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EXCITATORY EFFECT OF A NEW POLYPEPTIDE (ANTHOPLEURIN-B) FROM SEA ANEMONE ON THE GUINEA-PIG VAS DEFERENS

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1 Anthopleurin-B (AP-B, $> 3 \times 10^{-9}$ M), a newly isolated polypeptide from sea anemone (*Anthopleura xanthogrammica*), caused powerful rhythmic contractions in the guinea-pig isolated vas deferens. The other polypeptides anthopleurin-A from *A. xanthogrammica* and anthopleurin-C from *A. elegantissima*, elicited similar effects but in higher concentrations ($> 5 \times 10^{-8}$ M). Toxin II (10^{-6} M) isolated from the sea anemone, *Anemonia sulcata*, had no effect.

2 The rhythmic contractions induced by AP-B were inhibited by phentolamine, bretylium, guanethidine, reserpine, 6-hydroxydopamine, tetrodotoxin (TTX) and verapamil. Mecamylamine, atropine, methysergide, chlorpheniramine, and indomethacin had no effect.

3 AP-B (10^{-8} M ~ 10^{-5} M) caused a dose-dependent increase in the amount of endogenous noradrenaline (NA) released from the vas deferens. AP-B (10^{-5} M) increased the amount of NA released to approximately 310 times (12 μg/g tissue) that of untreated tissues.

4 The AP-B-induced release of NA was inhibited or abolished by TTX, verapamil, or incubation in Ca-free medium.

5 These results suggest that the AP-B-induced rhythmic contraction of the vas deferens is mediated through the release of NA from adrenergic nerve endings; AP-B is one of the most potent substances in stimulating NA release from the vas deferens.

Introduction

Anthopleurin-A (AP-A), a novel polypeptide was isolated recently from *Anthopleura xanthogrammica* (Shibata, Dunn, Kuchii, Kashiwagi & Norton, 1974; Shibata, Norton, Izumi, Matsuo & Katsuki, 1976) and its amino acid sequence determined (Tanaka, Haniu, Yasunobu & Norton, 1977). AP-A has a potent, positive inotropic action without producing effects on heart rate or blood pressure (Shibata *et al.*, 1976; Shibata, Izumi, Seriguchi & Norton, 1978; Blair, Peterson & Bishop, 1978; Scriabine, Van Arman, Morgan, Morris, Bennet & Bohidar, 1979; Shimizu, Iwamura, Toyama, Yamada & Shibata, 1979). Later, similar polypeptides, authopleurin-B (AP-B) also from *A. xanthogrammica* and anthopleurin-C (AP-C), from *A. elegantissima*, were isolated and their chemical structures characterized (Norton, Shibata, Kashiwagi & Bentley 1976; Norton, Kashiwagi & Shibata, 1978). Toxin II, a neurotoxic polypeptide with a primary structure similar to AP-A, was recently isolated from *Anemonia sulcata* (Beress, Beress, & Wunderer, 1975; Wunderer, Machleidt & Wachter, 1976; Bergman, Dubois, Rojas & Rathmayer, 1976). The effect of these polypeptides on smooth muscle has not been extensively studied.

The pharmacological properties of the neuro-effector junction of the vas deferens have received considerable attention. Following the observations of Huković (1961) two hypotheses concerning the nature of motor transmission in the vas deferens have been put forward: (1) adrenergic transmission (Birmingham & Wilson, 1963; Bhargava, Kar & Parmar, 1965; Jones & Spriggs, 1975); (2) non-adrenergic transmission (Ambache & Zar, 1971; von Euler & Hedqvist, 1975; Jenkins, Marshall & Nasmyth, 1977). Recently, McGrath (1978) demonstrated that even the response to single pulse field stimulation consisted of both adrenergic and non-adrenergic components. In this paper, we have shown for the first time that these new polypeptides, with the exception of toxin II, elicited a potent excitatory effect mediated through the release of endogenous noradrenaline from the adrenergic nerve terminals. A preliminary account of some of these findings has been presented elsewhere (Ohizumi, Shibata & Norton, 1979).

Methods

Mechanical responses

Male guinea-pigs (250 to 350 g) were used. The vas deferens was excised and set up as described by

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Birmingham & Wilson (1963). Preparations were suspended in a 20 ml organ bath containing Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl 120, KCl 4.8, $CaCl_2$ 1.2, $MgSO_4 \cdot 7H_2O$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 25.2 and glucose 5.8, pH 7.4. A Ca^{2+}-free solution was prepared by the omission of $CaCl_2$. For the low Ca^{2+}-high Mg^{2+} Krebs solution, $CaCl_2$ was reduced to 0.6 mM and the $MgSO_4 \cdot 7H_2O$ was increased to 2.6 mM. For a low Na^+-sucrose Krebs solution, 60 mM NaCl was replaced with 120 mM sucrose. For a low Na^+-LiCl Krebs solution, 120 mM NaCl was replaced with 120 mM LiCl. The bath medium was aerated with 95% O_2/5%CO_2 and the temperature maintained at 32°C. A resting tension of 1 g was applied to each strip. Tension was measured isometrically by a force displacement transducer and displayed on a polygraph. 6-Hydroxydopamine (6-OHDA, 150 mg/kg daily for 2 days) or reserpine (2 mg/kg daily for 2 days) was administered intraperitoneally. Experiments were performed 24 h after the last administration of drug.

Assay of noradrenaline (NA) released from the tissue

Chemical determination of NA released from the guinea-pig vas deferens into the incubation medium was carried out as follows. Guinea-pigs weighing 300 to 500 g were used. The vas deferens was excised and the serous membrane was carefully stripped away. Each vas deferens was suspended in a 4 ml organ bath containing the Krebs-Ringer bicarbonate solution maintained at 37°C and aerated with 95% O_2/5% CO_2. The tissues were equilibrated for 45 min during which time the solution was changed every 15 min. In the control experiments, control tissues were exposed to a fourth period of 15 min in Krebs-Ringer bicarbonate solution, to parallel the 15 min exposure period of the test tissues to the normal Krebs solution containing TTX or verapamil or a Ca-free Krebs solution. Finally, control tissues were transferred to the normal solution containing AP-B for 30 min, to parallel the 30 min exposure period of the test tissues to normal Krebs solution containing AP-B + TTX or AP-B + verapamil or a Ca-free Krebs solution containing AP-B. The pretreatment and test solutions contained phenoxybenzamine (5×10^{-5} M) to inhibit the reuptake of NA (Iversen & Langer, 1969; Wakade & Krusz, 1972). Separation of NA was carried out according to the method of Endo & Ogura (1973). The bath medium was chromatographed over *P*-cellulose equilibrated with 0.01 M phosphate buffer (pH 6.2). The elution buffers were exchanged stepwise. After development with 30 ml 0.01 M phosphate buffer to remove amino acids and lipids, NA was eluted with 21 ml 0.06 M phosphate buffer collected from the columns in 3 ml samples. Amount of NA in each fraction was determined by means of the trihydroxindole reaction according to Häggendal's method (1963). After 0.1 ml of 0.0025% $CuCl_2 \cdot 2H_2O$ solution was added to 3 ml of each sample, the oxidation was performed by addition of 0.1 ml of 0.25% $K_3Fe(CN)_6$ solution. After 2 min the oxidation was stopped with 0.4 ml of 1% mercaptoethanol in a sodium sulphite solution (5 g $Na_2SO_3 \cdot 7H_2O$/10 ml water). After 2 min 0.4 ml of 2 N NaOH was added to the solution. The fluorescence was measured at 330/500 nm (activating/fluorescent wavelengths) by means of an Aminco-Bowman spectrophotofluorometer (Aminco SPF 500). The sensitivity of the NA assay was 0.5 ng/3 ml. The mean recovery was about 95% through column chromatography.

Statistical analysis of the data

Three to five preparations from different animals were used for each experiment. Mean data are presented with their standard errors (s.e.mean). Student's *t* test was used to evaluate the results of the NA assay.

Isolation and purification of polypeptides

Anthopleurin-B (AP-B) (mol.wt. 4590, amino acid 42) isolated from *Anthopleura xanthogrammica*, and anthopleurin-C (AP-C) (mol.wt. 4875, amino acid 42) from *A. elegantissima* were purified as described previously (Norton *et al.*, 1976; 1978). The amino

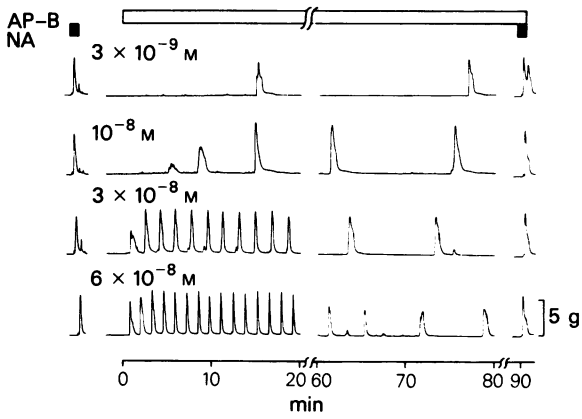

Figure 1 Isometric rhythmic contractile response to different concentrations of anthopleurin-B (AP-B) in the guinea-pig isolated vas deferens. AP-B was added 20 min after washing out the first standard dose of noradrenaline (NA, 3×10^{-5} M); 90 min after application of AP-B, the second dose of NA was added in the presence of AP-B. The presence of AP-B is indicated by the broken bar. Vertical calibration applies to each record.

acid analyses of newly isolated polypeptides, AP-B and AP-C, are as follows: AP-B; Asp_5, Thr_1, Ser_3, Glu_1, Pro_5, Gly_6, Ala_1, Cys_4, Val_1, Ile_2, Leu_2, Tyr_1, Phe_1, Lys_3, His_2, Arg_2, Trp_2. AP-C; Asp_4, Thr_2, Ser_4, Glu_1, Pro_4, Gly_8, Ala_2, Cys_6, Val_2, Ile_2, Leu_4, Lys_2, His_2, Arg_1, Trp_3 (Norton *et al.*, 1978).

Drugs

The following drugs were used. Toxin II isolated from *Anemonia sulcata* was purchased from Ferring GMBH (Kiel, West Germany). Other agents included tetrodotoxin (Sankyo), 6-hydroxydopamine (Sigma), reserpine (Apoplone, Daiichi Seiyaku), phentolamine methanesulphonate (Rogitine, Ciba-Geigy), bretylium tosylate (Burroughs-Wellcome), guanethidine sulphate (Ismeline, Ciba-Geigy), atropine sulphate (Tokyo Kasei), mecamylamine hydrochloride (Meiji Seika), chlorpheniramine maleate (Sankyo), indomethacin (Merk), verapamil hydrochloride (Eisai), noradrenaline bitartrate (Sigma), and methysergide (Sandoz).

Results

Mechanical response

AP-B, AP-A and AP-C caused powerful rhythmic contraction of the vas deferens at concentrations above 3×10^{-9} M, 5×10^{-8} M, 5×10^{-8} M, respectively. The shape of these responses was very similar. Toxin II had no effect even at a concentration of 10^{-6} M. An example of the contractile responses to different concentrations of AP-B is shown in Figure 1. The contractile response induced by AP-B (10^{-8} M) was comparable to the maximal response to noradrenaline (NA, 3×10^{-5} M). The frequency of the rhythmic contractions increased with AP-B concentrations in the range of 3×10^{-9} to 6×10^{-8} M in a dose-dependent manner. The frequency of the contractions decreased markedly between 20 and 60 min after exposure to AP-B at concentrations above 10^{-8} M but persisted for at least 3 h.

Figure 2 shows the effect of phentolamine (10^{-6} M), bretylium (3×10^{-4} M) and reserpine on the AP-B-induced contractions of the vas deferens. Pretreatment with phentolamine or bretylium abolished, while pretreatment with reserpine inhibited the AP-B-induced contraction. The AP-B-induced contraction was also inhibited markedly by either guanethidine (10^{-4} M) or 6-OHDA, but not affected by atropine (10^{-6} M), mecamylamine (3×10^{-5} M), chlorpheniramine (5×10^{-7} M), methysergide (10^{-6} M), or indomethacin (3×10^{-6} M). TTX (5×10^{-7} M), low Na^+ (85.2 mM)-sucrose medium, verapamil (3×10^{-6} M) or a low Ca^{2+} (0.6 mM)-high Mg^{2+} (2.6 mM) medium almost abolished the AP-B-induced contraction (Figure 3), while these treatments had no apparent effects on NA-induced contractions of the vas deferens. The

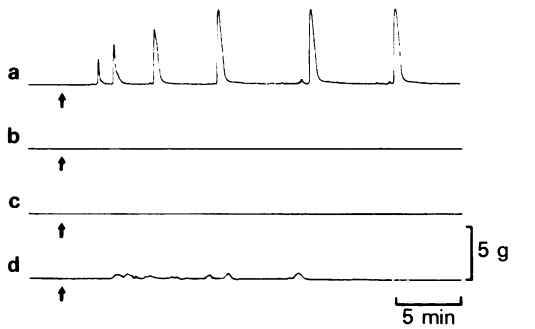

Figure 2 Effect of pretreatment with phentolamine, bretylium or reserpine on the anthopleurin-B (AP-B, 10^{-8} M)-induced (↑) rhythmic contractions in the guinea-pig isolated vas deferens. (a) Control; (b) phentolamine (10^{-6} M); (c) bretylium (3×10^{-4} M); (d) reserpine. Phentolamine and bretylium were added 15 min before AP-B. Reserpine (2 mg/kg i.p. daily) was given twice before the experiment and the vasa used 24 h after the last administration. Vertical and horizontal calibrations apply to each record.

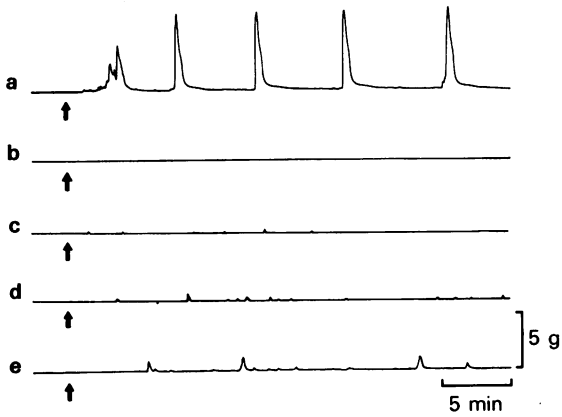

Figure 3 Effect of tetrodotoxin (TTX), low Na^+-sucrose medium, verapamil or low Ca^{2+}-high Mg^{2+} medium on the anthopleurin-B (AP-B, 10^{-8} M)-induced (↑) rhythmic contraction in the guinea-pig isolated vas deferens. (a) Control; (b) TTX (5×10^{-7} M); (c) low Na^+ (85.2 mM)-sucrose medium; (d) verapamil (3×10^{-6} M); (e) low Ca^{2+} (0.6 mM)-high Mg^{2+} (2.6 mM) medium. TTX and verapamil were added 15 min before AP-B. AP-B was added after incubation in both a low Na^+ and a low Ca^{2+}-high Mg^{2+} medium for 20 min. Vertical and horizontal calibrations apply to each record.

AP-B-induced contraction was also abolished by incubation of the vas deferens in low Na^+ (25.2 mM)-Li^+ (120 mM) medium for 90 min.

Assay of noradrenaline

AP-B ($10^{-8}\sim10^{-5}$ M) induced a dose-dependent increase in the amount of NA released from the vas deferens into the incubation medium (Figure 4). The maximum NA release was obtained with concentrations of AP-B in the 10^{-6} to 10^{-5} M range. The amount of the NA released following treatment with AP-B (10^{-5} M) was approximately 310 times (12.1 μg per g tissue during 30 min) greater than that released from unstimulated control vasa (39.3 ng per g tissue during 30 min). The NA release induced by AP-B (3×10^{-8} M) was inhibited or abolished after treatment with TTX (5×10^{-7} M) or verapamil (3×10^{-6} M) or after incubation in Ca-free medium (Table 1).

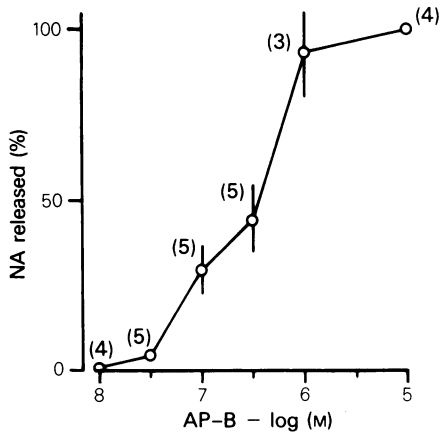

Figure 4 Increase in noradrenaline (NA) released by increasing doeses of anthopleurin-B (AP-B) from the guinea-pig vas deferens. The responses are expressed as % of the maximal amount of NA released by AP-B (10^{-5} M) which is 12.1 μg/g tissue. Symbols indicate the mean of the NA release; vertical lines show s.e.mean. Numbers in parentheses are number of experiments.

Discussion

AP-A, AP-B and AP-C, have similar primary amino acid sequences (Tanaka *et al.*, 1977; Norton *et al.*, 1978), and each elicited rhythmic contractions of the guinea-pig vas deferens of similar shape, the potency of AP-B being greatest. The AP-B-induced contraction was abolished by an α-adrenoceptor blocking agent and by pretreatment with reserpine or 6-OHDA, but was unaffected by anticholinergic, histamine or 5-hydroxytryptamine receptor blocking drugs or prostaglandin synthesis-inhibiting drugs. AP-B markedly increased the amount of NA released from the vas deferens. These data suggest that the AP-B-induced contraction is mediated by the release of endogenous NA.

In the presence of AP-B (10^{-6} M$\sim10^{-5}$ M), there was a profound increase in the NA levels in the medium (11.4$\sim$12.1 μg per g tissue). Since the endogenous NA content in the vas deferens was approximately 17 μg per g tissue (Wakade & Krusz, 1972), more than 50% of total tissue NA may have been released, into the medium following treatment with AP-B ($10^{-6}\sim10^{-5}$ M) for 30 min. The amount of NA released by 80 mM KCl was only 1 μg per g tissue under the same conditions (unpublished data).

Table 1 Effect of anthopleurin-B (AP-B) on the amount of noradrenaline (NA) released from guinea-pig vas deferens in the presence and absence of tetrodotoxin, verapamil or Ca^{2+}

Treatment	*Amount of NA released* (ng/g tissue)	n
None (control Krebs solution)	39.3 ± 6.3	5
AP-B (3×10^{-8} M)	437.0 ± 72.0	5
Tetrodotoxin (5×10^{-7} M) + AP-B (3×10^{-8} M)	28.7 ± 0.1*	4
Verapamil (3×10^{-6} M) + AP-B (3×10^{-8} M)	116.8 ± 18.2*	4
Ca-free solution + AP-B (3×10^{-8} M)	134.2 ± 28.5*	4

Values are mean ± s.e.mean.
Pretreatment and test solutions contained phenoxybenzamine (5×10^{-5} M). AP-B was added 15 min after treatment with tetrodotoxin and verapamil or incubation in Ca^{2+}-free medium. Measurements were made 30 min after treatment with AP-B.
Significantly different from the amount released in the presence of AP-B: $^*P<0.01$.
n = number of experiments.

The AP-B-induced contraction and the increased NA release from the tissues were markedly inhibited or abolished following treatment with TTX. The AP-B-induced release of NA increased with increasing (80–150 mM) external Na^+ concentration (unpublished data). Electrophysiological studies have indicated that the AP-A-induced depolarization of the crayfish giant axon was due to activation of TTX-sensitive Na^+ channels (Low, Wu & Narahashi, 1979). In the frog spinal cord, AP-B caused a marked enhancement of the stimulation-induced root potential and the L-glutamate-induced depolarization. These effects were completely blocked by TTX (Kudo & Shibata, 1980). Together these observations suggest that AP-B depolarizes by increasing the Na^+ permeability across the nerve cell membrane; this could play an important role in the release of NA from the adrenergic nerve fibres, and thus account for the rhythmic contractions of the vas deferens.

Ca^{2+} channels in vertebrate neurones are insensitive to verapamil or D600 (Haeusler, 1972; Van der Kloot & Kita, 1975; Gotgilf & Magazanik, 1977; Nachshen & Blaustein, 1979) which are potent antagonists of Ca^{2+} current in heart and smooth muscle. However, in the present experiments verapamil (3×10^{-6} M) or incubation in a low Ca^{2+}-high Mg^{2+} medium, abolished the AP-B-induced contraction and inhibited the AP-B-induced release of NA from the vas deferens. The release of NA by AP-B may therefore require extracellular Ca^{2+} in the medium and the inhibitory effect of verapamil or the low Ca^{2+}-high Mg^{2+} medium may be explained by their inhibition of Ca^{2+}-influx across the presynaptic nerve cell membranes, with inhibition of transmitter release from presynaptic terminals. Furthermore, it is assumed that in the present experiments verapamil (3×10^{-6} M) may have specifically inhibited Ca^{2+} channels on the nerve cell membrane of the vas deferens, since verapamil at relatively low concentrations ($10^{-6} \sim 10^{-5}$ M) had no apparent effect on Na^+ channels in the presynaptic nerve terminals (synaptosomes) from rat brains (Nachshen & Blaustein, 1979).

The present results have implicated NA in AP-A, AP-B and AP-C-induced contractions of the vas deferens. This is in contrast to previous reports suggesting that AP-A had no apparent effect on heart rate, blood pressure and vascular smooth muscle (Shibata *et al.*, 1976; 1978; Blair *et al.*, 1978; Scriabine *et al.*, 1979). Neither AP-B nor AP-C at concentrations of 10^{-8} M $\sim 10^{-6}$ M have any effects on the rabbit aorta and portal vein under the same conditions as described by Shibata *et al.* (1976) (unpublished data). The reason for this apparent discrepancy is unknown.

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