Amino acid neurotransmitters in the CNS: effect of thiopental

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Received 17 September 1984

Thiopental, a thiobarbiturate which partitions prefentially into the hydrophobic environment, inhibited transport of amino acid neurotransmitters, GABA, aspartate and glutamate, and of biogenic amine, dopamine, across the synaptosomal membrane. At a given protein and thiopental concentration GABA transport was more sensitive to the barbiturate than were the movements of aspartate and glutamate although the uptake of each amino acid was inhibited essentially to the same extent as was its K^+-stimulated release. By contrast, inhibition of dopamine uptake was larger than that of its release. Thiopental also inhibited the release of amino acid neurotransmitters caused by anaerobiosis. It is suggested that the barbiturate modifies the properties of the synaptosomal lipids and/or hydrophobic segments of proteins and thereby, simultaneously and independently, affects various membrane functions. The equal inhibition of uptake and release of amino acid neurotransmitters is consistent with the postulate that their transport occurs through the reversible membrane carriers which function efficiently in both the inward and outward directions.

Thiopental *Amino acid transport* *GABA* *Aspartate* *Glutamate* *Dopamine*

1. INTRODUCTION

It has long been known that barbiturates depress synaptic excitatory transmission [1,2] and enhance synaptic inhibitory mechanisms [3–5] in the mammalian central nervous system. The first of these phenomena occurs predominantly by a presynaptic decrease in the amount of neurotransmitter released [1,2,6–8] whereas enhancement of the synaptic inhibitory mechanisms takes place at both pre- [5,9] and postsynaptic [4,10,11] sites.

Barbiturates have also been reported to exert marked protective effects in cerebral hypoxia and ischemia (e.g., [12,13]). Synapses are particularly vulnerable to the lack of oxygen [14] and our studies have shown that the synaptic high-affinity uptake systems for the two putative excitatory amino acid neurotransmitters, glutamate and aspartate, which are responsible for termination of transmission modulated by these amino acids, were markedly inhibited by conditions mimicking hypoxia and ischemia [15]. Since these two excitatory amino acids have been suggested to act as neurotoxins [16], their increased levels in the synaptic cleft, caused by a damage to the transport proteins, could substantially contribute to irreversible neuronal damage. The objective of this investigation was to test the possibility that protective effects of barbiturates in hypoxia and ischemia are exerted, at least in part, through their inhibition of release of aspartate and glutamate.

2. MATERIALS AND METHODS

Male Sprague-Dawley rats were used throughout. Synaptosomes were isolated on discontinuous Ficoll gradients as in [17]. The final pellet was suspended in a modified Krebs-Henseleit buffer

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(140 mM NaCl, 5 mM KCl, 10 mM Tris–Hepes, 5 mM $NaHCO_3$, 1.3 mM $MgCl_2$, 1 mM $NaHPO_4$ and 1.25 mM $CaCl_2$), pH 7.4, containing 10 mM glucose.

Incubations were carried out as in [18]. The rates of neurotransmitter uptake and release were determined using radiolabeled derivatives [18]. Amino acids were measured by high-pressure liquid chromatography (HPLC) [15] and determinations of intrasynaptosomal potassium and calcium were made as in [19]. Protein was assayed according to [20].

3. RESULTS

3.1. *Effect of thiopental on amino acid release caused by anaerobiosis and high K^+*

The effect of thiopental on the release of putative amino acid neurotransmitters, GABA, aspartate and glutamate, was evaluated by measuring concentrations of these amino acids in supernatants from synaptosomes incubated either anaerobically or in the presence of 50 mM potassium. Table 1 shows that anaerobiosis increased the concentration of aspartate in the supernatant by 3.7-fold, that of glutamate by 4.5-fold and GABA by 11-fold. In samples incubated with thiopental the respective rises in external amino acids were considerably smaller (2.4-, 4.4- and 3.8-fold), the largest effect being seen with GABA and smallest with glutamate. Similar calculations for synaptosomes treated with 50 mM K^+ (last column in table 1) showed that thiopental was most effective in decreasing the depolarization-induced GABA release and least potent in preventing glutamate release. When veratridine (20 μM) was used to depolarize synaptosomal membrane by opening the Na^+ channels the results obtained were very similar to those with high potassium.

Changes in the external concentrations of 16 other amino acids were unaffected by the presence of thiopental (not shown).

3.2. *Effect of protein concentration on the inhibitory effects of thiopental*

It was noted that inhibition of release of neurotransmitter amino acids by thiopental was dependent on the amount of protein in the incubation mixture. Therefore the effect of thiopental concentration on the release of [^{14}C]GABA and [^{3}H]aspartate was investigated on the same synaptosomal suspension at a constant protein concentration and compared with the influence of the barbiturate on the rates of uptake of these two amino acids. Representative curves of uptake and release are shown in fig.1 and the summary of the results is given in table 2. Four aspects of the data are of interest: (i) thiopental caused inhibition of

Table 1

Effect of thiopental on the release of amino acid neurotransmitters caused by anaerobiosis and high K^+

Amino acid	Control	+ thiopental	Anaerobic	+ thiopental	50 mM K^+	+ thiopental
Asp	2.3 ± 0.7	2.1 ± 0.6	8.5 ± 1.6	5.1 ± 0.9[a]	14.1 ± 2.4	8.1 ± 1.3[a]
Glu	4.2 ± 0.9	3.8 ± 0.7	19.1 ± 3.1	16.6 ± 2.4[a]	25.9 ± 3.1	17.0 ± 2.0[a]
GABA	0.4 ± 0.13	0.42 ± 0.1	4.4 ± 1.2	1.6 ± 0.8[a]	5.1 ± 0.6	2.6 ± 1.0[a]

[a] $p < 0.01$

Synaptosomes were suspended at 1.5–2 mg protein/ml in Krebs-Henseleit medium, pH 7.3, containing 10 mM glucose and then divided into 3 parts. The first was incubated aerobically with and without 1 mM thiopental, the second was incubated anaerobically with and without thiopental, the third group was treated essentially like the first but the medium was supplemented with 50 mM K^+. All incubations were carried out for 40 min at 25°C. At the end of the incubation, 300-μl aliquots were withdrawn and centrifuged (Beckman microfuge) through a layer of silicone oil to separate synaptosomes from the external medium. The upper layer, containing the external medium, was pipetted off, acidified with trichloroacetic acid (3% final concentration) and used for the measurements of amino acids by HPLC. Values are presented as concentrations (μM) in the supernatants (means ± SD for 3 experiments). Results were evaluated statistically (paired t-test) within each group (i.e., with respect to samples incubated without thiopental)

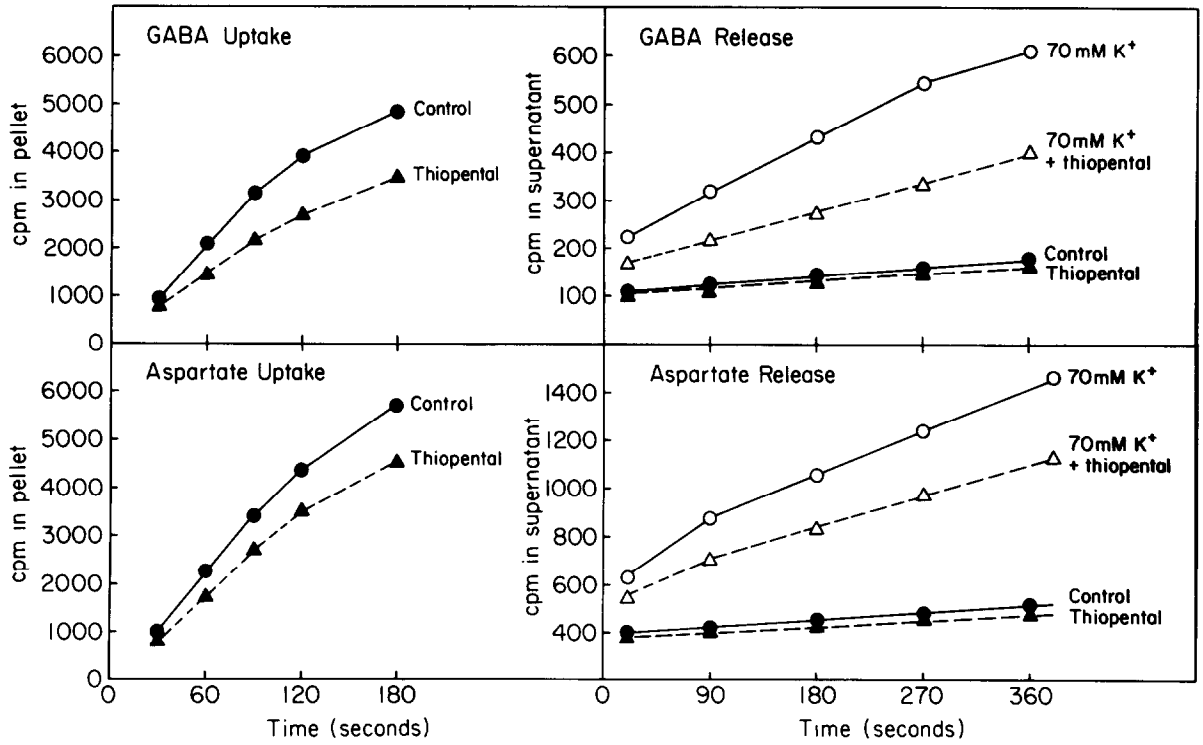

Fig.1. The effect of thiopental on uptake and release of GABA and aspartate. (Uptake) Synaptosomes were diluted to 1.2 mg protein/ml in Krebs-Henseleit buffer (pH 7.4) with and without 1 mM thiopental and uptake started by the addition of 2 μM [^{14}C]GABA and 2 μM D-[^{3}H]aspartate at 27°C. (Thiopental was always added immediately after the protein.) Aliquots (200 μl) were removed at the times indicated and rapidly centrifuged (Beckman microfuge) through a layer of silicone oil. Radioactivity was then measured in the pellets. (Release) Synaptosomes were preloaded with the amino acids by incubation for 20 min at 27°C with 2 μM [^{14}C]GABA and 2 μM D-[^{3}H]aspartate. Aliquots were withdrawn and diluted 10-fold (to about 1.2 mg protein/ml) into Krebs-Henseleit buffer containing 70 mM K^+, with and without 1 mM thiopental. Samples (200 μl) were removed and rapidly centrifuged through a layer of silicone oil. Radioactivity was then determined in the supernatants.

release as well as of uptake of the two amino acid neurotransmitters; (ii) inhibition increased with increasing concentration of thiopental; (iii) inhibition of uptake and release at a given concentration of thiopental was approximately the same; (iv) inhibition of aspartate uptake and release at the same barbiturate concentration was smaller than that of GABA. (Detailed kinetic evaluation of the inhibition of [^{14}C]GABA uptake by thiopental showed it to be competitive with respect to the amino acid with an apparent K_i of 1.0 mM.)

Since it can be argued from the results in table 2 that uptakes of radioactive GABA and aspartate were somewhat less inhibited than their releases we have measured the rates of net GABA uptake using HPLC. This method avoids possible interference from homoexchange which is a substantial component of the measured rates of uptake of radioactive amino acids. GABA (2 μM) was added at time zero to samples incubated at 1 mg protein/ml with and without 1 mM thiopental and aliquots were withdrawn at 20, 60 and 240 s to measure GABA concentration in the supernatants. 20 s after addition of the amino acid its level in control samples was 2.1 ± 0.36 μM and in incubations with thiopental it was 2.08 ± 0.23 μM (means ± SD for 3 experiments). After 4 min incubation, the respective levels were 1.44 ± 0.22 and 1.81 ± 0.15 μM. It can be calculated that in the absence of thiopental the concentration of GABA in the supernatant (external medium) decreased by 0.66 μM whereas in its presence it declined by 0.27 μM, which

Table 2

Concentration dependence of thiopental inhibition of amino acid transport

Amino acid	[Thiopental] (mM)	% Inhibition	
		Uptake	Release
GABA	0.25	8.2 ± 3.4	17 ± 1.8
	0.5	22	34 ± 4.6
	1.0	50 ± 18	58 ± 12
ASP	0.25	5.1 ± 1.3	11 ± 3.6
	0.5	13	20 ± 6.1
	1.0	29 ± 11	45 ± 16

Uptake and release of [^{14}C]GABA and D-[^{3}H]aspartate were measured as described in the legend to fig.1, in the incubation mixtures containing 1 ± 0.1 mg synaptosomal protein/ml. Both amino acids were added simultaneously to the synaptosomal suspensions. Inhibition (%) was calculated from the slopes of the initial (20–180 s) linear parts of the uptake or release curves. Values are means ± SD for 3 experiments except where SD is not given

represents a 60% inhibition of uptake. (The differences between the two sets of experimental values were significant at a level of P 0.01, as evaluated by the paired Student's t-test.) This value is in excellent agreement with the 58% inhibition of GABA release shown in table 2 and obtained at the same thiopental and protein concentrations.

3.3. *Effect of thiopental on dopamine transport*

The effect of thiopental on the uptake and release of radioactive dopamine was measured at 1 mg protein/ml and with 1 mM barbiturate. The uptake was inhibited by 41 ± 6.6% whereas the release was decreased by only 10 ± 4% (means ± SD for 9 experiments). The inhibition of uptake by thiopental was kinetically competitive with respect to dopamine with an apparent K_i of 0.9 mM.

3.4. *Effect of thiopental on intrasynaptosomal potassium and calcium*

Thiopental (1 mM) had no effect on intrasynaptosomal potassium concentration. Hence the transmembrane electrical potential, which in the synaptosomes is the K^+ diffusion potential [21], was unaltered. On the other hand, the barbiturate eliminated almost completely stimulation of calcium accumulation by synaptosomes depolarized with 50 mM K^+, in agreement with the results of others [22,23].

3.5. *Effect of thiopental on the synaptosomal membrane*

A fluorescent probe of the hydrophobic region of the membrane, 1,6-diphenyl-1,3,5-hexatriene [24], was used to measure the effect of thiopental on synaptosomal membranes. The results of 3 independent experiments carried out at about 1 mg protein/ml showed that as the thiopental concentration was increased the anisotropy of the probe's fluorescence decreased: it was 0.252 ± 0.011 (mean ± SD) at 0 thiopental, 0.214 ± 0.003 at 1 mM thiopental and 0.171 ± 0.005 at 2 mM thiopental. Since there was no change in the fluorescence lifetime (not shown) this decrease in the anisotropy indicates that the environment of the probe became progressively more fluid with increasing barbiturate.

4. DISCUSSION

Our results show that thiopental, a thiobarbiturate which partitions preferentially into the hydrophobic environment, inhibits transport of various neurotransmitters across the synaptosomal membrane as well as other membrane functions. The effect of thiopental was markedly dependent on the barbiturate and protein concentrations in the incubation mixture and at a given barbiturate concentration various processes exhibited different sensitivities to its action. This observation may explain some of the conflicting reports in which the same reactions were observed to be either sensitive or insensitive to barbiturate inhibition [9,25–27].

At a given protein and thiopental concentration the barbiturate inhibited movements of both the amino acid neurotransmitters and of dopamine but with different characteristics. The inhibition of uptakes of GABA, aspartate and glutamate required essentially the same thiopental concentration as blockage of their releases. This observation supports our postulate [28,29] that movements of amino acid neurotransmitters across the synaptosomal membrane occurs through the operation of reversible membrane carriers which function ef-

ficiently in either inward or outward directions. By contrast, the uptake of dopamine was considerably more sensitive to thiopental inhibition than was its release, consistent with the generally espoused view that uptake of biogenic amines by the nerve endings occurs through a membrane carrier whereas its release is primarily an exocytotic process [30].

The high-affinity uptake systems for GABA, acidic amino acids and dopamine are mediated by different membrane proteins. Hence inhibition of all these reactions implies that a common mechanism may be involved. Two possibilities can be entertained to explain the findings. The first is that thiopental, like other barbiturates, modifies properties of the synaptosomal membrane which, in turn, affects operation of various membrane functions. Such suggestions have been made [6,31] and our studies with the fluorescent probe seem to support this interpretation. However, it should be mentioned that although our results as well as those of others (e.g., [32]) indicate that barbiturates increase membrane fluidity, these compounds may also modify the hydrophobic segments of membrane proteins. Differential sensitivity of the various transport systems to thiopental at the same inhibitor and protein concentrations appears to be in agreement with the latter suggestion. The second possibility is that thiopental acts through a common effector of the neurotransmission event. It has been postulated that calcium fulfills such a role [33,34]. However, calcium is not required for uptake of various neurotransmitters, nor does its absence substantially inhibit amino acid release in our synaptosomal preparation [29]. These observations argue against calcium being responsible for the effects of thiopental on the movements of amino acid neurotransmitters. On the other hand, calcium influx caused by high potassium-induced membrane depolarization was inhibited by the barbiturate. Moreover, dopamine release, which is very sensitive to the presence of Ca^{2+}, was depressed by the anesthetic although to a much smaller extent than was the amine uptake. It is therefore tempting to postulate that blocking of calcium influx and consequences thereof contribute, to some extent, to the effects of barbiturates observed in this and other (e.g., [12,13,15]) studies. However, our results also suggest that the effects modulated through calcium require higher concentrations of thiopental than those exerted directly on the membrane.

The differential sensitivity of the 3 amino acid neurotransmitter uptake systems to thiopental is an interesting observation. Although the forces responsible for the maintenance of their concentration gradients across the synaptosomal membrane and hence their movements in both directions, are the same, GABA transport was much more sensitive to thiopental inhibition than were movements of aspartate and glutamate (see also [9]). This observation suggests that there are other mechanism(s) in addition to those mentioned above, which control movements of individual amino acid neurotransmitters. Whether this (these) mechanism(s) reside(s) in the membrane itself or is (are) exerted through intracellular second messengers remains to be established.

Finally, we have also shown that thiopental inhibits release of amino acid neurotransmitters caused by anaerobiosis. Since neurotransmission depends on the rate with which transmitters are released into the synaptic cleft our results mean that thiopental, by decreasing this rate, will lower the effective neurotransmitter concentration in response to the stimulus. If the concentration in the synaptic cleft falls below the threshold value, transmission may not even occur which, in the case of excitatory amino acids, will result in an overall suppression of electrical activity.

ACKNOWLEDGEMENT

Supported by USPHS grant HS 14504.

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