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Glucose transporters in isolated chromaffin cells

Effects of insulin and secretagogues

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1. Isolated chromaffin cells from bovine adrenal medulla were used to study glucose transport in a homogeneous neural tissue. 2. The affinity of glucose transporters was 1.20 ± 0.52 mM by the infinite-*cis* technique and 1.02 ± 0.09 mM by the direct transport experiments. 3. The affinity for 2-deoxyglucose of these transporters was 2.3 mM. 4. The glucose transporters, quantified by [^{3}H]cytochalasin B binding, were 419532 ± 120740 receptors/cell, which corresponds to about 7.2 ± 2 pmol/mg of protein, with $K_D = 0.1$ μM. 5. High-affinity insulin receptors with $K_D = 3.95$ nM were present at a density of 68400 ± 7500 per cell. 6. Insulin and secretagogues increased glucose transport, raising the transporter number at the plasma membrane without changes in the affinity.

INTRODUCTION

Chromaffin cells from adrenal medulla are specialized in the production, storage and secretion of catecholamines and are often used as a model to study the secretory process in neural cells (Burgoyne, 1984; Perrin & Aunis, 1985). They have also proved to be a very valuable approach to understanding the neuromodulatory effects of adenosine (Kumakura, 1984; Rotllan & Miras Portugal, 1985*a*,*b*; Miras Portugal *et al.*, 1986).

Under normal conditions, glucose serves as virtually the sole substrate for neural energy metabolism (Sokoloff *et al.*, 1977), this also being true for chromaffin cells (Pollard *et al.*, 1981; Millaruelo *et al.*, 1982, 1986), which moreover show a similar glycolytic isoenzyme pattern (Millaruelo *et al.*, 1986) to that found in brain.

Nevertheless, little attention has been paid to glucose transport across the chromaffin-cell membranes, although these could be a good model to provide valuable data to establish connections between neural cell stimulation and the increase in glucose consumption for energy metabolism (Kennedy *et al.*, 1975; Sokoloff, 1981).

On the other hand, insulin receptors can be found in neural tissues (Rees-Jones *et al.*, 1984; Zahniser *et al.*, 1984; Yorek *et al.*, 1985); their function, however, remains unclear. Basing our work on isolated chromaffin cells, our aims have been: (i) to study glucose transport by several techniques; (ii) the characterization and quantification of glucose transporters by radioligand-binding techniques; (iii) to study the effects of acetylcholine, nicotine and insulin on glucose transport; (iv) the characterization of insulin receptors.

MATERIALS AND METHODS

Materials

Collagenase, culture media, hexokinase, glucose-6-phosphate dehydrogenase, cytosine arabinofuranoside and insulin were obtained from Sigma Chemical Co. 2-Deoxy[U-^{14}C]glucose (303 mCi/mmol), [6-^{3}H]glucose (40 Ci/mmol), [4(n)-^{3}H]cytochalasin B (15.5 Ci/mmol) and ^{125}I-insulin (0.3 μCi/pmol) were from Amersham. [^{3}H]Phlorizin (56.5 Ci/mmol) was obtained from New England Nuclear. Cytochalasins B and E were purchased from Aldrich Chemical Co. and dissolved in ethanol. Silicone oils were from Siliconas Hispania (Barcelona, Spain). All other reagents were obtained from Merck.

Isolation and culture of chromaffin cells

Chromaffin cells were isolated from bovine adrenal glands essentially by the method of Miras Portugal *et al.* (1985). The cells were isolated by collagenase (EC 3.4.24.3) action and then purified through a Percoll gradient, carefully collected, and washed with Ca^{2+}/Mg^{2+}-free Locke's solution. Finally, cells were suspended in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal-calf serum and 50 μM-cytosine arabinofuranoside. Cells were plated in 24-well Costar cluster dishes and incubated at 37 °C in air/CO_2 (19:1) at a density of 500000 cells/well. Culture medium was changed every 2 days. All experiments were carried out with either recently isolated cells or ones that had been maintained in culture conditions.

Assay of glucose transport in isolated chromaffin cells

(*a*) Glucose transport was assessed with labelled 2-deoxyglucose and glucose, by the procedure of Decker & Lipmann (1981), as modified by us. The assays were carried out at room temperature (20 °C). Isolated chromaffin cells (1×10^6 cells) in 100 μl portions were washed twice with 1 ml of Hanks' salts (1.26 mM-$CaCl_2,2H_2O$, 5.37 mM-KCl, 0.44 mM-KH_2PO_4, 0.81 mM-$MgSO_4$, $7H_2O$, 137 mM-NaCl, 8.3 mM-Hepes and 0.4 mM-Na_2HPO_4) without glucose, by centrifugation in a Beckman Microfuge at 200 *g* for 2 min to remove the glucose from the medium. These packed cells were incubated with a solution containing 0.2 μCi of 2-deoxy[U-^{14}C]glucose (303 mCi/mmol; Amersham) or 0.2 μCi of [6-^{3}H]glucose (40 Ci/mmol; Amersham), and non-labelled product to give the required concentration in a final volume of 100 μl. All dilutions were carried out with Hanks' salts. At required times, transport was

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stopped by dilution 3-fold with ice-cold stopping solution containing 20 μM-cytochalasin B. This mixture was immediately layered over 200 μl of an oil mixture (Silicex 334/Silicex 342, 2:1, v/v; density = 1.04 g/ml) and centrifuged in a Beckman Microfuge (13000 *g*, 2 min) to pellet the cells under the oil. The pellet was resuspended in 100 μl of 1% Triton X-100 and radioactivity measured.

Cell water was determined as described by Miras Portugal *et al.* (1986), giving a value of 1.4 ± 0.2 μl of intracellular water/10^6 cells, the protein content being 105 μg/10^6 cells. These values allow comparison with data in the literature.

(*b*) Infinite-*cis* uptake assay. The isolated chromaffin cells were washed and centrifuged as previously described. Generally, 25×10^6–30×10^6 cells were mixed with 10 μCi of [6-^{3}H]glucose and non-labelled glucose (final concn. 60 mM) in Hanks' salts, in accordance with the procedure of Hankin *et al.* (1972). Incubations were undertaken at room temperature (20 °C). At required times, 100 μl portions (4×10^6–5×10^6 cells) of this solution were transferred to Eppendorff tubes containing 200 μl of an oil mixture and 1 ml of 0 °C stopping solution (0.1 mM-phloretin/1% ethanol in 0.9% NaCl/20 mM-Hepes, pH 7.4) (Hankin *et al.*, 1972; Robinson *et al.*, 1982). Transported glucose was recovered as a pellet under the oil layer, and radioactivity was counted as previously described.

Glucose-transporter quantification

Glucose transporters were identified by cytochalasin B-and phlorizin-binding experiments. Samples containing 2×10^6 isolated chromaffin cells were incubated in a final volume of 250 μl with 0.2 μCi of [^{3}H]cytochalasin B (15.5 Ci/mmol; Amersham) and non-labelled cytochalasin B to obtain a final concentration range from 50 to 500 nM, as described by Simpson *et al.* (1983). These binding experiments were undertaken in the absence or presence of 300 mM-glucose to determine the specific binding. After 15 min of incubation at 25 °C, samples were centrifuged for 2 min in a Beckman Microfuge at 13000 *g*. The remaining supernatant was aspirated and the pellet dissolved in 1% Triton X-100; radioactivity was then counted.

Cytochalasin B binding to chromaffin cells in culture was carried out in 24-well Costar cluster dishes containing 500000 cells/well, in the same ligand concentration range. Once equilibrium had been reached, usually after 15 min, cells were washed twice with 0.9% NaCl/20 mM-Hepes, pH 7.4, and were then carefully scraped off the plastic and counted for radioactivity in order to measure the bound form of cytochalasin.

Phlorizin binding was carried out in a similar way: 1 μCi of [^{3}H]phlorizin (56.5 Ci/mmol; New England Nuclear) and non-labelled product ranging in final concentration from 0.1 to 200 μM was used, in accordance with the original procedure of Moran *et al.* (1983).

Insulin-binding assays

Wells, containing 500000 cells, were incubated for 30 min at 25 °C with 0.3 μCi of ^{125}I-insulin (0.3 μCi/pmol; Amersham) and non-labelled product to obtain a final concentration range from 1 nM to 50 μM, essentially as described by Kono (1975). This incubation was stopped by removing the medium. The cells were then washed with 100 μl of 0.9% NaCl/20 mM-Hepes, pH 7.4, scraped off the plastic and counted for radioactivity in a γ-radiation spectrometer.

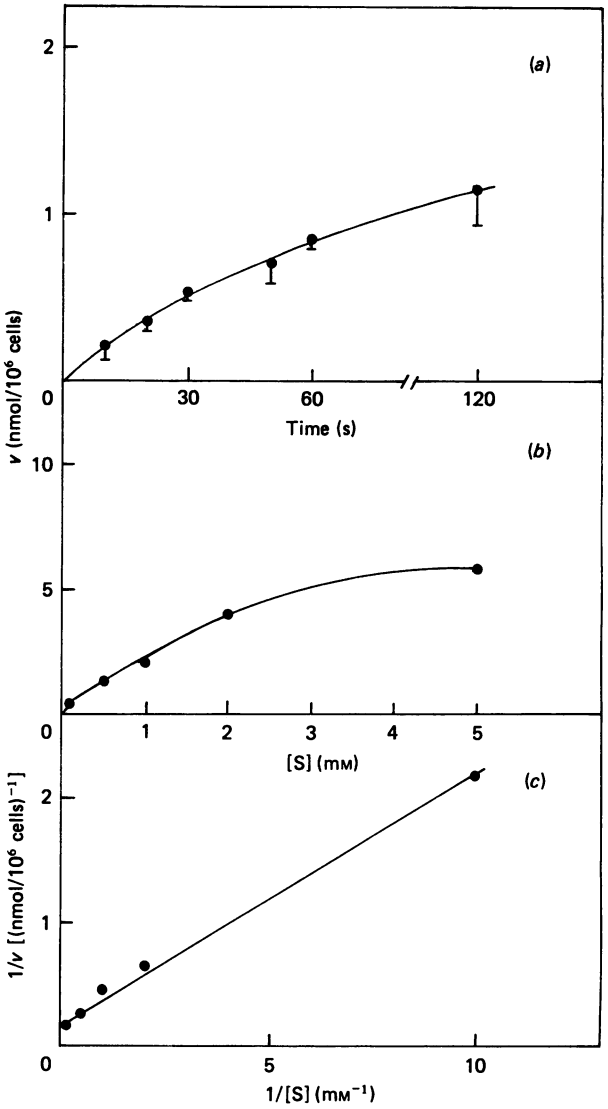

Fig. 1. 2-Deoxyglucose transport by chromaffin cells

(*a*) Time course of transport; (*b*) concentration-dependence of 2-deoxyglucose transport; (*c*) double-reciprocal plot of the initial rates of transport from (*b*). These results were from a typical experiment. In all cases 2×10^6 cells were used and experiments were performed as described in the Materials and methods section. Results are always expressed per 10^6 cells.

Owing to the presence of high catecholamine concentration, the glucose oxidase method is not a valid measure of glucose consumption. The latter was thus measured as described by Millaruelo *et al.* (1986), by the method of Bergmeyer *et al.* (1974), by using hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

Values are expressed as means $\pm$ S.D., with the numbers of experiments shown in parentheses. Linear-regression equations were calculated by the least-squares method, by using an Olivetti computer with a linear-regression program.

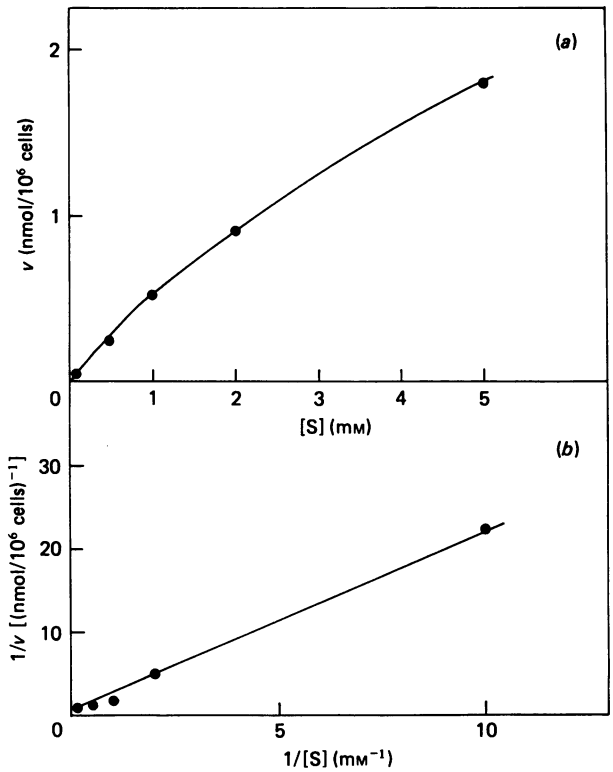

Fig. 2. Glucose transport by chromafin cells

(*a*) Concentration-dependence of glucose transport; (*b*) double-reciprocal plot of the initial rates of transport from (*a*). Results are shown of a typical experiment, carried out as described in the Materials and methods section.

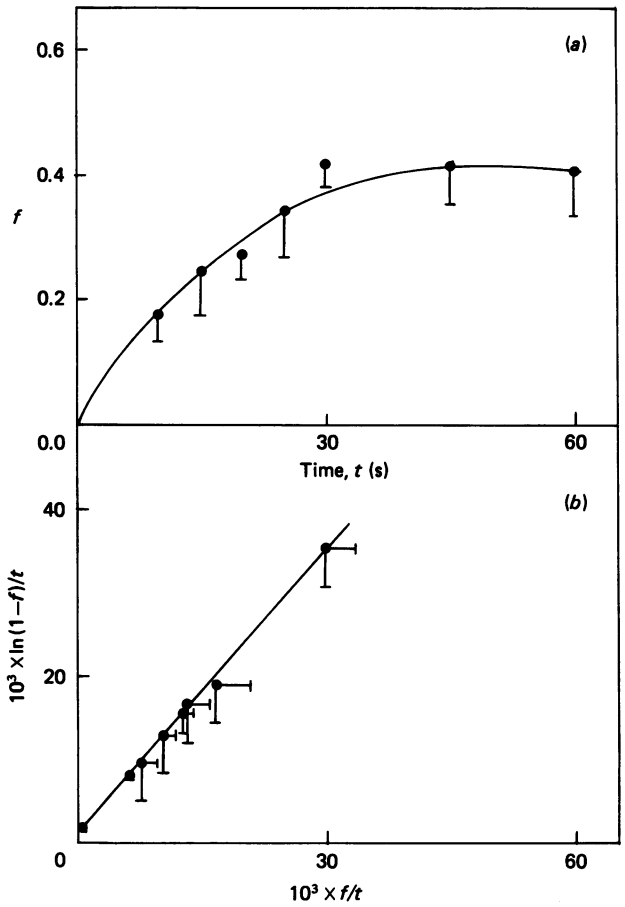

Fig. 3. Net influx of [6-^{3}H]glucose into recently isolated chromaffin cells under infinite-*cis* conditions.

(*a*) Time course of net glucose uptake, where f is the fractional filling with respect to equilibrium, as described in the Materials and methods section. (*b*) Linear plot resulting from an integrated-rate-equation transformation of these data, as described by Weiser *et al.* (1983). These results are means ± S.D. for five experiments.

RESULTS

Glucose transport in recently isolated chromaffin cells

The assays of glucose transport were performed in two different ways in order to study the kinetic properties, i.e. the affinity and capacity of transporters in the inner and outer face of the plasma membrane.

In order to characterize the kinetic properties of the hexose transporter in the outer face of the plasma membrane, the transport of glucose and its analogue 2-deoxyglucose, which is only metabolized to its phosphorylated derivative, were studied. Fig. 1 shows 2-deoxyglucose transport, this being nearly linear during the first 1 min (Fig. 1*a*). All experiments to characterize this transport kinetically were carried out during 1 min. A value of 2.3 ± 0.8 (4) mM for K_m was obtained, the $V_{max.}$ being 4.84 ± 1.2 (4) nmol/min per 10^6 cells. When a similar experiment was undertaken with [6-^{3}H]glucose, the values calculated were 1.02 ± 0.09 (6) mM for K_m and 1.83 ± 1.29 (6) nmol/min per 10^6 cells for $V_{max.}$ (range 2.96–0.42). Considering that 10^6 cells contain 1.4 μl of intracellular water, this value could be expressed as 1.307 mmol/litre of cells per min. The K_m value was very similar for all experiments; however, the $V_{max.}$ was highly variable, depending on the cell preparation (Fig. 2).

The infinite-*cis* procedure was carried out by measuring the intracellular [6-^{3}H]glucose increase for a high external concentration (60 mM). In Fig. 3 a typical experiment is shown, where f is the ratio of glucose transported at any time to the maximum glucose reached at equilibrium. In our experiments, equilibrium was reached at 30 min. The plot in Fig. 3(*a*) can be transformed into a linear plot by an integrated rate equation as described by Weiser *et al.* (1983) and Dustin *et al.* (1984), to obtain the K_m and $V_{max.}$ values for the inner face of the plasma membrane. These were 1.204 ± 0.52 (5) mM and 50.75 ± 22.45 (5) mmol/litre of cells per min respectively. When the graphical plot of Hankin *et al.* (1972) and Carruthers & Melchior (1984) was applied, similar values were obtained (K_m = 1.19 mM and $V_{max.}$ = 70.55 mmol/litre of cells per min). As with direct glucose-transport experiments, the $V_{max.}$ in the infinite-*cis* uptake assays depends on the cell preparation.

Identification and quantification of glucose transporters

The specific cytochalasin B binding was studied in recently isolated and cultured chromaffin cells.

Fig. 4 illustrates Scatchard plots of the [^{3}H]cytochalasin binding data in recently isolated chromaffin cells. The specific binding displaceable by D-glucose corresponds to 20% of the total ligand binding. The K_D obtained for cytochalasin was 0.09 ± 0.03 (8) μM. In order to quantify the number of

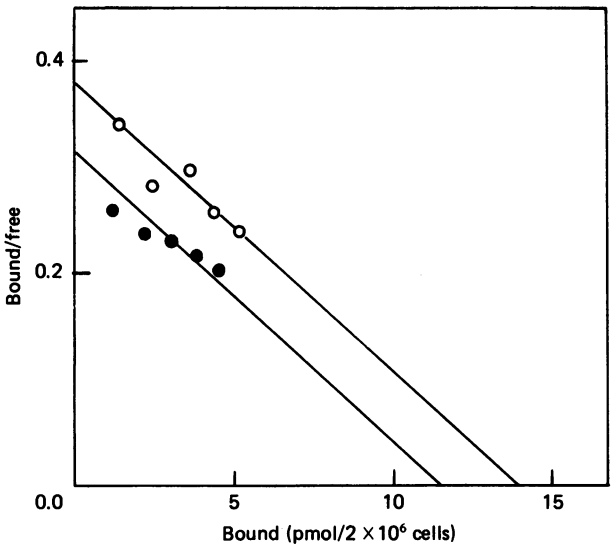

Fig. 4. Scatchard analysis of equilibrium [³H]cytochalasin B binding to isolated chromaffin cells

This binding was accomplished with 2×10^6 cells, as described in the Materials and methods section, and measured in the absence (○) or presence (●) of 300 mM-glucose. Results are shown of a typical experiment, with a specific maximal binding capacity of 1.2 pmol/10^6 cells.

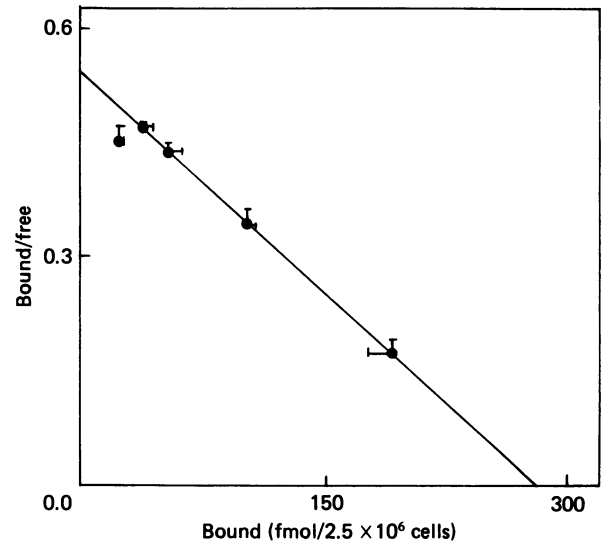

Fig. 5. Scatchard analysis of equilibrium ^{125}I-insulin binding to chromaffin cells in culture

Results are means ± S.D. for three determination in triplicate.

receptors, the maximal cytochalasin binding was measured in the presence and absence of glucose, the difference being due to the specific binding. A value of 0.75 ± 0.19 (8) pmol/10^6 cells was obtained. Taking into account the number of molecules/mol, this value becomes 419532 ± 120740 (8) receptors/cell. As 10^6 chromaffin cells contain 105 μg of protein, a value of 7.19 ± 1.85 (8) pmol/mg of protein was obtained. As in the literature units of pmol/mg of protein are used to express the binding capacity, the value arrived at is useful when making comparisons.

When 2-day-cultured chromaffin cells were studied, the K_D for specific binding of this ligand was 0.13 μM, which is not significantly different from that for recently isolated cells. The specific glucose-displaceable [³H]cytochalasin B binding was 429000 receptors/cell, which is close to that found in recently isolated chromaffin cells.

In our experimental conditions, phlorizin showed non-specific saturable binding, and was not therefore Na^+-dependent or displaceable by α-methyl D-glucoside (results not shown).

Insulin binding

Fig. 5 shows a Scatchard plot of ^{125}I-insulin binding to 4-day-cultured chromaffin cells. A linear plot is observed, showing a maximal binding capacity of 1.081 pmol/mg of protein, corresponding to about 68400 ± 7500 (3) receptors/cell (113 fmol/10^6 cells), the K_D being 3.95 ± 0.68 (3) nM.

Insulin, acetylcholine and nicotine effects on glucose transport and utilization

The effect of insulin and secretagogues on glucose transport was studied by the infinite-*cis* technique, the time course for glucose transport being represented as an integrated rate equation as mentioned above. Chromaffin cells, preincubated in the presence of insulin (8.33 nM) for 5 min, showed increase in the capacity of glucose transport, as shown in Fig. 6(*b*), where the K_m value remains unchanged. This increase ranges from 200 to 250%, depending on the cell preparation. The optimum insulin concentration needed to obtain this increase is approx. 8 nM, which corresponds to twice the K_D value for the hormone receptor.

Acetylcholine and nicotine, which are both potent secretagogues in the adrenal medulla, had a clear effect on glucose transport, as shown in Figs. 6(*c*) and 6(*d*). The affinity of these transporters, with respect to control, did not change; however, their capacity, expressed as a $V_{max.}$, always increased. As with insulin, each cell preparation showed a different $V_{max.}$ value, although in all cases the increase in response to the optimal concentrations of nicotine (10 μM) and acetylcholine (50 μM) was about 300%.

Cytochalasin-binding studies to chromaffin cells in the presence of insulin (30000 μunits/ml) showed the same affinity ($K_D = 0.1038$ μM) and $B_{max.}$ (maximal binding capacity) = 837452 receptors/cell, which corresponds to about a 2-fold increase in the specific maximal binding capacity.

Acetylcholine (50 μM) also produce an increase in the glucose transporters at the plasma-membrane level, to about $B_{max.} = 700000$ receptors/cell, without changing the dissociation constant ($K_D = 0.12$ μM). The changes in glucose transport and in cytochalasin-binding capacity produced by insulin or secretagogues that mimic acetylcholine, e.g. nicotine, correlate well with the increase in glucose consumption by these cells. The maximum effect is obtained at 8 nM, 50 μM and 10 μM for insulin, acetylcholine and nicotine respectively, which also corresponds to results of optimum transport and cytochalasin B-binding experiments.

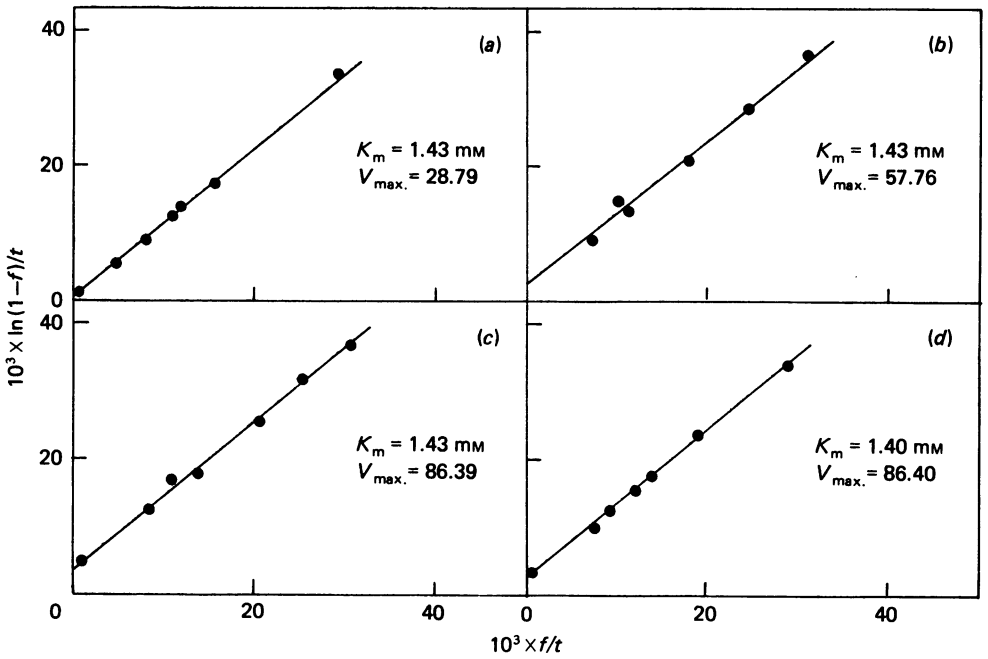

Fig. 6. Infinite-*cis* uptake in isolated chromaffin cells, submitted to several effectors

(*a*) Control, carried out as in Fig. 3. (*b*) Cells in the presence of insulin (8 nM) incubated for 5 min before starting the experiment. (*c*) Cells in the presence of 50 μM-acetylcholine incubated for 1 min before starting. (*d*) Cells treated with nicotine (10 μM) during 1 min before starting. $V_{max.}$ is expressed as mmol/litre of cells per min. This represents a typical experiment performed in triplicate with the same cell preparation. Differences between acetylcholine- or nicotine-treated cells and controls are more significant ($P < 0.01$) than those with insulin ($P < 0.05$).

DISCUSSION

These studies have demonstrated that chromaffin cells, like most mammalian cells, take up glucose by facilitated diffusion, but, to date, little attention has been paid to glucose transport in a homogeneous neural cell population. Although there have been very elegant approaches carried out with the giant axons of *Loligo*, in which the parameters of glucose, 2-deoxyglucose and 3-*O*-methylglucose transport were studied (Baker & Carruthers, 1981*a*,*b*), the results are difficult to extrapolate to mammalian neural cells. In the work reported here, when glucose was used, the affinity of the transporters at the outer face of the membrane [$K_m = 1.02 \pm 0.09$ (6) mM] agreed well with that reported by several authors for dissociated brain cells and other purified preparations of neural cells in primary cultures (Roeder & Tildon, 1984; Roeder *et al.*, 1985).

The K_m values obtained by the infinite-*cis* experiments, in which inhibition of net flux of saturating glucose was measured, were the same [$K_m = 1.204 \pm 0.52$ (5) mM] as those at the outer face of the membrane, as is the case with most models studied, such as erythrocytes and isolated rat adipocytes (Wheeler & Hinkle, 1985).

Nevertheless, the capacity expressed as a $V_{max.}$ was different for the two techniques. The $V_{max.}$ calculated at the outer face was 1.307 mmol/litre of cells per min, smaller than that found by the infinite-*cis* technique at the inner face [50.75 ± 22.45 (5) mmol/litre of cells per min], and also correlating well with the results in other cell lines, in which the exchange was faster than the net flux (Graff *et al.*, 1981; Plageman *et al.*, 1981).

Compared with glucose, the lower 2-deoxyglucose affinity of transporters ($K_m = 2.3$ mM) was compensated by a higher entrance rate, the $V_{max.}$. Similar findings were described by Weber (1973) and Baker & Carruthers (1981*a*) for cells of neural and non-neural origin. More recently, in isolated bovine adrenal chromaffin cells, glucose-transport characteristics have been studied with a non-metabolizable glucose analogue, 3-*O*-methyl-D-glucose (Bigornia & Bihler, 1986). The affinity described for the outer face of the membrane was 8.2 mM, which correlates well with values obtained with this sugar in other tissues (Whitesell & Gliemann, 1979).

To characterize and quantify the glucose transporters in chromaffin cells, two specific ligands have been used: (i) phlorizin, which showed no specific binding, and (ii) cytochalasin B. Our findings indicated the presence of cytochalasin B binding sites specifically displaceable by D-glucose in recently isolated and cultured chromaffin cells. The affinity of this ligand for the glucose transporters ($K_D = 0.09$ μM) is very similar to that reported in the literature for tissues from neural and non-neural origin (Simpson *et al.*, 1983; Dick *et al.*, 1984; Dick & Harik, 1986).

The isolated and cultured chromaffin cells had a high density of glucose transporters when the maximal binding capacity of cytochalasin B displaceable by D-glucose is considered, this being similar to that reported for adipocytes when expressed as number of receptors per cell (Cushman & Wardzala, 1980; Simpson *et al.* 1983). When the binding capacity was expressed as pmol/mg of protein, the value obtained was close to that found for particulate fractions of rat brain by Dick & Harik (1986).

The specific glucose-displaceable cytochalasin binding is an important tool with which to understand the biological action of insulin at the plasma-membrane

level. In the present results, the increase in glucose transport is well correlated with the rise in the number of transporters, both showing a 2-fold increase. Bigornia & Bihler (1986) have also reported a similar insulin effect on 3-*O*-methylglucose transport in these cells. Thus, although the influence of insulin on glucose transport in brain cells is controversial, there is increasing evidence suggesting that insulin has an effect on isolated chromaffin cells. The present paper shows that chromaffin cells have high-affinity insulin receptors (K_D = 3.95 nM), as demonstrated for other neural tissues by Raizada *et al.* (1982) in primary neuron-enriched cultures from fetal rat brain, and by Zahniser *et al.* (1984) in membranes isolated from specific areas of rat brain. The number of specific ^{125}I-insulin-binding sites [68000 ± 7500 (3) receptors/cell] is higher than that described in cells such as fibroblasts (Cynober *et al.*, 1985), myocytes (Standaert & Pollet, 1984) and also neural tumoral cells, such as Harding–Passey melanoma cells (Delicado *et al.*, 1986), which are not very sensitive to insulin. Nevertheless, the number of receptors was similar to that found in other cells, such as adipocytes (Flint *et al.*, 1979) and hepatocytes (Cech *et al.*, 1980), where the insulin effect at the molecular level is well known.

Central neural tissues in general do not appear to be dependent on insulin for carbohydrate or protein metabolism (Hendricks *et al.*, 1984), insulin and related substances being considered, at present, only as modulatory peptides or growth factors (Garcia Segura *et al.*, 1986).

Nevertheless, as chromaffin cells are submitted to the physiological peripheral blood changes in metabolite concentration, insulin must play an important role in the control of glucose transport and utilization. Other effectors, such as acetylcholine, also participate in chromaffin-cell glucose transport. Millaruelo *et al.* (1982) have reported the importance of protein kinases, Ca^{2+}- and cyclic-AMP-dependent, on glycogen storage and metabolism in these cells.

The results in the present paper enable us to suggest that adrenal chromaffin cells may prove to be a suitable model to study the regulation of sugar transport in a well-differentiated neural cell population.

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