British Journal of Pharmacology (1996) 118, 769-775

Desensitization of glucagon-like peptide 1 receptors in insulin-secreting βTC3 cells: role of PKA-independent mechanisms

[1]Jesper Gromada, *Steen Dissing & Patrik Rorsman

Department of Islet Cell Physiology, Novo Nordisk A/S, Symbion Science Park, Fruebjergvej 3, DK-2100 Copenhagen and *Department of Medical Physiology, The Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen, Denmark

1 The cellular processes involved in the desensitization of the glucagon-like peptide 1 receptors were investigated by measurements of the glucagon-like peptide 1(7-36)amide (GLP-1(7-36)amide)-induced increases in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in insulin-secreting βTC3 cells.

2 In the presence of 11.2 mM glucose, stimulation with GLP-1(7-36)amide led to a small membrane depolarization (< 10 mV), induction of electrical activity and a rapid increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ was not observed in the presence of the L-type Ca^{2+}-channel antagonist nifedipine. However, nifedipine was ineffective when applied after addition of GLP-1(7-36)amide.

3 The increase in $[Ca^{2+}]_i$ evoked by GLP-1-(7-36)amide was transient and even in the continued presence of the agonist, $[Ca^{2+}]_i$ returned to the basal value within 4-5 min. The latter process was slowed, but not prevented, by inhibition of protein kinase C (PKC) by staurosporine and Ro31-8220.

4 Short pretreatment of the cells with the phorbol ester, 4-β-phorbol-12-β-myristate-13-α-acetate (PMA), an activator of PKC, reduced the GLP-1(7-36)amide-evoked increase in $[Ca^{2+}]_i$ by 75%. This effect of PMA was fully reversed by staurosporine and Ro31-8220.

5 The ability of GLP-1(7-36)amide to increase $[Ca^{2+}]_i$ disappeared upon pre-exposure of the cells to the hormone (desensitization). This process was maximal within 5 min of exposure to the agonist. Following removal of the agonist from the medium, the ability to respond to subsequent stimulation by GLP-1(7-36)amide recovered gradually with time; half and complete recovery requiring >20 min and 60 min, respectively. The desensitizing action of GLP-1(7-36)amide persisted in the presence of either staurosporine or forskolin and did not require an elevation of $[Ca^{2+}]_i$.

6 Our data suggest that the GLP-1(7-36)amide-evoked increase in $[Ca^{2+}]_i$ is *initiated* by Ca^{2+}-influx though voltage-dependent and nifedipine-sensitive L-type Ca^{2+} channels but depends principally on Ca^{2+} mobilization from internal stores for its maintenance. The desensitization of the GLP-1 receptors that occurs in the continued presence of the agonist does not result from the activation of protein kinase A or Ca^{2+}-dependent kinases/phosphatases. Our data indicate that activation of PKC may contribute to the desensitization of the GLP-1 receptors but that other (PKC-independent) mechanisms also participate in this process.

Keywords: Insulin; β-cells; calcium; receptor desensitization; GLP-1(7-36)amide

Introduction

Glucagon-like peptide 1 (GLP-1), a potent insulinotropic gastrointestinal hormone, mediates its effect on the pancreatic β-cells via specific receptors (Fehmann & Habener, 1992; Thorens, 1992; Thorens & Waeber, 1993). Binding of GLP-1 to its receptor promotes the formation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and evokes a glucose-dependent increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and thereby promotes insulin secretion (Yada *et al.*, 1993; Göke *et al.*, 1993; Lu *et al.*, 1993; Cullinan *et al.*, 1994; Montrose-Rafizadeh *et al.*, 1994). The effect of GLP-1 on $[Ca^{2+}]_i$ in βTC3 cells is thought to involve mobilization of intracellular Ca^{2+} through Ca^{2+}-induced Ca^{2+} release (CICR) and Ca^{2+}-stimulated inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) production (Gromada *et al.*, 1995*a*). It has also been demonstrated that expression of GLP-1 receptors in HEK 293-cells is sufficient to reconstitute the entire cascade including CICR (Gromada *et al.*, 1995*b*).

Recent experiments have shown that the GLP-1 receptor in rat insulinoma HIT-T15 cells is subject to rapid and reversible homologous desensitization (Fehmann & Habener, 1991). Furthermore, it has been shown that high GLP-1 concentrations inhibit cyclic AMP production and insulin release (Drucker *et al.*, 1987; Göke & Conlon, 1988; Suzuki *et al.*, 1989; Gefel *et al.*, 1990). To characterize the cellular mechanisms involved in the desensitization of the GLP-1 receptors, we have monitored $[Ca^{2+}]_i$ in response to repetitive applications of glucagon-like peptide 1(7-36)amide [GLP-1(7-36)amide] in insulin-secreting βTC3 cells. We demonstrate that the desensitization of the GLP-1 receptors is a complex process which, unlike the actions on membrane potential, $[Ca^{2+}]_i$ and insulin secretion, is not mediated by cyclic AMP-induced activation of PKA. Desensitization of the GLP-1 receptors appears to be mediated, in part, by PKC-dependent mechanisms. In addition, there is evidence for a second component which is neither attributable to activation of PKA nor PKC.

Methods

Cell culture

βTC3 mouse insulinoma cells (Efrat *et al.*, 1988) were cultured in DMEM containing 1000 mg l^{-1} D-glucose, 10% heat-inactivated foetal calf serum, penicillin (50 units ml^{-1}), streptomycin (0.05 mg ml^{-1}), and 2 mM L-glutamine. Cells were passaged at 7 day intervals by washing once in Hanks solution

[1] Author for correspondence.

before detachment in trypsin, while media were changed twice a week. All experiments were performed with cells from passage 30–45.

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

Measurements of $[Ca^{2+}]_i$ in single βTC3 cells were obtained by use of a fluorescence microscope (Zeiss Axiovert 135) with a Xenon lamp, a CCD camera (Dage-MTI, USA) with an intensifier (Genesis, Dage-MTI, USA) and a digital image processing system (Universal Imaging, USA). βTC3 cells were loaded with 3 μM fura-2/AM for 40 min at 37°C. After incubation, the cells were resuspended and washed twice in a medium containing (in mM): Na^+140, K^+4, Ca^{2+} 2, Mg^{2+} 1, Cl^-150, (N-[2-hydroxyethyl]piperazine-N'-2-ethanesulphonic acid) HEPES 10, pH 7.3. We have previously demonstrated that GLP-1(7-36)amide causes glucose-dependent increase in $[Ca^{2+}]_i$ in βTC3 cells (Gromada *et al.*, 1995*a*). Therefore, all experiments in the present study were performed in a medium containing 11.2 mM glucose. During the experiments the cells were continuously superfused with control or test solution at rates between 2 ml min^{-1} at 37°C. Excitation wavelengths were 340 nm and 380 nm and emission was detected at 510 nm. Images were obtained by an oil-immersion u.v. objective (Zeiss Achrostigmat 40x, 1.30 numerical aperture) and transferred to a video tape recorder (Umatic SP, VO 9600, Sony). The images represent the average of 8 frames and the sample rate was 0.5 Hz. For analysis the data were replayed into the image processor and $[Ca^{2+}]_i$ was determined by averaging the pixel grey values inside a 4×4 μm window in the centre of the cells.

The $[Ca^{2+}]_i$ was calculated from the following formula (Grynkiewicz *et al.*, 1985): $[Ca^{2+}]_i = K_d(R\text{-}R_{min})/(R_{max}\text{-}R)(S_{f2}/S_{b2})$. Calibrations of intracellular fura-2 signals were done by adding ionomycin to a final concentration of 1 μM in order to equilibrate Ca^{2+} across the plasma membrane. The gray values obtained for R_{max} amounted to 241.R_{min} was obtained from a high-K^+ medium free of Ca^{2+} with 10 mM EGTA (ethylene glycol-O,O'-*bis*(2-aminoethyl)-*N',N,N',N'*-tetraacetic acid) and 1 μM fura-2 pentapotassium salt and amounted to 27. The proportionality constant (S_{f2}/S_{b2}) was determined to 9.8. A value for the apparent K_d of Ca^{2+} binding to fura-2 of 224 nM was used (Grynkiewicz *et al.*, 1985).

Measurements of membrane potential

The membrane potential of individual βTC3 cells was monitored by the perforated patch whole-cell configuration as previously described (Ämmälä *et al.*, 1993). Briefly, the membrane potential was monitored using the current-clamp mode of an EPC-9 patch-clamp amplifier and the programme Pulse (version 7.62; Heka Elektronik, Lambrecht/Pfalz, Germany). The pipette solution contained (in mM): K_2SO_4 76, NaCl 10, KCl 10, $MgCl_2$1 and HEPES 5 (pH 7.35 using KOH). Electrical contact with the cell interior was established by insertion of the pore-forming antibiotic amphotericin B (Rae *et al.*, 1991) into the patch membrane. The concentration of amphotericin B in the pipette solution was 0.24 mg ml^{-1}.

Chemicals

Fura-2 acetoxymethyl ester (fura-2/AM) and fura-2 pentapotassium salt were supplied by Molecular Probes (Eugene, OR, U.S.A.). Foetal calf serum was purchased from A. H. Diagnostic (Aarhus, Denmark). GLP-1(7-36)amide was synthesized at Novo Nordisk A/S (Bagsværd, Denmark). Dulbecco's modified Eagle medium (DMEM), Hanks' solution, trypsin, penicillin, streptomycin and L-glutamine were from Gibco BRL (Life Technologies, Inc, MD, U.S.A.). Ro31-8220 was obtained from Calbiochem (La Jolla, CA, U.S.A.). All other chemicals were obtained from Sigma Chemicals (St. Louis, MO. U.S.A.). PMA, forskolin, staurosporine and Ro31-8220 were all dissolved in dimethylsuphoxide (DMSO) (final conc. of DMSO: <0.1%).

Statistical analysis

Statistical evidence was evaluated by Student's *t* test. All data are expressed as mean values ± s.e.mean for *n* tested cells.

Results

The majority (80%) of the βTC3 cells do not respond to glucose stimulation with an increase in $[Ca^{2+}]_i$ but glucose responsiveness can be induced by addition of GLP-1(7-36)amide (Gromada *et al.*, 1995*a*). Figure 1 shows a membrane potential recording from a glucose non-responsive βTC3 cell in the presence of 11.2 mM glucose. Inclusion of 10 pM GLP-1(7-36)amide in the extracellular solution produced a prompt depolarization of 9 ± 1 mV ($n = 5$) and after a short delay, electrical activity consisting of Na^+ and Ca^{2+}-dependent action potentials was initiated (*cf.* Holz *et al.*, 1993).

These effects on electrical activity were associated with a rapid increase in $[Ca^{2+}]_i$ from a resting value of 187 ± 15 nM ($n = 189$) to ≈600 nM. This was followed by a gradual return of $[Ca^{2+}]_i$ to the prestimulatory level (Figure 2a). Similar effects on $[Ca^{2+}]_i$ were observed following stimulation with 10 nM GLP-1(7-36)amide (Table 1).

Addition of nifedipine (25 μM), an L-type Ca^{2+}-channel blocker, prior to the stimulation with GLP-1(7-36)amide abolished the increase in $[Ca^{2+}]_i$ (Figure 2b). However, it was ineffective in cells already challenged with the agonist (Figure 2(c)). By contrast, when βTC3 cells depolarized by high extracellular K^+ concentrations were exposed to nifedipine, $[Ca^{2+}]_i$ rapidly returned to the basal concentration. The same effects were obtained with 2.5 μM of the antagonist.

Effect of PKC on GLP-1(7-36)amide induced changes in $[Ca^{2+}]_i$

As already pointed out, the increase in $[Ca^{2+}]_i$ evoked by GLP-1(7-36)amide returns to basal within 4–5 min (average: 4.1 ± 0.5 min; $n = 50$) despite the continued presence of the agonist. This behaviour is obviously reminiscent of the process of desensitization of plasma membrane receptors. As a first attempt to elucidate the molecular mechanisms behind the spontaneous decrease in $[Ca^{2+}]_i$, we investigated the possible participation of PKC by adding staurosporine, a potent and fairly selective inhibitor of this kinase (Tamaoki *et al.*, 1986). As shown in Figure 3a, inhibition of PKC slightly prolonged the duration of the $[Ca^{2+}]_i$-increase evoked by GLP-1(7-36)amide. In this cell, 7 min was required for $[Ca^{2+}]_i$ to return to basal and in a series of 21 cells, a mean duration of the $[Ca^{2+}]_i$-transient of 7.3 ± 0.9 min ($P < 0.05$ when compared to

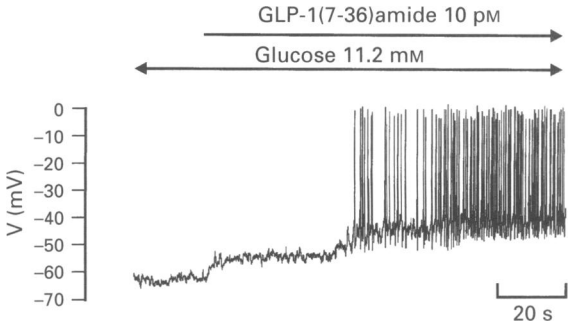

Figure 1 Effects of GLP-1(7-36)amide on electrical activity in a single βTC3 cells. The recording of the membrane potential was performed in the continuous presence of 11.2 mM glucose. GLP-1(7-36)amide (10 pM) was applied during the period indicated by the horizontal line.

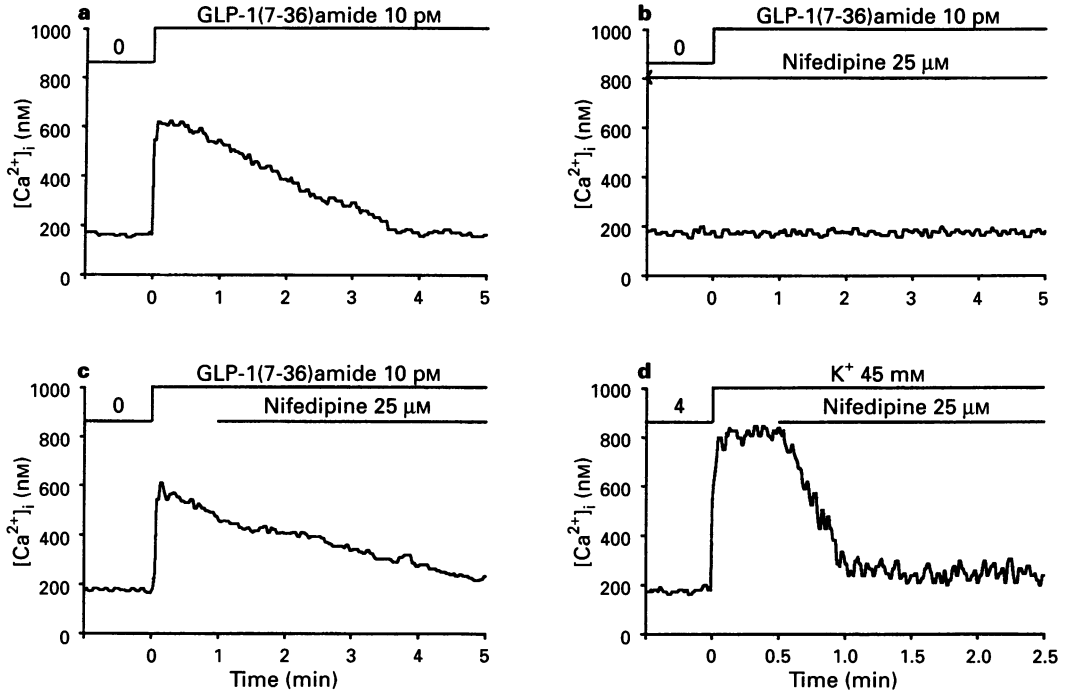

Figure 2 Effect of GLP-1(7-36)amide on $[Ca^{2+}]_i$ in βTC3 cells. (a) Typical Ca^{2+} response to GLP-1(7-36)amide (10 pM) in a single βTC3 cells in the presence of 11.2 mM glucose. (b) Nifedipine (25 μM) inhibits $[Ca^{2+}]_i$-response to stimulation with GLP-1(7-36)amide (10 pM). (c) $[Ca^{2+}]_i$ response to GLP-1(7-36)amide (10 pM) followed by application of nifedipine (25 μM). (d) Effect of 45 mM K^+ on $[Ca^{2+}]_i$ followed by application of nifedipine (25 μM). The tracings are representative of 20–34 cells in 4–8 different experiments.

Table 1 Modulation of GLP-1(7–36)amide induced increase in $[Ca^{2+}]_i$ by changes in cellular PKC activity

Condition#	n	$\Delta[Ca^{2+}]_i$
GLP-1(7-36)amide 10 pM	28	402 ± 35
GLP-1(7-36)amide 10 pM + PMA 100 nM	22	112 ± 39*
GLP-1(7-36)amide10 pM + PMA 16 nM	6	191 ± 23*
GLP-1(7-36)amide 10 pM + 4α-PMA 100 nM	18	420 ± 56
GLP-1(7-36)amide 10 pM + PMA 10 nM + staurosporine 100 nM	25	392 ± 57
GLP-1(7-36)amide 10 nM	33	446 ± 26
GLP-1(7-36)amide 10 nM + PMA 100 nM	17	152 ± 46*
GLP-1(7-36)amide 10 nM + 4α-PMA 100 nM	22	432 ± 58
GLP-1(7-36)amide 10 nM + PMA 100 nM + staurosporine 100 nM	34	456 ± 59

#Cells were pretreated for 5 min with 16-100 nM PMA and/or 100 nM staurosporine. The results were obtained in 4–8 different experiments. *$P < 0.05$.

the control value). When Ro31-8220 (500 nM) was used as the PKC-inhibitor, the corresponding value for the mean duration of the GLP-1(7-36)amide-induced $[Ca^{2+}]_i$-transient was 7.2 ± 0.1 min ($n = 10$). In the latter series of experiments, the mean duration under control conditions (i.e. absence of PKC-inhibitor) was 3.9 ± 0.2 min ($n = 8$). The amplitude of the $[Ca^{2+}]_i$-transients were the same, irrespective of the PKC-inhibitor used (Table 2).

To investigate further the role of PKC in the GLP-1(7-36)amide-induced increase in $[Ca^{2+}]_i$, PKC was activated by adding the phorbol ester, PMA (4-β-phorbol-12-β-myristate-13-α-acetate) (Nishizuka, 1984). Figure 3b shows a typical experiment in which a βTC3 cell was pretreated for 5 min with 100 nM PMA and subsequently stimulated with 10 pM GLP-1(7-36)amide. Pretreatment with PMA did not affect basal $[Ca^{2+}]_i$. However, the GLP-1(7-36)amide-induced increase in $[Ca^{2+}]_i$ was suppressed by 75% (Table 1). 4α-PMA (4-α-phorbol-12-β-myristate-13-α-acetate), an inactive phorbol ester, had no effect on the $[Ca^{2+}]_i$ response to GLP-1(7-36)amide stimulation (Table 1). The same effects were obtained with a lower concentration of PMA (16 nM; Table 1). The inhibitory action of PMA on the GLP-1(7-36)amide-induced increase in $[Ca^{2+}]_i$ could be reversed by addition of staurosporine (Figure 3(a)) and Ro31-8220 (not shown). The same pattern of $[Ca^{2+}]_i$ responses were observed following stimulation with a thousand-fold higher concentration of GLP-1(7-36)amide (Table 1). These data suggest that activation of PKC *contributes* to the desensitization of the GLP-1(7-36)amide responses. However, as will become clear below, this cannot be the only mechanism involved.

Homologous desensitization of GLP-1(7-36)amide-evoked rises in $[Ca^{2+}]_i$

Desensitization of membrane receptors can typically be reversed by extensive washing with agonist-free medium. Figure 4b illustrates the effects of GLP-1(7-36)amide on $[Ca^{2+}]_i$ in response to repetitive stimulation of the βTC3 cells. Following the first $[Ca^{2+}]_i$-transient, GLP-1(7-36)amide was removed from the perifusion medium for 5 min and the cell was subsequently exposed to the agonist again. It is clear that some recovery from desensitization occurred and the peak amplitude of the $[Ca^{2+}]_i$-transient was approx. 30% of the control value.

To investigate the influence of longer periods of recovery a slightly different experimental protocol was employed. The cells were first desensitized by a 5 min exposure to GLP-1(7-36)amide (10 pM or 10 nM) and then washed for 20 or 60 min with agonist-free medium. Figure 4b summarizes the recovery from desensitization. It can be seen that the recovery from desensitization for both concentrations of GLP-1(7-36)amide is half-maximal and complete within 20 and 60 min, respectively.

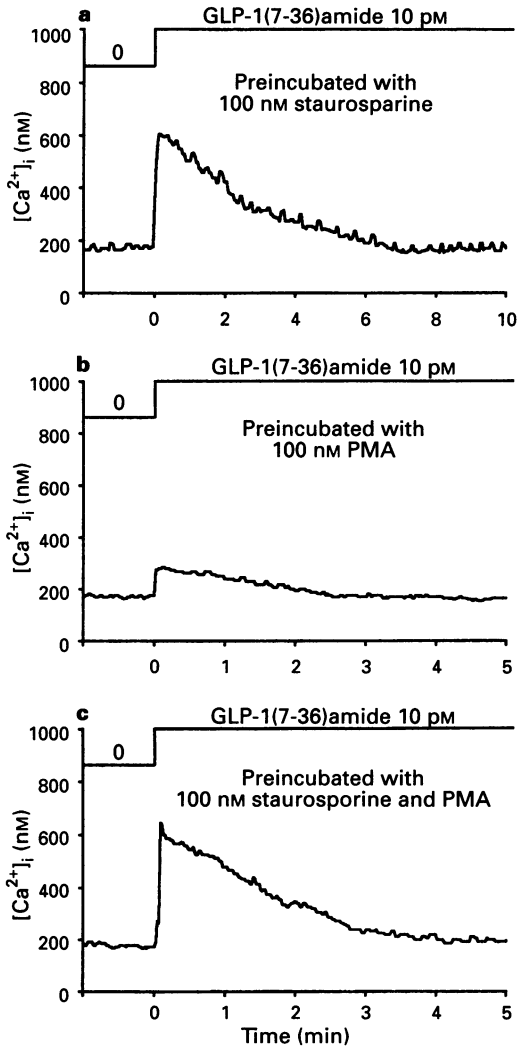

Figure 3 Effect of protein kinase C modulators on GLP-1(7-36)amide-evoked Ca^{2+} signalling in single βTC3 cells. (a) A βTC3 cell was pretreated for 10 min with staurosporine (100 nM) before stimulated with GLP-1(7-36)amide (10 pM). (b) Response from individual cell incubated for 10 min with PMA (100 nM) before stimulated with GLP-1(7-36)amide (10 pM). (c) βTC3 cell incubated with 100 nM of staurosporine and PMA for 10 min followed by application of 10 pM GLP-1(7-36)amide. The tracings are representative of 22–31 cells in 4–6 individual experiments.

Desensitization of GLP-1(7-36)amide evoked increases in $[Ca^{2+}]_i$ does not result solely from activation of PKC

The data of Figure 3 indicate that activation of PKC by GLP-1(7-36)amide participates in the desensitization process. Figure 5a shows an experiment where staurosporine (100 nM) was included before stimulation with GLP-1(7-36)amide. It is apparent that despite the presence of the PKC inhibitor, the $[Ca^{2+}]_i$ response remained transient and that $[Ca^{2+}]_i$ returned to basal within 7 min and a second application of GLP-1(7–36)amide 6 min later produced a $[Ca^{2+}]_i$ increase which was much reduced compared with the initial response. However, the relative amplitude of the second response (49 ± 11%; Table 2) was somewhat larger in the cells exposed to staurosporine (or Ro31-8220, not shown) than in their non-treated counterparts (26 ± 3%, $P<0.05$; Table 1). The difference we attribute to a PKC-dependent component of desensitization. Figure 5b shows that when desensitization was induced by both PMA and GLP-1(7-36)amide, 60 min was insufficient for complete recovery of the $[Ca^{2+}]_i$-response to renewed stimulation by GLP-1(7–36)amide. This is indeed consistent with the concept that a fraction of the desensitization is PKC-dependent as the recovery from the action of PMA is likely to be very slow considering the highly lipophilic nature of the compound.

Table 2 Effect of PKC and PKA activity on GLP-1(7–36)amide-induced changes in $[Ca^{2+}]_i$

Condition	n	$\Delta[Ca^{2+}]_i$
GLP-1(7-36)amide 10 pM + staurosporine 100 nM	21	427 ± 39
GLP-1(7-36)amide 10 pM + Ro31-8220 500 nM	10	415 ± 13
GLP-1(7-36)amide 10 pM + staurosporine[a] 100 nM, 5 min recovery period, GLP-1(7-36)-amide 10 pM	21	207 ± 43*
GLP-1(7-36)amide 10 pM + 100 nM PMA, 60 min recovery period, GLP-1(7-36)amide 10 pM	31	261 ± 78
Forskolin 4 μM	38	389 ± 36
Forskolin 4 μM, 10 min recovery period, GLP-1(7-36)amide 10 pM	34	372 ± 36
GLP-1(7-36)amide 10 pM + nifedipine[b] 25 μM, 8–10 min recovery period, GLP-1(7-36)-amide 10 pM	42	179 ± 77*

[a]βTC3 cells were pretreated for 10 min with 100 nM staurosporine or 500 nM Ro31-8220 before the first stimulation with GLP-1(7-36)amide. Staurosporine was present during the rest of the experiment. The results were obtained in 4-5 different experiments. [b]Nifedipine (25 μM) was added 5 min before the first pulse of forskolin or GLP-1(7-36)amide. Cells were washed in agonist and nifedipine-free medium (recovery period) and subsequently stimulated with GLP-1(7-36)amide in an extracellular solution without nifedipine. The results are the average of 6 individual experiments. *$P<0.05$.

Desensitization does not result from depletion of internal Ca^{2+}-stores

GLP-1(7-36)amide induced a marked stimulation of cyclic AMP production. We have therefore also considered the possibility that desensitization results from the activation of PKA. However, as illustrated in Figure 6a this was unlikely to be the case as forskolin, which elevates cyclic AMP to the same extent as GLP-1(7-36)amide in βTC3 cells (Gromada *et al.*, 1995*a*), did not mimic the action of GLP-1(7-36)amide on the desensitization process. It can also be observed that although $[Ca^{2+}]_i$ was markedly increased already in response to forskolin, this did not interfere with the magnitude of the GLP-1(7-36)amide-induced response (Table 2). This strongly argues that the desensitization observed during the $[Ca^{2+}]_i$-measurements does indeed result from true desensitization and is not simply the consequence of the depletion of intracellular Ca^{2+}-stores. Indeed, desensitization of the response to GLP-1(7-36)amide persisted even when nifedipine was included in the perifusion medium to block the increase in $[Ca^{2+}]_i$ (Figure 6b). Separate control experiments revealed that the K^+-induced increase in $[Ca^{2+}]_i$ recovered to 94 ± 1% ($n=4$) of its control amplitude within 8 min (i.e. the same interval as used in Figure 6b) of the withdrawal of nifedipine from the bath medium. From these data it can also be concluded that activation of Ca^{2+}-dependent protein kinases and/or phospatases is not involved in the process of desensitization of the GLP-1 receptors.

Discussion

Desensitization is a common feature of plasma membrane receptors. Here we have examined the properties of desensiti-

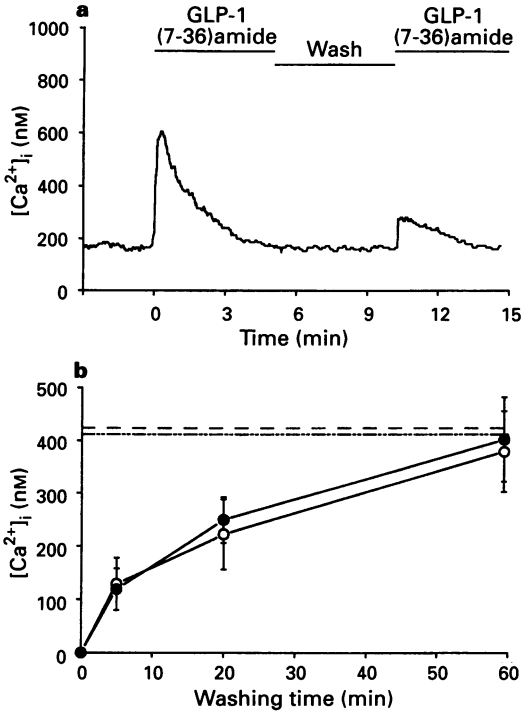

Figure 4 Desensitization of GLP-1(7-36)amide-evoked increase in $[Ca^{2+}]_i$. (a) βTC3 cells were stimulated with GLP-1(7-36)amide (10 pM) for 5 min followed by a 5 min recovery period where the cells were washed with extracellular solution without GLP-1(7-36)amide. The cells were subsequently stimulated with 10 pM GLP-1(7-36)amide. The tracing is representative of 22 cells from 5 individual experiments. (b) Recovery from desensitization of GLP-1(7-36)amide-evoked $[Ca^{2+}]_i$-response. βTC3 cells were stimulated for 5 min with 10 pM (●) or 10 nM (○) GLP-1(7-36)amide followed by washing in agonist-free medium for the indicated time periods. The amplitudes for control cells stimulated with 10 pM (dashed dotted line) or 10 nM (dashed line) GLP-1(7-36)amide are given. The points represent the mean ± s.e.mean for 12–32 cells from 4–6 individual experiments.

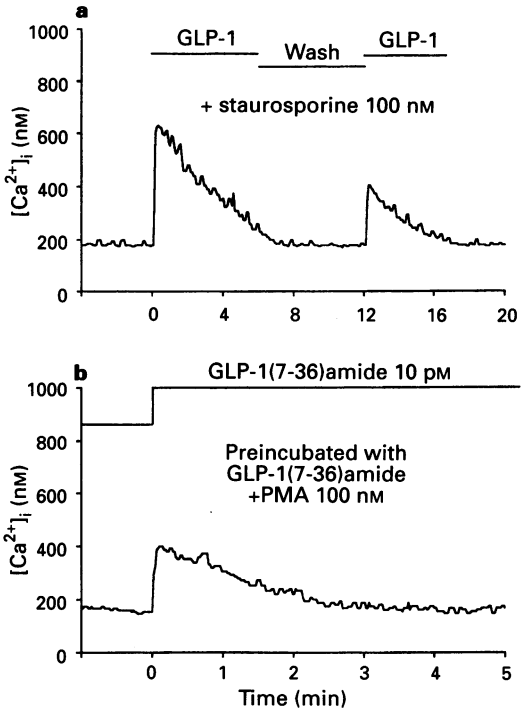

Figure 5 Protein kinase C contributed to the desensitization of the GLP-1(7-36)amide-evoked increase in $[Ca^{2+}]_i$. (a) Shows a representative experiment in a βTC3 cell that was pretreated with staurosporine (100 nM) for 10 min before sequential stimulation with 10 pM GLP-1(7-36)amide. (b) Ca^{2+} response to GLP-1(7-36)amide (10 pM) in a βTC3 cell pretreated with 10 pM GLP-1(7-36)amide and 100 nM PMA followed by a 60 min washout period. The tracings are representative of 21 and 29 cells from 4 and 5 individual experiments.

zation of the GLP-1 receptor by GLP-1(7-36)amide. Our data indicate that the desensitization produced by GLP-1(7-36)amide involves at least two distinct mechanisms: both PKC-dependent and PKC-independent pathways. Desensitization of the receptors does not involve activation of PKA (as suggested by the lack of effect of forskolin) and cannot be linked to activation of Ca^{2+}-dependent kinases/phosphatases. Desensitization can theoretically result from either a chemical modification of the receptors such that they cease to respond to the agonist, or a reduction in the number of receptors at the cell surface. We acknowledge that we cannot distinguish between these two alternatives but it appears that some conclusions can be drawn even without this distinction. Here we focus on a few particularly interesting features of our study.

GLP-1(7-36)amide causes mobilization of Ca^{2+} from intracellular stores

We have previously proposed that the insulinotropic action of GLP-1(7-36)amide results from its ability to increase $[Ca^{2+}]_i$ by mobilization of Ca^{2+} from intracellular stores. This is mediated by an initial limited influx of Ca^{2+} through voltage-dependent Ca^{2+}-channels. The resulting localized increase in $[Ca^{2+}]_i$ leads to the activation of phospholipase C and the formation of $Ins(1,4,5)P_3$. This mobilizes intracellular Ca^{2+} and eventually triggers a massive increase in $[Ca^{2+}]_i$ by Ca^{2+}-induced Ca^{2+}-release (Gromada *et al.*, 1995a). This idea is reinforced by the present observation that whereas the increase in $[Ca^{2+}]_i$ evoked by high-K^+ depolarization is abolished by the addition of nifedipine, this Ca^{2+}-channel blocker has little effect on the rise in $[Ca^{2+}]_i$ evoked by GLP-1(7-36)amide. This model is consistent with the present observation that GLP-1(7-36)amide produces a small depolarization in glucose non-re-

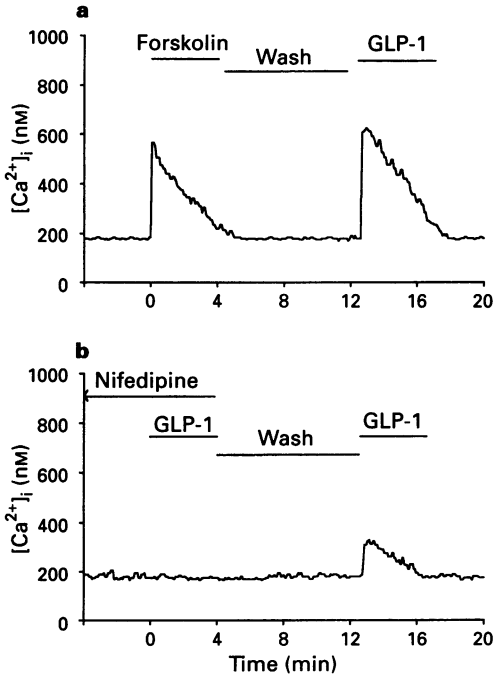

Figure 6 Desensitization of GAP-1(7-36)amide-evoked increase in $[Ca^{2+}]_i$ does not involve protein kinase A or a rise in $[Ca^{2+}]_i$. (a) Typical Ca^{2+} response in a βTC3 cell stimulated with forskolin (4 μM) and following a 8 min washout period with GLP-1(7-36)amide 10 pM. (b) The βTC3 cell was pretreated for 5 min with 25 μM nifedipine before stimulated with 10 pM GLP-1(7-36)amide followed by a 8 min washout period. The cell was subsequently stimulated with 10 pM GAP-1(7-36)amide. The tracings are representative of 26 and 42 cells in 5 and 6 individual experiments.

sponsive βTC3 cells and subsequently induces electrical activity. The transitory nature of the GLP-1(7-36)amide-induced $[Ca^{2+}]_i$-increase we explain as the depletion of CICR/Ins(1,4,5)P_3-sensitive intracellular Ca^{2+}-stores rather than the desensitization of the receptor. This is suggested by our observation that forskolin evokes a similar transient increase in $[Ca^{2+}]_i$: an effect which cannot be explained as the desensitization of a receptor. It follows from this that the amplitude of the $[Ca^{2+}]_i$-transients represent a more reliable indicator of the extent of desensitization of the GLP-1 receptors than the duration of the $[Ca^{2+}]_i$-transient.

Once CICR has been initiated, influx of extracellular Ca^{2+} is quantitatively only of minor importance thus accounting for the apparent resistance to nifedipine (Figure 2c). If the GLP-1(7-36)amide-induced increase in $[Ca^{2+}]_i$ is solely attributable to Ca^{2+}-influx through voltage-dependent Ca^{2+}-channels, it would have been expected to be as sensitive to nifedipine as that evoked by high-K^+ depolarization (Figure 2d). This is clearly not the case and we therefore conclude that the GLP-1(7-36)amide-induced increase in $[Ca^{2+}]_i$, although triggered by an initial Ca^{2+}-influx through voltage-dependent L-type Ca^{2+}-channels, is principally attributable to Ca^{2+} release from internal stores. The slow recovery of the $[Ca^{2+}]_i$-increase observed when nifedipine was applied after GLP-1(7-36)amide is unlikely to reflect the time required by Ca^{2+}-uptake and extrusion mechanisms to lower $[Ca^{2+}]_i$ back to the prestimulatory level. This is suggested by the observation that when Ca^{2+}-channels activated by high-K^+ depolarization are closed by nifedipine, $[Ca^{2+}]_i$ returns to basal within 30 s (Figure 2d) which is much shorter than the 4 min required following stimulation with GLP-1(7-36)amide (Figure 2c).

PKC-dependent desensitization of the GLP-1 receptors

Application of GLP-1(7-36)amide produces an increase in $[Ca^{2+}]_i$ which can be envisaged to activate the phospholipase C enzyme (Gromada *et al.*, 1995*a*). In addition to the generation of Ins(1,4,5)P_3, the hydrolysis of phosphatidylinositol 4,5-bisphosphate by the enzyme also leads to the production of 1,2-diacylglycerol. Diacylglycerol is the endogenous activator of PKC (Nishizuka, 1992) and we therefore investigated whether pharmacological modulation of this kinase influences the desensitization properties of the GLP-1 receptors. Such studies were prompted by the observations that PKC activity has been reported to modulate agonist-induced Ca^{2+} mobilization at the level of the plasma membrane receptors in the exocrine pancreas (Willems *et al.*, 1993). Indeed, activation of PKC by pretreatment of the cells with PMA markedly reduced the amplitude of the $[Ca^{2+}]_i$-transients that could subsequently be elicited by GLP-1(7-36)amide. Moreover, staurosporine and Ro31-8220 slowed desensitization and increased the duration of the $[Ca^{2+}]_i$-transient elicited by GLP-1(7-36)amide. We interpret these findings in terms of PKC modulating the GLP-1 receptor rather than affecting the intracellular Ca^{2+}-release mechanisms because in the cells exposed to the PKC-inhibitors, the amplitude of the GLP-1(7-36)amide-evoked $[Ca^{2+}]_i$-transient was unaffected. However, it is important to emphasize that the observed PKC-dependent changes of the duration of the GLP-1(7-36)amide-induced $[Ca^{2+}]_i$-transient do not necessarily imply that the rate of desensitization is affected. It is equally possible that PKC interferes with cellular Ca^{2+}-handling. For example, activation of PKC by PMA has been demonstrated to accelerate Ca^{2+}-extrusion across the plasma membrane (Berggren *et al.*, 1989). Such an effect would account for the lengthening of the GLP-1(7-36)amide-induced $[Ca^{2+}]_i$-transient in the presence of the PKC-inhibitors. Alternatively, there are two components of GLP-1 receptor desensitization: a rapid PKC-independent mechanism and a slower PKC-dependent process (cf. Barr & Watson, 1994). Such a concept would account for the prolongation of the GLP-1(7-36)amide-induced $[Ca^{2+}]_i$-transient observed after treatment with PKC-inhibitors (Figure 3).

PKC-independent mechanisms of desensitization

Although activation of PKC clearly can influence the desensitization of the GLP-1 receptors, it is obvious that there must be additional mechanisms of desensitization utilized by GLP-1. This is suggested by the observations: (1) that desensitization persists in the presence of maximally active concentrations of the PKC-inhibitor staurosporine and (2) that a fraction (25%) of the response to GLP-1(7-36)amide is resistant to pretreatment with PMA (Figure 3b). In the latter case, the GLP-1(7-36)amide-induced increase in $[Ca^{2+}]_i$ desensitizes with roughly the same time course as in the untreated control cells. The PKC-independent desensitization does not involve activation of PKA as suggested by the failure of forskolin to influence the $[Ca^{2+}]_i$-responses (Figure 6a). Likewise, we can exclude the involvement of Ca^{2+}-activated kinases and/or phosphatases as an increase in $[Ca^{2+}]_i$ is not required for desensitization to occur (Figure 6b). It is important to point out that desensitization as shown by a decreased amplitude of the $[Ca^{2+}]_i$-transient is not a consequence of depletion of intracellular Ca^{2+}-stores since the GLP-1(7-36)amide-induced $[Ca^{2+}]_i$-increase is not diminished in cells which have already been exposed to forskolin which produce a $[Ca^{2+}]_i$-transient of the same magnitude as GLP-1(7-36)amide itself. It thus appears that refilling of the intracellular Ca^{2+}-stores require only a few minutes for completion, whereas the recovery of the GLP-1 receptors from desensitization requires up to 1 h.

What then governs the PKC-independent desensitization of the GLP-1 receptor? The desensitization of the β-adrenoceptor has been demonstrated to result from its phosphorylation by β-adrenoceptor kinase (βARK) (Sibley *et al.*, 1986; Hausdorff *et al.*, 1990). The same kinase also appears to regulate the desensitization of the receptors for substance P (Mayor *et al.*, 1987). It is tempting to speculate that βARK, or at least a functionally related kinase, also control the PKC-independent desensitization of the GLP-1 receptor. It is of interest that vasopressin has been shown to elicit transient $[Ca^{2+}]_i$-responses in insulin secreting cells (Hughes *et al.*, 1992) similar to those we report here for GLP-1(7-36)amide. It is therefore tempting to speculate that the cellular processes that control desensitization of peptide receptors are the same.

Resensitization

The reappearance of functional GLP-1 receptors in the plasma membrane following exposure to and subsequent washout of GLP-1(7-36)amide is half-maximal within 20 min and requires up to 60 min for full recovery. This time course is similar to that of the recycling of internalized GLP-1 receptors or the slow dissociation of GLP-1 from the receptor (Widmann *et al.*, 1995). We emphasize again that the observed time course of resensitization is not simply the reflection of the time required for the refilling of the intracellular Ca^{2+}-stores which is completed within 4–5 min (Figure 6a).

Significance for pharmacotherapy of type 2 diabetes

Desensitization and resensitization of the GLP-1 receptor and the cellular signalling pathways involved are of relevance for the understanding of the insulinotropic action of GLP-1 in type 2 (non-insulin-dependent) diabetics. It is possible that the elevated GLP-1 concentrations found in patients with this form of diabetes (Ørskov *et al.*, 1991) may cause a reduced responsiveness to GLP-1 resulting in a reduced secretory capacity of the 'diabetic' β-cell. In this respect it would obviously be interesting to develop a GLP-1 analogue, which causes receptor activation but not receptor desensitization and internalization.

This work was supported in part by the Danish Natural Research Council (S.D.).

References

ÄMMÄLÄ, C., ASHCROFT, F.M. & RORSMAN, P. (1993). Calcium-independent potentiation of insulin release by cyclic AMP in single β-cells. *Nature*, **363**, 356–358.

BARR, A.J. & WATSON, S.P. (1994). Protein kinase C mediates delayed inhibitory feedback regulation of human neurokinin type 1 receptor activation of phospholipase C in UC11 astrocytoma cells. *Mol. Pharmacol.*, **46**, 266–273.

BERGGREN, P., ARKHAMMAR, P. & NILSSON, T. (1989). Activation of protein kinase C assists insulin producing cells in recovery from raised cytoplasmic Ca^{2+} by stimulating Ca^{2+} efflux. *Biochem. Biophys. Res. Commun.*, **165**, 416–421.

CULLINAN, C.A., BRADY, E.J., SAPERSTEIN, R. & LEIBOWITZ, M.D. (1994). Glucose-dependent alterations of intracellular free calcium by glucagon-like peptide-$1_{(7\text{-}36\text{amide})}$ in individual ob/ob mouse β-cells. *Cell Calcium*, **15**, 391–400.

DRUCKER, D.J., PHILIPPE, J., MOJSOV, S., CHICK, W. & HABENER, J.F. (1987). Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 3434–3438.

EFRAT, S., LINDE, S., KOFOD, H.,SPECTOR, D., DELANNOY, M., GRANT, S., HANAHAN, D., BAEKKESKOV, S. (1988). Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 9037–9041.

FEHMANN, H.C. & HABENER, J.F. (1991). Homologous desensitization of the insulinotropic glucagon-like peptide (7-37) receptor on insulinoma (HIT-T15) cells. *Endocrinology*, **128**, 2880–2888.

FEHMANN, H.C. & HABENER, J.F. (1992). Insulinotropic glucagon-like peptide-1(7-37)/(7-36)amide a new incretin hormone. *Trends Endocrinol. Metab.*, **3**, 158–163.

GEFEL, D., HENDRICK, G.J., MOJSOV, S., HABENER, J. & WEIR, G.C. (1990). Glucagon-like peptide-I analogs: effects on insulin secretion and adenosine 3′,5′-monophosphate formation. *Endocrinology*, **126**, 2164–2168.

GROMADA, J., DISSING, S., BOKVIST, K., RENSTRÖM, E., FRØKJÆR-JENSEN, J., WULFF, B.S. & RORSMAN, P. (1995*a*). Glucagon-like peptide 1 increases cytoplasmic calcium in insulin-secreting βTC3 cells by enhancement of intracellular calcium mobilization. *Diabetes*, **44**, 767–774.

GROMADA, J., RORSMAN, P., DISSING, S. & WULFF, B.S. (1995*b*). Stimulation of the cloned human glucagon-like peptide 1 receptor expressed in HEK 293 cells induces cAMP-dependent activation of calcium-induced calcium release. *FEBS Lett.*, **373**, 182–186.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

GÖKE, R. & CONLON, J.M. (1988). Receptors for glucagon-like peptide-1(7-36) amide on rat insulinoma-derived cells. *J. Endocrinol.*, **116**, 357–362.

GÖKE, R., WANGER, B., FEHMANN, H. & GÖKE, B. (1993). Glucose-dependency of the insulin stimulatory effect of glucagon-like peptide-1 (7-36) amide on the rat pancreas. *Res. Exp. Med.*, **193**, 97–103.

HAUSDORFF, W.P., CARON, M.G. & LEFKOWITZ, R.J. (1990). Turning off the signal: desensitization of β-adrenergic receptor function. *FASEB J.*, **4**, 2881–2889.

HOLZ, G.G., KÜHTREIBER, W.M. & HABENER, J.F. (1992). Pancreatic beta-cells are rendered glucose-competent by the insulinotropic hormone glucagon like peptide-1(7-37) *Nature*, **361**, 362–365.

HUGHES, S.J., CARPINELLI, A., NIKI, I., NICKS, J.L. & ASHCROFT, S.J.H. (1992). Stimulation of insulin release by vasopressin in the cloned β-cell line, HIT-T15: the role of protein kinase C. *J. Mol. Endocrinol.*, **8**, 145–153.

LU, M., WHEELER, M.B., LENG, X. & BOYD, A.E. (1993). The role of the free cytosolic calcium level in β-cell signal transduction by gastric inhibitory polypeptide and glucagon-like peptide I(7-37). *Endocrinology*, **132**, 94–100.

MAYOR, F., BENOVIC, J.F., CARON, M.G. & LEFKOWITZ, R.J. (1987). Somatostatin induces translocation of the β-adrenergic receptor kinase and desensitizes somatostatin receptors in S49 lymphoma cells. *J. Biol. Chem.*, **262**, 6468–6471.

MONTROSE-RAFIZADEH, C., EGAN, J.M. & ROTH, J. (1994). Incretin hormones regulate glucose-dependent insulin secretion in RIN 1046-38 cells: mechanisms of action. *Endocrinology*, **135**, 589–594.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature*, **308**, 693–698.

NISHIZUKA, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science*, **258**, 607–614.

ØRSKOV, C., JEPPESEN, J., MADSBAD, S. & HOLST, J.J. (1991). Proglucagon products in plasma of noninsulin-dependent diabetics and nondiabetic controls in the fasting state and after oral glucose and intravenous arginine. *J. Clin. Invest.*, **87**, 415–423.

RAE, J., COOPER, K., GATES, P. & WATSKY, M. (1991). Low access resistance perforated patch recordings using amphotericin B. *J. Neuorsci. Methods*, **37**, 15–26.

SIBLEY, D.R., STRASSER, R.H., BENOVIC, J.L., DANIEL, K. & LEFKOWITZ, R.J. (1986). Phosphorylation/dephosphorylation of the β-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 9408–9412.

SUZUKI, S., KAWAI, K., OHASHI, S., MUKAI, H. & YAMASHITA, K. (1989). Comparison of the effects of various C-terminal and N-terminal fragment peptides of glucagon-like peptide-1 on insulin and glucagon release from the isolated perfused rat pancreas. *Endocrinology*, **125**, 3109–3114.

TAMAOKI, T., NOMOTO, H., TAKAHASHI, I., KATO, Y., MORIMOTO, M. & TOMITA, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase. *Biochem. Biophys. Res. Commun.*, **135**, 397–402.

THORENS, B. (1992). Expression cloning of the pancreatic β cell receptor for the gluco-incretin hormone glucagon-like peptide-1. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 8641–8645.

THORENS, B. & WAEBER, G. (1993). Glucagon-like peptide-1 and the control of insulin secretion in the normal state and in NIDDM. *Diabetes*, **42**, 1219–1225.

WIDMANN, C., DOLCI, W. & THORENS, B. (1995). Agonist-induced internalization and recycling of the glucagon-like peptide-1 receptor in transfected fibroblasts and in insulinomas. *Biochem. J.*, **310**, 203–214.

WILLEMS, P.H.G.M., VAN HOOF, H.J.M., VAN MACKELENBERGH, M.G.H., HOENDEROP, J.G.J., VAN EMST-DE VRIES, S.E. & DE PONT, J.J.H.H.M. (1993). Receptor-evoked Ca^{2+} mobilization in pancreatic acinar cells: evidence for a regulatory role of protein kinase C by a mechanism involving the transition of high-affinity receptors to a low-affinity state. *Pflügers Arch.*, **424**, 171–182.

YADA, T., ITOH, K. & NAKATA, M. (1993). Glucagon-like peptide-1-(7-36)amide and a rise in cyclic adenosine 3′,5′-monophosphate increase cytosolic free Ca^{2+} in rat pancreatic β-cells by enhancing Ca^{2+} channel activity. *Endocrinology*, **133**, 1685–1692.

(Received January 2, 1996
Revised February 11, 1996
Accepted February 14, 1996)