Volume 229, number 1, 35-39 FEB 05612 February 1988

Glucose transporters in chromaffin cells: subcellular distribution and characterization

E.G. Delicado, M. Torres and M.T. Miras-Portugal*

Departamento de Bioquímica, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain

Received 13 November 1987; revised version received 4 January 1988

The glucose transporter was identified and characterized by cytochalasin B binding in subcellular membrane fractions of chromaffin tissue. The binding was saturable with a K_d of about 0.3 μM for each subcellular fraction. The B_{max} capacity was 12–16 pmol/mg protein for enriched plasma membrane fractions, 6.3 pmol/mg protein for microsomal membrane preparations and 5.4 pmol/mg protein for chromaffin granule membranes. Irreversible photoaffinity labelling of the glucose-protectable binding sites with [^{3}H]cytochalasin B followed by solubilization and polyacrylamide gel electrophoresis from enriched plasma membrane preparations demonstrated the presence of three molecular species: 97 ± 10, 51.5 ± 6 and 30 ± 4 kDa. The chromaffin granule membranes showed only a molecular species of 80 ± 10 kDa.

Glucose transporter; Chromaffin cell; Photoaffinity labeling; Cytochalasin B

1. INTRODUCTION

Chromaffin cells from adrenal medulla are a useful tool in understanding secretory processes in neural cells [1,2]. They have also proved to be an interesting model in metabolite transport studies and their modulation in homogeneous neural tissue [3–5].

Under normal conditions, glucose serves as virtually the sole substrate for neural energy metabolism [6], this also being true for chromaffin cells [7,8], which moreover show a similar glycolytic isoenzyme pattern to that found in brain and neural tissues [8,9].

The glucose transport and transporters in neural tissues have been characterized [10,11], even for chromaffin cells [12], and the stimulatory effects of secretagogues and insulin established [13].

Nevertheless, little attention has been paid to glucose transporter distribution in this tissue, which presents the peculiar characteristic of large quantities of storage granules being clearly different from fibroblasts and adipose tissue, where the transporters have been mainly characterized [14,15].

The present article deals with the glucose transporter subcellular distribution in chromaffin tissue and the molecular characterization, by using [^{3}H]-cytochalasin B as a radioligand.

Correspondence address: M.T. Miras-Portugal, Departamento de Bioquímica, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain

2. MATERIALS AND METHODS

[4(n)-^{3}H]Cytochalasin B (15.5 Ci/mmol) was obtained from Amersham. Unlabelled cytochalasin B and cytochalasin E were purchased from Aldrich. All other reagents were obtained from Merck, Sigma and Boehringer.

Subcellular fractions from adrenal medulla were obtained according to Zinder et al. [16] in the presence of 5 μM phenylmethylsulfonyl fluoride (PMSF) to avoid protease action. Enzymatic assays for different fractions were: 5'-nucleotidase (EC 3.1.3.5) according to Aronson and Touster [17] and acetylcholinesterase (EC 3.1.1.7) by the method of Low and Finean [18] as markers of plasma membranes; monoamine oxidase (EC 1.4.3.4) as described by Goridis and Neff [19], used as a mitochondrial marker enzyme and dopamine-β-hydroxylase (EC 1.14.17.1) according to Miras-Portugal et al. [20] as the enzymatic marker of chromaffin granules. Proteins were determined by the procedure of Bradford [21].

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00145793/88/$3.50

Glucose transporter quantification was performed by cytochalasin B binding assays, essentially as in [13,14].

Photolabelling with [^{3}H]cytochalasin B was carried out according to Shanahan [22]. Characterization of photolabelled polypeptides was effected by polyacrylamide gel electrophoresis according to Laemmli [23]. Values are expressed as means ± SD. Linear regression equations were calculated by the least-squares method, using a linear regression program.

3. RESULTS

3.1. *Enzyme marker distribution in chromaffin cells*

The data in table 1 list the activities of some conventional enzyme markers of plasma membranes, mitochondria and chromaffin granules. From these results the most characteristic constituent of chromaffin granules, dopamine-β-hydroxylase, was preferentially associated with the chromaffin granule fraction in the gradient, but a significant activity was also present in the G_3 fraction. The mitochondrial enzyme monoamine oxidase was principally associated with the G_3 fraction but significant amounts were also present in the chromaffin granule fraction. Acetylcholinesterase and 5′-nucleotidase showed the large distribution of plasma membranes through the gradient due to the heterogeneous size of vesicles formed from plasma membranes during the homogenization step procedure, as reported by others [16]. The G_1 and G_2 fractions showed the highest specific activities of acetylcholinesterase and 5′-nucleotidase with, at the same time, the lowest enzymatic activities of markers of mitochondria and chromaffin granules. Also the microsomal fraction (P 100 000 × g) was studied and its enzymatic levels

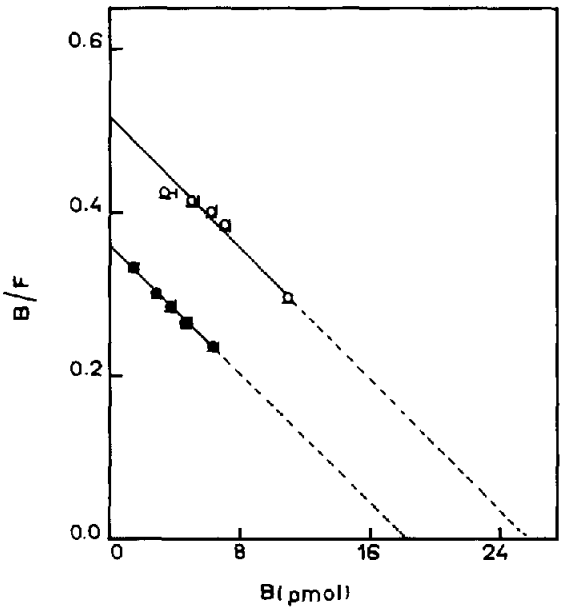

Fig.1. Scatchard analysis of equilibrium [^{3}H]cytochalasin B binding to enriched fraction of plasma membrane preparations, 0.4 mg protein (G_1 fraction) was incubated with [^{3}H]cytochalasin B (range 50–500 μM) and 5 μM cytochalasin E, in the presence (●—●) or absence (○—○) of 500 mM D-glucose. This plot is typically representative of four similar experiments performed in triplicate.

are given in table 1. Recently, acetylcholinesterase and other enzyme markers for plasma membrane have also been found as constituents of chromaffin granule membranes [24]. Nevertheless, these enzymatic markers will provide us with a real approach to study the subcellular distribution of glucose transporters.

Table 1

Distribution of marker enzymes in sucrose density fractions of adrenal medulla

Fraction	Protein (%)	5′-Nucleotidase	Acetylcholinesterase	Monoamine oxidase	Dopamine-β-hydroxylase
S 800 × g	100.0	2.3 (100)	17.1 (100)	1.7 (100)	31.5 (100)
P 10 000 × g	34.0	3.2 (45)	27.4 (42.5)	4.2 (80)	78.8 (81)
G_1	3.0	7.4 (10)	82.2 (15)	0.3 (0.5)	-
G_2	6.5	6.4 (18)	60.5 (23)	0.8 (3)	2.4 (0.5)
G_3	6.6	7.0 (20)	41.5 (16)	12.5 (48)	57.3 (12)
C.G.	14.8	0.7 (5)	8.8 (7.6)	1.6 (13)	155.0 (73)
P 100 000 × g	6.7	4.0 (22)	28.0 (11)	0.9 (3.5)	4.6 (1)
S 100 000 × g	53.0	0.2 (4.6)	0.9 (3)	-	2.4 (4)

Enzymes activities are expressed as specific activity in nmol/min per mg protein (% of total activity between parentheses). G_1, G_2 and G_3 fractions correspond to interfaces of 0.32–0.95, 0.95–1.34 and 1.34–1.6 M sucrose, respectively; C.G., chromaffin granules, obtained according to section 2

Table 2

Distribution of glucose transporters in sucrose density fractions of adrenal medulla, measured by [^{3}H]cytochalasin B binding

Fraction	K_d (μM)	B_{max} (pmol/mg protein)	% total bound
P 10000 × g	0.27 ± 0.03	5.09 ± 0.94	82.37
G_1	0.27 ± 0.09	16.27 ± 5.15	13.76
G_2	0.31 ± 0.02	12.16 ± 1.18	28.61
G_3	0.42 ± 0.08	7.09 ± 1.37	14.25
C.G.	0.20 ± 0.03	5.41 ± 0.38	22.44
P 100000 × g	0.26 ± 0.13	6.34 ± 0.56	17.64

B_{max} corresponds to the difference between the maximal bound capacity in the presence or absence of glucose (specific binding). Results are means ± SD of three experiments in duplicate

3.2. *Cytochalasin B binding distribution studies*

Fig.1 illustrates Scatchard plots of the [^{3}H]cytochalasin B binding data in plasma membranes (G_1 fraction) of chromaffin tissue. In order to quantify the number of glucose transporters, maximal cytochalasin binding was measured in the presence or absence of D-glucose, the difference being due to specific binding, that corresponds to about 20% of total ligand binding (specific binding = 16.28 ± 5.15 pmol/mg protein). The K_d value was 0.27 ± 0.09 μM.

Similar experiments were carried out with each fraction from the gradient and the results obtained are summarized in table 2. The maximal binding capacity correlates well with the presence of enzyme markers of plasma membranes, fractions G_1 and G_2. Also of interest is the high binding capaci-

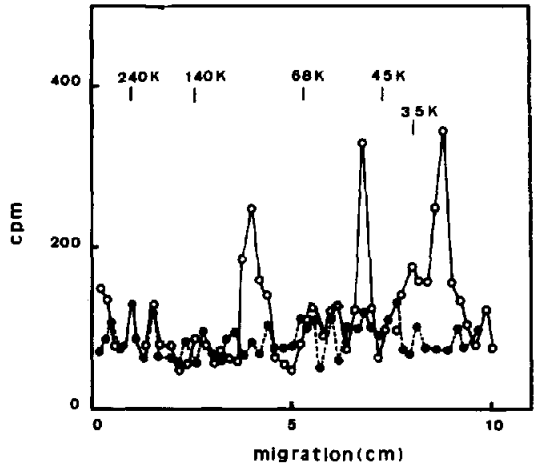

Fig.2. Distribution of radioactivity and proteins on SDS-polyacrylamide gels from [^{3}H]cytochalasin B-labelled plasma membrane proteins. Fraction G_1 membranes (1 mg/ml) were labelled in the presence of 0.5 μM [^{3}H]cytochalasin B (15.5 Ci/mmol) and 100 μM cytochalasin E, in the presence (●—●) or absence (○—○) of 500 mM D-glucose. The samples were photolyzed with ultraviolet light (280 nm, 5 min). In both cases, aliquots containing 50 μg protein were subjected to SDS gel electrophoresis using 10% polyacrylamide gels.

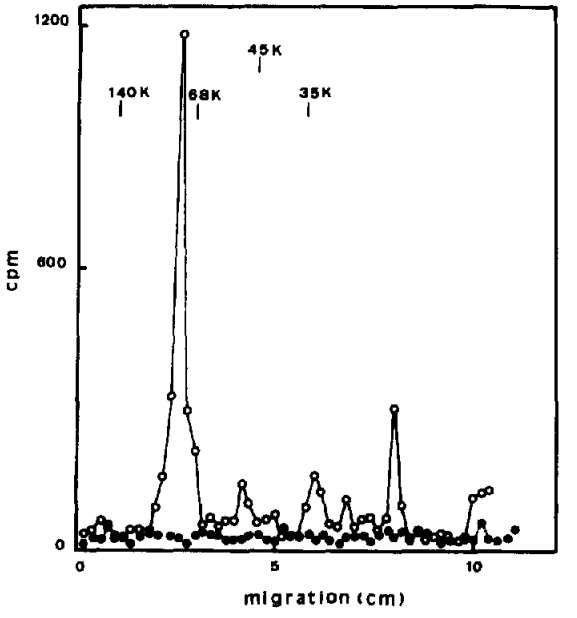

Fig.3. Distribution of radioactivity and proteins on SDS-polyacrylamide gels from [^{3}H]cytochalasin B-labelled chromaffin granule membrane proteins. Membranes (1 mg/ml) were labelled in the presence of 0.5 μM [^{3}H]cytochalasin B (15.5 Ci/mmol) and 100 μM cytochalasin E, in the presence (●—●) or absence (○—○) of 500 mM glucose. The samples were photolyzed with ultraviolet light (280 nm, 5 min). In both cases, aliquots containing 100 μg protein were subjected to SDS electrophoresis using 12% polyacrylamide gels.

ty of chromaffin granule membranes (specific binding = 5.41 ± 0.38 pmol/mg protein), that could not be explained by plasma membrane contamination.

3.3. *[^{3}H]Cytochalasin photoaffinity labelling of glucose-protectable binding sites*

Photoaffinity labelling studies were effected with enriched plasma membrane (G_1) and chromaffin granule membrane preparations. Fig.2 shows the photoincorporation of [^{3}H]cytochalasin B into plasma membrane polypeptides after ultraviolet light stimulation, which was analyzed by SDS-polyacrylamide gel electrophoresis. The pattern obtained for specific binding showed the presence of three molecular species with molecular masses of 97 ± 10, 51.5 ± 6 and 30 ± 4 kDa. When chromaffin granule membranes were studied (fig.3) only one molecular polypeptide with specific photolabelling for cytochalasin B was found, its molecular mass in SDS gel electrophoresis being 80 ± 10 kDa.

4. DISCUSSION

Specific glucose-displaceable cytochalasin B binding is an important tool for investigating quantitatively the glucose transporters in cellular and particulate tissue preparations. In all of the brain membranes the affinity constants are close to 0.3 μM [10]. Our results indicate the presence of a single class of saturable binding sites with a similar dissociation constant for each subcellular fraction. The maximal binding capacity of the P 10000 × g fraction present in chromaffin tissue correlates well with similar fractions from cerebral cortex and cerebellum from pig and rat brain [10,11]. When more enriched fractions from neuroblastoma and astrocytes were compared, the values reported were also very similar to that found in chromaffin tissue membrane preparations (12–16 pmol/mg protein) [25].

The maximal bound capacity throughout the gradient agrees well with the plasma membrane distribution, except for the chromaffin granule fraction, where there is a significant bound capacity not explainable by the plasma membrane contamination.

The insulin effect upon glucose transport by increasing the glucose transporters at the plasma membrane level through their translocation from a microsomal pool is well known [15,26]. The presence of these transporters in microsomal membrane preparations (P 100000 × g) correlates well with the insulin effect observed in this peripheral neural tissue, where insulin receptors have been described [13]. Furthermore, nicotine and other secretagogues increase the number of glucose transporters at the plasma membrane level of chromaffin cells [13]. However, the subcellular origin of these transporters is not well understood. In this way, the presence of high levels of glucose-displaceable cytochalasin B binding in the chromaffin granule fractions could shed some light and explain the increased number of glucose transporters when the regulated exocytotic event takes place. But the question arising is where and when the glucose transporters reach the chromaffin granule membranes. Two main possibilities exist (i) during the organelle biogenesis or (ii) after the exocytotic event when the plasma and chromaffin granule membranes undergo exocytosis for recycling. In both cases incomplete separation between the regulated and constitutive exocytotic pathways could be supposed [27,28].

The photolabelling studies effected with cytochalasin B showed a very different pattern when plasma membrane and chromaffin granule membranes were compared. In plasma membranes, three molecular species were photolabelled by cytochalasin B, suggesting the existence of three binding sites specifically protectable by glucose which could correspond to monomeric and dimeric forms of the transporter, as described in adipocytes and erythrocytes [29]. However, there is no clear proof that all are actually forms of the transporter. Nevertheless, the 30 kDa peptide could be the deglycosylated protein resulting from the action of glycosidases present as plasma membrane contamination, as confirmed elsewhere [30]. At the granule membrane level, only one molecular mass form was observed, which could correspond to the dimer. The differences in molecular masses of the labelled glucose transporters could be due to the degree of transporter glycosylation [31]. The possible existence of a different glucose transporter or other molecular species able to be photolabelled by [^{3}H]cytochalasin B in the presence of cytochalasin E and glucose could not be excluded. Additional studies are required to confirm the

identity of these transporters and their functionality or physiological functions when they are inside the cells.

Acknowledgements: This work was supported by grant no. PB86-0009 from the Spanish Comision Interministerial de Ciencia y Tecnologia, and by a fellowship from the Fondo de Investigaciones Sanitarias de la Seguridad Social to E.G.D.

REFERENCES

[1] Burgoyne, R.D. (1984) Biochim. Biophys. Acta 885, 335–344.
[2] Perrin, D. and Aunis, D. (1985) Nature 315, 589–593.
[3] Miras-Portugal, M.T., Torres, M., Rotllán, P. and Aunis, D. (1986) J. Biol. Chem. 261, 1712–1719.
[4] Torres, M., Molina, P. and Miras-Portugal, M.T. (1986) FEBS Lett. 201, 124–128.
[5] Torres, M., Bader, M.F., Aunis, D. and Miras-Portugal, M.T. (1987) J. Neurochem. 48, 233–235.
[6] Sokoloff, L., Fitzgerald, G.G. and Kaufman, E.E. (1977) in: Nutrition and the Brain (Wurtman, R.J. and Wurtman, J.J. eds) vol. 1, pp. 87–139, Raven, New York.
[7] Millaruelo, A.I., de Sagarra, M.R. and Miras-Portugal, M.T. (1982) J. Neurochem. 38, 470–476.
[8] Millaruelo, A.I., de Sagarra, M.R., Delicado, E.G., Torres, M. and Miras-Portugal, M.T. (1986) Mol. Cell. Biochem. 70, 67–76.
[9] Delicado, E.G., Torres, M. and Miras-Portugal, M.T. (1986) Cancer Res. 46, 3762–3767.
[10] Dick, A.P., Harik, S.I., Klip, A. and Walker, D.M. (1984) Proc. Natl. Acad. Sci. USA 81, 7233–7237.
[11] Dick, A.P. and Harik, S.I. (1986) J. Neurochem. 46, 1406–1411.
[12] Bigornia, L. and Bihler, I. (1986) Biochim. Biophys. Acta 885, 335–344.
[13] Delicado, E.G. and Miras-Portugal, M.T. (1987) Biochem. J. 243, 541–547.
[14] Salter, D.W. and Weber, M.J. (1979) J. Biol. Chem. 254, 3554–3561.
[15] Simpson, I.A., Yver, D.R., Hissin, P.J., Wardzala, L.J., Karnieli, D., Salans, L.B. and Cushman, S.W. (1983) Biochim. Biophys. Acta 763, 393–407.
[16] Zinder, O., Hoffman, P.G., Bonner, W.M. and Pollard, H.B. (1978) Cell. Tissue Res. 188, 153–170.
[17] Aronson, N.N. and Touster, O. (1974) Methods Enzymol. 31A, 92–93.
[18] Low, M.G. and Finean, J.B. (1977) FEBS Lett. 82, 143–146.
[19] Goridis, C. and Neff, N.H. (1971) J. Neurochem. 18, 1673–1682.
[20] Miras-Portugal, M.T., Aunis, D. and Mandel, P. (1973) FEBS Lett. 34, 140–142.
[21] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[22] Shanahan, M.F. (1982) J. Biol. Chem. 257, 7290–7293.
[23] Laemmli, U.K. (1970) Nature 227, 680–685.
[24] Burgun, C., Martinez-Muñoz, D. and Aunis, D. (1985) Biochim. Biophys. Acta 839, 219–227.
[25] Keller, K., Lange, K. and Malkewitz, J. (1986) J. Neurochem. 47, 1394–1398.
[26] Simpson, I.A. and Cushman, S.W. (1986) Annu. Rev. Biochem. 55, 1059–1089.
[27] Baker, P.K. and Knight, D.E. (1978) Nature 276, 620–622.
[28] Mellman, I., Fuchs, R. and Helenius, A. (1986) Annu. Rev. Biochem. 55, 663–700.
[29] Wheeler, T.J. and Hinkle, P.C. (1985) Annu. Rev. Physiol. 47, 503–517.
[30] Klip, A., Deziel, M. and Walker, D. (1984) Biochem. Biophys. Res. Commun. 122, 218–224.
[31] Baldwin, S.A., Baldwin, J.M., Gorga, F.R. and Lienhard, G.E. (1979) Biochim. Biophys. Acta 552, 183–188.