counter ion, TCA. If intracellular receptor site exists for methantheline, as is the case for the tertiary amine local anesthetics such as procaine[10,11] then a characteristic deviation in the log dose-response curve for acetylcholine should occur under conditions which improve the drug's accessibility to the intracellular space. However, as has been stated, the regression lines for acetylcholine in the presence of methantheline and methantheline-TCA did not differ statistically from one another. In light of the fact that the antimuscarinic activity of methantheline was unaffected by enhancing the drug's lipid solubility, it is unlikely that an intracellular locus of action exists. In addition, some inferences may be made concerning the nature of the membrane receptor environment for methantheline. Under the conditions employed in these experiments, the methantheline-TCA ion pair would exist only in a non-aqueous medium such as the cell membrane. Consequently, if the methantheline receptors were located in an aqueous environment, only free drug would be available and no further deviation in the log-dose response curve for acetylcholine would be expected when TCA was present[12,13]. The results presented in the figure support the hypothesis that formation of an ion pair with TCA did not influence the antimuscarinic activity of methantheline in the isolated tissue preparation.

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Inhibitory tripeptide, Lys-Phe-Tyr, as a fragment of physalaemin

H. Takeuchi, T. Morimasa and M. Matsumoto[1]

Institute for Neurobiology, Okayama University Medical School, 2–5–1 Shikata-cho, Okayama (Japan),
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Summary. Several peptides were separated from α-chymotrypsin-treated physalaemin by high voltage paper electrophoresis, and inhibition of the excitability of a molluscan giant neurone (tonically autoactive neurone) by the tripeptide, Lys-Phe-Tyr, was demonstrated.

Physalaemin is a hypotensive endecapeptide isolated by Erspamer et al.[2,3] from amphibian skin. Its biphasic effect (inhibitory-excitatory) on the excitability of a giant neurone (the TAN, tonically autoactive neurone)[4,5] in the subesophageal ganglia of the African giant snail (Achatina fulica Férussac) has been reported previously[6]. Excitation was much more marked than inhibition. Enzyme treatment of physalaemin showed that marked inhibition of the TAN could be produced by the α-chymotrypsin-(CT)-treated physalaemin[7]. In the present study, each fragment of this CT-treated physalaemin was separated by high voltage paper electrophoresis. Of the separated fragments, a tripeptide (Lys-Phe-Tyr) and an octopeptide including the tripeptide (Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr) (Pyr: L-pyroglutamic acid) were shown to have a strong inhibitory effect on the TAN.

Physalaemin (Protein Research Foundation, Osaka; 2.5×10^{-4} kg/l in 0.1 M ammonium acetate buffer, pH 8.2) was treated with CT (Worthington Biochemical Corporation, 55 units/mg; 5×10^{-5} kg/l; $^1/_{10}$ volume of physalaemin solution) at 37 °C for 6 h. After the treatment, trasylol (Bayer AG) was added to stop enzyme activity. High voltage paper electrophoresis of CT-treated physalaemin (Anastasi et al.[8]) was performed under the following conditions: paper, Toyo No. 51A; 50 V/cm; 90 min; pH, 1.9; running buffer, CH_3COOH–HCOOH(98%)–H_2O (87:25:888). The whole paper was stained with fluorescamine after electrophoresis, the guide strip being stained by ninhydrin and a peptide reagent[9]. Fragments of physalaemin were recovered from each of the peptide bands. The amino acid sequences of each fragment were determined after acid hydrolysis at 105 °C for 16 h.

A glass micropipette was implanted into the TAN soma, the intracellular biopotential recorded by a pen-writing galvanometer, and the number of spike discharges per minute counted with a spike counter. Substances to be examined were applied in 2 ways: by bath application (substances were dissolved in the snail's physiological solution[10], and applied to the dissected ganglia) and by microdrop application (a microdrop about 100 μm in diameter of the solution to be examined was formed at the tip of a second micropipette, and the microdrop placed precisely on the TAN surface) (diameter of this neurone was about 200 μm).

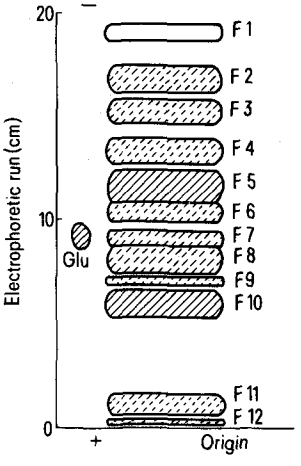

Fig. 1. Peptide bands of chymotrypsin-treated (for 6 h) physalaemin separated by high voltage paper electrophoresis (schematic drawing). Amino acid sequences of each fragment of physalaemin were determined to be as follows (Pyr: L-pyroglutamic acid): fragment (F) 1, Lys-Phe; F 2, Met-NH_2; F 3, Lys-Phe-Tyr; F 4, Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu; F 5, Gly-Leu-Met-NH_2; F 6, Gly-Leu-Met-NH_2; F 7, Tyr-Gly-Leu; F 8, Tyr-Gly-Leu-Met-NH_2; F 9, Pyr-Ala-Asp-Pro-Asn-Lys-Phe; F 10, Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr; F 11, Pyr-Ala-Asp-Pro-Asn and Gly-Leu-Met; F 12, Pyr-Ala-Asp-Pro-Asn and Gly-Leu-Met.

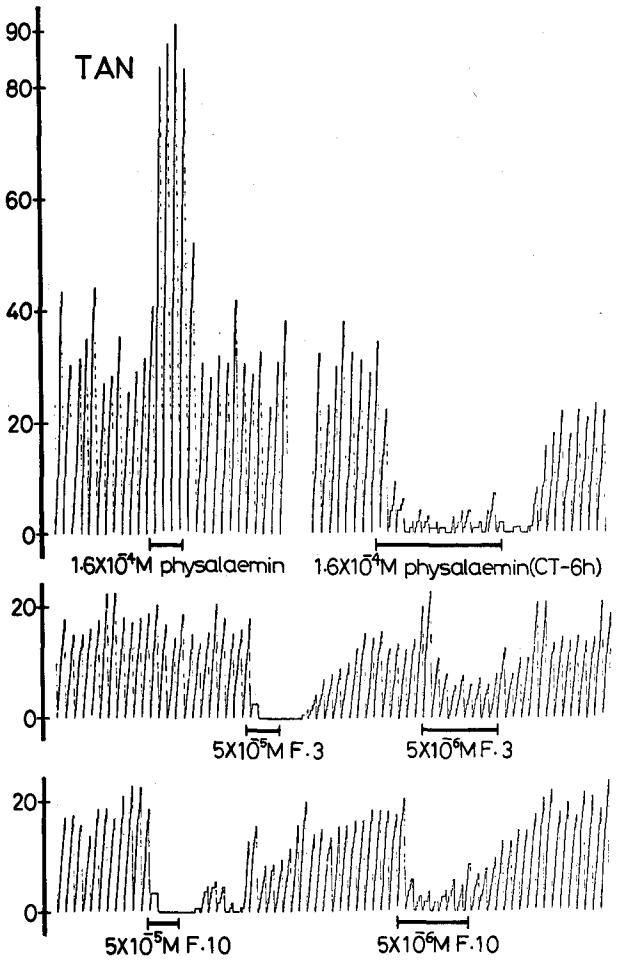

Fig. 2. Effects of untreated and chymotrypsin-treated physalaemin and its 2 fragments (F 3, Lys-Phe-Tyr; and F 10, Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr) on the TAN (tonically autoactive neurone) excitability (bath application). Ordinate, the number of spike discharges per min. Abscissa, time course, each histogram is 1 min. We applied untreated and chymotrypsin-treated physalaemin (1.6×10^{-4} M), F 3 (5×10^{-5} and 5×10^{-6} M) and F 10 (5×10^{-5} and 5×10^{-6} M).

After paper electrophoresis of CT-treated physalaemin, 12 separated peptide bands could be distinguished, as in figure 1. The amino acid sequences of these peptides were determined as described in the legend.

As shown in figure 2, the bath application of untreated physalaemin had a marked excitatory effect on the TAN (its slight inhibitory effect was masked by the excitatory one in the bath application). However, this substance, when treated by CT, showed an inhibitory effect on the same neurone, in contrast to that of untreated physalaemin. Of the fragments of physalaemin separated by high voltage paper electrophoresis, fragment (F) 3 (Lys-Phe-Tyr) and F 10 (Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr) showed a marked inhibitory effect. The critical concentration of both fragments in the production of this effect was about 5×10^{-6} M. The inhibitory active sites of the 2 substances are presumed, therefore, to be identical.

The inhibitory effect of the authentic tripeptide (Lys-Phe-Tyr, donated by Dr A. Inoue of Daiichi Pharmaceutical Co.) was confirmed on TAN excitability. A microdrop of 500 pg of the authentic tripeptide caused a marked inhibition of the TAN biopotential (figure 3). The biopotential returned completely to normal after washing the ganglia with physiological solution.

We have previously[11] reported that amino acids naturally occurring in the snail's ganglia[12], including Lys, Phe and Tyr, had no effect on the TAN, and, in the present study, also observed that fluorescamine-treated Lys, Phe and Tyr had no effect. It would appear, therefore, that the effect was produced by the tripeptide as a whole, rather than by the respective amino acids.

The question of whether or not the inhibitory active sites of the above-mentioned tripeptide are identical to those of the slight inhibitory effect of untreated physalaemin remains unanswered by the present study. However, the possibility that some oligopeptide having the same active sites as the tripeptide (Lys-Phe-Tyr) might play a physiological role in the nervous system, perhaps acting as a neurotransmitter of some neurones including the TAN of Achatina fulica, seems likely.

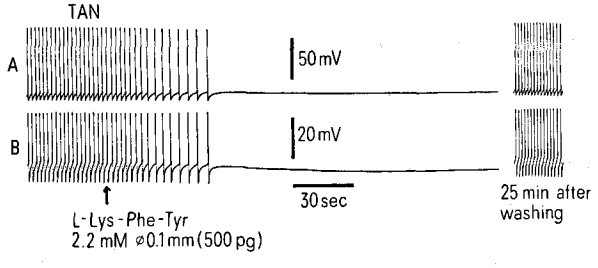

Fig. 3. Effects of authentic tripeptide (Lys-Phe-Tyr) on the TAN excitability (microdrop application). A, the full-spike recording of the TAN biopotential. B, the high gain recording of A (spike peaks were cut by an electronic voltage clipper). A microdrop (about 100 µm in diameter) of 2.2 mM authentic Lys-Phe-Tyr (total amount of this substance estimated to be about 500 pg) was placed on the TAN surface (arrow). 25 min after washing with the physiological solution, the biopotential returned to normal. Upper vertical bar, calibration for A (50 mV). Lower vertical bar, calibration for B (20 mV). Horizontal bar, time course (30 sec).

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