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Angiotensin AT1 and AT2 receptor antagonists modulate nicotine-evoked [^{3}H]dopamine and [^{3}H]norepinephrine release

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Abstract

Tobacco smoking is the leading preventable cause of death in the United States. A major negative health consequence of chronic smoking is hypertension. Untoward addictive and cardiovascular sequelae associated with chronic smoking are mediated by nicotine-induced activation of nicotinic receptors (nAChRs) within striatal dopaminergic and hypothalamic noradrenergic systems. Hypertension involves both brain and peripheral angiotensin systems. Activation of angiotensin type-1 receptors (AT1) release dopamine and norepinephrine. The current study determined the role of AT1 and angiotensin type-2 (AT2) receptors in mediating nicotine-evoked dopamine and norepinephrine release from striatal and hypothalamic slices, respectively. The potential involvement of nAChRs in mediating effects of AT1 antagonist losartan and AT2 antagonist, 1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid (PD123319) was evaluated by determining their affinities for α4β2* and α7* nAChRs using [^{3}H]nicotine and [^{3}H]methyllycaconitine binding assays, respectively. Results show that losartan concentration-dependently inhibited nicotine-evoked [^{3}H]dopamine and [^{3}H]norepinephrine release (IC_{50}: 3.9±1.2 and 2.2±0.7 μM; I_{max}: 82±3 and 89±6%, respectively). In contrast, PD123319 did not alter nicotine-evoked norepinephrine release, and potentiated nicotine-evoked dopamine release. These results indicate that AT1 receptors modulate nicotine-evoked striatal dopamine and hypothalamic norepinephrine release. Furthermore, AT1 receptor activation appears to be counteracted by AT2 receptor activation in striatum. Losartan and PD123319 did not inhibit [^{3}H]nicotine or [^{3}H]methyllycaconitine binding, indicating that these AT1 and AT2 antagonists do not interact with the agonist recognition sites on α4β2* and α7* nAChRs to mediate these effects of nicotine. Thus, angiotensin receptors

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contribute to the effects of nicotine on dopamine and norepinephrine release in brain regions involved in nicotine reward and hypertension.

Keywords

Angiotensin II receptors; dopamine; nicotinic acetylcholine receptors; norepinephrine; reward; smoking

1. INTRODUCTION

Tobacco smoking is the most preventable cause of death in the United States and is associated with increased cardiovascular disease, stroke, chronic lung disease, lung cancer and other cancers [1]. Nicotine, the most abundant alkaloid in tobacco, has intrinsic rewarding properties that contribute to tobacco dependence [2]. Activation of nicotinic acetylcholine receptors (nAChRs) by nicotine increases extracellular dopamine (DA) concentrations in brain reward circuits, including nucleus accumbens (NAc) and striatum [3, 4]. The NAc mediates primary reward, whereas the striatum is associated with habit formation and compulsivity [5-7]. Importantly, the transition from reward seeking to compulsive behavior associated with drug addiction appears to be mediated by a shift in activity from NAc to striatum [6, 7]. Consistent with this idea, smoking has been shown to be associated with decreased [^{11}C]raclopride (DA D2 receptor antagonist) binding in striatum, but not in NAc in humans, using positron emission tomography [8]. Therefore, hedonic response to nicotine appears to involve DA release in striatum.

Tobacco dependence is a potent risk factor for cardiovascular diseases, including hypertension, atherosclerosis, myocardial infarction and aortic abdominal aneurysm [9, 10]. Specifically, the paraventricular nucleus of the hypothalamus is an important site of integration of neuroendocrine and autonomic responses controlling blood pressure [11]. Microinjection of norepinephrine (NE) into the paraventricular nucleus increases systolic and diastolic blood pressure in rats [12]. Importantly, intraperitoneal and intracerebroventricular injections of nicotine induces NE release in the paraventricular nucleus [13]. Taken together, nicotine-induced hypertension is mediated, at least in part, by hypothalamic NE.

nAChRs are ligand-gated ion channel receptors consisting of homo- or heteropentameric transmembrane proteins with a diverse subunit composition [14, 15]. nAChR subtype diversity in function and pharmacological response are attributed to the specific subunit compositions, including α2-α10 and β2-β4 subunits encoded by individual subunit genes [16]. β2-Containing nAChRs mediate nicotine reward that results from presynaptic DA release following activation of α-conotoxin MII-sensitive nAChRs including α6β2β3*, α4α6β2β3*, α6β2* subtypes and α-conotoxin MII-insensitive α4β2* and α4α5β2* subtypes [17-20]. In accumbens shell, α-conotoxin MII-sensitive nAChRs are critical for nicotine reward [21]. In contrast, nicotine-evoked NE release is mediated by α3β4* nAChRs [22, 23]. Thus, distinct nAChRs subtypes mediate nicotine-evoked DA and NE release, resulting in nicotine reward and hypertension, respectively.

Pathology in the renin-angiotensin system (RAS) is known to underlie some types of cardiovascular diseases [24, 25]. The majority of physiological actions of angiotensin II (Ang II) are mediated by angiotensin type-1 (AT1) receptors, including, vasoconstriction, thirst, activation of the sympathetic nervous system, cellular differentiation and proliferation [26]. Angiotensinogen is the precursor for the RAS system [26, 27]. Importantly, both angiotensinogen mRNA as well as strong glial angiotensinogen immunoreactivity have been demonstrated in striatum and hypothalamus [28, 29]. Furthermore, both striatal and hypothalamic neurons express AT1 and angiotensin type-2 (AT2) receptors [30, 31]. The brain RAS mediates Ang II effects on fluid balance, thirst, blood pressure and cognitive function *via* AT1 receptors [31, 32]. Ang II activation of AT1 receptors results in release of NE from the hypothalamus [33]. AT1 receptor effects on blood pressure and water intake are counteracted by AT2 receptors [34, 35].

In addition to activation of the noradrenergic system, Ang II also modulates DA function as evidenced by decreases in expression of AT1 and AT2 receptors in the substantia nigra in Parkinson's patients and by Ang II-evoked striatal DA release *via* AT1 receptor activation [30, 36, 37]. Previous research shows that Ang II (0.1-1 μM) increased striatal DA released from superfused striatal slices *in vitro* and that Ang II (1-10 μM) increased striatal DA release into microdialysate in freely moving rats [37]. Losartan, an AT1 antagonist (1μM; administered *via* the microdialysis probe), inhibited the Ang II (10 μM)-induced increase in extracellular DA [37]. Other work shows that striatal DA levels were decreased following acute subcutaneous administration of losartan (10 mg/kg) [38]. The purpose of the current study was to extend the previous work by determining whether Ang II receptors serve as potential targets for intervention to ameliorate the addictive and cardiovascular effects of nicotine. Specifically, AT1 and AT2 receptor involvement in mediating nicotine-evoked DA and NE release from striatal and hypothalamic slices, respectively, was determined. Also, effects of Ang II-receptor ligands at nAChRs mediating DA and NE release were evaluated.

2. MATERIALS AND METHODS

2.1. Chemicals

Ang II, angiotensin peptides, cytisine, D-glucose, ethylenediaminetetraacetic acid (EDTA), L-ascorbic acid, mecamylamine hydrochloride, nomifensine maleate, pargyline hydrochloride, polyethyleneimine, *S*-(−)nicotine ditartrate and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO). [^{3}H]DA (3,4-ethyl-2 [*N*-^{3}H]dihydroxyphenylethylamine; specific activity, 33.7 Ci/mmol), [^{3}H]NE (levo-1-(3,4-dihydroxy-[ring-2,5,6-^{3}H]phenyl)-2-aminoethanol; specific activity, 14.0 Ci/mmol), [^{3}H]nicotine (L-(−)[*N*-methyl-^{3}H]; specific activity, 78.4 Ci/mmol) and tissue solubilizer (TS-2) were purchased from PerkinElmer Life Sciences (Boston, MA). [^{3}H]Methyllycaconitine ([1α4(S),6β,14α,16β]-20-ethyl-1,6,14,16-tetramethoxy-4-[[[2-([3-^{3}H]-methyl-2,5-dioxo-1-pyrrolidinyl)benozyl]-oxy]methyl]-aconitane-7,8-diol; specific activity, 25.4 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Losartan and PD123319 were generous gifts from Merck & Co. Inc. (Whitehouse Station, NJ) and Parke-Davis & Co. (Detroit, MI), respectively. Acetonitrile, calcium chloride, magnesium chloride, methanol, monosodium phosphate, potassium

chloride, sodium bicarbonate and trifluoroacetic acid were purchased from Fisher Scientific (Pittsburgh, PA). Sep Pak C18 columns were purchased from Waters (Milford, MA). Ang II polyclonal antibody was purchased from Abcam (Cambridge, MA).

2.2. Animals

Adult male Sprague Dawley rats (200-225 g) were obtained from Harlan Laboratories (Indianapolis, IN). All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky. Upon arrival, rats received standard rat chow (Teklad mouse/rat diet, Harlan Laboratories Inc., Indianapolis, IN) and water *ad libitum*.

2.3. [^{3}H]DA and [^{3}H]NE release assays

Nicotine-evoked [^{3}H]DA and [^{3}H]NE release using superfused striatal and hypothalamic slices, respectively, was determined using previously described methods with minor modifications [39-42]. Briefly, coronal striatal or hypothalamic slices were incubated for 30 min in Krebs' buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1 mM NaH_2PO_4, 1.3 mM $CaCl_2$, 11.1 mM glucose, 25 mM $NaHCO_3$, 0.11 mM L-ascorbic acid, and 0.004 mM EDTA, pH 7.4, saturated with 95% O_2/5% CO_2 at 34°C). Incubation continued for an additional 30 min in buffer containing 0.1 μM [^{3}H]DA or [^{3}H]NE. Then, each slice was transferred to a superfusion chamber and superfused (1 ml/min) with Krebs' buffer containing pargyline (10 μM), a monoamine oxidase inhibitor. Nomifensine (10 μM), a specific dopamine transporter inhibitor, was included only in the superfusion buffer for [^{3}H]DA release assays. After 60 min of superfusion, three samples (5 ml/sample) were collected to determine basal [^{3}H]DA or [^{3}H]NE outflow.

To determine the nicotine concentration response in hypothalamus, superfusion continued in the absence (control) or presence of nicotine (10, 30 and 100 μM) for 45 min. Using a repeated-measures design, each chamber containing a single slice was exposed to one nicotine concentration which remained in the buffer until the end of the experiment.

To evaluate AT1 and AT2 receptor-mediated modulation of nicotine-evoked [^{3}H]DA and [^{3}H]NE release, a within-subject design was employed such that each concentration of antagonist was evaluated in striatum or hypothalamus from each rat, and different groups of rats were employed for each antagonist. Initially, the effect of mecamylamine on nicotine-evoked [^{3}H]DA and [^{3}H]NE release was determined as a positive control, since this drug inhibits all known nAChRs. Subsequently, effects of losartan and PD123319, AT1 and AT2 receptor antagonists [43, 44], respectively, on nicotine-evoked [^{3}H]DA and [^{3}H]NE release were determined. Following collection of basal samples, superfusion continued for 45 min in the absence (control) or presence of mecamylamine (0.1-10 μM), losartan (1-30 μM) or PD123319 (0.01-10 μM), in order to determine intrinsic effects of the antagonists alone on basal [^{3}H]DA and [^{3}H]NE release. Subsequently, nicotine (10 μM for [^{3}H]DA release assays; 100 μM for [^{3}H]NE release assays) was added to the buffer and superfusate samples continued to be collected further for 45 min to determine inhibition of nicotine-evoked [^{3}H]DA and [^{3}H]NE overflow. Each superfusion chamber containing a single slice was exposed to only one concentration of mecamylamine, losartan or PD123319, which

remained in the buffer until the end of the experiment. At the end of the experiment, each slice was solubilized with TS-2 tissue solubilizer. Radioactivity in the superfusate samples and slices was determined by liquid scintillation spectrometry (Model B1600TR, Perkin Elmer Inc., Downers Grove, IL).

2.4. [^{3}H]Nicotine and [^{3}H]methyllycaconitine binding assays

The interaction of losartan and PD123319 with α4β2* and α7* nAChRs was evaluated in [^{3}H]nicotine and [^{3}H]methyllycaconitine binding assays, respectively, using a previously described method [45]. Membrane suspensions (100-140 μg protein/100 μl) were prepared from whole brain (excluding cortex and cerebellum) and incubated for 60 min (250 μl final volume) at room temperature in tubes containing losartan (1 nM-1 mM) or PD123319 (1 nM-1 mM) with either 3 nM [^{3}H]nicotine or 3 nM [^{3}H]methyllycaconitine. Nonspecific binding was determined in the presence of 10 μM cytisine for the [^{3}H]nicotine binding assay and 10 μM nicotine for the [^{3}H]methyllycaconitine binding assay. Reactions were terminated by addition of ice-cold buffer and rapid filtration through Whatman GF/B glass fiber filters presoaked in 0.5% polyethylenimine. Bound radioactivity was determined using liquid scintillation spectrometry (TopCount NXT; PerkinElmer Inc.).

2.5. Electrically-evoked Ang II release

Coronal striatal slices were transferred to superfusion chambers and superfused (1 ml/min) for 60 min with Krebs' buffer. Three samples (5 min intervals) were collected to determine basal Ang II outflow. Slices were electrically stimulated (30 volts, 3 msec duration, 5 Hz, 5 min) and 12 samples were collected to determine stimulation-evoked Ang II overflow. Superfusates from striatal slices were partially purified over Sep Pak C18 columns (changed every 30 minutes) preequilibrated with 4 ml methanol, 4 ml water, and 10 ml of Krebs' buffer. After washing columns with 10 ml of 0.1% trifluoroacetic acid, angiotensin peptides were eluted with 2 ml of 90% acetonitrile and 0.1% trifluoroacetic acid, followed by a second elution with 2 ml of 67% methanol, 33% acetonitrile, and 0.1% trifluoroacetic acid. Eluate was evaporated using vacuum centrifugation and reconstituted in radioimmunoassay buffer. Ang II was quantified using a polyclonal Ang II antibody as previously described [46]. This same method was employed to quantify Ang II content in striatal tissue homogenates.

2.6. Ang II-evoked [^{3}H]NE release from hypothalamus

Coronal slices from rat hypothalamus were incubated with [^{3}H]NE (0.01 μM) for 30 min. Slices were transferred to superfusion chambers and superfused (1 ml/min) for 60 min. Three samples (5 min intervals) were collected to determine basal [^{3}H]NE outflow. Slices were electrically stimulated (S1, 30 volts, 3 msec duration, 5Hz, 5 min) in the absence of Ang II, and 6 samples were collected to determine stimulation-evoked [^{3}H]NE overflow. Slices were superfused with buffer only, or buffer containing Ang II (1 nM–1 μM) for 30 min, and then, the electrical stimulus was repeated (S2). The ratio S2/S1 evoked [^{3}H]NE overflow was calculated. In separate studies, the same experimental design was employed to determine if losartan inhibited evoked [^{3}H]NE overflow from superfused hypothalamic slices pre-incubated with [^{3}H]NE. Radioactivity in the superfusate samples and slices was determined by liquid scintillation spectrometry.

2.7. Data analysis

Data are presented as mean values ± S.E.M., and 'n' represents the number of animals tested in each experiment. Data were analyzed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA) and SPSS version 9.0 (SPSS Science, Chicago, IL) software. Fractional [^{3}H]DA and [^{3}H]NE release were calculated by dividing the amount of [^{3}H] in each superfusate sample by the tissue-[^{3}H] at the time of sample collection; data are expressed as a percentage of basal. Basal [^{3}H]DA outflow was determined from the three samples collected prior to antagonist addition to the superfusion buffer. Nicotine-evoked total [^{3}H]DA and [^{3}H]NE overflow were calculated by summing the increases in fractional release above basal for an equivalent period of exposure to nicotine. Concentration- and time-dependent effects of the antagonists on nicotine-evoked [^{3}H]DA and [^{3}H]NE release were determined using two-way repeated measures ANOVA, with concentration and time as within-subject factors. One-way ANOVAs were used to determine concentration-dependent effects of the antagonists on total [^{3}H]DA and [^{3}H]NE release and concentration dependent effects of Ang II and losartan on [^{3}H]NE release. Dunnett's *post hoc* analyses were employed for subsequent comparisons of treatment samples to control. Nicotine and antagonist concentration-response data were fit by nonlinear least-squares regression using a variable slope, sigmoidal function. IC_{50} values and values for percent maximal inhibition (I_{max}) were determined. Student's t-test compared log IC_{50} values for mecamylamine inhibition of nicotine-evoked [^{3}H]DA and [^{3}H]NE release as well as basal and electrically-evoked Ang II release. For the binding assays, specific [^{3}H]nicotine and [^{3}H]methyllycaconitine binding were determined by subtracting nonspecific binding from total binding. Inhibition constants (K_i values) were determined using the Cheng-Prusoff equation [47].

RESULTS

3.1. In a concentration-dependent manner, nicotine evoked [^{3}H]NE overflow from superfused rat hypothalamic slices

Concentration- and time-dependent effects of nicotine on [^{3}H]NE overflow from superfused rat hypothalamic slices are illustrated in Fig. 1. Two-way ANOVA revealed a concentration × time interaction ($F_{33, 176} = 1.73$, $p < 0.05$; Fig 1, top). Across the time-course of the experiment, fractional [^{3}H]NE release increased following nicotine addition to the buffer, peaked 5 min after nicotine addition, and then declined towards basal over time despite the continued presence of nicotine in the buffer. The pattern of nicotine-evoked fractional [^{3}H]NE release was similar for each of the nicotine concentrations (10, 30 and 100 μM) evaluated, although the peak effect was dependent on concentration. Of the concentrations evaluated, only the highest nicotine concentration (100 μM) evoked a significant increase in total [^{3}H]NE overflow compared to the buffer control ($F_{3, 20} = 17.32$, $p < 0.001$; Fig.1, bottom).

3.2. Mecamylamine and losartan inhibited nicotine-evoked [^{3}H]DA and [^{3}H]NE overflow

Concentration- and time-dependent effects of mecamylamine and losartan on nicotine-evoked [^{3}H]DA and [^{3}H]NE overflow from superfused striatal and hypothalamic slices are illustrated in Figs. 2 and 3, respectively. Based on results from the nicotine concentration-

response studies (Smith et al., 2010; current results), antagonist inhibition of nicotine-evoked [^{3}H]DA and [^{3}H]NE fractional release was evaluated using 10 and 100 μM nicotine, respectively. Under control conditions (in the absence of mecamylamine), fractional [^{3}H]DA and [^{3}H]NE release increased following nicotine addition to the buffer, peaked after 10 and 5 min, respectively, and then declined over time towards basal despite the continued presence of nicotine in the buffer. In a concentration-dependent manner, mecamylamine inhibited nicotine-evoked fractional [^{3}H]DA and [^{3}H]NE release. Two-way ANOVA revealed a concentration × timeinteraction for mecamylamine inhibition of nicotine-evoked fractional [^{3}H]DA release. ($F_{24, 112} = 1.68$, $p < 0.05$; Fig 2: top-left). With respect to total [^{3}H]DA overflow, mecamylamine at 1 and 10 μM inhibited the effect of nicotine ($F_{3, 22} = 8.661$, $p < 0.001$; Fig 2: bottom-left). The IC_{50} value for mecamylamine was 1.0 ± 0.4 μM, and the I_{max} was 95 ± 1%. A concentration X time interaction ($F_{24, 112} = 2.954$, $p < 0.001$; Fig 2: top-right) was found also for mecamylamine inhibition of nicotine-evoked fractional [^{3}H]NE release. With respect to total [^{3}H]NE overflow, mecamylamine at 1 and 10 μM inhibited the effect of nicotine ($F_{3, 25} = 7.125$, $p < 0.001$; Fig 2: bottom-right). The IC_{50} for mecamylamine was 0.2 ± 0.03 μM, and the I_{max} was 85 ± 8%. Log IC_{50} values for mecamylamine inhibition of nicotine-evoked [^{3}H]DA and [^{3}H]NE overflow were not different ($t_{(9)} = 1.187$; $p > 0.05$).

In a concentration- and time-dependent manner, losartan inhibited nicotine-evoked fractional [^{3}H]DA and [^{3}H]NE release (Fig. 3). No intrinsic effects of losartan (1-30 μM) alone on fractional [^{3}H]DA ($F_{4, 29} = 1.85$; $p > 0.05$) and [^{3}H]NE release ($F_{4, 28} = 0.64$; $p > 0.05$) from striatum and hypothalamus, respectively, were found (Table 1). In these experiments, mecamylamine (10 μM) served as a positive control. Two-way ANOVA of the fractional [^{3}H]DA release data revealed main effects of concentration ($F_{3, 25} = 3.293$, $p < 0.001$) and time ($F_{8, 200} = 11.728$, $p < 0.001$); however, a concentration X time interaction was not found ($F_{24, 200} = 1.063$, $p > 0.05$; Fig 3: top-left). Losartan at 10 and 30 μM inhibited nicotine-evoked [^{3}H]DA overflow ($F_{3, 37} = 14.57$, $p < 0.001$; Fig 3: bottom-left). The IC_{50} for losartan was 3.9 ± 1.2 μM, and the I_{max} was 82 ± 3%. With respect to inhibition of nicotine-evoked fractional [^{3}H]NE release, two-way repeated measures ANOVA revealed a concentration X time interaction ($F_{24, 240} = 1.698$, $p < 0.05$; Fig 3: top-right), and a main effect of time ($F_{8, 240} = 7.851$, $p < 0.001$); however a main effect of concentration was not found ($F_{3, 30} = 2.24$, $p > 0.05$). Losartan also inhibited nicotine-evoked [^{3}H]NE overflow at 10 and 30 μM ($F_{3, 31} = 6.409$, $p < 0.001$; Fig 3: bottom-right). The IC_{50} for losartan was 2.2 ± 0.7 μM, and the I_{max} was 89 ± 6%.

3.3. PD123319 did not inhibit nicotine-evoked [^{3}H]NE overflow, but potentiated nicotine-evoked [^{3}H]DA overflow

Time course of the effect of PD123319 on nicotine-evoked [^{3}H]DA and [^{3}H]NEfractional release is illustrated in Fig. 4. No intrinsic effects of PD123319 (0.01-10 μM) alone on fractional [^{3}H]DA ($F = 0.31$; $p > 0.05$) and [^{3} ($F_{5, 46}$ H]NE release ($F_{5, 40} = 0.63$; $p > 0.05$) from striatum and hypothalamus, respectively, were found (Table 2). Two-way ANOVA of the fractional [^{3}H]NE release data revealed a PD123319 concentration X time interaction ($F_{32, 312} = 2.26$, $p < 0.0001$; Fig 4: top-right) and a main effect of time ($F_{8, 312} = 48$, $p < 0.001$); however, no main effect of concentration ($F_{4, 39} = 2.19$, $p > 0.05$) was found.

PD123319 (0.001-10 μM) did not inhibit nicotine-evoked [^{3}H]NE overflow ($F_{4, 38} = 0.3573$, $p > 0.05$; Fig 4: bottom-right).

With respect to nicotine-evoked fractional [^{3}H]DA release, two-way ANOVA revealed main effects of concentration ($F_{4, 53} = 4.139$, $p < 0.001$) and time ($F_{8, 424} = 43.602$, $p < 0.001$); however, the concentration X time interaction was not significant ($F_{32, 424} = 0.953$, $p > 0.05$; Fig 4: top-left). Surprisingly, low concentrations of PD123319 (0.01 and 0.1 μM) potentiated (about 40%) the effect of nicotine to increase [^{3}H]DA overflow ($F_{4, 39} = 3.328$, $p < 0.05$; Fig 4: bottom-left).

3.4. Losartan and PD123319 lacked affinity for α4β2* and α7* nAChRs

Affinity of losartan and PD123319 for α4β2* and α7* nAChRs was evaluated using [^{3}H]nicotine and [^{3}H]methyllycaconitine binding to whole brain membranes (Fig. 5). K_i values for nicotine were 3 nM and 370 nM at the [^{3}H]nicotine and [^{3}H]methyllycaconitine binding sites, respectively, consistent with previous reports [48]. At the concentrations evaluated, both losartan and PD123319 did not inhibit [^{3}H]nicotine and [^{3}H]methyllycaconitine binding (Fig. 5).

3.5. Ang II in striatal homogenates and superfusates

Ang II content in striatal tissue was 237 ± 22.7 pg/g tissue. Electrical stimulation of striatal slices evoked Ang II release in amounts (3.6 ± 0.36 pg/ml/min) significantly greater than basal (2.1 ± 0.18 pg/ml/min) ($t_{(4)} = p < 0.05$; Fig. 6). Basal Ang II release during each minute of superfusion constituted 1% of the striatal tissue content.

3.6. Ang II evokes [^{3}H]NE release from hypothalamus

Ang II (1 nM-1 μM) evoked [^{3}H]NE overflow following electrical stimulation of hypothalamic slices; however, one way ANOVA did not reveal a significant effect of concentration ($F_{(4, 34)} = 1.631$, $p > 0.05$; Fig. 7 top). Higher concentrations of losartan appeared to inhibit Ang II-evoked [^{3}H]NE overflow; however, this effect was not significant ($F_{(5, 37)} = 1.279$, $p > 0.05$; Fig. 7 bottom).

DISCUSSION

The role of AT1 receptors in Ang II-induced modulation of the DA reward system was evident from previous findings that losartan inhibits Ang II-mediated DA release from striatum [37]. The current study extends these previous findings by showing that losartan inhibits nicotine-evoked [^{3}H]DA and [^{3}H]NE release from rat striatum and hypothalamus, respectively, suggesting that AT1 receptors mediate, at least in part, both nicotine-evoked DA and NE release. PD123319 potentiates nicotine-evoked [^{3}H]DA release from rat striatum, suggesting that AT2 receptors exert tonic inhibition of nicotine-evoked DA release.

Since losartan and PD 123319 did not inhibit [^{3}H]nicotine and [^{3}H]methyllycaconitine binding, these antagonists do not appear to interact with α4μ2* and α7* nAChRs to elicit the inhibition and potentiation of nicotine-evoked neurotransmitter release. However, inhibition of the binding of these radioligands only interrogates interactions with the agonist binding

site on nAChRs. Thus, losartan may be inhibiting the effect of nicotine at these nAChRs through a negative allosteric mechanism of action. On the other hand, PD123319 may be potentiating the effect of nicotine in striatum through a positive allosteric interaction at nAChRs. Considerable evidence demonstrating the ability of pharmacological agents to allosterically modulate nAChRs supports the current interpretation. For example, desformylflustrabromine, a compound isolated from *Flustra foliacea*, has been identified as a positive allosteric modulator of α4β2 nAChRs [49]. Low micromolar concentrations of desformylflustrabromine potentiated (2–3-fold) agonist-evoked currents associated with human α4β2 nAChRs expressed in *Xenopus* oocytes, while higher desformylflustrabromine concentrations inhibited this α4β2-mediated response [49]. Also, the anthelminthic agent, ivermectin, was identified as a positive allosteric modulator of the α7 nAChR [50]. Another drug, UCI-30002 [N-(1, 2, 3, 4-tetrahydro-1-naphthyl)-4-nitroaniline], that reduces nicotine self-administration in rats, acts as a negative allosteric modulator at α4β2 nAChRs [51]. Thus, additional mechanistic studies are needed to rule out nAChR involvement in the response to these AT1 and AT2 antagonists.

The presence of Ang II in the striatum (22.7 pg/g tissue; current study) and hypothalamus (125 pg/g tissue [30]) indicates that the effects of losartan and PD123319 on nicotine-evoked DA and NE release may be through inhibition of the effects of endogenous and released (nicotine-evoked) Ang II. Ang II-containing neurons are present in striatum and hypothalamus [30, 31], and are the likely site of antagonist action. To summarize, a schematic of the tentative mechanism of action of losartan and PD123319 in striatum and hypothalamus is illustrated in Fig. 8.

In the hypothalamus, Ang II increases NE release as demonstrated previously as well as in the current study [33]. Further, losartan inhibits electrically-evoked NE release, although the level of inhibition did not reach statistical significance. Taken together, these results suggest that endogenous Ang II augments NE release in the hypothalamus. Further, losartan appears to inhibit the effect of endogenous Ang II and nicotine-stimulated Ang II release, collectively leading to inhibition of nicotine-evoked NE release (Fig. 8, right).

In contrast to *in vitro* binding studies that report losartan IC_{50} values in the nanomolar range [52], higher concentrations of losartan (10 and 30 μM) were required to inhibit the effect of nicotine on [^{3}H]DA and [^{3}H]NE release in the current study. Although, losartan 1 μM decreased nicotine-evoked [^{3}H]NE release from hypothalamic slices, statistical significance for this concentration was not obtained. Differences between the tissue preparations employed in the previously reported binding studies and the current neurotransmitter release study likely contribute to the observed differences in IC_{50} values for losartan. Such differences in IC_{50} values have been reported for other antagonists, including for example *S*-sulpiride, a DA D2 receptor antagonist. *S*-Sulpiride-induced changes in electrically-evoked [^{3}H]DA release from superfused striatal slices was reported at 10-fold greater concentrations than its affinity for DA D2 receptors determined using striatal membrane preparations [53, 54]. Membrane homogenate preparations allow greater access of drug to the receptor sites; whereas in comparison, intact striatal slice preparations restrict drug access to receptors, such that greater drug concentrations are required to penetrate the slice, reach the receptors and inhibit the functional response [54]. Apart from methodological differences, higher

losartan concentrations may be required for inhibiting nicotine-evoked DA and NE release to overcome a putative nicotine-stimulated Ang II release, that further increases DA and NE release in an AT1-dependent manner (Fig. 8).

In contrast to losartan, the selective AT2 antagonist PD123319 did not inhibit nicotine-evoked NE release from hypothalamus, suggesting that AT2 receptors are not involved in this response. Receptor autoradiography studies reveal a low density of AT2 receptors in rat hypothalamus relative to AT1 receptors [55, 56]. Furthermore, *in situ* hybridization in the hypothalamus detected AT1 receptor mRNA, but not AT2 receptor mRNA [57, 58]. Thus, the lack of inhibitory effect of PD123319 on nicotine-evoked NE release in hypothalamus is likely due to the low expression of AT2 receptors in this brain region.

In the striatum, electrical stimulation increases basal Ang II release, suggesting that neuronal stimulation, such as nicotinic receptor activation, may also increase Ang II release. Further, Ang II is shown to increase DA release in striatum *via* activation of AT1 receptors [37]. Thus, losartan may partially inhibit nicotine-evoked DA release *via* inhibition of nicotine-evoked Ang II release. In contrast to nicotine-evoked hypothalamic [^{3}H]NE release, losartan1 μM failed to decrease nicotine-evoked striatal [^{3}H]DA release. Differences in results between brain regions, may, in part, be due to differences in the amount of Ang II released by nicotine, such that greater concentration of losartan is required to attenuate nicotine-evoked striatal [^{3}H]DA release as compared to hypothalamic [^{3}H]NE release.

In contrast to hypothalamus, greater expression of AT2 receptors is observed in striatum [32]. Current results show that at low concentrations (0.01 and 0.1 μM), PD123319 potentiated nicotine-evoked DA release in striatum. The current study also demonstrates that 1% of striatal tissue content of Ang II constituted basal release from striatal slices during each minute of superfusion. Thus, activation of AT2 receptors by endogenous Ang II may suppress nicotine-evoked DA release (Fig. 8 left). Further, AT2 receptor activation counteracts AT1 receptor activation in striatum. Precedence for such counterbalances in the Ang II system comes from studies investigating effects of Ang II in the periphery in which AT2 receptor activation produces systemic vasodilation, counteracting AT1 receptor-mediated vasoconstriction induced by Ang II activation [26]. At high concentrations (1 and 10 μM), PD123119 did not alter nicotine-evoked DA release. Since selective inhibition of AT2 receptors occurs at low PD123319 concentrations, high concentrations of PD123319 may be acting non-selectively to also inhibit AT1 receptors. In support of this interpretation, PD123319 at nM concentrations has been shown to selectively inhibit AT2 receptors expressed in cultured rat mesangial cells, whereas μM concentrations of PD123319 also inhibited AT1B receptors [59]. Further, nonspecific actions of high concentrations of PD123319 at AT1 receptors have been suggested with respect to the peripheral Ang II system [60]. Taken together, PD123319-induced potentiation of the effect of nicotine on DA release in the current study appears to be due to inhibition of AT2 receptors, and the non-selective inhibition of AT1 receptors at high PD123319 concentrations appears to overcome the AT2 effect, such that the potentiation is not observed.

An alternative mechanism that may underlie the effects of losartan and PD123319 on nicotine-evoked DA release includes the presence of non-AT1 and non-AT2 receptors. In rat

striatum and hypothalamus, alternate Ang II binding sites have been identified using membrane binding assays and autoradiography [61, 62]. However, losartan and PD123319 have been reported to lack affinity for these alternate binding sites [61]. Nevertheless, the effects of losartan and PD123319 on nicotine-evoked striatal DA release and hypothalamic NE release may be mediated, in part, by an interaction at non-AT1 and non-AT2 receptors. Alternatively, effects of AT antagonists on DA and NE release may be secondary to effects on blood vessels.

In summary, the current findings suggest that AT1 receptors modulate nicotine-evoked DA and NE release in striatum and hypothalamus. However, in striatum, but not hypothalamus, AT2 receptors also appear to be involved. Importantly, crosstalk between AT1 and AT2 receptors in striatum appears to complicate Ang II modulation of the effect of nicotine on DA release. Importantly, the current results identify Ang II receptors as novel targets for intervention in the rewarding effects and cardiovascular complications of tobacco use.

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Abbreviations

Ang II	angiotensin II
DA	dopamine
MEC	mecamylamine
nAChR	nicotinic acetylcholine receptor
NE	norepinephrine
PD123319	1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid

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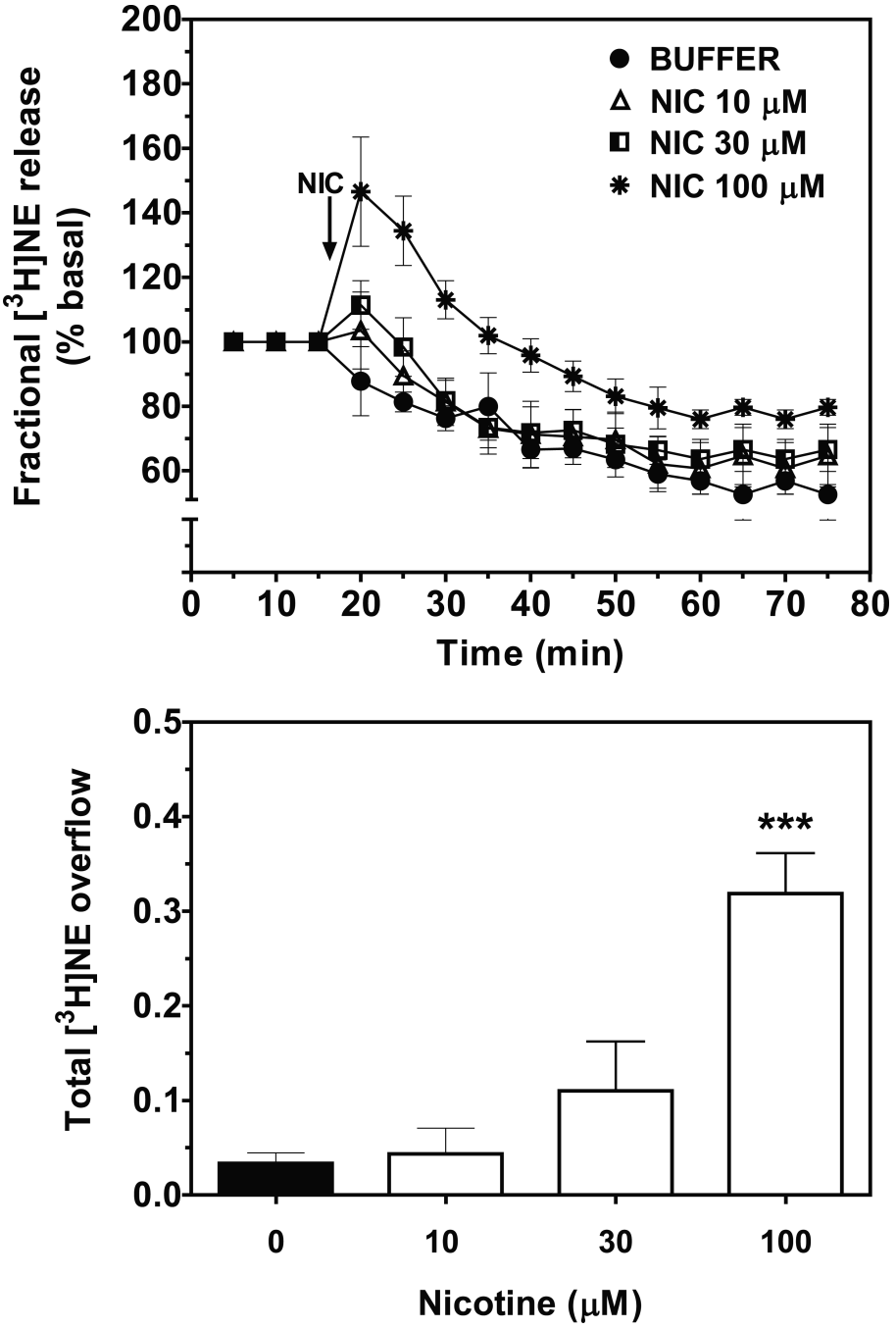

Figure 1.

Nicotine (NIC) stimulates fractional [^{3}H]NE release (top) and total [^{3}H]NE overflow (bottom) from superfused rat hypothalamic slices in a time-and concentration-dependent manner. Arrow indicates the time point at which NIC was added to the superfusion buffer. Data are expressed as mean ± S.E.M. Fractional release data are expressed as a percentage of basal. Basal fractional [^{3}H]NE release was 0.83 ± 0.098% tissue-[^{3}H]. n = 5 rats; ***$p < 0.001$, different from control (0 NIC; buffer only condition).

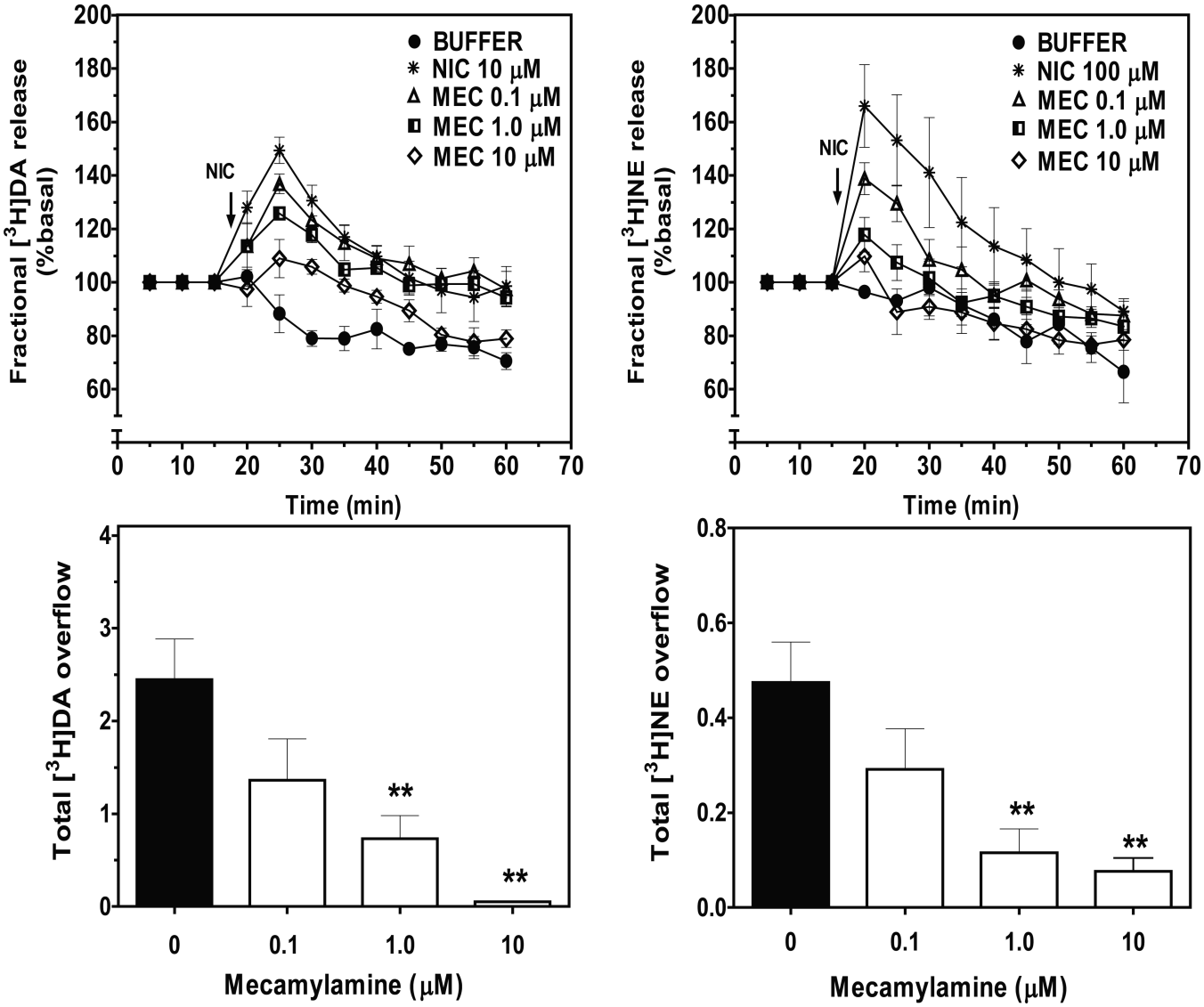

Figure 2.

Mecamylamine (MEC) inhibits NIC-evoked fractional [^{3}H]DA and [^{3}H]NE release (top) and total [^{3}H]DA and [^{3}H]NE overflow (bottom) from superfused rat striatal and hypothalamic slices, respectively. Arrow indicates the time point at which NIC was added to the superfusion buffer. Data are expressed as mean ± S.E.M. Fractional release data are expressed as a percentage of basal. Basal fractional [^{3}H]DA and [^{3}H]NE release were 0.86 ± 0.062 and 0.41 ± 0.034% tissue-[^{3}H], respectively. n = 5 rats; **p < 0.01, indicates different from control (0 MEC; effect of NIC in the absence of MEC).

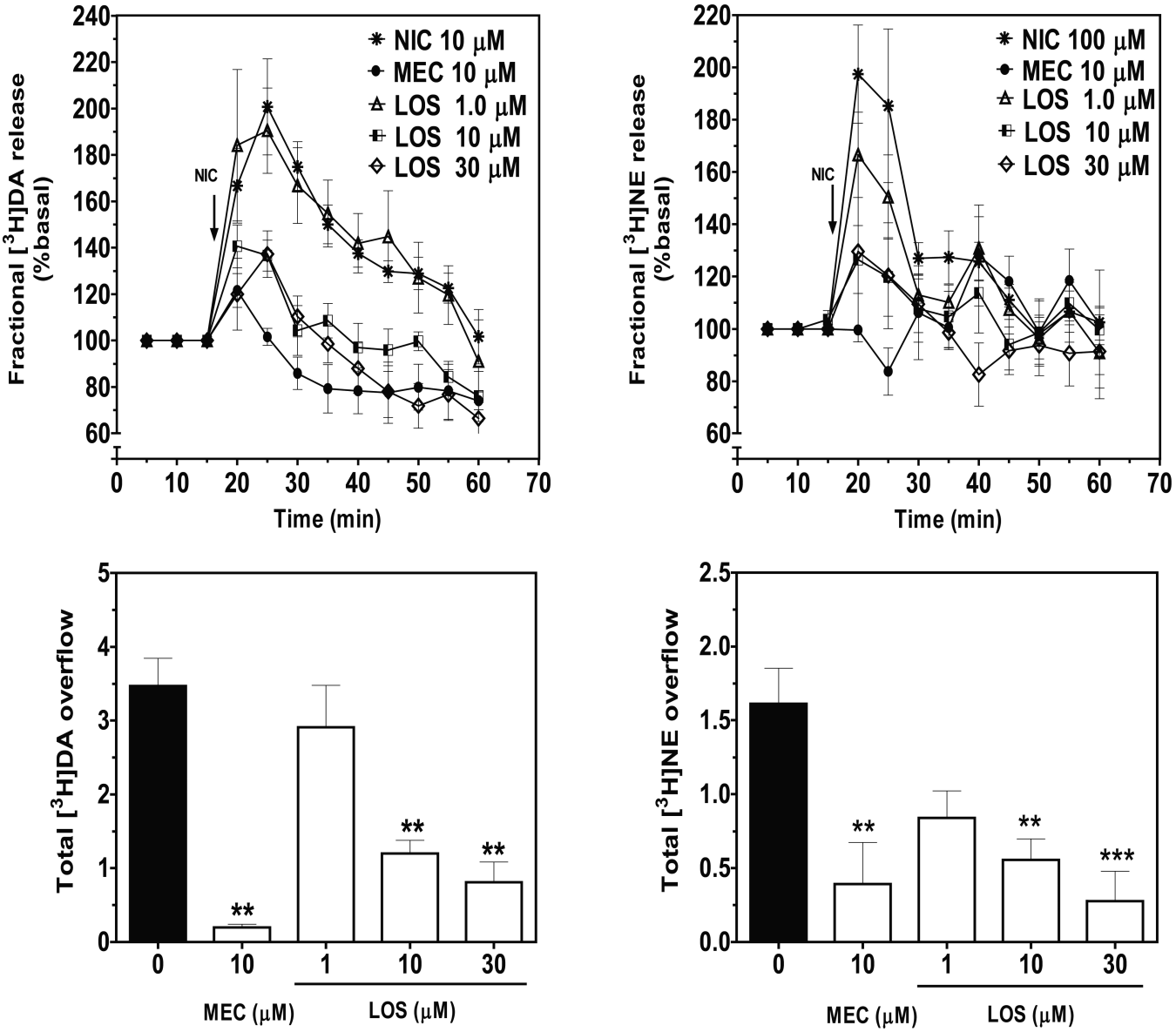

Figure 3.

Losartan (LOS) inhibits fractional NIC-evoked [^{3}H]DA and [^{3}H]NE release (top) and total [^{3}H]DA and [^{3}H]NE overflow (bottom) from rat striatal and hypothalamic slices, respectively. Arrow indicates the time point at which NIC (10 and 100 μM, for [^{3}H]DA and [^{3}H]NE release assays, respectively) was added to the superfusion buffer. Data are expressed as mean ± S.E.M. Fractional release data are expressed as a percentage of basal. Basal values for fractional [^{3}H]DA and [^{3}H]NE release were 0.76 ± 0.053 and 0.39 ± 0.028% tissue-[^{3}H],respectively. n = 6 rats; **p < 0.01, indicates different from control (0 LOS; effect of NIC in the absence of LOS or MEC).

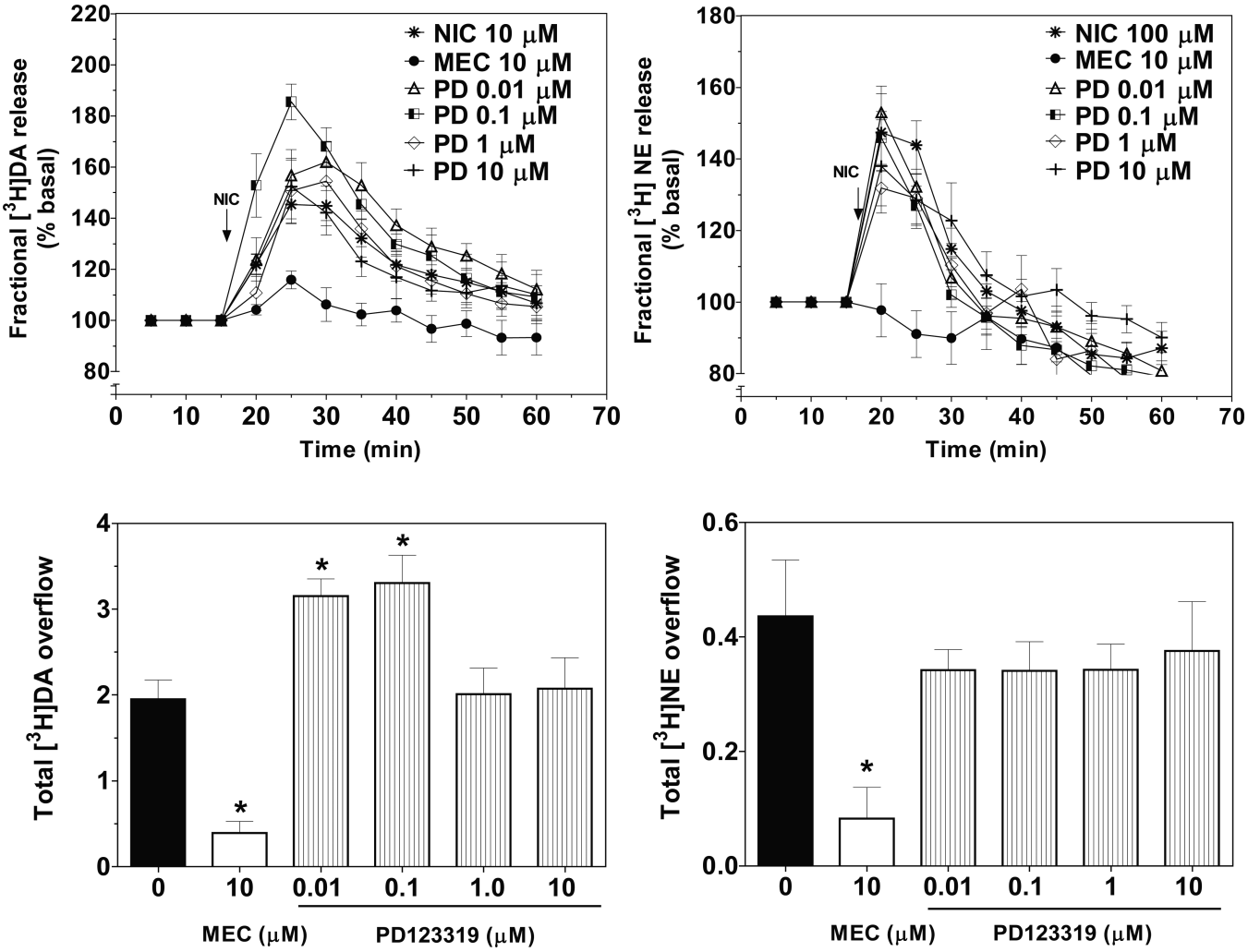

Figure 4.

PD123319 (PD), an AT2 antagonist, does not inhibit NIC-evoked [^{3}H]NE overflow from rat hypothalamic slices, but potentiates nicotine-evoked [^{3}H]DA overflow from rat striatal slices. Time course of PD effects on NIC-evoked fractional [^{3}H]DA (top-left) and fractional [^{3}H]NE (top-right) release. Effects of PD on total [^{3}H]DA (bottom-left) and total [^{3}H]NE (bottom-right) overflow. Arrow indicates the time point at which NIC was added to the superfusion buffer. Data are expressed as mean ± S.E.M. Fractional release data are expressed as a percentage of basal. Basal fractional [^{3}H]DA and [^{3}H]NE release are 0.86 ± 0.019 and 0.38 ± 0.013% tissue-[^{3}H], respectively. n = 6 rats; *p < 0.05, indicates different from NIC. (0 PD; effect of NIC in the absence of PD or MEC).

Figure 5

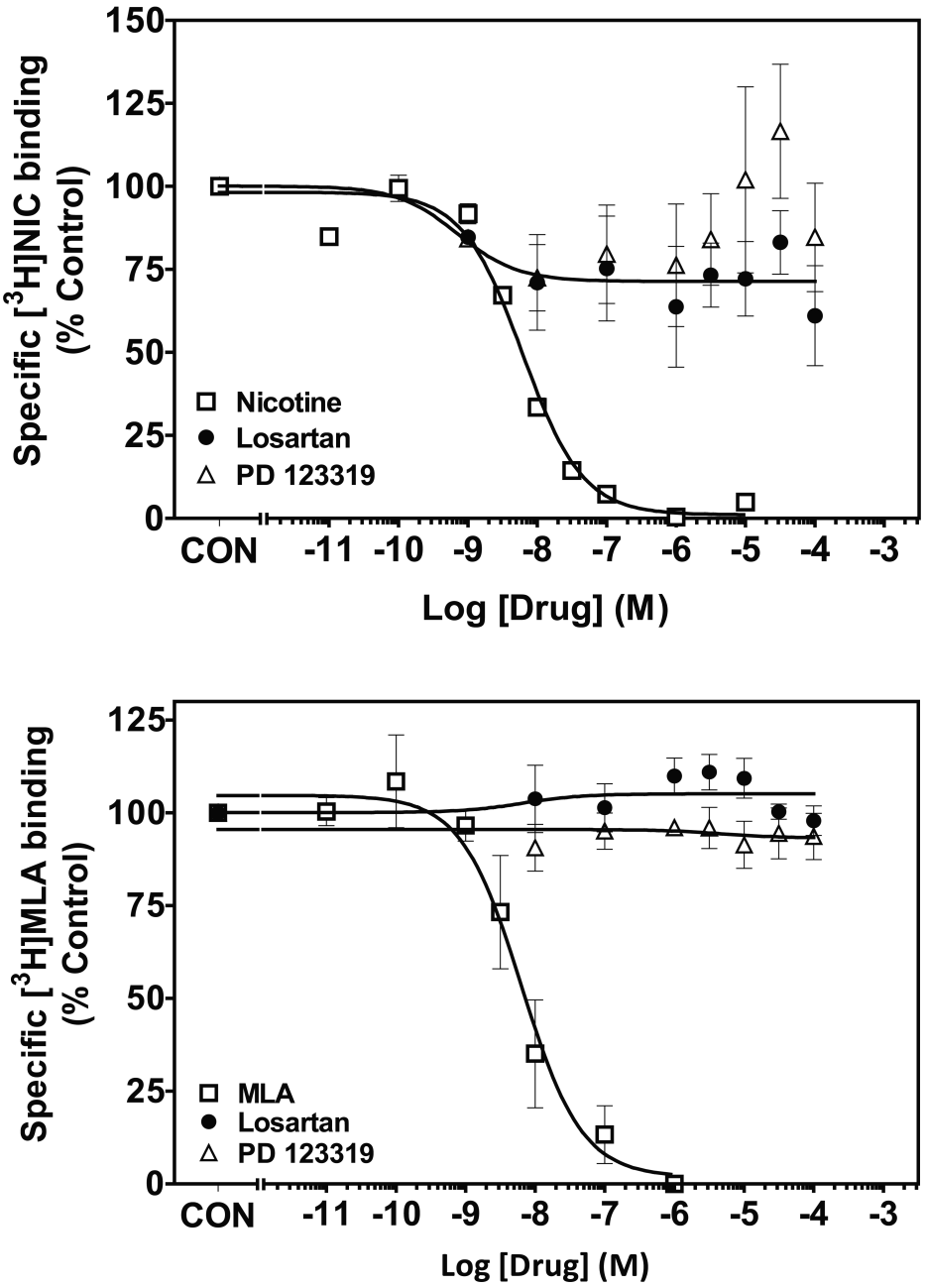

Figure 5.

Losartan and PD123319 concentration-response curves for inhibition of [^{3}H]nicotine (top) and [^{3}H]methyllycaconitine (bottom) binding. Data are expressed as mean ± S.E.M as a percentage of control. Control [^{3}H]nicotine and [^{3}H]MLA binding was (47.9 ± 5.18 and 54.9 ± 3.43 fmol/mg protein, respectively). n = 3-5 rats.

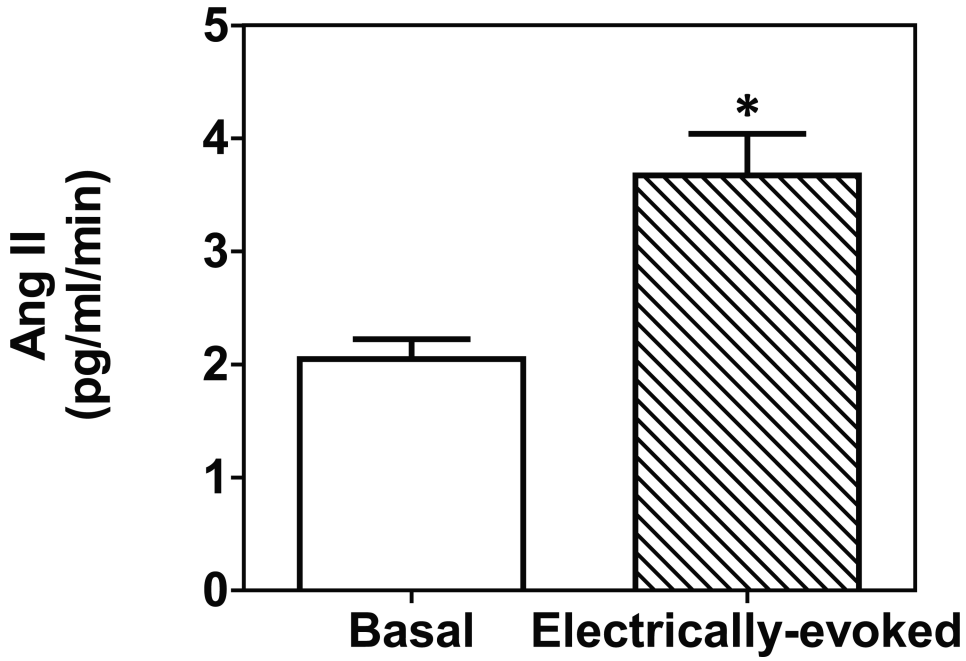

Figure 6.

Electrical stimulation of striatal slices induces Ang II release. Data are expressed as mean ± S.E.M. n = 3 rats; *p < 0.05, indicates different from basal.

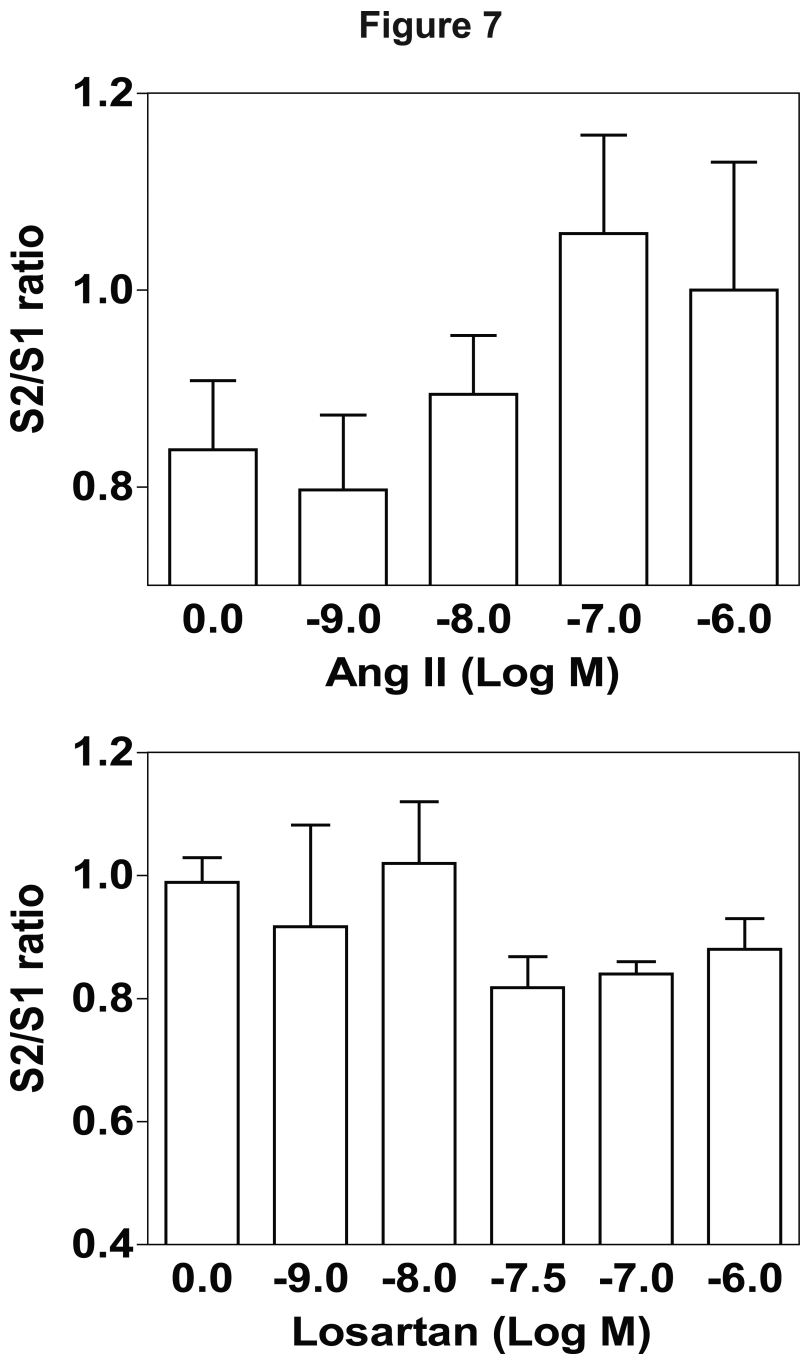

Figure 7.

Ang II evokes [^{3}H]NE release from rat hypothalamic slices (top). Losartan inhibits Ang II-evoked [^{3}H]NE release (bottom). Data are expressed as mean ± S.E.M. n = 5-6 rats; $p < 0.05$, indicates different from control (0).

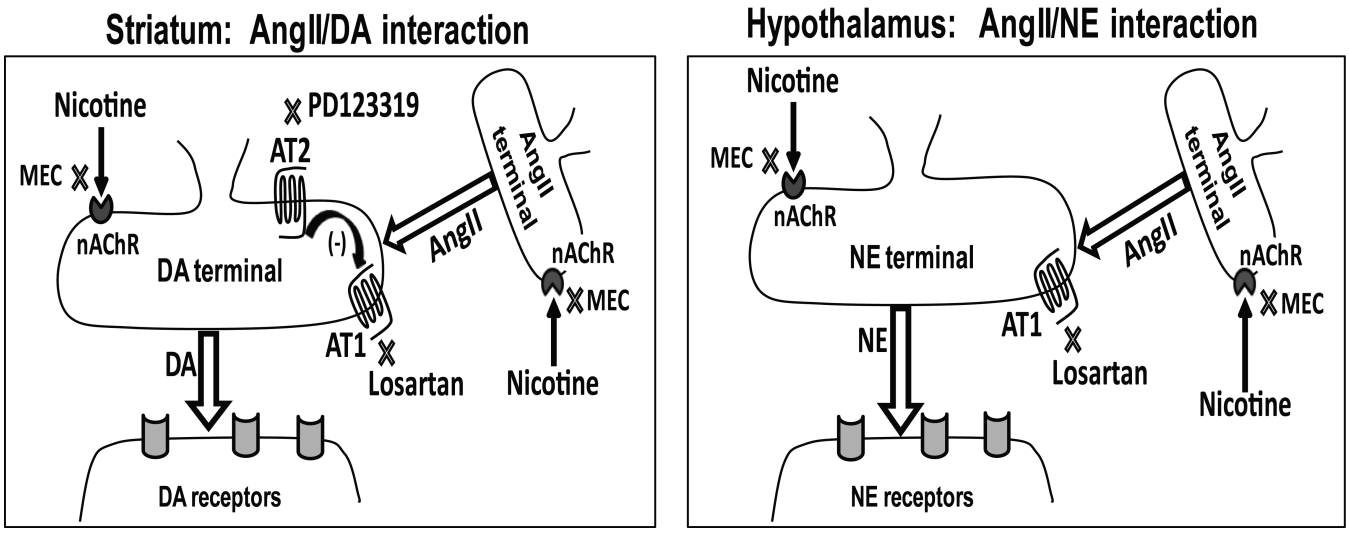

Figure 8.

Model for AT1 and AT2 receptors modulation of nicotine-evoked DA and NE release in striatum and hypothalamus, respectively. In the striatum (left) and hypothalamus (right), nicotine is shown to induce Ang II release *via* activation of nAChRs. Ang II stimulates DA and NE release from striatum and hypothalamus, respectively, in a AT1 receptor-mediated manner. Mecamylamine, a non-selective nAChR antagonist, inhibits nicotine-evoked neurotransmitter release by acting at nAChRs located on DA and NE terminals and/or at nAChRs located on Ang II containing neurons. Losartan inhibits nicotine-evoked neurotransmitter release *via* AT1 receptors located on DA and NE terminals. In striatum, low concentrations of PD123319 specifically inhibit AT2 receptors; thereby abolishing AT2-mediated tonic inhibition of AT1 receptors.

Table 1

Fractional [^{3}H]DA and [^{3}H]NE release from striatal and hypothalamic slices, respectively, in the presence of losartan.

Fractional release	Control	MEC 10 μM	LOS 1 μM	LOS 10 μM	LOS 30 μM	F-statistics[a]
[^{3}H]DA	0.98 ± 0.021	0.97 ± 0.054	0.99 ± 0.097	0.99 ± 0.093	1.22 ± 0.103	F[4, 29] = 1.85
[^{3}H]NE	0.54 ± 0.022	0.47 ± 0.055	0.61 ± 0.060	0.63 ± 0.163	0.47 ± 0.089	F[4, 28] = 0.641

[a] $p>0.05$

Table 2

Fractional [^{3}H]DA and [^{3}H]NE release from striatal and hypothalamic slices, respectively, in the presence of PD123319.

Fractional release	Control	MEC 10 μM	PD 0.01 μM	PD 0.1 μM	PD 1 μM	PD 10 μM	F-statistics[a]
[^{3}H]DA	3.2 ± 0.60	3.6 ± 0.34	3.8 ± 0.90	3.5 ± 0.74	3.5 ± 0.60	2.8 ± 0.29	F[5,46] = 0.31
[^{3}H]NE	2.6 ± 0.22	2.9 ± 0.69	1.9 ± 0.31	2.7 ± 0.55	2.3 ± 0.49	2.7 ± 0.26	F[5,40] = 0.63

[a] $p>0.05$