MECHANISMS OF SIGNAL TRANSDUCTION:
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doi: 10.1074/jbc.M212971200 originally published online January 15, 2003

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Mitochondria Efficiently Buffer Subplasmalemmal Ca\(^{2+}\) Elevation during Agonist Stimulation*

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In endothelial cells, local Ca\(^{2+}\) release from superficial endoplasmic reticulum (ER) activates BK\(_{ca}\) channels. The resulting hyperpolarization promotes capacitative Ca\(^{2+}\) entry (CCE), which, unlike BK\(_{ca}\) channels, is inhibited by high Ca\(^{2+}\). To understand how the coordinated activation of plasma membrane ion channels with opposite Ca\(^{2+}\) sensitivity is orchestrated, the individual contribution of mitochondria and ER in regulation of subplasmalemmal Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{pl}\)) was investigated. For organelle visualization, cells were transfected with DsRed and yellow cameleon targeted to mitochondria and ER. The patch pipette was placed far from any organelle (L1), close to ER (L3), or mitochondria (L2) and activity of BK\(_{ca}\) channels was used to estimate local [Ca\(^{2+}\)]\(_{pl}\). Under standard patch conditions, histamine increased [Ca\(^{2+}\)]\(_{pl}\) at L1 and L3 to 1.6 µM, whereas close to mitochondria [Ca\(^{2+}\)]\(_{pl}\) remained unchanged. If mitochondria moved apart from the pipette or in the presence of carbonyl cyanide-4-trifluoromethoxyphenylhyrazone, [Ca\(^{2+}\)]\(_{pl}\) at L2 increased in response to histamine. Under standard patch conditions Ca\(^{2+}\) entry was negligible due to cell depolarization. Using a physiological patch approach (5.6 mM K\(^{+}\) in the bath), changes in [Ca\(^{2+}\)]\(_{pl}\) could be monitored without cell depolarization and, thus, in conditions where Ca\(^{2+}\) entry occurred. Here, histamine induced an initial transient Ca\(^{2+}\) elevation to 3.5 µM followed by a long-lasting plateau at 1.2 µM in L1 and L3, whereas mitochondria kept neighboring [Ca\(^{2+}\)]\(_{pl}\) low during stimulation. Thus, superficial mitochondria and ER generate local domains of low and high Ca\(^{2+}\) allowing simultaneous activation of BK\(_{ca}\) and CCE, despite their opposite Ca\(^{2+}\) sensitivity.

In many cells, emptying of the endoplasmic reticulum (ER)\(^1\) represents an initial signal that triggers activation of the so-called capacitative Ca\(^{2+}\) entry through non-voltage-gated pathway(s) (CCE) (1). Remarkably, the CCE represents the main mechanism for Ca\(^{2+}\) entry in non-excitable cells and achieves long-lasting elevation of [Ca\(^{2+}\)]\(_{cyt}\). Although the actual protein(s) responsible for CCE is/are still under debate and matter of intense investigation, it has been clearly described that CCE is prevented by an elevation of Ca\(^{2+}\) at the mouth of the channel(s) (2–6). On the other hand, the amount of Ca\(^{2+}\) that actually enters the cells through CCE critically depends on activation of Ca\(^{2+}\)-activated K\(^{+}\) channels to achieve a membrane hyperpolarization and, thus, provide the driving force for Ca\(^{2+}\) entry (7, 8). Notably, in endothelial cells, superficial ER (sER) domains create spatial Ca\(^{2+}\) gradients beneath the plasma membrane (subplasmalemmal Ca\(^{2+}\) control unit, SCCU) that result in local activation of BK\(_{ca}\) channels (9–12). The existence of such localized Ca\(^{2+}\) elevation beneath the plasma membrane would explain, at least in part, the “Ca\(^{2+}\) paradox” that during cell stimulation activation of Ca\(^{2+}\)-activated ion currents occurs simultaneously with the Ca\(^{2+}\)-inhibitable CCE. However, we previously observed that, during a strong cell stimulation (i.e. 100 µM histamine), where BK\(_{ca}\) channels get activated also in regions far from the ER, a strong CCE still takes place (10). These findings emphasize that, although the sER contributes to Ca\(^{2+}\) influx by membrane hyperpolarization due to Ca\(^{2+}\)-activated K\(^{+}\) channels, another phenomenon, i.e. local lowering/buffering of the subplasmalemmal Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{sub}\)), has to occur simultaneously to facilitate CCE activity. Consequently, evidence was provided that mitochondria play a key role for CCE activity in non-excitable cells. In these experiments, in which mitochondria were depolarized by uncouplers of mitochondrial oxidative phosphorylation (i.e. the carbonyl cyanide phenylhydrazones FCCP and CCCP), which results in inhibition of mitochondrial Ca\(^{2+}\) uptake, the maintenance of CCE was prevented (2–4, 13). This phenomenon further referred to as “mitochondrial Ca\(^{2+}\) buffering” is thought to facilitate CCE by lowering subplasmalemmal Ca\(^{2+}\) at the mouth of this Ca\(^{2+}\)-inhibitable Ca\(^{2+}\)-entry pathway (14). However, these carbonyl cyanide-based mitochondrial uncouplers prevent mitochondrial Ca\(^{2+}\) signaling in a rather indirect way via abolishment of the H\(^{+}\) gradient, which results in a change of the mitochondrial pH and depolarization of the inner mitochondria membrane. Furthermore, these compounds have been found to affect the Ca\(^{2+}\) release from the ER (15) and result in a depolarization

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*This work was supported by the Austrian Funds (Grants SFB 714 and P-14586-PHA to W. F. G.), the Austrian National Bank (Grants P7542 to W. F. G., and P7902 to R. M., respectively), and the Swiss National Funds (Grant 31-56902.99). The Department of Medical Biochemistry & Medical Molecular Biology is a member of the Institutes of Basic Medical Sciences at the University of Graz and was supported by the infrastructure program (Grant UGP4) of the Austrian ministry of education, science and culture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: ER, endoplasmic reticulum; BK\(_{ca}\), large conductance Ca\(^{2+}\)-activated K\(^{+}\) channels; [Ca\(^{2+}\)]\(_{pl}\), free cytosolic Ca\(^{2+}\); [Ca\(^{2+}\)]\(_{pm}\), Ca\(^{2+}\) concentration at the inner side of the patch membrane; CCE, capacitative Ca\(^{2+}\) entry; FCCP, carbonyl cyanide-3-chlorophenylhyrazone; FCCP\(_{cyt}\), Ca\(^{2+}\) sensitivity of the BK\(_{ca}\) channel; sER, superficial endoplasmic reticulum; SCCU, subplasmalemmal Ca\(^{2+}\) control unit; YC4-ER, ER-targeted yellow cameleon 4.
of the plasma membrane (16). In view of the potential unspecific properties of mitochondrial uncouplers, ultimate proofs for the concept of mitochondrial Ca\textsuperscript{2+} buffering are necessary. Therefore, this study was designed to find further and direct evidence of mitochondrial Ca\textsuperscript{2+} buffering during cell stimulation in the human umbilical vein endothelial cell-derived cell line EA.hy926.

EXPERIMENTAL PROCEDURES

Materials—Cell culture chemicals were obtained from Invitrogen (Vienne, Austria) and fetal calf serum was from PAA Laboratories (Linz, Austria). Fura-2/AM was from Molecular Probes Europe (Leiden, Netherlands), and BCECF (carboxy-2,7-dichlorodihydro-1,3,3-dithylo-4,5-carboxyoxazolone) and histamine were from Sigma Chemicals (St. Louis, MO). Restriction enzymes and T4 DNA ligase were from New England BioLabs (Frankfurt, Germany) and the EndoFree Plasmid Maxi Kit was from Qiagen (Hilden, Germany). All other chemicals were from Roth, Karlsruhe, Germany.

Cell Culture—The human umbilical vein endothelial cell line, EA.hy926 (17) at passage 45 was used. Cells were grown on glass coverslips in Dulbecco’s minimum essential medium containing 10% fetal calf serum and 1% HAT (5 mM hypoxanthine, 20 μM aminopterin, 0.8 μM thymidine).

Plasmids and Transfection—YC4-ER (18, 19) and mtDsRed were cloned into pcDNA3 (Invitrogen, Groningen, Netherlands). For double transfection YC4-ER and mtDsRed were inserted into the two multiple cloning sites of the transfection vector pBludCE4.1 (Invitrogen). Cells of approximately 80% confluency were transiently transfected with 1.5–3 μg of purified plasmid DNA using TransFast\textsuperscript{TM} Transfection Reagent (Promega, Mannheim, Germany).

Organelle Visualization—Organelle organization was visualized in cells transiently transfected with YC4-ER or mtDsRed as described previously (10). Experiments were performed using a deconvolution microscope recently described (11, 12). A Nikon inverted microscope (Eclipse 300TE, Nikon, Vienna) was equipped with CFI Plan Fluor 40× objective (Nikon, Vienna, Austria), an epifluorescence system (150 W XBO, Optiquip, Highland Mills, NY), a computer controlled z-stage (Ludl Electronic Products, Hawthorne, NY), and a liquid-cooled charge-coupled device camera (–30 °C, Quantix KAF 1400G2, Roper Scientific, Acton, MA) that allowed image resolution of 0.17 μm pixel \(^{-1}\). Excitation/emission wavelengths were selected using a computer-controlled filter wheel (Ludl, Electronic Products, Hawthorne, NY). All devices were controlled either by Metafluor 4.0 (Visitron Systems, Puchheim, Germany) or ImagePro 3.0 (Media Cybernetics, Sliver Spring, MA) for deconvolution imaging. For organelle visualization, cells were illuminated alternatively at 440 nm (cameleon: 440AF21; Omega Optical, Brattleboro, VT) or 575 nm (mtDsRed: 528–633DBEM; dichroic XF53; Omega Optical). Image analysis and deconvolution were performed as previously described using ImagePro 3.0 (Media Cybernetics, Sliver Spring, MD) and constrained iterative deconvolution (Microtome, VayTek, Fairfield, IA) (10).

Ca\textsuperscript{2+} Measurements—Experiments were performed in Hepes-buffered solution containing (in mM) 138 NaCl, 5 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, and 10 Hepes acid, pH adjusted to 7.4. Nominal Ca\textsuperscript{2+}-free solution contained (in mM) 138 NaCl, 5 KCl, 1 MgCl\textsubscript{2}, 1 EGTA, and 10 Hepes acid, pH adjusted to 7.4. Ca\textsuperscript{2+}\textsubscript{\textsubscript{low}} values were measured using Fura-2, YC4-ER, and ratiometric-pericam-mt as described previously (10, 20). To determine \(\left[\text{Ca}^{2+}\right]_{\text{o}}\), cells were illuminated alternatively at 380 ± 15 and 380 ± 15 nm (Fura-2: 360HT15 and 380HT15; Omega Optical; Brattleboro, VT), and emission was monitored at 510 nm (510WB40; Omega Optical) as described previously (11).

Cytosolic Ca\textsuperscript{2+} Measurements during Cell Membrane Potential Clamp—As previously described (9, 21), cytosolic Ca\textsuperscript{2+} measurements were combined with the patch clamp technique (whole cell configuration) to control the cell membrane potential during cell stimulation. Experiments were performed using the Hepes-buffered solution mentioned above. The pipette solution contained (in mM) 130 KCl, 5 MgATP, 0.2 Na\textsubscript{2}GTP, 1 MgCl\textsubscript{2}, 10 Hepes, with pH adjusted to 7.2 with KOH.

Single Channel Recordings—Cell-attached and inside-out configurations of the patch clamp technique were used (22). Borosilicate glass pipettes (resistance of 6–10 MΩ) were pulled with a Narishige puller (Narishige Co. Ltd., Tokyo, Japan). Currents were recorded with an EPC-7 amplifier (List Medical, Darmstadt, Germany) filtered at 1 kHz (900C9L8L, Frequency Devices, Haverhill, MA), digitized by a digidata

![FIG. 1. Visualization of mitochondria and endoplasmic reticulum in endothelial cells for defined pipette positioning.](http://www.jbc.org/)

1320 interface (Axon Instruments, Union City, CA), and sampled by a PC running with pClamp 8.0 (Axon Instruments) at 5 kHz. Analysis of single currents was performed using Fetchan and pStat (Axon Instruments). The channel open state probability \((P_o)\) was expressed as the time spent in the open state \((t_o)\) divided by the total time of the recording \((t)\): \(P_o = t_o/t\). \(P_o\) was usually calculated on a 2-s sweep. When several identical channels \((N)\) were simultaneously open on the same patch, the open probability of one channel was calculated as follows: \(P_o = (t_o + 2t_{o2} + 3t_{o3} + \ldots + Nt_o)/Nt\), where \(t_o\) is the time spent by a channel at the open level \(N\). The pipette solution contained (in mM) 130 KCl, 1 MgCl\textsubscript{2}, 10 Hepes (pH 7.4 with KOH).

**Ca\textsuperscript{2+} Calibration of BK\textsubscript{Ca} Channel Activity**—The Ca\textsuperscript{2+} dependence of the BK\textsubscript{Ca} channel was tested within 0.5–30 μM free Ca\textsuperscript{2+} (calculated by MaxChelator; Dr. C. Patton, Hopkins Marine Station, Stanford University, CA). Consistent with our recent work (10) and with that of Barrett et al. (23), a correlation of the activity of BK\textsubscript{Ca} channels \((P_o)\) with the cell membrane potential \((V_{m})\) and \([\text{Ca}^{2+}]_{\text{im}}\) was extracted out of a series of in situ calibration procedures (see Fig. 7A).

**Standard Patch Protocol**—The standard experimental bath solution contained (in mM) 130 KCl, 1 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 10 Hepes (pH 7.45 with KOH; Fig. 6A). According to the Ca\textsuperscript{2+} calibration of the BK\textsubscript{Ca} channels, the \([\text{Ca}^{2+}]_{\text{im}}\) at each individual pipette location was calculated with the obtained \(P_o\) using the following equation:

\[
\log([\text{Ca}^{2+}]_{\text{im}}) = \log(EC_{50}^C) - \log(0.8305 - P_o)(P_o + 0.02181)
\]

where \(EC_{50}^C\) is the Ca\textsuperscript{2+} sensitivity of the channel. Based on our calibration published recently, \(EC_{50}^C = 5.5665\) (10).

**Physiological Patch Protocol**—In experiments using the physiological patch approach, the bath solutions contained (in mM) 130 NaCl, 5.6 KCl, 1 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 8 Hepes (pH 7.45 with NaOH). For more detail see Fig. 6. The procedure by which \([\text{Ca}^{2+}]_{\text{im}}\) was obtained using the physiological patch approach is shown in detail in Fig. 7B. Based on the linear relationship between \(V_{m}\) and the Ca\textsuperscript{2+} sensitivity of the BK\textsubscript{Ca} channel (EC\textsubscript{50}C), the obtained \(P_o\) and the actual \(V_{m}\) the Ca\textsuperscript{2+} concentration at the mouth of the BK\textsubscript{Ca} channel ([Ca\textsuperscript{2+}]\textsubscript{im}) under physiological patch clamp conditions was calculated as explained in Fig. 7B.

**Statistics**—Analysis of variance was performed, and statistical significance was evaluated using Scheffe’s post hoc F test. The level of significance was defined as \(p < 0.05\).

RESULTS

**Pipette Positioning in Proximity and Distance of Visualized Organelles**—Based on the expression of organelle-targeted DaRed (mtDsRed) and cameleon (YC4-ER) the organization of mitochondrial and ER network was visualized (Fig. 1). The patch pipette was placed far from any organelle (L1, Fig. 1), in the vicinity of mitochondrial rich domains (L2), or at locations close to ER structures (L3) as illustrated in the respective panels.
Mitochondria Effectively Buffer Cytosolic Ca\(^{2+}\) Rises and Ca\(^{2+}\) Entry Beneath the Plasma Membrane—In standard experimental bath solution, cells were stimulated with 100 \(\mu\)M histamine at a patch holding potential of +40 mV while the cell membrane potential was approximately 0 mV. Based on the monitored channel activity, the [Ca\(^{2+}\)]\(_{\text{pm}}\) at each individual pipette location was calculated as explained in detail under “Experimental Procedures.” With the pipette located far from mitochondria (i.e., \(\approx 5 \mu\)m; L1 and L2), BK\(_{\text{Ca}}\) activity, expressed as its maximal \(P_o\) (normalized) during stimulation, was 0.290 \(\pm\) 0.068 (\(n = 10\)), corresponding to \(\sim 1600 \text{nM} [\text{Ca}\(^{2+}\)]_{\text{pm}}\) (Fig. 2A). In agreement with our previous report, there was no considerable difference between the L1 and the L3 pipette location in response to histamine. In the vicinity of mitochondria (L2) BK\(_{\text{Ca}}\) activity was reduced by 91% (maximal \(P_o 0.025 \pm 0.013, n = 8, p < 0.05 \) versus L1 and L3) and represented an estimated [Ca\(^{2+}\)]\(_{\text{pm}}\) of \(\sim 160 \text{nM}\) (Fig. 2B).

The capacity of mitochondria to buffer neighboring subplasmalemmal Ca\(^{2+}\) and, thus, to prevent BK\(_{\text{Ca}}\) channel stimulation by histamine was further experienced under circumstances where the L2 pipette location was chosen initially but subplasmalemmal mitochondria occasionally moved away from the pipette (L1) during the experiment. If mitochondria were in the proximity of the patch (L2; Fig. 3A, left panel), the channel activation in response to 100 \(\mu\)M histamine was very small (\(P_o 0.015 \pm 0.011, n = 4\); Fig. 3B and C, tracings b) and comparable with resting cells (\(P_o 0.003 \pm 0.002, n = 4\); Fig. 3B and C, tracings a). However, after the respective mitochondrial domain moved approximately 4 \(\mu\)m from the patch location (Fig. 3A, right panel), a further histamine stimulation induced strong BK\(_{\text{Ca}}\) channel activation (\(P_o 0.252 \pm 0.073, n = 4; p < 0.05 \) versus previous L2 position; Fig. 3B and C, tracings c).

If mitochondrial Ca\(^{2+}\) uptake was prevented by 2 \(\mu\)M FCCP, the activity of neighboring BK\(_{\text{Ca}}\) channels in response to histamine increased 8-fold (maximal \(P_o 0.130 \pm 0.035, n = 5, p < 0.05 \) versus close to mitochondria without FCCP; Fig. 4, A and B). Notably, addition of 2 \(\mu\)M FCCP failed to initiate BK\(_{\text{Ca}}\) channel activation at L2 (data not shown). The time courses of BK\(_{\text{Ca}}\) channel activation did not differ in any conditions tested and represented a transient activation of BK\(_{\text{Ca}}\) channels (Fig. 4C) (10).
Fig. 4. Effect of FCCP on mitochondrial buffer function under isometric K⁺ conditions. In isometric K⁺, activation of BKCa channels in response to 100 μM histamine was monitored in the vicinity of mitochondria (L2) in the presence of 2 μM FCCP (A). The right panel represents channel activity before cell stimulation (a) and at the time of maximal stimulation after addition of 100 μM histamine (b and c). Maximal elevation of \([\text{Ca}^{2+}]_{\text{pm}}\) was calculated as described in Fig. 3 and is given in the left schema. B, statistical analysis on BKCa channel activation in response to 100 μM histamine at locations L1 and L3, L2, and L2 in the presence of 2 μM FCCP. *, \(p < 0.05\) versus L1 and L3 (L1 and L3, \(n = 10\); L2, \(n = 8\), and L2 + FCCP, \(n = 5\)). C, representative time courses of BKCa channel activation to 100 μM histamine at various pipette locations (L1 and L3, L2) and in the absence or presence of 2 μM FCCP.

Limitations of the Standard Patch Protocol—Notably, in the protocol above, high bath K⁺ concentration was used to prevent changes in the membrane potential during the recording. Under such conditions, plasma membrane K⁺ currents and, thus, membrane hyperpolarization that represents the driving force for \(\text{Ca}^{2+}\) entry are prevented (7, 8). Indeed, under physiological conditions endothelial cells hyperpolarized from \(-30.7 ± 3.1\) to \(-62.0 ± 2.8\) mV (\(n = 14\)) upon stimulation with 100 μM histamine. The pivotal role of the driving force for \(\text{Ca}^{2+}\) entry in endothelial cells was tested in experiments where the patch clamp technique (whole cell configuration) and single cell fluorometry were used simultaneously. Cells were stimulated with histamine and clamped at 0, −30, −60, or −90 mV. Although the initial transient remained unaffected, the \(\text{Ca}^{2+}\) plateau (measured 3 min after the onset of the response) increased with the amount of the hyperpolarization applied (Fig. 5). These findings demonstrate that in the experiments presented above (standard patch protocol) a very limited \(\text{Ca}^{2+}\) entry occurs due to the membrane depolarization in isometric K⁺, whereas under physiological conditions \(\text{Ca}^{2+}\) influx is facilitated by a significant membrane hyperpolarization.

The Physiological Patch Protocol—To estimate the impact of the driving force for \(\text{Ca}^{2+}\) entry on autacoid-induced BKCa stimulation, the effect of histamine on the activity of BKCa channels that were far from any organelle were compared under isometric (i.e. 130 mM) and physiological (i.e. 5.6 mM) extracellular K⁺ conditions (Fig. 6A). To allow a comparison of the BKCa channel activation under both patch clamp protocols, in the physiological patch protocol, a patch membrane potential was applied that compensated the actual cell membrane potential to achieve +40 mV at the channels in the patch. In 130 mM K⁺-containing buffer, histamine induced a transient BKCa channel stimulation, whereas under physiological conditions the activation of BKCa channels by histamine was biphasic and comprised an initial transient followed by a long lasting channel activation (Fig. 6B). These data clearly indicate that the conventional standard patch technique limits significantly the quantity and duration of the response of a cell to agonist stimulation.

Thus, it seems important to verify whether mitochondria buffer subplasmalemmal \(\text{Ca}^{2+}\) elevation even under conditions where hyperpolarization facilitates physiological \(\text{Ca}^{2+}\) entry. To address this point, experiments in physiological bath K⁺ concentration were performed. Notably, an experimental protocol was chosen (further referred to as “physiological patch”) that allowed the cell to hyperpolarize freely while single channel recordings were performed. The experimental procedure and analysis to estimate the \(P_{o}\) of the distinct BKCa channel at a certain pipette location, the whole cell membrane potential \(V_{\text{pm}}\), and \([\text{Ca}^{2+}]_{\text{pm}}\) are explained in detail in Fig. 7. Using the amplitude of the current and the current-voltage relationship of the BKCa channel, the actual membrane potential of the patch \(V_{\text{pm}}\) was calculated (Fig. 7B, steps 1 and 2). With \(V_{\text{pm}}\) and the applied potential \(V_{\text{applied}}\) the whole cell membrane potential \(V_{\text{wc}}\) was estimated (Fig. 7B, step 3). Following the open probability of the BKCa channels (Fig. 7, step 4), the \(\text{Ca}^{2+}\) concentration at the mouth of the channel \([\text{Ca}^{2+}]_{\text{pm}}\) was calculated according to the equation given (Fig. 7B, step 5). Thus, the physiological patch approach allows the measurements and quantitative analysis of distinct subplasmalemmal \(\text{Ca}^{2+}\) concentrations in stimulated cells, which are not handicapped by artificially imposed whole cell membrane potential.
Opposite Subplasmalemmal Ca$^{2+}$ Gradients under Physiological Conditions—According to this procedure, evaluations of the effect of histamine on BK$_{Ca}$ channel activity, $V_{oc}$, and $[\text{Ca}^{2+}]_{pm}$ under physiological conditions were completed in pipette locations L1, L2, and L3, and representative tracings of a respective recording are provided in Fig. 8 (L1, panel A; L2, panel B; L3, panel C). For further statistical evaluation and comparison, two distinct time periods (P1 and P2) were chosen as indicated in Fig. 8, where P1 represents the initial and transient phase and P2 the long lasting plateau phase where CCE takes place. Far from any organelle (L1), 100 μM histamine enhanced $[\text{Ca}^{2+}]_{pm}$ in P1 and P2 approximately 35 and 12 times, respectively (Fig. 8A and Table I). The response was biphasic and a strong transient P1 was followed by a long lasting $\text{Ca}^{2+}$ elevation during P2. Intriguingly, histamine-induced elevations of $[\text{Ca}^{2+}]_{pm}$ were completely buffered by neighboring mitochondria (L2), and only a small and transient $\text{Ca}^{2+}$ elevation was observed (Fig. 8B and Table I). In contrast, in the vicinity of sER (L3), $[\text{Ca}^{2+}]_{pm}$ rises upon histamine stimulation by approximately 63 in P1 and 13 times in P2 (Fig. 8C and Table I).

**DISCUSSION**

In this work we report that superficial domains of the mitochondria and the ER create opposite $\text{Ca}^{2+}$ gradients upon cell stimulation with the inositol 1,4,5-trisphosphate-generating agonist histamine. Using the combination of high resolution fluorescence microscopy for visualization of organelle-targeted fluorescent proteins and electrophysiology for locally defined

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Mitochondria effectively buffer neighboring subplasmalemmal Ca\textsuperscript{2+} under physiological conditions. Analysis of BK\textsubscript{Ca} channel activity (P\textsubscript{o}), whole cell membrane potential (V\textsubscript{em}), and [Ca\textsuperscript{2+}]\textsubscript{pm} at pipette location L1 (A), L2 (B), and L3 (C). D, schematic overview of the spatial distribution of subplasmalemmal Ca\textsuperscript{2+} elevations in response to 100 μM histamine. For statistical details see Table I.
single channel recordings, we found that superficial mitochondria effectively buffer subplasmalemmal Ca\textsuperscript{2+} during cell stimulation despite a large increase in cytosolic Ca\textsuperscript{2+}. On the contrary, superficial ER domains were observed to generate a high Ca\textsuperscript{2+} gradient beneath the cell membrane that results in cell hyperpolarization by activation of BK\textsubscript{Ca} channels.

One essential achievement of the present study was the simultaneous visualization of mitochondria and ER to allow an exact positioning of the patch pipette. Because the transfection efficiency of endothelial cells is rather low and the amount of expression of each individual fluorescent protein would need to be equal, a vector for double transfection was used. As shown in Fig. 1, a clear separation between mitochondria and ER could be realized by transfecting the cells with the vector pBudCE4.1 encoding ER-targeted YC4-ER and mitochondrial-targeted DaRed. Thus, this approach allowed distinct pipette positioning in respect to the ER and the mitochondria. Using the standard patch approach (i.e. 130 mM K\textsuperscript{+} outside) a strong activation of the BK\textsubscript{Ca} channels in response to 100 \mu M histamine was found at the ER (L3) and far from any organelle (L1). These findings are consistent with our previous report in which 100 \mu M histamine stimulated BK\textsubscript{Ca} channels in pipette locations L1 and L3, whereas only at low histamine concentration (i.e. 10 \mu M) a spatial subplasmalemmal Ca\textsuperscript{2+} elevation occurred between the plasma membrane and the sER (i.e. L3) (10). Localized Ca\textsuperscript{2+} events have been often reported in excitable and non-excitable cells (see Ref. 24 for review). Such localized elevations of subplasmalemmal [Ca\textsuperscript{2+}]\textsubscript{pm} have been clearly shown in pancreatic acinar cells (25), cardiac myocytes (26), smooth muscle (27), or HeLa cells (28) where Ca\textsuperscript{2+} signaling has been found to constitute a multitude of local, highly controlled processes that include ion channels, pumps, and organelles. All these reports deal with local elevation of Ca\textsuperscript{2+} in restricted areas of the cell. Although such high Ca\textsuperscript{2+} gradients have been found to constitute distinct triggers for the spatial modulation of Ca\textsuperscript{2+}-activated mechanisms (24), these studies fail to explain how during a cell stimulation that is accompanied with a large elevation in the cytosolic Ca\textsuperscript{2+} concentration Ca\textsuperscript{2+}-sensitive ion channels are still active despite the inhibitory action of Ca\textsuperscript{2+} on this pathway.

As in most other non-excitable cells, Ca\textsuperscript{2+} enters endothelial cells through the so-called CCE pathway. Notably, the CCE is sensitive to elevation of Ca\textsuperscript{2+} at the mouth of one or more of the channels (2–6, 29). However, in this study and our previous work (10) we demonstrate that the subplasmalemmal Ca\textsuperscript{2+} concentration elevates up to 1.6 \mu M free Ca\textsuperscript{2+} under standard patch clamp conditions, and, although such high Ca\textsuperscript{2+} concentration is known to prevent CCE, a large CCE took place during strong cell stimulation (10). Because this paradox situation was found in many cells, a phenomenon of local subplasmalemmal Ca\textsuperscript{2+} lowering was postulated. As mechanisms of such spatial subplasmalemmal Ca\textsuperscript{2+}-buffering plasma membrane Ca\textsuperscript{2+} pumps (30) and/or Ca\textsuperscript{2+} buffering by the mitochondria (2–6, 31–33) were suggested. The mitochondrial Ca\textsuperscript{2+} buffer function was predominantly investigated using uncouplers of mitochondrial oxidative phosphorylation (i.e. the carbonyl cyanide phenylhydrazones FCCP and CCCP) that result in inhibition of mitochondrial Ca\textsuperscript{2+} uptake (34) due to the depolarization of the mitochondria and consequently prevent CCE activity, monitored by conventional fluorometric Ca\textsuperscript{2+} measurements or whole cell currents (2–6, 31–33). However, because the uncouplers of mitochondrial oxidative phosphorylation have been reported to initiate Ca\textsuperscript{2+} release from the ER (15) and to depolarize the plasma membrane (16), mitochondrial Ca\textsuperscript{2+} buffering during cell stimulation needed to be studied directly.

Thus, our present findings, that in isometric K\textsuperscript{+} bath conditions BK\textsubscript{Ca} channel activation in response to 100 \mu M histamine was strongly reduced in the proximity of mitochondria, indicate for the first time that the increase in [Ca\textsuperscript{2+}]\textsubscript{pm} in response to histamine was effectively reduced by superficial mitochondria. This direct demonstration of mitochondrial “Ca\textsuperscript{2+} buffering” was further confirmed in experiments where BK\textsubscript{Ca} channel activity was restored after the superficial mitochondria dislocated from the patch during the experiments. Such mitochondrial movements have been reported frequently (for review see Refs. 35 and 36) and are thought to result from mitochondrial movements along the microtubular network (37). Considering our recent findings that the BK\textsubscript{Ca} channels are ubiquitously distributed in EA.hy926 cells (10), these data indicate that moving organelles affect the activity of neighboring plasma membrane channel. Thus, it seems possible that superficial organelles create their own distinct microenvironment along their way.

The contribution of mitochondria to local Ca\textsuperscript{2+} buffering monitored by using the BK\textsubscript{Ca} channels as Ca\textsuperscript{2+} sensors was further supported by our findings that FCCP restored BK\textsubscript{Ca} channel activation in patches close to mitochondria. Because mitochondrial Ca\textsuperscript{2+} buffering was measured in these experiments directly under controlled conditions (i.e. defined patch localization and clamped membrane potential), these data point to an elevation of [Ca\textsuperscript{2+}]\textsubscript{pm} in response to histamine due to the lack of mitochondrial Ca\textsuperscript{2+} sequestration under FCCP treatment. Also, although these data are consistent with previous experiments where uncouplers of mitochondrial oxidative phosphorylation were used to investigate the contribution of mitochondrial Ca\textsuperscript{2+} buffering to CCE (2–6, 31–33), this is the first time that FCCP was demonstrated to prevent subplasmalemmal mitochondrial Ca\textsuperscript{2+} buffering upon agonist stimulation on the single cell level.

Remarkably, the activation of BK\textsubscript{Ca} channels far from any organelle (L1), close to sER (L3) or next to mitochondria (L2) in the presence of FCCP, was found to be transient. This finding is quite surprising considering the long lasting cytosolic Ca\textsuperscript{2+} elevation and membrane hyperpolarization found in these cells in response to 100 \mu M histamine (9, 10, 21). The simplest explanation for the transient BK\textsubscript{Ca} channel activation in the

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<table>
<thead>
<tr>
<th>n</th>
<th>Number of channels</th>
<th>P\textsubscript{1}</th>
<th>P\textsubscript{2}</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Normal P\textsubscript{a}</td>
<td>V\textsubscript{oc}</td>
<td>[Ca\textsuperscript{2+}]\textsubscript{pm}</td>
</tr>
<tr>
<td></td>
<td>mV</td>
<td>\mu M</td>
<td></td>
</tr>
<tr>
<td>L1: far from any organelle</td>
<td>9</td>
<td>3.8 ± 0.5</td>
<td>0.413 ± 0.057\textsuperscript{a}</td>
</tr>
<tr>
<td>L2: close to mitochondria</td>
<td>8</td>
<td>2.3 ± 0.6</td>
<td>0.073 ± 0.024</td>
</tr>
<tr>
<td>L3: close to ER</td>
<td>5</td>
<td>2.2 ± 0.8</td>
<td>0.499 ± 0.069\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\( ^{a} p < 0.05 \) versus "close to mitochondria."
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standard patch protocol is that, in standard bath solution (i.e., isometric K\(^+\)) very little or no Ca\(^{2+}\) entry takes place as Ca\(^{2+}\) influx critically depends on the driving force that is most prominently provided by the activation of Ca\(^{2+}\)-activated K\(^+\) channels (8). This assumption is further supported by our data presented herein and previous reports that in endothelial cells Ca\(^{2+}\) entry depends critically on membrane hyperpolarization.

Thus, out of these findings we conclude that experiments in which the standard patch approach was used do not allow a proper evaluation of the kinetics and the magnitude of spatial Ca\(^{2+}\) gradients due to the strong reduction of CCE under the depolarizing conditions used. These findings raise a number of important questions: What is the subplasmalemmal Ca\(^{2+}\) concentration achieved by cell stimulation under physiological conditions? Do mitochondria still buffer Ca\(^{2+}\) under physiological conditions where CCE occurs? And, finally, is our SCCU concept still accurate, although one can expect higher transmembrane Ca\(^{2+}\) movements?

These aspects were verified in our experiments using a physiological patch that allowed the cell to manipulate its membrane potential freely while one can still follow single channel activity. Under these conditions, the driving force for Ca\(^{2+}\) entry is not diminished by artificial membrane depolarization, and, thus, a physiological CCE occurs. We believe that this approach, which reveals the actual patch potential (V\(_{pm}\), whole cell membrane potential (V\(_m\)), and [Ca\(^{2+}\)]\(_{pm}\), represents a landmark for progress in the evaluation of cellular Ca\(^{2+}\) homeostasis. Convincingly, under physiological but not standard patch conditions Ca\(^{2+}\) entry occurs, which was indicated by the second long lasting activation of the BK\(_c\) located far from any organelle (Figs. 6B, 8A, and 8C). This biphasic activation of the BK\(_c\) channels occurred despite a long lasting cell membrane hyperpolarization (Fig. 8, A and C), which was in the same range as that obtained in conventional current clamp protocol (i.e. approximately 30 mV). At pipette location L1, the estimated [Ca\(^{2+}\)]\(_{pm}\) elevations in response to histamine were also biphasic and revealed up to -3.5 and -1.2 \(\mu\)M during the initial transient (P1) and long lasting phase (P2), respectively. These levels of [Ca\(^{2+}\)]\(_{pm}\) correspond precisely to that found using membrane targeted ratiometric-pericam in pancreatic islet \(\beta\)-cells (38) and confirm our approach of monitoring [Ca\(^{2+}\)]\(_{pm}\) by BK\(_c\) channels.

When locating the pipette at sER domains (L3) the subplasmalemmal Ca\(^{2+}\) elevation in response to histamine exceeded that found in L1 (up to -6.3 and -1.3 \(\mu\)M during P1 and P2, respectively), whereas the onset of the second phase was faster. These data further support our previous concept on the specific role of the sER for local Ca\(^{2+}\) elevation (SCCU) (9–12). Moreover, by introducing the physiological patch approach we demonstrate that even under strong cell stimulation the SCCU builds up a subplasmalemmal Ca\(^{2+}\) gradient in which the Ca\(^{2+}\) concentration is higher than in areas without sER.

In our standard patch experiments the mitochondria have been found to buffer effectively neighboring Ca\(^{2+}\) in the subplasmalemmal area indicated by the lack of BK\(_c\) channel activation upon 100 \(\mu\)M histamine administration (Fig. 2). Using the physiological patch approach, it was of interest whether or not mitochondria are still able to buffer subplasmalemmal Ca\(^{2+}\) in their neighborhood, although we have found a 3- to 6-fold higher subplasmalemmal Ca\(^{2+}\) concentration at L1 and L3 compared with our standard patch experiments. Remarkably, despite such high subplasmalemmal Ca\(^{2+}\) elevation to histamine far from any organelle and close to ER domains, superficial mitochondria were still capable of buffering [Ca\(^{2+}\)]\(_{pm}\) during histamine stimulation to 0.25 and 0.10 \(\mu\)M in P1 and P2, respectively. These data demonstrate that, during strong cell activation, mitochondria buffer subplasmalemmal Ca\(^{2+}\) elevation by about 95 and 98\% compared with the L1 and L3 pipette positions. Furthermore, during P2, the phase where the Ca\(^{2+}\)-sensitive CCE takes place (29), subplasmalemmal mitochondria keep neighboring subplasmalemmal Ca\(^{2+}\) at basal levels. This is the first time that mitochondrial the “Ca\(^{2+}\) buffering” function was demonstrated directly under physiological conditions and without any pharmacological tools. Furthermore, these data convincingly prove the concept that superficial mitochondria indeed create a local microdomain of low Ca\(^{2+}\) that might sustain the activity of the Ca\(^{2+}\)-inhibitable CCE pathway.

Our findings, that even under physiological conditions, superficial organelles are able to create opposite Ca\(^{2+}\) gradients and build their own Ca\(^{2+}\) dynamics in their microenvironment, have important implications because Ca\(^{2+}\) operates as a crucial messenger for numerous pivotal functions in the cell. In endothelial cells, Ca\(^{2+}\) regulates the production of vasoactive compounds (for review see Ref. 39) and the activation of transcription factors (e.g. NF\(\kappa\)B) (40) and ion channels (41). Due to the opposite characteristics of Ca\(^{2+}\) gradients at superficial organelles during cell stimulation presented herein, the mechanisms for the versatility of Ca\(^{2+}\) as a ubiquitous second messenger becomes more transparent.

Acknowledgments—We thank Beatrix Petesch and Anna Schrölicer for excellent technical assistance; Prof. B. Y. Tsien, Dr. A. Miyawaki, and Prof. T. Pozzan for providing the cameleon and mtDsRed constructs; and Dr. C. J. S. Edgell for providing the EA.hy926 cells.