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Insulin-stimulated sodium transport in toad urinary bladder

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Mammalian and teleost insulins increase active sodium transport by the toad urinary bladder at subnanomolar concentrations. This stimulation is evident within 15 min and persists for hours. Porcine proinsulin and a cross-linked derivative of bovine insulin are less effective than porcine insulin in stimulating the short-circuit current (SCC), indicating the specificity appropriate for activation of sodium transport through an insulin receptor. The initial stimulation by insulin of the SCC is not blocked by pretreatment with actinomycin D, puromycin, cycloheximide, or tunicamycin. However, in the presence of any one of these inhibitors the sustained increase in SCC is blocked and the rise is short-lived, lasting only 45 to 90 min. In amphotericin-treated bladders, the addition of insulin did not further stimulate SCC.

Introduction

Insulin affects the transmembrane distribution of monovalent cations in many tissues, in particular, skeletal muscle. However, the hormone also stimulates the active transport of sodium in epithelial tissues. Herrera et al. [1] first demonstrated in 1963 that insulin increased the active sodium transport by frog skin and Herrera [2] subsequently reported a similar effect in the urinary bladder of the toad, *Bufo marinus*. These observations were confirmed by Crabbé and Francois [3], who also found an effect on the isolated colon of the toad. While a physiologic role for insulin in the regulation of sodium transport [4] should have been of considerable interest to both physiologists and clinicians, these initial reports were not vigorously pursued, presumably because the concentrations of hormone required were orders of magnitude greater than those normally found in the circulating plasma. In 1971 Nizet, Lefebvre, and Crabbé measured the effects of insulin upon the excretion of sodium, potassium, and water by the isolated dog kidney [5]. The addition of porcine insulin to the renal perfusate caused a significant reduction in the excretion of both cations, as well as water, and, unlike the studies in amphibian tissues, this effect was obtained with concentrations of insulin within the range observed in vivo, i.e., less than $6 \cdot 10^{-10}$ M (100 μU/ml). Subsequently, DeFronzo and co-workers [6] observed that infusions of insulin sufficient to elevate plasma levels of hormone (within the physiologic range) caused a sustained decrease in urinary sodium excretion in normal human subjects. Subsequently, this effect was dissociated from insulin-related changes in plasma glucose level [7], indicating a direct effect of physiologic levels of the hormone upon sodium transport by the renal tubular epithelium.

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The basic mechanisms leading to stimulation of sodium transport by insulin are unknown. Herrera et al. concluded that insulin stimulated the active transport of sodium at the serosal surface of the mucosal cells [1]. Crabbé and De Weer [8] compared the effects of aldosterone and insulin in the toad bladder and found that the peptide, unlike aldosterone, increased the rate of sodium transport relatively more than the pool size, presumably as a result of stimulating the sodium pump. Siegel and Civan [9] calculated that insulin, but not aldosterone, significantly increased the driving force (E_{Na}) of the sodium pump. Further evidence that insulin acted upon the sodium pump was obtained from more direct studies of $(Na^+ + K^+)$-ATPase [10]; increased activity of this enzyme has been correlated with elevations in the responsive form of the enzyme in some tissues [11,12]. Several authors reported that inhibitors of protein and RNA synthesis have no effect upon the stimulation of sodium transport by insulin [13,14]; however, Wiesmann et al. [15,16] observed that the sustained effect of insulin upon sodium transport is inhibited by actinomycin D, suggesting that insulin's long-term action on sodium transport requires the synthesis of protein(s).

To clarify some of these disparities in earlier studies of the mechanisms underlying this effect of insulin, we have examined the effects of inhibitors of protein, glycoprotein, and RNA synthesis and of amphotericin upon insulin-stimulated sodium transport in the toad's urinary bladder. We have also examined the dose-response relationships of mammalian, teleost, and chemically modified insulin analogs upon sodium transport by the tissue to probe the nature of the receptor mediating these effects.

Materials and Methods

Peptides and reagents. Porcine and bovine insulin and porcine proinsulin were provided by Dr. Ronald Chance, Eli Lilly & Co., Indianapolis. A_1-Diaminosuberoyl-B_{30} bovine insulin (Dsu-insulin) was synthesized [17] in the laboratory of Dr. Dietrich Brandenberg, who supplied the peptide for study. Anglerfish insulin was provided by Dr. Brian Noe. Tunicamycin (lot No. 361-26E-250A) was obtained from Eli Lilly & Co. Other chemicals were obtained from common commercial sources.

Measurement of sodium transport. Hemibladders were dissected from doubly pithed female Dominican toads (*Bufo marinus*) that had been kept in 0.6% NaCl for the previous 3 to 7 days, mounted on glass tubes and immersed in aerated amphibian Ringer's solution whose composition (in mM) was: NaCl, 85; KCl, 4; $NaHCO_3$, 17.5; $MgSO_4$, 0.8; KH_2PO_4, 0.8; $CaCl_2$, 1.5; and glucose, 10.5, (pH 8.2). The potential difference and short-circuit current (SCC) were measured using the method of Bentley [18] in tubes in which the external (serosal) bath was stirred by aeration. Puromycin (5–20 μg/ml), cycloheximide (1 μg/ml), actinomycin D (10–15 μg/ml), or tunicamycin (10 μg/ml) were added to the serosal bath 90 to 220 min prior to the addition of insulin. Amphotericin (10 μg/ml) was added to the mucosal surface as indicated. Chloramphenicol (30 μg/ml) was included in the Ringer's solution as described. Porcine, bovine or anglerfish insulins, proinsulin, or Dsu-insulin in dilute HCl (pH 3) were added to the serosal baths of the experimental tissues, while an equal volume of diluent was added to the control tissues. All experiments were performed a minimum of three times. In studies of inhibitors, each experiment was designed to achieve the following four pairings: (1) insulin vs. no addition; (2) inhibitor vs. no addition; (3) insulin plus inhibitor vs. insulin; and (4) insulin plus inhibitor vs. inhibitor.

Results

In a typical study, porcine insulin ($4.7 \cdot 10^{-8}$ M) added to the serosal surface of the in vitro toad urinary bladder increased the SCC within 15 min (Fig. 1). The rapid initial increment in SCC was followed by a slower, but more sustained, increase that approached a plateau approximately 2 h after the addition of hormone (Figs. 1–3, Table II). This pattern, a rapid rise in SCC followed by a sustained elevation that was maintained (or slightly increased) for periods of up to 24 h, was observed over a wide range of hormone concentrations. Preincubation of the tissues for at least 90 min with cycloheximide (cf. Ref. 19) or puromycin (Fig. 1) blocked the sustained increase in SCC but not the prompt increase seen shortly after the

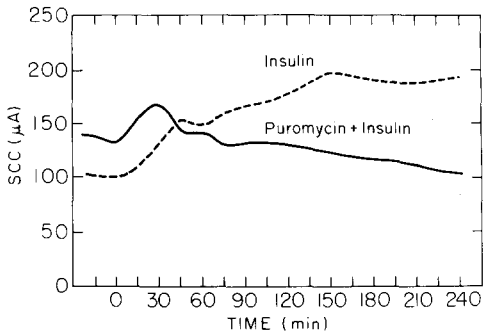

Fig. 1. The effect of puromycin on insulin-stimulated short-circuit current. The SCC was measured in paired hemibladders. Puromycin(5 μg/ml) was added to the serosal bath of one hemibladder. After 2 h of pre-incubation, porcine insulin ($4.7 \cdot 10^{-8}$ M) was added to both hemibladders (zero time).

addition of hormones. The same pattern in SCC was also obtained with actinomycin D (Fig. 2) and with tunicamycin (Fig. 3), which inhibits glycosylation of proteins [20]. Pre-incubation with cycloheximide inhibited the incorporation of radiolabeled amino acid into protein by 85–93%, and with puromycin, by 71–87%. The immediate insulin-stimulated increase in SCC of inhibitor treated tissues was observed regardless of either the length of time the tissues were preincubated with inhibitors (up to 4 h) or the concentrations of insulin used to stimulate the tissue ($6 \cdot 10^{-11}$ to $7 \cdot 10^{-7}$ M). After 2 h of exposure to insulin, the SCC of inhibitor-treated tissues was the same as inhibitor-treated tissues not exposed to insulin, suggesting that the apparent responsiveness of inhibitor-treated bladders to insulin did not result from an antibiotic-related decay in the basal current (Fig. 2 and Fig. 3, lower panel). Because any one of these inhibitors may have effects that may not be related to its generally accepted action, the effects of several different inhibitors which have in common the ability to block the incorporation of proteins into the plasma membrane, were evaluated. Since the results using all these inhibitors are similar, the observations are probably not due to selective effects of any one agent. However, a specific sensitivity of elements of the transport mechanisms to these agents has not been strictly ruled out.

Preincubation of the tissues with chloramphenicol, a bacterial protein synthesis inhibitor, has no effect upon either the baseline SCC or the response of the tissue to insulin or its analogs. 3-Deoxycytidine, an analog of uridine which inhibits the incorporation of uridine into ribosomal RNA [21,22], also failed to inhibit the stimulation of transport by insulin (data not shown).

Amphotericin increased the SCC by 3–5-fold when added to the solution bathing the muscosal surface of the toad urinary bladder [23], apparently by increasing the permeability of the muscosal (apical) membrane to sodium without

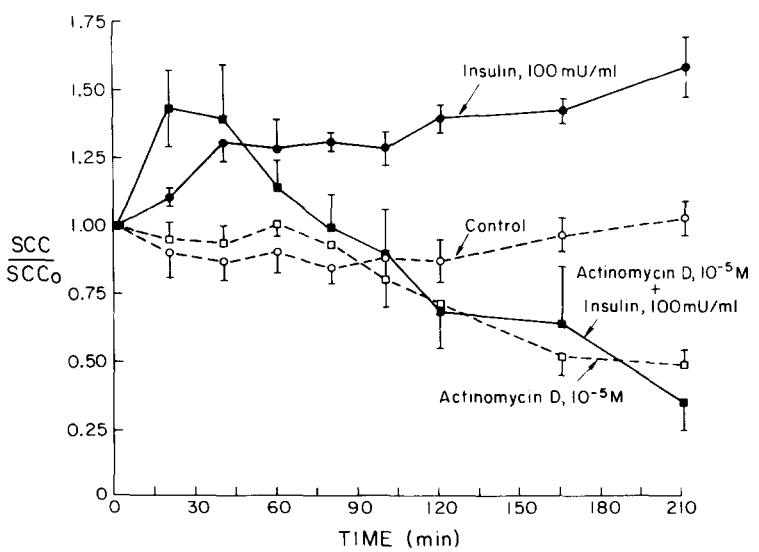

Fig. 2. Effect of actinomycin D on insulin-stimulated short-circuit current. The SCC was measured in 10 pairs of hemibladders. Both hemibladders of five pairs were treated with actinomycin D (10^{-5} M) (□,■), while the remaining five pairs were treated with the same volume of diluent (○,●). After 2 h porcine insulin ($6.7 \cdot 10^{-7}$ M) was added to one hemibladder of each pair (filled symbols, ■,●). The value at each interval is the mean ± S.E. of the SCC divided by the SCC at the time of addition of insulin. 'Insulin' hemibladders were paired with 'Control' tissues, while 'Actinomycin D plus Insulin' tissues were paired with 'Actinomycin D' tissues.

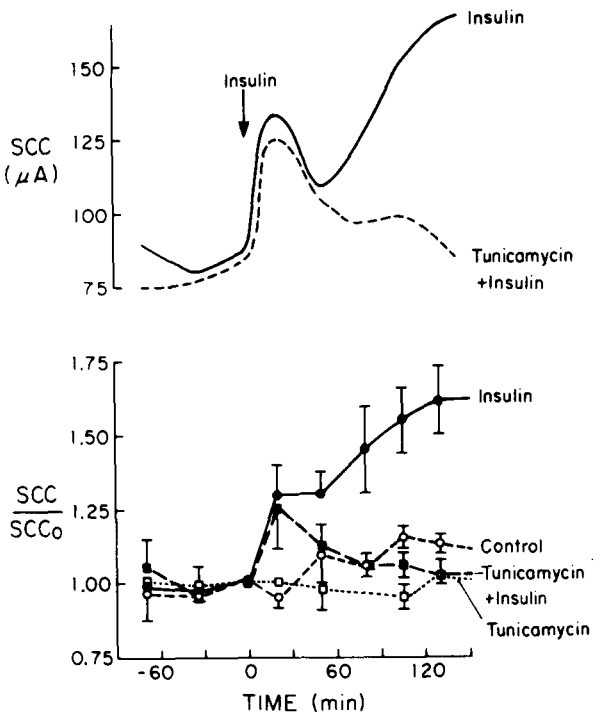

Fig. 3. Effect of tunicamycin on insulin-stimulated short-circuit current. The SCC was measured in eight pairs of hemibladders. Both hemibladders of four pairs were treated with tunicamycin (10 μg/ml) (□,■) and the remainder with diluent (○,●). After 100 min porcine insulin ($6.7 \cdot 10^{-7}$ M) (filled symbols) was added to certain tissues to obtain the following pairing of hemibladders: 'Insulin' vs. 'Control'; 'Tunicamycin' vs. 'Control'; 'Tunicamycin plus Insulin' vs. 'Tunicamycin'; and 'Tunicamycin plus Insulin' vs. 'Insulin'. The upper panel represents the SCC of one pair of hemibladders while the lower panel gives the mean ± S.E. of all hemibladders in each group.

affecting the sodium pump. The further addition of insulin ($6 \cdot 10^{-7}$ M) 90–135 min after treatment of the tissue with amphotericin did not further stimulate the SCC (Fig. 4). Incubations for longer periods were not attempted because of the unstable behavior of amphotericin-treated tissues.

Porcine insulin (Eli Lilly & Co.) stimulated SCC at concentrations as low as 5 to $7 \cdot 10^{-10}$ M (Fig. 5, Table I), while anglerfish insulin was effective at $3 \cdot 10^{-11}$ M (Table II). Although the maximal effect of either insulin was observed at approximately $(7–35) \cdot 10^{-8}$ M, anglerfish insulin produced a 50–100% larger increase in SCC than did the mammalian insulins at each concentration and time period tested. On the other hand, the potency

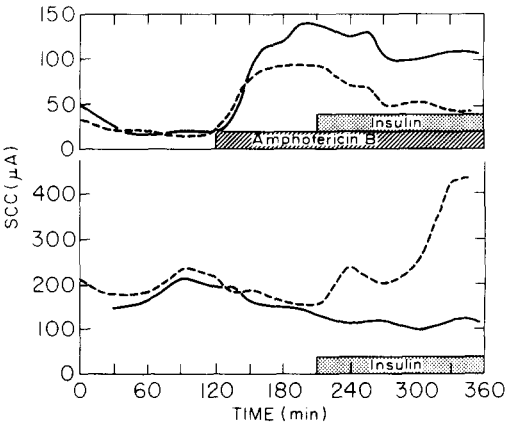

Fig. 4. Effect of amphotericin on insulin-stimulated short-circuit current. The SCC was measured in two pairs of hemibladders. Amphotericin (10 μg/ml) was added to the Ringers' solution bathing the mucosal surface of both hemibladders shown in the upper panel, but not to those of the lower panel. After 90 min porcine insulin ($6.7 \cdot 10^{-7}$ M) was added to one hemibladder of each pair (discontinuous lines).

of porcine proinsulin in stimulating SCC was considerably less than that of porcine insulin (Table I). Dsu-insulin, an analog of bovine insulin having the two constituent polypeptide chains covalently crosslinked by means of a suberimidate group, was similar to proinsulin in its effect upon sodium

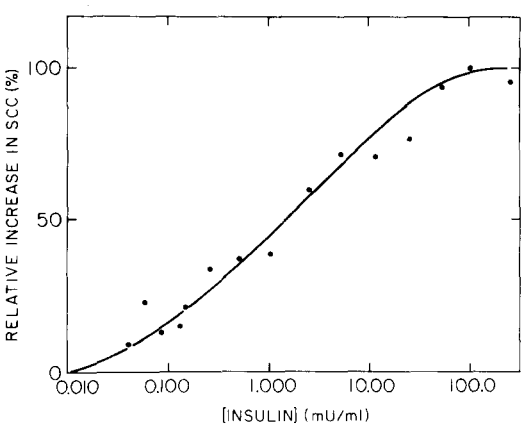

Fig. 5. Effect of porcine insulin concentration of the short-circuit current. SCC was measured in groups of bladders (n = 3–15) before and after 90 min exposure to porcine insulin in concentrations ranging from $3 \cdot 10^{-10}$ (45 μU/ml) to $6.7 \cdot 10^{-7}$ M (100 mU/ml). Each value is the average of the increase in the SCC at a given concentration of peptide expressed as the per cent of the maximum response produced by a saturating concentration of hormone at 90 or 120 min.

TABLE I

STIMULATION OF SODIUM TRANSPORT BY ANALOGS OF INSULIN

The % maximal increase in SCC was calculated as the % of the response to an analog at 120 min compared to the response to $7 \cdot 10^{-7}$ porcine insulin at 120 min measured on the same day. The actual increases in SCC ranged from 30 to 150% of the control values.

Analog	% maximal increase in SCC at an insulin concentration (M) of					
	$7 \cdot 10^{-11}$	$7 \cdot 10^{-10}$	$7 \cdot 10^{-9}$	$3 \cdot 10^{-8}$	$1 \cdot 10^{-7}$	$3 \cdot 10^{-7}$
Porcine insulin	0	21	38	72	77	100
Bovine insulin	–	43	55	–	98	–
Dsu-insulin	–	29	45	48	–	51
Proinsulin	–	10	21	33	66	53
Anglerfish insulin	10	28	40	70	–	100 [a]

[a] Maximum stimulation of SCC with anglerfish insulin was 167% of the maximal stimulation using porcine insulin.

TABLE II

EFFECT OF ANGLERFISH INSULIN ON SHORT-CIRCUIT CURRENT OF TOAD BLADDER

Time (min)	SCC_t/SCC_o									
	Control	insulin, type and concentration (M)								
		anglerfish								porcine
		$3 \cdot 10^{-11}$	$6 \cdot 10^{-11}$	$1.2 \cdot 10^{-10}$	$3 \cdot 10^{-10}$	$7 \cdot 10^{-10}$	$3 \cdot 10^{-9}$	$7 \cdot 10^{-9}$	$6 \cdot 10^{-8}$	$7 \cdot 10^{-7}$	$7 \cdot 10^{-7}$
30	0.93	0.94	1.25	1.07	1.04	1.10 [a]	1.26 [a]	1.29 [b]	1.32 [b]	2.04 [b]	1.26 [b,d]
60	0.97	0.94	1.25 [a]	1.28	1.15	1.11 [a]	1.30 [a]	1.52 [b]	1.27 [b]	2.04 [b]	1.26 [b,d]
90	0.99	0.95	1.00	1.31 [a]	1.47 [b]	1.31 [c]	1.40 [b]	1.59 [b]	1.37 [a]	1.94 [c]	1.42 [c,d]
120	0.95	1.00	1.01	–	1.17 [a]	1.40 [a]	1.41 [a]	1.56 [a]	1.26 [b]	2.29 [c]	1.58 [b,d]
690	0.87	1.25 [a]	1.18 [a]	–	1.71	1.55 [a]	1.22 [a]	–	1.24	1.93 [a]	1.31 [a,d]

Comparison of SCC in control tissues and in tissues treated with anglerfish insulin at the indicated concentrations: [a], $p < 0.05$; [b], $p < 0.01$; [c], $p < 0.001$.

Comparison of the SCC in tissues treated with porcine insulin with the SCC in tissues treated with the same concentration ($7 \cdot 10^{-7}$ M) of the anglerfish insulin: [d], $p < 0.25$.

transport. Porcine insulin obtained from Sigma Chemical Co. required concentrations two orders of magnitude greater than that of the peptide obtained from Eli Lilly to obtain a similar enhancement of SCC (data not shown), although the preparations were alleged to be equipotent. The reduced potency of the Sigma preparation in stimulating sodium transport is similar to that reported by other investigators who obtained the hormone from the same source [24].

Because differences in the zinc content of these insulin preparations might have contributed to their disparate activities, the effect of $ZnCl_2$ on the tissue was assessed. $ZnCl_2$ ($3 \cdot 10^{-7}$ M to 10^{-5} M) added to the serosal bathing solutions had no effect on SCC over a 14-h period. Higher concentrations of the metal inhibited SCC, confirming the results of Bentley [25]. In another set of studies, $ZnCl_2$ was added to the serosal bath concurrently with the addition of porcine insulin ($4.7 \cdot 10^{-8}$ M); the SCC values were equal to those of paired controls receiving insulin alone.

Discussion

Our results show that sodium transport in the toad bladder is responsive to concentrations of porcine insulin that approach the mammalian physiologic range, similar to a previous report by Cox and Singer [13]. Because the frog has been reported to be 10-fold more sensitive to its own insulin than to mammalian insulin [26], we next considered whether the toad bladder might be substantially more responsive to its endogenous

insulin. Lacking toad insulin we used anglerfish insulin, whose structure presumably more nearly resembles that of the amphibian peptide. We found that stimulation is discernible at concentrations of anglerfish insulin as low as $3 \cdot 10^{-11}$ M (4.5 μU/ml). This observation, together with the findings that the toad bladder responds to levels of mammalian peptide as low as 10^{-10} M, suggests that the toad bladder is responsive to its native hormone over a range of insulin concentrations similar to those experienced by the mammalian kidney ($3 \cdot 10^{-11}$ M to 10^{-9} M). If circulating levels of amphibian insulin have been correctly estimated at $8 \cdot 10^{-10}$ M, then these effects of insulin upon sodium transport would certainly be of physiologic significance. These data further endorse the amphibian bladder as a model for the effects of hormones upon the transport properties of the distal nephron.

Many of the effects of higher concentrations of insulin might possibly arise from its interaction with growth factor receptors. Because proinsulin has a higher affinity than insulin for insulin-like growth factor receptors in preparations from other tissues [27], we might expect that proinsulin would be at least as potent as insulin in stimulating transport if the peptides were increasing SCC as a result of binding to such growth factor receptors. The relative activities of both porcine proinsulin and the cross-linked analog, Dsu-insulin, approximate the relative efficacies (i.e., approx. 10% as effective as insulin) of these derivatives in stimulating lipogenesis in mammalian adipocytes [17], thus providing additional evidence that the stimulation by insulin of sodium transport is due to the interaction of insulin with specific insulin receptors rather than with receptors for other insulin-like growth factors.

We considered the possibility that zinc, which is used to prepare the hormone from porcine tissues, might affect the SCC. We found that the addition of $ZnCl_2$ in concentrations up to 10^{-5} M had no effect upon the baseline SCC. Since the zinc content of insulin prepared by Lilly is $4.4 \cdot 10^{-7}$ M at an insulin concentration of $6.7 \cdot 10^{-7}$ M, the addition of the cation is evidently not responsible for the increase in sodium transport.

Insulin increases SCC within 15 min and the stimulation persists for several hours. However, in our hands the SCC remains elevated much longer than previously reported, and we find that this sustained effect is not, as was reported by Wiesmann and co-workers [15,16], dependent on very high insulin concentrations. Although Wiesmann reported that the insulin-stimulated component persisted (for up to 3–4 h) only when concentrations of 100 mU/ml ($7 \cdot 10^{-7}$ M) or greater were used, we find that porcine insulin at subnanomolar concentrations causes the SCC to remain elevated for up to 24 h.

Our studies with inhibitors of protein and RNA synthesis indicate that while the initial increase in transport is not inhibited by these agents, the more sustained response is blocked. Since protein synthesis is greatly reduced in the presence of cycloheximide or puromycin, the sustained increase in sodium transport appears to require the synthesis of protein. Both Crabbé [14] and Cox and Singer [13] concluded that neither puromycin, actinomycin D nor cycloheximide had an effect on insulin-stimulated SCC, yet the graphs published by Crabbé [14] (in which actinomycin D and puromycin were tested) suggest that these inhibitors may have antagonized the insulin-induced SCC after 60 min of exposure to peptide, consistent with our findings. In addition, we find that tunicamycin, an inhibitor of glycoprotein biosynthesis [20], also prevents the long-term increase in SCC. The mechanism of this inhibition, which can be observed after a preincubation of 1–2 h, is unclear. Tunicamycin blocks insulin receptor biosynthesis [28], but insulin receptors, at least in some cells, are depleted much more slowly than the time course of decay of insulin-stimulated SCC, suggesting that loss of insulin receptors cannot account for this effect.

All of these results support the previous conclusion [15,16] that the effects of insulin upon sodium transport involve at least two distinct cellular processes: one of rapid onset and transient effect and a more sustained effect, apparently dependent upon protein and RNA synthesis. We have measured increases in the incorporation of labeled nucleotides into a broad range of polyadenylated RNAs [29]. In addition, we have also observed effects of insulin on the synthesis of some specific proteins [19]. The identity and function of these proteins and their relationship to insulin-mod-

ulated transport remain to be determined.

In analogy to the experiments of Lichtenstein and Leaf [23], we attempted to determine the site of insulin's action by studying its effects upon amphotericin-treated toad bladders. Our finding that insulin had no apparent effect on SCC after treatment of the tissues with amphotericin may be evidence that insulin's initial effect is to increase the permeability of the apical membrane to sodium. Other studies in our laboratory have shown that insulin causes alterations in the lactoperoxidase-catalyzed radioiodination of apical membrane proteins during the period encompassed by the initial, but not the sustained, increase in sodium transport [30]. Erlij and Schoen [31] found that insulin causes a rapid increase in the sodium permeability of the apical membrane of the frog skin. It has recently been found in hepatocytes that prior to stimulating the activity of $(Na^+ + K^+)$-ATPase, insulin increases the uptake of rubidium by an amiloride-sensitive pathway [32]. These findings raise the possibility that one of the processes by which insulin increases the transepithelial movement of sodium is to increase the sodium permeability of the apical membrane.

Acknowledgements

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