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EVIDENCE FOR NET UPTAKE OF GABA INTO MOUSE ASTROCYTES IN PRIMARY CULTURES—ITS SODIUM DEPENDENCE AND POTASSIUM INDEPENDENCE

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Content of GABA was measured in cultured, normal astrocytes (from the brain cortex of newborn mice) together with the effect of nonradioactive GABA on the efflux of labeled GABA from cells previously loaded with [^{14}C]GABA. An increase of external GABA concentration from 0 to 25 μM evoked a rise of the GABA content in the cells to a level which was approximately 50 times that of the incubation medium. Neither 200 nor 2000 μM nonradioactive GABA had any effect on the rate of release of radioactivity from cells loaded with [^{14}C]GABA. Both the high tissue/medium ratio and the lack of a GABA-induced enhancement of the release of radioactivity indicate that the previously observed high-affinity uptake of GABA in cultured astrocytes represents a net uptake and not a homoexchange with endogenous GABA. This uptake is sodium dependent but was found to be unaffected in potassium-free media; the quantitative correlation between GABA transport and sodium transport differed from that reported for synaptosomes.

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INTRODUCTION

Using bulk-prepared glial cells, Henn and Hamberger (1) were the first to demonstrate a high-affinity uptake of GABA into glial cells; such a glial transport system for GABA has since been demonstrated in peripheral ganglia (2–5), rat retina (6, 7), glioma cell lines (8–10), spinal cord explant cultures (11), and primary cultures of glial cells from the central nervous system (12, 13). Based upon such observations, it has repeatedly been suggested that uptake of GABA into glial cells may be of major importance for termination of the transmitter activity of this amino acid. In addition, an accumulation of GABA into nerve terminals has, however, also been observed (for references, see 14), and the original view, now challenged by the glial uptake of GABA and of other amino acids with transmitter function (e.g., 15, 16), was that high-affinity, sodium-dependent reuptake into nerve terminals was of prime importance for termination of transmitter activity of GABA and other putative transmitters (17, 18).

A cellular high-affinity uptake of GABA is only of importance for termination of transmitter activity provided it occurs with a reasonably high V_{max} and represents a net accumulation rather than a 1:1 homoexchange. There is little doubt that the GABA uptake into cerebral astrocytes in primary cultures (13) and into synaptosomal preparations (for references, see 19) is of sufficient intensity, compared to that in brain slices, to be of quantitative importance (13), whereas this may be questionable in case of the C-6 glioma cells (15, 16). Recent experiments by Levi and coworkers (20, 21) have indicated that the high-affinity uptake of GABA into synaptosomal preparations in vitro mainly, if not exclusively, is due to homoexchange with the endogenous pool; under in vivo conditions there may, however, be a concentrative net uptake (22). It has also been concluded that the uptake of GABA into satellite cells of sensory ganglia represents a homoexchange (5) and a similar process has been found to occur in bulk-prepared glial cells (23). In the latter preparation there is, in addition, a net uptake (24), and the observed uptake into C-6 glioma cells and rat retina seems to represent a net uptake (9, 25, 26).

In view of the large uptake capacity in the cultured astrocytes, it seemed of interest to establish whether this uptake represents a net accumulation or a homoexchange. In the present report this was investigated by studying the effect of an increase in the GABA concentration on the release of radioactive GABA from cultures of astrocytes previously loaded with [^{14}C]GABA. Under the conditions employed (an increase from 2.5–35 μM to 200 or 2000 μM GABA), a homoexchange

would be revealed by an increase in the rate of release of the radioactive GABA (cf. 5, 20, 21). To obtain further information about the ionic requirements for this uptake, results we have previously obtained on the sodium dependence of the GABA accumulation (13) were replotted in a Hill plot (to estimate the quantitative correlation between GABA transport and sodium transport) and the effect of the absence of potassium was studied.

EXPERIMENTAL PROCEDURE

Tissue Culture. Primary cultures of glial cells were prepared as described by Booher and Sensenbrenner (27) but with some minor modifications (28). Cortex-enriched parts of the cerebral hemispheres (i.e., the areas superficial to the lateral ventricles) were carefully dissected out from brains of newborn DBA mice and, after the meninges had been cleaned off, placed in a modified (13) Eagle's (29) minimum essential medium (MEM) containing 20% fetal calf serum (1 ml/brain), cut into small pieces, disintegrated by the Vortex method (30), and passed twice through sterile nylon nitex sieves with pore sizes of 80 (first sieving) and 10 (second sieving) μm. One ml of this cell suspension was introduced into a 50-ml Falcon plastic T flask together with 2 ml of the modified Eagle's MEM with serum and incubated at 37° in a 95/5% (v/v) mixture of atmospheric air and CO_2. The culture medium was changed after 3 days and subsequently 3 times per week. After 2 weeks the cultures reached confluency and were thereafter cultured for another week in the absence of calf serum and the presence of 0.25 mM [or in one set of experiments (GABA uptake in potassium-free media) 0.1mM] dibutyryl cyclic AMP (dBcAMP). This procedure led to the formation of morphologically differentiated astrocytes as previously demonstrated (28).

Uptake Experiments. For studying the possible effect on the GABA uptake by omission of potassium from the medium, uptake of GABA was measured as previously described (13). With tissue samples corresponding to about 30 μg protein (0.3 mg wet wt), 450 μl medium (13) and a probable potassium content in the tissue of about 100 μmol/g wet wt (31), the maximum potassium concentration in the potassium-free medium resulting from a possible loss from the tissue would be < 0.1 mM.

Release Experiments. Cultures, still attached to the Falcon flasks and kept at 37°, were "loaded" for 30 min with [1-^{14}C]GABA (0.2 μCi/ml) in the modified MEM (without serum) containing a low concentration of GABA (2.5, 12.5, or 35 μM). Subsequently, the cultures were rapidly washed with nonradioactive medium, and the release of radioactivity to a nonradioactive medium was determined as previously described (32, 33). Two ml portions of the nonradioactive medium were added (and subsequently removed) to each culture at 6-min intervals for a total period of 90 min. The washout medium contained either 2.5 or 25 μM GABA, except during a period of 24 min when the GABA concentration was increased to 200 or 2000 μM. The radioactivity in each wash-out sample was determined, and the activity remaining in the culture after the end of the 90 min wash-out period was added to that determined in all wash-out samples to indicate the total amount of radioactivity in the culture at the start of the wash-out. The remaining radioactivity at the end of each wash-out period was calculated as a percentage of the total amount of radioactivity accumulated by the tissue and plotted semilogarithmically (Figure 1). The slope of the resulting desaturation curve indicates the rate of the wash-out and an increased wash-out rate would be reflected by a steeper slope.

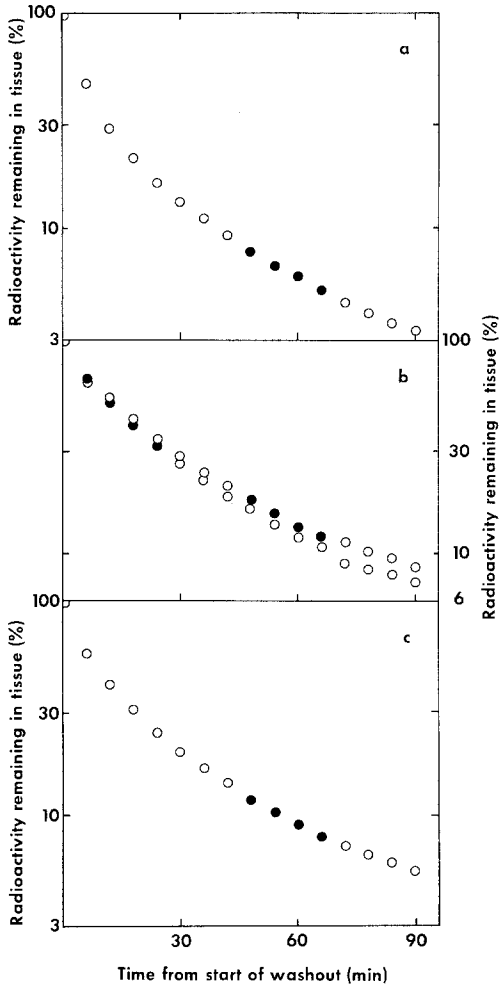

Fig. 1. Wash-out curve showing, as a function of time, release of radioactivity from astrocyte cultures loaded with ^{14}C-labeled GABA for 30 min. (a) the wash-out medium contained 25 μM (○) GABA except during the period 42–66 min (●) when the GABA concentration was increased to 200 μM. The curve is the average of 4 individual experiments. (b) Two individual experiments. The wash-out medium contained 25 μM (○) GABA except during the period 0–24 or 42–66 min (●) when the GABA concentration was increased to 2000 μM. (c) The wash-out medium contained 2.5 μM (○) GABA except during the period 42–66 min (●) when the GABA concentration was increased to 200 μM. The curve is the average of 5 individual experiments.

Corresponding experiments with tissue culture flasks without cultures showed that the contribution by the flask to the radioactivity released could be neglected.

Distribution of Radioactivity in Cultures after Loading with [^{14}C]GABA. In parallel experiments, cultures were loaded with 12.5 or 35 μM ^{14}C-labeled GABA for 30 min and immediately thereafter extracted and homogenized in a 2% (v/v) formic acid–acetone solution. Part of the extract was directly used for determination of total radioactivity and another part was chromatographed for 24 hr on No. 2 Whatman paper using an *n*-butanol–acetic acid–H_2O (120:30:50 v/v/v) solvent system. The spot corresponding to GABA was located by staining with ninhydrin–pyridine–acetic acid solution in acetone, cut out, extracted into 2 ml of H_2O, and used for determination of the radioactivity recovered as GABA.

Content of GABA. GABA contents in cultures exposed to 0 or 25 μM GABA were measured by conversion of GABA to dansyl-γ- butyrolactam (34) and determination of the lactam by a mass-spectrometric method using 2,2-dideutero-4-aminobutyric acid as an internal standard (D. A. Durden, P. H. Wu, and L. Hertz, unpublished experiments). The GABA content was measured in relation to protein which was determined as described by Lowry et al. (35), and recalculated to a wet weight basis using a protein content of 77 mg/g wet wt (36).

Supplies. Falcon plastic tissue culture T flasks (#3013) were purchased from Falcon (Oxnard, California), nylon nitex from Tobler, Ernst & Traber (New York, New York), and fetal bovine serum from Gibco/Biocult Laboratories, Ltd. (New York, New York). dBcAMP and all amino acids and vitamins were obtained from Sigma Chemicals Company (St. Louis, Missouri) and [1-^{14}C]GABA (specific radioactivity 49.4 mCi/mmol) from New England Nuclear Corp. (Boston, Massachusetts). All other chemicals were of purest grade available from regular commercial sources.

RESULTS

GABA Content and Uptake

The GABA content of astrocytes cultured in media without GABA was 0.05 ± 0.004 (SEM) μmol/g wet wt (n = 10) and after exposure to 25 μM GABA for 30 min it rose to 1.20 ± 0.10 μmol/g wet wt (n = 6), i.e., from almost nothing to an intracellular concentration about 50 times higher than that of the culturing medium.

Since the present cultures in general were grown in media containing 0.25 mM dBcAMP [compared to 0.1 mM in our previous report describing the kinetics of the GABA uptake (13)] and since they had been prepared in a manner which differs slightly from our earlier procedure (13), the kinetics of the GABA uptake were reinvestigated. The K_m and V_{max} values for the high-affinity uptake were found to be about 45 μM and 0.40 nmol/min/mg protein and thus correspond to the previous results (40 μM and 0.35 nmol/min/mg).

The potassium dependency of the GABA uptake was studied at a GABA concentration of 50 μM. After 5 min (initial uptake rates) the uptake in a potassium-free medium was 111 ± 5.6% (n = 8) of that in the presence of 5 mM potassium [100 ± 5.0% (n = 7)].

GABA Release

The fraction of the total amount of radioactivity in the cells, which is accounted for by GABA after incubation for 30 min, was 89.6 ± 2.0% (SEM) in media containing 12.5 μM [^{14}C]GABA and 90.9 ± 1.7% (n in both cases = 6) in media containing 35 μM [^{14}C]GABA. Thus, about 90% of the total radioactivity in the tissue represents unchanged GABA regardless of whether the medium contains GABA in a concentration which is low compared to the K_m value for the high-affinity uptake system or in a concentration similar to the K_m value. Since 80% of the accumulated GABA is released within 30 min of wash-out into nonradioactive media, it can be assumed also that the major part of the radioactivity found in the wash-out samples in the release experiments (Figure 1) represents untransformed GABA.

Figure 1a shows the efflux of [^{14}C]GABA from astrocytes which had been preloaded with ^{14}C-labeled GABA for 30 min. Initially and towards the end of the experiments the wash-out was into a medium containing 25 μM GABA (i.e., slightly below the K_m value for the uptake) but during the period 42–66 min (filled-in symbols in Figure 1a), the GABA concentration was increased to 200 μM (i.e., five times the K_m value). This increase in the external GABA concentration must have approximately doubled the uptake rate for GABA and, in case uptake and release had been coupled in a homoexchange, also the rate of the GABA release would have increased; from Figure 1a can be seen that this is not the case.

Similar experiments (Figure 1b) in which the GABA concentration was increased earlier during the wash-out (0–24 min) or in which it was increased from 25 to 2000 μM (i.e., to way above the high-affinity range) or (Figure 1c) from 2.5 μM (i.e., way below the K_m value) to 200 μM showed analogously a total lack of increase in the wash-out rate for GABA.

DISCUSSION

The observed content of GABA in the cultured astrocytes (0.05 μmol/g wet wt) was even lower than GABA contents reported for bulk-prepared (37–39) glial cells (0.4–0.7 μmol/g) or dorsal root ganglia [0.5 μmol/g

(40)]; this may be due to the virtual absence of GABA in the culturing medium and reflects the low activity of the L-glutamate decarboxylase (measured by CO_2 production) observed in primary cultures of glial cells (41, 42, C. B. Charington and L. Hertz, unpublished results). The considerable increase in GABA content subsequent to an increase in the GABA concentration in the medium strongly suggests a net inward transport of GABA.

That the previously described high-affinity uptake of GABA into cultured astrocytes (13) represents net uptake and not homoexchange with the endogenous pool of GABA is also indicated by the lack of stimulation of the efflux of [^{14}C]GABA by both 200 and 2000 μM nonradioactive GABA observed in the present study. This is in contrast to the dose-dependent stimulation of GABA efflux observed in sensory ganglia (5) and in bulk-prepared glial cells (23). It is, however, in agreement with the finding that GABA uptake into rat retinal glial cells (25) and C-6 glioma cells (26) seems to represent a net uptake rather than a homoexchange process. This demonstration of a net uptake of GABA with a high affinity further strengthens the possibility that glial cells are, indeed, deeply involved in termination of the transmitter activity of GABA. This may represent an analogy to the glial handling of other transmitters, e.g., glutamate and taurine, which also are taken up very efficiently by astrocytes (28, 42, 43) and which also seem predominantly or totally to be accumulated by net uptake processes (16, 28). However, the possibility cannot be excluded that the observed net uptake and subsequent metabolism of GABA (and glutamate) in astrocytes has to do primarily with "metabolic pools" rather than "transmitter pools" of these amino acids.

The lack of effect on the GABA uptake by potassium omission is at variance with findings in bulk-prepared glial cells (44) and synaptosomes (22). Based upon studies of GABA uptake in the latter preparation, it has been concluded that the energy for the GABA accumulation resides in the gradients of sodium and potassium and that three sodium ions (19, 22) and one potassium ion are translocated when one molecule of GABA is taken up. We have previously reported that the uptake of GABA into cultured astrocytes is sodium dependent (13). In order to study whether more than one sodium ion also is required for the uptake of each GABA molecule into cultured astrocytes, the previous results are presented in Figure 2 in a Hill plot (45–48):

$$\log [v/(V_{max} - v)] = n \log S - K$$

where S is the substrate concentration (in this case the concentration of sodium) and n the number of sites at which substrates bind cooperatively (46–48). The straight line in Figure 2 (calculated by the least-squares

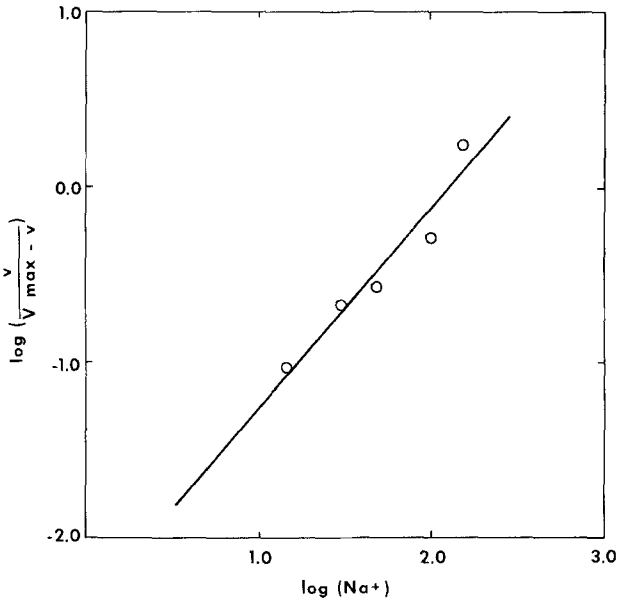

FIG. 2. Hill plot showing log $[v/(V_{max} - v)]$ as a function of the logarithm of the sodium concentration; v indicates initial uptake rates at the different sodium concentrations, V_{max} the initial uptake rate at infinitely high sodium concentration and the GABA concentration of 50 μM used for this set of experiments.

method) follows the equation $y = (1.15 \pm 0.19) \log x - K$, suggesting only one binding site for sodium (or a low degree of cooperativity), i.e., translocation of only one sodium ion for each molecule of GABA taken up. Further support for a one-to-one relation between GABA and sodium transport is given by the fact that the GABA content of 1.2 μmol/g wet wt after exposure to 25 μM GABA (cf. Results) corresponds to that which, according to Martin (19), can be expected on the basis of a membrane potential in cultured astrocytes of -70 to -80 mV (G. Moonen and P. G. Nelson, personal communication) and transport of one molecule of GABA together with *one* molecule of sodium. The possible physiological implication of this apparent difference between GABA transport in astrocytes and in synaptosomes is unknown; it could be related to the high concentration of GABA in certain synaptic endings since the theoretically maximum gradient between extra- and intracellular GABA concentration is considerably smaller (19, 22) when only one molecule of sodium is cotransported with one molecule of GABA than when three sodium molecules are involved.

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