Structure of a Molluscan Cardioexcitatory Neuropeptide

Abstract. *A cardioexcitatory substance from ganglia of the clam* Macrocallista nimbosa, *formerly designated peak C, is the tetrapeptide amide Phe-Met-Arg-Phe-NH_2. Its structure was determined by the combined use of Edman dansyl degradation and tryptic digestion. The structure was confirmed by synthesis. This neuropeptide is active at about 10^{-8}M when assayed on molluscan muscle.*

The neurohumors characteristic of the Mollusca are acetylcholine, 5-hydroxytryptamine, and dopamine. But aqueous extracts of molluscan ganglia also have cardioexcitatory activity attributable to a number of other unidentified, chromatographically separable substances (*1, 2*). One of these, designated peak C, is of particular interest because, in addition to augmenting the contractile force of molluscan cardiac muscle and inducing prolonged contractions of other muscles, it can regularize arrhythmic hearts or, indeed, induce beating in quiescent ones. Moreover, peak C has been found in all the major classes of the phylum Mollusca and is particularly concentrated in nervous tissue (*2, 3*). Therefore, it has been suggested that this substance may be a neurohormone (*2, 3*). Among the noncardiac muscles that contract when treated with peak C is the radula protractor of the whelk *Busycon contrarium* (*4*). Using this muscle together with the heart of the venerid clam *Mercenaria mercenaria* as a parallel bioassay system, we have isolated peak C from ganglion extracts of *Macrocallista nimbosa*, also a venerid bivalve, and have purified it by the combined use of solvent extractions, gel filtration, and ion-exchange chromatography (*4*). We now report experiments leading to the identification of peak C with a tetrapeptide amide having the sequence: Phe-Met-Arg-Phe-NH_2 (*5*).

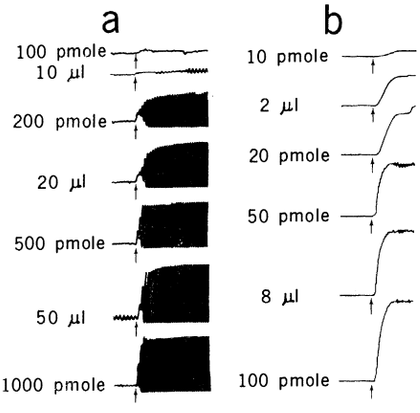

Fig. 1. Parallel bioassay of natural FMRFamide (purified peak C) and the synthetic peptide on (a) an isolated ventricle of *Mercenaria mercenaria* and (b) a radula protractor of *Busycon contrarium*. The muscles were isolated and suspended in seawater (27 parts per thousand) in a 5-ml aerated, temperature-controlled (22°C) organ bath. Contractions were recorded with force transducers (Grass FT 03C) connected to an inkwriting oscillograph (Grass model 7 polygraph). The doses were injected into the baths as indicated (arrows). The doses of synthetic FMRFamide were accurately known, and are expressed in picomoles added to the bath; a 50-pmole dose gives an actual bath concentration of $10^{-8}M$. The doses of the natural peptide are given in volumes of stock solution added to the bath. The stock solution used in this assay contained approximately 8 pmole/μl.

Purified peak C has a phenylalanine-like absorption spectrum and an isoelectric point greater than *p*H 10. It is inactivated by trypsin, chymotrypsin, and Pronase (*1, 4*), and gives a positive reaction with both ninhydrin and the Sakaguchi reagent for arginine. Its amino acid composition is $Phe_{2.00}Met_{0.81}Arg_{1.12}$ (*4, 5*). On the basis of the amino acid analysis, and assuming that the active agent is a tetrapeptide, we estimate that 1 g of dried ganglia dissected from 500 clams yields about 5 μg of material containing approximately 5 nmole of peak C (*6*).

Amino-terminal analysis and Edman dansyl degradation (*7, 8*) of purified peak C showed that the sequence begins Phe-Met-; but we were unable to determine a third residue with this method. Since the composition of peak C is $Phe_2Met_1Arg_1$, only two amino acid sequences are possible: Phe-Met-Phe-Arg or Phe-Met-Arg-Phe. However, the carboxyl terminal of peak C, like those of many biologically active peptides, might be an amide. Thus, there are really four possible structures, but each of them should yield different amino acids upon tryptic digestion (*9*), that is, Phe-Met-Phe-Arg-OH would yield no free amino acid and no ammonia; Phe-Met- Phe-Arg-NH_2 would give no amino acid but would yield ammonia; Phe-Met-Arg-Phe-OH would yield free phenylalanine; and Phe-Met-Arg-Phe-NH_2 would give phenylalanine amide. Therefore, we incubated a few nanomoles of peak C with trypsin, dansylated the incubation mixture, and chromatographed the product using the standard two-dimensional polyamide chromatographic system for separation of dansylated amino acids (*10*). In addition to a faint background of various dansyl amino acids, one intense spot appeared near the position of dansylproline which cochromatographed with an authentic sample of dansylated phenylalanine amide. Furthermore, this spot disappeared after acid hydrolysis of the dansylated tryptic digest, dansylated phenylalanine then becoming the most intense spot. Thus, of the four possibilities, the correct sequence for *Macrocallista* peak C appears to be Phe-Met-Arg-Phe-NH_2 (FMRFamide) (*11*).

We synthesized FMRFamide starting with the tripeptide Met-Arg-Phe (*12*). The tripeptide was converted to the methyl ester; *tert*-butoxycarbonyl phenylalanine was coupled to the tripeptide ester; the protected tetrapeptide ester was converted to the amide; and the product was deprotected. The resulting crude FMRFamide was purified by the same techniques used in the purification of the natural peptide (*4*).

Thin-layer chromatography of both the natural and synthetic materials revealed a major spot with an R_F value of 0.75 and a minor spot at 0.64 (*13*). The lower spot is probably an oxidation product of the upper one since its intensity is greater in older preparations and can be rapidly increased by exposing either the natural or synthetic material to oxidizing conditions, for example, dilute hydrogen peroxide.

The biological effects of natural and synthetic FMRFamide are qualitatively identical whether tested on the clam heart or the radula protractor (Fig. 1). Threshold on the clam heart is about $10^{-8}M$, and on the radula protractor about $10^{-9}M$. Log dose-response curves from the bioassay of the two peptides on the radula protractor were plotted, the concentration of the natural substance

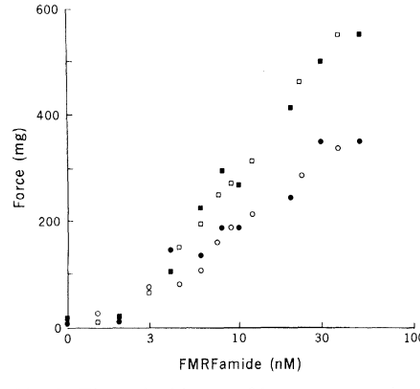

Fig. 2. The coincidence of log concentration-response curves for the action of synthetic and natural FMRFamide on the radula protractor muscle of *Busycon contrarium*. The responses of two muscles from the same animal are plotted separately. Doses of natural FMRFamide (peak C) were made up from a stock solution containing $8 \times 10^{-6}M$ peptide, a concentration estimated from relative staining intensity on thin-layer chromatograms and from amino acid analysis. Open symbols: FMRFamide (purified peak C). Closed symbols: synthetic FMRFamide.

being estimated from its staining intensity on thin-layer chromatograms. These curves are coincident (Fig. 2), and the effects of the natural and synthetic peptides are therefore quantitatively indistinguishable.

We have bioassayed an additional set of tetra-, tri-, and dipeptides containing various permutations of the same three amino acids found in *Macrocallista* FMRFamide (*14*). Of these, only Phe-Met-Arg-Phe-OH and Met-Arg-Phe-NH_2 had any activity; the relative potencies were no better than, respectively, 1/100 and 1/1000 that of FMRFamide. The dependence of activity like that of peak C on the specific structure and sequence proposed by analysis of the natural product suggests, again, the identity of *Macrocallista* peak C and FMRFamide.

FMRFamide resembles a synthetic gastrin-(14-17)-tetrapeptide amide analog, Phe-Met-Asp-Phe-NH_2 (*15*). But, since FMRFamide contains an arginyl residue in place of the essential aspartyl residue of the gastrin analog, it should not have any gastrin-like activity. Immunoreactive gastrin has been found in the blood, and gastrointestinal tissue of two species of gastropod molluscs, but this substance differs from peak C both in its size and tissue distribution (*16*).

Peak C activity has been observed in all the major molluscan classes (*3*), but there is no evidence as yet that all peak C's are FMRFamide. Moreover, although FMRFamide is a highly active agent, and is highly localized in *Macrocallista* ganglia (*3*), its normal physiological role has still to be defined. About 20 years ago, Welsh (*17*) noted that, for many neurotransmitters, there is a polypeptide neurohormone that mimics its action. He suggested that these polypeptides might be serving as stable, long-range, and long-term substitutes for the fast-acting neurohumors. Given the similarity between the actions of 5-hydroxytryptamine and FMRFamide on molluscan heart, and assuming that FMRFamide is, in fact, a neurosecretory product, its physiological role in molluscs might be as a long-term regulator of muscular rhythmicity and tone.

DAVID A. PRICE
MICHAEL J. GREENBERG
Department of Biological Sciences, Florida State University, Tallahassee 32306

References and Notes

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3. R. A. Agarwal, P. J. B. Ligon, M. J. Greenberg, *Comp. Gen. Pharmacol.* **3**, 249 (1972).
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5. Phe, phenylalanine; Met, methionine; Arg, arginine; Asp, aspartic acid; the term gastrin-(14-17)-tetrapeptide indicates the sequence at residues 14 to 17.
6. The yield of a number of peak C preparations was estimated by various methods: ultraviolet absorbance, amino acid analysis, and staining intensity on thin-layer chromatograms. The results were similar; that is, 5 nmole per gram of dried ganglia, or 10 pmole per clam.
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9. "Trypsin exhibits the highest degree of substrate specificity known for an endopeptidase; only those bonds involving the carboxyl groups of lysyl and arginyl residues are hydrolyzed" [C. B. Kasper, in *Protein Sequence Determination*, S. B. Needleman, Ed. (Springer-Verlag, New York, ed. 2, 1975), p. 132].
10. K. R. Woods and K. T. Wang, *Biochim. Biophys. Acta* **133**, 369 (1967), as modified for the microscale by Neuhoff (*8*).
11. F, M, and R are the one-letter abbreviations for the amino acids phenylalanine, methionine, and arginine approved by the IUPAC-IUB Commission on Biochemical Nomenclature [*Pure Appl. Chem.* **31**, 641 (1972)]. Thus, the designation FMRFamide conveys both the amino acid sequence of the peptide and the fact that the carboxyl terminal is substituted with a amide group.
12. Obtained from Research Plus Laboratories, Denville, N.J.
13. Eastman No. 6064 cellulose chromatogram sheets were used with a solvent consisting of *n*-butanol, acetic acid, and water (4 : 1 : 2), by volume. Spots were visualized with ninhydrin and the Sakaguchi reagent.
14. The following peptides were purchased: Met-Arg-Phe-OH (*12*), Arg-Phe-OH (*12*), and Phe-Arg-OH (Cyclo Chemical, Los Angeles, Calif.). Phe-Met-Arg-Phe-OH and its amide, Met-Arg-Phe-NH_2, and Arg-Phe-NH_2 were synthesized from these purchased peptides. Phe-Met-Phe-Arg-OH and Phe-Met-Arg-OH and its amide were synthesized from the component-protected amino acids.
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18. Supported by NIH grant HI09283 to M.J.G. This is contribution 70 from the Tallahassee, Sopchoppy, and Gulf Coast Marine Biological Association.

10 March 1977

DNA Strand Scission by Benzo[*a*]pyrene Diol Epoxides

Abstract. Syn-*and* anti-*benzo*[a]*pyrene diol epoxides elicit a concentration-dependent nicking of superhelical Col E1 DNA in an in vitro reaction monitored by agarose gel electrophoresis and electron microscopy. This strand scission represents less than 1 percent of the DNA modification by diol epoxide. Kinetic analysis implicates the formation of unstable phosphotriesters, hydrolysis of which nick the DNA.*

Benzo[*a*]pyrene is a widespread environmental pollutant possessing potent mutagenic and carcinogenic activity. This activity is dependent on metabolic activation of the hydrocarbon by microsomal monooxygenase. Recent evidence suggests that a 9,10-oxide of *trans*-7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene may be the ultimate carcinogenic metabolite (*1*). It appears that microsomal activation proceeds stereospecifically to give the *anti*-diol epoxide (*2, 3*). Both diastereomers, however, react with nucleic acids and are highly mutagenic (*3, 4*). Weinstein *et al.* (*5*), working with the *anti* isomer, and Koreeda *et al.* (*6*), working with the *syn* isomer, characterized the primary RNA adduct as a linkage between the C-2 amino group of guanine and the C-10 position of the hydrocarbon. Reaction of diol epoxide with DNA has not been characterized. We report here that both diastereomeric diol epoxides degrade DNA and RNA and present a mechanism for how this process might occur.

Covalently closed superhelical DNA, such as the *Escherichia coli* plasmid Col E1, is a sensitive probe for detecting strand scission. Form I Col E1 DNA, with a molecular weight of 4.2×10^6, contains more than 12,000 phosphodiester linkages (*7*); cleavage of any one permits the DNA strands to unwind, resulting in relaxed form II DNA. Introduction of another nick adjacent to the first but on the opposite strand gives linear form III DNA. All three forms are conveniently resolved by agarose gel electrophoresis. The Col E1 DNA used in this study contains 0.3 percent RNA as a discrete segment (*7*). Similar nicking, however, is seen with RNA-free SV40 DNA.

When Col E1 DNA is reacted with either diastereomeric diol epoxide in tris-HCl buffer, *p*H 8.0, gel electrophoresis shows substantial nicking (Fig. 1). A minimum ratio of diol epoxide to DNA mononucleotide of 0.01 is required for detectable nicking. Above this value nicking increases, until at a ratio of 0.5 to 1.0 no form I DNA remains. The effects of strand scission are also observed by electron microscopy (Fig. 2). When form I DNA is reacted with the *anti* isomer, relaxed circles result from a ratio of 0.5, while linear segments of random length result from a ratio of 5. At even higher ratios of diol epoxide to mononucleotide (that is, 15) a significant fraction of the DNA (~ 20 percent) becomes acid soluble, presumably due to the release of small oligonucleotides. This increased fragmentation suggests that nicks are occurring at many sites around the molecule and are not restricted to the RNA segment mentioned above. When the reaction is carried out in 20 m*M* phosphate