Two Distinct Mechanisms, Differentially Affected by Excitatory Amino Acids, Trigger GABA Release from Fetal Mouse Striatal Neurons in Primary Culture

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The mechanisms leading to Ca^{2+} -dependent and Ca^{2+} -independent GABA release were studied on highly purified striatal neurons developed in primary culture. Ca^{2+} -dependent GABA release, which represents about 75% of the 56 mM K^+ effect was totally inhibited when striatal neurons were first exposed to tetanus toxin (TnTx) (10 $\mu\text{g/ml}$) for 24 hr. The K^+ effect was potentiated when 1 mM nipecotic acid (an inhibitor of the GABA uptake system) was added during the stimulation period or when Na^+ was replaced by Li^+ . However, no difference in the GABA release measured under high- K^+ conditions was observed after a 22 min preincubation of the neurons in a medium containing nipecotic acid or Li^+ . Replacement of Cl^- ions by SO_4^{2-} did not modify K^+ -evoked GABA release.

Ca^{2+} -independent GABA release was stimulated by veratridine (20 μM), ouabain (3 mM), and monensin (20 μM), as well as the excitatory amino acids glutamate (100 μM), *N*-methyl-D-aspartate (100 μM), quisqualate (10 μM), and kainate (1 mM), drugs known to increase intracellular Na^+ concentration. The veratridine- or glutamate-evoked GABA release was neither inhibited when intracellular Ca^{2+} content was reduced by more than 90% nor by treatment of the neurons to TnTx. However, the Ca^{2+} -independent GABA release elicited by veratridine was inhibited by preincubation of the neurons in a medium containing 1 mM nipecotic acid and in a medium containing Li^+ instead of Na^+ or SO_4^{2-} instead of Cl^- .

These results strongly suggest that 2 different GABA release mechanisms exist in striatal neurons. The first, which is Ca^{2+} dependent and TnTx-sensitive, probably corresponds to the classical exocytotic process. The second, which is Ca^{2+} independent, not sensitive to TnTx, and stimulated by excitatory amino acids, is probably due to the reversal of the GABA uptake system.

Since the first proposal of the vesicular hypothesis by Katz 30 years ago (Del Castillo and Katz, 1957), numerous arguments have been made (Ceccarelli and Hurlbut, 1980; Heuser and Reese, 1981) suggesting that exocytosis of synaptic vesicles is responsible for neurotransmitter release. Whereas this hypothesis is now widely accepted, it cannot, however, explain all the results reported on neurotransmitter release studies (see Tauc, 1982; Israel et al., 1986). The vesicular hypothesis assumes that depolarization of the nerve terminals induces Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. The increase in cytoplasmic free Ca^{2+} concentration is then supposed to allow the exocytosis of synaptic vesicles. According to this mechanism, the extracellular Ca^{2+} $[(\text{Ca}^{2+})_o]$ dependence of neurotransmitter release is regarded as the main property of physiological release. However, GABA was rapidly recognized as a “particular” neurotransmitter, since, in contrast to many other transmitters, its release can be greatly and rapidly increased in the absence of $(\text{Ca}^{2+})_o$ (Srinivasan et al., 1969). In the last decade, 2 hypotheses have been proposed for this GABA release process. (1) Sandoval (1980) and Cunningham and Neal (1981) proposed that the release of Ca^{2+} from internal stores may, in fact, be involved in this GABA release process. (2) another interesting explanation was first presented by Haycock et al. (1978), who proposed that the $(\text{Ca}^{2+})_o$ -independent GABA release may result from the reversal of the GABA uptake system. This hypothesis is supported by more recent works of Yazulla (Yazulla, 1983; Yazulla and Kleinschmidt, 1983) and Schwartz (1982, 1987). These last studies were, however, performed on a particular model—retinal horizontal cells—in which no synaptic vesicles have been detected and from which no Ca^{2+} -dependent GABA release has been described. Therefore, the mechanism by which GABA can be released in a $(\text{Ca}^{2+})_o$ -independent manner from central neurons containing synaptic vesicles remained to be clearly established.

The aim of the present work was to further examine the $(\text{Ca}^{2+})_o$ -dependent and -independent GABA releases from central neurons, in order to determine if they correspond to 2 different release mechanisms. Recently, we developed a culture method that allows us to obtain highly differentiated neurons in primary culture after 14–16 d *in vitro* (DIV) (Weiss et al., 1986). For the following reasons, these neurons constitute a suitable model with which to study GABA release mechanisms: (1) The GABA release from these neurones has similar properties to that described using neuronal preparations differentiated *in vivo* (Pin et al., 1986); (2) in this preparation, GABA release from glial cells (Pearce and Dutton, 1981) is negligible since only 7% of the cell population is non-neuronal after 10 DIV (Bockaert et

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al., 1986); (3) endogenous amino acid release from these neurons can be measured easily using high-performance liquid chromatography (HPLC) (Pin et al., 1986). This latter point is particularly important since ^3H -GABA release does not always correlate with the release of endogenous GABA (Abe and Matsuda, 1983; see also Szerb, 1983b). We demonstrate here that GABA release from striatal neurons is not only triggered by the classical Ca^{2+} -dependent process but also by the reversal of the GABA uptake system allowing a nonvesicular, and probably extrasynaptic, GABA release.

Materials and Methods

Materials. Standard amino acids, veratridine, ouabain (octahydrate), monensin (sodium salt), glutamic acid, kainic acid, ruthenium red (oxychloride ammoniated), aminooxyacetic acid (hemi-hydrochloride), DL-allo glycine, thiosemicarbazide, ($\pm$)-nipecotic acid, poly(L-ornithine) ($M_w = 40,000$) and other chemicals of analytical grade, including hormones added in the culture medium, were obtained from Sigma (St. Louis, MO). TnTx was a gift from Dr. Bizzini (Paris) and TTX was from Boehringer Mannheim (FRG). Quisqualic acid and *N*-methyl-D-aspartic acid were from Cambridge Research Biochemicals Limited (Harston, England). Six-well clusters for tissue cultures were obtained from Costar (Cambridge, MA). Dulbecco's minimal essential medium and F-12 nutrient were obtained from Gibco Europe (Paris). DL-(3,4- ^3H) nipecotic acid (30 Ci/mmol) and 4-amino *n*-(2,3- ^3H)-butyric acid (^3H -GABA; 57 Ci/mmol) were from Amersham (France) and $^{45}\text{Ca}^{2+}$ was purchased as calcium chloride in water from New England Nuclear (Paris).

Striatal cell cultures. Neuronal cultures were prepared in serum-free media as previously described (Weiss et al., 1986). Striata were removed from 14–15-d-old Swiss Albino mouse embryos (Iffa Credo, Lyon, France) and mechanically dissociated with a fire-narrowed Pasteur pipette in serum-free medium. Cells were plated ($0.8\text{--}1.0 \times 10^6$ cells/ml, 2 ml/well) in 35-mm-diameter Costar culture dishes (6-well cluster), previously coated successively with poly(L-ornithine) (1.5 $\mu\text{g}/\text{ml}$) and culture medium containing 10% fetal calf serum. After withdrawing the last coating solution, cells were seeded in serum-free medium composed of a 1:1 mixture of Dulbecco's minimal essential medium and F-12 nutrient (Gibco Europe, Paris) and included glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM), and HEPES buffer (5 mM). Instead of serum, a defined hormone and salt mixture that included insulin (25 $\mu\text{g}/\text{ml}$), transferrin (100 $\mu\text{g}/\text{ml}$), progesterone (20 nM), estradiol (1 pM), putrescine (60 μM), and selenium salt (30 nM) was added.

Primary cultures of striatal glial cells from 14- to 15-d-old mouse embryos were performed essentially as neuronal cultures except that culture medium contained 10% fetal calf serum instead of the defined hormone and salt mixture. Moreover, culture medium was changed twice a week, and experiments were performed after 3 weeks *in vitro*, when the confluence was reached.

Amino acid release. Endogenous amino acid release experiments were performed essentially as previously described (Pin et al., 1986). Briefly, after 14–16 DIV, neuronal cultures were washed twice with 2 ml of prewarmed Krebs-Ringer solution devoid of amino acids (NaCl, 128 mM; KCl, 5 mM; CaCl_2 , 2.7 mM; MgSO_4 , 1.2 mM; Na_2HPO_4 , 1 mM; glucose, 10 mM; HEPES 20 mM, pH 7.35) and allowed to stand for 10 min in the same buffer at 37°C . The medium (2 ml) of each dish was then carefully renewed every 3 min. After 4 changes of medium, neuronal cultures were exposed to the stimulatory solution for 3 min. The removed media were collected and maintained at -20°C before HPLC analysis of amino acid content. In buffer containing an elevated concentration of K^+ , Na^+ concentration was decreased in order to keep osmolarity constant. For the experiments performed in Ca^{2+} -free medium, Krebs-Ringer solution devoid of added Ca^{2+} and with or without 1 mM EGTA was used from the beginning of the experiment.

Care was taken to avoid buffer contamination. Pyrolyzed water, devoid of any detectable amino acid was always used. Krebs-Ringer solution, concentrated 4-fold, was stored frozen without Ca^{2+} and glucose, which were added the day of the experiment when the final solutions were prepared.

Extraction of amino acids from striatal cells. The determination of the amino acid content of striatal cells developed in primary culture was determined as follows. Culture dishes were washed 3 times with 2 ml Krebs-Ringer solution. Striatal cells were then scraped twice in 300 μl of extracting medium (HCl, 0.05 N, 10 ml; absolute ethanol, 74 ml;

water, 16 ml). The 600 μl of extract was homogenized with a glass-glass potter (10 strokes) and then centrifuged at $180,000 \times g$ for 45 min. The resulting supernatant was stored at -20°C and diluted 10-fold in water before HPLC analysis of amino acid concentration.

Measurement of endogenous amino acids. Endogenous amino acid concentrations in each collected sample were determined by HPLC as previously described (Pin et al., 1986). A Beckman HPLC composed of two A 112 pumps and a model 421 system controller was used. Aliquots, 200 μl , were mixed with 70 μl orthophthalaldehyde-(OPA)/2-mercaptoethanol derivating solution and injected 1 min later using a Gilson automatic sample injector (model 231-401). OPA solution was made as follows: 100 mg OPA was dissolved in 2 ml ethanol, diluted to 20 ml in borate buffer (0.4 M, pH 10) before adding 100 μl 2-mercaptoethanol. OPA derivatives were then separated on a reverse-phase column (4.6 $\times$ 250 mm, 5 μm particle diameter, ALTEX ultrasphere ODS). The initial step was composed of 95% buffer A [tetrahydrofuran, 2%:acetonitrile, 2%:0.05 M phosphate acetate (pH 7.10), 96%] and 5% buffer B (methanol, 65%:water, 35%) at flow rate of 1 ml/min. The following gradient program was used [time (min), (% buffer B)]: 5,(5); 35,(54); 45,(55); 46,(100); 54,(100); 60,(5). Fluorescence was detected using a Shimadzu (RF 530) spectrofluorimeter. The area of each peak was determined with a Delsi (Enica 10) integrator and compared with those of external standards in order to determine the amino acid concentration.

^3H -GABA and (^3H)-nipecotic acid uptake. After 2 washes with 2 ml Krebs-Ringer solution and 10 min equilibration period, striatal neurons were exposed to 2 ml Krebs-Ringer solution containing 1 μCi ^3H -GABA (0.1 μM) or 1 μCi ^3H -nipecotic acid (1 mM) for the indicated time. Each dish was then rapidly washed 3 times with 2 ml of cold Krebs-Ringer solution. Tritium remaining in the cells was then extracted twice with 1 ml of 0.2 M NaOH, 0.5% Triton X100 solution. The total radioactivity accumulated in the neurons was determined by adding 6 ml of a scintillation cocktail (ACS II, Amersham), and 0.2 ml HCl 2 M.

Determination of Ca^{2+} content of striatal neurons. Measurement of $^{45}\text{Ca}^{2+}$ accumulation by striatal neurons was determined by adding 1 μCi $^{45}\text{Ca}^{2+}$ in the culture medium after 15–16 DIV. After 28 hr, neurons were rapidly washed 3 times with 2 ml Krebs-Ringer devoid of Ca^{2+} and containing 1 mM EGTA. $^{45}\text{Ca}^{2+}$ present in the neurons was determined after 3 rapid washes (<15 sec) of culture dishes with 2 ml cold buffer (NaCl 9 gm/liter; CaCl_2 , 5 mM; Tris HCl, 5 mM, pH 7.4). Total $^{45}\text{Ca}^{2+}$ remaining in the neurons was extracted with 2 ml of 0.2 M NaOH, 0.5% Triton X100 solution. This operation was repeated twice. Radioactivity was then determined as described above.

Determination of cyclic AMP and inositol phosphate production in striatal neurons. Intracellular levels of cyclic AMP and inositol phosphate in striatal neurons were determined as previously described (Sladeczek et al., 1985; Bockaert et al., 1986).

Statistics. In each experiment, GABA release was calculated as nmoles of GABA released per 3 min and per well. Results are given as means $\pm$ SEM and statistical differences were analyzed by Student's *t* test.

Results

(Ca^{2+})_o dependence of endogenous GABA release

Depolarization of striatal neurons with 56 mM K^+ resulted in a 15.6-fold increase in GABA release (Table 1). In the absence of (Ca^{2+})_o (1 mM EGTA was added), the basal release was enhanced and the K^+ -evoked GABA release was reduced still 4-fold (Table 1). In contrast, GABA release induced by veratridine (a specific activator of voltage-sensitive Na^+ channels), which was sensitive to TTX (Pin and Bockaert, 1987), was not inhibited in the absence of (Ca^{2+})_o (Table 1). A (Ca^{2+})_o-independent GABA release was also stimulated by monensin (a Na^+ carrier), ouabain (a specific inhibitor of the Na^+/K^+ pump), or by the excitatory amino acid neurotransmitter glutamate, which activates specific cationic conductances (Watkins and Evans, 1981; Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987) (Table 1). The Ca^{2+} -independent glutamate-evoked GABA release was probably mediated through the activation of the various subtypes of excitatory amino acid receptors (see Watkins and Evans, 1981) since the 3 classical agonists *N*-methyl-D-aspartate (NMDA), quisqualate, and kainate, when tested at the

Figure 1. Effect of excitatory amino acid receptor agonists on endogenous GABA release from striatal neurons. Each bar represents the GABA release during a 3 min period. The hatched bars represent the GABA release during the stimulation with NMDA (100 μM) (*a*, *b*), quisqualate (10 μM) (*c*, *d*), or kainate (1 mM) (*e*, *f*). Experiments with NMDA were performed in the absence of Mg²⁺. Experiments were performed either in the presence (*a*, *c*, *e*) or absence (*b*, *d*, *f*) of Ca²⁺ (1.1 mM). Results are expressed as the percentage of the basal release value and are means ± SEM of 4 (*a–d*) and 5 (*e*, *f*) experiments performed in triplicate. Basal GABA release was 0.10 ± 0.01 nmol/3 min/well (mean ± SEM of 8 triplicate determinations).

minimal concentration needed to induce a maximal response (Pin et al., 1988, and unpublished observations), all stimulated GABA release in a reversible and (Ca²⁺)_o-independent manner (Fig. 1). These results suggest that an increase in internal Na⁺ (Na⁺)_i concentration may be important for the (Ca²⁺)_o-independent GABA release.

Role of (Ca²⁺)_i in veratridine-evoked GABA release

Ruthenium red has been widely used to demonstrate the putative role of internal Ca²⁺ [(Ca²⁺)_i] in (Ca²⁺)_o-independent release of neurotransmitters because it inhibits the mitochondria

Ca²⁺ transporter (Cunningham and Neal, 1981; Schoffelemeier and Mulder, 1983). However, according to the multiple actions of ruthenium red (Baux et al., 1979), one has to be careful before attributing ruthenium red potentiation of the veratridine effect (data not shown; see Cunningham and Neal, 1981) to a facilitation of Ca²⁺ release from internal stores.

In order to test the possible role of (Ca²⁺)_i in veratridine-evoked GABA release, neuronal (Ca²⁺)_i stores were labeled with ⁴⁵Ca²⁺. Neurons, exposed to 1 μCi ⁴⁵Ca²⁺, rapidly accumulated ⁴⁵Ca²⁺, and a steady state was reached after 15–20 hr. After 28 hr, neurons rapidly lost their ⁴⁵Ca²⁺ content when the culture dishes were washed with a medium devoid of added Ca²⁺ and containing 1 mM EGTA (Fig. 2*a*). After a 2 hr wash, ⁴⁵Ca²⁺ remaining in the cells was only 7.4 ± 1.6% (*n* = 5) of the total accumulated ⁴⁵Ca²⁺, indicating an important loss of the neuronal (Ca²⁺)_i content. Under these conditions, we found that veratridine still stimulated GABA release (Fig. 2, *b*, *c*), the drug effect being 82.2 ± 7.9% of control (*n* = 5). Since basal release was also diminished (64.3 ± 4.9% of the control, *n* = 5), the inhibition of the veratridine effect was probably due to a loss in neuronal GABA content or to neuronal death induced by EGTA rather than to the massive decrease in (Ca²⁺)_i content observed. (Ca²⁺)_o-independent GABA release appears therefore to be largely independent of neuronal (Ca²⁺)_i content.

Inhibition of GABA release by TnTx

It was recently demonstrated that TnTx blocked the exocytotic process when injected into chromaffin cells (Penner et al., 1986). When striatal neurons were first exposed to TnTx (10 μg/well) for 24 hr, no modification of the basal or glutamate-evoked GABA releases was observed, either in the presence or absence of (Ca²⁺)_o (Fig. 3). The veratridine-stimulated release was only slightly inhibited when experiments were performed in the presence of (Ca²⁺)_o (Fig. 3*a*), but no inhibition was detectable in the absence of (Ca²⁺)_o (Fig. 3*b*). In contrast, TnTx treatment of the neurons resulted in a 75% inhibition of the K⁺-evoked GABA release measured in the presence of (Ca²⁺)_o (Fig. 3*a*), an inhibition equal to that obtained by the removal of (Ca²⁺)_o (Fig. 3*b*). Moreover, the K⁺ effect measured in the absence of (Ca²⁺)_o was not sensitive to the toxin (Fig. 3*b*). These results demonstrate that TnTx inhibited only the (Ca²⁺)_o-dependent part of the K⁺-evoked GABA release.

Effect of nipecotic acid on GABA release

It has been proposed that (Ca²⁺)_o-independent GABA release could be mediated by the GABA uptake system (Schwartz, 1982;

Table 1. Ca²⁺-dependency of GABA release elicited under various stimulatory conditions

Condition	GABA release (nmol/3 min/well)		<i>t</i> test
	1.1 mM Ca ²⁺	0 mM Ca ²⁺ (1 mM EGTA)	
Control	0.107 ± 0.007 (9)	0.159 ± 0.012 (10)	<i>p</i> < 0.05 ^a
K ⁺ (56 mM)	1.666 ± 0.031 (6)	0.649 ± 0.053 (4)	<i>p</i> < 0.001 ^a
Veratridine (20 mM)	4.001 ± 0.193 (7)	4.345 ± 0.198 (6)	NS
Ouabain (3 mM)	0.763 ± 0.042 (3)	1.176 ± 0.093 (3)	<i>p</i> < 0.02 ^a
Monensin (20 μM)	0.709 ± 0.018 (3)	0.971 ± 0.037 (3)	<i>p</i> < 0.005 ^a
Glutamate (100 μM)	0.707 ± 0.067 (6)	0.769 ± 0.066 (5)	NS

Endogenous GABA release from striatal neurons was determined as described under Material and Methods. Results are means ± SEM of (*n*) independent experiments. Superscript ^a indicates a significant increase and ^b indicates a significant decrease in the GABA release value in the absence of Ca²⁺ compared with the corresponding GABA release measured in the presence of Ca²⁺. All evoked GABA release values are significantly (*p* < 0.001) higher than basal release.

Figure 2. Role of $(\text{Ca}^{2+})_i$ in veratridine stimulation of GABA release from striatal neurons. *a*, $^{45}\text{Ca}^{2+}$ accumulation and release from striatal neurons. At time 0, $1\mu\text{Ci } ^{45}\text{Ca}^{2+}$ was added to the culture medium after 15 DIV. After 28 hr, striatal neurons were washed 3 times with Krebs Ringer devoid of added Ca^{2+} and containing 1 mM EGTA. At the indicated times, $^{45}\text{Ca}^{2+}$ present in the neurons was determined as described in the text. *b*, The hatched bar represents the basal release measured just before the veratridine stimulation and the open bar represents GABA release evoked by $20\mu\text{M}$ veratridine in the presence of Ca^{2+} . *c*, Same experiment as in *b* performed in the absence of Ca^{2+} and on neurons that were first exposed for 2 hr to a medium (2 ml) devoid of Ca^{2+} and containing 1 mM EGTA. Results are means $\pm$ SEM of triplicate determinations from typical experiments. Those experiments were repeated 5 times with similar results (see text).

Yazulla and Kleinschmidt, 1983). We therefore tested the effects on GABA release of various experimental conditions under which GABA uptake is inhibited.

Nipecotic acid is a classical competitive inhibitor of the GABA uptake system (Krogsgaard-Larsen, 1980; Kanner et al., 1983). In striatal neurons, 1 mM nipecotic acid totally blocked ^3H -GABA uptake (data not shown), and a rapid accumulation of ^3H -nipecotic acid (1 mM) was observed (Fig. 4*a*). In the presence of 1 mM nipecotic acid, basal release of endogenous GABA was rapidly increased (Fig. 4, *b*, *c*), as expected according to the inhibition of the GABA uptake system and to the heteroexchange process (Kanner et al., 1983).

Veratridine-evoked GABA release was inhibited as a function of the preincubation time of the neurons in a medium containing 1 mM nipecotic acid (Fig. 4*b*). This high concentration of nipecotic acid was chosen in order to allow a massive and rapid accumulation of this GABA uptake inhibitor by striatal neurons (Fig. 4*a*). The nipecotic acid inhibition of the veratridine effect was observed in both the absence and presence of $(\text{Ca}^{2+})_o$ (data not shown) and was almost complete when striatal neurons accumulated this inhibitor for 22 min. The glutamate response was also inhibited by nipecotic acid (data not shown), but, in contrast, K^+ depolarization still induced GABA release under these conditions (Fig. 4*c*).

The nipecotic acid inhibition was not due to a decrease in neuronal GABA content, since $90.9 \pm 5.5\%$ ($n = 5$) of the control amount of GABA remained in striatal neurons after a 22 min preincubation period in a 1 mM nipecotic acid containing medium (the control amount corresponded to that determined after a 22 min preincubation period in a control medium devoid of nipecotic acid).

Since nipecotic acid has a 10-fold lower affinity for the GABA carrier than GABA itself, one may expect that the intracellular concentration of nipecotic acid has to be at least 10-fold higher than that of GABA to inhibit the carrier-mediated GABA release process. After 25 min accumulation, we found that the ^3H -nipecotic acid content in the cells was 3.96 ± 0.07 -fold higher ($n = 9$) than the GABA content. This result is probably

an underestimation of the relative concentration of nipecotic acid and GABA on the cytoplasmic side of the carrier since GABA is known to be distributed among different compartments (Szerb, 1979, 1982, 1983a; Szerb et al., 1981; Abe and Matsuda, 1983).

These results strongly suggest that internal nipecotic acid preferentially inhibits veratridine-evoked GABA release.

Effect of Li^+ on GABA release

Choline and Li^+ are not able to replace Na^+ on the carrier molecule, and therefore, replacement of Na^+ by choline or Li^+ resulted in total inhibition of ^3H -GABA uptake in striatal neurons (data not shown). Whereas choline blocks voltage-sensitive Na^+ channels, the small cation Li^+ can pass through the open channel. Therefore, veratridine still depolarizes the cell when Na^+ was replaced by Li^+ . As shown in Figure 5*a*, the basal GABA release was enhanced in a low- Na^+ , high- Li^+ medium, and the veratridine-evoked GABA release was inhibited rapidly, as a function of the preincubation time. The inhibition was total after a 15 min preincubation period of the neurons in a Li^+ -enriched medium (Fig. 5*a*). This effect was not due to a decrease in the GABA content since (1) $88.1 \pm 7.0\%$ ($n = 4$) of the control intracellular amount of GABA remained in the neurons after a 22 min preincubation period in Li^+ -containing medium and (2) K^+ -evoked GABA release was not blocked by Li^+ (Fig. 5*b*). The veratridine effect is therefore dependent on Na^+ influx and is not a consequence of neuronal depolarization only.

Role of Cl^- in GABA release

The GABA carrier requires not only Na^+ but also Cl^- for net influx of GABA (Kanner et al., 1983). In striatal neurons, ^3H -GABA uptake was inhibited when Cl^- ions were replaced by the impermeant ions SO_4^{2-} (data not shown). When neurons were exposed, from the beginning of the experiment, to a medium containing SO_4^{2-} instead of Cl^- , the basal GABA release was increased, and, whereas the 56 mM K^+ -evoked GABA release was not affected, the veratridine effect was largely inhibited (Fig. 6).

Figure 3. Effect of TnTx on basal and evoked GABA release. Endogenous GABA release was measured during a 3 min exposition of striatal neurons to normal medium (basal) or medium containing 56 mM K⁺, 10 μ M veratridine (*Ver.*) or 100 μ M Glu. The hatched bars represent the results obtained with striatal neurons exposed to TnTx (10 μ g/well) for 24 hr before the experiment. Experiments were performed either in the presence (a) or absence (1 mM EGTA added) (b) of Ca²⁺. Results are means $\pm$ SEM of 4 independent experiments performed in triplicate. Statistical analysis (Student *t* test) revealed a significant difference from the respective control values with * *p* < 0.05, ** *p* < 0.001.

Role of GABA-metabolizing enzymes

The glutamic acid decarboxylase (GAD) activity seems to be increased during cell depolarization (Gold and Roth, 1979; Szerb, 1982; Giorgi and Meek, 1984). Moreover, it has been proposed that GABA produced by a subpopulation of GAD could be immediately released into the synaptic cleft (Tapia, 1983). Therefore, various inhibitors of the GABA-metabolizing enzymes GAD and GABA-transaminase (GABA-T) were tested for their effects on GABA release. When applied 9 min prior to stimulation, the GABA-T inhibitor aminooxyacetic acid (100 μ M) and two GAD inhibitors, allylglycine (100 μ M) or thiosemicarbazide (1 mM) were without effect either on basal release or on GABA release stimulated by K⁺, veratridine, or glutamate (data not shown).

Moreover, when total GABA present in the dish (in the neurons and in the medium) was determined after a 3 min stimulation period, no change could be detected between normal condition and K⁺ or veratridine depolarization (Table 2). Therefore, GABA release from striatal neurons was not due to an increase in GAD activity or a decrease in GABA-T activity.

Role of second-messenger systems

Second messengers cAMP, inositol phosphate, and/or diacylglycerol have been shown to increase neurotransmitter release (Mulder and Schoffeleer, 1985; Zurgil and Zisapel, 1985). We found that K⁺ (56 mM) and veratridine (20 μ M) did not signif-

Figure 4. Effect of nipecotic acid on GABA release. *a*, [³H]-nipecotic acid accumulation into striatal neurons. Neurons were washed 3 times and then exposed to 1 μ Ci [³H]-nipecotic acid (2, 1 ml). Tritium accumulated in the cells was determined at the indicated times. *b*, Basal (■) and 20 μ M veratridine-evoked (●) GABA releases were determined in the absence of Ca²⁺ (1 mM EGTA) and after exposure of the neurons to 1 mM nipecotic acid for the indicated time. Similar results were obtained in the presence of Ca²⁺ (data not shown). *c*, Basal (■) and 56 mM K⁺-evoked (●) GABA releases were determined in the presence of Ca²⁺ after exposure of the neurons to 1 mM nipecotic acid for the indicated time. In *b* and *c*, results are expressed as the percentage of the veratridine or K⁺ effect measured under control (C) conditions. Results are means $\pm$ SEM of 3–5 experiments performed in triplicate.

icantly stimulate cAMP formation, whereas vasoactive intestinal polypeptide and forskolin, which did not stimulate a massive release of GABA, induced a large increase in cAMP formation (data not shown).

Only a very small increase in the production of phosphatidylinositides was induced by veratridine. However, in contrast to the GABA release response, no effect of veratridine on inositol phosphate formation was measured in the absence of Ca²⁺ (1 mM EGTA was added in the medium) (data not shown).

These results suggest that (Ca²⁺)_o-independent GABA release is not due to the increase in activity of adenylate cyclase or phospholipase C.

Table 2. Effect of K⁺ and veratridine depolarizations on the GABA level in the culture dish

Condition	GABA (nmol/well)		
	Medium	Neurons	Total
Control	0.12 $\pm$ 0.01	13.39 $\pm$ 1.02	13.53 $\pm$ 1.01
K ⁺ 56 mM	1.16 $\pm$ 0.12	13.52 $\pm$ 0.67	14.69 $\pm$ 0.60
Veratridine	4.12 $\pm$ 0.30	8.28 $\pm$ 0.33	12.38 $\pm$ 0.50
Veratridine without Ca ²⁺	3.75 $\pm$ 0.33	8.22 $\pm$ 1.07	11.91 $\pm$ 0.84

Experiments were conducted as for the release experiments. After the 3 min stimulation period, GABA content in the medium and in the neurons was determined as described in Materials and Methods. Results are means $\pm$ SEM of 4 independent experiments performed in triplicate.

Figure 5. Effects of Li^+ on GABA release from striatal neurons. *a*, Basal (■) and 20 μM veratridine-evoked (●) GABA releases were determined in the absence of Ca^{2+} (1 mM EGTA) and after exposure of the neurons to a medium containing Li^+ instead of Na^+ for the indicated time. *b*, Basal (■) and 56 mM K^+ -evoked (●) GABA releases were determined in the presence of Ca^{2+} after exposure of the neurons to a medium containing Li^+ instead of Na^+ for the indicated time. Results are expressed as the percentage of the veratridine or K^+ effect measured under control (C) conditions and are means $\pm$ SEM of 3–5 experiments performed in triplicate.

Figure 6. Role of Cl^- in GABA release. Normal medium or a medium containing SO_4^{2-} instead of Cl^- was used from the beginning of the experiment. Experiments with veratridine were performed in the absence of Ca^{2+} (1 mM EGTA), whereas K^+ experiments were performed in the presence of Ca^{2+} . The hatched bars represent the basal release values determined just before stimulation with 10 μM veratridine or 56 mM K^+ (open bars). The asterisk (*) indicates a significant increase of the basal release in the absence of Cl^- ($p < 0.05$), and the triple asterisk (***) indicates a significant inhibition of the veratridine effect ($p < 0.001$) in the absence of Cl^- .

GABA release from striatal glial cells

In order to determine if Ca^{2+} -independent GABA release originated from the few glial cells present in neuronal cultures, the endogenous GABA content of, and GABA release from, glial cells developed in primary culture was examined.

Glial cells in primary culture were confluent after 21 DIV. At this developmental stage, no neurons could be observed with a phase-contrast microscope (data not shown), and glial cells appear as large, flattened, and epithelioid cells (data not shown).

Although these glial cells in primary culture were not immunostained with a GAD antibody (from Dr Tappaz, France; data not shown), they synthesize GABA (2.2 pmol/ μg protein). This GABA content was, however, 20-fold lower than that determined in neuronal cultures (37.9 pmol/ μg protein). Release of endogenous GABA from these glial cells could not, however, be elicited with either K^+ or veratridine (data not shown).

Discussion

The release of GABA has been widely studied in numerous models and using various types of stimulation (see Szerb, 1983b). It very soon appeared that the release of GABA behaved unlike that of other classical neurotransmitters such as ACh or nor-adrenaline since $(\text{Ca}^{2+})_o$ -independent GABA release could still be elicited under particular stimulatory conditions (Srinivasan et al., 1969; Benjamin and Quastel, 1972). The aim of the present work was to further examine the mechanisms leading to $(\text{Ca}^{2+})_o$ -dependent and independent GABA releases from striatal neurons differentiated in primary culture. We reported here that the $(\text{Ca}^{2+})_o$ -dependent part of the K^+ -evoked GABA release was sensitive to TnTx and was not (or slightly) affected by inhibitors of the GABA uptake system. Veratridine-stimulated, as well as excitatory amino acids-stimulated, GABA release was inhibited neither in the absence of external Ca^{2+} nor when the

internal Ca^{2+} content was reduced by more than 90%. This Ca^{2+} -independent GABA release was not sensitive to TnTx and was inhibited under various conditions known to prevent the functioning of the GABA uptake system. For 3 reasons the GABA release measured here is likely to originate from neurons and not from glial cells: (1) Only 7% of the cells were identified as astrocytes (Bockaert et al., 1986), (2) neither K^+ nor veratridine stimulated endogenous GABA release from striatal glial cells in primary culture, and (3) the GABA content of glial cells developed in primary culture is 20-fold lower than that determined from enriched neuronal cultures. Taken together, these results clearly indicate that 2 different mechanisms trigger GABA release from striatal neurons (see Fig. 7).

Ca^{2+} -dependent GABA release

We previously demonstrated that the Ca^{2+} -dependent GABA release, which represents about 75% of the K^+ effect, was probably due to the opening of various types of voltage-sensitive Ca^{2+} channels (Pin and Bockaert, 1987). Moreover, the Ca^{2+} -dependent GABA release became important after the 11th day *in vitro*, i.e., after the appearance of the synaptic vesicles (Pin et al., 1986; Weiss et al., 1986). Here we reported that the Ca^{2+} -dependent release was totally blocked by TnTx treatment of the neurons, in agreement with results reported by others (Collingridge et al., 1980; Pearce et al., 1983). The exact mechanism of action of TnTx remains unknown, but it is believed that TnTx binds to a cell membrane receptor and is then translocated/transported inside the cell (Parton et al., 1987) to induce modification of the intracellular target. Whatever the exact action of TnTx, the intracellular injection of this toxin into chromaffin cells clearly blocks the exocytotic process (Penner et al., 1986). One may therefore propose that the Ca^{2+} -dependent GABA release is due to the exocytosis of the synaptic vesicles present in the nerve terminals of striatal neurons (Bockaert et al., 1986; Weiss et al., 1986) (see type I, Fig. 7).

Figure 7. Schematic representation of the 2 types of GABA release mechanisms. The first mechanism of GABA release (Type 1) is Ca^{2+} -dependent and sensitive to TnTx, and is mainly stimulated by high- K^+ depolarization. The second GABA release process (Type 2) is Ca^{2+} -independent, not sensitive to TnTx, and mainly stimulated by EAA or veratridine under our experimental conditions.

The Ca^{2+} -independent GABA release mechanism

As discussed above, the mechanism leading to the Ca^{2+} -independent GABA release is different from the Ca^{2+} -dependent process, i.e., probably the exocytosis of synaptic vesicles, since it is independent of the intracellular Ca^{2+} content and is insensitive to TnTx. Interestingly, Ca^{2+} -independent GABA release was observed from cells that do not seem to contain synaptic vesicles (Schwartz, 1982; Yazulla and Kleinschmidt, 1983). Moreover, many authors have reported that GABA, released in a Ca^{2+} -independent manner, originated from a pool different from that giving rise to the Ca^{2+} -dependent release (Szerb, 1979, 1982, 1983a; Szerb et al., 1981; Abe and Matsuda, 1983).

The Ca^{2+} -independent GABA release is stimulated by various drugs known to increase intracellular Na^+ concentration through distinct mechanisms, i.e. veratridine, ouabain, monensin, and excitatory amino acid agonists. An increase in the intracellular Na^+ concentration may therefore play an important role in this particular GABA-release process, as previously reported by others (Sandoval, 1980; Cunningham et al., 1988).

We demonstrate here that the Ca^{2+} -independent GABA release may result from the reversal of the GABA uptake system (see type II, Fig. 7) as reported by Schwartz (1982, 1987), Yazulla and Kleinschmidt (1983), and Kato et al. (1985) on retinal horizontal cells. This conclusion is supported by the following results: (1) nipecotic acid, a competitive inhibitor of the GABA uptake system, inhibited veratridine-evoked GABA release as a function of its intracellular concentration; (2) replacement, from the beginning of the experiment, of Na^+ by Li^+ , or Cl^- by SO_4^{2-} in the medium resulted in an inhibition of the Ca^{2+} -independent GABA release, as well as of the ^3H -GABA uptake. Moreover, under all of these experimental conditions, the Ca^{2+} -dependent GABA release elicited by K^+ depolarization was slightly affected if at all.

It has been demonstrated that the GABA influx is driven by the inward gradients of Na^+ and Cl^- and is electrogenic since 2 Na^+ and one Cl^- ion are transported per GABA molecule (Kanner et al., 1983). The GABA carrier is totally reversible and may therefore serve to regulate the transmembrane GABA gradient $[(\text{GABA})_i/(\text{GABA})_o]$. According to this model of the GABA carrier functioning, the decrease in Na^+ gradient, as well as the depolarization of the plasma membrane induced by veratridine, ouabain, or monensin, may increase $(\text{GABA})_o$ concentration. Such a model also explains the Ca^{2+} -independent and TnTx-insensitive part of the K^+ -evoked GABA release. The increase in external K^+ concentration induced cell depolarization and is accompanied by a decrease in the Na^+ gradient

because Na^+ concentration in the medium was diminished in order to keep the osmolarity constant. However, the Ca^{2+} -independent component of the K^+ -evoked GABA release was very low compared with the veratridine effect. One possible explanation is that K^+ depolarization does not induce massive Na^+ influx because of the rapid inactivation of the voltage-sensitive Na^+ channels (Patlak and Horn, 1982). This latter hypothesis agrees with the absence of effect of TTx on K^+ -evoked GABA release (Pin and Bockaert, 1987).

Excitatory amino acid-evoked GABA release

We previously demonstrated that the activation of the NMDA (G_1) (Pin et al., 1988) and non-NMDA (quisqualate/kainate; the G_2 receptor according to O'Brien and Fischbach, 1986a) (J.-P. Pin, unpublished observations) receptor stimulated GABA release from striatal neurons developed in primary culture. Here we report that, under our experimental conditions, glutamate as well as the 3 classical excitatory amino acid agonists—NMDA, quisqualate, and kainate—mainly stimulated carrier-mediated GABA release (see Fig. 7) since this excitatory amino acid response was Ca^{2+} independent, was not sensitive to TnTx, and was inhibited by nipecotic acid. A similar conclusion has been reported by Yazulla (1983) and Kato et al. (1985) on retinal horizontal cells, which do not seem to contain synaptic vesicles. Our results extend this hypothesis concerning the GABA release mechanism stimulated by excitatory amino acids to central neurons that contain synaptic vesicles and that are also able to release GABA in a Ca^{2+} -dependent manner.

NMDA (G_1) and non-NMDA (G_2) receptors are associated to nonselective cationic channels (Watkins and Evans, 1981; O'Brien and Fischbach, 1986a; Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987) and their activation-induced nerve cell depolarization, as well as a large decrease in the transmembrane Na^+ gradient (Pumain et al., 1986), 2 conditions that favored the reversal of the GABA uptake system. However, excitatory amino acids also stimulate Ca^{2+} influx through voltage-sensitive Ca^{2+} channels and NMDA receptor-activated channels (Pumain and Heinemann, 1985; McDermott et al., 1986). Since excitatory amino acids mainly stimulated carrier-mediated Ca^{2+} -independent GABA release, one may conclude that the increase in $(\text{Ca}^{2+})_i$ induced by glutamate was not able to allow the exocytotic process. One possible explanation is that the excitatory amino acid receptors are mainly located far from the nerve terminals, i.e., on the soma and/or dendrites (O'Brien and Fischbach, 1986b). According to this hypothesis, GABA release stimulated by excitatory amino acid may be mediated by GABA carrier molecules located on the soma or dendrites of the GABAergic cells. Interestingly, dendritic GABA release has already been reported (Jaffe and Cuellar, 1980; Jahr and Nicoll, 1980).

Possible physiological role of the Ca^{2+} -independent GABA release

According to the proposed mechanism of the Ca^{2+} -independent GABA release, it is improbable that this release process is involved in fast inhibitory synapses. However, various arguments are in favor of a possible physiological relevance of the Ca^{2+} -independent GABA release mechanism stimulated by excitatory amino acids. First, an important cytoplasmic pool of GABA is present in neurons as indicated by the immunostaining of cytoplasm with a GABA antiserum (Ottersen and Storm-Mathisen, 1987). Second, this type of release mechanism leading to a rapid and massive release of neurotransmitter seems unique

to GABA. Dopamine or substance P release elicited by veratridine (Daguet et al., 1980; Jessel, 1978), and excitatory amino acid-evoked ACh or glutamate release (Lehmann and Scatton, 1984; Drejer et al., 1986) is $(\text{Ca}^{2+})_o$ -dependent. Even the release of another amino acid neurotransmitter such as glutamate from cerebellar granule cells is mainly TTx-sensitive and $(\text{Ca}^{2+})_o$ -dependent when stimulated by veratridine (J.-P. Pin, unpublished observations). Third, the rapid and great decrease in the Na^+ gradient required to reverse the GABA carrier can be triggered by excitatory amino acid neurotransmitters *in vivo* as measured with specific microelectrodes (Pumain et al., 1986).

Is the increase in the extracellular GABA concentration due to the reversal of the GABA uptake system sufficient to activate GABA receptors *in vivo*? This question must undoubtedly be answered in future experiments before a physiological role can be attributed to this particular GABA release mechanism. Interestingly, recent data indicate that GABA, released through the reversal of the uptake system could activate GABA receptors, at least in the toad retina (Schwartz, 1987), where neurotransmission can be measured even when Ca^{2+} was replaced by Co^{2+} , an inorganic Ca^{2+} channel blocker (Schwartz, 1986).

Since GABA can inhibit either glutamate release (Meier et al., 1984) or glutamate-induced excitation (Dingledine, 1986), we propose that this excitatory amino acid effect, which could rapidly increase the extracellular GABA concentration in the vicinity of the glutamate-activated receptors, may be involved in the regulation of glutamatergic synapses directed to GABAergic neurons. Such a regulatory mechanism may be particularly important to prevent the excess glutamatergic excitation involved in many brain diseases. Further *in vivo* experiments are needed to determine the extent of this speculative hypothesis, however.

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