Biochemical Pharmacology, Vol. 19, pp. 515–524. Pergamon Press. 1970. Printed in Great Britain

ENHANCEMENT OF VASOPRESSIN-INDUCED WATER LOSS IN TOAD BLADDER BY CERTAIN PHOSPHOLIPIDS

J. L. BOROWITZ

Department of Pharmacology, The Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, N.C. 27103, U.S.A.

(*Received* 30 *August* 1968; *accepted* 6 *June* 1969)

Abstract—Exposure of toad bladder halves to suspensions of phosphatidyl serine, phosphatidyl ethanolamine, or phosphatidyl inositide increases subsequent vasopressin-induced water loss from the bladders by approximately 40 per cent. Theophylline-induced water loss from toad bladder is also enhanced by phosphatidyl serine pretreatment of the bladders. The effect of phosphatidyl serine is evident in the absence of sodium transport, and is independent of any increased tissue metabolism. Preparations of lecithin, sphingomyelin, cholesterol, tripalmitin or fat soluble vitamins were ineffective in enhancing the response of toad bladder to vasopressin. It is suggested that the presence of certain phospholipids in anuran bladder tissue may be a factor in determining the extent of the physiological response of this tissue to antidiuretic hormone.

GENERALLY phospholipids are considered to be structural elements of biological interfaces. However, van Deenen points out that these substances cannot be considered inert since "in a number of membranes, the lipids are subject to exchange processes with environment and to subtle metabolic conversions which may contribute significantly to the dynamic character of the cellular boundary concerned".[1] Accordingly, it is possible that phospholipids may influence the actions of certain drugs by altering the nature of cell boundaries.

The data in this study show that previous exposure of toad bladder (*Bufo marinus*) to a colloidal suspension of phosphatidyl serine increases L-8 vasopressin-induced water loss from the bladder. This effect was found to be specific for phosphatidyl serine and closely related compounds since lecithin and a variety of other lipids or lipid-soluble substances did not enhance the response of the bladder to vasopressin.

METHODS AND MATERIALS

The method of Bentley[2] was used in which the two lobes of the bladder were tied on glass tubes and suspended in an aerated physiological solution of the following composition in grams per litre: NaCl, 8·61; KCl, 0·2; $NaH_2PO_4 . H_2O$, 0·05; Tris, 1·44; glucose, 0·01; $MgCl_2 . 6H_2O$, 0·01. The solution was adjusted to pH 7·4 with HCl. An hour equilibration in the isotonic solution was allowed before any treatment. Before each test period a fresh 3-ml portion of physiological solution diluted with 4 vols. of water was placed inside the bladders. The vials in which the bladders were placed generally contained a fresh 20-ml portion of the isotonic solution. In a few instances

10 ml was used to minimize the amount of phospholipid required. Treatment periods were held constant at 20 min and the experiments were carried out at room temperature. Water loss from the bladders was determined by weighing the bladders to the nearest mg (Mettler analytical balance, Type B) at the beginning and at the end of the 20-min periods.

Except where otherwise indicated, the general procedure used was as follows: After 1 hr equilibration, resting water loss in the absence of vasopressin was measured in both bladder lobes. Then the lobes were exposed to vasopressin. This was followed by exposure of one lobe to a suspension of a lipid or lipid soluble substance, while the control lobe was exposed to the physiological solution only. Both bladder lobes were then retested for their ability to respond to vasopressin, and this was followed by redetermination of resting water loss in each lobe in two successive 20-min periods.

In each experiment one bladder lobe was used as a control for the other lobe. To avoid any possible differences in the response of the left versus the right lobe, either left or right lobes were treated with phosphatidyl serine in alternate experiments. The difference between the responses to vasopressin before and after lipid treatment was compared to similar differences for controls. A P value of 0·05 or less was taken as significant by comparison of paired differences.

Phosphatidyl-L-serine was obtained from three sources: Mann Research Labs., Inc., New York, N.Y.; Nutritional Biochemicals Corp., Cleveland, Ohio and Sigma Chemical Co., St. Louis, Mo. The phosphatidyl serine from Mann Research Labs. is stated to be chemically pure whereas that from Nutritional Biochemicals is stated to be 92 per cent pure. Phosphatidyl serine from Sigma is Folch fraction III extracted from bovine brain. One sample of phosphatidyl serine from Sigma was found to have no effect on toad bladder. Phosphatidyl inositide (Folch fraction I), phosphatidyl ethanolamine (Folch fraction V) and synthetic *dl-α* lecithin were also obtained from Sigma Chemical Co.

Highly purified sphingomyelin (from beef brain) and tripalmitin were obtained from Mann Research Labs. The vitamin A (all-trans retinyl acetate) was obtained from Fisher Chemical Co. Vitamin E was obtained from Nutritional Biochemicals Corp., and calciferol was purchased from Eastman Organic Chemicals Corp.

Generally the lipids were suspended in physiological solution by first dissolving or mixing them in a small volume of ethanol or ethyl ether or warm physiological solution (50° or 80°) and then quickly adding the physiological solution. This procedure provided an even dispersion of the lipid (generally in a concentration of approximately 10^{-3} M) in the aqueous phase. Aeration of the samples maintained the homogeneous suspension throughout the treatment period.

The L-8 vasopressin used throughout this study was obtained from Sandoz, Inc., Hanover, N.J. Activity is expressed in terms of international pressor units. The designation mu indicates milliunits. Theophylline was obtained from Matheson, Coleman & Bell, Inc.

RESULTS

Resting water loss. Water loss in the absence of vasopressin was found to average about the same in phosphatidyl serine and control bladder halves at the beginning (33 mg) and at the end of the experiments (71 mg). Therefore, no correction for resting water loss was made in any of the data given.

Effect of phosphatidyl serine in the presence of vasopressin on vasopressin induced water loss in toad bladder. Early in this study, 6 dose–response curves of bladder lobes to 3, 10, 30 and 60 mu/ml of vasopressin were run in the presence and in the absence of phosphatidyl serine (four experiments with 1·67 mg/20 ml, and two experiments with 2·9 mg/20 ml). No significant difference in response of lobes exposed to both vasopressin and phosphatidyl serine simultaneously versus control lobes exposed to vasopressin alone was noted. Later, six experiments were done in which bladder lobes were exposed to 2·9 mg/20 ml of phosphatidyl serine and 30 mu/ml of vasopressin simultaneously and compared to controls exposed to vasopressin alone. Again no enhancement of the effect of vasopressin due to the presence of phosphatidyl serine in the medium was evident and on the average a slight decrease in water

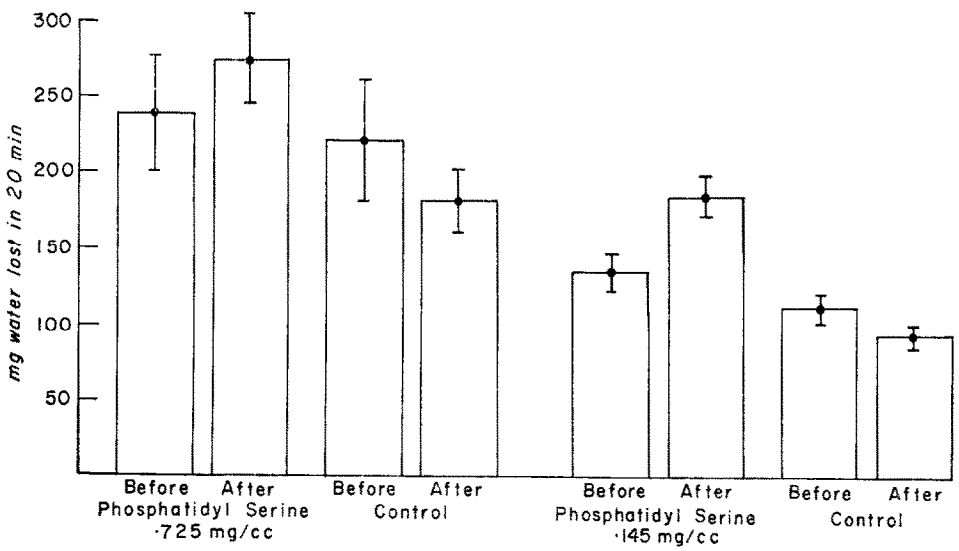

FIG. 1. Means ± S.E. are shown. Thirteen experiments were done at the 0·725 mg/ml concentration and the means of seven experiments are given for the 0·145 mg/ml concentration. Bladder halves from the same toads were used simultaneously in each experiment. One bladder half was exposed to a suspension of phosphatidyl serine in physiological solution for 20 min and the other to the physiological solution only. Water loss induced by L-8 vasopressin (30 mu/ml) was measured before and after exposure to the phospholipid suspension and control solution. The change in water flux produced by vasopressin after phosphatidyl serine treatment is significantly different compared to controls at both the high and low concentrations of phosphatidyl serine ($P < 0·01$ in both cases).

loss in the bladder lobes treated with phosphatidyl serine compared to controls was seen. It appeared that the effect of phosphatidyl serine could be shown more easily when the bladder was first soaked in a phosphatidyl serine suspension and then exposed to vasopressin. This procedure was adopted and used throughout the present study.

Effect of phosphatidyl serine pretreatment on vasopressin-induced water loss from toad bladder. Figure 1 shows the vasopressin-induced water loss from toad bladder before and after exposure to a suspension of phosphatidyl serine. The change in

water loss before and after exposure of one bladder lobe to phosphatidyl serine was compared to the change in water loss in similar periods in the control bladder lobe. By comparison of paired differences, the change in water loss in phosphatidyl serine (in a concentration of 0·725 mg/ml) versus control lobes is significant at the 1 per cent level. The analagous difference at the lower concentration of phosphatidyl serine (Fig. 1) is also significant at the 1 per cent level.

In Fig. 1, the experiments involving the low concentration of phosphatidyl serine were done at a different time of year and with different batches of animals than the experiments with the high concentration. These variables, time of year and batch of animals, are known to affect the response of toad bladder to vasopressin,[3] and they no doubt explain the difference in response to vasopressin evident in the two sets of experiments shown in Fig. 1.

It is also evident in Fig. 1 that the enhancement of the effect of vasopressin is no greater at the high dose of phosphatidyl serine than at the low concentration. Although this suggests that the effect of phosphatidyl serine is not dose related, it may also be related to the lower initial responses of the bladders in the experiments involving the low compared to those involving the high concentration of phosphatidyl serine.

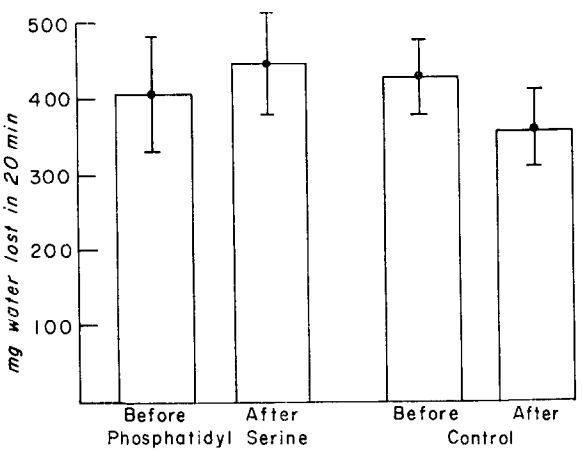

FIG. 2. Procedure is the same as for Fig. 1 except that ether was used to suspend the phosphatidyl serine (0·725 mg/ml). Means ± S.E. of ten experiments are shown. The effect of phosphatidyl serine is significantly different from control at the 5 per cent level.

Effect of phosphatidyl serine suspended with ether. Figure 2 shows that phosphatidyl serine suspended with ethyl ether is also effective in increasing the response of the toad bladder to L-8 vasopressin. The effect is unrelated to the presence of ether since an equivalent amount of ether was included in the media bathing control bladder lobes.

Effect of phosphatidyl serine in the absence of vasopressin. Nine experiments were done in which 1 lobe of the bladder was exposed to phosphatidyl serine (14·5 mg/20 ml suspended either with warm physiological solution or with ether) for 20 min, and the "resting water loss" measured in the following 20 min period in the absence of

L-8 vasopressin. Resting water loss in the 20-min period after exposure to phosphatidyl serine increased an average of 17 mg compared to resting water loss in a 20-min period immediately before exposure to phosphatidyl serine. The other half of the bladder was used simultaneously as the control and resting water loss in this half of the bladder increased an average of 11 mg in the same periods. Therefore, an increase in resting water loss cannot account for phosphatidyl serine's effect.

Effect of pH. In four experiments, no difference in pH of phosphatidyl serine suspensions, compared to physiological media without phosphatidyl serine, was found before or after exposure to the toad bladder. The pH, after exposure to phosphatidyl serine and control bladders, of media containing vasopressin was also found to be the same in experiments involving phosphatidyl serine as compared to control experiments. It is concluded that any difference in vasopressin-induced water loss related to phosphatidyl serine treatment cannot be due to a pH change in the media.

Effect of phosphatidyl serine on water loss from toad bladder independent of sodium transport. "Absence of potassium from the solution bathing the serosal side of the bladder nearly abolishes short circuit current and therefore sodium transport across the bladder."[4] This technique was used to study the effect of phosphatidyl serine on vasopressin-induced water movement in the absence of significant sodium transport. In 9 experiments, potassium was omitted from the solution bathing the serosal surfaces and the effect of phosphatidyl serine (0·725 mg/ml) studied as in the experiments of Fig. 1. Water transport, under these conditions, was enhanced an average of 45 per cent above control by pretreatment with phosphatidyl serine, and the effect was significant at the 0·1 per cent level. The effect of phosphatidyl serine on water loss induced by vasopressin in toad bladder therefore is not related to sodium transport.

Effect of phosphatidyl serine on oxygen consumption in toad bladder. To investigate the possibility that the effect of phosphatidyl serine might be related to a change in oxidative metabolism, oxygen uptake was measured in phosphatidyl serine (0·725 mg/ml) treated and control bladder homogenates in a Gilson respirometer at 25° in the presence of vasopressin (30 mu/ml). In five experiments, oxygen uptake in phosphatidyl serine treated bladders was found to be $1{\cdot}10 \pm 0{\cdot}22$ μl/mg dry wt./hr. In the control, untreated lobes oxygen uptake was $1{\cdot}20 \pm 0{\cdot}59$ μl/mg dry wt./hr. These values are close to those reported for untreated bladders of both *Bufo marinus*, 1·07 or 1·28 μl/mg dry wt./hr[5, 6] and *Bufo bufo* 1·31 μl/mg dry wt./hr.[7] Oxygen consumption of the bladder of *Bufo marinus* in the presence of arginine vasopressin is reported to be 1·40 μl/mg dry wt./hr.[5] It is concluded that phosphatidyl serine has no obvious effect on oxygen consumption and does not increase vasopressin-induced water transport in toad bladder by this mechanism.

Duration of effect of phosphatidyl serine. The effect of phosphatidyl serine is greater during the period immediately after exposure to the lipid than during an equal period 1 hr after exposure (Fig. 3). The response to vasopressin of bladder halves just after exposure to phosphatidyl serine are significantly greater ($P < 0{\cdot}01$) than controls. One hr after exposure to phosphatidyl serine, however, responses of the same bladders to vasopressin are approximately the same as controls. Apparently, enhancement of vasopressin-induced water loss from toad bladder after exposure to phosphatidyl serine is short lived, and can no longer be shown 1 hr after the exposure period.

However, when exposures to phosphatidyl serine are repeated, the enhancement

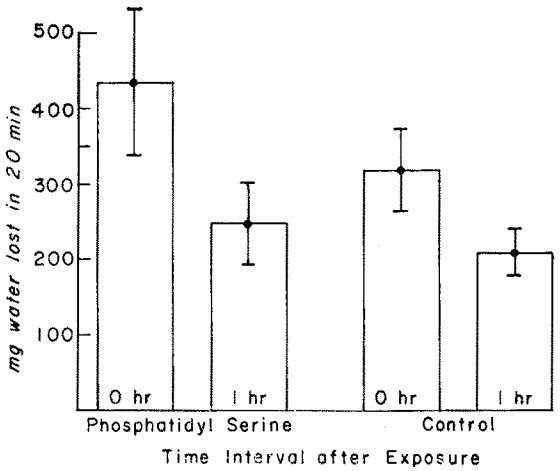

FIG. 3. Means ± S.E. of nine experiments are given. Phosphatidyl serine, 0·725 mg/ml, was dissolved in 1 ml of ethyl ether and 20 ml of physiological solution was added to give an homogenous suspension. Bladder halves were exposed to phosphatidyl serine or control solutions for 20 min. Water loss in response to 40 mu/ml of L-8 vasopressin was then measured immediately after and 1 hr after exposure to the phospholipid suspension or control solution. The response to vasopressin is significantly greater ($P < 0{\cdot}01$) immediately after exposure to the phospholipid compared to control, but 1 hr after exposure the phospholipid treated bladder is not significantly different from control.

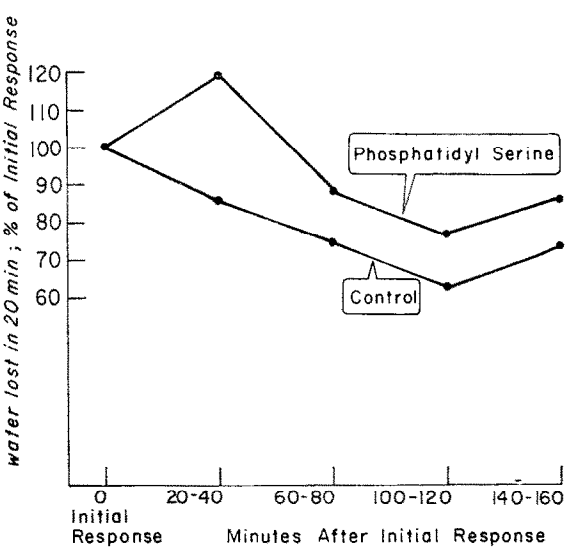

FIG. 4. The response to 30 mu/ml of L-8 vasopressin was tested immediately after exposure to suspensions of phosphatidyl serine or control solutions for 20 min and expressed as a per cent of the initial response before phosphatidyl serine treatment. The mean initial response for phosphatidyl serine treated bladder halves was 220 mg and for controls, 230 mg. Means of eight experiments are given. The responses obtained 60–100 min after the initial response were significantly greater ($P < 0{\cdot}01$) in phosphatidyl serine treated bladder halves than in controls by group comparison.

of vasopressin-induced water loss can be demonstrated for prolonged periods (Fig. 4). Group comparison of responses of phosphatidyl serine treated bladders with controls (60–160 min after the initial response) in terms of the per cent of the initial response reveals a significant difference ($P < 0{\cdot}01$). These data indicate that enhancement of vasopressin-induced water loss from toad bladder by phosphatidyl serine can be demonstrated repeatedly over a period of at least 2 hr.

Effect of phosphatidyl serine on the dose–response curve to vasopressin. Figure 5 shows that a wide range of doses of vasopressin is influenced by phosphatidyl serine and that the dose–response curve is apparently shifted to the left by this treatment.

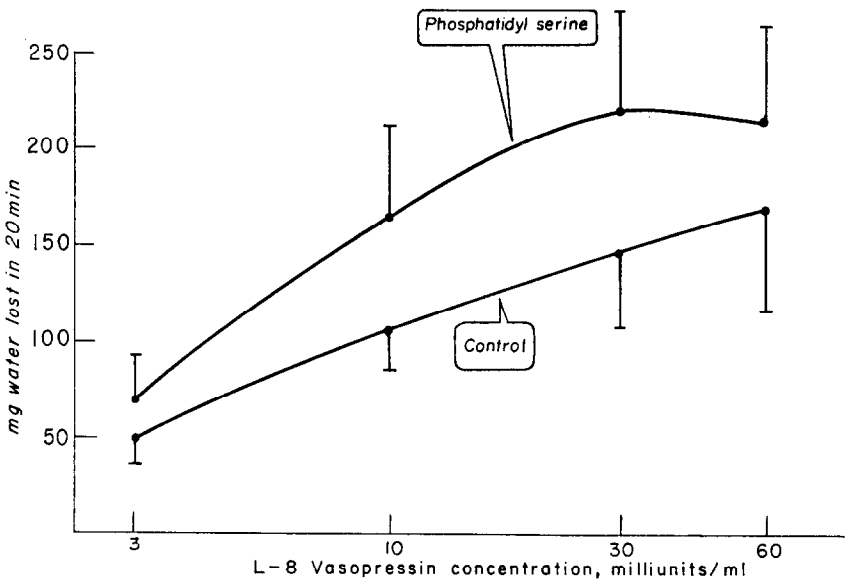

FIG. 5. Means ± S.E. of five experiments are shown for phosphatidyl serine (0·725 mg/ml) treated bladder lobes and for the paired control lobes. Bladders were pretreated for 20 min with either the phosphatidyl serine suspension or control prior to exposure to each of the doses of vasopressin. Differences in response of phosphatidyl serine treated and control lobes are significant at the 2 per cent level by comparison of paired differences including the 10, 30 and 60 mu/ml doses of vasopressin.

Effect of exposure of the mucosal surface of the bladder to phosphatidyl serine. In five experiments the effect of phosphatidyl serine (0·725 mg/ml) included in the 3 ml of diluted physiological solution bathing the mucosal surface of the bladder was tested on the response of the tissue to 30 mu/ml of L-8 vasopressin. The tissue was exposed to phosphatidyl serine, suspended by warming in diluted physiological solution, for the usual 20-min period before treatment with 30 mu/ml of L-8 vasopressin, The difference in response to vasopressin before and after exposure to phosphatidyl serine was compared to the difference in response to vasopressin before and after exposure to a control solution without phosphatidyl serine. Water loss increased an average of 74 mg in 20 min in the phosphatidyl serine treated bladder lobes

compared to the controls. The difference is significant at the 5 per cent level. It is concluded that exposure of either surface of toad bladder to phosphatidyl serine enhances subsequent vasopressin-induced water loss.

Effect of phosphatidyl serine on theophylline-induced water loss. Five experiments were done in which theophylline (10^{-2} M) was used to induce water loss in phosphatidyl serine and control bladder lobes. The experimental procedure used was the same as that for the data of Fig. 1. Pretreatment with lipid (0·725 mg/ml) enhanced water loss an average of 29·4 mg in treated lobes whereas water loss decreased an average of 5 mg in control lobes. The difference was significant at the 5 per cent level by comparison by paired differences.

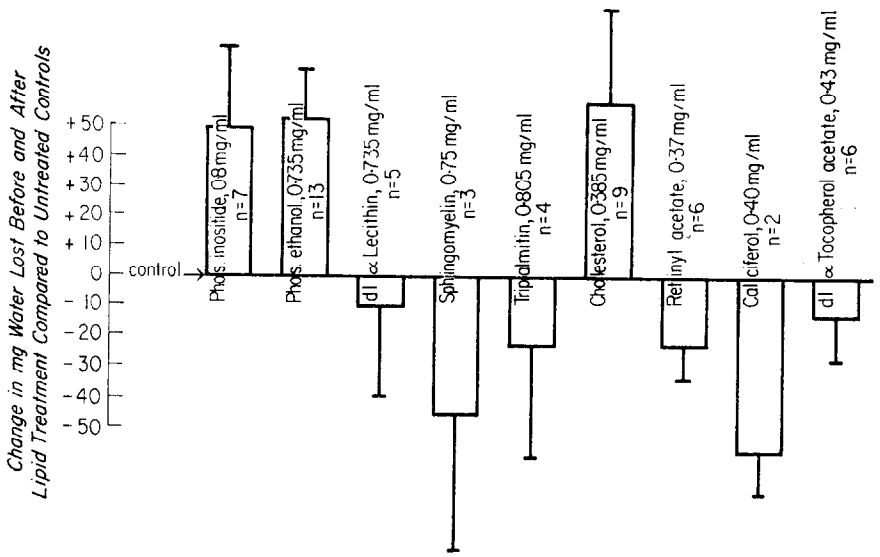

FIG. 6. Water loss during 20-min exposures to 30 mu/ml of L-8 vasopressin was measured before and after treatment with the lipid materials. The difference in these values was subtracted from similar differences in control untreated lobes. Increases or decreases (±S.E.) in water loss produced by lipid treatment compared to control bladders are shown in the graph. The response was significantly enhanced by phosphatidyl inositide ($P < 0·05$) and phosphatidyl ethanolamine ($P < 0·01$). The effect of cholesterol was not significant ($P < 0·10$), and none of the decreased responses was significant. Phosphatidyl inositide was suspended in warm physiological solution and *dl*-α-tocopherol was dissolved in the physiological solution at room temperature. The other lipids were dissolved in 1 ml of ethanol, with warming if necessary, and then 20 ml of the physiological solution was added quickly to produce an homogeneous suspension. Ethanol was included in controls when appropriate.

Effect of other lipids. Figure 6 shows that phosphatidyl inositide and phosphatidyl ethanolamine are also effective in enhancing vasopressin-induced water loss in toad bladder. However, lecithin and a number of other lipids and lipid soluble substances, all of which are present in kidney tissue, have no effect on water loss from the bladder. The effect therefore appears to be specific for phosphatidyl serine and closely related compounds.

DISCUSSION

It has been shown that phosphatidyl serine pretreatment enhances both theophylline- and vasopressin-induced water loss in toad bladder. Theophylline is thought to act by inhibiting phosphodiesterase thereby allowing adenosine-3′, 5′- monophosphate (cyclic AMP) to accumulate in bladder cells.[8] Vasopressin is thought to act by stimulating the formation of cyclic AMP.[8] Although an exogenously applied lipid substance had been shown to function as the serotonin receptor in smooth muscle,[9] exogenously applied phosphatidyl serine probably has no effect on the receptor mechanisms of the agonists employed in this study since theophylline and vasopressin have different sites of action. Instead it is likely that a mechanism common to both agonists is acted upon by phosphatidyl serine.

Thyroxin has been shown to enhance both oxygen consumption and water loss in the bladder of the toad *Bufo bufo* even when sodium of the medium was replaced with choline.[7] It was suggested that thyroxin increased cell metabolism which then had a manifold effect on permeability processes occurring across cell membranes. Thyroxin and vasopressin are known to act synergistically to increase water transfer across toad bladder.[10] The noted effect of phosphatidyl serine, therefore, could have been analogous to the effect of thyroxin and related to increased oxygen consumption. However, it was shown that phosphatidyl serine has no effect on oxidative metabolism in toad bladder. Thus, thyroxin and phosphatidyl serine probably act by different mechanisms to increase vasopressin-induced water loss in toad bladder.

Much of the data presented in this study suggests that phosphatidyl serine exerts its effect intracellularly. First, the effect of phosphatidyl serine is evident when the medium contains no phospholipid. Second, phosphatidyl serine is ineffective when included along with vasopressin in the medium bathing the bladder, suggesting that the phospholipid penetrates slowly into bladder cells, and does not accumulate in sufficient concentrations within the cell to exert a detectable effect under these conditions. Third, phosphatidyl serine treatment of either serosal or mucosal surfaces enhances subsequent vasopressin-induced water loss from the bladders. Apparently, penetration by phosphatidyl serine to its intracellular site of action can take place from either surface. Fourth, the effect of phosphatidyl serine is relatively short-lived and could not be detected 1 hr after treatment with the phospholipid. This suggests that the lipid is not tightly bound within the bladder cells and is washed out after prolonged exposure to a phospholipid-free medium. Last, the closely related compound, lecithin, did not enhance the response of toad bladder to vasopressin. Presumably, the quaternary ammonium portion of the lecithin molecule inhibits penetration of this substance into the bladder cells and this explains its lack of activity. It seems likely therefore that the noted effect of phosphatidyl serine is not exerted at an external membrane surface, but rather at an intracellular site.

One possible explanation for the effect of phosphatidyl serine is that this substance *per se* has agonistic properties in toad bladder. The concentrations of phosphatidyl serine occuring within the bladder in this study may have been subthreshold but still sufficient to enhance the effects of vasopressin. However, phosphatidyl serine bears little chemical resemblance to any of the known agonists for water loss in toad bladder. Also, no direct evidence is available to indicate that high concentrations of phosphatidyl serine have agonistic properties in the bladder of the toad.

In reference to general anesthetics, Ariens *et al.* state that, "A diffuse accumulation

of drug in the lipid phases of the biological object, for instance, the various membranes, seems feasible as a basis of explanation for the biological action".[11] A similar basis may serve to explain the action of phosphatidyl serine on vasopressin-induced water loss in toad bladder noted in this study. However, Ariens *et al.* further state that receptor principles are of little use in interpreting the actions of general anesthetics since these drugs have "low structural requirements and are devoid of active groups suitable for ionic or hydrogen bonds". The phospholipids employed in the present study do have active groups capable of forming chemical bonds and apparently also have certain structural requirements. It is proposed that the enhancement of vasopressin-induced water loss in toad bladder by phosphatidyl serine and related compounds is due to an alteration of lipoprotein phases within the toad bladder cells and that this effect is aided by the formation of weak chemical bonds.

Eggena *et al.* have shown that the extent of the vasopressin-induced water flux in toad bladder determines the duration and extent of the response to subsequent exposure to vasopressin.[12] The results of the present study showed that repeated exposure to phosphatidyl serine and to vasopressin produced a consistently elevated water loss above control with little evidence of a related increase in the "fatigue phenomenon". Apparently, enhancement of vasopressin-induced water flow by certain phospholipids is independent of the inhibitory influence of previous water flux, since the enhanced response can be demonstrated in the presence of the "fatigue phenomenon".

Acknowledgement—The able technical assistance of Mrs. Ann Vertenten is acknowledged. The author wishes to thank Dr. Camillo Artom for reading the manuscript and offering helpful suggestions. The L-8 vasopressin was obtained from Sandoz for use in this study by Dr. J. Maxwell Little. This work was supported by a grant from the North Carolina Heart Assoc.

REFERENCES

1. L. L. VAN DEENEN, *Ann. N.Y. Acad. Sci.* **137**, 717 (1966).
2. P. J. BENTLEY, *J. Endocr.* **17**, 201 (1958).
3. P. J. BENTLEY, *J. Endocr.* **26**, 295 (1963).
4. P. J. BENTLEY, *Biol. Rev.* **41**, 275 (1966).
5. H. RASMUSSEN, I. L. SCHWARTZ, M. A. SCHOESSLER and G. HOCHSTER, *Proc. natn. Acad. Sci. U.S.A.* **46**, 1278 (1960).
6. A. LEAF, L. B. PAGE and J. ANDERSON, *J. biol. Chem.* **234**, 1625 (1959).
7. A. J. MATTY and K. GREEN, *J. Endocr.* **25**, 411 (1963).
8. J. ORLOFF and J. S. HANDLER, *J. clin. Invest.* **41**, 702 (1962).
9. D. W. WOOLLEY and B. W. GOMMI, *Nature, Lond.* **202**, 1074 (1964).
10. E. MARUSIC and J. TORRETTI, *Nature, Lond.* **202**, 1118 (1964).
11. E. J. ARIENS, A. M. SIMONIS and J. M. VAN ROSSOM, in *Molecular Pharmacology*, vol. I (Ed. E. J. ARIENS) p. 345. Academic Press, New York (1964).
12. P. EGGENA, R. WALTER and I. L. SCHWARTZ, *Life Sci.* **7**, 59 (1968).