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ELECTROPHYSIOLOGICAL STUDIES OF 1-[2-(DODECYLOXY)ETHYL]PYRROLIDINE HYDROCHLORIDE (DEP) ON ASCARIS MUSCLE CELLS

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Abstract—The effects of 1-[2-(dodecyloxy)ethyl]pyrrolidine hydrochloride (DEP) on Ascaris muscle cells were investigated using electrophysiological techniques. The average resting potential of the muscle cells was 33.7±0.26 mV (S.E.M.). DEP caused depolarization and decreased spike frequency in single muscle cell. This DEP-induced depolarization was not antagonized by d-tubocurarine, other cholinolitics and tetrodotoxin. DEP reduced the membrane potential of the muscle cells bathed for 2 hr in the calcium-free solution containing EGTA but did not produce a contraction. In the presence of lecithin or cephalin in the medium, DEP did not cause depolarization, which suggests that DEP could produce a depolarization by changing the conformation of membrane lipoprotein and then by increasing the ion permeability. It is demonstrated that some divalent cations other than calcium ion could activate the contractile system of Ascaris muscle.

A large number of long-chain alkylamine derivatives have been synthesized and submitted to some screening tests for antiparasitic action (1, 2). Among these derivatives, 1-[2-(dodecyloxy)ethyl]pyrrolidine hydrochloride (DEP) has been found to have the strongest wormcidal action on Ascaris lumbricoides suum (2). It has been demonstrated in a previous paper (3) dealing with the pharmacological effects of DEP on Ascaris that DEP is absorbed through the cuticle of Ascaris body and produces a strong contraction leading to a rigidity in Ascaris body and to death of the worm. From the findings that DEP neither affected a release of acetylcholine from the nerve endings nor acted on the contractile proteins directly, together with other evidence, it has been assumed that the contraction produced by DEP may result from excessive release of calcium from the calcium stores (supposedly the sarcoplasmic reticulum) in Ascaris muscle cell (3).

In this paper, effects of DEP on the electrophysiological activities of Ascaris muscle cells were observed to investigate further the mechanism of the drug action.

MATERIALS AND METHODS

1. Preparations

Ascaris lumbricoides suum were obtained from a local slaughter house and kept overnight in the conventional Tyrode solution at 38°C. Only motile female adult worms were used for experiments. After the anterior 3 cm of the worm had been excised, 4 cm of
the next cylindrical segment was incised along one of the lateral lines and the gut was carefully removed (a rectangular preparation).

2. Solutions

Artificial perienteric fluid (4, 5) with the following composition was used: Na⁺, 129.0; K⁺, 24.5; Ca²⁺, 5.9; Mg²⁺, 4.9; Cl⁻, 50.0; acetate, 125.1; sucrose, 7.5 (mM). In some experiments, a calcium-free solution was employed, and was prepared by omitting the CaCl₂ from the normal solution and adding EGTA (2 mM).

3. Electrophysiological recording from muscle cells

One half of a rectangular preparation was pinned out flat, cuticle side down, on a slab of cork for the purpose of recording the electrical activity, and the other half was mounted horizontally to obtain the contraction. This preparation was placed in a double-walled acrylic organ bath filled with the solution at 38°C. Tension was recorded with a force-displacement transducer, the resting tension being approx. 2 g. Membrane potential and spike activity were recorded from muscle cell bellies with glass microelectrodes (10–20 MΩ) filled with 3 M KCl, displayed on an oscilloscope (Nihon Koden VC-7A) via a preamplifier (Nihon Koden MZ-3B) and photographed. The electrical activities were also recorded together with tension on a two channel penwriter (Nihon Koden RM-20). In all the experiments the solution was bubbled with nitrogen.

4. Recording of the isotonic contraction of isolated somatic muscle

A rectangular preparation was attached to a frontal writing lever, the load being approx. 2 g. The contraction of the longitudinal muscle was recorded on a smoked paper.

5. Drugs

Acetylcholine chloride (Ovisot, Daiichi Seiyaku), d-tubocurarine chloride (Amelisol, Takeda Yakuhin Kogyo), γ-aminobutyric acid (Wako Chemicals), piperazine hexahydrate (Daiichi Seiyaku), strychnine nitrate (Nissin Seiyaku), tetrodotoxin (Sankyo Seiyaku), sodium azide (Wako Chemicals), mersaryl (Igrosin, Takeda Yakuhin Kogyo), ethylenedioxibis(ethylenamino)tetraacetic acid (EGTA, Wako Chemicals). DEP was synthesized in our laboratory.

RESULTS

1. Effects of DEP on electrical activity of Ascaris muscle cell

The mean resting membrane potential of 237 cells tested in the artificial perienteric fluid was found to be 33.7 ± 0.26 mV (S.E.M.), inside negative. This value is in good

![Fig. 1. Spontaneous electrical activity recorded from the muscle cell bellies of Ascaris. (A) Slow waves; (B), (C) rhythmic spike potentials.](image-url)
agreement with that reported by del Castillo (4). Spontaneous rhythmic electrical activities are shown in Fig. 1. Large spikes were recorded from muscle bellies situated close to the nerve cord.

Effects of acetylcholine and DEP on the mechanical and electrical activities of Ascaris preparation are illustrated in Fig. 2A and 2B. DEP (2 × 10⁻⁴ g/ml) caused a sustained contraction and a depolarization similar to that produced by acetylcholine (2 × 10⁻⁵ g/ml). Acetylcholine increased spike frequency continuously, whereas DEP increased the frequency only at the first stage and thereafter gradually decreased it with the result of complete disappearance of the spontaneous activity. Both depolarization and contraction produced by DEP were persistent and could not be reversed by repeated washing of the preparation. Ten min after an addition of DEP (2 × 10⁻⁴ g/ml), the mean membrane potential showed 11 ± 0.6 mV (S.E.M., n = 45). Though DEP (5 × 10⁻⁵ g/ml) reduced the resting membrane potential by only about 2-3 mV, it remarkably decreased both amplitude and frequency of the spike. Correlation between depolarization produced by DEP and certain drugs involved in neuromuscular transmission of Ascaris was examined.

As shown in Fig. 2C, d-tubocurarine, a neuromuscular blocking drug in Ascaris muscle (4, 6, 7, 8), did not antagonize the response to DEP at a concentration of 3 × 10⁻⁵ g/ml, although at this concentration it blocked contractile and depolarizing action of acetylcholine almost completely. Effects of strychnine and procaine were also examined as they have been shown to have a curare-like action on neuromuscular transmission in vertebrate skeletal muscles (9, 10, 11). In Ascaris muscle cells strychnine (1.5 × 10⁻⁴ g/ml) produced a slight hyperpolarization and complete blockade of the spontaneous spike activity within 10 min (Fig. 3), and depressed depolarization and contraction by exogenously added acetylcholine (2 × 10⁻⁵ g/ml) almost completely, which suggests that it may act at a postsynaptic site of Ascaris neuromuscular junction. Procaine (2.5 × 10⁻⁴ g/ml) acted like strychnine on the muscle cells, but did not show any effect on the response to DEP. γ-Aminobutyric acid (GABA) causes powerful hyperpolarization and inhibition of the rhythmic spike activity with a resulting relaxation in Ascaris somatic muscle layer (4, 12).
When GABA (1 x 10⁻⁴ g/ml) was added to the solution after the cell membrane was depolarized by DEP, however, it produced a transient hyperpolarization followed by a gradual reduction of membrane potential (Fig. 4). Effects of piperazine (13) (2 x 10⁻⁴ g/ml) were found to be similar to those of GABA. Tetrodotoxin (1 x 10⁻⁶ g/ml) was shown to have no influence on the depolarizing action of DEP.

In the presence of lecithin (1 mM) or cephalin (1 mM) in the solution, DEP neither produced depolarization and contraction nor decreased the amplitude and frequency of the spontaneous spike, however, the response to DEP recovered as the concentration of the phospholipids was decreased.

In our previous paper (3) we stated that the contractile activity of DEP gradually diminished in *Ascaris* muscle preparation immersed in a calcium-free solution, therefore whether or not DEP depolarizes the cell membrane in a calcium-free solution was investigated. The resting potential increased fairly soon after the change to calcium-free solution and then slowly decreased, while the spontaneous spike activities disappeared gradually. As shown in Fig. 5, an addition of DEP (2 x 10⁻⁴ g/ml) to the solution 1 hr after the muscle preparation was immersed in a calcium-free solution produced a sustained depolarization and a transient contraction, but depolarization only occurred when DEP had been added 2 hr after the immersion.

2. Effects of DEP in the presence of sodium azide or mersaryl

As Fig. 6 shows, sodium azide, a muscle microsomal ATP-ase inhibitor (14, 15), inhibited a first rapid contraction produced by DEP (2 x 10⁻⁴ g/ml). On the other hand, DEP accelerated relaxation of the muscle preparation which had been brought to a state of contracture by the treatment with sodium azide (1 x 10⁻⁴ g/ml) in the normal solution (containing calcium ion) for 30 min and thereafter washed out with a calcium-free solution containing sodium azide (Fig. 7). Mersaryl (1 x 10⁻⁴ g/ml), an inhibitor of reticulum ATP-ase (16, 17), had a similar effect to that of sodium azide on the muscle preparation.
FIG. 6. Records of the isotonic contraction by DEP on Ascaris muscle preparation in the presence of sodium azide. (A) A normal solution was replaced by a calcium-free solution containing EGTA (2 mM) at the point of the thick arrow (○), in which the preparation was left for 2 hr and then DEP was added at 30 min after re-addition of CaCl\(_2\) (8 mM). (B) The condition is the same as (A) except that sodium azide was added to the bath at 30 min before Ca\(^{2+}\) re-addition. ACh; acetylcholine 1 \times 10^{-5} \text{ g/ml}, Ca\(^{2+}\); CaCl\(_2\) 8 mM, DEP; 2 \times 10^{-4} \text{ g/ml}, NaN\(_3\); 1 \times 10^{-4} \text{ g/ml}.

FIG. 7. Effects of DEP on the contracture produced by sodium azide. Sodium azide was added to the normal solution at the point of a closed circle (●), in which the preparation was left for 30 min after which the incubation medium was replaced by a calcium-free solution containing EGTA (2 mM) and NaN\(_3\). ACh; acetylcholine 1 \times 10^{-5} \text{ g/ml}, NaN\(_3\); 1 \times 10^{-4} \text{ g/ml}, DEP; 2 \times 10^{-4} \text{ g/ml}.

3. Effects of divalent cations on contractile system

Fig. 8 shows effects of divalent cations on Ascaris muscle contraction. Though DEP did not cause any contraction in the muscle preparation immersed for 2 hr in a calcium-free solution, it resumed contractile activity by an addition of some divalent cations to the solution at 10 min after DEP treatment. Ca\(^{2+}\) produced a very strong contracture. Sr\(^{2+}\) produced a slightly weaker contracture than Ca\(^{2+}\). The effect of Ba\(^{2+}\) was appreciably weak. The effect of Cd\(^{2+}\) was as strong as Ca\(^{2+}\). Mn\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\) and Hg\(^{2+}\) did not have any apparent effect. Fig. 9 demonstrates the result of the experiments in which the divalent cations were added to the solution 30 min before an addition of DEP. DEP produced the same magnitude of contracture on a re-addition of Ca\(^{2+}\) (8 mM) as in the normal solution. In Sr\(^{2+}\) solution, DEP did not cause the first rapid contraction, but slowly increased the degree of contracture. Cd\(^{2+}\) had an effect similar to that of Ca\(^{2+}\). Other divalent cations tested were ineffective. The replacement of Ca\(^{2+}\) ion in the normal solution for the equivalent moles of Sr\(^{2+}\) ion or Ba\(^{2+}\) ion resulted in a decrease of the tone of the muscle (relaxation). On the contrary, Cd\(^{2+}\) ion tended to increase the tone.
FIG. 8. Effects of divalent cations on Ascaris muscle preparation treated with DEP in a calcium-free solution containing EGTA (2 mM). Experimental conditions as described in Fig. 6A. Ten min after DEP addition, divalent cations were added. ACh; acetylcholine $1 \times 10^{-5}$ g/ml, DEP; $2 \times 10^{-4}$ g/ml, divalent cations; 8 mM each.

FIG. 9. Effects of DEP on Ascaris muscle in the solution with divalent cations substituted for Ca$^{2+}$ ion. Experimental conditions as described in Fig. 6A. Divalent cations were added at the point of the closed circle (○) and, at 30 min after that, DEP was added. ACh; acetylcholine $1 \times 10^{-5}$ g/ml, DEP; $2 \times 10^{-4}$ g/ml, divalent cations; 8 mM each.

DISCUSSION

In addition to the conventional biochemical (18) and pharmacological (19, 20) methods, recent application of electrophysiological method (4, 21, 22) to studies on anthelmintic actions makes it possible to comprehend the mechanism of drug action in relation to vital phenomena of parasitic helminths. In order to investigate the drug action Ascaris lumbricoides was employed on which electron microscopic and electrophysiological studies have widely been done, and on which the relationship between structure and function of neuromuscular junction, and also the contractile mechanism have been fairly well clarified (4, 19, 20, 23). The mononucleated somatic muscle cell has three parts: muscle fiber, muscle belly, and muscle arm which is divided into a number of fingers forming the muscle syncytium and finally neuromuscular junctions at the nerve cord. On the other hand, the contraction in the somatic muscle of Ascaris is elicited by rhythmic repetitive action potentials of myogenic origin which conduct toward muscle belly along the arm. The nerve cord fibers in close contact with the syncytium play the regulative role by two types of synapse: excitatory and inhibitory. On the physiological basis of Ascaris, we have investigated the electrophysiological effects of DEP on the muscle cell.
In our experiment DEP like acetylcholine reduced the membrane potential of the muscle cell bellies concomitant with the increase in tension. This suggests that the DEP-induced contraction may be triggered by a reduction of the resting potential. A previous experiment (3) with the depolarized muscle, however, showed that DEP causes a contraction regardless of changes in the resting potential. On the basis of these results DEP appears to act on, at least, the cytoplasmic membrane and the sarcoplasmic reticulum in *Ascaris* muscle cell. Following the study of DEP on the sarcoplasmic reticulum of *Ascaris* muscle cell reported in a previous paper (3), an attempt was made to investigate the mechanism of the depolarizing action of DEP.

It appears unlikely that the DEP-induced membrane depolarization depends on acetylcholine release from nerve endings, for it is not blocked by d-tubocurarine, procaine and strychnine. Since tetrodotoxin does not interfere with this depolarization, the mechanism is presumably different from that of batrachotoxin-induced depolarization, which has been reported to be mediated by a specific increase of sodium permeability and blocked by tetrodotoxin (24). The presence of lecithin or cepharin, major constituents of lipid layer in cell membrane, inhibited depolarization and contraction by DEP completely. This may be explained as follows; since DEP has a strong affinity to phospholipids, it easily interacts with phospholipids in membrane to break hydrophobic bindings between lipids and proteins, and to change the conformation of protein. This results in the breakage of ion balance across the membrane and a reduction of the membrane potential. It has been reported (25) that cationic surface-active agents strongly interact with the phospholipids in red cell membranes and remove the phospholipids out of the membranes to give rise to hemolysis. In our experiments with *Ascaris* muscle, however, removal of phospholipids was not observed with DEP.

DEP markedly increased \(\text{Ca}^{2+}\) contraction in calcium-free solution and accelerated the relaxation of the contracture produced by treatment with sodium azide or mersaryl, presumably due to inhibition of \(\text{Ca}^{2+}\) uptake to the calcium stores (supposedly the sarcoplasmic vesicles). These facts imply that DEP is apt to facilitate \(\text{Ca}^{2+}\) ion permeability in the cell membrane. Therefore, the depolarizing action of DEP appears to contribute at least partially to its contraction by increasing influx of \(\text{Ca}^{2+}\) out of the extracellular fluid into the cytoplasm.

It is generally known that \(\text{Sr}^{2+}\) ion, and \(\text{Ba}^{2+}\) ion in some cases, are able to substitute the specific \(\text{Ca}^{2+}\) functions in the contraction-relaxation cycle of various muscles. The effects of divalent cations other than \(\text{Ca}^{2+}\) on the *Ascaris* muscle preparation were examined under the assumption that permeability was also increased by DEP. The experiments in a calcium-free solution showed that some divalent cations could partly substitute \(\text{Ca}^{2+}\) ion in the activation of the contractile system of *Ascaris* muscle, the order of activation intensity being \(\text{Ca}^{2+} \geq \text{Cd}^{2+} > \text{Sr}^{2+} > \text{Ba}^{2+}\). Since, in the presence of \(\text{Cd}^{2+}\) substituted for \(\text{Ca}^{2+}\), DEP produced a contraction with the properties similar to the normal contraction, \(\text{Cd}^{2+}\) is assumed to accumulate in the calcium stores of *Ascaris* muscle cells. \(\text{Sr}^{2+}\) and \(\text{Ba}^{2+}\) appear, however, to exert very little effect. Since \(\text{Cd}^{2+}\) potentiates the twitch of frog
sartorius and toe muscles (26), and also stimulates the ATP-ase of actomyosin in rat skeletal muscle (27), an investigation of the physiological role of Cd\textsuperscript{2+} capable of producing a Ca\textsuperscript{2+}-like effect on *Ascaris* muscle should be most revealing.

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