Kinetic analysis of internalization, recycling and redistribution of atrial natriuretic factor–receptor complex in cultured vascular smooth-muscle cells

Ligand-dependent receptor down-regulation

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The kinetics of internalization, sequestration and metabolic degradation of atrial natriuretic factor (ANF)–receptor complex were studied in rat thoracic aortic smooth-muscle (RTASM) cells. These parameters were directly determined by measuring ^{125}I -ANF binding to total, intracellular and cell-surface receptors. Pretreatment of cells with the lysosomotropic agent chloroquine and the energy depleter dinitrophenol led to an increase in the intracellular ^{125}I -ANF radioactivity. After 60 min incubation at 37 °C, cell-associated ^{125}I -ANF radioactivity fell rapidly in chloroquine-treated cells (> 85 %) compared with the controls (< 45 %). ^{125}I -ANF radioactivity increased to a peak of 65 % of the initial level within 15 min in chloroquine-treated cells compared with only 22 % in the control cells. During the initial incubation period at 37 °C, chloroquine inhibited the release of both intact and degraded ^{125}I -ANF in a time-dependent manner. However, at later incubation times, the effect of chloroquine was diminished and release of both degraded and intact ligand was resumed. Extracellular unlabelled ANF did not affect the release of degraded ^{125}I -ANF but it accelerated the release of intact ANF by a retroendocytotic mechanism. After the endocytosis, about 30–40 % of ANF receptors were restored to the cell surface from the internalized pool of receptors. The restoration was blocked by chloroquine or dinitrophenol but not by cycloheximide. Exposure of RTASM cells to unlabelled ANF resulted in a time- and concentration-dependent loss of ANF receptors. Unlabelled ANF (10 nM) induced a loss of more than 52 % of ^{125}I -ANF binding, and a complete loss occurred at micromolar concentrations. It is inferred that ANF-induced down-regulation of its receptor resulted primarily from an increased rate in internalization and metabolic degradation of ligand–receptor complex by receptor-mediated endocytotic mechanisms.

INTRODUCTION

Atrial natriuretic factor (ANF), a peptide hormone produced in the specific granules of atrial myocytes, plays an important role in the regulation of extracellular fluid volume and electrolyte homoeostasis (deBold *et al.*, 1981). ANF stimulates the renal excretion of salt and water (Flynn *et al.*, 1983; Atlas *et al.*, 1984; Seidah *et al.*, 1984) and relaxation of vascular smooth-muscle cells (Currie *et al.*, 1983; Grammer *et al.*, 1983; Garcia *et al.*, 1984). It inhibits the release of aldosterone from adrenal gland (Atarashi *et al.*, 1984; DeLean *et al.*, 1984; Goodfriend *et al.*, 1984), renin from kidney (Burnett *et al.*, 1984; Maack *et al.*, 1984; Obana *et al.*, 1985a), vasopressin from anterior pituitary (Samson, 1985; Obana *et al.*, 1985b) and progesterone in Leydig tumour (MA-10) cells (Pandey *et al.*, 1985). ANF stimulates the release of testosterone in Leydig cells (Bex & Carbin, 1985; Pandey *et al.*, 1986a; Mukhopadhyay *et al.*, 1986), growth hormone in posterior pituitary (Harvath *et al.*, 1986) and progesterone in ovarian granulosa cells (Pandey *et al.*, 1987). It is believed that the intracellular action of ANF is exerted through its binding to specific cell-surface receptors [for reviews see Inagami (1989) and Garbers (1989)]. Multiple forms of ANF receptor subtypes have been proposed (Leitman *et al.*, 1986; Scarborough *et al.*, 1986; Pandey *et al.*, 1988). The structures of two ANF receptor subtypes, with guanylate cyclase activity (Chinkers *et al.*, 1989; Lowe *et al.*, 1989; Schulz *et al.*, 1989; Chang *et al.*, 1989; Pandey & Singh, 1990) and without guanylate cyclase activity (Fuller *et al.*, 1988), have been de-

termined. The prevalence of distinct ANF receptor subtypes seems to be specific to different ANF target-cell types (Pandey *et al.*, 1988; Leitman *et al.*, 1988). Photoaffinity-labelling and cross-linking studies have shown that smooth-muscle cells contain predominantly the non-guanylate cyclase-type of ANF receptor (Budzik *et al.*, 1987; Schenk *et al.*, 1987; Pandey *et al.*, 1988) which is also referred to as ANF clearance (ANF-C) receptor (Maack *et al.*, 1987). Contradictory to the hypothesis of a clearance role for these receptors, however, it has been reported that ANF-C receptors seem to be involved in the formation of inositol phosphate and diacylglycerol in cultured smooth-muscle cells (Hirata *et al.*, 1989) and also to be negatively coupled with the adenylate cyclase system, suggesting a role in the inhibition of the intracellular accumulation of cyclic AMP (Resink *et al.*, 1988; Anand-Srivastava *et al.*, 1990).

Despite extensive biochemical and molecular-biological studies, the mechanism and physiological significance of ANF receptor translocation after ligand binding is not yet clear. The binding of hormones to target cells seems to be highly regulated by several mechanisms including the ligand itself. Although, after long-term exposure of cultured smooth-muscle cells to ANF a decrease in ANF receptors was previously noticed (Hirata *et al.*, 1985b; Roubert *et al.*, 1987; Bellerman & Neuser, 1988; Hamet *et al.*, 1988; Cahill *et al.*, 1990), an interrelationship between the endocytotic mechanisms, intracellular sequestration of the ANF–receptor complex and ANF-dependent down-regulation in smooth muscle cells has not been demonstrated. This present report provides an insight into the pathways involved

in the intracellular sequestration of ANF-receptor complex and ligand-dependent receptor down-regulation in rat thoracic aortic smooth-muscle (RTASM) cells. In these studies, the lysosomotropic agent chloroquine and the energy depletor dinitrophenol, which are known to interfere with the intracellular routing and redistribution of the ligand-receptor complex, have been utilized. Chloroquine is known to inhibit lysosomal degradation and dinitrophenol disrupts energy-dependent intracellular trafficking of the ligand-receptor complex (Carpenter & Cohen, 1976; Stein & Sussman, 1986). In the absence of these agents, a large proportion of ANF receptors in RTASM cells were metabolized intracellularly, and a population of receptors recycled back to the cell surface. These present studies show that the ANF-receptor complex in RTASM cells is translocated from the cell surface to intracellular sites of metabolic degradation and redistribution which manifest the ligand-dependent receptor down-regulation.

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EXPERIMENTAL

Materials

Rat ANF-28 was obtained from the Peninsula Laboratories (Belmont, CA, U.S.A.) and ^{125}I -ANF was from Amersham Corporation (Arlington Heights, IL, U.S.A.). Chloroquine, dinitrophenol, cycloheximide and BSA were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tissue culture supplies were from Gibco Laboratories (Grand Island, NY, U.S.A.) and all other chemicals were of reagent grade.

Smooth-muscle cell culture

RTASM cell culture was established essentially as described before (Chamley *et al.*, 1977). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum and were grown at 37 °C in an atmosphere of 5% CO_2 /95% O_2 . Cultures were fed on alternate days and were subcultured every 6 days.

Analysis of cell-surface binding of ^{125}I -ANF

Binding of ^{125}I -ANF to RTASM cells was determined at 4 °C to ensure that ANF internalization did not occur at this temperature. The ANF-receptor-binding assay was performed in 6 cm^2 Petri dishes after 4-5 days of cell culture with slight modifications as described previously (Pandey *et al.*, 1986b). Before the binding, cells were washed three times (2 ml each wash) with assay medium (serum-free DMEM containing 0.1% BSA). The binding assay was performed in 2 ml of assay medium containing ^{125}I -ANF (1 nM). To determine non-specific binding, parallel dishes received unlabelled ANF (1 μM) in addition to ^{125}I -ANF. After the binding reaction at 4 °C, cells were washed four times (2 ml each wash) with ice-cold assay medium, dissolved in 0.5 M-NaOH, and ^{125}I -ANF radioactivity was counted in a Beckman Gamma-4000.

Release of surface-bound ^{125}I -ANF radioactivity by treatment with acid buffer

Cells in 6 cm^2 culture dishes were allowed to bind ^{125}I -ANF at 4 °C or at 37 °C for appropriate times and the binding reaction was terminated by aspirating the medium and washing the dishes four times (2 ml each wash) with ice-cold assay medium as described above. The dishes then received 1 ml of ice-cold glycine acidic buffer (50 mM-glycine/100 mM-NaCl, pH 3.5) and were incubated for 2 min at 4 °C. Acid eluates from the dishes were

collected and another 1 ml of cold acid buffer was used to wash the dishes. The two solutions were combined and acid-sensitive radioactivity was determined. The cells were dissolved in 0.5 M-NaOH which yielded acid-resistant radioactivity. The sum of the acid-sensitive and acid-resistant radioactivity was considered to be the total radioactivity.

Analysis of surface-bound, internalized and released ^{125}I -ANF in RTASM cells

Confluent culture dishes were washed twice (2 ml each wash) with assay medium (DMEM containing 0.1% BSA) and 2 ml of fresh medium was added to each dish. Cells were pretreated with either chloroquine (200 μM) or dinitrophenol (1 mM) for 30-45 min at 37 °C and then allowed to bind ^{125}I -ANF at 4 °C for 1 h, unless otherwise indicated. After completion of the binding reaction, cells were washed four times (2 ml each wash) with binding assay medium and each dish received 2 ml of fresh medium. All dishes were incubated at 37 °C to permit the internalization of bound ^{125}I -ANF. At the indicated times, the distribution of ^{125}I -ANF radioactivity between the cell surface, the cell interior and that released into the medium was determined. The amount of internalized ^{125}I -ANF was quantified by stripping the surface-bound ligand by treatment with glycine acidic buffer, pH 3.5. At appropriate time intervals, cells were removed from the 37 °C incubation and placed on ice. Medium was collected and cells were treated with acid buffer at pH 3.5 for 2 min as described above. The acid eluate was collected and cells were dissolved in 0.5 M-NaOH. The surface-bound, internalized and released ^{125}I -ANF radioactivity was counted in acid eluate, cell extract and culture medium respectively. The amount of intact and degraded ligand was determined by solubility of ^{125}I -ANF products in trichloroacetic acid. The ^{125}I -ANF precipitable in 5% trichloroacetic acid was considered to represent intact ^{125}I -ANF molecules. Samples of the culture media were applied to a C_{18} reversed phase h.p.l.c. column for further analysis of the nature of ^{125}I radioactivity as described elsewhere (Pandey *et al.*, 1986b).

Down-regulation of ANF receptors in RTASM cells

The confluent culture dishes were washed twice (2 ml each wash) with DMEM containing 0.1% BSA (assay medium) and a fresh 2 ml of medium was added to each dish. Cells were treated with various concentrations of ANF for various time periods at 37 °C. After completion of ANF treatment, cells were washed with acidic buffer, pH 3.5, to remove any remaining bound ANF. After the acid wash, cells were neutralized by thoroughly washing with assay medium (DMEM/0.1% BSA), and then ^{125}I -ANF binding was determined.

Ligand-dependent internalization and recycling of ANF receptors

Confluent cultures received cycloheximide for 2 h at 37 °C, after which ANF (1 μM) was added and dishes were further incubated for 1 h at 37 °C. Cells were washed free of ANF and further incubated in fresh medium with or without cycloheximide. Specific ^{125}I -ANF binding was determined at the indicated times in both cycloheximide-treated and untreated cells. Recycling of ANF receptors in RTASM cells was also determined after treatment of the cells with trypsin. Cells were washed with assay medium and then exposed to trypsin (0.025%) at 4 °C for 5 min. After trypsin treatment, cells were quickly washed with serum-containing medium followed by three washes with assay medium, and then incubated in fresh medium at 37 °C for the indicated time periods to determine ^{125}I -ANF binding. The trypsin treatment essentially abolished binding and did not cause any detachment of cells.

RESULTS

Release of acid-sensitive bound ^{125}I -ANF

Acid treatment rapidly released 85–90 % of bound ^{125}I -ANF from the cell surface within 2 min. In these experiments, acid treatment effectively separated surface-bound and internalized ^{125}I -ANF. At 4 °C, almost 95 % of bound ^{125}I -ANF was acid-sensitive irrespective of the incubation time (Fig. 1a). At 37 °C, 66 % of bound ^{125}I -ANF was acid-sensitive during the initial incubation times and this then declined rapidly (Fig. 1b).

Internalization and sequestration of ANF–receptor complex in RTASM cells

Culture dishes with and without chloroquine were incubated at 37 °C for 45 min, cooled at 4 °C and cell-surface receptors were labelled with ^{125}I -ANF for 1 h. After removal of the unbound ligand, cells were rapidly warmed to 37 °C in the fresh medium. At the indicated time intervals, the radioactivity associated with the cell surface, internalized into the cell interior and released into the culture medium was quantified by utilizing the acid wash procedure which specifically dissociated surface-bound

Fig. 1. Specific binding of ^{125}I -ANF to cultured RTASM cells as a function of time

Cells were incubated with ^{125}I -ANF at (a) 4 °C or (b) 37 °C in 2 ml of binding medium. At the indicated time, cells were washed and acid-sensitive (Δ) and acid-resistant (□) radioactivity was determined as described in the Experimental section. The total cell-bound radioactivity (○) was the sum of these two components. Each point represents the mean of three separate determinations.

Fig. 2. Quantitative analysis of ^{125}I -ANF radioactivity in RTASM cells after pretreatment with the lysotrophic agent chloroquine

Confluent monolayer cells in 6 cm² culture Petri dishes were pretreated with chloroquine for 30 min. Both (a) controls and (b) chloroquine-treated cultures were allowed to bind ^{125}I -ANF at 4 °C for 60 min, after which cells were washed with cold assay medium to remove the unbound ligand and warmed to 37 °C. At the indicated time intervals, the cell-surface (Δ), internalized (○) and released (□) radioactivity was determined in acid eluates, cell extracts and culture medium as described in the Experimental section. Each point represents the mean of three separate determinations.

^{125}I -ANF (Fig. 2a). At 37 °C, cell-associated radioactivity fell more rapidly in chloroquine-treated cells than in controls. After 1 h incubation, only 15 % ^{125}I radioactivity was associated with the cell surface in chloroquine-treated cells compared with 55 % in controls (Figs. 2a and 2b). After 15 min, in chloroquine-treated cells the acid-resistant (internalized) ^{125}I -ANF radioactivity accounted for 65 % compared with only 22 % in control cells. However, after 2 h incubation the effect of chloroquine was diminished and intracellular ^{125}I -ANF radioactivity decreased rapidly. The ^{125}I -ANF radioactivity in the culture medium increased steadily up to 4 h and then plateaued. In control cells, the internalized ^{125}I radioactivity was maintained at a steady-state level from 15 min to 4 h and then declined slightly (Figs. 2a and 2b).

Metabolic processing of internalized ANF–receptor complex in RTASM cells

RTASM cells were pretreated with chloroquine at 37 °C, exposed to ^{125}I -ANF at 4 °C, washed free of unbound ligand and

Fig. 3. Effect of unlabelled ligand on the accumulation of internalized ^{125}I -ANF

RTASM cells were preincubated in the absence or presence of chloroquine at 37 °C for 30 min and then cells were allowed to bind ^{125}I -ANF at 4 °C for 60 min. After being washed, one group of chloroquine-treated cells was exposed to unlabelled extracellular ANF (0.1 μM). Both treated and control cells were warmed to 37 °C. At the indicated times the amount of intracellular ^{125}I -ANF radioactivity was determined. The data shown represent the means $\pm$ S.E.M. of separate determinations from three to four experiments. $\circ$, Control; $\triangle$, chloroquine-treated; $\square$, chloroquine-treated + ANF.

reincubated at 37 °C for different time periods. One set of cultures also received unlabelled ligand (0.1 μM -ANF). The internalized ^{125}I -ANF radioactivity remained at a much higher level in chloroquine-pretreated cells than in the controls (Fig. 3). At 37 °C, approx. 85–90 % of internalized ^{125}I -ANF radioactivity was released into the culture medium. The nature of the radioactivity in the medium was analysed by h.p.l.c. (reversed phase C_{18} column) which indicated that a major proportion of the radioactivity was eluted in the pass-through fraction as degraded products and a small proportion was eluted at the position corresponding to ^{125}I -ANF (results not shown). Initially, chloroquine inhibited the release of degraded ^{125}I -ANF in a time-dependent manner (Fig. 4a), but, after a longer incubation time, the release of degraded radiolabelled ligand increased steadily. The release of intact ligand increased sharply after 2 h in chloroquine-treated cells (Fig. 4b). Extracellular unlabelled ANF did not affect the release of degraded ^{125}I -ANF, but it accelerated the release of intact ligand in chloroquine-treated cells (Figs. 4a and 4b). The treatment of RTASM cells with the energy depleter dinitrophenol for 30 min at 37 °C also inhibited the degradation of ^{125}I -ANF by approx. 50 % compared with untreated cells (Fig. 5).

ANF-dependent down-regulation of its receptor in RTASM cells

The exposure of RTASM cells to ANF (10 nM) caused a substantial decrease in ANF receptors leading to a loss of 50–60 % of total receptor number without changing the affinity of the binding sites (results not shown). RTASM cells were pretreated with various concentrations of unlabelled ANF for increasing time periods as shown in Fig. 6. ANF (at 10 nM) markedly decreased the surface binding in 45 min and the micromolar concentration of ANF produced almost a complete loss of cell surface binding (Fig. 6).

Internalization and recycling of ANF receptors in RTASM cells

In order to determine if the internalized ANF receptors are

Fig. 4. Quantitative analysis of intact and degraded ^{125}I -ANF in the culture medium

Confluent culture dishes were preincubated at 37 °C for 30 min in the absence ($\circ$, $\bullet$) or presence ($\triangle$, $\blacktriangle$) of chloroquine (200 μM) and then used for ^{125}I -ANF binding for 60 min at 4 °C. After being washed with assay medium to remove the unbound ligand, one group of chloroquine-treated cells was exposed to unlabelled ANF (10 nM) ($\square$, $\blacksquare$). All cultures were incubated at 37 °C and media were collected. Samples of medium from triplicate dishes were precipitated with trichloroacetic acid and both (a) degraded and (b) intact ^{125}I -ANF were quantified as outlined in the Experimental section. Data represent means $\pm$ S.E.M. of three separate determinations.

returned to the cell surface, RTASM cells were incubated with ANF (0.1 μM) for 1 h which produced an almost complete loss of specific cell-surface binding of ^{125}I -ANF. After pretreatment with unlabelled ANF, cells were washed with acid buffer to remove any bound ANF and incubated at 37 °C in fresh medium which exhibited a gradual return to cell-surface ^{125}I -ANF binding ($t_{1/2} = 20$ min). In 40 min the surface binding returned to approx. 35 % of the original level (Fig. 7). A parallel set of culture dishes was also incubated in the presence of cycloheximide (a protein-synthesis inhibitor) at 37 °C for 2 h preceding the ANF pretreatment and during the remainder of the incubation. The cycloheximide-pretreated cells also exhibited a return to cell-surface ^{125}I -ANF binding. In another experiment, RTASM cells were treated with a low concentration of trypsin (0.025 %) at 4 °C for 5 min which abolished cell-surface ^{125}I -ANF binding. After the cells were washed free of trypsin with serum containing DMEM and incubated in fresh assay medium at 37 °C, a return to cell-surface ^{125}I -ANF binding was observed in both cycloheximide-treated and untreated cells (Fig. 8).

Fig. 5. Effect of dinitrophenol on accumulation of intracellular ^{125}I -ANF

RTASM cells were labelled with ^{125}I -ANF at 4 °C for 60 min. After removal of unbound ligand, 2 ml of fresh medium was added to culture dishes and cells were treated with dinitrophenol (1 mM). The controls (○) and treated (●) culture dishes were incubated at 37 °C. At the indicated time intervals, intracellular ^{125}I -ANF radioactivity was determined. Values are means $\pm$ S.E.M. from three to four experiments.

Fig. 6. Time- and dose-dependent down-regulation of ANF binding in RTASM cells

Confluent cells in 6 cm² dishes were exposed to various concentrations of ANF for the indicated times. Cells were then washed with acid buffer to remove any surface-bound ANF, and assayed for specific ^{125}I -ANF binding as described in the Experimental section. The concentrations of ANF used were 10 pM (○), 10 nM (△) and 1 μM (□). Values are means $\pm$ S.E.M. of three to four experiments.

DISCUSSION

This present study was undertaken to investigate the processes involved in the endocytotic uptake, intracellular processing and ANF-dependent down-regulation of its receptors in a model RTASM cell system. Although the internalization of ANF receptors in vascular smooth-muscle cells has been previously documented (Hirata *et al.*, 1985a; Napier *et al.*, 1986; Nussenzweig *et al.*, 1990), the interrelationships between the endocytotic mechanisms, intracellular sequestration of ANF-receptor complex and ANF-dependent receptor down-regulation have

Fig. 7. Recycling of internalized ANF receptors in RTASM cells

Confluent cells were incubated in assay medium with cycloheximide (CX; 20 μg/ml) at 37 °C for 1 h and were then exposed to 1 μM-unlabelled ANF for 1 h. After being washed free of ANF with acid buffer, cells were recultured in fresh medium with or without cycloheximide, and specific ^{125}I -ANF binding was determined at various time intervals. The hatched bar represents the binding of ^{125}I -ANF in control cells which never received exogenous unlabelled ANF. Data represent means of three determinations and the data for the bar represent mean $\pm$ S.E.M. ○, Without cycloheximide; △, with cycloheximide.

Fig. 8. Trypsin-dependent loss of ^{125}I -ANF binding and recycling of ANF receptors

The confluent RTASM cells were incubated in assay medium at 4 °C in the presence of 0.025% trypsin for 5 min as described in the Experimental section. The parallel set of control cultures was incubated at 4 °C without trypsin. After trypsin treatment, cells were washed with medium containing 10% serum to stop the trypsin reaction. The cells were then incubated at 37 °C in fresh medium containing 10% serum and ^{125}I -ANF binding was determined in the presence or absence of cycloheximide. The hatched bar represents the binding of ^{125}I -ANF in control cells which were never exposed to trypsin. The data represent means $\pm$ S.E.M. of three separate experiments. ○, Without cycloheximide; △, with cycloheximide.

not been resolved. It is conceivable that the homeostatic regulation of ANF receptors and cellular sensitivity of ANF would be dependent on a dynamic equilibrium and reutilization of the ligand-receptor complex from cell surface to cell interior. Therefore to understand the dynamics of the interrelationship between these processes, the events that occur after binding in

ANF target cells were investigated. The results presented here show that ^{125}I -ANF binds to cell-surface receptors, enters through the process of receptor-mediated endocytosis and is delivered to the intracellular compartments. The majority of ^{125}I -ANF is degraded and rapidly released into the culture medium. The amount of endocytosed ^{125}I -ANF was found to increase continuously in the intracellular compartments until it reached a steady-state level after 60 min ($t_{1/2} = 30$ min). At this steady state, approx. 50% of the total radioactivity was present on the cell surface, 30% inside the cell and 20% released into the culture medium. The distribution of ^{125}I -ANF radioactivity between the cell surface, intracellular compartments and culture medium provides a dynamic equilibrium between the rates of ^{125}I -ANF uptake and its degradation and extrusion. The rate of both uptake and breakdown of ^{125}I -ANF are markedly decreased in the presence of metabolic inhibitors such as chloroquine and dinitrophenol and also at lower temperature, which suggest that the processing pathway of ^{125}I -ANF in RTASM cells is, in part, lysosomal.

A recent observation indicated that the Ca^{2+} ionophore A23187 decreased the internalization of the ANF-receptor complex in PC12 cells and suggested that the mobilization of the ligand-receptor complex in the cell interior may be dependent on extracellular Ca^{2+} (Rathinavelu & Isom, 1991). Similarly, Morton *et al.* (1989) have also previously documented that the low-affinity ANF-binding sites required Ca^{2+} in the plasma-membrane preparations of mesenteric artery from the sodium-depleted rats. However, Ca^{2+} did not affect the high-affinity ANF-binding sites in these plasma-membrane preparations. In our present studies, Ca^{2+} did not show any measurable effect on ^{125}I -ANF binding in RTASM cells. Since in the studies of Rathinavelu & Isom (1991) the PC12 cells were grown in RPMI medium which does not contain Ca^{2+} , it is possible that the exposure of the cells to a medium containing 1–2 mM extracellular Ca^{2+} might have evoked some effect of Ca^{2+} on the binding and internalization of the ANF-receptor complex in PC12 cells. However, it should be noted that the measurable effect was only 18–28%. The RTASM cells used in our present studies are grown in DMEM which contains Ca^{2+} (1 mM) and therefore Ca^{2+} may not have a measurable and significant effect on the kinetics of binding and internalization of ANF receptor in RTASM cells.

A major proportion of internalized ^{125}I -ANF was released into the culture medium which consisted of both degraded products and intact ^{125}I -ANF. It was found that approx. 80–85% of the released ^{125}I radioactivity constituted the degraded products and about 15–20% was intact ligand. These observations suggest that the majority of internalized ^{125}I -ANF is processed through the degradative compartments. Nevertheless, an alternative mechanism also seems to exist for the release of intact ligand in RTASM cells. Although the release of degraded ligand was blocked to a great extent by the lysosomotropic agent chloroquine, this agent was only partially effective in blocking the release of intact ^{125}I -ANF. The treatment of cells with unlabelled ANF further diminished the effect of chloroquine on the release of intact ligand, but it did not modify the effect of chloroquine in blocking the release of degraded products. Dual pathways for intracellular processing of peptide hormones have previously been described (Anderson *et al.*, 1977; Tietze *et al.*, 1982), suggesting the release of intact ligand through retroendocytosis and release of degraded products through simple diffusion into the culture medium (Marshall, 1985). It is also conceivable that retroendocytosis of a certain ligand could be the result of recycling of the receptor–ligand complex leading to the dissociation of the ligand from the receptor at the plasma membrane.

In these present studies the short-term exposure of RTASM cells with increasing concentrations of unlabelled ANF resulted

in a loss of cell-surface receptors (Fig. 6). The maximal effect of ANF on down-regulation of its receptor occurred between 30 and 60 min, suggesting the involvement of receptor-mediated endocytosis and subsequent metabolic degradation and redistribution of the ANF-receptor complex. Approx. 30% of ANF receptors returned to the cell surface in ANF-treated cells. Unlabelled ANF accelerated the release of intact ligand, indicating that ANF-dependent down-regulation of its receptor involves the internalization of the ligand-receptor complex which dissociates intracellularly and escapes the lysosomal degradative pathway. In addition to the ligand-dependent down-regulation of ANF receptors in vascular smooth-muscle cells, it has been shown that cyclic GMP also down-regulates ANF receptors in cultured vascular endothelial cells (Kato *et al.*, 1991). However, further studies are needed to support the notion that cyclic GMP is indeed involved in the down-regulation of ANF receptors in other tissues and cell systems.

The data presented here show that a proportion of internalized ANF receptors in RTASM cells recycle back to the plasma membranes. As a result, only 50% of surface receptors were lost in control cells at 37 °C after 8 h incubation, compared with an 85–90% receptor loss in chloroquine-pretreated cells (Figs. 2a and 2b). Since ANF at a concentration of 1 μM induced an almost complete loss of cell-surface binding at 37 °C, this strategy was utilized to examine the recycling of ANF receptors in these cells. After ANF treatment, cells were washed with acid buffer to remove any bound ligand and then incubated in the fresh assay medium at 37 °C; a gradual return to surface binding occurred ($t_{1/2} = 20$ min). A return of ^{125}I -ANF binding was also observed in cycloheximide-treated cells. These observations provided the evidence that the internalized ANF receptors in RTASM cells recycled back to the plasma membrane. In a parallel experiment, RTASM cells were treated with trypsin at 4 °C for 5 min, which abolished cell-surface ^{125}I -ANF binding. After the cells had been washed free of trypsin and incubated in fresh medium at 37 °C, a return to ^{125}I -ANF binding was observed. However, in cycloheximide-treated cells, binding of ^{125}I -ANF was 30–40% lower than in control cells. These findings further strengthened the view that a return to ANF binding in RTASM cells was due to recycling of the receptor molecules. Since a complete return to ANF binding did not occur, protein synthesis *de novo* may also be involved.

In conclusion, the present studies show that ANF receptors in RTASM cells are internalized and redistributed in the cell interior which provides an approach to the direct examination of the dynamics of ANF-receptor complex sequestration and processing in RTASM cells. The translocation of the ANF-receptor complex from the cell surface to the intracellular compartments seems to be linked to ANF-dependent receptor down-regulation. The ability of ANF to substantially decrease the level of ANF receptors in RTASM cells is linked to the internalization and accumulation of the ANF-receptor complex in the cell interior. Most of the internalized ^{125}I -ANF in RTASM cells is processed through the lysosomal degradative pathways, but an alternative mechanism also seems to exist for the release of intact ligand by retroendocytosis.

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