face and suggest that the catecholamine activates adenylate cyclase specifically through this receptor. The existence of β-receptors on the surfaces of cells of the rat myoblast cell lines has been established (*14–16*). However, Parent *et al.* (*17*) were unable to detect them on quail myoblasts before differentiation. The ability of the blocker to also prevent precocious fusion supports the idea that cyclic AMP is the positive intracellular regulator.

Finally, use was made of propranolol to determine whether the activation of adenylate cyclase through the β-adrenergic receptor is an obligatory step in the induction of cell fusion by PGE. We found that cyclic AMP accumulation in response to $10^{-5}M$ PGE was normal in the presence of propranolol (*18*) and that propranolol was not able to inhibit the precocious fusion provoked by PGE (Table 1). This result demonstrates that the positive regulation of myoblast fusion in vitro by a PGE-induced rise in cyclic AMP (*7, 8*) is independent of β-adrenergic stimulation. It was still possible that under control conditions of culture (no exogenous hormone), cyclic AMP generation was dependent on activation of adenylate cyclase through the β-adrenergic receptor. (Prostaglandins are modulators of hormone action, and the positive regulation of myoblast fusion might require PGE to act in conjunction with a hormone-activating adenylate cyclase through the β-adrenergic receptor.) To test this possibility, propranolol alone was added to the undifferentiated cultures. Table 1 shows that the β-antagonist has no observable effect on either cell numbers per field or cell fusion. Thus fusion in vitro is independent of β-adrenergic activation.

This finding is in contrast to the complete inhibition of cell fusion observed in the presence of inhibitors of prostaglandin production (*4*) and supports our proposal that a prostaglandin or related molecule is the endogenous signal responsible for generating the intracellular rise in cyclic AMP in vitro. Taken together, the findings presented here are consistent with our model of myoblast differentiation (*13*). That is, a prostaglandin or related molecule is generated within the myoblast culture and interacts with a myoblast in the G_1 phase of its cell cycle, provoking a transient rise in intracellular cyclic AMP. The cyclic nucleotide in turn triggers the intracellular events that culminate in cell fusion. The increase of ACh receptor numbers in response to agents that raise cyclic AMP, at times when cell fusion is normally complete (*9*), suggests that this effect of cyclic AMP is not contingent on stimulation of cell fusion. Instead, the effects of cyclic AMP on cell fusion and ACh receptors are consistent with cyclic AMP acting as a simultaneous initiator of a number of independent events that represent the different aspects of the expression of muscle's differentiated state. Cell fusion and ACh receptors both represent aspects of the differentiation of the myoblast membrane. It is not yet known whether cyclic AMP influences also the cytoplasmic differentiation of muscle and therefore how completely it is coordinating the expression of the differentiated state.

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19. This work was supported by the Muscular Dystrophy Group of Great Britain.
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9 February 1981; revised 14 July 1981

Epithelial Cell Volume Regulation: Bicarbonate Dependence

Abstract. *When* Necturus *gallbladder epithelial cells are osmotically shrunken, they rapidly return to their original volume despite the continued presence of a hypertonic bathing solution. This volume-regulatory process requires bicarbonate ions in the bathing solutions and is associated with the uptake of chloride ions. Volume-regulatory increase by epithelial cells is probably due to the parallel operation of sodium-hydrogen and chloride-bicarbonate exchangers in the apical cell membrane.*

Epithelial cells of *Necturus* gallbladder regulate their volume after a change in the osmolality of the solutions bathing either their apical or their basolateral surface (*1, 2*). When the osmolality of the solution bathing the apical (mucosal) surface is increased by the addition of mannitol, the cells shrink to a minimum size in about 40 seconds and then return to their original volume in about 90 seconds despite the continued hypertonicity of the apical bathing solution (Fig. 1, top). The rate of fluid flow into the *Necturus* gallbladder cell during volume-regulatory swelling is three to four times greater than the normal rate of fluid absorption by this tissue (*1–3*). Cell volume regulation after osmotically induced shrinkage probably involves the entry of NaCl from the mucosal medium into the cell across the apical cell membrane. Support for this conclusion comes from observations that volume-regulatory increase during perfusion of a hypertonic solution requires Na^+ in the mucosal bathing solution (*1–3*). The role of anions in volume regulation by epithelial cells has not been investigated.

We studied the HCO_3^- dependence of volume regulation by *Necturus* gallbladder epithelial cells in response to hypertonic solutions on their apical cell surface. Gallbladders were mounted in a miniature Ussing chamber and visualized by a light microscope equipped with differential interference contrast optics; the cell volume was measured by planimetry of optical sections of the epithelial cells (*2–4*). When both perfusion solutions contained 10 mM HCO_3^-, addition of 36-mOsm mannitol to the mucosal perfusion solution (an 18 percent increase in osmolality) caused typical cell shrinkage (14.4 percent) and subsequent volume-regulatory swelling (Fig. 1, top, and Table 1). Removal of all HCO_3^- from the perfusion solutions did not affect the osmotically induced cell shrinkage (15.4 percent) but prevented the volume-regulatory swelling (Fig. 1, bottom, and Table 1).

We punctured the cells of *Necturus* gallbladder with voltage and ion-sensitive microelectrodes to study the alterations in apical membrane potential difference (PD) and intracellular Cl^- activity (a_{Cl}) which accompany hypertonicity of the mucosal bath. The gallbladder was

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Table 1. Effect of 36-mOsm hypertonic solution in the mucosal bath; 0 to 40 seconds corresponds to the osmotic shrinkage period, and 40 to 130 seconds corresponds to the volume-regulatory period; J_v is volume flow across the apical cell membrane; J_{Cl} is flux across that membrane. Negative values indicate flows out of the cell. All values are the mean ± the standard error of the mean; the number of samples is given in parentheses. Control values of cell volume, PD, a_{Cl}, and Q_{Cl} are given in (*7*).

Time period (seconds)	(Experimental/control) × 100				J_v (cm sec^{-1} × 10^{-6})	J_{Cl} (M cm^{-2} sec^{-1} × 10^{-12})
	Volume	PD	a_{Cl}	Q_{Cl}		
			HCO_3^- present			
0 to 40	85.6* ± 0.9 (14)	103.3* ± 0.4 (35)	108.6* ± 1.8 (23)	92.8* ± 1.5	−11.4 ± 1.8 (10)	−98 ± 19 (23)
40 to 130	96.2‡ ± 1.4	104.9* ± 0.6	109.1† ± 2.5	106.5† ± 1.9	6.1 ± 1.3	75 ± 14
			HCO_3^- absent			
0 to 40	84.6* ± 1.1 (13)	99.6* ± 0.3 (31)	114.4* ± 1.9 (11)	93.6* ± 1.4	−10.7 ± 1.6 (10)	−70 ± 17 (11)
40 to 130	83.7* ± 1.2	99.2 ± 0.6	113.6* ± 2.7	91.4* ± 1.4	0.2 ± 0.2	−10 ± 6

*$P < .001$, experimental control different from 1.0. †$P < .01$. ‡$P < .05$.

mounted in a chamber designed to achieve rapid solution changes and minimum unstirred layer thickness (*5*). Two cells were punctured simultaneously across the apical cell membrane, one cell with a voltage-sensitive microelectrode and the other with a Cl^--sensitive microelectrode filled with liquid ion exchanger (*6*). The PD and a_{Cl} values were recorded continuously during exposure of the epithelium to a hypertonic mucosal solution. Shown in Fig. 2 are examples of PD and a_{Cl} for tissues bathed in HCO_3^- (top) and HCO_3^--free (bottom) Ringer solutions. The PD changes in HCO_3^- Ringer solution during hypertonicity were small (3 to 5 percent) and consistent with an increase of intracellular K^+ activity due to osmotic water loss.

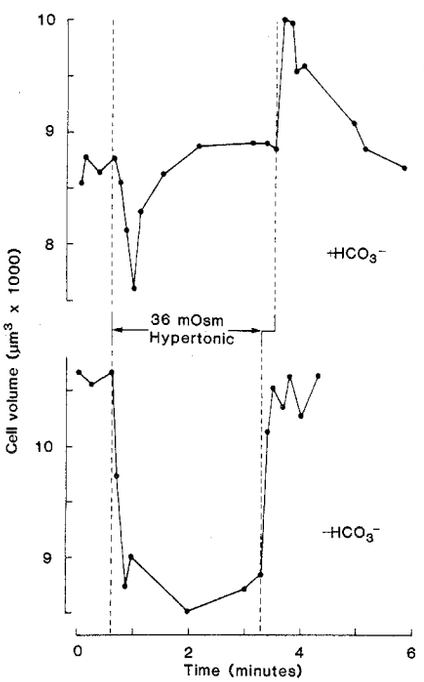

Fig. 1. *Necturus* gallbladder epithelial cell volume, determined by computerized planimetry of video images of the cells visualized in a light microscope, is shown during exposure to hypertonic perfusate on the mucosal surface. At the left dashed line, the osmolality of the mucosal bathing solution was increased by the addition of 36-mOsm mannitol. The responses of cells are shown for tissue bathed in HCO_3^- Ringer (top) or HCO_3^--free Ringer (bottom). The initial cell shrinkage was due to osmotically induced water loss; this was followed by volume regulation in the presence of HCO_3^- (top) but not in its absence (bottom). The mucosal perfusate was switched back to control osmolality at the dashed line on the right.

During the period of cell shrinkage in response to mucosal hypertonicity (< 40 seconds), increases in a_{Cl} were observed. Tissues bathed in HCO_3^- Ringer increased a_{Cl} by 8.6 percent; in HCO_3^--free Ringer solution the increase was 14.4 percent. Over the next 90 seconds, no further significant changes in a_{Cl} were observed either in the presence or absence of HCO_3^-, despite large differences in the response of the cell volume (see Fig. 2 and Table 1). From the combination of the cell volume and a_{Cl} records, it was possible to determine the osmotically induced changes in the quantity of Cl^- in the cell, Q_{Cl}, as well as the Cl^- flux across the apical cell membrane, J_{Cl} (*7*). During shrinkage, the quantity of intracellular Cl^- decreased 6 to 7 percent (probably because of solvent drag) in the presence or absence of HCO_3^-. Cells bathed in HCO_3^- Ringer solution then volume-regulated and regained twice the amount of Cl^- lost during the initial cell shrinkage. In contrast, cells in HCO_3^--free solutions failed to recover the lost Cl^- (see Table 1).

These data support the conclusion that gallbladder epithelial cells respond to osmotically induced shrinkage by gaining NaCl from the mucosal bathing medium. The mechanism by which NaCl enters the cell appears to be identical to that reported for volume-regulatory increase by the *Amphiuma* red blood cell in which NaCl enters by the exchange of Na^+ for H^+ and Cl^- for HCO_3^- (*8*). Water follows NaCl entry because H^+ and HCO_3^- are not osmotically active in the cell. These exchange processes are separate limbs of the NaCl entry mechanism and may be blocked by specific inhibitors such as amiloride for the Na^+-H^+ exchange and diisothiocyanostilbene-2,2′-disulfonic acid for the Cl^--HCO_3^- exchange (*8*). Similarly, volume-regulatory increase by *Necturus* gallbladder epithelial cells is blocked by the removal of mucosal Na^+ (*1, 2*), the addition of amiloride to the mucosal bathing medium (*9*), or the removal of Cl^- from the bathing solutions (*9*).

Evidence for the existence of Na^+-H^+ exchange at the apical membrane of *Necturus* gallbladder epithelium comes from a recent report of amiloride-sensitive, Na^+-dependent acidification of the mucosal bathing medium (*10*). Volume-

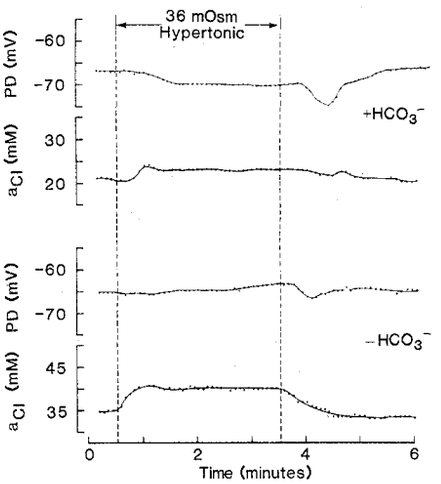

Fig. 2. Intracellular Cl^- activity (a_{Cl}) and the apical membrane potential difference (PD) are shown for cells during exposure to a hypertonic solution in the mucosal bath. When the perfusion solutions contain HCO_3^- (top), a small hyperpolarization occurs in the presence of the hypertonic perfusate; a small increase in a_{Cl} also occurs. In the absence of HCO_3^- (bottom), no significant PD change occurs, but a_{Cl} increases substantially. At the right dashed line the mucosal perfusate was returned to control Ringer; both PD records show a transient hyperpolarization, whereas a_{Cl} returns gradually to control levels.

regulatory swelling of *Necturus* gallbladder epithelial cells is the consequence of the transient stimulation of parallel Na^+-H^+ and Cl^--HCO_3^- exchangers in the apical membrane. On the other hand, NaCl entry into the cells during fluid absorption is by a different process in which there is coupled, carrier-mediated movement of NaCl across the apical membrane (*9*, *11*). This coupled NaCl entry is unaffected by amiloride (*9*) or by the absence of HCO_3^- (*11*). It occurs at about 25 percent of the rate of volume-regulatory flow. Thus, NaCl may enter the *Necturus* gallbladder epithelial cell across the apical membrane by two modes: (i) the coupled NaCl entry associated with fluid absorption and (ii) the rapid, but transient, exchanges triggered by alteration of the osmolality of the bathing solutions.

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4. The perfusion solutions had the compositions (in millimoles per liter) described here. The HCO_3^- Ringer contained: Na^+, 82.5; Cl^-, 80; K^+, 2.5; Ca^{2+}, 1.8; Mg^{2+}, 1.0; HCO_3^-, 10; $H_2PO_4^-$, 0.5; mannitol, 36; pH = 7.6 when gassed with 99 percent air and 1 percent CO_2; HCO_3^--free Ringer contained: Na^+, 82; Cl^-, 86; K^+, 2.4; Ca^{2+}, 1.0; Mg^{2+}, 1.0; HPO_4^{2-}, 1.0; $H_2PO_4^-$, 0.3; mannitol, 36; pH = 7.6 when gassed with 100 percent air. The control solution was 188 to 189 mOsm; the hypertonic solutions, 222 to 224 mOsm.
5. The chamber was constructed of plexiglass to create a narrow trough of fluid on both surfaces of the gallbladder. Fluid exchange in either bath was complete in 3 seconds. Further details of the design will be provided on request.
6. The PD and a_{Cl} values were measured by the use of conventional techniques as described in K. R. Spring and G. Kimura [*J. Membr. Biol.* **38**, 233 (1978)]. Since epithelial cells are electrically coupled and similar time-dependent changes of voltage in different cells were observed, PD and a_{Cl} measurements could be made in different cells.
7. Control values in the presence of HCO_3^- were as follows: cell volume, 10,416 ± 463 (14) μm^3; PD, −66.7 ± 1.3 (35) mV; a_{Cl}, 24.9 ± 2.0 (23) mM; total quantity of intracellular Cl^-, Q_{Cl}, 25.9 ± 2.6 (14) × 10^{-14} mole. Control values in the absence of HCO_3^- were as follows: cell volume, 11,289 ± 948 (13) μm^3; PD, −66.4 ± 1.3 (31) mV; a_{Cl}, 19.1 ± 2.2 (11) mM; Q_{Cl}, 21.6 ± 2.9 (11) × 10^{-14} mole.
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20 July 1981; revised 4 September 1981

Close-Range Attraction of Female Oriental Fruit Moths to Herbal Scent of Male Hairpencils

Abstract. *A blend of ethyl* trans*-cinnamate, methyl 2-epijasmonate, methyl jasmonate, and* (R)-(−)*-mellein, identified from the hairpencils of male Oriental fruit moths, attracts sex pheromone–releasing females several centimeters away. The chemicals thereby duplicate the behavioral effect elicited by hairpencil-displaying males during courtship; the chemicals also produce the herbal scent emanating from the hairpencils.*

Males of the order Lepidoptera often have accessory scent-producing organs, which usually consist of groups of elongated hairlike scales (hairpencils) that are bundled into special pouches and then everted and splayed in the vicinity of a female during courtship (*1*). Volatile chemicals identified from such structures (*2–4*) or from other specialized scales (*5*) have been described, without reference to the behavior elicited. Studies aimed at defining the behavioral roles played by scent scales, including ablation techniques (*6*), electroantennogram (EAG) assays (*7*), trapping experiments (*8*), or observational inferences (*9*), have revealed that, in most species, courtship pheromones exert a minimal observable effect on female behavior. The lack of an overt female response has hindered the identification of chemicals producing behavioral responses. In rare instances, behavior and chemistry have both been elucidated, but in those instances the compounds described evoked female "acceptance" through inferred quiescence (*10*) or abdominal extension (*11*), rather than attraction.

Courtship in the Oriental fruit moth, *Grapholitha molesta* (Busck), is unusual among the Lepidoptera in that males attract females after they themselves have been attracted to the vicinity of a female by a sex pheromone (*12*). A few centimeters from the female, the male turns away and repeatedly extrudes and retracts its abdominal hairpencils, propelling volatile chemicals over the female with wind generated from wing vibration (Fig. 1). The female immediately walks toward the source of the odor and with her head touches the tip of the male's abdomen, evoking from him a copulatory attempt (*12*). The overt movement of females toward displaying males in this species provided the opportunity to define a lepidopterous courtship pheromone that attracts females. With the use of behavioral and EAG assays, we were able to identify a blend of compounds that duplicates the activity of the natural pheromone. The compounds are ethyl *trans*-cinnamate (**1**), (*R*)-(−)-mellein (**2**), methyl jasmonate (**3**), and methyl 2-epijasmonate (**4**) (Fig. 2).

Fig. 1. Male Oriental fruit moth everting his hairpencil organs at the end of his abdomen and attracting a female that is walking toward him.

Approximately 5000 male equivalents (ME) of the hairpencil extract (*13*) were used for the isolation and identification (*14*). The crude extract on filter paper had a pleasant herbal odor, similar to that of the forcibly extruded hairpencils of living *G. molesta* males. Each 1000 ME was concentrated under nitrogen and fractionated on a gas-liquid chromatography (GLC) column [3 percent OV-101 (*15*)] into 12 fractions (Fig. 2). The only fraction to produce an EAG response from female antennae [mean ± standard deviation (S.D.) = 0.60 ± 0.20 mV; N = 9] above background (0.07 ± 0.05 mV; N = 9) was fraction 3 (the crude extract produced 1.25 ± 0.31 mV; N = 9) (*14*, *16*). The compound from this fraction was collected (approximate yield 0.5 ng per ME) and identified as ethyl *trans*-cinnamate (**1**) by GLC retention times, microhydrogenation, and diagnostic ultraviolet and mass spectra (*14*, *17*).

The fractions were tested for their attractiveness to calling females in an arena in moving air (*18*). The test samples on filter paper were placed 4 cm

0036-8075/81/1218-1359$01.00/0