selectively depletes (only) protein kinase C activity. HIT cells cultured with the phorbol ester still retained their normal secretory response to forskolin, in agreement with a previous study (Hii *et al.*, 1987). In addition, the increases in $[Ca^{2+}]_i$ induced by glucose and acetylcholine were normal in TPA-pre-treated cells. This observation does not support the suggestion that activation of protein kinase C in β -cells leads to depolarization and Ca^{2+} influx (Wollheim *et al.*, 1988). The poorer secretory response to glucose in TPA-pre-treated cells probably reflects the decreased insulin content of these cells, which in turn may result from prolonged exposure to a potent secretagogue.

We have previously suggested (Hughes *et al.*, 1987), as have other authors (Henquin *et al.*, 1987), that the mechanisms by which protein kinase C activation potentiates insulin release involves sensitization of the secretory apparatus to the existing level of intracellular Ca^{2+} . The observation that acetylcholine stimulates only a transient increase in $[Ca^{2+}]_i$, whereas cholinergic-induced insulin release can be abolished by protein kinase C depletion, suggests that the neurotransmitter may exert its effects primarily through such a sensitizing mechanism in HIT cells. More recently Hermans & Henquin (1989) assessed the relative importance of extracellular and intracellular Ca^{2+} for acetylcholine-stimulated insulin release. They concluded that extracellular Ca^{2+} was essential for sustained insulin release and that the neurotransmitter promotes secretion by a slight enhancement of Ca^{2+} influx associated with an increase in effectiveness of incoming Ca^{2+} on the release machinery. Thus it can be envisaged that acetylcholine binding to the muscarinic receptor in the β -cell membrane activates a two-stage process to potentiate glucose-stimulated insulin release. An initial increase in $[Ca^{2+}]_i$ through voltage-sensitive Ca^{2+} channels stimulates insulin release and primes the secretory mechanism for protein kinase C activation; this in turn sensitizes the secretory mechanism to Ca^{2+} , causing sustained insulin release.

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REFERENCES

- Adams, J. C. & Gullick, W. J. (1989) *Biochem. J.* **257**, 905–911
- Ashcroft, S. J. H. & Crossley, J. R. (1974) *Diabetologia* **11**, 274–279
- Ashcroft, S. J. H. & Stubbs, M. (1987) *FEBS Lett.* **219**, 311–315
- Ashcroft, S. J. H., Hammonds, P. & Harrison, D. E. (1986) *Diabetologia* **29**, 727–733
- Best, L. & Malaisse, W. J. (1984) *Endocrinology* (Baltimore) **115**, 1814–1820
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 148–154
- Burr, I. M., Slonim, A. E., Burke, V. & Fletcher, T. (1976) *Am. J. Physiol.* **231**, 1246–1249
- Christie, M. R. & Ashcroft, S. J. H. (1985) *Biochem. J.* **227**, 727–736
- Gagerman, E., Sehlin, J. & Taljedal, I.-B. (1980) *J. Physiol. (London)* **300**, 505–513
- Gaines, K. L., Hamilton, S. & Boyd, A. E. (1988) *J. Biol. Chem.* **263**, 2589–2592
- Garcia, M.-C., Hermans, M. P. & Henquin, J. C. (1988) *Biochem. J.* **254**, 211–218
- Griffey, M. A., Conaway, H. H. & Whitney, J. E. (1974) *Diabetes* **23**, 494–498
- Hellman, B. & Gylfe, E. (1986) *Pharmacology* **32**, 257–267
- Henquin, J. C., Bozem, M., Schmeer, W. & Nenquin, M. (1987) *Biochem. J.* **246**, 393–399
- Henquin, J. C., Garcia, M.-C., Bozem, M., Hermans, M. P. & Nenquin, M. (1988) *Endocrinology* (Baltimore) **122**, 2134–2142
- Hermans, M. P. & Henquin, J. C. (1989) *Diabetes* **38**, 198–204
- Hermans, M. P., Schmeer, W. & Henquin, J. C. (1987) *Endocrinology* (Baltimore) **120**, 1765–1773
- Hii, C. S. T., Jones, P. M., Persaud, S. J. & Howell, S. L. (1987) *Biochem. J.* **246**, 489–493
- Hill, R. S. & Boyd, A. E. (1985) *Diabetes* **34**, 115–120
- Hughes, S. J. & Ashcroft, S. J. H. (1988a) *J. Mol. Endocrinol.* **1**, 13–17
- Hughes, S. J. & Ashcroft, S. J. H. (1988b) *Biochem. J.* **249**, 825–830
- Hughes, S. J., Christie, M. R. & Ashcroft, S. J. H. (1987) *Mol. Cell. Endocrinol.* **50**, 231–236
- Hughes, S. J., Chalk, J. G. & Ashcroft, S. J. H. (1989) *Mol. Cell. Endocrinol.* **65**, 35–41
- Jones, P. M., Stutchfield, J. & Howell, S. L. (1985) *FEBS Lett.* **191**, 102–106
- Keahey, H. H., Rajan, A. S., Boyd, A. E. & Kunze, D. L. (1989) *Diabetes* **38**, 188–193
- Klip, A. & Ramlal, T. (1987) *Biochem. J.* **242**, 131–136
- Mathias, P. C. F., Carpinelli, A. R., Billaudel, B., Garcia-Morales, P., Valverde, I. & Malaisse, W. J. (1985) *Biochem. Pharmacol.* **34**, 3451–3457
- Metz, S. A. (1988) *Diabetes* **37**, 3–7
- Nelson, T. Y., Gaines, K. L., Rajan, A. S., Berg, M. & Boyd, A. E. (1987) *J. Biol. Chem.* **262**, 2608–2612
- Nenquin, M., Awouters, P., Mathot, F. & Henquin, J. C. (1984) *FEBS Lett.* **176**, 457–461
- Niki, I., Kelly, R. P., Ashcroft, S. J. H. & Ashcroft, F. M. (1989) *Pflugers Arch.* **415**, 47–55
- Nilsson, T., Arkhammar, P., Hallberg, A., Hellman, B. & Berggren, P.-O. (1987) *Biochem. J.* **248**, 329–336
- Persaud, S. J., Jones, P. M. & Howell, S. L. (1988) *Diabetic Med.* **5**, Suppl. 2, 24
- Prentki, M., Glennon, M. C., Thomas, A. P., Morris, R. L., Matschinsky, F. M. & Corkey, B. E. (1988) *J. Biol. Chem.* **263**, 11044–11047
- Rorsman, P. & Abrahamsson, H. (1985) *Acta Physiol. Scand.* **125**, 639–647
- Sanchez-Andres, J. V., Ripoll, C. & Soria, B. (1988) *FEBS Lett.* **231**, 143–147
- Santerre, R. F., Cook, R. A., Crisel, R. M. D., Sharp, J. D., Schmidt, R. J., Williams, D. C. & Wilson, C. P. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4339–4343
- Tamagawa, T., Niki, H. & Niki, A. (1985) *FEBS Lett.* **183**, 430–432
- Wollheim, C. B. & Biden, T. J. (1986) *J. Biol. Chem.* **261**, 8314–8319
- Wollheim, C. B., Siegel, E. G. & Sharp, G. W. G. (1980) *Endocrinology* (Baltimore) **107**, 924–929
- Wollheim, C. B., Dunne, M. J., Peter-Reisch, B., Bruzzone, R., Pozzan, T. & Petersen, O. H. (1988) *EMBO J.* **7**, 2443–2449
- Yamatani, T., Chiba, T., Kadowaki, S., Hishikawa, R., Yamaguchi, A., Inui, T., Fujita, T. & Kawazu, S. (1988) *Endocrinology* (Baltimore) **122**, 2826–2832