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BIPHASIC EFFECTS OF INSULIN AND OUABAIN ON FLUID TRANSPORT ACROSS RABBIT CORNEAL ENDOTHELIUM

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SUMMARY

1. Low levels of insulin stimulate transendothelial fluid transport from pre-swollen stroma to aqueous in rabbit corneal preparations. The rate of stromal thinning at the end of the first hour averages 30% faster with insulin, $3{\cdot}5 \times 10^{-11}$ M (4·8 μu./ml.), than that of the paired control. This concentration is about the physiological level in rabbit aqueous.

2. The stimulation with insulin is transient. Rates of thinning average higher but not significantly different from control rates by the second hour.

3. High levels of insulin between $3{\cdot}5 \times 10^{-9}$ M (480 μu./ml.) and $2{\cdot}0 \times 10^{-6}$ M ($2{\cdot}75 \times 10^{5}$ μu./ml.) inhibit fluid transport. The inhibition at the low end of this range of concentrations becomes more pronounced with longer perfusion times but appears not to exceed *ca.* 50% of the control rate.

4. Ouabain also induces a biphasic effect on fluid transport which is characteristically different from that with insulin. The maximal stimulation observed at all times occurred with a fixed concentration of 10^{-10} M. The stimulation is not transient but increases throughout the duration of the perfusion; the average rate is elevated 50% above the control rate by the third hour.

5. The transition from a stimulatory to an inhibitory effect occurs consistently at *ca.* 10^{-8} M with ouabain, while a similar transition with insulin occurs at *ca.* 10^{-9} M and appears to shift towards slightly higher concentrations during a 3 hr perfusion period.

6. Inhibition of fluid transport with ouabain, 3×10^{-7} M, is increased from *ca.* 50% after 1 hr to more than 70% at the end of the third hour of perfusion.

7. The combined presence of stimulatory concentrations of ouabain and insulin affects stromal thinning in a manner resembling the effect of ouabain alone more than that of insulin; additive effects could not be discriminated. Progressively raising the concentration of insulin to a level (10^{-8} M) that alone inhibits stromal thinning, ultimately abolishes the stimulatory effect of ouabain. Based on other evidence and current models of drug/hormone-membrane interaction, these results can be interpreted to indicate a concentration-dependent interaction between receptor complexes of ouabain and insulin with $(Na^+ + K^+)$-ATPase.

INTRODUCTION

The corneal endothelium is well suited to physiological studies of hormone action since it exists as a readily available single layer of homogenous cells. Isolation and culturing procedures that may yield mixed populations or transformed cells are avoided as well as problems of diffusion associated with connective tissues. Fluid transport can be monitored by changes in stromal thickness (Mishima & Kudo, 1967; Maurice, 1972) to provide an immediate and direct measurement of hormonal effects.

Reports that insulin is detectable by radioimmunoassay in aqueous of human (Greco, Ghirlanda, Fedeli, Fenici, Bertoni & Gambassi, 1973) and rabbit (Daniel & Henderson, 1967) at levels of approximately $\frac{1}{2}$ and $\frac{1}{5}$, respectively, those in the sera of these species raised the question of endothelial responsiveness to this hormone. Insulin has been shown to stimulate Na^+ efflux from skeletal muscles of frogs (Kernan, 1962; Grinstein & Erlij, 1974) and hypophysectomized rats (Zierler, Rogus & Hazelwood, 1966) in the presence of glucose as well as under conditions that rule out an induction of ionic translocation by osmotic changes (Creese, 1968; Moore, 1973). Insulin also increases the level of K^+ in liver (Kestens, Haxhe, Lambotte & Lambotte, 1963), uterus (Lostroh & Krahl, 1973) and, as shown with muscle, does so *in vitro* (Moore, 1973) and *in vivo* (Zierler & Rabinowitz, 1964). That insulin also enhances the activity of $(Na^+ + K^+)$-ATPase [Mg^{2+}-dependent, $(Na^+ + K^+)$-activated ATP phosphohydrolase, EC 3.6.1.3] in preparations of plasma membranes obtained from cultured human lymphocytes (Hadden, Hadden, Wilson, Good & Coffey, 1972) as well as in membranes from skeletal muscle and brain (Gavryck, Moore & Thompson, 1975) can be viewed as a mechanism whereby insulin could exert a regulatory influence over cation movements *in vivo*.

The effect of insulin on the rate of fluid transport across the corneal endothelium was investigated in the initial phase of the present study. This hormone was found to exert a biphasic effect, i.e. stimulation and inhibition at different ranges of concentration, similar to that reported for ouabain and other cardiotonic glycosides on the activity of $(Na^+ + K^+)$-ATPase in a variety of tissues from different species (cf. Bonting, 1970). At high concentrations ouabain and other cardiotonic glycosides are highly specific inhibitors of $(Na^+ + K^+)$-ATPase (cf. Skou, 1971). Blocking of insulin-stimulated Na^+ efflux by ouabain (Grinstein & Erlij, 1974) or acetylstrophanthidin (Moore, 1973) is consistent with most other evidence (cf. Lee & Klaus, 1971) substantiating a role of this enzyme in cation transport (cf. Baker, 1972). In the case of the cornea, the inverse relationship between the rate of stromal swelling (Trenberth & Mishima, 1968) and the activity of endothelial $(Na^+ + K^+)$-ATPase (Rogers, 1968) with the level of ouabain constitutes a priori evidence for an involvement of $(Na^+ + K^+)$-ATPase in transendothelial fluid transport. Additionally, this fluid transport is dependent on the level of Na^+ (Hodson, 1971; Dikstein & Maurice, 1972; Fischbarg & Lim, 1974) and K^+ (Fischbarg & Lim, 1974) in the solution bathing the endothelium as well as the cellular level of ATP (Anderson, Fischbarg & Spector, 1974). Since prior corneal studies detected no enhancement of either fluid transport (Trenberth & Mishima, 1968) or $(Na^+ + K^+)$-ATPase activity (Rogers, 1968) with ouabain concentrations of and above 10^{-8} M, the effect on fluid transport of ouabain concentrations that included a level below 10^{-8} M was reinvestigated. The

known capacity of ouabain and insulin to modify independently the activity of $(Na^+ + K^+)$-ATPase prompted experiments that provide results which reveal an antagonistic cooperativity between these agents on transendothelial fluid transport.

METHODS

Female albino rabbits weighing approximately 2·5 kg were sacrificed with 5 ml. of intravenously administered Nembutal and both eyes were immediately enucleated. One eye was kept in a moist container at room temperature during the 10–15 min interval required to dissect and mount the other eye. Epithelium was removed and the corneal preparations, mounted in two separate perfusion chambers (Fischbarg, 1973) were swollen concurrently from a normal thickness of 360 ± 15 μm (Dikstein & Maurice, 1972) to 500 ± 10 μm by imbibition from the denuded epithelial surfaces of a stationary basal salt solution containing glucose (BSG). The endothelial surfaces were perfused with the same solution at a rate of 49 μl./min. The BSG contained (mM) NaCl 110, $KHCO_3$ 3·8, $NaHCO_3$ 39, $MgSO_4$ 0·78, KH_2PO_4 1·0, $CaCl_2$ 1·7 and glucose 6·9 and was adjusted to 300 m-osmole with sucrose. Deturgescence was initiated by replacement of the BSG on the epithelial surface with silicone oil and stromal thickness was monitored microscopically as previously described in detail (Anderson, Fischbarg & Spector, 1973; Fischbarg & Lim, 1974).

The perfusing solutions for the test corneas differed from those for the respective paired controls by the addition of variable amounts of either crystalline bovine zinc insulin (0·05% Zn, *ca.* 24 u./mg) or ouabain obtained from Sigma Chemical Co., St Louis, Missouri. Solutions of zinc insulin were prepared either with BSG within the hour before use or as a stock solution, $2{\cdot}0 \times 10^{-5}$ M, in HCl, $1{\cdot}6 \times 10^{-3}$ N, and stored without detectable alteration of effectiveness as frozen aliquots at -20 °C for periods up to 2 weeks. Errors in weighing, incompleteness of insulin dissolution and/or losses before the perfusion experiments by non-specific adsorption (Gerschenson, Okigaki, Andersson, Molson & Davidson, 1972) were estimated to be less than 5% by spectrophotometric determination and comparison of the ϵ_M^{280} with published values (Lord, Gubensk & Rupley, 1973; Sperling & Steinberg, 1974). Although transendothelial fluid transport is not dependent on external Cl^- (Fischbarg & Lim, 1974) and preliminary studies showed that supplementation of BSG with HCl, $2{\cdot}0 \times 10^{-4}$ N, did not affect significantly either the rate or duration of fluid transport, control corneal preparations, nonetheless, were exposed to concentrations of HCl equivalent to those used in the test perfusing solution.

Before each experiment, solutions to be perfused were equilibrated at 38 °C and adjusted by gassing with CO_2 to pH 7·4 or a calculated P_{CO_2} of 57 mmHg. The P_{CO_2} was calculated by means of the Henderson–Hasselbach equation, $pH = pK_a + \log[HCO_3^-]/[CO_2]$, and the following values: pH, 7·4; $[HCO_3^-]$ in BSG solution, 0·043 M; pK_a, first ionization constant of carbonic acid, 6·025 and the solubility of CO_2, 0·024 mole per kg solvent/atm. Values for pK_a and CO_2 solubility, respectively, were obtained from published data (cf. Edsall & Wyman, 1958) either directly or by calculation and are based on solutions of NaCl, 0·15 M, at 38 °C. The solutions were immediately placed in 30 ml. syringes which were secured to a perfusion pump. Such small losses of CO_2 as may have occurred during transit of the solution through the 30 cm of polyethylene tubing connecting the syringe to the perfusion chamber would be expected to be offset, wholly or in part, by the 1·6-fold increase in solubility of CO_2 as the temperature of the solution falls to ambient (20 °C). More elaborate precautions to control the pH in the perfusion chamber were judged unnecessary for two reasons. First, the endothelium and stroma generate CO_2 which tends to acidify the contents of the 0·35 ml. perfusion chamber in a manner that resembles the physiological events in the ocular anterior chamber and, secondly, the transendothelial electrical potential difference which correlates closely with the rate of fluid transport remains approximately stable in the range $7{\cdot}2 \leqslant pH \leqslant 8{\cdot}1$ (cf. Fischbarg & Lim, 1974, fig. 18). Small shifts in this range of pH would be expected not to affect the results measurably.

RESULTS

Treatment of the data. An example of the primary data obtained from perfusion of paired endothelial preparations is shown in Fig. 1. Results are expressed as a plot of observed stromal thickness (ordinate) versus time (abscissa). In order to analyse quantitatively such primary data as this, an alternative treatment was necessary. The rate, i.e. the number of micrometers by which each preparation thinned during consecutive hourly intervals, was recorded. The average hourly rates for test

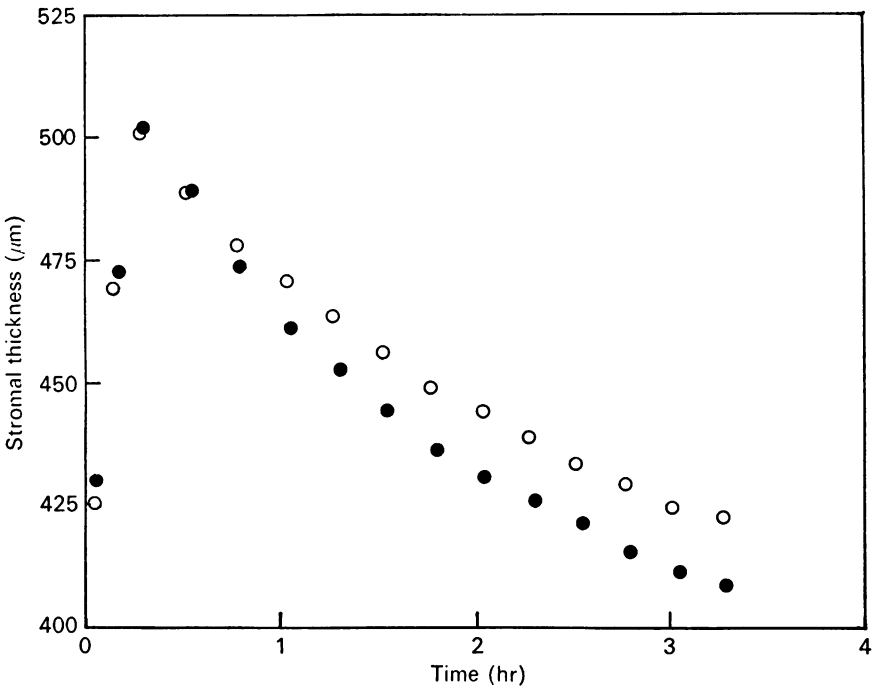

Fig. 1. Plot of stromal thickness *vs.* time for a typical perfusion experiment with a given pair of rabbit corneas denuded of epithelium. The stromal preparations were mounted in separate chambers, swollen *in situ* to *ca.* 500 μm and perfused according to techniques described and referred to in the text. The BSG solution used for perfusion of the test preparation (filled circles) differed from that for the control (open circles) by the addition of the tested agent which in this case was Zn-insulin, $3{\cdot}5 \times 10^{-11}$ M.

preparations are expressed in Figs. 2 and 3 as a percentage change of the respective, hourly control rates. These ratios normalize the rates of stromal thinning for test preparations with respect to those for the controls and also minimize such variability in thinning as may be encountered among different animals (Anderson *et al.* 1974). Under our experimental conditions, hourly thinning rates of paired preparations perfused with BSG alone agreed within 5 % up to 4 hr.

In Figs. 2 and 3 the mean percentage changes in stromal thickness are plotted as a function of the concentration of the test substance and standard errors are represented by the heights of the vertical bars. The curves were drawn by eye to fit the data points. The significance of the mean differences between the rates of stromal

thinning in μm/hr for pairs of test and control preparations was analysed statistically by means of the Student *t*-test for paired differences (Smith, 1968). The data in Fig. 4 was treated similarly to that in Figs. 2 and 3 but was obtained largely with controls perfused with BSG supplemented with either ouabain or insulin. The use of such controls as these provides a base line for comparison with test preparations supplemented with both ouabain and insulin as well as a means of detecting the effect of one agent acting in the presence of another.

Effects of insulin. The biphasic action of insulin on transendothelial fluid transport during 3 consecutive hours of perfusion is illustrated in Fig. 2. Stromal thinning in the first hour (Fig. 2*A*) is significantly stimulated by a low level of insulin, namely 16% with $4{\cdot}4 \times 10^{-12}$ M ($P < 0{\cdot}025$) and 30% with $3{\cdot}5 \times 10^{-11}$ M ($P < 0{\cdot}01$). The observed maximal stimulation diminished over the next 2 hr (Fig. 2*B*, *C*) to an average rate only 5% greater and not significantly different from that of the control.

With high concentrations of insulin between $1{\cdot}4 \times 10^{-8}$ and $2{\cdot}0 \times 10^{-6}$ M fluid transport is depressed to *ca.* 50% the control rate by the first hour and remains essentially unchanged through the rest of the perfusion period. Note that over the 3 hr period, with a concentration of insulin equal to or greater than 7×10^{-9} M, the number of original test preparations decreased by the third hour, i.e. certain preparations completely failed to sustain a measurable rate of deturgescence. More fluid leaked into than was outwardly transported from the stroma with the result that stromal thickness increased.

Concentrations of insulin between 7×10^{-10} and 7×10^{-9} M comprise a transition level that is characterized by large spreads in the S.E. of the average differences from the control thinning rate for the second and third hour. At the high end of these transition concentrations (i.e. insulin, $3{\cdot}5 \times 10^{-9}$ and 7×10^{-9} M) inhibition becomes more pronounced between the first and third hour while at the low end (insulin 7×10^{-10} M), the average rate change shows an opposite trend.

Effects of ouabain. Ouabain, like insulin, also exerts a biphasic effect on stromal thinning but the hourly rate curves (Fig. 3) exhibit characteristic differences from those for insulin. By 1 hr (Fig. 3*A*) stromal thinning with ouabain, 10^{-10} and 10^{-9} M, shows a mean stimulation of 23% ($P < 0{\cdot}05$) and 18% ($P < 0{\cdot}001$), respectively. The maximal stimulation observed with ouabain, 10^{-10} M, is thus about 7% less and occurs with a concentration about an order of magnitude higher than that observed with insulin. The stimulation, moreover, not only persists after the first hour but increases slightly (6%) by the end of the second hour (Fig. 3*B*) and then markedly (24%) by the end of the third hour (Fig. 3*C*). There also appears to be no tendency for the observed maximal stimulation to shift toward a higher concentration with perfusion times beyond 1 hr.

The transition with ouabain from a stimulatory to an inhibitory effect, unlike that with insulin, occurs sharply and remains at a fixed concentration (10^{-8} M) which is about an order of magnitude greater than that with insulin. In the presence of ouabain, 3×10^{-7} M, the rate of fluid transport is depressed 50% at the end of the first hour. Although the inhibition is sustained and even more marked by the third hour, no preparation entirely failed to deturgesce.

Modulation of the stimulatory effect of ouabain by insulin. Experiments were designed to detect whether or not ouabain and insulin act independently in the

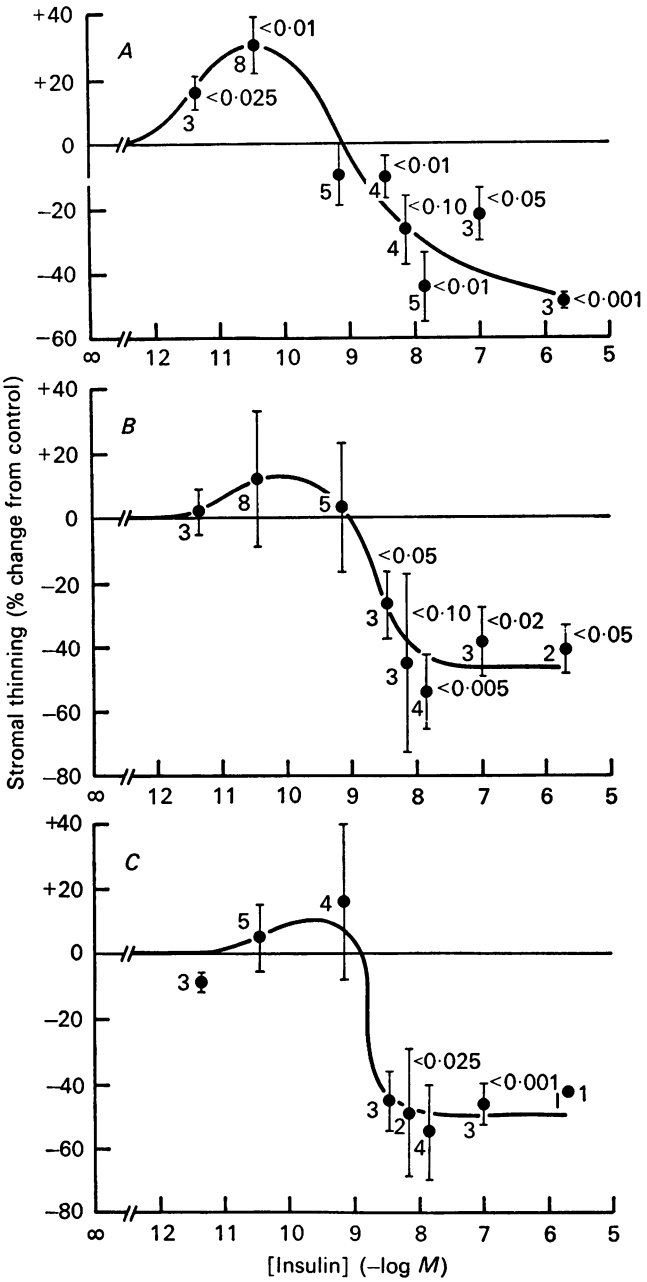

Fig. 2. Effect of insulin concentration on rates of stromal thinning during first (*A*), second (*B*) and third (*C*) hour of continuous perfusion. Each point and vertical bar represents the mean difference ± S.E. between paired test and control preparations. Numbers to left and right of bars indicate, respectively, number of paired experiments in which the test preparations sustained a measurable stromal deturgescence and the level of statistical significance, *P*, of mean paired differences in rates of thinning; values of $P \geqslant 0{\cdot}10$ are not shown. The horizontal line extending from zero on the ordinate represents the control rate (100%). Average thinning rates ± S.E. of all controls perfused with BSG were as follows: *A*, 38·6 ± 2·0 ($n = 35$); *B*, 28.3 ± 1·3 ($n = 31$) and *C*, 23·6 ± 1·4 μm/hr ($n = 25$).

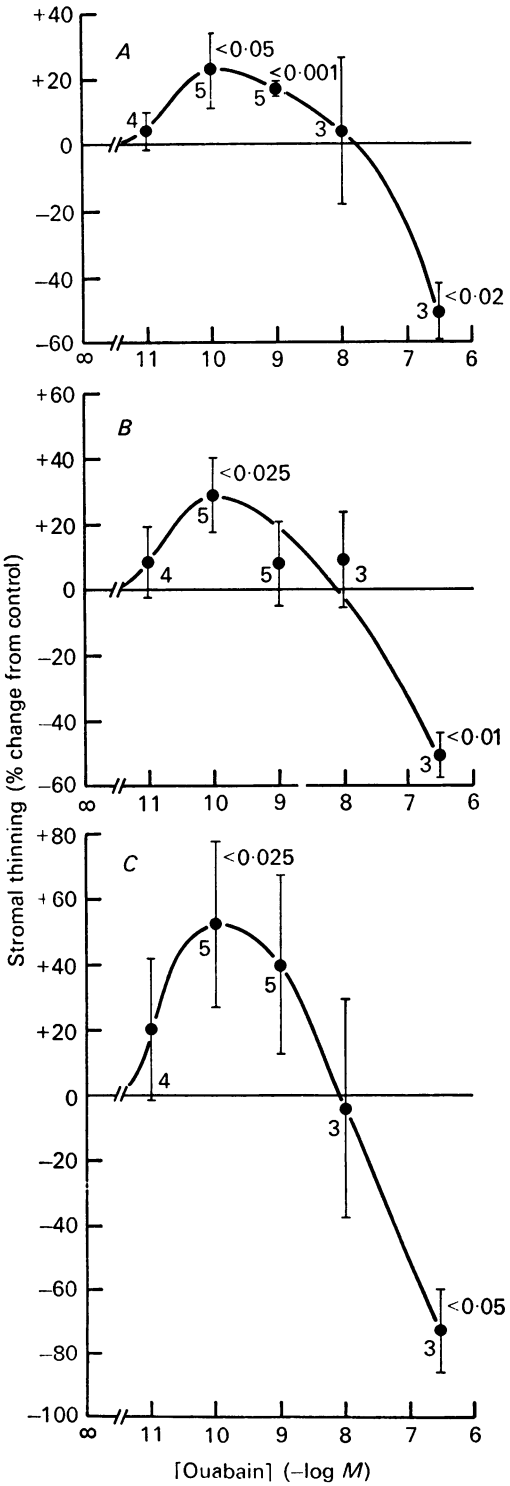

Fig. 3. Effect of ouabain concentration on rates of stromal thinning during first (*A*), second (*B*) and third (*C*) hour of continuous perfusion. Legend for Fig. 2 applies except that average thinning rates ± S.E. of all controls were as follows (n = 20): *A*, 27·7 ± 2·5; *B*, 23·8 ± 1·3; *C*, 19·6 ± 1·5 μm/hr.

presence of each other, i.e. are individual effects additive or not. The concentration of ouabain was held constant at 10^{-10} M since this level consistently induces a stimulation that is maximal for any hourly interval of the 3 hr perfusion period and yet is a level sufficiently low to permit examination of insulin over a range of concentrations spanning both stimulatory and inhibitory effects. Fig. 4 summarizes the observations and additionally includes data from Figs. 2 and 3 for ready comparison. Fig. 4, I*A* shows the combined effect of stimulatory levels of insulin, $3{\cdot}5 \times 10^{-11}$ M, and ouabain, 10^{-10} M, relative to BSG (Fig. 4, I*B*). The average stimulation tends, particularly by the third hour, to be lower than that which would be expected if the individual

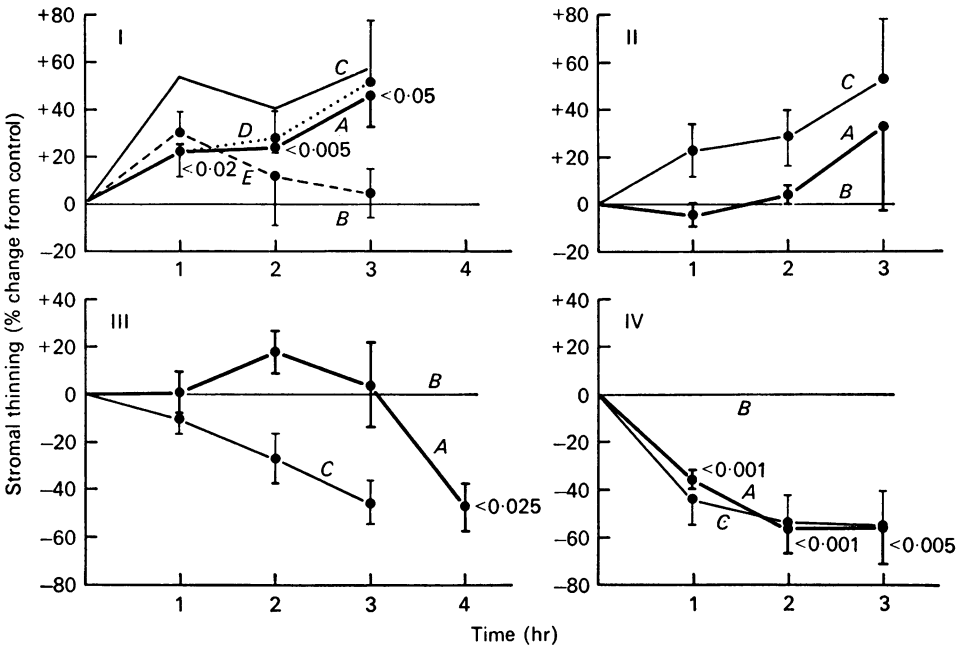

Fig. 4. Hourly rates of stromal thinning in the presence of ouabain, 10^{-10} M, perfused together with various concentrations of insulin. For each concentration of insulin tested in I–IV, *A* represents the observed rate relative to *B*, the paired preparation serving either as a control or an internal standard; *C* represents a rate for *A* calculated with data for Figs. 1 and 2 by assuming the effects of ouabain and insulin are additive. (I) *A*, insulin, $3{\cdot}5 \times 10^{-11}$ M, plus ouabain, 10^{-10} M; *B*, BSG; *C*, sum of *D* and *E*; *D*, ouabain, 10^{-10} M; *E*, insulin, $3{\cdot}5 \times 10^{-11}$ M. (II): *A*, insulin, $5{\cdot}6 \times 10^{-10}$ M, plus ouabain, 10^{-10} M; *B*, insulin, $5{\cdot}6 \times 10^{-10}$ M; *C*, ouabain, 10^{-10} M. (III) *A*, insulin, $3{\cdot}5 \times 10^{-9}$ M, plus ouabain, 10^{-10} M; *B*, ouabain, 10^{-10} M; *C*, insulin, $3{\cdot}5 \times 10^{-9}$ M. (*IV*) *A*, insulin, $1{\cdot}4 \times 10^{-8}$ M plus ouabain, 10^{-10} M; *B*, ouabain, 10^{-10} M; *C*, insulin, $1{\cdot}4 \times 10^{-8}$ M. Points, vertical bars and numbers to right of bars are defined in legend to Fig. 1. Average thinning rates of controls (n = 3 or 4) for I, II, III and IV were, respectively, $22{\cdot}7 \pm 2{\cdot}8$, $43{\cdot}0 \pm 10{\cdot}6$, $27{\cdot}0 \pm 0{\cdot}0$ and $22{\cdot}3 \pm 1{\cdot}2$ μm/first hour; $21{\cdot}7 \pm 2{\cdot}3$, $25{\cdot}0 \pm 1{\cdot}2$, $22{\cdot}3 \pm 0{\cdot}3$ and $23{\cdot}7 \pm 4{\cdot}6$ μm/second hour; $17{\cdot}0 \pm 2{\cdot}9$, $9{\cdot}7 \pm 3{\cdot}3$, $20{\cdot}7 \pm 1{\cdot}8$ and $20{\cdot}0 \pm 3{\cdot}8$ μm/third hour: III, $18{\cdot}0 \pm 1{\cdot}5$ μm/fourth hour.

effects were additive (Fig. 5, I*C*) and to resemble the effect of ouabain alone (Fig. 4 I*D*) more than that of insulin alone (Fig. 4, I*E*). Raising the level of insulin to $5{\cdot}6 \times 10^{-10}$ M, a transition concentration judged from Fig. 2 to be without a significant effect, produces a response in the presence of ouabain, 10^{-10} M

(Fig. 4, II*A*) by the first and second hour that resembles the thinning rate observed with this concentration of insulin alone (Fig. 4, II*B*) more than that expected if ouabain were fully effective (Fig. 4, II*C*). The presence of insulin thus appears to diminish the stimulation by ouabain. By the third hour the average rise of the thinning rate 30% above that of the control perfused with insulin alone suggests a latent manifestation of the ouabain stimulatory effect.

With insulin, $3{\cdot}5 \times 10^{-9}$ M, a transition concentration that is progressively more inhibitory after the first hour, no statistical discrimination between additive and modified ouabain effects is possible after 1 hr of perfusion with either ouabain or insulin serving as an internal standard. Throughout the second and third hour of perfusion (Fig. 4, III*A*), nevertheless, the influence of ouabain (Fig. 4, III*B*) dominates over that expected if insulin were solely effective (Fig. 4, III *C*; $P < 0{\cdot}025$ and $P < 0{\cdot}05$ for second and third hour, respectively). By the fourth hour the insulin effect is marked. All influence by ouabain, 10^{-10} M, (Fig. 4, IV*B*) is completely overwhelmed in the presence of insulin, $1{\cdot}4 \times 10^{-8}$ M (Fig. 4, IV*A*).

DISCUSSION

The concentration of insulin, namely $4{\cdot}4 \times 10^{-12}$ M or an amount equivalent to 4·8 μu./ml., which induced the observed optimal stimulation of transendothelial fluid transport interestingly approximates the physiological range of $6{\cdot}2 \pm 1{\cdot}4$ μu./ml. present in rabbit aqueous (Daniel & Henderson, 1967). This concentration of insulin is about 20 times less than that found to effect a significant 5% increase in ^{86}Rb uptake by human lymphocytes (Hadden *et al.* 1972) and 4–5 orders of magnitude less than the insulin concentrations routinely employed to influence the Na^+ and K^+ content of skeletal muscles (Zierler *et al.* 1966; Moore, 1973; Grinstein & Erlij, 1974). Since human lymphocytes are exposed to an insulin level in plasma that can fluctuate over a range of concentrations 4–20 times (Yalow & Berson, 1960; Cahill, 1971) the average concentration of insulin in the rabbit aqueous, the disparity in concentrations that effect a comparable physiological response in these two cell types probably reflects an adaptive device whereby the insulin level to which different cells are normally exposed will elicit a response of similar magnitude. An inverse correlation between the plasma insulin concentration and the concentration of insulin receptors has not only been observed in various tissues (cf. Gavin, Roth, Neville, De Meyts & Buell, 1974) but has been found, moreover, to be inducible in cultured human lymphocytes (Gavin *et al.* 1974). If, as some evidence indicates, the biological effects of polypeptide hormones are mediated subsequent to binding of the hormones with specific receptor sites on the cell surface (Cuatrecasas, 1974), the number of available receptors becomes significant. The very low level of insulin that stimulates fluid transport by the corneal endothelium would indicate that these cells are particularly rich in insulin binding sites.

The apparent lack of effect with ouabain, 10^{-8} M, and the 50% inhibition of fluid transport by the first hour with ouabain, 3×10^{-7} M, agrees with the findings of Trenberth & Mishima (1968). The stimulation of fluid transport presently detected with concentrations of ouabain below 10^{-8} M, in accordance with others' similar observations of $(Na^+ + K^+)$-ATPase activation (cf. Bonting, 1970), strengthens the inference that

transendothelial fluid transport and the activity of $(Na^+ + K^+)$-ATPase are linked (Rogers, 1968). The increased stimulatory effect of ouabain, 10^{-11} to 10^{-10} M, with increased time noted in this study has also been reported to occur with ouabain, 10^{-11} to 10^{-8} M, and microsomal $(Na^+ + K^+)$-ATPase of rabbit brain and chicken kidney (Palmer, Lassiter & Melvin, 1966). Transient stimulation followed by an inhibitory effect has occurred over a wide range of ouabain concentrations during potential difference measurements in frog skin (Wilbrandt & Weiss, 1960; MacRobbie & Ussing, 1961). Dahl & Hokin (1974) have reviewed the extensive studies of structure-activity and ligand effects on ouabain interaction with $(Na^+ + K^+)$-ATPase. They point out that the association of cardiac glycoside-ATPase complexes is relatively slow and the dissociation even slower than the association with rates for each process dependent on the ligands present. In this light, many if not all the above variable effects observed with increased time can be reconciled. The increased inhibition presently observed with prolonged perfusion may have a similar basis but non-specific binding at high concentrations should not be dismissed. Models of ouabain interaction with $(Na^+ + K^+)$-ATPase that propose a receptor allosterically related but not identical to the phosphorylated enzyme (Schwartz, Lindenmayer & Allen, 1972) or different receptors activated by different concentrations of the glycoside (McClane, 1965; Palmer, *et al.* 1966) could account for the biphasic effects.

The concerted effects of insulin and ouabain on transendothelial fluid transport indicate a coordinated interaction dependent on the relative concentrations of each agent. Previously discussed aspects of this process, namely, the individual, biphasic actions of insulin and ouabain on fluid transport, the cited evidence linking fluid transport with $(Na^+ + K^+)$-ATPase activity and the reported biphasic and stimulatory effects, respectively, of ouabain and insulin on $(Na^+ + K^+)$-ATPase, lend themselves to a consideration of the biphasic effect of insulin on fluid transport as a ouabain-like effect on $(Na^+ + K^+)$-ATPase. Although other functions of insulin cannot be discounted, observations that ouabain exhibits an insulin-like action on glucose uptake, glucose oxidation and glycogen synthesis (Ho & Jeanrenaud, 1967) as well as on levels of cyclic AMP and free fatty acids in adipose tissue (Ho, Jeanrenaud, Posternak & Renold, 1967) support this suggestion. Some evidence also indicates that certain of these (Blatt, McVerry & Kim, 1972) and other (Legros, Saines & Conard, 1973) functions shared by the hormone and glycoside are concentration-dependent and competitive. If, indeed, the activity of the enzyme, $(Na^+ + K^+)$-ATPase, is mutually modulated by ouabain and insulin, then the presently observed antagonistic action of these agents can be largely accommodated by the fluid membrane hypothesis for hormone-receptor complex formation by Cuatrecasas (1974). This flexible scheme permits different hormones, and presumably other agents, with either stimulatory or inhibitory effects to form multiple complexes simultaneously.

Several explanations can be offered for the transient stimulation of fluid transport during continuous perfusion with insulin. The most probable is that the concentration of insulin was reduced to an ineffective level as a result of non-specific binding to the perfusion apparatus. Losses of 76 % after a 2 hr incubation period have been reported (Gerschenson *et al.* 1972). This property would also explain the tendency for the stimulation to shift to higher concentrations with increased time. Inhibitory effects of insulin on fluid transport or any such other parameter associated with ionic

movement as membrane potential or short-circuit current have not been reported previously to our knowledge. That glucagon contamination of the insulin preparations is responsible for depressed fluid transport is unlikely. The usual contamination of 1% (Hadden *et al.* 1972) would amount to less than 10^{-3} μg/ml. in the presence of an inhibitory concentration of insulin (10^{-8} M) and this amount is appreciably less than 1 μg glucagon/ml. used to elicit a cellular response (Ho *et al.* 1967).

The choice of Zn-insulin in this and many other physiological studies is based on the wide usage by diabetics of the hormone in this form which has the therapeutically advantageous property of slow dissolution. That the amount of zinc in insulin at concentrations up to at least 7×10^{-9} M is without influence on either ($Na^+ + K^+$)-ATPase activity or glucose uptake by lymphocytes has been reported by Hadden *et al.* (1972). Whether or not the bound Zn present in insulin contributed to or was responsible for any of the present results requires re-examination with Zn-free insulin preparations.

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