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EFFECTS OF PEPTIDASE INHIBITORS ON BINDING AT ANGIOTENSIN RECEPTOR SUBTYPES IN THE RAT BRAIN

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Abstract—Sulfhydryl reducing agents affect angiotensin II (AII) receptor binding differentially at AT_1 and AT_2 sites. Consequently, sulfhydryl reducing agents are now used infrequently in AII receptor binding assays. In this regard, the present autoradiographic study evaluates the effects of additional peptidase inhibitors on AII receptor binding and radioligand integrity. EDTA at 5 mM enhanced binding similarly, by about 70%, at both AT_1 and AT_2 binding sites, whereas bacitracin (10^{-4} M) did not affect binding at either site. In contrast, addition of phenanthroline and bovine serum albumin (BSA) increased binding at AT_1 sites 2.3-fold, whereas binding at AT_2 sites was affected minimally. Degradation of ^{125}I-[Sar^1,Ile^8]-AII (^{125}I-SIAII) was determined by HPLC analysis of samples before and after incubation with tissue in each buffer. Omission of bacitracin from buffers reduced the recovery of intact radioligand to 83–87%, while recovery exceeded 94% in the presence or absence of all other buffer constituents. These results suggest that degradation of ^{125}I-SIAII is minimal in large volume *in vitro* receptor autoradiography studies of rat brain AII receptors. Further, the beneficial effects on radioligand binding caused by buffer constituents such as EDTA, phenanthroline, and BSA were not due to their ability to protect the radioligand from enzymatic degradation. Because these constituents (and possibly others) had differential effects on binding with respect to receptor subtypes, caution should be used when interpreting or comparing binding data obtained from various laboratories utilizing different buffer components.

Two subtypes of angiotensin II (AII)‡ receptors have been identified [1, 2]. The AT_1 receptor subtype is differentiated by high affinity for losartan (DuP753) and similar compounds, and relatively low affinity for CGP 42112A and PD123177. These compounds show the opposite pattern of selectivity for the receptor subtype designated AT_2. Receptor autoradiographic techniques have confirmed the presence of both AII receptor subtypes in discrete nuclei within the brain [3–7].

Several laboratories using membrane preparations or receptor autoradiography have employed a variety of procedures to investigate AII binding with the implicit assumption that the basic methodology does not have a detrimental effect on binding to the AII receptors of interest. This has not always been the case. For example, early studies indicated that sulfhydryl reducing agents were required to protect the radioligand from degradation [8, 9] and they increased binding affinity for ^{125}I-AII in brain tissue [10, 11]. Thus, sulfhydryl reducing agents were widely used in binding assays for brain tissue until it was revealed that they severely impair binding specifically at AT_1 but not AT_2 receptors [1, 12, 13]. Accordingly, most investigators now exclude sulfhydryl reducing agents when studying AII receptors.

Most investigators are in general agreement with respect to localization and distribution of AII receptor subtypes in brain tissue, and the relative affinities of various ligands at AT_1 and AT_2 sites. However, subtle differences are apparent with respect to relative binding density among brain nuclei in autoradiographic studies. Further, we have reported that the radioligand ^{125}I-[Sar^1,Ile^8]-AII (^{125}I-SIAII) has a 4-fold selectivity for brain AT_1 binding sites [14] which is not corroborated by other investigators [15, 16]. Prompted by the precedent set with sulfhydryl reducing agents, we hypothesized that different buffer constituents, utilized in binding assays in different laboratories, might affect binding differentially at AT_1 and AT_2 sites. Inclusion or exclusion of such a factor in incubation mixtures might alter results obtained in different laboratories. We began by investigating the peptidase inhibitors used routinely in our autoradiographic studies, bacitracin and EDTA, and then evaluated phenanthroline and bovine serum albumin (BSA), which have been used by other investigators [15, 17].

METHODS

Male Sprague–Dawley rats (240–300 g) were anesthetized with pentobarbital sodium (95 mg/kg, Nembutal, Abbott Laboratories, North Chicago, IL) and perfused intracardially with chilled phosphate-buffered saline. Whole brains were removed and frozen. Adjacent cryostat sections (20 μm thick) were thaw mounted on sets of six slides. Ten slides, each containing approximately eight brain sections, were preincubated in coplin jars containing 40 mL of one of five buffers (described below) for 30 min,

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‡ Abbreviations: AII, angiotensin II; BSA, bovine serum albumin; ^{125}I-SIAII, ^{125}I-[Sar^1,Ile^8]-angiotensin II; ^{125}I-Sar^1-AII, ^{125}I-[Sar^1]-angiotensin II.

at room temperature, before transfer to jars containing the same amount of the same buffer to which radioligand, ^{125}I-SIAII (360–375 pM) was added. After incubation for 1 hr, slides were rinsed, dried and apposed to X-ray film. Autoradiograms were evaluated by a video-based image analysis system (Imaging Research Inc., Ontario, Canada) utilizing 20 μm thick ^{125}I standards (Microscales Inc., Amersham, Arlington Heights, IL) to quantitate radioligand binding. Specific AII receptor binding was determined by subtraction of ^{125}I-SIAII binding in the presence of 10^{-6} M unlabeled AII from ^{125}I-SIAII binding in the absence of unlabeled AII (total binding). Brain nuclei were selected and categorized by subtype predominance (>90% AT_1 or AT_2) based on a previous study [14] to facilitate comparisons of buffer effects on the two subtypes. Subtype selective competitors were not used for this study.

Evaluation of EDTA and bacitracin. Studies in our laboratory have routinely used buffer containing 150 mM NaCl, 50 mM sodium phosphate (pH 7.1 to 7.2), 5 mM EDTA, and 0.1 mM bacitracin. Sets of six adjacent sections were prepared from each of six rat brains. These were incubated in either the above buffer (containing EDTA and bacitracin), a buffer from which bacitracin was omitted, or a buffer from which EDTA was omitted. Total and non-specific binding were determined for each of the three buffers.

Evaluation of phenanthroline and BSA. A second experiment compared our standard buffer (NaCl, $NaPO_4$, EDTA, and bacitracin) versus a buffer in which EDTA and bacitracin were replaced with 1,10-phenanthroline (5×10^{-4} M) and BSA (0.2%), and one in which no peptidase inhibitors were present (EDTA and bacitracin were omitted). Sets of six adjacent sections prepared from each of five rat brains as described above were incubated in each of the three study buffers with and without 1 μM unlabeled AII.

Binding was also compared in buffers containing phenanthroline or BSA alone (versus phosphate–saline buffer, N = 6) at the nucleus of the solitary tract (AT_1) and the inferior olivary nucleus (AT_2).

Analysis of radioligand degradation. For each animal, samples were taken from each buffer before and after a 60-min incubation of brain slices and analyzed for ^{125}I-SIAII and radiolabeled fragments by HPLC using a reverse phase C_{18} column (Microsorb, Rainen Instruments, Woburn, MA) and a radioisotope detector (Beckman model 171, Beckman Instruments, Palo Alto, CA). Peak areas for ^{125}I-labeled compounds were quantitated as height times width at half the peak height. Several peaks were resolved with 19% acetonitrile, 81% triethylamine phosphate (85 mM H_3PO_4 adjusted to pH 3.0 with triethylamine). The peak corresponding to ^{125}I-SIAII could not be further resolved by extending retention time using a mobile phase with 17% acetonitrile:83% triethylamine phosphate or 83% triethylamine acetate (104 mM acetic acid adjusted to pH 4.0 with triethylamine).

Compounds. Carrier-free monoiodinated ^{125}I-SIAII was prepared as described previously [18]. Peptides were obtained from Bachem (Torrance, CA). EDTA was obtained from Fisher Scientific (Pittsburgh, PA). Bacitracin, 1,10-phenanthroline and BSA (albumin, bovine, fraction V) were obtained from the Sigma Chemical Co. (St. Louis, MO).

Statistics. For each experiment, buffer effects on binding at each nucleus and recovery of intact radioligand were evaluated by a repeated measures analysis of variance and a modified Newman–Keuls test according to Winer [19].

Table 1. ^{125}I-[Sar^1,Ile^8]-angiotensin II recovery and binding in buffers containing bacitracin and/or EDTA

	+EDTA +Bacitracin	+Bacitracin (−EDTA)	+EDTA (−Bacitracin)
(A) Radioligand recovery	98 ± 1%	98 ± 3%	83 ± 7%*
(B) Specific radioligand binding†			
Predominantly AT_1 nuclei			
Piriform cortex	611 ± 134	311 ± 72‡	667 ± 171
Suprachiasmatic n.	1049 ± 79	715 ± 59‡	953 ± 63
Median preoptic n.	887 ± 111	493 ± 98‡	878 ± 79
Ventral hippocampus	700 ± 101	496 ± 67‡	679 ± 70
Paraventricular n.	1140 ± 87	604 ± 73‡	1014 ± 87
Anterior pituitary	4690 ± 484	2787 ± 359‡	4368 ± 419
Subfornical organ	1310 ± 193	724 ± 148‡	1286 ± 240
OVLT§	980 ± 208	640 ± 148‡	945 ± 172
Predominantly AT_2 nuclei			
Medial geniculate n.	468 ± 56	318 ± 59‡	441 ± 65
Superior colliculus	577 ± 56	304 ± 28‡	591 ± 52
Subthalamic n.	887 ± 80	581 ± 42‡	805 ± 52
Lateral septum	260 ± 24	141 ± 24‡	286 ± 24
Mediodorsal thalamus	372 ± 25	226 ± 19‡	346 ± 27

Values are means ± SEM, N = 6.
* Significantly different from value in the first column ($P < 0.05$).
† Binding values are reported in fmol/g of brain tissue.
‡ Significantly different from value in the first column ($P < 0.01$).
§ OVLT, organum vasculosum of the lamina terminalis.

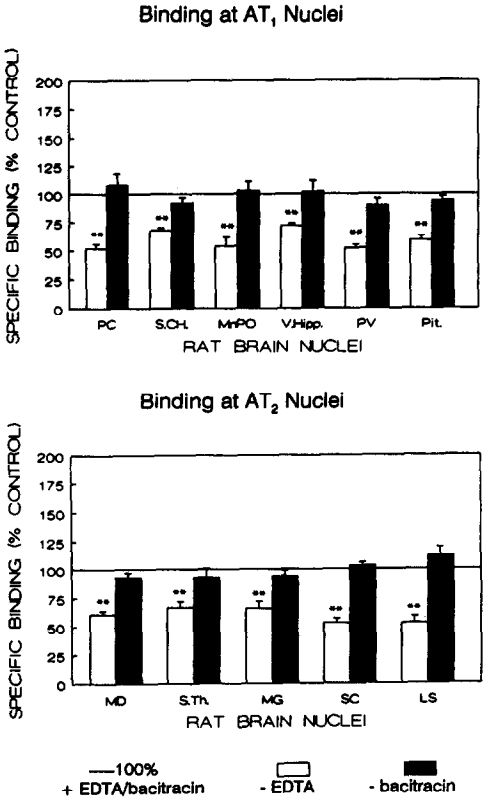

Fig. 1. Effects of EDTA (5 mM) and bacitracin (0.1 mM) on specific ^{125}I-SIAII binding. The 100% line represents specific ^{125}I-SIAII binding in the presence of bacitracin and EDTA, while the white bars depict binding in the absence of EDTA and the black bars depict binding in the absence of bacitracin. Key: (**) denotes a significant difference ($P < 0.01$) from the 100% control line. Data are expressed as means ± SEM ($N = 6$), and absolute values are given in Table 1B. Abbreviations: PC, piriform cortex; S.CH., suprachiasmatic n.; MnPO, median preoptic n.; V.Hipp., ventral hippocampus; PV, paraventricular n.; Pit., anterior pituitary; MD, mediodorsal thalamus; S.Th., subthalamic n.; MG, medial geniculate n.; SC, superior colliculus; and LS, lateral septum.

RESULTS

Table 1A describes radioligand integrity after incubation brain slices as a percentage of intact radioligand prior to incubation in three buffers differing with respect to the presence of EDTA and bacitracin. HPLC analysis determined nearly complete recovery of intact ^{125}I-SIAII from buffer containing both bacitracin and EDTA and in buffer containing only bacitracin following incubation of rat brain slices. However, intact radioligand recovery was decreased significantly ($P < 0.05$) in the absence of bacitracin. Table 1B compares specific ^{125}I-SIAII binding at rat brain nuclei in these three buffers. Figure 1 depicts specific binding as a percentage of specific binding in buffer containing both EDTA and bacitracin at predominantly AT_1 and AT_2 nuclei. The exclusion of EDTA consistently caused a significant ($P < 0.01$) reduction in specific ^{125}I-SIAII binding in all AT_1 and AT_2 predominant brain nuclei studied, with an average reduction of approximately 40%. Similarly, non-specific ^{125}I-SIAII binding (4–23% of total binding) was also consistently lower (average 17%) when EDTA was excluded, but the effect was significantly at only three of the nuclei surveyed. The exclusion of bacitracin had no significant effect on specific or non-specific binding at any brain nucleus.

Table 2A summarizes radioligand integrity in buffers containing phenanthroline and BSA, bacitracin and EDTA, or no peptidase inhibitors. The radioligand was well preserved by the bacitracin/EDTA buffer and by the phenanthroline/BSA buffer. Intact ^{125}I-SIAII recovery was reduced significantly in the buffer containing no peptidase inhibitors compared with the bacitracin/EDTA buffer (86 ± 4 vs 97 ± 1%, $P < 0.05$), but recovery in the phenanthroline/BSA buffer was not significantly different from either of the other two buffers. Table 2B details specific ^{125}I-SIAII binding in the three buffers. At all predominantly AT_1 nuclei (except the piriform cortex), binding was significantly higher in the phenanthroline/BSA buffer compared with both other buffers. In contrast, ^{125}I-SIAII binding at AT_2 nuclei in the phenanthroline/BSA buffer was significantly lower than EDTA/bacitracin and similar to the buffer with no additions. Figure 2 illustrates relative specific binding determinations in each of these buffers at predominantly AT_1 and predominantly AT_2 nuclei. Data are presented as a percentage of specific ^{125}I-SIAII binding in the bacitracin/EDTA buffer to facilitate comparison with Fig. 1.

A separate experiment evaluated the independent effects of phenanthroline or BSA. Specific binding at the nucleus of the solitary tract (AT_1) was 782 ± 52 fmol/g in phosphate–saline buffer, 1224 ± 93 with BSA (57% increase, $P < 0.01$), and 1122 ± 78 with phenanthroline (43% increase, $P < 0.01$). Specific binding at the inferior olivary nucleus (AT_2) was 410 ± 30 fmol/g in phosphate–saline buffer, 356 ± 23 with BSA (NS), and 325 ± 24 with phenanthroline (21% decrease, $P < 0.01$).

Thus, deletion of both EDTA and bacitracin (no additions) decreased binding by an average of 39% at all brain nuclei surveyed irrespective of subtype composition. Replacement of EDTA and bacitracin by phenanthroline and BSA caused a significant increase in ^{125}I-SIAII binding at all but one predominantly AT_1 nucleus (average 35%), while binding was reduced significantly (average 31%) at all AT_2 nuclei. A buffer effect on non-specific binding tended to parallel that for specific binding, but the differences were small and not significant.

DISCUSSION

Analysis of receptor binding studies ideally presupposes that ligand and receptor metabolism do not occur. Incubation constituents are normally selected to accomplish this goal with the expectation that these constituents will not interfere with the receptor–ligand reaction. The susceptibility of angiotensin II and its analogues to metabolism dictates the utilization of peptidase inhibitors in incubation mixtures. Many laboratories use radioligands such as ^{125}I-SIAII which are relatively resistant to aminopeptidase action but there is

Table 2. ^{125}I-[Sar^1,Ile^8]-angiotensin II recovery and binding in buffers containing phenanthroline/BSA, bacitracin/EDTA, or no additions

	+Phenanthroline +BSA	+Bacitracin +EDTA	No additions
(A) Radioligand recovery*	94 ± 4%	97 ± 1%	86 ± 4%*
(B) Specific radioligand binding†			
Predominantly AT_1 nuclei			
Piriform cortex	1061 ± 225	1026 ± 273	578 ± 112‡
Suprachiasmatic n.	1553 ± 334	1072 ± 248‡	769 ± 186‡
Median preoptic n.	1331 ± 217	1024 ± 170§	622 ± 51‡
Ventral hippocampus	1055 ± 117	817 ± 100‡	485 ± 43‡
Paraventricular n.	1542 ± 191	1015 ± 124‡	594 ± 89‡
Anterior pituitary	6285 ± 1449	4100 ± 780‡	2598 ± 750‡
Subfornical organ	3496 ± 843	1265 ± 265‡	641 ± 107‡
OVLT‖	2432 ± 266	1218 ± 172§	704 ± 123‡
Predominantly AT_2 nuclei			
Medial geniculate n.	245 ± 18	319 ± 24¶	189 ± 20§
Superior colliculus	347 ± 48	458 ± 62¶	253 ± 33§
Subthalamic n.	590 ± 75	825 ± 93¶	492 ± 48
Lateral septum	152 ± 9	239 ± 32¶	115 ± 8
Mediodorsal thalamus	167 ± 8	289 ± 13¶	240 ± 21¶

Values are means ± SEM (N = 5).

* Value for no additions was significantly different from (+Bacitracin + EDTA) ($P < 0.05$). All other comparisons were not significantly different.

† Binding values are reported in fmol/g of brain tissue.

‡,§ Significantly lower binding compared with values in the first column (‡$P < 0.01$ and §$P < 0.05$).

‖ OVLT, organum vasculosum of the lamina terminalis.

¶ Significantly higher binding compared with values in the first column ($P < 0.01$).

considerable variation in the selection of inhibitors for other peptidases and for other ionic buffer constituents. With regard to the criterion that constituents must not interfere with binding, many of these procedures were established before the discovery of AII receptor subtypes, and it is now apparent that this criterion was not satisfied independently for both AT_1 and AT_2 binding reactions. This important consideration was emphasized by the discovery that sulfhydryl reducing agents selectively interfere with binding at AT_1 sites [1, 12, 13].

In our studies of rat brain AII receptor binding, we have noted some subtle differences in data reported by various laboratories. For example, estimates of receptor subtype proportions within brain nuclei are not entirely consistent [4, 6, 7, 14]. Moreover, we found that ^{125}I-SIAII shows some selectivity for brain AT_1 receptors [14] which is at variance with findings from some laboratories [15, 16], but is similar to the findings of Tsutsumi and Saavedra [7] for ^{125}I-Sar^1-AII. It occurred to us that such discrepancies might result from one or more peptidase inhibitors exerting differential effects on binding at AT_1 and AT_2 receptors similar to that observed for sulfhydryl reducing agents. Table 3 itemizes incubation constituents used by several laboratories.

Comparison of binding at AT_1 and AT_2 sites was accomplished by sorting brain nuclei by predominant subtype (>90%) established in a previous study [14]. Thus, the results were not obtained from completely homogeneous receptor subtype populations. This approach was selected because utilization of subtype selective antagonists to mask each receptor subtype would introduce uncertainties with respect to possible differential metabolism or binding characteristics of antagonists in each experimental situation. We believe the approach is justified by the compelling internal consistency observed within the AT_1 and AT_2 categories.

We first evaluated the buffer constituents routinely used in our laboratory, EDTA and bacitracin. Addition of EDTA quantitatively increased specific binding by approximately 70% equally at both AT_1 and AT_2 binding sites. This is interesting because radioligand degradation was unaffected by the absence or presence of EDTA, suggesting that EDTA affects binding in a manner unrelated to radioligand preservation. Three possible mechanisms might be considered: (1) EDTA chelates ions that hamper the interaction of ^{125}I-SIAII with both AII receptors, (2) EDTA interacts directly with the radioligand (and/or receptors) to enhance binding affinity, or (3) EDTA inhibits metalloproteases that degrade both AT_1 and AT_2 receptors equally. The effect of EDTA to increase specific binding, and the tendency for a parallel increase in non-specific binding, suggest that EDTA affects ^{125}I–SIAII but not AII receptors. However, AII integrity was not monitored, and we cannot discount the possibility that derived non-specific binding includes some undisplaced specific binding.

Bacitracin appeared to be important for preserving radioligand, but had no effect on specific or non-specific binding. However, radioligand metabolism in the absence of bacitracin was minimal (13–17%) which probably accounts for our inability to observe differences in radioligand binding in the presence and absence of bacitracin. The presence of bacitracin

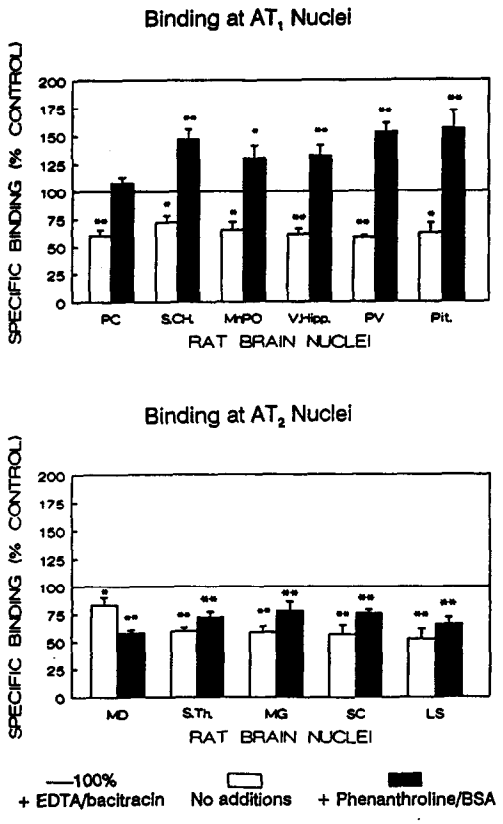

Fig. 2. Effects of phenanthroline (5×10^{-4} M) and BSA (0.2%) on specific ^{125}I-SIAII binding. The 100% line represents specific ^{125}I-SIAII binding in the presence of bacitracin and EDTA, while the white bars depict binding in the absence of peptidase inhibitors and the black bars depict binding with phenanthroline and BSA. Key: (*) and (**) denote significant differences ($P < 0.05$ and $P < 0.01$, respectively) from the 100% control line. Data are expressed as means ± SEM (N = 5), and absolute values are given in Table 2B. See brain nuclei abbreviations in the legend of Fig. 1.

may assume greater importance for the preservation of other angiotensin peptides and in binding assays where tissue/ligand ratios are higher. Parenthetically, concentrations of bacitracin greater than 0.1 mM inhibited ^{125}I-AII binding in rat brain homogenates (Speth RC, unpublished observation).

Some investigators utilize phenanthroline (0.1 to 1.0 mM) and BSA (0.2%) in binding assays (Table 3). Koziarz and Moore [17] evaluated ^{125}I-AII binding with bovine membrane preparations in the absence and presence of 1,10-phenanthroline (1 mM). Phenanthroline completely eliminated ^{125}I-AII degradation without affecting binding. Since similar results were obtained for both uterine (AT_2 predominant) and aortic (AT_1 predominant) preparations, we infer that binding was not differentially affected at AT_1 and AT_2 sites. These interesting observations prompted us to include phenanthroline in our autoradiographic system. We used a concentration of 0.5 mM phenanthroline which is lower that that used by Koziarz and Moore (1 mM) but higher than that used by Chang *et al.* [15] (0.09 mM). The excellent radioligand recovery even in the absence of inhibitors in our experiments may be attributed to the aminopeptidase resistance of the sarcosine-substituted radioligand and the low tissue/radioligand ratio in these autoradiographic studies. Interestingly, however, the phenanthroline/BSA combination had marked differential effects on binding at AT_1 nuclei as compared with AT_2 nuclei.

Unlike EDTA, addition of phenanthroline and BSA differentially affected binding at AT_1 and AT_2 sites. Phenanthroline chelates metal ions [20], but none that are not also chelated by EDTA. Thus, the chelating properties of phenanthroline are unlikely to account for the differential effects on binding. The combination of BSA and phenanthroline enhanced binding 2.3-fold at AT_1 nuclei when compared to phosphate–saline buffer. Since BSA alone enhanced binding by 57% and phenanthroline alone enhanced binding by 43% at the nucleus of the solitary tract (AT_1), it appears that both compounds independently enhance binding selectively at AT_1 receptors, and the effect is additive. The AT_1 receptor has disulfide bonds in its extracellular domain [21, 22] which are crucial for

Table 3. Itemized listing of buffer constituents for several laboratories

	Reference						
	[14]	[4]	[6]	[7]	[15]*	[16]*	[17]*
Sodium phosphate	50 mM	50 mM	10 mM	10 mM	10 mM		
Tris						50 mM	50 mM
NaCl	150 mM	150 mM	150 mM	120 mM	100 mM	125 mM	150 mM
$MgCl_2$		10 mM				6.5 mM	
EDTA	5 mM		5 mM	5 mM	5 mM	1 mM	5 mM
EGTA†		5 mM					
Bacitracin	0.1 mM		0.4 mM	0.07 mM	0.1 mM		
BSA		0.4%	0.2%	0.2%	0.2%	0.2%	0.2%
Phenanthroline					0.09 mM		1 mM

* Additional buffer constituents: [15] 0.2 mg/mL soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride; [16] 1.25 μg/mL each antipain, phosphoramidon, leupeptin, pepstatin A, bestatin and amastatin; [17] 1 mM phenylmethylsulfonyl fluoride.

† EGTA = ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

binding [1, 12]. Since BSA is stabilized by 17 disulfide bonds [23], it might serve as an antireductant and preserve the tertiary structure of the AT_1 receptor. We have not attempted a systematic analysis of each buffer constituent employed by multiple laboratories but we do provide evidence that procedural differences are likely to account for reported differences in binding characteristics.

This study indicates that degradation of ^{125}I-SIAII is minimal (<17%) in the complete absence of peptidase inhibitors in our autoradiographic procedures. It does not follow, however, that the observation is applicable to non-autoradiographic binding studies or to autoradiographic studies using non-sarcosine[1] angiotensin peptides or to studies with higher tissue/incubation medium ratios. Inclusion of bacitracin or phenanthroline/BSA leads to near total recovery of radioligand. EDTA enhances binding but does so uniformly at both AT_1 and AT_2 sites. In contrast, phenanthroline and BSA markedly enhance binding at AT_1 sites but not at AT_2 sites. It is clear that buffer constituents affect binding at AT_1 and AT_2 sites by mechanisms that are independent of radioligand metabolism. Therefore, relative binding characteristics between AT_1 and AT_2 receptor subtypes will necessarily differ and caution must be exercised in comparing data from different laboratories.

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