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Vasoactive Intestinal Peptide and Noradrenaline Exert Long-Term Control on Glycogen Levels in Astrocytes: Blockade by Protein Synthesis Inhibition

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Vasoactive intestinal peptide (VIP) and noradrenaline (NA) have been previously shown to promote glycogenolysis in mouse cerebral cortex (Magistretti, 1990). This action, which is fully expressed within a few minutes, is exerted on astrocytes (Sorg and Magistretti, 1991). In the present article, we report a second, temporally delayed, action of VIP or NA in primary cultures of mouse cerebral cortical astrocytes; thus, following glycogenolysis, an induction of glycogen resynthesis is observed, resulting, within 9 hr, in glycogen levels that are 6–10 times higher than those measured before the application of either neurotransmitter. This effect of VIP or NA is concentration dependent and, for NA, is mediated by adrenergic receptors of the β subtype. The continued presence of the neurotransmitter is not necessary for this long-term effect, since pulses as short as 1 min result in the doubling of glycogen levels 9 hr later. The induction of glycogen resynthesis triggered by VIP or NA is dependent on protein synthesis, since both cycloheximide and actinomycin D abolish it entirely. The ability to elicit glycogenolysis is not sufficient per se to trigger the induction of glycogen resynthesis. Thus, two glycogenolytic agents such as methoxamine, an α_1-adrenergic agonist, and phorbol 12,13-dibutyrate, both acting via protein kinase C activation, are unable to induce glycogen resynthesis. This observation, taken together with the fact that dibutyryl-cAMP application also results in enhanced glycogen resynthesis, strongly suggests that the long-term effect of VIP or NA is mediated by the cAMP second-messenger pathway. These results indicate that the same neurotransmitter, for example, VIP or NA, can elicit two actions with different time courses: (1) glycogenolysis, occurring within minutes, and (2) glycogen resynthesis, fully expressed after several hours. The two actions are mechanistically coordinated since the long-term one, that is, glycogen resynthesis, ensures that sufficient substrate is available for the expression of the short-term effect, that is, glycogenolysis. These results also indicate that the glycogen content of astrocytes in primary culture, a condition in which neurons are absent, can increase considerably; a parallel could therefore be drawn with the marked increases in brain glycogen content, particularly in astrocytes, that are observed in experimental neurodegeneration induced by brain trauma or x-irradiation (Shimizu and Hamuro, 1958; Lundgren and Miquel, 1970). In both conditions, the increase in glycogen occurs in reactive astrocytes that have been partially or totally deprived of their neuronal environment.

The glycogen content of the rodent brain ranges between 20 and 60 nmol/mg protein, depending on the region (Passonneau and Lauderdale, 1974; Sagar et al., 1987). In the cerebral cortex, glycogen levels are approximately 30 nmol/mg protein (Sagar et al., 1987). Glycogen is localized in astrocytes, to such an extent that this cell type can be positively identified at the ultrastructural level by the presence of glycogen granules in the cytoplasm (Peters et al., 1991). This astrocytic glycogen pool is metabolically very active, as indicated by its rapid turnover rate (Watanabe and Passonneau, 1973; Ibrahim, 1975; Siesjö, 1978) and by the fact that it is under the control of multiple regulatory mechanisms (Magistretti, 1988). Thus, decreased synaptic activity, achieved for example by anesthesia (Nelson et al., 1968; Brunner et al., 1971; Passonneau et al., 1971), markedly increases the glycogen content of the brain, particularly in astrocytes (Phelps, 1972). Administration of the glutamine synthetase inhibitor methionine sulfoximine has similar effects (Phelps, 1975). Glycogen levels are also increased during slow-wave sleep (Karnovsky et al., 1983). Under normal conditions, glycogen is hardly visible at the light microscopic level using standard staining procedures (Shimizu and Hamuro, 1958). However, a common finding reported following lesions of the brain parenchyma either of traumatic origin, for example, stab wound (Shimizu and Hamuro, 1958; Hager et al., 1967; Haymaker et al., 1970; Watanabe and Passonneau, 1974; Al-Ali and Robinson, 1982) or by x-ray irradiation (Wolfe et al., 1962; Lundgren and Miquel, 1970), is a massive deposition of glycogen granules, predominantly in glial cells at the periphery of the lesioned area. These histochemical observations have been confirmed by biochemical measurements of glycogen levels following brain trauma, which indicate an early (i.e., within 10 min) decrease followed by a delayed increase in glycogen after 2.5–24 hr (Watanabe and Passonneau, 1974). Another neurodegenerative condition in which glycogen accumulations are observed, particularly in the cerebral cortex, is Alzheimer's disease (Mann et al., 1987). Brain glycogen levels are also under the tight control of numerous neurohumoral factors (Magistretti, 1988). Thus, the monoamines noradrenaline (NA), histamine, and serotonin (5-HT) (Quach et al., 1978, 1980, 1982), as well as adenosine and the peptide

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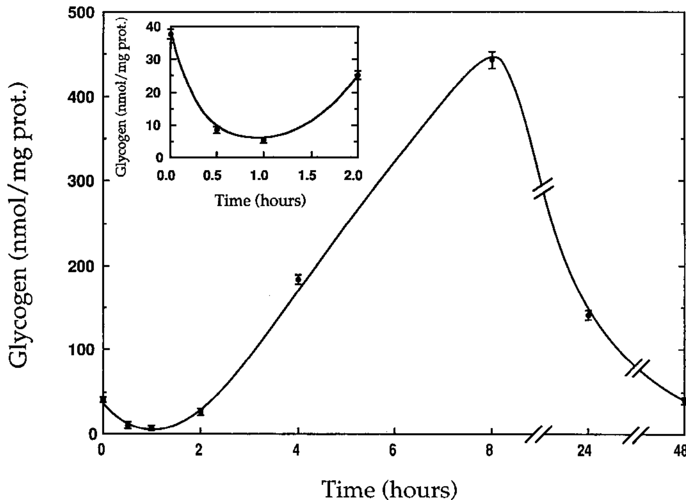


Figure 1. Time course of the effects of VIP on glycogen levels. VIP (1 μM) was applied for various periods of time. The *inset* shows in detail the curve for short periods of VIP application. Results are the mean ± SEM of quadruplicate determinations from one experiment, repeated once with similar results.

Table 1. Induction of glycogen resynthesis elicited by VIP as a function of pulse duration

Pulse duration	Glycogen levels (nmol/mg protein)
Experiment 1	
0	35 ± 7
10 min	149 ± 18
30 min	396 ± 29
1 hr	382 ± 25
2 hr	381 ± 13
9 hr	507 ± 12
Experiment 2	
0	27 ± 5
1 min	60 ± 4
5 min	69 ± 4
15 min	106 ± 7
30 min	135 ± 4
9 hr	365 ± 16

VIP (1 μM) was added for various periods of time. The medium was replaced with one not containing the neurotransmitter, and glycogen levels were assayed 9 hr after the beginning of VIP application. Results are the mean ± SEM of quadruplicate determinations for each separate experiment.

vasoactive intestinal peptide (VIP), exert a potent glycogenolytic action in slices of the cerebral cortex (Magistretti et al., 1981, 1986; Hof et al., 1988). More recently, most of these neurotransmitters have been shown to promote glycogenolysis in primary cultures of astrocytes (Sorg and Magistretti, 1991). In contrast, in this cellularly homogeneous preparation, insulin (Dringen and Hamprecht, 1992), glutamate, and methionine sulfoximine but not phenobarbital (Swanson et al., 1989, 1990) increase in a time- and concentration-dependent manner the glycogen content. These observations led us to propose that the primary action of certain neurotransmitters such as VIP or NA may be to regulate local energy homeostasis within the CNS by acting on non-neuronal cells such as astrocytes (Magistretti, 1990, 1991). From the foregoing set of observations, it is clear that the metabolism of brain glycogen occurs predominantly in astrocytes and that it is more dynamically regulated than previously thought.

As a further indication of the plasticity of brain glycogen metabolism, we report in the present article on a study conducted in primary cultures of mouse cerebral cortical astrocytes, in which two opposed and temporally regulated actions of a single neurotransmitter on glycogen levels are demonstrated. Thus, in addition to promoting glycogenolysis within a few minutes (Sorg and Magistretti, 1991), VIP or NA markedly stimulates the resynthesis of glycogen, resulting in a delayed increase in the glycogen content of astrocyte cultures. This long-term action of VIP or NA is sensitive to protein synthesis inhibition.

Materials and Methods

Cell culture. Primary cultures of cerebral cortical astrocytes were prepared from Swiss Albino newborn mice (1–2 d old) as previously described (McCarthy and de Vellis, 1980; Sorg and Magistretti, 1991). Briefly, 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 $10^5/cm^2$ on six-well plates (Sterilin) in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% fetal calf serum (FCS), in a final volume of 2 ml per well, and incubated at 37°C in an atmosphere containing 5% CO_2 at a 95% humidity. The culture medium was renewed after 4 d of seeding and subsequently twice per week. This procedure yields cultures in which 85–90% of cells are immunoreactive for glial fibrillary acidic protein (Stoyanov et al., 1988).

Glycogen assay. After 14–21 d in culture, the cells reached confluence and were used for the glycogen assay, as previously described (Sorg and Magistretti, 1991). Culture medium was replaced with a serum-free DMEM containing 5 mM glucose (instead of 25 mM for the culture medium). Four hours after having replaced the medium, pharmacological agents were added for various periods of time, during which cultures were maintained in the incubator. The reaction was stopped by washing the cells with ice-cold PBS (phosphate-buffered saline), and by adding 2 ml of 30 mM HCl. The cells were sonicated and the suspension was used to measure glycogen as described by Nahorski and Rogers (1972). Briefly, three 100 μl aliquots were sampled. In the first aliquot, 300 μl of acetate buffer were added. In the second, 300 μl of a solution containing 10% of amyloglucosidase (10 mg/ml) in acetate buffer (0.1 M, pH 4.65) were added, and the mixture was incubated at room temperature for 30 min. After incubation with amyloglucosidase, 2 ml of Tris HCl buffer (0.1 M, pH 8.1) containing $MgCl_2$ (3.3 mM), ATP (0.2 mM), NADP (25 μg/ml), hexokinase (4 μg/ml), and glucose-6-phosphate dehydrogenase (2 μg/ml) were added, and the mixture was incubated at room temperature for 30 min. The first aliquot was treated identically. The fluorescence of the NADPH formed was then read on a fluorometer (excitation, 340 nm; emission, 450 nm). The first aliquot gives the sum of glucose and glucose-6-phosphate, while the second gives the sum of glycogen, glucose, and glucose-6-phosphate; the amount of glycogen is then determined by the difference between the first two aliquots. The third aliquot was used to measure the protein content with the method of Bradford (1976). We should note that in this report, 1 mol of glycogen means 1 mol of glycosyl unit originating from glycogen.

ATP, NADP, and the enzymes were purchased from Boehringer Mannheim; VIP, from Bachem (Bubendorf, Switzerland); and all other reagents, from Sigma Chemical Co. (St. Louis, MO).

Statistical analyses were performed by the Student's *t* test.

Results

Application of VIP to primary cultures of mouse astrocytes results in two, time-dependent, effects on glycogen levels. During the first hour of application, the previously described (Sorg and Magistretti, 1991) glycogen hydrolysis is observed, leading to a virtually complete disappearance of glycogen from the cultures (Fig. 1, inset). From 1 hr on, a time-dependent resynthesis of glycogen occurs, resulting, within the third hour of incubation, in glycogen levels similar to those measured prior to the appli-

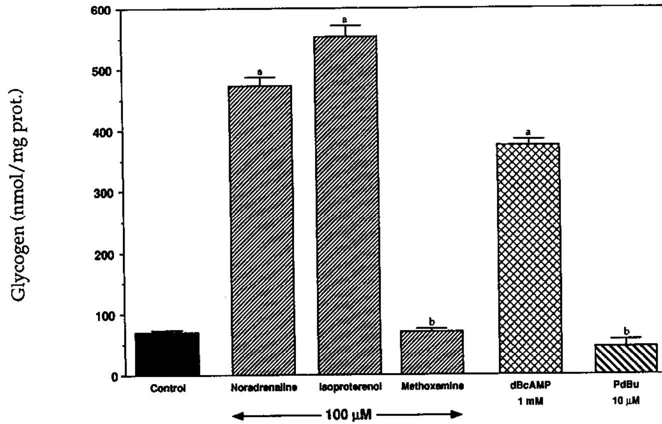
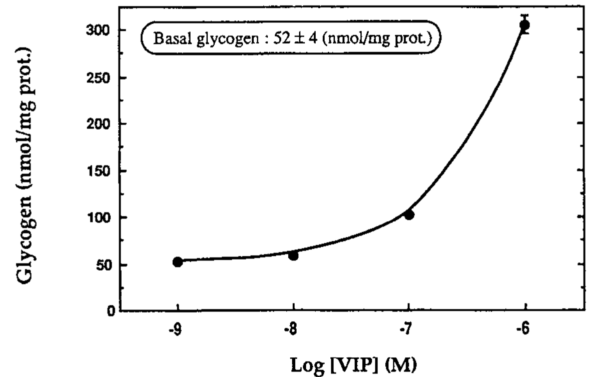


Figure 2. Effect of various glycogenolytic agents on glycogen resynthesis. Agents were applied for 30 min, the medium was replaced with one not containing the agents, and glycogen levels were assayed 8 hr 30 min later. Results are the mean ± SEM of quadruplicate determinations from one experiment, repeated twice for methoxamine and isoproterenol, three times for PdBu, seven times for dBcAMP, and 16 times for NA, with similar results. *a*, glycogen levels significantly different from control ($p < 0.01$); *b*, glycogen levels not significantly different from control ($p > 0.01$).



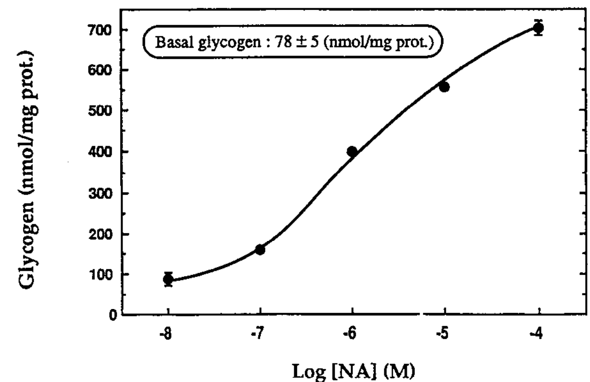


Figure 3. Concentration–response curves of the induction of glycogen resynthesis induced by VIP (*A*) and NA (*B*). VIP or NA were applied for 30 min, the medium was replaced with one not containing the neurotransmitters, and glycogen levels were assayed 8 hr 30 min later. Results are the mean ± SEM of quadruplicate determinations from one experiment, repeated once with similar results.

cation of VIP (Fig. 1). However, most unexpectedly, glycogen levels continue to rise until at least 8 hr, reaching levels 6–10 times higher than those present in the cultures under basal conditions (Fig. 1). This induction of glycogen resynthesis is reversible, since basal levels are reached within 48 hr (Fig. 1).

A puzzling consideration made in light of these results was that the stimulation of glycogen resynthesis could occur despite the continuous presence of a glycogenolytic neurotransmitter such as VIP. This fact led us to wonder whether the sustained exposure of the cultures to VIP was necessary or if a brief application of the peptide could also result in a delayed increase in glycogen levels. To test this possibility, we applied VIP pulses of various duration, changed the medium to remove the peptide, and measured glycogen levels 9 hr after the beginning of the exposure to VIP. As shown in Table 1, pulses of VIP as short as 10 min resulted, 9 hr later, in glycogen levels four times higher than those obtained under basal conditions. Even a 1 min pulse could approximately double glycogen levels (Table 1). In view of the foregoing observations, VIP pulses of 10 or 30 min were used in all further experiments.

At this stage of our analysis, the question was raised of whether the induction of glycogen resynthesis that followed VIP application was simply the consequence of the glycogenolytic action of the peptide or whether glycogenolysis and induction of glycogen synthesis could be dissociated. Results presented in Figure 2 clearly indicate that the latter mechanism occurs. Thus, methoxamine, an α_1-adrenergic agonist that promotes phosphatidylinositol turnover, and phorbol 12,13-dibutyrate (PdBu), a direct activator of protein kinase C (PKC), both promote glycogenolysis without overinducing glycogen synthesis. In contrast, NA and the β-adrenergic agonist isoproterenol, as well as dibutyryl-cAMP (dBcAMP), that is, three pharmacological agents that, like VIP, increase cAMP levels in astrocytes and promote a short-term glycogenolysis, all cause a marked overinduction of glycogen synthesis (Fig. 2).

The effect of VIP or NA in stimulating glycogen resynthesis is concentration dependent, becoming significant at concentrations over 100 nM for both neurotransmitters (Fig. 3).

Given the temporally delayed nature of glycogen synthesis overinduction promoted by VIP or NA, we considered the possibility that protein synthesis could be needed to elicit the observed effect. As shown in Figure 4*A*, this mechanism is likely to be operative, since inhibition of translation by cycloheximide (CX) completely inhibits the VIP- and NA-stimulated glycogen resynthesis. Experiments were carried out as follows: CX (100 μM) was applied 1 hr prior to a 30 min pulse of VIP or NA. After replacing the medium to remove VIP or NA, CX was added again at the same concentration, and glycogen was determined 8 hr and 30 min later. Figure 4*A* also shows that prolonged (up to 10 hr) exposure to CX at 100 μM is not toxic to astrocytes, at least as far as glycogen is used as an index, since levels of the polysaccharide in control and CX-treated cultures were not significantly different. However, a consistent observation was a marked decrease in glycogen levels in the cultures exposed to the glycogenolytic neurotransmitters in the presence of CX (Fig. 4*A*). Inhibition of transcription by actinomycin D at a concentration of 1 μM resulted in a similar inhibition of glycogen resynthesis elicited by VIP or NA (not shown).

In view of the known stimulating action of insulin on liver glycogen synthesis and of the previously described presence of insulin receptors functionally coupled to glucose transport in cultured astrocytes (Clarke et al., 1984), we examined the effect of insulin on glycogen levels. As shown in Figure 5*B*, incubation of astrocytes in the presence of insulin for 9 hr results in a

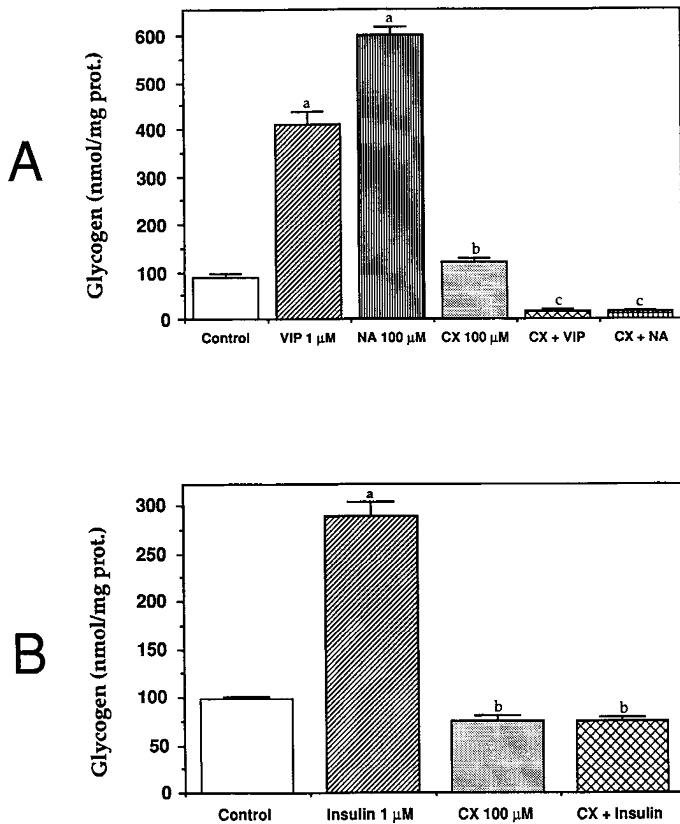


Figure 4. Suppression by cycloheximide (CX) of the induction by VIP (*A*), NA (*A*), or insulin (*B*) of glycogen resynthesis. CX (100 μM) was added 1 hr before application of VIP (1 μM), NA (100 μM), or insulin (1 μM); the neurotransmitters and insulin were maintained for 30 min; the medium was then replaced and CX added again; 8 hr 30 min later, glycogen levels were assayed. Results are the mean ± SEM of quadruplicate determinations from one experiment, repeated once with similar results. *a*, glycogen levels significantly higher than control ($p < 0.01$); *b*, glycogen levels not significantly different from control ($p > 0.01$); *c*, glycogen levels significantly lower than control ($p < 0.01$).

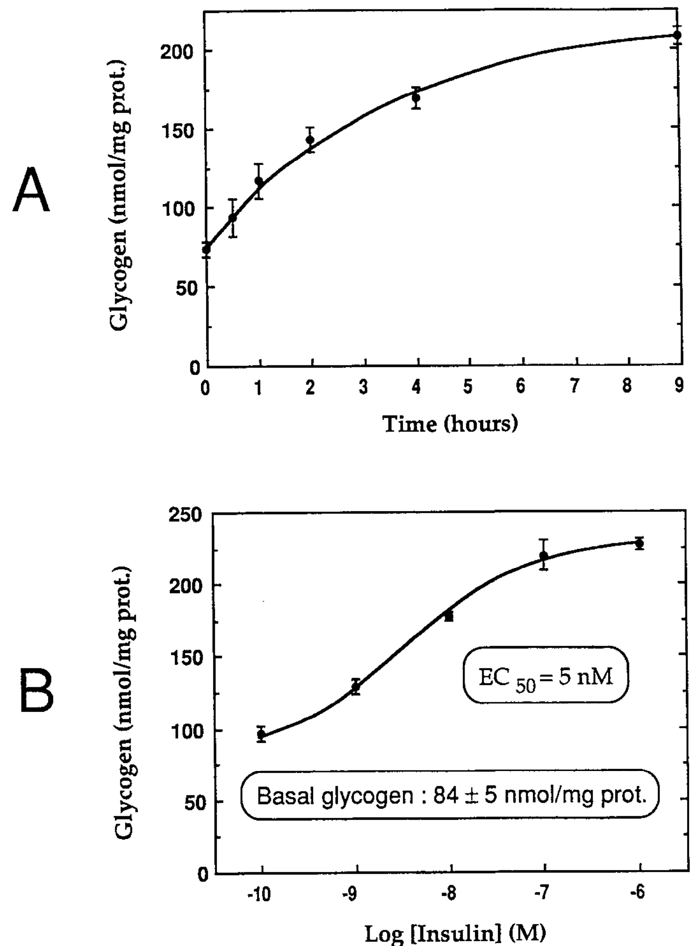


Figure 5. Effect of insulin on glycogen levels. *A*, Time course. Insulin was applied at 1 μM for increasing periods of time. *B*, Concentration–response curve. Insulin was applied for 9 hr at various concentrations. Results are the mean ± SEM of quadruplicate determinations from one experiment, repeated twice with similar results. The effect of insulin (1 μM) after application for 9 hr was measured in four other separate experiments.

concentration-dependent increase in glycogen levels. The effect is, however, less pronounced than that observed with NA or VIP, since over a number of separate experiments ($n = 5$), glycogen levels reached only two to three times control values, unlike the 6–10-fold increase consistently observed with NA or VIP. The effect of insulin is unidirectional (i.e., no glycogenolysis is elicited) as well as time dependent, with significant increases observed after 2 hr (Fig. 5*A*). The continuous presence of insulin is not necessary to stimulate glycogen synthesis. Thus, as shown in Figure 4*B*, a 30 min pulse of insulin (1 μM) can promote glycogen synthesis as measured 8 hr and 30 min later. As for NA and VIP, the effect of insulin, applied as a 30 min pulse, is abolished by CX (Fig. 4*B*).

The foregoing observations clearly indicated that glycogen levels could be increased up to 10-fold by brief exposure to appropriate agents. Since protein synthesis appeared to be involved in this phenomenon, we wondered whether other potential alterations in cell function reflected by the conversion of astrocytes from low- (40–80 nmol/mg protein) versus high- (300–600 nmol/mg protein) glycogen-containing cells would affect the glycogenolytic action of neurotransmitters. To this end, we examined glycogen hydrolysis elicited by VIP or NA in cultures pretreated for 48 hr with 1 mM dBcAMP, a treatment previously shown to increase glycogen levels six- to eightfold (Sorg and Magistretti, 1991). As shown in Figure 6, *A* and *B*, VIP or NA effectively hydrolyzed glycogen with EC_{50} values of 3 and 50 nM, respectively, which are values very similar, if not identical in the case of VIP, to those previously reported in cultures containing low glycogen levels, that is, around 30 nmol/mg protein (Sorg and Magistretti, 1991) instead of 284 (Fig. 6*A*) or 659 (Fig. 6*B*). A kinetic analysis of VIP- and NA-induced glycogenolysis in high-glycogen containing astrocytes indicates an initial rate of hydrolysis of 8 and 6 nmol/mg/min protein, respectively (Fig. 7*A*,*B*), which are values remarkably similar to those observed in low-glycogen-containing cells (Sorg and Magistretti, 1991). However, an important quantitative difference exists between the two conditions: in cultures containing low levels of glycogen, the rate of hydrolysis is linear for 2–5 min, reaches an asymptote within 10 min, and results in 60–80 nmol of glycogen/mg protein hydrolyzed over 30 min (Sorg and Magistretti, 1991). In contrast, in cultures with a high glycogen content, the initial rate of hydrolysis is linear over a 30 min period, resulting in 200–250 nmol of glycogen/mg protein hydrolyzed (Fig. 7*A*,*B*).

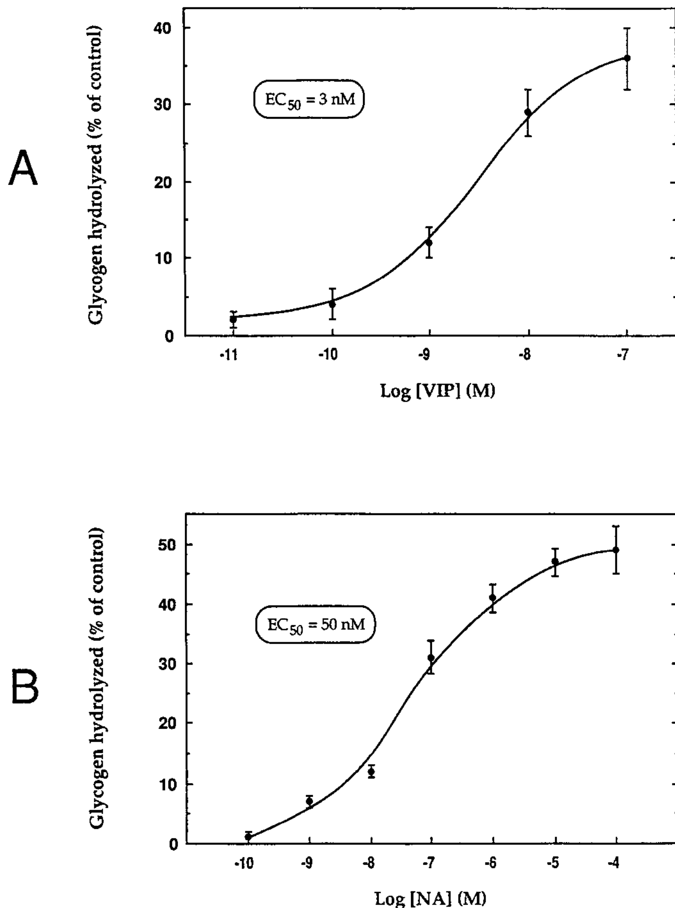


Figure 6. Concentration–response curves of the glycogenolytic effect of VIP (*A*) and NA (*B*) after a pretreatment with dBcAMP. Cultures were pretreated for 48 hr with 1 mM dBcAMP. The day of experiment, the medium was replaced by the standard one (5 mM glucose, no FCS, no dBcAMP); 4 hr later, various concentrations of VIP or NA were applied for 30 min. Basal glycogen levels were 284 ± 6 (*A*) and 659 ± 14 (*B*) nmol/mg protein for VIP and NA, respectively. Results are the mean ± SEM of quadruplicate (*A*) and triplicate (*B*) determinations from one experiment, repeated twice (*A*) with similar results.

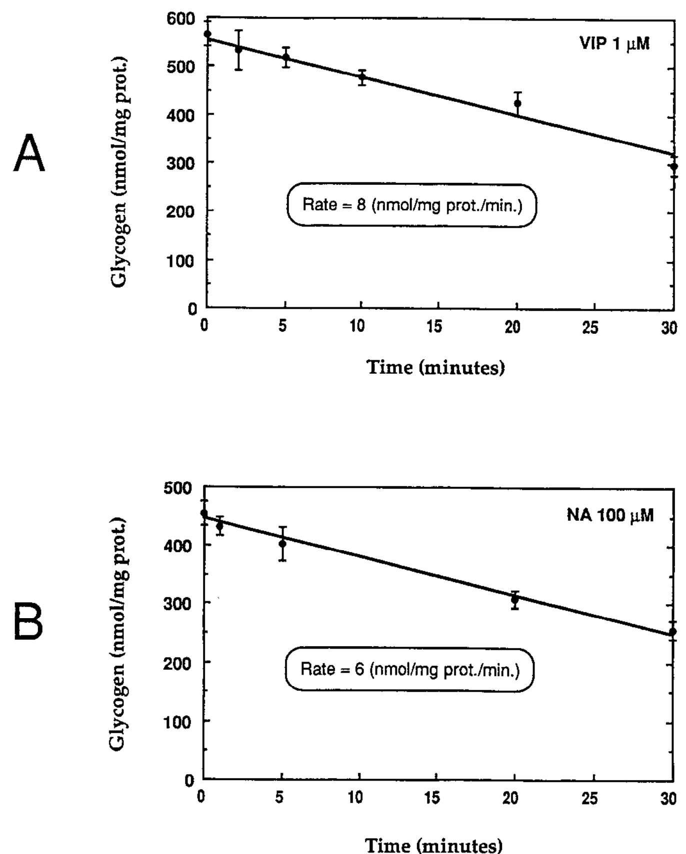


Figure 7. Time course of the glycogenolytic effect of VIP (*A*) and NA (*B*) after a pretreatment with dBcAMP. Cultures were pretreated for 48 hr with 1 mM dBcAMP. The day of experiment, the medium was replaced by the standard one (5 mM glucose, no FCS, no dBcAMP); 4 hr later, 1 μM VIP or 100 μM NA was applied for various periods of time. Results are the mean ± SEM of triplicate determinations from one experiment, repeated once with similar results.

Discussion

Results reported in the present study indicate that a given neurotransmitter can exert two opposed effects on glycogen levels in astrocytes. Thus, in addition to their well-documented glycogenolytic action (Sorg and Magistretti, 1991), VIP or NA induce glycogen resynthesis (Fig. 1). This action of VIP or NA occurs after glycogenolysis and can be triggered by brief exposure of the cultures to the neurotransmitters (Table 1). Interestingly, however, the ability of promoting glycogen hydrolysis and glycogen synthesis can be dissociated. Thus, methoxamine, an α_1-adrenergic agonist with glycogenolytic properties (Sorg and Magistretti, 1991), does not stimulate glycogen resynthesis (Fig. 2). Similarly, glycogenolysis triggered by PdBu (Sorg and Magistretti, 1991) is not followed by the induction of glycogen resynthesis (Fig. 2). α_1-adrenergic receptors are coupled to the phosphatidylinositol cascade that leads to PKC activation, and PdBu is a direct activator of PKC, while VIP and β-adrenergic receptors activated by isoproterenol promote cAMP formation (Quik et al., 1978). From the foregoing, it can be concluded that cAMP, and the subsequent activation of protein kinase A (PKA), triggers both glycogenolysis and glycogen resynthesis, while PKC activation is involved only in glycogenolysis. This is also confirmed by the dual action of dBcAMP, that is, stimulation of glycogen hydrolysis and resynthesis (Fig. 2; Sorg and Magistretti, 1991). A corollary to these comments is that induction of glycogen resynthesis is not the obligatory consequence of glycogenolysis. Furthermore, as illustrated by insulin, induction of glycogen synthesis may also occur without previous glycogen hydrolysis (Fig. 5*B*). The possible function of insulin in the brain has been long debated. Binding sites have been identified (Roth et al., 1986; Rosenfeld et al., 1987), and in particular functional receptors coupled to increased ^{3}H-2-deoxyglucose uptake have been demonstrated in cultured astrocytes (Clarke et al., 1984). Insulin-like immunoreactivity has been detected in the brain (Baskin et al., 1987; Wood et al., 1989), and an increase in glycogen levels, following insulin application *in vitro* to olfactory bulb slices, has been demonstrated (Coopersmith and Leon, 1987). Furthermore, a recent report has indicated a stimulatory effect of insulin on glycogen synthesis in astrocyte cultures (Dringen and Hamprecht, 1992), in a manner very similar to the observations reported here. Thus, a near doubling in glycogen levels was observed within 2 hr of insulin application, with an EC_{50} in the low nanomolar range (Fig. 5*A*,*B*; Dringen and Hamprecht, 1992). In contrast to the present report, however, the effect of insulin was revealed in cultures that had been starved of glucose for 2 hr, a treatment that decreased intracellular glycogen content by approximately 80% of normal levels (Dringen

and Hamprecht, 1992). In our study, we demonstrate that the effect of insulin is expressed in the presence of physiological concentrations of glucose. Furthermore, we have observed that a 30 min pulse of insulin is sufficient to promote glycogen synthesis through a mechanism that is sensitive to protein synthesis inhibition (Fig. 4*B*). Taken together, results reported here and in previous studies further stress the metabolic role played by insulin, or by insulin-like molecules, within the nervous system.

The effect of VIP, NA and also of insulin in increasing glycogen levels is observed, with a temporal delay, even after a brief application to the cultures. Thus, it is striking to note that a 1 min pulse of VIP leads to the doubling of glycogen levels within 9 hr after application. This observation strongly indicates that a cascade of events is triggered by the neurotransmitter, which progresses independent of receptor occupation. A likely step in this cascade of events is the increase in cAMP levels. Experiments in our laboratory indicate that following VIP application to astrocyte cultures, cAMP levels peak at 10 min and return to basal levels within 1 hr (P. J. Magistretti, M. Maillard, and J. L. Martin, unpublished observations). Evidence has been provided in recent years indicating that PKA can phosphorylate, among a number of substrates, a transcription factor called CREB (cAMP-responsive element–binding protein), which acts on specific DNA sequences, that is, cAMP-responsive elements, to regulate the expression of certain genes (Yamamoto et al., 1988; Montminy et al., 1990; Sheng et al., 1990). A clear example is the regulation by cAMP of the gene encoding for somatostatin (Gonzalez and Montminy, 1989) and for a number of G-protein–coupled membrane receptors, such as the substance P or the β-adrenergic receptors (Hershey et al., 1991; Collins et al., 1992). The fact that the increase in glycogen levels elicited by VIP or NA, two neurotransmitters that promote cAMP formation in astrocytes, is temporally delayed and sensitive to agents that inhibit DNA transcription and RNA translation strongly suggests that the synthesis of one or more enzymes involved in glycogen anabolism might be the molecular mechanism underlying the effect of VIP and NA. A number of enzymes are potential candidates for such regulation. Thus, glycogen synthase transcription could be enhanced by VIP or NA. Furthermore, since the activity of glycogen synthase is decreased, while that of phosphorylase is enhanced by phosphorylation, induction by VIP or NA of mRNA transcripts for phosphatases and their subsequent translation can also be envisioned. The dephosphorylation of glycogen synthase and phosphorylase that would result from increased phosphatase activity would favor glycogen synthesis. As to the effect of insulin, the synthesis of a number of mRNAs has been shown to be enhanced by the peptide in peripheral tissues, although the cascade of intracellular events leading to such effects is still largely unknown (Sasaki et al., 1984).

To begin to tackle the functional significance of increased glycogen content in astrocytes, the glycogenolytic effect of VIP or NA in cultures in which glycogen levels had been increased four- to sixfold by treatment with dBcAMP was examined. Analysis of the time and concentration dependence of the glycogenolytic action exerted by VIP or NA under those conditions clearly indicates that the potency of both neurotransmitters, as well as the rate/minute of glycogen hydrolysis, is virtually identical in astrocytes with low (Sorg and Magistretti, 1991) and high (Figs. 6*A,B*; 7*A,B*) glycogen content; however, the initial rate is maintained for at least 30 min in glycogen-rich astrocytes, indicating that considerably more glycosyl units are released. As a comparison, in astrocytes containing low glycogen levels, 30–80 nmoles of glycogen per mg of protein are hydrolyzed within the first 30 min after VIP or NA application, while in glycogen-rich astrocytes this value ranges between 200 and 250 (Fig. 7*A,B*). The question can therefore be raised of which of the two types of cultures reflects more closely *in vivo* conditions. The glycogen content of whole rodent brain ranges between 20 and 60 nmol/mg protein (Passonneau and Lauderdale, 1974; Sagar et al., 1987); a recent report in which microwave irradiation was used as an improved inactivation procedure indicated glycogen levels of 33 nmol/mg protein in rat cerebral cortex (Sagar et al., 1987). Under the experimental conditions used in this study, the mean glycogen content of primary astrocyte cultures was 62 ± 8 (n = 60), a value in good agreement with other recent reports (Swanson et al., 1989, 1990; Dringen and Hamprecht, 1992). When expressing the results of glycogen content *in vivo* per milligram of protein, one should bear in mind that glycogen is predominantly contained in astrocytes (Sotelo and Palay, 1968; Vaughn and Grieshaber, 1972), while other cells, namely neurons, which contain virtually no glycogen, contribute to the measured proteins. This means that *in vivo* studies underestimate the glycogen content of astrocytes, the question being by what factor. Since astrocytes constitute approximately half of the volume of the cerebral cortex (Kimelberg and Norenberg, 1989; Bignami, 1991; Peters et al., 1991), one could roughly estimate that glycogen content of astrocytes *in vivo* to be about 50–60 nmol/mg protein, that is, twice the value obtained when expressing the results by taking into account the protein content of cells that contain virtually no glycogen. An even more conservative estimate can be made by considering the fact that astrocytes outnumber neurons in the gray matter by approximately a factor of 10 (Bignami, 1991; Peters et al., 1991). This would indicate that levels of glycogen measured *in vivo* and expressed per milligram of protein should be corrected by only 10% to estimate astrocytic glycogen *in vivo*. Regardless of the correction factor used, it is clear that glycogen levels measured in the cultures after induction by VIP or NA, that is, values ranging between 250 and 600 nmol/mg protein, are considerably higher than those estimated *in vivo*.

What is, then, the significance of the induction of high glycogen levels by VIP or NA? One consideration that can be made is that astrocytes present in the cultures have not been exposed to neurotransmitters for 2–3 weeks, in contrast to their *in vivo* counterparts. Application of VIP or NA to neurotransmitter-naive astrocytes could therefore trigger the expression of developmentally regulated processes that result in glycogen levels normally found in the fully differentiated astrocytes present *in vivo*. However, as discussed earlier, the levels of glycogen *in vivo* are similar, after appropriate correction, to those observed in neurotransmitter-naive cultures rather than to those measured in cultures treated with VIP or NA. These considerations would therefore tend to discard the possibility that neurotransmitter-naive astrocytes are brought, by the exposure to VIP or NA, to a state of differentiation that results in glycogen levels observed *in vivo*. An obvious difference between the situation *in vivo* and in primary astrocyte cultures is that in the latter condition, neurons are missing. It is therefore likely that a subtle interplay between the glycogenolytic and synthetic effects of VIP or NA released *in vivo*, according to precise spatial and temporal modalities, results in levels of glycogen dynamically equilibrated around 30 nmol/mg of protein, that is, levels that are similar to those measured in unstimulated astrocytes *in vitro*. It is also

likely that other neuronally generated signals contribute to the stabilization of glycogen to the levels encountered *in vivo*. However, when astrocytes are exposed *in vitro* to VIP or NA in the absence of neurons, the full potential of the glycogen-synthetic effect of the two neurotransmitters is expressed undisturbed to yield glycogen levels in the hundreds of nmoles per milligram of protein. Slowly, however, that is, within 48 hr, the glycogen content of astrocytes returns to basal levels (Fig. 1).

These considerations may have a bearing to the increased glycogen content that is frequently encountered in certain types of reactive astrocytes, in particular following traumatic lesions of the brain parenchyma (Shimizu and Hamuro, 1958; Hager et al., 1967; Haymaker et al., 1970; Watanabe and Passonneau, 1974; Al-Ali and Robinson, 1982) and α-particle or x-irradiation (Wolfe et al., 1962; Lundgren and Miquel, 1970). Under these conditions, astrocytes, notably those that surround the lesioned area where neuronal death has occurred, are enriched in glycogen as assessed biochemically (Watanabe and Passonneau, 1974) and visualized by light and electron microscopy (Hager et al., 1967; Al-Ali and Robinson, 1982). The time course of the changes in glycogen levels has been examined following stab wounds in rat cerebral cortex (Watanabe and Passonneau, 1974). Within few minutes after the lesion, the glycogen content is markedly decreased. Concomitantly, cAMP levels reach peak values that are sevenfold higher than the control, returning to basal levels within 1 hr. In contrast, glycogen accumulation follows the initial decrease, reaching, already at 2.5 hr, values that are 2.5-fold higher than control levels (Watanabe and Passonneau, 1974). This increase in glycogen content in the vicinity of the lesion is maintained for at least 24 hr. The temporal pattern of glycogen decrease followed by increased glycogen deposition is strikingly similar to results reported in this article. Another study has indicated that localized lesions of the cerebral cortex caused by needle penetration result in an increase in glycogen levels not only at the site of reactive gliosis surrounding the insult, but also at distant sites throughout the neocortical mantle (Haymaker et al., 1970). Marked glycogen increases are also observed following inhibition of synaptic transmission by general anesthetics, that is, another condition in which neuronally generated signals to astrocytes are likely to be decreased (Nelson et al., 1968; Brunner et al., 1971; Passonneau et al., 1971). Recently, blockade of action potentials in the afferent nerve has been shown to produce reactive astrocytes in the chick cochlear nucleus (Canady and Rubel, 1992). It would be of interest to determine whether these reactive astrocytes also contain high glycogen levels.

The role of astrocytic glycogen in neural function has not yet been fully clarified. As noted earlier, the decrease in neuronal activity caused by lesions or by general anesthesia, markedly increases the glycogen content of astrocytes, indicating that under conditions of physiological synaptic activity, the turnover of the polysaccharide is rapid. In line with this view is the presence of corpora amylacea, that is, spherical bodies of 10–15 μm diameter revealed by standard glycogen stains, that are found in fibrous astrocytes of normal aging brains and in certain neurodegenerative disorders (Hume Adams et al., 1984). Like other cytoplasmic inclusions collectively denominated polyglucosan bodies (Robitaille et al., 1980; Thomas et al., 1980), such as Lafora and Bielschowsky bodies or amylopectin bodies of type IV glycogenosis, corpora amylacea are constituted by glucose polymers, implying that they are similar, if not identical, to glycogen. The mechanisms that lead to the accumulation of such glycogen-containing inclusions in astrocytes are unknown. It is conceivable that in pathological situations in which neuronal activity is decreased, glucose supplied by the circulation is in considerable excess of either the capacity of astrocytes to hydrolyze glycogen under the action of neurotransmitters such as VIP and NA, or of the energy requirements of the brain parenchyma. Supporting this view is the observation of increased glycogen deposition in cortical astrocytes of patients afflicted by Alzheimer's disease (Mann et al., 1987), a condition in which a considerable loss of neurons is at the forefront of the histopathological diagnosis (Hume Adams et al., 1984).

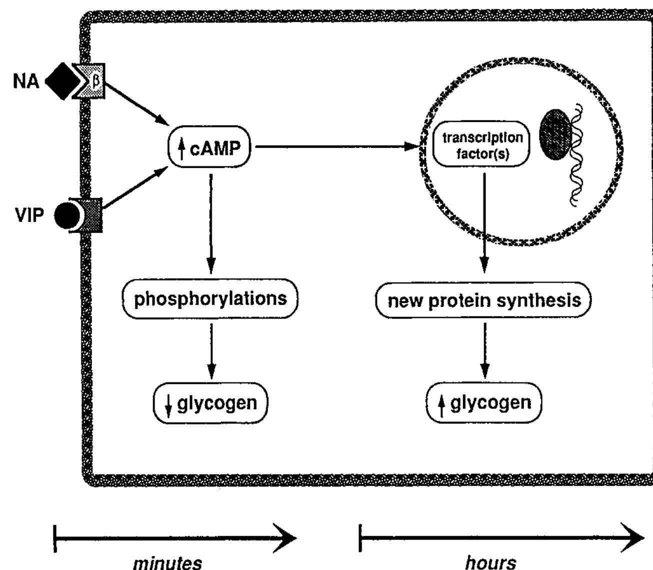


Figure 8. Bidirectional effects of VIP or NA on glycogen in astrocytes. With the short-term effect, within minutes after application, VIP or NA promote glycogenolysis. This effect is due to cAMP-dependent phosphorylation of preexisting proteins. With the long-term effect, within a few hours after application of VIP or NA, glycogen levels are increased 6–10 times above control levels. This effect is due to cAMP-dependent induction of new protein synthesis.

As previously reviewed, it is clear that glycogen represents an energy substrate reserve under the tight control of various neurotransmitters, including VIP and NA (Magistretti, 1990). In passing, one can note that the initial velocity of the glycogenolysis elicited by VIP or NA, that is, 7–9 nmol/mg protein/min (Sorg and Magistretti, 1991), is of the same order of magnitude as the glucose utilization of the rodent cerebral cortex measured with the 2-deoxyglucose technique, that is, 10–16 nmol/mg protein/min (Sokoloff et al., 1989). This correlation would tend to indicate that glycogen hydrolysis evoked by neurotransmitters may provide, in a quantitatively significant manner, energy substrates for neuronal function.

Regardless of the possible implications in brain pathophysiology, results reported in this study clearly indicate that the two neurotransmitters VIP and NA exert actions on glycogen metabolism, which, while appearing opposite in nature at superficial analysis, may in fact be endowed with a functional and teleologically directed complementarity. Thus, by increasing cAMP levels, VIP or NA simultaneously trigger a short-term effect, that is, glycogenolysis, as well a delayed one, that is, transcriptionally regulated glycogen resynthesis. This longer-term effect ensures that sufficient substrate is available for the continued expression of the short-term action of VIP or NA (Fig. 8).

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