Mechanisms of endothelial P2Y₁- and P2Y₂-mediated vasodilatation involve differential [Ca^{2+}]_i responses

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Marrelli, Sean P. Mechanisms of endothelial P2Y₁- and P2Y₂-mediated vasodilatation involve differential [Ca^{2+}]_i responses. Am J Physiol Heart Circ Physiol 281: H1759–H1766, 2001.—The present study was designed to evaluate the role of endothelial intracellular Ca^{2+} concentration ([Ca^{2+}]_i) in the difference between P2Y₁- and P2Y₂-mediated vasodilatations in cerebral arteries. Rat middle cerebral arteries were cannulated, pressurized, and luminally perfused. The endothelium was selectively loaded with fura 2, a fluorescent Ca^{2+} indicator, for simultaneous measurement of endothelial [Ca^{2+}]_i, and diameter. Luminal administration of 2-methylthioadenosine 5'-triphosphate (2-MeS-ATP), an endothelial P2Y₁ agonist, resulted in purely nitric oxide (NO)-dependent dilation and [Ca^{2+}]_i increases up to ~300 nM (resting [Ca^{2+}]_i = 145 nM). UTP, an endothelial P2Y₂ agonist, resulted in dilations that were both endothelium-derived hyperpolarizing factor (EDHF)- and NO-dependent with [Ca^{2+}]_i increases to >400 nM. In the presence of Nω-nitro-L-arginine-indomethacin to inhibit NO synthase and cyclooxygenase, UTP resulted in an EDHF-dependent dilation alone. The [Ca^{2+}]_i threshold for NO-dependent dilation was 220 vs. 340 nM for EDHF. In summary, the differences in the mechanism of vasodilatation resulting from stimulation of endothelial P2Y₁ and P2Y₂ purinoreceptors result in part from differential [Ca^{2+}]_i responses. Consistent with this finding, these studies also demonstrate a higher [Ca^{2+}]_i threshold for EDHF-dependent responses compared with NO.

endothelium-derived hyperpolarizing factor; calcium; cerebral; nitric oxide; purinergic

STUDIES IN THIS AND OTHER laboratories have previously shown that stimulation of endothelial P2Y₁ and P2Y₂ purinoreceptors in cerebral arteries produces a vasodilatation via different mechanisms (25, 38). In the rat middle cerebral artery, stimulation of P2Y₁ receptors results in a vasodilatation through a purely nitric oxide (NO)-dependent mechanism (25, 37, 38). In contrast, stimulation of P2Y₂ receptors results in a vasodilatation through endothelium-derived hyperpolarizing factor (EDHF)- and NO-dependent mechanisms (25, 38).

The role of endothelial intracellular Ca^{2+} concentration ([Ca^{2+}]_i) in the mechanism of NO production has been well demonstrated (for a review, see Ref. 27). In brief, an increase in endothelial [Ca^{2+}]_i results in Ca^{2+} binding to calmodulin, thus forming a Ca^{2+}-calmodulin complex capable of stimulating NO synthase (NOS). Endothelial NOS then catalyzes the conversion of arginine to citruline and NO.

The role of endothelial [Ca^{2+}]_i in the mechanism of the EDHF response is considerably less understood. However, an essential role for increased endothelial [Ca^{2+}]_i has gained acceptance based on experiments of three general types. First, agonists found to produce an EDHF response are also capable of increasing endothelial [Ca^{2+}]_i in cultured endothelial cells (6, 36). Second, an EDHF response can be elicited by Ca^{2+} ionophores such as A23187 (5, 30) and inhibited by removing extracellular Ca^{2+} (5, 12) or administering agents that prevent Ca^{2+} influx through nonselective cation channels (35). Third, inhibitors of calmodulin (a Ca^{2+}-sensitive protein) block the EDHF response in certain preparations (15, 29). Taken together, these data are suggestive of a requirement for increased endothelial [Ca^{2+}]_i in the production of EDHF-dependent dilations. However, none of these studies has directly shown a correlation between increased endothelial [Ca^{2+}]_i and EDHF-dependent dilations. Furthermore, no studies to date have directly addressed the relative requirement for increased [Ca^{2+}]_i for the EDHF response compared with other [Ca^{2+}]_i-dependent endothelial relaxing factors such as NO.

One of the primary reasons that direct evidence for a role of [Ca^{2+}]_i in the EDHF response is lacking is due to the difficulties in measuring EDHF. At this time, there is no consensus as to the chemical identity of EDHF despite a number of plausible candidates (11, 26). Furthermore, EDHF may be different depending on the particular tissues studied (7). Therefore, although the term EDHF is used throughout this article, it should be kept in mind that it is not presently clear whether EDHF represents one or more factors or even a phenomenon such as direct transfer of hyperpolarization (9). Because EDHF cannot be measured by the direct assay of a specific factor, the most reliable method for measuring EDHF has been via bioassay.

The present experiments were designed to test the hypothesis that the different mechanisms of endothelial P2Y₁- and P2Y₂-dependent vasodilatation in cerebral arteries are due to a differential endothelial [Ca^{2+}]_i response. Specifically, it was postulated that...
the P2Y2-dependent mechanism (which results in an EDHF-dependent dilation) would result in a greater increase in endothelial [Ca$^{2+}$]; than the purely NO-dependent mechanism. These experiments utilize a method whereby endothelial [Ca$^{2+}$]; can be selectively measured in the context of an intact pressurized cerebral artery (23). In addition to maintaining many physiological variables (see DISCUSSION), this method provides a means for monitoring both NO and EDHF responses (38). The simultaneous measurement of [Ca$^{2+}$]; and artery diameter allows the release of either “factor” to be monitored by bioassay and then directly correlated with endothelial [Ca$^{2+}$].

**METHODS**

All experiments were approved by the Animal Protocol Review committee at Baylor College of Medicine. Male Long-Evans rats (250–375 g) were anesthetized with isoflurane and decapitated. A total of 27 rats were used. Brains were removed and the right and left middle cerebral arteries (MCA) were dissected free and stored in Krebs solution until needed. All vessels were used within 4 h of harvesting.

**Mounting of pressurized/perfused cerebral arteries.** MCAs were cannulated with two glass micropipettes within a vessel chamber as previously described (23). Briefly, the MCA was secured on the micropipettes with 12-0 nylon sutures and the chamber was mounted on the stage of an inverted fluorescence microscope for diameter measurement and fluorescent measurement of endothelial [Ca$^{2+}$].

**Measurement of endothelial [Ca$^{2+}$]; in intact arteries.** The method for selective measurement of endothelial [Ca$^{2+}$]; in pressurized/perfused MCAs has recently been described in detail (23). Briefly, fura 2-AM was delivered lumina!ly to the endothelium. Fura 2-AM (0.67 μM) was administered with pluronic F127 (0.02%) to facilitate more even dispersion of fura 2-AM in solution. The artery was luminally perfused with the fura 2-AM solution for 5 min before being switched back to the standard Krebs buffer. The low concentration of fura 2-AM and short duration of loading ensured that the endothelial esterases were not overwhelmed, thus containing the Ca$^{2+}$-indicating dye to the endothelium. Deesterification of the dye followed for 20 to 30 min.

**Measurement of [Ca$^{2+}$] i** was achieved by rapidly alternating between 340- and 380-nm excitation wavelengths and measuring the resulting 510-nm emission from fura 2 (13, 23). The system for measuring [Ca$^{2+}$] i in intact arteries incorporated a beam splitter for the simultaneous measurement of the fura 2 signal and the vessel diameter (Intracellular Imaging; Cincinnati, OH). The beam splitter diverted the fura 2 signal to a photomultiplier and the red light image of the vessel to a charge-coupled device camera for measurement of vessel diameter. [Ca$^{2+}$] i was determined from a series of calibration curves based on the following equation

\[
[Ca^{2+}]_i = \beta[R - R_{\text{min}}]/(R_{\text{max}} - R)K_d
\]

For this equation, \(R\) is the ratio of the 340/380-nm-emission, \(R_{\text{min}}\) is the ratio in [Ca$^{2+}$];-free conditions, \(R_{\text{max}}\) is the ratio in saturating [Ca$^{2+}$]; conditions, \(\beta\) is the ratio of 380-nm unbound to 380-nm bound Ca$^{2+}$, and \(K_d\) is the determined dissociation constant for fura 2 to [Ca$^{2+}$]. An in situ calibration yielded the following values for the above equation: 1.52 (\(R_{\text{max}}\)), 0.18 (\(R_{\text{min}}\)), and 4.35 (\(\beta\)). The in situ \(K_d\) was determined to be 282 nM (19).

**Concentration-response curves.** Compounds were administered selectively to the endothelium or smooth muscle by adding the compound of interest either to the luminal perfusate or the abluminal perfusate, respectively. In situations where multiple concentrations of a drug were examined, cumulative concentration-response curves (CRCs) were performed, allowing for a steady state to be reached between concentrations.

Luminal CRCS were performed with 2-methylthioadenosine 5’-triphosphate (2-MeS-ATP, 10$^{-8}$ to 10$^{-5}$ M) and UTP (10$^{-7}$ to 10$^{-5}$ M). In some groups, Nω-nitro-l-arginine (l-NAME; 50 μM) and indomethacin (10 μM) were administered to inhibit the production of NO and prostacyclin, respectively (25). Previous studies have demonstrated that 2-MeS-ATP selectively stimulates endothelial P2Y1 purinoreceptors and produces dilation exclusively through the production of NO (25, 38). UTP selectively stimulates P2Y2 purinoreceptors and promotes dilation through a combination of NO- and EDHF-dependent mechanisms (25, 38).

**Chemicals and buffer compositions.** All drugs and chemicals were obtained from Sigma (St. Louis, MO) with the exceptions of fura 2-AM (TefLabs; Austin, TX), Br-A23187 (Molecular Probes; Eugene, OR), and 2-MeS-ATP (Research Biochemicals International). The Krebs buffer consisted of the following (in mM): 119 NaCl, 4.7 KCl, 21 NaHCO3, 1.18 KH2PO4, 1.17 MgSO4, 0.026 EDTA, 1.6 CaCl2, and 5.5 glucose (3).

**Statistical methods.** Values are reported as means ± SE. Comparison of single measurements between two groups was performed using a Student’s t-test or one-way ANOVA when more than two groups were evaluated. Comparison between two groups in which multiple conditions were evaluated was performed using a two-way repeated-measures ANOVA. When appropriate (significant group differences or significant group interactions), a Tukey test was used for individual comparisons between groups. Significance was defined as \(P < 0.05\). [Ca$^{2+}$] i tracings were low-pass filtered to reduce the effects of random noise.

**RESULTS**

Resting diameter (diameter after development of spontaneous tone) of the MCAs was 223 ± 4 μm with an endothelial [Ca$^{2+}$]; of 145 ± 9 nM (n = 9). In a separate group of arteries, incubation with l-NAME and indomethacin resulted in a significantly smaller resting diameter of 195 ± 6 μm (P = 0.003, t-test) with an endothelial [Ca$^{2+}$]; of 149 ± 6 nM (n = 16). Endothelial [Ca$^{2+}$]; did not differ significantly between the two groups. Maximal diameter, the diameter corresponding to that in the presence of a Ca$^{2+}$-free buffer, was 307 ± 4 and 302 ± 4 μm for the untreated and l-NAME-indomethacin groups, respectively.

Figure 1 shows representative experiments of endothelial [Ca$^{2+}$]; and diameter measurements after NO-mediated dilation via luminal delivery of a selective P2Y1 agonist (37), 2-MeS-ATP, alone (Fig. 1A) and in the presence of l-NAME-indomethacin (Fig. 1B). Note in Fig. 1A that 2-MeS-ATP (10 μM) produced an increase in [Ca$^{2+}$]; to ~300 nM, which was sufficient to cause a substantial NO-dependent dilation. In this
particular case, the dilation was maximal. However, in the presence of L-NAME-indomethacin, dilations to 2-MeS-ATP are virtually abolished despite significant increases in endothelial \([\text{Ca}^{2+}]_i\) (Fig. 1B). The horizontal line indicates the maximum achievable diameter (307 \(\mu\text{m}\)) for this particular artery.

A summary graph of the percent diameter change (A) and endothelial \([\text{Ca}^{2+}]_i\) (B) in response to 2-MeS-ATP is presented in Fig. 2. CRCs to 2-MeS-ATP alone (\(n = 4\) experiments) and after incubation with L-NAME-indomethacin (\(n = 4\) experiments) are plotted in black and gray, respectively. Luminal delivery of 2-MeS-ATP alone resulted in a dose-dependent increase in both endothelial \([\text{Ca}^{2+}]_i\) and artery diameter. Following inhibition of NOS and cyclooxygenase with L-NAME-indomethacin, however, 2-MeS-ATP resulted in an increase in endothelial \([\text{Ca}^{2+}]_i\) without an increase in diameter. This result is consistent with the finding that 1 \(\mu\text{M}\) UTP is found to involve the release of NO but no EDHF-dependent dilation (25, 38). Addition of 10 \(\mu\text{M}\) UTP resulted in a further increase in \([\text{Ca}^{2+}]_i\), accompanied by a maximal EDHF-dependent dilation.

A summary graph of the percent diameter change (A) and endothelial \([\text{Ca}^{2+}]_i\) (B) in response to UTP is presented in Fig. 4. CRCs to UTP alone (\(n = 5\) experiments) and after incubation with L-NAME-indomethacin (\(n = 5\) experiments) are plotted in black and gray, respectively. Luminal delivery of UTP alone resulted in an increase in endothelial \([\text{Ca}^{2+}]_i\) with a concomitant dilation. The addition of 10 \(\mu\text{M}\) UTP resulted in a further increase in \([\text{Ca}^{2+}]_i\) but no further increase in diameter because the artery had already reached maximal dilation. The presence of L-NAME-indomethacin, 1 \(\mu\text{M}\) UTP resulted in a significant increase in endothelial \([\text{Ca}^{2+}]_i\), without an increase in diameter. This result is consistent with the finding that 1 \(\mu\text{M}\) UTP is found to involve the release of NO but no EDHF-dependent dilation (25, 38). Addition of 10 \(\mu\text{M}\) UTP resulted in a further increase in \([\text{Ca}^{2+}]_i\), accompanied by a maximal EDHF-dependent dilation.

Fig. 2. Effect of L-NAME-Indo on 2-MeS-ATP diameter and endothelial \([\text{Ca}^{2+}]_i\) responses. A summary of percent diameter change (A) with corresponding endothelial \([\text{Ca}^{2+}]_i\), (B) is shown. Concentration-response curves (CRCs) were performed to 2-MeS-ATP alone or in the presence of L-NAME-Indo. Data are presented as means ± SE of steady-state values. *\(P < 0.05\) for individual comparisons between the two groups.
respectively. Luminal application of UTP alone resulted in a dose-dependent increase in both endothelial \([Ca^{2+}]_i\) and diameter. Following incubation with L-NAME-indomethacin, endothelial \([Ca^{2+}]_i\) increased, although dilations were essentially absent through 1 \(\mu\)M UTP \((P < 0.01,\) group difference; \(P < 0.05,\) Tukey test). However, at 10 \(\mu\)M UTP, maximal dilations comparable to UTP alone occurred. Similar to the CRC with 2-MeS-ATP, there was no endothelial \([Ca^{2+}]_i\) group difference attributable to L-NAME-indomethacin, although there was a significant interaction \((P < 0.05)\). Individual comparisons between groups demonstrated that endothelial \([Ca^{2+}]_i\) at 10 \(\mu\)M UTP was greater after L-NAME-indomethacin (Tukey test, \(P < 0.05)\).

To evaluate the \([Ca^{2+}]_i\) threshold required to elicit either a NO- or EDHF-dependent dilation, the endothelial \([Ca^{2+}]_i\) corresponding to the initiation of dilation was determined for NO- and EDHF-dependent agonists. A dilation of 15% was defined as the threshold for dilation based on the finding that 15% dilation reflected the smallest dilation that was conservatively above the inherent variability in diameter due to vasomotion. Therefore, the \([Ca^{2+}]_i\) threshold is defined herein as the endothelial \([Ca^{2+}]_i\) required to produce a 15% dilation.

Dilations were elicited by 2-MeS-ATP and UTP in the absence of L-NAME-indomethacin as well as by

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Fig. 3. Representative experiments depicting the simultaneous measurement of endothelial \([Ca^{2+}]_i\) and artery diameter to luminal administration of UTP alone (A) or UTP in the presence of L-NAME-Indo (B). Arrows indicate the points where UTP reached the endothelium at the indicated concentrations.

Fig. 4. Effect of L-NAME-Indo on UTP diameter and endothelial \([Ca^{2+}]_i\) responses. A summary of percent diameter change (A) with corresponding endothelial \([Ca^{2+}]_i\) (B) is shown. CRCs were performed to UTP alone or in the presence of L-NAME-Indo. Data are presented as means ± SE of steady-state values. *\(P < 0.05\) for individual comparisons between the two groups.

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Fig. 5. Endothelial \([Ca^{2+}]_i\) thresholds for nitric oxide (NO) and endothelium-derived hyperpolarization factor (EDHF) production. Comparison of the endothelial \([Ca^{2+}]_i\) thresholds for NO or EDHF release is shown. Data are presented as the means ± SE \([Ca^{2+}]_i\), required to elicit a 15% dilation. In the concentrations used and in the absence of L-NAME, UTP and 2-MeS-ATP produced a dilation through the production of NO (gray bars). In the presence of L-NAME-Indo, UTP and Br-A23187 produced a dilation through the production of EDHF (black bars). There is a significantly higher threshold for EDHF release compared with NO. *\(P < 0.05\) between the indicated groups by one-way ANOVA.
UTP and Br-A23187 in the presence of l-NAME/indomethacin (Fig. 5). 2-MeS-ATP results in the exclusive production of NO. UTP is believed to release exclusively NO at the concentration required to elicit a 15% dilation (38). In the presence of l-NAME-indomethacin, UTP and Br-A23187 result in EDHF-dependent dilations (24, 38). The [Ca\(^{2+}\)]_i thresholds for 2-MeS-ATP and UTP were 228 ± 23 nM (n = 5 experiments) and 218 ± 11 nM (n = 4 experiments). The [Ca\(^{2+}\)]_i thresholds for UTP and Br-A23187 in the presence of l-NAME-indomethacin were 330 ± 19 nM (n = 9 experiments) and 344 ± 20 nM (n = 6 experiments), respectively. The [Ca\(^{2+}\)]_i thresholds for EDHF-dependent dilations (UTP and Br-A23187 in the presence of l-NAME-indomethacin) were significantly greater than for NO-dependent dilations (2-MeS-ATP and UTP alone) (one-way ANOVA, P < 0.001; Tukey test, P < 0.05).

The following experiments were designed to determine whether a certain [Ca\(^{2+}\)]_i threshold was sufficient to produce an EDHF-dependent dilation. If the final endothelial [Ca\(^{2+}\)]_i achieved determines whether an agonist produces an EDHF-dependent dilation or not, then one would expect that a traditionally non-EDHF-producing agonist could be converted to an EDHF-producing agonist by augmenting the [Ca\(^{2+}\)]_i response.

2-MeS-ATP was utilized as a typically non-EDHF-producing agonist. Resting endothelial [Ca\(^{2+}\)]_i, was increased (or primed) to a new steady state by both receptor-dependent and -independent mechanisms through luminal administration of either UTP (1 \(\mu\)M) or Br-A23187 (2–3 \(\mu\)M) in the presence of l-NAME-indomethacin. Note that these priming concentrations of UTP and Br-A23187 are not sufficient to elicit significant dilations by themselves in the presence of l-NAME-indomethacin (see Fig. 7). When a new steady-state resting [Ca\(^{2+}\)]_i was reached, 2-MeS-ATP (10 \(\mu\)M) was administered luminally. Figure 6 shows a representative experiment in which 2-MeS-ATP was added after priming with a subdilating concentration of UTP. Note that the addition of 2-MeS-ATP resulted in a further increase in [Ca\(^{2+}\)]_i with a subsequent maximal dilation. Addition of abluminal charybdotoxin (70 nM) completely inhibited these dilations to 2-MeS-ATP (Fig. 7). As a control, the charybdotoxin-treated vessels were subsequently diluted by abluminal K⁺ (15 mM, data not shown), indicating that nonselective impairment of dilation did not result. Abluminal K⁺ has been demonstrated to dilate cerebral arteries through stimulation of smooth muscle inward rectifying K⁺ channels (16, 20). These data indicate that the dilation to 2-MeS-ATP was indeed a result of an EDHF-dependent mechanism.

**DISCUSSION**

This study evaluated the hypothesis that the different mechanisms of endothelial P2Y\(_1\) and P2Y\(_2\)-dependent vasodilation in cerebral arteries are due to a differential endothelial [Ca\(^{2+}\)]_i response. Furthermore, it was speculated that the EDHF-dependent mechanism (UTP/P2Y\(_2\)) would result in a greater increase in endothelial [Ca\(^{2+}\)]_i, than the purely NO-producing mechanism (2-MeS-ATP/P2Y\(_1\)) due to a presumed higher [Ca\(^{2+}\)]_i threshold for EDHF-dependent dilation. This hypothesis was addressed in the following three steps: 1) simultaneous measurement of endothelial [Ca\(^{2+}\)]_i and artery diameter in response to a NO-dependent agonist and an EDHF-dependent agonist, 2) correlating endothelial [Ca\(^{2+}\)]_i with both NO and EDHF responses, and 3) potentiating the endothelial [Ca\(^{2+}\)]_i response of a non-EDHF-producing agonist such that the higher [Ca\(^{2+}\)]_i threshold for EDHF-dependent dilation was achieved.

**Simultaneous measurement of endothelial [Ca\(^{2+}\)]_i and artery diameter in response to a NO-producing agonist and an EDHF-dependent agonist.** The present study is the first to report endothelial [Ca\(^{2+}\)]_i in an intact artery with simultaneous confirmation of EDHF-dependent dilation. The significance of measuring endothelial [Ca\(^{2+}\)]_i in the context of an intact artery is twofold. First, dilation of the artery itself serves as a bioassay for the EDHF response. Because the chemical identity of EDHF is still undetermined (11), bioassay remains the best method for monitoring EDHF-dependent dilations. Second, an intact artery maintains significant myoendothelial (smooth muscle/endothelium) couplings that may be critical for ion flux or transfer of small-molecular-weight mediators that...
increases in endothelial \[\text{Ca}^{2+}\]. Or, alternatively, agonists that produce greater A23187 in the presence of L-NAME-Indo. Summary of percent diam-
priming with sub-EDHF-producing concentrations of UTP or Br-

may be critical for EDHF-dependent dilations (4, 8, 9, 22, 33). However, due in large part to the complexities of selectively measuring endothelial \[\text{Ca}^{2+}\] in an intact artery, endothelial \[\text{Ca}^{2+}\] in a pressurized artery has been measured in very few instances (8, 10, 14, 17, 18, 23, 28, 34).

In the present study, UTP (a P2Y2-selective, EDHF-

By using artery dilation under specific conditions as a bioassay for relaxing factor release, the effect of increased \[\text{Ca}^{2+}\] could be correlated with either NO- or EDHF-dependent responses. From the threshold data presented in Fig. 5, it is clear that EDHF-dependent relaxations have a higher requirement for endothelial \[\text{Ca}^{2+}\] than NO. The present studies are consistent with others regarding the range of \[\text{Ca}^{2+}\] required for expressed endothelial cell NOS (ecNOS) activity. For instance, in coronary endothelium, ecNOS activity increased steeply until reaching a plateau at around 300 nM \text{Ca}^{2+} (18). This reported \text{Ca}^{2+} sensitivity of ecNOS is highly congruent with the \text{Ca}^{2+} sensitivity of the NO-dependent dilation reported herein. Because this study is the first to report EDHF-dependent relaxations as a function of endothelial \[\text{Ca}^{2+}\], no direct comparison from the literature is possible. However, Nagao et al. (30) reported that a higher concentration of A23187 (a \text{Ca}^{2+} ionophore) was required to dilate rat mesenteric arteries when L-NAME-indomethacin was present. Although these studies were suggestive of a higher \[\text{Ca}^{2+}\], requirement for EDHF release, other possible explanations cannot be ruled out. For instance, without measuring \[\text{Ca}^{2+}\], it cannot be assumed that L-NAME-indomethacin does not affect the effectiveness of A23187 to increase \[\text{Ca}^{2+}\]. Furthermore, because A23187 was not administered selectively to the endothelium, potential effects of A23187 on smooth muscle could complicate interpretation. If, however, one assumes that the
increased concentration of A23187 resulted simply in a greater endothelial \([Ca^{2+}]_i\), then the study of Nagao et al. (30) and the present study qualitatively agree.

Potentiation of the endothelial \([Ca^{2+}]_i\) response of a non-EDHF-producing agonist such that the higher \([Ca^{2+}]_i\) threshold for EDHF-dependent dilation was achieved. The present studies found that the purely NO-producing agonist (2-MeS-ATP) (25, 37) failed to increase endothelial \([Ca^{2+}]_i\) to the apparent threshold for EDHF-dependent dilation (Figs. 1, 2, and 5). If the hypothesis that EDHF-dependent dilation has a higher \([Ca^{2+}]_i\) threshold is correct, then one might expect to elicit an EDHF response with 2-MeS-ATP provided the increase in \([Ca^{2+}]_i\) reached that critical threshold. To augment the 2-MeS-ATP \([Ca^{2+}]_i\) response, the resting \([Ca^{2+}]_i\) was increased (or primed) via receptor-dependent (UTP/P2Y2) and independent (Br-A23187) mechanisms just before 2-MeS-ATP addition. Importantly, both of these priming agents resulted in significant increases in endothelial \([Ca^{2+}]_i\), with minimal changes in diameter (Figs. 6 and 7). Subsequent addition of 2-MeS-ATP resulted in a further increase in \([Ca^{2+}]_i\), thus reaching the \([Ca^{2+}]_i\) threshold for EDHF-dependent dilation. On reaching the higher threshold, the arteries dilated through an EDHF-dependent mechanism as confirmed by the sensitivity to the selective Ca-sensitive K+ channel blocker charybotoxin (Fig. 7). In this artery preparation (25, 38), as well as in guinea pig middle cerebral artery (7), charybotoxin alone has been shown to be effective in completely blocking the EDHF-dependent dilation. Interestingly, a combination of charybotoxin and apamin is required to completely block the EDHF-dependent dilation in guinea pig basilar artery (32) as well as the many peripheral arteries studied (7).

In summary, these studies demonstrate that differences in the mechanism of vasodilatation resulting from stimulation of endothelial P2Y1 and P2Y2 purinoceptors result from differential endothelial \([Ca^{2+}]_i\) responses. In particular, stimulation of the EDHF-linked receptor system (P2Y2) results in a significantly greater increase in \([Ca^{2+}]_i\) than the purely NO-linked receptor system (P2Y1). Additionally, these studies provide the first direct evidence of separate endothelial \([Ca^{2+}]_i\) thresholds for NO- and EDHF-dependent dilations. These studies further suggest that the final endothelial \([Ca^{2+}]_i\) elicited by an agonist determines whether that agonist will produce an EDHF-dependent dilation.

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