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Evidence for a K_{ATP} ion channel link in the inhibition of hypercapnic dilation of pial arterioles by 7-nitroindazole and tetrodotoxin ☆

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Abstract

7-Nitroindazole, an inhibitor of neuronal nitric oxide synthase, reportedly inhibits hypercapnic dilation, but tetrodotoxin, an inhibitor of neuronal transmission, reportedly does not. Thus, evidence does not uniformly support the hypothesis of a neurogenic link to the hypercapnic response. Others suggest the hypercapnic response is mediated by a K_{ATP} ion channel. In the following studies, we observed that topically administered tetrodotoxin inhibited dilations produced by hypercapnia. In addition, topical tetrodotoxin and either topical or intraperitoneal 7-nitroindazole, inhibited dilations produced by the K_{ATP} channel openers, cromakalim and pinacidil. Inhibition of hypercapnic dilation and inhibition of dilation by the openers of the K_{ATP} channel was immediately reversed by either L-lysine or L-arginine, amino acids previously shown to facilitate opening of the channel. The data strongly supports the previous conclusion that there is a K_{ATP} ion channel link in the response of pial arterioles to hypercapnia. The location of the channel is not established by these data, nor is it known whether the action of tetrodotoxin on the channel was direct or indirect. © 2001 Published by Elsevier Science B.V.

Keywords: Cerebral circulation; Hypercapnia; Vasodilatation; Pial arteriole; K_{ATP} channel; Tetrodotoxin; 7-Nitroindazole; L-Arginine; L-Lysine; Pinacidil; Cromakalim; Sodium nitroprusside; Nitric oxide (NO)

1. Introduction

Evidence reviewed elsewhere suggests a neurogenic component in the chain of events causing cerebral blood vessels to dilate in response to CO_2 (Iadecola et al., 1994). Supporting evidence includes the ability of inhibitors of nitric oxide synthase to interfere with the response (Okamoto et al., 1997; Wang et al., 1995, 1998; Iadecola 1992; Iadecola and Xu 1994; Iadecola and Zhang, 1994; Irikura et al., 1994; Ma et al., 1996). This evidence is consistent with the belief that nitric oxide (NO), the product of nitric oxide synthase action on its substrate L-arginine (Palmer et al., 1988), is a mediator or modulator of the hypercapnic response (Iadecola et al., 1994; Ma et al., 1996). However, the inhibitors of nitric oxide synthase that were initially used in such studies inhibit both the nitric oxide synthase that exists in blood vessels (eNOS) and the isoform found in nerve cell bodies and their processes (nNOS). Therefore, inhibition of a response by these nitric oxide synthase inhibitors may not by itself establish that the response is mediated by nitric oxide (NO) released from the nervous system.. However, 7-nitroindazole is a nitric oxide synthase inhibitor believed to have, in vivo, a selective action on neuronal nitric oxide synthase (Moore and Bland-Ward, 1996; Zagvazdin and Benter, 1998). Therefore, when this inhibitor was found to also inhibit the response to CO_2 (Okamoto et al., 1997; Wang et al., 1995, 1998) the evidence supporting a neurogenic link in the response was strengthened.

In contrast, a relatively small number of studies using tetrodotoxin (Fabricius et al., 1995; Yang and Iadecola, 1998; Pelligrino et al., 1995; You et al., 1994; Fabricius and Lauritzen, 1994) failed to demonstrate inhibition by tetrodotoxin of the hypercapnic response. Since, because of its ability to block sodium channels, tetrodotoxin is a well-established blocker of action potentials (Dryer, 1994; Kao, 1966; Narahashi et al., 1964), the failure of tetrodotoxin to interfere with the hypercapnic response is evidence against a neurogenic link in that response, although one cannot rule out the possibility that hypercapnia

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might in some way release NO from nerves without triggering an action potential.

Additional evidence against a role for NO in mediating the response has appeared in a recent series of papers (Kontos and Wei, 1996, 1998; Wei and Kontos, 1999) which demonstrated, in rats and cats, a dependence of the hypercapnic response on the opening of a K_{ATP} channel. In addition, those studies demonstrated that arginine analogs, which inhibit nitric oxide synthase and which inhibit the response to CO_2, have the additional property of interfering with the dilating effect on pial arterioles of well-established openers of the K_{ATP} channels. Therefore, those studies offered an alternative hypothesis to explain the inhibitory action of such nitric oxide synthase inhibitors on the hypercapnic response; namely, that they were working via a blockade of the K_{ATP} channel. Moreover, in those studies, the K_{ATP} channel was found to require either L-arginine or L-lysine to maintain its open state. This, rather than its function as a substrate for nitric oxide synthase, explained the ability of L-arginine to reverse the inhibitory effect of arginine analog inhibitors of nitric oxide synthase on the response to CO_2.

Very recent studies (Xu et al., in press) support the hypothesis that the K_{ATP} ion channel mediates the response to CO_2. This new data was gathered from the in vitro study of cloned K_{ATP} channels and showed that the class of channel found in vessel walls has within it a pH sensitive site. As the pH was lowered from 7.4 to 6.6, the K_{ATP} channels shifted from the closed to the open state. This reduction in pH is like that which accompanies hypercapnia. It is well established that the response to the change in CO_2 during hypercapnia is mediated not by the CO_2 per se but by the shift in pH (Kontos et. al., 1977).

In the in vivo studies just described, 7-nitroindazole was not tested. The first aim of the present study was to see whether its inhibitory action on the hypercapnic response might also be explained by an ability to prevent the opening of the K_{ATP} channel. After finding that 7-nitroindazole could prevent dilation of pial arterioles by well-established openers of the channel, and after demonstrating that this inhibition could be reversed by amino acids previously shown to be required for the dilating action of conventional K_{ATP} channel openers, we proceeded to the second aim of the study. This was to test the ability of tetrodotoxin to interfere with the response to CO_2. In view of the handful of negative studies in the literature (Fabricius et al., 1995; Yang and Iadecola, 1998; Pelligrino et al., 1995, You et al., 1994; Fabricius and Lauritzen, 1994), we expected no effect, but it was important to perform the studies because those in the literature did not, for the most part, look directly at surface arterioles but were either flow studies or in vitro studies of a large artery. When we found that tetrodotoxin, in our hands, did inhibit the hypercapnic response, we then asked whether it, like 7-nitroindazole and the arginine analog inhibitors of nitric oxide synthase, might also inhibit the opening of K_{ATP} channels. We found that this was the case, and that its inhibitory action both on dilation of pial arterioles by K_{ATP} channel openers and by CO_2, could be reversed by an amino acid shown previously to be required for the dilating action of conventional K_{ATP} channel openers.

2. Methods

2.1. Preparation

Experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. Male Sprague–Dawley rats (250–350 g body weight) were anesthetized with sodium pentobarbital (55 mg/kg i.v.). 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. Mean $\pm$ standard error for the blood gases obtained from all the animals were: $PaO2 = 89 \pm 3$ mm Hg, $PaCO_2 = 37 \pm 1$ mm Hg. The mean pH was 7.43 ± 0.01 and mean arterial blood pressure was 115 ± 1 mm Hg.

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 10 min prior to measurement of vessel caliber.

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 (MCSF) (Raper et al., 1972). There were three outlets from the window. Two were inflow and outflow paths used only to replace the control MCSF with MCSF 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% 0_2, 6% CO_2 and the balance N_2. Diameter of pial arterioles was measured with a Vickers image-splitting device. Usually three to six pial arterioles were measured in each rat. All monitoring took place with the MCSF 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

Cromakalim, pinacidil, L-arginine, L-lysine and sodium nitroprusside were obtained from Sigma. The sodium salt of 7-nitroindazole and tetrodotoxin were obtained from Calbiochem. 7-Nitroindazole was obtained from Cayman Chemical. It was dissolved in vegetable oil and administered intraperitoneally. All other drugs were dissolved in the MCSF for topical application. Although 7-nitroindazole-sodium salt Na arrived labeled as water soluble it was in fact only soluble in alcohol. After making a stock solution in ethanol subsequent dilution could be made in MCSF resulting in a final concentration of 0.5% ethanol. At this concentration, the solvent alone had no effect on diameter or on the response to openers of the K_{ATP} channel, nor did the vegetable oil used as the vehicle in the i.p. experiments (see Results).

2.5. Experimental design

The space under the window was filled with MCSF and the vessel diameters at resting state were measured. In studies employing K_{ATP} channel openers as the dilating stimulus, the fluid under the window was then replaced with MCSF containing various drugs. Vessel caliber was measured again at a new steady state. Responses to topically applied drugs were determined 2–4 min following each application (the time elapsing to reach a steady state). In each rat, the response to one K_{ATP} channel opener (either cromakalim or pinacidil) was determined in the presence or absence of either tetrodotoxin or 7-nitroindazole-sodium salt. Topical treatment with either 1 μM tetrodotoxin or 100 μM 7-nitroindazole-sodium salt lasted for 15 min. After topical tetrodotoxin or 7-nitroindazole-sodium salt, the fluid under the window was replaced with mock CSF containing either cromakalim or pinacidil. In some experiments, either 5 μM L-lysine or 1 μM L-arginine was then applied to see whether the inhibitory action of tetrodotoxin or 7-nitroindazole on the response to a K_{ATP} channel opener would be reversed. These amino acids were chosen because a previous study showed that one or the other is required in order for the openers of the channel to be effective (Kontos and Wei, 1998).

The effect of intraperitoneal 7-nitroindazole on the response to K_{ATP} channel openers was tested 1 h after its injection. After demonstrating the ability of the i.p treatment to inhibit dilation caused by a K_{ATP} channel opener, the ability of L-lysine to reverse the inhibition was tested.

In studies employing hypercapnia as the dilating stimulus, the response to hypercapnia was tested in the absence and then in the presence of either tetrodotoxin or 7-nitroindazole-sodium salt as described above or before and 1 h after i.p injection of 7-nitroindazole. The ability of either L-lysine or L-arginine to reverse the inhibitory effect of tetrodotoxin, 7-nitroindazole-sodium salt or 7NI was then tested as described above.

In each rat, the effect of a potentially inhibitory drug was also tested against dilation produced by sodium nitroprusside. The latter was chosen as the control dilator because its action depends upon the release of NO and subsequent activation of guanylate cyclase. Thus, we could rule out an action on either NO or on guanylate cyclase as the basis for any inhibitory effects produced by either tetrodotoxin or 7-nitroindazole.

Twelve separate studies were performed in 12 different groups of rats. In study #1, the responses to pinacidil and to sodium nitroprusside were tested before and after treatment with tetrodotoxin. Study #2 tested the response to cromakalim before tetrodotoxin, after tetrodotoxin and immediately again but with lysine added to the cromakalim. Study #3 began the series of studies using 7-nitroindazole or 7-nitroindazole-sodium salt (the "soluble" form of 7-nitroindazole). In study #3, pinacidil and then sodium nitroprusside were tested before and after 7-nitroindazole-sodium salt. In study #4, cromakalim was tested alone, then after 7-nitroindazole-sodium salt, and then immediately again but with L-lysine added to the cromakalim. Study #5 tested pinacidil and cromakalim before and after 7-nitroindazole (i.p.). Study #6 tested pinacidil before and after 7-nitroindazole (i.p.) and then immediately again with L-arginine added to the pinacidil. Study #7 tested pinacidil before and after 7-nitroindazole and then immediately again with L-lysine added to the pinacidil. Studies #8 and #9 tested the vehicles for 7-nitroindazole-sodium salt (0.5% ethanol) and for 7-nitroindazole (vegetable oil, i.p.) against the response to the openers of the K_{ATP} channel. Studies #10 and #11 tested the duration of the inhibition produced by a single 15-min application of 7-nitroindazole-sodium salt and of tetrodotoxin. Study #12 tested the effect of tetrodotoxin against dilation produced by hypercapnia.

2.6. Statistics

Dilations were expressed as a percent of base diameter. Since more than one arteriole was monitored in each rat, the responses obtained in each rat were averaged and the mean response of each rat was then used in the statistical analysis of that particular experiment. In the text and figures, the mean responses for each rat in an experiment are averaged and the mean% increase in diameter ± standard error of these mean values are shown for the rats in that experiment. However, all treatment effects were determined by using each rat as its own control and comparing pre and post treatment mean% increases in diameter using the paired *t* test. Differences were to be considered significant when the *P* value was equal to or less than 0.05, but, in fact, in most cases the differences were significant at the 0.01 level as shown in the Results section.

3. Results

The results were marked and consistent. Over 200 separate arterioles were observed during the experiments reported below and in each experiment the results of that experiment were qualitatively identical in every monitored arteriole in every rat without exception.

Fig. 1 displays the dose-dependent dilations to both pinacidil, an opener of K_{ATP} channels, and to sodium nitroprusside. Immediately after application of 1 μM tetrodotoxin, the response to pinacidil was virtually eliminated ($N = 5$, $P < 0.0l$ for each dose of pinacidil) while the response to sodium nitroprusside was not affected. Tetrodotoxin did not change the basal diameter of the vessels in this or in any of the following studies.

The duration of the inhibitory effect of 1 μM tetrodotoxin was tested in three additional rats. Prior to the 15-min application of tetrodotoxin, the dilation caused by 1 and 2 μM pinacidil was $8 \pm 2\%$ and $17 \pm 2\%$, respectively. Forty-five minutes after the tetrodotoxin was washed out, the response to pinacidil was still markedly suppressed ($2 \pm 1\%$ and $4 \pm 1\%$; $P < 0.05$).

Fig. 2 shows dose-dependent dilation produced by cromakalim, another opener of the K_{ATP} channel. The response to each of the two doses was almost eliminated by topical pretreatment with 1 μM tetrodotoxin ($N = 5$ rats; $P < 0.01$ for the effect of tetrodotoxin on each dose of pinacidil). Moreover, as the third set of data in this figure shows, the inhibitory action of tetrodotoxin on cromakalim was eliminated if 5 μM L-lysine, one of the amino acids that permits cromakalim to dilate these vessels (Kontos and Wei, 1998) was added to the mock CSF containing this opener of K_{ATP} channels. Lysine by itself had no effect on diameter.

Fig. 3 shows that the dose-dependent dilations produced by pinacidil were virtually eliminated by pretreatment with 100 μM 7-nitroindazole-sodium salt ($N = 5$; $P < 0.01$ for tetrodotoxin effect on each dose of pinacidil). The dose-dependent dilation produced by sodium nitroprusside was not affected by 7-nitroindazole-sodium salt. The 7-nitroindazole-sodium salt had no effect on the resting diameter in this or in any of the following studies.

The duration of the effect of 100 μM 7-nitroindazole-sodium salt was tested in another experiment using three additional rats and a single dose (2 μM) of pinacidil. Dilation was $16 \pm 1\%$ of resting diameter before pinacidil and was only $3 \pm 1\%$ immediately after topical 7-nitroindazole-sodium salt ($P < 0.0l$). After 90 min had elapsed, the response was still only $4 \pm 2\%$. At 120 min, the response was almost fully restored ($14 \pm 3\%$).

Fig. 4 shows that the dose-dependent dilation by the K_{ATP} channel opener cromakalim was also blocked by pretreatment with topical 7-nitroindazole-sodium salt, l00 μM ($N = 5$ rats; $P < 0.01$ for effect of 7-nitroindazole-sodium salt on either dose of cromakalim) The figure also shows that, as was the case with tetrodotoxin, the inhibitory action of 7-nitroindazole-sodium salt was ameliorated by L-lysine.

Fig. 5 shows the dilations produced first by a single dose of pinacidil and then by a single dose of cromakalim. The rats were then injected with 20 mg/kg 7-nitroindazole. One hour later, the intraperitoneal 7-nitroindazole

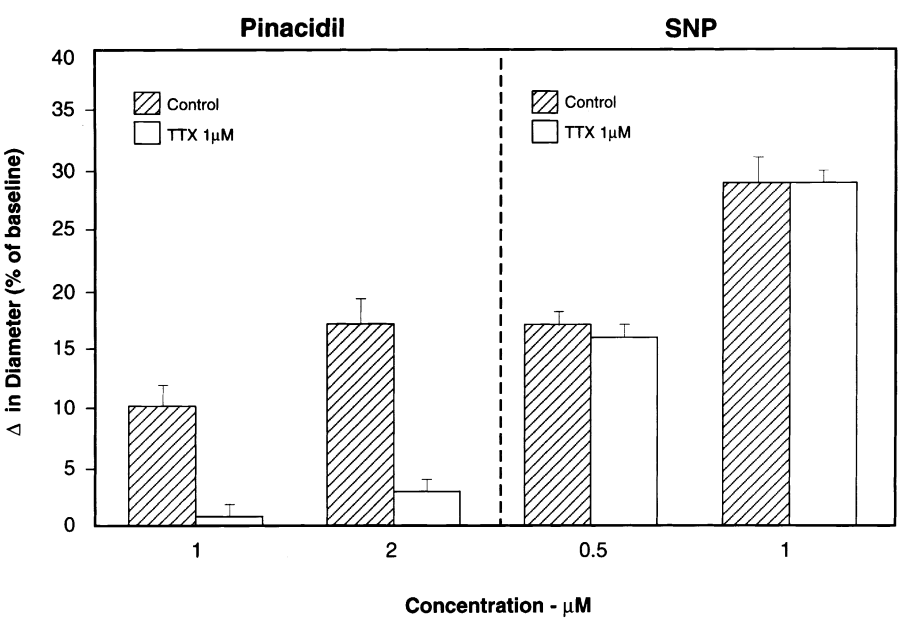

Fig. 1. Five rats; mean diameter of 28 arterioles was 46 ± 3 μm. Dose-dependent dilation was produced by both pinacidil and sodium nitroprusside (SNP). After topical treatment with 1 μM tetrodotoxin (tetrodotoxin), the response to each dose of pinacidil ($P < 0.01$) was blocked or markedly inhibited but that to sodium nitroprusside remained intact.

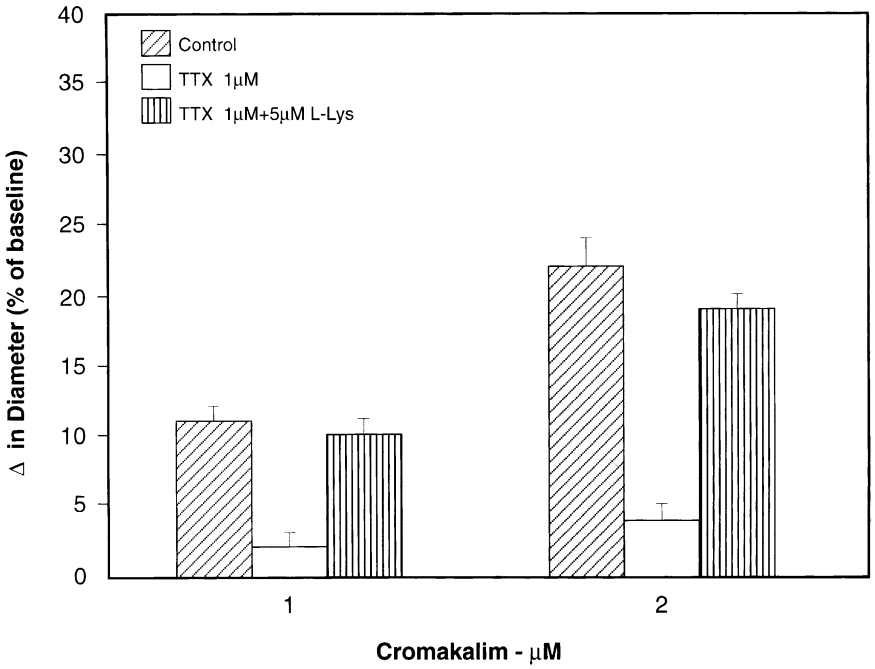

Fig. 2. Five rats; mean diameter of 23 arterioles was 39 ± 3 μm. They displayed dose-dependent dilation to cromakalim prior to topical tetrodotoxin (TTX) 1 μM. After TTX treatment, the response to each dose of cromakalim was greatly reduced ($P < 0.01$) but could be restored by adding 5 μM L-lysine to the solution containing cromakalim.

was found to have blocked the response to both openers of the K_{ATP} channel ($N = 5$ rats; $P < 0.0l$ for effect on either pinacidil or cromakalim).

The reproducibility of the effect of 7-nitroindazole i.p. is illustrated in Fig. 6 which displays data from five additional rats. It shows that the dose-dependent dilation produced by pinacidil was blocked after i.p. 7-nitroindazole ($P < 0.01$ for effect on each dose of pinacidil). The figure also shows that the response to pinacidil was restored when 1 μM L-arginine was added to the solution under the cranial window. Arginine by itself had no effect on resting diameter.

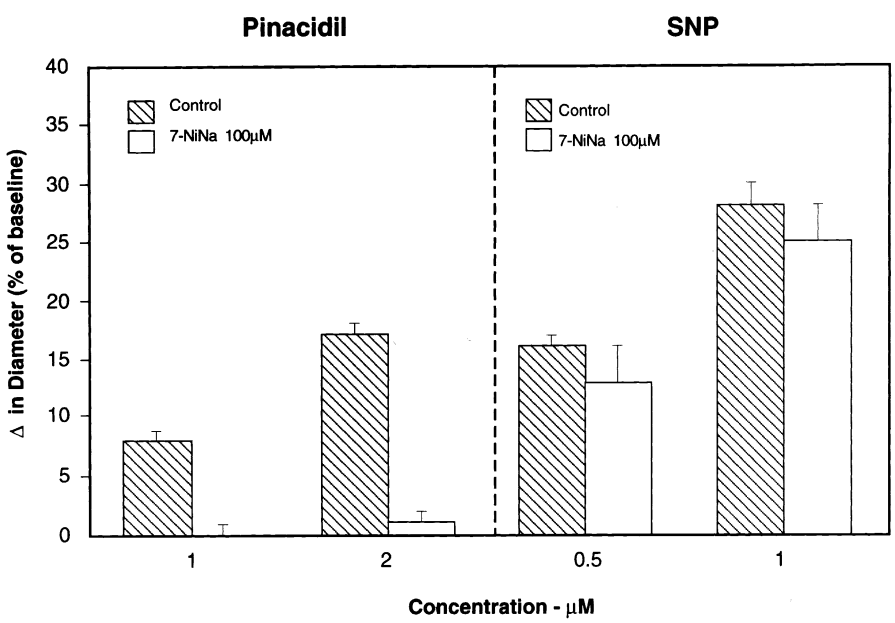

Fig. 3. Five rats; 24 arterioles, diameter = 47 ± 2 μm. Dose-dependent dilations were produced by pinacidil and by sodium nitroprusside (SNP). After application of 100 μM 7-nitroindazole-sodium salt (7-nitroindazole-sodium salt), the response to each dose ($P < 0.01$) of pinacidil was essentially abolished while responses sodium nitroprusside were not significantly altered.

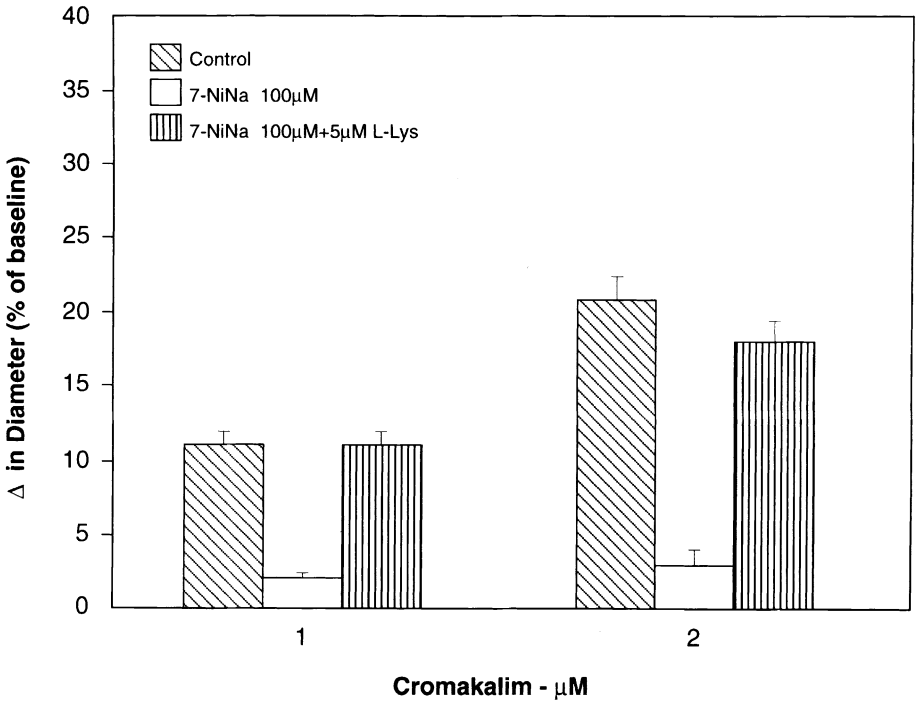

Fig. 4. Five rats, 24 arterioles, diameter = 39 ± 2 μm. Dose-dependent dilation was produced by cromakalim and the response to each dose ($P < 0.01$) was virtually abolished after topical application of 100 μM 7-nitroindazole-sodium salt (7-nitroindazole-sodium salt). The response to cromakalim was restored when 5 μM L-lysine was added to the solution containing cromakalim.

Reproducibility of results is again shown in Fig. 7 which indicates that in five additional mice 20 mg/kg i.p. 7-nitroindazole abolished the response to each of two doses of pinacidil ($P < 0.01$ for effect on each dose of pinacidil). In this study, as shown in the figure, L-lysine, 5 μM, abolished the inhibitory action of the 7-nitroindazole.

The vehicle (ethanol 0.5% final concentration) for 7-nitroindazole-sodium salt was tested on 10 arterioles in three rats. It had no effect, the dilation to 1 and 2 μM pinacidil being 10 ± 1% and 20 ± 2% of resting diameter before and 12 ± 1% and 20 ± 2% after application of the ethanol.

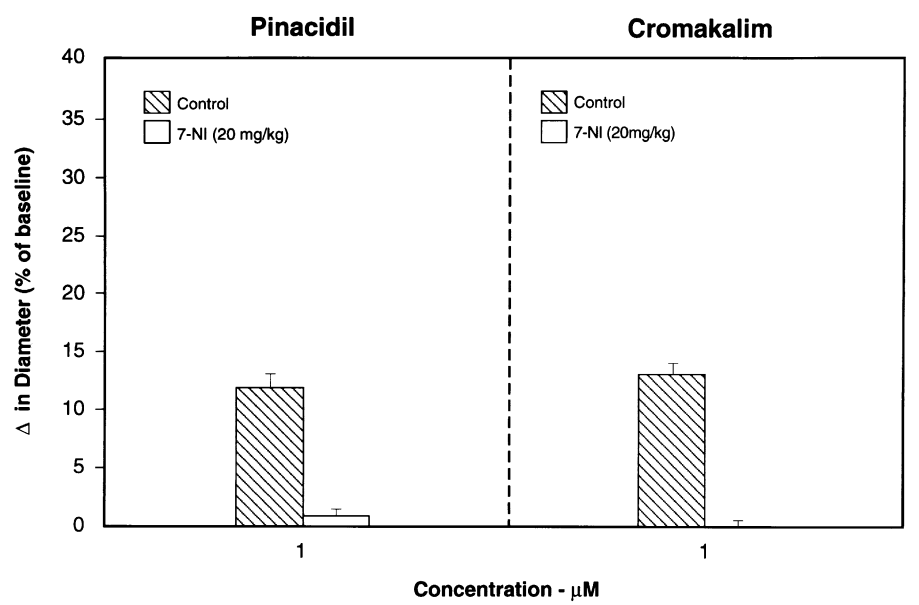

Fig. 5. Five rats; 30 arterioles, diameter = 42 ± 2 μm. Pinacidil and cromakalim dilated the arterioles. Then 20 mg/kg nitroindazole (7-nitroindazole) was injected i.p. One hour later, retest with pinacidil and cromakalim showed that these responses had been blocked ($P < 0.01$).

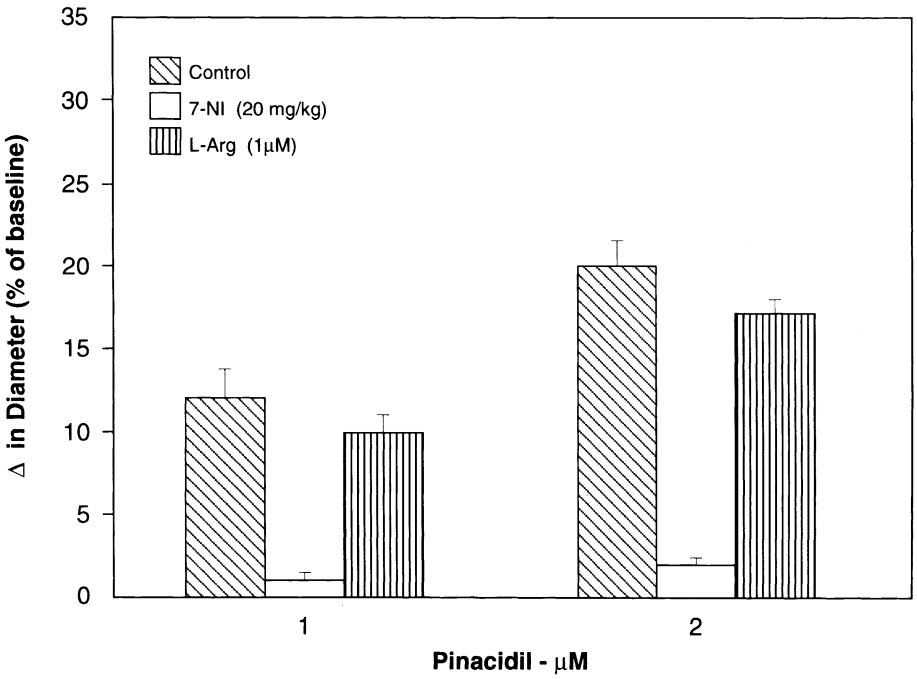

Fig. 6. Five rats; 34 arterioles, diameter = 43 ± 2 μm. Pinacidil produced dose-dependent relaxation. One hour after i.p. 7-nitroindazole, 20 mg/kg, pinacidil was unable to elicit dilation ($P < 0.01$). The ability to produce dilation was restored by adding 1 μM L-arginine to the solution containing pinacidil.

Likewise, the vegetable oil vehicle for the 7-nitroindazole given i.p. had no effect on the response to pinacidil. In three rats, the dilations before vehicle were 8 ± 1% and 19 ± 2%. One hour after injection of the vegetable oil, the retested responses were 8 ± 1% and 18 ± 2%.

To examine the effect of tetrodotoxin on the response to CO_2, we tested 28 arterioles, diameter 40 ± 2 μm, from six rats. Prior to respiring the CO_2-enriched mixture, the blood gases in the six rats (M ± S.E.M.) were PaO_2 = 80 ± 4 mm Hg, $PaCO_2$ = 38 ± 1 mm Hg, pH = 7.44 ± 0.01.

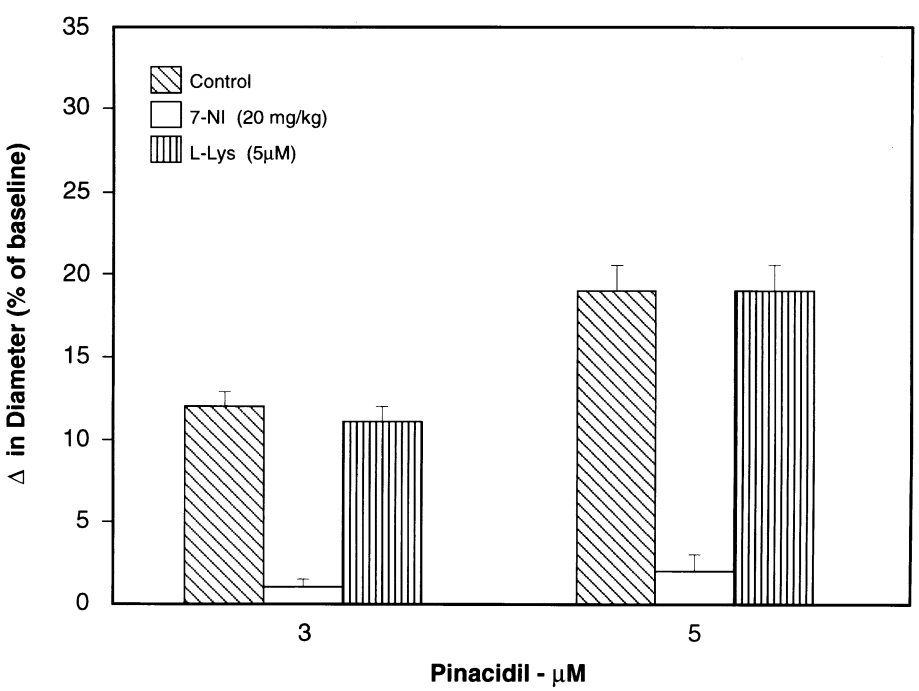

Fig. 7. Five rats; 28 arterioles, diameter = 40 ± 2 μm. Pinacidil produced dose-dependent dilation of the pial arterioles. One hour after 7-nitroindazole, 20 mg/kg i.p., pinacidil was unable to elicit dilation ($P < 0.01$). This ability was restored as soon as 5 μM L-lysine was added to the solution containing pinacidil.

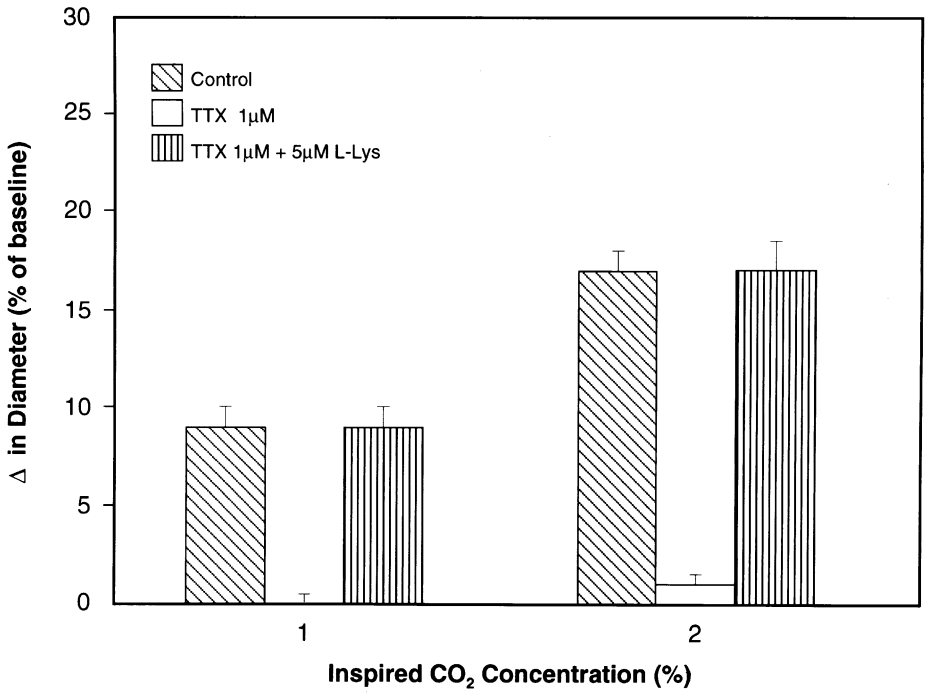

Fig. 8. Five rats; 24 arterioles, diameter = 41 ± 2 μm. While breathing 3% and 5% CO_2 they dilated by 8% and 17% (±1%), respectively. Following the application of 1 μM tetrodotoxin (TTX) neither degree of hypercapnia resulted in dilation ($P < 0.01$). However, the ability of hypercapnia to relax the arterioles was immediately and fully restored by adding 5 μM L-lysine to the mock CSF.

When breathing 3% CO_2, the $PaO_2 = 83 \pm 3$ mm Hg, $PaCO_2 = 53 \pm 2$ mm Hg, pH = 7.34 + 0.01 when breathing 5% CO_2, $PaO_2 = 92 \pm 2$ mm Hg, $PaCO_2 = 66 \pm 2$; pH = 7.26 ± 0.01. The mean arterial blood pressure in this set of experiments was 119 ± 1 mm Hg during the control period, 121 ± 1 mm Hg while breathing 3% CO_2 and 121 ± 2mm Hg while breathing 5% CO_2. Inspiring 3% CO_2 and 5% CO_2 dilated the vessels by 8 ± 1% and 17 ± 1% of resting diameter (40 ± 2 μm). Immediately after the 15-min application of 1 μM tetrodotoxin, the response to 3% and 5% CO_2 was only 1 ± 1% and 1 ± 1% ($P < 0.01$ for each concentration of inspired CO_2).

The results were replicated in an additional study of 24 arterioles in five rats shown in Fig. 8. In this experiment, the control $PaCO_2$ was 37 ± 1 and was raised to 53 ± 2 and to 70 ± 3 mm Hg by 3% and 5% inspired CO_2, respectively. Here, after the response to CO_2 was blocked by the 15-min application of 1 μM tetrodotoxin ($P < 0.01$ for each concentration of inspired CO_2), the tetrodotoxin was washed out and the fluid under the window was replaced with mock CSF containing 5 μM L-lysine. The response to CO_2 was retested and was found to be restored to normal.

4. Discussion

These data reveal novel findings: first, both 7-nitroindazole (in either its alcohol/water soluble or water insoluble form) and tetrodotoxin prevented well known openers of the K_{ATP} channel from dilating rat pial arterioles. The 7-nitroindazole was affective when given either topically or intraperitoneally. Second, tetrodotoxin inhibited the dilation produced by hypercapnia; third, the effects of 7-nitroindazole and tetrodotoxin were reversed by either L-arginine or L-lysine.

Two important questions must be discussed in light of this data. First, are the active drugs used here really acting on a K_{ATP} channel? Second, where is that channel?

The belief that the K_{ATP} channel is involved is supported by several lines of evidence: (a) the fact that each agent could prevent the dilation produced by pinacidil and cromakalim, two well-known openers of the channel (Quayle et al., 1997; Faraci et al., 1994); (b) the effects of 7-nitroindazole and tetrodotoxin on the response to K_{ATP} channel openers and the inhibitory action of tetrodotoxin on the response to CO_2 were reversed by L-arginine or L-lysine. Either of these same amino acids was previously shown to permit dilation of pial arterioles by openers of the K_{ATP} channel (Kontos and Wei, 1998). These amino acids were also previously shown (Kontos and Wei, 1996) to reverse the inhibitory effect of glibenclamide on the dilation caused by well-established openers of the channel. Glibenclamide is an established closer of the K_{ATP} channel (Quayle et al., 1997; Faraci et al., 1994); (c) previous studies demonstrated that arginine analogs, shown by others to inhibit the response to hypercapnia (Wang et al., 1995; Iadecola, 1992; Iadecola and Xu, 1994; Iadecola and Zhang, 1994; Irikura et al., 1994; Ma et al., 1996), can also prevent dilation by openers of the K_{ATP} channel (Kontos

and Wei, 1996, 1998). Moreover, earlier studies showed that the response of pial arterioles to changes in CO_2 could be inhibited by established blockers of the K_{ATP} channel and that this effect, too, was reversed not only by L-arginine but also by L-lysine (Kontos and Wei, 1996, 1998). The facilitory effect of these amino acids cannot be explained on the basis of their role as substrates for nitric oxide synthase because only arginine is such a substrate (Palmer et al., 1988); (d) as reviewed elsewhere (Kontos and Wei, 1996; Quayle et al., 1997) data from some other, though not all other laboratories, has implicated potassium channels as mediators of hypercapnic dilation, in a variety of blood vessels. The importance of the K_{ATP} channel may vary with species and with the size of the vessel, with the organ and with the place of the vessel within the vascular bed of a given organ (Quayle et al., 1997; Faraci et al., 1994).

The response of pial arterioles to changes in CO_2, the inhibition of the response by arginine analogs (Kontos and Wei, 1996), by 7-nitroindazole (Okamoto et al., 1997; Wang et al., 1995, 1998) or by tetrodotoxin as reported here, and restitution of the response by L-arginine or L-lysine can all be explained by postulating dependence of the response on an arginine/lysine-dependent K_{ATP} channel that can be closed by any of the inhibitory agents in question. Ultimate proof of our hypothesis would rest upon the difficult electrophysiological determinations of the open state of K_{ATP} channels observed with patch clamp studies performed on pial arterioles in vivo. To our knowledge no one has published such studies.

Alternatively, one might postulate that the identical inhibitory effects of tetrodotoxin, 7-nitroindazole and glibenclamide on both K_{ATP} channel openers and on hypercapnic dilation are an irrelevant coincidence. Then the inhibitory action on hypercapnic dilation of either tetrodotoxin reported here or of 7-nitroindazole reported by others would be independent of any action on the K_{ATP} channel. In that case, one would also have to postulate that the ability of L-lysine or L-arginine to reverse all these inhibitory actions is also coincidental and not dependent upon the apparent reported necessity of one or the other of these amino acids to permit the dilating action of K_{ATP} channel openers (Kontos and Wei, 1996, 1998).

The series of coincidences just mentioned seems highly unlikely. Nevertheless, their possibility cannot be totally eliminated since the mechanism(s) by which tetrodotoxin and 7-nitroindazole could prevent opening of the K_{ATP} channel is (are) not revealed by this study.

However, there is now definitive support for the conclusion that was drawn from the cited studies of Wei and Kontos and of Kontos and Wei; namely, that the response to hypercapnia is dependent upon or modulated by a K_{ATP} ion channel. This is the conclusion that provided the rationale, in the present study, for testing the ability of tetrodotoxin or of 7-nitroindazole to block the action of K_{ATP} channel openers after it was known that both of these drugs blocked the response to CO_2. The definitive support for the conclusion that the response to CO_2 is dependent upon a K_{ATP} ion channel comes from the recent work of Xu et al. They used a cloned portion of the class of K_{ATP} channel known to occur in vascular smooth muscle. They showed that the probability of having these channels in the open state was increased as the pH declined from 7.4 to 6.6. As the CO_2 levels increase during hypercapnia, the pH is reduced over this same range and it is that change in pH which is the cause of the vasodilatation (Kontos et al., 1977).

Kontos and Wei inhibited the response to hypercapnia with glibenclamide. Glibenclamide as an agent which selectively inhibits the sulfonylurea sensitive site (Quayle et al., 1997). However, the recent work of Xu et al. shows that the pH sensitive portion of the K_{ATP} ion channel is not the sulfonylurea sensitive site. They showed this by using a cloned channel that lacked this moiety and responded, nevertheless, to reductions in pH. Thus, when Kontos and Wei inhibit the response of pial arterioles to hypercapnia with glibenclamide they are simply showing that activation of the channel at the pH sensitive site cannot trigger vasodilatation if the sulfonylurea sensitive site is blocked. Kontos and Wei also showed that the inhibitory effect of glibenclamide on hypercapnic vasodilatation was reversed by L-lysine or L-arginine. And they showed that L-arginine or L-lysine was essential for the K_{ATP} channel to be opened by pinacidil or cromakalim (Kontos and Wei, 1998). Since the sulfonylurea sensitive site is not the pH sensitive site of the channel (Xu et al., in press), the ability of these amino acids to reverse the effects of glibenclamide in previous studies or to reverse the inhibitory effects of tetrodotoxin or of 7-nitroindazole in the present study must indicate either an interaction between the two sites or the ability of arginine and lysine to affect multiple sites on the channel. It is also possible that these amino acids exert an indirect effect on the channel through some metabolic effect on the arteriolar wall. Structural and steric similarities between arginine and lysine provide a basis for suggesting that they can both act on the same site (Kontos and Wei, 1998). Indeed, these similarities provide the basis for the fact that both amino acids share an uptake mechanism into tissue (Furesz et al., 1991).

If tetrodotoxin in the present study or 7-nitroindazole in the reports of other authors (Okamoto et al., 1997; Wang et al., 1995, 1998) inhibits hypercapnic dilation via an effect on the K_{ATP} ion channel, it is also possible that, rather than a direct action on the pH or sulfonylurea sensitive sites, they act indirectly, instead, via an action on one or more of the many factors essential for channel activation (Quayle et al., 1997). For example, an action of tetrodotoxin on a sodium channel in the vascular smooth muscle could affect the resting potential and consequently the resting and the open state of the potassium channel. In fact, tetrodotoxin sensitive sodium channels have been

detected in vascular smooth muscle of at least one microvascular bed, that in the heart (Walsh et al., 1998), and have been reported in other tissues as well (Aggarwal et al., 1997).

However, as the present data and other studies show (Kontos and Wei, 1996; Quayle et al., 1997; Ibbotson et al., 1993), drugs with very diverse structures can prevent the opening of a variety of potassium channels. For example, drugs containing either a guanidino or imidazoline moiety can block a delayed rectifying potassium channel (Ibbotson et al., 1993; Corpus et al., 1994) and also block the effects of levocromakalim (Ibbotson et al., 1993), a well-established opener of the K_{ATP} channel (Quayle et al., 1997). In this regard, it is of interest to note that tetrodotoxin is a guanidino compound (Kao, 1966).

It should also be noted that we used 1 μM tetrodotoxin, a dose chosen because that is the dose used by those showing an effect of tetrodotoxin on cerebrovascular responses in vivo (Fabricius and Lauritzen, 1994; Meng et al., 1996). However, much lower doses are all that are required to block nerve impulses in vitro (Narahashi et al., 1964) and have been reported to block effects of transmural electrical stimulation of isolated cerebral arteries (Liu and Lee 1999; Chen and Lee, 1993). It is possible that the 1 μM dose might be less selective than nanomolar doses and affect other channels in some preparations.

The data presented here are not the first to indicate that either tetrodotoxin or 7-nitroindazole may have targets in addition to those traditionally ascribed to them. Other laboratories have shown that 7-nitroindazole can have targets in addition to nitric oxide synthase. In a recent study, 7-nitroindazole in doses which had no effect on NO production, nevertheless protected neurons from delayed death following transient ischemia (Lei et al., 1999). 7-Nitroindazole has also been shown to relax smooth muscle in vitro (Medhurst et al., 1994). 7-Nitroindazole has long been known to block both nNOS and eNOS with equal efficacy in vitro (Moore and Bland-Ward, 1996) and its apparently selective action on nNOS in vivo has both been questioned (Zagvazdin and Benter, 1998) and remains unexplained (Moore and Bland-Ward, 1996).

With respect to tetrodotoxin, there have been other, albeit controversial, reports that tetrodotoxin can block potassium channels (Dryer, 1994). These are sodium activated potassium channels whose function is uncertain.

Having discussed the evidence favoring the opinion that all the active agents in the present study were acting on a K_{ATP} channel, we must now discuss whether or not the channel is located in the vessel wall. Such channels are known to exist in vascular smooth muscle (Faraci and Heistad, 1998; Nagao et al., 1996). Nevertheless, there is still the hypothetical possibility that the K_{ATP} channel we are dealing with is located in the brain or in perivascular nerves.

In considering the possibility of a target in the brain, one must point out that this study, like most of the work with pial arterioles published by others, including immediately relevant literature from other laboratories (e.g. Iadecola et al., 1994; Wang et al., 1998; Pelligrino et al., 1995; Armstead, 1996; Bari et al., 1996; Irikura et al., 1995; Sobey et al., 1997; Taguchi et al., 1994; Veltkamp et al., 1998), administered drugs topically in the fluid over the surface of the brain. We may ask whether in such studies one or more of the agents interacted with a target in the brain rather than in the blood vessel wall. Such a possibility cannot be definitively ruled out for our study or any of the others. But against this possibility are three facts: (a) the responses began "immediately"; (b) most in vivo responses of suffused pial arterioles, even when compared across species, are qualitatively identical to in vitro responses of cerebral vessels (Edvinsson et al., 1993; Rosenblum 1998); (c) use of micropipettes to apply minute amounts of drug in immediate juxtaposition to the arteriolar wall and presumably dramatically limiting exposure of the brain surface to the drug, has generally produced the same qualitative results as studies in which vessels were observed following diffusion of the drug over the entire craniotomy site (Edvinsson et al., 1993). In addition to this evidence suggesting that the target of tetrodotoxin and of 7-nitroindazole in the present study was not in the brain, we may also point out that the type of K_{ATP} ion channel found in neurons reacts to reductions in pH in a manner opposite that found for the K_{ATP} channel found in blood vessels (see Xu et al., in press, for references).

Our finding that tetrodotoxin inhibits the response to hypercapnia is at variance with six reports from three laboratories (Yang and Iadecola, 1998; Pelligrino et al., 1995; You et al., 1994; Fabricius and Lauritzen, 1994; Akgoren et al., 1994; Yang and Iadecola, 1996). However, those studies themselves do not provide consistent results. Four utilized laser-Doppler measurements of cerebral blood flow, rather than measurements of pial arteriolar diameter, and reported that tetrodotoxin either increased or had no effect on the response to CO_2. Since the flow measured by the laser-Doppler technique is affected by the response of parenchymal as well as pial arterioles to CO_2, and since the laser-Doppler probe is always placed between pial vessels in order to avoid artifactual recordings, the laser-Doppler study cannot serve to refute the present findings. One in vivo study of pial arterioles showed no effect of tetrodotoxin on hypercapnic vasodilatation (Pelligrino et al., 1995). There the arterioles were observed two days after implantation of the cranial window and the study employed continuous suffusion of artificial cerebrospinal fluid during the observations rather than stopped flow as used here. These features may represent important differences from our own procedures. One other study failed to find an effect of tetrodotoxin on the response to CO_2 (You et al., 1994), but this was an in vitro study of a much larger cerebral blood vessel than those observed here. Therefore, it too cannot serve as refutation of the present data. Moreover, if it did so serve, then it along with several

negative in vivo studies would stand in opposition to the belief that the response to CO_2 was modulated by nerves, including nitroxidergic nerves.

So far as we know, tetrodotoxins classical action inhibits only the development of action potentials and hence would not alter basal (i.e. unstimulated) release of NO from nerves or nerve cell bodies. All workers who have used tetrodotoxin to test for the existence of a neural mechanism underlying the response to CO_2 have thus assumed that hypercapnia might activate that neural mechanism. Our use of tetrodotoxin has made the same assumptions.

A direct action of tetrodotoxin or of 7-nitroindazole on NO or on guanylate cyclase, the well-established molecular target of NO, cannot explain the present data because neither drug affected the response to sodium nitroprusside, an NO donor and activator of guanylate cyclase.

The evidence that NO and hence nitroxidergic nerves are not involved in hypercapnic dilation also includes the report that LY83583, an inhibitor of guanylate cyclase failed to block hypercapnic dilation of pial arterioles in vivo (Kontos and Wei, 1996).Others (Yang and Iadecola, 1998) point out that LY83583 has effects other than direct inhibitory inactivation of guanylate cyclase; for example, it produces reactive oxygen species which can inactivate NO. However, even if that were its mode of action, its failure (Kontos and Wei, 1996) to inhibit hypercapnic dilation would still serve as evidence that NO is not involved in the response.

In contrast to the failure of LY83583, the hypercapnic response has been inhibited by 1*H*[1,2,4]oxadiazolo[4,3-*a*]quinoxalifl 1-one (ODQ), a supposedly selective inhibitor of guanylate cyclase (Garthwaite et al., 1995) and this has been offered as support of the hypothesis that NO is involved. However, when two drugs with a common biochemical target, in this case guanylate cyclase, have different actions on the response to CO_2, the failure of one of the drugs to impair the response should not lead to dismissal of the experiment employing the failed drug. Rather, it is logical to suggest that the effect of ODQ, the drug which inhibited the response, might be due to an action of ODQ on some site other than guanylate cyclase. Belief in this hypothesis is encouraged by the fact that experiments employing ODQ to inhibit the response to CO_2 used concentrations that are 1000 times greater than those required to inhibit guanylate cyclase in vitro (Garthwaite et al., 1995).

The observations reported here may be dependent upon the state of the ion channels at the start of the experiment and this in turn might depend upon the resting potential of the pial arterioles. This is unknown in our studies and in almost all in vivo studies by others. Resting potential or some other factor relevant to the initial state of the ion channels could depend upon one or more of the following parameters: type of anesthesia (Crystal et al., 1997; Gibbons et al., 1996; Seki et al., 1997; Sturaitis et al., 1994; Koslowski and Ashford, 1991) presence and type of paralytic agent; composition of artificial cerebrospinal fluid; the use of a stagnant suffusate during measurements of arteriolar diameter as was done here versus the use of a continuously flowing suffusate which may wash out lysine or arginine, essential for ion channel functioning (Kontos and Wei, 1998). One or more of these factors may explain the failure of one laboratory (Golding et al., 2000) to replicate earlier findings (Kontos and Wei, 1998; Wei and Kontos, 1999) that glibenclamide, a K_{ATP} ion channel blocker, inhibits the dilation produced by hypercapnia.

We are aware of studies which, unlike those of Wei and Kontos, found marked responses to K_{ATP} ion channel openers in spite of continuous suffusion of the pial window (Wang et al., 1998).We cannot determine whether this difference in results reflects one or more of the other factors discussed above. However, with regard only to the matter of continuous suffusion of the pial window versus stopped flow during periods of observation, washout of essential endogenous amino acids in the study of Kontos and Wei (1998) was determined by both the rate of flow and its duration. It is theoretically possible that in cases where responses to channel openers remain in spite of flow, the flow on the cerebral surface is slow or is laminar so that the bottom layer remains relatively unstirred and necessary amino acids are removed so slowly that they can be continuously replenished from the plasma.

Finally, in some studies, species differences or age of animal may also play a role. One or both factors may account, in a study of immature pigs (piglets) (Armstead, 1996), for the absence of an inhibitory effect of 7-nitroindazole on dilation of pial arterioles induced by aprikalim, an opener of the K_{ATP} channel and a close chemical relative of cromakalim, one of the channel openers used in the present study. However, with respect to the dependence of the response to CO_2, at least in part, on the K_{ATP} ion channel, it should be noted that such a dependence has been demonstrated in at least two other species (Kontos and Wei, 1996; Wei and Kontos, 1999; Faraci et al., 1994) in addition to the rats used here. In rabbits (Faraci et al., 1994), only the effect of the lesser degree of hypercapnia was inhibited by glibenclamide in contrast to the results presented here where both the effects of 3% and of 5% CO_2 were inhibited.

In conclusion, the results presented here suggest that the inhibitory action of tetrodotoxin and 7-nitroindazole on the response to hypercapnia is due to interaction with a K_{ATP} ion channel. Strictly speaking, this conclusion can extend, at present, only to the experimental conditions described here in which the model is pial arteriolar responses in rats. However, in light of our findings, and those previously reported by Kontos and Wei, it appears reasonable to suggest that in any study demonstrating an effect of a treatment upon the response of pial arterioles to CO_2, it would be prudent for the investigator to also test the ability of that treatment to block the action of K_{ATP} channel

openers before assuming that the treatment effect is due to some other previously defined target of the treatment.

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