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CALCIUM STIMULATION OF GLUTAMINE HYDROLYSIS IN SYNAPTOSOMES FROM RAT BRAIN†

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Calcium stimulates the hydrolysis of glutamine in synaptosomes prepared from rat brain both by the sucrose- (12) and the Ficoll/sucrose-gradient techniques (13). The calcium activation is phosphate-dependent and maximal effect is obtained at a calcium concentration of 0.5–1.0 mM. It is reduced by increasing the numbers of synaptosomes in the incubation mixture, and abolished by the product inhibitors of glutaminase, glutamate and ammonia, but unaffected by the uncoupler 2,4-dinitrophenol which inhibits the mitochondrial proton pump. Moreover, since the hydrolysis of glutamine is mediated by glutaminase (EC 3.5.1.2), and calcium does not activate the purified enzyme, an indirect phosphate-dependent effect of calcium on glutaminase is most likely. Calcium activates preferentially the N-ethylmaleimide insensitive fraction of glutaminase. The calcium activation is not dependent on synaptosomal membranes as it is found in synaptosomes subject to previous freezing. It is also found in isolated synaptosomal mitochondria and is thus a property of nerve endings. The calcium activation of glutaminase is unaffected by potassium in depolarizing concentrations, and may not be directly involved in the neurotransmission processes, but possibly in replenishing depleted stores of transmitter glutamate.

INTRODUCTION

Since it has been suggested that phosphate-activated glutaminase (PAG) (L-glutamine amidohydrolase (EC 3.5.1.2)) is mainly responsible for production of the transmitter candidates glutamate and GABA from glutam-

† This issue is dedicated to Donald B. Tower.
Abbreviations used: NEM, N-ethylmaleimide; PAG, phosphate-activated glutaminase.

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ine (1–4), the regulation of this enzyme is essential. Similar to purified PAG (5), PAG in the synaptosomal-enriched fraction is not only activated by phosphate, but also by many other anionic compounds such as succinate and citrate (6). Rather high concentrations of organic anions are however, required so that it is uncertain if they play any role in the regulation of PAG. Otherwise, main regulators of tissue-bound PAG appear to be the reaction products, glutamate and ammonia, as well as the pH (6–7). We have described in preliminary reports that calcium is a potent activator of the PAG-mediated glutamine hydrolysis in synaptosomal-enriched preparations, and kidney mitochondria (6, 8). Similar findings have recently also been reported in brain homogenate and slices (9) and in cultured astrocytes (10).

In this paper we have studied in more detail the mechanism of the calcium effect in synaptosomal-enriched preparations and particularly, the combined effect of calcium and physiologically active effectors on PAG, such as phosphate, glutamate and ammonia, as well as that of NEM and depolarizing concentrations of potassium.

EXPERIMENTAL PROCEDURE

Materials. Wistar rats 2 months of age were obtained from the National Public Health Institute, Oslo. L-[U-^{14}C]glutamine was obtained from New England Nuclear corp Boston, Massachusetts. Antimycin A, oligomycin and calmodulin (phosphodiesterase 3′-5′ cyclic nucleotide activator) from bovine heart were products of Sigma Chemical Co. St. Louis Missouri. The other reagents from commercial sources were of purest grade available.

The Preparation of Synaptosomal-Enriched Fractions and Assay of Glutamate Formation from Glutamine. The synaptosomal-enriched fraction was either prepared as described by Kvamme and Olsen (11), by sucrose-gradient fractionation according to Whittaker and Barker (12), or by the Ficoll/sucrose-gradient technique by Booth and Clark (13). A purer synaptosomal fraction has been postulated to be obtained by the latter method (13). However, this has not been confirmed by others (14), and the yield is lower which makes this method more cumbersome. Synaptosomal mitochondria were prepared according to Lai and Clark (15).

The glutamate formation from glutamine, which is a measure of the PAG activity of the synaptosomal-enriched preparations, was assayed by measuring labelled glutamate formed from labelled glutamine after incubation for 2 min at 25°C and pH 7.4 if not otherwise stated, following a preincubation period of the synaptosomes without labelled glutamine for 5 min at 25°C. The reaction was thus started by addition of 2 mM L-[U-^{14}C]glutamine and terminated by adding ice-cold ethanol to a final concentration of 67% followed by centrifugation (18,000 *g*, 1 min).

The assay mixture contained synaptosomal-enriched fraction (usually 4 mg protein per ml), 90 mM NaCl, 56 mM KCl, 100 mM D-mannitol, 30 mM sucrose, 4 mM Hepes (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) and 5 mM $MgCl_2$. The content of Na-phosphate and other compounds are indicated in the figure legends. 10 mg/l oligomycin and 0.6 mg/l antimycin A were added to prevent oxidation of glutamate.

Using the Ficoll/sucrose-gradient method for isolation of synaptosomes, the initial rate of glutamine hydrolysis was constant for about 5 min under our conditions (Figure 1), which agrees with our previous findings, employing the sucrose-gradient technique (11, 16). Moreover, in our assay the variation in rate of glutamine hydrolysis with the substrate (0.5–2.0 mM) and phosphate concentration (5–10 mM) followed closely first order kinetics, indicating

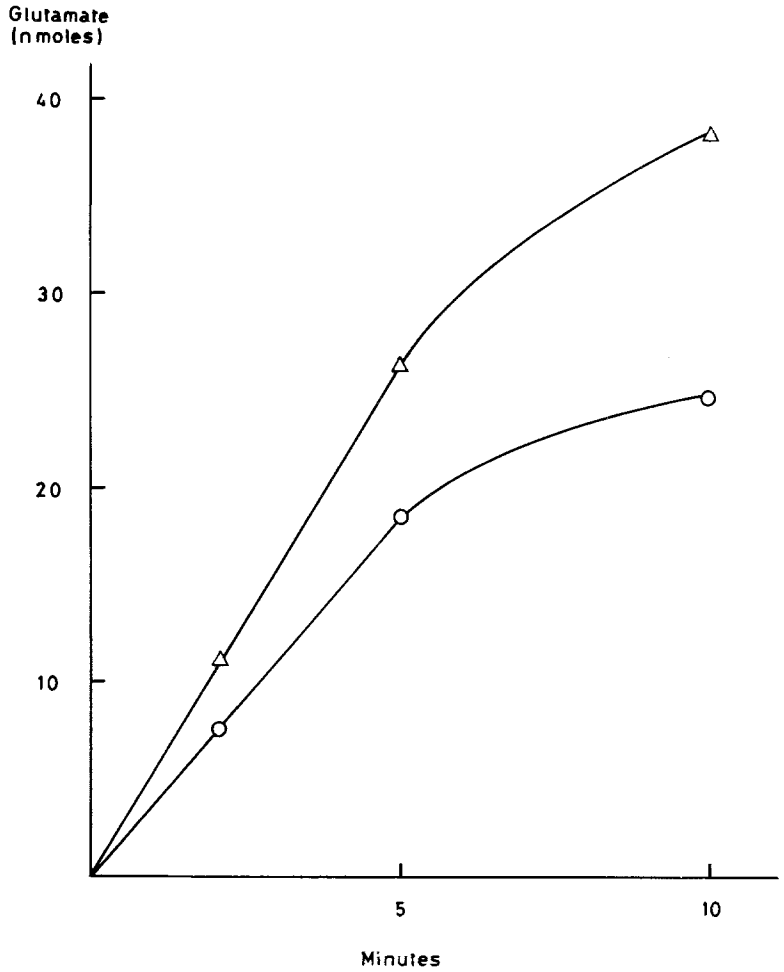

FIG. 1. Glutamate formation from glutamine as a function of time in synaptosomal preparations. The synaptosomal-enriched preparation which was prepared by the Ficoll/sucrose-gradient technique (13), was preincubated for 5 min at 25°C and pH 7.4 with 10 mM phosphate and with and without 1 mM calcium, as indicated. Thereafter the preparation was incubated for the time indicated at 25°C and pH 7.4 with 2 mM L-[U-^{14}C]glutamine. Other conditions were as described in Experimental Procedure. Mean of 2 experiments. ○: no calcium added; △: calcium added.

that the synaptosomal membrane represents no important barrier when the concentration of these compounds are not below the lower limits shown in the brackets.

Paper Chromataographic Determination of Amino Acids. Since glutamine in synaptosomal-enriched preparations is initially almost exclusively metabolized by the PAG reaction to yield glutamate and ammonia, it is of utmost importance to get a good separation of glutamine and glutamate and to keep the "blank values" of glutamate formed by non-enzymatic hydrolysis of glutamine, as low as possible. This is achieved by paper chromatography in *n*-butanol-acetic acid-water (100:22:50, v/v), using the "drip and dry" method (5). The processing of the chromatograms and counting were as described before (11). If not otherwise stated, the results are expressed as means of 6 experiments ± standard error of means and the 100% values of the figures are within the ranges shown in Table I.

Ammonia was measured enzymatically by determining NADPH oxidized in the glutamate dehydrogenase reaction (5).

Protein was determined by the biuret method of Zamenhof (17).

RESULTS

As demonstrated in Figure 1, calcium activates glutamine hydrolysis, and the initial rate of hydrolysis is constant for about 5 min, also with calcium present (Figure 1). Maximal activation is obtained by 0.5–1.0 mM calcium (Figure 2) and no further activation has been obtained by increasing the calcium concentration beyond 1 mM. It should be noted that the relative increase in ammonia formation on addition of calcium corresponds closely to that of glutamate (Figure 2 A). As expected, EGTA abolishes the effect of calcium (not shown). Calcium promotes the activation by phosphate, because this activation is not additive but almost proportional to that of calcium (Figure 2 B). Furthermore, the calcium activation of glutamine hydrolysis is completely dependent on phosphate, as calcium has no effect in the absence of this ion (Table I). Synaptosomes prepared both by the sucrose- (12) (Figure 2 B, Table I A) and the Ficoll/sucrose-gradient techniques (13) (Figure 1, 2 A) show calcium activation of the hydrolysis of glutamine, but the rate of hydrolysis appears to be somewhat lower in the latter preparation, and slightly higher calcium concentrations are required to obtain activation.

The hydrolysis of glutamine to yield glutamate and ammonia is catalyzed by mitochondrial PAG and no other enzyme appears to have any appreciable effect on this reaction in synaptosomes (11). Since the synaptosomal membrane contains active transport mechanisms for compounds which might influence the activity of mitochondrial PAG, we tested synaptosomes where the membranes were damaged by freezing. As shown in Table I A b synaptosomes kept frozen for 4 months at −80°C show no appreciable loss in hydrolytic activity and the calcium activation does not appear to be dependent on an intact synaptosomal membrane.

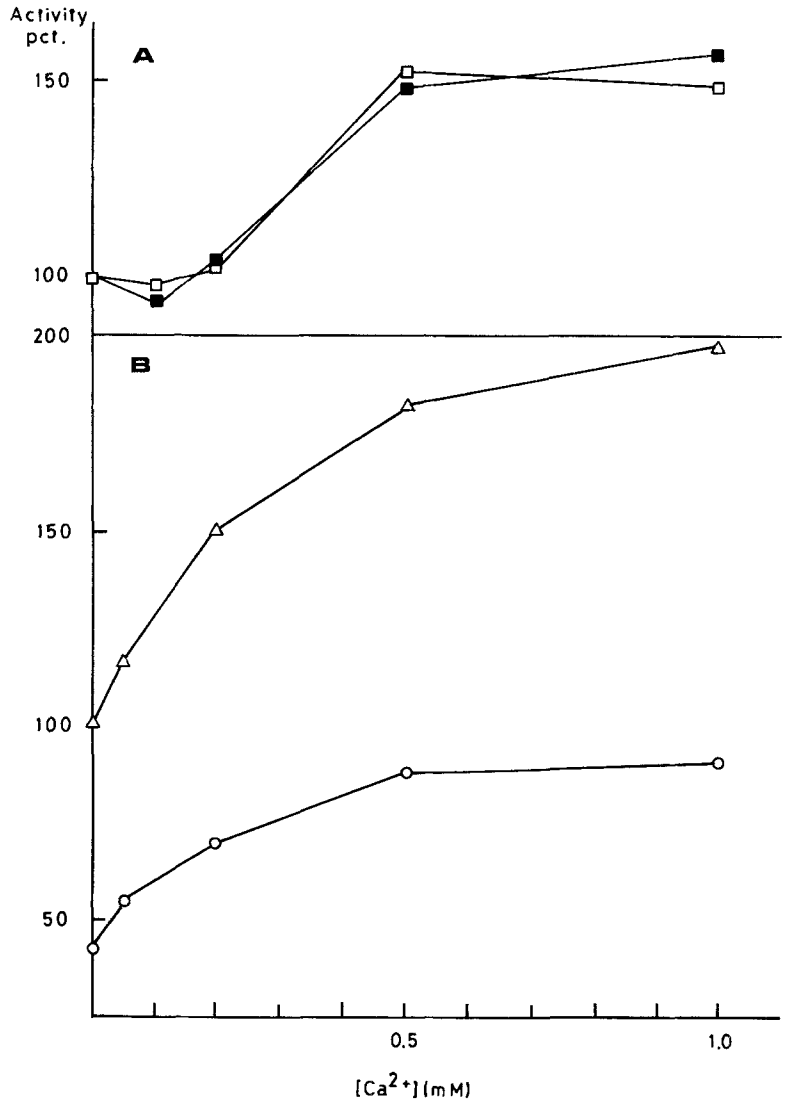

FIG. 2. The effect of calcium on the formation of glutamate and ammonia from glutamine. The assay conditions were as in Figure 1. The activity is calculated as glutamate or ammonia formed per min in percent of that in assays with no calcium and 10 mM phosphate added. A: Synaptosomes prepared by the Ficoll/sucrose-gradient technique (13). 10 mM phosphate added. NH_4^+ (■) and Glu (□) formed were determined in the same experiment. B: Synaptosomes prepared by the sucrose-gradient technique (12). Mean of 4 experiments. Added phosphate: ○, 5 mM; △, 10 mM.

TABLE I
PHOSPHATE DEPENDENCE OF THE CALCIUM ACTIVATION OF PAG

		nmol Glu·min^{-1}·mg protein^{-1}				
		A			B	
		a		b		
[Pi] (mM)		0	10	10	0	10
[Ca^{2+}] (mM)	0	2.3 ± 0.1	6.1 ± 0.4	6.1	5.6 ± 2.0	15.9 ± 2.3
	0.5	2.2 ± 0.1	10.7 ± 0.7	9.2	4.3 ± 1.3	32.7 ± 5.3

The synaptosomal-enriched preparation was preincubated either with and without calcium or with and without phosphate and calcium before the other additions. The preincubation was performed for 5 min at 25°C and pH 7.4. Thereafter the preparation was incubated for 2 min at 25°C and pH 7.4 with 2 mM L-[U-^{14}C]glutamine. Other conditions were as described in Experimental Procedure A: Synaptosomes prepared by the sucrose-gradient technique (12). a: Freshly prepared synaptosomes, $n = 6$. b: Synaptosomes kept frozen for 4 months at −80°C, $n = 2$. B: Synaptosomal mitochondria prepared according to Lai and Clark (15), $n = 4$.

Moreover, the synaptosomal enriched preparations are known to be contaminated with free mitochondria which might be responsible for the effect of calcium. Table I B demonstrates that although the specific activity of PAG is 2-3 fold higher in isolated synaptosomal mitochondria than in the synaptosomal-enriched preparation, the relative activation by calcium is little affected. However the calcium activation seems to be dependent on structural binding of PAG because calcium has no effect on purified PAG (not shown).

Since the products of the PAG reaction, glutamate and ammonia, are the most important inhibitors of the synaptosomal enzyme (7, 16), the effect of these compounds on the calcium activation was investigated. As shown in Figure 3, the calcium activation is abolished by the reaction products of PAG. Calcium does not counteract the inhibition by either compound, but unexpectedly appears to increase the inhibition by glutamate. Figure 4 demonstrates that the extent of calcium activation also is dependent on the concentration of synaptosomes, as determined by the protein concentration in the incubation mixture, and that the activation can be abolished by increasing the protein concentration. Thus, the specific activity of PAG decreases on increasing the protein concentration, and more so when calcium is present. This decrease in specific activity is difficult to explain, but is assumed to be caused by accumulation of the reaction products of PAG (7, 16), whereby the calcium activation also is counteracted (cf. Figure 3). In addition, the concentration of free calcium ions is likely to be somewhat reduced when the protein concentration is increased, due to unspecific protein binding of calcium.

By use of thiol reagents NEM and p-mercuribenzoate evidence has been produced to show that PAG in the synaptosomal-enriched fraction is compartmentalized within the inner mitochondrial membrane and localized to a NEM-sensitive and -insensitive compartment (8, 11). For that reason it was considered of interest to investigate the effect of calcium on the NEM-sensitive and -insensitive fraction of PAG. The NEM-sensitive PAG is the fraction of PAG inhibited by preincubation with NEM, and the NEM-insensitive PAG is the uninhibited fraction. As demonstrated in Figure 5, only the NEM-insensitive PAG of the synaptosomal-enriched preparations is activated by calcium, and the NEM-sensitive PAG is un-

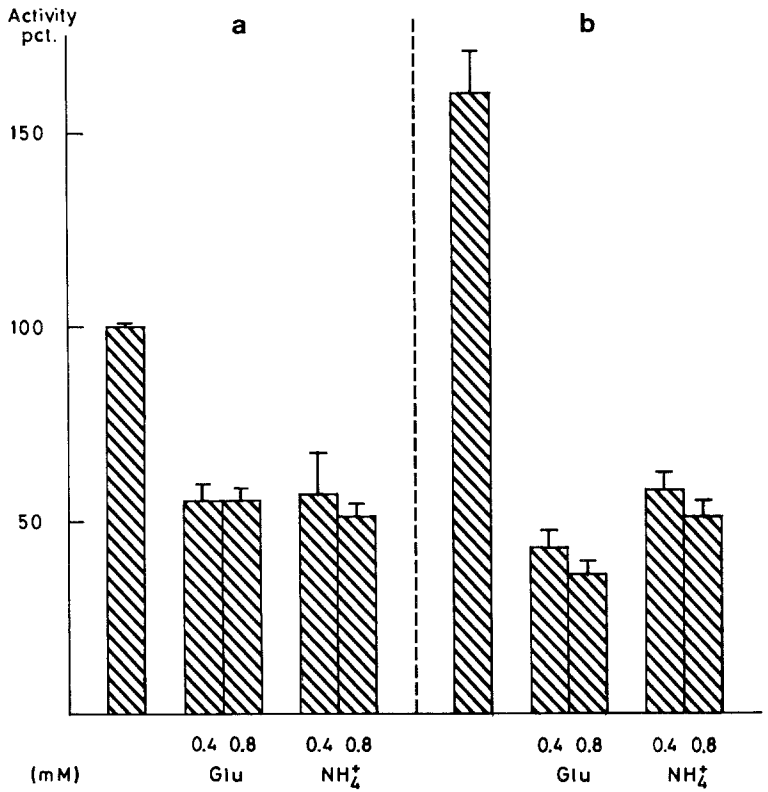

FIG. 3. The effect of calcium on the inhibition of glutamate formation from glutamine by added ammonia and glutamate. The synaptosomes were prepared by the sucrose-gradient technique (12) and 10 mM phosphate was added. When indicated, 0.5 mM calcium and 0.4 and 0.8 mM of ammonia and glutamate were also added. Other conditions were as in Figure 1. The activity is calculated as glutamate formed per min in percent of that in assays with no calcium, ammonia or glutamate added. Estimation of the significance (using Student's *t*-test) of the reduced hydrolytic activity as caused by calcium in the presence of 0.08 mM glutamate showed, $p < 0.001$. a: no calcium added; b: calcium added.

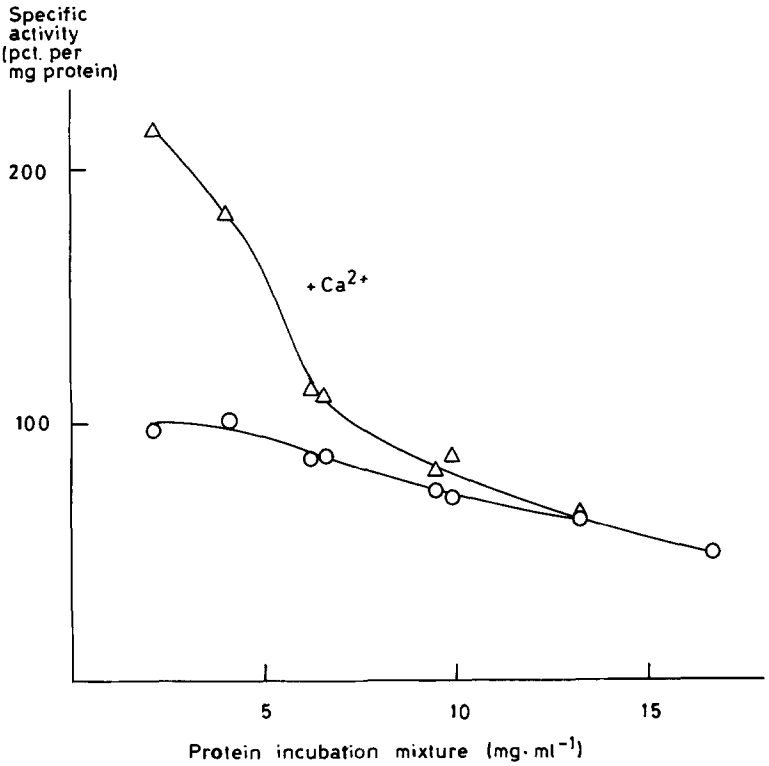

FIG. 4. The effect of the synaptosomal concentration on the calcium activation of glutamate formation from glutamine. The synaptosomes were prepared by the sucrose-gradient technique (12). Increasing amounts of the synaptosomal-enriched fraction, as indicated by the protein concentrations, were added to the incubation mixture. Phosphate (10 mM) was also added and 0.5 mM calcium, when indicated. Other conditions were as in Figure 1. The specific activity is calculated as glutamate formed per min per mg protein in percent of that with 2 mg protein and no calcium added.

changed, or reduced in some experiments. The activation of PAG can therefore be fully accounted for by the activation of the NEM-insensitive fraction, which amounts to 400–500%, by 0.5 mM calcium under these conditions. For comparison, total PAG is activated about 80% by calcium in the absence of NEM (Figure 5).

As calcium only activates the tissue-bound PAG and does not affect the purified enzyme, it may have an indirect effect on PAG. Thus, calcium may decrease the mitochondrial proton concentration because the pH optimum of purified PAG is about 9.0 (18). This possibility has been tested by using the uncoupling agent 2,4-dinitrophenol which is known to inhibit

the mitochondrial proton pump and making the mitochondrial inner membrane permeable to protons (19). As shown in Table II, 2,4-dinitrophenol does not inhibit the calcium activation, which therefore may be associated with phosphate transport or the sensitivity of PAG to phosphate.

The effect of potassium on the hydrolysis of glutamine and on the cal-

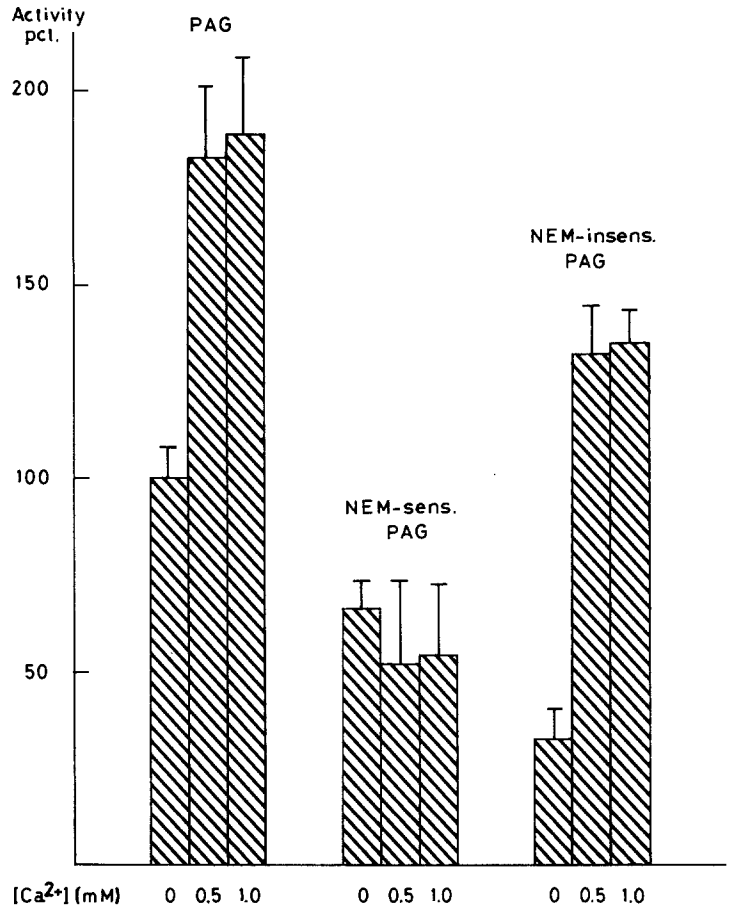

FIG. 5. The effect of calcium on the NEM-sensitive and-insensitive PAG. The synaptosomes were prepared by the sucrose-gradient technique (12) and preincubated with and without 0.5 mM NEM before the other additions. The preincubation was performed for 5 min at 25°C and pH 7.4. Thereafter the preparation was incubated for 2 min at 25°C and pH 7.4. 2 mM L-[U-^{14}C]glutamine, 10 mM phosphate, and where indicated, 0.5 mM calcium were added to the incubation mixture. Other conditions were as described in Experimental Procedure. NEM-sensitive PAG is calculated as the difference between the activity of PAG without and with (NEM-insensitive PAG) preincubation with NEM. The activity is calculated as glutamate formed per min in percent of that in assays with no calcium and NEM added.

TABLE II

THE EFFECT OF 2,4-DINITROPHENOL (DNP) ON THE CALCIUM ACTIVATION OF PAG

Additions	nmol Glu·min^{-1}·mg protein^{-1}		
	No Ca^{2+}	Ca^{2+}	*P*
No	6.5 ± 0.7	11.2 ± 0.4	<0.01
DNP	5.0 ± 0.4	11.1 ± 0.2	<0.001

The composition of the incubation mixture and other conditions were as described in Figure 1 and in Experimental Procedure. The synaptosomes were prepared by the sucrose-gradient technique (12). 10 mM phosphate was added, and when indicated, $5 \cdot 10^{-4}$ M DNP and 0.5 mM calcium. The significance of the calcium activation is shown (Student's *t*-test).

cium activation of this reaction has been investigated because potassium in high concentrations is known to have a depolarizing action on nervous tissue. As demonstrated in Table III, magnesium seems to have no effect on the activity, and the effects of potassium and calcium are largely dependent on the presence of phosphate in the incubation medium. Thus, 56 mM potassium in the presence of high sodium concentration (90 mM), and 10 mM phosphate inhibits the reaction without affecting the calcium activation (Table III A, B). The effect of potassium disppears on omitting

TABLE III

THE EFFECT OF HIGH POTASSIUM CONCENTRATION OF THE CALCIUM ACTIVATION OF PAG

[Ca] (mM)	[K] (mM)	nmol Glu·min^{-1}·mg protein^{-1}		
		A	B No Mg added	C No phosphate added
0	3	9.3 ± 0.8	10.4 ± 0.3	2.6 ± 0.5
		$p < 0.05$	$p < 0.01$	ns
	56	6.2 ± 1.0	6.8 ± 0.3	2.4 ± 0.7
0.5	3	13.7 ± 0.6	13.2 ± 1.1	3.3 ± 0.5
		$p < 0.01$	$p < 0.05$	ns
	56	9.7 ± 0.4	8.9 ± 0.7	3.0 ± 0.2

The composition of the incubation mixture (containing 1 mM Mg and 10 mM phosphate) was as described in Experimental Procedure and Table I, with the changes indicated. The synaptosomes were prepared by the sucrose-gradient technique (12). The variation in ionic composition was compensated by addition of equimolecular amounts of the suspending buffer for the synaptosomes (0.25 M D-mannitol, 0.07 M sucrose and 0.01 M Hepes, pH 7.4). *p* values (Student's *t*-test) are shown for the effect of K^+. The calcium activation is significant ($p < 0.05$), except in C when it is non significant (ns).

phosphate from the incubation medium, and so does the calcium activation (Table III C). However, when the sodium concentration of the incubation medium is lowered from 90 mM to 3 mM with 2 mM phosphate present, the hydrolysis of glutamine is increased both by calcium and by high potassium (not shown).

Since a great many enzymes have been described which are activated by calcium in the presence of the protein activator calmodulin (20), the lack of calcium activation of purified PAG might be caused by a loss of calmodulin during the purification. For that reason we investigated the effect of a calmodulin preparation (phosphodiesterase 3′,5′ cyclic nucleotide activator from bovine heart) on pig brain PAG, purified as described previously (5). However, the calmodulin preparation showed no effect on the purified enzyme, neither in the presence nor absence of added calcium (not shown). Therefore, PAG does not appear to belong to the family of calmodulin-activated enzymes.

DISCUSSION

It has been shown that calcium stimulates glutamine hydrolysis in synaptosomal preparations, affecting the relative formation similarly of both the reaction products, glutamate and ammonia. It has also been shown that the calcium stimulation cannot be ascribed to an effect on transport mechanisms in the synaptosomal membrane of substances influencing the PAG activity. Moreover, since calcium activates glutamine hydrolysis as mediated by PAG, in synaptosomes prepared both by the sucrose- (12) and the Ficoll/sucrose-gradient techniques (13), as well as in isolated synaptosomal mitochondria, the effect is likely to be a true property of nerve endings.

The activators of PAG are generally of anionic nature and calcium being a cation, is exceptional in this respect. Since calcium does only activate the NEM-insensitive PAG which is suggested to be localized in the inner part of the mitochondria (11) and has no effect on the purified enzyme, calcium may stimulate tissue-bound PAG in some indirect way. The PAG activity is strongly pH-dependent, but as described above, a calcium-mediated reduction of the mitochondrial proton concentration is rendered unlikely and other explanations, such as an effect of calcium on the mitochondrial phosphate transport (24) or the sensitivity of PAG to phosphate activation have to be considered.

Since a major fraction of phosphate enters the mitochondria by a NEM-sensitive carrier (25), and the calcium activation is phosphate-dependent also in the presence of NEM, calcium must increase the phosphate entry

by a NEM-insensitive carrier if the activation is coupled to phosphate transport. It appears, however, to be more likely that calcium increases the concentration of an activator of PAG which makes PAG more sensitive to phosphate (21, 23), because some ionic activators are known to affect purified PAG in this way. Thus the dye bromothymol blue (21, 22) and several acyl-CoA derivatives (21, 23) activate purified kidney and brain PAG by cooperating with phosphate. In support of an acyl CoA-mediated mechanism, calcium has recently been reported to inhibit the hydrolysis of acyl-CoA by acyl-CoA hydrolase (26). Moreover, it has been suggested that intramitochondrial acyl-CoA or acylcarnitine modulates the calcium-induced mitochondrial swelling which is accompanied by liberation of free fatty acids (27).

It is of interest that the PAG-inhibitors glutamate and ammonia, also abolish the activation by calcium, particularly because glutamate preferentially inhibits the NEM-sensitive PAG (7), whereas calcium activates NEM-insensitive PAG. As a possible explanation it may be suggested that calcium increases the permeability of the inner mitochondrial membrane to glutamate and NH_4^+ whereby the NEM-insensitive PAG is inhibited and the calcium activation prevented. Our finding that calcium actually enhances the inhibition by glutamate may favor this explanation.

The physiological role of the calcium activation of PAG is obscure. It is tempting to speculate that the suggested calcium-dependent release of transmitter glutamate derived from glutamine (1–4), is mediated by calcium activation of PAG. However, this possibility is unlikely for many reasons. Thus the calcium activation is unaffected by potassium in depolarizing concentrations and potassium activates PAG only at unphysiologically low extrasynaptosomal sodium concentrations. Furthermore, since calcium activates the NEM-insensitive PAG, which presumably is localized in the inner part of the mitochondria, the glutamate formed in response to calcium must penetrate the mitochondrial inner membrane, representing a permeability barrier, in order to be released from the nerve endings.

Another and more likely possibility is that the effect of calcium on PAG is secondary to its effect on transmitter release, and thereby serves to replenish depleted stores of transmitter glutamate (6). Calcium may in addition play a role in maintaining the normal electrical activities of brain cells by regenerating glutamate from electrophysiologically inert glutamine, as suggested by Benjamin (9). Since the glutamate formed in the inner part of the mitochondria is readily available to glutamate dehydrogenase and the enzymes of the tricarboxylic acid cycle, the calcium activation of PAG may also serve to satisfy rapid demands for energy, e.g. in relation to neurotransmission. Thus, glutamate has been shown to be oxidized by

an initial reaction with glutamate dehydrogenase in brain slices (28) as well as in astrocytes (29).

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