EFFECT OF NAPHTHALENE ON SODIUM TRANSPORT IN THE FROG SKIN

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CHAPTER I

INTRODUCTION

Skin of the amphibian such as the frog is a generally utilized and widely accepted animal model for transport physiology. The frog skin is a sheet of epithelium that works as a syncytium. It exhibits electro-chemical properties that are easily monitored and manipulated. Work done on the frog skin will promote a better understanding of the transport mechanisms which can be extrapolated to other systems, such as the mammalian renal system, that are not fully understood at this point and are extremely difficult to use in experimental situations.

When the frog skin is chamber-mounted and the bathing solutions on both sides of the skin are identical, one can apply enough electrical current to change the potential difference to zero. This condition is called short-circuited. In 1951 Ussing and Zerahn showed that the short-circuited conditions are well correlated to the net Na^+ ion flux across the frog skin. Then in 1958, Koefoed-Johnsen and Ussing proposed a model that explained sodium transport in the frog skin. The KJU model, now widely accepted, is described briefly. In the frog skin, Na^+

ions pass from the pond-side to the serosal-side. The Na^+ ions enter the cells through a passive entry site on the apical surface of the cell. The Na^+ ions diffuse across the cells and are actively transported out by a Na^+/K^+ ATP'ase pump located on the basolateral surface of the cells. The crossing of these ions creates a potential difference (PD). Using these principles one can alter the bathing solutions of a short-circuited frog skin and determine their effect on Na^+ transport.

Due to the increase in our societal demands for petroleum products, there has been an increase of petroleum by-products found in the environment. One of these by-products is naphthalene. Naphthalene is the single most toxic component of coal tar and is found in most other petroleum products. With the increase of naphthalene in the environment comes the increase in the concern for its effect on organisms. There have been several studies on the effects of naphthalene and other polyaromatic hydrocarbons on bacteria, cultured cells, invertebrates, freshwater and marine fish, and a variety of other vertebrates including humans.

The purpose of this research was to study the effect of naphthalene on sodium transport in the frog skin. This study provides insight in the effect of naphthalene, and possibly other polyaromatic hydrocarbons, on transport in

an intact tissue system. Accompanying the naphthalene experiments, there has been some additional work done with the frog skin. This collection of work is considered to be relevent not only to understanding the model for sodium transport but also in appreciating the complexity of such a system.

CHAPTER II

REVIEW OF THE LITERATURE

Frog As An Experimental Animal

At first the use of the frog skin may seem inappropiate but it is the traditionally used animal for modeling systems involved in the transport of salts and water. As early as 1799 (Townson) it was known that amphibian skin regulated and transported water. It was not until 1938 that Krough showed that the amphibian skin transported salts. In 1949(a,b) Ussing showed that the frog skin exhibited a PD in an *in vitro* preparation for several hours. This transport also went against a concentration gradient. Ussing and Zehran (1951) went on to show that the I_{sc} was directly correlated with the net Na^+ flux. The frog skin not only could be used as a model for transport systems but also exhibited electro-chemical properties (eg. PD,I,R) that could be measured. It was then possible to monitor and manipulate these parameters and also to have total control of the composition and duration of the bathing solutions that were in contact with the skin. These features alone make the frog skin an excellent choice as an animal model for transport systems,

yet the frog skin exhibits some other favorable characteristics. First, the frog skin (and to some extent bladders) responds physiologically like the tubules of the kidney (Herrera 1971). Therefore it may serve as model for the mammalian renal system and the fetal skin system, which operates similarly to the renal system. Second, the frog skin is loosely attached and therefore easily removable from the animal with minimal tissue damage (Kidder 1973). Last of all, the frog is relatively inexpensive and easily maintained.

The epidermis of the skin is organized similar to that of the human skin. The frog epidermis consists of 5-8 layers of cells divided into four distinct strata. Beginning with the outermost layer, they are: (1) statum cornium, (2) stratum granulosum, (3) stratum spinosum, and (4) stratum germinativum which rests upon the basement membrane that separating the epidermis from the dermis (Farquhar and Palade 1964). The stratum cornium is not considered to be important in Na^+ transport since it consists layer of dead cells. The other layers are implemented to varying degrees in different models that describe Na^+ transport mechanisms.

All the cells of the different strata are held together with different types of junctions. There are three types: tight junctions, gap junctions, and

desmosomes (Farquhar and Palade 1963). The various functions of these junctions are briefly described. The tight junctions are responsible for the fusion of adjacent cells and are generally found near the lumen of tissues or in those layers closest to the outside (eg. stratum granulosum). Tight junctions acts as a barrier. Gap junctions are found in cells that are tightly packed and are thought to aid in the transmission of nerve impulses and the passage of small ions. The desmosomes are credited with mechanical adhesions of cells not in close proximity. The distribution of these junctions varies with the tissue type and species.

KJU Model

Reid (1892,1901) made the first double flux chamber that allowed the skin to be monitored between pond-side and serosal-side bathing solutions. There was a great deal of work done after that time to establish the characteristics and repeatability of such experiments. In 1949(a,b) Ussing showed that the frog skin, when chamber-mounted, exhibited a potential difference for a long time. In 1951 Ussing and Zerahn showed that the short-circuited conditions are well correlated to the net Na^+ ion flux across the frog skin. Using these principles one can alter the bathing solutions of a short-circuited frog skin

and determine their effect on Na^+ transport. It was not until 1958 when Koefoed-Johnsen and Ussing proposed a model for the transport of Na^+ that became widely accepted. In this model of the frog skin, Na^+ ions pass from the pond-side to the serosal-side. The Na^+ ions enter the cell through a passive entry site on the apical surface of a stratum germinativum cell. The Na^+ ions diffuse across the cell and are transported out by a "pump" located on the basolateral surface of that cell.

The model has undergone some modifications throughout the years. The first modification was by Ussing and Windhager in 1964. They suggested that the cell layer mainly responsible for the entry of the Na^+ ions was the stratum granulosum. They proposed that the majority of Na^+ moves from cell to cell through the different layers via junctions (now called gap junctions). Once the Na^+ diffused to the stratum germinativum cells it was pumped out into the intercelluar space by Na^+/K^+ ATP'ase. This pathway is known as the paracellular (or shunt) pathway. In 1968 Cereijido and Rotunno proposed the intramembraneous pathway. In this pathway the stratum granulosum cell layer is responsible for the entry of Na^+ and the active transport of the Na^+ out of the cell by Na^+/K^+ ATP'ase. The Na^+ then travels around, not through, the cells of the other layers by a saltatory mechanism

until it enters the serosa. The most recent modification was the multi-compartmental model introduced by Huf and Howell (1974). They suggesed that there are five separate compartments between the cell layers. These compartments form different sodium pools that achieve two Na^+ transporting functions. One is the transepithelial transport that was originally suggested by the KJU model. And the second function is to maintain a reserve of Na^+ ions at all the strata so that there is always a PD even though it may be undetectable.

Although there is still a debate about how Na^+ transport should be modelled, there seem to be two schools of thought. The first maintains that only one cell layer is responsible for the entry and active transport of Na^+. Because the morphology of the frog skin is now better understood through electron microscopy studies, the stratum granulosum is assumed to be that cell layer. The second school of thought is that all the epithelial layers equally share the responsibility of transporting Na^+. The cornerstone of this work was provided by to Farquhar and Palade (1964). By electron microscopy, they localized the ATP'ase activity primarily in the stratum granulosum and stratum spinosum with some in the stratum germinativium cells. Although the debate continues about the details of the Na^+ transport mechanism, the KJU model with the shunt

modification is the generally accepted model for the transport of Na^+ by the frog skin.

Naphthalene

Physical and Chemical Properties

Naphthalene is a polycyclic aromatic hydrocarbon (PAH) with a molecular formula of $C_{10}H_8$ and a molecular weight of 128.16. Its melting point is 80.2 C° and it boils at 218 C°. It has a density of 1.0253 and is soluble in alcohol and benzene. Although Bohan (1951) reported that 30 mg/L naphthalene were soluble in water, and Josephy and Radt (1948) reported its solubility in water to be 40 mg/L, naphthalene in its pure form is a white crystal at room temperature. It is also a "catalytic air oxident" and it is steam volatile and reacts explosively with N_2O_5.

Where It Is Found and Its Uses

Naphthalene occurs naturally in petroleum and is considered the most toxic component of coal tar (Boylan and Tripp 1971). Although naphthalene is considered toxic, it is used in industrial manufacturing. Its major use is in the production of phthalic anhydride which is in turn used for the production of dyes, pigments, pharmaceuticals, and insecticides (USEPA). Naphthalene is

also used for making mothballs and to make napthols that are used in solvents, lubricants, and motor fuels (USEPA).

Naphthalene enters into the environment primarily in industrial wastes and oil spills. Naphthalene has been found in water supplies, in sewage, and in cigarette smoke.

Toxic Effects on Organisms

Naphthalene is considered to be toxic in an aqueous environment. There is little work done on the effect of naphthalene on sodium transport in any species. There is ample information not only on the toxicity and characteristics of naphthalene as described above but also on various effects of PAH on different systems of a wide variety of organisms. In most of the experiments in which naphthalene was used it was dissolved in alcohol.

Bernhein (1974) studied the effect of alcohols and hydrocarbons on *Pseudomonas aeruginosa*. In this Gram-negative bacterium, both the aromatic and aliphatic alcohols cause an increase of potassium efflux. Bernhein showed that the effect on potassium efflux was directly correlated to the hydrophobicity of the molecules. He also showed that certain aromatic hydrocarbons increased the influx of salt with minimal effect on potassium. This suggests that the mechanism of action is on the salt entry

site.

In 1982, Harmon and Sanborn investigated the effect of naphthalene on isolated mitochondria and on cultured cells. They reported that O_2 consumption was inhibited in both by as little as 10 ppm naphthalene. They also observed that at concentrations greater than 7.5 ppm the cultured cell rounded up and separated from the flask surface. They also reported that naphthalene does not interfere with ATP'ase activity in any way. In a subsequent study, Harmon (1988) reinvestigated the effect of naphthalene on cytochrome oxidase. He found were that both the K_m and V_{max} increased in the presence of naphthalene. And even though naphthalene was actually altered by an enzyme, it still had no apparent effect on cytochrome a_3.

Daphnia magna exposed to concentration of 5 mg/L naphthalene showed immediate behavioral changes (Crider et al 1982). *D. magna* also exhibited an LC_{50} for 24 and 48 hours of 6.6 and 4.1 mg/L respectively. The oxygen consumption of *D. magna* decreased when they were exposed to various level of naphthalene concentrations for a 24-hour period. However the most interesting results Crider et al, (1982) reported are the effects of a 24-hour exposure of naphthalene on hemoglobin concentration. At low levels, 1 mg/L, the hemoglobin concentration increased

to 102 nmoles/animal. But at higher concentrations of naphthalene, ranging from 3-9 mg/L, the hemoglobin concentration decreased to as low as 67 nmoles/animal. In 1983 Trucco et al, showed naphthalene was not only extremely toxic to *Daphnia pulex* but also had the highest accumulation ratio compared to other varieties of PAH used in the experiments.

The results of Darville and Wilhm (1984) are comparable to those of Crider et al, (1982). For *Chironomus* and *Tanytarsus*, the LC_{50} was approximately 13 mg/L and oxygen consumption decreased with 1-hour exposures to naphthalene concentration ranging from 1-12 mg/L. The hemoglobin concentration increased when *Chironomus* was exposed to 1 and 5 mg/L naphthalene for 4 hours and it decreased with 10 and 12 mg/L. Darville et al, (1983) also studied the effect of naphthalene on hemolymph ionic concentration of *Chironomus*. He observed that the Na^+, K^+, and Cl^- hemolymph concentrations increased with naphthalene exposure. Darville et al, then speculated that the possible mode of action of naphthalene occurs at Na^+/K^+ ATP'ase pump although this only explains the K^+ increase and not the Na^+ and Cl^- increase.

Morrow et al, (1975) suggested that aromatic hydrocarbons, and particularly the mono-cyclic aromatics, have toxic effects on young coho salmon at 100 ppm or

higher concentrations. He observed behavioral changes beginning at 100 ppm and when concentrations were increased to 500 ppm and 1000 ppm the symptoms persisted for approximately 72 hours. He also observed an increase in Na^+, K^+, and Cl^- in the blood of these animals for up to 3 hours after exposures to toluene, xylene, and benzene. He speculated that the mechanism for the toxicity of these mono-cyclic aromatics was related to a change in membrane structure, and specifically in the gill. From the increase in monovalent ions due to the change in membrane permeability, the carbonate and pH systems deteriorated, leading to ionic imbalance which caused behavioral changes and eventually death.

McKeown and March (1977) reported similar results in saltwater (acclimated) rainbow trout. When these fish were treated with a dispersant, they showed an significant increase in Na^+ level in the blood. However, when the freshwater rainbow trout were exposed to the same treatment, they showed a decrease in Na^+ levels. McKeown and March concluded that the variation they observed is possibly due to an interference with the Na^+/K^+ ATP'ase pump.

There are several reported cases of hemolytic anemia in children who ingested mothballs. Zuelzer and Apt (1949) investigated the effect of naphthalene ingestion on

dogs. They reported that naphthalene was absorbed and it produced hemolytic anemia in the dogs with as little as a 3 gram dose. Mackell et al, (1951) also reported cases of hemolytic anemia in children who accidently swallowed mothballs. They concluded that the toxicant is the oxidation products of naphthalene metabolism, not the naphthalene itself.

Drugs Used In Transport Studies

Amiloride is a diuretic that immediately eliminates the I_{sc} when administered to the pond-side bathing solution and has no effect on the serosal-side bathing solution (Nagel and Dorge 1970). Amiloride is thought to interact with the membrane proteins on the apical border of the cell in such a way as to prohibit Na^+ ions from entering the epithelial cells. Because no Na^+ ions enter the cell, no Na^+ ions are pumped from the cell, and thus no PD exists. Once the amiloride is removed from the pond-side bathing solution, the, I_{sc} will gradually reappears. If additional doses of amiloride are repeated on the frog skin, the recovery time decreases between each applications.

Ouabain is a cardiac glycoside that eliminates the I_{sc} upon application to the serosal-side bathing solution. It is known that ouabain interferes with the ATP'ase

located on the basolateral membrane of the cell (Fortes 1977). Once the Na^+/K^+ pump is shut down, no Na^+ ions are actively transported out of the cell, thus no PD. Ouabain is not as reversible as amiloride in the sense that it takes a considerable amount of time for recovery.

CHAPTER III

EFFECT OF NAPHTHALENE ON SODIUM TRANSPORT IN THE FROG SKIN

Introduction

Isolated abdominal frog skin, when mounted on an in vitro chamber, actively transports sodium from the pond-side bathing solution to the serosal-side when the skin is chamber mounted. Active transport of sodium causes an electrical potential difference (PD), up to 140 mv, that is proportional to the log of the pond-side sodium concentration (Koefoed-Johnsen and Ussing 1958). When the solutions bathing each side of the skin are identical and enough external current is applied to change the PD to zero, the skin is "short-circuited". Under short-circuit conditions, no driving force for ions or water exists across the skin. Thus, any net flux of ions (or water) must be due to processes internal to the skin and equal to the short-circuit current (I_{sc}). Ussing and Zerahn (1950) showed that the I_{sc} correlated well with the measured net sodium flux. I_{sc} is abolished by low concentrations of amiloride, a diuretic, which blocks the entry of sodium from the pond-side, thus causing the disappearance of Na^+

transport and a dramatically reduced I_{sc}. The currently accepted model for sodium active transport in the frog skin is that proposed by Koefoed-Johnsen and Ussing (1958) and modified through the years (Ussing and Windhager 1964) to include the paracellular pathway. This model proposes that sodium enters through a passive channel in the pond-side membrane of the first layer of living cells. Sodium diffuses through the cytoplasm of all of the epithelial cells of the epithelium and is pumped out in exchange for potassium by Na^+/K^+ ATP'ases on the basolateral membranes. Net transport of sodium is accomplished by the differential permeabilities of the pond-side membrane and the basolateral membrane resulting in no net potassium transport and a measurable net sodium transport towards the basolateral side of the skin.

The pond-side membrane is mainly permeable to sodium whereas the basolateral membrane is mainly permeable to potassium. Thus the net flux of potassium is near zero and the net flux of sodium differs considerably from zero (typically 20 microamps/cm^2). Some isolated frog skins exhibit considerable chloride conductance, which can be abolished by exchanging the chloride in the bathing solution for sulfate.

Naphthalene, a polycyclic aromatic hydrocarbon (PAH), occurs in oil byproducts and in crude oil (Boylan and

Tripp 1971). Naphthalene and similar compounds have been identified as acutely toxic petroleum products (Anderson et al. 1974). Naphthalene has been shown to modify Na^+/K^+ ATP'ase of aquatic invertebrates (Darville et al. 1983). Mitochondrial respiration is inhibited at levels of 15 ppm naphthalene (Harmon and Sanborn 1982). We have investigated the effect of naphthalene on the active transport of sodium by the frog skin.

Materials and Methods

Frogs, *Rana pipiens*, were purchased from Wm. Lemberger (Oshkosh, WI) and kept unfed in tap water until use. Generally the frogs were used within two weeks of arrival. Frogs were anesthetized by injection of 10% urethane. The abdominal skin was excised and placed in frog Ringer's solution which contained 110 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 2.5 mM TRIS buffer and were adjusted to pH 8.3. Some solutions were made with $NaSO_4$ replacing NaCl. Solutions containing naphthalene were freshly prepared for each experiment. In all solutions, naphthalene was dissolved by agitation and heating (70C°) overnight. A modified Ussing chamber made of glass was used to mount the skin. The PD was measured by two 3% agar-frog Ringer's bridges; current (I, I_{sc}) was passed by Ag-AgCl electrodes placed so the current density was

uniform across the skin. An automatic voltage clamp was used to maintain the PD at preset values. Values of PD and I_{sc} were read from a digital panel meter. A chart recorder (Schlumberger) was used to obtain time-based records.

Results

Experiments described in Figure 1 showed the effect of naphthalene when applied in separate doses. The initial dose (1 mg/L) is near the minimal dose for which we could detect a response. Often doses at the 1 mg/L would not elicit responses on the initial dose. Also shown in Figure 1 is a probable long term effect of the low dose of naphthalene, the steady rise in I_{sc} during the 220 to 250 minute record. Subsequent application of the 30 mg/L dose produced a strong response in I_{sc} and a recovery to near control I_{sc}.

Once we established the clear effect of naphthalene on the I_{sc}, we then asked whether a dose-response relationship existed. Figure 2 records the results of an experiment using sequential doses of varying concentrations of naphthalene. In this figure the initial 1 mg/L dose of naphthalene caused a drop in I_{sc} (compare to the rise in Figure 1). Subsequent applications of naphthalene evinced more predictable responses with the

Fig. 1 demonstrates the effect of 1 mg/L naphthalene and 30 mg/L naphthalene solution upon the I_{sc} of the frog skin. After the I_{sc} stabilized, 1 mg/L of naphthalene was added to the pond-side for 30 minutes. After rinsing with control frog Ringer's the pond-side skin was then exposed to a 30 minute dose of 30 mg/L naphthalene solution.

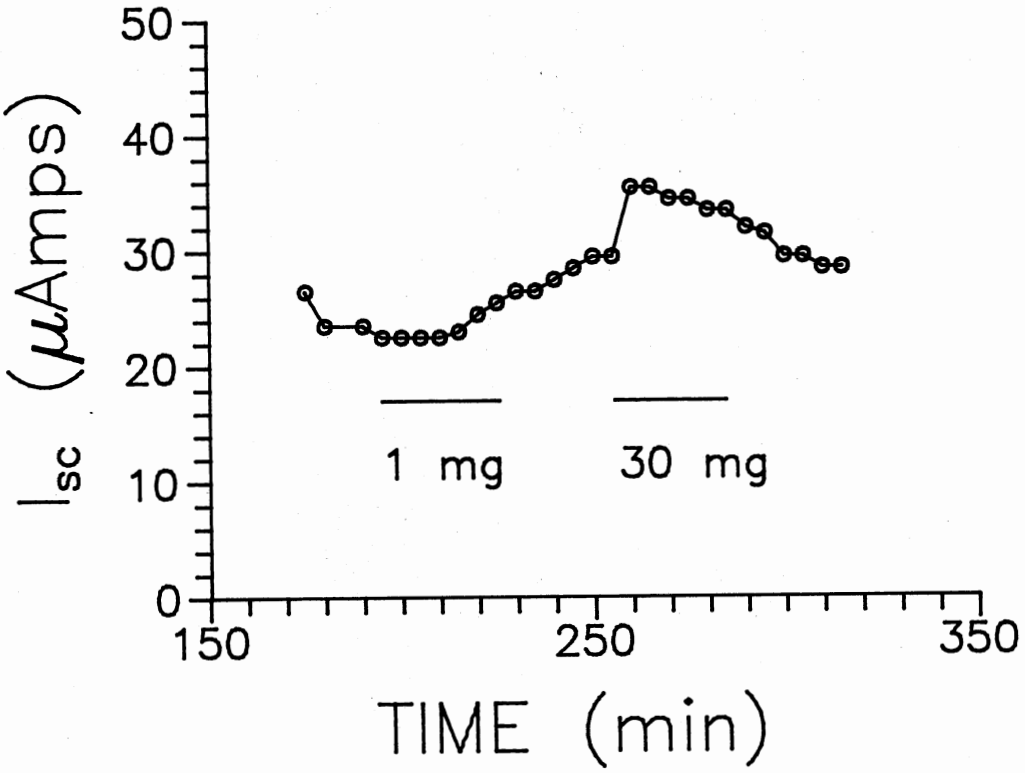

50
40
30
20
10
0
I_{sc} (μAmps)
1 mg
30 mg
150
250
350
TIME (min)

Fig. 2 is the result of a stepwise experiment using 1 mg/L, 10 mg/L, and 30 mg/L solution of naphthalene. The first set of doses were administered and the skin allowed to recover in frog Ringer's before the next 15 minute exposure of naphthalene. The last naphthalene sequence (385 minutes) is an increase from 1 mg/L to 30 mg/L naphthalene without rinsing with frog Ringer's solution.

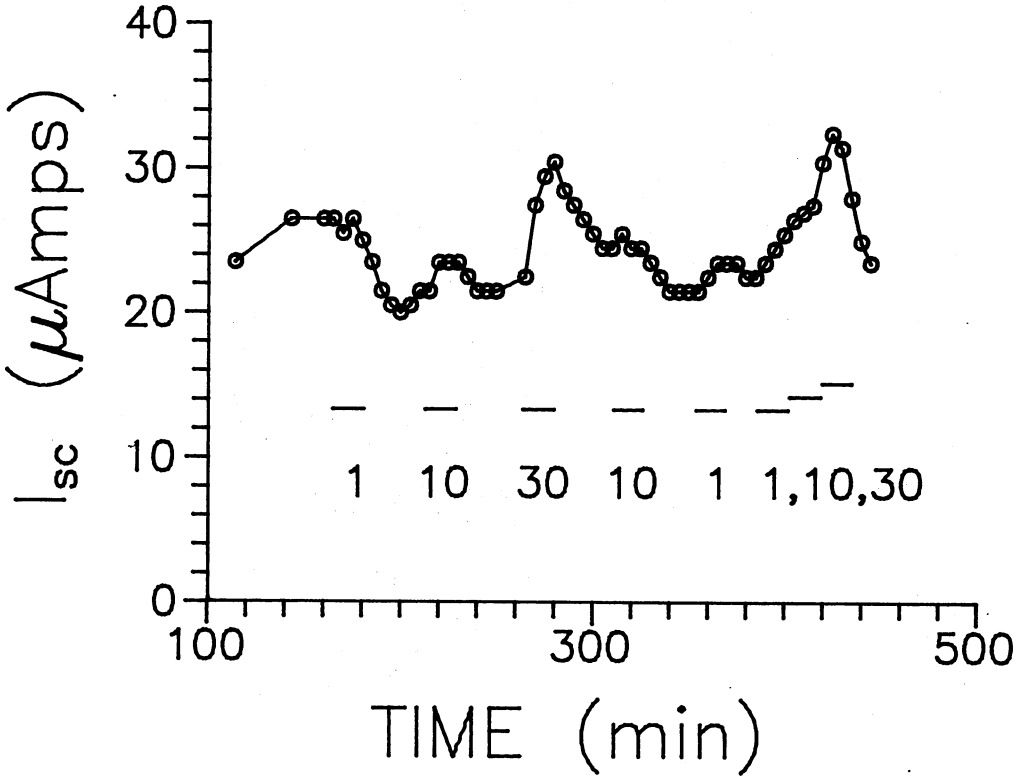
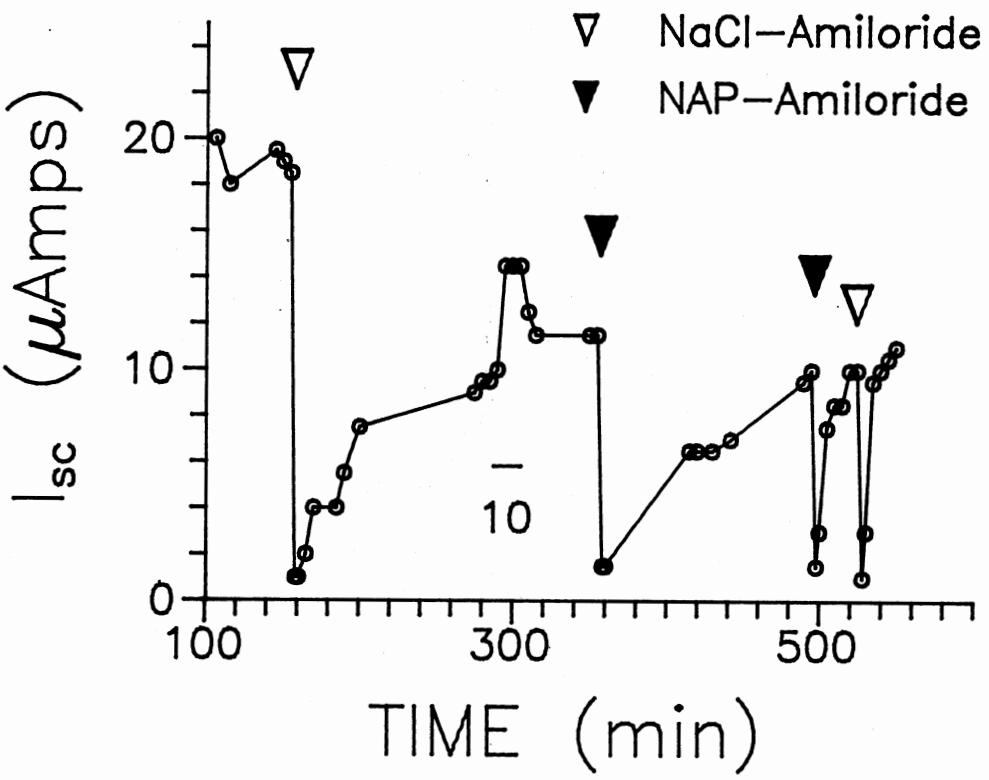

40
30
20
10
0
I_{sc} (μAmps)
1 10 30 10 1 1,10,30
100
300
500
TIME (min)

Fig. 3 shows the effect of the interaction of naphthalene and amiloride on the I_{sc}. The first application shows the typical response of amiloride in frog Ringer's. After a period of recovery, the pond-side bathing solution was changed to 10 mg/L naphthalene for 15 minutes. After another period of recovery the pond-side bathing solution was changed to a 10 mg/L naphthalene solution containing 10 micromolar amiloride.


NaCl–Amiloride
NAP–Amiloride
I_{sc} (μAmps)
20
10
0
100
300
500
10
TIME (min)

final stepwise naphthalene administration producing graded responses of I_{sc}.

Amiloride blocks the passive entry of sodium. In the experiment described in Figure 3, we tested the interaction of amiloride and naphthalene. If naphthalene caused the entry of sodium via a separate route, amiloride should not block the naphthalene-mediated sodium entry. As seen in Figure 3, amiloride rapidly drops I_{sc}. In the latter section of the figure (ie. 360 minutes), amiloride and naphthalene solution also produced a rapid drop in I_{sc}, showing that the entry path enhanced by naphthalene is probably the same pathway through which sodium normally enters the skin.

Discussion

Summarizing the effect of naphthalene on the frog skin, we found that naphthalene has an EC_{50} of 4.4 mg/L determined from the eleven frogs. Two of those frogs did not respond to naphthalene or amiloride and were not used in the data set. The response of the skins to 10 mg/L naphthalene was 17 $\pm$ 4 (SEM) which was significant at the 1% level. Naphthalene had no effect when administered to the blood side of the frog skin.

The experiments reported here demonstrate an

unequivocal effect of naphthalene on the sodium transport of frog skin. Figure 1 suggests not only does naphthalene increase I_{sc} but that the possibility of a dose-response relationship exists. It is also important to note that the 1 mg/L effect did not appear that until the end of the 30 minute dose. Therefore the overall conclusion from these experiments is that at all concentrations of naphthalene, the I_{sc} increased corresponding to an increase in sodium transport. The only exception is demonstrated in Figure 2. The initial dose of 1 mg/L naphthalene causes the I_{sc} response to decrease. Subsequent exposures of naphthalene, regardless of concentration or time administered, cause an increase in I_{sc}. Similiar results of naphthalene have also been observed in our experiments using ethanol as a solvent for naphthalene. Since the frog skin is a widely accepted model for vertebrate sodium active transport (eg. mammalian kidney, human fetal skin, mammalian bladder) inferences can be drawn about one of the modes of action of naphthalene (and possibly other PAH) on sodium transport systems. Although the earlier work of Darville et al (1983) reported effects of naphthalene on Na^+/K^+ ATP'ase isolated from cells of Chironomus, the effects reported here were on intact cells and on an intact epithelial preparation. It is surprising then that the

epithelial preparation is more sensitive to naphthalene than the isolated components. We can also infer from the rapidity of response that the mode of action in these experiments was probably on the entry of sodium into the cell. This evidence is seen in Figure 3. Regardless of the bathing solution used, whether frog Ringer's or naphthalene solution, amiloride caused the I_{sc} to drop dramatically. Though the possibility exists that naphthalene acts on the Na^+/K^+ ATP'ase, the data support a primary effect on the sodium entry pathway. If one accepts the premise that amiloride acts stoichometrically with the sodium receptors, then we may speculate that naphthalene, since the concentration of naphthalene is in the same order of magnitude as amiloride, is modifying the properties of the receptors on a one to one basis.

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References

Anderson JW, et al (1974) Characteristics of dispersions and water-soluble extracts of crude and refined oils and their toxicity to estuarine crustaceans. Mar Biol 27:75-88

Boylan BW, Tripp DB (1971) Determination of hydrocarbons in seawater extracts of crude oil and crude oil fractions. Nature 230:44-47

Darville RG, et al (1983) Effect of naphthalene on the hemolymph ion concentrations of *Chironomus attenuatus* and the possible mode of action. Environ Tox & Chem 2:423-429

Harmon HJ, Sanborn MR (1982) Effect of naphthalene on respiration in heart mitochondria and intact cultured cells. Environ Res 29:160-173

Koefoed-Johnsen V, Ussing HH (1958) The nature of the frog skin potential. Acta Physiol Scand 42:298-308

Ussing HH, Windhager K (1964) Nature of shunt path and active sodium transport path through frog skin epithelium. Acta Physiol Scand 61:484-504

Ussing H, Zerahn K (1950) Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta Physiol Scand 23:110-127

CHAPTER IV

ETHANOL AS A SOLVENT FOR NAPHTHALENE

Introduction

Preliminary experiments similar to those desribed in Chapter III were performed using ethanol to dissolve the naphthalene. Others in this laboratory had attempted to solve the problem associated with the use of ethanol (JT Blankemeyer and B Stringer 1988). Upon successful completion of the naphthalene experiments, it seems appropiate report the preliminary observations that lead abandoning ethanol as a solvent.

Method and Materials

The experimental procedure was identical to that followed in Chapter III with the following exception. The naphthalene was dissolved in 10 ml of 95% ethanol and then added to the NaCl frog Ringer's prior to the dilution to one liter. The solutions were made on the day of the experiment and were stored in the dark until used.

Results

No consistent patterns in I_{sc} were observed. In some stepwise exposures to naphthalene, there was an increase, in others a decrease, and in some no change in the I_{sc}. Results were not reprodicible upon repetition.

Discussion

These observations suggest that the ethanol has a separate effect on the frog skin. The possibility exists that ethanol and naphthalene interact in such a way to change the properties of skin, or even that ethanol may elicit a response from a system (eg. second messanger), while the naphthalene may affect another system. The condition of the skin prior chamber-mounting could influence the results observed. Whatever the mechanism, further investigations are needed of the effect of ethanol and its interaction with naphthalene on the frog skin.

CHAPTER V

ADDITIONAL EXPERIMENTS

Introduction

Prior to the success of the naphthalene experiments, I investigated several aspects of the generalized scheme for Na^+ transport discussed in Chapter II. Of particular interest was whether one cell layer or all cell layers are actually responsible for the transport of Na^+. Three separate experiments were conducted and outlined below.

Methods and Materials

Cesium Experiment

The procedures for these experiments are those found in Chapter III with the following exceptions. The frog skin was placed on the chamber in NaCl frog Ringer's. Then the pond-side bathing solution was changed to CsCl frog Ringer's (made by subsituting 110mM NaCl for 110 mM CsCl). After the frog skin was exposed to CsCl frog Ringer's for 5 minutes, the chamber was disassembled and the skin frozen with liquid nitrogen. The skin was then cut into 1-mm^2 pieces and stored in liquid nitrogen until cryosectioning

and the electron microscopy X-ray microprobe analysis could be performed at the O.S.U. Electron Microscopy Laboratory.

Anthroylouabain Experiment

The fluorescent dye anthroylouabain was used to locate the site of ATP'ase. The procedure followed was that skin was removed from the abdomen of an anesthetized *Rana pipiens* and placed in 10 ml of NaCl frog Ringer's with 1 mg of anthroylouabain added. The skin was exposed to the anthroylouabain for two hours and the bathing solution aerated with 95% O_2. The skin was then cut into 1-mm^2 pieces and then frozen using O.C.T. compound and placed in a cryostat. The frozen skin was cryosectioned into approximately 5-8 micron sections and placed on glass slides. The slides were warmed to room temperature and viewed under a Nikon fluorescence microscope and photographed.

Electron Microscopy

Electron microscopy was wsed to locate the gap junctions in the frog skin using the work done by Shahin (1986) as a comparision. Freshly removed abdominal skin of a double-pithed *Rana pipiens* was fixed in 0.02 M glutaraldehyde, and embedded with 100% DER (Kocan et al 1980). The blocks were thick-sectioned and stained with

Mallory's stain (Richardson et al 1960) and viewed under a light microscope. Ultrathin sections (prepared by K.M. Kocan) were stained with uranyl acetate and lead citrate (Venable and Coggeshall 1965). The grids were then viewed by TEM and photographed.

Results

Cesium Experiment

Cs^+ was found in all the cells of the different strata. The entire found to be procedure was unreliable, expensive, and extremely time consuming. Of the 15 frogs that were frozen and examined by TEM, only 6 sets of results were recorded. And only two of these showed Cs^+ in all levels.

Anthroylouabain Experiment

The majority of fluorescent particles were concentrated on the basolateral side of the stratum granulosum cells. The photographs taken were underexposed making this uncertain.

Electron Microscopy

In our examination with TEM desmosomes were abundant. Gap junctions, if present, were not apparent.

Discussion

Results from the Cs^+ experiment support the model proposed by Ussing and Windhager (1964), that is, that the cells communicate in such a way that the Cs^+ would pass from cell to cell. The Cs^+ must enter the stratum granulosum first because of its location. However, these experiments do not exclude the possiblity that Cs^+ could be passing via the intramembraneous pathway or through the intracellular spaces simultaneously. These experiments also do attempt to explain how the cells communicate, it simply suggests that they do.

The anthroylouabain results strongly support the suggestion that the stratum granulosum is the main layer responsible for the entry and the exit (via active transport) of Na^+. This aspect is common to all three model modifications introduced in the 60's and 70's (Ussing and Winghager 1964, Cereijido and Rotunno 1968, Huf and Howell 1974). However, the anthroylouabain observations do not support the portion of the KJU model (1958) that predicts the stratum germinativum as mainly responsible for Na^+ uptake. This experiment does not differentiate among the three model modifications with respect to how the Na^+ is transported through the other strata and eventually enters the serosa. It only supports the common idea that

the stratum granulosum is instrumental in the transport of Na^+.

Shahin and Blankemeyer (1989) stated that gap junctions were found in all the strata of the epidermis with the smallest number found in the stratum granulosum. Their work supports the hypothesis of a paracellular pathway and the multi-compartment hypothesis. However based on the electron micrographs from the experiments we performed, gap junctions do not exist at a "true" zero time, that is, when the frog skin was immediately removed and directly placed in glutaraldehyde. In fact, a number of desmosomes were observed. Desmosomes function to hold cells together despite intracellular spaces. So not only were no gap junctions observed, the cells did not even appear close enough to one another to possess them. Our work supports the concept that the Na^+ is transported by the intramembraneous pathway or through the intracellular spaces.

Although the results of the naphthalene experiments do not offer any clarification of the model, they do suggest alternative perspectives. Given the rapidity of the response to naphthalene concentrations greater than 1 mg/L and understanding that the frog skin is a tight epithelium, two explanations are possible: (1) the stratum granulosum

is the sole layer reponsible for transporting Na^+ and (2) the cells are coupled so intricately that all strata respond as if they were one layer. In either case, the stratum granulosum is an important part, if not the sole transport layer, of the skin, thus partially supporting all the modifications of the model described earlier.

Based on all the experiments conducted, I am convinced that the stratum granulosum is the layer responsible for the regulation of Na^+ transport. That is the location of the passive entry of Na^+ and the greatest active transport of Na^+ takes place in this layer. However I am not persuaded that we can clearly conclude whether Na^+ travels through the cell or around the cells of the different strata.

CHAPTER VI

CONCLUSIONS

Based on the experiments in which naphthalene is dissolved in NaCl frog Ringer's, naphthalene causes an increase in I_{sc}, which corresponds to an increase in Na^+ transport. The only exception to this conclusion is the inconsistent effect of the initial 1 mg/L dose of naphthalene. In some experiments, this concentration was associated with an immediate increase in I_{sc}, in some, with a delayed increase, while in others a decrease. Another conclusion from this data is that one of the possible modes of action of naphthalene is via the amiloride-sensitive entry site for Na^+. The naphthalene-ethanol experiments suggests a need for further investigation of the individual effect of ethanol on the frog skin and the interaction between naphthalene and ethanol.

Based on the experiments dealing with general model for Na^+ transport, I am convinced that the stratum granulosum is the layer responsible for the regulation of Na^+ transport. That is the location of the passive entry of Na^+ and the greatest active transport of Na^+ take place

in this layer. However I am not persuaded that we can clearly conclude whether Na^+ travels through the stratum granulosum layer or through all different strata.

LITERATURE CITED

Anderson JW, et al (1974) Characteristics of dispersions and water-soluble extracts of crude and refined oils and their toxicity to estuarine crustaceans and fish. Mar Biol 27:75-88

Bernheim F (1974) Effect of aromatic hydrocarbons, alcohols, ketones and aliphatic alcohols on cell swelling and potassium efflux in *Pseudomonas aeruginosa*. Cytobios 11:91-95

Bohn RL, Claussen WF (1951) The solubility of aromatic hydrocarbons in water. J Amer Chem Soc 73:1571-1578

Boylan DB, Tripp BW (1971) Determination of hydrocarbons in seawater extracts of crude oil and crude oil fractions. Nature 230:44-47

Cereijido M, Rotunno CA (1968) Fluxes and distribution of sodium in frog skin; a new model. J Gen Physiol 51:280s-289s

Crider JL, et al (1982) Effects of naphthalene on the hemoglobin concentration and oxygen uptake of *Daphnia magna*. Bull Envir Contam Toxicol 28:52-57

Darville RG, et al (1983) Effect of naphthalene on the hemolymph ion concentrations of *Chironomous attenuatus* and the possible mode of action. Envir Tox and Chem 2:423-429

Darville RG, Wilhm JL (1984) The effect of naphthalene on oxygen consumption and hemoglobin concentration in *Chironomus attenuatus* and on oxygen consumption and life cycle of *Tanytarsus dissimilis*. Envir Tox and Chem 3:135-141

Farquhar MG, Palade GE (1963) Junctional complexes in various epithelia. J Cell Biol 17:375-412

Farquhar MG, Palade GE (1964) Functional organization of amphibian skin. Zoology 51:569-577

Fortes G (1977) Anthroylouabain: a specific fluorescent

probe for the cardiac glycoside receptor of the Na-K ATPase. Biochem Vol 16 3:531-540

Harmon HJ (1988) Effect of naphthalene on cytochrome oxidase activity. Bull Envir Contam Toxicol 40:105-109

Harmon HJ, Sanborn MR (1982) Effect of naphthalene on respiration in the heart mitochondria and intact cultured cells. Envir Res 29:160-173

Herrera FC (1971) Ion movement in asymmetrical cells: frog skin and toad bladder. Membrane and Ion Transport E.E.Bittar (ed) A Wiley-Interscience puplication: New York Vol 3 pp 1-47

Huf EG, Howell JR (1974) Comuter stimulation of sodium fluxes in frog skin epidermis. J Memb Biol 15:47-66

Josephy E, Radt F (1948) Encyclopedia of organic chemicals, series III. Elsevier Publishing Co Inc: New York p 459

Kidder III GW (1973) Electrical measurements in epithelial tissues: theory and practice. J Biol Phys 1:143-174

Kocan KM, et al (1980) Ultrastructure of *Anaplasma marginale* Theiler in *Dermacentor andersoni* Stiles and *Dermacentor variabilis* (Say). Am J Vet Res 41:1966-1976

Koefoed-Johnsen V, Ussing HH (1958) The nature of frog skin potential. Acta Physiol Scand 42:298-308

Krough A (1938) The active absorption of ions in some fresh water animals. Z Vergl Physiol 25:335-350

Larsen EH (19) Effect of amiloride, cyanide and oubain on the active transport pathway in toad skin. Transport Mechanisms in Epithelia: Munksgaard, Copenhagen. pp 131-143

Mackell JV, et al (1951) Acute hemolytic anemia due to ingestion of naphthalene moth balls. Pediatrics 7: 722-728

McKeown BA, March GL (1978) The acute effect of bunker c oil and oil dispersant on: 1 serum glucose, serum sodium and gill morphology in both freshwater and

seawater acclimated rainbow trout (*Salmo Gairdneri*). Water Res 2:157-163

Morrow JE, et al (1975) Effects of some components of crude oil on young coho salmon. Copeia 2:326-331

Nagel W, Dorge A (1970) Effect of amiloride on sodium transport of frog skin. Plugers Arch 317:84-92

Reid EW (1892) Experiments upon "absorption with out osmosis". Brit Med J 1:323-326

Reid EW (1901) Transport of fluid by certain epithelia. J Physiol 26:436-444

Richardson KC, et al (1960) Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol 35:313-323

Rick R, et al (1981) Electron microprobe analysis of frog skin epithelium: pathway of transepithelial sodium transport. Ion Transport by Epithelia: Raven Press: New York pp 197-208

Shahin SH, Blankemeyer JT (1989) Demostration of gap junctions in the frog skin epithelium. Am J Physiol : in press

Townson R (1799) Tracts and observations in natural history and biology. J White London

Trucco RG, Engelhardt FR, Stacey B (1983) Toxicity, accumulation and clearance of aromatic hydrocarbons in *Daphnia pulex*. Envir Poll (Series A) 31:191-202

U S E P A (1980) Publication. 440/5-80-059

Ussing HH (1949a) The active ion transport through the isolated frog in the light of tracer studies. Acta Physiol Scand 17:1-37

Ussing HH (1949b) The distinction by means of tracers between active transport and diffusion. Acta Physiol Scand 19:43-56

Ussing HH, Zerahn K (1951) Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta Physiol Scand 23:110-127

Ussing HH, Windhager EE (1964) Nature of shunt path and active sodium transport path through frog skin epithelium. Acta Physiol Scand 61:484-504

Venable JH, Coggeshall R (1965) A simplified lead citrate stain for use in electron microscopy. J Cell Biol 25:407-408

Zuelzer WW, Apt L (1949) Acute hemolytic anemia due to naphthalene poisoning. J A M A 141:185-190

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