The EMBO Journal vol.8 no.9 pp.2481 – 2488, 1989

The adenylate cyclase-coupled vasopressin V_2-receptor is highly laterally mobile in membranes of LLC-PK_1 renal epithelial cells at physiological temperature

David A.Jans, Reiner Peters, Josef Zsigo[1] and Falk Fahrenholz

Max-Planck-Institut für Biophysik, Kennedy Allee 70, D-6000 Frankfurt am Main 70, FRG

[1]Present address: Institute of Medical Chemistry, Medical University of Szeged, H-6720 Szeged, Hungary

Communicated by B.Hamprecht

The lateral mobility of membrane-associated hormone receptors has been proposed to play an important role in signal transduction. Direct measurements, however, have shown that the receptors for insulin, epidermal growth factor and β-adrenergic antagonists exhibit low mobility at physiological temperature. The present study, which represents the first report of lateral mobility of a polypeptide hormone receptor coupled to adenylate cyclase, yielded quite different results. The lateral mobility of the vasopressin renal-type (V_2)-receptor was measured in the basal plasma membrane of cells of the LLC-PK_1 porcine epithelial line, using the technique of fluorescence microphotolysis (photobleaching) and a rhodamine-labelled analogue of vasopressin. The analogue, 1-deamino[8-lysine(N^6-tetramethylrhodamyl-aminothiocarbonyl)]vasopressin (TR-LVP) was synthesized and shown to have binding properties and biological activities very similar to those of Arg^8-vasopressin (AVP). TR-LVP could be used to label specifically the V_2-receptor of living LLC-PK_1 cells, whereby LLC-PK_1 cells incubated with TR-LVP in the presence of a 100-fold excess of AVP, or cells from the LLC-PK_1 V_2-receptor-deficient line M18 incubated with TR-LVP could be used as controls for non-specific binding. Using optical sectioning, specific receptor mobility could be measured both in the absence and presence of free TR-LVP. The V_2-receptor was found to be largely mobile at 37°C: the mobile fraction (f) was ~0.9, and the apparent lateral diffusion coefficient (D) ~3.0×10^{-10} cm^2/s. V_2-receptor mobility greatly decreased with decreasing temperature: at 10°C f was reduced to ~0.1. Lipid probe mobility demonstrated that receptor immobility at 10°C was not attributable to a temperature-induced phase transition of the plasma membrane lipid bilayer.

Key words: vasopressin/V_2-receptor/epithelial cell line/lateral mobility/photobleaching

Introduction

The important role of the neurohypophyseal polypeptide hormone vasopressin in fluid and electrolyte homeostasis in mammals through its antidiuretic action on kidney (Grantham and Burg, 1966) is mediated via the adenylate cyclase-coupled renal (V_2)-receptor (Dousa *et al.*, 1971; Jard, 1983; Skorecki *et al.*, 1986). Conversion of the signal represented by the ligand–receptor complex into activation of adenylate cyclase is mediated by guanyl-nucleotide binding (G)-proteins, which represent a mechanism of signal transduction common to many biological systems, such as the well-studied β-adrenergic system (Ross and Gilman, 1980; Gilman, 1987; Casey and Gilman, 1988).

The exact mechanism of G-protein-mediated signal transduction remains unclear. Some authors have proposed that the lateral mobility of the hormone–receptor complex in the plasma membrane is an important factor whereby the activation of adenylate cyclase could be induced through random collision with the ligand–receptor complex (Cuatrecasas, 1974; De Meyts *et al.*, 1976; Kahn, 1976; Tolkovsky and Levitzki, 1978). This view is supported by the observation that within one cell type the total pool of adenylate cyclase molecules can be activated by receptors with different ligand specificities. Furthermore, receptors of one cell type can activate the cyclase pool of a different cell type after cell fusion (Orly and Schramm, 1976). On the other hand, direct measurements of hormone receptor lateral mobility reveal that receptor mobility is frequently quite low. Polypeptide hormone receptors such as the insulin receptor display a substantial mobile fraction only at reduced temperature, becoming rapidly immobilized at physiological temperature (Schlessinger *et al.*, 1978a; Zidovetski *et al.*, 1981). The high-affinity binding sites for epidermal growth factor are immobile at both physiological and reduced temperatures (Rees *et al.*, 1984). The adenylate cyclase-coupled β-adrenergic receptor has also been found to be largely immobile in cultured liver cells (Barkadjieva *et al.*, 1980; Henis *et al.*, 1982). For the red blood cell membrane, a system which is very well characterized with respect to structure, lateral mobility and hormone-dependent adenylate cyclase activation, it has been suggested (Peters, 1988) that coupling of receptor and cyclase may be mediated by lateral diffusion of G-proteins in the aqueous phase (cytoplasm).

We report here on photobleaching measurements of the lateral mobility of the V_2-receptor in the basal plasma membrane of the LLC-PK_1 pig kidney epithelial cell line (Hull *et al.*, 1976). Because membrane-localized hormone receptors occur at extremely low area densities, photobleaching measurements of receptor lateral mobility generally pose severe technical problems. In this study, a rhodamine-labelled analogue of vasopressin (TR-LVP) was synthesized which showed binding properties and biological activity very similar to those of vasopressin, and which possessed sufficient fluorescence intensity to enable reliable estimates of receptor mobility. The experimental system used in this study is distinguished by two rather unique advantages: (i) since the vasopressin receptor is located in the basolateral plasma membrane of LLC-PK_1 cells (Kinoshita *et al.*, 1987), optical sectioning could be employed to measure receptor mobility in both the presence and absence of free ligand; (ii) the vasopressin receptor deficient LLC-PK_1 mutant cell line M18 (Jans *et al.*, 1986)

©IRL Press

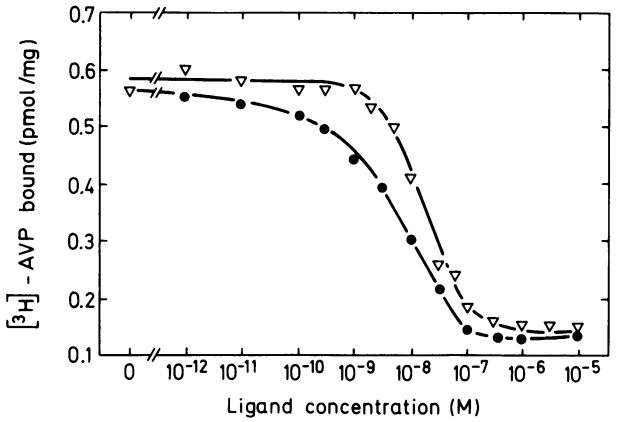


Fig. 1. Competition of [^{3}H]AVP binding to LLC-PK$_1$ cells by AVP and the rhodamine-labelled vasopressin analogue TR-LVP. LLC-PK$_1$ cells were incubated with 10^{-8} M [^{3}H]AVP and the indicated concentrations of AVP (●——●) or TR-LVP (▽——▽) for 30 min. Cells were filtered, washed and filters counted as described (Jans *et al.*, 1986). Results are shown for a single typical experiment performed in triplicate for which the SEM was <10% the value of the mean.

was available as a negative control. By these means, it was found that the V_2-receptor, in contrast to results for other hormone receptors, was highly mobile at physiological temperature (mobile fraction ~0.9 and apparent lateral diffusion coefficient about $\sim 3.0 \times 10^{-10}$ cm^2/s). Surprisingly, V_2-receptor mobility greatly decreased with decreasing temperature (mobile fraction ~0.1 at 10°C). Measurements of lipid probe mobility indicated that temperature-dependent receptor immobility was not due to a liquid–crystalline phase transition in the plasma membrane lipid bilayer.

Results

Binding properties of the rhodamine-labelled vasopressin analogue

The binding affinity of the rhodamine-labelled vasopressin analogue 1-deamino[8-lysine(N^6-tetramethylrhodamyl-aminothiocarbonyl)]vasopressin (TR-LVP) for the V_2-receptor of LLC-PK$_1$ cells was determined by incubating cells with 10^{-8} M [^{3}H]AVP and various concentrations of non-radioactive TR-LVP or AVP (Figure 1). The estimated K_D value for TR-LVP was $6.9 \pm 1.5 \times 10^{-9}$ M ($n = 3$), ~4 times that for AVP ($1.9 \pm 0.5 \times 10^{-9}$ M, $n = 3$).

Biological activity of the rhodamine-labelled vasopressin analogue

Agonistic stimulation of adenylate cyclase activity leads to the elevation of intracellular cAMP levels, and activation of the cAMP-dependent protein kinase (cAMP-PK) (Bechtel *et al.*, 1977; Flockhart and Corbin, 1982). TR-LVP was compared to AVP with respect to its ability to promote the activation of cAMP-PK in LLC-PK$_1$ cells. Cell monolayers were treated with various ligand concentrations in the presence or absence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). Both AVP and TR-LVP exhibited comparable concentration dependence of activation of cAMP-PK both in the presence and absence of IBMX (Figure 2A). AVP induced half-maximal response at

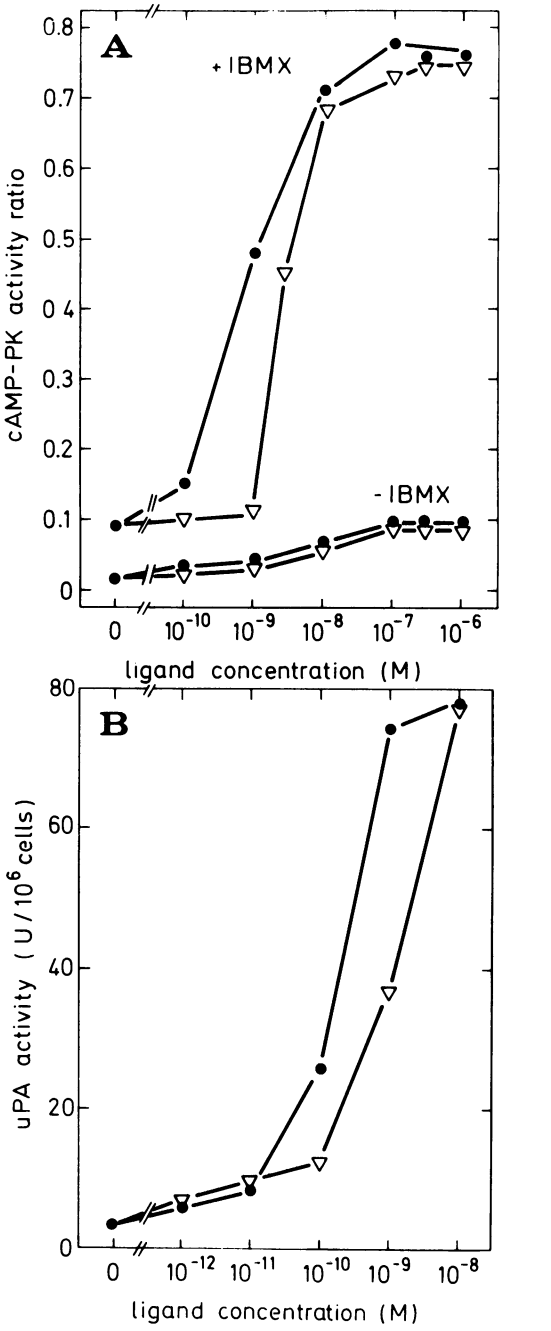


Fig. 2. Biological activity of TR-LVP. (**A**) Concentration dependence of activation of cAMP-PK in LLC-PK$_1$ cells by AVP (●——●) or TR-LVP (▽——▽). LLC-PK$_1$ cell monolayers were incubated for 30 min with the indicated ligand concentrations in the presence or absence of 500 μM IBMX and then washed and cell extracts prepared and assayed as described in Materials and methods. The cAMP-PK activity ratio represents the cAMP-PK activity measured in the absence of exogenously added cAMP (agonist-induced cAMP-PK activation) relative to that in the presence of 10 μM cAMP (total cAMP-PK), for duplicate determinations from a single typical experiment. (**B**) Concentration dependence of the stimulation of uPA production in the LLC-PK$_1$ cell line by AVP (●——●) or TR-LVP (▽——▽). Monolayers (95–100% confluent) were washed four times with serum-free DMEM and incubated with various concentrations of ligand in serum-free DMEM (2 mg/ml BSA). Medium was collected after 24 h and uPA activity (expressed per 10^6 cells) determined as described in Materials and methods. Results represent the average of at least three separate determinations, performed in triplicate, where the SEM was <15% the value of the mean. Maximal response has been attained in the case of TR-LVP at 10^{-8} M.

Table I. Binding of the rhodamine-labelled vasopressin analogue TR-LVP to cells of the LLC-PK_1 and the LLC-PK_1 receptor-deficient M18 cell lines as determined by single-cell fluorescence measurements

Treatment	LLC-PK_1	M18
(A) Fluorescence of fixed cells (arbitrary units)[a]		
(a) None	3865 ± 213	2917 ± 249
(b) 10^{-8} M TR-LVP	9708 ± 833	4084 ± 199
(c) 10^{-8} M TR-LVP + 10^{-6} M AVP	5100 ± 425	3806 ± 173
(d) Specific fluorescence (b − c)	4608	278
(e) 10^{-7} M TR-LVP	12 524 ± 588	4763 ± 213
(f) 10^{-7} M TR-LVP + 10^{-5} M AVP	8983 ± 648	6501 ± 345
(g) Specific fluorescence (e − f)	3641	0
(B) Fluorescence of unfixed cells (arbitrary units)[a]		
(1) None	1560 ± 131	2334 ± 151
(2) 10^{-8} M TR-LVP	3143 ± 60	2814 ± 240
(3) 10^{-8} M TR-LVP + 10^{-6} M AVP	2253 ± 190	2775 ± 189
(4) Specific fluorescence (2 − 3)	890	39
(5) 10^{-7} M TR-LVP	3964 ± 444	2866 ± 226
(6) 10^{-7} M TR-LVP + 10^{-5} M AVP	2376 ± 231	3100 ± 251
(7) Specific fluorescence (5 − 6)	1588	0

[a]Cells were grown for 3 days on coverslips (in 12-well Costars) and then treated for 30 min in serum-free medium as indicated. Cells were then washed four times with NaCl/Pi (phosphate buffered saline) at 4°C, prior either to direct mounting in 87% glycerol with 2% propylgallate (**B**), or fixation with 4% *p*-formaldehyde prior to mounting (**A**). Measurements of fluorescence intensity were performed as described in the text. Results represent the mean ± SEM for at least 10 separate determinations. The experiments with fixed and unfixed cells were performed on different days and are by no means to be quantitatively compared.

8.1×10^{-10} M, whereas TR-LVP induced half-maximal response at 3.4×10^{-9} M, the difference in concentration dependence comparable to that in binding affinity (Figure 1). In identical fashion to AVP, which fails to promote cAMP-PK activation in the LLC-PK_1 receptor-deficient mutant M18 (activity ratio of 0.12 after 30 min treatment with 10^{-7} M AVP and 500 μM IBMX), TR-LVP failed to activate the cAMP-PK of M18 cells (activity ratio of 0.11 after 30 min treatment with 10^{-7} M TR-LVP and 500 μM IBMX).

In addition to adenylate cyclase and cAMP-PK activation, AVP induces the production of the extracellularly secreted protease urokinase-type plasminogen activator (uPA) in LLC-PK_1 cells at the level of transcription (Nagamine *et al.*, 1983). LLC-PK_1 and M18 cells were treated with various concentrations of AVP or TR-LVP, and uPA activity quantitated in a coupled assay with the chromogenic substrate S-2251 (Figure 2B). In similar fashion to the results with cAMP-PK activation, a small but reproducible difference was evident in the concentration of AVP or TR-LVP required to induce half-maximal response (Figure 2B). Treatment with 10^{-8} M TR-LVP, like AVP, induced no uPA response in M18 (not shown). Half-maximal response by LLC-Pk_1 cells was induced by 2.1×10^{-10} M AVP and 1.1×10^{-9} M TR-LVP (Figure 2B).

The results for cAMP-PK activation and uPA induction demonstrated the biological activity of the rhodamine-labelled vasopressin analogue TR-LVP. The concentration dependence of the responses, with TR-LVP inducing half-maximal response at ~4–6 times the concentration of AVP, was completely consistent with the difference in binding affinity of the two ligands.

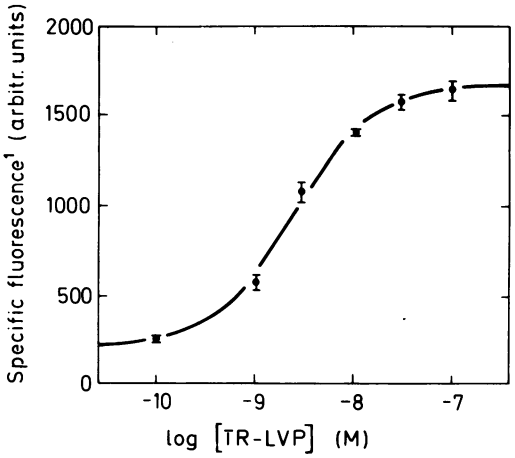


Fig. 3. Concentration dependence of TR-LVP binding to LLC-PK_1 cells as determined by single-cell fluorescence measurements. Cells were grown and treated as described in legend to Table I, except that cells were not fixed. Each measurement represents the mean ± the standard error of the mean for at least 12 separate determinations. [1]Specific fluorescence denotes the difference in fluorescence intensity of cells treated in the absence and presence of a 100-fold excess of AVP. Autofluorescence was 41% of the maximal fluorescence intensity, and non-specific fluorescence (that in the presence of TR-LVP and a 100-fold excess of AVP) was 31% that of maximal fluorescence, after correction for autofluorescence.

The rhodamine-labelled analogue of vasopressin as a fluorescent label specific for the V_2-receptor of LLC-PK_1 cells

The concentration and time dependence of TR-LVP binding was measured on single cells using the fluorescence microphotolysis (photobleaching) apparatus. Cell monolayers were incubated with TR-LVP, washed to remove unbound ligand, and then mounted and cell-associated fluorescence measured in circular areas of 3.15 μm radius. Maintaining the measurement parameters constant, parallel series of measurements were performed to derive values for non-specifically bound fluorescence and autofluorescence. Non-specific binding was determined using cells incubated with TR-LVP in the presence of a 100-fold higher AVP concentration, and autofluorescence determined by measuring cells incubated in the absence of ligand. Table I shows results from cells which were fixed (A) or unfixed (B) subsequent to incubation. Subtraction of non-specific fluorescence (above) from total fluorescence enables estimation of specific fluorescence. M18 cells exhibit greatly reduced specific fluorescence in comparison to LLC-PK_1 cells (Table I), consistent with previous experiments with radioactive ligand (Jans *et al.*, 1986, 1987c) (M18 AVP binding <8% that of LLC-PK_1). Where higher TR-LVP concentrations (10^{-7} M) were used (Table I), no specific fluorescence could be detected in M18 cells. The TR-LVP analogue appeared to be specific for the vasopressin receptor of LLC-PK_1 cells, in that the binding could be competed with excess AVP, and that the V_2-receptor-negative mutant M18 exhibited markedly reduced specific binding. Using fluorescence measurements, the concentration dependence of TR-LVP binding to LLC-PK_1 cells was determined both with and without (Figure 3) subsequent fixation of cells. Cells were incubated for 30 min with various concentrations of TR-LVP, in the absence and presence (non-specific binding) of 100-fold excess AVP. From the measurements

of specific fluorescence (Figure 3) the concentration of TR-LVP resulting in half-maximal binding could be estimated. The value of $6.6 \pm 1.6 \times 10^{-9}$ M ($n = 5$) compared well with the estimated binding constant from the competition studies above, and did not differ significantly for cells that were fixed or unfixed subsequent to incubation with ligand. The time course of the binding of TR-LVP to LLC-PK_1 cells was examined using fluorescence measurements (Figure 4B) and compared to the time course of the binding of [^{3}H]AVP (Figure 4A). Total [^{3}H]AVP binding reached a maximum within 15 min, whereas internalization—defined as bound [^{3}H]AVP not dissociable from the cells by pH 3 treatment (Schneider *et al.*, 1988; Segaloff and Ascoli, 1981)—was maximal (64% total binding) after 60 min (Figure 4A). Fluorescence measurements revealed that TR-LVP showed kinetics of binding to LLC-PK_1 cells very similar to those of [^{3}H]AVP (Figure 4B), total specific binding being maximal within 15 min and internalization 53% of total binding within 30 min. These results with fluorescence indicated that TR-LVP exhibited binding and internalization kinetics completely comparable to those of [^{3}H]AVP.

Visualization of binding of the rhodamine-labelled vasopressin analogue to LLC-PK_1 cells

Binding of TR-LVP to LLC-PK_1 and M18 cells was visualized by video-enhanced fluorescence microscopy of washed cells (Figure 5). Marked differences were evident in the intensity of the fluorescence associated with LLC-PK_1 cells incubated with 10^{-7} M TR-LVP (Figure 5B), and either M18 cells incubated with 10^{-7} M TR-LVP (Figure 5D), or LLC-PK_1 cells incubated with 10^{-7} M TR-LVP in the presence of 10^{-5} M AVP (not shown). Fluorescence was diffuse, as well as concentrated in points or aggregates (not present in the controls for non-specific binding), and did not appear to be associated with distinguishable subcellular structures.

Lateral mobility of the vasopressin V_2-receptor

The technique of photobleaching (Peters *et al.*, 1974; Axelrod *et al.*, 1976; Edidin *et al.*, 1976; Jacobson *et al.*, 1976; Peters, 1981, 1986; Petersen *et al.*, 1986) was used to measure the lateral mobility of the V_2-receptor in the basal plasma membrane of living cells of the LLC-PK_1 cell line. A circular illuminated area of 2.0 μm radius was used. Since there are ~40 000 V_2-receptors per LLC-PK_1 cell (Jans *et al.*, 1986), which are presumably diffusely distributed in the basolateral plasma membrane (Kinoshita *et al.*, 1987), it can be estimated that only 1000–2000 receptors were present in the illuminated area. In order to measure fluorescent signals originating from such small populations, and to discriminate them reliably from non-specific and autofluorescence, special methods had to be employed. (i) Mobility measurements were performed at saturating concentrations of TR-LVP (10^{-7} M). (ii) The microscope optics were focused on the basal cell surface which faces the glass support, the depth of focus having a half-width of about ± 1.5 μm, as previously determined (Scholz *et al.*, 1985). It was accordingly possible to measure fluorescence from the basal membrane with reasonable spatial selectivity. (iii) Relatively large excitation energies were employed to elicit a sufficiently strong fluorescent signal. In order to avoid (unintentional) bleaching occurring during the course of the

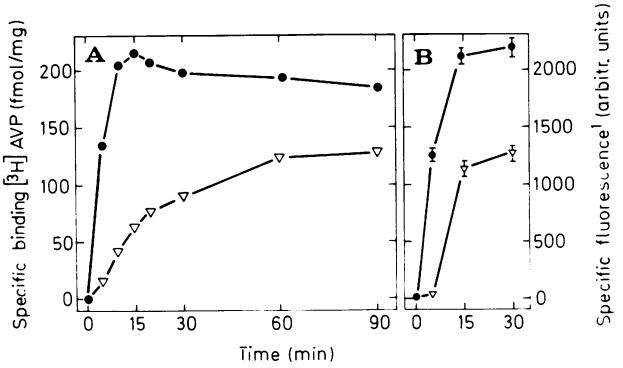


Fig. 4. Time course of binding and internalization of [^{3}H]AVP (**A**) and TR-LVP (**B**) in LLC-PK_1 cells. (**A**) Cells were grown for 3 days in 12-well Costars, washed twice with serum-free medium and then total (10^{-8} M [^{3}H]AVP) and non-specific (10^{-8} M [^{3}H]AVP and 10^{-6} M AVP) binding incubations carried out at 37°C for the times indicated. Monolayers were then washed four times at 4°C and the cells lysed with NaCl/Pi containing 0.2% Triton X-100. Lysates were counted, and also determined for protein concentration. To determine internalized hormone, monolayers were treated for 3 min with 200 mM Gly-HCl, pH 3, 200 mM NaCl (subsequent to binding incubation) to remove hormone still bound to the exterior of the cell (Segaloff and Ascoli, 1981) and then the cell lysates prepared as above. (●—●) Total specific [^{3}H]AVP binding; (▽—▽) specific [^{3}H]AVP binding resistant to acid treatment (internalized). Non-specific binding was <24% total [^{3}H]AVP binding. (**B**) Cells were grown on coverslips as described in footnote to Table I. Total and non-specific (10^{-8} M TR-LVP in the absence and presence of 10^{-6} M AVP) incubations were carried out as described in legend to Figure 3 except that after washing cells were fixed and fluorescence intensity measured as described in footnote to Table I. Internalized (acid-resistant) fluorescence was determined on cells which were treated as in (A) prior to fixation. (●—●) Total specific TR-LVP fluorescence; (▽—▽) specific fluorescence subsequent to pH 3 treatment above. Each measurement represents the mean ± SEM for at least 11 separate determinations. [1]Specific fluorescence was as defined in the legend to Figure 3. Autofluorescence represented 20% of the maximal fluorescence intensity; and non-specific fluorescence 30 and 10% of the maximal fluorescence after subtraction of the autofluorescence for total and internalized ligand respectively.

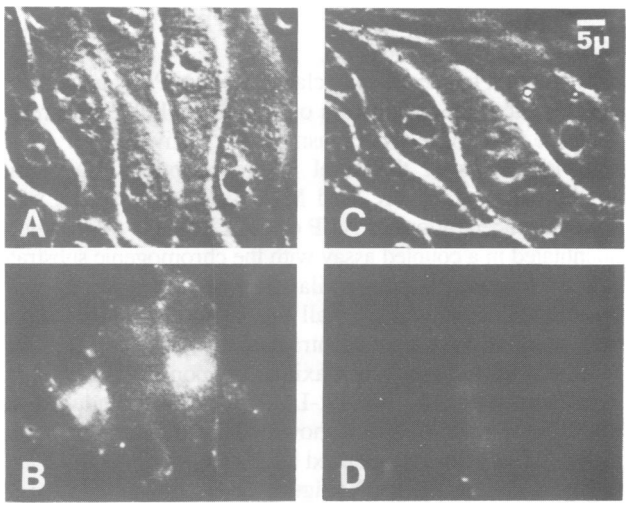


Fig. 5. Visualization of TR-LVP binding to LLC-PK_1 and M18 cells. Cells were incubated for 10 min with ligand at 37°C, washed twice, and mounted in serum-free medium. Cells were photographed using a 40 × oil immersion objective. (**A,B**) LLC-PK_1 cells incubated with 10^{-7} M TR-LVP and photographed under normal (**A**) or fluorescent (**B**) illumination. (**C,D**) M18 cells incubated with 10^{-7} M TR-LVP under normal (**C**) or fluorescent (**D**) illumination.

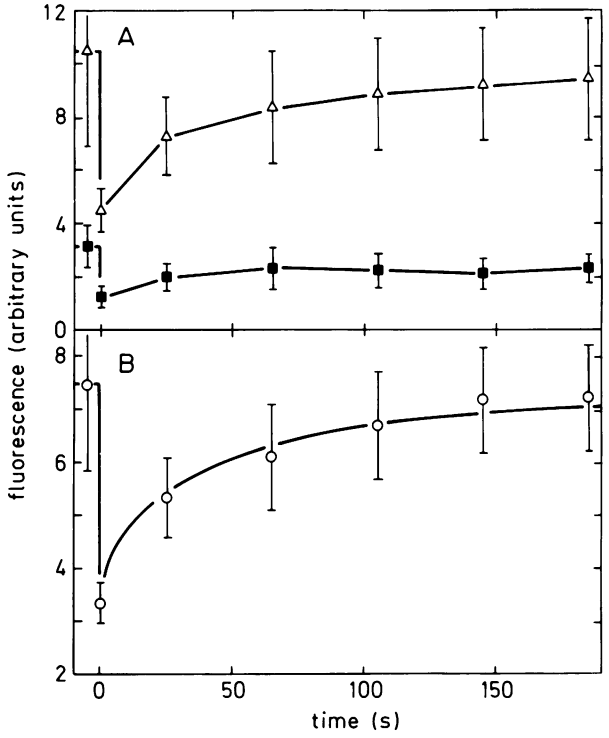


Fig. 6. Lateral mobility of the V_2-receptor in the basal plasma membranes of LLC-PK_1 cells at 37°C. Measurements were performed as described in the text on living cells which were washed subsequent to a 10 min incubation with 10^{-7} M TR-LVP and mounted in serum-free medium at 37°C. Measurements were carried out for 5–15 min subsequent to washing. (**A**) ∇—∇ total fluorescence (LLC-PK_1 cells treated with 10^{-7} M TR-LVP); and ■—■ non-specific fluorescence (M18 cells treated with 10^{-7} M TR-LVP medium; identical results for non-specific fluorescence were derived using LLC-PK_1 cells treated with 10^{-7} M TR-LVP together with 10^{-5} M AVP). Each point represents the average of at least five separate measurements, with the standard deviation indicated. (**B**) Specific fluorescence (○—○), representing non-specific subtracted from total fluorescence. The full line represents the least-square best fit (see text for details).

measurements, fluorescence intensity was monitored at only a few short intervals of the fluorescence recovery process, rather than in the usual continuous fashion. Maintaining the measurement parameters constant, parallel series of measurements were performed in this manner on several cells for each experimental treatment. The measurements for each time interval were then pooled and averaged.

The experimental procedure in measurements of receptor mobility is demonstrated in Figure 6. A series of photobleaching measurements was made on LLC-PK_1 prelabelled with 10^{-7} M TR-LVP, which yielded the recovery of total bound fluorescence after photobleaching (Figure 6A, triangles). At identical measurement conditions, photobleaching measurements were made either on M18 cells incubated with 10^{-7} M TR-LVP (Figure 6A, squares), or on LLC-PK_1 cells incubated with 10^{-7} M TR-LVP together with 10^{-5} M AVP (not shown), both of which yielded the recovery of non-specific fluorescence after photobleaching (identical in both cases). Finally, the values for the photobleaching recovery for non-specific fluorescence were subtracted from those for total bound fluorescence, to yield the photobleaching recovery of specifically bound fluorescence (Figure 6B, circles). The latter values were evaluated according to Axelrod *et al.* (1976) for two components, a mobile fraction f with an apparent lateral diffusion coefficient, D; and an 'immobile' fraction, with $D < 10^{-12}$ cm²/s. The experimental data were fitted by theoretical curves (Figure 6B, full line) employing the method of Soumpasis (1983) and a non-linear least-square algorithm.

Table II. Lateral mobility of the V_2-receptor and of a lipid probe in the basal plasma membrane of LLC-PK_1 cells

Temperature (°C)	Free ligand present during measurement	Parameter of mobility[a]		
		D (10^{-10} cm²/s)	f	n[a]
(**A**) V_2-receptor mobility (TR-LVP)				
37	no[b]	3.0	0.85	2
37	yes[b]	2.5	0.92	4
23	yes[b]	1.5	0.65	3
10	no[b]	UD[c]	0.10	2
Fixed cells[d]				
23	no	UD[c]	0.05	1
(**B**) Lipid probe mobility [$DiOC_{14}$ (3) probe][e]				
37	no	70.5	0.97	12
10	no	18.2	0.92	10

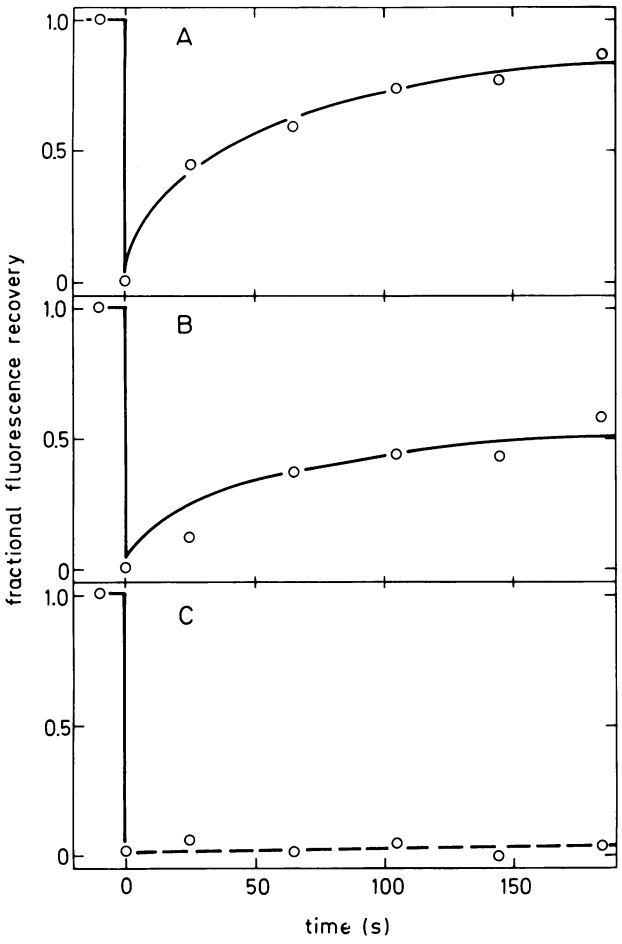
[a] n is the number of experiments used to derive the values for D, apparent lateral diffusion coefficient, and f, the mobile fraction. Each experiment for TR-LVP comprised at least five separate determinations for each of auto-, non-specific and total fluorescence, as described in the text and in the legend to Figure 6. The standard deviation between calculated values for different experiments (where appropriate) was <28 and <8% the value of the mean for D and f respectively.
[b] Cell monolayers were treated with ligand for 10 min at the temperature indicated, mounted with or without prior washing, and measurements performed at the indicated temperature for an additional 20 min. In the case of the measurements at 10°C, monolayers were treated for 30 min with TR-LVP at 4°C prior to washing. Measurements were then performed at 10°C for an additional 20 min (see Figure 7C).
[c] Unable to be determined.
[d] Cells were fixed as described in footnote to Table I, prior to mobility measurements.
[e] Cells were incubated with $DiOC_{14}(3)$ as described in legend to Figure 8, and measurements performed up to 30 min after mounting.

Results of mobility measurements are summarized in Table II. Measurements on cells that had been washed subsequent to a short incubation with TR-LVP yielded values for f and D of 0.85 and 3.0×10^{-10} cm^{-2}/s respectively at 37°C. The measured mobile fraction represents an exceptionally high value for a membrane-localized hormone receptor at physiological temperature. The D value here, on the other hand, is quite comparable to published results for integral membrane proteins (see Edidin, 1987; Jacobson *et al.*, 1987). For cells that had been mounted in the incubation medium, i.e. in the presence of 10^{-7} M TR-LVP, the photobleaching measurements (Figure 7A) yielded values very similar to those for washed cells (Table II), demonstrating that rapid association/dissociation of the ligand was absent, and did not influence the mobility measurements.

Mobility measurements at 37, 23 and 10°C (Figure 7, Table II) revealed that receptor mobility strongly decreased with decreasing temperature. At 10°C, f was ≤0.1. In cells that were fixed subsequent to incubation with TR-LVP, no mobility could be detected (Table II).

Mobility of a lipid probe in LLC-PK_1 cells

For comparison, the lateral mobility of the lipid probe 3,3-ditetradecyloxacarbocyanine [$DiOC_{14}(3)$] was deter-



Fig, 7. Temperature dependence of lateral mobility of V_2-receptor in the basal plasma membrane of LLC-PK_1 cells. Measurements were performed on cells in the continued presence of 10^{-7} M TR-LVP at 37°C (**A**) or 23°C (**B**). In **C**, cells were initially incubated with ligand for 30 min at 4°C (>80% maximal binding with 0% internalization), then washed, and mounted in medium without ligand. Measurements were carried out for 5–25 min subsequent to washing. The curves (derived as per Figure 6) represent the least-square best fits to the experimental data for specific fluorescence. The standard deviation between measurements was <25, <30 and <20% the value of the mean in **A**, **B** and **C** respectively ($n > 5$). Measurements on cells at 10°C were also performed in the presence of ligand (not shown). Identical results to those in **C** were obtained, although a reduced photobleaching efficiency was achieved.

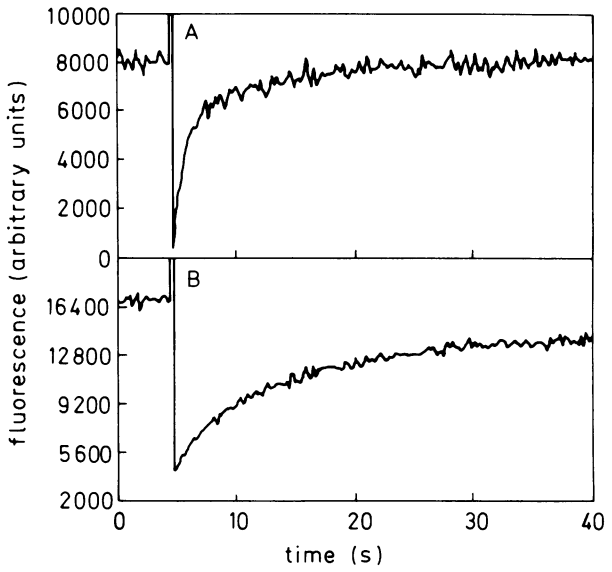


Fig. 8. Temperature dependence of lipid mobility in the basal plasma membrane of LLC-PK_1 cells. Cells were grown on coverslips as in footnote to Table I, and then treated with the lipid probe $DiOC_{14}(3)$ (7 μg/ml) for 5 min at 37°C. Cells were washed four times, and then counted in serum-free medium. Measurements were performed at 37°C (**A**) or 10° C (**B**). Results show single measurements, representative of a series of similar measurements (for average D and f values, see Table II).

mined in LLC-PK_1 cells at 10 and 37°C (Figure 8, Table II), with fluorescence recovery monitored in the usual continuous fashion (Peters, 1986). At 37°C, the lipid probe was completely mobile (f close to 1.0), and exhibited an apparent diffusion coefficient of $\sim 70 \pm 10 \times 10^{-10}$ cm^2/s ($n = 12$), which compares well with published measurements on A6 toad kidney epithelial cells (Dragsten *et al.*, 1981). At 10°C, f was still close to unity, whilst the D value had dropped to $18 \pm 6 \times 10^{-10}$ cm^2/s ($n = 10$). This showed clearly that the plasma membrane was still in a highly fluid (mobile) state at 10°C, and that V_2-receptor immobility at this temperature could not be attributed to a liquid–crystalline phase transition in the lipid bilayer.

Discussion

This study reports the first measurement of the lateral mobility in membranes of a polypeptide hormone receptor that is coupled to adenylate cyclase. We have succeeded in measuring V_2-receptor lateral diffusion at low receptor density in an epithelial cell line using a biologically active analogue of vasopressin whose binding properties, in terms both of affinity and kinetics of binding and internalization, resemble those of the natural hormone in all respects. We have been able to use an LLC-PK_1 vasopressin receptor-deficient mutant cell line M18 as an additional negative control for specificity. We have succeeded in measuring mobility in the presence of the fluorescent ligand, and hence were able to control for ligand dissociation at higher temperatures. Our results with washed cells were completely comparable to those with cells measured in the continuous presence of ligand (see Table II), indicating that a rapid on–off rate is unlikely to play a role in the measurements here either with or without ligand, at least over the time course of the measurement. The relatively weak intensity of the fluorescence (only one chromophore per ligand molecule) meant that visualization of specific V_2-receptor association with subcellular structures or of the time course of internalization was not possible. The use however of auto- and non-specific fluorescence controls enabled a quantitative estimation of specific V_2-receptor movement in the basal plasma membrane of the LLC-PK_1 cell line.

This study shows a clear dependence of the receptor mobile fraction on temperature, the fraction of mobile receptors being maximal at physiological temperature ($f = 0.9$) and low at 10°C ($f = 0.1$). The value of the diffusion coefficient paralleled this trend, also being highest at 37°C, and lowest at 10°C (Table II). These results for the mobile fraction contrast to those derived for the polypeptide hormone (non-cyclase coupled) receptors for insulin and epidermal growth factor (EGF) where almost no mobility could be detected at 37°C, and a maximal mobile fraction was determined at 4°C (Schlessinger *et al.*, 1978a; Zidovetzki *et al.*, 1981; Rees *et al.*, 1984). Diffusion

coefficients for these receptors (Schlessinger *et al.*, 1978a) are comparable, however, to those determined in this study for the V_2-receptor. The low mobile fraction of EGF and insulin receptors has been attributed to receptor aggregation and internalization, which occurs rapidly at 37°C ($t_{1/2}$ = 6 min for EGF; Zidovetzki *et al.*, 1981). The kinetics of V_2-receptor internalization is somewhat slower ($t_{1/2}$ = 14 min; Figure 4), perhaps explaining the fact that we succeeded in detecting a high fraction of mobile receptors.

The results for the measurements of lipid probe mobility in this study are comparable to published values with respect to both apparent lateral diffusion coefficient and mobile fraction in biological membranes (Schlessinger *et al.*, 1977; Dragsten *et al.*, 1981). Importantly, the mobile fraction at 10°C was close to unity, and the diffusion coefficient 18×10^{-10} cm^2/s, which is much higher than the D values for lipid probes in apparent crystalline phase ($< 1 \times 10^{-10}$ cm^2/s; Derzko and Jacobson, 1980). We conclude that the lipid bilayer of LLC-PK$_1$ cells is in a predominantly fluid state at 10°C and that the marked temperature dependence of V_2-receptor mobility can accordingly not be explained by a simple liquid–crystalline transition of the plasma membrane. Rather, that the mobile fraction and lateral diffusion of the V_2-receptor is highest at 37°C argues for its physiological importance, and a possible role in signal transduction.

An interesting aspect in considering the temperature dependence of receptor mobility is the fundamental difference in signal transduction mechanism of the adenylate cyclase-coupled V_2-receptor and the tyrosine kinase receptors for EGF and insulin. Clearly, a cyclase-activating receptor must interact with other membrane components of the system to bring about signal transduction and stimulation of cAMP production by the catalytic subunit of the adenylate cyclase. Diffusion of a single ligand–receptor complex for a certain length of time subsequent to ligand binding would enable the activation of a number of G_S and adenylate cyclase molecules, and result in amplification of the agonistic signal, which is a well-documented property of the cyclase membrane transduction system (Brandt and Ross, 1986; Ransnäs and Insel, 1988). In stark contrast, ligand binding on the part of the EGF or insulin receptor directly activates its kinase activity and signal transduction, with no essential prerequisite for receptor mobility in this process. Schlessinger (1980, 1988) has proposed that EGF receptor-mediated signal transduction requires receptor oligomerization in the membrane (i.e. the autophosphorylation event leading to signal transduction occurs inter- rather than intra-molecularly). Receptor aggregation (rapid immobilization) would be a prerequisite for transduction in this system, and has indeed been observed (Schlessinger *et al.*, 1978b)–only short-term receptor diffusion would be required, sufficient to generate the signal transducing receptor aggregates. The differences between the EGF- and V_2-receptor mobilities could accordingly be a direct consequence of their different signal transduction mechanisms, and in this sense support the mobile receptor model of Cuatrecasas (1974) and others for adenylate cyclase-coupled receptors (De Meyts *et al.*, 1976; Kahn, 1976; Tolkovsky and Levitzki, 1978).

On the basis of the results for V_2-receptor mobility, and the apparent role of the cytoplasmic fraction in G-protein signal transduction (see Chabre, 1987; Peters, 1988), it can be postulated that lateral diffusion of the V_2-receptor is necessary for receptor collision and interaction with the membrane-associated G_S complex, which then results in dissociation of the $G_{S\alpha}$ subunit into the aqueous phase. Interaction of the soluble activating $G_{S\alpha}$ (Lynch *et al.*, 1986) with the adenylate cyclase would then be a comparatively rapid process (cytoplasmic diffusion) meaning that the rate-limiting step in hormone-dependent cyclase activation would be the rate of V_2-receptor lateral diffusion in the plasma membrane lipid bilayer. This interesting possibility is currently under investigation in this laboratory.

Materials and methods

Materials were as described previously (Jans *et al.*, 1987a–c).

Cell culture

Cells of the LLC-PK$_1$ porcine kidney epithelial line (Hull *et al.*, 1976) and LLC-PK$_1$ receptor-deficient mutant M18 (Jans *et al.*, 1986, 1987c) were cultured as described previously in DMEM supplemented with 10% (v/v) foetal calf serum, 0.2 mg/ml streptomycin and 50 U/ml penicillin (Jans *et al.*, 1987a–c).

Synthesis of rhodamine-labelled vasopressin analogue

The rhodamine-labelled analogue of vasopressin, 1-deamino[8-lysine(N^6-tetramethylrhodamylaminothiocarbonyl)]vasopressin (TR-LVP) was synthesized using a modification of the procedure of Buku *et al.* (1985). 1-Deamino 8-lysine (dLVP) was prepared as described (Fahrenholz *et al.*, 1985) using the solid-phase method. Fifteen milligrams (12 μmol) of dLVP was dissolved in 3 ml methanol and 15 μl of 0.8 M triethylamine (in methanol) added. Four millilitres of 5 mg/ml tetramethylrhodamine-isothiocyanate (Serva isomer R) (42 μmol) in methanol was added and the mixture stirred overnight in the dark. Insoluble material was removed by centrifugation and the supernatant applied to silica plates (silica gel 100 F_{254}, Merck, Darmstadt) and run in chloroform/methanol/water (65:25:4 v/v). A red coloured band (R_f = 0.59) was removed by scraping and extracted with methanol. After concentration, the solution was applied to a Sephadex LH-20 column in methanol, and the main peak concentrated to dryness. The yield as estimated by absorbance spectrum and amino acid analysis was 14% of the starting material dLVP. Spectral maxima were at 272 and 545 nm (minor maximum 355.2 nm).

Assays

Extracts for the assay of cAMP-dependent protein kinase (cAMP-PK) catalytic activity were prepared and assayed with Kemptide (Leu-Arg-Arg-Ala-Ser-Ala-Gly) as substrate as described (Jans *et al.*, 1986, 1987a–c). The cAMP-PK activity ratio expresses the catalytic subunit activity present in cell extracts (assayed in the absence of cAMP) relative to the total stimulatable activity (assayed in the presence of cAMP) (Devis *et al.*, 1985; Jans, 1986, 1987a–c). The ratio gives a direct measure of the extent of cAMP-PK activation produced by treatment with different cAMP agonists and reflects the level of cAMP in tissues (Keely *et al.*, 1975). Hormone-induced cAMP-PK activity could be completely inhibited by the inclusion of 100 nM protein kinase inhibitor peptide 5–24 (Scott *et al.*, 1986) in the assay mix. It is assumed that the activity ratio measures events occurring intracellularly rather than during the preparation of cell extracts (Soderling *et al.*, 1974; Corbin, 1983; Jans *et al.*, 1987b).

Cells to be assayed quantitatively for uPA induction were treated and uPA activity in medium measured as outlined previously (Jans *et al.*, 1986, 1987b,c) using the chromogenic substrate S-2251 (D-Val-Leu-L-Lys-*p*-nitroanilide) (Bachem AG, Bubendorf, Switzerland) in a coupled assay with human plasminogen (Sigma Chemical Co.). Human urokinase (EC 3.4.32.31, Calbiochem) was used as standard, with uPA activities expressed as Ploug units/mg of cellular protein. Protein was estimated using the dye binding assay of Bradford (1976) with BSA (fatty-acid-free) as standard. Vasopressin binding was measured on whole cells as described previously (Jans *et al.*, 1986, 1987c). Dissociation constants (K_D), the concentration of hormone corresponding to 50% maximal binding, were determined as described previously (Fahrenholz *et al.*, 1984, 1985; Jans *et al.*, 1986).

Fluorescence measurements

Cells to be used for fluorescence measurements were grown on coverslips (15 × 15 mm) for 3–4 days to ~50% confluence. After incubation with ligand, cells were washed with NaCl/Pi containing 0.5 mg/ml BSA, and mounted in glycerol containing 2% propylgallate with or without prior fixation with 4% *p*-formaldehyde (in NaCl/Pi). Cells for lateral diffusion

measurements were mounted (without fixation) in the incubation medium, in the presence or absence of ligand (see text). The methods used in measurements of fluorescence intensity and the fluorescence microphotolysis apparatus used have been described previously in detail (Peters, 1986).

Acknowledgements

The authors thank Patricia Jans for capable and patient technical assistance, especially the frustrations of endless coverslip manipulation. Dr B.A.Hemmings and CIBA-Geigy Basel are thanked for making the M18 cell line available. This work was supported by grants of the Deutsche Forschungsgemeinschaft to R.P. and F.F.

References

Axelrod,D., Koppel,D.E., Schlessinger,J., Elson,E.L. and Webb,W.W. (1976) *Biophys. J.*, **16**, 1055–1069.

Barkadjieva,A., Peters,R., Hekman,M., Hornig,M., Burgermeister,W. and Helmreich,E.J.M. (1980) In Holzer,H. (ed.), *Metabolic Interconversion of Enzymes*. Springer Verlag, Heidelberg, pp. 378–392.

Bechtel,P.J., Beavo,J.A. and Krebs,E.G. (1977) *J. Biol. Chem.*, **252**, 2691–2697.

Bradford,W.M. (1976) *Anal. Biochem.*, **72**, 248–249.

Brandt,D.R. and Ross,E.M. (1986) *J. Biol. Chem.*, **261**, 1656–1664.

Buku,A., Schwartz,I.L., Gazis,D., Ma,C.L. and Eggena,P. (1985) *Endocrinology*, **117**, 196–200.

Casey,P.J. and Gilman,A.G. (1988) *J. Biol. Chem.*, **263**, 2577–2580.

Chabre,M. (1987) *Trends Biochem. Sci.*, **12**, 213–215.

Corbin,J.D. (1983) *Methods Enzymol.*, **99**, 227–232.

Cuatrecasas,P. (1974) *Annu. Rev. Biochem.*, **43**, 169–214.

De Meyts,P., Bianco,A.R. and Roth,J. (1976) *J. Biol. Chem.*, **251**, 1877–1888.

Derzko,Z. and Jacobson,K. (1980) *Biochemistry*, **19**, 6050–6057.

Devis,P.E., Grohol,S.H. and Taub,M. (1985) *J. Cell Physiol.*, **125**, 23–25.

Dousa,T., Hechter,O., Schwartz,J.L. and Walter,R. (1971) *Proc. Natl. Acad. Sci. USA*, **68**, 1693–1697.

Dragsten,P.R., Blumenthal,R. and Handler,J.S. (1981) *Nature*, **294**, 718–722.

Edidin,M. (1987) *Curr. Top. Membrane Transport*, **29**, 91–127.

Edidin,M., Zagyansky,Y. and Lardner,T.Y. (1976) *Science*, **191**, 466–468.

Fahrenholz,F., Boer,R., Crause,P., Fritzsch,G. and Grzonka,Z. (1984) *Eur. J. Pharmacol.*, **100**, 47–58.

Fahrenholz,F., Boer,R., Crause,P. and Toth,M.V. (1985) *Eur. J. Biochem.*, **152**, 589–595.

Flockhart,D.A. and Corbin,J.D. (1982) *CRC Crit. Rev. Biochem.*, **12**, 133–186.

Gilman,A.G. (1987) *Annu. Rev. Biochem.*, **56**, 615–649.

Grantham,J.J. and Burg,M.B. (1966) *Am. J. Physiol.*, **211**, 255–259.

Henis,Y.I., Hekman,M., Elson,E.L. and Helmreich,E.J.M. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2907–2911.

Hull,R.N., Cherry,W.R. and Weaver,G.W. (1976) *In Vitro*, **12**, 670–677.

Jacobson,K., Wu,E.-S. and Poste,G. (1976) *Biochim. Biophys. Acta*, **433**, 215–223.

Jacobson,K., Ishihara,A. and Irman,R. (1987) *Annu. Rev. Physiol.*, **49**, 163–175.

Jans,D.A., Resink,T.J., Wilson,E.R., Reich,E. and Hemmings,B.A. (1986) *Eur. J. Biochem.*, **160**, 407–412.

Jans,D.A., Gajdas,E.L., Dierks-Ventling,C., Hemmings,B.A. and Fahrenholz,F. (1987a) *Biochim. Biophys. Acta*, **930**, 392–400.

Jans,D.A., Resink,T.J. and Hemmings,B.A. (1987b) *Biochem. J.*, **243**, 413–418.

Jans,D.A., Resink,T.J. and Hemmings,B.A. (1987c) *Eur. J. Biochem.*, **162**, 571–576.

Jard,S. (1983) *Curr. Top. Membrane Transport*, **18**, 255–285.

Kahn,C.R. (1976) *J. Cell Biol.*, **70**, 261–286.

Keely,S.L., Corbin,J.D. and Park,C.R. (1975) *J. Biol. Chem.*, **250**, 4832–4840.

Kinoshita,Y., Fukase,M., Kubota,T. and Fujita,T. (1987) *Hormones Metab. Res.*, **19**, 393–394.

Lynch,C.J., Morbach,L., Blackmore,P.F. and Exton,J.H. (1986) *FEBS Lett.*, **200**, 333–336.

Nagamine,Y., Sudol,M. and Reich,E. (1983) *Cell*, **32**, 1181–1190.

Orly,J. and Schramm,M. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 4410–4416.

Peters,R. (1981) *Cell Biol. Intern. Rep.*, **5**, 733–760.

Peters,R. (1986) *Biochim. Biophys. Acta*, **864**, 305–359.

Peters,R. (1988) *FEBS Lett.*, **234**, 1–7.

Peters,R., Peters,J., Tews,K.H. and Bähr,W. (1974) *Biochim. Biophys. Acta*, **357**, 282–294.

Petersen,N.O., Felder,S. and Elson,E.L. (1986) In Weir,D.M., Herzenberg,L.A., Blackwell,C. and Herzenberg,L.A. (eds), *Handbook of Experimental Immunology*. Blackwell Scientific, Oxford, Vol. 1, pp. 24.1–24.23.

Ransnäs,L.A. and Insel,P.A. (1988) *J. Biol. Chem.*, **263**, 17239–17242.

Rees,A.R., Gregoriou,M., Johnson,P. and Garland,P.B. (1984) *EMBO J.*, **3**, 1843–1847.

Ross,E.M. and Gilman,A.G. (1980) *Annu. Rev. Biochem.*, **49**, 533–564.

Schlessinger,J. (1980) *Trends Biochem. Sci.*, **5**, 210–214.

Schlessinger,J. (1988) *Trends Biochem. Sci.*, **13**, 443–447.

Schlessinger,J., Axelrod,D., Koppel,D.E., Webb,W.W. and Elson,E.L. (1977) *Science*, **195**, 307–309.

Schlessinger,J., Schechter,Y., Cuatrecasas,P., Willingham,C. and Pastan,I. (1978a) *Proc. Natl. Acad. Sci. USA*, **75**, 5353–5357.

Schlessinger,J., Schechter,Y., Willingham,M.C. and Pastan,I. (1978b) *Proc. Natl. Acad. Sci. USA*, **75**, 2659–2663.

Schneider,H.-G., Raue,F., Zink,A., Koppold,A. and Ziegler,R. (1988) *Mol. Cell. Endocrinol.*, **58**, 9–15.

Scholz,M., Schulten,K. and Peters,R. (1985) *Eur. Biophys. J.*, **13**, 37–44.

Scott,J.D., Glaccum,M.B., Fischer,E.H. and Krebs,E.G. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1613–1616.

Segaloff,D.L. and Ascoli,M. (1981) *J. Biol. Chem.*, **256**, 11420–11423.

Skorecki,K.L., Verkman,A.S. and Ausiello,D.A. (1986) *Mineral Electrolyte Metab.*, **12**, 64–70.

Soderling,T.R., Corbin,J.D. and Park,C.R. (1974) *Methods Enzymol.*, **38**, 358–367.

Soumpasis,D.M. (1983) *Biophys. J.*, **41**, 95–97.

Tolkovsky,A.M. and Levitzki,A. (1978) *Biochemistry*, **17**, 3759–3810.

Zidovetzki,R., Yarden,Y., Schlessinger,J. and Jovin,T.M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6981–6985.

Received on April 10, 1989; revised on June 12, 1989