Identification and Characterization of Three Distinct Atrial Natriuretic Factor Receptors

EVIDENCE FOR TISSUE-SPECIFIC HETEROGENEITY OF RECEPTOR SUBTYPES IN VASCULAR SMOOTH MUSCLE, KIDNEY TUBULAR EPITHELIUM, AND LEYDIG TUMOR CELLS BY LIGAND BINDING, PHOTOAFFINITY LABELING, AND TRYPTIC PROTEOLYSIS*

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Three distinct atrial natriuretic factor (ANF) receptors have been identified and characterized from rat thoracic aortic cultured vascular smooth muscle (RTASM) cells, kidney tubular epithelium (MDCK), and Leydig tumor (MA-10) cells. These include 1) a disulfide-linked 140-kDa protein found in RTASM cells, which was reduced by dithiothreitol (DTT) to 70 kDa, 2) a 120–135-kDa single polypeptide protein, specific to MDCK and MA-10 cells whose M, was not reduced by DTT, and 3) a 66–70-kDa protein prevalent in both RTASM and MDCK cells, which was not reduced by DTT. After incubation of RTASM cells with 4-azidobenzoyl 125I-ANF, labeling of the 140-kDa protein was blocked by both full-length ANF(1-126) and truncated ANF(103-123). In contrast, the labeling of the 120-kDa receptor in MDCK cells was blocked only by full-length ANF(99-126). However, labeling of the 68–70-kDa receptor in both RTASM and MDCK cells was blocked by full-length ANF(99-126) and truncated ANF(103-123). Binding of 125I-ANF(99-126) to RTASM and MDCK cells was rapid, specific, and saturable with a Kd of 1.5 X 10^-10 M and binding capacity (Bmax) of 2.1 X 10^6 sites/RTASM cell and Kd 4.5 X 10^-10 M and Bmax 5 X 10^4 sites/MDCK cell, respectively. Binding of 125I-ANF(99-126) to RTASM cells was displaced with both full-length ANF(99-126) and truncated ANF(103-123), however, binding to MDCK cells was efficiently displaced only with full-length ANF. Both ANF(99-126) and ANF(103-123) stimulated cGMP in RTASM cells but only ANF(99-126) elicited cGMP in MDCK cells. Tryptic proteolysis of the high M, single chain receptor produced only a 68-kDa fragment, whereas disulfide-linked 140-kDa receptor yielded 52-, 38-, 26-, and 14-kDa fragments. These data provide direct biochemical evidence for three distinct ANF receptors which might be linked to diverse physiological functions of ANF such as natriuresis in the kidney, vasorelaxation in vascular smooth muscle, and steroiogenic responsiveness in Leydig cells.

Atrial natriuretic factor (ANF)1 is a newly discovered peptide hormone, synthesized in the specific granules of mammalian atrial cardiocytes (1). ANF elicits multiple physiological responses largely directed to the reduction of blood pressure and blood volume. In addition to its effects on renal excretion of sodium and water (2-4) and vasorelaxation of vascular smooth muscle cells (5-7), ANF has been shown to inhibit aldosterone release from adrenal glands (8-10), renin release from kidney (11-13), and vasopressin from neurohypophysis (14, 15) and to stimulate androgen secretion from testicular Leydig cells (16, 17). ANF stimulates membrane-bound guanylate cyclase (18, 19) and inhibits adenylate cyclase (20), thereby resulting in an increase in cGMP and a decrease in cAMP; however, the precise mechanism by which ANF elicits multiple physiological responsiveness is still unknown.

The cellular responsiveness of ANF action is manifested through its binding to specific cell surface receptors and the existence of ANF receptors of 66–70 kDa and 120–140 kDa have been shown in the membrane preparations and on intact cells (21-25). Recently, ANF receptor proteins of 120–180 kDa have also been isolated from different tissue sources (26-30). These studies have employed varying methodology to identify or isolate ANF receptors. The controversy regarding the diversity of the M, of the ANF receptor molecules has not yet been resolved. It is not clear whether the variation in the M, and the structural properties of ANF receptor is due to tissue-specific differences or due to methods of plasma membranes and cell preparation or receptor identification and isolation. To have a better understanding of this question, we have employed standard photoaffinity labeling techniques in combination with ligand binding and tryptic proteolysis to identify or characterize ANF receptors. The controversy regarding the diversity of the M, of the ANF receptor molecules has not yet been resolved. It is not clear whether the variation in the M, and the structural properties of ANF receptor is due to tissue-specific differences or due to methods of plasma membranes and cell preparation or receptor identification and isolation. To have a better understanding of this question, we have employed standard photoaffinity labeling techniques in combination with ligand binding and tryptic proteolysis to identify or characterize ANF receptors.
To determine the nonspecific binding, parallel dishes received unlabeled ANF (1 x 10^{-7} M) in addition to radiolabeled ANF. After the binding reaction, the dishes were quickly washed four times (2 ml each wash) with ice-cold assay medium. Cells were then dissolved in 2 ml of 0.5 M NaOH and cell-bound ^125I radioactivity was determined. Scatchard plot and binding parameters were analyzed by the Ligand program (11).

**Photoaffinity Labeling of ANF Receptors on the Intact Cells—**Cells in 6-cm² Petri dishes were washed four times (3 ml each wash) with assay medium and incubated in 2 ml of this medium containing 1.1 x 10^{-4} M AZB-[^125]I-ANF in the presence or absence of unmodified ANF (1 x 10^{-4} M) for 10 min at 0°C in the dark. After completion of the binding, cells were washed two times (2 ml each wash) with ice-cold assay medium and then placed in 2 ml of fresh medium. Photoysis was carried out at 0°C for 10 min using a 250-W General Electric sunlamp at a distance of 15 cm. Cells were then washed four times (2 ml each wash) with assay medium and then lysed in 1 ml of % SDS containing phenylmethylsulfonyl fluoride (1 mM) and N-ethylmaleimide (1 mM). The cell lysates were incubated with 10 μl of deoxyribonuclease at 37°C for 30 min.

**Tryptic Digestion of Photoaffinity Labeled ANF Receptors—**Cells were photoaffinity labeled at 4°C and were incubated at 16°C for 10 min. Tryptic digestion was performed in the presence of trypsin (100 μg/ml) at 16°C for indicated time periods, and the reaction was stopped by adding soybean trypsin inhibitor (200 μg/ml). Medium was removed and cells were solubilized in 1% SDS containing phenylmethylsulfonyl fluoride (1 mM) and N-ethylmaleimide (1 mM).

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Autoradiography—** SDS-PAGE was carried out by a method described before (25). The autoradiographs were scanned in a LKB Ultrascan Laser Densitometer attached with the Hewlett-Packard integrator.

**Hormonal Treatment of the Cells and cGMP Assay—**Cultured cells were allowed to grow to confluency in 6-cm² Petri dishes. Culture medium was removed and cells were washed with three changes (4 ml each wash) of serum-free medium containing 0.1% BSA (assay medium). Cells were treated in 2 ml of assay medium with ANF (10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 10^{0}, 10^{1}, 10^{2}, 10^{3}, 10^{4}, 10^{5}, 10^{6}, 10^{7}, 10^{8}, 10^{9}, 10^{10}, 10^{11}, 10^{12} M) in the presence or absence of radiolabeled ANF. After the incubation, the cells were treated with trypsin-EDTA, cells were washed and then dissolved in 2 ml of 0.5 N NaOH and cell-bound ^125I radioactivity was determined. Scatchard plot and binding parameters were analyzed by the Ligand program (11).
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Data using a one-site model indicated a single class of high affinity binding sites. Analysis of the same binding data using a two-site model did not significantly improve the curve fitting.

Then we compared full-length ANF(99-126) and truncated ANF(103-123), for their ability to increase intracellular cGMP in RTASM and MDCK cells. Both full-length ANF(99-126) as well as truncated ANF(103-123) stimulated cGMP in RTASM cells with an EC50 (effective concentration required to give 50% stimulation of cGMP at the Kd concentration) approximately 15 and 50 nM, respectively (Fig. 2A). On the other hand, in MDCK cells only full-length ANF(99-126) stimulated cGMP formation with an EC50 of 12 nM, whereas truncated ANF(103-123) did not show any effects on cGMP stimulation in these cells (Fig. 2B).

Identification of ANF Receptor Subtypes by Photoaffinity Labeling—Incubation of intact RTASM cells with AZB-125-I-ANF(99-126) yielded essentially two radiolabeled protein bands with apparent M, of 140 and 70 kDa as determined by SDS-PAGE and autoradiography (Fig. 3, lane a). The labeling of both of these bands was completely abolished by the inclusion of either ANF(99-126) or ANF(103-123) in the incubation medium at 1 µM concentrations (Fig. 3, lanes b and c). (The apparent band of 67 kDa is an artifact due to labeled BSA). The specificity of the receptor labeling was observed as angiotensin II or ACTH were unable to inhibit labeling of 140- and 70-kDa bands (Fig. 3, lanes d and e). After treatment of the same samples (as in lanes c-e) with DTT (100 mM), the 140-kDa protein band was reduced to a 70-kDa band (Fig. 3, lanes f-j); however, the 70-kDa band was not reduced by DTT treatments. Increasing concentrations of ANF(99-126) inhibited the labeling of both 140- and 70-kDa bands in a dose-dependent manner (Fig. 4, lanes a-f). Again, when the same samples were pretreated with DTT, the 140-kDa band was completely reduced to a 70-kDa band (Fig. 4, lanes g-i). Truncated ANF(103-123) also inhibited the labeling of both 140- and 70-kDa bands in a dose-dependent manner (Fig. 5, lanes a-f), and the 140-kDa band was again reduced to a 70-kDa band after DTT treatments (Fig. 5, lanes g-i). Quantitative determination using densitometric scanning of the 140- and 70-kDa bands shown in Figs. 4 and 5 revealed that full-length ANF(99-126) suppressed the labeling of these two bands slightly more than the truncated peptide. However, these differences were not statistically significant.

Incubation of MDCK cells with AZB-125-I-ANF(99-126) also yielded high M, (120,000) and low M, (65,000-68,000) receptor bands after photoaffinity labeling (Fig. 6, lane a). Full-length ANF(99-126) at 0.5 × 10^-7 M concentration inhibited the labeling of both 120- and 66-68-kDa bands (Fig. 6, lane b). Hormones unrelated to ANF such as ACTH and angiotensin II were unable to displace these radiolabeled bands (data not shown). After treatment with DTT, in these cells, however, neither the 120- nor the 66-68-kDa protein bands were reduced to a

### TABLE I

Summary of the ANF receptor binding sites, Kd values, and ANF-dependent stimulation of cGMP by 1 × 10^-7 M of full-length ANF(99-126) and truncated ANF(103-123) in cultured cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Total binding sites/cell (Bmax)</th>
<th>Dissociation constant (Kd)</th>
<th>Cyclic GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pmol/1 × 10^6 cells</td>
</tr>
<tr>
<td>RTASM</td>
<td>2.1 × 10^6</td>
<td>1.5 × 10^-10</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>MDCK</td>
<td>5.0 × 10^6</td>
<td>4.5 × 10^-10</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>MA-10</td>
<td>1.0 × 10^6</td>
<td>5.0 × 10^-8</td>
<td>0.76 ± 0.09</td>
</tr>
</tbody>
</table>

*Values in the parentheses indicate the percentage of the nonreducible high M, receptor subtype.
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Fig. 2. Dose-response curves of cGMP accumulation in response to full-length ANF(99-126) or truncated ANF(103-123) in RTASM cells (A) and MDCK cells (B). The confluent cultured cells were treated with indicated concentrations of peptides for 5 min at 37 °C in the presence of 0.2 mM 3-isobutyl-1-methylxanthine as described under "Experimental Procedures." The concentration of cGMP was determined by radioimmunooassay as described in the text. Each point represents the mean and bars indicate the S.E. from four determinations, •, ANF(99-126) and ○, ANF(103-123).

Fig. 3. Autoradiograms showing the specificity of 140-kDa disulfide-linked and 70-kDa disulfide-unlinked ANF receptors in RTASM cells. Confluent cells were allowed to bind AZB-[125I]-ANF(99-126) in the dark at 4 °C in the absence (lane a) or presence of ANF(99-126) (lane b), ANF(103-123) (lane c), ANG II (lane d), or ACTH (lane e) each at 1 μM concentration. After washing with fresh medium, cells were photolyzed as described under "Experimental Procedures." Samples were electrophoresed under unreduced (lanes a-e) and reduced (lanes f-j) conditions on 7.5% gels, and radiolabeled bands were visualized by autoradiography. Arrows indicate the position and left margin of M, 140- and 70-kDa radiolabeled bands. Bars on the right and left margin indicate the M, of marker proteins (myosin = 205,000, β-galactosidase = 116,000; phosphorylase b = 97,000, bovine serum albumin = 67,000, ovalbumin = 45,000, and carbonic anhydrase = 29,000).

lower M, band (Fig. 6, lanes c and d). It should be noted that in MDCK cells, under nonreducing conditions, the low M, ANF receptor migrated as a 68-kDa band and its labeling was blocked by cold ANF. The 67-kDa band is an artifact due to labeled BSA. Under reducing conditions, the low M, ANF receptor migrated at a somewhat faster rate than BSA, positioned as a 66-kDa band, and its labeling was blocked by cold ANF (Fig. 6, lanes c and d). The faster mobility of low M, ANF receptor, under reducing conditions, is presumably due to reduced internal disulfide linkages. Increasing concentrations of ANF(99-126) inhibited the labeling of both 120- and 66-kDa bands in a dose-dependent manner (Fig. 7, lanes a-f).

However, at 1 × 10⁻⁷ M to 1 × 10⁻⁵ M ANF concentrations, the 120-kDa band was inhibited more effectively than the 66-kDa band (Fig. 7, lanes e and f). In contrast, truncated ANF(103-123) at 1 μM concentration almost completely inhibited the labeling of the 66-68-kDa band but only minimally the labeling of the higher M, band corresponding to 120 kDa (Fig. 8, lanes b and d). Again, after treatment of the samples with DTT, neither the 120- nor the 66-68-kDa bands were reduced (Fig. 8, lanes c and d).

Tryptic Proteolysis of Photoaffinity Labeled ANF Receptor—To further examine the structural heterogeneity of ANF receptors, tryptic proteolysis was employed. Fig. 9A shows the photoaffinity labeled 140- and 70-kDa receptors in RTASM cells before limited proteolysis under unreduced (lane a) or reduced (lane b) conditions. Trypsin treatments of photoaffinity labeled RTASM cells produced multiple distinct bands of 52, 38, 26, and 14 kDa (Fig. 9B, lanes a-c). These fragments most likely were originated from the disulfide-linked 140-kDa protein as the 70-kDa band was not affected by a 5-min tryptic proteolysis (Fig. 9B, lanes a-f). After DTT treatments, the M, of the tryptic fragments remained virtually unchanged (Fig. 9B, lanes d-f). Then, MA-10 and LLC-PK₁ cells were used to examine the proteolytic pattern of the high M, disulfide-unlinked protein as these cells have only one high M, ANF receptor (Fig. 10, lanes a and c). Tryptic proteolysis of photoaffinity labeled MA-10 and LLC-PK₁ cells
under "Experimental Procedures." Samples in the dark at 4 °C for 10 min in the absence (lane a) or presence of ANF(99-126) (lane b), as described under "Experimental Procedures." In lanes a and b, samples were not treated with DTT. Lanes c and d, same as lanes a and b, except samples were treated with DTT. Arrows indicate the Mᵢ and position of radiolabeled ANF receptors, and bars indicate the Mᵢ of marker proteins.

produced only one 68-kDa fragment from the single chain 135-kDa protein (Fig. 10, lanes b and d).

**DISCUSSION**

These results demonstrate the existence of three distinct ANF receptor subtypes in cultured cell lines of renal and vascular origin. These include the larger Mᵢ receptor of 140-kDa in RTASM cells which is completely reduced by DTT treatments to a lower Mᵢ receptor of the 70-kDa band. The second type is another high Mᵢ receptor of 120–135 kDa present in MDCK and MA-10 cells which is not reduced by treatments with sulfhydryl reagents such as DTT or β-mercaptoethanol. The third type is a lower Mᵢ receptor of 66–70 kDa, also a single polypeptide chain, prevalent in both RTASM and MDCK cells. These ANF receptor subtypes exhibited distinct binding characteristics for full-length ANF(99-126) and truncated ANF(103-123) molecules. Photoaffinity labeling of the 140-kDa receptor band in RTASM cells was inhibited by either full-length ANF(99-126) or truncated ANF(102-123); however, labeling of the 120-kDa band in MDCK cells was inhibited only by full-length ANF(99-126) but not by truncated ANF(103-123). On the other hand, the 66–70-kDa labeled bands in both MDCK and RTASM cells were effectively inhibited by either ANF(99-126) or ANF(103-123). It has been shown that affinity cross-linking of 125I-ANF to cultured bovine endothelial cells resulted in two distinct bands of 66 and 130 kDa (45). ANF(99-126) blocked labeling of both the 66- and 130-kDa bands, whereas ANF(103-123) failed to completely block the labeling of the 130-kDa band. These endothelial cells possess similar high Mᵢ ANF receptor to MDCK cells shown in our present studies.

Studies in the structure-activity relationships of various length ANF peptides have shown the differential degree of responsiveness for both natriuretic and vasorelaxant activities. ANF(103-123) was found to be 10 times less potent than ANF(99-126) in natriuretic activity (32, 46). On the other hand ANF(103-123) was either equally or only 2-fold less potent than ANF(102-123) or ANF(99-126) for vasorelaxant activity (32, 47). In our study, truncated ANF(103-123) is comparable or only slightly less potent than ANF(99-126) in displacing radiolabel from the

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**Fig. 6. Specificity of photoaffinity labeled disulfide-unlinked 120- and 68-kDa ANF receptor subtypes on the intact MDCK cells.** Confluent cells were labeled with AZB-125I-ANF(99-126) in the dark at 4 °C for 10 min in the absence (lane a) or presence of ANF(99-126) (lane b), as described under "Experimental Procedures." Lanes a and b represent the photoaffinity labeled cells with AZB-125I-ANF(99-126) in the absence or presence of unmodified ANF(103-123), respectively. The labeled 67-kDa bands are artifactual due to BSA in assay media used in these experiments. Presumably, after DTT treatment, ANF receptor moved faster and was positioned below the BSA-labeled band. Arrows indicate the position and Mᵢ of ANF receptors, and bars indicate the Mᵢ of marker proteins.

**FIG. 7. Competition of ANF(99-126) with AZB-125I-ANF(99-126) receptor complexes in intact MDCK cells.** Confluent monolayer cells were incubated with varying concentrations of ANF(99-126) and were labeled with AZB-125I-ANF(99-126) in the dark at 4 °C as described under "Experimental Procedures." Samples in lanes a-f were treated with DTT, and radiolabeled bands were analyzed by SDS-PAGE and autoradiography. Arrows indicate the Mᵢ of labeled receptors and bars indicate the Mᵢ of marker proteins.

**FIG. 8. Specificity of truncated ANF(103-123) for the 68-kDa receptor band in MDCK cells.** Confluent monolayer cells were photolyzed in the dark at 4 °C as stated under "Experimental Procedures." Lanes a and b represent the photoaffinity labeled cells with AZB-125I-ANF(99-126) in the absence or presence of unmodified ANF(103-123), respectively. The labeled 67-kDa bands are artifactual due to BSA in assay media used in these experiments. Presumably, after DTT treatment, ANF receptor moved faster and was positioned below the BSA-labeled band. Arrows indicate the position and Mᵢ of ANF receptors, and bars indicate the Mᵢ of marker proteins.

**FIG. 9. Limited tryptic proteolysis of 140-kDa disulfide-linked receptor in photoaffinity labeled intact RTASM cells.** A, photoaffinity labeled ANF receptors before limited proteolysis under unreduced (lane a) or reduced (lane b) conditions. B, photoaffinity labeled receptors after limited proteolysis. Cells were photolyzed and treated with trypsin (100 μg/ml) as described under "Experimental Procedures." After solubilizing the cells in 1% SDS, samples were electrophoresed under unreduced (lanes a–c) and reduced (d–f) conditions. Arrows indicate the position and Mᵢ of receptor bands before and after trypsin treatments.
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140-kDa band in RTASM cells. This is consistent with previous reports of full-length and truncated ANF effects on vasorelaxation (32, 46). In the MDCK cells, which are a model system for sodium transport, ANF(103-123) does not displace the 120-kDa ANF receptor band. Thus the differences in the ability of ANF(103-123) to bind with larger M, receptors in RTASM and MDCK cells may explain the differences observed in the natriuretic and vasorelaxant properties of truncated ANF(103-123) reported previously (32, 46, 47). Heterogeneity of the ANF receptor has been postulated based on different binding characteristics and ability to generate cGMP by varying lengths of ANF peptides (45, 48, 49). Recent evidence suggests that the majority of renal ANF receptors seem to be biologically inactive, silent (50). Whether any of the three ANF receptors observed in the present studies is a silent receptor subtype remains to be seen. Our results indicate that multiple forms of high MI ANF receptors possibly exist separately in cells originating from the renal and vascular systems. It seems possible that the single chain disulfide-unlinked high M, ANF receptor in MDCK cells is coupled to guanylate cyclase, similarly to one of the two isolated receptors in bovine adrenal cortical cells (51). The disulfide-linked 140-kDa high M, ANF receptor found in RTASM cells seem to be a non-guanylate cyclase containing receptor and may be a dimer of 70-kDa proteins linked by a disulfide bridge(s). Hence, whether this is a homodimer or heterodimer is yet not clear.

In previous studies, we have shown that ANF(96-126) (1 X 10^{-7} M) stimulated cGMP approximately 1500-fold in MA-10 cells (52) and only one predominant radiolabeled 135-kDa band was observed which was not reduced by DTT and that its labeling was not protected by truncated ANF(100-123) (25). After photoaffinity labeling of MA-10 cells with AZB-^{125}I-ANF, followed by treatment with ANF(96-126), cGMP production was reduced by 99% as compared to the nonphotolyzed cells. These findings suggest that guanylate cyclase-coupled ANF receptor molecule may consist of a single polypeptide chain and is polymerized by disulfide linkages. In MA-10 and LLC-PK1 cells, the 135-kDa receptor showed characteristics similar to that of 120-kDa high M, ANF receptor of MDCK cells, it was not reduced by DTT, and its labeling was not inhibited by ANF(100-123).

Our findings illustrate two extreme types of tissues (cells); the first, RTASM cells with a predominantly reducible high M, ANF receptor subtype, and the second, testicular Leydig tumor cells (MA-10) with an exclusively nonreducible high M, receptor subtype. Cells derived from the kidney tubular epithelium (MDCK) contain both nonreducible high and low M, ANF receptor subtypes. Table I summarizes the results of the present work, relating cGMP production with the receptor number of the nonreducible high M, receptor. Large differences exist between different cells in the nonreducible high M, receptors (1000 sites/cell in RTASM to 10^6 sites/cell in mouse Leydig cells). As the receptor number increases, the cGMP production (stimulated by 10^{-7} M ANF) increases. A linear relationship is observed between MDCK cells and Leydig cells, while RTASM cells deviate from the linearity. This may be due to an error in the estimation of the nonreducible high M, receptor in these cells (because of its very small proportion or the complicated kinetics of cGMP production). Alternatively, it may imply an additional mechanism of cGMP production in RTASM cells. The characteristic cell lines identified in the present studies will permit further examination of these alternative mechanisms. Such studies are currently in progress.

In MA-10 cells, the nonreducible high M, ANF receptor was found which exhibits biochemical and pharmacological properties similar to the nonreducible 120-kDa receptor of MDCK cells. The variation between the 120 and 135 kDa mass of the single chain disulfide-unlinked receptor in these cells may be explained by differences in the carbohydrate moieties of the proteins due to culture conditions. The proteolytic cleavage of disulfide-unlinked 135-kDa band in LLC-PK1 and MA-10 cells produced only one additional band of 68 kDa, which suggests that only one cleavage site of the disulfide-unlinked receptor is accessible at the extracellular surface of the plasma membrane. In contrast, in RTASM cells the proteolytic cleavage produced multiple smaller distinct bands. After trypsin treatment of photoaffinity labeled RTASM cells, 70-kDa ANF receptor was persistent; however, the 140-kDa band was completely abolished. It is not yet clear whether smaller tryptic fragments were generated from the 140-kDa or from the 70-kDa receptor bands, since the apparent persistence of 70-kDa band might have been due to the appearance of tryptic products from the 140-kDa band (similar to MA-10 or LLC-PK1 cells). Nevertheless, our speculation that smaller tryptic fragments might have been originated from the 140-kDa disulfide-linked receptor in RTASM cells is based on the following observations: (i) after 2.5 min of tryptic proteolysis, the 140-kDa receptor band was almost completely digested and the 70-kDa band was not affected by the trypsin treatments. However, at the same time, the low M, tryptic fragments were already distinctly present. (ii) Even after 5 min of tryptic digestion, the 70-kDa ANF receptor protein was still protected. (iii) In MA-10 and LLC-PK1 cells, only the 135-kDa ANF receptor band was predominantly labeled, and after 10 min of tryptic proteolysis, only the 68-kDa band was generated. Since in both MA-10 and LLC-PK1 cells, not even fainter low M, bands (smaller than 66-68 kDa) were seen, we predict that in RTASM cells low M, tryptic fragments may have been generated from the 140-kDa ANF receptor protein. However the complete answer to these questions awaits the purification and structure determination of these receptor molecules. At least at present, the differences...
between the single chain disulfide-unlinked 120–135-kDa and disulfide-linked 140-kDa receptors in terms of reducing properties and binding characteristics to full-length and truncated ANF strongly support the differences in the structural properties of the two high M, ANF receptors in MDCK, LLC-PK1, and MA-10 versus RTASM cells. Nevertheless, if the high M, ANF receptor in RTASM cells is predominantly a non-glycylated cyclase-containing receptor protein, the mechanism of the generation of cGMP by ANF in RTASM cells as found in the present study or reported previously (53, 54) is not known. It is possible that in the presence of an agonist hormone, the ANF receptor might be coupled in a stimulatory fashion to guanylate cyclase through some regulatory proteins such as guanine nucleotide regulatory proteins (G-proteins) which might be involved in the stimulation of cGMP by ANF in RTASM cells. Recently, the involvement of G-protein in the ANF-mediated inhibition of adenylate cyclase was shown in rat aortic particles (55). However, the coupling of ANF receptors to such regulatory protein(s) remains to be established.

The structural basis of ANF binding to its receptor is unknown. However, Scatchard analysis of ANF binding supports the existence of a single site. The full-length ANF99-126 and truncated ANF105-123 do not appear to recognize the same site of the molecule of the disulfide-unlinked receptor which seem to be present in the kidney and endocrine cells such as MDCK, LLC-PK1, and MA-10 cells. The truncated ANF105-123 did not significantly inhibit the binding of radiolabeled ANF99-126 and was unable to inhibit the labeling of the 120-kDa high M, receptor in MDCK and MA-10 cells. Hitherto, the full-length ANF99-126 peptide molecule seems to provide the structural framework for the binding site whose specificity is probably defined by unique amino acid sequences of Phe-Arg-Tyr at the carboxyl terminus end which is lacking in truncated ANF105-123. The spasmylocytic effects of ANF are most pronounced in the renal artery and aorta with high affinity (Kd = 1 × 10⁻¹⁰ M) binding sites for ANF which are specific and saturable (56). These findings correlate well with the present results of ANF receptors in cultured RTASM and MDCK cells.

In conclusion, findings of this investigation suggest that the variation in ANF receptor subtypes resides with specific ANF target cell types. ANF receptor subtypes appear to exist that at least three forms of ANF receptor subtypes are present in renal, testicular, and vascular cells. The presence of high density ANF binding sites, yet low cGMP stimulation in RTASM cells as compared to MDCK cells, suggests that cyclase-uncoupled ANF receptor subtypes may be linked to a signal transduction system in RTASM cells producing different second messenger in addition to cGMP. The dramatic differences between the biochemical properties of three ANF receptor subtypes obtained in the present study suggest distinct evolutionary origin of multiple ANF receptor proteins in different target cells.

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