Small- and intermediate-conductance Ca^{2+}-activated K^+ channels directly control agonist-evoked nitric oxide synthesis in human vascular endothelial cells

Jian-Zhong Sheng and Andrew P. Braun
Smooth Muscle Research Group, Department of Pharmacology and Therapeutics,
Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada

Sheng J-Z, Braun AP. Small- and intermediate-conductance Ca^{2+}-activated K^+ channels directly control agonist-evoked nitric oxide synthesis in human vascular endothelial cells. *Am J Physiol Cell Physiol* 293: C458–C467, 2007. First published April 25, 2007; doi:10.1152/ajpcell.00036.2007.—The contribution of small-conductance (SK_{Ca}) and intermediate-conductance Ca^{2+}-activated K^+ (IK_{Ca}) channels to the generation of nitric oxide (NO) by Ca^{2+}-mobilizing stimuli was investigated in human umbilical vein endothelial cells (HUVECs) by combining single-cell microfluorimetry with perforated patch-clamp recordings to monitor agonist-evoked NO synthesis, cytosolic Ca^{2+} transients, and membrane hyperpolarization in real time. ATP or histamine evoked reproducible elevations in NO synthesis and cytosolic Ca^{2+}, as judged by 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) and fluo-3 fluorescence, respectively, that were tightly associated with membrane hyperpolarizations. Whereas evoked NO synthesis was unaffected by either tetraethylammonium (10 mmol/l) or $BaCl_2$ (50 μmol/l) + ouabain (100 μmol/l), depleting intracellular Ca^{2+} stores by thapsigargin or removing external Ca^{2+} inhibited NO production, as did exposure to high (80 mmol/l) external KCl. Importantly, apamin and charybdotoxin (ChTx)/ triarylmethane (TRAM)-34, selective blockers SK_{Ca} and IK_{Ca} channels, respectively, abolished both stimulated NO synthesis and membrane hyperpolarization and decreased evoked Ca^{2+} transients. Apamin and TRAM-34 also inhibited an agonist-induced outwardly rectifying current characteristic of SK_{Ca} and IK_{Ca} channels. Under voltage-clamp control, we further observed that the magnitude of agonist-induced NO production varied directly with the degree of membrane hyperpolarization. Mechanistically, our data indicate that SK_{Ca} and IK_{Ca} channel-mediated hyperpolarization represents a critical early event in agonist-evoked NO production by regulating the influx of Ca^{2+} responsible for endothelial NO synthase activation. Moreover, it appears that the primary role of agonist-induced release of intracellular Ca^{2+} stores is to trigger the opening of both K_{Ca} channels along with Ca^{2+} entry channels at the plasma membrane. Finally, the observed inhibition of stimulated NO synthesis by apamin and ChTx/TRAM-34 demonstrates that SK_{Ca} and IK_{Ca} channels are essential for NO-mediated vasorelaxation.

calcium; endothelium; hyperpolarization; small-conductance calcium-activated potassium channel; intermediate-conductance calcium-activated potassium channel channel

THE VASCULAR ENDOTHELIUM exerts precise control over the contractile state of the vessel wall through the stimulated synthesis and release of both constrictor and dilatory substances. Stimulus-induced vasodilation appears to occur principally via cellular mechanisms that involve the synthesis and release of nitric oxide (NO) or EDRF (20) and prostacyclin along with a non-NO, non-prostanoid EDHF, whose identity remains a matter of debate. As recently discussed (5, 40), potential candidates for EDHF include K^+, cytochrome *P*-450 metabolites of arachidonic acid, hydrogen peroxide, and C-type naturetic peptide as well as electrical coupling between cells via myoendothelial gap junctions. As the EDHF-type response appears to display differing pharmacological sensitivity in a variety of vascular preparations and species, it is possible that more than one cellular mechanism may contribute to this phenomenon.

In endothelial cells (ECs), a number of vasodilatory agonists, such as acetylcholine, bradykinin, and ATP, elevate cytosolic free Ca^{2+} as a result of intracellular release and external entry and further evoke a hyperpolarization of membrane potential (45). Several studies (14, 36, 37) have further demonstrated that these events were closely associated with the release of EDRF from isolated ECs. Collectively, such key observations led to the hypothesis that membrane hyperpolarization contributed to the agonist-induced production of EDRF by increasing the electrical driving force for Ca^{2+} influx. Observations of agonist-evoked changes in cytosolic free Ca^{2+} and membrane hyperpolarization in ECs from a number of vascular beds and species have indicated that these events are widespread (45) and are thus likely to be of physiological importance in endothelial function.

Through the use of electrophysiological recordings, pharmacological agents, and the detection of mRNA species, it is now evident that ECs isolated from various sources express Ca^{2+}-activated K^+ (K_{Ca}) channels, which are capable of producing membrane hyperpolarization in response to elevations of cytosolic $[Ca^{2+}]$. The channel types commonly observed are small-conductance (SK_{Ca}) and intermediate-conductance K_{Ca} (IK_{Ca}) channels, whereas the expression of the large-conductance K_{Ca} (BK_{Ca}) channel appears to be more variable (1, 45). In isolated ECs, pharmacological inhibitors of SK_{Ca} channels [such as apamin (59)] and IK_{Ca} channels [i.e., charybdotoxin (ChTx) (59) and triarylmethane-34 (TRAM-34) (61)] have been shown to block agonist-induced K^+ currents (4, 8, 30, 39, 49), and, in intact arteries, such blockers have been further reported to inhibit selectively non-NO/non-prostanoid or EDHF-induced relaxations (5, 12, 15, 17, 25).

Although a number of reports have highlighted a role for SK_{Ca} and IK_{Ca} channels in the phenomenon of EDHF-mediated vasorelaxation (for reviews, see Refs. 5 and 40), few

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Address for reprint requests and other correspondence: A. P. Braun, 3330 Hospital Dr. NW, Calgary, AB, Canada T2N 4N1 (e-mail: abraun@ucalgary.ca).

studies have specifically examined the direct contribution of these same channels in agonist-evoked NO production. This has been largely due to the difficulty of simultaneously monitoring NO synthesis and functional responses (e.g., vasodilation, membrane hyperpolarization, and cytosolic Ca^{2+} transients) in a single preparation. Most recently, however, Stankevicius et al. (56) showed that blockade of SK$_{Ca}$ and IK$_{Ca}$ channels by apamin and ChTx, respectively, interfered with acetylcholine-induced NO production and vasorelaxation in rat mesenteric arteries, thereby establishing a functional role for SK$_{Ca}$ and IK$_{Ca}$ channels in NO-mediated vasodilation. In the present study, we sought to define mechanistically the functional role(s) of SK$_{Ca}$ and IK$_{Ca}$ channels in agonist-induced NO production. To do so, we utilized highly selective SK$_{Ca}$ and IK$_{Ca}$ channel inhibitors in combination with single-cell microfluorimetry and patch-clamp electrophysiology to examine directly agonist-induced NO synthesis, changes in cytosolic free [Ca^{2+}], and membrane hyperpolarizations in single human vascular ECs. Using this strategy, we acquired well-resolved temporal and spatial data that reveal novel insights into the cellular mechanism underlying stimulated NO synthesis by Ca^{2+}-mobilizing agonists and define the critical role of SK$_{Ca}$ and IK$_{Ca}$ channels in this process.

MATERIALS AND METHODS

Cell culture and fluorescence measurements. The EC line EA.hy926 (16), derived from the human umbilical vein [human umbilical vein ECs (HUVECs)], was cultured and loaded with the membrane-permeable forms of the fluorescent dyes 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) or fluo-3, as recently described (54). In our isolated EC preparations, agonist-stimulated increases in DAF-FM fluorescence were abolished in the presence of the NO synthase (NOS) inhibitor *N*-nitro-L-arginine methyl ester (0.1 mmol/l), consistent with the reported specificity of this fluorescent reporter (31). Fluorescence measurements were performed in a ~0.3-ml bath chamber mounted on the stage of a Nikon TE300 inverted microscope equipped with a 75-W xenon arc lamp and SFX-1 microfluorimeter. Both DAF-FM and fluo-3 fluorescence signals were measured using excitation and emission band-pass filters centered on 488 and 520 nm, respectively; data were acquired using AxoScope software and analyzed with pCLAMP 7 and SigmaPlot software suites. As the fluorescent intensity of the triazole- or NO-bound form of DAF-FM originating from a single cell was typically quite modest, the strong excitation light needed to observe reliable fluorescent signals often resulted in some photobleaching of the NO-modified form of DAF-FM during continuous cell illumination. Exposure of the cell to intermittent illumination through the use of a timer-driven, optic shutter reduced but did not completely eliminate the photobleaching of NO-modified DAF-FM. A manually controlled diaphragm was used to restrict the region of light collection to the cell of interest.

Electrophysiology. Voltage- and current-clamp measurements were performed using perforated patch-clamp methodology in combination with an Axopatch 200B amplifier, Digidata 1200B analog-to-digital interface, and Clampex 7 software. Electrical signals recorded under current clamp and voltage clamp were typically sampled at 1 Hz and 5 KHz, respectively. Borosilicate glass micropipettes (2–4 MΩ tip resistance) were first briefly dipped into standard filling solution [final concentration (in mmol/l) 100 K-aspartate, 30 KCl, 1 MgCl$_2$, 2 Na$_2$-ATP, and 10 HEPES (pH 7.2) with 1 mol/l KOH] and then back filled with the same filling solution containing nystatin (50 mg/l final concentration). The bath solution for both fluorescence and electrophysiological recordings contained (in mmol/l) 135 NaCl, 5 KCl, 1 MgCl$_2$, 1.5 CaCl$_2$, and 10 HEPES (pH 7.4) with 1 mol/l NaOH. The

high-KCl bath solution was prepared by an equimolar substitution of NaCl with KCl; for the Ca^{2+}-free solution, CaCl$_2$ was omitted and replaced by 2 mM EGTA. Cells in the bath chamber were constantly superfused at ~1 ml/min, and solution changes were performed by gravity flow from a series of elevated solution reservoirs using manually controlled solenoid valves. All fluorescence and electrophysiological recordings were performed at 35°C.

Reagents. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were of ACS grade or higher. DAF-FM diacetate and Fluo-3 AM were obtained from Molecular Probes (Eugene, OR). TRAM-34 was kindly provided by Dr. Heike Wulff (UC Davis).

RESULTS

In HUVECs loaded with the NO-sensitive fluorescent dye DAF-FM diacetate (31), histamine, or the purinergic agonist ATP evoked reproducible increases in cellular fluorescence under control conditions (Fig. 1*A*); however, agonist-evoked increases in fluorescence were inhibited in the presence of apamin and ChTx, blockers of SK$_{Ca}$ and IK$_{Ca}$ channels (59), respectively (Fig. 1, *B* and *C*). Brief exposure of dye-loaded cells to the direct NO donor sodium nitroprusside (SNP) at the end of each experiment demonstrated that apamin and ChTx did not interfere with DAF-FM activation and fluorescence in loaded cells. Exposure of cells to either apamin or ChTx alone produced only partial (20–40%) inhibition of agonist-evoked increases in DAF-FM fluorescence (data not shown). These observations thus demonstrate that both apamin and ChTx act directly on the endothelium to block agonist-evoked NO production. In contrast to the observed inhibitory effects of apamin + ChTx, agonist-induced NO production was unaffected by a bath application of tetraethylammonium (TEA; 10 mmol/l), which would be expected to block BK$_{Ca}$ channels along with some types of voltage-gated K$^+$ channels (i.e., Kv1) (Fig. 2*A*) (23, 44). We also observed only a very modest (~10%) inhibition by TEA of agonist-evoked Ca^{2+} transients in single fluo-3-loaded HUVECs (see Supplemental Fig. 1).[1] Collectively, these data are consistent with the rather low-affinity block by external TEA of both native and recombinant IK$_{Ca}$ channels (IC$_{50}$ value: 8–10 mmol/l) (2, 27, 35) along with the SK$_{Ca}$2 and SK$_{Ca}$3 channel isoforms detected in the vascular endothelium (IC$_{50}$ values: ~3 and ~9 mmol/l, respectively) (4, 43). Similar to TEA, we also observed that evoked NO production was unaltered in the presence of 50 μmol/l BaCl$_2$ and 100 μmol/l ouabain, which block inwardly rectifying K$^+$ (Kir) channels (23) and Na$^+$-K$^+$-ATPase (29), respectively (Fig. 2*B*). Taken together, these data suggest that BK$_{Ca}$, Kv1, and Kir channels, along with Na$^+$-K$^+$-ATPase, do not functionally contribute to agonist-induced NO production in single HUVECs.

To characterize the Ca^{2+} dependence of agonist-evoked NO production, intracellular endoplasmic reticulum (ER) Ca^{2+} stores were disrupted by exposure to the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump inhibitor thapsigargin (TG). Following initial NO responses to ATP and histamine, rapid application of 0.5 μM TG abolished further increases in NO upon reexposure to either agonist (Fig. 3, *A* and *C*). However, brief application of SNP to TG-treated cells still produced large fluorescence signals, indicating that TG did not

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[1] Supplemental material for this article is available online at the *American Journal of Physiology-Cell Physiology* website.
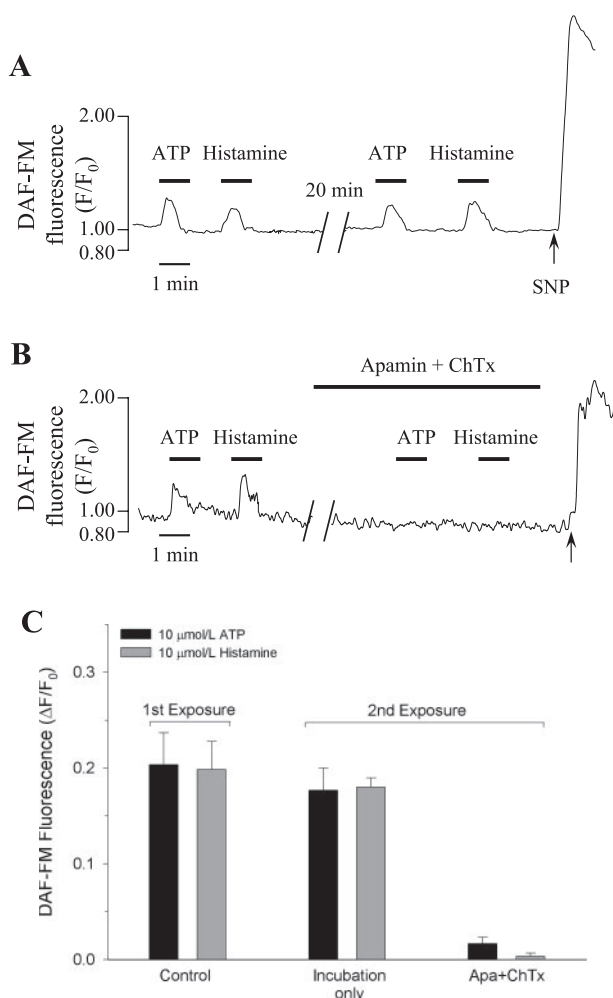
A

B

C

Fig. 1. Agonist-stimulated nitric oxide (NO) production is inhibited by apamin (Apa) and charybdotoxin (ChTx). *A*: fluorescence tracing recorded from a single human umbilical vein endothelial cell (HUVEC) loaded with 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) dye. Exposure of the cell to either ATP (10 μmol/l) or histamine (10 μmol/l) is indicated by the horizontal bars above the tracing. The break in the recording indicates the ~20-min control incubation period prior to reapplication of ATP and histamine. In *B*, a single HUVEC was exposed to 1 μmol/l Apa and 0.1 μmol/l ChTx for ~20 min prior to a second application of ATP and histamine. The addition of sodium nitroprusside (SNP; 10 μmol/l) at the end of each experiment is denoted by the arrow beneath the fluorescence tracings. Changes in cellular DAF-FM fluorescence (ΔF/F₀) in response to the first (control) and second agonist applications, in either the absence (incubation only) or presence of Apa and ChTx, are shown in *C*. Data are presented as means ± SE of fluorescence tracings recorded from 4–6 individual cells for each agonist under each condition.

external solution containing 1.5 mmol/l Ca^{2+} completely restored agonist-evoked NO synthesis (Fig. 3, *B* and *C*). This latter finding is thus consistent with previous results showing that stimulated EDRF/NO production is inhibited in the absence of external Ca^{2+} (13, 26, 32–34, 37).

While the above data indicate that intra- and extracellular Ca^{2+}, along with SK_{Ca} and IK_{Ca} channels, play critical roles in agonist-stimulated NO synthesis, exactly how these components are interrelated temporally and mechanistically is unclear. To establish such relations, we carried out dual recordings of membrane potential and either cytosolic free Ca^{2+} or NO production using microfluorimetry in single patch-clamped HUVECs. In fluo-3-loaded cells, ATP and histamine evoked rapid elevations in cytosolic free Ca^{2+} and membrane hyperpolarizations that reversed upon agonist washout (Fig. 4). Following exposure of the same cell to apamin and the highly
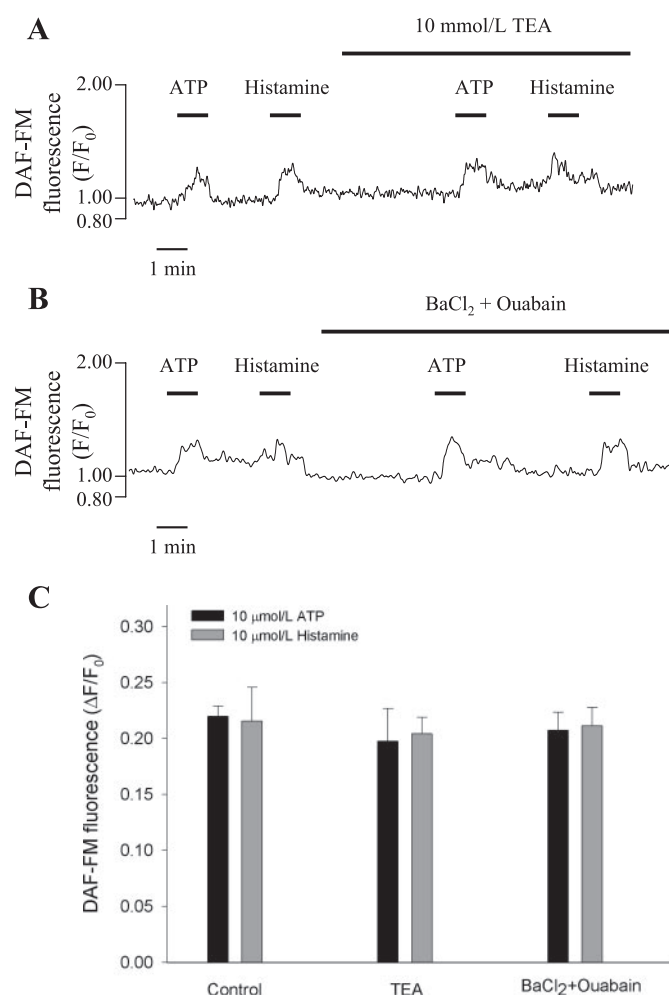
A

B

C

Fig. 2. Exposure to either tetraethylammonium (TEA) or $BaCl_2$ and ouabain does not prevent agonist-induced NO production. Stimulation of single DAF-FM-loaded HUVECs by either ATP (10 μM) or histamine (10 μM) produced characteristic increases in cellular fluorescence. Following the addition of either 10 mM TEA (*A*) or 50 μM $BaCl_2$ and 100 μM ouabain (*B*), the same cell was reexposed to first ATP and then histamine, as indicated by the horizontal bars. The histogram in *C* quantifies the agonist-induced DAF-FM fluorescence signals (ΔF/F₀) for both ATP and histamine in the absence and presence of either TEA or $BaCl_2$ and ouabain. Data are presented as means ± SE of fluorescence tracings from 4 individual cells for each agonist under each condition.
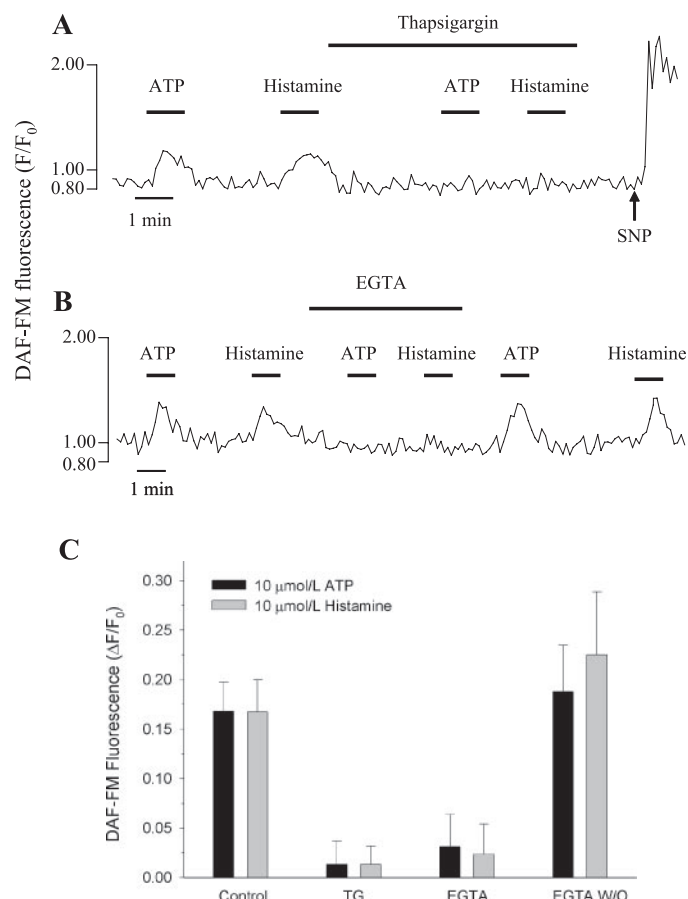
compromise the NO sensitivity of DAF-FM in the intact cell. As cells were typically exposed to TG immediately following agonist washout, intracellular Ca^{2+} stores may not have been sufficiently full to support a TG-evoked NO response. In separate experiments, however, we observed that an application of 0.5 μM TG alone produced a significant increase in DAF-FM fluorescence (data not shown), as recently reported (13). The contribution of external Ca^{2+} to agonist-evoked NO production was examined in DAF-FM-loaded cells by brief exposure to a bath solution containing 0 Ca^{2+}-2 mM EGTA. Rapid removal of external Ca^{2+} abolished ATP- and histamine-induced NO production' however, reintroduction of the

Fig. 3. Interfering with intracellular Ca^{2+} release or preventing external Ca^{2+} entry blocks agonist-induced NO production. *A*: fluorescence tracing recorded from a single DAF-FM-loaded HUVEC exposed to either 10 μmol/l ATP or 10 μmol/l histamine, as indicated by the horizontal bars above the tracing. Continuous application of 0.5 μmol/l thapsigargin (TG) is shown by the long horizontal bar. The arrow at the end of the tracing indicates the addition of the NO donor SNP (10 μmol/l). *B*: changes in DAF-FM fluorescence in a single dye-loaded HUVEC in response to either ATP or histamine. Exposure of the cell to an external saline solution containing 2 mmol/l EGTA and no added Ca^{2+} is indicated by the horizontal bar. Following washout of the EGTA-containing solution and return to physiological saline solution, the same cell was reexposed to both ATP and histamine. The effects of TG exposure or external Ca^{2+} removal on agonist-evoked DAF-FM fluorescence signals (ΔF/F$_0$) are quantified in the histogram shown in *C*. Data are presented as means ± SE for each agonist; control responses to either agonist were recorded from 8–9 individual cells, whereas agonist-evoked responses in the presence of either TG or EGTA were taken from 4–5 cells under each condition.

due to Ca^{2+}-dependent Cl$^-$ channel activity, as previously reported (48).

A major benefit of carrying out such dual recordings of agonist-evoked membrane hyperpolarizations together with either Ca^{2+} or NO signals is that we were able to determine the precise temporal pattern among these three cellular events, which has not been previously described. In response to either ATP or histamine, increases in DAF-FM fluorescence lagged behind the onset of membrane hyperpolarization by 8–12 s (see Fig. 6*A*); in the case of TG-induced NO production, this delay was typically only 2–3 s (Table 1). In contrast, agonist-evoked increases in fluo-3 fluorescence typically preceded membrane hyperpolarization by 2–5 s (Fig. 6*B*). In agreement
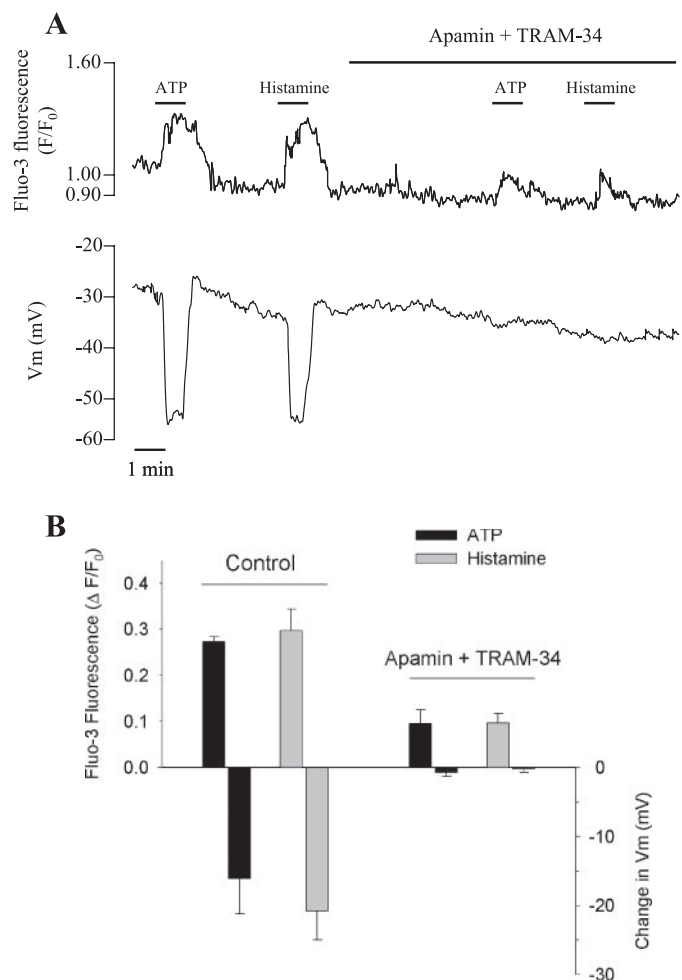

Fig. 4. Blockers of small-conductance (SK$_{Ca}$) and intermediate-conductance Ca^{2+}-activated K$^+$ (IK$_{Ca}$) channels interfere with agonist-stimulated increases in cytosolic free Ca^{2+} and transient membrane hyperpolarizations. Single HUVECs loaded with the Ca^{2+}-sensitive dye fluo-3 were held under current clamp using a nystatin-permeabilized patch-clamp method. *A*: simultaneous and continuous recordings of fluo-3 fluorescence (*top* trace) and membrane potential (V_m; *bottom* trace) from a single cell. Brief exposure of the cell to either 10 μmol/l ATP or 10 μmol/l histamine is indicated by the bars above the fluorescence tracing. Continuous exposure of the cell to 1 μmol/l Apa and 1 μmol/l triarylmethane (TRAM)-34 is shown by the long horizontal bar. Agonist-stimulated changes in both fluo-3 fluorescence (ΔF/F$_0$) and V_m (ΔV_m) in the absence and presence of Apa and TRAM-34 are quantified by the *top* and *bottom* axes, respectively, of the histogram shown in *B*. Data are presented as means ± SE of simultaneous recordings from 5 individual cells for each agonist.

selective IK$_{Ca}$ channel blocker TRAM-34 (61), agonist-evoked membrane hyperpolarizations were abolished and changes in fluo-3 fluorescence were significantly reduced, as shown in Fig. 4*B*. This latter finding is thus consistent with earlier studies demonstrating that agonist-evoked Ca^{2+} transients in isolated vascular ECs are decreased with membrane depolarization (9, 33, 51). In parallel experiments, ATP- and histamine-evoked increases in DAF-FM fluorescence were also found to be closely associated with membrane hyperpolarizations (Fig. 5). The addition of apamin and TRAM-34 to the same DAF-FM-loaded cell inhibited both of these responses upon reexposure to ATP and histamine. Interestingly, transient membrane depolarizations were typically observed upon agonist withdrawal (Figs. 4*A* and 5*A*), which may be
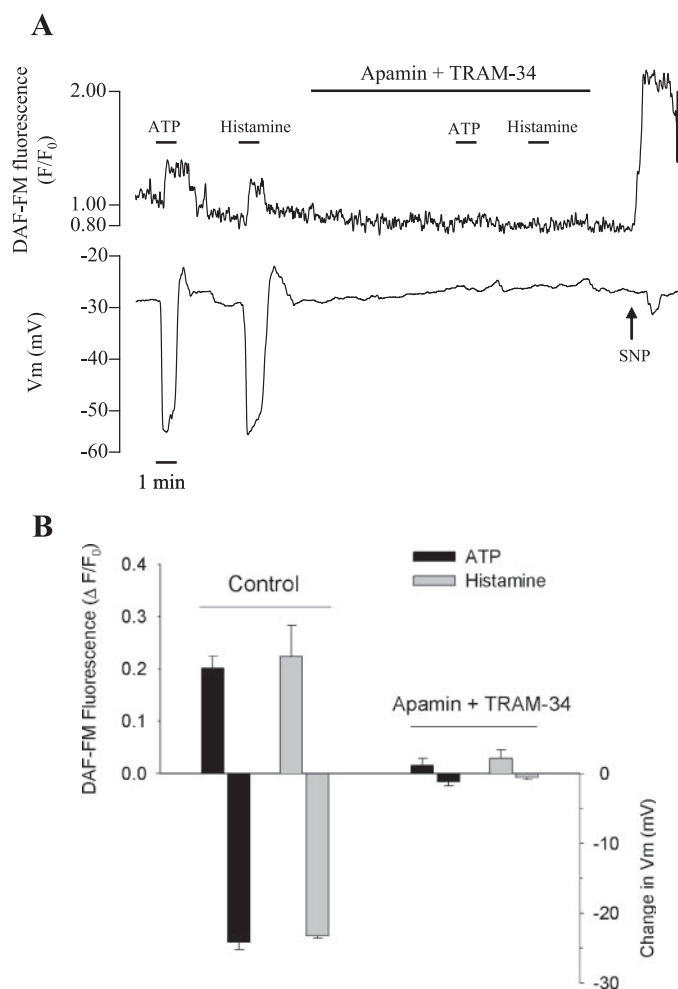
A

B

Fig. 5. Blockade of SK_{Ca} and IK_{Ca} channels prevents agonist-stimulated increases in NO production and transient membrane hyperpolarizations. Single HUVECs loaded with DAF-FM dye were held under current clamp using a nystatin-permeabilized patch clamp method. *A*: simultaneous and continuous recordings of DAF-FM fluorescence (*top* trace) and V_m (*bottom* trace) from a single cell. Brief exposure of the cell to either ATP (10 μmol/l) or histamine (10 μmol/l) is indicated by the bars above the upper tracing. Continuous exposure of the cell to 1 μmol/L Apa and 1 μmol/l TRAM-34 is shown by the long horizontal bar. Exposure of the cell to the NO donor SNP (10 μmol/l) at the end of the experiment is shown by the arrow beneath the V_m trace. Agonist-stimulated changes in both cellular DAF-FM fluorescence ($\Delta F/F_0$) and ΔV_m in the absence and presence of Apa and TRAM-34 are quantified in *top* and *bottom* axes, respectively, of the histogram shown in *B*. Data are presented as means $\pm$ SE of recordings taken from 5 individual cells for each agonist.

with our observations, Li et al. (62) have shown using simultaneous fura-2 and DAF-2 fluorescence measurements that the onset of agonist-evoked increases in cytosolic Ca^{2+} preceded NO production in the coronary artery endothelium. These data sets thus define a distinct temporal pattern in which an agonist-evoked increase in cytosolic $[Ca^{2+}]$ is followed by membrane hyperpolarization, which is then followed by NO synthesis.

To confirm that apamin and TRAM-34 were indeed acting to block endothelial K_{Ca} channels, whole cell membrane currents were recorded from single HUVECs stimulated by ATP in the absence and presence of apamin and TRAM-34. Under basal conditions, single HUVECs displayed a modest outwardly rectifying macroscopic current that typically reversed near

-40 mV (Fig. 7). ATP increased the magnitude of the outward current and shifted the reversal potential to values near -70 mV, consistent with the activation of membrane K^+ channels. This ATP-stimulated outward current was largely inhibited in the presence of apamin and TRAM-34 (Fig. 7, *inset*).

As the above data pointed to a critical role for membrane hyperpolarization in agonist-stimulated NO production, we exposed single DAF-FM-loaded HUVECs to a high KCl-containing bath solution to "clamp" the membrane voltage near 0 mV and prevent agonist-induced hyperpolarization. As expected, brief exposure to 80 mmol/l external K^+ depolarized the endothelial membrane potential and abolished agonist-stimulated NO synthesis (Fig. 8). Upon washout and return to 5 mmol/l external K^+, membrane potential recovered to the control level, and both ATP and histamine induced typical membrane hyperpolarizations that were associated with increases in DAF-FM fluorescence. If we further hypothesize
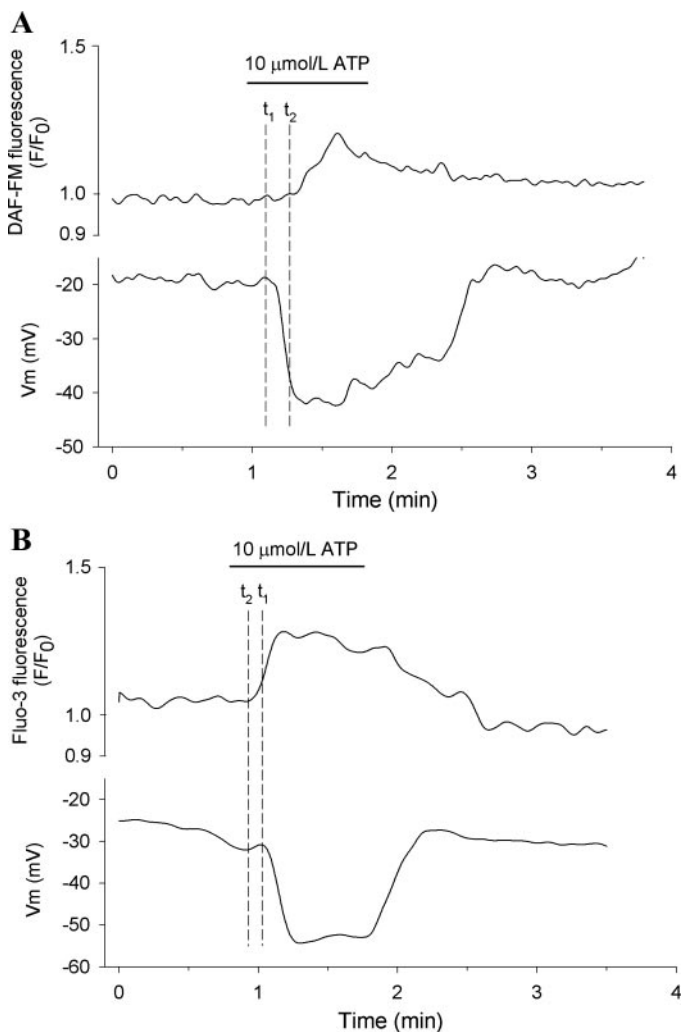
A

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Fig. 6. Kinetic relations between agonist-stimulated changes in V_m and cytosolic free Ca^{2+} and NO production. Simultaneous recordings of V_m and either DAF-FM or fluo-3 fluorescence from a single HUVEC are shown in *A* and *B*, respectively. Temporal differences between the onsets of agonist-evoked hyperpolarization (denoted by the vertical dashed line marked t_1) and stimulated increases in either the DAF-FM or fluo-3 fluorescence signals (denoted by the vertical dashed line marked t_2) were quantified and are shown in Table 1.

Table 1. *Temporal relations between the onset of stimulus-driven membrane hyperpolarization and increases in either DAF-FM or fluo-3 cellular fluorescence induced by ATP, histamine, or thapsigargin*

Stimulus	n	T1 − T2, s
DAF-FM fluorescence		
ATP	8	−12.5±6.2*
Histamine	9	−8.5±4.6*
Thapsigargin	3	−2.3±0.3*
Fluo-3 fluorescence		
ATP	5	2.1±3.6
Histamine	5	4.5±3.3*

Calculated differences are expressed as means ± SE; *n*, numbers of cells used under each condition. T1, onset of stimulus-driven membrane hyperpolarization; T2, onset of increases in either 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) or fluo-3 cellular fluorescence induced by ATP (10 μmol/l), histamine (10 μmol/l), or thapsigargin (0.5 μmol/l). Time differences between the starts of the hyperpolarizing event and the fluorescence signal were calculated as T1 − T2 (refer to Fig. 6) and are expressed in seconds. Negative values indicate that the hyperpolarizing response preceded the associated fluorescence signal, whereas a positive value indicates membrane hyperpolarization followed the fluorescence increase. *The observed difference was statistically different than zero using a paired Student's *t*-test ($P < 0.05$).

that NO production evoked by Ca^{2+}-mobilizing agonists is absolutely dependent on membrane hyperpolarization, it should then be possible to regulate agonist-evoked NO production in real time by directly controlling endothelial membrane potential. To test this possibility, single DAF-FM-loaded HUVECs were voltage clamped at potentials ranging from 0 to −80 mV, and ATP was then briefly applied at each potential. In agreement with the above hypothesis, the magnitude of agonist-evoked NO production was observed to increase at increasingly negative membrane potentials, reaching a maximum between −60 and −80 mV (Fig. 9). These data thus provide the first direct real-time demonstration that evoked
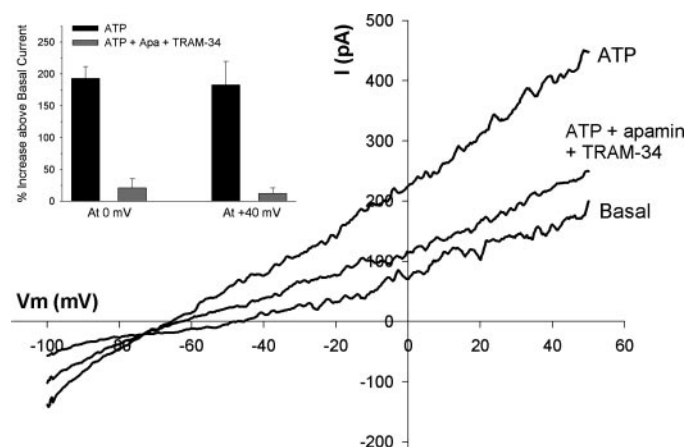

Fig. 7. Sensitivity of ATP-activated membrane currents (*I*) to Apa and TRAM-34. Whole cell currents were recorded from single voltage-clamped HUVECs in response to a 50-ms voltage ramp (−100 to +50 mV) using a nystatin-perforated patch-clamp technique. Membrane currents were first recorded under basal conditions and then in response to 10 μM ATP, followed by 10 μM ATP + 1 μM TRAM-34 and 1 μM Apa. The mean percentage increases (±SE) in current magnitude above baseline evoked by ATP in the absence and presence of Apa and TRAM-34 are shown in the *inset*. Current tracings represent the averages of 3–4 single sweeps under each condition and are representative of 5 similar experiments.
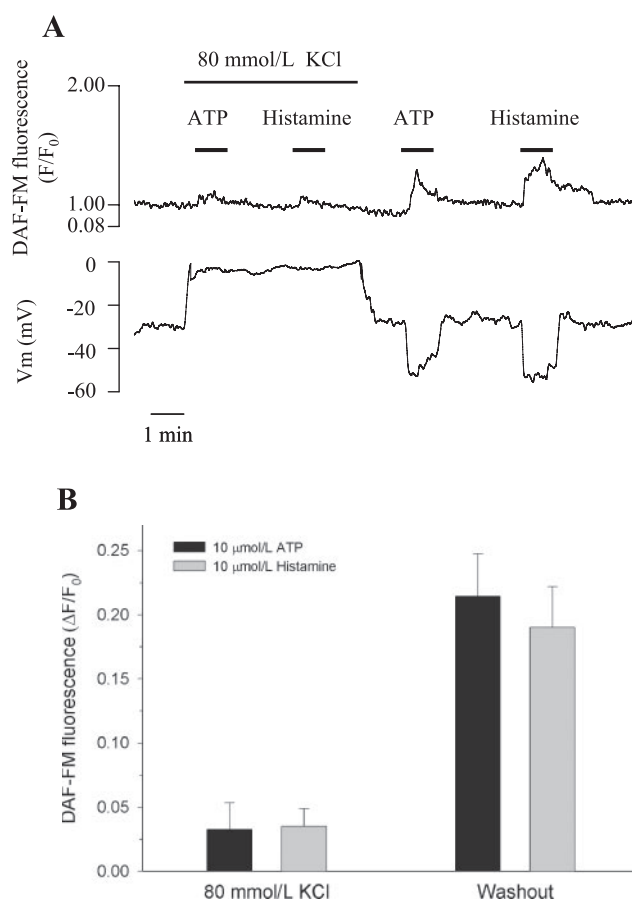

Fig. 8. Elevation of extracellular KCl prevents agonist-induced NO production. Single HUVECs loaded with DAF-FM dye were held under current clamp as described in Fig. 7. *A*: simultaneous and continuous recordings of DAF-FM fluorescence (*top* trace) and V_m (*bottom* trace) from a single cell. ATP or histamine was briefly applied to the cell, as indicated by the horizontal bars, in either the presence or absence of 80 mmol/l external KCl. Agonist-induced elevations in DAF-FM fluorescence ($\Delta F/F_0$) under both normal and elevated KCl conditions are quantified in *B*. Mean data (±SE) represent recordings from 4 individual cells for each agonist.

membrane hyperpolarization acts as a critical, rate-limiting factor for stimulated NO production by Ca^{2+}-mobilizing agonists in single vascular ECs.

DISCUSSION

The release of NO from vascular ECs in response to vasorelaxant hormones, such as acetylcholine and histamine, is known to be affected by changes in intracellular and external Ca^{2+} levels along with membrane potential. To date, however, the precise mechanistic and temporal patterns linking these three cellular parameters have not been rigorously established by means of direct experimental measurements. In the present study, we utilized nystatin-perforated patch-clamp recordings in combination with single-cell microfluorimetry to monitor directly, in real time, agonist-stimulated membrane hyperpolarization, NO synthesis, and cytosolic Ca^{2+} transients in single HUVECs. In addition, we specifically addressed the contributions of endothelial SK$_{Ca}$ and IK$_{Ca}$ channels to these events by using the selective inhibitors apamin and ChTx/TRAM-34, respectively (59, 61). In doing so, this study provides novel experimental insights that define the mechanistic
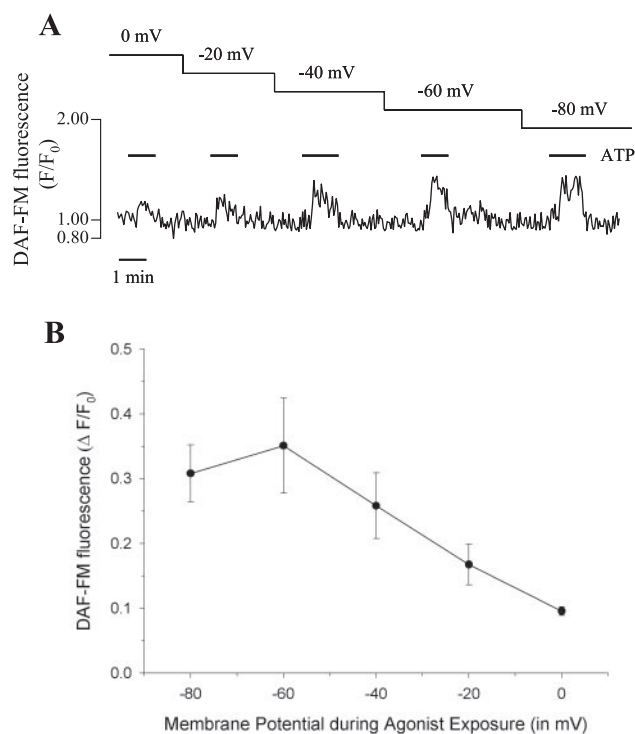
Fig. 9. The magnitude of agonist-induced NO synthesis is directly dependent on V_m. Single HUVECs loaded with DAF-FM dye were voltage clamped via a nystatin-perforated patch and held at V_ms ranging from 0 to -80 mV, as denoted by the step-wise series of horizontal bars (*A*). At each test potential, the voltage-clamped cell was briefly exposed to 10 µmol/l ATP as indicated by the horizontal bars. The data tracing beneath the stimulation protocol represent the continuous DAF-FM fluorescence signal simultaneously recorded from the single voltage-clamped cell at each V_m and in response to each application of ATP. *B*: quantification of the ATP-induced elevation in DAF-FM fluorescence ($\Delta F/F_0$) observed at each V_m. Results are expressed as means ± SE of fluorescence signals recorded from 5 voltage-clamped cells.

role of K_{Ca} channels in hormone-stimulated NO production at the level of a single EC.

Our fundamental observation that acute, agonist-evoked NO production is blocked by a combination of apamin and either ChTx or TRAM-34 in DAF-FM-loaded HUVECs (Figs. 1 and 5) reveals two novel and important insights. First, it implicates a critical role for endothelial SK_{Ca} and IK_{Ca} channels in evoked NO synthesis, which is consistent with functional data showing that apamin + ChTx interferes with agonist-stimulated, endothelium-dependent vasorelaxation (11, 56). Second, it strongly suggests that manipulations designed to decrease NO production by disrupting intracellular Ca^{2+} levels will interfere directly with the activation of SK_{Ca} and IK_{Ca} channels, which would be expected to mimic the toxin-induced inhibition of NO production shown in Fig. 1. In line with this latter point, we observed that prior depletion of ER stores by TG inhibited agonist-evoked NO production, whereas transient removal of external Ca^{2+} produced a similar effect (Fig. 2), as reported earlier by investigators using indirect measurements of EDRF release (33, 37). Although endothelial NOS (eNOS) itself may be sensitive to manipulations of intracellular free Ca^{2+}, these observations are also consistent with earlier data showing that both intracellular release and external Ca^{2+} entry strongly influence the magnitude and/or duration of evoked Ca^{2+} transients (45) along with the duration of membrane hyperpolar-

ization (3, 10) in stimulated ECs. In an elegant study using fluorescent probes to report simultaneous changes in cytosolic free Ca^{2+} and NO production, Isshiki et al. (26) demonstrated that agonist-evoked NO synthesis is strongly dependent on external Ca^{2+} entry and largely insensitive to Ca^{2+} released from intracellular stores in bovine aortic ECs. Taken together, these data highlight and contrast the functional roles of both intracellular Ca^{2+} release and Ca^{2+} entry for NO production evoked by Ca^{2+}-mobilizing agonists.

While the above findings imply critical roles for intra- and extracellular Ca^{2+} and SK_{Ca} and IK_{Ca} channels in stimulated NO synthesis, they do not establish a precise temporal relation among these three parameters. To define such a pattern, we performed simultaneous recordings of membrane potential and either fluo-3 or DAF-FM fluorescence in single HUVECs (Figs. 4 and 5). The results of this approach demonstrated that agonist-evoked increases in both cytosolic Ca^{2+} levels and NO synthesis were tightly associated with transient membrane hyperpolarizations, such that membrane hyperpolarization closely followed cytosolic Ca^{2+} elevations but preceded increases in agonist-evoked NO production (Fig. 6 and Table 1). This temporal pattern thus establishes membrane hyperpolarization as an essential intermediate step in stimulated NO production. Importantly, apamin and the highly selective IK_{Ca} blocker TRAM-34 (61) abolished both agonist-evoked membrane hyperpolarization and increases in NO synthesis and significantly reduced elevations in cytosolic free Ca^{2+}. The modest Ca^{2+} transient remaining in the presence of apamin and TRAM-34 likely reflects the combination of Ca^{2+} release from intracellular stores and the residual entry of external Ca^{2+}. Our observation that apamin and TRAM-34 inhibited an agonist-evoked, outwardly rectifying current in single HUVECs (Fig. 7) is further consistent with the activation of SK_{Ca} and IK_{Ca} channels in ECs and agrees with the reported presence of these channels in the vascular endothelium (4, 8, 22, 39, 45, 49, 50).

If agonist-evoked membrane hyperpolarization is truly an essential upstream event regulating NO synthesis, then preventing hyperpolarization by means other than blockade of SK_{Ca} and IK_{Ca} channels would also be expected to interfere with NO synthesis. As shown in Fig. 8, "clamping" membrane potential to ~0 mV by a brief exposure to high external KCl blocked both agonist-induced membrane hyperpolarization and NO synthesis. This finding is thus consistent with the above prediction and provides a direct link between membrane potential and NO synthesis in a single EC. High external KCl has been reported previously to reduce EDRF release from populations of agonist-stimulated ECs (36), whereas Stankevicius et al. (56) recently demonstrated a similar inhibition of stimulated NO production by 80 mmol/l KCl in the rat mesenteric artery. Based on such data, we hypothesized that endothelial membrane hyperpolarization represents a critical, rate-limiting process regulating NO synthesis by Ca^{2+}-mobilizing stimuli. By using voltage clamp to accurately control endothelial membrane potential in DAF-FM-loaded HUVECs, we observed that the magnitude of agonist-stimulated NO synthesis increased in a linear manner with the degree of membrane hyperpolarization between 0 and -80 mV (Fig. 9). This singular result thus establishes a direct quantitative relation between membrane potential and stimulated NO synthesis at the level of a single EC. In related experiments, it has already been reported that the amplitudes of agonist-evoked Ca^{2+} transients in isolated ECs

are lower at more depolarized membrane potentials (6, 9, 33, 51, 60) and that such changes in Ca^{2+} transients appear to be linearly related to membrane voltage over the range of -80 to $+40$ mV (28, 53).

Mechanistically, our data suggest that NO synthesis evoked by Ca^{2+}-mobilizing stimuli can be described by a pathway of discrete cellular events that feed forward in a positive manner (Fig. 10). While such a model incorporates many of the key findings reported in previous studies, it makes two important distinctions critical to stimulated NO production. First, agonist-mediated release of intracellular Ca^{2+} stores triggers not only the Ca^{2+}-dependent activation of SK$_{Ca}$ and IK$_{Ca}$ channels (38, 52) but also initiates the entry of external Ca^{2+}, which is primarily responsible for eNOS activation. Second, stimulus-evoked membrane hyperpolarization, mainly via SK$_{Ca}$ and IK$_{Ca}$ channels and possibly BK$_{Ca}$ channels (45), is absolutely required for stimulated NO synthesis, likely due to its influence on external Ca^{2+} entry. It is noteworthy, however, that in intact arterial preparations, agonist-evoked elevations in EC cytosolic Ca^{2+} are reported to be unaltered in the presence of apamin and ChTx/TRAM-34 (21, 41, 56). Although the reason(s) behind such observations is unclear at present, it is possible that there may be unrecognized differences in the dynamics and/or detection of intracellular Ca^{2+} transients in isolated ECs versus cells present in an intact endothelial layer. As shown by our data (Fig. 3) and earlier results (26, 32, 37), stimulated Ca^{2+} entry, rather than agonist-induced Ca^{2+} release from ER stores, appears to be principally responsible for eNOS activation and further influences the duration of agonist-induced membrane hyperpolarization (3, 42). Our model thus distinguishes ER store Ca^{2+} release as the "trigger" that initiates both the opening of K$_{Ca}$ channels and the opening of store-operated Ca^{2+} entry channels [i.e., transient receptor potential (TRP) channels] in the plasma membrane. The ensuing membrane hyperpolarization acts to support Ca^{2+} entry, which
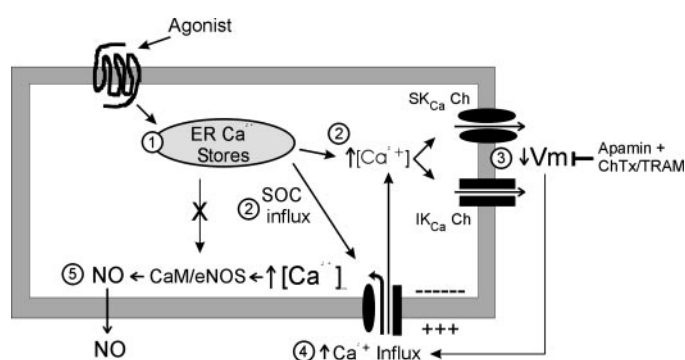
would be influenced by external [Ca^{2+}] and the magnitude of the hyperpolarizing event. Based on the above observations, we can further suggest that these two sources of mobilized Ca^{2+} carry out distinct and largely noninterchangeable roles in the multistep process of agonist-evoked NO synthesis. Differences in the spatial distribution of Ca^{2+}-sensitive molecules may further contribute to the greater efficiency of eNOS activation by Ca^{2+} entry compared with release from intracellular stores; for example, both eNOS and TRP channels are reported to be colocalized in membrane caveolae (19, 46).

Finally, can we rationalize why membrane potential has such a dominant influence on Ca^{2+}-dependent eNOS activation? Although membrane hyperpolarization would increase the electrical driving force for agonist-induced Ca^{2+} entry (6, 7, 9, 24, 36, 51), the degree of change in driving force (i.e., $+160$ mV under rest to $+180$ mV; see Fig. 4) based on Nernst-type calculations would be modest. More recently, however, the possibility that Ca^{2+}-permeable TRP channels themselves may display voltage-dependent gating has been discussed (47). For the inwardly rectifying, Ca^{2+}-permeable TRP channels in the endothelium (46), such voltage sensitivity would mean that TRP channel open probability and Ca^{2+} entry would be exponentially related to changes in membrane voltage. Current-voltage relations observed for agonist-evoked inward current through some Ca^{2+}-permeable TRP channels (57) and native endothelial store-operated Ca^{2+} channels (18) appear consistent with such a possibility. As a result of such an exponential relationship, a modest hyperpolarization of -20 mV could produce a significantly larger increase in Ca^{2+} influx compared with a strictly linear relation predicted by a modest increase in the electrical driving force for external Ca^{2+} alone. Furthermore, the position along the voltage axis of the rectifying current-voltage relation for stimulated Ca^{2+} entry relative to the cell's resting potential may also influence the magnitude of hyperpolarization-induced Ca^{2+} influx.

In summary, our data indicate that the activation of SK$_{Ca}$ and IK$_{Ca}$ channels, leading to membrane hyperpolarization, represents an essential early event in the cellular pathway underlying agonist-stimulated NO production. Consistent with this conclusion, genetic knockout of either the endothelial SK$_{Ca}$3 or IK$_{Ca}$ channels in mice gives rise to systemic hypertension and reduces hormone-induced, endothelium-dependent vasorelaxation (55, 58). Very recent observations demonstrating that apamin- and ChTx-sensitive K$_{Ca}$ channels regulate acetylcholine-evoked NO production in the intact rat superior mesenteric artery (56) are further consistent with our data and strongly suggest that the mechanistic insights described in our study are relevant to the native vascular endothelium. Finally, the inhibition of agonist-evoked NO synthesis by apamin and ChTx/TRAM-34 observed in this study and by Stankevicius et al. (56) demonstrate a critical functional role for SK$_{Ca}$ and IK$_{Ca}$ channels in the cellular mechanisms underlying hormone-induced, NO-dependent vasorelaxation.

Fig. 10. Model summarizing the relations between agonist-induced changes in cytosolic free Ca^{2+}, V_m, and NO production in a single HUVEC. Hormonal activation of a G protein-coupled receptor causes the generation of inositol (1,4,5)-trisphosphate and the release of intracellular Ca^{2+} stores [endoplasmic reticulum (ER)] (*step 1*). Store release elevates cytosolic [Ca^{2+}] (*step 2*) and further triggers store-operated channel (SOC)-mediated Ca^{2+} influx. Increased cytosolic [Ca^{2+}] results in the activation of SK$_{Ca}$ and IK$_{Ca}$ channels (*step 3*), and the ensuing membrane hyperpolarization increases Ca^{2+} influx via SOCs (*step 4*). Enhanced Ca^{2+} influx is critical for stimulating adequate NO production by membrane-associated endothelial NO synthase (eNOS) (*step 5*) and further promotes K$_{Ca}$ channel activity. Blockade of SK$_{Ca}$ and IK$_{Ca}$ channel-mediated membrane hyperpolarization by Apa and ChTx/TRAM-34, respectively, reduces store-operated Ca^{2+} influx, thereby preventing eNOS activation. CaM, calmodulin.

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