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Release of endogenous benzodiazepine receptor ligands (endozepines) from cultured neurons

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Endozepines are naturally occurring small organic molecules, devoid of peptidic bonds and halogens, that act as allosteric modulators of the $GABA_A$ receptor through their actions at the benzodiazepine binding site. Endozepines are present in physiologically significant amounts in the brain and can act as potent positive allosteric modulators of the $GABA_A$ receptor. In this study, 3 endozepines present in cultured cerebellar granule cells were found to be released from neurons in a potassium-stimulated, calcium-dependent fashion. This release could also be mimicked by increasing concentrations of veratridine. Although endozepines were also found in cultured astrocytes, they could not be released in significant amounts by potassium depolarization. Differential release under depolarizing conditions and granule cell content of the various endozepines suggested a possible metabolic relationship between these two processes.

The $GABA_A$ receptor is a hetero-oligomeric protein that is composed of subunits that contribute to the allosteric regulation of GABA action [12]. The α subunit contains a binding site for benzodiazepines, which can act as positive allosteric regulators of GABA's actions at the $GABA_A$ receptor [7]. An endozepine is defined as any non-peptide naturally occurring small organic molecule, devoid of halogens, that can act as an allosteric modulator of $GABA_A$ chemical gating by GABA acting via the benzodiazepine binding site. Recently, halogenated 1,4-benzodiazepines and non-benzodiazepine substances have been identified in the CNS in animals and man [1, 5, 6, 11, 13]. Although halogenated benzodiazepines are present, their concentrations are physiologically irrelevant; by contrast, the non-halogenated endozepines appear to be present in potentially physiologically significant amounts [8]. Moreover, their content is increased in the spinal fluid of patients with hepatic encephalopathy [6]. Recently, several of these endozepines have been purified [8]. Mass spectrometric analysis established that they are not halogenated compounds and thereby differ from benzodiazepines used therapeutically. The structure of at least one of them has a quinoline core [8]. They are present in rat and human brain, and their content varies within different brain structures. Like neurotransmitters or neuromodulators, the endozepine content of the particulate fraction of brain homogenates is greater than that of the supernatant. When tested on primary cultures of neonatal rat cortical neurons using voltage patch-clamp technology, they act as potent positive allosteric modulators of the native $GABA_A$ receptors that are present on these cells [8].

If endozepines act as natural ligands for the benzodiazepine binding site, then their release from cellular storage sites in calcium-dependent manner following depolarization might be expected, as it is described for other neurotransmitters and neuromodulators. The purpose of the present experiments was to determine whether the extraneuronal availability of endozepines is controlled in a manner similar to that of neurotransmitters/neuromodulators. Previously, we had shown that the cerebellum, as well as other brain regions, contains endozepines [9]. To precisely control cellular conditions, the present studies were performed on cultured cerebellar granule cells (essentially a culture of monotypic cells), which are also known to have $GABA_A$ related benzodiazepine receptors. These studies demonstrate that several endozepines can be released from primary cultures of cerebellar granule cells, but not from primary cultures of cere-

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bellar glial cells, in a high potassium-stimulated, calcium-dependent fashion.

Cultured cerebellar granule cells were prepared from 8 day old rats, as described by Gallo et al. [3]. Briefly, cells obtained from trypsin digest of cerebellum were resuspended and plated in freshly prepared culture medium (Basal Eagle's Medium) (BME, Flow Labs), 10% fetal calf serum (Gibco), 25 mM KCl, 2 mM glutamine, and 0.1% (w/v) gentamicin (Gibco), at a density of $1–1.2 \times 10^6$ cells/ml into 100 mm Nunc culture dishes coated with poly-L-lysine, 5 μg/ml. Cells were incubated at 37°C with humidity, in an atmosphere of 5% CO_2/95% air. Cytosine arabinofuranoside (Ara-C; 10 μM) was added to cultures after 16 h of culture to inhibit cell division and attachment of non-neuronal cells.

Cerebellar astrocytes were prepared as described by Woodhams et al. [14]. For those cultures, the culture medium consisted of BME, 10% fetal calf serum, 2 mM glutamine, and 0.1% (w/v) gentamicin. The KCl concentration for glial cultures was maintained at 4.7 mM and Ara-C was not added.

After 8 days in culture, cells were washed 3 times with 5 ml of Locke's solution (in mM: KCl 5.6, NaCl 154, $NaHCO_3$ 3.6, $CaCl_2$ 2.3, $MgCl_2$ 1, D-glucose 5.5, HEPES 5). Cells were then incubated for 5–10 min with Locke's solution containing 50 mM KCl, with an appropriate reduction in NaCl to maintain osmolality. Calcium free Locke's solution was prepared without $CaCl_2$, and contained 1 mM EGTA. For veratridine experiments, washed cells were incubated in Locke's solution containing veratridine at concentrations ranging from 2.5 to 30 μM. Following veratridine, the incubation medium was collected and lyophilized for endozepine analysis. Cultured granule cells and astrocytes were collected by adding 5 ml of acetic acid (1 M) and then scraping cells from plates. For protein analysis aliquots of either incubation medium or cell scrapings were saved. There was no measurable protein in incubation medium, while collected cells usually had 400 μg protein/100 mm dish. Typically each experimental protocol was performed on 10 plates of cells.

Endozepines were quantified in cells or in incubation medium by HPLC purification as detailed previously. Pooled incubation medium or cells were lyophilized, then reconstituted in a small volume of distilled deionized water (DDW) containing 0.1% trifluoroacetic acid (TFA). Samples were then filtered (0.45 μm, nylon Acrodisk, Gelman) and passed through C_{18} Sep-Pak cartridges. The cartridges were washed with 5 ml water/0.1% TFA (Pierce, IL, USA) and the endozepines were eluted with 3 ml acetonitrile/0.1% TFA. These samples were vacuum dried (Savant Speed-Vac), reconstituted with water/0.1% TFA and purified by HPLC as described previously [6]. Endozepines were eluted from a reverse phase column (250 × 4 mm, Bio-Sil ODS 10, Bio-rad) using a water/0.1% TFA and acetonitrile/0.1% TFA gradient at 1%/min from 0 to 70% acetonitrile/0.1% TFA. All fractions were lyophilized and reconstituted in water or 50 mM sodium phosphate buffer (pH 7.4) prior to radioreceptor binding assays. Extraction efficiency was monitored by adding trace amounts (1,000 cpm) of [^{3}H]flumazenil (NEN) to medium or cells prior to extraction. Recovery averaged 83.9 ± 4.1% (S.E.M.).

Previous studies have shown that the endozepines purified with this methodology are devoid of peptide bonds, or are previously identified chemicals capable of altering benzodiazepine binding such as β-carbolines, inosine, DBI, ammonia, nicotinamide, porphyrin or hypoxanthine [8].

Endozepine activity was quantified by the ability of reconstituted HPLC fractions enriched with endozepines to inhibit the binding of [^{3}H]flunitrazepam (spec. act. 87 Ci/mmol, NEN, Boston, MA, USA) to rat cerebellar membranes as described previously [6]. Membrane protein was measured by the method of Bradford [2].

Aliquots of reconstituted HPLC fractions (50 μl) were incubated in 50 mM sodium phosphate buffer (pH 7.4)

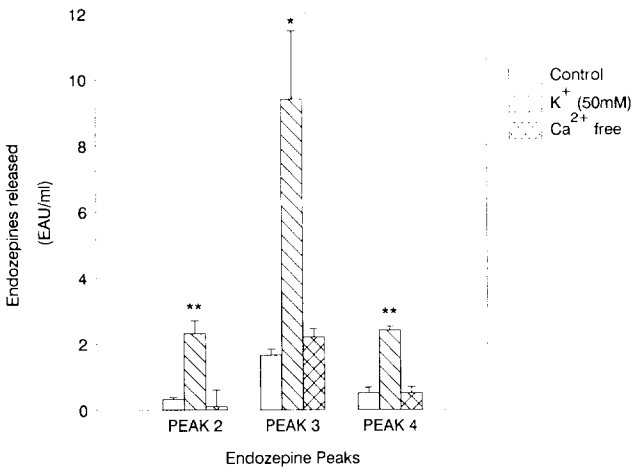

Fig. 1. Release of endozepines from cultured cerebellar granule cells following treatment with KCl (50 mM). Cultured cells were washed with Locke's medium, then incubated 5 min with a depolarizing concentration of KCl (50 mM). In some cases, experiments were performed with calcium free depolarizing medium which contained 1 mM EGTA. Medium was collected from 5–10 culture dishes for each treatment, then pooled, lyophilized and extracted as described in the text. Reconstituted specimens were purified with reverse-phase HPLC, and HPLC fractions were tested for the ability to displace [^{3}H]flunitrazepam from rat cerebellar membranes. Three peaks of endozepine activity were identified. Endozepine activity was expressed in endozepine arbitrary units (EAU), which is the number of picomoles of diazepam required to inhibit [^{3}H]flunitrazepam binding to rat cerebellar membranes to the same extent as endozepines. Error bars represent S.E.M. from 3 experiments. $^*P<0.05$ and $^{**}P<0.01$ vs. control.

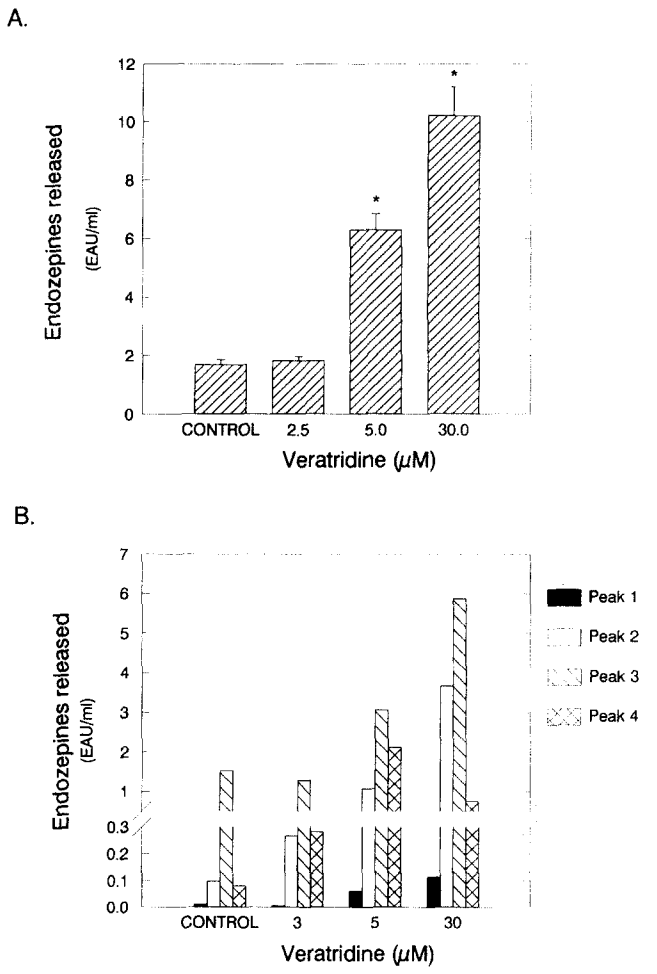

Fig. 2. Effects of veratridine-induced cell depolarization on the release of endozepines from cultured granule cells. Total endozepine release is shown in A. The effects of veratridine on individually purified endozepines is shown in B. Purification and quantification of endozepines was as described in the text. Error bars represent S.E.M. from 3 experiments. *$P<0.01$ vs. control.

which contained 5 μM GABA, 1 nM [^{3}H]flunitrazepam, and rat cerebellar membranes (100 μg) in a final assay volume of 0.25 ml for 1 h at 4°C. The reaction was terminated by vacuum filtration through glass fiber filters (type C, Cambridge Technology, Watertown, MA, USA) using a cell harvester (Cambridge Technology). The filters were transferred to scintillation vials (Wheaton Omnivials, Millville, NJ, USA) with scintillation cocktail (Safety Solve; Research Products International) and radioactivity was measured. The endozepine activity was quantified by an arbitrary unit (EAU, endozepine arbitrary unit) defined as the number of picomoles of diazepam required to inhibit [^{3}H]flunitrazepam binding to rat cerebellar membranes to the same extent as endozepines.

All reagents were obtained from Sigma Chemical Co., unless indicated otherwise. HPLC mobile phases were obtained from Fisher Chemical company and were all HPLC grade.

Statistical analysis between groups was performed by Student's t-test. A P value of <0.05 was considered significant. Data are expressed as means ± S.E.M., unless indicated otherwise. All experiments were performed 2–4 times.

Endozepines are present in the cerebellum in vivo [9] and could be measured in cerebellar granule cells in vitro. To exclude a contamination by the serum used to culture the granule cells and astrocytes we performed the assay after repeated washings (up to 5) with Locke's solution, and found that endozepines were still present in the cultured cells.

Depolarization of cultured granule cells by potassium produced a marked increase in the incubation medium content of endozepines. Total endozepines released into medium significantly ($P<0.01$) increased 6-fold following treatment with 50 mM KCl, from 2.27 ± 0.31 (S.E.M.) EAU/ml in controls to 14.1 ± 2.7 (S.E.M.) EAU/ml in medium from treated cells. Cell content of total endozepines did not significantly change in control cells (20.4 ± 12.4 EAU/mg protein) following depolarization with potassium (51.4 ± 9.0 EAU/mg protein).

Under baseline conditions, HPLC analysis of extracted medium, granule cells and astrocytes revealed the presence of 3 endozepines (peaks 2–4), with retention times of 60–62 min (peak 2), 65–67 min (peak 3) and 73–75 min (peak 4). Peak labeling was based on previously identified endozepine retention profiles from rat brain [8]. Depolarization of granule cells leads to a release of all three endozepines into the medium with a 14-fold increase in peak 2, a 6-fold increase in peak 3 and a 5-fold increase in peak 4 (Fig. 1).

When granule cells were exposed to increasing concentrations of veratridine there was a clear dose–response relationship. Increasing amounts of veratridine lead to an increase in total endozepine release (Fig. 2A) with a 6-fold increase in endozepine release by 30 μM veratridine. Similarly, there was a dose-dependent increase in peaks 2-4 following treatment with veratridine, and at high doses, small amounts of peak 1 were detectible (Fig. 2A). Although the concentration of medium endozepines increased with veratridine treatment, total granule cell content of endozepines decreased with increasing concentrations of veratridine, except with a 30 μM dose, when there was a large apparent increase in cell content (Fig. 3A). This increase was not statistically significant due to variability in cell content of endozepines. When the ratio of medium-to-cell endozepine content was calculated, the dose-dependent increase in medium release was still evident, except at 30 μM veratridine (Fig. 3B).

Finally, to determine if the release of endozepine was

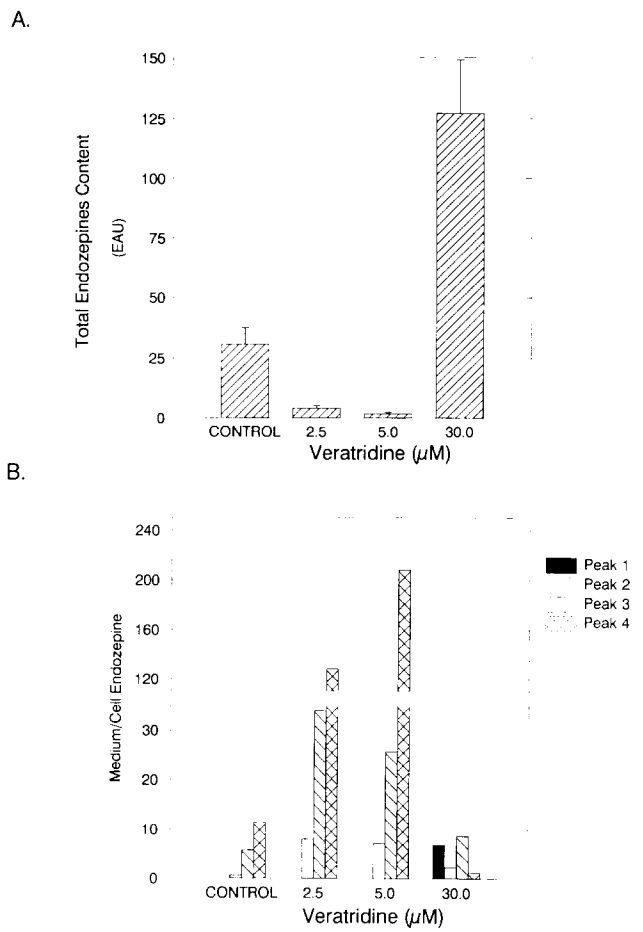

Fig. 3. Effect of veratridine on granule cell endozepine content. Total granule cell endozepines are shown in A. A comparison of the ratio of total medium endozepines to total cell endozepine content for each peak is shown in B. Error bars represent S.E.M. from 3 experiments.

calcium-dependent, the depolarization induced release was studied in the presence and absence of calcium. As shown in Fig. 1, potassium-stimulated endozepine release was markedly diminished in the absence of calcium.

Only small amounts of endozepine (peaks 2 and 4) could be extracted from glial cells and there was no appreciable release of glial cell endozepines into the medium under depolarizing conditions (data not shown).

Since the original discovery of high affinity binding sites for halogenated 1,4-benzodiazepines on the $GABA_A$ receptor, there have been multiple attempts to identify an endogenous ligand for this site. The cloning of the $GABA_A$ receptor and identification of subunits that confer benzodiazepine sensitivity further support the possibility of natural benzodiazepine receptor ligands [12]. Endozepines are a group of endogenous substances that by definition act as allosteric modulators of GABA action at $GABA_A$ receptors. Recently several non-peptide, low molecular weight endozepines have been identified in human and rat brain [8]. Although the exact chemical structure is not yet known, at least one possesses a quinoline core. None are halogenated benzodiazepines, which have been identified by others in trace amounts in the CNS [13]. Regionally, endozepines are present in variable amounts in the CNS [9]. Endozepines are also present in brain in potentially physiologically relevant concentrations (50–100 nM). For these reasons, a study of the physiological regulation of endozepines was necessary.

The present study convincingly demonstrates that several endozepines can be released from primary cultures of cerebellar granule cells by potassium-stimulated depolarization. Furthermore, this release can be mimicked by veratridine-induced neuronal depolarization in a dose-dependent manner. Significantly, the release of all peaks is clearly dependent on the presence of calcium, thus similar to neurotransmitter release. Quantitatively, the effects of either potassium or calcium on endozepine release varies for each peak, suggesting that the intracellular pools of these substances may be under differential control.

Since the exact chemical identity of the peaks is not known, the metabolic relationship between the peaks is not yet clear. It is likely that certain peaks can act as precursors for others. With increasing depolarization, peak 4 maintained the highest medium to cell ratio, while peaks 2 and 3 increased only slightly or decreased. This suggests that the latter two peaks could, although not necessarily, act as precursors for peak 4.

The localization of released endozepines appears to be restricted to neurons, since cultured astrocytes were unable to release endozepines under depolarizing conditions. Thus the present experiments demonstrate that brain endozepines can be released from neurons in a manner similar to other neurotransmitters and neuromodulators. Comparable physiologic regulation of brain endozepines may occur in vivo. Recent preliminary studies examining microdialysis dialyzates from rat cortex have demonstrated the presence of similar endozepines that also appear to be increased by depolarization (unpublished data).

The origin of brain endozepines is not yet known. Granule cells may synthesize these compounds from a substrate present in the cells, or they may use precursors present in the culture medium. The structure of one endozepine, peak 2, was recently shown to have a molecular weight of 265, was not halogenated, and included a quinoline core [8]. It is likely that either aromatic amino acids, or dietary precursors with a quinoline core structure, may serve as precursors. In fact, a number of dietary quinolines and isoquinolines are available from

plant (e.g. anthranilic acid) and protein sources (e.g. tryptophan) [4, 10].

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