Differential inactivation of vasopressin receptor subtypes in isolated membranes and intact cells by *N*-ethylmaleimide

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Vasopressin receptors in plasma membranes and on cell monolayers were treated with sulfhydryl reagents. Specific binding of [^{3}H]AVP to renal V_2 receptors in membranes from bovine and porcine kidney and on LLC-PK$_1$ cells was markedly (80–90%) reduced after treatment with NEM but that to V_1 receptors on rat liver membranes and A7r5 smooth muscle cells only slightly (10–30%). Inactivation of receptors by NEM reduced the number of binding sites without altering the affinity of unmodified receptor molecules. High affinity ligands (agonists and antagonists), in complex with the V_2 receptor, protected against its inactivation. The results suggest that one or more cysteine residues are located in the ligand-binding site of the V_2 receptor, and are essential for hormone binding. Furthermore, it is possible to use NEM to differentiate between vasopressin isoreceptors.

Vasopressin; Vasopressin receptor; Sulfhydryl reagent; *N*-ethylmaleimide

1. INTRODUCTION

Two types of vasopressin receptor, with differing ligand specificity, second messenger system, and function, have so far been characterised. V_1 receptors, which are mainly found in vascular smooth muscle cells and in hepatocytes, play an important role in regulation of the vascular system and in carbohydrate metabolism. V_1 receptors function via the phospholipase C signal transfer system. V_2 receptors play a role in water resorption in the mammalian kidney collecting tubule and the amphibian urinary bladder. This process is mediated by cyclic AMP (for review see [1]).

Several recent observations suggest that sulfhydryl reagents (e.g. NEM, p-CMBS, IAA) may modify vasopressin-induced water permeability [2–4]. In these studies, the effect of these cysteine-modifying reagents on receptor-ligand interaction was not investigated. Other types of membrane receptor (e.g. opiate, acetylcholine, adrenergic and dopaminergic receptors) have been shown to have altered ligand binding properties after treatment with sulfhydryl reagents [5]. Binding studies with mutant receptors produced by expression of modified human β_2-adrenergic receptor genes showed that substitution of a number of conserved cysteine residues resulted in reduced affinity of the receptor for its ligand [6–7].

In this report we demonstrate the effect of NEM and other sulfhydryl reagents on the binding properties of V_1 and V_2 vasopressin receptor subtypes in isolated membranes and intact cells. V_1 receptors are relatively stable towards NEM whereas V_2 receptors are very sensitive both to NEM and to the other sulfhydryl reagents tested. The inactivation of the V_2 receptor, due to NEM, can be prevented by receptor-ligand complex formation prior to treatment with the sulfhydryl reagent.

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Abbreviations: AVP, [8-arginine]vasopressin; NEM, *N*-ethylmaleimide; IAA, iodoacetamide; p-CMBS, *p*-chloromercuribenzenesulfonic acid; Mca, 3-mercapto-3,3-cyclopentamethylene propionic acid; Asu, L-2-aminosuberic acid; EPPS, *N*-(2-hydroxyethyl)-piperazine-*N'*-(3-propanesulfonic acid)

2. MATERIALS AND METHODS

2.1. *Materials*

[^{3}H]AVP (53.6 Ci/mmol) was purchased from Du Pont NEN, Bad Homburg FRG; p-CMBS, NEM and IAA were from Sigma Chemie GmbH, Deisenhofen FRG, [Asu^{1,6},Arg^8]vasopressin was from Peninsula Laboratories, Belmont, CA, USA. The other peptides were produced by solid phase synthesis. All other chemicals were of analytical grade.

2.2 *Membrane preparations and receptor binding assays*

Bovine kidney inner medulla plasma membranes were prepared by a two-step centrifugation procedure [8]. The 10000 × *g* pellet resulting from this procedure had a specific binding capacity of approximately 1.3 pmol [^{3}H]AVP/mg protein. Porcine kidney membrane preparations obtained via a similar method [9] had a capacity of 0.55 pmol [^{3}H]AVP/mg protein. Rat liver plasma membranes, containing 0.65 pmol V_1 receptor/mg protein, were prepared using an aqueous two-phase polymer system [10]. Binding tests with plasma membranes were performed by incubating membranes (containing 100 μg of protein) in 100 μl of the corresponding binding buffer [8–10] containing 10 nM [^{3}H]AVP in presence (non-specific binding) or in the absence (total binding) of 1 μM AVP for 30 min at 30°C. To terminate the binding tests, the mixtures were diluted with 5 ml of ice-

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cold washing buffer [8–10], immediately filtered under vacuum through Whatman GF/F filters, and rinsed twice with 5 ml of ice-cold washing buffer. The filters were placed in counting vials with scintillation liquid and analysed by liquid scintillation spectrometry. Protein determination was performed by means of modified fluorescamine assay [11].

The effect of sulfhydryl reagents on specific binding was tested by performing the binding test after preincubation of the membranes (100 μg protein in 100 μl of binding buffer [8–10]) with sulfhydryl reagents, with or without subsequent removal of the sulfhydryl reagents by centrifugation prior to the binding test. In order to investigate receptor-ligand complex stability, bovine kidney membranes (100 μg protein in 100 μl of binding buffer: 50 mM EPPS, 5 mM $MgCl_2$, pH 8.2 [8]) were incubated with 10 nM [^{3}H]AVP for 30 min at 30°C and then incubated for a further 30 min in the presence or absence (control) of 1 mM NEM. For the ligand protection studies, bovine kidney membranes (100 μg protein in 100 μl binding buffer) were preincubated with saturating concentrations of the peptide ligand for 30 min at 30°C. After incubation with ligand, the membrane suspension was incubated with 1 mM NEM for a further 30 min. The membranes were then collected by centrifugation, and resuspended in an equal volume (100 μl) of 250 mM ammonium-acetate buffer, pH 5.5, to allow bound ligand to dissociate from the receptor. After 5 min at 30°C, these membranes were washed with binding buffer and binding tests were performed. For the receptor binding saturation assays, increasing concentrations of [^{3}H]AVP were used in the presence (non-specific binding) or in the absence (total binding) of an 100-fold excess of AVP.

The LLC-PK_1 porcine kidney epithelial, and A7r5 rat aortic smooth muscle cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum, 0.2 mg/ml steptomycin and 50 U/ml penicillin, and [^{3}H]AVP binding tests were performed on cell monolayers as previously described [12]. Cell monolayers were preincubated in the presence or absence (control) of 1 mM NEM for 30 min at 30°C, and after removal of the sulfhydryl reagent containing medium they were further incubated with 10 nM [^{3}H]AVP in the presence (non-specific binding) or in the absence (total binding) of 1 μM AVP in DMEM for 60 min at 4°C. (This incubation was performed at a lower temperature to prevent internalization of the receptors.)

All assays were performed in triplicate. The experiments were performed two times. Binding curves were fitted to a logistic function with a weighted iterative least-squares procedure based on the method of steepest descent [10]. The figures and tables show representative experiments. The standard deviation from the mean is indicated in the figures.

2.3 *Determination of the stability of [^{3}H]AVP by HPLC*

Membranes (containing 1 mg of protein) in 1 ml of binding assay buffer were incubated with 1 mM NEM and 10 nM [^{3}H]AVP for 30 min at 30°C. After incubation, the membranes were pelletted by centrifugation for 10 min at 10000 × *g*. The supernatant was then subjected to chromatography (Varian 5000 Liquid Chromatograph) on a Knauer LiChrosorb 10 RP18 HPLC column (250 × 4 mm) with a 20 ml linear gradient of 18% to 72% acetonitrile in 0.09% trifluoroacetic acid at a flow rate of 1 ml/min. The fractions were analysed for radioactivity in order to determine the retention time of the radioactive material. The retention time for unlabelled AVP (8.9 min) was determined separately under the same conditions, using absorbance at 220 nm to detect the eluting AVP.

3. RESULTS

3.1. *Effect of pretreatment with sulfhydryl reagents on the specific binding of [^{3}H]AVP to bovine V_2 receptors*

Preincubation of bovine kidney membrane with p-CMBS, NEM and IAA gives rise to concentration-dependent inactivation of the V_2 binding sites (Fig. 1).

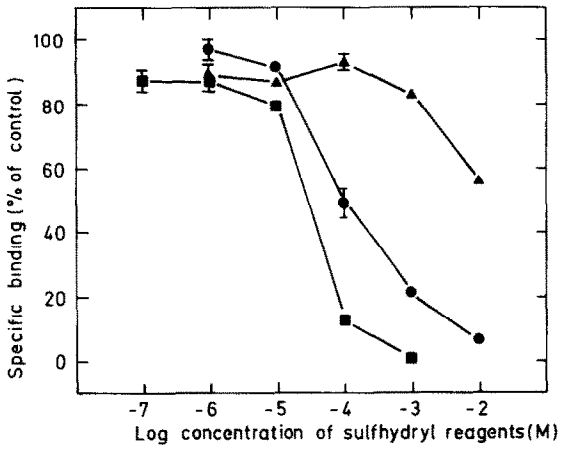

Fig. 1. Effect of sulfhydryl reagents on the specific binding of [^{3}H]AVP to the V_2 vasopressin receptor in bovine kidney membranes. Membranes were preincubated with increasing concentrations of IAA (▲), NEM (●) and p-CMBS (■) as described in section 2 before measuring [^{3}H]AVP binding capacity. Results are expressed as a percentage of the control (100% = 1.3 pmol/mg protein).

With all three reagents, a maximal, irreversible effect occurs within 30 min. The most effective inactivating agent is p-CMBS. Higher concentrations of p-CMBS, and also of NEM, lead to total inactivation of the receptor. IAA, as a less potent sulfhydryl reagent, decreases specific binding to a lesser extent, even at higher concentrations. All experiments shown here were performed in buffer at pH 8.2, the optimal pH for the receptor-ligand interaction. The experiments were also performed at pH 7.0, the optimum pH for sulfhydryl group alkylation by the reagents [5]. The results (data not shown), were similar to those obtained at pH 8.2. When membranes were concurrently treated with [^{3}H]AVP and NEM and then extracted, all of the radioactivity recovered had the same retention time as unlabelled AVP, on HPLC, excluding the possibility that the radioactive AVP is chemically modified after incubation with NEM.

Treatment of bovine renal membranes with 1 mM NEM causes a 79.1% reduction in vasopressin binding sites (Fig. 2). The dose-dependent binding experiments demonstrate that alkylation of sulfhydryl groups completely abolishes binding to those V_2 receptors that are modified. The affinity of the remaining bovine kidney V_2 receptors for AVP in NEM-treated membranes is unchanged. The remaining vasopressin binding sites appear to be homogenous, as the dose-dependent binding curves generated linear Scatchard plots (results not shown). The difference between the equilibrium dissociation constants of the untreated V_2 receptors ($K_d = 0.98 \pm 0.19$ nM) and the receptors treated by NEM ($K_d = 1.35 \pm 0.21$) is not significant.

3.2. *Effect of preincubation with various ligands on NEM-induced V_2 receptor inactivation*

The receptor-ligand complexes in bovine kidney

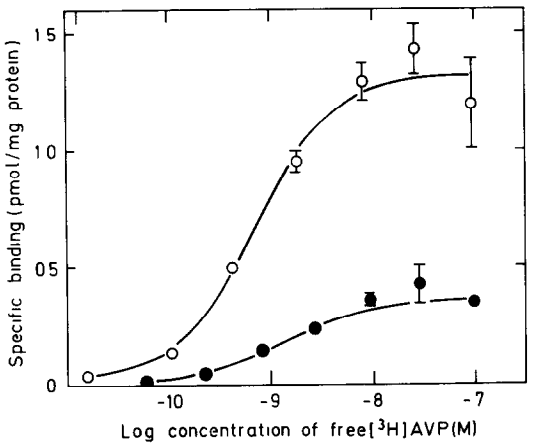

Fig. 2. Effect of NEM on the dose-dependent binding of [^{3}H]AVP to the bovine renal V_2 vasopressin receptor. The results show the concentration of specific [^{3}H]AVP binding sites of the untreated membranes (○) and of membranes after treatment with 1 mM NEM (●). The reaction of membranes with 1 mM NEM and binding of [^{3}H]AVP was performed as described in section 2.

membranes, formed by preincubation with 10 nM [^{3}H]AVP, do not dissociate after 30 min treatment with 1 mM NEM, 105.8 ± 14.6% of the receptor binding sites being occupied with respect to the untreated control. Pretreatment of the bovine kidney membranes with agonists (ligands 1–3, Table I), or antagonists (ligands 4 and 5 [13], in Table I) at concentrations which correspond to approximately 10 times their K_d-values virtually completely protects the V_2 receptor from NEM-induced inactivation (Table I).

3.3. *Comparison of NEM-treated V_1 and V_2 vasopressin receptors in isolated membranes and in established cell lines*

The V_2 receptor of porcine kidney membranes is inactivated by pretreatment with 1 mM NEM to a similar extent as the V_2 receptor of bovine membranes. The rat liver V_1 receptor, on the other hand, is relatively stable to pretreatment with 1 mM NEM (Table II). In all cases, treatment with NEM results in a reduced number of binding sites but does not affect the affinity for AVP (K_d values not shown) of the remaining binding sites. The sensitivity of the V_1· receptor of A7r5 rat smooth muscle cells and of the V_2 receptor of LLC-PK$_1$ porcine kidney epithelial cells to NEM treatment correlates with the results found in membrane preparations. Generally, V_2 receptors are inactivated to a much greater extent (approximately 10–20% of binding activity remaining) than V_1 receptors (approximately 70–90% of binding activity is remaining, Table II).

4. DISCUSSION

We have studied the influence of several sulfhydryl group-alkylating agents, on V_1 and V_2 receptors in intact cells and in isolated membranes investigating the effect of NEM in greater detail. We found that NEM, p-CMBS and IAA inactivate V_2 binding sites in a dose-dependent manner in bovine kidney membranes. This inactivation by NEM markedly reduces the number of binding sites without altering the affinity of unmodified receptor molecules. Our results suggest that this effect is due to a direct reaction of NEM with V_2 receptor

Table I

Protection against NEM-induced inactivation of bovine V_2 membrane receptors by pretreatment with vasopressin agonists and antagonists (control: binding capacity without NEM treatment)

Ligand	Concentration (nM)	Receptor binding remaining after preincubation with ligands (% of control)
No ligand		17 ± 4
1 [Arg^8]vasopressin	10	95 ± 2
2 [1-deamino,D-Arg^8]vasopressin	30	91 ± 4
3 [Asu^{1,6},Arg^{8}vasopressin	30	97 ± 2
4 desH$_2$NGly^9[Mca^1,D-Ile^2,Ile^4,Arg^8]vasopressin	100	96 ± 6
5 [Mca^1,D-Phe^2,Ile^4,Arg^8,Lys^9]vasopressin	100	101 ± 5

Table II

Effect of pretreatment with 1 mM NEM on the binding of [^{3}H]AVP to V_2 and V_1 vasopressin isoreceptors (control: binding capacity without NEM pretreatment)

Receptor subtype	Binding capacity (fmol/mg protein)		Receptor binding remaining after treatment with NEM (% of control)
	Control	1 mM NEM pretreatment	
V_2 (bovine kidney membranes)	1312 ± 16	274 ± 4	20.9
V_2 (pig kidney membranes)	549 ± 9	50 ± 12	9.1
V_2 (LLC-PK$_1$ cells)	204 ± 7	33 ± 5	16.2
V_1 (rat liver membranes)	653 ± 8	570 ± 10	87.3
V_1 (A7r5 cells)	148 ± 5	107 ± 7	72.3

molecules and not with G proteins, since intact G proteins are necessary for the receptor conformation with highest binding affinity. GTP and non-hydrolysable analogues, via G proteins, reduce the ligand affinity of bovine kidney membrane V_2 binding sites by a factor of 2–3 [14]. Other V_2 receptors (in porcine kidney membranes and in LLC-PK_1 cells) are similarly inactivated by NEM. Our results show that is is possible to use NEM to differentiate between vasopressin isoreceptors.

The different reactivities of V_1 and V_2 receptors may be explained in terms of difference in primary structures. The V_2 receptor molecules could have one or more exposed cysteine residues which should be intact for the binding activity. Our data suggest that sulfhydryl groups are located in, or close to, the ligand-binding domain of the V_2 receptor and can be protected by preincubation with vasopressin agonists or antagonists. Another possible explanation of the results of the ligand protection experiments is that the binding of a specific ligand to the V_2 receptor can change the receptor conformation in such a way that critical sulfhydryl groups become less accessible to NEM. The protection against inactivation with the carba analogue [$Asu^{1,6}$,Arg^8]vasopressin lacking a disulfide moiety suggests that protection by vasopressin analogues is not through a direct chemical reaction of the analogues' sulfhydryl groups with a sulfhydryl group of the receptor molecule.

Sulfhydryl group-alkylating agents have been used to investigate vasopressin induced water channels. The direct effects of NEM, p-CMBS, $HgCl_2$, IAA and other reagents on the water pathways in the luminal membrane of kidney proximal tubule [15] and in the luminal membrane of the frog urinary bladder [2–4] are well established. Less attention was paid, in these studies, to the direct effect of the sulfhydryl-alkylating agents on the vasopressin receptors. In an earlier study it was shown that NEM added to the serosal solution of the isolated toad bladder in concentrations greater than 1 mM completely inhibited the effect of vasopressin [16]. Our results suggest that this effect could be explained by a direct inactivation of vasopressin receptor molecules.

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