Characterization and Function of the Bovine Kidney Epithelial Angiotensin Receptor Subtype 4 Using Angiotensin IV and Divalinal Angiotensin IV as Receptor Ligands

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ABSTRACT

125I-Angiotensin (Ang) IV and 125I-divalinal Ang IV [AT receptor subtype 4 (AT4)] receptor agonist and putative antagonist, respectively] were used to characterize the AT4 receptor in Mardin-Darby bovine kidney epithelial cells (MDBK cell line). Both 125I-Ang IV and 125I-divalinal Ang IV bound to a single high-affinity site (KD = 1.37 and 1.01 nM, respectively) and to a comparable density of binding sites (Bmax = 1335 and 1407 fmol/mg protein, respectively). Competition of either radiolabeled ligand with several Ang related peptides demonstrated similar displacement affinities in the following affinity order: Ang IV > divalinal Ang IV > Ang III > Ang II > losartan = PD 123177. Guanosine-5’-O-(3-thio)triphosphate or sulfhydryl reducing agents did not affect the binding of either radiolabeled ligand. Brief exposure of MDBK cells to Ang IV or divalinal Ang IV (0.1 nM to 1 μM) caused a concentration-dependent rise in intracellular calcium concentration levels with a reduced calcium response observed with Ang IV at micromolar concentrations. These results indicate that Ang IV and divalinal Ang IV bind with high affinity to the same receptor and that the MDBK AT4 receptor is not coupled to a classic G protein, nor are sulfhydryl bonds important in regulation of receptor affinity. The MDBK AT4 receptor appears to be pharmacologically similar to that described in nonrenal tissues. Functional studies suggest that AT4 receptor activation can increase intracellular calcium concentration levels in MDBK cells and that divalinal Ang IV possesses agonist activity with respect to this particular intracellular signaling system.

The renin-angiotensin system is composed of a cascade of biochemical reactions that have a pivotal role in the homeostatic regulation of the internal environment of organisms. The numerous actions of the renin-angiotensin system throughout the body are likely due to the formation of angiotensin (Ang) II and several shorter Ang II fragments [e.g., Ang(2-8), Ang(1-7), and Ang(3-8)] that can act on Ang II receptor subtype 1 (AT1), AT2, or atypical (non-AT1, non-AT2) AT receptor subtypes to elicit biological responses (Ardaillou and Chansel, 1998). Elucidation of some of these AT4-dependent functions has relied on the ability of the putative partial nonpeptide AT4 receptor antagonist divalinal Ang IV to interact with the same receptor sites as Ang IV (Krebs et al., 1996; Handa et al., 1998) and to selectively block the actions of Ang IV (Kerins et al., 1995; Coleman et al., 1998; Handa et al., 1998; Patel et al., 1998). Despite the growing use of divalinal Ang IV as a tool to elucidate the physiology of the AT4 receptor system, no study has yet characterized both the binding and functional properties of divalinal Ang IV in the same tissue or cell type.

AT4 receptors are abundant in the kidney and have been shown to be expressed in cultured rat mesangial cells.

ABBREVIATIONS: Ang, angiotensin; MDBK, Mardin-Darby bovine kidney; AT receptor, angiotensin receptor; GTPγS, guanosine-5’-O-(3-thio)triphosphate; [Ca2+]i, intracellular calcium concentration.
(Ardaillou and Chansel, 1996; Chansel et al., 1998), rat and rabbit proximal tubules (Dulin et al., 1994; Handa et al., 1998), cultured opossum proximal tubule cells (Dulin et al., 1995), and cultured SV-40 transformed human collecting duct cells (Czekalski et al., 1996). To date, functions associated with the renal Ang IV AT₄ receptor system include increased cortical renal blood flow (Coleman et al., 1998), modulation of mesangial cell contractility (Ardaillou and Chansel, 1996), and inhibition of energy-dependent solute transport in the proximal tubule (Handa et al., 1998). The renal epithelial AT₄ receptor has been partially characterized in opossum proximal tubule (OKT7A) cells and SV-40 transformed human collecting duct cells and has yielded conflicting results on ligand/receptor-coupling mechanisms and intracellular signal-transduction pathways (Dulin et al., 1995; Czekalski et al., 1996). In the present study, we used a Mardin-Darby bovine kidney (MDBK) epithelial cell line to explore the properties of the renal epithelial AT₄ receptor because most of the known facts on ligand binding pharmacology, physiology, and protein analysis of the AT₄ receptor has been performed in bovine tissues (e.g., Swanson et al., 1992; Kerins et al., 1995; Bernier et al., 1998; Zhang et al., 1998). In addition, preliminary results revealed that MDBK cells expressed abundant amounts of the AT₄ receptor with no detectable AT₁ or AT₂ receptors. Consequently, the aims of this study were 3-fold: first, to pharmacologically characterize the AT₄ receptor in a bovine renal epithelial cell line (MDBK cells); second, to provide data demonstrating that the putative AT₄ receptor antagonist divalinal Ang IV binds exclusively and with high affinity to the renal epithelial AT₄ receptor; and third, to examine functional responses to Ang IV and divalinal Ang IV in the same cell line used to characterize the AT₄ receptor.

Materials and Methods

Cell Culture. MDBK cells are a distal tubular-like epithelial cell line (Ishikawa et al., 1978; Gagnon et al., 1994). The cells were grown in an atmosphere of 95% air/5% CO₂ at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 5% FBS, 5% calf bovine serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 5 μg/ml amphotericin B. Cultures were re-fed with fresh media every 2 days. All the experiments presented in this study were performed at passages 3 to 12 on confluent cells that had been cultured for 5 to 7 days.

Cell Membrane Preparation. Confluent MDBK cells grown in 75-cm² flasks were washed once with ice-cold PBS followed by the addition to the flask of 2 ml of ice-cold isotonic buffer [150 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane, 50 μM Plummer’s inhibitor (carboxypeptidase inhibitor), 20 μM bestatin (aminopeptidase inhibitor), 5 mM EDTA, 1.5 mM 1,10-phenanthroline (divalent ion chelators), and 0.1% heat-treated BSA at pH 7.4]. The cells were dislodged by scraping with a rubber policeman, collected in a centrifuge tube, and homogenized in 10 ml of isotonic buffer for −10 s. The homogenate was centrifuged at 40,000 g for 30 min at 4°C. The supernatant was discarded, the pellet was rehomogenized in 10 ml of isotonic buffer, and the high-speed centrifugation was repeated. The final pellet was resuspended in isotonic buffer to a working concentration of 1 mg protein/ml.

Liver and adrenal medulla tissues were obtained from decapitated adult male Sprague-Dawley rats. The tissues were immediately homogenized in 10 ml of hypotonic buffer (50 mM Tris, 1 mM EDTA, pH 7.4, at 4°C) for −10 s. The homogenates were then centrifuged at 500g for 10 min at 4°C; the supernatant saved on ice, the pellet resuspended in 10 ml of hypotonic buffer, rehomogenized, and recen trifuged. The supernatants were combined and centrifuged at 40,000g for 30 min at 4°C. The resulting pellet was then resuspended in 10 ml of isotonic buffer, homogenized, and the high-speed centrifugation was repeated. The final pellet was resuspended in isotonic buffer to a working concentration of 1 mg protein/ml.

Radioreceptor Assays. All MDBK experiments were performed on freshly isolated cell membranes because freezing of membranes at −20°C or −70°C for 24 h resulted in ≥30% loss in specific binding to the AT₄ receptor (not shown). MDBK cell membranes (25 μg of protein unless stated otherwise) were incubated in a total volume of 250 μl of assay buffer (isotonic buffer). Incubations were performed at 37°C for 60 min with 0.6 nM ¹²⁵I-Ang IV or 90 min with 0.6 nM ¹²⁵I-divalinal Ang IV, and nonspecific binding was assessed in the presence of 1 μM unlabeled Ang IV or divalinal Ang IV, respectively. Bound and free radioligands were separated by vacuum filtration in a cell harvester using 32 glass fiber filters and washed with 5 × 4 ml of PBS (pH 7.2 at room temperature). Radioactivity retained by the protein-bound filters was counted using an ICN 10/880 gamma counter (77% efficiency).

To investigate whether the AT₄ receptor was G protein linked, the membrane preparations were first preincubated at 22°C for 60 min in the assay buffer supplemented with 5 mM MgCl₂ and containing increasing concentrations of a nonhydrolyzable analog of GTP [guanosine-5’-O-(3-thio)triphosphate (GTPγS), 1 nM to 1 mM]. Pretreated membranes were then studied in equilibrium binding experiments. The effect of a sulfhydryl-reducing agent on ¹²⁵I-peptide binding was examined by incubating the membranes in the assay buffer containing increasing concentrations of dithiothreitol (0.1 μM to 1 M).

Measurements of Cytosolic Calcium. All studies were performed using either a standard bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 6 mM glucose (pH 7.4 with NaOH) or a Ca²⁺-deficient bath solution containing 0 mM CaCl₂ and 3 mM MgCl₂. Fluorometric determination of cytosolic calcium used subconfluent monolayers of MDBK cells grown on glass coverslips. Cells were loaded with 2 μM Fura 2-acetoxyethyl ester and pluronic acid for 30 min and then washed for 30 min in standard bath solution at room temperature. Coverslips were placed in a temperature-regulated chamber that sat on the stage of an inverted microscope that was mounted with a micropipette perfusion system. Studies were performed at 37°C on groups of two to six cells whose fluorescence was delimited by an adjustable window. Fura 2 was alternatively excited at wavelengths of 340 and 380 nm, and the emission at 510 nm was measured with a photomultiplier tube using a Nikon Photomoscan-2 fluorescence photometry system. To assess whether all cells responded to Ang IV, we examined individual cell intracellular calcium concentration ([Ca²⁺]i) measurements in groups of cells with digital imaging fluorochrome microscopy using Metafluor Imaging system software. We confirmed that Ang peptide-induced alteration in [Ca²⁺]i was due to 340- and 380-nm emission intensities changing in reciprocal directions and that the peptides did not exhibit autofluorescence. The ratio of the 340/380-nm emission intensity was converted to an actual calcium concentration using a Fura 2 calibration table without determination of minimum and maximum values in individual cells. Consequently, the cytosolic calcium values must be interpreted as approximate concentrations.

Iodination of Peptides. Ang IV and divalinal Ang IV were iodinated by incubating each peptide (50 μg) in 0.2 M sodium phosphate buffer (pH 7.2), containing 50 μg of chloramine T and −2 μCi of Na¹²⁵I for 25 s at room temperature (total volume of 120 μl). The reaction was terminated by the addition of 500 μg of Na₂S₂O₅ in 50 μl of sodium phosphate buffer. Moniodinated peptides were separated from unlabeled and diiodinated peptides by HiPLC using a reversed phase C₃₀ column and a linear acetoniitrite (solvent A) gradient of 9 to 26% over 90 min. Solvent B was 83 mM triethylamine phosphate at pH 3.0.
**Drugs.** We received gifts of losartan (DuP 753) from DuPont/Merck Pharmaceuticals and PD 123177 and PD 123319 from Parke-Davis. Norleucine-1 Ang IV, D-Val1 Ang IV, divalinal Ang IV [Vα(CH2)NH ψVα(CH2)NHPP], and norleucine-Tyr-Ile-(6)-hexamidine were prepared in our laboratory (J.W.H.). Fura 2-acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR). Ang IV, Ang II, GTPγS, dithiothreitol, and other reagents were acquired from Sigma-Aldrich Co. (St. Louis, MO), Bachem California (Torrance, CA), or Peninsula Laboratories Inc. (Belmont, CA).

**Statistics.** All values represent mean ± S.E. One- and two-way ANOVAs and an appropriate post hoc test were used to analyze multiple groups. Differences between mean values were taken to be significant at the .05 level. CRunch Interactive Statistical Package (CRISP), SigmaStat, and InPlot4 computer software programs were used to analyze results.

**Results**

**Radioligand Binding Studies.** Figure 1 illustrates the comparative binding of 125I-Ang IV, 125I-divalinal Ang IV (both AT4 receptor ligands), and 125I-Sar1, Ile8 Ang II (AT1/AT2 receptor ligand) to MDBK cell membranes as a function of protein concentration. Both 125I-Ang IV and 125I-divalinal Ang IV displayed high specific binding to MDBK cell membranes, suggesting the possible presence of AT4 receptors, whereas 125I-Sar1, Ile8 Ang II bound negligibly and likely reflected the absence or low density of AT1/AT2 receptors in this renal epithelial cell line. Specific binding of 125I-Ang IV and 125I-divalinal Ang IV to MDBK membranes increased as a function of time, and apparent steady state was reached between 60 and 90 min for both 125I-peptides and remained stable until 120 to 150 min, after which time we generally found an upward drift in binding. Consequently, incubation times of >90 min were not performed. Under these radioreceptor binding conditions, the amount of nonspecific 125I-peptide bound to the tissue was ≤10% of total binding. Specific binding of 125I-peptide was reversible after the addition of 1 μM unlabeled peptide to steady-state conditions (Fig. 2).

Calculation of the $K_d$ value from association and dissociation rate constants indicated that 125I-Ang IV and 125I-divalinal Ang IV bound with high affinity to the membrane receptor with kinetic $K_d$ values of 52.6 and 25.7 pM, respectively. The results from saturation binding isotherms and Scatchard plots are shown in Fig. 3 and demonstrate that both radioligands bound to a single class of high-affinity binding sites (apparent $K_d$: Ang IV, 1.37 ± 0.37 nM; divalinal Ang IV, 1.01 ± 0.01 nM; $n = 6$ each) and to a similar maximum number of receptors ($B_{max}$: Ang IV, 1335 ± 148 fmoI/mg protein; divalinal Ang IV, 1407 ± 161 fmoI/mg protein; $n = 6$ each).

The similarity seen in kinetic experiments and saturation isotherms for 125I-Ang IV and 125I-divalinal Ang IV was mirrored by nearly identical rank order affinities and $K_i$.
values for several competing Ang-related peptides for $^{125}$I-Ang IV and $^{125}$I-divalinal Ang IV binding (Table 1). All competition curves were best fit with a single-site binding model. Ligand structure-binding studies revealed that amino-terminal extensions or deletions of Ang IV produced peptides with less affinity for the binding protein than native Ang IV. We also obtained a reduction in binding affinity for carboxyl-terminal-extended or -deleted analogs of Ang IV. In agreement with previous reports (Sardinia et al., 1993), Val-Tyr-terminal-extended or -deleted analogs of Ang IV and NleYI-6-hexamide (both putative AT$_4$ receptor antagonists) were insensitivity to the presence of the sulfhydryl-reducing agent dithiothreitol at concentrations that abolished $^{125}$I-Ang II binding to rat liver membranes (Fig. 4A). Only the $^{125}$I-Ang II binding to rat liver membranes could be inhibited further by exposing membranes to GTPyS for longer periods. Binding of $^{125}$I-Ang IV or $^{125}$I-divalinal Ang IV to MDBK cell membranes was also insensitive to the presence of the sulfhydryl-reducing agent dithiothreitol at concentrations that abolished $^{125}$I-Ang II binding to rat liver Ang AT$_1$ receptors (Fig. 4B). Similar responses were observed with a second sulfhydryl-reducing agent, mercaptoethanol (not shown, $n = 3$), except that its potency to inhibit $^{125}$I-Ang II binding to rat liver AT$_1$ receptors was one order of magnitude less than that of dithiothreitol (mercaptoethanol: IC$_{50} = 6.94$ mM; dithiothreitol: IC$_{50} = 0.88$ mM). These results suggested that the radioligand-bound receptor protein studied in MDBK cell membranes was distinct from the AT$_1$ receptor and that the binding site of the AT$_4$ receptor did not require G protein or sulfhydryl bonds for binding affinity.

We also examined whether Ang IV and divalinal Ang IV demonstrated high affinity for the Ang II type AT$_1$ or AT$_2$ receptors as defined, respectively, by $^{125}$I-Sar$_1$Ile$_8$Ang II or $^{125}$I-divalinal Ang IV binding to rat adrenal medulla membranes treated with 10 mM losartan. As shown in Fig. 5, A and B, AT$_1$ receptor ligands (Ang II and losartan)
and AT2 receptor ligands (Ang II, PD 123177, and PD 123319) displayed high affinity for their respective receptors, whereas Ang IV and divalinal Ang IV exhibited either no affinity or extremely low affinity for the AT1 and AT2 receptor.

**Intracellular Calcium Studies.** [Ca2+]i response experiments were performed at 37°C in MDBK cells that were briefly exposed (~20 s) to a concentration of Ang IV or divalinal Ang IV that varied from 0.1 nM to 1 mM. Temperature appeared to be one critical factor in these studies 1) because Ca2+ responses to Ang IV were observed in 38% of experiments performed at 22°C and increased to 75% of experiments when performed at 37°C and 2) because in Ang IV-responsive cells, where temperature of the bath solution was randomly ramped-up or ramped-down between 22°C and 37°C, we found that the [Ca2+]i response to Ang IV at 22°C was ~60% less than the corresponding responses recorded at 37°C. As shown in Figs. 6 and 7, both Ang IV and divalinal Ang IV were capable of producing a rise in [Ca2+]i. Although the concentration-response curves (0.1–10 nM) for Ang IV and divalinal Ang IV were not significantly different from each other (as shown in Fig. 7), they diverged at the highest concentration tested, with 1 μM Ang IV eliciting a dramatic diminished [Ca2+]i response compared with an enhanced [Ca2+]i response with 1 μM divalinal Ang IV (P < .01). The small but detectable [Ca2+]i response to 1 μM Ang IV was not due to receptor desensitization because it occurred even if the highest concentration of Ang IV was administered first, followed immediately by lower concentrations that produced greater [Ca2+]i responses (not shown), or interspersed between repeated 10 nM concentrations that produced maximal increases in [Ca2+]i (as shown in Fig. 6A). Although elevated concentrations of Ang IV metabolites could poten-
tially compete with the parent peptide for the membrane AT₄ receptor and result in a diminished [Ca²⁺]ᵢ response, the local concentration of Ang IV metabolites around the cell would most likely be low because MDBK cells were continuously perfused with Ang IV. Also, smaller peptide fragments of Ang IV had less affinity than Ang IV for the MDBK AT₄ receptor and, therefore, were unlikely to effectively compete for membrane binding sites (Table 1). Exposure of cells to Ang IV for longer periods (~3 min) resulted in an initial transient increase in [Ca²⁺]ᵢ, that fell to a plateau level and remained constant until the exposure to Ang IV was terminated (far right response shown in Fig. 6A). Incubation of cells with a Ca²⁺−free buffer for 5 min resulted in a greatly diminished (~90%) transient and plateau response to Ang IV that could be fully restored by incubating cells with the Ca²⁺−containing bath solution (not shown). Short- or long-term exposure of cells to divalinal Ang IV, in the presence or absence of extracellular Ca²⁺, produced a similar [Ca²⁺]ᵢ response pattern (except at micromolar concentrations) as that described for Ang IV (Figs. 6B and 7).

Because the photomultiplier tube averaged the calcium response from the group of cells under investigation, it was unclear whether all cells or subgroups of cells responded to the Ang peptides. Consequently, we used digital imaging fluorescence microscopy, which allowed simultaneous measurements of [Ca²⁺]ᵢ from individual cells within groups of cells. These experiments could only be performed with MDBK cells and buffers that were at room temperature and revealed that all cells within a group responded to Ang IV, with the magnitude of the response being highly variable from cell to cell (Fig. 6C).

Discussion

In the present study, we identified the presence of a single, high-affinity AT₄ receptor in a bovine renal epithelial cell line (MDBK), a line that expresses distal tubule cell characteristics (Ishizuka et al., 1978; Ganong et al., 1994). MDBK cells appear to exclusively express the AT₄ receptor subtype (Ishizuka et al., 1978; Ganong et al., 1994). The MDBK AT₄ receptor has similar pharmacological and coupling characteristics as the AT₄ receptor described in immortalized human collecting duct cells and in nonrenal tissues (e.g., receptor was unlikely to be coupled to a "classic" G protein, did not require sulphydryl bonds for binding affinity, and had similar structural requirements for the Ang IV molecule to bind to the AT₄ receptor; Bernier et al., 1995; Wright et al., 1995; Czekalski et al., 1996). However, ¹²⁵I-Ang IV binding to the opossum proximal tubule (OK7A cells) AT₄ receptor was inhibited by GTPgS and dithiothreitol (Dulin et al., 1995), suggesting the possible existence of an AT₄ receptor subtype that was clearly distinct from the AT₄ receptor described in more distal epithelial segments of the nephron (Czekalski et al., 1996; present study). Several investigators have estimated that the molecular mass of the major AT₄ receptor expressed in several bovine tissues (hippocampus, thymus, adrenals, kidney, aorta, cultured aortic endothelial cells) under dithiothreitol-reducing conditions is 165 to 186 kDa with ~20 to 30% of the receptor protein N-glycosylated (Bernier et al., 1998; Zhang et al., 1998, 1999). The AT₄ receptor appears to have a long extracellular domain (Bernier et al., 1998) and is associated with additional protein subunits in its nonreduced or native form (Bernier et al., 1998; Zhang et al., 1998). There also is the possibility that multiple AT₄ receptor isoforms may exist (Zhang et al., 1999). Analysis of Scatchard plots, as well as Ang-related compounds competing for ¹²⁵I-Ang IV or ¹²⁵I-divalinal Ang IV binding sites on MDBK cell membranes, indicated that the data best fit a one-binding-site model. These results would be consistent with the presence of a single high-affinity AT₄ receptor subtype on MDBK cells and is supported by the observation that SDS-polyacrylamide gel electrophoresis analysis (under reducing conditions) of ¹²⁵I-benzoylphenylalanine-Ang IV cross-linked to the solubilized AT₄ receptor from bovine kidney tissue (Zhang et al., 1998, 1999) or MDBK cell membranes (R.K.H., unpublished results) resulted in the labeling of a single protein band.

The intracellular signaling mechanisms associated with AT₄ receptor activation are unknown. Our results suggest that a rise in [Ca²⁺]ᵢ, is at least one mechanism by which ligand binding to the membrane AT₄ receptor is transduced into an intracellular signal. Furthermore, extracellular Ca²⁺ appears to be a major source contributing to the observed transient and sustained elevation in [Ca²⁺]ᵢ, in response to Ang IV and divalinal Ang IV. The pattern and sources of the [Ca²⁺]ᵢ response to Ang IV may be cell specific (e.g., Ang IV produced only a sustained elevation in [Ca²⁺]ᵢ, in cultured rat vascular smooth muscle cells that was related to the influx of extracellular calcium and possibly the generation of intracellular inositol phosphates; Dostal et al., 1990), whereas only a rapid, transient [Ca²⁺]ᵢ response was observed in cultured opossum OK7A proximal tubule cells (Dulin et al., 1995) and cultured rat mesangial cells (Chansel et al., 1998), which was solely due to an influx of extracellular calcium. In contrast, there are reports that high concentrations of Ang IV (~0.1 µM) had no effect on [Ca²⁺]ᵢ, in neuroblastoma cells (Ranson et al., 1992), chick cardiac myocytes (Baker and Aceto, 1990), bovine aortic endothelial cells (Briand et al., 1998), or human collecting duct cells (Czekalski et al., 1996). Because in these latter studies cells were exposed to only a single, high con-
centration of Ang IV, it is unclear whether the absence of a change in [Ca\(^{2+}\)]\(_i\) was related to the fact that the AT\(_4\) receptor may not be linked to an intracellular Ca\(^{2+}\) signaling mechanism in these particular cell types or, alternatively, related to the high concentration of Ang IV used in their studies. Our results emphasize the importance of examining [Ca\(^{2+}\)]\(_i\) responses over a range of Ang IV concentrations and revealed major increases in [Ca\(^{2+}\)]\(_i\) at low nanomolar concentrations of Ang IV. The reasons accounting for a diminished [Ca\(^{2+}\)]\(_i\) response to high micromolar concentrations of Ang IV are unknown but do not appear to be due to receptor desensitization or the binding of Ang IV metabolites to the AT\(_4\) receptor. One possibility is that micromolar concentrations of Ang IV activate a non-AT\(_4\) receptor or perhaps a low-affinity AT\(_4\) receptor (either on the same receptor protein as the high-affinity AT\(_4\) receptor site or on a separate receptor protein) that interferes or opposes the rise in [Ca\(^{2+}\)]\(_i\), induced by high-affinity AT\(_4\) receptor stimulation. Such low-affinity Ang IV binding sites would not be readily detected by the filtration separation technique used in the present study. In contrast, micromolar concentrations of divalinal Ang IV did not exhibit a diminished [Ca\(^{2+}\)]\(_i\) response and may be related to the fact that divalinal Ang IV appears to have less affinity than Ang IV for low-affinity Ang IV binding sites as demonstrated by their respective affinity for AT\(_4\) type AT\(_1\) and AT\(_2\) receptors (shown in Fig. 5, A and B). The concentration-dependent biphasic action of Ang IV on [Ca\(^{2+}\)]\(_i\), is not restricted to MDBK cells in that it has also been observed in the human HK-2 proximal tubule cell line (R.K.H., unpublished results). The mechanisms accounting for the biphasic [Ca\(^{2+}\)]\(_i\) response pattern to increasing concentrations of Ang IV inrenal epithelial cells and its biological significance are unknown and require further investigation.

Several investigators have successfully used divalinal Ang IV as an antagonist of Ang IV-mediated actions in a variety of physiological systems; including fibrinolysis (Kerins et al., 1996), blood flow regulation (Krebts et al., 1996; Coleman et al., 1998; Patel et al., 1998), and solute transport (Handa et al., 1998). The use of divalinal Ang IV as an AT\(_4\) receptor antagonist is based on the premise that it is specific to the AT\(_4\) receptor, that it competes for the same group of receptors as the AT\(_4\) receptor agonist, and that it has no intrinsic AT\(_4\) receptor agonist activity. We found that the Ang II type AT\(_1\) or AT\(_2\) receptor had little or no affinity for Ang IV and divalinal Ang IV, whereas the MDBK AT\(_4\) receptor displayed comparable values for either ligand with respect to high-affinity binding, receptor density, and inhibition constants (K\(_i\)) for competing Ang-related peptides. These results are in agreement with an earlier report that compared 125I-Ang IV and 125I-divalinal Ang IV binding sites in bovine adrenal membranes (Krebts et al., 1996). We reported previously that relatively high concentrations of divalinal Ang IV can block the actions of Ang IV on cerebral and renal cortical blood flow or energy-dependent proximal tubule solute transport, without manifesting any intrinsic biological activity in these physiological systems (Krebts et al., 1996; Coleman et al., 1998; Handa et al., 1998). However, a comparison of functional properties of Ang IV and divalinal Ang IV in MDBK cells revealed that both peptides could significantly elevate [Ca\(^{2+}\)]\(_i\). Consequently, the apparent physiological activity (agonism or antagonism) of divalinal Ang IV may depend on the physiological process and/or cell type that is being examined.

In conclusion, a single high-affinity AT\(_4\) receptor subtype is expressed in the MDBK epithelial cell line and appears to be pharmacologically similar to the AT\(_4\) receptor described in human collecting duct cells and in nonrenal tissue. Although divalinal Ang IV has many of the attributes necessary for it to be an AT\(_4\) receptor antagonist, it possesses intrinsic AT\(_4\) receptor agonist activity with respect to intracellular calcium signaling. Consequently, care must be taken to evaluate the agonist/antagonist activity of divalinal Ang IV in the physiological system under investigation.

References


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