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Ca^{2+}-independent insulin exocytosis induced by α-latrotoxin requires latrophilin, a G protein-coupled receptor

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α-Latrotoxin (α-LTX) induces exocytosis of small synaptic vesicles (SSVs) in neuronal cells both by a calcium-independent mechanism and by opening cation-permeable pores. Since the basic molecular events regulating exocytosis in neurons and endocrine cells may be similar, we have used the exocytosis of insulin-containing large dense core vesicles (LDCVs) as a model system. In primary pancreatic β-cells and in the derived cell lines INS-1 and MIN6, α-LTX increased insulin release in the absence of extracellular calcium, but the insulin-secreting cell lines HIT-T15 and RINm5F were unresponsive. α-LTX did not alter membrane potential or cytosolic calcium, and its stimulatory effect on exocytosis was still observed in pre-permeabilized INS-1 cells kept at 0.1 μM Ca^{2+}. Consequently, pore formation or ion fluxes induced by α-LTX could be excluded. The Ca^{2+}-independent α-LTX-binding protein, latrophilin, is a novel member of the secretin family of G protein-coupled receptors (GPCR). Sensitivity to α-LTX correlated with expression of latrophilin, but not with synaptotagmin I or neurexin Iα expression. Moreover, transient expression of latrophilin in HIT-T15 cells conferred α-LTX-induced exocytosis. Our results indicate that direct stimulation of exocytosis by a GPCR mediates the Ca^{2+}-independent effects of α-LTX in the absence of altered ion fluxes. Therefore, direct regulation by receptor-activated heterotrimeric G proteins constitutes an important feature of the endocrine exocytosis of insulin-containing LDCVs and may also apply to SSV exocytosis in neurons.

Keywords: calcium/exocytosis/insulin/latrophilin/α-latrotoxin

Introduction

The black-widow spider (*Latrodectus tredecimguttatus*) neurotoxin α-latrotoxin (α-LTX) is known to bind specifically to presynaptic neuronal membranes (Valtorta *et al.*, 1984) and to cause extensive neurotransmitter release from small synaptic vesicles (SSVs) (Grasso *et al.*, 1980). α-LTX induces exocytosis by two mechanisms. First, massive influx of cations including Ca^{2+} occurs through non-specific cation-permeable pores (Rosenthal and Meldolesi, 1989; Rosenthal *et al.*, 1990). Secondly, α-LTX stimulates exocytosis when Ca^{2+} is omitted from extracellular solutions (Meldolesi *et al.*, 1984). This indicates an alternative, Ca^{2+}-independent mechanism which may, however, require other divalent cations (Rosenthal *et al.*, 1990).

Purification of α-LTX-binding sites led to the discovery of neurexins, a highly polymorphic family of neuronal cell surface proteins (Ushkaryov *et al.*, 1992). Neurexin Iα constitutes a specific high-affinity binding site for α-LTX (Davletov *et al.*, 1995) and physically interacts with the vesicle protein synaptotagmin I (Hata *et al.*, 1993). The role of synaptotagmin as a calcium sensor in neuronal exocytosis has been demonstrated through its molecular characterization and extensive genetic analysis (Littleton and Bellen, 1995; Sudhof, 1995). These findings suggested that α-LTX may cause neuroexocytosis by direct activation of synaptotagmin via neurexin, thus bypassing the requirement for Ca^{2+} to activate synaptotagmin. However, binding of α-LTX to neurexin Iα requires Ca^{2+} (Davletov *et al.*, 1995), whereas the toxin is known to bind and elicit its effects on neuroexocytosis also in the absence of extracellular Ca^{2+} (Meldolesi *et al.*, 1984). In addition, synaptotagmin I-deficient mice remain sensitive to α-LTX, indicating that the toxin can exert its effect in the absence of neurexin–synaptotagmin interactions (Geppert *et al.*, 1994). More recently, a novel α-LTX-binding protein was purified, termed latrophilin, which does not require Ca^{2+} for its interaction with the toxin (Davletov *et al.*, 1996; Krasnoperov *et al.*, 1996). Latrophilin has been cloned and shown to be a G protein-coupled receptor (GPCR) of the secretin family (Krasnoperov *et al.*, 1997; Lelianova *et al.*, 1997). The immediate control of exocytosis by inhibitory GPCRs has been shown in insulin secretion (Lang *et al.*, 1993, 1995). These observations raise the possibility that GPCR-mediated direct control of exocytosis constitutes a general mechanism.

As in neuroexocytosis, the endocrine release of insulin occurs by exocytotic fusion with the plasma membrane and is triggered by a rise in intracellular calcium (Theler *et al.*, 1992; Wollheim *et al.*, 1996). Molecular dissection of the exocytotic process in yeast, flies and mammals has prompted the SNARE hypothesis to account for the detailed molecular events (Rothman, 1996). Vesicle docking and fusion require the assembly of specific proteins into a multisubunit complex. The functional role of several of its components has also been demonstrated in the endocrine β-cells including synaptobrevin/VAMP (Regazzi *et al.*, 1995; Wheeler *et al.*, 1996), syntaxin (Martin *et al.*, 1995; Lang *et al.*, 1997a), α-SNAP (Kiraly-Borri *et al.*, 1996), SNAP-25 (Sadoul *et al.*, 1995) and synaptotagmin (Lang *et al.*, 1997b). Thus, the general exocytotic machinery seems to be conserved in endocrine

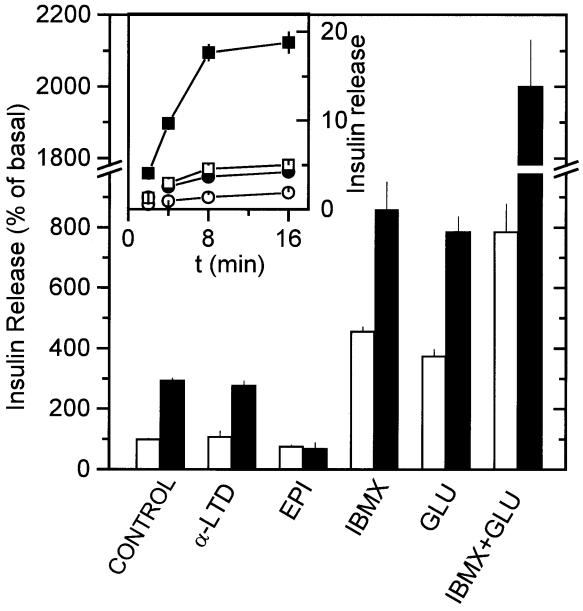

Fig. 1. α-Latrotoxin induces insulin release in INS-1 cells. INS-1 cells grown in plastic microwells (10^5 cells/well) were washed twice with KRBH and subsequently exposed for 16 min to the following agents in KRB in the absence (open bars) or presence (filled bars) of 4 nM α-LTX: 2 mM glucose (CON), α-latrodectin 10 nM (LTD), epinephrine 10 μM (EPI), 0.1 mM IBMX/10 μM forskolin (IBMX) and 20 mM glucose in the absence (GLU) or presence of 0.1 mM IBMX/10 μM forskolin (IBMX + GLU). Data were obtained from three separate experiments with distinct cell passages and values were normalized to insulin release at 2 mM glucose in the absence of α-LTX. n = 9–12 for each condition; *$2P<0.05$ as compared with the absence of α-LTX. Inset: time dependence of α-LTX effect. Cells kept on ice were exposed to 2 mM glucose (circles) or 0.1 mM IBMX/10 μM forskolin (squares) in the absence (open symbols) or presence (closed symbols) of 4 nM α-LTX. Cells subsequently were shifted to 37°C for the indicated time period. Values are expressed as insulin secreted as a percentage of cell content. *$2P<0.05$ as compared with the absence of α-LTX. n = 4 from two independent experiments.

and neuronal cells, and the basic regulation of vesicle docking and fusion may be similar.

In view of their well-characterized physiology and ease of culture, endocrine cells could serve as a convenient model to study general features of the release machinery. We therefore decided to investigate whether α-LTX stimulates insulin exocytosis in pancreatic β-cells. Our data demonstrate that α-LTX evokes Ca^{2+}-independent exocytosis without induction of cation fluxes. This provides a versatile model for the study of α-LTX action. Moreover, sensitivity to α-LTX required the expression of the GPCR latrophilin, but not of neurexin Iα or synaptotagmin I in clonal β-cell lines. Finally, transient expression of latrophilin in HIT-T15 cells conferred α-LTX-stimulated insulin release. This lends further support to the role of heterotrimeric G proteins in the receptor-mediated direct control of exocytosis.

Results

α-LTX induces Ca^{2+}-independent insulin secretion from primary and clonal β-cells

α-LTX induced a >2-fold increase of basal insulin release in the highly differentiated clonal β-cell line INS-1 (Figure 1). Insulin release is positively regulated in β-cells by glucose, which induces a rise in cytosolic Ca^{2+} (Theler *et al.*, 1992; Wollheim *et al.*, 1996), and can be potentiated further by raising cAMP levels (Gillis and Misler, 1993). As can be seen in Figure 1, the addition of 20 mM glucose to INS-1 cells increased insulin secretion, an effect that was markedly enhanced by the cyclic nucleotide phosphodiesterase inhibitor IBMX. α-LTX enhanced the release evoked by 20 mM glucose, 0.1 mM IBMX or the combination of glucose and IBMX. A similar effect was observed with BWSV, the crude venom gland extract of black-widow spiders. BWSV (diluted 1:200) increased basal and glucose-stimulated insulin release by 322 ± 26% (n = 4, $2P< 0.01$) and 289 ± 42% (n = 4, $2P<0.01$), respectively. To investigate further the specificity of α-LTX action, we determined whether the observed stimulation could be inhibited by activation of receptors known to inhibit insulin exocytosis (Lang *et al.*, 1995). Indeed, the addition of 10 μM epinephrine completely abolished the effect of the toxin (Figure 1). Moreover, we directly tested the effect of latrodectin (LTD), a low molecular weight component of unknown function commonly found in preparations of α-LTX and which shares structural homology with crustacean hyperglycaemic hormone (Gasparini *et al.*, 1994). Recombinant α-LTD was incapable of causing secretion of insulin on its own or in conjunction with α-LTX itself (Figure 1). The time course of the α-LTX effect is shown in the insert of Figure 1. α-LTX caused a rapid rise in insulin secretion, which was already significant after 2 min and largely complete within 8 min. Although the effect of α-LTX on insulin release may appear modest as compared with neurotransmitter release in PC12 cells or neurons (Meldolesi, 1982; Meldolesi *et al.*, 1983), it should be noted that endocrine secretion always proceeds at a slower rate than neuroexocytosis (Heidelberger *et al.*, 1994; Bokvist *et al.*, 1995; Eliasson *et al.*, 1997).

In neuronal preparations, α-LTX elicits its effect at pico- to nanomolar concentrations (Grasso *et al.*, 1980; Rosenthal and Meldolesi, 1989; Grasso and Mercanti Ciotti, 1993). We therefore tested the concentration dependency of the toxin at basal and stimulated insulin release from the well-differentiated INS-1 and MIN6 cells as well as the less differentiated cell lines RINm5F and HIT-T15 (Figure 2). α-LTX stimulated the basal release from primary β-cells and the derived cell lines INS-1 or MIN6, in both the absence and presence of extracellular Ca^{2+} (Figure 2, upper and middle panel). In the absence of Ca^{2+} the observed release was slightly reduced, which may be related to the fact that pre-incubation of cells with EGTA had already increased basal secretion by 50–70%. However, these data clearly demonstrate that the toxin effect is mediated by an α-LTX receptor, which is largely insensitive to Ca^{2+} in terms of toxin binding and effect on secretion. The toxin also induced a 3-fold increase in insulin secretion evoked by 20 mM glucose in primary cells or by 20 mM glucose/0.1 mM IBMX in INS-1 or MIN6 cells (Figure 2, lower panel). The effects were clearly present at 0.1 nM toxin under basal conditions and, in the case of stimulated secretion, were fully expressed at 1 nM with an EC_{50} of ~0.2 nM. Comparable results were obtained with toxin preparations from distinct commercial and non-commercial sources (see Materials and methods). In contrast, the hamster β-cell line HIT-T15 displayed

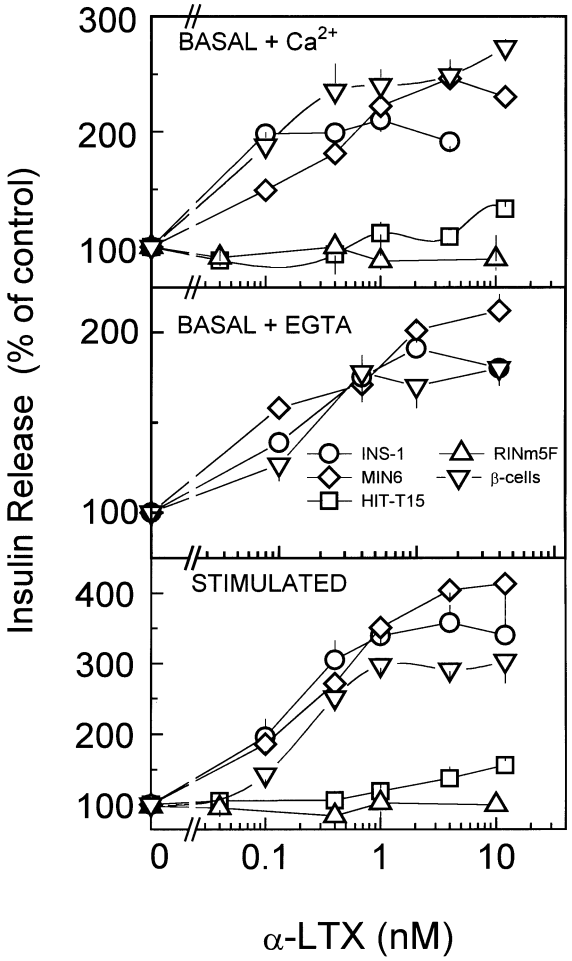

Fig. 2. Concentration dependence of α-LTX effects on insulin secretion in different clonal cell lines in the presence or absence of extracellular Ca^{2+}. Primary islet cells (▽), INS-1 (○), MIN6 (◇), HIT-T15 (□) or RINm5F (△) cells were tested for the effects of α-LTX at basal conditions with Ca^{2+} (upper panel: KRBH containing 3.4 mM Ca^{2+}) or without Ca^{2+} (middle panel: no Ca^{2+} but 1 mM EGTA added) or under stimulating conditions (lower panel: INS-1 and MIN6, 20 mM glucose + 0.1 mM IBMX; islet cells, 20 mM glucose; HIT-T15, leucine, glutamine and glucose 5 mM each; RINm5F, 20 mM alanine). Values were normalized to the release in the absence of α-LTX. n = 6–15 from 2 or 3 separate experiments.

only a marginal response, requiring concentrations of at least 10 nM (Figure 2), and rat insulinoma RINm5F cells were always unresponsive to the toxin.

Binding of α-LTX to insulin-secreting cells

To ensure that the observed secretory activity of α-LTX is associated with specific binding of the neurotoxin to the cell surface, we exposed cells to the toxin and subsequently added an antibody directed against α-LTX (Cattaneo and Grasso, 1986; Grasso and Mercanti Ciotti, 1993). As shown in Figure 3A, a patchy distribution of immunofluorescence was apparent in all cells of the toxin-sensitive insulin-secreting cell line INS-1. No staining was observed when α-LTX, but not the antibody directed against α-LTX, was omitted (Figure 3B). As evident under higher magnification, staining is confined to the plasma membrane (Figure 3C). Furthermore, α-LTX binding was not detected in HIT-T15 and RINm5F cells (data not shown). In contrast, α-LTX binding was also found in

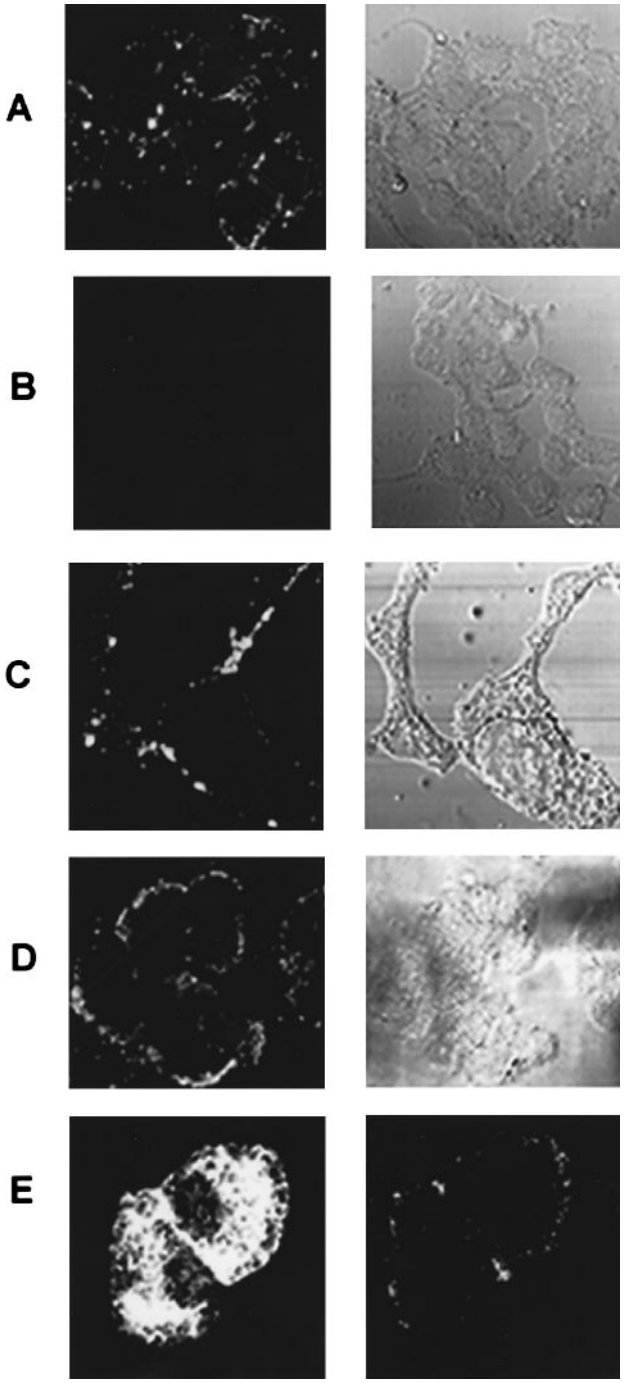

Fig. 3. α-LTX binds to INS-1 cells and primary β-cells. Cells seeded on coverslips were fixed briefly, exposed for 10 min to 10 nM α-LTX and subsequently exposed to a monoclonal antibody against α-LTX followed by incubation with a fluorescein-labelled second antibody. (**A**) INS-1E cells; (**B**) INS-1E cells exposed to buffer only in the absence of α-LTX and subsequently incubated with anti-α-LTX antibody; (**C**) INS-1E cells at higher magnification; (**D**) primary islet cells; (**E**) primary islet cells exposed to α-LTX, permeabilized and incubated with anti-α-LTX antibody and anti-insulin antibodies. Left panel: anti-insulin staining. Right panel: anti-α-LTX staining.

primary β-cells (Figure 3D). As preparations of primary pancreatic islet cells also contain non-β cells, the preparation was permeabilized and simultaneously stained for insulin (Figure 3E, left panel) and bound α-LTX (Figure 3E, right panel). The presence of both immunostains on

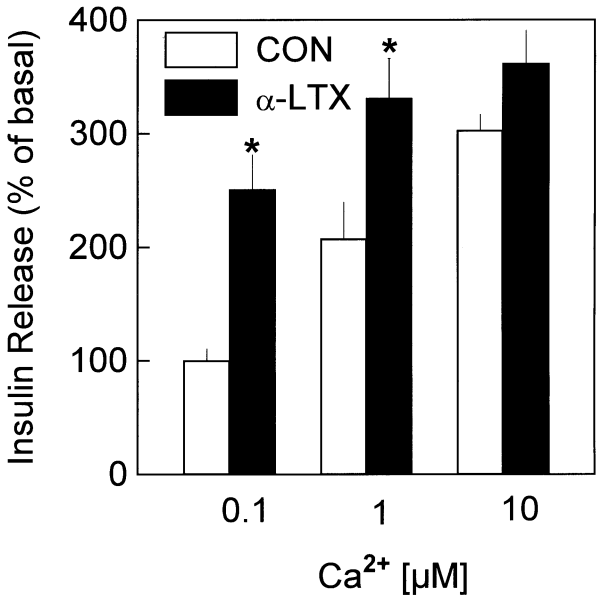

Fig. 4. α-LTX induces exocytosis in pre-permeabilized INS-1 cells. INS-1 cells in suspension were permeabilized with *Staphyloccocus aureus* α-toxin, washed and pre-equilibrated to the indicated Ca^{2+}-concentrations in the absence (open bars) or presence (filled bars) of 4 nM α-LTX at 4°C. Subsequently cells were shifted for 10 min to 37°C and secretion was terminated by centrifugation at 4°C. Data from three experiments (n = 6–9 for each point) were normalized to insulin release at 0.1 μM Ca^{2+} in the absence of α-LTX; *2P<0.05.

the same cells specifically demonstrates α-LTX binding to insulin-secreting β-cells.

α-*LTX induces exocytosis from cells permeabilized with Staphylococcus aureus* α-*toxin*

In neuronal cells, α-LTX causes a rapid, massive and almost complete release of neurotransmitters which is, at least in part, due to its hypothetical pore-forming activity and the resulting Ca^{2+} influx (Nicholls *et al.*, 1982; Sabirov *et al.*, 1993). The secretory effect observed here in insulin-secreting cells is clearly more modest, suggesting that pore formation may not be the main mechanism for inducing exocytosis in INS-1 and MIN6 cells. We therefore measured α-LTX effects in cells already permeabilized with staphylococcal haemolysin α-toxin, which creates pores in plasma membranes and allows the exchange of small molecules up to ~2 kDa (Ahnert Hilger *et al.*, 1993; Jonas *et al.*, 1994; Kiraly-Borri *et al.*, 1996; Maechler *et al.*, 1997). Under this condition, ionic gradients are abolished and the cytosolic Ca^{2+} is clamped to defined values by the use of Ca^{2+}/EGTA buffers. Permeabilized INS-1 cells secrete in response to levels of free Ca^{2+} >0.5 μM, and hormone release is maximally stimulated at 10 μM (Jonas *et al.*, 1994). As shown in Figure 4, permeabilized INS-1 cells responded with a 2- to 3-fold increase in insulin release when the cytosolic Ca^{2+} was raised from basal levels (0.1 μM Ca^{2+}) to 1 or 10 μM. Addition of α-LTX (4 nM) to permeabilized cells still caused an increase in insulin release (Figure 4). The effect was significant at 0.1 μM (basal) and 1 μM Ca^{2+}, whereas no significant effect of α-LTX was observed under maximal stimulation of secretion by Ca^{2+} (10 μM). Hence, insulin release induced by α-LTX does not require alterations in the levels of cytosolic Ca^{2+}.

α-*LTX does not alter cytosolic* Ca^{2+} *or plasma membrane potential*

The results from permeabilized cells do not exclude a contribution of pore formation by α-LTX to its effect on insulin secretion from intact cells. We therefore tested directly whether changes in cytosolic Ca^{2+} or pore formation might be observed in β-cell lines upon exposure to α-LTX. As shown in Figure 5, α-LTX did not alter cytosolic Ca^{2+} in INS-1 (Figure 5A) or HIT-T15 cells (Figure 5B) as measured with fura-2 in cell suspensions. Moreover, α-LTX did not modify the membrane potential in INS-1 cells (Figure 5C) as the fluorescence of the potential-sensitive probe bisoxonol remained unchanged. Clearly this neurotoxin does not alter the membrane integrity of insulin-secreting cells in suspension. As the secretion experiments were performed on cells in monolayers, we also examined whether α-LTX could have an effect on Ca^{2+} permeability only in attached cells. In the single cell recording from INS-1 cells shown (Figure 5D), glucose elicited a pronounced increase in cytosolic Ca^{2+} which was not affected further by α-LTX. Moreover, in single cell experiments where cells responded only by slow oscillatory rises in cytosolic Ca^{2+} after exposure to 20 mM glucose, α-LTX did not change this pattern and did not induce additional rises in cytosolic Ca^{2+} (n = 8, data not shown). Therefore α-LTX-induced non-specific ion channels, described in neuronal cells and model membranes, are not encountered in β-cells.

Expression of latrophilin, neurexin I and synaptotagmin I in insulin-secreting cells

The effect of α-LTX has been linked to three synaptic proteins, namely synaptotagmin I and two α-LTX-binding proteins, neurexin Iα and latrophilin. Synaptotagmin I immunoreactivity was present in all cell lines (Figure 6A) regardless of their toxin sensitivity. Expression of neurexins and latrophilin was examined by Northern blot and, as standard, a probe for cyclophilin was used (Figure 6B) that is ubiquitously expressed in all tissues (Lad *et al.*, 1991; Ryffel *et al.*, 1991). Neurexin Iα (Figure 6B, middle blot) was found in toxin-sensitive and -insensitive cell lines. It is noteworthy that the toxin-insensitive HIT-T15 cells expressed more neurexin Iα than rat brain. In the case of neurexin Iβ, high levels of mRNA were found in HIT-T15 and INS-1 cells but were undetectable in MIN6 cells (Figure 6B, middle blot). In contrast to synaptotagmin and neurexins, latrophilin mRNA is abundant only in brain and in those cell lines which responded well to α-LTX, i.e. INS-1 and MIN6 cells (Figure 6B, upper blot). Indeed, latrophilin mRNA expression in these cells was comparable with that in whole brain. Interestingly, a weak message was also detected in HIT-T15 cells, which responded marginally to very high concentrations of α-LTX (Figure 2). Latrophilin expression was also confirmed at the protein level after enrichment by wheat germ agglutinin (WGA) affinity chromatography (Figure 6C). Again, latrophilin was only found in INS-1 and MIN6 cells, and was almost undetectable in the toxin-insensitive HIT-T15 cells.

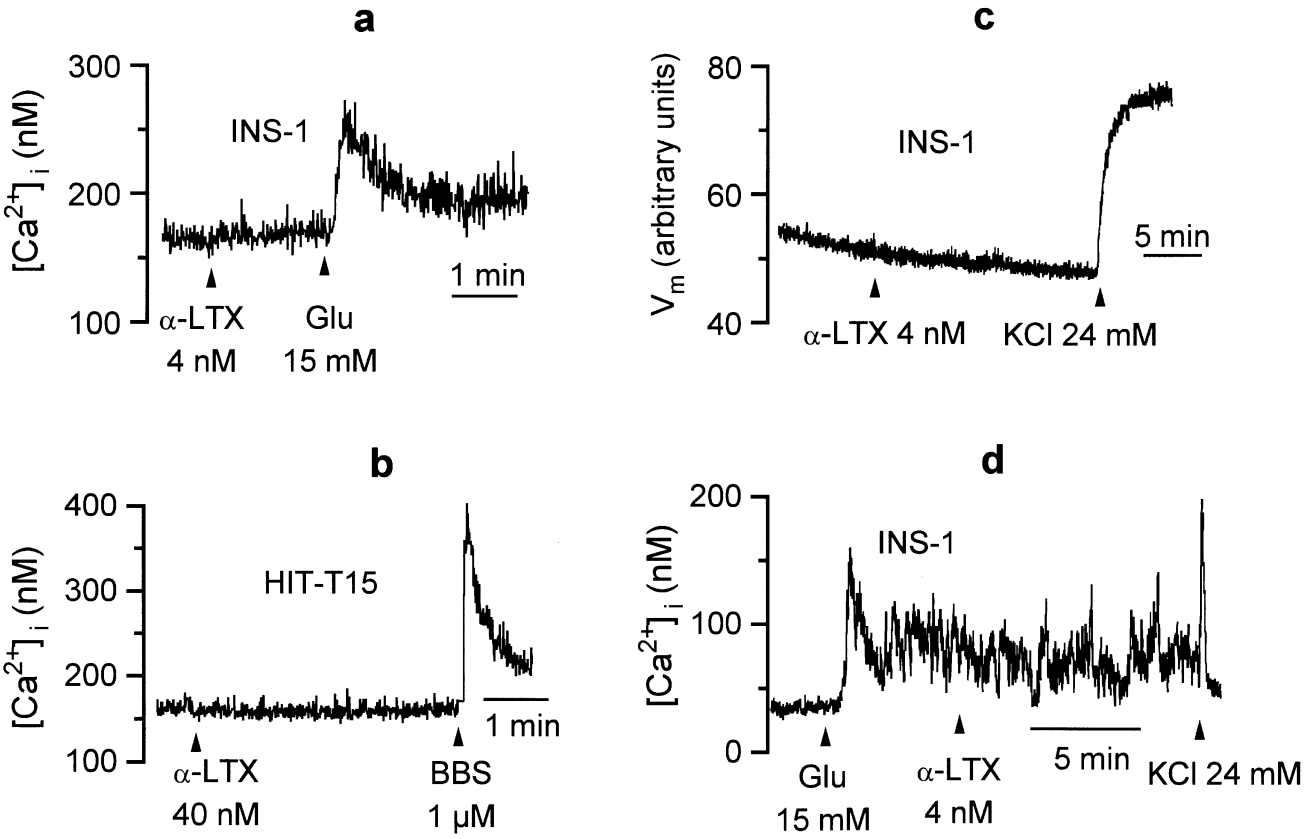

Fig. 5. α-LTX does not alter cytosolic Ca^{2+} or membrane potential. Cytosolic Ca^{2+} was measured with fura-2 in cells in suspension (a and b) or in single cells (d) and bisoxonol was used to determine changes in membrane potential (c). Additions of α-LTX, glucose (Glu), KCl or bombesin (BBS) are indicated by arrows. (**a**) Cytosolic Ca^{2+} of INS-1 cells in suspension; (**b**) cytosolic Ca^{2+} of HIT-T15 cells in suspension; (**c**) membrane potential of INS-1 cells in suspension; (**d**) single cell measurements of cytosolic Ca^{2+} in attached INS-1 cells. Representative traces from five (a, b and c) or 10 (d) independent experiments are shown.

Transient expression of latrophilin in HIT-T15 cells confers α-LTX-sensitive insulin release

Having established the correlation between latrophilin expression and α-LTX effects in clonal β-cells, we investigated whether expression of latrophilin in a toxin-insensitive cell line could confer insulin exocytosis inducible by α-LTX. To this end, we chose HIT-T15 cells for two reasons. First, HIT-T15 cells are largely toxin insensitive and express only minute amounts of latrophilin. Secondly, we have established previously a transient co-transfection system which permits the study of transgene effects on insulin secretion (Lang *et al.*, 1995, 1997a,b,c). This system relies on the use of human insulin C-peptide as a reporter gene for insulin release in a hamster cell line. Using the appropriate enzyme-linked immunosorbent assay (ELISA), insulin release can be measured exclusively from transiently transfected cells only, without interference by the endogenous hamster insulin C-peptide. To validate that latrophilin (LPH) is required for α-LTX effects, we co-transfected HIT-T15 cells with plasmids encoding human insulin (phINS) and pcDNA3/LPH containing the open reading frame of latrophilin (Lelianova *et al.*, 1997). Co-transfection with phINS and pCDNA3 was used as a control. α-LTX increased insulin release only in cells co-transfected with pcDNA3/LPH and phINS but not in control cells transfected with pcDNA3 and phINS (Figure 7). Similarly to the effect of α-LTX on INS-1 cells (see Figure 1), the toxin again potentiated insulin secretion induced by glucose.

Discussion

We have used the neurotoxin α-LTX to address the question of whether endocrine secretion of peptide hormones such as insulin from LDCVs shares regulatory features with neuroexocytosis. The binding of α-LTX to a high affinity site has been described to be specific for neuronal cell membranes (Valtorta *et al.*, 1984) and to constitute the first step leading to the release of neurotransmitters (Meldolesi, 1982). Here we report that the neurotoxin is also capable of binding specifically to membranes of primary β-cells and the well differentiated derived cell lines INS-1 and MIN6, but not to the less differentiated β-cell lines HIT-T15 and RINm5F. Therefore, the sensitivity towards this neurotoxin correlates with the degree of cellular differentiation and probably reflects the expression of a physiological process. Although we did not establish the binding kinetics, the demonstration of a functional effect at picomolar toxin concentrations compares well with published values (Meldolesi, 1982; Meldolesi *et al.*, 1983). Moreover, the action of the toxin could not be reproduced by LTD, the low molecular weight component often present in α-LTX preparations. In addition to its action on secretion, α-LTX is known to exert a cytotoxic effect. However, cytotoxicity requires high concentrations of toxin and depends on the massive influx of calcium (Watanabe *et al.*, 1983). We can rule out such a phenomenon, as exocytosis in INS-1 cells was evident at picomolar concentrations in the absence of ion

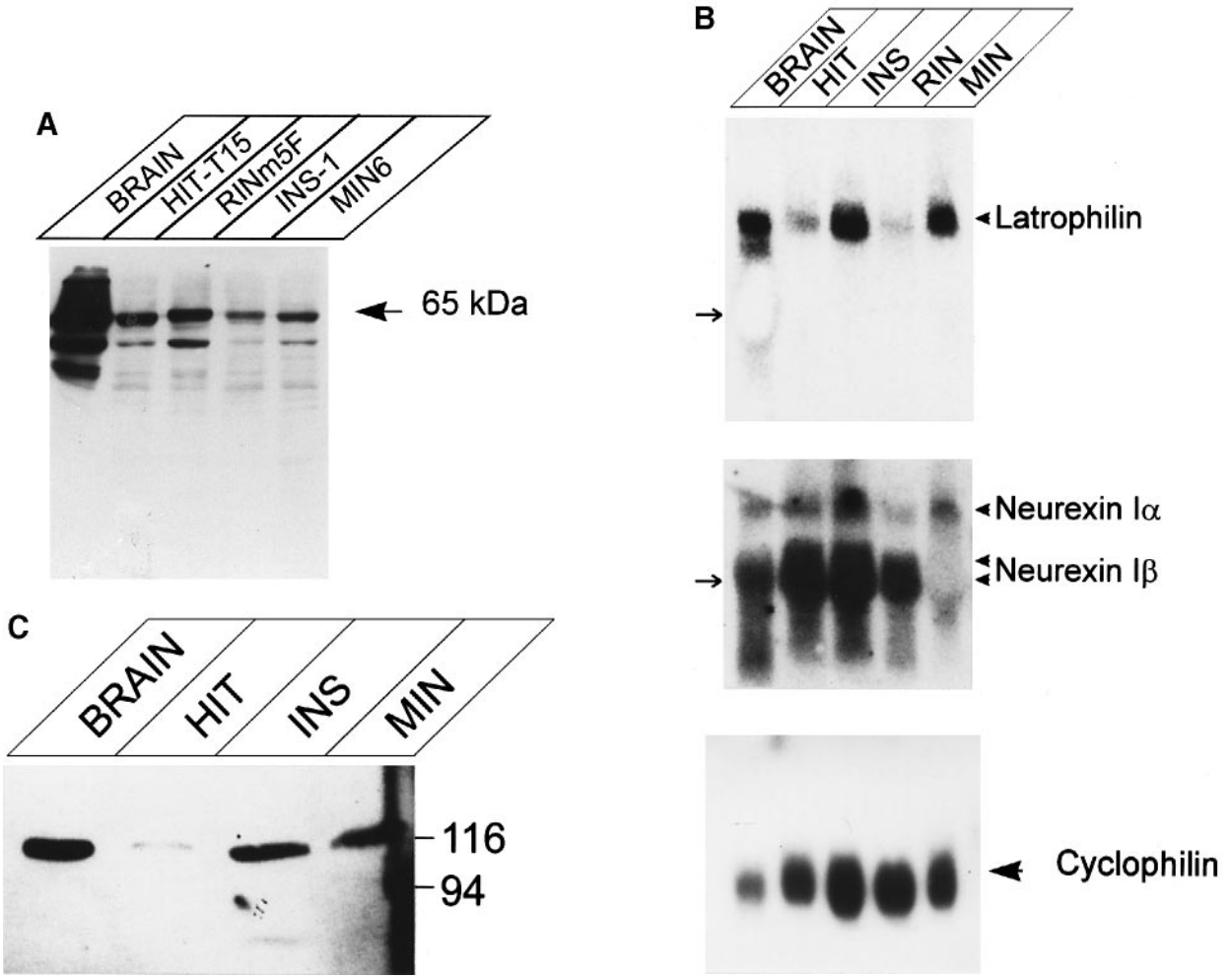

Fig. 6. Sensitivity to α-LTX correlates with expression of latrophilin, but not of synaptotagmin or neurexin. (**A**) Synaptotagmin I immunoreactivity in clonal β-cell lines. Crude membranes (30 μg/lane) from rat brain (BRAIN), RINm5F (RIN), HIT-T15 (HIT), MIN6 (MIN) or INS-1 cells (INS) were separated by SDS–PAGE and subsequently immunoblotted with antibodies against synaptotagmin (mab 41.1, 1:4000). (**B**) Northern blot analysis of neurexin and latrophilin expression. Total RNA (30 μg/lane) from whole rat brain or β-cell lines (as specified) was separated on agarose gel, transferred onto nylon membranes and hybridized with a probe for latrophilin (upper blot), neurexin Iα or neurexinIβ (middle blot) and for cyclophilin (lower blot), a ubiquitously expressed cytosolic protein. The positions of 18S and 28S rRNA are indicated by arrows. Densitometric measurements of latrophilin expression reveal a 2-fold increase in HIT-T15 cells and an 8-fold increase in brain, INS-1 and MIN6 in comparison with RINm5F. (**C**) Immunoblot of latrophilin. Lubrol extracts of crude membranes from rat brain or specified cell lines were enriched in latrophilin by affinity chromatography on WGA–Sepharose. Specifically eluted material subsequently was separated by SDS–PAGE and immunoblotted with anti-latrophilin antibody (dilution 1:500). Latrophilin expression in HIT-T15 cells is 13 times lower than in the brain and 11 times lower than in INS-1 cells. Expression in MIN6 cells was not fully proportional to the level of latrophilin message present; however, these cells still expressed 7.5 times more protein than HIT-T15 cells.

influx. Furthermore, the secretory response was inhibited by the receptor agonist epinephrine, a physiological regulator of insulin release (Lang *et al.*, 1995; Sharp, 1996).

α-LTX has been reported to stimulate not only exocytosis of neurotransmitter-containing SSVs but also that of LDCVs in neuroendocrine cells (Meldolesi *et al.*, 1986; Barnett *et al.*, 1996) and in peptide-releasing peripheral neurons (Waterman and Maggi, 1995; De Potter *et al.*, 1997). The peptide hormone insulin is released from LDCVs (Wollheim *et al.*, 1996), and the sensitivity of insulin exocytosis to α-LTX suggests the presence of a general mechanism. Several explanations have been proposed to account for the massive neuroexocytosis induced by the toxin. The opening of non-specific cation-permeable pores by the toxin profoundly changes the intracellular ion composition (Nicholls *et al.*, 1982; Robello *et al.*, 1987; Hurlbut *et al.*, 1994). Clearly the subsequent rapid rise in intracellular Ca^{2+} is capable of triggering neuroexocytosis. The most interesting feature of the toxin is, however, its capacity to elicit neuroexocytosis even in the absence of extracellular Ca^{2+} (Nicholls *et al.*, 1982). In fact, α-LTX-induced changes in cytosolic Ca^{2+} and stimulation of release could be partially dissociated by the use of functional antibodies against α-LTX (Pashkov *et al.*, 1993). This suggests an immediate mechanism of regulation of exocytosis. However, it remains a matter of debate whether the influx of cations other than Ca^{2+} may be responsible for the neurotransmitter release (Misler and Falke, 1987; Rosenthal *et al.*, 1990; Adam Vizi *et al.*, 1993; Hurlbut *et al.*, 1994). Our results indicate a direct regulation of exocytosis independently from alterations in cytosolic calcium or ion fluxes. First, α-LTX stimulated insulin release to a similar extent in the absence or presence of extracellular calcium.

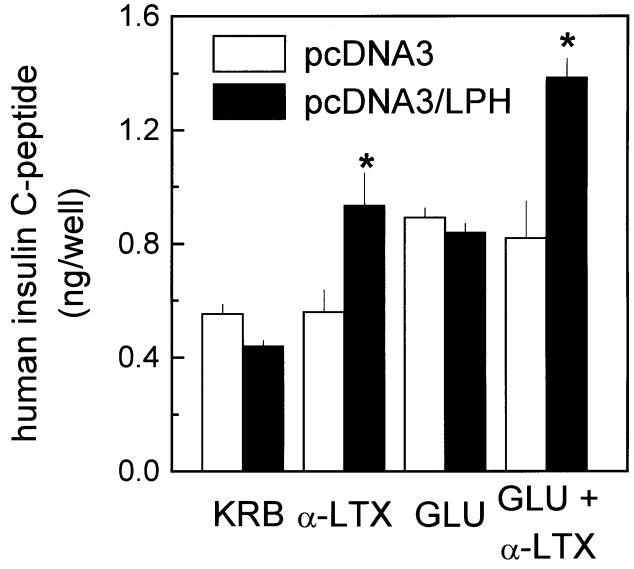

Fig. 7. Transient expression of latrophilin in HIT-T15 cells confers α-LTX-sensitive insulin release. HIT-T15 cells were co-transfected with reporter gene encoding human insulin and latrophilin encoding pcDNA3/LPH. After 48 h, cells were exposed for 16 min to Krebs–Ringer buffer (KRB), 1 nM α-LTX in KRB (α-LTX), 3 mM glucose (GLU) or glucose and 1 nM α-LTX in KRB (GLU + α-LTX). Transfection with pcDNA3/LPH did not change the content of the human insulin C-peptide as compared with the control (26.2 ± 3.6 versus 25 ± 1.3 pg/0.8×10^6 cells, respectively). n = 6–12 from three experiments.

Secondly, we could exclude changes in ion fluxes or non-specific membrane damage as a cause for insulin release, since these would have resulted in major alterations in the signal of the fluorescent indicators. Most importantly, the exocytotic response was still present after previous permeabilization of cells with α-toxin from *S.aureus*, which constitutes a true exocytotic model (Ahnert-Hilger *et al.*, 1989; Jonas *et al.*, 1994; Maechler *et al.*, 1997). It should be noted that the molecular size of α-LTX prevents its entry through the pores formed by *Staphylococcus* α-toxin (Sabirov *et al.*, 1993). This preparation excludes any contribution from channels created by α-LTX to the observed stimulation of exocytosis. The absence of pore-forming activity of α-LTX in insulin-secreting cells and the conserved toxin activity in permeabilized cells demonstrate for the first time the direct regulation of exocytosis by an α-LTX-induced process. A similar mechanism of action may underlie the increase of spontaneous and evoked transmitter release in hippocampal pyramidal cells observed at subnanomolar concentrations of α-LTX (Capogna *et al.*, 1996a,b). The occurrence of cation-independent toxin effects in β-cell lines may also explain the less pronounced stimulation of exocytosis reported here as compared with cerebellar granular cells (Cattaneo and Grasso, 1986) or PC-12 cells (Shoji Kasai *et al.*, 1994). However, in the latter cells, the omission of Ca^{2+} from the extracellular medium reduced the degree of neurohormone release to close to that observed in our system. Moreover, in the primary β-cells, insulin release was stimulated to the same degree by α-LTX as by glucose, the main regulator of insulin secretion. It should indeed be noted that endocrine secretion of LDCVs, as encountered in the β-cells, always proceeds at a slower rate than neuroendocrine secretion from chromaffin cells or melanotrophs and neuroexocytosis of synaptic vesicles (Heidelberger *et al.*, 1994; Proks *et al.*, 1996; Eliasson *et al.*, 1997).

We have delineated some of the protein components required for α-LTX action. The toxin is known to bind to neurexin Iα (Ushkaryov *et al.*, 1992; Davletov *et al.*, 1995), which in turn may interact with the carboxy-terminus of the vesicular Ca^{2+}-sensing protein synaptotagmin I (Petrenko *et al.*, 1991; Hata *et al.*, 1993). Surprisingly, we found appreciable and uniform expression of the Ca^{2+}-dependent α-LTX-binding protein neurexin Iα in all cell lines tested. In view of its distribution and the Ca^{2+} independence of α-LTX effects in INS-1 and MIN6 cells, we can exclude neurexin Iα as a functional receptor in exocytosis. Moreover, synaptotagmin I was always expressed in all cell lines, in contrast to previous reports (Jacobsson *et al.*, 1994; Wheeler *et al.*, 1996). However, the biological activity of α-LTX in clonal β-cells coincided with expression of the recently identified Ca^{2+}-independent α-LTX receptor latrophilin/CIRL (Ca^{2+}-independent receptor of α-LTX) (Davletov *et al.*, 1996; Krasnoperov *et al.*,1997; Lelianova *et al.*, 1997). We have not determined latrophilin expression directly in primary islet cell preparations by Northern blots. Indeed, these preparations always contain non-β cells which will skew the analysis. However, in the derived cell lines, α-LTX binding correlated with expression of latrophilin. We therefore assume that also in primary β-cells, binding of α-LTX (as demonstrated by secretion and by immunofluorescence) is most likely due to the expression of latrophilin. In addition, the observed EC_{50} of 0.2 nM in the toxin-sensitive cells correlated most closely with the reported K_d of 0.6 nM for toxin binding to purified latrophilin (Davletov *et al.*, 1996), whereas the K_d for α-LTX binding to neurexin Iα is one order of magnitude higher (Davletov *et al.*, 1995). Most importantly, transient expression of latrophilin/CIRL in the normally toxin-insensitive cell line HIT-T15 conferred α-LTX-induced insulin exocytosis. These findings clearly indicate a pivotal role for latrophilin/CIRL in mediating Ca^{2+}-independent α-LTX effects in the absence of pore formation in insulin-secreting cells.

The functional importance of latrophilin/CIRL in chromaffin cells has also been reported very recently (Krasnoperov *et al.*, 1997). Primary chromaffin cells respond to α-LTX, and the ensuing stimulation of exocytosis is strictly calcium-dependent (Barnett *et al.*, 1996; Krasnoperov *et al.*, 1997). Overexpression of latrophilin/CIRL considerably increased the sensitivity of LDCV exocytosis to α-LTX (Krasnoperov *et al.*, 1997). Although the toxin binding was Ca^{2+}-independent, as expected for latrophilin/CIRL, secretion required the presence of the cation in contradistinction to our results in clonal β-cells. These observations suggest a cell type-specific coupling of latrophilin/CIRL to downstream effectors: the effectors are Ca^{2+}-dependent in chromaffin cells (Barnett *et al.*, 1996; Krasnoperov *et al.*, 1997), but Ca^{2+}-independent in β-cells, as demonstrated here. In neurons, both forms are probably present (Nicholls *et al.*, 1982; Meldolesi *et al.*, 1984; Capogna *et al.*, 1996a,b).

Latrophilin/CIRL is a GPCR (Krasnoperov *et al.*, 1997;

Lelianova *et al.*, 1997). We and others have shown that GTP, which is generated by glucose in β-cells (Detimary *et al.*, 1996), induces insulin exocytosis even when levels of $[Ca^{2+}]_c$ are kept in the subnanomolar range (Vallar *et al.*, 1987; Jonas *et al.*, 1994; Proks *et al.*, 1996). It is of interest that the effect of GTP is independent of synaptotagmin (Lang *et al.*, 1997b) or of soluble cytosolic factors (Kiraly-Borri *et al.*, 1996). The nucleotide probably regulates a step subsequent to the action of Ca^{2+} (Proks *et al.*, 1996). It is tempting to speculate that α-LTX induces insulin exocytosis by interaction with such a mechanism and thereby bypasses the requirement for Ca^{2+}. Most of the receptors of the secretin/calcitonin family, to which latrophilin/CIRL belongs, bind biologically active peptides and participate in various secretion processes. Thus, α-LTX may mimic a natural ligand of latrophilin/CIRL. Such ligands could physiologically regulate exocytosis from both neuronal and endocrine cells by activating the receptor-mediated signal transduction mechanism coupled to components of the release sites. We have previously characterized such a direct receptor-mediated regulation for inhibitory neurohormones at a late step in LDCV exocytosis (Lang *et al.*, 1995). Future research should address the nature of the latrophilin effector(s) and the ligands that may participate in controlling the basic exocytotic functions of all secretory cells.

Materials and methods

Materials

BWSV and α-LTX were prepared as described in Grasso and Mastrogiacomo (1992) or Lelianova *et al.* (1997). In addition, a commercial source was used (Latoxan, Apt, France). The low molecular weight component LTD was expressed and purified from Sf9 cells (Pescatori *et al.*, 1995). All toxins were stored at –80°C, and different batches of toxins gave quantitatively comparable results. Antibody mab 41.1 against the first C2 domain of synaptotagmin I was kindly provided by Dr R.Jahn, New Haven, CT.

Insulin secretion, cell permeabilization and transient co-transfection

The insulin-secreting cell lines HIT-T15, RINm5F, MIN6 and INS-1 were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum as described previously (Asfari *et al.*, 1992; Sakurada *et al.*, 1993; Lang *et al.*, 1993, 1995). Cells cultured in microtitre plates were washed twice with Krebs–Ringer bicarbonate/HEPES (KRBH) supplemented with 0.1% bovine serum albumin (BSA). The cells were then incubated for 15 min in the presence of the indicated agents; subsequently supernatants were removed, centrifuged at 4°C and kept at –20°C until insulin assay (Lang *et al.*, 1995). Permeabilization was performed as described previously (Kiraly-Borri *et al.*, 1996; Maechler *et al.*, 1997; Lang *et al.*, 1997b) using *S.aureus* α-toxin and Ca^{2+}/EGTA buffers. Levels of free Ca^{2+} were determined with a microelectrode. HIT-T15 cells were transiently co-transfected as published (Lang *et al.*, 1995, 1997a,b,c), except for the use of 1 µl of Transfectam and 5 µg of cDNA/well (3 µg phINS encoding human insulin together with 2 µg of pcDNA3 or 2 µg of pcDNA3/LPH). pCDNA3/LPH encodes the open reading frame of latrophilin (Lelianova *et al.*, 1997) under the control of a cytomegalovirus promoter. At 48 h after co-transfection, secretion experiments were performed and human C-peptide determined by ELISA (DAKO, Zug, Switzerland).

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

Average membrane potential was measured by exposing cells to the fluorescent probe bisoxonol at a final concentration of 100 nM (Lang *et al.*, 1993). For determination of $[Ca^{2+}]_i$, cells were incubated for 30 min at 37°C with 1 µM fura-2/acetoxymethyl ester (Lang *et al.*, 1993). Measurements of cytosolic Ca^{2+} in single cells were performed as described (Theler *et al.*, 1992).

Immunofluorescence and immunoblots

Cells were grown on glass coverslips for 3 days. For α-LTX binding, they were washed three times for 5 min with KRBH, fixed for 2 min in 2% paraformaldehyde in KRBH, washed again three times and incubated for 20 min in KRBH/0.1% BSA with or without 10 nM α-LTX (Cattaneo and Grasso, 1986). After three washes with KRBH containing 5% BSA, cells were exposed for 1 h to anti-α-LTX antibody (mouse ascites, diluted 1:50 in KRBH/1% BSA) or permeabilized, blocked (Lang *et al.*, 1997b) and exposed to a rabbit polyclonal anti-insulin antibody (Linco) and a monoclonal anti-α-LTX antibody. Cells were subsequently washed three times for 5 min with KRBH/0.1% BSA and mounted on glass slides. Confocal microscopy was performed with a Zeiss Axiophot fluorescent microscope and a BioRad MRC-600 confocal scanner (Richmond, CA). SDS–PAGE and immunoblotting of crude membranes were performed as described (Lang *et al.*, 1995) using enhanced chemiluminescence (Amersham; Zurich, Switzerland) for detection. Latrophilin was enriched as described previously using Lubrol extracts of crude membranes and WGA affinity chromatography (Davletov *et al.*, 1996). A monoclonal antibody against synaptotagmin I (mab 41.1) was kindly provided by Dr R.Jahn (Goettingen, Germany).

Northern blot and digital imaging

Total RNA from specified cell lines or rat brain was separated by 1.5% agarose gels and electrotransferred to nylon membranes (Lang *et al.*, 1997b). Radioactively labelled probes used to detect messages were, respectively: (i) the insert from a full-length latrophilin clone (RBCR9-15; Lelianova *et al.*, 1997; DDBJ/EMBL/GenBank accession No. U78105); and (ii) a *Not*I fragment (1118–5095) of rat neurexin Iα cDNA hybridizing with neurexin Iα and Iβ.

The images of Figure 6B–D were captured using a Umax scanner (PowerLook II) and Adobe Photoshop software at a 1200×1200 d.p.i. resolution. For Northern blots, several autoradiographic exposures were made and scanned to determine the region of maximum linearity of film and scanner response. Those exposures were chosen in which the weak hybridization signals were above the film sensitivity limit and, at the same time, strong signals were not saturated. The digitized images were then transferred into SigmaGel gel analysis software (Jandel) and the bands compared using peak intensity or area measurements.

Statistical analysis

Results are presented as means ± SE from experiments performed on at least three independent cell preparations, unless otherwise stated. Statistical analysis was performed by Student's two-tailed *t*-test for unpaired data (2*P*).

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