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SHORT COMMUNICATION

High-Affinity, Divalent Ion-Specific Binding of ^{3}H-ATP to Homogenate Derived from Rabbit Urinary Bladder

Comparison with Divalent-Ion ATPase Activity

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SUMMARY

There is circumstantial evidence that ATP may be an excitatory neurohumoral transmitter in the urinary bladder of several species. Although it is controversial whether or not ATP is acting as a neurohumoral transmitter, it is generally believed that ATP stimulates contractility via the stimulation of a specific "ATP receptor." Using radioligand filtration methodology we have studied the characteristics of binding of ^{3}H-ATP to homogenate preparations of the rabbit urinary bladder. We have compared the binding characteristics with the enzyme characteristics of divalent ion-specific ATPase. The results can be summarized as follows: (1) ^{3}H-ATP binding showed divalent ion-specific (80% of total binding) and divalent-independent binding. The cation-specific binding was saturable and displayed nonlinear kinetics, whereas the divalent cation-independent binding was not saturable at the concentrations studied. (2) β,γ-methylene ATP was (a) approximately 100 times more potent than disodium ATP at stimulating contraction, (b) approximately 75 times more potent than disodium ATP at displacing ^{3}H-ATP from specific binding sites, and (c) a poor inhibitor of divalent ion-specific ATPase activity. (3) ^{3}H-ATP binding is maximal between pH 5 and pH 7, whereas ATPase activity is maximal between pH 8 and pH 10. (4) ^{3}H-ATP binding is maximal at an ATP concentration of 180 nM, whereas ATPase activity requires an ATP concentration of 4 mM for maximal activity. From these studies we conclude that the binding site observed in these studies is not the substrate-binding site on calcium/magnesium ATP and may be related to the contractile site on the bladder membrane.

There is circumstantial evidence that ATP is a neurohumoral transmitter in certain specific smooth-muscle organ systems (1–4). In the rabbit urinary bladder, ATP has been demonstrated to produce a direct stimulation of contraction and there is evidence that ATP may mediate the contractile effect of low-frequency field stimulation (3). One of the major difficulties in establishing ATP as a specific neurohumoral transmitter concerns the relatively high concentrations of ATP which are required to stimulate contraction (3, 5). These concentrations are greater than one might expect if ATP acts via a neurohumoral receptor system. One explanation might be that the tissue rapidly hydrolyzes the ATP, thus requiring high concentrations of ATP in order to stimulate contraction (4, 6). A second explanation might be that the actual transmitter is an analogue of ATP which is considerably more potent than ATP itself (similar to α,β-methylene ATP or β,γ-ATP, which are 100 times more potent than ATP in stimulating the contraction of rabbit bladder (7).

In order to investigate the possible transmitter role of ATP, we have studied the characteristics of ^{3}H-ATP binding to homogenates of the rabbit urinary bladder. Since one of the many complicating factors in the demonstration of an ATP "synaptic receptor" is the presence of membrane-bound ATPase enzymes (which would also be expected to have membrane-binding sites for ATP), this study compares specific characteristics between ^{3}H-ATP binding and divalent ion-specific ATPase activity.

The urinary bladders of 28 adult male New Zealand White rabbits were removed under light ketamine/xylazine anesthesia. The bladder was dissected free of fat and serosa, and the dome was separated from the base at the level of the ureteral orifices. The bladder body was homogenized in ice-cold Tris buffer (pH 7.4, 50 mM) to a final concentration between 1 and 3 mg/ml (wet weight).

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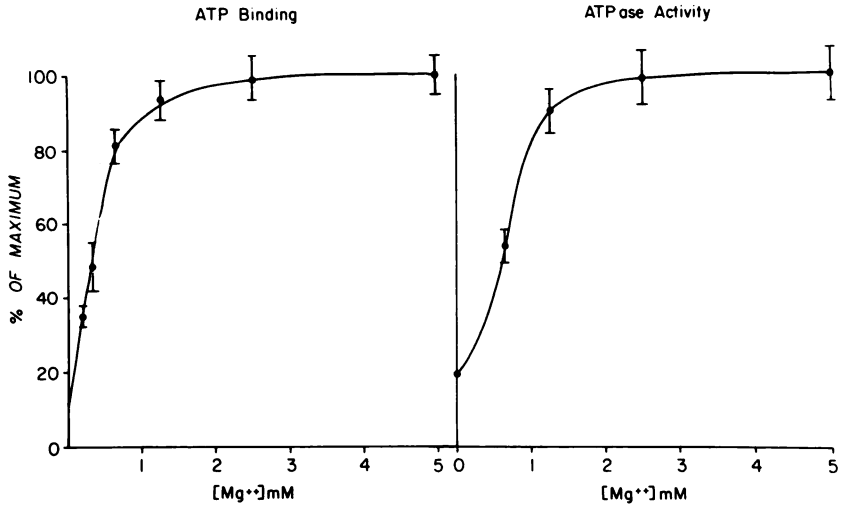

FIG. 1. *Effect of magnesium*

The effect of magnesium on both ^{3}H-ATP binding (180 nM ^{3}H-ATP) and ATPase activity (2 mM ATP) was determined as described in the text. Each *point* represents the mean ± standard error of five separate determinations.

The homogenate was warmed to 37° and incubated for 10 min to allow the hydrolysis of any endogenous ATP (endogenous ATP was monitored by luciferin-luciferase photometry). Thereafter the homogenate was maintained at 0°.

In general, the binding assay was as follows: to 100 μl of homogenate were added 20 μl of buffer (50 mM Tris, pH 7.4) alone or containing various concentrations of agents under study. The reaction was started with the addition of 20 μl of ^{3}H-ATP in buffer (various concentrations). The samples were vortexed thoroughly and maintained at 0° with agitation for 10 min. At the end of the incubation period, 3 ml of ice-cold buffer were added to the samples, which were then passed through a Millipore filtration apparatus (24-mm Whatman GF/C glass-fiber filters) and washed with 20 ml of ice-cold buffer. The filters were removed and placed in a vial with 5 ml of scintillation fluid. The radiation content was then assayed in a Packard scintillation counter.

The urinary bladders of 10 adult male New Zealand White rabbits were removed under light ketamine/xylazine anesthesia. The bladders were prepared in a manner identical with that employed for the homogenates used for the binding studies.

The activity of ATPase was determined as follows (8): Each reaction vessel contained 50 mM Tris (pH 7.4) and varying concentrations of ions and protein in a total volume of 650 μl. The reaction was started with the addition of Tris/ATP (varying concentrations). Samples were incubated between 0° and 37° for varying periods of time, and the reaction was terminated with the addition of 1 ml of 15% trichloracetic acid. The protein concentration and incubation time were adjusted to assure that less than 10% of the ATP was hydrolyzed. Precipitated protein was removed by centrifugation at 2000 × *g* for 10 min, and inorganic phosphate was measured spectrophotometrically by the method of Akera and Brody (8).

In experiments where hydrolysis of ATP was measured at substrate concentrations below 1 μM, ATP was meas-

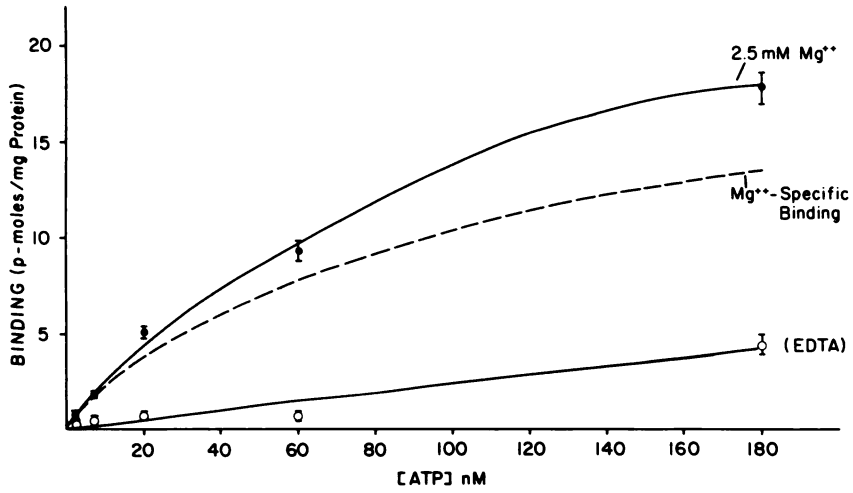

FIG. 2. *Binding as a function of ^{3}H-ATP concentration*

The binding of ^{3}H-ATP to bladder homogenates was determined as described in the text, utilizing various concentrations of ^{3}H-ATP in the presence of either 1 mM EDTA or 1 mM EDTA and 2.5 mM $MgCl_2$. Each *point* represents the mean ± standard error of six separate determinations.

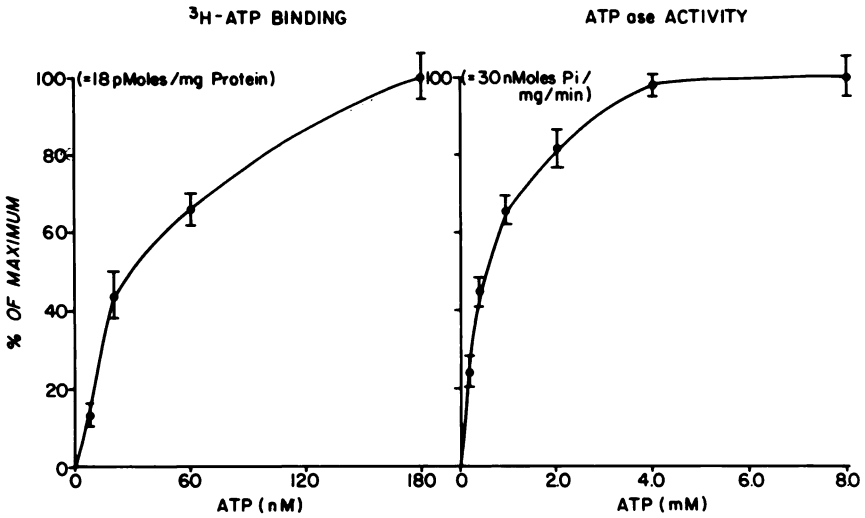

FIG. 3. *Comparative binding and ATPase activity*

The binding of ^{3}H-ATP and ATPase activity of bladder homogenates was determined in the presence of 2.5 mM Mg^{2+} and various concentrations of ATP. Each *point* represents the mean ± standard error of five separate determinations.

ured directly by utilizing the luciferin-luciferase assay for ATP. The use of luciferin-luciferase can quantify accurately the decrease in ATP concentration at low substrate levels.

^{3}H-ATP (23.9 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). ATP and β,γ-methylene ATP were purchased from Sigma Chemical Company (St. Louis, Mo.). Other chemical compounds were obtained from general commercial sources.

Preliminary studies (9) demonstrated that the rate of ^{3}H-ATP binding to bladder homogenate membranes was extremely fast. Binding was maximal within 15 sec and remained constant for 20 min at 0°. Studies on the hydrolysis of ATP (200 nM–1 mM) by bladder homogenates (1–3 mg/ml) demonstrated that less than 10% of the ATP was hydrolyzed within 10 min at 0°. Increasing the temperature above 0° or increasing the tissue concentration above 10 mg/ml increased the ATP hydrolysis rapidly. The binding at 0° was stable and allowed us to utilize glass-fiber filters to separate bound from free ^{3}H-ATP in a manner similar to that employed in the determination of adrenergic and cholinergic receptors. Washing the filters with up to 50 ml of ice-cold buffer produced no significant change in binding.

The effect of Mg^{2+} both on the binding of ^{3}H-ATP to bladder homogenates and on ATPase activity is shown in Fig. 1. The ATP binding and ATPase activity can be divided into Mg^{2+}-specific and Mg^{2+}-independent activity. The ED_{50} was approximately 0.8 mM for both binding and ATPase activity, and maximal activity was observed at approximately 1.5 mM for both. Similar data were obtained for calcium (data not presented).

Sodium (1 mM–100 mM), potassium (0.5 mM–40 mM), and sodium/potassium (various combinations) either alone or in the presence of magnesium (2.5 mM) had no effect on the ATP binding (data not shown). Similarly,

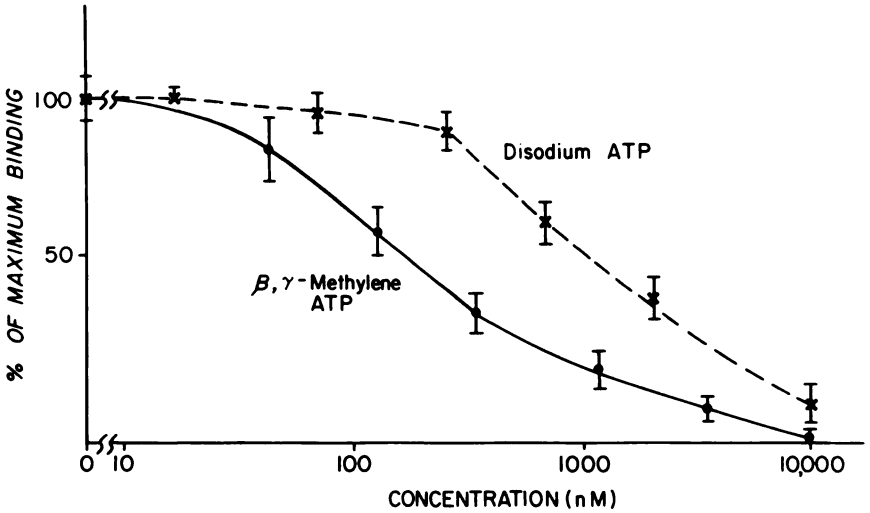

FIG. 4. *Displacement of ^{3}H-ATP*

The binding of ^{3}H-ATP (180 nM) was determined in the presence of various concentrations of β,γ-methylene ATP and disodium ATP. Each *point* represents the mean ± standard error of between four and six determinations.

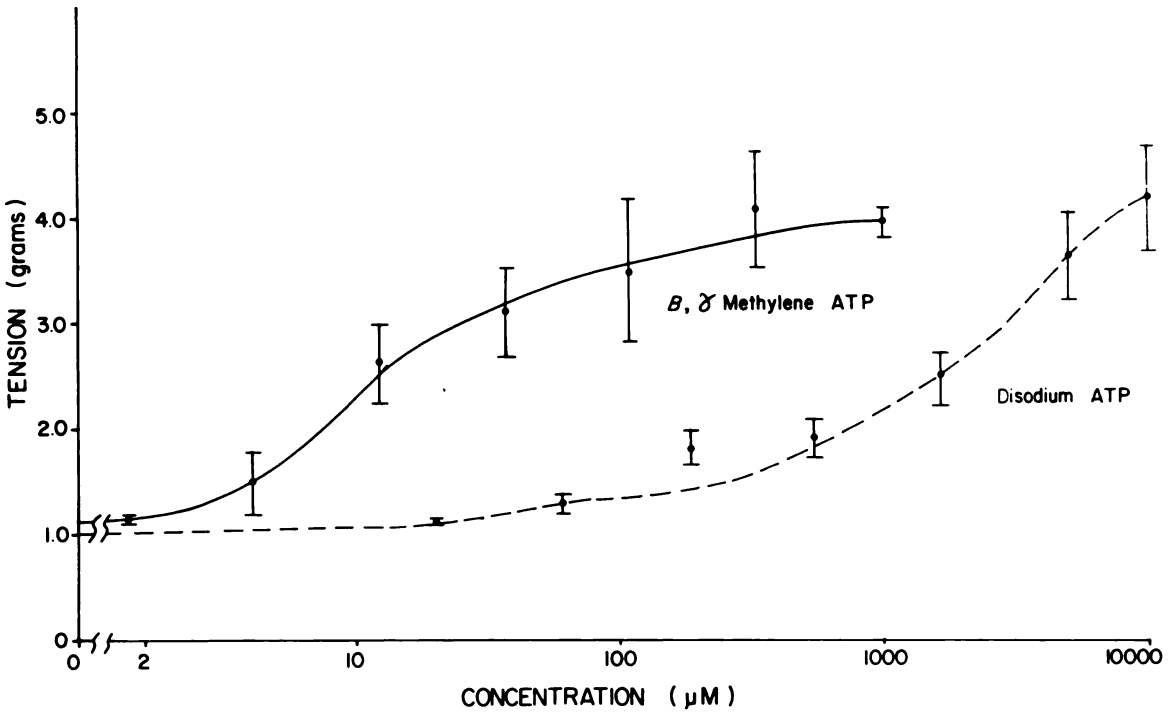

FIG. 5. *Effect of β,γ-methylene ATP and ATP on bladder strip contraction*
The effect of the cumulative addition of β,γ-methylene ATP and disodium ATP on isolated muscle strips is presented. Each *point* represents the mean ± standard error of four to six separate determinations.

the ATPase activity of our preparation (utilizing either 2 mM or 1 μM ATP as substrate) was not stimulated by either Na^+, K^+ or Na^+ and K^+.

The binding of ^{3}H-ATP, and ATPase activity, as a function of the concentration of ATP are presented in Figs. 2 and 3. The Mg^{2+}-dependent binding is saturable, whereas the Mg^{2+}-independent binding did not saturate at the concentrations of ^{3}H-ATP employed. Identical binding curves were obtained for Ca^{2+} specific (data not shown).

Figure 3 also displays the Mg-dependent ATPase activity as a function of ATP concentration. Whereas maximal binding occurred at nanomolar concentrations of ATP, maximal ATPase activity required millimolar concentrations of ATP.

Figure 4 displays the displacement of ^{3}H-ATP by both disodium ATP and β,γ-methylene ATP. β,γ-Methylene ATP was approximately 75 times more potent than disodium ATP at displacing ^{3}H-ATP from bladder membranes. This is a similar order of potency observed in the

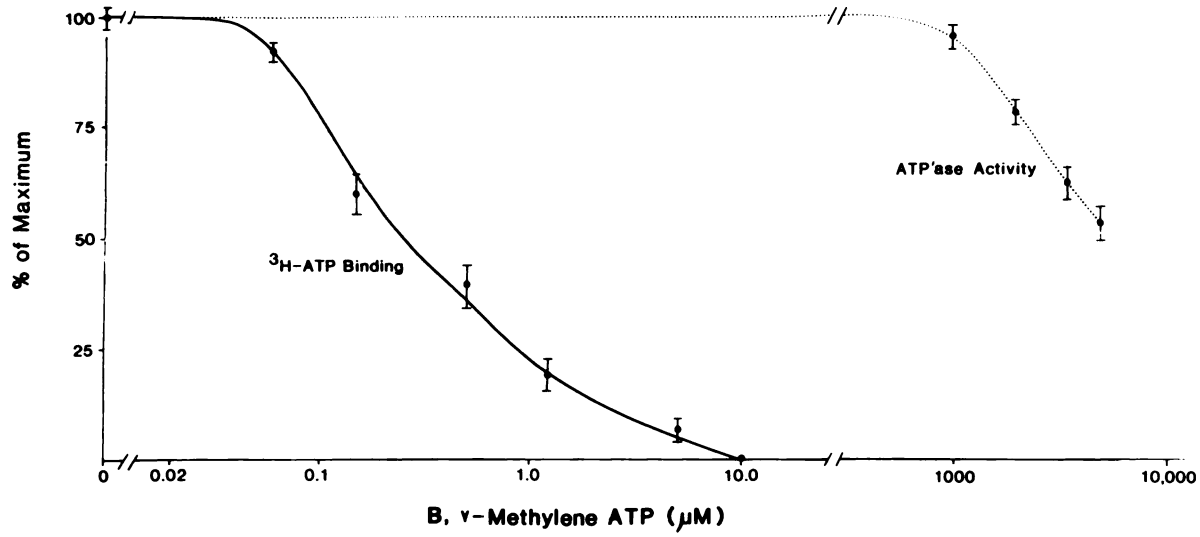

FIG. 6. *Effect of β,γ-methylene ATP on ^{3}H-ATP binding and ATPase activity*
The effect of β,γ-methylene ATP on ^{3}H-ATP binding (180 nM ^{3}H-ATP) and ATPase activity (100 μM ATP) was determined as described in the text. Each *point* represents the mean ± standard error of four to six separate determinations.

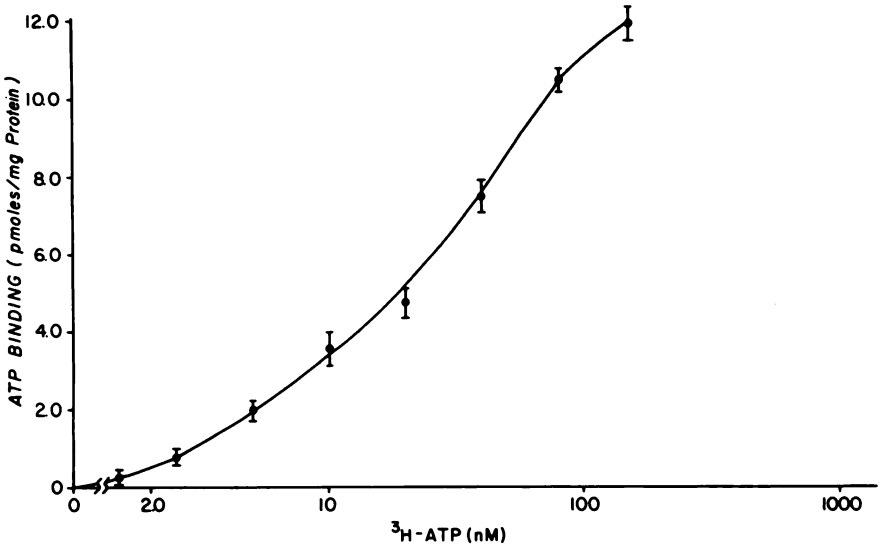

FIG. 7. *Specific binding of ^{3}H-ATP*

The binding of ^{3}H-ATP in the presence and absence of 5 μM β,γ-methylene ATP was determined as described in the text. The specific binding, i.e., the difference between the amount bound in the absence of the competitor (total bound) and the amount bound in the presence of the competitor (nonspecific binding) was determined at each of eight concentrations of ^{3}H-ATP. Each *point* represents the mean ± standard error of five separate determinations.

ability of these two agonists in stimulating contractility (Fig. 5) (7). In contrast, β,γ-Methylene ATP was a weak inhibitor of divalent ion-specific ATPase (Fig. 6). Adenine and adenosine (1 μM → 1 mM) did not compete for the ^{3}H-ATP binding site (data not shown).

Utilizing 5 μM β,γ-methylene as a competing agent, specific binding of ^{3}H-ATP to bladder was determined (Fig. 7). Scatchard analysis (Fig. 8) demonstrated nonlinear kinetics consistent with the presence of multiple sites of ^{3}H-ATP binding. The Hill plot (Fig. 9) for cooperativity shows a slope of 0.966, which demonstrates a noncooperative binding.

The binding of ATP as a function of pH is shown in Fig. 10. There was a broad pH optimum for binding between pH 5 and pH 8; above pH 8 the binding decreased sharply. In contrast, ATPase had a pH maximum of 9.0.

There are many intracellular membrane-bound binding sites for ATP, including ATPase, adenylate cyclase, and the site responsible for contractile stimulation. Each binding site would be expected to have its own kinetic characteristics.

As previously stated, it was not the purpose of this study to identify a specific "ATP synaptic receptor" but to characterize the binding to homogenates derived from the rabbit urinary bladder and to compare the binding with divalent ion-specific ATPase activity.

Divalent ion-specific ATPase represents the major

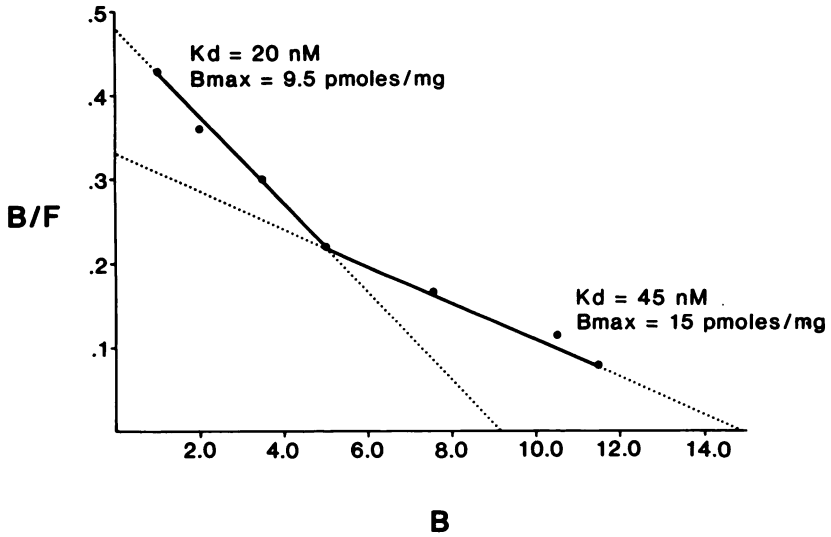

FIG. 8. *Scatchard analysis*

The data obtained from Fig. 7 were plotted according to the Scatchard equation. The lines of best fit were determined by linear regression analysis.

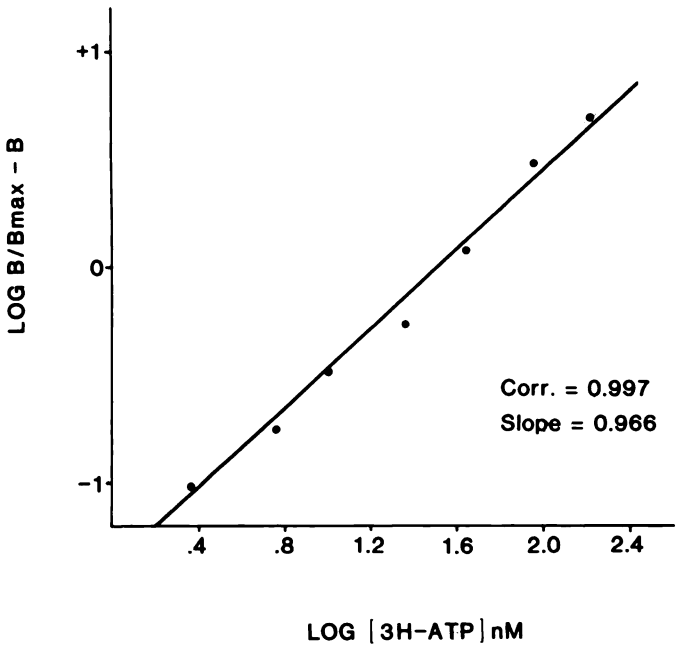

FIG. 9. *Hill analysis*

The data obtained from Fig. 7 were plotted according to the Hill equation. The line of best fit was determined by linear regression analysis.

form of ATPase activity present in the bladder and thus represents a major group of ATP membrane-binding sites. Because both the observed binding and ATPase activity are divalent ion-specific, the biochemical comparison of these two systems was chosen for these initial studies.

The bladder membranes within the homogenate bind ^{3}H-ATP rapidly, and the binding is stable enough to separate free from bound ^{3}H-ATP using standard filter separation. The temperature must be maintained at 0° throughout the assay to prevent the hydrolysis of ATP. Additionally, low tissue concentrations must be utilized (1–3 mg/ml wet weight). Higher tissue concentrations hydrolyze the low levels of ^{3}H-ATP present and thus result in nonlinear binding versus protein curves (9).

The majority of the observed binding is divalent ion-specific. This is in contrast to the divalent ion-independent ATP binding demonstrated in brain microsomes by Norby and Jensen (10, 11) and Hegyvary and Post (12). The specific binding shows saturation, and Scatchard analysis demonstrated the presence of two classes of binding sites with K_d values of 45 nM and 20 nM and B_{max} values 15 and 9.5 pmoles/mg of protein, respectively. It is interesting to note that the density of ATP binding sites is 300–1000 times greater than the density of either muscarinic cholinergic receptors (22 fmoles/mg of protein) or *beta*-adrenergic receptors (91 fmoles/mg of protein) in the bladder dome (5, 13). Similar high-density binding of ATP was reported by Hegyvary and Post (12) for brain microsomes. Whereas Hegyvary and Post reported that their binding site was related to Na,K-ATPase activity, we cannot at this time ascribe the binding demonstrated in these studies to any specific set of binding sites.

In view of the marked effect of divalent cations on the specific ^{3}H-ATP binding, these initial studies compared the characteristics of binding of ^{3}H-ATP to bladder membranes and the divalent ion-specific ATPase activity of the same membrane preparation. The results of these studies showed that (a) both binding and ATPase activity displayed the same sensitivity to Ca^{2+} and Mg^{2+}. (b) The effect of pH on both functions demonstrated that maximal binding occurred at pH values at which ATPase activity was very low (pH 5–7), whereas at pH values at which ATPase activity was highest, binding was less than 50% of maximal (pH 9–10). (c) β,γ-Methylene ATP was

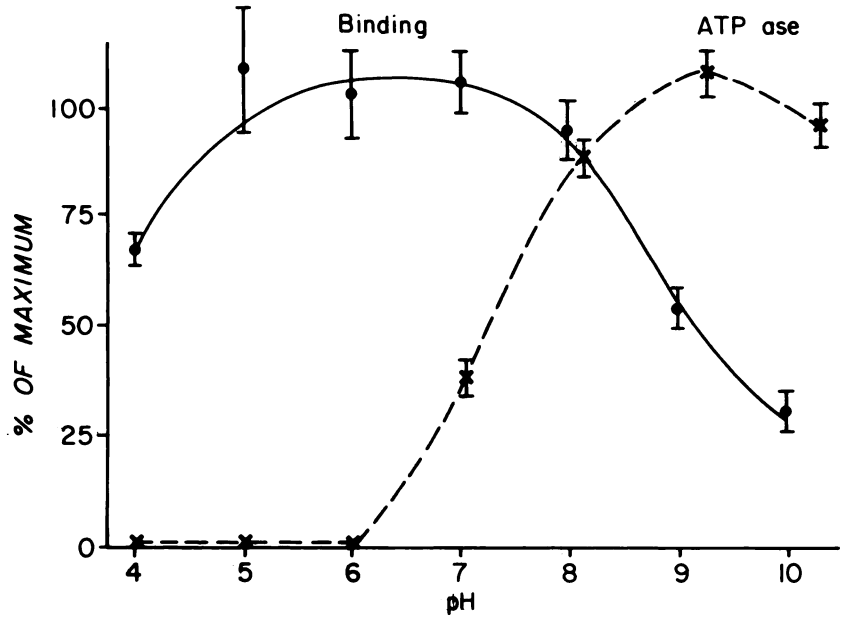

FIG. 10. *Effect of pH*

The effects of various pH values on both ^{3}H-ATP binding (180 nM ^{3}H-ATP) and ATPase activity (2 mM ATP) are presented. Each *point* represents the mean ± standard error of four to six separate determinations.

a potent competitor for ^{3}H-ATP binding, whereas this compound was a very poor inhibitor of ATPase activity. Even though β,γ-methylene ATP is not a substrate, these studies demonstrate that it has a very poor affinity for the ATPase substrate binding site but a relatively high affinity for the ^{3}H-ATP binding sites. (d) Maximal binding occurred at nanomolar concentrations of ^{3}H-ATP, wherease millimolar concentrations of ATP were required for maximal ATPase activity. No high-affinity, divalent ion-specific ATPase activity was observed. We conclude from these studies that the binding sites observed in these studies are not related to divalent ion-specific ATPase activity.

The major criticism in relating the high-affinity binding observed in these studies to the contractile function of ATP is the discrepancy in potency. Binding to membranes is maximal at nanomolar concentrations, whereas millimolar concentrations of ATP are required for maximal stimulation of bladder contraction.

The contractile effects of ATP on bladder strips may present barriers to ATP penetration which may not be present in membrane preparations. One possibility is that ATPase activity on the surface of the bladder muscle may explain the high concentrations of ATP necessary to stimulate contraction (4). Previous studies in our laboratory demonstrated that bladder strips have a fairly low ATPase activity and that, in the time necessary to contract the bladder, only a negligible amount of ATP is hydrolyzed (7). However, it is possible that a high concentration of ATPase localized at the receptor site could explain the high concentrations of ATP required in the absence of a significant breakdown of ATP.

Our demonstration that the potency of β,γ-methylene ATP at displacing ^{3}H-ATP correlates fairly well with the potency of β,γ-methylene ATP in contracting the bladder is suggestive that the binding site may be related to the contractile effect of ATP.

Our radioligand filtration method for the study of ^{3}H-ATP binding has several advantages over the dialysis methodology utilized by Hegyvary and Post (12) and Norby and Jensen (10, 11). Filtration binding analysis is considerably faster, thus allowing a greater number of analysis per unit time. Additionally, the more rapid the analysis is, the less likely the ATP is to be hydrolyzed. Filtration binding is technically simpler, requiring less equipment and fewer solutions, as well as being more versatile.

Further detailed studies on the nature of the binding of ^{3}H-ATP to bladder membranes and correlation of the binding with enzymatic studies of both ATPase and adenylate cyclase activity, and with studies on the physiological response of the bladder to ATP, are required to ascribe a function to the divalent ion-specific ATP binding observed in these studies.

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