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(Na^+,K^+)ATPase LEVELS IN PREADIPOCYTE CELL LINES ESTABLISHED FROM GENETICALLY-OBESE AND NON-OBESE MICE

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SUMMARY : (Na^+,K^+)ATPase levels were examined in clonal cell lines established from C57 BL/6J ob/ob (clone Ob17) and +/? (clone HGFu) mice. Both the number of [^{3}H]ouabain binding sites and the rate of [^{86}Rb] uptake showed no increase in HGFu cell line. These results, obtained on preadipocyte cells grown and differentiated under identical culture conditions, are not in favor of "intrinsic" differences between adipose cells from lean and obese mice regarding the Na^+/K^+-transport catalyzed by the (Na^+,K^+)ATPase.

INTRODUCTION

The contribution of the (Na^+,K^+)ATPase in the energy expenditure of animals is still being debated, with figures ranging from 5-6% to 45-90% (1,2). The reduction of the number of Na^+/K^+-pump units in the genetically-obese mouse has been proposed as a primary defect in the development of obesity (3). Particulate fractions obtained from hindlimb muscles of the ob/ob mouse were reported to contain significantly less total [^{3}H]ouabain binding sites than those obtained from lean animals (4). Moreover a decrease in the rate of active Na^+ and K^+ transport as well as a decrease in the number of Na^+/K^+-pump units were also reported for erythrocytes in obese patients (5). In contrast to these results a recent report indicates no significant difference in the number of [^{3}H]ouabain binding sites and in the [^{42}K] uptake determined

ABBREVIATIONS : PBS, phosphate-buffered saline (no Ca^{++} and Mg^{++}) ; PMSF, phenylmethylsulfonyl fluoride ; DTT, dithiothreitol ; TEA, triethanolamine ; ATP, adenosine-5'-triphosphate ; FCS, fetal calf serum ; DME medium, Dulbecco's modified Eagle's medium.

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in different muscles from lean and obese mice (6). All these investigations were performed under conditions where the substrate and the hormonal environment of the cells *in vivo* are different between obese and non-obese animals or patients.

We have previously reported the establishment of a preadipocyte cell line from the adult C57 BL/6J ob/ob mouse (7). A preadipocyte cell line was also established by the same isolation procedure from the adult lean C57 BL/6J +/? mouse (C. Forest *et al.*, submitted). Both cell lines respond to physiological concentrations of insulin and triiodothyronine (unpublished results) and present after differentiation both the morphological and biochemical properties of mature adipocytes. The availability of these cell lines led us to carry out a comparative study of their number of [^{3}H]ouabain binding sites under identical culture conditions. These measurements were combined with determinations of [$^{86}Rb^+$]uptake. The results presented in this paper argue against the existence of "intrinsic" differences in (Na^+,K^+)ATPase levels between cell lines established from genetically-obese and non-obese mice.

MATERIALS AND METHODS

Cell Culture

Methods of cell growth, cell numbering and determination of cell protein content were as previously described (7). At confluence were added 17 nM insulin and 1.5 nM triiodothyronine to standard medium (changed every other day), with inclusion of biotin (8 $\mu g.ml^{-1}$) and pantothenate (4 $\mu g.ml^{-1}$).

Preparation of Crude Adipocyte Membrane

19 to 32 culture dishes (100-mm diameter) of each condition were rinsed twice with PBS and once with the homogenization buffer (Medium I : 10 mM Tris-HCl pH 7.4/1 mM EDTA/0.25 M sucrose/5 µM PMSF/1 mM DTT). Cells were scraped with a rubber policeman in ice-cold Medium I and homogenized in a prechilled Polytron homogenizer (2x6s at a settting of 3). The homogenate was centrifuged at 40,000 g for 60 min and the pellet was resuspended in ice-cold Medium II (50 mM TEA pH 7.5/2 mM $MgCl_2$/100 mM NaCl/2 mM ATP/5 µM PMSF/1 mM DTT) and homogenized in a Potter-Elvejehm homogenizer (20 strokes), resulting in a suspension containing from 1.0 to 1.5 mg of protein per ml.

[^{3}H]Ouabain Binding Assay

Crude membrane protein (1 mg per assay) was incubated in Medium II with [^{3}H]ouabain (11.5 Ci/mmol at the concentrations indi-

cated) at 25°C in a total volume of 1.6 ml (unless specified). At the indicated times triplicate aliquots of 0.5 ml were immediatly filtered through Whatman GF/B filters. Filters were then washed rapidly with three 5 ml portions of ice-cold PBS. Filtration and washing required a total time less than 30 s. Filters were counted in the presence of 10 ml of Biofluor (New England Nuclear Co.). In each experiment, "non-specific" binding to protein was determined by measuring the amount of radioactivity bound to filters when incubations were performed in the presence of 0.1 mM unlabeled ouabain. The binding values reported refer to "specific" binding after substraction of the "non-specific" binding from the total binding. "Specific" binding was proportional to the amount of membrane protein under these conditions.

[$^{86}Rb^{+}$] Uptake

Ob17 and HGFu cells were grown to confluence in DME medium supplemented with 10% FCS using 24-well plates (16-mm diameter). Two days after confluence, cells in each well were rinsed twice at 37°C with 1 ml of medium III (10 mM Hepes buffer pH 7.4/140 mM NaCl/2 mM $CaCl_2$/1 mM $MgCl_2$/5 mM glucose). Cells were then incubated for 3 hours at 37°C with 0.2 ml of medium III containing from zero to 1 mM ouabain. [^{86}Rb] Cl was added (5 mM final concentration ; 5 µCi/ml) and uptake was performed for 30 min at 37°C. [^{86}Rb] uptake (linear under these conditions) was arrested by rinsing the cells four times with 1.5 ml of ice-cold medium III. Cells were treated with 1 ml of 0.1N NaOH and radioactivity was determined with a Gamma-counter. Control experiments showed that, after 3 hours in the presence of 1 mM ouabain, more than 95% of the cells remained viable as shown by erythrosin exclusion.

Materials

Dulbecco's Modified Eagle's Medium was purchased from Gibco (catalogue n° H21). FCS was a product of Seromed. [$^{86}Rb^{+}$] and [^{3}H]ouabain were obtained from Amersham Centre and New England Nuclear Co., respectively. All other compounds were from Sigma Chemical Co.

RESULTS AND DISCUSSION

Figure 1 shows that binding of [^{3}H]ouabain to membranes of differentiated Ob17 and HGFu cells is a saturable process. The non-specific binding varies from 25 to 75% of the total binding capacity. Scatchard plots computed from the curves of specific binding (Fig.1, inset) indicate the existence of high-affinity sites in both cell lines, with a K_d value of 50 nM. The maximal binding capacity is 460 fmol.mg^{-1} and 350 fmol.mg^{-1} of membrane protein for Ob17 and HGFu cells respectively. These figures correspond to 140,000 and 110,000 [^{3}H]ouabain binding sites per cell of Ob17 and HGFu lines respectively.

Rates of association (k_a) and dissociation (k_d) were calculated from curves of Fig. 2 obtained on crude membrane proteins

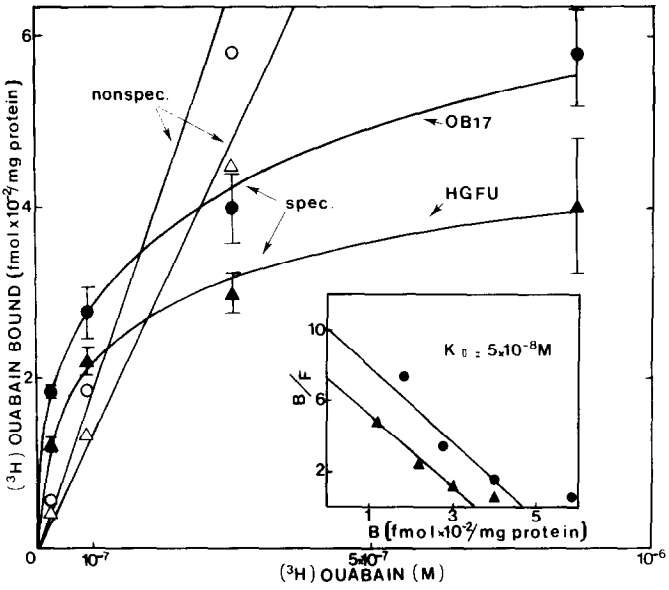

Fig.1 : Binding of [^{3}H]ouabain to Crude Membrane Protein of 12 Day Post-confluent Ob17 and HGFu Cells

Increasing concentrations of [^{3}H]ouabain were incubated 180 min at 25°C. Experiments were carried out in the presence of 0.1 mM unlabeled ouabain for the determination of the "non-specific" binding.
Inset : Scatchard plots computed from specific binding curves. B/F is the ratio of bound over free ligand and B is bound ligand.

from differentiated cells. The association process is very similar in both cases, with k_a values of $3.6 \times 10^5 M^{-1}.min^{-1}$ and $4.4 \times 10^5 M^{-1}.min^{-1}$ for Ob17 and HGFu cells, respectively. The dissociation process was followed by first incubating crude membranes with 80 nM [^{3}H]ouabain and then with 0.1 mM unlabeled ouabain in order to displace the radioactive ligand. The first-order rate constant of dissociation k_d is found to be 0.023 min^{-1} for the two cell lines. Values of the equilibrium dissociation constants, calculated from k_a and k_d ($K_d = \frac{kd}{ka}$), are 60 nM for Ob17 cells and 52 nM for HGFu cells. These values are in agreement with those obtained at equilibrium (Fig.1).

Taken together, these results show the existence in both cell lines after adipose conversion of a family of high-affinity binding sites for ouabain. It should be pointed out that these sites are

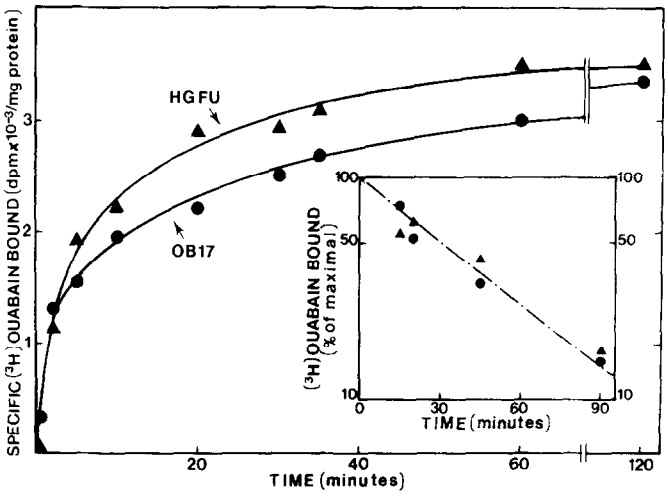

Fig.2 : Time Course of Association and Dissociation (inset) of Labeled Ouabain to Ob17 and HGFu Cells

Crude membrane proteins (4.5 mg total) of 10 day post-confluent Ob17 and HGFu cells were incubated (7 ml total volume) in the presence of 80 nM [^{3}H]ouabain. The separation of bound and free radioactive ligand, as well as the determination of the "non-specific" binding, were performed as described in "Materials and Methods".

Inset : [^{3}H]ouabain (80 nM) was first associated with crude membrane proteins of Ob17 and HGFu cells for 60 min at 25°C as above. Dissociation of the radioactive ligand was followed after addition of 0.1 mM unlabeled ouabain. 0.5 ml aliquots were taken at the indicated times to measure the radioactivity which remains bound to membranes.

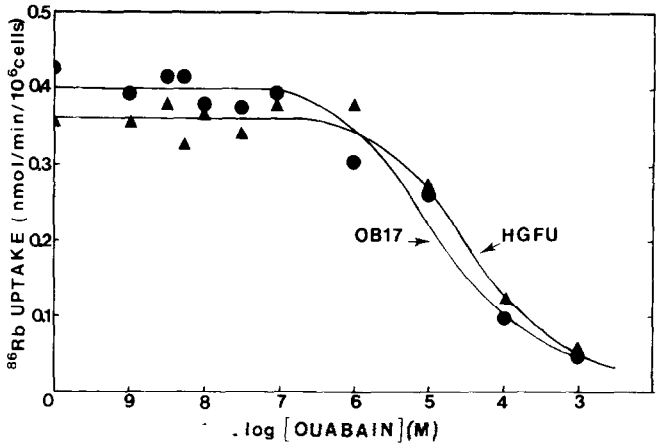

Fig.3 : Ouabain-Suppressible [^{86}Rb] Uptake in Ob17 and HGFu Cells

The uptake of [^{86}Rb] was measured after preincubating confluent cells for 3 hours at 37°C with varying ouabain concentrations, under conditions described in "Materials and Methods". Each point represents the mean value of duplicate wells. The apparent dissociation constants are indicated by arrows.

already present in exponentially-growing cells and in bromodeoxyuridine-treated cells (not shown) which both are not differentiated into adipose cells (7).

Figure 3 shows that more than 85% of the [^{86}Rb] influx is inhibited at 1mM ouabain. The ouabain-suppressible component of [^{86}Rb] uptake is very similar in confluent (undifferentiated) Ob17 cells and in HGFu cells (0.34 and 0.3 nmol/min/10^6 cells respectively). The apparent dissociation constant for the ouabain-receptor complex obtained from [^{86}Rb]influx experiments was 20 µM for Ob17 cells and 40 µM for HGFu cells.

These values of apparent dissociation contants are nearly 3 orders of magnitude higher than those found from binding data with [^{3}H]ouabain. Therefore there seems to be 2 classes of ouabain binding sites : i) a first class which is of a high-affinity type, which is identified with [^{3}H]ouabain, and which is not seen in [$^{86}Rb^{+}$] influx experiments ; their functional role remains to be determined. ii) a second class of low-affinity binding sites which are functional for [$^{86}Rb^{+}$] (and therefore for K^{+}) transport.

Whether one considers the high-affinity binding sites ($K_d \simeq 50$-60 nM) or the low-affinity binding sites (apparent $K_d \simeq 20$-40 µM), it is clear that no significant difference is found between cell lines established from genetically-obese and non-obese mice. Therefore it is unlikely that a reduction in the levels of (Na^{+},K^{+}) ATPase is the primary defect in the development of obesity in the ob/ob mouse.

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REFERENCES

1. Ismail-Beigi, F. (1977) Current topics in membranes and transport, vol.9, p. 367, Academic Press, New-York.
2. Clark, D.G., Brinkman, M., Filsell, O.H., Lewis, S.J., and Berry, M.N. (1982) Biochem.J. 202, 661-665.
3. Bray, G.A., and York, D.A. (1979) Physiol. Reviews 59, 719-809.
4. Lin, M.H., Van der Tuig, J.R., Romsos, D.R., Akera, T., and Leveille, G.A. (1979) Amer.J.Physiol. 238, E193-3199.
5. De Luise, M., Blackburn, G.L., and Flier, J.S. (1980) New Engl.J.Med. 303, 1017-1022.
6. Clausen, T., and Hansen, O. (1982) Biochem.Biophys.Res.Commun. 104, 357-362.
7. Nègrel, R., Grimaldi, P., and Ailhaud, G. (1978) Proc.Natl. Acad.Sci.USA 75, 6054-6058.