Phospholipase C Activation by Prostacyclin Receptor Agonist in Cerebral Microvascular Smooth Muscle Cells (44462)

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Abstract. **The mechanism through which iloprost permits cerebral vasodilation induced by specific stimuli is incompletely understood. Previous study suggests there might be interplay between the adenylyl cyclase and phospholipase C (PLC) systems. Coupling of the prostacyclin receptor with the PLC pathway system was investigated. Iloprost, a stable prostacyclin analog, was used as a prostacyclin receptor agonist. We investigated the effects of iloprost (10^{-12}–10^{-6} *M*) on inositol 1,4,5-trisphosphate (IP_3) production by piglet cerebrovascular smooth muscle cells in primary culture. Iloprost caused concentration- and time-dependent increases in IP_3 production in control cells and in cells pretreated with LiCl (to prevent further IP_3 metabolism). Iloprost treatment (10^{-12} *M*) of cerebrovascular smooth muscle cells, in the absence and presence of 20 m*M* LiCl, resulted in 2-fold and 4-fold increases in the formation of IP_3, respectively. In contrast, 10^{-10} *M* to 10^{-6} *M* iloprost, either in the presence or absence of LiCl, induced moderate or no increase in IP_3 formation. Iloprost (10^{-10}–10^{-12} *M*) strongly stimulated diacylglycerol (DAG) generation, whereas higher concentrations (10^{-8} *M*) did not induce an increase. In conclusion, the results suggest that prostacyclin receptors on cerebromicrovascular smooth muscle can couple to PLC, generating the second messengers, IP_3 and DAG.** [P.S.E.B.M. 2000, Vol 223]

In newborn pigs, hypercapnia induces cerebral vasodilation that is accompanied by an increase in prostanoid synthesis (1). Indomethacin, a prostaglandin cyclooxygenase and prostacyclin receptor (IP receptor) inhibitor, blocks both hypercapnia-induced production of prostanoids (2) and cerebral vasodilation (1, 3–5). However, hypercapnia, prostanoids, and vasodilation do not interact in a classical mediator, second messenger, response paradigm. Instead, prostacyclin (PGI_2) agonists, at low concentrations that produce no dilation, restore cerebromicrovascular dilation to hypercapnia that had been blocked by indomethacin (1, 4). Prostacyclin is involved in the hypercapnia-induced cerebral vasodilation through a permissive action in newborn pigs because increasing levels are not necessary to cause dilation directly (4). The permissive effect is not limited to only hypercapnia; iloprost also permits cerebral vasodilation to histamine (6), and epoxyeicosatrienoic acids (7). In addition, low concentrations of iloprost also have been found to restore hypercapnia-induced pial arteriolar dilation lost after light/dye microvascular injury (6).

The mechanism by which IP receptor agonists permit hypercapnic and other dilatory cerebral vasodilation in newborn pigs is not completely understood. Hypercapnia induces dose- and time-dependent increases in cyclic nucleotides that can be blocked by indomethacin (5) at concentrations that also inhibit vasodilation (4). These results suggest that indomethacin, at least in part, inhibits vasodilation by blocking the elevation of adenosine 3′,5′-cyclic monophosphate (cAMP) induced by hypercapnia in the cerebral microvasculature. Thus, the mechanism by which PGI_2 permits vasodilation to hypercapnia and other positive vasodilators may involve the amplification of cAMP production in response to the primary stimulus in the cerebral vasculature of newborn pigs. The ability of PGI_2 to amplify cAMP

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responses to other stimuli does not appear to involve direct elevation of cAMP. Treatment with isoproterenol, which also elevates cAMP levels in the cerebral microvasculature of the piglet (8), does not permit hypercapnia-induced vasodilation (4). However, application of iloprost, at doses that are subthreshold for cAMP elevation, restores vasodilator-induced dilation that had been blocked by indomethacin (4, 6, 7). Therefore, PGI_2 interaction with its receptor may amplify cAMP levels, and thereby produce its permissive actions on cerebral vasodilation in newborn pigs.

In addition to IP receptor agonists, the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate also permits hypercapnia-induced vasodilation. In this regard, the phorbol ester has been shown to restore hypercapnia-induced arteriolar dilation and the increase in cortical cAMP following indomethacin treatment (9). Furthermore, down-regulation of PKC resulted in the inhibition of vasodilation induced by hypercapnia (9). Therefore, these results and the above findings provide evidence that there might be an interplay between the adenylyl cyclase (AC) and phospholipase C systems. If the IP receptors were coupled to production of diacylglycerol, PGI_2 could increase the gain of the AC system *via* a PKC-dependent mechanism.

In this regard, the present study was undertaken to address the hypothesis that IP receptors are coupled to the phospholipase C pathway in newborn pig cerebral microvascular smooth muscle cells.

Materials and Methods

Isolation and Culture of Microvessel Smooth Muscle Cells from Newborn Pig Brain. The animal protocols used were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee (Memphis, TN). In newborn pigs and premature human infants, the effects of indomethacin treatment on cerebral blood flow appear to be similar (3). Therefore, piglets were used to address the stated hypothesis. The isolation of cells was performed aseptically.

Primary cultures of microvascular smooth muscle cells from newborn pigs were performed as previously described with modification (10, 11). Briefly, newborn brain cortex was removed under ketamine hydrochloride (40 mg/kg) and acepromazine (4 mg/kg) anesthesia. The tissue was minced and homogenized in M199 isolation solution. The microvessels were isolated by differential filtration of cerebral cortex homogenate through 300-μm and 60-μm nylon mesh screens, and collected by centrifugation (400*g* for 5 min). The microvessels were placed on Matrigel-coated Costar plates in Cellgro Dulbecco's modification of Eagles Medium 1X with 4.5 gm/l glucose, L-glutamine, 20% fetal bovine serum, antibiotic antimycotic mixture, and 200 U/ml nystatin. Cultures were maintained in 5% CO_2/air at 37°C, and medium was changed every 2 days. Smooth muscle cells that grew out from the ends of adherent microvessels were grown for 14 days before study. Such cell cultures consist of >95% vascular smooth muscle cells as identified by fluorescent staining with antibodies specific to α-smooth muscle actin (10).

Measurement of IP_3. All experiments were conducted on confluent quiescent cells. The culture medium was changed to serum-free medium 24 hr before the experiment. Prior to the experiment, the cells were washed with Dulbecco's phosphate-buffered saline and used to determine the effects of iloprost on IP_3 formation. In our experiments, we used untreated cells or cells treated with LiCl, an inhibitor of inositol monophosphatase, to prevent further metabolism of IP_3. Cells (untreated or pretreated with 20 m*M* LiCl for 10 min at 37°C) were incubated with the agonists (10^{-7} *M* endothelin-1 and 10^{-12}–10^{-6} *M* iloprost) in artificial cerebrospinal fluid (aCSF) equilibrated with 5% CO_2/21% O_2/74% N_2. After agonist or aCSF had been added for the indicated duration (0–180 sec), the reaction was stopped by addition of 0.2 ml ice cold 100% trichloroacetic acid (TCA) for each 1 ml of solution. The cells were scraped, suspended, and transferred to test tubes. The acid suspension was homogenized on ice and centrifuged for 10 min at 1000*g*. Following centrifugation, the supernatant was decanted and incubated for 15 min at room temperature. Trichloroacetic acid was removed from the extracts by addition of 2 ml of a mixture of three volumes of 1,1,2-trichloro-1,2,2-trifluoroethane plus 1 volume trioctylamine for each 1 ml of TCA extract. Following 15 sec of mixing, samples were incubated for 3 min at room temperature. The aqueous top layer was removed and samples were stored at –20°C until assay. The IP_3 radioreceptor kit from Dupont/NEN was used to determine cellular IP_3 levels in the samples. Samples and standards were placed in 5 ml of Poly Fluor scintillation solution and counted in a β-counter for 2 min. The IP_3 concentrations in the samples were determined from the standard curve within the range of 0.12–12.0 pmol/tube. Protein contents in the cell cultures were determined by a modification of the method of Bradford (12) whereby samples and standards were dissolved in 1.8 *M* NaOH instead of 0.1 *N* HCl.

Measurement of sn-1,2-Diacylglycerol. Cellular diacylglycerol (DAG) was measured using a commercial kit from Amersham (Arlington Heights, IL). The assay is based on the methods described by Kennerly *et al.* (13) and by Preiss *et al.* (14). Cultured cells planted on Matrigel-coated petri dishes (protein content 0.8–1 mg/dish) were stimulated with 10^{-12}–10^{-8} *M* iloprost for 0–30 min at 37°C. The reaction was stopped with 2 ml methanol. Cells were scraped into suspension, transferred to test tubes, and 1 ml of chloroform was added. Cells were homogenized in the chloroform: methanol mixture(1:2 v/v). After the addition of 1 ml of NaCl to break the phases, the cell extract was centrifuged at 5000*g* for 2 min. The lower chloroform phase was evaporated under nitrogen, and stored at –20°C for no longer than 72 hr prior to DAG determination. The cell extracts were incubated with DAG kinase and [^{32}P]-ATP for 30 min at 25°C. The [^{32}P]-phosphatidic acid formed was separated from unreacted [^{32}P]-ATP by a number of extraction steps

using chloroform:methanol mixture (1:2 v/v), and 1% (v/v) perchloric acid. The extracts were transferred to scintillation vials containing Poly Fluor scintillation solution, and counted in a β-counter. The resultant [^{32}P]-phosphatidic acid was calculated based on the results of the standard curve measured in a range of 31–1000 pmol/tube.

Statistical Analysis. Data are expressed as mean ± SEM. Statistical comparisons were made with ANOVA. Fisher's protected least-significant differences test was used for multiple comparisons. Differences in mean values were considered significant at a value of $P < 0.05$.

Materials. Iloprost was a gift from Schering (Berlin, Germany). The IP_3 radioreceptor assay kit was obtained from DuPont/NEN (Boston, MA). Fetal bovine serum was purchased from HyClone (Logan, UT). Cellgro Dulbecco's Modification of Eagles Medium 1X was from Gibco Laboratories (Grand Island, NY). Disposable culture plates were from Costar (Cambridge, MA). Nylon mesh screens were obtained from Spectrum (Houston, TX). Poly Fluor scintillation solution was purchased from Packard (Meriden, CT). The DAG assay kit and [^{32}P]-ATP were purchased from Amersham. Chloroform, methanol, and perchloric acid were obtained from commercial sources. All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Results

Effect of Endothelin-1 on IP_3 Formation. Endothelin-1 (ET-1) has been demonstrated to induce inositol phosphate turnover in rat atria (15); hence, ET-1 was used as a positive control/reference for IP_3 production in our cultured cerebral smooth muscle cells. ET-1(10^{-7} *M*) rapidly increased IP_3 in cerebromicrovascular smooth muscle cells (Figs. 1 and 2). Figure 1 shows the time dependence of the effect of ET-1 on IP_3 formation. In the absence of LiCl, this stimulation was statistically significant with respect to time zero, reaching a maximum at 15 sec, and this maximal level was maintained throughout the time period. Similar experiments were performed in the presence of 20 m*M* LiCl to prevent further IP_3 conversion due to inositol monophosphatase inhibition (16). The quantity of IP_3 formation, in the presence of LiCl, reached a plateau at ≈ 30 sec. The accumulation of IP_3 induced by ET-1 was also significant with respect to aCSF, the control (Figs. 1 and 2). The level of IP_3 formation, in the absence or presence of LiCl, resulted in a 2-fold increase over the control. The time course of ET-1 stimulation of IP_3 formation in the presence of LiCl was extended to 3 min (Fig. 2). The modest and transient increase in IP_3 production induced by aCSF might be due to the exposure of the cells to gas (5% CO_2 in air, see Materials and Methods) and/or the basal cellular level.

Effect of Iloprost on IP_3 Accumulation. To determine whether IP receptors are coupled to the phospholipase C (PLC) pathway, cell cultures were stimulated with iloprost, an IP receptor agonist. Cells were treated with either 10^{-12}, 10^{-10}, 10^{-8}, or 10^{-6} *M* iloprost for the time indicated in the absence or presence of 20 m*M* LiCl. In the absence of LiCl, iloprost at a concentration of 10^{-12} *M* induced a rapid and time-dependent increase in the accumulation of IP_3 in cultured smooth muscle cells (Fig. 3), resulting in a 2-fold increase over the control. Iloprost at 10^{-10} *M* also increased IP_3, but this elevation was very short-lived. Iloprost at 10^{-8} *M* and 10^{-6} *M* showed slight to no effect on IP_3 formation in cultured smooth muscle cells (Fig. 3). As can be seen, IP_3 levels were maintained throughout the 60-sec time period.

The pretreatment of cell cultures with 20 m*M* LiCl greatly enhanced the production of IP_3 upon stimulation with 10^{-12} *M* iloprost (Fig. 4) vs that in the absence of LiCl

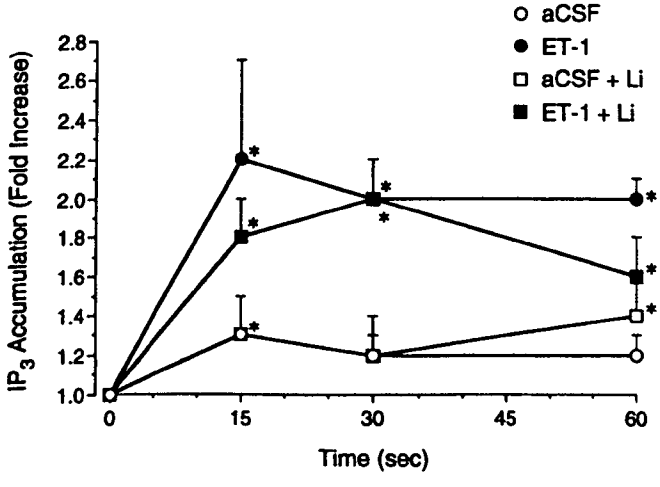

Figure 1. Time course of IP_3 accumulation after stimulation with endothelin-1. Cultures were stimulated with 10^{-7} *M* endothelin-1 for 0–60 sec either in the presence or absence of 20 m*M* LiCl. Results are expressed as fold increase relative to time 0. Each point represents the mean ± SEM of at least three experiments performed in duplicate. *$P < 0.05$ compared with time 0.

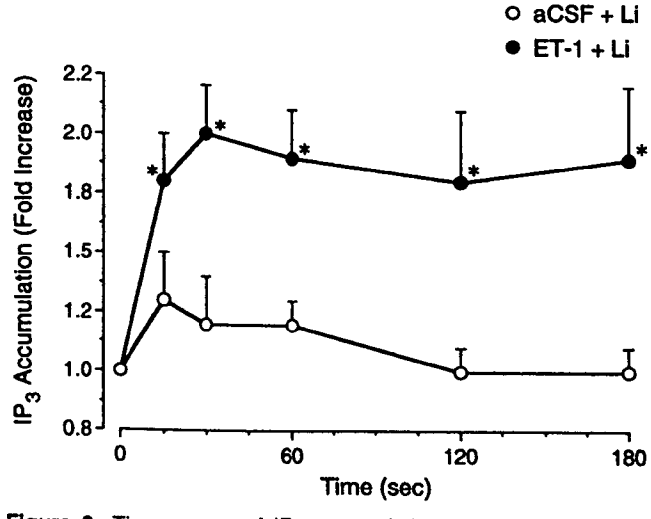

Figure 2. Time course of IP_3 accumulation after stimulation with endothelin-1 in the presence of 20 m*M* LiCl. Cultures were stimulated with 10^{-7} *M* endothelin-1 for 0–180 sec after pretreatment of cells with 20 m*M* LiCl for 10 min. Results are expressed as fold increase relative to time 0. Each point represents the mean ± SEM of at least six experiments performed in duplicate. *$P < 0.05$ compared to time 0.

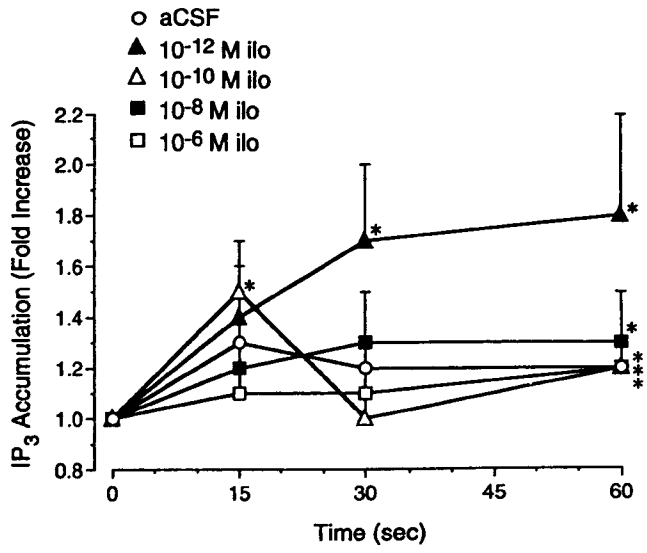

Figure 3. Stimulation of IP_3 accumulation by iloprost in the absence of LiCl. Cultures were stimulated with 10^{-6} *M* to 10^{-12} *M* iloprost for 0–60 sec in the absence of 20 m*M* LiCl. Results are expressed as fold increase relative to time 0. Each point represents the mean ± SEM of six experiments performed in duplicate. $^*P < 0.05$ compared to time 0.

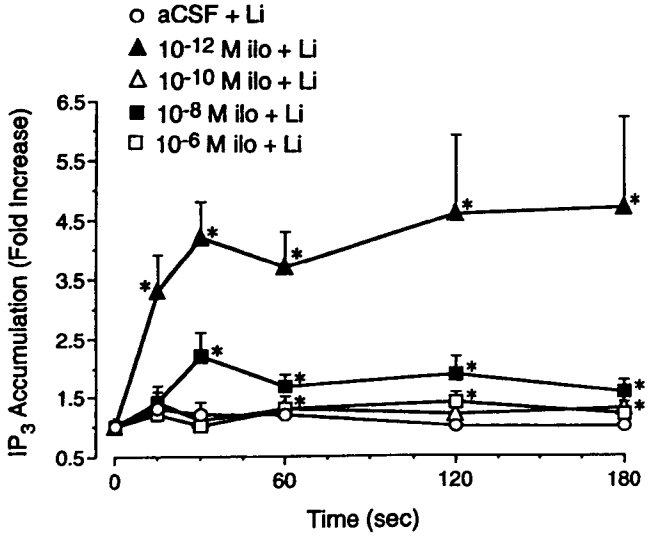

Figure 4. Stimulation of IP_3 accumulation by iloprost in the presence of 20 m*M* LiCl. Cultures were stimulated with 10^{-6} *M* to 10^{-12} *M* iloprost for 0–180 sec in the presence of 20 m*M* LiCl for 10 min. Results are expressed as fold increase relative to time 0. Each point represents the mean ± SEM of at least six experiments performed in duplicate. $^*P < 0.05$ compared to time 0.

(Fig. 3) a 4-fold versus a 2-fold increase, respectively), presumably by inhibiting the enzymatic action of inositol monophosphatase (16). The results in the presence of 20 m*M* LiCl were similar to those found in the absence of LiCl. Specifically, low concentrations of iloprost (10^{-12} *M*) mediated a rapid and time-dependent elevation in the formation of IP_3, whereas higher concentrations (10^{-10} *M* to 10^{-6} *M*) affected elevation of IP_3 minimally (Fig. 4).

Effect of Iloprost on DAG Accumulation. Iloprost produced dose- and time-dependent effects on DAG formation (Fig. 5). Stimulation of cultured smooth muscle

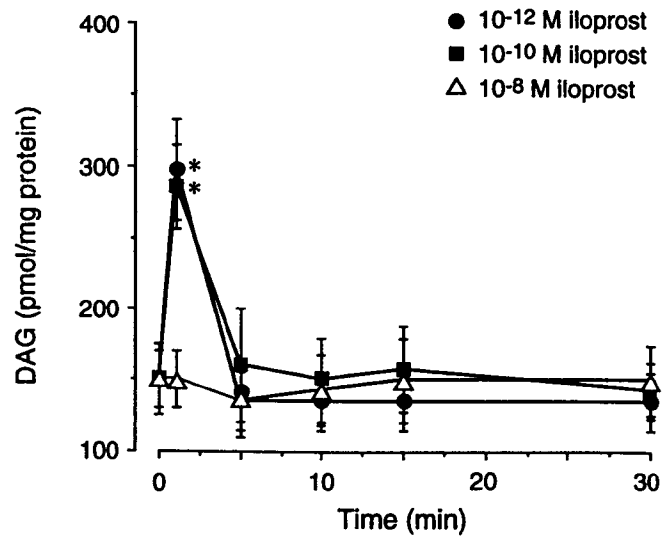

Figure 5. Time course of DAG accumulation with iloprost. Cultures were stimulated with 10^{-8} *M* to 10^{-12} *M* iloprost for 0–30 min. Results are expressed as pmol DAG/mg protein. Each point represents the mean ± SEM of two experiments performed in duplicate. $^*P < 0.05$ compared to time 0.

cells with 10^{-12} *M* and 10^{-10} *M* iloprost induced a rapid but transient increase in DAG, peaking at 1 min and decaying by 5–10 min, and which was significantly different from time 0. In contrast, 10^{-8} *M* iloprost did not evoke an increase in DAG accumulation in cultured smooth muscle cells. Thus, the effects of iloprost on IP_3 and DAG are similar with stimulation at very low doses of iloprost, but little to no effect occurred at higher doses. These results suggest that iloprost at low concentrations is coupled to phosphatidylinositol hydrolysis, but the effects are abrogated at higher concentrations.

Discussion

The present study demonstrates for the first time that low concentrations of iloprost (10^{-12} *M*) will induce rapid and time-dependent increases in inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) formation, whereas iloprost at higher concentrations (10^{-8}–10^{-6} *M*) evokes minimal to no alteration in IP_3 or DAG in cultured vascular smooth muscle cells. It is a surprise that 10^{-8} *M* iloprost in the presence of 20 m*M* LiCl stimulated an increase in IP_3 production (Fig. 4) while not affecting DAG levels (Fig. 5). This increase in IP_3 could be due to variation among the experiments performed, or a biphasic release of IP_3 (15). Additionally, these results do not exclude the possibility that activation of prostacyclin receptors (IP receptors) are coupled to the activation of phospholipase D and/or the breakdown of other polyphosphates. Therefore, the rates of synthesis and metabolism of either IP_3 or DAG may vary due to the differences in enzymatic rates, the physiological activation and inhibition of signal transduction pathways, the variation in cellular production, and the variation in recovery due to the extraction procedure. Although different assays were employed in evaluating the mass levels of IP_3

or DAG, these assays are specific for these two second messengers. However, the extraction procedure may allow for differences in the production level of IP_3 or DAG between experiments. Clearly, there might be other physiological and biochemical explanations that have not been elucidated.

The complex mechanisms of IP_3 and DAG release are not completely understood; however, these interactions could produce different mass levels of IP_3 or DAG. The present study clearly shows that low concentrations of iloprost evoke the formation of IP_3 and DAG, whereas higher doses of iloprost elicit minimal effect. These data support the hypothesis that iloprost is coupled to phosphatidylinositol 4, 5-bisphosphate (PIP_2) hydrolysis through activation of PLC in the cerebral vasculature of newborn pigs, which suggest the possibility that the permissive action of prostacyclin involves activation of a PKC by DAG.

Recent studies have shown that the permissive mode of action in regulating vasodilation might be a common mode of action (17–19). In this regard, in adult rat cerebral cortex, inhibitors of nitric oxide (NO) synthase attenuate hypercapnia-induced vasodilation, and this attenuation can be reversed by a guanosine 3′,5′-cyclic monophosphate (cGMP) analog (18) or NO donors (18, 19). Moreover, it has been found that after removal of the endothelium or inhibition of NO synthase, α2-adrenoreceptor-mediated cerebral vasodilation is blocked in rat cerebral arteries (17). This blockade can be reversed by the addition of subdilator levels of NO donors and by the addition of cGMP analogs (17). These findings collectively suggest that NO is a permissive modulator of cerebral vasodilation in adult rat, since only a basal level of NO is required for expression of dilation to another stimulus (17–19). Further, it is suggested that NO acts by maintaining cGMP levels in smooth muscle cells (17–19).

Although PGI_2 does not appear to provide permissive input for vasodilation in the adult rat cerebral cortex (19), it does provide such input in the piglet cerebral cortex as mentioned before (4, 6, 7, 9). Such a discrepancy could relate to species and/or age. In this regard, vasodilator responses to low concentrations of prostanoids decrease as age increases in primates, whereas vasoconstrictor responses to prostanoids in higher concentrations increase markedly with age (20). In addition, it has been shown that with maturation in pigs, acetylcholine causes NO-dependent cerebral vasodilation not seen in the newborn (21, 22). These results show that the age of a given individual might affect vasodilator and vasoconstrictor actions to a given stimulus and may also affect the signaling mechanisms involved.

Prostacyclin receptors are coupled to the adenylyl cyclase (AC) and PLC systems (Present Study) in smooth muscle cells cultured from the cortex of piglet brains (2, 11). Therefore, PGI_2 could permit augmented cerebral adenosine 3′,5′-cyclic monophosphate (cAMP) responses and, thus, arteriolar vasodilation by activating PKC (9). The increases in IP_3 and DAG content generated in smooth muscle cells in the present study suggest that iloprost binding to its receptor will activate PLC and the hydrolysis of PIP_2 leading to the generation of the second messengers, IP_3 and DAG (23). The coupling of the IP receptors to both the AC and PLC systems has also been demonstrated in another cell line. Prostacyclin receptors have been shown to be coupled to the AC pathway as well as to the PLC pathway in cultured mast cells (24). It is tempting to suggest that there might be different IP receptor subtypes in smooth muscle cells that might be involved in a "cross-talk" between the AC system and the phosphoinositide system.

This cross-talk is suggested in experiments performed in our laboratories and others. First, it has been shown that hypercapnia elevates cAMP levels in isolated smooth muscle cell cultures, but this increase in cAMP is markedly augmented in co-cultures of endothelial and smooth muscle cells (11). Additionally, *in vivo*, it has been demonstrated that phorbol 12-myristate 13-acetate can reverse the indomethacin-induced blockade of both cerebral vasodilation and cAMP production evoked by hypercapnia (9). Furthermore, downregulation of PKC with long-term phorbol ester treatment, abolished vasodilation and blocked the ability of iloprost to restore cerebral vasodilation to hypercapnia (9). Finally, PKC has been shown to sensitize the AC pathways to various stimuli through phosphorylation (25, 26). Although the molecular target of PKC in its permissive role in vasodilation remains to be established, the inhibitory phosphorylation of a $G_i\alpha$ coupled to AC appears to have been excluded (27). However, phosphorylation of an AC or a $G_s\alpha$ to enhance the production of intercellular cAMP in response to alternative stimuli remains plausible (26).

There is evidence that cAMP might alter phosphoinositide hydrolysis in smooth muscle cells (28). Such effects could explain why only low concentrations of iloprost stimulate increases in IP_3 and DAG content whereas higher concentrations have minimal effects. Iloprost does produce dose-dependent increases in cAMP (11). We speculate that the elevated concentrations of cAMP, produced by high concentrations of iloprost, may provide negative feedback to inhibit hydrolysis of PIP_2 (28), thereby attenuating or abolishing the generation of IP_3 and DAG. As a result, the activation of low and high iloprost concentrations may determine molecular physiological coupling of the IP receptors. In this regard, higher concentrations of iloprost do not require PKC-dependent mechanisms to enhance cAMP because higher concentrations can stimulate the AC pathway directly, whereas lower concentrations require the activation of the PKC pathway to enhance the production of cAMP. Therefore, depending on the strength of the stimulus, the permissive action of prostanoids may be converted to more conventional coupling producing dilation by directly activating the AC pathway system.

In conclusion, iloprost at low concentrations stimulates DAG and IP_3 generation, indicating coupling to PLC signaling systems in cultured microvascular smooth muscle cells from piglet brains. These data add another piece to the

puzzle of the mechanism of permissive actions of PGI_2 in cAMP-dependent cerebral dilations in newborn pigs, and other experiments are being performed to understand the regulatory mechanism(s) of PGI_2 permissive actions more accurately in the cerebral microvasculature of piglets. This finding has potential importance in the understanding of the mechanism(s) of cerebral blood flow regulation in the newborn.

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