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Arachidonic acid stimulates glucose uptake in cerebral cortical astrocytes

(norepinephrine/vasoactive intestinal peptide/glia/energy metabolism)

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ABSTRACT **Arachidonic acid (AA) has recently been shown to influence various cellular functions in the central nervous system. Here we report that AA increases, in a time- and concentration-dependent manner, 2-deoxy-D-[1-^{3}H]glucose ([^{3}H]2DG) uptake in primary cultures of astrocytes prepared from the cerebral cortex of neonatal mice. This effect is mimicked by an unsaturated fatty acid such as linolenic acid, while palmitic and arachidic acids, two saturated fatty acids, are inactive. Pharmacological agents that increase the endogenous levels of AA by stimulating AA release (melittin) or by inhibiting its reacylation (thimerosal) also promote [^{3}H]2DG uptake by astrocytes. We also report that norepinephrine (NE) stimulates the release of [^{3}H]AA from membrane phospholipids, with an EC_{50} of 3 μM; this effect is accompanied, with a temporal delay of $\approx$4 min, by the stimulation of [^{3}H]2DG uptake, for which the EC_{50} of NE is 1 μM. Since the cerebral cortex, the brain region from which astrocytes used in this study were prepared, receives a massive noradrenergic innervation, originating from the locus coeruleus, the effects of NE reported here further stress the notion that certain neurotransmitters may play a role in the regulation of energy metabolism in the cerebral cortex and point at astrocytes as the likely targets of such metabolic effects.**

Astrocytes play an important role in the maintenance of local homeostasis within the central nervous system (1, 2). In addition to clearing the extracellular space from K^+, glutamate, or γ-aminobutyric acid, whose levels increase as a result of neuronal activity (3–5), astrocytes may contribute to energy metabolism homeostasis, a function already postulated over a century ago by Golgi (6). For example, astrocytes are the cell type in which glycogen is almost exclusively stored within the central nervous system (7, 8), and certain neurotransmitters can readily mobilize this energy reserve (9, 10). Thus, vasoactive intestinal peptide (VIP) and norepinephrine (NE), two neurotransmitters contained in discrete neuronal populations in the cerebral cortex (11), promote, within minutes of application, glycogenolysis in primary cultures of astrocytes (12); within a longer time frame (4–8 hr), these two neurotransmitters promote glycogen resynthesis (13). These actions of VIP and NE on astrocyte energy metabolism are mediated by the cAMP-generating signal transduction cascade (12, 13). These observations are part of a growing body of experimental evidence indicating the presence in astrocytes of specific neurotransmitter receptors that are coupled to second messenger-generating cascades (14), further elucidating the nature of neuron–glia interactions. A second messenger cascade that has emerged in recent years as being involved in several aspects of neural functions is the receptor-coupled phospholipase A2 (PLA2), whose activation leads to the release of arachidonic acid (AA) from membrane phospholipids; AA may in turn become the substrate for specific cyclooxygenases and lipoxygenases to generate prostaglandins and leukotrienes, respectively (15). Accordingly, a role for AA has been considered in long-term potentiation, ischemia, and seizures (16, 17). AA is released by astrocytes (18), where it has been shown to inhibit the uptake of glutamate (19–21). Recently, studies in Swiss 3T3 fibroblasts have demonstrated that AA is an efficient stimulator of glucose uptake (22, 23). Glucose uptake in astrocytes has been successfully examined by using 2-deoxy-D-[1-^{3}H]glucose ([^{3}H]2DG) as a tracer (24–27) and shown to be modulated by various agents, including insulin (24), ouabain (26), monensin (28), thyroid hormones (29), and elevated extracellular K^+ (30). Given the role that astrocytes play in the regulation of energy metabolism in the central nervous system, we set out to determine the consequences of manipulating AA metabolism on glucose uptake in astrocytes.

MATERIALS AND METHODS

AA was purchased from Nu Chek Prep (Elysian, MN); [^{3}H]2DG (specific activity, 17.3 Ci/mmol; 1 Ci = 37 GBq) and [^{3}H]AA (specific activity, 200 Ci/mmol) were from Amersham. Fetal calf serum (FCS) was purchased from Seromed (Berlin), while Dulbecco's modified Eagle's medium (DMEM) and all other chemicals were from Sigma. AA was dissolved in hexan (1 mg/ml) and stored as a stock solution at −70°C. On the day of the experiment, an aliquot was taken, evaporated under a stream of nitrogen, resuspended in 1 ml of diluted [^{3}H]2DG solution (final concentration, 48 nM), and briefly (5 sec) sonicated. Arachidic and linolenic acids were prepared by the same procedure, while palmitic acid was readily dissolved in 100% ethanol.

Preparation of Primary Cultures of Mouse Cerebral Cortical Astrocytes. Primary cultures of cerebral cortical astrocytes were prepared from Swiss albino newborn mice (1–2 days old) by a modification of the method described by McCarthy and de Vellis (31). Forebrains were removed aseptically from the skulls, the meninges were excised carefully, and the neocortex was dissected. The cells were dissociated by passage through needles of decreasing gauges (1.2 × 40 mm, 0.8 × 40 mm, and 0.5 × 16 mm) with a 5-ml syringe. No trypsin was used for dissociation. The cells were seeded at a density of 10^5 cells per cm^2 on six-well plates in DMEM containing 10% FCS and 25 mM glucose in a final vol of 2 ml per well and incubated at 37°C in an atmosphere containing 5% CO_2 at 95% humidity. The culture medium was renewed after 4 days of seeding and subsequently twice per week.

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Abbreviations: [^{3}H]2DG, 2-deoxy-D-[1-^{3}H]glucose; [^{3}H]AA, [^{3}H] arachidonic acid; NE, norepinephrine; VIP, vasoactive intestinal peptide; NDGA, nordihydroguaiaretic acid; PLA2, phospholipase A2.

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These conditions yield astrocyte cultures containing 85–90% glial fibrillary acidic protein-positive cells (32).

[^{3}H]2DG Uptake. Conditions were similar to those previously used in our laboratory for other metabolic studies in primary astrocyte cultures (12, 13). Experiments were conducted 14–21 days after cultures were plated. On the day of experiment, the medium was removed and cells were incubated for 4 hr in 2 ml of low glucose (5 mM instead of 25 mM) serum-free DMEM (DMEM-5) at 37°C in an atmosphere containing 5% CO_2 at 95% humidity. At the end of this 4-hr preincubation, the medium was replaced by 1 ml of DMEM-5 containing [^{3}H]2DG (final concentration, 48 nM). Pharmacological agents were added as 10 μl (10 μl of DMEM-5 was added in control plates), and cells were further incubated for 20 min (unless otherwise stated). A concentration of 5 mM glucose in the medium is sufficient to saturate the uptake process (refs. 26 and 28; this study; data not shown). The incubation was terminated by washing the cells three times with 4 ml of ice-cold phosphate-buffered saline. Astrocytes were then lysed by adding 2 ml of 10 mM NaOH containing 0.1% Triton X-100, and a 500-μl aliquot was assayed for ^{3}H by liquid scintillation counting (efficiency, 35%). The protein content was measured by the method of Bradford (33) in 100 μl of the remaining lysate. [^{3}H]2DG uptake was expressed in fmol per mg of protein.

[^{3}H]AA Release. [^{3}H]AA release was measured according to the method described by Tencé et al. (34). Using this method, agonist-induced release of radioactivity in the extracellular medium corresponds to 1–2% of cellular ^{3}H content (34). Astrocyte cultures were preincubated with [^{3}H]AA (final concentration, 5 nM) in DMEM-5 for 5 hr at 37°C. Cultures were then washed four times at 37°C with 2 ml of DMEM-5 containing 1 mg of fatty acid-free bovine serum albumin per ml and further incubated at 37°C in 2 ml of the same medium for 15 min (unless otherwise stated) in the presence or absence of pharmacological agents. At the end of the incubation, the medium was collected and centrifuged at 100 × *g* for 5 min. An aliquot of the supernatant (500 μl) was assayed for ^{3}H by liquid scintillation counting (efficiency, 35%). The protein content was measured by the method of Bradford (33). [^{3}H]AA release was expressed in fmol per mg of protein.

Statistical analysis was performed by Student's *t* test.

RESULTS

As shown in Fig. 1, basal [^{3}H]2DG uptake increases linearly during 20 min, at a calculated rate of 49 fmol of [^{3}H]2DG per min per mg of protein. Linearity of [^{3}H]2DG uptake continues at least up to 60 min (data not shown). Approximately 75% of [^{3}H]2DG uptake by astrocytes is inhibited by the specific glucose transporter inhibitor cytochalasin B (35) at a concentration of 10 μM (Table 1). The [^{3}H]2DG taken up and phosphorylated by astrocytes remains largely within the cells, as theoretically predicted (36), previously demonstrated experimentally (28), and confirmed in this study (see below). For instance, after 20 min of incubation in the presence of [^{3}H]2DG, the medium was removed and replaced by a fresh one not containing the tracer; the cultures were further incubated for increasing periods of time. The radioactivity remaining within the cells was monitored in parallel wells and shown to decrease only marginally over a period of 40 min {intracellular [^{3}H]2DG in fmol per mg of protein (n = 4): t = 0 min, 502 ± 12; t = 20 min, 418 ± 4; t = 40 min, 412 ± 8}. On the basis of these observations, an incubation time of 20 min was selected for further experiments (except for time-course experiments).

When astrocytes were incubated in the presence of 100 μM AA, a time-dependent increase in [^{3}H]2DG uptake was observed (Fig. 1). Thus, the absolute increase in [^{3}H]2DG uptake over a 20-min period in the presence of 100 μM AA corresponds to ≈600 fmol per mg of protein (Fig. 1 *Inset*). The increase in [^{3}H]2DG uptake elicited by 100 μM AA is completely inhibited by 10 μM cytochalasin B, as indicated by the fact that the amount of radioactivity remaining in the cells under these conditions is similar to that observed in the presence of cytochalasin B alone (Table 1).

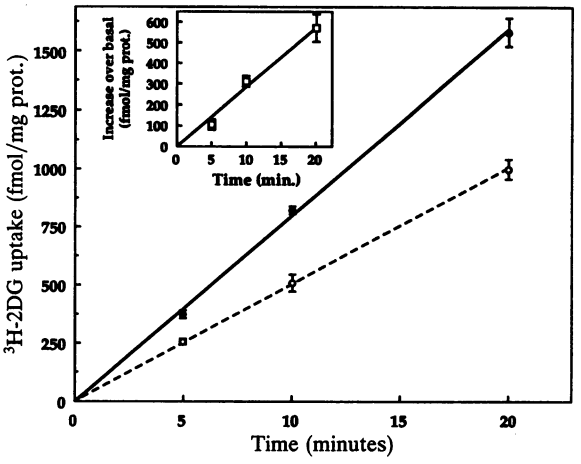

FIG. 1. Time course of basal and AA-stimulated [^{3}H]2DG uptake by primary cultures of mouse cerebral cortical astrocytes. Astrocytes were incubated for various periods of time with (●) or without (○) 100 μM AA, and [^{3}H]2DG uptake was determined as described. Results, expressed as fmol per mg of protein, are means ± SEM of triplicate determinations from one experiment repeated twice with similar results. At each time point, the difference between basal and AA-stimulated [^{3}H]2DG uptake was statistically significant (P < 0.001). (*Inset*) Absolute increase in [^{3}H]2DG uptake elicited by 100 μM AA.

The effect of AA is concentration-dependent, with an EC_{50} of ≈50 μM (Fig. 2*A*).

The specificity of the effect of AA was tested by examining the action of other fatty acids with different chain lengths or degrees of unsaturation. As indicated in Fig. 2*B*, linolenic acid (18 carbons and 3 double bonds; i.e., 18:3) also stimulated [^{3}H]2DG uptake, with an EC_{50} of ≈100 μM, while arachidic and palmitic acids (20:0 and 16:0, respectively) were inactive (Fig. 2*B*).

To determine whether the observed effect of AA was direct or dependent on the formation of some of its biologically active metabolites, notably prostaglandins or leukotrienes, the effect of AA on [^{3}H]2DG uptake was examined in the presence of specific inhibitors of the cyclooxygenase and lipoxygenase pathways. As shown in Table 2 the effect of AA on [^{3}H]2DG uptake was not inhibited by either indomethacin, a specific cyclooxygenase inhibitor, or by nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, both at 10 μM.

Table 1. Astrocytes were exposed to 100 μM AA in the presence or absence of 10 μM cytochalasin B and [^{3}H]2DG uptake was determined

Agent(s) added	[^{3}H]2DG uptake, fmol per mg of protein
None	748 ± 112
AA (100 μM)	1226 ± 73
Cytochalasin B (10 μM)	188 ± 41*
AA (100 μM) and cytochalasin B (10 μM)	111 ± 9†

Results are means ± SEM of triplicate determinations from one experiment repeated once with similar results.
*Significantly different from control (P < 0.001).
†Significantly different from 100 μM AA alone (P < 0.001).

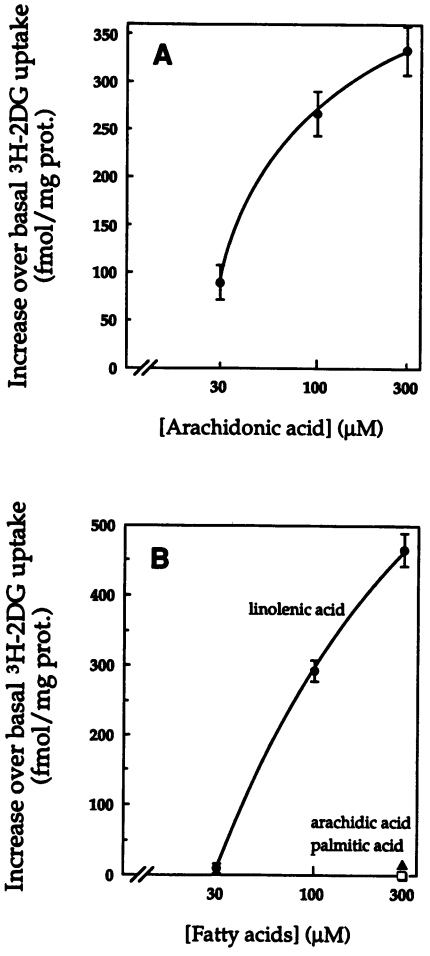

FIG. 2. Concentration–response of the stimulatory effect of AA (*A*) and linolenic acid (*B*) on [³H]2DG uptake by primary cultures of mouse cerebral cortical astrocytes. Astrocytes were incubated in the presence of increasing concentrations of AA (*A*), linolenic acid (*B*) or in 300 μM arachidic or palmitic acid (*B*). [³H]2DG uptake was determined as described. Results are means ± SEM of triplicate determinations from one experiment repeated three times (*A*) or twice (*B*) with similar results. Results are expressed as absolute increases in fmol per mg of protein over basal level. Basal [³H]2DG uptake was 427 ± 18 fmol per mg of protein.

In fact, NDGA potentiated, although marginally, [³H]2DG uptake evoked by AA (Table 2).

The levels of free AA in cells are determined by the balance between AA release from membrane phospholipids resulting from the action of specific phospholipases, notably PLA2, and by the degree of reesterification of fatty acid moieties into membrane phospholipids, a process that is catalyzed by

Table 2. Astrocytes were exposed to 100 μM AA in the presence or absence of 10 μM indomethacin or 10 μM NDGA and [³H]2DG uptake was determined

Agent(s) added	[³H]2DG, fmol per mg of protein
None	733 ± 16
AA (100 μM)	1457 ± 2
AA (100 μM) and NDGA (10 μM)	1746 ± 8*
AA (100 μM), and indomethacin (10 μM)	1568 ± 70

Results are means ± SEM of triplicate determinations from one experiment repeated once with similar results.
*Significantly different from 100 μM AA alone ($P < 0.001$).

Table 3. Astrocytes were incubated in the presence of either melittin or thimerosal and [³H]2DG uptake and [³H]AA releases were determined

Agent added	[³H]2DG uptake, fmol per mg of protein	[³H]AA release, fmol per mg of protein
None	1650 ± 39	1239 ± 11
Melittin (330 ng/ml)	5273 ± 6*	7444 ± 506†
Thimerosal (10 μM)	2258 ± 32*	4649 ± 19†

Results are means ± SEM of triplicate determinations from one experiment repeated once with similar results.
*Significantly different from [³H]2DG uptake in the absence of added agents ($P < 0.001$).
†Significantly different from [³H]AA release in the absence of added agents ($P < 0.001$).

acyltransferases (37). We therefore examined the effect of the PLA2 activator melittin (38) and of the acyltransferase inhibitor ethylmercurithiosalicylate (thimerosal) (39) on [³H]2DG uptake, reasoning that both pharmacological manipulations should enhance the levels of endogenous AA and hence of [³H]2DG uptake. As shown in Table 3, both melittin at 330 ng/ml and thimerosal at 10 μM stimulate [³H]2DG uptake; this effect is accompanied by an enhancement of AA levels as measured by the accumulation in the medium of [³H]AA previously incorporated into membrane phospholipids (ref. 34; see also *Materials and Methods*). The results reported in Table 3 clearly indicate that mobilization of endogenous AA by pharmacological manipulations can promote [³H]2DG uptake.

To further characterize the physiological significance of AA-mediated [³H]2DG uptake, we tested the effect of NE and of VIP, two neurotransmitters for which we have previously demonstrated a role in the regulation of energy metabolism in astrocytes (12, 13). As shown in Fig. 3*A*, NE at 10 μM increases [³H]AA release in a time-dependent manner over a 15-min period. Fig. 3*B* illustrates the concentration dependency of the effect elicited by NE, which reveals an EC_{50} of ≈3 μM.

NE promotes [³H]2DG uptake in a time-dependent manner (Fig. 4*A*). Thus, following a lag period of ≈4 min after NE application, [³H]2DG uptake increases markedly during the next 6 min, to reach equilibrium thereafter.

The effect of NE on [³H]2DG uptake is also concentration dependent (Fig. 4*B*) with an EC_{50} of ≈1 μM, a value remarkably close to that obtained for the stimulation of [³H]AA release (see Fig. 3*B*).

In contrast, VIP is without effect on both indices ([³H]AA in fmol per mg of protein: control = 938 ± 9.4, 1 μM VIP = 754 ± 64.1; [³H]2DG in fmol per mg of protein: control = 967 ± 4; 1 μM VIP = 966 ± 10; $n = 7$ for all conditions).

DISCUSSION

In the present study, we have examined the regulation by AA of [³H]2DG uptake in astrocytes. We have shown that AA increases, in a time- and concentration-dependent manner, [³H]2DG uptake. This effect is mimicked by pharmacological manipulations that increase the levels of endogenous AA, such as the activation of PLA2, inhibition of AA reacylation, or application of NE.

Previous studies in a number of cell types, including astrocytes (22–30, 40), have established the suitability of [³H]2DG for the assessment of glucose uptake. The characteristics of basal [³H]2DG uptake by cultured astrocytes described in this study are in good agreement with previous reports (26, 28, 30). In particular, the linearity over time of [³H]2DG uptake, and an uptake rate ranging between 3 and 9 nmol per mg of protein per min (calculated from the specific activity of [³H]2DG) are similar to what has been previously

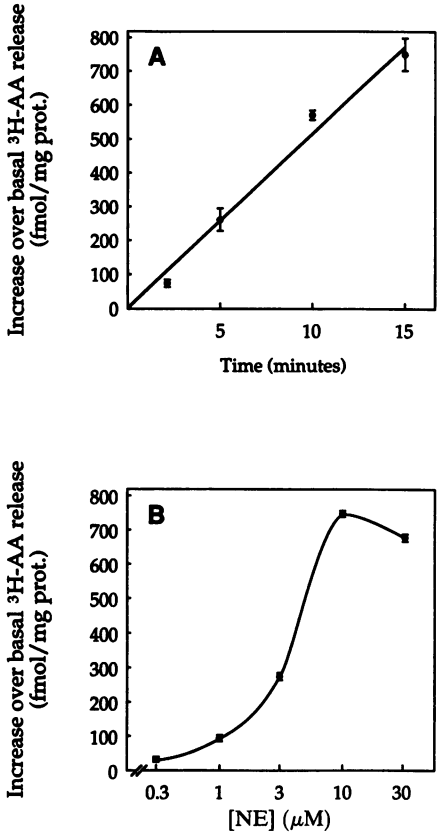

FIG. 3. Time course (*A*) and concentration–response (*B*) of the stimulatory effect of NE on [^{3}H]AA release from primary cultures of mouse cerebral cortical astrocytes. (*A*) Astrocytes were incubated for various periods of time in the presence of 10 μM NE, and [^{3}H]AA release was determined as described. Results, expressed as fmol per mg of protein, are means ± SEM of triplicate determinations from one experiment repeated once with similar results. Results are expressed as absolute increases in fmol per mg of protein over basal levels. Basal [^{3}H]AA release at each time point was (in fmol per mg of protein) 285 ± 4, 422 ± 10, 850 ± 5, and 1175 ± 43 at, respectively, 2, 5, 10, and 15 min. (*B*) Astrocytes were incubated in the presence of increasing concentrations of NE, and [^{3}H]AA release was determined as described. Results, expressed as fmol per mg of protein, are means ± SEM of triplicate determinations from one experiment repeated once with similar results. Results are expressed as absolute increases in fmol per mg of protein over basal level. Basal [^{3}H]AA release was 1175 ± 43 fmol per mg of protein.

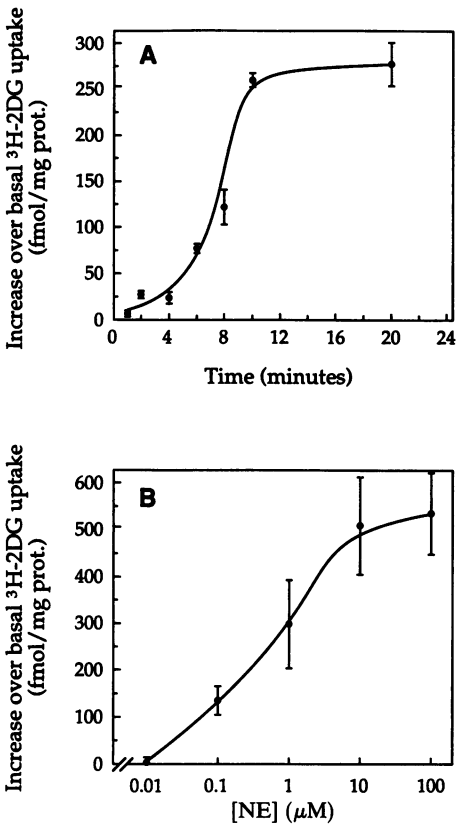

FIG. 4. Time course (*A*) and concentration–response (*B*) of the stimulatory effect of NE on [^{3}H]2DG uptake by primary cultures of mouse cerebral cortical astrocytes. (*A*) Astrocytes were incubated for various periods of time in the presence of 10 μM NE, and [^{3}H]2DG uptake was determined as described. Results, expressed as fmol per mg of protein, are means ± SEM of triplicate determinations from one experiment repeated once with similar results. Results are expressed as absolute increases in fmol per mg of protein over basal levels. Basal [^{3}H]2DG uptake at each time point was (in fmol per mg of protein) 147 ± 5, 236 ± 7, 357 ± 10, 423 ± 7, 599 ± 33, 713 ± 13, and 1203 ± 38 at, respectively, 1, 2, 4, 6, 8, 10, and 20 min. (*B*) Astrocytes were incubated in the presence of increasing concentrations of NE, and [^{3}H]2DG uptake was determined as described. Results, expressed as fmol per mg of protein, are means ± SEM of triplicate determinations from one experiment repeated once with similar results. Results are expressed as absolute increases in fmol per mg of protein over basal level. Basal [^{3}H]2DG uptake was 1004 ± 54 fmol per mg of protein.

reported (26, 30). This rate of [^{3}H]2DG uptake is of the same order as glucose utilization of the grey matter as determined by the 2-deoxyglucose autoradiography technique in rodent cerebral cortex, assuming a protein content of 10% for brain tissue (36). This observation would tend to suggest that glucose utilization in the cerebral cortex as measured by the 2-deoxyglucose autoradiography technique (36) may reflect, at least in part, glucose uptake in astrocytes.

Results reported in this article have revealed two stimulators of [^{3}H]2DG uptake in astrocytes—namely, AA and NE. The effect of AA is expressed significantly between 30 and 100 μM, similar to the concentrations at which AA stimulates glucose transport in 3T3 fibroblasts (22, 23) and inhibits glutamate uptake in astrocyte cultures (20). Experiments with cyclooxygenase and lipoxygenase inhibitors indicate that further metabolism of AA into prostaglandins or leukotrienes is not required and that AA *per se* exerts the action on [^{3}H]2DG uptake (Table 2). In fact, NDGA significantly increases the effect of AA, suggesting that by blocking lipoxygenase activity more AA is available to enhance glucose uptake. The presence of double bonds in the fatty acid chain appears to be an absolute requirement for the expression of the effect, since, as noted earlier, arachidic acid (20:0; i.e., same chain length as AA but no double bond) was without effect, while another unsaturated fatty acid, linolenic acid (18:4), also promoted [^{3}H]2DG uptake. Similar structural requirements have been reported for the effects of long-chain fatty acids on glutamate and γ-aminobutyric acid uptake inhibition (41) as well as for Na/K-ATPase activity inhibition in the nervous system (42).

Results reported in this article indicate that releasing AA from endogenous cellular sources results in effects similar to the application of exogenous AA on [^{3}H]2DG uptake by astrocytes (Table 3). Thus, both direct activation of PLA2 by melittin and inhibition of AA reacylation by thimerosal increase free [^{3}H]AA levels and [^{3}H]2DG uptake. These observations indicate that intercellular signals that trigger AA release from endogenous stores or inhibit AA reacylation could also increase [^{3}H]2DG uptake in their target cells. This hypothesis has been demonstrated by the observation that NE, a monoamine for which a clear neurotransmitter function has been established in the cerebral cortex (43)—i.e., the

brain region from which astrocytes used in this study have been prepared—releases [^{3}H]AA and promotes [^{3}H]2DG uptake with EC_{50} values that are remarkably similar (Figs. 3 and 4). The effect of NE on [^{3}H]2DG uptake is temporally delayed (≈4 min after its application), while the increase in [^{3}H]AA release is already significant at 2 min; this is consistent with the fact that a time-dependent accumulation of sufficient AA concentrations may be needed before the effect on glucose transport becomes apparent. In view of the effect of thimerosal and melittin, both inhibition of reacylation and PLA2 activation can be envisioned as potential mechanisms of action of NE to increase AA levels in astrocytes. Regardless of the molecular mechanism involved, results reported in this article demonstrate that NE can increase AA levels in astrocytes.

In the cerebral cortex, NE has been shown to stimulate the formation of cAMP followed, among other cAMP-dependent cellular actions, by glycogenolysis (11). This regulation of glycogen metabolism is exerted in astrocytes (12). Given the metabolic nature of glycogenolysis and of [^{3}H]2DG uptake, a causal relation between these two actions of NE in astrocytes could be considered. However, VIP, another neurotransmitter that triggers cAMP-dependent glycogenolysis in astrocytes (9, 12), is without effect on [^{3}H]AA levels and [^{3}H]2DG uptake. These results further support the notion that the activation of glucose uptake elicited by NE is related to the capacity of the monoamine to increase free AA levels, and not to its effect on cAMP or on glycogen metabolism.

The NE-containing neuronal system originates in the locus coeruleus (43, 44). Within the cerebral cortex, the noradrenergic system adopts a general trajectory that is parallel to the pial surface, thus endowing NE-containing axons with the capacity to exert their actions throughout the cortical mantle, spanning across functionally and cytoarchitectonically distinct areas (44). Studies in freely moving rodents and primates (44) have shown that the NE-containing neurons of the locus coeruleus are activated when the animal is exposed to unexpected, nonnoxious sensory stimulation (45). In the course of such behavioral situations, the noradrenergic tone of the cerebral cortex is enhanced and is likely to trigger, among other actions, the increase in glucose uptake by astrocytes described here. This action, in parallel with the previously described glycogenolytic effect of NE, which also occurs in astrocytes (12), would represent a coordinated regulatory mechanism to provide an adequate supply of metabolic substrates when energy demands of the active neuropil are increased.

Astrocyte end-feet surround intraparenchymal blood vessels (8); it is therefore likely that at least part of the glucose entering the brain parenchyma is taken up by astrocytes. It remains to be determined whether glucose crosses the astrocyte barrier unmetabolized or whether it is first stored as glycogen and subsequently released by glycogenolytic neurotransmitters such as VIP or NE to provide a readily available metabolic substrate for neurons.

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