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Effects of Phenylsuccinate on Potassium-Stimulated Taurine Release in Cultured Neurons and Astrocytes and in Rat Hippocampus In Vivo

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Swelling-induced release of taurine was investigated in vivo in hippocampus by microdialysis or in vitro in cultured neocortical neurons or astrocytes. Swelling was induced either by increasing the extracellular K^+ concentration or by exposing the cells to hyposmotic conditions. It was found that the drug phenylsuccinate, which inhibits the mitochondrial dicarboxylate carrier as well as biosynthesis of neurotransmitter glutamate, inhibits swelling-induced taurine release both in vivo and in cultured cells. Thus, phenylsuccinate may be used to investigate the mechanism involved in taurine release associated with regulatory volume decrease in cells. © 1996 Wiley-Liss, Inc.

Key words: hippocampus, neurons, astrocytes, swelling, phenylsuccinate, regulatory volume decrease

INTRODUCTION

Phenylsuccinate was originally described as an inhibitor of the mitochondrial dicarboxylate carrier (Passarella et al., 1987) and based on this observation it was recently introduced as an inhibitor of biosynthesis of neurotransmitter glutamate both in cultured glutamatergic neurons and in rat hippocampus in vivo (Palaiologos et al., 1988, 1989; Christensen et al., 1991). Additionally, phenylsuccinate has been shown to inhibit the biosynthesis of neurotransmitter gamma aminobutyric acid (GABA) (Cobo et al., 1993). Release of neurotransmitter amino acids is induced by depolarization of the tissue either by K^+ concentrations of 25 mM or more or by excitatory amino acids (Belhage et al., 1993; Schousboe et al., 1996). However, exposure of nervous tissue or nerve cells to a high concentration of K^+ not only leads to depolarization but also to cell swelling (Hertz and Schousboe, 1975). Swelling induced by anisosmotic conditions or elevated K^+ concentrations has been shown to be associated with a pronounced release of taurine (Pasantes-Morales and Schousboe, 1988, 1989; Solis et al., 1988; Wade et al., 1988; Kimelberg et al., 1990; Pasantes-Morales and Martin del Rio, 1990; Schousboe et al., 1990) and to a lesser extent release of glutamate and other amino acids including GABA (Pasantes-Morales and Schousboe, 1988; Kimelberg et al., 1990; Schousboe and Pasantes-Morales, 1992). Therefore, the possibility exists that phenylsuccinate might interfere with processes related to cell volume regulation. In order to gain information about this, taurine release, primarily if not exclusively reflecting cell volume changes (Pasantes-Morales and Martin del Rio, 1990), was followed in the absence or presence of phenylsuccinate in cultured neurons and astrocytes exposed to high concentrations of K^+ or hyposmotic conditions (see Pasantes-Morales and Schousboe 1988, 1989; Schousboe and Pasantes-Morales, 1989) as well as in rat hippocampus in vivo using the microdialysis technique (Benveniste et al., 1984) and infusion of K^+ to elicit cell swelling.

MATERIALS AND METHODS

Materials

Pregnant (gestational day 15) or newborn NMRI mice and Wistar rats were obtained from the animal quarters at the Panum Institute, University of Copenhagen. Plastic tissue culture dishes were purchased from NUNC A/S (Roskilde, Denmark) and fetal calf serum

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was from Sera-Lab (Crawley Down, Sussex, UK). Cytosine arabinoside, poly-L-lysine, trypsin, trypsin inhibitor, DNAse, amino acids, vitamins, and insulin were obtained from Sigma (St. Louis, MO). [^{3}H]taurine (specific radioactivity 20.9 Ci/mmol) was purchased from Du Pont Research Products (Frankfurt, Germany). All other chemicals were of the highest purity available from regular commercial sources.

Cell Culture

Cerebral cortical (neocortical) neurons were cultured essentially as described by Hertz et al. (1989a). Astrocytic proliferation was curtailed by addition of the mitotic inhibitor cytosine arabinoside (20 μM) after 48 hr of culture (Dichter, 1978; Larsson et al., 1985). The neurons were routinely cultured for 5–7 days before experiments were performed (for further details see Schousboe et al., 1985; Drejer et al., 1987).

Astrocytes were cultured essentially as described by Hertz et al. (1989b). Prefrontal cortex was taken from newborn mice. The tissue samples were passed through sterile 80 μm pore-size Nitex nylon sieves into a slightly modified Dulbecco's medium containing 20% (vol/vol) fetal calf serum and plated onto poly-L-lysine-coated Petri dishes and culture flasks during the last culture week. The astrocytes were grown for a total of 3 weeks, changing the medium 2 days after inoculation and subsequently twice a week.

Superfusion of Cells

Cultures grown in either 35 mm Petri dishes or 24-well multitest dishes were preloaded for 30 min with [^{3}H]taurine as previously described (Schousboe and Pasantes-Morales, 1989). Subsequently, fractional release (35 mm Petri dishes) was determined in experiments carried out according to the procedure described by Drejer et al. (1987). After loading with labeled taurine, cells were superfused with a 37°C HEPES-buffered saline solution (135 mM NaCl, 5 mM KCl, 0.6 mM $MgSO_4$, 1.7 mM KH_2PO_4, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES), pH 7.4, continuously delivered by a peristaltic pump at a rate of 2 ml/min. After a washing period of 10 min, 1 ml fractions were collected directly into scintillation vials. Results are expressed as fractional release, i.e., the radioactivity released in the 1 ml fractions as a percentage of total radioactivity present in the cells immediately before release. Accumulated radioactivity at the start of the superfusion was calculated by adding the radioactivity in all fractions to that remaining in the cells at the end of the superfusion. The latter was determined in ethanol (70%, vol/vol) extracts of the cells. In another set of experiments (24-well multitest dishes) release was followed during 10 min periods without superfusion. After loading, excess radioactivity was removed by three successive rapid washes, and the cells were further washed during two periods of 10 min each and then with isosmotic medium (spontaneous release) or with media of reduced osmolarity or with an increased K^+ concentration (stimulated release).

Microdialysis

Male Wistar rats weighing 300–350 g were used. Throughout the experiment the animals were intubated and mechanically ventilated, using 0.5–0.75% halothane in nitrous oxide/oxygen (2:1) for anesthetic purposes. The animals were placed in a stereotaxic frame, and bregma located. Bilateral holes were trepanned in the skull corresponding to the locations of the dorsal hippocampal formations, and two microdialysis probes (membrane length: 2 mm, diameter 0.24 mm; CMA Microdialysis, Sweden) were implanted. Stereotaxic coordinates in relation to bregma were −3.8 mm anteroposterior, 2.4 mm mediolateral, and 3.9 mm ventral to the dura surface. By way of two liquid switches, the inlets of the probes were each connected to a microdialysis pump, and the outlets were led to a microfraction collector. Before experimental procedures and dialysate sampling, the dialysis probes were perfused with Krebs-Ringer bicarbonate (KRB) buffer for 1.5 hr (Benveniste and Diemer, 1987). On the right side, 50 mM phenylsuccinate was added to the KRB buffer. At all times, the probes were perfused at a flow rate of 4 μl/min. Based on the in vitro recovery of molecules with similar molecular weight as phenylsuccinate, the extracellular concentration of phenylsuccinate immediately surrounding the dialysis membrane was estimated to be approximately 5 mM (Collin and Ungerstedt, 1988). Compounds containing a succinate moiety have been reported to chelate Ca^{++}. In order to test a possible Ca^{++} chelating effect of phenylsuccinate, the level of free Ca^{++} was measured in KRB buffer with and without 5 mM phenylsuccinate. Using a calcium electrode (Radiometer, Denmark), phenylsuccinate was shown not to alter the level of free Ca^{++} in KRB buffer (results not shown).

The experimental protocol was as follows. First, dialysate samples representing basal levels of taurine were collected on both sides (control and phenylsuccinate) over a period of 20 min (4 samples). Using the liquid switches, 3 consecutive 10 min ON + 20 min OFF perfusions with 50 mM KCl (isosmotic reduction in NaCl) were subsequently performed. Throughout the experiment, the dialysate was fractionated every 5 min, yielding 20 μl/sample. At the end of the experiment, the brains were removed, frozen in isopentane, and stored at −80°C. One or 2 days later, the brains were cut in 20 μm frontal sections on a cryostat (Leitz 1720, Leica, Germany), and stained with hematoxylin-eosine. Histological evaluation verified the correct location of the dialysis

membranes in the hippocampal formations of all experimental animals. All animal experiments were approved by the Danish Animal Experiment Inspectorate.

Dialysate Amino Acid Analysis

The level of taurine in the dialysis samples was measured by high pressure liquid chromatography (HPLC) with fluorimetric detection essentially as described by Lerma et al. (1986). Quantitation was carried out by the external standard procedure from peak areas (Lab Calc., Galactic Industries Corporation). The approximate limit of detection for taurine was 10 nM.

RESULTS

Taurine Release From Hippocampus

On the control side, perfusion of the dialysis probe with 50 mM KCl resulted in distinct increases in the level of taurine. Compared to the basal level (average of the first 4 samples), peak increases of taurine measured 143%, 153%, and 134% for the 1st, 2nd, and 3rd KCl perfusions, respectively. On the side where phenylsuccinate (50 mM) was added to the perfusate, the effect of KCl was completely abolished (Fig. 1).

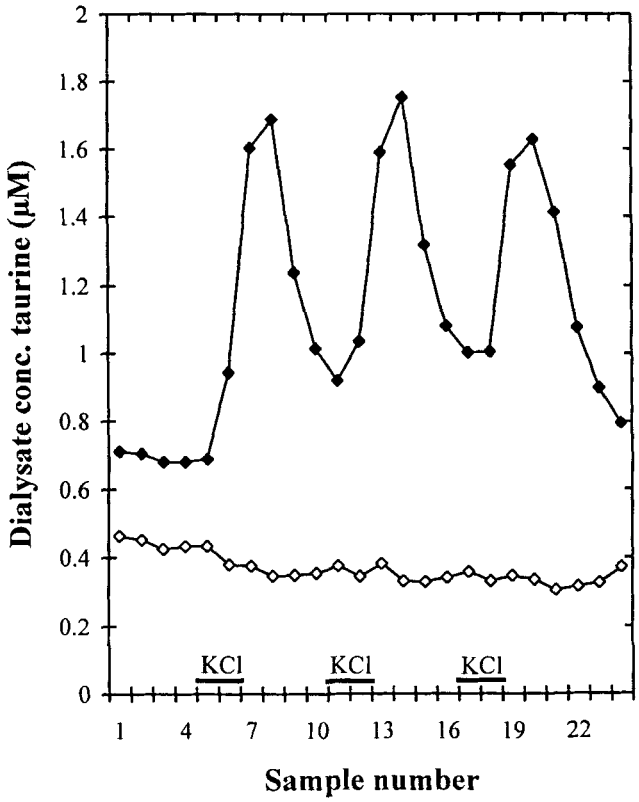

Fig. 1. Effect of phenylsuccinate on in vivo release of taurine induced by KCl. Results were obtained by bilateral microdialysis in the hippocampi of anesthetized rats (N = 5) and represent the means with SEM values less than 24%. KCl (50 mM) was introduced as indicated by the horizontal bars (for further details, see Materials and Methods). Bolus perfusions (10 min/bolus) of the dialysis probes with 50 mM KCl were associated with increases in the level of taurine on the control side (◆) only, whereas the taurine level remained stable on the side containing 50 mM phenylsuccinate (◇). The data were tested statistically using the Wilcoxon matched pairs test by comparing peak increases during KCl perfusion (samples 8, 14, and 20) with the basal level of taurine (mean of the first 4 samples). The K^+-induced increases in taurine overflow in the control side were statistically significantly different ($P < 0.05$) from those on the phenylsuccinate side.

Taurine Release From Cultured Cells

Taurine release from cultured cerebral cortical neurons preloaded with [^{3}H]taurine was monitored continuously in the superfusion system using a superfusion medium with a physiological composition (HBS) or media with added phenylsuccinate (5 mM) or KCl (100 mM isosmolar reduction in NaCl) in the presence or absence of phenylsuccinate (Fig. 2A–C). It can be seen that during superfusion of the neurons in HBS, exposure to 5 mM phenylsuccinate had essentially no effect on the fractional release of taurine which was approximately 0.05%/30 sec (Fig. 2A). Exposure of the neurons to 100 mM KCl under isotonic conditions (Fig. 2B) led to a pronounced increase in taurine release (10-fold) with a slow onset. This K^+-stimulated release of taurine was substantially reduced by the simultaneous exposure to 5 mM phenylsuccinate (Fig. 2C). In order to quantify these effects, release of taurine was studied in a fixed time assay system where released taurine was accumulated for 10 min (Table I). It can be seen that both 100 mM KCl (isotonic) and a hyposmotic medium (50% osmolarity) caused a large increase in taurine release. Under both conditions, addition of 5 mM phenylsuccinate led to a statistically significant reduction in the taurine release. It should be noted that in this experimental paradigm, addition of phenylsuccinate (5 mM) alone had no effect on the taurine release.

Similar experiments were performed on cerebral cortical astrocytes in culture. When superfused in a physiological medium, addition of 5 mM phenylsuccinate had no effect on taurine release (Fig. 3A). Figure 3B shows that exposure of the astrocytes to 100 mM KCl under isosmotic conditions led to a large increase in taurine release from the cells. This K^+-stimulated taurine release could be reduced by simultaneous addition of 5 mM phenylsuccinate in the superfusion medium (Fig. 3C). Table I shows that in experiments utilizing 10 min washout periods, addition of 5 mM phenylsuccinate inhibited both K^+ (100 mM)- and hyposmolarity (50%)-induced taurine release from astrocytes preloaded with [^{3}H]taurine. The inhibitory effect of phenylsuccinate (5

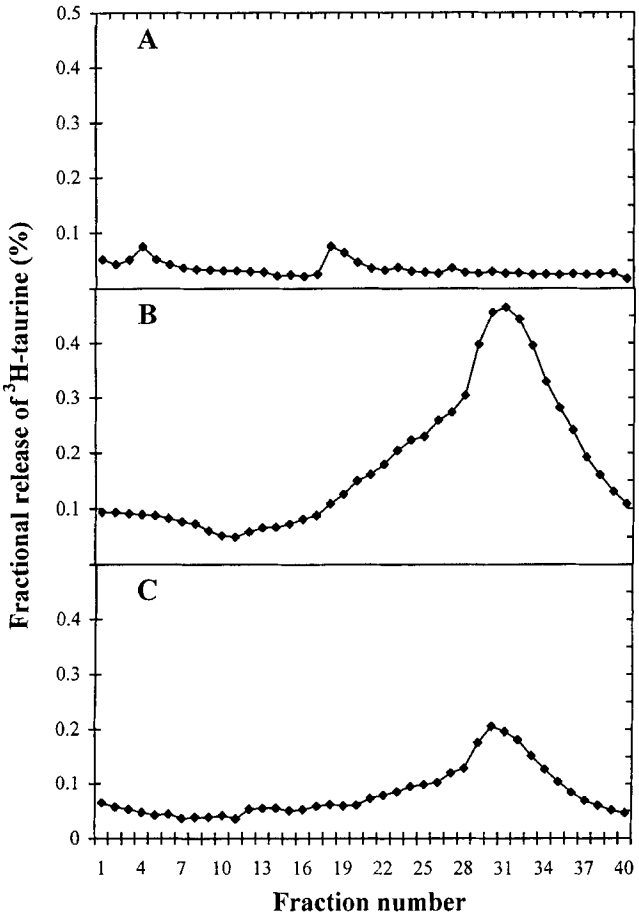

Fig. 2. Release of [^{3}H] taurine from cultured cerebral cortical neurons during superfusion of the cells with a physiological buffer (HBS) + 5 mM phenylsuccinate (**A**), HBS + 100 mM KCL (**B**), or HBS + 100 mM KCl and 5 mM phenylsuccinate (**C**). Cells were cultured (7 days) from embryonic cerebral cortex, preloaded for 30 min with 10 μM [^{3}H]taurine (1 μCi/culture), and subsequently superfused (2 ml/min) as detailed in Materials and Methods. Release is presented as fractional release, i.e., percent of that remaining in the cells at that time. Fractions were collected every 30 sec. KCl and/or the drug was present in the superfusion medium from fraction 12 to fraction 28. Results are averages of 3–4 experiments with SEM values less than 15%.

mM) was almost the same (approximately 40%) whether taurine release was elicited by 100 mM K^+ or by exposure of the cells to hyposmotic conditions.

DISCUSSION

Phenylsuccinate has been used previously as a tool to differentiate between glutamate being released from a cytoplasmic, non-neurotransmitter-related pool or a neurotransmitter-related pool presumably located in vesicles (Palaiologos et al., 1988; Christensen et al., 1991). Its

TABLE I. Effect of Phenylsuccinate (5 mM) on K^+ (100 mM)- or Hypotonicity (160 mOsm)-Induced Taurine Release From Cultured Cerebral Cortical Neurons and Astrocytes†

	Fractional release (%)	
Washout medium	Neurons	Astrocytes
HBS	4.9 ± 0.3	3.5 ± 0.3
Hypotonic (50%)	57.9 ± 1.2	62.9 ± 1.0
KCl (100 mM)	16.6 ± 0.5	14.2 ± 0.4
HBS + phenylsuccinate (5 mM)	3.9 ± 0.3	2.2 ± 0.1
Hypotonic + phenylsuccinate	40.9 ± 1.0*	40.7 ± 0.4*
100 mM KCl + phenylsuccinate	11.5 ± 0.4*	7.9 ± 0.4*

†Neurons and astrocytes were cultured from mouse cerebral cortex in 24-well plates as described in Materials and Methods. Prior to the experiment, cells were loaded for 30 min with [^{3}H]taurine (0.1 μCi/culture) and after a brief wash in HBS to remove excess radioactivity the cultures were incubated for 10 min periods in HBS, experimental solution, and again HBS. At the end of the experiment, radioactivity remaining in the cells was determined in ethanol (70%) extracts and this was added to the radioactivity present in the washout media to give the radioactivity present in the cells at the start of the three 10 min washout periods. The radioactivity present in the medium at the end of each 10 min washout period was expressed as fractional release (see Materials and Methods). Results are averages ± SEM of 3–6 experiments.

*$P < 0.05$, Student's t-test (statistically significant differences between taurine release elicited by K^+ or hyposmolarity in the absence or presence of phenylsuccinate).

mechanism of action in this regard involves an inhibition of transfer of α-ketoglutarate from mitochondria to the cytoplasm where it is transaminated to form glutamate (Palaiologos et al., 1988). Thus, phenylsuccinate was used recently to demonstrate that ischemia-induced glutamate overflow to the extracellular space in rat hippocampus involves release from a non-neurotransmitter-related glutamate pool as this overflow was not affected by phenylsuccinate while K^+-stimulated glutamate release could be blocked completely by this drug (Christensen et al., 1991). It could, however, be argued that interference with cellular volume regulatory processes could play a role in the action of phenylsuccinate as glutamate can be released from brain cells during cell volume regulation (Kimelberg et al., 1990). Taurine has been shown to function as one of the major osmoregulatory compounds in astrocytes and neurons (for references see Schousboe and Pasantes-Morales, 1992) as well as in many other cells and organs (Hoffmann and Simonsen, 1989). The observation in the present study that phenylsuccinate inhibited taurine overflow in hippocampus during exposure of the tissue to a high concentration of K^+ administered through the dialysis fiber is best explained by an action of phenylsuccinate on the cell membrane-associated mechanism responsible for taurine release under osmotic stress. That this indeed may be the case can be deduced from the results of the experiments performed on cultured neurons and astrocytes. In

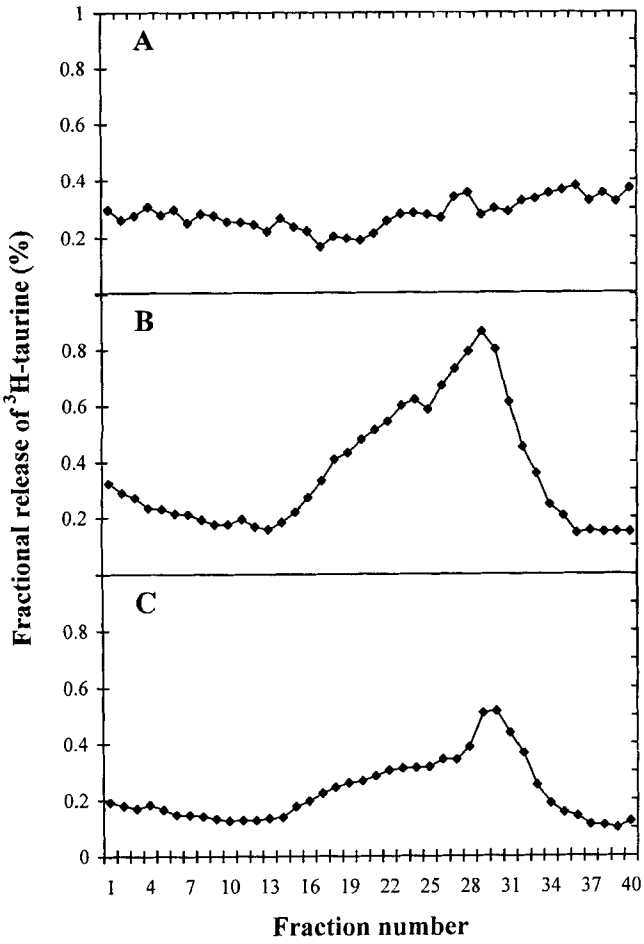

Fig. 3. Release of [^{3}H]taurine from cultured cerebral cortical astrocytes during superfusion of the cells with a physiological buffer (HBS) + 5 mM phenylsuccinate (**A**), HBS + 100 mM KCl (**B**), or HBS + 100 mM KCl and 5 mM phenylsuccinate (**C**). Cells were cultured (21 days) from neonatal cerebral cortex, preloaded for 30 min with 10 μM [^{3}H]taurine (1 μCi/culture), and subsequently superfused (2 ml/min) as detailed in Materials and Methods. Release is presented as fractional release, i.e., percent of that remaining in the cells at that time. Fractions were collected every 30 sec. KCl and/or the drug was present in the superfusion medium from fraction 12 to fraction 28. Results are averages of 3–4 experiments with SEM values less than 15%.

these cells, K^+- or hyposmolarity-induced taurine release is mediated exclusively by swelling sensitive channels in the plasma membrane allowing taurine to be released from the cell during regulatory volume decrease (Schousboe and Pasantes-Morales, 1992, and references therein). The exact nature of these channels remains to be elucidated but it has been shown recently that sulfhydryl groups play an essential role in the release process (Martinez et al., 1994). Whether phenylsuccinate may react with such groups is presently unknown, but this drug may be used to further characterize volume sensitive taurine efflux at the molecular level. It should be mentioned that this mechanism does not seem to involve the cell membrane taurine carrier (Sánchez-Olea et al., 1991; Schousboe et al., 1991). Whether phenylsuccinate actually partly prevents K^+-induced cell swelling is not clear, but blockers of Cl^- transport have been shown to inhibit both volume regulation and efflux of taurine in astrocytes (Sánchez-Olea et al., 1993).

The present findings raise the question as to whether or not the inhibitory action of phenylsuccinate on K^+-stimulated glutamate overflow in rat hippocampus previously described by Christensen et al. (1991) may be related to inhibition of volume regulation or to inhibition of synthesis of neurotransmitter glutamate. It is difficult to rule out the possibility that at least part of the K^+-stimulated glutamate overflow could originate from a non-neurotransmitter glutamate pool sensitive to cell swelling. In this context it is important that in cultured cerebellar granule neurons, where K^+-stimulated taurine release occurs exclusively subsequent to K^+-induced cell swelling (Schousboe et al., 1990), phenylsuccinate blocks release of endogenous glutamate but not that of exogenously supplied [^{3}H]D-aspartate (Palaiologos et al., 1989). Since [^{3}H]D-aspartate would have been released by cell swelling in case this process were responsible for the K^+-induced glutamate release, this finding strongly suggests that the inhibitory action of phenylsuccinate on K^+-stimulated glutamate release is unrelated to its inhibition of the volume sensitive taurine release mechanism. In spite of its different mechanisms of action, phenylsuccinate or structurally related compounds may be useful in elucidating the nature of the mechanism responsible for taurine release activated by cell swelling.

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