Ifenprodil reduces excitatory synaptic transmission by blocking presynaptic P/Q type calcium channels

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Ifenprodil reduces excitatory synaptic transmission by blocking presynaptic P/Q type calcium channels. J Neurophysiol 107: 1571–1575, 2012. First published December 28, 2011; doi:10.1152/jn.01066.2011.—Iafenprodil is a selective blocker of NMDA receptors that are heterodimers composed of GluN1/GluN2B subunits. This pharmacological profile has been extensively used to test the role of GluN2B-containing NMDA receptors in learning and memory formation. However, ifenprodil has also been reported to have actions at a number of other receptors, including high voltage-activated calcium channels. Here we show that, in the basolateral amygdala, ifenprodil dose-dependently blocks excitatory transmission to principal neurons by a presynaptic mechanism. This action of ifenprodil has an IC50 of ~10 μM and is fully occluded by the P/Q type calcium channel blocker α-agatoxin. We conclude that ifenprodil reduces synaptic transmission in the basolateral amygdala by partially blocking P/Q-type voltage-dependent calcium channels.

NMDA receptors are ionotropic glutamate receptors that are present at most excitatory synapses in the mammalian central nervous system where they play key roles in synapse formation and synaptic plasticity (Cull-Candy et al. 2001; Paoletti and Neyton 2007). Like most ionotropic receptors, NMDA receptors are heteromeric ion channels assembled from several distinct subunits (Paoletti and Neyton 2007). Functional receptors are thought to be tetramers that contain two obligatory GluN1 subunits, and two of either GluN2 (GluN2A - GluN2D) or GluN3 subunits (Paoletti and Neyton 2007). Of these, in the mature brain, GluN2A/B subunits are the predominant subunits in forebrain, whereas GluN2C and GluN2D subunits appear to be restricted to some interneurons (Monyer et al. 1994). The subunit composition of synaptic NMDA receptors undergoes a developmental program such that at birth only GluN1/GluN2B heterodimers are present, and, with maturation, GluN2B subunits are gradually replaced by GluN2A (Monyer et al. 1994). At adult synapses, while both GluN1/GluN2A and GluN1/GluN2B diheteromeric receptors are thought to be present (Cull-Candy et al. 2001; Paoletti and Neyton 2007), coimmunoprecipitation and molecular electrophysiological studies indicate that triheteromeric GluN1/GluN2A/GluN2B receptors are also present (Al-Hallaq et al. 2007; Rauner and Kohr 2011; Sheng et al. 1994).

NMDA receptors play a central role in the induction of different forms of synaptic plasticity that are thought to be the cellular mechanism that underlies learning and memory formation (Malenka and Bear 2004). The presence of different subtypes of NMDA receptors has raised the question of whether different types of receptors may be responsible for different types of synaptic plasticity and learning (Yashiro and Philpot 2008). The phenylethanolamine ifenprodil [2-(4-benzyl-piperidino)-1-(4-hydroxyphenyl)-1-propanol] was initially used as a vasodilator and anti-ischemic (Young et al. 1981) but was subsequently found to be a selective antagonist at GluN2B-containing NMDA receptors (Williams 1993). GluN2B subunits have a high affinity binding site for ifenprodil (Perin-Dureau et al. 2002), and expression studies have shown that ifenprodil blocks GluN1/GluN2B heterodimers with 400 times greater selectivity than GluN1/GluN2A heterodimers (IC50 ~0.3 μM for GluN1/GluN2B compared with ~140 μM for GluN1/GluN2A) (Williams 1993). Consequently, ifenprodil has been extensively used to study the contribution of GluN1/GluN2B NMDA receptors in synaptic physiology and learning and memory formation (Cull-Candy et al. 2001; Paoletti and Neyton 2007; Yashiro and Philpot 2008).

The amygdala is a temporal lobe structure that plays a central role in the acquisition and expression of fear memories (Pape and Pare 2010; Sah et al. 2003). Neurons in the basolateral amygdala receive glutamatergic inputs from both cortical and subcortical structures, and NMDA receptor-dependent synaptic plasticity in both the basolateral amygdala and associated structures underlies fear learning and its extinction (Pape and Pare 2010; Sah et al. 2003). Electrophysiological and immunohistochemical studies have shown that NMDA receptors containing GluN2A as well as GluN2B subunits are present at excitatory synapses in the amygdala (Lopez de Armentia and Sah 2003; Mahanty and Sah 1999; Weisskopf and LeDoux 1999). When using ifenprodil as a selective antagonist of GluN1/GluN2B heterodimers, it has been suggested that synaptic plasticity that mediates fear learning results from specific activation of these receptors (Rodrigues et al. 2001; Sotres-Bayon et al. 2007; Walker and Davis 2008; Zhang et al. 2008). However, NMDA receptor antagonism is not the only reported pharmacological effect of ifenprodil. At concentrations similar to those used to block NMDA receptors, ifenprodil has also been found to inhibit alpha-adrenoceptors (Hondo et al. 1988), 5-hydroxytryptamine3 (5-HT3) receptors (McCoo and Lovinger 1995), and high voltage-activated voltage-dependent calcium channels (VDCCs) (Bath et al. 1996; Church et al. 1994).

Here we show that in the basolateral amygdala, ifenprodil attenuates excitatory synaptic transmission by blocking presynaptic P/Q-type VDCC.
METHODS

Acute brain slices were prepared from 21- to 28-day-old Wistar rats in accordance within the guidelines of the University of Queensland Institutional Animal Ethics Committee. The study was approved by the Animal Ethics Committee. Rats were anesthetized using isoflurane and were decapitated, and their brains were removed into ice-cold artificial cerebral spinal fluid (ACSF) solution containing (in mM) 118 NaCl, 25 NaHCO3, 10 glucose, 2.5 CaCl2, 1.2 Na2HPO4, and 1.3 MgCl2. Brains were sliced into 350-μm coronal sections using a Leica VT1000S vibratome at 0°C, transferred to a chamber containing ACSF at 33–34°C for 30 min, and then maintained for several hours in ACSF at room temperature.

Whole cell recordings were made from brain slices maintained in a recording chamber continuously perfused with oxygenated ACSF at 32–33°C. Recording electrodes (3–5 MΩ) were filled with pipette solution containing (in mM) 135 CsMeSO4, 8 NaCl, 10 HEPES, 2 Mg2ATP, and 0.3 Na3GTP (pH 7.2 with CsOH), osmolarity 290 mOsm/kg. Voltage clamp recordings were made using a patch-clamp amplifier (Multiclamp 700A, Molecular Devices) controlled by an Apple iMac computer running Axograph (Axograph X). Current signals were filtered at 4–8 kHz and digitized at 20 kHz (Instrutech ITC18), acquired, stored, and analyzed using Axograph. Access resistance was monitored and maintained at 5–15 MΩ throughout the experiment. GABAergic transmission was blocked with picrotoxin (100 μM) and CGP53855A (1 μM) in the ACSF. CGP538551 was added to block GABA(B) receptors, as these receptors are well known to also be coupled to presynaptic calcium channels (Reid et al. 1998).

Excitatory postsynaptic currents (EPSCs) were evoked using a bipolar stimulating electrode placed just over the external capsule. For paired pulse ratio (PPR) experiments, paired stimuli were delivered with an interpulse interval of 50 ms. PPR was calculated by dividing the amplitude of the second EPSC of the pair by the amplitude of the first. Illustrated responses represent the average of 10–50 individual trials. Unless otherwise indicated, Student’s t-tests were used for statistical comparisons between groups. Spontaneous synaptic events (minis) were detected using a scaled template consisting of a sum of two exponentials (Clements and Bekkers 1997) implemented in Axograph. For each condition, more than 30 segments each 0.8- to 1.8-s duration were scanned. All results are expressed as means ± SE. Picrotoxin, CGP538545A, and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzo[1:4]quinazoline-7-sulfonamide (NBQX) were prepared as stock solutions in DMSO and diluted in ACSF when required. Cytochrome c (0.1 mg/ml) was added to the perfusate prior to application of ω-agatoxin IVA and ω-agatoxin TK to reduce binding of these compounds to the perfusing tubes. ω-agatoxin IVA and ω-agatoxin TK were purchased from Alomone. Picrotoxin and cytochrome c were purchased from Sigma-Aldrich. All other drugs were purchased from Tocris.

RESULTS

Wholecell recordings were made from principal neurons in the basolateral amygdala, and EPSCs were evoked at a holding potential of −65 mV by electrical stimulation in the external capsule. Stimuli were delivered using paired pulse stimulation with an interstimulus interval of 50 ms. At this holding potential, EPSCs in basolateral amygdala neurons are mediated AMPA-type glutamate receptors, and the contribution of NMDA receptors is minimal due to the block by extracellular magnesium (Mahanty and Sah 1999). Application of ifenprodil produced a dose-dependent block of AMPA receptor-mediated EPSCs with an IC50 of 10.8 μM (Fig. 1, A and B). On washout of ifenprodil, application of NBQX fully blocked the EPSC, confirming that these currents were mediated solely by AMPA receptors. Blockade of the AMPA receptor-mediated EPSC was accompanied by a clear change in PPR (Fig. 1A). The mean control PPR was 1.10 ± 0.11, increasing to 1.29 ± 0.15 in 10 μM ifenprodil (P < 0.05, n = 9), suggesting that the attenuation of the EPSC in ifenprodil results from a reduction in the probability of transmitter release from these terminals (Delaney et al. 2007).

At these synapses, it has been suggested that NMDA receptors are also present presynaptically where they respond to glutamate released from thalamic afferents to the amygdala (Humeau et al. 2003). It is thus conceivable that ifenprodil’s actions could be due to inhibition of presynaptic NMDA receptors. To test this possibility, we applied the broad spectrum NMDA receptor antagonist D-APV. Application of D-APV (30 μM) that fully blocks NMDA receptor-mediated EPSCs at these synapses (Mahanty and Sah 1999) had no significant effect on AMPA receptor-mediated EPSCs (3.5 ± 3.5% change in EPSC amplitude at Vm −65, P = 0.71, n = 5). Moreover, in the presence of D-APV, ifenprodil continued to block AMPA receptor-mediated EPSCs in these cells (Fig. 1C). These results show that ifenprodil reduces excitatory synaptic transmission at these synapses by a mechanism independent of NMDA receptors.

To test if ifenprodil may be having a direct effect on postsynaptic AMPA receptors, we examined its effects on AMPA receptor-mediated currents evoked by direct iontophoretic application of glutamate. Recordings were made at holding potential of −65 mV and in the presence of the noncompetitive NMDA receptor antagonist MK801 (10 μM) to prevent activation of NMDA receptors and picrotoxin to block inhibitory transmission. Glutamate-evoked currents were insensitive to ifenprodil (10 μM, Fig. 2D) with a change of 1.9 ± 2.1% (n = 4, P = 0.35), showing that the reduction in the AMPA receptor-mediated EPSC by ifenprodil is not due to an action at postsynaptic AMPA receptors. The change in PPR in the presence of ifenprodil suggests that the inhibitory effect on the EPSC was likely due to an inhibitory effect on transmitter release (Zucker 1989). Another measure of presynaptic release is the frequency of spontaneous synaptic events. We therefore recorded AMPA receptor-mediated spontaneous EPSCs (sEPSCs) in the presence of APV and picrotoxin to block NMDA, GABA, and glycine receptors. Application of ifenprodil (10 μM) led to significant reduction in the frequency of sEPSCs (Fig. 2). The mean sEPSC frequency decreased from 8.1 ± 2.1 Hz in control conditions to 6.3 ± 1.6 Hz in the presence of ifenprodil (n = 5), an average decrease of 22 ± 5% (Fig. 2C; P < 0.05). Consistent with the lack of effect on postsynaptic AMPA receptors, the mean amplitudes of sEPSCs was not affected by ifenprodil (Fig. 2, B and C; 2.6 ± 6.2% increase, P > 0.05, n = 5). Together, these results show that ifenprodil reduces excitatory transmitter release independent of its actions on NMDA receptors.

Several receptors and channels that can regulate transmitter release have been found to be inhibited or inactivated by ifenprodil, including alpha-adrenoceptors (Honda et al. 1988), 5-HT3 receptors (McCool and Lovinger 1995), and high voltage-activatedVDCCs (Bath et al. 1996; Church et al. 1994). We found that evoked synaptic currents were unaffected by the 5-HT3 receptor antagonist Y25130 (10 μM; 1.8 ± 6.1% increase, P > 0.05, n = 5; not shown), the alpha-1 specific adrenoceptor antagonist prazosin (5 μM), and the alpha-2 specific adrenoceptor antagonist yohimbine (10 μM; 0.1 ±
Transmitter release is evoked by calcium influx via activation of presynaptic VDCCs during the action potential (Katz 1966). These channels form a large gene family, and, in neurons, N-, P-, and Q-type VDCCs are major contributors to evoked transmitter release (Reid et al. 2003). The effect of ifenprodil on high voltage-activated calcium channels has been, in part, localized to P-type VDCCs (Biton et al. 1995). We therefore tested if the actions of ifenprodil on AMPA receptor-mediated EPSCs could result from effects on P-type VDCCs. Application of the specific P/Q type calcium channel blockers ω-agatoxin IVA (200–300 nM) or ω-agatoxin TK (200 nM) (Mintz et al. 1992) led to a suppression of transmitter release (Fig. 3) reducing the EPSC by 72 ± 10% (n = 4; P = 0.03). In the presence of ω-agatoxin, application of ifenprodil had little effect on the EPSC (9 ± 11% reduction; P = 0.18; n = 5), significantly less than the 36.6 ± 4.4% reduction in the absence of ω-agatoxin (P < 0.01; n = 10). The occlusion of ifenprodil’s actions is consistent with its effects being mediated by blockade of presynaptic P-type VDCCs.

DISCUSSION

In this study, we have shown that application of ifenprodil to excitatory synapses in the basolateral amygdala reduces synaptic transmission in a dose-dependent manner. This effect can be seen as a reduction in evoked glutamatergic synaptic currents, as well as a reduction in the frequency of spontaneous EPSCs. The concentration dependence of this blockade is quite steep, with an IC₅₀ of ~10 μM. The reduction in EPSC amplitude was accompanied by an increase in the PPR, indicating that the effect of ifenprodil was presynaptic. Application of the P-type calcium channel blocker ω-agatoxin also reduced transmitter release and occluded the actions of ifenprodil. Ifenprodil has previously been reported to partially block voltage-gated calcium channels in cultured hippocampal neurons (Church et al. 1994), although the identity of the calcium channel was not tested in that study. Moreover, the same concentration of ifenprodil (10 μM) has also been shown to reduce potassium-mediated ⁴⁵Ca uptake into brain synaptosomes (Honda et al. 1989), a signal that was blocked by P-type calcium channel blockers (Mintz et al. 1992). Together, these results suggest that ifenprodil reduces transmitter release by an action on presynaptic P-type calcium channels.

Cortical inputs to basolateral amygdala neurons have been proposed to express NMDA receptors (Humeau et al. 2003). However, these NMDA receptors were found to not be activated by glutamate released from these terminals but instead sense glutamate released from nearby thalamic afferents to the same neurons. Our findings that APV had no effect on basal synaptic transmission (Fig. 2, see also Faber et al. 2005) are consistent with this proposal.

The effects of ifenprodil on transmitter release were seen at concentrations that selectively block NMDA receptors (Chenard and Menniti 1999; Lopez de Armentia and Sah 2003) and well...
within the range often used in slice-recording based studies (3–10 μM). However, ifenprodil is also widely used in testing potential roles of GluN2B-containing NMDA receptors in a variety of amygdala-dependent learning studies. In these studies, it is typically dissolved at a concentration of 3–4 μg/μl and infused to selected targets in vivo (Laurent and Westbrook 2008; Rodrigues et al. 2001; Sotres-Bayon et al. 2007; Zhang et al. 2008). This represents a concentration of ifenprodil of 10 mM at the tip of the infusion pipette, and despite the dilution of the agent as it is infused, our results suggest that it would be difficult to test for a specific effect of ifenprodil on GluN2B containing NMDA receptors without also affecting synaptic transmission at synapses where P-type calcium channels are present. For example, in hippocampal dentate gyrus granule cells, where presynaptic P/Q type channels are present at excitatory synapses, ifenprodil has been reported to reduce the frequency of spontaneous EPSCs (Dalby and Mody 2003).

We have shown that ifenprodil has presynaptic inhibitory actions at excitatory synapses in the BLA by acting at P-type voltage-dependent calcium channels. The potency of block of GluN2B containing receptors and calcium channels is very close, and given the fourth power relationship between calcium

**Fig. 3. Blockade of P/Q-type VDCC occludes the actions of ifenprodil.** A: time course plot showing evoked EPSC amplitude and PPR before and during application of the P/Q-type calcium channel blocker ω-agatoxin IVA. Average EPSCs before and after application of ω-agatoxin IVA are shown. B: time course plot showing EPSC amplitude and PPR before and during application of ifenprodil (indicated by bar) in the presence of ω-agatoxin IVA. Average EPSCs before and after application of ifenprodil are shown. C: bar graph showing average effect of agatoxin on evoked EPSCs and the effect of ifenprodil applied in the presence of ω-agatoxin (**P < 0.01). Recordings were obtained in the presence of D-APV (μM).
influx and transmitter release (Dodge and Rahamimoff 1967), our results indicate that off-target actions of ifenprodil can be a major confound in interpreting the actions of ifenprodil at NMDA receptors.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.J.D. and P.S. conception and design of research; A.J.D. and J.M.P. performed experiments; A.J.D. and J.M.P. analyzed data; A.J.D., J.M.P., and P.S. prepared figures; P.S. drafted manuscript; P.S. approved final version of manuscript.

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