Potentialiation by Endotoxin of Responses Associated with Increases in Calcium Conductance

(corticosteroid)

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ABSTRACT Bacterial endotoxin at concentrations of 0.03 $\mu\text{g/ml}$ enhanced the following responses, each of which reflects calcium influx: (1) contraction of spiral intestine retractor muscle of dogfish; (2) amplitude of postsynaptic potential of squid giant synapse as a function of presynaptic depolarization; (3) membrane responses of deep abdominal extensor muscle of crayfish, stimulated intracellularly; and (4) slow transient (calcium) current in trabeculae of frog atrium under voltage clamp. Corticosteroid antagonized the endotoxin effect on the smooth muscle, not on the frog atrial current. One mechanism of action of endotoxin may be to increase transmembrane calcium currents.

The mechanisms by which bacterial endotoxins inflict lethal damage are not fully understood. When a lethal dose is injected into the circulation there is generalized vasoconstriction, progressive hypotension, release of vasoactive agents, increased permeability of the peripheral vessels, with loss of plasma and erythrocyte extravasation, especially into the lungs and gastrointestinal mucosa, and progressive decline in venous return and in cardiac output until vital centers can no longer be sustained.** Much evidence indicates that in mammals the local release of norepinephrine is an essential step in the process of tissue injury by endotoxin (1-3). Liver, spleen, heart, adrenal, and hypothalamus of animals killed by endotoxin show a substantial depletion of the stores of norepinephrine (4). Since denervated visceral tissues escape injury due to endotoxin and show no depletion of norepinephrine, it appears that endotoxin is primarily a neurotoxin.

In a recent study of a neuromuscular synapse of the crayfish, endotoxin was shown to act presynaptically and to cause an increase in transmitter release (5), but no mechanism for this enhancement was given. Further information is needed concerning the action of endotoxin on excitable membranes

** The biological activity of the endotoxins used in these studies was determined in two ways: (1) the median lethal dose when given intravenously to adult white New Zealand rabbits, i.e., 100 ng/kg; and (2) the least amount (0.1 ng) in a series of 2-fold dilutions (standard error = one tube dilution more or less) required to produce a solid clot when interacted with a lysate of the blood cell from horseshoe crab (*Limulus polyphemus*), i.e., 0.1 ng/kg. These values are nearly the same for most endotoxins prepared by the conventional extraction technique. Nucleic acids and other contaminants in these preparations do not exhibit any of the pharmacological effects of endotoxin.

and synapses. We present evidence that in four different preparations, each of which depends for a certain response on increases in calcium conductance, endotoxin enhanced the physiologic response. These preparations and responses are: (1) tonic contraction of smooth muscle from a dogfish mesentery; (2) postsynaptic response in squid giant fiber synapses; (3) response of lobster and crayfish muscle fibers to direct stimulation; and (4) calcium current in frog atrial muscle fibers.

Dogfish mesenteric muscle

The mesenteric smooth muscle of a dogfish can give either brief phasic or prolonged tonic contractions in response to either electrical or mechanical stimulation; these responses are determined by action potentials that appear to result from increases in calcium conductance (7). Phasic contraction is associated with a single action potential, and tonic contraction with repetitive muscle spikes; these responses occur in the presence of tetrodotoxin, an agent that selectively blocks the regenerative sodium conductance increase in nerve and muscle (8), and result from direct stimulation of the muscle. Effects of endotoxin were tested on 12 preparations. Muscle strips were mounted in a 30-ml bath so that contractions were recorded with a force transducer and action potentials by floating microelectrodes (for details see ref. 7). Each muscle was initially stimulated with pulses of 5-msec duration at 60-sec intervals so that only repeated phasic contractions of constant amplitude were obtained (9); then endotoxin was added to the bath to give final concentrations of 0.03-0.1 $\mu\text{g/ml}$. Fig. 1 shows that endotoxin enhanced contraction and caused a preparation that was giving only phasic contractions (Fig. 1A) to go into prolonged tonic contractions (Fig. 1B and C). The enhancement was noted within 2-5 min and reached a maximum at 5-10 min. During the tonic contraction, rhythmic action potentials were observed (Fig. 1C). The enhanced smooth muscle response due to endotoxin was reversible by washing and could be repeated several times on the same preparation. Addition of corticosteroids (methylprednisolone, *Solu-Medrol*, Upjohn, or *Decadron*, Merck) at concentrations of 0.1 $\mu\text{g/ml}$ caused immediate recovery from the endotoxin effect (Fig. 1D and H). If a preparation was pretreated with steroid, the endotoxin was ineffective. It is possible that endotoxin may act by way of autonomic nerve endings since the dogfish muscle is sensitive to norepinephrine by way of

FIG. 1. Effect of endotoxin on spiral intestine retractor (mesenteric) muscle of dogfish. A-D were from the same preparation. (A) Control response in normal saline, phasic mechanical response to single shock. (B) Mechanical response 5 min after application of 0.03 µg/ml of endotoxin. Note enhanced tonic response. (C) Sections from an electrical (upper) and mechanical (lower) recording to show repetitive intracellularly recorded spikes during tonic contraction in response to a single shock after endotoxin. Total duration of contraction was 7 min, 5 sec; At left is rising phase of contraction, electrode dislocated by movement; middle shows electrode in another cell firing repetitively. Such prolonged responses could be obtained repeatedly from the same preparation. (D) Mechanical response 1 min after application of the steroid preparation Solumedrol (0.1 µg/ml). Note that after addition of steroid a single stimulus did not evoke tonic contraction despite the presence of endotoxin. Calibration: A, B, D, and F, 3 g 10 sec. C, 3 g 1 mV, 2 sec. E-H were from another preparation. (E) Control contraction; (F) response 5 min after endotoxin (0.03 µg/ml) was added; (G) 10 min after endotoxin was added, contraction lasted 1.5 min; (H) 5 min after steroid Decadron (6 µg/ml) was added, now single shock does not elicit tonus. Calibration: 3 g, 10 sec.

alpha receptors. However, the effect of endotoxin was not abolished by phenoxybenzamine, an alpha receptor blocker, or tetrodotoxin. This result suggests that endotoxin acts di-

FIG. 2. Effect of endotoxin (0.03 µg/ml) on the relationship between presynaptic depolarization and postsynaptic potential amplitude in squid giant synapse. Perfusate contained 10^{-7} M tetrodotoxin throughout the experiment. Each set of measurements was done after the preparation had been perfused for 15-20 min with about 25 ml of the test solution. Solid circles, control values; open circles, responses after addition of 0.03 µg/ml of endotoxin; triangles, recovery after washing.

FIG. 3. (1) Effects of 0.05 µg/ml of endotoxin on the spike-like response of the deep abdominal extensor muscle of crayfish produced by intracellularly applied current of 22-msec duration. (A) Before application of endotoxin; (B) response 3 min after application of endotoxin; (C) after washing with van Harreveld's solution. Calibration: 44 mV, 22 msec. (2) Effects of 0.05 µg/ml of endotoxin on the graded response of the deep abdominal extensor muscle of crayfish produced by stimulation by 20-msec intracellular pulses in choline-substituted solution. (A) Control response; (B) 2 min after application of endotoxin; (C) several successive responses with constant injected current, 4 min after endotoxin. Duration of response declines on repetition. Calibration: 44 mV, 22 msec.

rectly on the smooth muscle. Additional effects on nerve endings that cannot be detected by these methods are not excluded.

Squid giant synapse

In order to study a calcium system more directly, we selected the giant fiber synapse of squid since preparations poisoned with tetrodotoxin, which blocks Na currents, show responses to presynaptic depolarization which depend on calcium entry at presynaptic endings (10, 11). Squid stellate ganglia were mounted as described by Kusano (10) and perfused with artificial sea water. Stimulating and recording intracellular microelectrodes were placed on both the presynaptic and postsynaptic sides of the synapse. The preparation was treated with tetrodotoxin to abolish pre- and postsynaptic action potentials; the concentration of tetrodotoxin (10^{-7} M) has no effect on transmitter release (11). Postsynaptic potential responses were measured as a function of presynaptic depolarization, and pre- and postsynaptic membrane resistance was measured. A wide range of input-output relations of the synapse was established; then endotoxin was applied to a final concentration of 0.03 µg/ml. Resting membrane potential and membrane resistances of the pre- and postsynaptic fibers were not altered by the endotoxin. The action potential was not modified in the endotoxin-containing saline. The delayed rectification due to the depolarizing K-activation was also not modified. Nevertheless, there was evidence that the endotoxin enhanced synaptic transmission by augmenting the input-output relation (Fig. 2). The augmentation was more evident in those preparations in which transfer characteristics were lower than average (10). The endotoxin effect on the squid giant synapse was nearly completely reversible when the toxin was removed from the perfusate. The increase of transmitter release was observed in preparations in which sodium currents were blocked; hence it is postulated that this increase in efficacy of synaptic transmission may have resulted from an increase in the Ca-current, which is necessary for electrosecretory coupling elicited by presynaptic depolarization (11). However, the possibility is not excluded that the same amount of transmitter is released under endotoxin as normally and that it causes a larger change in postsynaptic conductance.

Fig. 4. Voltage step (lower beam) and associated membrane current (upper beam) from a trabecula of frog atrium bathed in control saline (left) and in saline containing 0.03 µg/ml of endotoxin (right), sucrose gap records. Scales: current, 20 nA; voltage, 20 mV; time, 10 msec.

Lobster and crayfish muscle fibers

A synaptic effect of endotoxin in the crayfish, *Orconectes* sp., has been reported (5). In those experiments, an increased frequency of miniature excitatory postsynaptic potentials, decreased facilitation, and increased evoked excitatory postsynaptic potential were observed after the application of endotoxin.

To examine further the effects of endotoxin on excitable properties, we investigated the spike-like response to intracellularly injected current of the medial deep abdominal extensor muscle of the crayfish *Procambarus clarkii* (12). The ventral abdomen containing the deep abdominal extensor muscle was secured in a lucite chamber and bathed with van Harreveld's crayfish saline (13). For certain experiments choline chloride was substituted for sodium chloride. Two microelectrodes were inserted in the muscle fiber, one for stimulating with current pulses of varying duration and magnitude, the other for recording the potential responses, in the manner of Fatt and Katz (14).

Stimulation of fibers of the deep abdominal extensor muscle of *Orconectes* sp. produces propagated spikes (15). We found that many muscle fibers of *Procambarus clarkii* gave only a graded response (12). Stimulation of other fibers of *Procambarus* resulted in a spike-like response, which was not propagated (Fig. 3-1). Addition of 0.05 µg/ml of endotoxin brought about an increase in amplitude of the spike-like response (Fig. 3-1B). In six out of nine fibers with a distinguishable spike-like response, the amplitude increased with endotoxin. The endotoxin effect was reversible (Fig. 3-1C).

In the crayfish *Orconectes* sp., the spike in the deep abdominal extensor muscle appears to be caused primarily by calcium ions (15). To determine whether the increase in amplitude in the response in *Procambarus* results from increased influx of calcium or of sodium ions, we performed five experiments in solutions with choline substituted for sodium. As shown in Fig. 3-2, addition of 0.05 µg/ml of endotoxin increased both the amplitude and duration of the response in choline, indicating that endotoxin could be acting by increasing calcium influx (Fig. 3-2B and 2C).

We monitored membrane resistance by using hyperpolarizing pulses. Four fibers showed essentially no change in input resistance after addition of endotoxin. Evidently endotoxin affects primarily calcium activation.

From these experiments we conclude that endotoxin increases the amplitude of the spike-like response in the deep abdominal extensor muscle; that the endotoxin effect is reversible; and that from the experiments in choline, endotoxin affects calcium influx rather than sodium influx.

We have also observed potentiation of the action potential in response to nerve stimulation of the deep abdominal extensor muscle of lobster *Homarus americanus* with endotoxin application.

Fig. 5. Frog atrial bundle in sucrose gap. Plot of peak slow inward current (ordinate) against magnitude of step depolarization from resting potential (abscissa). (O) Control; (Δ) 0.03 µg/ml of endotoxin; (□) 0.03 µg/ml of endotoxin and twice the control calcium concentration.

Frog atrial muscle

Since the preceding observations suggest that endotoxin increases calcium currents, we tested the agent on a system in which calcium currents can be measured directly by means of voltage clamping. There is considerable evidence that one component of the transmembrane current that flows during amphibian cardiac action potential is carried by calcium ions (16-19). This component is large and exists in addition to and independent of sodium and potassium conductance mechanisms similar to those found in nerve and striated muscle (20, 21). Under voltage-clamp conditions the current for which Ca^{++} is at least a partial carrier is a transient, which is activated by depolarizations greater than 50 mV and is inwardly directed for depolarizations between this value and about 130 mV. The time-course of this transient is about 10 times longer than that of the sodium transient; the Ca current will be referred to as the slow transient. Tetrodotoxin has little or no effect on this slow transient but does abolish the fast sodium-carried transient. There is some evidence for fluxes of both Na and Ca in the slow inward transient since Rougier *et al.* (17) found a sizeable slow transient in Ca-free Ringer's solution. Our data (Connor, unpublished) and those of Tarr (16) indicate that for normal concentrations of Ca, sodium flux is at most a minor carrier of the slow transient, with calcium flux predominant.

Voltage clamp measurements were made in a double sucrose gap (22) on single atrial trabeculae, 50-80 µm in diameter, 3-4 mm in length, from *Rana pipiens*. In the chamber that was used, the central or test node was formed by flowing sucrose-saline interfaces and was 60-80 µm wide. The physiological saline contained twice the usual calcium concentration (2.2 mM) in order to augment the voltage clamp current of the slow transient and contained also 10 mM tetraethylammonium chloride to reduce delayed outward current (23). In some experiments tetrodotoxin was added to abolish the early fast currents.

Fig. 4 (left) gives the time-course of a control slow transient under voltage clamp conditions in a sucrose gap. The fast sodium transient has been blocked by tetrodotoxin (3 ×

10^{-7} M). Fig. 4 (*right*) shows the membrane current associated with an identical voltage step after a 4-min exposure to saline containing endotoxin ($0.03 \mu\text{g}/\text{ml}$) in addition to tetrodotoxin. The magnitude of the slow current is increased over that of the control, but other aspects of the time course are unchanged within the limits of experimental detection. The enhancing effect of endotoxin was reversible by prolonged washing with normal or tetrodotoxin-containing Ringer's solution. The observations in Fig. 4 are typical of a series made on 15 trabeculae which displayed a sizeable slow transient in the control saline; i.e., the magnitude of the current was increased by a factor of 2–4 with little or no change in other parameters. Preparations in which the slow transient current was negligibly small or absent at the beginning showed little response to the toxin.

Fig. 5 shows the peak inward current of the slow transient as a function of clamp voltage under three conditions. No correction for leakage current has been made. The control is the response in saline containing 2.2 mM Ca. The curve of results after exposure of the preparation to endotoxin, demonstrates a considerable increase in peak inward current over the control. When the external calcium concentration was increased from 2.2 to 4.4 mM, a further increase in peak inward current resulted. This increase is consistent with evidence that calcium ions are carrier of some of the slow transient current since the electrochemical driving force would be greater in 4.4 mM than in 2.2 mM Ca. Corticosteroid (Solumedrol) was applied at concentrations up to $2 \mu\text{g}/\text{ml}$ along with endotoxin. In contrast to the observations on dogfish smooth muscle, no antagonism was observed. This finding suggests that not all calcium channels are identical.

The effect of endotoxin on the fast sodium current was not quantitatively ascertained. However, five trabeculae that were tested in Na-free Ringer's solution (Tris replacement) showed increments in the slow transient on exposure to endotoxin, which were within the range of those seen in normal Ringer. Hence, the effect of endotoxin on any slow sodium current must be very small or negligible. This result is in agreement with observations on squid axons (Narahashi, T. & Fine, J., unpublished) and on crayfish and frog axons (Parnas, unpublished) which showed no obvious effect of endotoxin; hence action on Na-conductance changes, if it occurs, is negligible.

Conclusion

We conclude that in four dissimilar preparations, responses that were known to depend on calcium conductance are enhanced by endotoxin. It is quite possible that endotoxin increases calcium currents in general and that this effect may explain many of the clinical actions of endotoxin. Further, endotoxin should be a useful tool for the study of calcium conductance changes in other systems.

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