Biochimica et Biophysica Acta, 971 (1988) 307–316
Elsevier

BBA 12327

Fluorescent glucagon derivatives. II. The use of fluorescent glucagon derivatives for the study of receptor disposition in membranes

Larry D. Ward, Richard C. Cantrill, Helmuth Heithier, Reiner Peters * and Ernst J.M. Helmreich

Department of Physiological Chemistry, University of Würzburg, Würzburg (F.R.G.)

(Received 29 January 1988)
(Revised manuscript received 1 June 1988)

Key words: Glucagon receptor; Glucagon derivative; Fluorescence; Hepatocyte; Video intensification microscopy; (Rat)

When monolayer cultured hepatocytes were incubated with 1 nM [^{125}I]glucagon at 30 °C, equilibrium was reached after 10 min, whereas at 4 °C, equilibrium was reached after 60 min. At the higher temperature, 11.2% of the bound ligand was broken down after 60 min, at the lower temperature, the amount of degradation was negligible. At 30 °C, acid-washing did not remove specifically bound ligand; thus, it was assumed that the ligand was internalised at this temperature, since some of the specifically bound ligand could be washed off at lower temperatures. This was confirmed in experiments when monolayer cultures of hepatocytes were incubated with fluorescein-labelled derivatives of glucagon. The distribution of specific binding on the cell surface was studied at both 30 and 4 °C using video intensification microscopic techniques. In keeping with studies using radiolabelled glucagon, more fluorescence was detected following incubation at 4 °C than at 30 °C and it could be removed by washing the cells. Video intensification microscopy indicated that at the lower temperature, the bound ligand was distributed all over the cell surface. At the higher temperature, ligand-derived fluorescence could only be detected in mobile intracellular vesicles.

Introduction

Information on the mobility of hormone receptors in intact cells and on the putative interactions of such receptors with other membrane components and cytoskeletal structures is of fundamental importance to the understanding of the mechanism of hormonal activation and desensitisation. The few data available indicate unexplained discrepancies in the relationship of lateral mobility and position of receptors in the membrane and their function (for a review see Ref. 1). Fluorescence photobleaching recovery experiments with EGF, for example, indicated that the high-affinity EGF receptor population on A431 cells, which seems to be responsible for the biological activity of EGF, is immobile in the cell membrane. Whereas, the predominant lower-affinity receptor population, which is initially freely mobile, is in-

* Present address: Max Planck Institute for Biophysics, 6000 Frankfurt/Main 71 (F.R.G.).

Abbreviations: EGF, epidermal growth factor; ATP, adenosine triphosphate; BSA, bovine serum albumin; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; FRAP, fluorescence recovery after photobleaching; NBD, 4-nitrobenz-2-oxa-1,3-diazole.

Correspondence: R.C. Cantrill, Physiologisch-Chemisches Institut, Koellikerstrasse 2, 8700 Würzburg, F.R.G.

ternalised when EGF is bound [2]. Conversely, insulin receptors, which are initially mobile, formed clusters in the presence of ligand at physiological temperatures and were internalised [3]. β-Adrenergic receptors, probed with the fluorescent antagonist, NBD-alprenolol, were predominantly immobile and preaggregated in Chang liver cells and were only slowly released to a more mobile state on activation by an agonist [4]. This was a surprising finding when interpreted in terms of the widely accepted floating receptor hypothesis which emphasises the importance of diffusional encounters between hormone-receptor complexes [5] and other components of the cyclase system [6]. Reconciliation of these results would require that the other components of the system are freely mobile.

The synthesis of biologically active fluorescent glucagon derivatives, described in the preceding paper [7], provides a new tool to investigate the disposition in the membrane of a polypeptide hormone receptor which is linked to the adenylate cyclase system. A fluorescent hormone agonist also offers distinct advantages over the fluorescent β-antagonist previously used [4] allowing mobility and receptor distribution to be measured directly as a consequence of activation, desensitisation and internalisation.

This communication documents the visualisation of the glucagon receptor of the rat hepatocyte in monolayer culture. Conditions were established first using [^{125}I]glucagon and then used for the reproducible measurement of the disposition of receptor bound fluorescein-labelled glucagon derivatives by video intensification microscopy.

Preliminary data were presented at the Spring Meeting of the Bunsen-Gesellschaft für Physikalische Chemie, 'Mechanisms of Membrane Transport' Königstein, Taunus, F.R.G., 21–23 March, 1988.

Materials and Methods

Chemicals. Bacitracin, and bovine serum albumin were Sigma products. Glucagon was obtained from Serva, Heidelberg, F.R.G. [^{125}I]-Glucagon (New England Nuclear) was a generous gift from Dr. H. Schoene, Hoechst Company, Frankfurt, F.R.G. Fluorescein-Trp^{25}-glucagon was prepared and characterised as described [7].

Preparation of rat hepatocytes. Rat liver hepatocytes were isolated as described in the preceding paper [7], and either used immediately for suspension binding assays or seeded in petri dishes (3.5 cm diameter) containing sterile glass cover slips at a density of 35000/dish for microscopic studies or seeded at a density of 40000/well in NUNC 24-well plates for monolayer binding studies. Cells were cultured in Ham's F12 medium supplemented with 10% fetal calf serum, insulin ($5 \cdot 10^{-8}$ M) and dexamethasone ($1 \cdot 10^{-7}$ M) for 2 days prior to experimentation. The medium was changed twice daily.

Binding studies. [^{125}I]Glucagon binding in suspension was carried out as described in the preceding paper [7]. Freshly prepared hepatocytes ($(2–5) \cdot 10^5$ cells/ml) were incubated for appropriate times with 40 pM [^{125}I]glucagon in PBS buffer containing 0.1% BSA/0.1% bacitracin (pH 7.4). Non-specific binding was determined in the presence of 10^{-6} M native glucagon.

Cells in monolayer culture were first washed three times with ice-cold PBS buffer before incubation in 1.0 ml PBS buffer containing 0.1% BSA/0.1% bacitracin/1 nM [^{125}I]glucagon. At the end of the incubation the hepatocyte monolayers were washed with cold PBS buffer and the cell-bound radioactivity was released with 1 ml 0.1 M NaOH and determined by gamma counting. Non-specific binding was determined in the presence of 10^{-6} M glucagon. Internalised and surface-bound [^{121}I]glucagon was determined on hepatocytes in monolayer culture. After incubation with 1 nM [^{125}I]glucagon, monolayers were washed with 1 ml ice-cold PBS containing 0.1% BSA, treated with 0.5 M NaCl in 0.2 M acetic acid (pH 2.5) for 6 min before washing them again with 1 ml ice-cold PBS [8]. Radioactivity liberated into the aqueous phase was considered to be surface-bound, whereas that remaining with the cells was considered to be internalised. The degradation of glucagon was determined by precipitation with 10% TCA [9].

The dissociation of [^{125}I]glucagon from cultured hepatocytes was determined as follows: after 30 min incubation with the labelled ligand at 4 or 30 °C, the monolayers were washed twice with

PBS buffer and then further incubated in 1 ml PBS buffer containing 0.1% BSA/0.1% bacitracin (pH 7.4) at the same temperature for up to 40 min. At various times, both the supernatant and cell-bound radioactivity were determined. Non-specific binding was measured in the presence of 10^{-6} M native glucagon.

Video intensification microscopy. Washed hepatocytes, cultured for 2 days on glass coverslips, were incubated in PBS buffer (pH 7.4) containing 0.1% BSA/0.1% bacitracin at 30 °C and at 4 °C for 30 min with $5 \cdot 10^{-8}$ M or 10^{-7} M fluorescent ligand. The cells were washed three times with PBS/BSA/bacitracin buffer to remove excess ligand and viewed in PBS. Cells were viewed on a Zeiss Universal microscope using a water immersion lens (40 ×). Images were stored and analysed using a video intensification system (Hamamatsu Photonic Microscope System C1966). After storage of the fluorescent image, native glucagon was added to give a final concentration of 10^{-5} M. The same cells were again viewed after 15 and 30 min. The image obtained at each time point was subtracted from the original image and sites of fluorescence loss were recorded. The high concentration of glucagon derivative used in these experiments precluded the measurement of ligand association by video intensified image analysis.

Results

Glucagon binding

As described previously [10], [^{125}I]glucagon binding to cells in suspension at 30 °C showed a distinct peak after 10 min; at 20 °C, the maximum observed after 20 min was less distinct. No peak was seen with cells in suspension at 4 °C or with cells in monolayer culture at either 4 °C or 20 °C: steady-state binding was reached after 60 and 20 min, respectively. The peaks in the kinetic plots could have arisen from the proteolytic breakdown of [^{125}I]glucagon, since degradation rates varied with temperature and were 0.4%, 3.6% and 11.2% per h, respectively, for cells in suspension at 4, 20 and 30 °C (Fig. 1). However, with monolayer cultures, the breakdown of [^{125}I]glucagon was negligible at 4 °C and only 0.6% per h at 20 °C (data not shown). The rate of breakdown was neither concentration-dependent at the glucagon concentrations used nor a function of receptor occupancy, since the rate of degradation remained unchanged in the presence of 1 μM unlabelled glucagon. The increased rate of glucagon breakdown in suspension assays at higher temperatures paralleled a decrease in the viability of the hepatocytes and, similarly, a decrease in non-specific binding. Non-specific binding increased slightly at 30 °C over the first 30 min before decreasing linearly at a rate of 12.8% per h. At 20°C, the rate of decrease of non-specific binding was 6.6% per h, whereas at 4 °C in suspension and with cells in monolayer culture at any temperature, there was no time-dependent change in non-specific binding (data not shown). The viability of suspended cells, as judged by Trypan blue exclusion, after incubation for 5 h at 30, 20 and 4 °C was 55%, 75% and 95%, respectively. The viability of adhered cells under assay conditions could not be accurately determined as they could not be removed quantitatively from the culture dish without damage.

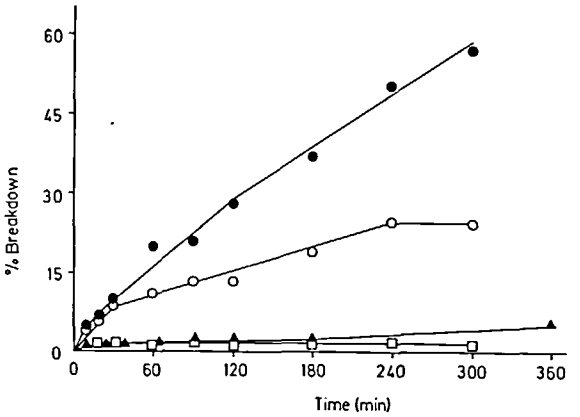

Fig. 1. Time- and temperature-dependent breakdown of [^{125}I]glucagon bound to hepatocytes in suspension and monolayer culture. At each time point, TCA-soluble radioactivity was determined and the data are given as percent of the initial [^{125}I]glucagon concentration (1 nM). (●) Hepatocytes incubated in suspension at 30 °C, (○) hepatocytes incubated in suspension at 20 °C, (▲) hepatocytes incubated in suspension at 4 °C, (□) hepatocytes monolayers incubated at 20 °C. These experiments were repeated three times with comparable results and a representative example is shown.

Acid washing cells has been used before for monitoring the internalisation of receptor-bound ligand [8]. At 30 °C, the amount of specifically bound ligand was approximately the same in control as in acid-washed cells (Fig. 2). However, an

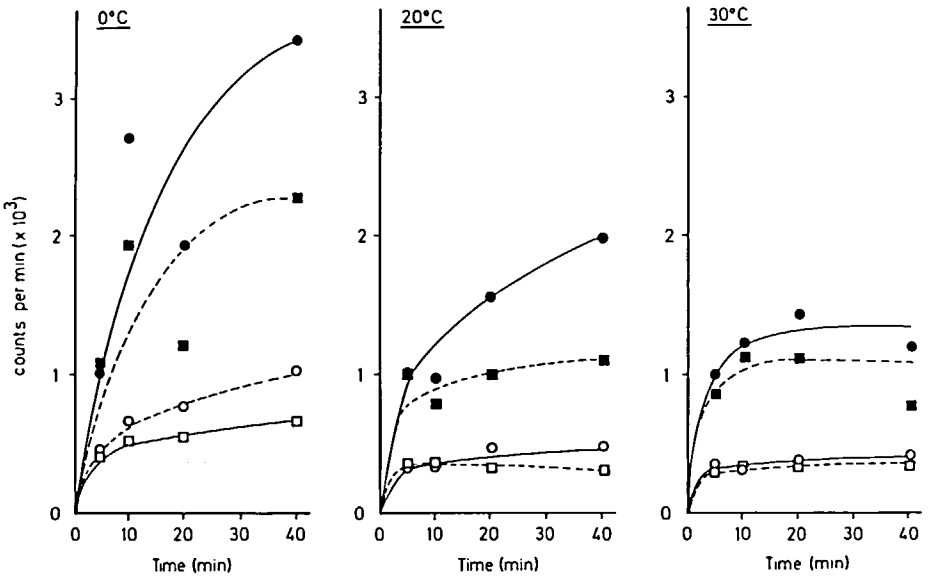

Fig. 2. [^{125}I]Glucagon binding to primary hepatocytes in monolayer culture. Total binding was performed in the presence (○) and absence (●) of 10^{-6} M native glucagon. Surface-bound ligand was measured by acid washing the cells incubated in the presence (□) and absence (■) of 10^{-6} M native glucagon as described in Materials and Methods [8]. These experiments were repeated three times with comparable results.

increasingly greater proportion of specifically bound ligand could be removed by acid-washing at lower temperatures. These results are best explained as a rapid removal of ligand-bound receptors from the cell surface at higher temperatures, rendering them less accessible to acid-washing.

Radiolabelled glucagon rapidly dissociated from cells incubated at 4° C and appeared in the supernatant both in the presence and absence of native glucagon. The loss of radioactivity was biphasic; only the latter part of the exponential decline in bound glucagon is shown in Fig. 3. Following incubation at 30° C the loss of radioactivity was slower (Fig. 3) and, after an initial slight decline, became negligible. During both phases, that proportion of the radioactivity attributable to specific receptor binding did not change (data not shown). The slow dissociation of ligand at 30° C and the constant specific binding provide further evidence for the internalisation of the receptor-bound ligand during the 30 min incubation period at this temperature.

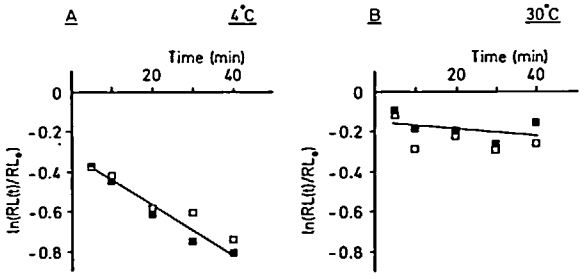

Fig. 3. Dissociation of [^{125}I]glucagon from hepatocyte monolayers at 4 and 30° C. RL(t) represents the amount of ligand bound to the cell at time (t) and RL_0 is the amount of ligand bound at time zero. Data are shown for both total (■) and non-specifically bound ligand (□), which was not displaceable in the presence of 10^{-6} M native glucagon. The data are representative of four separate determinations. In all cases the counts bound and the percent of specific binding were similar to those presented in Fig. 2.

Video intensification microscopy

The relatively large total number of high- and low-affinity binding sites on primary hepatocytes in culture makes them suitable for the measurement of fluorescent-ligand binding (see accompanying paper [7]). Following incubation at 4° C for 30 min in the absence of added ligand, the autofluorescence of a hepatocyte in monolayer culture varies little over the following 30 min even though the cell is slowly warming up to the temperature of the viewing medium (15–20° C) (Fig. 4). Sub-

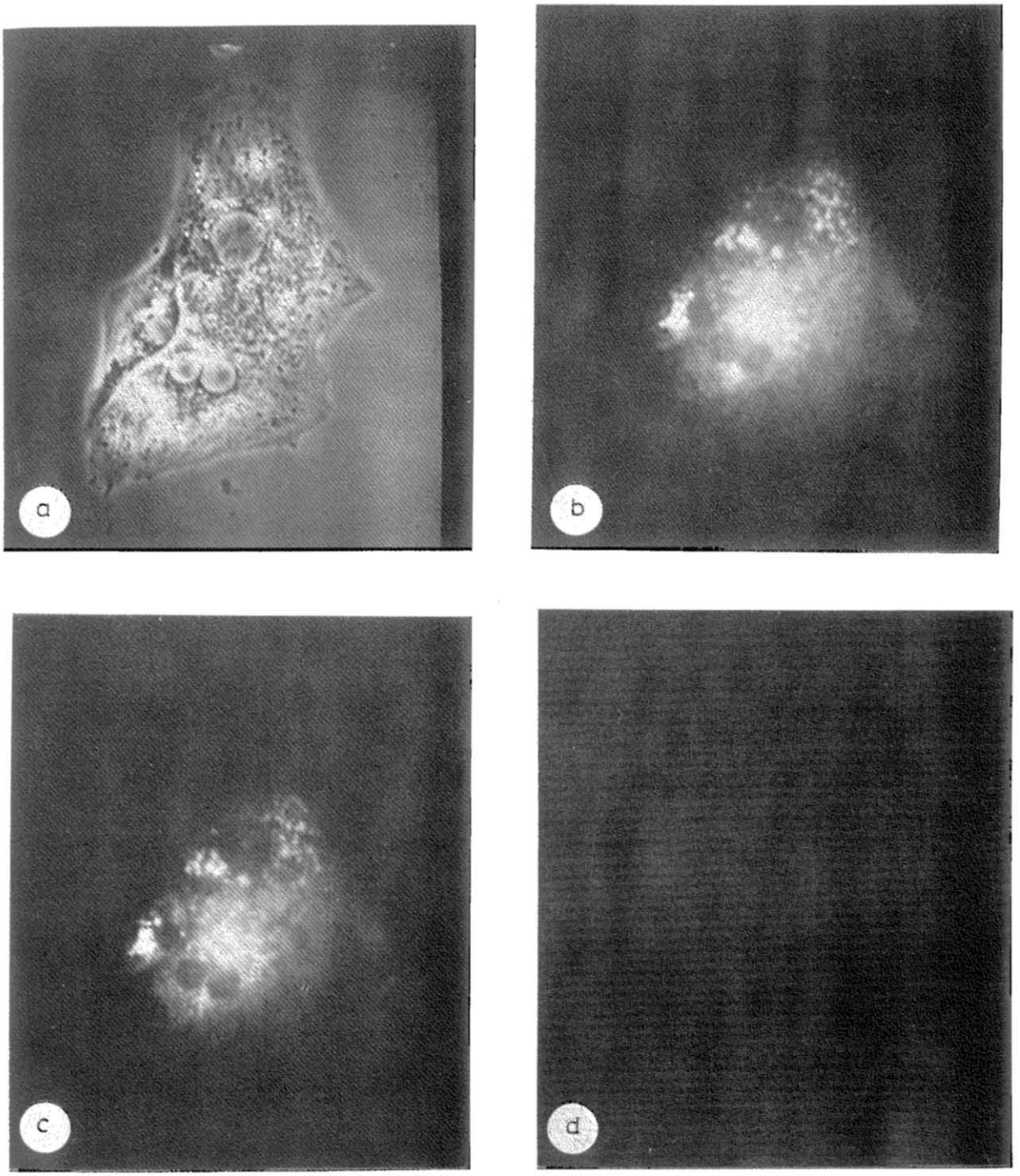

Fig. 4. Video intensified images of primary hepatocytes cultured in monolayers following incubation at 4°C without ligand for 30 min. (a) Phase contrast; (b) initial autofluorescence; (c) autofluorescence after 30 min in viewing medium; (d) decay of autofluorescence at 15–20°C in the viewing medium expressed as the difference between the initial cellular fluorescence at time zero and after 30 min. The photographs shown here and in Figs. 5–7 are representative of many cells viewed under the conditions specified.

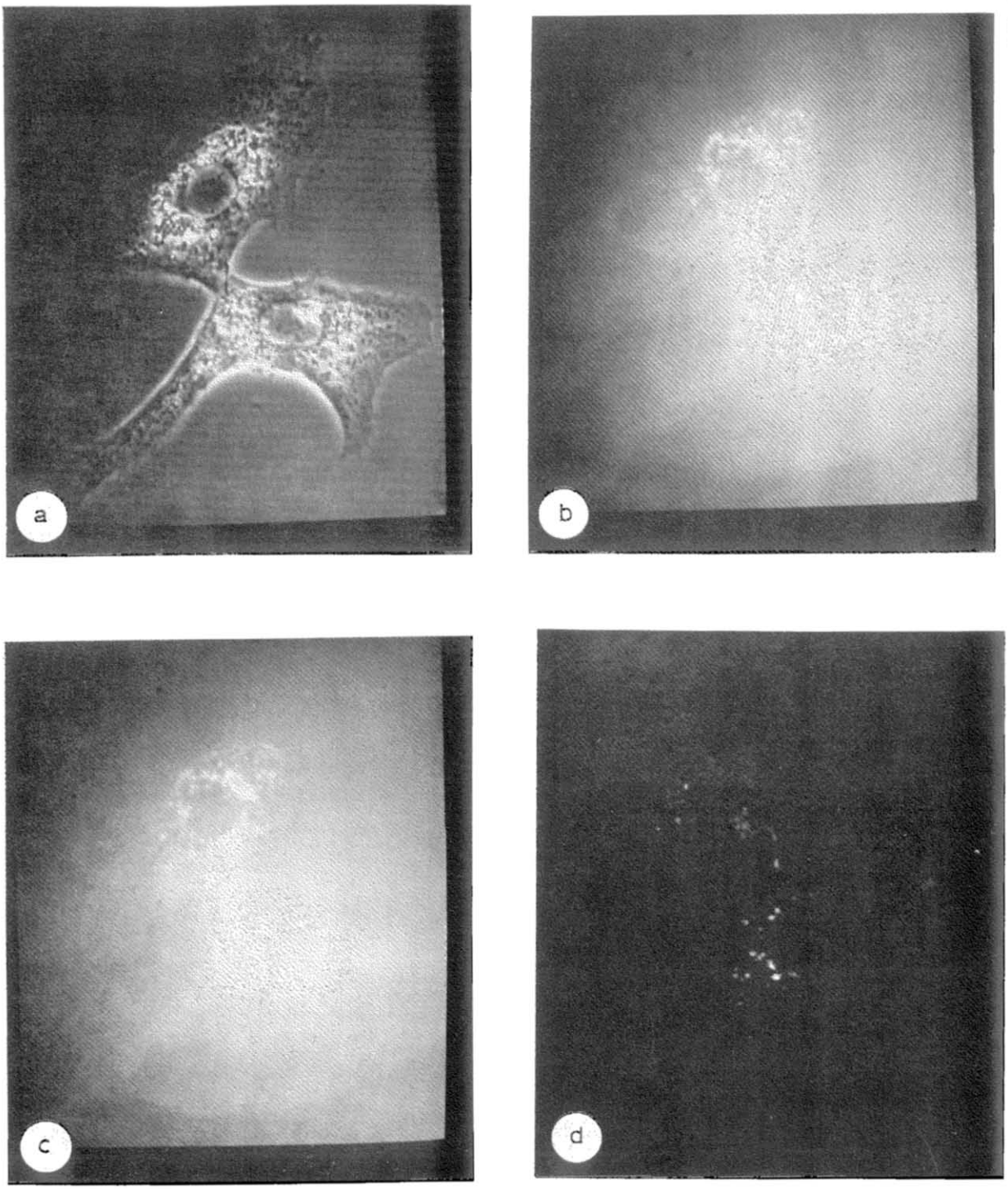

Fig. 5. Video intensified images of primary hepatocytes cultured in monolayers following incubation at 30 °C without ligand for 30 min. (a) Phase contrast; (b) initial autofluorescence; (c) Autofluorescence after 20 min; (d) Difference between the initial cellular fluorescence seen at time zero and after 20 min.

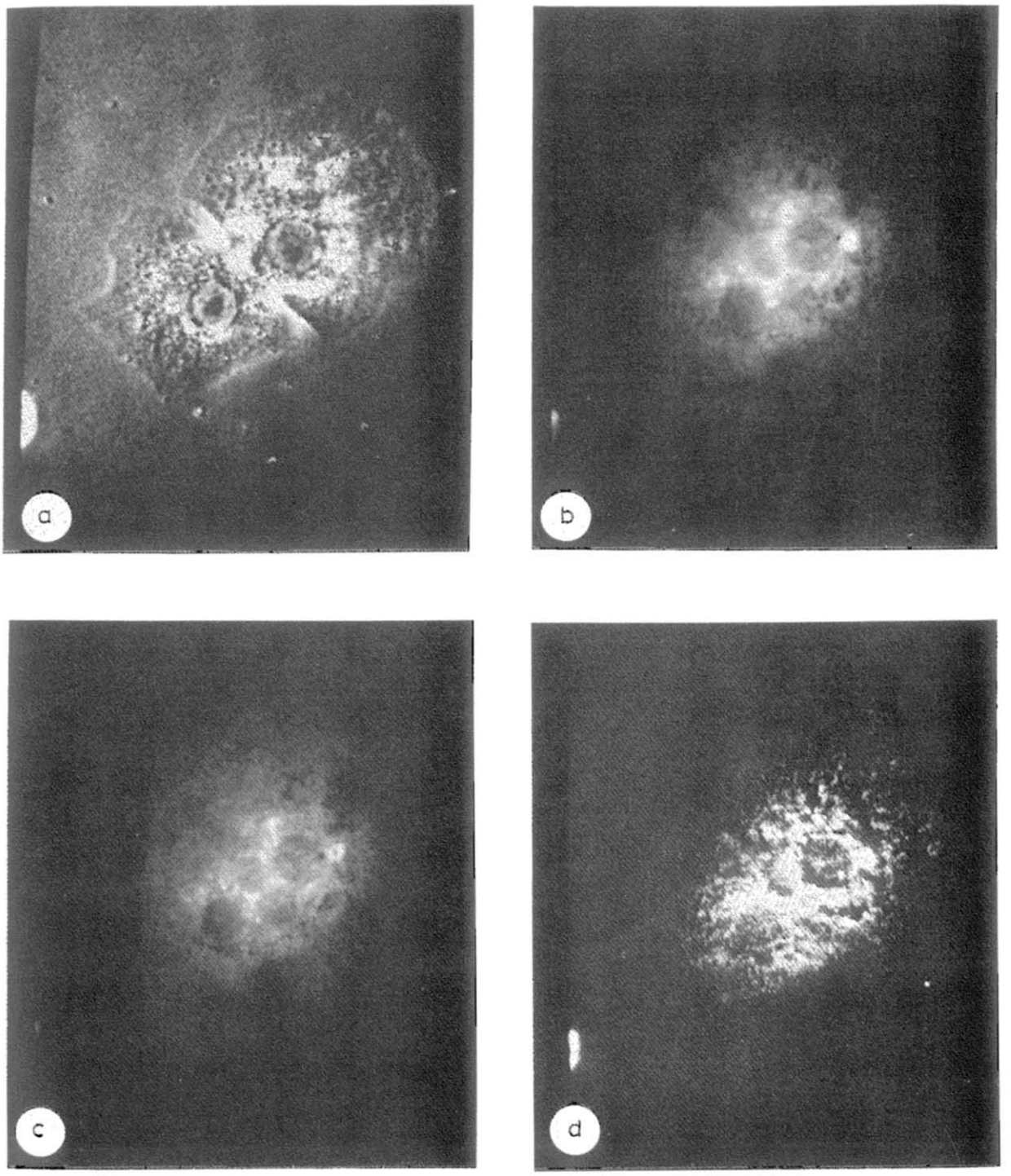

Fig. 6. Video intensified images of primary hepatocytes cultured in monolayers following incubation at 4°C with 10^{-7} M fluorescein-Trp^{25}-glucagon for 30 min. (a) Phase contrast; (b) initial fluorescence at time zero; (c) fluorescence after 30 min; (d) difference between the initial fluorescence at time zero and after 30 min.

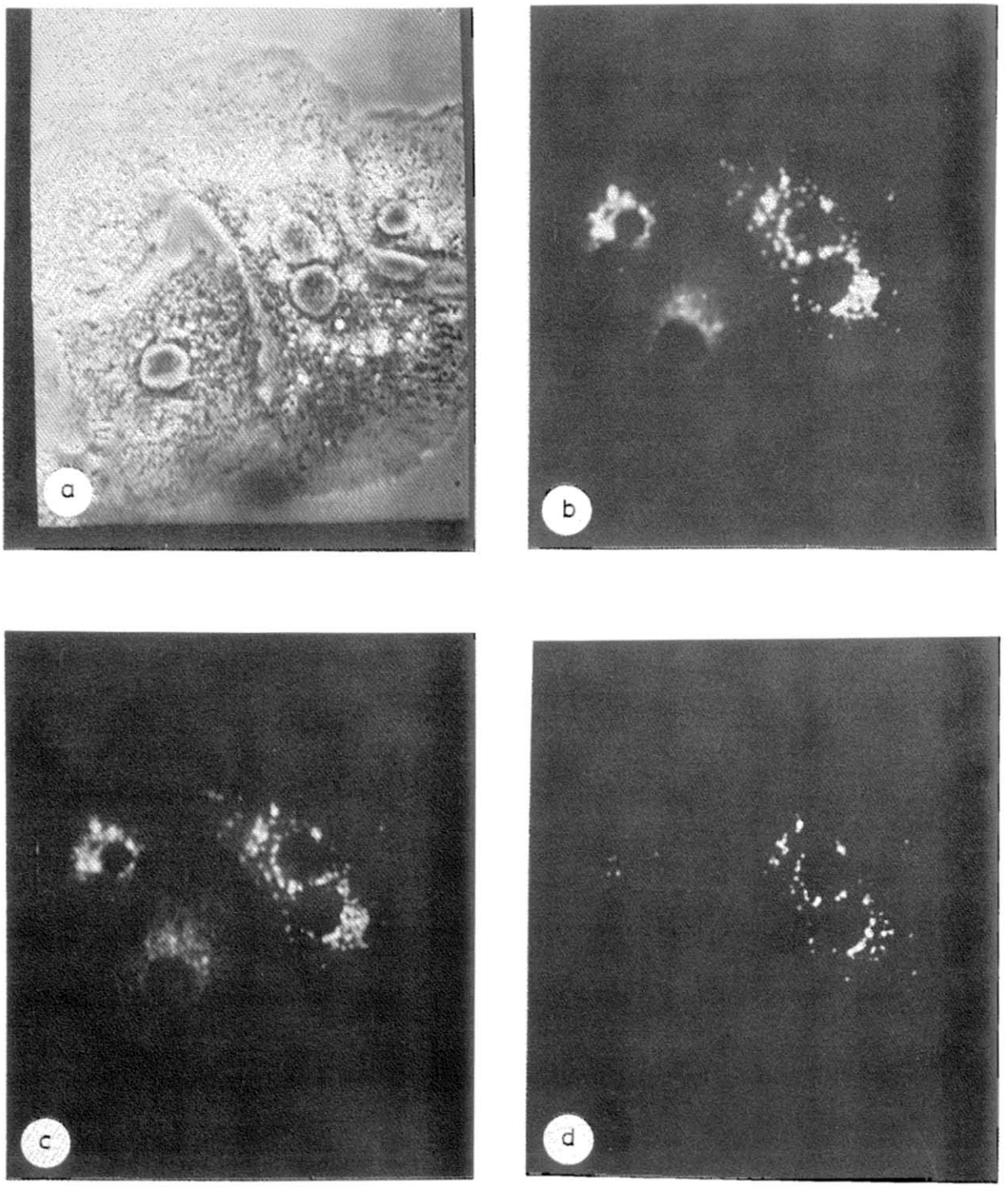

Fig. 7. Video intensified images of primary hepatocytes cultured in monolayers following incubation at 30 °C with 10^{-7} M fluorescein-glucagon for 30 min. (a) Phase contrast; (b) initial fluorescence at time zero; (c) fluorescence after 30 minutes; (d) difference between the initial fluorescence at time zero and after 30 min.

traction of the video images stored during the 30-min period following incubation at 30 °C showed alterations in the fluorescence distribution (Fig. 5d). These changes are consistent with the movement of intracellular vesicles although the cells were slowly cooling to the temperature of the viewing medium. Hepatocytes became more fluorescent following incubation with 10^{-7} M fluorescein-Trp^{25}-glucagon (Figs. 6, 7) or $5 \cdot 10^{-8}$ M difluorescein(His^{1}, Lys^{12})-glucagon (not shown) for 30 min at either 4 or 30 °C. After incubation at 4 °C, fluorescence labelling appeared all over the cell surface although more could be seen in the perinuclear region (Fig. 6). Fluorescence labelling at 30 °C was confined to the perinuclear regions and was not homogeneous amongst the cells of the monolayer (Fig. 7). Fluorescence slowly declined at 4 °C and at higher temperatures, even without the addition of unlabelled glucagon, 10^{-5} M final concentration (Figs. 6 and 7). Following incubation at 4 °C, the loss of fluorescence occurred from all over the cell surface (Fig. 6), probably resulting from slow dissociation of the fluorescent ligand from receptor sites. However, at 30 °C, the fluorescence loss was more localised. The change in fluorescence observed at these temperatures is consistent with the movement of highly fluorescent intracellular vesicles and can be rationalised assuming internalisation of receptor-bound fluorescent ligand and its final appearance in the lysosomal compartment where the high concentration of fluorescein and the acid medium may lead to quenching, thus explaining the reduction in fluorescence. Due to the relatively high concentration of fluorescein-glucagon (10^{-7} M) used in these experiments, it is likely that both the high- and low-affinity sites were visualised [7].

Discussion

In this paper, it was established that primary hepatocytes in monolayer culture are suited for the measurement of glucagon receptor distribution by microscope imaging techniques. Monolayer cultures seem to offer advantages over cells in suspension, since we showed that the rate of glucagon breakdown in monolayer cultures is reduced and the non-specific binding component remains unchanged with time. Furthermore, no peak was seen in the [^{125}I]glucagon-binding curve as was observed with cells in suspension (this study and Ref. 11). These findings, in general, indicate a greater stability and viability of adhered cells compared with cells in suspension. Using video intensification microscopy, we have examined the fate of two of the fluorescein-labelled glucagon derivatives whose preparation and properties were described in the previous paper [7]. The distribution of monofluorescein-Trp^{25}-glucagon was followed in primary hepatocyte monolayer cultures at different temperatures: after incubation at 4 °C, fluorescence was lost more randomly with time from all areas of the cell, although a greater loss appeared to occur in the perinuclear region where the greater part of the fluorescent intracellular vesicles were also concentrated (see Fig. 6). The fluorescence decay at the higher temperature (30 °C) was mainly confined to the highly fluorescent vesicles. These vesicles were shown to move during the measuring period, suggesting fairly rapid receptor internalisation and lysosomal accumulation with $t_{1/2} < 10$ min. Furthermore, at low temperatures, a considerable amount of the specifically bound glucagon could be removed by acid-washing, whereas little or no washout occurred at 30 °C (Fig. 2) and dissociation of the radiolabelled ligand was slower at this temperature (Fig. 3). Autoradiographic studies using [^{125}I]glucagon indicated that more than 30% of the specifically bound radioactive-labelled ligand was internalised in 60 min at 37 °C by rat hepatocytes in suspension and was concentrated in the lysosomal compartment [12]. Therefore, internalisation of glucagon receptor complexes occurs with much greater efficiency in hepatocytes in monolayer culture than in suspension. Such differences have been reported previously for EGF binding and A431 cells [13]. However, this comparison remains provisional because a distinction between internalised endocytic vesicles and plasma membrane-associated vesicles cannot be made based on the effect of acid-washing [14], nor is it possible to separate the effects of slow ligand dissociation which occurs following longer incubation periods with glucagon from internalisation and degradation [15–17].

Rapid internalisation in the perinuclear region and lysosomal accumulation was also seen with

ferritin-labelled EGF [8]. Thus, the data at the higher temperature are consistent with rapid internalisation of the ligand-bound receptor, but give no indication of the route taken by those receptors. The relatively high concentration of fluorescent ligand which had to be used in these experiments precluded, however, measurements of ligand association kinetics, as were previously made in the case of vasopressin binding to isolated kidney tubules [18].

Receptor clustering and aggregation is well documented for EGF and insulin binding to cells in culture [1,3,19]. The accumulation of fluorescence in the perinuclear region at 30 °C shown in this study could also be interpreted to indicate receptor clustering prior to internalisation. However, Fig. 6 shows that following incubation with fluorescein-Trp^{25}-glucagon at 4 °C, there is also some increase in the fluorescence of the perinuclear vesicles. However, since cells had to be viewed at 15–20 °C in order to prevent fogging of the optical system, it is probable that the changes in general cell surface fluorescence are the result of both ligand dissociation and, perhaps, receptor internalisation as the cells warm up. However, no attempt was made to distinguish between these possibilities and further investigation is required.

The results from video intensification microscopy of fluorescein-Trp^{25}-glucagon bound to primary hepatocytes in monolayer culture demonstrate the suitability of these ligands for the study of binding and internalisation of fluorescent glucagon receptor complexes. Such studies have previously only been possible using the electron microscope [12]. The results obtained with fluorescently labelled glucagon are in agreement with electron microscopic studies and show that these derivatives may be used to label the glucagon receptors on a living cell and monitor the fluorescence through the processes of receptor internalisation and eventual relocation in the lysosomal compartment.

However, the specifically bound fluorescence from these glucagon derivatives on primary hepatocytes did not give sufficient signal to allow quantitative receptor lateral-mobility measurements using the FRAP method. Currently, we are investigating the possibility that permanent cell-lines with lower autofluorescence and non-specific binding may be suitable for FRAP measurements using these fluorescent glucagon derivatives.

Acknowledgements

This study was supported by the D.F.G., Project grant A4, S.F.B. 176, University of Würzburg, the Fonds der chemischen Industrie e.V. and the Hoechst Company, Frankfurt. L.D.W. was the recipient of an Alexander von Humboldt award from the Humboldt Foundation.

References

1 Helmreich, E.J.M. and Elson, E.L. (1984) Adv. Cycl. Nuc. Prot. Phos. Res. 18, 1–62.
2 Rees, A.R., Gregoriou, M., Johnson, P. and Garland, P.B. (1984) EMBO J. 3, 1843–1847.
3 Schlessinger, J., Schechter, Y., Cuatrecasas, P. Willingham, M.C. and Pastan, I. (1978) Proc. Natl. Acad. Sci. USA 75, 5353–5357.
4 Henis, Y.I., Hekman, M., Elson, E.L. and Helmreich, E.J.M. (1982) Proc. Natl. Acad. Sci. USA 79, 2907–2911.
5 Cuatrecases, P. (1974) Annu. Rev. Biochem. 43, 169–214.
6 Tolkovsky, A.M. and Levitzki, A. (1978) Biochemistry 17, 3795–3810.
7 Heithier, H., Ward, L.D., Cantrill, R.C., Klein, H.W., Im, M.-J., Pollak, G., Freeman, B., Schiltz, E., Peters, R. and Helmreich, E.J.M. (1988) Biochim. Biophys. Acta 971, 298–306.
8 Haigler, H.T., Maxfield, F.R., Willingham, M.C. and Pastan, I. (1980) J. Biol. Chem. 255, 1239–1241.
9 Lipson, K.E., Kolhatkar, A.A., Cherskey, B.D. and Donner, D.B. (1986) Biochemistry 25, 2612–2620.
10 Bonnevie-Nielsen, V., Polonsky, K.S., Jaspan, J.J., Rubenstein, A.H., Schwartz, T.W. and Tager, H.S. (1982) Proc. Natl. Acad. Sci. USA 79, 2167–2171.
11 Mahan, L.C., Motulsky, H.J. and Insel, P.A. (1985) Proc. Natl. Acad. Sci. USA 82, 6566–6570.
12 Barazzone, P., Gorden, P., Carpentier, J.-L., Orci, L., Freychet, P. and Canivet, B. (1980) J. Clin. Invest. 66, 1081–1093.
13 Zidovetski, R., Yarden, Y., Schlessinger, J. and Jovin, T.M. (1981) Proc. Natl. Acad. Sci. USA 78, 6891–6895.
14 Hertel, C. and Perkins, J.P. (1987) J. Biol. Chem. 262, 11407–11409.
15 Horwitz, E.M., Jenkins, W.T., Hoosein, N.M. and Gurd, R.S. (1985) J. Biol. Chem. 260, 9307–9315.
16 Horwitz, E.M., Wyborski, R.J. and Gurd, R.S. (1986) J. Biol. Chem. 261, 13670–13676.
17 Corin, R.E., Ferriola, P. and Donner, D.B. (1982) J. Biol. Chem. 257, 1626–1631.
18 Kirk, K.L., Buku, A. and Eggena, P. (1987) Proc. Natl. Acad. Sci. USA 84, 6000–6004.
19 Schlessinger, J. (1980) Trends Biochem. Sci. 5, 210–214.