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SEQUESTERED A431 CELL β-ADRENOCEPTORS REMAIN FUNCTIONAL AFTER HOMOLOGOUS DESENSITIZATION

DEIRDRE COONEY and ALAN K. KEENAN*
Department of Pharmacology, University College, Belfield, Dublin 4, Republic of Ireland

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Abstract—This study seeks to investigate the functional state of sequestered β-adrenoceptors in A431 cells which have undergone homologous desensitization. Incubation of cells with isoprenaline under desensitizing conditions caused a reduction (a) in the number of cell surface β-receptors as measured by ^{3}H-CGP 12,177 binding, and (b) in the extent of agonist-stimulatable adenylate cyclase activity in membranes prepared from desensitized cells. We infer that those receptors have been sequestered to an internal membrane site since they were detectable by the lipophilic ligand ^{125}I-CYP but not by the more hydrophilic ^{3}H-CGP 12,177. We confirmed that sequestration had occurred by fractionation on non-linear sucrose density gradients of membranes prepared from desensitized cells. The lighter density membrane fraction contained up to 50% of the total receptor pool after desensitization, but only 5–7% of membrane (fluoride-stimulatable) adenylate cyclase. Fusion of desensitized cells in the presence of polyethylene glycol caused a reassociation of sequestered β-receptors with their biochemical effector Gs since agonist-dependent adenylate cyclase stimulation was restored to the levels measurable after fusion of non-desensitized cells. We conclude that sequestered β-receptors in desensitized A431 cells are fully functional.

Homologous desensitization of the β-adrenergic receptor-adenylate cyclase system is thought to involve sequestration of cell surface receptors into a membrane compartment in which the receptor has no contact with its biochemical effector Gs† which functions normally [1, 2]. While there has been some controversy in the past as to whether receptor–Gs uncoupling precedes sequestration [3, 4], the balance of evidence now favours a parallel time-course for the two processes in a number of cell systems [5, 6]. Using the hydrophilic ligand ^{3}H-CGP 12,177 the disappearance of cell surface β-receptors in WEH17 lymphoma and C6 glioma cells has been shown to occur with half times of the order of 2 min and to parallel the decrease in hormone-stimulated adenylate cyclase activity [7–9].

A major limitation in the interpretation of these data and the establishment of a distinction between uncoupled and sequestered states of the receptor has been the technical difficulty in assessing receptor sequestration. In this study we investigate the relationship between β-receptor sequestration and desensitization in A431 cells, following fractionation of membranes prepared from untreated and desensitized cells on non-linear sucrose density gradients. We demonstrate that the functional activity of desensitized receptor is fully restored after fusion together of desensitized cells with polyethylene glycol (PEG).

* To whom reprint requests and correspondence should be addressed.

† Abbreviations used: Gs, stimulatory guanine nucleotide binding protein; Con A, concanavalin A; CYP, cyanopindolol; PEG, polyethylene glycol.

MATERIALS AND METHODS

Materials. ^{3}H-CGP 12,177 and ^{125}I-cyanopindolol (^{125}I-CYP) were obtained from Amersham Buchler. α-^{32}P-ATP and ^{125}I-pindolol were from New England Nuclear (Boston MA). ICI 118,551 was a gift from ICI. All other chemicals were of the highest purity commercially available. A431 cells (E III subclone) were a gift from the Department of Virology and Immunology, University of Wurzburg Medical School, F.R.G.

Cell culture. A431 cells were grown in RPMI-1640 medium (Biochrome) supplemented with 5% fetal calf serum (Boehringer, Mannheim) and $NaHCO_3$ was added to a final concentration of 23.8 mM. Routinely cells were seeded at a density of 6×10^6 cells/flask (650 ml/175 cm^3) or 145,000 cells per petri dish. Trypsin (0.25%) was used to detach cells during subculture. Cells were grown for 1–2 days in serum-containing medium which was then replaced by serum-free medium and the cells allowed to grow for 1 more day before use. Cultures were regularly screened for mycoplasm infections using the fluorescent DNA stain bisbenzimidazole (Hoechst).

Desensitization and preparation of membranes. Monolayer cultures of A431 cells were desensitized by incubation in phosphate buffered saline (PBS) containing 10 μM 1-isoprenaline and 0.1 mM ascorbic acid at 37° for 30 min. The incubation was ended by removal of the medium and the cell layers were washed 4 times with PBS at 37°. Ice-cold PBS was then added and cells were detached using a rubber policeman. Cells were collected by centrifugation at 500 *g*, resuspended in buffer A

(150 mM NaCl, 20 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.8) and disrupted by nitrogen cavitation using a Parr cell disruption bomb (500 psi N_2 for 20 min). The lysate was centrifuged at 600 *g* for 10 min and the resulting supernatant recentrifuged at 30,000 *g* for 30 min. Membranes were washed in 90:10 buffer (90 mM NaCl, 10 mM Tris–HCl, pH 7.4) and suspended in the appropriate assay buffer or stored in liquid nitrogen in buffer B (10 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, pH 7.4).

Binding of ^{3}H-*CGP* 12,177 *to intact cells.* Monolayer cultures of cells were washed 4 times with PBS at 37° and all subsequent procedures were carried out at 4°. Samples were incubated for 12 hr in PBS containing increasing concentrations of ^{3}H-CGP 12,177 (0.05–10 nM). Non-specific binding for this and all other experiments was determined in the presence of 1 μM l-propranolol. Incubations were ended by aspiration of the supernatant, the cells were washed 4 times with cold PBS and removed from the dish with 0.2 M NaOH. Sample radioactivity was measured in a total volume of 3 ml NaOH to which was added 20 ml scintillant. Cell number per dish was determined either using a hemocytometer (in which case cells were removed from the dish using PBS containing 2 mM EDTA) or by the Peterson method of protein determination [10] and using the standard of 71 μg protein being equivalent to 10^5 cells.

Binding of ^{3}H-*CGP* 12,177 *to membranes.* Samples containing 100–150 μg protein were incubated for 45 min at 37°, in a final volume of 4 ml, in buffer C (150 mM NaCl, 10 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4), with increasing concentrations of ligand (as above). Samples were then passed over Whatman GF/C filters and the filters were washed 4 times with 4 ml ice-cold 90:10 buffer before addition of 10 ml scintillant and counting.

Binding of ^{125}I-CYP *to membranes.* Samples containing 2–10 μg protein were incubated for 60 min at 37° in a final volume of 600 μl or 1 ml, in buffer C, with ligand (5–250 pM). After filtration on GF/C filters as described for ^{3}H-CGP 12,177, radioactivity was measured by gamma counting.

Displacement experiments. Incubations (500 μl) contained 25–50 pM ^{125}I-pindolol, 5–15 μg membrane protein and increasing concentrations of isoprenaline (1 nM–100 μM) in buffer C. After incubation at 37° for 60 min, samples were treated as described for ^{125}I-CYP binding to membranes.

Adenylate cyclase activity in membranes. The method of Salomon *et al.* [11] was used. Routinely samples (150 μl) contained 20–50 μg protein, 0.5 mM ATP, 1–2 μCi α-^{32}P-ATP, 0.4 mg/ml theophylline, 0.3 mg/ml creatinine phosphokinase, 3 mg/ml creatinine phosphate and 3 mM $MgCl_2$ in TME buffer (50 mM Tris–HCl, 2 mM $MgCl_2$, 1 mM EDTA). Stimulated activity was determined in the presence of l-isoprenaline (10 μM) with or without guanyl nucleotides (10 μM) or with NaF (10 mM) or forskolin (0.1 mM). Assays were ended after 20 min at 37°, by addition of 200 μl stopping solution (10 mM Tris–HCl, 2% SDS, 2 mg/ml ATP and 0.45 mg/ml cAMP pH 7.5). After chromatography of cAMP, specific activities were expressed as pmol cAMP/mg protein/min.

Membrane fractionation on sucrose density gradients. Samples were prepared for sucrose density gradient centrifugation by incubating cells prior to lysis with 0.25 mg/ml Con A for 30 min on ice. Following lysis the samples were centrifuged at 500 *g* for 5 min to remove unlysed cells and the supernatant (10–20 mg protein) was layered onto a non-linear sucrose gradient (5–50% sucrose, w/w, containing an intermediate plateau at 31%, to improve separation in this region). Sucrose solutions were prepared in 10 mM HEPES, 1 mM EDTA, pH 8. Gradients were centrifuged at 100,000 *g* for 90 min using a Beckman SW-27 rotor. After removal of a volume corresponding to that of the applied sample, 1 ml fractions were collected from the top of the gradient by pumping a solution of 60% sucrose (1.5 ml/min) through the bottom of the centrifuge tube. Receptor binding and fluoride-stimulated adenylate cyclase activities in fractions were determined as described above and sucrose concentrations were measured using a refractometer (Zeiss).

Fusion experiments. A431 cells were fused to each other essentially as described by Strasser and Lefkowitz [12] except that asolectin was substituted for phosphatidylcholine. Briefly, washed cell pellets were suspended in a final volume of 600 μl with ice-cold fusion buffer (130 mM NaCl, 5 mM KCl, 4.8 mM $MgCl_2$, 10 mM Tris–HCl, 5 mM ATP, 5 mM glucose, pH 7.4) at 30°. A lipid mixture (50 μl) containing 10 mg/ml asolectin, 0.5 mg/ml lysolecithin (type VI from soybean) and 20 μl of 1 M $MgCl_2$, was then added and the mixture was incubated on ice for 5 min. A 50% (w/w) PEG solution (500 μl) in fusion buffer (or for control incubations fusion buffer alone) was added to the cell suspension after both had been equilibrated at 30° for 1 min. After further incubation for 90 sec, the fusion mixture was serially diluted at 100-sec intervals by the addition of 0.35, 0.5, 1.5, 3.5 and 7.5 ml fusion buffer. Cells were then pelleted by centrifugation (500 *g*, 5 min, 4°) and resuspended in 12 ml buffer A for preparation of membranes as described above.

RESULTS

Desensitization of the β-adrenoceptor–adenylate cyclase system

Following treatment of cell monolayers with l-isoprenaline for 30 min, a reduction of over 50% was observed in the number of cell binding sites for ^{3}H-CGP 12,177 without a change of affinity (Fig. 1). After preparation of crude membrane fractions from desensitized and untreated cells, membrane binding activities were compared using ^{3}H-CGP 12,177 or the more liphophilic ^{125}I-CYP. In desensitized membranes there were 50% fewer binding sites as measured by ^{3}H-CGP 12,177 binding, similar to the observation for intact cell binding, while no such reduction was seen with ^{125}I-CYP binding (Fig. 2 and Table 1A). The K_D values for either ligand in membranes were unaltered after desensitization. In desensitized membranes there was also a marked reduction in hormone-stimulated adenylate cyclase activity though not in the stimulations by Gpp(NH)p.

Table 1. Mean paired differences between (A) receptor binding (fmol/mg) and (B) adenylate cyclase activities (pmol cAMP/mg/min) in membranes prepared from control and desensitized cells, together with 95% confidence limits

		Mean difference	95% Confidence limits
A.	^{3}H-CGP 12,177 binding	191.30	±73.30
	^{125}I-CYP binding	0.30	±86.75
B.	Basal	−0.36	±2.32
	Isoprenaline	32.38	±8.55
	Gpp(NH)p	10.36	±5.57
	NaF	−5.70	±55.0
	Forskolin	33.60	±132.85

NaF or forskolin (Fig. 3 and Table 1B). Further confirmation of receptor–Gs uncoupling was obtained from the observed reduction in the ability of GTP to cause a rightward shift of the agonist displacement curve after desensitization (Fig. 4).

Separation of membrane and "light density" fractions after desensitization

It was decided to resolve the crude membrane fraction further, with a view to separation of pure plasma membranes from other cellular membranes. Such a separation would assist in determining whether the uncoupling of receptor and Gs was due to an inherent decrease in receptor functionality or to microsequestration into either a domain of the plasma membrane or another membrane compartment as suggested by the observed reduction in ^{3}H-CGP 12,177 binding. The preparation of plasma membranes in the presence of Con A has been used by others to separate cell fractions containing β-

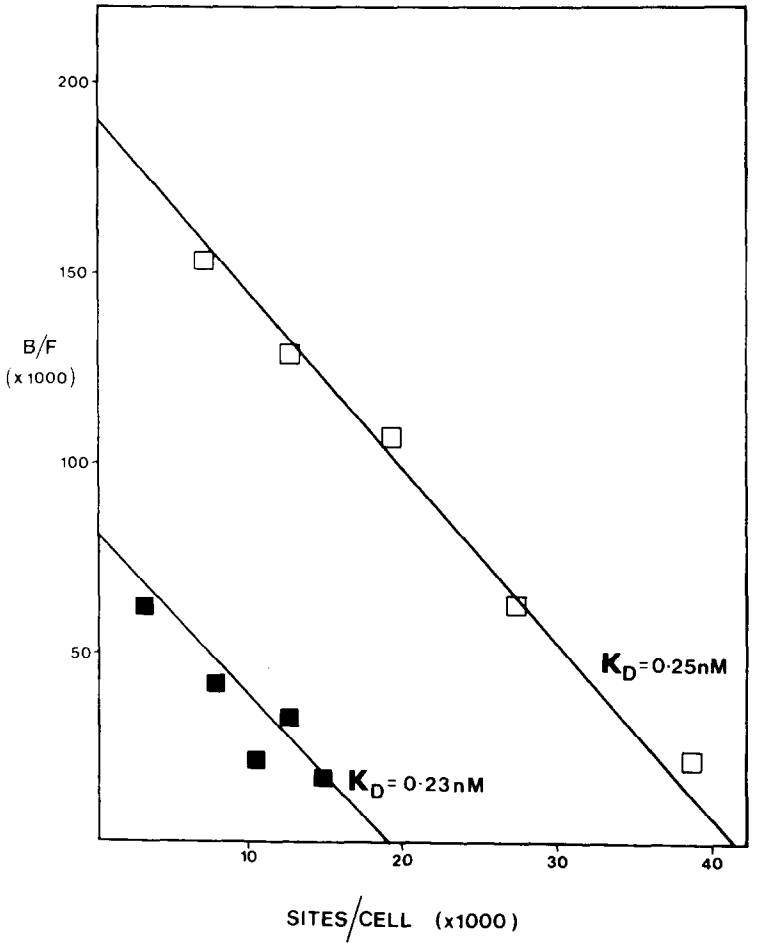

Fig. 1. Scatchard analysis of ^{3}H-CGP 12,177 binding to control □ and desensitized ■ A431 cells.

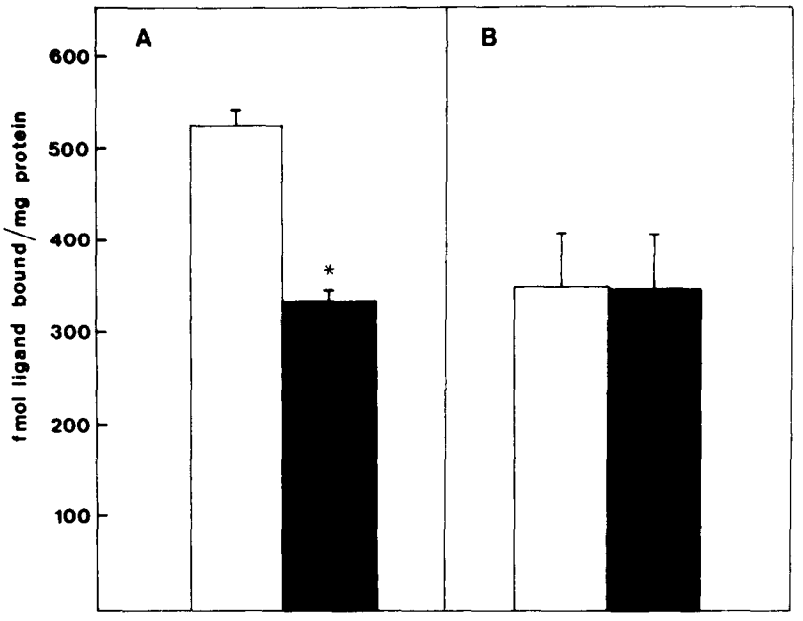

Fig. 2. Comparison of ^{3}H-CGP 12,177 (A) and ^{125}I-CYP (B) binding to membranes prepared from control (□) and desensitized (■) A431 cells. Data shown are means ± SEM of 3 sets of experiments. *Significantly different from control, $P < 0.05$ (paired t-test).

receptor binding activity [3]. The apparent basis of the usefulness of Con A in this method involves a crosslinking reaction which stabilises the plasma membranes to fragmentation and vesiculation during cell lysis. Thus lysis of cells treated with Con A results in the formation of plasma membrane fragments which migrate as more uniform particles to heavier densities on sucrose density gradients.

After preparation of homogenates from control and desensitized cells, and resolution on sucrose density gradients, two peaks containing ^{125}I-CYP binding were detected. The "heavy density" membrane fraction migrated between sucrose densities of 35 and 40% (w/w) and coincided with the plasma membrane marker adenylate cyclase; a second light density membrane fraction containing 5–7% of

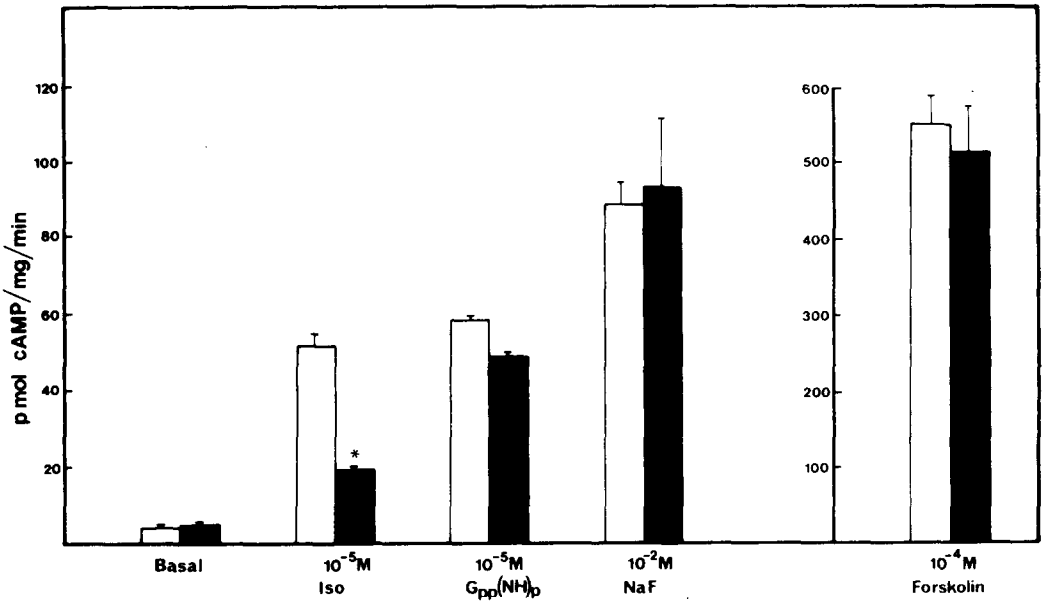

Fig. 3. Adenylate cyclase activity in membranes prepared from control (□) and desensitized (■) A431 cells. Values are means ± SEM of 3 independent experiments performed in duplicate. *Significantly different from control, $P < 0.01$ (paired t-test).

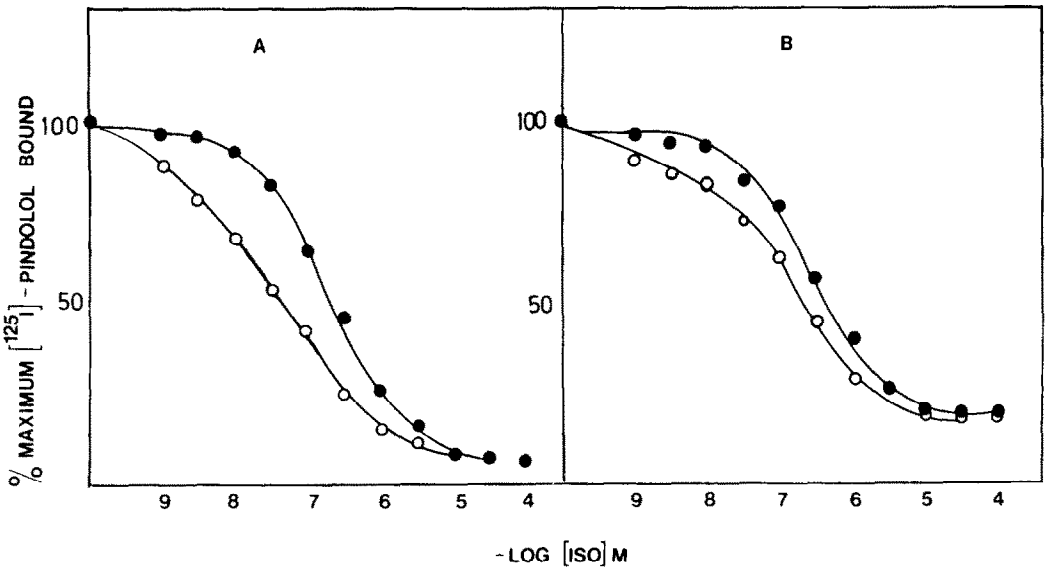

Fig. 4. GTP-induced shift of high affinity agonist (isoprenaline) binding to membranes prepared from control (A) and desensitized (B) cells: open circles—GTP; closed circles + 10 μM GTP.

cyclase activity migrated at lower sucrose densities (23–26%, w/w; see Figs 5 and 6). In untreated cells the majority of receptor (80–85%) was present in the plasma membrane while after desensitization a translocation of receptors from the plasma membrane fraction to the light density fraction (50% of receptor binding in each fraction) had occurred (Fig. 5).

Restoration of receptor–Gs coupling after fusion of desensitized cells

via Gs or the catalytic sub-unit (Fig. 3). Further desensitized cells were subjected to the fusion procedure in the absense of PEG, there was no significant effect of the treatment of adenylate cyclase activity in membranes. There was likewise no effect on the extent of desensitization of isoprenaline-stimulated cyclase activity (compare Fig. 3 with Fig. 7A). Although fusion of control cells in the presence of PEG caused a marked increase in basal and stimulatable cyclase activities, the fold stimulation over basal remained constant (isoprenaline 8-fold, NaF 25-fold, Fig. 7B). When desensitized cells were fused in the presence of PEG, similar absolute values were obtained to those seen in fused controls including

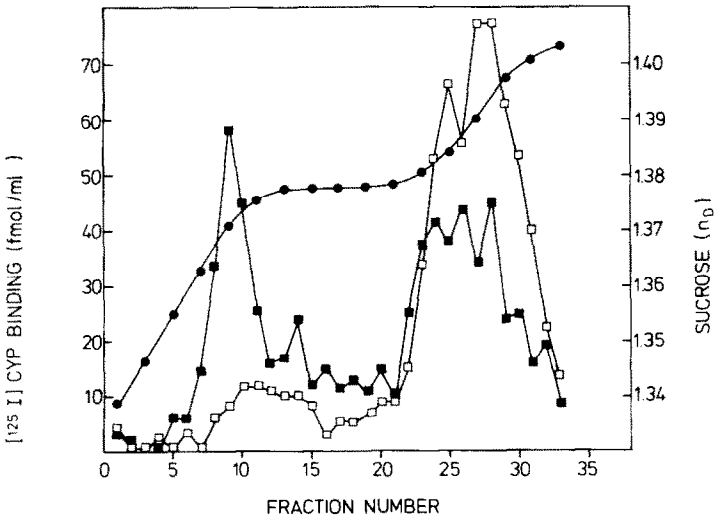

Fig. 5. Non-linear sucrose density gradient fractionation of homogenates prepared from control (□) and desensitized (■) A431 cells. Specific binding of fractions was measured using 50 pM ^{125}I-CYP. Sucrose concentrations in fractions (closed circles) are expressed in refractive index units (n_D).

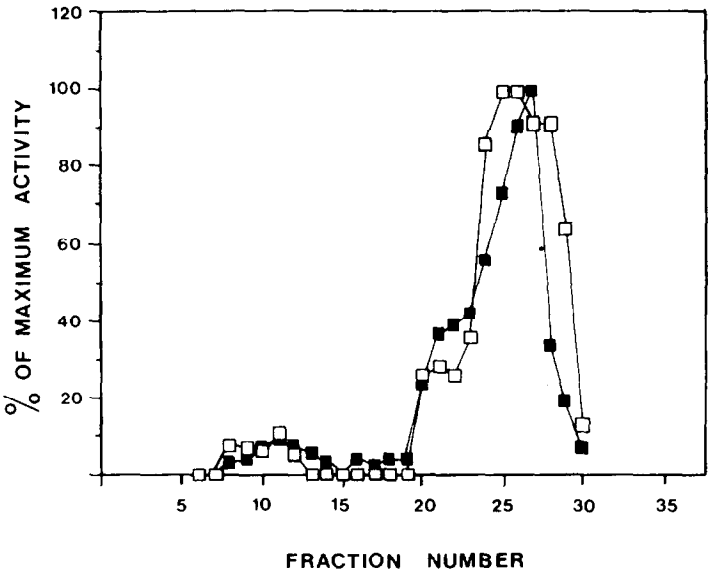

Fig. 6. NaF-stimulated adenylate cyclase activity in fractions recovered from sucrose gradient centrifugation of control (□) and desensitized (■) cell homogenates.

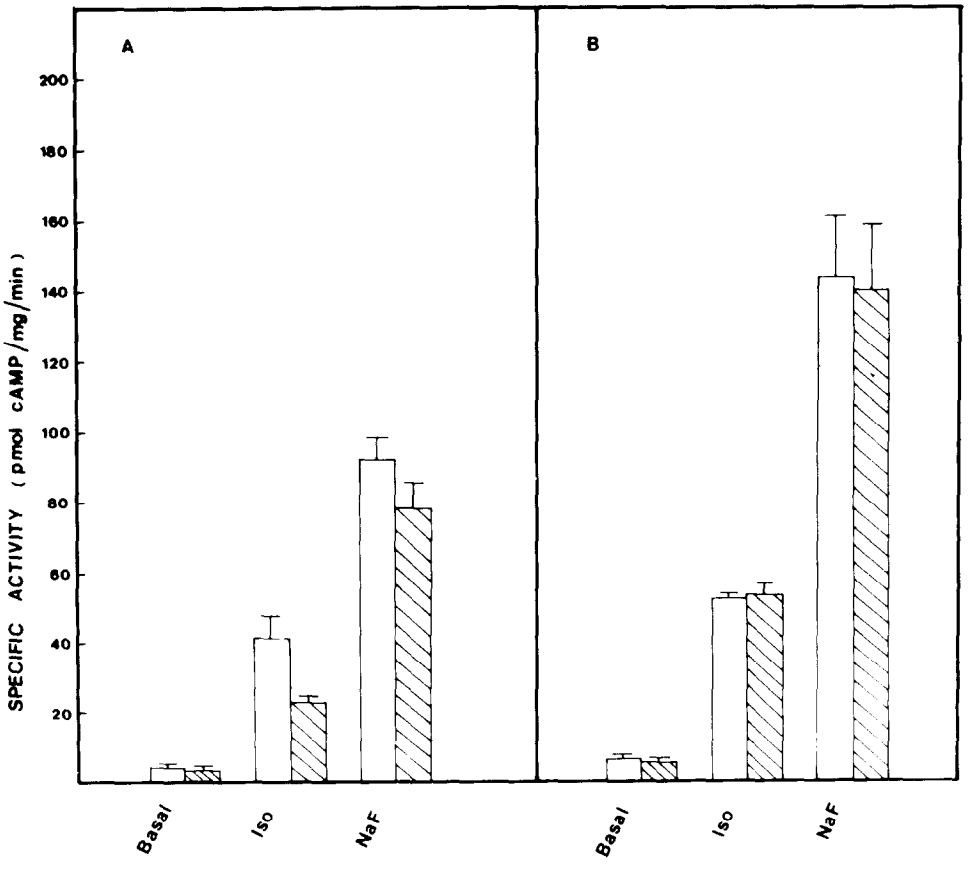

Fig. 7. Adenylate cyclase activity in membranes prepared from control (□) and desenitized (■) A431 cells following treatment of cells in the absence (A) and presence (B) of PEG. Activities were measured with 10 μM GTP (Basal), 10 μM GTP + 10 μM l-isoprenaline (Iso) or 10 mM NaF. Data shown are means ± SEM of 3 sets of experiments.

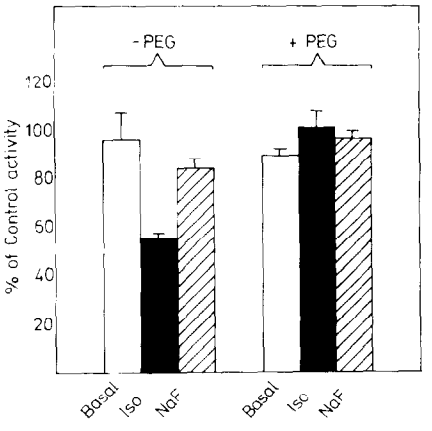

Fig. 8. Adenylate cyclase activity in membranes prepared from desensitized A431 cells following treatment of cells in the absence or presence of PEG. Measurements were made as in Fig. 7 (Basal □, Iso ■, NaF ▨) and results were expressed as a percentage of the corresponding value before desensitization. Data shown are means ± SEM of 3 sets of experiments.

full isoprenaline-stimulated cyclase activity (Fig. 7B and Fig. 8).

DISCUSSION

This study shows sequestration of β-receptors to be the critical event in their uncoupling from adenylate cyclase during homologous desensitization. We have shown that the isoprenaline-induced desensitization in A431 cells is homologous since diminished hormone responsiveness was not accompanied by a change in the level of adenylate cyclase activation via Gs or the catalytic sub-unit (Fig. 3). Further evidence that the uncoupling occurred at receptor–Gs level was obtained from the apparent conversion of high to low affinity agonist binding sites (Fig. 4). Receptors were not degraded under desensitizing conditions but were translocated from the cell surface to an internal membrane site where they were detectable by the lipophilic ligand ^{125}I-CYP but not by the more hydroyphilic ^{3}H-CGP 12,177 (see Figs 1 and 2). Both of these binding sites would seem to be tightly associated with the membrane since they co-purified following preparation of membrane from desensitized cells. Furthermore, separation of these binding sites was achieved only using non-linear sucrose density gradients, in agreement with the findings of other workers who separated β-receptor sub-populations after desensitization in a number of mammalian cell lines [13]. Although the compartment in which the sequestered receptors were found (the light density fraction) was virtually devoid of plasma membrane marker adenylate cyclase activity, the possibility cannot be ruled out that in the intact cell this compartment is somehow physically associated with the plasma membrane.

When desensitized cells were fused together with PEG, reassociation of sequestered receptors with their biochemical effector Gs occurred as evidenced by the ability of hormone to once more stimulate adenylate cyclase activity as in control cells (Figs 7 and 8). The strong causal link between homologous desensitization and receptor sequestration implicit in our results obtained with A431 cells has been the subject of much discussion in regard to homologous desensitization in general. Strulovici *et al.* [14] have shown internalised β-receptors isolated from desensitized frog erythrocytes to be fully active in coupling with Gs after fusion with *Xenopus laevis* erythrocytes. Similar conclusions have been reached in studies using S49 cells [15] and desensitized mammalian lung β-receptors were equieffective with controls in coupling to human erythrocyte Gs after co-reconstitution into phospholipid vesicles [16]. In contrast, Kassis and Fishman [17] using membrane–membrane and membrane–cell fusion techniques, found that homologous desensitization in a number of cell types led to a functional alteration of the β-receptor which was not reversed by reassociation of components in the presence of fusogens. It is possible, however, that these workers used conditions which were not optimal for fusion, as suggested by Strasser and Lefkowitz [12].

It should be pointed out that the conclusions reached from this study do not exclude the possibility that an early functional receptor alteration such as phosphorylation precedes sequestration in homologous desensitization as has previously been proposed [3]. We, however, have been unable to detect any change in the apparent molecular weight of desensitized A431 cell β-receptors on SDS–PAGE after photoaffinity labelling with ^{125}I-CYPazide (data not shown). Similar observations have been made by others in frog erythrocytes [18] and in rat lung [19]. Although there have been reports of enhanced receptor phosphorylation during homologous desensitization [20], this does not appear to result in decreased mobility on SDS gels, in contrast to the observations described for the desensitized turkey erythrocyte β_1-receptor [21, 22].

Recently some doubt has been cast on the contribution of receptor phosphorylation to homologous desensitization by the finding that the carboxyl terminus of the hamster β-receptor expressed in mouse L cells was not required for hormone-induced receptor sequestration or functional desensitization of adenylate cyclase [23]. Putative phosphorylation sites on the carboxyl terminus have been identified for both cAMP-dependent and β-receptor-specific protein kinases and deletion mutants lacking these sites did not show any impaired ability to undergo hormone-mediated sequestration of β-receptors.

Whatever the phosphorylation state of sequestered β-receptor in A431 cells, we have shown that its functionality is restored by the probable increase in membrane fluidity produced by PEG during fusion of desensitized cells and that sequestration *per se* accounts for the desensitization.

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