PTX-sensitive G proteins and permissive action of prostacyclin in newborn pig cerebral circulation

BORUCH ZUCKER AND CHARLES W. LEFFLER
Laboratory for Research in Neonatal Physiology, Department of Physiology and Biophysics, University of Tennessee, Memphis, Tennessee 38163

Zucker, Boruch, and Charles W. Leffler. PTX-sensitive G proteins and permissive action of prostacyclin in newborn pig cerebral circulation. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H259–H263, 1998.—The present study of newborn pig cerebral circulation investigated the role of pertussis toxin (PTX)-sensitive GTP binding proteins in the permissive action of prostacyclin in specific dilator responses. Pial arterioles of anesthetized piglets were observed through closed cranial windows. The piglets were treated topically with PTX and intravenously with indomethacin. The effects of hypercapnia (10% CO2 ventilation) and topical 5,6-epoxyeicosatrienoic acid (5,6-EET) on pial arteriolar diameters were noted before and after the intervention. Samples of the artificial cerebrospinal fluid (aCSF) were collected from beneath the cranial windows for determination of the aCSF concentration. After administration of PTX, indomethacin still abolished pial arteriolar dilation to both hypercapnia and 5,6-EET and also inhibited the aCSF elevation caused by hypercapnia. The addition of phorbol 12-myristate 13-acetate (PMA), but not iloprost, restored the increase in cAMP and undesirable responses in piglets. Prostacyclin (PGI2), derived from cyclooxygenase (22). PKC could phosphorylate a Gi protein associated with adenylyl cyclase. Therefore, in newborn pig cerebral microvasculature, PTX appears to inhibit a G protein involved in the permissive action of prostacyclin. However, the protein kinase C (PKC) activator PMA appears to act downstream from the block, and, therefore, the permissive action of PMA is not affected by PTX. We suggest that the prostacyclin IP receptor may be coupled to phospholipase C via a PTX-sensitive G protein that normally permits vasodilation to specific stimuli via activation of a PKC, resulting in phosphorylation of a component of the adenylyl cyclase pathway.

cerebrovascular circulation; hypercapnia; epoxyeicosatrienoic acids; adenosine 3',5'-cyclic monophosphate; protein kinase C

PROSTANOIDs are involved in a variety of vascular responses in piglets. Prostacyclin (PGI2), derived from vascular endothelium, plays an important role in preventing vasoconstriction and platelet aggregation. Actions of extracellular PGI2 are mediated by the cell surface IP receptors. Prostanoids contribute "permissively," rather than classically, in the cerebral vasodilation that accompanies hypercapnia (16). "Permissive" refers to the requirement for IP-receptor activation to initiate a series of events that allow the vascular smooth muscle to respond to hypercapnia as well as several other stimuli. The current hypothesis has PGI2 binding to its receptor and activating phospholipase C (PLC) (12). PLC generates diacylglycerol, which activates a protein kinase C (PKC). Without PGI2, PKC may be inhibited by an upstream protein tyrosine kinase (22). PKC could phosphorylate a G protein [thereby inhibiting it (9)] or adenylyl cyclase [thereby activating it (10)], either of which would result in increased production of cAMP in response to stimulation. The resultant increase in cAMP would lead to cerebral vasodilation.

Arachidonic acid can also be metabolized to epoxyeicosatrienoic acids (EETs) via cytochrome P-450 epoxygenase. EETs, like hypercapnia, require the permissive action of PGI2 to produce vasodilation in the newborn pig cerebral circulation (15). The cyclooxygenase inhibitor indomethacin has been used to inhibit the vascular response to hypercapnia (13, 14, 18, 25) and EETs (15). In indomethacin-treated piglets, responses to EETs and hypercapnia are restored by treatment with PGI2 analogs (15, 16). The PKC activator phorbol 12-myristate 13-acetate (PMA) restores cerebral vasodilation in response to hypercapnia in indomethacin-treated piglets (22).

The present study was designed to address the hypothesis that PGI2 initiates a cascade that culminates in the phosphorylation and inhibition of G1α associated with adenylyl cyclase. Therefore, in newborn pigs, the role of G1α was assessed by treating the pial arterioles topically with pertussis toxin (PTX). PTX catalyzes the ADP-ribosylation of a cysteine residue found near the carboxy terminal of certain G protein subunits: the known PTX-sensitive G proteins are Gαi1,3, Gαo1,2, and Gβ1,2 (23).

METHODS

Surgery. The animal protocols used were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee (Memphis, TN). Newborn pigs (1–3 days old) were anesthetized initially with ketamine hydrochloride (33 mg/kg im) and acepromazine (3.3 mg/kg im) and maintained on α-chloralose (50 mg/kg iv). The animals were ventilated with air via a tracheotomy. Catheters were inserted in the femoral vein for administering drugs and fluids and in the femoral artery for recording blood pressure and drawing samples for blood gas and pH analysis. Core temperature was monitored with a rectal probe. A heating pad was used to maintain core body temperature between 37 and 38°C. Blood and gas levels were maintained within physiological limits except during experimentally induced hypercapnia.

The head was immobilized, the scalp was retracted, and a hole 2 cm in diameter was made in the skull over the parietal cortex. The dura was cut without touching the brain, and all cut edges were retracted over the bone so that the periarchnoid space was not exposed to bone or damaged membranes. A stainless steel and glass cranial window was placed in the hole and cemented into place with dental acrylic. The space

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under the window was filled with artificial cerebrospinal fluid (aCSF; 150 meq/l Na, 3 meq/l K, 2.5 meq/l Ca, 1.2 meq/l Mg, 32 meq/l Cl, 3.7 mM glucose, 6 mM urea, 25 meq HCO₃, pH 7.32–7.44, Pco₂ 40–46 mmHg, Po₂ 45–50 mmHg) through stainless steel injection ports incorporated into the sides of the window. The volume of fluid directly beneath the window was ~500 µl and was contiguous with the periarchnoid space. Pial arterioles were observed with a dissecting microscope. Diameters were measured with a video micrometer coupled to a television camera mounted on the microscope and a video monitor. In most piglets, two arterioles of different sizes were measured.

Materials. 5,6-Epoxyeicosatrienonic acid (5,6-EET; 100 µg/ml; Cayman Chemical) was diluted to yield working dilutions of 10⁻⁷ M and 10⁻⁶ M. Isoproterenol (Sigma Chemical) was dissolved to yield a working dilution of 10⁻⁶ M. Pertussis toxin (50 µg/ml; Sigma Chemical) was diluted to yield a working dilution of 1 µg/ml. Indomethacin trihydrate, a gift from Merck Sharp & Dohme Research Laboratories, was dissolved in normal saline and given intravenously (5 mg/kg). Iloprost (3 × 10⁻⁴ M), a gift from Schering Pharmaceutical Research, was diluted to yield a working dilution of 10⁻¹² M. PMA (Sigma Chemical) was dissolved in DMSO to give a 1 mM stock solution. A final dilution of 1 µM was made in aCSF. Unless stated otherwise, all dilutions were made in aCSF.

Experimental design. The experimental design consisted of the initial recording of the pial arteriolar responses to hypercapnia and 5,6-EET (10⁻⁷ M and 10⁻⁶ M), both of which are known to require a permissive contribution by prostacyclin, and to isoproterenol, which is known to be independent of prostanoids. Hypercapnia was produced by ventilation with a 10% CO₂–21% O₂–69% N₂ mixture, causing an increase in arterial Pco₂ (PaCO₂) from 33.8 ± 0.4 to 73.5 ± 2.4 mmHg. Hypercapnic treatment was administered for 10 min, whereas all other dilator agonists were administered topically for 5 min. At the end of each tested response, an arterial blood gas measurement was taken and a sample of cortical periarchnoid CSF (300 µl) was collected (see Measurements of CSF cAMP). The area under the cranial window was flushed with aCSF to remove the previous stimulus and allow the vessels to return to baseline diameters. Injections were made under the cranial window through a 0.22-µm filter to maintain sterility. When 5,6-EET or isoproterenol was applied, pial arteriolar diameters were measured at 1, 3, and 5 min. Hypercapnia produces a sustained vasodilation that is typically maximal from 7 to 10 min. The maximal dilation obtained during a given treatment was recorded as the response. Isoproterenol (10⁻⁹ M) was used to test any generalization change in vascular reactivity, because the response to isoproterenol is consistent over time and is not associated with prostanoids or endothelium dependent [not affected by indomethacin treatment or light/dye endothelial injury (17)].

After determination of responses to hypercapnia, 5,6-EET, and isoproterenol, PTX (1 µg/ml) was administered topically for 1 h and subsequently washed off with aCSF. After PTX treatment, a bolus of indomethacin was administered (5 mg/kg iv).

The second half of the protocol was a repeat of the first half, with the exception that either iloprost (10⁻¹² M, a prostacyclin analog) or PMA (10⁻⁶ M, a PKC stimulator) was added to the aCSF. Both iloprost and PMA have been shown to return the vasodilation response to hypercapnia and EETs in the presence of the cyclooxygenase inhibitor indomethacin (15, 22).

Measurements of CSF cAMP. Cortical periarchnoid CSF (300 µl) was collected from under the window by slowly infusing aCSF into an inlet port of the cranial window and allowing the CSF to drip freely into a collection tube from an outlet port. The first drop was not collected, because it was in the port during the treatment and not under the window. The collection tubes contained EDTA (5 mM) buffered in Tris base to pH 7.4. Immediately after collection, the samples were frozen and stored at –60°C. cAMP content was determined by RIA with the use of antibodies we produced in rabbits and using commercial ¹²⁵I-labeled CAMP (Amersham) as a radioligand. Acetylation was performed before assay.

Statistical Analysis. Values for each variable are presented as means ± SE. Comparison between treatments used ANOVA with repeated measures. Fishers protected least significant differences test was used for multiple comparisons. P < 0.05 was regarded as significant.

RESULTS

No significant changes in blood gases and pH were observed within or between groups before, during, or after topical administration of 5,6-EET, isoproterenol, or PTX. With the exception of the hypercapnic periods, when PaCO₂ was 73.4 ± 2.3 mmHg and arterial pH was 6.97 ± 0.01, the arterial pressure, blood gas, and pH values were within normal limits for newborn pigs and were not affected by the interventions. Isoproterenol stimulated vasodilation in the pial arterioles in both groups before and after treatment with PTX and indomethacin. Vasodilation to isoproterenol (10⁻⁶ M) was not affected by PTX [64 ± 4 to 91 ± 7 µm before and 65 ± 4 to 89 ± 7 µm after (n = 8)] or by PTX and indomethacin [66 ± 5 to 90 ± 9 µm before and 69 ± 4 to 84 ± 7 µm after (n = 6)]. Thus the vessels retained reactivity in general following the treatments.

Although the diameters of larger (60–100 µm) and smaller (35–50 µm) pial arterioles were measured, the data from only one arteriole of ~60 µm in diameter are presented for each piglet to simplify presentation of the results, because no differences of significance to the current question were observed relative to vessel size.

The pial arteriolar diameters during normocapnia and hypercapnia are shown in Fig. 1. Although not shown in Fig. 1, PTX alone had no effect on vasodilation in response to hypercapnia: before PTX, pial arterioles dilated from 62 ± 8 to 99 ± 9 µm, and after PTX, the same arterioles dilated from 63 ± 7 to 104 ± 14 µm in response to hypercapnia (n = 3). Treatment with PTX and indomethacin blocked the hypercapnia-induced vasodilation (Fig. 1). After iloprost was added to the aCSF in piglets treated with PTX and indomethacin, the arterioles were still unable to respond to hypercapnia. However, when PMA was added to the aCSF, the pial arterioles did vasodilate in response to hypercapnia, even in piglets pretreated with PTX and indomethacin.

The cAMP concentrations during normocapnia and hypercapnia are shown in Fig. 2. During hypercapnia, the concentration of cAMP increases. After treatment with PTX and indomethacin, there were no significant changes in cAMP. The addition of iloprost to aCSF did not return the cAMP response to hypercapnia in PTX and indomethacin-treated piglets. Conversely, there was a significant increase in cAMP during hypercapnia.
when PMA was added to the aCSF, even after treatment with PTX and indomethacin.

The pial arteriolar diameters before and during treatment with 5,6-EET (10^{-7} M and 10^{-6} M) are shown in Fig. 3. The pial arterioles dilated in response to 5,6-EET (10^{-7} M and 10^{-6} M). After iloprost was added to the aCSF in piglets treated with PTX and indomethacin, the arterioles were unable to respond to 5,6-EET. However, when PMA was added to the aCSF, the pial arterioles did dilate in response to 5,6-EET, even in piglets pretreated with PTX and indomethacin.

**DISCUSSION**

The new findings in this study are that 1) PMA can remove the block of 5,6-EET-induced vasodilation by indomethacin, 2) inhibition of a G\(_i\) protein associated with adenylyl cyclase is not involved in the mechanism of the permissive action of prostacyclin in dilations to hypercapnia and 5,6-EET, and 3) a PTX-sensitive G protein appears to be present in the permissive pathway between the IP receptor and PKC activation.

The concept of endothelium-derived relaxing factors (EDRF) serving permissive functions in the regulation of vascular tone, instead of being directly coupled to the dilation by producing the second messenger, is becoming generally appreciated as a prominent role of EDRF, which may convey information relative to the functional integrity of the vessel wall (12). It has been speculated that a similar function regarding the functional integrity of vascular innervation could be played by neuron-derived nitric oxide (NO) (11). Although permissive actions of EDRF have been described in other circulations (5, 6), contributions to cerebral hemodynamic function are the most often studied, including the actions of NO in adult rodents (4, 7, 8) and prostacyclin in newborn pigs (15, 16, 20, 24). A permissive role of the thromboxane TP receptor in the constrictor response to ACh in the piglet cerebral microvasculature was demonstrated earlier (1). Whether permissive actions of NO and/or TP agonists function by increasing the gain of the primary second messenger system, as appears to be the case with prostacyclin, remains to be investigated.

EETs are potent vasodilators of newborn pig cerebral arterioles and could play a role in the control of
cerebral circulation (15). Furthermore, indomethacin blocks dilation to EETs, and iloprost can restore the vasodilation to EETs, in indomethacin-treated piglets. These findings led to the conclusion that the permissive role of prostacyclin in vasodilation to EETs is similar to the role in the dilation of piglet arterioles to hypercapnia. In the present study, PMA treatment could be substituted for the prostacyclin analog (iloprost) in reversing the indomethacin-induced blockade of EET cerebral vasodilation, similar to the case of hypercapnia (22).

The permissive mechanism of the influence of prostacyclin on cerebral microvascular reactivity appears to provide an explanation for the unique ability of indomethacin, among the myriad of cyclooxygenase inhibitors, to inhibit cerebral vascular responses to specific stimuli, notably hypercapnia. Indomethacin shares with many pharmacological agents inhibitory actions on both cyclooxygenase isoforms. However, 100% inhibition of cyclooxygenase activity with any inhibitor is not accomplished. Therefore, residual prostacyclin may be sufficient to serve the permissive function. In contrast to other cyclooxygenase inhibitors, indomethacin is also a weak antagonist of the IP receptor (19) and inhibits organic add transport, and thus prostanoids, from the cell (2). Therefore, indomethacin is uniquely capable of removing the influence of prostacyclin by inhibiting its synthesis, trapping residually produced prostacyclin intracellularly, and, finally, interfering with receptor binding of remaining low extracellular prostacyclin.

A hypothetical model has been proposed to explain the hypercapnic permissive cascade in the neonatal pig cerebral microcirculation (12). This model was based on the following findings: 1) indomethacin and endothelial injury can block the pial arteriolar dilation in response to hypercapnia, 2) IP-receptor agonists can selectively restore dilations blocked by indomethacin or endothelial injury, and 3) protein tyrosine kinase inhibition or protein kinase C stimulation will also return the pial arteriolar dilator responses. Two potential mechanisms appeared most obvious: PKC acting on an inhibitory G protein [inactivating it (9)] or on adenylyl cyclase [activating it (10)]. In the present study, treatment with PTX and indomethacin blocked vasodilation to both hypercapnia and 5,6-EET (10⁻⁷ M and 10⁻⁶ M). If the IP-receptor/PKC permissive action resulted from inhibitory phosphorylation of a Gα associated with adenylyl cyclase, then PTX should allow vasodilation to hypercapnia and EETs in the presence of indomethacin. However, this did not occur. In sharp contrast, PTX blocked the ability of iloprost to restore dilation. Therefore, it appears that the necessary target for PKC to exert its permissive action is not a Gα protein coupled to adenylyl cyclase. The second possibility, that PKC acts on the adenylyl cyclase (increasing its intrinsic activity), is still viable and remains to be tested. By priming the adenylyl cyclase, a given increase in H⁺ concentration may cause greater amounts of cAMP to be produced by the smooth muscle cell, perhaps enough to stimulate vasodilation. We envision that the mechanisms involved in the permissive action of prostacyclin in EET- and histamine-induced dilations are similar to the mechanisms involved in vasodilation to hypercapnia.

In piglets treated only with indomethacin, iloprost will allow hypercapnia and EETs to stimulate vasodilation (15, 16). In the present study, iloprost did not return the vasodilation after PTX treatment. This leads to the conclusion that somewhere in the permissive pathway there is a PTX-sensitive G protein (probably Gα or Gβ) (3, 20, 21) that is downstream from the IP receptor but upstream from PKC. The probable location for this protein would be coupling the IP receptor with phospholipase C, as envisioned in the cartoon representation of our current hypothesis (Fig. 4). We suggest that IP-receptor-mediated activation of PKC results in increased gain of the adenylyl cyclase system on stimulation. This IP-receptor-mediated activation of PKC appears to involve coupling via a PTX-sensitive G protein.

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Address for reprint requests: C. W. Leffler, Dept. of Physiology and Biophysics, Univ. of Tennessee, Memphis, 894 Union Ave., Memphis, TN 38163.

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