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Cyclic AMP and Intracellular Ionic Activities in *Necturus* Gallbladder

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Summary. Open-tip and liquid ion-exchanger microelectrodes were used to study the effects of cAMP (6 mM, added to the serosal medium) on apical membrane potential (E_m) and intracellular sodium, potassium, and chloride activities (a^i_{Na}, a^i_K, a^i_{Cl}) in *Necturus* gallbladder under open-circuit conditions. Transepithelial potential difference (E_{Tr}) was also measured. In the presence of cAMP, a^i_{Cl} fell from about 1.5 times its equilibrium value to a level that corresponded to electrochemical equilibrium across the apical and basolateral cell membranes. Under these conditions a^i_{Na} decreased and a^i_K increased, E_m was unchanged and E_{Tr} increased from virtually zero to a small but significant serosal positive value. The cAMP-induced increase in a^i_K was abolished when Cl^--free incubation media were used. Addition of the Ca^{++}-ionophore A23187 (0.5 μg/ml) to the serosal medium had no effect on E_m, E_{Tr}, or a^i_{Cl}. When A23187 was added to the mucosal medium, E_m and the basolateral membrane potential hyperpolarized by about 20 mV and an increase in the outwardly directed electrochemical driving force for Cl^- was observed. These results indicate that cAMP inhibits coupled transapical Na–Cl entry into epithelial cells of *Necturus* gallbladder and suggest that this inhibition may not be mediated by an increase in intracellular Ca^{++} concentration.

Key words: *Necturus* gallbladder, cyclic AMP, calcium ionophore, intracellular ionic activities, membrane potentials, liquid ion-exchanger microelectrodes

It is well known that cAMP, or agents that increase intracellular cAMP levels (e.g. theophylline, vasoactive intestinal peptide, cholera and heat-labile *E. coli* enterotoxins) induce fluid and electrolyte secretion by intestinal tissue (Nellans, Frizzell & Schultz, 1974; Field, 1978). The action of cAMP on the intestine appears to involve two different effects. These are (*i*) inhibition of coupled electroneutral Na–Cl transport across the apical membranes of the absorptive cells (an anti-absorptive effect), and (*ii*) direct stimulation of anion secretion (Field, 1979). It has been suggested that these two effects occur in different cell types of the intestinal epithelium, the anti-absorptive effect in the villous cells and the secretory process in the crypt cells (Field, 1979).

Addition of the Ca^{++}-ionophore, A23187, to the serosal side of stripped, isolated rabbit ileum (Bolton & Field, 1977) or to the mucosal side of the rabbit colon (Frizzell, 1977) produced changes in ionic fluxes that were qualitatively similar to those elicited in the same tissues by cAMP. However, unlike the action of cAMP, the effects induced by A23187 were obtained only when Ca^{++} was present in the external medium. These results suggest that the action of cAMP on ionic fluxes in intestinal epithelia is mediated by an increase in cytoplasmic Ca^{++} levels (Bolton & Field, 1977; Frizzell, 1977). However, the question whether the anti-absorptive effect of cAMP, its secretory effect, or both of these are mediated by an increase in cytoplasmic Ca^{++} remains open. The present experiments were designed to obtain further information on this point. Since only the anti-absorptive effect of cAMP is observed in the epithelial cells of the gallbladder (Frizzell, Dugas & Schultz, 1975; Heintze, Petersen, Olles, Saverymuttu & Wood, 1979) we compared the effects of cAMP and A23187 on the transepithelial potential difference (E_{Tr}), apical cell membrane potential (E_m), and intracellular K^+, Na^+ and Cl^- activities (a^i_K, a^i_{Na}, a^i_{Cl}) in this tissue.

Materials and Methods

Necturus maculosus were obtained from Graska Biological Supplies (Oshkosh, Wis.). They were kept in a large aquarium at 4 °C and

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Table 1. Intracellular chloride, potassium and sodium activities in *Necturus* gallbladder: effect of 6 mM cAMP

	E_m (mV)	E_{Tr} (mV)	a^i_{Cl} (mM)	a^i_K (mM)	a^i_{Na} (mM)
Control	-52 ± 2 (13)	-0.1 ± 0.2 (13)	14 ± 2 (4)	84 ± 5 (5)	9 ± 1 (4)
+6 mM cAMP	-50 ± 2 (13)	$+0.8\pm0.2$ (13)	9 ± 1 (4)	113 ± 6 (5)	6 ± 1 (4)
P	n.s.	<0.01	<0.05	<0.01	<0.05

The paired *t*-test was used. Mean values ±SEM are given. Number in parentheses is the number of tissues studied.

were fed live minnows. Animals were killed by a blow on the head. The gallbladder was removed and mounted as a flat sheet in a divided chamber (White & Armstrong, 1971) at 23 °C. The mucosal and serosal sides of the tissue were perfused continuously and independently. The perfusion system permitted the solutions bathing the mucosal and serosal aspects of the tissue to be changed rapidly. The reservoirs containing these solutions were bubbled with 100% O_2 throughout the experiment. The control Ringer's solution contained, in mM, NaCl 100, KH_2PO_4 0.8, K_2HPO_4 2.3, Ca gluconate 1.8, and mannitol 21. The pH was 7.2. In experiments with Cl^--free media, Na gluconate was substituted for NaCl. Adenosine 3′:5′-cyclic-monophosphoric acid (cAMP; Sigma, St. Louis, M.) was added, at a concentration of 6 mM, to the serosal bathing medium. When this was done, the pH was adjusted to 7.2 with Tris and, to maintain the same osmolality as that of the mucosal medium, the mannitol content was reduced to 9 mM. Stock solutions of A23187 (Calbiochem, La Jolla, Calif.) were prepared by dissolving the ionophore in 95% ethanol. The final concentration of ethanol in the bathing medium was 0.1%. It was established by separate experiments that ethanol, at this concentration, did not affect the electrophysiological characteristics of the tissue.

E_{Tr} was measured by two calomel half-cells connected through 3 M KCl/agar bridges to the mucosal and serosal solutions, respectively. The outputs of these half-cells were connected to a digital voltmeter (model 1350, Data Precision, Wakefield, Mass.) and to one channel of a dual-channel strip chart recorder (Sargent-Welch Scientific Co., Skokie, Ill.). The mucosal half-cell was grounded and used as a reference. The same reference was used for measuring the potentials recorded with microelectrodes. These were connected through an Ag/AgCl wire and a guarded cable to the input of a high impedance preamplifier (model 311J, Analog Devices, Norwood, Mass.; input resistance $> 10^{14}$ Ω) with unit gain. The potentials recorded by the microelectrodes were recorded simultaneously on a second model 1350 digital voltmeter, the second channel of the strip chart recorder, and an audiomonitor.

Single-barreled micropipettes were drawn to a tip diameter of about 1 μm in a Narishige model PD-5 horizontal puller from "Kwik-Fil" borosilicate glass capillary tubes (o.d. 1.2 mm, i.d. 0.68 mm; W.P. Instruments, New Haven, Conn.) previously cleaned by boiling in a concentrated detergent solution (Garcia-Diaz & Armstrong, 1980). Micropipettes for fabricating open-tip and liquid ion-exchanger microelectrodes were drawn under identical conditions. Open-tip micropipettes for measuring apical membrane potentials (E_m) were back-filled with 3 M KCl. Their tip resistances ranged from 20–30 MΩ in normal Ringer's. Liquid ion exchanger microelectrodes were used to measure a^i_K, a^i_{Na}, and a^i_{Cl}. Corning 477317 and 477315 exchangers were used in K^+- and Cl^--selective microelectrodes. Na^+-selective microelectrodes were as described elsewhere (O'Doherty, Garcia-Diaz & Armstrong, 1979). Ion-selective microelectrodes were prepared by the method of Fujimoto and Kubota (1976) as modified by Garcia-Diaz and Armstrong (1980).

Liquid ion-exchanger microelectrodes were calibrated in electrolyte solutions with concentrations covering the physiological range. Details of the calibration procedure, determination of selectivities, and the measurement of intracellular ionic activities have been given elsewhere (Garcia-Diaz & Armstrong, 1980). In the present study, it was found by direct measurement that the addition of 6 mM cAMP to the calibrating solutions did not affect the response of K^+-, Na^+-, or Cl^--selective microelectrodes. To correct for interference by intracellular K^+, Na^+-selective microelectrodes were calibrated in solutions containing different amounts of Na^+ together with a fixed amount of K^+. The K^+ concentrations of these solutions were chosen to approximate that of the cytoplasm. Since an increase in a^i_K, in the presence of cAMP, was found in the present study, Na^+-selective microelectrodes were calibrated in two sets of solutions containing different K^+ concentrations. For control conditions, the calibrating solutions contained 110 mM K^+. For experiments with cAMP, 145 mM K^+ was used. These K^+ concentrations correspond closely to the mean a^i_K values found under these two sets of conditions (Table 1).

Individual experiments were performed as follows: after mounting the tissue under open-circuit conditions in the chamber, 60 min were allowed for the establishment of a steady state under control conditions. E_m and the intracellular activity of one ion (K^+, Na^+ or Cl^-) were then measured. Following this, one of the bathing solutions was changed for another that contained either cAMP (serosal side only) or A23187 (either the mucosal or serosal side), 45–60 min were allowed for the establishment of a new steady state, and the above parameters were again measured. E_{Tr} was recorded continuously throughout the experiment. Microelectrodes were mounted perpendicularly to the tissue on a micromanipulator (MM 33, Narishige, Japan). This was used to bring the microelectrode close to the mucosal surface of the tissue. Final movement of and cell impalement with the microelectrode were accomplished under microscopic observation (Stereo-microscope III, Zeiss, New York, N.Y.) with a hydraulic drive micromanipulator (MO-10, Narishige, Japan). During experiments with open-tip microelectrodes a repetitive current pulse (0.5 nA, 1.2 sec) was applied at 3-sec intervals. In this way the input resistance of the microelectrodes used to measure E_m was continuously monitored. Criteria for accepting impalements with open-tip and ion-selective microelectrodes were as reported elsewhere (Garcia-Diaz & Armstrong, 1980). For each intracellular measurement, a minimum of 4 or 5 acceptable impalements was recorded with individual tissue preparations.

Results

Table 1 shows the effect of 6 mM cAMP, added to the serosal medium, on E_m, E_{Tr}, a^i_{Na}, a^i_{Cl}, and a^i_K in *Necturus* gallbladder. It is apparent from this Table that E_m is not significantly affected by cAMP. E_{Tr} changed from virtually zero to a small but significant serosal positive value. Similar effects of cAMP on E_m and E_{Tr} in isolated bullfrog small intestine were reported by Armstrong and Youmans (1980).

Table 1 also shows that both a^i_{Cl} and a^i_{Na} were significantly decreased by cAMP. The intracellular Cl^- activity (a^{eq}_{Cl}) that is in electrochemical equilibrium with the mucosal bathing medium is given by the equation:

$$E_m = (RT/F) \ln (a^{eq}_{Cl}/a^o_{Cl}) \quad (1)$$

where a^o_{Cl} is the Cl^- activity in the bathing medium and R, T and F have their usual meanings. For $E_m = -50$ mV and $a^o_{Cl} = 77$ mM, as in the present experiments, one obtains $a^{eq}_{Cl} = 10$ mM. Therefore, a^i_{Cl} in the presence of cAMP (9 ± 1 mM) did not differ from its equilibrium value across the apical cell membrane. Furthermore, since E_m was small (< 1 mV) under these conditions, intracellular Cl^- was also in electrochemical equilibrium with respect to the basolateral cell membrane. This, together with the observed decrease in a^i_{Na} (Table 1), strongly suggests that cAMP inhibits coupled transapical Na−Cl entry into the epithelial cells of *Necturus* gallbladder. In media containing Cl^-, cAMP significantly increased a^i_K (Table 1). However, when external Cl^- was completely replaced by gluconate (Table 2), cAMP had no effect on a^i_K.

Table 3 summarizes the results obtained with the Ca^{++} ionophore A23187. It is clear from this Table that significant effects were obtained only when A23187 was present in the mucosal medium. Under these conditions, E_m was hyperpolarized by about 20 mV. Since E_{Tr} did not change, it may be concluded that the basolateral membrane potential was hyperpolarized to the same extent. Although the absolute value of a^i_{Cl} decreased, its ratio to the equilibrium value (a^i_{Cl}/a^{eq}_{Cl}) increased significantly. In other words, the outwardly directed driving force for Cl^-, defined as $(E_{Cl} - E)$ where $E_{Cl} = (RT/F) \ln a^i_{Cl}/a^o_{Cl}$, and E is the apical or basolateral cell membrane potential, increased from about 11 to about 20 mV.

Table 2. Effect of cAMP on intracellular potassium activity in Cl-free media

	N	E_m (mV)	E_{Tr} (mV)	a^i_K (mM)
Control	3	-48 ± 2	$+1.0 \pm 0.1$	80 ± 5
+6 mM cAMP	3	-48 ± 1	$+1.0 \pm 0.1$	80 ± 5
P		n.s.	n.s.	n.s.

N is the number of tissues studied. The paired t-test was used. Values are given as mean ± SEM.

Discussion

The average value of E_m found in the present study under control conditions was about 50 mV (inside negative). This agrees with the value (52.6 mV) previously reported for the same preparation under identical conditions (Garcia-Diaz & Armstrong, 1980). Somewhat higher values for E_m in *Necturus* gallbladder have been reported by others (Frömter & Diamond, 1972; Reuss & Finn, 1975; Van Os & Slegers, 1975), but when the high K^+ permeability of the apical cell membrane (Reuss & Finn, 1975) and the different external K^+ concentrations used are taken into account, these values are consistent with that reported in the present study.

Under control conditions a^i_{Cl} exceeded the level corresponding to electrochemical equilibrium across the apical cell membrane. This agrees with the results of earlier studies with *Necturus* gallbladder (Reuss & Grady, 1979; Garcia-Diaz & Armstrong, 1980) and other leaky epithelia (Armstrong, Bixenman, Frey, Garcia-Diaz, O'Regan & Owens, 1979; Duffey, Turnheim, Frizzell & Schultz, 1978; Spring & Kimura, 1978; Duffey, Thompson, Frizzell & Schultz, 1979). Under these conditions, the ratio of a^i_{Cl} to its equilibrium value, a^i_{Cl}/a^{eq}_{Cl} found in the present experiments ranged from 1.4 to 1.6. This is close to the value

Table 3. Effect of A23187 (0.5 μg/ml) on intracellular chloride accumulation

	N	E_m (mV)	E_{Tr} (mV)	a^i_{Cl} (mM)	a^{eq}_{Cl} (mM)	a^i_{Cl}/a^{eq}_{Cl}
Group 1						
Control	5	-46 ± 2	$+0.1 \pm 0.4$	19 ± 1	12 ± 2	1.6 ± 0.2
+A23187 (mucosal side)	5	-67 ± 1	$+0.1 \pm 0.4$	12 ± 1	5 ± 0.2	2.4 ± 0.2
P		< 0.1	n.s.	< 0.05	< 0.01	< 0.05
Group 2						
Control	5	-50 ± 2	-0.1 ± 0.1	18 ± 2	11 ± 1	1.6 ± 0.1
+A23187 (serosal side)	5	-50 ± 3	-0.1 ± 0.1	20 ± 2	11 ± 2	1.8 ± 0.1
P		n.s.	n.s.	n.s.	n.s.	n.s.

The paired t-test was used. Values are means ± SEM N is the number of tissues studied.

(1.8) reported earlier from this laboratory for *Necturus* gallbladder under identical conditions (Garcia-Diaz & Armstrong, 1980). Reuss and his associates (Reuss & Grady, 1979; Reuss & Weinman, 1979) reported a value of 4.3 for this ratio. It was suggested (Garcia-Diaz & Armstrong, 1980) that this discrepancy could be due to the absence of HCO_3^- from the bathing media used in our laboratory. In addition, it should be noted that the medium pH was higher in the studies of Reuss and his co-workers than it was in our experiments. It has been reported (Smith, Orellana & Field, 1980) that Na−Cl absorption by the isolated intestine of the winter flounder *(Pseudopleuronectes americanus)* is enhanced when the pH of the medium is increased.

The mean a^i_K and a^i_{Na} values found in the present experiments under control conditions (Table 1) agree with previous estimates of these parameters in isolated *Necturus* gallbladder (Graf & Giebisch, 1979; Reuss & Weinman, 1979; Garcia-Diaz & Armstrong, 1980).

The results summarized in Table 1 strongly indicate that cAMP inhibits coupled transapical Na−Cl entry in *Necturus* gallbladder as it does in other leaky epithelia (Nellans, Frizzell & Schultz, 1974; Frizzell, Dugas & Schultz, 1975). Because of this coupled entry process a^i_{Cl} is normally above its equilibrium value and intracellular Cl^- accumulation depends on the presence of Na^+ in the external medium. Thus, in rabbit gallbladder (Duffey et al., 1978), flounder intestine (Duffey et al., 1979), renal proximal tubule (Spring & Kimura, 1978) and *Necturus* gallbladder (Garcia-Diaz & Armstrong, 1980), a^i_{Cl}, in the absence of external Na^+, declines to the level corresponding to electrochemical equilibrium. In similar fashion (Table 1), a^i_{Cl} in the presence of cAMP decreased to a level that did not differ significantly from its equilibrium value. Furthermore, under these conditions (Table 1), a^i_{Na} decreased to a value close to that observed in Cl^--free media (Armstrong, Garcia-Diaz & Diez de los Rios, 1980). The constancy of E_m under these conditions (Table 1) confirms the fact that the transport process affected by cAMP is electroneutral. Finally, a mucosal-positive diffusion potential of about 1.2 mV across the tight junctions of isolated, actively transporting *Necturus* gallbladder has been reported (Curci & Frömter, 1979). The origin of this potential can be related to the cation-selective properties of the shunt pathway (Van Os & Slegers, 1975) and the hypertonic NaCl solution present in the intercellular spaces as predicted by the standing osmotic gradient theory of isotonic fluid absorption (Diamond & Bossert, 1967). Inhibition of coupled transapical Na−Cl entry could cause the solution in the intercellular spaces to become approximately isotonic. This, in turn, could abolish the diffusion potential across the tight junctions. The change in E_{T_r} produced by cAMP (0.9 mV serosal positive, Table 1) is consistent with the abolition of such a diffusion potential resulting from inhibition, by this agent, of coupled Na−Cl transport across the apical cell membrane. It may also reflect a small but finite electrogenic Na^+ absorption under these conditions since, in addition to coupled NaCl entry, some Na^+ crosses the apical cell membrane by a diffusive process (Reuss & Finn, 1975; Van Os & Slegers, 1975; Graf & Giebisch, 1979). Although A23187, added to the mucosal medium, reduced a^i_{Cl} below its control value (Table 3), a closer inspection of these results suggests that this reduction, unlike that caused by cAMP, may not be due to inhibition of coupled transapical Na−Cl entry. In the presence of cAMP (Table 1) a^i_{Cl} had declined, within 1 hr, to a value that corresponded to an equilibrium distribution of this ion across the apical and basolateral cell membranes ($a^i_{Cl}/a^{eq}_{Cl}=1$). By contrast, after exposure of the apical surface of the tissue to A23187 for 1–2 hr the ratio a^i_{Cl}/a^{eq}_{Cl} was 2.4. Since E_{T_r} was essentially zero, this corresponds to an outwardly directed driving force for Cl^-, across the basolateral cell membrane, of about 20 mV. Since the basolateral cell membrane of *Necturus* gallbladder has a finite Cl^- conductance (Reuss & Finn, 1975) it is difficult to reconcile a sustained electrochemical potential gradient of this magnitude with a situation in which transapical Cl^- entry is completely inhibited, or virtually so. Therefore, the results of the present experiments may be interpreted as indicating that A23187 does not mimic the anti-absorptive effect of cAMP in *Necturus* gallbladder.

Since a^i_{Cl} is normally above its equilibrium value (Table 1 and 3), diffuse Cl^- exit across the basolateral cell membrane is energetically downhill. Measurement of the basolateral Cl^- conductance in *Necturus* gallbladder indicates that only about 3 per cent of the measured rate of net transepithelial Cl^- transport in this tissue can be accounted for by simple diffusion across the basolateral cell membrane (Reuss, 1979). Similarly, it has been estimated that the paracellular route cannot account for more than about 8 per cent of the total transepithelial Cl^- flux (Reuss, 1979). Different electroneutral processes such as neutral transport of NaCl, neutral KCl transport, and electroneutral Cl^-/HCO_3^- exchange have been proposed to account for the observed Cl^- fluxes (Henin & Cremaschi, 1975; Van Os & Slegers, 1975; Reuss, 1979). The a^i_K values reported in the present paper (Tables 1 and 2) are consistent with the existence of electroneutral KCl transport across the basolateral cell membrane (Reuss, 1979; Reuss, Weinman & Grady, 1980). In the presence of cAMP, there is a significant increase in a^i_K (Table 1). This could be due

to the fact that cAMP reduces the outwardly directed driving force ($\Delta\mu_K+\Delta\mu_{Cl}$) for coupled KCl transport across the basolateral cell membrane. Under these conditions coupled KCl exit across this membrane could be reduced resulting in accumulation, to greater than normal levels, of intracellular K^+. In support of this conclusion it was found (Table 2) that, in the absence of extracellular Cl^-, cAMP did not affect a^i_K.

It might be argued that, if a reduction in coupled basolateral KCl transport could, as suggested above, result in enhanced accumulation of cellular K^+, then a^i_K should be significantly greater under Cl^--free conditions than in normal media. This was not the case in the experiments reported herein (Table 2). However, following a 60-min exposure to a Cl^--free medium it may be assumed that a^i_{Cl} has fallen to a very low level, not more than 3–4 mM at most (Garcia-Diaz & Armstrong, 1980). If, as seems likely, gluconate does not readily penetrate the cell membrane, electroneutrality considerations suggest that, during a relatively long incubation period in a Cl^--free medium, some cellular K^+ is also lost.

If the apical membrane of the absorptive cells in *Necturus* gallbladder behaved as a K^+ electrode, one would expect the cAMP-induced increase in a^i_K (Table 1) to be accompanied by a hyperpolarization of about 8–9 mV in E_m. The fact that E_m, in this and in an earlier study under identical conditions (Garcia-Diaz & Armstrong, 1980), was at all times well below the corresponding value of E_K, suggests a lower sensitivity of E_m to the transapical K^+ activity ratio than would be predicted by the Nernst equation. Nevertheless, the absence of any observable change in E_m during the experiments summarized in Table 1 is puzzling. It is however consistent with earlier observations under similar conditions (Garcia-Diaz & Armstrong, 1980). In that study it was found that E_m was unaffected by complete removal of Na^+ from the bathing medium even though a^i_K decreased by about 15 mM following exposure of the tissue to Na^+-free media.

In media containing Ca^{++}, cAMP and A23187 produce qualitatively similar changes in the net transport of Na^+ and Cl^- by rabbit ileum and in net Cl^- transport by rabbit colon (Bolton & Field, 1977; Frizzell, 1977). However, analysis of the effects of these agents on the unidirectional fluxes of Na^+ and Cl^- shows that this similarity arises from the fact that, in both tissues, cAMP and A23187 increase the movement of Cl^- in the secretory ($s \rightarrow m$) direction. These results and those reported in the present paper raise the interesting speculation that the anti-absorptive effects of cAMP in *Necturus* gallbladder are not Ca^{++}-mediated and that only secretory responses to this agent are related to cytosolic Ca^{++} levels. Further work, in particular a comparison, by direct measurement, of the effects of cAMP and A23187 on intracellular Ca^{++} activity, is required to test this hypothesis.

The mucosal hyperpolarization produced by A23187 (Table 3) has also been reported by Reuss, Bello-Reuss and Grady (1980) who attribute it to an increase in the K^+ conductance of both the apical and basolateral cell membranes produced by this agent. These increases in K conductance will increase the conductive K fluxes across both cell membranes. This could result in a decreased exit of K^+ and Cl^- across the basolateral cell membrane *via* a KCl coupled transport pathway. Because of this, and the relatively low Cl^- conductance of the basolateral cell membrane (Reuss, 1979) it is possible that, under these conditions, coupled transapical Na−Cl entry could sustain a higher than normal steady-state a^i_{Cl}/a^{eq}_{Cl} ratio. This agrees with the findings reported in Table 3. Another possibility is that intracellular Ca^{++} directly stimulates electroneutral Na−Cl coupled entry across the apical cell membrane. However, in the light of present evidence that Ca^{++} is a secretagogue and is implicated in a variety of secretory processes in many different tissues (Berridge, 1979), this possibility seems remote. Direct measurement of intracellular Ca^{++} activities in *Necturus* gallbladder and of the effect of cAMP on this parameter should provide further information on the relationship between cytosolic Ca^{++} levels and the effect of cAMP in this tissue.

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