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Studies on the Interactions between Arachidonic Acid Metabolism and Calcium in Regulating Vasopressin-Induced Water Permeability in the Toad Urinary Bladder

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STUDIES ON THE INTERACTIONS BETWEEN ARACHIDONIC ACID METABOLISM AND CALCIUM IN REGULATING VASOPRESSIN-INDUCED WATER PERMEABILITY IN THE TOAD URINARY BLADDER

by

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RONALD M. BURCH. Studies on the Interactions Between Arachidonic Acid Metabolism and Calcium in Regulating Vasopressin-Induced Water Permeability in the Toad Urinary Bladder. (Under the direction of PERRY V. HALUSHKA).

The toad urinary bladder and epithelial cells isolated from it were found to synthesize prostaglandin E (PGE) and thromboxane A_2 (TXA_2). The syntheses of both of these compounds were found to be stimulated by arginine-vasopressin and by its non-pressor antidiuretic analog 1-deamino-8-D-arginine-vasopressin. cAMP, a putative second messenger for vasopressin in the toad bladder, when added to the incubation media, did not affect TXA_2 synthesis. However, in the isolated cells both TXA_2 and PGE syntheses were found to be stimulated by calcium.

In studies using the isolated toad bladder, inhibition of TXA_2 synthesis with imidazole (1 mM) and 7-(1-imidazolyl)-heptanoic acid (50-100 μM) was found to inhibit vasopressin-stimulated water flow to a maximum of 30%. Similarly, an antagonist of TXA_2 action, trans-13-azaprostanoic acid (50-300 μM) inhibited vasopressin-stimulated water flow in a dose-dependent fashion to a maximum of 35%, while the biologically inactive cis isomer was without effect.

Several compounds which exhibit TXA_2-like effects in other systems, (15Z)-hydroxy-9α,11α-(epoxymethano)prosta-5Z,13E dienoic acid (U44069), its 9α,11α-(methanoepoxy) isomer (U46619), and TXB_2, mimicked the hydroosmotic effect of vasopressin. This action was inhibited in a dose-dependent fashion by trans-13-azaprostanoic acid while cis-13-azaprostanoic acid was without effect.

Vasopressin was found to elicit an enhancement in ^{45}Ca efflux from prelabelled toad bladder epithelial cells. Compartmental analysis of ^{45}Ca efflux from prelabelled cells revealed three components. Studies using EGTA, lanthanum, and mitochondrial inhibitors suggested that the first component of efflux (S_1) represented ^{45}Ca bound to heterogenous sites on the extracellular surface of the plasma membrane, the second component (S_2) represented ^{45}Ca bound to some intracellular site(s), and the third component (S_3) was composed of two sites, a slowly exhanging site near the plasma membrane, and mitochondria.

Vasopressin reduced the sizes of calcium pools S_1 and S_3. Exogenous cAMP also reduced the size of S_3, but increased the size of S_2. The TXA_2 mimetic, U46619, acted similarly to cAMP; it decreased the size of pool S_3 and increased the size of pool S_2. The TXA_2 synthesis inhibitor 7-(1-imidazolyl)-heptanoic acid blocked the effect of vasopressin to reduce the size of S_3. The TXA_2 antagonist trans-13-azaprostanoic acid, in concentrations not lethal to the isolated cells, inhibited vasopressin-stimulated water flow only slightly (17%) and did not significantly alter vasopressin's effects on ^{45}Ca kinetics. However, trans-13-azaprostanoic acid significantly blocked the effect of U46619 to decrease the size of S_3.

Exogenous PGE_1, 1 μM, a concentration which completely inhibits vasopressin-stimulated water flow, enhanced both the efflux and influx of ^{45}Ca. However, net cellular ^{45}Ca was increased. The increased ^{45}Ca was located entirely in pool S_3.

These results are consistent with a hypothesis wherein vasopressin causes a net release of calcium from an intracellular storage site, perhaps mitochondria. PGE and TXA_2 syntheses are stimulated, perhaps

by the altered calcium fluxes. TXA_2, like vasopressin, reduces the size of a putative mitochondrial calcium compartment, and inhibition of TXA_2 synthesis, or antagonism of TXA_2 action, inhibits vasopressin-stimulated water flow. PGE enhances ^{45}Ca uptake into the epithelial cells and increases the size of pool S_3, resulting in inhibition of vasopressin-stimulated water flow.

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CHAPTER I

GENERAL INTRODUCTION

The molecular mechanism of vasopressin's antidiuretic action is unclear. This is due, in part, to the difficulty in obtaining sufficient quantities of pure vasopressin-sensitive tissue from the structurally complex kidney. The toad urinary bladder has been extensively used as an alternative model for the study of vasopressin-mediated alterations in water permeability for a number of reasons: 1) it is responsive to vasopressin, water permeability being enhanced up to 100-fold, 2) the tissue is large, several square centimeters, and easy to isolate, 3) the whole bladder, or epithelial cells derived from it, remain viable for many hours in vitro, 4) it is composed of a single layer of epithelial cells which is supported by only a thin layer of connective tissue, and, most important, 5) it appears that where studied, the biochemical and physiological responses to vasopressin in the toad bladder are similar to those which occur in the mammalian collecting tubule.

Vasopressins and oxytocins of anuran or mammalian origin act to increase water permeability across the toad bladder (Bentley, 1966). Permeability to urea is also enhanced by vasopressin (Maffly, et al., 1960). The toad urinary bladder actively transports sodium and this transport is also enhanced by vasopressin (Leaf, et al., 1958). While the stimulation of water, urea, and sodium transport are similar in many respects, they are independent and can be dissociated by means of a number of chemical modifications of the membrane and by drugs (Andreoli and Schafer, 1976). Vasopressin is active only when

placed on the serosal surface of the bladder (Bentley, 1958). However, all available evidence points to the mucosal surface as the permeability barrier to water, urea, and sodium (Andreoli and Schafer, 1976). Thus, an information transfer system must exist to enable vasopressin to influence the permeability of the opposite surface of the cell. Several candidate mediators have been suggested and this dissertation presents data gathered concerning two of these putative mediators, thromboxane A_2 and calcium.

Vasopressin and cyclic adenosine 3'-5'-monophosphate (cAMP) in the toad bladder

Vasopressin enhances the cellular accumulation of cAMP in toad bladder epithelial cells (Handler et al., 1965) and enhances the activity of adenylate cyclase in broken epithelial cell preparations from toad bladder (Bär et al., 1970). Further, exogenous cAMP and theophylline, a cyclic nucleotide phosphodiesterase inhibitor, mimic the actions of vasopressin on water, urea, and sodium transport (Orloff and Handler, 1962). Together, these data provide evidence that cAMP plays a role in the mediation of the response to vasopressin. However, investigations into the molecular mechanism of action of cAMP in the toad bladder have met only very limited success to date (Strewler and Orloff, 1977). Greengard and associates (DeLorenzo et al., 1973; Greengard, 1976) have identified a protein which is dephosphorylated in response to vasopressin or cAMP. The dephosphorylation appears to be correlated to the rate of sodium transport and not to alterations in water permeability (Walton et al., 1975). The identity of this protein remains unknown as does its significance to transport.

Vasopressin and calcium in the toad bladder

While calcium has for some time been implicated in the mediation of vasopressin's actions, studies of its role are hampered because, in addition to possibly serving as a second messenger, calcium plays a role in maintaining the structural integrity of the tight junctions between the epithelial cells. If calcium is removed from the bathing medium, the tight junctions become permeable, allowing enhanced water permeability unrelated to vasopressin's effect (Hays et al., 1965). Further, calcium is necessary for the transduction of information from the vasopressin receptor to the adenylate cyclase. Absence of calcium or high calcium concentrations inhibit vasopressin receptor-mediated stimulation of adenylate cyclase activity (Bockaert et al., 1972; Hynie and Sharp, 1971). However, calcium has no effect on the binding of vasopressin to its receptor (Roy et al., 1973).

High concentrations of calcium in the serosal bathing medium inhibit vasopressin-stimulated water flow without affecting vasopressin-stimulated sodium transport (Petersen and Edelman, 1964; Argy et al., 1967). Similarly, other divalent and trivalent cations, added serosally, inhibit vasopressin-stimulated water flow (Bentley, 1959; Weitzerbin et al., 1974). The effects of exogenous cAMP are not altered by high serosal calcium (Argy et al., 1967). These data led to the hypothesis that vasopressin stimulates cAMP formation at two sites, one calcium-sensitive and responsible for enhanced water permeability, and the other insensitive to calcium and responsible for sodium transport (Petersen and Edelman, 1964).

A second hypothesis concerning the role of calcium in vasopressin's actions considers calcium to be not only a coupling factor,

but incorporates it as a second messenger for vasopressin (Berridge, 1975). Thorn and Schwartz (1965) showed that the efflux of ^{45}Ca from prelabelled bladders increased after the addition of vasopressin. Snart and Dalton (1972) found only a single activation energy for the stimulation of cAMP synthesis by vasopressin and suggested that the dual adenylate cyclase hypothesis might be better replaced by a hypothesis in which vasopressin directly elicits a release of membrane calcium which leads to enhanced water permeability. Cuthbert and Wong (1974) confirmed the findings of Thorn and Schwartz and further reported that the enhancement of efflux occurs only from the mucosal surface of the bladder. They considered the release to be from the plasma membrane since hemibladders labelled on only the serosal side with ^{45}Ca exhibited no enhancement of efflux from the mucosal side upon the addition of vasopressin. This observation was also made by Walser (1970). Exogenous cAMP did not mimic vasopressin, causing instead an inhibition of efflux from the side to which it was added. Interestingly, phospholipase C incubation on the serosal surface inhibited vasopressin-stimulated water flow and vasopressin-stimulated ^{45}Ca efflux (Cuthbert et al., 1971; Cuthbert and Wong, 1973), but did not inhibit the accumulation of cAMP in response to vasopressin.

Recent unconfirmed work of Hardy (1978) demonstrates that alterations in calcium flux alone can mimic the water permeability response to vasopressin. In bladders pretreated with the divalent cation ionophore A23187 and then exposed to an increased serosal calcium concentration, a water flow response was generated which was comparable to that elicited by vasopressin. Lowering the serosal calcium concentration during the water flow response to vasopressin or A23187

inhibited the response. Further, the effects of a submaximal concentration of vasopressin on water flow were enhanced when the ionophore was added together with vasopressin. These results suggest that vasopressin may stimulate calcium uptake. This hypothesis is supported by the observations of Humes et al. (1980), who found that verapamil, an organic calcium uptake inhibitor, inhibited vasopressin-stimulated water flow across the toad bladder in a dose-dependent manner.

In contrast to the studies cited above, a number of indirect studies have suggested that vasopressin causes a decrease in the concentration of cytoplasmic ionized calcium. When toad bladders were pretreated with A23187 a slight enhancement in basal water flow was observed (Taylor et al., 1979). However, A23187 inhibited the enhancement in water flow elicited by vasopressin (Taylor et al., 1979). This effect was observed over a wide range of serosal calcium concentrations, and, when A23187 was added to the mucosal bathing medium, a nominally calcium-free solution in these experiments, a similar inhibition of vasopressin-stimulated water flow was observed (Taylor et al., 1979). These observations suggest that A23187 acted to release calcium from intracellular binding sites to increase cytoplasmic ionized calcium. However, Humes and Weinberg (1980) observed that A23187 inhibited respiration in isolated toad bladder epithelial cells and reduced viability as assessed by trypan blue exclusion. Thus, A23187 may have inhibited vasopressin-stimulated water permeability by a mechanism separate from any effect on calcium metabolism.

Taylor et al. (1979), on the assumption that lowered serosal sodium concentration would enhance calcium uptake into the isolated

toad bladder, an assumption confirmed by Grinstein and Erlij (1978) in frog skin, assessed the effects of lowered serosal sodium concentration on vasopressin-stimulated water flow. They found that as sodium concentration was progressively lowered, vasopressin-stimulated water flow was increasingly inhibited. This effect was dependent upon serosal calcium concentration, suggesting that lowered serosal sodium concentration may inhibit calcium efflux by some sort of sodium-calcium exchange process (Taylor et al., 1979). In fact, Chase and Al-Awqati (1981) have found a sodium-dependent calcium pump in serosal plasma membrane vesicles isolated from the toad bladder. These results suggest that a low intracellular calcium concentration is required for vasopressin to exert its antidiuretic action.

Studies have been carried out using Ringer's solutions without added potassium to inhibit the sodium pump (Taylor et al., 1979; Finn et al., 1966). Lack of potassium was found to inhibit vasopressin-stimulated water flow, presumably by inhibition of the sodium pump to increase intracellular sodium concentration, which would in turn increase intracellular calcium concentration by means of a sodium-calcium exchanger pumping the excess sodium out of the cell. As with the experiments with lowered extracellular sodium concentration, the effect of lowered extracellular potassium on water flow was calcium-dependent (Taylor et al., 1979). However, the mechanism of inhibition of vasopressin-stimulated water flow by lack of potassium may not be so readily explained. Ouabain, which also inhibits the sodium pump, does not appear to affect vasopressin-stimulated water flow (Davis et al., 1978) across the toad bladder, although one study did report an inhibition by ouabain (Finn et al., 1966). Of course

potassium removal would also result in a more negative membrane potential, which would also increase the activity of the sodium-calcium exchanger.

Several other agents including acetylcholine and several of its analogs, seem to act in many tissues to increase cytoplasmic ionized calcium. These agents also inhibit vasopressin-stimulated water flow in the toad bladder (Wiesmann et al., 1978; Arruda and Sabatini, 1980b). All enhance ^{45}Ca uptake into isolated toad bladder epithelial cells (Wiesmann et al., 1978; Arruda and Sabatini, 1980b). Further, these agents do not block vasopressin-stimulated water flow when the extracellular calcium concentration is lowered, or in the presence of low concentrations of lanthanum ion or pentobarbital, agents which block the enhanced uptake of ^{45}Ca in response to these agents (Wiesmann et al., 1978; Arruda and Sabatini, 1980b).

Ramwell and Shaw (1970) noted that PGE_1 or high serosal calcium concentration inhibited vasopressin-stimulated water flow, while PGE_1 or low serosal calcium concentration enhanced sodium transport in frog skin and toad bladder. They preloaded frog skin with ^{45}Ca and added PGE_1 at the beginning of desaturation. PGE_1 was found to enhance ^{45}Ca efflux from the skins, leading to the suggestion that PGE_1 may exert its effects on transport by modifying calcium metabolism. Cuthbert and Wong (1974) confirmed that PGE_1 enhances efflux of ^{45}Ca from prelabelled bladders, but the experimental protocol used by each group precluded determination of whether the enhanced efflux was due to net extrusion of calcium from the epithelium or enhanced exchange due to increased membrane fluxes.

In summary, calcium appears to play a second messenger role in the antidiuretic action of vasopressin in the toad bladder. While data are limited, it seems clear that vasopressin mobilizes calcium from some unknown site. Whether increased ^{45}Ca efflux noted in several studies represents net calcium extrusion or enhanced exchange rate is unknown. The results with A23187 and verapamil suggest that calcium uptake may also play a role in the action of vasopressin. However, no studies have examined ^{45}Ca uptake into the toad bladder in response to vasopressin.

Metabolism of arachidonic acid in the isolated toad bladder

Arachidonic acid, the precursor for both prostaglandins and thromboxanes of the 2 series, is stored primarily in membrane phospholipids (Hong and Levine, 1976). The rate-limiting step in the synthesis of prostaglandins and thromboxanes is the catalysis by phospholipases of the release of arachidonic acid from its membrane storage sites (Kunze and Vogt, 1971) (Fig. 1). Arachidonate is metabolized by fatty acid cyclooxygenase (prostaglandin synthetase) to produce the labile endoperoxides, prostaglandin G_2 (PGG_2) and PGH_2 (Samuelsson et al., 1978). These endoperoxide intermediates serve a pivotal role since all prostaglandins and thromboxanes are synthesized from them. The toad urinary bladder synthesizes PGE_2 (Zusman et al., 1977) and thromboxane A_2 (TXA_2) (this dissertation). The synthesis of PGE_2 is catalyzed by prostaglandin endoperoxide E isomerase, while the synthesis of TXA_2 is catalyzed by an as yet poorly characterized enzyme, thromboxane synthetase. TXA_2 is the biologically active thromboxane but it is unstable in aqueous media, with a half-life of

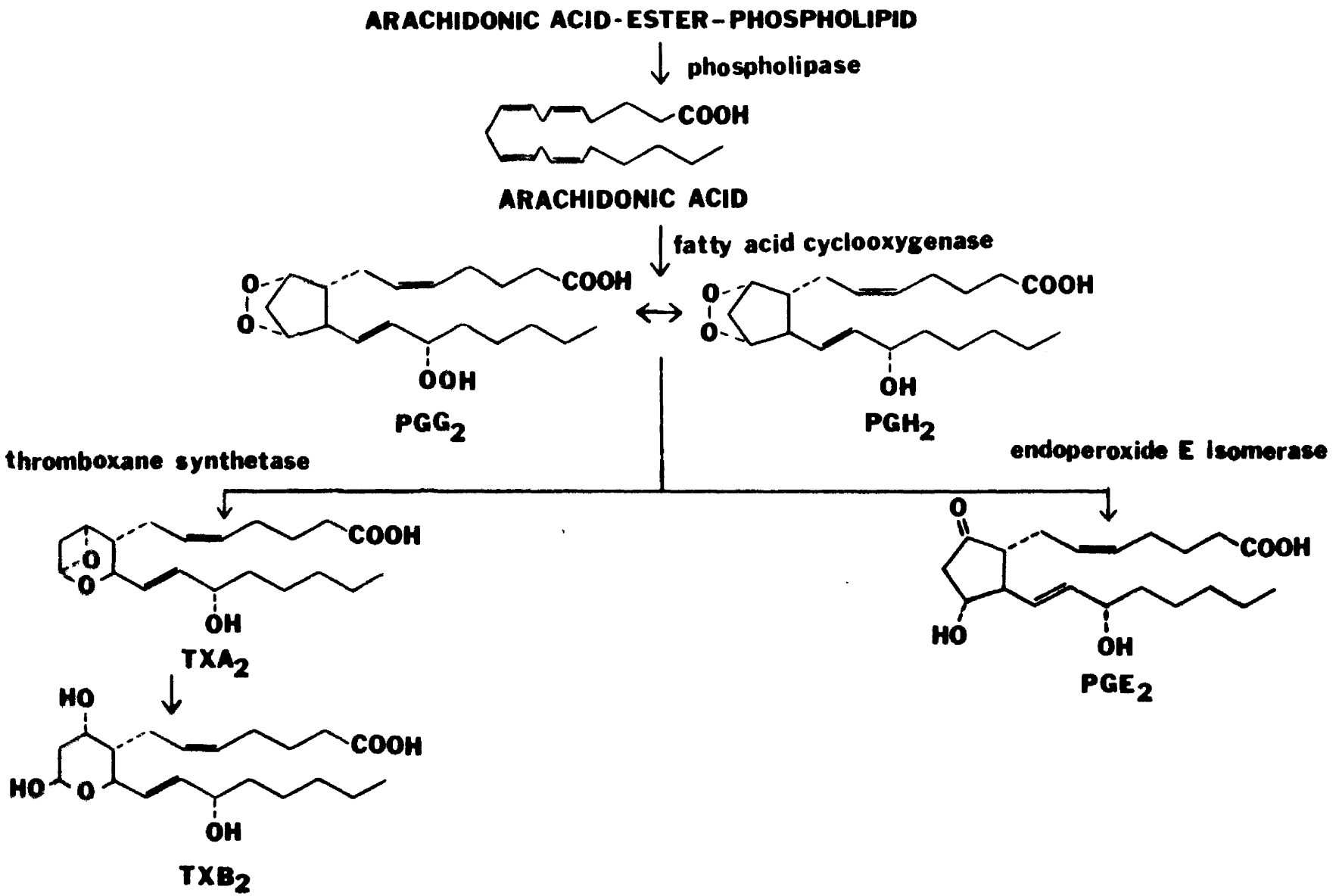


Figure 1. Pathways of arachidonic acid metabolism in the toad urinary bladder.

37 sec, undergoing spontaneous hydrolysis to the stable, much less active TXB_2 (Hamberg et al., 1975).

Bisordi et al. (1980) reported that the toad bladder does not synthesize PGE_1 or $PGF_{2\alpha}$ based on radioimmunoassay data. However, in thin-layer chromatogram scans of [^{14}C]-arachidonate metabolites from intact hemibladders and from bladder homogenates, other peaks were observed (Zusman et al., 1977; this dissertation). Thus, the question of whether the toad bladder synthesizes other prostaglandins remains open.

Role of arachidonate metabolites in vasopressin's actions

Exogenous PGE_1 and other prostaglandins (Urakabe et al., 1975) have two effects in the isolated toad urinary bladder, inhibition of vasopressin-stimulated water flow (Orloff et al., 1965) and stimulation of sodium transport (Lipson and Sharp, 1971). PGE_1 also inhibits theophylline-stimulated water flow, but not cAMP-stimulated water flow, suggesting an inhibitory effect on an adenylate cyclase pool affecting water permeability (Orloff et al., 1965). Zusman et al. (1977) found that vasopressin stimulates PGE synthesis in the toad bladder by activating a phospholipase A_2. However, an earlier study (Wong et al., 1972) and a recent study (Bisordi et al., 1980) found no stimulation of PGE biosynthesis by vasopressin. These differing findings will be discussed in detail in Chapter II.

Inhibition of PGE biosynthesis by cyclooxygenase inhibitors or phospholipase inhibitors results in enhanced vasopressin-stimulated water flow (Zusman et al., 1977). Further, addition of arachidonic acid causes inhibition of vasopressin-stimulated water flow while addition of arachidonic acid plus cyclooxygenase inhibitors is without

effect (Zusman et al., 1977). Neither theophylline nor exogenous cAMP enhance PGE biosynthesis (Zusman et al., 1977). Thus, it appears that vasopressin stimulates phospholipase activity independent of cAMP.

Exogenous PGE_1 inhibits the cellular accumulation of cAMP in response to vasopressin (Omachi et al., 1974) although this observation has not been confirmed in other laboratories (Lipson et al., 1971; Wong et al., 1972). This observation supports the widespread hypothesis that PGE_1 inhibits vasopressin-sensitive adenylate cyclase (Orloff et al., 1965; Lipson and Sharp, 1971). However, several laboratories have reported that PGE_1 has no effect on vasopressin-sensitive adenylate cyclase activity in broken cell preparations (Bär et al., 1970). These data suggest that PGE_1 inhibits vasopressin-stimulated cAMP accumulation by an indirect mechanism. Similar findings have been reported for the effects of PGE_1 on hormone-sensitive adenylate cyclases in adipocytes (Ramwell and Shaw, 1970).

Prostaglandins, thromboxanes, and calcium

Prostaglandins and thromboxanes have been suggested to exert their actions through calcium-dependent mechanisms in a variety of tissues (Rubin and Laychock, 1978). That calcium and prostaglandin metabolism are related is undoubted, since many of the phospholipases which catalyze the rate-limiting step in prostaglandin and thromboxane synthesis require calcium for activity (Kunze and Vogt, 1971), and the increased prostaglandin synthesis observed in response to many peptide hormones is dependent on increased phospholipase activity (Zusman and Keiser, 1977). Further, the divalent cation ionophore

A23187 mimics phospholipase activation in a number of tissues and is associated with increased prostaglandin and thromboxane synthesis (Oelz et al., 1978).

PGE_1 has been reported to be a "calcium ionophore" based on its ability to stimulate calcium uptake into isolated rat mitochondria (Kirkland and Baum, 1972). Since the publication of that report many studies have been undertaken in attempts to implicate prostaglandins and thromboxanes as calcium ionophores. Several prostaglandins, especially TXA_2, and PGH_2 and its stable analogs U46619 and U44069, compare favorably with A23187 in extracting calcium into organic solvents under limited conditions (Gerrard and White, 1978; Reed and Knapp, 1978). When tested in a mitochondrial system, A23187, PGG_2, and U44069 all stimulated uncoupled oxidation of succinate which was inhibited by ruthenium red, La^{3+}, or EGTA (Reed and Knapp, 1978). Further, a number of prostaglandins were found to release calcium from isolated mitochondria (Malmstrom and Carafoli, 1975; McNamara et al., 1980). In guinea pig myometrium PGE_1 was found to depolarize the plasma membrane, leading to enhanced Ca^{2+} influx (Clegg et al., 1966). In contrast, no natural prostaglandins nor U46619 were able to transport calcium into phosphatidylcholine vesicles while A23187 was readily able to do so (Weissmann et al., 1980).

In direct studies of calcium binding to membrane preparations, several prostaglandins have been reported to inhibit calcium binding to a fraction of uterine smooth muscle ascribed to the sarcoplasmic reticulum (Carsten and Miller, 1977). In addition, in an intact smooth muscle preparation, PGE_1 has been shown to inhibit calcium

binding to a high affinity site presumed to be intracellular in location (Wheeler and Weis, 1980).

Thus, it is not certain whether prostaglandins and thromboxanes exert their effects on Ca^{2+} by means of a direct ionophoretic action or by a receptor-mediated event. These data tend to support a hypothesis wherein the actions of TXA_2 and prostaglandins are mediated via interactions with receptors. While no receptor has yet been reported for TXA_2, receptors have been characterized for a number of prostaglandins (Hammarström et al., 1975; Morrell, 1976; Schafer et al., 1979). The availability of receptor level antagonists such as trans-13-azaprostanoic acid and 9,11-epoxyimino-prostanoic acid, TXA_2 antagonists (LeBreton et al., 1979; Fitzpatrick et al., 1978), and 7-oxa-13-prostanoic acid, a PGE antagonist (Fried et al., 1969) provide useful tools to test which of the possibilities is more tenable. It will be important to determine whether the TXA_2 and prostaglandin antagonists block the TXA_2- and prostaglandin-mediated calcium movements as well as the physiological responses to the prostaglandins.

Statement of Specific Aims

The objective of this study was to further examine the interactions between calcium and arachidonate metabolites in regulating the enhancement in permeability to water of the toad urinary bladder by vasopressin. This dissertation considers the following specific aims:

1. To determine the effects of vasopressin and its putative second messenger calcium on the synthesis of TXA_2 in the isolated toad urinary bladder and in suspensions of isolated toad urinary bladder epithelial cells.

2. To determine the effects of vasopressin on ^{45}Ca non-steady state influx and efflux, and on ^{45}Ca steady state compartmental analysis in suspensions of isolated toad urinary bladder epithelial cells.

3. To determine the effects of the TXA_2-like compound, U46619, the effects of the TXA_2 synthesis inhibitor, 7-(1-imidazolyl)-heptanoic acid, and the effects of the TXA_2 antagonist, trans-13-azaprostanoic acid, on ^{45}Ca non-steady state influx and efflux, and on ^{45}Ca steady state compartmental analysis in suspensions of isolated toad urinary bladder epithelial cells.

4. To determine the role of extracellular and intracellular calcium in the antidiuretic response to vasopressin and TXA_2-like compounds in the toad urinary bladder.

CHAPTER II

THE EFFECTS OF VASOPRESSIN AND CALCIUM ON THE SYNTHESIS OF TXA_2 AND PGE IN THE ISOLATED TOAD URINARY BLADDER AND IN TOAD BLADDER EPITHELIAL CELLS IN SUSPENSION, AND THE ROLE OF TXA_2 IN VASOPRESSIN-STIMULATED WATER FLOW

INTRODUCTION

Zusman et al. (1977) found that the isolated toad urinary bladder synthesized PGE and that vasopressin stimulated the synthesis of PGE by enhancing the release of arachidonate from membrane-bound stores by a phospholipase. Burch et al. (1979) confirmed that PGE synthesis in the toad bladder was stimulated by vasopressin and, in addition, that the toad bladder synthesized TXA_2, and that the synthesis of this metabolite was also stimulated by vasopressin. Bisordi et al. (1980) confirmed the observations that the isolated toad urinary bladder, and epithelial cells derived from it, synthesize PGE and TXA_2. However, these authors failed to detect any increase in the synthesis of either metabolite in the presence of vasopressin or its non-pressor analog, 1-deamino-8-D-arginine vasopressin (dDAVP). They attributed the earlier reports of Zusman et al. and Burch et al. to artifacts obtained through the use of an indirect radioimmunoassay for PGB, instead of direct assay for PGE_2. Thus, the objective of this study was to confirm that the syntheses of PGE and TXA_2 are stimulated by vasopressin. Further, since phospholipases are calcium-requiring enzymes, the effects of altered calcium on PGE and TXA_2 syntheses were also assessed. Since the possible role of TXA_2 in modulating vasopressin-stimulated water flow has not been described, experiments were carried out in an attempt to characterize its effect on basal and vasopressin-stimulated water flow.

MATERIALS AND METHODS

The following were generous gifts: indomethacin, Merck, Sharp, and Dohme Research Laboratory, Rahway, NJ; PGE_2, $PGF_{2\alpha}$, PGE_1, TXB_2, U46619, and U44069, Drs. J. Pike and U. Axen, The Upjohn Co., Kalamazoo, MI; trans and cis isomers of the 13-azaprostanoic acid, Drs. G. LeBreton and D. Venton, University of Illinois, Chicago, Ill., and Drs. R. Broersma, P. Anzenveno, and B. Cregge, Dow Chemical Co., Indianapolis, Ind. The following were purchased from commercial sources: spectral grade solvents, Burdick-Jackson Co., Muskegon, MI; imidazole, Aldrich Chemical Co., Milwaukee, WI; [5,6-^{3}H]-PGE_1 (90-150 Ci/mmol), [5,6,8,11, 14,15-^{3}H]-PGE_2 (90-150 Ci/mmol), and [5,6,8,9,11,12,14,15-^{3}H]-TXB_2 (60-150 Ci/mmol), and Omnifluor®, New England Nuclear Corp., Boston, MA; [1-^{14}C]-arachidonic acid (50-55 Ci/mmol), Applied Science Laboratories, State College, PA; Silicic acid (Biosil A 200-400 mesh), Biorad Laboratories, Richmond, CA; Machery-Nagel Sil G-25 thin-layer chromatography plates, Brinkmann Instruments, Westbury, NY; charcoal and dextran, Schwarz/Mann, Orangeburg, NY; bis(trimethylsilyl)acetamide, and o-methyl-hydroxylamine hydrochloride, Pierce Chemical Co., Rockford, IL; 3',5'-cyclic monophosphate, sodium salt, ethylenediamine tetraacetic acid, disodium salt, tris(hydroxymethyl) aminomethane-HCl (tris), and indomethacin, Sigma Chemical Co., St. Louis, MO; Pitressin, a pituitary extract of arginine-vasopressin, Parke Davis and Co., Detroit, MI; deamino-8-D-arginine-vasopressin, Ferring Pharmaceuticals, New York, NY; collagenase, type IV (165-200 U/mg), Worthington Enzyme Division, Millipore Corp., Bedford, ME; Prosil®, PCR Chemical Division, SCM Corp., Gainesville, FL, Bio-Solv®, Beckman Instruments, Fullerton, CA.

The Ringer's solutions were of the following compositions, bicarbonate buffered: 90 mM NaCl, 3.0 mM KCl, 25 mM $NaHCO_3$, 1.0 mM $MgSO_4$, 0.5 mM KH_2PO_4, 1.0 mM $CaCl_2$, and 6.0 mM glucose, pH 7.4; phosphate-buffered: 110 mM NaCl, 4.0 mM KCl, 0.5 mM $CaCl_2$, 1.0 mM $NaHPO_4$, 4.0 mM NaH_2PO_4, and 6.0 mM glucose, pH 7.4; tris-buffered: 112 mM NaCl, 5.0 mM KCl, 1.0 mM $CaCl_2$, 20 mM tris-HCl, and 6.0 mM glucose, pH 7.4. The bicarbonate-Ringer's solution was constantly aerated with 95% O_2:5% CO_2 and the phosphate- and tris-Ringer's solutions were constantly aerated with compressed air. All Ringer's solutions used for cell suspensions contained penicillin G, 25,000 U/liter, and streptomycin sulfate, 0.05 g/liter.

Indomethacin, 36 mg, was dissolved in 10 ml 0.1 M phosphate buffer, pH 8, and diluted into 2 liters Ringer's solution to yield a concentration of 50 μM. Control Ringer's solution contained an equivalent amount of phosphate buffer. Meclofenamate, 7-(1-imidazolyl)-heptanoic acid, and imidazole were dissolved directly into Ringer's solution. U46619, U44069, and TXB_2 were dissolved in ethanol to make stock solutions (10 mM), which were stored at -20°C. When these were added to experimental Ringer's solutions, the control media contained an equivalent volume of ethanol. 13-Azaprostanoic acid (3.2-19 mg) was wetted with 25 μl of ethanol and dissolved in 10 ml of Ringer's solution at pH 9. This solution was diluted into 200 ml Ringer's solution at pH 7.4 to obtain concentrations of 50 to 300 μM. Control media contained the ethanol and pH 9 Ringer's vehicle.

Radioactivity counting. Radioactivity of ^3H and ^{14}C was counted in a Beckman LS-355 liquid scintillation counter (Beckman Instruments, Fullerton, CA). The scintillation cocktail consisted of 40g Omnifluor®

(Beckman) and 2 liters Bio-Solv® (Beckman), in 10 liters of toluene. Aqueous samples were dissolved up to 1 ml per 10 ml cocktail. Radioactivity was counted to an instrumental error of 3% maximum, standardized to an internal standard. In the experiments described no attempt was made to correct for the counting efficiency of the instrument (ca. 30% for ^{3}H and 75% for ^{14}C).

Water flow. Water flow was measured gravimetrically by the method of Bentley (1958). Hemibladders were mounted with polyester thread on glass tubes 6 cm in length, 6 to 8 mm o.d., which had been slightly flared on one end, and 3 to 4 ml of one-fifth osmolar Ringer's solution (diluted with distilled water) was placed into each bag. The serosal bathing chamber consisted of a 40 ml polycarbonate centrifuge tube (3 cm in diameter) which had been cut to 8 to 10 cm in length (Fig. 2). A small hole had been bored through the tube near the bottom and a stainless-steel tube of 2 mm o.d. mounted through it with an epoxy cement. To the stainless-steel tube was attached catheter tubing which was connected in turn to a manifold. By this means the serosal bath (15 ml Ringer's solution) was constantly aerated. The free end of the glass tube holding the hemibladder was placed through a #5 1/2 rubber cork and the whole assembly mounted into the polycarbonate tube. Osmotic water flow was measured as the weight loss from the hemibladder monitored by removing the rubber stopper-glass tube-hemibladder assembly, blotting the hemibladder 3 times on a Kimwipe®, and suspending it in a Mettler Type H16 balance. Generally, hemibladders were allowed to stabilize for at least 2 hours during which time the serosal media were changed every 30

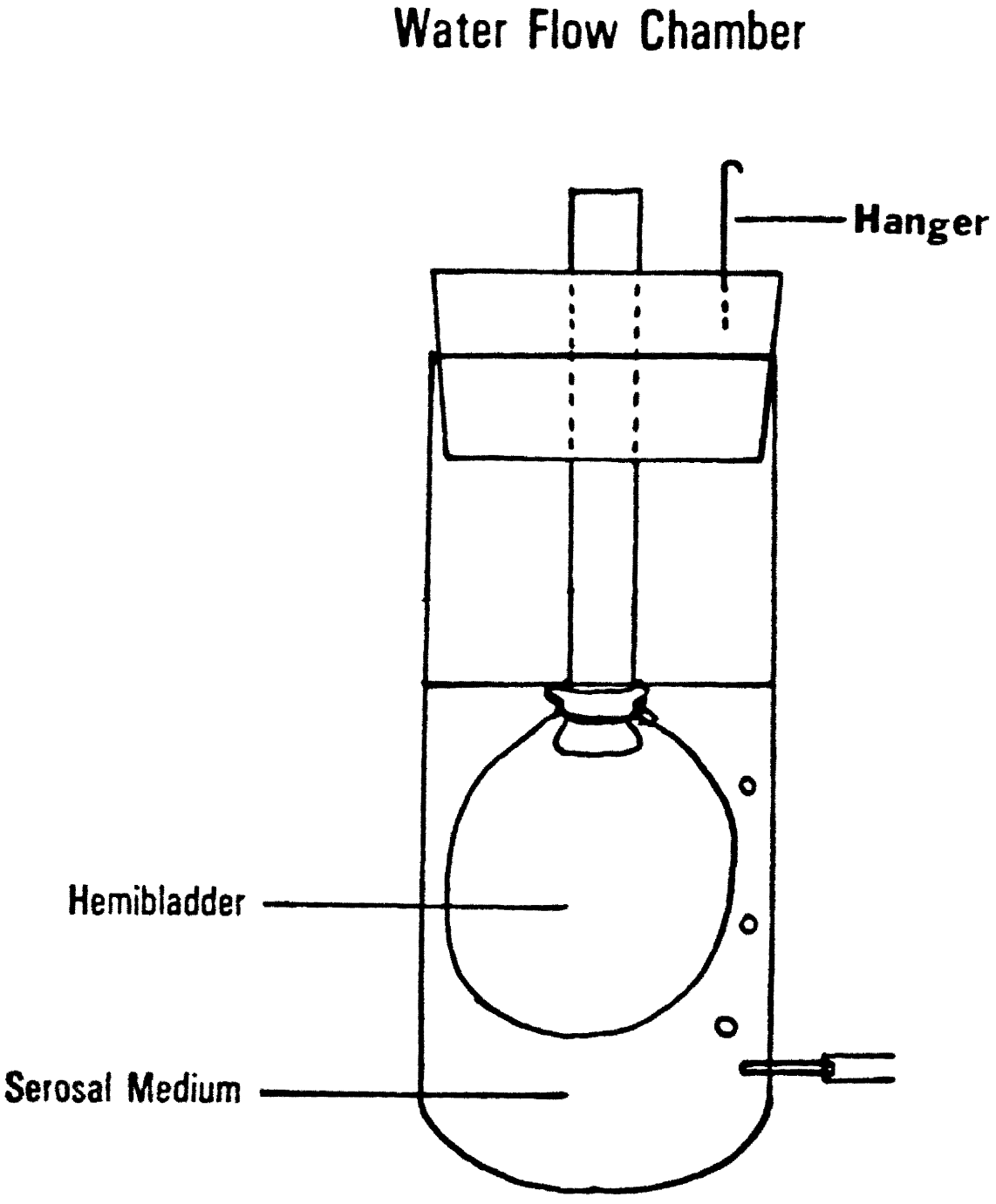


Figure 2. Water flow chamber. The hemibladder was mounted in the chamber mucosal surface inward. The rubber stopper did not fit tightly to allow air to escape from the chamber.

minutes and the mucosal media changed twice, before any experimental manipulations were commenced.

Water flow was generally normalized to the mean weight of the hemibladders used in the experiments by use of the following formula: water flow = $[(mass_i - mass_f)/\Delta t]/(mass_{hemibladder})\ (mass_{\bar{x},hemibladders})$ or, in units

$$= [(mg\ lost/min)/mg]\ (mg/hemibladder)$$

$$= mg/min/hemibladder$$

where $mass_i$ and $mass_f$ are the masses of the hemibladder assembly at the beginning of and the end of the time period, respectively, Δt is the time interval between the mass determinations, $mass_{hemibladder}$ is the mass of the hemibladder alone after it has been thoroughly blotted on a Kimwipe®, and $mass_{\bar{x},hemibladder}$ is the mean mass of all the hemibladders used in the experiment, usually arbitrarily chosen to be 150 mg.

Normalization of water flow to hemibladder surface area. U46619, U44069, and TXB_2 all contracted hemibladders. Therefore, water flow was normalized to mean hemibladder surface area in experiments using these agents. Water flow was measured as weight loss/minute per hemibladder and expressed as mg/minute/10cm^2 surface area. The mean surface area for the hemibladders used in these experiments was 10.4±2.8cm^2, n=38. Surface area was calculated assuming the hemibladder sacs were spheres where volume = $(4/3)\pi r^3$ and surface area = $4\pi r^2$, where r is radius in cm. The volume was determined by submerging a hemibladder in a beaker of Ringer's solution containing the experimental agent, up to the level of the thread used to secure it to the glass tube. The hemibladder was then filled with the usual mucosal

solution of diluted Ringer's solution to the point where the bottom of the water meniscus was level with the thread on the glass tube. At this point the pressure inside the hemibladder equalled the pressure outside the hemibladder so no distension occurred due to hydrostatic pressure and the hemibladder appeared spherical. The mucosal solution was drawn up into a graduated syringe and volume was determined to within 0.1 ml. The procedure was then carried out a second time. The coefficient of variation of the differences between the two determinations was 1.4% (n=16). In hemibladders treated with U44069, U46619 or TXB_2, after the experiment was completed the volume was determined, then the hemibladders were washed three times with Ringer's solution at 10 minute intervals to remove the drug, and the procedure repeated.

Since water is assumed to move through pores in the apical membrane (Koefoed-Johnsen and Ussing, 1953), a highly folded contracted hemibladder may impede water flow, leading to an underestimate of water flow when compared to a hemibladder of equal weight which is not contracted. Determination of surface area, on the other hand, serves as an index of the amount of epithelium available for unimpeded flow (Kachadorian and Levine, 1979). To test the hypothesis that hemibladder surface area is a valid normalization parameter for water flow, one member of each of eight pairs of hemibladders was filled with 3 ml of diluted Ringer's solution while the other was fully distended by adding mucosal medium over a one hour period, until no more could be accomodated, usually 20 to 30 ml. The distended hemibladders were emptied and 3 ml diluted Ringer's solution were added. These hemibladders remained distended after emptying. The hemibladders were

allowed to stabilize 30 minutes, then vasopressin (50 mU/ml) was added and water flow was determined for 30 minutes. The weights of the hemibladders were not significantly different between the 2 groups (149±8 mg in the undistended hemibladders and 133±7 mg in the distended hemibladders, n=8 pairs). At the end of the vasopressin treatment period, the measured surface areas of the distended hemibladders were significantly greater than their paired controls (10.0±3.0 cm^2 in the undistended hemibladders and 16.1±3.0 cm^2 in the distended hemibladders, $p<0.01$, n=8 pairs). Water flows normalized to weight were significantly greater in the distended hemibladders than in their paired undistended controls (0.28±0.04 mg/minute per mg hemibladder in undistended hemibladders and 0.50±0.07 mg/minute per mg hemibladder in the distended hemibladders, $p<0.01$, n=8 pairs). However, when water flow was normalized to surface area, there was no difference between the 2 groups (3.6±0.2 mg/minute per cm^2 hemibladder in undistended hemibladders and 3.7±0.2 mg/minute per cm^2 hemibladder in the distended hemibladders). Similar observations have been reported by Kachadorian and Levine (1979). While the data demonstrate that normalization of osmotic water flow to surface area is preferable in situations where surface areas may be different, for several representative experiments, water flow was normalized to mean hemibladder wet weight in addition to the normalization to surface area. The mean hemibladder wet weight for these experiments was 164±12 mg (n=38). Since weight was not changed by the agents used in these studies, this further confirms that <u>absolute</u> water flow was increased by each experimental agent.

Collagenase method of cell preparation. The procedure described is modified from Gatzy and Berndt (1968) and Wiesmann et al. (1977). After double pithing the toad, the abdomen was opened and the abdominal vein leading from the bladder was severed and the bladder was perfused through the ventricle antegrade with 50 ml of Ca,Mg-free bicarbonate-Ringer's solution. The bladder was then perfused retrograde through the abdominal vein with approximately 15-20 ml of Ringer's solution, until the perfusate was no longer sanguinous. The bladders were excised, cut into quarter bladders, and placed into a Ca,Mg-free bicarbonate-Ringer's solution containing type IV collagenase (2 mg/ml). Since trypsin damages toad bladder cells (Gatzy and Berndt, 1968), type IV collagenase was used since it is claimed by the manufacturer to be essentially trypsin-free. The bladder pieces were gently agitated with 95% O_2:5% CO_2 for 60 min after which the collagenase solution was replaced with bicarbonate-Ringer's solution containing 1 mM $CaCl_2$ and 1 mM $MgSO_4$ for 30 min. The bladder pieces were removed from the solution and placed mucosal-side up on a piece of Parafilm®-covered styrofoam. The epithelial cells were dislodged by very gently scraping the surface of the bladder only once with the edge of a microscope slide. Cells from several bladders were pooled in a 40 ml polycarbonate centrifuge tube and pelleted at 200 x g at room temperature in a floor model IEC Clinical Centrifuge for 5 min. The supernatant was poured off, replaced, and the gelatious pellet resuspended by passing the pellet through a plastic Eppendorf pipet tip (2 ml) attached to 1 ml adjustable pipet. At times the end of the pipet tip was cut to obtain a wider inside diameter for ease in pipeting the initial suspension. The suspension

was passed into the pipet several times as necessary to resuspend the pellet. Any small pieces of smooth muscle, opaque and white in color, were removed from the suspension with the pipet. The centrifugation and pipeting procedures were repeated twice more, by which time the major portion of mucus had been removed. After the pellet had been resuspended a third time, the suspension was passed through 2 layers of U.S.P. type VII gauze to remove the remaining mucus. The suspension was pelleted once more, resuspended, and diluted to final volume (3 mg protein/ml). The gelatinous material surrounding the cells was probably not DNA released from damaged cells. The epithelial surface of toad bladders contains goblet cells, ca. 5% total epithelial cells, which secrete a mucus. Upon incubation of the intact hemibladder for even a few minutes in Ringer's solution mucus can be detected on the epithelial surface. Thus, the gelatinous material reported here probably represents secretion from goblet cells during the 90 min incubation of the intact hemibladder. Viability of cells prepared by this method was 96±2%, n=4, by trypan blue exclusion.

Scraping method of cell preparation. This method is essentially that described by Bisordi et al. (1980). Bladders were perfused as described above with phosphate-Ringer's solution containing $CaCl_2$. Bladders were excised and immediately placed mucosal-side up on Parafilm®-covered styrofoam. The epithelial cells were scraped off the submucosa with the edge of a microscope slide and placed into phosphate-buffered Ringer's solution at 4°C for 60 min. Viability of cells prepared by this method was 52±9%, n=4, by trypan blue exclusion.

EDTA method of cell preparation. The method was modified from Scott and Sapirstein (1974). Bladders were perfused as described above with bicarbonate-Ringer's solution or tris-Ringer's solution, then excised and cut into small pieces (approximately 3 mm). These were placed into a 50 ml Ehrlenmeyer flask (which had been previously silanized with Prosil®) containing Ca, Mg-free Ringer's solution plus 100 μM EDTA. The bladder pieces were stirred moderately with a teflon-coated magnetic stirbar for 10 min. The medium was decanted and discarded. Fresh EDTA-Ringer's solution was added and the bladder pieces stirred for 10 min. The medium, containing detached cells, was decanted and filtered through 2 layers of gauze into a polycarbonate centrifuge tube. Fresh EDTA-Ringer's solution was added to the bladder pieces and the harvesting procedure was repeated, usually 3 or 4 times in total, until the cells were all collected. The cells were centrifuged at 200 x g for 5 min and resuspended to a final protein concentration of approximately 3 mg/ml, in either bicarbonate-Ringer's solution containing 1 mM $CaCl_2$ and 1 mM $MgSO_4$ or tris-Ringer's containing varying amounts of calcium. Viability of cells prepared by this method was 75±12%, n=4, by trypan blue exclusion.

Experimental protocol for vasopressin and dDAVP experiments. In all experiments, bladders from 6-8 toads were pooled to allow for the simultaneous incubation of triplicate samples for controls, vasopressin, dDAVP, and indomethacin or meclofenamate incubates. This was considered to be a single experiment with the mean of the 3 measurements being averaged. Cells prepared by the collagenase or EDTA methods were incubated at room temperature (22-25°C) with constant gentle agitation from a teflon-coated magnetic stirbar for 60 min

under an atmosphere of 95% O_2:5% CO_2 or compressed air as appropriate. Aliquots of 3 ml were placed into 10 ml polycarbonate centrifuge tubes. Suspensions were pelleted at high speed in an IEC Model CL tabletop clinical centrifuge for 45 sec and resuspended in Ringer's solution or Ringer's solution plus indomethacin or meclofenamate for 15 min with stirring. Then the suspensions were pelleted once again. Resuspension was into Ringer's solution or Ringer's solution plus vasopressin (5 mU/ml or 10 mU/ml), dDAVP (130 nM), indomethacin (50 μM), or meclofenamate (10 μM). Incubation with stirring was continued for 15 min after which the suspensions were pelleted, the media saved for radioimmunoassay of PGE and TXB_2, and the pellet retained for protein assay by the method of Lowry et al. (1951). Viability at the end of the experiment was not reduced from the initial estimate, being 94±5%, n=4, and 75±8%, n=4, for cells isolated by the collagenase or EDTA methods, respectively.

After a 60 min incubation at 4°C, cell suspensions prepared by the scraping method described by Bisordi et al. (1980) were pelleted at 200 x g in a Sorvall RC-2B refrigerated centrifuge for 1 min and resuspended in phosphate-Ringer's solution at room temperature. Aliquots (1.5 ml) of the gelationous cell mass were placed into 1.5 ml phosphate-Ringer's solution or phosphate-Ringer's solution plus vasopressin, indomethacin, or meclofenamate, at room temperature and incubated with stirring for 15 min. The suspensions were pelleted as described above, and the media and protein processed as described above.

Protocol for experiments with altered calcium concentration. The cell suspension from a single preparation of 6-8 toads was divided

into 10 aliquots in 40 ml polycarbonate centrifuge tubes. The suspensions were pelleted as described above and resuspended in 2 ml Ringer's solution containing 0.1, 0.5, 1.0, or 2.0 mM calcium and no magnesium, or 0.1 mM calcium plus 0.1 mM EGTA for the 0 calcium point (Blackmore et al., 1978). The suspensions were repelleted and resuspended in identical Ringer's solutions. The suspensions were incubated with gentle agitation by means of teflon-coated magnetic stirbars under an atmosphere of 95% O_2:5% CO_2 or compressed air as appropriate for 30 min. The suspensions were repelleted, resuspended a final time, and incubated for 15 min under the appropriate atmosphere. The suspensions were pelleted, the media taken for assay of TXB_2 and PGE, and the pellet retained for protein determination.

Radioimmunoassay of immunoreactive (i)PGE. To an aliquot (5 to 10 ml) of serosal media was added [^{3}H]-PGE_1(1500 cpm) to account for recovery losses. The media were acidified to pH 3.5 with formic acid and extracted twice with 2 volumes of ethyl acetate, the ethyl acetate then being evaporated under a stream of N_2. In the experiments using cell suspensions an aliquot (0.5 ml) was taken and diluted to 2 ml with distilled water to facilitate processing, acidified, and extracted once with 20 ml ethyl acetate and the ethyl acetate evaporated under a stream of N_2. The dried extract was redissolved and applied to a silicic acid column (0.5 g) (Webb et al., 1978). The prostaglandin E fraction was eluted (Webb et al., 1978) and converted to PGB using 0.1 N methanolic KOH, then assayed using a previously described radioimmunoassay procedure (Alexander et al., 1975). The term iPGE is used because the antibody cross-reacts with both

the 1 and 2 series. The antibody does not significantly cross-react with other prostaglandins (Table 1).

Radioimmunoassay of $iTXB_2$. To an aliquot of serosal media was added [^{3}H]-TXB_2 (1500 cpm) to account for recovery losses. The media were acidified to pH 3.5 with formic acid and extracted twice with 2 volumes of ethyl acetate, the ethyl acetate then being evaporated under a stream of N_2. In the experiments using cell suspensions an aliquot (0.5-1.0 ml) was taken and diluted to 2 ml with distilled water to facilitate processing, [^{3}H]-TXB_2 was added for recovery, the media were acidified and extracted once with 20 ml of ethyl acetate, and the ethyl acetate was evaporated under a stream of N_2. The dried extract was redissolved in 50 µl of methanol and 950 µl of chloroform and applied to a silicic acid column (0.5 g). TXB_2 was eluted and the eluate evaporated under a stream of N_2. The residue was dissolved in gelatin phosphate buffer (Alexander et al., 1975) and assayed by a previously described radioimmunoassay procedure (Burch et al., 1979). The antibody was kindly supplied by Dr. J.B. Smith, Cardeza Foundation, Philadelphia, PA. After incubation at 37°C for 4 hours the free [^{3}H]-TXB_2 was separated from the bound using a charcoal-dextran solution (Alexander et al., 1975) and the bound [^{3}H]-TXB_2 was counted in a liquid scintillation spectrometer (Beckman). The interassay variability was 17% (n=10). Cross-reactivities of various prostaglandins are shown in Table I.

Because U44069 and U46619 were found to cross-react with the TXB_2 antibody, the dried extracts were redissolved in methanol and subjected to silica gel thin-layer chromatography using the solvent system chloroform-methanol-acetic acid-water (90:8:1:0.8) (Moncada

TABLE I

CROSS-REACTIVITIES OF THE PGB AND TXB_2 ANTIBODIES

Prostaglandin	Picograms required for 50% displacement of (^3H)-PGB_2	% Cross-reactivity
PGB Antibody[a]		
PGB	170	100
PGE_2	1×10^3	17
$PGF_{2\alpha}$	5×10^5	0.03
6-oxo-$PGF_{1\alpha}$	3.6×10^4	0.47
PGD_2	4.4×10^4	0.38
TXB_2	$>5 \times 10^5$	<0.03
15-oxo-PGB_2	1.8×10^5	0.09
TXB_2 Antibody[b]		
TXB_2	140	100
6-oxo-$PGF_{1\alpha}$	1.8×10^6	0.008
PGD_2	4.6×10^5	0.03
$PGF_{2\alpha}$	4.0×10^5	0.04
PGE_2	9.0×10^5	0.02
PGE_1	$>2.5 \times 10^6$	<0.001

[a] taken from Webb et al. (1978)
[b] taken from Burch et al. (1979)

et al., 1977). The retardation factor (R_f) for the analogs was scraped and TXB_2 was eluted from the silica gel using 5% acetic acid in methanol (vol/vol) (Green, 1978) and the solvent was evaporated under a stream of N_2. The residue was subjected to column chromatography as described above and assayed.

[^{14}C]-Arachidonic acid metabolism in toad bladder homogenates. Bladders were homogenized in 100 mM phosphate buffer, pH 7.4 (1 g bladder wet weight to 3 ml of buffer) with a Tekmar Model SDT tissue homogenizer at medium speed for 30 sec at 0°C. [^{14}C]-Arachidonic acid (0.3 mM, 0.7 μCi) was added to each flask (3 ml) and the homogenates were incubated with shaking under an atmosphere of 95% O_2:5% CO_2 at 25°C for 30 min. The media were acidified to pH 3.5 with formic acid and extracted twice with 10 ml ethyl acetate. The ethyl acetate was evaporated under a stream of N_2. The dried extracts were redissolved in methanol and 25 μg each of PGE_2, $PGF_{2\alpha}$, and TXB_2 were added as markers. The solution was applied to a silicic acid column (0.5 g) and the prostaglandin fraction was eluted (Bills et al., 1976). This fraction was evaporated under a stream of N_2 and subjected to silica-gel thin-layer chromatography as described above. The plates were scanned with a Packard Model 7201 radiochromatogram scanner.

Gas chromatographic-mass spectrometric confirmation of TXB_2 synthesis from endogenous precursor. Media (2.2 liters) were collected from 56 hemibladders which had been incubated in bicarbonate-Ringer's solution for 30 min periods. The media were acidified to pH 3.5 with formic acid and extracted twice with 3 volumes of diethyl ether. The ether was flash-evaporated and [^{3}H]TXB_2 (10,000 cpm) was added

to the residue to serve as a marker. A blank was prepared by adding [^{3}H]TXB_2 (10,000 cpm) to another tube and subjecting it to the same procedures as the sample. The dried extract was redissolved and applied to a silicic acid column (0.5 g), 10 ml of chloroform were added to elute fatty acids, and 12 ml of chloroform-methanol (95:5) were added to elute the TXB_2 fraction. The eluate was concentrated under a stream of nitrogen and subjected to thin-layer chromatography as described above. The portion of the plate corresponding in R_f to authentic TXB_2 was scraped and eluted with methanol, and an aliquot was taken to determine radioactivity. Ethereal diazomethane was added to the methanolic extract to form the thromboxane methyl ester. After 5 min the excess reagent was evaporated under a stream of nitrogen and the residue was subjected to thin-layer chromatography as described above. The portion of the plate corresponding in R_f (0.68) to authentic thromboxane methyl ester was scraped and eluted with methanol, and an aliquot was taken to determine radioactivity. The solvent was evaporated under a stream of nitrogen and the thromboxane methyl ester methoxime tritrimethylsilyl (tri-TMS) derivative was formed (Fitzpatrick, 1977). The derivative was subjected to combined gas chromatography-mass spectrometry using a Finnigan 3200 gas chromatograph-mass spectrometer equipped with a glass column 2 m i.d. x 2 m coated with 3% OV-1 on Supelcoport 80/100, operated isothermally at 210°C, with electron energy 70 eV. Carrier gas was helium flowing at a rate of 25 ml/min. TXB_2 levels in the blank were below the limits of detectibility by this method.

Statistical procedures. Most experiments were designed such that the two hemibladders from a single toad were compared to one

another, one serving as a control and the other, an experimental hemibladder. Differences between means was assessed using Students t-test for unpaired observations (Steel and Torie, 1960). Statistical analysis of the data in Figure 11 was performed by Dr. Boyd Loadholt, Department of Biometry, Medical University of South Carolina, using an analysis of covariance computer program (Dixon, 1975).

RESULTS

METABOLISM OF ARACHIDONATE IN THE TOAD URINARY BLADDER AND ITS MODIFICATION BY VASOPRESSIN

[^{14}C]-Arachidonate metabolism in toad bladder homogenates. In the studies performed prior to these experiments, only PGE had been reported to be synthesized by the toad bladder (Zusman et al., 1977). Thus, we chose first to establish whether other prostaglandins are synthesized by this tissue. In particular, we wished to determine whether the toad bladder synthesizes TXA_2, since Puig Muset et al. (1972) had reported that the thromboxane synthetase inhibitor imidazole inhibited vasopressin- stimulated water flow. Bladders were homogenized to maximally stimulate endogenous phospholipases and to minimize acylation of the added [^{14}C]-arachidonate into the membrane phospholipids. [^{14}C]-Arachidonate was incubated with homogenates of toad urinary bladder in 100 mM phosphate buffer, pH 7.4. Radioactive products were observed by thin-layer chromatography to comigrate with authentic standards: $PGF_{2\alpha}$ (R_f=0.41), TXB_2 (R_f=0.45), and PGE_2 (R_f=0.58) (Fig. 3). Zones (0.5 cm) were scraped from the thin-layer plates and radioactivity was eluted from the silicic acid with methanol. Radioactivity was measured by liquid scintillation counting. In the control homogenates the product peaks accounted for 15% of the total radioactivity added as [^{14}C]-arachidonic acid. The peaks corresponding to $PGF_{2\alpha}$, PGE_2, and TXB_2 contained 5.3, 5.5, and 4.2% of the total added radioactivity, respectively. After treatment with 10 mM indomethacin the product peaks together contained only 3.2% of the total radioactivity, confirming that the control peaks resulted from enzymatic conversion of the [^{14}C]-arachidonic acid.

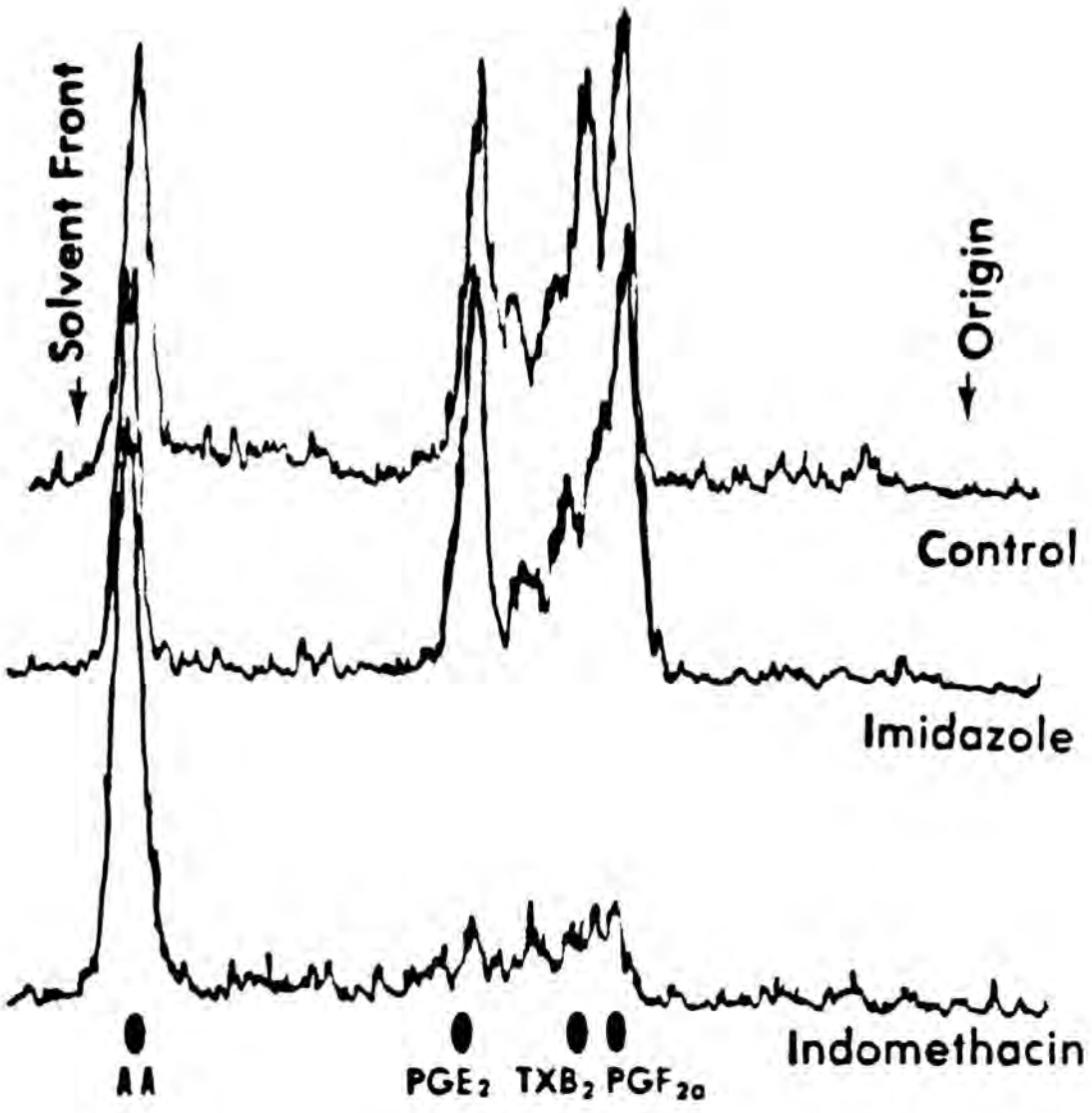


Figure 3. Products of [^{14}C]-arachidonic acid in toad urinary bladder homogenates. The upper scan is a radiochromatogram obtained from a control homogenate incubated in phosphate buffer for 30 minutes. The middle scan is of a homogenate incubated with imidazole (1 mM) for 30 minutes. The lower scan is of a homogenate incubated with indomethacin (10 mM) for 30 minutes. The spots indicate migration of authentic samples of arachidonic acid (AA), PGE_2, TXB_2, and $PGF_{2\alpha}$.

Addition of 1.0 mM imidazole reduced the amount of radioactivity contained in the TXB_2 peak by 54% compared to the control incubation. The decrease in radioactivity associated with the R_f of authentic TXB_2 in the presence of imidazole raised the possibility that the isolated toad urinary bladder possesses thromboxane synthetase. Therefore, we sought to identify unequivocally the presence of TXB_2 synthesized from endogenous arachidonic acid in the media bathing the toad urinary bladders.

Mass spectral confirmation of TXB_2 in the media bathing the hemibladders. A methyl ester methoxime tri-TMS derivative was formed from the partially purified ethyl acetate extract of the pooled media bathing the hemibladders (see Methods). A single peak corresponding in retention time (12 min) to a sample of authentic TXB_2 methyl ester methoxime tri-TMS was eluted (Fig. 4a) and its mass spectrum was identical to that obtained using authentic TXB_2 (Fig. 4b) to prepare the derivative. In addition, these mass spectra contained fragments identical to those formed from the same derivative of TXB_2 isolated from other sources (Chang et al., 1977; Smith et al., 1977).

Time-course of vasopressin-stimulated $iTXB_2$ and iPGE syntheses and water flow. In an effort to characterize the role of TXA_2 in the toad bladder we first determined the effects of vasopressin on its synthesis in the isolated toad bladder. Also measured for comparison were iPGE synthesis and osmotic water flow. Within 8 min after its addition, vasopressin caused a significant ($p < 0.02$, $n=6$) stimulation of $iTXB_2$ synthesis (Fig. 5). Maximum rate of synthesis was reached by 16 min and fell rapidly thereafter, returning to basal level by 32 min. Vasopressin-stimulated water flow reached its

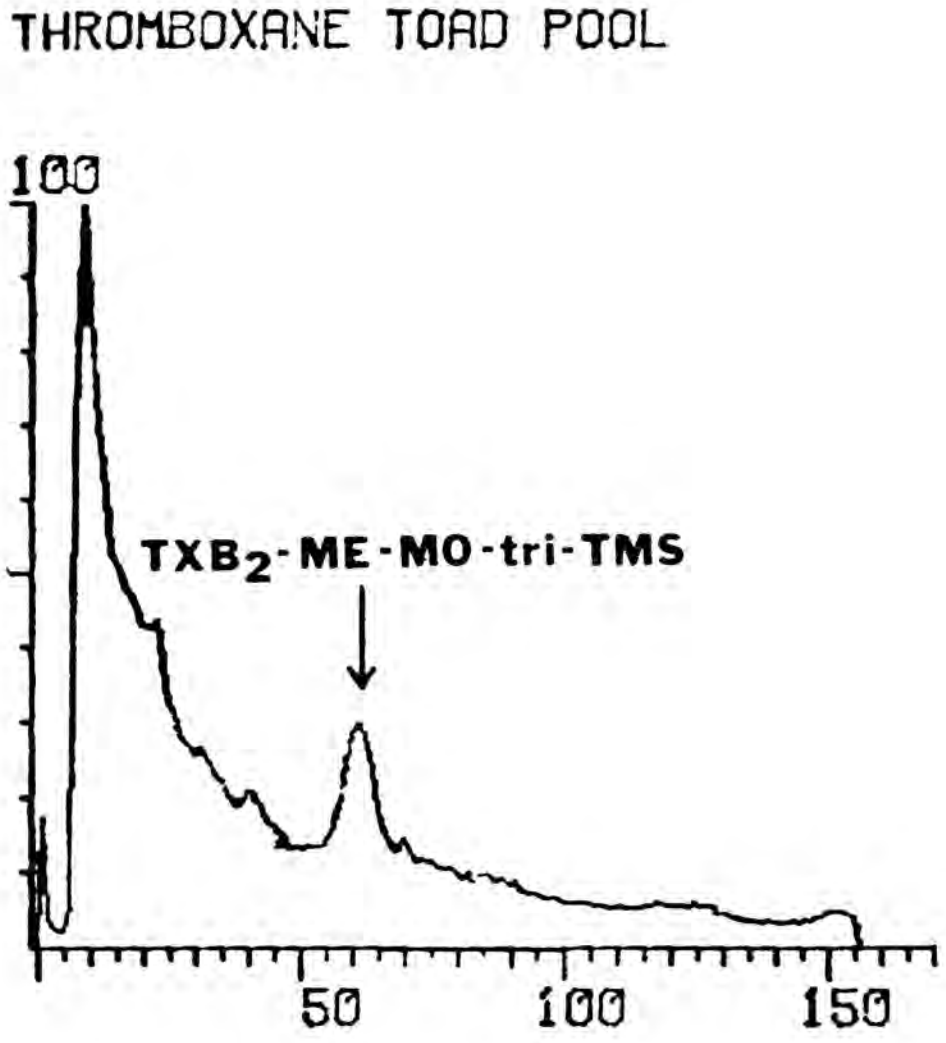


Figure 4a. Total ion current from the derivatized extract obtained from toad bladder incubation media. The mass spectrum is contained in the lower trace in Figure 4b.

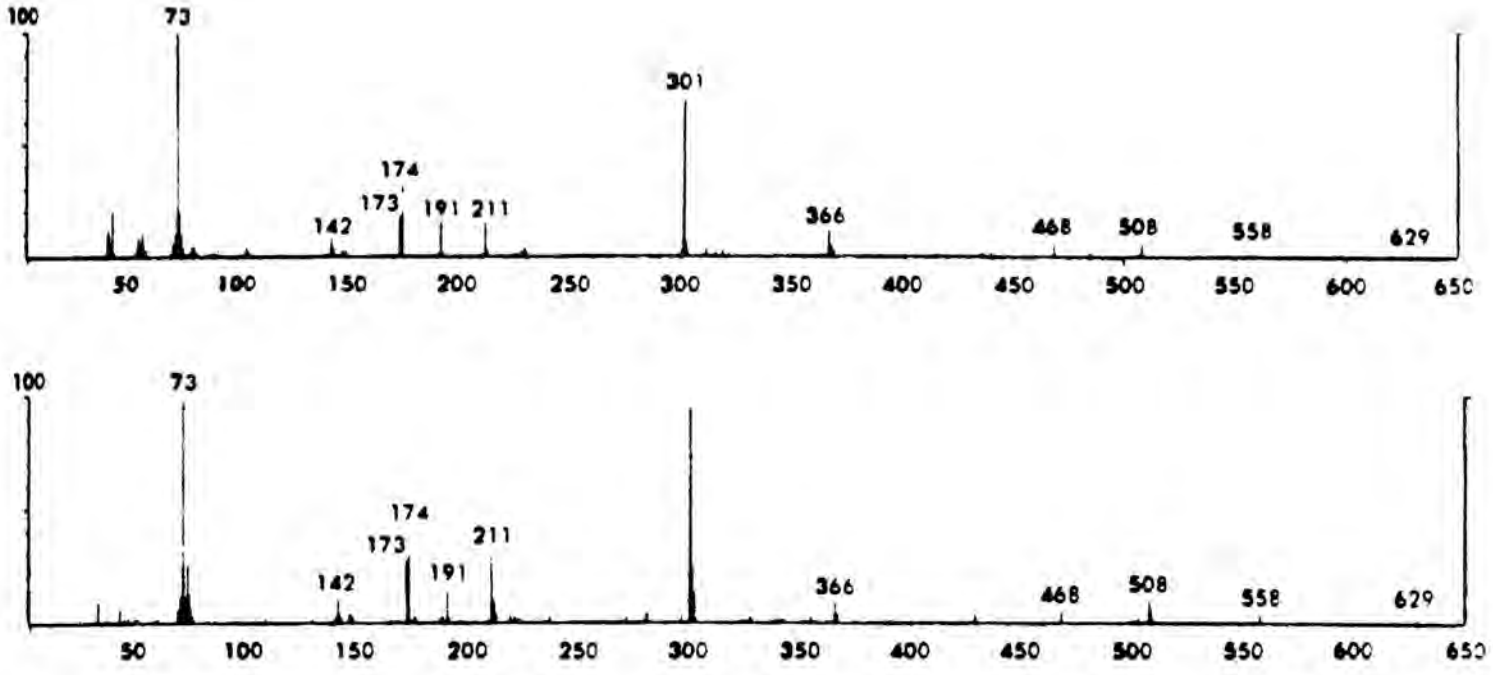


Figure 4b. Mass spectra of TXB_2 methyl ester methoxime tri-TMS derivatives. The upper scan is the mass spectrum of a derivatized sample of authentic TXB_2. The lower scan is the mass spectrum of a derivatized sample isolated from toad bladder bathing media.

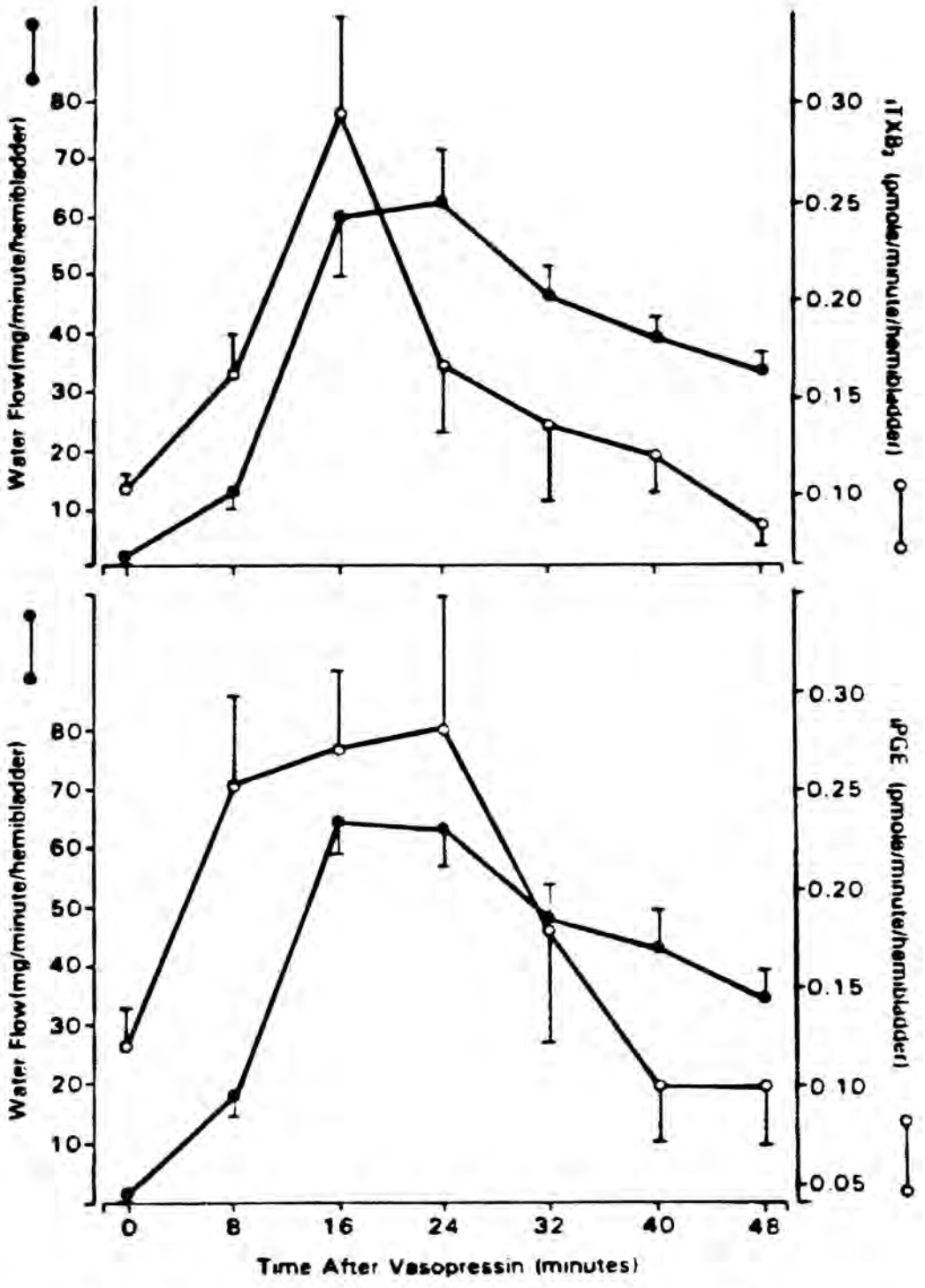


Figure 5. Time course of vasopressin stimulation of water flow and $iTXB_2$ and iPGE synthesis. Vasopressin (25 mU/ml) was added at time 0. At 8 minute intervals the serosal media were collected and replaced with fresh serosal media containing vasopressin. The upper figure shows vasopressin-stimulated $iTXB_2$ synthesis and vasopressin-stimulated water flow in the same hemibladders. Media from 2 hemibladders (not from the same toad) were pooled for assay of $iTXB_2$. Each point represents the synthetic or flow rate of 6 pairs of hemibladders. The lower figure shows vasopressin-stimulated water flow and vasopressin-stimulated iPGE synthesis in the same hemibladders. Each point represents the flow rate of synthetic rate for 5 hemibladders.

maximum during the 16-to 24-min time period. Vasopressin also significantly stimulated ($p < 0.05$, n=5) iPGE synthesis within 8 min, but the synthesis rate exhibited a prolonged plateau and decreased to basal level by 32 min (Fig. 5).

In one group of hemibladders vasopressin-stimulated water flow was compared to vasopressinstimulated $iTXB_2$ and iPGE synthesis. In these hemibladders $iTXB_2$ synthesis was positively correlated with vasopressin-stimulated water flow ($p<0.001$, n=21, $r=0.75$) (Fig. 6). There was no significant correlation between vasopressin-stimulated water flow and iPGE synthesis.

Effects of an osmotic gradient on vasopressin-stimulated $iTXB_2$ synthesis. To confirm that vasopressin's stimulation of $iTXB_2$ synthesis was not secondary to the enhancement in water flow, the dilute mucosal Ringer's solution was replaced with full-strength Ringer's solution. Basal $iTXB_2$ synthesis was identical under the 2 conditions: 0.058±0.010 pmol/min/hemibladder in the presence of the gradient and 0.055±0.006 pmol/min/hemibladder in the absence of a gradient, respectively, n=6 pairs. Vasopressin stimulated $iTXB_2$ synthesis in the 2 groups of hemibladders to the same extent, to 0.087±0.008 and 0.083± 0.008 pmol/min/ hemibladder, gradient and no gradient, respectively. In the absence of the gradient, vasopressin-stimulated water flow was almost completely inhibited, being stimulated from 5.8±1.3 to 47.9±6.7 mg/min/hemibladder in the presence of a gradient and from -2.1±0.9 to 0.3±0.8 mg/min/hemibladder in the absence of an osmotic gradient.

Effects of exogenous cAMP on $iTXB_2$ synthesis. Exogenous cAMP enhances water permeability but does not alter basal PGE synthesis

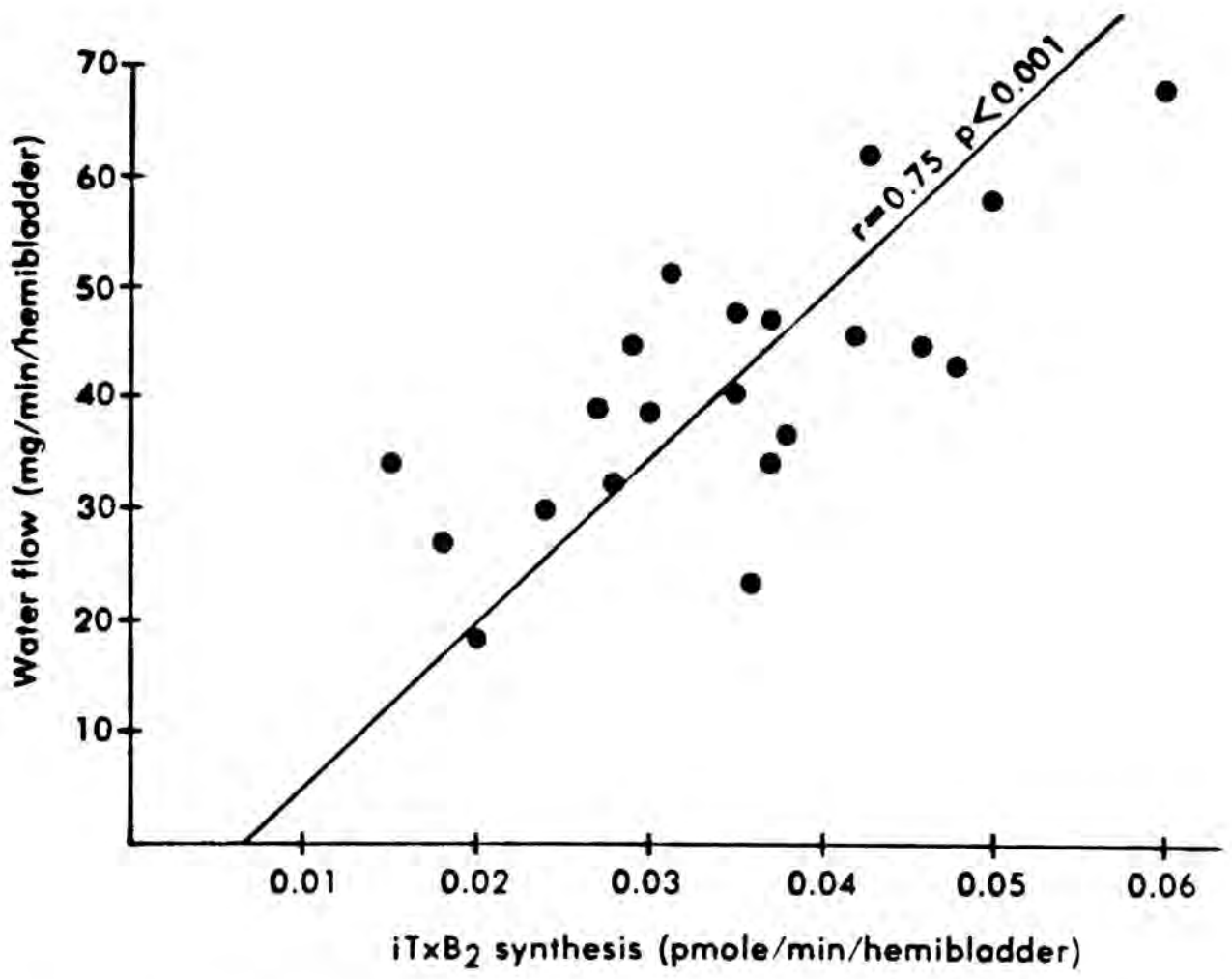


Figure 6. Correlation of vasopressin-stimulated water flow with $iTXB_2$ synthesis in the toad urinary bladder. Vasopressin (5 mU/ml) was added to the serosal media and water flow and $iTXB_2$ synthesis were measured for 30 minutes, beginning 15 minutes after vasopressin was added.

(Zusman et al., 1977). In the present study, cAMP (0.5 mM) increased water flow from 1.1±0.7 to 19.2±2.3 $mg \cdot min^{-1} \cdot hemibladder^{-1}$ ($p<0.01$, $n=5$), while $iTXB_2$ synthesis was not affected, (being 0.054±.006 pmol/min/hemibladder basally and 0.056±0.007 pmol/min/hemibladder in the presence of cAMP ($n=5$).

iPGE and $iTXB_2$ syntheses in toad bladder epithelial cells. The toad bladder consists not only of epithelial cells but also supporting stroma and smooth muscle. Therefore, we prepared suspensions of epithelial cells to document that they are responsible for the increased synthesis of iPGE and $iTXB_2$ in response to vasopressin. Epithelial cells prepared by all three methods synthesized iPGE and $iTXB_2$ (Table II). In the cells prepared by the collagenase and EDTA methods and incubated in bicarbonate-Ringer's buffer, iPGE syntheses were similar, 0.27±0.05 and 0.25±0.03 pmol/min/mg protein, respectively. However, iPGE synthesis was nearly 10-fold higher ($p<0.001$) in the cells prepared by the scraping method (2.02±0.53 pmol/min/mg protein) compared to the above methods. $iTXB_2$ synthesis was only about one-tenth (0.032± 0.004 pmol/min/mg protein) ($p<0.001$) of iPGE synthesis in cells prepared by the collagenase method and one-half (1.11±0.39 pmol/min/mg protein) ($p<0.05$) of iPGE synthesis in the scraped cells. In contrast, in cells prepared using the EDTA method, $iTXB_2$ synthesis was about two-fold greater (0.59±0.07 pmol/min/mg protein ($p<0.01$) than iPGE synthesis.

Incubation of the cells with indomethacin (50 μM) or meclofenamate (10 μM) significantly inhibited iPGE and $iTXB_2$ synthesis (Table II). iPGE synthesis was inhibited 78±14% ($p<0.02$), 91±18% ($p<0.001$), and 83±6% ($p<0.01$) and $iTXB_2$ synthesis was inhibited 53±8% ($p<0.02$),

TABLE II

EFFECTS OF VASOPRESSIN AND dDAVP ON iPGE AND $iTXB_2$ SYNTHESIS IN ISOLATED TOAD BLADDER EPITHELIAL CELLS

	Basal	Vasopressin	Basal	dDAVP	Meclofenamate or Indomethacin
			(pmol/min/mg protein)		
			Collagenase Method		
iPGE	0.27 (6) ±0.05	0.53[c] (6) ±0.10	0.27 (6) ±0.05	0.43[b] (6) ±0.09	0.059[b] (3) ±0.010
$iTXB_2$	0.032 (6) ±0.004	0.054[b] (6) ±0.009	0.034 (6) ±0.004	0.054[a] (6) ±0.01	0.015[b] (3) ±0.002
			Scraping Method		
iPGE	2.02 (6) ±0.53	2.22 (6) ±0.45	-	-	0.18[d] (3) ±0.030
$iTXB_2$	1.11 (6) ±0.39	0.83 (6) ±0.23	-	-	0.053[d] (3) ±0.002
			EDTA Method		
iPGE	0.25 (12) ±0.03	0.26 (12) ±0.02	-	-	0.043[c] (3) ±0.003
$iTXB_2$	0.59[e] (12) ±0.07	0.62[e] (12) ±0.08	-	-	0.047[c] (3) ±0.004

[a] $p < 0.05$, [b] $p < 0.02$, [c] $p < 0.01$, [d] $p < 0.001$, compared to basal; [e] $p < 0.01$, compared to iPGE; number of observations in each group is given by the number in parentheses.

The medium for the suspensions prepared by the collagenase and EDTA methods was bicarbonate-Ringer's containing 1 mM calcium and 1 mM magnesium; the medium for the cells prepared by the scraping method was phosphate-Ringer's solution containing 0.5 mM calcium. Incubation time was 15 min.

For isolation procedures, see Methods.

Concentration of AVP was 5 mU/ml; dDAVP, 130 nM; meclofenamate, 10 μM; and indomethacin, 50 μM.

95±10% ($p<0.001$, and 92±10% ($p<0.01$) in cells prepared by the collagenase, scraping, and EDTA methods, respectively.

Effects of vasopressin and dDAVP on iPGE and $iTXB_2$ synthesis in isolated epithelial cells. In a previous report (Bisordi et al, 1980) vasopressin (5 mU/ml) and dDAVP (130 nM) stimulated hydroosmotic water flow to the same extent in isolated toad bladders. Similarly, in our laboratory vasopressin (5 mU/ml) stimulated water flow from 1.2±0.5 to 29.8±2.9 mg/min/hemibladder (n=4), while dDAVP (130 nM) stimulated water flow from 1.4±0.4 to 30.4±2.6 mg/min/hemibladder (n=4). In cells prepared via the collagenase method, vasopressin (5 mU/ml) stimulated iPGE synthesis from 0.27±0.05 to 0.53±0.1 pmol/min/mg protein ($p<0.01$, n=6) and $iTXB_2$ synthesis from 0.032±0.004 to 0.054±0.009 pmol/min/mg protein ($p<0.02$, n=6) (Table II). dDAVP (130 nM) stimulated iPGE synthesis from 0.27±0.05 to 0.43±0.09 pmol/min/mg protein ($p<0.02$, n=6) and $iTXB_2$ synthesis 0.034±0.004 to 0.054 ±0.011 pmol/min/mg protein ($p<0.05$, n=6). dDAVP stimulated iPGE synthesis significantly ($p<0.05$) less than vasopressin but both stimulated $iTXB_2$ to the same extent. In cells prepared by the scraping or EDTA methods, vasopressin failed to stimulate iPGE or $iTXB_2$ synthesis (Table II).

Effects of extracellular calcium on iPGE and $iTXB_2$ synthesis in isolated epithelial cells. In cells prepared via the collagenase method, in the absence of exogenously added calcium in the medium iPGE synthesis was approximately 35% greater than $iTXB_2$ synthesis. Increasing the calcium concentration to 0.1 mM caused an increase in iPGE synthesis (Fig. 7), while raising the calcium concentration to 0.5 or 1.0 mM was without further effect. At the same time that

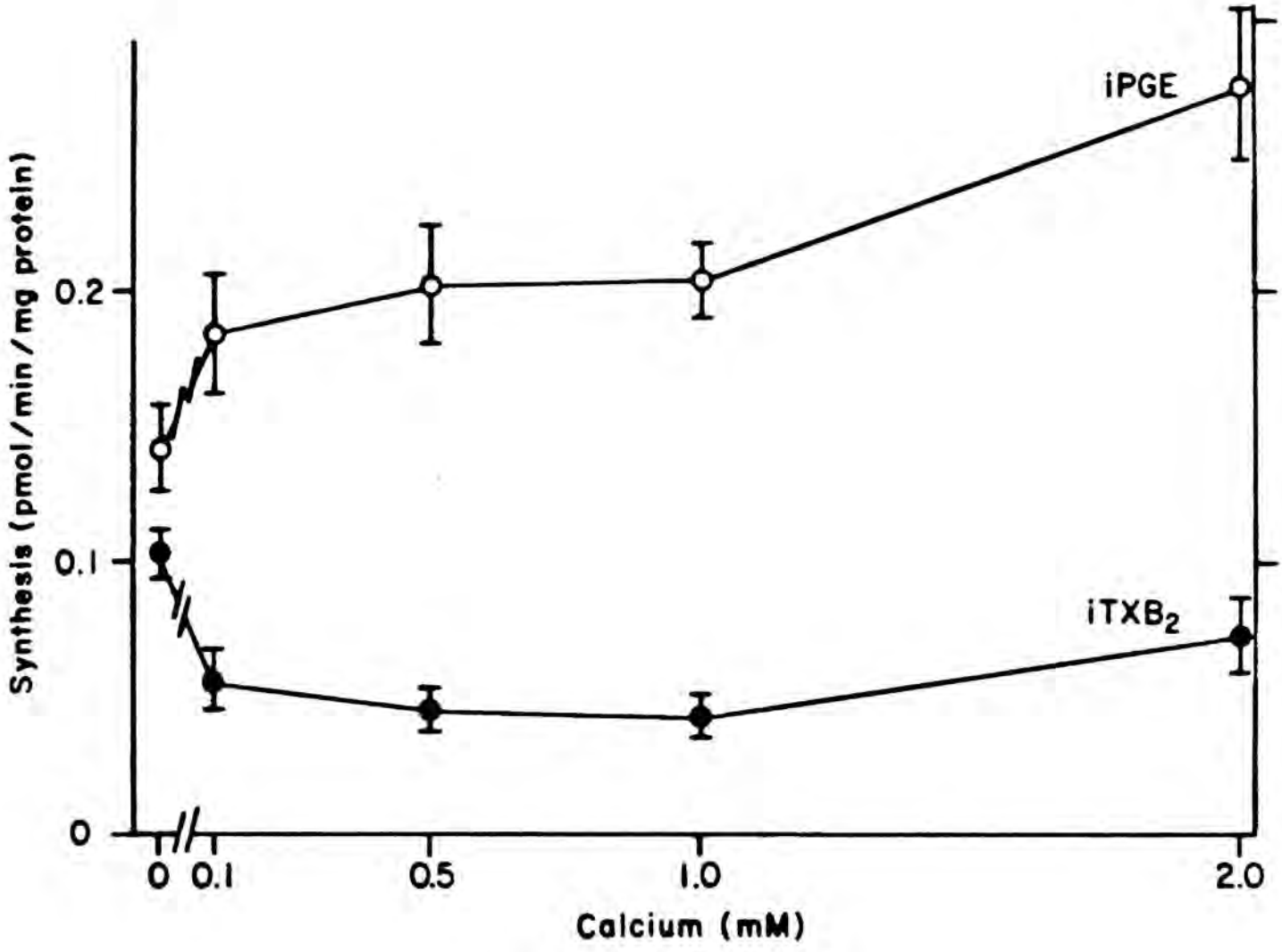


Figure 7. Effects of extracellular calcium on $iTXB_2$ and iPGE synthesis in toad bladder epithelial cells isolated by the collagenase method. See Methods for details of cell preparation and experimental protocol. Both iPGE and $iTXB_2$ were quantitated in the bathing media of the same suspensions by radioimmunoassay procedures. Each value represents the mean $\pm$ SEM for 6 suspensions. Synthesis of iPGE was the same in the presence of 0.1, 0.5, and 1.0 mM added calcium, but these were significantly different from synthesis in the absence of exogenously added calcium ($p < 0.05$) and in the presence of 2.0 mM added calcium ($p < 0.05$). Synthesis of $iTXB_2$ was the same in the presence of 0.1, 0.5, and 1.0 mM added calcium, but these were significantly different from synthesis in the absence of exogenously added calcium ($p < 0.02$) and in the presence of 2.0 mM added calcium ($p < 0.05$).

iPGE synthesis increased, $iTXB_2$ synthesis decreased by the same amount, such that the sum of arachidonate metabolism to iPGE plus $iTXB_2$ was the same at all calcium concentrations from 0 to 1.0 mM: 0.25 pmol/ min/mg protein at no added calcium, 0.24 pmol/min/mg protein at 0.1 mM calcium, 0.25 pmol/ min/mg protein at 0.5 mM calcium, and 0.25 pmol/min/mg protein at 1 mM calcium. However, at 2 mM calcium, the synthesis of both metabolites was significantly increased ($p<0.05$) such that net conversion of arachidonate to iPGE plus $iTXB_2$ increased to 0.35 pmol/min/mg protein.

In the cells prepared by the EDTA method, a qualitatively different effect of calcium was observed. iPGE and $iTXB_2$ syntheses were not significantly different in phosphate-Ringer's solution (Fig. 8a) or bicarbonate-Ringer's solution (Fig. 8b), in the absence of exogenously added calcium. Increasing the calcium concentration to 0.1 mM increased the syntheses of both iPGE and $iTXB_2$. The increase in $iTXB_2$ was greater than the increase in iPGE in both buffers and the increase in $iTXB_2$ was greater in the phosphate-Ringer's than the bicarbonate-Ringer's. Upon raising the concentration of calcium to 0.5, 1.0, or 2.0 mM the synthesis rates of both iPGE and $iTXB_2$ fell compared to the rates in the presence of 0.1 mM calcium. At 0.5 and 1.0 mM calcium, $iTXB_2$ synthesis remained greater than iPGE synthesis, but at 2.0 mM calcium the synthetic rates had returned to the level seen in the absence of exogenously added calcium and iPGE and $iTXB_2$ syntheses were again identical.

In the above experiments, the cells incubated in phosphate-Ringer's solution (5 mM phosphate) had higher maximum $iTXB_2$ synthesis rates than did the cells incubated in bicarbonate-Ringer's solution (0.5

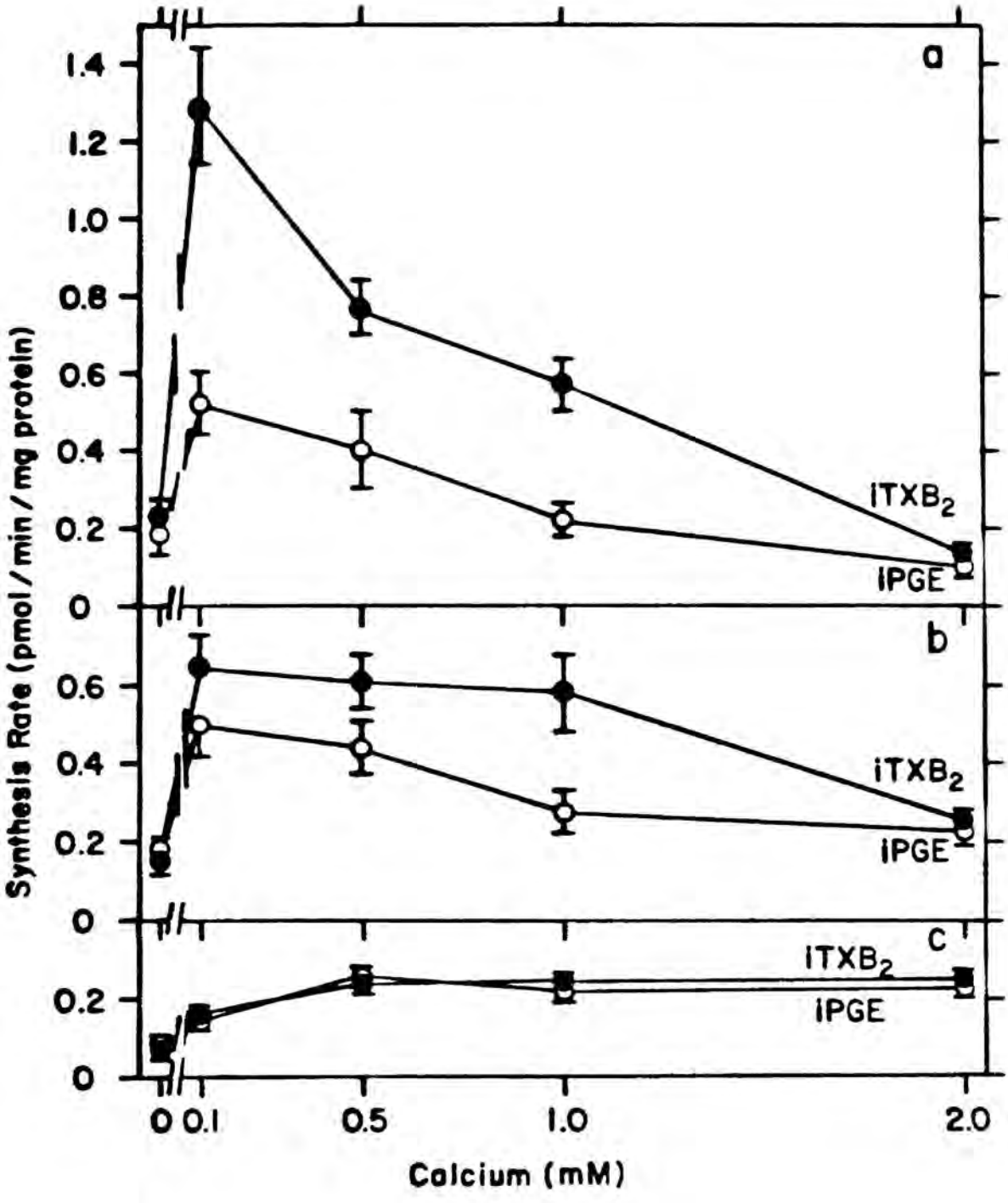

a
b
c
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
iTXB2
iPGE
Synthesis Rate (pmol / min / mg protein)
0 0.1 0.5 1.0 2.0
Calcium (mM)

Figure 8. Effects of extracellular calcium on $iTXB_2$ and iPGE synthesis in toad bladder epithelial cells isolated by the EDTA method. See Methods for details of cell preparation and experimental protocol. Both iPGE and $iTXB_2$ were quantitated in the bathing media of the same suspensions by radioimmunoassay procedures. Each value represents mean $\pm$ SEM for 6 suspensions. (a) Cells were incubated in phosphate-Ringer's solution. Synthesis of $iTXB_2$ was elevated above that in the absence of exogenously added calcium at 0.1 mM calcium ($p < 0.01$), 0.5 mM calcium ($p < 0.02$) and 1.0 mM calcium ($p < 0.05$), but was not different from that found at 2.0 mM calcium. Synthesis of iPGE was elevated above that in the absence of exogenously added calcium at 0.1 and 0.5 mM added calcium ($p < 0.05$), but was not at 1.0 mM or 2.0 mM calcium. At 0.1 mM, 0.5 mM, and 1.0 mM added calcium, $iTXB_2$ synthesis was greater than iPGE synthesis ($p < 0.02$). (b) Cells were incubated in bicarbonate-Ringer's solution. Synthesis of $iTXB_2$ was elevated above that in the absence of exogenously added calcium at 0.1 mM, 0.5 mM, and 1.0 mM added calcium ($p < 0.05$), but was not different at 2.0 mM added calcium. iPGE synthesis was elevated above that in the absence of exogenously added calcium at 0.1 mM and 0.5 mM added calcium ($p < 0.05$) but was not different at 1.0 mM or 2.0 mM added calcium. At 0.1 mM, 0.5 mM, and 1.0 mM calcium, $iTXB_2$ synthesis was greater than iPGE synthesis ($p < 0.05$). (c) Cells were incubated in tris-Ringer's solution. iPGE and $iTXB_2$ syntheses were not different from one another at any concentration of added calcium. Syntheses of iPGE and $iTXB_2$ were greater at 0.1 mM added calcium than in the absence of exogenously added calcium ($p < 0.05$) and the syntheses of iPGE and $iTXB_2$ were greater at 0.5 mM, 1.0 mM, and 2.0 mM calcium than at 0.1 mM calcium ($p < 0.05$).

mM phosphate). Extracellular phosphate concentration promotes calcium uptake into cells (Borle, 1972). Therefore, cells were incubated in tris-Ringer's solution to minimize the influx of calcium into the cells. In these cells iPGE and $iTXB_2$ syntheses were only about one-half those seen in the bicarbonate-Ringer's and phosphate-Ringer's solutions in the absence of exogenously added calcium (Fig. 8c). In tris-Ringer's solution raising the calcium concentration from 0 to 2 mM increased both iPGE and $iTXB_2$ synthesis by identical amounts and the absolute amounts synthesized were less than those found in the other 2 buffer media containing phosphate.

EFFECTS OF THROMBOXANE SYNTHETASE INHIBITORS ON VASOPRESSIN-STIMULATED WATER FLOW AND ARACHIDONIC ACID METABOLISM

Effects of imidazole. Imidazole at low concentrations is a selective inhibitor of thromboxane synthetase (Moncada et al., 1977; Needleman et al., 1977). In addition, imidazole has been shown to inhibit vasopressin-stimulated water flow (Puig Muset et al., 1972). In the presence of 1 mM imidazole, basal $iTXB_2$ synthesis was not different from control (Table III). In control hemibladders vasopressin (5 mU/ml) increased $iTXB_2$ synthesis 136.3±18.4% ($p<0.001$, n=12). In imidazole-pretreated hemibladders $iTXB_2$ synthesis was stimulated by vasopressin 72.8±11.3% ($p<0.001$, n=12), that is, 44±10% less ($p<0.001$, n=12 pairs) than the stimulation of control rate. In the control hemibladders vasopressin-stimulated water flow was 47.7± 5.0 mg/min/hemibladder, compared to 37.0±3.5 mg/min/hemibladder in the imidazole-pretreated hemibladders, an inhibition of 23.2±4.4% ($p < 0.02$, n=12 pairs). Since imidazole has been reported to cause shunting of endoperoxide intermediates from TXA_2 to other prostaglandins (Nijkamp et al., 1977), iPGE synthesis was measured in these hemibladders. Basal iPGE synthesis was not affected by imidazole (1 mM) (Table III). However, vasopressin-stimulated iPGE synthesis was 0.30±0.04 pmol/min/ hemibladder in control hemibladders compared to 0.41±0.04 pmol/min/hemibladder in the imidazole pretreated hemibladders ($p < 0.001$, n=11 pairs). Thus, the reduction in vasopressin-stimulated water flow observed in this experiment may have been caused, not by an inhibition of $iTXB_2$ synthesis, but by enhanced iPGE synthesis.

In further experiments imidazole was found to stimulate vasopressin-stimulated iPGE synthesis in a dose-dependent manner at

TABLE III

WATER FLOW, $iTXB_2$ AND iPGE SYNTHESIS IN THE PRESENCE OF IMIDAZOLE AND VASOPRESSIN

Imidazole	$iTXB_2$		iPGE		Water Flow	
	Control	Imidazole	Control	Imidazole	Control	Imidazole
mM	pmol/min/hemibladder		pmol/min/hemibladder		mg/min/hemibladder	
			Basal			
1	0.034±0.005	0.026±0.004(12)	0.16±0.02	0.17±0.02(11)	0.4±0.3	0.8±0.3(12)
50	0.28±0.02	0.20±0.01[b](6)	0.22±0.01	0.23±0.02(6)	1.3±0.3	2.8±0.3[b](6)
			Vasopressin-stimulated			
1	0.080±0.023	0.045±0.013[c](12)	0.30±0.04	0.41±0.04[c](11)	47.7±5.0	37.0±2.5[b](12)
50	0.87±0.08	0.14±0.02[c](6)	3.6±0.3	0.42±0.30[c](7)	33.4±6.4	47.0±4.4[a](6)

[a] $P < .05$, [b] $P < .02$, [c] $P < .001$, compared to control.

Water flow was measured gravimetrically, $iTXB_2$ and iPGE by radioimmunoassay. All values are mean ± S.E.M.; n given in parentheses. The experiments carried out in the presence of 1 mM imidazole were performed in January, those in the presence of 50 mM imidazole, in May. Endogenous steroid levels are considered to be high in the winter, resulting in high vasopressin-stimulated water flow (Handler et al., 1969) and these observations may account for absolute differences in water flow and PGE synthesis among controls in this study.

concentrations from 0.01 to 1.0 mM (Fig. 9) Imidazole at high concentrations has been reported to inhibit cyclooxygenase as well as thromboxane synthetase. In toad bladders imidazole (50 mM) inhibited vasopressin-stimulated $iTXB_2$ and iPGE syntheses (Table III) while stimulating vasopressin-stimulated water flow (Table III). Thus, imidazole, at a high concentration (50 mM) acted in the same manner as other cyclooxygenase inhibitors (Zusman et al., 1977) to enhance the response to vasopressin, presumably by inhibiting PGE synthesis.

Effects of 7-(1-imidazolyl)-heptanoic acid (7IHA). 7IHA has been reported to be a specific thromboxane synthetase inhibitor which has no effects on cyclooxygenase activity (Yoshimoto et al., 1978). 7IHA was synthesized by Mr. Tom Eller in the laboratory of Dr. Daniel R. Knapp as previously described (Burch et al., 1980). 7IHA caused a dose-dependent inhibition of vasopressin-stimulated water flow, reaching a maximum inhibition of 30±10% ($p < 0.05$) at a concentration of 50 μM (Fig. 10). 7IHA also inhibited $iTXB_2$ synthesis in a dose-dependent manner (Fig. 10). Vasopressin-stimulated iPGE synthesis was not affected by 7IHA, even at concentrations as high as 500 μM (Fig. 10).

To determine whether 7IHA inhibited vasopressin-stimulated water flow by a mechanism not related to TXA_2 synthesis inhibition, the effect of 7IHA on vasopressin-stimulated water flow was assessed in the presence of fatty acid cyclooxygenase inhibitors. Hemibladders were pretreated with indomethacin (50 μM) or meclofenamate (1 μM) for 1 hour prior to water flow measurements. Vasopressin was added at a low concentration (0.2 mU/ml) since cyclooxygenase inhibitors increase the sensitivity of the toad bladder to vasopressin (Zusman

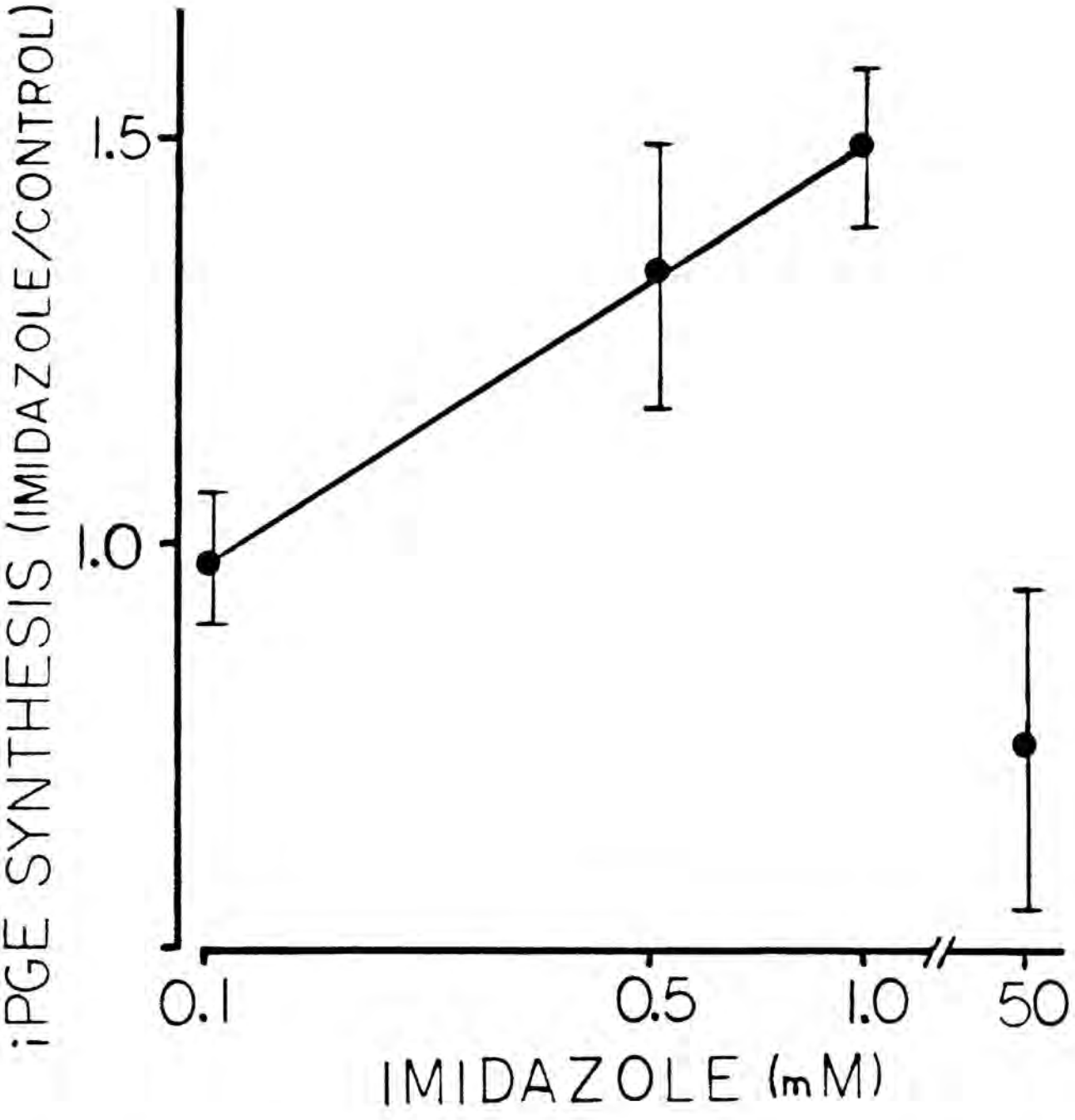


Figure 9. Effect of imidazole on vasopressin-stimulated iPGE synthesis. Hemibladders were incubated in flasks in the presence of imidazole for 30 minutes. Paired hemibladders were used as controls. Vasopressin (5 mU/ml) was added and synthesis was measured for 30 minutes.

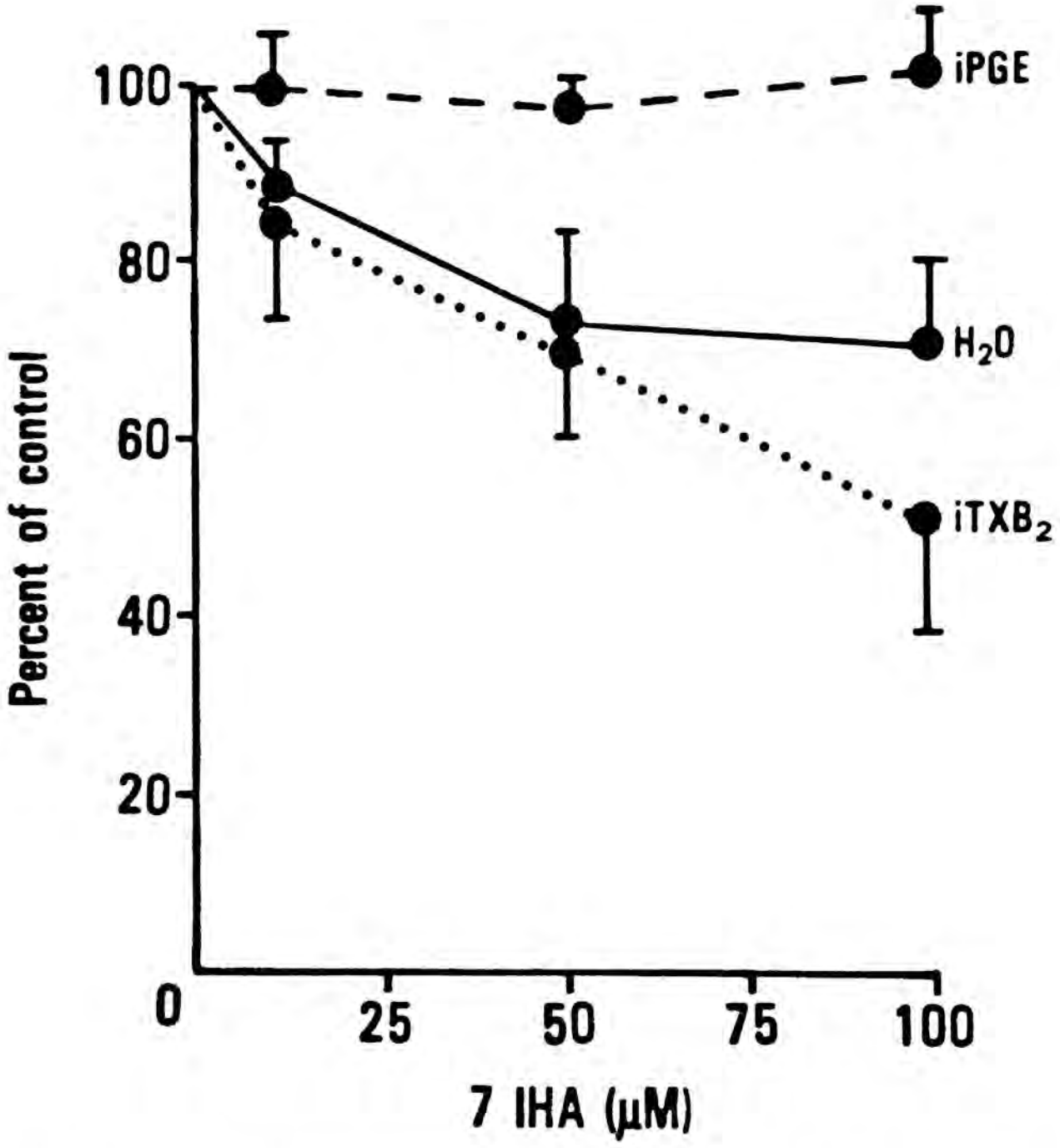


Figure 10. Dose-response relationships for the inhibition of vasopressin-stimulated water flow, and $iTXB_2$ and iPGE syntheses by 7-(1-imidazolyl)-heptanoic acid. Inhibition of water flow and $iTXB_2$ synthesis became significant ($p < 0.05$) at 50 μM 7IHA. No significant inhibition of iPGE synthesis occurred.

et al., 1978). 7IHA (100 μM) had no effect on basal or vasopressin-stimulated water flow in the presence of indomethacin or meclofenamate (Table IV). The magnitude of the water flow response to vasopressin in the presence of meclofenamate was less ($p < 0.02$) than the response in the presence of indomethacin, a phenomenon often attributed to phosphodiesterase inhibition by indomethacin (Flores and Sharp, 1972). In addition, the effects of 7IHA in the presence of meclofenamate on a high concentration of vasopressin was assessed. Again, 7IHA had no effect (Table IV). Exogenous cAMP stimulates water flow across the toad urinary bladder but does not affect $iTXB_2$ synthesis. Pre-treatment of hemibladders with 7IHA (100 μM) for 1 hour did not significantly alter the water flow response to cAMP compared to control hemibladders (Table IV).

EFFECTS OF TXA_2 ANTAGONISTS ON VASORPESSIN-STIMULATED WATER FLOW

trans-13-Azaprostanoic acid. LeBreton et al. (1979) described trans-13-azaprostanoic acid (t13APA) as an antagonist of either TXA_2 or PGH_2 in the platelet, which does not alter arachidonic acid metabolism. Two series of experiments were performed with t13APA. In the first, 50 μM and 100 μM concentrations of t13APA were found to inhibit vasopressin (5 mU/ml)-stimulated water flow in a dose-dependent manner, reaching a maximum inhibition of 33±7% ($p < 0.05$, n=6 pairs) (Fig. 11). In the second experiment, concentrations of t13APA of 100 μM and 300 μM also inhibited vasopressin-stimulated water flow in a dose-dependent manner, reaching a maximum inhibition of 30±6% ($p < 0.05$, n=9 pairs). Two different shipments of toads were used in these experiments, consequently, the absolute magnitudes of responses in the 2 experiments were different. However, in each experiment

TABLE IV

EFFECTS OF 7-(1-IMIDAZOLYL)-HEPTANOIC ACID ON PROSTAGLANDIN-INDEPENDENT WATER FLOW

	Water Flow (mg/min/hemibladder)			
	Basal		Vasopressin, 0.1 mU/ml	
	Control	7IHA	Control	7IHA
Meclofenamate	2.7±0.7	3.4±0.6	8.6±1.5	10.1±1.7(5)
Indomethacin	1.5±0.5	1.2±0.3	37.1±6.4	34.6±6.2(10)
			Vasopressin, 5 mU/ml	
Meclofenamate	1.8±0.3	1.4±0.4	65.9±10.4	63.1±8.6(8)
			cAMP, 0.5 mM	
	1.1±0.7	1.3±0.6	19.2±2.3	18.6±4.3(5)

Values are mean ± S.E.M. Number of hemibladders in each group is given in parentheses. Concentration of 7IHA was 100 μM, meclofenamate, 1 μM, and indomethacin, 50 μM. Preincubation was for 1 hour. The experiments were performed in bicarbonate-Ringer's solution.

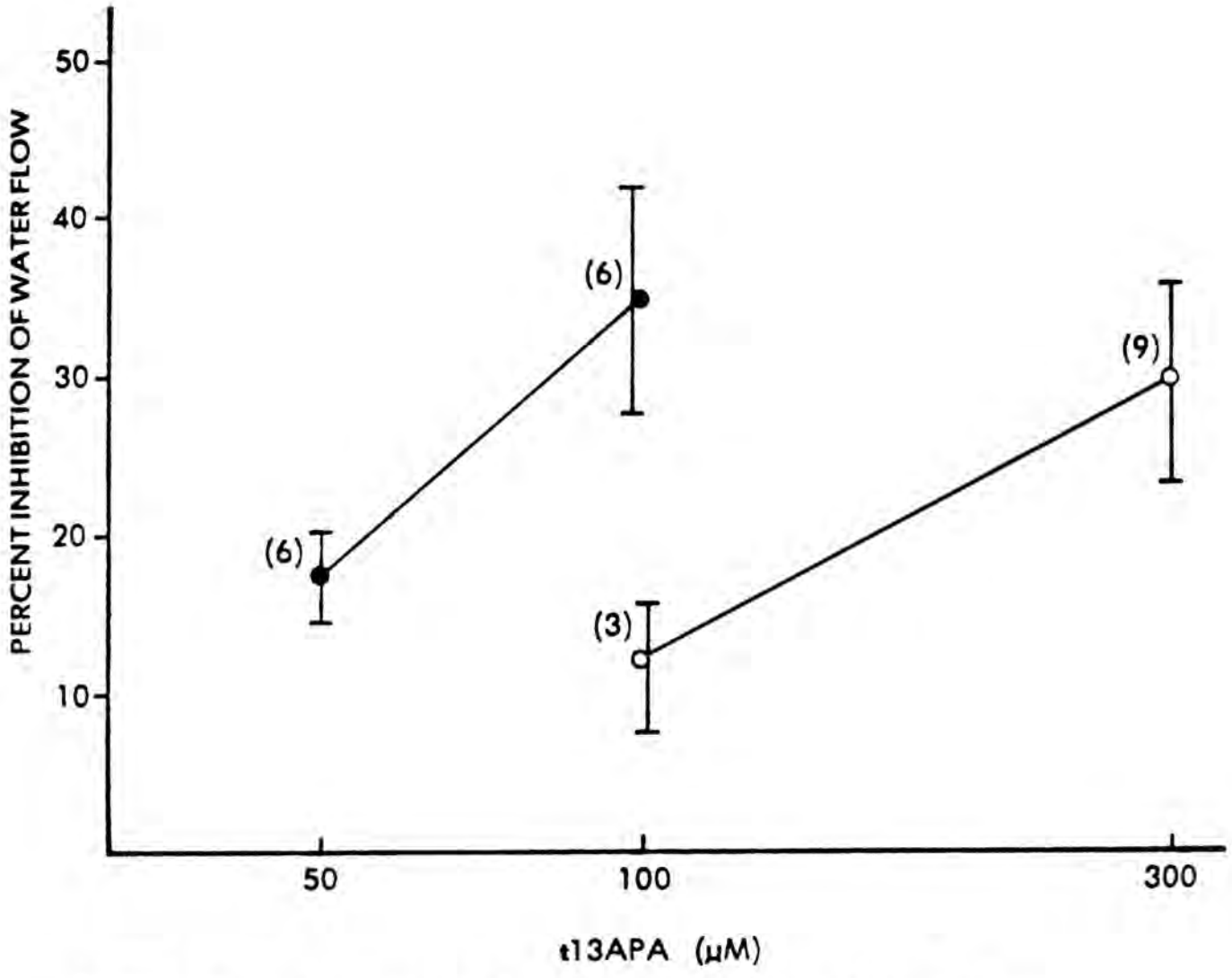


Figure 11. Dose-response relation for inhibition of vasopressin-stimulated water flow by *trans*-13-aza-prostanoic acid. Slopes obtained for the two series of experiments were not significantly different ($p > 0.05$). The two series were pooled, yielding a common line of inhibition of vasopressin-stimulated water flow $y = 45.7x - 68.9$. Inhibition was significantly ($p < 0.05$) greater for each higher concentration of t13APA.

vasopressin-stimulated water flow was significantly inhibited compared to control, and in each experiment a dose-response relationship was evident.

In an effort to determine whether t13APA depends on TXA_2 synthesis to inhibit vasopressin-stimulated water flow several experiments were performed to assess the effects of t13APA (300 μM) on vasopressin-stimulated water flow in the presence of indomethacin or meclofenamate. Like 7IHA, t13APA did not affect vasopressin-stimulated water flow in the absence of TXB_2 or PGE synthesis (Table V). Similarly, t13APA (300 μM) did not affect cAMP-stimulated water flow (Table V).

cis-13-azaprostanoic acid. In the platelet the cis isomer of 13-azaprostanoic acid is inactive as an antagonist of TXA_2 (LeBreton et al., 1979). In the toad bladder, at a concentration of 300 μM, c13APA had no effect on water flow stimulated by 1.0 mU/ml vasopressin. In the control hemibladders water flow was stimulated from 1.4±0.4 to 42.5 mg/ min/hemibladder and in the hemibladders pretreated with c13APA (300 μM) water flow was stimulated from 1.5±0.5 to 40.3±3.2 mg/min/hemibladders (n=6 pairs) (difference between stimulated water flows not significant).

EFFECTS OF TXA_2 MIMETICS ON WATER FLOW

Since TXA_2 synthesis inhibitors and TXA_2 antagonists inhibited vasopressin-stimulated water flow it became of interest to assess the effects of TXA_2 itself on vasopressin's actions. However, the extreme lability of TXA_2 precluded direct testing of its effects on water flow. Thus, stable compounds which mimic TXA_2's actions were sought. The stable prostaglandin endoperoxide analogs (15Z)-hydroxy-9α,11α-(epoxymethano)prosta-5Z, 13E-dienoic acid (U44069) and its

TABLE V

EFFECTS OF trans-13-AZAPROSTANOIC ACID ON PROSTAGLANDIN-INDEPENDENT WATER FLOW

Agent	Water Flow (mg/min/hemibladder)			
	Basal		Vasopressin, 0.2 mU/ml	
	Control	t13APA	Control	t13APA
Meclofenamate	1.8±0.8	2.0±1.1	12.2±2.2	10.8±3.1(10)
Indomethacin	2.9±1.0	2.6±0.6	28.6±4.2	26.1±3.4(10)
			cAMP, 0.5 mM	
	2.2±1.1	2.3±1.8	11.0±3.4	9.5±3.2(5)

Values are mean ± S.E.M. Number of hemibladders in each group is given in parentheses. Concentration of t13APA was 300 μM, meclofenamate, 1 μM, and indomethacin, 50 μM. Preincubation was for 1 hour. The experiments were performed in bicarbonate-Ringer's solution.

11α,9α-(epoxymethano) analog, U46619, possess biological activity that mimic the actions of TXA_2 and/or the prostaglandin endoperoxides in platelets and aortic strips (Malmsten, 1976). TXB_2, the stable metabolite of TXA_2, has also been shown to exhibit actions similar to TXA_2 in the cardiovascular system (Friedman et al., 1979).

Effects of U44069 and U46619 on $iTXB_2$ synthesis. U44069, in addition to acting as a prostaglandin endoperoxide/TXA_2 agonist, has been reported to be a thromboxane synthetase inhibitor (Diczfalusy and Hammarström, 1977). At a concentration of 1 μM, U44069 had no effect on basal $iTXB_2$ synthesis (Table VI). However, U44069 significantly inhibited vasopressin (5 mU/ml)-stimulated $iTXB_2$ synthesis 46.8±6.2% ($p<0.01$, n=6) (Table VI). U46619 (1 μM) had no effect on either basal or vasopressin-stimulated $iTXB_2$ synthesis (Table VI).

Effects of TXB_2, U44069, and U46619 on basal water flow. In hemibladders not pretreated with indomethacin or meclofenamate, U46619 (1 μM) caused a significant increase in basal water flow, from 2.8±0.5 to 5.7± 0.9 mg/min/10cm^2 ($p<0.05$, n=5) when measured over a 30 min period, whereas U44069 tended to increase water flow (3.0±0.6 to 4.4±1.2 mg/min·10cm^2, $0.10 < p > 0.05$, n=5). To reduce the contribution of variable amounts of endogenous TXA_2 to the water flow response, hemibladders in the remaining experiments were pretreated with indomethacin (50 μM) or meclofenamate (10 μM) for 1 hr before the addition of the agonists. Both U44069 and U46619 stimulated basal water flow in indomethacin-pretreated hemibladders (Fig. 12). U44069 (1 μM) stimulated basal water flow from 4.2±0.8 to 12.4±1.2 mg/min/10cm^2 ($p< 0.05$, n=5), or from 4.2±0.8 to 9.1±1.1 mg/min/150 mg hemibladder wet weight ($p<0.05$, n=5) (Methods). U46619 was more effective than

TABLE VI

EFFECTS OF U44069 AND U46619 ON BASAL AND VASOPRESSIN-STIMULATED $iTXB_2$ SYNTHESIS

	Basal	Vasopressin
U44069, 1 μM		
	pmol/min/hemibladder	
Control	0.075±0.014	0.121±0.020[a]
U44069	0.066±0.012	0.088±0.023[b]
U46619, 1 μM		
	pmol/min/hemibladder	
Control	0.066±0.011	0.094±0.009[a]
U46619	0.064±0.010	0.104±0.011[a]

[a] $P < 0.05$, compared with basal rate.
[b] $P < 0.01$, compared with vasopressin-stimulated controls.

Vasopressin concentration was 5 mU/ml. Hemibladders were preincubated with U44069 or U46619 for 30 min, n=6 pairs, each group.

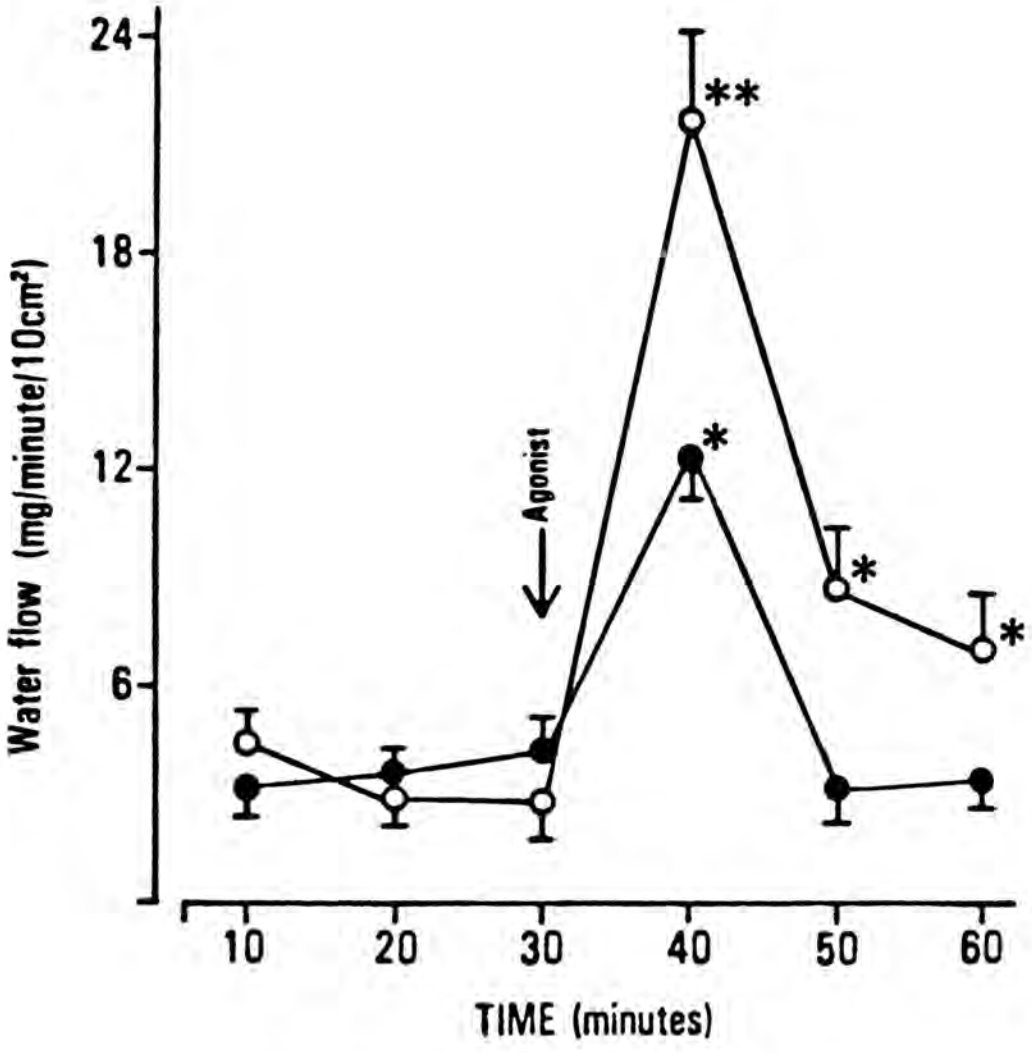


Figure 12. Stimulation of water flow in response to the endoperoxide/ TXA_2 agonists U44069 and U46619. Hemibladders were pretreated with indomethacin (50 μM) for one hour. Basal water flow was measured for 30 minutes, then U44069 (filled circles) or U46619 (open circles) (1 μM) was added to the serosal bathing media and water flow was measured for 30 minutes. Each point represents the mean ± SEM for 5 hemibladders. *$p < 0.05$, **$p < 0.01$, compared with basal.

U44069, increasing water flow from 2.8±1.0 to 21.8±2.2 mg/min/10cm^2 ($p<0.01$, n=5), or from 2.6± 1.0 to 17.1±1.9 mg/min/150 mg ($p<0.01$, n=5). A lower concentration U46619 (0.1 μM) stimulated basal water flow from 2.7± 1.1 to 13.4±1.0 mg/min/10cm^2 ($p<0.02$, n=5), or from 2.5±1.0 to 10.1±0.9 mg/min/150 mg ($p<0.05$, n=5).

TXB_2 stimulated basal water flow in a dose-dependent manner (Fig. 13). The highest concentration of TXB_2 used, 50 μM, stimulated basal water flow from 2.1±0.3 to 15.9±0.8 mg/min/10cm^2 ($p<0.02$, n=4), or from 2.1±0.3 to 14.3±0.7 mg/min/150 mg ($p<0.05$, n=4). To determine that the stimulation of basal water flow was not due to some nonspecific effect of prostanoic acid derivatives, the effect of PGE_1 was assessed on basal water flow. PGE_1 (2.5 μM) caused no change in basal water flow (2.1±0.8 to 2.3±0.6 mg/min/10cm^2, n=5), confirming previous observations (Lipson and Sharp, 1971).

Effects of trans and cis-13-azaprostanoic acid on TXB_2- and U46619- stimulated water flow. As described above, in the toad bladder, t13APA, a TXA_2 antagonist (LeBreton et al., 1979), had no effect on basal water flow, but inhibited vasopressin-stimulated water flow. Further, t13APA had no effect on vasopressin-stimulated water fow in the presence of inhibitors of fatty acid cyclooxygenase. Thus, the effects of t13APA on water flow stimulated by TXB_2 and U46619 in the presence of indomethacin (50 μM) were assessed. t13APA (300 μM) inhibited TXB_2 (25 μM)- stimulated water flow 79±9% ($p<0.02$, n=5). t13APA also antagonized the effects of U46619 in a dose-dependent fashion (Fig. 14). At a concentration of 300 μM, t13APA completely inhibited the water flow response to 0.1 μM U46619 (Fig. 14). Since indomethacin inhibits cyclic nucleotide phosphodiesterase in addition

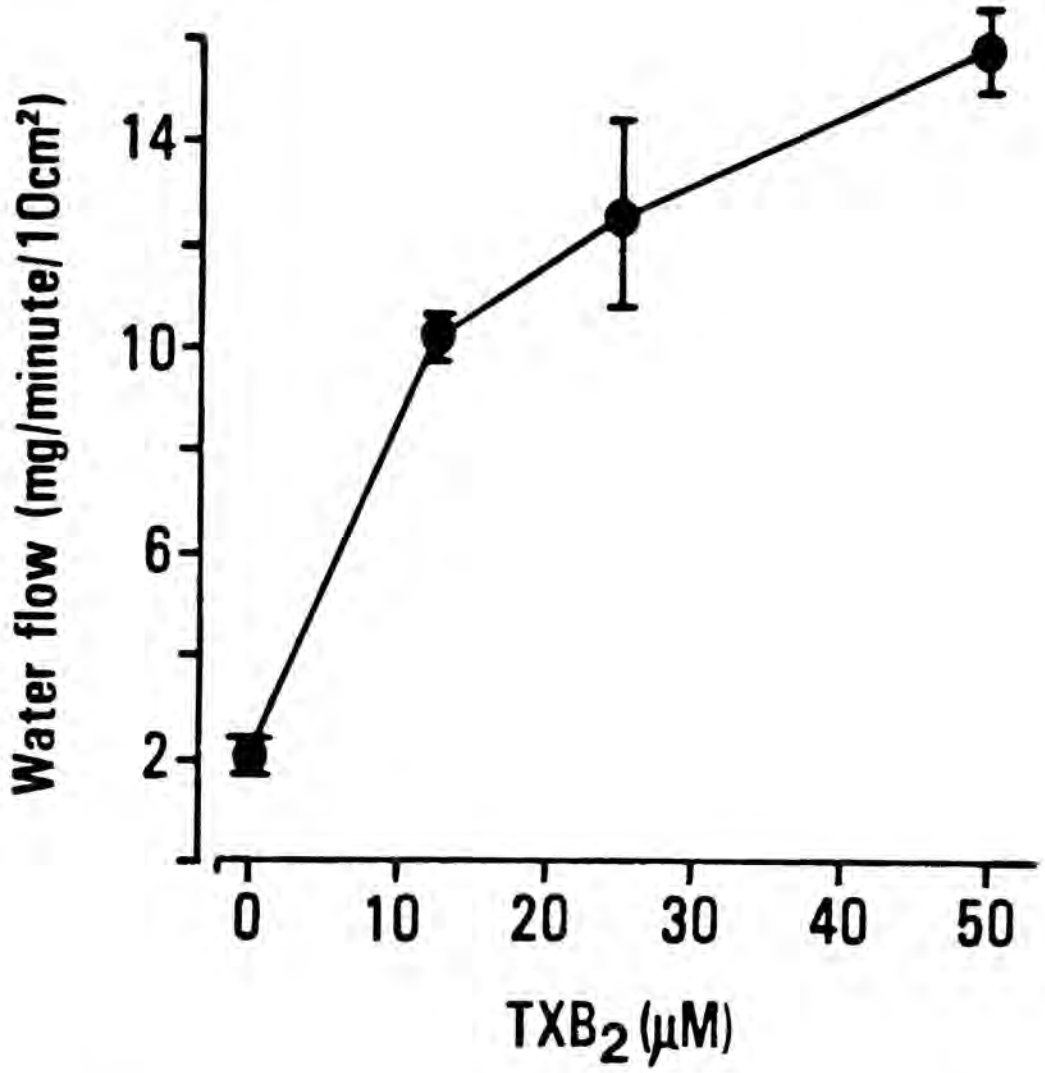


Figure 13. Dose-response relation for stimulation of osmotic water flow by TXB_2. Hemibladders were pretreated with indomethacin (50 μM) for 1 hour. Basal water flow was measured for 30 min (n=10). TXB_2 was added to the serosal bathing media and water flow was measured for three consecutive 10 min periods. Each point represents the mean ± SEM for 3 or 4 heimibladders during the first 10 min period. Each point is significant at $p < 0.05$ or $p < 0.02$ compared with basal.

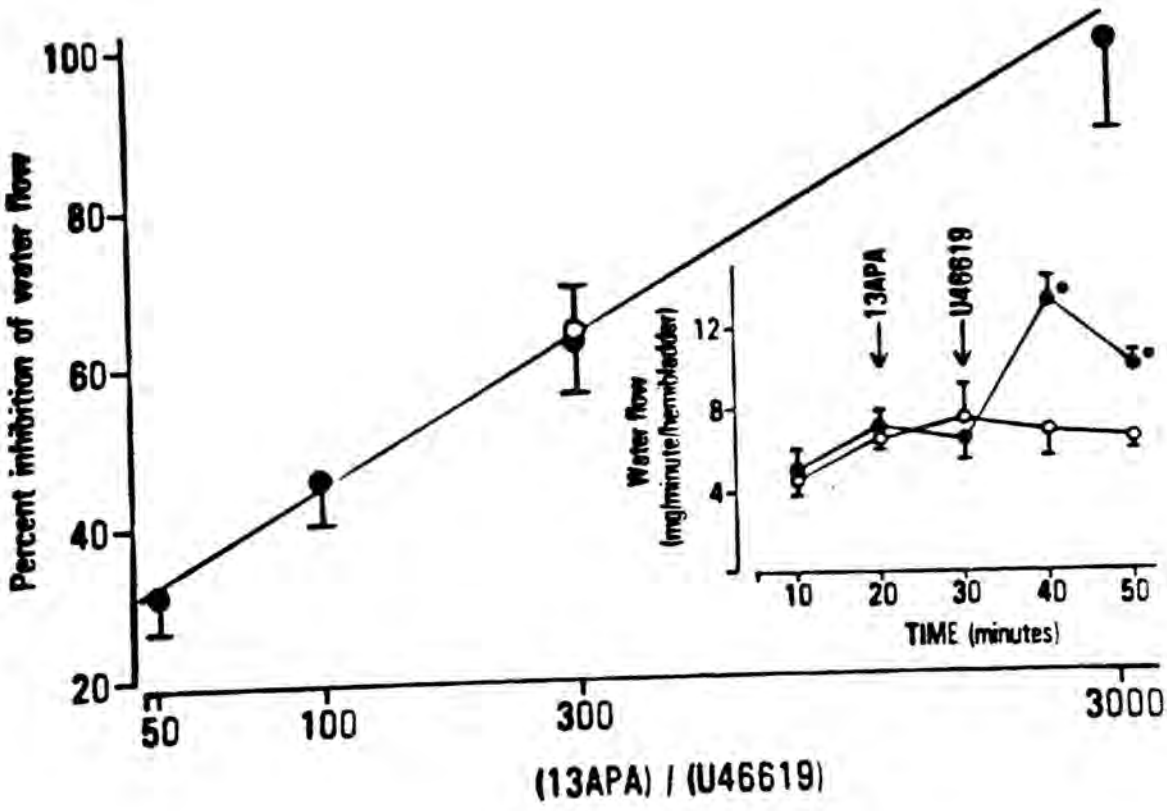


Figure 14. Inhibition of U46619-stimulated water flow by trans-13-azaprostanoic acid. The abscissa represents the molar concentration ratio of trans-13-azaprostanoic acid (13APA) to U46619. The filled circles represent hemibladders pretreated with indomethacin (50 μM) and the open circle represents hemibladders pretreated with meclofenamic acid (1 μM) for 1 hour. The hemibladders were then preincubated for 10 minutes with trans-13-azaprostanoic acid prior to the addition of U46619. Each point represents the mean ± SEM for 5 hemibladders. Inset: Inhibition of U46619 (0.1 μM)-stimulated water flow by trans-13-azaprostanoic acid (300 μM). 13APA was added to the experimental hemibladders (open circles) and U46619 (closed circles) was added to the control hemibladders. *$p < 0.05$, $n = 5$ pairs.

to cyclooxygenase (Flores and Sharp, 1972), the effect of t13APA on U46619-stimulated water flow was assessed in the presence of another cyclooxygenase inhibitor, meclofenamate, which does not inhibit phosphodiesterase (Alexander et al., 1975). At a molar concentration ratio of 300:1 t13APA inhibited U46619-stimulated water flow 65±6% ($p<0.02$, n=5) in meclofenamate- (1 μM) pretreated hemibladders while U46619-stimulated water flow was inhibited 64±6% in indomethacin (50 μM)-pretreated hemibladders (Fig. 14). The absolute levels of TXB_2- and U46619-stimulated water flow were significantly ($p<0.05$) greater in the indomethacin-pretreated hemibladders (22.3±1.8 mg/min/10 cm^2 in the presence of U46619) than in the meclofenamate-pretreated hemibladders (12.6± 1.2 mg/min/10 cm^2 in the presence of U46619). Since this concentration of indomethacin has been previously shown to inhibit cyclic nucleotide phosphodiesterase in the toad bladder (Flores and Sharp, 1972), the increased water flow in the presence of indomethacin compared with meclofenamate suggests that these agents could be activating adenylate cyclase.

The cis isomer of 13-azaprostanoic acid is inactive as an antagonist of TXA_2 or the endoperoxide/TXA_2-like agent U46619 (LeBreton et al., 1979). Thus, we tested the effects of c13APA on U46619-stimulated water flow. At a concentration of 300 μM, c13APA had no effect on water flow stimulated by 0.1 μM U46619. In control hemibladders U46619 stimulated water flow from 2.4±0.5 to 14.8±1.3 mg/min/10cm^2, and in the c13APA-pretreated hemibladders, water flow was stimulated from 1.7±0.6 to 13.6±3.2 mg/min/10cm^2.

Effect of phosphodiesterase inhibition on U46619-stimulated water flow. The observation that U46619 elicited a greater water flow

response in the presence of indomethacin than meclofenamate suggested that U46619 may mimic vasopressin by enhancing the intracellular accumulation of cAMP since indomethacin has been shown in the toad bladder to be a phosphodiesterase inhibitor (Flores and Sharp, 1972). In the mammalian uterus U44069 has been shown to enhance cAMP accumulation (Vesin et al., 1979). To test this hypothesis hemibladders were pretreated with meclofenamate, which has been shown to not inhibit phosphodiesterase. Three separate experiments were then performed. In the first, one hemibladder of each pair was treated with theophylline (0.5 mM), a concentration which only minimally affected water flow itself (Table VII). Next, to both hemibladders of each pair was added U46619 (1 μM). By itself, U46619 stimulated water flow from 1.8±0.4 to 12.2±0.8 mg/min/10 cm^2 (Δ= 10.2±0.5) in the first 10 min. In the hemibladders which had been pretreated with theophylline, U46619 stimulated water flow from 4.0±0.6 to 19.4±1.2 mg/min/ 10 cm^2 (Δ=15.6±0.8). The Δ in the presence of theophylline was statistically different from U46619 alone ($p<0.01$, n=6 pairs). Since the possibility remained that the greater response to U46619 in the presence of theophylline was due to an effect of theophylline added to an effect of U46619, additional experiments were performed. In one series of experiments theophylline was added to one hemibladder of each pair and U46619 to the other. Theophylline elicited a slight water flow response which was maintained (Table VII) while U46619 elicited its usual transitory response. After 10 min the Δ for water flow across the hemibladders pretreated with U46619 was 11.2±0.9 mg/min/ 10 cm^2 while the Δ for water flow was 2.0±0.9 mg/min/10 cm^2 in the theophylline-pretreated hemibladders. In another series of

TABLE VII

EFFECTS OF PHOSPHODIESTERASE INHIBITION ON U46619-STIMULATED WATER FLOW

Treatment	n	WATER FLOW (mg/min/10 cm^2) Basal[a]	Basal ± Theophylline[b]	Theophylline ±U46, 10 min[c]	Theophylline ±U46 20 min[d]
U46619	(6)	1.5±0.2	1.8±0.4	12.2±0.8[g]	3.2±0.5[e]
U46619 + Theophylline	(6)	1.3±0.3	4.0±0.6[f]	19.4±1.2[g,l]	7.8±1.2[g,m]
Theophylline (0.5 mM)	(9)	1.9±0.4	4.1±0.9[e,f]	4.0±0.9[3]	3.4±0.6[e]
U46619 (1 μM)	(9)	2.1±0.3	2.4±0.4	13.1±0.9[g,h]	3.6±0.4[e]
Theophylline	(9)	1.6±0.3	4.1±1.0[e]	4.1±1.2[e]	3.7±0.9[e]
U46619 + Theophylline	(9)	1.7±0.4	4.3±1.0	21.9±3.0[i,j]	8.3±1.6[g,k]

[a] Basal water flow is the average of two 10 min periods, [b] theophylline (T) (0.5 mM) was added to the appropriate hemibladders and water flow measured for 10 min. [c] U46619 (1 μM) was added to the appropriate hemibladders and water flow was measured for 10 min, [d] a second 10 min period of incubation with U46619, [e] $p < 0.05$ compared to Basal, [f] $p < 0.05$ compared to U46619, [g] $p < 0.01$ compared to basal, [h] $p < 0.02$ compared to theophylline, [i] $p < 0.001$, compared to theophylline, [j] $p < 0.02$ compared to U46619, above (unpaired analysis), [k] $p < 0.02$ compared to theophylline, [l] $p < 0.02$ compared to U46619, [m] $p < 0.05$ compared to U46619.

All hemibladders were pretreated with meclofenamate (10 μM) for 1 hour prior to the beginning of the basal periods. Paired hemibladders were used.

experiments run concurrently with the last, theophylline was added to both hemibladders of each pair. Then U46619 was added to one hemibladder of each pair. Again, theophylline elicited a slight, sustained increase in water flow (Table VII) while U46619 elicited a transitory increase. After 10 min the Δ for water flow response to U46619 plus theophylline was 17.8±3.0 mg/min/10 cm^2 while the Δ for the response to theophylline alone was 2.4±0.2 mg/ min/10 cm^2. By unpaired t analysis, the Δ for U46619 plus theophylline (17.8±3.0) was significantly different ($p<0.02$, $n=18$) from the Δ for U46619 alone (11.2±0.9) while Δ for theophylline alone was the same in the 2 experiments (2.0±0.9 versus 2.4±0.2) using this protocol. Thus, U46619 appeared to have a greater effect in the presence of theophylline, at least when the response is considered as the absolute increment in water flow.

Effects of U46619 on vasopressin-stimulated water flow. Because TXA_2 may be a positive modulator or partial mediator of vasopressin-stimulated water flow, the effects of the endoperoxide/TXA_2 agonist U46619 were assessed on vasopressin-stimulated water flow in indomethacin-pretreated hemibladders. Vasopressin and U46619 (1 μM) were added simultaneously to the experimental hemibladders. Water flow was determined for 30 min. Vasopressin stimulated water flow in a dose-dependent manner from 0.1 to 2.0 mU/ml vasopressin (Fig. 15). U46619 (1 μM) significantly increased water flow in this concentration range of vasopressin, but, by paired-t analysis the effect was not significant at the 2.0 mU/ml concentration. The dose-response curve was significantly shifted to the left ($p<0.001$) in a parallel fashion (slopes of 0.64 and 0.62, control and U46619, respectively) suggesting

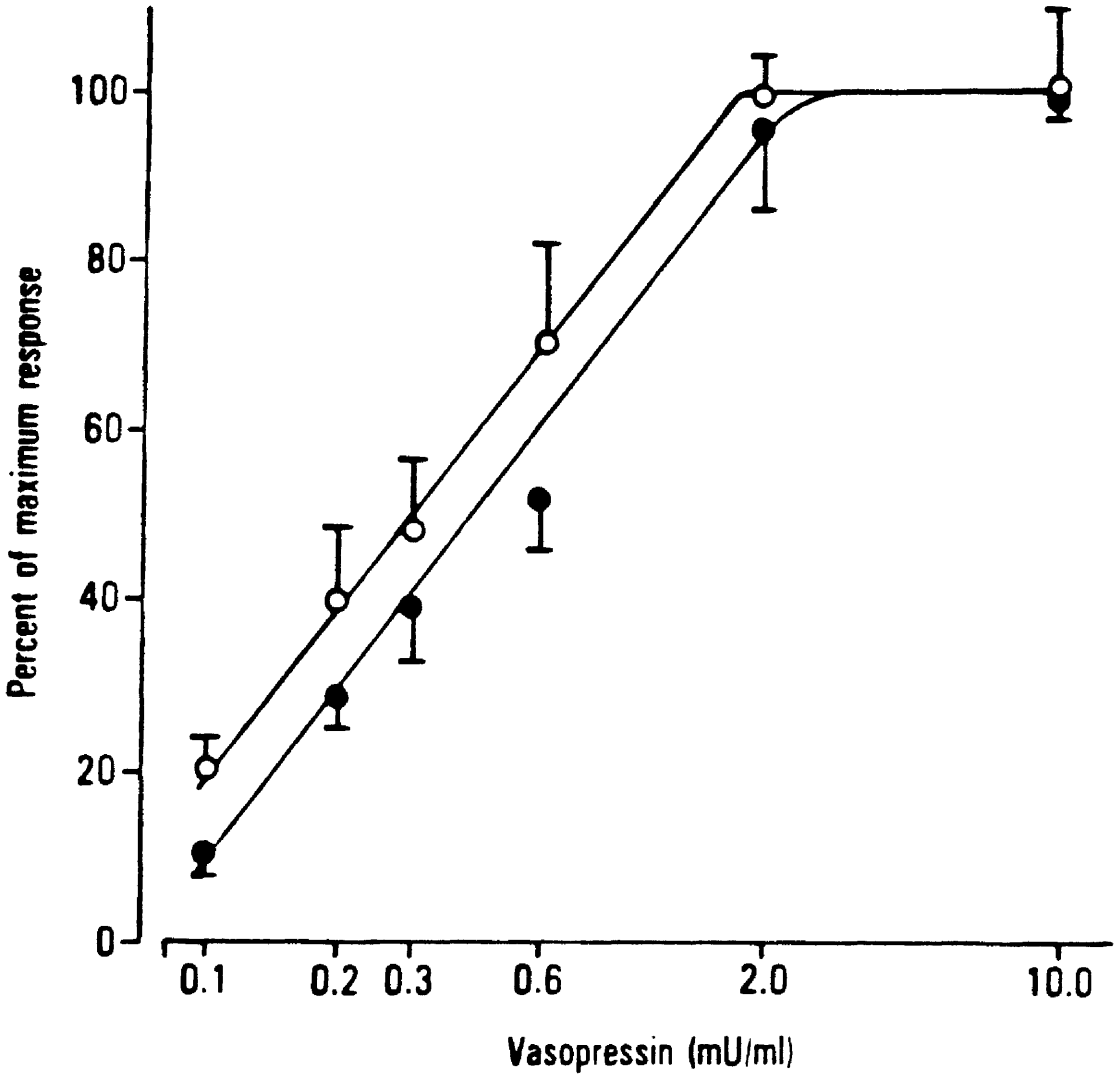


Figure 15. Effects of U46619 on vasopressin-stimulated water flow. Hemibladders were pretreated with indomethacin (50 μM) for 1 hour. Vasopressin (closed circles) or vasopressin plus U46619 (1 μM) (open circles) was added to the serosal baths and water flow was measured for 30 minutes. After thorough washing to return water flow to basal level and a 30 minute recovery period, a supramaximal dose of vasopressin (10 mU/ml) or vasopressin plus U46619 was added and water flow was measured for another 30 minutes. Water flow is expressed as a percentage of this maximal response (64.3±4.6 mg/minute per 10 cm^2, n=72). Each point represents the mean±SEM for 6 hemibladders. U46619 significantly ($p<0.001$) increased vasopressin-timulated water flow and significantly shifted the dose-response curve to the left ($p<0.001$) in a parallel fashion (slopes were 0.64 for the control hemibladders and 0.62 for the U46619-treated hemibladders).

that vasopressin and U46619 are acting on the same sequence of events to increase water flow.

DISCUSSION

In these studies incubation of toad urinary bladder homogenates with [^{14}C]-arachidonic acid resulted in the synthesis of at least 2 radioactive products corresponding to [^{14}C]-PGE and [^{14}C]-TXB_2. The synthesis of PGE by the isolated toad urinary bladder has been described previously (Zusman et al., 1977). In this study, the presence of TXB_2 synthesized from endogenous arachidonic acid was verified by gas chromatography-mass spectrometry and quantitated using a specific radioimmunoassay.

In a previous study of [^{3}H]-arachidonic acid metabolism by the toad urinary bladder, Zusman et al. (1977) demonstrated an increased release of [^{3}H]-arachidonic acid induced by vasopressin and increased [^{3}H]-PGE synthesis. However, they did not report any synthesis of [^{3}H]-TXB_2. However, the synthesis of TXB_2 by the isolated toad urinary bladder and epithelial cells derived from it was recently confirmed by another laboratory (Bisordi et al., 1980). This study confirms that vasopressin stimulates iPGE synthesis and demonstrates that iTXB_2 synthesis is also stimulated by vasopressin. Thus, vasopressin, by stimulating an acyl hydrolase, resulting in the increased release of arachidonate from membrane-ester storage sites, increased the synthesis of not only PGE_2, but also TXA_2. In a very recent study Kirschenbaum et al. (1981) reported that rabbit cortical collecting tubules synthesize PGE_2 and TXA_2, and that syntheses of both compounds were rapidly stimulated by vasopressin.

Exogenous cAMP stimulated water flow but did not affect TXA_2 synthesis. Thus, these studies suggest that vasopressin stimulates TXA_2 synthesis separate from cAMP synthesis, similar to its effect

on PGE_2 synthesis (Zusman et al., 1977). TXA_2 synthesis was stimulated by vasopressin whether or not an osmotic gradient existed across the bladders. Thus, stimulation of TXA_2 synthesis appeared to be a direct effect of vasopressin and not secondary to enhanced water movement.

Vasopressin-stimulated iPGE synthesis reached a maximum during the first 8-min period after the addition of vasopressin (20 mU/ml) and remained unchanged until after water flow began to decline toward basal rate. This observation is qualitatively similar to the observations of Zusman et al. (1977) who also reported that vasopressin (1 mU/ml) stimulated iPGE synthesis within the first 10 min and remained unchanged over a period of 40 min. The vasopressin-stimulated synthetic rate reported by Zusman et al. (1977) was 10 times higher than basal, whereas that in the present study was only 2 times higher. Although an explanation for the differences is not known, geographical source, housing, and season, 3 variables that may influence synthetic rate, must be considered. However, the observations made in the 2 studies are consistent with PGE playing a part in a negative feedback loop where vasopressin stimulates PGE synthesis which in turn negatively modulates vasopressin's action.

Vasopressin stimulation of $iTXB_2$ synthesis and water flow occurred in parallel, but $iTXB_2$ synthesis peaked prior to maximum water flow. This is consistent with a positive modulator role for TXA_2 in vasopressin-stimulated water flow in which stimulation of TXA_2 synthesis leads to enhanced water flow. Since the bladders responded to TXB_2 with a water flow response, TXB_2 may not be excluded as the active metabolite. However, in many systems it is found that

TXA_2 is much more active than TXB_2 (Friedman et al., 1977). In the present studies high concentrations of TXB_2 were required to elicit the water flow response.

Epithelial cells isolated from toad urinary bladders via several methods synthesize iPGE and $iTXB_2$ and the rates of synthesis of these substances vary with respect to the method of isolation and the extracellular calcium concentration. The collagenase method for isolating epithelial cells from toad bladders was described by Gatzy and Berndt (1968) who compared it to an EDTA method. They found that cells isolated using collagenase retained vasopressin responsiveness as judged by enhanced oxygen consumption and that the cells appeared to be anatomically intact as judged by electron microscopy. Modifications of this method have been widely used to prepare toad bladder epithelial cells which respond to vasopressin (Wiesmann et al., 1977; MacKnight et al., 1971) and other agents (Arruda and Sabatini, 1980a,b). Using this isolation technique, we found that vasopressin as well as its non-pressor-antidiuretic analog, dDAVP (Zaoral et al., 1967; Vavra et al., 1968; Sawyer et al., 1974), stimulate iPGE and $iTXB_2$ synthesis in epithelial cells, confirming previous observations in intact hemibladders (Zusman et al., 1977; Burch et al., 1979).

Vasopressin increases urinary excretion of PGE in normal rats and in rats with congenital diabetes insipidus (Walker et al., 1978; Dunn et al., 1978); vasopressin also stimulates PGE_2 synthesis in cultured renomedullary intersitial cells and isolated perfused rabbit kidneys, but, in contrast, dDAVP does not (Beck et al., 1980; Zipser et al., 1981). This has led to the hypothesis that enhanced urinary PGE_2 excretion is associated only with the pressor component of

vasopressin and is unrelated to its antidiuretic action (Beck et al., 1980). However, it has been shown that dDAVP enhances iPGE excretion in rats with congenital diabetes insipidus (Dunn et al., 1980; Walker & Frölich, 1980; Walker & Frölich, 1981) and in human patients with central diabetes insipidus (Dunn et al., 1980). The previous observations, coupled with the present study demonstrating that vasopressin and dDAVP stimulate iPGE and TXB_2 synthesis in toad bladder epithelial cells, are consistent with the notion that in vivo the antidiuretic component of these peptides is associated with stimulation of the synthesis of prostaglandins and thromboxanes in collecting tubules.

It was reported in a previous study that vasopressin did not stimulate iPGE or $iTXB_2$ synthesis in scraped epithelial cells (Bisordi et al., 1980). Utilizing the same procedure, basal synthesis rates for iPGE and $iTXB_2$ similar to that study were obtained, and no stimulatory effect of vasopressin was observed. The basal synthetic rates of iPGE and $iTXB_2$ in the scraped cells were 10- and 30-fold greater than those observed in the cells prepared by using collagenase. Scraping the bladders to prepare the cells is clearly deleterious as evidenced by the much lower viability, i.e. 52% trypan blue exclusion. Trauma is an effective nonspecific stimulus for activating phospholipase (McMurray and MaGee, 1972) and since vasopressin appears to stimulate PGE and TXB_2 synthesis via activation of this group of enzymes trauma may mask vasopressin's effect. Thus, it would appear that isolating cells via a scraping procedure is not suitable either for studying the effects of vasopressin and dDAVP or other hormones

on endogenous arachidonic acid metabolism or studies on the effects of prostaglandins and thromboxanes on cellular functions.

Using the EDTA technique to prepare cell suspensions, we observed no stimulation of iPGE or $iTXB_2$ synthesis with AVP. Gatzy and Berndt (1968) found this technique to damage toad bladder cells anatomically and to render them insensitive to vasopressin or other agents as assessed by a failure of these agents to alter oxygen consumption. Scott and Sapirstein (1974) reported that vasopressin caused enhanced accumulation of cAMP in toad bladder epithelial cells prepared by this method, but Handler and Preston (1976) were unable to confirm those observations. Thus, it appears that the EDTA technique is associated with irreproducible results and may not be appropriate for use in obtaining consistently viable cells.

$iTXB_2$ synthesis was nearly 20-fold greater in the cells prepared by the EDTA method compared to the collagenase method when incubated in bicarbonate-Ringer's solution with 1 mM calcium. In contrast, iPGE synthesis was similar in the cells prepared via either method. The reason for the reversal in the ratio of these 2 arachidonic acid products, produced by the preparative methods, is unknown. EDTA does not appear to have an effect on the catalysis of the endoperoxide intermediates to TXA_2 by a purified thromboxane synthetase (Hammarström and Falardeau, 1977). Since EDTA is a commonly-used agent for preparing cell suspensions from a variety of tissues, the potential interactions between EDTA and arachidonic acid metabolism should be more carefully evaluated.

Many phospholipases, the rate-limiting enzymes in prostaglandin and thromboxane synthesis, which catalyze the release of arachidonic

acid from various membrane storage sites, require calcium (Kunze and Vogt, 1971). Not surprisingly, in the present study iPGE and $iTXB_2$ syntheses in toad bladder epithelial cells were influenced by the calcium concentration in the bathing media. Previous studies of the effect of calcium on the metabolism of arachidonic acid by renal tissue have usually measured only a single end product. Therefore, a possible dual effect of calcium on arachidonate metabolism would not have been discovered. In rat inner medullary kidney slices, preincubation with calcium-free media and EGTA reduced PGE synthesis compared with control media (2.5 mM Ca^{2+}) while the addition of the calcium ionophore A23187 to the control media enhanced arachidonate release and PGE synthesis (Zenser and Davis, 1978; Zenser et al., 1980). When, the formation of two products was monitored in minced renal papillae, addition of A23187 enhanced PGE synthesis 5-fold while $PGF_{2\alpha}$ synthesis was enhanced only 2-fold (Oelz et al., 1978). Thus, when A23187 was used as the means to alter intracellular calcium, the synthesis of both PGE_2 and $PGF_{2\alpha}$ were increased. Surprisingly, in the cells isolated by the collagenase technique, increasing the calcium concentration from 0 to 1 mM did not alter the net amount of arachidonate metabolized to iPGE plus $iTXB_2$, but rather resulted in enhanced iPGE synthesis at the expense of $iTXB_2$. This unique effect of calcium on arachidonic acid metabolism has not been previously reported.

This effect of calcium on arachidonic acid metabolism is consistent with several possibilities. A single source of arachidonate may have been released by two different phospholipases, one controlling the release of arachidonate and coupled to the enzymatic pathway

to PGE, and the other coupled to the pathway to TXB_2. Alternatively, the availability of the prostaglandin endoperoxide intermediates to the enzymes thromboxane synthetase and prostaglandin endoperoxide E isomerase may be influenced by the intracellular calcium content. While to our knowledge no precedent for the latter possibility has been reported, the recent work of Billah et al. (1980) demonstrates that multiple phospholipases may differentially control arachidonate release within a single cell. Obviously, additional explanations for the present observations are tenable. Clearly, additional studies are necessary to determine if calcium can produce this unique effect on arachidonic acid metabolism in other intact cell systems.

In the cells prepared by the EDTA method and incubated in bicarbonate- or phosphate-Ringer's solution, increasing the calcium concentration from 0 to 0.1 mM increased iPGE and $iTXB_2$ syntheses, but further increasing the calcium concentration was associated with a decline of iPGE and $iTXB_2$ syntheses toward the rates seen in the media with no added calcium. The cyclooxygenase has been reported to be localized to the endoplasmic reticulum and nuclear membranes (Rollins and Smith, 1980). Black et al. (1981) have reported that endoplasmic reticulum vesicles isolated from adipocytes and incubated in extracellular media containing varying amounts of calcium, sequester more calcium when the cells were incubated in media containing moderately low concentrations of calcium compared to no added calcium. However, when extracellular calcium was raised to several mM, calcium sequestration by the subsequently isolated endoplasmic reticulum vesicles was inhibited toward the zero-calcium level. Similarly, Waite and Sisson (1972) found that increasing calcium concentration

from 0 to 1.0 mM was associated with an enhanced phospholipase A_2 activity in isolated rat liver mitochondrial membranes; however, increasing the calcium concentration further to as high as 5 mM caused a progressive inhibition of the phospholipase A_2 activity. Thus, the present findings may be rationalized by suggesting that altered extracellular calcium concentration may modify intracellular calcium metabolism. This suggestion is strengthened by the observation that the calcium-induced increases in iPGE and $iTXB_2$ synthesis in the phosphate-Ringer's solution were much greater than the increases in the bicarbonate-Ringer's solution. This phenomenon may be explained by the well-established effect of phosphate to increase intracellular calcium concentrations (Borle, 1972). That enhanced cellular calcium uptake may be involved is further supported indirectly by the present observation that in tris-Ringer's solution, which contains no anion to complex calcium, increasing extracellular calcium was associated with only a slight increase in iPGE and $iTXB_2$ syntheses compared to the other 2 buffer media. That the large increases in synthetic rates were observed in the cells prepared by the EDTA method but not the collagenase method is consistent with the notion that the EDTA method produces cells which are damaged (Grenier and Smith, 1978) compared to the cells isolated by the collagenase method, damage which may be reflected by increased calcium permeability of the plasma membrane.

It has been demonstrated previously that exogenously administered PGE inhibits vasopressin-stimulated water flow (Orloff et al., 1965) while administration of any of a number of cyclooxygenase inhibitors results in potentiation of vasopressin-stimulated water flow (Ozer

and Sharp, 1972; Zusman et al., 1977). Puig Muset et al. (1972) found that imidazole caused an inhibition of vasopressin-stimulated water flow in isolated urinary bladders of Bufo bufo. We have confirmed their observation using urinary bladders of B. marinus. Concentrations of imidazole (1 mM) which increased iPGE synthesis inhibited vasopressin-stimulated water flow and $iTXB_2$ synthesis. Imidazole, 50 mM, a concentration which inhibited iPGE and $iTXB_2$ synthesis by 88% and 84%, respectively, acted as a typical cyclooxygenase inhibitor to potentiate vasopressin-stimulated water flow. The biphasic action of imidazole on vasopressin-stimulated water flow is consistent with the accepted mechanism of PGE modulation of the permeability response to vasopressin (Orloff et al., 1965; Zusman et al., 1977). However, the possibility that TXA_2 may be playing a role opposite to that of PGE_2 in affecting vasopressin-stimulated water flow could not be ruled out. This proposal was attractive since Samuelsson et al. (1978) proposed that thomboxanes and prostaglandins may act antagonistically toward one another as modulators of hormone action.

Imidazole inhibited vasopressin-stimulated water flow. While this effect is consistent with a positive modulatory role for TXA_2 on vasopressin-stimulated water flow, the results could easily be accounted for by the enhanced iPGE synthesis caused by imidazole. In addition, imidazole has a variety of effects other than thromboxane synthetase inhibition. Imidazole activates phosphodiesterase (Butcher et al., 1962). Imidazole is also known to reduce intracellular mobilization of calcium (Yu et al., 1967) while vasopressin increases the release of ^{45}Ca from prelabelled hemibladders (Thorn

and Schwartz, 1968). Thus, imidazole may have antagonized the action of vasopressin by decreasing the mobilization of intracellular calcium by a direct effect on calcium stores. The potential decreased availability of calcium at high concentrations of imidazole may play a role in the decreased synthesis of both PGE and TXB_2, since phospholipases, the enzymes catalyzing the rate-limiting step in prostaglandin and thromboxane synthesis (Kunze and Vogt, 1971) are often calcium-requiring enzymes (McMurray and Magee, 1972). Imidazole is also known to inhibit cyclooxygenase at high concentrations (Moncada et al., 1977). Thus, decreased activity of a phospholipase(s) or cyclooxygenase may account for the decreased synthesis of TXB_2 and PGE found with imidazole (50 mM) in these studies.

In an attempt to clarify any role for TXA_2 in the action of vasopressin on water permeability in the toad urinary bladder, another thromboxane synthetase inhibitor, 7-(1-imidazolyl)-heptanoic acid (7IHA) was used. 7IHA, at concentrations of 10 μM to 100 μM, inhibited vasopressin-stimulated $iTXB_2$ synthesis and water flow, but did not affect iPGE synthesis. This is in contrast to imidazole, which caused increased iPGE synthesis at the same time that $iTXB_2$ and water flow were inhibited. However, at a concentration of 500 μM, 7IHA, while further inhibiting $iTXB_2$ synthesis, did not further inhibit vasopressin-stimulated water flow.

In contrast to 7IHA, t13APA does not affect arachidonic acid metabolism (LeBreton et al., 1979). Furthermore, in several different tissues previously examined, t13APA only antagonized the actions of TXA_2 or PGH_2, and not PGE_2 or PGI_2 (LeBreton et al., 1979). Thus, the use of t13APA, a TXA_2 or PGH_2 antagonist, which does not alter

arachidonic acid metabolism yet is dependent upon it to exert its antagonistic actions, permitted a further assessment of the role of TXA_2 in vasopressin-stimulated water flow. t13APA inhibited vasopressin-stimulated water flow in a dose-dependent manner. That TXA_2 is the active arachidonate metabolite and that PGH_2 is not, is supported by the observations that imidazole and 7IHA, agents that reduce the synthesis of TXA_2 inhibited vasopressin-stimulated water flow, which would not be expected if PGH_2 were the active compound. While PGH_2 was not measured no evidence has been presented to suggest that imidazole or 7IHA affected PGH_2 synthesis. The observations that imidazole increased PGE synthesis and that 7IHA had no effect on iPGE synthesis suggest that PGH_2 synthesis was not altered. That TXA_2 is not an absolute requirement for vasopressin-stimulated water flow is supported by the observations that a high concentration of imidazole (50 mM) almost completely inhibited $iTXB_2$ synthesis but increased vasopressin-stimulated water flow, and that fatty acid cyclooxygenase inhibitors also enhance vasopressin-stimulated water flow (Flores and Sharp, 1972; Zusman et al., 1977). The enhancement of vasopressin-stimulated water flow by imidazole (50 mM) and fatty acid cyclooxygenase inhibitors is presumably due to inhibition of PGE synthesis.

To further assess the specificity of their action, the effects of 7IHA and t13APA were assessed on cAMP-stimulated water flow. cAMP does not affect the synthesis of PGE (Zusman et al., 1977) or $iTXB_2$. Neither 7IHA nor t13APA affected cAMP-stimulated water flow, further attesting to the specificity of the actions of these agents.

TXA_2 is a short-lived intermediate of arachidonic acid metabolism; therefore, direct demonstration of its effects in the toad bladder is technically difficult. Although TXB_2 is generally considered to be an inactive product of hydrolysis of TXA_2, at higher concentrations it has been shown to exhibit actions similar to TXA_2 (Friedman et al., 1979). Thus, in the present studies, the effects of TXB_2 and the stable endoperoxide/TXA_2-like agonists U44069 and U46619 were assessed on the water flow response of the toad bladder. U46619 stimulated water flow in bladders that were not pretreated with cyclooxygenase inhibitors, while U44069 did not. The failure of U44069 to stimulate water flow may have been due to its simultaneous inhibition of TXA_2 synthesis, which would perhaps, negate its agonistic properties. All three of these compounds stimulated basal water flow in indomethacin- or meclofenamate-pretreated hemibladders. The relative order of potency for the three compounds was similar to that which has previously been reported (Malmsten, 1976; Friedman et al., 1979). U46619 was two fold more potent than U44069, an observation that has been previously made in aortic strips (Malmsten, 1976). That the stimulation of water flow was not a nonspecific effect of prostanoic acid derivatives is borne out by the observation that PGE_1 did not stimulate water flow.

These compounds may be mimicking the action of either the prostaglandin endoperoxides or TXA_2. That the active metabolite in the toad bladder is TXA_2 and not the endoperoxide was discussed above. The complete pharmacology of these agents is not known. We have attempted to characterize some of their actions in the isolated toad bladder. U46619, U44069, and TXB_2 three compounds of different

structure, but which in a variety of tissues have similar actions (Malmsten, 1976; Friedman et al., 1979), all exhibited similar actions in the toad bladder, at concentration ratios similar to those used in other tissues (Malmsten, 1976; Friedman et al., 1979). These actions have been assumed in the past to mimic TXA_2. However, definitive evidence supporting this notion is lacking, and other actions of these compounds may exist. The effects of these compounds are antagonized by t13APA, but not by c13APA, properties consistent with specific receptor agonist activity (LeBreton et al., 1979). Finally, these agents were studied in the absence of endogenous arachidonic acid metabolism to avoid the influence of possible alterations in the cellular actions of endogenous prostaglandins. Thus, while alternative explanations may exist for the observations reported herein, the data are consistent with the hypothesis that TXA_2 may partially mediate the water flow response to vasopressin in the toad bladder.

U46619 enhanced vasopressin-stimulated water flow to submaximal concentrations of vasopressin, resulting in a parallel shift in the dose-response curve. The parallel shift, while not ruling out other possibilities, supports the notion that vasopressin and U44619 are acting on the same pathway(s) leading to increased water flow. Since the response to vasopressin is enhanced, the step affected by the agonist may be rate-limiting at low doses. However, this step is not rate-limiting at high concentrations of vasopressin since the agonist did not enhance water flow to supramaximal doses of vasopressin.

In contrast to these observations, in a preliminary study, Ludens and Taylor (1979) found that U44069 and U46619 inhibited vasopressin-

stimulated water flow in hemibladders not pretreated with cyclooxygenase inhibitors. Their findings may be reconciled with ours in part, since U44069 inhibited vasopressin-stimulated $iTXB_2$ synthesis (vide supra), which may account for the decrease in the vasopressin-stimulated water flow response. However, TXB_2, and U44069 and U46619 also cause marked, sustained contraction of the hemibladders that reduced their surface areas ca. 5% and 25%, respectively. Therefore, simply weighing the empty hemibladder at the end of the experiment leads to a substantial overestimate of transporting surface in the agonist-treated hemibladders, with concomitant underestimation of water flow. Indeed, in preliminary experiments using the conditions of Ludens and Taylor (1979), without correcting for the decrease in surface area, we also observed a reduction in vasopressin-stimulated water flow in the presence of U44069 and U46619, compared with control hemibladders. However, in these experiments, TXB_2, U44069, and U46619 all increased the absolute magnitude of basal water flow.

In indomethacin- or meclofenamate-pretreated hemibladders, t13APA antagonized the increase in water flow due to U46619 or TXB_2. In contrast, the inactive c13APA had no effect on U46619-stimulated water flow. Thus, these studies support the concept that t13APA specifically antagonizes a site of action common to both vasopressin and U46619.

The significance of TXA_2 as a mediator or modulator of vasopressin-stimulated water flow is presently unknown. TXA_2 synthesis inhibition or antagonism inhibit vasopressin-stimulated water flow to approximately 30%. Fatty acid cyclooxygenase inhibitors such as meclofenamate inhibit the synthesis of both PGE_2 and TXA_2 in the

toad bladder and augment vasopressin-stimulated water flow 40% (Bisordi et al., 1980) to approximately 100% (Zusman et al., 1977). In the toad bladder the synthesis rate of PGE is generally greater than that of TXA_2. Thus, the net result of complete inhibition of fatty acid cyclooxygenase would be expected to be augmentation of vasopressin-stimulated water flow. The studies with fatty acid cyclooxygenase inhibitors also point out that TXA_2 synthesis is not an absolute requirement for vasopressin-stimulated water flow in the toad urinary bladder.

Figure 16 summarizes the hypothesis constructed from the data which have been described. Vasopressin increases a phospholipase activity (Zusman and Keiser, 1977) in the epithelial cells of the toad bladder, resulting in the release of arachidonate from its storage pool in membrane phospholipids. Fatty acid cyclooxygenase catalyzes the conversion of arachidonate into the prostaglandin endoperoxides which have at least two possible fates, conversion to either PGE_2 or TXA_2. PGE_2 acts as a negative modulator of vasopressin-stimulated water flow (Orloff et al., 1965), and the present studies provide evidence consistent with a positive modulator role for TXA_2. Inhibition of prostaglandin synthesis by mepacrine, a phospholipase inhibitor (Zusman and Keiser, 1977), or by inhibitors of cyclooxygenase (Flores and Sharp, 1972; Zusman et al., 1977), result in increased vasopressin-stimulated water flow because of the removal of the negative modulator, PGE_2. Inhibition of thromboxane synthesis by imidazole or 7IHA, or thromboxane antagonism by t13APA, inhibit vasopressin-stimulated water flow.

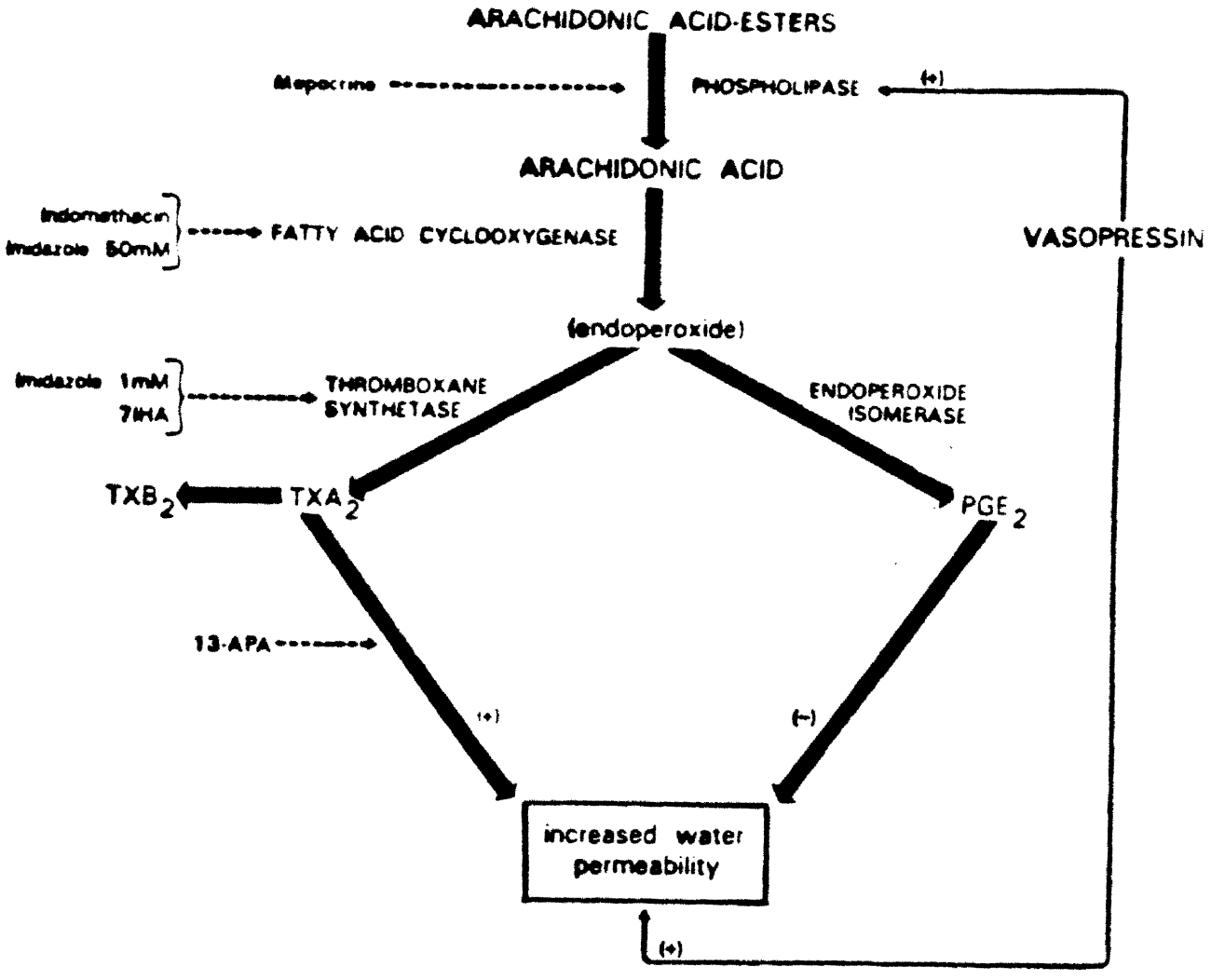


Figure 16. Vasopressin-stimulated arachidonic acid metabolism and its modulation of vasopressin-stimulated water flow. Vasopressin stimulates (+) phospholipase activity and water permeability. The released arachidonic acid is converted to TXA_2 which acts as a positive modulator (+) or to PGE which acts as a negative modulator (-) of vasopressin-stimulated water flow. Agents which inhibit each enzyme are denoted by dashed arrows directed toward the enzyme; 13APA is an antagonist of TXA_2's action.

CHAPTER III

THE EFFECTS OF VASOPRESSIN AND TXA_2-LIKE COMPOUNDS ON ^{45}Ca EFFLUX FROM ISOLATED TOAD URINARY BLADDER EPITHELIAL CELLS IN SUSPENSION

INTRODUCTION

Vasopressin has been shown to enhance ^{45}Ca efflux from preloaded intact toad urinary bladders (Thorn and Schwartz, 1965; Schwartz and Walter, 1969; Cuthbert and Wong, 1974). This chapter describes experiments examining ^{45}Ca efflux from isolated toad urinary bladder epithelial cells. Epithelial cell suspensions were used instead of intact bladders because the agents which were to be tested are known to have effects on smooth muscle as well as epithelial cells in the toad bladder. Vasopressin is known to cause relaxation of toad bladder smooth muscle (Dibona and Civan, 1970) while U46619 was shown (Chapter II) to cause a marked contraction of smooth muscle in the toad bladder. Thus, use of isolated epithelial cells allowed any effects of the agents on ^{45}Ca fluxes to be attributed soley to the epithelial cells, the presumed target for vasopressin's antidiuretic effect.

MATERIALS AND METHODS

The following were purchased from commercial sources: $^{45}CaCl_2$ (0.65-0.77 Ci/mmole), and $Na^{125}I$ (17 Ci/mg), The Radiochemical Center, Amersham, England; [methoxy-^{3}H]-inulin (14 mCi/g), New England Nuclear Corp., Boston, MA; bovine serum albumin, RIA grade, and antimycin A, Type III, isolated from Streptomyces kitazawaensis mycellia, KCN, and sodium metabisulfite, Sigma Chemical Co., St. Louis, MO; chloramine T, Eastman Kodak Co., Rochester, NY; other chemicals and reagents were supplied by the sources listed in Chapter II, Materials and Methods.

[^{125}I]-Albumin was prepared by the chloramine T method: 2 µg bovine serum albumin was dissolved in 20 µl of 0.5 M phosphate buffer (pH 7.4) and 5 µl (100 µ Ci/µl) of $Na^{125}I$ solution was added directly from the shipping container. Chloramine T (17.5 mg chloramine T in 5 ml of 0.05 M phosphate buffer (pH 7.4) in a light-tight container), 5 µl, was added and the solution was allowed to incubate for 60 sec. Stop solution (12 mg sodium metabisulfite in 5 ml of 0.5 M phosphate buffer), 40 µl, was added and the tube shaken for 15 sec. The reaction mixture was poured over a column (30 cm x 1 cm) of Sephadex G100 which had been equilibrated with 0.05 M phosphate buffer (pH 7.4) containing 2 µg/ml bovine serum albumin and fractions collected at 1 ml/tube. The labelled albumin was collected in fraction 8. The nominal activity of [^{125}I]-albumin was 200 mCi/g. The author wishes to thank Dr. Alfred Crosswell for the chloramine T methodology.

Perturbation of ^{45}Ca efflux. To each of 2 polycarbonate centrifuge tubes (15 ml) were added 3 ml of suspended cells (approximately 2-3 mg protein) which had been isolated by the collagenase method.

The suspensions were placed into a water bath maintained constantly at 25°C. and stirred with teflon-coated magnetic stirbars under an atmosphere of 95% O_2, 5% CO_2. To each suspension was added 10 μCi ^{45}Ca. Uptake of ^{45}Ca label proceeded for 90 min (Fig. 17). At this time the suspensions were centrifuged in an IEC Model CL tabletop clinical centrifuge at high speed for 35 sec. The supernatant was decanted and retained for specific activity determination. The pellets were resuspended in nonradioactive media and centrifuged again to clear the intercellular spaces of trapped ^{45}Ca. The cells were centrifuged, the media collected, and the pellets resuspended at the end of 1,5,10 and each 10 min thereafter in a protocol suggested by Borle (1975). At the end of the experiment the pellet was dissolved in 0.1 N NaOH for several hours and aliquots were taken for ^{45}Ca and protein determinations. Use of the Biosolve®-liquid scintillation cocktail (Chapter II) with the Beckman LS-355 counter was not associated with any quench, even in the presence of the unneutralized NaOH (cpm=98±2% of predicted dpm at a range of 50 cpm to 200,000 cpm per vial). ^{45}Ca remaining in the cells at each time point was determined by summing the radioactivity in the pellet with the radioactivity lost to the bathing media over each period. Experimental manipulations were performed by adding the agents to one of the suspensions after efflux had been carried out for a specific period of time. The rate of ^{45}Ca efflux was monitored as the percent ^{45}Ca remaining in the cells which has been lost per time period, or "instantaneous" first order rate constant (Butcher, 1980), as follows:

$$k_{app} = cpm_w/[(cpm_{cell(t)} + cpm_{cell(t+\Delta t)})/2]\Delta t$$

where cpm_w is the radioactivity lost from the cells during the time

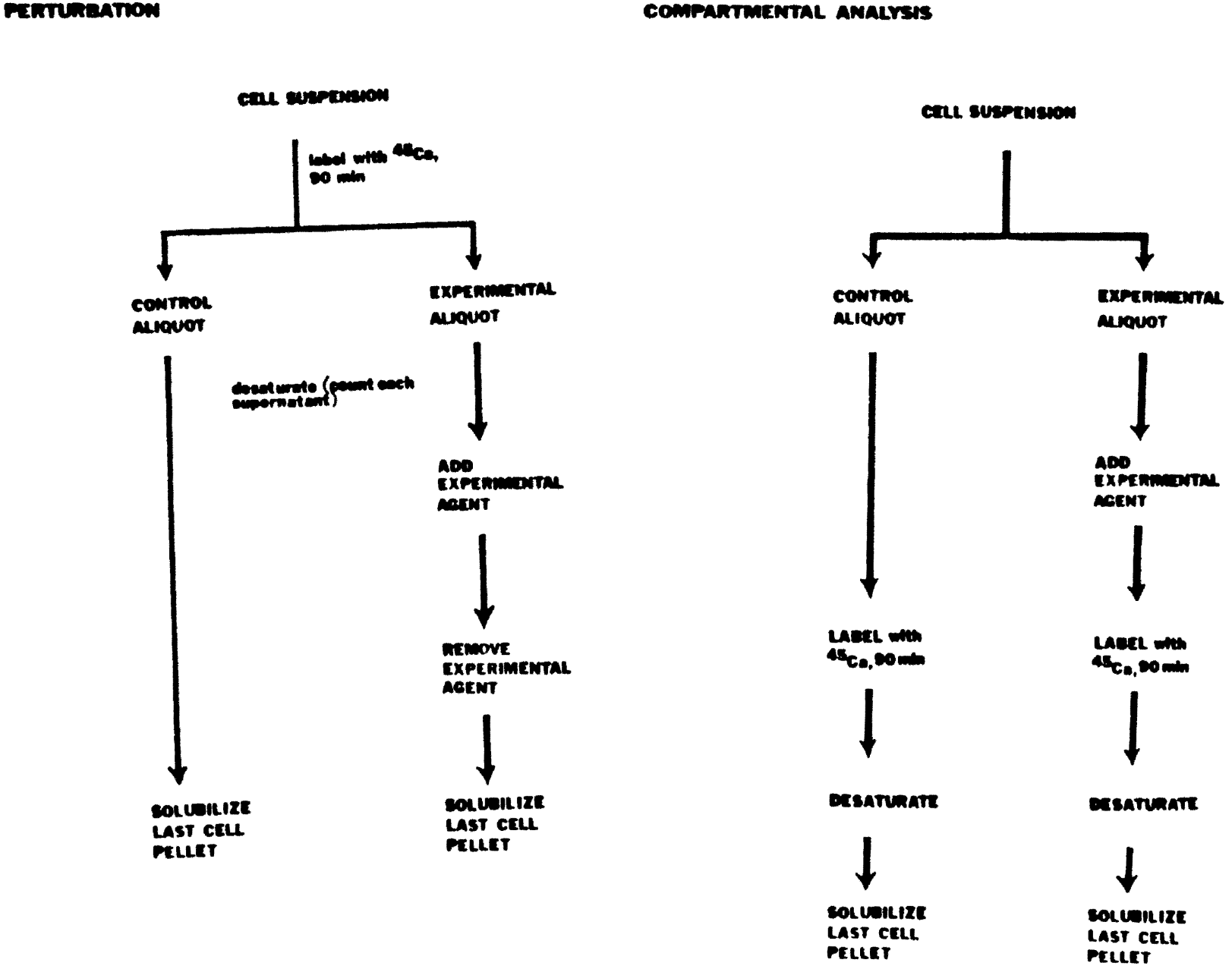


Figure 17. Flow scheme for the two types of ^{45}Ca experiments. Experimental details are contained in the Methods.

interval Δt, and $[(cpm_{cell(t)} + cpm_{cell(t+\Delta t)})/2]$ is the mean amount of radioactivity remaining in the cells during Δt. Usually the results are expressed as the ratio $k_{app,experimental}/k_{app,control}$, but referred to as $efflux_{experimental}/efflux_{control}$.

Steady state ^{45}Ca content. Cell suspensions were labelled for 90 min with ^{45}Ca (10 μCi/4 ml suspension). Suspensions were then centrifuged in the table top centrifuge at high speed for 45 sec and resuspended in bicarbonate-Ringer's solution containing ^{45}Ca of the same specific activity. Each suspension was then divided into four 1 ml aliquots, one served as control, the remainder were exposed to vasopressin (0.1, 1.0, or 20 mU/ml). The "lanthanum method" described by van Breeman et al. (1972) was used to separate extracellular ^{45}Ca. The procedure described here is quite similar to that modification described by Lee and Auersperg (1980) for isolated fibroblasts. After vasopressin was added to the experimental suspensions, 300 μl aliquots were taken from each suspension after 1, 5, or 15 min and injected into a 40 ml polycarbonate centrifuge tube containing 30 ml of ice-cold La-Ringer's solution (90 mM NaCl, 10 mM tris-HCl, 10 mM $LaCl_3$, pH 7.4), and centrifuged at 500 x g for 1 min at 4°C in a Sorvall IC-2B centrifuge. The supernatant was decanted, the pellets were resuspended in 30 ml La-Ringer's solution and the suspensions placed into an ice bucket for 30 min. The suspensions were recentrifuged, the pellets dissolved in 0.1 N NaOH, and aliquots were taken for determination of ^{45}Ca and protein.

Compartmental analysis of ^{45}Ca efflux. The suspensions were labelled with ^{45}Ca and desaturated as described above (Fig. 17). In these experiments experimental agents were always added to one of

the suspensions before uptake of the label and the suspensions were incubated for a period of time which would be sufficient for establishment of a stable physiological response in intact hemibladders. This last condition was required since after 90 minutes of uptake the slow compartment of efflux to be described was not loaded to equilibrium specific activity. Equilibration of specific activity would require at least 14 hours in these cells, a condition which clearly could not be tolerated by isolated cells without subjection to temporary culture. Thus, by assuring equilibrium to be achieved in response to experimental agents, reasonable assurance was obtained that calcium pools and fluxes into and out of the pools would also be in equilibrium. From the rate coefficients of efflux, the mathematical models could then correct the measured pool sizes to those which would have been measured if equilibrium specific activity had been reached. The amount of ^{45}Ca remaining in the cells was calculated for each time during desaturation as described above and the data were further analyzed by the mathematical models to be presently described.

Compartmental models. The equations for calculating the kinetic parameters will be presented for a parallel system where each compartment independently exchanges with the bathing medium compartment (Fig. 18,a-c). The derivations necessitate that the system be in a steady state. Thus, the respective fluxes, compartment sizes, and rate constants of transfer are considered to be constant and equal during the uptake of isotope and during desaturation. During the labelling period the system can be considered to be closed. That is, all compartments are of finite size and no calcium is added or subtracted from the system consisting of the bathing medium and the various cellular compartments. During the labelling period the change in specific activity a_i, in each compartment i can be described by

$$da_i/dt = k_{0i}a_{i(\infty)}$$

or, rearranging and taking the Laplace transform (Robertson, 1957)

$$a_i/a_{i(\infty)} = 1 - e^{-\lambda_i t} \qquad (1)$$

where t is labelling time in minutes, λ_i is the exponential constant of calcium influx into the ith compartment during the labelling period, and $a_{i(\infty)}$ is the specific activity of compartment i at infinite time, or, equilibrium specific activity.

Since the bathing medium compartment in these experiments is very large, fluxes into and out of the medium are so small as to be negligible (in the experiments described here the cell exchangeable calcium is approximately 2×10^{-8} mole and the bathing medium pool is 3×10^{-6} mole, or, the cellular pool is 0.7% of the bathing medium pool). Thus, the specific activity of the medium is constant during the labelling phase and can be accepted to be equal to $a_{i(\infty)}$.

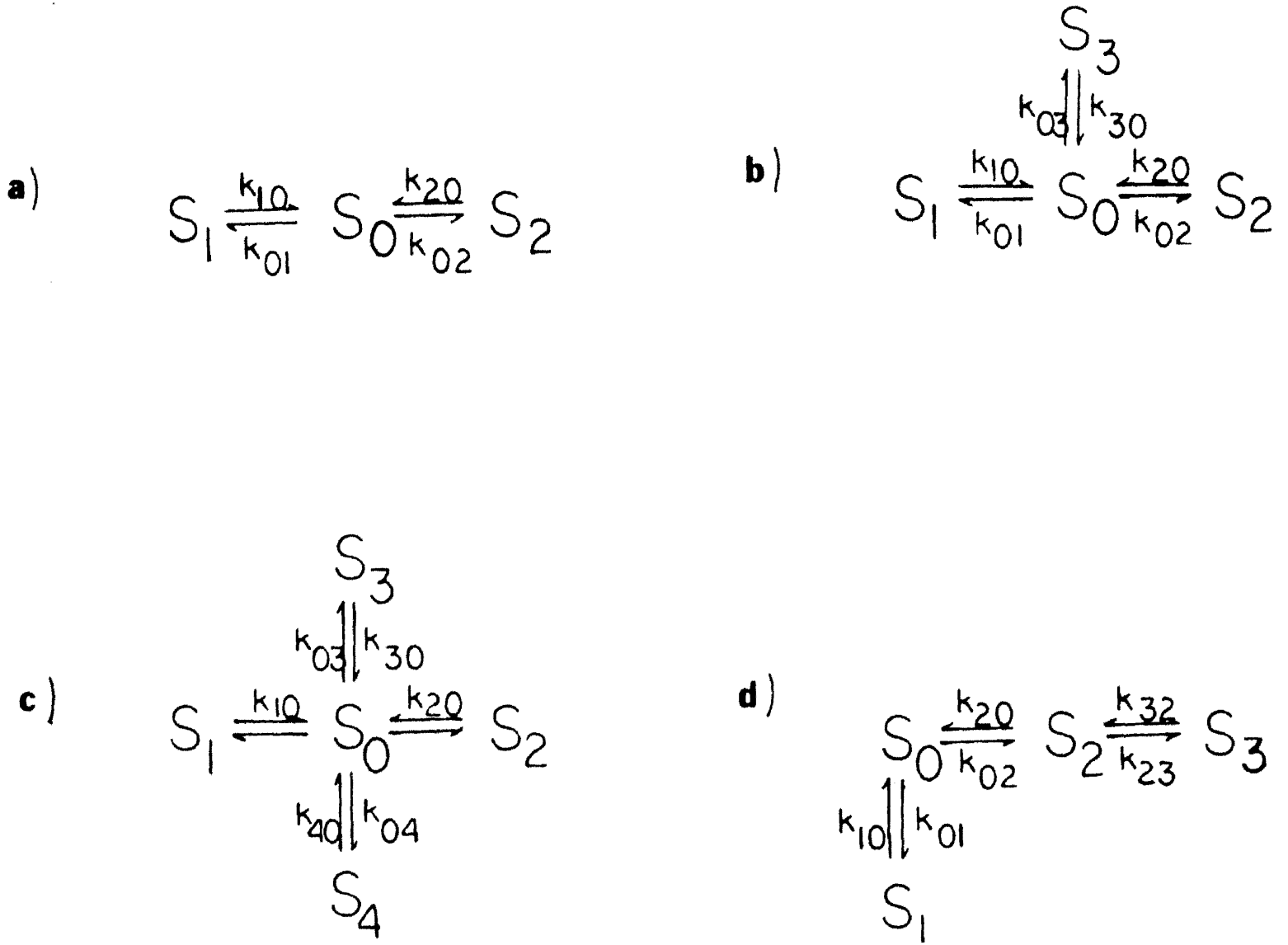


Figure 18. Models used in the compartmental analyses of ^{45}Ca efflux from prelabelled isolated toad urinary bladder epithelial cells. a) 2-compartment parallel model (S_1 and S_2) plus the bathing medium compartment (S_0), b) 3-compartment parallel model, c) 4-compartment parallel model, and d) a modified series 3-compartment model. Experimental data were fitted to the 4-compartment parallel model, the most rapidly exchanging compartment was discarded, and the constants for the modified series 3-compartment model were mathematically derived (see METHODS).

By definition

$$a_i = R_i/S_i \tag{2}$$

where R_i is the radioactivity in compartment i, and S_i is the amount of traced substance, here calcium, in compartment i. Substituting Equation (1) into Equation (2)

$$R_i/S_i = a_{i(\infty)}(1 - e^{-\lambda_i t}) \tag{3}$$

or

$$S_i = R_i/[a_{i(\infty)}(1 - e^{-\lambda t})] \tag{4}$$

During desaturation the system can be described as a linear combination of exponentials (Sheppard, 1962)

$$X_T = X_1 e^{-\lambda_1 t} + X_2 e^{-\lambda_2 t} + \cdots + X_n e^{-\lambda_n t} \tag{5}$$

where X_i are coefficients of the exponential terms, in these experiments equivalent to R_i. To graphically determine X_1 through X_n and λ_1 through λ_n the natural logarithm is taken from each side of Equation (5)

$$\ln X_T = \ln X_1 - \lambda_1 t + \ln X_2 - \lambda_2 t + \cdots + \ln X_n - \lambda_n t \tag{5a}$$

One may plot $\ln X_T$ versus time and obtain a series of linear functions each with slope λ_i and y-intercept $\ln X_i$. Alternatively, the method used in these experiments was a reiterative nonlinear least squares program (Nelder and Mead, 1965) to obtain the experimental intercepts and slopes.

Experimentally, the amounts of ^{45}Ca released into the medium during each time period, R_{mt}, during desaturation are summed and to this is added the amount of ^{45}Ca remaining in the cells at the end of desaturation, R_c, or

$$R_{m,t(0)} = R_{m1} + R_{m5} + \cdots + R_{m,t(160)} + R_c = R_m + R_{cells} \tag{6}$$

where $R_{m,(160)}$ is the amount of radioactivity lost into the medium

during the period from 150 to 160 minutes.

A value R_t is calculated to represent total exchangeable calcium remaining in the cells at any time during the desaturation

$$R_t = [(R_{mt}/\text{mg cell protein})]/a_{i(\infty)} \quad (7)$$

Each R_{mt} is obtained by subtracting from $R_{mt(0)}$ the total amount of ^{45}Ca released into the medium up to time t. Thus, R_t is X_T normalized to specific activity and cell protein, having the units cpm or moles, if the specific activity of the medium $a_{i(\infty)}$ is known.

The following demonstration that $\lambda_i = k_{i0}$ is adapted from Borle (1975). During the desaturation the system is said to be open, that is, the medium compartment, since it is periodically renewed, can be considered to be infinite in size. Thus, the slope λ_i of each kinetic phase represents the rate constant of efflux k_{i0} from compartment i to medium 0, or

$$dR_i/dt = - \rho_{i0}a_i \quad (8)$$

where ρ_{i0} is the rate of exchange or flux from compartment i to medium 0.

Since

$$a_i = R_i/S_i \quad (2)$$

$$dR_i/dt = - \rho_{i0}(R_i/S_i) \quad (9)$$

or

$$dR_i/dt = - (\rho_{i0}/S_i)R_i \quad (10)$$

At time 0 of desaturation all radioactivity is in compartment i and is equal to $R_{i(0)}$, and, at infinite time $R_i = 0$. Thus

$$R_i = R_{i(0)}\exp[-(\rho_{i0}/S_i)t] \quad (11)$$

or, taking the natural logarithms

$$\ln R_i = \ln R_{i(0)} - (\rho_{i0}/S_i)t \qquad (12)$$

Plotting $\ln R_i$ versus time the slope is $-(\rho_{i0}/S_i)$ and the y-intercept is $R_{i(0)}$ (the X_i of Equation (5)).

Since

$$\rho_{i0}/S_i = k_{i0} \qquad (13)$$

the slope of component i represents the rate constant of efflux from compartment i to medium 0. Equation (4) is an expression of the labelling period occurring in a closed system and the exponential constant λ_i is not a rate constant. The equation describing ^{45}Ca uptake in such a closed system can be developed as follows for a medium compartment S_0 and a cellular compartment S_i.

$$R_0 = a_0 S_0 \qquad (14a)$$

$$R_i = a_i S_i \qquad (14b)$$

Since S_0 and S_i are constant

$$dR_0/dt = S_0(da_0/dt) \qquad (15a)$$

$$dR_i/dt = S_i(da_i/dt) \qquad (15b)$$

Also

$$dR_0/dt = \rho_{i0}a_i - \rho_{0i}a_0 \qquad (16a)$$

$$dR_i/dt = \rho_{0i}a_0 - \rho_{i0}a_i \qquad (16b)$$

Since the system is in steady state

$$\rho_{0i} = -\rho_{i0} = \rho \qquad (17)$$

Therefore

$$dR_0/dt = S_0(da_0/dt) = \rho(a_i - a_0) \qquad (18a)$$

$$dR_i/dt = S_i(da_i/dt) = \rho(a_0 - a_i) \qquad (18b)$$

Combining Equations (18a) and (18b)

$$dR_0/dt - dR_i/dt = -\rho(1/S_0 + 1/S_i)(a_0 - a_i) \qquad (19)$$

Since a_i ultimately approaches a_0, the terminal value of $(a_0 - a_i)$ approaches zero. Moreover, since all the radioactivity is initially

in compartment 0 with specific activity $a_0(0)$, the initial $(a_0 - a_i) = a_0(0)$. The solution is then

$$a_0 - a_i = a_0(0)\exp -\rho[(S_0 + S_i)/(S_0S_it)] \quad (20)$$

From Equation (18b)

$$a_0 - a_i = (dR_i/dt)/\rho \quad (21)$$

and combining Equations (20) and (21)

$$(dR_i/dt)/\rho = a_0(0)\exp -\rho[(S_0 + S_i)/(S_0S_it)] \quad (22)$$

Since $a_0(0) = a_{i(\infty)}$

$$(dR_i/dt)/a_{i(\infty)} = \rho\exp -\rho[(S_0 + S_i)/(S_0S_it)] \quad (23)$$

or, in logarithmic form

$$\ln (dR_i/dt)/a_{i(\infty)} = \ln \rho - \rho[(S_0 + S_i)/(S_0S_i)]t$$

The slope of the semilogarithmic plot of Equation (23) versus time is equal to λ_i or

$$\lambda_i = \rho[(S_0 + S_i)/S_0S_i] \quad (24)$$

Therefore

$$\lambda_i/\rho = (S_0 + S_i)/S_0S_i \quad (25)$$

Since at steady state

$$\rho_{i0} = k_{i0}S_i = \rho \quad (26)$$

Equations (25) and (26) give

$$\lambda_i/k_{i0}S_i = (S_0 + S_i)/S_0S_i \quad (27)$$

or

$$\lambda_i = k_{i0}S_i[(S_0 + S_i)/S_0S_i] \quad (28)$$

or

$$\lambda_i = k_{i0}[(S_0 + S_i)/S_0] \quad (29)$$

In the experiments performed in this dissertation S_0 is more than 100 times larger than S_i (see the second paragraph of this section). Thus

$$(S_0 + S_i)/S_0 = (>100 + 1)/(>100) \simeq 1 \quad (30)$$

Therefore, under the conditions of the present experiments

$$k_{i0} \simeq \lambda_i \tag{31}$$

Thus, the size, S_i, of each compartment can be calculated from Equation (4), and since λ_i is also k_{i0}, the exchange rate (flux) into and out of the compartment is

$$\rho_{i0} = \rho_{0i} = k_{i0}S_i = \lambda_i S_i \tag{32}$$

The equations above describe the kinetics of a parallel system. If, instead, the system is in series (Fig. 17d) the specific activity in the more slowly exchanging compartments will rise due to influx from the more rapidly exchanging compartments (due to ρ_{12} and ρ_{23}) during the initial portion of desaturation; that is, until the specific activity in the more rapidly exchanging compartments falls to that in the more slowly exchanging compartments (Uchikawa and Borle (1978) for a discussion of this phenomenon and derivation of equations to quantitate it). Thus, analyzing the system as if it were in parallel would overestimate the sizes of the slowly exchanging compartments and underestimate the sizes of the rapidly exchanging compartments. The error incurred from such an analysis may be calculated according to Huxley (1960)

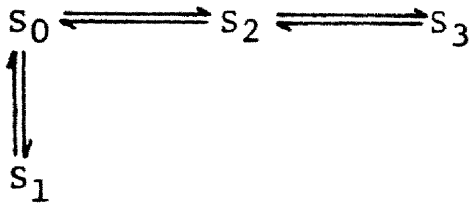
$$R_{s,true} = [R_f R_s(\lambda_f - \lambda_s)^2]/(R_f\lambda_f^2 + R_s\lambda_s^2) \tag{33}$$

where the subscripts s and f refer to the more slowly exchanging compartment and the more rapidly exchanging compartment, respectively. In the present experiments the 2 more slowly exchanging compartments have the characteristics $R_f = 1.2$, $R_s = 6.1$, $\lambda_f = 0.06$, and $\lambda_s = 0.006$. If these 2 compartments are actually in series, then $R_{s,true} = 0.78R_s$, or, an error of 22% exists. Thus, equations will next be presented by which the kinetic parameters for a modified

series case may be calculated.

The following derivations are taken directly from Uchikawa and Borle (1978), being modified only to use of the same symbols which have already been described.

The system of exchangeable calcium compartments in isolated cells is represented as follows



where S_0 is the medium compartment, S_1 is calcium bound to the glycocalyx, and S_2 and S_3 are the two intracellular pools.

1. Labelling period.

According to the general kinetic equation, one can write

$$a_{1-3,t'} = Ka_{1-3,t'} + I \qquad 0 \leq t' \leq t \qquad (34)$$

where $a_{1-3,t'}$ is the specific activity of S_1, S_2, and S_3 at a time, t', during labelling, t' is any time during the labelling period t, and I is tracer input into the system, where

$$K = \begin{bmatrix} -k_{10} & 0 & 0 \\ 0 & -k_{20}+k_{23} & k_{23} \\ 0 & k_{32} & -k_{32} \end{bmatrix} \qquad (35)$$

$$a_{0,t'} = [a_{1,t'} \quad a_{2,t'} \quad a_{3,t'}]^t \qquad (36)$$

and

$$I = a_{i(\infty)}[k_{10} \quad k_{20} \quad 0]^T \qquad (37)$$

At the beginning of the labelling period, the system contains no tracer so that

$$a_{i(\infty)} = 0 \qquad (38)$$

Assuming that K has three distinct eigen values, $-\lambda_i$, where $i = 1, 2, 3$, the solution of these differential equations is

$$a_{1-3,t'} = a_{i(\infty)} U F_{t'} \tag{39}$$

where

$$U = \begin{bmatrix} 1 & 0 & 0 \\ 0 & (\lambda_3 - k_{20})/(\lambda_3 - \lambda_2) & (\lambda_2 - k_{20})/(\lambda_2 - \lambda_3) \\ 0 & \lambda_3/(\lambda_3 - \lambda_2) & \lambda_2/(\lambda_2 - \lambda_3) \end{bmatrix} \tag{40}$$

and

$$F_{t'} = [\exp(-\lambda_1 t' - 1) \quad \exp(-\lambda_2 t' - 1) \quad \exp(-\lambda_3 - 1)] \tag{41}$$

where

$$\lambda_1 = k_{10} \tag{42}$$

$$\lambda_2 \lambda_3 = k_{32} k_{20} \tag{43}$$

$$\lambda_2 + \lambda_3 = k_{20} + k_{23} + k_{32} \tag{44}$$

2. Transition period

The specific activity of the cells at the end of the labelling period is the same as their specific activity at the beginning of the desaturation period so that

$$A_0 = a_{1-3,t} \tag{45}$$

where

$$A_{t'} = [A_{1,t'} \quad A_{2,t'} \quad A_{3,t'}] \tag{46}$$

3. Desaturation period

During desaturation, tracer input I into the system is zero. The differential equation describing it is

$$A_{t'} = K A_{t'} \qquad t \geq 0 \tag{47}$$

and

$$A_0 = a_{1-3,t} \tag{45}$$

The solution to these equations is

$$A_{t'} = a_{1\text{-}3,t}UG_{t'} \tag{48}$$

where

$$G_{t'} = \begin{bmatrix} [\exp(-\lambda_1 t - 1)]\exp(-\lambda_1 t') \\ [\exp(-\lambda_2 t - 1)]\exp(-\lambda_2 t') \\ [\exp(-\lambda_3 t - 1)]\exp(-\lambda_3 t') \end{bmatrix} \tag{49}$$

4. Relative radioactivity of the system

Let R_{it} be the amount of tracer in compartment i, R_t the sum of R_{it} where $i = 1, 2, 3$.

One can write

$$r(t) = R_t/a_{i(\infty)} \tag{50}$$

$$= \sum_{i=1}^{3} a_{i(\infty)}C_i[\exp(-\lambda_i t - 1)][\exp(-\lambda_i t')] \tag{51}$$

where C_i has the same meaning as S_i in the previous parallel case. Thus, the values S_i derived here will have the "same" meaning as S_i in the parallel case, that is, amount of traced substance in compartment i. Thus,

$$C_1 = S_1 \tag{52}$$

$$C_2 = [(S_2 + S_3)\lambda_3 - k_{20}S_2]/(\lambda_3 - \lambda_2) \tag{53}$$

$$C_3 = [(S_2 + S_3)\lambda_2 - k_{20}S_2]/\lambda_2 - \lambda_3) \tag{54}$$

Let $R_{i0} = C_i[\exp(-\lambda_i t' - 1)]$, then

$$r(t) = \sum_{i=1}^{3} R_{i0}(\exp -\lambda_i t') \tag{55}$$

The solutions for equations (52 through (54) are as follows

$$S_1 = C_1 \tag{56}$$

$$k_{10} = \lambda_1 \tag{57}$$

$$\rho_{10} = k_{10}S_1 \tag{58}$$

$$S_2 + S_3 = C_2 + C_3 \tag{59}$$

$$\rho_{20} = C_2\lambda_2 + C_3\lambda_3 \tag{60}$$

$$S_2 = (\rho_{20})^2/(C_2\lambda_2^2 + C_3\lambda_3^2) \tag{61}$$

$$S_3 = (C_2 + C_3) - S_2 \tag{62}$$

$$\rho_{23} = S_2S_3(\lambda_2\lambda_3/\rho_{20}) \tag{63}$$

$$k_{ij} = \rho_{ij}/S_i \tag{64}$$

Data fitting. A reiterative least squares program (Nelder and Mead, 1965) was used to obtain the kinetic parameters λ_i and R_i. The fitting procedures were executed on a Hewlett-Packard HP 2100A computer with 32K memory. The models used were series of 2, 3 or 4 exponentials (Equation 5). An estimate of error, "functional error," was obtained for each model

$$\text{functional error} = \sum_{t=0}^{t=n} [(\text{observed } R_t - \text{predicted } R_t)/(\text{observed } R_t)]^2$$

For the 12 experiments reported in Table VIII, the functional errors were 0.040021±0.000057, 0.003402±0.000008, and 0.001014±0.000003, for the 2, 3, and 4 component models, respectively (Fig. 18). An F-ratio test (Boxenbaum et al., 1974) to determine whether the reduced functional errors obtained when the model was expanded were statistically different was performed. The use of a 3-compartment model was significantly better than a 2-compartment model ($p<0.01$). However, a 4-compartment model was nearly statistically significantly better than a 3-compartment model ($p<0.07$). Based on washout data to be described below, a 4-compartment model was chosen, only the 3 more slowly exchanging compartments being attributed to the cells. The author wishes to acknowledge the invaluable aid rendered by Ms. Martha Stroud who programmed the computer and offered instruction in the use of the programs.

Statistics. Most experiments were performed in such a manner that a single cell suspension was divided into 2 equal volumes and used for control and experimental measurements. In these experiments differences between means was tested with Student's t-test for paired observations (Steel and Torrie, 1960) using either a Texas Instruments

TABLE VIII

KINETICS OF THE CELLULAR CALCIUM POOLS

Parallel Case

Fast Pool (S_1)

S_1	ρ_{10}	k_{10}
1.52 ±0.16	0.364 ±0.062	0.226 ±0.020

Intermediate Pool (S_2)

S_2	ρ_{20}	k_{20}
1.16 ±0.06	0.080 ±0.007	0.073 ±0.006

Slow Pool (S_3)

S_3	30	k_{30}
6.01 ±0.60	0.038 ±0.004	0.0065 ±0.0004

Series Case

Fast Pool (S_1)

S_1	ρ_{10}	k_{10}
1.52 ±0.16	0.364 ±0.062	0.226 ±0.020

Intermediate Pool (S_2)

S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
2.35 ±0.16	0.123 ±0.010	0.042 ±0.004	0.055 ±0.006	0.016 ±0.002

Slow Pool (S_3)

S_3	ρ_{32}	k_{32}
4.78 ±0.50	0.042 ±0.004	0.0092 ±0.0010

n=12. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM.

TI-59 calculator with statistics chip or a Monroe Statistician® preprogrammed calculator. Several limitations are inherent in the methods used to analyze the data. The cumulative amount of ^{45}Ca in the cells was obtained by summing the individual media measurements. Thus, each point contains not only its own experimental error but also the error from each of the other points. Thus, the least squares program is not strictly appropraite for analysis of the data (Boxenbaum et al., 1974). Boxenbaum et al. (1974) have pointed out that comparisons between fitted parameters using a t-test may not be reliable, especially when more than one model may be fitted to the data.

In several of the ^{45}Ca efflux experiments the data were analyzed as $\ln^{45}Ca$ remaining versus time and the several components of efflux were separated by "curve peeling" (Casteels and Droogmans, 1975). The linear portion of the slowest component (from 110 minutes to the end of the experiment) was analyzed with the linear regression program of the TI-59 calculator and residuals calculated from the best-fit line. The linear portion of the next component (50-110 minutes) was then analyzed similarly. The values for the control and experimental suspensions for each component were then subjected to analyses of covariance to determine whether the lines were different (Dixon and Massey, 1969).

RESULTS

EFFECTS OF VASOPRESSIN AND cAMP ON ^{45}Ca EFFLUX FROM PRELABELLED EPITHELIAL CELLS

Effects of vasopressin on ^{45}Ca efflux from isolated toad bladder epithelial cells in suspension. Thorn and Schwartz (1965), Schwartz and Walter (1969), and Cuthbert and Wong (1974) have shown that vasopressin causes an enhancement of ^{45}Ca release from prelabelled intact hemibladders. To determine whether epithelial cells isolated by the collagenase method responded to vasopressin in the same manner an attempt was made to reproduce those results. Desaturation was commenced simultaneously in 2 aliquots of a cell suspension which had been labelled with ^{45}Ca. After the 10 min decanting vasopressin (10 mU/ml) was added to one tube of each pair of aliquots and remained for three 10 min periods. As shown in Fig. 19, vasopressin elicited an enhancement in ^{45}Ca efflux which lasted as long as vasopressin was present in the bathing media. Maximum enhancement was 39±11%. Efflux returned toward control rate after the removal of vasopressin. Efflux of ^{45}Ca from the isolated cells was from 3 compartments (to be discussed below). The rate constants of efflux were such that by 90 min the first 2 compartments were completely desaturated. In an additional series of experiments, vasopressin was added after 120 min of desaturation. Vasopressin (10 mU/ml) enhanced ^{45}Ca efflux to 28±8% after 20 min (Fig 20). Efflux returned to basal rate after vasopressin was removed. Thus, vasopressin appeared to elicit enhanced ^{45}Ca efflux from at least the third, most slowly exchanging compartment.

Effects of vasopressin on steady state ^{45}Ca efflux. The enhancement in ^{45}Ca efflux observed when vasopressin was added to cells already

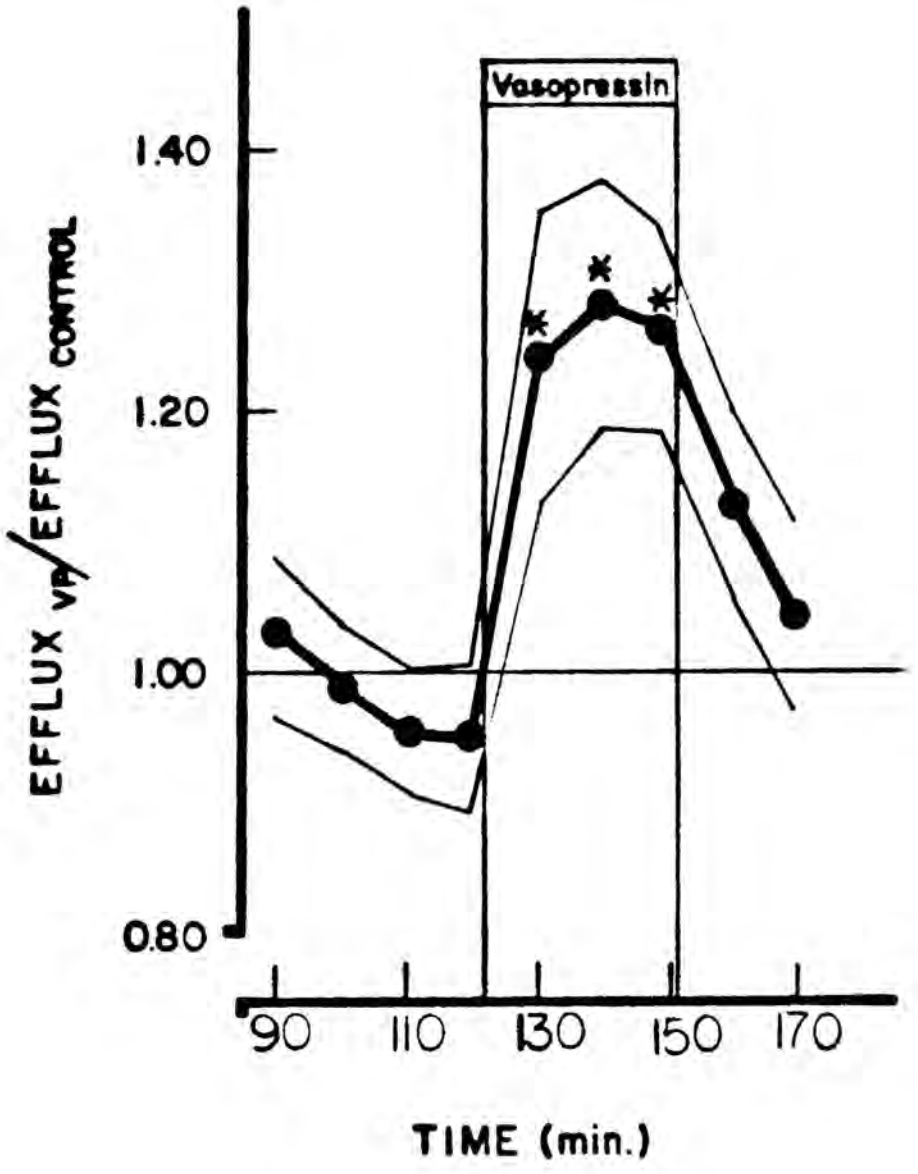


Figure 20. Effect of vasopressin on ^{45}Ca efflux from pool S_3. The protocol was that described in the legend to Figure 19 except that vasopressin (10 mU/ml) was added after 120 minutes of desaturation. *$p < 0.05$, $n = 4$ pairs.

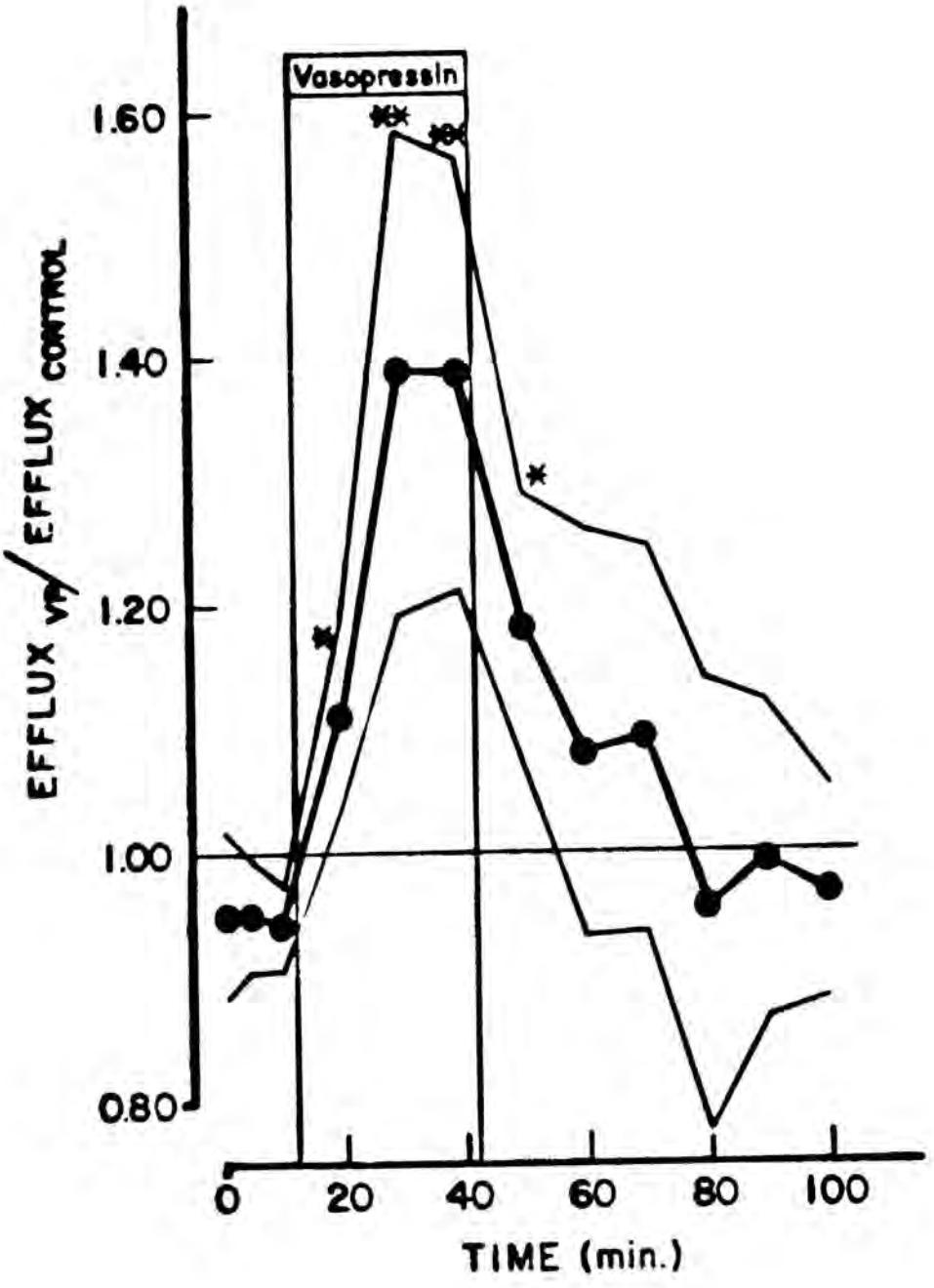


Figure 19. Effect of vasopressin on ^{45}Ca efflux from epithelial cells isolated from toad urinary bladder. Cell suspensions were labelled for 90 minutes with ^{45}Ca. Each suspension was then divided into 2 aliquots and desaturated simultaneously. To one of each pair of aliquots was added vasopressin (10 mU/ml) after 30 minutes of desaturation. Data are plotted as the ratio of efflux from cells incubated with vasopressin to control (see METHODS). The values are expressed as mean (circles) $\pm$ SEM (bracketing lines). *p <0.05, **p <0.02, n = 6 pairs.

undergoing desaturation may be accounted for by at least 2 processes. Vasopressin may have caused a net efflux of calcium from the cells, or, the rate of calcium exchange may have increased. In an attempt to distinguish between these 2 possibilities, the following experiment was performed. Suspensions were labelled with ^{45}Ca for 90 min. The suspensions were then divided into 4 aliquots, one served as control, the remainder received vasopressin (0.1, 1.0, or 20.0 mU/ml). Aliquots were taken from these suspensions 1, 5, and 15 minutes after the addition of vasopressin and the ^{45}Ca content of the cells determined (see Methods). In these experiments ^{45}Ca was present in the bathing media at all times. Therefore, any decrease in ^{45}Ca content of the cells would represent net calcium efflux while simple enhancement in calcium-calcium exchange would cause no change in ^{45}Ca content. As shown in Fig. 21 vasopressin elicited a net loss of ^{45}Ca at concentrations of 1 and 20 mU/ml, concentrations which are physiologically active, while vasopressin, 0.1 mU/ml, a concentration which only rarely causes an enhancement in water permeability in intact toad bladders, did not elicit any change in ^{45}Ca content of the cells. These experiments suggested that the enhanced ^{45}Ca efflux observed in the perturbation experiments described previously was due to net efflux of calcium.

Effects of preincubation with vasopressin on ^{45}Ca content and efflux. Fig. 22 demonstrates that ^{45}Ca exchange in the cells is best described by a series of exponential components. The steady state experiment just described cannot distinquish the effects of vasopressin on any particular component of ^{45}Ca exchange. Thus, experiments were performed in an attempt to gain a more quantitative

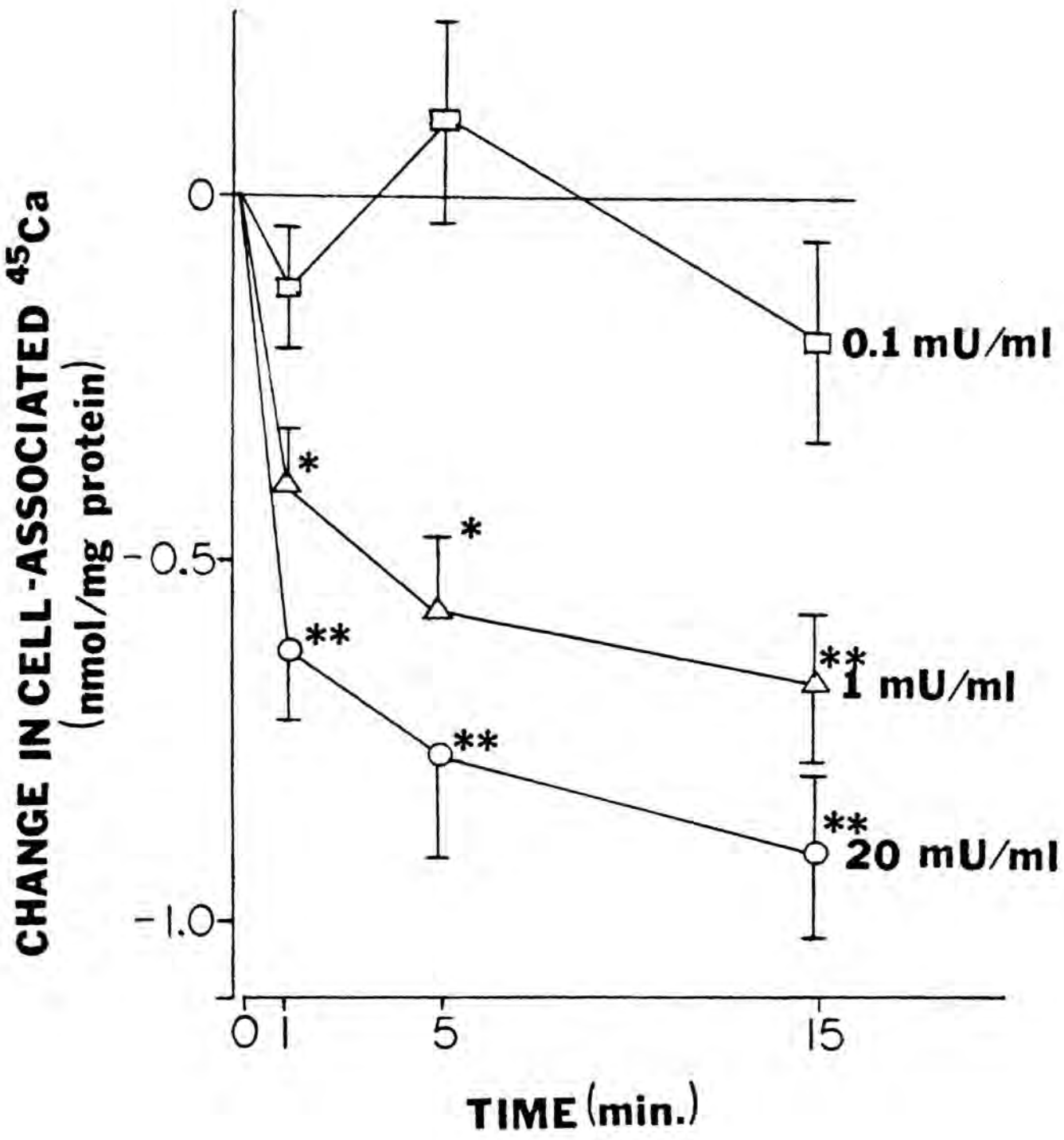


Figure 21. Effect of vasopressin on steady state ^{45}Ca content of isolated toad urinary bladder epithelial cells. Cells were labelled with ^{45}Ca for 90 minutes. Vasopressin (0.1, 1, or 20 mU/ml) was then added to the experimental suspensions (time = 0 on the graph) and at various times afterward aliquots were taken from both the experimental and control suspensions and ^{45}Ca content of the cells was determined by the lanthanum method. ^{45}Ca concentration in the control cells was 2.87 $\pm$ 0.42, 2.90 $\pm$ 0.33, 2.97 $\pm$ 0.38, and 3.10 $\pm$ 0.26 nmol/mg protein at time = 0, 1, 5, and 15 minutes. (*$p < 0.05$, **$p < 0.02$, compared to control, n = 5 for each point).

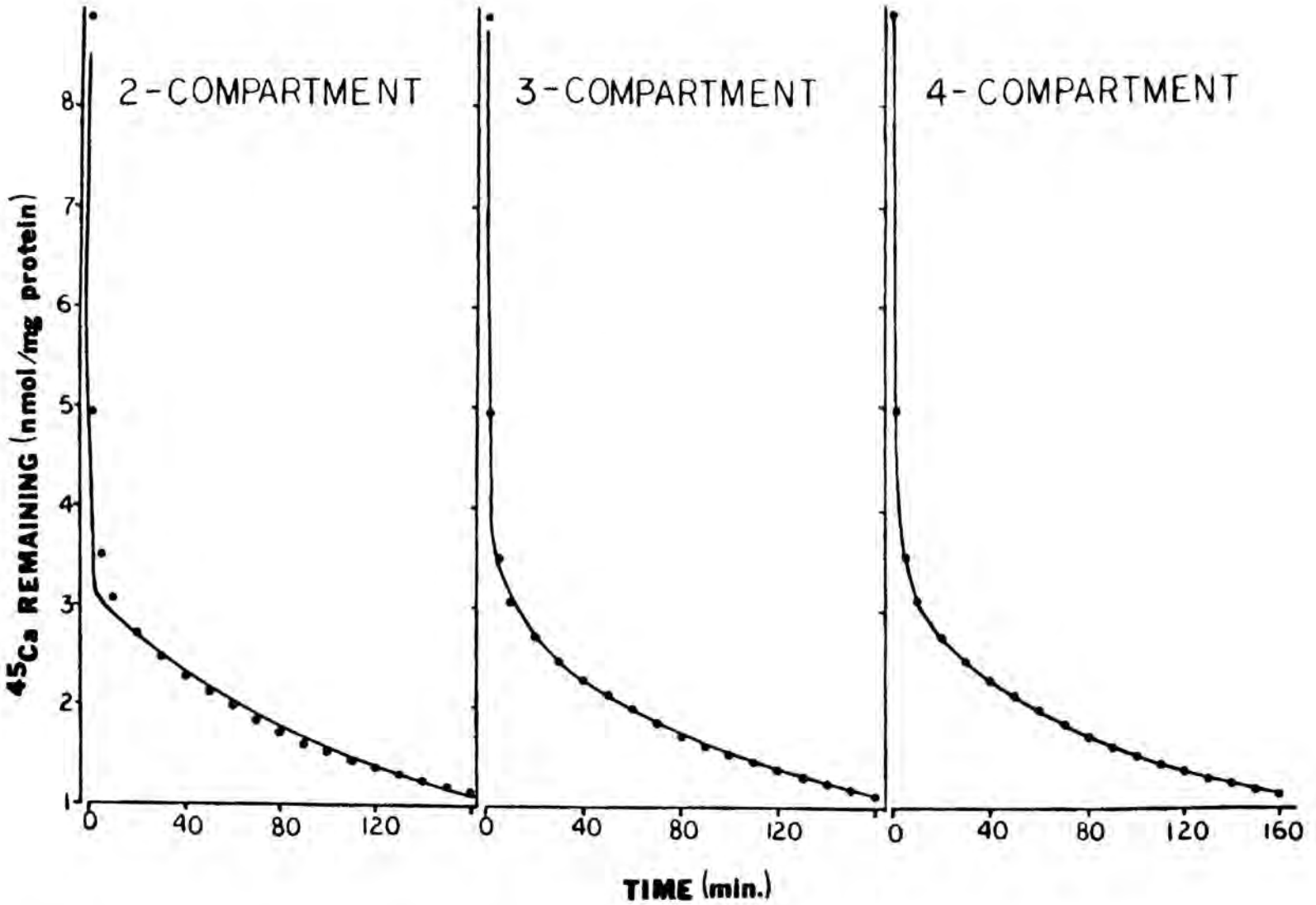


Figure 22. ^{45}Ca efflux from epithelial cells isolated from the toad urinary bladder. Points represent the actual data for a single experiment. The curves were generated by computer and are the best fits for each case (see Methods). Estimates of the relative differences, functional error, of the fitted curves to the actual data were calculated (see Methods) for each case: 0.040134, 0.004212, and 0.000983, for the 2, 3, and 4 compartment cases, respectively, where a lower functional error signifies a better fit.

estimate of the effect of vasopressin on calcium content of the several components of calcium exchange in the cells.

In these experiments vasopressin was added to the experimental aliquots of the suspensions before the addition of the ^{45}Ca label. After 15 min preincubation the control and experimental aliquots were simultaneously labelled with ^{45}Ca for 90 min. Vasopressin remained in the experimental aliquots during the labelling and desaturation periods. The effects of preincubation of the cells with vasopressin (10 mU/ml) are shown in Fig. 23. Preincubation with vasopressin (10 mU/ml) resulted in significantly less total uptake of ^{45}Ca during the labelling period, 7.76±0.62 nmol/mg protein, compared to the paired control suspensions, 9.95±0.58 nmol/mg protein ($p<0.05$, n=6 pairs). Fig. 23 demonstrates that the efflux of ^{45}Ca from the cells can be well described by a series of 4 exponential components. Vasopressin decreased the time-0 intercepts of 2 of the 4 components. The intercept of the most rapidly exchanging component, here called X_0 (X_i refers to the actual intercepts of each component while S_i is the pool size calculated from X_i), was not affected by vasopressin (3.77±0.49 nmol/mg protein in the control suspensions compared to 3.52± 0.52 nmol/mg protein in the suspensions incubated in vasopressin). The intercept of the second component, X_1, was reduced in size from 2.03± 0.47 to 0.96±0.14 nmol/mg protein ($p< 0.025$, n=6 pairs). The intercept of the third component, X_2, was not affected by vasopressin (1.38±0.28 versus 1.43±0.34 nmol/mg protein in controls). The size of the fourth component, X_3, was decreased by vasopressin from 2.72±0.26 to 1.90±0.34 nmol/mg protein ($p<0.01$, n=6) pairs). While vasopressin enhanced the apparent rate

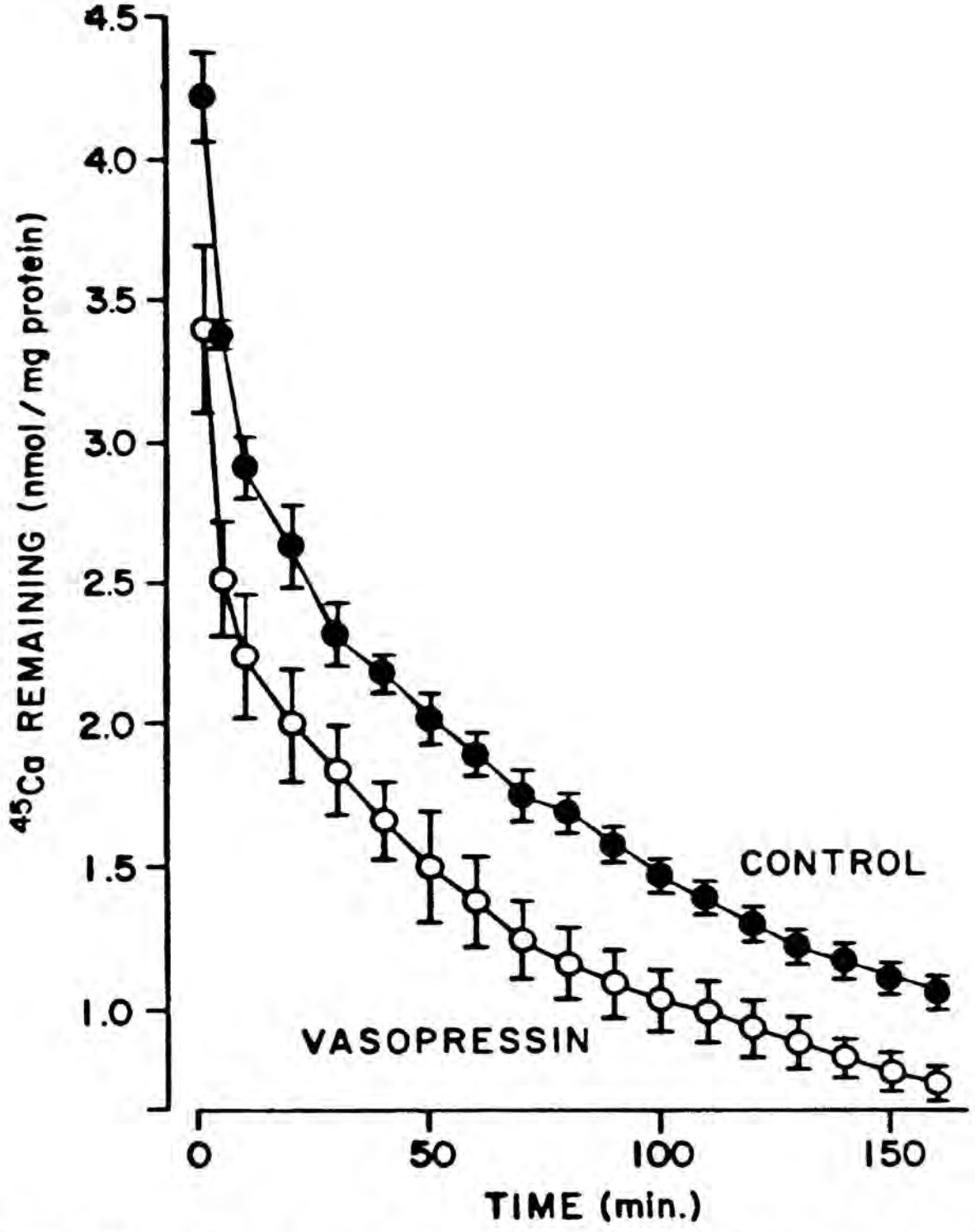


Figure 23. ^{45}Ca efflux from isolated toad bladder epithelial cells preincubated with vasopressin (10 mU/ml). Cell suspensions were divided into 2 aliquots, 1 of which was incubated for 15 minutes with vasopressin (10 mU/ml). Then both aliquots were labelled with ^{45}Ca for 90 minutes and desaturated. The experimental aliquots contained vasopressin during the labelling and desaturation periods. Each vasopressin point is different from the respective control point ($p < 0.02 - 0.05$, $n = 6$ pairs). ^{45}Ca content at time = 0, not shown for the sake of clarity, were 9.95 ± 0.58 and 7.76 ± 0.62 nmol/mg protein, control and vasopressin, respectively.

constant of efflux of ^{45}Ca from cells undergoing desaturation (Fig. 19), it did not significantly change the steady state rate constants of ^{45}Ca efflux of any of the components (Table XIII). In the experiments of Cuthbert and Wong (1974), the enhancement rate constant of efflux was transitory, supporting the present observations.

In a separate series of experiments the effects of a lower concentration of vasopressin (1 mU/ml) were assessed on steady state ^{45}Ca efflux. In these experiments vasopressin (1 mU/ml) reduced the size of X_1 from 1.64±0.30 to 1.14±0.23 nmol/mg/protein ($p<0.05$, n=6 pairs), and X_3 was reduced in size from 2.48±0.22 to 2.06±0.28 nmol/mg protein ($p<0.05$, n=6 pairs). Neither X_0 nor X_3 were affected.

Effects of exogenous cAMP on ^{45}Ca efflux from prelabelled cells. cAMP is generally considered to be the second messenger for vasopressin-stimulated water permeability (Orloff and Handler, 1967), and exogenous cAMP and several of its analogs mimic the effect of vasopressin on water permeability (Orloff and Handler, 1962). However, Cuthbert and Wong (1974) found that in intact hemibladders, exogenous cAMP actually inhibited ^{45}Ca efflux from preloaded hemibladders. To determine whether exogenous cAMP mimicks vasopressin in isolated epithelial cells, pertubation and steady state experiments were performed.

Fig. 24 shows the effect of adding cAMP (10 mM) to cells already undergoing desaturation. During the first 10 min cAMP caused a slight inhibition of ^{45}Ca efflux, 16±4% ($p<0.05$, n=6). This inhibition was not exhibited at 20 or 30 min after the addition of cAMP. Upon the removal of cAMP from the bathing media, ^{45}Ca efflux transiently increased to 125±9% ($p<0.05$, n=5 pairs) of control at 10 min

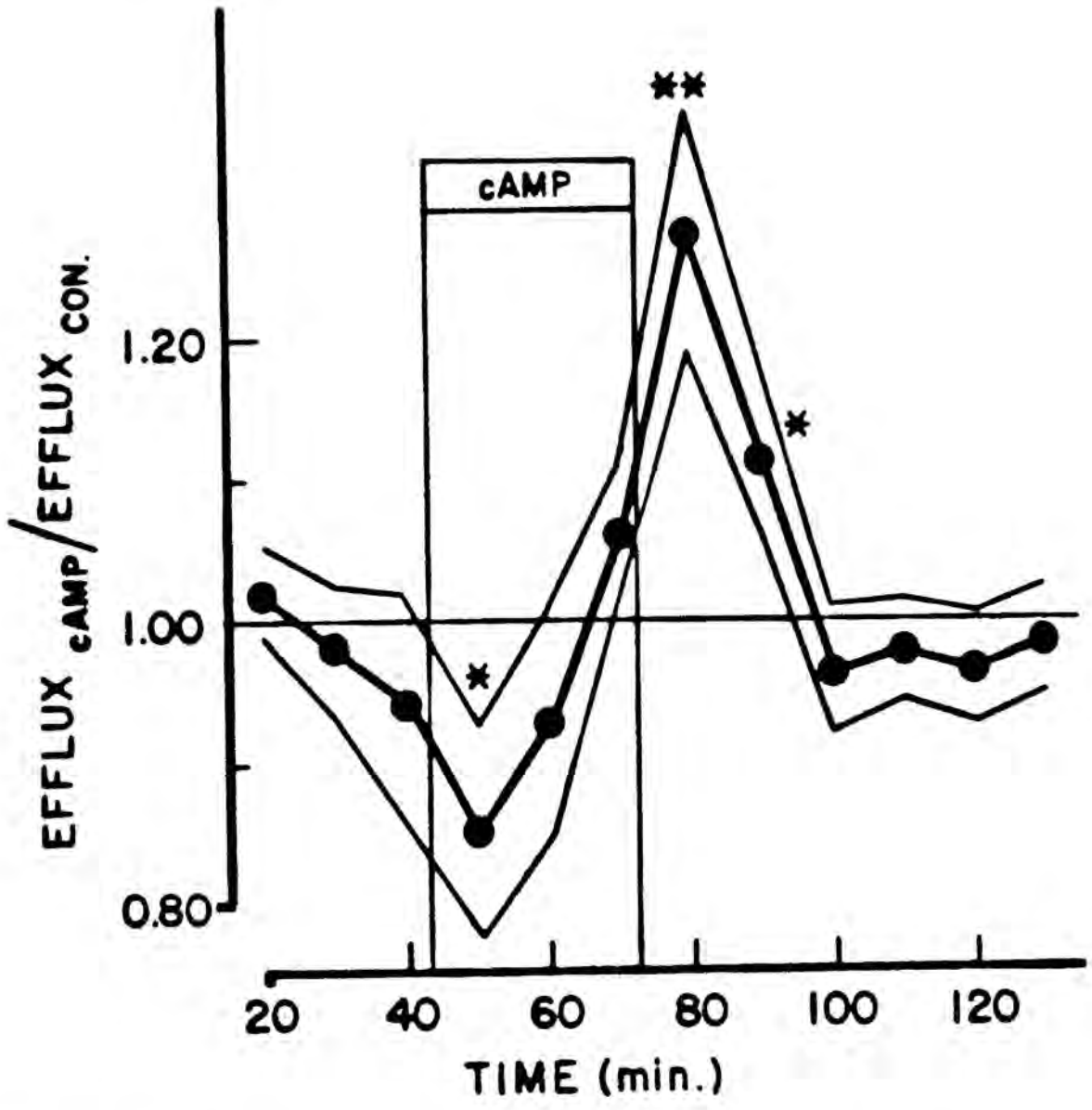


Figure 24. Effect of cAMP on ^{45}Ca efflux from isolated toad bladder epithelial cells. Suspensions were labelled with ^{45}Ca for 90 minutes. Each suspension was divided into 2 aliquots and desaturated simultaneously. After 40 minutes of desaturation exogenous cAMP (10 mM) was added to 1 of each pair of aliquots (*$p < 0.05$, **$p < 0.02$, $n = 6$ pairs).

and 111±5% ($p<0.05$, n=5 pairs) of control at 20 min after the removal of cAMP. This effect of cAMP to enhance ^{45}Ca efflux after its removal was also observed in the figures published by Cuthbert and Wong (1974).

In an additional series of experiments, cAMP was added to the cells for 15 min prior to the prelabelling period with ^{45}Ca. Analysis of the desaturation curves (Fig. 25) showed that in contrast to vasopressin, cAMP did not affect total uptake of ^{45}Ca (9.32±0.62 nmol/mg protein versus 8.97±0.72 nmol/mg protein in control aliquots). However, the intercepts of the third, X_2, and fourth, X_3, components of efflux were affected by cAMP. In contrast to vasopressin, cAMP increased the intercept of the third component to 2.04±0.41 nmol/mg protein compared to 1.30± 0.32 nmol/mg protein in the control suspensions ($p< 0.05$, n=5 pairs) while, like vasopressin, cAMP decreased the intercept of the fourth component from 2.68±0.28 nmol/mg protein in the control suspensions to 2.21±0.26 nmol/mg protein in the suspensions preincubated with cAMP ($p< 0.05$, n=5 pairs).

COMPARTMENTAL ANALYSIS OF ^{45}Ca IN ISOLATED TOAD BLADDER EPITHELIAL CELLS AND THE EFFECTS OF VASOPRESSIN AND cAMP

Characteristics of the steady state ^{45}Ca efflux curve. The efflux of ^{45}Ca from toad bladder epithelial cells labelled for 90 min with ^{45}Ca was measured for 140-160 min. Fig. 22 shows a typical efflux curve. Graphical analysis yielded 4 calcium pools. The most rapidly exchanging pool, termed S_o', was assigned as extracellular ^{45}Ca trapped in the bathing medium remaining with the cell pellet after the initial washing. This assignment was made based on the

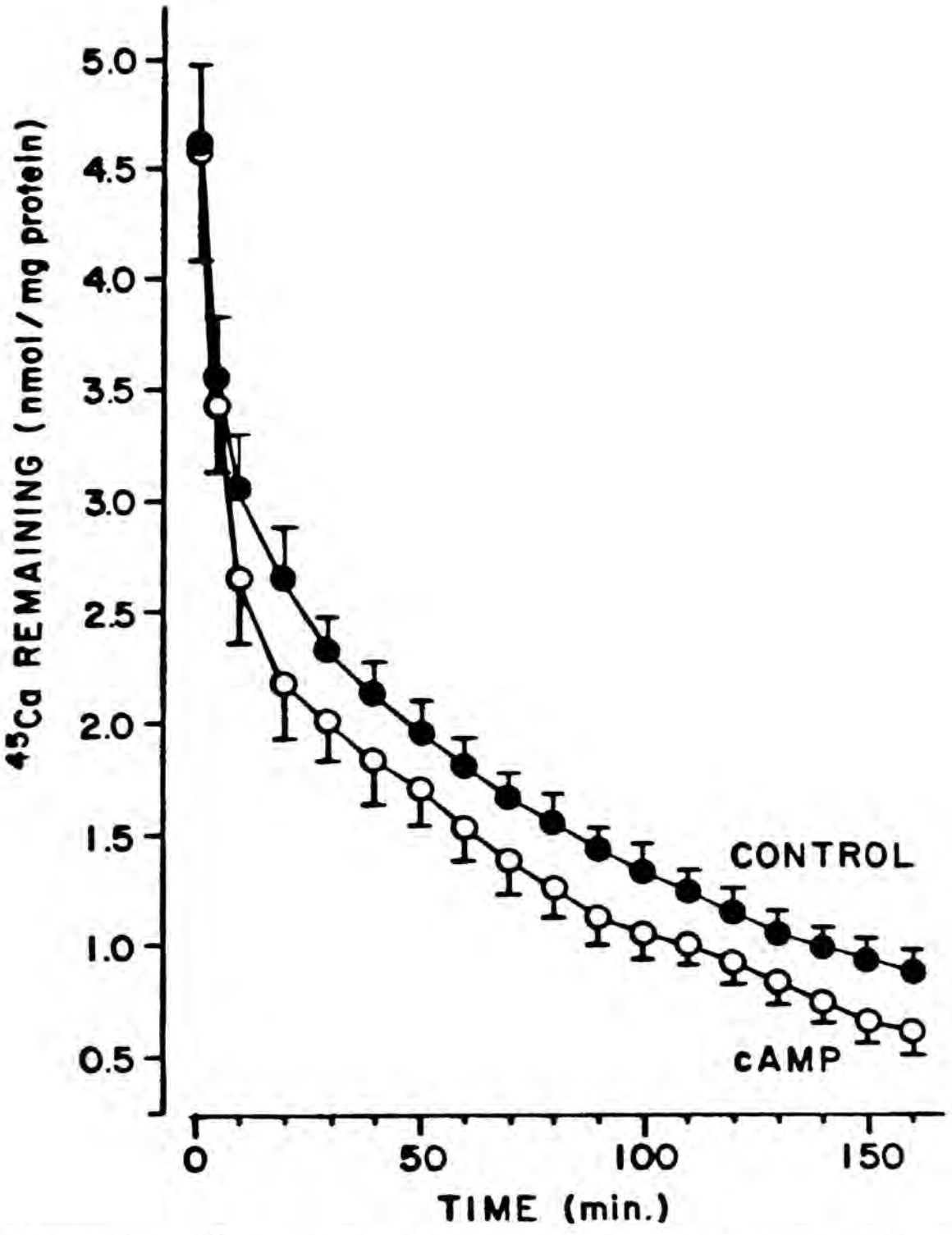


Figure 25. ^{45}Ca efflux from isolated toad bladder epithelial cells preincubated with exogenous cAMP (10 mM). Cell suspensions were divided into 2 aliquots, 1 of which was incubated for 15 minutes with cAMP (10 mM). Then both aliquots were labelled with ^{45}Ca for 90 minutes and desaturated. The experimental aliquots contained cAMP during the labelling and desaturation periods. Curves were analyzed as ln ^{45}Ca remaining versus time and divided into linear components (see Methods). Analysis of covariance revealed significant differences between the control and experimental values for components S_2 ($p < 0.01$, $n = 5$ pairs) and S_3 ($p < 0.02$, $n = 5$ pairs). ^{45}Ca content at time = 0 were 8.79 ± 0.72 and 9.32 ± 0.62 nmol/mg protein, control and cAMP, respectively.

similarities in both rate constant of exchange and in the "space" occupied by this ^{45}Ca pool and those for [^{3}H]-inulin and [^{125}I]-bovine serum albumin. Thus, the rate constant of exchange for ^{45}Ca was 2.60±0.42 min^{-1} (n=12), for [^{3}H]-inulin 2.55±0.56 min^{-1} (n=4), and for [^{125}I]-albumin 2.86±0.48 min^{-1} (n=4). Similarly, ^{45}Ca space for pool S_o' was 28±5 µl (n=12), for [^{3}H]-inulin 21±4 µl (n=4), and for [^{125}I]-albumin 23±6 µl (n=4).

The remaining 3 exchangeable calcium pools had time constants [$1/k_{ij}$, that is, average "residence time" in the pool (Atkins, 1974)] of 4.4, 14, and 154 min, and contained 1.52, 1.16, and 6.01 nmol calcium/ mg protein, respectively (Table VIII). Thus, in the present experiments where uptake time was 90 min, the most rapidly exchanging pool, S_1, and the intermediate pool, S_2, were 100% loaded before commencement of desaturation, and the slowly exchanging pool, S_3, was 44% loaded. Thus, the 90 min labelling period was chosen since it is the minimum time required to reach 100% loading of pool S_2. Upon commencement of desaturation, the calculated time taken to reach 1% of initial radioactivity was 20 min for S_1, 63 min for S_2, and 710 min for S_3, and to reach 0.1% of initial radioactivity, 30 min for S_1, 95 min for S_2, and 1050 min for S_3.

Arrangement of the Calcium Pools. As demonstrated in the Methods, the calculations for the actual rate constants of desaturation and pool sizes from the apparent values obtained from the desaturations depend upon whether the calcium pools are arranged in parallel without interconnections, or in series, such that the calcium contained in the more slowly exchanging pools must pass successively through the more rapidly exchanging pools to reach the

bathing medium. Thus, several experiments were performed in an effort to gain some insight into the structural identities of the exchangeable calcium pools, so that inference might be made concerning arrangement of the pools.

Effects of lanthanum on ^{45}Ca efflux. La^{3+} has greater affinity for membrane Ca^{2+}-binding sites than does Ca^{2+} (Sanborn and Langer, 1970). Thus, La^{3+} is often used to displace Ca^{2+} from membrane binding sites (Sanborn and Langer, 1970; van Breeman et al., 1972). Further, La^{3+} does not penetrate most biological membranes (Langer and Frank, 1972) and has been shown by electron microscopy to not cross the plasma membrane of toad urinary bladder epithelial cells (Davis, 1981). Thus, La^{3+} was used in an attempt to displace any ^{45}Ca bound to the extracellular surface of the epithelial cells. In physiological experiments, it was found that 1 mM La^{3+} in tris-Ringer's solution containing 1 mM Ca^{2+}, completely inhibited vasopressin-stimulated water flow. Thus, 1 mM La^{3+} was used in the present experiments. In 4 experiments, simultaneous desaturation was commenced in 2 aliquots of a single cell suspension which had been prelabelled with ^{45}Ca for 90 min. After the 1 min decanting, the Ringer's solution added to the experimental suspension contained 1 mM La^{3+} in addition to 1 mM Ca^{2+}. This resulted in an enhanced efflux to 182%±24 ($p< 0.01$, $n=4$) of control (Fig. 26). Upon removal of the La^{3+} at the 10 min decanting, efflux returned toward the control rate. In 4 similar experiments La^{3+} was added after the 50 min decanting, when only pools S_2 and S_3 were still labelled. In these experiments ^{45}Ca was not displaced, instead ^{45}Ca efflux was inhibited to 21±4% ($p<0.01$, $n=4$) of control (Fig 26). These results suggest

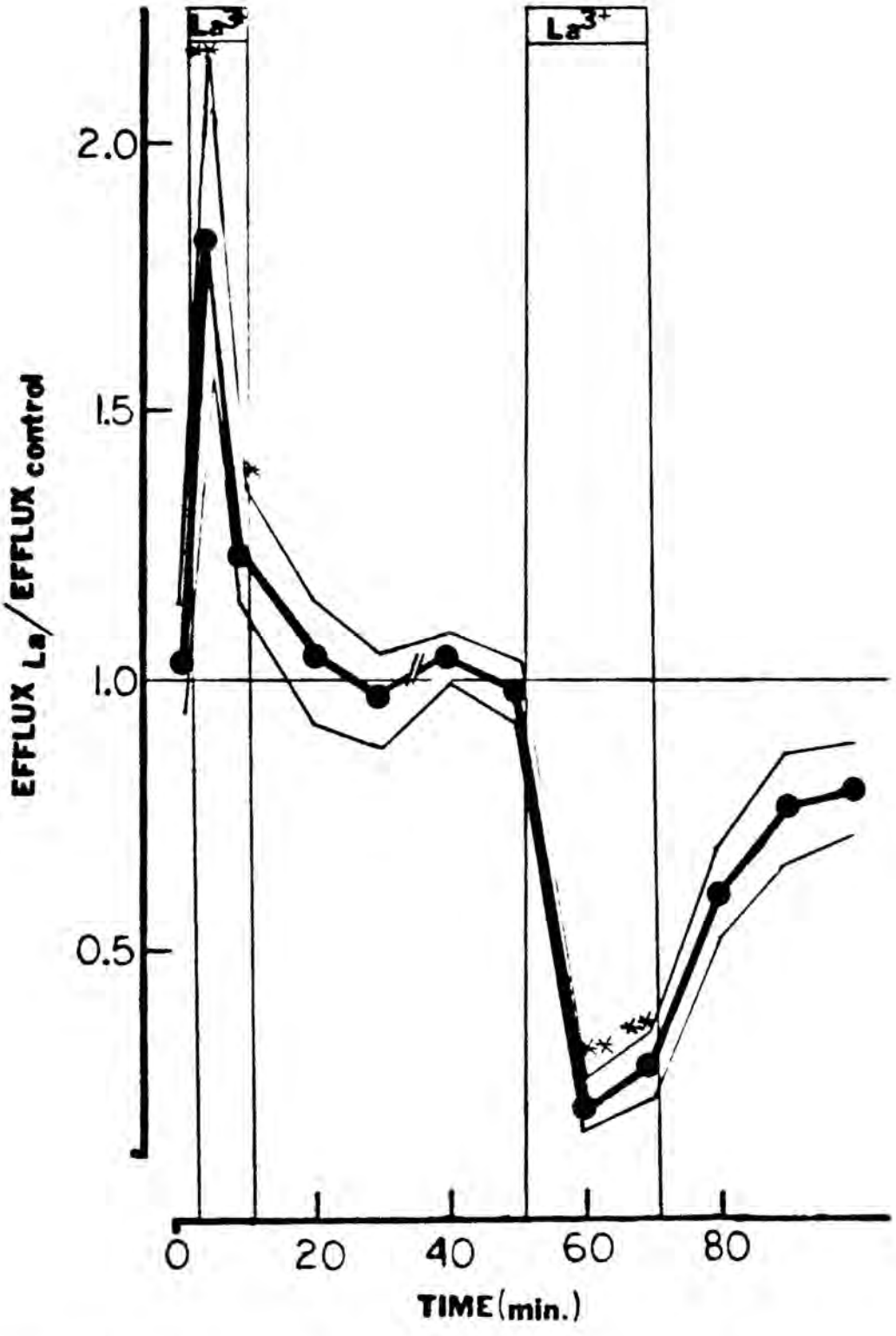


Figure 26. Effect of La^{3+} on ^{45}Ca efflux from isolated toad bladder epithelial cells. Suspensions were labelled with ^{45}Ca for 90 minutes. Each suspension was divided into 2 aliquots which were desaturated simultaneously. After 1 minute or 50 minutes of desaturation La^{3+} (1 mM) was added to 1 of each pair of aliquots (*$p < 0.05$, **$p < 0.01$, $n = 4$ pairs).

that pool S_1 consists, at least partly, of superficially bound ^{45}Ca and that ^{45}Ca efflux from pool S_2 or S_3 consists, at least in part, of ^{45}Ca which must cross the plasma membrane from inside the cells to reach the bathing medium, and that this transport is blocked by 1 mM La^{3+}.

In an additional 4 experiments the effects of La^{3+} on ^{45}Ca compartmental analysis were studied. Claret-Bethon et al. (1977) found that the displacement of ^{45}Ca from pool S_1 by La^{3+} is transient, being complete within only 30 sec. In the present experiments labelling with ^{45}Ca took place for the usual 90 min. The cells were pelleted, and to the wash solution of the experimental suspensions was added 1 mM La^{3+}. The suspensions were stirred for 30 sec, pelleted, and normal Ringer's solution was added to both control and experimental suspensions and desaturation was commenced in the usual fashion. La^{3+} displaced 48% of the ^{45}Ca in pool S_1 (Table IX). The absolute decrement calculated from these experiments is somewhat suspect since the rate constant of efflux from pool S_1 was greatly increased; thus, the rate constants of efflux from pools S_o' and S_1 were within 3-fold of one another. Myhill (1967) has calculated that if only 5% error exists in the experimental data, then 2 rate constants of efflux can be resolved if only they differ by a factor of 4. In the present experiments the experimental error is probably greater than 5%, since, for example, the radioactivity was determined to a precision of only 3%. In these experiments the sizes of pools S_2 and S_3 were not affected by La^{3+} (Table IX). However, even though La^{3+} was not nominally present during the desaturations, the rate constants of efflux from these compartments were significantly

TABLE IX

EFFECTS OF RINSING WITH La^{3+} ON ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			Fast Pool (S_1)				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Control	1.64[a] ±0.18	0.398[a] ±0.047	0.248 ±0.031	1.34[a] ±0.18	0.398[a] ±0.047	0.248 ±0.031		
La^{3+}	0.82 ±0.10	0.728 ±0.033	0.903 ±0.044	0.82 ±0.10	0.228 ±0.033	0.263 ±0.044		
	Intermediate Pool (S_2)			Intermediate Pool (S_2)				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Control	1.01 ±0.21	0.079 ±0.006	0.077[a] ±0.007	2.00 ±0.22	0.111[a] ±0.011	0.028 ±0.003	0.057[a] ±0.006	0.014 ±0.002
La^{3+}	1.22 ±0.30	0.055 ±0.008	0.045 ±0.006	2.04 ±0.24	0.072 ±0.008	0.021 ±0.003	0.035 ±0.004	0.009 ±0.002
	Slow Pool (S_3)			Slow Pool (S_3)				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Control	4.38 ±0.37	0.029[a] ±0.004	0.0062[a] ±0.0007	3.36 ±0.38	0.028 ±0.003	0.0081[a] ±0.0010		
La^{3+}	4.40 ±0.44	0.018 ±0.003	0.0044 ±0.0009	3.54 ±0.40	0.021 ±0.003	0.0056 ±0.0008		

n=4. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, La^{3+} = 1 mM.

[a] $p < 0.05$ compared to La^{3+}.

decreased. Thus, these "steady state" experiments tend to confirm the results obtained with the perturbation experiments, that pool S_1 represents, at least in part, calcium bound to extracellular sites and that pools S_2 and S_3 represent calcium which is located inside the cells, or at least, which is bound to sites not accessable to lanthanum.

In another series of experiments La^{3+} (1 mM) remained in the experimental suspensions for the entire desaturation. In these suspensions S_2 was undetectable while S_3 was significantly larger than in control suspensions (Table X). Thus, the effects of La^{3+} on ^{45}Ca efflux in these experiments was similar to that which was noted by Langer and Frank (1972) in fibroblasts and myoblasts in culture, where La^{3+} slowed efflux of ^{45}Ca from one compartment to a rate only about 10% as rapid.

Effects of EGTA on ^{45}Ca efflux. In several other tissues a slowly exchanging calcium pool is observed with a time constant of efflux similar to that of the slowly exchanging pool, S_3, observed in the present experiments (Sanborn and Langer, 1970; Claret-Berton et al, 1977). The latter have concluded that this slowly exchanging pool represents extracellular calcium based on displacement by the calcium-chelating agent, EGTA. Indeed, in intact isolated toad bladders, Cuthbert and Wong (1974) concluded that a slowly exchanging calcium pool which may be identical to the pool S_3 in the present experiments, is extracellular in location based on displacement by EGTA. Thus, several perturbation experiments were performed using EGTA. Simultaneous desaturation was commenced in 2 aliquots from prelabelled cell suspensions. After the 100 min decanting, a time

TABLE X

EFFECTS OF La^{3+} ON ^{45}Ca KINETICS

	Parallel Case		
	Fast Pool (S_1)		
	S_1	ρ_{10}	k_{10}
Control	1.48[a] ±0.20	0.337[a] ±0.042	0.232 ±0.035
La^{3+}	0.80 ±0.10	0.702 ±0.023	0.856 ±0.042
	Intermediate Pool (S_2)		
	S_2	ρ_{20}	k_{20}
Control	1.25 ±0.22	0.086 ±0.006	0.072 ±0.007
La^{3+}	N.D.	N.D.	N.D.
	Slow Pool (S_3)		
	S_3	ρ_{30}	k_{30}
Control	4.25[a] ±0.40	0.029[a] ±0.004	0.0068[a] ±0.0007
La^{3+}	5.34 ±0.62	0.019 ±0.004	0.0038 ±0.0006

n=4. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, La^{3+} = 1 mM.

[a] $p < 0.05$ compared to La^{3+}.

at which only pool S_3 contained ^{45}Ca, the Ringer's solution added to the experimental suspensions contained no added calcium and 2 mM EGTA. This resulted in an enhanced efflux to 340±46% of control efflux (Fig. 27). EGTA was allowed to remain for only one time period since other experiments have demonstrated that toad bladder epithelial cells do not appear viable after prolonged incubation with EGTA in the absence of calcium. These experiments suggest that S_3 may represent slowly exchanging extracellular calcium.

In additional experiments, the effects of EGTA on "steady state" ^{45}Ca efflux was studied. Suspensions were labelled for 90 min. The cells were pelleted, and the rinsing Ringer's solution for the experimental suspensions contained no added Ca^{2+} and 2 mM EGTA. The suspensions were pelleted again and normal Ringer's solution was added to both control and experimental suspensions and desaturation was commenced as usual. EGTA displaced essentially all the ^{45}Ca in pool S_1 (Table XI). The size of pool S_2 was not significantly affected, while the size of pool S_3 was decreased by 56-70% (Table XI). Similarly, the time constants of efflux were lowered from 4.3 to 1.0 min, 14 to 11 min, and 150 to 74 min, for pools S_1, S_2, and S_3, respectively.

Intracellular calcium. The data obtained with La^{3+} and EGTA suggest that calcium pool S_1 is completely extracellular. Neither La^{3+} nor EGTA affected the size of pool S_2. This suggests that pool S_2 may represent intracellular calcium. Borle (1972) and Borle and Uchikawa (1978) have evidence to suggest that at least part of S_3 can be attributed to mitochondria. In the present experiments EGTA decreased the size of S_3 by about 60% while La^{3+} was without any

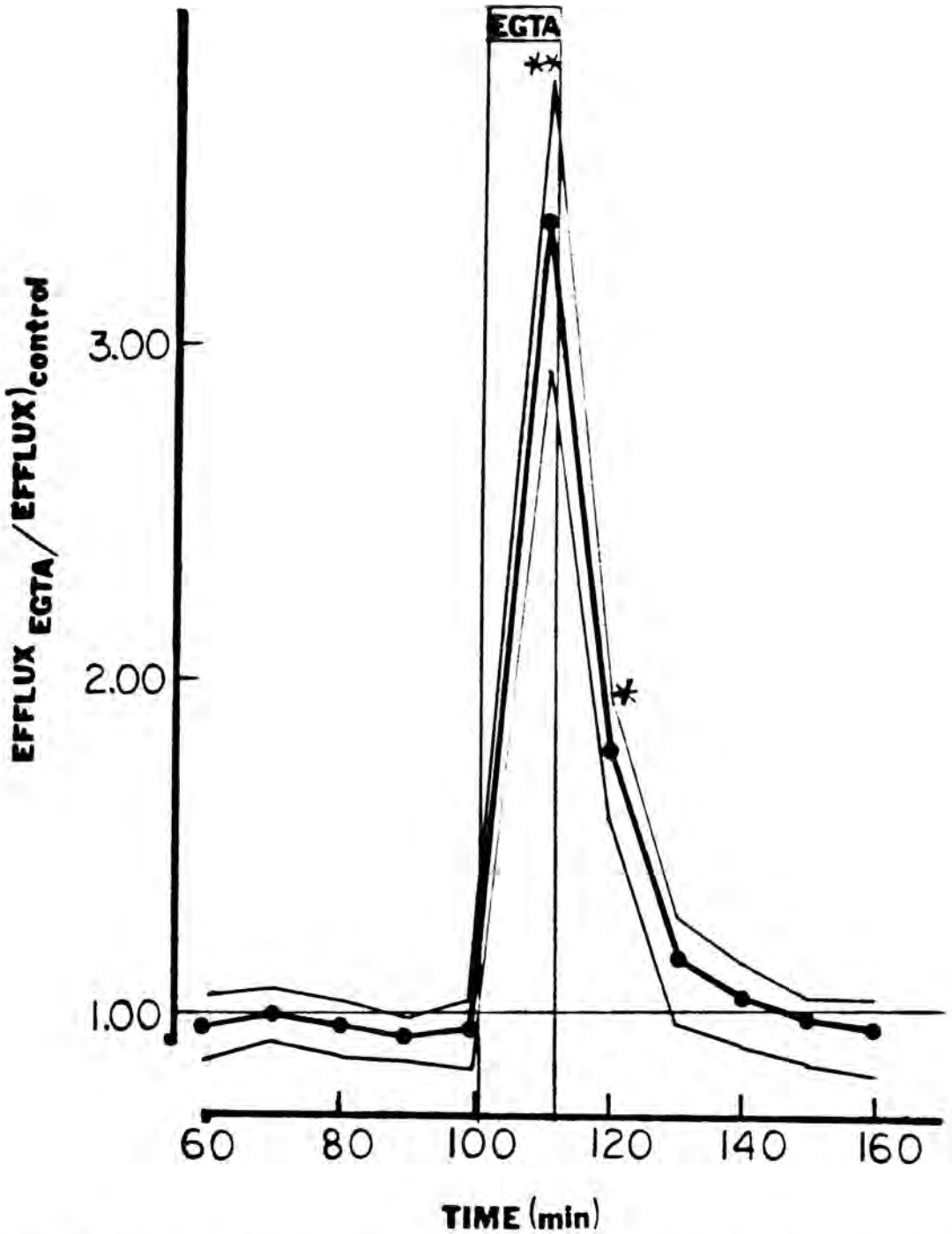


Figure 27. Effect of EGTA on ^{45}Ca efflux from isolated toad bladder epithelial cells. Suspensions were labelled with ^{45}Ca for 90 minutes. Each suspension was divided into 2 aliquots which were desaturated simultaneously. After 100 minutes of desaturation Ca^{2+}-free Ringer's solution containing EGTA (2 mM) was added to 1 of each pair of aliquots (*$p < 0.01$, **$p < 0.001$, $n = 4$ pairs).

TABLE XI
EFFECTS OF RINSING WITH EGTA ON ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			**Fast Pool (S_1)**				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Control	1.83[c] ±0.18	0.372 ±0.059	0.206[b] ±0.021	1.83[c] ±0.18	0.372 ±0.059	0.206[b] ±0.021		
EGTA	0.34 ±0.02	0.360 ±0.040	1.06 ±0.042	0.34 ±0.02	0.360 ±0.040	1.06 ±0.042		
	Intermediate Pool (S_2)			**Intermediate Pool (S_2)**				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Control	1.17 ±0.31	0.075[c] ±0.007	0.064[c] ±0.004	2.36 ±0.11	0.118[b] ±0.009	0.043[a] ±0.004	0.050[b] ±0.006	0.018 ±0.003
EGTA	1.26 ±0.31	0.312 ±0.042	0.248 ±0.025	2.30 ±0.37	0.456 ±0.120	0.031 ±0.003	0.198 ±0.028	0.013 ±0.002
	Slow Pool (S_3)			**Slow Pool (S_3)**				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Control	6.23[c] ±0.49	0.038 ±0.004	0.0061[b] ±0.0004	4.84[c] ±0.35	0.043[a] ±0.004	0.0089[b] ±0.0009		
EGTA	2.30 ±0.23	0.033 ±0.002	0.0143 ±0.0009	2.08 ±0.17	0.031 ±0.003	0.0149 ±0.0013		

n=4. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, EGTA = 2 mM.

[a] $p < 0.05$, [b] $p < 0.02$, [c] $p < 0.01$, compared to EGTA.

effect. Since mitochondria are considered to play an important role in buffering intracellular calcium, the effects of two inhibitors of mitochondrial function, antimycin A and cyanide, were assessed on steady state calcium metabolism. The effects of these 2 agents were similar and the data from the 2 were pooled (Table XII). The mitochondrial inhibitors decreased the size of pool S_3 ca. 40%. At the same time the size of pool S_2 tended to be increased. This effect is similar to that described for the effect of mitochondrial inhibitors on calcium metabolism in whole liver (Carafoli, 1967). In that tissue inhibition of mitochondrial function was associated with decreased stores of calcium in the mitochondria, while microsomal calcium was increased.

Effects of vasopressin on ^{45}Ca compartmental analysis. While most of the data gathered on the arrangement of the exchangeable calcium compartments in the isolated toad bladder epithelial cells are consistent with pool S_2 and at least part of S_3 being internal and thus probably in series with one another, the possibility that the compartments may actually be in parallel cannot be ignored. Therefore, all compartmental analyses were performed for both a modified series and parallel cases. The modified series case of Uchikawa and Borle (1978) was used. In this model the most rapidly exchanging compartment, S_1, is not considered to be in series with compartments S_2 and S_3 since it appears to be composed of loosely associated extracellular calcium. The present data with EGTA treatment supports the use of this model. EGTA displaced S_1 without affecting the size of S_2. Further, the first component, S_0', was not

TABLE XII

EFFECTS OF MITOCHONDRIAL INHIBITORS ON ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			Fast Pool (S_1)				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Control	1.83 ±0.20	0.430 ±0.058	0.247 ±0.025	1.83 ±0.20	0.430 ±0.058	0.247 ±0.025		
Inhibitors	1.72 ±0.18	0.444 ±0.060	0.283 ±0.031	1.72 ±0.18	0.444 ±0.060	0.283 ±0.031		
	Intermediate Pool (S_2)			Intermediate Pool (S_2)				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Control	1.42[a] ±0.28	0.094[a] ±0.015	0.064[a] ±0.008	2.42a ±0.26	0.143 ±0.048	0.042 ±0.008	0.042[a] ±0.004	0.011 ±0.001
Inhibitors	1.90 ±0.32	0.142 ±0.035	0.105 ±0.029	3.18 ±0.41	0.201 ±0.040	0.034 ±0.008	0.106 ±0.022	0.014 ±0.002
	Slow Pool (S_3)			Slow Pool (S_3)				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Control	5.31[b] ±0.68	0.034 ±0.004	0.0075[a] ±0.0005	3.95[c] ±0.52	0.042 ±0.008	0.0101[a] ±0.0010		
Inhibitors	3.15 ±0.72	0.039 ±0.010	0.0111 ±0.0012	2.35 ±0.50	0.034 ±0.008	0.0141 ±0.0011		

n=6. 3 KCN, and 3 antimycin A. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, KCN = 2 mM, antimycin A = 10 μM.

[a] $p < 0.05$, [b] $p < 0.02$, [c] $p < 0.01$, compared to inhibitors.

considered since it appeared to represent trapped bathing media calcium.

As shown previously, incubation of the cells with vasopressin prior to ^{45}Ca labelling caused a reduction in the total ^{45}Ca uptake into the cells (Fig 23). When these data were subjected to compartmental analysis vasopressin was seen to greatly reduce the amount of ^{45}Ca binding in 2 pools, S_1 and S_3, while analysis of the series case also suggested a small reduction in the size of pool S_2 (Table XIII). This observation is consistent with that reported in Fig. 21, which also demonstrated a reduction in cellular ^{45}Ca in response to vasopressin. The rate constants of ^{45}Ca flux were not different from control with the exception of k_{23} (Table XIII) in the series model, which was reduced in size ($p<0.02$, n=6 pairs), suggesting that the permeability of a membrane controlling flux from S_2 into S_3 was reduced by vasopressin. The fluxes of ^{45}Ca were reduced between all compartments except ρ_{20} in the parallel model (Table XIII). Reduction in steady state fluxes was not surprising in view of the reduced compartment sizes. In additional experiments, a lower concentration of vasopressin (1 mU/ml) was also found to significantly reduce the size of pool S_3 (Table XIV).

Effects of cAMP on ^{45}Ca compartmental analysis. Compartmental analysis of the ^{45}Ca efflux curves in Fig 24 demonstrate that cAMP (10 mM) did not affect the kinetic parameters of S_1; however, S_3 was significantly reduced in size ($p<0.05$) (Table XV). The flux of ^{45}Ca from S_2 to S_0 (ρ_{20}) was significantly ($p<0.02$-0.05) increased in both the parallel and series cases, while k_{20} was slightly elevated in both cases, but not significantly. The enhanced size of S_2

TABLE XIII

EFFECTS OF VASOPRESSIN (10 mU/ml) ON ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			**Fast Pool (S_1)**				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Control	1.67[a] ±0.20	0.429[b] ±0.067	0.254 ±0.010	1.67[a] ±0.20	0.429[b] ±0.067	0.254 ±0.010		
Vasopressin	0.86 ±0.12	0.194 ±0.028	0.230 ±0.033	0.86 ±0.12	0.194 ±0.028	0.230 ±0.033		
	Intermediate Pool (S_2)			**Intermediate Pool (S_2)**				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Control	1.11 ±0.06	0.071 ±0.009	0.064 ±0.006	2.30a ±0.26	0.102 ±0.012	0.035[b] ±0.008	0.046 ±0.006	0.014[b] ±0.002
Vasopressin	1.21 ±0.11	0.052 ±0.012	0.047 ±0.007	1.85 ±0.052	0.069 ±0.012	0.016 ±0.002	0.040 ±0.004	0.008 ±0.001
	Slow Pool (S_3)			**Slow Pool (S_3)**				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Control	5.52[b] ±1.06	0.032[b] ±0.006	0.0058 ±0.0007	4.33[b] ±0.79	0.035[b] ±0.008	0.0080 ±0.0010		
Vasopressin	2.71 ±0.36	0.017 ±0.002	0.0058 ±0.0010	2.21 ±0.41	0.016 ±0.002	0.0074 ±0.0010		

n=6. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM.

[a] $p < 0.05$, [b] $p < 0.02$, compared to vasopressin.

TABLE XIV

EFFECTS OF VASOPRESSIN (1 mU/ml) ON ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			Fast Pool (S_1)				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Control	1.64[a]	0.384[a]	0.236	1.64[a]	0.384[a]	0.236		
	±0.29	±0.042	±0.031	±0.29	±0.042	±0.031		
Vasopressin	1.12	0.260	0.228	1.12	0.260	0.228		
	±0.34	±0.034	±0.026	±0.34	±0.034	±0.026		
	Intermediate Pool (S_2)			Intermediate Pool (S_2)				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Control	1.28	0.089	0.074	2.06	0.122	0.042[a]	0.055	0.019[a]
	±0.18	±0.010	±0.006	±0.24	±0.010	±0.007	±0.007	±0.002
Vasopressin	1.34	0.084	0.065	2.14	0.112	0.032	0.054	0.013
	±0.22	±0.009	±0.007	±0.29	±0.009	±0.006	±0.007	±0.002
	Slow Pool (S_3)			Slow Pool (S_3)				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Control	5.94[a]	0.036	0.0064	5.08[a]	0.042[a]	0.0082		
	±0.52	±0.004	±0.0007	±0.50	±0.007	±0.0006		
Vasopressin	4.73	0.032	0.0066	3.90	0.032	0.0080		
	±0.61	±0.003	±0.0008	±0.56	±0.006	±0.0008		

n=6. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM.

[a] $p < 0.05$, compared to vasopressin.

TABLE XV

EFFECTS OF cAMP ON ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			Fast Pool (S_1)				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Control	2.02 ±0.30	0.447 ±0.073	0.223 ±0.018	2.02 ±0.30	0.447 ±0.073	0.223 ±0.018		
cAMP	1.83 ±0.17	0.373 ±0.062	0.234 ±0.023	1.83 ±0.17	0.373 ±0.062	0.234 ±0.023		
	Intermediate Pool (S_2)			Intermediate Pool (S_2)				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Control	1.30[a] ±0.32	0.104[a] ±0.012	0.078 ±0.009	2.42[a] ±0.32	0.144[a] ±0.012	0.042 ±0.006	0.058 ±0.007	0.017[a] ±0.002
cAMP	2.04 ±0.41	0.226 ±0.018	0.112 ±0.013	3.24 ±0.45	0.264 ±0.022	0.036 ±0.005	0.078 ±0.006	0.011 ±0.002
	Slow Pool (S_3)			Slow Pool (S_3)				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Control	5.41[a] ±0.63	0.043 ±0.004	0.0078 ±0.0005	4.23[a] ±0.63	0.042 ±0.006	0.0096 ±0.0010		
cAMP	4.62 ±0.54	0.036 ±0.004	0.0082 ±0.0009	3.41 ±0.52	0.036 ±0.005	0.0101 ±0.0014		

n=5. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, cAMP = 10 mM.

[a] $p < 0.05$, compared to cAMP.

coupled with enhanced ρ_{20} suggested that cAMP induced a net uptake of extracellular calcium into S_2. This may account for the reduction in ^{45}Ca efflux when exogenous cAMP was added to prelabelled cells (Fig. 24). However, since pool S_3 was reduced in size, the enhancement in size of S_2 may merely represent transfer of ^{45}Ca from S_3 to S_2 and the increased ρ_{20} may be a reflection of enhanced permeability of the membrane. The rate constants of exchange and fluxes into and out of S_3 while reduced, were not changed significantly by cAMP (Table XV).

EFFECTS OF TXA_2 SYNTHESIS INHIBITORS, ANTAGONISTS, AND MIMETICS ON ^{45}Ca EFFLUX FROM TOAD BLADDER EPITHELIAL CELLS

Effect of 7IHA on ^{45}Ca efflux in the presence of vasopressin.

7IHA was shown (Fig 10) to inhibit vasopressin-stimulated water flow and TXA_2 synthesis. To determine whether 7IHA also inhibited vasopressin-induced alterations in ^{45}Ca metabolism the following experiments were performed. As would be predicted from the water flow experiments, control experiments showed that 7IHA (100 μM), by itself, had no effect on ^{45}Ca compartmental analysis (Table XVI). Thus, cells were preincubated with 7IHA (100 μM) for 30 min, followed by addition of vasopressin (1 mU/ml) for 15 min. The cells were labelled with ^{45}Ca for 90 min still in the presence of both 7IHA and vasopressin. 7IHA blocked the alteration in ^{45}Ca metabolism in response to vasopressin (Fig 28). Specifically, it increased the ^{45}Ca content at time = 0 and increased the size of pool S_3. Upon performance of compartmental analysis, 7IHA was found to not affect vasopressin's reduction in the size of pool S_1 (Table XVII). The

TABLE XVI

EFFECTS OF 7-(1-IMIDAZOLYL)-HEPTANOIC ACID ON ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			Fast Pool (S_1)				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Control	1.60 ±0.18	0.381 ±0.048	0.243 ±0.021	1.60 ±0.18	0.381 ±0.048	0.243 ±0.021		
7IHA	1.48 ±0.16	0.341 ±0.079	0.231 ±0.028	1.48 ±0.16	0.341 ±0.079	0.231 ±0.028		
	Intermediate Pool (S_2)			Intermediate Pool (S_2)				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Control	1.20 ±0.12	0.091 ±0.008	0.078 ±0.008	2.96 ±0.24	0.142 ±0.016	0.040 ±0.006	0.048 ±0.004	0.015 ±0.001
7IHA	1.26 ±0.10	0.094 ±0.007	0.074 ±0.006	3.12 ±0.29	0.148 ±0.010	0.038 ±0.005	0.044 ±0.005	0.012 ±0.002
	Slow Pool (S_3)			Slow Pool (S_3)				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Control	6.32 ±0.64	0.052 ±0.007	0.0096 ±0.0007	4.62 ±0.46	0.040 ±0.006	0.0086 ±0.0006		
7IHA	6.12 ±0.62	0.058 ±0.006	0.0099 ±0.0006	4.41 ±0.48	0.038 ±0.005	0.0084 ±0.0007		

n=6. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, 7IHA = 100 μM.

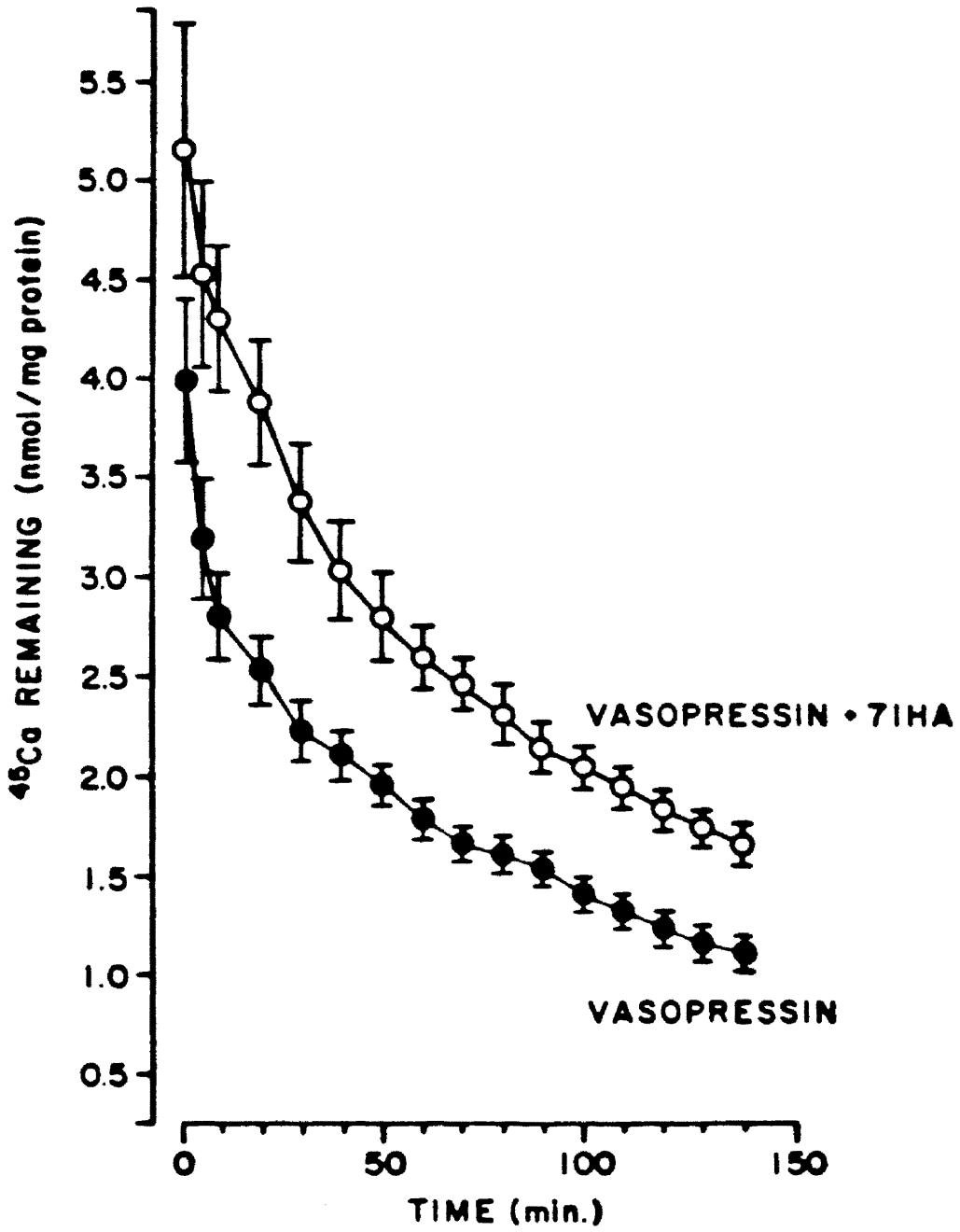


Figure 28. ^{45}Ca efflux from isolated toad bladder epithelial cells preincubated with vasopressin (1 mU/ml) or vasopressin plus 7-(1-imidazolyl)-heptanoic acid (100 μM). Cell suspensions were divided into 2 aliquots, 1 of which was incubated for 15 minutes with 7IHA (100 μM). Then both aliquots were incubated with vasopressin (1 mU/ml) for 15 minutes. Both aliquots were labelled with ^{45}Ca for 90 minutes and desaturated. The aliquots contained the experimental agents during the labelling and subsequent desaturation periods. Each vasopressin plus 7IHA point is significantly different from the respective vasopressin point ($p < 0.02$-0.05, $n = 6$ pairs). ^{45}Ca content at time = 0 were 8.44 ± 0.68 and 11.86 ± 1.13 nmol/mg protein ($p < 0.05$), vasopressin and vasopressin plus 7IHA, respectively.

TABLE XVII

EFFECTS OF 7-(1-IMIDAZOLYL)-HEPTANOIC ACID ON VASOPRESSIN-INDUCED ALTERATIONS IN ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			Fast Pool (S_1)				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Vasopressin	1.72[a] ±0.17	0.345[a] ±0.043	0.208 ±0.027	1.72[a] ±0.17	0.345[a] ±0.043	0.208 ±0.027		
Vasopressin plus 7IHA	1.09 ±0.26	0.278 ±0.037	0.261 ±0.021	1.09 ±0.26	0.278 ±0.037	0.261 ±0.021		
	Intermediate Pool (S_2)			Intermediate Pool (S_2)				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Vasopressin	1.42 ±0.37	0.068 ±0.022	0.074 ±0.009	1.83 ±0.58	0.132 ±0.015	0.066 ±0.006	0.073 ±0.015	0.034 ±0.005
Vasopressin plus 7IHA	1.66 ±0.28	0.096 ±0.031	0.092 ±0.023	1.56 ±0.49	0.152 ±0.028	0.070 ±0.007	0.097 ±0.013	0.041 ±0.005
	Slow Pool (S_3)			Slow Pool (S_3)				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Vasopressin	5.03[a] ±0.59	0.064 ±0.007	0.0130[a] ±0.0008	4.62[a] ±0.51	0.066 ±0.006	0.0140[a] ±0.0018		
Vasopressin plus 7IHA	8.10 ±1.48	0.065 ±0.006	0.0089 ±0.0011	8.08 ±1.64	0.070 ±0.007	0.0089 ±0.0012		

n=6. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM vasopressin = 1 mU/ml, 7IHA = 100 μM.

[a] $p < 0.05$, compared to 7IHA.

effect of vasopressin to reduce the size of pool S_3 was completely blocked by 7IHA (Table XVII).

Effects of cis- and trans-13APA on ^{45}Ca efflux. t13APA, but not c13APA was shown to inhibit vasopressin-stimulated water flow in the isolated, intact toad bladder (Chapter II). The effect of t13APA appeared to be antagonism of the action of TXA_2, since t13APA, but not c13APA, also antagonized the water flow response to U46619 (Fig. 14). Therefore, the effects of t13APA and c13APA on ^{45}Ca efflux were assessed. In the initial experiments the concentration of 13APA was 300 μM, the maximum concentration which had been used in the water flow experiments. This concentration of either isomer of 13APA was associated with massive uptake of ^{45}Ca into the isolated cells (Table XVIII). Either isomer of 13APA, when dissolved in the Ringer's solution at a concentration of 300 μM, was observed to cause a great amount of foaming when the solution was aerated. Thus, the massive ^{45}Ca uptake observed in the presence of 13APA was assumed to be caused by a detergent-like degradation of cell membranes.

Lower concentrations of 13APA were tested on ^{45}Ca efflux. 13APA (100 μM) was associated with somewhat enhanced ^{45}Ca uptake in pools S_2 and S_3 (Table XVIII). However, 13APA (50 μM), while enhancing ^{45}Ca uptake into pool S_2, had no effect on pool S_3 (Table XIX and XX).

Effects of cis- and trans-13APA on ^{45}Ca efflux in the presence of vasopressin. Since t13APA (50 μM) had caused only a small inhibition of water flow in response to vasopressin (5 mU/ml) (Chapter II) a pilot experiment was performed to assess the effects of t13APA (50 μM) on water flow stimulated by a lower dose of vasopressin (1

TABLE XVIII

EFFECTS OF cis- AND trans-13-AZAPROSTANOIC ACID ON ^{45}Ca KINETICS

	S_2	k_{20}	S_3	k_{30}
13APA (300 μM)[a]				
Control	1.12±0.14	0.084±0.007	5.84±0.42	0.0075±0.0006
c13APA	18.73±4.21	0.148±0.018	46.82±7.82	0.0143±0.0016
Control	1.24±0.13	0.078±0.006	6.23±0.58	0.0082±0.0008
t13APA	12.47±3.92	0.134±0.023	31.76±4.95	0.0148±0.0017
13APA (100 μM)[b]				
Control	1.06±0.12	0.072±0.007	6.24±0.42	0.0076±0.0006
c13APA	2.42±0.42	0.122±0.024	18.74±3.24	0.0102±0.0011
Control	1.12±0.14	0.068±0.007	5.98±0.38	0.0069±0.0006
t13APA	2.65±0.82	0.110±0.018	14.37±2.87	0.0098±0.0012

Suspensions were incubated with 13APA or vehicle for 30 minutes prior to labelling with ^{45}Ca.

[a] n=2 pairs, [b] n=3 pairs.

TABLE XIX

EFFECTS OF trans-13-AZAPROSTANOIC ACID ON ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			**Fast Pool (S_1)**				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Control	1.58 ±0.23	0.327 ±0.090	0.231 ±0.034	1.58 ±0.23	0.372 ±0.090	0.231 ±0.034		
t13APA	1.83 ±0.28	0.428 ±0.118	0.242 ±0.028	1.83 ±0.28	0.428 ±0.118	0.242 ±0.028		
	Intermediate Pool (S_2)			**Intermediate Pool (S_2)**				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Control	1.18[a] ±0.12	0.084[a] ±0.008	0.072 ±0.007	2.33[a] ±0.22	0.128[a] ±0.014	0.051 ±0.004	0.055 ±0.006	0.019 ±0.004
t13APA	1.52 ±0.14	0.149 ±0.012	0.098 ±0.007	2.83 ±0.25	0.198 ±0.016	0.053 ±0.004	0.070 ±0.009	0.019 ±0.003
	Slow Pool (S_3)			**Slow Pool (S_3)**				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Control	5.82 ±0.62	0.041 ±0.004	0.0073 ±0.0007	4.73 ±0.38	0.051 ±0.004	0.0094 ±0.0008		
t13APA	5.92 ±0.60	0.044 ±0.003	0.0082 ±0.0008	4.65 ±0.41	0.053 ±0.004	0.0114 ±0.0012		

n=6. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, t13APA = 50 μM.

[a] $p < 0.05$, compared to t13APA

TABLE XX

EFFECTS OF cis-13-AZAPROSTANOIC ACID ON ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			**Fast Pool (S_1)**				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Control	1.82 ±0.31	0.413 ±0.123	0.242 ±0.031	1.82 ±0.32	0.413 ±0.123	0.242 ±0.031		
c13APA	1.73 ±0.24	0.370 ±0.078	0.219 ±0.028	1.73 ±0.24	0.370 ±0.078	0.219 ±0.028		
	Intermediate Pool (S_2)			**Intermediate Pool (S_2)**				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Control	1.12 ±0.09	0.078[a] ±0.006	0.070 ±0.006	2.20[a] ±0.24	0.128 ±0.012	0.46 ±0.004	0.052 ±0.005	0.019 ±0.002
c13APA	1.30 ±0.12	0.114 ±0.009	0.088 ±0.007	2.72 ±0.29	0.152 ±0.018	0.52 ±0.004	0.062 ±0.005	0.018 ±0.003
	Slow Pool (S_3)			**Slow Pool (S_3)**				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Control	6.02 ±0.54	0.042 ±0.003	0.0081 ±0.0008	4.92 ±0.42	0.046 ±0.004	0.0091 ±0.0013		
c13APA	6.24 ±0.61	0.044 ±0.004	0.0074 ±0.0007	4.84 ±0.48	0.052 ±0.004	0.0120 ±0.0016		

n=6. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, c13APA = 50 μM.

[a] $p < 0.05$, compared to c13APA.

mU/ml). The control hemibladders were incubated with c13APA (50 μM). In the control hemibladders vasopressin stimulated water flow from 1.2±0.5 to 50.4±10.1 mg/min/hemibladder, while in the hemibladders preincubated with t13APA for 30 min vasopressin stimulated water flow from 1.4±0.1 to 42.0±8.8 mg/min/hemibladder. Thus, in the control hemibladders, the increment in water flow due to vasopressin was 49.2±10.2 mg/min/hemibladder, and in the hemibladders preincubated with t13APA the increment in water flow due to vasopressin was 40.6±8.7 mg/min/hemibladder, an inhibition of 16.9±3.8 % ($p<0.02$, n=6 pairs).

In the ^{45}Ca efflux experiments the cells were incubated with t13APA (50 μM) for 30 min, then vasopressin (1 mU/ml) was added for an additional 15 min. The cells were labelled with ^{45}Ca for 90 min still in the presence of t13APA and vasopressin, then desaturated. Control suspensions contained c13APA (50 μM). No significant difference was found in ^{45}Ca efflux between control suspensions (c13APA plus vasopressin) and those preincubated with t13APA and vasopressin (Fig 29). Compartmental analysis revealed no significant differences between control and t13APA-pretreated suspensions, although the size of pool S_3 tended to be increased in the presence of t13APA ($0.10>p>0.05$, n=6 pairs) (Table XXI). This trend was confirmed by analysis of covariance of the ln ^{45}Ca content versus time lines for pool S_3. When analysis of covariance was performed pool S_3 in the presence of vasopressin plus t13APA was significantly larger ($p<0.05$) than in the presence of vasopressin plus c13APA. However, it must be re-emphasized that the differences were quite small.

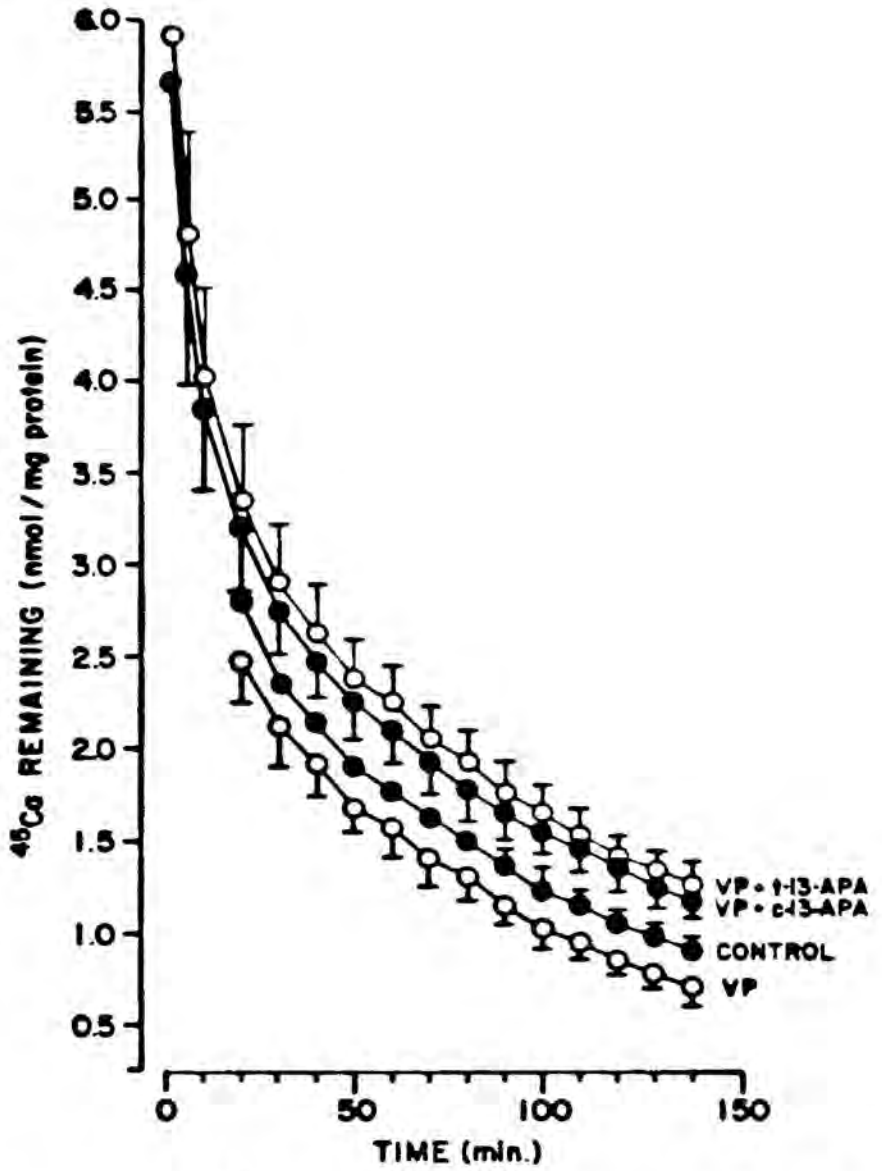


Figure 29. ^{45}Ca efflux from isolated toad bladder epithelial cells preincubated with vasopressin (1 mU/ml) plus *trans*-13-azaprostanoic acid (50 μM) or vasopressin plus *cis*-13-azaprostanoic acid. Cell suspensions were divided into 2 aliquots, 1 of which was incubated for 30 minutes with t13APA (50 μM) and the other with c13APA (50 μM). Then both aliquots were incubated with vasopressin (1 mU/ml) for 15 minutes. Both aliquots were labelled for 90 minutes and desaturated. The aliquots contained the experimental agents during the labelling and desaturation periods. When the data were analyzed as ln ^{45}Ca remaining versus time and analysis of covariance performed for each component, pool S_3 was found to be significantly larger in the presence of vasopressin plus t13APA than in the presence of vasopressin plus c13APA ($p < 0.05$ for 6 pairs of aliquots). Also shown is the effect of vasopressin (1 mU/ml) on ^{45}Ca efflux. Each vasopressin point shown is significantly different from the respective control point ($p < 0.05$, $n = 6$ pairs).

TABLE XXI

EFFECTS OF trans-13-AZAPROSTANOIC ACID ON VASOPRESSIN-INDUCED ALTERATIONS IN ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			Fast Pool (S_1)				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Vasopressin	1.64	0.438	0.257	1.64	0.438	0.257		
plus c13APA	±0.34	±0.052	±0.030	±0.34	±0.052	±0.030		
Vasopressin	1.42	0.372	0.248	1.42	0.372	0.248		
plus t13APA	±0.41	±0.043	±0.027	±0.41	±0.043	±0.027		
	Intermediate Pool (S_2)			Intermediate Pool (S_2)				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Vasopressin	1.48	0.164	0.114	2.04	0.201	0.043	0.097	0.022
plus c13APA	±0.28	±0.012	±0.010	±0.29	±0.024	±0.005	±0.010	±0.003
Vasopressin	1.37	0.168	0.122	1.89	0.205	0.042	0.101	0.022
plus t13APA	±0.34	±0.014	±0.012	±0.39	±0.026	±0.006	±0.013	±0.002
	Slow Pool (S_3)			Slow Pool (S_3)				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Vasopressin	4.98[a]	0.044	0.0088	4.42[a]	0.043	0.0095		
plus c13APA	±0.44	±0.004	±0.0007	±0.40	±0.005	±0.0008		
Vasopressin	5.24	0.041	0.0082	4.76	0.042	0.0084		
plus t13APA	±0.40	±0.003	±0.0007	±0.42	±0.004	±0.0011		

n=6. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, vasopressin = 1 mU/ml, c13APA = 50 μM, t13APA = 50 μM.

[a] $p < 0.05$, when compared to t13APA by analysis of variance.

Effects of U46619 on ^{45}Ca efflux from prelabelled cells. U46619 (0.1-1 μM) was shown to mimic the water permeability response to vasoprssin (Fig. 12). U46619 appeared to act by mimicking TXA_2 since the effect was blocked by t13APA (Fig 14). The present experiments were performed to determine whether U46619 also mimicked vasopressin's effect on ^{45}Ca fluxes. When U46619 (1 μM) was added to suspensions already undergoing desaturation, no effect on ^{45}Ca efflux was noted (Fig. 30). However, after U46619 was removed from the bathing media ^{45}Ca efflux transiently increased. This effect was similar to that observed when exogenous cAMP was added to the cells during desaturation (Fig. 24).

Effects of U46619 on ^{45}Ca compartmental analysis. In suspensions which had been preincubated with U46619 (1 μM) for 15 min prior to labelling with ^{45}Ca for 90 min, uptake of ^{45}Ca into the cells was identical to uptake into control cells (10.62±0.80 nmol/mg protein compared to 10.72±0.97 nmol/mg protein in the controls). However, by 70 min after commencement of desaturation the cells incubated with U46619 contained significantly less ^{45}Ca than did the control cells (Fig 31). When these curves were analyzed by compartmental analysis, it was found that U46619 (1 μM) had increased the size of pool S_2 while decreasing the size of pool S_3 (Table XXII). In addition to increasing the size of S_2, the flux of calcium into and out of S_2 (ρ_{20}) was increased, as well as the rate constant of efflux from S_2 (Table XXII). These effects are similar to those observed with cAMP (Table XIII). A lower concentration of U46619 (0.1 μM) was observed to cause similar alterations in ^{45}Ca metabolism (Table XXIII), although of lower magnitude.

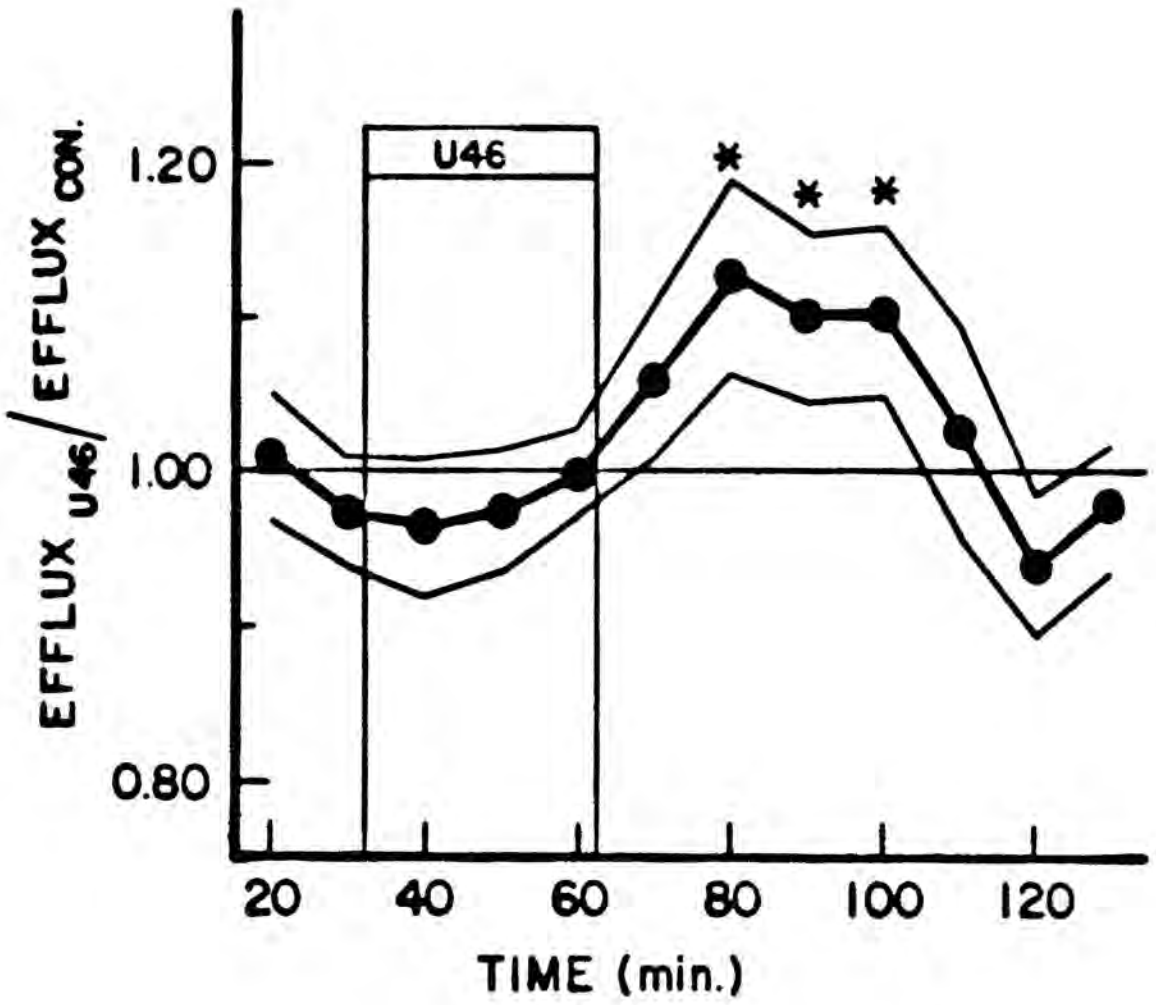


Figure 30. Effect of U46619 on ^{45}Ca efflux from isolated toad bladder epithelial cells. Suspensions were labelled with ^{45}Ca for 90 minutes. Each suspension was divided into 2 aliquots which were desaturated simultaneously. After 30 minutes of desaturation U46619 (1 μM) was added to 1 of each pair of aliquots (*$p < 0.05$, $n = 6$ pairs).

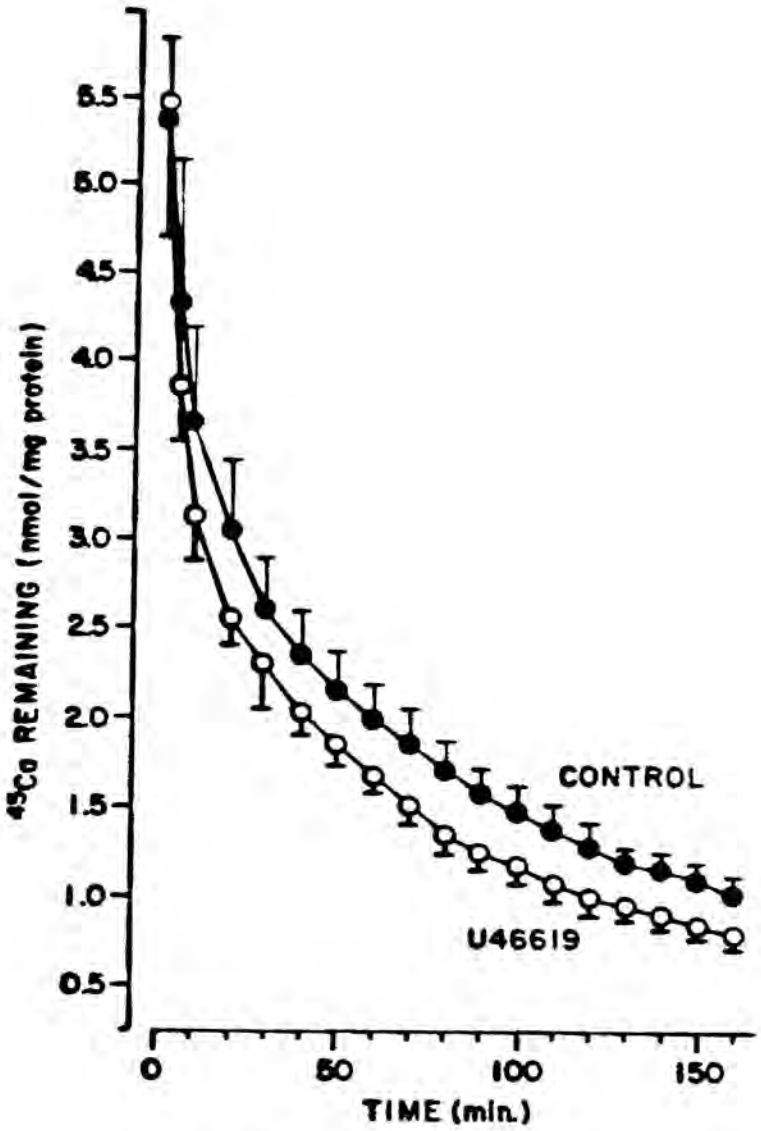


Figure 31. ^{45}Ca efflux from isolated toad bladder epithelial cells preincubated with U46619 (1 μM). Cell suspensions were divided into 2 aliquots, 1 of which was incubated for 15 minutes with U46619 (1 μM). Then both aliquots were labelled for 90 minutes with ^{45}Ca and desaturated. The experimental aliquots contained U46619 during the labelling and desaturation periods. Curves were analyzed as ln ^{45}Ca remaining versus time and divided into linear components (see Methods). Analysis of covariance revealed that in the presence of U46619 component S_2 was larger ($p < 0.01$ for 6 pairs of aliquots) and component S_3 was smaller ($p < 0.02$ for 6 pairs of aliquots) than control. ^{45}Ca content at time = 0 were 10.62 $\pm$ 0.80 and 10.72 $\pm$ 0.97 nmol/mg protein, control and U46619, respectively.

TABLE XXII

EFFECTS OF U46619 (1 μM) ON ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			Fast Pool (S_1)				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Control	1.40 ±0.25	0.371 ±0.109	0.214 ±0.030	1.40 ±0.25	0.371 ±0.109	0.214 ±0.030		
U46619	1.83 ±0.42	0.304 ±0.090	0.173 ±0.032	1.83 ±0.42	0.304 ±0.090	0.173 ±0.032		
	Intermediate Pool (S_2)			Intermediate Pool (S_2)				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Control	1.20[a] ±0.09	0.087[b] ±0.009	0.081[b] ±0.007	2.38[a] ±0.20	0.139[a] ±0.009	0.048 ±0.009	0.063 ±0.008	0.017 ±0.003
U46619	2.27 ±0.32	0.230 ±0.054	0.120 ±0.011	3.36 ±0.33	0.268 ±0.059	0.038 ±0.005	0.091 ±0.018	0.013 ±0.003
	Slow Pool (S_3)			Slow Pool (S_3)				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Control	6.40[a] ±0.72	0.043 ±0.003	0.0071 ±0.0003	5.15[a] ±0.58	0.048 ±0.009	0.0101 ±0.0001		
U46619	5.34 ±0.41	0.038 ±0.005	0.0080 ±0.0007	4.23 ±0.40	0.038 ±0.005	0.0082 ±0.0010		

n=6. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, U46619 = 1 μM.

[a] $p < 0.05$, [b] $p < 0.02$, compared to U46619.

TABLE XXIII

EFFECTS OF U46619 (0.1 μM) ON ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			**Fast Pool (S_1)**				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Control	2.05 ±0.24	0.418[a] ±0.058	0.242 ±0.028	2.05 ±0.24	0.418[a] ±0.058	0.242 ±0.028		
U46619	2.53 ±0.38	0.584 ±0.071	0.237 ±0.026	2.53 ±0.38	0.584 ±0.072	0.237 ±0.026		
	Intermediate Pool (S_2)			**Intermediate Pool (S_2)**				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Control	1.28[a] ±0.09	0.091[a] ±0.011	0.073[a] ±0.006	2.54[a] ±0.22	0.140[a] ±0.011	0.053 ±0.004	0.054 ±0.005	0.019 ±0.003
U46619	1.52 ±0.13	0.138 ±0.018	0.091 ±0.012	3.02 ±0.32	0.196 ±0.013	0.050 ±0.006	0.063 ±0.007	0.016 ±0.002
	Slow Pool (S_3)			**Slow Pool (S_3)**				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Control	6.32[a] ±0.62	0.049 ±0.003	0.0082 ±0.0007	5.08[a] ±0.51	0.053 ±0.004	0.0094 ±0.0010		
U46619	5.94 ±0.48	0.039 ±0.004	0.0078 ±0.0006	4.62 ±0.42	0.050 ±0.006	0.0106 ±0.0011		

n=6. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, U46619 = 0.1 μM.

[a] $p < 0.05$, compared to U46619.

Effects of t13APA on ^{45}Ca efflux in the presence of U46619.

Because of the effect of high concentrations of t13APA to markedly enhance ^{45}Ca uptake into the cells (Table XVI), a low concentration of t13APA, 50 μM, was used in these experiments. Since the concentration ratio of t13APA to U46619 must be quite high to achieve blockade of U46619-stimulated water flow (Chapter II), a low concentration of U46619 (0.1 μM) was used. Examination of Fig. 14 suggested that at this concentration ratio (500) t13APA should inhibit U46619-stimulated water flow ca. 70%. The cell suspensions were incubated with t13APA (50 μM) (c13APA in controls) for 30 min. U46619 (0.1 μM) was added to the suspensions and 15 min later labelling with ^{45}Ca began. After 90 min labelling, desaturation of the suspensions was commenced. The total uptake of ^{45}Ca was not different between control suspensions (c13APA plus U46619) and those preincubated with t13APA plus U46619 (9.87±0.54 nmol/mg protein versus 9.72±0.68 nmol/mg protein in the control suspensions). However, by 80 min of desaturation the control suspensions contained significantly less ^{45}Ca (Fig. 32). Compartmental analysis of the curves revealed that preincubation with t13APA increased the size of S_2 ($p<0.05$, n=6) and t13APA tended to increase the size of pool S_3 ($0.10>p>0.05$, n=6 pairs) (Table XXIV). When the data were analyzed using the modified series case the size of pool S_3 was significantly ($p<0.05$, n=6 pairs) increased in the suspensions incubated with t13APA (Table XXIV). When the data for the parallel and series cases were analyzed as ln ^{45}Ca content versus time and analysis of covariance performed on each component pool S_3 was found to be significantly larger in

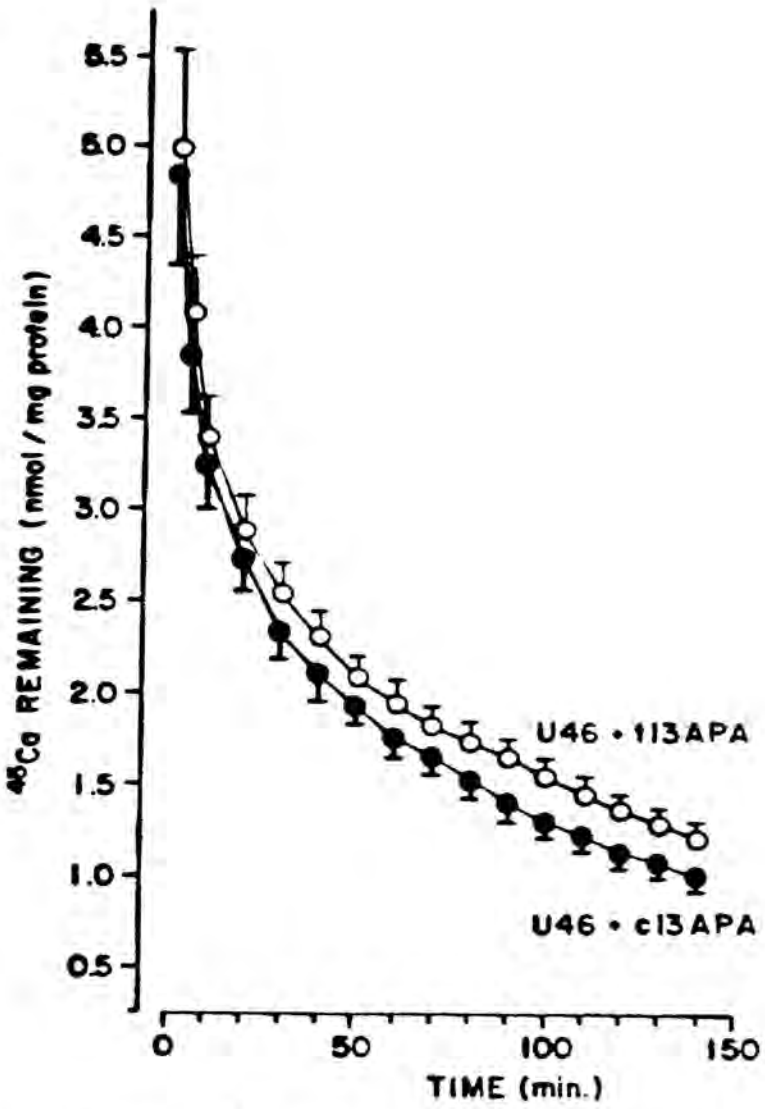


Figure 32. ^{45}Ca efflux from isolated toad bladder epithelial cells preincubated with U46619 (0.1 μM) plus *trans*-13-azaprostanoic acid (50 μM) or U46619 plus *cis*-13-azaprostanoic acid. Cell suspensions were divided into 2 aliquots, 1 of which was incubated for 30 minutes with t13APA (50 μM) and the other with c13APA (50 μM). Then both aliquots were incubated with U46619 (0.1 μM) for 15 minutes. Both aliquots were labelled with ^{45}Ca for 90 minutes and desaturated. The aliquots contained the experimental agents during the labelling and desaturation periods. When the data were analyzed as ln ^{45}Ca remaining versus time and analysis of covariance performed, pool S_3 was found to be significantly larger in the presence of U46619 plus t13APA than in the presence of U46619 plus c13APA ($p < 0.02$ for 6 pairs of aliquots).

TABLE XXIV

EFFECTS OF trans-13-AZAPROSTANOIC ACID ON U46619-INDUCED ALTERATIONS IN ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			Fast Pool (S_1)				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
U46619	2.22	0.472	0.216	2.22	0.472	0.216		
plus c13APA	±0.24	±0.058	±0.022	±0.24	±0.058	±0.022		
U46619	2.14	0.494	0.230	2.14	0.494	0.230		
plus t13APA	±0.32	±0.062	±0.028	±0.32	±0.062	±0.028		
	Intermediate Pool (S_2)			Intermediate Pool (S_2)				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
U46619	1.43[a]	0.094[a]	0.092	3.87	0.148	0.070	0.043	0.018
plus c13APA	±0.12	±0.007	±0.006	±0.32	±0.018	±0.007	±0.004	±0.002
U46619	1.87	0.128	0.088	4.24	0.164	0.076	0.038	0.014
plus t13APA	±0.20	±0.012	±0.008	±0.46	±0.016	±0.007	±0.003	±0.002
	Slow Pool (S_3)			Slow Pool (S_3)				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
U46619	6.90	0.052	0.0083	4.48[a]	0.070	0.0104		
plus c13APA	±0.68	±0.006	±0.0005	±0.46	±0.007	±0.0012		
U46619	7.21	0.056	0.0078	4.96	0.080	0.0114		
plus t13APA	±0.74	±0.005	±0.0006	±0.48	±0.007	±0.0010		

n=6. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, U46619 = 0.1 μM, c13APA = 50 μM, t13APA = 50 μM.

[a] $p < 0.05$, compared to U46619 plus t13APA.

the presence of U46619 plus t13APA than in the presence of U46619 plus c13APA ($p<0.02$ for 6 pairs of suspensions).

DISCUSSION

The present studies demonstrate that vasopressin, exogenous cAMP, and U46619, all of which enhance water permeability of the intact, isolated toad urinary bladder, all affect ^{45}Ca metabolism in epithelial cells isolated from the toad bladder. Further, several agents which inhibit water permeability also block the alterations in ^{45}Ca metabolism in response to vasopressin and U46619.

Effects of vasopressin on ^{45}Ca efflux

Vasopressin enhanced the instantaneous rate constant of efflux from cells undergoing ^{45}Ca desaturation. In this regard, the isolated cells behaved in a manner qualitatively similar to intact hemibladders (Thorn and Schwartz, 1968). In the cell suspensions vasopressin caused a peak enhancement of 39±11% in the rate of efflux during the first 20 min in which it was present. In the experiments of Cuthbert and Wong (1974), using intact hemibladders, the maximal enhancement in ^{45}Ca efflux due to vasopressin was approximately 150%, and in the experiments of Schwartz and Walter (1969) vasopressin elicited an enhancement in ^{45}Ca efflux of somewhat less than 100%. The sampling frequency used by these 2 groups was much greater and the peak enhancement in ^{45}Ca efflux was observed only 1-3 minutes after vasopressin addition. Thus, the quantitative differences between the present experiments and those in which intact hemibladders were used may be partially accounted for by the sampling frequencies, or they may result from alterations to the plasma membranes of the isolated cells caused by collagenase or the absence of extracellular calcium during the isolation procedure.

The enhanced ^{45}Ca efflux observed when vasopressin was added to suspensions already undergoing desaturation may be explained by at least 2 processes. Vasopressin may have caused a net efflux of calcium from the cells, or, the rate of exchange of calcium may have been increased. In addition, the size of the exchangeable pool may have been increased (Sha'afi et al., 1981). The latter possibility may be less likely since the bathing medium was unlabelled, and the effect was seen when vasopressin was added late in the desaturation when only pool S_3 was labelled. Thus, any calcium entering a larger exchangeable pool would be ^{40}Ca and dilute the specific activity of the exchanging pool, making it less likely that enhanced efflux of ^{45}Ca would be observed, even if exchange with the bathing medium increased.

The experiments which examined the effect of vasopressin on the steady state concentration of ^{45}Ca in the cells support the explanation that the effect of vasopressin is to cause a net extrusion of calcium from the cells. The cells lost a maximum of 0.90±0.11 nmoles of calcium per mg cell protein after exposure to 20 mU/ml vasopressin based on the specific activity of the bathing medium. This loss was largely complete within 1 min (0.63±0.09 nmol/mg protein), confirming the observation of Cuthbert and Wong (1974). Vasopressin (0.1 mU/ml), a concentration which does not enhance osmotic water flow, did not have any effect on the amount of ^{45}Ca in the cells. Vasopressin (1 mU/ml), which in separate experiments elicited a water flow response of about 70% that seen in the presence of a supramaximal concentration, 20 mU/ml, caused a reduction in ^{45}Ca content (0.67±0.10 nmol/mg protein) 76% of that observed in the

presence of vasopressin at 20 mU/ml (0.90±0.11 nmol/mg protein), suggesting parallel responsiveness of the 2 effects to vasopressin.

When the cells were incubated with vasopressin (10 mU/ml) and subsequently labelled with ^{45}Ca and desaturated, 3 components of efflux were observed. Vasopressin enhanced efflux from 2 of these, causing a net decrease in cellular calcium of 1.89 nmol/mg protein, (Legend to Fig. 23) much more than observed in the steady-state experiment (0.90 nmol/ mg protein). However, the most rapid component of efflux may be calcium bound to the external surface of the plasma membrane. In the steady state experiments where lanthanum was used to displace extracellular calcium this first component would not have been detectable due to displacement by lanthanum. When the first component is ignored, preincubation with vasopressin (10 mU/ml) was found to cause a reduction in cellular calcium of 0.82 nmol/mg protein, quite similar to the 0.90 nmol/mg protein observed in the steady state experiments.

Compartmental Analysis

Several experiments were performed in an attempt to identify the structural correlates of the several components of ^{45}Ca efflux. Efflux was observed to occur from four pools. The most rapidly exchanging pool was assigned as that residual ^{45}Ca contained in the bathing media which was trapped between the cells in the first pellet. This assignment was based on the observation that this pool exchanged with the same rate and occupied the same space as the extracellular markers inulin and bovine serum albumin.

The rate constant for exchange from pool S_1 (0.2 min^{-1}) was in the range usually considered to be the ^{45}Ca bound to the extracellular

surface of the plasma membrane (Borle, 1970; Sandborn and Langer, 1972). Predictably La^{3+} (1 mM) displaced about 50% of this pool. This displacement was quite similar in magnitude to that reported for La^{3+} displacement of ^{45}Ca from the glycocalices of cultured heart cells and fibroblasts (Langer and Frank, 1972). EGTA displaced essentially all of the ^{45}Ca in this pool. Thus, "pool" S_1 appeared to be composed of 2 pools of extracellular membrane-bound ^{45}Ca which exchanged with similar time constants which, due to the poor resolution of the present method, were not able to be dissociated kinetically.

In the experiments in which the cells were incubated with La^{3+} the rate of efflux from pool S_2 slowed to a rate similar to that for efflux from pool S_3. This suggests that the ^{45}Ca from pool S_2 and perhaps pool S_3 must cross the plasma membrane to reach the bathing medium. Langer and Frank (1972) observed in heart cells and in fibroblasts that incubation with high concentrations of La^{3+} reduced the rate of efflux from a pool with characteristics similar to S_2 to a rate which is similar to that of S_3 in these experiments. Further suggesting that S_2 was intracellular in location was the observation that EGTA did not affect the size of pool S_2.

Pool S_3 exchanged only slowly with bathing medium calcium. Borle has suggested that pools with characteristics similar to pool S_3 in a variety of cells are mitochondrial in origin. In the present experiments mitochondrial inhibitors were observed to reduce the size of pool S_3 by about 40%. At the same time the size of pool S_2 was increased. A similar effect of mitochondrial inhibitors was noted by Carafoli (1967) in liver. Uncoupling of mitochondrial

function reduced mitochondrial calcium content while enhancing microsomal calcium content, presumably by allowing the microsomal calcium uptake systems to compete more favorably with the mitochondria for available calcium. However, in the present experiments EGTA displaced about 70% of ^{45}Ca from pool S_3. However, EGTA increased the rate constants of efflux from pools S_2 and S_3 from 0.064 and 0.0061 min^{-1} to 0.248 and 0.0143 min^{-1} so that the enhanced efflux observed in the perturbation experiment may represent the enhanced rate constants. However, the possibility cannot be excluded that up to 70% of pool S_3 may be extracellular in location.

In preliminary experiments not reported in this dissertation, an attempt was made to determine the Q_{10}'s of efflux from pools S_2 and S_3. The Q_{10} of efflux from pool S_2 was 2.88 in one experiment and 3.24 in another, suggesting that a membrane must be crossed by an energy-requiring process. The Q_{10} for pool S_3 was apparently higher since little efflux was observed from this pool under the conditions of the experiment.

The model derived from the foregoing data may be summarized as follows. The bathing medium, pool S_0, may be considered of infinite size since it is periodically changed. Another bathing medium, pool S_0' represents that bathing medium which remains with the cell pellet on centrifugation of the suspensions. "Pool" S_1 is composed of 2 pools of extracellular bound calcium. Pool $S_{1,L}$ is that pool which may be displaced by La^{3+} and represents about 50% of pool S_1. Pool $S_{1,E}$ is that pool which is displaced by EGTA but not by La^{3+}.

S_2 is considered to be the cytoplasmic pool except for a possible mitochondrial component. Thus, calcium extrusion from the cell must

take place only via flux from S_2 into the bathing media, S_0. For a large variety of isolated cells the plasma membrane surface area is about 30 cm^2/mg protein (Borle, 1970; Claret-Berthon et al., 1977). Thus, calcium flux across the plasma membrane, $S_2 \rightarrow S_0$, amounts to some 44 femptomol/cm^2/sec in these cells, well within the range of 14-76 fentomol/ cm^2/sec published for transport of calcium across the plasma membranes of a wide variety of cells (Borle and Anderson, 1976, for a review). Further, the rate constant of efflux from pool S_2 was also virtually identical to those from "cytoplasmic pools" from a variety of cell types (Hodgkin and Keynes, 1957; Blaustein and Hodgkin, 1969; Borle, 1970; Sanborn and Langer, 1970; Langer and Frank, 1972; Claret-Berthon et al., 1977). The isolated toad bladder epithelial cells are spherical (Eggena, 1978), thus their volume is about 15.3 µl/mg protein. If the free ionized calcium concentration in the cytoplasm is assumed to be 0.1 µM, then the portion of S_2 which may be accounted for by free ionized calcium is 0.0015 nmol/mg protein or less than 0.1% of S_2. Thus, S_2 must consist primarily of sites such as endoplasmic reticulum and binding proteins. Pietras et al. (1976) found that total cellular calcium in bullfrog bladder epithelial cells was 3.7 mM. Their method of determination would presumably have included pools S_1, S_2, and S_3. In this study, exchangeable calcium in all 3 pools would amount to about 0.6 mM calcium. Thus, one may expect that a large non-exchanging or very slowly-exchanging calcium fraction exists in these cells, perhaps amounting to 80% of total cellular calcium.

"Pool" S_3, like pool S_2 appears to be composed of 2 pools. Pool $S_{3,E}$ is the 60-70% of S_3 which may be displaceable by EGTA and

which is not affected by mitochondrial inhibitors. It appears to be close to the extracellular space since it is displaced by EGTA, but since La^{3+} did not affect it, it must be on the inside of the cells. Perhaps binding to the inside surface of the plasma membrane to phospholipid head-groups would satisfy the requirement of close association to the extracellular space and inaccessibility to La^{3+}. Pool $S_{3,M}$ is that 30-40% of pool S_3 which is affected by mitochondrial inhibitors and which is not displaced by La^{3+}. Presumably, it represents mitochondrial calcium.

Effects of cAMP and U46619 on ^{45}Ca Efflux

Unlike vasopressin, exogenous cAMP caused a transient inhibition of ^{45}Ca efflux. This effect of cAMP is similar to that reported by Cuthbert and Wong (1974) except that in their studies, using intact hemibladders, the inhibition of ^{45}Ca efflux in response to cAMP was about 75%, and lasted as long as cAMP was present in the bathing media. While the effect of cAMP on ^{45}Ca efflux was different from that seen in response to vasopressin, compartmental analysis revealed that cAMP, like vasopressin, caused a significant reduction in the size of the calcium pool S_3. The decrease in the size of pool S_3 (0.82±0.13 nmol/mg protein) was accompanied by a similar increase in the size of pool S_2 (0.73±0.10 nmol/mg protein), consistent with the notion that calcium was transferred from pool S_3 to pool S_2 in response to cAMP. However, in these experiments cAMP was present for 15 min plus 90 min during labelling of the cells. Thus, the alteration in pool S_2 may not have occurred simultaneously with the alteration in S_3, and the changes may not be associated. In fact, compartmental analysis showed that exchange of calcium between pool

S_2 and the bathing medium was enhanced by cAMP. This type of biphasc effect of agents on ^{45}Ca flux has been observed in pancreatic acinar cels (Schulz, 1980) and appears to be common to many agents.

Like cAMP, U46619 also did not enhance ^{45}Ca efflux from the cells undergoing desaturation. The effects of U46619 on ^{45}Ca compartmental analysis were quite similar to those seen in response to cAMP. Pool S_3 was decreased in size and pool S_2 was increased in size by a similar amount. A similar effect of U46619 to enhance the size of one cellular calcium pool by shifting from another pool without affecting net efflux has been observed by LeBreton (personal communication, in press in Am. J. Physiol.). Further, calcium exchange between pool S_2 and bathing medium was enhanced by U46619. The similarities in the effects of U46619 and cAMP on ^{45}Ca compartmental analysis suggests that U46619 may act to increase water permeability across the toad bladder and alter ^{45}Ca metabolism by enhancing the cellular accumulation of cAMP. In support of this notion is the observation that U44069 enhances cAMP accumulation in the uterus (Vesin et al., 1979). Further, experiments described in Chapter II suggest that the magnitude of the water flow response to U46619 may be increased by inhibition of cyclic nucleotide phosphodiesterase.

Effects of 7IHA and 13APA on the ^{45}Ca Response of Vasopressin and U46619.

In order to explore the role of TXA_2 in vasopressin's stimulation of water permeability the effects of 7IHA, a TXA_2 synthesis inhibitor, and t13APA, a TXA_2 antagonist, were assessed on vasopressin-induced alterations in ^{45}Ca metabolism. 7IHA (100 μM) blocked the effect of

vasopressin to reduce the size of pool S_3. While vasopressin (1 mU/ml) decreased the size of S_3 by 1.14±0.28 pmol/mg protein. 7IHA blocked the effect of vasopressin completely, the size of pool S_3 in the presence of 7IHA plus vasopressin was 2.97±0.62 pmol/mg protein greater than in the presence of 7IHA alone. Since at this concentration 7IHA (100 μM) inhibits vasopressin-stimulated water flow only 20-25% no direct correlation appears to exist between the inhibition of ^{45}Ca efflux brought about by 7IHA and its inhibition of vasopressin-stimulated water flow. However, experiments to be described in the next chapter suggest that the "extra" increase in size of S_3 in response to 7IHA may be mediated by PGE_2 acting unopposed by TXA_2.

t13APA is a TXA_2 antagonist which inhibited vasopressin-stimulated water flow, while its cis isomer, which is inactive as a TXA_2 antagonist, had no effect on vasopressin-stimulated water flow. Preliminary experiments showed that at the concentration (300 μM) used in the water flow studies both trans- and cis-13APA acid caused a massive uptake of ^{45}Ca into the isolated epithelial cells. When the Ringer's solutions containing the compounds were aerated a large amount of foaming occurred. Thus, the massive ^{45}Ca uptake observed in response to these compounds was assumed to be due to enhanced permeability of the cells to calcium caused by a non-specific detergent effect. Similar results have been obtained when isolated cells have been incubated with other detergents (Murphy et al., 1980). At a concentration of 50 μM neither isomer of 13APA affected the size of pool S_3. However, both compounds did increase the size of pool S_2 significantly. In spite of this nonspecific effect of these compounds, an attempt was made to characterize the effect of t13APA

on vasopressin-induced alterations in calcium metabolism. To control for the independent effect of the compounds on pool S_2, the control suspensions contained c13APA. In preliminary experiments t13APA (50 μM) inhibited vasopressin (1 mU/ml)-stimulated water flow only 17±4% ($p<0.02$, n=6 pairs). In the isolated cells vasopressin (1 mU/ml) decreased the size of pool S_3 by 1.1±0.28 nmol/mg protein. Incubation of the cells with t13APA in addition to vasopressin blocked the effect of vasopressin by 0.28±0.08 nmol/mg protein (26% reduction). While this amount corresponded to the 17% inhibition of vasopressin-stimulated water flow, the effect was not statistically significant. Perhaps placing the cells into temporary culture and labelling with ^{45}Ca to isotopic equilibrium would allow an effect of t13APA to be discerned. In the present experiments pool S_3 was labelled only to about 40% of equilibrium, thus, a small error in measurement would produce a large error in the estimate of pool size, especially when the expected change is of the order of 15-25%.

U46619 is thought to enhance water permeability by acting as a TXA_2 mimetic. This assumption based in part on the observation that the water permeability effect could be antagonized by trans- but not cis-13APA. Further, the action of U46619 was enhanced by phosphodiesterase inhibtors, an effect also observed for vasopressin-stimulated water flow. Thus, the effect of t13APA (50 μM) was determined on U46619 (0.1 μM)-stimulated water flow. Interpolation of the dose-response curve for inhibition of U46619-stimulated water flow by t13APA suggested that at the stated concentrations of each agent, water flow might be inhibited about 70%. U46619 (0.1 μM) decreased the size of pool S_3 by 0.46±0.12 nmol/mg protein ($p<0.05$) when the

data were analyzed using the series case. In the presence of t13APA the effect of U46619 was blocked by 0.52±0.21 nmol/mg protein ($p<0.05$), or t13APA blocked the effect of U46619 to reduce the size of pool S_3. The 100% blockade of the effect of U46619 on the size of pool S_3 is somewhat greater than would be predicted on the basis of the water flow studies (70%). However, the inprecision of the method may well account for the differences.

The experiments described in this study suggest that vasopressin, cAMP, and U46619, agents which enhance water permeability across the toad bladder, all reduce the size of an intracellular calcium pool, S_3. This pool may be mitocondrial in location. Vasopressin in the liver is thought to release calcium from mitochondria as part of its action in that tissue (e.g., Babcock et al., 1979). It is also interesting to note that U46619 has been shown to release calcium from mitochondria isolated from liver (Reed and Knapp, 1978) and from mitochondria isolated from smooth muscle (McNamara et al., 1980). Exogenous cAMP has been shown to release calcium from mitochondria isolated from several sources (Borle, 1974; Mattib and O'Brien, 1974). Other physiological data supporting the notion that release of mitochondrial calcium may play a role in the antidiuretic response to vasopressin is the observation that mitochondrial inhibitors greatly prolong the action of vasopressin in the toad bladder (Masters and Fanestil, 1976).

cAMP and U46619 also caused uptake of calcium into another pool, S_2, which may represent endoplasmic reticulum and other cytoplasmic sites. Thus, these two agents decrease the size of S_3 and increase the size of S_2 without affecting ^{45}Ca efflux. LeBreton et

al. (1981) have in press a manuscript describing an identical action of U46619 on blood platelets, to a cause shift of calcium from one pool into the dense tubular system of the platelet without affecting calcium efflux. The uptake of calcium into pool S_2 caused by cAMP and U46619 may be similar to the cAMP-dependent uptake into sarcoplasmic reticulum (Tada et al., 1974).

Thus, the present data tend to support the hypothesis that vasopressin may alter water permeability across the toad bladder by a mechanism that includes release of membrane-bound calcium. Further, other agents that enhance the water permeability of the toad bladder may also act to release calcium from the same site. Finally, the source of the released calcium may be mitochondria.

CHAPTER IV

THE ROLE OF EXTRACELLULAR CALCIUM IN THE ANTIDIURETIC RESPONSE TO VASOPRESSIN AND TXA_2-LIKE COMPOUNDS IN THE TOAD URINARY BLADDER

INTRODUCTION

The role of extracellular calcium in vasopressin's antidiuretic effect is unclear. La^{3+} blocks vasopressin-stimulated water flow (Weitzerbin et al., 1974) as do several divalent cations which may substitute for calcium at its binding site(s) and block its effect (Bentley, 1959). The organic calcium uptake blocker, verapamil, blocks vasopressin's antidiuretic effect in vivo in the dog (Berl, 1981) and one report suggested that verapamil also inhibited vasopressin-stimulated water flow in the toad bladder (Humes et al., 1980). However, in another report (Bentley, 1974) verapamil was found to have no effect on vasopressin-stimulated water flow in the toad bladder. Increasing extracellular calcium in the presence of A23187 mimics the water flow response to vasopressin in the toad bladder (Hardy, 1978) although this observation has not been confirmed (Taylor et al., 1979). However, high serosal calcium concentration inhibits vasopressin-stimulated water flow (Petersen and Edelman, 1964). Since the experiments with cAMP and U46619 demonstrated that these compounds may significantly increase the size of one intracellular ^{45}Ca pool in toad bladder epithelial cells, it became of interest to determine the role of extracellular calcium in vasopressin and U46619's antidiuretic effects.

In the initial experiments designed to examine the effect of vasopressin on ^{45}Ca metabolism, it was found that vasopressin, when added to cells previously loaded with ^{45}Ca, caused a marked increase

in the fraction of ^{45}Ca which appeared in the bathing medium (Fig. 19, Chapter III). This increase in ^{45}Ca could have represented an enhancement in net calcium extrusion from the cells or, since the ^{45}Ca efflux is representative in these experiments of calcium-calcium exchange across the plasma membrane, could have represented an increase in cytosolic calcium concentration from either internal stores or from enhanced rate of exchange into the bathing medium. The experiments which examined ^{45}Ca compartmental analysis suggested that vasopressin caused a decrease in cellular exchangeable calcium. However, as noted previously, intracellular free ionized calcium could change dramatically but not be apparent from the compartmental analysis. Thus, to answer the question of whether the action of vasopressin requires an extracellular source of calcium, several groups of experiments were performed: 1) physiological, in which extracellular calcium was modified and resultant effects on vasopressin-stimulated water flow determined, 2) biochemical, in which the effects of vasopressin on ^{45}Ca movement were assessed, and 3) studies of the effects of inhibitors of vasopressin-stimulated water flow on ^{45}Ca influx.

MATERIALS AND METHODS

Verapamil and D-600 were the generous gifts of Knoll Pharmaceuticals; the following were purchased from commercial sources: ionophore A23187, Calbiochem Corp., San Diego, CA; carbamylcholine (carbachol) and quinidine, Sigma Chemical Co., St. Louis, MO. Other chemicals and reagents were described in previous chapters.

^{45}Ca uptake into epithelial cells isolated from toad urinary bladders. Epithelial cells were prepared by the collagenase method. The method to be described is similar to that described by Wiesmann et al. (1977). Cells were finally suspended to a concentration of 1 mg cell protein/ml Ringer's solution. The suspensons were incubated with stirring in 15 ml polycarbonate centrifuge tubes at 25°C under an atmosphere of 95% O_2:5% CO_2. Experimental manipulations were performed as described in the text. Aliquots of cells from the same suspensions were always run in parallel as controls. The controls always contained any vehicle used. ^{45}Ca equivalent to 0.5 μCi/ml Ringer's solution was added to each tube and incubation was continued for 5, 10, 30, 60, or 90 min. Aliquots of 1 ml were taken and injected into 20 ml of ice-cold Ca-free Ringer's solution containing 10 mM EGTA and centrifuged for 2 min at 4°C. The supernatants were decanted and the pellet resuspended and washed twice more with 20 ml aliquots of ice-cold Ca-free-EGTA Ringer's solution. The pellets were dissolved in 0.1 N NaOH and aliquots taken for measurement of ^{45}Ca radioactivity and cell protein.

Water flow studies were performed by the method of Bentley (1958) as described in Chapter II.

Extracellular calcium was measured by the titrametric method of Borle and Briggs (1968).

RESULTS

Effects of altered mucosal calcium concentration on vasopressin-stimulated water flow. In these experiments serosal calcium concentration was maintained at 1 mM while the mucosal bathing solution was 1 mM Ca^{2+} in 1:5 Ringer's solution in the controls or no added calcium in the experimental hemibladders. After incubation for 1 hour and changing the mucosal baths every 20 min, neither basal nor vasopressin (1 mU/ml)-stimulated water flow was affected by the lack of calcium in the mucosal bathing media. In the control hemibladders vasopressin stimulated water flow from 1.6±0.7 to 66.7±6.0 mg/min/hemibladder and in the experimental hemibladders vasopressin stimulated water flow from 1.6±0.5 to 66.9±6.3 mg/min/ hemibladders, n=4 pairs.

Effects of altered serosal calcium concentration on vasopressin-stimulated water flow. In these experiments one hemibladder of each pair was bathed in a bicarbonate-Ringer's solution containing no Mg^{2+} and 1 mM Ca^{2+}. The experimental hemibladder was bathed in a bicarbonate-Ringer's solution containing a different concentration of calcium. In all cases, mucosal calcium concentration was maintained at 0.2 mM for both control and experimental hemibladders to assure that the epithelial cells would not become detached. In these experiments both high and low calcium concentrations were found to inhibit vasopressin-stimulated water flow. The response to vasopressin in the presence of 10 mM calcium was only 65±8% of that in the presence of 1 mM calcium ($p<0.02$, n=5 pairs) (Fig. 33). The response in the presence of 0.2 mM calcium was not depressed. However, in the presence of 0.025 mM calcium, the response was only 73±12% ($p<0.05$, n=5 pairs) and in the presence of 10 μM calcium, i.e., in the absence

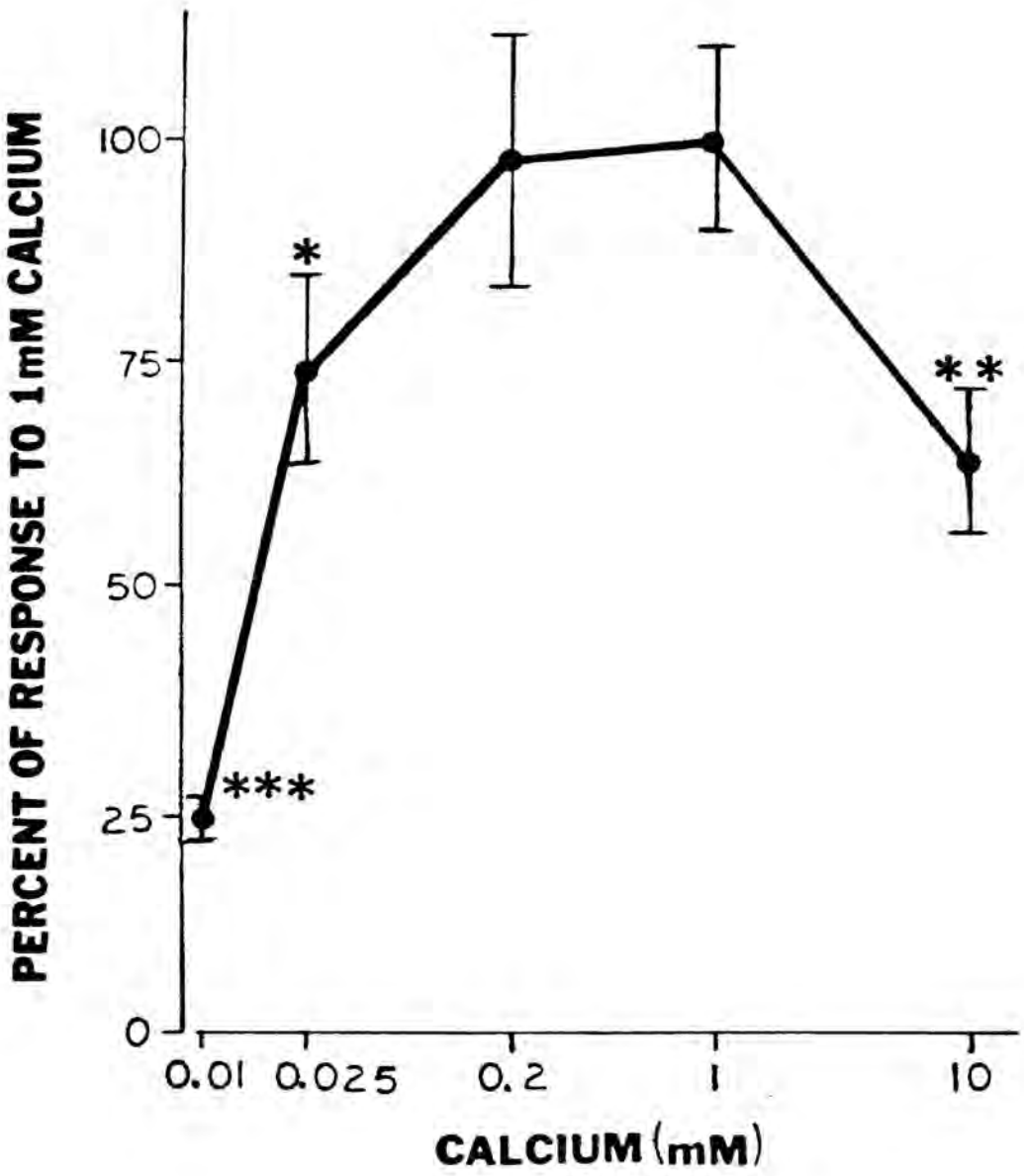


Figure 33. Effects of altered serosal calcium concentration on vasopressin-stimulated water flow. One member of each pair of hemibladders was incubated in the presence of an altered serosal calcium concentration while the contralateral hemibladder was incubated in 1 mM serosal calcium. Incubation was for 60 minutes with serosal calcium being changed every 20 minutes. All hemibladders were bathed with 0.2 mM calcium in the mucosal bath. Vasopressin (1 mU/ml)-stimulated water flow was measured for 30 minutes (*$p < 0.05$, **$p < 0.02$, and ***$p < 0.01$, compared to 1 mM calcium, n = 5 pairs, each point).

of exogenously-added calcium, the response was only 24±2% ($p<0.01$, n=5 pairs) of that observed in the presence of 1 mM Ca^{2+}.

To assess the reversibility of decreased calcium concentration on the water flow response to vasopressin after the initial 30 min period with vasopressin in calcium-free serosal media, the vasopressin was washed out and after a 30 min recovery period, 1 mM Ca^{2+} was added to the calcium-depleted hemibladders. After 10 min, vasopressin was added again. In these hemibladders vasopressin still did not elicit a water flow response (Table XXV). In a similar experiment 1 mM Ca^{2+} was added during the 30 min recovery period, and the hemibladders were incubated an additional 30 min, for a total of 60 min with 1 mM calcium. In these hemibladders a small amount of recovery occurred. Water flow in response to vasopressin after the recovery period was 11±3 mg/min/hemibladder compared to 6±1 mg/min/hemibladder in the absence of added calcium ($p< 0.05$, n=5) (Table XXV). However, control hemibladder response was 36±8 mg/min/hemibladder. Thus, simply the lack of extracellular calcium did not appear to be the cause of the lack of a response to vasopressin at low calcium concentrations. To assess the generality of the defect caused by a lack of serosal calcium other experiments were performed to assess the effects of exogenous cAMP and U46619 on water flow. The response to cAMP was also abolished in the absence of serosal calcium (Table XXVI). In addition, the water flow response to U46619 was inhibited (Table XXVI).

Effect of altered extracellular calcium concentration on vasopressin-stimulated water flow in the presence of magnesium or strontium. In many tissues removal of extracellular calcium is associated with

TABLE XXV
REVERSIBILITY OF THE EFFECTS OF DECREASED SEROSAL CALCIUM CONCENTRATION ON VASOPRESSIN-STIMULATED WATER FLOW

	WATER FLOW (mg/min/hemibladder	
READDITION OF CALCIUM FOR 10 MINUTES		
	First Vasopressin Period	
	Basal	Vasopressin (1 mU/ml)
Control (1 mM Ca, Mg-free)	1.8±.0	35.2±8.7
Ca, Mg-free Ringer's Solution	1.7±0.6	5.2±1.2[a]
	Second Vasopressin Period	
Control (1 Mm Ca, Mg-free)	-	34.2±4.8
Readdition of Ca (1 mM)	-	6.0±0.9[a]
READDITION OF CALCIUM FOR 60 MINUTES		
	First Vasopessin Period	
Control (1 mM Ca, Mg-free)	1.8±0.4	35.4±6.1
Ca, Mg-free Ringer's Solution	1.6±0.2	5.1±2.1[a]
	Second Vasopressin Period	
Control (1 mM Ca, Mg-free)	1.4±0.4	36.1±7.8
Ca, Mg-free (One-half of above group)	1.8±0.4	6.0±1.3[a]
Readdition of Ca (1 mM)	2.2±0.8	11.0±2.9

[a] $p < 0.01$, compared to control; [b] $p < 0.05$, compared to no calcium, 60 min.

Experimental hemibladders were incubated in Ringer's solution without added calcium for 60 min, the Ringer's solution being changed every 20 min. Control hemibladders were incubated in Ringer's solution containing 1 mM calcium. Vasopressin-stimulated water flow was measured for 30 min.

In the upper 2 experiments n=5 pairs, each group. In the lower experiment n=10 pairs for control and no calcium. During the recovery period, the no calcium group was divided into 2 groups (n=5 in each) to yield the no calcium, 60 min, and calcium, 60 min groups.

TABLE XXVI

EFFECTS OF DECREASED SEROSAL CALCIUM ON cAMP- AND U46619-STIMULATED WATER FLOW

	WATER FLOW (mg/min/hemibladder)	
	Basal	cAMP (10 mM)
Control (1 mM Ca; Mg-free)	1.6±0.3	39.0±7.2
Ca, Mg-free Ringer's Solution	2.2±0.6	5.5±1.4[a]
	Basal (mg/min/10 cm^2)	U46619 (1 μM)
Control (1 mM Ca; Mg-free)	1.6±0.8	16.2±1.8[b]
Ca, Mg-free Ringer's Solution	1.8±0.6	9.4±1.8[b]

[a] $p < 0.01$, compared to control; [b] $p < 0.05$, compared to control, n=5 pairs, each group.

Experimental hemibladders were incubated in Ringer's solution without added calcium for 60 min, with Ringer's solution being changed every 20 min. Control hemibladders were incubated in Ringer's solution containing 1 mM calcium. cAMP-stimulated water flow was measured for 30 min. U46619-stimulated water flow was measured for 10 min in hemibladders which had been preincubated with indomethacin (50 μM) for 1 hour.

structural damage. However, the role of calcium may still be studied in physiological processes by substituting another divalent ion such as magnesium. This ion will substitute for calcium in maintaining structural integrity, but will not substitute for calcium as a stimulus transducer (Putney and Askeri, 1980). In the toad bladder it has been reported that either Mg^{2+} or Sr^{2+} may substitute for Ca^{2+} in maintaining vasopressin responsiveness (Bentley, 1959; Schwartz and Walter, 1972). Thus, several experiments were conducted to further investigate the necessity of extracellular calcium in the response to vasopressin without incurring the apparent structural alterations of the experiments described above.

Control hemibladders were preincubated for 60 min in Ringer's solution containing 1 mM Ca^{2+} and no Mg^{2+}, while experimental hemibladders were preincubated in Ringer's solution containing no Ca^{2+}, but containing 1 mM Mg^{2+} or 1 mM Sr^{2+}. Basal water flows were no different in control hemibladders compared to hemibladders incubated with either Mg^{2+} or Sr^{2+}. Further, when vasopressin was added, water flow was not inhibited as it had been in the absence of any divalent cation. Sr^{2+} preserved vasopressin responsiveness completely (Table XXVII), while Mg^{2+} preserved two-thirds responsiveness (Table XXVII).

After vasopressin had been present for 30 min, it was washed out and the hemibladders were allowed to recover for 60 min. The control hemibladders were still incubated in Ringer's solution containing 1 mM Ca^{2+} while the hemibladders which had been incubated in 1 mM Mg^{2+} or 1 mM Sr^{2+} were divided into 2 groups. In half, incubation continued in the presence of 1 mM Mg^{2+} or 1 mM Sr^{2+}. In the other group the Mg^{2+} or Sr^{2+} was replaced with 1 mM Ca^{2+}. Thus, in

TABLE XXVII

EFFECTS OF Mg^{2+} OR Sr^{2+} SUBSTITUTION FOR CALCIUM ON VASOPRESSIN-STIMULATED WATER FLOW

	WATER FLOW (mg/min/hemibladder)	
	Basal	Vasopressin (1 mU/ml)
Initial Period		
Control	1.2±0.4	41.8±5.7
Mg^{2+}	1.6±0.6	32.9±4.2[a]
Control	1.8±0.7	38.4±4.8
Sr^{2+}	1.2±0.4	36.8±4.2
Second incubation with vasopressin		
Control	1.8±0.9	44.6±5.0
Mg^{2+}	1.4±0.8	5.8±2.7[b]
Control	2.1±0.6	42.4±3.7
Sr^{2+}	1.6±0.4	5.2±1.6[b]
Second incubation with vasopressin with calcium replacement		
Control	1.8±0.9	44.6±5.0
Ca^{2+} replacing Mg^{2+}	1.2±0.6	40.2±6.8
Control	2.1±0.6	42.4±3.7
Ca^{2+} replacing Sr^{2+}	1.7±0.5	46.4±5.2

[a] $p < 0.05$, [b] $p < 0.01$, compared to control.

Experimental hemibladders were incubated in Ringer's solution with 1 mM Mg^{2+} or Sr^{2+} substituting for 1 mM Ca^{2+} for 60 min, the Ringer's solutions being changed every 20 min. The control hemibladders were incubated in Ringer's solution containing 1 mM Ca^{2+}.

In the initial period n=10 pairs for each group. The Mg^{2+} and Sr^{2+} hemibladders were then divided into 2 groups (n=5, each). In one group incubation in the presence of Mg^{2+} or Sr^{2+} continued for 60 min; in the other group 1 mM Ca^{2+} was substituted for Mg^{2+} or Sr^{2+} and incubation continued for 60 min. In each case, Ringer's solutions were changed every 20 minutes.

these hemibladders the Ringer's solution during the recovery period was identical to the Ringer's solution bathing the control hemibladders.

After the 60 min recovery period vasopressin was again added and vasopressin-stimulated water flow was measured for 30 min. In those experimental hemibladders which were incubated in Ringer's solution containing Mg^{2+} or Sr^{2+} responsiveness was significantly reduced during the second incubation with vasopressin (Table XXVII). However, in those experimental hemibladders in which Ca^{2+} had been substituted for Mg^{2+} or Sr^{2+}, full responsiveness to vasopressin was retained (Table XXVII). Thus, these experiments demonstrated that extracellular calcium is not an absolute requirement to maintain the water flow response to vasopressin. The presence of Mg^{2+} or Sr^{2+} was required probably only to maintain intact connections among cells and basement membranes. However, hemibladders exposed to Mg^{2+} or Sr^{2+}, in substitution for Ca^{2+} were able to respond to vasopressin only one time. This suggests that subsequent responses to vasopressin may require a refilling of an intracellular calcium compartment to maintain responsiveness.

Effect of stoichiometric chelation of serosal calcium on vasopressin-stimulated water flow. Since in the previous experiments, hemibladders were incubated for extended periods of time in Ringer's solutions with altered divalent cation concentrations an additional experiment was performed in which the divalent cation concentrations were altered more acutely. In these experiments hemibladders were incubated in Ringer's solution containing 1 mM Ca^{2+} and 1 mM Mg^{2+}. To one member of each pair of hemibladders was added 1 mM EGTA to

stoichiometrically bind the serosal calcium and 5 min later vasopressin was added and water flow determined for 30 min. The addition of EGTA did not affect basal water flow in control experiments during which EGTA was present for 30 min (Table XXVIII).

Stoichiometric calcium chelation did not inhibit vasopressin-stimulated water flow (Table XXVII). Further, when vasopressin was subsequently washed out but EGTA remained, water flow returned to basal rate (Table XXVIII). Thus, under conditions where no net calcium influx could occur, vasopressin was able to exert its full effect on water flow.

Effects of organic calcium antagonists on vasopressin-stimulated water flow. Verapamil and its methoxy derivative D-600 are organic calcium antagonists which act by inhibiting the uptake of calcium through the plasma membrane of many cell-types. Bentley (1974) reported that verapamil in concentrations up to 100 μM had no effect on vasopressin-stimulated water flow across the isolated toad bladder. Recently, however, Humes et al. (1980) reported that verapamil, after 90 min preincubation at a concentration of 100 μM, does inhibit vasopressin-stimulated water flow. Since our data with the substitution of magnesium or strontium for serosal calcium suggested that extracellular calcium is not required for vasopressin action we also assessed the effects of verapamil and D-600 on vasopressin-stimulated water flow.

Hemibladders were incubated with verapamil (1-100 μM) for 60 min. Basal water flow was measured during this period. Vasopressin (1 mU/ml) was then added and vasopressin-stimulated water flow was measured for 30 min. Only at the highest concentration tested, 100

TABLE XXVIII

EFFECT OF STOICHIOMETRIC CALCIUM CHELATION ON VASOPRESSIN-STIMULATED WATER FLOW

	WATER FLOW (mg/min/hemibladder)	
	Basal	EGTA (1 mM)
Control (no EGTA)	1.4±0.4	1.5±0.4
Experimental (EGTA, 1 mM)	1.2±0.6	1.4±0.4
	Basal	Vasopressin (1 mU/ml)
Control (no EGTA)	1.8±0.6	36.8±5.4
EGTA (1 mM) during the vasopressin period	1.6±0.2	38.4±6.0
Subsequent washout of vasopressin		
Control	2.0±0.4	
EGTA	1.8±0.6	

Experimental hemibladders were incubated with EGTA (1 mM) for 5 min to chelate the serosal calcium (1 mM). Then vasopressin was added and stimulated water flow was measured for 30 min., n=5 pairs, each group.

μM, was verapamil found to have any effect on vasopressin-stimulated water flow (Table XXIX). Similar experiments were performed using D-600. D-600 was also observed to inhibit vasopressin-stimulated water flow to a small extent (Table XXIX).

Since Humes et al. (1980) found that long periods of preincubation with verapamil were necessary to produce an inhibition in vasopressin-stimulated water flow the possibility arose that the effect was due not to inhibition of calcium uptake per se, but to a reduction in the calcium concentration at some critical site, presumably intracellular, perhaps identical to that which appeared necessary to elicit a second response to vasopressin in the presence of magnesium or strontium. Further, verapamil does not act immediately to inhibit calcium uptake; calcium must first leave its binding site, after which it is replaced by verapamil (Langer et al., 1975). Thus, a series of experiments was performed in which the hemibladders were incubated for 30 min with D-600 (100 μM), stimulated with vasopressin in the presence of D-600, washed in the presence of D-600 to remove vasopressin, allowed to recover for 30 min, and restimulated with vasopressin a second time, still in the presence of D-600. The rationale was that vasopressin should deplete intracellular calcium, and since D-600 would inhibit calcium uptake to "reload" the cells, the hemibladders would not recover within the 30 min period allowed.

In the presence of 1 mM serosal calcium and 30 min preincubation, D-600 (100 μM) did not significantly inhibit vasopressin-stimulated water flow, compared to paired control hemibladders (Fig. 34). However, upon restimulation with vasopressin, D-600 did elicit a significant inhibition of water flow (28±6%, $p<0.05$, n=6 pairs). In the

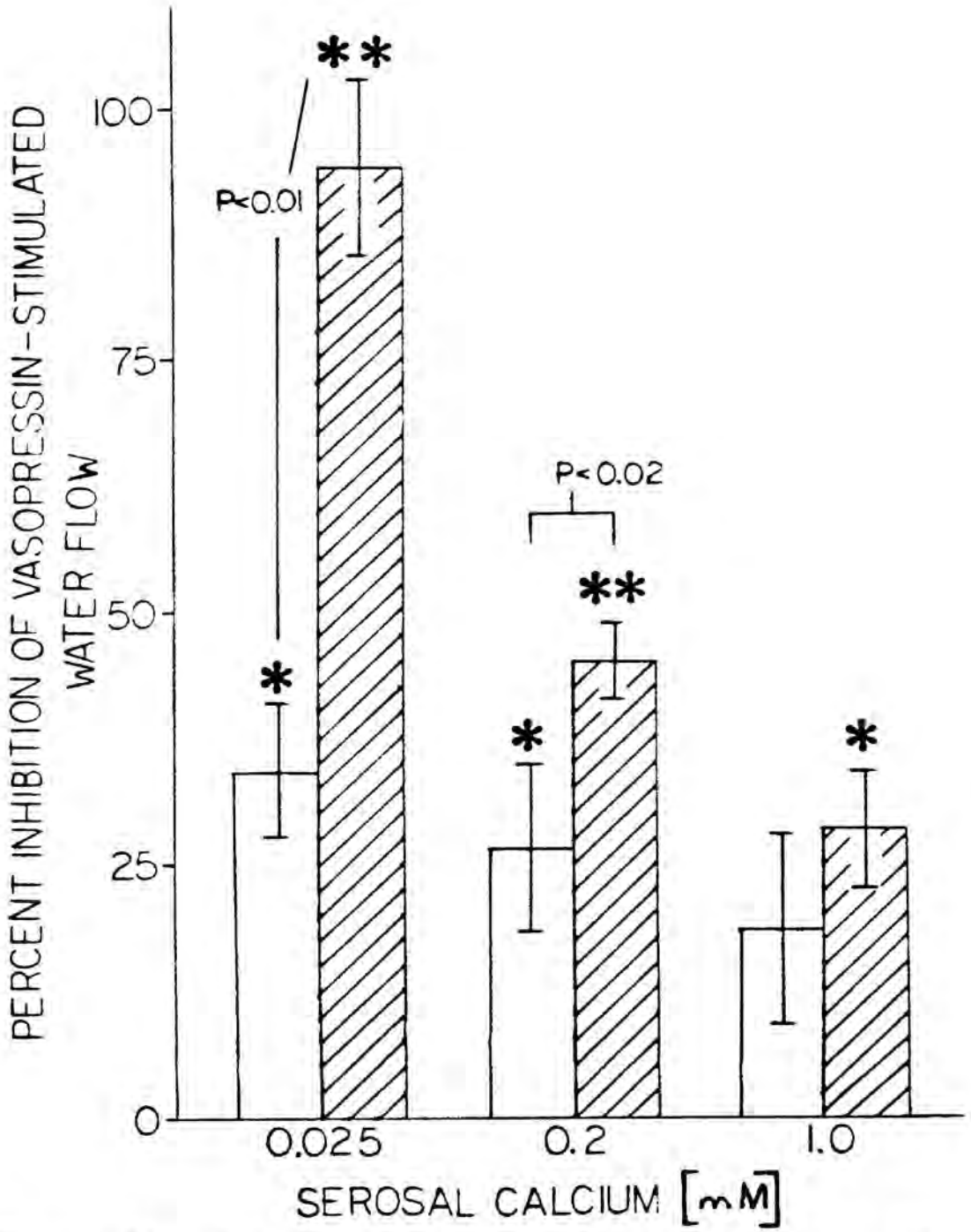


Figure 34. Calcium dependence of the inhibition of vasopressin-stimulated water flow by D-600. Hemibladders were incubated in Ringer's solutions with altered serosal calcium concentrations for 1 hour, the Ringer's solutions being changed every 20 min. D-600 (100 μM) was added to 1 member of each pair and basal water flow was measured for 30 minutes. Then vasopressin (1 mU/ml) was added and stimulated water flow was measured for 30 minutes (open bars). Vasopressin was washed out by changing the serosal media every 10 minutes for 30 minutes, still in the presence of D-600 where appropriate. Then vasopressin was added again and stimulated water flow was measured for 30 minutes (hatched bars).
*$p < 0.05$, **$p < 0.01$, compared to control hemibladders.

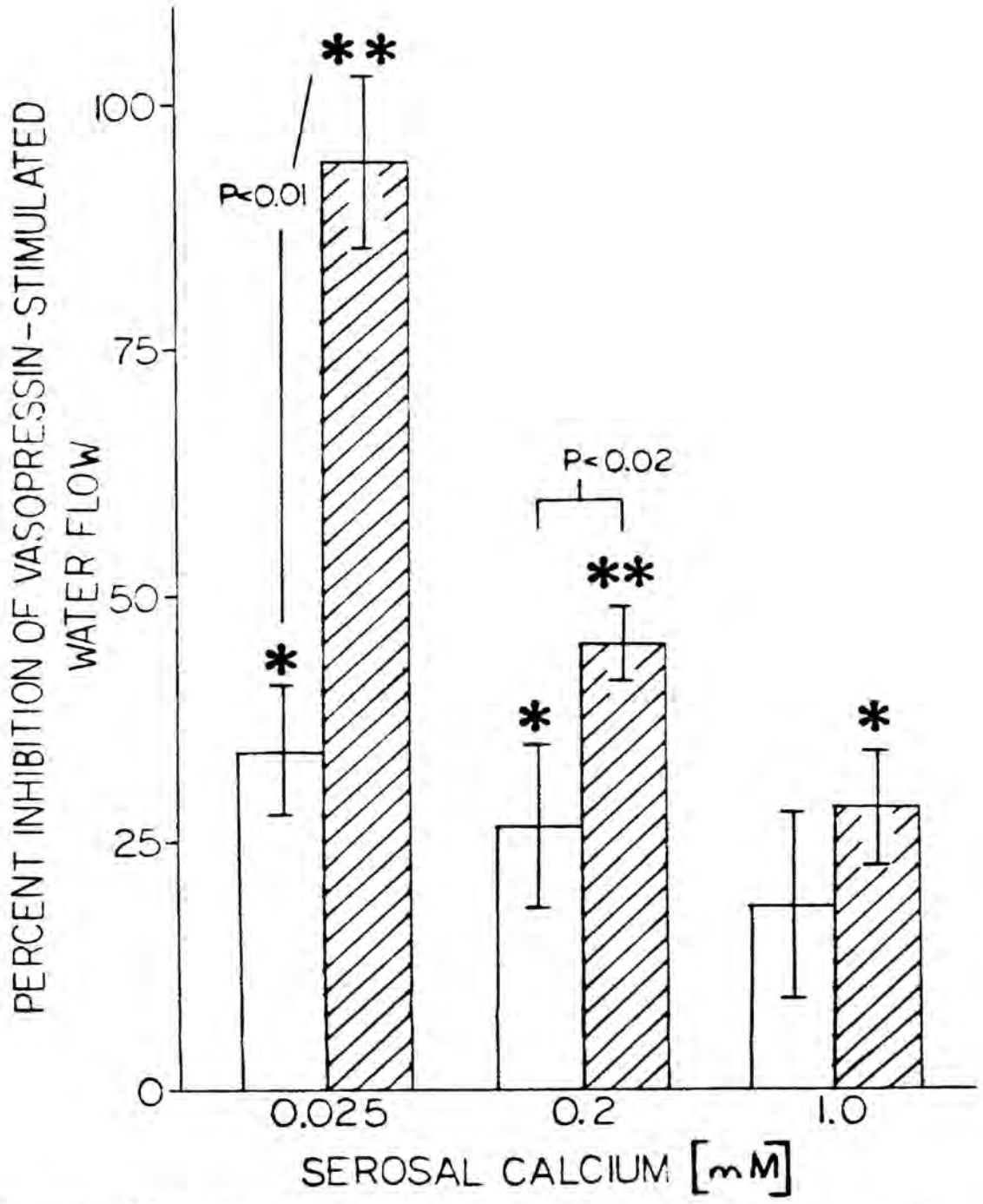


Figure 34. Calcium dependence of the inhibition of vasopressin-stimulated water flow by D-600. Hemibladders were incubated in Ringer's solutions with altered serosal calcium concentrations for 1 hour, the Ringer's solutions being changed every 20 min. D-600 (100 μM) was added to 1 member of each pair and basal water flow was measured for 30 minutes. Then vasopressin (1 mU/ml) was added and stimulated water flow was measured for 30 minutes (open bars). Vasopressin was washed out by changing the serosal media every 10 minutes for 30 minutes, still in the presence of D-600 where appropriate. Then vasopressin was added again and stimulated water flow was measured for 30 minutes (hatched bars).
*$p < 0.05$, **$p < 0.01$, compared to control hemibladders.

presence of 0.2 mM calcium, 30 min preincubation with D-600 (100 μM) produced a significant inhibition of vasopressin-stimulated water flow (27±8%, $p<0.05$, n=6 pairs). Upon restimulation with vasopressin the inhibition rose ($p<0.02$) to 45±4% ($p<0.01$ compared to control, n=6 pairs). When serosal calcium concentration was reduced to 0.025 mM, 30 min incubation with D-600 (100 μM) produced a 34±7% inhibition in vasopressin-stimulated water flow ($p<0.05$, n=6 pairs). However, when the hemibladders were restimulated with vasopressin, the inhibition rose ($p<0.01$) to 94±9% ($p<0.01$ compared to control, n=6 pairs). Thus, D-600 was effective in inhibiting vasopressin-stimulated water flow, especially in the presence of low serosal calcium, or after the hemibladders had been depleted of calcium by previous exposure to vasopressin.

Effects of lanthanum on vasopressin-stimulated water flow. Lanthanum, when added in low concentration (50 μM) to Ringer's solution containing 1 mM calcium, does not inhibit basal ^{45}Ca uptake but does inhibit the stimulation in ^{45}Ca uptake observed when the cells are incubated with A23187 or carbachol (Wiesmann et al., 1977, 1978). At high concentration (1-88 mM) lanthanum has been reported to inhibit ^{45}Ca efflux from several types of isolated cells as well (Langer and Frank, 1972; Weiss, 1978). Lanthanum (1 mM) was found to inhibit ^{45}Ca efflux from prelabelled toad bladder epithelial cells (Chapter III). Thus, experiments were performed to determine whether a low concentration (50 μM) of lanthanum affected vasopressin-stimulated water flow by inhibiting calcium influx, or whether a high concentration (1 mM) of lanthanum, by inhibiting calcium efflux, inhibited vasopressin-stimulated water flow.

These experiments were performed in tris-Ringer's solution. The Ringer's solutions contained 1 mM Ca^{2+} but no added Mg^{2+}. During incubation with La^{3+} (50 μM) for 30 min basal water flow was unchanged (Table XXX). Vasopressin (1 mU/ml) was then added and water flow measured for 30 min. La^{3+} (50 μM) had no effect on vasospressin-stimulated water flow (Table XXX). Incubation with La^{3+} (1 mM) caused a small, but significant enhancement in basal water flow (Table XXX). When vasopressin (1 mU/ml) was added water flow was enhanced in the control hemibladders, but La^{3+} (1 mM) completely blocked the effect of vasopressin to enhance water flow (Table XXX).

^{45}Ca uptake by isolated toad bladder epithelial cells. Using the EGTA technique for removing extracellular calcium described in the Methods, ^{45}Ca uptake into the cells was assessed. Even though this uptake method has been described in the literature (Wiesmann et al., 1977, 1978; Arruda et al., 1980 a,b; Humes et al., 1980) experiments were performed to test the validity of the method. The divalent cation ionophore A23187 has been reported to enhance ^{45}Ca uptake into isolated toad bladder epithelial cells (Wiesmann et al., 1977). In the isolated epithelial cells used in the present experiments, preincubation with A23187 (10 μM) for 30 min increased ^{45}Ca uptake from 1.48±0.12 nmol/10 min/mg protein in control suspensions to 5.04± 0.70 nmol/10 min/mg protein ($p<0.01$, n=4 pairs). Similarly, replacement of bathing medium sodium with choline has been reported to enhance uptake of ^{45}Ca into intact frog skin, a related epithelium (Grinstein and Erlij, 1978). In the isolated epithelial cells used in the present experiments, replacement of bathing medium sodium with choline for 30 min enhanced ^{45}Ca uptake from 1.16±0.06 nmol/10

TABLE XXX

EFFECTS OF LANTHANUM ON VASOPRESSIN-STIMULATED WATER FLOW

	WATER FLOW (mg/min/hemibladder)	
	Basal	Vasopressin (1 mU/ml)
Control	1.4±0.2	34.8±3.6
Lanthanum (50 μM)	1.6±0.4	35.6±2.8
Control	0.6±0.2	28.2±1.7
Lanthanum (1 mM)	1.5±0.2[a]	1.6±0.4[b,c]

[a] $p < 0.05$, [b] $p < 0.001$, compared to control; [c] not significantly different from basal, n=5 pairs, each group.

Lanthanum was added to the serosal medium (tris-Ringer's, 1 mM Ca, Mg-free) and basal water flow measured for 30 min. Vasopressin (1 mU/ml) was then added and stimulated water flow was measured for 30 min.

min/mg protein to 2.84±0.60 nmol/10 min/mg protein ($p<0.05$, n=4 pairs). ^{45}Ca uptake was 0.34±0.04 nmol/10 min/mg protein in the presence of 0.1 mM extracellular calcium, 1.08±0.11 nmol/10 min/mg protein in the presence of 1 mM extracellular calcium, and 2.12±0.31 nmol/ 10 min/mg protein in the presence of 10 mM extracellular calcium (n=4, each group).

^{45}Ca uptake increased with time to a pseudo-steady state level: 0.98±0.12 nmol/mg protein after 5 min to 1.72±0.12 nmol/mg protein after 10 min to 2.80±0.52 nmol/mg protein after 30 min to 2.90±0.22 nmol/mg protein after 60 min to 2.97±0.20 nmol/mg protein after 90 min (n=5). Thus, it appeared that the EGTA method of assessment of ^{45}Ca uptake into the cells was valid.

Effects of vasopressin on ^{45}Ca uptake into toad bladder epithelial cells. Several protocols were devised to determine whether vasopressin causes enhanced labelling of cellular calcium wth ^{45}Ca. In the first experiments vasopressin (10 mU/ml) was added to the experimental suspension together with the ^{45}Ca. As shown in Fig. 35a, vasopressin had no significant effect on ^{45}Ca uptake at any individual point. Upon transforming the data to $\ln(^{45}Ca_{\infty}, -^{45}Ca_t)$ versus time, two linear components were observed, 5 to 30 min and 30 to 90 min. An analysis of variance performed from 5 to 30 min or 30 to 90 min demonstrated that in the presence of vasopressin less ^{45}Ca was accumulated by the cells ($p<0.02$ for 5 to 30 min and $p<0.01$ for 30 to 90 min) compared to the controls. In similar experiments vasopressin (10 mU/ml) was added to the experimental suspensions 15 min prior to the addition of ^{45}Ca. In these experiments (Fig. 35b) the effect of vasopressin to reduce ^{45}Ca uptake was apparent at the 30

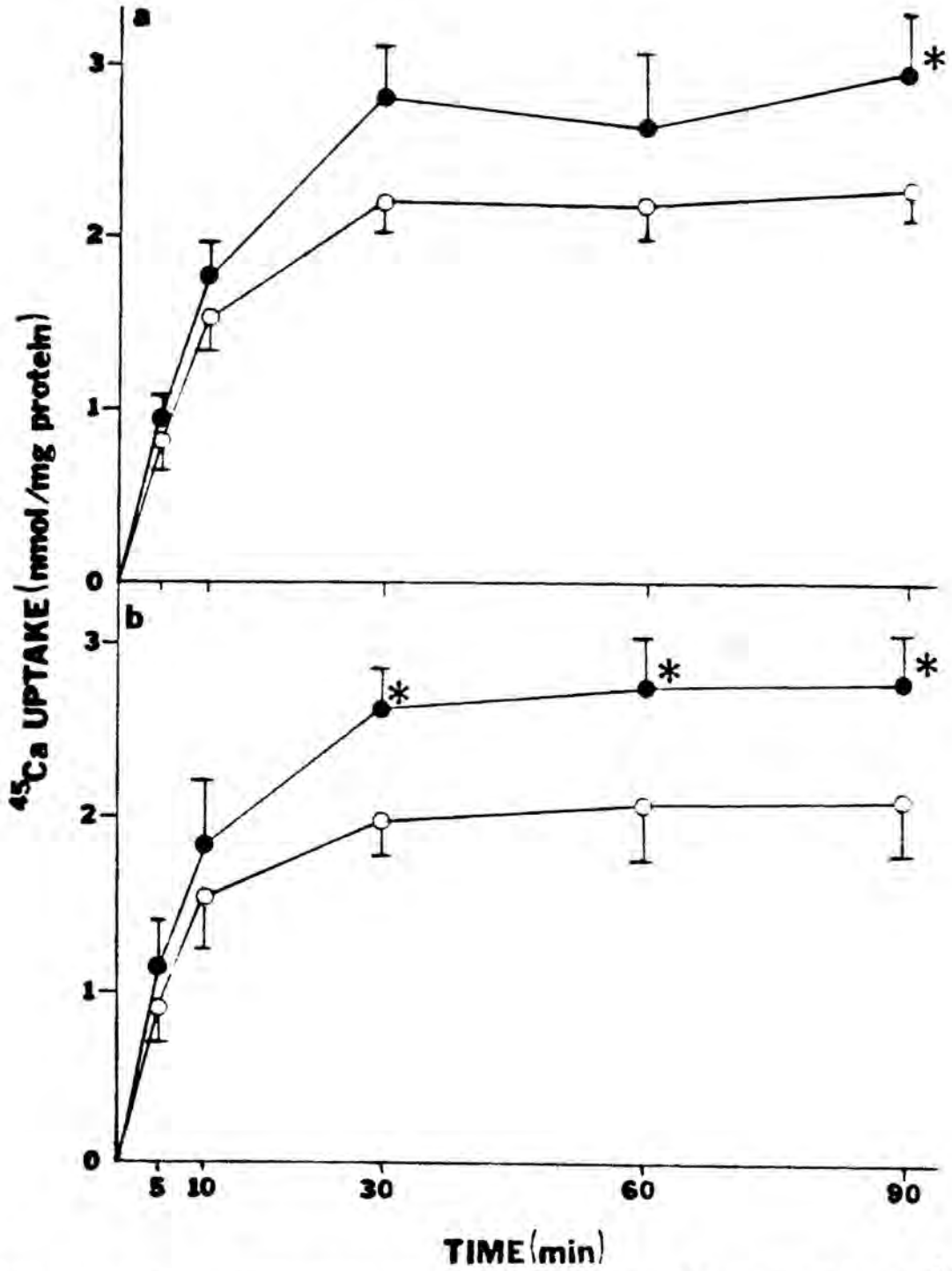


Figure 35. Effects of vasopressin (10 mU/ml) on ^{45}Ca uptake. a) Vasopressin was added at time = 0, n = 5 pairs of suspensions, b) Vasopressin was added 15 minutes prior to ^{45}Ca, n = 6 pairs of suspensions. Filled circles represent uptake into control suspensions and open circles represent uptake into vasopressin-treated suspensions. (*$p < 0.05$, by paired-t analysis).

to 90 min points by paired-t analysis at each time ($p<0.05$, each time) or by analysis of variance ($p<0.01$ for 5 to 30 min and $p<0.001$ for 30 to 90 min).

Effects of U46619 on ^{45}Ca uptake into toad bladder epithelial cells. When U46619 (1 μM) was added to the epithelial cells at the same time as ^{45}Ca, no significant effect on ^{45}Ca uptake was observed at any individual time point (Fig. 36). However, when the 5 to 30 min points were analyzed together by an analysis of variance, the cells which were incubated with U46619 took up significantly less ^{45}Ca over this period ($p<0.01$). By the 60 and 90 min time points, it is clear that the cells incubated with U46619 took up a similar amount of ^{45}Ca to the control cells.

EFFECTS OF COMPOUNDS WHICH INHIBIT VASOPRESSIN-STIMULATED WATER FLOW ON ^{45}Ca METABOLISM

Effects of PGE_1 on ^{45}Ca efflux from isolated toad bladder epithelial cells in suspension. PGE_1 inhibits vasopressin-stimulated water flow (Orloff et al., 1965). Since prostaglandins have been implicated in the regulation of cellular calcium metabolism in a variety of tissues (see Chapter I), the effects of PGE_1 on ^{45}Ca metabolism in toad bladder epithelial cells were assessed. PGE_1 (1 μM) was added to epithelial cells undergoing desaturation. PGE_1 (1 μM) caused an enhancement in ^{45}Ca efflux of 34±8% after 10 min (Fig. 37). Efflux remained enhanced the entire 30 min during which PGE_1 was present, but had decreased to only 20±5% above control by 30 min ($p<0.05$, n=5 pairs) (Fig. 37).

Effects of PGE_1 on steady state ^{45}Ca content and efflux. The enhancement in ^{45}Ca efflux observed upon addition of PGE_1 (1 μM) to

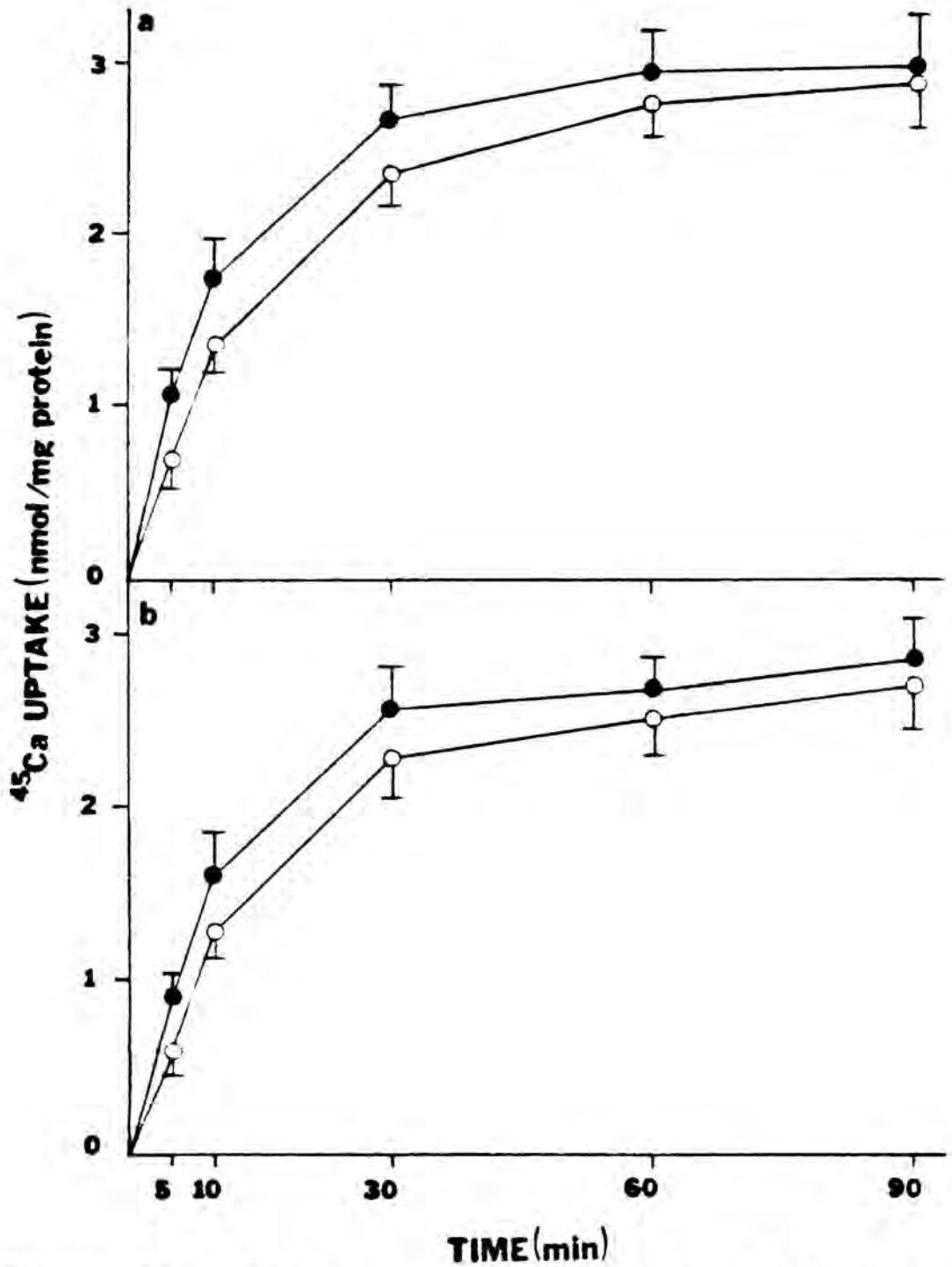


Figure 36. Effects of U46619 (1 μM) on ^{45}Ca uptake. a) U46619 was added at time = 0, n = 6 pairs of suspensions, b) U46619 was added 15 minutes prior to ^{45}Ca, n = 6 pairs of suspensions. Filled circles represent uptake into control suspensions and open circles represent uptake into U46619-treated suspensions.

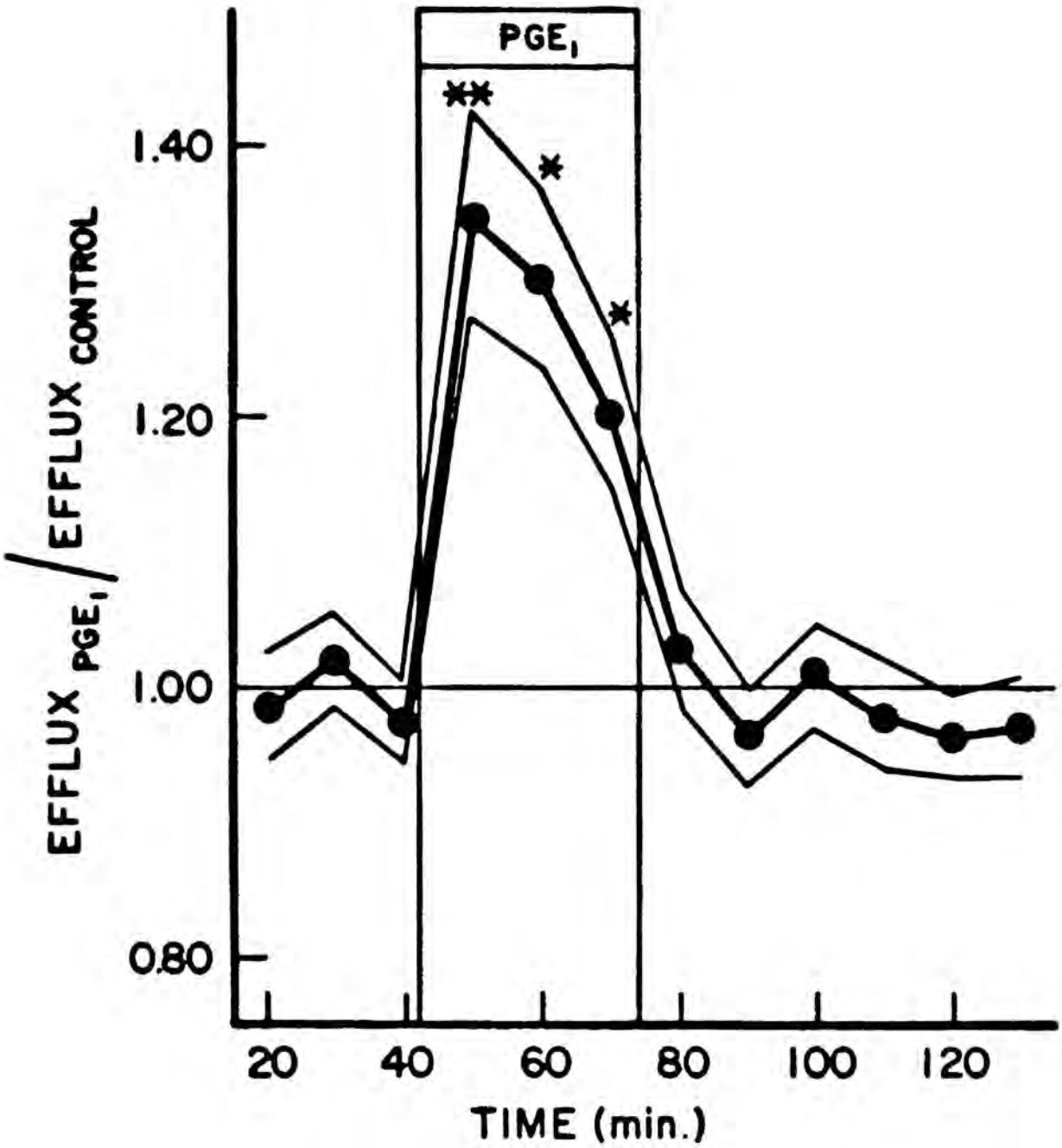


Figure 37. Effect of PGE_1 on ^{45}Ca efflux from isolated toad bladder epithelial cells in suspension. Cell suspensions were labelled with ^{45}Ca for 90 minutes. The suspensions were each divided into 2 aliquots and desaturated simultaneously. After 40 minutes of desaturation PGE_1 (1 μM) was added to 1 member of each pair of aliquots. ($^{*}p < 0.05$, $^{**}p < 0.02$, n = 6 pairs)

epithelial cells undergoing ^{45}Ca desaturation suggested that PGE_1 may affect cellular calcium metabolism in a manner similar to that observed in the presence of vasopressin. Thus, the effect of PGE_1 was determined on ^{45}Ca efflux under steady state conditions. Suspensions were incubated with PGE_1 (1 μM) prior to labelling with ^{45}Ca. Preincubation with PGE_1 resulted in significantly greater total uptake of ^{45}Ca (11.36±1.23 nmol/ mg protein versus 7.82±0.85 nmol/mg protein in control suspensions) ($p<0.01$, n=4 pairs) (Fig. 38). Compartmental analysis revealed that the size of pool S_3 was greatly increased by PGE_1 ($p<0.01$, n=4 pairs) (Table XXXI). Further, the rate constants of exchange from the second and third compartments were increased in the presence of PGE_1. The sizes of pools S_1 and S_2 were not significantly affected (Table XXXI).

Effects of cylcooxygenase inhibitors on steady state ^{45}Ca content and efflux. Indomethacin and other non-steroidal antiinflammatory agents have been reported to inhibit calcium binding to several cellular membranes (Northover, 1973) and to inhibit several calcium-dependent cellular processes (Northover, 1977a). Thus, the effects fo the non-steroidal antiinflammatory agents/cyclooxygenase inhibitors, indomethacin and meclofenamate were assessed on ^{45}Ca kinetics in the isolated epithelial cells. Cells were pretreated with either indomethacin (50 μM) or meclofenamate (10 μM) for 30 minutes, then labelled with ^{45}Ca. The results were pooled since the effects of the two agents were similar. Neither indomethacin nor meclofenamate had a significant effect on the calcium pool S_1 (Table XXXII). However, the sizes of pools S_2 and S_3 were significantly reduced in the

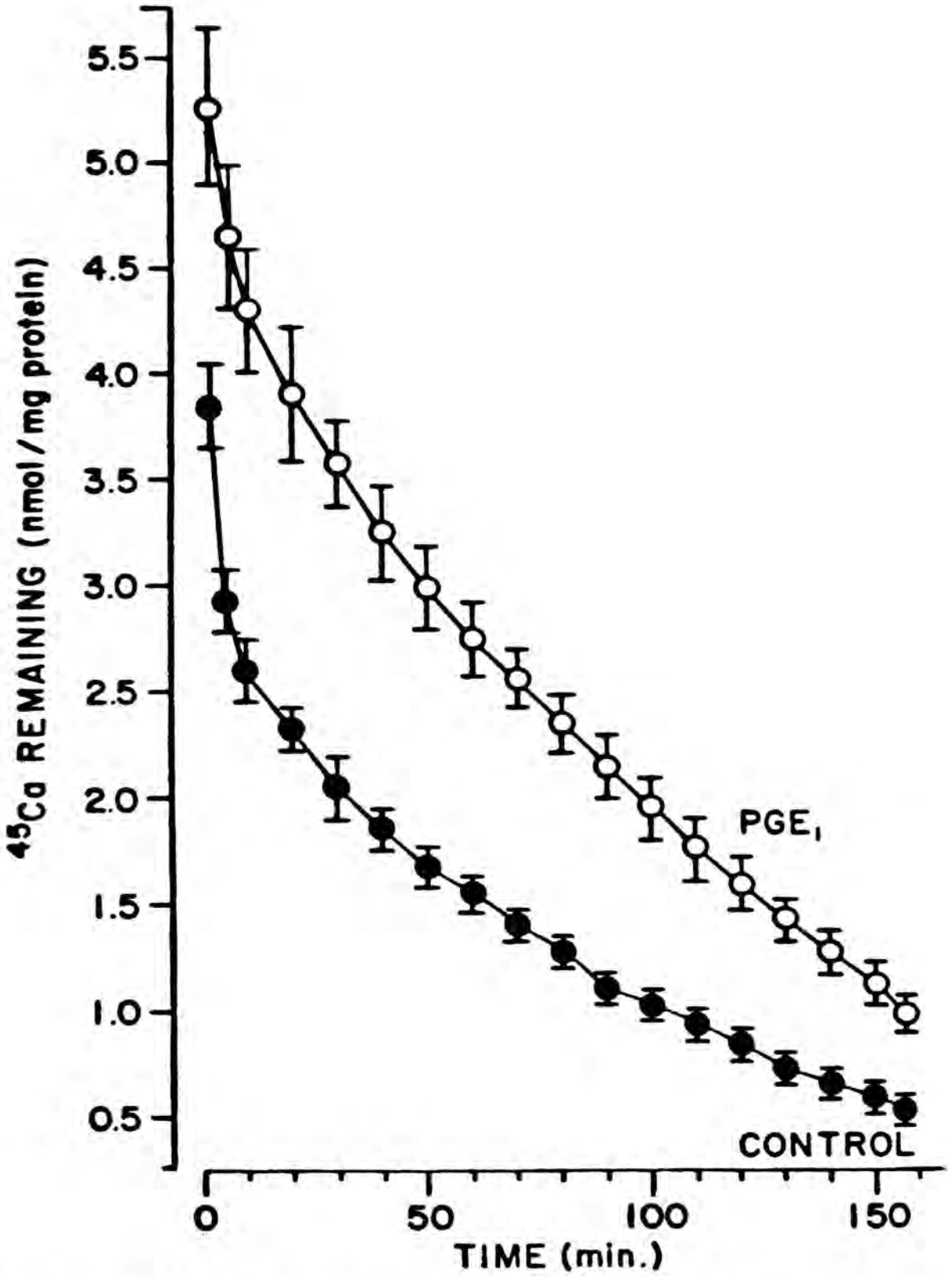


Figure 38. ^{45}Ca efflux from isolated toad bladder epithelial cells preincubated with PGE_1 (1 μM). Cell suspensions were divided into 2 aliquots, 1 of which was incubated for 15 minutes with PGE_1 (1 μM). Then both aliquots were labelled with ^{45}Ca for 90 minutes and desaturated. The experimental aliquots contained PGE_1 during the labelling and desaturation periods. Each PGE_1 point is different from the respective control point ($p < 0.02 - 0.05$, $n = 4$ pairs). ^{45}Ca content at time = 0 were 7.82 ± 0.85 and 11.36 ± 1.23 nmol/mg protein, control and PGE_1, respectively.

TABLE XXXI

EFFECTS OF PGE_1 ON ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			Fast Pool (S_1)				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Control	1.58 ±0.26	0.437 ±0.028	0.276 ±0.034	1.58 ±0.16	0.437 ±0.028	0.276 ±0.034		
PGE 1	1.74 ±0.26	0.526 ±0.047	0.296 ±0.038	1.74 ±0.26	0.526 ±0.047	0.296 ±0.038		
	Intermediate Pool (S_2)			Intermediate Pool (S_2)				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Control	1.02 ±0.34	0.072 ±0.006	0.074[a] ±0.007	2.20 ±0.29	0.114 ±0.011	0.057[b] ±0.007	0.050 ±0.006	0.024[c] ±0.003
PGE 1	0.82 ±0.32	0.076 ±0.008	0.092 ±0.010	2.03 ±0.38	0.128 ±0.014	0.118 ±0.014	0.065 ±0.008	0.059 ±0.009
	Slow Pool (S_3)			Slow Pool (S_3)				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Control	6.81[b] ±0.84	0.044[a] ±0.002	0.0068[a] ±0.0006	5.57[c] ±0.58	0.057[c] ±0.007	0.0099[b] ±0.0014		
PGE 1	9.92 ±0.98	0.091 ±0.011	0.0094 ±0.0011	8.62 ±0.74	0.118 ±0.014	0.0143 ±0.0018		

n=4. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, PGE_1 = 1 μM.

[a] $p < 0.05$, [b] $p < 0.02$, [c] $p < 0.01$, compared to PGE_1.

TABLE XXXII

EFFECTS OF CYCLOOXYGENASE INHIBITORS ON ^{45}Ca KINETICS

	Parallel Case			Series Case				
	Fast Pool (S_1)			Fast Pool (S_1)				
	S_1	ρ_{10}	k_{10}	S_1	ρ_{10}	k_{10}		
Control	1.42 ±0.34	0.362 ±0.048	0.254 ±0.042	1.42 ±0.34	0.362 ±0.048	0.254 ±0.042		
Cyclooxygenase Inhibitors	1.16 ±0.28	0.324 ±0.054	0.276 ±0.054	1.16 ±0.28	0.324 ±0.054	0.276 ±0.054		
	Intermediate Pool (S_2)			Intermediate Pool (S_2)				
	S_2	ρ_{20}	k_{20}	S_2	ρ_{20}	ρ_{23}	k_{20}	k_{23}
Control	1.23[a] ±0.18	0.101[a] ±0.013	0.086[b] ±0.010	2.70[b] ±0.16	0.161 ±0.012	0.064[a] ±0.008	0.058[b] ±0.007	0.022 ±0.002
Cyclooxygenase Inhibitors	1.07 ±0.12	0.132 ±0.015	0.126 ±0.018	1.83 ±0.14	0.178 ±0.013	0.048 ±0.006	0.094 ±0.012	0.025 ±0.003
	Slow Pool (S_3)			Slow Pool (S_3)				
	S_3	ρ_{30}	k_{30}	S_3	ρ_{32}	k_{32}		
Control	7.12[a] ±0.64	0.054[a] ±0.006	0.0080 ±0.0007	5.68[a] ±0.63	0.064[a] ±0.008	0.0112 ±0.0014		
Cyclooxygenase Inhibitors	5.03 ±0.70	0.042 ±0.005	0.0088 ±0.0009	4.28 ±0.58	0.048 ±0.006	0.0111 ±0.0017		

n=5. All results are expressed as mean ± S.E.M. S_i = pool size, nmol/mg protein; ρ_{ij} = exchange rate (flux) from pool i to pool j, nmol/min/mg protein; k_{ij} = rate constant of efflux from pool i to pool j, min^{-1}. Bathing media calcium = 1 mM, Meclofenamate = 10 μM; indomethacin = 50 μM.

[a] $p < 0.05$; [b] $p < 0.02$, compared to cyclooxygenase inhibitors.

presence of these agents. Further, the rate constant of efflux from pool S_2 was increased (Table XXXII).

Effects of agents which inhibit vasopressin-stimulated water flow on ^{45}Ca uptake. A number of compounds which inhibit vasopressin-stimulated water flow are thought to act by increasing the concentration of free ionized calcium in the cytoplasm. Among these are the divalent cation ionophore A23187 (Wiesmann et al., 1977; Hardy, 1978; Taylor et al., 1979), acetylcholine and its analog carbamylcholine (Wiesmann et al., 1978; Arruda and Sabatini, 1980a), and quinidine (Arruda et al., 1980b). In addition, substitution of choline for serosal sodium (Davis et al., 1978; Grinstein and Erlij, 1978), removal of serosal potassium (Bentley, 1959; Davis et al., 1978; Taylor et al., 1979), and high serosal calcium concentration (Petersen and Edelman, 1969; Argy et al., 1967) all inhibit vasopressin-stimulated water flow and may enhance calcium uptake. Since many of these experiments measured the effects of these agents or maneuvers on only vasopressin-stimulated water flow and not calcium uptake, and others were performed using allied epithelia such as frog skin, turtle bladder, and frog bladder, the following experiments were performed in order to test all of these compounds on both vasopressin-stimulated water flow and ^{45}Ca uptake.

The ^{45}Ca uptake studies were all of a single kind. Since all of the drugs or maneuvers are thought to enhance cytoplasmic free calcium concentration, ^{45}Ca uptake was performed for 5 and 10 minutes to gain an assessment not of intracellular pool size but of inward directed permeability to calcium. That the measurement of uptake represented a measure of relative permeability was assumed because

uptake was linear between 5 and 10 minutes for each agent. No attempt was made to measure intracellular calcium pools since some of these agents may actually decrease total exchangeable calcium but increase cytoplasmic free calcium (Shelby et al., 1976).

The data are contained in Table XXXIII. A23187 (10 μM) significantly stimulated basal water flow. However, vasopressin-stimulated water flow was inhibited 46.8±5.7% ($p<0.02$, n=6 pairs). A23187 enhanced ^{45}Ca uptake 3.4-fold, the uptake being linear over the 10 min range. Carbachol (100 μM) inhibited vasopressin-stimulated water flow 52.6±5.2% ($p<0.01$, n=6 pairs) while enhancing ^{45}Ca uptake 1.90-fold ($p<0.02$, n=4 pairs). Quinidine (1 mM) inhibited vasopressin-stimulated water flow 60.3±8.2% ($p<0.01$, n=6 pairs) while ^{45}Ca uptake was not altered by this agent. Epinephrine (1 μM) inhibited vasopressin-stimulated water flow 38.2±4.6% ($p<0.02$, n=6 pairs), while enhancing ^{45}Ca uptake 1.82-fold ($p<0.02$, n=6 pairs). PGE_1 (1 μM) inhibited vasopressin-stimulated water flow 98.6± 2.0% ($p<0.001$, n=6 pairs), while enhancing ^{45}Ca uptake 1.5-fold ($p<0.02$, n=6 pairs).

Substitution of choline for serosal sodium inhibited vasopressin-stimulated water flow 66±11% ($p<0.02$, n=5 pairs), while enhancing ^{45}Ca uptake 2.45-fold ($p<0.02$, n=6 pairs). To assure that this effect was not due to stimulation of a cholinergic receptor, another experiment was performed using sucrose to substitute for NaCl. In the hemibladers incubated in sucrose-Ringer's solution (180 mM sucrose) vasopressin-stimulated water flow was inhibited 53±11% ($p<0.02$, n=5 pairs) while ^{45}Ca uptake was enhanced 2.21-fold (Table XXXIII). Removal of serosal potassium inhibited vasopressin-stimulated water flow 48±7% ($p<0.01$, n=6 pairs), while enhancing ^{45}Ca uptake 1.48-

TABLE XXXIII

EFFECTS OF SEVERAL AGENTS ON VASOPRESSIN-STIMULATED WATER FLOW AND ^{45}Ca UPTAKE

	WATER FLOW (mg/min/hemibldder)		^{45}Ca UPTAKE (pmol/min/mg/protein)	
Agent	Basal	Vasopressin (1 mU/ml)		
Control	1.2±0.4[b]	42.4±3.6[b] (6)	148±12[c] (4)	
A23187 (10 μM)	3.4±0.8	21.8±2.8	504±70	
Control	1.2±0.6	38.1±4.3[c] (6)	174±9[b] (4)	
Carbachol (100 μM)	1.0±0.4	18.3±2.4	312±38	
Control	1.2±0.4	34.5±4.0[c] (6)	115±11 (6)	
Quinidine (1 mM)	1.3±0.4	13.7±2.1	122±18	
Control	0.4±0.3	35.6±2.8[b] (6)	132±21[b] (6)	
Epinephrine (1 μM)	0.7±0.2	22.0±2.0	238±20	
Control	1.8±0.5	38.2±3.6[d] (6)	168±14[b] (6)	
PGE_1 (1 μM)	2.1±0.4	2.4±0.8	246±46	
Control	1.2±0.3	32.4±2.5	126±18 (6)	
Ouabain	1.6±0.2	33.9±4.1	132±14	
Control	0.9±0.2	32.8±2.6[b] (5)	108±11[b] (4)	
Calcium (10 mM)	1.1±0.3	20.6±2.2	212±31	
Control	2.9±0.9	43.6±4.3[c] (6)	118±12[a] (6)	
Na^+ replacing K^+	2.1±0.4	23.7±4.0	172±24	
Control	1.0±0.3	36.4±3.6[b] (5)	116±6[a] (6)	
Choline replacing Na^+	1.4±0.5	17.8±1.4	284±60	
Control	1.2±0.6	32.8±2.8[b] (5)	124±16[c] (6)	
Sucrose replacing Na^+	1.1±0.7	14.6±2.0	276±20	

[a] $p < 0.05$, [b] $p < 0.02$, [c] $p < 0.01$, [d] $p < 0.001$, compared to experimental.

fold ($p<0.05$, n=6 pairs). Incubation with ouabain (100 μM) for 1 hour had no effect on vasopressin-stimulated water flow and did not affect ^{45}Ca uptake (Table XXXII).

DISCUSSION

These studies have shown that uptake of calcium from the extracellular bathing media is not required for vasopressin to exert its antidiuretic effect in the toad bladder. However, intracellular calcium does seem to be required, either to exert some direct effect or to allow the tissue to recover the ability to respond to vasopressin a second time. Further, vasopressin does not cause enhanced uptake of ^{45}Ca into toad bladder epithelial cells. Finally, several inhibitors of vasopressin-stimulated water flow cause enhanced ^{45}Ca uptake into epithelial cells isolated from the toad urinary bladder.

Removal of all calcium from the Ringer's solution bathing the mucosal surface of the toad bladder did not affect the hydroosmotic response to vasopressin. This observation was not unexpected since the calcium concentration in urine would be expected to vary tremendously and in random fashion. Further, it is common practice to bath the mucosal surface with distilled water during water flow experiments. However, when calcium was progressively removed from the serosal Ringer's solution without adding any other divalent cation, vasopressin-stimulated water flow was progressively inhibited. This observation suggested that calcium uptake might be necessary for vasopressin to exert its effect. The inhibition was not reversible, even when the usual amount of calcium was added back to the serosal Ringer's solution for 1 hour. Further, since the phenomenon of inhibition of water flow was also exhibited in response to cAMP and U46619, two other agents which stimulated water permeability in the control hemibladders, a nonspecific effect of decreased calcium concentration was suspected. In fact, Hardy and DiBona in a recent

abstract (1981) found that bathing toad bladders in a serosal medium devoid of calcium or other divalent cation caused marked detachment of the mitochondria-rich and granular cells from the basal cells and supporting connective tissues while the cells remained interconnected among themselves by tight junctions. Further, they found that while osmotic water flow in response to vasopressin was inhibited, the diffusional permeability response was not lost.

Since calcium appears to be required for maintenance of bladder structure (Hays et al., 1965), it was thought that inclusion of another divalent ion might preserve structural integrity and allow the determination of whether extracellular calcium is, in fact, required for vasopressin action. Magnesium often substitutes well for calcium in maintaining structural integrity, while it is not able to substitute for calcium in transducing biochemical messages (Putney and Askari, 1980). Thus, in additional experiments when the Ringer's solution was devoid of calcium, magnesium (1 mM) was included. When magnesium was present to substitute for calcium, vasopressin-stimulated water flow was only slightly inhibited by the reduced extracellular calcium. Further, if calcium was then substituted for magnesium, the hemibladders responded to vasopressin normally. However, if vasopressin was washed out of the hemibladders which were bathed in magnesium-Ringer's solution (no calcium), the hemibladders did not respond to vasopressin a second time. Similar results were obtained when strontium was substituted for calcium. When strontium was used the initial response to vasopressin was not inhibited at all. However, when vasopressin was added a second time to hemibladders incubated with strontium, no water permeability response

was observed. When calcium was added back and the hemibladders were stimulated with vasopressin, the full permeability response was restored. Thus, strontium appeared to not be substituting for calcium as a biochemical messenger, but only in the maintenance of structural integrity.

Verapamil and its methoxy derivative, D-600, are organic calcium antagonists which act by inhibiting the uptake of calcium through the plasma membrane of many types of cells (Triggle and Swamy, 1980). Bentley (1974) reported that verapamil, in concentrations up to 100 μM, had no effect on vasopressin-stimulated water flow across the isolated toad bladder. Recently, however, Humes et al. (1980) reported that verapamil, after 90 min preincubation, does inhibit vasopressin-stimulated water flow in a dose-dependent fashion. Since the data with cation substitution suggested that extracellular calcium is not required for vasopressin to enhance water permeability, the effects of verapamil and D-600 were assessed on vasopressin-stimulated water flow. Only at very high concentrations after long preincubation were these agents observed to slightly inhibit vasopressin-stimulated water flow.

Since these organic calcium antagonists bind to the same sites as does calcium, it was thought that stimulation of water flow and calcium efflux with vasopressin might allow the agents to more readily gain access to their binding sites and thus inhibit the water flow response to a second challenge with vasopessin. Thus, hemibladders were incubated with vasopressin for 30 min in the presence of D-600. No inhibition of water flow was observed during the first vasopressin period. The hemibladders were then allowed to recover

for 30 min and then incubated with vasopressin a second time. Water flow was inhibited 28% ($p<0.05$) during the second vasopressin period. When the same protocol was used with hemibladders incubated with lower serosal concentrations of calcium, a greater effect of D-600 was observed. Thus, in the presence of 200 μM serosal calcium inhibition of the water flow response during the first vasopressin period was 27% and during the second vasopressin period water flow was inhibited 45%. In the presence of 25 μM serosal calcium inhibition of water flow during the initial vasopressin period was 34% and increased to 94% during the second inhibition. These results are consistent with more ready access of D-600 to its binding site after vasopressin has displaced calcium. However, since D-600 inhibited basal ^{45}Ca uptake 35%, these data are also consistent with the hypothesis invoked to explain the results with magnesium and strontium substitution. That is, vasopressin induces the release of intracellular calcium. D-600, during the 30 min recovery period, inhibited complete reuptake of the calcium. Thus, when the hemibladders were restimulated with vasopressin, the full response could not be developed since some critical intracellular calcium pool was not reloaded. Thus, when the serosal calcium concentration was 1 mM a large uptake still occurred and little inhibition was noted when the hemibladders were restimulated with vasopressin. However, when the serosal calcium concentration was reduced, reuptake of calcium was more readily compromised by D-600 and the response to the second stimulation with vasopressin was markedly reduced.

Lanthanum at low concentration (50 μM) has been shown to not affect basal ^{45}Ca uptake into toad bladder epithelial cells (Wiessmann

et al., 1977, 1978; Arruda and Sabatini, 1980b). However, it inhibits enhanced ^{45}Ca uptake in response to carbachol or A23187 (Wiesmann et al., 1977, 1978; Arruda and Sabatini, 1980b). In the present experiments serosal lanthanum, 50 μM, did not affect vasopressin-stimulated water flow. At a high concentration ($\geq$ 1 mM) lanthanum inhibits ^{45}Ca efflux as well as influx from several types of cells (Langer and Frank, 1972; Lee and Auersperg, 1980). In the experiments described in Chapter III, lanthanum (1 mM) was observed to significantly inhibit basal ^{45}Ca efflux from isolated toad bladder epithelial cells. Lanthanum, 1 mM, was found to inhibit vasopressin-stimulated water flow completely, suggesting that blockade of calcium efflux may contribute to this inhibitory effect. However, the significance of this observation must be held suspect. Cuthbert and Wong (1974) reported that calcium release in response to vasopressin occurs only from the mucosal surface. Since lanthanum does not cross the toad bladder plasma membrane (Davis, 1981), its effect in this experiment may not have been due to inhibition of calcium efflux, unless inhibition of basal calcium efflux via the serosal sodium-calcium exchange (Chase and Al-Awqati, 1981) had increased cytoplasmic calcium to a concentration which vasopressin could not displace. The effect of lanthanum in these experiments may have been at the level of vasopressin receptor-adenylate cyclase coupling. Coupling of these proteins has been reported to be calcium-dependent (Bockaert et al., 1972). Of course, some other site may have been affected.

Neither vasopressin nor U46619 enhanced calcium uptake into isolated toad bladder epithelial cells. Vasopressin significantly

reduced the equilibrium amount of ^{45}Ca taken up into the cells and the rate of influx during the linear portion of uptake (to 10 min). The equilibrium calcium uptake was about 0.7 nmol/mg protein less than control, similar to the findings of the experiments described in Chapter III. U46619 reduced slightly the rate of ^{45}Ca influx during the linear portion of influx (to 30 min). However equilibrium ^{45}Ca uptake was identical in control cells and in cells incubated in U46619. Thus, when the present observations are combined with those in Chapter III, it appears that vasopressin has no effect on calcium influx into the cells; however, an enhancement in the rate of efflux results in a net reduction in the amount of calcium in one cellular compartment (S_3). U46619, on the other hand, reduces influx of calcium while not affecting efflux. Thus, total cell calcium is little changed. However, the amount of calcium in one cellular compartment (S_3) is reduced, similar to the effect observed with vasopressin.

Several agents which are thought to increase cytoplasmic calcium concentration inhibit vasopressin-stimulated water flow. PGE_1 was found to enhance ^{45}Ca influx and efflux, resulting in net calcium accumulation. The accumulation was into that compartment from which vasopressin released calcium. Indomethacin and meclofenamate, at concentrations which nearly completely inhibit PGE synthesis, reduced the sizes of pools S_2 and S_3. Thus, PGE may be implicated in maintaining cellular calcium homeostasis. However, these agents have been implicated as cellular calcium antagonists, an action which may be independent of prostaglandin synthesis inhibition (Northover, 1977b).

Epinephrine and carbachol both enhanced ^{45}Ca uptake to an extent similar to PGE_1. However, while PGE_1 inhibited vasopressin-stimulated water flow completely, epinephrine and carbachol inhibited water flow only about 50%. Thus, PGE_1 may have some additional effect. However, since dose-response experiments were not performed in these studies, differences among the apparent effectiveness of the agents must remain in question. The enhanced net cellular accumulation of calcium in response to PGE_1 may explain the greater effect of this compound, since in other systems carbachol has been shown to enhance cytoplasmic free calcium concentration, and thus ^{45}Ca uptake while actually reducing the amount of membrane-bound calcium (Shelby et al., 1976).

Quinidine inhibited vasopressin-stimulated water flow confirming the observations of Taylor et al. (1979). However, no enhancement in ^{45}Ca uptake was noted in response to this compound. Recently, Arruda and Sabatini (1980b) found that quinidine did not enhance ^{45}Ca uptake into turtle bladders. However, they found that quinidine did enhance ^{45}Ca efflux from prelabelled hemibladders. Thus, quinidine may act to inhibit vasopressin-stimulated water flow by enhancing the release of calcium from an internal membrane to enhance cytoplasmic free calcium.

Substitution of serosal sodium with choline or sucrose inhibited vasopressin-stimulated water flow and enhanced ^{45}Ca uptake. These results may be due to the existence of a sodium-calcium exchange on the serosal plasma membrane (Chase and Al-Awqati, 1981). Removal of serosal potassium also inhibited vasopressin-stimulated water flow and enhanced ^{45}Ca uptake. This result may have been due to inhibition

of the sodium pump by lack of potassium associated with enhanced intracellular sodium which is exchanged for extracellular calcium by means of the sodium-calcium exchange. Alternatively, lack of potassium may also have reduced the membrane potential to a more negative value, which also would be expected to enhance the activity of the sodium-calcium exchanges. However, incubation with ouabain did not inhibit vasopressin-stimulated water flow or enhance ^{45}Ca uptake. Thus, removal of potassium may have inhibited vasopressin-stimulated water flow and enhanced ^{45}Ca uptake by some mechanism other than inhibition of the sodium pump.

Thus, the present studies suggest that vasopressin-stimulated water flow is not dependent on extracellular calcium or stimulation of calcium influx. However, intracellular calcium does appear to be required for vasopressin to elicit its antidiuretic effect.

CHAPTER V

GENERAL CONCLUSIONS: A HYPOTHETICAL MODEL FOR THE INTERACTIONS AMONG PROSTAGLANDINS, THROMBOXANES, CALCIUM, AND CYCLIC NUCLEOTIDES IN THE ANTIDIURETIC RESPONSE TO VASOPRESSIN, AND PROPOSED DIRECTIONS FOR FUTURE RESEARCH

A MODEL TO DESCRIBE THE INTERACTIONS AMONG PROSTAGLANDINS, THROMBOXANES, CALCIUM, AND CYCLIC NUCLEOTIDES IN RESPONSE TO VASOPRESSIN

As a result of the work described in this dissertation and a review of the literature, a working model has been developed in an attempt to describe the possible relationships between vasopressin's stimulation of water permeability and alterations in prostaglandin, thromboxane, calcium, and cyclic nucleotide metabolism. The model is presented in Fig. 39 and the following text is keyed to the figure.

(1) Vasopressin binds to receptors on the serosal surface of the epithelial cells of the toad urinary bladder. Two classes of receptors have been identified based on [^{3}H]-oxytocin binding (Bockaert et al., 1970). One receptor class is coupled to adenylate cyclase as demonstrated by activation of adenylate cyclase activity by vasopressin in broken cell preparations from the toad bladder (Bär et al., 1970; Bockaert et al., 1972). That the other class of receptors may be physiologically relevant is supported by several observations: a) several vasopressin analogs differentially activate water permeability and sodium transport (Bourguet and Morel, 1967); b) a number of vasopressin analogs are able to completely activate water permeability at concentrations below which any enhancement may be observed in adenylate cyclase activity (Kirchberger et al., 1977); and c) vasopressin activates PGE and TXA_2 synthesis (Zusman et al., 1977; Burch et al., 1980) while theophylline and cAMP both stimulate water

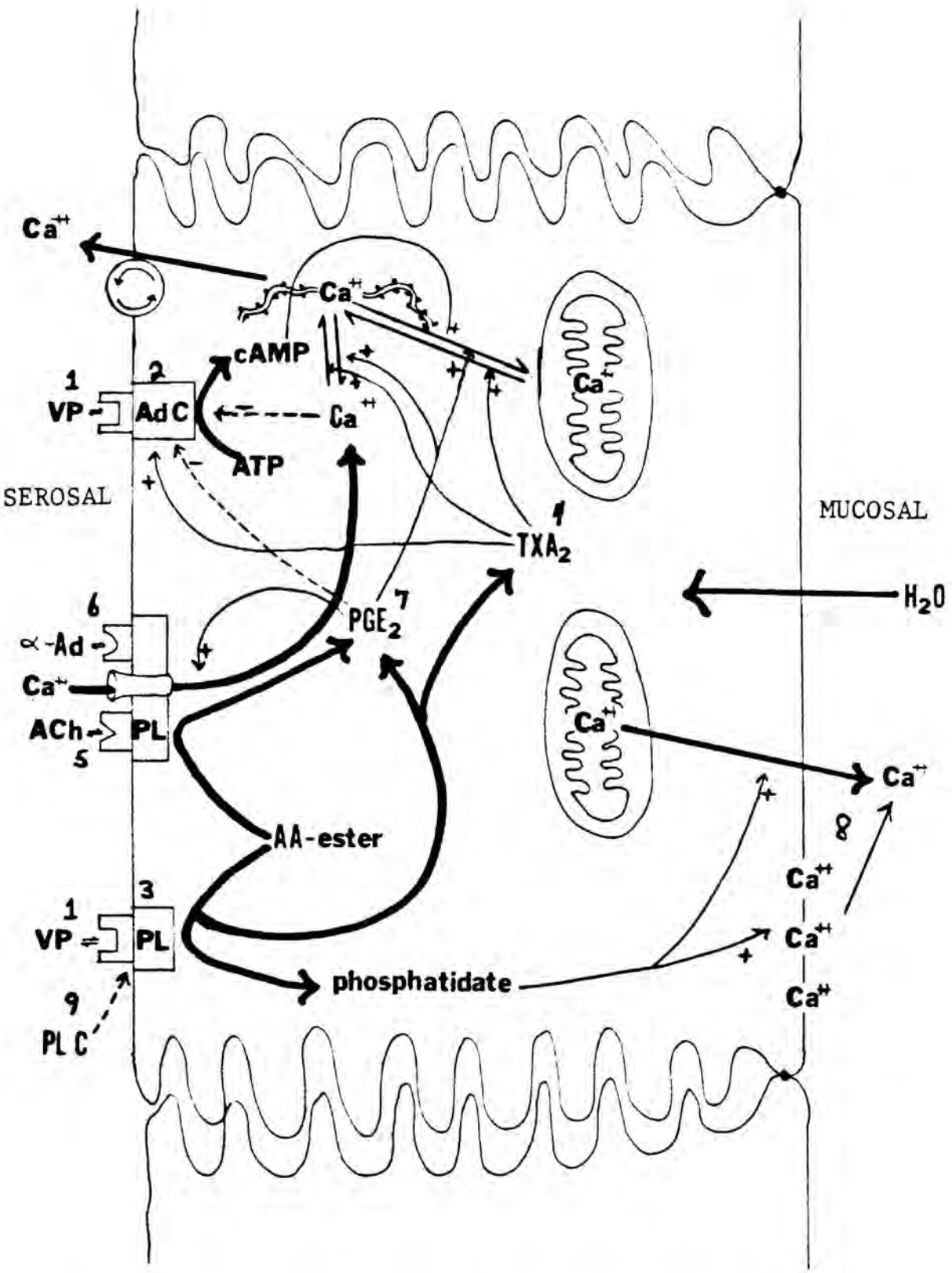


Figure 39. Scheme of postulated events may modify vasopressin's hydroosmotic effect in the toad urinary bladder. Pictured is an epithelial cell. The serosal surface is backed by connective tissue/smooth muscle layer. The epithelial cells are 20 μm across maximally, while the entire tissue including the supporting layer is 100 μm across. The numbers are keyed to the text.

flow via an enhancement in cellular cAMP concentration without affecting PGE or TXA_2 synthesis (Zusman et al., 1977; Burch et al, 1980).

(2) The vasopressin receptor coupled to adenylate cyclase induces an enhancement in the metabolism of ATP to cAMP (Handler et al., 1965). cAMP is generally thought to act by altering the level of phosphorylation of proteins. Greengard and associates have found that addition of vasopressin or exogenous cAMP to intact toad bladder epithelial cells or broken epithelial cell preparations from the toad bladder is associated with a reduction in the level of phosphorylation of at least one protein (DeLorenzo et al., 1973; Greengard, 1976). The identity of this protein remains unknown. However, its dephosphorylation appears to correlate more closely with enhanced sodium transport than with altered water permeability (Walton et al., 1975). The results of the present experiments suggest that cAMP may alter calcium kinetics in the epithelial cells of the toad bladder. Thus, exogenous cAMP reduced the size of exchangeable calcium pool S_3, while increasing the size of pool S_2. Hormones acting through cAMP such as ACTH have been reported to cause an intracellular shifting of calcium from one cellular pool to another while inhibiting ^{45}Ca efflux (Jaanus and Rubin, 1971). Pool S_3 may represent mitochondrial calcium. cAMP has been reported to release calcium from mitochondria isolated from several sources (Borle, 1974; Matlib and O'Brien, 1974). Pool S_2 may represent primarily calcium bound to endoplasmic reticulum and other cytoplasmic structures. cAMP has been reported to enhance calcium uptake into the sarcoplasmic reticulum in heart (Tada et al., 1974). Further, fibroblasts, hepatocytes, and adipocytes, among other cell types, contain active calcium

uptake systems in their endoplasmic reticuli (Moore and Pastan, 1978; Becker et al., 1980; Black et al., 1981), and these uptake systems have been shown to increase calcium-binding activity in response to peptide hormones (Andia-Waltenbaugh et al., 1980; Reinhart and Bygrave, 1981). Calcium ion potently inhibits vasopressin-sensitive adenylate cyclase in the toad bladder (Hynie and Sharp, 1971). Thus, vasopressin may induce some conformational change in its receptor such that coupling to adenylate cyclase occurs (Rodbell, 1980) resulting in enhanced cAMP synthesis. The initially-formed cAMP may act to lower the concentration of cytoplasmic calcium near the adenylate cyclase, perhaps by dephosphorylating a membrane protein in the endoplasmic reticulum to induce calcium uptake by this organelle. The lowered cytoplasmic calcium concentration may further activate the vasopressin-receptor-adenylate cyclase complex.

(3) There are two classes of vasopressin receptors present on the epithelial cells. Since vasopressin appears to enhance PGE_2 and TXA_2 synthesis independent of cAMP (Zusman et al., 1977; Burch et al., 1980), the second class of vasopressin receptors may be coupled to a phospholipase. The phospholipase may include at least 2 pools of enzymes based on the studies which demonstrate that calcium alters PGE and TXA_2 synthesis ratios. At least 2 products, PGE_2 and TXA_2, are formed.

(4) TXA_2 acts in a manner similar to exogenous cAMP. That is, it decreases the size of calcium pool S_3 and increases the size of pool S_2. A similar effect of PGI_2 to cause an intracellular shift of calcium from one pool to another has been postulated (Rubin et al., 1980). The ultimate effect of TXA_2 may well be enhancement of

cAMP production (Vesin, 1979), perhaps by lowering the concentration of calcium near the vasopressin-sensitive adenylate cyclase.

(5) Acetylcholine and its active analogs inhibit vasopressin-stimulated water flow (Wiesmann et al., 1978). Acetylcholine generally is considered to increase cellular influx of calcium through a specific channel (Berridge, 1980). Recent observations of Cuthbert and associates (Cuthbert and Wilson, 1981) suggest that acetylcholine's actions may involve prostaglandins. Since current hypotheses suggest that the calcium channel is opened by activation of a phospholipase C to enhance phosphatidylinositol turnover (Berridge, 1980), acetylcholine may activate a phospholipase which releases arachidonic acid to synthesize PGE_2.

(6) The toad bladder has an α-adrenergic receptor on the serosal surface of the epithelial cells (Handler et al., 1968). Occupation of this receptor by α-agonists markedly inhibits vasopressin-stimulated osmotic water flow (Handler et al., 1968; Cuthbert and Wong, 1972). The water flow response to theophylline was also inhibited by α-agonists, while these agents had no effect on cAMP-stimulated water flow (Handler et al., 1968). α-Agonists often enhance calcium uptake (Berridge, 1980) and, since, theophylline-stimulated water flow is inhibited by these agents, basal adenylate cyclase activity must be inhibited. Calcium and PGE are the 2 endogenous agents most often associated with this effect in the toad bladder. α-Agonists have been shown to enhance prostaglandin synthesis in the MDCK line of cultured kidney epithelial cells (Levine and Moskowitz, 1979). Further, clonidine, an α_2 agonist, has been shown to antagonize the antidiuretic effects of vasopressin in man and animals, and to

stimulate renal PGE_2 synthesis (Ercan et al. 1979). Recently (Cuthbert and Wilson, 1981) cyclooxygenase inhibitors have been shown to abolish the effects of α-adrenergic agonists on sodium transport across isolated frog skin.

(7) Exogenous PGE_2 inhibits vasopressin-stimulated water flow (Orloff et al., 1965). Its action has been suggested to include inhibition of the vasopressin-sensitive adenylate cyclase (Lipson and Sharp, 1971). Indeed, exogenous PGE_1 has been reported to inhibit the cellular accumulation of cAMP in response to vasopessin (Omachi et al., 1974). However, PGE_1 did not inhibit vasopressin-stimulated adenylate cyclase activity in broken cell preparations from toad bladder (Bär et al., 1970). Thus, its action appears to be indirect. The present studies demonstrate that exogenous PGE increases the calcium permeability of the epithelial cell plasma membranes, resulting in net calcium accumulation. Since calcium inhibits vasopressin-sensitive adenylate cyclase activity (Hynie and Sharp, 1971) PGE may inhibit adenylate cyclase activity by increasing the calcium concentration near the vasopressin-sensitive adenylate cyclase. This hypothesis has been presented as a general mechanism for PGE inhibition of cAMP accumulation by Silver and Smith (1975).

(8) Vasopressin induces a net release of calcium from the epithelial cells, apparently from the mucosal side (Cuthbert and Wong, 1974). However, neither exogenous cAMP nor U46619 do so, suggesting that the calcium release occurs by some mechanism other than accumulation of cAMP. Some messenger molecule must mediate the calcium release since release occurs from the mucosal side while vasopressin binds to the serosal side. Phosphatidate is here invoked as the

messenger. There is no experimental evidence for phosphatidate mediation of the calcium release, however, phosphatidate is a potent and effective calcium ionophore (Tyson et al., 1976; Serhan et al., 1981), and a vasopressin receptor appears to be coupled to a phospholipase in the toad bladder. Further, vasopressin causes a shift of [^{3}H]-arachidonate from one phospholipid pool to another in the toad bladder (Halushka, et al., unpublished observations). In platelets, a similar shift is observed in response to some agonists, and an intermediate of the shift appears to be phosphatidate (Lapetina et al., 1980). The calcium release may occur from mitochondria or from the mucosal plasma membrane.

Plasma membrane release is favored by the observation that EDTA displaces ^{45}Ca from the same slowly exchanging pool as does vasopressin (Cuthbert and Wong, 1974). Further, the presence of lanthanum in the mucosal bathing solution did not inhibit vasopressin-stimulated water flow (Weitzerbin et al., 1974). However, if lanthanum was present in the mucosal bathing solution while vasopressin was present, and remained while vasopressin was washed out, inhibition of water flow to a second challenge with vasopressin occurred. Thus, lanthanum may have been able to bind to calcium sites from which vasopressin had displaced calcium which usually was only slowly exchanged.

Calcium release from mitochondria is favored by the following data. Incubation of toad baldders with mitochondrial inhibitors did not inhibit the induction of vasopressin-stimulated water flow (Masters and Fanestil, 1979). However, the "off-time," that is, the time taken for water flow to return to basal level after washing out

vasopressin, was significantly lengthened in the presence of mitochondrial inhibitors (Masters and Fanestil, 1979). Mitochondrial inhibitors decreased the size of calcium pool S_3 (the pool from which vasopressin released calcium), suggesting that the lengthened "off-time" in the presence of the mitochondrial inhibitors may have been due to impaired ability of the mitochondria to take up calcium.

Amiloride inhibited the release of calcium from intact hemibladders in response to vasopressin (Cuthbert and Wong, 1974). Further, in toad bladder epithelial cells, amiloride appeared to have marked effects on mitochondrial structure (D. Baron, unpublished observations). No data exist which demonstrate whether amiloride blocks the vasopressin-induced calcium release directly, or depletes this pool of calcium prior to the addition of vasopressin. It would be interesting to determine the effect of amiloride on the off-time of the vasopressin response. Since EDTA depleted only about half of pool S_3 and mitochondrial inhibitors depleted only about half, pool S_3 may represent 2 calcium pools with similar rate constants of efflux, one mitochondrial and the other slowly-exchanging plasma membrane-bound calcium. Thus, both sites may have been affected by vasopessin.

(9) Incubation of intact hemibladders on the serosal surface with a nonspecific phospholipase C inhibited vasopressin-stimulated water flow and calcium release (Cuthbert et al., 1971; Cuthbert and Wong, 1973). However, phospholipase C did not block intracellular accumulation of cAMP in response to vasopressin (Cuthbert et al., 1971). Thus, phospholipase C affected the vasopressin-induced alteration in water permeability of the mucosal membrane by an action on the serosal membrane. The step affected was not the vasopressin

receptor-adenylate cyclase coupling. It is suggested that phospholipase C acted to destroy coupling of the second vasopressin receptor to its specific phospholipase. It may be argued that the incubation with phospholipase C would have caused formation of phosphatidate, which, according to the model, should have enhanced calcium release and perhaps water flow. However, hormone-sensitive phospholipases C which have been described are found free in the cytosol and probably catalyze formation of phosphatidate from intracellular membranes (Billah et al., 1980; DiRenzo et al., 1981). The exogenously applied phospholipase C would only have cleared phosphatidate from the external face of the serosal plasma membrane.

The model which has been presented provides a hypothesis which may be subjected to experimental testing. Several components are supported by only little data or indirect data, however, the model does account for many phenomena which have been described. It is the first attempt since that of Berridge (1975) to bring together all of the disparate data which have been collected concerning possible interactions among cyclic nucleotides, calcium, and now, arachidonate metabolites in controlling vasopressin-stimulated water flow. A great shortcoming of the model is that it does not explain how the interactions of these myriad "second messengers" bring about enhanced water permeability. No arrows may be drawn to the water entry step at the mucosal membrane. In this regard, after 23 years of intense study of the "mechanism" of vasopressin's effect on the water permeability of the toad bladder, only a "black box" can be depicted.

DIRECTIONS FOR FUTURE RESEARCH

The basic molecular mechanism by which vasopressin elicits an enhancement of water permeability across several epithelia remains a mystery. The data presented in this dissertation suggest that TXA_2 and intracellular calcium may play roles. A role for calcium has been suggested often in the past but definitive experiments have yet to be performed. TXA_2 is a new putative mediator.

The studies presented here demonstrate that the toad bladder synthesizes TXA_2 in response to vasopressin. This observation has recently been extended to isolated mammalian collecting tubules (Kirschenbaum et al., 1981). This dissertation suggests that TXA_2 may be a positive modulator of vasopressin-stimulated water flow based on experiments using TXA_2 synthesis inhibitors, TXA_2 antagonists, and TXA_2 mimetics. Unfortunately, none of the experiments were conclusive. The TXA_2 synthesis inhibitors, imidazole and an imidazole analog, 7IHA, may well have inhibited vasopressin-stimulated water flow by an interaction with calcium (Yu et al., 1967).

The TXA_2 antagonist, t13APA appeared to destroy isolated toad bladder cells by means of some detergent effect at concentrations found to inhibit vasopressin-stimulated water flow. Thus, inhibition of vasopressin-stimulated water flow may have occurred in response to nonspecific interactions of t13APA with the hemibladders. This possibility is weakened by the observation that c13APA, which also destroyed the isolated cells, did not affect vasopressin-stimulated water flow across the intact hemibladders.

The TXA_2 mimetics U46619, U44069, and TXB_2 mimicked vasopressin's hydroosmotic effect. However, U46619 and U44069 are actually endo-

peroxide analogs. Thus, a locus for their effects is uncertain. Further, the duration of action of these compounds was quite short compared to vasopressin. That the compounds were acting as TXA_2 mimetics is suggested by the observation that t13APA but not c13APA antagonized the water permeability response to these compounds. U46619 mimicked the effect of cAMP on ^{45}Ca kinetics in the isolated cells. In another system U46619 appears to act by enhancing cellular accumulation of cAMP. Thus, these compounds may have acted to enhance water permeability by enhancing cAMP accumulation.

The shortcomings of the studies described above suggest several areas in need of further investigation. 1) More specific TXA_2 synthesis inhibitors are needed to determine whether TXA_2 synthesis inhibition is associated with inhibition of vasopressin-stimulated water flow, 2) more specific TXA_2 antagonists are needed to determine the role of TXA_2, 3) studies should be performed to determine whether U46619, U44069, and TXB_2 enhance water flow by enhancing cAMP accumulation. If this is found to be the case then further experiments with these compounds need to be performed to determine how they are able to enhance cAMP accumulation.

Perhaps the most important area for research concerning the role of TXA_2 is the determination of whether the mammalian kidney synthesizes TXA_2 in response to vasopressin and whether TXA_2 may play a role in modulating vasopressin-stimulated water flow. If the toad bladder is studied as an alternative model for the mammalian kidney, then, until evidence that TXA_2 plays a role in free water clearance in the kidney has been obtained, it seems unwarranted to pursue the mechanism of action of TXA_2 in the toad bladder.

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