Activation of visceral afferents by bradykinin and ischemia: independent roles of PKC and prostaglandins

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Guo, Zhi-Ling, J. David Symons, and John C. Longhurst. Activation of visceral afferents by bradykinin and ischemia: independent roles of PKC and prostaglandins. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1884–H1891, 1999.—We have shown that the cyclooxygenase (COX) and protein kinase C (PKC) systems both contribute to afferent activation in response to bradykinin (BK) and abdominal ischemia. Because the contribution from PKC to C fiber activation may depend, in part, on prostaglandin production, we hypothesized that an intact COX system is required for PKC-induced activation of ischemically sensitive abdominal visceral afferents by BK and abdominal ischemia. Single-unit activity of abdominal visceral C fibers was recorded from the right thoracic sympathetic chain of anesthetized cats. Three repeated injections of BK (1–2 µg/kg iv) produced similar increases in afferent activity from the baseline of 1.32 ± 0.24, 1.37 ± 0.32, and 1.41 ± 0.24 impulses/s (n = 5). In another group of animals (n = 5), the second and third BK injections were performed after COX inhibition (indomethacin; 5 mg/kg iv) and then combined COX + PKC inhibition (PKC-19–36, 20 µg/kg iv), respectively. Inhibition of COX reduced (P < 0.05) the afferent response to BK (0.59 ± 0.12 impulses/s) compared with the unblocked condition (1.14 ± 0.27 impulses/s), whereas combined COX + PKC inhibition further attenuated the increase from baseline (0.18 ± 0.09 impulses/s; P < 0.05). Similar results were obtained in a third group of cats when the antagonists were administered in reverse order (n = 7). In a fourth group of cats (n = 9) that were pretreated with indomethacin, ischemia increased afferent activity (0.78 ± 0.17 impulses/s). However, neural activity was attenuated (0.51 ± 0.14 impulses/s; P < 0.05) during a second bout of ischemia in the presence of indomethacin + PKC (19–36). These results suggest that the contribution from PKC to the activation of ischemically sensitive C fibers, particularly by BK, does not require an intact cyclooxygenase system.

sympathetic afferents; nociception; cat; phosphoinositide system; protein kinase C

ABDOMINAL ISCHEMIA causes profound cardiovascular reflexes characterized by increased blood pressure, heart rate, and myocardial contractility. The function of these reflexes likely is to increase the chronotropic and inotropic properties of the heart to augment cardiac output and maintain blood pressure and thus facilitate perfusion to ischemic visceral organs. Abdominal visceral C fibers form the afferent limb of this reflex and are stimulated by a variety of substances produced during mesenteric ischemia (9, 10, 20, 23, 26, 27). We have shown previously that bradykinin (BK) is produced locally during abdominal ischemia and that this peptide is important in activating abdominal visceral nerves (23). Elucidation of the signal transduction processes whereby BK stimulates C fiber afferents has been a recent focus of our laboratory.

We have demonstrated that BK-mediated increases in afferent activity are attenuated after cyclooxygenase (COX) blockade (23). These data suggest the importance of prostaglandins in BK-mediated afferent activation during abdominal ischemia. Phospholipase A2 (PLA2) is the primary enzyme regulating arachidonic acid release from membrane phospholipids. By stimulating PLA2 to produce more arachidonic acid, BK may stimulate the production of prostaglandins, which ultimately may be responsible for sensitizing afferent endings to this peptide and/or other metabolic stimuli (1, 2).

BK stimulates ischemically sensitive abdominal visceral afferents by activating kinin B2 receptors (23), leading to G protein-mediated activation of a phosphatidylinositol-specific phospholipase C (PLC). Two second messengers generated by activating PLC are diacylglycerol and δ-myo-inositol 1,4,5-trisphosphate. Diacylglycerol activates protein kinase C (PKC), whereas δ-myo-inositol 1,4,5-trisphosphate mobilizes calcium from intracellular stores. Together, these processes initiate phosphorylation of cellular components and are key steps in the process of signal transduction (1, 4).

Recent findings from our laboratory suggest that the phosphoinositide system is one of the intracellular signaling pathways used when abdominal visceral C fibers are stimulated by BK during ischemia (13). For example, increases in impulse activity of ischemically sensitive abdominal visceral C fibers caused by ischemia and BK were attenuated after PKC inhibition. These data indicate that PKC contributes to the activation of abdominal visceral afferents during ischemia and specifically to BK-mediated activation of these afferents (13). The PKC-related contribution to the activation of afferents by BK may depend on the ability of PKC to stimulate prostaglandin production (17). In this regard, PKC has been documented to activate PLA2, leading to arachidonic acid release and the subsequent production of prostaglandins (8, 11, 16, 17, 28, 29). Prostaglandins are important because they can sensitize afferent nerve endings to BK and/or other metabolic stimuli that accumulate during ischemia (1, 2, 19). The purpose of the present study, therefore, was to investigate whether the contribution from PKC to BK and ischemia-induced activation of C fibers is
dependent on the production of COX metabolites. We hypothesized that an intact COX system is required for PKC-induced activation of ischemically sensitive abdominal visceral afferents by BK and abdominal ischemia. A preliminary report of this work has been presented (14).

METHODS

Surgical Preparation

Adult cats (2.5–3.8 kg) of either sex were anesthetized initially using ketamine (20–30 mg/kg im) and maintained with α-chloralose (30–40 mg/kg iv). After intubation of the trachea, respiration was maintained artificially (model 661, Harvard pump, Ealing, South Natick, MA). Arterial blood gases were analyzed frequently throughout the surgical instruments and the experimental protocols (model ABL-3, Radiometer, Westlake, OH) and were maintained within physiological limits (Po2, 100–150 mmHg; Pco2, 28–35 mmHg; pH 7.35–7.45) by adjusting the respiration rate and/or tidal volume, altering the inspired O2, and/or administering NaHCO3 (1 M iv). A femoral artery and vein were cannulated for measurement of arterial pressure (Statham P231 D) and administration of fluids and drugs, respectively. Body temperature was maintained between 36 and 38°C using a heating pad and lamp. Surgical and experimental protocols used in this study were approved by the Animal Use and Care Committee at the University of California, Davis.

The method for recording abdominal sympathetic afferent activity has been described previously in detail (13, 23). Briefly, after a midline sternotomy was made, the third through eleventh right ribs and the middle and lower lobes of the right lung were removed. An inflatable occlusion cuff was placed around the descending thoracic aorta proximal to the diaphragm after both phrenic nerves were cut. After the overlying fascia was removed, the right paravertebral sympathetic chain was isolated, draped over a Plexiglas platform, and covered with warm mineral oil. With the use of an operating microscope (Zeiss, Germany), small nerve filaments were dissected gently from the sympathetic chain between T6 and T10, and the caudal end was placed across a thread placed from the receptive field along the supposed afferent pathway through the paravertebral ganglion along the course of the major splanchnic nerve through the sympathetic chain to the recording electrode. The conduction time was determined by measuring the delay between the triggered artifact from the electrical stimulation and the action potential of the afferent detected by the recording electrode. Conduction velocity (CV) of each afferent was calculated by dividing conduction distance by conduction time. C fibers were classified as those with a CV < 2.5 m/s and had CV values ranging between 0.27 and 1.24 m/s in this study. Each C fiber had a receptive field that could be located precisely.

Experimental Protocols

Effect of COX and PKC inhibition on BK-mediated activation of ischemically sensitive abdominal visceral afferents. After an ischemically sensitive C fiber was identified (see Data Analysis), the discharge activity of the afferent was measured during a 2-min control period and in response to the injection of BK (1–2 µg/kg) through the femoral artery catheter into the descending thoracic aorta (n = 5). After 15–20 min, the COX pathway was inhibited using indomethacin (5 mg/kg iv). This dose has been shown previously to effectively inhibit cyclooxygenase activity in cats (21). Fifteen minutes later, BK was injected and afferent activity recorded as described. After a 30- to 40-min stabilization period, a third injection of BK was performed in the presence of COX + PKC inhibition [PKC-(19–36); 20 µg/kg iv]. We have documented that this dose of PKC-(19–36) is sufficient to attenuate increases in discharge activity of abdominal visceral afferents in response to ischemia and BK (1 µg/kg iv; 13).

Identical procedures were performed using a second group of animals (n = 7), with the exception that administration of indomethacin and PKC-(19–36) was reversed following the initial injection of BK. When these two protocols were completed, the receptive field of the afferent nerve was manipulated mechanically or stimulated electrically to document its viability.

Time/vehicle/reproducibility controls. In five cats, afferent activity was measured before and during three injections of BK that were separated by 30–40 min. Vehicles for indomethacin and PKC-(19–36) (i.e., 2–3 ml of 100 mM Na2CO3 and 0.9% NaCl, respectively) were administered before the second and third injections of BK.

Effect of COX and PKC inhibition on afferent activity during abdominal ischemia. Nine cats were pretreated with indomethacin (5 mg/kg iv; 21), and an ischemically sensitive afferent was identified by observing the response to 10 min of abdominal ischemia. After 30–40 min, BK was inhibited [PKC-(19–36), 20 µg/kg iv; 13] and abdominal ischemia was repeated.

Drugs and Solutions

BK, indomethacin (Sigma, St. Louis, MO), and PKC-(19–36) (Research Biochemicals International, Natick, MA) were purchased commercially. BK was reconstituted using 0.9% saline to a concentration of 10 µg/ml (23). Indomethacin was dissolved in 100 mM Na2CO3, diluted to a concentration of 25 mg/ml, and administered at a dose of 5 mg/kg (21). PKC-(19–36) was dissolved in distilled water to a concentration of 100 µg/ml and diluted further using 0.9% saline to an administration dose of 20 µg/kg in 1 ml (13).
Data Analysis

The peak discharge rate of ischemically sensitive afferents was averaged over 60 s during 5–10 min of control and ischemia when the greatest number of spikes occurred. Afferents were considered to be ischemically sensitive if the increase in discharge activity during 5–10 min of abdominal ischemia was sustained at least twofold above baseline. BK-induced increases in afferent activity were measured by averaging the discharge rate during the entire response period (usually 30–60 s). This period of measurement was maintained constant in each afferent after the injection of the antagonist. The latency of response to BK or ischemia was measured from the time of injection or arterial occlusion to the time when a sustained increase of at least 10% over baseline activity occurred.

Statistical Analysis

Data are expressed as means ± SE. The increase in impulse frequency of ischemically sensitive C fibers from control induced by injection of BK or ischemia was compared among interventions in each group using a one-way repeated-measures ANOVA with a Tukey post hoc test. If the data were not normally distributed, as determined by the Kolmogorov-Smirnov test, they were compared using the Friedman repeated-measures ANOVA on ranks, followed by a Dunnett’s post hoc test. The onset latency of discharge activity was compared using a one-way repeated-measures ANOVA. All statistical calculations were performed with commercially available software (Jandel Scientific Software, San Rafael, CA). Values were considered significantly different when P < 0.05.

RESULTS

BK-Mediated Activation of Ischemically Sensitive Abdominal Visceral Afferents in Presence of COX and PKC Inhibition

In the first group of cats (n = 5), BK (1–2 µg/kg ia) increased the discharge activity of ischemically sensitive C fibers (CV = 0.50 ± 0.07 m/s) from 0.02 ± 0.01 to 1.14 ± 0.27 impulses/s (P < 0.05) after an onset latency of 16 ± 5 s (Fig. 1A). After COX inhibition (indomethacin, 5 mg/kg iv), BK increased afferent activity from 0.05 ± 0.01 to 0.59 ± 0.12 impulses/s (P < 0.05), after an onset latency of 19 ± 6 s. The peak response, however, was attenuated (P < 0.05) after COX inhibition compared with the unblocked condition. After combined COX + PKC inhibition [PKC-(19–36), 20 µg/kg iv], the response to BK was reduced further (P < 0.05) than after COX inhibition alone, such that increases from baseline no longer were observed (i.e., 0.06 ± 0.02 to 0.18 ± 0.09 impulses/s). Concerning the onset latency after combined inhibition, no responses were observed in three of five fibers. In the remaining two fibers, onset latencies were 58 and 26 s. An original tracing of an ischemically sensitive C fiber (CV = 0.37 m/s) that underwent COX and PKC inhibition is shown in Fig. 2.

Similar results were observed when the order of antagonists was reversed. BK increased the discharge activity of ischemically sensitive C fibers (CV = 0.64 ± 0.08 m/s, n = 7) from 0.04 ± 0.02 to 1.21 ± 0.22 impulses/s (P < 0.05), after an onset latency of 15 ± 6 s (Fig. 1C). In the presence of PKC inhibition, BK increased afferent activity from 0.05 ± 0.02 to 0.77 ± 0.17 impulses/s (P < 0.05), after an onset latency of 36 ± 11 s. The peak response was attenuated (P < 0.05) after PKC inhibition compared with the untreated condition. After combined PKC + COX inhibition, the maximal response to BK was less (P < 0.05) compared with the PKC inhibition alone such that increases from baseline no longer were observed (0.07 ± 0.03 to 0.21 ± 0.06 impulses/s). Concerning the onset latency after combined inhibition, responses were not observed in two of seven fibers. In the remaining five fibers, onset latencies averaged 40 ± 11 s. An original tracing of an ischemically sensitive C fiber subjected to PKC and COX inhibition (CV = 0.42 m/s) is shown in Fig. 3.
**Time/Vehicle/Reproducibility Controls**

BK (1–2 µg/kg ia) increased the afferent activity of ischemically sensitive C fibers (CV = 0.56 ± 0.18 m/s, n = 5) to a similar extent in response to the first (0.01 ± 0.01 to 1.32 ± 0.24 impulses/s), second (0.08 ± 0.03 to 1.37 ± 0.32 impulses/s), and third (0.06 ± 0.03 to 1.41 ± 0.24 impulses/s) injections (Fig. 1A). Likewise, similar onset latencies were obtained after the first (13 ± 2 s), second (18 ± 3 s), and third (22 ± 5 s) injections of BK. The location of afferents studied in these protocols is shown in Table 1.

**Ischemia-Induced Activation of Abdominal Visceral Afferents in Presence of COX and PKC Inhibition**

After treatment with indomethacin (5 mg/kg iv), abdominal ischemia increased the discharge activity of C fiber afferents (CV = 0.67 ± 0.08 m/s) from 0.03 ± 0.01 to 0.78 ± 0.17 impulses/s (P < 0.05, n = 9), after an onset latency of 122 ± 30 s. Approximately 30–40 min later in the presence of combined COX and PKC inhibition, a second bout of ischemia increased the neural activity of C fiber afferents from 0.04 ± 0.02 to 0.51 ± 0.14 impulses/s (P < 0.05), after an onset latency of 201 ± 53 s. However, the magnitude of the ischemia-induced increase in afferent activity was less (P < 0.05; Fig. 4), and the onset latency of the response was greater during ischemia after combined COX + PKC inhibition, compared with ischemia in the presence of COX inhibition alone.

**DISCUSSION**

Findings from the present study do not support our hypothesis that an intact COX system is required for PKC-induced activation of ischemically sensitive abdominal visceral afferents by BK or abdominal ischemia. Instead, we observed that PKC inhibition attenuated BK-induced increases in the afferent activity even in the presence of COX inhibition. Furthermore, increases in C fiber activity in response to abdominal ischemia were blunted by combined PKC + COX inhibition compared with those observed after indomethacin alone. Therefore, the contribution from PKC to activation of ischemically sensitive abdominal visceral C fibers by BK or abdominal ischemia, in part, occurs independently from the production of prostaglandins.

We reported previously that BK produced during abdominal ischemia stimulates ischemically sensitive C fiber afferents by activating kinin B₂ receptors.
Moreover, BK-mediated increases in afferent activity were observed to be attenuated by the inhibition of the COX system (23). Because this peptide can stimulate PLA2 and thus liberate arachidonic acid for subsequent prostaglandin synthesis, we concluded that prostanoid production is required for the afferent response to BK. The importance of prostaglandins likely arises from their ability to sensitize abdominal visceral afferent nerves to BK and/or other metabolic stimuli that accumulate during ischemia (1, 2, 19).

The phosphoinositide system also plays an important role in stimulating ischemically sensitive afferents by BK. In this regard, exogenous activation of PKC stimulates, while inhibition of PKC attenuates, the discharge rate of ischemically sensitive abdominal visceral afferents (13). Concerning BK, we observed that activation of ischemically sensitive afferents by exogenous administration of this peptide was reduced after inhibition of PKC. These findings suggest that both the COX and phosphoinositide systems contribute to the activation of abdominal visceral afferents during ischemia. However, results from these studies do not address the issue of whether PKC works in concert with, or independently from, the COX system.

Table 1. Location of abdominal visceral C fiber afferents

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Values are numbers of afferent endings; PKC-(19—36), protein kinase C-(19—36).

Fig. 3. Responses of an ischemically sensitive C fiber (CV = 0.42 m/s) to BK (1 µg/kg ia) before (A), after PKC-(19—36) (20 µg/kg iv; B), and PKC-(19—36) + indomethacin (5 mg/kg iv; C). Neurograms 1–6 represent original tracings obtained at points 1–6 shown by arrows in A (1, 2), B (3, 4), and C (5, 6). Receptive field of this afferent was in the gallbladder.

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Values are numbers of afferent endings; PKC-(19—36), protein kinase C-(19—36).

Fig. 4. Responses of ischemically sensitive C fibers to ischemia after indomethacin (5 mg/kg iv) and after indomethacin + PKC-(19—36) (20 µg/kg iv; n = 9). Histograms represent means ± SE. *P < 0.05 vs. respective control value. †P < 0.05 vs. first ischemia.
The magnitude of the PKC-related component could depend on prostaglandin production. In this regard, activation of PKC by compounds that are similar structurally to diacylglycerol (i.e., phorbol esters) can stimulate arachidonic acid release and prostaglandin production in various cell types (6–8, 29). Also, the ability of phorbol 12-myristate 13-acetate to mimic a PKC-related event can be reduced by two different COX inhibitors (6). Finally, phorbol 12,13-dibutyrate is not capable of increasing renal afferent activity in rats that have consumed a diet deficient in essential fatty acids, the precursors of arachidonic acid (17). These findings indicate the potential importance of COX-derived products in PKC-related events.

Evidence that the PKC-related contribution to BK-mediated increases in afferent activity depends on the production of prostaglandins is based largely on data obtained in vitro and involves a sequence of observations. BK initially stimulates kinin B2 receptors of sensory neurons through G protein-mediated activation of PLC. Activation of PLC then generates the intracellular second messenger diacylglycerol that subsequently activates PKC (1, 22, 25). PKC then is available to activate PLA2 by one or more of a number of ways (3, 12, 15, 18, 24, 30). First, PKC can directly activate PLA2 by phosphorylating this enzyme (12, 24). Second, PKC could phosphorylate and thereby inactivate a protein that normally inhibits PLA2 (3, 15). Third, PKC could act synergistically with calcium to activate PLA2 (12, 16, 18, 24, 30). In any case, PLA2 then liberates arachidonic acid from membrane phospholipids, leading to the subsequent production of prostaglandins. Taken together, the possibility therefore exists that the contribution of PKC to afferent activation could depend, at least in part, on prostaglandins (Fig. 6).

Figure 5. Responses of C fiber (CV = 0.43 m/s) to 10 min of ischemia before (A) and after (B) inhibition of PKC using PKC-(19—36) (20 µg/kg iv) in presence of COX inhibition (indomethacin 5 mg/kg iv). Neurograms 1–4 represent original tracings obtained at points 1–4 shown by arrows in A (1, 2) and B (3, 4). This particular afferent innervated the gallbladder.

Figure 6. Potential mechanisms for BK-mediated activation of ischemically sensitive abdominal visceral afferents. BK activates phospholipase C (PLC) by stimulating kinin B2 receptors, then induces diacylglycerol (DG) production, which activates PKC. By combining with Ca2+, the activation of PKC stimulates afferents by increasing the concentration of prostaglandins (PGs) by activating phospholipase A2 (PLA2) through stimulation of kinin B2 receptors and/or through synergistic action of PKC with Ca2+. The importance of stimulation of PLA2 by PKC with regard to activation of the afferent response is labeled with a question mark because this formed the hypothesis that was tested in this study. We found that BK and ischemia-induced increases in afferent activity were blunted by PKC inhibition, even in the presence of COX inhibition. Therefore, the contribution from PKC to afferent activation by these stimuli does not depend on PLA2 activation and subsequent prostaglandin production. IP3, inositol 1,4,5-trisphosphate; COX, cyclooxygenase; AA, arachidonic acid; PIP2, phosphatidylinositol 4,5-bisphosphate.
From our previous studies and the existing literature, we hypothesized that an intact COX system is required for PKC-induced activation of ischemically sensitive abdominal visceral afferents by BK. We reasoned that inhibition of PKC would not attenuate BK-mediated increases in afferent activity in the presence of COX inhibition. This was not observed. Instead, our findings indicated that reductions in afferent activity after COX inhibition were reduced further by PKC inhibition. In fact, BK was unable to augment afferent activity at all following combined blockade. These findings suggest that the contribution from PKC to BK-mediated activation of ischemically sensitive C fibers does not depend on an intact cyclooxygenase pathway.

We then examined whether an intact COX system is required for PKC-induced activation of ischemically sensitive C fibers in response to endogenous stimuli produced during abdominal ischemia. Our hypothesis that afferent activity would be similar during 10 min of abdominal ischemia after COX inhibition and combined COX and PKC inhibition was not supported. Instead, we observed that inhibition of PKC reduced neural activity in response to the second bout of ischemia in the presence of indomethacin. This reduction in discharge activity during the second exposure to abdominal ischemia was not due to reduced fiber responsiveness, tachyphylaxis, and/or a time/vehicle/volume effect. In this regard, we have demonstrated previously that, after administration of vehicle for indomethacin (NaHCO₃), two bouts of abdominal ischemia (10 min each) evoke similar increases in C fiber activity when separated by 30–40 min (10). Moreover, we have observed that the vehicle for PKC-(19–36) (i.e., 0.9% saline) does not reduce the discharge activity of afferents during abdominal ischemia (13). Taken together, our results suggest that PKC contributes independently to activation of C fibers during ischemia, irrespective of its ability to stimulate prostaglandin production.

Our data indicate that the phosphoinositide system contributes to activation of ischemically sensitive abdominal visceral afferents. We suggest that this contribution from PKC to cell signaling potentially could result from the ability of this substance to phosphorylate cellular proteins, including cell surface receptors, cytoskeletal proteins, ion channels, and cytosolic and nuclear proteins (1, 22). By phosphorylating these proteins, PKC facilitates cell signaling both locally, at the site of activation, and globally by amplifying other signal transduction pathways such as cAMP, cGMP, calcium, and arachidonic acid metabolites (22). PKC also may control ion movement (e.g., Ca²⁺, Na⁺, and K⁺), thereby modulating the concentration of intracellular ions, membrane potential, and electrical signals (22). Each of these PKC-related events represent key steps in the process of signal transduction (1) and may contribute to activation of sensory nerves. Future research will be necessary to define the importance of these two pathways through which PKC may operate in its contribution to activation of sensory nerves during ischemia.

In summary, we observed that the role of PKC in BK-mediated and ischemia-induced activation of ischemically sensitive C fibers is not dependent on the production of COX products. Therefore, both prostaglandins (the COX system) and PKC (the phosphoinositide system) contribute independently to increases in sensory nerve activity in response to BK and abdominal ischemia. These findings provide new information that increases our understanding of the signal transduction pathways involved in the activation of abdominal visceral afferent nerve endings to mesenteric ischemia.

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