J. Membrane Biol. 90, 107–113 (1986)

The Journal of
Membrane Biology

Binding of ^{3}H-Phenamil, an Irreversible Amiloride Analog, to Toad Urinary Bladder: Effects of Aldosterone and Vasopressin

J.L. Garvin,* S.A. Simon, E.J. Cragoe,† and L.J. Mandel
Departments of Physiology and Anesthesiology, Duke University Medical Center, Durham, North Carolina 27710 and † Merck, Sharp and Dohme Research Laboratory, West Point, Pennsylvania 19486

Summary. Phenamil, an analog of amiloride, has previously been shown to bind specifically to sodium channels in toad bladder (J.L. Garvin et al., *J. Membrane Biol.* **87**:45–54, 1985). In this paper, ^{3}H-phenamil was used to measure sodium channel density in both isolated epithelial cells and intact bladders. From the specific binding to intact bladders, a channel density of 455 ± 102 channels/μm^2 was calculated. No correlation between specific binding and the magnitude of irreversible inhibition of short-circuit current was found. Pretreatment of intact bladders with 1 mg/ml trypsin reduced specific binding to isolated cells by 82 ± 5%. In isolated cells, neither aldosterone nor vasopressin had any significant effect on specific phenamil binding. It is inferred that phenamil binds to both open and closed channels which may be either in the mucosal membrane or in the submembrane space. Finally, and rather surprisingly, we found that ^{3}H-phenamil binds irreversibly to the basolateral membrane at concentrations as low as 4×10^{-7} M. Therefore, care must be used in interpreting binding studies with amiloride or its analog at such concentrations.

Key Words amiloride · toad bladder · phenamil · sodium channels · binding

Introduction

The study of the changes in sodium channel density in the mucosal membrane of toad urinary bladder has been useful as a tool for elucidating molecular mechanisms by which the hormones aldosterone and vasopressin increase mucosal membrane permeability to sodium ions. To date, three different approaches have been utilized: ^{14}C-amiloride binding (Cuthbert & Shum, 1975), noise analysis (Li et al., 1982; Palmer et al., 1982), and trypsinization of the apical membrane (Garty & Edelman, 1983). Use of these three methods has resulted in different explanations for the responses to aldosterone and vasopressin. Additionally, different values for the number of sodium channels are obtained upon comparison of noise analysis and binding data.

Cuthbert and Shum (1975) found that aldosterone increased the number of ^{14}C-amiloride binding sites in epithelial cells isolated from the toad bladder. This result was consistent with an earlier work of Cuthbert et al. (1974) using ^{14}C-amiloride in frog skin. From these studies the authors concluded that aldosterone increased the number of sodium channels in the mucosal membrane. This increase presumably accounted for the greater influx of sodium through the mucosal membrane observed upon addition of aldosterone (Leaf & MacKnight, 1972).

Cuthbert and Shum (1975) also reported that vasopressin did not increase the number of ^{14}C-amiloride binding sites. Since the flux and short-circuit current (I_{sc}) increase upon vasopressin addition, they concluded that vasopressin increased the conductance of existing sodium channels rather than their number in the membrane. They reported a sodium channel density that ranged from 800 to 1400 channels/μm^2.

Noise analysis only partially supports the findings of Cuthbert and Shum (1975). In a recent noise analysis study, Palmer et al. (1982) found that aldosterone increased the number of channels, a result consistent with the ^{14}C-amiloride binding data. However, the sodium channel density they reported (1 channel/μm^2) was about 1000 times less than the value reported by Cuthbert and Shum (1975). Li et al. (1982), also using noise analysis, showed that vasopressin caused an increase in channel density, whereas the aforementioned ^{14}C-amiloride binding data did not show such an increase. Again, the channel densities reported by the two methods differed by about three orders of magnitude. These differences cannot be accounted for by the high KCl

* *Present address:* Laboratory of Kidney and Electrolyte Metabolism, National Institutes of Health, Bethesda, Maryland 20205.

concentrations placed on the basolateral side during the noise measurements (Abrahamcheck et al., 1985).

An interesting report by Garty and Edelman (1983) showed that the addition of trypsin to the mucosal surface of toad bladders reduced I_{sc}. Also, after trypsinization, the addition of vasopressin increased I_{sc}, suggesting that additional amiloride-sensitive sodium channels were recruited from an intracellular pool presumably in the form of vesicles. However, these results are not consistent with a study that showed that membrane capacitance (which is proportional to plasma membrane area) does not increase upon addition of vasopressin (Stetson et al., 1982).

Garty and Edelman (1983) also showed that after trypsinization the addition of aldosterone did not increase I_{sc} to the same extent as it did before trypsinization, suggesting that the aldosterone-stimulated Na channels were already present in the membrane prior to the addition of the hormone. Consistent with this paradigm is the recent work of Sariban-Sohraby et al. (1983), who presented evidence showing that aldosterone increases sodium influx across cultured A6 cells by methylating an already present sodium channel. Thus, there are several inconsistencies between the ^{14}C-amiloride binding data and the results obtained by noise analysis and chemical modification of the sodium channel. In an attempt to clarify some of these inconsistencies we used the irreversible radiolabeled sodium channel inhibitor ^{3}H-phenamil (Garvin et al., 1985) to determine the sodium channel density in the absence and presence of aldosterone or vasopressin.

This study represents the first in which an irreversible inhibitor of the mucosal sodium channel was utilized to measure channel density. A channel density of about 450 channels/μm^2 was calculated. This value does not significantly change upon either the addition of aldosterone or vasopressin. Our results also suggest that ^{3}H-phenamil permeates the mucosal membrane and therefore can bind to sodium channels inside epithelial cells. From our analysis of the specific binding one can rationalize the discrepancy between channel densities calculated from noise analysis and phenamil binding data by considering that ^{3}H-phenamil may bind to both open and closed channels as well as other specific binding sites. Surprisingly, it was found that ^{3}H-phenamil exhibits specific binding to the basolateral membrane (which is presumably devoid of the "usual" epithelial sodium channel) at concentrations as low as 4×10^{-7} M. Therefore, care must be used in interpreting binding studies with amiloride or its analog used at such concentrations.

Materials and Methods

Toads, *Bufo marinus*, were obtained from Carolina Biological Supply (Burlington, N.C.). The toads were kept in aquaria containing tap water at room temperature until they were sacrificed by double pithing.

Solutions

Two saline solutions were utilized in this study. The first solution, called C (*see* Garvin et al., 1985), contained (in mM): 100 NaCl; 4.0 KCl; 1.5 $CaCl_2$; 0.8 $MgSO_4$; 2.5 $NaHCO_3$; 0.8 NaH_2PO_4, and 10.5 glucose, pH 8.1. The other solution, called E (30 mM Na), was similar to solution C except that it contained only 29.2 mM NaCl and no $NaHCO_3$ at pH 6.4. In ^{3}H-phenamil binding experiments using isolated cells, choline Cl (recrystallized) was added to solution E to increase the osmolarity to ~220 mOsm.

All chemicals were reagent grade. Amiloride, phenamil, and ^{3}H-phenamil were synthesized at Merck, Sharp and Dohme Research Laboratory. Trypsin, type IX (EC no. 3.4.21.4) and aldosterone were purchased from Sigma Chemical Company (St. Louis, Mo.). Vasopressin was obtained from Parke-Davis (Morris Plains, N.J.).

Binding of ^{3}H-Phenamil to Isolated Cells

The ^{3}H-phenamil specific binding dose response isotherm, vasopressin, aldosterone and trypsin experiments were performed with isolated epithelial cells. To obtain isolated cells, hemibladders were incubated in solution C (with no Ca added) containing 1 mM EDTA at 4°C for 15 min, whereupon the epithelial cells were scraped from the supporting tissue by two passes of a blunted microscope slide. The cell sheet was dispersed by pipetting for 5 min. The resulting suspension was layered on a Percoll® (Pharmacia, Piscataway, N.J.) cushion, density 1.091, and centrifuged at $1000 \times g$ to remove red blood cells. This process was repeated three times. The resulting cells were washed twice to remove traces of Percoll and resuspended in solution E, to which 65 mM choline Cl were added. The cell suspension was then filtered through a 74 μm nylon mesh (Small Parts, Miami, Fla.), pelleted, and resuspended in the same solution. The cells were then divided in half and placed into two vials containing stir bars, in which the binding studies were performed at room temperature. One sample (2 ml) was used to measure total binding and the other to measure nonspecific binding. Specific binding was obtained by subtracting the nonspecific binding from the total binding. At the beginning of the experiment, unlabeled phenamil was added to the nonspecific binding vial at 1000 times the concentration of the ^{3}H-phenamil (to be added later). After 15 min, ^{3}H-phenamil (range 5×10^{-9} to 10^{-6} M) was added to both vials and incubated for 40 min (Garvin et al., 1985). The binding experiments were terminated by centrifugation through 300 μl dioctyl phthalate (4°C) on a 50 μl Percoll cushion in an Ependorf tabletop centrifuge. Dioctyl phthalate was selected because its density is 1.035 at 4°C and it is immiscible with water. The pellet was then washed three times in solution E containing 65 mM choline Cl, and twice in distilled water for 15 min to remove the unbound phenamil. The resulting pellet was then dissolved in 5% deoxycholate in 0.1 M NaOH. Aliquots (100 μl) were counted in a Beckman LS-250 liquid scintillation counter using Aquasol (New England Nuclear, Boston, Mass.) as the scintillation fluid.

Protein content was measured using the method of Lowry et al. (1951).

Effect of Aldosterone

Toads were kept in aquaria containing 0.6% NaCl for at least three days (Benjamin & Singer, 1974) or hemibladders were incubated in solution C containing 1 mg/ml penicillin for 16 hr prior to exposure to aldosterone in order to reduce endogenous aldosterone levels (Edelman et al., 1963). At least two toads were used for each experiment. One hemibladder from each toad was incubated in either solution C plus diluent (as the control) or solution C containing 10^{-6} M aldosterone for 6 hr. After this incubation, the binding experiments were performed as described previously, using ^{3}H-phenamil at a concentration of 10^{-7} M.

To insure that the bladders treated with aldosterone responded by increasing Na transport, the I_{sc} was measured in intact bladders in the presence and absence of aldosterone (*see* Garvin et al., 1985, for methods). We found that aldosterone (10^{-6} M) caused I_{sc} to double in about 90 min.

Effect of Vasopressin

One hemibladder from each of two toads was incubated for 15 min in 100 mU/ml vasopressin (Parke-Davis, Detroit, Mich.) in solution C. During the remainder of the experiment this tissue was exposed to vasopressin. The other two hemibladders were incubated in solution C only and served as controls. Isolated epithelial cells were prepared and binding experiments performed as described earlier. The effect of vasopressin was found to reach a maximum at 15 min and decay with a $t_{1/2}$ of 110 min from I_{sc} measurements.

Effect of Trypsin

One hemibladder from each of two toads was incubated for 40 min in solution C containing 1 mg/ml trypsin type X (Sigma, St. Louis, Mo.) in a petri dish. The other two hemibladders were incubated in C only and served as controls. Isolated epithelial cells were prepared and binding experiments performed as described previously.

^{3}H-Phenamil Binding to Apical Membrane of Intact Bladder

Hemibladders were mounted in chambers having an area of 10 cm^2. The serosal half of the chamber was perfused with solution E and the mucosal half of the chamber was perfused with solution C. I_{sc} was recorded during the experiment. Two chambers were used in each experiment, one to measure total binding and the other to measure nonspecific binding. After the I_{sc} reached steady state, excess unlabeled phenamil was added to the mucosal solution of the chamber in which nonspecific binding was being measured. After 15 min, 4×10^{-7} M ^{3}H-phenamil was added to the mucosal solution of both chambers. At the end of 40 min, the drug was removed from both chambers by washing. When I_{sc} reached steady state, the hemibladders were removed and isolated epithelial cells were prepared as described in the section on cell binding.

The concentration of ^{3}H-phenamil on the basolateral side was obtained by counting a 100-μl sample after the bladder was incubated for 40 min with 4×10^{-7} M phenamil on the apical side. Under these conditions the basolateral concentration was 3×10^{-10} M.

Channel density was calculated from a knowledge of: the specific binding, the specific activity of phenamil (2.15 Ci/mmol), and a measured protein-to-bladder area ratio of 0.16 mg protein/cm^2. The protein-to-bladder area conversion was obtained by measuring the amount of protein in epithelial cells scraped from the area of tissue exposed in the chamber.

Binding of ^{3}H-Phenamil to the Basolateral Surface of Intact Bladder

Experiments were performed as above except that solution C bathed both sides of the tissue and phenamil was added to the basolateral solution. ^{3}H-phenamil concentrations ranged from 10^{-7} M to 2×10^{-6} M. These concentrations of phenamil have been previously shown not to affect I_{sc} when added to the basolateral side (Garvin et al., 1985).

Statistics

All data are reported as the mean ± SEM. Data were analyzed using the student's t-test. Differences with $P < 0.1$ were considered significant. All hemibladders reaching a steady-state I_{sc} were utilized.

Results

Binding of ^{3}H-Phenamil to Isolated Cells

Figure 1 shows the concentration dependence of ^{3}H-phenamil binding to isolated epithelial cells in solution E (30 mM Na, pH 6.4). The three curves represent the total, nonspecific, and specific binding. The specific binding isotherm is composed of two distinct regions. The low concentration region, <0.1 μM, which exhibits saturation, is expanded in the inset. In the high concentration region, >0.1 μM, the specific binding increases monotonically with increasing phenamil concentration. The same behavior was seen by Cuthbert and Shum (1975) in toad bladder using ^{14}C-amiloride.

The saturation seen in the low concentration region was assumed to represent binding to the mucosal sodium channels (*see* Discussion), since 0.1 μM phenamil inhibits I_{sc} in intact bladders more than 50% under identical conditions (Garvin et al., 1985). The specific binding at 0.005 μM phenamil is not statistically different from zero ($P > 0.1$). However, as the nonspecific binding was about 5 times background, the absence of specific binding cannot be attributed to the inability to detect ^{3}H-phenamil. The specific binding in the high concentration re-

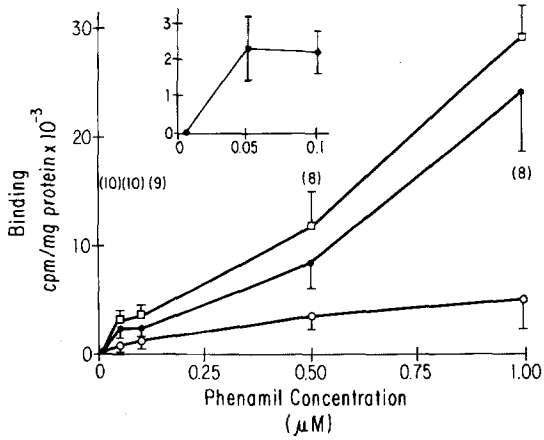

Fig. 1. Concentration dependence on ^{3}H-phenamil binding to isolated epithelial cells. Total (□), nonspecific (○), and specific (●) binding curves are shown. Binding was performed in a solution containing 30 mM sodium at pH 6.4 with tissue that had been incubated for at least 16 hr in solution C. The inset shows an expanded view of the 0 to 0.1 μM range of specific binding. Specific binding saturates at 0.05 μM phenamil. Specific binding at 0.005 μM phenamil was not significantly different from zero ($P > 0.3$). The point at this concentration masks the error bars. The number of experiments is given in parentheses. Data are reported as the mean ± SEM

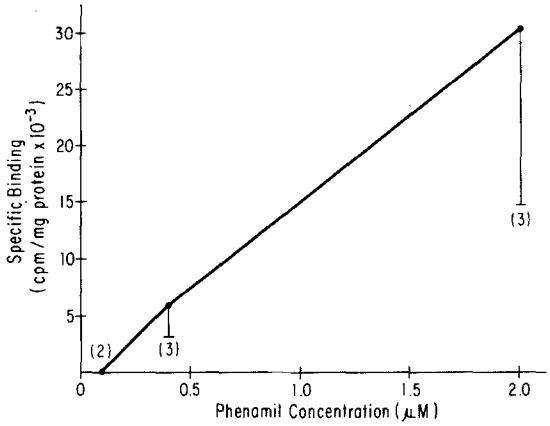

Fig. 2. A graph of the specific binding of ^{3}H-phenamil to the basolateral surface of a toad bladder mounted in a Ussing chamber *vs.* the concentration of phenamil in the solution bathing the basolateral surface. The numbers in parentheses indicate the number of experiments. The specific binding at 0.4 and 2.0 μM are significantly greater than zero ($P < 0.1$)

gion likely represents the association of phenamil with "sites" other than the sodium channel. Such sites may be any of the ones reported to be affected by amiloride and its analogs at concentrations higher than 0.1 μM (Benos, 1982). This proposition is supported by the data of Fig. 2, which shows specific binding of ^{3}H-phenamil to the intact bladder when ^{3}H-phenamil is applied to the basolateral side. At a concentration of 0.1 μM there is no specific binding. However, at higher concentrations, the binding increases linearly with phenamil concentration and is comparable (albeit slightly larger) in magnitude to that observed in the isolated cells.

Since Garty and Edelman (1983) showed that both the I_{sc} and the ability of amiloride to inhibit I_{sc} are significantly inhibited by trypsin, the effects of trypsin on ^{3}H-phenamil binding were studied. Trypsin reduced the specific binding of 10^{-7} M phenamil to isolated cells from 687 ± 168 to 142 ± 88 cpm/mg or 82 ± 5% in six paired experiments ($P < 0.01$).

Effects of Vasopressin and Aldosterone on ^{3}H-Phenamil Binding on Isolated Cells

The effects of vasopressin and aldosterone on phenamil binding are shown in the Table. The ^{3}H-phenamil concentration was 10^{-7} M in both sets of experiments. No statistically significant difference in specific ^{3}H-phenamil binding in paired experiments between control and vasopressin and/or aldosterone-treated cells were found.

Binding of ^{3}H-Phenamil to Intact Bladders

^{3}H-phenamil binding experiments were performed with intact bladders mounted in Ussing chambers so that irreversible inhibition of I_{sc} could be measured together with specific binding. The results of nine experiments (Fig. 3) show that no significant correlation existed between specific binding and the magnitude of the I_{sc} irreversibly inhibited. Specific binding also did not correlate with initial I_{sc} or percentage of I_{sc} irreversibly inhibited (*data not shown*). The specific binding, which averaged 936 ± 210 cpm/mg protein, corresponds to a channel density of 455 ± 102 channels/μm^2.

Discussion

In a previous paper (Garvin et al., 1985), we reported that phenamil inhibited the I_{sc} of toad urinary bladder irreversibly, specifically and with high affinity, and is competitive with amiloride. Furthermore, it was reported that the extent of irreversible inhibition increased as the mucosal sodium concentration and pH decreased. In the present communication, we used this information to investigate further the interaction of phenamil with the epithelial sodium channel by using tritiated phenamil. From these measurements, the density of mucosal sodium

Table. Effects of vasopressin and aldosterone on the specific binding of ^{3}H-phenamil to isolated toad bladder cells from paired bladders in cpm/mg protein

	cpm/mg/protein	n	
Control	2344 ± 1115	(5)	NS
Vasopressin	2212 ± 645	(5)	
Control	2807 ± 576	(9)	NS
Aldosterone	3576 ± 1018	(9)	

channels in the presence and absence of aldosterone and vasopressin was calculated.

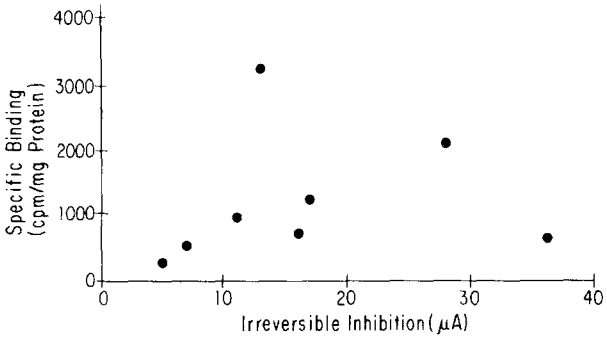

Fig. 3. A plot of specific binding of ^{3}H-phenamil *vs.* irreversibly inhibited I_{sc}. Experiments were performed in a solution containing 30 mM sodium at pH 6.4 in chambers of area 10 cm^2. No significant correlation exists between specific binding and the magnitude of the irreversibly inhibited I_{sc} (n = 9)

Binding of ^{3}H-Phenamil

Binding studies proved easier to perform in isolated epithelial cells than in Ussing chambers. The major problem with the former approach was the possibility that ^{3}H-phenamil bound to sites other than the mucosal sodium channel. The experiments shown in Figs. 1 and 2 were performed to define the concentration range within which ^{3}H-phenamil binding would be specific only for the sodium channels. Both the saturation of cellular binding at 0.1 μM and the lack of binding from the basolateral side at this concentration suggest that most of the specific ^{3}H-phenamil binding at 0.1 μM is to the mucosal side. That this binding is predominantly to the sodium channel is provided by three pieces of evidence: (a) at this concentration, more than 50% of the I_{sc} is inhibited by mucosal phenamil in intact bladders under similar experimental conditions; (b) phenamil seems to bind to the same site as amiloride since excess amiloride prevents specific binding of ^{3}H-phenamil; also the relationship between specific binding of phenamil *vs.* concentration of ^{3}H-phenamil (*see* Fig. 1) is very similar to that reported for specific amiloride binding *vs.* concentration of ^{14}C-amiloride (Cuthbert & Shum, 1975); and (c) trypsin, which appears to decrease the number of amiloride-sensitive mucosal sodium channels (Garty & Edelman, 1983), decreases specific binding of ^{3}H-phenamil by about 80%. Furthermore, this result suggests that at least 80% of ^{3}H-phenamil is bound to the cell exterior since trypsin is not believed to enter the cell.

The specific binding of ^{3}H-phenamil observed at concentrations higher than 0.1 μM probably represents the lower affinity sites, such as the Na-K-ATPase, Na-Ca exchange, Na-H exchange, and Na-Na exchange, as well as other sites still to be determined. It is important to emphasize that at concentrations as low as 4×10^{-7} M there is significant binding ($P < 0.1$) to the basolateral membrane, a membrane putatively devoid of sodium channels. Consequently, the concentration range over which investigators can be certain that they are only investigating the apical sodium channel is limited to concentrations $\leq 10^{-7}$ M. In this regard, the experiments with intact bladders were performed at an apical phenamil concentration of 4×10^{-7} M (Fig. 3), in order to obtain a statistically significant inhibition of I_{sc}. The justification for these experiments is that the phenamil concentration in the basolateral solution was measured to be 3×10^{-10} M. This concentration is a factor of 1000 less than the concentration where specific binding was detected. Therefore, the binding data for the intact bladders rests on justifiable assumptions.

Channel Density

Channel densities could be calculated from the binding data, provided that the binding stoichiometry was known. Although the stoichiometry of phenamil binding could not be determined in these experiments due to the limited concentration range over which specific binding could be obtained, it is reasonable to assume that phenamil binds with a 1 : 1 stoichiometry to the sodium channel. This stoichiometry is consistent with the data obtained for amiloride using noise analysis (Lindemann & Van Driessche, 1977) although other investigators, using analogs of amiloride that stimulate I_{sc}, suggested that there may be two amiloride binding sites on the sodium channel (Benos & Whatthey, 1981; Li & Lindemann, 1983). If the stoichiometry is greater than one phenamil molecule per channel, the estimate of channel density will be lower by that ratio.

However, it is important to note that the estimate of channel density from noise analysis and ^{14}C-amiloride binding data differ by a factor of 1000 and not by a factor of two. Such a situation exists for gap junctions where there is a large excess of junctions over that required for cell communication (Meyer et al., 1981).

From the binding data to intact bladder, a channel density of 450 channels/μm^2 is calculated. This value is two to three times lower than the previously reported values of 800 to 1400 channels/μm^2 (Cuthbert & Shum, 1975, who also assumed a 1 : 1 binding stoichiometry) in the toad bladder. The most likely source of this difference is the inability of Cuthbert and Shum (1975) to wash adequately their preparation due to the high dissociation rate constant of amiloride from the channel (Lindemann & Van Driessche, 1977). This problem is not encountered with phenamil due to its irreversible binding (or low dissociation rate constant).

Using noise analysis to obtain the sodium channel density of toad bladder, Li et al. (1982) and Palmer et al. (1982) reported a value of about 1 channel/μm^2 under control conditions. This value is much lower than that reported in this study (450 channels/μm^2). Various possibilities for this are:

(1) Actual membrane area: Based on capacitance measurements, Li et al. (1982) found that 1 cm^2 of chamber area corresponds to 2 cm^2 of actual apical membrane area.

(2) Hormonally recruitable channels: Noise analysis data have shown that aldosterone increases channel density twofold (Palmer et al., 1982) and vasopressin nearly fivefold (Li et al., 1982). Additionally, other channels may exist which are hormonally or chemically recruitable as will be discussed below.

(3) Stoichiometry of binding: The stoichiometry of ^{3}H-phenamil binding was assumed to be 1 to 1; the stoichiometry may be greater (Benos & Whatthey, 1981; Li & Lindemann, 1983).

(4) Binding to other sites: Unfortunately, the binding of phenamil to sites other than the sodium channel is a possibility. An analysis of the number of high-affinity sites could not be completed due to the limited range over which specific binding could be measured. However, as discussed previously, phenamil binding at 0.1 μM displays the properties expected for binding specifically to the sodium channel.

The present experiments do not follow further differentiation among these possibilities. Nevertheless, the large excess of binding sites over conducting channels makes it improbable that variations in the number of conductive channels by hormonal or chemical stimulation would be observable as an alteration in the number of phenamil binding sites. If sodium channels were located in the cytoplasm, phenamil would in all probability also bind to them. This occurs because phenamil [like amiloride (Benos et al., 1983)] apparently permeates through plasma membranes and can therefore bind to dormant channels existing in cytoplasmic vesicles that are incorporated into the plasma membrane upon appropriate stimulation (Garty & Edelman, 1983). The 20% of phenamil binding sites that are trypsin-insensitive may be associated with these dormant cytoplasmic channels. Consequently, it is not surprising that there is no correlation between phenamil binding and I_{sc} inhibition and that neither aldosterone or vasopressin alter the number of phenamil binding sites.

The present results with aldosterone are not in agreement with those of Cuthbert and Shum (1975), who reported a doubling in the number of ^{14}C-amiloride binding sites. On the other hand, our results with vasopressin are in agreement with Cuthbert and Shum in that no change in the number of binding sites was observed. It is unclear why this discrepancy in results was observed with aldosterone. Perhaps their inability to adequately wash their preparation interfered with their experimental results. As discussed earlier, we think that the large excess of phenamil binding sites makes it highly unlikely that conditions which alter sodium conductance would be reflected in changes in binding. Clearly, these types of binding experiments do not distinguish among the various proposed models for aldosterone or vasopressin action. However, these binding studies raise the extremely interesting possibility that a large excess of sodium channels may be present in these cells.

This work was supported by North Carolina Heart Association and Sigma Xi grants to J.L. Garvin and NIH Grant GM 29256 to L.J. Mandel and NS 20669 to S.A. Simon. We thank Dr. Dale Benos for critically reading this paper and the referees for making several useful suggestions. We further acknowledge Ms. Gay Blackwell for typing this manuscript.

References

Abrahamcheck, F.J., Van Driessche, W., Helman, S.I. 1985. Autoregulation of apical membrane Na^+ permeability of tight epithelia. Noise analysis with amiloride and CGSS 4270. *J. Gen. Physiol.* **85**:555–582

Benjamin, W.B., Singer, I. 1974. Aldosterone-induced proteins in toad urinary bladder. *Science* **186**:269–272

Benos, D.J. 1982. Amiloride: A molecular probe of sodium transport in tissues and cells. *Am. J. Physiol.* **242**:C131–C145

Benos, D.J., Reyes, J., Shoemaker, D.G. 1983. Amiloride fluxes across erythrocyte membranes. *Biochim. Biophys. Acta* **734**:99–104

Benos, D.J., Whatthey, J.W.H. 1981. Inferences on the nature of

the apical sodium entry site in frog skin epithelium. *J. Pharmacol. Exp. Ther.* **219:**481–488

Cuthbert, A.W., Okpako, D., Shum, W.K. 1974. Aldosterone, moulting, and the number of sodium channels in frog skin. *Brit. J. Pharmacol.* **51:**128P

Cuthbert, A.W., Shum, W.K. 1975. Effects of vasopressin and aldosterone on amiloride binding in toad bladder epithelial cells. *Proc. R. Soc. London B* **189:**543–575

Edelman, I.S., Bogoroch, R., Porter, G.A. 1963. On the mechanism of action of aldosterone on sodium transport: The role of protein synthesis. *Proc. Natl. Acad. Sci. USA* **50:**1169–1177

Garty, H., Edelman, I.S. 1983. Amiloride-sensitive trypsinization of apical sodium channels. *J. Gen. Physiol.* **81:**785–803

Garvin, J.L., Simon, S.A., Cragoe, E.J., Jr., Mandel, L.J. 1985. Phenamil: An irreversible inhibitor of sodium channels in the toad urinary bladder. *J. Membrane Biol.* **87:**45–54

Leaf, A., Macknight, A.D.C. 1972. The site of the aldosterone-induced stimulation of sodium transport. *J. Steroid Biochem.* **3:**237–245

Li, J.H.-Y., Lindemann, B. 1983. Chemical stimulation of Na transport through amiloride-blockable channels of frog skin epithelium. *J. Membrane Biol.* **75:**179–192

Li, J.H.-Y., Palmer, L.G., Edelman, I.S., Lindemann, B. 1982. The role of sodium-channel density in the natriferic response of the toad urinary bladder to an antidiuretic hormone. *J. Membrane Biol.* **64:**77–89

Lindemann, B., Van Driessche, W. 1977. Sodium-specific membrane channels of frog skin are pores: Current fluctuations reveal high turnover. *Science* **195:**292–294

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275

Meyer, D.J., Yancy, S.B., Revel, J.-P. 1981. Intercellular communication in normal and regenerating rat liver: A quantitative analysis. *J. Cell Biol.* **91:**505–523

Palmer, L.G., Li, J. H.-Y., Lindemann, B., Edelman, I.S. 1982. Aldosterone control of the density of sodium channels in the toad urinary bladder. *J. Membrane Biol.* **64:**91–102

Sariban-Sohraby, S., Burg, M.G., Turner, R.J. 1983. Apical sodium uptake in the toad kidney epithelial cell line A6. *Am. J. Physiol.* **245:**C167–C171

Stetson, D.L., Lewis, S.A., Alles, W., Wade, J.B. 1982. Evaluation by capacitance measurements of antidiuretic hormone induced membrane area changes in toad bladder. *Biochim. Biophys. Acta* **689:**267–274

Received 15 August 1985