

# Vasopressin $V_2$ -Receptor Mobile Fraction and Ligand-dependent Adenylate Cyclase Activity Are Directly Correlated in LLC-PK<sub>1</sub> Renal Epithelial Cells

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**Abstract.** The role of hormone receptor lateral mobility in signal transduction was studied using a cellular system in which the receptor mobile fraction could be reversibly modulated to largely varying extents. The G-protein-coupled vasopressin  $V_2$ -type receptor was labeled in LLC-PK<sub>1</sub> renal epithelial cells using a fluorescent analogue of vasopressin, and receptor lateral mobility measured using fluorescence microphotolysis (fluorescence photobleaching recovery). The receptor mobile fraction ( $f$ ) was  $\sim 0.9$  at  $37^\circ\text{C}$  and  $<0.1$  at  $10^\circ\text{C}$ , in accordance with previous studies. When cells were incubated for 1 h at  $4^\circ\text{C}$  without hormone, and then warmed up to  $37^\circ\text{C}$  and labeled with the vasopressin analogue,  $f$  increased from  $\sim 0.4$  to  $0.8$  over  $\sim 1$  h. The apparent lateral diffusion coefficient was not markedly affected by temperature pretreatment.

Studies with radiolabeled vasopressin indicated that temperature pretreatment influenced neither receptor number nor binding/internalization kinetics. F-actin staining revealed that temperature change resulted in reversible changes of cytoskeletal structure. The maximal rate of in vivo cAMP production at  $37^\circ\text{C}$  in response to vasopressin, but not to forskolin (receptor-independent agonist), was also markedly influenced by preincubation of cells at  $4^\circ\text{C}$ , thus paralleling the effects of temperature preincubation on  $f$ . A linear correlation between  $f$  and maximal cAMP production was observed, suggesting that the receptor mobile fraction is a key parameter in hormone signal transduction in vivo. We conclude that mobile receptors are required to activate G-proteins, and discuss the implications of this for signal transduction mechanisms.

VARIOUS models have been proposed for the activation of adenylate cyclase (AC)<sup>1</sup> by hormones binding to specific membrane integral receptors on the external surface of the plasma membrane lipid bilayer (Cuatrecasas, 1974; Kahn, 1976; Tolkovsky and Levitzki, 1978). All models must account for the mediating role of the GTP-binding (G-) protein complexes, which are responsible for modulating AC activity through their stimulating (G<sub>s</sub>-protein) or inhibiting (G<sub>i</sub>-protein) action. The fact that the G<sub>sa</sub> polypeptide component appears capable of redistribution from the membrane, where it is probably anchored by the  $\beta\gamma$ -complex, to the cytosolic fraction upon agonist binding to receptor (Stryer and Bourne, 1986; Lynch et al., 1986; McArdle et al., 1988; Ransnäs et al., 1988; Ransnäs and Insel, 1989) implies that the rate-limiting steps in signal transduction are likely to be the protein-protein interactions occurring within the plasma membrane lipid bilayer fraction (Chabre, 1987; Peters, 1988). The collision coupling or "mobile receptor" hypothesis (Cuatrecasas, 1974; Kahn, 1976; De Meyts et al.,

1976; Tolkovsky et al., 1978) and variations thereof (e.g., Swillens and Dumont, 1980; Swillens, 1982; Sobolev et al., 1988) propose that these interactions occur through transient collisionary contacts between protein components as the result of lateral diffusion of receptors and G-proteins within the plasma membrane. This is presumably then sufficient to activate the G-protein complex via liberation of G<sub>sa</sub> into the aqueous phase.

We have reported direct lateral mobility measurements, using the technique of fluorescence microphotolysis (fluorescence photobleaching recovery or FRAP), for the G-protein-coupled vasopressin  $V_1$ - and  $V_2$ -type receptors (Jans et al., 1989, 1990a-c). The  $V_2$ -receptor of LLC-PK<sub>1</sub> renal epithelial cells shows a marked temperature dependence of receptor-mobile fraction ( $f$ ), whereby  $f$  is highest (almost 1.0) at physiological temperature, and lowest ( $<0.1$ ) at  $10^\circ\text{C}$  (Jans et al., 1989). We have also observed (Jans et al., 1990c) that prolonged treatment with the acidotrophic agent ammonium chloride results in almost total immobilization of the  $V_2$ -receptor ( $f = 0.2$ ) concomitant with changes in the actin cytoskeleton.

The present study furthers the above observations, showing in particular that receptor immobilization by a low-temperature pretreatment is reversible. This effect, which was

1. *Abbreviations used in this paper:* AC, adenylate cyclase; AVP, arginine vasopressin; IBMX, isobutylmethylxanthine; R-PH, rhodamine-labeled phalloidin; TR-LVP, 1-deamino[8-llysine( $N^6$  tetramethylrhodamylaminothiocarbonyl) vasopressin.

accompanied by reversible changes within the cytoskeleton as revealed by F-actin staining, provided a unique opportunity to investigate the role of  $f$  in signal transduction. A linear correlation was observed between  $f$  and maximal cAMP production in response to AVP, providing strong evidence for a mechanistic role for  $V_2$ -receptor lateral mobility in AC activation *in vivo*.

## Materials and Methods

Materials were as described previously (Jans et al., 1989, 1990a,c). The rhodamine-labeled analogue of vasopressin, 1-deamino [8-lysine ( $N^6$ -tetra-methylrhodamylaminothiocarbonyl)] vasopressin (TR-LVP) was prepared as described (Jans et al., 1989). Cells of the LLC-PK<sub>1</sub> pig kidney epithelial cell line (Hull et al., 1976) were cultured in DMEM supplemented with 10% (vol/vol) fetal calf serum, 0.2 mg/ml streptomycin, and 50 U/ml penicillin (Jans et al., 1986, 1989).

### Vasopressin Binding

Vasopressin binding was measured on whole cell monolayers using tritium-labeled [ $^3$ H]vasopressin (AVP) as described (Jans et al., 1989). Internalized hormone was determined using a 3-min treatment with 200 mM Gly-HCl, pH 3, 200 mM NaCl subsequent to the binding incubation (Jans et al., 1989, 1990a).

### In Vivo cAMP Production

Cell monolayers were grown in 12-well microtiter plates to 90–95% confluence. With or without prior pretreatment either at 4°C (1 h) or 4°C (1 h) followed by 37°C (1 h) in serum-free DMEM containing 2 mg/ml BSA, or with prior pretreatment at 37°C for 2 d with 10 mM NH<sub>4</sub>Cl in normal medium (Jans et al., 1990c), cells were treated with agonists in the presence of 500  $\mu$ M isobutylmethylxanthine (IBMX) (in the absence of NH<sub>4</sub>Cl) in serum-free DMEM/BSA, and the incubation stopped by washing at 4°C with NaCl/Pi, and the addition of boiling 2 mM IBMX/0.05 M Na<sup>+</sup> acetate, pH 6.2 (Steinberg et al., 1979; Jans et al., 1987). Extracts were then boiled for 3 min, and kept at -20°C before the determination of cAMP using the competitive protein binding assay of Tovey et al. (1974), and of protein concentration, using the dye binding assay of Bradford (1976) with BSA (fatty-acid-free) as standard.

### Fluorescence Measurements

Cells to be used for fluorescence and lateral diffusion measurements were grown on coverslips (15 × 15 mm) for 3–4 d to ~50% confluence (Jans et al., 1989). After incubation with ligand, cells were washed with NaCl/Pi (containing 0.5 mg/ml BSA), and mounted in the incubation medium in the absence of ligand. The methods used in measurements of fluorescence intensity and the fluorescence microphotolysis apparatus used have been described previously in detail (Peters, 1986) and in particular for LLC-PK<sub>1</sub> cells (Jans et al., 1989, 1990b,c).

### F-actin Staining

Cells were grown on coverslips, and fixed with 4% *p*-formaldehyde in NaCl/Pi subsequent to various pretreatments. Cells were then stained with 1  $\mu$ g/ml rhodamine-labeled phalloidin (R-PH) as described (Wulf et al., 1979; Jans et al., 1990c). Staining could be completely competed with 50  $\mu$ g/ml nonlabeled phalloidin (Jans et al., 1990c).

## Results

### $V_2$ -receptor Lateral Mobility

We were interested in further investigating the marked temperature dependence of the  $V_2$ -receptor mobile fraction ( $f$ ), and in particular the low  $f$  (~0.1) measured at 10°C (Jans et al., 1989). As previously, the technique of fluorescence microphotolysis (photobleaching) (Axelrod et al., 1976; Peters,

1986), together with the rhodamine-labeled vasopressin analogue TR-LVP (Buku et al., 1985; Jans et al., 1989), was used, with fluorescence monitored at 20-s intervals, rather than in the usual continuous fashion, to avoid (unintentional) bleaching during the course of the measurements. A series of measurements under identical conditions were performed for photobleaching recovery in LLC-PK<sub>1</sub> cells subsequent to treatment with 10<sup>-7</sup> M TR-LVP in the absence (total binding) or presence (nonspecific binding) of 10<sup>-5</sup> M AVP. Measurements for each time interval were pooled and averaged. Subtraction of the measurements for nonspecifically bound from those for total bound fluorescence enabled estimation of the recovery of specifically bound fluorescence after photobleaching. Experimental data were then fitted by theoretical curves (Soumpasis, 1983) for two components: a mobile fraction ( $f$ ) with apparent lateral diffusion coefficient ( $D$ ) and an immobile fraction ( $D < 10^{-12}$  cm<sup>2</sup>/s) (Axelrod et al., 1976).

We initially tested whether the low  $V_2$ -receptor mobile fraction previously determined at 10°C was due to the fact that the incubation with ligand was performed at 4°C (1 h) before the measurements (Jans et al., 1989). It seemed possible that low temperature itself may induce receptor immobilization. To address this question, cells were incubated at 37°C with ligand (10 min), and then washed and lateral mobility measurements performed at 10°C. The mobile fraction was 0.23 (Table I) indicating that the  $V_2$ -receptor is largely immobile at 10°C. This value is significantly higher than that for 4°C-preincubated cells, however ( $f$  of ~0.1) (Jans et al., 1989), implying that treatment at 4°C may have an immobilizing effect on the  $V_2$ -receptor. This observation was further examined by pretreating cells for 1 h either at 37°C or 4°C in the absence of ligand, prior to incubation with ligand, washing and measurement of  $V_2$ -receptor lateral mobility, all at 37°C (Table I, Fig. 1). Interestingly, a marked reduction of  $V_2$ -receptor lateral mobility was evident in the 4°C-pre-

Table I. Lateral Mobility of the  $V_2$ -Receptor in the Basal Plasma Membrane of LLC-PK<sub>1</sub> Cells

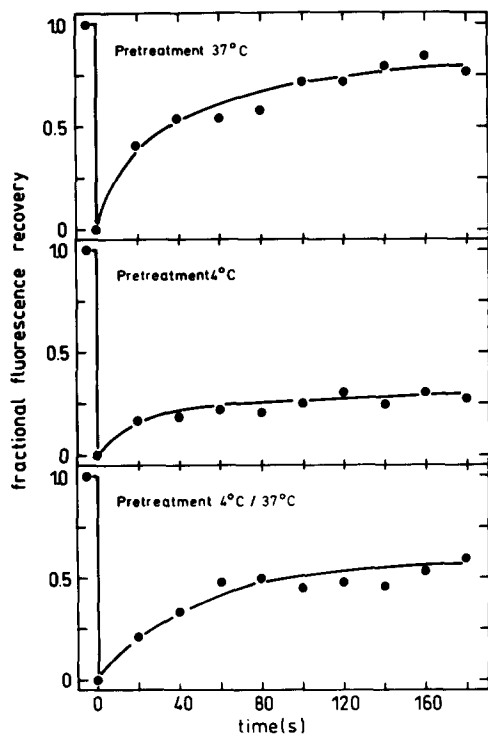
Pretreatment (without ligand)*	Parameter of mobility‡		
	$D$	$f$	$n$
	10 <sup>-10</sup> cm <sup>2</sup> /s		
A) Measurements performed at 10°C (10–30 min)§			
37°C	UD¶	0.23 ± 0.01	3
B) Measurements performed at 37°C (10–30 min)§			
1) 37°C	2.70 ± 0.12	0.94 ± 0.02	27
2) 4°C	2.19 ± 0.21	0.42 ± 0.02	10
3) 4°C/37°C	2.37 ± 0.17	0.78 ± 0.04	10
C) Measurements performed at 37°C (30–60 min)§			
i) 37°C	3.01 ± 0.45	0.72 ± 0.05	8
ii) 4°C	2.64 ± 0.17	0.62 ± 0.03	11

\* Pretreatment involved incubation at the indicated temperature in serum-free DMEM (without ligand) for 1 h. Treatment B3 involved 1 h at 4°C followed by 1 h at 37°C.

‡ Values for the apparent lateral diffusion coefficient ( $D$ ) and mobile fraction ( $f$ ) represent the mean ± SEM, where  $n$  is the number of experiments. Each experiment comprises at least five separate measurements on different cells for total and nonspecifically bound fluorescence (see text and legend to Fig. 1).

§ In all cases, cells were washed at 37°C, subsequent to the appropriate pretreatment, and then incubated with ligand for 10 min at 37°C, washed at 37°C, and mounted in serum-free, ligand-free DMEM. Measurements were then performed at the temperatures and for the times after ligand addition, as indicated.

¶ Unable to be determined.



**Figure 1.** Lateral mobility of the  $V_2$ -receptor in the basal plasma membranes of LLC-PK<sub>1</sub> cells with or without pretreatment at 4°C. Pretreatments were for 1 h at 4°C or 37°C or at 4°C followed by 37°C (1 h) without ligand in serum-free DMEM. Photobleaching measurements were subsequently performed on cells incubated with ligand for 10 min at 37°C, washed, and mounted in serum-free medium at 37°C. The curves represent the least square best fits to the experimental data for specific fluorescence, calculated from at least five individual measurements for each of total and nonspecifically bound fluorescence (see Results).

treated cells, whereby both  $D$  ( $2.2 \times 10^{-10}$  cm<sup>2</sup>/s compared with  $2.7 \times 10^{-10}$  cm<sup>2</sup>/s for 37°C-pretreated cells) and in particular  $f$  (0.42 compared with 0.94 for 37°C-pretreated cells) were reduced (Table I, B2; Fig. 1). This effect of 4°C pretreatment appeared to be transient in that longer incubation at 37°C resulted in an increase in  $f$  (0.62) and  $D$  ( $2.6 \times 10^{-10}$  cm<sup>2</sup>/s) (Table I, Cii), values almost comparable to those of 37°C-pretreated cells (Table I, Ci). As shown previously (Jans et al., 1990b) internalization is associated with  $V_2$ -receptor immobilization with time at 37°C ( $f = 0.72$ , Table I, Ci) where internalized ligand accounts for ~40% of total bound hormone after ~45 min at 37°C (Jans et al., 1989). The reduction of  $f$  by 4°C pretreatment could be largely reversed by a 1-h treatment at 37°C subsequent to the 4°C treatment (Table I, B3; Fig. 1). In this case the values for  $D$  and  $f$  were intermediate between those for exclusively 4°C pretreated or 37°C pretreated cells. It thus appeared that pretreatment of LLC-PK<sub>1</sub> cells at 4°C effected a reduction in  $V_2$ -receptor lateral mobility, which could be reversed by subsequent incubation at 37°C.

Some experiments (not shown), performed using longer incubation times at 4°C, indicated that 4°C pretreatment for up to 3 h had essentially the same maximal effect (60% reduction of  $f$ ). The reversibility of this effect by subsequent 37°C pretreatment, however, seemed to be influenced by the

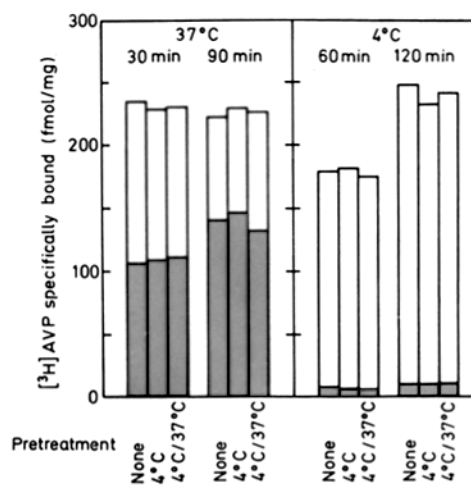
duration of 4°C pretreatment. Longer times at 37°C were required in the case of cells treated for longer at 4°C to bring about reversal of the "immobile" phenotype, whereas 30 min 4°C pretreatment reduced  $f$  to a somewhat lesser extent ( $f \sim 0.6$ ). The time-consuming nature of the diffusion measurements (25–30 min for a series of five to six measurements for a single experimental condition) prevented closer examination of the kinetics of this phenomenon.

### [<sup>3</sup>H]AVP Binding

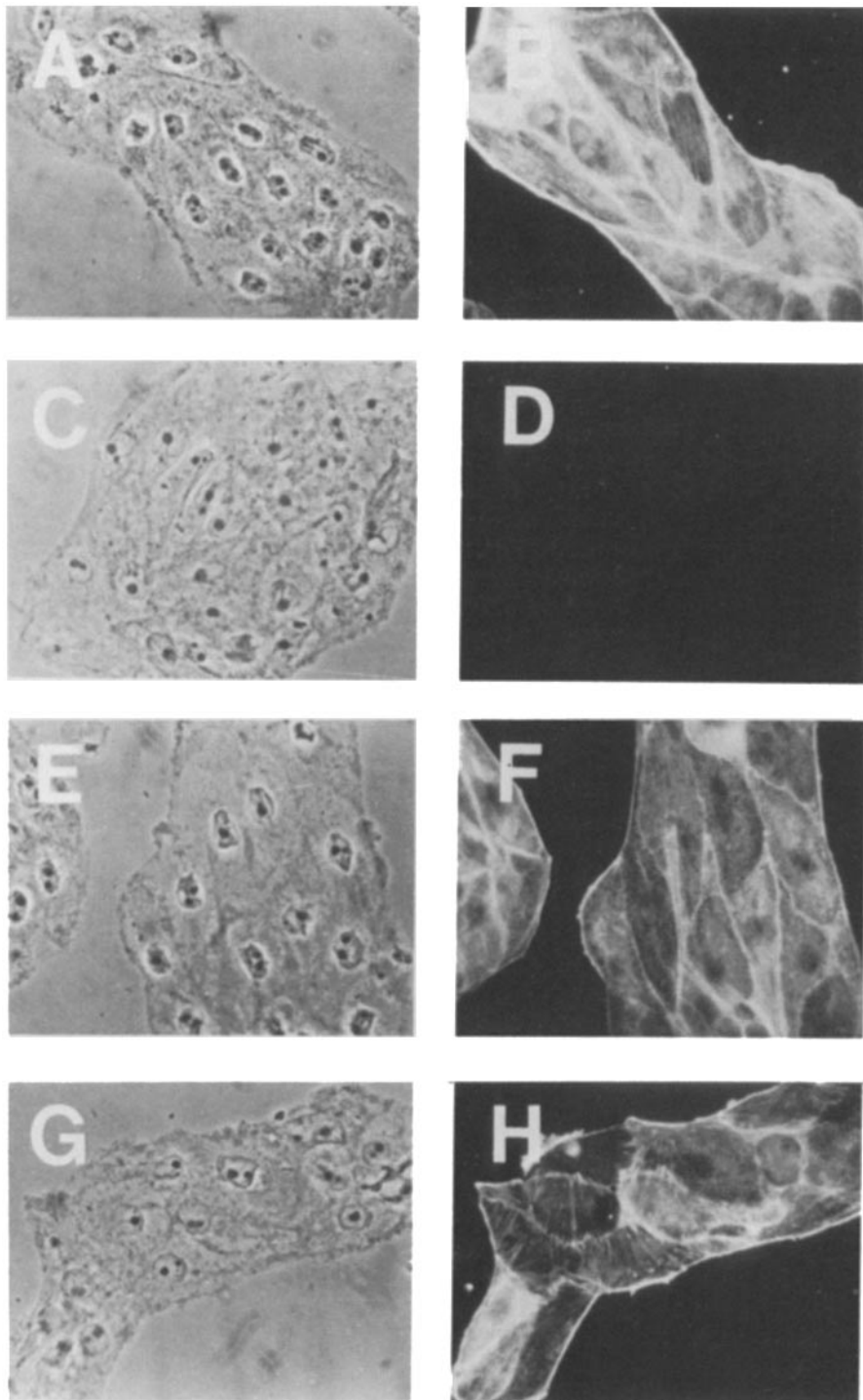
To ensure that the temperature effects on  $V_2$ -receptor lateral mobility did not result from altered ligand binding properties, maximal specific [<sup>3</sup>H]AVP binding and internalization at both 4°C and 37°C were measured on cells pretreated for 1 h at 4°C or 37°C, or 4°C followed by 37°C as above. No differences were observed in maximal binding or internalization at either temperature for cells with or without 4°C or 4°C/37°C pretreatment over the time intervals used in the lateral diffusion measurements (Fig. 2), and no differences were detected in the binding affinity for AVP (results not shown). The effects of 4°C pretreatment on  $V_2$ -receptor lateral mobility were accordingly not the result of altered ligand binding or internalization.

### F-actin Staining

A discernible effect of the 4°C pretreatment of LLC-PK<sub>1</sub> cells was a morphological change, whereby cells became rounder and cell-cell contact regions more distinct. We investigated this in more detail by staining the actin cytoskeleton of non- and pretreated cells with R-PH. Cells were fixed with *p*-formaldehyde subsequent to the temperature pretreat-



**Figure 2.** Specific [<sup>3</sup>H]AVP binding and internalization by LLC-PK<sub>1</sub> cells with or without temperature pretreatments. Cell monolayers in 12-well plates (Costar Corp., Cambridge, MA) were pretreated as in Fig. 1, and then washed and incubated with  $10^{-8}$  M [<sup>3</sup>H]AVP in the absence or presence (nonspecific) of  $10^{-6}$  M AVP at the indicated temperature for the indicated times. Duplicate cultures were then analyzed for total binding (□) or internalized ligand (■), determined as described in Materials and Methods, using a pH3 treatment (Jans et al., 1989, 1990a). Results represent the means from a single typical experiment performed in triplicate (SEM < 12% the value of the mean).



**Figure 3.** Visualization of the actin cytoskeleton of LLC-PK<sub>1</sub> cells subsequent to various pretreatments. Cells were grown on coverslips and pretreated as follows, before fixation and staining with 1  $\mu\text{g}/\text{ml}$  R-PH (see Materials and Methods) (Wulf et al., 1979; Jans et al., 1990c). *A–D*, untreated; *E* and *F*, 4°C (1 h); *G* and *H*, 10 mM NH<sub>4</sub>Cl (2 d). Cells were photographed with a 40 $\times$  water immersion objective under normal (*A*, *C*, *E*, *G*) or fluorescent (*B*, *D*, *F*, *H*) illumination. In *C* and *D*, cells were incubated with 1  $\mu\text{g}/\text{ml}$  R-PH in the presence of 50  $\mu\text{g}/\text{ml}$  unlabeled phalloidin.

ments described above, stained with R-PH at room temperature (30 min), washed and photographed using fluorescence microscopy (Fig. 3). LLC-PK<sub>1</sub> cells revealed the typical actin filament or stress fiber staining previously observed (Jans et al., 1990c) (Fig. 3 *B*). Interestingly, 4°C-pretreated cells exhibited a quite different staining pattern, in that fibers were markedly reduced in both prevalence and distinctness (Fig. 3 *F*) resulting in a cloudy or fuzzy appearance. Treatments as short as 15 min at 4°C (not shown) were sufficient to bring about an unequivocal declarification of actin microfilament

structure as visualized with R-PH here. This effect could be almost completely reversed in a time-dependent manner by subsequent treatment at 37°C before actin staining (not shown), in similar fashion to the effect on *f* above. Specificity of the staining for the actin cytoskeleton was demonstrated by almost total competition of the labeling by 50-fold higher concentrations of unlabeled R-PH (Fig. 3 *D*). It could accordingly be concluded that the treatment of cells at 4°C led to alterations in the actin cytoskeleton, presumably as a result of the inhibition of actin polymerization (Howard and

Oresajo, 1985). 4°C treatment thus appeared to concomitantly affect both V<sub>2</sub>-receptor lateral mobility and the actin cytoskeleton of LLC-PK<sub>1</sub> cells, thereby resembling prolonged treatment of LLC-PK<sub>1</sub> cells with 10 mM NH<sub>4</sub>Cl (Jans et al., 1990c). The latter treatment also reduces *f* (to 0.2) and concomitantly alters the actin cytoskeleton, such that stress fibers are more pronounced and thicker than in untreated cells (shown for comparison in Fig. 3 H). These results show that alteration of cytoskeletal structure, either through primary or secondary effects, can strongly influence lateral mobility of the V<sub>2</sub>-receptor.

### *In Vivo* cAMP Production in LLC-PK<sub>1</sub> Cells Pretreated to Reduce V<sub>2</sub>-Receptor Mobile Fraction

That treatments could be used in the absence of ligand to effect different V<sub>2</sub>-receptor *f* values at 37°C presented a unique possibility to test for the role of *f* in signal transduction in vivo. Cells were pretreated as above at 37°C, 4°C, 4°C then 37°C, or 10 mM NH<sub>4</sub>Cl (2 d), before washing at 37°C, and treatment without or with different concentrations of either AVP or forskolin (nonreceptor-mediated AC activator) in the presence of IBMX. The latter amplifies cAMP production through inhibiting phosphodiesterase activity. Representative results are presented in Fig. 4, and summa-

rized in Table II and Fig. 5. A quite striking result was obtained for AVP-mediated stimulation of cAMP production in that, without exception, the maximal rate of cAMP production positively correlated with V<sub>2</sub>-receptor mobile fraction (Fig. 4, Table II; see Fig. 5). The treatments reducing *f* resulted in lower maximal rates of cAMP production, with the effects being more pronounced at AVP concentrations below the K<sub>D</sub> of ligand binding ( $3.2 \times 10^{-9}$  M; Jans et al., 1986), in terms of the percent reduction compared with untreated cells. For example, the maximal cAMP responses of 4°C-pretreated cells to 10<sup>-7</sup>, 10<sup>-9</sup>, and 10<sup>-10</sup> M AVP were 20, 31, and 62% reduced respectively, compared with untreated cells (Fig. 4 a; Table II). The AVP concentrations inducing half-maximal response ( $\sim 1.5 \times 10^{-9}$  M) were the same in all cases (not shown). In contrast, forskolin-induced (nonreceptor mediated) cAMP production was unaffected by the various treatments, with the possible exception of NH<sub>4</sub>Cl treatment (Fig. 4 b, Table II), implying that the effects of the treatments on AVP-induced cAMP production were not attributable to effects on AC itself. It was concluded that the reduced AVP-stimulated AC activation was the direct result of reduction of *f* induced by the various temperature and NH<sub>4</sub>Cl pretreatments. The direct correlation between the V<sub>max</sub> of AVP-stimulated cAMP production at the three AVP concentrations tested is shown in Fig. 5. The linear re-

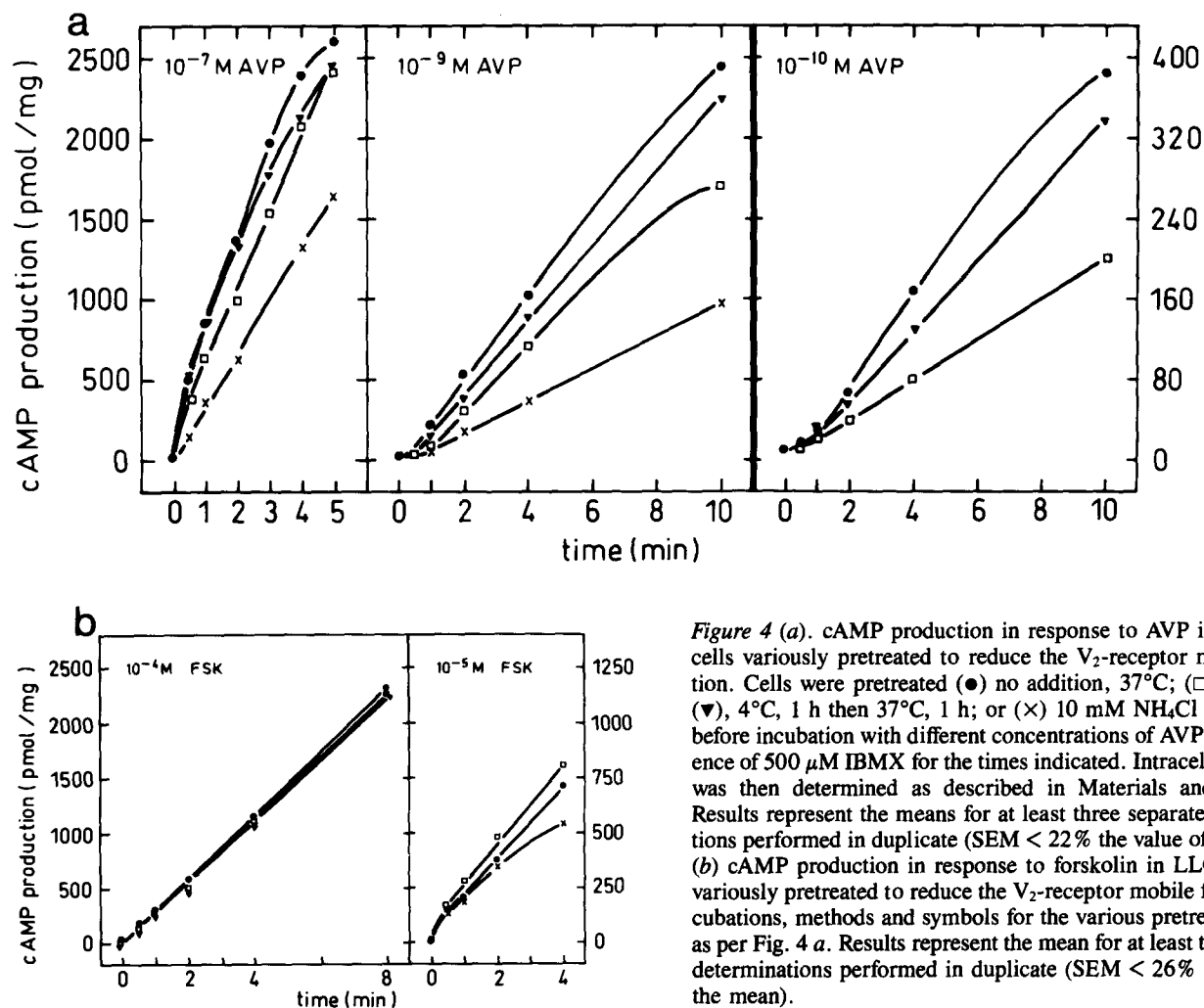


Figure 4 (a). cAMP production in response to AVP in LLC-PK<sub>1</sub> cells variously pretreated to reduce the V<sub>2</sub>-receptor mobile fraction. Cells were pretreated (●) no addition, 37°C; (□) 4°C, 1 h; (▼) 4°C, 1 h then 37°C, 1 h; or (×) 10 mM NH<sub>4</sub>Cl (37°C, 2 d) before incubation with different concentrations of AVP in the presence of 500 μM IBMX for the times indicated. Intracellular cAMP was then determined as described in Materials and Methods. Results represent the means for at least three separate determinations performed in duplicate (SEM < 22% the value of the mean). (b) cAMP production in response to forskolin in LLC-PK<sub>1</sub> cells variously pretreated to reduce the V<sub>2</sub>-receptor mobile fraction. Incubations, methods and symbols for the various pretreatments are as per Fig. 4 a. Results represent the mean for at least two separate determinations performed in duplicate (SEM < 26% the value of the mean).

**Table II. Maximal Velocities of cAMP Production In Vivo in Response to AVP or Forskolin in LLC-PK<sub>1</sub> Cells Subsequent to Various Pretreatments Reducing V<sub>2</sub>-Receptor Mobile Fraction**

Pretreatment <sup>§</sup>	f <sup>§</sup>	Maximal velocity*				
		Treatment <sup>‡</sup>				
		AVP		Forskolin		
		10 <sup>-7</sup> M	10 <sup>-9</sup> M	10 <sup>-10</sup> M	10 <sup>-4</sup> M	10 <sup>-5</sup> M
		pmol/mg per min				
37°C	0.94	585	290	48.4	285	170
4°C/37°C	0.78	535	240	36.2	280	ND
4°C	0.42	470	200	18.5	285	188
10 mM NH <sub>4</sub> Cl (2 d)	0.21	330	95	ND	ND	148

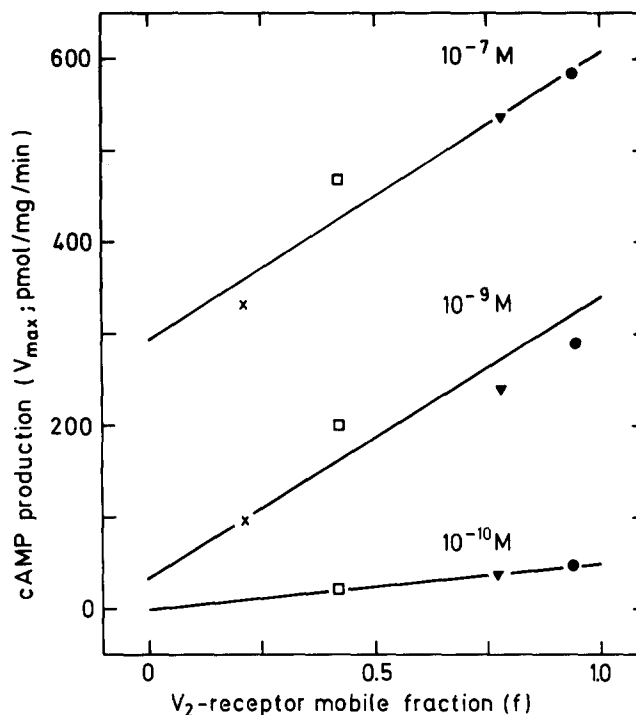
\* Calculated from Fig. 4, *a* and *b*, and other data. Results represent the average of at least two separate determinations performed in duplicate, for which the SD was <28% the value of the mean.

‡ Treatments with AVP or forskolin were in the presence of 500 μM IBMX.  
<sup>§</sup> Pretreatments were as per Table I, except that incubation with 10 mM NH<sub>4</sub>Cl was for 2 d (Jans et al., 1990c). The *f* values for V<sub>2</sub>-receptor mobile fraction are from Table I and Jans et al. (1990c).

gression was almost 1.0 for all three cases, implying the direct dependence of V<sub>max</sub> on *f*, and arguing for a mechanistic role of *f* in AVP-mediated signal transduction.

## Discussion

This study represents the first direct investigation of the relationship between receptor lateral diffusion and signal transduction in vivo. The perhaps surprising observation that treatment of LLC-PK<sub>1</sub> cells without ligand at 4°C reversibly immobilizes V<sub>2</sub>-receptor provided the means to test for the role of receptor lateral mobility, and, in particular, of the receptor mobile fraction, in stimulation of cAMP production by AC. Briefly, cells could be pretreated to reduce *f* (as measured at 37°C), and then treated with hormone or forskolin at 37°C to examine activation kinetics. Importantly, forskolin (nonreceptor mediated) response was unaffected, indicating no influence of the pretreatments on AC itself. AVP responses were markedly reduced in contrast, in terms of the maximal rates of cAMP production. The extent of response to AVP correlated directly with the magnitude of *f*, the effects of reducing *f* being more pronounced in terms of cAMP response at lower (sub-K<sub>D</sub>) AVP concentrations. This close correlation of *f* with AVP-stimulated AC response (see Fig. 5) suggests a possible role for V<sub>2</sub>-receptor lateral mobility in AVP mediated AC activation, in support of previous data from this laboratory (Jans et al., 1989, 1990c) and of the hypothesis that the process of interaction between receptor-hormone complex, G-proteins, and the AC catalytic subunit are diffusion controlled (Cuatrecasas, 1974; De Meyts et al., 1976; Kahn, 1976; Bergman and Hechter, 1978; Tolkovsky and Levitzki, 1978; Hanski et al., 1979; Tolkovsky et al., 1982). From the recent data suggesting that the AC-stimulating G<sub>sa</sub> polypeptide is redistributed from the membrane to the cytosolic (aqueous) fraction upon agonist binding to receptor (Stryer and Bourne, 1986; Lynch et al., 1986; McArdle et al., 1988; Ransnäs and Insel, 1988; Ransnäs et al., 1989), it can be concluded that the rate-limiting step of AC activation may be lateral diffusion within



**Figure 5.** Direct correlation of V<sub>2</sub>-receptor mobile fraction and AVP-stimulated cAMP production. The data for the various pretreatments (symbols as per Fig. 4) are from Table II. Linear regression analyses were *r* = 0.96 (*x* = 319, *y* = 292.5) for 10<sup>-7</sup> M AVP; *r* = 0.97 (*x* = 289.5, *y* = 28.9) for 10<sup>-9</sup> M AVP; and *r* = 0.996 (*x* = 50.3, *y* = -1.11) for 10<sup>-10</sup> M AVP, respectively. The *f* values measured using 10<sup>-7</sup> M ligand (Table I) are also used for the lower ligand concentrations, since it was not possible to measure *f* directly at lower TR-LVP concentrations (<800 chromaphores/illuminated area).

the plasma membrane. That the interactions between the receptor-hormone complex and G<sub>saβr</sub> occur through diffusion-controlled collision also explains the well-established amplifying property of the AC system, i.e., that one receptor-hormone complex can activate many G<sub>sa</sub> and subsequently AC molecules (Orly and Schramm, 1975; Brandt and Ross, 1986; Ransnäs and Insel, 1988). In this respect, one would expect the receptor mobile fraction to be a more important factor at low (physiological) hormone concentrations, where only a minor fraction of receptors are occupied. Our results, that low V<sub>2</sub>-receptor *f* results in a more pronounced reduction of maximal ligand-stimulated AC activation at sub-K<sub>D</sub> AVP concentrations compared with 10<sup>-7</sup> M (100% receptor occupancy), are consistent with this. It should be stressed that the conclusions here concerning a role for receptor mobility in signal transduction are not necessarily applicable to in vitro/membrane vesicle systems where local concentrations of all membrane components as well as of GTP, GDP, ATP, Pi, kinase, phosphatase, et cetera, are unlikely to reflect the dynamic in vivo situation. Our results are all the more relevant because they reflect physiological conditions, including the complex membrane and cytosolic activation/downregulation apparatus. In this complex in vivo system, receptor lateral mobility clearly is a very important factor. Interestingly, the only other direct measurement of lateral mobility of an AC-coupled receptor indicated that whilst a β-receptor

agonist may induce receptor mobility, the receptor-antagonist complex is largely immobile (Henis et al., 1982). The intriguing possibility that antagonists of the AC system may function by receptor immobilization also argues for the physiological importance of receptor lateral mobility. The role of receptor lateral mobility in signal transduction by the polypeptide hormone receptors for insulin and epidermal growth factor has been discussed elsewhere (Jans et al., 1989, 1990a,; and see Schlessinger, 1988, 1989).

The mechanism whereby the V<sub>2</sub>-receptor is immobilized by treatment at 4°C remains unclear. As mentioned above, forskolin responses are not affected, indicating that the results are not explicable in terms of effects on AC itself. AVP binding is also completely unaffected, but a destabilization of the actin stress fibers is clearly effected, as revealed by staining of the actin cytoskeleton. The exact explanation of the effect is unclear, but probably is the result of the inhibition of actin polymerization, which has been observed at low temperature (Howard and Oresajo, 1985). The concomitant effects of 4°C treatment on both the actin cytoskeleton and V<sub>2</sub>-receptor mobility in LLC-PK<sub>1</sub> cells is comparable to that of prolonged treatment of the same cells with NH<sub>4</sub>Cl or cytochalasin B (see Jans et al., 1990c). It can be concluded that the cytoskeleton has an important influence on V<sub>2</sub>-receptor mobility, as has been reported for a number of other integral membrane proteins (Edelman, 1976; Schlessinger et al., 1976; Koppel et al., 1981; Tank et al., 1982). It should be remembered that the alterations to the cytoskeleton induced by low temperature or NH<sub>4</sub>Cl treatment probably have pleiotropic effects on the cell, possibly including membrane structural integrity. It is accordingly unlikely that the actin cytoskeleton is the sole mediator of the effects on V<sub>2</sub>-receptor mobile fraction. A role for lipid bilayer fluidity in structure and function has been documented for many membrane proteins and receptors, including the β-adrenergic receptor (Orly and Schramm, 1975; Axelrod et al., 1978; Hanski et al., 1979; Bakardjjeva et al., 1979; Helmreich and Elson, 1984).

The results here imply that V<sub>2</sub>-receptor lateral mobility may be a determining factor for AVP-dependent AC activation. Since this activation is known to be mediated by the G-protein components, which are in a 100- to 150-fold excess (Pfeuffer, 1977; Jans, D. A., unpublished results) with respect to the V<sub>2</sub>-receptor, our results imply either (a) that the G<sub>s</sub> complex is essentially immobile, and that activation of the latter is solely brought about by lateral diffusion of the mobile hormone-receptor complex; or (b) that both G<sub>s</sub> and the V<sub>2</sub>-receptor are mobile and that the domain of the receptor that interacts with/activates G<sub>s</sub> is masked under conditions of low mobility, e.g., at low temperature. Since the cytoskeleton appears to play an active role in the system, based on the results here, it is possible that the receptor is in some way linked to cytoskeletal or cytoskeleton-attached components, such as actin, spectrin, ankyrin, et cetera (see Peters, 1988). At 37°C, the G-protein interaction domains of the receptor are/become accessible, so that lateral diffusion of and collisionary contacts between both mobile G-protein and hormone-receptor can result in activation of G<sub>s</sub>. A variant of hypothesis b is that G<sub>s</sub> and receptor are freely mobile, but in separate microcompartments of the plasma membrane, and only at 37°C are the constraints to movement between the microcompartments less strict. Again, an active role of the cytoskeleton in regulating these processes could be en-

visaged, and evidence indeed exists for a spectrin-actin meshwork structure of the red blood cell plasma membrane skeleton whose rigidity as a lateral diffusion barrier is reduced at 37°C (Golan and Veatch, 1980; Lin et al., 1987). Experimental differentiation between hypotheses a and b above will be achieved via direct in vivo measurement of the lateral mobility of the G-protein complex and polypeptide components in both membrane and aqueous phases.

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