Journal of Supramolecular Structure 4:289 (249)–303 (263) (1976)

VASOPRESSIN-SENSITIVE KIDNEY ADENYLATE CYCLASE: Modulation of the Hormonal Response

Christian Roy

Laboratoire de Physiologie Cellulaire, Collège de France, Paris, Cedex 05, France

Vasopressin-sensitive pig kidney adenylate cyclase is sensitive to several effectors, such as Mg^{2+}, other divalent cations, and guanyl nucleotides. The purpose of the present study was to compare the main characteristics of adenylate cyclase activation by vasopressin, Mg^{2+}, and GMPPNP, respectively. Mg^{2+} ions were shown to exert at least three different effects on adenylate cyclase. The substrate of the adenylate cyclase reaction is the Mg-ATP complex. Mg^{2+} interacts with an enzyme regulatory site. Finally, Mg^{2+} can modulate the hormonal response, with Mg^{2+} ions affecting the coupling function – that is, the quantitative relationship between receptor occupancy and adenylate cyclase activation. At all the magnesium concentrations tested, from 0.25 mM to 16 mM, adenylate cyclase activation was not a direct function of receptor occupancy. At low Mg^{2+} concentrations, adenylate cyclase activation dose-response curve to the hormone tended to be superimposable to the hormone dose-binding curve. These results suggest a role of magnesium at the coupling step between the hormone-receptor complex and adenylate cyclase response. Cobalt, but not calcium, ions could exert the same effects as Mg^{2+} ions on this coupling step.

GMPPNP induced considerable adenylate cyclase activation (15 to 35 times the basal value). Activation by GMPPNP was highly time and temperature dependent. At 30°C, a 20 to 60 min preincubation period in the presence of GMPPNP was needed to obtain maximal activation. The higher the dose of GMPPNP in the medium, the longer it took to reach equilibrium. At 15°C, activation was still increasing with time after 3 hr preincubation in the presence of the nucleotide. GMPPNP was active in a 10^{-8} M to 10^{-5} M concentration range. Unlike the results obtained with lysine vasopressin, the kinetic characteristics of dose-dependent adenylate cyclase activation curves by GMPPNP were unaffected by varying Mg^{2+} concentrations except for the increase in velocity when raising Mg^{2+} concentration. It was not clear whether or not the activation processes by the hormone and by GMPPNP had common mechanisms.

Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether) N, N′-tetraacetic acid; cyclic AMP, cyclic adenosine 3′, 5′ monophosphate; GMPPNP, guanyl-5′-yl imidodiphosphate; Km, concentration eliciting 50% of the maximal effect.

INTRODUCTION

An acellular preparation from the medulla-papillary region of mammalian kidney was found to contain antidiuretic hormone-sensitive adenylate cyclase (1–5). The system exhibits high sensitivity toward antidiuretic hormone and structurally related molecules. Furthermore, it was possible to eliminate from this acellular preparation the enzymic activities responsible for hormone degradation or inactivation (2, 6, 7), thus allowing accurate kinetic analysis of hormonal binding and action. So far, mammalian kidney adenylate cyclase appears to be the most convenient system for studying the molecular events involved in the early steps of the action of antidiuretic hormone on the kidney.

Using highly labeled and biologically active tritiated lysine vasopressin (8), we demonstrated the presence of specific hormone binding sites on membranes obtained from pig (2, 3) and rat (4) kidney medulla (Table I). The maximal binding capacity was 2 to 4 times higher in the pig system than in the rat system. This difference may reflect differences in the membrane purification procedure used on the two kinds of kidney medulla. In both systems the apparent Km for lysine-vasopressin binding (hormonal concentration leading to 50% saturation of binding sites) was close to 10^{-8}M. Binding was time and temperature dependent. The time required to reach half-equilibrium value (i.e., association half-time) could be reduced, either by increasing the concentration of free hormone in the

TABLE I. Characteristics of Lysine-vasopressin Binding on Plasma Membranes Prepared From Rat or Pig Kidney

	Pig kidney	Rat kidney preparation A	Rat kidney preparation B
Maximal binding capacity (pmol/mg protein)	0.8–1.0	0.2–0.4	0.2
Apparent Km (nM)	20	8	14
Half-time (min) for association at			
20 nM LVP	2	–	–
30 nM LVP	–	1.5	–
Km AVP/Km LVP	1.7	0.25	0.11
Hill coefficient	1.4	–	–

Pig medulla plasma membranes were prepared as previously described (2). The medulla was homogenized in an isotonic medium kept at 4°C containing 250 mM sucrose, 10 mM Tris Hcl pH 8, 3.3 mM $MgCl_2$, and 1 mM EDTA-Tris pH 8. The 600 X g pellet was washed 5 to 6 times in the same medium rendered hypotonic by omitting the sucrose.

Preparation A for rat enzyme was the same as that described above for pig enzyme except that the pH of the isotonic and hypotonic media was 7.4 and the number of washings limited to three.

Preparation B was as follows: medulla was homogenized in isotonic medium (pH 7.4). Homogenate was then centrifuged for 5 min at 500 X g and the pellet discarded. The supernatant was centrifuged at 1500 X g and the pellet was washed three times in hypotonic medium. Final pellet is taken as a source of enzyme.

The apparent Km for arginine vasopressin was calculated taking into account the dose of [^{3}H] lysine vasopressin used and assuming that arginine vasopressin behaved like a pure competitive inhibitor of tritiated peptide binding. Results are the average of at least three different experiments performed on distinct enzyme preparations.

Apparent Km = dose of peptide leading to 50% occupation of binding sites.

AVP: arginine vasopressin; LVP: lysine vasopressin.

incubation medium or by raising the temperature. These observations indicated that the rate-limiting step for the lysine-vasopressin binding observed was the hormone receptor interaction itself. The major difference between the pig and rat receptors concerns their respective specificities toward the natural antidiuretic principles. In the pig, lysine vasopressin, the natural hormone in this species, has a higher affinity than arginine vasopressin for the renal receptor, whereas the reverse is observed in the rat, where arginine vasopressin is the natural hormone. All the above-mentioned kinetic studies of hormone binding indicate that the hormone interacts with a homogeneous population of binding sites (2, 3).

Numerous correlations were established between vasopressin binding and adenylate cyclase activation by the hormone. They led to the conclusion that the detected binding sites constitute the antidiuretic hormone receptors involved in enzyme activation. The correlations were as follows. 1) The membranes prepared from the cortical portions of the kidney were nearly unresponsive to antidiuretic hormone. The response of the cortical adenylate cyclase to lysine-vasopressin never exceeded 20% of that measured on medullary enzymes. Moreover, the cortical membrane fraction did not contain any appreciable amounts of lysine-vasopressin binding sites. On the contrary, adenylate cyclase obtained from the medullary part of the kidney was highly responsive to the hormone. On such fraction, it was also possible to detect hormone binding sites exhibiting the expected properties (see below). 2) Both the binding and adenylate cyclase activation processes exhibited the expected zoological specificity. The lysine-vasopressin: arginine-vasopressin potency ratio is of comparable magnitude whether it is measured by the relative ability of these two peptides to induce an antidiuretic response in vivo, to activate medullary adenylate cyclase, or to interact with the renal vasopressin receptors (Table I). 3) Medullary adenylate cyclase responsiveness to vasopressin and hormone binding capacity, respectively, followed closely similar evolution patterns during postnatal maturation of the kidney function in the rat. Both the vasopressin binding capacity and response, which were very low during the first days of life, increased rapidly between days 10 and 25 and reached adult values between days 30 and 45 after birth (9). 4) Kinetic analysis of vasopressin binding and adenylate cyclase response in the pig system showed that the enzyme activation was time dependent. Using a submaximal lysine-vasopressin concentration, enzyme activation and hormonal binding had identical time courses. 5) Chemical modification(s) of the vasopressin molecules affected in a corresponding way the ability of modified hormonal molecules to bind to the receptor on the one hand and to modulate adenylate cyclase activity on the other (10, 11). This is illustrated in Table II in the case of vasopressin structural analogs. For the entire series of analogs tested, the peptide concentration needed to obtain saturation of the binding sites was very close to that leading to maximal enzyme activation. Thus it is clear that the recognition pattern of the binding sites detected is strikingly similar to that of the physiological receptor involved in the adenylate cyclase response. This conclusion was fully confirmed by further studies using 21 analogs of the oxytocin series (11). Two of the tested oxytocin analogs (carbamoyl-O-methyl tyrosine oxytocin and pivaloyl-O-methyl tyrosine oxytocin) behaved like pure competitive inhibitors. They were ineffective in stimulating adenylate cyclase activity but blocked the activation induced by vasopressin in a dose-dependent manner. They inhibited [^{3}H] lysine-vasopressin binding to the same maximal extent as did unlabeled lysine vasopressin. The concentration of inhibitor leading to 50% saturation of the binding sites was very close to that needed to double the apparent Km for adenylate cyclase activation by lysine vasopressin.

TABLE II. Structural Requirements for Binding and Action of Lysine Vasopressin Analogs

[8-lysine] vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-GlyNH$_2$				Concentration of peptide needed for:	
	Chemical modification in [Lys^8] vasopressin molecule					
Compound	Ring closure	Amino group of cysteine in position 1	Modification of tyrosine 2 hydroxyl	Lateral side chain	80% adenylate cyclase activation*	80% receptor occupancy†
[8-lysine] vasopressin	-S-S-	+	–	-Pro-Lys-GlyNH$_2$	5×10^{-8}	8×10^{-8}
[8-arginine] vasopressin	-S-S-	+	–	-Pro-Arg-GlyNH$_2$	6×10^{-8}	1.3×10^{-7}
[deamino- 8-D-arginine] vasopressin	-S-S-	–	–	-Pro-[D-Arg]-GlyNH$_2$	9×10^{-6}	6.5×10^{-6}
[1,6-α-deaminocystathionine, 8-ornithine] vasopressin	-S-CH$_2$-	–	–	-Pro-Orn-GlyNH$_2$	1.4×10^{-8}	2.6×10^{-8}
[1, 6-α-deaminocystathionine, 8-arginine] vasopressin	-S-CH$_2$-	–	–	-Pro-Arg-GlyNH$_2$	2×10^{-8}	2.6×10^{-8}
[1, 6-α-deaminocystathionine, 8-D-arginine] vasopressin	-S-CH$_2$-	–	–	-Pro-[D-Arg]-GlyNH$_2$	7.6×10^{-6}	7.2×10^{-6}
[1, 6-α-deaminocystathionine, 2-tertbutyl tyrosine, 8-N-tosyl arginine] vasopressin	-S-CH$_2$-	–	O-C(CH$_3$)$_3$	-Pro-Arg(Tos)-GlyNH$_2$	2.3×10^{-6}	3×10^{-6}
Des-9-glycineamide, [8-lysine] vasopressin	-S-S-	+	–	-Pro-Lys	2×10^{-6}	6×10^{-6}
vasopressin-(1-6)-hexapeptide amide	-S-S-	+	–	-NH$_2$	10^{-4}	10^{-4}
deamino-vasopressin-(1-6)-hexapeptide amide	-S-S-	–	–	-NH$_2$	inactive	

*Peptide concentrations eliciting 80% maximal adenylate cyclase activation were directly measured on the dose-response curves for each peptide.

†Since none of the lysine-vasopressin analogs were available in labeled form, their affinity for hormonal receptor was deduced from competition experiments. Increasing amounts of unlabeled peptide were added to a constant dose of [^{3}H]lysine vasopressin (10^{-8} M = Km/2). The unlabeled peptide concentration necessary to occupy 80% of the vasopressin binding sites is equal to 2.66 times the concentration of the same peptide, inducing a 50% drop in [^{3}H]lysine vasopressin binding. These calculations assume that each analog behaved like a pure competitive inhibitor of [^{3}H]lysine vasopressin for binding.

By measuring lysine-vasopressin binding and enzyme activation under identical conditions on the same membrane preparation, it was possible to define the quantitative relationship between receptor occupancy and response. When the enzyme response had been measured after a preincubation period, in the presence of hormone sufficient to allow the binding process to reach equilibrium, it was observed that the dose-response curve for lysine vasopressin exhibited a high degree of apparent negative cooperativity, as indicated by the low value (0.30–0.35) of the Hill coefficient (12). For enzyme activation by the hormone, dose-dependent enzyme activation was observable within a very wide concentration range, from 10^{-12}M to 10^{-7}M. Maximal adenylate cyclase activation and receptor saturation were obtained for the same hormonal concentrations. Conversely, the dose-binding curve exhibited positive cooperativity as indicated by a Hill coefficient above unity (Table I). Thus the activation and binding curves were not superimposable. The apparent Km for activation (5×10^{-10}M) was much lower than for binding (2×10^{-8}M, Table I). Considerable enzyme activation occurred for very low degrees of receptor occupancy. In other words, enzyme activation is not a linear function of receptor occupancy (nonlinear coupling). The simplest interpretation of the apparent negative cooperativity observed for the dose-response curve was to assume that several categories of receptor exist which differed in their affinity for the hormonal molecule. If the receptors exhibiting the highest affinity for vasopressin were more effective in eliciting adenylate cyclase activation, this would account for the results observed. However, such an interpretation proved unacceptable since kinetic studies of [^{3}H]lysine-vasopressin binding failed to reveal such heterogeneity among the receptor site population. It was thus concluded that the nonlinear coupling was the result of the molecular events involved in the transfer of the information carried by the hormone-receptor complex to the adenylate cyclase catalytic site, resulting in an increase in cyclic AMP production. This increase is not a direct function of receptor occupancy by the hormone. For a given increment in receptor occupancy, the increase in cyclic AMP production decreased when tending toward saturation of binding sites.

It has already been established that the transduction process can be affected 1) by varying the magnesium concentration in the incubation medium (see below); 2) by chemically modifying the hormonal molecule (10, 17); and 3) by the hormonal state of the animal from which the membrane preparation is derived. In the rat, adrenalectomy leaves the number and properties of renal vasopressin receptors almost unchanged but strongly reduces adenylate cyclase activation by vasopressin. This observation suggests an impairment in the efficacy of receptor enzyme coupling. Its efficacy was restored by treating the animals with dexamethasone. Dexamethasone was also able to enhance coupling efficacy in normal rats. Pig kidney ADH receptor exhibited a high degree of stereospecificity toward structural analogs of vasopressin (Table II). Binding of these analogs was studied using an indirect method based on analysis of the dose-dependent inhibitions of [^{3}H] lysine-vasopressin binding (see legend to Table II). For these analogs the dose dependency of the binding process appears to be very similar to that observed for lysine vasopressin, despite a large variation in the dissociation constants measured. However, the shape of the dose-response curve, as indicated by the value of their Hill coefficient, depended largely on the structure of the analog, suggesting differences in the coupling process among the analogs studied. For the most potent peptides, and especially for natural antidiuretic hormones, the coupling function is typically a nonlinear one as already mentioned. For

analogs with lower affinity for the hormonal receptor, it tends to become linear. There is no strict correlation between the affinity for the receptor and the nonlinearity of the coupling function.

Kidney medulla adenylate cyclase activity can be modulated by several effectors such as Mg^{2+}, other divalent cations (cobalt, calcium), nucleotides, and sodium fluoride. The purpose of the present study is to compare the characteristics of activation induced by these different effectors and to test the possibility that they have a common mechanism with the hormone-induced activation process.

METHODOLOGICAL REMARKS

Since hormonal binding to the receptor, adenylate cyclase activation and possibly the action of regulatory agents are time dependent, correlations between receptor occupancy and/or concentration of regulatory agents and adenylate cyclase activity can only be established under equilibrium conditions. Accurate analysis of the time course of adenylate cyclase activation by several effectors might also be a useful approach to study the mechanisms of their action. It is thus necessary to be able to measure the instantaneous velocity of cyclic AMP production. This can only be done by measuring enzyme velocity during the short period following preincubation of the enzyme with the regulatory agent.

The basic procedure chosen for the measurement of adenylate cyclase activity was as follows. Plasma membranes were preincubated for various periods of time in a medium identical to that used for the final assay. At the end of the preincubation period a tracer amount of [α-^{32}P] ATP was added and the reaction allowed to proceed for a short time. The presence of an ATP regenerating system (creatine kinase 1 mg/ml, phosphocreatine 20 mM) maintained the ATP concentration constant during the entire experiment (preincubation and final assay periods). Destruction of the labeled cyclic AMP formed was prevented by the presence of 1 mM unlabeled cyclic AMP. A detailed description of the experimental techniques used was given in an earlier article (13).

EFFECTS OF Mg^{2+} AND OTHER DIVALENT CATIONS

Increasing the Mg^{2+} concentrations enhanced basal adenylate cyclase activity when the amount of ATP was kept constant (Fig. 1A). It may be calculated from the dissociation constant of the ATP-Mg^{2+} complex (14) that under our experimental conditions (Tris-HCl pH 8,100 mM) the Mg-ATP complex was the predominant species even for the lowest Mg^{2+} concentrations tested. Thus the effect of Mg^{2+} cannot mainly be accounted for by an increase in the substrate concentration required for the adenylate cyclase reaction. As indicated by Fig. 1B, Mg^{2+} increased the maximal velocity of the reaction without affecting the Km for ATP. This indicates that, as in other systems (15), Mg^{2+} exerts a regulatory effect on kidney adenylate cyclase. The stimulating effect of Mg^{2+} exhibited non-Michaelian kinetics (see Fig. 1C): It was not possible to determine any apparent affinity of Mg^{2+} for its regulatory site on adenylate cyclase, as illustrated by Eadie's plot which did not yield a straight line.

During hormonal stimulation of adenylate cyclase, both lysine vasopressin and Mg^{2+} increased the maximal velocity of the reaction without changing the Km for ATP. In addition, Mg^{2+} interfered with hormonal stimulation. When the Mg^{2+} dependency of renal

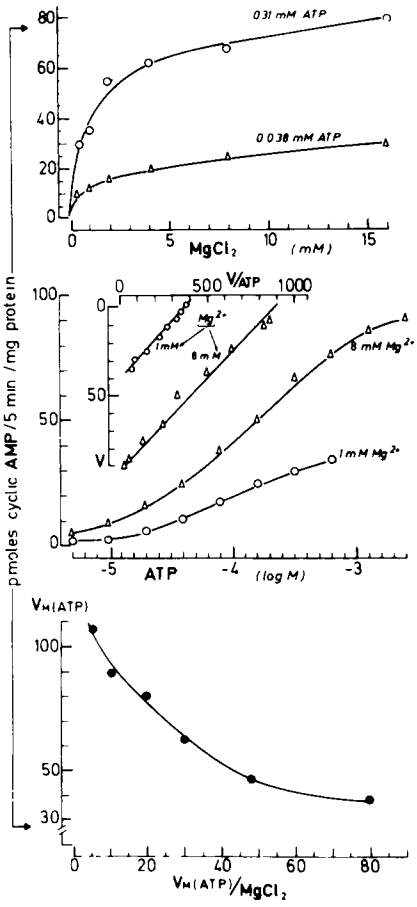

Fig. 1. ATP and magnesium-dependent adenylate cyclase activity under basal conditions. In order to cover a wide concentration range for Mg^{2+}, the enzyme which contained 3 mM $MgCl_2$ (see legend to Table I), was diluted in a Mg^{2+}-free medium containing 1 mM EDTA and 10 mM Tris-HCl, pH 8 so as to lower its Mg^{2+} content to 0.5 mM.

The upper graph (Fig. 1A), illustrates typical dose dependency of basal adenylate cyclase activity at two different ATP concentrations as a function of the Mg^{2+} content in the incubation medium. The concentrations of ATP choosen were equal to 0.32 and 3.1 times the Km for ATP (10^{-4} M, see middle panel). These concentrations led to 24% and 76%, respectively, of the maximal velocity inducible by ATP. Within this range of velocity, dose dependency toward Mg^{2+} was similar. This was also checked (not shown) for ATP concentrations ranging from 5×10^{-6} M to 5×10^{-3} M, under conditions where Mg^{2+} concentration was at least as high as ATP concentration.

The middle panel shows the dose dependency of adenylate cyclase activity for ATP at two different Mg^{2+} concentrations. Similar results were obtained using Mg^{2+} concentrations ranging from 0.25 mM to 32 mM in conditions where ATP concentration never exceeded Mg^{2+} concentration. The inset represents Eadie's plot at both the concentrations tested, ATP being taken as the substrate; y intercept is referred to as V_M(ATP) (see lower panel).

Lower panel: Eadie's representation of maximal velocity for ATP at different Mg^{2+} concentrations. Mg^{2+} is taken as the substrate.

adenylate cyclase was measured under basal conditions and in the presence of threshold, submaximal, and maximal doses of lysine vasopressin, it was clear (Fig. 2) that the Mg^{2+} effect depended on the hormonal doses tested. For the lowest hormonal concentrations the presence of Mg^{2+} was necessary for enabling hormonal activation of the enzyme. From Fig. 2 (right part, top panel), it is clear that in the presence of 10^{-11} M lysine vasopressin, no stimulation of adenylate cyclase was apparent at 0.3 mM Mg^{2+} (ATP concentration being 0.25 mM). However, raising Mg^{2+} concentration by only 3-fold was enough to elicit a significant response to 10^{-11} M lysine vasopressin (2-fold increase over basal value). For submaximal doses of lysine vasopressin an optimum in the activation ratio was observed between 2 to 4 mMg^{2+}. Higher concentrations reduced the activation ratio. This reflects the fact that for the highest Mg^{2+} concentrations tested, Mg^{2+} increased basal adenylate cyclase activity when vasopressin stimulated activity has already reached its maximum. Thus, the dose dependency of adenylate cyclase activation by lysine vasopressin was affected by the Mg^{2+} concentration in the incubation medium. As illustrated by Fig. 3,

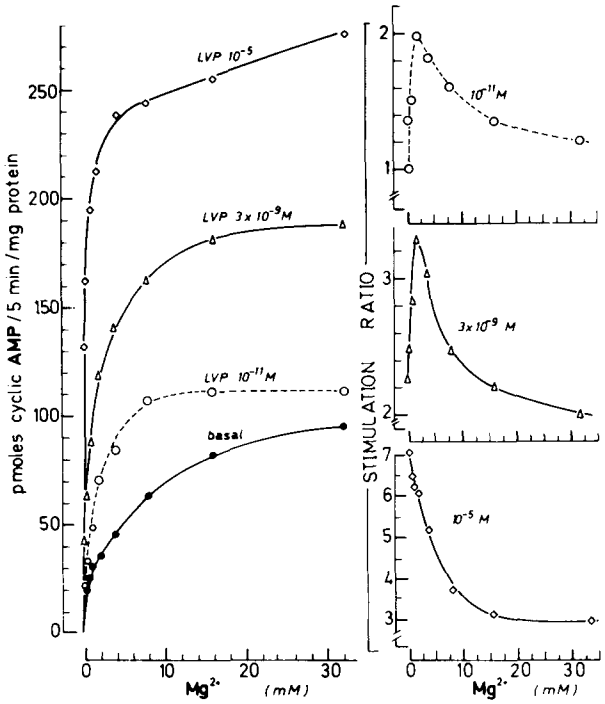

Fig. 2. Dose-dependent adenylate cyclase activation by Mg^{2+} under basal and hormonally stimulated conditions. As indicated in the legend to Fig.1, most of the Mg^{2+} was removed from the enzyme. The lowest Mg^{2+} concentration tested was 0.30 mM.

Preincubation of membranes was carried out at 30°C for 15 min in a medium containing 0.25 mM ATP, 1 mM cyclic AMP, 100 mM Tris-HCl pH 8, 1 mg/ml creatine kinase, 20 mM creatine phosphate, and the indicated amounts of Mg^{2+} and vasopressin (total volume 95 µl). After preincubation, 10 µl [α-^{32}P] ATP (1µCi) were added and the reaction allowed to proceed for 5 min. The samples were then diluted with unlabeled ATP and filtered on aluminum oxide columns as previously described (13).

Left panel: adenylate cyclase activation was measured as a function of the Mg^{2+} concentration under 4 experimental conditions, under a basal condition, a threshold dose of lysine vasopressin, a submaximal dose of hormone, and a maximal dose.

Right panel: stimulation ratio (stimulated/basal activity) at different doses of lysine vasopressin as a function of Mg^{2+} concentration.

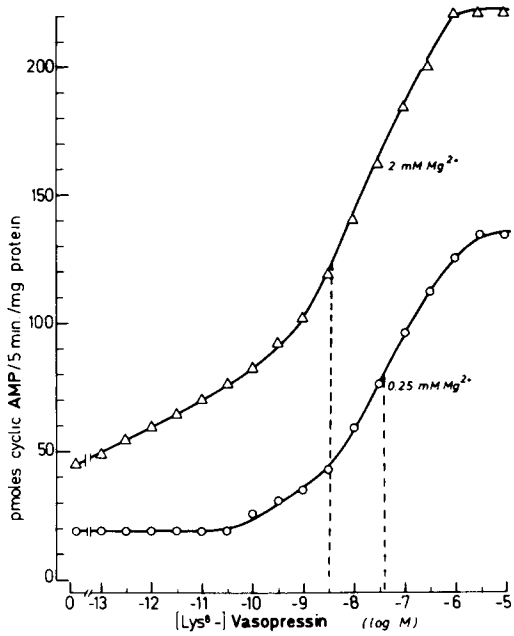

Fig. 3. Dose-response curve for vasopressin at different Mg^{2+} concentrations. The same membrane preparation was used for both dose-response curves. Mg^{2+} was first eliminated from the enzyme (see legend to Fig. 1), and its concentration was then adjusted to the desired values. Magnesium concentrations chosen were derived from the experiment described in Fig. 2.

Note that when 0.25 mM Mg^{2+} is present in the incubation mixture no response is observable for hormone concentrations below 10^{-10} M.

Dotted lines indicate concentrations of peptide eliciting 50% of the maximal response.

lowering the Mg^{2+} concentration in the incubation medium reduced the range of hormonal concentrations for which dose-dependent adenylate cyclase activation was observable. In the presence of 2 mM Mg^{2+} a dose-dependent response to lysine vasopressin was observable from 10^{-12}M to 10^{-6}M hormone, while at low Mg^{2+} (0.25 mM; ATP concentration, 0.20 mM), the concentration range in which the enzyme response occurred was restricted (from 10^{-10} M to 10^{-6} M). At high magnesium concentration, the response covered 6 orders of magnitude instead of 3–4 orders of magnitude at low Mg^{2+} content in the incubation mixture. As a consequence, the value of the Hill coefficient rose from 0.30 at 2 mM Mg^{2+} to 0.58 at 0.25 mM Mg^{2+}. At both these Mg^{2+} concentrations, maximal enzyme activation was obtained for the same hormonal concentration. Provided that it is demonstrated under these experimental conditions, different only with respect to the magnesium, and that the characteristics of hormonal binding are unchanged (16), then these observations suggest that the effect of Mg^{2+} on hormonal responsiveness is located at the coupling step.

It was not possible to account for hormonal activation of adenylate cyclase by an increase in the affinity of the enzyme regulatory site for Mg^{2+} due to vasopressin. Such a mechanism cannot explain either the presence of an optimal Mg^{2+} concentration for the stimulation ratio (Fig. 2) or the additional effect of vasopressin on the maximal velocity of the reaction measured in the presence of saturating amounts of Mg^{2+}.

It may also be noted from Fig. 3, that by raising Mg^{2+} concentration in the basal adenylate cyclase medium, activity was increased by about 2-fold, while the activity due

to the hormone was only increased by about 50%. Thus, despite the fact that when increasing Mg^{2+} the stimulation ratio dropped, there was still a potentialization of the response due to the hormone.

In conclusion, Mg^{2+} exerts at least three different effects on vasopressin-sensitive renal adenylate cyclase: the substrate of the adenylate cyclase is the Mg-ATP species; it increases the maximal velocity of the reaction under conditions where this increase cannot be accounted for by an increase in Mg-ATP; and finally, Mg^{2+} appears to be able to modulate the relation between receptor occupancy and adenylate cyclase response. Concerning this last point, the conclusion was mainly derived from the fact that whatever the Mg^{2+} concentration in the incubation medium, the concentrations of lysine vasopressin eliciting maximal response were the same. Already published data substantiate this conclusion (16).

An analysis of the effects of other divalent cations might provide additional arguments for distinguishing between the different effects of Mg^{2+}. If these effects are mediated by distinct Mg^{2+} binding sites, it is reasonable to assume that these sites exhibit different stereospecificities and reactivities toward divalent cations.

Figures 4 and 5 describe the effect of Ca^{2+} on pig kidney adenylate cyclase. Raising Ca^{2+} concentrations above 0.1 mM led to dose-dependent inhibition of basal- and vasopressin-stimulated activities (Fig. 4A). For a given Ca^{2+} concentration the respective percentage inhibition of basal and stimulated activities were similar. Contrary to the results obtained with frog bladder adenylate cyclase (13), removal of Ca^{2+} by treating the enzyme

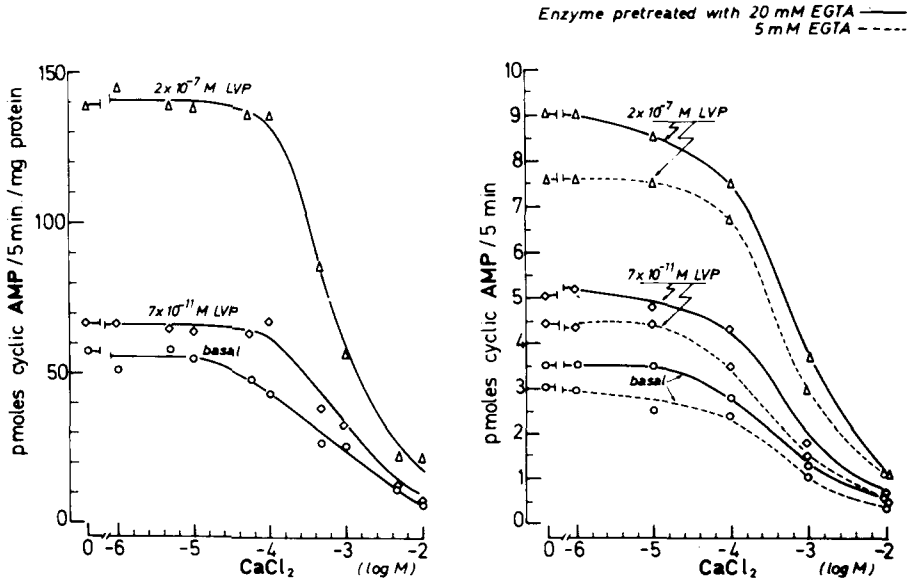

Fig. 4. Effects of increasing calcium concentrations and EGTA treatment on adenylate cyclase responsiveness to hormone. Hormonal concentrations used are maximal (2×10^{-7} M) and threshold doses (7×10^{-11} M).

Enzyme treatment by EGTA. Right panel: 1.5 ml membrane fraction was incubated for 15 min min at room temperature in 20 ml of a medium containing 3.3 mM $MgCl_2$, 10 mM Tris-HCl pH 8, and 5 or 20 mM EGTA. Membranes were then centrifuged at 1600 X g for 10 min. The pellet was dispersed and washed twice in 100 ml of a medium containing 10 mM Tris-HCl pH 8 and 3.3 mM $MgCl_2$. The final pellet was used as a source of enzyme. Left panel: no enzyme treatment.

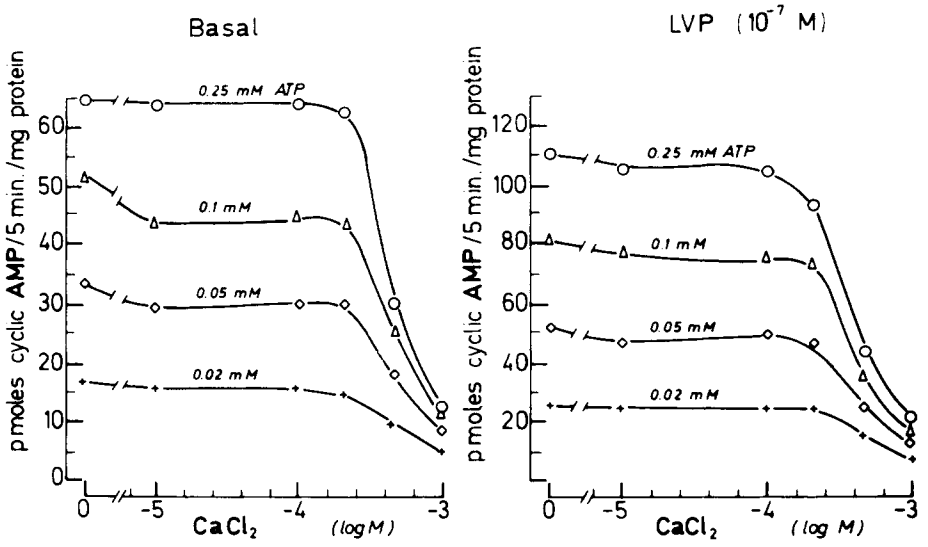

Fig. 5. Effects of increasing calcium concentrations at different levels of ATP in the incubation medium. The Mg^{2+} concentration in the incubation medium was 0.25 mM after washing the enzyme as indicated in the legend to Fig. 1. Since a maximal dose of lysine vasopressin leading to almost instantaneous occupation of the receptor was used, the reaction was carried out immediately after addition of the enzyme. No preincubation was performed.

with either 5 or 20 mM EGTA did not eliminate the stimulating effect of lysine vasopressin on the pig kidney enzyme (Fig. 4B). The Ca^{2+} concentration range, for which inhibition of basal and maximally stimulated adenylate cyclase was observed, was independent of the ATP concentration in the incubation medium (ATP varies from 0.2 to 2.5 Km). This observation makes it unlikely that the inhibition is the result of the formation of an ATP-Ca^{2+} complex behaving like a competitive inhibitor at the catalytic site of the enzyme. In that case, one would expect the inhibitory effect of a given Ca^{2+} concentration to be more pronounced at high than at low ATP concentrations – that is, that the inhibition by Ca^{2+} would occur in a lower concentration range when using high ATP concentrations. The ratio vasopressin stimulated: basal adenylate cyclase activity was independent of Ca^{2+} concentration whether the hormone was added at a threshold or maximal amount. This observation is hardly compatible with the assumption that Ca^{2+}could compete with Mg^{2+} to affect the coupling step. Finally, it is possible to account for the Ca^{2+} effect observed by the Ca^{2+} interaction with the adenylate cyclase regulatory site where it blocks the stimulatory effect of Mg^{2+} on the maximal velocity of the reaction (see above).

Figure 6 compares the effects of $Co^{2+}$and Sr^{2+} ions on the dose dependency of adenylate cyclase activation by lysine vasopressin. It is clear that 0.3 mM Sr^{2+} or 0.3 mM Co^{2+}does not change basal adenylate cyclase activity. However, concentration range in which adenylate cyclase response occurred was much more restricted in the presence of Sr^{2+} (10^{-9} M–10^{-6} M) than in the presence of Co^{2+} (10^{-11} M–10^{-6} M). In the presence of Sr^{2+}, a situation similar to that observed at low Mg^{2+} concentration is found. On the contrary, Co^{2+} appears to be able to mimic the effects of Mg^{2+} on the dose-response curves to the hormone (Fig. 3). This may be taken as a further confirmation of the specific effect of Mg^{2+} ions on the coupling step.

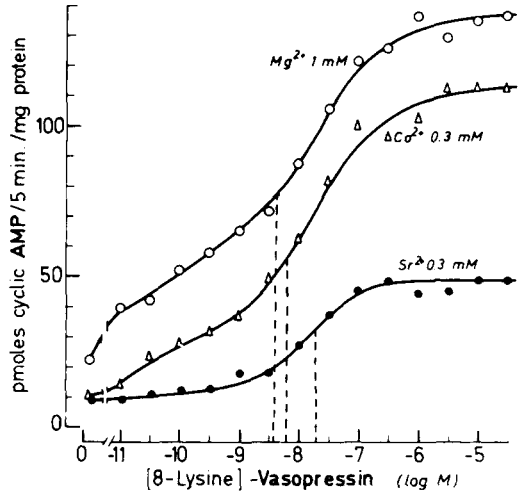

Fig. 6. Effects of Co^{2+} and Sr^{2+} on lysine-vasopressin dose-response curves. The Mg^{2+} concentration in the enzyme was lowered as indicated in the legend to Fig. 1, the final concentration in the incubation medium being 0.1 mM. The effects of divalent cations on lysine-vasopressin dose-response curves were tested for 5 min after a 15 min preincubation period at 30°C to allow hormone-receptor interaction to approach equilibrium.

The concentrations of strontium and cobalt used were chosen so that the divalent cation concentration was slightly in excess over that of ATP. Dotted lines correspond to the concentration of lysine vasopressin eliciting 50% of the maximal response.

EFFECTS OF GMPPNP

Different groups (17, 18) have shown that GMPPNP is a potent activator of several adenylate cyclase systems. We recently observed that GMPPNP was also able to activate pig kidney adenylate cyclase. The enzyme activation was time, temperature, and concentration dependent (Fig. 7). Activation by GMPPNP was a slow process. After 3 hr incubation at 15°C activation still increased with time. When the enzyme was preincubated in the presence of GMPPNP at 30°C, an equilibrium state for adenylate cyclase activation was reached within 20 to 60 min, depending on the concentration of GMPPNP tested.

The time required to reach equilibrium was shorter at low (10^{-7} M) than at high (10^{-5} M) GMPPNP concentrations. The maximal stimulation induced by GMPPNP varied from 15 to 35 times the basal value. It is possible that the apparent equilibrium for activation observed results from the combination of a slow activation process and a time-dependent denaturation of the enzyme. When GMPPNP was removed from the incubation medium during the onset of the activation process and the adenylate cyclase activity was then measured as a function of time, the adenylate cyclase activity remained stable and equal to the activity measured just before GMPPNP removal. Thus the activation process of pig kidney adenylate cyclase by lysine vasopressin and GMPPNP, respectively, are strikingly different as regards time and dose dependency, as well as reversibility (2).

Whether or not the membranes were preincubated in the presence of GMPPNP, raising the Mg^{2+} concentrations in the incubation medium enhanced adenylate cyclase activity (Fig. 8). However, in contrast to the results obtained under hormonal stimulation by

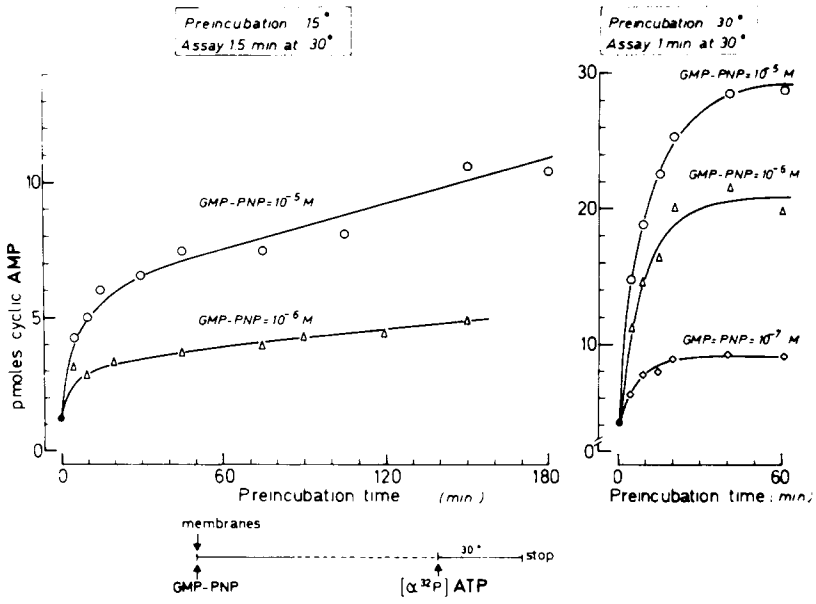

Fig. 7. Time course of adenylate cyclase activation by GMPPNP at 15°C and 30°C. The enzyme was preincubated at 15°C and 30°C in 100 mM Tris-HCl pH 8 containing the indicated amounts of GMPPNP (total volume 30 µl). After preincubation, 10 µl of a medium containing 1 mM ATP, 4 mM cyclic AMP, 0.4 µCi [α-^{32}P] ATP, 4 mg/ml creatine kinase, and 80 mM creatine phosphate were added. Samples were then incubated at 30°C for either 1 min (preincubation at 30°C) or 1.5 min (preincubation at 15°C). The reaction was stopped by diluting samples with unlabeled ATP. Points on the curves refer to the velocity of the adenylate cyclase reaction after different periods of time following mixing of membranes and GMPPNP. Assays were performed at 30°C whatever the temperature at which preincubation occurred. However, in order to get similar velocities, samples which have been preincubated at 15°C were incubated for 1.5 min at 30°C instead of 1 min, in order to compensate for thermic equilibration.

lysine vasopressin, the shape and characteristics of the dose-response curves were independent of the Mg^{2+} concentrations; threshold doses were identical; concentrations of GMPPNP eliciting 50% of the maximal observed effect were the same; and finally, when expressed as percent of the maximal effect, whatever the Mg^{2+} concentration, curves were superimposable. In any case, the dose dependency of adenylate cyclase activation by GMPPNP should be analyzed with caution since, as stated above, it was difficult to reach time equilibrium conditions for enzyme activation. Saturability of enzyme activation by high GMPPNP concentrations, which seemed apparent when adenylate cyclase activity was measured for a 5-min period following addition of the nucleotide to the incubation medium, was far less pronounced when the enzyme was preincubated for 20 min with GMPPNP. In both cases, dose-dependent activation was observable throughout concentration range covering more than 3 orders of magnitude.

Further experiments are needed to ascertain whether GMPPNP and vasopressin have a common action mechanism and whether guanyl nucleotides are physiologically involved in hormonal action.

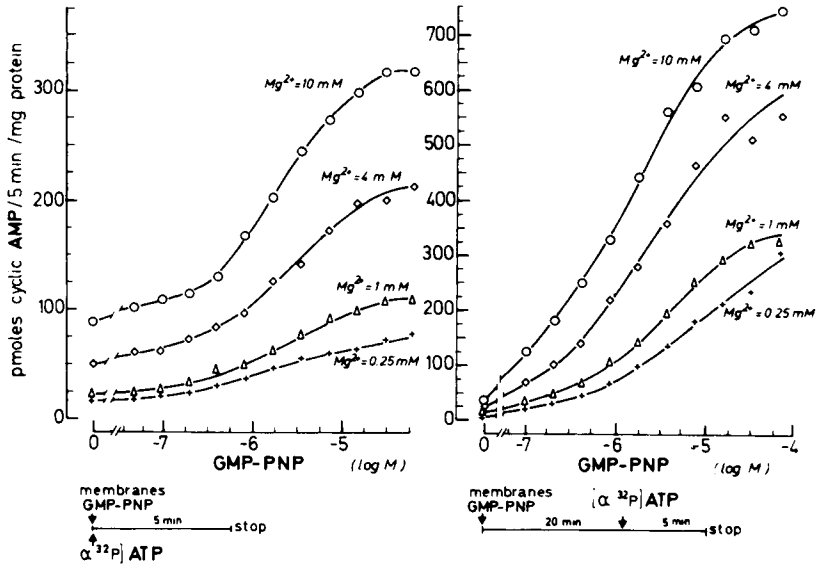

Fig. 8. Effects of Mg^{2+} on dose-dependent adenylate cyclase activation by GMPPNP. The Mg^{2+} concentration in the incubation medium was lowered as indicated in the legend to Fig. 1. Dose dependency for adenylate cyclase activation by GMPPNP was measured for a 5 min incubation period at 30°C either immediately after addition of the enzyme (left) or after a 20 min preincubation period (right).

ACKNOWLEDGMENTS

This research was supported by grants no. 73-31206 from the Délégation Générale à la Recherche Scientifique et Technique and R.C.P. 220, L. A. 219 from the Centre National de la Recherche Scientifique.

REFERENCES

1. Dousa, T., Hechter, O., Schwartz, I. L., and Walter, R., Proc. Natl. Acad. Sci., U.S.A., 68:1693-1697 (1971).
2. Bockaert, J., Roy, C., Rajerison, R., and Jard, S., J. Biol. Chem. 248:5922-5931 (1973).
3. Bockaert, J., Roy, C., Rajerison, R., and Jard, S., C. R. Hebd. Séances Acad. Sci., Paris, 276:649-652 (1973).
4. Rajerison, R., Marchetti, J., Roy, C., Bockaert, J., and Jard, S., J. Biol. Chem. 249:6390-6400 (1974).
5. Campbell, B. J., Woodward, G., and Borberg, V., J. Biol. Chem. 247:6167-6175 (1972).
6. Barth, T., Roy, C., Rajerison, R., and Jard, S., Mol. Cell. Endocrinol. 2:69-79 (1975).
7. Barth, T., Rajerison, R., Roy, C., and Jard, S., Mol. Cell. Endocrinol. 2:81-90 (1975).
8. Pradelles, P., Morgat, J. L., Fromageot, P., Camier, M., Bonne, D., Cohen, P., Bockaert, J., and Jard, S., Fed. Eur. Biochem. Soc. Lett. 26:189-192 (1972).
9. Rajerison, R., Butlen, D., and Jard, S., Mol. Cell. Endocrinol. (in press).
10. Roy, C., Barth, T., and Jard, S., J. Biol. Chem. 250:3149-3156 (1975).
11. Roy, C., Barth, T., and Jard, S., J. Biol. Chem. 250:3157-3168 (1975).
12. Hill, A. V. Biochem. J. 7:471-480 (1913).
13. Bockaert, J., Roy, C., and Jard, S., J. Biol. Chem. 247:7073-7081 (1972).
14. O'Sullivan, W. J., and Perrin, D. D., Biochemistry 3: 18-26 (1964).
15. Birnbaumer, L., Biochim. Biophys. Acta 300:129-158 (1973).

16. Jard, S., Roy, C., Barth, T., Rajerison, R., and Bockaert, J., in "Advances in Cyclic Nucleotides Research," G. I. Drummond, P. Greengard, and G. A. Robison (Eds.), vol. 5, Raven Press, New York (1975).
17. Londos, C., Salomon, Y., Lin, M. C., Harwood, J. P., Schramm, M., Wolff, J., and Rodbell, M., Proc. Natl. Acad. Sci. U.S.A. 71:3087-3090 (1974).
18. Spiegel, A. M., and Aurbach, G. D., J. Biol. Chem. 249:7630-7636 (1974).