The effect of thiol reagents on GABA transport in rat brain synaptosomes

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The nature of γ-aminobutyric acid (GABA) transport has been investigated in preparations of rat brain synaptosomes using a number of thiol reagents with varying membrane permeabilities. *N*-Ethylmaleimide, *p*-chloromercuribenzoate and *p*-chloromercuriphenylsulfonate effectively inhibited GABA transport in both directions (i.e., uptake and release) whereas 5,5′-dithiobis-2-nitrobenzoate, mercaptopropionate and *N*-nitroethylenediamine were much less effective, or ineffective, even at millimolar concentrations. For each of the thiol reagents, the inhibition profile for GABA uptake was approximately the same as that for its release. The effectiveness of the reagents indicates that there is an external, reactable SH-group on the transporter, that the thiol reagent must be somewhat lipophilic for it to react with the SH-group(s), and that the same synaptosomal transport system is responsible for both uptake and release of GABA.

Synaptosome *γ-Aminobutyric acid* *Thiol reagent* *Neurotransmitter transport*

1. INTRODUCTION

It is generally agreed that γ-aminobutyric acid (GABA) is one of the primary inhibitory neurotransmitters in the mammalian central nervous system [1–3]. Experiments have indicated that GABA may function at up to 50% of the synapses in the brain, depending on the region investigated [4–8]. The properties of GABA uptake have been studied extensively in brain slices and isolated nerve endings (synaptosomes). Two systems for the neuronal accumulation of radiolabeled GABA have been demonstrated, one with a relatively high affinity, a K_m value of 2–10 μM, and the other with a relatively low affinity, a K_m value of approx. 1 mM [9–12]. The high affinity uptake system is almost exclusively neuronal and is dependent on the presence of sodium [13,14]. In preparations of isolated brain synaptosomes it has been demonstrated that two sodium ions accompany the movement of one GABA molecule from outside to inside and that the energy utilized for this transport is provided by a combination of the sodium concentration gradient, $[Na^+]_o/[Na^+]_i$, and the transmembrane electrical potential [15].

Depolarization with high external potassium concentrations or veratrine alkaloids results in the release of GABA. A number of studies have shown that the stimulus-secretion coupled release of GABA has a substantial calcium-independent component [13,16–19]. These observations have led to the suggestion that neuronal membranes possess a reversible sodium-dependent transport system which may be responsible for both GABA uptake and release [13,18]. One method of testing the symmetry suggested by this hypothesis is to use a non-competitive inhibitor of GABA uptake and observe its effect on stimulus-secretion coupled GABA release. If the membrane transport system is symmetric, one would expect a non-competitive inhibitor of GABA influx to have the same or a parallel effect on GABA efflux.

Here we report the effects of a variety of thiol

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reagents on the transport of GABA across synaptosomal membranes isolated from rat brains. Thiol reagents were chosen for study because they are known to be potent non-competitive inhibitors of GABA uptake [20]. The data provide evidence that GABA uptake and release utilize a reversible transport system.

2. MATERIALS AND METHODS

Synaptosomes were isolated from the cortex and midbrain of 200–250 g, male Sprague-Dawley rats as in [21]. The synaptosomes were suspended in a Krebs-Henseleit–Hepes buffer (140 mM NaCl, 5 mM KCl, 10 mM Tris–Hepes 5 mM $NaHCO_3$, 1.3 mM $MgSO_4$ and 1 mM $NaHPO_4$ (pH 7.4) at about 7 mg protein/ml. All incubations were done at 27°C and were supplemented with 2.5 mM $CaCl_2$ and 10 mM glucose. Protein concentration was determined as in [22] using bovine serum albumin as the standard.

2.1. *Measurements of GABA uptake and steady state gradients*

After preincubating the synaptosomes for 5 min a thiol reagent was added and incubation continued for 5 more min. The synaptosomes were diluted 5-fold into the same buffer containing 1.2 μM [^{14}C]GABA (New England Nuclear). Aliquots (200 or 300 μl) were withdrawn, rapidly centrifuged and radioactivity measured as in [16]. Maximal (steady state) GABA gradients were measured by removing aliquots 15 min after the 5-fold dilution of synaptosomes after ascertaining that the maximal gradient had been attained.

2.2. *Measurements of GABA release*

Synaptosomes were preincubated with 1.25 or 2.5 μM [^{14}C]GABA plus 2 mM amino-oxyacetic acid for 10–15 min. Thiol reagents were then added and incubation continued 5 min. The synaptosomes were diluted 10-fold into either the same buffer as described above or a depolarizing Krebs-Henseleit–Hepes buffer containing 40 mM KCl plus 60 μM veratridine. Aliquots (200 or 300 μl) were withdrawn and handled as described above. The initial rates of depolarized release were calculated by linear regression analysis of the measured radioactivity of both supernatant and pellet fractions.

2.3. *Measurements of intrasynaptosomal water*

Synaptosomes were preincubated, thiol reagent was added and incubation continued for 5 min. After diluting the synaptosomes 5-fold into non-depolarizing Krebs-Henseleit–Hepes buffer and incubating 10–15 min, intrasynaptosomal water was measured as in [16].

2.4. *Measurements of membrane potential*

Synaptosomes were incubated in the presence or absence of thiol reagent, as described above, diluted 5-fold with non-depolarizing Krebs-Henseleit–Hepes buffer, and further incubated 10–15 min. The transmembrane electrical potentials were calculated from the steady state distribution of potassium as in [16].

2.5. *Measurements of oxygen consumption*

Synaptosomes were preincubated 5 min, thiol reagents were added and incubation continued 5 min, and the rates of oxygen consumption were measured with a Clark-type oxygen electrode at 25°C.

2.6. *Reversal of the effects of p-chloromercuribenzoate*

2.6.1. GABA uptake and steady state gradients

Synaptosomes were incubated with *p*-chloromercuribenzoate (PCMB) for 5 min. The synaptosomes were then diluted 5-fold into non-depolarizing buffer containing mercaptoethanol, at 1.5 times the final concentration of the thiol reagent, and incubation was continued for 5 more min. [^{14}C]GABA was added to the incubation mixture and uptake and steady state gradient measurements were made as described above.

2.6.2. GABA release

Synaptosomes were loaded with [^{14}C]GABA and treated with PCMB for 5 min as described. Mercaptoethanol, at 1.5 times the PCMB concentration, was added and incubation continued for 5 more min. The synaptosomes were then diluted 10-fold into either depolarizing or non-depolarizing buffer and aliquots were withdrawn and treated as described above.

3. RESULTS

3.1. *The effect of thiol reagents on GABA uptake and release*

Fig.1 shows the effects of various SH-reagents on GABA uptake and release. PCMB and *p*-chloromercuriphenylsulfonate (PCMS) were equally effective at inhibiting GABA transport with IC_{50} values for both influx and efflux of about 200 μM. *N*-Ethylmaleimide (NEM) was somewhat less effective as an inhibitor, with an IC_{50} for transport in both directions across the synaptosomal membrane of 500 μM. 5,5′-Dithiobis-2-nitrobenzoate (DTNB) at the maximal concentration utilized (1 mM) decreased GABA transport only 20–40%. Mercaptopropionate and *N*-nitroethylenediamine, the two least lipophilic agents used, were ineffective, even at millimolar concentrations (not shown).

It was noted that the small efflux of accumulated [^{14}C]GABA which is usually observed in synaptosomes diluted into non-depolarizing media was increased to varying degrees by incubating with thiol reagents. At the IC_{50} values for inhibition of GABA transport PCMB caused no significant increase in this efflux whereas PCMS stimulated it 2-fold. At higher concentrations both compounds stimulated the rate of efflux of the ac-

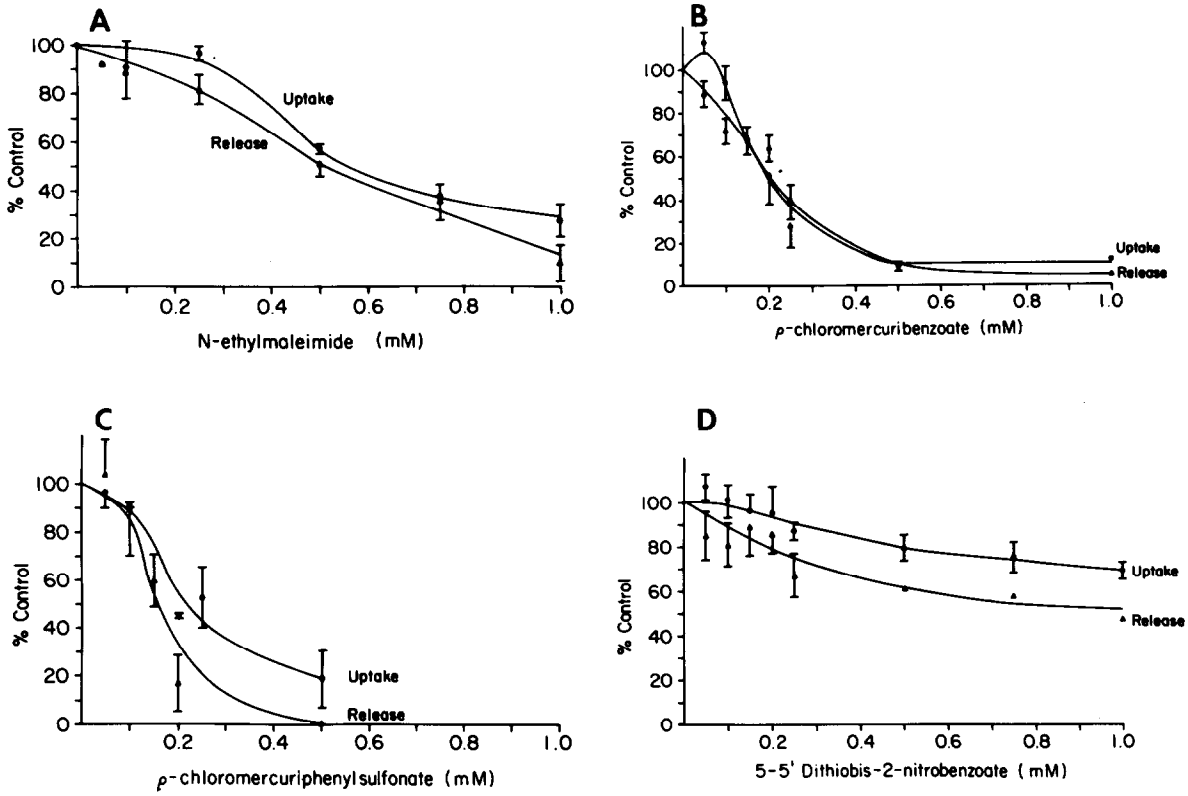

Fig.1. The effect of thiol reagents on GABA uptake and release. Synaptosomes were suspended to a protein concentration of about 7 mg/ml in Krebs-Henseleit–Hepes buffer (pH 7.4). After preincubating the synaptosomes, thiol reagents were added and incubation was continued for 5 min. The reaction mixtures were again diluted 5-fold for uptake experiments. Aliquots (200 or 300 μl) were rapidly centrifuged through silicone oil 30, 60, 90, 120 and 180 s after either the addition of 1.2 μM [^{14}C]GABA (uptake) or diluting synaptosomes 10-fold into depolarizing or normal Krebs-Henseleit–Hepes buffer (release). See section 2 for details. Each point represents the mean % of control ± SE for individual experiments with *n* values as follows: (A) *N*-ethylmaleimide-uptake, 3, release, 6; (B) *p*-chloromercuribenzoate-uptake, 15, release, 14; (C) *p*-chloromercuriphenylsulfonate-uptake, 2, release, 4; (D) 5,5′-dithiobis-2-nitrobenzoate-uptake, 3, release, 4. The mean control value for uptake is 232.75 ± 16.44 pmol/mg protein per min.

cumulated amino acid. NEM in the range of 250–500 μM increased GABA leakage by about 50%, and 1.5-fold at 1 mM. DTNB appeared to have no significant effect at the concentrations utilized. Because the effects of thiol reagents on stimulation-secretion coupled release were of particular interest, the values for depolarized release presented in fig.1 were corrected for that not due to depolarization of the synaptosomes, i.e., the efflux which occurred upon dilution into non-depolarizing media.

3.2. *The effect of thiol reagents on synaptosomal respiration*

The effect of various thiol reagents on synaptosomal respiration is illustrated in fig.2. It can be seen that, of the agents tested, only NEM produced a significant inhibition of oxygen consumption. The other compounds did not affect O_2 uptake in the concentration range employed.

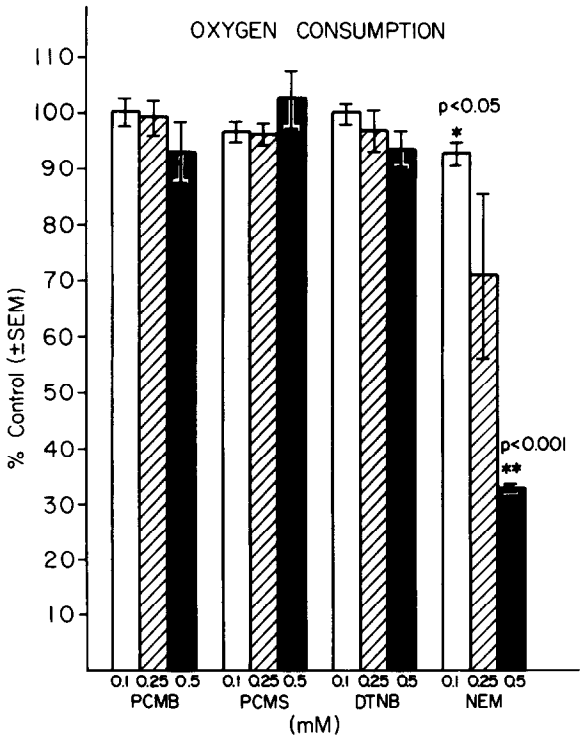

Fig.2. The effect of thiol reagents on synaptosomal respiration. Synaptosomes were suspended in Krebs-Henseleit–Hepes buffer to a final concentration of about 7 mg protein/ml, and preincubated for 5 min. Thiol reagents were then added and the rates of oxygen consumption were measured with a Clark-type oxygen electrode at 25°C. The values are the means of 3 experiments ± SE. The *p*-values from the Student's *t*-test are indicated.

3.3. *The effect of PCMB and PCMS on synaptosomal membrane potential and the maximal GABA gradient*

The effects of PCMB and PCMS on the transmembrane electrical potential and maximal GABA gradients were investigated in order to establish whether the inhibition of uptake was due to a decrease in the driving forces for GABA accumulation [13–15]. It can be seen (table 1) that the IC_{50} concentration of PCMB had no significant effect on the membrane potential. At 250 μM, this compound decreased the steady-state GABA gradient from approx. 600 to 400, without much change in the electrical potential. Both the membrane potential and the $[GABA]_i/[GABA]_o$ were decreased at 500 μM PCMB.

PCMS caused a slightly greater depolarization of synaptosomes and a more pronounced decrease in the maximal GABA gradient than did PCMB. The larger decrease in the maximal GABA equilibrium gradient with PCMS as compared to PCMB may have been caused by higher non-specific leakage of the amino acid induced by the former compound.

Neither PCMS nor PCMB had any effect on the sodium gradient when measured by atomic absorption or by the equilibrium distribution of ^{22}Na (not shown).

3.4. *Reversibility of the effects of PCMB*

The reversibility of the effects of PCMB was studied using mercaptoethanol at concentrations 1.5 times greater than the PCMB concentration utilized. Fig.3 shows that after treating the synaptosomes with 250 μM PCMB, mercaptoethanol was able to fully restore GABA transport. The steady state GABA gradient was also returned to its original value, within the limits of experimental error. At 500 μM PCMB, GABA uptake and release were restored to about 75% of their respective controls and $[GABA]_i/[GABA]_o$ to 500 ± 24 (i.e., somewhat lower than control, table 1). Mercaptoethanol alone had no significant effect on either GABA transport or the steady state GABA gradient.

Table 1

Effect of *p*-chloromercuribenzoate and *p*-chloromercuriphenylsulfonate on transmembrane electrical potential and GABA gradient

Concentration (μM)	Membrane potential (mV)		GABA gradient (i/o)	
	PCMB	PCMS	PCMB	PCMS
0	54.8 ± 2.0	54.8 ± 2.0	594.0 ± 59.2	594.0 ± 59.2
100	52.8 ± 3.4	50.6 ± 7.9	589.6 ± 62.9	640.0 ± 203.6
200	53.8 ± 2.2	53.2 ± 9.2	478.4 ± 55.0	179.5 ± 15.5[b]
250	52.1 ± 2.4	48.1 ± 9.0	389.7 ± 42.3[b]	121.2 ± 15.5[c]
500	46.1 ± 3.1[a]	38.9 ± 9.9[b]	98.3 ± 3.1[e]	26.8 ± 7.5[d]

[a] $p < 0.050$
[b] $p < 0.025$
[c] $p < 0.010$
[d] $p < 0.005$
[e] $p < 0.001$ (Student's *t*-test)

Synaptosomes were suspended in Krebs-Henseleit–Hepes buffer, preincubated, and incubated with PCMB or PCMS as described. The synaptosomes were then diluted 3-fold into buffer with (for determining the GABA gradient) or without (for the potassium distribution measurement) 1 μM [^{14}C]GABA plus 2 μM amino-oxyacetic acid and allowed to equilibrate for 15 min. Aliquots (200 or 300 μl) were withdrawn and handled as described in section 2. The *n* values for PCMB and PCMS were 7 and 2, respectively

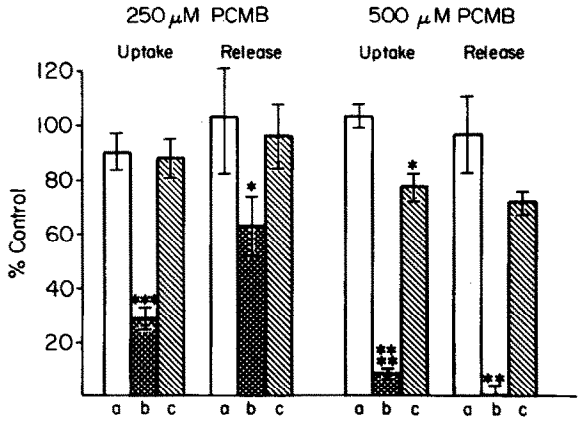

Fig.3. The reversibility of the effect of *p*-chloromercuribenzoate on GABA transport. Synaptosomes were suspended in Krebs-Henseleit–Hepes buffer, preincubated and incubated for 5 min with PCMB as described above. The effects of the thiol reagent were reversed by incubating the synaptosomes with 1.5 times the final PCMB concentration for 5 min. See section 2 for details. The values are the means of the % of control ± SE for each of the 3 uptake and 4 release experiments. (a) Mercaptoethanol, (b) PCMB, (c) PCMB plus mercaptoethanol. * $p < 0.025$, ** $p < 0.010$, *** $p < 0.005$, *** $p < 0.001$ (Student's *t*-test).

4. DISCUSSION

The results presented here show that thiol reagents are effective inhibitors of GABA transport. The effectiveness of these compounds in interfering with the transport of the amino acid depended, to a certain degree, on their lipophilicity. The ability of DTNB, PCMB, and PCMS, reagents which are only partially membrane-permeable, to successfully block GABA uptake and release indicates that the reactive SH-group(s) on the transport protein is oriented toward the external surface of the plasma membrane. On the other hand, the fact that the least lipophilic reagents, mercaptopropionate and *N*-nitroethylenediamine, were ineffective suggests that the SH-group(s) is not completely exposed to the external milieu but is likely to be buried within a hydrophobic part of the transporter.

Furthermore, our results demonstrate that all the thiol reagents which inhibited GABA uptake also blocked the amino acid's release, and the extent to which the two processes were decreased was approximately the same. This pattern of effectiveness indicates that the GABA transporter of synaptosomes mediates both the influx and

depolarization-stimulated efflux of this amino acid.

The most effective inhibitors of GABA transport were PCMB and PCMS which at their IC_{50} values affect neither synaptosomal respiration nor the transmembrane electrical potential or sodium concentration gradient. By contrast, NEM at its IC_{50} for GABA transport also caused a decrease in synaptosomal respiration, in agreement with the known sensitivity of mitochondria to this reagent [23]. It should be pointed out that a decrease in respiration and the consequent reduction in ATP synthesis would ultimately lead to an influx of Na^+ and membrane depolarization (i.e., it would decrease the driving forces for GABA accumulation). However, a decrease in the driving forces for GABA accumulation would be expected to result in a decline in the uptake and a stimulation of the release of the amino acid neurotransmitter. Hence, the effect of NEM on GABA transport seen in this work is not likely to be due only to an indirect action through energy depletion but rather appears to be a combination of its effect on transport and on mitochondrial function.

The IC_{50} values for the effective SH-group reagents were in the range of 200–500 μM which, at the protein concentration utilized here, corresponds to 30–70 nmol/mg protein. These figures compare with studies on the mitochondrial transport system which is inhibited by 50% at a concentration of thiol reagents between 10–50 nmol/mg protein [23].

It is also worth mentioning that higher concentrations of both PCMB and PCMS affect other synaptosomal functions: they increase non-specific leakage of GABA and decrease the transmembrane electrical potential. Although the mechanism(s) which is responsible for the latter changes is not clear at the present time, this observation is not unexpected because the thiol reagents are non-specific in that they can react with any accessible SH-group and modify many related functions.

The extrapolation of our in vitro findings to in vivo situations is difficult. In in vitro studies of isolated synaptosomes, the release of GABA appears to occur from the cytosolic compartment [24] and contains a large calcium-insensitive component. Thus, in our experimental system, vesicular release of GABA, if it exists, contributes negligibly to the observed pattern of release of this amino acid. If the in vitro model is valid, there exists in vivo a substantial non-vesicular component for the depolarization-stimulated release of GABA which occurs via reversal of the membrane potential and Na-dependent transport system.

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