Characterization and localization of Ac-SDKP receptor binding sites using 125I-labeled Hpp-Aca-SDKP in rat cardiac fibroblasts

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Zhuo JL, Carretero OA, Peng H, Li XC, Regoli D, Neugebauer W, Rhaleb NE. Characterization and localization of Ac-SDKP receptor binding sites using 125I-labeled Hpp-Aca-SDKP in rat cardiac fibroblasts. Am J Physiol Heart Circ Physiol 292: H984–H993, 2007. First published October 6, 2006; doi:10.1152/ajpheart.00776.2006.—We have shown that the tetrapeptide N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) inhibited endothelin-1 (ET-1)–induced cell proliferation and collagen synthesis in cultured rat cardiac fibroblasts (CFs) and reduced left ventricle collagen deposition in rats with aldosterone (salt)- and ANG II-induced hypertension. However, it is not known whether these effects are mediated by receptor binding sites specific for Ac-SDKP. We hypothesized that Ac-SDKP exerts antifibrotic effects by binding to specific receptor sites in cultured rat CFs, which mediate the inhibitory effects of Ac-SDKP on ET-1-stimulated collagen synthesis. Ac-SDKP binding sites in rat CFs and hearts were characterized by a specific radioligand, 125I-labeled 3-(p-hydroxyphenyl)-propionic acid (or desaminotyrosine) (Hpp)-Aca-SDKP, a biologically active analog of Ac-SDKP. 125I-labeled Hpp-Aca-SDKP bound to rat CFs and fractionated membranes with similar affinities and specificity in a concentration- and time-dependent fashion. Scatchard plot analyses revealed a single class of high-affinity Hpp-Aca-SDKP binding sites (maximal binding: 1.704 ± 198 fmol/mg protein; dissociation constant: 3.3 ± 0.6 nM). 125I-labeled Hpp-Aca-SDKP binding in CFs was displaced by unlabeled native peptide Ac-SDKP (inhibition constant: 0.69 ± 0.15 nM) and the analog Hpp-Aca-SDKP (inhibition constant: 10.4 ± 0.2 nM) but not the unrelated peptide ANG II or ET-1 (10 μM). In vitro, both Ac-SDKP and Hpp-Aca-SDKP inhibited ET-1-stimulated collagen synthesis in CFs in a dose-dependent fashion, reaching a maximal effect at 1 nM (control: 7.5 ± 0.4; ET-1: 19.9 ± 1.2; ET-1 + Ac-SDKP: 7.7 ± 0.4; ET-1 + Hpp-Aca-SDKP: 9.7 ± 0.1 μg/mg protein; P < 0.0001). Ac-SDKP also significantly attenuated ET-1-induced increases in intracellular calcium and MAPK ERK1/2 phosphorylation in CFs. In the rat heart, in vitro autoradiography revealed specific 125I-labeled Hpp-Aca-SDKP binding throughout the myocardium, primarily interstitially. We believe that these results demonstrate for the first time that Hpp-Aca-SDKP is a functional ligand specific for Ac-SDKP receptor binding sites and that both Ac-SDKP and Hpp-Aca-SDKP exert antifibrotic effects by binding to Ac-SDKP receptors in rat CFs.

collagen synthesis; heart; autoradiography; receptor

N-ACETYL-SERYL-ASPARTYL-LYSYL-PROLINE (Ac-SDKP) is a naturally occurring inhibitor of pluripotent hematopoietic stem cell entry into the S phase of the cell cycle and hepatic protein proliferation (4, 15). Ac-SDKP is normally present in nanomolar concentrations in human plasma and circulating mononuclear cells (24). This tetrapeptide is released primarily from its precursor thymosin–β4 and hydrolyzed exclusively by angiotensin I-converting enzyme (ACE) (2). Because chronic ACE inhibition in humans is associated with a four- to fivefold increase in serum Ac-SDKP levels, it has been suggested that changes in serum Ac-SDKP levels could be used as a marker of effective ACE inhibition in humans (2, 3). However, we and others have recently shown that Ac-SDKP exerts antifibrotic effects in cultured rat cardiac fibroblasts (CFs) in vitro and in rat hearts in vivo, thus uncovering a new role for Ac-SDKP (22, 25–27). In vitro, Ac-SDKP inhibits DNA and collagen synthesis by rat CFs when stimulated with FBS or endothelin (26). In rats with renovascular or mineralocorticoid hypertension, Ac-SDKP inhibits collagen deposition and cell proliferation in the heart (fibroblasts) and kidney (22, 27). In addition, our group (25) showed that, after myocardial infarction, Ac-SDKP both prevented and regressed collagen synthesis and deposition in the interstitial and perivascular spaces of the rat heart and inhibited infiltration of monocytes and macrophages in the left ventricle. Together, these studies suggest that Ac-SDKP plays an important role in the regulation of cardiac remodeling and function beyond its involvement as a biological marker of ACE inhibition.

Although the antifibrotic effects of Ac-SDKP are well documented, the cellular mechanisms involved are not fully understood. Ac-SDKP does not alter systemic blood pressure (22, 25, 27) and has no effect on cardiomyocyte hypertrophy after myocardial infarction, nor does it significantly affect cardiac function (25, 30). However, its antifibrotic actions appear to involve inhibition of macrophage infiltration, transforming growth factor-β expression (14), and MAPK activity (26, 23), which are important mechanisms of cytokine-induced tissue fibrosis. However, at present, we do not know whether the effects of Ac-SDKP are mediated by specific receptor binding sites present in CFs, since no receptors (or binding sites) for Ac-SDKP have been identified or localized in any cardiovascular cells or tissues because of the lack of a specific radioligand. In the present study, we tested the hypothesis that Ac-SDKP-induced antiproliferation and anticollogen deposition in rat CFs are mediated by specific Ac-SDKP receptor binding sites. To characterize these binding sites, we developed a novel radioligand called 3-(p-hydroxyphenyl)-propionic acid (or desaminotyrosine) (Hpp)-Aca-SDKP, an 125I-labeled analog of Ac-SDKP, for radioreceptor assays in cultured rat CFs.

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and cardiac tissue. We believe our results demonstrate for the first time that Hpp-Aca-SDKP is a functional ligand specific for Ac-SDKP receptor binding sites and that both Ac-SDKP and Hpp-Aca-SDKP exert antifibrotic effects by binding to Ac-SDKP receptors in CFs.

MATERIALS AND METHODS

Primary culture of rat CFs. CFs from adult Sprague-Dawley rats were isolated according to our own protocols and those described by Eghbali et al. (10, 26). Briefly, two or three hearts from adult Sprague-Dawley rats weighing 200–250 g (8–12 wk old) were rapidly excised under pentobarbital sodium anesthesia (50 mg/kg ip), and atrial tissue was removed. All protocols or procedures involving use of rats were approved by the Institutional Animal Care and Use Committee of Henry Ford Health System. Ventricles were minced with scissors and placed in tubes containing 5 ml calcium bicarbonate-free Hanks’ solution with HEPES (CBFH) buffer with 0.067% collagenase B (Boehringer Mannheim) along with a small magnetic bar. Tubes were kept on Magnetic Stir 1 (setting 2) for 15 min; 5 ml of CBFH buffer were added to each tube, and minced tissue was pipetted up and down 30 times. Cell suspensions from three digestion periods were pooled together in a 50-ml tube containing 5 ml FCS on ice and pelleted at 1,000 rpm for 5 min. The pellet was washed twice with 10% FCS-DMEM. Suspensions from six sequential digestions were combined, centrifuged, and resuspended in 8 ml 10% FCS-DMEM and then passed through a 60 mesh screen (Sigma) into a 100-mm tissue culture dish. Cells were incubated for 45 min at 37°C in 5% CO2. Those that remained unattached were discarded, whereas attached cells were washed twice with 10% FCS-DMEM and allowed to grow to confluence before passage. Cell passage was performed with a trypsin-based solution in 1:3 dilutions. All cells used in these experiments were from passages 3–4 (10, 23, 26).

Synthesis of Hpp-Aca-Ser-Asp-Lys-Pro-OH. In preliminary binding experiments using [3H]Ac-SDKP as a radioligand, we found very high nonspecific binding (∼50%), which could not be easily displaced with unlabeled Ac-SDKP (10 μM) and made it difficult to construct saturation binding curves and perform Scatchard analyses. To develop a functional ligand for Ac-SDKP, we synthesized an Ac-SDKP analog by adding desaminotyrosine to the sequence of the tetrapeptide (Hpp-Aca-SDKP), which can be labeled with 125I (20, 21). Briefly, Hpp-Aca-Ser-Asp-Lys-Pro-OH was synthesized on solid phase support with a peptide synthesizer (Applied Biosystems 430A), using a Merrifield resin (substitution 0.885 meq/g grain size, 100–200 mesh, 1% divinylbenzene) with the first amino acid (t-proline) attached (Chem-Impex International). N-t-Boc-protected amino acid [Boc-Lys(2-CIZ)-OH, Boc-Asp(OBzl)-OH, and Boc-Ser(Bzl)-OH; Chem-Impex], used for coupling, were activated by DCC/HOBT in 1-methyl-2-pyrrolidinone as solvent. Couplings of Boc-Aca-OH (N-t-butyloxycarbonyl-6-amino caproic acid; Aldrich) and Hpp (Sigma) were achieved with TBTU/HOBt [O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (Chem-Impex) and 1-hydroxybenzotriazole (Peptides International)], using N-methylmorpholine as a base. The final peptide was cleaved from the resin with anhydrous hydrogen fluoride, using anisole as a scavenger (20, 21). The resulting crude peptide was purified by reverse-phase (C18) chromatography, and its identity was confirmed by mass spectrometry, which yielded a mass of 707 Da. Aca-OH, N-t-butyloxycarbonyl-6-amino caproic acid (Aldrich); Hpp, 3-(p-hydroxyphenyl)-propionic acid or desaminotyrosine; SDKP, N-acetyl-seryl-aspartyl-tyrosyl-proline. The added desaminotyrosine can be labeled with 125I for Ac-SDKP radioreceptor assays in cultured cells and receptor localization in tissues.

In the end of the experiment, the medium was examined for collagen synthesis by hydroxyproline assay (26). Cells were harvested, and protein content was determined. Collagen synthesis was expressed as micrograms collagen per milligram protein (26).

125I-labeled Hpp-Aca-SDKP receptor binding in cultured rat CFs. Fibroblasts were split and subcultured in six-well plates in DMEM + 10% FCS until 80% confluent (3–5 days culture; ∼5 × 105 cells) (23, 26). Subconfluent cells were washed twice with 2 ml PBS, pH 7.4, and then incubated with the radioligand for 60 min at 37°C in 2 ml of 10 mM sodium phosphate buffer, pH 7.4, containing 0.3 μCi/ml (∼1 nM)125I-labeled Hpp-Aca-SDKP (125I-Hpp-Aca-SDKP, specificity of 4,565 Ci/mmol; Bachem), 150 mM NaCl, 5 mM Na2EDTA, 0.2% NaN3, 0.2% BSA, 0.5 mg/ml bacitracin, 10 μM captopril, and 1 μM peptatin A. After incubation, cells were washed twice with 2 ml of ice-cold PBS and scraped with a cell lifter. Cell-bound and unbound radioligands were separated by rapid filtration with a 48-well filtration device (Brandel) followed by four washes with 1 ml of ice-cold buffer (31, 34, 36). Bound radioactivity on the filter disks was counted. To exclude the possibility that 125I-Hpp-Aca-SDKP binding in rat CFs may reflect receptor uptake or internalization, parallel binding assays were separately performed in membrane fractions prepared from fractionated rat CFs as described (31, 36). Total binding was determined in the presence of 125I-Hpp-Aca-SDKP (∼1 nM) without competition by unlabeled compounds. Nonspecific binding was determined in the presence of 10 μM unlabeled Hpp-Aca-SDKP. Specific binding was taken as the difference between total and nonspecific binding as described (31–33).

Saturation 125I-Hpp-Aca-SDKP receptor binding and Scatchard plot analyses. Saturation radioligand binding experiments measure specific radioligand binding at equilibrium at various concentrations,
allowing us to determine maximal binding sites (Bmax) and dissociation constant (Kd). To carry out these experiments, cultured rat CFs were subcultured and assayed with the binding buffer and conditions described above. Specific 125I-Hpp-Aca-SDKP binding was determined in a six-well plate containing increasing concentrations of 125I-Hpp-Aca-SDKP (0, 0.03, 0.1, 0.3, 1.0, 3.0, and 10 nM) in the incubation buffer for 60 min at 37°C. In separate experiments, a constant concentration (10 μM) of unlabeled Ac-SDKP was added into incubations to determine nonspecific binding. Cells were washed and scraped, and bound and unbound radioligands were separated by rapid filtration. Binding data were analyzed with GraphPad Prism 4 as described (31–33, 35, 36).

**Competition for 125I-Hpp-Aca-SDKP binding by unlabeled SDKP analogs and unrelated peptides.** To determine the specificity of 125I-Hpp-Aca-SDKP binding sites in cultured rat CFs, binding experiments were performed with increasing concentrations of unlabeled Hpp-Aca-SDKP and Ac-SDKP (10−10 to 10−4 M) or 10 μM each of Hpp-Aca-SDKP, Ac-SDKP, SDKP (dehydro), or triptide SDK at 37°C for 60 min. Additionally, we also tested the effects of unrelated peptides ANG II (10−8 M) or ET-1 (10−10 M) on 125I-Hpp-Aca-SDKP binding in CFs to determine whether these peptides compete for Ac-SDKP binding. After incubation, the cells were washed and scraped, and bound and free radioligands were separated by rapid filtration. Radioactivity of each sample was counted as described (31–33, 35, 36).

**Effects of Ac-SDKP on ET-1-induced intracellular calcium responses in rat CFs.** Our group (25–27) has previously shown that Ac-SDKP significantly attenuated ET-1-induced cell proliferation and collagen synthesis in rat CFs. However, we do not know the cellular mechanisms involved in these effects. ET-1 is a potent vasoconstrictor and growth factor, and increased intracellular calcium concentration ([Ca2+]i) induced by ET-1 is one of the major signaling pathways (1, 9, 18). To determine whether Ac-SDKP attenuates ET-1-stimulated collagen synthesis in part by inhibiting [Ca2+]i, responses to ET-1, we pretreated subconfluent CFs plated on glass coverslips with serum-free medium, Ac-SDKP (10 nM), or thapsigargin (1 μM), an inhibitor of sarco(endo)plasmic reticulum Ca2+/ATPase (SERCA), to deplete intracellular calcium stores and loaded these with the calcium indicator fura 2 (2 μM) at 37°C for 30 min as described previously (16, 17, 33). After two washes with PBS, coverslips were placed inside a perfusion chamber maintained at 37°C, mounted on a Nikon Eclipse TE 2000-U fluorescence microscope coupled with a Lambda DG4 illumination system (Sutter Instruments). Fura 2-loaded cells were alternately excited at 340 and 380 nM every 3 s. Basal ratiometric calcium imaging was first recorded for 5 min with serum-free medium alone. CFs were then stimulated with ET-1 (1 nM), and 340/380 ratiometric calcium images were continuously recorded at 3-s intervals for up to 10 min. At the end of experiment, CFs were tested on whether they responded to the calcium ionophore A-23187 (10 μM) or with thapsigargin (1 μM), a potent inhibitor of SERCA ATPase to deplete intracellular calcium stores (16, 17, 33). After treatment, the medium was removed and CFs were washed twice with ice-cold PBS and lysed with a modified RIPA buffer containing an inhibitor cocktail (Roche). Protein was measured with the use of a bicinchoninic acid kit (Pierce). Protein (10 μg) from each sample was separated on 8%–16% SDS-PAGE and transferred semi-dry onto an Immobilon-P membrane (Millipore). Total and phosphorylated ERK1/2 were detected by immunoblotting with a rabbit ERK1/2 monoclonal antibody (Cell Signaling Technology) or a mouse antiphosphorylated ERK1/2 monoclonal antibody against an amino acid sequence containing phosphorylated Tyr-204 of human ERK1/2 (Santa Cruz) (16, 17). Immunoblots were visualized by enhanced chemiluminescence using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz), scanned, and analyzed with a microcomputer imaging device (MCID, Imaging Research, Ontario, Canada) (16, 17).

**125I-Hpp-Aca-SDKP receptor binding in rat heart sections visualized by quantitative in vitro autoradiography.** Because we have demonstrated specific 125I-Hpp-Aca-SDKP binding sites in cultured rat CFs in vitro, we next wanted to determine whether specific 125I-Hpp-Aca-SDKP binding sites are present in the rat heart in vivo. Quantitative in vitro autoradiography was employed to visualize the anatomic distribution of Ac-SDKP binding sites, as described for localization of ACE and ANG II receptor subtypes in rat and human blood vessels and hearts (31, 32, 35, 36). Rats were decapitated, and the hearts were removed quickly. The cardiac chambers were filled with tissue medium and snap frozen in isopentane on ice. Heart sections (10 μm thick) were cut on a Cryostat and mounted on glass slides, which were dried overnight under reduced pressure. Tissue sections were first preincubated for 15 min with 10 nM sodium phosphate buffer to clear the endogenous ligand and then incubated with 125I-Hpp-Aca-SDKP (1 nM) for 60 min at 37°C in 10 ml of 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, 5 mM Na2EDTA, 0.2% NaN3, 0.2% BSA, 0.5 mg/ml bacitracin, 10 μM captopril, and 10 μM pepstatin A. After incubation, sections were washed four times with fresh buffer without BSA (1 min each) and air dried. Sections were then exposed to X-ray film in light-tight cassettes with a set of radioactivity standards for up to 30 days. To enable cellular localization, some sections were coated with LM-1 liquid emulsion (Amersham), dried, and exposed in light-tight cassettes for 60 days as described previously (31–33, 34, 36). After exposure, the films and emulsion-coated sections were developed, and autoradiograms were analyzed by a microcomputer imaging device (Image Research). To determine whether Ac-SDKP binding is localized to cardiomyocytes or the cardiac interstitium, adjacent sections were stained with fluorescein-labeled peanut lectin (Vector Laboratories) to identify the myocyte border as described previously (22, 25, 27, 30).

**Statistical analysis.** Results are expressed as means ± SE. Ac-SDKP receptor binding constant (Kd) and Bmax in cultured CFs were analyzed by GraphPad Prism 4. Total and nonspecific binding were compared by unpaired t-test.

**RESULTS**

**Effects of Hpp-Aca-SDKP on ET-1-stimulated collagen synthesis in cultured rat CFs.** To determine whether the synthesized analog Hpp-Aca-SDKP is a functional agonist of its native tetrapeptide Ac-SDKP, we compared their inhibitory effects on ET-1-stimulated collagen synthesis in cultured rat CFs. As shown in Fig. 2, Hpp-Aca-SDKP inhibited ET-1-induced collagen synthesis in a dose-dependent fashion, reaching maximum inhibition at a concentration of 1 nM. However, Hpp-Aca-SDKP lost its inhibitory effect on ET-1-stimulated collagen synthesis at 1 μM. As our group reported previously (26), Ac-SDKP (1 nmol/l) completely blocked ET-1-induced collagen synthesis (Fig. 2) but also lost its inhibitory activity at
Fig. 2. Effects of Ac-SDKP and its analog Hpp-Aca-SDKP on endothelin-1 (ET-1)-stimulated collagen synthesis in cultured rat cardiac fibroblasts (CFs). Note that ET-1 (10 nM) markedly increased CF collagen synthesis (solid bar) compared with control (open bar). Hpp-Aca-SDKP (hatched bars) had no effect on ET-1-induced collagen synthesis at 0.05 nM but inhibited the response at 0.1 and 1 nM in a concentration-dependent manner. However, Hpp-Aca-SDKP lost its inhibitory activity at 1 μM. Ac-SDKP (1 nM) also potently inhibited ET-1-induced collagen synthesis (cross-hatched bar), as described previously (26).

Fig. 3. Total and nonspecific binding in the presence of excess unlabeled Hpp-Aca-SDKP (10 μM) (A), saturation binding isotherms with increasing concentrations of 125I-labeled Hpp-Aca-SDKP (125I-Hpp-Aca-SDKP, 0–10 nM) (B), and Scatchard plot analysis (C) of 125I-Hpp-Aca-SDKP receptor binding sites in intact CFs (left) or fractionated cell membranes (right) (n = 6 from 3 experiments). Note that both intact CFs and fractionated CF membranes bound 125I-Hpp-Aca-SDKP with similar maximal binding site (B_max) and dissociation constant (K_d) results.
Hpp-Aca-SDKP (10 μM), which displaces 125I-Hpp-Aca-SDKP binding sites. Under equilibrium conditions, both intact CFs and isolated membranes displayed highly specific 125I-Hpp-Aca-SDKP binding (>85% of total binding), whereas nonspecific binding represented only ~15% of the total (Fig. 3A). Thus the level of nonspecific binding is consistent with those found in ACE and AT1-receptor binding using 125I-labeled radioligands (31–36).

Saturation binding experiments and Scatchard plot analyses of 125I-Hpp-Aca-SDKP binding in cultured rat CFs and fractionated membranes. Figure 3B shows one of three representative saturation binding isotherms with 125I-Hpp-Aca-SDKP in intact rat CFs or fractionated membranes. Specific Hpp-Aca-SDKP binding was increased in a concentration-dependent manner, reaching a plateau at 10 nM. Analysis of the binding data with nonlinear regression (curve fit) and one-site binding (hyperbola) showed a single class of binding sites with high affinity for 125I-Hpp-Aca-SDKP in both intact CFs and fractionated membranes. As shown in Fig. 3C, the level (Bmax) of 125I-Hpp-Aca-SDKP binding was slightly lower in CF membranes, but it was not significantly different from that of intact CFs. In either CFs or fractionated membranes, we were unable to curve fit the two-site binding data (hyperbola). Best-fit values for Bmax and Kd are shown in the inserted Scatchard plots (Fig. 3C).

Competition for 125I-Hpp-Aca-SDKP receptor binding by unlabeled Hpp-Aca-SDKP and other Ac-SDKP analogs and in cultured rat CFs. To confirm the specificity of 125I-Hpp-Aca-SDKP binding in CFs, we compared the inhibitory potencies of Hpp-Aca-SDKP, the native peptide Ac-SDKP, and other SDKP analogs (Fig. 4). Both Hpp-Aca-SDKP (Fig. 4A) and Ac-SDKP (Fig. 4B) displaced 125I-Hpp-Aca-SDKP binding in CFs in a concentration-dependent manner, but the native peptide [inhibition constant (Ki) = 0.69 ± 0.2 nM] was more potent than the synthesized analog (Ki = 10.4 ± 0.2 nM). Other analogs such as SDKP (dehydro) and the tripeptide SDK were less potent in displacing 125I-Hpp-Aca-SDKP binding than Ac-SDKP or Hpp-Aca-SDKP, whereas the nonrelated peptides ET-1 or ANG II had no effect on 125I-Hpp-Aca-SDKP binding even at 10 μM.

Effects of Ac-SDKP on ET-1-induced [Ca2+]i mobilization in rat CFs. ET-1 binds to its cell surface receptors to induce a variety of cellular responses, with increased [Ca2+]i, being one of the most important signaling pathways (1, 9, 18). We have shown that Ac-SDKP inhibited ET-1-stimulated collagen synthesis in rat CFs (26), but the cellular mechanisms by which Ac-SDKP exerts its effect are not known. In the present study, Ac-SDKP alone did not significantly alter basal [Ca2+]i (basal: 93 ± 26 nM vs. Ac-SDKP: 116 ± 33 nM; not significant) (Fig. 5A). However, ET-1 induced a rapid increase in [Ca2+]i, from basal levels (328 ± 52 nM; P < 0.01) (Fig. 5B), which was significantly attenuated by pretreatment of CFs with Ac-SDKP (10 nM) (156 ± 38 nM; P < 0.01 vs. ET-1) (Fig. 5C). Pretreatment of CFs with thapsigargin to deplete [Ca2+]i stores and Ac-SDKP also abolished [Ca2+]i responses to ET-1 (88 ± 22 nM; P < 0.01 vs. ET-1) (Fig. 5D).

Effects of Ac-SDKP on ET-1-induced activation of MAPK ERK1/2 in rat CFs. Activation or phosphorylation of MAPK ERK1/2 by growth-promoting and vasoactive factors is well recognized as an important intracellular signaling pathway in cell proliferation, protein synthesis, and transformation (5, 13, 16, 17, 19, 29). Figure 6 shows that ET-1 (1 nM) induced more than threefold increases in phosphorylation of ERK1/2 (control: 100% vs. ET-1: 320 ± 39%; P < 0.01), as expected. Pretreatment of CFs with Ac-SDKP (10 nM) for 30 min inhibited ET-1-induced ERK1/2 phosphorylation by almost 50% (205 ± 29%; P < 0.01 vs. ET-1). However, Ac-SDKP alone appeared to have a small effect on basal phosphorylated ERK1/2 (132 ± 16%; P < 0.05 vs. ET-1). Depletion of
intracellular calcium stores with thapsigargin attenuated ET-1-stimulated ERK1/2 phosphorylation (ET-1: 282 ± 13% vs ET-1 + thapsigargin: 158 ± 38%; P < 0.01), whereas inhibition of PLC with U-73122 had no effect on ERK1/2 response to ET-1 stimulation (252 ± 10% vs. ET-1; not significant) (Fig. 7).

Autoradiographic and cellular localization of 125I-Hpp-Aca-SDKP binding sites in the rat heart. Figure 8 shows anatomic localization of 125I-Hpp-Aca-SDKP binding in the rat heart. Specific binding occurred throughout the heart, including atria and ventricles, septum, and blood vessels, with relatively higher levels in the left ventricle and septum (Fig. 8A). Emulsion autoradiographs revealed that silver grains clearly outlined cardiomyocytes (Fig. 8C, arrows) and overlapped with fluorescein-labeled peanut lectin staining, primarily in the interstitium (Fig. 8E). Nonspecific binding was only 14% of total binding, suggesting that specific 125I-Hpp-Aca-SDKP binding could be displaced by an excess of unlabeled Hpp-Aca-SDKP (10 μM) (Fig. 8, B and D). Relative quantitative levels of 125I-Hpp-Aca-SDKP in the rat heart are shown in Table 1 (n = 6).

DISCUSSION

Our group has previously shown that Ac-SDKP potently inhibits DNA and collagen synthesis in cultured rat CFs in vitro (25, 26) and attenuates the fibrotic effects of aldosterone (salt)- and ANG II-induced hypertension in rats in vivo (22, 25, 27). This tetrapeptide also prevents and reverses cardiac fibrosis in rats with chronic heart failure after myocardial infarction (30). The antifibrotic effects of Ac-SDKP appear to involve multiple signaling pathways, including inhibition of transforming growth factor-β and connective tissue growth factor expression in CFs mediated via p42/p44 MAPK (14, 23, 26) and inhibition of plasminogen activator-1 expression in human mesangial cells (mediated via Smads) (14). However, we do not know whether these effects involve specific Ac-SDKP receptor binding sites. Using a new radioligand, 125I-Hpp-Aca-SDKP, we demonstrated for the first time that there is a single class of high-affinity receptor binding sites for Hpp-Aca-SDKP in cultured CFs. Because 125I-Hpp-Aca-SDKP receptor binding was potently displaced by unlabeled Hpp-Aca-SDKP and

Fig. 6. Effects of Ac-SDKP on ET-1-induced MAPK ERK1/2 phosphorylation in rat CFs. CFs were stimulated with ET-1 (1 nM) in the absence or presence of Ac-SDKP (10 nM) at 37°C for 5 min. Top: Western blots of phosphorylated and total ERK1/2. Bottom: semi-quantitative data as % of control from triplicate blots for each protocol. Note that ET-1 markedly increased ERK1/2 phosphorylation, which was attenuated by pretreatment of cells with Ac-SDKP (10 nM). Total ERK1/2 was not changed by any treatment and also served to confirm equal protein loading. Note that, in double bands, bottom represents ERK2 (p44) and top band represents ERK1 (p44). p, Phosphorylated ERK1/2; t, total ERK1/2. *P < 0.05 and **P < 0.01 vs. control; #P < 0.05 vs. ET-1.
Although we performed binding assays using different buffers (pH 7.4, 7.0, or 6.5) used to displace [3H]SDK*P binding. Similar high levels of nonspecific binding were also observed in rat cardiac sections as visualized by quantitative in vitro autoradiography. Because we added a high concentration of captopril (10 μM) to the binding buffer to inhibit ACE, it is unlikely that the persistent high levels of nonspecific binding were due to degradation of the radioligand by ACE, which almost exclusively breaks down Ac-SDKP both in vivo and in vitro (6, 8, 11, 12). These results suggest that [3H]SDK*P is not an ideal radioligand for Ac-SDKP receptor binding, probably due to the chemical properties of the [3H]-labeled compound.

To develop a functional ligand for pharmacological characterization of Ac-SDKP receptor binding sites, we synthesized an Ac-SDKP analog for the first time by adding desaminotyrosine to the sequence of the tetrapeptide, which can be labeled with 125I (20, 21). To exclude the possibility that the analog may lose its biological activity when desaminotyrosine is added, we first examined whether it would exert effects similar to its native tetrapeptide on ET-1-stimulated collagen synthesis in cultured rat CFs. At 1 nM, both Ac-SDKP and Hpp-Aca-SDKP potently attenuated ET-1-induced collagen synthesis (34). However, their inhibitory effects on ET-1-stimulated collagen synthesis were lost at micromolar concentrations (Fig. 2) (26). The cellular mechanisms or signaling pathways underlying these biphasic effects of Ac-SDKP and Hpp-Aca-SDKP are not fully understood at present, but it may relate to the physiological levels (nM) of endogenous Ac-SDKP in the circulation and tissues (6, 24, 25, 27). Furthermore, persistent exposure of receptor binding sites to these ligands may lead to full occupancy and subsequent desensitization of the receptors. Nevertheless, our data confirm that Hpp-Aca-SDKP is pharmacologically similar to Ac-SDKP and can be used as a functional ligand for Ac-SDKP receptor binding in cells and tissues. Indeed, in radioreceptor binding assays using 125I-labeled Hpp-Aca-SDKP, we were able to reduce nonspecific binding substantially, from ~50% with [3H]Ac-SDK-P to <15% with 125I-labeled Hpp-Aca-SDKP (Fig. 3A). Levels of specific 125I-labeled Hpp-Aca-SDKP receptor binding were comparable to those reported for other peptide receptors such as ANG II, ET-1, or bradykinin (31–33, 35, 36). In addition to increasing the level of specific binding, this radioligand made it possible to perform saturation studies to determine the binding characteristics of Ac-SDKP receptors, including Bmax and Kd (Fig. 3). Saturation binding and Scatchard plot analyses revealed only a single class of receptor binding sites, since the best curve fit was with the one-site rather than the two-site model. Specificity of 125I-Hpp-Aca-SDKP binding was examined further with different Ac-SDKP analogs and other unrelated peptides. Again, Ac-SDKP and Hpp-Aca-SDKP potently displaced 125I-Hpp-Aca-SDKP binding, with a Kd of ~0.69 and 10.4 nM, respectively (Fig. 4). The Kd values of the tetrapeptide and its analog Hpp-Aca-SDK are close to the concentrations at which they exert their maximal inhibitory effects on ET-1-induced collagen synthesis (23, 26, 34). Interestingly, the tripeptide analog SDK also significantly blocked 125I-Hpp-Aca-SDKP binding, probably due to its similar chemical structure to Ac-SDKP (Fig. 4). In fact, Thierry et al. (28) showed that SDK was even more potent than Ac-SDKP in biological activity. We also found that SDK exhibited inhibitory effects on ET-1-stimulated collagen synthesis in CFs (Peng H, Carrero OA, and Rhaleb NE, unpublished observations). By contrast, the nonrelated peptides ET-1 and ANG II had no effect on 125I-labeled Hpp-Aca-SDKP binding in CFs. Thus we believe that our results confirm for the first time the presence of a single class of high-affinity and high-specificity Ac-SDKP receptor binding sites in rat CFs.
Although the use of $^{125}$I-Hpp-Aca-SDKP enabled us to characterize Ac-SDKP receptor binding sites in cultured rat CFs, it is important to determine whether there are specific Ac-SDKP receptor binding sites in the heart and localize them at the cellular level. We have shown that Ac-SDKP prevents and reverses cardiac fibrosis in rats with aldosterone (salt-) and ANG II-induced hypertension, as well as rats with myocardial infarction (22, 25, 27, 30). These antifibrotic effects of Ac-SDKP in vivo may be mediated via specific Ac-SDKP receptor binding sites, but such sites have not been reported in any tissues to our knowledge. Localization of Ac-SDKP receptors in tissues is important to study the cellular mechanisms by which this tetrapeptide exerts beneficial effects on cardiac tissue fibrosis or target organ damage (25, 27, 30). To achieve this goal, we employed quantitative in vitro autoradiography to localize $^{125}$I-Hpp-Aca-SDKP receptor binding sites in the rat heart, which is a powerful tool to localize peptide receptors at the tissue and cellular levels. Indeed, we have used this technique to extensively study vasoactive peptide receptor distribution in the rat heart, blood vessels, and kidneys (31–36). As shown in Fig. 8, $^{125}$I-Hpp-Aca-SDKP receptor binding occurred at moderate levels throughout the rat heart, including the atria, septum, and ventricles, with maximal binding in the left ventricle and septum. The pattern of $^{125}$I-Hpp-Aca-SDKP binding in the rat heart indicates a homogeneous distribution, but this does not permit cellular localization. Further studies

Table 1. Cardiac distribution of specific $^{125}$I-Hpp-Aca-SDKP receptor binding in rats as visualized by quantitative in vitro autoradiography

<table>
<thead>
<tr>
<th>Structure</th>
<th>Specific binding, dpm/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left Atrium</td>
<td>25.8±3.5</td>
</tr>
<tr>
<td>Right Atrium</td>
<td>23.6±1.6</td>
</tr>
<tr>
<td>Left Ventricle</td>
<td>34.7±2.6</td>
</tr>
<tr>
<td>Right Ventricle</td>
<td>29.6±3.9</td>
</tr>
<tr>
<td>Septum</td>
<td>29.3±2.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. Specific binding was determined as the difference between total binding and binding in the presence of excess unlabeled Hpp-Aca-SDKP (10 μM). Hpp, 3-(p-hydroxyphenyl)-propionic acid; SDKP, N-acetyl-seryl-aspartyl-lysyl-proline. There were no significant differences in specific $^{125}$I-labeled Hpp-Aca-SDKP receptor binding between different cardiac structures (one-way ANOVA).
with emulsion light microscopic autoradiography revealed binding at the border of cardiomyocytes, illustrating a pattern consistent with the cardiac interstitium. The presence of specific 125I-Hpp-Aca-SDKP binding in the rat cardiac interstitium is therefore consistent with our findings with cultured rat CFs, further supporting our hypothesis that there are high-affinity and high-specificity binding sites for Ac-SDKP in CFs.

The significance of the finding that specific Ac-SDKP receptor binding sites are present in rat CFs remains to be determined. At present, we still do not know the precise size or structure of the receptor protein that binds Ac-SDKP, nor do we know which class of peptide receptor it belongs to. However, we did find that Ac-SDKP consistently inhibited ET-1-stimulated collagen synthesis and cell proliferation in rat CFs in vitro and prevented and reversed cardiac fibrosis induced by aldosterone (salt)- or ANG II-induced hypertension in vivo (22, 25–27, 30). There are two important mechanisms or signaling pathways that mediate the vasoconstrictor and growth effects of ET-1 (1, 9, 18). One is receptor-mediated mobilization of [Ca2+]i, and the other is activation of various MAPKs such as ERK1/2 or JNK (1, 13, 18, 29). Increased [Ca2+]i is also associated with MAPK activation in renal microvascular cells and mesangial cells (16, 17). In the present study, Ac-SDKP did not alter basal [Ca2+]i, but it significantly attenuated [Ca2+]i responses to ET-1, an effect comparable to thapsigargin, an inhibitor of SERCA ATPase (Fig. 5). This suggests that the effects of Ac-SDKP may involve modulation of Ca2+ release from intracellular calcium stores, although the precise mechanisms are not known. Indeed, we further found that ET-1-induced ERK1/2 phosphorylation, as expected (13, 29), and this effect of ET-1 was significantly attenuated by Ac-SDKP (Fig. 6). Thapsigargin, a potent inhibitor of SERCA ATPase, attenuated ET-1-induced ERK1/2 phosphorylation, whereas U-73122, an inhibitor of PLC, did not (Fig. 7). One plausible explanation for this finding would be that the inhibitory effects of Ac-SDKP on ET-1 increased [Ca2+]i, and activation of MAPK ERK1/2 were probably mediated in part by intracellular mechanisms other than PLC-activated signaling. However, more work is required to study how Ac-SDKP binds and activates its receptor binding sites to inhibit cardiac fibroblast proliferation and collagen synthesis. Only when the receptor is cloned or specific receptor antagonists are developed will we be able to fully elucidate the cellular mechanisms (including signaling pathways) involved in the antifibrotic effects of Ac-SDKP in CFs in vitro, as well as the role of Ac-SDKP in cardiac remodeling during target organ damage due to hypertension and myocardial infarction. Nevertheless, our work on specific Ac-SDKP receptor binding sites in rat CFs will likely open up a new field in studying interactions between this novel peptide, ACE inhibition, and cardiac remodeling during hypertension and chronic heart failure.

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