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CYCLIC-AMP-INDUCED WATER UPTAKE IN A MOTH OVARY: INHIBITION BY BAFILOMYCIN AND ANTHRACENE-9-CARBOXYLIC ACID

YUREN WANG* AND WILLIAM H. TELFER†

Department of Biology, University of Pennsylvania, Philadelphia, PA 19104-6018, USA

*Present address: Department of Pharmacology, University of Pennsylvania, Philadelphia, PA 19104-6084, USA
†Author for correspondence (e-mail: wtelfer@sas.upenn.edu).

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Summary

The ion physiology of osmotic swelling and a consequent loss of epithelial patency was examined in the ovaries of the moth *Hyalophora cecropia*. After 30 min in the presence of an activator of cyclic-AMP-dependent protein kinase (PKA), the membrane potentials of both oocyte and follicle cells had hyperpolarized by approximately 30 %, cytoplasmic pH had dropped from 7.26 to 7.06, a normally low Cl^- conductance had increased and the follicle cells had begun to swell. Since ion distribution studies have indicated that conductance increases should depolarize membranes in this system, it is proposed that hyperpolarization may be effected by an azide-inhibitable component of the membrane potential. Nanomolar levels of bafilomycin, an inhibitor of H^+ V-ATPase, blocked the active component and prevented osmotic swelling in response to PKA activation. Under a variety of circumstances, correlations were seen between membrane potential and cytoplasmic pH, suggesting that substrate availability to the proton pump may contribute to hyperpolarization. H^+ V-ATPases are known to energize ion and water transport across many epithelia, but in this case they generate water absorption by the epithelium. The increase in Cl^- conductance was also required for the swelling response: the Cl^- channel blocker anthracene-9-carboxylic acid prevented both swelling and hyperpolarization, as did Cl^- substitution in the medium. Differences in isotope loading rates between $^{36}Cl^-$ and $^{86}Rb^+$ suggested that, after PKA activation, Cl^- functions other than as a counterion for K^+ uptake.

Key words: Cl^- conductance, $^{36}Cl^-$, follicle, follicle cell, patency, H^+ V-ATPase, $^{86}Rb^+$, Sp-cAMPS.

Introduction

In ovarian follicles of the cecropia moth, vitellogenesis ends when the follicle cells surrounding the oocyte transform from a porous layer into a more conventional tight epithelium. The transformation entails three changes that prevent further access of hemolymph proteins to the oocyte surface. The follicle cells dismantle a sulfated glycosaminoglycan matrix that contributes to patency during vitellogenesis (Telfer, 1979), they close the intercellular spaces by increasing their volume, and they form tight-junction-like diffusion barriers at their apical ends (Rubenstein, 1979).

Cell-permeant analogs of cyclic AMP stimulate follicle cells to undergo these changes *in vitro* by activating cyclic-AMP-dependent protein kinase (PKA) (Wang and Telfer, 1996, 1997). After a lag period of over an hour, vitellogenin uptake stops, the epithelium becomes impermeable to small molecules as well as to vitellogenin, and production of the intercellular matrix is inhibited. Most relevant to the present study, cellular swelling also occurs, so that each cell now presses against its lateral neighbors.

In a number of other systems, transepithelial water and ion transport are energized by a proton-translocating, vacuolar-type ATPase (H^+ V-ATPase) (Harvey and Wieczorek, 1997; Harvey *et al.* 1998) that is selectively inhibited by nanomolar concentrations of bafilomycin (Bowman *et al.* 1988; Drose and Altendorf, 1997). We present evidence here that the hydration phase of development in *Hyalophora cecropia* follicles, an example of developmentally regulated cellular swelling, also requires an H^+ V-ATPase. In particular, we propose that membrane hyperpolarization, an obligatory early step towards epithelial swelling, results from an increased concentration of protons available to a bafilomycin-sensitive mechanism. An early increase in membrane conductance, which could be inhibited with 9-AC and depended on the presence of Cl^- in the medium, was also an obligatory step in the pathway towards swelling.

Materials and methods

Preparation and incubation of follicles

Vitellogenic follicles were dissected from *Hyalophora cecropia* (L.) females showing early wing pigmentation (day 18 of the 22 day pupal–adult molt at 25 °C). Follicles were dissected in a 300 mosmol l^{-1} physiological salt solution (PSS) empirically developed to optimize yolk formation and

electrophysiological stability (Anderson and Telfer, 1969; Woodruff *et al.* 1992). It contained 40 mmol l^{-1} KCl, 15 mmol l^{-1} $MgCl_2$, 4 mmol l^{-1} $CaCl_2$, 110 mmol l^{-1} Tris-succinate (pH 6.5) and 70 mmol l^{-1} sucrose. The composition of PSS reflects the unusual inorganic ion composition of the hemolymph in Lepidoptera whose larvae develop on Na^+-poor, K^+-rich diets (Harvey *et al.* 1975). Chloride substitution experiments utilized the gluconate salt of K^+ and the SO_4^{2-} salts of Mg^{2+} and Ca^{2+}. Choline chloride was used in place of KCl in K^+ substitution experiments.

For each experiment, matched chains of ten vitellogenic follicles were dissected from the eight ovarioles of a single female. Hemolymph proteins were soaked out of the intercellular spaces by two 20 min rinses with shaking in PSS. For PKA activation, the Sp-isomer of adenosine-3′,5′-monophosphorothioate (Sp-cAMPS) was used at 1 mmol l^{-1}. This cyclic AMP analog is cell-permeant, resistant to degradation by phosphodiesterases and promotes dissociation of the regulatory and catalytic subunits of PKA (Rothermel and Botelto, 1988). At the concentrations used, Sp-cAMPS induces the termination of vitellogenin uptake with a lag period of approximately 1 h (Wang and Telfer, 1996).

Each chain of ten follicles was incubated in 200 µl of PSS containing specified isotopes, Sp-cAMPS and/or inhibitors. Stock solutions of inhibitors with low solubility in water (bafilomycin and anthracene-9-carboxylic acid, 9-AC) were prepared in dimethylsulfoxide (DMSO) at concentrations high enough so that the final incubation medium contained less than 1 % DMSO. The same concentration of DMSO was added to control cultures not exposed to these inhibitors. For measurements of electrical properties and proton or isotope contents, the chain of ten was divided into individual follicles and each result is expressed as a mean of the ten (± S.E.M.).

Isotope loading and unloading

Isotopic ion loading was measured by incubating follicles in $^{36}Cl^-$ (3.7 MBq ml^{-1}) or $^{86}Rb^+$(3.7 MBq ml^{-1}). After incubation, the chain was dropped into 50 ml of PSS for several seconds, during which individual follicles were separated and transferred for counting to scintillation vials containing 250 µl of a tissue solubilizer (50 mmol l^{-1} Tris-HCl, pH 7.0, 1 % SDS and 0.35 % 2-mercaptoethanol). To measure unloading rates, follicles were first incubated in $^{36}Cl^-$ for 2.5 h, by which time the amount of label per follicle had reached a steady state. They were then transferred to 5 ml of fresh PSS without isotope. After 10 min, the follicles were individually dissolved and counted as in the loading experiments.

Electrophysiology

Electrical measurements were made in a 0.25 ml open-topped chamber grounded through a 3 mol l^{-1} KCl bridge to a Ag/AgCl wire (Woodruff *et al.* 1992). Standard 3 mol l^{-1} KCl microelectrodes were attached to S-7071A electrometers (World Precision Instruments, New Haven, CT, USA) for membrane potential measurements or to an S-7071A ionophoresis unit (also from World Precision Instruments) to provide current pulses. Proton-selective microelectrodes were constructed using a liquid ion-exchange resin from World Precision Instruments, as described by Ammann *et al.* (1981) and Palmer and Civan (1977). Calibrations and measurements were performed as in Woodruff *et al.* (1992). Prior to impalement with microelectrodes, follicles were treated for 1 min with 1 mg ml^{-1} collagenase (type 1A, Sigma) to soften the basement membrane (Woodruff and Telfer, 1990).

At the stages studied, the cytoplasmic bridges to the nurse cells have disintegrated, and an epithelium of follicle cells completely surrounds the oocyte. The follicle cells thus form a closed ellipsoid, differing in this regard from the tubular or flat-sheet configurations of the more intensively studied insect epithelia. A second important difference is that, prior to treatment with Sp-cAMPS and during the subsequent lag phase, oocyte and follicle cells communicate *via* ion- and fluorescent-dye-permeable gap junctions (Woodruff, 1979). Properties measured with microelectrodes or isotopes in one type of cell therefore reflect the contributions of both.

Follicle cell swelling

Epithelial cell height was used as the index of swelling (Wang and Telfer, 1997). It was measured in living follicles viewed through the low-power objective lens of an inverted microscope with the focal plane set halfway through the follicle, where its diameter appeared widest. Epithelial height was measured with a filar micrometer as the distance at this level between the edge of the opaque yolk mass and the basement membrane forming the surface of the follicle.

Reagents

The Sp-isomer of adenosine-3′,5′-monophosphorothioate (Sp-cAMPS) was purchased from Biolog Life Science Institute (Bremen, Germany); $^{36}Cl^-$ and $^{86}Rb^+$ were purchased from New England Nuclear (Newark, DE, USA); bafilomycin-A1 was the gift of Dr Philip Rea, University of Pennsylvania; anthracene-9-carboxylic acid (9-AC) and all other reagents came from Sigma (St Louis, MO, USA).

Results

Early responses to Sp-cAMPS

Three changes were detected during the lag phase in Sp-cAMPS-induced termination of vitellogenin uptake. In an earlier report, the height of the follicle cells at the end of this 1.5 h period was, on average, 20 % greater than that of follicles incubated in PSS alone, and cellular cross section was 25 % greater, yielding a calculated volume increase of 50 % (Wang and Telfer, 1997). In the time course study shown in Fig. 1, the increase in height was first detected after 30 min in Sp-cAMPS and reached a stable, maximum value by 3 h. Epithelial height in treated follicles was 20 % greater than that in follicles incubated for 2 h in PSS without Sp-cAMPS. Cell widths were not measured but, as in the earlier study, swelling had obliterated the intercellular spaces by this time, so that a volume increase of 50 % can be assumed here also. Since the

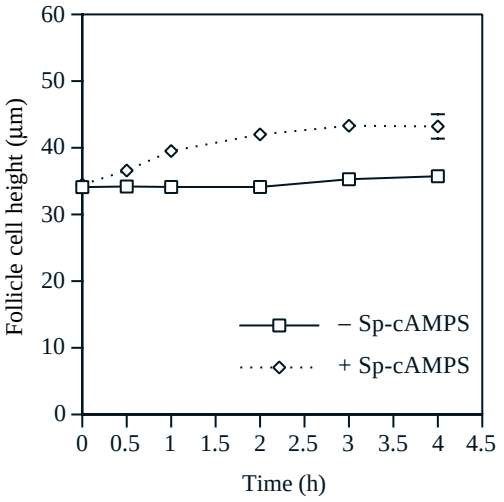

Fig. 1. Follicle cell height, measured between the oocyte surface and the basement membrane of living follicles, as a function of time of incubation in PSS, with or without 1 mmol l^{-1} Sp-cAMPS. For each point, *N*=10; values are means ± S.E.M.; where an error bar is not visible, the standard error falls within the diameter of the symbol.

overall dimensions of the follicles studied did not change detectably, epithelial swelling must have occurred primarily at the expense of the intercellular spaces.

A plot of current injected *versus* voltage change indicated that, within 30 min of treatment with Sp-cAMPS, there was an increase in general membrane conductance (filled squares *versus* filled diamonds in Fig. 2). The difference largely disappeared in Cl^--free PSS (open circles), but was unaffected by K^+-free PSS (open triangles). As would be expected, the increase in Cl^- conductance was accompanied by increases in the loading and unloading rates of $^{36}Cl^-$. The amount of isotope taken up in 10 min began to increase within 15 min of exposure to Sp-cAMPS and continued to rise over the next 1 h (Fig. 3A). Unloading accelerated on a similar time course (Fig. 3B). The Cl^- channel blocker 9-AC (Palade and Barchi, 1977; Welsh, 1984) had no effect on $^{36}Cl^-$ uptake in unstimulated follicles, but prevented the increase that occurred in response to Sp-cAMPS (Table 1).

Table 1. *The effects of 9-AC and Sp-cAMPS on the uptake of $^{36}Cl^-$ and on membrane potentials*

Additions to PSS	$^{36}Cl^-$ taken up in 10 min (cts min^{-1} $follicle^{-1}$)	ΔΨ (mV)
None	376±16	−37.5±0.6
Sp-cAMPS	499±27*	−45.5±1.5*
9-AC	374±17	−33.3±0.2
9-AC + Sp-cAMPS	372±27	−34.5±0.9

Follicles were incubated in PSS with or without Sp-cAMPS and/or 9-AC, as shown in the left-hand column, for 30 min. $^{36}Cl^-$ (3.7 MBq ml^{-1}) was then added to the medium, and 10 min later the follicles were briefly rinsed and dissolved for counting. Concentrations for both Sp-cAMPS and 9-AC were 1 mmol l^{-1}.

Membrane potentials were measured between the cytoplasm of the oocyte and the medium.

*Significantly different from the value obtained in the absence of Sp-cAMPS (P<0.01).

9-AC, anthracene-9-carboxylic acid; ΔΨ, membrane potential.

Values are means ± S.E.M., *N*=10.

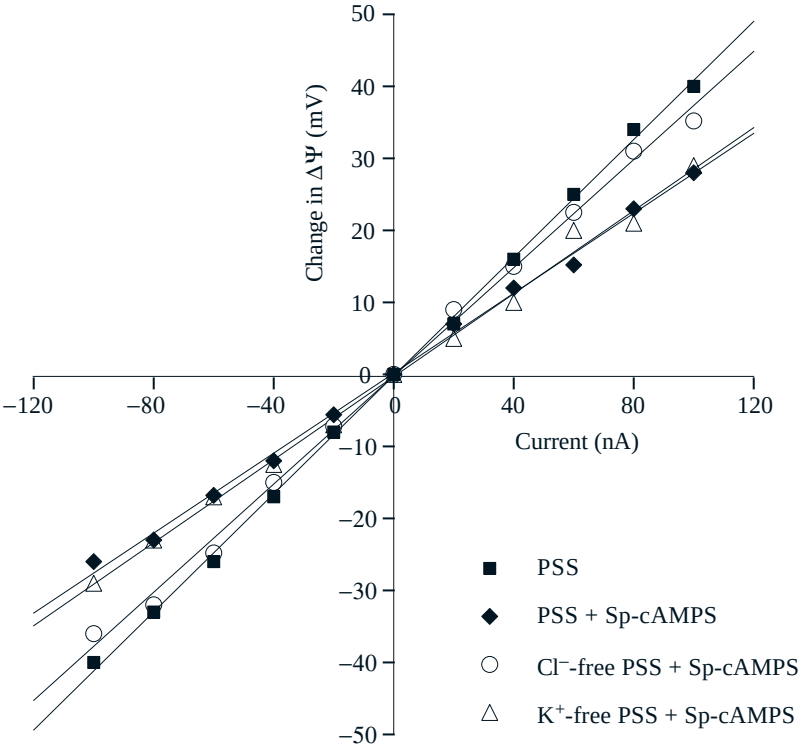

Fig. 2. Current–voltage change relationship for cell membranes in vitellogenic follicles treated for 30 min with 1 mmol l^{-1} Sp-cAMPS. Current-carrying and voltage-recording microelectrodes were inserted into the cytoplasm of the oocyte. The oocyte and follicle cells are joined by gap junctions, so that both compartments contribute to the changes in membrane potential (ΔΨ). The increase in Cl^- conductance seen in this experiment was confirmed by the isotope loading and unloading experiments reported in Fig. 3.

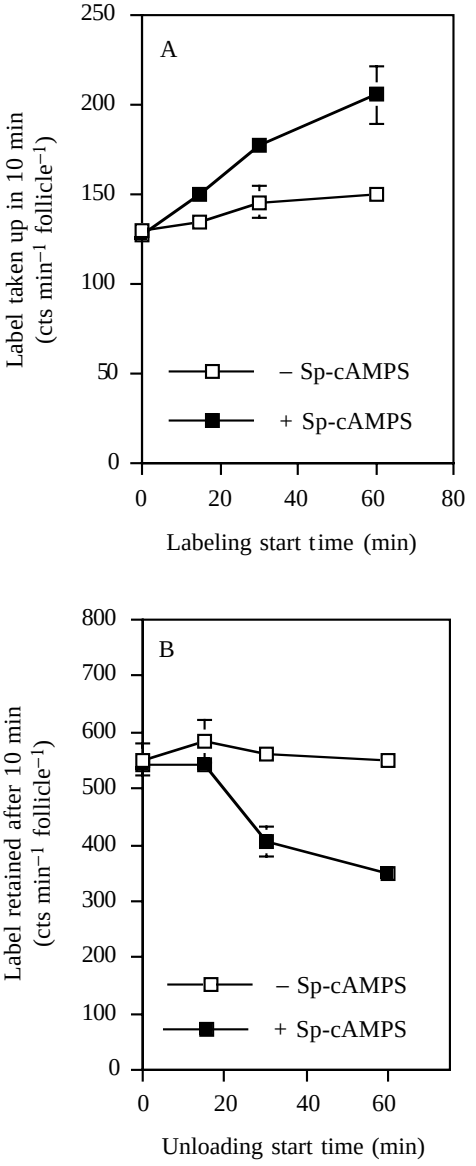

Fig. 3. Effects of 1 mmol l^{-1} Sp-cAMPS on the loading and unloading of follicles with $^{36}Cl^-$. (A) Matched sets of vitellogenic follicles were incubated in PSS for the times shown, in the absence or presence of Sp-cAMPS; $^{36}Cl^-$ was added to the medium (3.7 MBq ml^{-1}) for 10 min, and the follicles were then rinsed, dissolved and counted as described in Materials and methods. (B) Matched sets of follicles were preloaded for 2 h in PSS containing $^{36}Cl^-$, by which time an equilibrium between follicle and medium had been reached (see Fig. 4B). Sp-cAMPS, or an equivalent volume of PSS without SP-cAMPS, was then added and, at the times shown, the follicles were transferred from the labeling medium to a large volume of PSS that lacked the isotope. After 10 min of unloading, the follicles were individually dissolved for scintillation counting. For each point, N=10; values are means ± S.E.M.; where an error bar is not visible, the standard error falls within the diameter of the symbol. The differences between –Sp-cAMPS and +Sp-cAMPS in A at 20, 30 and 60 min and in B at 30 and 60 min were significant (P<0.01).

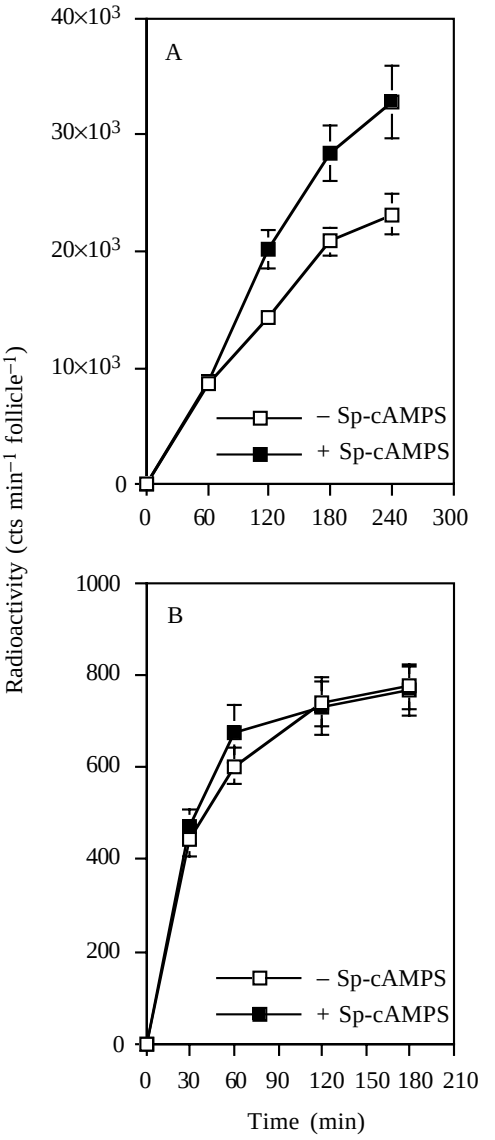

Fig. 4. Effects of 1 mmol l^{-1} Sp-cAMPS on the time course of accumulation by follicles of (A) $^{86}Rb^+$ (37 MBq ml^{-1} PSS) and (B) $^{36}Cl^-$ (3.7 MBq PSS). Isotope and Sp-cAMPS were added to the incubation medium simultaneously at t=0. For each point, N=10; values are means ± S.E.M.; in A, the effects of Sp-cAMPS at 120–240 min are significant (P<0.01).

As might be expected for fluid uptake, the increase in follicle cell height required Cl^- and K^+, the major inorganic ions in PSS. In this experiment, Sp-cAMPS elicited a 25% greater follicle cell height during incubation for 2 h in PSS, whereas in Cl^--free PSS, swelling was reduced to 6% and in K^+-free PSS it was reduced to 3% (Table 2).

The dependencies of swelling on Cl^- and K^+ were based on separate principles, however, since Sp-cAMPS affected labeling with $^{36}Cl^-$ and $^{86}Rb^+$ very differently. For the first hour of incubation in $^{86}Rb^+$, Sp-cAMPS did not significantly affect the amount of label accumulated (Fig. 4A), but uptake subsequently accelerated, as would be expected for cells that

Table 2. *Effects of ion substitutions on the ability of Sp-cAMPS to promote an increase in follicle cell height and membrane hyperpolarization*

	Follicle cell height		ΔΨ	
Incubation medium	(μm)	Difference (%)	Oocytes (mV)	Follicle cells (mV)
PSS	46.1±1.1	–	–37.1±1.4	–40.9±0.5
PSS + Sp-cAMPS	57.5±1.8*	+24.7	–51.8±2.4*	–54.2±1.8*
Cl^--free PSS	44.5±1.0	–	–38.6±2.2	–40.4±2.2
Cl^--free PSS + Sp-cAMPS	47.3±2.0	+6.3	–43.4±2.0*	–45.4±2.8*
K^+-free PSS	44.8±1.1	–	–61.1±2.3	–64.3±2.8
K^+-free PSS + Sp-cAMPS	46.2±1.5	+3.1	–89.9±1.1*	–91.0±1.3*

Membrane potential, ΔΨ, was measured after 30 min and epithelial height after 2 h of incubation under the conditions shown. Cl^-- and K^+-free media were prepared as described in Materials and methods. The concentration of Sp-cAMPS was 1 mmol l^{-1}.

*Significantly greater than the corresponding value in the absence of Sp-cAMPS (P<0.001).

Values are means ± S.E.M., N=10.

were utilizing the osmotic effects of K^+ to absorb water. In contrast, $^{36}Cl^-$ reached the same level during 3 h in the presence of Sp-cAMPS as in its absence (Fig. 4B). Other ions must therefore neutralize the electrical effects of the increased accumulation of K^+.

The early responses to Sp-cAMPS also included a temporary membrane hyperpolarization. The response remained strong until 1 h and then disappeared by 2 h. The example shown in Fig. 5 was seen with electrodes positioned in an oocyte, but the same response was seen in follicle cells (not shown). That the two compartments appeared to be coupled in this regard is not surprising since, during vitellogenesis, fluorescent compounds and ion currents readily pass between them *via* gap junctions (Woodruff, 1979; Woodruff and Telfer, 1990).

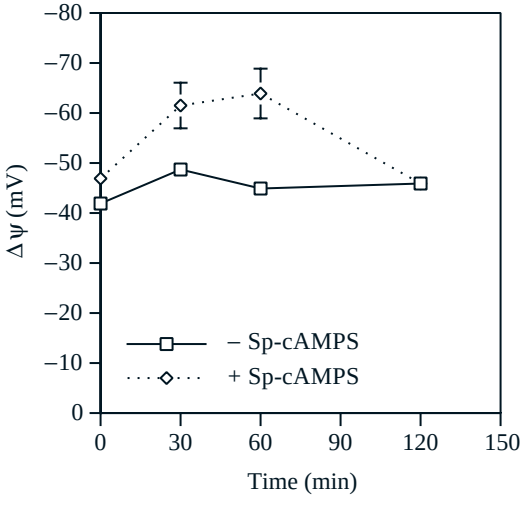

Fig. 5. An example of the transitory membrane hyperpolarization (oocyte to medium) induced by 1 mmol l^{-1} Sp-cAMPS in vitellogenic follicles. The differences between −Sp-cAMPS and +Sp-cAMPS at 30 and 60 min were significant (P<0.01). Follicle cells, in which membrane potential (ΔΨ) is generally 1–3 mV more negative than in the oocyte (see Tables 2 and 3), undergo similar changes. Values are means ± S.E.M., N=10.

The basis of hyperpolarization

ΔΨ, the membrane potential measured between the cytoplasm of either the oocyte or follicle cells and PSS, includes a passive component that varies between different females from −12 to −20 mV and an azide-inhibitable, active component of −20 to −25 mV (Woodruff *et al.* 1992). Hyperpolarization is unlikely to result from the increase in Cl^- conductance, because this would be expected to depolarize follicle membranes; in a set of vitellogenic follicles in which ΔΨ averaged −35 mV, the equilibrium potential for Cl^- was −22 mV (Woodruff *et al.* 1992). In addition, since K^+ substitution did not affect the change in conductance induced by Sp-cAMPS (Fig. 2), opening channels accessible to this ion should not account for hyperpolarization either. A change in the active component of the membrane potential is therefore more likely to underlie this response.

Bafilomycin, an inhibitor of H^+ V-ATPases that acts at low nanomolar concentrations (Bowman *et al.* 1988; Drose and Altendorf, 1997), depolarized oocyte and follicle cell membranes to nearly the same extent as azide (Table 3). At a bafilomycin concentration of 150 nmol l^{-1}, depolarization began in 5 min and was complete at 20 min (not shown). Inhibition of the active component was approximately 50 % in 30 nmol l^{-1} bafilomycin and reached a maximum at 1 μmol l^{-1} (Fig. 6). This result suggests that H^+ V-ATPase activity becomes a candidate for energization of cell membranes in *H. cecropia* follicles, as has been established for goblet cells in the midgut of *Manduca sexta* (reviewed by Harvey and Wieczorek, 1997).

Two models of how an increase in bafilomycin-sensitive proton pumping could be elicited by Sp-cAMPS were considered. One called for a molecular modification of the pump itself or its translocation to the cell surface, and the second for an increase in the availability of protons serving as

Table 3. *The effects of Sp-cAMPS, bafilomycin-A and azide on membrane potentials and the cytoplasmic pH of vitellogenic follicles*

Additions to PSS	Follicle cells ΔΨ (mV)	Oocytes ΔΨ (mV)	Oocytes pH
None	−38.0±1.3	−36.0±1.3	7.26±0.01
Sp-cAMPS	−52.6±1.9	−47.0±1.5	7.06±0.01
Bafilomycin	−14.3±1.0	−13.0±0.8	7.02±0.02
Sp-cAMPS and bafilomycin	−20.8±1.1	−20.0±1.7	6.92±0.02
Azide	−13.0±0.7	−11.5±1.2	6.79±0.02
Sp-cAMPS and azide	−21.7±2.2	−16.0±1.3	6.69±0.02

Each entry is a mean value for 8–10 follicles measured after incubation for 30 min in PSS containing the substances shown. In every case, values obtained in Sp-cAMPS were significantly different from those obtained in its absence (P<0.01, except in the case of membrane potential (ΔΨ) for the oocytes, where P<0.05).

Concentrations were 1 mmol l^{-1} for Sp-cAMPS, 150 nmol l^{-1} for bafilomycin-A and 10 mmol l^{-1} for sodium azide.

Similar results were seen in follicles from two other females.

Values are means ± S.E.M.

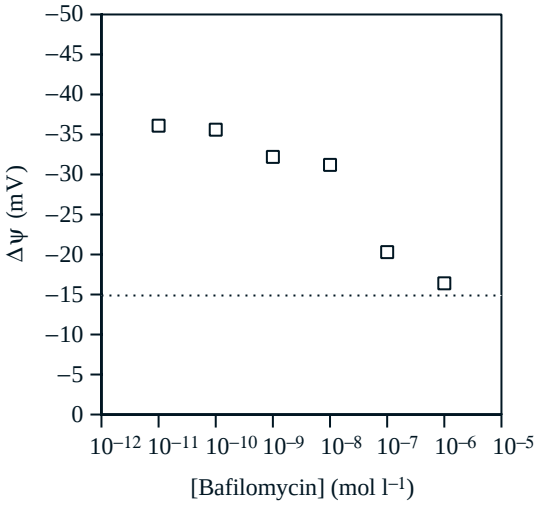

Fig. 6. Sensitivity of the membrane potential (ΔΨ) to bafilomycin-A. Each point is the average for 8–10 follicles (values are means ± S.E.M.; error bars are not visible because standard errors are smaller than the diameter of the points). The broken line represents the passive membrane potential seen in follicles incubated in 10 mmol l^{-1} sodium azide. Electrode tips were positioned in the cytoplasm of the oocyte.

substrate. Since the two would entail opposite effects on cytoplasmic pH, an experimental distinction between them was possible: molecular modification that increased the efficiency of the pump, or insertion of new units into the cell surface, should produce a rise in cytoplasmic pH, whereas an increase in the amount of protons should be detected as a fall in pH. After 30 min in Sp-cAMPS, when hyperpolarization was at a maximum, cytoplasmic pH in the oocyte had dropped from 7.26±0.01 to 7.06±0.01 (Table 3) (P<0.001), suggesting that cytoplasmic acidification promotes hyperpolarization.

Sp-cAMPS induced partial but significant membrane repolarization in follicles treated with either 150 nmol l^{-1} bafilomycin or 10 mmol l^{-1} azide, and here again cytoplasmic acidification occurred (Table 3). Since the concentration of bafilomycin used in this experiment would not have completely suppressed the active component of ΔΨ (Fig. 6), acidification would presumably allow residual electrogenic enzyme activity to produce the observed partial repolarization. An increase in proton availability should also have enhanced electrogenic enzyme activity in 10 mmol l^{-1} azide, since this reagent should inhibit only mitochondrial ATP production and not the generation of ATP by glycolysis. A fundamental question arising from this model, therefore, is the origin of the protons produced in response to Sp-cAMPS.

Further effects of 9-AC

That the increase in Cl^- conductance plays a pivotal role in the response to PKA activation was indicated by additional effects of 9-AC. This Cl^- channel blocker prevented not only the increase in 10 min loading of Cl^- (Table 1), but also hyperpolarization (Table 1, right-hand column), follicle cell swelling (Table 4) and the termination of vitellogenin uptake (not shown). Like the differing effects of PKA activation on

Table 4. *Inhibition of the effect of Sp-cAMPS on follicle cell height*

Additions to PSS	Follicle cell height (μm)	Follicle cell height (% change due to Sp-cAMPS)
None	44.5±1.5	–
Sp-cAMPS	54.5±1.5*	+22.5
9-AC	45.4±1.6	–
9-AC + Sp-cAMPS	43.6±0.6	–0.2
Furosemide	43.0±2.7	–
Furosemide + Sp-cAMPS	54.8±2.2*	+27.4
None	40.3±1.8	–
Sp-cAMPS	49.6±1.0*	+23.1
Bafilomycin	38.1±1.5	–
Bafilomycin + Sp-cAMPS	39.4±1.8	+3.4
Amiloride	36.1±0.3	–
Amiloride + Sp-cAMPS	50.4±1.0*	+39.6

Measurements were made after incubation for 2 h in PSS containing the additions shown in the left-hand column. Concentrations were 2 mmol l^{-1} for Sp-cAMPS, 1 mmol l^{-1} for 9-AC, 1 mmol l^{-1} for furosemide, 150 nmol l^{-1} for bafilomycin-A and 0.5 mmol l^{-1} for amiloride.

The two sections of the table represent experiments carried out on different dates.

*Value obtained in the presence of Sp-cAMPS was significantly greater than that obtained in its absence (P<0.01).

Values are means ± S.E.M., N=10.

$^{36}Cl^-$ and $^{86}Rb^+$ uptake (Fig. 4), the variety of responses blocked by 9-AC implies that the role of Cl^- in water absorption is more complex than simply as a counterion for K^+ uptake.

Contrast with some water-secreting epithelia

In a number of insect epithelia concerned with fluid secretion, an amiloride-sensitive cation/H^+ antiporter couples the export of cations to the down-gradient return of H^+ to the cytoplasm. The secreted cation is replaced in the cytoplasm from the basal extracellular environment through any of a variety of membrane channels or pumps (reviewed in Harvey *et al.* 1998.) In Malpighian tubules of *Rhodnius prolixus*, for instance, 5-hydroxytryptamine-stimulated fluid secretion is retarded both by amiloride acting at the apical surface of the epithelium (Maddrell and O'Donnell, 1992) and by furosemide, an inhibitor of $Na^+/K^+/Cl^-$ cotransport at the basal surface (O'Donnell and Maddrell, 1984). However, neither amiloride nor furosemide affected Sp-cAMPS-induced fluid uptake in follicle cells (Table 4). Despite their mutual reliance on an H^+ V-ATPase for energization of fluid movements, ancillary mechanisms of cation transport are presumably very different between these cases of fluid uptake and transcellular secretion.

Discussion

Sites of PKA activation and hyperpolarization

The restriction of swelling to the follicle cells during the 4 h incubations used in this study is consistent with the normal timetable of post-vitellogenic development. *In situ*, the oocyte also undergoes a 50 % volume increase, but not until several hours after follicle cell swelling has been completed (Y. Wang and W. Telfer, unpublished observation). That the follicle cells initiate the response to PKA activation *in vitro* is consistent with the finding that they contain 90 % of the total follicular PKA activity (Wang, 1996).

When sections of vitellogenic follicles from *Manduca sexta* were stained with monoclonal antibodies against the 20 kDa subunit of an H^+ V-ATPase (Janssen *et al.* 1995), the stain was localized both in the cortex of the oocyte and at the outer or basal surface of the follicular epithelium. The former would be expected, since vesicular proton loading, a general feature of endocytosis, is required for the transfer of vitellogenin from endosomes to yolk bodies (Stynen *et al.* 1988). The follicle cell surface, by contrast, is much less active in endocytosis, and localization here is more consistent with a role for H^+ V-ATPase in generating follicular membrane potentials.

Follicle cells and oocyte communicate through gap junctions (Woodruff, 1979), and any ionic changes induced in one should therefore be transmitted to the other. This supposition is supported by the observations recorded in Tables 2 and 3 that $\Delta\Psi$ measured with electrodes in the follicle cells was consistently within 1–4 mV of that recorded in the oocyte. The epithelium was always the more negative of the two compartments, however, and this in turn is consistent with a role in generating the active component of $\Delta\Psi$.

The consequences of PKA activation

Vitellogenic follicles responded to incubation in Sp-cAMPS by increasing their Cl^--based membrane conductance, by cytoplasmic acidification, by a membrane hyperpolarization strong enough to overcome the expected depolarizing effects of Cl^- conductance and by an increase in the rate of K^+ uptake, as indicated by accelerated $^{86}Rb^+$ loading. The challenge now is to determine how these changes are related to each other and to the 50 % increase in follicle cell volume.

The increase in Cl^- conductance in response to cyclic AMP analogs is a fundamental change in the ion physiology of vitellogenic follicles, since ion substitution experiments have shown that Cl^- contributes little to $\Delta\Psi$ during yolk deposition (Woodruff *et al.* 1992 for *H. cecropia*; O'Donnell and Sharda, 1994, for *Rhodnius prolixus*). It is a necessary step in the response to Sp-cAMPS because, as noted above, 9-AC and Cl^--free medium blocked both hyperpolarization and follicle cell swelling.

An earlier study using ion-selective microelectrodes (Woodruff *et al.* 1992) showed that an increase in the conductance for Cl^-, H^+, Ca^{2+} or Mg^{2+} should depolarize vitellogenic follicles. The equilibrium potential for K^+, the principal permeant cation, is close enough to $\Delta\Psi$ so that a change in conductance would in this case have little effect. K^+ substitution, in any event, indicated that this cation contributes less than Cl^- to the early increase in conductance (Fig. 2). Hyperpolarization is therefore more probably due to the active fraction of $\Delta\Psi$ that can be inhibited with azide.

The finding that bafilomycin depolarized cell membranes in the follicle to approximately the same degree as azide suggests that the electrogenic effects of H^+ extrusion account for most of this fraction of $\Delta\Psi$. Observations on cytoplasmic pH have supported this idea. In an earlier study (Stynen *et al.* 1988), $\Delta\Psi$ became more negative in synchrony with a fall in cytoplasmic pH when follicles were incubated with the cation/H^+ exchange ionophores nigericin and monensin. The response was inhibited by azide and thus depended on metabolic energy. Interpretation of this relationship focused at the time on the ability of proton ionophores to inhibit endosome processing during vitellogenin uptake. Subsequently, O'Donnell and Sharda (1994), working with follicles of *R. prolixus*, showed that experimental acidification was accompanied by hyperpolarization of follicle membranes. Their analysis went on to provide evidence for a proton pump that generated approximately 10 % of the membrane potential. The fraction of $\Delta\Psi$ attributable to the proton pumps in *H. cecropia* was greater than in *R. prolixus*, approximately 60 % as opposed to 10 %, but this may be a manifestation of evolutionary adaptation to a Na^+-poor diet which, in moths, has led to a high-$[K^+]$/low-$[Na^+]$ hemolymph and to the use of proton pumps to energize cell membranes in place of the more conventional ouabain-inhibitable Na^+/K^+-ATPase (Wieczorek *et al.* 1986).

The drop in pH that occurs after PKA activation should by itself promote electrogenesis since it indicates an increase in substrate availability to the H^+ V-ATPase. An additional

Table 5. *Summary of physiological responses evoked by PKA activation*

Response	Source of evidence
Activation of PKA with Sp-cAMPS	Wang and Telfer (1996, 1997)
Increased Cl^- conductance (Cl^- required for swelling)	Figs 2, 3 (Tables 1, 4)
Acidification of cytoplasm	Table 3
Hyperpolarization (correlated with acidification)	Tables 1–3; Fig. 5 (Table 3; Stynen *et al.* 1988; O'Donnell and Sharda, 1994, for *Rhodnius prolixus* follicles)
Increased uptake of K^+ (K^+ required for swelling)	Fig. 4A (Table 2)
Fluid uptake	Fig. 1; Tables 2, 4; Wang and Telfer (1997)

PKA, cyclic-AMP-dependent protein kinase.

Parentheses in the right-hand column enclose the sources of evidence for the statement enclosed in parentheses in the left-hand column.

mechanism is also possible, however. In mammalian nephrons and a number of other epithelia, acidification promotes the insertion of cytoplasmic vesicles containing H^+ V-ATPase into apical cell membranes of mitochondria-rich cells. In the nephron, this mechanism promotes proton removal from the blood and secretion into the urine (reviewed by Brown and Breton, 1996). While acidification following PKA activation of follicles is generated by the cells themselves, rather than being imposed externally, vesicle insertion into plasma membranes could well be an additional factor in hyperpolarization.

The steps leading to fluid uptake in response to Sp-cAMPS are summarized in Table 5. Activation of PKA by this and other analogs of cyclic AMP was demonstrated in the references indicated and was highlighted by the inability of the Rp-stereoisomer of cAMPS to block vitellogenin uptake or to induce follicle cell swelling. The increase in Cl^- conductance is presumably the consequence of a phosphorylation cascade initiated by PKA activation in the follicle cells. For technical reasons, cytoplasmic acidification was measured only in the oocyte; we presume that it reflects primarily proton diffusion across gap junctions, possibly from even more strongly acidified follicle cells. For the reasons described above, hyperpolarization is presumed to result from an increased substrate availability to the electrogenic proton pump, although the possibility that the lower pH promotes vesicle insertion into the plasma membranes is not ruled out. Hyperpolarization could, in principle, account for the accelerated uptake of K^+, but the specific channels involved are undefined and the possibility of another mode of energization is not ruled out. Swelling occurs first in the follicle cells because PKA and plasma-membrane-associated H^+ V-ATPase are concentrated there, and perhaps also because these cells are surrounded by spaces into which they can readily expand; oocyte swelling, in contrast, would be constrained by the vitelline envelope, whose mechanical loosening would be essential for the final swelling of the follicle that is observed *in situ*.

Several basic questions are left unanswered by these considerations. Are the increases in Cl^- conductance and acidification separate effects of PKA activation, or does one generate the other? Does acidification result from metabolic changes that increase organic acid production or from proton leakage across membranes? How is K^+ uptake accelerated and how are its electrical effects neutralized? And, central to understanding hydration in the context of ovarian development *in situ*, how are individual follicles stimulated to generate these changes at an appropriate stage of egg formation?

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