J. Membrane Biol. 88, 113–121 (1985)

The Journal of
Membrane Biology

Intracellular Calcium Activity in Split Frog Skin Epithelium: Effect of cAMP

Ellie Kelepouris, Zalman S. Agus, and Mortimer M. Civan
Departments of Medicine and Physiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Summary. Measurement of intracellular calcium activity (a^c_{Ca}) by ion-selective microelectrodes has previously been technically limited to relatively large cells ($\gtrsim 20$ μm). We now report results obtained with this technique in the small epithelial cells ($\lesssim 10$ μm) of split frog skin using microelectrodes having an outer tip diameter of < 0.2 μm. The basolateral membrane potential was measured with Ca^{2+}-selective microelectrodes (E^{sc}_{Ca}) and with reference micropipettes (ψ^{sc}) either sequentially or simultaneously in 15 successful experiments. Under baseline conditions, a^c_{Ca} was measured to be 215 ± 39 nM (mean ± SE), in close agreement with the mean values estimated from published data obtained with *Necturus* proximal tubule. Stimulation of Na^+ transport across six skins with 1 mM serosal 8 *p*-chlorophenylthio-3′,5′ cyclic AMP (CPTcAMP) increased a^c_{Ca} by a factor of 2.6 ± 0.6. The increase in a^c_{Ca} preceded the CPTcAMP-induced increase in I_{sc}. The results of the present study indicate that electrometric determination of intracellular calcium activity is now feasible in a much wider range of cell systems than heretofore possible. CPT cAMP elevates intracellular Ca^{2+} activity; this phenomenon is an early event, preceding the natriferic effect of CPTcAMP.

Key Words ion-selective microelectrodes · short-circuit current · membrane potential · barium · basolateral impalements · cyclic-3′,5′ AMP

Introduction

Cytosolic calcium activity is a critical regulator of a wide range of cellular functions, including excitation-contraction and excitation-secretion coupling, intercellular communication, and exocytosis. Calcium may also play an important role in regulating transepithelial transport of solutes and water. For these reasons, considerable effort has been devoted to developing techniques for measuring intracellular calcium activity (a^c_{Ca}) (Tsien & Rink, 1983).

Optical techniques are not readily applied to classical models of transepithelial transport, such as frog skin and toad urinary bladder, because of the presence of multiple tissue layers within the preparation. Although, in principle, calcium-selective microelectrodes should permit direct monitoring of a^c_{Ca}, the technique has, until now, been successfully applied only to large epithelial cells, greater than 20 μm in diameter (Berridge, 1979; Lee et al., 1980*a*).

We now report the development of microelectrodes with very fine tips, and yet possessing high sensitivity and selectivity for calcium and adequately fast response times. The current manuscript reports the first measurements of intracellular calcium activity using these microelectrodes in small epithelial cells ($\lesssim 10$ μm in diameter) both under baseline transporting conditions and following stimulation of sodium transport with CPTcAMP.

Materials and Methods

Preparations and Solutions

The ventral abdominal skins were excised from doubly pithed frogs *Rana pipiens pipiens* obtained from West Jersey Biological Supply (Wenonah, N.J.). Before mounting, the underlying corium was removed using a modification of the technique of Fisher, Erlij and Helman (1980). The split frog skins were mounted inner surface up between the two halves of a Lucite® chamber and the skins were supported by a fine mesh stainless steel screen. Tissue areas of 1.9 cm^2 were exposed for experimental study. The serosal solution was aerated and both serosal and mucosal media were circulated separately, superfused across the tissue surfaces, and then discarded.

The serosal and mucosal media consisted of a standard Ringer's solution containing in mM: NaCl 116, $NaHCO_3$ 2.3, KCl 3.4, NaH_2PO_4 0.15, Na_2HPO_4 0.9, $MgCl_2$ 2, $CaCl_2$ 0.9. The solution had a pH of 7.6 and an osmolality of 226 mOsm per kg water.

Transepithelial potential (ψ^{ms}, serosa with respect to mucosa) was measured with calomel half-cells connected to the solutions by 3 M KCl-agar bridges. Transepithelial current (I_T, positive mucosa to serosa) was applied with two AgCl-coated silver wires. During the experiments, the tissue was continuously short-circuited except for brief periods (3 to 10 sec) of hyperpolarization during which ψ^{ms} was clamped to 10 mV. The resulting change in transepithelial current was used to calculate tissue resistance (R_T).

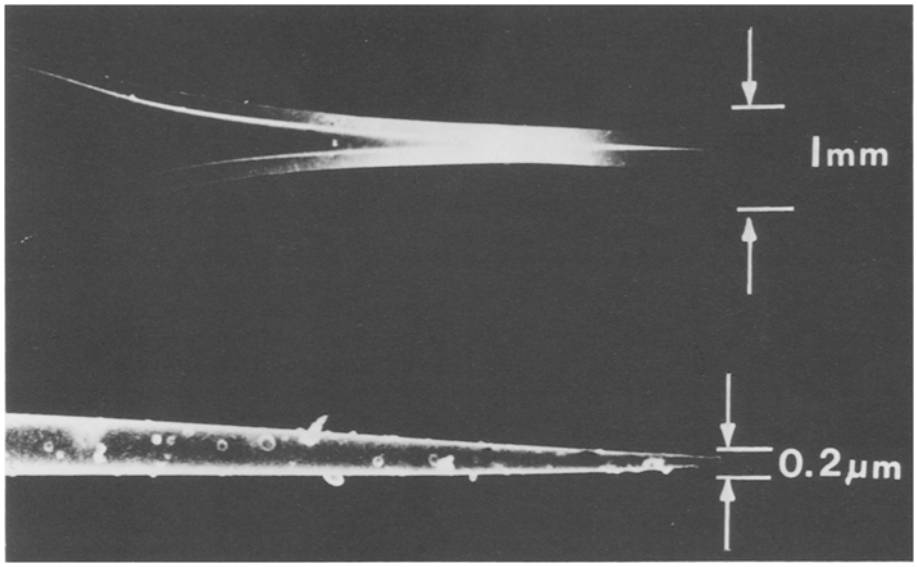

Fig. 1. Scanning electron micrograph of a single representative micropipette tip at two different magnifications (courtesy of Paul Norton and Dr. Donald R. DiBona). The outer tip diameter is <0.18 μm

Intracellular potentials (ψ^{sc}) were measured with reference to the serosal bathing solution. The outputs of the micropipettes and microelectrodes were introduced into a differential-input preamplifier (Model FD 223, WP-Instruments, Inc., New Haven, Conn.). The transepithelial current and the voltage outputs of the calcium-selective microelectrode (E^{sc}_{Ca}) and reference micropipette (ψ) were continuously monitored with two dual-pen, strip chart recorders and a storage oscilloscope.

Further details concerning the biological preparation and electronics employed have been published elsewhere (DeLong & Civan, 1983).

Micropipettes and Microelectrodes

Single-barrelled micropipettes and microelectrodes were drawn from omega-dot fiber-containing borosilicate capillary glass tubing having an outer diameter of 1.5 mm and an inner diameter of 0.75 mm (Glass Company of America, Millville, N.J.). A Brown-Flaming micropipette puller (Model P-77, Sutter Instrument Company, San Francisco, Calif.) was used. A scanning electron micrograph of the tip of a representative micropipette is shown in Fig. 1 demonstrating an outer diameter of < 0.18 μm. The reference open-tip micropipettes were filled with 0.5 M KCl solution and when tested in Ringer's solution, displayed resistances of 60 to 100 MΩ.

Ca^{2+}-selective microelectrodes were prepared by first baking the micropipettes at 170°C for 2 hr and then exposing the inner surface of the glass to hexamethyldisilazane vapor (Petrarch Systems Inc., Levittown, Pa.). The microelectrodes were subsequently baked at 170°C for an additional 30 min and then backfilled with a small amount of calcium-selective resin (WP-Instruments, New Haven, Conn.). After the tip filled spontaneously to a length of 400 μm, the barrel was backfilled with a reference solution of 100 mM $CaCl_2$.

Solutions of 1 to 2×10^{-3} through 1 to 2×10^{-7} M free Ca^{2+} concentration were used to calibrate the microelectrodes. In 13 of the 15 experiments, calibrations were performed both before and after impalements. In the initial experiments (1 to 6, Table 1), the calibration solutions were similar to those of Lee et al. (1980*a*), made from a Ca^{2+} buffer containing EGTA. The Ca^{2+} concentrations of the solutions were calculated with an apparent dissociation constant of 3.80×10^{-7} for Ca-EGTA (Marban et al., 1980) at a pH of 7.0. Additional solutions containing 10^{-3} and 10^{-4} Ca^{2+} were made by serial dilution of 0.1 M $CaCl_2$ solution. All the solutions contained 100 mM K^+, 10 mM HEPES, and 1 mM Mg^{2+} and had a pH of 7.0. The Ca-EGTA buffers additionally contained 10 mM acid EGTA (Sigma Chemical Company, St. Louis, Mo.). Ultrapure chemicals were used whenever possible. In experiments 7 through 15 (Table 1), the calibration solutions of Marban et al. (1980) were used. All solutions contained 100 mM K^+, 5 mM Ca^{2+}, and 10 mM calcium ligand: nitrilotriacetic acid for pCa 5, N-Hydroxyethylethylene-diaminetriacetic acid (HEDTA) for pCa 6, and EGTA for pCa 7. The solutions also contained 10 mM HEPPS (pCa 5), HEPES (pCa 6) and BES (pCa 7) and were titrated with N/10 KOH to the appropriate pH of 8.42, 7.70 and 7.29, respectively.

In the text below, the Ca^{2+} concentrations of solutions will be expressed either directly in mol · liter^{-1} or as the negative logarithm of that number (pCa).

The solutions all had the same ionic strength and hence, probably the same activity coefficient of Ca^{2+}. In this study, the Ca^{2+} activity coefficient of 0.35, calculated by the extended Debye-Hückel equation (Buttler, 1964) was used to obtain the calibration curve shown in Fig. 3. This procedure is justified because calculated and measured Ca^{2+} activity coefficients at various ionic strengths are in reasonably good agreement (Schonhorn & Gregor, 1961; Moore & Ross, 1965; Lee et al., 1980*a*).

The voltage outputs from both reference micropipettes and calcium-selective microelectrodes were sensed with chlorided silver wires. Cells were impaled across the basolateral membranes. Minimal criteria for acceptability of penetrations included: (1) rapid changes in voltage on entering and leaving the cell; (2) a steady potential, constant within 1 mV for at least 1 min; (3) constancy of the voltages measured by the reference micropipette and Ca^{2+}-selective microelectrode in the external Ringer's solution, before and after impalement, to within 3 mV

Table 1. Summary of intracellular and transepithelial electrophysiologic measurements in split frog skin epithelium

Preparation	I_{sc} ($\mu A \cdot cm^{-2}$)	R_T ($k\Omega \cdot cm^2$)	ψ^{sc} (mV)	$(f_o^{bl})_\psi$	E_{Ca}^{sc} (mV)	$(f_o^{bl})_E$	a_{Ca} (nM)
I	7.4	4.35	−57.6	0.37	−137.0	0.33	282
II	13.7	1.70	−67.6	0.22	−157.0	0.16	361
III	7.9	7.87	−72.6	0.12	−172.4	0.14	228
IV	7.4	3.01	−80.3	0.22	−168.0	0.09	490
V	4.2	3.01	−66.1	0.07	−189.0	0.09	17
VI	7.4	2.61	−78.9	0.05	−187.0	0.04	273
VII	13.7	3.17	−60.1	0.21	−179.9	0.08	63
VIII	21.1	4.22	−62.9	0.45	−173.5	0.35	126
IX	31.6	1.19	−63.2	0.20	−156.8	0.17	316
X	13.7	2.71	−71.8	0.12	−188.2	0.11	224
XI	15.8	2.38	−66.4	0.36	−181.5	0.30	50
XII	17.9	5.43	−84.8	0.12	−200.2	0.11	398
XIII	7.4	2.24	−75.4	0.08	−188.8	0.18	314
XIV	18.4	1.73	−65.7	0.18	−187.9	0.07	35
XV	4.2	1.65	−64.9	0.11	−191.2	0.08	48
Mean	13	3.2	−69	0.19	−177	0.15	215
± SE	±2	±0.5	±2	±0.03	±4	±0.03	±39

(usually less than 2 mV); and (4) similar estimates of basolateral fractional resistance measured with the reference micropipette and the Ca^{2+}-selective microelectrode. In addition, the intracellular position of many of the tips was verified by the transient addition of serosal $BaCl_2$ (DeLong & Civan, 1983) which reversibly depolarizes the membrane.

The intracellular calcium activity (a_{Ca}^c) was calculated utilizing the empirical relationship:

$$E_{Ca}^{sc} = S \log (a_{Ca}^c / a_{Ca}^s) + \psi^{sc} \quad (1)$$

where S is the sensitivity of the microelectrode and where the superscript (s) refers to the serosal phase. In general, S is not constant throughout the activity range of interest. Therefore, the calculation was carried out by relating the measured value of $(E_{Ca}^{sc} - \psi^{sc})$ and the known value of a_{Ca}^s to the calibration curve for E_{Ca}^{sc} as a function of a_{Ca}^s. All values recorded below are presented as the mean ± SE.

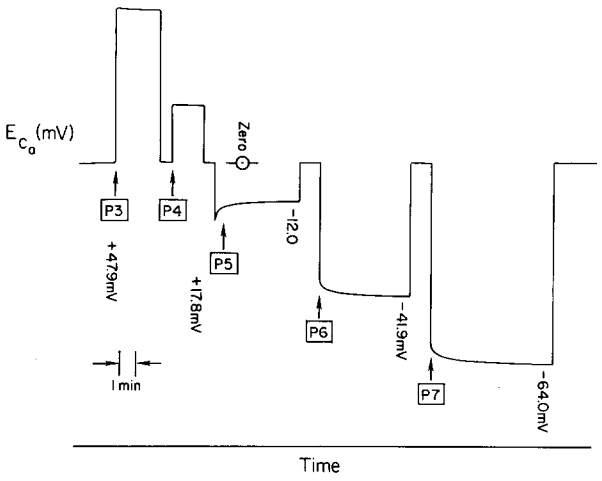

Fig. 2. Time courses of voltage output (E_{Ca}) from Ca^{2+}-selective microelectrode following changes in calibrating solutions

Results

The calibrating procedure for each microelectrode is illustrated in Figs. 2 and 3. Figure 2 demonstrates the time courses of the potential recordings of one representative microelectrode measured at the different concentrations of free Ca^{2+} from 1 to 2×10^{-3} through 1 to 2×10^{-7} M. Figure 3 presents three sets of calibration data obtained with the microelectrode of Fig. 2. The potentials recorded by the microelectrode are plotted against the calcium activity before and after intracellular impalements; the two post impalement calibrations were performed an hour apart. The slopes of the three calibration lines are the same to within 1 mV. Averaging the results for all 15 experiments, a decade change in Ca^{2+} concentration produced an electrode response of 33.5 ± 1.0 mV over the range 1 to 2×10^{-3} to 1 to 2×10^{-6} M, slightly in excess of the ideal Nernst slope of 29.6 mV; this phenomenon has been previously noted with some Ca^{2+}-selective microelectrodes (Lee & Im, 1984). Over the range in Ca^{2+} concentration of 1 to 2×10^{-6} to 1 to 2×10^{-7} M, the slope was 30 ± 2 mV. The ratio of the microelectrode sensitivity at pCa 6 to 7 to that at pCa 3 to 6 was 0.90 ± 0.04. As illustrated by Fig. 3*B*, the electrode response in the Ringer's solution was close to that

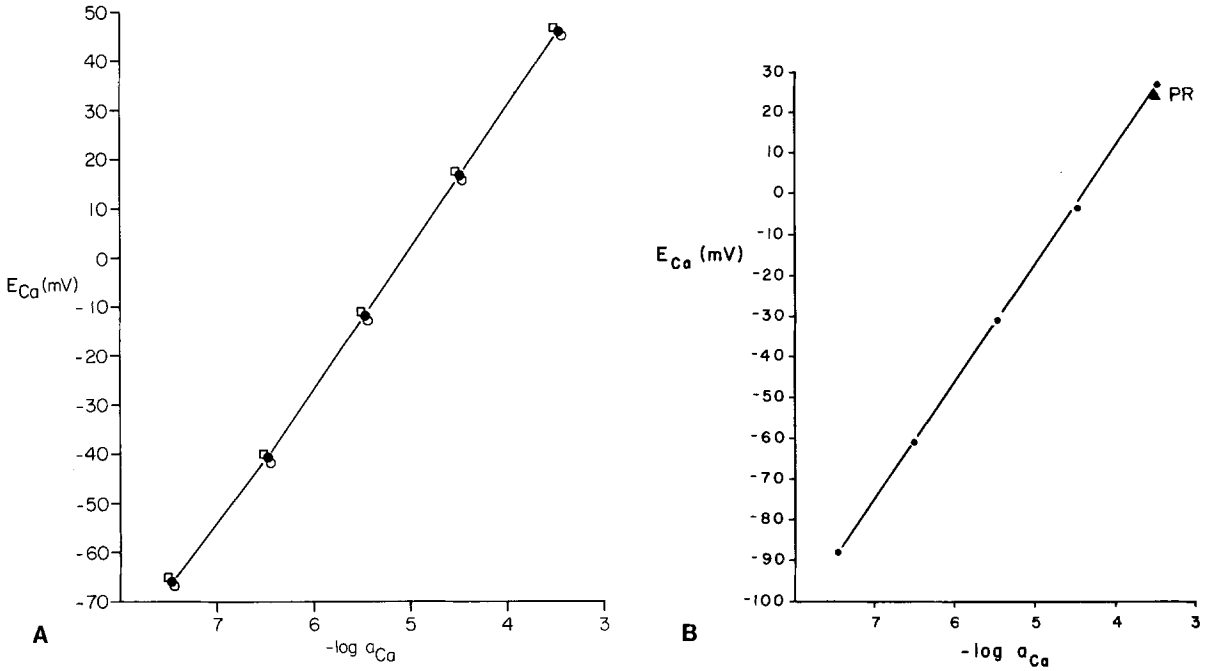

Fig. 3. *A.* Calibration relationship between voltage output from Ca^{2+}-microelectrode (E_{Ca}) and calcium activity (a_{Ca}) before (solid circles) and after (open square and circles) intracellular impalements. *B.* Calibration relationship between voltage output from Ca^{2+} microelectrode (E_{Ca}) and calcium activity in various calcium concentrations including Ringer's (PR) solution containing 0.9 mM Ca^{2+}

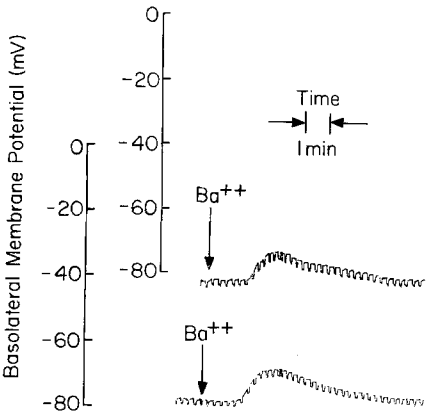

Fig. 4. Simultaneous recordings of basolateral membrane potentials using two reference micropipettes in two different cells before, during and after transient administration of 0.5 mM serosal Ba^{2+}

noted in the calibration solution at a pCa of 3. The adequacy of the response times of the electrodes can be assessed from Figs. 5 and 6; upon withdrawal from the cells, the 90% response times were 18 and 23 sec, respectively.

The syncytial properties of frog skin epithelium have been previously documented by electron probe X-ray microanalysis (Rick et al., 1978) and intracellular electrical recordings (Nagel, 1976; De-Long & Civan, 1983). This forms a rational basis for intracellular measurements using separate micropipettes and microelectrodes in different cells. In order to confirm this in our preparation, basolateral membrane potentials were recorded simultaneously with two reference micropipettes in separate cells (Fig. 4). The potentials recorded were −80 mV (identical within 1 mV) and stable for several minutes. The intracellular position of the micropipettes was verified by the addition of $BaCl_2$ to the serosal medium. This resulted in a reversible and similar depolarization of each membrane potential.

Our initial studies were performed by measuring ψ^{sc} with reference micropipettes and E^{sc}_{Ca} with the Ca^{2+}-selective microelectrode sequentially within the short-circuited frog skin epithelium. Subsequently, it proved possible to measure ψ^{sc} and E^{sc}_{Ca} simultaneously in 8 of the 15 preparations studied.

A representative experiment with simultaneous recordings is illustrated in Fig. 5, which presents ψ^{sc}, E^{sc}_{Ca} and I_{sc} as functions of time before, during and after applying 0.5 mM Ba^{2+}. With the reference micropipette within a cell interior, the Ca^{2+} microelectrode was introduced without resulting in a displacement of the ψ^{sc} signal. Following the rapid penetrations, the microelectrode voltages remained stable for a period of 5 min. Serosal $BaCl_2$ reversibly depolarized the membrane potential, sensed by the Ca^{2+} microelectrode and reference micropipette

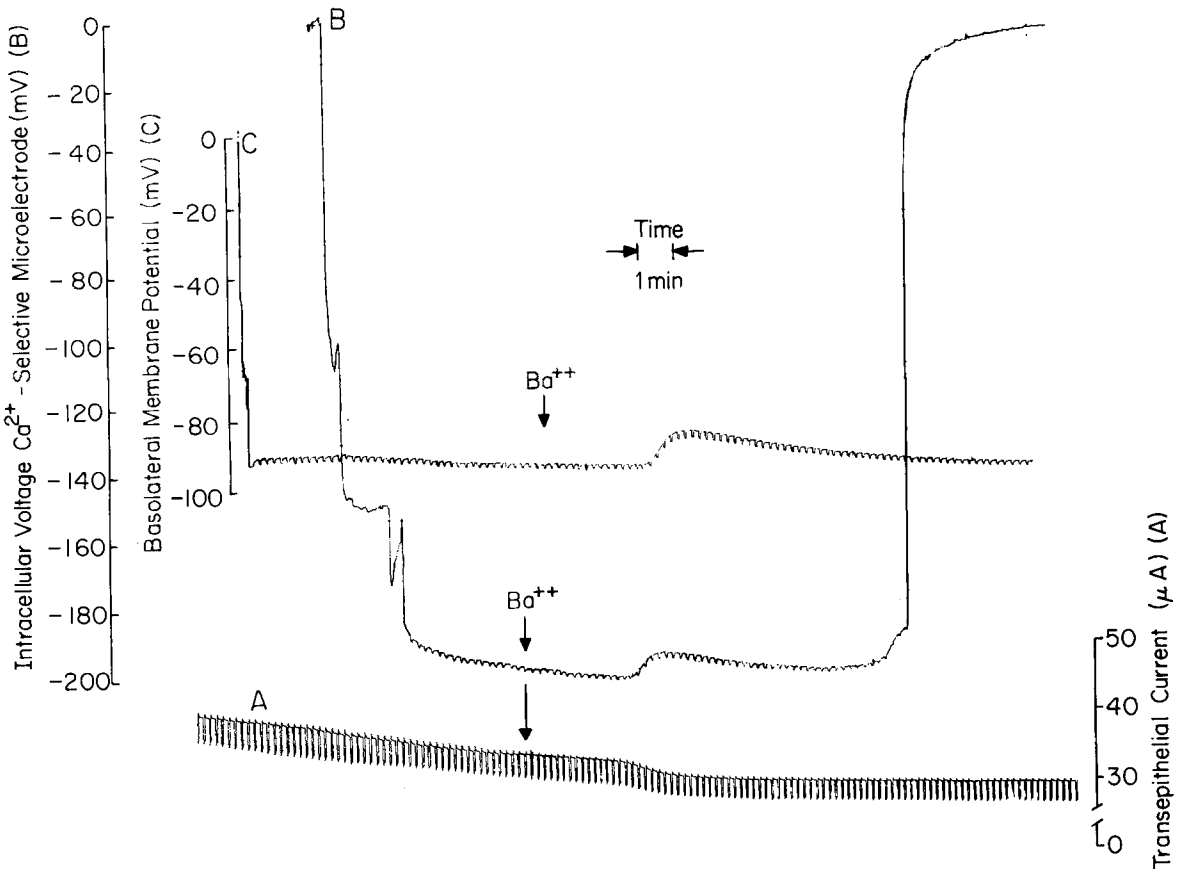

Fig. 5. Simultaneous recordings of transepithelial current (*A*) and the intracellular potentials measured with a Ca^{2+}-selective microelectrode (*B*) and a reference micropipette (*C*) before, during and after the serosal administration of 0.5 mM Ba^{2+}. The apparent displacement of the arrows for the E^{sc}_{Ca} and ψ^{sc} traces reflects the physical displacement of the pens on the chart recorder. Note that the mV/cm scale for the Ca^{2+}-selective microelectrode is slightly smaller than that depicted for the reference micropipette

to a similar extent, coincident with its effect to decrease the transepithelial current. When the microelectrodes were withdrawn from the cells, the voltage outputs of both the reference micropipette and Ca^{2+} microelectrode returned completely to their respective baseline values.

Table 1 summarizes the results obtained from the 15 experiments under baseline conditions. The short-circuit current (I_{sc}) was 13 ± 2 μA · cm^{-2} and the total tissue resistance (R_T) was 3.2 ± 0.5 kΩ · cm^2. The mean basolateral membrane potential in the short-circuited state (ψ^{sc}) was −69 ± 2 mV and the mean value of E^{sc}_{Ca} recorded under the same conditions was −177 ± 4 mV. As described above, one of the minimal criteria for acceptability of intracellular penetrations was the requirement that the basolateral fractional resistance recorded with the reference micropipette $(f^{bl}_o)_\psi$ and with the Ca^{2+}-selective microelectrode $(f^{bl}_o)_E$ be similar. The mean value for $(f^{bl}_o)_\psi$ of 0.19 ± 0.03 was not significantly different from that of 0.15 ± 0.03 for $(f^{bl}_o)_E$; the paired difference was 0.04 ± 0.02. Assuming an extracellular calcium activity coefficient of 0.35, the absolute value of the intracellular calcium activity (a^c_{Ca}) obtained in the present studies was 215 ± 39 nM under baseline transporting conditions.

Given the hypothesis that intracellular free calcium and cAMP are interrelated second messengers in many cells (Rasmussen & Tenenhouse, 1968; Rasmussen, 1970; Rasmussen & Goodman, 1977), it was of considerable interest to measure a^c_{Ca} not only under baseline conditions, but also following stimulation with cAMP. A total of six experiments were conducted before, during and after adding 1 mM CPTcAMP to the serosal medium. Of these, 5 skins were successfully studied with simultaneous penetrations and continuous monitoring of the membrane potential and E^{sc}_{Ca} during the course of introducing CPTcAMP. As illustrated by Fig. 6, CPTcAMP depolarized both E^{sc}_{Ca} and ψ^{sc}. However, the depolarization of the calcium microelectrode trace was larger than that of the reference micropipette, so that the calculated value of a^c_{Ca} in this particular experiment rose from 398 to 668 nM. In each

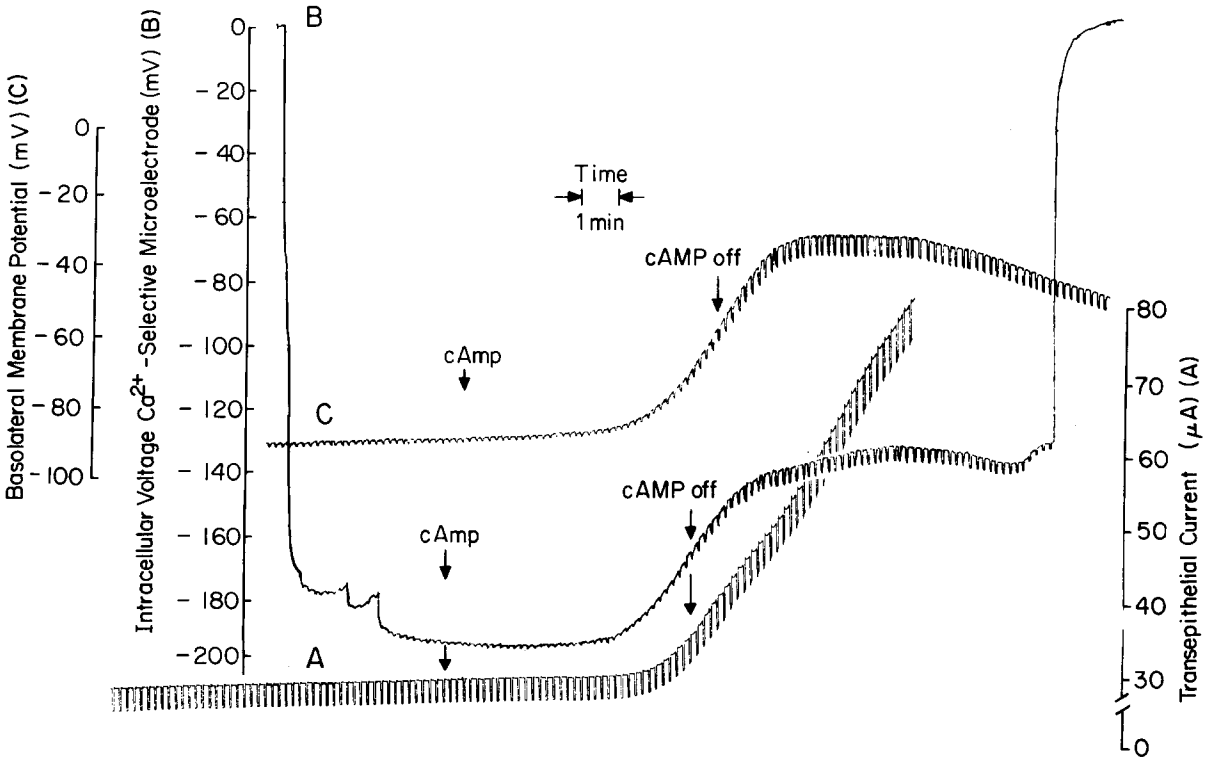

Fig. 6. Simultaneous recordings of transepithelial current (*A*) and the intracellular recordings measured with a Ca^{2+}-selective microelectrode (E^{sc}_{Ca}) (*B*) and reference micropipette (ψ^{sc}) (*C*) following the addition of cAMP. The cAMP was added at a single point in time; the displacement of the two arrows for the E_{Ca} and ψ^{sc} traces reflects the displacement of the pens on the chart recorder. Note that the mV/cm scale for the Ca^{2+}-selective microelectrode is slightly smaller than that depicted for the reference micropipette

Table 2. Summary of intracellular and transepithelial electrophysiologic measurements in split frog skin epithelium: Effect of CPT cAMP

	Baseline transporting conditions							CPTcAMP stimulation						
Preparation	I_{sc} ($\mu A \cdot cm^{-2}$)	R_T ($k\Omega \cdot cm^2$)	ψ^{sc} (mV)	$(f^{bl}_o)_\psi$	E^{sc}_{Ca} (mV)	$(f^{bl}_o)_E$	a_{Ca} (nM)	I_{sc} ($\mu A \cdot cm^{-2}$)	R_T ($k\Omega \cdot cm^2$)	ψ^{sc} (mV)	$(f^{bl}_o)_\psi$	E^{sc}_{Ca} (mV)	$(f^{bl}_o)_E$	a_{Ca} (nM)
III	7.9	7.87	−72.6	0.12	−172.4	0.14	228	19.7	4.75	−27.7	0.34	−113.1	0.21	643
V	4.2	3.01	−66.1	0.07	−189.0	0.09	17	6.4	2.10	−52.2	0.12	−175.7	0.14	32
XI	15.8	2.38	−66.4	0.36	−181.5	0.30	50	21.6	1.31	−52.0	0.28	−144.8	0.47	282
XII	17.9	5.43	−84.8	0.12	−200.2	0.11	398	44.2	1.90	−34.8	0.51	−139.9	0.51	668
XIII	7.4	2.24	−75.4	0.08	−188.8	0.18	314	19.5	1.09	−55.2	0.27	−158.1	0.28	588
XV	4.2	1.65	−64.9	0.11	−191.2	0.08	48	18.4	0.66	−57.3	0.40	−176.3	0.40	79
Mean	9.5	3.76	−72	0.14	−187	0.15	176	22	2.0	−47	0.32	−151	0.29	382
± SE	±4	±0.98	±3	±0.04	±4	±0.03	±65	±5	±0.6	±5	±0.05	±10	±0.07	±117

of the six experiments (Table 2), CPTcAMP was found to increase baseline a^c_{Ca} by factors of 1.65 to 5.64; the mean ± SE was 2.6 ± 0.6. Therefore, even by nonparametric analysis, the probability of the null hypothesis was less than 0.02.

In order to consider the possible physiologic significance of the CPTcAMP-induced rise in a^c_{Ca}, it was of particular interest to compare the times of onset of the increases in short-circuit current and intracellular calcium activity. Caution must be exercised in drawing such comparisons when dealing with a multilayered preparation like frog skin. For this reason, an analysis was first made of the relative times of onset of the changes in E^{sc}_{Ca}, ψ^{sc} and I_{sc} after adding serosal Ba^{2+}; these times of onset are symbolized by t_{Ca}, t_ψ and t_I, respectively. As illustrated by Fig. 5, the three times of onset were closely similar following addition of Ba^{2+}. In a series of seven comparisons, $(t_{Ca} - t_I)$ was 0.03 ± 0.05 min, and in a series of 29 experiments, $(t_\psi - t_I)$ was

0.02 ± 0.03 min. Thus, the depolarizations of E^{sc}_{Ca} and ψ^{sc} and fall in short-circuit current following the Ba^{2+} occurred simultaneously. In contrast, in each of the five experiments in which simultaneous measurements were obtained, depolarization of E^{sc}_{Ca} induced by cAMP was noted before any increase in short-circuit current could be observed. The mean ± SE values for $(t_{Ca} - t_I)$ was 1.92 ± 0.67 min, significant at the 0.05 probability level. The onset of depolarization of E^{sc}_{Ca} can be taken to be a direct index of the onset of rise in a^c_{Ca}, since this depolarization preceded that of ψ^{sc} in four of the five experiments.

Discussion

Intracellular calcium activity (a^c_{Ca}) may be a critical regulator of transepithelial sodium and water transport (Grinstein & Erlij, 1978; Taylor & Windhager, 1979; Windhager & Taylor, 1983). Cystosolic Ca^{2+} (a^c_{Ca}) is presumed to modify, directly or indirectly, the sodium permeability of the apical membrane and thus play an important messenger role mediating the effects produced by cyclic AMP in many biological cells (Rassmussen & Tenenhouse, 1968; Rasmussen, 1970; Rasmussen & Goodman, 1977). Given the possible central role of calcium, not only in transepithelial transport but also in a great range of cellular function, its measurement assumes appreciable importance. Because of various technical difficulties encountered in measurements of a^c_{Ca}, only circumstantial evidence exists to support such a role and conclusions regarding the role of a^c_{Ca} in modifying transepithelial Na^+ transport remain conflicting. It has been of particular interest to measure a^c_{Ca} in frog skin. This model system has been under investigation at least as long (Matteucci & Cima, 1845) and by as broad a range of experimental techniques (Civan, 1983) as any other tight epithelium.

Although Ca^{2+}-selective microelectrodes permit direct measurement of intracellular calcium activity (Oehme et al., 1976; Armstrong et al., 1980; Lee et al., 1980*a*; Marban et al., 1980), miniaturization of the electrode tip to permit impalement of small cells has presented a technical challenge. In the present report, a method is presented for preparing microelectrodes with tips of < 0.2 μm in outer diameter. The 90% response times upon withdrawal of the microelectrodes from the cells are as short as 18 to 23 sec (Figs. 5 and 6), and display satisfactory sensitivity and selectivity to concentrations of Ca^{2+} as low as 10^{-7} M (Fig. 3). Most importantly, stable impalements can be performed with these microelectrodes (Figs. 5 and 6). Given the small size of the epithelial cells of frog skin ($\lesssim 10$ μm in diameter), it is likely that the microelectrodes described here can be applied to a variety of other cell systems whose intracellular calcium activity has not yet been directly measured.

Using these microelectrodes, the mean intracellular calcium activity in 15 succesful experiments was measured to be 215 ± 39 nM (Table 1). This value is close to the estimates of 217 ± 60 nM and 133 ± 13 nM which can be calculated from the published data obtained with *Necturus* proximal tubule. The reported values of 116 ± 32 nM (Lee et al., 1980*a*) and of 71 ± 7 nM (Lorenzen et al., 1984), were based on a presumed value of 2.041×10^{-7} M for the apparent dissociation constant of the EGTA buffer used in their calibration solutions. The currently accepted estimate is 3.8 to 4.0×10^{-7} M (Blinks et al., 1982; Bers, 1982; Tsien & Rink, 1983), so that their reported values of a^c_{Ca} should be revised upward by a factor of approximately 1.87.

In sodium-transporting epithelia, the apical membrane permeability to Na^+ is the rate-limiting step in the regulation of transepithelial Na^+ transport. It has long been appreciated that in tight epithelia such as frog skin and toad bladder, cellular cyclic AMP is one mediator of hormonal effects on apical Na^+ permeability. However, the exact steps following cyclic AMP generation that directly modulate the rate of Na^+ entry, remain incompletely understood. In particular, the generation of cAMP may be associated with a change in a^c_{Ca} (Rasmussen & Tenenhouse, 1968; Rasmussen, 1970; Rasmussen & Goodman, 1972); the finally expressed permeability changes may reflect the interplay of both Ca^{2+} and cAMP. In order to examine this problem directly, we have measured a^c_{Ca} before, during and after the serosal addition of CPTcAMP. In each of six experiments, intracellular calcium activity was indeed found to be increased; the ratio of the activities after to before stimulation was 2.6 ± 0.6.

In principle, the increase in a^c_{Ca} and the natriferic effect induced by CPTcAMP could be related in one of three ways: (1) the natriferic effect could cause the increase in a^c_{Ca}, presumably by slowing the rate of sodium-calcium exchange by the putative antiport at the basolateral membrane (Blaustein, 1974; Grinstein & Erlij, 1978; Taylor & Windhager, 1979); (2) the elevation in cytosolic Ca^{2+} activity may play a role in generating the natriferic response, or (3) the two effects could be parallel unrelated phenomena. The present data suggest that the first possibility is unlikely. Slowing of antiport activity could arise from the membrane depolarization and enhanced apical Na^+ entry produced by CPTcAMP. However, the depolarization followed the increase in a^c_{Ca} in four of the five experiments where simultaneous measurements were

conducted. In all five preparations, enhanced Na^+ entry (as reflected by the rise in I_{sc}) also followed, rather than preceded, the increase in a^c_{Ca}.

The data do not allow a distinction between the second and third possibilities. The temporal relationships, however, are certainly consistent with an effect of cytosolic calcium to modulate sodium transport, at least in part, by an increase in apical sodium conductance. Evidence for a relationship between a_{Ca} and apical Na^+ conductance has also been obtained in studies using vesicles from another tight epithelium, the urinary bladder of the toad (Chase & Al-Awqati, 1983). In this preparation, however, intravesicular calcium was reported to inhibit Na^+ transport across the vesicular walls. The mechanism by which changes in cytosolic calcium could alter apical membrane conductance and the precise role of Ca^{2+} in modifying the final natriferic effect are currently not understood and could involve both direct and indirect actions. These studies also do not address the source of the calcium and the mechanism by which CPTcAMP results in elevation of cytosolic calcium. Finally, it should be emphasized that these studies only report effects of an exogenous analog of cyclic AMP. Whether or not similar alterations in cytosolic calcium activity would occur with hormonal stimulation remains to be directly evaluated.

In summary, we have been able to measure cytosolic calcium activity in split frog skin epithelium. The data indicate that CPTcAMP produces a significant increase in a^c_{Ca} which is detectable prior to a detectable change in membrane conductance and short-circuit current. Further studies to evaluate the mechanisms of this effect, the linkage between these events and the effects of hormonal stimulation, are underway.

We are grateful to Drs. Chin O. Lee and Hiroshi Yamaguchi for their advice in preparing calcium-selective microelectrodes, for visiting our laboratory and graciously permitting us to visit theirs, and to Paul Norton and Dr. Donald R. DiBona for the preparation of scanning electron micrographs. We are also most appreciative of early gifts of calcium resin by Professor W. Simon. We thank Mary Beth Pope for her participation at an early stage of the project in beginning to establish a calibration procedure, and Kim Peterson-Yantorno for her expert technical assistance. The work was supported in part by research grants AM 20632 and AM 33138 from the National Institutes of Health. Dr. Kelepouris is a recipient of the Clinican-Scientist Award of the American Heart Association (#83-408).

References

Armstrong, W. McD., Youmans, S.J., O'Doherty, J. 1980. Calcium regulation during stimulus-secretion coupling; Continuous measurement of intracellular calcium activities. *Science* **209:**510–513

Berridge, M.J. 1979. Relationship between the cyclic nucleotides in ion secretion. *In:* Mechanisms of Intestinal Secretion. H.J. Binder, editor. pp. 65–81. Liss, New York

Bers, D.M. 1982. A simple method for the accurate determination of free [Ca] in Ca-EGTA solutions. *Am. J. Physiol.* **242:***C*404–*C*408

Blaustein, M.P. 1974. The interrelationship between sodium and calcium fluxes across cell membranes. *Rev. Physiol. Biochem. Pharmacol.* **70:**33–82

Blinks, J.R., Wier, W.G., Hess, P., Prendergast, F.G. 1982. Measurement of Ca^{2+} concentrations in living cells. *Prog. Biophys. Molec. Biol.* **40:**1–114

Buttler, J.N. 1964. Ionic Equilibrium: A Mathematical Approach. pp. 428–468. Addison-Wesley, Reading, Massachusetts

Chase, H.S., Al-Awqati, Q. 1983. Calcium reduces the sodium permeability of luminal membrane vesicles from toad bladder. Studies using a fast-reaction apparatus. *J. Gen. Physiol.* **81:**643–665

Civan, M.M. 1983. Epithelial Ions and Transport. Application of Biophysical Techniques. Wiley Interscience, New York

DeLong, J., Civan, M.M. 1983. Microelectrode study of K^+ accumulation by tight epithelia: I. Baseline values of split frog skin and toad urinary bladder. *J. Membrane Biol.* **72:**183–193

Fisher, R.S., Erlij, D., Helman, S. 1980. Intracellular voltage of isolated epithelia of frog skin: Apical and basolateral cell punctures. *J. Gen. Physiol.* **76:**447–453

Grinstein, S., Erlij, D. 1978. Intracellular calcium and the regulation of sodium transport in the frog skin. *Proc. R. Soc. London B.* **202:**353–360

Lee, C.O., Im, W.B. 1984. Measurement of intracellular free calcium with Ca^{2+}-selective microelectrodes. Problems and techniques. *In:* Mechanisms of Intestinal Electrolyte Transport and Regulation by Calcium. M. Donowitz and C.W. Sharp, editors. Vol. 17, pp. 157–168. A.R. Liss, New York

Lee, C.O., Taylor, A., Windhager, E.E. 1980*a*. Cytosolic calcium ion activity in epithelial cells of *Necturus* kidney. *Nature (London)* **287:**859–861

Lee, C.O., Uhm, D.Y., Dresdner, K. 1980*b*. Sodium-calcium exchange in rabbit heart muscle cells; Direct measurement of sarcoplasmic Ca^{2+} activity. *Science* **209:**699–701

Lorenzen, M., Lee, C.O., Windhager, E.E. 1984. Cytosolic Ca^{2+} and Na^+ activities in perfused tubules of *Necturus* kidney. *Am. J. Physiol.* **247:**F93–F102

Marban, E., Rink, T., Tsien, R.W., Tsien, T.Y. 1980. Free calcium in heart muscle at rest and during contraction measured with Ca^{2+}-selective microelectrodes. *Nature (London)* **286:**845–850

Matteucci, C., Cima, A. 1845. Mémoire sur l'endosmose. *Ann. Chim. Phys.* **13:**63–86

Moore, E.W., Ross, J.W. 1965. NaCl and $CaCl_2$ activity coefficients in mixed aqueous solutions. *J. Appl. Physiol.* **20:**1332–1336

Nagel, W. 1976. Intercellular junctions of frog skin epithelial cells. *Nature (London)* **264:**469–471

Oehme, M., Kessler, M., Simon, W. 1976. Neutral-carrier Ca^{2+}-microelectrode. *Chimia* **30:**204–206

Rasmussen, H. 1970. Cell communication, calcium ion, and cyclic adenosine monophosphate. *Science* **170:**404–412

Rasmussen, H., Goodman, D.B.P. 1977. Relationships between calcium and cyclic nucleotides in cell activation. *Physiol. Rev.* **57:**421–509

Rasmussen, H., Tenenhouse, A. 1968. Cyclic-adenosine monophosphate, calcium and membranes. *Proc. Natl. Acad. Sci. USA* **59:**1364–1370

Rick, R., Dörge, A., Arnim, E. von, Thurau, K. 1978. Electron microprobe analysis of frog skin epithelium: Evidence for a syncytial sodium transport compartment. *J. Membrane Biol.* **39:**313–331

Schonhorn, H., Gregor, H.P. 1961. Multilayer membrane electrodes. III. Activity of alkaline earth salts in mixed electrolytes. *J. Am. Chem. Soc.* **83:**3576–3579

Taylor, A., Windhager, E.E. 1979. Possible role of cytosolic calcium and Na-Ca exchange in regulation of transepithelial sodium transport. *Am. J. Physiol.* **236:**F505–F512

Tsien, R.Y., Rink, T.J. 1983. Measurement of free Ca^{2+} in cytoplasm. *In:* Current Methods in Cellular Neurobiology. J.L. Barber, editor. pp. 249–312. Wiley, New York

Windhager, E.E., Taylor, A. 1983. Regulatory role of intracellular calcium ions in epithelial Na transport. *Annu. Rev. Physiol.* **45:**519–532.

Received 16 April 1985; revised 24 June 1985