Chlorpropamide upregulates antidiuretic hormone receptors and unmasks constitutive receptor signaling

JACQUES A. DURR,1,2 JÖHANNES HENSEN,3
TOBIAS EHNIS,4 AND MARY S. BLANKENSHIP5

(With the Technical Assistance of C. Klein)

1Division of Nephrology, Department of Veterans Affairs Medical Center, Bay Pines 33744;
2Department of Medicine, University of South Florida College of Medicine, Tampa 33612;
3Klinikum Hannover Nordstadt, Medizinische Klinik, Hannover 30167; 4Department of Medicine IV, University of Erlangen-Nuernberg, Erlangen 8520, Germany; and 5Medical Research and Development, Department of Veterans Affairs Medical Center, Bay Pines, Florida 33744


The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
The CP-supplemented culture media and the sterile control media were filtered and handled similarly.

Cell cultures. LLC-PK₁ cells (ATCC CRL 1392; 1.5 × 10⁵ cells/ml) were seeded in 75-cm² tissue culture flasks (Falcon) and grown at 37°C in a humidified CO₂ incubator (95% air-5% CO₂) in Ham’s F-12 medium supplemented with 6% inactivated fetal bovine serum, 2 mmol/l l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The culture medium was replaced every 48 h.

LLC-PK₁ cells reach ~40% confluence after 2 days, 70% after 3 days, and 95% after 4–5 days, with dome formations by days 6–8. Exposure of matched subcultures to 100 µM CP for 24 h did not affect cell viability (trypan blue), growth pattern, or membrane protein content of confluent monolayers (0.32 ± 0.02 vs. 0.33 ± 0.02 mg/ml). Inhibition of cell growth required chronic exposure to CP concentrations that were 20–50 times higher (2–5 mM). Therefore, chronic CP exposure consisted of supplementing culture media of matched subcultures with 100 µM CP for 24 h. Cells were harvested in exposure consisted of supplementing culture media of matched subcultures with 100 µM CP for 24 h. Cells were harvested in

Analysis. ADH binding and cAMP production rates were analyzed with the computer programs LIGAND (55) and ALLFIT (17). The ALLFIT program statistically tests whether two or more sigmoidal dose-response curves share common parameters (16, 17). Other software used were Excel (Microsoft), Framework (AshtonTate), Scientist (Micromath), and Sigma Plot (Jandel). The Mann-Whitney U-test and the Wilcoxon matched pairs test were used where appropriate. Computer modeling of ADH-responsive AC activity, based on the extended ternary complex model of hormone signaling, was performed by using the program ALLFIT. This analysis and the derivation of the A-O plots are provided in the APPENDIX.

RESULTS

CP as an ADH antagonist. The present study confirms our earlier finding (24, 25) that the binding of our N'-lysyl-conjugated ADH ligands to LLC-PK₁ cell membranes is specific and saturable (Fig. 1). Here we show that CP displaces the ADH ligand from LLC-PK₁ cell membranes in a competitive manner (Fig. 2) with an IC₅₀ of 2.5 ± 0.5 mM. Analysis with LIGAND revealed an inhibition constant (Kᵢ) of 2.8 ± 0.5 mM (n = 6). In addition to displacing ADH from its receptor, CP also competitively inhibited ADH-stimulated AC activity (Fig. 3). CP inhibited ADH-stimulated AC, with an inhibition constant (Kᵢ) of 250 ± 6 µM (n = 6). The CP concentration of 333 µM was used because it is in the vicinity of the Kᵢ for inhibition and within the therapeu-

Radioligands. Conjugation labeling of ADH is described elsewhere (23, 24, 32). Owing to the large difference in HPLC retention times between unconjugated and N'-conjugated α-amino-protected and deprotected lysine vasopressin (LVP) derivatives, the specific activity of the tracer matches that of the carrier-free [¹²⁵I] label used in the preparation (23–25). In the presence of bacitracin, ligand binding to LLC-PK₁ cell membranes was rapid, saturable, and reversible (24, 25).

Binding. For the “cold saturation” (55) studies, cells or membranes were incubated with a fixed amount of ADH tracer (~60,000 counts·min⁻¹·tube⁻¹) and increasing concentrations of ADH, ranging from 0 to 5 × 10⁻⁵ M. Nonspecific binding was assessed in the presence of excess ADH (~10⁻³ M). The binding buffer consisted of 100 mM Tris·HCl, 5 mM MgCl₂, 0.1% BSA, and 0.1% bacitracin, pH 7.4. After centrifugation in a high-speed microcentrifuge, the pellet was resuspended once more in hypotonic buffer to yield the final pellet. Protein content was determined with the Bio-Rad protein kit by using IgG as the standard.

Binding kinetics. Binding studies were performed in membranes from matched pairs of control (●) and 24-h CP-exposed (●) LLC-PK₁ subcultures. Scatchard plots (inset) suggested a single class of binding sites that increased with 24-h exposure to 100 µM CP. Moreover, CP treatment did not alter affinity for ADH (identical slopes). This was confirmed by the program LIGAND, which, in this example, revealed a higher ADH receptor density [Bmax; 909 vs. 803 fmol/mg protein, or 140,432 vs. 124,048 copies of receptors per cell, respectively, in control and CP-exposed cells, but identical affinities (Kᵢ); i.e., 1.2 vs. 1.3 nM, respectively]. B/F, ratio of bound to free ADH.

Fig. 1. Antidiuretic hormone (ADH) saturation binding studies in membranes from control and chloropropamide (CP)-treated cells. ADH receptor saturation studies were performed in membranes from matched pairs of control (○) and 24-h CP-exposed (●) LLC-PK₁ subcultures. Scatchard plots (inset) suggested a single class of binding sites that increased with 24-h exposure to 100 µM CP. Moreover, CP treatment did not alter affinity for ADH (identical slopes). This was confirmed by the program LIGAND, which, in this example, revealed a higher ADH receptor density [Bmax; 909 vs. 803 fmol/mg protein, or 140,432 vs. 124,048 copies of receptors per cell, respectively, in control and CP-exposed cells, but identical affinities (Kᵢ); i.e., 1.2 vs. 1.3 nM, respectively]. B/F, ratio of bound to free ADH.
Competitive inhibition of ADH-stimulated AC by CP (i.e., unchanged ADH affinity (K_i), the same ADH receptor density (B_max), and the ED_50 (d) were significantly different (16) (Fig. 5). Moreover, a nonessential mode of activation (72) of ADH-sensitive AC (i.e., | V_{max} | K_a), by 24-h exposure to 100 µM CP, was also suggested by the double reciprocal plots, corrected for basal activity (Fig. 5, inset). The normalized (i.e., in %maximum) fractional AC activation-fractional receptor occupation (A-O) plots constructed with the present results (APPENDIX) are depicted in Fig. 6.

As the A-O plots for both controls (Figs. 3 and 5) were identical, one single plot representing their arithmetic mean was constructed (○, middle curve). The top curve (●) depicts the A-O plot obtained for cells chronically exposed to CP (Fig. 5), and the bottom curve (■) represents the A-O plot in the presence of 333 µM CP (Fig. 3). The A-O plot pattern of the cells exposed for 24 h to CP suggests enhanced receptor-to-AC stoichiometry or increased receptor density, as predicted by the "random hit matrix model" proposed for ADH receptor-AC coupling (4), or an enhanced agonist "efficacy" (14). Conversely, the A-O plot pattern obtained in the presence of CP is characteristic of decreased coupling between receptor and cyclase units (4), or lower intrinsic

**Fig. 3. Acute effects of CP on ADH-stimulated cAMP production rate in LLC-PK_1 cells. CP (333 µM, ○) displaced the normal (●) ADH-AC dose-response curve to the right without affecting maximum response (competitive inhibition). Indeed, CP increased ED_50 from C_50 = 0.075 ± 0.020 nM to C_50 = 0.255 ± 0.059 nM but did not affect maximum response of adenylyl cyclase (AC) to ADH (d_1 = d_2 = maximum velocity (V_{max}) = 6,906.96 ± 240.07 fmol cAMP·min^{-1}·mg protein^{-1}). Conventional Lineweaver-Burk plots (inset), corrected for basal activity, also suggest competitive inhibition (72) of ADH-stimulated AC activity by CP (unchanged y-intercept, i.e., 1/V_{max}, but a different x-intercept, i.e., -1/K_a). Results obtained with ALLFIT; see APPENDIX.
The mechanism by which CP potentiates ADH-mediated antidiuresis (1, 6, 7, 34, 44, 45, 48, 49, 52–54, 56, 76) has eluded any explanation for the past 30 years. Our findings that CP competitively inhibits ADH binding and AC stimulation in LLC-PK1 cells (Figs. 2 and 3) were surprising, although the paradoxical in vitro inhibition by CP of ADH binding (57) and AC stimulation (3, 43, 54) were previously reported in rat renal membranes. Because CP has no intrinsic ADH-like activity (1, 6, 34, 45, 48, 49, 52, 53, 56, 76) and inhibits ADH- but not fluoride-stimulated AC (54), it behaves as a specific competitive ADH antagonist. And as the in vivo (6, 45, 48, 49, 52–54, 56, 76) and in vitro (Figs. 1–6) (52–54, 57) CP concentrations were identical (30), the seemingly irreconcilable ADH potentiation in vivo but ADH antagonism in vitro cannot merely be explained by a dose effect. Any inherent differences between the in vivo and in vitro models can be excluded because the paradox of ADH potentiation (6, 48, 49, 52–54, 76) and antagonism (3, 43, 54, 57) is now documented within the same model (Figs. 1–6). Thus although Lineweaver-Burk plots suggest competitive inhibition of ADH-sensitive AC (unchanged $V_{\text{max}}$ higher apparent $K_a$) when CP is added acutely to the assay, nonessential activation (72) of ADH-sensitive AC ($V_{\text{max}}$ $\frac{K_a}{[H_+]}$), is seen when CP is added to the culture medium for 24 h before cell harvesting (Figs. 3 and 5, insets). The same nonessential mode of activation of ADH-sensitive AC was observed in renal membranes of CP-treated rats (52, 53), a model found by us to be also associated with ADH receptor upregulation (32).

ADH receptor upregulation in rats (32) and LLC-PK1 cells (Figs. 1 and 4) alone, however, cannot explain, at least within the framework of the classical G protein-coupled receptor-signaling theory, the enhanced basal AC activity in both models (Fig. 5) (52, 53) and the inability of CP-treated rats to dilute their urine after a sustained oral water load (6 $\times$ 10 ml/8 h), the latter of which was designed to suppress ADH completely (32). An inability to fully dilute the urine after a sustained water load was also observed in CP-treated normal volunteers, and even in patients with ADH-deficient DI (28, 48, 60). Moreover, sensitive radioimmunoassays for ADH and neurophysine also suggest that the impaired free water clearances after CP treatment in rats and humans are ADH independent (60). Finally, the fact that impaired maximal free water clearance is also observed in CP-treated DI rats (39) is compelling evidence that it is ADH independent. Thus, although CP-mediated antidiuresis requires residual ADH (6, 34, 45, 48, 49, 56), the impaired diluting ability after CP treatment (32, 39, 48, 60) is ADH independent.

This puzzling phenomenon is reminiscent of the antidiuretic state that arises during chronic infusions of peptidic ADH antagonist in DI rats (37, 46, 74). Indeed, ADH receptor upregulation also occurs in this model (10) where the antidiuresis, by definition, is ADH independent, because it appears in rats unable to produce ADH (DI rat). That it is also independent of other putative endogenous ADH agonists such as oxytocin is further suggested by the fact that this antidiuresis arises during infusion of saturating doses of “neutral” ADH antagonists (i.e., in a situation where there are no free ADH receptors available). In fact, this antidiuresis correlated with the relative potency of the antagonists used; therefore, intrinsic partial agonism is also unlikely. Moreover, their half-life is several orders of magnitude shorter (<20 min) than the protracted (days) antidiuresis (10) that persisted after their infusion was discontinued. That this paradoxical antidiuresis could be due to an acquired intrinsic ADH agonist activity of the ADH antagonist is also unlikely, because these antagonists have no demonstrable agonistic activity when tested acutely in the same rats (74). Such confounding questions could be avoided with the present in vitro model, where the same constellation of increased basal AC activity and ADH receptor upregulation was elicited by CP (Figs. 1 and 5), which also behaved as a competitive antagonist, for both ADH binding (Fig. 2) and AC stimulation (Fig. 3). ADH receptor upregulation (Fig. 4) (10, 32) is the common finding in the above states and is characterized by increased basal ADH-like signaling activity, i.e., either an increased basal AC activity (Fig. 5) and/or an impaired free water clearance (10, 32, 52, 53) during ADH suppression. Thus constitutive, hormone-independent receptor-signaling activity (14, 18) has to be considered for the antidiuretic V2 ADH isoreceptor.

This constellation induced by CP and ADH antagonists in vivo (10, 32) and CP in vitro (Figs. 2, 3, 5) may be explained by the extended G protein-coupled receptor-signaling hypothesis (14, 42). This model challenges the traditional view that inactive receptors R are converted, on hormone (H) binding, into active HR* complexes (H $+$ R $\rightarrow$ HR*) (18). Rather, agonists merely stabilize the active state R* of receptors that spontaneously isomerize into their inactive (silent) R and active (signaling) R* conformations (R $\rightleftharpoons$ R*), as they display higher affinity for R* than R (APPENDIX).

Pure neutral competitive antagonists, on the other hand, have no R/R* preferences and hence do not affect...
the equilibrium $R \rightleftharpoons R^*$, i.e., basal receptor-signaling activity; they only compete with agonist for binding. Finally, those antagonists that display a higher affinity for $R$, and thus stabilize the inactive receptor conformation, are termed inverse agonists (71). Inverse agonists inhibit “basal” signaling activity because they decrease the number of receptors that spontaneously reside in the productive conformation $R^*$. However, as the inactive state $R$ predominates in the absence of agonists, hormone-independent signaling activity (due to $R^*$) is difficult to detect. Initially, computer simulations were used to predict the behavior of such models (14, 41).

Eventually, receptor mutations that spontaneously favor the active transition state ($R \rightarrow R^*$) (67, 68), or models that overexpress the wild-type receptor ($R \rightarrow R^*$) in vitro (2, 12, 75) and in transgenic mice (9), i.e., models that stochastically increase the active receptor conformation $R^*$, were found to consistently display increased basal, i.e., hormone-independent receptor-signaling activity (41, 50). Although the significance of tonic receptor-signaling activity in normal physiology is still debated (8, 50, 71), it is increasingly recognized in vitro in cell cultures (40), where inverse agonists have become invaluable probes for unmasking this phenomenon (13).

Several independent lines of evidence suggest constitutive signaling activity of the renal ADH isoreceptor. First, the association of receptor upregulation with ADH-independent, ADH-like effects in vivo (10, 32) and in vitro (52) (Fig. 5) point to some constitutive signaling activity of unliganded ADH receptors. Tonic ADH receptor-signaling activity is further suggested by the unique inhibitor properties of CP. Indeed, the inhibition of ADH binding and activation of AC in LLC-PK1 cells by CP is of a competitive nature because CP increases the apparent $K_d$ and $K_a$ (Figs. 2 and 3) but has no effects on $B_{max}$ and $V_{max}$ and is restricted to the effect of ADH. Indeed, CP inhibits AC only when stimulated by ADH (3, 43, 54) (Fig. 3) but not by postreceptor stimuli such as fluoride, GTP, or 5'-guanylyl imidodiphosphate (43, 52–54). Because the competitive ADH inhibitor CP also inhibits basal, i.e., ADH-independent AC, activity in rat renal membranes (3, 43) and LLC-PK1 cells (Fig. 3) and, similarly, increases urine flow rate or free water clearance in the absence of endogenous ADH in dogs (76) and rats (52), it acts as an ADH inverse agonist. These findings imply that the ADH receptor displays constitutive signaling activity in vivo as well as in vitro. Moreover, the computer simulations of agonist dose-response curves in the allosteric receptor model (14, 41) predict that inverse agonists lower the initial plateau.

---

**Fig. 4.** Effect of CP on ADH receptor density in 14 LLC-PK1 cell subcultures. ADH receptor density was assessed in preparations from 14 matched pairs of LLC-PK1 subcultures exposed (+CP; hatched bars) or not exposed (-CP; open bars) to 100 µM CP for 24 h before harvesting. Individual saturation studies (Fig. 1) revealed that membranes from CP-exposed subcultures had a higher ADH receptor density ($1,040 \pm 82$ vs. $899 \pm 58$ fmol/mg protein, $n = 14$, $P < 0.01$) but the same ADH affinity ($1.14 \pm 0.096$ vs. $1.14 \pm 0.113$ nM, $n = 14$, not significant).

**Fig. 5.** Chronic effects of CP on ADH-stimulated cAMP production rate in LLC-PK1 cells. Exposure to 100 µM CP for 24 h potentiated subsequent AC response to ADH. In this study, $ED_{50}$ of ADH for AC activation was $C_2 = 0.112 \pm 0.025$ nM in control cells (●) compared with $C_2 = 0.053 \pm 0.009$ nM for cells preexposed to CP (○). Maximal stimulation of AC (i.e., parameter $d = V_{max}$) was significantly higher in 24-h CP exposed cells than in control ($d_2 = 8,414.77 \pm 219.80$ vs. $d_1 = 5,748.22 \pm 238.67$ fmol cAMP·min⁻¹·mg protein⁻¹, respectively). Conventional double reciprocal plots ($1/c_{AMP}$ vs. $1/LVP$, inset), corrected for basal activity, suggest nonessential activation (72) of ADH-sensitive AC by CP.

**Fig. 6.** Effect of CP on ADH receptor density in 14 LLC-PK1 cell subcultures. ADH receptor density was assessed in preparations from 14 matched pairs of LLC-PK1 subcultures exposed (+CP; hatched bars) or not exposed (-CP; open bars) to 100 µM CP for 24 h before harvesting. Individual saturation studies (Fig. 1) revealed that membranes from CP-exposed subcultures had a higher ADH receptor density ($1,040 \pm 82$ vs. $899 \pm 58$ fmol/mg protein, $n = 14$, $P < 0.01$) but the same ADH affinity ($1.14 \pm 0.096$ vs. $1.14 \pm 0.113$ nM, $n = 14$, not significant).
V upper plateau (water loading, hence ADH suppression, in CP-treated impaired ability to maximally dilute the urine after explained (6, 48, 49, 52–54, 56, 76). Similarly, the antidiuresis is well known but has hitherto never been explained (6, 48, 49, 52–54, 56, 76). The need for residual ADH release in the CP-mediated ADH would have been required for the antidiuresis. And, like for CP, residual agonists (APPENDIX). If these antagonists were also inverse antagonists, because it occurred during their infusion (10, 37, 46, 74). Constitutive receptor-signaling activity is resistant, by definition, to ADH antagonists, because it occurred during their infusion (10, 37, 46, 74). Constitutive receptor-signaling activity is resistant, by definition, to neutral competitive antagonists (APPENDIX). If these antagonists were also inverse agonists, then receptor upregulation alone may not have led to the antidiuresis and, like for CP, residual ADH would have been required for the antidiuresis. The need for residual ADH release in the CP-mediated antidiuresis is well known but has hitherto never been explained (6, 48, 49, 52–54, 56, 76). Similarly, the impaired ability to maximally dilute the urine after water loading, hence ADH suppression, in CP-treated rats (32, 39, 60) and humans (48, 60), has not yet received an adequate explanation. The allosteric receptor model predicts that receptor upregulation alone (32) could explain the impaired diluting ability uncovered by ADH suppression and medullary washout (48, 32, 28, 60) of CP during water loading, if CP had weak ADH inverse agonist properties, or its corollary, if the ADH receptor displayed ADH-independent activity.

The set point of the allosteric receptor equilibrium (R = R*), crucial in hormone signaling, depends on the ionic strength (41, 42), and triggering of G protein-coupled receptor-signaling by salts (35) has recently been attributed to an allosteric receptor transition R → R* (14). This phenomenon is of particular relevance to the renal ADH isoreceptor, strategically located in the renal medulla, in an ionic milieu affected by the state of hydration and the antidiuresis itself.

Potentiation of the ADH-sensitive AC by NaCl occurs in the rat (15, 19, 27), rabbit (21, 22), pig (63–66, 73), and bovine (31, 58) kidneys, where NaCl not only enhances ADH-stimulated but also basal AC activity (15, 19, 21, 22, 27, 31, 58, 63–66, 73). Dose-response curves in LLC-PK1 membranes (Fig. 7) reveal an

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig6}
\caption{Fractional (%V_max vs. %B_max) AC activation-ADH receptor occupancy (A-O) plots. Middle curve, ○ normal A-O relationship in LLC-PK1 cells as derived from 2 control experiments (● in Figs. 3 and 5) (4) (see APPENDIX); ■, A-O plot pattern in presence of 333 µM CP (Fig. 3); ●, A-O plot pattern for cells grown in 100 µM CP (Fig. 5), a treatment that results in receptor upregulation (Fig. 4). Line of identity (dashed line; fractional A = fractional O) represents 1/1 A-O coupling pattern. For points above this line, receptor “reserve” is invoked.

 Constitutive, ADH-independent ADH receptor-signaling activity may explain other paradoxes of antidiuresis. Thus the antidiuresis arising with receptor upregulation in the DI rats chronically infused with peptidic ADH antagonists was resistant, by definition, to ADH antagonists, because it occurred during their infusion (10, 37, 46, 74). Constitutive receptor-signaling activity is resistant, by definition, to neutral competitive antagonists (APPENDIX). If these antagonists were also inverse agonists, then receptor upregulation alone may not have led to the antidiuresis and, like for CP, residual ADH would have been required for the antidiuresis. The need for residual ADH release in the CP-mediated antidiuresis is well known but has hitherto never been explained (6, 48, 49, 52–54, 56, 76). Similarly, the impaired ability to maximally dilute the urine after water loading, hence ADH suppression, in CP-treated rats (32, 39, 60) and humans (48, 60), has not yet received an adequate explanation. The allosteric receptor model predicts that receptor upregulation alone (32) could explain the impaired diluting ability uncovered by ADH suppression and medullary washout (48, 32, 28, 60) of CP during water loading, if CP had weak ADH inverse agonist properties, or its corollary, if the ADH receptor displayed ADH-independent activity.

The set point of the allosteric receptor equilibrium (R = R*), crucial in hormone signaling, depends on the ionic strength (41, 42), and triggering of G protein-coupled receptor-signaling by salts (35) has recently been attributed to an allosteric receptor transition R → R* (14). This phenomenon is of particular relevance to the renal ADH isoreceptor, strategically located in the renal medulla, in an ionic milieu affected by the state of hydration and the antidiuresis itself.

Potentiation of the ADH-sensitive AC by NaCl occurs in the rat (15, 19, 27), rabbit (21, 22), pig (63–66, 73), and bovine (31, 58) kidneys, where NaCl not only enhances ADH-stimulated but also basal AC activity (15, 19, 21, 22, 27, 31, 58, 63–66, 73). Dose-response curves in LLC-PK1 membranes (Fig. 7) reveal an

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig7}
\caption{Effect of NaCl on LVP-cAMP dose-response in intact LLC-PK1 monolayers. Culture medium (Ham's F-12) of confluent monolayers in 24-well plates was aspirated and replaced with 1 ml culture medium supplemented with 1 mM IBMX and 0.1 g% bacitracin, and plates were incubated at 37°C for 10 min. This medium was then aspirated and replaced with 0.5 ml of similar medium that contained serial dilution of LVP and was obtained by adding to 9 vol of preincubation medium 1 vol of either 150 (●) or 1,500 (○) mM NaCl. After 15 min at 37°C, plates were placed on ice and 1 ml of iced ethanol-acetic acid (99:1) was added to each well. After gentle stirring, solution was aspirated and diluted in assay buffer. cAMP standards contained the same final ratio of ethanol and acetic acid. Protein content of each well was measured by the Lowry method directly on “ethanol-acetic acid-fixed” monolayer. cAMP production rate was expressed in fmol·µg⁻¹·min⁻¹. Normal Ham's F-12 medium contains 130 mM NaCl. Basal activity (no LVP added) was 1.168 ± 0.109 and 2.427 ± 0.174 fmol·µg⁻¹·min⁻¹ in control and NaCl-supplemented wells, respectively (P < 0.001).}
increase in parameters a and d (i.e., basal AC activity and $V_{\text{max}}$) but not in c (EC$_{50}$), consistent with an R $\rightarrow$ R* transition. At each dose of ADH the ratio of productive (R* + HR*) to total receptor $R_t$, (R* + HR*)/$R_t$, an index of activity, is increased in the presence of NaCl. NaCl promotes the high-affinity state of the ADH receptor in LLC-PK$_1$ cells but has no effect on $R_t$ (64, 65), a phenomenon coined “receptor transition” (64, 65). That this may be an allosteric effect is further suggested by the finding that mannitol inhibits the salt effect in LLC-PK$_1$ cell membranes (73). Indeed, mannitol is known to act as a “compatible” solute that stabilizes the native conformation of proteins and prevents their salting out by chaotropic agents like NaCl. The “salting out” phenomenon is nothing more than an extreme allosteric transition. Furthermore, the rapid reversibility of the NaCl effect (65), together with the fact that NaCl increased basal and ADH-stimulated AC activities by 50 and 100%, respectively, but affected fluoride-stimulated activity by <10% (73), also points to an activating allosteric transition at a step preceding AC and G proteins, hence by exclusion, that takes place at the ADH receptor itself (R $\rightarrow$ R*). It is now well accepted that receptor transitions (R $\rightarrow$ R*) that favor the high-affinity receptor conformation R* not only potentiate the AC response to hormone but also increase the hormone-independent basal AC activity (41).

A salt-induced allosteric transition of the ADH receptor in favor of ADH-independent signaling activity may explain why ADH-deficient rats concentrate their urine during dehydration (26), a state that elevates the ionic strength of the renal medulla. A receptor transition in the opposite direction (R $\rightarrow$ R*) has been postulated to account for the initial ADH resistance of water-replete DI rats (64). The effect of NaCl on the ADH-AC dose-response curve in LLC-PK$_1$ cells supports this hypothesis (Fig. 7). Our present knowledge of the physiology of antidiuresis suggests that the renal ADH receptor may have evolved into an exquisitely salt-sensitive allosteric transducer. This could compensate for the lack of redundancy in the antidiuretic mechanism (5), which rests primarily on one single hormone and one single ADH-regulated water channel (36), compared with the diversity of the renal antinatriuretic mechanisms (20). Clearly, although outside the scope of the present report on CP, similar studies based on the allosteric receptor isomerization model will be required to further assess the effects of NaCl on ADH signaling (Fig. 7). Of relevance to both CP (Figs. 1–6) and NaCl (Fig. 7) is the recent report of ADH-independent constitutive signaling activity and ADH inverse agonism of the antagonist SR-121 463A in the D136A mutated human V$_2$ ADH receptor (51). Relevance to CP resides in the fact that its chemical structure, with a benzene-sulfonamide residue at one end, linked, via the amino-carbonyl bridge, to a hydrophobic moiety at the other, closely resembles the structure of this nonpeptide ADH inverse agonist. Reminiscent of the salt effect in ADH signaling (Fig. 7) is the general rule that mutations that confer constitutive activity ($R \rightleftharpoons \uparrow R*$) destabilize receptors, hence rendering them susceptible to denaturation (29, 51). Substitution of the aspartic acid in the conserved DRH/Y sequence, like in this case (51), consistently activates receptors (69, 70). This anionic residue has the potential of forming “constraining salt bridges” that stabilize the inactive conformation R of receptors (61, 69, 70). Moreover, aspartic acid residues are known to play a key role in the allosteric modulation of receptor activity by Na$^+$ (38, 59, 62). That the salt effect (Fig. 7) occurs specifically at the level of the V$_2$ receptor within the V$_2$ receptor-G protein-adenylyl cyclase signaling cascade in LLC-PK$_1$ is further suggested by the fact that although NaCl markedly magnifies (Fig. 7, and Ref. 73) cAMP stimulation by ADH, it has very little (<10%) effect on postreceptor stimulation of AC by NaF (73). Moreover, although in rat renal papillary collecting tubule cells NaCl markedly potentiates the cAMP response to ADH, it has no effect on cAMP stimulation by forskolin or prostaglandin E$_2$ (47).

In summary, this study provides evidence that CP is a weak inverse ADH agonist for the V$_2$ ADH renal receptor. This explains why CP upregulates ADH receptors in vivo (32) and in vitro (this study) and why CP treatment ameliorates water handling in partial central DI but has no effect in patients with nephrogenic DI due to mutated ADH receptors incapable of constitutive signaling activity (1). Because the corollary of inverse ADH agonism is constitutive ADH receptor activity, the inability of rats and humans to maximally dilute their urine after CP treatment (32, 39, 48, 60), and presumably upregulated ADH receptors (32), suggests the presence of ADH-independent, but ADH receptor-dependent, signaling in normal renal water handling.

APPENDIX

The Extended Model of G Protein-Coupled Receptor Signaling

\[
\begin{align*}
R & \rightleftharpoons R^* \\
+ & + \\
H & H \\
\Uparrow & \Uparrow \\
HR & \leftarrow HR^*
\end{align*}
\]

In this model (2, 42) the receptor spontaneously assumes an allosteric equilibrium (transitions) between a silent R and an active R* conformation. The signal [i.e., adenylyl cyclase (AC) activity] is a function of the ratio (R* + HR*)/$R_t$, where R* and HR* are the active conformations of the free and hormone-bound receptor that interact productively with G proteins, and where total receptor $R_t$ is given by the mass conservation $R_t = R + R^* + HR + HR^*$. G protein interactions are not depicted. The agonist has a higher affinity for the active conformation R* and hence will stabilize it. In the absence of hormone H, the silent conformation R predominates, and thus basal signaling activity is minimal.

Any nonhormonal perturbation that increases R* will enhance basal and ADH-responsive AC activity. Because neutral antagonists (I) bind equally well to the R and R* conformations, they do not affect the allosteric equilibrium or the total amount of active species of the receptor in the absence of H. At the extreme, in the presence of an excess of
neutral antagonist I, the allosteric receptor equilibrium $R = \leftrightarrow R^*$ is merely replaced with the equivalent equilibrium $IR = \leftrightarrow IR^*$, and because $IR^*$ is active, the basal signaling activity is not affected. Thus neutral antagonists neither inhibit basal signaling activity nor prevent the increase in signaling activity seen with receptor upregulation ($R = \leftrightarrow R^*$) or induced by external, nonhormonal factors such as NaCl ($R = \leftrightarrow IR^*$). However, as they compete with $H$ for binding to $R$ and $R^*$, they inhibit the stimulation mediated by $H$. On the other hand, antagonists that have a higher affinity for $R$ than for $R^*$, i.e., inverse agonists, decrease basal signaling activity in the absence of agonists. Computer simulations of this model (14, 41) predict that receptor upregulation should be associated with 1) an enhanced maximal agonist response ($V_{\text{max}}$) to agonist, 2) an increased basal signaling activity, and 3) a lower $ED_{50}$ for agonist stimulation. Moreover, computer simulations based on this model also predict that inverse agonists should 1) decrease basal activity, 2) increase the $ED_{50}$ for agonist, and 3) leave unaltered the maximal response to agonists. This was the pattern observed for the ADH-AC signaling system in LLC-PK1 cells and for the antagonistic characteristics of CP. As inverse agonists stabilize preferentially the inactive receptor conformation $R$, i.e., perturb the allosteric equilibrium $R = \leftrightarrow R^*$ in favor of $R$, they may also affect the trajectory of the fractional activation-occupation (A-O) plots. This was observed for CP, and may in part account for the finding that its $K_i$ for inhibition of ADH binding was higher than its $K_i$ for inhibition of ADH-stimulated AC. Clearly, studies will be required to elucidate the molecular mechanism.

Computer Modeling of ADH-Dependent AC Activity

The program ALLFIT, which allows to test statistically the hypothesis that families of dose-response curves share common parameters (16, 17), was used to verify in the ADH-responsive AC system of LLC-PK1 cells, the predictions of the extended ternary complex model (2, 8, 9, 12–14, 40–42, 50, 67, 68, 71, 75) of hormone signaling.

ALLFIT fits the empirical four-parameter logistic equation to the data. The advantage of this method is that no assumptions are required concerning the mechanism(s) underlying the phenomenon under observation. Thus by testing whether dose-response curves share common parameters, ALLFIT allows extraction of objective information not obtainable by standard graphical assessment of dose-response curves, including the double reciprocal plots (Lineweaver-Burk plots) (72).

The conventional notation for the four-parameter logistic equation of a dose ($X$)-response ($Y$) relationship is

$$Y = \frac{a - d}{1 + \frac{X}{c}} + d$$

where $a$ is the lower plateau ($X = 0$) or basal activity, $d$ is the upper plateau ($X \to \infty$) or maximal response ($V_{\text{max}}$), $c$ is the $ID_{50}$, and $b$ is the slope factor. In the last version of ALLFIT (16), the roles of $a$ and $d$ have been exchanged to ensure that the fraction is positive; hence $b$ assumes a negative value (16).

**A-O Plots**

The dependency of AC activation ($A = \%V_{\text{max}}$) on receptor occupancy ($O = \%B_{\text{max}}$) has been extensively assessed for the renal ADH receptor (4, 31, 58). Computer modeling of this relationship has led to a random-hit matrix model of hormone signaling in which ADH receptors are assumed to interact with a set of vicinal AC units (4). Although the possibility of an ADH-independent, spontaneous ADH receptor-signaling activity has been considered initially, this then-novel concept was not pursued in this model (4). This idea, however, has recently been revived for G protein-coupled receptors (2, 8, 9, 12–14, 40–42, 50, 67, 68, 71, 75). To model the A-O relationship, $A$ and $O$ have to be recorded under similar experimental conditions (i.e., same buffer and at equilibrium) (4, 31, 58). Therefore, all the AC stimulation studies were performed in binding buffer under the same conditions as ADH binding, with the sole exception that $0.5 \text{ mM IBMX}$ was present during AC stimulation. IBMX, however, had no affects on ligand binding.

Because the A-O plots for the renal ADH-signaling system, published by others, were constructed with the basal AC activity subtracted (4, 31, 58), for comparison we used the same representation (Fig. 8). Thus $A$, the fractional activation of AC, expressed as the % maximal activation ($d = V_{\text{max}}$), corrected for basal activity $a$, was obtained by the equation

$$A = 100 \cdot \frac{Y}{X}$$

Similarly, a normalized expression for receptor occupancy ($O = \%B_{\text{max}}$) was derived from the saturation-binding equation for a ligand $X$ in the presence of an inhibitor $I$ (23), by writing

$$O = 100 \cdot \frac{X}{K_d \left(1 + \frac{I}{K_i} + X\right)}$$

The parameters $b$, $K_d$, and $K_i$ were determined with the programs LIGAND and ALLFIT. Note that although the independent variable $X$, in the four-parameter logistic equation, represents the total ADH concentration ([ADH]$_t$) dose used to stimulate AC, in the normalized binding equation it represents free ADH concentration ([ADH]$_f$). Thus the equation relating $A$ to $O$ [i.e., $A = f(O)$; A-O plots] may not be derived by simple elimination of $X$ between the two equations. However, because the amount of receptor used in the AC stimulation studies was small, and given the concentrations of ADH used and the $K_d$ of 1.14 nM, the approximation $[ADH]_f \approx [ADH]_t$ was allowed. Indeed, as calculated from the binding isotherm, the ratio $[ADH]_f/[ADH]_t$ ranged from 0.9790 to 0.9997 (i.e., ~0.01); thus $[ADH]_f \approx [ADH]_t$.

When the ratio of $O$ to $A$ is plotted against the ratio of $X$ to $I$, the curves of the same graph, Fig. 8.

---

**Fig. 8.**
This work was supported in part by a Deutsche Forschungsgemeinschaft Grant (He 1472/3–1) and by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs, VA Merit Review Grant.

The technical help of C. Klein is greatly acknowledged.

Address for reprint requests and other correspondence: J. A. Durr, Mail Code (111), Medical Service, Div. of Nephrology, Bay Pines VA Medical Center, PO Box 5005, Bay Pines FL 33744.

References


