Cellular/Molecular

Presynaptic I_1-Imidazoline Receptors Reduce GABAergic Synaptic Transmission in Striatal Medium Spiny Neurons

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Imidazoline receptors are expressed widely in the CNS. In the present study, whole-cell patch-clamp recordings were made from medium spiny neurons in dorsal striatum slices from the rat brain, and the roles of I_1-imidazoline receptors in the modulation of synaptic transmission were studied. Moxonidine, an I_1-imidazoline receptor agonist, decreased the $GABA_A$ receptor-mediated IPSCs in a concentration-dependent manner. However, glutamate-mediated EPSCs were hardly affected. The depression of IPSCs by moxonidine was antagonized by either idazoxan or efaroxan, which are both imidazoline receptor antagonists containing an imidazoline moiety. In contrast, yohimbine and SKF86466 (6-chloro-2,3,4,5-tetrahydro-3-methyl-1*H*-3-benzazepine), which are α2-adrenergic receptor antagonists with no affinity for imidazoline receptors, did not affect the moxonidine-induced inhibition of IPSCs. Moxonidine increased the paired-pulse ratio and reduced the frequency of miniature IPSCs without affecting their amplitude, indicating that this agent inhibits IPSCs via presynaptic mechanisms. Moreover, the sulfhydryl alkylating agent *N*-ethylmaleimide (NEM) significantly reduced the moxonidine-induced inhibition of IPSCs. Thus, the activation of presynaptic I_1-imidazoline receptors decreases GABA-mediated inhibition of medium spiny neurons in the striatum, in which NEM-sensitive proteins such as $G_{i/o}$-type G-proteins play an essential role. The adenylate cyclase activator forskolin partly opposed IPSC inhibition elicited by subsequently applied moxonidine. Furthermore, the protein kinase C (PKC) activator phorbol 12,13-dibutyrate attenuated and the PKC inhibitor chelerythrine potentiated the moxonidine-induced inhibition of IPSCs. These results suggest that IPSC inhibition via presynaptic I_1-imidazoline receptors involves intracellular adenylate cyclase activity and is influenced by static PKC activity in the striatum.

Key words: **imidazoline receptors; GABAergic IPSCs; G-proteins; *N*-ethylmaleimide; protein kinase C; striatum**

Introduction

Accumulating evidence suggests that there are specific binding sites recognized by imidazolines and their structurally related compounds in the peripheral and central tissues. Such binding sites have been separated into at least three subclasses, defined as I_1-, I_2-, and non-I_1/I_2- (= I_3) imidazoline receptors (for review, see Eglen et al., 1998). Although the α2-adrenergic receptor ligands containing an imidazoline moiety display high affinity for imidazoline receptors, there has been no reported correlation between the distribution pattern of the imidazoline receptors and α2-adrenergic receptors in many brain regions (De Vos et al., 1994; Piletz et al., 2000).

The physiological functions of imidazoline receptor subtypes are not fully determined, but it seems likely that I_1-imidazoline receptors in the brainstem participate in the regulation of arterial blood pressure (Bousquet, 1995; Ernsberger and Haxhiu, 1997). It has been reported that I_1-imidazoline receptors are subcellularly located on the plasma membrane (Piletz and Sletten, 1993; Ernsberger and Shen, 1997). In contrast, some populations of I_2-imidazoline receptors are located intracellularly on the outer membrane of mitochondria and inhibit monoamine oxidase activity (Carpéné et al., 1995; Ozaita et al., 1997; Lalies et al., 1999). I_3-imidazoline receptors have been shown to act peripherally in the regulation of ATP-sensitive K^+ channels coupled with insulin secretion (Chan et al., 1994; Olmos et al., 1994; Zaitsev et al., 1999) and centrally in the modulation of firing activity of locus ceruleus neurons (Ugedo et al., 1998) and spinal reflexes (Kino et al., 2005).

It is widely accepted that I_1- and I_2-imidazoline receptors are distributed in the CNS (Bricca et al., 1989; Ernsberger et al., 1995; Lione et al., 1998; Ruggiero et al., 1998). A previous study by De Vos et al. (1994) using autoradiography has revealed that the striatum is one of the regions of the human brain that is richest in I_1- and I_2-imidazoline receptors. Similarly, in rodents, a moderate density of these receptor subtypes has been demonstrated (Kamisaki et al., 1990; Ruggiero et al., 1998) (but see Vauquelin et al., 1999). Evidence that I_2-imidazoline receptor binding is altered in the putaminal tissue taken postmortem from patients with Huntington's disease (Reynolds et al., 1996) and that the I_2-imidazoline receptor ligand 2-(2-benzofuranyl)-2-imidazoline increases dopamine release in the striatum shown *in vivo* (Sastre-Coll et al., 2001) indicates that imidazoline receptors may influence neuronal function in the striatum. However, no study has provided direct evidence of modulatory roles for I_1- and/or I_2-imidazoline receptors detected electrophysiologically in the striatum. In the

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study presented here, we focused on I_1-imidazoline receptors and explored their roles in the modulation of excitatory and inhibitory synaptic transmission in the dorsal striatum by using moxonidine, a mixed I_1-imidazoline and α2-adrenergic receptor agonist with at least 40-fold selectivity for I_1-imidazoline receptors over α2-adrenergic receptors (Ferry et al., 1988; Ernsberger et al., 1993) and demonstrated that activation of I_1-imidazoline receptors reduces GABAergic inhibitory synaptic transmission by *N*-ethylmaleimide (NEM)-sensitive protein-mediated presynaptic mechanisms.

Materials and Methods

All of the experimental protocols used here were approved by the Animal Care and Use Committee of Nagoya City University and were performed according to the guidelines of the National Institutes of Health and the Japanese Pharmacological Society.

Slice preparation. Wistar/ST rats (SLC, Shizuoka, Japan) 6- to 14-d-old were killed by cervical dislocation under ether anesthesia. The brains were then quickly removed and placed in ice-cold low-sodium artificial CSF (ACSF), pH 7.4 (after bubbling with 95% O_2 and 5% CO_2) containing the following (in mM): 215.5 sucrose, 3 KCl, 1 NaH_2PO_4, 25 $NaHCO_3$, 11 D-glucose, 1 $CaCl_2$, and 5 $MgCl_2$. Coronal slices 150 μm thick containing the striatum were prepared using a vibratome (DSK-1000; Dosaka, Kyoto, Japan) and then maintained for at least 60 min at room temperature (22–24°C). The slices were then transferred to a recording chamber mounted on the stage of a microscope (Axioskop; Zeiss, Jena, Germany) and superfused with standard ACSF (at 33–34°C, pH 7.4 after bubbling with 95% O_2 and 5% CO_2) containing the following (in mM): 113 NaCl, 3 KCl, 1 NaH_2PO_4, 25 $NaHCO_3$, 11 D-glucose, 2 $CaCl_2$, and 1 $MgCl_2$ (at a rate of 3 ml/min).

Patch-clamp electrophysiology. Whole-cell voltage-clamp recordings were made from visually identified medium spiny neurons in the dorsal striatum using an upright microscope with a water-immersion objective lens (40×; Zeiss) with Nomarski optics. The patch electrodes (2.5–3 μm tip diameter) were pulled from borosilicate glass capillaries (Harvard Apparatus, Edenbridge, UK) and had a resistance of 3–5 MΩ when filled with the internal solution containing the following (in mM): 140 CsCl, 10 HEPES, 1.1 EGTA, 2 $MgCl_2$, 3 MgATP, and 0.3 Tris-GTP, pH 7.4 adjusted with CsOH. Synaptic currents were evoked by stimulating the neighboring area (within a 40–100 μm radius of the recorded neuron) via a glass pipette filled with 1 M NaCl. A voltage pulse 0.2 ms in duration at 0.1 Hz was applied at suprathreshold intensity via the stimulating electrode. Glutamate-mediated EPSCs and GABAergic IPSCs were recorded in the presence of (−)-bicuculline methiodide [10 μM (referred to simply as bicuculline)] and 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX) (10 μM), respectively, at a holding potential of −60 mV. When spontaneous miniature IPSCs (mIPSCs) were recorded, tetrodotoxin (TTX) (0.5 μM) was further added to the extracellular solution containing CNQX (10 μM). Under these experimental conditions, EPSCs, IPSCs, and mIPSCs were detected as inward deflections (Momiyama and Koga, 2001). Whole-cell currents were amplified (EPC-7; List Medical, Darmstadt, Germany), low-pass filtered at 4 kHz, and digitized at 10 kHz for computer analysis with pClamp7 software (Molecular Devices, Union City, CA). The access resistance was monitored by measuring capacitative transients obtained in response to a hyperpolarizing voltage step (10 mV, 10 ms) from a holding potential of −60 mV. All experiments were performed at 33–34°C.

The effects of moxonidine on evoked IPSCs were evaluated by comparing averaged IPSCs taken during the peak responses to the drug (six traces for 1 min) with those before drug application (18 traces for 3 min). mIPSCs were analyzed using N software (kindly provided by Dr. S. F. Traynelis, Emory University, Atlanta, GA), and the frequency and amplitude distribution of the events for 5–10 min during the peak response to moxonidine were compared with those obtained before application of the drug. The concentration–inhibition curve of IPSCs obtained with moxonidine was fitted, and the IC_{50} value was determined using nonlinear regression analysis with the embedded logistic function in Origin (Microcal Software, Northampton, MA): $\%I = E_{max}/\{1 + (IC_{50}/[D])^n\}$, where $\%I$ is the percentage inhibition of IPSCs, $[D]$ is the concentration of moxonidine, E_{max} is the maximal percentage inhibition of IPSCs, and n is the Hill slope.

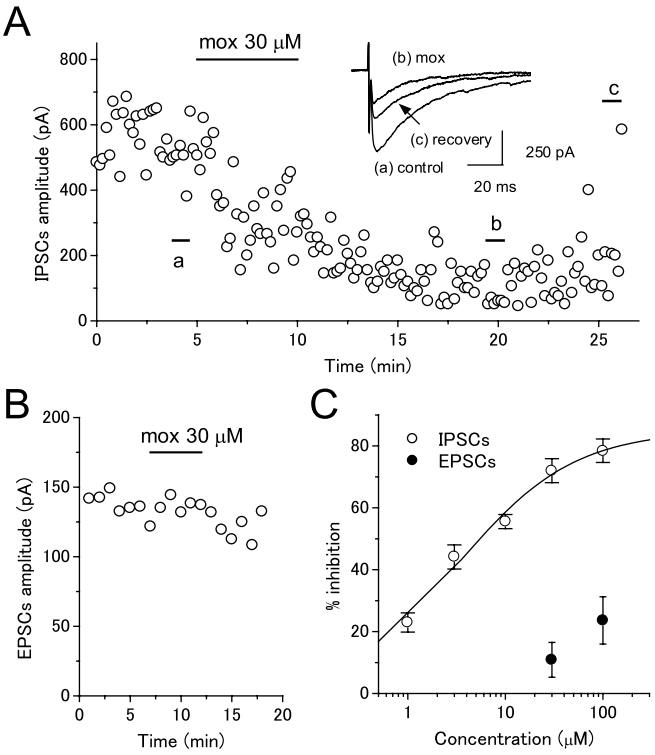

Figure 1. Selective inhibition of GABAergic IPSCs by moxonidine in striatal neurons. Whole-cell patch-clamp recordings were made from medium spiny neurons in slices of the rat brain dorsal striatum. The cell was clamped close to −60 mV, and either GABAergic IPSCs or glutamatergic EPSCs were evoked at 0.1 Hz. ***A***, A representative time course of the inhibition of IPSCs by moxonidine (mox; 30 μM) bath applied for 5 min. Each point represents individual IPSCs. Inset, Example averaged traces of six consecutive IPSCs recorded during the times indicated on the graph in ***A***. ***B***, A representative time course showing almost no effect of moxonidine (30 μM for 5 min) on EPSCs. Each point represents the mean amplitude of six consecutive IPSCs evoked at 0.1 Hz. ***C***, Concentration–inhibition relationship for the effect of moxonidine on IPSCs (open circles; $n = 4$–5) and EPSCs (filled circles; $n = 4$–5). Each point represents the mean ± SEM.

All data are expressed as means ± SEM. Student's *t* test (two-tailed) was used to compare the data for two groups. Two-tailed *t* test with Bonferroni's correction following one-way ANOVA was used for multiple comparisons between the control and the treated groups (Wallenstein et al., 1980). The Kolmogorov–Smirnov test was used for comparison of the cumulative probability distributions of mIPSCs. Differences at $p < 0.05$ were considered to be significant.

Drugs were administered by bath application. (−)-Bicuculline methiodide, CNQX, moxonidine hydrochloride, efaroxan hydrochloride, idazoxan hydrochloride, yohimbine hydrochloride, 6-chloro-2,3,4,5-tetrahydro-3-methyl-1*H*-3-benzazepine (SKF86466), noradrenaline, NEM, forskolin, phorbol 12,13-dibutyrate (PDBu), and chelerythrine chloride were purchased from Sigma (St. Louis, MO, USA). TTX was obtained from Wako (Osaka, Japan).

Results

Inhibition of $GABA_A$ receptor-mediated synaptic currents by moxonidine in striatal neurons

Bath application of moxonidine inhibited GABAergic IPSCs elicited in cells voltage clamped at a holding potential of −60 mV in a concentration-dependent manner. The recorded GABAergic IPSCs were mediated by $GABA_A$ receptors, because they were abolished by bicuculline (10 μM; data not shown). Figure 1*A* illustrates the typical time course of IPSC suppression in response to moxonidine (30 μM). This concentration of moxonidine superfused for 5 min reduced IPSCs by 72.0 ± 3.9% ($n = 5$), and the

effect was slowly reversed during washout. The concentration–inhibition curve, constructed at a concentration range between 1 and 100 μM ($n = 4$–5), revealed an IC_{50} of 3.4 μM (Fig. 1*C*). In contrast, moxonidine was much less effective on glutamatergic EPSCs and reduced EPSCs by only 10.9 ± 5.6% ($n = 6$) and 23.6 ± 7.6% ($n = 5$) at concentrations of 30 and 100 μM, respectively (Fig. 1*B*,*C*). Therefore, only the effect of moxonidine on GABAergic IPSCs was studied further.

Pharmacological characterization of moxonidine-induced inhibition of IPSCs

Because moxonidine has an affinity for both I_1-imidazoline and α2-adrenergic receptors (Ferry et al., 1988; Ernsberger et al., 1993), we next determined pharmacologically which receptors mediate the moxonidine-induced inhibition of GABAergic IPSCs. In the presence of efaroxan (10 μM) or idazoxan (10 μM), which block both imidazoline receptors (I_1 for efaroxan and $I_{1,2}$ for idazoxan) and α2-adrenergic receptors, moxonidine (30 μM) reduced IPSCs by only 10.4 ± 2.9% [efaroxan, $n = 5$ (Fig. 2*A*,*E*)] or 22.2 ± 7.3% [idazoxan, $n = 5$ (Fig. 2*B*,*E*)], being significantly weaker compared with moxonidine alone (72.0 ± 3.9%; $p < 0.01$; $n = 5$). In contrast, yohimbine (10 μM) and SKF86466 (10 μM) (Hieble et al., 1986), both of which are α2-adrenergic receptor antagonists with no affinity for imidazoline receptors, did not impair the inhibitory effect of moxonidine (30 μM) on IPSCs (inhibition by 72.4 ± 6.3%, $n = 4$ and 62.4 ± 6.3%, $n = 5$ in the presence of yohimbine and SKF86466, respectively) (Fig. 2*C*–*E*). We confirmed that yohimbine and SKF86466 at this concentration essentially diminished the noradrenaline (30 μM)-induced inhibition of GABAergic IPSCs elicited under respective blockade of α1- and β-adrenergic receptors with prazosin (10 μM) and propranolol (10 μM) ($p < 0.01$; $n = 4$ each) (Fig. 3). Thus, moxonidine inhibits IPSCs via I_1-imidazoline receptors but not α2-adrenergic receptors.

Determination of the synaptic site of action of moxonidine-induced inhibition of IPSCs

To determine the synaptic site of action of moxonidine, we first examined the effect of moxonidine on the ratio of the amplitude of the second IPSC divided by that of the first one [paired-pulse ratio (PPR)] elicited by two successive stimuli of identical strength at an interval of 50 ms. A change in the PPR is considered to be attributable to a presynaptic change in release probability (Zucker, 1989; Manabe et al., 1993). A typical example of IPSCs in Figure 4, *A* and *B*, shows a change in the PPR from depression to facilitation by application of moxonidine (30 μM). In four cells tested, moxonidine (30 μM) increased the PPR from 0.60 ± 0.05 to 1.13 ± 0.10 ($p < 0.01$), suggesting that moxonidine decreases the release probability of GABA presynaptically.

To further establish the mode of presynaptic action of moxonidine in the inhibition of GABAergic inhibitory synaptic transmission, the effects of moxonidine (30 μM) on action potential-independent spontaneous mIPSCs recorded in the presence of CNQX (10 μM) and TTX (0.5 μM) were examined. Sample recordings before and during application of moxonidine (30 μM) in Figure 5*A* show that application of moxonidine (30 μM) reduced the frequency of mIPSCs. This was clearly evidenced by the cumulative probability distributions of mIPSC interevent intervals and amplitude (Fig. 5*B*,*C*, respectively) constructed from the same cell before and during application of moxonidine, demonstrating that moxonidine shifted significantly the interevent interval distribution to longer intervals without altering the amplitude distribution. In five cells, moxonidine (30 μM) decreased the mean frequency of mIPSCs from 1.6 ± 0.1 to 0.5 ± 0.1 Hz ($p < 0.01$) (Fig. 5*D*). In contrast, the mean amplitude of mIPSCs before and during application of moxonidine were 35.9 ± 6.2 and 35.0 ± 5.9 pA, respectively ($p > 0.05$) (Fig. 5*D*). Together, these results demonstrate that moxonidine reduced GABAergic synaptic transmission in the medium spiny neurons by acting on presynaptic I_1-imidazoline receptors.

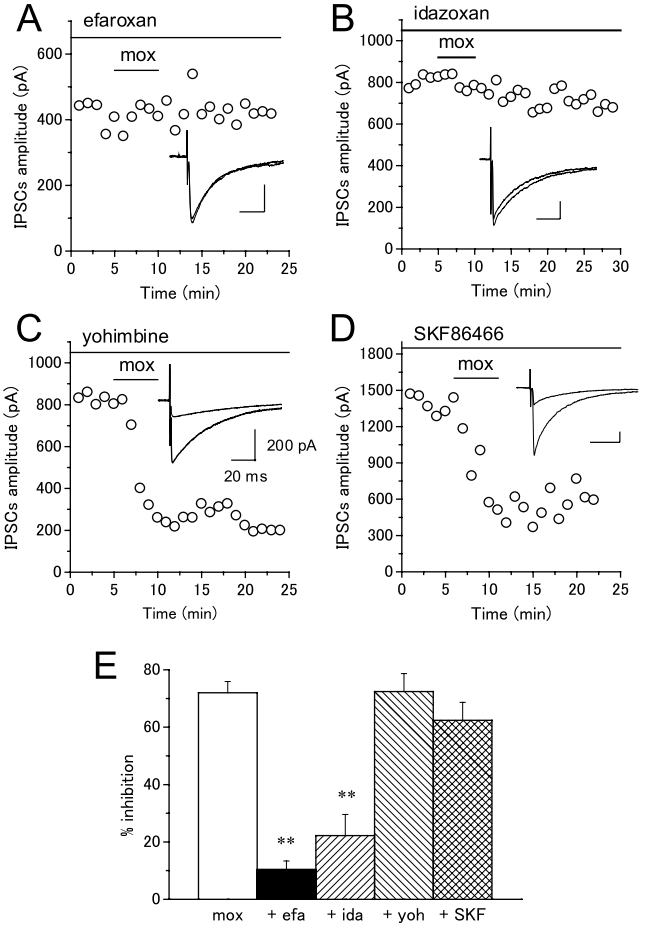

Figure 2. I_1-imidazoline receptors mediate the moxonidine-induced inhibition of IPSCs. ***A***, ***B***, Moxonidine (mox; 30 μM) was applied in the presence of efaroxan (efa; 10 μM) or idazoxan (ida; 10 μM), which block both imidazoline receptors (I_1 for efaroxan and $I_{1,2}$ for idazoxan) and α2-adrenergic receptors. The inhibitory effect of moxonidine on IPSCs was primarily attenuated. ***C***, ***D***, No impairment of the moxonidine (30 μM)-induced inhibition of IPSCs in the presence of yohimbine (yoh; 10 μM) and SKF86466 (SKF; 10 μM), both of which are α2-adrenergic receptor antagonists with no affinity for imidazoline receptors. Each point represents the mean amplitude of six consecutive IPSCs evoked at 0.1 Hz. Example traces on each graph are the averaged IPSCs in control and in moxonidine (smaller trace). Calibration: 500 pA, 20 ms. ***E***, Summary of the effects of imidazoline and/or α2-adrenergic receptor antagonists on the moxonidine-induced inhibition of IPSCs. Each column represents the mean ± SEM. The significance of differences between the values for the moxonidine alone ($n = 5$) and antagonist-treated groups ($n = 4$–5) was determined with the two-tailed multiple *t* test with Bonferroni's correction following ANOVA (4 comparisons in 5 groups). $^{**}p < 0.01$.

Effects of NEM on moxonidine-induced inhibition of IPSCs

Previous studies have suggested that I_1-imidazoline binding sites are coupled with G-proteins (Molderings et al., 1993; Ernsberger and Shen, 1997; Takada et al., 1997; Greney et al., 2000). To assess whether inhibition of IPSCs mediated by presynaptic I_1-imidazoline receptors involves G-proteins, the effect of moxonidine on IPSCs was examined in the presence of the sulfhydryl alkylating agent NEM, which attacks sulfhydryl groups of cys-

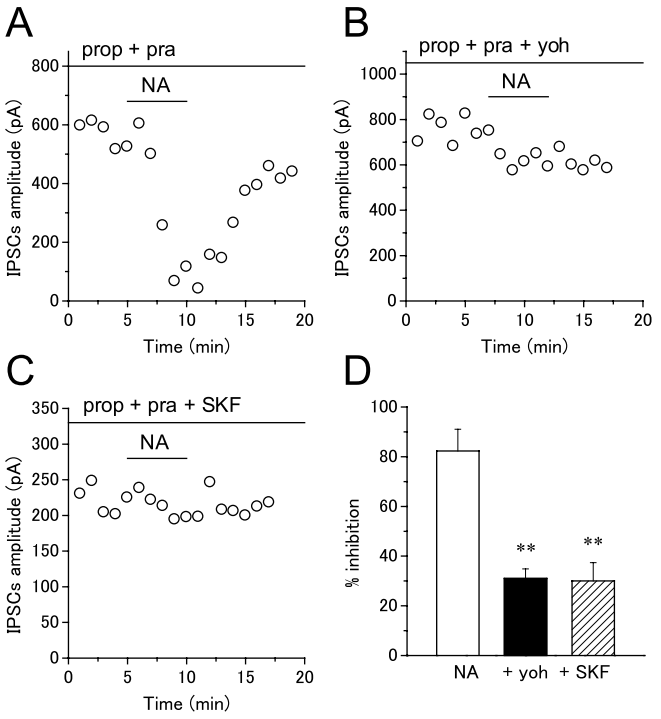

Figure 3. Yohimbine and SKF86466 antagonize the noradrenaline-induced inhibition of IPSCs. ***A***, Noradrenaline (NA; 30 μM) applied for 5 min in the presence of propranolol (prop; 10 μM) and prazosin (pra; 10 μM) reduced IPSCs. ***B***, ***C***, Attenuation of the noradrenaline-induced inhibition by yohimbine (yoh, ***B***; 10 μM) and SKF86466 (SKF, ***C***; 10 μM). Each point represents the mean amplitude of six consecutive IPSCs evoked at 0.1 Hz. ***D***, Summary of the effects of the α2-adrenergic receptor antagonists yohimbine and SKF86466 on the noradrenaline-induced inhibition of IPSCs. Each column represents the mean ± SEM. The significance of differences between the values for noradrenaline in the presence of propranolol and prazosin and α2-adrenergic receptor antagonist-treated groups ($n = 4$ each) was determined with the two-tailed multiple *t* test with Bonferroni's correction following ANOVA (2 comparisons in 3 groups). $^{**}p < 0.01$.

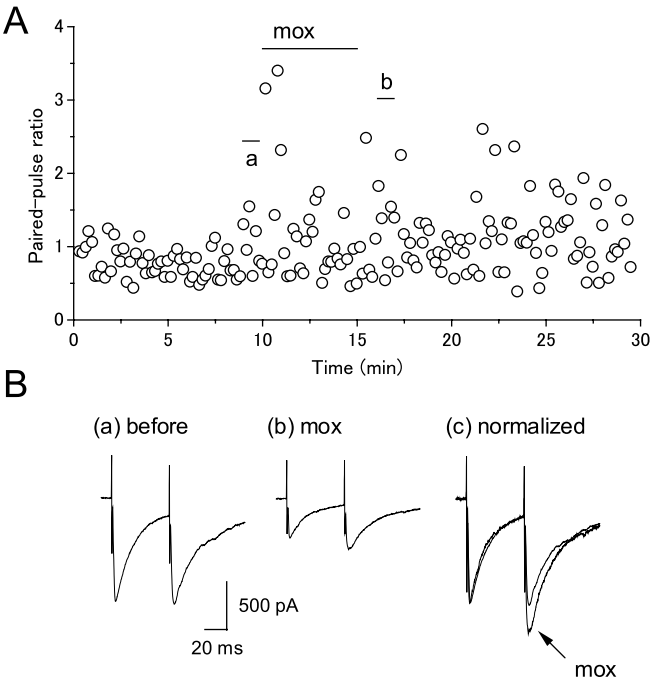

Figure 4. Moxonidine increases the paired-pulse ratio. To assess the effect of moxonidine (mox; 30 μM) on the PPR, two successive stimuli of identical strength at an interval of 50 ms were applied (0.1 Hz). ***A***, A typical time course of the increase in the PPR in response to moxonidine. Each point represents the individual PPR. ***B***, Example averaged traces of six consecutive paired IPSCs recorded during the times indicated on the graph in ***A***.

teine residues of the proteins, including pertussis toxin (PTX)-sensitive $G_{i/o}$-type G-proteins (Shapiro et al., 1994; Momiyama and Koga, 2001). As shown in Figure 6*A*, NEM (50 μM) alone did not affect IPSCs. However, subsequent application of moxonidine (30 μM) resulted in only slight depression of IPSCs. In five cells recorded in the presence of NEM, moxonidine (30 μM) reduced IPSCs by 10.4 ± 8.2%, which was significantly smaller than the inhibition elicited by moxonidine alone (67.6 ± 5.4%; $p < 0.01$; $n = 5$). Thus, NEM-sensitive proteins such as $G_{i/o}$-type G-proteins are involved in the presynaptic I_1-imidazoline receptor-mediated inhibition of IPSCs. Although we need additional evidence to conclude that presynaptic I_1-imidazoline receptors are coupled with $G_{i/o}$-type G-proteins, the final set of experiments reported here assessing the intracellular signaling was made partly based on the assumption that $G_{i/o}$-type G-proteins are involved.

Intracellular signaling mediating and affecting the moxonidine-induced inhibition of IPSCs

Because $G_{i/o}$-type G-proteins are coupled negatively to adenylate cyclase activity, we first explored the possibility that a decrease in adenylate cyclase activity was the intracellular element involved in the inhibition of IPSCs by moxonidine. Bath application of forskolin (10 μM), an activator of adenylate cyclase, increased IPSCs by 170.7 ± 25.8% (data not shown; $n = 6$). Subsequent application of moxonidine (30 μM) in the presence of forskolin reduced IPSCs by 53.7 ± 3.5% ($n = 6$) (Fig. 7*A*, left, *B*), which was significantly different from the control value for moxonidine alone (68.1 ± 5.1%; $p < 0.05$; $n = 5$). This result suggests that forskolin may have opposed the decrease in adenylate cyclase activity that was involved partly in the inhibition of IPSCs via presynaptic I_1-imidazoline receptors.

We then examined whether an increase in protein kinase C (PKC) activity, which would also be expected in the effect mediated by $G_{i/o}$-type G-proteins, was involved in the inhibition of IPSCs in response to moxonidine. However, bath application of the PKC activator PDBu (0.5 μM) increased IPSCs by 217.5 ± 26.3% (data not shown; $n = 7$), suggesting that PKC activation does not function in downstream signaling leading to inhibition of IPSCs after occupation of presynaptic I_1-imidazoline receptors. Surprisingly, application of moxonidine (30 μM) in the presence of PDBu decreased IPSCs by only 29.2 ± 2.9% ($n = 7$) (Fig. 7*A*, middle, *B*), which was significantly different from the control value for moxonidine alone (70.3 ± 4.5%; $p < 0.01$; $n = 5$). Moreover, the PKC inhibitor chelerythrine (5 μM), which alone did not significantly change IPSCs, augmented the moxonidine-induced inhibition of IPSCs (88.1 ± 3.7%; $n = 5$) (Fig. 7*A*, right, *B*), which was significantly different from the control value for moxonidine alone (65.7 ± 4.1%; $p < 0.01$; $n = 6$). Thus, it is likely that the presynaptic inhibition of IPSCs mediated by I_1-imidazoline receptors is influenced by how PKC is statically activated.

Discussion

There has been a complete lack of studies on the roles of imidazoline receptors in the regulation of neuronal activities in the striatum, despite the fact that the striatum has one of the highest density of I_1- and I_2-imidazoline receptors in the human brain (De Vos et al., 1994) and also has a moderate density of there

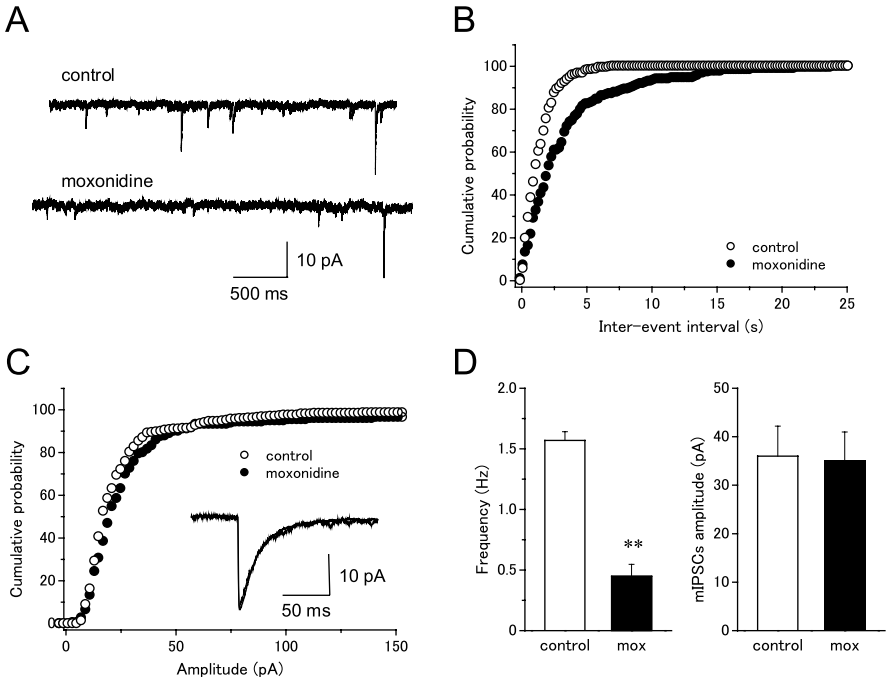

Figure 5. Moxonidine reduces the frequency but not the amplitude of mIPSCs. The mIPSCs were recorded in the presence of CNQX (10 μM) and TTX (0.5 μM). ***A***, Typical example traces recorded in control and during application of moxonidine (mox; 30 μM). ***B***, ***C***, Cumulative probability distribution of interevent intervals (***B***) and peak amplitudes (***C***) of mIPSCs in control (open circles; 395 events) and in moxonidine (filled circles; 189 events) from the same cell as that shown in ***A***. The interevent interval was increased, and the amplitude of IPSCs was unaffected by moxonidine. Inset on graph ***C*** shows superimposed traces of the averaged mIPSCs in control (395 events) and in the presence of moxonidine (189 events). ***D***, Summary graphs showing that moxonidine decreased the mean frequency, without altering the mean amplitude, of mIPSCs. Each column represents the mean ± SEM ($n = 5$ each). Student's t test; $^{**}p < 0.01$.

receptor subtypes in the rodent brain (Kamisaki et al., 1990; Ruggiero et al., 1998) (but see Vauquelin et al., 1999). In the present study focusing on I_1-imidazoline receptors, we demonstrated for the first time the novel role of I_1-imidazoline receptors in the modulation of GABAergic inhibitory synaptic transmission in the medium spiny neurons of the dorsal striatum. This inhibitory effect on IPSCs was mediated by presynaptic mechanisms coupled with NEM-sensitive proteins culminating partly in a decrease of intracellular adenylate cyclase activity and was influenced by static PKC activity.

I_1-imidazoline receptors mediate moxonidine-induced inhibition of IPSCs

We used moxonidine as a selective agonist of I_1-imidazoline receptors. Nevertheless, its weak affinity for α2-adrenergic receptors (Ferry et al., 1988; Ernsberger et al., 1993) may have accounted for the observed inhibition of IPSCs. Hayar and Guyenet (2000) revealed that inhibition of both EPSCs and IPSCs by moxonidine in bulbospinal neurons of the rostral ventrolateral medulla is mediated via α2-adrenergic receptors. Indeed, in the present study, application of noradrenaline under blockade of both α1- and β-adrenergic receptors with prazosin and propranolol, respectively, resulted in α2-adrenergic receptor-mediated inhibition of IPSCs, as was demonstrated by the apparent lack of inhibition by noradrenaline when combined with either yohimbine or SKF86466, which are non-imidazoline antagonists selective for α2-adrenergic receptors. However, this was not the case for the inhibition of IPSCs by moxonidine in the striatum. Neither yohimbine nor SKF86466 influenced the effect of moxonidine on IPSCs. Only in the presence of efaroxan or idazoxan, which block both imidazoline receptors (I_1 for efaroxan and $I_{1,2}$ for idazoxan) and α2-adrenergic receptors, was the inhibitory effect of moxonidine on IPSCs essentially lost. We therefore conclude that moxonidine reduces IPSCs via I_1-imidazoline receptors.

Presynaptic mechanisms underlie moxonidine-induced inhibition of IPSCs

Paired-pulse protocol and analysis of mIPSCs are the most widely used approaches for assessing the synaptic sites of drug action. Moxonidine elicited an increase in the PPR concurrently with a reduction of IPSCs, which is considered to reflect a presynaptic change in release probability (Zucker, 1989; Manabe et al., 1993). More importantly, we have observed a decrease in the frequency of mIPSCs after application of moxonidine without any change in their amplitudes. From these two lines of evidence, it is most likely that the moxonidine-induced inhibition of IPSCs is primarily presynaptic in origin and not attributable to postsynaptic reduction in the sensitivity of $GABA_A$ receptors. Our results not only support the immunocytochemical study by Ruggiero et al. (1998), who revealed the presence of imidazoline receptors in axon terminals of the rat CNS, but demonstrate electrophysiologically, for the first time, the functional existence of presynaptic imidazoline receptors that are coupled with neurotransmitter release in the CNS.

G-proteins and intracellular signaling

NEM uncouples PTX-sensitive $G_{i/o}$-type G-proteins from receptors (Shapiro et al., 1994; Momiyama and Koga, 2001). The present result clearly demonstrates that PTX-sensitive $G_{i/o}$-type G-proteins couple with presynaptic I_1-imidazoline receptors. Previous studies have suggested that the coupling of imidazoline receptors with G-proteins is based on the sensitivity of imidazoline-specific binding to GTP or nonhydrolyzable analogs (Molderings et al., 1993; Ernsberger and Shen, 1997; Greney et al., 2000). Moreover, Takada et al. (1997) have demonstrated in rats that intracerebroventricular pretreatment with PTX prevented the antidysrhythmic effect of systemically injected rilmenidine, a selective I_1-imidazoline receptor agonist, on halothane-adrenaline dysrhythmias. Our present study may provide more details about the functional linkage of I_1-imidazoline receptors and G-proteins, especially in the modulation of GABA release at GABAergic terminals in the dorsal striatum. However, we should take a possible involvement of other proteins that contain active cysteine residues and therefore are attacked by NEM into consideration. This remains to be clarified.

Our finding that the moxonidine-induced inhibition of IPSCs was reduced by forskolin suggests that the cAMP pathway is an intracellular target of I_1-imidazoline receptors, and this is also in line with the suggested coupling with $G_{i/o}$-type G-proteins (but see Greney et al., 2000). Because the activation of PKC with PDBu did not mimic the inhibitory action of moxonidine on IPSCs, at

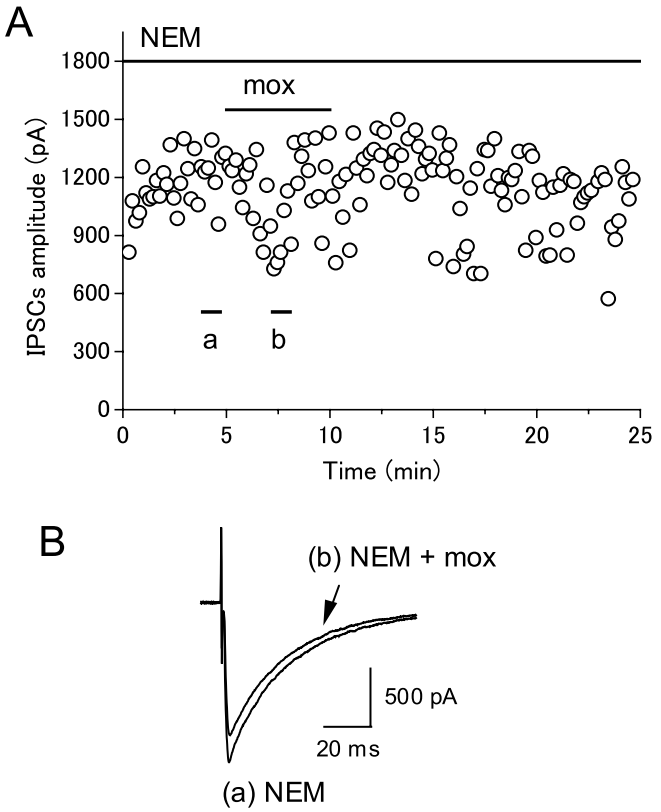

Figure 6. Effects of NEM on moxonidine-induced inhibition of IPSCs. ***A***, A representative time course of the effect of moxonidine (mox; 30 μM for 5 min) on IPSCs in the presence of NEM (50 μM). Each point represents individual IPSCs evoked at 0.1 Hz. ***B***, Example averaged traces of six consecutive IPSCs recorded during the times indicated on the graph in ***A***.

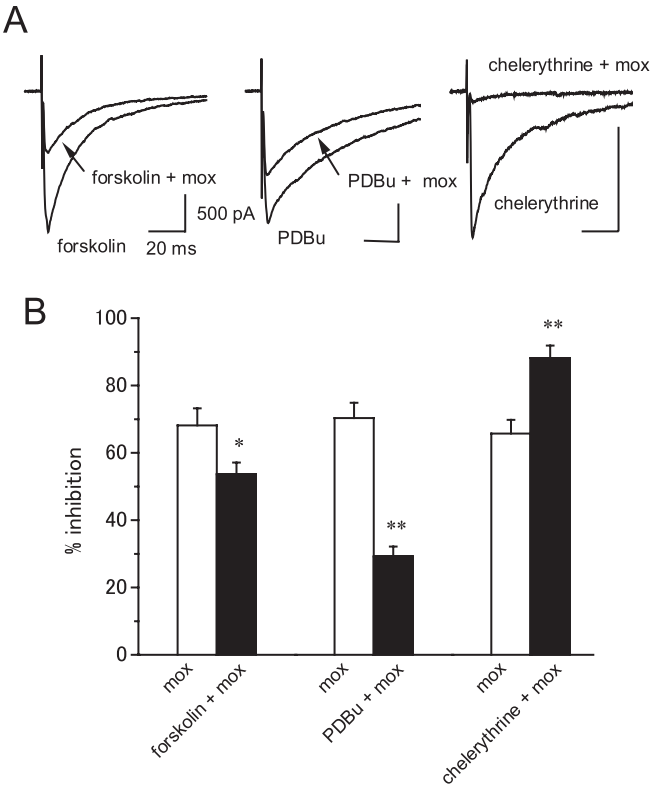

Figure 7. Involvement of the cAMP pathway and modulation by PKC in the moxonidine-induced inhibition of IPSCs. ***A***, Typical averaged traces of six consecutive IPSCs showing that the moxonidine-induced inhibition of IPSCs was attenuated by the adenylate cyclase activator forskolin (10 μM; left) and the PKC activator PDBu (0.5 μM; middle) and potentiated by the PKC inhibitor chelerythrine (5 μM; right). Calibration: 500 pA, 20 ms. ***B***, Summary of the effects of forskolin, PDBu, and chelerythrine on moxonidine-induced inhibition of IPSCs. Each column represents the mean ± SEM ($n = 5$–7). Student's t test; $^*p < 0.05$, $^{**}p < 0.01$.

least diacylglyceride-sensitive PKC is unlikely to play a role in this action of presynaptic I_1-imidazoline receptors. Rather, the finding that PDBu attenuated and the PKC inhibitor chelerythrine potentiated the moxonidine-induced inhibition of IPSCs could be explained by a mechanism similar to that demonstrated in the modulation of the coupling of metabotropic glutamate receptors to G-proteins by PKC (Macek et al., 1998; Poisik et al., 2003). Additional study is needed to explore this mechanism.

As a transduction pathway for I_1-imidazoline receptors, activation of phosphatidylcholine-specific phospholipase C, which results in downstream activation of mitogen-activated protein kinase along with PKC, has been proposed previously in PC12 cells (Edwards et al., 2001; Zhang et al., 2001) and recently in the rostral ventrolateral medulla *in vivo* (Zhang and Abdel-Rahman, 2005). This pathway may be linked to the imidazoline binding protein showing characteristics of I_1-imidazoline receptors whose gene has been recently cloned (Piletz et al., 2000). However, whether or not this pathway is associated with G-proteins remains undetermined. Because I_1-imidazoline receptors could be coupled to more than one transduction pathway, as was demonstrated by Greney et al. (2000), the involvement of this pathway as well as participation of the atypical subtype of PKC (Edwards et al., 2001) should be also assessed in the presynaptic modulation of IPSCs via I_1-imidazoline receptors.

Physiological implications

$GABA_A$ receptor-mediated IPSCs in the dorsal striatum arise from intrinsic GABAergic inputs through either neighboring medium spiny neurons via axon collaterals or interneurons. Removal of GABAergic inhibition on the medium spiny neurons by presynaptic I_1-imidazoline receptors may result in augmentation of the striatal GABAergic output signals. At present, however, we are unable to conclude whether I_1-imidazoline receptor-mediated disinhibition of the striatal output neurons results in a net increase or decrease of neuronal activities in the internal globus pallidus and substantia nigra, the basal ganglia output nuclei that send inhibitory projections to the glutamatergic thalamocortical neurons (Alexander and Crutcher, 1990). Decreased activities of the thalamocortical neurons in Parkinson's disease should be clinically normalized to achieve stimulation of movement via the cortex (Gerfen, 1992; Di Chiara et al., 1994). Considering that tizanidine, an imidazoline agent in clinical use for spasticity (Wagstaff and Bryson, 1997) with a higher affinity for I_1-imidazoline receptors over α2-adrenergic receptors (Piletz et al., 1996), reduces muscle rigidity by acting at the supraspinal level (Honda et al., 2002; Kino et al., 2005), the therapeutic potential of specific agonists of I_1-imidazoline receptors appears to be further established for the treatment of motor dysfunction related to the basal ganglia.

In our preliminary experiment, the I_1-imidazoline receptor antagonist efaroxan alone did not alter the amplitude of IPSCs at higher concentrations (data not shown). The postulated endogenous ligands for imidazoline receptors, including agmatine (Reis and Regunathan, 2000), harmane (Parker et al., 2004), and imidazoleacetic acid-ribotide (Prell et al., 2004), could be easily washed out of slice preparations, thereby resulting in failure to

detect their tonic inhibitory effects on IPSCs. Agmatine and imidazoleacetic acid-ribotide are considered to be synthesized from L-arginine (Reis and Regunathan, 2000) and histamine (Thomas and Prell, 1995), respectively, and released synaptically in the brain (Reis and Regunathan, 2000; Prell et al., 2004). However, the striatum is not enriched with agmatine (Reis and Regunathan, 2000), and the presence of imidazoleacetic acid-ribotide has been studied only in the brainstem neurons (Prell et al., 2004). Moreover, the formation and distribution of harmane in the brain have not been fully determined. The endogenous ligands may reach both I_1- and I_2-imidazoline receptors in the striatum. Activation of I_2-imidazoline receptors inhibits monoamine oxidase (Carpéné et al., 1995; Ozaita et al., 1997; Lalies et al., 1999) and elicits dopamine release (Sastre-Coll et al., 2001), both of which can result in an increase of extracellular dopamine. Hence, it is plausible that the endogenous ligands facilitate the striatal direct output pathway via both I_1-imidazoline receptor-mediated disinhibition and I_2-imidazoline receptor-mediated increase in dopamine. Additional studies to address the role of the endogenous ligands for imidazoline receptors will provide new insights into drug research in the field of neurodegenerative diseases related to the basal ganglia.

References

Alexander GE, Crutcher MD (1990) Functional architecture of basal ganglia circuits: neural substrates of parallel processing. Trends Neurosci 13:266–271.

Bousquet P (1995) Imidazoline receptors: from basic concepts to recent developments. J Cardiovasc Pharmacol 26:S1–S6.

Bricca G, Dontenwill M, Molines A, Feldman J, Tibirica E, Belcourt A, Bousquet P (1989) Rilmenidine selectivity for imidazoline receptors in human brain. Eur J Pharmacol 163:373–377.

Carpéné C, Collon P, Remaury A, Cordi A, Hudson A, Nutt D, Lafontan M (1995) Inhibition of amine oxidase activity by derivatives that recognize imidazoline I_2 sites. J Pharmacol Exp Ther 272:681–688.

Chan SL, Brown CA, Scarpello KE, Morgan NG (1994) The imidazoline site involved in control of insulin secretion: characteristics that distinguish it from I_1- and I_2-sites. Br J Pharmacol 112:1065–1070.

De Vos H, Bricca G, De Keyser J, De Backer JP, Bousquet P, Vauquelin G (1994) Imidazoline receptors, non-adrenergic idazoxan binding sites and α_2-adrenoceptors in the human central nervous system. Neuroscience 59:589–598.

Di Chiara G, Morelli M, Consolo S (1994) Modulatory functions of neurotransmitters in the striatum: ACh/dopamine/NMDA interactions. Trends Neurosci 17:228–233.

Edwards L, Fishman D, Horowitz P, Bourbon N, Kester M, Ernsberger P (2001) The I_1-imidazoline receptor in PC12 pheochromocytoma cells activates protein kinases C, extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK). J Neurochem 79:931–940.

Eglen RM, Hudson AL, Kendall DA, Nutt DJ, Morgan NG, Wilson VG, Dillon MP (1998) "Seeing through a glass darkly:" casting light on imidazoline "I" sites. Trends Pharmacol Sci 19:381–390.

Ernsberger P, Haxhiu MA (1997) The I_1-imidazoline-binding site is a functional receptor mediating vasodepression via the ventral medulla. Am J Physiol 273:R1572–R1579.

Ernsberger P, Shen IH (1997) Membrane localization and guanine nucleotide sensitivity of medullary I_1-imidazoline binding sites. Neurochem Int 30:17–23.

Ernsberger P, Damon TH, Graff LM, Schafer SG, Christen MO (1993) Moxonidine, a centrally acting antihypertensive agent, is a selective ligand for I_1-imidazoline sites. J Pharmacol Exp Ther 264:172–182.

Ernsberger P, Graves ME, Graff LM, Zakieh N, Nguyen P, Collins LA, Westbrooks KL, Johnson GG (1995) I_1-imidazoline receptors. Definition, characterization, distribution, and transmembrane signaling. Ann NY Acad Sci 763:22–42.

Ferry D, Armah BI, Goll A, Glossmann H (1988) Characteristics of the binding of the antihypertensive agent moxonidine to α_2-adrenoceptors in rat brain membranes. Arzneimittelforschung 38:1442–1445.

Gerfen CR (1992) The neostriatal mosaic: multiple levels of compartmental organization. Trends Neurosci 15:133–139.

Greney H, Ronde P, Magnier C, Maranca F, Rascente C, Quaglia W, Giannella M, Pigini M, Brasili L, Lugnier C, Bousquet P, Dontenwill M (2000) Coupling of I_1 imidazoline receptors to the cAMP pathway: studies with a highly selective ligand, benazoline. Mol Pharmacol 57:1142–1151.

Hayar A, Guyenet PG (2000) Prototypical imidazoline-1 receptor ligand moxonidine activates alpha2-adrenoceptors in bulbospinal neurons of the RVL. J Neurophysiol 83:766–776.

Hieble JP, DeMarinis RM, Fowler PJ, Matthews WD (1986) Selective alpha-2 adrenoceptor blockade by SK&F 86466: *in vitro* characterization of receptor selectivity. J Pharmacol Exp Ther 236:90–96.

Honda M, Sekiguchi Y, Sato N, Ono H (2002) Involvement of imidazoline receptors in the centrally acting muscle-relaxant effects of tizanidine. Eur J Pharmacol 445:187–193.

Kamisaki Y, Ishikawa T, Takao Y, Omodani H, Kuno N, Itoh T (1990) Binding of [^{3}H]*p*-aminoclonidine to two sites, α_2-adrenoceptors and imidazoline binding sites: distribution of imidazoline binding sites in rat brain. Brain Res 514:15–21.

Kino Y, Tanabe M, Honda M, Ono H (2005) Involvement of supraspinal imidazoline receptors and descending monoaminergic pathways in tizanidine-induced inhibition of rat spinal reflexes. J Pharmacol Sci 99:52–60.

Lalies MD, Hibell A, Hudson AL, Nutt DJ (1999) Inhibition of central monoamine oxidase by imidazoline$_2$ site-selective ligands. Ann NY Acad Sci 881:114–117.

Lione LA, Nutt DJ, Hudson AL (1998) Characterisation and localisation of [^{3}H]2-(2-benzofuranyl)-2-imidazoline binding in rat brain: a selective ligand for imidazoline I_2 receptors. Eur J Pharmacol 353:123–135.

Macek TA, Schaffhauser H, Conn PJ (1998) Protein kinase C and A3 adenosine receptor activation inhibit presynaptic metabotropic glutamate receptor (mGluR) function and uncouple mGluRs from GTP-binding proteins. J Neurosci 18:6138–6146.

Manabe T, Wyllie DJ, Perkel DJ, Nicoll RA (1993) Modulation of synaptic transmission and long-term potentiation: effects on paired pulse facilitation and EPSC variance in the CA1 region of the hippocampus. J Neurophysiol 70:1451–1459.

Molderings GJ, Moura D, Fink K, Bonisch H, Gothert M (1993) Binding of [^{3}H]clonidine to I_1-imidazoline sites in bovine adrenal medullary membranes. Naunyn Schmiedebergs Arch Pharmacol 348:70–76.

Momiyama T, Koga E (2001) Dopamine D_2-like receptors selectively block N-type Ca^{2+} channels to reduce GABA release onto rat striatal cholinergic interneurones. J Physiol (Lond) 533:479–492.

Olmos G, Kulkarni RN, Haque M, MacDermot J (1994) Imidazolines stimulate release of insulin from RIN-5AH cells independently from imidazoline I_1 and I_2 receptors. Eur J Pharmacol 262:41–48.

Ozaita A, Olmos G, Boronat MA, Lizcano JM, Unzeta M, Garcia-Sevilla JA (1997) Inhibition of monoamine oxidase A and B activities by imidazol(ine)/guanidine drugs, nature of the interaction and distinction from I_2-imidazoline receptors in rat liver. Br J Pharmacol 121:901–912.

Parker CA, Anderson NJ, Robinson ES, Price R, Tyacke RJ, Husbands SM, Dillon MP, Eglen RM, Hudson AL, Nutt DJ, Crump MP, Crosby J (2004) Harmane and harmalan are bioactive components of classical clonidine-displacing substance. Biochemistry 43:16385–16392.

Piletz JE, Sletten K (1993) Nonadrenergic imidazoline binding sites on human platelets. J Pharmacol Exp Ther 267:1493–1502.

Piletz JE, Zhu H, Chikkala DN (1996) Comparison of ligand binding affinities at human I_1-imidazoline binding sites and the high affinity state of alpha-2 adrenoceptor subtypes. J Pharmacol Exp Ther 279:694–702.

Piletz JE, Ivanov TR, Sharp JD, Ernsberger P, Chang CH, Pickard RT, Gold G, Roth B, Zhu H, Jones JC, Baldwin J, Reis DJ (2000) Imidazoline receptor antisera-selected (IRAS) cDNA: cloning and characterization. DNA Cell Biol 19:319–329.

Poisik OV, Mannaioni G, Traynelis S, Smith Y, Conn PJ (2003) Distinct functional roles of the metabotropic glutamate receptors 1 and 5 in the rat globus pallidus. J Neurosci 23:122–130.

Prell GD, Martinelli GP, Holstein GR, Matulic-Adamic J, Watanabe KA, Chan SL, Morgan NG, Haxhiu MA, Ernsberger P (2004) Imidazoleacetic acid-ribotide: an endogenous ligand that stimulates imidazol(in)e receptors. Proc Natl Acad Sci USA 101:13677–13682.

Reis DJ, Regunathan S (2000) Is agmatine a novel neurotransmitter in brain? Trends Pharmacol Sci 21:187–193.

Reynolds GP, Boulton RM, Pearson SJ, Hudson AL, Nutt DJ (1996) Imidazoline binding sites in Huntington's and Parkinson's disease putamen. Eur J Pharmacol 301:R19–R21.

Ruggiero DA, Regunathan S, Wang H, Milner TA, Reis DJ (1998) Immunocytochemical localization of an imidazoline receptor protein in the central nervous system. Brain Res 780:270–293.

Sastre-Coll A, Esteban S, Miralles A, Zanetti R, Garcia-Sevilla JA (2001) The imidazoline receptor ligand 2-(2-benzofuranyl)-2-imidazoline is a dopamine-releasing agent in the rat striatum in vivo. Neurosci Lett 301:29–32.

Shapiro MS, Wollmuth LP, Hille B (1994) Modulation of Ca^{2+} channels by PTX-sensitive G-proteins is blocked by *N*-ethylmaleimide in rat sympathetic neurons. J Neurosci 14:7109–7116.

Takada K, Hayashi Y, Kamibayashi T, Mammoto T, Yamatodani A, Kitamura S, Yoshiya I (1997) The involvement of pertussis toxin-sensitive G proteins in the post receptor mechanism of central I_1-imidazoline receptors. Br J Pharmacol 120:1575–1581.

Thomas B, Prell GD (1995) Imidazoleacetic acid, a γ-aminobutyric acid receptor agonist, can be formed in rat brain by oxidation of histamine. J Neurochem 65:818–826.

Ugedo L, Pineda J, Ruiz-Ortega JA, Martín-Ruiz R (1998) Stimulation of locus coeruleus neurons by non-I_1/I_2-type imidazoline receptors: an *in vivo* and *in vitro* electrophysiological study. Br J Pharmacol 125:1685–1694.

Vauquelin G, De Backer JP, Ladure P, Flamez A (1999) Identification of I_1 and I_2 imidazoline receptors in striatum membranes from different species. Ann NY Acad Sci 881:135–143.

Wagstaff AJ, Bryson HM (1997) Tizanidine. A review of its pharmacology, clinical efficacy and tolerability in the management of spasticity associated with cerebral and spinal disorders. Drugs 53:435–452.

Wallenstein S, Zucker CL, Fleiss JL (1980) Some statistical methods useful in circulation research. Circ Res 47:1–9.

Zaitsev SV, Efanov AM, Raap A, Efanova IB, Schloos J, Steckel-Hamann B, Larsson O, Östenson CG, Berggren PO, Mest HJ, Efendic S (1999) Different modes of action of the imidazoline compound RX871024 in pancreatic β-cells. Blocking of K^+ channels, mobilization of Ca^{2+} from endoplasmic reticulum, and interaction with exocytotic machinery. Ann NY Acad Sci 881:241–252.

Zhang J, Abdel-Rahman AA (2005) Mitogen-activated protein kinase phosphorylation in the rostral ventrolateral medulla plays a key role in imidazoline (I_1)-receptor-mediated hypotension. J Pharmacol Exp Ther 314:945–952.

Zhang J, El-Mas MM, Abdel-Rahman AA (2001) Imidazoline I_1 receptor-induced activation of phosphatidylcholine-specific phospholipase C elicits mitogen-activated protein kinase phosphorylation in PC12 cells. Eur J Pharmacol 415:117–125.

Zucker RS (1989) Short-term synaptic plasticity. Annu Rev Neurosci 12:13–31.