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Ammonia-Induced Taurine Release From Cultured Rabbit Müller Cells Is an Osmoresistant Process Mediated by Intracellular Accumulation of Cyclic AMP

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A previous study demonstrated the release of newly loaded radiolabelled taurine (Tau) from cultured rabbit Müller glia not only following typical cell volume-increasing treatments with high (65 mM) potassium ions or hypotonic media, but also with ammonium chloride (further referred to as ammonia), in a dose-dependent manner, at doses ranging from physiological (0.25 mM) to those accompanying hyperammonemic coma (5 mM) (Faff-Michalak et al., Glia 10:114–120, 1994). Stimulation of Tau release by ammonia, but not by 65 mM potassium, was correlated with a dose-dependent increase of intracellular cAMP levels. The release, as measured at 5 mM ammonia, was abolished by compounds that prevented cAMP increase: an adenylate cyclase inhibitor, miconazole, a protein kinase A inhibitor HA 1004, an anion channel blocker, niflumic acid, and a Tau transport site agonist, β-alanine. The release by ammonia differed from potassium-induced release in its resistance to 1) increase of medium tonicity by addition of 50 mM sucrose; 2) addition of the anion/cation cotransport blocker, furosemide; and 3) removal of calcium from the superfusion medium. The results suggest that ammonia-induced Tau release is mediated by intracellular accumulation of cAMP and may occur either via an osmoresistant, cAMP-controlled channel or a cAMP-activated Tau transporter. The release observed at the physiological concentration of ammonium chloride suggest a role for ammonia as a signal molecule. © 1996 Wiley-Liss, Inc.

Key words: Müller glia, taurine release, ammonia, cyclic AMP

INTRODUCTION

The neuroinhibitory sulfur amino acid taurine (Tau) abounds in the glial cells of the brain (Martin et al., 1988) and Müller cells, the predominant glial cell of retina (Pow and Crook, 1993). Studies with cultured glial cells have demonstrated Tau release into the extracellular space in response to a variety of external stimuli. Tau is co-released with osmotically obligated water and ions under conditions that cause cell swelling (hypoosmotic low sodium or isoosmotic high potassium media), suggesting osmoregulatory or osmosensory function of the amino acid (Martin et al., 1990a; Pasantes-Morales and Schousboe, 1988, 1989; Vitarella et al., 1994). The swelling-induced Tau release appears to occur via specific osmosensitive anion channels and is thus abolished by different anion transport blockers (Jackson and Strange, 1993; Kimelberg et al., 1990; Pasantes-Morales et al., 1994a,b; Sanchez-Olea et al., 1992), albeit the ionic requirements for Tau release and regulatory volume decrease do not match perfectly (O'Connor and Kimelberg, 1993). In addition, the high potassium-dependent Tau release appears to be dependent upon extracellular calcium (Philibert et al., 1988), an ion indispensable in glial cell volume regulation (O'Connor and Kimelberg, 1993; for another viewpoint, see Pasantes-Morales and Schousboe, 1989). On the other hand, Tau is released from glial cell in response to stimulation by β-adrenergic receptor agonists (isoproterenol) (Shain et al., 1986), or adenosine (Madelian et al., 1988) through a cAMP-mediated mechanism, supposedly involving a stretch-activated channel or a selective Tau transporter. Such a release is thought to represent a feedback neuromodulatory response of glial cells to the activity of adjacent neurons (for reviews, see Martin et al., 1988; Martin and Shain, 1993). The cAMP-mediated release also appears to be modulated by osmolarity, may be reflecting an osmosen-

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sory function of Tau (Martin and Shain, 1993) but is independent of extracellular calcium (Martin et al., 1989).

Ammonia is a neurotoxin whose action is primarily targeted at glial cells: It induces profound morphological and metabolic changes astrocytes in the brain (Norenberg, 1981), and Müller glia in the retina (Reichenbach et al., 1995a,b). Recent studies have demonstrated that ammonia added at doses ranging from physiological (0.25 mM) to those accompanying hyperammonemic coma (5 mM) stimulates the release of Tau from rat astrocytes (Albrecht et al., 1994) and rabbit Müller glia cultured in vitro (Faff-Michalak et al., 1994). Here we investigated the possibility that, by analogy to the receptor agonist-induced release, ammonia-induced Tau release may be mediated by cAMP. Therefore, we measured paralelly cAMP levels and Tau release in cultures treated with different concentrations of ammonium chloride and/or with various compounds that inhibit or modulate cAMP accumulation an/or protein kinase A activity. We also tested the stimulatory effect of ammonia for its sensitivity to a) changes in medium osmolarity, b) anion transport blockers, c) a Tau carrier agonist (β-alanine), and d) absence of extracellular calcium. We compared ammonia-induced Tau release with the release elicited by high (65 mM) K^+, whose mechanism in other cell systems has been relatively well established.

MATERIALS AND METHODS

Preparation of Müller Cell Cultures

The cultures were prepared from the eyeballs of ether-aneasthetized rabbits of the third postnatal day exactly as earlier described (Scherer and Schnitzer, 1988; Reichelt et al., 1989; Faff-Michalak et al., 1994). Briefly, the retinae were isolated and small retinal pieces were mechanically dissociated and grown at 37°C in a humidified 5% CO_2/95% air, in minimal essential medium with 10% fetal bovine serum. Confluent monolayers of vimentin-positive epithelial cells representing Müller cells were obtained after 14 days in vitro (DIV).

Loading and Release of [^{3}H]Tau

The loading and release of radiolabeled Tau were carried out exactly as described in the previous report (Faff-Michalak et al., 1994). Cells at 14 DIV in 35 mm dishes were incubated in 1 ml of a Krebs-Ringer medium with 2.5 mM $CaCl_2$ (pH 7.4) containing 1 μCi of [^{3}H] Tau (35 mCi/mmol, Amersham, UK), for 15 min at 37°C. The cultures were then washed three times for 3 min and five times for 5 min each with 1 ml of the medium without Tau. The next 13 washes, each lasting 5 min were collected for radioactivity measurements. Stimuli were added twice, each time for 5 min, during the 4th and 9th wash, respectively (cf. Faff-Michalak et al., 1994). All changes in the medium composition and/or pharmacological agents were likewise introduced during the 4th and 9th wash. All the values are expressed as % total radioactivity, which refers to the sum of radioactivity released in the peak poststimulus fractions (5–7 and 10–12) as compared with the initial radioactivity, which was the sum of the released radioactivity plus the radioactivity of the pellet.

cAMP Content Measurements

The cells were incubated for 5 min in stimulus media without radiolabeled Tau. Immediately after incubation the cells from each dish were scraped off with a plastic rod into 1 ml of Krebs-Ringer medium containing 0.4 mM EDTA. The cell suspension was homogenized with a hand-driven glass-teflon homogenizer, and then boiled for 1 min and centrifuged (5 min, 10,000 rpm) to remove proteins. The cAMP content was measured in 20 μl samples of the supernatant using a cAMP radioimmunoassay kit (Amersham, UK).

LDH Assay

To evaluate cell membrane leakage, LDH was assayed in cell extracts prepared with 0.1% Triton in the Krebs Ringer buffer, and in the various stimulation media after 5 min incubation (see above), with the spectrophotometric method of Bergmeyer (1970), as adapted from Frandsen and Schousboe (1987).

Statistical Analysis

Statistical analysis was performed with one-way ANOVA followed by Duncan's test for multiple comparisons.

RESULTS

Increasing Concentrations of NH_4Cl Promote Dose-Dependent cAMP Accumulation and Tau Release: KCl Stimulates Tau Release Without Affecting cAMP Content

Treatment of the cells with NH_4Cl at concentrations ranging from 0.25–5 mM produced a dose-dependent increase of cAMP content in these cells (Fig. 1, lower panel), which was accompanied by the stimulation of Tau release (Fig. 1, upper panel). Treatment with 65 mM KCl did not affect the cAMP content of the cells (Fig. 1, lower panel). However, as shown previously (Faff-Michalak et al., 1994), the treatment stimulated Tau release from 4.8% to 11.3% of total radioactivity (Fig. 1, upper panel).

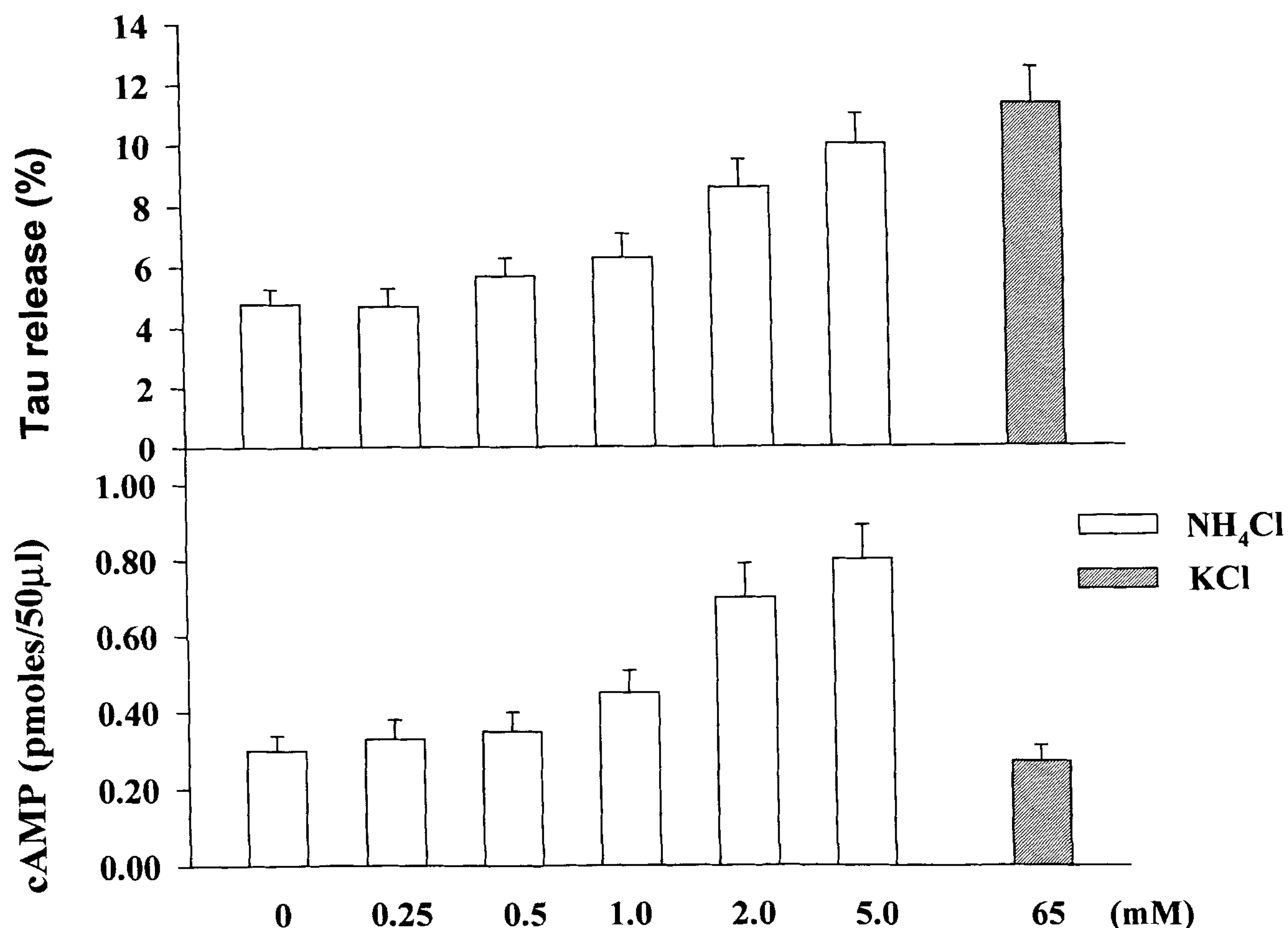

Fig. 1. Effects of increasing concentrations of NH_4Cl, and of 65 mM KCl on Tau release and the cAMP content in cultured rabbit Müller glia. Results are means ± SD, for 3–6 independent tests.

Inhibition of cAMP Accumulation or Protein Kinase A Is Correlated With Suppression of NH_4Cl-Dependent, but Not of KCl-dependent Tau Release

An adenylate cyclase inhibitor, miconazole, and a protein kinase A inhibitor, HA 1004, substantially reduced the stimulatory effects of 5 mM NH_4Cl on both cAMP accumulation (Fig. 2, lower panel) and Tau release (Fig. 2, upper panel). B-alanine, which shares with Tau the transport site at the cell membrane, and an anion transport inhibitor niflumic acid, inhibited both effects of NH_4Cl as well (Fig. 2). B-alanine did not affect Tau release evoked by 65 mM KCl, and HA 1004 was only slightly inhibitory (Fig. 2, upper panel).

NH_4Cl-Dependent Tau Release Is Independent of Extracellular Calcium: Release by KCl Is Extracellular Calcium Dependent

Treatment with a calcium-free medium containing EGTA and ionomycin only insignificantly reduced stimulation of Tau release by 5 mM NH_4Cl but prevented stimulation by 65 mM KCl (Fig. 3; "+ ionomycin"). Superfusion with a medium in which calcium was replaced by 10 mM Mg^{2+} increased basal Tau release from 4.9% to 11.5% of total radioactivity, and addition of 5 mM NH_4Cl to the superfusion medium produced a further stimulation of Tau release to 18.3% of total radioactivity, whereas 65 mM KCl did not enhance Tau release in this medium (Fig. 3; "+ Mg^{2+}").

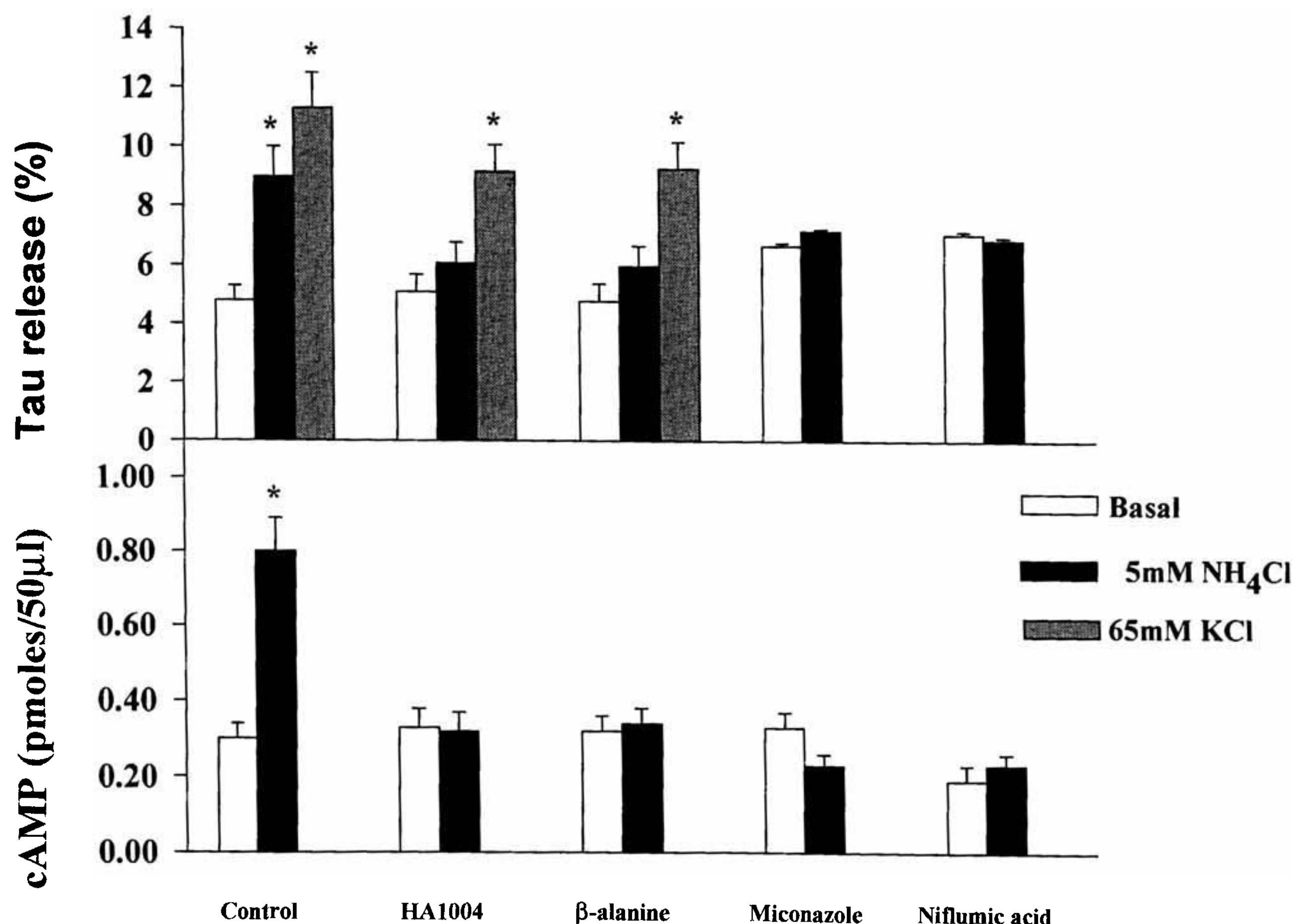

Fig. 2. Effects of various metabolic inhibitors on the cAMP accumulation and Tau release in the presence or absence of 5 mM NH_4Cl or 65 mM KCl. The concentrations used were HA 1004, 5 μM; β-alanine, 1 mM; miconazole, 200 μM, niflumic acid, 50 μM. Results are means ± SD, for 3–6 independent tests. *$P < 0.05$ as compared to basal release ("basal").

NH_4Cl-Dependent Tau Release Is Not Suppressed by Increased Medium Osmolarity: Release by KCl Is Osmosensitive

In our routine procedure, Tau release was stimulated by NH_4Cl in a slightly hyperosmolar medium: addition of NH_4Cl was not compensated for by reduction of NaCl (see Methods). No stimulation of Tau release was observed when 5 mM NH_4Cl was replaced by equimolar amount of KCl, the final concentration of KCl being 8 mM (not shown). In the experiments with a calcium-free medium containing magnesium, which did not suppress the stimulatory effect of NH_4Cl on Tau release (Fig. 3), the medium was hyperosmolar as well: 2.5 mM $CaCl_2$ was replaced by 10 mM $MgCl_2$. Addition to the superfusion medium of 50 mM sucrose did not affect stimulation of Tau release by 5 mM NH_4Cl, but abolished stimulation by 65 mM KCl (Fig. 3).

NH_4Cl-Dependent Tau Release Is Not Suppressed by Furosemide: Release by KCl Is Furosemide-Sensitive

Addition of a K^+, Cl^--cotransport blocker, furosemide (Kimelberg et al., 1979), only slightly inhibited 5 mM NH_4Cl- dependent release, but blocked the release by 65 mM KCl (Fig. 3).

Stimulation With 5 mM NH_4Cl Is Not Accompanied by Measurable LDH Leakage From the Cells, Irrespective of Other Additions to the Medium

The LDH activity of supernatants after incubation with the stimuli was in all instances less than the detection threshold of 0.001 IU/litre, as compared to at least 0.045 IU/litre measured in the respective cell homogenates (data not shown).

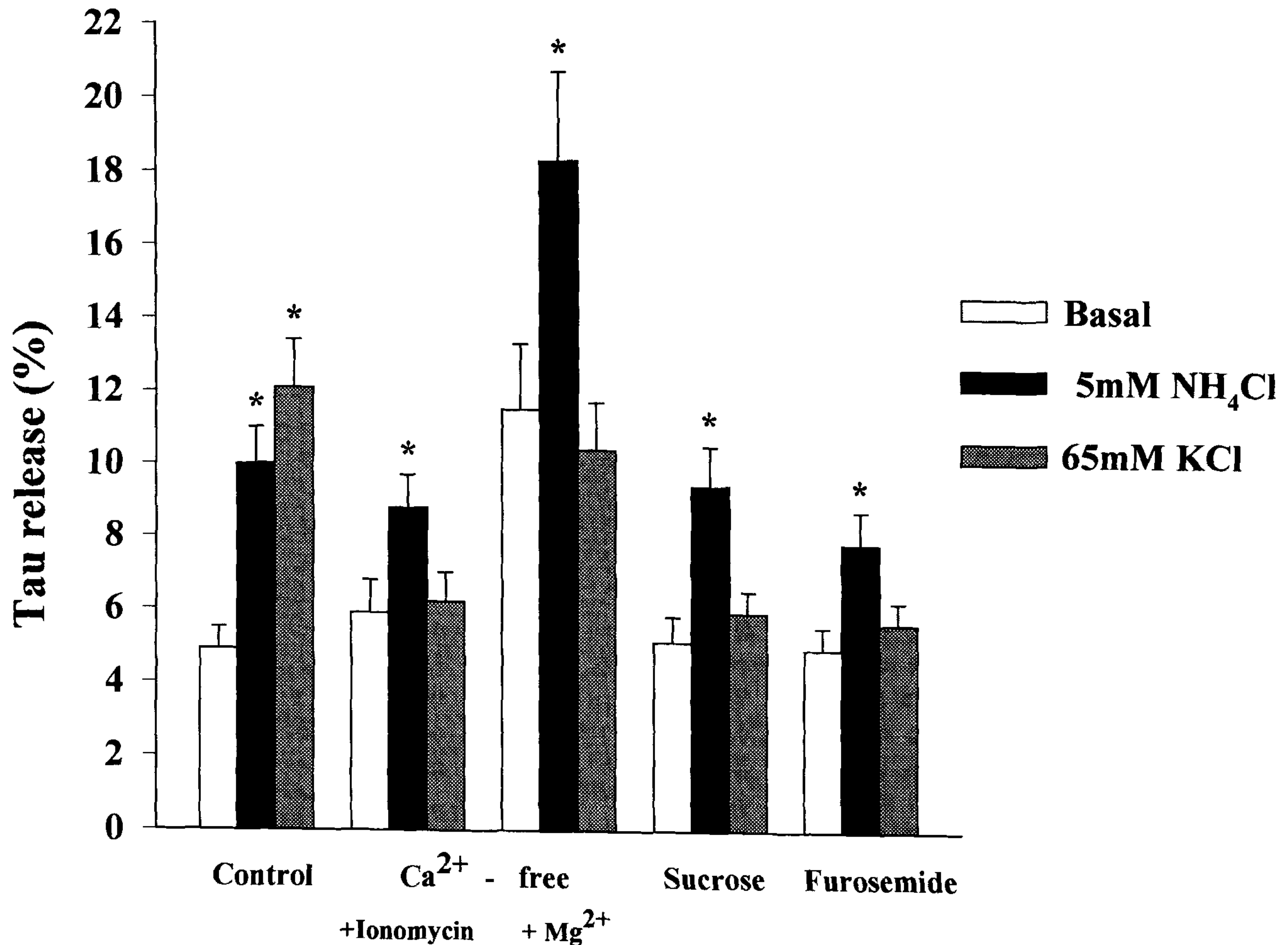

Fig. 3. Effects of removal of calcium, and of addition of 50 mM sucrose or 5 mM furosemide to the superfusion media, on Tau release in the presence or absence of 5 mM NH_4Cl or 65 mM KCl. In the superfusion medium with ionomycin (5 μM), 2.5 mM $CaCl_2$ was replaced by 1 mM EGTA, 2.5 mM $MgSO_4$, and 0.2 mM $CaCl_2$; in the medium with Mg^{2+}, 2.5 mM $CaCl_2$ was replaced by 10 mM $MgCl_2$. Results are means ± SD, for 3–6 independent tests. *$P < 0.05$ as compared to basal release ("basal").

DISCUSSION

High Potassium-Induced Tau Release

The characteristics of Tau release from cultured Müller glia evoked by high potassium ions noted in the present study, resembled very closely the release by the same stimulus described by others in cultured astrocytes, or LRM 55 glial cells. The similarities included susceptibility to changes in medium osmolarity (Martin et al., 1990b), dependence on extracellular calcium (Philibert et al., 1988), and inhibition by a potassium/chloride cotransport blocker, furosemide (Martin et al., 1990a). Thus, like in the other tissues, the release in Müller cells is understood to reflect Tau transport out of the cells with osmotically obligated water and ions via osmosensitive ion channels, as a reaction to cell swelling (see Introduction). The absence of stimulation of Tau in a hyperosmotic medium is in keeping with an earlier finding that cell swelling is the exclusive mediator of potassium-induced Tau release in cultured astrocytes (Pasantes-Morales and Schousboe, 1989) and neurons (Schousboe et al., 1990). The calcium-dependence of the potassium-induced release would suggest in addition that the release is a part of an osmoregulatory response (O'Connor and Kimelberg, 1993). It must be noted however that, the calcium requirement and the involvement of potassium/chloride cotransport in regulatory volume decrease may differ in cells originating from different species (Pasantes-Morales and Schousboe, 1989; see also the introductory paragraph). It may be recalled from our previous study that Müller cells in culture significantly increase their volume in 65 mM KCl (Faff-Michalak et al., 1994). The noninvolvement of cAMP in the potassium-induced

release is not surprising, as there is no reason to implicate cAMP in activation of anion-permeable channels (Jackson et al., 1994).

Ammonia-Induced Tau Release

The present study revealed that the mechanism of ammonia-induced release of Tau from cultured Müller cells differs in all the aspects studied from the release elicited by high potassium ions: It turned out to be calcium-independent, insensitive to furosemide or medium hypertonicity, and cAMP-dependent.

The most important observation of the present study was that the release of Tau by ammonia was correlated with intracellular accumulation of cAMP. This result in association with the suppression of the release by preventing this accumulation, strongly supports the hypothesis that cAMP mediates a significant component of the Tau-releasing activity of ammonia. While suppression of cAMP accumulation by an adenylate cyclase inhibitor, miconazole (Watson, 1990), is self explanatory, the mechanisms involved in HA 1004, β-alanine, or niflumic acid action are obscure. HA 1004 is an inhibitor of cAMP-dependent protein kinase (Hirai and Okada, 1994), and its interference with the cAMP synthesis or degradation has not been reported. B-alanine is a substrate/inhibitor of the Tau carrier in various CNS tissues (Larsson et al., 1986; Liu et al., 1992) and as such could be expected to stimulate Tau release by heteroexchange. However, the lack of effect of β-alanine on basal Tau release may indicate that, the heteroexchange is poor in Müller glia, as it is in LRM55 cells (Martin et al., 1990a). Niflumic acid, an anion transport inhibitor, has been shown to suppress Tau efflux associated with hypoosmotic or hyponatremic swelling of cultured glial cells (Sanchez-Olea et al., 1992), or brain slices (Law, 1994). The question whether niflumic acid inhibited ammonia-induced release of Tau by blocking Tau transport through an anion channel or by suppressing cAMP accumulation remains to be resolved: the two possibilities are not mutually exclusive.

Ammonia-induced Tau release was only insignificantly affected by furosemide, which is a chloride/potassium cotransport blocker. This contrasts both with the furosemide-sensitivity of the release by high potassium ions, and with the cAMP-dependent release mediated by β-adrenergic receptors in LRM55 glia (Martin et al., 1990a). The furosemide-insensitivity of ammonia-induced Tau release from Müller glia is consistent with the previous demonstration of a chloride-independent component of Tau release evoked by 2–5 mM ammonia (Faff-Michalak et al., 1994). It cannot be excluded therefore that ammonia activates a separate Tau channel which is not a chloride channel: a specific Tau channel which does not conduct chloride efflux, is activated in Ehrlich mouse ascites tumour cells subjected to hypoosmotic swelling (Lambert and Hoffmann, 1994).

In the experiments analyzing the effect of extracellular calcium, two kinds of calcium-free media were employed: a) a medium with EGTA, also including ionomycin to facilitate calcium escape from the cell (Purpura et al., 1994) and b) a medium in which calcium was replaced by 10 mM Mg^{2+} (Martin et al., 1989). Independence of the presence calcium in the perfusion media is a feature common to the cAMP-mediated component of ammonia-induced Tau release in Müller glia and cAMP-dependent release elicited by stimulation of β-adrenergic or other neurotransmitter receptors in LRM55 cells or cortical astrocytes (Martin et al., 1989; Shain et al., 1989). However, the resistance to changes in medium osmolarity distinguishes ammonia-induced Tau release in Müller glia from the cAMP-mediated release in other types of glia (Martin et al., 1990a,b, 1993), but also in tissues outside the nervous system, for instance in flounder erythrocytes, where Tau release performs an osmoregulatory function (Thoroed et al., 1995). Interestingly, the difference in osmosensitivity between Tau release from Müller cells and LRM55 cells came to light in experiments dealing with calcium dependence: Replacement of calcium with 10 mM magnesium, which renders the medium hyperosmolar, abolished the cAMP-mediated release in LRM55 cells (Martin et al., 1989), but not in the present system (Fig. 3). The increase of basal Tau release in the calcium-free medium with magnesium is difficult to explain: Notably, a similar effect of this medium on Tau release was earlier noted in cultured cerebellar astrocytes (Holopainen et al., 1984).

Tsacopoulos and Poitry (1995) have proposed that in honey bee retina, ammonia may serve as carrier of metabolic signal sent by photoreceptors to glial cells, the concept being based on the observation of transient increases of ammonia levels in retinal slices upon light stimulation. The lowest ammonia concentration found to induce cAMP accumulation and Tau release in cultured Müller glia (0.25 mM) is close to that measured in light-activated bee retina (Tsacopoulos and Poitry, 1995). Transient rises in ammonia concentration have also been measured in brain slices (Szerb and O'Regan, 1985) or synaptosomes under conditions simulating active neurotransmission (Erecińska et al., 1990). It is thus tempting to assume that in retina, ammonia is an actual signal molecule transmitted from neurons (photoreceptors) to glial cells to synchronize some of the functions of the two cell types: Ammonia released from activated photoreceptors would induce cAMP accumulation in the adjacent Müller cells, resulting in Tau release. Tau released to the extracellular space would, as an inhibitory amino acid, negatively modulate the photoreceptor activity, in accordance with its postulated gliotransmitter role.

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