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ORIGINAL ARTICLE

# Evidence for an angiotensin-(1-7) neuropeptidase expressed in the brain medulla and CSF of sheep

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**Abstract**

Angiotensin-(1-7) [Ang-(1-7)] is an alternative product of the brain renin-angiotensin system that exhibits central actions to lower blood pressure and improve baroreflex sensitivity. We previously identified a peptidase that metabolizes Ang-(1-7) to the inactive metabolite product Ang-(1-4) in CSF of adult sheep. This study purified the peptidase 1445-fold from sheep brain medulla and characterized this activity. The peptidase was sensitive to the chelating agents o-phenanthroline and EDTA, as well as the mercury compound p-chloromercuribenzoic acid (PCMB). Selective inhibitors to angiotensin-converting enzyme, neprilysin, neurolysin, and thimet oligopeptidase did not attenuate activity; however, the metallopeptidase agent JMV-390 was a potent inhibitor of Ang-(1-7) hydrolysis (Ki = 0.8 nM). Kinetic studies using $^{125}$I-labeled Ang-(1-7), Ang II, and Ang I revealed comparable apparent $K_m$ values (2.6, 2.8, and 4.3 μM, respectively), but a higher apparent $V_{max}$ for Ang-(1-7) (72 vs. 30 and 6 nmol/min/mg, respectively; $p < 0.01$). HPLC analysis of the activity confirmed the processing of unlabeled Ang-(1-7) to Ang-(1-4) by the peptidase, but revealed < 5% hydrolysis of Ang II or Ang I, and no hydrolysis of neurotensin, bradykinin or apelin-13. The unique characteristics of the purified neuropeptidase may portend a novel pathway to influence actions of Ang-(1-7) within the brain.

**Keywords:** Ang-(1-7), metabolism, neuropeptidase, Renin-angiotensin system.

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Angiotensin-(1-7) [Ang-(1-7)] is a bioactive hormone of the renin-angiotensin system (RAS) that we originally identified in the brain and circulation of the rat 25 years ago (Chappell *et al.* 1989). Since that time, accumulating scientific evidence suggests that Ang-(1-7) opposes the actions of Ang II and may constitute an endogenous buffering system to the angiotensin-converting enzyme (ACE)-Ang II-Ang II type 1 ($AT_1$) receptor axis in the regulation of autonomic function, blood pressure, and blood pressure-independent metabolic pathologies (Chappell 2007; Xu *et al.* 2011; Chappell *et al.* 2014). Indeed, administration of the Ang-(1-7) antagonist D-Ala$^7$-Ang-(1-7) (A779) decreases baroreflex sensitivity (Arnold *et al.* 2011; Kar *et al.* 2011) and increases inflammation (Capettini *et al.* 2012), implying a significant degree of endogenous Ang-(1-7) tone in the brain. Moreover, the Ang-(1-7) receptor antagonist reduces the actions of either angiotensin-converting enzyme (ACE, EC 3.4.15.1) or $AT_1$ receptor inhibition, further suggesting a key role of the peptide in the therapeutic benefits of RAS blockade (Chappell 2007).

Ang II is primarily formed through the direct processing of Ang I by the dipeptidyl carboxypeptidase ACE, although other peptidases including chymase (EC 3.4.21.39) and cathepsin G (EC 3.4.21.20) may contribute under pathological conditions (Sadjadi *et al.* 2005; Rykl *et al.* 2006). In contrast, Ang-(1-7) can be processed directly from Ang I by

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*Abbreviations used*: ACE, angiotensin-converting enzyme; ang, Angiotensin; DEAE, diethylaminoethyl; DTT, dithiothreitol; JMV-390, *N*-[3-[(hydroxyamino) carbonyl]-1-oxo-2(*R*)-benzylpropyl]-L-leucine; PCMB, p-chloromercuribenzoic acetate; TOP, thimet oligopeptidase.

 

several endopeptidase including neprilysin (EC 3.4.24.11), thimet oligopeptidase (TOP, EC 3.4.24.15), and prolyl endopeptidase (PE, EC 3.4.21.26) (Welches *et al.* 1991; Campbell *et al.* 1998; Allred *et al.* 2000; Alzayadneh and Chappell, in press). In addition, Ang-(1-7) is efficiency generated directly from Ang II by the mono-carboxypeptidases ACE2 (EC 3.4.15.1) and prolyl carboxypeptidase (EC 3.4.16.2) (Grobe *et al.* 2013; Pereira *et al.* 2013). Although ACE constitutes the major Ang II-forming pathway, the peptidase also degrades a number of biological active peptides including Ang-(1-7) (Chappell *et al.* 1998). The impact of ACE inhibitors to increase circulating levels of Ang-(1-7) reflects the marked reduction in Ang-(1-7) metabolism to Ang-(1-5), as well as the alternative processing of Ang I by the metallopeptidase neprilysin (Campbell *et al.* 1998; Chappell *et al.* 1998; Yamada *et al.* 1998). We recently observed that ACE activity was higher in CSF from adult offspring of betamethasone-exposed pregnant ewes, a model of glucocorticoid-induced fetal programming characterized by higher blood pressure and reduced baroreflex function (Marshall *et al.* 2013b). Although the hydrolysis of Ang-(1-7) in sheep CSF *ex vivo* was attenuated by ACE inhibition, the majority of the peptide-degrading activity in the CSF was due to a thiol-sensitive endopeptidase that cleaved Ang-(1-7) to Ang-(1-4) (Marshall *et al.* 2013b). The Ang-(1-7) peptidase activity was significantly higher in the CSF of the betamethasone-exposed group and the CSF content of Ang-(1-7) was inversely correlated with peptidase activity (Marshall *et al.* 2013b). Selective inhibitors against the endopeptidases neprilysin, TOP, and neurolysin (EC 3.4.24.16) did not attenuate the hydrolysis of Ang-(1-7) to Ang-(1-4) in the CSF possibly suggesting a unique Ang-(1-7)-degrading activity in brain. To address this possibility, this study sought to obtain a sufficient amount of the purified activity from brain medullary tissue to achieve a more complete characterization of the peptidase. A 1445-fold enrichment of the peptidase was achieved from the brain medulla of sheep and the purified activity evaluated against angiotensins and other neuropeptide substrates. We report that the medullary peptidase appears similar to the activity in the CSF to metabolize Ang-(1-7) to Ang-(1-4), exhibits marked sensitivity to mercury-based inhibitors, chelating agents, and the metalloendopeptidase agent JMV-390, and hydrolyzes Ang-(1-7) to a greater extent than Ang II and Ang I, while other bioactive peptides including bradykinin, neurotensin, and apelin-13 were not cleaved.

## Methods

### Animals

Mixed breed sheep (obtained from a private local vendor) were delivered at term, farm raised, and weaned at 3 months of age. At 10–12 months of age, male offspring were brought to our Association for Assessment and Accreditation of Laboratory Animals Care approved facility, where they were maintained on a normal diet with free access to tap water and a 12-h light/dark cycle (lights on 7 AM–7 PM). Sheep were anesthetized with ketamine and isoflurane and killed by exsanguination. Brain medullae were removed and immediately covered with optimal cryosection media and stored at −80°C. CSF (~3 mL per animal) was extracted, taking care to avoid contamination with blood, and tubes were stored at −80°C. All procedures were approved by the Wake Forest University School of Medicine ACUC for animal care.

### Homogenization of sheep brain

Brain medullae were cut 4 mm rostral and 2 mm caudal to the obex and divided in half along the dorsoventral axis to isolate the dorsal medulla including the solitary tract nucleus. The dorsal medullae from two animals were pooled (2.5 g) for each purification and homogenized in HEPES buffer (25 mM Na+ free HEPES, 10 μM ZnCl2, 0.05% Triton, pH 7.0) using a Power Gen 1000 tissue grinder (Fisher Scientific, Pittsburgh, PA, USA) on setting 5 for 60 s and centrifuged at 25 000 *g* for 30 min at 4°C. Supernatant was retained for the subsequent purification steps.

### Cerebrospinal fluid concentration

CSF (5 mL) was pooled and concentrated from five animals 1 : 5 using molecular weight filtration tubes to remove small proteins and endogenous angiotensin peptides (30 kDa, Millipore, Bedford, MA, USA). Concentrated CSF was resuspended in a final volume of 5 mL HEPES buffer (25 mM HEPES, 125 mM NaCl, 10 μM $ZnCl_2$, pH = 7.4) and protein concentration was measured using a Bradford protein assay.

### DEAE-cellulose chromatography

Diethylaminoethyl Sepharose (DEAE, Sigma-Aldrich, St Louis, MO, USA) was equilibrated with HEPES buffer (25 mM Na+ free HEPES, 10 μM $ZnCl_2$, 0.05% Triton, pH 7.0) and incubated with the medullary supernatant for 60 min at 21°C. The DEAE gel was pelleted at 1000 g for 60 s, and supernatant was removed. The gel was subsequently washed in HEPES buffer with an increasing step gradient of NaCl (100, 250, 500 mM, 1M for 30 min) and the gel pelleted at 1000 *g*. The eluted material from the gradient was assayed for protein content and enzyme activity.

### Cibacron blue chromatography

Fractions containing the highest activity from the DEAE gel were combined (250 and 500 mM NaCl), concentrated on 50 kDa molecular weight concentrations tubes (Millipore), washed with Na+ free HEPES buffer, and incubated with equilibrated Cibacron Blue Sepharose Fast Flow (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) for 60 min. The flow-through fractions from the Cibacron gel contained the majority of activity and were applied directly to the Q-Sepharose column.

### Q-sepharose chromatography

Q-Sepharose Fast Flow (Sigma-Aldrich) was equilibrated with Na-free HEPES buffer in a 2.5 × 10 cm column. The unbound Cibracron fraction was applied directly to the Q-Sepharose column and washed with a step gradient of increasing NaCl (0, 100, 200, 400, 600 mM, and 1 M NaCl). Fractions were collected and assayed for protein content and enzyme activity.

### Gel electrophoresis

Purified protein eluted from the Q-Sepharose column was diluted 1 : 1 in Laemmli buffer containing β-mercaptoethanol and boiled for 5 min to induce denaturing conditions. Proteins were separated on 10% Mini-PROTEAN TGX gels for 80 min at 120 V in Tris-glycine buffer. Gels were stained with Imperial™ Blue Stain (Thermo Fisher Scientific, Rockford, IL, USA) or silver stain (PlusOne™ Silver Stain Kit, GE Healthcare Bio-Sciences) according to manufacturer's instructions.

### Inhibitor and pH profile

Using aliquots of partially purified peptidase, various inhibitors were tested for their ability to inhibit $^{125}$I-Ang-(1-7) metabolism including p-chloromercuribenzoic acid (PCMB), E-64, EDTA, o-phenanthroline, N-[N-[1-(S)-carboxyl-3-phenylpropyl]-(S)-phenyl-alanyl]-(S)-isoserine e (SCH), N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-p-aminobenzoate (CFP), Z-prolyl prolinal (ZPP), dithiothreitol (DTT), the dipeptide Pro-Ile, phenylmethylsulfonyl fluoride (PMSF), aprotinin, soybean trypsin inhibitor (SBTI), and the metallopeptidase inhibitor *N*-[3-[(hydroxyamino)carbonyl]-1-oxo-2(*R*)-benzylpropyl]-L-leucine (JMV-390). Each reaction was conducted in the presence of 0.5 nM $^{125}$I-Ang-(1-7), 100 nM Ang-(1-7), 92 ng of the Q-Sepharose fraction or 25 μL of CSF, and a cocktail of inhibitors (metabolism cocktail) containing the amino-peptidase inhibitors amastatin (2 μM) and bestatin (10 μM), the chymase inhibitor chymostatin (10 μM), the carboxypeptidase A inhibitor benzylsuccinate (10 μM), and the ACE inhibitor lisinopril (10 μM). All inhibitors were obtained from Sigma (St.Louis, MO, USA) except SCH 39370 (gift from Schering Plow), CFP (Bachem, King of Prussia, PA, USA), and JMV-390 (Tocris bioscience, Bristol, UK). A pH profile was conducted as described previously (Marshall *et al.* 2013c). Purified peptidase (10 μL) was added to buffers ranging from pH 3.5 to 5.5 using 25 mM 2-ethanesulfonic acid, 125 mM NaCl, and pH 7.5–9.5 using 25 mM HEPES, 125 mM NaCl. Iodinated products generated by the incubation of peptidase and $^{125}$I-Ang-(1-7) were separated using HPLC.

### Peptidase kinetics

Kinetic or competition assays were performed with $^{125}$I-Ang-(1-7), -Ang II or -Ang I as substrates, and increasing concentrations of corresponding unlabeled peptides as described previously (Shaltout *et al.* 2009; Marshall *et al.* 2013b). Reaction velocities for generation of $^{125}$I-Ang-(1-4) from the radiolabeled peptides were expressed as pmol/min/mg or nmol/min/mg protein. The kinetic assays were performed with the metabolism cocktail to prevent the contribution of other peptidases and preserve the Ang-(1-4) product. Apparent kinetic constants ($K_m'$, $V_{max}'$) were determined using Michaelis–Menten kinetics in the GraphPad Prism 5 (San Diego, CA, USA) statistical program. Peptides were obtained from Bachem (Torrance, CA, USA) or custom synthesized (Genscript, Piscataway, NJ, USA).

### HPLC separation

Metabolism reactions were conducted at 37°C in reaction buffer using 1445-fold purified peptidase (92 ng in a final volume of 250 μL) or 25 μL CSF. Each reaction included 0.5 nM $^{125}$I-Ang-(1-7) and 0.1 μM non-iodinated Ang-(1-7) (Shaltout *et al.* 2009). The reactions were stopped after 60 min by addition of ice-cold 1.0% phosphoric acid and centrifuged at 16 000 *g*. The supernatant was immediately filtered for separation by reverse-phase HPLC on a Shimadzu equipped with an Aeris Peptide XB-C18 3.6 μm (2.1 × 100 mm, Phenomenex, Torrance, CA, USA). The $^{125}$I-products were monitored by a Bioscan flow-through γ detector as described (Marshall *et al.* 2013b). Products were identified by comparison of their retention times to $^{125}$I-standard peptides and sensitivity to peptidase inhibitors. Peptides were iodinated by the chloramine T method and purified by HPLC to a specific activity > 2 000 Ci/mmol (Chappell *et al.* 1989).

### HPLC separation

Metabolism reactions were conducted at 37°C in reaction buffer using 1445-fold enriched peptidase (185 ng) and 50 nmol of unlabeled Ang I, Ang II, Ang-(1-7), Ang-(1-6) or Ang-(1-5), neurotensin, bradykinin, bradykinin-(1-7), bradykinin-(1-5), or apelin-13 in a final volume of 125 μL (final concentration of 0.4 mM). The reactions were stopped after 24 h by addition of 1.0% phosphoric acid and separated by HPLC using a 0.1% phosphoric acid solvent system performed under a gradient of 0–15–25% phase B (80% acetonitrile) at a flow rate of 0.35 mL/min at ambient temperature. Peptides were monitored at 220 nm and the products identified by the retention time of standard peptides.

### Statistics

Data are expressed as mean ± SEM. Unpaired *t*-tests and one-way repeated measures ANOVA with Bonferroni post-tests were used for the statistical analysis of the data with GraphPad Prism. The criterion for statistical significance was set at $*p < 0.05$.

## Results

### Comparison of CSF and medullary metabolism of Ang-(1-7)

We initially compared the metabolism of $^{125}$I-Ang-(1-7) in the cytosolic fraction of the brain medulla to metabolism in the CSF. As shown in Fig. 1, $^{125}$I-Ang-(1-7) was metabolized primarily to $^{125}$I-Ang-(1-4) with a minor peak corresponding to $^{125}$I-Ang-(3-4) in the medullary supernatant fraction; the $^{125}$I-Ang-(1-4) peak was abolished by addition of the thiol inhibitor PCMB (10 μM). Comparison of the Ang-(1-7) saturation curves revealed similar apparent $K_m$ ($K_m'$) values for the CSF and brain medulla activities, but a 6-fold higher apparent $V_{max}$ ($V_{max}'$) for the medullary peptidase (Fig. 1c).

### Purification of Ang-(1-7) peptidase activity from sheep medulla

In lieu of the higher tissue activity, the soluble fraction of the brain medulla was used as the initial source for the purification of peptidase activity. Soluble activity from 2.5 g of dorsal brain medulla was subjected to consecutive steps on DEAE, Cibacron Blue, and Q-Sepharose chromatography (Table 1). Peptidase activity was monitored by the conversion of $^{125}$I-Ang-(1-7) to $^{125}$I-Ang-(1-4) and sensitivity to the metalloendopeptidase inhibitor JMV-390 (see inhibitor sensitivity, below). The peptidase activity eluted in

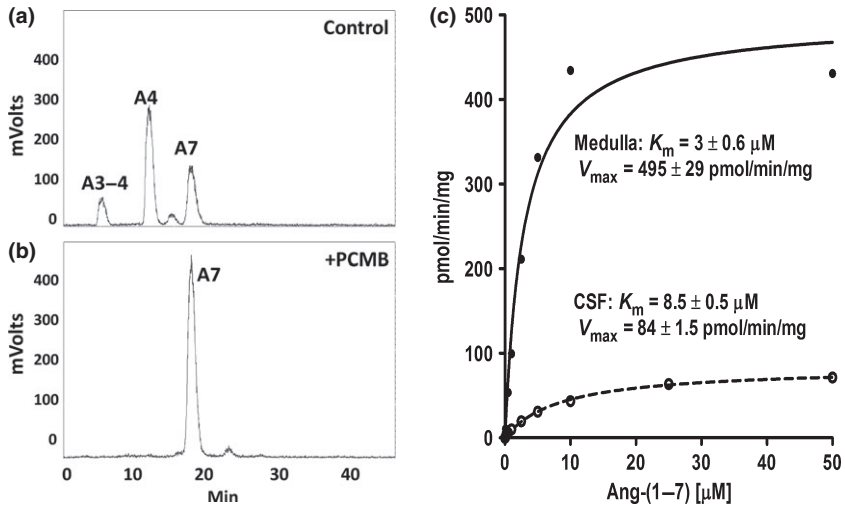


**Fig. 1** Ang-(1-7) metabolizing peptidase activity in the brain medulla and CSF. (a) $^{125}$I-Ang-(1-7) [A7] is metabolized to $^{125}$I-Ang-(1-4) [A4] and $^{125}$I-Ang-(3-4) [A3-4]. (b) A7 metabolism is abolished with the addition of p-chloromercuribenzoic acetate (PCMB) (10 μM). (c) Comparison of apparent kinetics of $^{125}$I-Ang-(1-7) metabolizing peptidase in brain medulla and CSF. Reactions were run in the presence of an inhibitor cocktail [amastatin (AM), bestatin (BS), chymostatin (CHYM), benzylsuccinate (BSC), lisinopril (LIS)] for 60 min at 37°C.

**Table 1** Four-step purification of brain peptidase

| Stage | Activity (pmol/mL) | Protein (mg/mL) | Specific activity (pmol/mg) | Yield (%) | Purification factor (fold) |
|---|---|---|---|---|---|
| Supernatant (25 000 *g*) | 142 | 244 | 0.58 | 100% | 1 |
| DEAE | 127 | 17 | 7.5 | 89% | 13 |
| Cibacron blue | 183 | 4 | 44.5 | 128% | 77 |
| Q-Sepharose | 40 | .047 | 831.9 | 25% | 1445 |

A combination of centrifugation, DEAE, Cibacron Blue, and Q-Sepharose chromatography was used to achieve 1445-fold purification over starting material.

250 mM NaCl on DEAE, was not retained on the Cibacron Blue gel, and eluted at 200 mM NaCl on Q-Sepharose following extensive washing with the 100 mM NaCl buffer. Fractions were collected in 15 mL increments from the 200 mM NaCl elution on the Q-Sepharose column; fraction 3 had the highest specific activity and was used to calculate purity and yield. We attained a 1445-fold purification with a 25% yield of the active peptidase using this protocol (Table 1). Peptidase activity apparently increased following the Cibacron Blue column (89–128%) which may reflect the separation of inhibitory substances that compete for the metabolism of Ang-(1-7). A second purification of the medullary soluble fraction obtained from the medullary tissue of different animals resulted in a similar degree of enrichment (1970-fold) and enzyme yield (31%) following the Q-Sepharose fractionation (preparation 2). Sodium dodecyl sulfate gels of the Q-Sepharose purified fraction stained with Imperial™ Protein Stain or the silver stain revealed approximately five protein bands in the 50–80 kDa range indicating that complete purification of the Ang-(1-7) peptidase was not achieved. Since the denatured gels abolished activity, it remains unclear what band corresponds to the peptidase.

## Inhibitor sensitivity

The sensitivity of the enriched peptidase from the Q-Sepharose column or concentrated CSF to various inhibitors was assessed by the conversion of $^{125}$I-Ang-(1-7) to $^{125}$I-Ang-(1-4). Similar to the CSF activity, the medullary peptidase was insensitive to specific inhibitors against neprilysin (10 μM, SCH 39370), TOP (10 μM, CFP), neurolysin (1 mM, Pro-Ile), the general cysteine peptidase inhibitor E-64 (10 μM), and the serine protease inhibitors PMSF (100 μM), aprotinin (80 μM), and SBTI (100 μM) (Fig. 2a, Table 2). PCMB (10 μM) and o-phenanthroline (1 mM) abolished peptidase activity while DTT (5 mM) and EDTA (5 mM) exhibited partial inhibition (Fig. 2b). The optimal pH for the peptidase purified from medullary tissue was pH 7.5 (Fig. 2c). As compared to control conditions (Fig. 3a), 1 nM JMV-390 reduced the Ang-(1-4) peak and 100 nM JMV-390 essentially abolished activity (Fig. 3b and c, respectively). A dose–response curve for JMV-390 inhibition revealed an $IC_{50}$ of 0.8 ± 0.2 nM with a $r$ value of 0.996 for a one-site competition curve (Fig. 3d). This was not significantly different from the $IC_{50}$ for JMV-390 of the CSF activity (1.1 ± 0.4 nM) (Table 2).

## Kinetic properties

Saturation studies on the purified peptidase activity were performed using $^{125}$I-labeled Ang-(1-7), Ang II and Ang I to compare the apparent kinetic constants among the three peptides. The kinetic analysis revealed apparent $K_m$ ($K_m'$) and $V_{max}'$ values of 2.6 ± 0.3 μM and 72 ± 2.2 nmol/min/mg for Ang-(1-7), 2.8 ± 0.7 μM and 30 ± 3 nmol/min/mg for Ang II, and 4.3 ± 0.7 μM and 6 ± 0.3 nmol/min/mg for Ang I (Fig. 4a–c, respectively). Although the $K_m'$ values were similar for all three peptides, Ang-(1-7) exhibited the

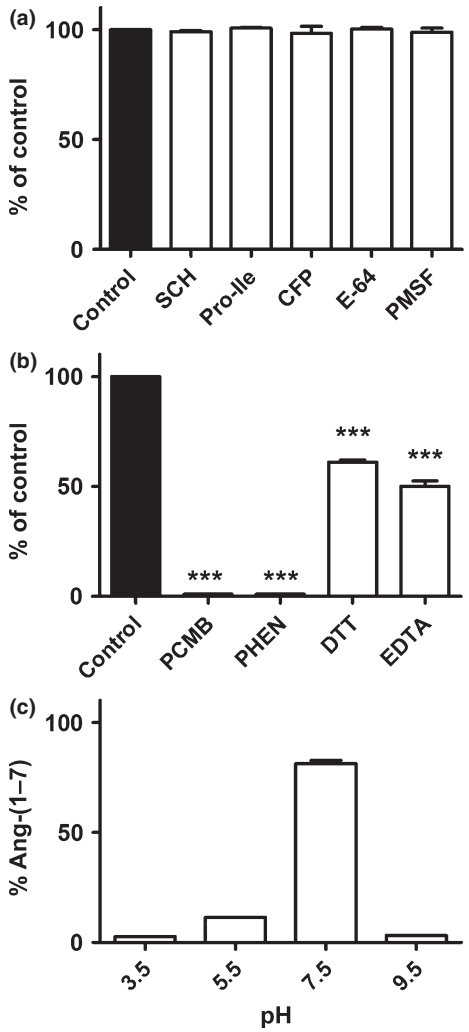


**Fig. 2** Inhibitor and pH profiles of the enriched Ang-(1-7) brain medullary peptidase. (a) Specific inhibitors SCH 39370 (10 μM, SCH), Pro-Ile (1 mM), CFP (10 μM), E-64 (10 μM), or phenylmethylsulfonyl fluoride (PMSF) (100 μM) did not inhibit activity. (b) Peptidase was sensitive to the chelating agents p-chloromercuribenzoic acetate (PCMB) (10 μM), o-phenanthroline (1 mM, PHEN), dithiothreitol (5 mM, DTT), and EDTA (5 mM). (c) pH profile reveals an optimal pH of 7.5 for peptidase activity. Reactions were carried out in the presence of an inhibitor cocktail [amastatin (AM), bestatin (BS), chymostatin (CHYM), benzylsuccinate (BSC), lisinopril (LIS)] for 60 min at 37°C. $N = 3$ separate determinations from the Q-Sepharose purified fraction of preparation 1, $^{***}p < 0.0001$ vs. control.

highest $V_{max}'$ as compared to Ang II or Ang I ($p < 0.01$ vs. Ang I or Ang II, $N = 3$). Apparent kinetic values for $^{125}$I-labeled Ang-(1-7), Ang II and Ang I were also derived from the second batch of purification. While the $K_m'$ values for Ang-(1-7) (2.0 μM), Ang II (7.4 μM) and Ang I (1.7 μM) were similar to the first purification, the $V_{max}'$ values were > 2-fold higher (174, 69, and 20 nmol/min/mg protein for Ang-(1-7), Ang II, and Ang I, respectively) and likely reflect the greater degree of purity in preparation 2 that yields a higher $V_{max}'$.

**Table 2** Comparison of CSF and the purified medullary peptidase activities for hydrolysis of $^{125}$I-Ang-(1-7)

| | CSF | Medullary tissue |
|---|---|---|
| Ang-(1-7) Affinity | | |
| *Km'* (μM) | 8.5 ± 0.5 | 3.0 ± 0.6 |
| *Vmax'* (pmol/min/mg) | 84 ± 1.5 | 495 ± 29 |
| Inhibitor sensitivity (% of control) | | |
| JMV-390 | 0 ($IC_{50}$ = 1.1 ± 0.3 nM) | 0 ($IC_{50}$ = 0.8 ± 0.2 nM) |
| PCMB (10 μM) | 0 | 0 |
| APMA (10 μM) | 0 | 0 |
| EDTA(5 mM) | 61 ± 2 | 79 ± 5 |
| DTT (5 mM) | 50 ± 4 | 73 ± 3 |
| o-phenanthroline (1 mM) | 1 ± 2 | 4 ± 0.3 |
| E-64 (10 μM) | 100 ± 1 | 100 |
| SCH (10 μM) | 100 | 99 ± 0.6 |
| CFP (10 μM) | 102 ± 3 | 98 ± 3 |
| Pro-Ile (1 mM) | 101 ± 2 | 101 ± 0.5 |
| Aprotinin (80 μM) | 100 | 103 ± 0.9 |
| SBTI (100 μM) | 101 ± 2 | 101 ± 0.3 |
| Lisinopril (10 μM) | 100 | 100 |
| Optimal pH | 7.5 | 7.5 |

Values for each inhibitor represent percent of control. $N = 3$ separate determinations from the Q-Sepharose purified fraction.

## Substrate specificity

We compared the metabolism of $^{125}$I-Ang-(1-7) in the purified medullary fraction to the initial tissue supernatant in the absence of any peptidase or protease inhibitors. Essentially complete metabolism of $^{125}$I-Ang-(1-7) to $^{125}$I-Ang-(3-4) was evident in the medullary supernatant fraction that likely reflects dipeptidyl aminopeptidase activity (Fig. 5a). In contrast, $^{125}$I-Ang-(1-4) was the primary product following a 60- or 120-min incubation with the purified peptidase (Fig. 5b and c, respectively). Addition of the inhibitor JMV-390 essentially abolished the $^{125}$I-Ang-(1-4) peak, and did not reveal additional metabolites suggesting the absence of other activities that metabolize Ang-(1-7) (Fig. 5d). Given the apparent lack of other peptidase activities in the purified preparation, we determined its specificity against various unlabeled angiotensins, as well as the bioactive peptides neurotensin, bradykinin, and apelin-13 (Figs 6 and 7). Ang-(1-7) was exclusively hydrolyzed to Ang-(1-4) and addition of JMV-390 abolished the peak of Ang-(1-4) (Fig. 6a and b, respectively). Ang II and Ang I were metabolized to Ang-(1-4); however, the extent of metabolism was markedly less than that of Ang-(1-7) (Fig. 6c and d). Ang-(1-6) and Ang-(1-5) were also metabolized to Ang-(1-4), but at lower rates than Ang-(1-7) (Table 3). Neurotensin, bradykinin, and the

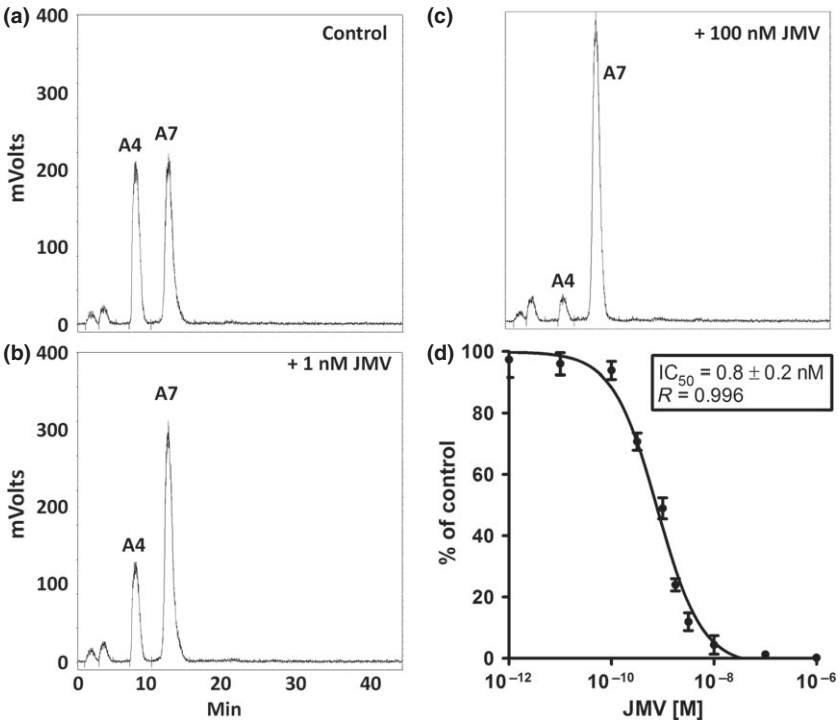


**Fig. 3** JMV-390 inhibits the Ang-(1-7) peptidase activity. (a–c) $^{125}$I-Ang-(1-7) was incubated with purified enzyme in the presence of 0, 1 nM, or 100 nM JMV-390. (d) A dose–response curve was conducted to determine the $IC_{50}$ for JMV-390. Reactions were carried out in the presence of an inhibitor cocktail [amastatin (AM), bestatin (BS), chymostatin (CHYM), benzylsuccinate (BSC), lisinopril (LIS)]. $N = 3$ separate determinations from the Q-Sepharose purified fraction of preparation 1.

bradykinin metabolites bradykinin-(1-7) and bradykinin-(1-5) were not hydrolyzed by the peptidase as reflected by the absence of intermediate peaks distinct from the parent peptides (Fig. 7 a–d). Based on the extent of hydrolysis of the unlabeled peptides, we calculated the rate of metabolism for each peptide substrate (Table 3). Ang-(1-7) was hydrolyzed to a greater extent than Ang II (11-fold) or Ang I (27-fold). The rate of Ang-(1-7) metabolism was 2-fold higher than Ang-(1-6) and 26-fold greater than the pentapeptide Ang-(1-5). There was no apparent metabolism of neurotensin, bradykinin or bradykinin metabolites, or apelin-13 (chromatograph not shown) following the 24-h incubation conditions.

## Discussion

In this study, we isolated and characterized an Ang-(1-7) metabolizing activity in the brain medulla of sheep that extends the original identification of the peptidase activity in the CSF (Marshall *et al.* 2013c). With respect to the similar kinetic parameters, substrate specificity, inhibitor sensitivity, and optimal pH, it is likely that the medullary peptidase and the CSF peptidase activities are identical (Table 2). Using a combination of DEAE Sepharose, Cibacron Blue, and Q-Sepharose chromatography, the peptidase was purified 1445- and 1950-fold from supernatant fraction of the brain medulla in two preparations using tissues pooled from multiple animals. The isolated activity is optimally active at pH 7.5 and sensitive to the mercury compounds PCMB and aminophenylmercuric acetate, and chelating agents o-phenanthroline, EDTA, and DTT; however, peptidase activity is not reduced by selective inhibitors against the metalloendopeptidases neprilysin, neurolysin, and TOP nor the serine protease inhibitors PMSF, aprotinin, or SBTI. The peptidase appears to preferentially cleave the Tyr$^4$-Ile$^5$ bond of Ang-(1-7) in comparison to other angiotensin peptides or the bioactive peptides neurotensin, bradykinin, and apelin-13. Kinetic analyses using [$^{125}$I]-labeled angiotensins revealed similar $K_m'$ values ($2.6 \pm 0.3$, $2.8 \pm 0.7$, and $4.3 \pm 0.7$ μM, for Ang-(1-7), Ang II, and Ang I, respectively), but a significantly higher $V_{max}'$ for Ang-(1-7) ($72 \pm 2.2$ nmol/min/mg) versus Ang II ($30 \pm 3$ nmol/min/mg) or Ang I ($6 \pm 0.3$ nmol/min/mg). These data likely represent a novel pathway for specific regulation of central Ang-(1-7) levels.

Metallopeptidases are the major class of enzymes involved in extracellular peptide metabolism (Shrimpton *et al.* 2002). ACE, ACE2, neprilysin, TOP, and neurolysin belong to the same class of thermolysin-like zinc-dependent peptidases that hydrolyze peptide substrates (< 40 amino acids) and are maximally active at a neutral pH (Rawlings and Barrett 1993; Shrimpton *et al.* 2002). The characterization of a soluble metalloendopeptidase in the CSF and brain medulla is consistent with this class of peptidases, yet appears to be more restricted regarding the substrate length and the site of hydrolysis. For example, angiotensin peptides larger than Ang-(1-7) such as Ang II and Ang I exhibited an 11- and 27-fold lower rate of hydrolysis to generate Ang-(1-4). Interestingly, neurotensin contains a Tyr-Ile site at positions 12 and 13 of the peptide but was not cleaved by the peptidase; this may reveal a more constrained effect of

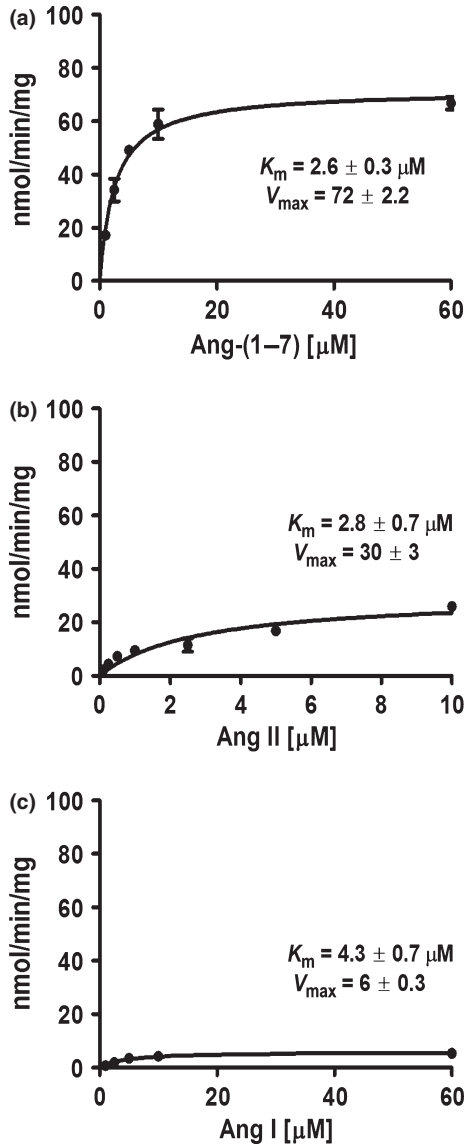


**Fig. 4** $^{125}$I-Ang-(1-7) exhibits the highest apparent $V_{max}$′ (nmol/min/mg protein) for the brain medullary peptidase. (a–c) Apparent kinetic values were determined for $^{125}$I-Ang-(1-7), $^{125}$I-Ang II, and $^{125}$I-Ang I. Kinetic values were determined based on the conversion of radiolabeled peptide to $^{125}$I-Ang-(1-4) in the presence of an inhibitor cocktail [amastatin (AM), bestatin (BS), chymostatin (CHYM), benzylsuccinate (BSC), lisinopril (LIS)]. $N = 3$ separate determinations from the Q-Sepharose purified fraction of preparation 1.

peptides > 10 residues in length. In this regard, both neurolysin and TOP exhibit restriction on the length of their peptide substrates that may reflect the limited access to the active sites of these peptidases (Shrimpton and Smith 2000). Peptides that lack the Tyr-Ile site such as bradykinin and apelin-13 were not cleaved by this peptidase. Moreover, the seven and five amino acid metabolites of bradykinin were not hydrolyzed by the medullary peptidase. The shorter fragments of Ang-(1-7) including Ang-(1-6) and Ang-(1-5) that are generally considered biologically inactive were hydrolyzed at a lower rate (2- and 25-fold, respectively) than Ang-(1-7), again suggesting an optimal length for recognition by the peptidase. Finally, we note that while the velocity rates for the hydrolysis of $^{125}$I-Ang-(1-7) and Ang-(1-7) were similar (72 vs. 54 nmol/min/mg), the velocity rate for $^{125}$I-Ang II was 6-fold higher than that of Ang II (30 vs. 5 nmol/min/mg) and the rate for $^{125}$I-Ang I was 3-fold higher than Ang I (6 vs. 2 nmol/min/mg). These data suggest an apparent influence of the addition of the $^{125}$I-group on the Tyr$^4$ residue, particularly for Ang II that may enhance the rate of hydrolysis of the peptide. Although additional kinetic studies are required to elucidate the mechanism for this effect on the Tyr residue, the use of $^{125}$I-labeled peptides other than Ang-(1-7) may not accurately reflect the substrate characteristics for the peptidase.

The Ang-(1-7) peptidase was isolated from the supernatant fraction of the medullary tissue, consistent with the soluble activity detected in the CSF (Marshall *et al.* 2013c). Metallopeptidases such as ACE, ACE2, neprilysin, and neurolysin are predominantly membrane associated, thus they are situated to hydrolyze bioactive peptides in the extracellular space. In contrast, TOP is a soluble enzyme that is primarily intracellular with no hydrophobic membrane-spanning domain (Acker *et al.* 1987; Woulfe *et al.* 1992; Shrimpton *et al.* 2003; Jeske *et al.* 2004). Several studies suggest that TOP localizes to the nucleus, consistent with the identification of a nuclear localization sequence for the peptidase (Healy and Orlowski 1992; Thompson *et al.* 1995; Massarelli *et al.* 1999; Alzayadneh and Chappell, in press). TOP reportedly undergoes both stimulated secretion and constitutive release from neuronal cells and is known to associate with lipid rafts in the plasma membrane (Ferro *et al.* 1999; Jeske *et al.* 2003, 2004). Once in the extracellular space, TOP participates in the extracellular metabolism of neuropeptides such as gonadotropin-releasing hormone, bradykinin, and neurotensin (Ferro *et al.* 1999). We have not established the intracellular distribution of the Ang-(1-7) peptidase nor the specific cell type (i.e., neuronal vs. glia) that express the peptidase within the medulla. Increasing evidence supports an intracellular RAS including the expression of intracellular Ang II and Ang-(1-7), as well as their respective receptors (Singh *et al.* 2007; Ellis *et al.* 2012; Gwathmey *et al.* 2012; Chappell *et al.* 2014). Indeed, we quantified the peptide content of Ang-(1-7), Ang II and Ang I in the brain medulla of sheep (Marshall *et al.* 2013a). Krob *et al.* (1998) demonstrated intense Ang-(1-7) immunostaining in hypothalamic neurons of the mRen2(27) transgenic rats suggesting the intracellular localization of the peptide. Furthermore, Gironacci and colleagues reported intracellular Ang-(1-7) expression in primary neuronal cells cultured from the hypothalamic-brainstem areas (Verrilli *et al.* 2009). In this regard, the peptidase may potentially influence the local processing of Ang-(1-7) within the cells.

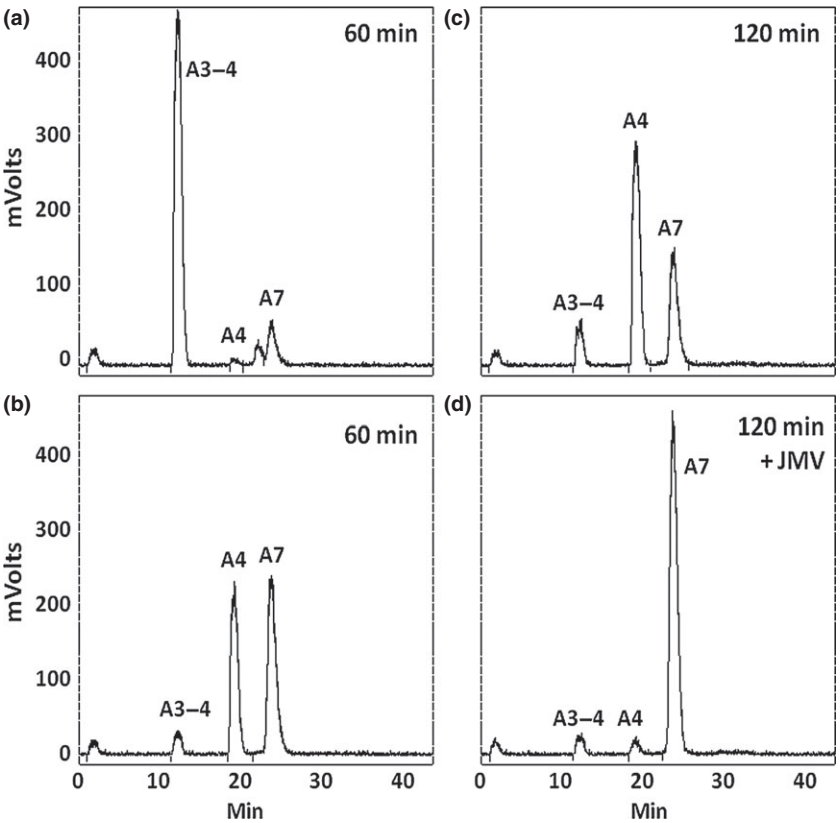


**Fig. 5** Purified peptidase lacks other Ang-(1-7) metabolizing enzyme activity. (a) Medullary supernatant incubated with $^{125}$I-Ang-(1-7) and no inhibitors for 60 min. (b) Q-Sepharose purified fraction incubated with $^{125}$I-Ang-(1-7) and no inhibitors for 60 min. (c) Q-Sepharose purified fraction incubated with $^{125}$I-Ang-(1-7) and no inhibitors for 120 min. (d) Q-Sepharose purified fraction incubated with $^{125}$I-Ang-(1-7) and 1 nM JMV for 120 min.

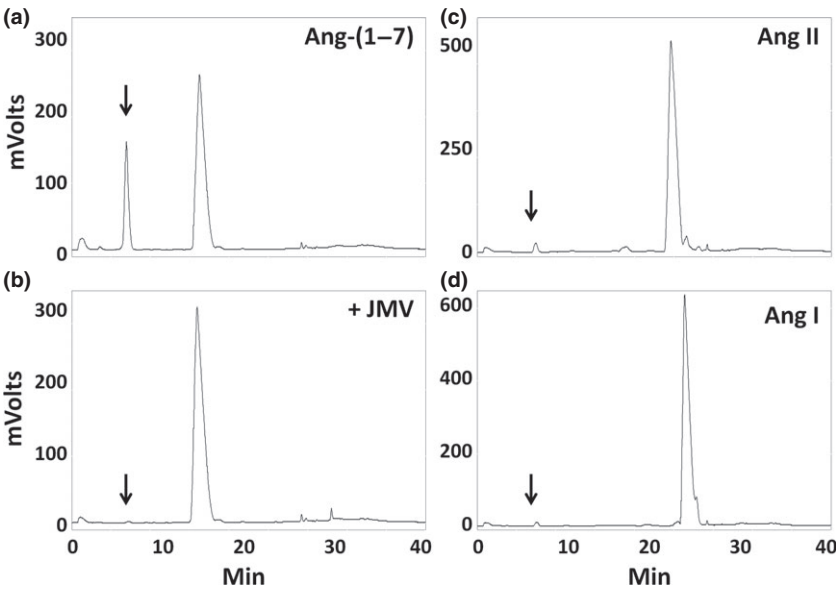


**Fig. 6** Purified medullary peptidase preferentially hydrolyzes Ang-(1-7) to Ang-(1-4) (arrow). Purified peptidase was incubated with 50 nmol (final concentration of 400 μM) of (a) Ang-(1-7), (b) Ang-(1-7) + 10 μM JMV-390, (c) Ang II, (d) Ang I for 24 h at 37°C in the presence of no inhibitors.

Alternatively, the peptidase may be secreted or released from medullary tissue to degrade extracellular Ang-(1-7) or perhaps other substrates that are unidentified at this time. Indeed, the presence of the soluble peptidase in the CSF may reflect the secretion of the enzyme into this compartment. Additional studies are necessary to identify the cell types that contain the peptidase and address whether the enzyme activity is actively secreted from medullary tissues.

The current chromatographic approach was not intended to achieve a preparation of pure enzyme, but to obtain sufficient activity to characterize the peptidase; thus, we cannot establish the identity of the protein with this preparation at the present time. Nevertheless, the studies of the enriched enzyme suggest a metalloendopeptidase-like activity to convert Ang-(1-7) to Ang-(1-4). Although the peptidase activity was not blocked by selective inhibitors to neprilysin,

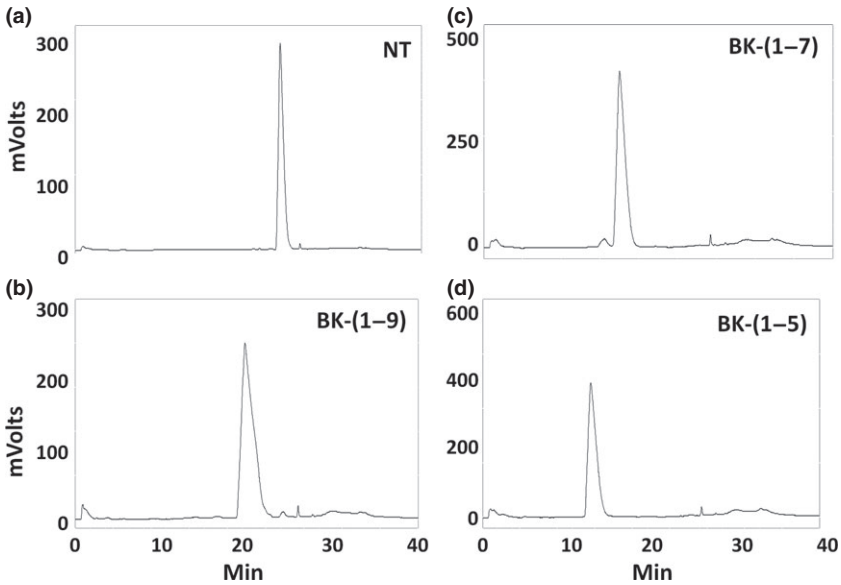


**Fig. 7** Purified medullary peptidase does not hydrolyze other selected neuropeptides. Purified peptidase was incubated with 50 nmol (final concentration of 400 μM) of (a) Neurotensin (NT), (b) Bradykinin (BK)-(1-9), (c) BK-(1-7), or (d) BK-(1-5) for 24 h at 37°C in the presence of no inhibitors.

**Table 3** Comparison of the metabolism velocities for angiotensins and other peptides

| Peptide | Amino acid sequence | Velocity (nmol/min/mg) |
|---|---|---|
| Ang I | DRVYIHPFHL | 2.0 |
| Ang II | DRVYIHPF | 4.9 |
| Ang-(I-7) | DRVYIHP | 54 |
| Ang-(I-6) | DRVYIH | 27 |
| Ang-(I-5) | DRVYI | 2.1 |
| Neurotensin | ELYENLPRRPYIL | NC |
| Bradykinin-(I-9) | RPPRFSPFR | NC |
| Bradykinin-(I-7) | RPPRFSP | NC |
| Bradykinin-(I-5) | RPPRF | NC |
| Apclin 13 | KFRRQRPRLSHKG | NC |

Velocity is an approximation of $V_{max}$, as reactions are carried out in the presence of excess peptide substrate. NC = not cleaved within 24 h of digestion.

ACE, neurolysin, or TOP, the enzyme exhibits marked sensitivity to the inhibitor JMV-390 ($IC_{50}$ = 0.80 nM). In contrast, the reported $IC_{50}$ values for JMV-390 against neprilysin, neurolysin, TOP, and leucine aminopeptidase (EC 3.4.11.1) are 30–60 nM, and a far higher value of 70 μM against ACE (Doulut *et al.* 1993). JMV-390 was originally synthesized to inhibit metalloendopeptidase activity thereby extending the functional benefits of neurotensin and neuromedin in brain (Doulut *et al.* 1993). The molecular design of JMV-390 was based on the recognition of the leucine–leucine sequence that corresponds to neurotensin, and the amino-terminal hydroxy amino group for an interaction with the zinc cofactor in the active site (Doulut *et al.* 1993).

ACE constitutes a prominent pathway for the metabolism of Ang-(1-7) in the circulation (Chappell 2007). ACE inhibition significantly increases the half-life of Ang-(1-7) in the circulation by blocking the hydrolysis of Ang-(1-7) to Ang-(1-5) (Chappell *et al.* 1998; Yamada *et al.* 1998). The marked increase in circulating Ang-(1-7) following administration of ACE inhibitors also reflects an increase in neprilysin-dependent processing of Ang I to Ang-(1-7) (Campbell *et al.* 1998; Chappell *et al.* 1998). We reported that soluble ACE contributes to Ang-(1-7) metabolism in sheep CSF *ex vivo*; however, the majority of the Ang-(1-7)-degrading activity was ACE independent and this activity inversely correlated with Ang-(1-7) content in the CSF (Marshall *et al.* 2013c). The presence of an Ang-(1-7) peptidase in the CSF and brain medulla may represent an alternative pathway to metabolize the peptide, particularly if the local expression of ACE is low or there is restricted access to ACE. Additional studies are necessary to compare the relative expression of ACE and the Ang-(1-7) peptidase in other areas of the brain, as well as the presence of the metallopeptidase in the circulation and peripheral tissues such as the heart and kidney that express Ang-(1-7).

## Perspective and significance

Central Ang-(1-7) is critically involved in blood pressure control and autonomic regulation. Low levels of Ang-(1-7) are associated with hypertension, reduced baroreflex sensitivity for control of heart rate, inflammation, cell proliferation, and oxidative stress (Averill and Diz 2000; Diz *et al.* 2008, 2011). The peptidase characterized in this study may potentially represent a novel Ang-(1-7) metabolizing pathway involved in the pathological reduction of central Ang-(1-7) levels (Marshall *et al.* 2013c). Moreover, we identified JMV-390 as a potent inhibitor of the Ang-(1-7) peptidase that exhibits an $IC_{50}$ value far below that reported for neprilysin,

neurolysin, TOP, leucine aminopeptidase, and ACE. JMV-390, or more selective analogs, may constitute a novel selective inhibitor against the Ang-(1-7) peptidase to prevent metabolism of Ang-(1-7) in brain. Enhanced peptidase activity in CSF of animals exposed *in utero* to glucocorticoids, where Ang-(1-7) levels were significantly lower than control animals, further suggests that the peptidase participates in physiological regulation of the brain RAS.

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All experiments were conducted in compliance with the ARRIVE guidelines.

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