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Megalin binds and internalizes angiotensin-(1-7)

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Abstract

Megalin is a multiligand receptor heavily involved in protein endocytosis. We recently demonstrated that megalin binds and mediates internalization of ANG II. Although there is a strong structural resemblance between ANG II and ANG-(1-7), their physiological actions and their affinity for the angiotensin type 1 receptor (AT_1R) are dissimilar. Therefore, the hypothesis of the present work was to test whether megalin binds and internalizes ANG-(1-7). The uptake of ANG-(1-7) was determined by exposure of confluent monolayers of BN/MSV cells (a model representative of the yolk sac epithelium) to fluorescently labeled ANG-(1-7) (100 nM) and measurement of the amount of cell-associated fluorescence after 4 h by flow cytometry. Anti-megalin antisera and an AT_1R blocker (olmesartan) were used to interfere with uptake via megalin and the AT_1R, respectively. ANG-(1-7) uptake was prevented by anti-megalin antisera (63%) to a higher degree than olmesartan (13%) ($P < 0.001$). In analysis by flow cytometry of binding experiments performed in brush-border membrane vesicles isolated from kidneys of CD-1 mice, anti-megalin antisera interfered with ANG-(1-7) binding more strongly than olmesartan ($P < 0.05$ against positive control). Interactions of megalin with ANG-(1-7) at a molecular level were studied by surface plasmon resonance, demonstrating that ANG-(1-7) binds megalin dose and time dependently and with an affinity similar to ANG II. These results show that the scavenger receptor megalin binds and internalizes ANG-(1-7).

Keywords

endocytosis; epithelial cells; angiotensin type 1 receptor; olmesartan

THE RENIN-ANGIOTENSIN SYSTEM (RAS) is a hormonal cascade with coordinated actions in the brain, cardiovascular system, and the kidneys that regulates water and electrolyte balance as well as blood pressure. During the past three decades, the involvement of the RAS has been ascertained for various forms of hypertension and target organ damage, particularly in the heart and the kidneys. Moreover, its role in the pathogenesis of hypertension and other cardiovascular diseases has been further substantiated by the therapeutic efficacy of angiotensin-converting enzyme inhibitors and ANG II receptor type 1 (AT_1R) blockers (ARBs) (14).

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ANG II is classically recognized as the main effector of the RAS. In the last few years, however, several reports have shown that other metabolites generated by alternative enzymatic cleavage of ANG I and/or ANG II, like ANG III, ANG IV, and ANG-(1-7), are also biologically active (6). Of these, ANG-(1-7) is now recognized as a separate mediator of RAS actions. The effects of ANG-(1-7) have been extensively studied, and they can be similar, opposite to, or separate from those of ANG II (for reviews, see Refs. 10 and 24). Meanwhile, the receptor for ANG-(1-7) remains unclear (6). Whereas Santos et al. (27) have shown that ANG-(1-7) binds with high affinity to the G protein-coupled receptor Mas, others have demonstrated that in some cases the effects of ANG-(1-7) can be prevented by the use of ARBs (6).

We recently reported that the scavenger receptor megalin binds and internalizes ANG II (12). Furthermore, we showed that megalin provides the predominant pathway for uptake of ANG II at high concentrations for at least the first 24 h in a cell line (BN/MSV) expressing at its apical side megalin and the AT_1R. Megalin is a ~600-kDa transmembrane protein belonging to the low-density lipoprotein-receptor family of single-transmembrane-domain receptors. This multiligand receptor, heavily involved in protein endocytosis, is expressed in several tissues, including the epithelium of placenta and the brush border of the proximal tubule in the kidney (7). The main renal function of megalin is thought to be the nonspecific uptake of proteins and peptides that escape through the glomerular filtration barrier (7). However, our recent report suggests that megalin can participate in the regulation of intratubular ANG II levels by binding and internalizing ANG II.

Because ANG II (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8) strongly resembles ANG-(1-7) (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7) as these two peptides differ only in the presence of a phenylalanine in *position 8*, we sought to determine whether the endocytic properties of the receptor megalin would include binding and uptake of ANG-(1-7). Second, because the affinities of these two peptides for the AT_1R are quite dissimilar, we compared their affinities for megalin.

MATERIALS AND METHODS

Animals, reagents, and antibodies

Mice (CD-1 strain, 2-3 mo old, $n = 24$) were purchased from Charles River Laboratories (Wilmington, MA). These animals were kept in a temperature-controlled room and fed standard Purina mouse chow diet with free access to tap water. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the New Orleans Veterans Affairs Medical Center, where the animal experiments were performed. All reagents were from Sigma (St. Louis, MO) unless otherwise stated. Boron dipyrromethene difluoride fluorophore (BODIPY)-conju-gated ANG-(1-7) [BODIPY-ANG-(1-7)] was custom synthesized by Century 21st Biochemicals (Marlboro, MA). Unlabeled ANG-(1-7) and the antagonist $_D$-Ala^7-Ang-(1-7) (A-779) (25) were purchased from Phoenix Pharmaceuticals (Belmont, CA). Purified human megalin and polyclonal antibodies against megalin were provided by Dr. Pierre J. Verroust (Institut National de la Santé et de la Recherche Medicale, Paris, France). These antibodies were raised against proteins purified by immunoaffinity chromatography using previously reported monoclonal antibodies coupled to Sepharose 4B (13,20,22,23). Antibodies were determined to be monospecific by immunoblotting on whole brush-border preparations and by immunoprecipitation of biosynthetically labeled yolk sac epithelial cells in culture. Importantly, these antibodies recognize mouse, rat, and human megalin (22,23). Anti-neurokinin-1/ substance-P receptor antiserum (anti-NK1 antibodies) was a gift of Dr. Jacques Couraud, Gif-sur-Yvette, France (3). Sankyo (Tokyo, Japan) provided RNH-6270 (active form of olmesartan, hereafter referred to as olmesartan).

Cell culture

Cell experiments were conducted using immortalized yolk sac cells from the Brown Norway rat (BN/MSV; *passages 3-9*) (18). These cells share several characteristics with renal proximal tubule cells that make them suitable for endocytotic studies, including a well-developed brush border and a similarly specialized endosomal pathway, with abundant expression of the proteins of interest including megalin and the AT_1R, as well as surface epithelial markers specific for brush border (12,18). The expression, localization, internalization, and intracellular trafficking of megalin in BN/MSV have been demonstrated previously by immunocytochemical methods (15,19). BN/MSV cells were cultured in conventional T flasks (T75) in a humidified incubator with 5% CO_2 and environmental air using DMEM/F-12 (GIBCO/Invitrogen, Carlsbad, CA) supplemented with heat-inactivated fetal calf serum (10%) and an antibiotic cocktail [ciprofloxacin (Bayer, West Haven, CT) and fungizone (GIBCO/ Invitrogen)]. Cells were fed every 48 h and divided on reaching confluency.

ANG-(1-7) uptake in BN/MSV cells analyzed by flow cytometry

In uptake experiments, monolayers of BN/MSV were exposed to labeled ANG-(1-7) [BODIPY-ANG-(1-7), 100 nM] for 4 h with or without a competitive antagonist. To block AT_1R-dependent BODIPY-ANG-(1-7) uptake, the specific AT_1R blocker olmesartan was used at a concentration 1,000-fold greater than ANG-(1-7) (10 μM). To block megalin-dependent BODIPY-ANG-(1-7) uptake, a polyclonal antiserum against megalin that recognizes the holoprotein was used in stepped 100- to 3,000-fold dilutions (2,12,13,22,23). Previous studies showed that these antibodies effectively prevent the uptake of known ligands for megalin, including ANG II and metallothionein (12,17). Other additional experimental groups were included for further analysis: a combination of olmesartan/anti-megalin antibodies was added to search for an additive effect; A-779 was examined, as this agent has been suggested as a specific blocker for the Mas receptor (26). Anti-NK1 peptide antibodies were chosen as negative controls for nonspecific binding because they bind brush-border membrane vesicles at the same titer as the anti-megalin antisera (12,17). Finally, chloroquine (10 mM) was used to demonstrate that BODIPY-ANG-(1-7) uptake is dependent on acidification of the endosomal compartment (12,16,17).

BN/MSV cells were seeded in 96-well plates (20,000 cells/well) and allowed to grow to confluence. Each experimental group was run in triplicate, and the experiments were repeated a minimum of three times. To prevent nonspecific binding, ovalbumin (0.1%) was added to serum-free medium (SFM) and this solution (SFM-ovalbumin 0.1%) was used for all steps during the experiments. On the day of the experiment, cells were washed with SFM-ovalbumin 0.1% and allowed to equilibrate for 2 h at 37°C. The competitors were then added, and the cells were incubated for 1 h. After that, the cells were treated with BODIPY-ANG-(1-7) (100 nM) and incubated for an additional 4 h. To stop the uptake reaction, and remove unbound BODIPY-ANG-(1-7), the well plate was first placed on ice and the cells were washed twice with ice-cold PBS. Then, the cells were incubated for 5 min with an acid solution (50 mM acetic acid, 150 mM NaCl, pH 3.0) to release membrane-bound BODIPY-ANG-(1-7). At this stage, the cells were washed again with PBS and trypsinized, transferred to flow cytometry tubes, and stored on ice briefly until the cell-associated fluorescence [an indication of internalized BODIPY-ANG-(1-7)] was determined by flow cytometry. Samples were analyzed using a FACS-Vantage flow cytometer (Becton Dickinson Immunocytochemistry, San Jose, CA) using a dedicated Macintosh computer. Excitation was at 488 nm using a coherent 2-W argon ion laser. For each particle, emission was measured using photomultipliers at 530 ± 30 nm. Data were collected as 4,000 observations-mode files and analyzed using CellQuest software (Becton Dickinson Immunocytochemistry).

ANG-(1-7) binding to brush-border membrane vesicles analyzed by flow cytometry

On the day of the experiment, mice were killed by CO_2 inhalation in a closed chamber. The kidneys were then extracted via median incision, randomly distributed in four groups (~12 kidneys/group), and kept in ice-cold PBS until further processing. The kidneys were decapsulated, and brush-border membrane vesicles (BBMV) were isolated according to the divalent cation precipitation method (2,13,20). We chose to study the binding of BODIPY-ANG-(1-7) to this surface as it represents mainly the apical side of the proximal tubule, a surface rich in megalin. Furthermore, although the exofacial portion of megalin is found predominantly on the internal surface of renal intermicrovillar clefts (13), it remains on the outside of >90% of membrane vesicles prepared from BN/MSN cells and BBMV (15). The binding of BODIPY-ANG-(1-7) to BBMV was investigated in the presence of competitors for AT_1R (10 μM olmesartan), megalin (anti-megalin antibodies, 1:100 dilution), or both. Anti-NK1 peptide antibodies were used as negative controls for nonspecific interference by binding (17). BBMV were preincubated for 1 h with SFM-ovalbumin 0.1% at 4°C, treated with the various competitors, and incubated for 1 h at 37°C before the addition of BODIPY-ANG-(1-7) (100 nM). Finally, after an additional 4 h, the binding of BODIPY-ANG-(1-7) to BBMV was analyzed by flow cytometry as described except that the data were collected as 10,000 observations-mode files.

Surface plasmon resonance experiments

A molecular interaction of ANG-(1-7) with megalin was assayed using a BIACORE 3000 biosensor system (Biacore). For these experiments, highly purified human renal megalin was immobilized to a dextran-coated gold surface (CM5 biosensor chip) by covalent conjugation. In surface plasmon resonance (SPR), injection of a soluble protein produces a signal change that is directly proportional to the mass of bound protein and is reported in real time as a change in resonance units (RU). Thus SPR allowed us to explore qualitatively an interaction between megalin and ANG-(1-7). Megalin (0.025 mg/ml in 10 mM acetate, pH 4.53) was immobilized (6,000-8,000 RU) in one flow cell on a CM5 chip using standard primary amine-coupling methods (12,17). A second flow cell, activated and blocked with ethanolamine but lacking immobilized megalin, provided a real-time reference correction for instrumental artifacts and nonspecific binding events. A solution of pure ANG-(1-7) or pure ANG II (0.5 mg/ml each in HEPES-buffered saline, pH 7.4, containing 2 mM Ca, 2 mM Mg, and 0.005% surfactant P20) was injected over both flow cells at room temperature. Maximum reproducibility was obtained when 0.0008% sodium dextran sulfate (cat. no. 17-0340-01, Pharmacia Biotech) was also included in the buffer. ANG-(1-7) or ANG II was injected at a flow of 5 μl/min for 25 min and then allowed to dissociate for 15 min. Each sample was injected at least three times. The Biacore KINJECT sequence was used to minimize mechanical noise and to maintain sample consistency. The small size of ANG-(1-7) (~1 kDa) enabled us to use a slow flow without encountering problems associated with diffusion (mass transport). No regeneration (removal of bound protein by injection of a second, typically harsh solvent) was necessary due to the low affinity of the ligands tested. BIAEvaluation software (V. 3.1)was used to analyze the results. The “double-referencing” technique of Myszka (21) was used to eliminate additional instrumental artifacts. The blank injections used for this procedure were identical to sample solutions except for the omission of the tested peptides.

The receptor cubilin was immobilized under virtually identical conditions to provide a negative control for binding. Cubilin (0.025 mg/ml in 10 mM acetate, pH 4.78) was immobilized (6,000-8,000 RU) in one flow cell on a CM5 chip using standard primary amine-coupling methods as detailed by the manufacturer. A second flow cell was activated and blocked with ethanolamine but lacked immobilized cubilin, providing a real-time reference correction for instrumental artifacts and nonspecific binding events. ANG-(1-7)

(0.5 mg/ml) was injected over both flow cells at room temperature in HEPES-buffered saline, pH 7.4 containing 2 mM Ca, 2 mM Mg, and 0.005% surfactant P20. Because no binding was observed, regeneration (removal of bound protein by injection of a second, typically harsh, solvent) was unnecessary. Additional procedures were as described alone for megalin.

Statistical analysis

Data are expressed as means ± SE throughout the manuscript. Statistical analysis was performed using GraphPad Prism Software 4.0 (GraphPad Software, San Diego, CA) by one-way ANOVA and Bonferroni's post hoc comparison as appropriate. Flow cytometry data were also analyzed by Kolgomorov-Smirnov summation statistics (29). A value of $P < 0.05$ was considered statistically significant.

RESULTS

BODIPY-ANG-(1-7) uptake of BN/MSV cells determined by flow cytometry

Figure 1 shows that BN/MSV cells take up BODIPY-ANG-(1-7) after a single-dose exposure. Using data from previous experiments with ANG II (12), we chose to incubate the cells for 4 h.

Figure 1 also shows the effect of the different agents tested on BODIPY-ANG-(1-7) uptake ($P < 0.001$, 1-way ANOVA). Individually, only anti-megalin antibodies significantly inhibited BODIPY-ANG-(1-7) uptake. This inhibition was concentration dependent and ranged approximately from 63 to 30% at the lowest dilution (1:100 dilution) to the highest dilution (1:3,000) of the antisera. These results are consistent with our hypothesis that megalin binds and internalizes ANG-(1-7).

Olmesartan alone did not have a significant effect on BODIPY-ANG-(1-7) uptake (~13% in reduction). This is despite the fact that olmesartan concentration was 10,000 times higher than BODIPY-ANG-(1-7). However, there was an additive effect when anti-megalin antibodies and olmesartan were combined, as reflected by a >70% in reduction in uptake [$P < 0.05$ by Bonferroni's post hoc test against the anti-megalin antibodies group (1:100 dilution)]. Moreover, preincubation with A-779 had no significant effect on ANG-(1-7) uptake, suggesting that it is unlikely that the Mas receptor participates in the uptake process, at least on the apical side of BN/MSV cells and at the concentration and time tested. Consistent with previous reports, chloroquine, which inhibits endosomal acidification, completely abolished the uptake of the ANG-(1-7) ligand by BN/MSV cells (12,17). Accordingly, chloroquine-treated cells were considered negative controls for the current analysis. When used at the same dilution as anti-megalin antisera (1:100), anti-NK1 antibodies did not produce any significant effect on BODIPY-(ANG-1-7) uptake.

BODIPY-ANG-(1-7) binding to BBMV analyzed by flow cytometry

Figure 2 shows the results of experiments analyzing BODIPY-ANG-(1-7) binding to BBMV. A gated analysis was implemented to isolate a homogenous population of single vesicles based on the size-dependent parameters (forward and side-scatter parameters). This population included 80-90% of the total sample in each series. Considered collectively, the different agents caused a general variability detectable by one-way ANOVA ($P < 0.05$). Separately, anti-megalin antisera (1:100 dilution) produced the strongest binding inhibition. Olmesartan slightly reduced binding, although this effect was not significant by post hoc tests. The combination of anti-megalin antibodies plus olmesartan suggested a possible additive effect; however, this effect was not statistically different compared with the anti-megalin group. Moreover, variability was the greatest in the results for the groups treated

with olmesartan, a finding compatible with the long-term recognized low affinity of ANG-(1-7) for AT_1R (5).

Molecular studies of ANG II-megalin binding using SPR

SPR analysis of the ANG-(1-7) binding to megalin is displayed in Figure 3. Surface mass changes compatible with a low-affinity interaction between megalin and ANG-(1-7) were detected. Moreover, these responses uniformly increased with time. The observed variations and noise are normal for the very low signal levels used to optimize a study of binding constants when the sizes of the two interacting species are quite different. In turn, no interactions were observed between ANG-(1-7) and cubilin, another protein expressed in the apical side of the proximal tubule and yolk sac epithelium (data not shown). To produce signal changes large enough for qualitative detection of binding, we were compelled to use high surface coverage by megalin on the SPR chip. As megalin is ~500 times larger than ANG-(1-7), we conclude that the binding stoichiometry is 1:1. However, in the absence of a true equilibrium (which would not be reached except after significantly longer injection periods), we cannot rule out the possibility of the presence of more than one binding site. During the dissociation of ANG-(1-7) from megalin, the signal decayed exponentially with a half-life of ~15 s, consistent with a low-affinity binding interaction. As can be appreciated in Fig. 3, the binding kinetics of ANG II and ANG-(1-7) are fairly similar and of low affinity. Using the BIAEvaluation software, the K_d for each ligand was estimated to be ~3×10^{-2} M. These calculations reflect the low affinity observed for other known megalin ligands (16,17). In thermodynamic terms, ANG II and ANG-(1-7) produce responses with the same order of magnitude, as would be expected for ligands of similar size; in kinetic terms, however, ANG II equilibrates more rapidly with megalin than does ANG-(1-7).

DISCUSSION

The hypothesis of the present work is that megalin binds and mediates ANG-(1-7) uptake. Three lines of evidence are presented to support this hypothesis. First, in BN/MSV cells, a cell model with stable megalin expression, the use of anti-megalin antibodies strongly interfered with labeled ANG-(1-7) uptake. Second, in BBMV isolated from mouse kidneys, the use of the same antisera also prevented labeled ANG-(1-7) binding. Finally, SPR experiments consistently show an interaction, at a molecular level, between pure ANG-(1-7) and megalin.

Our results suggest that ANG-(1-7) may be internalized and is not limited to surface receptor binding. We also demonstrate that this process is mediated by megalin at the concentration and time tested. When BN/MSV cells were pretreated with anti-megalin antibodies, there was a strong inhibition in ANG-(1-7) uptake that was specific and dose dependent, as demonstrated by a lesser effect at lower concentrations of the antisera and the lack of effect of control antibodies (anti-NK1). Even though we did not find a significant role for the AT_1R-mediated pathway in the uptake of ANG-(1-7), further work is needed to test the role and relevance of each pathway at different concentrations and times of exposure to ANG-(1-7), especially at lower ANG-(1-7) concentrations that may be more physiological. The weak nature of the interactions between megalin and angiotensin peptides, as demonstrated by the SPR experiments, makes it all the more remarkable that anti-megalin antibodies so thoroughly inhibit ligand uptake. The high level of megalin expression in the renal proximal tubule and yolk sac epithelia, combined with rapid receptor recycling, may allow such a weak interaction to produce efficient ligand scavenging under normal conditions.

Flow cytometry only allows assay of the presence of the fluorochrome conjugated to the angiotensin peptide before addition to the cell cultures. However, because measures were

taken to remove free-floating as well as surface peptide, cell-associated fluorescence detection by flow cytometry is considered an indication of the internalized peptide. However, the metabolism and distribution of the peptide during intracellular trafficking remain to be determined.

Anti-Nk1 antisera were included in these studies to minimize the limitations inherent in making comparisons between an angiotensin peptide-positive control and antibody interference, due to binding of megalin to some of its other ligands present in serum (7,8). Specifically, serum contains varying concentrations of potential inhibitors other than the specific antibodies including immunoglobulin light chains, which are known ligands for megalin (15), and the concentrations of nonspecific immunoglobulins vary between antisera (7,8,12). As NK-1 has no known role in the megalin pathway (7), anti-NK-1 antisera provided a control for specific and nonspecific binding of serum ligands to megalin. Our results support these assumptions as they demonstrated a stronger interference in ANG-(1-7) uptake by anti-megalin antibodies at all dilutions compared with anti-NK1-antibodies.

Interplay between megalin and, perhaps, the AT_1R as endocytic receptors for ANG-(1-7) might be important in several tissues where these two receptors are coexpressed, such as the apical side of the proximal tubule in the kidney (4,7). Although a consensus has not been reached in this matter, ANG-(1-7) seems to induce diuresis and natriuresis in the kidney, except in water-loaded rats (10,24). The overall sodium and water balance, renal nerve activity, as well the overall activity of the RAS appear to influence ANG-(1-7) effects in the kidney (10,24).

In contrast to its modest effects on kidney hemodynamics, ANG-(1-7) has several actions on different segments of the nephron (1,11,28). Furthermore, an intratubular production of ANG-(1-7) has also been suggested (9,19). These observations suggest that ANG-(1-7), either filtered from the general circulation or locally produced by the proximal tubule, can act on the apical side of this segment or be delivered to more distal areas. Megalin-mediated uptake of ANG-(1-7) might contribute to regulate the tubular levels of this hormone as well as the amount excreted in urine. Another possibility is that binding of ANG-(1-7) to megalin may also elicit separate intracellular signals, but this has yet to be determined. Nevertheless, the present study demonstrates that ANG-(1-7) does bind to megalin on the apical side of the proximal tubule. Whether this interaction triggers its internalization, although likely, has yet to be determined.

The Mas receptor is a putative specific receptor for ANG-(1-7) in diverse tissues, including the kidney and vasculature (27), but to date its expression in the proximal tubule or the placental epithelium has not been clearly established. As reported by Santos et al. (27), there is low-level specific binding of ^{125}I-ANG-(1-7) in the kidneys of wild-type mice. However, this kind of analysis relies on high-affinity binding between the receptor and its ligand. That is not the case with megalin and ANG-(1-7) in the present work. Furthermore, the current analysis does not diminish the role of the Mas receptor or any other ANG-(1-7)-specific receptor as mediators of the physiological effects of ANG-(1-7). Rather, we extend and complement this understanding by showing that in the megalin-dependent BN/MSV cells and renal brush-border membrane systems examined and at the conditions tested, megalin acts as a binding and uptake mechanism for ANG-(1-7). Moreover, ANG-(1-7) is known to have a low affinity for the AT_1R (5), and our data are consistent with these findings.

Comparable to our earlier findings that the predominant uptake mechanism for ANG II, at high concentrations and after a short-time exposure, is not AT_1R, but megalin in tissues with coexpression of both receptors (11), the same has now been demonstrated for ANG-(1-7). The similar low affinity of these two ligands suggests that megalin acts as a scavenger.

Therefore, these data would indicate a role for megalin, one of the most abundant proteins on the apical side of the renal proximal tubule (8), in the removal of active peptides of the renal angiotensin cascade from the tubular fluid.

In summary, we present evidence to support the concept that megalin binds and internalizes ANG-(1-7). Moreover, in BN/MSV cells, a model for epithelium with coexpression of megalin and the AT_1R, megalin serves as the predominant ANG-(1-7) uptake pathway. The observation that ANG-(1-7) can be internalized widens the area in which to explore the pleiotropic effects of this hormone. These findings also suggest a role for megalin not only as a scavenger receptor but also as a regulator of local activity of RAS in those tissues where these system and megalin expressions coexist.

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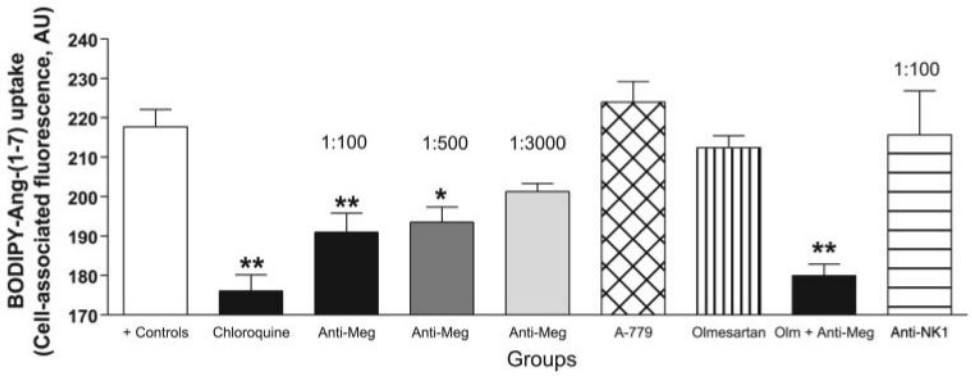

Fig. 1.

Flow cytometry analysis of BODIPY-ANG-(1-7) uptake by Brown Norway rat cells (BN/MSV). Cells were preincubated with the different agents for 2 h before the addition of BODIPY-ANG-(1-7) (100 nM except for the positive control). Anti-Meg, anti-megalin antibodies; Olm, olmesartan; A-779, D-Ala^7-ANG-(1-7); anti-Nk1, anti-NK1 antibodies (negative controls); AU, arbitrary units. Nos. above the bars represent corresponding antibody dilutions, and bars are accumulative means ± SE of several samples (n = 6-9, 4,000 observations/sample). *P < 0.05 vs. positive controls. **P < 0.001 vs. positive controls (Bonferroni's post hoc test, 1-way ANOVA).

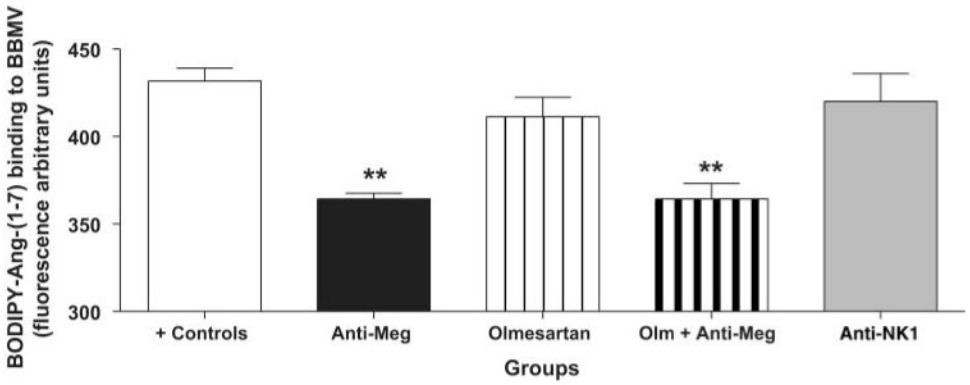

Fig. 2.

BODIPY-ANG-(1-7) binding to mouse renal brush-border membrane vesicles (BBMV). BBMV were isolated from CD-1 mice and preincubated with competitors for 1 h before addition of BODIPY-ANG-(1-7) (100 nM). Olm+Anti-Meg, combination of olmesartan and anti-megalin antibodies; + control, positive controls. Bars represent accumulative means ± SE of 3 series of samples (10,000 individual cell measurements/series). $^{**}P < 0.05$ vs. positive control (Bonferroni's post hoc test).

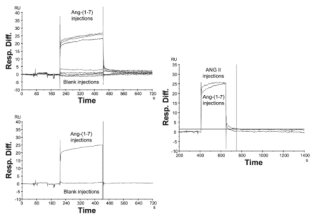

Fig. 3.

Surface plasmon resonance (SPR) analysis of ANG-(1-7)-megalin interaction. *Top left*: results of 10 superimposed experiments performed at separate times: 5 ANG-(1-7) injections vs. 5 blank injections. All are corrected in real time for buffer-related arti-facts as described elsewhere (21). *Bottom left*: average of the same 5 injections is presented after blank correction. *Right*: comparison of the results obtained for injections of ANG II and ANG-(1-7). Although the similarly sized peptides produce comparable final responses, their rates of equilibration, represented by the rising and falling of the signal, are clearly different. All SPR experiments represent the effect of a continuous injection of pure ANG-(1-7) or ANG II (both 0.5 mg/ml) over immobilized megalin.