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ORIGINAL ARTICLE

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Forskolin increases apical sodium conductance in cultured toad kidney cells (A6) by stimulating membrane insertion

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Abstract The role of membrane traffic in the stimulation of apical Na^+ permeability caused by increases in cytoplasmic cyclic AMP was assessed by measuring the effects of forskolin on transepithelial capacitance (C_T), transepithelial conductance (G_T), and short-circuit current (I_{sc}) in A6 cultured toad kidney cells. Apical water permeability was probed by recording cell volume changes after reducing the osmolality of the apical bath. We found that forskolin does not increase the osmotic water permeability of the apical membrane of A6 cells, and thus does not stimulate the insertion of water channels. Comparison of the effects of forskolin and insulin on Na^+ transport demonstrated that both agents produce reversible increases in C_T, G_T and I_{sc}. G_T and C_T increased proportionally during the rising phase of the insulin response. However, a non-linear relationship between both parameters was recorded when forskolin was given in NaCl Ringer's solution. The relationship between C_T and G_T became linear after the effects of forskolin on Cl^- conductances were eliminated by substituting Cl^- by an impermeant anion. In contrast, in Cl^--containing Na^+-free solutions, the nonlinearity became more pronounced. Successive additions of insulin and forskolin caused additive increases in C_T. Because increases in C_T and Na^+ transport occurred in the absence of stimulation of water permeability and increases of C_T and G_T were directly proportional when Na^+ was the major permeating ion across the apical membrane, we suggest that the increase in apical Na^+ permeability in the presence of either forskolin or insulin is due to the insertion of channels residing in intracellular pools. In contrast, the increased Cl^- permeability caused by forskolin may be related to the activation of channels already present in the membrane.

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Introduction

Changes in membrane composition by insertion of specific transporters is the major mechanism for control of water permeability by neurohypophysial hormones and other agents that increase the cytoplasmic adenosine 3',5'-cyclic monophosphate (cAMP) content in tight epithelia. This conclusion is based on a vast amount of evidence using morphological, biochemical and electrophysiological techniques to demonstrate insertion of water channels into the apical membrane [3, 5, 27]. Insertion of Na^+ channels as the mechanism of the increased apical Na^+ permeability caused by stimulation of cytoplasmic cAMP production is a more controversial issue. The insertion hypothesis is supported by measurements of blocker-induced noise in whole epithelia [10, 17] and by measurements of single-channel activity in cell-attached patches [20] that show an increased number of Na^+ channels in the apical membrane during conditions that increase cytoplasmic cAMP. In addition, studies with proteolytic enzymes also indicate that new Na^+ channels appear in the apical membrane after treatment with vasopressin [14]. However, a totally different view, suggesting that phosphorylation of channels already residing in the membrane is the cause of increased Na^+ permeability [1], has been proposed based mainly on studies of a purified 300-kDa polypeptide.

Garty and Palmer [15] have recently reviewed the evidence supporting both hypotheses. They point out that the 300-kDa and other similarly purified polypeptides cannot be identified with the apical Na^+ channel of tight epithelia, because the biophysical profile of these purified polypeptides does not correspond to that of any of the channels thus far studied in intact cells. Moreover, they also note that the number and sizes of the subunits of the purified polypeptides do not correspond to those

of the epithelial Na^+ channel (ENaC), the channel cloned by expression in *Xenopus laevis* oocytes [15]. Garty and Palmer [15] noted that the evidence supporting membrane insertion as the mechanism of the increased apical Na^+ conductance caused by stimulation of cAMP production is also incomplete. They express a specific concern: that measured increases in membrane capacitance, a parameter used to asses membrane insertion because it is proportional to membrane area, may correlate better with the stimulation of water permeability than with the stimulation of apical Na^+ permeability caused by increasing cytoplasmic cAMP.

In this study we have measured the effects of stimulating cAMP production in A6 cells on membrane capacitance, ion and water transport. These measurements demonstrate that osmotic water permeability does not increase in response to stimulation of cytoplasmic cAMP production. Thus, studies in these cells may provide a useful test of whether changes in apical Na^+ permeability are correlated with changes in membrane traffic in the absence of insertion of water channels. Another reason to study the relationship between membrane insertion and the stimulation of Na^+ transport is that previous studies indicate that insulin increases apical Na^+ permeability by promoting insertion of Na^+ channels [11]; therefore, it is interesting to determine whether increased cAMP acts in a similar fashion in A6 cells. In addition, in A6 cells, stimulation of cytoplasmic cAMP production also increases apical Cl^- permeability [4, 26, 30] making it possible to compare whether changes in either Na^+ or Cl^- permeability are related to changes in transepithelial capacitance (C_T).

Materials and methods

Preparation

In this study we used passages 103–106 of a subclone (jA6) of A6 cells obtained from Dr. J. P. Johnson (University of Pittsburgh, Pittsburgh, Pa., USA). Cells were cultured on permeable Anopore filter supports (pore size 0.2 µm) (Nunc Intermed, Roskilde, Denmark). The epithelial monolayers were mounted in Ussing-type chambers [8] that enabled continuous perfusion of both compartments and rapid changes of the composition of the solutions. The DC value of the transepithelial voltage was clamped to zero with a fast-voltage-clamp device. The gain of the voltage amplifier was 50 and the current amplifier could be operated at either 50 or 10 mV/µA. The frequency responses of the current and voltage amplifier were matched by reducing the bandwidth of the current amplifier with a trim capacitor across the feedback resistance. In the range of 1 Hz to 8 kHz the phase shift between current and voltage amplifier was less than 0.1 degree. At higher frequencies it increased to reach approximately 0.3 degrees at 22 kHz.

Transepithelial conductance and capacitance measurements

Hardware for C_T measurements was based on two Digital Signal Processing (DSP) boards (Model 310B, Dalanco Spry, Rochester, N.Y., USA). One DSP board was used to record transepithelial conductance (G_T) and short-circuit current (I_{sc}). G_T was measured by imposing a 1 Hz sine wave voltage of 5 mV to the tissue. Transepithelial current (I_T) and voltage (V_T) changes caused by this sine wave were sampled at a rate of 25,000 measurements per second. I_{sc} was calculated as mean value of I_T data collected for the G_T measurements. The system enabled us to update every 7 s the G_T, I_{sc} and C_T values. C_T was recorded with five sine waves with frequencies of 2, 2.7, 4.1, 5.4 and 8.2 kHz. The figures in this paper illustrate records at 4.1 kHz.

Measurement of the membrane capacitance of epithelia is hampered by the complicated structure of this type of tissue. In its simplest form the electrical equivalent circuit of an epithelium consists of the series arrangement of two membranes shunted by a parallel resistance that represents the conductive properties of the paracellular pathway. The determination of the capacitance of the individual membranes from transepithelial impedance measurements is only possible in conditions where the time constants of the apical and basolateral membranes are quite different [24]. This condition is achieved when the apical membrane is permeabilized with an ionophore [11, 23]. This treatment results in the appearance of two semicircles in the Nyquist plot, each reflecting the properties of the apical and basolateral membrane. The impedance plot of A6 epithelia not subjected to such treatment contains only one semicircle that displays only small deviations from the impedance plot of a single membrane. Because, under most physiological conditions, it is impossible to determine the capacitance of each border of the epithelium, we decided to measure the equivalent capacitance of the epithelium and interpret the results assuming a simple RC network. At high frequencies, C_T thus calculated equals the equivalent capacitance of the serial arrangement of apical and basolateral membranes (Eq. 1):

$$\frac{1}{C_T} = \frac{1}{C_{ap}} + \frac{1}{C_{bl}} \tag{1}$$

where C_{ap} and C_{bl} are the apical and basolateral membrane capacitances, respectively. Because C_{ap} is at least 10 times smaller than C_{bl} [11, 29], changes in C_T will be mainly determined by alterations of apical membrane area. The procedure to calculate C_T is based on the equations (Eqs. 2, 3) for the real (Re z) and imaginary (Im z) parts of the impedance of the single RC circuit:

$$\mathrm{Re}\, z = R_S + \frac{R_T}{1 + R_T C_T \omega^2} \tag{2}$$

and

$$\mathrm{Im}\, z = \frac{-R_T^2 C_T \omega}{1 + R_T C_T \omega^2} \tag{3}$$

where R_T and C_T represent the equivalent transepithelial resistance and capacitance of the epithelium. The determination of these parameters requires an estimation of the resistance in series with the epithelium (R_S). Using the impedance data of two frequencies one can calculate R_S as (Eq. 4):

$$R_S = \frac{\mathrm{Re}\, z_1 \frac{\mathrm{Im}\, z_2}{\omega_2} - \mathrm{Re}\, z_2 \frac{\mathrm{Im}\, z_1}{\omega_1}}{\frac{\mathrm{Im}\, z_2}{\omega_2} - \frac{\mathrm{Im}\, z_1}{\omega_1}} \tag{4}$$

R_S was obtained as mean value of ten possible combinations of the set of five frequencies. With this value of R_S we could determine C_T as (Eq. 5):

$$C_T = \frac{-\mathrm{Im}\, z}{\omega\left(\mathrm{Re}\, z - R_S\right)^2 + \left(\mathrm{Im}\, z\right)^2} \tag{5}$$

The use of the DSP system enabled us to update the C_T values every 2 s.

Cell thickness measurement

The method used for recording cell thickness has been previously described in detail [25]. The filter supports were coated with fluorescent microspheres of 1 µm diameter (L5081, Molecular Probes, Eugene, Ore., USA) embedded in a thin gelatin layer. The apical membrane of the epithelium was labelled with fluorescent avidin-

coated microbeads (F8776, Molecular Probes) by exposing the apical surfaces for 30 min to 1 ml of an isotonic NaCl Ringer's solution which contained 2 µl/ml of the beads. The microbeads that did not attach to the membranes were washed off by rinsing the apical compartments with NaCl Ringer's solution. The preparation was mounted on the microscope stage in a modified Ussing's chamber and short-circuited. The distance between a basolateral reference bead and an apical bead was recorded as cell thickness (T_c). Focusing of the microbeads was done by recording the fluorescent light intensity at different focal distances obtained by moving the objective with a piezo focusing device (PIFOC, Physik Instrumente, Waldbronn, Germany). The position with maximal light intensity was considered as the recorded position of the microbead. The system was controlled by a personal computer equipped with a frame grabber. Taking into account the size of the microsphere, the T_c values were corrected by subtracting 1 µm from the measured heights. T_c values were expressed as a percentage of the control. An improved version of the software used in the present experiments enabled us to record simultaneously and continuously T_c of up to 20 cells. Only records where T_c was followed over the entire duration of the experiments were used for evaluation of cell volume.

Solutions

NaCl Ringer's solutions contained (in mM) 135 Na^+, 2.5 K^+, 2.5 HCO_3^-, 1 Ca^{2+} and 137 Cl^- (pH=8.0, osmolality 260 mosmol/kg H_2O). Na^+-free solutions were prepared by replacing Na^+ by *N*-methyl-D-glucamine ($NMDG^+$). Aspartate was used as a substitute for Cl^- to prepare Cl^--free Ringer's solutions. All chemicals were obtained from either Sigma (St. Louis, Mo., USA), Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany). Insulin (Actrapid HM) was purchased from Novo Nordisk and, if not stated otherwise, added at a concentration of 20 nM to the basolateral side. Cellular cAMP was stimulated by adding 5 µM forskolin to the basolateral compartment.

Results

Water permeability

Neurohypophysial hormones and other agents increase cytoplasmic cAMP and thus control water permeability of the apical membrane of a number of tight epithelia by promoting the insertion of water channels into the apical membrane [3, 5, 27]. To evaluate the water permeability of either the apical or the basolateral membranes of A6 cells, we measured changes in cell height, a parameter directly proportional to cell volume [25], when the osmolality of either the apical (π_{ap}) or basolateral (π_{bl}) solution was modified. Effects of dilution of the apical and basolateral solutions in forskolin-treated epithelia are illustrated in Fig. 1. After a control period, in which the tissues were bilaterally perfused with 260 mosmol/kg H_2O NaCl Ringer and 5 µM forskolin on the basolateral side, π_{ap} was reduced to 54 mosmol/kg H_2O. This reduction in π_{ap} did not change cell height. In contrast, when π_{bl} was reduced to 140 mosmol/kg H_2O, cell height rapidly increased and after reaching a peak value returned to values slightly above control. The lack of cell volume increase upon the drastic reduction of π_{ap} shows that the apical membrane is impermeable to water even when cAMP production is stimulated by forskolin. The transient response in cell volume caused by reducing π_{bl} resembles that found in previous studies from our laboratory [7]. It demonstrates that the basolateral membrane is highly permeable to water and that the expansion of cell volume triggers a regulatory volume decrease in A6 cells.

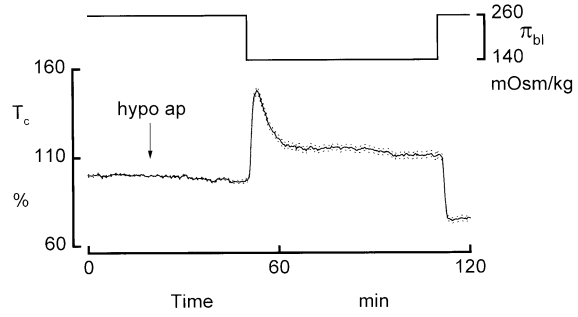


Fig. 1 Effects of reducing the osmolality of the apical (π_{ap}) or basolateral (π_{bl}) solution on the cell height of A6 epithelia incubated in forskolin. The *solid line* is the mean of 55 records obtained in 5 experiments; the *dotted lines* are the SEM. The tissues were incubated throughout the experiment with 5 µM forskolin in the basolateral solution. Except for the presence of forskolin the tissues were initially incubated in symmetrical Ringer's solution with an osmolality of 260 mosmol/kg H_2O. First π_{ap} was reduced to 54 mosmol for the rest of the experiment. Next π_{bl} was lowered to 140 mosmol for a period of 1 h

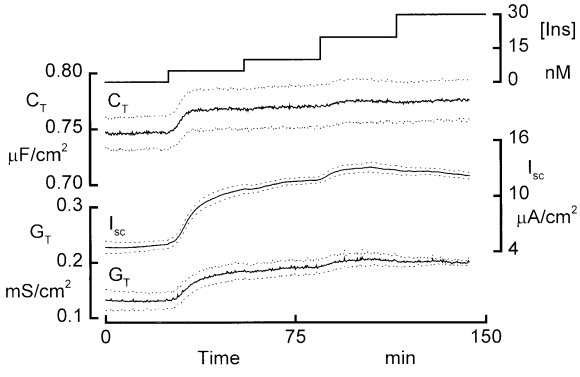


Fig. 2 Concentration dependence of insulin's action. The *upper line* shows the changes in insulin concentration in the basolateral solution while short-circuit current (I_{sc}), transepithelial conductance (G_T) and capacitance (C_T) were simultaneously measured in A6 cells. The *solid lines* are averages (n=5) and the *dotted lines* represent SEM. At a concentration of about 15 nM, insulin reached its maximal effect

Insulin

Comparison of the effects of forskolin with those of insulin was carried out because previous observations indicate that insulin increases the Na^+ conductance of the apical membrane of A6 cells by increasing insertion of Na^+ channels [11]. We reexamined the effects of insulin with the improved method for rapid sampling of C_T and verified whether the effects of insulin, originally observed using high doses [11], are also observed with doses that do not interact with insulin growth factor (IGF) receptors. Figure 2 shows the responses of I_{sc}, G_T and C_T when the concentration of insulin was increased in a

stepwise fashion. All three parameters were stimulated by insulin and concentrations beyond 20 nM did not further increase the response on any of the measured parameters. Five nanomolar insulin caused an increase in I_{sc} that was more than 60% of the magnitude of the response observed when 30 nM of the hormone was used. This observation indicates that the responses to insulin described here are not due to activation of IGF receptors, since much higher concentrations of insulin are required to activate IGF receptors in A6 cells [2, 20]. Figure 3 shows that the effects of insulin on these parameters are reversed when the hormone is removed from the perfusion fluid. The figure and the data summarized in Table 1 also show that the effects of the hormone are similar when it is given either to untreated tissues or after the parameters have recovered to their original levels following a prior period of perfusion with forskolin.

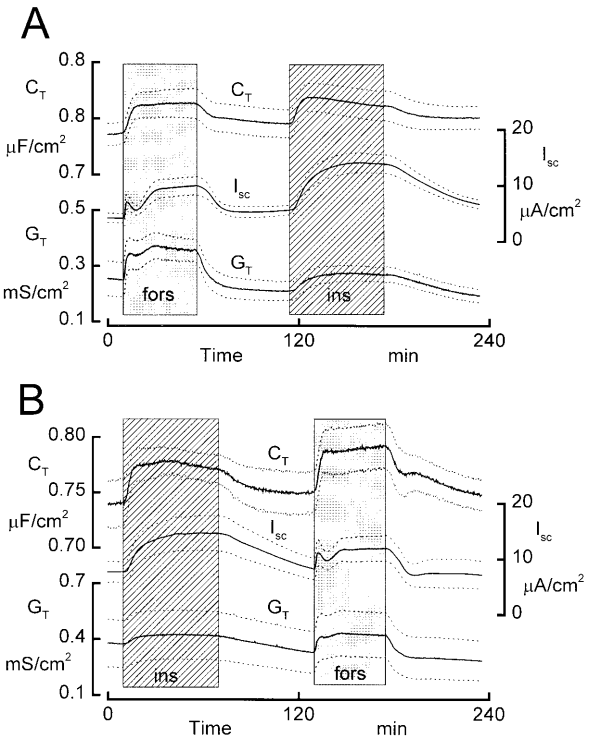


Fig. 3A,B Comparison of the effects of insulin and forskolin. Twenty nanomolar insulin and 5 μM forskolin were added separately to the basolateral solution. **A, B** Experiments in which the order of addition of agonists was reversed. The *solid lines* are averages (n=5) and the *dotted lines* represent SEM. The effects of both insulin and forskolin are reversible and there is little influence on the response of prior addition of each agent

In a previous study we suggested that insulin increases apical Na^+ permeability by promoting insertion of membranes containing Na^+ channels [11]. This hypothesis was further explored by the simultaneous and repeated sampling of G_T and C_T carried out with the new technique used in this study. By plotting the values of G_T as a function of C_T during the rising phase of the stimulation induced by the hormone, we could further examine whether changes of these parameters are correlated. Values collected during the transition from the resting basal level to the stimulated value were plotted as illustrated in Fig. 4. These plots show that G_T increases in direct proportion to C_T.

We previously suggested that almost all the effects of insulin on G_T and I_{sc} are due to increased apical Na^+ conductance. This conclusion was based on the finding that administration of insulin to epithelia perfused with apical Na^+-free solutions does not produce significant increases in G_T and I_{sc} [11]. Here we repeat this type of experiment to verify the correlation between G_T and C_T. This analysis enables us to detect possible effects of insulin on the apical Cl^- conductance as described by Marunaka and Eaton [20] in experiments utilizing patch-clamp methods. Figure 5 shows that perfusion of the apical side with Na^+-free solutions blocks the increase in I_{sc} and G_T caused by insulin while C_T still increases in response to the hormone. The lack of G_T increase shows that in this clone of A6 cells, insulin does not increase apical Cl^- conductance. Plots of G_T as a function of C_T (Fig. 5B) further emphasize the fact that the change in G_T is absent in Na^+-free solutions.

Forskolin

Forskolin stimulates cAMP production by directly activating adenylate cyclase. In A6 cells forskolin increases

Table 1 Separate and cumulative effects of forskolin and insulin on short-circuit current (I_{sc}), transepithelial capacitance (C_T) and conductance (G_T). Increases in I_{sc} (ΔI_{sc}), C_T (ΔC_T) and G_T (ΔG_T) elicited by insulin and forskolin. The order of administration of these compounds is indicated at the *top* of each *column*. The data in rows labelled "Separate" were obtained from experiments as shown in Fig. 3. The data in the rows labelled "Cumulative" were obtained from experiments as depicted in Fig. 9. The values in the *columns* labelled *I+F* (insulin followed by forskolin) and *F+I* (forskolin followed by insulin) for the rows labelled "Separate" were calculated by adding the increases caused by forskolin and insulin administered separately (see Fig. 3). The corresponding values for the rows labelled "Cumulative" were recorded in the same experiments as shown in Fig. 9 when both agents were present simultaneously in the basolateral solution

		Insulin		Forskolin		Insulin+Forskolin	
		First	Second	First	Second	I + F	F + I
ΔI_{sc} (μA/cm²)	Separate	9.44±1.41	9.55±0.82	6.87±0.83	4.12±0.26	13.56±1.66	16.42±1.72
	Cumulative	7.02±0.75	2.85±0.19	3.93±0.38	1.44±0.26	8.46±0.91	6.48±0.50
ΔC_T (μF/cm²)	Separate	0.050±0.004	0.040±0.005	0.050±0.008	0.037±0.003	0.087±0.007	0.090±0.013
	Cumulative	0.033±0.002	0.058±0.008	0.054±0.002	0.058±0.003	0.090±0.004	0.114±0.015
ΔG_T (mS/cm²)	Separate	0.05±0.02	0.07±0.01	0.11±0.01	0.09±0.01	0.14±0.03	0.17±0.03
	Cumulative	0.06±0.02	0.02±0.01	0.11±0.01	0.08±0.01	0.14±0.02	0.13±0.02

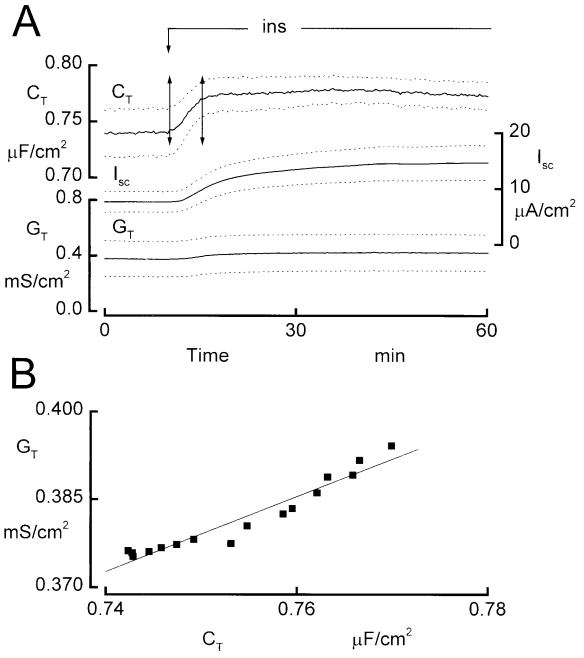


Fig. 4A,B Relationship between G_T and C_T during insulin treatment. To examine whether increases in G_T are correlated with increases in C_T we plotted the values of G_T as a function of C_T during the rising phase of the stimulation induced by the hormone. The interval used to collect the data for the plot shown in **B** are marked by *arrows* in the tracing shown in part **A**. The slope of the regression line through the data points is 0.69±0.04 mS/μF

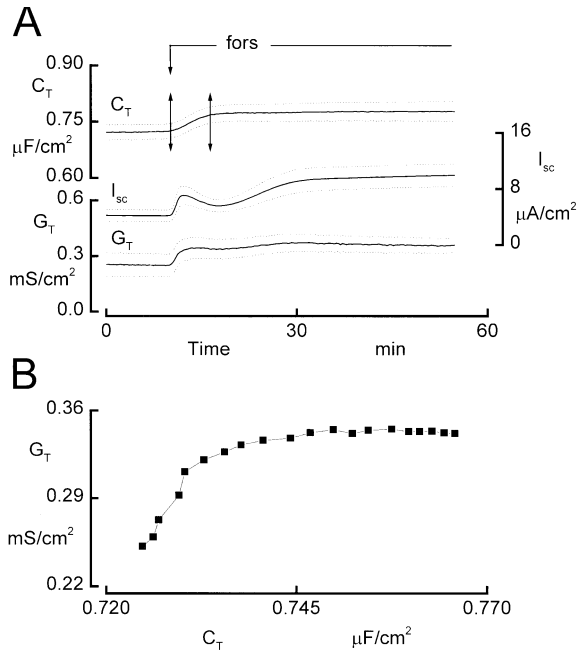


Fig. 6A,B Relationship between G_T and C_T during forskolin treatment. Tissues were incubated in NaCl Ringer's solution. To examine whether increases in G_T are correlated with increases in C_T we plotted the values of G_T as a function of C_T during the rising phase of the stimulation induced by forskolin. The interval used to collect the data for the plot shown in part **B** are marked by *arrows* in the tracing shown in **A**. During the action of forskolin the increase in G_T was a non-linear function of C_T, a relationship that is markedly different from the effects of insulin shown in Fig. 4

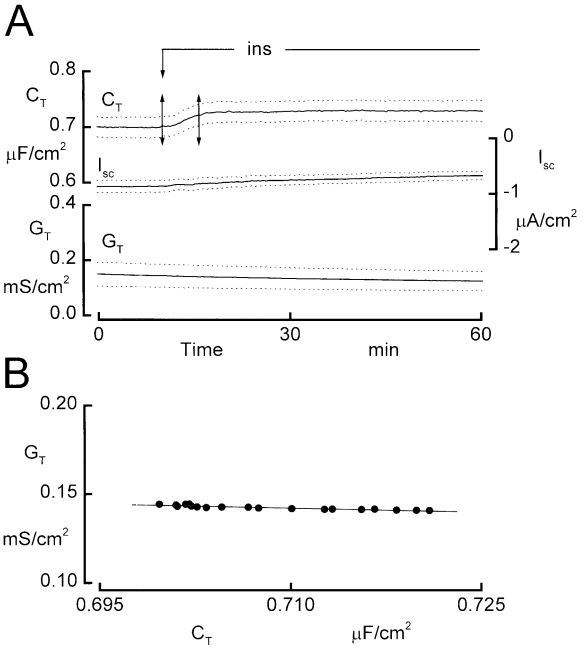


Fig. 5A,B Response to insulin in the absence of Na^+ transport. Effects of insulin were assayed after replacing apical Na^+ by *N*-methyl-D-glucamine ($NMDG^+$). **A** Time course of the effect of insulin on I_{sc}, G_T and C_T. **B** G_T as a function of C_T. *Arrows* in part **A** show the interval used to collect data plotted in panel **B**. These data show that insulin increases C_T in tissues incubated with apical Na^+-free solutions while increases in I_{sc}, and G_T are abolished. The slope of the regression line through the data points is –0.15±0.02 mS/μF

the cAMP content [4]. Figure 3 shows that forskolin increases I_{sc}, G_T and C_T in A6 cells in a reversible fashion. The stimulation of I_{sc} and G_T is biphasic: forskolin first produces an early rise that rapidly reaches a peak. This peak is followed by a brief recovery, and then a second sustained increase of both parameters is observed. The biphasic responses of I_{sc} are in agreement with previous findings in studies of the same cells [4, 26]. In contrast, the increase in C_T displays a monophasic response.

A plot of the increases in G_T as a function of the changes in C_T sampled during the rising phase of forskolin stimulation is shown in Fig. 6B. In contrast with the results with insulin (Fig. 4), this relationship was not linear. The reason for the difference between insulin and forskolin is that insulin has a major effect only on Na^+ transport while forskolin causes major activation of two separate transport processes in A6 cells. One is an amiloride-sensitive transport of Na^+ from apical to basolateral solution, the other involves Cl^- movements in the opposite direction [4, 26, 30]. The major component of each response appears to be an increase in ion movements through apical membrane channels.

The contribution of each of these channels was explored by using either Na^+-free or Cl^--free solutions to separate the effects of forskolin on Na^+ and Cl^- conductances, denoted as G_{Na} and G_{Cl}, respectively. Figure 7 shows the results obtained when epithelia were perfused with Na^+-free solutions on the apical side. Removing

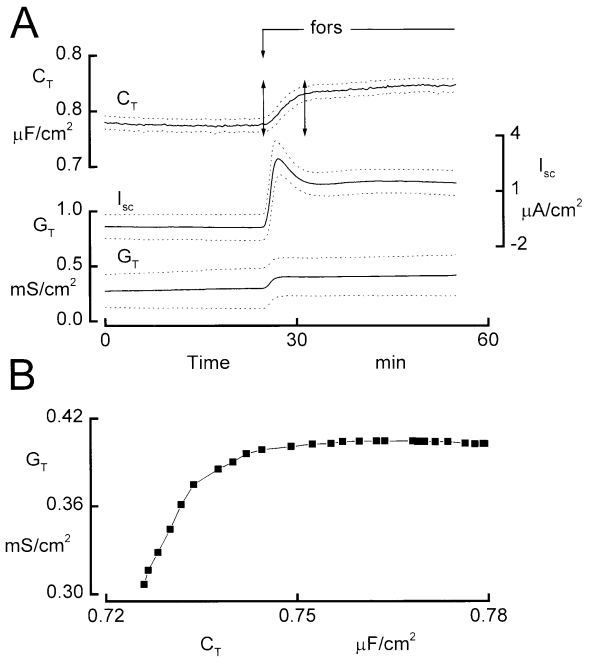
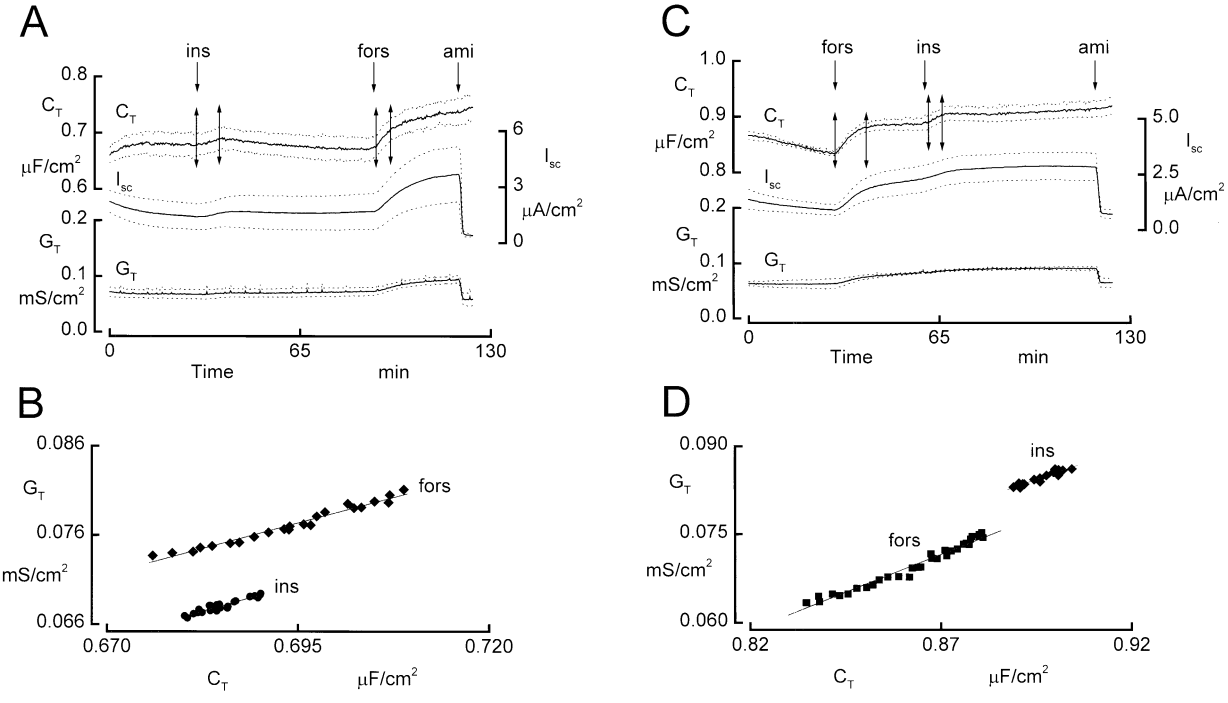


Fig. 7A,B Response to forskolin in the absence of Na^+ transport. Apical Na^+ was replaced by $NMDG^+$. **A** Time course of I_{sc}, G_T and C_T. **B** Plot of G_T as a function of C_T during the rising phase of the action of forskolin. *Arrows* in **A** show the interval used to collect data plotted in **B**. These data show that forskolin increases C_T, I_{sc}, and G_T in tissues incubated with apical Na^+-free solutions. In contrast with the effects of insulin that were abolished in Na^+-free solutions, forskolin still increased I_{sc} and G_T. The increase in G_T caused by forskolin was again a non-linear function of the change in C_T

apical Na^+ (Fig. 7A) did not modify the increase in C_T caused by forskolin. Forskolin also produced a marked increase of I_{sc} and G_T despite that fact that basal I_{sc} was markedly depressed by Na^+-free solutions. The initial time course of the stimulation of I_{sc} was virtually identical to the early part of the response recorded in Na^+-containing solutions (see Fig. 6A), although the late component of the increase in I_{sc} did not occur. This finding is in agreement with previous observations showing that during forskolin's action the increase in Na^+ permeability develops later than the increase in Cl^- permeability [4, 26]. A plot of the increase in G_T as a function of the increase in C_T (Fig. 7B) under these incubation conditions shows that the relationship is not linear.

The effects of forskolin and insulin on I_{sc}, G_T and C_T in epithelia perfused with Cl^--free solutions on both sides are shown in Fig. 8. Perfusion with Cl^--free solutions depressed I_{sc} and G_T, by 69±4 and 52±5% (n=6), respectively. The reductions of I_{sc} and G_T have been observed in other studies and are probably due to reduction of Na^+ currents as a consequence of reductions in cell volume and/or osmolality [4, 22, 28]. The effects of insulin on I_{sc}, G_T and C_T in epithelia perfused with Cl^--free

Fig. 8A–D Relationship between G_T and C_T studied in Cl^--free solutions. Apical and basolateral Cl^- was replaced by aspartate. **A** Effect of insulin and forskolin on the time course of I_{sc}, G_T and C_T. **B** Plot of G_T as a function of C_T during the action of insulin and forskolin. **C** Effect of forskolin and insulin on I_{sc}, G_T and C_T. **D** Corresponding plot of G_T as a function of C_T. *Arrows* in parts **A** and **C** show the interval used to collect data plotted in part **B** and **D**. The slopes of the regression lines through the data points in **B** are 0.24±0.02 and 0.23±0.009 mS/μF for insulin (*INS*) and forskolin (*FORS*), respectively. The slopes of the regression lines in **D** are 0.26±0.01 mS/μF for forskolin and 0.23±0.02 mS/μF for insulin



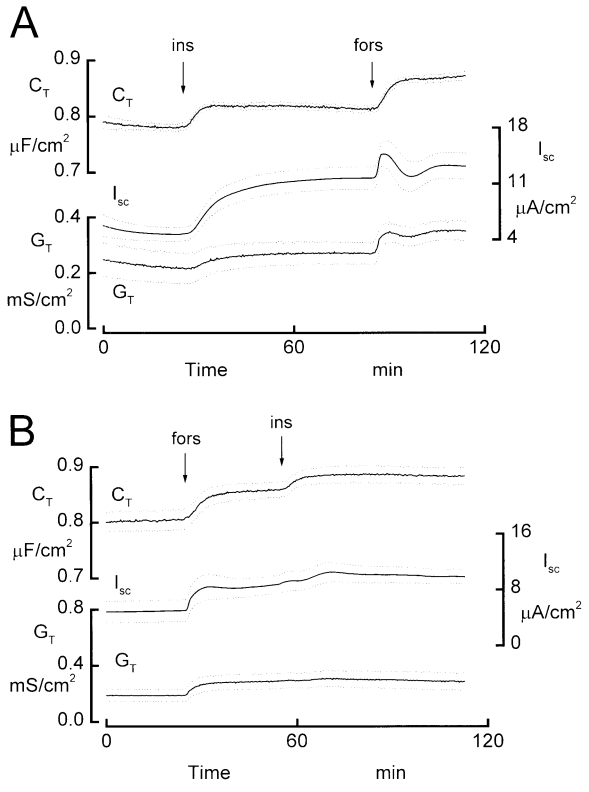


Fig. 9A,B Additive effects of insulin and forskolin on I_{sc}, G_T and C_T. Experiments were performed in NaCl Ringer's solutions. **A, B** Experiments in which the order of addition of agonists was reversed

solutions were markedly reduced. However, plots of G_T as a function of C_T show that these parameters increase in direct proportion during the rising phase of insulin's action (Fig. 8B). This response is in contrast with the dissociation observed when insulin is given to epithelia incubated in Na^+-free solutions (Fig. 5).

Figure 8A, C shows that forskolin still has large stimulatory effects on I_{sc}, G_T and C_T in epithelia perfused with Cl^--free solutions. Figure 8C, D shows a plot of the increase in G_T as a function of the increase in C_T in Cl^--free solutions. Increases of G_T become directly proportional to increases in C_T when the contribution of Cl^- conductance to the forskolin-induced response is eliminated, i.e. Na^+ conductance (G_{Na}) is the dominant component of G_T.

Combined effects

The finding that both insulin and forskolin increase G_{Na} in direct proportion to the increase in C_T suggests that these agents may act on a common effector. One test of this suggestion is to find out whether maximally active doses of these agents have additive effects. We examined this point by comparing experiments as shown in Fig. 3, in which each agonist was administered separately, with the responses of both agents when simultaneously present as in Fig. 9. In the latter group, one agonist was first added to the basolateral perfusate followed, after 1 h, by a solution containing both agents. A comparison of these results with those of experiments in which either insulin or forskolin was given separately (see Fig. 3), is presented in Table 1. Forskolin and insulin had additive effects on C_T regardless of the order of addition of these compounds. However, the effects of these agonists on I_{sc} and G_T were not always additive. Insulin had significantly smaller effects when added to forskolin-containing solutions than those observed when insulin was given alone.

Discussion

Our results show that stimulation of cAMP production with forskolin increases the Na^+ conductance of the apical membranes of A6 cells without increasing their water permeability. This observation is in contrast with the proposal that increases in C_T caused by stimulation of cAMP are solely related to insertion of water channels. More remarkable, C_T and G_{Na} increased in direct proportion during the rising phase of the forskolin response, suggesting that increases in G_{Na} and membrane area are linked. During the rising phase of the insulin response C_T and G_{Na} also increased in direct proportion, suggesting that insertion of Na^+ channels is a common mechanism for stimulating apical Na^+ permeability by different agonists (see Figs. 4 and 8). However, increases in channel insertion do not appear to be the only mechanism involved in the regulation of apical membrane conductance: the stimulation of G_{Cl} caused by forskolin does not bear any direct relationship to increases in C_T.

C_T and membrane area

The major assumption in our interpretation of the current results is that changes in C_T represent changes in apical membrane area. Previous studies [11, 29] showed that the basolateral membrane capacitance is about 10–12 times larger than the apical. Therefore, C_T will be dominated by the apical side of the epithelium. Furthermore, the assumption that changes in membrane capacitance reflect alterations in membrane area seems to be the best interpretation for our results. Indeed, in a great variety of situations it has been observed that membrane capacitance provides reliable estimates of membrane area. For example, stimulation of insertion of membranes as a mechanism of stimulation of transport by insulin and agents that increase cAMP production has been demonstrated in other systems, both with morphological methods and with determinations of transfer of molecules from cytoplasmic compartments to the surface membrane [3].

It should be noted that changes in apparent membrane capacitance (the amount of charge stored in the membrane per unit change in membrane potential) have been observed when the conformation of a variety transporters

is modified during different physiological conditions [16, 19]. However, similar increases in apparent membrane capacitance were not observed during agonist-induced changes in channel conformation that resulted in increased conductance [19]. Indeed, thus far there is no compelling reason to suggest that the agonists used in this study do not act by increasing membrane area but that they instead systematically modify the other parameters that determine membrane capacitance, i.e. membrane thickness and dielectric constant.

Our interpretation that the C_T measurements reflect changes in membrane area during the action of forskolin is supported by recent results of cytological experiments. An acceleration of the retrieval of fluid phase markers and fluorescent membrane markers from the apical membrane after removal of forskolin has been observed in A6 cells [9]. The concordance of evidence obtained with totally independent methods provides strong support for the conclusion that insertion of channels is the mechanism for stimulation of apical Na^+ conductance caused by forskolin. In further agreement with this proposal are observations showing that neurohypophysial hormones and insulin increase the number of amiloride-sensitive Na^+ channels in the apical membrane of tight epithelia [10, 11, 17].

Changes in G_{Na} and C_T are closely linked

The direct proportionality in the increases in G_{Na} and C_T caused by insulin and by forskolin when given in Cl^--free solutions implies that the kinetics of the changes of the two parameters are almost the same. This similarity in kinetics is remarkable because a large number of intermediate steps occur between the arrival of each agonist to its membrane receptor and the changes in the two parameters. Moreover, the signalling pathways involved in the action of each agonist are quite different since they are blocked by different types of inhibitors. Indeed, the responses to insulin involve steps that are inhibited by blockers of phosphatidylinositol 3-kinase (PI-3K) and protein synthesis [12, 13], while the effects of forskolin are blocked by interfering with production of cAMP. The finding that such different and involved regulatory sequences lead to changes in C_T and G_{Na} that are so closely correlated suggests that the changes in these two parameters are linked by a cause-effect relationship.

Increase in G_{Cl}

In contrast with the increases in G_{Na} that were closely linked to the augmentation of C_T, the relation between G_{Cl} and C_T is more complicated. Evidently, our findings do not support the notion that G_{Cl} is regulated by membrane traffic in A6 cells. Therefore, a different kind of mechanism may be involved; for example, an effect on channel conformation that leads to increased open probability. One possibility for such an effect is that stimulation of cAMP production initiates a signalling sequence that eventually leads to channel phosphorylation. However, the possibility that the increased G_{Cl} is linked to insertion of a very small population of vesicles with a high density of Cl^- channels that occurs very rapidly after the increase in cytoplasmic cAMP is not ruled out by our findings. Indeed, there is evidence from other sources compatible with this view. Thus Ling and coworkers [18] concluded that the increase in Cl^- conductance caused by agents that increase cytoplasmic cAMP in A6 cells and rabbit collecting tubules are mediated by an increase of the number of active Cl^- channels in the membrane. Along similar lines are observations on the stimulation of cystic fibrosis transmembrane regulator (CFTR) chloride conductance in *Xenopus laevis* oocytes. In this case G_{Cl} regulation is mediated by membrane insertion of transport sites, and stimulation of cAMP production causes increases of G_{Cl} and membrane capacitance that are closely linked [21].

Additive effects

Do insulin and forskolin promote insertion of membranes originating from separate intracellular pools of membranes? An answer to this question can be found in the additive effects of insulin and forskolin (in Fig. 9 and Table 1) together with a model that describes membrane trafficking. The process that controls apical membrane area, and therefore apical capacitance, may be represented, in an extremely simplified form, by the following scheme (Eq. 6):

$$M_c \underset{k_{ret}}{\overset{k_{ins}}{\rightleftarrows}} M_a \qquad (6)$$

where M_c is the area of the membrane in the cytoplasmic compartments, M_a is the amount of apical membrane, k_{ins} and k_{ret} are the rate constants for insertion and retrieval, respectively. In this model the probability of a vesicle being in the apical membrane is (Eq. 7):

$$P_{mem}=k_{ins}/(k_{ret}+k_{ins}) \qquad (7)$$

Assuming that in the steady state $k_{ins}=k_{ret}$ and that agonists modify apical membrane area M_a by acting only on either one or both rate constants k_{ins} and k_{ret} we can make specific predictions on the additivity of the effects of agonists.

According to the model, if two agonists promote membrane insertion from the same intracellular pool, maximal doses of two agonists that increase k_{ins} will not have additive effects on C_T. Therefore, the additive effects of insulin and forskolin suggest that the inserted membranes originate from separate pools. The finding of additive effects on C_T together with the model also excludes the possibility that one of the agonists stimulates membrane insertion and the other inhibits membrane retrieval. Again the model predicts in this case that if the inserted membranes originate from a single intracellular pool, the effects of maximal stimu-

lation with two agonists will not be additive. The conclusion that emerges from these considerations is that separate pools of epithelial Na^+ channels participate in the regulation of apical permeability. Evidently, this conclusion is preliminary because of the simplifications involved in selecting initial conditions and constructing the model.

A separate issue is raised by the finding that the effects of insulin and forskolin on I_{sc} and G_T are not always additive. There are several possible reasons why this might be the case. Changes in I_{sc} caused by the successive addition of two agonists may not be proportional to the number of additional channels recruited because the driving forces across the apical membrane are different in basal conditions and when transport has already been stimulated by the first agonist. Changes in conductance ought to be additive when additional channels are recruited. One possible cause for our results is that the data were collected in conditions where tissues were incubated with NaCl solutions in which forskolin activates conductances to both Na^+ and Cl^-. This activation may lead to changes in cell composition that in turn affect subsequent responses. Unfortunately, this problem could not be solved by measurements of additive effects in Cl^--free solutions because the insulin response was depressed under these conditions.

Conclusion

The close correlation between the increases in G_{Na} and C_T caused by forskolin and insulin suggests that these effects are linked by a cause-effect relationship. The most likely explanation for the changes in C_T is that they are due to changes in membrane area; therefore, the close linkage between changes in G_{Na} and C_T indicates that hormonally induced increases in apical G_{Na} are caused by the transfer of Na^+ channels, perhaps residing in two separate cytoplasmic compartments, to the surface membrane. In contrast, the relationship between changes in G_{Cl} and C_T leaves open the possibility that G_{Cl} is controlled by a mechanism that does not involve membrane traffic. A change in membrane composition caused by insertion of transporters or channels residing in cytoplasmic compartments appears to be a rather common mechanism for the effects of insulin and increased cytoplasmic cAMP on membrane function. Cytological studies and measurements of C_T indicate that the stimulations of glucose transport [6] and water permeability [3, 27] caused by these agents are also caused by a similar mechanism.

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