Possible obligatory functions of cyclic nucleotides in hypercapnia-induced cerebral vasodilation in adult rats

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Hypercapnia is one of the most potent vasoconstricting stimuli in the cerebral circulation of mammals. However, the specific mechanisms involved in the cerebral vascular smooth muscle (VSM) relaxation induced by hypercapnia, in particular the contributions from the cyclic nucleotide cascades, are far from settled. In the adult and neonate the cyclooxygenase (COX) inhibitor indomethacin has been shown to block hypercapnia-induced cerebral vasodilation (17), indicating a role for vasoconstricting prostanoids (PGs), mainly prostacyclin, in the regulation of cerebral blood flow during hypercapnia. Because these PGs have been linked to enhanced cyclic nucleotide production (1, 2), especially cAMP (23), one might also suspect a role for cyclic nucleotides in CO2-induced cerebral vasodilation. Additional evidence in the neonate suggested that PGs play a permissive role in hypercapnic cerebral vasodilation. That is, if one administers a PG in the presence of indomethacin, at a dose that produces no vasodilation by itself, cerebrovascular CO2 reactivity can be restored to normal levels (12, 24). Thus, rather than CO2 increases eliciting vascular relaxation via enhanced generation of PGs, a certain basal level of vasodilator PGs must be present to provide an “optimal environment” for vasodilation to occur. Whether cAMP plays a permissive role in the CO2 response in the neonate is controversial (12, 24). In adults such a permissive function for cAMP has been reported (22).

In adult and juvenile animals, but not neonates (30), nitric oxide (NO) may participate in hypercapnia-induced cerebrovasodilation. Recent reports also indicate that NO, via cGMP, has a permissive function in the CO2 response (7, 15, 25). Furthermore, evidence points to the involvement of NO being the neural rather than the endothelial isoform of NO synthase (NOS) (15, 22, 27). Still other publications have indicated cross talk in the suspected permissive roles of the cyclic nucleotides. That is, NO supplementation, in the presence of indomethacin (24), and cAMP analog suffusion, in the presence of a NOS inhibitor (22), have been shown to reverse the diminished pial arteriolar CO2 reactivity. However, the functions of the cyclic nucleotides may not be entirely permissive. Hypercapnia can increase cyclic nucleotide levels in the brain, with those changes blocked by indomethacin and NOS inhibitors (6, 9, 17, 24, 29). Therefore, it is possible that cAMP and/or cGMP also play obligatory roles in hypercapnia-induced cerebral vasodilation.

To date, no studies have addressed whether hypercapnia increases both cAMP and cGMP in adult brains. The objectives of the present study were to examine, in adult rats, whether cGMP and cAMP play obligatory roles in hypercapnia-induced cerebral arteriolar dilation and to identify the cellular source of the neuronal NOS (nNOS)-induced NO generation during hypercapnia. In vivo and in vitro (primary rat neuronal and astrocyte cultures) approaches were applied. For the former, we used a closed cranial window system that permitted measurements of pial arteriolar diameters and periarachnoid cerebrospinal fluid (pCSF) cyclic nucleotide levels. The following questions were addressed: 1) Are the hypercapnia-induced pial arteriolar diameter changes in the absence and presence of nNOS or COX inhibition mirrored by changes in the levels of cGMP and cAMP in pCSF? 2) Is the source of the NO

7-nitroindazole; indomethacin; astrocyte; neuron; primary culture; periarachnoid cerebrospinal fluid
produced during hypercapnia neurons, astrocytes, or both?

METHODS

The study received approval of the Institutional Animal Care and Use Committee. For the in vivo study, 19 male Sprague-Dawley rats (~400 g) were employed. Animals were anesthetized with halothane, followed by paralysis (curare, 1 mg/kg), tracheotomy, and connection to a rodent respirator. Bilateral femoral arterial and venous catheters were then inserted under 0.7% halothane-70% N2O–30% O2 anesthesia. The rats were then placed in a prone position, with the head immobilized, and a craniotomy (~10 mm diameter) was performed over the midline of the skull. The dura was carefully removed keeping the sagittal sinus intact. An acrylic cranial window (11 mm diameter, 1 mm thick) equipped with three ports [inflow, outflow, and intracranial pressure (ICP) monitoring] was fixed to the skull with cyanoacrylate gel, and the rats were permitted to recover for about 2 h. After the surgery halothane was discontinued, the rats were maintained on 70% N2O–30% O2–fentanyl (10 µg/kg loading dose, 25 µg·kg\(^{-1}·h^{-1}\) thereafter) and mechanical ventilation until the end of the experiments. Wound sites were infiltrated with bupivacaine. A microscope (Nikon), with attached color video camera (Sony), equipped with an epi-illumination, dark-field system (Fryer, Huntley, IL) was positioned over the cranial window. Magnifications of ~800 were displayed on a video monitor. Precise measurements of vessel diameter were made directly from the monitor using a calibrated video microscaler (Optech). Except during a period of hypercapnia, arterial PCO2 was maintained at 35–40 mmHg. Body temperature (Optech) was controlled at 5–10 mmHg by adjusting the height of the outflow cannula. Initial diameter measurements were made (in 25- to 60-µm pial arterioles) following a 30-min period of cortical cannula. The diameter was then reimposed. For each challenge pCSF was collected from Amersham (Arlington Heights, IL). Briefly, pCSF was slowly infused (0.2 ml/min) into the inlet port of the cranial window, and the CSF was allowed to drip freely from the outlet cannula into a collection tube (containing 50 µl 200 mM EDTA buffered in Tris-base to pH 7.4). Immediately after collection the CSF samples were frozen and stored at ~80°C.

To identify whether increased CO2 elicits NO-dependent increases in cGMP in specific brain cells, we measured cGMP concentrations in primary cultures of cortical neurons or astrocytes. Primary cortical neuronal cultures were prepared from 16- to 18-day fetal Sprague-Dawley rats. The brains were removed and freed of meninges, and the cerebral cortices were isolated. The tissues were minced and treated with 0.1% trypsin for 20 min at 37°C. The cell suspension was then centrifuged, and the pellet was resuspended in DMEM plus 10% heat-inactivated horse serum and plated at a density of 3 × 10^5 cells/well in six-well poly-l-lysine-coated plates.

One-half of the culture medium was replaced with astrocyte conditioned medium the second day after plating, and on the third day cytosine arabinoside (3 µM) was added to each well to inhibit the proliferation of astrocytes. In pilot studies it was found that earlier addition of cytosine arabinoside (17), which produced >95% neuronal purity, did not permit survival for a period of time sufficient to permit maximal neuronal NADPH diaphorase expression. Cultures were kept at 37°C in a humidified incubator with a 5% CO2 atmosphere without further medium changes. Cells were used on days 11–15, when NADPH diaphorase staining showed that the nNOS containing neurons had reached the maximal level. Astrocytes were prepared from 1- to 3-day-old neonatal Sprague-Dawley rats using the procedures described for the neuronal cultures, except the pellet was resuspended in DMEM plus 10% fetal bovine serum (FBS) and plated at a density of 5 × 10^5/well. The cells were fed twice weekly with DMEM plus 10% FBS and used, when confluent, on days 11–15. For cell identifications, cultures were stained with neuron-specific enolase and glial fibrillary acidic protein staining, but only high percentage neuronal survival, i.e., survival for a period of time sufficient to permit maximal neuronal NADPH diaphorase expression. Cultures were kept at 37°C in a humidified incubator with a 5% CO2 atmosphere without further medium changes. Cells were used on days 11–15, when NADPH diaphorase staining showed that the nNOS containing neurons had reached the maximal level. Astrocytes were prepared from 1- to 3-day-old neonatal Sprague-Dawley rats using the procedures described for the neuronal cultures, except the pellet was resuspended in DMEM plus 10% fetal bovine serum (FBS) and plated at a density of 5 × 10^5/well. The cells were fed twice weekly with DMEM plus 10% FBS and used, when confluent, on days 11–15. For cell identifications, cultures were stained with neuron-specific enolase and glial fibrillary acidic protein staining. As stated previously, the neuronal cultures showed an 80–90% purity (remainder astrocytes). The purity of astrocyte cultures was >95%.

Cells in gas-tight 2-ml sister-culture wells were exposed to the NO donor sodium nitroprusside (SNP) at a concentration of 25 µM for 1 min, to confirm the presence of endogenous guanylyl cyclase in neurons and astrocytes. The Ca\(^{2+}\)-dependent NOS inhibitor A-23187 (10 µM) was added in the presence or absence of the nonselective NOS inhibitor N\(^{G}\)-nitro-l-arginine (l-NNA, 10 µM) to establish the presence of Ca\(^{2+}\)-dependent NOS in both preparations. In each case, the DMEM-FBS medium was removed and replaced with an acSF solution containing the test agents. Control cells (for baseline cyclic nucleotide measurements) were given drug-free acSF. For hypercapnia studies, the DMEM-FBS medium was removed and replaced with an acSF solution containing the test agents. Control cells (for baseline cyclic nucleotide measurements) were given drug-free acSF. For hypercapnia studies, the DMEM-FBS medium was removed and replaced with an acSF solution containing the test agents. Control cells (for baseline cyclic nucleotide measurements) were given drug-free acSF. For hypercapnia studies, the DMEM-FBS medium was removed and replaced with an acSF solution containing the test agents. Control cells (for baseline cyclic nucleotide measurements) were given drug-free acSF.
Radioimmunoassay, and the pellets were dissolved in 1 N NaOH for measurement of protein using the Bradford method (20). The cGMP contents were normalized to the protein content.

The l-NNA, A-23187, and SNP were obtained from Sigma (St. Louis, MO). 7-NI was from ICN Biologics (Aurora, OH). In most cases, a paired t-test was used for statistical analyses. Otherwise, a repeated-measures ANOVA with a post hoc Tukey analysis (A-23187 experiments) was used. All values are reported as means ± SE.

RESULTS

Cranial Window Studies

Arterial blood variables. In all animals arterial Po2 levels were maintained at >100 mmHg and mean arterial blood pressure was maintained at ~130 mmHg throughout the experiments. The measured arterial pH and PCO2 ranges were 7.35–7.42 and 34–40 mmHg, respectively, during normocapnia, and 7.01–7.16 and 58–70 mmHg, respectively, during hypercapnia. Over the ~180-min course of each experiment, no significant variations in PCO2, pH, or mean arterial blood pressure were observed in any group between periods of hypercapnic exposure. The PCO2 increases with each hypercapnic episode were similar in each group.

Baseline pial arteriolar diameters and cyclic nucleotides. The initial pial arteriolar diameters measured in groups 1 and 2 were 43.3 ± 2.3 and 44.9 ± 1.8 µm, respectively. Baseline cGMP and cAMP values were 115 ± 6 and 589 ± 45 fmol/ml in group 1 and 140 ± 14 and 635 ± 34 fmol/ml in group 2, respectively. Exposure to 7-NI or indomethacin did not significantly affect resting pial arteriolar diameters and cyclic nucleotide contents in any of the groups.

The pial arteriolar responses to hypercapnia, expressed as CO2 reactivity (% increase in diameter/mmHg change in PCO2), and changes in pCSF cyclic nucleotide levels before and after 7-NI or indomethacin are summarized in Figs. 1, 2, and 3, respectively. Before 7-NI administration, hypercapnia increased pial arteriolar diameter 40 ± 6% (which yields a CO2 reactivity of 1.50 ± 0.3, Fig. 1). Hypercapnia was accompanied by significant elevations in cGMP (by 73%, Fig. 2) and cAMP (by 79%, Fig. 2). Inhibition of nNOS did not change the basal cGMP and cAMP content but significantly attenuated hypercapnia-induced arteriolar dilation (67% reduction in CO2 reactivity) and reduced hypercapnia-induced cGMP and cAMP increases by 84 and 66%, respectively (Fig. 2).

In the COX inhibition study, hypercapnia elicited a 42 ± 6% pial arteriolar dilation before indomethacin (CO2 reactivity = 1.7 ± 0.2, Fig. 1) and significant increases in pCSF cGMP (by 92%, Fig. 3) and cAMP (by 71%, Fig. 3). Indomethacin did not change the baseline cAMP but reduced hypercapnia-induced arteriolar dilation (by 82%, Fig. 1) and substantially attenuated the pCSF cAMP increase (by 80%, Fig. 3). The cGMP increase accompanying hypercapnia was completely blocked by indomethacin (Fig. 3).

The normal NMDA-induced cGMP increase of 45% was reduced by 68% after 7-NI administration (Fig. 4).

Fig. 1. Arteriolar response to hypercapnia (expressed as %diameter increase/mmHg change in PCO2) before and after 7-nitroindazole (7-NI; n = 10 observations; 1 observation/rat) or cyclooxygenase inhibitor indomethacin (Indo; n = 9 observations). Hypercapnia-induced relaxation was significantly blocked by 7-NI and indomethacin. *P < 0.05 vs. initial. Values are means ± SE.

Fig. 2. Percent changes in cyclic nucleotides (means ± SE; n = 10 observations) in periarachnoid cerebrospinal fluid (pCSF) when going from normo- to hypercapnia in absence or presence of neuronal nitric oxide synthase (nNOS) inhibitor l-NNA (10 μM; Fig. 5). The basal cGMP content in astrocytes was ~3% of the content measured...
in neurons, although the cAMP levels were similar. Exposure to 14% CO₂ did not affect cGMP content in astrocytes. Because of this we were confident that the hypercapnia-induced cGMP increases in neuronal cultures were not derived from the 10–20% astrocytes contaminating the cultures. Administration of the NO donor SNP (25 µM) significantly increased cGMP in both neurons and astrocytes (Fig. 6). The Ca²⁺-dependent NOS activator A-23187 (10 µM) significantly increased cGMP in neurons (5-fold) and in astrocytes (2-fold) (Fig. 7). The increase with A-23187 was also completely inhibited by L-NNA, demonstrating the presence of NO-stimulatable guanylyl cyclase and Ca²⁺-dependent NOS in both preparations. No significant changes in cAMP were seen in neurons when switching CO₂ from 5 to 14% (Fig. 8).

DISCUSSION

There were four principal findings in this study. First, based on the effects of 7-NI and indomethacin, nNOS-derived NO and vasodilator PGs overlap considerably in their capacity to support hypercapnia-induced pial arteriolar dilation. Second, hypercapnia is accompanied by significant increases in pCSF levels of the principal NO and vasodilator PG “second messengers” cGMP and cAMP, respectively. Third, mirroring the overlap seen with nNOS and COX inhibitor effects on CO₂ reactivity, the CO₂-induced increases in both cyclic nucleotides could be prevented by administration of either 7-NI or indomethacin. Fourth, exposure to elevated CO₂ levels, in vitro, elicits a transient NOS-dependent increase in neuronal (but not astrocytic) cGMP levels, suggesting that the cellular source of the NO responsible for CO₂-induced increases in cGMP in vivo is neurons.

A number of studies have suggested that cGMP and cAMP act in a “permissive” manner in supporting hypercapnia-induced cerebral vasodilation (7, 8, 22, 24, 25). That is, rather than NOS or COX inhibitors suppressing CO₂ reactivity via blocking the ability to increase cGMP or cAMP synthesis, those agents may act by reducing cyclic nucleotide levels below a critical threshold. The strategy for identifying a permissive
function of a substance like cGMP (i.e., repletion of the reduced cGMP levels, accompanying NOS inhibition, through administration of a cGMP analog) assumes that the cellular system has been restored to a state that existed before NOS inhibition. However, in a recent report, we found that this was not the case (25). In that study, exogenous cGMP reversed the 7-NI-induced attenuation of cerebrovascular CO2 reactivity, but the normally K-channel independent nature of the hypercapnic response converted to one that was repressed by multiple K-channel blockers. Clearly, the factors participating in CO2 reactivity in the latter case (i.e., NOS inhibition cGMP repletion) were different, and perhaps more complicated, compared with those present under conditions of normal NOS activity. This compelled us to reevaluate the permissive function of cGMP.

The role of cAMP as a permissive entity in hypercapnia is also unsettled. In adult rats addition of a cAMP analog restored pial arteriolar CO2 reactivity in the presence of a nNOS inhibitor (22). However, in the neonate, one cAMP-elevating agent (adenosine) was found to partially restore pial arteriolar CO2 reactivity in the presence of indomethacin, whereas another cAMP-elevating drug, isoproterenol, was not (12, 24). There is no clear explanation for these conflicting results, although species and age-related factors cannot be ruled out. There are a number of factors that could influence the responses to the above agents, independent from their intended actions as permissive agents. One possibility that could account for cAMP being effective in one study and not another is the phosphodiesterase (PDE)-resistant nature of the 8-bromo analogs compared with “natural” (i.e., agonist-induced) cAMP (22). A second possibility relates to a differential compartmentalization of adenylyl cyclase, PDEs, and cAMP “targets” (e.g., cyclic nucleotide kinases and the protein targets of those kinases), in relation to specific receptors (22). For example, if the receptor-adenylyl cyclase combination were located in close proximity to the permissive target of cAMP, then the cAMP formed would be less susceptible to PDE actions. This could account for different effects of one receptor agonist vs. another (e.g., adenosine vs. isoproterenol vs. PGs).

Ostensibly, present results would appear to be more in line with the classic or “obligatory” model of NO- or prostanoid-induced cerebral vasodilation, where the synthesis of those substances is increased in the presence of a vasodilating stimulus. Thus we found that hypercapnia elicited NOS-dependent increases in cGMP levels in primary neuronal cultures and 7-NI- and indomethacin-inhibitable increases in pCSF cGMP and cAMP concentrations. This is similar to data obtained in the newborn pig, where hypercapnia-induced pial arteriolar dilation was accompanied by significant increases in both cAMP and cGMP production, as measured in pCSF (17). Those responses were repressed by indomethacin but, in contrast to adults, were unaffected by NOS inhibition (17, 30). Furthermore, consistent with an obligatory role for cAMP and/or cGMP, inhibition of cyclic nucleotide hydrolysis both augmented the pCSF cyclic nucleotide increases and the vasodilating response to hypercapnia (18). In adult rats hypercapnia has been reported to increase brain tissue NO and cGMP levels, with both responses blocked in the presence of NOS inhibitors (6, 9, 29).
The transient increase in neuronal cGMP levels, when increasing CO₂ levels, is rather curious but not without precedent. Thus Pearce et al. (21) found a similar transient increase in cGMP levels in cerebral vessels in the continued presence of NO donors. Like current findings, the peak cGMP elevations were achieved by ~1 min, followed by a return to baseline values by ~2 min. Moreover, maximum relaxation of the vessels was achieved at a time when the cGMP content was back at baseline levels. In another report (16) it was shown that exposing cerebral VSM cells (in noncontact coculture with endothelial cells) to a steady level of hypercapnia produced a transient cAMP increase in the VSM, with a time course similar to that seen in the present study and the study by Pearce et al. Reductions in cyclic nucleotide levels intracellularly are generally attributable to PDE actions and the presence of cyclic nucleotide export mechanisms (5, 14). These data would suggest that persisting vascular relaxation is controlled by factors occurring downstream from cGMP·cAMP synthesis (i.e., phosphorylation and dephosphorylation events; contractile protein Ca²⁺ sensitivity). There is one additional finding in the study by Parfenova and Leffler (16) that has some relevance to the current discussion. That is, despite the transient rise and then fall in cAMP levels within VSM, their sites of synthesis in perivascular and endothelial cells and will interact with their adenylyl cyclase-linked receptors in all nearby cells, especially VSM cells, which contain a high density of such receptors. Thus the indomethacin-sensitive rise in pCSF cAMP levels should be mirrored by similar changes in VSM.

A second cautionary note relates to the possibility that hypercapnia-induced increases in cyclic nucleotides do not necessarily obviate permissive roles for these agents. The fact that the levels of the cyclic nucleotides increase may have little to do with vasodilation, as long as a minimum level is maintained (i.e., the permissive concept). In fact, in the neonate, there is evidence to support such a possibility. Thus the COX inhibitors aspirin (19) and ibuprofen (24) were able to reduce or block the hypercapnia-induced rise in pCSF cAMP and vasodilator PG levels but had no effect on hypercapnia-induced pial arteriolar dilation. Indomethacin blocked both the vascular and pCSF responses, with the arteriolar response (in the presence of indomethacin) being restored by addition of a vasodilator PG (12, 24). These findings could be taken to suggest that the increase in cAMP is superfluous and unnecessary for CO₂-induced arteriolar relaxation. In adult animals it remains to be shown whether cAMP behaves in a similar manner in hypercapnia.

An additional possibility that merits consideration is that the cyclic nucleotides may participate in hypercapnic cerebral vasodilation in both permissive and obligatory manners. As an extension of that potentiality, it might also be considered that the intracellular content of one cyclic nucleotide may need to be maintained (the permissive function) to allow levels of the other to increase (the "conventional" or obligatory function). For example, this might occur at the level of cyclic nucleotide hydrolysis, where a cGMP-inhibitable cAMP-PDE and perhaps even a cAMP-inhibitable cGMP-PDE have been suggested to participate in cerebrovascular regulation (22). Thus simply maintaining the level of one cyclic nucleotide may permit the other to rise in the presence of an appropriate stimulus.

Finally, one might consider that cyclic nucleotides may not be the permissive or even the obligatory agents in VSM relaxation. The presumption made in most studies (including the present one) is that the vasodilating responses to PGs and NO are mediated by cAMP and cGMP, respectively. This may not always be true. CAMP-independent vasodilating actions of NO have been reported (10), and prostanoid-mediated vasodilations may occur independently from cAMP changes (1). Furthermore, the permissive action of vasodilator PGs may be more direct, not necessarily occurring through cAMP (12, 13). Although cAMP and cGMP remain the most likely candidates as mediators of PG and NO-related vasodilations, respectively, it cannot be presumed that they act exclusively in each pathway.

There was considerable overlap in the abilities of 7-NI and indomethacin to attenuate hypercapnia-induced pial arteriolar dilation. This was similar to results reported in an earlier study from our laboratory regarding the influence of indomethacin and the nonse-
lective NOS inhibitor L-NNA on cortical CBF changes during hypercapnia (28). Furthermore, not only was hypercapnia accompanied by approximately twofold increases in pCSF, cGMP, and cAMP levels in the present study, but those changes were also completely blocked by either indomethacin or 7-NI. The fact that blocking the hypercapnia-induced rise in one cyclic nucleotide prevented the rise in the other implies that any characterization of cyclic nucleotide contributions to the cerebrovascular CO2 response as obligatory is an oversimplification. That is, the present data suggest that both cGMP and cAMP must increase for hypercapnic cerebrovasodilation to occur. That scenario also implies a capacity for each cyclic nucleotide to regulate the levels of its counterpart and is strongly suggestive of cross talk between the two cyclic nucleotide pathways. The reasonable possibility of cross talk regulation occurring at the level of cyclic nucleotide breakdown (i.e., the PDEs) was discussed earlier. On the other hand, there is little or no compelling evidence suggesting that cGMP and cAMP can influence each other synthesis (22).

The discussion above suggests a mechanism whereby cGMP can prevent the loss of cAMP and vice versa. It does not necessarily explain the substantial overlap in the abilities of NOS and COX inhibitors to repress CO2-induced cerebrovasodilation. That overlap may occur at the level of the protein kinase targets of cGMP and cAMP actions (PKG and PKA, respectively). We recently found that PKG and PKA inhibitors attenuated pial arteriolar CO2 reactivity in a nonadditive fashion (22). The targets of the kinase actions were not identified, but the fact that inhibitor actions were nonadditive and superimposable could be attributed to common serine-threonine targets for the two kinases. On the other hand, PKG and PKA are known to phosphorylate separate sites as well (4). Thus one might also consider the possibility that these kinases may act in a cooperative manner, in that phosphorylation of one site via one kinase is needed before another site can be phosphorylated by the other kinase. Consequently, both sites need to be phosphorylated to permit hypercapnia-induced increases in H+ to elicit VSM relaxation.

In conclusion, we found that administration of the nNOS inhibitor 7-NI or the COX inhibitor indomethacin each produced ~80% reductions in the CO2 reactivity of pial arterioles in adult rats. Furthermore, the hypercapnia-induced increases in cGMP and cAMP generation in the brain in vivo were completely blocked when either of the inhibitors was given, suggesting that increased synthesis of both cGMP and cAMP are important to the hypercapnic response and that cross talk exists between the cyclic nucleotide pathways. Studies using brain neurons and astrocytes in culture further identified neurons as the source of the NO participating in the CO2 response. The source of the vasodilator PG involved in hypercapnia-induced cerebrovasodilation was not identified.

We gratefully acknowledge the expert technical assistance of Anthony Sharp and Xielu Wei.

This work was supported by the National Heart, Lung, and Blood Institute Grants HL-56162 and HL-52594.

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