Role of phospholipase C in development of myogenic tone in rat posterior cerebral arteries

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Received 23 July 2002; accepted in final form 19 August 2002

JARAJAPU, Yagna P. R., and Harm J. Knot. Role of phospholipase C in development of myogenic tone in rat posterior cerebral arteries. Am J Physiol Heart Circ Physiol 283: H2234–H2238, 2002—Earlier studies have implicated phospholipase C (PLC) in the development of myogenic tone (MT) based on pharmacological studies in larger arteries. In the present study, we further investigated the cellular effects of PLC inhibition using pharmacological and electrophysiological approaches to provide more quantitative functional evidence for the involvement of PLC in the genesis of MT in small cerebral arteries. The phosphatidylinositol-selective PLC (PI-PLC) inhibitor U-73122 decreased MT by 87% in posterior cerebral arteries from Sprague-Dawley rats with pIC50 of 6.2 ± 0.09 (n = 5). Similar potency (pIC50 of 6.2 ± 0.04, n = 5) was observed in arteries with MT that were further constricted with 30 nM serotonin. The phosphatidylinositol-specific (PC-PLC) inhibitor D609 had no effect on MT. U-73343, the inactive analog of U-73122, did not show any relaxant effect, but at higher concentrations (>1 μM) it reduced MT. In the presence of 125–500 nM U-73122, the pressure-diameter curves shifted toward that obtained in Ca-free conditions. U-73122-mediated decrease in MT was accompanied by a decrease in mean arterial wall calcium (maximum effect: 77 ± 3% of 16 mM KCl-mediated decrease, n = 4). This was due to a simultaneous membrane potential hyperpolarization of ~9 mV or from −44 ± 1 to −53 ± 2 mV (10 μM, P < 0.001, n = 8). In summary, this study provides the first quantitative data suggesting a critical involvement of PI-PLC in the genesis of pressure-induced MT in rat cerebral arteries via membrane potential depolarization and increased calcium influx.

serotonin; diacylglycerol; myogenic constriction; U-73122; D609

MYOGENIC TONE is defined as the constrictor response of arteries to an increase in the intraluminal pressure (2). It is well accepted that this mechanism is particularly prominent in small arteries and is a major determinant of peripheral vascular resistance and thus plays a major role in the regulation of systemic blood pressure. Also, a large component of autoregulation in regional blood flow in vital organs such as the brain, heart, and kidney in response to changes in blood pressure is attributed to myogenic tone (8).

Even though this phenomenon was first described in 1902 (13), the mechanisms underlying this response have not been fully elucidated and are likely to be complex. Activation of phospholipase C (PLC) isoforms has been implicated in the generation of myogenic response in small arteries. Narayanan et al. (15) provided direct biochemical evidence by showing an increased production of inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] and diacylglycerol (DAG), the products of PLC-mediated metabolism of phosphatidylinositol bisphosphate, in dog renal arteries. Osol et al. (17) showed pharmacological evidence for the involvement PLC in eliciting myogenic response in rat posterior cerebral arteries by using the phosphatidylinositol-selective PLC (PI-PLC) inhibitor U-73122. Recent pharmacological studies also provide supportive evidence for the functional involvement of PLC using the less selective inhibitor 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate in rat skeletal muscle arterioles and using U-73122 in human subcutaneous arteries (1, 5).

Despite these proposed roles and the generally accepted view that PLC activation takes place during myogenic constriction, there has been no study to determine the role or mechanism by which PLC activity may lead to myogenic constriction. Determining the intracellular response to PLC inhibition may therefore provide a first step to understand the role of PLC in myogenic constriction. In this study the effects of PLC inhibition on membrane potential, intracellular calcium, and diameter were investigated to provide a more quantitative functional analysis of the involvement of PLC in the genesis of myogenic constriction. The PI-PLC-selective inhibitor U-73122 and the phosphatidylcholine (PC)-selective PLC inhibitor D609 were pharmacologically quantified, and the intracellular effects were studied by using imaging and electrophysiological approaches (14, 19).

METHODS

Animal, preparation of cerebral arteries, and measurement of arterial diameter. Male Sprague-Dawley (SD) rats (250–300 g) were anesthetized by an intraperitoneal injection of

H2234 0363-6135/02 $5.00 Copyright © 2002 the American Physiological Society http://www.ajpheart.org
pentobarbital sodium (160 mg/kg) and killed by decapitation. The brain was removed and placed in an ice-cold oxygenated physiological cerebrospinal fluid (pCSF; see Drugs, chemicals, and solutions). Posterior cerebral arteries were quickly isolated and dissected free of connective tissue. Secondary or tertiary branches were cut and transferred to a custom-built arteriograph filled with ice-cold pCSF, cannulated with glass pipettes, and secured with nylon thread as described previously (9). The arteriograph was then placed on the stage of an inverted microscope, and the artery visualized with a monochrome CCD camera coupled to a calibrated video caliper system to measure arterial diameter. The arteries were slowly pressurized to 70 mmHg under no-flow conditions by using a pressure servo-null system (Living Systems; Burlington, VT) and warmed to 37°C while being continuously superfused (5 ml/min) with pCSF bubbled with 21% O₂-5% CO₂-74% N₂ (pH 7.35–7.40 in the bath).

**Experimental protocol.** After an equilibration period of ~20 min, arteries showed stable myogenic tone at 70 mmHg. Afterwards, the effect of different pharmacological agents on myogenic tone was evaluated. Concentration-response curves were generated first by cumulative addition to the superfusate. Some arteries were assessed for receptor-independent contraction to 60 mM KCl after treatment with U-73122. In additional experiments, arteries with myogenic tone were superfused with 30 nM 5-hydroxytryptamine (5-HT, serotonin) before the cosuperfusion with cumulative addition of U-73122. Obtaining the maximal dilated diameter in a calcium-free pCSF (see Drugs, chemicals, and solutions) concluded all experiments. Pressure-diameter curves were obtained by increasing the pressure in 20-mmHg steps from 10 to 130 mmHg. Some arteries were pretreated with 125 and 500 nM U-73122 or 500 nM U-73343 for 30 min at 70 mmHg before pressure-diameter curves were obtained. Pressure-diameter curves were repeated in the presence of calcium-free pCSF.

**Calcium imaging by using fura 2 in isolated arteries.** Arteries with stable myogenic tone were loaded with the calcium-sensitive dye fura 2 by using the following protocol: arteries were allowed to return to room temperature for 15 min, and then superfusion was stopped. The pCSF was removed from the bath and replaced with that containing 5 μM fura 2-AM in DMSO and 2 μg/ml pluronic F-127. After 30 min, the fura 2-loading solution was washed out at room temperature, and then the temperature was raised back to 37°C. All arteries were allowed to recover myogenic tone completely before the experiment was started. Internal diameter was monitored by using a video caliper before and after acquisition of calcium images.

Fura 2 ratio images and Ca²⁺ measurements were obtained by using either 1) the IoNOptix SoftEdge acquisition package for simultaneous recording of arterial diameter and vascular wall Ca²⁺ at 250 Hz (IoNOptix; Milton, MA) or 2) a computer-controlled monochromater excitation light source (TILL Polychrome II) synchronized to a cooled programmable CCD camera with exposure control (PCO SensiCam, TILL-photonics; Martinsried, Germany). During excitation at 340 or 380 nm, emission at 510 nm was collected by the camera for 300 ms. A 340-to-380 nm ratio image was generated following background subtraction, and one or several areas of interest were used to quantify the ratio values after each treatment. To minimize photo-leaching and photodamage, calcium images were only obtained when arterial diameter was at steady state after each treatment. The effect of U-73122 on the arterial wall calcium was evaluated by cumulative addition to the superfusate. The decrease in the fura 2 ratio was expressed as the percentage of that produced by 16 mM KCl, which causes a maximum decrease in the arterial wall calcium due to hyperpolarization secondary to the activation of inward-rectifier potassium channels (11, 12).

**Membrane potential measurements.** Membrane potential was measured as described previously (9) by using the sharp microelectrode technique in intact pressurized arteries using conventional intracellular glass microelectrodes filled with 3 M KCl solution and tip resistances of 40–60 MΩ. Cerebral arterial smooth muscle cells were impaled from the cleaned adventitial side of the pressurized artery. Membrane potentials were recorded with an Axoclamp 2B Amplifier at an acquisition rate of 1 kHz.

**Data analysis and statistics.** Results were expressed as means ± SE; n indicates the number of independent experiments, which equals the number of animals used for experimentation. Means were compared by Student’s t-test and a P value <0.05 was considered as statistically significant. CRCs were analyzed using the software program GraphPad Prism by fitting the data to a four-parameter sigmoidal logistic equation. The potency of the contractile agonist and enzyme inhibitors was expressed as the negative logarithm of the concentration of the agonist or the inhibitor to produce the 50% of the maximum effect (pEC₅₀ or pIC₅₀, respectively) as calculated by GraphPad Prism.

**Drugs, chemicals, and solutions.** U-73122, U-73343, D609, and EGTA were purchased from Sigma (St. Louis, MO), and 5-HT (serotonin HCl) was purchased from Fluka (Steinheim, Germany). Fura 2 was purchased from Molecular Probes (Eugene, OR). Stock solutions (10 mM) of U-73122 and U-73343 were prepared in DMSO, and those of 5-HT and D609 were prepared in distilled water. The composition of pCSF (mmol/l) was 120 NaCl, 3 KCl, 24 NaHCO₃, 1.2 NaH₂PO₄, 1.25 CaCl₂, 1.2 MgSO₄·7H₂O, and 4 glucose. pCSF with 16 and 60 mM KCl was prepared by replacing NaCl with an equimolar quantity of KCl. Calcium-free pCSF was prepared by replacing CaCl₂ with an equimolar quantity of MgSO₄·7H₂O with 2 mM EGTA.

**RESULTS**

**General properties of the cerebral arteries used in this study.** The average passive diameter (fully dilated considered to be 0% constriction) of the rat posterior cerebral arteries used in this study was 145 ± 4 (n = 35) μm. At 70 mmHg intravascular pressure the arteries developed myogenic tone and constricted to an average diameter of 88 ± 2 μm representing an average myogenic tone of 38 ± 2%. Under these conditions, elevation of extracellular potassium to 16 mM (hyperpolarization to approximately −59 mV) caused a near-maximal dilation to 133 ± 3 μm or equal to 20 ± 2% of the myogenic constriction, and a further elevation to 60 mM (depolarization to approximately −20 mV) caused a constriction to 55 ± 2 μm or 165 ± 6% of myogenic tone (62% constriction). These two responses were used as benchmarks for dilation, calcium concentration, and constriction, respectively, as well as to verify the viability of the arteries during the experimental protocols and to assess possible nonselective or time-dependent effects of the pharmacological agents before and after their use.

**Effect of PLC inhibition on myogenic tone.** The PI-PLC inhibitor U-73122 produced a concentration-dependent decrease in myogenic tone of the arteries up to...
87% with a potency (pIC\textsubscript{50}) of 6.2 ± 0.09 (0.6 μM) \((n = 5)\) in the rat posterior cerebral arteries (Fig. 1A). The inactive analog U-73343 did not affect myogenic tone up to a concentration of 1 μM but did inhibit myogenic tone at higher concentrations \((n = 5, \text{Fig. 1A})\) in these arteries. The PC-PLC inhibitor D609 did not affect myogenic tone in these arteries in the concentration range of 10 nM to 10 μM \((n = 5, \text{Fig. 1A})\).

Preincubation with increasing concentrations of U-73122 caused a progressive loss of the myogenic response in these arteries at all pressures considered to be in the range of autoregulation (50–110 mmHg) in these arteries as assessed with pressure-diameter curves \((n = 4, \text{Fig. 1A})\).

**Effect of PLC inhibition on 5-HT-induced vasoconstriction.** The inhibitory effect of U-73122 was also evaluated in the arteries with myogenic tone after activating PI-PLC to a higher extent by 5-HT, which is known to produce vasoconstriction via the \(G_{q/G_{11}}\) protein-PLC pathway. 5-HT produced an additional concentration-dependent sustained constriction of the posterior cerebral arteries with stable myogenic tone (Fig. 2A). The maximum constriction observed was 131 ± 4% \((n = 5)\) of the myogenic tone with a potency (pEC\textsubscript{50}) of 8.5 ± 0.20 (3.2 nM). In the presence of 30 nM 5-HT, a concentration that produced a constriction of 124 ± 4% \((n = 5)\) of myogenic tone, U-73122 dilated the arteries with a pIC\textsubscript{50} of 6.2 ± 0.04 (0.64 μM) (Fig. 2B), which is similar to that observed in the absence of 5-HT.

In a separate set of experiments, we further assessed possible nonselective effects of U-73122. In these experiments, four arteries with myogenic tone (constricted from 186 ± 2.5 to 113 ± 7 μm or by 39%) were preincubated with 10 μM U-73122 for 30 min. This resulted in a loss of 73% of myogenic tone (dilation from 113 to 166 ± 7 μm). Under these conditions, elevation of extracellular potassium to 60 mM resulted in a...
Further constriction from 113 to 72 ± 7 μm or a total constriction of 61%, which is similar to nontreated arteries. However, long-term continued incubation (>2 h) with this drug did result in a progressive loss of responsiveness to 60 mM KCl.

Effect of PLC inhibition on the arterial wall calcium and membrane potential. Arteries with myogenic tone dilate to 94–98% of their passive diameter in response to elevation of extracellular potassium to 16 mM. This is due to activation of inward rectifier K⁺ channels resulting in hyperpolarization of ~15 mV. The resulting hyperpolarization reduces the open probability of voltage-dependent Ca²⁺ channels resulting in ~80% decrease in arterial wall calcium concentration when expressed as the fura 2 ratio (9, 11). Application of 10 μM U-73122 to the superfusate of the arteries resulted in an arterial wall membrane potential hyperpolarization of ~9 mV or from −44 ± 1 to −53 ± 2 mV (P < 0.001, n = 8). This hyperpolarization-mediated decrease in myogenic tone was accompanied by a proportional decrease in the arterial wall calcium. The observed decrease in arterial wall calcium was 77 ± 3% (n = 3, Fig. 3A) of that produced by 16 mM KCl.

DISCUSSION

Summary of key findings in this study. Our results suggest that PI-PLC plays an early key role in the genesis of myogenic response in small cerebral arteries from rats. We further provide evidence that membrane potential depolarization and activation of Ca²⁺ entry are involved in the downstream pathway leading to the myogenic constriction. We also provide evidence that PC-PLC does not play a significant role in myogenic tone. The potency of the inhibition of myogenic tone by U-73122, the membrane potential effects, and reduction of arterial wall Ca²⁺ are consistent with the ability of U-73122 to inhibit the catalytic activity of PI-PLC (19).

Role of PLC in the regulation of pressure-induced myogenic tone. Previous studies using the PI-PLC inhibitor U-73122 as a pharmacological tool suggested a role for PLC in pressure-induced myogenic tone and in arterial constriction to vasoconstrictor hormones based on the ability of U-73122 to cause nearly maximal vasodilation (1, 5, 17). Additional support of a role for activation of PLC in myogenic vasoconstriction is studies that observed a pressure- or stretch-induced activation of PLC as well as pressure-dependent increases in the enzyme’s main products, Ins(1,4,5)P₃ and DAG (15). However, the selectivity of this compound in vascular smooth muscle and the possible mechanisms whereby PLC inhibition may lead to vasodilation remained unexplored. In the present study, U-73122 showed similar potency in reversing myogenic tone as well as 5-HT-mediated constriction in the arteries with myogenic tone that served as a positive control. This observation suggests that the dilation produced in the arteries with myogenic tone was exclusively dependent on the inhibition of PI-PLC. Furthermore, it suggests that maximum possible activation of PI-PLC is not required to maintain basal myogenic tone. We used intact arteries in this study. The possible release of vasodilator substances from the endothelium is generally secondary to PLC activation in these cells (e.g., for acetylcholine or bradykinin). Therefore, contrary to the observed dilation resulting from PLC inhibition with U-73122, PLC inhibition in the endothelium is expected to produce constriction. Although we cannot exclude an effect of U-73122 on PLC activity in the endothelium, our results strongly suggest that the major effect of U-73122 is directly on the smooth muscle.

This is the first study demonstrating the importance of PLC in regulating the membrane potential and intracellular calcium during myogenic contractions. Membrane potential depolarization and voltage-regulated calcium channel activity are obligatory mechanisms in the regulation of arterial diameter by pressure in this preparation and small arteries from other vascular beds (9). Whereas data from our lab as well as
others suggest a role for K+ channel inhibition as a major mechanism of depolarization in smooth muscle, it remains unclear how intravascular pressure regulates membrane potential (13).

Nature of the PI-PLC activity-induced depolarization cerebral arteries. Our results are consistent with a sequence of events whereby pressure through a yet unknown mechanism or “pressure-sensor” activates PI-PLC leading to depolarization of the arterial wall that leads to activation of voltage-dependent Ca2+-channels and an increase in global arterial wall Ca2+ resulting in vasoconstriction.

Activation of PI-PLC generally results in the generation of the second messengers Ins(1,4,5)P3 and DAG. Because the final response in this cerebral artery preparation is a significant depolarization (minimally 9 mV from 10 to 70 mmHg can be contributed to PLC activation), either of these second messengers could be responsible for the observed depolarization. In this context Ins(1,4,5)P3-induced Ca2+ release from the sarcoplasmic reticulum could contribute to inhibition of K+ channels, such as members of delayed rectifier voltage-dependent K+ channels family via a direct Ca2+-dependent block (6, 7) or inhibition of Ca2+-activated K+ channels via graded reduction of luminal sarcoplasmic reticulum Ca2+ content, critical for activation of these channels in arteries with myogenic tone (10, 16, 21). Ins(1,4,5)P3-induced Ca2+ release from the sarcoplasmic reticulum could also lead to activation of “store-operated or store-depletion” activated channels of the transient receptor potential potential (TRP) family in smooth muscle or activation of Ca2+-activated chloride channels (18, 20).

DAG is generally thought to activate protein kinase C (PKC) isozymes. PKC activation has been shown to inhibit delayed rectifier in vascular smooth muscle (4) and to inhibit Ca2+ release channels (ryanodine-receptors) leading to inhibition of the activity of Ca2+-dependent K+ channels via inhibition of Ca2+ sparks (3). Alternatively (or concomitantly), DAG has been shown to directly activate TRP channels in other tissues. Future studies by our laboratory are directed to delineate the pathways that are initiated by PLC and to investigate the possible role of PLC activity in pathological conditions.

In conclusion, our results provide strong support for the hypothesis that PI-PLC acts as a critical early signaling element in the pressure-induced generation of myogenic tone in small cerebral arteries. Our observations are consistent with the following cascade of events: pressure leads to activation of PI-PLC; PLC activity leads to membrane potential depolarization and activation of Ca2+-channels and Ca2+ entry; and the increase in arterial wall Ca2+ leads to an increase in arterial (myogenic) tone.

These studies were supported by the American Heart Association Grant GIA-9860037T and by the National Eye Institute Grant RO1 EY-12601.

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