Selective Loss of Phorbol-12,13-Dibutyrate–Facilitated L-Glutamate Transport in Forebrain Neurons of Aged Rats

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Phorbol-12,13-dibutyrate (PDBu), an activator of protein kinase C, was used to evaluate potential age-related changes in phosphorylation-dependent facilitation of high-affinity L-glutamate uptake in the rat central nervous system (CNS). Forebrain homogenates from male Fisher-344/brown Norway F1 hybrid rats were separated into glia-enriched (glial plasmalemmal vesicles) and neuron-enriched fractions (synaptosomes) and assayed for sodium-dependent transport of L-[3H]glutamate. Gial fractions from rats aged 5, 25, 31, and 37 months exhibited similar rates of basal L-[3H]glutamate transport and demonstrated no significant age-related differences with respect to the maximal facilitatory effect of PDBu (1–100 μM). In contrast, neuronal fractions exhibited an age-related decline in both indices, with basal L-[3H]glutamate transport decreasing from 710 ± 31 to 560 ± 40 pmoL/mg protein/90 s for the 5- and 37-month groups, respectively (p < .03) and PDBu having a significantly attenuated effect in aged animals. Together, these results provide support for the hypothesis that aging is associated with a decrease in the number of neuronal L-glutamate transporters as well as a diminished capacity to up-regulate these transporters through a protein kinase C–dependent pathway.

In addition to the widely-recognized role of L-glutamate (GLU) as the primary transmitter at excitatory central nervous system (CNS) synapses, a substantial body of evidence has implicated this excitatory substance in the generation of certain pathophysiologic sequelae and neuronal death (excitotoxicity) which occur following cerebral ischemia, stroke, and other traumatic insults as well as the progressive loss of CNS neurons during aging. Support for a possible role of GLU in age-related cell death derives from several observations, including the increased vulnerability of aged individuals to excitotoxic effects by selected GLU agonists (1,2) and the marked increase in extracellular levels of GLU in certain forebrain regions of aged rats (3,4). Although the latter observation may be an important contributor to some age-related changes, the underlying basis for elevated GLU levels in cerebral extracellular fluid is unknown. Based upon studies in isolated tissues, several investigators have speculated that enhanced neuronal efflux could account for elevated GLU levels in aged animals (5,6). In addition, in view of the recognized importance of high-affinity GLU uptake as a major determinant of extracellular GLU levels in vivo (7), it seems equally plausible that attenuated uptake could contribute to the rise in GLU levels in aged animals. However, previous investigations have yielded inconsistent and contradictory results regarding age-related changes in GLU uptake (8–10). Nevertheless, based upon the more recent discovery of multiple GLU transporter subtypes which are expressed differentially by neurons and glia (reviewed in ref. 11), it is possible that measurements of GLU uptake in mixed cell homogenates or brain slices could mask age-related changes in specific populations of GLU transporters. Therefore, the present study was undertaken in order to evaluate possible age-related changes in glial and neuronal GLU transporters by using a novel method for simultaneous isolation of highly-purified membrane-encapsulated vesicles derived from glia (glial plasmalemmal vesicles or GPV) and neurons (synaptosomes) in rat brain.

METHOD

Male Fisher-344/brown Norway F1 rats (National Institute On Aging colony) aged 5, 25, 31, and 37 months were used for this study. Animals were housed in pairs on a 12-hour light/dark cycle with food and water available ad libitum under “pathogen-free” conditions for 5–10 days prior to use. Forebrain tissues (without cerebellum) from two rats of the same age were combined for each experiment and used to prepare GPV and synaptosomal fractions according to our previously published method (12). Sodium-dependent L-[3H]glutamate uptake was measured in aliquots (50 μL) of isolated GPV or synaptosomal fractions in 0.5 mL (final volume) of a solution containing 140 mM NaCl, 5 mM KCl, 1.0 mM CaCl2, 1.0 mM MgCl2, 1.2 mM NaH2PO4, 10 mM D-glucose, and 20 mM HEPES at pH 7.4 (25°C). Sodium-independent uptake was measured in parallel using a buffer in which NaCl was replaced by an iso-osmotic concentration of choline chloride. Uptake was initiated by addition of 50 μL of L-[2,3,4-3H]-glutamic acid (60 Ci/mmol; American Radio labeled Chemicals, Inc., St. Louis, MO) at a final concentration of 5 μM and was terminated after 90 seconds by rapid vacuum filtration onto glass fiber filters as described previously. For experiments in which the effects of phorbol-12,13-dibutyrate (PDBu) were examined, tissues were preincubated with drug or vehicle (0.01% acetone v/v) for 5 minutes prior to initiation of uptake. All values are reported as arithmetic means (± SEM) for five fractions isolated on separate experimental days. Statistical significance was accepted at a level of p < .05 as determined by repeated measures (two-way) analysis of variance (ANOVA) with one within-subject factor (concentration of PDBu) and one between-subject factor (animal age). Tukey’s multiple pair-

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wise comparison was used to identify significant differences among individual groups.

RESULTS

As shown in Figure 1 (top), L-[\(^{3}\)H]glutamate uptake in GPV fractions did not differ significantly among animals at 5, 25, 31, or 37 months of age (range of group means was 179 ± 19 to 193 ± 23 pmol/mg protein/90 s). Retrospective power calculations indicated that a pairwise difference of 47 pmol/mg protein/90 s in GLU uptake by GPV fractions (i.e., 26% of the mean control value for 5-month-old rats) could have been detected between any two age groups with 80% power at a two-tailed significance level of .05. Therefore, in view of the number of animals used for this study, any age-related differences smaller than this may not be reported as significant. In contrast, GLU transport in synaptosomal fractions exhibited an age-related decline among the four groups with uptake being significantly reduced in 37-month-old animals \(F(3,16) = 4.20, p < .03\) relative to 5-month-old rats \((710 ± 31 \pm 560 ± 40 \text{ pmol/mg protein/90s})\). In addition, significant differences were detected with respect to the effect of PDBu in GPV and synaptosomal fractions. In GPV fractions, brief pretreatment with PDBu, an activator of protein kinase C, produced a significant concentration-dependent facilitation of GLU transport in all age groups with maximal increases of 52% (5 months), 103% (25 months), 81% (31 months) and 83% (37 months) over vehicle-treated controls. The stimulatory effect of PDBu on GLU transport was statistically significant \((p < .05)\) at all drug concentrations of 3 \(\mu\)M or above in all age groups, whereas 1 \(\mu\)M drug also produced a significant increase in 31-month-old animals. Although there appeared to be a trend toward enhanced facilitated GLU transport by PDBu in GPV fractions from older versus young animals, no significant interaction was detected between animal age and the concentration of drug \(F(15,80) = 2.05; p > .05\) for any age groups. By comparison, synaptosomal fractions exhibited a substantially smaller response to PDBu treatment with average maximal increases of 14–34% over age-matched control values. Although the stimulatory effect of PDBu was statistically significant \((p < .05)\) in synaptosomal fractions, higher concentrations of the drug were needed to achieve a significant effect in 5- and 37-month-old animals (10 \(\mu\)M) as well as 25-month-old animals (30 \(\mu\)M). Furthermore, in contrast to GPV fractions, significant differences were detected between the responses to PDBu in tissues from the youngest and oldest groups. As shown in Figure 1 (filled symbols), synaptosomal fractions from 37-month-old rats exhibited significantly smaller increases in GLU uptake in the presence of PDBu than tissues from 5-month-old rats. This difference between 5- and 37-month-old groups was significant \((p < .05)\) throughout the entire range of PDBu concentrations \(F(3,16) = 12.91, 3.46, 7.95, 7.44, \text{and} 4.37\) for 1, 3, 10, 30, and 100 \(\mu\)M, respectively).

DISCUSSION

The major conclusions of this study are the following: (a) the basal rate of GLU transport is significantly reduced in forebrain neurons of aged rats; (b) GLU transport in glia is unchanged in aged rats; and (c) glia maintain their capacity to respond to protein kinase C–mediated activation of GLU transport, whereas this regulatory pathway is markedly attenuated in neurons of aged rats. The selective decrease in neuronal GLU transport reported here is noteworthy insofar as the method used for simultaneous isolation of synaptosomal and glial fractions is highly effective at removing glial and neuronal contaminants from each respective fraction (12). In view of the differential expression of GLU transporter subtypes in neurons and glia, this methodologic improvement could account for our ability to detect an age-related decrease in neuronal GLU uptake, whereas most previous investigations using slices or crude synaptosomal...
homogenates have failed to detect such a change. Conversely, it should be noted that the apparent preservation of GLU transport in glial fractions from aged rats does not preclude the possibility that individual subtypes of glial GLU transporters (glutamate transporter type 1 [GLT-1] and glutamate/aspartate transporter [GLAST]) undergo mutually opposed changes during aging. This possibility must be considered in view of the uncertainty regarding relative levels of GLT-1 and GLAST transporters within isolated GPV fractions. However, despite the absence of direct measurements of GLT-1 and GLAST levels, it would appear that a substantial component of GLU transport in GPV fractions is mediated by GLT-1. This conclusion is based in part on the enhanced rate of GLU transport following treatment of glial fractions with the protein kinase C activator PDBu (Figure 1) as well as the recent report that GLU transport by GLAST is inhibited following protein kinase C-mediated phosphorylation (13). Finally, it is important to consider the relative levels of GLU uptake measured in synaptosomal and glial-enriched fractions. Although the level of GLU uptake in synaptosomes of young adult rats is nearly fourfold greater than uptake in glial fractions (Figure 1), this difference is a direct consequence of the different recovery efficiencies for glia-derived and neuron-derived elements achieved with the multistep isolation procedure used here (12). Consequently, this data should not be viewed as evidence that neurons possess a greater capacity for GLU uptake than glia in forebrain tissues of living animals. Indeed, several recent reports have demonstrated the predominant role exhibited by glial transporters (specifically GLT-1) with regard to the regulation of extracellular forebrain GLU levels in vivo (14,15). In light of these reports, the age-related decline in synaptosomal GLU transport reported here is unlikely to be responsible for elevated extracellular levels of GLU in aged animals.

The response to PDBu treatment provided further evidence for the differential effect of aging on glial and neuronal GLU transporters. Although GPV fractions from 5-, 25-, 31- and 37-month-old rats exhibited no net change with respect to the stimulatory effect of PDBu, synaptosomal fractions displayed an age-dependent reduction in the response to this protein kinase C activator (Figure 1). The stimulatory action by PDBu appears to be a consequence of protein kinase C activation insofar as the effect is blocked completely by calphostin C (500 nM), a selective protein kinase C inhibitor, and is not observed following treatment with the inactive analog 4-a-phorbol-12-myristate 13-acetate (authors, unpublished data). The basis for this diminished capacity to up-regulate synaptosomal GLU transport following PDBu treatment is currently not known, but it could be a reflection of impaired protein kinase C translocation which is known to occur in cortical and hippocampal structures of aged rats (16,17).

In conclusion, we have provided novel evidence that GLU transporters in neurons are preferentially impaired in aged rats. In view of the maintenance of GLU transport in glia, it is unclear whether the decline in neuronal GLU transport could be a contributory factor for the increase in extracellular GLU which occurs in aged animals. In addition, our findings raise the possibility that synaptic actions of GLU could be facilitated as a consequence of the impaired termination of GLU by neuronal transporters.

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