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INHIBITION OF ACCUMULATIVE TRANSPORT BY A PROTEIN FROM COBRA VENOM

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Abstract—A heat-stable, phospholipase A-free protein from cobra venom (*Naja naja*) inhibits amino acid and 3-*O*-methyl-D-glucose transport by small intestine and the uptake of para-aminohippurate and amino acids by kidney cortex slices. Although accumulation of these substances is thought to be linked to cation concentration or movement, Na^+ efflux from human erythrocytes or the inhibition of this process by ouabain is unaffected by this protein. The possibility of an effect on the Na^+ pump of other tissues is not, however, ruled out.

DURING recent attempts to study the role of membrane phospholipids in iodide transport of thyroid tissue,[1] a protein fraction was isolated from cobra venom (*N. naja*) that inhibited iodide transport but was free of phospholipase A activity and a number of other enzymes known to be present in the crude venom.[2] Although ^{32}P incorporation into thyroid phospholipids or the stimulation of these reactions by thyrotropin was not influenced by this protein, K^+ concentration in treated thyroid tissues was decreased by 30 per cent. Since transport of many substances across the cell membrane appears to be linked to the movement or concentration of cations, or to both, it was important to obtain information on the activity of this inhibitor on a variety of substrates and tissues. The present study reports results on the accumulation of several amino acids, 3-*O*-methyl glucose, *p*-aminohippurate, and $^{24}Na^+$ in a variety of tissues. The transport processes believed to be linked to Na^+ movement were inhibited by the protein from *N. naja* venom. However, Na^+ efflux from erythrocytes was unaffected.

MATERIALS AND METHODS

Compounds labeled with ^{14}C were obtained from the New England Nuclear Corp. All amino acids were of the L form. $^{24}Na^+$ was obtained as neutral NaCl from the Isoserve Division of the Cambridge Nuclear Corp. Para-aminohippuric acid was from Eastman Kodak Co., and *N*-(1-naphthyl) ethylenediamine-2HCl from Fisher Scientific Co. All other chemicals were of reagent grade. 3-*O*-methyl-D-glucose-^{14}C was >99 per cent pure by chromatography in pyridine:ethyl acetate:water (1:3·6:1·15) and in butanol:ethanol:water (5:1:4).

The inhibitory protein, protein B, was obtained by column chromatography of crude cobra (*N. naja*) venom on carboxymethylcellulose as described elsewhere.[1, 2] The material is stable to heating for 15 min at 100° in 0·10 M acetate buffer (pH 5·1), gives a single peak on sephadex G-50 gel filtration, and its inhibitory activity is

proportional to the optical density at 280 mμ and to the Biuret reaction. It is inactivated by pronase, but is relatively resistant to trypsin.

For intestinal transport studies amino acids were used at a concentration of 1/30 of the K_m where this was known.[3] All concentrations used are listed in the tables. Gut sacs were prepared from the terminal 30 cm of rat ileum as described previously.[3] The initial serosal and mucosal amino acid concentrations were identical. Protein B was added to the mucosal side. Experimental and control values were always compared by using sacs made from adjacent segments. At complete inhibition the initial serosal to mucosal ratio of 1·0 would be present. Therefore, 1·0 was subtracted from the observed ratio, assuming that the remainder of the amino was transported by an 'active' process or processes. Thus by 'active' we refer only to the fraction present in excess of that accumulated under anaerobic conditions.

Amino acid accumulation by rat kidney slices was carried out and corrected to intracellular water, as described by Rosenberg *et al.*[4] Concentrations used were 1/30 of the K_m for glycine and phenylalanine.[5] A 90-min incubation period was used.

3-*O*-methyl glucose accumulation by strips of rabbit ileal mucosa was measured under conditions similar to those described by Schultz *et al.*[6] in male rabbits weighing 1·5–2·0 kg and killed by air embolism.

Para-aminohippurate accumulation by rabbit kidney cortex slices was carried out according to Burg and Orloff.[7] The medium concentration of the acid was 8×10^{-5} M in all experiments.

Sodium efflux was measured after preloading washed human erythrocytes for 4 hr at 37° with $^{24}Na^+$ in a high K^+ medium, according to Tosteson and Hoffman.[8] (We should like to thank Dr. Floyd Kregenow for his advice in these studies.) Sodium concentrations of packed red cells were determined by flame photometry with a 4·0 per cent correction for trapped medium after centrifugation for 15 min at 40,000 *g*. Hemoglobin concentrations were calculated from the absorbancy at 540 mμ. All values are corrected for the contribution due to hemolysis. Counting was carried out in a well-type scintillation spectrometer with the 2·76 MeV peak. In the conversion of $^{24}Na^+$ efflux to m-moles of Na^+/l. cells, the per cent $^{24}Na^+$ extracellular at time zero was determined by extrapolation. This value (3–5 per cent) was subtracted from the observed efflux at each time point prior to calculation of the Na^+ equivalent.

RESULTS

Amino acid transport

Rat intestinal sacs. The transport of leucine, lysine, phenylalanine, and the non-metabolized amino acid, cycloleucine, is inhibited 63–77 per cent by protein B concentrations of 71 μg/ml (Table 1). No difference was observed in the degree of inhibition whether sacs were preincubated for 10, 20 or 40 min with protein B followed by washing and incubation, and those in which the protein was present during the amino acid accumulation period. A similar effect of protein B, present 'only' during a brief preincubation, has previously been shown in thyroid slices.[1] Hence, for convenience, protein B was present throughout the incubation period. Amino acid transport was inhibited 'only' when protein B had been added to the mucosal side of the gut sac. Dose-response curves using leucine-^{14}C as a model revealed 50 per cent inhibition over a range of 62–78 μg protein B/ml (Fig. 1). Accumulation of this amino acid was completely inhibited by 150–550 μg/ml of protein B.

Rat kidney cortex slices. Comparable degrees of amino acid transport inhibition were observed in rat kidney cortex slices (Table 2). Control concentration ratios were similar to those previously reported.[5] Thirty-one to 58 per cent inhibition of accumulation of these amino acids was obtained during incubation with 100 μg/ml protein B (Table 2). After a 90-min incubation, the inulin space of protein B-treated slices was 38 per cent of the wet weight as opposed to 29 per cent in controls. Tissue water was 77 per cent of wet weight in the controls and 80 per cent in the toxin-treated tissue. However, this change in the inulin space is not responsible for the observed inhibition of amino acid accumulation.

3-O-*methyl glucose transport in rabbit ileum.* Intracellular to extracellular concentration ratios of 8·8–9·4 were consistently found in mucosal strips incubated for 30–40 min in 2×10^{-3} M 3-*O*-methyl glucose (Table 3). These values could be reduced to 1·0 by incubation in an atmosphere of nitrogen or in 5×10^{-5} M ouabain. The

TABLE 1. EFFECT OF *N. naja* PROTEIN B ON AMINO ACID TRANSPORT BY EVERTED RAT GUT SACS

Amino acid*	Initial concn. (M)	Serosal concentration/mucosal concentration†				Mean inhibition (%)
		Control		With toxin‡		
		Mean	Range	Mean	Range	
Leucine (6)	$7{\cdot}3 \times 10^{-5}$	13·6	4·0–23·0	3·8	1·6–7·0	77
Lysine (4)	$2{\cdot}3 \times 10^{-5}$	9·2	5·2–18·2	3·2	2·0–4·4	67
Phenylalanine (4)	$4{\cdot}7 \times 10^{-5}$	7·1	4·5–8·8	2·4	1·5–3·1	76
Cycloleucine (3)	$6{\cdot}4 \times 10^{-5}$	6·1	4·8–7·5	2·9	2·2–3·5	63

* Number in parentheses refers to the number of rats.
† These values represent accumulation in excess of a ratio of 1·0.
‡ Protein B, 71 μg/ml on mucosal side.

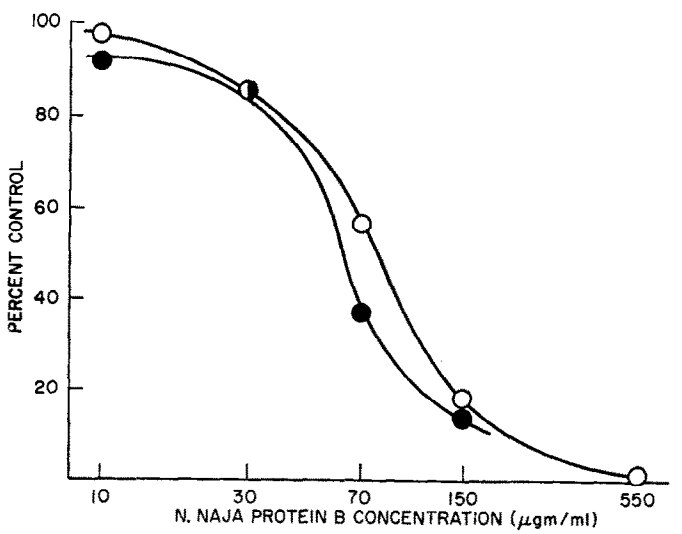

FIG. 1. The effect of *N. naja* protein B on L-leucine transport by rat everted intestinal sacs. Results obtained with 2 animals are depicted. Inhibition was calculated as a percentage of the mean ratio of the 2 adjacent control sacs. In curve ○—○, the mean of 5 controls = 20·9, range 18·3–21·9. In curve ●—●, the mean of 4 controls = 18·3, range 17·4–19·5.

control ratios were rather higher than those reported by Schultz *et al.*;[6] we have no explanation for this difference. The inulin space was 21 per cent of the wet tissue weight in control strips and 29 per cent during incubation with protein B. Tissue water was 80 per cent of the wet weight of controls and 81 per cent in toxin-treated strips. A 46–55 per cent reduction in the concentration gradients was produced by 150 μg/ml of *N. naja* protein B, whereas 400 μg/ml resulted in 70 per cent inhibition.

TABLE 2. INHIBITION OF AMINO ACID TRANSPORT IN RAT KIDNEY CORTEX SLICES BY *N. naja* PROTEIN B

Amino acid*	Initial concn. (M)	Intracellular conc./medium concn.†				Mean inhibition (%)
		Control		With toxin‡		
		Mean	Range	Mean	Range	
Glycine (6)	$5{\cdot}6 \times 10^{-5}$	8·1	7·1–9·9	5·5	4·5–6·8	31
Cycloleucine (5)	$5{\cdot}5 \times 10^{-5}$	1·6	1·0–1·9	1·0	0·4–1·4	39
Phenylalanine (6)	$1{\cdot}3 \times 10^{-5}$	2·2	1·2–3·5	0·9	0·5–1·3	58

* Number in parentheses refers to the number of rats used.
† These values represent accumulation in excess of a ratio of 1·0.
‡ Protein B, 100 μg/ml.

TABLE 3. INHIBITION BY *N. naja* PROTEIN B OF 3-*O*-METHYL-D-GLUCOSE TRANSPORT IN RABBIT MUCOSAL STRIPS

Exp. no.	ntracellular concn./medium concn.*				Mean inhibition (%)
	Control		With toxin†		
	Mean	Range	Mean	Range	
1	9·1	8·2–9·7	4·9	4·1–6·4	46
2	9·4	9·1–9·8	4·2	3·1–4·8	55
3	8·8	8·6–9·9	4·1	3·7–4·8	53

* These values represent accumulation in excess of a ratio of 1·0.
† Protein B, 150 μg/ml. All experiments were performed in triplicate.

Transport of p-*aminohippurate in rabbit kidney slices.* In order to compare amino acid and sugar transport with yet another class of substances, the effect of protein B on the accumulation of the organic anion, *p*-aminohippurate, was investigated in rabbit kidney cortex slices. Control ratios varied from 8·4 to 11·3 (mean, 9·3), in good agreement with values reported by Burg and Orloff.[7] Thirty μg/ml of protein B led to a 59 per cent reduction in the concentration ratio (uncorrected for intracellular water). As expected, 2×10^{-4} M ouabain reduced the *p*-aminohippurate concentration by >90 per cent.

Na^+ efflux from erythrocytes. On the assumption that an effect on the Na^+ pump could provide a common mechanism for inhibition by protein B of the accumulation of these diverse substances, $^{24}Na^+$ efflux from preloaded human erythrocytes was studied. This tissue was chosen because of the ease with which it is preloaded with $^{24}Na^+$.

After correction for ~5 per cent hemolysis caused by protein B, no difference could be demonstrated between control efflux rates of Na^+ and those occurring in the

presence of either 10 or ~100 μg/ml of the *N. naja* protein (Fig. 2). A concentration as great as 150 μg/ml (not shown) also was ineffective. It should be noted that the expected inhibition in Na^+ efflux produced by 1×10^{-4} M ouabain was in no way altered by the presence of 100 μg protein B/ml medium.

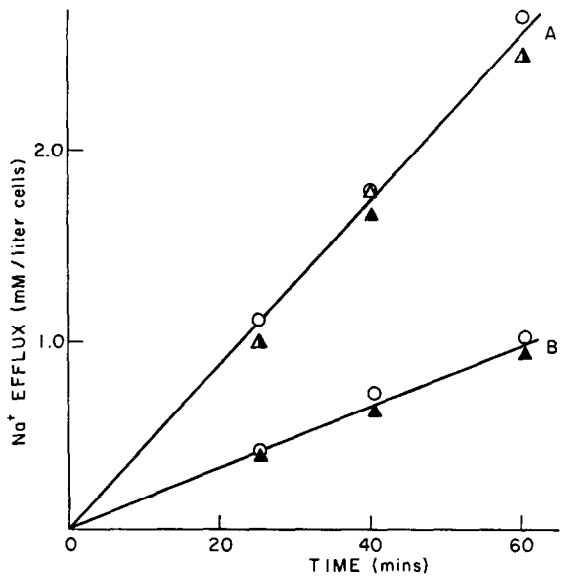

FIG. 2. The effect of *N. naja* protein B on Na^+ efflux from human erythrocytes. Line A, buffer-saline media; line B, same media with $1 \cdot 10^{-4}$ M ouabain; control ○—○; protein B (10 μg/ml) △—△; protein B (100 μg/ml) ▲—▲.

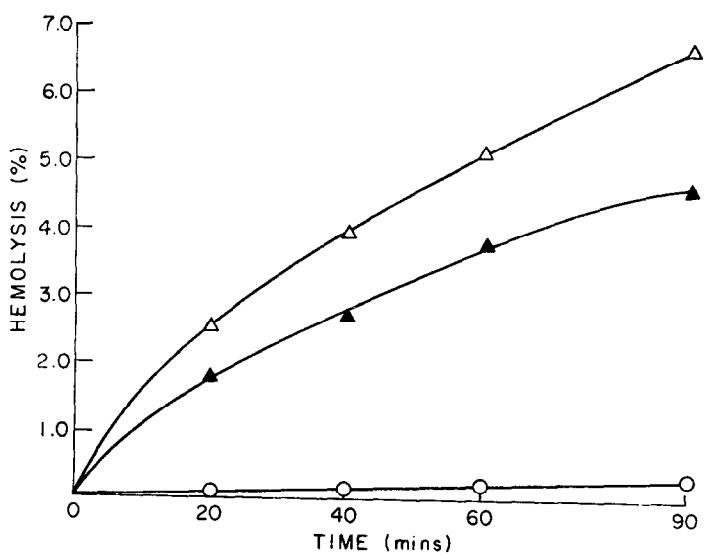

FIG. 3. Hemolysis of washed human erythrocytes produced during a 90-min incubation with *N. naja* protein B in buffer-saline media, pH 7·4. Control ○—○; protein B (30 μg/ml) ▲—▲; protein B (150 μg/ml) △—△.

That protein B 'could' act on the erythrocyte was shown by the slight but definite hemolysis depicted in Fig. 3. It is clear, therefore, that the Na^+ pump of the human erythrocyte is not sensitive to the action of this venom component.

DISCUSSION

It is clear from the above results and previous work[1, 2] that a protein component of cobra venom (*N. naja*) can inhibit the accumulation in various tissues of four different classes of compounds: (1) small anions (I^-, ReO_4^-); (2) amino acids (both utilizable and nonutilizable); (3) the nonmetabolized hexose, 3-O-methyl glucose; and (4) the organic anion *p*-aminohippurate. The factor is inhibitory in thyroid, small intestine, and kidney, and is effective in all species so far tried (cow, dog, guinea pig, rat, and rabbit). The transport inhibition produced by this venom factor thus appears to be of a general nature. Since we have observed only the accumulation of the compounds after attainment of the steady state, a decrease could be due to an effect on either influx or efflux, and the present results do not differentiate between these possibilities.

It has become apparent in the last few years that the transport of many substances is linked, in some manner which is not understood, to an intact and functioning "Na^+ pump". In the case of thyroidal iodide transport, a linear dependence on the external Na^+ concentration has been demonstrated.[9] In addition, ouabain, which inhibits the Na^+ pump,[10] is also a potent inhibitor of iodide transport.[11] The accumulation of amino acids and sugars by small intestine is also dependent on the integrity of this pump,[12, 13] and the uptake of *p*-aminohippurate by the rabbit kidney is inhibited by strophanthin.[7] A unifying hypothesis for the diverse inhibitory properties of this protein was therefore sought in an effect on the Na^+ pump. To our surprise, protein B was entirely without effect on the Na^+ pump of the human erythrocyte, despite a weak hemolyzing action. Although this suggests that the Na^+ pump may not be involved in the effect of protein B, the erythrocyte pump differs in some respects from that of most tissues, e.g. in its energy source. For this reason, Na^+ transport in the toad bladder is of interest, since it derives its energy from oxidative reactions. Preliminary results with the isolated toad bladder (carried out by Dr. S. Mendoza) show that the Na^+ pump of this tissue is inhibited by the venom protein B, 10 μg/ml, as is the resting potential difference.

The above findings do not permit differentiation between the more likely sites for an effect of protein B: interaction with a transport or 'carrier' site; coupling to an energy source or another transport system; or interaction with the cell membrane producing permeability changes such as a leak. Studies are in progress on the mechanism of the inhibition.

The importance of using purified venom fractions needs emphasis. Despite the fact that fractionation studies have shown that the effects on the red cell,[14] muscle[15] or thyroid tissue[1, 2] are associated with proteins 'other' than phospholipase A, the assumption is still frequently made that results obtained with crude or heated venom reflect phospholipase A activity.[16, 17] The above results make it clear, however, that such conclusions are not necessarily justified even when simultaneous phospholipid hydrolysis can be demonstrated.

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