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Different Calcium-Mobilizing Receptors Share the Same Guanine Nucleotide-Binding Protein Pool in Hepatocytes

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SUMMARY

High affinity binding of epinephine to the α_1-adrenoceptor reflects the association of the ligand-receptor complex with a guanine nucleotide-binding protein (G protein) and thereby allows the receptor-G protein interaction to be assessed by radioligand binding methods. We have used [^{3}H]prazosin/epinephrine competition binding to rat liver plasma membranes to examine the effects of other Ca^{2+}-mobilizing hormones on the interaction between the α_1-adrenoceptor and its G protein. The aim of our experiments was to test whether the different Ca^{2+}-mobilizing receptors in liver share the same limited pool of G proteins. [Arg^8] Vasopressin (AVP) caused a concentration-dependent (EC_{50} = 0.49 ± 0.03 nM) inhibition of the extent to which epinephrine formed a high affinity complex with the α_1-adrenoceptor; antagonist binding was unaffected by AVP. The effect of AVP was competitively antagonized (K_d = 0.27 ± 0.10 nM) by a selective peptide antagonist of the V_1 vasopressin receptor. We conclude that, in rat hepatocytes, α_1-adrenoceptors and V_1 vasopressin receptors converge to interact with the same pool of G proteins.

Hepatocytes respond to a variety of extracellular stimuli with repetitive transient elevations in cytoplasmic free Ca^{2+} concentration, Ca^{2+} spikes, the frequency of which increases as the concentration of extracellular stimulus is increased (1). Many of the receptors that evoke these responses do so by first activating a G protein, one of the family of G_q proteins (2), which then stimulates phosphoinositidase C activity, leading to inositol-1,4,5-trisphosphate formation and consequent mobilization of intracellular Ca^{2+} stores (3). Although many receptors share these features in hepatocytes, the final intracellular Ca^{2+} signals are characteristic of the receptor that evoked them. Phenylephrine, an α_1-adrenergic agonist, for example, gives rather sharp intracellular Ca^{2+} spikes, whereas AVP evokes spikes in which the falling phase is more prolonged (4). Understanding the mechanisms that generate these agonist-specific Ca^{2+} signals requires that we establish whether the different receptors converge at some point in the signaling pathway. Earlier work (5) suggests that norepinephrine, AVP, and angiotensin II stimulate a shared Ca^{2+} entry pathway in rat hepatocytes, but the possibility that the receptors converge at an earlier stage in the signaling pathway has not yet been addressed.

G proteins are the first elements of signaling cascades with the potential to integrate signals from different types of receptors and direct them to different effector systems. Experiments *in vitro* have established that the functional interactions between receptors and G proteins can be surprisingly promiscuous; for example, the β-adrenoceptor can, in phospholipid vesicles, activate both G_i and G_s (6). In the native membrane environment, topological and stoichiometric constraints are likely to limit such promiscuity (7). Indeed, in intact cells receptor-G protein interactions can be remarkably specific. In GH_3 cells, for example, both somatostatin and acetylcholine inhibit voltage-dependent Ca^{2+} channels, but the effects of somatostatin are mediated by G_{o2}, whereas the muscarinic receptor acts via the very closely related, but distinct, G protein G_{o1} (8). Where distinct receptors can activate the same G protein species, it is important to resolve whether they interact with the same or distinct G protein pools. Subsequent steps in the signaling pathway will inevitably lose the ability to identify the receptor if different receptors converge to the same pool of G proteins, but topographical restraints may allow receptors to interact with the same G protein species and still retain information that identifies the nature of the activated receptor. Conclusive evidence that different receptors can couple to the same pool of G proteins in native cells comes from studies of

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; AVP, [Arg^8]vasopressin; B_{max}, concentration of binding sites; EC_{50}, concentration causing 50% of the maximal effect; $[G]_t$, total concentration of G proteins; IC_{50}, concentration of competing drug that causes 50% inhibition of specific radioligand binding; K_d, equilibrium dissociation constant of a ligand; K_H and K_L, K_d for high and low affinity binding sites, respectively; R_H and R_L, fraction of binding sites in high and low affinity states, respectively; $[R]_t$, total concentration of receptors; d(CH_2)$_5$Tyr(Me)AVP, [1-(β-mercapto-β,β'-cyclopentamethylenepropionic acid), 2-(*O*-methyl)tyrosine][Arg^8]vasopressin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid.

receptor-stimulated adenylyl cyclase activity in turkey erythrocytes (9) and receptor-mediated inhibition of adenylyl cyclase activity in rat adipocytes (10).

The ability of an agonist to form a high affinity complex with its receptor only when the receptor is associated with its G protein (11) provides a convenient radioligand binding assay for assessing receptor-G protein interactions. We have used this method to examine the possibility that the different receptors of liver plasma membranes that stimulate polyphosphoinositide hydrolysis share the same limited pool of G proteins. We have argued that, if two different receptor classes compete for the same pool of G proteins, then addition of the agonist for one of the receptors will allow those receptors to bind to G proteins and thereby prevent the other class of receptor from binding to the G protein. The method assumes that agonist-occupied receptors are able to sequester a substantial fraction of the G protein pool available to the other receptor class (i.e., that $[G]_t$ is not appreciably greater than $[R]_t$). Modeling based on the ternary complex model (11) suggests that an agonist will identify two affinity states of its receptor in the absence of guanine nucleotides only if $[G]_t \leq [R]_t$ (12, 13). Earlier studies with rat hepatocyte plasma membranes (14, 15) have shown that epinephrine identifies two interconvertible affinity states of the α_1-adrenoceptor, suggesting that the requirement for our experiments, namely, that $[G]_t \leq [R]_t$, is satisfied. We have, therefore, examined the effects of AVP on the ability of the α_1-adrenoceptor of liver plasma membranes to form a high affinity complex with epinephrine, to test whether the different receptors share the same G protein pool.

Materials and Methods

Rat liver plasma membranes were prepared by Percoll gradient centrifugation, as previously described (15). The protein content of the membranes was determined by the method of Bradford (16), with bovine serum albumin as the standard.

Binding of [^{3}H]prazosin was measured as previously described (15). Briefly, liver membranes (50 μg of protein) were incubated with 3 nM [^{3}H]prazosin, in a final volume of 250 μl (except for the experiments shown in Figs. 2 and 3, where the volume was increased to 500 μl) containing 20 mM HEPES, 1 mM EGTA, 10 mM $MgCl_2$, 0.8 mM ascorbic acid, 3 mM catechol, 0.1% bovine serum albumin, and 0.1% bacitracin, pH 7.4, for 30 min at 37°, in the presence of the indicated concentrations of unlabeled ligands. Incubations were terminated by addition of 4 ml of ice-cold wash buffer (5 mM HEPES, 1 mM EGTA, 10 mM $MgCl_2$, pH 7.4), followed by filtration through Whatman GF/C filters. The filters were washed twice with 4 ml of wash buffer, and their radioactivity was measured by liquid scintillation counting at 50% efficiency. In all experiments, <5% of the total added radioligand was bound and specific binding (typically 2000 dpm/tube) was about 75–80% of total binding.

[^{3}H]AVP binding was measured as described by Fahrenholz *et al.* (17), with several modifications. Liver membranes (20 μg of protein) were incubated in the presence of [^{3}H]AVP, in a final volume of 250 μl containing 20 mM HEPES, 1 mM EGTA, 10 mM $MgCl_2$, 0.1% bovine serum albumin, and 0.1% bacitracin, pH 7.4, for 20 min at 37°. In competition binding studies, the final concentration of [^{3}H]AVP was 1 nM, and specific binding (typically 2900 dpm/tube) was 70–80% of total binding. In saturation binding experiments, the concentration of [^{3}H]AVP was between 0.05 and 30 nM. Nonspecific binding was determined in the presence of 10 μM unlabeled AVP.

Saturation data were analyzed using the iterative nonlinear curve-fitting program LIGAND (18). The experimentally determined value for nonspecific binding was used as an initial estimate for computer analysis. Competition curves were analyzed using the iterative program GraphPAD InPlot 3.15 (GraphPAD Software, San Diego), which estimates maximal and nonspecific binding from the extrapolated upper and lower limits of the competition curve. The IC_{50} values were transformed to K_d values by using the equation of Cheng and Prusoff (19). Statistical comparison between one- and two-site models was made according to the 'extra sum of squares' test, calculating the F statistic as described by Munson and Rodbard (18); p values of <0.05 were considered significant.

[^{3}H]Prazosin (25 Ci/mmol) was from Amersham. [^{3}H]AVP (75.8 Ci/mmol) was from New England Nuclear. AVP, $d(CH_2)_5Tyr(Me)AVP$, (−)-epinephrine bitartrate, isoproterenol hydrochloride, phenylephrine hydrochloride, bacitracin, and bovine serum albumin were from Sigma. GTP was from Boehringer-Mannheim. Percoll was from Pharmacia. HEPES was from Calbiochem. Phentolamine was from Research Biochemicals Inc. All other chemicals were of the highest grade commercially available.

Results

[^{3}H]Prazosin/epinephrine competition binding curves in rat liver plasma membranes were best fitted by a two-site model, indicating the interaction of epinephrine with two affinity states of the α_1-adrenoceptor (K_L = 1300 ± 110 nM, R_L = 67 ± 2%, K_H = 31 ± 6 nM, R_H = 33 ± 2%, n_H = 0.65 ± 0.01, 17

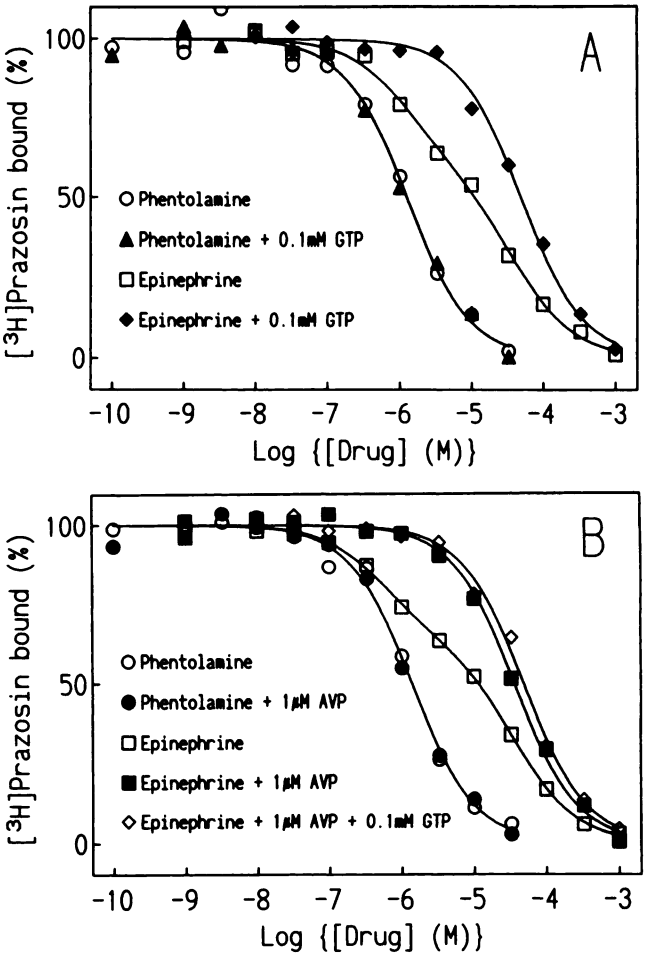

Fig. 1. Effect of AVP and GTP on agonist and antagonist binding to α_1-adrenoceptors. Membranes (50 μg) were incubated with 3 nM [^{3}H]prazosin and increasing concentrations of epinephrine or phentolamine, in the presence or absence of 0.1 mM GTP and/or 1 μM AVP. The data are plotted as percentages of specific binding. Results shown are from representative experiments, which were repeated at least three times.

experiments) (Fig. 1A). In the presence of GTP (0.1 mM), the competition curves revealed that epinephrine bound to a single class of low affinity sites (K_d = 1960 ± 120 nM, n_H = 0.97 ± 0.03, four experiments). The antagonist phentolamine bound to a single class of sites, and its binding was unaffected by guanine nucleotides (Fig. 1A). These results confirm earlier work (14, 15), from which it has been concluded that high affinity binding of an agonist occurs when the receptor is associated with a G protein with no bound guanine nucleotide.

In our earlier work (15), we consistently observed a small difference in the affinities of the low affinity conformation of the receptor for epinephrine in the presence and absence of GTP. The difference is preserved in our present results and, although small, it is now statistically significant (K_{L-GTP} = 1300 ± 110 nM, K_{L+GTP} = 1960 ± 120 nM); other workers have reported a similar discrepancy in both the α_1-adrenoceptor and other adrenergic receptors (for example, see Refs. 20 and 21). We cannot explain these observations, but we do not feel that this discrepancy affects the interpretation of our subsequent experiments, where we have examined the influence of ligands on formation of the high affinity complex between agonists and the α_1-adrenoceptor.

In the presence of 1 μM AVP, the [^{3}H]prazosin/epinephrine competition binding curves revealed that epinephrine bound to a single low affinity state of the α_1-adrenoceptor (K_d = 1222 ± 110 nM, n_H = 0.96 ± 0.02, 10 experiments) (Fig. 1B). The K_d of this state was indistinguishable from the low affinity state of the control curve (K_L = 1300 ± 110 nM). AVP (1 μM) and GTP (0.1 mM) together had no greater effect on epinephrine binding than did GTP alone (Fig. 1B). There was no change in the B_{max} of the receptor for epinephrine as a result of any of these additions (data not shown).

The effect of AVP on epinephrine binding was concentration dependent (EC_{50} = 0.49 ± 0.03 nM, three experiments) (Fig. 2). Computer-assisted analysis showed that AVP caused a concentration-dependent decrease in the fraction of α_1-adrenoceptors in the high affinity state (percentage of R_H), consistent with a progressive uncoupling of the α_1-adrenoceptor from its G protein with increasing concentrations of AVP. The EC_{50} for the effect of AVP calculated from the decrease in the fraction of α_1-adrenoceptors in the high affinity state (percentage of R_H) (0.44 ± 0.18 nM, three experiments; data not shown) was in excellent agreement with the EC_{50} calculated directly from the inhibition of epinephrine binding (0.49 nM). The same range of AVP concentrations (1 pM to 1 μM) had no effect on binding of the α_1-adrenergic antagonists [^{3}H]prazosin or phentolamine (Figs. 1B and 2). The effect of AVP on agonist binding does not, therefore, result from a direct interaction with the α_1-adrenoceptor ligand-binding site.

Further evidence supporting the specificity of the AVP effect is provided by our results with a synthetic peptide antagonist of the V_1 vasopressin receptor, d(CH_2)$_5$Tyr(Me)AVP (22), which competitively inhibited the effect of AVP on the binding of epinephrine to α_1-adrenoceptors (Fig. 3A). d(CH_2)$_5$Tyr(Me)AVP evoked concentration-dependent shifts to the right in the AVP concentration-response curve. Each of the curves had a Hill coefficient of unity. Schild analysis of these data (23) yielded a straight line with a slope of unity (1.02 ± 0.05, three experiments), indicating that d(CH_2)$_5$Tyr(Me)AVP is a competitive antagonist of AVP (K_d = 0.27 ± 0.10 nM, three experiments) (Fig. 3B). The close correspondence of the K_d of

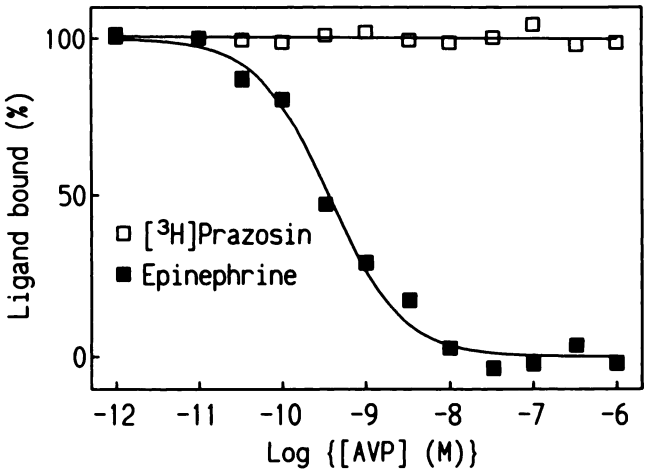

Fig. 2. Concentration dependence of the effect of AVP on agonist and antagonist binding to α_1-adrenoceptors. Inhibition of ligand binding to rat liver plasma membranes by increasing concentrations of AVP was measured as described in Materials and Methods. The effect of increasing concentrations of AVP on antagonist binding (□) was determined directly, by measuring [^{3}H]prazosin (3 nM) specific binding. The effect of AVP on agonist binding (■) was assessed by measuring inhibition of 10 μM epinephrine binding (corresponding to the IC_{50} for the control curve) in competition with 3 nM [^{3}H]prazosin. Under these conditions, the amount of [^{3}H]prazosin bound in the presence of 10 μM epinephrine was 2930 ± 85 dpm/tube (three experiments), and the amount of [^{3}H]prazosin bound increased to 3792 ± 132 dpm after addition of a maximally effective concentration of AVP. Data are plotted as percentages of specific epinephrine binding (100% = 2930 dpm of bound [^{3}H]prazosin; 0% = 3792 dpm of bound [^{3}H]prazosin). Results are representative of three independent experiments.

AVP for the V_1 receptor from [^{3}H]AVP binding (K_d = 0.48 ± 0.08 nM, six experiments, data not shown) and the EC_{50} for its effects on α_1-adrenoceptors (EC_{50} = 0.49 ± 0.03 nM), together with the potent antagonism by d(CH_2)$_5$Tyr(Me)AVP (K_d = 0.27 ± 0.10 nM, three experiments, from Schild analysis and K_d = 0.26 ± 0.02 nM, three experiments, from competition binding analysis, data not shown), suggest that in our experiments the effects of AVP are specifically mediated via its interaction with the V_1 vasopressin receptor.

We next investigated the effects of an agonist (isoproterenol) that activates a receptor (β-adrenoceptor) linked to a G protein (G_s) known to be distinct from that which regulates polyphosphoinositide hydrolysis. In this experiment, we used phenylephrine as a selective α_1-adrenergic agonist, to avoid the activation of β-adrenoceptors that would accompany activation of α_1-adrenoceptors by the nonselective agonist epinephrine. Fig. 4 demonstrates that 100 μM isoproterenol, a concentration that causes >98% occupancy of liver β-adrenoceptors, had no effect on phenylephrine binding to α_1-adrenoceptors. This result is consistent with the suggestion that AVP influences the binding of agonists to only those receptors that stimulate polyphosphoinositide hydrolysis.

Discussion

We have confirmed earlier results (14, 15) by demonstrating that, in liver plasma membranes incubated in the absence of guanine nucleotides, agonists bind to two conformations of the α_1-adrenoceptor, a low affinity site and a high affinity site that is sensitive to guanine nucleotides. These results, together with earlier modeling studies (12, 13), are consistent with the sug-

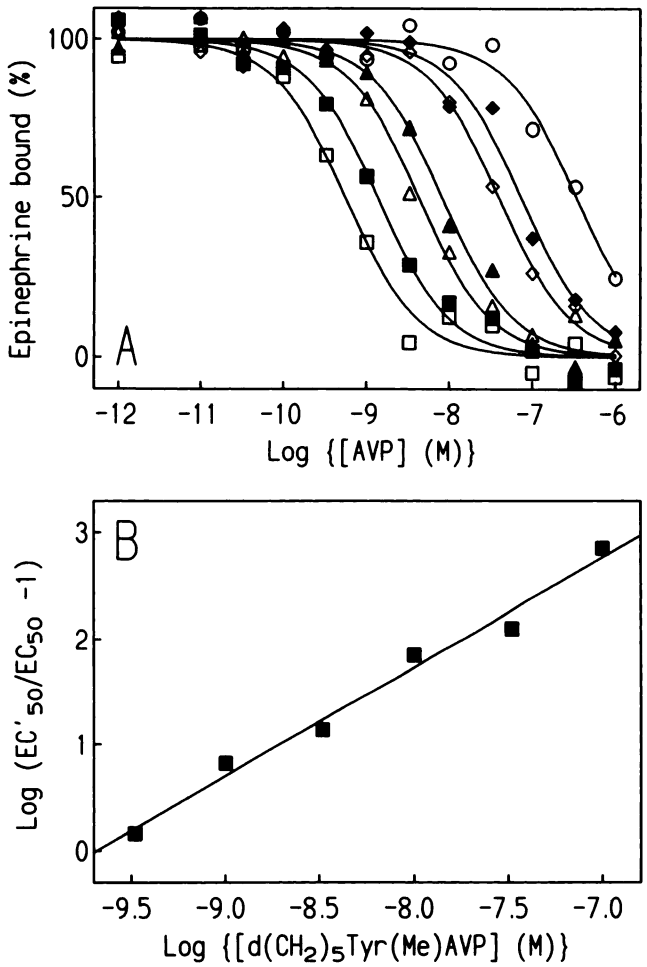

Fig. 3. Inhibition by $d(CH_2)_5Tyr(Me)AVP$ of the effects of AVP on agonist binding to α_1-adrenoceptors. A, Membranes (50 μg) were incubated with 3 nM [^{3}H]prazosin, 10 μM epinephrine, and increasing concentrations of AVP, exactly as in Fig. 2, but in the absence (□) or presence of 0.33 nM (■), 1 nM (△), 3.3 nM (▲), 10 nM (◇), 33 nM (◆), and 100 nM (○) $d(CH_2)_5Tyr(Me)AVP$. Data are plotted as percentages of specific binding. A representative experiment of a series of three is shown. B, Schild plot of the data shown in A. EC_{50} is the AVP concentration causing 50% of the maximal effect upon epinephrine binding to the α_1-adrenoceptor. EC'_{50} is the EC_{50} of the effect of AVP in the presence of different concentrations of $d(CH_2)_5Tyr(Me)AVP$.

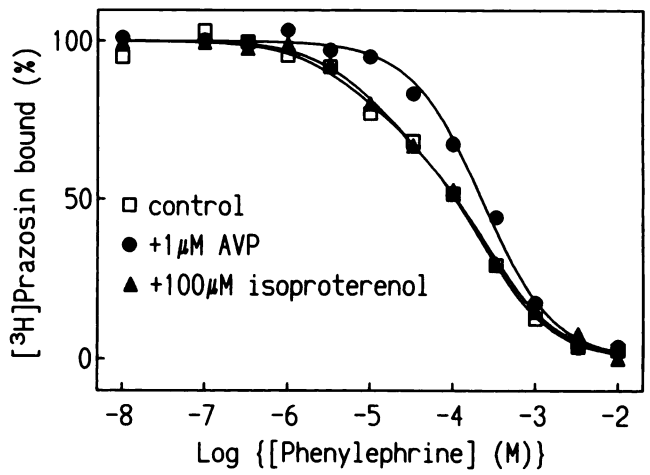

Fig. 4. Inhibition by AVP but not isoproterenol of phenylephrine binding to α_1-adrenoceptors. Membranes (50 μg) were incubated with 3 nM [^{3}H]prazosin and increasing concentrations of phenylephrine in the presence or absence of 1 μM AVP or 100 μM isoproterenol. Data are plotted as percentages of specific binding. A representative experiment of a series of three is shown.

gestion that the number of G proteins available for interaction with α_1-adrenoceptors is not substantially larger than the number of α_1-adrenoceptors. This is a necessary condition for our subsequent experiments, in which we examined the possibility that Ca^{2+}-mobilizing receptors share the same G protein pool as the α_1-adrenoceptor by testing the effects of agonists of those receptors on the high affinity binding of epinephrine to the α_1-adrenoceptor. We predicted that, if different receptors share the same limited pool of G proteins, then activation of one class of receptor may substantially deplete the G protein pool and prevent α_1-adrenoceptors from binding to G proteins and so from forming the high affinity complex of epinephrine with the receptor-G protein complex.

AVP, through its interaction with the V_1 vasopressin receptor, destabilized the high affinity conformation of the α_1-adrenoceptor-epinephrine complex. The effect is closely related to receptor occupancy, because the half-maximal effect of AVP (EC_{50} = 0.49 ± 0.03 nM) is very similar to the concentration required for half-maximal receptor occupancy (K_d = 0.48 ± 0.08 nM). We were concerned that this inhibitory effect of AVP could be mediated by the signaling pathway activated by the V_1 receptor rather than through its effects on depleting the G protein pool. That explanation is, however, very unlikely, because the binding assays were performed under conditions (1 mM EGTA, no ATP, and no GTP) where G proteins, phosphoinositidase C, and protein kinases could not be activated. This suggests that the effects of AVP are mediated not by activation of a signaling pathway but by the ability of the AVP-bound V_1 receptors to bind to G proteins and thereby decrease their availability for other receptors. We conclude, therefore, that in hepatocytes the V_1 receptor and the α_1-adrenoceptor share the same limited pool of G proteins.

In contrast to the effects of AVP on epinephrine binding to α_1-adrenoceptors, neither equilibrium binding of [^{3}H]AVP nor the rate of [^{3}H]AVP dissociation from V_1 receptors was affected by activation of α_1-adrenoceptors (data not shown). We are now examining two alternative explanations for these results; it could be that activated V_1 receptors bind to their G proteins with higher affinity than do activated α_1-adrenoceptors, or the pool of G proteins available to V_1 receptors may be larger than that available to α_1-adrenoceptors. Either explanation would be consistent with both our present conclusion and our observation that, in the absence of guanine nucleotides, V_1 receptors show a uniformly high affinity for AVP.

Wange *et al.* (24) recently reported that activation of the V_1 receptor in liver stimulated γ-azidoanilido[^{32}P]GTP-labeling of two proteins (42 and 43 kDa), which were immunochemically identified as belonging to the G_q family (2). Our present results suggest that at least one of these G proteins is shared with the α_1-adrenoceptor; it is, however, possible that only one is shared and the other is activated only by the V_1 receptor.

Our results showing that V_1 receptors and α_1-adrenoceptors share the same limited G protein pool in hepatocytes suggest that subsequent steps in the polyphosphoinositide signaling pathway can have no means of identifying the nature of the receptor that caused the G protein to be activated. This early convergence of receptors will need to be incorporated into the

models that seek to explain how the Ca^{2+} spikes evoked by activation of α_1-adrenoceptors and V_1 receptors retain features that identify the nature of the receptor that evoked them.

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