Presence of insulin receptors in cultured glial C6 cells

Regulation by butyrate

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The presence of insulin receptor and its regulation by butyrate and other short-chain fatty acids was studied in C6 cells, a rat glioma cell line. Intact C6 cells bind ^{125}I -insulin in a rapid, reversible and specific manner. Scatchard analysis of the binding data gives typical curvilinear plots with apparent affinities of approx. 6 nM and 70 nM for the low-affinity (approx. 90 % of total) and high-affinity (approx. 10 % of total) sites respectively. Incubation with butyrate results in a time- and dose-dependent decrease of insulin binding to C6 cells. A maximal effect was found with 2 mM-butyrate that decreased the receptor by 40–70 % after 48 h. Butyrate decreased numbers of receptors of both classes, but did not significantly alter receptor affinity. Other short-chain fatty acids, as well as keto acids, had a similar effect, but with a lower potency. Cycloheximide caused an accumulation of insulin receptors at the cell surface, since insulin binding increased and receptor affinity did not change after incubation with the inhibitor. Simultaneous addition of butyrate and cycloheximide abolished the loss of receptors produced by the fatty acid. In cells preincubated with butyrate, cycloheximide also produced a large increase in receptor numbers, showing that in the absence of new receptor synthesis a large pool of receptors re-appears at the surface of butyrate-treated cells.

INTRODUCTION

Insulin and insulin receptors are abundant in brain cells [1,2], which suggests that this hormone could play a role in the regulation of growth, development and function of the nervous system.

In mixed cultures (glial and neuronal) of fetal rat brain cells the presence of functionally active insulin receptors has been shown [3]. More specifically, glial cells appear to be a target for insulin action, since homogeneous glial cultures from neonatal rat brain possess insulin receptors, and insulin stimulates 2-deoxy-D-glucose uptake in these cultures [4].

As characterized by insulin-binding studies, the brain receptor appears similar [5], although with somewhat lower affinity than non-neural receptors [6]. Photoaffinity labelling revealed that there may be structural differences between receptors from brain cells and those from peripheral cells [6], which may reflect differences in the carbohydrate components [7]. However, the insulin receptor of the rat brain is also coupled with tyrosine kinase activity, which, as in other types of cells, phosphorylates the receptor as well as exogenous substrates [8]. In addition, receptor number might not be regulated in the same way as the peripheral receptor by insulin [6,9].

Cellular and membrane lipid composition may have profound effects on insulin binding and action. It has been reported that incubation of cells in medium enriched in fatty acids with chain length between 10 and 18 carbon atoms leads to changes in insulin receptor number [10–13]. Short-chain fatty acids, such as butyrate, profoundly affect proliferation and differentiation of mammalian cells in culture [14], including C6 cells (a rat glioma cell line) [15,16], and are therefore potential modulators of insulin binding in those cells. Butyrate is

a natural C_4 fatty acid normally present in lipids, which constitutes 10 % of milk fatty acids [14]. This compound acts at relatively low concentrations (around 5 mM), and other short-chain fatty acids are much less active than butyrate.

In this study we have examined the regulation of the insulin receptor by butyrate in C6 cells. Our results indicate that C6 cells bind insulin specifically, and that butyrate and other short-chain fatty acids down-regulate surface insulin-receptor number. On the other hand, inhibition of protein synthesis with cycloheximide results in accumulation of receptors at the cell membrane. The finding that this accumulation also occurs in cells previously incubated with butyrate strongly suggests, although does not unequivocally prove, that the fatty acid could modulate the cellular distribution of receptors.

MATERIALS AND METHODS

Hormones and chemicals

Bovine and porcine insulin were from Sigma. Insulin-like growth factor I (IGF-I) was obtained from Amgen Biologicals (Thousand Oaks, CA, U.S.A.), and human proinsulin from Lilly. ^{125}I for protein iodination, L-[3,4,5- ^3H]leucine (sp. radioactivity 142 Ci/mmol) and 2-deoxy-D-[2,6- ^3H]glucose (^3H deoxyglucose) (sp. radioactivity 33 Ci/mmol) were from Amersham. Short-chain fatty acids, cycloheximide, bovine serum albumin and 2-deoxy-D-glucose were from Sigma. Culture medium and sera were from Gibco and plastic flasks and plates were purchased from Nunc or Linbro (Flow Laboratories).

Cell cultures

C6 glial cells were grown as monolayers as previously described [17]. For the experiments the cells were plated

Abbreviations used: IGF-I, insulin-like growth factor I; PBS, phosphate-buffered saline.

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at an initial density of 40 000–60 000 cells in six-well 9 cm² plates. After 3 days the medium was replaced by a medium containing 10% (v/v) newborn-calf serum treated with the ion-exchange resin AG 1X8 (Bio-Rad) and charcoal as previously described [18]. With this treatment more than 90% of exogenously added ¹²⁵I-insulin is removed from the serum. The cells were grown in this medium for 24 h before the addition of butyrate or other compounds that were used at the concentrations and for the times indicated in the text. The treatment with butyrate was initiated in rapidly growing cells, and at the end of the experimental period a control culture normally contains 700–1000 µg of protein, which is equivalent to 6×10^6 – 10×10^6 cells.

Binding studies

Insulin was iodinated with chloramine-T to a specific radioactivity of 150–200 µCi/µg [19] and purified on cellulose CF-11 columns. The purity of the preparation was checked by h.p.l.c. on a C₈ Ultrasphere column (150 mm × 4.2 mm internal diam.; Altex) under the conditions described by Ilondo *et al.* [20]. All radioactivity was present essentially in a single peak with the same mobility as the native unlabelled hormone. The bindability of the iodinated insulin was assessed by two successive incubations of a tracer amount of ¹²⁵I-insulin (25 pM) with a large number of cells (approx. 200×10^6 cells) as previously described [20]. The maximal bindability of the preparation was about 70–80% both before and after re-purification by h.p.l.c., and the hormone was not repurified for the experiments, since the fraction of insulin denatured by the labelling procedure seems to be small.

Before binding experiments, cell monolayers were washed with a buffer containing 0.1 M-Hepes, 120 mM-NaCl, 1.2 mM-MgSO₄, 5 mM-KCl, 15 mM-sodium acetate, 10 mM-glucose and 0.2% (w/v) bovine serum albumin. The presence of acetate in the buffer did not influence binding. Unless otherwise stated, the binding assay was carried out in the monolayers in 1 ml of this buffer containing 0.3–0.4 nM-¹²⁵I-insulin at 20–22 °C. Binding achieved a maximum between 70 and 120 min, and a 2 h incubation time was used in the experiments. Non-specific binding was determined in the presence of 15 µg of unlabelled insulin/ml. The non-specific binding was subtracted from the total to determine the receptor-associated hormone. After binding, the medium was removed by aspiration and the cells were quickly washed with 5 ml of ice-cold 0.01 M-phosphate/0.15 M-NaCl, pH 7.6 (PBS), containing 0.1% albumin, and once with PBS without albumin. The cells were then lysed in 0.4 M-NaOH and transferred to tubes for determination of cell-associated radioactivity. A portion of the lysate was used for determination of cell protein by the method of Lowry *et al.* [21]. All binding data were normalized to 100 µg of cell protein and represent the mean of triplicate cultures from which the non-specific binding has been subtracted.

Insulin degradation was monitored by assessing the ability of ¹²⁵I-insulin to be precipitated with 15% (w/v) trichloroacetic acid. Trichloroacetic acid-soluble radioactivity was considered to be degraded insulin.

Measurement of precursor uptake

The uptake of deoxyglucose was measured in 2 cm² 24-well plates as described with monolayers of cultured glial cells [4]. Cell monolayers were grown in the regular

medium and shifted to serum-free medium 15 h before uptake was measured. The cultures received 0.3 ml of binding buffer without glucose and albumin, and were incubated with insulin for 8 min at 37 °C. Thereafter, 0.2 mM unlabelled deoxyglucose and 0.8 µCi of [³H]-deoxyglucose were added. The reaction was allowed to proceed at room temperature for 5 min and was terminated by aspiration and extensive washing with ice-cold PBS.

Protein synthesis was studied by incubation of the cells with 2 µCi of [³H]leucine/ml for 2 h at 37 °C. The cells were washed with PBS and then received 1 ml of 15% trichloroacetic acid. The trichloroacetic acid-soluble material was discarded, and the incorporation was measured in the trichloroacetic acid-insoluble material.

In both types of studies, the cells were digested with 0.2 M-NaOH, and samples of the digests were taken for protein and radioactivity measurements.

Calculations

All data points presented are means or means ± S.D. of at least triplicate cultures that did not vary by more than 10–15%. Scatchard plots were analysed according to a two-sites model with an Apple IIe computer by using the ISIS 12 program from Rodbard *et al.* [22].

RESULTS

¹²⁵I-Insulin binding to C6 cells

Incubation of C6-cell monolayers with ¹²⁵I-insulin resulted in a rapid binding, with a maximum attained between 70 and 120 min at 20–22 °C. After incubation with 0.3 nM-iodoinsulin for 120 min, 1–2 fmol of insulin/

Fig. 1. Kinetics of ¹²⁵I-insulin dissociation from C6 cells

The cultures were incubated with ¹²⁵I-insulin for 2 h at 20 °C. The cells were then thoroughly rinsed to remove unbound hormone, and subsequently incubated with (○) or without (●) 10 µg of insulin/ml for the indicated time periods. The labelled hormone that remained bound to the cells was measured at each time. The results are represented as percentages of the initial binding, and represent means of triplicate cultures with variation less than 10%.

100 μg of cell protein was bound specifically. The non-specific binding was 10–15 % of the total binding, and under these binding conditions iodoinsulin degradation was small (less than 5–10 %). Fig. 1 shows the kinetics of dissociation of ^{125}I -insulin either by dilution alone or in the presence of 10 μg of unlabelled insulin/ml. As observed in other systems, unlabelled insulin induced a marked acceleration of the dissociation, and in these conditions 50 % of the ^{125}I -insulin was dissociated at 9–10 min and more than 85 % at 60 min.

Fig. 2 shows the result of a typical competition study in C6 cells. The concentration of insulin that inhibited 50 % of the binding (ED_{50}) was 60 ng/ml in this particular experiment. By Scatchard analysis the maximal binding capacity was calculated to be 67 fmol/100 μg of cell protein. By using a two-site model, the curvilinear plot was resolved into two components. The binding parameters obtained by averaging the results from seven different experiments were as follows: the estimated binding capacity was 10.1 ± 2.5 and 81 ± 18 fmol/100 μg of protein for the high- and low-affinity sites respectively, which represents approx. 7500 and 60000 sites/cell, and the dissociation constants of the receptor sites were 6 ± 1 nM for high-affinity receptors and 70 ± 7 nM for low-affinity receptors.

Fig. 3 shows the specificity of insulin binding to C6 cells. Unlabelled insulin was more potent in competing for binding to receptors than was IGF-I, and proinsulin was less potent. Other peptides, such as glucagon, growth hormone or prolactin, did not compete at all.

Fig. 2. ^{125}I -insulin binding to C6 glioma-cell monolayers

Cells were plated and grown as described in the Materials and methods section. Before binding studies, the cells were shifted to an insulin-depleted medium and grown for a further 48 h. The cells were incubated for 2 h with ^{125}I -insulin and various concentrations of unlabelled insulin. Hormone-binding activity was measured, and all values were corrected for non-specific binding. The Figure shows a Scatchard plot of the results of a typical cell-monolayer insulin-binding study, and the inset represents the competition for binding of ^{125}I -insulin by the unlabelled hormone. Each data point represents the mean of three different cultures. B_0 is maximum label bound.

Fig. 3. Specificity of insulin binding to C6 cells

The cells were incubated with ^{125}I -insulin with or without the following unlabelled peptides: pig insulin ($\circ$), bovine insulin ($\bullet$), IGF-I ($\square$), proinsulin ($\triangle$), glucagon ($\blacktriangle$), prolactin ($\blacksquare$), growth hormone ($\star$). Binding is expressed as a percentage of the maximum binding obtained in the absence of unlabelled peptides. Each point represents the average of triplicate cultures.

Fig. 4. Effect of butyrate on regulation of insulin receptors in cultured C6 cells

Cells were exposed to various concentrations of butyrate for 48 h (a) or to 2 mM butyrate for the times indicated (b). Specific binding of ^{125}I -insulin to intact cells was determined. The results are plotted as percentages of binding by control cells that did not receive butyrate, and are means of three separate cultures that did not vary by more than 5–10 %.

Effect of butyrate on ^{125}I -insulin binding

Fig. 4(a) shows the effect of incubation for 48 h with different concentrations of butyrate on ^{125}I -insulin binding to C6 cells. Butyrate produced a dose-dependent decrease in cell-associated radioactivity. Half-maximal inhibition was obtained at 0.2 mM-butyrate, and the maximal decrease (40–50 % of the control) was found

Table 1. ¹²⁵I-insulin resistant to mild acid wash at 4° and 20 °C in control and butyrate-treated cells

C6 cells were incubated with or without 2 mM-butyrate for 48 h. Binding studies were performed either for 2 h at 20 °C or for 18 h at 4 °C. The cultures were then washed twice with PBS, pH 7.6 or pH 6.0, and incubated for 15 min at 20 °C. The buffer was changed again and the monolayers were incubated for a further 5 min at 37 °C. The buffer was then removed by aspiration, the cells were lysed with NaOH, and the cell-associated radioactivity was determined.

	Binding at 20 °C (fmol/100 µg of protein)	
	pH 7.6	pH 6.0
Control	1.78 ± 0.04	0.23 ± 0.01
Butyrate	0.84 ± 0.03	0.11 ± 0.01
	Binding at 4 °C (fmol/100 µg of protein)	
	pH 7.6	pH 6.0
Control	2.17 ± 0.20	0.25 ± 0.04
Butyrate	1.43 ± 0.15	0.14 ± 0.03

with 2 mM fatty acid. The inhibition of insulin binding was due to a decrease in specific binding, with little or no change in non-specific binding, and does not represent an artifact owing to increased degradation of the ¹²⁵I-insulin.

Fig. 4(b) shows that the effect of butyrate is also time-dependent. Insulin binding increased slightly during the first hours of incubation with butyrate, and decreased thereafter, being 60% of control at 24 h and 40% of control at 48 h. The decrease in receptor was maximal between 48 and 72 h, and longer incubation times did not elicit a further decrease (results not shown).

As shown in Table 1, the effect of butyrate was also observed when the binding studies were performed at 4 °C. In addition, only a small fraction of radioligand (less than 15%) was resistant to acid-wash [23] after binding either at 4° or 20 °C, both in control cells and in cells incubated with butyrate. This suggests that ligand internalization is not quantitatively important at 20 °C, and that the decrease in cell-associated radioactivity produced by the fatty acid represents a decrease in cell-surface binding.

As shown in Table 2, and in agreement with our previous results showing that butyrate inhibits DNA synthesis in C6 cells [15,16], 2 mM-butyrate inhibited cell growth. This concentration of butyrate decreased [³H]thymidine incorporation by more than 90% (results not shown), but it did not appreciably change protein content per cell or [³H]leucine incorporation into total cell protein. Additionally, as assessed by Trypan Blue exclusion, this concentration of the fatty acid did not affect cell viability.

Saturation binding studies of ¹²⁵I-insulin to cell monolayers were performed in untreated cells and in cells incubated for 48 h with 2 mM-butyrate. Fig. 5 shows that the Scatchard plots from control and butyrate-treated cells were roughly parallel. Butyrate decreased receptor concentration of both high- and low-affinity sites. The number of binding sites decreased from 12.2 and 80 fmol/100 µg of protein in untreated to 6.2 and 33 fmol/100 µg of protein in butyrate-treated cells, which represents a 49 and 59% decrease respectively, whereas affinity did not change much (7 versus 8 nM and 80 versus 77 nM). Similar results were obtained in two additional experiments, in which affinity was not altered but receptor number decreased by 40–70%.

Effect of different short-chain fatty acids and ketone bodies on insulin binding to C6 cells

Table 3 compares the effect of a 48 h incubation with C₃–C₅ fatty acids. Butyrate was the most active compound, and propionate and valerate were also effective, although less potent than butyrate. The influence of the fatty acids on protein content per culture paralleled the decrease in insulin binding, and acetate and isobutyrate, an isomer form of butyrate, did not appreciably affect protein content, nor did they alter receptor numbers. β-Hydroxybutyrate and acetoacetate also altered insulin binding, but their potency was much lower than that of butyrate. At 10 mM the effect of acetoacetate on the receptor and protein content was similar, and that of β-hydroxybutyrate less pronounced, than that caused by 2 mM-butyrate.

Effect of the inhibition of protein synthesis on the response to butyrate

To study whether protein synthesis was necessary for the effect of butyrate to occur, insulin binding was measured in C6 cells incubated for different time periods with butyrate, cycloheximide and the combination of both. Fig. 6 shows that incubation with cycloheximide, at a concentration that inhibits protein synthesis in C6 cells [17], does not decrease cell-associated radioactivity,

Table 2. Effect of butyrate on C6-cell proliferation and protein synthesis

C6 cell cultures were grown under the conditions described in the Materials and methods section. When the cultures contained $4.2(\pm 0.4) \times 10^6$ cells and 417 ± 35 µg of protein, they were incubated with or without 2 mM-butyrate for 48 h. The cells were then counted with a haemocytometer and their protein content was measured. [³H]Leucine incorporation into protein was determined in parallel cultures as described in the Materials and methods section. Results are means ± S.D. of three or four cultures.

	10 ⁻⁶ × No. of cells/culture	Protein (µg/culture)	Protein/cell (pg)	[³ H]Leucine incorporation (c.p.m./100 µg of protein)
Control	10.4 ± 0.9	1013 ± 75	98 ± 19	20092 ± 1037
Butyrate	7.2 ± 0.3	800 ± 70	111 ± 6	21167 ± 3173

Fig. 5. Scatchard plot of binding of ^{125}I -insulin to untreated and butyrate-treated C6-cell cultures

The cells were incubated without (●) or with (○) 2 mM-butyrate. After 48 h the cultures were incubated with ^{125}I -insulin in the presence of increasing concentrations of unlabelled insulin, and cell-associated radioactivity was determined. The results shown are representative of one experiment, which was repeated three separate times with similar results.

but rather produces a paradoxical increase in insulin binding to C6 cells. After long-term incubations (24–48 h) cycloheximide normally caused a 20–40 % decrease in total cellular protein, but did not induce important changes in the protein/DNA ratio or in the protein content per cell (results not shown).

Fig. 6 also shows that butyrate decreased insulin binding, and that when the cells received butyrate and cycloheximide simultaneously the inhibitor totally blocked the receptor loss produced by butyrate.

As shown in Fig. 7, the increase in receptor numbers caused by cycloheximide was also dose-dependent. A half-maximal response was found at 2 μM , and concentrations higher than 25 μM did not induce further increases.

Fig. 8 compares the Scatchard plots of ^{125}I -insulin binding to control cells and to cells incubated with 5 μM -cycloheximide for 20 h. The treatment increased high- and low-affinity receptor concentrations by 40 and 60 % respectively, but did not significantly alter the affinity of the receptor.

As shown in Fig. 9, cycloheximide also produced a time-dependent increase in binding in cells preincubated for 48 h with 2 mM-butyrate. At the maximal time interval of incubation with the inhibitor (9 h) studied in this experiment, receptor numbers in the cells receiving butyrate+cycloheximide were lower than in the cells incubated with cycloheximide alone. However, after longer times of incubation (24 h) with cycloheximide the effect of butyrate was totally reversed (Table 4).

Next, we studied the ability of C6 cells to regenerate receptors after incubation with butyrate in the presence and the absence of protein synthesis *de novo* (Fig. 10). Cells were incubated in the absence or the presence of 2 mM-butyrate for 48 h. The cultures were then washed free of butyrate, and the cells were incubated further for up to 48 h with butyrate, and without butyrate and/or cycloheximide. When cells preincubated with butyrate were resuspended in butyrate-free medium, receptor numbers did not reach control values even after 48 h of

Table 3. Effect of short-chain fatty acids and keto acids on insulin binding and protein content

C6 cells were incubated with the concentrations of the compounds indicated for 48 h in three separate experiments. Insulin binding was measured after incubation with 0.3 nM- ^{125}I -insulin as described in the Materials and methods section. All data are means $\pm$ S.D. of triplicate cultures.

Expt. no.	Compound tested	Concn. (mM)	^{125}I -insulin bound (fmol/100 μg of protein)	Protein (μg /culture)
I	Control		1.8 ± 0.04	825 ± 33
	Butyrate	2	0.9 ± 0.04	523 ± 23
	Propionate	2	1.2 ± 0.11	685 ± 37
		10	0.6 ± 0.02	509 ± 35
	Valerate	2	1.2 ± 0.14	712 ± 32
		10	0.7 ± 0.01	598 ± 57
II	Control		1.5 ± 0.11	1308 ± 67
	Butyrate	2	1.0 ± 0.03	932 ± 28
	Acetate	2	1.6 ± 0.05	1320 ± 46
		10	1.3 ± 0.08	1219 ± 22
	Isobutyrate	2	1.4 ± 0.04	1296 ± 39
		10	1.3 ± 0.05	1187 ± 57
III	Control		1.7 ± 0.09	665 ± 59
	Butyrate	2	0.8 ± 0.05	360 ± 36
	β -Hydroxybutyrate	2	1.5 ± 0.18	653 ± 30
		10	1.1 ± 0.03	581 ± 23
	Acetoacetate	2	1.6 ± 0.09	660 ± 53
		10	0.9 ± 0.12	335 ± 34

Fig. 6. Effect of inhibition of protein synthesis on butyrate-induced receptor loss

C6 cells were incubated with 2 mM-butyrate (○), 5 μ M-cycloheximide (■) or both (□) for different time periods. The cells were then incubated with 125 I-insulin and specific binding was determined. In this and in the following Figures each data point represents the mean of three different cultures with variation less than 5–15%.

Fig. 7. Effect of cycloheximide on the concentration of insulin receptors in C6 cells

The cells were incubated with the concentration of cycloheximide indicated for 24 h. Receptor concentrations were determined after incubation with 125 I-insulin.

butyrate removal. If, however, the cells were resuspended in fresh medium plus cycloheximide the number of insulin receptors was the same as the values observed with control cells incubated with cycloheximide alone. These results are similar to those found when cycloheximide was added with the fatty acid present in the culture medium, and show that after inhibition of protein synthesis the full complement of insulin receptors appears in the cells incubated with butyrate.

The effect of butyrate on the receptor was not irreversible, since in separate experiments we have observed that 96 h after butyrate removal receptor numbers were already normalized.

Fig. 8. Scatchard plot of insulin binding in the presence of cycloheximide

The cells were incubated with (■) or without (●) 5 μ M-cycloheximide for 20 h. Thereafter, specific binding of 125 I-insulin was determined in the presence of increasing concentrations of unlabelled hormone.

Fig. 9. Effect of cycloheximide on insulin binding to C6 cells preincubated with butyrate

The cultures were incubated in the presence or absence of 2 mM-butyrate for 48 h. Cycloheximide (at 5 μ M) was then added to half of the control and butyrate-treated cultures, and the incubation was continued for the times indicated in the Figure. At 4.5, 7 and 9 h after adding cycloheximide, receptor amounts were measured by incubation with 125 I-insulin. ●, Control; ○, butyrate; ■, control + cycloheximide; □, butyrate + cycloheximide.

Effect of butyrate on deoxyglucose uptake

Deoxyglucose uptake was determined in control C6 cells and in cells treated with butyrate. Basal rates of uptake and the maximal response to insulin in C6 cells were very similar to those found in non-transformed glial

Table 4. Receptor concentrations in cells incubated with butyrate and cycloheximide

Control cells and cells that had received 2 mM-butyrate for 48 h were incubated in the absence or the presence of 5 μ M-cycloheximide for a further 24 h, and 125 I-insulin binding was determined. Data are means $\pm$ S.D. for triplicate cultures.

	125 I-insulin bound (fmol/100 μ g of protein)
Control	1.3 $\pm$ 0.1
Butyrate	0.7 $\pm$ 0.1
Cycloheximide	3.7 $\pm$ 0.4
Butyrate + cycloheximide	3.3 $\pm$ 0.2

Fig. 10. Insulin binding following butyrate removal in the presence and absence of cycloheximide

The cells were incubated with and without 2 mM-butyrate for 48 h. At the end of this period all cultures were washed (time 0 in the Figure). A third of the cultures that had been incubated with butyrate received again the same concentration of the fatty acid (○), another third received the medium alone (Δ) and the remaining third received 5 μ M-cycloheximide (□). Receptor concentrations were determined at the times indicated in these groups, as well as in groups of control cells (●) and control cells incubated with cycloheximide (■) for the same periods of time.

cells [4]. As shown in Fig. 11, butyrate did not change basal uptake, but produced a very significant decrease in the insulin-stimulated component of deoxyglucose uptake.

DISCUSSION

C6 glial cells bind insulin specifically in a rapid, saturable and reversible manner, which suggests that the binding represents a true insulin-receptor interaction. In addition, binding parameters were similar to those found in cultured brain cells [3,6], and Scatchard plots were

Fig. 11. Effect of butyrate on deoxyglucose uptake

The cells were incubated in serum-containing medium for 48 h in the presence or absence of 2 mM-butyrate. The cells were then shifted to serum-free medium, butyrate was added again, and deoxyglucose uptake was measured 15 h later, after incubation with the concentrations of insulin indicated as described in the Materials and methods section. All data points are means $\pm$ S.D. of five separate cultures: ●, control; ○, + butyrate.

curvilinear as those of insulin receptors from neural and non-neural cells, suggesting the existence of either negative co-operativity or two separated binding sites.

In C6 cells insulin increased deoxyglucose uptake, a classic metabolic event typical of the biological action of insulin in non-neural cells, although a relatively high concentration of hormone is necessary for this effect to occur. This increase is also found in non-transformed glial cells in culture [4], and shows that C6 cells could be a good model for the study of insulin binding and action in glia. Others have found that insulin did not significantly increase deoxyglucose uptake in mixed cultures of brain cells [6], which might reflect the heterogeneity of the cellular composition of the brain.

Fatty acids are important modulators of the growth and metabolism of brain cells. These cells are able to metabolize short-chain fatty acids, which are rapidly incorporated into glutamine and related amino acids [24]. Short-chain fatty acids have also a role in the synthesis of long-chain fatty acids, and it has been described that C6 cells can elongate exogenous acetate and other fatty acids and incorporate them into complex lipids, phospholipids and triacylglycerol [25].

Butyrate causes a time- and dose-dependent decrease in insulin binding to C6 cells, and other fatty acids, such as propionate or valerate, also decrease the receptor, although they are less potent. Butyrate does not significantly alter the affinity of the receptor for insulin, but rather decreases the estimated number of binding sites. This effect does not seem to reflect a generalized toxic action, since protein synthesis was not altered by the fatty acid.

The mechanism(s) by which the fatty acid modulates receptor number in C6 cells has not yet been determined. Butyrate might alter the physical state of the plasma membrane, which in turn might affect the receptor. Related to this, it has been shown that butyrate influences

membrane ganglioside composition [26], and that other fatty acids added exogenously to the culture medium change membrane lipid composition, and as a consequence membrane fluidity and insulin binding and action in different cell types [10–12].

On the other hand, the variety of effects of butyrate and other short-chain fatty acids has been normally attributed to the hyperacetylation of histones [27] and other chromatin proteins caused by the inhibition of the nuclear deacetylases [28]. Butyrate inhibits histone deacetylation in C6 cells [29,30], but the present data do not suggest a close relationship between the inhibition of histone deacetylation and the modulation of insulin receptor concentrations, since acetoacetate and β -hydroxybutyrate decreased insulin receptor concentrations, despite being inactive in changing histone acetylation [28], and isobutyrate, which produces histone acetylation [28–30], did not decrease insulin binding to C6 cells.

In other cultured cells the concentration of insulin receptors has been reported to vary with cell density and the stage of growth of the cultures, as well as with the stage of the cell cycle [13,31–33]. Butyrate, which decreased the receptor concentrations, also affected proliferation of C6 cells; other fatty acids and keto acids lowered insulin receptor concentrations only at concentrations that also decreased proliferation, and isobutyrate and acetate, which did not change the receptor concentrations, also did not alter C6-cell growth appreciably. In addition, we have very recently demonstrated that glucocorticoids and cyclic AMP, which inhibit C6-cell proliferation [16], also modulate the insulin receptor in these cells [34,35]. Therefore an inhibition of cell replication might be related to the regulation of receptor concentrations by butyrate in C6 cells. Interestingly, both butyrate and cyclic AMP produce an early increase of insulin binding, followed by a significant decrease after longer periods (24–48 h). The reason for this biphasic modulation is unknown at present.

For homologous down-regulation by insulin, an increase in receptor degradation [36–38] or a translocation of receptor to an intracellular compartment without altering total cellular contents of receptors [39, 40] have been described in different cell types, although insulin-receptor down-regulation may not require internalization in IM-9 lymphocytes [41]. Therefore, in addition to a decrease in receptor synthesis *de novo* or to an increase in receptor degradation, butyrate could modulate the distribution of pre-existing receptor. It is also possible that in butyrate-treated cells the receptor is still associated with the plasma membrane, but in a conformation that does not allow for hormone binding.

Inhibition of protein synthesis with cycloheximide is associated with an accumulation of insulin receptor at the cell surface of C6 cells. Whereas in IM-9 lymphocytes cycloheximide slowly decreases insulin receptors [42], this compound does not change insulin binding in erythrocytes or human fibroblasts [40,43] and, in complete agreement with our results in C6 cells, significantly increases receptor number in the surface of mouse fibroblasts [44]. This increase in the absence of new receptor synthesis could arise from a decrease of receptor degradation [45–47], an externalization of receptors from the intracellular compartment [48], and/or an inhibition of receptor internalization [47], and

would be only observed in cells having a substantial intracellular pool of insulin receptors [48].

Protein synthesis *de novo* is necessary for the insulin-induced down-regulation of receptors [42,43], and our results show that this is also the case with the butyrate-induced receptor loss in C6 cells, since cycloheximide completely blocked the effect of the fatty acid when both compounds were added together. Cycloheximide also increased receptor concentrations in cells that had been pre-treated with butyrate, and that therefore had a decreased number of receptors at the cell membrane. We have very recently shown that cycloheximide also blocks the effect of dexamethasone when both are added simultaneously to C6 cells, but, in contrast with that found with butyrate, this increase was not present in cells that had been pre-treated with glucocorticoids [43], suggesting that different mechanisms might mediate the effect of glucocorticoids and short-chain fatty acids on the receptor. The number of insulin receptors available for reinsertion in the cell surface cannot be supplied by receptor synthesis *de novo* in the presence of cycloheximide, and therefore the re-appearance of insulin receptors in the butyrate-treated cells is probably supplied by a preformed pool of receptors, which may be intracellular, or may be 'hidden' in the plasma membrane [40]. The most likely explanation for the effect of cycloheximide on the cells incubated with butyrate is that the fatty acid could decrease surface insulin receptors while having little effect on total cellular binding capacity. If this is so, butyrate would affect receptor internalization and/or would slow the rate of receptor recycling to the plasma membrane. Future studies are needed to clarify the role of fatty acids on each of these phenomena.

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REFERENCES

1. Havrankova, J., Schemmchel, D., Roth, J. & Brownstein, M. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5737–5741
2. Hendricks, S. A., Roth, J., Rishi, S. & Becker, K. L. (1983) in *Brain Peptides* (Krieger, D. T., Brownstein, M. J. & Martin, J. B., eds.), pp. 903–939, John Wiley and Sons, New York
3. Raizada, M. K., Yang, J. W. & Fellows, R. E. (1980) *Brain Res.* **200**, 389–400
4. Clarke, D. W., Boyd, F. T., Jr., Kappy, M. S. & Raizada, M. K. (1984) *J. Biol. Chem.* **259**, 11672–11675
5. Yip, C. C., Moule, M. L. & Yeung, C. W. T. (1980) *Biochem. Biophys. Res. Commun.* **96**, 1671–1678
6. Ciaraldi, T., Robbins, R., Leidy, J. W., Thamm, P. & Berhanu, P. (1985) *Endocrinology (Baltimore)* **116**, 2179–2185
7. Heidenreich, K. A., Zahniser, N. R., Berhanu, P., Brandenburg, D. & Olefsky, J. M. (1983) *J. Biol. Chem.* **258**, 8527–8530
8. Rees-Jones, R. W., Hendricks, S. A., Quarum, M. & Roth, J. (1984) *J. Biol. Chem.* **259**, 3470–3474
9. Pacold, S. T. & Blackard, W. G. (1979) *Endocrinology (Baltimore)* **105**, 1452–1457
10. Ginsberg, B. H., Brown, T. J., Simon, I. & Spector, A. A. (1981) *Diabetes* **30**, 773–780
11. Grunfeld, G., Baird, K. L. & Kahn, C. R. (1981) *Biochem. Biophys. Res. Commun.* **103**, 219–226

12. Ginsberg, B. H., Jabour, J. & Spector, A. A. (1982) *Biochim. Biophys. Acta* **690**, 157–164
13. Ginsberg, B. H. (1984) in *Biological Regulation and Development* (Goldberger, R. F. & Yamamoto, R. F., eds.), vol. 3B, pp. 59–97, Plenum Press, New York
14. Kruh, J. (1982) *Mol. Cell. Biochem.* **42**, 65–82
15. Ortiz-Caro, J., Yusta, B., Pascual, A. & Aranda, A. (1988) *Dev. Brain Res.* **42**, 229–237
16. Ortiz-Caro, J., Montiel, F., Yusta, B., Pascual, A. & Aranda, A. (1988) *Endocrinology* (Baltimore) **122**, 2597–2603
17. Montiel, F., Aranda, A., Villa, A. & Pascual, A. (1986) *J. Neurochem.* **47**, 1336–1343
18. Samuels, H. H., Stanley, F. & Shapiro, L. E. (1979) *Biochemistry* **18**, 715–721
19. Freychet, P., Kahn, C. R., Roth, J. & Neville, D. M., Jr. (1972) *J. Biol. Chem.* **247**, 3953–3961
20. Ilondo, M. M., Dehart, I. & De Meyts, P. (1986) *Biochem. Biophys. Res. Commun.* **134**, 671–677
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
22. Rodbard, D., Thakur, A. K. & Jaffe, M. L. (1980) BCTIC Computer Code Collection, Med-52, Biomedical Computing Technology Information Center (BCTIC) R-1302, Vanderbilt Medical Center, Nashville
23. Kahn, C. R. & Baird, K. (1978) *J. Biol. Chem.* **253**, 4900–4906
24. Reijnierse, G. L., Veldstra, H. & Van den Berg, C. J. (1975) *Biochem. J.* **152**, 477–484
25. Cook, H. W., Clarke, J. T. R. & Spence, M. W. (1982) *J. Lipid Res.* **23**, 1292–1300
26. Fishman, P. H., Simmons, J. L., Brady, R. O. & Freese, E. (1974) *Biochem. Biophys. Res. Commun.* **59**, 292–299
27. Riggs, M. G., Whittaker, R. G., Neumann, J. R. & Ingram, V. M. (1977) *Nature* (London) **268**, 462–464
28. Sealy, L. & Chalkley, R. (1978) *Cell* **14**, 115–121
29. Kumar, S., Weingarten, D. P., Callahan, J. W., Sachar, K. & de Vellis, J. (1984) *J. Neurochem.* **43**, 1455–1463
30. Ortiz-Caro, J., Montiel, F., Pascual, A. & Aranda, A. (1986) *J. Biol. Chem.* **261**, 13997–14004
31. Thomopoulos, P., Roth, J. & Lovelace, E. (1976) *Cell* **8**, 417–423
32. Reed, B. C., Kaufman, S. H., Mackall, J. C., Student, A. K. & Lane, M. D. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4876–4880
33. Karlsson, F. A., Grunfeld, C., Kahn, C. R. & Roth, J. (1979) *Endocrinology* (Baltimore) **104**, 1383–1392
34. Montiel, F., Ortiz-Caro, J., Villa, A., Pascual, A. & Aranda, A. (1987) *Endocrinology* (Baltimore) **121**, 258–265
35. Montiel, F., Aranda, A. & Pascual, A. (1988) *Mol. Cell. Endocrinol.*, in the press
36. Kasuga, M., Kahn, C. R., Hedro, J. A., Van Obberghen, E. & Yamada, K. M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6917–6921
37. Ronnet, G. V., Knutson, V. P. & Lane, M. D. (1982) *J. Biol. Chem.* **257**, 4285–4291
38. Marshall, S., Garvey, W. T. & Geller, M. (1984) *J. Biol. Chem.* **259**, 6376–6384
39. Krupp, M. & Lane, M. D. (1981) *J. Biol. Chem.* **256**, 1689–1694
40. Peterson, S. W., Miller, A. L., Kelleher, R. S. & Murray, E. F. (1983) *J. Biol. Chem.* **258**, 9605–9607
41. Ilondo, M. M., Courtoy, P. J., Geiger, D., Carpentier, J.-L., Rousseau, G. G. & De Meyts, P. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6460–6464
42. Kosmakos, F. C. & Roth, J. (1980) *J. Biol. Chem.* **255**, 9860–9869
43. Prince, M. J., Baldwin, D., Tsai, P. & Olefsky, J. M. (1981) *Endocrinology* (Baltimore) **109**, 1754–1759
44. Kadle, R., Kalter, V. G., Raizada, M. K. & Fellows, R. E. (1983) *J. Biol. Chem.* **258**, 13116–13119
45. Knutson, V. P., Ronnett, G. V. & Lane, M. D. (1985) *J. Biol. Chem.* **260**, 14180–14188
46. Reed, B. C., Ronnett, G. V., Clements, P. R. & Lane, M. D. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2909–2912
47. Heidenreich, K. A., Branderburg, D., Berhanu, P. & Olefsky, J. M. (1984) *J. Biol. Chem.* **259**, 6511–6515
48. Deutsch, P. J., Rosen, O. M. & Rubin, C. S. (1982) *J. Biol. Chem.* **257**, 5350–5358

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