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Colocalisation of insulin and IGF-1 receptors in cultured rat sensory and sympathetic ganglion cells

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ABSTRACT

Peripheral sensory and autonomic neurons are known to possess insulin receptors. These have been considered to be of the peripheral type, i.e. similar to those of hepatic and fat cells rather than of the brain type which show dual specificity for both insulin and insulin-like growth factor (IGF-1). We have examined the localisation of insulin and IGF-1 receptors in cultured sensory and sympathetic ganglion cells using confocal microscopy and indirect labelling with FITC (fluorescein isothiocyanate) and TRITC (tetramethyl rhodamine isothiocyanate) respectively. We have shown that in cultured U266B1 multiple myeloma cells these receptors display separate localisation, whereas they are colocalised in IM-9 lymphocytes which are known to possess hybrid receptors. We have confirmed the sequestration of insulin and IGF-1 receptors in the cytoplasm of sensory and sympathetic neurons, consistent with a brain-type receptor. The colocalisation of insulin and IGF-1 receptors in sensory and sympathetic ganglion cells is consistent with the view that they are hybrid receptors, similar to those present in the CNS. The function of these receptors, as suggested for the CNS, may be related to trophic support for neurons.

Key words: Neurotrophic support; diabetic neuropathy; hybrid receptors.

INTRODUCTION

Insulin and IGF-I are homologous growth promoting peptides (LeRoith & Roberts, 1993). Insulin is produced in the pancreas and regulates the metabolism of fats, carbohydrates and proteins. The main source of circulating IGF-1 is the liver but it is also produced locally in tissues. IGF-1 has broad growth promoting activity during development and it is considered to be an autocrine regulator of skeletal growth and protein metabolism and to possess local autocrine/paracrine activity (Delafontaine & Lou, 1993). A family of soluble peptides, the IGF binding proteins (IGFBPs) each specific for the IGFs, plays an important role in modulating activity, regulating circulating levels, sequestering, delivering and targeting of the IGFs (Conover & Powell, 1991; Rechler & Brown, 1992; Cohick & Clemmons, 1993; Rechler, 1993).

In the nervous system IGF-1 appears to regulate tissue growth, supporting differentiation of fetal neurons in culture, stimulating protein synthesis and promoting neurite outgrowth (Recio-Pinto et al. 1984; Ishii & Recio-Pinto, 1987; Werner et al. 1989, 1994; Mozell & McMorris, 1991; Heidenreich, 1993). Because of its ability to influence neural function, IGF-1 has been explored as a candidate for the treatment of neurological disorders. It is known that IGF-1 levels are reduced in human diabetes (Tan & Baxter, 1986; Arner et al. 1989) and in streptozotocin-diabetic rats (Phillips & Young, 1976) but this does not appear to be related to hyperglycaemia (Tan & Baxter, 1986). Insulin restores IGF-1 levels in rats (Taylor et al. 1987) and it appears to function via the IGFBPs (Gelato et al. 1992). In diabetic rats, full nerve fibre growth can be restored by continuous subcutaneous administration of insulin (McCallum et al. 1986) and infusion of IGF-1 can restore rat somatic growth (Scheiwiller et al. 1986) but it has not been established whether IGF-1 can also correct the deficit in growth in nerve fibre diameter (Sharma et al. 1981) although it is known that both IGF-1 and IGF-2 can ameliorate the impairment in sensory nerve regeneration (Ishii & Lupien, 1995).

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The receptors for insulin and IGF-1 are also structurally similar, synthesised as single chains that become glycosylated and cleaved to produce α and β subunits (Ulrich et al. 1986). The insulin receptor is a tetrameric transmembrane glycoprotein composed of 2 α/β subunit pairs covalently linked between the α chains via disulphide bridges (Morrison et al. 1988; Rosen, 1989). The α chains are extracellular and the β subunits have extracellular, transmembrane and cytoplasmic domains. The cytosolic domains of the β subunits are tyrosine kinases that become autophosphorylated on insulin binding (Goldfine, 1987). The receptor for IGF-1 is also a tetrameric protein with 2 distinct α/β subunit disulphide-linked pairs, spanning the membrane and possessing tyrosine kinase activity (Ullrich et al. 1986; Wilden et al. 1989). The highest levels of IGF-1 receptor expression are found during embryonic development but the receptor is continuously expressed in adult tissues (Garofalo & Rosen, 1989; Werner et al. 1989). Despite the structural similarity between the IGF-1 and insulin receptors, each binds to its specific ligand with 100 to 1000-fold higher affinity (Moxham et al. 1989). The kinetics of each receptor binding to its ligand are also different, suggesting distinct interactions and functions for each pair (DeMeyts et al. 1994). Studies report the existence of hybrid receptors composed of one half ($\alpha\beta$) insulin receptor polypeptide and the other half of ($\alpha'\beta'$) IGF-1. These receptors have been observed in cells such as NIH3T3 mouse fibroblasts, IM-9 lymphocytes, HepG2 hepatoma cells and human placental membranes (Moxham et al. 1989; Soos & Siddle, 1989).

Insulin and IGF-1 receptors are present in the central nervous system (Havrankova et al. 1978; Hill et al. 1986; Lowe & LeRoith, 1986; Adamo et al. 1989; Unger et al. 1991). Despite the blood-brain barrier insulin can reach the brain, probably via receptor-mediated transport in vascular endothelial cells (VanHouten et al. 1979; Woods et al. 1985). Although insulin does not control glucose utilisation in the central nervous system (CNS), it appears to have a growth promoting role (Puro & Agardh, 1984; Unger et al. 1991). Insulin receptors also exist in the peripheral nervous system (Waldbillig & LeRoith, 1987), in dorsal root and sympathetic ganglion cells (Llewelyn et al. 1988).

Structural differences exist between brain insulin receptors and nonneural peripheral tissue insulin receptors (Raizada et al. 1988). Brain type receptor subunits α and β appear to have lower molecular weights (Yip et al. 1980; Heidenreich et al. 1983; Hendricks et al. 1984), partly due to differences in carbohydrate moieties (Heidenreich & Brandenberg, 1986). Similarly, neuronal cells express the brain type IGF-1 receptor with lower molecular weight than the peripheral type receptor that is expressed by glial cells in the CNS (Burgess et al. 1987; Shemer et al. 1987). Altered kinetics of binding reported between the brain and peripheral type IGF-1 receptors have been regarded as indicative of a distinct function for IGF-1 in the CNS (Nielson, 1991). It has recently been shown that brain-type insulin receptors are in fact hybrids, consisting of an $\alpha\beta$ insulin receptor subunit and an $\alpha'\beta'$ IGF-1 receptor subunit (Moss & Livingston, 1993). Peripheral sensory and autonomic neurons also possess insulin receptors but based on the molecular weights of their subunits they have been considered homotetramers (peripheral type) (Waldbillig & LeRoith, 1987). However, various studies point towards brain-type behaviour of peripheral neuronal insulin receptors (Unger et al. 1991; James et al. 1993; Patel et al. 1993).

To shed more light in the controversy of the existence and localisation of insulin and IGF-1 receptors in the peripheral nervous system, we have used immunofluorescence and confocal microscopy to localise insulin and IGF-1 binding in primary neuronal cultures derived from dorsal root and superior cervical ganglia. We then compared the intracellular localisation of these peptides with those in a multiple myeloma cell line known to possess brain type hybrid receptors and with those in a lymphocyte cell line with peripheral type receptors for insulin and IGF-1.

MATERIALS AND METHODS

Hormones and antibodies

Human insulin was obtained from Boehringher Mannheim, East Sussex, UK and human recombinant IGF-1 was a gift from Cephalon Inc, USA. Polyclonal antibodies to insulin and IGF-1 were from Peninsula Laboratories Europe Ltd, Merseyside, UK, and are known not to cross-react with the other ligand. Secondary antibodies were from Jackson Immunoresearch Laboratories Inc, USA.

Preparation of neuronal culture substrates

Permanox chamber slides (NUNC, TCS Biologicals Ltd, Buckingham, UK) were coated with 0.5 mg/ml poly-D-lysine (Sigma) in 0.1 M borate buffer pH 8.4 (Sigma) for 1 h at room temperature. After 3 washes in tissue culture grade water (Sigma), they were aseptically dried and incubated with 30 μg/ml laminin

(Life Technologies Ltd, Paisley, UK) in Ca^{2+}-Mg^{2+}-free Hanks' balanced salt solution (CMF HBSS, Sigma) overnight at 4 °C (Orr & Smith, 1988). They were washed once in CMF HBSS and incubated with 0.3 mg/ml collagen III (Collagen Corporation, California, USA) in HBSS for 2 h at 37 °C, washed twice with HBSS, once in growth medium and filled with medium for cell plating.

Operative procedures

Sprague–Dawley rats were reared in the Comparative Biology Unit of the Royal Free Hospital School of Medicine. To obtain dorsal root ganglia (DRG), Sprague–Dawley rats aged 1–4 d were killed humanely by cervical dislocation and placed on ice. Dorsal root ganglia ($\sim$ 15) were dissected under aseptic conditions, removed, washed 3 times and placed in cold CMF HBSS. For superior cervical ganglia (SCG), Sprague–Dawley rats aged 1–4 d were killed by intraperitoneal injection of sodium pentobarbitone (Sagatal, veterinary grade, Rhone Merieux) administered at 6 mg/kg body weight. Neonates were then placed on ice. SCG (2 per neonate) were dissected, removed, washed 3 times and placed in cold CMF HBSS under aseptic conditions.

Dissociation and culturing of dorsal root and superior cervical ganglion neurons

Dissected ganglia were cleaned of connective tissue and washed in HBSS. They were then incubated in 0.2% collagenase type IV (Life Technologies) in HBSS for 20 min, followed by a brief wash and a further incubation with 0.25% trypsin (Sigma) in HBSS for 18 min (Millaruelo et al. 1988). Both enzyme digestions were performed at 37 °C. After rinsing in buffer, the trypsin reaction was arrested by immediate addition of culture medium DMEM/F12 (Sigma)-0.8% fatty-acid-free bovine serum albumin (FAF-BSA, Sigma). The ganglia were carefully triturated by 10 passages with glass pipettes and immediately plated on precoated multiwell chamber slides. The monolayer culture was incubated in a humid environment at 37 °C in 4% CO_2. The cells were maintained for up to 7 d in DMEM/F12 medium-0.8% FAF-BSA, 5µg/ml iron-rich transferrin, 0.625% glucose, 2 mM glutamine, 3×10^{-8} M sodium selenite, 100 iu/ml penicillin and 100 ng/ml NGF (modified from Dreyfus & Black, 1990; James et al. 1993) (all from Sigma). The growth media were changed on alternate days. DRG from 1 rat yielded 12 cultures, whereas SCG ganglia yielded 3–4 cultures.

Immunofluorescent labelling of insulin and IGF-1

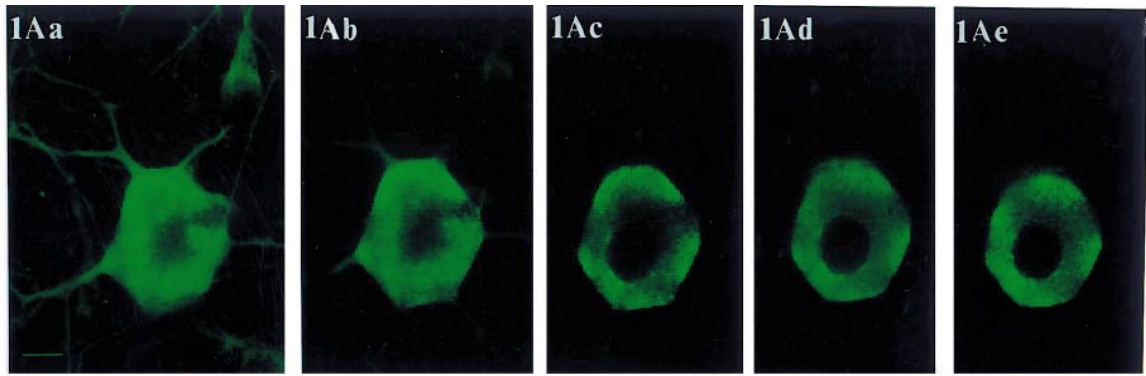
Cells in chamber slides were fixed in freshly prepared 4% paraformaldehyde-PBS pH 7.4 for 12 min at room temperature. Following 3 washings in PBS, cells were permeabilised in Simultest IMK Plus lysing solution (Becton Dickinson UK Ltd, Oxford, UK) for 15 min at room temperature ($<$ 1.5% formaldehyde-5% diethylene glycol-PBS). After 5 washes, cells were blocked in the universal blocker polyvinylpyrrolidone (PVP, MW: 40,000 Da, Sigma) (1% PVP-PBS) for 30 min. The blocking agent was drained and cells were incubated with 30 ng/ml IGF-1–0.1% PVP-PBS or with 20 ng/ml insulin-0.1% PVP-PBS for 70 min (Cowley & Pratten, 1993). After 3 washings, they were treated with guinea pig anti-insulin polyclonal antibody or rabbit anti-IGF-1 polyclonal antibody in 0.1% PVP-PBS (1:150 dilution) for 1 h. The antibodies are known not to crossreact with the other ligand. Following 3 washings, slides were incubated with antiguinea pig-FITC polyclonal antibody or antirabbit-FITC antibody, in 0.1% PVP-PBS (1:50 dilution) for 45 min. For 2-colour immunofluorescence, following 3 washes, cell nuclei were labelled with 2 µg/ml propidium iodide-PBS-100 µg/ml DNase-free RNase at 37 °C for 20 min. Slides were washed 3 times, mounted in Citifluor fluorescence preserver (Agar Scientific Ltd, Essex, UK) and sealed with nail varnish. Fluorescently labelled neuronal cultures were observed under an MRC 600 confocal microscope.

Colocalisation of insulin and IGF-1

Slides were washed in PBS, fixed, permeabilised and blocked as above. Wells were drained and incubated with 30 ng/ml IGF-1-CMF PBS-0.1% PVP for 70 min at room temperature, followed by 3 washes and a further incubation with 20 ng/ml insulin in the above buffer for 70 min. Cells were washed 3 times and incubated with anti-IGF-1 rabbit and guinea pig anti-insulin polyclonal antibodies (Peninsula) (1:150 dilution) in CMF PBS-0.1% PVP for 1 h. Following 4 washings, they were incubated in antiguinea pig-FITC antiserum and antirabbit-TRITC antiserum (1:50 dilution) in 0.1% PVP-PBS for 45 min. Coverslips were washed 3 times, mounted in Citifluor and sealed with nail varnish.

Cultures of U266B1 and IM-9 cell lines

U266B1 myeloma cells and IM-9 lymphocytes were obtained from the European Collection of Cell Cultures. Both lines were grown in suspension at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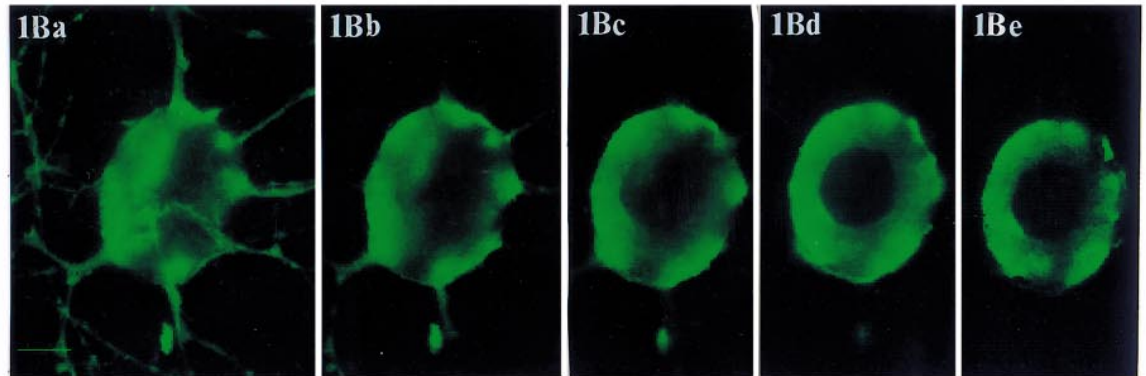
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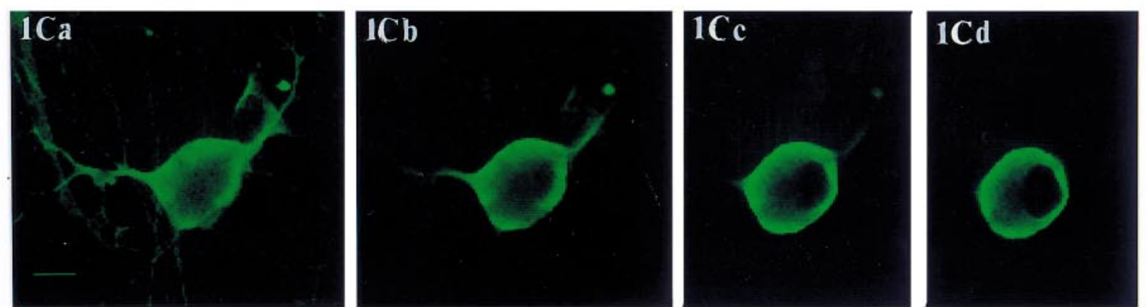
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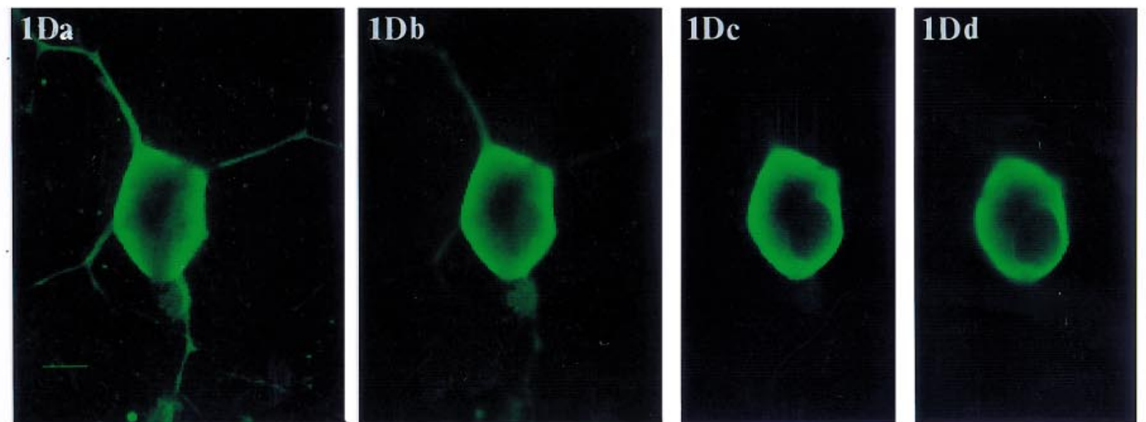
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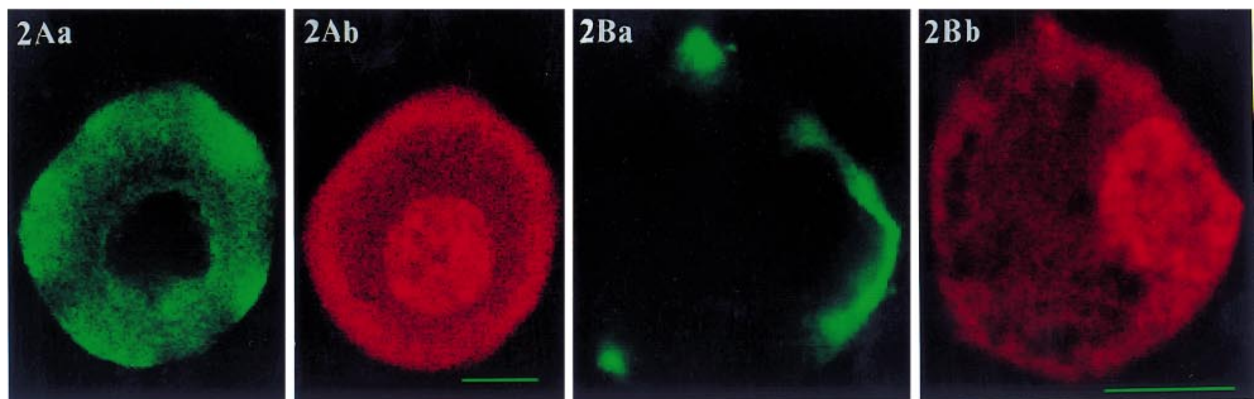
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37 °C in an 8% CO_2 humidified incubator in RPMI 1640 medium, supplemented with 10% heat inactivated FCS (Life Technologies), 2 mM glutamine, 100 iu/ml penicillin, 100 µg/ml streptomycin (Sigma). U266B1 cells were divided 1:1 every 3 d, whereas IM-9 were split 1:4 every 2 d.

U266B1 and IM-9 cell attachment

Prior to immunocytochemistry, cells were serum-depleted by suspension in RPMI 1640–2% FAF BSA overnight (Freund et al. 1994). For immunostaining, chamber slides were coated with 0.1 mg/ml poly-D-lysine-0.1 M borate buffer pH 8.4, for 1 h at room temperature, followed by 3 washes with tissue culture-grade water and allowed to dry. To the slides, 0.75 ml of a confluent (5×10^5 cells/ml) cell suspension was added and the cells were allowed to attach for 20 min in a 37 °C incubator.

Colocalisation of insulin and IGF-1 in U266B1 and IM-9 cell lines

Cells were observed under a phase contrast microscope to confirm that they had attached to the coated surface of the slide. They were washed once with CMF PBS by carefully flooding the slide chambers. They were fixed with 4% paraformaldehyde-PBS, permeabilised, blocked, incubated with insulin, IGF-1 and labelled for both their antibodies as described for the primary neuronal cultures.

RESULTS

Monolayer neuronal cell cultures both from superior cervical and from dorsal root ganglia attached and flattened to the coated surface of the chamber slide within 1 h after plating. The majority started developing neurite extensions within 3–4 h following plating. The growth media lacked fetal bovine serum (Dreyfus & Black, 1990) in order to eliminate the influence of circulating insulin, IGF-1 and any IGFBPs from the serum and serum supplements during growth and in immunostaining experiments. FAF-BSA used as a serum supplement did not appear to contain traces of IGF-1 or insulin as is true of media supplemented with other types of BSA (authors' observations). For the same reason, blocking with normal donkey or goat sera during immunocytochemical labelling was substituted by the general blocker PVP which does not have a physiological origin. The growth media and buffers for immunostaining were therefore clear of detectable levels of insulin and IGF-1.

The use of the above serum-free culture system also eliminated the need for addition of cytosine arabinoside used to arrest growth of nonneuronal cells, Schwann cells and fibroblasts which undergo mitosis (Aguayo et al. 1975; Wood, 1976). Fibroblasts in particular, proliferate within 2–4 d and interfere with the development of the primary neuronal culture system (Godfrey et al. 1975). It was necessary to avoid the use of cytosine arabinoside as this causes apoptosis in postmitotic neurons by disrupting the topoisomerase II repair system (Tomkins et al. 1994). In our monolayer neuronal cultures, serum-free media supplemented with FAF-BSA discouraged the growth of fibroblasts by over 90%, but some Schwann cells grew along with the neurons and on some occasions were observed aligned with neurite extensions.

The dissociated cultures both from SCGs and DRGs exhibited high survival and attachment. Monolayer cultures of single neurons interconnected through their neurite extensions were produced from dissociated DRG. SCG cultures showed single isolated neurons but also clusters of up to 15 cell bodies. DRGs were easier to dissociate successfully into single cells and their attachment and subsequent survival rate in a monolayer culture was slightly better than for SCGs. This may be due to the nature of sympathetic ganglion cells as well as the action of proteases used for dissociation. In vivo as well as in culture, SCGs had more neurite contacts than DRGs and if these are damaged during dissociation, this may make the neurons more susceptible to protease action.

Cultures used in this study were permeabilised with Simultest Lysing solution, thus allowing intracellular access of insulin and IGF-1 to their receptors. In single immunofluorescent experiments for each hormone, the majority of DRG neurons were immunopositive for insulin and IGF-1 binding sites (Fig. 1,

Fig. 1. Series of consecutive confocal optical sections of primary neuronal cultures from dissociated dorsal root ganglia (panels *A*, *B*) and superior cervical ganglia (panels *C*, *D*). The presence of insulin (panels *A*, *C*) and IGF-1 (panels *B*, *D*) binding sites is indicated by indirect immunolabelling with fluorescein (green). Insulin and IGF-1 in DRGs are seen in neurite extensions (*a*) and concentrate in the cytoplasm (*b*–*e*) and in the cytoplasmic areas proximal to the neuronal nucleus (*c*–*e*). The localisation of insulin and IGF-1 in SCGs appears in neurite extensions (*a*), the cytoplasmic areas around the cell surface (*b*) and close to cell nuclei (*c*, *d*). The cell nuclei are dark nonfluorescent areas in the middle of the cell sections. Bar, 10 µm. Section thickness ~ 2 µm.

Fig. 2. Confocal optical section of a primary neuronal culture from dissociated dorsal root ganglia confirming the localisation of insulin (panels *A*) and IGF-1 (panels *B*) binding sites. Insulin or IGF-1 binding is indicated by fluorescein labelling (*a*, in green) and the neuronal nucleus indicated by propidium iodide staining (*b*, in bright red). Bar, 10 µm. Section thickness ~ 1 µm.

panels *A*, *B*). Neurite extensions appeared prominently stained for insulin and the immunofluorescence extended far from the cell body (panels *A*, *a*). Insulin appeared to bind very strongly to the cell cytoplasm, particularly in the area around the nucleus and internal to the surface membrane (panels *A*, *c–e*). It therefore appears that insulin staining in DRG neuronal cultures is predominantly cytoplasmic, in agreement with previous observations made for SCG monolayer cultures (James et al. 1993). It is further worthwhile noting that our attempts to label DRGs with insulin without permeabilising neurons led to background immunolabelling of neuronal bodies and neurite extensions. Schwann cells were free of strong fluorescent labelling both for IGF-1 and insulin. A low expression of insulin receptors in glial cells has also been observed previously in SCG cultures (James et al. 1993). Interestingly, studies of brain neuronal and glial cells have reported that neurons possess brain-type hybrid receptors whereas glial cells express the peripheral type (Burgess et al. 1987; Shemer et al. 1987). It is possible that a similar difference between neurons and Schwann cells exists in peripheral nervous tissue.

The pattern of IGF-1 binding in dorsal root ganglia was very similar to that of insulin (Fig. 1, panels *B*). Strong concentration of IGF-1 immunoreactivity was seen in neurite outgrowths, in the cytoplasmic areas leading to neurite extensions (*a*, *b*) as well as the cytoplasmic areas close to the cell surface (*c*) and adjacent to the nucleus (*d*, *e*).

Similar observations were made by immunolabelling of SCG monolayer cell cultures. SCG neurons were positive both for insulin (panels *C*) and IGF-1 (panels *D*). Strong immunofluorescence was observed in neurite outgrowths (*a*, *b*), on the cell surface in a capping-like fashion and in the cell cytoplasm with complete absence of immunostaining in the nuclear regions (*c*, *d*). The capping-like location of insulin and IGF-1 close to the cell surface was seen more commonly in SCG cells, whereas the concentration around the nuclear area appeared to be a predominant feature in DRGs.

In order to confirm the cytoplasmic localisation of insulin and IGF-1, propidium iodide was utilised in a dual imunofluorescence experiment (Fig. 2). Propidium iodide binds to double stranded DNA, marking the position of the cell nucleus and aiding the identification of insulin and IGF-1 binding sites. However, nonspecific labelling of at least part of the neuronal cytoplasm by propidium iodide was observed for neurons in all cultures. No such observation was made for fibroblasts or Schwann cells which showed strong fluorescence for cell nuclei only. Despite this limitation, confocal optical sections of DRG cells showed that insulin (panels *A*) and IGF-1 (panels *B*) receptors (*a*, in green) were not located inside the neuronal nuclei (*b*, in bright red) but instead in close proximity to them on many occasions. Propidium iodide labelling also confirmed the presence of nuclei within the dark, nonfluorescent areas seen in the middle of cell sections in Figure 1.

IGF-1 binding thus showed similar trends to that for insulin both in sympathetic and sensory neuronal cultures. It is thus possible that the binding sites for both are localised in the same areas. To further study the location of IGF-1 and insulin binding sites in sensory and sympathetic ganglion neurons, double immunofluorescence labelling and confocal microscopy was employed to colocalise the fluorescent signals of insulin and IGF-1 within the same cells. Figure 3 shows confocal optical sections of DRG and SCG neurons labelled both for insulin (in green) and IGF-1 (in orange-red). The nuclei are indicated by dark nonfluorescent areas located within the cell sections. These areas showed minimal fluorescent background. In both sensory (*a*) and sympathetic (*b*, *c*) ganglion cells, the binding sites of insulin and IGF-1 were concentrated together (in yellow). They were observed in the cell cytoplasm, often in cytoplasmic areas leading to neurite outgrowths, to the cell membrane and around the nuclei. The 2 fluorescent signals appeared colocalised. Fibroblasts in SCG (Fig. 3*c*, top cell) and DRG (Fig. 3*d*) cultures appeared lightly labelled with insulin and IGF-1, whereas some showed separate binding areas for IGF-1. The pattern of insulin and IGF-1 binding in fibroblasts appeared granular and dispersed whereas neurons showed strong local concentration of both IGF-1 and insulin in specific cytoplasmic areas. There thus appear to be fewer insulin and IGF-1 binding sites in fibroblasts. The binding pattern of insulin to DRG and SCG fibroblasts was strikingly different to that of neurons, an observation in agreement with previous reports on primary SCG cultures (James et al. 1993).

In order to gain some insight into the nature of the colocalisation of insulin and IGF-1 binding in sensory and sympathetic ganglion cells, IM-9, a lymphocyte cell line known to possess hybrid insulin-IGF-1 receptors was studied (Soos & Siddle, 1989). The localisation of insulin and IGF-1 binding sites was explored using dual immunofluorescence and confocal microscopy (Fig. 4*a*). The results were subsequently compared with simultaneous localisation of insulin and IGF-1 in U266B1 (Fig. 4*b*), a myeloma cell line

with separate insulin and IGF-1 receptors (Freund et al. 1994). Confocal optical sections of immunostained IM-9 lymphocytes showed a pattern of insulin (in green) and IGF-1 (in red) binding similar to that of sensory and sympathetic neuron cultures. Immunofluorescence for both was concentrated and localised in the same cytoplasmic areas (Fig. 4*a*, in yellow). This was expected, since the majority of insulin receptors in IM-9 lymphocytes have been shown to be IGF-1/insulin hybrids (Soos & Siddle, 1989) and therefore insulin and IGF-1 have, in effect, a common receptor. In contrast, the dual immunofluorescence labelling pattern in U266B1 cells was quite different. The localisation of insulin binding sites was quite distinct from that for IGF-1 (Fig. 4*b*). Insulin immunoreactivity was present in the cytoplasm in rounded fluorescent patches of various sizes (in green), whereas IGF-1 labelling was seen in the cytoplasmic areas around the nucleus (orange-red). There was some cell surface and cytoplasmic IGF-1 immunofluorescence. Insulin and IGF-1 binding sites were sometimes dispersed and others concentrated in a capping-like manner, possibly suggesting receptor endocytosis. The location of the insulin receptors in these cells is consistent with the role of insulin in metabolism of carbohydrates, fats and proteins in peripheral-type tissues. It is also consistent with the known property of the insulin receptor to undergo endocytosis, accumulation into endosomal cytoplasmic bodies and recycling to the cell surface (Marshall et al. 1981; Smith & Jarett, 1987). IGF-1 and its receptor may have a different, trophic role in peripheral tissues. A trophic function for IGF-1 may explain the accumulation of IGF-1 binding sites in the cytoplasmic area around the nucleus.

It is thus apparent that immunocytochemical insulin and IGF-1 colocalisation observed in IM-9 lymphocytes which possess hybrid receptors is also true of sensory and sympathetic neurons. This contrasts with the myeloma U266B1 cell line with separate receptors for insulin and IGF-1, and which exhibits separate binding areas for each hormone. The colocalisation of insulin and IGF-1 receptors in peripheral nervous tissue primary cultures supports the view that these tissues possess hybrid, 'brain-type' receptors.

DISCUSSION

The central nervous system is considered insulin-insensitive in vivo and in vitro (Goodner & Berrie, 1977, 1978) and insulin does not affect energy balance or glucose utilisation in peripheral nerve trunks (Greene & Winegrad, 1979). This similarity of the peripheral and central nervous systems is related to the blood-nerve and perineurial diffusion barriers that restrict access of insulin and fatty acids, rendering nerves dependent on glucose metabolism for energy. The presence of fenestrated capillaries render the blood-nerve barrier deficient in sensory and sympathetic ganglia (Jacobs et al. 1976, 1977); however, glucose and amino acid uptake by rat dorsal root ganglia is not influenced by insulin (Patel et al. 1993). This suggests that ganglia behave similarly to other peripheral and central nervous tissue.

Brain insulin receptors are hybrids, with dual specificity for insulin and IGF-1 (Moss & Livingston, 1993). Their regulation differs from peripheral type receptors. Brain receptors are not upregulated in rat streptozotocin-induced diabetes (Paclod & Blackard, 1979) unlike some peripheral tissues such as hepatic plasma membranes (Davidson & Kaplan, 1979) although this is not observed in adipocytes (Vann Bennett & Cuatrecasas, 1972).

Peripheral sensory and autonomic neurons also possess insulin receptors but these have been considered to be of the peripheral type, based on the molecular weights of their subunits (Waldbillig & LeRoith, 1987). However, a number of factors point towards the possible existence of brain-type receptors in the peripheral nervous system. Peripheral neuronal insulin receptors behave much like insulin brain-type receptors (Unger et al. 1991) and, as already stated, glucose and amino acid uptake is independent of insulin in dorsal root ganglia (Patel et al. 1993). Immunocytochemical studies in the rat brain have demonstrated the cytoplasmic localisation of insulin receptors (Unger et al. 1991). Similarly, recent evidence has shown their intracytoplasmic localisation in superior cervical ganglia (James et al. 1993).

The present study complements previous findings that suggest the existence of a brain-type receptor in the peripheral nervous system. Immunocytochemical evidence points towards the colocalisation of insulin and IGF-1 binding sites both in sensory and sympathetic ganglion cells grown in primary dissociated cultures. Along with the observation of colocalised binding areas in IM-9 lymphocytes, known to possess hybrid receptors, and the separate cellular location of each in a myeloma cell line known to have peripheral-type receptors, our results point towards the existence of brain-type, hybrid receptors for insulin and IGF-1 in the peripheral nervous system. The existence of hybrids in peripheral nerves may have interesting implications for the function of such receptors in a system that does not depend on insulin for its glucose

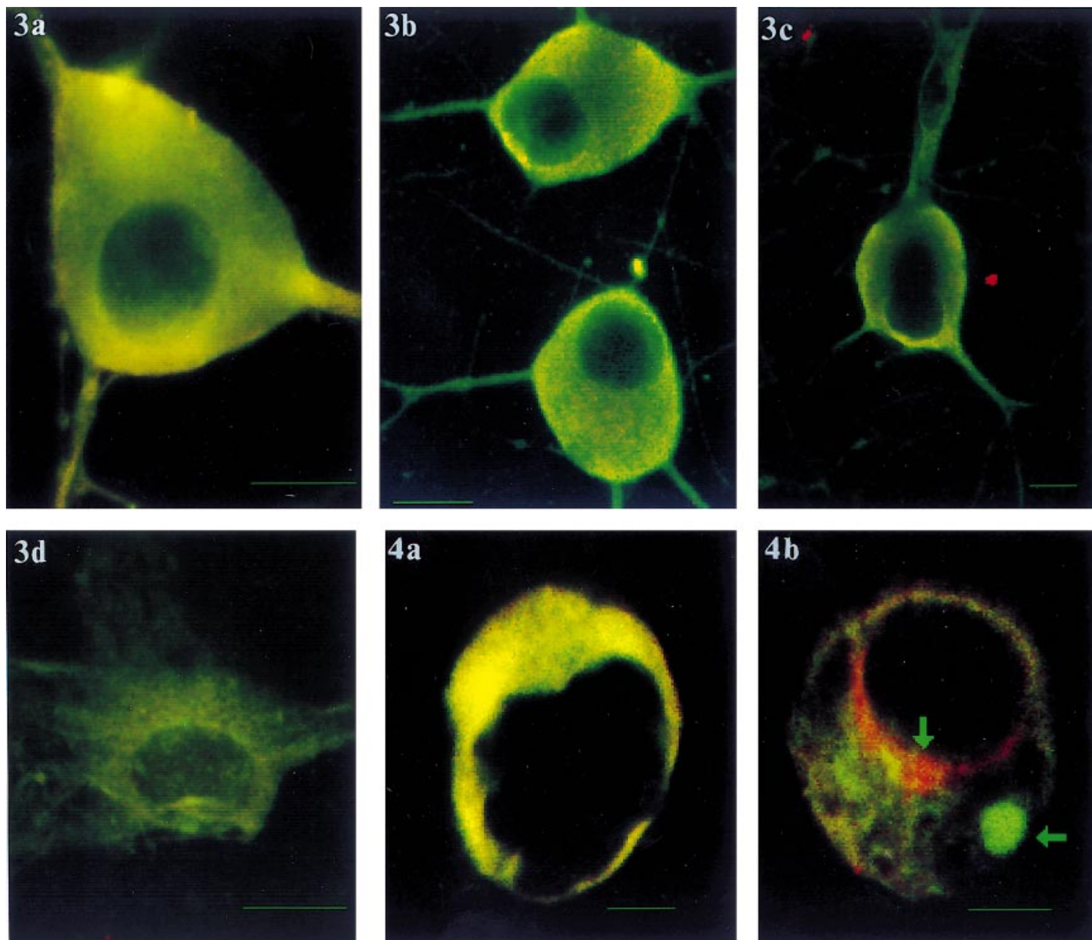

Fig. 3. Double immunofluorescence and confocal imaging for the localisation of insulin (green) and IGF-1 (red) binding sites in DRG (*a*) and SCG (*b*) ganglion cells grown in a primary serum-free culture system. Insulin and IGF-1 appear localised in the same cytoplasmic areas (in yellow) in both types of neuron. Dual immunolabelling of fibroblasts in SCG (*c*, top cell) and DRG primary cultures (*d*) resulted in a granular labelling pattern and binding of IGF-1 (red) appeared slightly reduced and at times separate from the insulin binding areas (green). Bar, 10 µm. Section thickness ∼ 1 µm.

Fig. 4. Confocal optical sections of cell lines known to possess insulin and IGF-1 receptors. Double immunofluorescence reveals the colocalisation (in yellow) of insulin and IGF-1 in IM-9 lymphocytes (*a*) which have hybrid brain-type heterotetrameric receptors for IGF-1 and insulin. Separate binding sites (insulin in green, IGF-1 in red, shown by arrows) are seen in U266B1 multiple myeloma cells (*b*) that have peripheral-type receptors for the 2 hormones. Bar, 10 µm. Section thickness ∼ 1 µm.

and amino acid uptake. Growth promoting and trophic effects of insulin and IGF-1 may point towards an important role in axonal growth and maintenance and therefore in peripheral nerve disease.

Postnatally, circulating insulin has access to the brain where it stimulates protein synthesis via the brain subtype of the insulin receptor (Kappy et al. 1984; Heidenreich & Toledo, 1989). If this role continues into adult life and peripheral sensory and autonomic neuron receptors behave much like brain-type receptors, insulin and its receptor may contribute to the development of diabetic polyneuropathy, a distal length-related degenerative axonopathy, occurring in diabetes mellitus (Said et al. 1983). A dying-back type axonopathy could be the result of impaired trophic support for the parent neurons, resulting in a reduction in essential protein synthesis and a failure of axonal maintenance (Thomas, 1994). A possible role for insulin and IGF-1 in the development of diabetic neuropathy may therefore be linked to the existence, type and function in structural maintenance of their receptors in the peripheral nervous system.

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