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Zinc Protoporphyrin-IX Blocks the Effects of Metabotropic Glutamate Receptor Activation in the Rat Nucleus Tractus Solitarii

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SUMMARY

The effects of the metabotropic glutamate receptor agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid [(1*S*,3*R*)-ACPD] on ionic current responses produced by ionotropic glutamate and γ-aminobutyric acid $(GABA)_A$ receptor activation in the nucleus of the tractus solitarius (NTS) were examined. Recordings were made in the dorsomedial subdivision of the NTS adjacent to the area postrema in transverse brainstem slices of the rat. (1*S*,3*R*)-ACPD produced a small inward current (I_{ACPD}) associated with a decrease in conductance in approximately 50% of recordings. Monosynaptic excitatory postsynaptic currents (EPSCs) evoked by electrical stimulation in the region of the tractus solitarius in the presence of D-amino-5-phosphonopentanoic acid and bicuculline were reversibly reduced by (1*S*,3*R*)-ACPD in >90% of cells. The inward current evoked by pressure application of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (I_{AMPA}) was potentiated in the presence of (1*S*,3*R*)-ACPD, whereas the outward current evoked by the $GABA_A$ receptor agonist muscimol (I_{MUSC}) was inhibited. We have previously demonstrated that these effects may involve the activation of soluble guanylate cyclase. The diffusible second messengers nitric oxide and carbon monoxide are known to activate soluble guanylate cyclase. The nitric oxide synthase inhibitor L-ω-nitroarginine failed to inhibit responses to (1*S*,3*R*)-ACPD. The selective heme oxygenase inhibitor Zn-protoporphyrin-IX, which would be expected to block the production of carbon monoxide, antagonized the effects of (1*S*,3*R*)-ACPD on EPSCs, I_{AMPA}, and I_{MUSC}. However, I_{ACPD} was not blocked. A relatively inactive metalloprotoporphyrin, Cu-protoporphyrin-IX, was ineffective. A cell-permeant form of cGMP, 8-Br-cGMP, inhibited EPSCs, I_{AMPA}, and I_{MUSC} in the presence of Zn-protoporphyrin-IX but did not induce an inward current. These results further support the hypothesis that multiple metabotropic glutamate receptors exist in the NTS, and they suggest that one of these may be coupled to the activation of a soluble guanylate cyclase via the liberation of an easily diffusible second messenger such as carbon monoxide.

In vivo and *in vitro* evidence indicates that the excitatory amino acid glutamate is the principle transmitter of vagal projections to the NTS, mediating the afferent arm of the baroreceptor reflex (1–3). Glutamate can act on two principle classes of receptor, i.e., 1) ionotropic glutamate receptors, which can be distinguished pharmacologically by their selective activation by AMPA, kainate, or *N*-methyl-D-aspartate, and 2) mGluRs, which can be selectively activated by (1*S*,3*R*)-ACPD (4, 5). A number of signal transduction pathways linked to mGluR activation have been identified (6–8). In many cases, however, the mechanisms underlying mGluR effects remain to be elucidated (9).

mGluRs are widely distributed in the central nervous system (10). (1*S*,3*R*)-ACPD has been shown to modulate neurotransmission in a number of brain regions (11–14). In our previous studies, we examined the effects of mGluR activation on the actions of glutamate and GABA in the dorsomedial NTS (15, 16). We observed diverse modulatory effects of (1*S*,3*R*)-ACPD on glutamatergic transmission evoked from the region of the TS, an area that includes vagal afferent projections to the NTS (1, 17, 18). The ability of (1*S*,3*R*)-ACPD to reduce monosynaptic, DNQX-sensitive EPSCs appeared to be mediated at a presynaptic locus, inasmuch as the postsynaptic sensitivity of the AMPA receptor population, determined by examining

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ABBREVIATIONS: NTS, nucleus of the tractus solitarius; (1*S*,3*R*)-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP5, D-amino-5-phosphonopentanoic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; aCSF, artificial cerebrospinal fluid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; TTX, tetrodotoxin; NO, nitric oxide; EPSC, excitatory postsynaptic current; I_{ACPD}, inward current evoked by (1*S*,3*R*)-ACPD; I_{AMPA}, inward current evoked by AMPA; I_{MUSC}, outward current evoked by muscimol; mGluR, metabotropic glutamate receptor; TS, tractus solitarius; GABA, γ-aminobutyric acid; Zn-P, zinc-protoporphyrin-IX; Cu-P, copper-protoporphyrin IX; L-nitro-Arg, L-ω-nitroarginine; EPSP, excitatory postsynaptic potential; AP, area postrema.

I_{AMPA}, was actually enhanced in the presence of (1*S*,3*R*)-ACPD (16).

In addition to its effects on glutamatergic transmission, (1*S*,3*R*)-ACPD also induces a direct excitatory effect on a subpopulation of dorsomedial NTS neurons (I_{ACPD}), resulting from the inhibition of a K^+ conductance (15). This effect can be produced not only by (1*S*,3*R*)-ACPD but also by endogenously released transmitter after low frequency tetanus in the region of the TS (15).

Glutamate receptors can also modulate transmission mediated by GABA, the principal inhibitory transmitter of the central nervous system (19). A possible role for mGluRs in this response has recently been demonstrated (14). The NTS provides a unique opportunity for investigating this phenomenon, because there appear to be no presynaptic interactions between GABAergic interneurons and glutamate-containing terminals of vagal afferents (20). Thus, the (1*S*,3*R*)-ACPD-mediated reductions in monosynaptic inhibitory postsynaptic currents evoked from the region of the TS (15) probably result entirely from the observed potent decrease in the sensitivity of postsynaptic $GABA_A$ receptors (I_{MUSC}) produced by (1*S*,3*R*)-ACPD.

We previously noted that known activators of guanylate cyclase in the NTS, such as atrial natriuretic peptide (3), brain-derived natriuretic peptide (17, 21), or a cell-permeant cGMP analog (8-Br-cGMP), mimicked the ability of (1*S*,3*R*)-ACPD to inhibit EPSCs, inhibitory postsynaptic currents, or I_{MUSC} and to enhance I_{AMPA}, while failing to affect I_{ACPD} (16). We hypothesized that a diffusible second messenger, such as NO, which would be expected to stimulate guanylate cyclase (22), might couple mGluR activation to cGMP production in the NTS (16). However, the NO-releasing agent sodium nitroprusside did not mimic the actions of (1*S*,3*R*)-ACPD, nor were they blocked by extracellularly applied hemoglobin (16). These data raised the possibility that another diffusible activator of guanylate cyclase, generated and acting within the cell, could mediate some of the observed effects of (1*S*,3*R*)-ACPD.

Recently, the possibility that CO may be a major physiological regulator of guanylate cyclase in the brain has emerged (23–25). The distribution of heme oxygenase-2, which can potentially produce CO release in neurons, is more highly correlated with the localization of guanylate cyclase than is that of NO synthase (26, 27). We now report that the compound Zn-P, which inhibits heme oxygenase (25, 27), is an effective antagonist of several (1*S*,3*R*)-ACPD effects in the NTS. This appeared to be due to selective inhibition of heme oxygenase, inasmuch as the relatively inactive metalloprotoporphyrin Cu-P (28) failed to block the same effects.

Materials and Methods

Experiments were performed on 51 neurons in the dorsomedial subdivision of the NTS at the level of the AP, in 225–250-μ transverse brainstem slices prepared from Holtzman rats of age 26–37 days, as described previously (15, 16).

Slices were continuously perfused (4–6 ml/min) with aCSF (in mM: NaCl, 126; $NaHCO_3$, 26.2; NaH_2PO_4, 1; KCl, 3; $MgSO_4$, 1.5; $CaCl_2$, 2.5; glucose, 10) and continuously gassed with 95% O_2/5% CO_2. Experiments were performed under conditions of voltage clamp, with the exception of three of nine experiments examining evoked excitatory transmission. In these cells, V_m was maintained at a constant level via direct current injection. Currents or potentials were recorded in the discontinuous single-electrode current- or voltage-clamp mode of the amplifier (V_{hold}, −50 to −80 mV) with whole-cell patch electrodes containing the following (in mM): potassium gluconate, 145; $MgCl_2$, 2; $CaCl_2$, 0.1; HEPES, 5; EGTA, 1.1; and K_2ATP, 5; pH 7.2. A bipolar stimulating electrode was placed in the ipsilateral TS. EPSPs/EPSCs were evoked (0.1 Hz) in the presence of the *N*-methyl-D-aspartate antagonist AP5 (50 μM) and the $GABA_A$ agonist bicuculline (10 μM). I_{AMPA} was evoked by brief pressure ejection (15–20 msec, 3–7 psi) of (*S*)-AMPA (1–10 mM), in bicuculline/AP5-containing aCSF supplemented with 1 μM TTX, from a blunt patch pipette directed at the soma under visual (400× magnification) guidance. I_{MUSC} was evoked by brief pressure ejection of muscimol (10 mM) in AP5/DNQX-containing aCSF. In some cases, I_{MUSC} was evoked in the presence of TTX. Results in the presence or absence of TTX were indistinguishable and are pooled in the present study. I_{ACPD} was observed in approximately 50% of cells exposed to (1*S*,3*R*)-ACPD at a concentration of 25 μM or greater.

Zn-P, Cu-P, or L-nitro-Arg was continuously applied for 2–6 min before (1*S*,3*R*)-ACPD, to ensure adequate cellular permeation. The mean percentages of control current in the presence of 25 μM (1*S*,3*R*)-ACPD [(peak control current/peak current with (1*S*,3*R*)-ACPD) × 100] were pooled and compared with the values recorded in the presence of Zn-P (3 μM) or with the combined application of Zn-P and 8-Br-cGMP (100 μM). Control and treatment values were calculated separately for Cu-P (3 μM) and L-nitro-Arg (100 μM). Recovery of (1*S*,3*R*)-ACPD effects were observed in some cases after >30-min washout of Zn-P.

Results and Discussion

As illustrated in Fig. 1, (1*S*,3*R*)-ACPD produced a reversible reduction in the amplitude of the EPSP evoked by stimulation

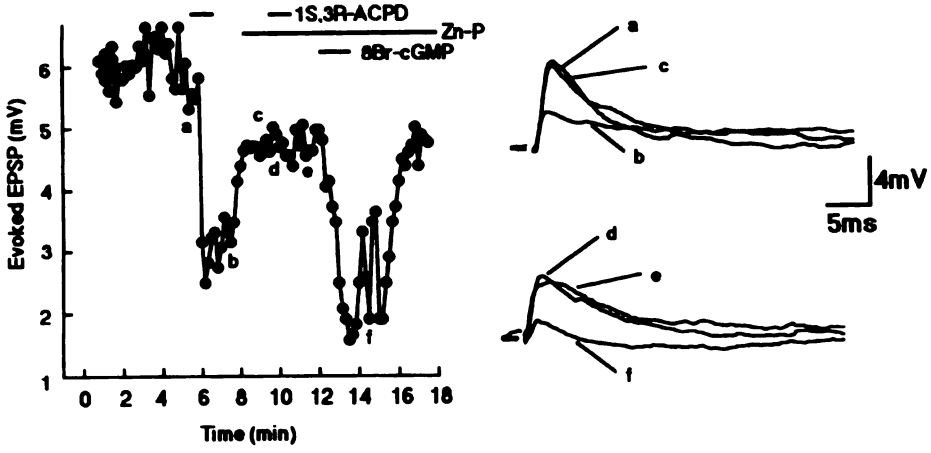

Fig. 1. *Left*, plot of the peak amplitude of monosynaptic EPSPs evoked from the region of the TS. The results illustrate the reversible inhibition of the EPSP by the addition of 25 μM (1*S*,3*R*)-ACPD. Addition of 3 μM Zn-P blocked the inhibitory effects of (1*S*,3*R*)-ACPD on the EPSP. At 100 μM, 8-Br-cGMP reversibly reduced the EPSP in the continued presence of Zn-P. *Right*, high-time resolution traces of the EPSP and effects of (1*S*,3*R*)-ACPD, Zn-P, and 8-Br-cGMP, taken from the plot (*left*) at the indicated positions. Control V_m in this cell was −57 mV.

in the region of the TS. In the presence of Zn-P, (1*S*,3*R*)-ACPD failed to diminish the evoked EPSP. However, in the continued presence of Zn-P, 8-Br-cGMP was able to completely mimic the ability of (1*S*,3*R*)-ACPD to reduce excitatory transmission. As shown in Fig. 1, the addition of 3 μM Zn-P itself occasionally produced a small reduction in the amplitude of the EPSP. This diminution of whole-cell potentials/currents by Zn-P was observed in approximately 50% of cells in the present study. As summarized in Fig. 5 (see below), evoked EPSP/EPSCs were identically inhibited by (1*S*,3*R*)-ACPD alone (46.1 ± 2.0% of control, $n = 9$) or by 8-Br-cGMP in the presence of Zn-P (47.2 ± 1.0% of control, $n = 3$). In contrast, (1*S*,3*R*)-ACPD was ineffective in the presence of Zn-P (91.0 ± 2.6% of control, $n = 9$). The reduction in the effectiveness of (1*S*,3*R*)-ACPD was not due to desensitization, because multiple applications of this glutamate agonist have been shown to be equally effective in this paradigm (15). Although the effective concentration of Zn-P used in the present study was 1 order of magnitude higher than the K_i for inhibition of heme oxygenase-2 in rat brain microsomal membranes (25), this may reflect the lower accessibility of this compound to its locus of action in brain slices. Lower concentrations of Zn-P (50–100 nM) proved only moderately effective in preliminary studies, and higher concentrations could not be used due to solubility limitations. The largely inactive Cu-P (3 μM) failed to antagonize (1*S*,3*R*)-ACPD effects on the EPSC [(1*S*,3*R*)-ACPD alone, 49.9 ± 15.1% of control; with Cu-P, 46.3 ± 14.9% of control; $n = 3$].

Our previous investigations demonstrated that the effects of (1*S*,3*R*)-ACPD on glutamatergic transmission were mediated at both pre- and postsynaptic loci (15, 16). To isolate the postsynaptic effects of (1*S*,3*R*)-ACPD on ionotropic glutamate receptors, brief pressure pulses of AMPA were used. We previously reported that I_{AMPA} could be enhanced in a reversible and nondesensitizing manner by (1*S*,3*R*)-ACPD (16). As shown in Fig. 2, the postsynaptic enhancement of I_{AMPA} by (1*S*,3*R*)-ACPD was abolished in the presence of Zn-P. I_{AMPA} was enhanced to 157.5 ± 10.9% of control by (1*S*,3*R*)-ACPD but to only 100.2 ± 2.7% of control ($n = 8$) in the presence of Zn-P. A small and variable decrease in the size of I_{AMPA} was noted in some cells in the presence of Zn-P alone. As with the EPSP/EPSC, however, 8-Br-cGMP fully mimicked the effects of (1*S*,3*R*)-ACPD in the presence of Zn-P (155.9 ± 11.1% of control, $n = 3$; see Fig. 5). As before, Cu-P was completely ineffective [(1*S*,3*R*)-ACPD alone, 120.2 ± 9.3% of control; with Cu-P, 129.4 ± 3.6% of control; $n = 3$].

(1*S*,3*R*)-ACPD or a brief, low frequency tetanus applied to the region of the TS in DNQX/AP5/bicuculline-containing aCSF produces an inward current (I_{ACPD}) in approximately half the neurons of the dorsomedial NTS (15). As shown in Fig. 3, I_{ACPD} produced by exogenous (1*S*,3*R*)-ACPD had identical properties in the absence or presence of Zn-P. Furthermore, in this study and our previous investigation, 8-Br-cGMP was not observed to mimic the ability of (1*S*,3*R*)-ACPD to produce I_{ACPD} (15). The mean current produced by (1*S*,3*R*)-ACPD alone (11.1 ± 1.1 pA) did not differ from that produced in the presence of Zn-P (10.2 ± 2.9 pA, $n = 14$).

In addition to effects on excitatory transmission, the majority of dorsomedial NTS neurons receive inhibitory inputs from the local GABAergic interneurons (15). Curiously, these cells do not appear to make synaptic contacts with the glutamatergic afferent terminals of the TS (20). (1*S*,3*R*)-ACPD is a potent blocker of monosynaptic inhibitory transmission in this region of the NTS (15, 29). This effect seems to be mediated largely at a postsynaptic locus, inasmuch as the actions of the $GABA_A$ agonist muscimol on dorsomedial NTS neurons are strongly depressed in the presence of (1*S*,3*R*)-ACPD (16). In the present study, we examined the effects of Zn-P on the postsynaptic I_{MUSC}. As shown in Fig. 4, Zn-P completely blocked the (1*S*,3*R*)-ACPD-mediated reduction in I_{MUSC}. The mean effect of (1*S*,3*R*)-ACPD on I_{MUSC} (68.5 ± 0.7% of control) was inhibited by Zn-P (99.5 ± 1.9% of control, $n = 7$). As with the EPSP/EPSC and I_{AMPA}, 8-Br-cGMP mimicked the effects of (1*S*,3*R*)-ACPD in the presence of Zn-P (77.7 ± 4.4% of control, $n = 7$; summarized in Fig. 5). Once again, (1*S*,3*R*)-ACPD was equally effective in the presence (37.7 ± 8.2% of control) or absence (39.0 + 9.0% of control, $n = 3$) of Cu-P.

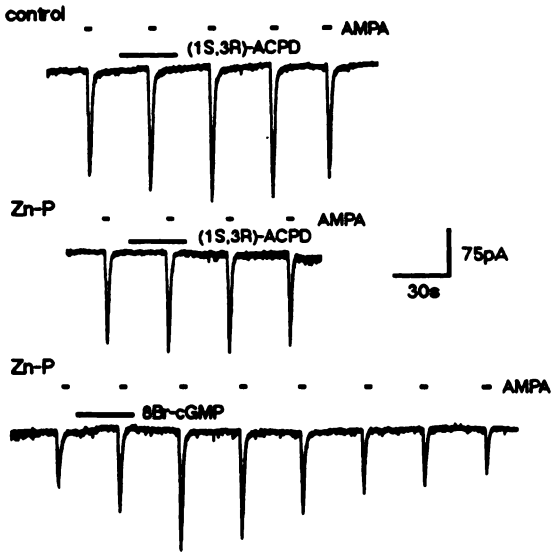

Fig. 2. *Top*, I_{AMPA} in an NTS neuron was enhanced in the presence of 25 μM (1*S*,3*R*)-ACPD. *Middle*, the potentiation of I_{AMPA} produced by (1*S*,3*R*)-ACPD in this cell was markedly reduced in the presence of 3 μM Zn-P. *Bottom*, in the continued presence of Zn-P, addition of 100 μM 8-Br-cGMP reversibly potentiated I_{AMPA} in this cell. V_{hold}, −70 mV.

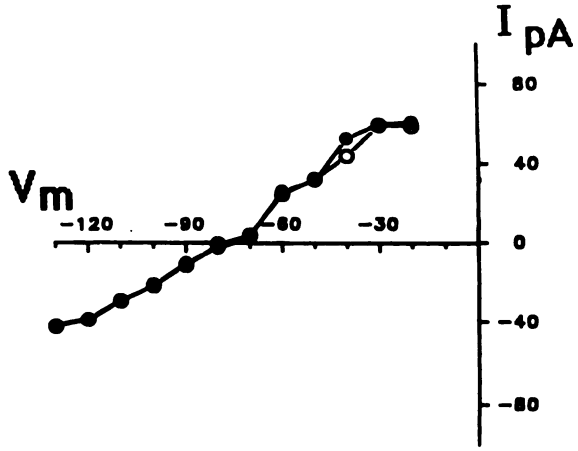

Fig. 3. Steady state current/voltage relationship for I_{ACPD} in an NTS neuron in the absence (○) and presence (●) of Zn-P (3 μM). I_{ACPD} was recorded as the difference between the whole cell current in the absence and presence of 25 μM (1*S*,3*R*)-ACPD, after correction for the control leak current. I_{ACPD} in the presence of Zn-P was determined as the difference between whole-cell current with Zn-P alone and in the presence of both Zn-P and (1*S*,3*R*)-ACPD, after correction for leak current.

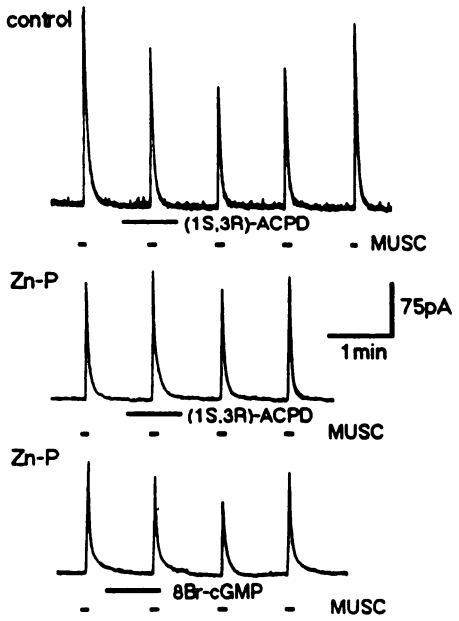

Fig. 4. *Top*, I_{MUSC} was reversibly reduced in the presence of 25 μM (1*S*,3*R*)-ACPD. *Middle*, in the same cell, the response to (1*S*,3*R*)-ACPD was blocked in the presence of 3 μM Zn-P. *Bottom*, in the continued presence of Zn-P, 100 μM 8-Br-cGMP inhibited I_{MUSC}.

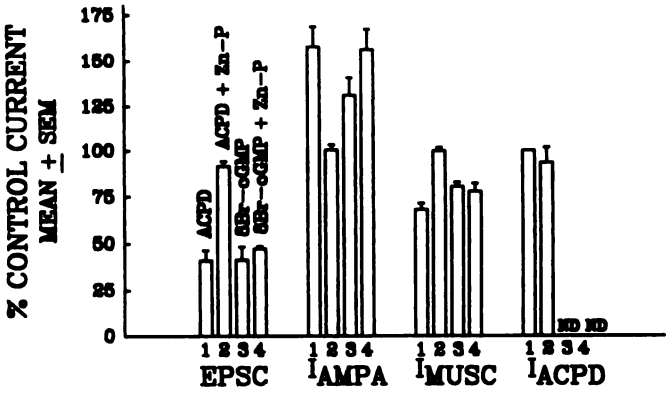

Fig. 5. Summary of (1*S*,3*R*)-ACPD (25 μM) effects in NTS neurons (*bar 1*). Zn-P (3 μM) (*bar 2*) blocked (1*S*,3*R*)-ACPD effects on evoked EPSP/EPSCs (*EPSC*), I_{AMPA}, and I_{MUSC}. Control I_{ACPD} (11.1 ± 1.1 pA, n = 14) was normalized to 100% and compared with the value obtained in the presence of Zn-P (3 μM). 8-Br-cGMP (100 μM) (*bar 3*) substantially mimicked the effects of (1*S*,3*R*)-ACPD; however, I_{ACPD} was not detected (*ND*) in the presence of 8-Br-cGMP (100 μM) or Zn-P. Zn-P failed to block the effects of 8-Br-cGMP (*bar 4*) ($3 \leq n \leq 14$).

These results shed additional light on the mechanism of action of (1*S*,3*R*)-ACPD in the NTS and may have important implications for its actions elsewhere in the brain. As shown here and in our previous studies (15, 16, 30), (1*S*,3*R*)-ACPD produces both pre- and postsynaptic effects in the NTS. All of these, with the exception of I_{ACPD}, can be mimicked by 8-Br-cGMP, as summarized in Fig. 5. This suggests that cGMP might be involved in the signal transduction pathway linking mGluR activation with some of its cellular effects. How then might cGMP be produced in the NTS? The possible role of NO in this respect has been widely discussed, and evidence for its participation in certain phenomena has been presented (13, 18). However, our previous efforts to establish a role for NO in the NTS proved negative (16). In the present study, we confirmed these results with the NO synthase inhibitor L-nitro-Arg. (1*S*,3*R*)-ACPD produced identical effects on I_{MUSC} or EPSCs in the absence or presence of L-nitro-Arg (78.5 ± 11.1 versus 75.9 ± 9.3% of control I_{MUSC}, n = 3; 55.5 ± 7.3 versus 56.7 ± 4.0% of control EPSC, n = 3). Rather than being blocked, (1*S*,3*R*)-ACPD effects on I_{AMPA} were somewhat potentiated in the presence of L-nitro-Arg (179.5 ± 40.7 versus 140.3 ± 9.6% of control, n = 3). These results further suggest that NO is unlikely to mediate the observed (1*S*,3*R*)-ACPD effects in the NTS.

It has recently been pointed out that endogenous CO production could also lead to cGMP synthesis because, like NO, CO can activate guanylyl cyclase (24, 25). The enzyme heme oxygenase could act as a source of CO in neurons. Two forms of this enzyme are found in the brain. Heme oxygenase-1 normally shows only a limited distribution, but its synthesis can be selectively increased in certain neurons and glia through activation of heat shock elements by diverse stimuli (26, 27). In contrast, heme oxygenase-2 is widely distributed in the brain under all conditions (26, 27). Notably, both forms of the enzyme are found in abundance in the brainstem (26).

Zn-P has previously been demonstrated to be an effective inhibitor of heme oxygenase in brain microsomes (25, 27). Thus, the inhibitory effects of Zn-P observed in the present studies, as summarized in Fig. 5, are compatible with a CO/cGMP link in the signal transduction pathway used by (1*S*,3*R*)-ACPD. Activation of mGluRs has been shown to increase cGMP synthesis in the brain in some cases (22). However, stimulation of CO synthesis in the brain by glutamate or any other neurotransmitter receptor agonist remains to be demonstrated. It is interesting to note that activation of mGluRs leads to the potentiation of ionotropic glutamate receptor effects in several areas of the brain in addition to the NTS. These include the cerebellum (29), spinal cord (12), and hippocampus (11). It is not known whether these effects also involve a CO/cGMP link. However, it has been recently reported that Zn-P blocks the production of long term potentiation in the hippocampus (23). This is of interest considering current speculation concerning a role for mGluRs in this phenomenon (13, 31). Finally, we also do not know how cGMP produces the changes in ionotropic receptor activity we have observed. One possible mechanism concerns activation of a protein phosphatase by cGMP-dependent protein kinase, as has been reported recently (32).

Our data with Zn-P also support previous conclusions about the heterogeneity of signal transduction pathways linked to mGluR activation in the NTS (16, 30). Thus, Zn-P failed to reduce I_{ACPD}. This current was also not produced by 8-Br-cGMP, a compound that mimics all of the other effects of (1*S*,3*R*)-ACPD in the NTS. I_{ACPD} is produced by the inhibition of a K^+ conductance, presumably using a different signal transduction pathway. Whether these diverse effects are due to the activation of one or more mGluR subtypes is also unclear at this point. However, recent pharmacological data generated in our laboratory using novel mGluR antagonists have suggested that this may be the case (30).

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