

Adenosine Stimulates Glycogenolysis in Mouse Cerebral Cortex: A Possible Coupling Mechanism Between Neuronal Activity and Energy Metabolism

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Adenosine promotes a concentration-dependent hydrolysis of ^3H -glycogen newly synthesized from ^3H -glucose by mouse cerebral cortical slices. The EC_{50} for this effect is $7 \mu\text{M}$. Theophylline antagonizes the glycogenolysis induced by adenosine with an EC_{50} of $80 \mu\text{M}$. The rank-order of potencies of adenosine agonists is adenosine 5'-cyclopropyl-carboxamide > 2-chloroadenosine \gg N^6 -cyclohexyladenosine = adenosine, suggesting that adenosine promotes glycogenolysis via receptors of the A_2 type. This contention is substantiated by the weak stereospecificity observed for the glycogenolytic action of D- and L-(phenylisopropyl)-adenosine. The glycogenolysis elicited by adenosine at 10 and $100 \mu\text{M}$ is inhibited by ouabain at $10 \mu\text{M}$, a concentration of the cardiac glycoside not significantly affecting ^3H -glycogen levels *per se*. Interestingly, the previously demonstrated glycogenolytic action of vasoactive intestinal peptide (Magistretti et al., 1981, 1984) and of norepinephrine (Quach et al., 1978) is also antagonized by ouabain.

These results demonstrate the existence of a metabolic action of adenosine, which is sensitive to ouabain and appears to be mediated by A_2 receptors. The concentrations at which adenosine promotes glycogenolysis are of the same order of magnitude as those reached extracellularly by the nucleoside during neuronal depolarization (Pull and McIlwain, 1972). This set of observations therefore supports the notion that adenosine plays a modulatory role in the coupling between neuronal activity and energy metabolism in the CNS.

In recent years, a large body of experimental evidence has been accumulated delineating a role for adenosine as mediator of intercellular communication in the CNS (for a recent review, see Snyder, 1985, and references therein). Such a view has emerged in part from the demonstration of a number of cellular actions of adenosine (Phillis and Wu, 1981; Snyder, 1985), which include the inhibition (at nanomolar concentrations) and stimulation (at micromolar concentrations) of adenylate cyclase (van Calcar et al., 1978, 1979). The actions of adenosine are mediated by 2 types of receptors, A_1 and A_2 , located on the extracellular surface of adenosine's target cells (van Calcar et al., 1978, 1979; Bruns et al., 1980; for reviews, see Daly et al., 1981, and Daly, 1985). Furthermore, adenosine is released into the extracellular space from active neurons during electrically or chemically in-

duced depolarizations (Schubert and Kreutzberg, 1974, 1975; Shimizu et al., 1970a), where concentrations of adenosine can reach $150\text{--}400 \mu\text{M}$ (Pull and McIlwain, 1972).

Certain neurotransmitters have been shown to display metabolic actions in the CNS, in addition to their effects on neuronal excitability (Magistretti and Morrison, 1985; Morrison and Magistretti, 1983). Thus, vasoactive intestinal peptide (VIP) (Magistretti et al., 1981) and the monoamines norepinephrine, histamine, and 5-HT (Quach et al., 1978, 1980, 1982) hydrolyze ^3H -glycogen newly synthesized from ^3H -glucose by mouse cerebral cortical slices. Interestingly, this energy-mobilizing process appears to occur, at least in part, in astrocytes (Magistretti et al., 1983), where glycogen is predominantly stored (Lajtha et al., 1981).

In the present set of investigations we have examined the effect of adenosine on glycogen levels in mouse cerebral cortical slices and have observed that (1) adenosine promotes glycogenolysis in the cerebral cortex, (2) this effect appears to be mediated by A_2 receptors, and (3) the glycogenolytic action of adenosine is antagonized by ouabain.

Materials and Methods

Swiss male albino mice (20–25 g) were used throughout this study; they were maintained in an alternating 12 hr light/12 hr dark cycle and had free access to food and water.

Cerebral cortical slices were prepared as follows (Magistretti et al., 1981). Mice were decapitated and their brains rapidly removed. The cerebral cortex was then dissected on ice and immediately placed in a modified Krebs-Ringer bicarbonate buffer, pH 7.4 (KRG), containing (in mM concentration): NaCl, 120; KCl, 3; CaCl_2 , 2.6; MgSO_4 , 0.67; KH_2PO_4 , 1.2; glucose, 3; NaHCO_3 , 27.5, previously gassed with $\text{O}_2:\text{CO}_2$ (95:5). The dissected cortices (usually 2 per experiment) were then placed on a McIlwain tissue chopper, their ventral aspect facing the chopping plate, and $250 \mu\text{m}$ slices were prepared. The plate was then rotated by 90° , and the cortices were cut again. The slices were then resuspended in ice-cold KRG (6 ml/cortex) and incubated during 15 min at 37°C under continuous gassing ($\text{O}_2:\text{CO}_2$, 95:5) and vigorous shaking. After replacing the medium, $270 \mu\text{l}$ of the slice suspension was distributed into individual polypropylene test tubes, and $10 \mu\text{l}$ ^3H -glucose (20 nmol) was added. After gassing with a stream of $\text{O}_2:\text{CO}_2$ (95:5), the tubes were capped and incubated at 37°C . After 30 min, drugs ($20 \mu\text{l}$) were added for 10 min. Antagonists were added 3 min before the agonists. The tubes were then centrifuged, and the supernatant was removed and replaced with fresh KRG ($300 \mu\text{l}$). The slice suspension was then sonicated for 5 sec, boiled for 10 min, and centrifuged again.

^3H -Glycogen was isolated from the supernatants by ethanol precipitation on filter paper as previously described (Magistretti et al., 1981; Quach et al., 1978; Solling and Esmann, 1975). Aliquots ($150 \mu\text{l}$) of the deproteinized supernatants were pipetted on Whatman 31-ET filter paper disks (24 mm diameter) and immersed into an ice-cold 60% (vol/vol) ethanol/10% (wt/vol) trichloroacetic acid solution (10 ml/filter) for 10 min. The filters were then placed into 66% ethanol solutions (10 ml/filter) at room temperature for 6 successive 10 min periods. After a final 5 min in acetone, filters were dried, placed into scintillation counting vials, and counted for ^3H with an efficiency of 34%. Using this isolation

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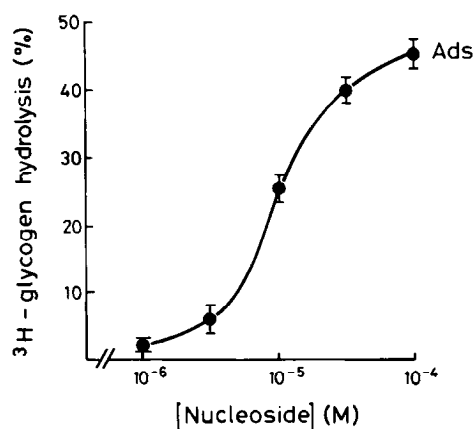


Figure 1. Concentration–response curve of the glycogenolytic effect of adenosine. Mouse cerebral cortical slices were incubated as described in Materials and Methods. Results are the means \pm SEM of 7 separate experiments and are expressed as percentages of basal ³H-glycogen levels hydrolyzed. Basal ³H-glycogen levels (cpm/mg protein): 18,124 \pm 1610.

technique, contamination by glucose was <0.5% and the glycogen recovery >99% (Magistretti et al., 1984).

cAMP levels were determined as follows. Slices were incubated and treated in conditions identical to those used for the ³H-glycogenolysis assay, except for a KCl concentration of 5 mM in KRG. Aliquots of the supernatants (10–100 μ l) were lyophilized and resuspended in 20 μ l of Tris/EDTA (0.05 M/4 mM) buffer. cAMP was then determined with the saturation assay method described by Brown et al. (1971). Experimental values were calculated by computer from a standard curve ranging from 0 to 16 pmol of cAMP.

Proteins were determined in the pellets as described by Lowry et al. (1951).

Adenosine 5'-cyclopropyl-carboxamide was a generous gift of Abbott Laboratories (North Chicago, IL), D- and L-N⁶-(S-phenylisopropyl)-adenosine and adenosine were purchased from Boehringer Mannheim (Mannheim, FRG); theophylline, 2-chloroadenosine, and N⁶-cyclohexyladenosine from Sigma (St. Louis, MO), and ouabain (g-strophanthin) from Merck (Darmstadt, FRG). 6-³H-glucose (0.5 Ci/mmol) was purchased from Amersham International.

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

Results

Mouse cerebral cortical slices maintained in KRG at 37°C will synthesize ³H-glycogen from ³H-glucose in a time-dependent manner (Magistretti et al., 1981; Quach et al., 1978); within 30 min of incubation, stable levels of ³H-glycogen are reached, and the potential glycogenolytic effect of a given substance can be tested. As shown in Figure 1, adenosine promotes a concentration-dependent hydrolysis of the newly synthesized ³H-glycogen. The EC₅₀ of adenosine in eliciting this action is 7 μ M, with a maximal efficacy of 46% hydrolysis of basal ³H-glycogen levels (Fig. 1).

Figure 2 shows that the glycogenolytic effect of adenosine is antagonized in a concentration-dependent manner by theophylline (EC₅₀, 80 μ M), an antagonist of adenosine at the adenosine receptor level (Bruns et al., 1980; Sattin and Rall, 1970).

On the basis of the rank-order of potencies of adenosine agonists and of the nature of their cellular actions, 2 types of adenosine receptors have been described, denominated A₁ and A₂ (van Calker et al., 1978, 1979; Daly, 1985). As shown in Figure 3A, we observed that the glycogenolytic action of adenosine is mimicked by the metabolically stable analogs adenosine 5'-cyclopropyl-carboxamide (ACC), 2-chloroadenosine (2CIA), and N⁶-cyclohexyladenosine (CHA). Thus, ACC promotes ³H-glycogen hydrolysis with an EC₅₀ of 150 nM and a maximal efficacy of 72%, whereas these parameters are 600 nM and 67% for 2CIA and 7 μ M and 66% for CHA (Fig. 3A). This pharmacological

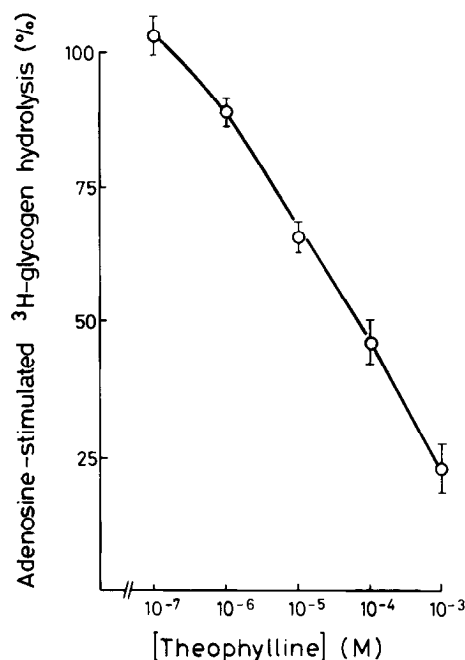


Figure 2. Concentration–response curve of the antagonism by theophylline of adenosine-stimulated ³H-glycogen hydrolysis. Mouse cerebral cortical slices were incubated as described in Materials and Methods. Results are the means \pm SEM of 3 separate experiments and are expressed as percentages of the maximal glycogenolytic effect of 100 μ M adenosine alone.

profile of potencies of ACC, 2CIA, and CHA in eliciting glycogenolysis is suggestive of an adenosine effect mediated by the occupation of adenosine receptors of the A₂ type (Daly et al., 1981; Snyder, 1985). A finer tool to dissect the receptor type mediating adenosine's effects is provided by the use of the stereoisomers of phenylisopropyl-adenosine (PIA). Thus, at A₁ receptors L-PIA is approximately 70–100-fold more potent than D-PIA, while at A₂ receptors the stereospecificity is markedly reduced, with the L-isomer being only 3–5-fold more potent than the D-isomer (Daly et al., 1981; Smellie et al., 1979a). This latter profile of potencies was observed for the glycogenolytic effect of L-PIA and D-PIA (Fig. 3B), thus substantiating the notion that adenosine promotes glycogenolysis via A₂ receptors.

We have subsequently examined the effect of the cardiac glycoside ouabain, an inhibitor of the Na⁺/K⁺-ATPase (Skou, 1965), on adenosine-induced glycogenolysis. As shown in Figure 4, ouabain, 10 μ M, inhibited the hydrolysis of ³H-glycogen elicited by 10 and 100 μ M adenosine without significantly affecting ³H-glycogen levels when tested alone. In contrast to its inhibitory action on adenosine-induced glycogenolysis, ouabain did not antagonize the stimulatory effect of adenosine on cAMP levels; in fact, ouabain, 100 μ M, and adenosine, 100 μ M, stimulated in a more than additive manner cAMP formation in mouse cerebral cortical slices incubated in conditions identical to those used for the ³H-glycogenolysis assay (Table 1).

As previously reported (Magistretti et al., 1981, 1984; Quach et al., 1978), the VIP and the monoamine norepinephrine (NE) promote a concentration-dependent ³H-glycogen hydrolysis in mouse cerebral cortex, with EC₅₀ values of 26 and 500 nM, respectively. It therefore seemed of interest to examine the effect of ouabain on the glycogenolytic action of VIP and NE. As shown in Table 2, 10 μ M ouabain inhibited almost completely the ³H-glycogen hydrolysis elicited by VIP, 0.5 μ M, and NE, 1 μ M.

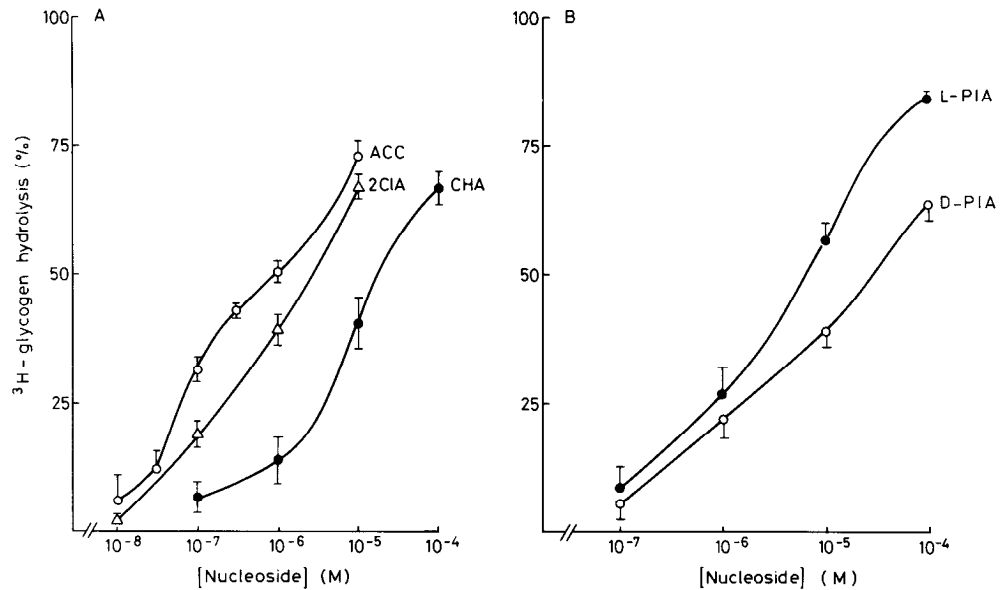


Figure 3. Concentration-response curves of the glycogenolytic effect of ACC, 2CIA, CHA (A) and of L-PIA and D-PIA (B). Mouse cerebral cortical slices were incubated as described in Materials and Methods. Results are the means \pm SEM of 3–5 separate experiments. Results are expressed as percentages of basal ³H-glycogen hydrolyzed. Basal ³H-glycogen levels (cpm/mg protein): 28,434 \pm 1880 (A) and 17,345 \pm 1787 (B).

Discussion

In the series of experiments reported in this article we observed that adenosine promotes a concentration-dependent hydrolysis of ³H-glycogen newly synthesized from ³H-glucose by mouse cerebral cortical slices. This adenosine-mediated glycogenolysis is ouabain-sensitive.

The cellular actions of adenosine, mediated by the occupation of specific adenosine receptors, are antagonized by alkylxanthines such as theophylline (Bruns et al., 1983; Fredholm and Persson, 1982; Smellie et al., 1979b). Thus, the inhibitory effect of adenosine on the spontaneous and stimulus-evoked firing rate of neurons as well as the inhibition of the release of certain neurotransmitters is antagonized by theophylline (Kuroda,

1978; Phillis and Barraco, 1985; Phillis et al., 1979). Furthermore, the alkylxanthines antagonize both the inhibitory and stimulatory effects of adenosine on cAMP generation (Daly et al., 1981; Smellie et al., 1979b), and theophylline competes (IC₅₀ in the low micromolar range) with ³H-ligands that specifically label adenosine receptors (Bruns et al., 1980). Thus, the observation reported here, that the glycogenolytic action of adenosine is antagonized by theophylline, indicates that adenosine stimulates glycogenolysis through an interaction with specific receptors located on the extracellular surface of target cells.

The rank-order of potencies of adenosine agonists, i.e., ACC > 2CIA \gg CHA = adenosine, the reduced stereospecificity for PIA isomers, and the activity of adenosine at micromolar concentrations represent indications that adenosine promotes glycogenolysis in mouse cerebral cortex through occupation of receptors of the A₂ type (Daly et al., 1981; Snyder, 1985).

The inhibition of the glycogenolytic action of adenosine by ouabain raises several points for discussion. A first hypothesis to explain this observation is that ouabain antagonizes the stimulatory effect of adenosine on cAMP formation; the cyclic nucleotide's levels would therefore not increase after the occupation of adenosine A₂ receptors, hence preventing the activation of cAMP-dependent phosphorylation cascades. One of these cAMP-dependent phosphorylation cascades leads to the conversion of the glycogen-hydrolyzing enzyme phosphorylase from its *b* (less active) to its *a* form (Siesjö, 1978). However, the interaction observed between ouabain and adenosine on cAMP levels is of an additive (or more than additive) nature, rather than of an inhibitory one (Shimizu and Daly, 1972, and Table 1). This first hypothesis appears therefore unlikely.

A second possibility is that ouabain, by inhibiting the activity of Na⁺/K⁺-ATPase and hence modifying ionic gradients across the cell membrane, inhibits the activation of cAMP-dependent enzymes distal to cAMP formation, thus preventing the expression of the glycogenolytic action of adenosine. This mechanism is supported by the observation indicating that ouabain inhibits hormone-stimulated glycogenolysis in adipocytes (Ho and Jeanrenaud, 1967). Furthermore, in the presence of ouabain or in K⁺-free media, glycogen synthesis is stimulated in adipocytes (Ho and Jeanrenaud, 1967) and in skeletal muscle (Clausen, 1966). The observation that ouabain antagonizes the glycogenolytic action of VIP and NE (Table 2) further supports the notion of a general inhibitory effect of the cardiac glycoside on the enzymatic process that leads to the breakdown of glycogen.

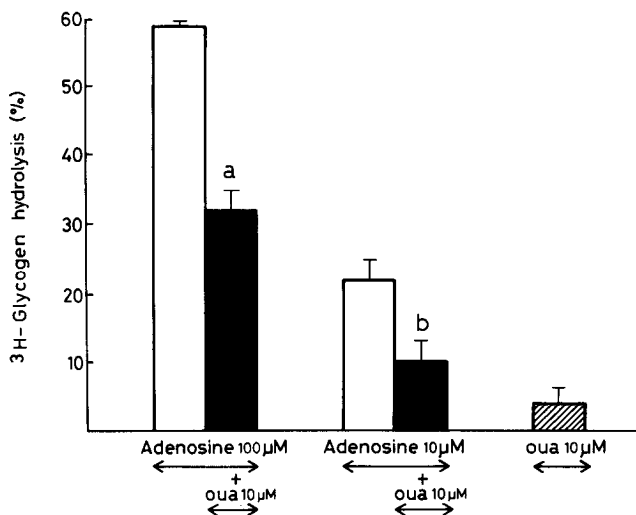


Figure 4. Inhibition by 10 μ M ouabain of the glycogenolytic effect of 10 and 100 μ M adenosine. Mouse cerebral cortical slices were incubated as described in Materials and Methods. Results are the means \pm SEM of one experiment ($n = 4$ for each value). Similar results were obtained in 3 separate experiments. Results are expressed as percentages of basal ³H-glycogen hydrolyzed. Basal ³H-glycogen levels (cpm/mg protein): 20,202 \pm 672. *a*, Significantly different from ³H-glycogenolysis observed in the presence of 100 μ M adenosine alone ($p < 0.001$). *b*, Significantly different from ³H-glycogen hydrolysis observed in the presence of 10 μ M adenosine alone ($p < 0.05$). SEM of basal levels, 3.32%.

Table 1. Stimulatory effect of adenosine, ouabain, and adenosine plus ouabain on cAMP levels in mouse cerebral cortical slices

Agent(s) added	Concentration (μM)	cAMP (pmol/mg protein)
None	—	8.11 \pm 0.91
Adenosine	100	210.9 \pm 18.7 ^a
Ouabain	100	145.7 \pm 11.11 ^a
Adenosine + ouabain	100	825.37 \pm 36.3 ^b

Experimental conditions as in Figures 1–4, described in detail in Materials and Methods. Results are the means \pm SEM of 8 separate determinations.

^a Significantly different from cAMP levels observed in the absence of added agents, ($p < 0.001$).

^b Significantly different from cAMP levels observed in the presence of adenosine, 100 μM , or ouabain, 100 μM , tested alone ($p < 0.001$).

It is also conceivable that adenosine could activate a ouabain-sensitive, energy-consuming cellular process such as a Na^+/K^+ -ATPase; this would in turn stimulate the mobilization of glycosyl units from glycogen by causing a decrease in the energy charge of the cell (Siesjö, 1978). However, this view seems unlikely, since no evidence exists for a direct activation of Na^+/K^+ -ATPase by adenosine.

It is worth noting here that adenosine and ouabain have been shown to display antagonistic interactions on at least 2 other cellular actions. First, adenosine has been shown to antagonize the ouabain-induced release of ACh from the neuromuscular junction of the frog (Branisteanu et al., 1979). This inhibitory action of adenosine has been proposed to be the consequence of a decrease in intracellular free Ca^{2+} concentration (Branisteanu et al., 1979). Second, ouabain significantly decreased the efficacy of adenosine in relaxing rabbit coronary and femoral arteries *in vitro* (Foley, 1984).

In summary, the observations presented in this article would indicate that one of the actions of ouabain in the CNS may be to impair neurotransmitter-mediated mobilization of energy stores.

Two further questions can be raised regarding adenosine-mediated glycogenolysis, i.e., when could it become operative and within which cell types? Adenosine has been shown to be released in the extracellular space upon electrically and chemically induced excitation of cerebral cortical slices (Pull and McIlwain, 1972, 1973; Shimizu et al., 1970a). The concentrations reached by adenosine in the extracellular space following such manipulations have been estimated to be between 150 and 400 μM (Pull and McIlwain, 1972). Furthermore, adenosine derivatives are released from dendrites and axon terminals, thus pointing to a neuronal origin of release (Schubert and Kreutzberg, 1975). In addition, direct evidence for a stimulation-dependent release of ^3H -adenosine from axon terminals in the dentate gyrus *in vivo* has been described (Schubert et al., 1976). Experiments reported in this article indicate that adenosine promotes glycogenolysis between 1 and 100 μM (Fig. 1), i.e., concentrations that are reached in the extracellular space following depolarization of neurons. Regarding the cellular localization of the adenosine-induced glycogenolysis, an indication is offered by experiments in which the effect of adenosine on ^3H -glycogen levels was examined in primary cultures of astrocytes (Magistretti et al., 1983). In this cell type, 100 μM adenosine hydrolyzed over 50% of the newly synthesized ^3H -glycogen (Magistretti et al., 1983). This observation, if representative of interactions occurring also *in vivo*, indicates the existence of a functional interaction between neurons and astrocytes whereby, upon depolarization, neuronally released adenosine would promote

Table 2. Inhibitory effect of ouabain on the glycogenolytic action of VIP and NE

Agent(s) added	Concentration (μM)	^3H -glycogen hydrolysis (%)
None	—	3.9 \pm 1.3
Ouabain	10	5.7 \pm 1.5 ^a
VIP	0.5	53.4 \pm 2.7
VIP + ouabain	10	7.3 \pm 2 ^b
NE	1	50.6 \pm 2
NE + ouabain	10	9 \pm 2.7 ^c

Experimental conditions as in Figures 1–4 and Table 1, described in detail in Materials and Methods. Results are the means \pm SEM of 12–24 determinations from 4 separate experiments and are expressed as percentages of basal ^3H -glycogen hydrolyzed. Basal ^3H -glycogen levels (cpm/mg protein): 44,427 \pm 1192.

^a Not significantly different from ^3H -glycogen hydrolysis in the absence of added agents ($p > 0.05$).

^b Significantly different from VIP, 0.5 μM , alone ($p < 0.001$).

^c Significantly different from NE, 1 μM , alone ($p < 0.05$).

glycogenolysis in astrocytes and mobilize energy reserves for the active neuronal environment.

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