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Differential internalization and processing of atrial-natriuretic-factor B and C receptors in PC12 cells

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PC12 cells express two atrial-natriuretic-factor-(ANF)-receptor subtypes with molecular masses of 130000 (B receptor) and 70000 (C receptor). The B-receptor subtype constitutes 65% of the cell-surface receptor population, and the remaining 35% are C receptors as determined by saturation binding studies in the presence of C-ANF, a C-receptor-selective analogue. ANF-(99-126)-peptide [ANF(99–126)], which can bind to both B- and C-receptor subtypes, was rapidly internalized into the cells after incubation at 37 °C. Internalization of ^{125}I-ANF(99–126) was used as an index of the receptor-mediated endocytosis and to quantify receptor internalization. In the presence of a saturating concentration of C-ANF, receptor-mediated internalization of ^{125}I-ANF(99–126) was reduced by 24%, indicating B receptors mediate 76% of ligand internalization. Incubation of cells with 10 μM-ANF at 37 °C down-regulated both receptor subtypes as reflected by decreased surface binding. Time-dependent studies suggest that B- and C-receptor subtypes undergo differential down-regulation. Incubation of down-regulated cells for 120 min in ANF-free medium produced a recovery of 35% of the original cell-surface binding. Affinity cross-linking of ^{125}I-ANF to the receptors on the plasma membrane in re-incubated (up-regulated) cells demonstrated expression of predominantly the B-receptor subtype. Monensin blocked 72% of receptor up-regulation, whereas cycloheximide inhibited 43%, suggesting an active recycling mechanism involved in mediating up-regulation of the B receptors. The present study demonstrates a rapid internalization and intracellular recycling mechanism for B receptors in PC12 cells. C receptors also undergo internalization and down-regulation, but recycling of this receptor subtype into the plasma membrane occurs at a lower rate and to a lesser extent than is the case for the B receptor.

INTRODUCTION

Atrial natriuretic factor (ANF) binds to two receptor subtypes with M_r 130000 and 70000 which have been characterized in a variety of tissues and cell types [1]. The high-M_r receptor is a bifunctional protein with an extracellular ANF-binding domain and an intracellular guanylate cyclase domain [2]. The guanylate cyclase-coupled receptor subtype is termed the B receptor, since a number of biological actions of ANF are thought to be mediated through this receptor subtype [3–9]. The low-M_r receptor is a functionally and morphologically distinct homodimeric protein [10,11]. It has been proposed that this receptor type clears endogenous ANF from the circulation after ligand binding [11,12] and is widely termed the 'C receptor'. Many target tissues contain both ANF receptor subtypes in variable proportions [1], and it appears that the multiple receptor subtypes generate diverse ANF responses. Since the two receptor subtypes appear to modulate different biological responses to the hormone, it is possible the cellular expression and regulation of receptor subtypes also could differ.

ANF-receptor internalization and intracellular processing have been observed in a variety of cell types, including vascular smooth muscle [13] anterior pituitary [14], pancreatic [15], corticotropin-secreting AtT-20 cells [16] and in rat adrenal-medullary cells [17]. In phaeochromocytoma (PC12) cells, we observed rapid internalization of ANF receptors, subsequent to ligand binding [18]. All the above studies were conducted either with anti-ANF antibodies for intracellular localization of internalized ANF or with radiolabelled ANF to determine intracellular distribution. These studies reflect only the total internalization processes, which have been proposed to be primarily mediated by C receptors [11–19]. The ability of both B and C receptors to undergo internalization and to modulate cell-surface receptor populations has not been reported.

In the present study the cell-surface expression and internalization of ANF receptor subtypes are characterized, and the differential cellular regulation of the receptor subtypes has been determined. It appears that an active recycling pathway plays a role in modulating the cell-surface B- and C-receptor populations in the PC12 cells.

MATERIALS AND METHODS

Synthetic ANF-(99–126)-peptide [ANF(99–126)] was obtained from Sigma Chemical Co., and an ANF-C-receptor-selective analogue (C-ANF) from Cambridge Research Biochemicals (New York, NY, U.S.A.). Atriopeptin-II(103–125) and atriopeptin-I(103–123) were purchased from Peninsula Research Laboratories (Belmont, CA., U.S.A.) and ^{125}I-ANF (2000 Ci/mmol) was obtained from the Amersham Corp. (Arlington Heights, IL, U.S.A.).

PC12 cell culture

PC12 cells were obtained from the American Type Culture collection (CRL No. 1721) and maintained in RPMI medium with 10% (v/v) heat-inactivated horse serum, 5% (v/v) fetal-bovine serum, 50 units of penicillin/ml and 50 μg of streptomycin/ml. The cells were grown in a humidified air/CO_2 (19:1) atmosphere at 37 °C.

Abbreviations used: ANF, atrial natriuretic factor; ANF(99–126) etc., ANF-(99–126)-peptide etc.; C-ANF, an ANF-C-receptor-selective analogue; AP, atriopeptin.

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Receptor binding assays

For binding assays, PC12 cells were harvested and washed twice with serum-free RPMI medium. The cells were re-suspended in medium containing 0.2% BSA and 1.0 mg of bacitracin/ml, 20 μg of leupeptin/ml and aprotinin. This medium was used for diluting the labelled and unlabelled ANF. ^{125}I-ANF was incubated with 1×10^5 cells in a volume of 100 μl at 0 °C for 120 min. All assays were performed in polystyrene tubes and the cells were pre-cooled before the addition of ^{125}I-ANF. At the end of incubation the bound and unbound radiolabelled ligands were separated by rapid filtration using GF/C glass-fibre filters treated with 0.05% Tween-20 in phosphate-buffered saline (10 mM-phosphate/145 mM-NaCl, pH 7.4). The filters were washed rapidly with an additional 10 ml of ice-cold 0.15 M-NaCl. Non-specific binding was determined in the presence of 10 μM unlabelled ANF. For competition binding studies, PC12 cells were incubated with ^{125}I-ANF in the presence of unlabelled ANF analogue such as atriopeptin-I(103–123) [AP-I(103–123)] and C-ANF.

Affinity cross-linking of ANF receptors

Confluent cells were washed with serum-free RPMI medium and homogenized using a Polytron instrument by suspending the cells in ice-cold medium containing 0.25 M-sucrose. The homogenate was centrifuged at 1500 *g* for 5 min and the supernatant collected was centrifuged again at 8000 *g* for 10 min. The supernatant was centrifuged at 40000 *g* for 45 min and the pellet was re-suspended in the serum-free RPMI medium containing proteinase inhibitors and centrifuged again at 40000 *g* for 30 min to remove the sucrose. Washing was repeated twice and the final pellet was re-suspended in binding medium. The membrane suspension (100 μg/tube) was incubated with ^{125}I-ANF(99–126) for 120 min at 0 °C in a total volume of 100 μl. At the end of incubation, an equal volume of 2 mM-bis(sulphosuccinimidyl) suberate was added and the incubation was continued for an additional 120 min at room temperature. Finally, the samples were solubilized and electrophoresed by the method of Laemmli [20] using a 7.5% (w/v)-acrylamide separating gel under reducing conditions. The gels were dried and exposed to Kodak X-Omat X-ray film at −70 °C for 7 days. The band densities of the autoradiograms were determined by scanning on a Hoefer transmittance/reflectance densitometer. Each study was repeated three or four times and the variation among studies was < 10%.

Measurement of receptor internalization

PC12 cells were washed in pre-warmed, serum-free, RPMI medium and loosely pelleted by centrifugation at 300 *g* for 5 min. Washed PC12 cells (1×10^5 cells/tube) were incubated with ^{125}I-ANF(99–126) at 37 °C for different times. At the end of specific incubation periods, cells were washed immediately by adding ice-cold 0.15 M-NaCl and centrifuging at 300 *g* for 2 min at 0 °C. ANF bound to the cell surface was determined by extracting radioactivity from intact cells with 0.2 M-acetic acid in 0.5 M-NaCl [21]. Acid-washed cells were solubilized in 1 M-NaOH and the radioactivity associated with the cells was taken to represent the internalized ligand.

Quantification of receptor down-regulation and recycling

ANF receptors were down-regulated by incubating cells with 10 μM of unlabelled ANF(99–126) for 120 min at 37 °C. At the end of the incubation, cells were washed with serum-free RPMI medium and re-incubated in ANF-free medium at 37 °C for 120 min. To assess receptor recycling, cells were re-incubated with and without monensin (25 μM), cytochalasin B (10 μM) or cycloheximide (10 μg/ml) at 37 °C for 120 min. At the end of incubation, cells were washed and receptor binding was determined by incubating with ^{125}I-ANF(99–126) at 0 °C. For affinity cross-linking experiments, plasma membranes were prepared from the re-incubated cells and the receptor cross-linking was performed as described above.

RESULTS

PC12 cells exhibited specific, saturable binding of ^{125}I-ANF (99–126) with an apparent K_d of 3.28×10^{-10} M and $B_{max.}$ of 1.45×10^{-10} M for 1×10^5 cells (Fig. 1). Affinity cross-linking of radiolabelled ligand to receptors confirmed the presence of two receptor subtypes, in the M_r ranges of 130000 and 70000 (Fig. 2). Incubation of plasma membranes with 200 pM of ^{125}I-ANF(99–126) produced maximum cross-linking as compared with 100 pM, indicating maximum binding at saturating concentration (Fig. 2). The proportion of ^{125}I-ANF(99–126) cross-linking to B and C

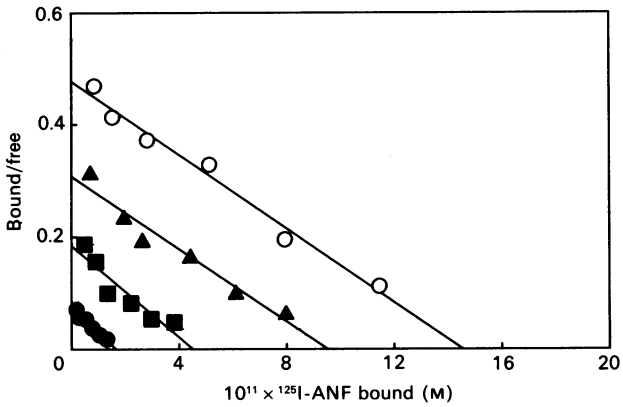

Fig. 1. Scatchard analysis of ^{125}I-ANF binding to receptors

PC12 cells (1×10^5) were incubated with ^{125}I-ANF(99–126) for 120 min at 0 °C in the presence of 10 μM each of C-ANF (▲) and AP-I (■). Binding was analysed in down-regulated cells (●) by incubating with 10 μM unlabelled ANF(99–126) for 120 min at 37 °C. Total binding was determined with control cells in the absence of analogues (○).

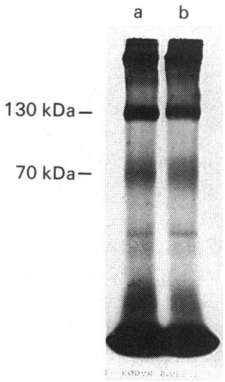

Fig. 2. Affinity labelling of ANF receptors

^{125}I-ANF(99–126) was cross-linked to receptors by incubation of plasma membranes at 0 °C for 120 min, followed by addition of bis(sulphosuccinimidyl) suberate. The plasma membranes were incubated with 200 pM (lane a) or 100 pM (lane b) of ^{125}I-ANF. The membranes were solubilized and electrophoresed by the method of Laemmli [20], using a 7.5%-(w/v)-acrylamide separating gel under reducing conditions. The gels were dried and exposed to Kodak X-Omat X-ray film at −70 °C for 7 days. A representative radiogram from four experiments is shown.

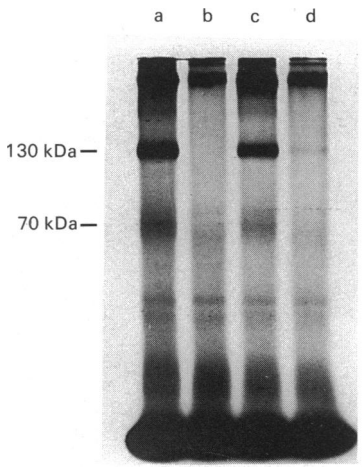

Fig. 3. Effect of ANF analogues on receptor binding

PC12 cell plasma membranes were incubated with ^{125}I-ANF(99–126) in the presence or absence of 10 μM of unlabelled ANF analogues. Lanes: (a) membranes incubated with ^{125}I-ANF(99–126) alone; (b) unlabelled ANF(99–126)+^{125}I-ANF(99–126); (c) membranes incubated in the presence of C-ANF+^{125}I-ANF(99–126); (d) the membrane incubated with AP-I+^{125}I-ANF. A representative autoradiogram from one of three experiments is shown.

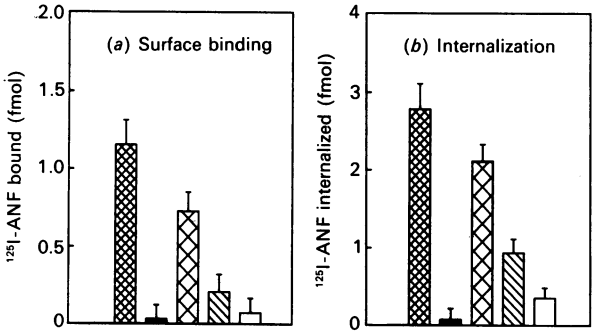

Fig. 4. Surface binding (*a*) and internalization (*b*) of ^{125}I-ANF in presence of ANF analogues

PC12 cells (1×10^5) were incubated with ^{125}I-ANF(99–126) at 37 °C in the absence and presence of 10 μM unlabelled ANF(99–126), C-ANF, AP-I and AP-II for 60 min. At the end of the incubations, surface-bound ligand radioactivity was removed by washing with acetic acid/NaCl, and the radioactivity associated with the acid-washed cells, which represented the amount of ^{125}I-ANF(99–126) internalized, was determined. ^{125}I-ANF(99–126) content of the acid extract represented the corresponding cell-surface binding. ⊠, Control; ■, ANF(99–126); ⊠, C-ANF; ⧅, AP-I; □, AP-II.

receptors in all control membranes was 66±8% and 30±6% respectively as determined by measuring the band densities in the autoradiograms.

Affinity cross-linking of ^{125}I-ANF to receptors in the presence of unlabelled ANF(99–126), C-ANF and AP-I(103–123) enabled the determination of ligand specificity for the receptor subtypes. Unlabelled ANF(99–126) displaced both high- and low-M_r receptor binding in the PC12 cell plasma membrane (Fig. 3). C-ANF (10 μM) selectively displaced only the low-M_r receptor binding in PC12 cells. Lower concentrations of C-ANF (< 10 μM) did not produce maximal displacement of the low-M_r receptor binding in the affinity cross-linking experiment. Interestingly, 10 μM-AP-I(103–123) displaced low-M_r receptor binding and a significant portion of high-M_r receptor binding. Competitive, saturation binding studies with 10 μM of unlabelled atriopeptin-I produced a displacement of 85% of the ^{125}I-ANF(99–126) from the specific binding sites. ^{125}I-ANF(99–126) bound to the remaining binding sites with an apparent K_d of 2.54×10^{-10} M and $B_{max.}$ 1.74×10^{-11} M. C-ANF displaced 35% of ^{125}I-ANF(99–126) binding to the PC12 cells, indicating this portion of the receptor population in PC12 cells represents the low-M_r clearance receptor and those remaining are the cyclic GMP coupled high-M_r B receptors. When the cells were down-regulated with 10 μM of unlabelled ANF(99–126), the $B_{max.}$ value decreased to 4.69×10^{-11} M and the K_d value was 5.4×10^{-10} M, indicating disappearance of specific binding sites from the cell surface.

Internalization of ANF receptors

Incubation of PC12 cells with ^{125}I-ANF(99–126) at 37 °C resulted in a rapid receptor-mediated internalization of the radiolabelled ligand. In order to identify and quantify the receptor subtype(s) undergoing rapid internalization, the receptor-mediated internalization was studied in the presence of different ANF analogues. Internalization of ^{125}I-ANF was blocked by 10 μM of unlabelled ANF(99–126) by displacing labelled ligand from specific binding sites (Fig. 4). In the presence of C-ANF, internalization of ^{125}I-ANF(99–126) was 24% less than the undisplaced control at the end of 60 min. This suggested the high-M_r receptors are responsible for 76% of the internalization process in PC12 cells. The corresponding surface binding was 35% less as compared with the surface binding observed in the absence of C-ANF, in good agreement with our saturation binding data. On the other hand, atriopeptin-II (AP-II) reduced the internalization by 87% and the corresponding surface binding to 93% less than the respective control. The percentage decrease in the total internalization of labelled ligand in the presence of AP-II correlates with the decreased surface binding, and this percentage is higher than the C-receptor population, suggesting AP-II reduces the internalization of ^{125}I-ANF by displacing binding from both B and C receptors. AP-I reduced the internalization by 69% over 60 min. This inhibition of internalization by AP-I is also greater than that by C-ANF, which can be the result of low-affinity binding of AP-I to the B receptors at higher concentrations, as reported previously [22]. The decrease in ^{125}I-ANF internalization in the presence of unlabelled ANF analogues correlates with the observed displacement of the ^{125}I-ANF(99–126) surface binding by the respective analogues.

Quantification of receptor down-regulation and intracellular recycling

Incubation of cells with 10 μM unlabelled ANF(99–126) for various times (60–180 min) produced a differential down-regulation of receptor subtypes (Fig. 5). Both receptor subtypes were down-regulated in a time-dependent manner, and C-receptor down-regulation was maximal at 60 min (Fig. 5, lane b), whereas maximum down-regulation of the B receptor was noted at 120 min (Fig. 5, lane c). Incubation of cells for 180 min with ANF(99–126) did not produce an additional decrease in the density of the B receptor (Fig. 5, lane d).

Monensin, which inhibits receptor recycling from the intracellular pools, was used to characterize receptor recycling in PC12 cells. When down-regulated cells were re-incubated at 37 °C in ANF-free medium, the cells recovered 35% of the original binding sites (Fig. 6). In the presence of monensin, 72% of the recovery process was inhibited. Inhibition of the re-expression of the cell-surface binding by monensin strongly suggests a cellular receptor recycling process controlling the cell-surface receptor population. This conclusion was further sup-

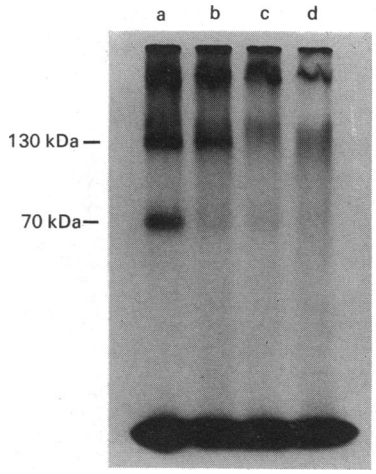

Fig. 5. Down-regulation of ANF receptor subtypes

PC12 cells were incubated with 10 μM unlabelled ANF(99–126) for 60, 120 and 180 min, followed by affinity labelling with ^{125}I-ANF(99–126). Lanes: (a) receptor labelling in plasma membrane prepared before down-regulation, at zero time; (b) receptor labelling in plasma membrane of cells incubated for 60 min; (c) cells incubated for 120 min; (d) cells incubated for 180 min. A representative autoradiogram from three individual experiments is shown.

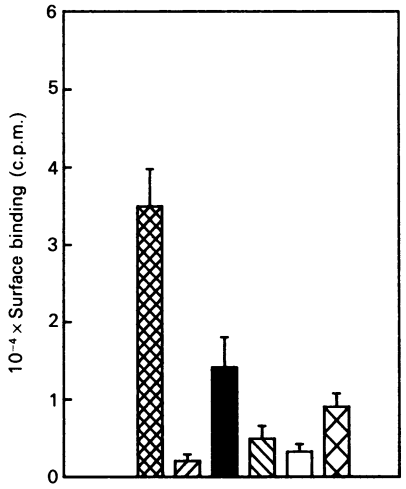

Fig. 6. Effect of monensin, cytochalasin B and cycloheximide on recovery of cell-surface binding in down-regulated cells

PC12 cells were incubated with 10 μM unlabelled ANF(99–126) at 37 °C for 120 min. After incubation the cells were washed to remove unbound excess ANF and re-incubated without any treatment. The cells were re-incubated with monensin (25 μM), cytochalasin-B (10 μM) and cycloheximide (5 μg/ml) for another 120 min, and cell-surface binding was determined. Total surface binding was determined with the cells that were not down-regulated. ⊠, Total binding; ▨, down-regulated; ■, up-regulated; ▧, cytochalasin B; □, monensin; ⊠, cycloheximide.

ported by our results from affinity cross-linking experiments. Affinity cross-linking of receptors in plasma membranes of up-regulated cells demonstrated reappearance of B receptors on the plasma membrane. Monensin in re-incubation medium blocked this reappearance of B receptors, indicating that B receptors are recycled back to the plasma membrane from an internal pool after down-regulation by a monensin-sensitive mechanism (Fig. 7). Re-incubation of down-regulated cells with cycloheximide also resulted in 43 % inhibition of the receptor recovery.

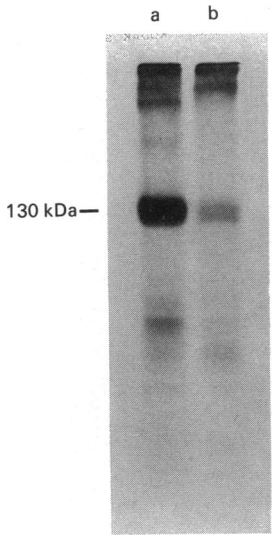

Fig. 7. Effect of monensin on ANF receptor subtype recycling

PC12 cells were down-regulated by incubating with 10 μM unlabelled ANF(99–126) at 37 °C for 120 min. Cells were then washed with serum-free RPMI medium to remove excess unbound ANF and re-incubated in the presence or absence of 25 μM-monensin for another 120 min. At the end of incubation, cells were washed, homogenized and the plasma membrane separated. ^{125}I-ANF(99–126) was used to determine the receptors on the plasma membrane by affinity cross-linking of the ligand to ANF receptors. Lane a, membrane from cells re-incubated without monensin; lane b, membrane from cells re-incubated with monensin. A representative autoradiogram from three experiments is shown.

Greater inhibition by monensin may be due to its interference with the transport of newly synthesized receptors to the plasma membrane [23], in addition to the inhibition of the recycling process. Incubation of down-regulated cells in the presence of cytochalasin B produced 57 % inhibition of receptor recovery and indicates that cytoskeletal elements are also involved in the transport of ANF receptors from the intracellular pools to the cell surface.

DISCUSSION

PC12 cells exhibit rapid receptor-mediated internalization of ^{125}I-ANF(99–126) when incubated at 37 °C. By internalizing receptors from the cell surface, the number of receptors on the plasma membrane can be modulated and cellular responsiveness to the hormone regulated. This mechanism is similar to that observed for epidermal growth factor and insulin receptors in which the distribution of receptors between cell surface and intracellular pools in target organs is modulated after ligand stimulation [24,25]. Since ANF receptors exist as two functionally distinct subtypes, differential regulation of the subtypes represent a unique model for understanding cellular regulation of receptor expression.

In the present study, C-ANF was used as a tool to selectively bind and identify C-receptor-mediated events. Receptor-mediated internalization of ^{125}I-ANF(99–126) was studied in the presence of a saturating concentration of C-ANF which would prevent binding of ^{125}I-ANF(99–126) to C receptors. Under this condition, if only B receptors mediated the ligand internalization process, internalization would be expected to remain unaltered. On the other hand, if a significant portion of internalization was mediated by C receptors, internalization of ^{125}I-ANF(99–126) would be maximally reduced. Our results clearly demonstrate that both B and C receptors are internalized subsequent to ligand

binding in PC12 cells. Saturating concentrations of C-ANF reduced receptor-mediated internalization by only 24%. This decrease in internalization correlates with the cell-surface C-receptor population. The remaining 76% of the internalization is mediated by the B receptors. Other hormone analogues, including AP-I and AP-II, produced greater inhibition of internalization than C-ANF, reflecting their relative affinity for the B receptors.

Affinity cross-linking studies in our experiments revealed down-regulation of both B- and C-receptor subtypes. C receptors were almost completely down-regulated by a 60-min incubation with ligand, but B receptors were not down-regulated as extensively and rapidly as did C receptors at 60 min. Maximal down-regulation of B receptors was observed at 120 min. At this point, receptor synthesis and recycling stabilize the surface B-receptor population. This is analogous to the receptor steady state resulting from an equal rate of receptor up-regulation and down-regulation that has been reported for muscarinic receptors [26]. As a result of this steady-state mechanism, total down-regulation of B receptors could not be achieved in our experiments, even up to 180 min.

Reversibility of ANF receptor loss from the cell surface by up-regulation was demonstrated in vascular smooth-muscle cells [27], but the events leading to the up-regulation of ANF receptors have not been defined clearly. On the basis of the inhibitory effects of actinomycin D and cycloheximide, protein synthesis was proposed as one of the possible mechanisms underlying this process. In the present study, monensin, a carboxylic ionophore, which inhibits recycling of a variety of receptors by disrupting function of intracellular vesicles [23], potentially inhibited the up-regulation of ANF receptors. Inhibition by monensin was greater than that by cycloheximide, and hence it is evident that recycling of receptors may contribute greater to the up-regulation of ANF receptors in PC12 cells than acquisition of newly synthesized receptors. Our study also suggests the rate and mode of down-regulation of ANF receptors depend on the continuous externalization of internalized receptors from an internal pool. Faster down-regulation of C receptors from the cell surface may be due to the inability to recycle back to the plasma membrane immediately after internalization, unlike the B receptors. In contrast, a portion of the B receptors down-regulated from the plasma membrane may be stored in an internal pool which can be readily mobilized to the cell surface. This is in accordance with the mechanism existing for many receptor systems, such as epidermal-growth-factor receptors, which are continuously externalized from internal pools through a monensin-sensitive, but not chloroquine-sensitive, pathway [24].

Inhibition of up-regulation by cytochalasin-B suggests a cytoskeletal element participation in the transport of B receptors from the internal pools to the cell surface. Up-regulation of C receptors by either synthesis or recycling did not occur within the time frame of our studies. It appears the C receptors may be transported to the lysosomes after internalization and hydrolysed, leading to down-regulation of C receptors from the membrane.

These results demonstrate the differential regulation of B and C receptors in PC12 cells. B receptors constitute the major portion of the receptor-mediated internalization process and undergo an active recycling which is sensitive to cytochalasin-B. The C receptors are down-regulated more quickly than the B receptors, since they are not recycled or up-regulated as quickly as the B receptors. Recycling of B receptor is supported by two observations. First, total B-receptor down-regulation could not be achieved. Secondly, monensin, which is shown to disrupt the recycling of many receptor types, inhibited up-regulation of ANF B receptors.

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