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The Potent Excitatory Effect of a Novel Polypeptide, Anthopleurin-B, Isolated from a Sea Anemone (*Anthopleura Xanthogrammica*) on the Frog Spinal Cord[1]

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ABSTRACT

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The effect of a novel polypeptide, anthopleurin-B (AP-B), isolated from a sea anemone (*Anthopleura xanthogrammica*) was studied on the isolated, intra-arterially perfused spinal cord of the bullfrog and was compared with those of three other polypeptides isolated from sea anemones [anthopleurin-A (AP-A), anthopleurin-C (AP-C) and neurotoxin II of *Anemonia sulcata* (ATX-II)]. AP-B (10^{-9}–10^{-7} M) caused a marked augmentation of the ventral and dorsal root potentials induced by the stimulation of the dorsal root, whereas AP-A, AP-C and ATX-II, even at a concentration of 10^{-7} M, showed no apparent effect. At a higher concentration of 10^{-6} M, these polypeptides caused a depolarization in both ventral and dorsal roots, but had no excitatory actions on the root potentials. AP-B markedly prolonged the half-decay time of the excitatory postsynaptic potential and elevated the membrane resistance, although it had no effect on the duration of motoneuronal action potentials. AP-B facilitated the depolarization induced by L-glutamate or substance-P in Ca^{++}-free, Mg^{++} (3.6 mM) Ringer solution. The excitatory action of AP-B was much greater in low Ca^{++} media (0.9 mM) than in high Ca^{++} media (3.6 mM). The results suggest that AP-B acts on the subsynaptic membrane to make it more sensitive to neurotransmitters and that the interaction between AP-B and Ca^{++} on the neuronal membrane may play a significant role on the mode of its action.

A novel polypeptide anthopleurin-A (AP-A) with a potent cardiostimulant action was recently isolated from a sea anemone (*Anthopleura xanthogrammica*) (Shibata *et al.*, 1974, 1976, 1978; Norton *et al.*, 1976; Tanaka *et al.*, 1977, Blair *et al.*, 1978, Shimizu *et al.*, 1979). More recently Norton *et al.* (1978) isolated the polypeptides anthopleurin-B (AP-B) and anthopleurin-C (AP-C) from the sea anemones, *Anthopleura-xanthogrammica* and *Anthopleura-elegantissima*, respectively, and showed that these polypeptides also had potent cardiostimulant action. Previous experiments indicated that AP-A had selective, potent, positive inotropic effect on the myocardium but had no apparent effect on vascular smooth muscle (Shibata *et al.*, 1976). Neurotoxin II (ATX-II), a neurotoxic polypeptide with a primary structure very similar to AP-A, was recently isolated from a sea anemone (*Anemonia sulcata*) (Beress *et al.* 1975, Wunderer *et al.*, 1976; Bergman *et al.*, 1976). Their reports lead us to speculate that the polypeptide isolated from sea anemone might have neuroactive properties. Therefore, the present experiments were undertaken to define the properties of AP-A, AP-B and AP-C on the frog spinal cord and compared to those of ATX-II. The preliminary report of this experiment has been presented elsewhere (Kudo and Shibata, 1979).

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Materials and Methods

Isolated, intra-arterially perfused spinal cord of the bullfrog. Bullfrogs (*Rana catesbiana*) weighing 80 to 200 g were obtained from May to December, 1978. The technique for preparing the isolated, intra-arterially perfused spinal cord preparation was almost the same as those described by Matsuura *et al.* (1969) and Kudo *et al.* (1975). Frogs were cooled in crushed ice until in the anesthetic state. All the blood was flushed out by perfusing with fresh Ringer's solution *via* a cannula in the ascending artery. The spinal cord was exposed from the dorsal surface by dissecting away the vertebrae and the dura mater. The spinal cord was isolated and arranged in a chamber. A glass cannula (about 200 μm in tip diameter) was inserted into the anterior spinal artery. The spinal cord was then perfused with amphibian Ringer's solution composed of (millimolar composition): NaCl, 115; KCl, 2.7; $CaCl_2$, 1.8 (in some experiments, $CaCl_2$, 0.9 and $MgCl_2$, 0.9); and glucose, 5.5, with a pH adjusted to 7.6 by adding $NaHCO_3$. The perfusion rate was about 0.3 ml/min. The temperature of the chamber was kept constant at 16°C.

Recording of the root potentials and root reflexes by sucrose-gap method. The sucrose-gap method applied in the present study has already been reported (Kudo *et al.*, 1975). The 9th or 10th ventral and dorsal roots were arranged in two separate pools divided by a sucrose stream, thus isolating the spinal cord and the peripheral stump of the ventral or dorsal root by a sucrose-gap. Potential differences between the spinal cord and the peripheral root stumps were detected by a pair of Ag-AgCl electrodes and recorded by a two pen DC-recorder (Technicorder, type 3047, Yokokawa). An adjacent dorsal root was

stimulated by a pair of Ag-AgCl electrodes (0.1 Hz, duration 0.5 msec, 3–8 V, Nihonkohden SEN 3101). The evoked ventral and dorsal root potentials and reflexes were displayed on an oscilloscope (Nihonkohden VC-9).

Intracellular study. A glass microelectrode filled with 1 M potassium citrate (20–30 megohms in tip resistance) was inserted into a motoneuron. The resting membrane potential and action potentials evoked by the stimulation of a dorsal root were detected by a microelectrode amplifier (WPI model M707) and displayed on an oscilloscope (Tektronix 7613). In some experiments, the membrane resistance was tested with small pulses of inward current that produced 5 to 20 mV of electrotonic potentials.

Materials. Drugs used were AP-A (49 amino acids, MW 5183), AP-B (42 amino acids, MW 4590), AP-C (47 amino acids, MW 4875), ATX-II (47 amino acids, MW 4770), tetrodotoxin (TTX), L-glutamate and substance P. All were dissolved in Ringer's solution and applied through the anterior spinal artery by exchanging the perfusing medium with a drug containing solution (polypeptides and TTX) or by perfusing through a fine polyethylene cannula inserted into a glass cannula at a rate of 0.2 ml/min for 15 sec once every 10 min (L-glutamate and substance P).

Chemistry. AP-B was obtained from the sea anemone *A. xanthogrammica* from Bodega Bay, CA. Hydrolysis with 6 N HCl and with 0.2 M Ba $(OH)_2$ for tryptophan at three different times gave the following absolute amino acid analyses: 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 and TRP_2. The details of isolation and characterization of AP-B were reported elsewhere (Norton *et al.*, 1978).

Results

Root potentials. Figure 1 shows the characteristic actions of four polypeptides (AP-A, AP-B, AP-C and ATX-II) on root potentials. In one of four experiments, AP-B at a concentration of 10^{-9} M caused effective augmentation of the amplitudes of ventral and dorsal root potentials induced by the stimulation of the dorsal root. In three of six preparations, AP-B (10^{-8} M) markedly augmented the amplitudes of ventral and dorsal root potentials (fig. 1A and fig. 2). After the application of AP-B, the d.c.-base line in the ventral root became unstable and initially shifted in a little negative direction (depolarized) and then in a positive direction (hyperpolarized). The effect of AP-B was diminished to only a half of maximum even after prolonged washing (60 min). In a higher concentration (10^{-7} M), AP-B produced depolarization in both ventral and dorsal root and after washing the d.c.-potential in ventral and dorsal root reversed to hyperpolarization which was accompanied by an increase in the amplitudes of evoked root potentials (fig. 1A). Spontaneous oscillations occurred in all the preparations treated with AP-B (10^{-7} M). However, the other polypeptides (AP-A, AP-C and ATX-II) had no effect except at a higher concentration (more than 10^{-6} M) which only caused depolari-

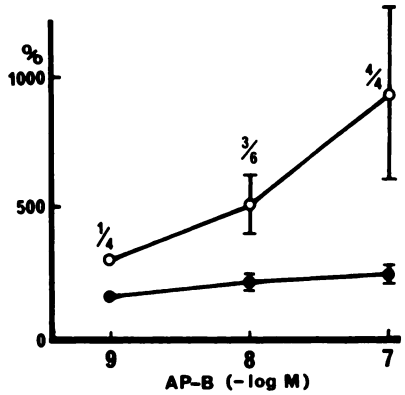

Fig. 2. Dose-response relationships for AP-B on the ventral and dorsal root potentials induced by the stimulation of the dorsal root. Percentage of increase in the ventral root potentials (open circle) and dorsal root potentials (filled circle) (Ca^{++}, 1.8 mM). Each point represents the mean of responses in the AP-B sensitive preparations with S.E.. The number above each concentration indicates the number of AP-B sensitive preparations. Ordinate, percentage of increase in the root potentials 20 min after the application of AP-B. Abscissa, the concentration of AP-B in a logarithmic scale.

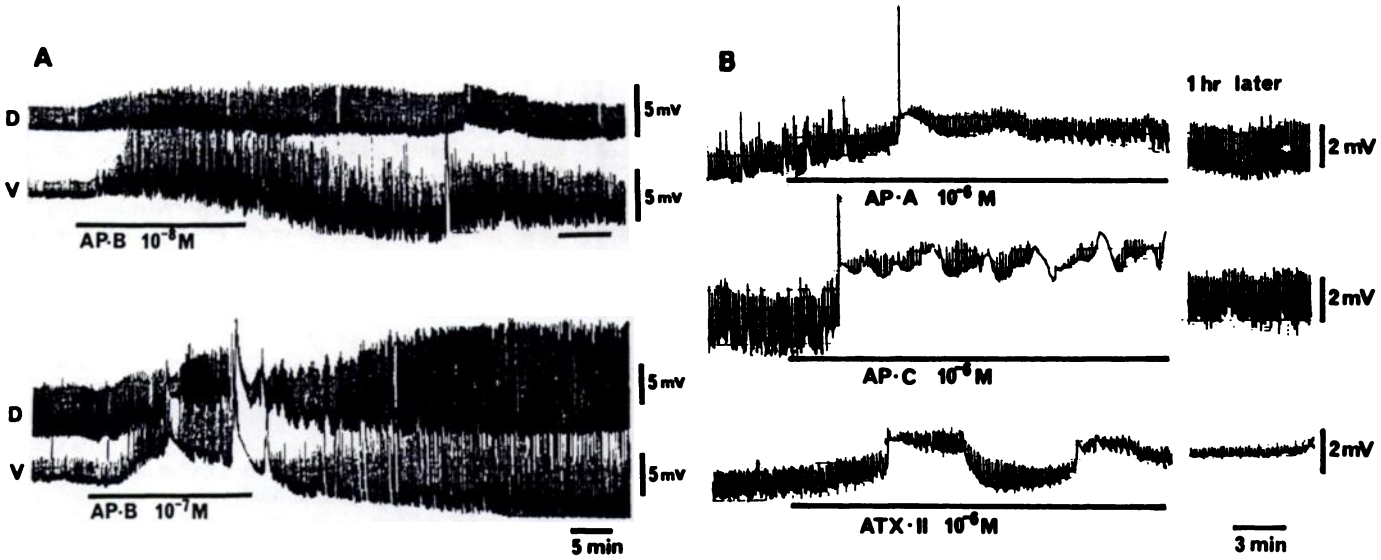

Fig. 1. Representative effects of AP-A, AP-B, AP-C and ATX-II on the ventral and dorsal root potentials induced by the stimulation of the dorsal root. A, the effects of AP-B. Upper trace in each set of figures shows the dorsal root potentials of the 10th dorsal root. Lower trace shows the ventral root potentials of the 9th ventral root. The stimulations were given on the 9th dorsal root at a rate of 0.1 Hz (0.5 msec, 3–8 V). AP-B was perfused for 20 min (Ca^{++}, 1.8 mM). B, effects of AP-A, AP-C and ATX-II. The ventral root potentials in 9th ventral root were induced by stimulating the 9th dorsal root. Each polypeptide was applied for 20 min (Ca^{++}, 1.8 mM).

zation of ventral and dorsal roots (fig. 1B). Figure 2 shows the dose-response relationships for AP-B on the evoked root potentials in the AP-B sensitive preparations.

Figure 3 shows the effect of AP-B on the root reflexes and potentials displayed on an oscilloscope; AP-B (10^{-8} M) caused a marked augmentation of amplitudes of root reflexes and potentials induced by the stimulation of the dorsal root. A half-decay time of the root potential was prolonged markedly. Even at 60 min after the perfusion with drug-free Ringer's solution, excitatory effects of AP-B still remained. Such effects of AP-B (10^{-8} M) on root reflexes and potentials were observed in all three AP-B sensitive preparations.

After treatment with TTX (10^{-7} M), AP-B (10^{-8} M) failed to produce any effect on the root potentials. Post-treatment with TTX (10^{-7} M) abolished the augmented root potentials induced by AP-B (10^{-8} M). Moreover, the excitatory effects of AP-B on the root potentials which were difficult to attenuate by prolonged washing were easily abolished by the addition of TTX (10^{-7} M) for only 5 min during the washing.

Influences of extracellular divalent cations. After perfusing control tissues with Ca^{++}-free medium containing Mg^{++} (0.9 mM), root potentials were nearly abolished. In three such preparations tested, the treatment with AP-B (10^{-8} M) produced rhythmic oscillatory potentials. The size of the oscillatory potentials decreased during the development of depolarization and then increased as d.c.-base line shifted in the positive direction (fig. 4A). These oscillatory potentials were abolished by treatment with TTX (10^{-7} M). In preparations perfused with Ca^{++}-free medium containing Mg^{++} (3.6 mM), which also abolished root potentials, AP-B did not cause oscillatory potentials even in a higher concentration of 2×10^{-8} M ($n = 3$) (fig. 4B).

The influence of extracellular Ca^{++} concentration was tested in three preparations which were confirmed to be sensitive to AP-B (10^{-8} M) in 0.9 mM Ca^{++} solution. Extracellular Ca^{++} concentration was increased to 1.8 mM, 3.6 mM and then reduced to 0.9 mM again. As shown in figure 5, the excitatory effect of AP-B on the root potentials was more remarkable in a lower Ca^{++} medium (0.9 mM) than in 1.8 mM Ca^{++} medium. When the preparation was exposed to 3.6 mM Ca^{++} medium, the excitatory effect of AP-B almost disappeared. Although the range of augmentation of root potentials by AP-B varied from preparation to preparation, such influence of extracellular Ca^{++} was observed in all preparations. In preparations perfused with modified medium [Ca^{++} (0.9 mM) and Mg^{++} (0.9 mM)], the excitatory action of AP-B was more potent than in normal Ringer's solution.

Augmentation of the depolarization induced by L-glutamate and substance P. In preparations perfused with Ca^{++}-free medium containing Mg^{++} (3.6 mM), AP-B (2×10^{-8} M) caused augmentation of the amplitude of depolarization induced by L-glutamate (5×10^{-8} mol) ($n = 5$) or substance P (5×10^{-10} mol) ($n = 3$) (more than 200% of that of control). In four of eight experiments, a little hyperpolarization in the root potential was observed during the application of AP-B but the

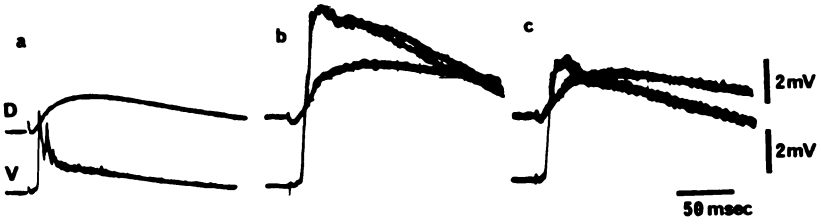

Fig. 3. Effects of AP-B on the root reflexes and potentials. Oscilloscopic display of dorsal root (D, upper traces) and ventral root (V, lower traces) reflexes and potentials measured by sucrose-gap method. Three traces were superimposed in each recording. a, control; b, 20 min after the application of AP-B (10^{-8} M); and c, 60 min after the washing.

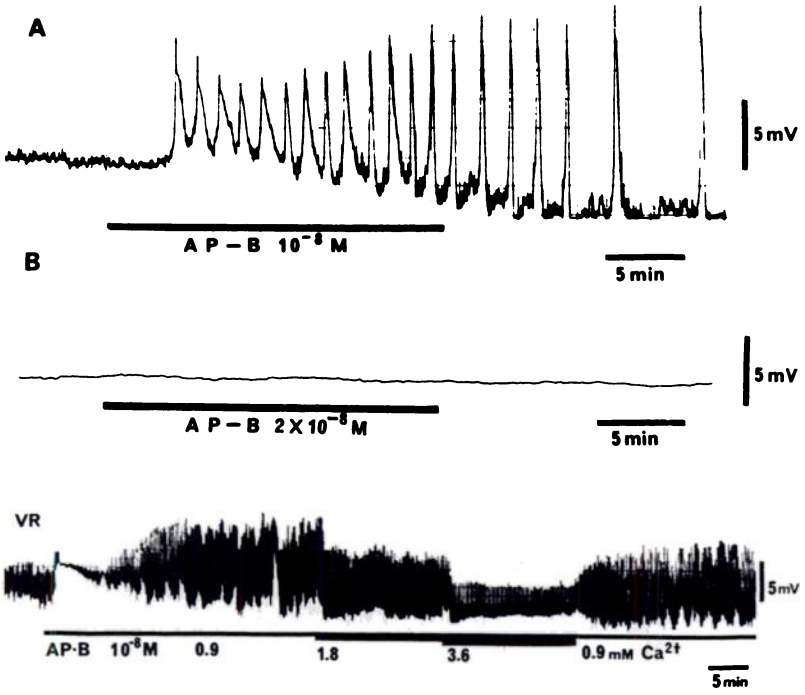

Fig. 4. Effect of AP-B on the ventral root potential in the Ca^{++}-free medium. A, 0.9 mM of Mg^{++} was added to the Ca^{++}-free medium. AP-B (10^{-8} M) was applied for 20 min. B, 3.6 mM of Mg^{++} was added to the Ca^{++}-free medium. AP-B (2×10^{-8} M) was applied for 20 min.

Fig. 5. Influence of extracellular Ca^{++} concentration on the effects of AP-B on the ventral root potentials. During the time period indicated by the horizontal bar, AP-B (10^{-8} M) was perfused, where the Ca^{++} concentration was 0.9, 1.8 or 3.6 mM.

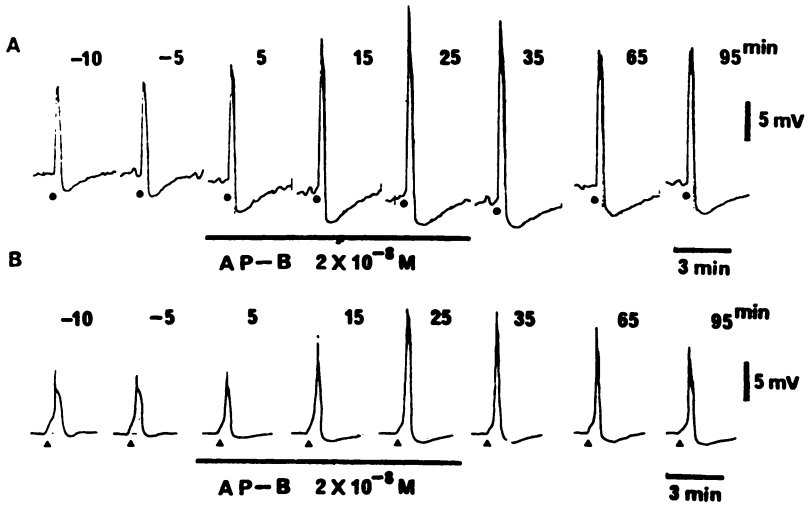

Fig. 6. The augmentation by AP-B of the depolarizing effects of putative excitatory neurotransmitters. The preparation was perfused with Ca^{++}-free medium containing Mg^{++} (3.6 mM). A, the effect on depolarization induced by L-glutamate perfused through a fine polyethylene cannula inserted into the arterial cannula (10^{-3} M, 0.2 ml/min, for 15 sec, 5×10^{-8} mol). B, the effect on the depolarization induced by substance P (10^{-5} M, 0.2 ml/min, for 15 sec, 5×10^{-10} mol). L-Glutamate or substance P was infused once every 10 min.

augmentation of the effects of L-glutamate and substance P were not always accompanied by a hyperpolarization (fig. 6).

Figure 7 shows the time course of the effect of AP-B on L-glutamate-induced depolarization in preparations treated with Ca^{++}-free medium containing high Mg^{++} (9.0 mM) or TTX (10^{-7} M) as the neurotransmission blocker. Although AP-B caused a marked augmentation of L-glutamate-induced depolarization in Ca^{++}-free medium containing Mg^{++} (9.0 mM), it was without effect in the preparations treated with TTX (10^{-7} M).

Microelectrode studies. The effect of AP-B on the activity of the single motoneuron activated by the stimulation of dorsal root was tested in preparations perfused with Ca^{++} (0.9 mM) and Mg^{++} (0.9 mM) containing Ringer's solution. AP-B (10^{-8} M) was applied for 20 min after a stable resting membrane potential (approximately −70 mV) was recorded for about 30 min and then perfused with drug-free Ringer's solution for 30 min. The intracellular microelectrode can be placed in six motoneurons of six separate spinal cords throughout the experiments. Figure 8 indicates a representative recording of the effect of AP-B (10^{-8} M) on the action potentials and excitatory postsynaptic potentials (EPSP). Treatment with AP-B (10^{-8} M) made the membrane potential unstable. In four neurons, AP-B caused a slight depolarization, but had no or a slight hyperpolarizing effect in the other two. The duration of the action potential was not changed by the treatment with AP-B (10^{-8} M), whereas the half-decay time of the EPSP was gradually prolonged and accompanied by an increase in the firing rate of motoneuron (figs. 8, A and B). In addition, the membrane resistance of the motoneuron was elevated (fig. 8B, inset). These effects were obvious in four neurons but were not clear in the other two neurons.

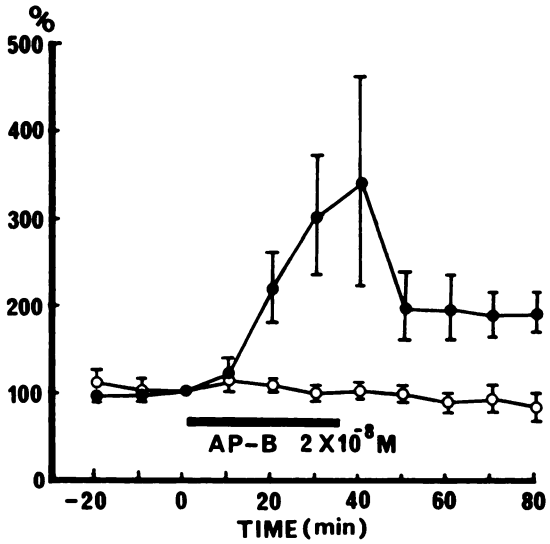

Fig. 7. The time course of the augmentation of L-glutamate-induced depolarization by AP-B. The preparation was perfused with Ca^{++}-free medium containing Mg^{++} (0.9 mM) (filled circle), or TTX (10^{-7} M) (open circle). AP-B (2×10^{-8} M) was applied for 35 min. Ordinate, L-glutamate-induced depolarization in percentage of the response obtained in 0 min. Each point represents the mean ± S.E. of five separate experiments.

Discussion

Although four polypeptides obtained from sea anemones have quite similar amino acid sequences (Tanaka *et al.*, 1977; Norton *et al.*, 1978; Wunderer *et al.*, 1976), only AP-B had a potent excitatory action on the root potentials of the frog spinal cord. AP-B also had a stronger depolarizing action on both ventral and dorsal roots than the other tested polypeptides. Although three dimensional structures of these polypeptides have not yet been determined, the structure of AP-B with four cysteine residues and two disulfide bridges (Norton *et al.*, 1978) can be estimated to be quite different from those of the other polypeptides with six cysteine residues and three disulfide bridges (Tanaka *et al.*, 1977; Norton *et al.*, 1978; Wunderer, 1978; Ishizaki *et al.*, 1979). This lead us to speculate that the difference in the action of AP-B on the spinal cord from the other polypeptides might be associated with differences in three dimensional structure.

ATX-II has been shown to cause a prolongation of the duration of action potential in frog myelinated nerve fibers (Romey *et al.*, 1976; Rathmayer and Beress, 1976). These authors suggested that the polypeptide may have selective blocking action on the inactivation of the open Na^{+}-channel. Recently, Low *et al.* (1979) reported that AP-A has a potent and specific action on sodium inactivation. However, the augmentation of the root potential caused by AP-B in the moto-

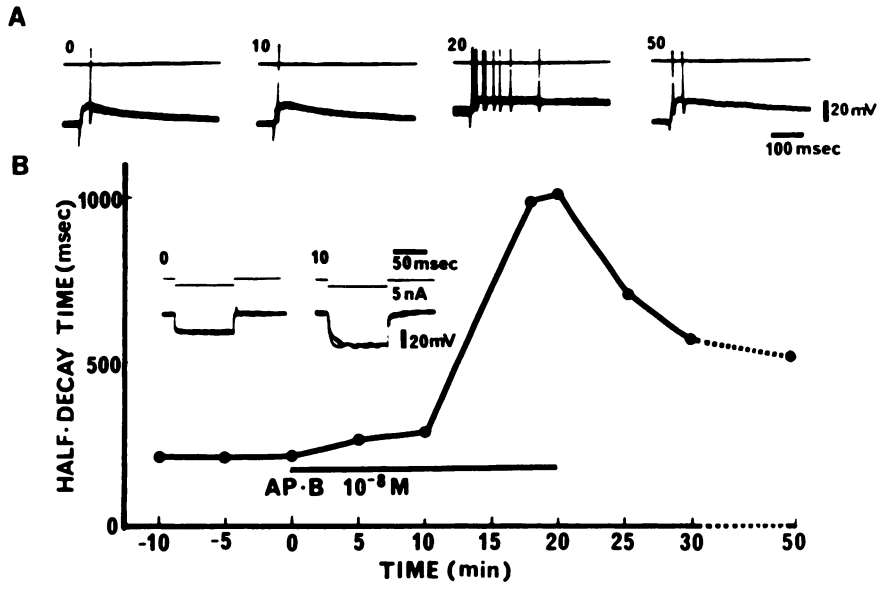

Fig. 8. Effects of AP-B on a motoneuron electrical activity. The preparation was perfused with Ca^{++} (0.9 mM) and Mg^{++} (0.9 mM) containing Ringer's solution. A, the intracellular recording of the motoneuronal action potential and excitatory postsynaptic potential induced by the stimulation of a dorsal root. AP-B was added for 20 min. Three traces were superimposed in each recording. The numbers above the tracings correspond to the time marks on the graph B. B, the time course of the half-decay time of EPSP after the application of AP-B. Ordinate, half-decay time of EPSP. Abscissa, time after the application of AP-B. Insets, recordings of membrane resistance detected by passing constant current pulses (5 nA) for 100 msec. Three traces were superimposed.

neuron was accompanied by a prolongation of the EPSP not the action potential. Thus, the excitatory actions of AP-B on the frog spinal cord seem to differ from those of ATX-II and AP-A. There are at least two possible mechanisms in the augmentation of EPSP: 1) the alteration in the properties of receptors or its surrounding structures to exaggerate the neurotransmitter-receptor interaction (postsynaptic) and 2) the facilitation of neurotransmitter release (presynaptic).

In preparations perfused with Ca^{++}-free medium containing Mg^{++} (0.9 mM), AP-B (10^{-8} M) caused a spontaneous oscillation in the membrane potential of the postsynaptic membrane. A similar phenomenon was observed in the frog spinal cord during the perfusion with Ca^{++}-free medium (Kim *et al.*, 1978). When extracellular Mg^{++} was increased to 3.6 mM, the depolarizing action of AP-B was abolished. These data strongly suggest that AP-B causes the labilization of the postsynaptic membrane by antagonizing the stabilizing actions of divalent cations. In the present experiments, the depolarizing actions of L-glutamate and substance P were found to be augmented by AP-B in the preparations perfused with the Ca^{++}-free medium with Mg^{++} (3.6 or 9.0 mM). Thus, it seems likely that AP-B sensitized the subsynaptic membrane to neurotransmitters.

ATX-II was suggested to cause transmitter release from rat brain synaptosomes (Romey *et al.*, 1976) and from frog neuromuscular junction (Tazieff-Depierre *et al.*, 1978). Also, AP-A was found to release ATP from rat brain synaptosomes (Dr. T. D. White, Faculty of Medicine, Dalhausie University, personal communication). However, the present experiments provide no available evidence for discussing whether AP-B stimulates the transmitter release in the frog spinal cord or not.

Romey *et al.* (1976) reported that pretreatment with TTX blocked the binding of ATX-II, but the binding of ATX-II to the open channel was not affected by post-treatment with TTX. On the other hand, Low *et al.* (1979) demonstrated that AP-A was fully active on crayfish axon pretreated with TTX and suggested that pretreatment with TTX does not prevent subsequent interaction of AP-A with its receptor. In the present study, the effect of AP-B was completely blocked by both pre- and post-treatment with TTX. Although the excitatory effect of AP-B on the root potential was difficult to revert by washing, it was easily attenuated by the application of TTX for only 5 min during washing. Moreover, the augmentation by AP-B of the L-glutamate-induced depolarization was not observed in the preparation treated with TTX as a neurotransmisson blocker. Thus, it seems likely that TTX might interfere with the binding of AP-B on the neuronal membrane. Further study is being undertaken to define the possible mechanisms of effects of AP-B and the interaction with TTX.

Acknowledgments

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