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GAMMA-GLUTAMYL-TRANSPEPTIDASE (GGTP) AND NA+K+-ATPase ACTIVITIES IN DIFFERENT SUBPOPULATIONS OF CLONED CEREBRAL ENDOTHELIAL CELLS: RESPONSES TO GLIAL STIMULATION

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Summary: Glial stimulation of Na+K+-ATPase and gamma-glutamyl-transpeptidase was taken as parameter for blood brain barrier function in cloned cerebral endothelial cells of different phenotypes. In type I cells ("cobblestone" phenotypus) gamma-glutamyl-transpeptidase activity increased 10-12 fold and Na+K+-ATPase activity was 2-fold increased after glial stimulation. In type II cells ("spindle-form" phenotype) gamma-glutamyl-transpeptidase was only 2-fold increased, whereas Na+K+-ATPase was even depressed. K+-(^{86}Rb) uptake was twice as high in type I cells. These data indicate that type I cells are involved in blood brain barrier function.

Endothelial cell growth supplement (ECGS) together with heparin has been reported to influence growth and differentiation of cloned cerebral endothelial cells (cEC) leading to a characteristic cobblestone-like appearance of the cells (in the following referred to as "phenotype I"). On the contrary, depletion of ECGS and heparin resulted in an elongated, spindle formed phenotype ("phenotype II") (1). Comparable results have been obtained by use of exogenous fibroblast growth factor (FGF) in cloned adrenal capillary enodothelial cells (2). Studies on biochemical and functional characteristics of the different cell types (1,2,3) gave rise to the idea that bbb function may be attributed to cEC of phenotype I, whereas cEC which are involved in neovasculogenesis probably express phenotype II. To evaluate this hypothesis we studied the expression of bbb associated transport enzyme activities (4,5-8) in cloned cEC of both phenotypes: activity of gamma-glutamyl-transpeptidase (GGTP) was spectrophotometrically determined (9), Na+K+-ATPase activity was monitored using ^{86}Rb as

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tracer substance (19). For induction of enzyme activities we used glial plasma membranes (gPM), since it is well known that bbb related functions are enhanced by glial stimulation in-vivo as well as in-vitro (3,10-13).

MATERIALS AND METHODS

Materials

Bovine brain was obtained from the local slaughter house. Fetal calf serum and cell culture media were purchased from Gibco and Seromed, endothelial cell growth supplement (ECGS) was from Collaborate Research. ^{86}RbCl was bought from Du Pont NEN. The GGTP-kit was from Sigma as well as ouabain an all other chemicals. Liquid scintillation fluid (Ecoscint) was obtained from National Diagnostics (U.S.A)

Cell culture

C6 rat glioma cells were kept in 75 cm^3 flasks and maintained in HAM F10 medium plus 10% FCS.
Cerebral capillary endothelial cells were prepared from pure capillaries derived from bovine fore brain as described by Tontsch and Bauer (1). In short, vessels were collagenized (0.075%, 10 min, room temperature), washed in culture medium (medium 199 with Earle's salts, L-glutamin plus 10% FCS; ECGS, 20 µg/ml and heparin, 100 µg/ml) and plated at a density of approximately 2.5 x 10^4 cells/ml. Stable cell lines from cloned cells were established and characterized as described previously (1). Two phenotypes were used: cEC with "cobblestone-like" appearance and cells which were "spindle" shaped (grown in culture medium without ECGS and heparin).

Plasma membrane preparation

Plasma membranes were prepared from confluent C6 glioma cultures and from confluent chick fibroblast cultures according to Bauer and Hurtenbach (14). Briefly, cells were trypsinized, centrifuged and the pellet homogenized with a tightly fitting glas pestle in isotonic sucrose phosphate buffer. After differential centrifugation the microsomal fraction was washed in 5 mM Tris/HCl/1 mM EDTA , pH 8.1 and pelleted at 55ooo x g. Plasma membranes were resuspended in Dulbecco's phosphate buffered saline (DPBS) and added to confluent cEC of both phenotypes for 3 days.

Enzyme assays

i)GGTP

Cells were washed 3 times with DPBS at room temperature and processed according to Maxwell et al. (8). GGTP activity was determined with a colorimetric assay, based essentially on the method of Naftalin et al. (9) using measurement of liberated p-nitroaniline for quantification. Enzyme activity was related to protein concentration, measured by the method of Lowry et al.(15).

ii) Na^+K^+-ATPase

Confluent cells were washed twice at room temperature with influx buffer (for detail see ref. 16). 2 µCi/ml ^{86}Rb were added for influx studies. Influx was performed for 15 min at room temperature. Then cells were washed 3 times with isotonic saline and 500 µl of 0,5% triton X-100 were added to each well. After 12 h extraction time the supernatants were transfered to scintillation vials and counted in a liquid scintillation counter (Packard).
Na^+K^+ATPase activity was estimated as the ouabain-inhibitable part of ^{86}Rb-uptake. Ouabain was used at a final concentration of 1 mM, solved in influx buffer.

RESULTS

The two EC phenotypes, type I ("cobblestone"-like appearance) and type II ("spindle" form) derived from cloned cerebral capillary EC differ significantly in their ^{86}Rb uptake rates (Fig. 1). In both cell types, ^{86}Rb influx was linear up to one hour but the uptake rate was twice as high in type I cells. Based on these data ^{86}Rb influx for detection of Na^+K^+-ATPase activity was performed for 15 min. in all experiments. Fig. 2 shows the activity of Na^+K^+-ATPase in type I cEC cultured with and without glial plasma membranes (gPM). ATPase activity in type I cells was nearly two-fold increased after glial stimulation for two days, whereas in type II cEC it was decreased by more than 50 % after incubation with gPM (Fig. 3). GGTP also exhibited marked differences in its activity when compared in both cEC types. In type I cells GGTP activity was elevated about 10-12 times after gPM incubation, whereas in type II cEC enzyme activity was only twofold increased. In untreated cells GGTP activity was nearly identical, i.e. about 200 units/mg protein, within the two phenotypic groups. Plasma

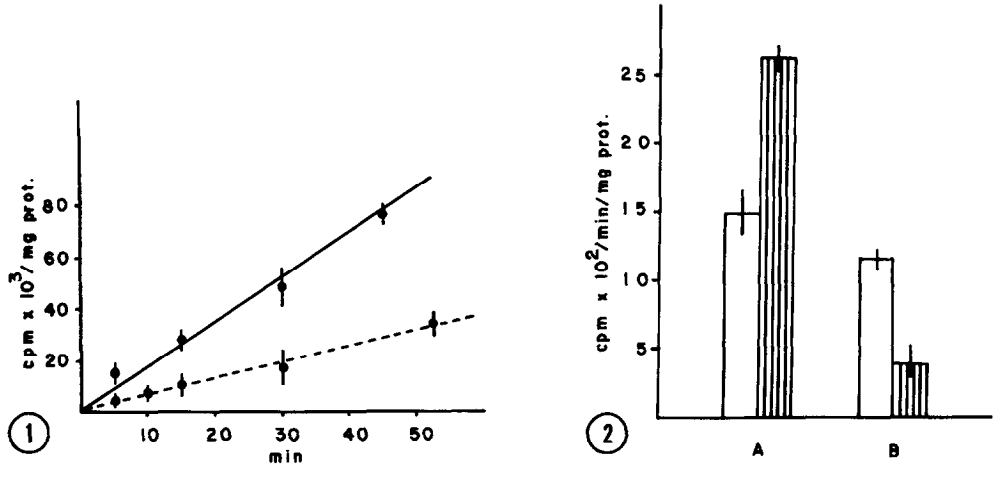

Fig. 1: Time course of ^{86}Rb uptake in the two cEC types.
——— type I CEC,
- - - - type II cEC. Means and s.d. are given; n=6

Fig. 2: Total ^{86}Rb influx and ouabain inhibitable part of ^{86}Rb uptake (Na^+K^+-ATPase activity) with and without incubation with gPM.
A: type I cEC; B: type II cEC.
☐ control; ▤ gPM incubation
Means and s.d. are given; n=6

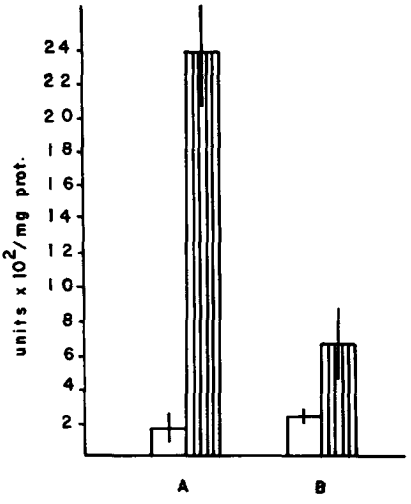

Fig. 3: Activity of GGTP in cEC with and without gPM incubation.
A: type I cEC, B: type II cEC.
▭ control; ▤ gPM incubation
means and s.d. are given; n=4-10

membranes from fibroblasts had no effect on enzyme activities as observed in control studies (data not shown).

DISCUSSION

Phenotypic diversity of EC in-vivo is a common phenomenon (17,18). cEC differ markedly in morphology and function from other EC, since they specialize at a distinct developmental stage, acquiring selective transport properties. From this stage onwards they are referred to as the blood brain barrier (bbb) (4,19-21).

The usually high activities of bbb associated transport enzymes in-vivo decrease when cEC are kept in culture (3,22) - probably due to the loss of glial environment which is known to promote bbb function (3,10-13,23).
In a previous study we have found that addition of ECGS and heparin to the culture medium led to cobblestone like appearance of the cells. This phenotype switched to spindle form within 3 days when ECGS and heparin were absent in the medium (1). Similar morphologic diversity of cloned EC derived from bovine adrenal cortex have been reported by Tsuboi et al. (2) who showed that cellular phenotypes were determined by endogenous and

exogenous fibroblast growth factor. In these experiments EC of cobblestone like appearance (type I) did not migrate or invade in contrast to EC of spindle form (type II).

We now report that inducibility of bbb associated enzyme activities differ significantly in cloned cEC of the two phenotypes described above. Incubation with glial membranes led to a marked increase of GGTP and Na^+K^+-ATPase activity in type I cEC, whereas enzyme activities in type II cEC were left completely unaffected. Moreover, Na^+K^+-ATPase activity in the latter cell type was even depressed after glial contact. $K^+(^{86}Rb)$-permeability of plasmamembranes in type I cells was generally higher as concluded from total ^{86}Rb uptake studies.

Based on these data we present evidence that functional characteristics can be induced in cultured cEC of that phenotype, which is considered to be responsible for bbb function in-vivo.

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