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► **To cite this version:**

Patricia Pierson, Brigitta Peteri-Brunbäck, Didier Pisani, Maria Pia Abbracchio, Jean-Marc Mienville, et al.. A2b receptor mediates adenosine inhibition of taurine efflux from pituicytes. *Biology of the Cell*, 2007, 99 (8), pp.445-454. 10.1042/BC20070028 . hal-03009126

**HAL Id: hal-03009126**

**<https://hal.science/hal-03009126>**

Submitted on 10 Dec 2021

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# **Adenosine A<sub>2b</sub> receptor-mediated inhibition of taurine efflux from pituicytes**

Patricia Pierson, Brigitta Peteri-Brunbäck, Didier Pisani<sup>1</sup>,  
Maria Pia Abbracchio<sup>2</sup>, Jean-Marc Mienville and Lia Rosso<sup>3</sup>

CNRS UMR 6548, Laboratoire de Physiologie Cellulaire et Moléculaire  
Université de Nice-Sophia Antipolis, Nice, France

<sup>2</sup> Dipartimento di Scienze Farmacologiche, Università di Milano, Milan, Italy

<sup>1</sup> Present address: Institut Gustave Roussy, 39 rue Camille Desmoulins, 94805 Villejuif, France

<sup>3</sup> Present address: Centre Intégré de Génomique, Université de Lausanne, CH-1015 Lausanne,  
Switzerland

Running title: Adenosine inhibits taurine efflux from pituicytes

Correspondence: Jean-Marc Mienville

Laboratoire de Physiologie Cellulaire et Moléculaire  
CNRS UMR 6548  
Faculté des Sciences  
Université de Nice-Sophia Antipolis  
28 Avenue Valrose  
06108 Nice Cedex 2, France  
Tel: (33) 492 076 861  
Fax: (33) 492 076 850  
Email: mienvill@unice.fr

**Background and purpose:** As a continuation of our ongoing work on the modulation of neurohypophysial hormone output by pituicytes, this study analyses the effects of adenosine and other purinergics on the ability of these cells to release taurine, an inhibitor of neurohormone secretion.

**Experimental approach:** We measured radioactive efflux from cultured pituicytes and whole neurohypophyses preloaded with [<sup>3</sup>H]taurine. Cultured pituicytes were also used to study adenosine receptor mRNA expression and intracellular calcium variations.

**Key results:** Taurine efflux elicited by hypotonic shocks is ~30-50% smaller in the presence of 10  $\mu$ M adenosine or 1  $\mu$ M NECA. Both compounds also inhibit basal efflux in a manner not immediately reversible. Agonists of the A<sub>1</sub>, A<sub>2a</sub> or A<sub>3</sub> receptor subtypes have no relevant effect on basal taurine release, and the A<sub>1</sub> antagonist DPCPX has no effect at all on NECA's inhibition of basal or hypotonicity-induced release. In turn, the A<sub>2b</sub> antagonists MRS 1706 or alloxazine partially reverse NECA's inhibition of basal or hypotonicity-evoked efflux, respectively. Both A<sub>1</sub> and A<sub>2b</sub> receptor mRNAs are expressed in pituicytes, which is consistent with an A<sub>1</sub>-mediated effect on cell morphology, and an A<sub>2b</sub>-mediated effect on taurine release. Forskolin and dibutyryl cAMP mimic the inhibitory effects of purinergics on basal taurine efflux, and the adenylylase inhibitor DDA partially reverses NECA's inhibition of the hypotonic response. Intracellular calcium is unlikely to be implicated in efflux inhibition.

**Conclusions and implications:** Our results suggest that purinergic inhibition of taurine efflux from pituicytes operates through A<sub>2b</sub> receptors coupled to intracellular cAMP increase. They point to a possible modulation of neurohypophysial hormone output by endogenous adenosine released in either physiological or pathological situations.

## Introduction

The output of the neurohypophysial hormones vasopressin (VP) and oxytocin (OT) appears to be regulated in a complex manner at different levels of the hypothalamo-neurohypophysial axis. The primary factor that controls secretion is the electrical activity of the magnocellular neurones of the hypothalamic supraoptic and paraventricular nuclei. This activity is modulated by physiological states such as parturition and lactation, which stimulate OT secretion, and by departures from the hydromineral and volume balance of the organism, which modulate VP secretion. A second level of control is through activation of various receptors present on neurohypophysial terminals by both afferent fibers and systemically circulating substances (reviewed by Falke, 1991). Finally, a third possibility for modulating VP and OT output involves pituicytes, the astroglial cells of the neurohypophysis. This particular regulation may be exerted both via glial morphological changes that facilitate or impede hormone release into the blood (reviewed by Theodosis and MacVicar, 1996 ; Hatton, 1999), and via pituicyte release of the inhibitory amino acid taurine. The latter activates glycinergic receptors present on neurohypophysial terminals, which results in inhibition of evoked VP release most likely through blockade of the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) rise necessary for exocytosis (Hussy *et al.*, 2001). Thus, hypotonic shocks induce taurine efflux from pituicytes (Hussy *et al.*, 2001; Rosso *et al.*, 2004b), consistent with the need to inhibit VP secretion and increase diuresis in hypotonic conditions. Recently, we showed that VP also powerfully activates taurine efflux from pituicytes in culture, an effect weakly mimicked by OT. Moreover, this result was extended to the intact neural lobe preparation, in which we demonstrated the stimulatory effect of endogenous VP on taurine release (Rosso *et al.*, 2004b). This suggests the existence of a negative feedback loop whereby VP may inhibit its own secretion (Rosso *et al.*, 2004a).

An important aspect to consider in neurohormone secretion is the corelease of ATP present in millimolar concentration in secretory vesicles (Gratzl *et al.*, 1980). Although ATP may exert a number of effects per se, both on neurohypophysial terminals (Troadek *et al.*, 1998) and on pituicytes (Troadek *et al.*, 1999; 2000), the presence of ectoATPases in the neurohypophysial

environment (Thirion *et al.*, 1996) makes it likely that extracellularly released ATP is quickly broken down to adenosine. Indeed, we have shown that ATP-mediated pituicyte stellation is actually due to breakdown of the nucleotide to adenosine, which per se induces even more potent and dramatic effects than ATP (Rosso *et al.*, 2002a). Further, the fact that adenosine-induced morphological changes can be reversed by VP (Rosso *et al.*, 2002a,b) suggests a dynamic balance between two endogenous compounds, adenosine and VP, with opposite actions on pituicytes. Therefore, it seemed important to investigate any potential effect of adenosine on the ability of pituicytes to release taurine. Here we show that in contrast to VP, adenosine decreases both basal and hypotonicity-evoked taurine efflux. Interestingly, the pharmacology of this effect suggests involvement of the A<sub>2b</sub> receptor, at variance with A<sub>1</sub> receptor-mediated morphological changes of the same cells (Rosso *et al.*, 2002a). Thus, we also studied the expression of adenosine receptor mRNAs in pituicytes, which to our knowledge constitutes the first characterization of this type in these cells. Finally, we attempted to elucidate the transduction mechanisms involved in the inhibition of taurine release by purinergics.

## **Materials and Methods**

### *Pituicyte cultures and [<sup>3</sup>H]taurine efflux*

Pituicyte explant cultures were prepared as described previously (Rosso *et al.*, 2002a) from adult Wistar rats (150-200 g) anesthetized with CO<sub>2</sub> and decapitated in accordance with French/European ethical guidelines. General methods for [<sup>3</sup>H]taurine efflux, including in the whole neurohypophysis preparation, have been detailed previously (Rosso *et al.*, 2004b). A slight modification of the protocol concerns data presentation: due to frequent rundown, basal efflux (i.e. the first 5-6 points) is now fitted with a monoexponential function (Kaleidagraph software; [www.synergy.com](http://www.synergy.com)); subsequently, the whole data set is divided by the fit and multiplied by 100 to express release as a percentage of basal (Deleuze *et al.*, 2000). Efflux data are presented as mean ± SEM and, unless otherwise stated, n represents the number of culture dishes counted.

### *RT-PCR experiments*

Hearts were taken from some of the animals used for cultures; they were cleaned and immediately frozen in liquid N<sub>2</sub> for RNA analysis. RNAs from rat hearts and from pituicyte cultures were prepared with RNA Instapure (Eurogentec) according to the manufacturer's procedure. Reverse transcription was carried out with M-MLV reverse transcriptase (Invitrogen) and oligodT on 1 µg of total RNA. Taq polymerase (Invitrogen) was used for PCR amplification of partial cDNA with the forward primer 5'-CCTCTGGGTAGCGGACAC-3' and reverse primer 5'-TTCACAGCCCAAATCACC -3' for A<sub>1</sub> receptor, the forward primer 5'-TGTCCTGGTCCTCACGCAGAG -3' and reverse primer 5'-CGGATCCTGTAGGCGTAGATGAAG -3' for A<sub>2a</sub> receptor, the forward primer 5'-TTTACAGACCCCCACCAA -3' and reverse primer 5'-GGAGCCAACACAGAGCAA -3' for A<sub>2b</sub> receptor, and the forward primer 5'-TCCTTTCTGGTGGGACTGAC -3' and reverse primer 5'-AGGGTTCATCATGGAGTTTCG -3' for A<sub>3</sub> receptor. A partial β-actin cDNA fragment was amplified as control with the forward primer 5'-AGCCATGTACGTAGCCATCC-3' and the reverse primer 5'-CAGTGAGGCCAGGATAGAGC-3'.

### *Calcium imaging*

[Ca<sup>2+</sup>]<sub>i</sub> variations were estimated with the ratiometric, membrane-permeable, fluorescent probe Fura-2 AM. Pituicytes were incubated 45 min at 37°C in the presence of 5 µM Fura-2 AM + 0.01% pluronic acid and subsequently rinsed. During experiments, culture dishes were continuously superfused with Locke's medium (4 ml/min), and the same bath perfusion was used to apply drugs. Experiments were performed on the stage of an inverted microscope (Zeiss Axiovert 10) equipped with a Xenon lamp and a rotating filter set allowing 350/380 nm excitation. Axon Imaging Workbench 2.2 software was used to drive the filter wheel, acquire fluorescence images and process data. For any given experiment, fluorescence signals were obtained from 10-15 cells and used to

calculate F350/380 emission ratio as an index of  $[Ca^{2+}]_i$  fluctuations. Using Kaleidagraph software, average ratio data were smoothed with a running average over 10-20 time points; this attenuated noise due to small shifts of sampling period (5 to 15 s) among experiments. SEM data were 90-97% reduced to allow for figure clarity.

### *Statistical analysis*

Confidence interval was computed and two-way ANOVA was performed with Statistica software (www.statsoft.com).

### *Drugs*

$[^3H]$ Taurine was purchased from Amersham Pharmacia Biotech (Orsay, France). Adenosine, forskolin, dibutyryl cAMP, alloxazine, NECA (N-ethylcarboxamidoadenosine), CPA (N<sup>6</sup>-cyclopentyladenosine), IB-MECA (N<sup>6</sup>-(3-iodobenzyl)-9-[5-(methylcarbamoyl)- $\beta$ -D-ribofuranosyl]adenine), DPCPX (1,3-dipropyl-8-cyclopentylxanthine), CGS 21680 (2-*p*-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine) and DDA (2',5'-dideoxyadenosine) were purchased from Sigma-RBI. MRS 1706 (N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]acetamide) was obtained from Tocris, UK.

## **Results**

### *Effects of purinergics on hypotonic shock-induced taurine efflux from pituicytes*

During our previous work (Rosso *et al.*, 2004b), we found that VP potentiates the taurine efflux evoked by a mildly hypotonic shock in pituicytes. Here we found that adenosine (10  $\mu$ M) has the opposite effect, decreasing the efflux induced by a 270-mOsm medium to less than 50% of control (Figure 1a). The effect on a 200-mOsm shock is less pronounced, resulting in 32% inhibition (Figure 1b). We thereafter switched to NECA, a potent and stable agonist commonly used to

investigate adenosine receptors though it does not readily discriminate among their subtypes. As shown in Figure 1c, 1  $\mu$ M NECA is as potent as 10  $\mu$ M adenosine, reducing the hypotonic response to 68% of control.

#### *Effects of purinergics on basal taurine efflux from pituicytes*

It is obvious from Figure 1a that adenosine initially decreases basal efflux soon after its application. Therefore, we subsequently focused on the inhibitory effect of purinergics on basal taurine efflux, which indeed appears to be decreased by adenosine as shown in Figure 2a. At the resolution level used here, variability in basal efflux is more apparent and might hinder detection of a real effect. Therefore, wherever appropriate we plot the 99% confidence interval of the baseline (horizontal lines). Figure 2b shows that NECA also decreases basal taurine efflux in cultured pituicytes. A common finding for both adenosine and NECA is that recovery of efflux back to control after agonist removal is not readily obtained within the time frame of the experiment. To examine whether this might be due to culturing conditions, and at the same time validate our results in a more physiological model, we tested the effects of NECA in the whole neurohypophysis preparation, for which it has previously been shown that pituicytes constitute the source of taurine release (Miyata *et al.*, 1997). This experiment resulted in the same type of effect as that seen in cultures (Figure 2c).

#### *Search for the adenosine receptor subtype involved in taurine efflux inhibition*

Adenosine receptors comprise A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub> and A<sub>3</sub> subtypes, which all belong to the metabotropic receptor family and differentially couple to various G proteins (Ralevic and Burnstock, 1998). We sought to determine the receptor subtype involved in the inhibition of taurine efflux by purinergics, using agonists and antagonists at a maximum concentration of 1  $\mu$ M to optimize selectivity. For instance, IB-MECA, a preferential A<sub>3</sub> agonist, does not induce any significant effect at 0.1 (n = 5; not shown) or 1  $\mu$ M (Figure 3a), whereas it does inhibit taurine efflux at 10  $\mu$ M (n = 5; not shown),



a concentration that corresponds to its EC<sub>50</sub> at A<sub>2b</sub> receptors (Ralevic and Burnstock, 1998). Taurine release slightly drops below confidence interval after application of CPA, a selective A<sub>1</sub> agonist, although the effect is delayed and clearly different from that seen with NECA (Figure 3b). To nevertheless investigate whether A<sub>1</sub> receptors might be involved, we preincubated pituicytes with DPCPX, a selective A<sub>1</sub> antagonist, and coapplied the latter with NECA. The traces obtained in the presence of NECA alone or in combination with DPCPX are virtually superimposable (Figure 3c), suggesting that A<sub>1</sub> receptors are not involved in NECA's effect. This view is further supported by the fact that DPCPX does not prevent NECA from inhibiting the hypotonic response (Figure 3d). We conclude that CPA may have a slight non-specific (i.e. non-A<sub>1</sub>) effect. Given the known potency of NECA at A<sub>2</sub> receptors (Ralevic and Burnstock, 1998), we set out to investigate any involvement of their A<sub>2a</sub> or A<sub>2b</sub> subspecies. CGS 21680, a selective A<sub>2a</sub> agonist, does not decrease basal taurine efflux and, if anything, may initially raise it slightly above confidence limits (Figure 3e), as occasionally seen with non-specific agonists (e.g. Figure 2a).

#### *Purinergics likely inhibit taurine efflux via A<sub>2b</sub> receptors*

As there are no available agonists of A<sub>2b</sub> receptors (Ralevic and Burnstock, 1998), we resorted to specific antagonists to test the involvement of this receptor subtype in taurine efflux inhibition. Figure 4a displays responses obtained with either NECA alone or in combination with 100 nM MRS 1706, an antagonist of A<sub>2b</sub> receptors. Even though the responses with MRS 1706 appear to lie above those with NECA alone, with mostly non-overlapping error bars, we felt that a definite conclusion as to the antagonist's effect might need a more stringent analysis, and opted for analysis of variance. To validate this approach, we decided to focus on the six time points recorded in the presence of drugs (i.e. 40-65 min), and to perform a two-way analysis with time and drug as the two impacting factors. This of course yields a significant time effect ( $p = 10^{-5}$ ), but also a significant drug effect ( $p = 0.02$ ), while interaction is not significant ( $p = 0.75$ ). Our attempt to optimize inhibition of NECA's effect by increasing antagonist concentration failed, as the responses obtained

with or without 1  $\mu$ M MRS 1706 virtually superimpose ( $n = 4$ ; not shown). We thus switched to both another model (inhibition of a hypotonic shock) and another drug, namely the  $A_{2b}$  antagonist alloxazine. The result is shown in Figure 4b. Although the response in the presence of 1  $\mu$ M alloxazine + NECA does not reach control peak amplitude (see Figure 1c), it is significantly larger than that in the presence of NECA alone ( $p = 0.006$ ; no interaction effect; 2-way ANOVA). While NECA's response is not affected by 100 nM alloxazine ( $n = 3$ ), it turns out to be potentiated by 10  $\mu$ M antagonist ( $n = 6$ ; not shown), again emphasizing potential non-specific effects or perhaps uncontrolled interactions between adenosine receptor subtypes (Ogata *et al.*, 1996; Ralevic and Burnstock, 1998).

#### *Adenosine receptor expression in pituicytes*

To get a clearer picture of the adenosine receptors potentially expressed in pituicytes, and thus virtually involved in taurine efflux inhibition, we resorted to the RT-PCR technique. Our results demonstrate the presence of mRNAs for  $A_1$  and  $A_{2b}$  but not  $A_{2a}$  or  $A_3$  receptors in pituicytes, whereas all four subspecies are detected in rat heart (Figure 5). The detection of  $A_1$  receptor mRNA in pituicytes is not surprising given our previous data showing involvement of this receptor in adenosine-induced pituicyte stellation (Rosso *et al.*, 2002a). The detection of  $A_{2b}$  receptor mRNA and the lack of detection of  $A_{2a}$  or  $A_3$  transcripts nicely support our contention that taurine efflux inhibition is likely due to  $A_{2b}$  receptor activation. As to the possibility that efflux inhibition is linked to stellation, this can be ruled out based on the widely different time courses of the two events (see Rosso *et al.*, 2002a), and on our unpublished observation that 1  $\mu$ M NECA does not induce stellation (whereas 10  $\mu$ M does). Incidentally, this suggests that at 1  $\mu$ M NECA is rather specific for  $A_2$  receptors and has little, if any,  $A_1$  effect in pituicytes.

#### *Transduction mechanisms involved in taurine efflux inhibition*

Adenosine A<sub>2b</sub> receptors are known to activate adenylylase and increase intracellular cAMP (cAMP<sub>i</sub>; Ralevic and Burnstock, 1998). If this was the mechanism for taurine efflux inhibition then any agent raising cAMP<sub>i</sub> should be able to mimic the effect of purinergics. We found this to be the case for forskolin, a potent adenylylase activator (Figure 6a). To verify that cAMP<sub>i</sub> increase was responsible for the effect, we directly applied the cell-permeable analog dBcAMP to pituicytes, and indeed observed inhibition of efflux though the effect is somewhat delayed, perhaps because of slow diffusion of dBcAMP inside cells (Figure 6b). Note that in both cases recovery back to baseline is not obtained 25-30 min after agonist removal, similar to results obtained with purinergics.

If purinergics inhibit taurine release via adenylylase activation and subsequent increase in cAMP<sub>i</sub>, then blocking this pathway should prevent or attenuate their effect. Figure 6c shows hypotonic responses inhibited by NECA alone or after preincubation and coapplication with 100 μM DDA, a cell-permeable adenylylase inhibitor that blocks cAMP<sub>i</sub> rise. Again, the hypotonic response in NECA + DDA does not reach control amplitude but it is significantly larger than that in NECA alone ( $p = 0.008$ ; no interaction effect; 2-way ANOVA), suggesting that adenylylase and cAMP<sub>i</sub> transduce the effect of NECA. This provides additional support in favor of an involvement of A<sub>2b</sub> receptors, which are known to ubiquitously stimulate adenylylase, whereas A<sub>1</sub> receptors rather couple to inhibition of the enzyme (Ralevic and Burnstock, 1998). At 10 μM, DDA has no effect on the inhibition by NECA of hypotonicity-evoked taurine efflux, as the responses with or without DDA are virtually superimposable ( $n = 3$ ; not shown).

As we established previously that an increase in free [Ca<sup>2+</sup>]<sub>i</sub> stimulates taurine release (Rosso et al., 2004a), we hypothesized that, on the contrary, NECA might decrease release by reducing [Ca<sup>2+</sup>]<sub>i</sub>. We thus attempted to study [Ca<sup>2+</sup>]<sub>i</sub> variations induced by NECA with the same methods as used previously (Rosso et al., 2002a, 2002b). However, the responses obtained were too heterogeneous to draw a clear conclusion, apart from the proposal that Ca<sup>2+</sup> is unlikely to be involved in taurine-efflux inhibition.

## Discussion

Our data epitomize the recurrent difficulties encountered by researchers working on cells or tissues expressing several adenosine receptor subtypes. Such difficulties may be due to concentration-dependent selectivity of available compounds and/or physiological interactions among these receptor subtypes (Ralevic and Burnstock, 1998). An example of the former is provided by our finding that IB-MECA has no effect on taurine release at 0.1 or 1  $\mu\text{M}$  whereas it is inhibitory when used at 10  $\mu\text{M}$ , a concentration that corresponds to its  $\text{EC}_{50}$  at  $\text{A}_{2b}$  receptors (Ralevic and Burnstock, 1998). More difficult to account for is the fact that increasing the concentration of the  $\text{A}_{2b}$  antagonists MRS 1706 or alloxazine results in loss of efficacy or potentiation, respectively. Possible interpretations include a concentration-dependent antagonist-to-agonist reversal of action, non-specific blockade of taurine channels, or complex interactions between  $\text{A}_1$  and  $\text{A}_2$  receptors as previously reported (Ogata *et al.*, 1996). Despite these difficulties, we believe we have gathered sufficient evidence through pharmacological, expression, and signal transduction studies to propose that purinergic inhibition of taurine release from pituicytes operates via  $\text{A}_{2b}$  receptors. Somehow at variance with our conclusion, Saransaari and Oja (2000) found mainly  $\text{A}_1$  receptor-mediated effects of purinergics on taurine release from mouse hippocampal slices. However, perhaps due in part to tissue heterogeneity, these effects are fairly complex as low agonist concentrations potentiate or decrease basal efflux in developing vs. adult tissue, respectively, and have disparate effects on stimulated efflux, while higher concentrations are ineffective. Thus, a developmental issue may have to be added to the above mentioned pitfalls.

The signaling mechanism by which  $\text{A}_{2b}$  receptors inhibit taurine release in pituicytes appears to involve an increase in  $\text{cAMP}_i$ . Contrary to us, Deleuze *et al.* (2000) found no effect of  $\text{dBcAMP}$  on basal or evoked taurine efflux from supraoptic tissue where glial cells are assumed to be the sole source of taurine. Interestingly, in this preparation taurine release is not affected by VP or OT either (N. Hussy, personal communication; see also Rosso *et al.*, 2004b), indicating substantial differences

between supraoptic cells in situ and pituicytes in culture. Our initial hypothesis that a rise in  $cAMP_i$  might result in a decrease in resting  $[Ca^{2+}]_i$  must be forgone as it turns out that the average steady-state  $[Ca^{2+}]_i$  does not appear to be affected by NECA. On the other hand, the transient increase in  $[Ca^{2+}]_i$  at the beginning of NECA's application may explain the small initial rise in taurine efflux occasionally observed with this compound or with adenosine (Figures 1b, 2a and b, 3d). Indeed, according to our calibrations in pituicytes, the F350/380 ratio increase observed in Figure 7e ( $<0.1$  unit) roughly corresponds to a 25 nM  $[Ca^{2+}]_i$  rise. Matching this value against our previous VP concentration/ $[Ca^{2+}]_i$  response curve (Rosso *et al.*, 2002b) yields a VP concentration around 0.3 nM, which, on our VP concentration/response curve for taurine efflux stimulation, corresponds to the limit of detection (Rosso *et al.*, 2004b). Incidentally, the heterogeneity of steady-state  $[Ca^{2+}]_i$  response patterns once again emphasizes the possibility of uncontrolled interactions among adenosine receptors. Alternatively, it may be due to differential expression of  $[Ca^{2+}]_i$ -modulating enzymes such as cAMP-sensitive/cGMP-dependent protein kinase (Lincoln *et al.*, 1990). Although the converse possibility of an impact of  $[Ca^{2+}]_i$  on  $cAMP_i$  might be considered (Ogata *et al.*, 1996), a safe account of our results may consist of dissociating decreased taurine efflux from  $[Ca^{2+}]_i$  fluctuations. One obvious alternative then is phosphorylation of any target(s) coupled to the taurine channel or of the channel itself. In the latter perspective, phosphorylation-dependent internalization would be consistent with the kinetics observed in our experiments.

Whatever the precise molecular mechanism by which adenosine inhibits taurine release, our present results add a new piece to the intricate puzzle surrounding the regulation of neurohypophysial hormone output. At the pituicyte level, there seems to exist a balance between a stimulatory, self-sustaining process handled by purinergic modulators, and a negative feedback operated by the neurohormones themselves. This also may have significance for pathological situations. During brain ischemia for example, there is an increase in VP levels without any osmotic or pressure changes, which so far could not be explained easily (Franceschini *et al.*, 2001). As adenosine is massively released during brain ischemia or trauma (Phillis and Goshgarian, 2001), its

inhibition of taurine release by pituicytes via low-affinity A<sub>2b</sub> receptors might be a factor for the increased secretion of VP. The latter, in turn, appears to worsen the outcome of brain ischemia by promoting both vasospasm and edema (Franceschini *et al.*, 2001). Therefore, as there already is evidence that A<sub>1</sub> receptor activation is neuroprotective (Phillis and Goshgarian, 2001), A<sub>1</sub>-agonist / A<sub>2b</sub>-antagonist combinations may provide novel and perhaps more efficient therapies for the ischemic brain condition.

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## Figure legends

**Figure 1:** Purinergic inhibition of hypotonicity-evoked taurine efflux in pituicytes. Shocks at the indicated osmolarity were elicited in control conditions (open circles) or in the presence of 10  $\mu$ M adenosine (Ado; a and b) or 1  $\mu$ M NECA (c; filled circles) for the time indicated by sequences above traces. (a) n = 5 in control, 4 in Ado; (b) n = 9 in control, 6 in Ado; (c) same controls as in (b), n = 16 in NECA.

**Figure 2:** Purinergic inhibition of basal taurine efflux in pituicytes. Adenosine (Ado) or NECA were applied for the time indicated by bars above traces onto cultured pituicytes (a [n = 7] and b [n = 16]) or whole neural lobes (c; n = 3). Horizontal lines represent 99% confidence limits of basal efflux calculated over the first 6-7 time points before drug application.

**Figure 3:** Adenosine receptor-subtype specificity of taurine efflux inhibition in pituicytes. (a, b and e) Tests performed with agonists of  $A_3$  (a, n = 11),  $A_1$  (b, n = 13) or  $A_{2a}$  receptors (e, n = 3) indicate little fluctuation off confidence limits (horizontal lines). (c and d) The  $A_1$  receptor antagonist



DPCPX has no effect on NECA-mediated inhibition of basal taurine efflux (c; same controls as Figure 2b; n = 7 in NECA + DPCPX) or hypotonic response (d; n = 3 in control, 4 in NECA + DPCPX, both at 1  $\mu$ M).

**Figure 4:** Effects of A<sub>2b</sub> receptor antagonism on NECA-induced inhibition of taurine efflux in pituicytes. (a) shows the effect of NECA on basal efflux in control conditions (open circles; same controls as in Figure 2b) vs. with pre- and co-application of 100 nM MRS 1706 (filled circles; n = 11). (b) shows the effect of NECA on hypotonicity-evoked (cf. bar above traces) efflux in control conditions (open circles; same controls as NECA data in Figure 1c) vs. with pre- and co-application of 1  $\mu$ M alloxazine (filled circles; n = 6).

**Figure 5:** Expression of adenosine receptor subtype mRNA in cultured pituicytes vs. rat heart.

**Figure 6:** Implication of adenylyclase and intracellular cAMP in purinergic inhibition of taurine efflux from pituicytes. (a and b) Forskolin (n = 8) or dBcAMP (n = 6) decrease basal efflux in a manner similar to adenosine or NECA. (c) 100  $\mu$ M 2',5'-dideoxyadenosine (DDA; filled circles; n = 6) partially reverses the inhibitory effect of 1  $\mu$ M NECA (open circles; same data as in Figure 1c) on the taurine efflux elicited by a 200-mOsm medium (compare with Figure 1c).

Table 1. Primer list.

	forward primer	reverse primer
<b>A<sub>1</sub> receptor</b>	5'-CCTCTGGGTAGCGGACAC-3 '	5'-TTCACAGCCCAAATCACC-3 '
<b>A<sub>2a</sub> receptor</b>	5'-TGTCTGGTCCTCACGCAGAG-3 '	5'-CGGATCCTGTAGGCGTAGATGAAG-3 '
<b>A<sub>2b</sub> receptor</b>	5'-TTTACAGACCCCCACCAA-3 '	5'-GGAGCCAACACAGAGCAA-3 '
<b>A<sub>3</sub> receptor</b>	5'-TCCTTTCTGGTGGGACTGAC-3 '	5'-AGGGTTCATCATGGAGTTCG-3 '
<b>β-actin</b>	5'-AGCCATGTACGTAGCCATCC-3 '	5'-CAGTGAGGCCAGGATAGAGC-3 '

Figure 1.

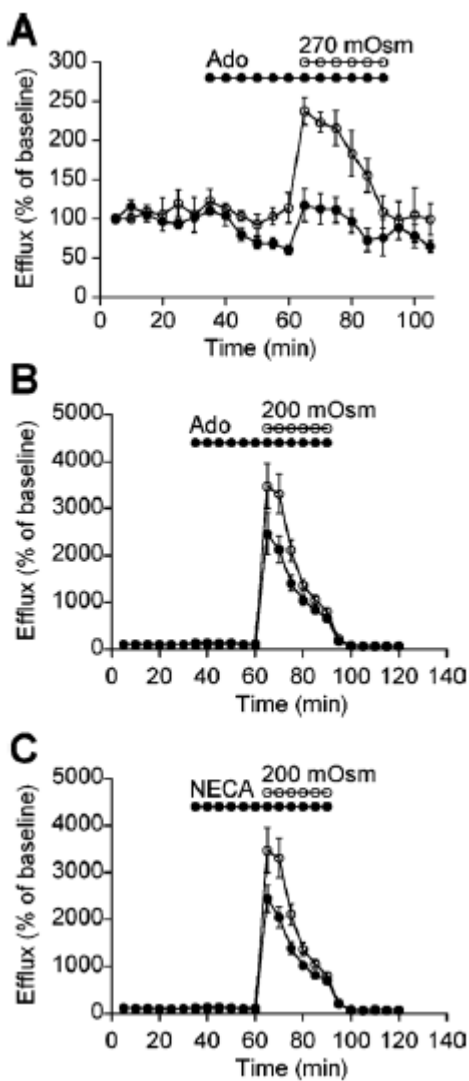


Figure 2.

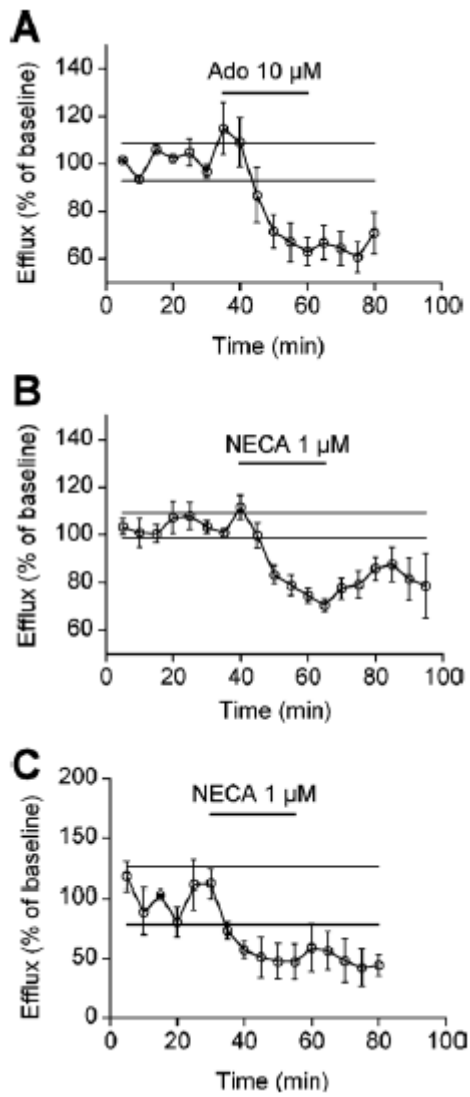


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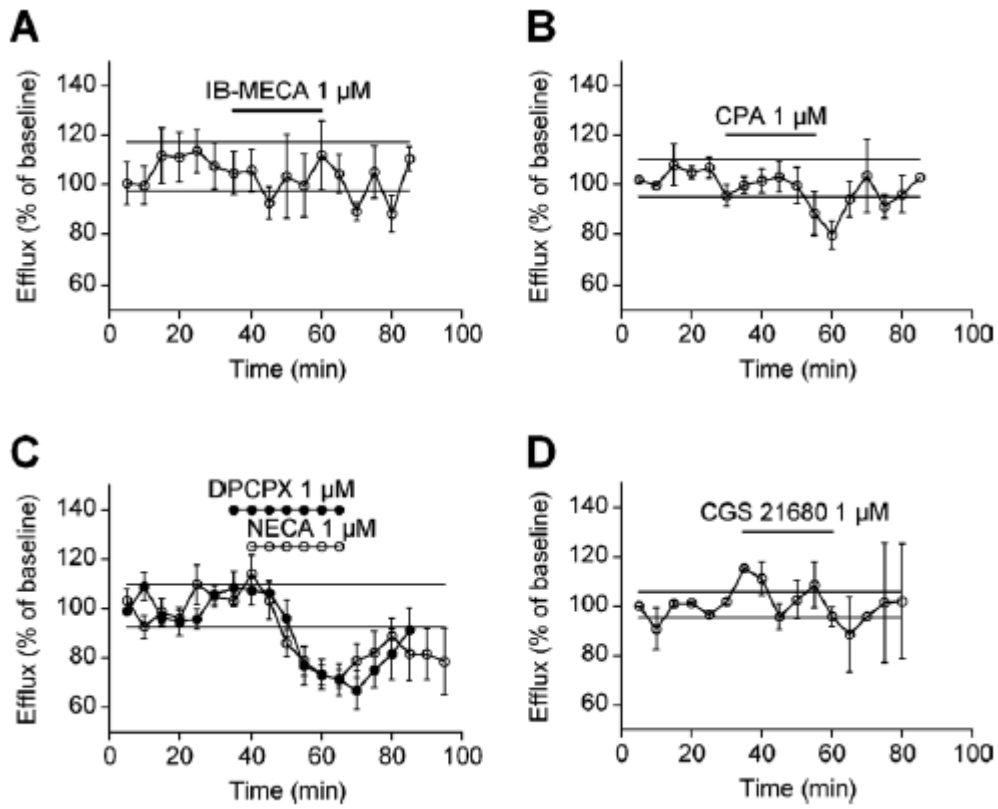


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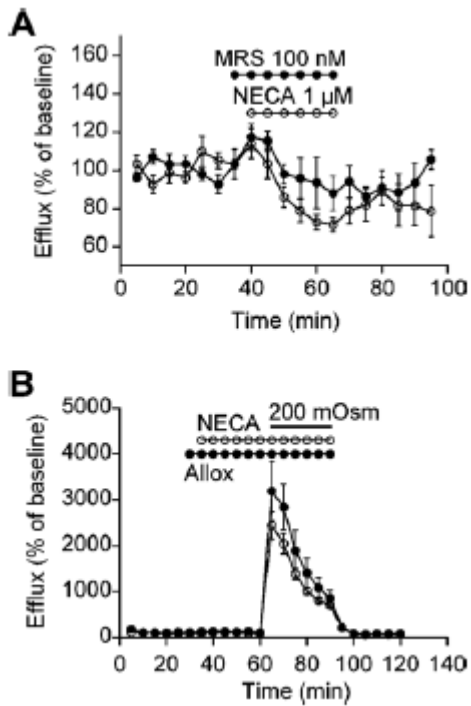


Figure 5.

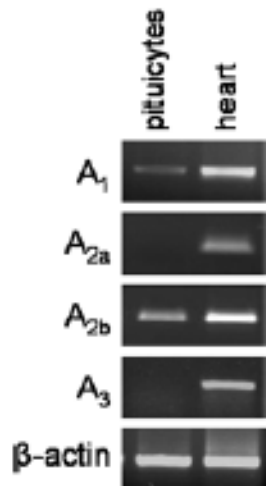


Figure 6.

