

## **Potassium-stimulated GABA release is a chloride-dependent but sodium- and calcium-independent process in cultured astrocytes**

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**Abstract.** Depolarization of cultured astrocytes by KCl stimulated  $\gamma$ -aminobutyric acid (GABA) release in a dose-dependent manner. At 60 mM KCl, the stimulatory effect was calcium- and sodium- independent, and was not altered by the presence of  $\beta$ -alanine. The potassium-evoked GABA release was inhibited by furosemide and 4-acetamido-4'-isothiocyano-2,2'-stilbene disulfonic acid (SITS), blockers of the chloride transporter across the plasma membrane, as well by chloride ion replacement with glucuronate. Other depolarizing agents, such as veratridine and ouabain, decreased basal GABA release; ouabain also inhibited the stimulatory effect of 60 mM KCl. The high  $K^+$ -induced GABA release may affect CNS excitability and may represent an important aspect of glial-neuronal interactions.

**Key words:** calcium, chloride, cortical astrocytes, depolarizing agents, GABA release, sodium

## INTRODUCTION

Release of neurotransmitter or neuromodulatory amino acids is one of the ways by which astrocytes react to high, depolarizing concentrations of extracellular potassium ( $K^+$ ). High  $K^+$ -evoked release in astrocytic preparations has been characterized in considerable detail with regard to the neuromodulator taurine, and special attention has been paid to the role of calcium ( $Ca^{2+}$ ) and chloride ( $Cl^-$ ) ions in taurine release (Philibert et al. 1988, 1989, Pasantes-Morales and Schousboe 1989, Martin et al. 1990). By contrast, there is a controversy as to the release of the inhibitory amino acid GABA. Studies with mixed glial-neuronal preparations derived from different regions of the CNS (Sarthy 1983) or PNS (Bowery et al. 1979), and with bulk-isolated cerebral astrocytes (Sellstrom and Hamberger 1977, Albrecht and Rafałowska 1987) have indicated that glial cells release GABA upon treatment with high concentrations of potassium chloride (KCl). However, such a release was not observed in cultured rat cerebellar (Pearce et al. 1981), and mouse cerebral cortical astrocytes (Larsson et al. 1983). In the present study, the effect of increasing the concentration of KCl in the medium on GABA release was investigated in cultured astrocytes from neonatal rat cerebral cortex. The dependence of the high  $K^+$ -stimulated GABA release on  $Ca^{2+}$  ions and the involvement of  $Cl^-$  and sodium ( $Na^+$ ) were investigated.

## METHODS

### Astrocytic culture

Neonatal rat cortical astrocytes were grown as previously described in the presence of dibutyl-AMP (Gregorios et al. 1985). Based on immunocytochemical analysis, astrocytes accounted for at least 95% of the cell population (Gregorios et al. 1985). Cultures ranging in age from 3.5-6 weeks were used for experiments.

### Loading and release of GABA

GABA loading and release was carried out as described by Gallo et al. (Gallo et al. 1989). Cells in 35 mm dishes were preincubated for 15 min at 37°C in 1 ml Krebs-Ringer medium (140 mM NaCl; 3 mM KCl; 0.6 mM  $MgCl_2$ ; 1 mM  $CaCl_2$ ; 10 mM glucose; 50 mM HEPES, pH 7.4), and then incubated in a Krebs-Ringer

medium containing 10  $\mu$ M aminooxyacetic acid, an inhibitor of GABA deamination, with 1  $\mu$ Ci [ $^3H$ ]-GABA (40 Ci/mmol, New England Nuclear), for 16 min at 37°C, in a rotating water bath. The cultures were then washed 3 times for 3 min and 5 times for 5 min each with 1 ml of the medium without radiolabelled GABA. The subsequent 9 washes were collected for monitoring radioactive GABA. The cells were treated with various concentrations of KCl as indicated in the legends, between the 3rd and 4th medium change (cf. Fig. 1). To maintain isotonicity, when the KCl concentration in the stimulation medium was increased, NaCl concentration was equivalently reduced. At the end of the release the cells were solubilized in 1 ml of 1 N NaOH. Aliquots of the collected medium and of the NaOH extracts were used for radioactivity measurements.

### Calculations

To illustrate the kinetics of  $K^+$ -stimulated GABA release (Fig. 1), the release data collected during 5 min wash periods were expressed as fractional release (f.r.) i.e., percentage of the radioactivity lost from the cells, referred to as the total radioactivity present in the cells at time of collecting the fraction. All the results except those of Fig. 1 were expressed as % basal release, which is equal to: f.r. during stimulation/f.r. before stimulation  $\times$  100.

Statistical analysis was performed with the two-tailed Students' *t*-test.

## RESULTS

$K^+$ -stimulated GABA release from astrocytes in a dose-dependent manner, in the range of 7.5-60 mM (Fig. 1). There were variations in the maximum stimulation attained at 60 mM KCl (note different mean values in Table I and Figs. 2 and 3), but the dose-dependence of the effect was evident throughout. As measured at 60 mM KCl, the stimulatory effect was  $Ca^{2+}$ -independent (Table I). The stimulation by KCl was abolished when  $Cl^-$  in the medium was replaced by a nonpermeable ion glucuronate, and the release was decreased to a level slightly below basal in the presence of either of the two different  $Cl^-$  transport blockers: furosemide and SITS, both at concentration of 1 mM (Fig. 2). Addition of 100  $\mu$ M  $BaCl_2$  did not inhibit the  $K^+$ -induced GABA release (Fig. 2). The stimulation of GABA release by KCl was not affected by substitution of NaCl in the stimulation

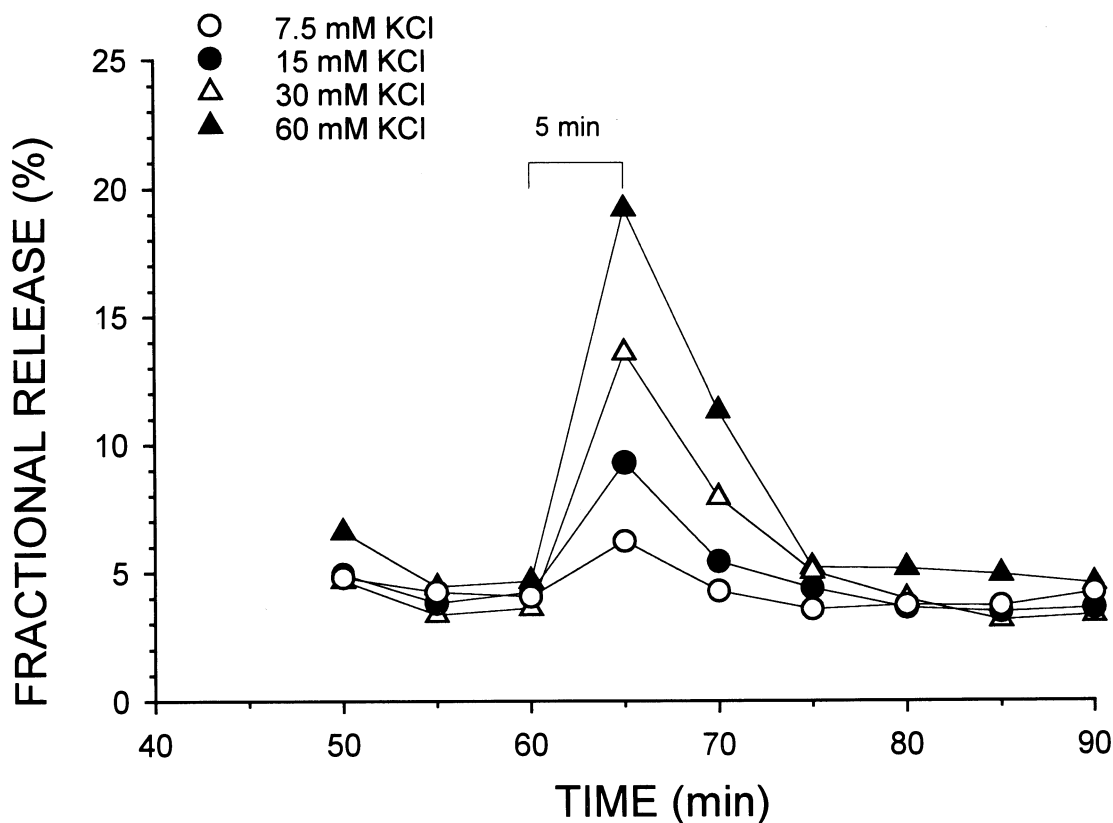


Fig. 1. Dose-dependence of [<sup>3</sup>H]-GABA release from astrocytes at KCl concentrations ranging from 7.5–60 mM. Bracket indicates the time period of KCl stimulation. A representative experiment out of 4 experiments is shown in the Figure.

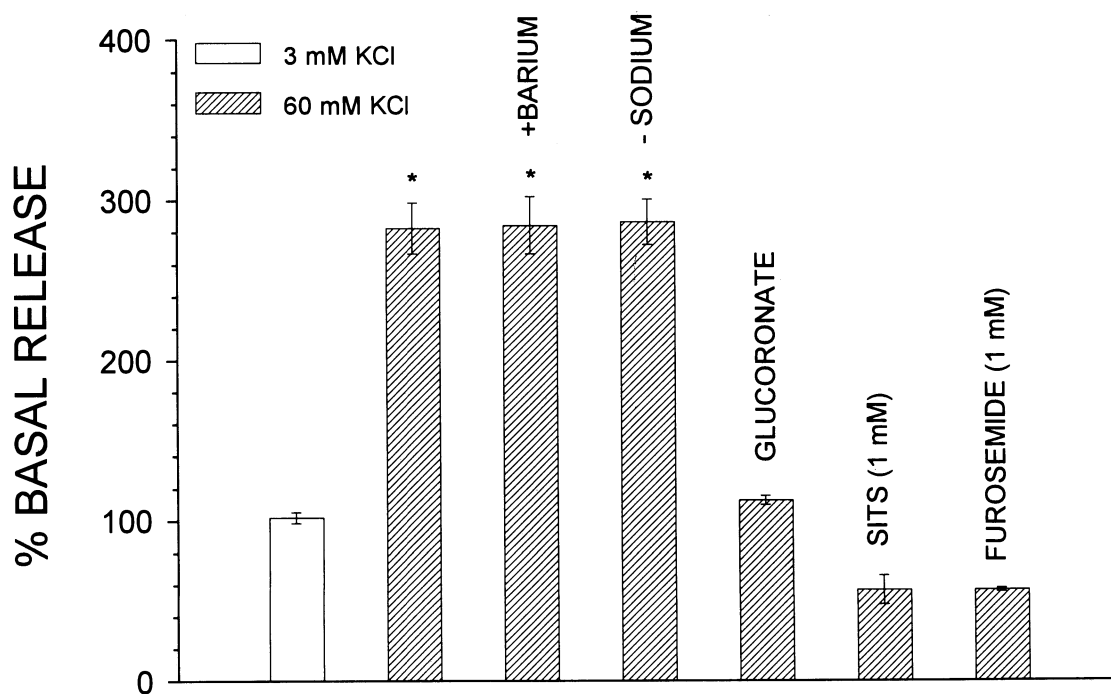


Fig. 2. The basal (3 mM KCl) and K<sup>+</sup>-stimulated (60 mM KCl) GABA release and the effect of 100  $\mu$ M BaCl<sub>2</sub>, equimolar replacement of sodium chloride with choline chloride, replacement of chloride salts with glucuronate, 1 mM SITS and 1 mM furosemide. Results are mean  $\pm$  SEM,  $n = 6$ . \*Significantly different from control. For  $P$  values see Results.

TABLE I

Effect of extracellular  $\text{Ca}^{2+}$  removal and  $\beta$ -alanine on  $\text{K}^{+}$ -stimulated GABA release in astrocytes

| Experimental conditions      | $\text{K}^{+}$ (mM) | $\text{Ca}^{2+}$ (mM) | $\beta$ -Alanine (mM) | % Basal Release   |
|------------------------------|---------------------|-----------------------|-----------------------|-------------------|
| Removal of $\text{Ca}^{2+}$  | 3                   | 1                     | 0                     | $102 \pm 3.5$ (6) |
|                              | 60                  | 1                     | 0                     | $397 \pm 17$ (6)  |
|                              | 60                  | 0                     | 0                     | $346 \pm 23$ (6)  |
| Addition of $\beta$ -alanine | 3                   | 1                     | 0                     | $99 \pm 6$ (5)    |
|                              | 3                   | 1                     | 1                     | $99 \pm 3$ (3)    |
|                              | 60                  | 1                     | 0                     | $372 \pm 23$ (4)  |
|                              | 60                  | 1                     | 1                     | $316 \pm 26$ (4)  |

Results are mean  $\pm$  SEM, with the number of experiments in parentheses. Student's *t*-test showed that there was not statistically significant difference in  $\text{K}^{+}$ -stimulated GABA release in the absence or presence of extracellular  $\text{Ca}^{2+}$  or in the absence or presence of  $\beta$ -alanine.

medium with choline chloride (Fig. 2), and was only insignificantly affected by 1 mM  $\beta$ -alanine, a GABA up-

take inhibitor (Table I). The effect of 60 mM KCl on GABA release was reduced by 32% (the two-tailed *t* test  $P=0.0008$ ) in the presence of 1 mM ouabain (Fig. 3). Ouabain (1 mM) and/or 100  $\mu\text{M}$  veratridine reduced the basal GABA release (Fig. 3). Ouabain inhibited basal GABA release by 23% ( $P=0.0015$ ) and veratridine by 25% ( $P=0.0008$ ), whereas the combination of ouabain and veratridine inhibited basal GABA release by 34% ( $P<0.0001$ ) (see Fig. 3).

## DISCUSSION

The present study clearly shows that in contrast to cultured rat cerebellar astrocytes (Pearce et al. 1981), or mouse cortical astrocytes (Larsson et al. 1983), astrocytes derived from neonatal rat cerebral cortex possess the ability to release GABA upon stimulation with high  $\text{K}^{+}$ . This discrepancy is likely to reflect both regional and species difference, since astroglia-enriched fractions bulk-isolated from adult rat cerebral cortex showed a high  $\text{K}^{+}$ -stimulated release (Sellstrom and Hamberger 1977, Albrecht and Rafałowska 1987). The observed  $\text{Ca}^{2+}$ -independence of GABA release in this study is in agreement with earlier observations in bulk-isolated astrocytes (Sellstrom and Hamberger 1977) and sympath-

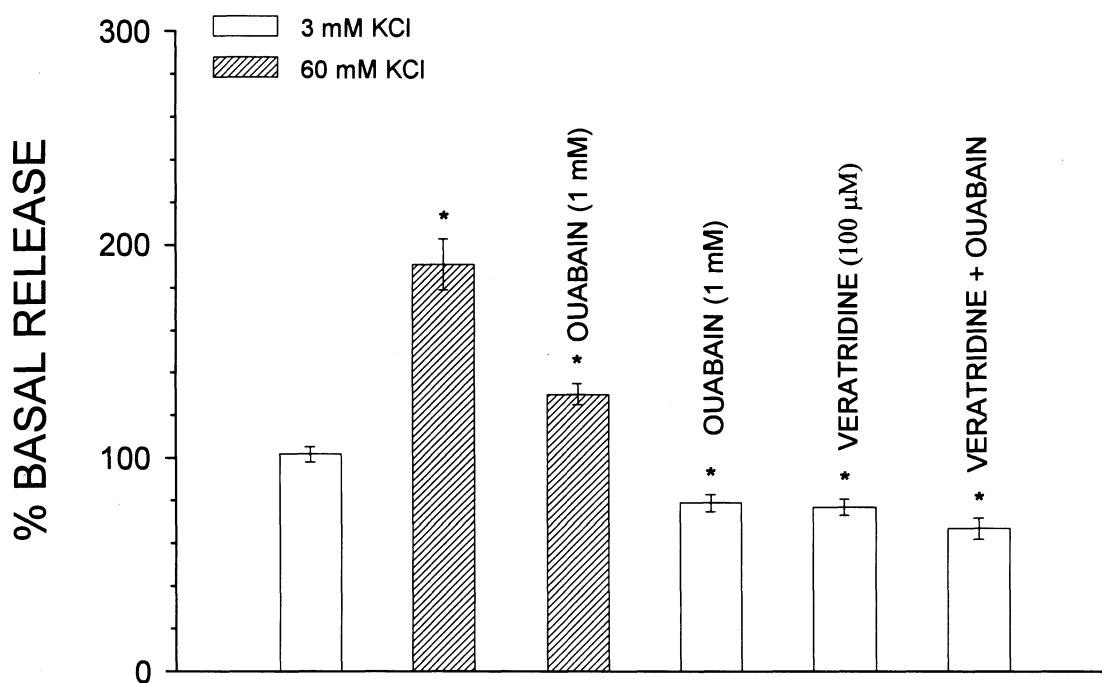


Fig. 3. The effects of depolarizing agents on GABA release. Depolarizing agents, such as KCl (60 mM), ouabain (1 mM), veratridine (100  $\mu\text{M}$ ) and the combination of ouabain and veratridine, and ouabain with 60 mM KCl were tested for their effects on GABA basal release (3 mM KCl). Results are mean  $\pm$  SEM,  $n=6$ . \*Significantly different from control. For *P* values see Results.

etic glial cells (Bowery et al. 1979), but does not apply to all types of glial cells. For instance, Müller glial cells show both the Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent component of GABA release (Sarthy 1983). The controversies concerning the role of Ca<sup>2+</sup> are not confined to GABA release. K<sup>+</sup>-induced taurine release has been demonstrated to be Ca<sup>2+</sup>-independent in mouse cerebral astrocytes (Pasantes-Morales and Schousboe 1989), but Ca<sup>2+</sup>-dependent in rat cerebral (Philibert et al. 1988) and cerebellar astrocytes (Philibert et al. 1989), and Müller glia (Faff-Michalak et al. 1994).

The lack of 60 mM K<sup>+</sup> effect on GABA release when Cl<sup>-</sup> ions were replaced by a nonpermeable glucuronate ions and the total inhibition of the effect by two different Cl<sup>-</sup> transport inhibitors- furosemide and SITS (Fig. 2) demonstrate Cl<sup>-</sup>-dependence of the K<sup>+</sup>-stimulated GABA release. Furosemide inhibits the Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> cotransporter, blocking the coinflux of these ions in astrocytes in primary culture (Kimelberg 1981) and in a glioma cell line (Wolpaw and Martin 1984). The disulfonic stilbene SITS inhibits the HCO<sub>3</sub><sup>-</sup>/Cl<sup>-</sup> exchanger, and Cl<sup>-</sup>/Cl<sup>-</sup> exchange (Kimelberg et al. 1979, Kimelberg 1981, Wolpaw and Martin 1984), but also blocks a Cl<sup>-</sup> channel that becomes activated when the cell membrane is depolarized and is impermeable to glucuronate (Gray and Richie 1986; for a review see Walz 1989).

The independence of K<sup>+</sup>-induced GABA release on the presence of Na<sup>+</sup> in the stimulation medium appears to exclude the contribution of a reversal of Na<sup>+</sup>-driven GABA uptake in the K<sup>+</sup>-stimulated GABA release. The absence of a significant effect of β-alanine on K<sup>+</sup>-stimulated GABA release (Table I) is also consistent with the noninvolvement of Na<sup>+</sup>/GABA cotransport in GABA release. Although β-alanine is now believed to be an agonist of the taurine transporter (Holopainen 1988, Schousboe et al. 1988), earlier studies have demonstrated that it inhibits Na<sup>+</sup>-dependent GABA uptake as well (Schon and Kelly 1975). The lack of Na<sup>+</sup> effect on astrocytic GABA release is, however, further complicated by the observation that veratridine and ouabain did affect basal GABA release and ouabain inhibited K<sup>+</sup>-evoked GABA release. These two compounds, in synaptosomes, stimulate the Ca<sup>2+</sup>-independent GABA release by collapsing the Na<sup>+</sup> electrochemical potential (Sihra and Nicholls 1987). The mechanism by which the two compounds inhibit GABA release in astrocytes is unclear.

The results taken together indicate that K<sup>+</sup>-driven Cl<sup>-</sup> influx into astrocytes and its subsequent efflux are the

prerequisites of KCl-stimulated GABA release from astrocytes. According to Walz and coworkers (Walz and Hertz 1983, Walz and Mukerji 1988), an increase in extracellular K<sup>+</sup> to the level used in the present study causes massive influx of K<sup>+</sup> and Cl<sup>-</sup> into the cells, followed by an outward transport of Cl<sup>-</sup>. It should be noted that Cl<sup>-</sup>-driven GABA efflux has been demonstrated in different CNS cells (Pasantes-Morales et al. 1988; and references therein).

The effects of the different stimulating media on the transmembrane potential of astrocytes have not been measured in the present study. However, earlier studies have demonstrated astrocytic cell membrane depolarization by ouabain (Bowman et al. 1984) and high K<sup>+</sup> in the absence of Cl<sup>-</sup> (Pasantes-Morales and Schousboe 1989), the two conditions that did not stimulate GABA release in the present study. Accordingly, astrocytic cell membrane depolarization *per se* is unlikely to be a factor in the KCl-stimulated GABA release. It is known that depolarization- and Ca<sup>2+</sup>-independence, but Cl<sup>-</sup>-dependence also is a property of taurine release from mouse cortical astrocytes during high K<sup>+</sup>-induced swelling (Pasantes-Morales and Schousboe 1989). Moreover, swelling induced by hypoosmotic solutions was accompanied by taurine release which, like GABA release in the present study, was Na<sup>+</sup>-independent (Pasantes-Morales and Schousboe 1988).

Both the KCl-induced taurine release in mouse astrocytes (Pasantes-Morales and Schousboe 1989) and GABA release in rat astrocytes (Fig. 2) were unaffected by 100 μM BaCl<sub>2</sub>, which blocks the inward rectifier K<sup>+</sup>-channel (Walz et al. 1984). However, barium has a much smaller effect on K<sup>+</sup> fluxes in rat than in mouse astrocytes (Walz and Kimelberg 1985).

The physiological function of high KCl-induced GABA release from cortical astrocytes is uncertain. Taurine release in response to hypotonicity- or high KCl-induced cell swelling has been ascribed an osmoregulatory role (Pasantes-Morales and Schousboe 1988, 1989), although this view has been questioned as the taurine concentration accompanying astrocytic swelling is perhaps too small (Martin et al. 1990). Since GABA concentration in astrocytes cultured *in vitro* is very low (Larsson et al. 1983, Pasantes-Morales and Schousboe 1988), an osmoregulatory role of GABA would appear unlikely. However, K<sup>+</sup>-stimulated GABA release could be of importance in neuronal-astrocytic interactions. The concept that astrocytes *in situ* use GABA as a neuromodulatory signal deserves further attention. It could be

of significance during the states of brain excitability, when  $K^+$  is massively released from neurons into extracellular space; the released GABA could modulate brain excitability.

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