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The Osmotic Component of Ethanol and Urea Action is Critical for Their Immediate Stimulation of Thyrotropin-Releasing Hormone (TRH) Release from Rat Brain Septum

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Summary

There is considerable evidence linking alcohol consumption and sedation and TRH in the brain septum. Moreover, innate septal TRH concentration is inversely related to the degree of ethanol preference. Recently we demonstrated in rats that four-week ethanol drinking increased the septal TRH content by 50 %. We had shown previously that ethanol induces neuronal swelling, which is known to evoke the secretion of hormones, peptides and amino acids from various types of cells. We have therefore explored the effect of hyposmotic medium and of 80 and 160 mM ethanol and 80 mM urea (both permeant molecules) in isosmotic and hyperosmotic (preventing cell swelling) media on the *in vitro* release of TRH by the rat septum. Lowering medium osmolarity resulted in a hyposmolarity-related increase in TRH secretion. Both ethanol and urea stimulated TRH release only in isosmolar solution. Our data indicate that ethanol in clinically relevant concentrations can induce TRH release from the septum by a mechanism involving neuronal swelling.

Key words
Ethanol • TRH • Septum • Cell volume • Urea

Introduction

Thyrotropin-releasing hormone (TRH), originally isolated and characterized on the basis of its role in the regulation of pituitary thyrotropin secretion, is a wide-spread CNS peptide (Oliver *et al.* 1974) possessing numerous other biological functions (for review see Lechan 1993, Štrbák *et al.* 2000). TRH in the septum is present in both neuronal perikarya and fibers (Merchenthaler *et al.* 1988, Ishikawa *et al.* 1986). Septal TRH is of special interest in relation to alcohol preference: the TRH content is significantly reduced in the brain septum of alcohol-naive alcohol-preferring rats compared with alcohol-naive alcohol-non-preferring rats (Morzorati and Kubek 1993). TRH injected into the medial septum also antagonizes the depressant action of ethanol (McCown, *et al.* 1985).

We previously demonstrated in rats that four-week ethanol drinking increased septal TRH content by 50 % but did not affect hypothalamic hypophysiotropic TRH (Nikodémová *et al.* 1998). We also showed that ethanol induces neuron swelling within seconds

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(Nikodémová *et al.* 1997). Changes in cell volume can be powerful links in transduction chains regulating cellular function (Haussinger *et al.* 1994) including the modulation of secretory activity (Inukai *et al.* 1992, 1993). Cell swelling evokes the secretion of hormones, peptides, and amino acids from various types of cells (Blackard *et al.* 1975, Greer, *et al.* 1990, Sato *et al.* 1990a, b, c, d, Nikodémová *et al.* 1999, Štrbák and Nikodémová 1999, Benický *et al.* 1998, 2000). In the present experiments, we have evaluated whether ethanol directly affects septal TRH secretion *in vitro* in a short-term static incubation system. We have also explored whether the mechanism of ethanol effect in septum involves cell volume changes and compared its effect with other stimuli known to increase cell volume (e.g. urea and hyposmolarity).

Methods

Male Wistar rats (Charles River, Sulzfeld, Germany) weighing 300-350 g were kept under controlled temperature (22-24 °C) and a constant 12 h light/dark cycle, fed with Purina Chow and tap water *ad libitum*.

Tissue incubations

After decapitation, the brain was rapidly removed and the entire septum dissected (Palkovits and Brownstein 1988). The incubations were performed in stoppered Eppendorf tubes at 37 °C in 5 % CO_2/95 % O_2 atmosphere. After a 60-min preincubation period, the septum was incubated for four 15-min periods in 150 µl medium according to the following sequence: 1) basal medium, 2) stimulating medium (with either ethanol, urea, hyposmotic medium or high KCl), 3) basal medium, 4) high KCl to stimulate TRH secretion by evoking membrane depolarization at the end of each experiment. All media were assayed immediately for determination of released TRH by radioimmunoassay. In one experiment two successive 15 min incubations with the same medium were used for each of the four periods.

Composition of media

Basal medium contained 6 mM $NaHCO_3$, 130 mM NaCl, 5.6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, 1.5 mM ascorbic acid, 2 mM HEPES, pH 7.4, osmolarity 284 mosm/kg H_2O. To minimize degradation of released TRH into the medium, 30 mg of bacitracin/100 ml medium was added. The stimulating isosmolar media were prepared from the basal medium by changing NaCl concentration to maintain physiological osmolarity by the following procedures: plasma membrane-depolarizing medium (77.6 mM NaCl, 56 mM KCl); isosmotic media containing various concentrations of ethanol – 80 mM (91.8 mM NaCl), 160 mM (55.6 mM NaCl) or 80 mM urea (Lachema Brno) (91.8 mM NaCl); hyperosmotic media (concentration of NaCl was not changed) containing either 80 mM ethanol or urea or 160 mM ethanol, osmolarity was 360 and 430 mosmols, respectively; hyposmotic media – 30 % or 50 % hyposmotic medium (202 and 142 mosm/kg H_2O, respectively) was achieved by appropriate dilution of basal medium with water. All media were gassed with 5 % CO_2/95 % O_2 atmosphere for 20 min.

Thyrotropin-releasing hormone determination

TRH released into the media was determined by specific radioimmunoassay (Eskay *et al.* 1976) with several modifications. The TRH antibody was prepared in our laboratory by immunization of rabbits with synthetic TRH coupled to bovine serum albumin and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

Cross-reactivity of the TRH antiserum with TRH degradation products (Sigma, TRH free acid, His-Pro-diketopiperazin, pGlu-Glu-Pro-NH_2, Glu, His, Pro) or TRH precursors (Sigma, TRH-Gly, Lys-Arg-Glu-His-Pro-Gly-Lys-Arg and Lys-Arg-Glu-His-Pro-Gly) was <0.01 %. The crossreactivity with pGlu-Glu-Pro-NH_2 was 8 %. Synthetic TRH (a gift from Prof. Kasafirek, Research Institute of Pharmacology and Biochemistry, Prague) was labeled with $Na^{125}I$ using the chloramine-T method and purified on Sephadex G-15 column (Pharmacia, 60×1 cm). All assays were performed in a total volume 400 µl 0.01 M PBS (pH 7.6). After overnight incubation at 4 °C bound and free peptide was separated by cold 200 µl dextran-coated charcoal (500 mg Norit + 50 mg dextran in 100 ml H_2O). The sensitivity of the assay was 1 pg of TRH per tube. TRH standards were prepared in each utilized medium. Thus, correction for recovery was included. All samples from each experiment were analyzed in the same assay to avoid interassay variation. The intraassay coefficient of variance was 4.2 %.

Data analysis

Data are expressed as mean ± S.E.M., the number of samples (n) are indicated in the legends to the figures. Data were compared by paired Student's t-test and analysis of variance (ANOVA) for repeated measures. For independent groups, ANOVA followed by

Newman-Keuls multiple comparisons were used. Data were considered significantly different at $p<0.05$.

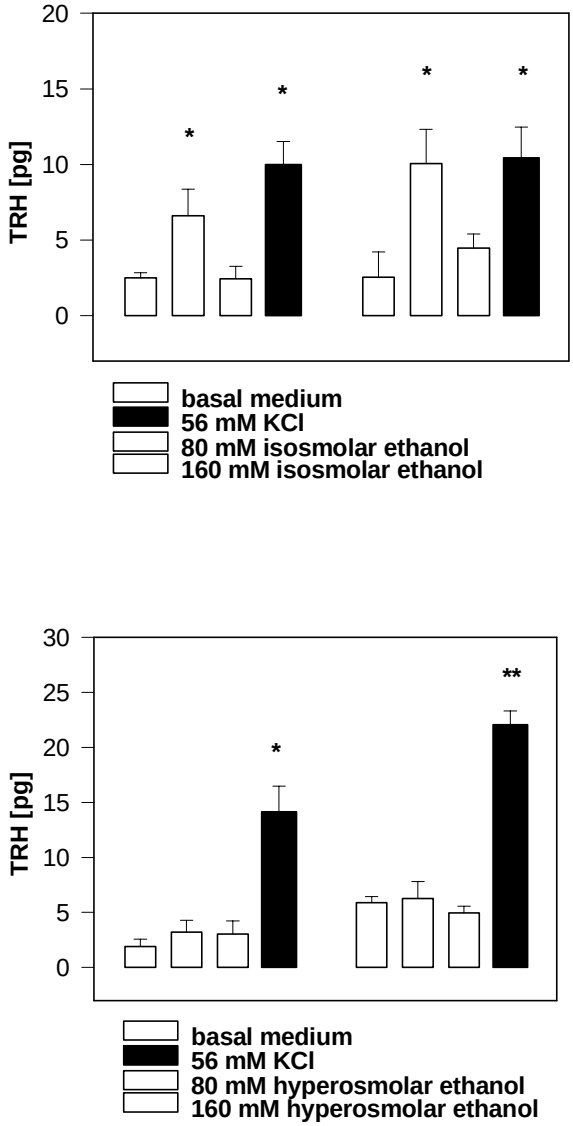

Fig. 1. *Stimulation of TRH secretion by ethanol. The four bars represent four successive 15-min incubations of the same tissue alternatively in basal and stimulating medium. Left, 80 mM ethanol, right 160 mM ethanol. Upper panel: ethanol in isosmolar medium, lower panel: ethanol was simply added to the basal medium, thus increasing its osmolarity by 80 or 160 mosmol/kg. TRH secretion was stimulated by high KCl concentration evoking membrane depolarization at the end of each experiment. Values are means ± S.E.M., n=7 (pooled from two independent experiments) and n=4 for 80 mM and 160 mM ethanol, respectively. * p<0.05, ** p<0.001 compared with the preceding basal secretion.*

Results

Effect of ethanol on TRH release

Ethanol in the medium at both concentrations (80 and 160 mM) stimulated the release of TRH from the septum when applied in isosmolar medium (Fig. 1, upper panel) compared to that released in the preceding or following incubation in basal medium. The functional integrity of the septum was confirmed at the end of incubation by application of high medium K^+ as a nonspecific stimulus (Fig. 1). The simple addition of

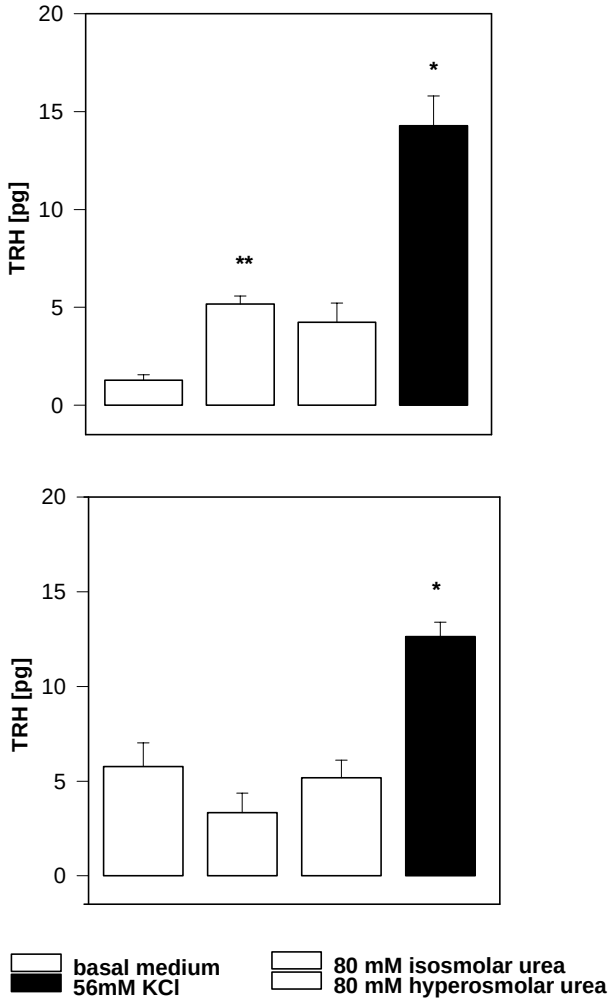

Fig. 2. *Stimulation of TRH secretion by urea. Upper panel: isosmolar, medium lower panel: hyperosmolar medium (80 mM urea). Experimental design as described in Fig. 1. n=4, *p<0.05, ** p<0.001 compared with the preceding basal secretion.*

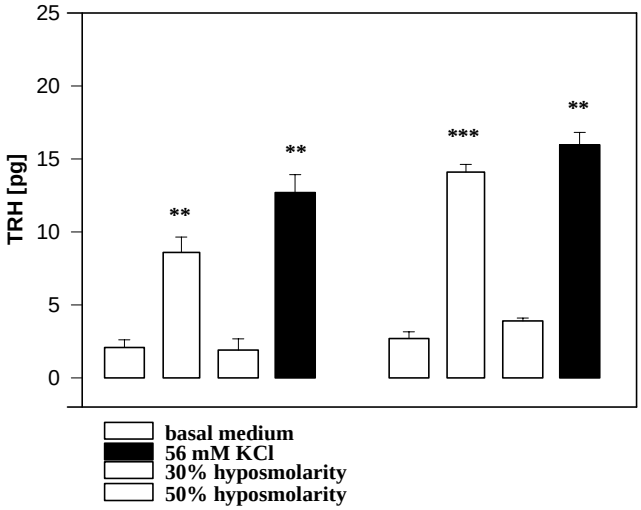

Fig. 3. *Stimulation of TRH secretion by hyposmotic medium. Left – 30 % dilution (n=8, pooled from two independent experiments), right – 50 % dilution (n=3). Experimental design as described in Fig. 1. **p<0.01, ***p<0.001 compared with the preceding basal secretion.*

ethanol to the basal medium, thus making it hyperosmolar, failed to stimulate TRH release at either concentration (Fig. 1, lower panel). Secretion remained low during the following incubation period in the basal medium and responded readily to the nonspecific stimulus at the end of the experiment. Even two successive periods with hyperosmolar ethanol failed to stimulate TRH secretion (data not shown).

Effect of urea on TRH release

Urea in an 80 mM concentration stimulated the release of TRH when applied in isosmolar medium (Fig. 2, upper panel). No response was present if urea was applied in the hyperosmolar medium (Fig.2, lower panel). The clear response to nonspecific stimulation at the end of incubation demonstrated the functional capacity of the tissue.

Effect of hyposmolarity on TRH release

Decreasing medium osmolarity resulted in a hyposmolarity-related increase in TRH secretion (Fig. 3, left and right sections) which returned to basal values in isosmolar basal medium. The control stimulus (56 mM) depolarizing K^+ solution at the end of the experiment was effective in each case.

Discussion

There is considerable evidence linking alcohol consumption and sedation and TRH in the brain septum. A TRH analog attenuates alcohol preference in alcohol-preferring rats (Rezvani *et al.* 1992) and monkeys (Rezvani *et al.* 1997). Injection of TRH into the medial septum significantly shortens ethanol impairment of the righting reflex (McCown *et al.* 1985). TRH is effective in the medial septum but not in the nucleus accumbens or raphe obscurus (McCown *et al.* 1986). There is a lower content of septal TRH in alcohol-naive alcohol-preferring than in non-alcohol-preferring rats. Recovery of the righting reflex after a sedating dose of ethanol is associated with an increase of septal TRH (Morzorati and Kubek 1993). These data strongly suggest that endogenous septal TRH may be involved in arousal from drug-induced sedation and that innate differences in septal TRH may be associated with a preference for ethanol. However, data on the mechanism of the effect of ethanol on septal TRH were lacking. We have now shown that ethanol directly stimulates *in vitro* TRH release from the septum.

Ethanol and urea are osmotically active substances that easily cross the plasma membrane. Because their entrance into the cell is accompanied by water influx, they can impair the cell volume dependent on an imbalance between intra- and extracellular osmotic pressure. We previously demonstrated (Nikodémová *et al.* 1997) that isosmotic 80 mM ethanol or hyposmolar medium induces neuronal swelling within several seconds, a 12 % increase was reached in 40 seconds. However, when applied in a hyperosmolar medium, 80 mM ethanol did not change the cell volume and in higher concentrations caused cell shrinkage (Nikodémová *et al.* 1997). It has recently been recognized that changes in cell volume can act as powerful links in the transduction chains regulating cellular function including secretory activity (Haussinger *et al.* 1994, Lang *et al.* 1998). Cell swelling (induced by hyposmolarity, ethanol or urea) evokes the secretion of hormones and peptides from various types of cells (Blackard *et al.* 1975, Greer *et al.* 1990, Sato *et al.* 1990 a,b,c,d, Nikodémová *et al.* 1999, Benický *et al.* 2000). Isosmolar ethanol and urea in the present experiments produced comparable effects to that induced by medium hyposmolarity. After restoration of the isosmolar medium, secretion returned to basal

values. These results indicate that the changes induced in the septum by the permeants and hyposmolarity during a 15-min incubation are short-lasting and reversible.

The stimulating effect of ethanol and urea in an isosmolar (but not in a hyperosmolar medium) on septal TRH secretion in our present study indicates that this effect involves cell swelling. This is in accordance with our previous data showing stimulation of the release of hypophysiotropic hypothalamic TRH (Nikodémová *et al.* 1997) as well as TRH and insulin from isolated pancreatic islets (Benický *et al.* 1997, 2000) by isosmotic ethanol in a dose-dependent manner. The release of another hypophysiotropic hypothalamic neurohormone GnRH is also stimulated by only isosmolar (but not by hyperosmolar) ethanol (Inukai *et al.* 1993), medium hyposmolarity or isosmolar urea (Inukai *et al.* 1992). In conclusion, our data indicate that ethanol in clinically relevant concentration induces TRH release from the septum by a mechanism involving neuronal swelling.

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Reprint requests

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