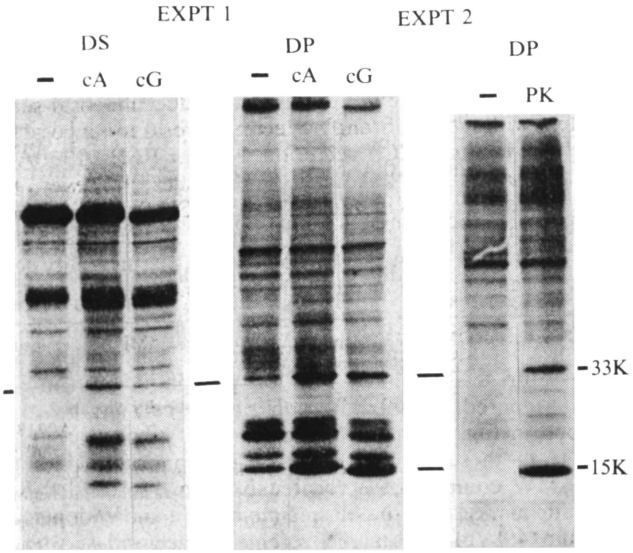

Fig. 3 The effect of cyclic AMP, cyclic GMP and the catalytic subunit of the cyclic AMP-dependent protein kinase on phosphorylation of membrane and cytoplasmic proteins. Membrane vesicles (DP) and cytoplasmic fraction (DS) were prepared as described in Fig. 3. Before phosphorylation the membrane vesicles shown in experiment 2 were lysed in 10 mM HEPES *p*H 7.3, pelleted and resuspended. This procedure removes from the membrane residual cytoplasmic proteins and loosely associated peripheral proteins. Samples were incubated (after a 2 min incubation at 37 °C) for 1 min (expt 1) or 1.5 min (expt 2) at 37 °C in 10 mM (expt 1) or 5 mM (expt 2) $MgCl_2$, 10 μM [γ-^{32}P]ATP and 10 mM HEPES *p*H 7.3. The phosphorylation reaction was stopped by adding an equal volume of gel buffer (4% w/v) SDS, 20% glycerol, 4% (v/v β-mercaptoethanol, 118 mM Tris-PO_4 *p*H 6.9, 0.008% bromophenol blue). The samples were heated at 98 °C for 2 min then frozen before analysis by SDS gel electrophoresis. Autoradiograms of SDS gels of phosphorylated DP and DS are shown. Control channels are indicated (–). Additions present during phosphorylation were 10^{-6} M cyclic AMP (cA), 10^{-3} M cyclic GMP (cG), 30 nanomolar units per ml of catalytic subunit of the cyclic AMP-dependent protein kinase (PK). [Where 1 nmolar unit is the amount of enzyme which catalyses the transfer of 1 nmol of ^{32}P label from ATP to 1 mg of protamine sulphate per min.] The catalytic subunit of the cyclic AMP-dependent protein kinase was prepared from bovine lung according to the method of Rosen and Erlichman[23] and assayed according to the method of Walsh *et al.*[24].

ribonuclease reduces metabolically incorporated, trichloroacetic acid-precipitable ^{3}H-uridine by 96%.

The 33,000-MW protein can be phosphorylated *in vitro* by endogenous kinases in isolated cell fractions (DP and DS). The phosphorylation of this component is dependent on the addition of either cyclic AMP or cyclic GMP (Fig. 3). Higher concentrations of cyclic GMP (10^{-3} M) than cyclic AMP (10^{-6} M) are required for maximal phosphorylation of this 33,000-MW protein. The 33,000-MW protein, phosphorylated in the presence of cyclic AMP *in vitro* (Fig. 3), comigrates on SDS gel electrophoresis with the 33,000-MW protein, the phosphorylation of which is increased after addition of FGF to ^{32}P-labelled cells. The 33,000-MW protein is also phosphorylated when the membranes are incubated with a purified cyclic AMP-dependent protein kinase or its catalytic subunit (Fig. 3). The phosphorylation of a protein with an apparent MW of 15,000 is also stimulated *in vitro* by cyclic nucleotides and is increased by the cyclic AMP-dependent protein kinase or its catalytic subunit (Fig. 3). The phosphorylation of this protein is low in ^{32}P-labelled cells and is not increased after the addition of FGF or serum to intact cells (Figs 1 and 2). Because the addition of FGF to intact, ^{32}P-labelled 3T3 cells results in a specific increase in phosphorylation of the 33,000-MW protein mainly in the fraction enriched in plasma membrane, and phosphorylation of both the 33,000-MW protein and a 15,000-MW protein can be increased in all fractions made accessible to cyclic AMP or protein kinase by cell breakage, it appears that, in the intact cell, local regulation of phosphorylation in response to FGF occurs at the membrane.

In summary, the addition of serum or a highly-purified fibroblast growth factor to intact, quiescent 3T3 cells causes a rapid increase in phosphorylation of membrane proteins, with the most dramatic change in phosphorylation of a protein of MW 33,000. Studies with broken-cell preparations show that the phosphorylation of the 33,000-MW protein is stimulated by cyclic nucleotides, particularly by cyclic AMP.

Possible mechanisms for the increased phosphorylation of the 33,000-MW protein are: (1) an increase in the local concentration of cyclic GMP[12,13] or cyclic AMP[14]; (2) a direct stimulation of a membrane-associated protein kinase by the mitogen, as demonstrated by Carpenter *et al.*[15]; they find that epidermal growth factor increases phosphorylation of membrane preparations from human epidermoid carcinoma A-431 cells; (3) translocation of a protein kinase from one cellular compartment to another[16–18]; (4) inhibition of a specific phosphatase; or (5) exposure of phosphorylatable sites on the 33,000-MW protein substrate as a result of rearrangement of the protein in the membrane.

The rapidity of the increase in phosphorylation of the 33,000-MW protein after addition of mitogens raises the possibility that phosphorylation of specific membrane proteins may be the initial metabolic event which occurs when a mitogen stimulates quiescent 3T3 cells to synthesise DNA.

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Role of Ca^{2+}-dependent regulator protein in intestinal secretion

AFTER exposure to secretagogues the small intestine changes from a tissue that absorbs fluid and electrolyte from lumen to blood into a tissue that secretes electrolyte and fluid into the lumen[1–4]. It has been shown that this secretion results from an increase in the passive Cl^- permeability of the mucosal border, which permits NaCl to leak passively from the lateral intercellular spaces, where it is present at hypertonic concentrations[5], into the mucosal bathing solution. Na^+ and water, electro-

Table 1 Fluxes across sheets of rabbit ileum stripped of serosa and external muscle layers

	$J_{ms}^{Cl^-}$ (μmol cm^{-2} h^{-1})				
	Control	Choleragen (2 μg ml^{-1})	Theophylline (10 mM)	A23187 (2 μg ml^{-1})	A23187 (2 μg ml^{-1}) + Theophylline (10 mM)
No addition	11.93 ± 0.60 (45)	8.64* ± 0.60 (11)	7.03† ± 0.34 (14)	7.12† ± 0.37 (26)	7.86† ± 0.34 (12)
Stelazine (0.1 mM)	11.27 ± 1.26 (6)	13.13‡ ± 0.20 (7)	11.14‡ ± 1.15 (4)	13.88‡ ± 0.60 (5)	12.00‡ ± 0.82 (7)
RMI 12330A (0.1 mM)	11.96 ± 0.43 (17)	11.45‡ ± 0.54 (9)	11.65‡ ± 0.84 (6)	12.22‡ ± 0.40 (25)	11.02‡ ± 0.45 (5)

The exposed area was 1.76 cm^2 (ref. 10). Bathing solutions contained in addition to secretagogues, mM: 140 NaCl, $KHCO_3$, 2.4 K_2HPO_4, 0.4 KH_2PO4, 1.2 $CaCl_2$ and 1.2 $MgCl_2$. Solutions were maintained at 37 °C and gassed with 5 % CO_2: 95 % O_2. ^{36}Cl was added to mucosal solution, to measure m–s flux, or serosal solution for s–m flux; the cold sides were sampled at 30-min intervals for 1.5 h. All secretagogues were present at equal concentrations on both sides of the tissue. The choleragen was supplied by Schwarz-Mann. No significant effects of treatment on $J_{sm}^{Cl^-}$ were observed. The pooled mean of fluxes measured in all conditions gave $J_{sm}^{Cl^-} = 7.88 \pm 0.16(60)$ μmol cm^{-2} h^{-2}. Values are mean ± s.e.m.

* $P < 0.005$; test compared with control (colurnn 1).

† $P < 0.001$; test compared with control.

‡ $P < 0.001$; test compared with treated without modifier.

osmotically coupled to Na^+ movement, leak through the tight junctions[1,2], and Cl^- leaks through relatively anhydrous anion-selective channels, induced within the mucosal border by secretagogues. The increased reflux of NaCl from the lateral intercellular space accounts for both the apparent decrease in electroneutral NaCl uptake across the mucosal border induced by secretagogues and the apparent increase in active Cl^- secretion and short-circuit current[3,6,7]. We have investigated the mechanism by which intestinal secretagogues increase passive Cl^- permeability and thereby cause secretion. Cl^- permeability is increased by several secretagogues, some of which, such as theophylline and choleragen, increase intracellular cyclic AMP concentration, and others, such as A23187, the Ca^{2+} ionophore, or carbachol, do not[8]. Thus there has been no known common mode of secretory induction. To investigate this problem we used two drugs that prevent intestinal secretion *in vitro*, RMI 12330A (Richardson Merrell), and the antipsychotic phenothiazine trifluoperazine (Stelazine, Smith, Kline and French). RMI 12330A prevents secretion by inhibiting choleragen-induced adenylyl cyclase activity[9]. Stelazine inhibits phosphodiesterase in tissues[11,12] by preventing the activation of the enzyme by Ca^{2+}-dependent regulator protein, CDR. We report here that it also inhibits Cl^- secretion and binds to CDR.

Table 2 Passive Cl^- flux across sheets of rabbit ileum with serosa and muscle layers removed

	J_{ms}^{Cl} (μmol cm^{-2} h^{-1}) ± s.e.m.	
	Control	Theophylline (10 mM)
No addition	10.65 ± 0.34 (56)	14.00* ± 0.55 (32)
Stelazine (0.1 mM)	11.65 ± 0.78 (3)	11.81† ± 0.47 (8)
RMI 12330A (0.1 mM)	10.65 ± 0.41 (30)	10.20† ± 0.60 (24)

The exposed area was 1.76 cm^2. The mucosal bathing solution contained mM: 200 NaCl, 10 $KHCO_3$, 2.4 K_2HPO_4, 0.4 KH_2PO_4, 1.2 $CaCl_2$ and 1.2 $MgCl_2$. The serosal solution contained: 280 mannitol, 10 $KHCO_3$. 2.4 K_2HPO_4, 0.4 KH_2PO_4, 1.2 $CaCl_2$ and 1.2 $MgCl_2$. Both mucosal and serosal solutions contained 0.1 mM ouabain to inhibit active transport. The solutions were maintained at 37 °C and gassed with 5 % CO_2: 95 % O_2. ^{36}Cl was added to the mucosal solution and the serosal side was sampled at 30-min intervals for 1.5 h. All other reagents were present at equal concentrations in both bathing solutions.

* $P < 0.001$; test compared with control.

† $P < 0.001$; test compared with theophylline.

Table 1 shows that theophylline (10 mM), choleragen (1 μg ml^{-1}) and A23187 (2 μg ml^{-1}) all reduce mucosal-serosal Cl^- flux, without significantly affecting serosal to mucosal flux, thereby inducing net Cl^- secretion. Neither RMI 12330A nor Stelazine affect Cl^- flux in control tissues; however, both prevent the decreases in mucosal-serosal Cl^- fluxes induced by the secretagogues. Table 2 shows that Stelazine and RMI 12330A prevent the increase in passive Cl^- permeability across the mucosal border induced by theophylline.

Table 3 Effect of RMI 12330A and Stelazine on cyclic AMP after exposure to secretagogues

	cyclic AMP (pmol per mg tissue dry weight)				
	Control	Choleragen (2 μg ml^{-1})	Theophylline (10 mM)	A23187 (2 μg ml^{-1})	A23187 (2 μg ml^{-1}) + Theophylline (10 mM)
No addition	9.50 ± 0.57 (17)	19.00* ± 0.88 (7)	44.60* ± 2.95 (9)	9.67 ± 0.33 (19)	70.00* ± 5.35 (8)
Stelazine (0.1 mM)	9.75 ± 0.34 (6)	20.26* ± 1.24 (4)	51.21* ± 5.10 (9)	13.00*‡ ± 0.27 (13)	67.44* ± 6.60 (3)
RMI 12330A (0.1 mM)	7.70 ± 0.84 (12)	10.48† ± 0.94 (6)	9.30† ± 0.61 (12)	7.80† ± 0.30 (5)	41.36*† ± 3.20 (4)

Tissues were incubated for 30 min before extraction, except where exposed to choleragen with or without modifiers, when they were incubated for 60 min. Approximately 0.1-g pieces of tissue were homogenised in a glass homogeniser with a Teflon pestle in 2 ml of ice-cold 4 mM EDTA, *p*H 7.4. The homogenates were then boiled for 2 min and the cyclic AMP of aqueous extract was measured using a cyclic assay kit (Radiochemical Center, Amersham). Values are mean ± s.e.m.

* $P < 0.001$; test compared with control (column 1).

† $P < 0.001$; test compared with treated without modifier (row 1).

‡ $P < 0.005$; test compared with treated without modifier.

Table 3 shows the effect of RMI 12330A and Stelazine on the induced changes in intracellular cyclic AMP after exposure to various secretagogues. Theophylline and choleragen increased tissue levels of cyclic AMP; A23187 had no effect on them. With theophylline present, A23187 increased cyclic AMP levels above those found with theophylline alone, suggesting that raised intracellular Ca^{2+} increases both adenylyl cyclase and phosphodiesterase activity. RMI 12330A prevented the increase in tissue levels of cyclic AMP seen with theophylline and choleragen. This effect is consistent with its reported effects as an inhibitor of adenylyl cyclase activity[9]. Stelazine had no effect on tissue levels of cyclic AMP in controls, or in conditions where they were already high due to the presence of theophylline or choleragen.

These results indicate that Cl^- secretion is independent of tissue levels of cyclic AMP because secretion was observed either with high levels of cylic AMP, in the presence of theophylline or choleragen, or with low levels of cyclic AMP, in the presence of A23187. And this secretion was in all conditions inhibited by Stelazine, which did not affect intracellular cyclic AMP levels. However, our results do indicate that adenylyl cyclase is activated whenever secretion is observed.

A single event leading to activation of secretion is probable. A likely common event in all stimulating modes is a rise in free Ca^{2+} within the tissue. With A23187 this can be assumed from its well known action in promoting increased membrane permeability to Ca^{2+} (ref. 10). However, as the small intestine has a very active Ca^{2+} pump, it is hard to demonstrate increased uptake even of labelled Ca^{2+} with this agent, and very hard to show directly any change in intracellular free Ca^{2+} levels. CDR has a raised affinity for Stelazine in the presence of 1 μM Ca^{2+} (refs 11, 12). This high affinity for Stelazine is specific to the Ca–CDR complex. CDR alone has only low affinity for Stelazine. We therefore examined the uptake of ^3H-labelled

Table 4 Uptake of ^{3}H-Stelazine

	Stelazine (μmol per g wet weight)				
	Control	Choleragen (3μg ml^{-1})	Theophylline (10 mM)	A23187 (2μg ml^{-1})	A23187 (2μg/ml) + EGTA (0.5 mM)
No addition	1.00 ± 0.03 (38)	3.31* ± 0.12 (9)	2.02* ± 0.11 (20)	2.36* ± 0.15 (19)	1.06 ± 0.06 (6)
RMI 12330A (0.1 mM)	1.08 ± 0.06 (9)	0.26† ± 0.02 (8)	1.14§ ± 0.05 (3)	1.10§ ± 0.37 (3)	0.68† ± 0.07 (5)

Uptake of ^{3}H-Stelazine into pieces of tissue of approximately 0.1 g after incubation in Ringer solution for 30 min, except with choleragen where incubation was for 50 min. Stelazine required 15 min to reach equilibrium with the tissue. The drug was extracted for 18 h in 2 ml of 0.1N HNO_3. The initial concentration of ^{3}H Stelazine was 0.3 μM. (34.9 mCi mM^{-1}). Values are mean ± s.e.m.

* $P < 0.001$; test compared with control (column 1).

† $P < 0.001$; test compared with treated without modifier (row 1).

‡ $P < 0.005$; test compared with control.

§ $P < 0.005$; test compared with treated without modifier.

Stelazine into rabbit ileum to see if Ca^{2+}-dependent Stelazine-binding could be used as an indicator of raised free intracellular Ca^{2+} levels in the secretory state.

Table 4 shows that Stelazine uptake by rabbit ileum was stimulated by A23187, choleragen and theophylline and inhibited by RMI 12330A. The A23187-stimulated uptake was dependent on the presence of Ca^{2+} in the external Ringer solution. The apparent dissociation constant of Stelazine for intracellular sites in theophylline-stimulated rabbit intestine was 10^{-6} M. This is in close agreement with previous work on the affinity of Stelazine for purified CDR[11-13].

These results are consistent with the view that intracellular free Ca^{2+} is raised to approximately 10^{-6} M during all types of secretory stimuli, as indicated by the increase in high affinity Stelazine-binding within the tissue. Because the stimulation by A23187 of intestinal Cl^- secretion can be blocked by Stelazine or RMI 12330A, it is clear that raised intracellular Ca^{2+} by itself does not stimulate secretion, but that the Ca^{2+}-activated form of CDR may directly activate the increase in mucosal border Cl^- permeability.

To ascertain whether RMI 12330A and Stelazine act on Ca–CDR, we extracted CDR from rabbit small intestine by the method of Dedman *et al.*[14]. We found that RMI 12330A competed with ^{3}H-Stelazine for the Ca^{2+}-dependent binding sites on the protein extract.

We conclude that activation of intestinal secretion involves the following sequence: secretagogues, or ionophore, bind to the brush-border and increase passive Ca^{2+} uptake into the epithelial cells; the increased Ca^{2+} uptake increases intracellular Ca^{2+} activity to approximately 10^{-6} M. At this concentration Ca^{2+} binds to CDR to form Ca–CDR, which then activates the brush-border Cl^- conductance, thus permitting increased NaCl leakage from the lateral intercellular space—hence secretion. Additionally, Ca–CDR may activate adenylyl cyclase and phosphodiesterase, but the steady state concentration of cyclic AMP seems not to be an important parameter in the control of secretion.

Binding of antipsychotic phenothiazines to Ca^{2+}–CDR prevents the activation of several enzyme reactions[11,15] and here is shown to inhibit the activation of brush-border Cl^- conductance. It has been reported that chlorpromazine, a weak antipsychotic phenothiazine, reduces intestinal fluid loss caused by cholera in human subjects[16]. This drug, like trifluoperazine, binds to Ca–CDR, albeit with a lower affinity[11,17]. Our results suggest that it might be worth investigating a range of phenothiazines for antidiarrhoeal activity, having regard to their affinities for Ca–CDR, rather than their effectiveness as inhibitors of adenylyl cyclase.

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Concanavalin A binds to puffs in polytene chromosomes

CHANGES in transcriptional activity at defined loci are often correlated with significant local structural changes in the genome[1], and in polytene chromosomes, such changes are thought to be associated with compositional or conformational changes in the protein complement at these particular bands[2,3]. Thus, various studies on Balbiani rings and specific 'puffs' in such chromosomes are useful for elucidating the role of defined chromosomal components in both chromosome structure and gene activity. Such studies require specific probes which will allow *in situ* localisation of a chromosomal component during the various stages of puffing. Antibodies specific to purified histone fractions[4-7], HMG proteins[8], RNA polymerase[9] and non-histone protein subfractions[10] have been used in studies on chromatin and chromosome structure. We reported previously that concanavalin A (Con A) specifically binds to three types of non-histone proteins present in chromatin purified from rat liver nuclei and suggested that derivatives of Con A might serve as specific probes to study the *in situ* organisation of these non-histone proteins[11]. We have now reacted fluorescein-labelled Con A with polytene chromosomes isolated from different developmental stages of *Chironomus thummi* and visualised the location of the bound Con A by fluorescence microscopy. We observed that the fluorescent lectin, which has an affinity for glucose- and mannose-containing molecules, specifically bound to the transcriptionally active regions of chromosome IV. The extent of binding of Con A to the Balbiani rings present in regions b and c of chromosome IV is proportional to the size of the respective ring. Our results indicate that glucose- or mannose-containing molecules are present in these Balbiani rings and that the availability of these sugars to interact with Con A can be correlated with the developmental stage of a puff. We suggest that lectins can be useful cytological tools with which to study the *in situ* organisation of defined chromosomal components during various functional states of the genome.

To avoid background staining due to Con A binding sites present in plasma membranes[12], it was necessary to isolate unfixed nuclei containing polytene chromosomes from *Chironomus* salivary glands. As shown in Fig. 1*a*, these nuclei are completely free of cytoplasmic material. When such nuclear