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Research Article

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Effect of Purified Staphylococcal Alpha Toxin on Active Sodium Transport and Aerobic Respiration in the Isolated Toad Bladder

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ABSTRACT Purified staphylococcal alpha toxin was found to inhibit the active transport of sodium across the isolated toad bladder when applied to the serosal but not to mucosal surface. Heating or the addition of specific antitoxin abolished this effect. Low temperatures reduced this activity significantly. Application of vasopressin to the bladder serosa shortly after toxin resulted in only weak and transient stimulation of sodium transport; once maximal toxin activity had been established, exposure to the hormone was without effect. Transport in bladders previously stimulated by vasopressin was rapidly inhibited by alpha toxin. Concentrations that suppressed active sodium transport completely within 30–45 min produced a significant increase in oxygen consumption by minced bladder tissue within the same period; antitoxin neutralized this activity. 2,4-dinitrophenol also inhibited sodium transport and stimulated oxygen consumption by the toad bladder. The addition of 2,4 dinitrophenol to bladder tissue in which respiration was maximally stimulated by alpha toxin resulted in a further increase in respiratory rate. The addition of toxin to bladder tissue after its exposure to a concentration of 2,4 dinitrophenol known to uncouple oxidative phosphorylation produced a significant stabilization but no increment in respiratory rate. The data are consistent with the previously suggested action of staphylococcal alpha toxin on cell membranes and suggest that energy-dependent transport processes are inhibited. The stimulation of oxygen consumption may be due to an additional effect on oxidative phosphorylation.

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INTRODUCTION

Purified staphylococcal alpha toxin has been shown to have hemolytic, dermatonecrotic, and lethal effects in experimental animals (1–6). Several investigations have suggested that the primary site of action of this toxin is the cell membrane. This hypothesis has been based on morphologic evidence of cytolysis or ballooning of cell surfaces, and on the demonstration of escape of intracellular material after exposure to the toxin (7–14). Studies indicating that artificial liposomes lose incorporated anions or glucose after the application of toxin support the assumption that the cytotoxicity of this substance results from a primary action on membrane lipids (15).

The isolated toad bladder was used as an experimental model in the studies reported in this paper in order to investigate the action of alpha toxin on active transport and metabolism in intact tissue. Inhibition of transepithelial potential difference (PD) and short circuit current (SCC) by the application of alpha toxin to the serosal bathing medium of the isolated bladder has been reported previously (16). In addition, oxygen consumption by minced bladder tissue was found to be stimulated by the toxin (16). The purpose of this paper is to record the results of continued

study of this problem designed to determine (*a*) the effect of toxin on active sodium transport, as measured by isotopic labeling, (*b*) the relationship of dose and temperature to the activity of toxin, and (*c*) the influence of vasopressin and 2,4 dinitrophenol on toxin-induced changes in active sodium transport and oxygen consumption.

METHODS

Purified staphylococcal alpha toxin was prepared by a modification of the method of Madoff and Weinstein (1). The Wood 46 strain of *Staphylococcus aureus* was grown in 6 liters of yeast dialysate supplemented with casamino acids, glucose, thiamine, and nicotinic acid at pH 7.1. The medium was divided into 550-ml aliquots, inoculated with organisms and placed on a rotary shaker at 37°C for 18–24 hr. The bacteria were removed by centrifugation at 15,000 rpm in a Servall continuous flow, refrigerated centrifuge, Ivan Sorvall, Inc., Norwalk, Conn., at 4°C. The supernatant fluid was treated by the addition of ammonium sulfate to 90% saturation. The precipitate present after overnight storage at 4°C was centrifuged in the manner described above and the sediment dissolved in 250-ml veronal buffer (0.02 mole/liter, pH 8.6). This solution was dialyzed for 24–48 hr against 30 liters of the same buffer and applied to a refrigerated Spinco continuous flow paper curtain electrophoresis, Beckman Instruments Inc., Spinco Div., Fullerton, Calif., at a feed rate of 3 ml/hr. The alpha toxin fraction was concentrated between tubes 7–12, as demonstrated by determination of the hemolytic activity against a 2% suspension of rabbit erythrocytes. The concentrated toxin fraction was dialyzed against 0.005 M sodium phosphate buffer (pH 7.4) until pH equilibration was reached. The partially purified preparation was then applied to a *O*-diethylaminoethyl (DEAE)-cellulose ion exchange column (1.2 × 40.0 cm) at the same pH. The filtrate was collected and dialyzed against 0.1 M phosphate buffer at pH 5.0. It was then adsorbed on a carboxymethyl cellulose ion exchange column at a rate of 8 ml/hr. The column was then washed with the solvent buffer for 24 hr and the toxin eluted with 0.1 M phosphate buffer, pH 7.0. Because this material was relatively unstable in this buffer, it was immediately dialyzed against 70% ammonium sulfate. The bag containing a silky white precipitate was transferred to a dialysis bath of 0.03 M borate buffer at pH 8.6. The final preparation was frozen in borate buffer and stored in 0.5-ml aliquots at −60°C.

The specific activity of the toxin, the results of antigenic analysis by the Ouchterlony technique, and the technique of measurement of SCC and PD across the isolated toad bladder have been described previously (16). Purified staphylococcal alpha toxin is maximally hemolytic at a temperature of 34 to 42°C (17). The effect of this toxin on toad bladder ion transport was studied at 32°C because a progressive decrease in SCC in the absence of toxin was noted to occur at temperatures above 34°C. The temperature of the baths was maintained at 32°C by a rubber heating coil wrapped around the chambers. Bidirectional sodium fluxes were determined simultaneously with ^{22}Na and ^{24}Na (Isoserve Inc., Cambridge, Mass.) using the double chamber described by Sharp and Leaf (cross-sectional area-2.88 cm^2) (18). ^{22}Na (40 μc) and ^{24}Na (750 μc), each in a 0.5 ml NaCl solution (40 meq/liter), were added to 10 ml of mucosal or serosal bathing media, respectively. Adequate mixing was achieved by rapid bubbling of 100% oxygen. An equilibration period of 30 min was allowed for isotope distribution before each experiment was started. Aliquots of 250 μl were then withdrawn from each chamber at 0, 30, 60, 90, 120, and 150 min and delivered to separate counting tubes. Toxin (12,000 hemolytic units [HU] in 100 μl) was added to the serosal medium at 60 min, and vasopressin (2 pressor units in 100 μl) was added to the same side at 120 min. The aliquots of isotope were counted in a Nuclear-Chicago dual channel gamma scintillation counter, Nuclear-Chicago Corporation, Des Plaines, Ill., immediately after each experiment. Settings were adjusted so that ^{24}Na counts (as a result of serosal to mucosal flux) could be determined in the mucosal samples with a contamination of 0.04% of the ^{22}Na activity present. Mucosal to serosal flux of ^{22}Na was determined 3 wk later when the activity of ^{24}Na in serosal samples was negligible. Undirectional fluxes (in $\mu eq/sec$) were calculated according to the following equations:

$$\text{Permeability coefficient } (K \text{ trans}) = \frac{\text{increase in counts on the unlabeled side}}{\text{concentration of counts on the labeled side} \times \text{area of membrane} \times \text{time}}$$

$$\text{Flux} = K \text{ trans} \times \text{area} \times \text{concentration.}$$

The short circuit current during each 30-min time period was converted from μamp-sec to μeq/sec using the relationship,

$$1 \text{ equivalent} = 96{,}500 \text{ amp-sec.}$$

Oxygen consumption by toad bladder was measured manometrically in either a Warburg or Gilson respirometer. Bladders were excised and finely minced at 4°C in tris buffer (0.1 mole/liter, pH 7.1) containing 0.012 M NaCl, 0.004 M $MgCl_2$, and 0.25 M sucrose (sucrose-buffer solution). The volume of minced tissue was adjusted so that 2 ml contained the equivalent of a single bladder (400 mg wet weight). Each flask received 2 ml of bladder suspension. Sodium succinate (0.08 mole/liter) was used as substrate by adding 0.3 ml of a 0.8 M solution in tris buffer. Toxin, 16,000 HU in 0.4 ml, and sucrose-buffer solution were added to make a final volume of 3.0 ml. 2,4 dinitrophenol (1.5×10^{-4} moles/liter) was either included in the initial incubation mixture or added from a sidearm as 0.15 ml of a 3.0×10^{-3} M solution in tris buffer. The incubating medium was made hypertonic (795 mOsm) to provide an osmotic environment approximating intracellular fluid because of the high per cent of broken cells in finely minced tissue (19). The results are expressed as microliters of O_2 uptake per flask (400 mg wet weight) per minute.

RESULTS

Effect of purified toxin on short circuit current, transmembrane potential, and active sodium transport. Preliminary studies were done by adding 500 μl of purified toxin (40,000 HU/ml) to the serosal bathing medium of one hemibladder. The same quantity of toxin was inactivated by heating at 56°C for 30 min and was similarly applied to the control. A rapid decline in both SCC and PD was observed after the addition of toxin in 10 experiments. The heated material produced no significant change. These experiments were repeated with toxin neutralized by antitoxin as control, in order to exclude the possibility of a nonspecific effect. Six studies were carried out with 150 μl of alpha toxin (6000 HU). One set of bladders was treated with toxin alone and another with the same quantity of toxin previously incubated with a fully neutralizing dose of staphylococcal antitoxin (75 μl). In order to maintain equal volumes in all of the preparations, 75 μl of Ringer's solution was added to the untreated toxin in three experiments and 75 μl of normal horse serum in three others. Vasopressin, 0.1 U/ml, was applied to the serosa of both the experimental and control preparations 45 min after exposure to toxin or at the time that the SCC of either hemibladder fell below 10 μamp. An abrupt but small rise in SCC occurred within 1–2 min after treatment with toxin in half of the experiments; this occurrence was followed by a rapid fall to less than 10 μamp in 30–45 min in all. In a typical experiment, SCC rose from 79–84 μamp 2 min after the addition of toxin. It then declined to 50 μamp after 10, 25 μamp after 20, and less than 10 μamp after 30 min (Fig. 1). PD fell steadily after exposure to toxin. The addition of vasopressin after 30 min did not significantly stimulate either

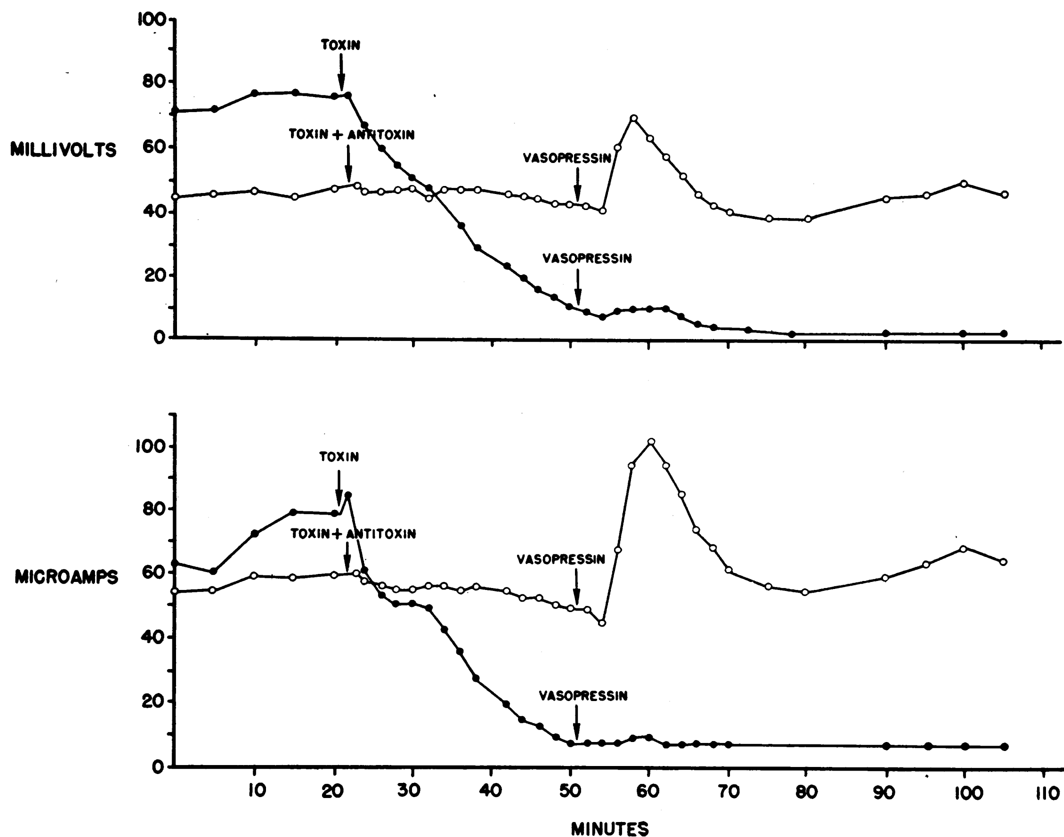

FIGURE 1 Effect of the addition of staphylococcal alpha toxin to the serosal bathing medium on short circuit current and transmembrane potential. 6000 hemolytic units (HU) (0.150 ml) of toxin was used. The effect of toxin plus antitoxin is representative of that obtained in experiments using either antitoxin alone, heated toxin, or Ringer's solution as control.

SCC or PD. The control hemibladder treated with neutralized toxin remained essentially unchanged until the hormone was added, after which SCC and PD rose and resistance fell. The difference in SCC and PD between toxin-treated and control preparations after 30 min was significant by *t* test analysis ($P < 0.01$). No consistent difference was observed when Ringer's solution or normal horse serum was employed for volume equilibration; however, in some instances, the serum appeared to retard the activity of the toxin. The results of these studies are summarized in Table I.

Six experiments were carried out to determine the effect of toxin added to the mucosal bathing medium. The quantity of toxin used (18,000 HU) was three times greater than that employed in the studies described above. No effects were noted when either toxin or Ringer's solution was applied

TABLE I

*Effect of the Addition of Staphylococcal Alpha Toxin to the Serosal Bathing Medium on Short Circuit Current and Transmembrane Potential**

Expt. no.	Substance added to bath‡	Short circuit current (microamperes) and potential difference (millivolts)					
		Initial		After 30 min		Decline§	
		SCC	PD	SCC	PD	SCC	PD
1	Toxin + Ringer's	79	77	<10	10	>69	67
	Toxin + Antitoxin	60	48	49	42	11	6
2	Toxin + Ringer's	94	61	16	13	78	48
	Toxin + Antitoxin	203	97	185	91	18	6
3	Toxin + Ringer's	57	26	20	9	37	17
	Toxin + Antitoxin	58	23	54	25	4	−2
4	Toxin + Horse serum	73	82	53	43	20	39
	Toxin + Antitoxin	72	46	65	45	7	1
5	Toxin + Horse serum	42	28	23	16	19	12
	Toxin + Antitoxin	58	25	57	26	1	−1
6	Toxin + Horse serum	89	92	12	16	77	76
	Toxin + Antitoxin	69	62	63	59	6	3

SCC, short circuit current; PD, transepthelial potential difference.

* The cross-sectional area of each hemibladder was 7.02 cm^2.

‡ 6000 HU (0.150 ml) of toxin was used in each experiment.

§ The addition of toxin with Ringer's solution or horse serum to the serosal bathing medium produced a significant decline in SCC and PD as compared to the addition of toxin plus antitoxin ($P < 0.01$ by the *t* test).

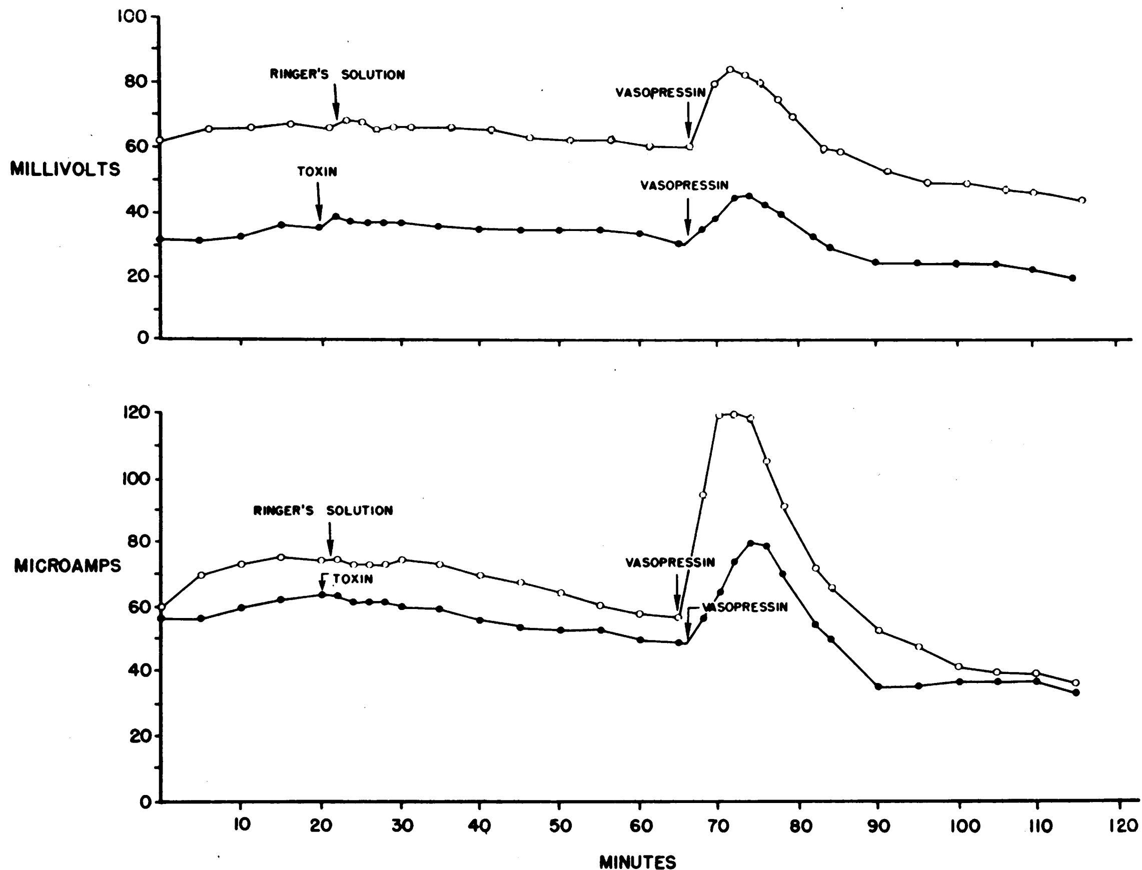

FIGURE 2 Effect of addition of staphylococcal alpha toxin to the mucosal bathing medium on short circuit current and transmembrane potential. 18,000 HU (0.450 ml) of toxin was used.

to the bladder mucosa. Analysis of the difference in SCC and PD between the experimental and control hemibladder at 30 min revealed a P value of > 0.4. The change in PD was parallel to that of the SCC in each experiment. The addition of vasopressin to the serosal surface produced a characteristic stimulation of SCC and PD in both the control and toxin-treated hemibladders (Fig. 2).

Leaf, Anderson, and Page have demonstrated that the SCC under aerobic conditions can be quantitatively accounted for by net active transport of sodium (20). The question arose whether the SCC measured after exposure of the serosal surface of the bladder to toxin continues to equal net active sodium transport. In six experiments, the mean net sodium flux was not significantly different from the short circuit current either before or after the administration of toxin (Table II). A minimal rise in short circuit current and fall in net sodium flux occurred after the addition of vasopressin. The fall in net sodium flux after the addition of toxin was primarily due to a decline in mucosal to serosal flux, whereas serosal to mucosal flux increased only slightly. These results indicate that the fall in SCC caused by the toxin represents a quantitatively equal inhibition of net active sodium transport.

Relationship of ammonia content to toxin effect. Grady, Madoff, Duhamel, Moore, and Chalmers (21) and Lyng and Spaun (22) have independently presented evidence that the inhibition of SCC in frog skin by cholera toxin is due to its ammonia content. Neptune and Mitchell (23) have warned that this artifact may be present in any toxin purified by ammonium sulfate precipitation. Consequently, after dialysis of the purified alpha toxin against 0.03 M borate buffer at pH 8.6, the dialysate was studied for an effect on the SCC and PD of the isolated bladder. No inhibition was found when 100 μl was added to the serosal bathing

TABLE II

Comparison of the Net Sodium Flux and Short Circuit Current across the Toad Bladder before and after the Addition of Toxin and Vasopressin to the Serosal Medium

Time period	Mean sodium flux		Mean net sodium flux	Mean short circuit current	P
	M to S	S to M			
min	*meq/sec* $\times 10^{-6}$ ±SEM				
Control					
0– 30	164.6±8.8	5.4+0.3	159.2±8.8	157.3± 7.9	0.9
30– 60	155.4±7.7	8.6±1.0	146.8±7.2	149.4± 6.7	0.8
After toxin					
60– 90	113.9±7.4	20.2±3.9	93.7±6.8	93.0± 7.8	0.9
90–120	69.1±4.9	15.4±3.6	53.7±5.0	49.7±10.9	0.7
After vasopressin					
120–150	69.6±6.5	19.1±3.5	50.8±6.5	56.5± 9.7	0.6

M, mucosal compartment; S, serosal compartment.

medium. The toxin contained less than 0.2×10^{-3} M ammonia when analyzed by the method of Berthelot (24). The addition of 100 µl of a 0.2 $\times 10^{-3}$ M ammonium sulfate solution to the serosal side of the isolated bladder had no effect. These results indicate that the effect of alpha toxin on the isolated toad bladder is not due to the presence of ammonia or ammonium ion.

Dose–response relationship. A single lot of toxin containing 50,000 HU/ml of borate buffer (pH 8.6) was used in 10 experiments. Paired hemibladders were exposed to five dose levels (25,000, 15,000, 5000, 2500, and 1250 HU) and the responses analyzed statistically. No attempt was made to determine the minimally active concentration.

Since the SCC and PD of the bladders were variable during the period of pretreatment, the SCC was expressed as a percentage of the value recorded at the time toxin was added. The data were subjected to a one-way analysis of variance based on times to 50% reduction in initial SCC (Fig. 3). The *F* test indicated a significant difference between the five doses at the 1% level. The Duncan test revealed a similar response at the 5% level within the three dose ranges shown in brackets in Fig. 3. The use of 15,000 and 25,000 hemolytic units produced similarly rapid effects; SCC declined to 10–20% in 40–50 min. The administration of 1250, 2500, and 5000 hemolytic units produced a fall in SCC to 30–40% of initial values in 60 min. The responses to 5000 and 15,000 U were found to overlap the other two dose ranges. Despite the presence of overlap, these studies suggest a direct relationship between dose of toxin and rate of decline of SCC.

Relation of temperature to activity of toxin on bladder. Purified staphylococcal alpha toxin is maximally hemolytic at 34–42°C (17), and its lethal effect in toads has been shown to be decreased at 4°C (25). In order to determine whether there is a relation between temperature and activity of toxin on bladder tissue, several experiments were carried out at 15 and 32°C. In five separate studies, paired hemibladders were mounted and the Ringer's baths maintained at 15°C. 6000 hemolytic units of alpha toxin in a volume of 150 µl were added to the serosal side of one hemibladder and 150 µl of Ringer's solution to the control. Both SCC and PD of the untreated bladders were decreased at this temperature. A negligible effect of alpha toxin was observed. In a representative experiment the SCC of the toxin-treated bladder fell from 70 to 65 µamp in 50 min and then to 43 µamp after a total of 100 min (Fig. 4 A). In a series of five experiments, no significant effect of the toxin at 15°C could be consistently demonstrated. In four other studies, one hemibladder was maintained at 15°C and the other at 32°C; both were exposed to the same quantity of toxin as above. As can be seen in Fig. 4 b, a rapid decline in SCC (from 183 to 25 µamp) occurred at 32°C while less than 20% inhibition developed at 15°C after 50 min (from

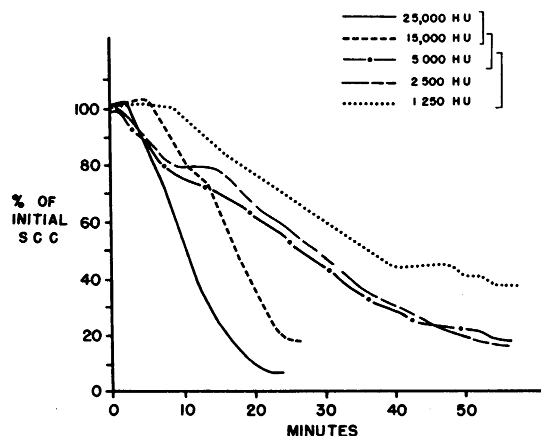

FIGURE 3 Dose activity relationship of the effect of staphylococcal alpha toxin on short circuit current (*SCC*). Toxin, in the doses indicated, was added at zero time after a 20 min stabilization period.

44 to 37 μamp). Parallel changes in PD occurred in each experiment.

Effect of vasopressin on SCC and PD of toxin-treated bladders. Since bladder serosa exposed to toxin appeared unable to respond to vasopressin once SCC had fallen to less than 10 μamp (Fig. 1), two groups of four experiments each were carried out in an attempt to delineate this phenomenon. In the first study, vasopressin was added 10 min after the administration of toxin, regardless of the level of SCC and PD at the time. A variable rise in SCC but little or no increase in PD was observed. This did not appear to reverse the ultimate inhibitory effect of the toxin since both the subsequent rate and degree of decline of SCC and PD were similar to those resulting without the addition of vasopressin. In a typical experiment (Fig. 5), SCC fell from 235 to 155 μamp 10 min after the addition of toxin. Vasopressin was then administered, resulting in a rapid rise to 242 μamp and a subsequent decline to 10 μamp within a total time of 50 min. PD declined steadily from 93 to 10 mv and was not increased by vasopressin. The control was stimulated to a much greater degree by the hormone, and both SCC and PD remained at higher than starting levels throughout

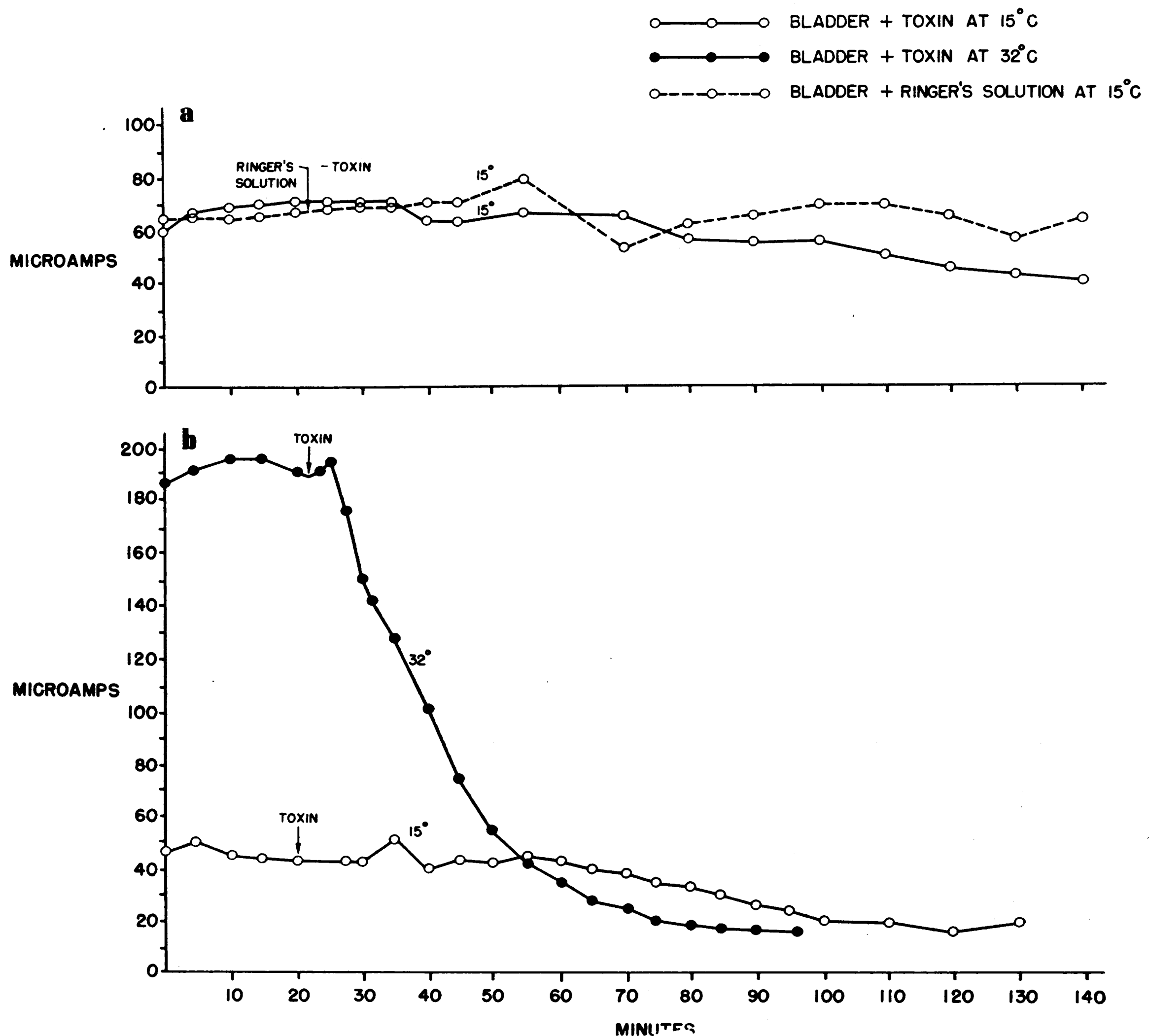

FIGURE 4 Effect of low temperature on the activity of staphylococcal alpha toxin added to the serosal bathing medium. (*a*) represents an experiment done at 15°C using 6000 HU (0.150 ml) of toxin. (*b*) represents an experiment in which toxin was added to the serosal bathing medium of bladders maintained at 15 and 32°C.

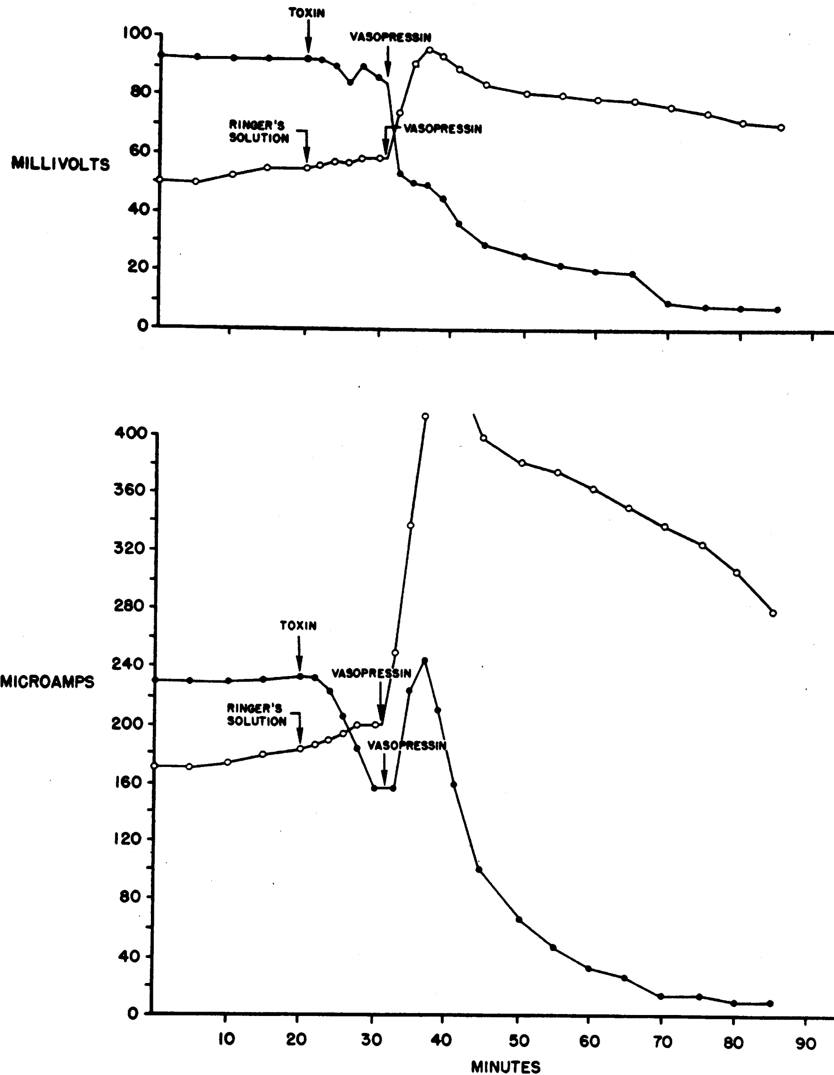

FIGURE 5 Effect of vasopressin on short circuit current and transmembrane potential when administered to the serosal bathing medium 10 min after toxin. 6000 HU (0.150 ml) of toxin and 2 U (0.1 ml) of vasopressin were used.

the experiment. In the second study, vasopressin was first added to the serosal side of the bladder. When maximal stimulation had occurred in both the experimental and control preparations, and SCC and PD were stable for 30 min, toxin was administered. This led to a rapid decline of SCC and PD as in the first group of experiments (Fig. 6).

These data suggest that stimulation of SCC and PD by vasopressin neither prevents nor significantly alters the inhibitory effects of staphylococcal alpha toxin. Once it has acted upon the bladder for a sufficient period of time, exposure to vasopressin fails to produce a response. Addition of the hormone shortly after toxin results in a transient stimulation of SCC. Since vasopressin has been shown to have an effect on the double permeability barrier of the mucosal surface of the toad bladder, it seems possible that an effect of alpha toxin on the mucosal surface of the bladder might be induced by prior or subsequent administration of vasopressin serosally. In two such experiments, carried out in duplicate, no effect of mucosally administered toxin occurred either before or after the serosal application of vasopressin.

Effect of alpha toxin on oxygen consumption by

the toad bladder. Several studies were carried out to determine whether inhibition of active transport by staphylococcal alpha toxin was secondary to an effect on oxidative metabolism. A dose (16,000 HU) that produced maximal suppression of sodium transport was incubated with respiring bladder tissue in the 7-unit Warburg respirometer.[1] This produced significant stimulation of respiration. The mean oxygen consumption of 4.4–4.8 μl/min maintained by tissue treated with toxin plus antitoxin, antitoxin alone, or either Tris or borate buffer was increased to 6.5 μl/min by the addition of the toxin. ($P < 0.001$) (16). 2.4 dinitrophenol (DNP), a compound which uncouples oxidative phosphorylation, has been found to cause a similar fall in SCC and PD and an increase in oxygen consumption by isolated frog skin (26). Studies were therefore undertaken to compare the effects on respiration of alpha toxin and DNP alone and those produced by their sequential application.

Minced toad bladder tissue was incubated with succinate (0.08 mole/liter) in tris buffer and oxygen consumption measured in a Gilson respirometer for a 45 min period. At that point, alpha toxin (16,000 HU) was tipped in. In a second flask, 2,4 DNP (1.5×10^{-4} moles/liter) was tipped in, and tris buffer (0.01 mole/liter, pH 7.4) was added to a third (control) flask. In each instance, the oxygen consumption in the succeeding 45 min was compared with the initial period, and the result expressed as percent change in microliters of oxygen consumed (Table III A). Alpha toxin caused a $13.0 \pm 3.0\%$ increase in oxygen uptake, DNP an $18.8 \pm 3.3\%$ increase, and tris buffer a $10.5 \pm 1.6\%$ decrease. Both toxin

[1] Will Scientific, Inc., Cambridge, Mass.

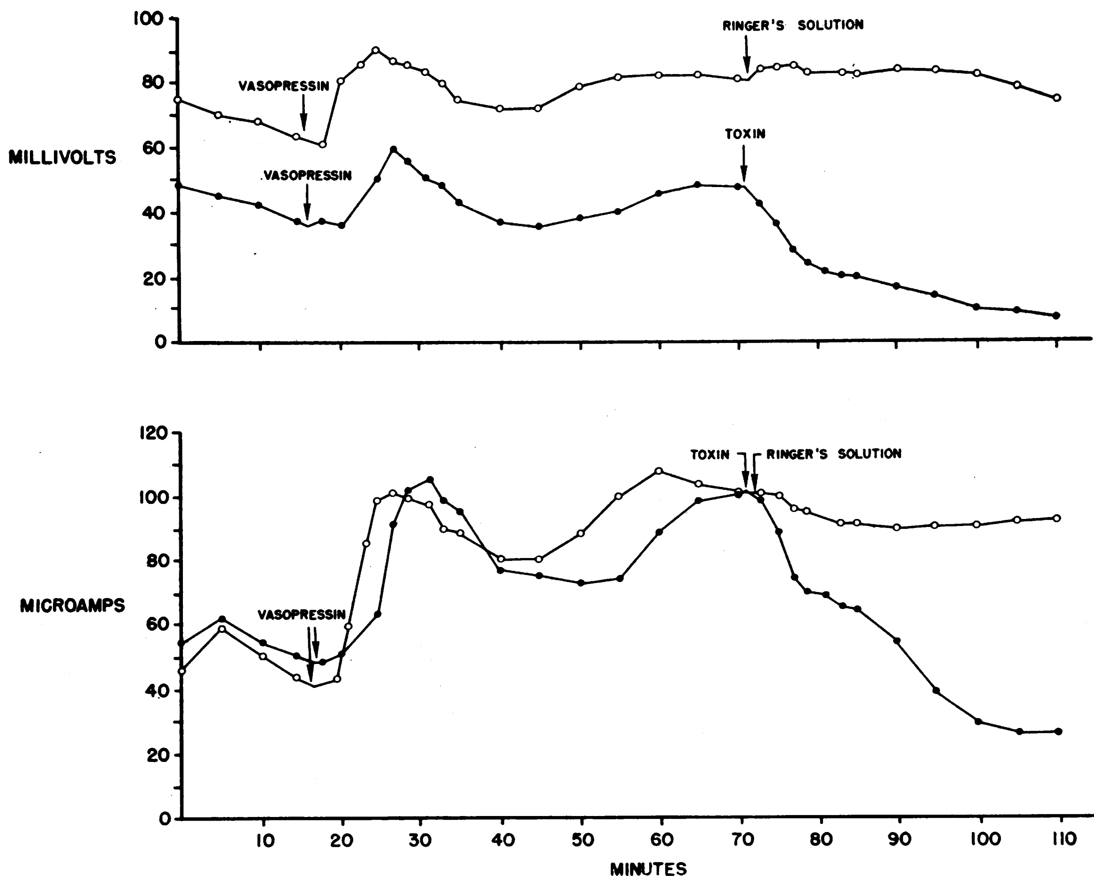

FIGURE 6 Effect of staphylococcal alpha toxin on short circuit current and transmembrane potential when administered to the serosal bathing medium after vasopressin. 6000 HU (0.150 ml) of toxin and 2 U (0.100 ml) of vasopressin were used.

and DNP significantly stimulated oxygen uptake when compared with tris buffer ($P < 0.001$).

This group of experiments was repeated, and an additional 45 min period of oxygen uptake was measured (Table III B). Alpha toxin was added at 45 min and DNP from a second sidearm at 90 min. This order was reversed in duplicate flasks. Again, alpha toxin stimulated oxygen consumption ($+14.9 \pm 4.0\%$). When DNP was added to toxin-treated tissue, a further stimulation occurred ($+18.5 \pm 1.8\%$). In the reverse experiment, DNP stimulated oxygen uptake ($+12.0 \pm 2.7\%$), but when toxin was added to DNP treated tissue, respiration was decreased ($-12.8 \pm 2.4\%$). These results indicated that DNP caused a further stimulation in respiratory rate in toxin-treated tissue as

TABLE III A

Effect of Staphylococcal Alpha Toxin and 2,4 Dinitrophenol on Oxygen Consumption by Minced Toad Bladder Tissue

Material added*	Time period	Mean O_2 consumed‡	Mean % change‡	P
	min	μl		
Buffer (22)	0–45	168 ± 6.5	−10.5 ±1.6	
Buffer (22)	45–90	149 ± 5.2		
Buffer (13)	0–45	177 ± 7.8	+13.0 ± 3.0	<0.001
Toxin (13)	45–90	198 ± 7.5		
Buffer (6)	0–45	182 ±14.1	+18.8 ±3.3	<0.001
DNP (6)	45–90	216 ±17.7		

DNP, 2,4 dinitrophenol.
* Borate buffer (0.03 mole/liter, pH 8.6); alpha toxin (16,000 HU) in borate buffer; and DNP (1.5×10^{-4} moles/liter) were added. The number in parentheses indicates the number of experiments done.
‡ Expressed as ± standard error of the mean.

TABLE III B

Effect of Staphylococcal Alpha Toxin and 2,4 Dinitrophenol on Oxygen Consumption by Minced Toad Bladder Tissue

Material added*	Time period	Mean O_2 consumed‡	Mean % change‡	P§
	min	μl		
Buffer (10)	0–45	148 ± 7.1	+14.9 ±4.0	
Toxin (10)	45–90	168 ± 6.9	+18.5 ±1.8	
DNP (10)	90–135	199 ± 6.8		
Buffer (8)	0–45	176 ±10.8	+12.0 ±2.7	<0.6
DNP (8)	45–90	198 ±14.7	−12.8 ±2.4	<0.001
Toxin (8)	90–135	172 ±13.3		

*Borate buffer (0.03 mole/liter, pH 8.6); alpha toxin (16,000 HU) in borate buffer; and DNP (1.5×10^{-4}) moles/liter) were added. The number in parentheses indicates the number of experiments done.
‡ Expressed as ± standard error of the mean.
§ *P* values compare the mean per cent change between comparable time periods.

TABLE III C

Effect of Staphylococcal Alpha Toxin and 2,4 Dinitrophenol on Oxygen Consumption by Minced Toad Bladder Tissue

Material added*	Time period	Mean O_2 consumed‡	Mean % change‡	P
	min	μl		
DNP (10)‡	0–45	295 ±17.1	−11.8 ±3.1	<0.05
Toxin (10)	45–90	258 ±12.2		
DNP (10)	0–45	290 ± 8.6	−23.8 ±4.2	
Buffer (10)	45–90	219 ±13.7		

* Borate buffer (0.03 mole/liter; pH 8.6); alpha toxin (16,000 HU) in borate buffer; and DNP (1.5×10^{-4} moles/liter) were added. The number in parentheses indicates the number of experiments done.
‡ Expressed as ± standard error of the mean.

compared with the effect of toxin on DNP-treated tissue ($P < 0.001$). A third group of experiments was done to determine whether toxin had any effect different from buffer when added to DNP-treated tissue (Table III C). Bladder tissue was allowed to respire in the presence of DNP, and alpha toxin or tris buffer was added from the sidearm of duplicate flasks. In this study, the addition of toxin resulted in a decrease in oxygen uptake ($-11.8 \pm 3.1\%$), which was significantly smaller than that observed after buffer ($-23.8 \pm 4.2\%$) ($P < 0.5$). Thus while toxin did not stimulate oxygen consumption in DNP-treated tissue, it did cause a significantly sustained respiratory rate.

Effect of 2,4 DNP on the isolated toad bladder. In 6 experiments the addition of DNP (2.5 ×

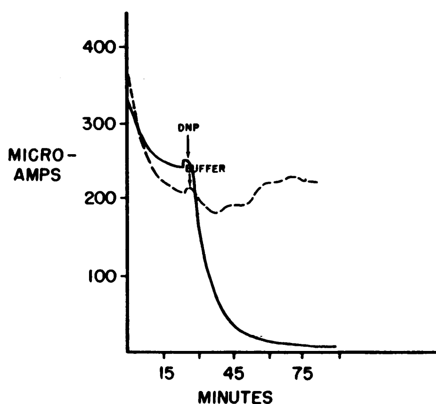

FIGURE 7 Effect of the addition of 2, 4 dinitrophenol (*DNP*) to the serosal bathing medium on short circuit current. The concentration of DNP was 2.5×10^{-4} moles/liter. The change in PD paralleled that of SCC. Addition of DNP to the mucosal bathing medium produced identical changes.

10^{-4} moles/liter) to either the serosal or mucosal surfaces of the bladder produced a rapid decline in both SCC and PD (Fig. 7).

DISCUSSION

Since active sodium transport is considered to be a cell membrane function dependent upon energy metabolism (27–30), examination of the effects of purified staphylococcal alpha toxin on both active transport and O_2 consumption in the isolated toad bladder provides a useful method for the investigation of its action on living tissue. Inhibition of active sodium transport in the isolated bladder by alpha toxin under aerobic conditions may result from changes in (*a*) the oxidative metabolism of substrate necessary to provide utilizable energy, (*b*) the coupling of this energy to the transport process, or (*c*) the mechanisms of active transport located at the membrane site. The observed stimulation of oxygen consumption by the toxin suggests that it does not inhibit substances involved in the metabolism of succinate, i.e., succinic dehydrogenase and the electron transport system. The fact that alpha toxin and DNP both stimulate oxygen consumption and suppress sodium transport raises the possibility that the toxin may cause uncoupling of oxidative phosphorylation, an effect known to be produced by DNP. In a comparative study of the effects of three classes of metabolic inhibitors (electron transport inhibitors, energy transfer inhibitors, and uncouplers of oxidative phosphorylation) on the short circuit current and oxygen consumption of the isolated toad bladder, only uncouplers of oxidative phosphorylation were found to stimulate oxygen consumption while inhibiting short circuit current (31). However, since DNP alters short circuit of the bladder when applied to either the mucosal or serosal surface, its activity appears to be different from that of alpha toxin which acts only when in contact with serosa. This may be related to the ease of penetration of DNP due to its smaller molecular size and greater lipid solubility. A more definite distinction between the mode of action of these two substances is suggested by the fact that the addition of DNP leads to an increase in oxygen consumption by bladder tissue even after it has been maximally stimulated by toxin, but that the latter is unable to stimulate oxygen consumption in DNP-treated tissue. Thus the stimulatory action of alpha toxin on oxygen consumption appears to be dependent upon the presence of coupled respiration, although the uncoupling effect of DNP is not inhibited by the action of toxin. These data suggest that alpha toxin may uncouple oxidative phosphorylation but by a mechanism different from that of DNP. To test this hypothesis, its effects on mitochondrial swelling, adenosine triphosphatase (ATPase) activity, and P:O ratio are currently being investigated.

A possible action of alpha toxin on the active transport mechanisms located in the serosal membrane of the bladder epithelial cell is suggested by the finding that it is effective when added to the serosal but not to the mucosal bathing medium. Vasopressin has been shown to alter the mucosal barriers of the epithelial cell layer of the isolated bladder despite the fact that it also is effective only when added to the serosal side (32). Failure of vasopressin to stimulate sodium transport after maximal inhibition by alpha toxin or to block the action of toxin suggests different sites of action for these substances. Our findings are therefore consistent with either an effect on oxidative phosphorylation or on active transport mechanisms located at the serosal membrane. These possibilities are not mutually exclusive if the toxin were to produce an alteration of both cell and mitochondrial membranes. Niselovskaya and Paderina have demonstrated uncoupling of oxidative phosphorylation in the liver of guinea pigs poisoned with staphylococcal toxin (33). Although these studies were done with crude toxin, the results are consistent with the stimulation of oxygen consumption by purified alpha toxin demonstrated in our studies and the inhibition of this effect by 2,4 DNP.

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