Plasmid pCCK, kindly donated by Dr. Jack Dixon, was derived from the CCK cDNA plasmids pCK2 AB5 and pCK16AB5 (Deschenes et al., 1984) and contains a 535 bp cDNA fragment complementary to the mRNA of rat preproCCK. A double digest with HindIII and EcoRI restriction enzymes excised the cDNA fragment, which was separated from the rest of the plasmid by gel electrophoresis and labelled with [35S]PtdCTP (Feinberg & Vogelstein, 1982; 1984) to a specific activity of 1-3 x 109 c.p.m. /μg. The labelled DNA was separated from unincorporated labelled nucleotides using Sephadex G-50, ethanol precipitated and left overnight at −20°C. The probe was sedimented and resuspended in prehybridization buffer to the desired concentration and denatured before hybridization. A 201 bp DNA fragment of the human β-globin gene (donated by P. J. Nicholls) was labelled to the same specific activity and used as a control probe.

Cryostat sections (15 μm) of frozen rat brain were thaw mounted on to gelatinized slides, fixed in 4% formalin (10 min), washed twice in phosphate-buffered saline (5 min), ethanol (2 min) and left to air dry. Prehybridization buffer (2 x SSC, 5 x Denhardt’s, 0.5% SDS, 50 mM phosphate buffer pH 7.0, 50% formamide, 100 μg of denatured salmon sperm DNA/μl) was applied to the sections and left overnight at −20°C. The probe was sedimented and labelled DNA was separated from unincorporated labelled nucleotides using Sephadex G-50, ethanol precipitated and left overnight at −20°C. The specificity of hybridization under these experimental conditions was tested by treatment with ribonuclease and by use of a cDNA probe for β-globin mRNA which is not expressed in brain. The pattern of labelling was completely lost in the presence of ribonuclease and with the cDNA probe for β-globin mRNA only background levels of labelling were seen. It was unlikely that the probe was binding to protein as treatment with proteinase K was without effect. The protocol described above allows visualization of preprocholecystokinin mRNA within the rat brain. High levels of mRNA were found in cerebral cortex (especially in the caudate, piriform cortex, cingulate, hippocampus and thalamus). This distribution correlates well with that of CCK immunoreactivity within cell bodies and with results of hybridization in situ histochemistry recently obtained by Siegel & Young (1985) using a [35S]-labelled complementary RNA probe.


Larsson, L.-I. & Rehfeld, J. F. (1979) Brain Res. 165, 201-211

Siegel, R. E. & Young, W. S., III (1983) Neuropeptides 6, 573-580


Received 10 June 1986

Release of neurokinin A-like immunoreactivity from rat substantia nigra slices in vitro

F. JAVIER DIEZ-GUERRA and PIERS C. EMSON


The tachykinins are a family of peptides whose members share the common C-terminal sequence Phe-X-Gly-Leu-Met-NH2. These include the non-mammalian peptides edeoinisin, kassinin and physalaemin, and substance P (SP), one of the best characterized peptide neurotransmitter/modulators in the mammalian nervous system (Jessel & Womack, 1985). Recently, this family of peptides has been considerably enlarged by the isolation and characterization of three novel tachykinins, namely neurokinin A (NKA) (also called neurokinin-α, substance K or neuropeptide K), neurokinin B (NKB) (also called neurokinin-β or neuromedin L) (Kangawa et al., 1983; Kimura et al., 1983) and neuropeptide K (NPK) (Tatemoto et al., 1985), an N-terminally extended form of NKA. Molecular genetic studies have revealed the presence of two bovine preprotachykinins strongly (e.g. lateral thalamus, lateral and medial geniculate, dorso-medial thalamus, ventrodorsal thalamus and the ventral nucleus). Within the cerebral cortex a laminar distribution was evident, high levels of mRNA CCK being present in an outer band (layers I to III), no labelling in layer IV and a second band of heavy labelling (layers V and VI). The CA1-3 cell layers of hippocampus also showed up as intense bands of labelling. Low levels of mRNA CCK were found in caudate/putamen, kidney and adrenals.

The specificity of hybridization under these experimental conditions was tested by treatment with ribonuclease and by use of a cDNA probe for β-globin mRNA which is not expressed in brain. The pattern of labelling was completely lost in the presence of ribonuclease and with the cDNA probe for β-globin mRNA only background levels of labelling were seen. It was unlikely that the probe was binding to protein as treatment with proteinase K was without effect. The protocol described above allows visualization of preprocholecystokinin mRNA within the rat brain. High levels of mRNA were found in cerebral cortex (especially in the caudate, piriform cortex, cingulate, hippocampus and thalamus). This distribution correlates well with that of CCK immunoreactivity within cell bodies and with results of hybridization in situ histochemistry recently obtained by Siegel & Young (1985) using a [35S]-labelled complementary RNA probe.

Abbreviations used: SP, substance P; NKA, neurokinin A; NKB, neurokinin B; NPK, neuropeptide K; PPT, preprotachykinin; GABA, γ-aminobutyric acid.
small plastic chambers (150 µl internal volume) which trapped the tissue between two pieces of nylon mesh and fitted into a special purpose built perfusion system. The chambers contain a pair of platinum foil electrodes (5 mm apart), which are connected to a Grass SS-88 stimulator via stimulus isolation and constant current units. After 30 min perfusion (0.1 ml/min) with warm (37°C) Yamamoto’s Heps medium supplemented with 0.1% bovine serum albumin, a collection of 5-min fractions started and progressed for a maximum of 90 min. The perfusate was collected in tubes containing 0.25 ml of 0.04 M-HCl, frozen immediately on dry ice and freeze-dried before radioimmunoassay. Once the experiment was completed, the tissue remaining in the chambers was collected, homogenized in 1 ml of 0.1 M-HCl, centrifuged for 5 min and the supernatant removed, frozen, freeze-dried and assayed for NKA.

The basal release levels of NKA-like immunoreactive material were just on the limit of detection of the assay (1–5 pg) and amounted to less than 0.1% of the tissue content. Perfusion with high K+ (45 mM, 2 min) or electrical stimulation (25 Hz, 4 mA, 2 min) both evoked Ca2+-dependent release. Electrical stimulation increased consistently the basal release levels to 1% of the tissue stores and was completely blocked by the addition of tetrodotoxin. K+ depolarization elicited release ranging from 5 to 10% of the tissue content. The recovery of released immunoreactivity was significantly enhanced by the addition of a mixture of peptidase inhibitors to the perifusion medium (1 µm-thiorphan or 1 µm-phosphoramidon were added as inhibitors of metalloendopeptidase 24.11, 10 µM-bestatin was added to inhibit aminopeptidase, and 10 µM-captopril was used to inhibit angiotensin-converting enzyme). A second stimulation period, either electrical or K+ results in much reduced evoked release, about 30–50% of the material released in response to the first stimulation.

When 2 min stimulation periods were applied at 5, 10, 25 and 50 Hz, increasing amounts of immunoreactivity were detected in the perifusate. However, analysis of the material released per unit pulse indicated a clear facilitation phenomenon between 5 and 25 Hz and no further increase at higher frequencies. Reversed-phase h.p.l.c. of the perifusates after stimulation revealed the presence of NKA, NKB and NPK along with their oxidized forms, suggesting stimulated release of all three peptides although NKA was the major immunoreactive form released. Finally, the addition of picrotoxin (50 µM), a γ-aminobutyric acid (GABA) blocker, to the perifusion medium increased both basal and stimulated release, whereas muscimol (50 µM), a GABA-A receptor agonist, reduced electrically evoked release.

In the presence study we provide experimental evidence for the release of tachykinin-like peptides from the rat substantia nigra. The fact that the release (especially that electrically evoked) was Ca2+-dependent, tetrodotoxin-sensitive, showed frequency facilitation and was modulated by GABA-related drugs, suggests the release is of physiological significance.

Thyrotrophin-releasing hormone analogue binding to central thyrotropin-releasing hormone receptors

ROBERT G. ROBERTSON,* JULIE A. KELLY† and EWAN GRIFFITHS*

*Department of Physiology, University of Manchester Medical School, Manchester M13 9PT, U.K.; †Joined Chemistry Department, Manhattan College, Riverdale NY 10471, U.S.A.

The actions of thyrotrophin-releasing hormone (TRH; Glp-His-ProNH₂) in the central nervous system are mediated by specific TRH receptors (Griffiths, 1985). Several analogues of TRH have been synthesized with enhanced central activity, though the precise reasons for this enhanced activity is not fully understood (Metcalfe, 1982). To define their mechanisms of actions, the ability of various TRH analogues to displace [125I]-TRH from receptors in rat brain has been investigated together with their stability to degradation by rat brain peptides. Coronal sections (15 µm) were cut by cryostat from brain frozen in isopentane at −40°C, mounted on clean microscope slides and dried for several minutes. These were then incubated in 50 mM-Tris/HCl (pH 7.4) containing 0.1% bovine serum albumin and 0.4 µg of bacitracin/ml with 30 nM [125I]-TRH (Amersham International; 54 Ci/mM). After incubation, the slides were rinsed twice with 50 mM-Tris/HCl (pH 7.4) containing 0.1% bovine serum albumin at 4°C for 2 min. Ligand binding was measured by transferring the sections to scintillant (PCS/toluene, 1:1) and subsequent fl-emission counting. Specific binding was calculated as the difference in radioactivity bound in the presence and absence of 20 µM-TRH (Bachem U.K.). For analogue studies, coronal sections of rat brain extending through the hypothalamus were incubated with 30 nM-[125I]-TRH and increasing amounts of each TRH analogue. Stability to degradation by rat brain peptides was determined by incubation with two subcellular fractions (soluble and particulate), followed by separation of metabolites using h.p.l.c. (Griffiths et al., 1985).

For coronal sections in the hypothalamic region, Woolf plots from saturation analysis of [125I]-TRH binding showed a binding affinity (Kd) of 7.2 ± 1.5 nM (n = 9; ± S.E.M.) and a density of binding sites (Bmax) of 10.6 ± 1.0 fmol/mg of protein. Coronal sections at the level of the brain stem displayed a similar binding affinity (7.1 nM) but a lower density of receptors (Bmax, 3.9 fmol/mg of protein). Displacement studies with various TRH analogues revealed the following IC50 values: [3-methyl-His]-TRH, 0.87 nM; TRH, 10.3 nM; [Glp-His-(3,3'-dimethyl)ProNH₂], 35 nM; CG3703 (6-[400]-aminoadipyl-His-thiazolidine-4-carboxamide), 400 nM. A qualitative autoradiographic study showed highest TRH receptor levels in the amygdala, nucleus accumbens, periaqueductal grey, sensorimotor and temporal cortex, pontine nuclei and facial nuclei, all sites at which TRH may have a physiological significance.

Abbreviation used: TRH, thyrotrophin-releasing hormone.