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Further Evidence for the Regulation of the Tight Junction Ion Selectivity by cAMP in Goldfish Intestinal Mucosa

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Summary. It has been reported that cAMP controls the transepithelial Cl^- conductance in fish intestine (Bakker, R., Groot, J.A., 1984, *Am. J. Physiol.* **246:**G213–G217; Krasny, E.J., Madara, J.L., DiBona, D.L., Frizzell, R.A., 1983, *Fed. Proc.* **42:**1100). In both studies, the cAMP effect was interpreted as an increase in tight junction Cl^- conductance, because cAMP did not change the membrane potential or membrane resistance ratio. However, the activation of a Cl^- conductance in the membranes of a subset of the epithelial cells might be difficult to discern from an increase in tight junction Cl^- conductance. Here we report experiments that were designed to distinguish a tight junction Cl^- conductance from a membrane Cl^- conductance in a subpopulation of the epithelial cells. The effect of hypotonicity on the cAMP-induced increase in transepithelial conductance showed that cAMP-induced conductance is located in series with the lateral intercellular spaces. Transepithelial serosa to mucosa direct current caused an increase in resistance due to so-called transport number effects. Forskolin abolished the transport number effects, indicating that cAMP increases the Cl^- conductance of the tight junctions. Increasing cAMP did not change mannitol fluxes, whereas Cl^- fluxes more than doubled. Changes in dilution potential and transepithelial resistance demonstrated that the cAMP-induced conductance is specific for Cl^- and Br^- as opposed to I^-, NO_3^-, SO_4^{2-} and gluconate$^-$. In contrast, cytochalasin D also decreased the transepithelial resistance and dilution potential in Nagluconate Ringer's. This demonstrates that cAMP acts on the tight junctions in a more specific manner than cytochalasin D.

Key Words chloride permeability · cyclic AMP · cytochalasin D · intestine · ion selectivity · leaky epithelia · tight junctions · transport number effects

I. Introduction

In 1972, Frömter and Diamond [15] showed that the tight junctions in a number of epithelia provide the main route for transepithelial current flow. In these leaky epithelia, the ionic permeability properties of the tight junctions primarily determine transepithelial ionic diffusion. As the tight junctions represent a direct interface between the mucosal solution and the intraepithelial compartment (the lateral intercellular spaces and the basal interstitium) where coupling of water to salt transport takes place [9], modulation of the ion permeability of the tight junctions may provide a means of regulating epithelial water transport.

Therefore, an important question is whether the tight junction permeability is controlled by the epithelial cells, and if so, what mechanism is involved in this regulation.

Changes in paracellular ion permeability due to an increase in intracellular cAMP were reported for rabbit ileum [23, 33] and *Necturus* gallbladder [10]. Similar effects on ion permeability induced by plant cytokinins [6], which alter microfilament morphology, suggest that the cytoskeleton could be involved in regulation of tight junction permeability.

However, in these reports the decrease in cation selectivity was accompanied by an increase in transepithelial resistance. Model calculations [7, 21] show that an increase in the resistance of the lateral spaces will decrease the paracellular cation selectivity. Therefore, it cannot be ruled out that the reported changes in ion permeability are due to a collapse of the lateral spaces, as in fact was shown experimentally by Ewald and Frömter [11] for *Necturus* gallbladder.

Moreover, it has been shown for a variety of epithelia including intestinal [16, 36] and gallbladder epithelium [32] that cAMP induces the opening of Cl^- channels in the apical membrane. A possible effect of cAMP on the tight junctions may thus be obscured by a dramatic effect on apical Cl^- conductance [22].

The increase in paracellular Cl^- permeability due to an increase in cellular cAMP reported for fish intestinal epithelia [4, 24, 34] provides more direct evidence for an effect on the tight junctions, since in these epithelia the transepithelial resistance *decreased* and no change in membrane potential or membrane resistance ratio occurred.

In goldfish intestinal epithelium, drugs that elevate intracellular cAMP concentration lead to (*i*) an increase in transepithelial conductance, (*ii*) an increase in both unidirectional Cl^- fluxes (the Na^+ fluxes remaining unchanged), and (*iii*) a decrease in both mucosal and serosal NaCl dilution potentials [20]. In combination with the lack of an effect on mucosal membrane Cl^- conductance, these observations suggest that the cAMP-induced Cl^- conductance is located in the tight junctions.

However, the effects on Cl^- fluxes, dilution potentials and transepithelial resistance result from the entire epithelial cell population, whereas the measured membrane potentials might only reflect the properties of the cells that are accessible to microelectrode impalements and/or have stable membrane potentials. Even though fish intestine is more homogenous in cell types than mammalian small intestine (e.g., there are no crypts in fish intestine), it cannot be completely excluded that cAMP induces the opening of Cl^- channels in the membrane(s) of a small subpopulation of the epithelial cells, which was overlooked in microelectrode experiments. In these cells, cAMP would then either open Cl^- channels in both the apical and the basolateral membrane or open Cl^- channels in the cell membrane opposing the membrane with a normally very large Cl^- conductance (*cf.* the mitochondria-rich cells in amphibian skin [13, 25] or Cl^- cells in fish operculum [12]).

We designed experiments to unequivocally assign the cAMP-induced increase in transepithelial Cl^- conductance to either the tight junctions or the cell membranes of special cells.

In section III.A, we show the effect of modulating the resistance of the lateral intercellular spaces on the cAMP-induced increase in transepithelial conductance. The results demonstrate that the cAMP-induced conductance is located in series with the lateral intercellular spaces, in agreement with a change in tight junction conductance.

In section III.B, we show that direct current passage across goldfish intestine leads to so-called transport number effects due to the (under normal conditions) cation selectivity of the tight junctions.

When cellular cAMP is increased, the transport number effects are completely abolished. This is evidence for a cAMP-induced Cl^- conductance in the tight junctions, instead of a Cl^- conductance in a novel cellular pathway, spatially separated from the tight junctions.

In section III.C, we examined the effect of cAMP on dilution potentials in Na^+ salts of various anions, and on mannitol fluxes. We show that the cAMP-induced increase in tight junction conductance is specific for Cl^- and Br^-.

In section III.D, we show experiments in which we examined the effect of cytochalasin D on transepithelial resistance, on NaCl and Nagluconate dilution potentials, and on current-induced transport number effects. The results indicate that cytochalasin D leads to a more general permeability increase than evoked by cAMP. We speculate that the action of cAMP is also mediated by the cytoskeleton, though in a more subtle way than in the case of the cytochalasins.

We have provided evidence that the epithelial cells in goldfish intestine are capable of regulating tight junction Cl^- conductance by changes in the intracellular second messenger cAMP.

II. Materials and Methods

Goldfish intestinal mucosa was stripped of its underlying muscular layers and mounted in tissue holders as described previously [19] leaving an exposed area of 0.2 cm^2. The tissue holders were clamped between two Lucite chambers with a volume of 0.5 ml, which were continuously perfused. Solutions containing 8-Br-cAMP, forskolin or cytochalasin D were recirculated, the total volume being 2 ml. Potential changes were measured using 3M KCl agar bridges connected to Ag-AgCl electrodes. Resistance was calculated from the voltage changes caused by current pulses of +10 and −10 μA of 1-sec duration. Corrections were made for the resistance of the perfusion solutions. The input resistance of the amplifiers used was higher than $10^9\ \Omega$. To study the effect of direct current passage, a constant current source was connected to the current passing electrodes used for resistance measurements. This made it possible to follow the transepithelial resistance during direct current passage.

In the flux experiments, mucosal strips mounted on tissue holders were used to separate two compartments filled with 3 ml of the desired solution. A stream of humidified 95% O_2 and 5% CO_2 was continuously passed over the solutions. The solutions were stirred by magnetic buttons. After addition of $^{36}Cl^-$ (NaCl, the Radiochemical Company) and ^{14}C-mannitol (NEN Research Products), a 45-min period was allowed to reach a steady flux. After four aliquots (0.1 ml) at 15-min intervals were drawn, theophylline (1 mM) was added to the serosal solution and aliquots were drawn for four consecutive 15-min periods. Six mucosal strips were used in one experiment, allowing for time controls in which no theophylline was added.

In some experiments, membrane potentials were measured with intracellular microelectrodes as described earlier [1].

The perfusion solutions were used at room temperature and were gassed by 95% O_2 and 5% CO_2. The composition of the control NaCl Ringer's was (in mM): NaCl 117.5, KCl 5.7, $NaHCO_3$ 25, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, and mannitol 27.8. The pH was 7.3. All chemicals used were of analar grade. In the glucose Ringer's, mannitol was replaced by glucose. As compared to the control Ringer's, the isotonic low NaCl Ringer's contained 80 mM less NaCl, which was osmotically compensated for by mannitol. The hypotonic low NaCl Ringer's was identical with the isotonic low NaCl Ringer's in ion composition, but contained no extra mannitol; the osmolarity was 170 mOsM. In the Ringer's with various monovalent anions, NaCl and KCl were replaced by the Na^+ and K^+ salt of the anion, and $CaCl_2$ was

replaced by $CaSO_4$. In the sulfate Ringer's, NaCl and KCl were replaced by half the concentration of Na_2SO_4 and K_2SO_4, while mannitol was used to attain the normal osmolarity. Dilution potentials were evoked by replacing 59 mM of the Na^+ salt of the present anion (39 mM in the case of Na_2SO_4) by 111 mM mannitol. In NMG^+ Ringer's, Na^+ was replaced by N-methyl-D-glucamine and the bicarbonate buffer by 10 mM Tris-HEPES, pH 7.3. All Ringer's, except the hypotonic Ringer's, had an osmolarity of 310 mOsM. Forskolin (Sigma Chemical) was used at a concentration of 0.01 mM, theophylline (Sigma) at 1 mM, 8-Br-cAMP (Sigma) at 1 mM, and cytochalasin D (Sigma) at 5 μg/ml in DMSO (the final DMSO concentration was 0.25%).

Table 1. Effect of cAMP (1 mM 8-Br-cAMP) on transepithelial conductance (G_{ms}) under isotonic and hypotonic conditions[a]

	Isotonic	Hypotonic
G_{ms}	29.7 ± 0.9	15.9 ± 0.7
G_{ms}, cAMP	36.1 ± 1.7	17.7 ± 0.8
n	12	6
ΔG_{ms}	+6.4 ± 0.9	+1.8 ± 0.2[b]

[a] Values in mS cm^{-2}.
[b] Significantly different from ΔG_{ms} under isotonic conditions at $P < 0.001$.

III. Results

A. Effect of Hypotonicity on the cAMP-Induced Change in Transepithelial Conductance

The transepithelial conductance equals the sum of the paracellular and the transcellular conductance. The paracellular conductance is determined by the tight junctions in series with the lateral spaces; the transcellular conductance is determined by the mucosal membrane in series with the serosal membrane.

The "leakiness" of an epithelium depends on its relative shunt conductance: the paracellular (or shunt) conductance as a percentage of the total transepithelial conductance.

As the relative shunt conductance in leaky epithelia is typically 90% or larger, the transepithelial conductance primarily reflects the paracellular conductance. An observed change in transepithelial conductance is, therefore, usually interpreted as a change in paracellular conductance. In particular, a decrease in transepithelial conductance, which exceeds the transcellular conductance (as observed in hypotonic bathing solutions, *see below*), must primarily result from a decreased paracellular conductance.

An increase in conductance, however, could be due to an increase in either paracellular or transcellular conductance. (As the transcellular conductance represents the lumped conductance of all cells, an increase in transcellular conductance may be equivalent with the addition of a novel cellular conductance.)

An obvious way to trace the origin of an experimentally evoked increase in transepithelial conductance is to examine the effect of varying one particular conductance (tight junction, lateral space, mucosal membrane, or serosal membrane) on the evoked increase in conductance.

We used this approach to gain insight in the location of the cAMP-induced increase in transepithelial conductance in goldfish intestine. Since the lateral spaces can be excluded as a site of direct action of cAMP (*see* Discussion), we examined the effect of modulating the lateral space resistance on the cAMP-induced conductance increase.

As the lateral spaces represent a resistance in series with the tight junctions, the contribution of the tight junction conductance to the paracellular and, hence, the transepithelial conductance can be influenced by varying the lateral space resistance.

We expect, therefore, that if cAMP activates a novel cellular conductance, the contribution of this conductance to the transepithelial conductance will be independent of the resistance of the lateral spaces, since a transcellular conductance by-passes the series array of the tight junction and the lateral space.

If, on the other hand, cAMP increases tight junction conductance, the increase in transepithelial conductance due to cAMP will be attenuated when the width of the lateral spaces has been reduced. It should be noted, however, that the latter conclusion is only valid when the paracellular and transcellular pathway can be viewed as distinct and separate conductive pathways, and if one assumes that the effect of cAMP on either the tight junction or the transcellular conductance does not depend on the experimental technique used to modulate lateral space width (*see* Discussion).

The most obvious ways to modulate lateral space width is by using hypotonic solutions. Moreover, from previous experiments, we learned that goldfish enterocytes respond osmometrically to hypotonicity, and that there is a linear relationship between cell volume and transepithelial resistance in goldfish intestine [18]. This indicates that the lateral spaces gradually decrease in diameter with increasing hypotonicity.

Table 1 shows the effect of cAMP on the transepithelial conductance (G_{ms}) in isotonic and hypotonic low NaCl Ringer's. The two solutions were

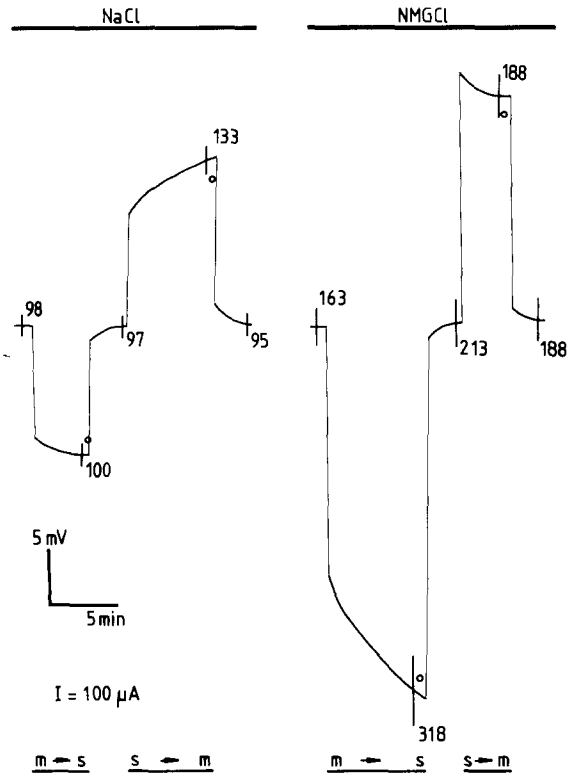

Fig. 1. Time course of the change in transepithelial potential difference (Ψ_{ms}) in stripped intestinal epithelium of the goldfish caused by a direct current of 100 μA from mucosa to serosa ($m \rightarrow s$) and serosa to mucosa ($s \rightarrow m$) in NaCl Ringer's and in a Ringer's where all Na^+ has been replaced by NMG^+. Vertical excursions in the Ψ_{ms} trace are due to current pulses of +10 and −10 μA superimposed on the direct current. Indicated values for the transepithelial resistance (in Ω) were corrected for the resistance of the solutions, which was 35 Ω in NaCl Ringer's and 75 Ω in NMGCl Ringer's. Open circles indicate the value of Ψ_{ms} expected from the ohmic *IR* drop

identical in ion composition and differed only in mannitol concentration.

In normal NaCl Ringer's, the relative shunt conductance of goldfish intestine is 95% [1]. Assuming that by lowering the NaCl of the Ringer's the decrease in paracellular conductance is at most proportional to the decrease in conductivity of the solution and that the transcellular conductance does not increase, the relative shunt conductance in low NaCl Ringer's can be estimated to be larger than 90%. Therefore, the 46% decrease in G_{ms} due to hypotonicity can only result from a decrease in paracellular conductance (i.e., a reduction in lateral space width).

The observed attenuation of the cAMP-induced increase in transepithelial conductance under hypotonic conditions shows that the effect of cAMP cannot unequivocally be assigned to a novel cellular Cl^- conductance. This is in agreement with an effect of cAMP on the tight junctions.

However, although the results are in favor of a tight junction effect, we cannot completely exclude a conductive pathway in the membranes of a subset of the epithelial cells. The experiments described below use an entirely different approach to locate the cAMP effect. Therefore, the combined results permit us to decide whether the site of action of cAMP is in the tight junctions or in special Cl^- conducting cells.

B. Transport Number Effects

Current passage through a barrier to ion diffusion will generally lead to accumulation and depletion of salt on the respective borders of that barrier. This occurs because the transport number of an ion (i.e., the current carried by that ion as a fraction of the total current) in general differs between the barrier and the aqueous solution (*see* Barry and Diamond [5] for an overview). Therefore, current passage through a compartment with boundaries differing in transport number will, depending on the direction of the current, result in accumulation or depletion of salt in the compartment. The mucosal border (the cation-selective tight junctions) and the serosal border (a free solution boundary) of the lateral intercellular space differ in transport numbers so that upon serosa to mucosa ($s \rightarrow m$) current passage the lateral spaces will be depleted and collapse, leading to an increase in transepithelial resistance. The collapse of the lateral spaces depends on the difference in transport numbers between the two boundaries, and will, therefore, depend on the cation selectivity of the tight junctions.

1. Effect of Current on Transepithelial PD *and Resistance in NaCl and NMGCl Ringer's Solution*

The effect of passing current through goldfish intestinal epithelium is shown in Fig. 1. This is a typical recording of the changes in transepithelial PD (Ψ_{ms}) and transepithelial resistance (R_{ms}) induced by the passage of a current of 100 μA from mucosa to serosa ($m \rightarrow s$) and from serosa to mucosa ($s \rightarrow m$), respectively. The recording shows a progressive increase in ψ_{ms} due to $s \rightarrow m$ current. This was mainly due to an increase in R_{ms}, although the change in Ψ_{ms} exceeded the change expected from the voltage drop across R_{ms} (further denoted as "*IR* drop"). This difference represents the polarization potential, which reflects the depletion of NaCl in the lateral spaces. In fact, this is the driving force for wa-

ter movement out of the lateral spaces, which leads to the collapse and the rise in R_{ms}.

Mucosa to serosa current did not change R_{ms}. The deviation of the change in Ψ_{ms} from the expected *IR* drop may again be accounted for by a polarization potential, now of the opposite polarity as due to $s \rightarrow m$ current.

These results agree with the effects expected from the difference in transport numbers between the cation-selective tight junctions and the free solution.

To confirm that the observed effects depend on the ion selectivity of the tight junctions, experiments were performed in which Na^+ was replaced by the less permeable cation N-methyl-D-glucamine. From dilution potentials across the stripped epithelium (7.8 ± 0.5 mV; $n = 6$) and across a free solution boundary (5.9 mV; $n = 2$), we deduced that the transport number of Cl^- is larger than that of NMG^+ in the tight junctions, and that the Cl^- transport number in the tight junctions, when bathed in NMGCl Ringer's, is larger than in free solution. So in NMGCl Ringer's, the tight junctions are in fact *an*ionselective, and as shown in Fig. 1, the effects of passing current are reversed. Now, a secondary voltage change due to an increase in transepithelial resistance with time resulted from a *mucosa to serosa* current, whereas no change in resistance was observed upon $s \rightarrow m$ current.

In NaCl Ringer's, a $s \rightarrow m$ current of 100 μA increased R_{ms} in 5 min from 19 ± 1 to 30 ± 2 Ωcm^2 ($n = 5$). In NMGCl Ringer's, a 100 μA $m \rightarrow s$ current increased the resistance in 5 min from 44 ± 1 to 58 ± 3 Ωcm^2 ($n = 5$). (The average resistance increase is given for a period of 5 min of current flow, because the resistance continued to increase for prolonged periods of time, and we did not wait for the resistance to attain a plateau value.)

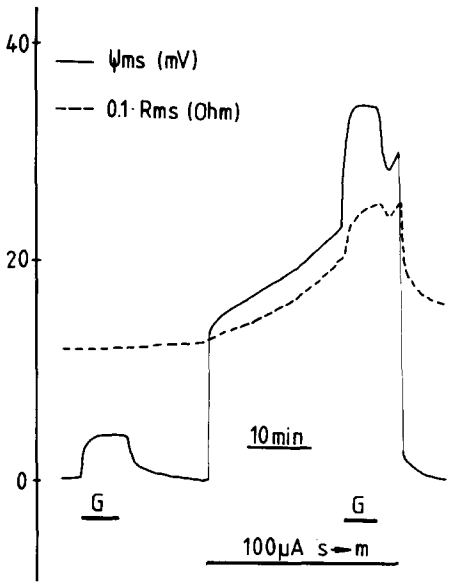

Fig. 2. Redrawing of an original recording of the transepithelial PD (Ψ_{ms}) and resistance (R_{ms}) in an experiment in which the effect of mucosal glucose (indicated by *G*) was measured under control conditions and during a serosa to mucosa direct current (100 μA $s \rightarrow m$). R_{ms} was determined every 30 sec by +10 and −10 μA current pulses of 1-sec duration, and was corrected for the resistance of the solutions (45Ω). As R_{ms} is plotted at a 10 times compressed scale, and the current was 0.1 mA, the trace also represents the contribution of the *IR* drop (in mV) to Ψ_{ms} during direct current flow

2. Effect of $s \rightarrow m$ Current on the Glucose-Evoked Potential

Similar effects have been reported for frog gallbladder by Bindslev, Tormey and Wright [8]. There is little doubt that in the flat gallbladder epithelium the increase in R_{ms} induced by $s \rightarrow m$ current is caused by a collapse of the lateral spaces. However, the increase in R_{ms} in goldfish intestine could, due to the folded structure, partially result from a collapse of the subepithelial tissue. To answer this question, we studied the effect of mucosal glucose during $s \rightarrow m$ current.

Figure 2 shows the effect of switching the mucosal perfusate to a glucose-containing solution on Ψ_{ms} and R_{ms} before and during current flow from serosa to mucosa. During $s \rightarrow m$ current, an increased glucose-evoked potential (GEP) was observed. Under open circuit conditions, glucose induces an intraepithelial current. The effect of glucose on Ψ_{ms} can be equated with the voltage drop across the paracellular resistance caused by this current. The effect of glucose on the intracellular PD was equal under control and $s \rightarrow m$ current conditions (*not shown*), indicating that no considerable change in glucose-evoked current occurred. Apparently, the increased GEP during $s \rightarrow m$ current is caused by an increase in the resistance of the paracellular pathway. A collapse of the subepithelial tissue in the folds would represent an increase in a resistance in series with the epithelium. Such an increase could not have led to a larger GEP.

Moreover, in contrast with control conditions, glucose also enhances the resistance increase. This could be due to the contribution of the glucose-induced current to the $s \rightarrow m$ current and/or to cell swelling, which would now increase R_{lis}, since the lateral spaces are already partially collapsed.

Therefore, both the enhanced GEP and the additional increase in R_{ms} during $s \rightarrow m$ current leaves

Table 2. Mean values ± SEM of four experiments for transepithelial resistance R_{ms}, the glucose-evoked potential change (GEP), and for the glucose-evoked change in R_{ms} ($\Delta R_{ms,G}$) under control conditions and during 100 μA $s \rightarrow m$ current

	Control	100 μA $s \rightarrow m$	Ratio
R_{ms} (Ωcm^2)	25 ± 2	71 ± 18	2.7 ± 0.5[a]
GEP (mV)	2.6 ± 0.5	4.5 ± 1.3	1.6 ± 0.2[a]
$\Delta R_{ms,G}$ (%)	0 ± 2	+8 ± 1[b]	

[a] Significantly different from 1 at $P < 0.02$.
[b] Significantly different from 0 at $P < 0.02$.

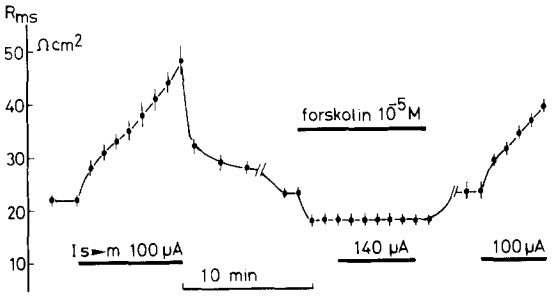

Fig. 3. Time course of the change in transepithelial resistance (R_{ms}) induced by serosa to mucosa ($s \rightarrow m$) current in the absence and presence of forskolin. The curves represent the mean ± SEM of five experiments. In the presence of forskolin, the current was increased to 140 μA so that the *IR* drop equaled the initial *IR* drop in the absence of forskolin. The length of the time gaps in the recording is about 10 min

no doubt that the current-induced resistance increase is caused by a collapse of the lateral spaces.

The average of four experiments in which the effect of glucose is compared under control and $s \rightarrow m$ current conditions is given in Table 2.

3. cAMP Abolishes Transport Number Effects

If cAMP increases the Cl^- conductance of the tight junctions, one would expect a reduction of the current-induced transport number effects. If, on the other hand, cAMP induces a Cl^- conductance in special cells spatially separated from the Na^+ conductive tight junctions, one would expect that the transport number effects would still occur, provided that the transepithelial current is increased to compensate for the extra conductance through the presumed special cells.

Figure 3 shows the effect of $s \rightarrow m$ current on R_{ms} in the absence and presence of forskolin (a direct activator of adenylate cyclase). In the presence of forskolin, a $s \rightarrow m$ current of 140 μA had no effect at all on R_{ms}. The current was increased to 140 μA to evoke an *IR* drop across the epithelium equal to the initial *IR* drop (due to 100 μA) under control conditions, thereby assuring that in the presence of forskolin the current through the tight junctions is at least as large as under control conditions. Further increasing the current to 200 μA (n = 5) or 350 μA (n = 2) had no effect on R_{ms}. After forskolin had been washed away, $s \rightarrow m$ current did induce an increase in R_{ms} showing that the cAMP effect is fully reversible. In the presence of forskolin, both $s \rightarrow m$ and $m \rightarrow s$ current caused changes in Ψ_{ms}, which remained constant during current passage and equaled the *IR* drop.

Apparently, an increase in cellular cAMP can completely, but reversibly abolish the transport number effects. It may, therefore, be concluded that cAMP affects the cation selectivity of the tight junctions.

C. Selectivity of the cAMP-Induced Conductance

Table 3 shows the effect of a rise in intracellular cAMP (induced by 1 mM theophylline) on the paracellular conductance and the dilution potential in Ringer's solutions containing Na^+ salts of various anions. The paracellular conductance was determined from the measured transepithelial resistance after correction for a subepithelial series resistance of 24% of the control value [1], and by assuming a 95% relative shunt conductance under control conditions [1]. From the dilution potentials, the transport numbers for the various anions were calculated using the Henderson equation. From these values, it can be deduced that cAMP changes the selectivity sequence of the tight junctions from $SO_4^{2-} = NO_3^- > Cl^- = Br^- = I^- = Gluconate^-$ to $Cl^- = Br^- > SO_4^{2-} = NO_3^- > I^- = Gluconate^-$.

From the change in selectivity sequence and the observed increase in paracellular conductance, it may be concluded that cAMP specifically increases the conductance for Cl^- and Br^-.

Double-label experiments in which $^{36}Cl^-$ and ^{14}C-mannitol $s \rightarrow m$ fluxes were measured showed that the mannitol flux remained unchanged in the presence of theophylline, while the Cl^- flux increased threefold (Table 4). Also, no correlation between the mannitol and Cl^- fluxes could be detected, either under control conditions or in the presence of cAMP.

D. Effects of Cytochalasin D

Similar to the effect of cAMP, cytochalasin D (5 μg/ml) decreased the transepithelial resistance to 74 ±

Table 3. Effect of 1 mM theophylline ("theo") on paracellular conductance and dilution potential in Ringer's containing Na salts of various anions[a]

Anion	Paracellular conductance (mS cm^{-2})			n	Dilution potential (mV)		Anionic transport number	
	−theo	+theo	Δ		−theo	+theo	−theo	+theo
Cl^-	43 ± 3	57 ± 5	14 ± 1[b]	(11)	−8.4 ± 1.2	−1.1 ± 0.2	0.15	0.42[c]
Br^-	34 ± 4	40 ± 5	6 ± 1[b]	(4)	−7.5 ± 0.5	−0.7 ± 0.1	0.18	0.42[c]
I^-	37 ± 4	38 ± 5	0 ± 1	(5)	−7.7 ± 0.4	−7.6 ± 0.5	0.17	0.17
NO_3^-	39 ± 2	45 ± 2	5 ± 1[b]	(8)	−4.7 ± 0.4	−3.9 ± 0.4	0.28	0.31
SO_4^{2-}	42 ± 3	44 ± 3	3 ± 1	(6)	−6.2 ± 0.3	−6.1 ± 0.3	0.31	0.32
Gluconate$^-$	37 ± 2	40 ± 3	3 ± 1	(5)	−8.3 ± 0.4	−8.2 ± 0.4	0.15	0.15

[a] The paracellular conductance was derived from the measured transepithelial resistance as indicated in the text. The dilution potentials were corrected for the change in liquid junction potential across the 3M KCl agar bridges. The transport number of the tight junctions for the various anions was calculated from the dilution potential using Henderson's equation. Only Na^+, K^+ and Cl^- or the substituting anion were taken into account. Including HCO_3^- (with a transport number equal to that in the Ringer's) in the calculations did not change the selectivity sequence under either control or cAMP conditions. The ionic mobilities needed for the calculations were taken from Robinson and Stokes [35], and for gluconate ($u_{gluconate} = 2.88 \times 10^{-4}$ cm^2 sec^{-1} V^{-1}) from Zuidema, Dekker and Siegenbeek van Heukelom [37]. 8-Br-cAMP (1 mM) instead of theophylline was used in the iodide experiments.
[b] Significantly different from 0 at $P < 0.01$.
[c] Significantly different from control at $P < 0.01$.

2% of the control value ($n = 7$) and the dilution potential to 22 ± 1% ($n = 7$). Cytochalasin D (and in earlier experiments cytochalasin B (10 μg/ml)) also abolished current-induced transport number effects.

However, whereas cAMP acts instantaneously (<1 min), the time required to attain a maximal effect of cytochalasin D was 10 to 30 min. Furthermore, the action of cytochalasin D appeared to be irreversible.

In contrast with the effect of increasing intracellular cAMP (*see* Table 3), the effect of cytochalasin D in NaGluconate Ringer's was a reduction of the transepithelial resistance to 73 ± 2% ($n = 6$) and of the dilution potential to 22 ± 1% ($n = 7$). This indicates that cytochalasin D induces a more general increase in permeability than cAMP.

Table 4. Effect of 1 mM theophylline on serosa to mucosa $^{36}Cl^-$($J_{Cl,sm}$) and ^{14}C-mannitol ($J_{mannitol,sm}$) fluxes[a]

	Control	Theophylline (1 mM)	P
$J_{Cl,sm}$	3.0 ± 0.5	8.6 ± 1.5	<0.01
$J_{mannitol,sm}$	0.36 ± 0.10	0.37 ± 0.07	NS

[a] Values (in μmol cm^{-2} hr^{-1}) represent the mean ± SEM of 10 experiments. Least squares analysis of the data showed no correlation between Cl^- and mannitol fluxes ($R = 0.14$).

IV. Discussion

The present paper reports the results of our studies on the localization of the cAMP-induced Cl^- conductance in goldfish intestinal epithelium.

First we will discuss the interpretation of the change in transepithelial conductance due to cAMP under isotonic and hypotonic conditions. Secondly, we will consider the effects evoked by transepithelial direct current flow and the influence of cAMP on these effects. Finally, we will address the selectivity of the cAMP-induced conductance, the possible underlying mechanisms and the physiological significance of the modulation of tight junction permeability.

A. The cAMP-Induced Conductance is Located in Series with the Lateral Spaces

The lateral spaces can be excluded as a site of action of cAMP, because the resistance of the lateral spaces under control conditions is too small to account for the observed decrease in transepithelial resistance [4]. A simple calculation based upon morphological and electrophysiological data shows that, provided that the diameter of the lateral spaces is larger than 0.05 μm, the resistance of the lateral spaces is maximal 25% of the tight junction resistance [3]. Experimental evidence for a negligible contribution of the lateral spaces to the paracellular resistance is provided by the observation that $m \rightarrow s$ direct current, which will distend the lateral spaces, does not lead to a decrease in R_{ms} (*see* section

III.B). Also, the cAMP-induced increase in conductance in combination with the loss of cation selectivity [4] is incompatible with an effect of cAMP on lateral space width.

As the increased R_{ms} in isotonic low NaCl Ringer's as compared to normal Ringer's matches the value expected from the increased resistivity of the low NaCl Ringer's, one may assume that the lateral space resistance remains negligible in the low NaCl Ringer's, which is used in the experiments described in section III.A.

The hypothesis that cAMP acts on the tight junctions could have been rejected straight away if the observed increase in transepithelial conductance due to cAMP had been comparable under isotonic and hypotonic conditions. Although the observation that hypotonicity attenuates the cAMP-induced increase in transepithelial conductance is in favor of this hypothesis, a definitive validation of the hypothesis also depends on the assumption that the cAMP effect *per se* is comparable under isotonic and hypotonic conditions. Unfortunately, we cannot exclude that hypotonicity prematurely opens the conductive pathway, which is normally activated by cAMP. Studying dilution potentials in isotonic and hypotonic Ringer's would not give a clue, because the increase in lateral space resistance will in itself reduce the transepithelial cation selectivity [7, 21]. However, the linear relation between transepithelial resistance and increasing hypotonicity [18] does not suggest a sudden anomalous conductance increase.

Moreover, an enclosure of special cells by the epithelial cells or effects of cable-like properties of the lateral spaces adjacent to these presumed special cells may add uncertainty to the conclusions.

B. Transport Number Effects in Goldfish Intestine Are Suppressed by cAMP

In leaky epithelia, the paracellular pathway conducts the greater part of transepithelial current. Therefore, the lateral spaces will be subject to transport number effects. The effects of direct current passage through a leaky epithelium with cation-selective tight junctions are well described for *Necturus* gallbladder [14] and frog gallbladder [8]. The changes in transepithelial PD caused by $s \rightarrow m$ current in these epithelia showed three distinct transients: the first transient with a half time in the range of milliseconds is related to the electrical capacity of the epithelium (due to the slow time scale not visible in Figs. 1 and 2), the second transient, with a half time in the range of seconds to minutes is the polarization potential due to the transport number effect. The polarization potential reflects respectively the accumulation and depletion of salt on the mucosal and lateral space sides of the tight junctions. The depletion of salt in the lateral spaces is the driving force for water movement from the lateral spaces. The movement of fluid from the lateral spaces will lead to a collapse and increase in resistance of the lateral spaces, which causes the third transient in transepithelial PD.

Indeed, it has been shown with morphological techniques [8, 14] that the resistance increase is associated with a collapse of the lateral spaces. Recently, electrophysiological evidence has been presented that in *Necturus* gallbladder during $s \rightarrow m$ current the resistance of the lateral spaces increases depending on the density and the duration of the current [26].

NaCl dilution potentials show that the tight junctions in goldfish intestinal epithelium are cation selective [4]. As expected, $s \rightarrow m$ direct current flow through goldfish intestine led to an increase in transepithelial resistance. The polarization potential is reflected by the difference between the calculated *IR* drop and the observed change in Ψ_{ms} (*see* Fig. 1).

After replacing Na^+ by NMG^+, the direction of the current had to be reversed ($m \rightarrow s$) to induce a resistance increase. This is in agreement with the observation that the transport number of NMG^+ is smaller in the tight junctions than in free solution, which in fact means that the tight junctions are anion selective in NMGCl Ringer's. Bindslev et al. [8] showed that the effect of current on transepithelial PD and resistance are reversed after La^{3+} treatment, which renders the junctions anion selective. In goldfish intestine, La^{3+} has nonspecific and irreversible effects (e.g., decrease in GEP) suggesting that La^{3+} is toxic to this tissue.

Serosa to mucosa current flow across an epithelium with cation-selective tight junctions will lead to salt deprivation and subsequent collapse of the lateral spaces. In goldfish intestine, the correlation of the GEP and R_{ms} during $s \rightarrow m$ current demonstrates that the increase in R_{ms} is indeed caused by a collapse of the lateral spaces. The smaller relative increase in GEP as compared to the relative increase in resistance (shown in Table 2) might be explained by the fact that a collapsed lateral space should be considered as a distributed resistance. The glucose-induced current, therefore, returns through this distributed resistance, with an equivalent value considerably smaller than the increase in resistance determined from transepithelial current pulses.

It should be noted that in contrast to rat and hamster intestine, where glucose can alter tight

junction permeability [30], in goldfish intestine glucose does not alter R_{ms} [1] or mannitol fluxes (*see* section IV.D).

Our results show that in goldfish intestine, transport number effects are absent when intracellular cAMP is increased by forskolin. In the presence of forskolin, even a current of 350 μA does not induce transport number effects, indicating that in the tight junctions the transport number for Cl^- has increased considerably. This is in agreement with the observed reduction in NaCl dilution potential due to 8-Br-cAMP [4], from which an increase in transport number for Cl^- from 0.12 to 0.46 can be derived.

C. Specificity of the cAMP-Induced Tight Junction Conductance

We have used theophylline as a drug to increase the cellular cAMP concentration. Theophylline may increase both cAMP and cGMP, but because 8-Br-cGMP has no effect on transepithelial resistance or Cl^- fluxes [17], we can assign the effect of theophylline to cAMP. The effect of cAMP on exclusively the transport numbers for Cl^- and Br^-, combined with the increase in both unidirectional Cl^- fluxes [4] and the lack of an effect on Na^+ [4] and mannitol fluxes demonstrate that cAMP specifically increases the tight junction conductance for the small anions Cl^- and Br^-.

D. Possible Mechanism of Regulation of Tight Junction Permeability

In 1980, Bentzel et al. [6] suggested regulation of tight junction permeability by the cytoskeleton. The authors reported effects of plant cytokinins and two other cytoskeleton-active drugs cytochalasin B and phalloidin on tight junction ion permeability and structure in *Necturus* gallbladder. Duffey et al. [10] reported similar effects due to cAMP, and suggested that cytosolic cAMP may act on cytoskeletal elements to regulate tight junction permeability.

This hypothesis is corroborated by the finding that exposure of many cell types to cAMP results in an alteration of the actin component of the cytoskeleton [29].

Our experiments with goldfish intestine show that the effect of cAMP is more specific than the effect of cytochalasin. Although, like cAMP, cytochalasin D increases transepithelial conductance, reduces NaCl dilution potentials and abolishes transport number effects, these effects show a much slower onset, are not reversible and are not specific for Cl^-. A direct comparison between the effect of cAMP and cytochalasin in other intestinal epithelia is not available. Recently, Madara, Barenburg and Carlson [27] have reported effects of cytochalasin D on tight junction structure and function in the guinea pig ileum. They observed an increase in transepithelial conductance and serosa to mucosa Na^+ and mannitol fluxes concomitant with a decrease in cation selectivity. They do not present data on Cl^- fluxes. However, the increase in transepithelial conductance in conjunction with the decrease in cation selectivity requires an increase in Cl^- conductance, which is even larger than the increase in Na^+ conductance. If it is assumed that cytochalasin D has no effect on the Cl^- conductance of the apical membrane, then it must be concluded that the paracellular Cl^- conductance is increased, suggesting that goldfish and guinea pig enterocytes react to cytochalasin D in a similar way.

Recent work of Pappenheimer, Reiss and Madara [28, 30, 31] has drawn attention to cytoskeleton-mediated regulation of the tight junction permeability for charged and uncharged solutes by activation of Na^+-substrate cotransport.

This mechanism could be responsible for the self-activation of absorption of sugars and amino acids through the paracellular pathway by solvent drag in rat and hamster intestine. However, measurements in both perfused isolated segments (similar to the technique used by Pappenheimer) and stripped sheets of goldfish intestine show no effect of Na^+-substrate cotransport on transepithelial resistance [1, 2] and mannitol fluxes (*unpublished observations*). Although Pappenheimer [30] suggests that insufficient oxygenation might be the reason that changes in transepithelial resistance due to Na^+-substrate cotransport have not been observed earlier, insufficient oxygenation in our preparation is highly unlikely, since the preparation remained viable (as judged from the GEP) for periods up to 6 hr, and the experiments were performed at room temperature (assuring a 1.3-fold higher O_2 content of the perfusate). The comparison between fish and rat or hamster intestine shows that modulation of tight junction permeability by activation of Na^+-substrate cotransport is not a intrinsic property of the cotransport system. This suggests that a secondary mechanism, probably mediated by intracellular messengers, is involved in this effect in the two rodents, which is not present in the goldfish. As far as we know, no ultrastructural studies of the effect of cAMP or other intracellular messengers have been made in mammalian or fish intestine. Such studies may give further information about the mechanism by which the enterocytes regulate the permeability properties of the tight junctions and

about the role of the cytoskeleton in this regulation. Because the tight junctions constitute part of the main diffusional pathway between the lumen and the interstitial space, the regulation of the properties of this barrier is important for the modulation of transepithelial salt and water transport and paracellular solvent drag.

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