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Dilation of rat brain arterioles by hypercapnia in vivo can occur even after blockade of guanylate cyclase by ODQ

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Abstract

1*H*-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) is an inhibitor of guanylate cyclase and has been reported to inhibit dilation of cerebral blood vessels by hypercapnia. This supports the hypothesis that this dilation is dependent upon guanylate cyclase, activated by nitric oxide (NO) released from neural tissue. However, there are conflicting reports concerning the role of guanylate cyclase in response to hypercapnia. Therefore, we tested the effect of topically applied ODQ (10 μM) on rat pial arterioles observed with a microscope through a closed cranial window. In one study, we tested ODQ ability to inhibit both the dilation produced by hypercapnia (3% and 5% inspired CO_2) and, in the same rats, the dilation produced by *N*-methyl-D-aspartate (NMDA). In another experiment, we tested the ability of ODQ to inhibit dilation produced by hypercapnia and the dilation produced by 3-morpholinosydnonimine (SIN-1), a donor of NO. The responses to NMDA and to NO are known to depend upon activation of guanylate cyclase and were both blocked in the present study. However, the response to hypercapnia was not affected. These findings provide evidence that hypercapnic dilation can occur independently of guanylate cyclase activation.

Keywords: Hypercapnia; Cerebral circulation; Arteriolar diameter; Pial arterioles; Guanylate cyclase; Nitric oxide (NO); NMDA (*N*-methyl-D-aspartate); ODQ; SIN-1 (3-morpholinosydnonimine)

1. Introduction

1*H*-[1,2,4] oxadiazolo [4,3,-a] quinoxalin-1-one (ODQ) has been considered a selective inhibitor of soluble guanylate cyclase (Garthwaite et al., 1995). Nitric oxide (NO) is thought to relax vascular smooth muscle by activating soluble guanylate cyclase (Moncada et al., 1991). Many investigators believe that NO is released by neurons or nerve processes when CO_2 is elevated and that the release of the NO with the subsequent activation of guanylate cyclase is responsible for the dilation of brain blood vessels and the increase in blood flow that results from hypercapnia (Iadecola et al., 1994). ODQ has been used to test this hypothesis and, as predicted, inhibited the cerebrovascular response to hypercapnia (Yang and Iadecola, 1998). This result contrasts with the failure of LY83583, another inhibitor or inactivator of guanylate cyclase (Mulsch et al., 1989) to inhibit the response to hypercapnia (Kontos and Wei, 1996). It also contrasts with the failure to demonstrate involvement of guanylate cyclase in the dilation by hypercapnia of a large cerebral blood vessel in vitro (You et al., 1994).

Because of these contradictory results, we decided to try to confirm the ability of ODQ to inhibit hypercapnic dilation of pial arterioles in vivo. However, to be certain that the ODQ was actually inhibiting guanylate cyclase, we also tested its ability to block the dilation produced by topical application of either NMDA (*N*-methyl-D-aspartate) or 3-morpholinosydnonimine (SIN-1). Both of these drugs are known to produce dilation by activating guanylate cyclase. NMDA does so by interacting with receptors in neural tissue. This in turn causes the release of neural NO which then activates guanylate cyclase in the smooth muscle of overlying pial arterioles which leads to the dilation of these blood vessels (Faraci and Breese, 1993; Meng et al., 1995; Faraci and Sobey, 1999). SIN-1 directly releases NO, which in turn activates guanylate cyclase (Feelisch et al., 1989) to produce the dilation. As the following data show the dose of ODQ that we used virtually abolished the ability of either

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NMDA or SIN-1 to produce dilation even though that same dose had no effect on the response to hypercapnia.

2. Methods

2.1. Preparation

Experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. Male Sprague–Dawley rats (300–380 g body weight) were anesthetized with sodium pentobarbital (55 mg/kg i.p.). A femoral vein was cannulated for additional anesthetic as needed. A femoral artery was cannulated for continuous measurement of blood pressure and periodic determination of blood gas and blood pH values. After completion of a tracheotomy, each rat was ventilated on a positive pressure ventilator following paralysis by pancuronium bromide (3 mg/kg i.v.). During experiments with normocapnic conditions, $PaCO_2$ was adjusted to between 35 and 40 mm Hg and maintained constant throughout each experiment.

2.2. Hypercapnia

The rats were ventilated with a mixture of air containing either 3% or 5% CO_2. Each level of hypercapnia was maintained for at least 7 min prior to the measurement of vessel caliber. In the first experiment, PaO_2, $PaCO_2$ and arterial pH before CO_2 were (M±SD): 98±10, 36±3 mm Hg and 7.48±0.02 pH units, respectively. After 3% CO_2, the values were: 99±11, 52±2 mm Hg and 7.36±0.02. After 5% CO_2, the values were 106±5, 66±2 mm Hg and 7.26±0.02. In the second experiment, the values before CO_2 were 90±11, 36±2 mm Hg and 7.46±0.02. After 3% CO_2, they were 91±9, 52±1 mm Hg and 7.34±0.02. After 5% CO_2, they were 99±11, 63±11 mm Hg and 7.28±0.02. Blood pressure in the experiments was 114±5 and 116±3 mm Hg, respectively, and remained essentially unchanged throughout the experiment.

2.3. Cranial window

Cerebral microcirculation of the parietal cortex was observed with a Wild microscope through an acutely implanted, closed, cranial window (Ellis et al., 1983; Levasseur et al., 1975) filled with mock cerebrospinal fluid (mock CSF) (Raper et al., 1972). There were three outlets from the window. Two were inflow and outflow paths used only to replace the control mock CSF with mock CSF containing a drug, or to wash out such solutions in order to reestablish a baseline. The pH of the fluid placed under the window was always adjusted to 7.35 by equilibration with a mixture of 6% O_2, 6% CO_2 and the balance N_2. Diameter of pial arterioles was measured with a Vickers image-splitting device. All monitoring took place with the mock CSF stationary under the window. The third opening in the window was used to continuously monitor the intracranial pressure, which was maintained at 5 mm Hg with a fluid column also connected to this port and kept at a predetermined height.

2.4. Drugs

ODQ, SIN-1 and NMDA were obtained from Sigma. ODQ is not soluble in water so stock solutions are generally made in either ethanol or dimethylsulfoxide (DMSO). However, we previously demonstrated (Rosenblum et al., 2001a) that in rats prepared as in the current study, DMSO prevents dilation of pial arterioles by openers of the K_{ATP} ion channel. This inhibition occurred with final concentrations of DMSO that would be present if we were to use DMSO to make stock solutions of ODQ. We were unable to find a concentration of DMSO which would dissolve ODQ and yet would, by itself, fail to inhibit the response to openers of K_{ATP} channels when diluted with mock cerebrospinal fluid to the same concentration as that present in the test dose of ODQ. We wished to avoid using a diluent that created this abnormal situation in this preparation. Therefore, we used ethanol instead of DMSO. Ethanol can also block the effect of K_{ATP} channel openers under the experimental conditions used here (Rosenblum et al., 2001a). However, the inhibitory effect of ethanol on the response to K_{ATP} openers diminished as concentrations of ethanol were raised from less than 0.01% to over 0.3%. A final concentration of 0.5% was innocuous (Rosenblum et al., 2001a). Therefore, in the present study, stock solutions of ODQ were made in ethanol at concentrations that, when diluted with mock cerebrospinal fluid, gave a final concentration of 10 μM ODQ in 0.5% ethanol. The dose of ODQ was chosen because others found it effective in blocking cerebrovascular responses that are dependent upon either NO or guanylate cyclase (Sobey and Faraci, 1997; Meng et al., 1998; Faraci and Sobey, 1999). Moreover, others have shown no greater effect of ODQ at doses higher than 10 μM (Brunner et al., 1996; Schrammel et al., 1996).

2.5. Experimental design

The space under the window was filled with mock CSF and the vessel diameters at resting state were measured. Three separate experiments were conducted, each with five rats. In each study, cumulative dose responses to 3% and 5% CO_2 were obtained. Several minutes were required to reach the plateau response to each concentration of CO_2. After the last test of CO_2, the rat is switched back to room air and the experiment resumes once the arterioles have returned to basal diameter. Cumulative responses were also obtained to either two doses of NMDA [experiments one and two] or to two doses of SIN-1 [experiment three]. When NMDA was used, the cumulative response to 100 and 200 μM was tested

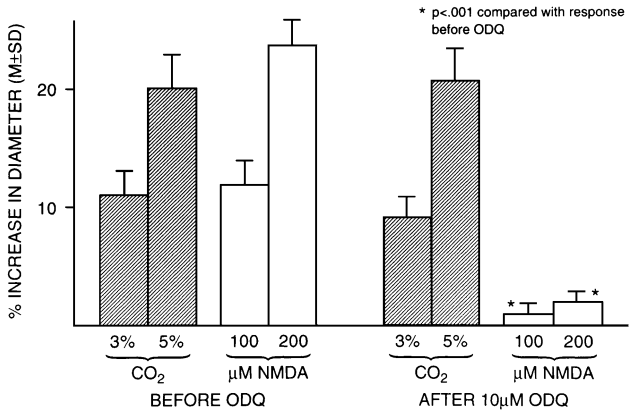

Fig. 1. Results in five rats whose arterioles were 41±4 μm (M±SD) in diameter. Before ODQ, there was dose-dependent dilation to both CO_2 and to NMDA. ODQ had no effect on the response to CO_2 but blocked the response to NMDA.

by replacing the fluid under the window with mock CSF containing the NMDA. When SIN-1 was tested, the cumulative response to doses of 0.5 and 1.0 μM was determined. These doses of NMDA or of SIN-1 gave dilations similar in magnitude to those produced by CO_2.

After these normal responses to CO_2 and either NMDA or SIN-1 were observed, we tested the effect of ODQ on the responses. To do this, the fluid under the window was replaced by mock CSF containing 10 mM ODQ. This was left under the closed window for 15 min after which we retested CO_2 or a dilating drug. During the test of CO_2, the ODQ remained under the window. Then the window was flushed with mock CSF until arterioles returned to basal diameter. To test the effect of ODQ on NMDA [experiments one and two] or on SIN-1 [experiment 3], the mock CSF with ODQ was also placed under the window for 15 min. However, then this fluid was replaced with mock CSF containing either NMDA [experiments one and two] or to SIN-1 [experiment three]. Thus, ODQ was not present during the actual test of these drugs. Since it takes up to 6 min to obtain a two-dose cumulative dose–response, the results below, showing an inhibitory effect of 15 min of ODQ on these two dilators, represent an effect which lasted at least 6 min beyond the removal of the ODQ. No experiments were performed to determine how much longer inhibition would continue.

Though experiments one and two each tested CO_2 and NMDA, there were two differences in design between these experiments. In the first experiment, the 0.5% ethanol [final concentration of diluent in mock CSF] was only present in the solution of ODQ, whereas in the second experiment, 0.5% ethanol was present throughout the experiment. This was done to make sure that the results of the first experiment did not depend, in some way, on the absence of the ethanol from the solutions of NMDA or from the mock CSF during tests of CO_2. The second experiment also differed from the first with respect to the sequence of tests performed. In the first experiment, we always tested the response to CO_2 before the response to NMDA. This meant that by the time the NMDA was tested, the vessels in each rat had already been exposed to ODQ during the prior test of its effect on the response to CO_2. To avoid this potential source of bias, in the second experiment, we alternated the sequence so some rats were tested with NMDA first and CO_2 second. We also alternated the sequence of tests during the third experiment in which the response to SIN-1 substituted for the response to NMDA as an indication of successful blockade of guanylate cyclase by ODQ. So in some rats, the response to CO_2 came before the response to SIN-1 and in some, it came after the test of SIN-1. The sequence of tests had no effect on the results in either experiment two or three. In the experiment with SIN-1, we followed the same procedure as in the second experiment with NMDA and kept 0.5% ethanol present in the mock CSF throughout the experiment, thus avoiding any possible bias produced by having this component of the diluent present only during one portion of the experiment.

Each of the three experiments used five rats. Three to five arterioles were monitored in each rat. In each rat, the responses to the vessels in that rat were averaged for each treatment, and the mean responses for each treatment were used for statistical analysis. Thus, although there were more than 15 arterioles monitored in each experiment, the *N* for the purposes of statistical analysis was 5, in each study. Each rat served as its own control for comparison of pre- and post-ODQ responses. The paired *t*-test was used to compare a response before ODQ with that response after ODQ.

3. Results

3.1. ODQ blocks NMDA but not hypercapnia

As described under Methods, there were two experiments in which NMDA was tested. In the first experiment, shown

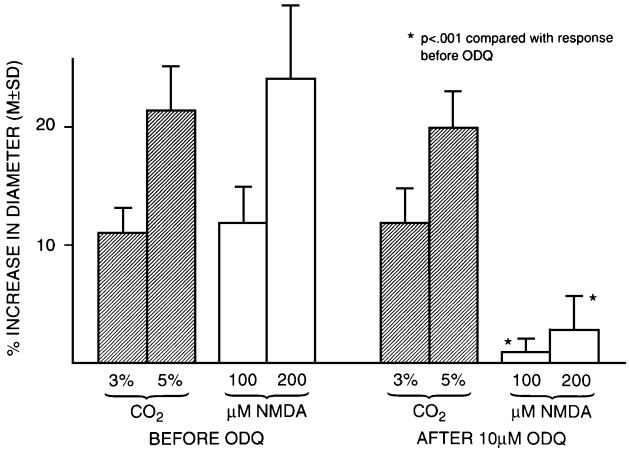

Fig. 2. Results in five rats whose arterioles were 40±4 μm in diameter. Unlike the study illustrated in Fig. 1, there was 0.5% ethanol present, not only in the solution containing ODQ, but throughout the entire study. Nevertheless, the results were exactly the same as in Fig. 1. That is, there was no effect of ODQ on the response to CO_2, but ODQ virtually abolished the response to NMDA.

in Fig. 1, five rats were tested first to CO_2 and then to NMDA. Following this, the same rats were treated with 10 μM ODQ, placed under the pial window for 15 min. Then they were retested with CO_2 and finally retested with NMDA by replacing the fluid under the window with mock CSF containing the NMDA. The arteriolar diameters were 41±4 μm (M±SD) and were not affected by ODQ. The figure shows that there was dose-dependent dilation to CO_2 and to NMDA before ODQ and that after ODQ, the response to CO_2 was not affected but the response to NMDA was virtually abolished.

In this experiment, when the effect of a 15-min application of ODQ was tested, the ODQ in mock CSF with ethanol was not washed out from under the window during the test of the response to CO_2. However, the solution containing ODQ was replaced with ordinary mock CSF immediately prior to the test of NMDA. To rule out the absence of ethanol in the solution of NMDA as a reason for the difference between the effect of ODQ on CO_2 and the effect of ODQ on NMDA, a second experiment was performed. In this experiment, 0.5% ethanol was present throughout the entire series of pre- and post-ODQ tests. Five rats were used. The arteriolar diameter was 40±4 μm (M±SD) and was not affected by the ODQ. Fig. 2 shows that the results were exactly the same as those of the first experiment.

3.2. ODQ blocks SIN-1 but not hypercapnia

Fig. 3 shows the results of the experiment in which tests of CO_2 and of SIN-1 were performed before and after ODQ. SIN-1 produced dose-dependent dilations at doses of 0.5 and 1 μM. Once again, ODQ failed to affect the response to CO_2, while the response to SIN-1 was blocked. The diameter of the arterioles was 45±4 μm (M±SD) and was not affected by ODQ.

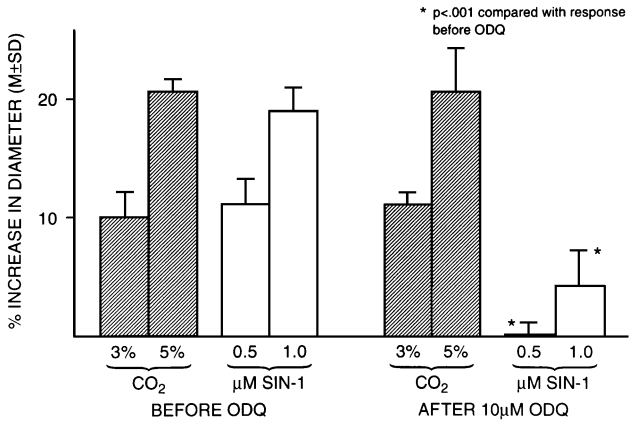

Fig. 3. Results in five rats whose arterioles were 45±4 μm in diameter. SIN-1 and CO_2 both produced dose-dependent dilation. However, only SIN-1 was inhibited by 10 μM ODQ.

4. Discussion

ODQ failed to affect the response to hypercapnia. In contrast, in two experiments, the same dose of ODQ blocked the response to NMDA in the same rats. In the third experiment, the ODQ blocked the response to SIN-1. Since both NMDA and SIN-1 cause dilation by activating guanylate cyclase, we conclude that this enzyme was indeed blocked by the ODQ. Therefore, we conclude that the response to hypercapnia does not require guanylate cyclase activity. Moreover, both NMDA and SIN-1 activate guanylate cyclase by releasing nitric oxide. NMDA does so by releasing nitric oxide from neurons (Faraci and Breese, 1993; Faraci and Sobey, 1999; Meng et al., 1995) while SIN-1 is a nitric oxide donor (Feelisch et al., 1989). Therefore, our results also indicate that nitric oxide is not a necessary mediator of the response to hypercapnia.

These conclusions receive some support from several studies. In one, the basilar artery of the rat was examined in vitro. The response to CO_2 was not accompanied by a rise in cGMP, the product of guanylate cyclase activation (You et al., 1994). In the rat and the cat (Kontos and Wei, 1996), anesthetized and paralyzed with the agents used in the present study, the response of pial arterioles to hypercapnia was not inhibited by LY 83583, another inhibitor of guanylate cyclase (Mulsch et al., 1989). In another study, the response of rat cerebral blood flow to low and to moderate levels of CO_2 was apparently mediated by nitric oxide but the response to very high levels was not (Iadecola and Zhang, 1994). When rabbits were used (Faraci et al., 1994) and the diameter of pial arterioles was measured in vivo, an inhibitor of nitric oxide synthase completely blocked dilation produced by low levels of hypercapnia but inhibited only 60% of the response to a high level of CO_2. Finally, and most convincingly, are experiments (Irikura et al., 1995) using knockout mice that lack the neuronal isoform of nitric oxide synthase. This is the isoform that is thought to support the cerebrovascular response to hypercapnia. In these knockout mice, the response to hypercapnia is normal and unaffected by nonselective inhibitors of nitric oxide synthase. Thus, some mechanism has come into play, which is independent of any nitric oxide synthase. An NO-independent mechanism apparently operated in the present investigations since here, the responses to low or moderate elevations of CO_2 were independent of guanylate cyclase, the mediator of NO-driven responses.

Nevertheless, there is much data to suggest an important role for nitric oxide and for guanylate cyclase in the response to hypercapnia. Of major significance were reports by others (Iadecola et al., 1994) showing that inhibitors of nitric oxide synthase (NOSI) inhibited the response to hypercapnia.

We also found that NOSI inhibited dilation of pial arterioles by hypercapnia in the present preparation (Kontos and Wei, 1996; Rosenblum et al., 2001b). However, in the latter studies, it was also shown that the NOSI had an

additional property; they not only prevented dilation of pial arterioles by CO_2 but also prevented dilation by openers of K_{ATP} ion channels. This effect was demonstrable in rats and cats using the anesthetic and paralytic agent used in the present study. The response to hypercapnia was also blocked by glibenclamide in concentrations known to make it a selective inhibitor of K_{ATP} channels. L-Arginine, the substrate for nitric oxide synthase, was found to reverse the effect of glibenclamide, but was also found to have a permissive effect on the dilation of the arterioles by openers of the K_{ATP} channel (Kontos and Wei, 1998). Thus, in the rat preparation used in the present study, there is a second way to interpret the inhibitory effects of the nitric oxide synthase inhibitors and its "rescue" (Iadecola et al., 1994) by L-arginine. This alternative links the opening of the K_{ATP} channels to the dilation produced by hypercapnia. In support of the hypothesis linking opening of the K_{ATP} channel to dilation produced by hypercapnia, there is now evidence that the K_{ATP} channels contain a moiety with a pH sensitive site. As the pH is reduced by CO_2, the open state probability of the channels increases across the range of pH changes known to occur during hypercapnia (Xu et al., 2001). The dilation of pial arterioles by hypercapnia is known to be dependent upon the reduction in pH elicited by the increase in CO_2 (Kontos et al., 1977).

However, even if K_{ATP} channels do mediate the response to hypercapnia in the present preparation, one cannot invoke an action on the K_{ATP} channel to reconcile all of the discrepancies between publications. For example, in rats, some workers (Golding et al., 2000) fail to inhibit the response to CO_2 with glibenclamide, the selective inhibitor of the K_{ATP} channel. Moreover, in the study of knockout mice that demonstrated an alternative, NO-independent path to hypercapnic dilation, an arginine analog inhibitor of nitric oxide synthase was used to demonstrate that the hypercapnic response was now independent of the synthase (Irikura et al., 1995). If, in those mice, the arginine analog had inhibited opening of K_{ATP} channels, and if these channels were mediating the response to hypercapnia, then that response should have been blocked by the analog in spite of the genetic deletion of nitric oxide synthase.

With respect to the effects of ODQ and in contrast with the present results, some laboratories (Yang and Iadecola, 1998; Lindauer et al., 2001) report that ODQ inhibited the cerebrovascular response to hypercapnia. However, it is possible that some responses to ODQ may be due to other actions of the drug because of the high dose used. In the initial report of its inhibitory effect on the hypercapnic response (Yang and Iadecola, 1998), the dose was 100 mM, or 10 times the dose used here and 10 times greater than the dose reported to be maximally affective in inhibiting guanylate cyclase in blood vessels (Brunner et al., 1996). In fact, such a high dose is 1000 times greater than the 100 nM dose originally reported to inhibit 80% of cGMP production in cerebellum by the enzyme (Garthwaite et al., 1995). Use of such a high dose might have resulted in some effect of ODQ, other than inhibition of or in addition to the inhibition of guanylate cyclase.

It is also possible that, in some studies, the diluent for ODQ rather than the drug was responsible for the inhibition of the response to hypercapnia. However, at least one study (Lindauer et al., 2001), very carefully conducted on a small artery in vitro, with apparently appropriate vehicle controls, not only showed the dependence of the hypercapnic response on guanylate cyclase and the inhibition of both by ODQ, but also failed to show an effect of the diluent ethanol, even at the lower concentrations that blocked dilation by K_{ATP} openers in vivo in a different preparation (Rosenblum et al., 2001a).

These discrepant findings raise a third possibility or set of possibilities, namely that the results are dependent upon such aspects of the experimental design as the nature of the model—in vivo vs. in vitro—the size of the blood vessels, the type of anesthetic, the presence and type of paralytic agent as well as the species being investigated. A report by Wang et al. (1998) supports the concept that the mechanisms controlling the response to hypercapnia can be altered by experimental conditions and also indicates that under some circumstances, the K_{ATP} channel can be involved. They could not show an effect of the channel blocker glibenclamide under normal circumstances but were able both to inhibit the response to hypercapnia with an inhibitor of nitric oxide synthase and to restore the response by adding back cGMP, the product of guanylate cyclase activity. However, once the inhibition of nitric oxide synthase occurred, glibenclamide was able to block the restitution of the response by cGMP. Moreover, under the conditions that enabled glibenclamide to act, iberiotoxin, a selective inhibitor of the calcium-activated potassium ion channel was also affective.

In the present paper, the data do not, and were not meant to, identify the modulators of alternative mechanisms that might be responsible for the hypercapnic response. The study was designed only to test whether the response persists even when guanylate cyclase is inhibited. Under the experimental conditions described here, the response of rat pial arterioles, examined in vivo, does indeed persist in the absence of functional guanylate cyclase. The response remained unaffected when the enzyme was inhibited by ODQ in a dose sufficient to prevent dilation by nitric oxide that was directly supplied by SIN-1 or indirectly supplied through the activation of NMDA sensitive neurons. The data confirm prior results from the same preparation, which indicated that the response to hypercapnia was not being mediated by nitric oxide or guanylate cyclase (Kontos and Wei, 1996, 1998; Rosenblum et al., 2001b).

Acknowledgements

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