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AWARD NUMBER DAMD17-96-1-6315

TITLE: Fluid Transport Mechanisms in Breast Gross Cystic Disease

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REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 4

19990811 102

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1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Annual (1 Sep 97 – 31 Aug 98)

4. TITLE AND SUBTITLE
Fluid Transport Mechanisms in Breast Gross Cystic Disease

5. FUNDING NUMBERS
DAMD17-96-1-6315

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The goal of this research is to develop a pharmacological strategy for reducing or eliminating the pathological accumulation of fluid that occurs in breast gross cystic disease (GCD). Over the last year, we have analyzed several of the transport proteins and intracellular signaling mechanisms that mediate the movement of fluid (and ions) across confluent monolayers of 31EG4 cultured mammary epithelial cells. We hope eventually to be able to analyze cells cultured from: (1) reduction mammioplasties in normal individuals; and (2) patients with gross cystic disease. In all cases, we will use antibodies and immunocytochemistry to identify the transport proteins that are located on the apical and basolateral membranes of these cells. At the same time, in a complementary set of experiments, confluent monolayers of each cell type will be mounted in modified Ussing chambers to measure the transport rates of the pumps, cotransporters, exchangers, and channels that are located at each membrane. **The present preliminary experiments have provided the first measurements of fluid transport across any mammary epithelia.** In particular, the magnitude and direction of the fluid transported across each preparation has been directly measured. As shown in the data (see below) we have been able to make solution composition changes and add secretagogues or transport inhibitors to the apical or basal baths in order to help identify which particular apical or basolateral membrane proteins and/or which signaling pathways mediate the vectorial transport of fluid across **31EG4** mammary cell line (Sjaasted *et al.*, 1993).

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	27
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRA
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The goal of this research is to develop a pharmacological strategy for reducing or eliminating the pathological accumulation of fluid that occurs in breast gross cystic disease (GCD). Over the last year, we have analyzed several of the transport proteins and intracellular signaling mechanisms that mediate the movement of fluid (and ions) across confluent monolayers of 31EG4 cultured mammary epithelial cells. We hope eventually to be able to analyze cells cultured from: (1) reduction mammioplasties in normal individuals; and (2) patients with gross cystic disease. In all cases, we will use antibodies and immunocytochemistry to identify the transport proteins that are located on the apical and basolateral membranes of these cells. At the same time, in a complementary set of experiments, confluent monolayers of each cell type will be mounted in modified Ussing chambers to measure the transport rates of the pumps, cotransporters, exchangers, and channels that are located at each membrane. **The present preliminary experiments have provided the first measurements of fluid transport across any mammary epithelia.** In particular, the magnitude and direction of the fluid transported across each preparation has been directly measured. As shown in the data (see below) we have been able to make solution composition changes and add secretagogues or transport inhibitors to the apical or basal baths in order to help identify which particular apical or basolateral membrane proteins and/or which signaling pathways mediate the vectorial transport of fluid across **31EG4** mammary cell line (Sjaasted *et al.*, 1993**).**

BODY

Background/Significance

GCD is the most common benign breast disease and its etiology is unknown. Seven to ten percent of women in Western countries develop macrocysts of the breast, mainly in the premenopausal decade, and there is now strong evidence to indicate that these women are at a higher risk of later developing breast cancer, at a rate 2 - 4 times that of the general population (Malatesta et al, 1998, Angeli et al., 1994; Leis, 1993; Bodian, 1993; Bodian et al., 1992; Bundred et al., 1991; Ciatto et al., 1990).

In breast gross cystic disease (GCD) there are two types of epithelial cells that line the cysts, apocrine (tall, columnar) and attenuated (flat); it is the apocrine cells that serve as a marker for precancerous changes in the surrounding tissue (Leis, 1993; Petrakis et al., 1993). These cysts can be formed in one of two ways: either by a fibrocystic obstruction in the duct or by excessive fluid secretion across the ductal-alveolar epithelia (Dogliotti et al., 1990). In the former case cysts do not usually reoccur after aspiration, but in the latter case - cysts frequently do reoccur (Angeli et al., 1987; Molina et al., 1990). Recent evidence suggests that the apocrine cells may provide the paracrine or autocrine signals that lead to the development of breast epithelial hyperplasia (Athanassiadou et al., 1992; Leis, 1993; Cassoni et al., 1995). If, as in many organ systems, the progression from benign to malignant epithelial -cell phenotype is regulated in part by inhibitory signals then fluid accumulation in the cysts

would **reduce the activity** of these inhibitory signals and speed the onset of tumor growth. For example, in breast carcinomas, E-cadherin mediated adhesion between cells is significantly reduced because the expression level of this protein is reduced (Fish and Molitoris, 1994; Shiozaki et al., 1991; Eidelman et al, 1989).

Breast cyst fluid from GCD patients contains many different chemical components at activity levels that are quite unusual for any ventricular space. These abnormal secretory products include electrolytes, hormones, growth factors, cytokines, immunoglobulins and other proteins (Bradlow et al., 1985; Miller, 1989; Wick et al, 1989; Molina et al., 1990; Dogliotti et al., 1990; Reed et al., 1992; Lai et al., 1993; Angeli et al, 1987, 1992, 1994; Cassoni et al., 1995). Approximately 70% of the total protein content of the breast GCD fluid is made up of only three proteins with molecular weights of 15, 24 and 44kd (gross cystic disease fluid proteins - GCDFP- 15,24,& 44). Of these three polypeptides, GCDFP-15 is the one consistently present in carcinomas of the breast (Wick et al., 1989; Mazoujian et al., 1989; Petrakis et al., 1993; Cassoni et al., 1995). We intend to measure the activity GCDFP-15 and its ability to alter fluid transport rate because: (1) its secretion is increased in breast cancer (Umaar et al., 1994); (2) it is mitogenic for normal human and breast cancer cell lines (Cassoni et al., 1995), but **not** colon carcinoma cell lines (HT29 or NIC-H716), neuroblastoma cell line-IMB32 or NIC-H209 (small cell lung carcinoma cells). The chemical activity of this secreted protein and its role in the development of breast epithelia hyperplasia may be regulated, in part, by the amount of fluid secreted into the cyst.

In patients with breast cystic disease, the electrolyte composition of cystic fluid is quite abnormal compared to most secretions (high K and low Na, Cl & pH; Angeli et al., 1994; Molina et al., 1990; Dixon et al., 1984), but it bears a striking resemblance to the endolymph fluid produced by the apocrine - like dark cells that line the semicircular canal of the ear (Marcus et al., 1992). Because the dark cells also secrete some of the same proteins (GCDFP-15), they provide a possible model for fluid (and ion) transport across mammary cells. Previous work using mammary cells from animal tissues also provides a helpful starting point (Shennan, 1990), but most of these studies were carried out using isolated epithelial cells, from mouse or bovine mammary glands or vesicle preparations (Shennan et al., 1994; Furuya et al., 1993; Shennan, 1992). In one case, confluent monolayers were used to study the influence of hormonal regulation on the polarized distribution of Na/H exchange and Na·HCO_3 cotransport (Sjaastad et al., 1993). Some ion transport information has been obtained using confluent monolayers of cultured mouse mammary epithelia (Bisbee, 1981a,b), but, except for the data summarized below, nothing is known about the mechanisms that drive fluid secretion across the epithelia that line the ducts and lobules of the mammary glands.

In most gland systems fluid secretion is driven by a chloride transport pathway consisting of an apical membrane Cl channel and a basolateral membrane Na, K, 2Cl cotransporter. There is some evidence that this is also true for two of the human breast cancer cell lines, MCF-7 and BC19/3 (Altenberg et al., 1994). These authors showed that intracellular Cl concentration in both cell lines is quite high, $\approx$ 40 mM, strongly suggesting that chloride is actively accumulated, perhaps via furosemide-sensitive

Na,K,2Cl cotransporters (Shennan, 1989, 1990); Cl efflux in MCF - 7 cells occurs via Ca^{2+} -dependent Cl channels (Flezar and Heisler, 1993). These results suggest the presence of a chloride transport pathway that could drive fluid secretion into the ducts. Therefore our first experiments have probed for the presence of apical membrane Cl channels and basolateral Na, K,2Cl cotransporters, because this pathway will most likely provide the basis for a therapeutic approach to the prevention of fluid-filled cysts in gross cystic disease patients.

Previously we determined the membrane and intracellular signaling mechanisms that regulate fluid transport across the retinal pigment epithelium (Hughes et al., 1984; Edelman and Miller, 1991; Joseph and Miller, 1992; Edelman et al., 1994; Gallemore et al., 1997; Peterson and Miller, 1997; Peterson et al., 1998). We also demonstrated that these same mechanisms serve to control the hydration of the small extracellular space that lies between the RPE apical membrane and the distal portion of the neural retina (Bialek and Miller, 1994; Peterson et al., 1997; Evans et al, 1998; Uyekubo et al., 1998). Recently we provided the first measurements of fluid transport across monolayers of cultured human airway surface and gland cells and determined some of the mechanisms that could provide therapeutic hydration in the airways of cystic fibrosis patients (Jiang et al., 1993; Jiang et al., 1997). Based on this experience, we have sought to develop a pharmacological strategy that can be used to prevent or reduce the fluid that accumulates in the mammary gland ducts during gross cystic disease. The potential benefits of preventing this accumulation of fluid would be three-fold: (1) a possible reduction in cancer risk or disease progression; (2) a reduction or removal of the pain that inevitably accompanies the fluid - induced intracystic tension; (3) the removal of an impediment that continues to cause misdiagnosis of breast cancer by primary care physicians (Leis, 1993).

Methods

Mammary culture samples; 31EG4 mouse mammary cells obtained from Professor Firestone (UC Berkeley) were maintained in DMEM/F12, 5% FBS, 5µg/mL insulin, 50 µg/mL gentamicin sulfate. Upon reaching confluencey, cells were plated onto Transwells at a density of 1.0 x 10^5 cells/well. They were grown in DMEM/F12, glutamine, 2% Fetal bovine serum (FBS), 5µg/mL insulin, 1 µM Dexamethasone, 5µg/mL prolactin and 50 µg/mL gentamicin sulfate Transwells were seeded at a density of 5xl0^5 cells/well. Transepithelial resistance measurements were taken with an EVOM (Epithelial Voltammeter, World Precision Instruments, New Haven CT).

Electrophysiology: Transwells with confluent monolayers and an R_t over 300 $\Omega \cdot cm^2$, were then used for the electrophysiology measurements. The tissue was mounted on a nylon mesh support and clamped into a modified Ussing chambers, as previously described. The transepithelial potential (TEP) was measured by calomel electrodes in series with Ringer-agar bridges. TEP is the voltage difference between the apical and basal bath electrodes (V_b-V_a). The transepithelial resistance (R_t) were

obtained by passing current pulses, usually of 1μA across the tissue and measuring the change in TEP (R_T= ΔTEP/ΔI). The ΔTEP/ΔI was multiplied by the area of the chamber (0.075 cm^2) and the resistance expressed in units of units Ω•cm^2.

The control ringer (1 liter) was made of: 5g KCl, 0.8g $MgSO_4 \cdot 7H_2O$, 113.4g NaCl, 26.2g $NaHCO_3$, 1.0g NaH_2PO_4 anhydrous, 5.6g D-glucose, 5g taurine, and 1.8g $CaCl_2$, at pH 7.4. For 8.6 mM Cl ringer, 113.4g methanesolufonic acid was substituted for NaCl. MCF-7 cells were treated with low Cl ringer, a cAMP cocktail, made up of 500 μM IBMX, 100 μM 8-(4-chlorophenylthio) adenosine 3';5'-cyclic monophosphate (CPT), and 12μM forskolin, as well as 10^{-8} M epinephrine, and 2 mM barium chloride. 31EG4 cells were treated with the cAMP cocktail, 10μM amiloride, and 20 μM ionomycin.

Fluid and ion transport: Transepithelial fluid flows were measured using an extremely sensitive capacitance probe technique developed in this laboratory (Hughes et al, 1984; Jiang et al., 1993) in which a sheet of epithelium (0.5 cm^2 exposed area) is mounted between two water-impermeable Kel F half-chambers. A very sensitive oscillator circuit is connected to a pair of capacitance probes placed on each side of the tissue to measure the changes in capacitance between the probe tips and the fluid meniscuses that are produced as ion-linked fluid is osmotically driven across the tissue from its apical to basolateral surfaces (or vice versa). Fluid movement across the epithelium is recorded by the changes in probe output voltage. Ports in the bottom of the half-chambers allow for solution composition changes. Voltage sensing and current-passing bridges built into each half chamber permit continuous monitoring of transepithelial voltage (V_T) and resistance (R_T), the latter from the voltage deflections in response to current pulses of known magnitude. This technique has an accuracy of ± l μl/min, corresponding to a fluid rate approximately 1/10th of the average baseline fluid transport rate seen in these experiments.

PCR: RT-PCR was used to see if phospholamban, CFTR, the Na-K-2Cl cotransporter (NKCC1 and NKCC2), the amilloride sensitive Na channel, and CHIP28 are present in the 31EG4 mammary cell line. The cells were homogenized and total RNA was extracted using the RNAzol B method. cDNA was made using 0.5 mg of total RNA, oligo dt primers, and MMLV reverse transcriptase.

Westerns: 31EG4 cells were cultured as described above. Protein was then isolated by placing the cells into 100 ul of protein isolation buffer (nM): 65 NaCl, 2 MgCl, 1 EDTA, 5 Tris-caetate, pH 7.4 containing the following protease inhibitors (ug/ml): 2 aprotinin, 2 leupeptin, 1pepstatin A, 2 antipan, 100 PMSF, 50 TLCK, and 100TPCK. The cells were homogenized by sonicating (Branson, Danbury,CT) on ice for 60 seconds. Protein concentration was determined using the bicinchonic act protein assay (BCA Protein Assay, Pierce, Rockford, IL). To separate protein from lipid membrane, the sample was incubated for 4 hours at room temperature in 4 % digitonin (w/v) in 0.2M sodium phosphate buffer, pH 8.6. Electrophoresis of the samples was carried out using Bio-Rad Mini Protein ready gels (7.5% TRIS-HCL) and the Bio Rad Mini-Protean cell (Bio-Rad, Hercules, CA). The running buffer contained 25 mM TRIS, 200 mM Glycine, 01% SDS. Electroblotting of the proteins onto PVDF membranes was carried

out in a Bio-Rad Mini-Trans-Blot call at 4*C. The transfer buffer contained 25 mM TRIS, 200mM Glycine, pH 8.2-8.5.

For immunodetection, the blotted PVDF membranes were then blocked in 2% casein (in 0.1% (v/v) Tween-20 in PBS) for 1 hour at RT then incubated with 1:1000 dilution of primary anti-CFTR antibody overnight at 4 C. The membranes were rinsed 3 X 5 minutes with blotto and then incubated with 1:5000 dilution of biotinylated anti-rabbit secondary antibody (Sigma, St. Louis, MO) for 1 hour at RT followed by 3 X 5 minutes with blotto then 5 minutes with PBS. The labeled membranes were developed by the enhanced chemiluminescence method (Amersham, Arlighton Heights, IL)

Results

Electrophysiology Experiments: The 31EG4 mammary cell line has allowed us to start the process of testing our model of mammary epithelia fluid and ion transport (see Figure 1). Electrophysiology and fluid transport measurements were used to test the validity of this model. Table 1 is a summary of all experiments on the 31GE4 monolayer. The mean apical membrane potential (V_A) in 28 different tissues is -41±11 (mV). The average TEP is - 4.8 ±1.6 mV (apical side negative as in the model shown in Fig. 1). This mean TEP demonstrates that V_B is ≈ 5 mV hyperpolarized relative to V_A. The mean total tissue resistance (R_t) is 857±256 ($\Omega \cdot cm^2$) and the mean value of R_A/R_B is 2.7 ±0.3, indicating that the apical membrane resistance is almost a factor of three greater than the basolateral membrane resistance.

Untreated tissue (n=28)				Amiloride (apical) (n=17 for 10 tissues)				cAMP (apical) (n=12 for 9 tissues)			
TEP (mV)	R_t ($\Omega \cdot cm2$)	V_A (mV)	R_A/R_B	TEP (mV)	ΔR_t ($\Omega \cdot cm2$)	ΔV_A (mV)	R_A/R_B	TEP (mV)	ΔR_t ($\Omega \cdot cm2$)	ΔV_A (mV)	R_A/R_B
-4.8 ±1.6	857±256	-41±11	2.7 ±0.3	2.7±1.1	122±58	-13±5	30±18	-2.4±0.7	-156±45	15±4.6	0.82 ±0.67

Table 1. Summary of electrophysiology experiments with cAMP and Amiloride added to the apical bath. Columns 1-4 show the baseline data for 28 cell cultures. Columns 5 – 8 and 9 - 12 summarize the amiloride and cAMP induced changes in membrane voltage and resistance, respectively.

The first series of electrophysiology experiments tested the effects of elevated levels of cAMP on intracellular voltage and membrane resistance. For example, Figure 2 (top) shows that the addition of cAMP cocktail to the apical bathing solution (black bar) depolarized the apical (V_A) and basolateral (V_B) membranes (≈ 10 mV) and decreased the total tissue resistance (R_t) by ≈ 300 $\Omega \cdot cm^2$. The cAMP- induced increase in TEP has only one possible interpretation - that the secretogogue-induced rate of membrane

depolariztion was greater at the apical membrane compared to the basolateral membrane. Therefore, elevating cAMP in mammary cells has a predominant effect at the apical membrane. This voltage change is consistent with an increase in apical conductance for an ion channel whose equilibrium potential is depolarized relative to the apical membrane resting potential (≈ -51 mV). This interpretation is corroborated by the rapid and large decrease in R_t (300 $\Omega \cdot cm^2$) and a two fold decrease in R_A/R_B. These resistance changes are also consistent with a cAMP- induced increase in apical membrane conductance (a decrease in R_A). In many epithelia this kind of cAMP response is produced by the presence of a protein kinase A (cAMP) – dependent **Cl channel**, the so called cystic fibrosis membrane conductance regulator (CFTR) in the apical membrane. In the experiment summarized in Figure 3, we test this notion by adding NPPB (50μM), a known CFTR blocker, to the apical bath.

The data in Figure 3 is a continuous recording obtained from the same tissue shown in Fig 2. The first part of this experiment shows that the addition of NPPB to the apical bath has no appreciable effect on membrane voltage and resistance. However, when cAMP was added in the presence of NPPB the response was quite different compared to that seen in Fig. 2. In this case, cAMP **hyperpolarized** both membranes and R_A/R_B **increased**. As in Figure 2, R_t decreased suggesting an increase in tissue conductance. In Figure 3, the TEP increase means that V_B hyperpolarized at a greater rate than V_A and that finding, along with the observed increase in R_A/R_B, indicates that in the presence of NPPB the dominant effect of cAMP was at the basolateral membrane. These voltage and resistance changes are constant with the opening of a channel at the **basolateral** membrane- perhaps a response that is normally masked by the electrical response of apical CFTR to an elevation in cell cAMP. When the tissue is returned to normal Ringer's (NHR) and then treated again with apical cAMP the control response is restored (see Figure 2).

In the next series of experiments we tested for the presence of an amiloride sensitive sodium channel. Figure 4 shows a characteristic set of voltage and resistance changes that occur when 20μM amiloride is added to the apical bath. Apical amiloride decreased the TEP by 2mV, increased R_t, by 100 $\Omega \cdot cm^2$ and caused a large 25mV, hyperpolarization of the apical (V_A) and basolateral (V_B) membranes. Concomitant with these voltage changes, the ratio of apical to basolateral membrane resistance, R_A/R_B, increased by almost a factor of 15; from 2.5 to 37. The amiloride-induced decrease in TEP demonstrates that the rate of membrane hyperpolarization was greater at the apical membrane compared to the basolateral membrane. These changes in voltage and resistance provide strong evidence for the conclusion that apical amiloride produced a large decrease in apical membrane conductance for a Na channel whose equilibrium potential is depolarized relative to the apical membrane resting potential (≈ -51 mV). The magnitude of the electrical responses and the well – known specificity of amiloride for Na channels makes it practically certain that all of these electrical changes were caused by the closure of sodium channels at the apical membrane. To test for the presence of Na sodium channels at the basolateral membrane, 20 μM amiloride was added to the basal bath (Figure 5). In contrast to the apical responses, basal amiloride caused a small, 5 mV depolarization of the apical (V_A) and basolateral (V_B) membranes, a 50 $\Omega \cdot cm^2$

increase in R_t, and a slight drop in the R_A/R_B while the TEP remained constant. These changes are consistent with a basolateral membrane response, possibly secondary to the inhibition of the putative Na/H exchanger on the basolateral membrane (see Fig. 1) and a change in cell pH.

Next we sought to determine if alterations in the functional activity of one of the two apical membrane channels, either CFTR or the amiloride sensitive Na channels, could affect the activity of the other. In Fig. 6 we first blocked Na channel activity with amiloride. In this experiment, 50 μM amiloride was added to the apical bath and produced voltage and resistance changes expected for the closure of the apical Na channels (compare with control data in Figure 4). Cyclic AMP was then added to the apical bath and we measured the subsequent membrane voltage and resistance changes that presumably occurred following the increase in cell cAMP. The voltage and resistance changes shown in Figure 6 clearly indicate that CFTR activity is unaltered in when the Na channels are blocked. In comparing Figures 2 and 6 we see that the membrane voltage changes induced by cyclic AMP in the presence of amiloride are more than twice those observed in its absence (Fig. 2). The reason for this difference is that amiloride significantly hyperpolarized the apical membrane and greatly increased the driving force for Cl exit across the apical membrane. Thus activation of CFTR by cAMP in this case produced a much larger change in voltage. In the presence of amiloride, the cAMP – induced change in R_A/R_B was also quite large, larger than in its absence suggesting conductance changes for other channels in addition to CFTR.

In the converse set of experiments shown in Figure 7, the addition of the cAMP cocktail induced a large 40 mV depolarization of the apical (V_A) and basolateral (V_B) membranes and decreased the total tissue resistance (R_t) by 125 $\Omega \cdot cm^2$. This is consistent with the opening of CFTR on the apical membrane (as seen in Figure 2). With CFTR activated, we next tested if the Na channel is functional. In this case the tissue was incubated in a cAMP cocktail that included 50 μM amiloride. In the presence of cAMP the amiloride responses were almost completely abolished. There was no significant changes in R_t, R_A/R_B and TEP. V_A and V_B hyperpolarized only slightly (5 mV). This result could have occurred because there is an **inhibitory interaction** between CFTR and the Na channels such that when CFTR is active the Na channels are normally quiescent. This result could help explain the observed increase in Na and fluid absorption that occurs in cystic fibrosis when CFTR is degraded in the endoplasmic reticulum and does not traffic to the apical membrane. On the other hand, this result could have occurred because cAMP depolarized the apical membrane and significantly reduced the driving force for Na entry. This hypothesis will be tested by current clamping the apical membrane potential to the depolarized level produced by cAMP and then adding apical amiloride. If the amiloride response is again absent (CFTR inactivated) then the latter hypothesis is strengthened. If the amiloride response is present under current clamp conditions then we need to more seriously consider the former hypothesis, which is that normal CFTR activity could down regulate Na channel activity as has been postulated for airway epithelia.

Fluid Transport Experiments: In the next series of experiments we mounted the 31EG4 monolayers in a specially designed fluid transport chamber (Hughes et al., 1984; Jiang et al., 1993; Peterson and Miller, 1997; Peterson et al., 1998) that allowed us to simultaneously measure J_V, TEP and R_t. **It should be emphasized that these are the only extant measurements of fluid transport across any mammary cell preparation.**

Net steady-state fluid transport across any epithelium is determined by a balance of active ion – coupled transport pathways whose vectorial direction allows either the absorption or secretion of fluid (Jiang et al., 1997; Peterson and Miller, 1997; Peterson et al., 1998). In the tissues that we studied this balance could be tipped either way; some tissues absorbed fluid (apical to basolateral flow) in the unstimulated steady – state while others secreted fluid in the basolateral to apical direction. Figures 8 and 9 summarize the data for two tissues (unstimulated) in which net transport was in the **secretory direction**. In each case, the addition of cAMP to the apical bath caused a significant increase in fluid secretion (by a factor of approximately two) and a decrease in R_t and TEP (not shown). These results suggest that in these cultures, net Cl secretion is the dominant active-ion pathway for driving fluid across the cell and that fluid secretion increased because cAMP activated apical membrane CFTR. As shown in Fig. 1 Cl entry at the basolateral membrane is probably via a Na,K,2Cl cotransporter but this hypothesis remains to be tested.

Figures 10 and 11 summarize the data for two tissues in which the pattern of response to activation by cAMP was in the opposite direction. In Fig. 10 the unstimulated tissue was secreting fluid ($\approx 2\ \mu l \cdot cm^{-2} \cdot hr^{-1}$) as in the tissues shown in Fig.'s 8 and 9. In this case, however, the elevation of cell cAMP **increased** the rate of fluid absorption - opposite to the effects seen in Fig.'s 8 and 9. This increase in fluid absorption was blocked by apical NPPB, implicating a role for CFTR. This result suggests that in some cultures the electrochemical driving force for Cl is directed inward at the apical membrane. That notion will need to be directly confirmed using intracellular microelectrode recording techniques with conventional and double barreled Cl sensitive microelectrodes. Figure 11 shows a tissue in which the unstimulated rate of net steady - state fluid transport was in the absorption direction. This result was also obtained in several other cultures (n = 3, not shown) and suggests the dominance of an absorbtion pathway. The electrophysiology data illustrated above (see also the model in Fig. 1) implicates the Na absorption transport pathway, consisting of amiloride sensitive apical membrane Na channels and basolateral membrane Na/K pumps (Fig. 1). The data summarized in Fig. 11 also indicates that this pathway is cAMP sensitive since elevating cell cAMP more than doubled the rate of fluid absorption.

Westerns: Using a polyclonal antibody to CFTR (Genzyme), two dominant bands, of approximate molecular weight of 170 and 130 kD, were detected.

Pcr: The message for CFRT and phospholamban was found in 31EG4 cells. The primers designed for the identification of ENAC have yet to work. New primers are being designed in an attempt to find the message.

Conclusions

Electrophysiology Experiments: The electrophysiology data obtained using 31EG4 cultures is important because it provides the first evidence in human mammary cells for apical membrane Cl channels (cAMP and Ca^{2+}-activated) and amiloride sensitive Na channels. The presence of these transport mechanisms provides the physiological basis for understanding how fluid and milk are transported into the mammary ducts. The excessive secretion of fluid seen in breast cystic disease could be pharmacologically or genetically reduced by upregulating fluid absorption out of the ducts into the blood via the amiloride sensitive Na channels or down regulating secretion via the apical membrane Cl channels.

***Westerns and PCR*:** The mRNA and protein for CFTR have been found in 31EG4 cells and provide further evidence that this membrane protein is functioning in this mouse mammary cell line.

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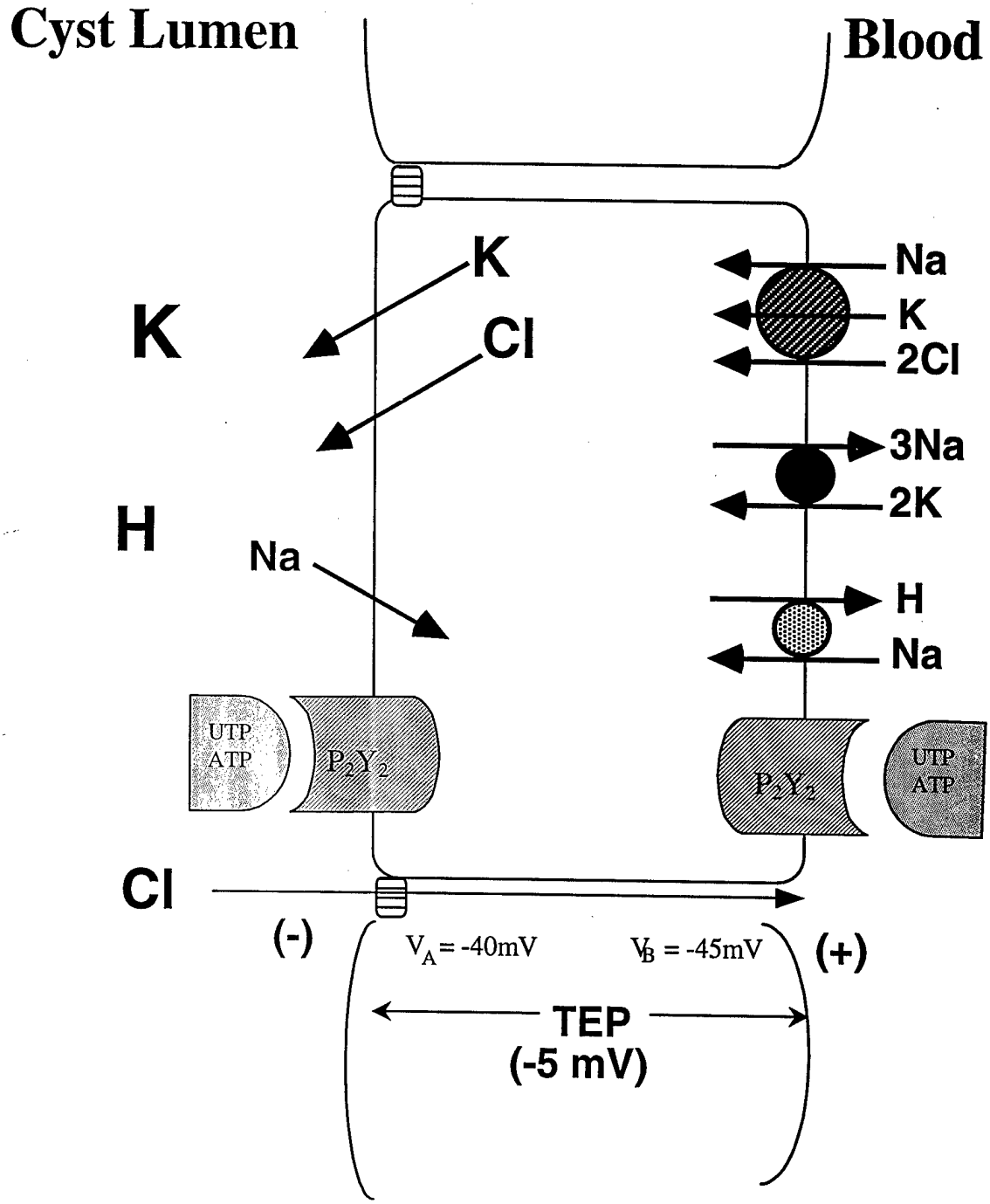

Figure 1. Ion transport model for 31EG4 mammary epithelia.

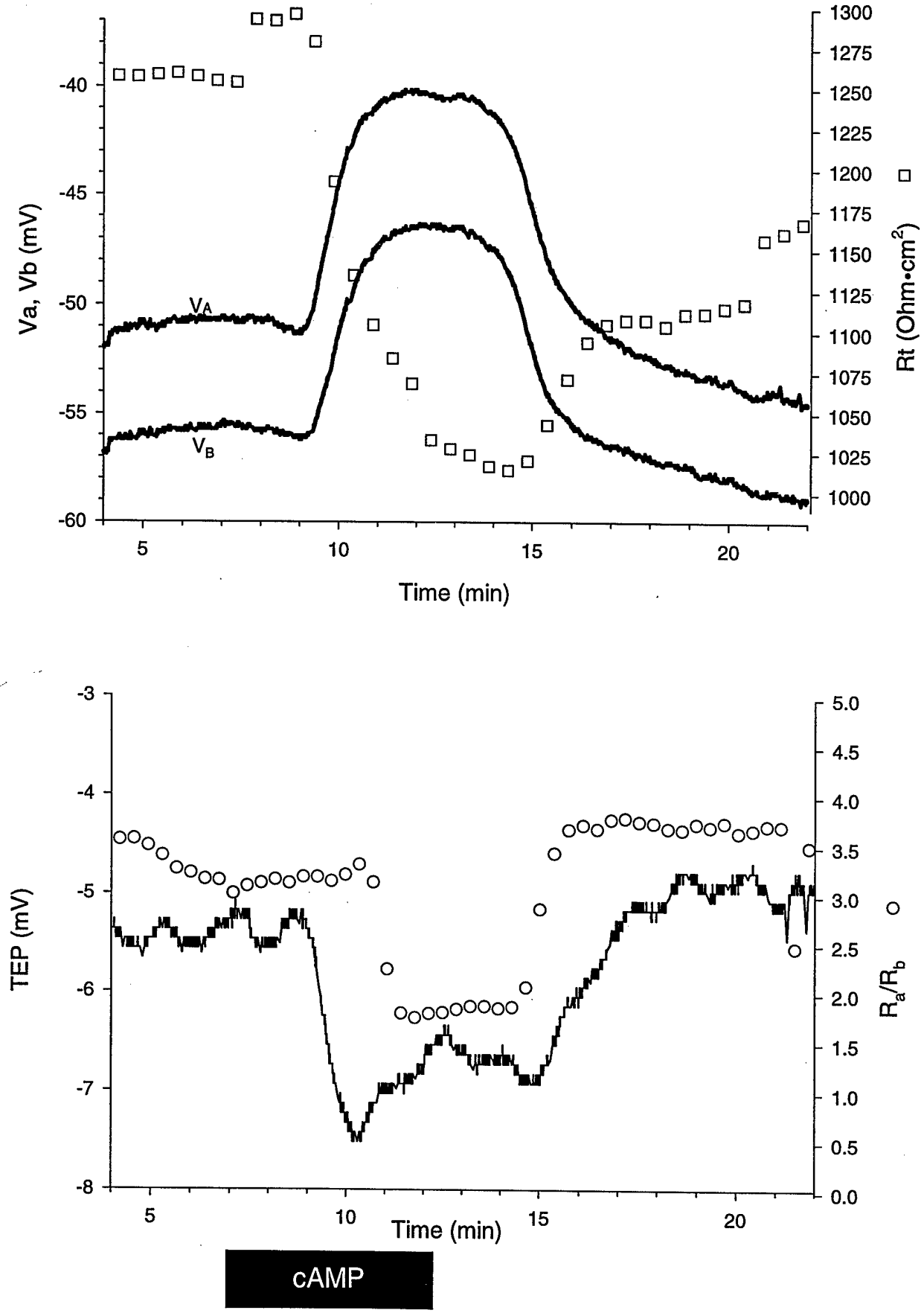

Figure 2. Electrical and resistance response to cAMP cocktail applied to the apical bath.

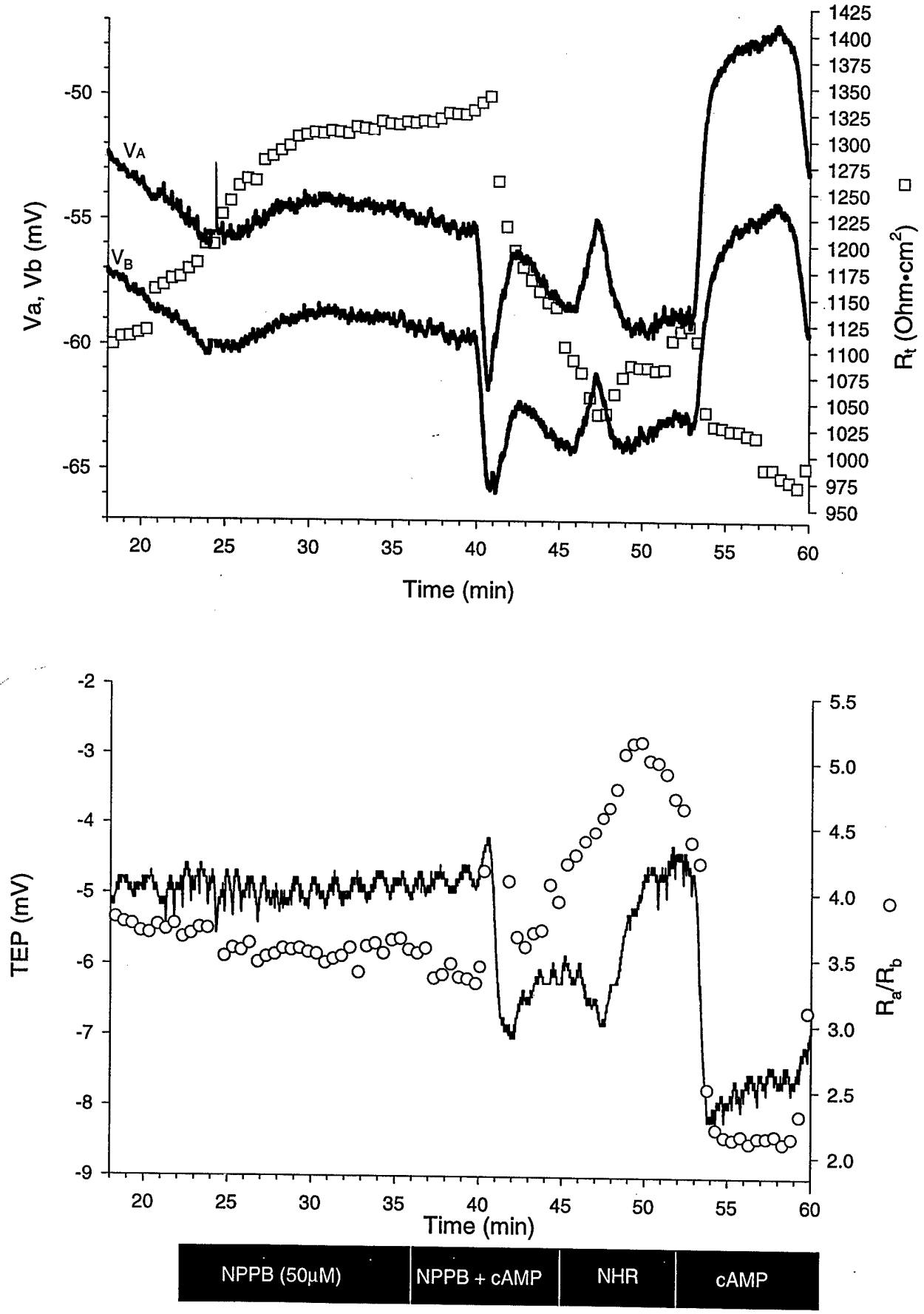

Figure 3. Electrical and resistance changes to NPPB+cAMP in the apical bath.

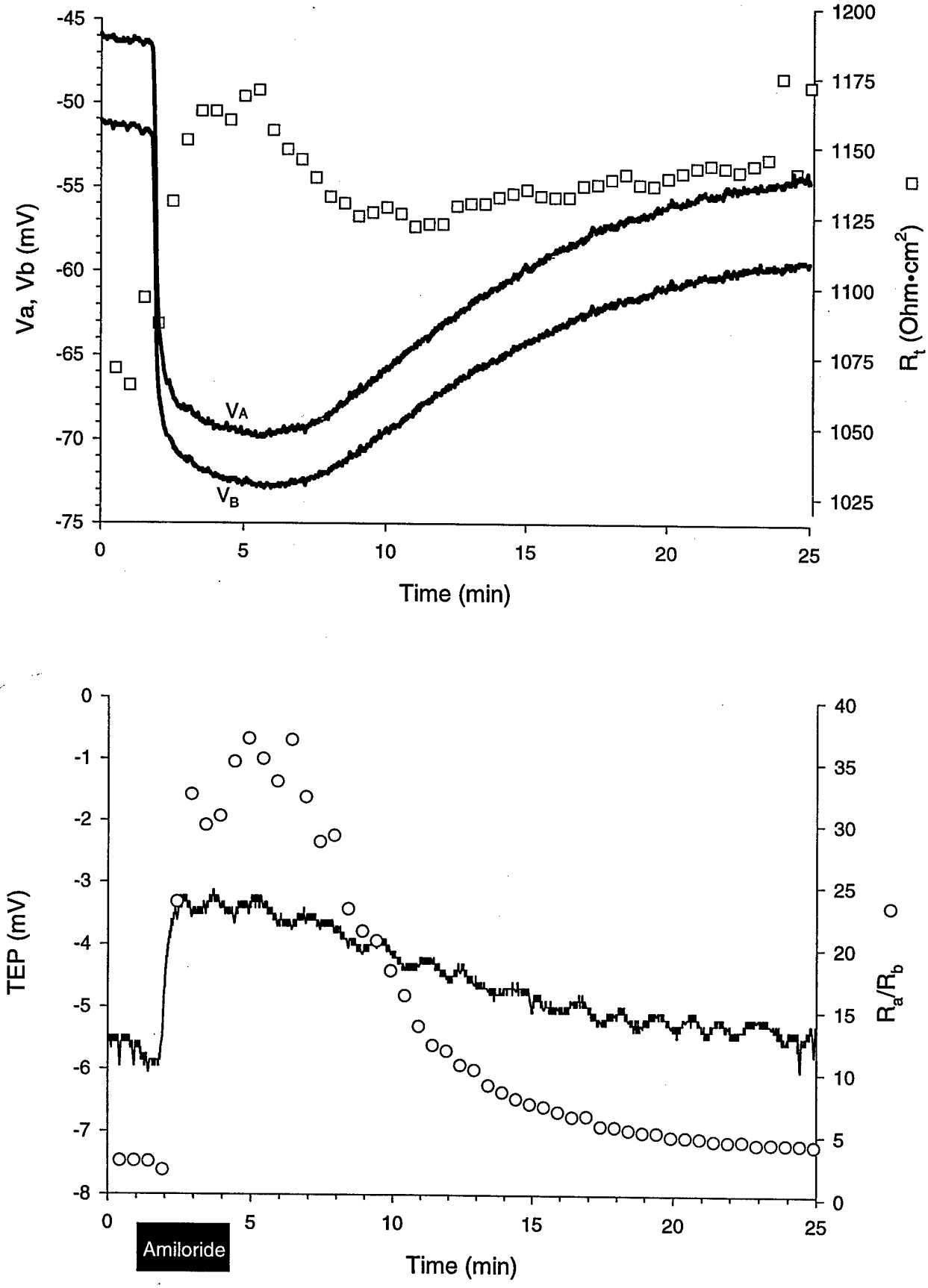

Figure 4. Electrical and resistance response to apically apllied 20 μM amiloride.

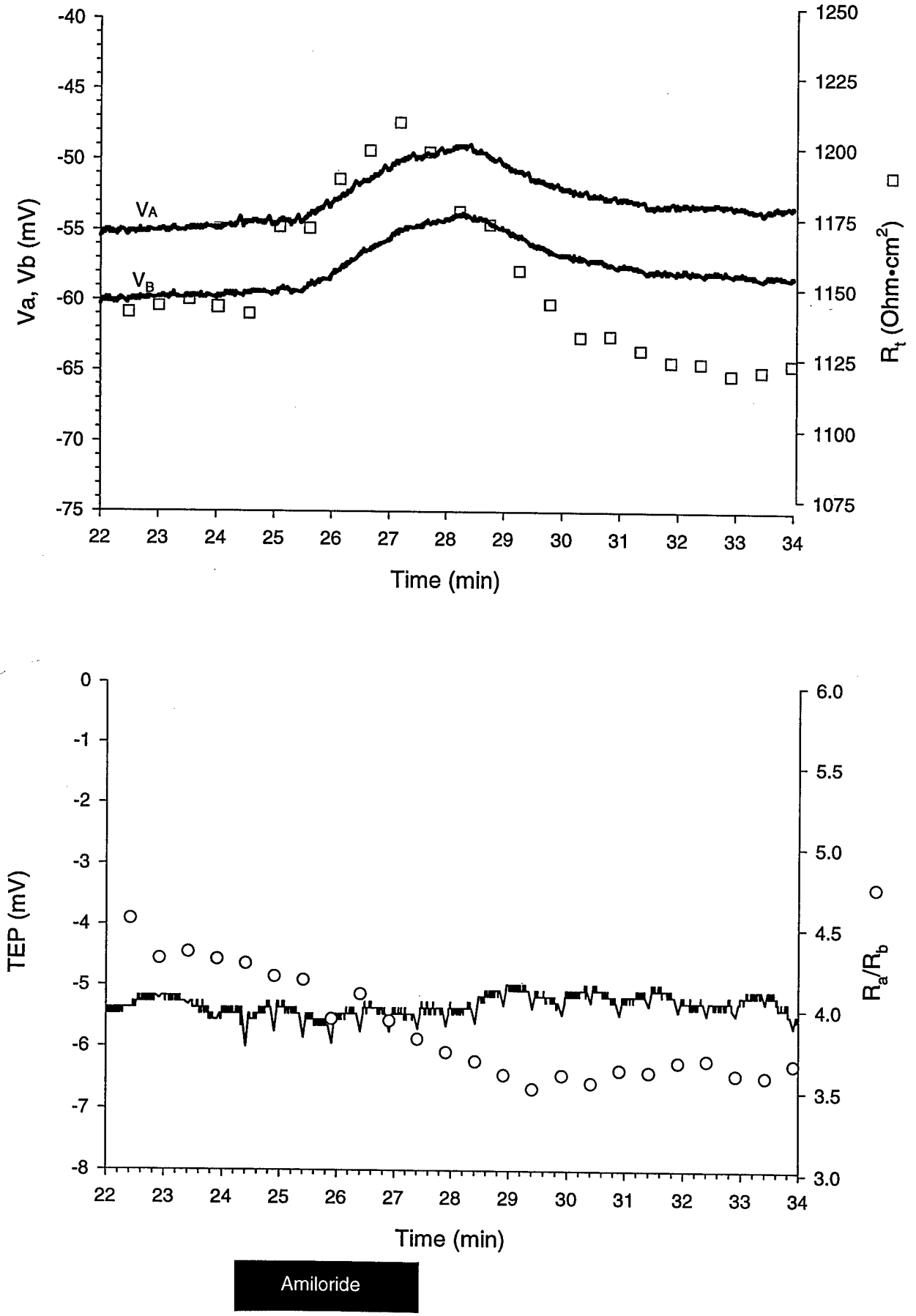

Figure 5. Electrical and resistance response to 20 μM amiloride applied to the basal bath.

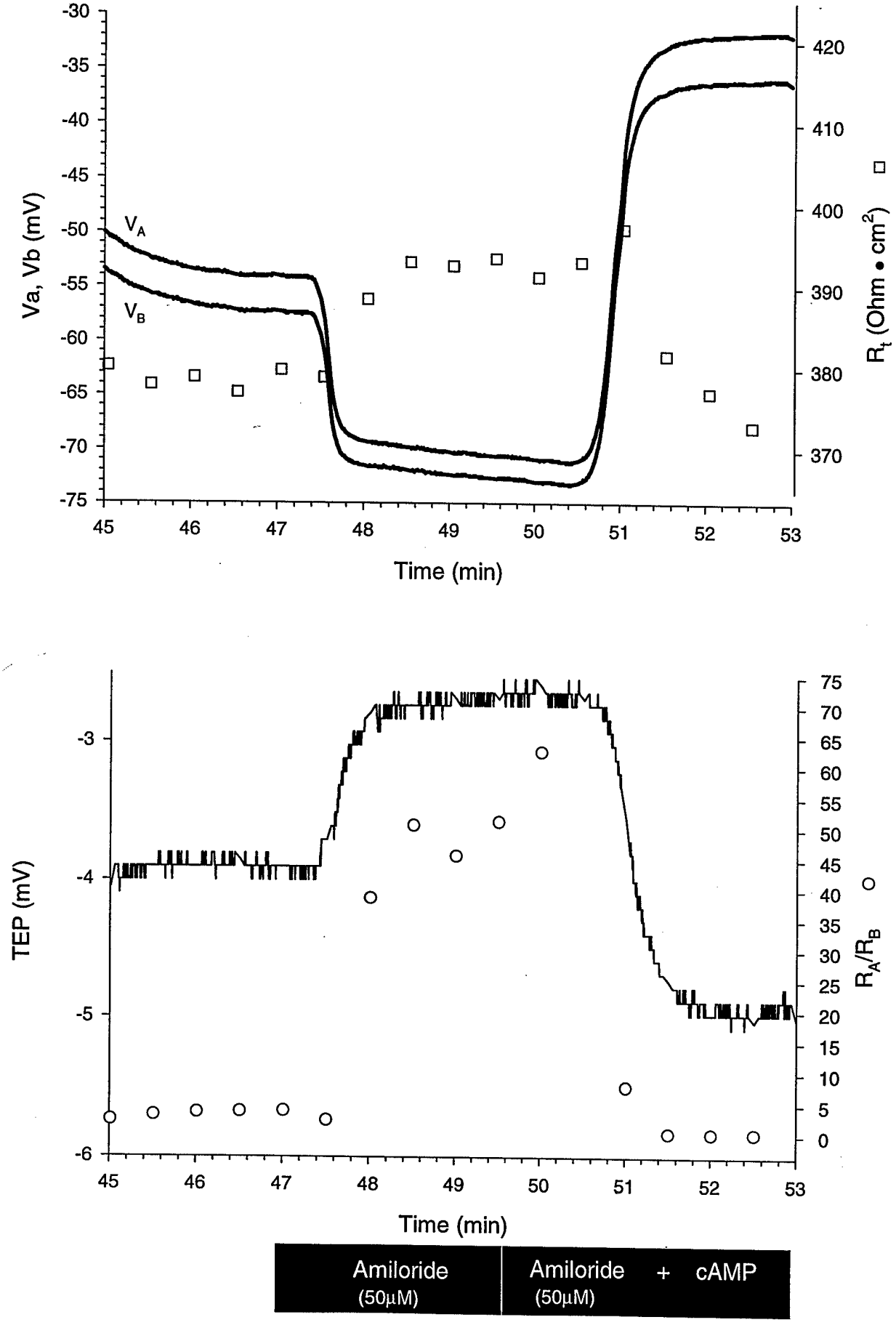

Figure 6. Electrical and resistance response to 50µM amiloride and 50µM amiloride+cAMP applied to the apical bath.

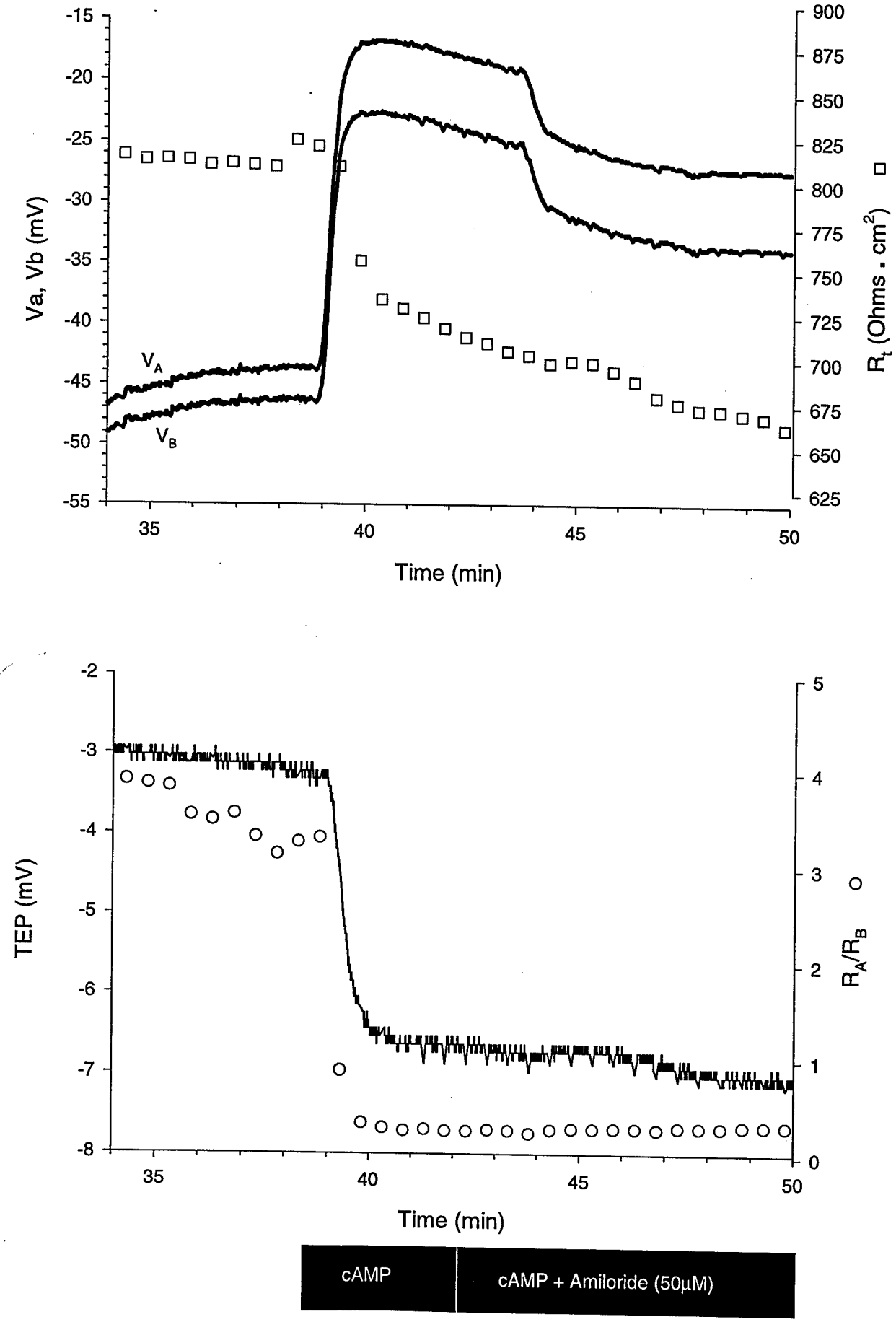

Figure 7. Electrical and resistance response to cAMP and cAMP + amiloride applied to the apical bath.

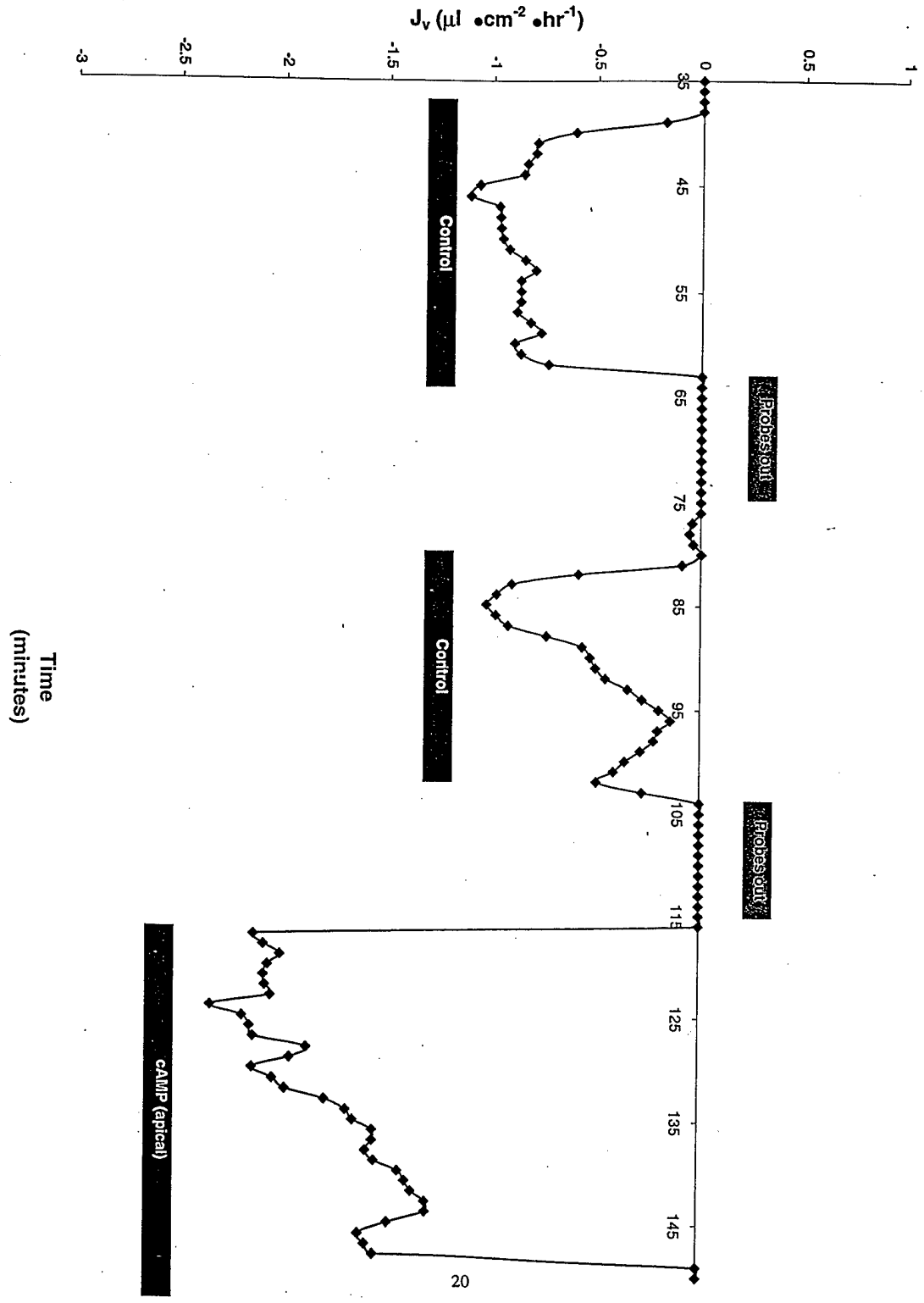

Figure 8. Fluid transport experiment. Fluid transport (J_V) is plotted with respect to time. "Control" is normal ringers solution. The addition of cAMP to the apical bath caused an increase in fluid secretion.

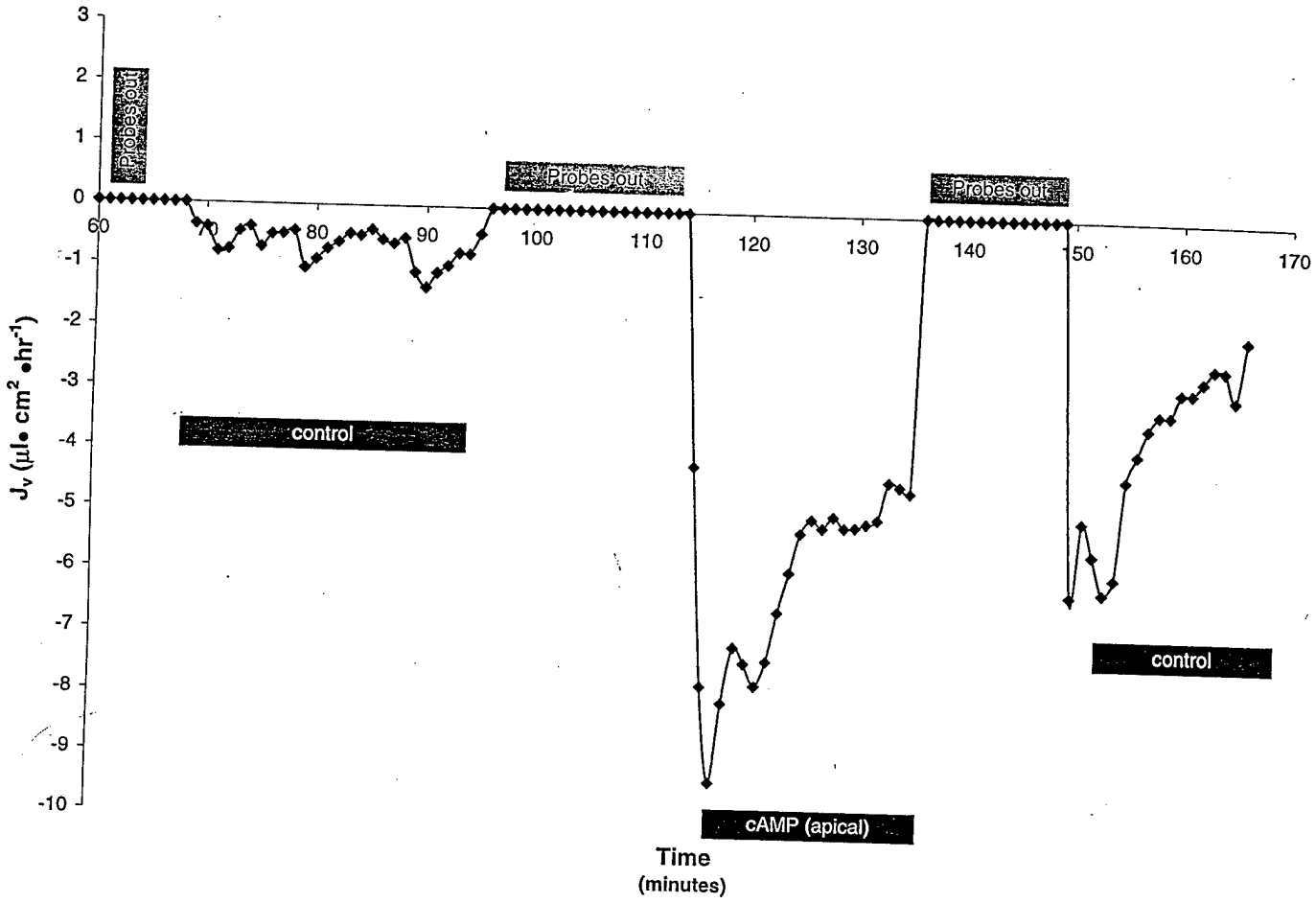

Figure 9. Fluid transport experiment. Fluid transport (J_v) is plotted with respect to time. "Control" is normal ringers solution. The addition of cAMP to the apical bath caused an increase in fluid secretion.

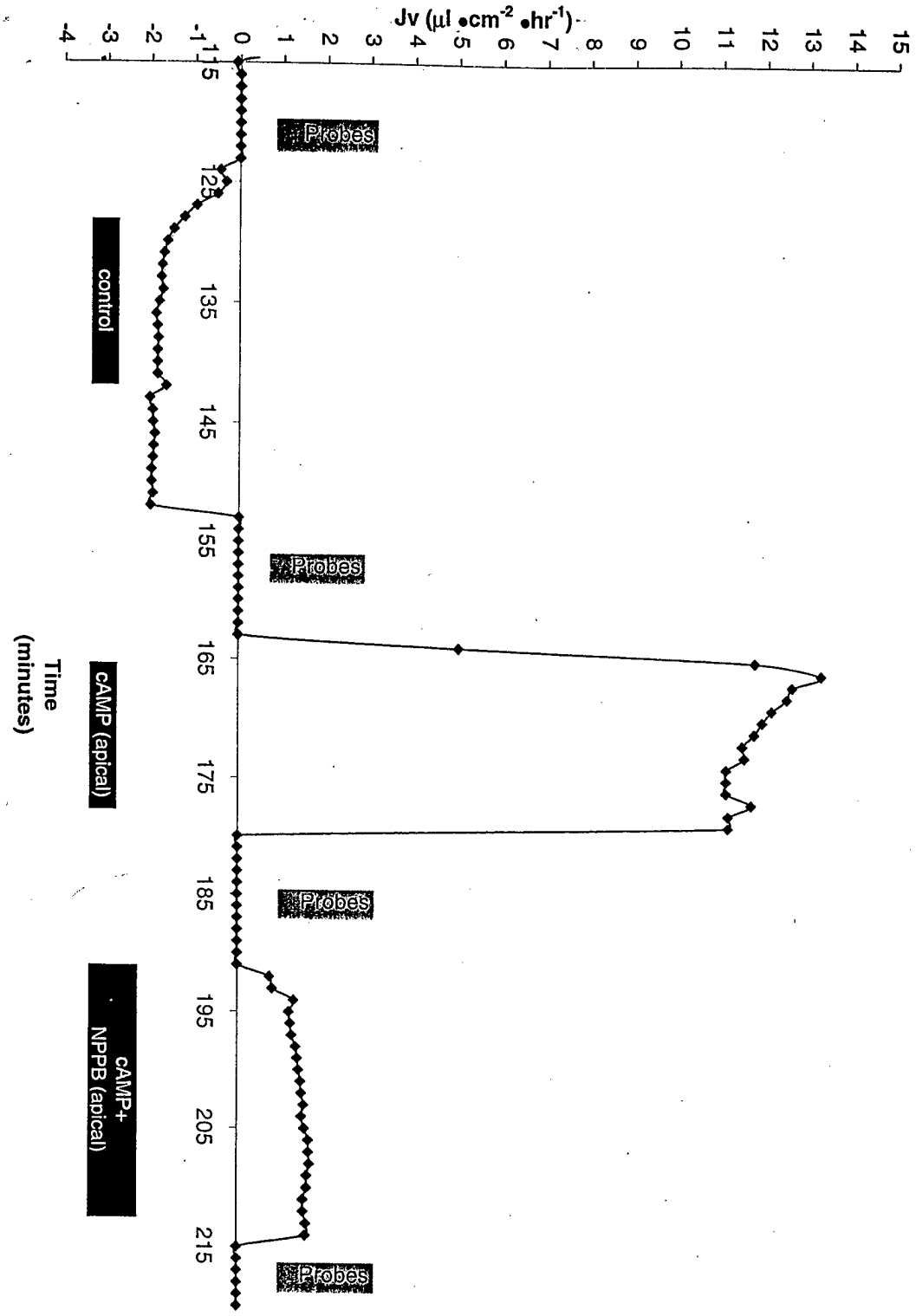

Figure 10. Fluid transport experiment. Fluid transport (J_V) is plotted with respect to time. "Control" is normal ringers solution. The addition of cAMP to the apical bath caused a net fluid absorption.

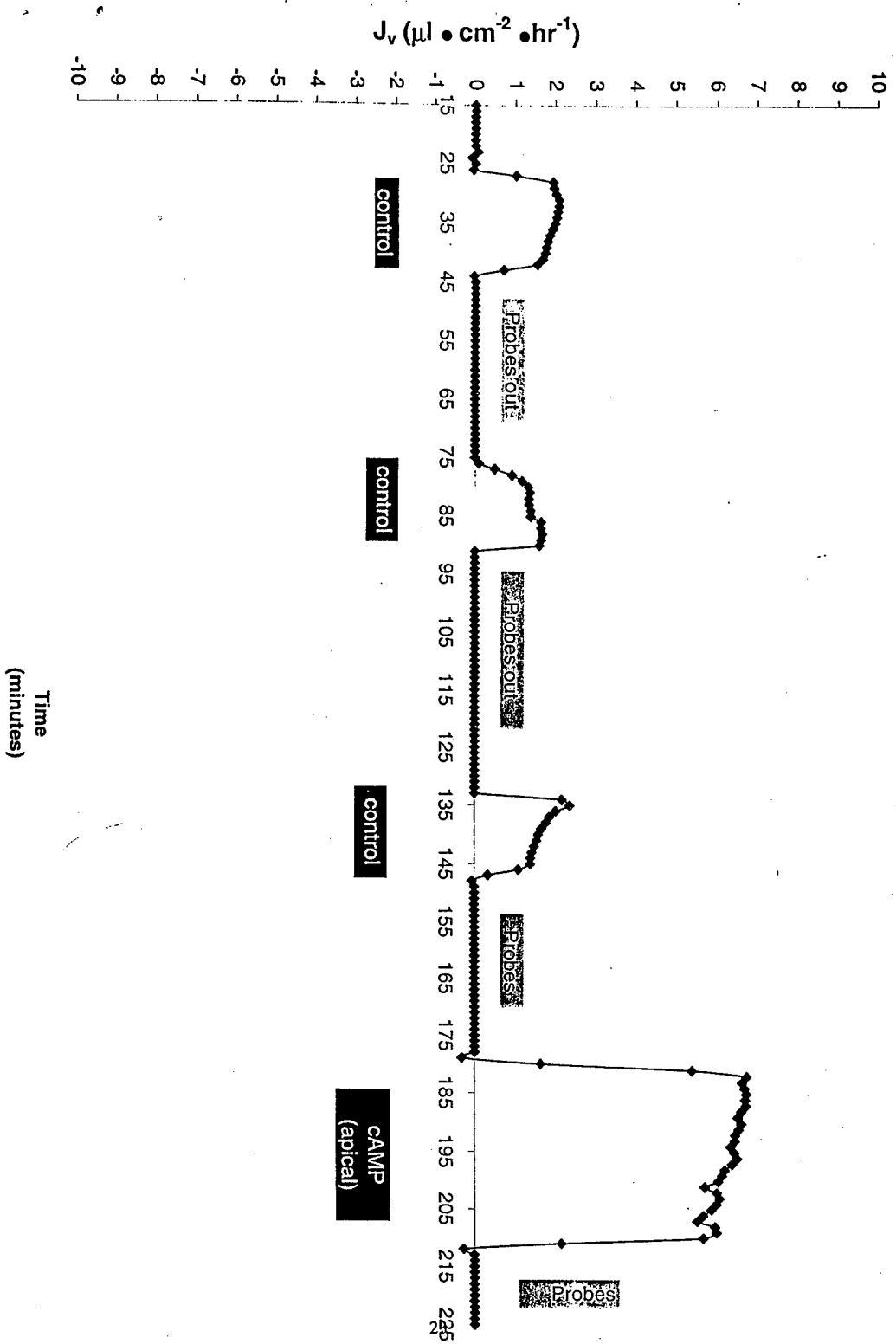

Figure 11. Fluid transport experiment. Fluid transport (J_v) is plotted with respect to time. "Control" is normal ringers solution. The addition of cAMP to the apical bath caused an increase in fluid absorption.