Transport of γ -aminobutyrate and L-glutamate into synaptic vesicles

Effect of different inhibitors on the vesicular uptake of neurotransmitters and on the Mg^{2+} -ATPase

Else M. FYKSE* and Frode FONNUM

Norwegian Defence Research Establishment, Division for Environmental Toxicology, P.O. Box 25, N-2007 Kjeller, Norway

The uptakes of γ -aminobutyrate (GABA) and L-glutamate into synaptic vesicles isolated from rat brain were compared with respect to the effects of 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid (SITS), 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (DIDS) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (N144), agents known to block anion channels. The uptake of glutamate was inhibited by low micromolar concentrations of SITS, DIDS and N144. GABA uptake was much less sensitive to these agents than was glutamate uptake. SITS and N144 inhibited the vacuolar H^+ -ATPase of synaptic vesicles to a smaller extent than the glutamate uptake. The uptake of GABA was not affected by the permeant anions Cl^- and Br^- , whereas the uptake of glutamate was highly stimulated by low concentrations of these ions. The uptakes of both glutamate and GABA were inhibited by similar, but not identical, concentrations of the lipophilic anion SCN^- .

INTRODUCTION

Mammalian brain synaptic vesicles actively accumulate γ -aminobutyrate (GABA), glycine and glutamate by a Mg^{2+} -ATP-dependent process. A vesicular Mg^{2+} -ATPase generates an electrochemical proton gradient which is important for uptake of neurotransmitters (Disbrow *et al.*, 1982; Naito & Ueda, 1985; Fykse & Fonnum, 1988; Hell *et al.*, 1988; Maycox *et al.*, 1988; Kish *et al.*, 1989; Christensen *et al.*, 1990). The Mg^{2+} -ATPase of synaptic vesicles belongs to a class of vacuolar H^+ -ATPases which are responsible for acidification of different, subcellular organelles (Nelson, 1986, 1987; Maycox *et al.*, 1988; Cidon & Sihra, 1989; Shioi *et al.*, 1989; Floor *et al.*, 1990). GABA and glutamate are taken up into different populations of synaptic vesicles (Fykse & Fonnum, 1989) with slightly different affinities. The K_m value for the uptake of glutamate was about 1 mM (Naito & Ueda, 1985; Maycox *et al.*, 1988), and the K_m value for GABA was determined to about 6 mM (Fykse & Fonnum, 1988; Kish *et al.*, 1989). The evidence for a specific vesicular uptake of GABA, glycine and glutamate supports the notion that synaptic vesicles play an important role in amino acid synaptic transmission.

The uptake of glutamate is stimulated by low concentrations of Cl^- (Naito & Ueda, 1985; Maycox *et al.*, 1988; Fykse *et al.*, 1989), whereas the uptake of GABA is insensitive to variations in the concentration of Cl^- (Fykse *et al.*, 1989; Kish *et al.*, 1989). Different groups have reported that ATP hydrolysis generated a large proton gradient across the synaptic-vesicle membrane at high concentrations of Cl^- . A small proton gradient and a large membrane potential are reported at low Cl^- concentrations (Maycox *et al.*, 1988; Cidon & Sihra, 1989). The uptake of glutamate is driven by the membrane potential, since collapsing the membrane potential by high concentrations of Cl^- inhibited the uptake of glutamate (Maycox *et al.*, 1988; Cidon & Sihra, 1989; Shioi *et al.*, 1989). On the basis of these experiments, a direct involvement of Cl^- in the uptake of glutamate would be a reasonable explanation. The purpose of the present study was to

investigate the differences in the mechanisms of the uptake of GABA and glutamate in detail, and specially to examine the role of Cl^- and other permeant ions in the uptake of GABA and glutamate. We have compared the effect of the anion-channel blockers 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (DIDS), 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid (SITS) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (N144) on the uptake of GABA and glutamate and on the vesicular Mg^{2+} -ATPase activity.

EXPERIMENTAL

Materials

GABA, L-glutamate (dipotassium salt), ATP (disodium salt), SITS, DIDS and CPG-3000 controlled-pore glass beads of nominal pore diameter 271.7 nm (lot 36F-0650, mesh 120/200) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $[2,3-^3H]$ GABA (40 Ci/mmol) and L- $[2,3-^3H]$ glutamate (25 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). N144 was a gift from Dr. J. J. Nordmann, Centre de Neurochimie, Strasbourg, France.

Preparation of synaptic vesicles

Synaptic vesicles were purified from male Wistar rats (200–250 g) obtained from Møllegaard, Ejby, Denmark. For each experiment, 10–15 rats were killed by decapitation, and the brains were quickly removed and kept on ice. Synaptic vesicles were isolated as described in principle by Whittaker *et al.* (1964) and in detail by Fykse & Fonnum (1988). The crude synaptosomal fraction (P_2) was osmotically shocked by resuspension in 10 mM-Tris/maleate (pH 7.4)/0.1 mM-EGTA and centrifuged at 13 000 g for 30 min. The supernatant was laid on the top of 0.4 M- and 0.6 M-sucrose solutions and centrifuged in a Contron TST 28.38 rotor at 65 000 g for 2 h. The vesicle fraction was isolated from the band containing 0.4 M-sucrose (D-fraction). The vesicle preparations were stored in liquid N_2 without loss of activity. This vesicle preparation was used in most of the uptake experi-

Abbreviations used: AChE, acetylcholinesterase (EC 3.1.1.7); DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid; GABA, γ -amino-n-butyric acid; IC_{50} , concn. giving 50% inhibition; NEM, N-ethylmaleimide; N144, 5-nitro-2-(3-phenylpropylamino)benzoic acid; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid.

* To whom correspondence should be addressed.

ments. The vesicular uptake was not stimulated by Na^+ (Fykse & Fonnum, 1988). Any contamination by plasma membranes therefore cannot be of any significance. Furthermore, the uptake was dependent on Mg^{2+} and ATP and inhibited by proton ionophores (Fykse *et al.*, 1989).

To compare the effect of the inhibitors on the Mg^{2+} -ATPase activity and the uptake of GABA and glutamate, the D-fraction was further purified by chromatography on a column (44 cm $\times$ 1.6 cm) of CPG-3000 controlled-pore glass beads in 110 mM-potassium tartrate/10 mM-Hepes (pH 7.4)/0.1 mM-EGTA. The elution rate was 1 ml/min, and 3 ml fractions were collected.

Vesicular uptake of GABA and glutamate

This was done mainly as described by Fykse & Fonnum (1988). The standard incubation mixture (total volume 0.3 ml) for assaying vesicular uptake contained 0.25 M-sucrose, 10 mM-Tris/maleate (pH 7.4) and 4 mM- MgCl_2 (if not otherwise stated). Synaptic vesicles (about 0.1 mg of protein) were preincubated at 30 °C for 15 min, and [^3H]GABA (1 μCi ; final concn. 1 mM) or L-[^3H]glutamate (0.5 μCi ; final concn. 1 mM) and ATP (final concn. 2 mM; disodium salt neutralized by Tris base) were added, and the mixture was further incubated for 3 min at 30 °C. The reaction was stopped by addition of 7 ml of ice-cold 0.15 M-KCl, followed by rapid filtration through a Millipore HAWP filter (diameter 25 mm, pore size 0.45 μm). The incubation tubes were further washed twice with the KCl solution. The filters were dissolved in 10 ml of Filter Count (Packard), and the radioactivity was determined in a Packard Tri-Carb 2200 liquid-scintillation counter with a counting efficiency of 50–56%. Blanks were identical with the test solution in each case, but were incubated at 0 °C. Blank values corresponded to about 10 and 15% of the radioactivity retained on the filters for the uptakes of glutamate and GABA, and the blank values were 266 ± 10 and 434 ± 19 c.p.m. (both $n = 20$) respectively. The different inhibitors and anions tested were included in the preincubation mixtures.

Uptakes of GABA and glutamate were also studied on the fractions eluted from the CPG-3000 controlled-pore glass column. In these experiments the uptakes of GABA and glutamate were measured in the elution buffer, which also was used for the Mg^{2+} -ATPase experiments.

Assays for Mg^{2+} -ATPase activity, acetylcholinesterase (AChE) activity and protein

The Mg^{2+} -ATPase activity was measured at 30 °C mainly as described by Penefsky & Bruist (1984). The incubation mixture for measuring Mg^{2+} -ATPase contained synaptic vesicles (10–40 μg of protein), 10 mM-Hepes (pH 7.4), 110 mM-potassium tartrate, 4 mM- MgCl_2 , 2 mM-phosphoenolpyruvate, 20 units each of lactate dehydrogenase and pyruvate kinase/ml, 0.06 mM- K_2NADH and 3 mM-ATP (pH 7.4) in a total volume of 1225 μl . The conversion of NADH into NAD^+ was measured at 340 nm in a Beckman DU 50 spectrophotometer, and 1 nmol of NADH converted corresponds to 1 nmol of phosphate used.

The AChE activity was measured by a radiochemical method (Sterri & Fonnum, 1978). The protein content were measured as described by Lowry *et al.* (1951), with BSA as standard.

Statistics

The results are expressed as mean values ($\pm$ S.E.M.) of absolute uptake or as relative uptakes (as percentage of controls). Groups of data were analysed by Student's *t* test. The IC_{50} values (concn. giving 50% inhibition) were calculated from 3 or 4 different experiments with a Multiple Drug Effect Analysis Program (Chou & Chou, 1985).

RESULTS

Effect of inhibitors on vesicular uptake

Owing to the ability of anions to influence vesicular glutamate uptake activity, the effects of SITS and DIDS, agents known to affect plasma-membrane anion channels (Cabantchik *et al.*, 1978), were examined (Figs. 1a and 1b). Both compounds inhibited the uptake of glutamate better than the uptake of GABA. The IC_{50} values for the uptake of glutamate were calculated as 1.4 μM for DIDS and 3.1 μM for SITS. For the uptake of GABA, the IC_{50} values for DIDS and SITS were calculated as 9.9 μM and 40 μM respectively. In the absence of Cl^- the effect of SITS on the uptake of glutamate was decreased, and the IC_{50} value was calculated as 13 μM . The effect of SITS on the uptake of GABA was not affected by removing Cl^- . In crude synaptosomal fractions of rat brains, the IC_{50} values of SITS for the high-affinity uptake of GABA and glutamate were about 1 mM (E. M. Fykse, unpublished work). This is consistent with results of Fosse *et al.* (1989) on glutamate uptake. Similar results were obtained with the more specific Cl^- -channel blocker N144 (Wangemann *et al.*, 1986) on the vesicular uptake of GABA and glutamate (Fig. 1c). The IC_{50} value for glutamate uptake was calculated as 15 μM , whereas for GABA it was calculated as 150 μM .

Thiocyanate anion (SCN^-) is membrane-permeant and is

Fig. 1. Effects of SITS, DIDS, N144 and KSCN on the uptake of GABA and glutamate

The activity was assayed as stated in the Experimental section. SITS (a), DIDS (b), N144 (c) and KSCN (d) were added just before the preincubation, which lasted for 15 min. In the absence of inhibitors, the uptakes of GABA and glutamate in the presence of 8 mM- Cl^- were 820 ± 80 ($n = 13$) and 2900 ± 200 ($n = 19$) pmol/min per mg of protein respectively; in the absence of Cl^- the glutamate control value was 500 ± 100 pmol/min per mg of protein ($n = 5$) (n = no. of experiments). Key: ●, glutamate (+ Cl^-); ▲, glutamate (– Cl^-); ○, GABA (+ Cl^-).

Fig. 2. Chromatography of synaptic vesicles on a column of CPG-3000 controlled-pore glass beads

The recovery of protein was 51 %. (a) Distribution of Mg^{2+} -ATPase and AChE. (b) Distribution of the uptakes of GABA and glutamate and protein content. I and II refer to the pooled fractions mentioned in the Results section.

Table 1. Effects of inhibitors on the Mg^{2+} -ATPase activity

The D-fraction was purified by chromatography on a controlled-pore glass column (CPG-3000). Fractions 14–17 (I) and 19–24 (II) were pooled, and the Mg^{2+} -ATPase activity was measured as described in the Experimental section. In all experiments, 50 μ M-vanadate, 1 mM-ouabain and 5 μ g of oligomycin B/ml were included. The enzyme activity is expressed as % of control, and the data are means $\pm$ S.E.M. of three independent experiments. The control values were 190 ± 50 and 180 ± 20 nmol of P_i /min per mg of protein for fractions I and II respectively. The significance of difference was calculated by Student's *t* test: **P* < 0.05, ***P* < 0.005.

Inhibitor	Mg^{2+} -ATPase [relative activity (%)]	
	Fraction I (14–17)	Fraction II (19–24)
SITS (1 μ M)	101 $\pm$ 8	95 $\pm$ 5
SITS (10 μ M)	90 $\pm$ 8	83 $\pm$ 1*
SITS (25 μ M)	80 $\pm$ 11	69 $\pm$ 6*
N144 (1 μ M)	102 $\pm$ 7	103 $\pm$ 3
N144 (10 μ M)	93 $\pm$ 7	101 $\pm$ 22
N144 (25 μ M)	89 $\pm$ 9	104 $\pm$ 4
NEM (100 μ M)	48 $\pm$ 7*	34 $\pm$ 2**

known to abolish the inside vesicular positive electrochemical potential. The Mg^{2+} -ATP-dependent uptakes of both GABA and glutamate were inhibited by thiocyanate in a dose-dependent manner (Fig. 1d). The IC_{50} values were calculated as 3.9 mM and 1.9 mM for uptake of GABA and glutamate respectively.

Vesicular uptake and Mg^{2+} -ATPase activity in a more purified synaptic-vesicle fraction

It has been reported (Diebler & Lazereg, 1985; Rothlein & Parsons, 1982) that the anion-transport blocker SITS also inhibits

Table 2. Effects of SITS and N144 on the uptake of GABA and glutamate into vesicles purified on a controlled-pore glass column

Fractions were pooled as described in the Results section and in Table 1. In the uptake experiments, only fraction II was used, owing to the low uptake activity in fraction I. The uptake was performed as described in the Experimental section. The results are expressed as means $\pm$ S.E.M. of three or four independent experiments: **P* < 0.05, ***P* < 0.005 by Student's *t* test. The control values for fraction II were calculated as 6600 ± 600 and 1110 ± 160 pmol/min/mg of protein for the uptakes of glutamate and GABA respectively.

Inhibitor	Vesicular uptake [relative activity (%)]	
	Glutamate	GABA
SITS (1 μ M)	85 $\pm$ 4*	111 $\pm$ 12
SITS (10 μ M)	46 $\pm$ 6**	82 $\pm$ 12
SITS (25 μ M)	22 $\pm$ 1**	51 $\pm$ 4**
N144 (1 μ M)	91 $\pm$ 6	—
N144 (10 μ M)	61 $\pm$ 9*	84 $\pm$ 13
N144 (25 μ M)	31 $\pm$ 5*	55 $\pm$ 8*

the Mg^{2+} -ATPase of *Torpedo*. We have therefore studied the effect of SITS on the vesicular Mg^{2+} -ATPase of rat brain. In these experiments we used a more purified synaptic vesicle fraction, i.e. synaptic vesicles chromatographed on a controlled-pore glass column. A typical run is shown in Fig. 2. The Mg^{2+} -ATPase was eluted in two peaks. Traces of AChE, a plasma-membrane marker, were co-eluted with the first peak. The uptake of GABA and glutamate was co-eluted with the second peak, which was almost free of AChE contamination. In the vesicle fraction, specific activity of the Mg^{2+} -ATPase was enriched by a factor 2, as compared with 3-fold enrichment in the uptake of GABA and glutamate.

To obtain enough material, fractions 13–17 (I) and 19–24 (II) were pooled. The effects of SITS and N144 on the Mg^{2+} -ATPase (Table 1) and the vesicular uptake (Table 2) were examined. In these experiments both the vesicular uptake and the ATPase were examined in 110 mM-potassium tartrate and 10 mM-Hepes, pH 7.4. The ATPase experiments were done in the presence of 50 μ M-vanadate, 1 mM-ouabain and 5 μ g of oligomycin B/ml. Ouabain and vanadate inhibit plasma-membrane ATPase, and oligomycin B is known to inhibit the mitochondrial H^+ -ATPase. The Mg^{2+} -ATPase of fractions I and II was inhibited by 42 and 27% respectively by including the three inhibitors. N144 had almost no effect on the ATPase activity at all, neither fraction I nor II (Table 1). The uptake of glutamate into fraction II was inhibited by 78%, whereas the uptake of GABA was inhibited by 49%, by 25 μ M-SITS (Table 2). The vesicular Mg^{2+} -ATPase activity was inhibited by 31% by 25 μ M-SITS.

The vesicular Mg^{2+} -ATPase is known to be inhibited by *N*-ethylmaleimide (NEM) (Grønberg & Flatmark, 1987). In the presence of 100 μ M-NEM the ATPase activity in fraction II was inhibited by 65%, whereas that in fraction I was inhibited by 45%. If SITS (25 μ M) was added in the presence of NEM (100 μ M) no further inhibition was seen.

Ion-sensitivity of the vesicular uptake of GABA and glutamate

Different anions were tested for their capacity either to stimulate or to inhibit the uptake of GABA and glutamate (Tables 3 and 4). The experiments were performed in the presence of 4 mM- $MgSO_4$, and different anions were successively added. Low concentrations of either KCl or KBr hardly affected the uptake of GABA (Table 3), whereas the uptake of glutamate was

Table 3. Effects of various anions on the vesicular uptake of GABA

A vesicle fraction (D-fraction) was incubated in 0.25 M-sucrose/10 mM-Tris/maleate (pH 7.4)/4 mM-MgSO₄/2 mM-ATP/1 mM-[³H]GABA (2.9 mCi/mmol) for 3 min at 30 °C. The control value in the absence of any of the salts in the table was 830 ± 70 pmol/min per mg of protein (*n* = 6). Data (means ± S.E.M.) are percentages relative to the control (nos. of determinations in parentheses). The significance of differences was calculated by Student's *t* test: **P* < 0.05, ***P* < 0.005.

Salt added	Concn. ...	GABA uptake [relative activity (%)]		
		1 mM	5 mM	50 mM
KF		99 ± 4 (4)	95 ± 5 (4)	78 ± 7 (4)*
KCl		94 ± 5 (4)	91 ± 3 (5)	76 ± 3 (4)**
KBr		95 ± 5 (4)	90 ± 8 (4)	53 ± 4 (4)**
KI		95 ± 7 (4)	84 ± 7 (4)	24 ± 3 (4)**
KNO ₃		71 ± 7 (4)*	36 ± 5 (4)**	12 ± 3 (4)**

Table 4. Effects of different anions on the vesicular uptake of glutamate

A vesicle fraction (D-fraction) was incubated in 0.25 M-sucrose/10 mM-Tris/maleate (pH 7.4)/4 mM-MgSO₄/2 mM-ATP/1 mM-L-[³H]glutamate (1.9 mCi/mmol) for 3 min at 30 °C. The control value was 560 ± 70 (*n* = 7) pmol/min per mg of protein. The uptake is expressed as percentage of control, and the data are means ± S.E.M. (nos. of determinations in parentheses): **P* < 0.05, ***P* < 0.005 by Student's *t* test.

Salt added	Concn. ...	Glutamate uptake [relative uptake (%)]		
		1 mM	5 mM	50 mM
KF		93 ± 9 (5)	55 ± 3 (4)**	13 ± 7 (4)**
KCl		320 ± 50 (5)**	400 ± 70 (6)**	127 ± 14 (4)
KBr		410 ± 80 (6)**	450 ± 80 (5)**	54 ± 13 (4)*
KI		200 ± 40 (6)*	160 ± 40 (4)	11 ± 4 (4)**
KNO ₃		180 ± 30 (6)*	118 ± 34 (4)	12 ± 2 (4)**

stimulated about 3- and 4-fold respectively (Table 4). Previously we have shown that K⁺ had no effect on the uptake of GABA and glutamate (Fykse *et al.*, 1989). KF was a much more potent inhibitor of the glutamate uptake than of the GABA uptake. Low concentrations of KI and KNO₃ stimulated the uptake of glutamate significantly, whereas high concentrations inhibited both uptake systems.

To examine if SO₄²⁻ had any effect on the vesicular uptake, we used Mg²⁺-ATP salt in the incubation mixture. Also in this case the uptake of GABA was not stimulated by addition of low concentrations of Cl⁻, whereas the uptake of glutamate was highly stimulated, as before.

DISCUSSION

In the present study we have shown that the agents SITS, DIDS and N144 were about 10 times more potent in inhibiting the Mg²⁺-ATP-dependent uptake of glutamate than the corresponding uptake of GABA into vesicles. The effect of SITS and N144 could not be explained by an effect on the vesicular Mg²⁺-ATPase, since the Mg²⁺-ATPase was less inhibited than the vesicular uptake of glutamate. In addition, we have extended the previous findings of Naito & Ueda (1985), Fykse *et al.* (1989) and Kish *et al.* (1989) that the vesicular uptakes of GABA and glutamate have different sensitivity to permeant anions. We have

also shown that the inhibitory effect of SITS on the uptake of glutamate was less potent in the absence of Cl⁻.

Since SITS and N144 inhibited the glutamate uptake more than hydrolysis of ATP, it is likely that their inhibitory effect was due to an effect on the glutamate carrier and not on the vesicle H⁺-ATPase. This agrees with the general view that the vesicle H⁺-ATPase is a well-preserved structure belonging to a class of enzyme (vacuolar type) responsible for acidification of different intracellular organelles (Nelson, 1986, 1987; Cidon & Sihra, 1989). The vesicle H⁺-ATPase is immunologically related to the chromaffin-granule enzyme (Cidon & Sihra, 1989), and the mammalian vesicle H⁺-ATPase closely resembled the vacuolar ATPase of chromaffin granules (Floor *et al.*, 1990). It is therefore reasonable to assume that SITS, DIDS and N144 did not primarily affect the Mg²⁺-ATPase in the concentration range used in the present experiments.

The effect of SITS on the synaptosomal high-affinity uptake of glutamate was more than 100-fold less potent (Fosse *et al.*, 1989) than the effect on the vesicular uptake. This shows the large differences in the structure of the vesicular transporter and the high-affinity glutamate transporter of synaptosomes.

Agents which affect the Mg²⁺-ATPase, NEM and *NN'*-dicyclohexylcarbodi-imide ('DCCD'), or the electrochemical proton gradient, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), nigericin and gramicidin seems to inhibit the uptake of GABA and glutamate more similar than SITS, DIDS and N144 (Fykse *et al.*, 1989). The anion SCN⁻ have been shown to inhibit the uptake of glutamate and GABA (Shioi *et al.*, 1989; Hell *et al.*, 1990), and in the present work the uptake of GABA and glutamate was inhibited nearly to the same extent by SCN⁻.

The point then arises whether SITS, DIDS and N144 may affect the glutamate transporter. The three compounds, although only to a small extent N144, have two negatively charged groups, as in glutamate. The distance between the groups (C₆) is, however, larger than in glutamate, and should not give inhibition (Christensen *et al.*, 1991). In other biological systems such as red blood cells, the stilbenedisulphonate derivatives SITS and DIDS are known to be blockers of anion transport (Cabantchik *et al.*, 1978), and Cabantchik *et al.* (1978) have shown that 5–8 μM-DIDS almost completely inhibited anion exchange in red blood cells. This is in agreement with the effect of DIDS on the uptake of glutamate. In the absence of Cl⁻, the effect of SITS on the uptake of glutamate was less potent. These results indicate that a Cl⁻ channel or a Cl⁻-binding site might be involved in the uptake of glutamate. This is in agreement with the fact that different anions affected the uptake of GABA and glutamate differently. Low concentrations of several permeant anions such as Cl⁻, Br⁻ and I⁻ stimulated the uptake of glutamate, which are in agreement with Naito & Ueda (1985). Low concentrations of F⁻ inhibited the uptake of glutamate significantly. In contrast, low concentrations of permeant anions did not stimulate the uptake of GABA in the preparations, which is consistent with the effect of Cl⁻ on the uptake of GABA (Fykse *et al.*, 1989; Kish *et al.*, 1989). In contrast, Hell *et al.* (1990) found a 40% decrease in the GABA uptake in the absence of Cl⁻; maximal uptake was observed in the range of 4–50 mM-Cl⁻ ions. Even in the experiments where we added Mg²⁺ as Mg²⁺-ATP to avoid SO₄²⁻ in the incubation mixture, we did not find any stimulation of the GABA uptake by Cl⁻. The uptake of glutamate was highly stimulated as before. All results indicate at least a different anion-sensitivity of the uptake systems for GABA and glutamate.

We also tested the more specific anion-channel blocker N144 (Wangemann *et al.*, 1986), since some of the present results support the idea that a Cl⁻ channel might be involved in the uptake of glutamate. The effects on the uptake of GABA and glutamate were in agreement with the effects of SITS and DIDS.

Dayanithi & Nordmann (1989) found that N144 caused release of vasopressin from rat permeabilized neurohypophysial nerve endings, and they concluded that a Cl^- channel was involved in the release process. They found the IC_{50} value to be $5 \mu\text{M}$. Release of adrenaline from chromaffin granules has also been shown to be inhibited by SITS, and anions such as Cl^- were required for the release process (Pazoles & Pollard, 1978). Cidon & Nelson (1982) investigated the effect of SITS on the Mg^{2+} -ATPase of chromaffin granules and concluded that up to a concentration of $10 \mu\text{M}$ -SITS the inhibition was due to a blockage of a Cl^- channel.

To explain the different effects of SITS, DIDS and N144 on the neurotransmitter uptakes, a direct effect on at least part of the glutamate transporter seems to be a reasonable explanation. The effects of low concentrations of permeant anions on the uptake of glutamate, and the more potent effect of anion-channel blockers, indicate involvement of an anion channel or an anion site. The lack of effect of these anions on the uptake of GABA, and the less potent effect of the anion-channel blockers, indicate that permeant anions are not directly involved in the uptake of GABA. One possible mechanism for the uptake of glutamate is that a glutamate/ Cl^- antiport exists. More experiments need to be done to show clearly whether a Cl^- channel, a Cl^- -binding site and a glutamate/ Cl^- antiport are involved in the uptake of glutamate.

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