Imidazoleacetic acid-Ribotide Induces Depression of Synaptic Responses in Hippocampus Through Activation of Imidazoline Receptors

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ABSTRACT

Imidazole-4-acetic acid-ribotide (IAA-RP), an endogenous agonist at imidazoline receptors (I-Rs), is a putative neurotransmitter/regulator in mammalian brain. We studied the effects of IAA-RP on excitatory transmission by performing extracellular and whole-cell recordings at Schaffer collateral-CA1 synapses in rat hippocampal slices. Bath-applied IAA-RP induced a concentration-dependent depression of synaptic transmission that, after washout, returned to baseline within 20 min. Maximal decrease occurred with 10 µM IAA-RP, which reduced the slope of field extracellular postsynaptic potentials (fEPSPs) to 51.2 ±5.7% of baseline at 20 min of exposure. Imidazole-4-acetic acid-riboside (IAA-R; 10 µM), the endogenous dephosphorylated metabolite of IAA-RP, also produced inhibition of fEPSPs. This effect was smaller than that produced by IAA-RP (to 65.9±3.8% of baseline), and occurred after a further 5-8 min delay. The frequency, but not the amplitude, of miniature excitatory postsynaptic currents was decreased, and paired-pulse facilitation (PPF) was increased after application of IAA-RP, suggesting a principally presynaptic site of action. Since IAA-RP also has low affinity for α<sub>2</sub>-adrenergic receptors (α<sub>2</sub>-ARs), we tested synaptic depression induced by IAA-RP in the presence of α<sub>2</sub>-ARs, I<sub>1</sub>-Rs or I<sub>3</sub>-Rs antagonists. The α<sub>2</sub>-AR antagonist rauwolscine (100 nM), which blocked the actions of the α<sub>2</sub>-AR agonist clonidine, did not affect either the IAA-RP-induced synaptic depression or the increase in PPF. In contrast, efaroxan (50 µM), a mixed I<sub>1</sub>-R and α<sub>2</sub>-AR antagonist, abolished the synaptic depression induced by IAA-RP and abolished the related increase in PPF. KU-14R, an I<sub>3</sub>-R antagonist, partially attenuated responses to IAA-RP. Taken together, these data support a role for IAA-RP in modulating synaptic transmission in the hippocampus through activation of I-Rs.
INTRODUCTION

Imidazole-4-acetic acid-ribotide (IAA-RP) is an endogenous agonist at imidazoline receptors (I-Rs) and has been proposed to serve as a modulator of neural activity in various CNS regions (Friedrich et al. 2007b; Martinelli et al. 2007b; Prell et al. 2004). Several lines of evidence suggest that IAA-RP may have an important role in blood pressure regulation (Eglen et al. 1998; Ernsberger et al. 1993; Regunathan and Reis 1996) and may underlie the association between hypertension and diabetes (Bousquet et al. 1984; Chan 1998; Morgan 1999). IAA-RP derives from the conjugation of phosphoribosyl-pyrophosphate to imidazoleacetic acid (IAA) catalyzed by imidazoleacetic acid-phosphoribosyl transferase (IPRT; E.C. 6.3.4.8) via a rare mechanism in mammals in which ATP serves as an energy source rather than as a substrate for the reaction (Crowley 1964). In mammalian brain, IAA appears to derive mainly from the transamination of L-histidine by histidine-pyruvate aminotransferase isoenzyme II (HPAT-II; E.C. 2.6.1.38 (Noguchi et al. 1976; Okuno et al. 1990)); the resultant imidazolepyruvate can be oxidized to produce IAA (Okuno et al. 1998). IAA-RP is dephosphorylated by phosphatases and 5'-nucleotidases to produce imidazole-4-acetic acid-riboside (IAA-R) (Crowley 1964; Prell et al. 2004; Thomas and Prell 1995).

IAA-RP binds with high affinity to I-Rs. The existence of I-Rs in the CNS and in peripheral tissues was initially proposed on the basis of observations that the anti-hypertensive effects of clonidine and related imidazole- and imidazoline-containing drugs were not mediated exclusively by $\alpha_2$-adrenergic receptors ($\alpha_2$-ARs) (Atlas 1991; Bousquet et al. 1984; Karppanen 1977), and therefore involved additional binding sites. Thus far, three I-R subtypes have been recognized pharmacologically and designated I$_{1-3}$-R (Eglen et al. 1998; Head 1999; Morgan 1999). I$_1$-Rs are localized to plasma membranes and have high affinity for a group of anti-hypertensive drugs containing a synthetic imidazoline ring; such drugs include clonidine, moxonidine and rilmenidine (Bousquet et al. 1984; Chan et al. 2005; Ernsberger et al. 1990). I$_2$-Rs, which appear to be mainly allosteric sites on amine oxidases, may be involved in...
modulating behavioral states such as depression, anxiety and feeding behavior (Eglen et al. 1998; Garcia-Sevilla et al. 1990; Holt et al. 2004; Parini et al. 1996). I$_3$-Rs were originally identified in pancreatic beta cells and have been shown to mediate insulin secretion (Morgan 1999); I$_3$-Rs or I$_3$-like receptors may also be involved in central blood pressure regulation (Prell et al. 2004). While the precise molecular identities of the I-Rs have yet to be established fully, one candidate I$_1$-R protein termed Nischarin or IRAS (imidazoline receptor antisera-selected protein) has been cloned from human hippocampus (Ivanov et al. 1998; Piletz et al. 2000; Sun et al. 2007; Wu et al. 2005) and a second potential candidate I$_1$-R belonging to the sphingosine-1-phosphate receptor family has been suggested more recently (Molderings et al. 2007).

The physiologically-active endogenous ligands for CNS I-Rs are thus far uncertain. A number of studies have shown that agmatine, an endogenous ligand at multiple binding sites including I$_1$-Rs (Reis and Regunathan 2000), alters neurotransmission and behavior (for reviews see (Halaris and Piletz 2007; Wu et al. 2008)). IAA-RP stimulates the release of arachidonic acid from PC12 cells, and potentiates glucose-induced insulin release from beta cells in the pancreas (Prell et al. 2004). In addition, IAA-RP undergoes depolarization-induced Ca$^{2+}$-dependent release from synaptosomes, and microinjection of IAA-RP into the blood pressure regulatory region of the medullary reticular formation alters systemic blood pressure (Prell et al. 2004). Moreover, intense IAA-RP immunoreactivity has been localized in cells in various regions of the CNS, including the hippocampal formation (Friedrich et al. 2007a; Friedrich et al. 2007b; Martinelli et al. 2007a; Martinelli et al. 2007b).

The only electrophysiological analyses to date that directly address the synaptic actions of IAA-RP are our preliminary reports demonstrating inhibitory effects on transmission in hippocampal (Bozdagi et al. 2006) and corticostriatal (Artis et al. 2007) pathways. The present study was conducted to examine the effects of IAA-RP and its natural metabolite IAA-R on neurotransmission in the Schaffer collateral-commissural pathway of the hippocampus. This region shows intense IAA-RP immunolabeling and contains a high density of I-R binding sites.
Our data strongly suggest that IAA-RP interacts with I-Rs to inhibit excitatory transmission from Schaffer collaterals to CA1 pyramidal neurons, and further suggest that these effects may be mediated, at least in part, through a presynaptic mechanism.

**MATERIALS AND METHODS**

**Animals**

Four to six-week old outbred male Sprague-Dawley (SD) rats were used as a source of tissue for the electrophysiology experiments. Young (4-6 week) as well as older (8-12 week) SD rats were used for immunocytochemical studies. Animals were housed in an AAALAC-accredited facility and allowed free access to food and water. The Institutional Animal Care and Use Committee of the Mount Sinai School of Medicine approved all experiments.

**Tissue collection and preparation for immunocytochemistry and immunofluorescence**

Rats were placed under deep anesthesia (pentobarbital, 55 mg/kg) and perfused intracardially with phosphate buffered saline (PBS, pH 7.4, 10 mM, 80 ml/min for 1 min) followed by room temperature (RT) fixative (4% paraformaldehyde with 0.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, 80 ml/min) for 1 min, and then the same fixative (10 ml/min) for 25-30 minutes. Brains were harvested and stored in cold PBS with 0.02% NaN₃ as a preservative. Blocks of brain tissue were sectioned (50 µm) coronally by vibrating microtome (Vibratome) and the serial sections were stored in wells of cold PBS containing 0.02% NaN₃ at 4°C.

**Immunocytochemistry procedures**

For immunocytochemical staining with diaminobenzidine (DAB), sections were incubated in blocking buffer (PBS containing 10% heat-inactivated normal goat serum, 0.1% Triton X-100 and 0.02% NaN₃) for 5-16 hr at RT. Buffer was then removed and sections were resuspended in rabbit anti-IAA-RP primary antiserum diluted in blocking buffer. The production, extensive characterization and specificity of this antiserum have been published previously (Friedrich et al. 2007; Martinelli et al. 2007; Prell et al. 2004). After overnight incubation at RT on an orbital
sections were washed five times with PBS over a period of 8 hr, and then resuspended in blocking buffer (without Na$_3$) containing 1-2 µg/ml of anti-rabbit IgG conjugated to horseradish peroxidase (Jackson Immunoresearch). After overnight incubation at RT and five subsequent rinses (1 hr or more each) in PBS, sections were reacted for 5-10 minutes with 0.5 mg/ml of DAB (Sigma) in 0.05 M TRIS buffer (pH 7.6) containing 0.03% H$_2$O$_2$. The reaction was stopped by removing the DAB solution and replacing it with PBS. Sections were mounted from water onto glass slides, dehydrated in graded water-ethanol mixtures (50, 75, 95, 100%), clarified with xylene and coverslipped using DPX (Electron Microscopy Sciences) as mounting medium.

**Immunofluorescence**

Following pre-incubation in blocking buffer (5-16 hrs), sections were incubated overnight in rabbit anti-IAA-RP antiserum (1:600 in blocking buffer) alone or combined with mouse monoclonal anti-synapsin-1 (1/250; BD Pharmingen) or mouse monoclonal anti-MAP-2 (microtubule associated protein; 1/1000; Chemicon). After multiple rinses in PBS, sections were incubated overnight with AlexaFluor®-labeled goat anti-rabbit and (for double labeling experiments) goat anti-mouse IgG secondary antibodies (Invitrogen), each at a concentration of 2-5 µg/ml in blocking buffer. Sections were then washed thoroughly, stained with DAPI (Invitrogen, 1 µg/ml), washed again, and wet-mounted on slides using Prolong (Invitrogen) mounting medium.

**SDS PAGE**

Samples of SD rat brain homogenate (20µg protein/lane) were electrophoresed (200V, 60 min) on a Criterion precast 10% polyacrylamide gel (BioRad, Hercules, CA). After blotting the gel on a PVDF membrane (Immun-Blot, BioRad), the latter was blocked overnight in blocking buffer (PBS with 10% normal goat serum and 0.05% Tween-20), and cut into strips. Individual strips were incubated overnight with primary antibodies diluted in blocking buffer, washed and incubated again overnight with peroxidase-labeled goat anti-rabbit antibodies (1:5000, Jackson
Immunoresearch, West Grove, PA). After washing, the strips were developed with diaminobenzidine (5mg/ml in 0.1M Tris buffer pH: 7.6 with 0.03% H₂O₂). The primary antibodies utilized were rabbit anti-IAA-RP (1: 5000) produced in our laboratory (Prell et al. 2004), rabbit anti-pan-cadherin (1:5000, Sigma, St Louis, MO), and rabbit anti-mouse calretinin (1:5000, LabVision, Freemont CA; species reactivity: mouse, rat and human). The lane stained with rabbit anti-IAARP was identical to the negative control lane, indicating that the anti-IAARP antibody does not bind to rat brain proteins (Fig. 1A).

**Microscopy**

Tissue sections were examined and images collected using a Zeiss Axioplan 2 microscope equipped for structured illumination imaging (ApoTome®). Images were processed and publication images prepared using Adobe Photoshop®. Intensity adjustments were made equally to all parts of each image.

**Slice Preparation and Electrophysiology**

Acute hippocampal slices (350 µm) were prepared from brains of 4-6 week old SD rats for extracellular and intracellular recordings. Throughout all experiments, slices were perfused with Ringer's buffer containing (mM): NaCl, 125; KCl, 2.5; MgSO₄, 1.3; NaH₂PO₄, 1; NaHCO₃, 26.2; CaCl₂, 2.5; glucose, 11. During extracellular recordings (electrode solution: 3 M NaCl), Ringer's buffer was saturated with 95% O₂/5% CO₂ and maintained at 32°C. Brain slices were incubated for 1 hr prior to establishing a baseline of field-EPSPs (fEPSPs). These were evoked by stimulation of the Schaffer collateral-commissural afferents (100 µs pulses every 30 s) with bipolar tungsten electrodes placed into area CA3 (Bozdagi et al. 2000), and with recordings obtained from stratum radiatum or stratum pyramidale in area CA1. Test stimulus intensity was adjusted to obtain fEPSPs with amplitudes that were one-half of the maximal response. The fEPSP initial slope (mV/ms) was determined from the average waveform of four consecutive responses. Reagents were applied to the bath for various durations, which are indicated in the figures. Paired-pulse responses were measured with inter-stimulus intervals of 50 ms, and were
expressed as the ratio of the averaged responses from the second stimulation pulses (FP2) to those from the first (FP1). To compare the membrane input resistance before and after exposure to IAA-RP, constant current pulses (200 ms, -1.0 to +0.5 nA) were passed, and the current-voltage (I-V) values were measured from the averages of four recordings at the steady state of the transients (170 ms after the onset of the pulse). The input resistance was derived from the linear portion of the I-V curve between 0 and -0.5 nA and was measured before and after exposure to reagents.

For whole-cell recordings, coronal brain slices (300 μm) were obtained from 4 week old SD rats and placed in 4°C artificial cerebral spinal fluid (ACSF) containing (in mM): 127 NaCl, 1,25 NaH₂PO₄, 25 NaHCO₃, 2.5 KCl, 25 Glucose, 2 CaCl₂ and 1 MgCl₂ and bubbled with 95% O₂/5% CO₂. The slices were allowed to recover for 30 min at 32°C. The slices were then transferred to a recording chamber and perfused with ACSF in the presence of 0.5 μM tetrodotoxin (TTX) at a rate of 1-2 ml/min at 30-32°C. The recording patch pipettes were filled with (in mM): 120 cesium methane sulfonate, 10 TEA, 5 NaCl, 4 Lidocaine, 10 HEPES, 1.1 EGTA, 4 Mg-ATP and 0.3 Na-GTP. Miniature excitatory postsynaptic currents (mEPSCs) were recorded with an Axon 700B amplifier (Molecular Devices, Palo Alto, CA). Neurons were held at -70 mV throughout the experiment. mEPSCs were analyzed off line with Mini analysis software and statistical significance was determined using the Kolmogorov-Smirnov test.

**Agonist and antagonist compounds.**

All compounds were added to the perfusion buffer to achieve the final concentrations indicated in the figures. Disodium IAA-RP and IAA-R'HCl, previously synthesized in our laboratory (Matulic-Adamic and Watanabe 1991; Prell et al. 2004), were used at final concentrations also specified in the figure legends. Efaroxan, a combined I₁-R antagonist/I₃-R agonist (Chan and Morgan 1990), rauwolscine, an α₂-AR antagonist, and bicuculline
methiodide, a GABA\textsubscript{A}-R antagonist, were purchased from Sigma. KU-14R, an I\textsubscript{3}-R antagonist (Chan et al. 1997), was a gift from Dr. Noel Morgan (Peninsula Medical School, Plymouth, UK).

**Statistics**

Throughout this report, data are expressed as mean ± SD. Statistical analyses were performed using either a paired t-test or ANOVA (where appropriate), with \( p<0.05 \) considered statistically significant.

**RESULTS**

**IAA-RP is present in hippocampal pyramidal neurons.**

IAA-RP immunoreactivity is present in both the hippocampal and dentate gyri. Within the hippocampus proper, IAA-RP immunolabeling is prominent in the cell bodies of pyramidal neurons (Fig. 1B). In addition, both the apical and basal dendrites of these neurons are immunostained, as confirmed by labeling with an antibody against a microtubule-associated protein (Fig. 1C, D). The IAA-RP-labeled dendrites are tightly ensheathed by synapsin-I-related immunoreactivity (data not shown), verifying the dense innervation of IAA-RP-positive pyramidal cell dendrites.

**IAA-RP and its metabolite, IAA-R, suppress synaptic transmission of the Schaffer collateral-commissural afferents in rat hippocampal slices.**

In order to determine whether IAA-RP has a direct effect on synaptic transmission in the hippocampus, CA1 stratum radiatum field potentials were recorded from acute slices in response to CA3 Schaffer collateral-commissural afferent stimulation. Bath application of IAA-RP for 20 min induced a reversible and concentration-dependent depression of synaptic transmission (Fig. 2A); fEPSP slopes were reduced to 51.2 ± 5.7% of baseline with 10 \( \mu \text{M} \) IAA-RP (\( p<0.01 \) vs baseline, \( n=12 \)). Recovery of the response began immediately at the start of IAA-RP washout, and returned to baseline within approximately 20 min (Fig. 2A). Extending the duration of bath application of IAA-RP up to 35 min (data not shown) did not induce further
inhibition of synaptic transmission (fEPSP slope= 54 ± 3% of baseline, n=3, p=0.6 compared to 20 min). We also tested the effect of the IAA-RP dephosphorylated metabolite IAA-R, which also binds to I-Rs although with lower affinity than that of IAA-RP (Prell et al. 2004). IAA-R (10 µM) likewise induced a decrease in the slope of fEPSPs (Fig. 2B, C). However, IAA-R at equal concentrations elicited a less marked suppression (reduction to 65.9 ± 3.8% of baseline at 20 min of application), and its action started after a delay of approximately 5 min relative to that of IAA-RP. The time-course for return to baseline after IAA-R washout was similar to that of IAA-RP (Fig. 2B). The concentration-dependent depression of synaptic transmission measured from 30 nM to 100 µM at the end of each IAA-RP application had an IC50 of approximately 1.6 µM, which was significantly different from that of IAA-RP (2 µM, p=0.04, t-test) (Fig. 2C).

**IAA-RP–mediated synaptic depression appears to be I-R-dependent.**

Since IAA-RP binds to I-Rs and α2-ARs, albeit with very different affinities (Prell et al. 2004), we tested whether the synaptic depression induced by IAA-RP (10 µM) was affected by blockers of I-Rs or α2-ARs. We found that rauwolscine (100 nM), a potent and highly selective α2-AR antagonist, did not significantly influence IAA-RP-induced synaptic depression (49.4 ± 2.8% of baseline at 20 min of application, Fig. 3A), although it completely blocked synaptic depression induced by the α2-agonist clonidine (10 µM, Fig. 3B). In contrast, efaroxan (50 µM), which is a combined I1-R and α2-AR antagonist, nearly abolished the synaptic depression induced by IAA-RP (98.8 ± 1.8% of the baseline at 20 min of application, Fig. 3C). The concomitant application of efaroxan (50 µM) and rauwolscine (100 nM) blocked the IAA-RP effect in a manner that was indistinguishable from that produced by efaroxan alone (data not shown). In the presence of rauwolscine (100 nM) and the I3-R antagonist KU-14R (100 nM), the synaptic depression produced by 10 µM IAA-RP was inhibited by approximately 30% with respect to the maximal IAA-RP response (Fig. 3D), suggesting the involvement of an I3-R-mediated effect.
IAA-RP-induced synaptic depression is unlikely to be mediated by modulation of GABAergic transmission or intrinsic membrane properties.

Since fast inhibitory neurotransmission in the hippocampus is mediated by GABA_A receptors (Scimemi et al. 2005; Semyanov et al. 2004), we assessed whether the synaptic depression induced by IAA-RP was produced by modulation of GABAergic inhibition. Bath application of the selective GABA_A receptor antagonist bicuculline methiodide (10 μM) led to a small but significant increase in the slope of the fEPSPs to 106 ± 3% of baseline (n=4, p=0.002, Fig. 4A). However, once the slope of the fEPSPs stabilized, addition of IAA-RP (10 μM) reduced the fEPSP amplitude to a value (54 ± 5%) similar to that observed in absence of bicuculline (Fig. 4A, n=4, p=0.6 relative to Fig. 2A). This indicates that GABA_A receptors are not involved in the rate-limiting process of IAA-RP-induced synaptic depression.

The depressive effects of exogenous IAA-RP are not likely to reflect changes in intrinsic membrane properties or excitability of the CA1 pyramidal neurons since intracellular recordings prior to and following washout of bath-applied 10 μM IAA-RP showed no changes in resting membrane potential (-67.0 ± 0.2 vs -66.0 ± 0.3 before and 20 minutes after IAA-RP application, respectively; n=12, Student’s t-test, p=0.28, Fig. 4B, lower histogram). Similarly, IAA-RP had no effect on the mean input resistance (controls: 37.6± 2.4 MΩ, 39.3 ± 2.8 MΩ in the presence of 10 μM IAA-RP, n=6, p=0.49, Fig. 4B, upper histogram). The input-output function of these cells (n=6, Fig. 4C) decreased during bath application of IAA-RP, but returned to control levels by 20 minutes after washout, indicating that IAA-RP application did not produce lasting changes in synaptic transmission. Presynaptic fiber volley amplitude corresponding to a given stimulus intensity was unaffected with IAA-RP treatment, as shown in the I/O function plotted as fiber volley/stimulus intensity, indicating that the number of stimulated afferents was not affected (Fig. 4D).

IAA-RP-mediated depression of synaptic transmission involves a presynaptic mechanism.
We next asked whether the depression in synaptic transmission was caused by alterations in presynaptic and/or postsynaptic parameters. We performed whole-cell patch-clamp recordings in hippocampal slices and monitored spontaneous mEPSCs in the presence of tetrodotoxin (TTX). The amplitude of mEPSCs from CA1 pyramidal neurons did not change after IAA-RP application but the frequency of mEPSCs was significantly lower (n=8, p<0.05, Fig 5 A, B), suggesting a potential presynaptic alteration following IAA-RP application. Paired-pulse facilitation (PPF), another measure of presynaptic function (Katz and Miledi 1968; Zucker and Regehr 2002), was used to examine this further. PPF was calculated as the ratio of the slope of the second to that of the first fEPSPs (FP₂/FP₁). At 50 ms ISI (see Methods), PPF increased from 1.31 ± 0.06 to 1.44 ± 0.07 (n=11, p<0.01; Fig. 5C) in the presence of 10 µM IAA-RP, consistent with a presynaptic effect. The increase in PPF was completely blocked by the combined I₁-R and α₂-AR antagonist efaroxan (FP₂/FP₁ = 1.31 ± 0.08 and 1.32 ± 0.07 before and after exposure to IAA-RP, respectively; n=6, p=0.23 relative to control), but was not prevented by application of the α₂-AR antagonist rauwolscine (100 nM) (FP₂/FP₁ = 1.31 ± 0.08 and 1.43 ± 0.08, before and after exposure to IAA-RP, respectively; n=6, p=0.001). These data indicate that the IAA-RP-mediated modulation of PPF involves I₁-Rs, but not α₂-ARs.

DISCUSSION

The present study provides the first demonstration that IAA-RP suppresses excitatory synaptic transmission at Schaffer collateral-CA1 pyramidal neuron synapses. This suppression appears to be attributable primarily to activation of I-Rs and may include an I₁-R-mediated presynaptic effect. Mechanistically, a decrease in mEPSC frequency, but not amplitude, and an increase in the paired-pulse ratio with IAA-RP application are both consistent with a presynaptic locus involving diminished release.

Our conclusion that IAA-RP is an endogenous ligand at I-Rs is based primarily on previous research from our laboratory showing that IAA-RP is an agonist in I-R model systems and that I-
R-related antagonists block its effects (Prell et al. 2004). However, it is also possible that IAA-RP interacts with other receptors, notably $\alpha_2$-ARs, and in theory some of our observed effects could be attributable to $\alpha_2$-AR activation rather than I-R mediated effects alone (although IAA-RP has a thousand-fold greater affinity for I-Rs than for $\alpha_2$-ARs (Prell et al. 2004)). We addressed this issue by comparing the synaptic inhibition resulting from bath application of IAA-RP alone with that occurring from combined application of IAA-RP and antagonists to both classes of receptors. Efaroxan, a mixed I$_1$-R/$\alpha_2$-AR antagonist with forty-fold selectivity for I$_1$-Rs over $\alpha_2$-ARs (Head and Mayorov 2006), completely blocked the IAA-RP-mediated inhibition of synaptic transmission in CA1. In contrast, the depression of synaptic transmission was entirely unaffected by the very potent and highly selective $\alpha_2$-AR blocker rauwolscine, which is an alkaloid compound lacking imidazole/imidazoline rings and which exerts negligible effects on I-Rs (Ernsberger and Haxhiu 1997). To determine if the inability of rauwolscine to alter the IAA-RP effect in CA1 was due to unrecognized experimental artifacts, we also tested it in control experiments using the prototypical $\alpha_2$-AR agonist clonidine. Although clonidine successfully attenuated fEPSPs in our preparation, these effects were abolished by rauwolscine, thus confirming that this antagonist functioned as an effective $\alpha_2$-AR blocker in our experimental paradigm. Hence, these results support the view that IAA-RP-induced suppression does not occur via the stimulation of $\alpha_2$-ARs.

It is also possible that, in addition to I$_1$-Rs, IAA-RP interacts with a novel non-I$_1$-R, non-I$_2$-R imidazoline receptor in brain (Prell et al. 2004). This hypothesis was suggested by the high affinity radioligand binding profile of IAA-RP in brain as compared with its lower affinity in peripheral tissues and by the seemingly paradoxical rise in blood pressure observed following IAA-RP microinjections into rat brainstem, where I$_1$-R agonist activity was expected to lower blood pressure. Such observations are quite distinct from those obtained in I$_1$-R subtype models (Ernsberger and Haxhiu 1997), and led us to propose that IAA-RP in brain may also interact
with an I-R subtype similar to the peripheral \( I_3 \)-Rs described in the pancreas (Chan and Morgan 1990). To evaluate that proposal within the context of synaptic transmission in the hippocampus, we utilized the selective \( I_3 \)-R antagonist KU-14R in the present study. This antagonist partially suppressed the IAA-RP-mediated inhibition effect, suggesting that \( I_3 \)-like receptors may also contribute to the IAA-RP synaptic effects. \( I_3 \)-Rs were originally described in pancreatic beta cells (Chan and Morgan 1990); our previous studies showed that IAA-RP is a potent insulin secretagogue in human and rat pancreatic beta cells (human IC\(_{50}\): 3 nM; rat IC\(_{50}\): 30 nM) and that its actions are blocked by KU-14R (Prell et al. 2004). Based on these data, we presently attribute the effects of IAA-RP on synaptic transmission in the Schaffer collateral-commissural pathway to the actions of \( I_1 \)-R and \( I_3 \)-R or an \( I_3 \)-R -like receptor. Our current hypothesis is that the rate-limiting step of the IAA-RP effect is driven mainly by \( I_1 \)-Rs, while \( I_3 \)-Rs may be involved in a non-rate-limiting process.

The conclusion that IAA-RP acts via I-Rs is bolstered by three additional experiments performed in the hippocampal slices. Since suppression of synaptic transmission could also result from an alteration in GABAergic transmission, IAA-RP was applied to the bath in the presence of bicuculline, a potent antagonist of ionotropic GABA\(_A\) receptors. Bicuculline did not affect the suppression of synaptic transmission mediated by IAA-RP, indicating that changes in fast GABAergic transmission are unlikely to be directly responsible for the IAA-RP effects. In addition, our findings suggest that changes in intrinsic membrane properties and excitability of the CA1 pyramidal neurons do not appear to mediate the depressive effects of IAA-RP. Thirdly, since the dephosphorylated metabolite of IAA-RP, IAA-R, binds to I-Rs, albeit with lower affinity (Prell et al. 2004), we investigated its effects on fEPSP. Although IAA-R also depresses fEPSP, this depression is less pronounced than that produced by IAA-RP at the same concentration, and the onset of the inhibition is consistently delayed with respect to that observed for IAA-RP. Although the mechanisms dictating this delay remain unclear, one hypothesis is that IAA-RP is the more effective agonist, and the delayed effect may reflect the time needed for the
processing (e.g. local uptake, diffusion and phosphorylation) of IAA-R to generate IAA-RP. Exploration of this issue will require additional studies.

We initially proposed that IAA-RP functions as a modulator of neural transmission because it is released in a Ca\(^{2+}\)-dependent manner from synaptosomal preparations following depolarization, it is present within neurons of brainstem regions involved in the regulation of sympathetic output, and it alters blood pressure when micro-injected into the rostral ventrolateral medulla (RVLM), a brainstem center involved in the regulation of sympathetic outflow and cardiovascular functions (Prell et al. 2004). Subsequent immunocytochemical mapping studies localized IAA-RP in neurons of numerous other brain regions, suggesting that its putative modulatory role is not limited to the brainstem (Friedrich et al. 2007). In fact, IAA-RP immunoreactivity is prominent in both CA3 and CA1 pyramidal cells, and high densities of I-R binding sites are present in the hippocampus (De Vos et al. 1994; Piletz et al. 2000; Ruggiero et al. 1998). The presence of IAA-RP in dendrites of CA1 cells raises the possibility that dendritic IAA-RP, if released (Bergquist and Ludwig 2008), could act presynaptically on the contacts formed by the Schaffer collaterals. However, the present data do not exclude the alternative or additional possibility that IAA-RP is present within, and is released from, the Schaffer collateral endings themselves.

Indeed, the results of our experiments examining the frequency and amplitude of mEPSCs and PPF in the presence or absence of IAA-RP, efaroxan and/or rauwolscine suggest that the synaptic depression of IAA-RP may be mediated, at least in part, through a presynaptic mechanism. Generally, changes in frequency are interpreted as a presynaptic mechanism involving altered release, while changes in amplitude are interpreted as a postsynaptic mechanism involving changes in glutamate receptor number or signaling. PPF is an enhancement in the amplitude of an EPSP elicited by the second of a pair of closely spaced (<1 sec) stimuli. The increase in amplitude of the second response is thought to result from the increased probability of transmitter release due to the additive effects of calcium remaining in
the presynaptic cytosol after the first stimulus of the pair, and of calcium inflow into the terminal caused by the second stimulus (Katz and Miledi 1968; Zucker and Regehr 2002). In the present study, we observed a significant decrease in mEPSC frequency and an increase in PPF in the presence of IAA-RP. This could reflect altered presynaptic calcium sequestration in response to IAA-RP signaling, or decreased transmitter release induced by the first stimulus that would in turn increase the store of transmitter available for release by the second stimulus.

Studies on the effects of synthetic imidazoline agonists acting in the reticular formation on sympathetic outflow and cardiovascular function have also suggested that I-Rs are located presynaptically (Chan et al. 2005; Head 1999), and an electrophysiological study in acute slices of rat striatum has provided evidence that presynaptic I1-Rs are involved in the inhibition of GABA_A-mediated inhibitory postsynaptic currents (IPSCs) by the imidazoline drug moxonidine (Tanabe et al. 2006). Moreover, we have presented preliminary data showing that IAA-RP has similar inhibitory effects on IPSCs in striatal slices (Artis et al. 2007). Nonetheless, postsynaptic effects are also likely to contribute to shaping the inhibitory synaptic response to IAA-RP obtained in the acute hippocampal slices, and additional studies will be necessary to identify the mechanisms underlying these effects.

The mechanisms of the fEPSP depression and the changes in mEPSC frequency and paired-pulse ratio elicited by IAA-RP are likely to be complex and may involve multiple receptors. Two potential candidates for I1-Rs are IRAS (Piletz et al. 1999), the homologue of human Nischarin (Piletz et al. 2003), an integrin-binding cytosolic protein involved in cytoskeletal structure and organization (Alahari et al. 2004), and the S1P1/S1P3 (Molderings et al. 2007) receptors for phosphorylated sphingosine, an autocrine and paracrine mediator of multiple cell functions (Alvarez et al. 2007). Both of these receptors have been shown to be present in hippocampus (Toman and Spiegel 2002) and may be involved in mediating the IAA-RP effects. In this context, it is interesting to note a recent report showing that activation of
S1P1 receptors leads to the depression of spontaneous excitatory postsynaptic currents in cultured cortical pyramidal neurons (Sim-Selley et al. 2009).

Functionally, several lines of evidence suggest that IAA-RP may have an important and widespread role in blood pressure regulation, and more generally in sympathetic drive: i) IAA-RP displaces clonidine from its binding sites in membranes of the RVLM, a region that is rich in IRs and α2ARs and is an important site of action of anti-hypertensive (imidazoline-containing) agents such as clonidine, moxonidine and rilmenidine (Eglen et al. 1998; Ernsberger et al. 1993; Regunathan and Reis 1996); ii) IAA-RP has been visualized by immunocytochemistry in neurons, processes and axon terminals in the RVLM (Friedrich et al. 2007; Prell et al. 2004); and iii) microinjections of IAA-RP into the RVLM of rats produced a transient hypertensive response (Prell et al. 2004). We have previously proposed that IAA-RP may serve different roles in forebrain and mid/hindbrain (Friedrich et al. 2007; Martinelli et al. 2007). In the forebrain, ribotide might exert a homeostatic modulatory influence on neuronal activity, while in more caudal regions IAA-RP may have a more direct neurotransmitter/modulator role. In particular, since injections of IAA-RP into the RVLM alter blood pressure, and since IAA-RP immunoreactivity is present in all of the major autonomic cell groups of the brainstem, we surmise that the ribotide is an endogenous regulator of general sympathetic drive, and in particular systemic blood pressure, through its actions at IRs and possibly α2ARs. Moreover, since IAA-RP stimulates the release of both insulin and arachidonic acid, and IAA-R is present in plasma and urine (see (Prell et al. 2004)), IAA-RP may also exert a hormone-like function on peripheral tissues. Taken together, this suggests that IAA-RP could putatively serve as a neurochemical linking the disorders of diabetes and hypertension.

Lastly, a recent biochemical study in hippocampal slices (Dahmani et al. 2008) addressing the expression of pERK1 and pERK2, two kinases involved in intracellular signaling pathways related to synaptic plasticity and long-term potentiation (Roux and Blenis 2004), provides a more general context for our observations on I1-Rs. Exposure of hippocampal slices harvested
from rats as well as $\alpha_2$-AR-knockout mice to dexmedetomidine (a synthetic agonist with affinity for $\alpha_2$-ARs and I-Rs) increased the expression of both pERK1 and pERK2 via an I$_1$-R-dependent mechanism (Dahmani et al. 2008). Since the IAA-RP-mediated depression of fEPSPs is likely to be due to a decrease in glutamate release from the Schaffer collaterals, and since glutamate-mediated synaptic plasticity is essential for learning and memory (Bliss and Collingridge 1993; Dingledine et al. 1999; Malenka and Bear 2004; Morris 1989), our data more generally suggest that IAA-RP may act presynaptically at I-Rs to modulate the release of neurotransmitters critical for synaptic plasticity, learning and memory formation.
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**FIGURE LEGENDS**

**Figure 1.** IAA-RP immunoreactivity. **A.** Immunoblots of rat brain homogenate stained with rabbit anti-IAARP, left lane), rabbit anti-pan cadherin (left-center lane; positive control 1), rabbit anti-calretinin (right-center lane; positive control 2) and with peroxidase labeled goat anti-rabbit antibodies only (right lane; negative control). The lane stained with rabbit anti-IAARP was identical to the negative control lane, indicating that the anti-IAARP antibody does not bind to rat brain proteins. **B:** A maximum intensity projection from a confocal image stack of the CA3 hippocampal region immunostained for IAA-RP, with MAP-2 co-localization used to identify neuronal dendrites. DAPI nuclear counterstain is shown in blue. The CA3 pyramidal cell perikarya are strongly immunoreactive for IAA-RP, whereas the intense MAP-2 staining obscures the IAA-RP signal present in most dendritic profiles. **C, D:** The individual channels of a single image plane from the z-stack shown in panel C are enlarged to illustrate the co-localization of MAP-2 (C) in IAA-RP (D)-immunolabeled dendrites (arrows). Scale bars: A: 200 µm; B, C: 20 µm.

**Figure 2.** IAA-RP and IAA-R depress CA1 stratum radiatum field potential amplitude in a concentration-dependent manner. **A:** Hippocampal slices (n=12) were treated with IAA-RP (0.1, 1 and 10 µM) and mean ± SD fEPSP slope was plotted. The horizontal bar indicates the duration of IAA-RP application after achieving a stable baseline for at least one hour. Inset: Representative traces before (arrow) and 20 min after application of 10 µM IAA-RP. Calibration: 10 ms, 0.5 mV. **B:** Mean ± SD fEPSP slope was determined after application of 10 µM IAA-RP (closed circles) or 10 µM IAA-R (open circles). IAA-R-induced depression started with an approximately 5 min delay relative to that of IAA-RP. **C:** dose–response relationship for the depression of field potential amplitude by IAA-RP (closed circles) and IAA-R (open circles).

**Figure 3.** IAA-RP depresses excitatory synaptic transmission in the CA1 region through a
mechanism that is dependent on I-Rs, but not $\alpha_2$-ARs. In all panels, the lower horizontal bars designate the duration of antagonist applications and the upper horizontal bars represent the duration of IAA-RP application. **Panel A:** hippocampal slices were pre-incubated with the $\alpha_2$-AR antagonist rauwolscine (100 nM) for 20 min before starting the IAA-RP perfusion. In the presence of rauwolscine, the 10 µM IAA-RP-induced depression was not different from that of IAA-RP alone. **Panel B:** Clonidine (10 µM) suppressed field EPSP slope measurements in the hippocampal slices (closed circles). However, this clonidine-induced synaptic depression was abolished by 100 nM rauwolscine (open circles). **Panel C:** pretreatment of the hippocampal slices with efaroxan (50 µM; open circles) profoundly reduced the IAA-RP-induced synaptic depression. **Panel D:** The $I_3$-R antagonist KU-14R (100 nM; open circles) inhibited the synaptic depression produced by 10 µM IAA-RP.

**Figure 4.** IAA-RP does not alter intrinsic membrane properties or hippocampal excitability via a GABA$_A$ receptor-mediated mechanism. **Panel A:** Mean data showing that the depression of field potentials by IAA-RP (10 µM; closed circles) was not prevented in the presence of the GABA$_A$ receptor antagonist bicuculline methiodide (BIC, 10 µM; n=4, open circles). The horizontal bars indicate the durations of IAA-RP and BIC application. **Panel B:** All input resistance (IR; upper panel) and resting membrane potential (RMP; lower panel) measurements shown are mean ± SD. **Panel C:** Input-output curves plotting stimulus strength against fEPSP slope are shown in control slices (open circles), after 20 min of IAA-RP application (closed circles), and 20 min following washout of IAA-RP (open triangles) (n=12). The inset shows representative traces for the given stimulus intensities in the input-output graph. Scale: 10 ms, 0.5 mV. **Panel D:** Pooled data (mean ± SD) showing the relationship between stimulus intensity and fiber volley in control slices (open circles), 20 min of IAA-RP application (closed circles), and following washout of IAA-RP (open triangles).
**Figure 5.** IAA-RP decreases the frequency, but does not change the amplitude, of mEPSCs, and also increases paired-pulse facilitation in the Schaffer collateral-commissural pathway.

**Panel A:** Sample traces of mEPSCs from the baseline, 20 min after IAA-RP perfusion and 20 min after washout in a hippocampal neuron in the presence of 0.5 µM TTX. Scale bars: 10 pA and 1 sec. **Panel B:** IAA-RP reversibly decreased the frequency but not the amplitude of mEPSCs in the hippocampus CA1 pyramidal neurons. Left panel shows the cumulative distribution plots of mEPSC inter-event intervals and the right panel shows the amplitude before, 20 min after and rinse of IAA-RP. IAA-RP reversibly shifted the frequency distribution to longer inter-event intervals ($P < 0.05$, $n = 8$, Kolmogorov-Smirnov test) but had no effect on the amplitude distribution. **Panel C:** Mean data for the IAA-RP effect on FP2/FP1 ratio ($n = 11$, $p<0.01$). The IAA-RP-induced increase in PPF (black bar) relative to control (white bar) was completely blocked by efaroxan (50 µM; light gray bar), but was not prevented by rauwolscine (100 nM; dark gray bar). Inset: Representative traces for PPF (50 ms IPI) for control (black) and at 20 min after IAA-RP application (red), with traces normalized to the first response for comparison.
Figure A: Graph showing the depression of fEPSP slope (% vs. time (min)).

Figure B: Graph comparing Field EPSP (%) at different times for 10 μM IAA-RP and 10 μM IAA-R.

Figure C: Graph plotting Depression of fEPSP slope (%) against Log (μM).