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Solubilisation partial characterisation of the α-MSH receptor on primary rat Schwann cells

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The ACTH/MSH_{4-10} melanocortin core peptide sequence possesses neurotrophic properties in peripheral nerve. During functional neuroanatomical recovery after damage to peripheral nerves, Schwann cells play a significant role in facilitating regeneration. Here we employ a modified super-potent α-MSH analogue to solubilise α-MSH receptor proteins from cultured primary rat Schwann cells. [^{125}I-Tyr^2,Nle^4,D-Phe^7,ATB-Lys^{11}]-α-MSH photoaffinity labelled proteins from Schwann cells were analyzed by SDS-PAGE followed by autoradiography. The results indicate that the α-MSH receptor proteins labelled have a molecular weight of 42–45 kDa. These data are the first to demonstrate solubilisation and characterisation of α-MSH receptors from non-melanoma cells.

α-Melanocyte stimulating hormone; Receptor isolation; Melanocortin; Schwann cell; Nerve regeneration

1. INTRODUCTION

α-Melanocyte stimulating hormone (α-MSH) (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2), has a multitude of well-recognised in vivo actions, both in the peripheral [1,2] and the central [3] nervous systems. Its mechanism of action is thought to be receptor mediated [4,5], resulting, in melanoma cell lines and melanocytes in the elevation of intracellular concentrations of cyclic-AMP [6–8], the activation of protein kinase C [9] and the elevation of intracellular calcium [10]. Stimulation of these cells by melanocortin peptides results in the activation of tyrosine kinases and the subsequent production of melanin.

After nerve injury, caused by chemical [11], diabetic [12] or mechanical [13] trauma, α-MSH and its metabolically stable α-MSH_{4-9} analogue Org2766 (Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH) can enhance functional regeneration of the nerve [14]. The characterisation of an Org2766 binding site on Schwann cells in vitro [15] has indicated that the Schwann cell may be a site for the neurotrophic actions of melanocortin-derived peptides.

In this report we describe the isolation and partial characterisation of a plasma membrane-associated receptor protein for α-MSH on primary rat Schwann cells by photoaffinity labelling using a monoiodinated photoreactive analogue of α-MSH, [Nle^4,D-Phe^7]α-MSH, a technique which has successfully employed to identify α-MSH binding proteins on various melanoma cell lines [16,17].

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2. MATERIALS AND METHODS

All protease inhibitors were from Boehringer Mannheim. Enzymes for tissue dissociation were obtained from Sigma and DMEM from Northumbria Biologicals. [^{125}I-Tyr^2,Nle^4,D-Phe^7,ATB-Lys^{11}]-α-MSH was synthesized and iodinated as described previously [18].

2.1. *Tissue culture*

Schwann cell cultures were obtained from the sciatic nerves of 1–2-day-old Wistar-type rats of both sexes, according to the method of Brockes et al. [19], with modifications [15]. Briefly, the sciatic nerves were dissected out, pooled in cold, sterile PBS and finely minced (1 mm lengths). The tissue was then enzymically dissociated by treatment with Collagenase-type III (0.05%, 5 min, 37°C) followed by trypsin (0.25%, 15 min, 37°C). Collagenase was removed by low speed centrifugation and trypsin activity inhibited by the addition of FBS. Tissue was then dissociated mechanically by passage through 22- and 23-gauge needles.

After pelleting cells were resuspended in high glucose (4500 mg/l) Dulbecco's Modified Eagles' Medium, supplemented with 10% FBS, 2 mM glutamate, 25,000 U/ml penicillin and 25 mg/ml streptomycin.

The presence of rapidly dividing contaminating fibroblasts was reduced by the addition of cytosine β-D-arabinofuranoside, at a concentration of 10 μM, 24 h after plating for 48–72 h.

2.2. *Photoaffinity labelling and preparation of membranes*

Primary rat Schwann cells were grown on Ezin tissue culture flasks (175 cm^2; Flow) to count of approximately 2×10^6 cells per flask, at 37°C in a humidified atmosphere of 5% CO_2/95% air. Growth medium was removed and the cell were then incubated with [^{125}I-Tyr^2,Nle^4,D-Phe^7,ATB-Lys^{11}]-α-MSH (0.1 nM) in binding buffer (DMEM; 25 mM HEPES, 0.2% (w/v) BSA and 0.3 mM 1,10-phenanthroline) in the presence or absence of 100 nM [Nle^4,D-Phe^7]α-MSH. After 4 h incubation at 4°C in the dark, the upper surface of the flask was peeled off and the cells were irradiated, on ice, with UV light of 320 nm (2×15 W fluorescent tubes, FL15E UV-320) at a distance of about 10 cm for 5 min. After irradiation the cells were scraped from the flask and washed by low-speed centrifugation ($700 \times g$) to remove unbound ligand. The cells were resuspended in homogenisation buffer (10 mM Tris-HCl, pH 7.4, 0.3 mM 1,10-phenanthroline, 1 mM phenylmeth-

ylsulphonylfluoride). They were kept on ice for 30 min and homogenised by five passages through a 21-gauge needle and five passages through a 25-gauge needle. The homogenate was centrifuged at 20,000 × *g* for 30 min, at 4°C, to obtain crude membrane fractions. The pellet was resuspended in solubilisation buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1.5% (v/v) Triton X-100 and protein inhibitors at 0.1 μg/ml) and kept on ice for 1 h. The crude membrane fraction was then centrifuged at 50,000 × *g* for 1 h, at 4°C to obtain purified membrane fractions.

2.3. *SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

Purified membrane fractions were solubilised and SDS-PAGE was performed according to the method of Laemmli [20], using homogeneous slab gels (20 × 16 cm, 1 mm thick) containing 10% acrylamide in the separating gel. Following electrophoresis the gels were stained with Coomassie blue, dried and exposed on pre-flashed X-ray films with intensifying screens, at −70°C for 1 week.

The apparent molecular weights of the labelled proteins were estimated by a graphical method in which the molecular weights of a series of ^{14}C-labelled protein standards were plotted against their relative mobilities. The standards used were myosin (200 kDa), phosphorylase B (97.5 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa).

2.4. *Analysis of SDS-PAGE autoradiographs*

Autoradiographs were quantified by scanning on a BioRad GS-670 imaging densitometer and the scan results for receptor labelling in the absence or presence of excess [Nle^4-D-Phe^7]-α-MSH from 30 kDa to 200 kDa molecular weight proteins analysed in the BioRad Molecular Analyst software.

3. RESULTS

These experiments were performed using [^{125}I-Tyr^2, Nle^4,D-Phe^7,ATB-Lys^{11}]-α-MSH, a monoiodinated photoreactive α-MSH derivative. It has previously been demonstrated that this compound binds specifically to B16 melanoma cells [18]. Photoaffinity labelling was performed in the presence and absence of a large excess (100 nM) of unlabelled peptide to demonstrate the specificity of binding.

Incubation of primary rat Schwann cells with this monoiodinated photoreactive probe, followed by UV irradiation, SDS-PAGE, autoradiography and densitometric analysis demonstrates the specific radioactivity was localised in an apparent doublet protein band corresponding to molecular weight of 42–45 kDa photoaffinity labelled proteins (Fig. 1). A small amount of radioactivity was also observed at a higher and lower molecular weights, corresponding to 69 kDa and 29 kDa. The presence of excess unlabelled [Nle^4,D-Phe^7]α-MSH completely removed the 42–45 kDa protein doublet, but did not affect these other bands, indicating that the doublet represents specific labelling of an α-MSH receptor or subunits of an α-MSH receptor complex, the other radiolabelled bands being proteins which bind α-MSH non-specifically. The signal generated in Fig. 1 was associated with protein isolated from approximately 6 × 10^7 cells.

Densitometric analysis of the gel autoradiograph revealed a difference in ligand radioactive signal density

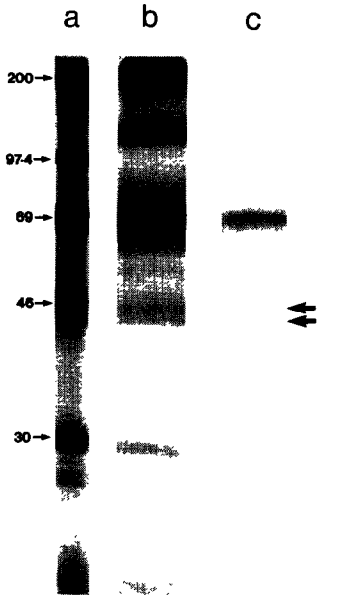

Fig. 1. Autoradiography of SDS-PAGE treated [^{125}I-Tyr^2,Nle^4,D-Phe^7,ATB-Lys^{11}]-α-MSH labelled Schwann cell membrane proteins. (Lane a) ^{14}C-labelled molecular weight markers; (lane b) membranes labelled with [^{125}I-Tyr^2,Nle^4,D-Phe^7,ATB-Lys^{11}]-α-MSH; (lane c) membranes labelled in the presence of excess cold unlabelled [Nle^4,D-Phe^7]-α-MSH.

(Fig. 2), between membrane preparations that have been exposed to the photoreactive ligand in the presence and absence of excess [Nle^4,D-Phe^7]α-MSH, that is in agreement with the low signal observed in the gel autoradiograph.

4. DISCUSSION

In this paper we report the first isolation and partial characterisation of α-MSH receptor proteins from cells of non-melanocyte origin emploing a novel ligand. Rat Schwann cell [^{125}I-Tyr^2,Nle^4,D-Phe^7,ATB-Lys^{11}]-α-MSH photoaffinity labelled proteins were isolated and analysed by SDS-PAGE followed by quantitative autoradiography in order to determine the molecular weight of the α-MSH receptor.

The results indicate that the proteins isolated have a molecular weight of 42–45 kDa. The abolition of these bands by incubation in the presence of excess unlabelled peptide indicates that this labelling was specific. Other bands, of higher (and one lower) molecular weights are also labelled. Labelling is reduced by the presence of excess unlabelled ligand, but is not wholly abolished. These non-specific bands cannot be identified at this stage.

The band observed may or my not be a true doublet, whether caused by differences in the primary structure of the protein or the result of post-translational modifications of the protein, or a solubilisation-induced artifact, remains to be established. However, preliminary studies on the receptor isolated from B16 melanoma

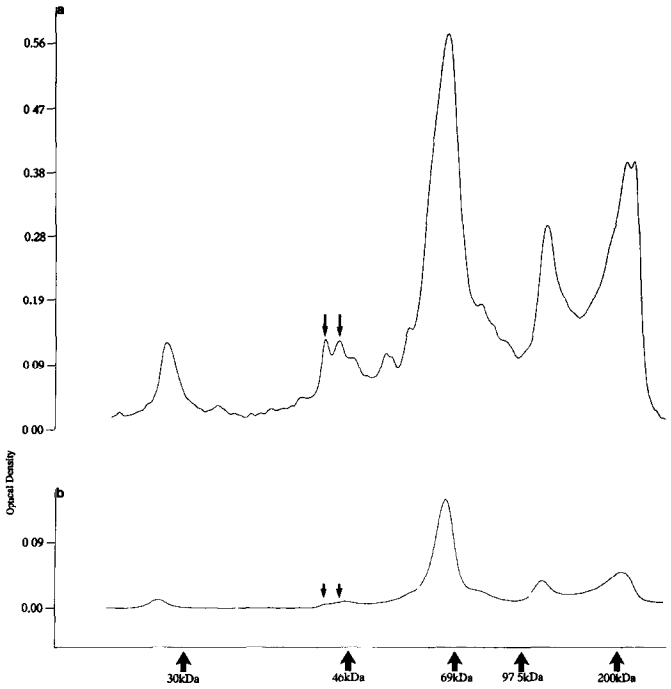

Fig. 2. Densitometric analysis of SDS-PAGE Autoradiograph. (a) Non-specific binding. (b) Specific labelling of doublet. Scans aligned with centres of 46 kDa marker. Proteins of interest arrowed.

cells [18] indicates the receptor may exist in different states of *N*-glycosylation, with a core protein having an apparent molecular weight of 28 kDa [21], observed as a single band after SDS-PAGE.

[^{125}I]Naps-MSH labelling of B16 cells [18] revealed a labelled protein of 45 kDa, with a core weight of 42 kDa after neuramidinase treatment. This labelling was specific, as controls of incubation in the presence of 3,000-fold excess α-MSH or Naps-MSH, the incubation of [^{125}I]Naps-MSH in the dark, without UV irradiation or with UV photolysis of the ligand prior to the experiment proved to be negative. The Cloudman S91 cell line also bound the photolabelled ligand, but expressed a receptor protein with an apparent molecular weight of 50 kDa, i.e. slightly larger than that on B16 cells, possibly due to a different glycosylation state.

Receptors for melanocortin-derived peptides have also been identified previously on amphibian melanocytes in skin preparations [22], in the mammalian brain [23] and on primary cultures of rat Schwann cells [15] and neurones [24] in binding studies using a variety of radio- or biotin-labelled ligands. In all these studies isolation of the receptor protein(s) and their biochemical characterisation has not been reported.

Pharmacological binding and functional assays [14,22,23,25], however, have indicated that multiple melanocortin receptor types may exist. This has been supported with the recent cloning of the gene and expression of the protein for melanocortin-derived peptide receptors [26–28] and the characterisation of four sub-types of the melanocortin receptor. The four receptors have distinct profiles of pharmacological activity, though all are common in their membership of the G-protein coupled family of receptors. The melanocortin (MC)-1 receptor, which is the α-MSH receptor, is expressed in melanocytes and melanoma tissues, the adrenocorticotrophin (ACTH) preferring MC-2 receptor has been localised to the adrenal cortex [26]. More recently the MC-3 and MC-4 receptors [27,28] have been localised to the hypothalamus (but not in melanoma cells) and other regions of the brain associated with behavioural effects of the melanocortin peptides [3]. The MC-3 receptor has a requirement for an ACTH/MSH core sequence, namely Met^4-Glu/Gly-His-Phe-Lys-Trp-Gly/Asp^{10}, to be present in its ligands for binding and biological activity. This requirement is similar to that seen previously in primary culture of rat Schwann cells [15] where Met$(O_2)^4$-Glu-His is required for binding. The MC-4 sub-class also requires such a core sequence, however, there is an additional requirement for Tyr^2 for binding, and Pro^{12} for full biological activity. Due to the localisation of this protein, almost exclusively within the hippocampus [28] (the MC-3 receptor is found in extra-hippocampal nervous tissues also) the MC-3 receptor is likely to be responsible in the mediation of behavioural responses by the classical pro-opiomelanocortin-derived peptides.

Binding analysis with the superpotent ligand and receptor number determination on Schwann cells was not possible, due to the low numbers of receptors on the

cultured cells. In light the of the low signal generated in the autoradiograph the lack of this data is not surprising. The development of an immortalised Schwann cell line that expresses this receptor and a more sensitive assay procedure for this cell type will be required to elucidate this data.

We postulate that the receptor isolated and partially characterised from primary rat Schwann cells, as a 42–45 kDa protein doublet similar to that of the B16 melanoma cell line, is likely to be of the MC-1 subclass of the melanocortin receptor family and not of the MC-2, -3 or -4 subclasses.

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REFERENCES

[1] Bär, P.R.D., Schrama, L.H. and Gispen, W.H. (1990) in: Neuropeptides. Basics and Perspectives (D. De Wied ed.) pp. 175–211, Elsevier, Amsterdam, The Netherlands.
[2] Strand, F.L., Rose, K.J., King, J.A., Segarra, A.C. and Zuccarelli, L.A. (1989) Progr. Neurobiol. 33, 45–85.
[3] De Wied, D. and Jolles, J. (1982) Physiol. Rev. 62, 972–1059.
[4] DiPasquale, A., Varga, J.M., Moellmann, G. and McGuire, J. (1978) Anal. Biochem. 84, 37–48.
[5] Mac Neil, S., Johnson, S.K., Bleehen, S.S., Brown, B.L. and Tomlinson, S. (1981) Regulatory. Peptides. 228, 339–334.
[6] Buffey, J.A., Thody, A.J., Bleehen, S.S. and MacNeil, S. (1992) J. Endocrinol. 133, 333–340.
[7] Bitensky, M.W. and Demopoulos, H.B. (1970) J. Invest. Dermatol. 54, 83.
[8] Gerst, J.E., Sole, J., Mather, J.P. and Salomon, Y. (1986) Mol. Cell. Endocrinol. 46, 137–147.
[9] Mac Neil, S., Dobson, J., Bleehen, S.S. and Buffey, J.A. (1990) Br. J. Dermatol. 123, 828.
[10] Eberle, A.N., Kriwaczek, M. and Schwyzer, R. (1987) Bull. Schweitz. Akad. Med. Wiss. 34, 99–111.
[11] Sporel-Özekat, R.E., Edwards, P.M., Gerritsen van der Hoop, R. and Gispen, W.H. (1990) Eur. J. Pharmacol. 186, 181–187.
[12] Van der Zee, C.E.E.M., Gerritsen van der Hoop. R. and Gispen, W.H. (1989) Diabetes 38, 225–230.
[13] Tonnaer, J.A.D.M., Schuijers, G.J.P.T., Van Diepen, H.A. and Peeters, B.W.M.M. (1992) Eur. J. Pharmacol. 214, 33–37.
[14] Bijlsma, W.A., Jennekens, F.G.I., Schotman, P. and Gispen, W.H. (1981) Eur. J. Pharmacol. 76, 73–79.
[15] Dyer, J.K., Philipsen, H.L.A., Tonnaer, J.A.D.M. and Haynes, L.W. (1993) Ann. NY Acad. Sci. 680, 496–498.
[16] Plummer, T.H., Elder, D.H., Alexander, S., Phelan, A.N. and Tarentio, A.L. (1984) J. Biol Chem. 259, 10700–10704.
[17] Solca, F., Siegrist, W., Drozdz, R., Girand, J. and Eberle, A.N. (1989) J. Biol. Chem. 264, 14277–14281.
[18] Ahmed, A.R.H., Oliver, G.W., Adams, G., Kinsman, R.G., Branch, S.K., Moss, S.H., Notarianni, L.J. and Pouton, C.W. (1992) Biochem. J. 286, 377–382.
[19] Brockes, J.P., Fields, K.L. and Raff, M.C. (1979) Brain Res. 165, 105–118.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Ahmed, A.R.H., Oliver, G.W., Adams, G., Branch, S.K., Moss, S.H., Notarianni, L.J. and Pouton, C.W. (1993) Biochem. J. Submitted.
[22] Sawyer, T.K., Staples, D.J., Castrucci, A.M.L., Hadley, M.E., Al-Obeidi, F.A., Cody, W.L. and Hruby, V.J. (1990) Peptides 11, 351–357.
[23] Tatro, J.B. (1990) Brain Res. 536, 124–132.
[24] Van Huizen, F., Philipsen, H.L.A. and Tonnaer, J.A.D.M. (1991) Soc. Neurosci. Abstr. 17, 95.14.
[25] Verhaagen, J., Edwards, P.M., Jennekens, F.G.I., Schotman, P. and Gispen, W.H. (1987) Brain Res. 404, 142–150.
[26] Mountjoy, K.G., Robbins, L.S., Mortrud, M.T. and Cone, R.D. (1992) Science 257, 1248–1251.
[27] Gantz, I., Konda, Y., Tashiro, T., Shimoto, Y., Miwa, H., Munzert, G., Watson, S.J., DelValle, J. and Yamada, T. (1993) J. Biol. Chem. 268, 8246–8250.
[28] Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S.J., DelValle, J. and Yamada, T. (1993) J. Biol. Chem. 268, 15174–15179.