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Identification of α- and β-species of calcitonin gene-related peptide in the rat amygdala after separation with capillary zone electrophoresis

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ABSTRACT

Calcitonin gene-related peptide (CGRP) may be present in two forms in nervous tissue. Reversed-phase high-performance liquid chromatography has previously been found to be insufficient to clearly separate α-CGRP and β-CGRP. A method for the separation of CGRPs by capillary zone electrophoresis has been developed. Separation of human or rat α-CGRP and β-CGRP was achieved at pH values between 3.5 and 4.5 and a potential of 20 kV in a fused-silica capillary. Electrophoresis of an extract of rat amygdala in a micropreparative way, with subsequent radioimmunoassay, revealed for the first time the presence of α-CGRP and β-CGRP in this brain area. The method may thus be used for separation of CGRPs, to reveal the distribution of α-CGRP and β-CGRP, and for purity control.

INTRODUCTION

Calcitonin gene-related peptide (CGRP) is a member of a peptide family with a highly conserved amino acid sequence, occurring in a variety of species [1,2]. In rats and humans a separate gene from that encoding calcitonin and α-CGRP encodes a peptide with a close structural homology to α-CGRP [3,4]. This peptide is called β-CGRP and differs from α-CGRP only by the substitution of one amino acid in rats and by three amino acids in humans (see Table I). The messenger ribonucleic acid (mRNA) for rat β-CGRP has previously been shown to occur in the brain and sensory ganglia. Although human β-CGRP has been isolated from the spinal cord [5], it remains to be established whether expression of a specific mRNA necessarily results in production of the predicted final peptide. The exact distribution of α-CGRP and β-CGRP in the brain has not yet been established, since the hitherto available antisera lack the specificity to discriminate unambiguously between α-CGRP and β-CGRP, and a clear separation by reversed-phase high-performance liquid chromatography (HPLC) has not yet been achieved. Mulderry *et al.* [1] separated rat α-CGRP and β-CGRP by cation-exchange chromatography using a fast protein liquid chromatography gradient system. Recently, Andrew *et al.* [2] raised monoclonal antibodies distinguishing between human α-CGRP and β-CGRP. Their usefulness for measuring rat or human α-CGRP and β-CGRP separately in nervous tissue has, however, not yet been tested.

Capillary zone electrophoresis has become a powerful tool for the separation of ionic species including proteins, peptides and polynucleotides during the last decade [6,7]. Recently, the separation of neuropeptides, such as structurally closely related opioid peptides, as well as of bradykinin and Lys-bradykinin, by capillary zone electrophoresis has been reported [8].

This paper describes the separation of human and rat α-CGRP and β-CGRP by capillary zone electrophoresis. Furthermore, we separated an extract of rat amygdala in a micropreparative

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TABLE I

PRIMARY STRUCTURE OF CGRPs USED IN THE PRESENT STUDY

The amino acids that differ in rat α-CGRP and rat β-CGRP, in human α-CGRP and in human β-CGRP, and in human β-CGRP and in chicken CGRP, respectively, are printed in bold type.

Compound	Structure
Rat α-CGRP	SCNTATCVTH RLAGLLSRSG GVVKDNFVPT NVGSEAF
Rat β-CGRP	SCNTATCVTH RLAGLLSRSG GVVKDNFVPT NVGSKAF
Human α-CGRP	ACDTATCVTH RLAGLLSRSG GVVKNNFVPT NVGSKAF
Human β-CGRP	ACNTATCVTH RLAGLLSRSG GMVKSNFVPT NVGSKAF
Chicken CGRP	ACNTATCVTH RLADFLSRSG GMVKSNFVPT NVGSKAF

manner and identified α-CGRP and β-CGRP by radioimmunoassay in collected fractions.

EXPERIMENTAL

Chemicals

For all experiments, HPLC-grade water from a four-stage purification system (E-pure Barnstead, UK) was used. Calcitonin gene-related peptides (rat α-CGRP, rat β-CGRP, human α-CGRP, human β-CGRP, chicken CGRP) were purchased from Peninsula (Heidelberg, Germany). The primary structures of the used peptides, as declared by the supplier, are listed in Table I. Hydroxyphenoxymethylcellulose (HPMC) was obtained from Sigma (Munich, Germany). All other reagents were of analytical grade and purchased from Merck (Darmstadt, Germany).

Instrumentation

The capillary electrophoresis (CE) system P/Ace 2000 (Beckman Instruments, Palo Alto, CA, USA) was used with Beckman system software controlled by an IBM PS/2 computer. This system contained built-in 214, 254-, 280- and 415-nm narrow-band filters for on-line detection. Electrophoreses were performed in a fused-silica tube, 75 μm I.D., 57 cm total length, or 50 cm from inlet to detector. The 214-nm filter was used for all the experiments described here.

Separation of standard peptides

Samples were placed on the inlet tray of the CE instrument. The system was programmed for pressure injection of the sample for 5 s (standards) or 15 s (extract from rat amygdala). The capillary column was washed and reconditioned after each run. The separation buffer was 50 m*M* NaH_2PO_4 containing 0.1% trifluoroacetic acid and 0.05% HPMC to minimize adsorption. Various pH values between 1.5 and 4.5 were adjusted with sodium hydroxide, since the selectivity of peptide separations by capillary zone electrophoresis has previously been reported to be critically dependent on the pH [9]. A borate buffer (pH 8.5) was used for preliminary experiments. Since with this buffer system no satisfactory results were obtained with CGRPs, *i.e.* no narrow peaks were produced within a reasonable analysis time, all presented data were obtained with the phosphate separation buffer at an acidic pH. The standard peptides were dissolved in a concentration of 0.5 mg/ml in water and diluted 1:10 (v/v) with separation buffer. The applied potential was 20 kV, which resulted in a current of *ca.* 70 μA with the used buffers.

Extraction of CGRP from rat amygdala

Ten Sprague–Dawley rats were killed by decapitation and the brains rapidly removed. The amygdalae were dissected bilaterally as described by Haring *et al.* [10], pooled and frozen at −80°C. This brain region was chosen because the central nucleus of the amygdala was reported to be among the brain regions containing the highest concentrations of CGRP. The pooled tissue was then homogenized in ten volumes of 2 *M* acetic acid by sonication. After centrifugation, the supernatant was concentrated on Sep-Pak cartridges (Millipore), essentially as described previously for other neuropeptides [11]. This procedure resulted in a recovery of *ca.* 70% and

made it possible to reconstitute the extract in a volume as low as 30 μl of distilled water. This sample was filtered through a 0.45-μm microfilter and subjected to micropreparative CE and subsequent radioimmunoassay, as described below.

Separation of the brain extract by CE in the micropreparative mode

Fraction collection is performed differently with CE than with HPLC. With electroelution, the end of the capillary must stay in contact with a solution containing buffer and an electrode during the collection in order to maintain the electric field that drives the separation. Though most custom-built and commercially available CE systems are not designed to perform fraction collection in an automated fashion, the instrument used in this study was specifically designed to work as an automatic fraction collector for micropreparative applications. We followed the instructions of the manufacturer [12] for micropreparative separation of the acidic extract from rat brain.

Initial runs were carried out to establish the precision of the migration time. This was particularly important since repeated runs were used to increase the total sample load (see below). In our case, the relative standard deviation of the retention times of rat α-CGRP and rat β-CGRP was *ca.* 0.9%. The instrument was programmed first to rinse the system, then to inject the sample under pressure for 15 s and then to separate the extract for 12 min at 20 kV. The voltage was then reduced to 5 kV, as recommended by Biehler and Schwartz [12], in order to counteract a possible recovery loss at high voltage. Furthermore, we observed a frequent voltage leakage of the system when the outlet vial was changed during separation at 20 kV. During the separation at 5 kV, the outlet vial was changed three times. These microvials contained 30 μl of separation buffer (pH 3.93). The first collection period until 17 min should contain no CGRP immunoreactivity (IR). The second collection period from 17 to 30.6 min included the retention time of rat β-CGRP, and the third collection period included the retention time of α-CGRP. Thereafter, the system was pressure-rinsed with distilled water and separation buffer for 5 min each. The separation of the extract was repeated five times to increase the total sample load. At the end, the system was calibrated again with synthetic rat α-CGRP and rat β-CGRP under exactly the same conditions, to verify that the collected fractions indeed contained the proper peptides.

Radioimmunoassay

The radioimmunoassay for CGRP-IR was carried out with the commercially available antiserum RAS 6009 (Peninsula, Munich, Germany) essentially as described by Saria *et al.* [13]. The cross-reactivity of the used antiserum for rat α-CGRP and rat β-CGRP was found to be virtually the same. The collected fractions (*ca.* 30 μl) were used as a radioimmunoassay sample without further sample preparation.

RESULTS

The migration times of CGRPs were found to be very sensitive to changes in pH values of the separation buffer in the acidic range. The migration behaviour of human α-CGRP, human β-CGRP, rat α-CGRP, rat β-CGRP and chicken CGRP is demonstrated in Fig. 1. Whereas pH values below 3 resulted only in a separation of rat α-CGRP from the other peptides, at higher pH values a clear separation of human α-CGRP from human β-CGRP and of rat α-CGRP from rat β-CGRP was achieved. Under the conditions used, human α-CGRP could not be separated from rat β-CGRP. It has to be noted that rat β-CGRP contained a minor peak that migrated similarly to human β-CGRP (Fig. 2). Since we did not have the means to determine the amino acid sequence of this compound we refer to it as "impurity of rat β-CGRP".

Electrophoresis of commercially available standards of human α-CGRP and human β-CGRP revealed single peaks that could be well separated with buffers of pH 3.70 or above (Fig. 3). Using borate buffer (pH 8.5), no clearcut peaks of CGRPs were obtained at 20 kV with the same capillary (not shown). To determine which type of CGRP was present in the rat amygdala, UV detection is not sensitive enough. Therefore we determined CGRP by radioimmunoassay in fractions collected in the micropreparative mode.

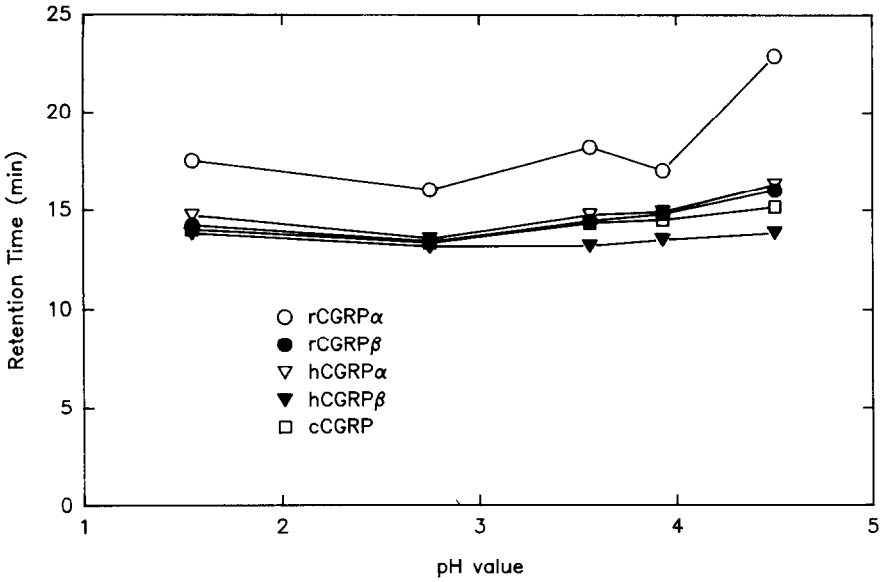

Fig. 1. Migration times at 20 kV/70 μA (retention time) of rat α-CGRP (rCGRPα), rat β-CGRP (rCGRPβ), human α-CGRP (hCGRPα), human β-CGRP (hCGRPβ) and chicken CGRP (cCGRP) at different pH values of the separation buffer of a composition described in Experimental. The relative standard deviation of the retention times after repeated injection was less than 0.9%.

Fraction 1 contained less CGRP-IR than the detection limit of *ca.* 5 fmol (Fig. 4). The second fraction collected contained over 20 fmol, and the third fraction collected *ca.* 58 fmol of CGRP-IR (Fig. 4). Fractions obtained from five consecutive collection runs with separation buffer only, prior to the separation of the rat brain extract, did not contain any measurable CGRP-IR (not shown). The injection of standards in the micropreparative mode at 5 kV after the separation of the extract verified that fraction 2 contained rat β-CGRP and that fraction 3 contained rat α-CGRP. The migration positions of these standards are marked with arrows in Fig. 4.

DISCUSSION

It is generally accepted that reversed-phase HPLC is a useful method to examine the identity of a neuropeptide measured with radioimmu-

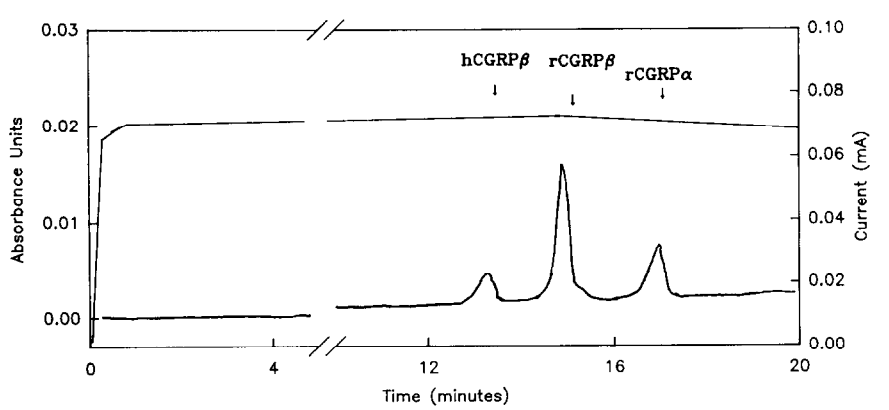

Fig. 2. Electropherogram (UV absorbance at 214 nm) of a mixture of synthetic rat α-CGRP (rCGRPα) and rat β-CGRP (rCGRPβ) in a concentration of 13 nmol/ml at 20 kV with a separation buffer of pH 3.93. The peak migrating close to, but not identical with the position of human β-CGRP (hCGRPβ), was a peak that occurred in the synthetic rat β-CGRP sample and may be an impurity. It is also clear that the UV absorbance of rat α-CGRP at 214 nm was lower than that of the other CGRPs, although the same amount should have been injected. This leaves some doubt about the actual amount of rat α-CGRP in the purchased 0.5-mg sample.

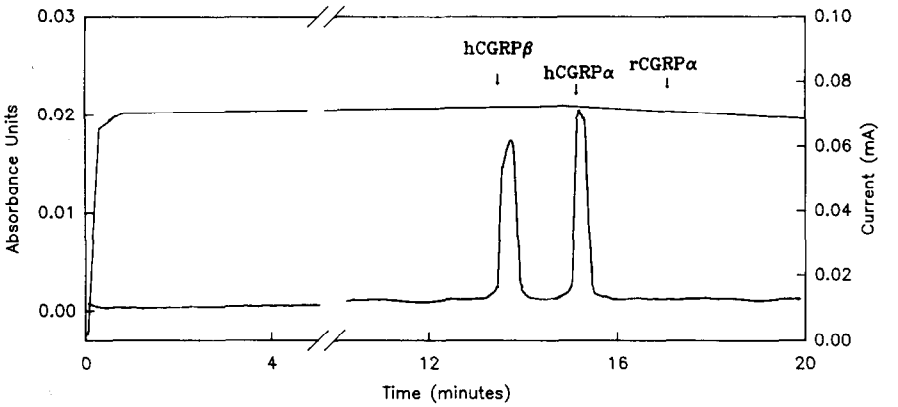

Fig. 3. Electropherogram (UV absorbance at 214 nm) of a mixture of synthetic human α-CGRP (hCGRPα) and human β-CGRP (hCGRPβ) in a concentration of 13 nmol/ml at 20 kV with a separation buffer of pH 3.93. The migration position of rat α-CGRP (rCGRPα) under the same conditions is also indicated.

noassay or to check the purity of commercially available synthetic peptides. In the case of CGRPs, reversed-phase HPLC has been found to be insufficient for the separation of either rat or human α-CGRP and β-CGRP. A clearcut separation has so far been achieved with only ion-exchange HPLC, and only for rat α-CGRP and β-CGRP [1]. In the present paper we describe for the first time an efficient and rapid method, that is capillary zone electrophoresis, as an alternative to HPLC separation. The use of a commercially available instrument with the possibility of complete automation is very easy and fast. If the amount of sample is limited, another crucial advantage of CE may be the requirement of only extremely small sample volumes. According to information from the manufacturer of the instrument, a 5-s pressure injection into a capillary of 75 μm I.D. should amount to a volume of only *ca.* 30 nl. Therefore, only minimal sample loss occurs. Another clear advantage of CE over conventional liquid chromatography is the superior separation efficiency, which may be one to three orders of magnitude higher [8]. Furthermore, the

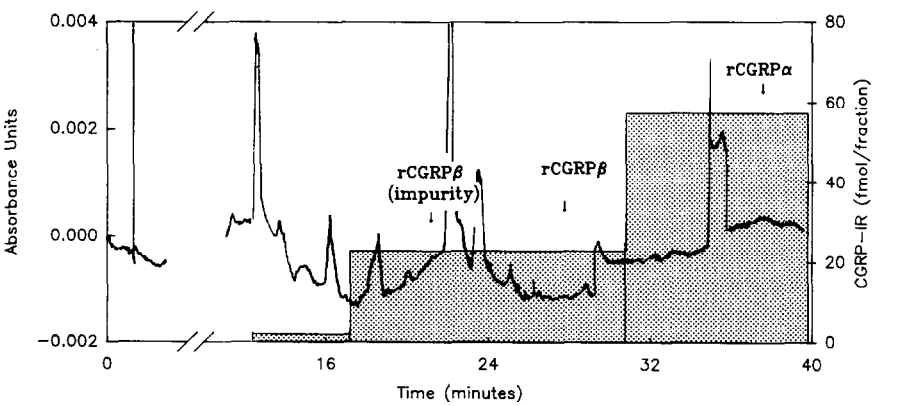

Fig. 4. Electropherogram, and CGRP-IR in fractions, of an extract of rat amygdala. The separation was performed at 20 kV for 12 min, then at 5 kV until 40 min. The sample and standards were dissolved in the separation buffer at pH 3.93. The outlet vial was changed three times with the controlling software. For details of the preparation of the sample see Experimental. The curve shows the UV absorbance at 214 nm (left ordinate) of one of the five runs. The dotted bars indicate CGRP-IR in the collected fractions, where the bar-width marks the collection period and the bar-height the amount CGRP-IR in the respective vial according to the right ordinate. The migration positions of rat α-CGRP (rCGRPα), rat β-CGRP (rCGRPβ) and the impurity of rat β-CGRP [rCGRPβ (impurity)], which were determined after the samples run, are indicated. The peak-width of CGRPs at 5 kV was less than 2 min.

separation depends on physicochemical properties of molecules different from those responsible for separation in HPLC. Therefore, this method should be at least useful to supplement information obtained with HPLC. Our CE separations of commercial peptide samples revealed that one substance, *i.e.* rat β-CGRP, contained two peaks, which could be traced with UV detection at 214 nm. According to the manufacturer's information, this peptide sample had undergone purity control with reversed-phase HPLC prior to sale, resulting in a single narrow peak. This indicates that CE may be a powerful and particularly important tool in addition to, or under certain circumstances instead of HPLC, to establish the purity of synthetic peptides.

To investigate the question of whether a certain neuronal or endocrine cell type produces α-CGRP or β-CGRP, three approaches may be suitable. One is to measure the expression of the specific messenger RNAs with Northern blot analysis or *in situ* hybridization technique. For instance, Noguchi *et al.* [14] reported the presence and, in some neurons, even the co-localization, of the mRNAs for α-CGRP and β-CGRP in dorsal root ganglia. The presence of the final gene products in the terminal regions of those neurons, *i.e.* the dorsal horn of the spinal cord, cannot, however, be certainly deduced from such experiments. Another drawback of this approach in the brain is the lack of knowledge of the origin of CGRP-containing fibres in some brain regions, since mRNAs can be found only in the cell bodies. A different approach is the production of highly specific antibodies for either α-CGRP or β-CGRP. So far, only one publication reports such antibodies [2] which have, however, not yet been tested in tissue extracts. Moreover, CGRP-IR has been found to be heterogeneous in blood [15] and in cerebrospinal fluid [16]. Therefore, a third approach to identify the type of CGRP produced in a particular cell type may be recommended. Thus, it seems useful to check CGRP-IR in any new biological matrices with chemical methods, no matter how specific the antiserum is assumed to be. Such precautions for immunological measurements of peptides in biological matrices are now even required by the scientific journal *Peptides*. Capillary zone electrophoresis may be an excellent tool to identify the CGRPs, as it may be for other neuropeptides.

As outlined in the introduction, Mulderry *et al.* [1] have used ion-exchange HPLC in combination with radioimmunoassay to identify α-CGRP and β-CGRP in dorsal root ganglia and in the colon of the rat, respectively. In the amygdala, with reversed-phase HPLC combined with radioimmunoassay, only a single peak of immunoreactivity has previously been found. This indicates that CE is superior to reversed-phase HPLC for the separation of CGRPs.

Our results with micropreparative CE and subsequent radioimmunoassay indicate for the first time the presence of both α-CGRP and β-CGRP in the rat amygdala. According to the immunoreactivities measured, the molecular ratio of the two forms is *ca.* 3:1 (α-CGRP/β-CGRP). It may be obvious that we collected rather broad fractions during micropreparative CE. This was to obtain a safety margin in case the minor peak of the synthetic rat β-CGRP, which we assumed to be an impurity, was in fact the authentic β-CGRP and the larger one was the impurity. In any case, both peaks were included in the fraction referred to as the β-CGRP-containing one, and both peaks were clearly separated from α-CGRP. Unfortunately, we did not have the means to determine the amino acid sequence of the two peaks. Our assumption that the major peak of the β-CGRP sample represents authentic rat β-CGRP is, however, supported by the observation of a similar cross-reactivity of this β-CGRP sample and the α-CGRP sample with our antiserum, which is known to exhibit full cross-reactivity with human or rat α-CGRP and β-CGRP (data not shown). Whether the two CGRP species are present in the same nerves, or occur in separate neurons with possibly different anatomical origins, should be addressed in further studies. Micropreparative CE can certainly be used to extend our present studies to other brain regions or peripheral organs. It has to be mentioned that the amount of sample that can be collected during one separation run is limited owing to the small volume applicable. Nevertheless, the possibility of sample concentration, and repeated collection runs in an automated mode combined with a sensitive radioimmunoassay, should be

sufficient to measure α-CGRP and β-CGRP in smaller amounts of tissue or biological fluids of rats and humans.

CONCLUSION

Capillary zone electrophoresis has been found to be a powerful tool to separate CGRPs, particularly rat and human α-CGRP and β-CGRP. Using this method in a micropreparative mode, combined with subsequent radioimmunoassay, has resulted for the first time in the demonstration of the presence of both α-CGRP and β-CGRP in the rat amygdala. This approach may be used for identification of either type of CGRP, for chemical characterization of immunologically measured CGRP and for purity control of synthetic peptides.

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