Role of Calcium-Sensitive K⁺ Channels and Nitric Oxide in In Vivo Coronary Vasodilation From Enhanced Perfusion Pulsatility

Nazareno Paolocci, MD, PhD; Pasquale Pagliaro, MD, PhD; Takayoshi Isoda, MD; Federico W. Saavedra, MD; David A. Kass, MD

Background—In vitro studies support K⁺Ca channel–induced smooth muscle hyperpolarization as underlying acetylcholine-mediated (or bradykinin-mediated) vasodilation that persists despite combined nitric oxide (NO) and PGI₂ inhibition. We tested the hypothesis that these channels are activated by enhanced pulsatile perfusion in vivo and contribute substantially to vasodilation from this stimulus.

Methods and Results—The canine left descending coronary artery was perfused with whole blood at constant mean pressure, and physiological flow pulsatility was set at 40 or 100 mm Hg by computer servo-pump. Cyclooxygenase was inhibited by indomethacin. Mean flow increased +18±2% (P<0.0001) with enhanced pulsatility. This response declined ~50% by blocking NO synthase (L-NMMA) or K⁺Ca [charybdotoxin (CbTX)+apamin (AP)]. Combining both inhibitors virtually eliminated the flow rise. Inhibiting either or both pathways minimally altered basal coronary flow, whereas agonist-stimulated flow was blocked. Bradykinin-induced dilation declined more with CbTX+AP than with L-NMMA (~66% versus ~46%, P=0.03) and was fully blocked by their combination. In contrast, acetylcholine-induced dilation was more blunted by L-NMMA than by CbTX+AP (~71% versus ~44%, P<0.0002) and was not fully prevented by the combination. Substituting iberiotoxin (IbTX) for CbTX greatly diminished inhibition of pulse pressure and agonist flow responses (with or without NOS inhibition). Furthermore, blockade by IbTX+AP was identical to that by AP alone, supporting a minimal role of IbTX-sensitive large-conductance K⁺Ca channels.

Conclusions—K⁺Ca activation and NO comodulate in vivo pulsatility-stimulated coronary flow, supporting an important role of a hyperpolarization pathway in enhanced mechanovascular signaling. Small- and intermediate-conductance K⁺Ca channels are the dominant species involved in modulating both pulse pressure– and bradykinin-induced in vivo coronary dilation. (Circulation. 2001;103:119-124.)

Key Words: circulation ■ ion channels ■ nitric oxide ■ bradykinin ■ endothelium-derived factors

In vivo coronary arterial tone is regulated by local metabolic demand and neurohormones,¹ mean and phasic pressures (ie, myogenic response),² and endothelium-mediated shear stress.³⁻⁶ NO is well established as a prominent modulator of the latter.⁷,⁸ However, growing evidence supports an additional pathway resistant to NO synthase (NOS) and cyclooxygenase (COX) inhibition, involving a hyperpolarizing factor (EDHF).⁹,¹⁰ Although the precise nature of EDHF remains somewhat controversial,¹¹⁻¹³ a common footprint is its activation of calcium-dependent potassium (K⁺Ca) channels inhibitable by charybdotoxin (CbTX) plus apamin (AP).¹⁴,¹⁵ These channels may also be activated by NO and cGMP,¹⁶,¹⁷ suggesting some role of K⁺Ca channel conductance to NO¹⁸ and shear-stress signaling as well.

The presence of an EDHF pathway is based largely on in vitro studies in which vascular hyperpolarization is directly observed with increased shear¹⁹ or pulsatile stretch.²⁰ Existing in vivo data are scant and have reported only on agonist stimulation.²¹ Furthermore, translating in vitro findings to the intact heart is nontrivial, because the latter shows unaltered basal coronary tone despite K⁺Ca+NOS-COX blockade,²¹,²² whereas in vitro, this combination constricts. Because intact vessels are continually exposed to mechanical stimuli, K⁺Ca activation may play a greater role when vascular stresses are enhanced, as from higher pulsatility with exertion. Increased pulsatility alone elevates in vivo coronary flow,²³,²⁴ despite unaltered regional function and metabolism, an effect blunted by half by NOS inhibition yet amplified by concomitant low-dose adenosine.²³,²⁴ The role for K⁺Ca activation in this response is unknown but is suggested by previous in vitro data²⁰ and studies showing exercise training enhancement of endothelium-mediated vasorelaxation by NO and hyperpolarization-dependent signaling.²⁵

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The present study tested the hypothesis that K<sup>+</sup><sub>Ca</sub> channels contribute prominently to coronary flow modulation by increased perfusion pulsatility, separate from that due to NOS stimulation. We further probed channel subspecificity for this pulsatile signaling by comparing it to that for bradykinin (BK) and acetylcholine (ACH). We show a prominent contribution of K<sup>+</sup><sub>Ca</sub> channels to in vivo pulsatile flow modulation and reveal signaling similarities with BK-induced dilation, with a dominant role of intermediate- and small-conductance channels.

**Methods**

The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University. Coronary pulse pressure (PP) was varied with a computer-controlled servo-system previously reported.<sup>23,24</sup> Adult mongrel dogs (n=25 to 30 kg) were anesthetized with pentobarbital (30 mg/kg IV) and fentanyl (50 mg/kg IV) (n=25) or chloralose/urethane (0.1/1.0 g/kg IM) (n=5) and ventilated with enhanced inspired oxygen to maintain physiological arterial Po<sub>2</sub>, Pco<sub>2</sub>, and pH. The latter tested specific effects of pentobarbital anesthesia on coronary vascular responses. Responses with ether anesthesia were similar, so data were combined for analysis. COX activity was inhibited by indomethacin (5.0 mg/kg IM). After heparinization (8000 IU bolus, 1000 IU/h), arterial blood withdrawn from a femoral artery was pressurized at 100 mm Hg constant pressure was digitally recorded, modified in computer memory to provide feedback for the servo-system. Central aortic pressure and coronary pressure and mean coronary flow at 40 or 100 mm Hg pulsatility. Elevating PP augmented mean flow by 5.3% to 30.4% (Figure 1A displays example steady-state tracings of phasic coronary pressure and mean coronary flow at 40 or 100 mm Hg pulsatility). PP was set to 40 mm Hg, and the preparation was allowed to stabilize 8 minutes before servo-pump regulation. 23,24 In preliminary studies, this dose combined with L-NMMA fully prevented maximal BK-induced dilation, supporting the adequacy of the concentration.

In 7 dogs, the protocol was conducted with IbTX (4.7 nmol/min, 131 nmol/L) substituted for CbTX. IbTX is a highly selective high-affinity blocker of large K<sup>+</sup><sub>Ca</sub> channels that does not affect intermediate- or small-conductance K<sup>+</sup><sub>Ca</sub> channels.<sup>25,30</sup> This dose was 3 times higher than that previously studied in vivo.<sup>21,29</sup> Last, AP alone was tested (n=6) and PP and agonist testing performed. Animals receiving IbTX did not receive CbTX. Not all portions of the protocol were obtained in each animal; hence, different sample sizes are noted for each condition.

**Pharmaceuticals**

Nor-Monomethyl-l-arginine (L-NMMA) was from CalBiochem. AP, CbTX, Iberiotoxin (IbTX), ACh, BK, and sodium nitroprusside were from Sigma. Adenosine (Adenocard) was from Fujisawa USA. Diethylamine/NO was provided by Dr David A. Wink (Radiation Biology Branch, NIH, Bethesda, MD). Each drug was dissolved in isotonic saline at 37°C just before use and administered intracoronarily through a side port in the servo-perfusion cannula at ≤2 mL/min. Coronary flow was unaltered by saline alone at such rates.

**Experimental Protocols**

PP was set to 40 mm Hg, and the preparation was allowed to stabilize for 15 to 20 minutes. Data were subsequently obtained with PP set to 40 or 100 mm Hg at the same mean pressure. Steady state was always observed after 30 to 60 seconds.<sup>24</sup> Data were recorded over 60 to 90 seconds, starting 2 minutes after a PP change. PP was varied between the two levels repeatedly to obtain multiple files for each condition, and results were averaged. Basal flow, PP-altered flow, and agonist-altered flow (ACH 150 μg/30 s IC and BK 100 μg/30 s IC) were assessed under the following conditions: (1) baseline (n=28); (2) 5-minute pretreatment and continued infusion of AP (15 nmol/min IC; mean concentration of 417 nmol/L at 36 min/average coronary blood flow) and CbTX (1.5 nmol/min IC, 42 nmol/L) to broadly block K<sup>+</sup><sub>Ca</sub> channels (n=20); (3) recontrol after CbTX+AP was continued (10 to 15 minutes after both agents were discontinued); (4) L-NMMA (n=20) (0.5 mg·kg<sup>−1</sup>·min<sup>−1</sup> IC) administered 20 minutes before and continuing during PP and agonist testing; and (5) combined CbTX+AP+L-NMMA (n=11).

**Baseline Coronary Flow in Isolated Servo-Perfused Left Anterior Midproximal Descending Artery Under Various Experimental Protocols**

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Baseline</th>
<th>Inhibitor</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbTX+AP</td>
<td>19</td>
<td>33.9±3.8</td>
<td>34.9±3.8</td>
<td>NS</td>
</tr>
<tr>
<td>IbTX+AP</td>
<td>5</td>
<td>43.8±5.2</td>
<td>41.3±6.6</td>
<td>NS</td>
</tr>
<tr>
<td>AP</td>
<td>6</td>
<td>44.1±6.6</td>
<td>48.3±7.0</td>
<td>NS</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>20</td>
<td>41.5±4.4</td>
<td>35.8±3.8</td>
<td>0.04</td>
</tr>
<tr>
<td>CbTX+AP+L-NMMA</td>
<td>11</td>
<td>32.6±5.3</td>
<td>30.4±4.5†</td>
<td>0.07</td>
</tr>
<tr>
<td>IbTX+AP+L-NMMA</td>
<td>6</td>
<td>40.2±6.1*</td>
<td>38.6±6.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

Flow is in mL/min.
*Data are post-L-NMMA.
†P=0.02 vs pre-L-NMMA baseline.

The dose of CbTX was based on in vitro studies showing the effectiveness of a 10 to 100 nmol/L concentration for inhibiting maximal ACh or BK responses, as well as previous in vivo studies.<sup>21,29</sup> In preliminary studies, this dose combined with L-NMMA fully prevented maximal BK-induced dilation, supporting the adequacy of the concentration.

**Results**

**K<sup>+</sup>Ca and NO Modulation of Basal Coronary Tone**

Basal flow at 40 mm Hg PP in the left anterior descending coronary artery territory averaged 36.7±17.7 mL/min (n=28). This is close to 1.0 mL·g<sup>−1</sup>·min<sup>−1</sup> for these hearts, similar to flows measured before servo-pump regulation.<sup>23,24</sup> Inhibition of K<sup>+</sup><sub>Ca</sub> by CbTX-AP, IbTX-AP, or AP alone did not significantly alter basal flow (Table). Mean flow declined slightly but significantly with L-NMMA. Subsequent addition of K<sup>+</sup><sub>Ca</sub> channel blockers did not reduce flow further, although the combination of CbTX-AP+L-NMMA was also lower than the pre-L-NMMA baseline.

**K<sup>+</sup>Ca and NO Modulation of Pulsatile-Stimulated Flow**

Figure 1A displays example steady-state tracings of phasic coronary pressure and mean coronary flow at 40 or 100 mm Hg pulsatility. Elevating PP augmented mean flow by +18.3±2.4% (36.0±4.5 to 41.8±4.9 mL/min, n=20, P<0.0001). CbTX+AP blunted this response to +11.9±2.9% (P<0.001) (Figure 1B and 1C). It was fully restored on rebaseline to +20.6±3.9% but declined similarly with L-NMMA (+11.7±2.3%, P<0.0001). Importantly,
CbTX-AP blunted BK responses by AP or L-NMMA alone.

*P<0.001 vs control response. †P<0.001 vs baseline or rebaseline by ANOVA, ‡P<0.0001, 40 vs 100 mm Hg PP. CON indicates control. C, Summary data.

Figure 1. A, Increase in mean coronary flow at higher perfusion pulsatility in vivo. Top, Arterial pressure generated by servo-system in isolated left anterior descending coronary artery. Mean pressure is constant (dashed line) despite rise in pulsatility. Middle, Corresponding coronary flow waveforms. With higher pulsatility, systolic flow rises while diastolic flow is preserved, resulting in a net increase in mean flow. Bottom, Mean change in coronary flow: P<0.0001, 40 vs 100 mm Hg PP. B, Influence of KCa channel, NOS, and combined blockade on coronary flow increase from enhanced perfusion pulsatility. Example from 1 dog, with flow data normalized to baseline at 40 mm Hg PP, CON indicates control. C, Summary data. *P<0.001 vs baseline or rebaseline by ANOVA, †P<0.001 vs CbTX+AP or L-NMMA alone.

Comparison With BK- and ACh-Stimulated Flow

Although KCa inhibition did not alter basal coronary tone and NOS inhibition had modest effects, each alone and in combination profoundly altered agonist-stimulated dilation. Figure 2A displays coronary flow at 40 mm Hg PP before and after agonist administration, and Figure 2B summarizes these data. Both ACh and BK achieved near-maximal flow responses (ACh +274.6±22.7%, BK +274.5±24.6%). CbTX+AP blunted BK responses by ~66.3±5.2% (residual +89±20% flow rise, n=13), whereas the ACh response declined to a lesser extent (~44.5±7.2%, P=0.016 versus BK, n=11). The opposite was observed with L-NMMA: NOS inhibition reduced ACh more than BK flow elevation (BK ~45.8±7.0%, ACh ~70.6±3.1%, P<0.01 versus BK). Disparities were also observed when these agents were combined, revealing full inhibition of BK flow responses, similar to PP signaling, but residual ACh-induced flow elevation (~41.3±8.1%, P<0.001).

Subchannel Selectivity of KCa Response

To selectively test the role of large-conductance KCa channels in PP and agonist-stimulated signaling, we substituted combining NOS and KCa blockade (lower panel) virtually eliminated PP flow responses (31.1±4.4 versus 31.9±4.2, P=NS, n=11). The latter was not paralleled by inhibition of direct NO-dependent dilation, because nitroprusside or diethylamine/NO still induced vasodilation (~140% postblockade versus +110% preblockade).

Discussion

This study demonstrates for the first time that in vivo, KCa channels blocked by CbTX-AP are important comodulators, along with NO, in regulating coronary flow by increased perfusion PP. The combined pathways are sufficient to totally or almost totally explain mechanical and BK-induced dilation. Selective large-conductance KCa channel blockade by IbTX did not alter PP or agonist-induced dilation. This and data showing that AP-sensitive channels contribute modestly to both PP and BK but not ACh responses support similarities between PP and BK signaling, principally via small- and intermediate-conductance KCa channels. Although direct measurement of vascular hyperpolarization could not be performed in vivo, the sensitivity to CbTX+AP (with comitant NOS-COX inhibition) is a hallmark of EDHF-
mediated hyperpolarization. Therefore, the present results further provide novel critical support that such pathways acting via K\textsuperscript{+}Ca channels play an important role in modulating coronary flow.

Comparison to Previous In Vivo Studies
Several studies have explored the role of K\textsuperscript{+}Ca channels in vivo, although to date this has focused only on agonist- or ischemia-dependent signaling. For example, Nishikawa et al\textsuperscript{22} reported that residual dilation to ACh in <100-\mu m diameter arterioles was inhibited by suffusion with K\textsuperscript{+} buffer or by K\textsuperscript{+}Ca blockade. NOS-COX inhibition alone was effective in blocking ACh dilation in less distal arterioles, supporting a more prominent role of NO signaling in such vessels.\textsuperscript{31} Node et al\textsuperscript{1} reported that CbTX or IbTX combined with L-NAME prevented or blunted BK and (BK-dependent) postischemic dilation. Ischemic protection by 17\beta-estradiol, which appeared largely modulated by NO and BK, was inhibited by IbTX+L-NAME,\textsuperscript{29} suggesting that K\textsuperscript{+}Ca channel activation, like K\textsuperscript{+}ATP-channel activation,\textsuperscript{32} plays an important role in modifying the impact of coronary supply/demand imbalance. Pulse-perfusion signaling may be relevant in this regard, helping to explain beneficial effects of exercise (with enhanced PP) on flow reserve.\textsuperscript{33}

Pulse Perfusion Signaling Mechanisms
Although pulse frequency (heart rate) has been shown to influence in vivo coronary flow,\textsuperscript{30} this alters metabolic demand and chamber load in addition to flow pulsation. Our preparation facilitated the study of PP effects alone, confirming a role of NO release,\textsuperscript{23} the lack of accompanying change in regional and global myocardial function or regional myocardial oxygen consumption.\textsuperscript{23,24} Although modest under basal (autoregulating) conditions, PP flow augmentation is amplified by low levels of adenosine or K\textsuperscript{+}ATP channel agonists,\textsuperscript{24} suggesting that the mechanism may play a more prominent role when PP normally rises, as during exertion.

Increasing PP in vivo alters both phasic shear and vascular distension, and as with NO, K\textsuperscript{+}Ca channel activation or EDHF signaling appears to be involved with both stimuli in vitro.\textsuperscript{3,20} Static stretch of smooth muscle cells stimulates K\textsuperscript{+}Ca channels,\textsuperscript{34} whereas pulsatile stretch of porcine coronary vessels with NOS/COX inhibition releases EDHF\textsuperscript{20} inhibitable by K\textsuperscript{+}Ca channel blockade. This may counter Ca\textsuperscript{2+}-dependent increases in vascular tone from higher mean distending pressures. The latter is linked to smooth muscle depolarization triggering Ca\textsuperscript{2+} entry via voltage-gated channels,\textsuperscript{2} but the increase in [Ca\textsuperscript{2+}]\textsubscript{i} also activates K\textsuperscript{+}Ca channels.\textsuperscript{35,36}

Another possible mechanism should be considered. Residual ACh responses despite NOS inhibition have been reported to directly correlate with persistent NO release in some studies,\textsuperscript{13,37} suggesting incomplete NOS blockade. If true in our study, residual NO would have to preferentially act via K\textsuperscript{+}Ca activation. Although NO and cGMP can activate these channels,\textsuperscript{16,17} this has been documented only for large-conductance channels, requiring substantial NO levels. Such channels played a minor role in contrast to small- and intermediate-conductance channels in our preparation. Furthermore, the diminutions of PP responses by L-NMMA and CbTX-AP were nearly equal (separately and combined), which would not be expected if substantial cross-talk between NO- and K\textsuperscript{+}Ca-dependent signals occurred. Finally, the L-NMMA dose was 4 times that shown to fully prevent in vivo ACh-mediated dilation in conductance arterioles (>100 \mu m).\textsuperscript{32}

Last, coronary microvessels and larger arteries undergo sustained dilation in response in increases in PP\textsuperscript{38,39} that might lower coronary resistance to enhance flow. However, both mean pressure and PP were identical for all protocols, despite near-complete inhibition of PP flow responses with CbTX-AP+L-NMMA. This is probably not due to prevention of smooth muscle distensibility, because basal coronary flow was little altered, and vasorelaxation to NO donors was preserved.

Role of Small- and Intermediate-Conductance K\textsuperscript{+}Ca Channels
The pharmacological inhibitor sensitivities of the PP-mediated flow changes most closely matched those observed
with BK stimulation. Unlike ACh, both PP and BK responses were nearly or fully blocked by combined CbTX-AP+L-NMMA and partially diminished by AP alone. IbTX altered none of these responses. BK induces vasorelaxation in the coronary microcirculation principally by membrane hyperpolarization.\textsuperscript{30,44} Combined NOS inhibition and CbTX-AP consistently inhibits this, whereas diminished blockade is observed with IbTX.\textsuperscript{42} CbTX blocks both intermediate-\textsuperscript{28,30} and large-conductance channels,\textsuperscript{26} whereas IbTX selectively inhibits the latter.\textsuperscript{30} At concentrations greater than those used in the present study, CbTX can also inhibit voltage-gated K\textsuperscript{+} channels; however, these are not thought to play a prominent role in coronary endothelium.\textsuperscript{43} AP is relatively selective for small-conductance channels. Thus, our results support a dominant role for intermediate- and small-conductance K\textsuperscript{+} channels to the PP response.

The observed selectivity for inhibiting BK, ACh, and PP responses to CbTX-AP but not IbTX-AP is supported by several previous in vitro studies in renal vessels\textsuperscript{44} and rat hepatic artery.\textsuperscript{45} However, some discrepancies with earlier data have been reported in coronary vessels. For example, IbTX inhibited NOS-COX-independent ACh-induced dilation\textsuperscript{22} and BK-induced dilation in canine coronary arteries.\textsuperscript{21} The specific nature of the preparation and/or dose of agonist may play a role. For example, the BK-agonist dose used by Node et al\textsuperscript{41} was \(\approx 5\%\) that in the present study. At this dose, L-NMMA also fully inhibited BK flow increases (33.9 versus 33.0 mL/min, mean \(\Delta\text{CBF} = -0.96 \pm 1.0\), \(P = \text{NS}\)) in 3 of 4 studies, whereas addition of IbTX blocked the residual response in the fourth study. This suggests a greater involvement of IbTX-insensitive channels at higher BK doses. Importantly, this selectivity is similar to that observed for pulsatile perfusion.

Residual ACh-mediated dilation despite full inhibition of BK (or PP) signaling with CbTX-AP+L-NMMA is a novel observation that suggests an NO-, K\textsuperscript{+}C\textsubscript{a}, and COX-independent pathway. Such an alternative pathway is supported by recent data showing ACh induction of a hyperpolarizing K\textsuperscript{+} current different from K\textsubscript{C\textsubscript{a}} currents.\textsuperscript{12}

**Study Limitations**

Regional myocardial metabolism was not directly measured to test whether K\textsuperscript{+}C\textsubscript{a} blockade specifically altered PP metabolic interactions and thus, potentially, flow responses. However, previous data revealed no effect of elevating PP on local myocardial oxygen consumption under basal conditions or with concomitant adenosine, BK, verapamil,\textsuperscript{24} or epinephrine (unpublished data), making such interaction unlikely. Only one CbTX (or IbTX) dose was studied, largely because of the expense of these agents even for intracoronary in vivo studies. Although this cannot confirm maximal inhibition, full blockade of high-dose BK dilation by CbTX+AP+L-NMMA is compatible with an adequate if not maximal inhibitory effect.

Enhancing in vivo perfusion PP alters both phasic stretch and flow, and one cannot separate the primary stimulus in this setting. It remains possible that different findings would be observed with less compliant vessels (ie, aging), where pulse distension would be lower. Furthermore, the in vivo preparation cannot definitively identify whether vascular smooth muscle or endothelial K\textsuperscript{+}C\textsubscript{a} channels are the dominant effectors of the dilatory response. In vitro studies\textsuperscript{12,27} applying intraluminal CbTX have shown a greater role for the endothelial channels; other studies showed that both barium and ouabain were necessary to inhibit the vascular smooth muscle hyperpolarization, whereas CbTX+AP was sufficient in endothelial cells.\textsuperscript{14}

**Conclusions**

Like NO signaling, K\textsuperscript{+}C\textsubscript{a} channel activation plays a minimal role in modulating basal coronary tone, yet a prominent one when stimulated by agonists such as ACh and BK or by pulsatile perfusion. This suggests that along with K\textsuperscript{+}ATP channels, adenosine, and NO, K\textsubscript{C\textsubscript{a}} channels most likely contribute to vasodilator reserve during exertional stress. This is the first study to reveal a role for these channels in vivo for non-agonist-stimulated flow. Recent studies suggest that EDHF-dependent vascular dilation maybe upregulated when NO signaling is downregulated, such as in congestive heart failure\textsuperscript{46} or, conversely, downregulated when inducible NO expression is enhanced, ie, sepsis and atherosclerosis.\textsuperscript{37} This and the present data support the importance of determining those signals that activate this pathway in vivo in normal and diseased hearts.

**Acknowledgments**

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