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ION CHANNELS, RECEPTORS AND TRANSPORTERS

Electrophysiological characterization of store-operated and agonist-induced Ca^{2+} entry pathways in endothelial cells

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Abstract In endothelial cells, agonist-induced Ca^{2+} entry takes place via the store-operated Ca^{2+} entry pathway and/or via channel(s) gated by second messengers. As cell stimulation leads to both a partial Ca^{2+} store depletion as well as the production of second messengers, these two pathways are problematic to distinguish. We showed that passive endoplasmic reticulum (ER) depletion by thapsigargin or cell stimulation by histamine activated a similar Ca^{2+}-release-activated Ca^{2+} current (CRAC)-like current when 10 mM Ba^{2+}/2 mM Ca^{2+} was present in the extracellular solution. Importantly, during voltage clamp recordings, histamine stimulation largely depleted the ER Ca^{2+} store, explaining the activation of a CRAC-like current (due to store depletion) upon histamine in Ba^{2+} medium. On the contrary, in the presence of 10 mM Ca^{2+}, the ER Ca^{2+} content remained elevated, and histamine induced an outward rectifying current that was inhibited by Ni^{2+} and KB-R7943, two blockers of the Na^+/Ca^{2+} exchanger (NCX). Both blockers also reduced histamine-induced cytosolic Ca^{2+} elevation. In addition, removing extracellular Na^+ increased the current amplitude which is in line with a current supported by the NCX. These data are consistent with the involvement of the NCX working in reverse mode (Na^+ out/Ca^{2+} in) during agonist-induced Ca^{2+} entry in endothelial cells.

Keywords Receptor-activated Ca^{2+} entry · Store-operated Ca^{2+} entry · Na^+/Ca^{2+} exchanger · Endothelial cells · Ionic channels

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Abbreviations used

SOCE	Store-operated Ca^{2+} entry
RACE	Receptor-activated Ca^{2+} entry
IP_3	Inositol 1,4,5-trisphosphate
ER	Endoplasmatic reticulum
SERCA	Sarco-endoplasmic reticulum Ca^{2+} ATPase
TG	Thapsigargin
NCX	Na^+/Ca^{2+} exchanger
$D1_{ER}$	ER-targeted cameleon

Introduction

Calcium signaling in non-excitable cells relies, for a large part, on the Ca^{2+} entry phase that occurs through non-voltage-gated channel(s). Store-operated Ca^{2+} entry (SOCE), which is activated by the depletion of the endoplasmic reticulum (ER), is the best-characterized and most studied pathway leading to Ca^{2+} influx. Initially, the SOCE phenomenon was described in acinar cells [45], and it was then extensively studied in T lymphocytes and mast cells (review in [42]). While the mechanism coupling ER Ca^{2+} depletion to ion channel activation remained unresolved for more than 20 years, it was recently shown that the ER transmembrane protein stromal interaction molecule (STIM1) is the Ca^{2+} sensor that aggregates and redistributes toward the plasma membrane upon store depletion [29, 46, 64]. The Orai1 protein on the other side is postulated to be the Ca^{2+} entry channel that is getting activated by STIM1 clustering [9, 59, 63]. In most experiments, SOCE is activated by a large ER Ca^{2+} depletion achieved by, e.g., blocking the sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCA) with drugs like thapsigargin (TG). Besides SOCE, other routes for Ca^{2+} entry exist that are not linked

to store depletion but require the production of second messengers to be activated, a pathway that we called receptor-activated Ca^{2+} entry (RACE; [22]). For instance, diacylglycerol [17], arachidonic acid [32, 51], or Ca^{2+} itself ([3, 23]) are known activators of these store-independent cation channels.

The endothelium is a multifunctional "organ" involved in multiple processes like the regulation of coagulation, vessel permeability, angiogenesis, or the local control of the vascular tone. Most, if not all, of these regulatory mechanisms imply a cytosolic Ca^{2+} signal that crucially depends on the Ca^{2+} entry phase [15]. While SOCE is frequently referred to as the main pathway for Ca^{2+} entry and thus essentially contributes to a proper function of endothelial cells [1, 10], several channels from the transient receptor potential (TRP) family are present on endothelial cells and have been implicated in numerous functions (review [61]). It is reasonable to postulate that during a physiological cell stimulation, two major events take place that account for Ca^{2+} entry. On the one hand, the Ca^{2+} content of the ER decreases (to a level that is often unknown), leading possibly to SOCE activation, and on the other hand, second messengers are produced as a consequence of mechanisms downstream to receptor activation that eventually stimulate store-independent Ca^{2+} entry channels. Recently, we showed that during endothelial cell stimulation by histamine, the ER Ca^{2+} depletion is very minor [22], reaching about 15% of what is produced upon SERCA inhibition with TG. Malli et al. obtained a similar level of store depletion upon agonist stimulation [31]. Even though these measurements reflected global ER Ca^{2+} changes, these findings strengthen our hypothesis that other Ca^{2+} entry pathways than SOCE are engaged during agonist-induced cell activation that are of physiological relevance. However, using cytosolic Ca^{2+} measurements, it is difficult to separate RACE from SOCE mainly due to the lack of specific inhibitors for one or the other pathway [22]. In addition, it is more than likely that if the RACE pathway is inhibited, the ER Ca^{2+} depletion that would develop will increase the SOCE pathway, and the resulting cytosolic Ca^{2+} signal would remain almost unaffected. Thus, the best approach to separate both entries is to measure directly the currents on intact cells using the patch-clamp technique. In blood cells, the current supporting SOCE, known as Ca^{2+}-release-activated Ca^{2+} current (I_{CRAC}), is well-characterized and presents some key features like a high selectivity for Ca^{2+}, an inward rectification, a tiny unitary conductance, as well as a complex regulation by Ca^{2+} itself ([42]). For blood cells, at least, it is now firmly established that I_{CRAC} is supported by Orai1 channel [44, 58, 62]. Besides cells from the immune system, a SOCE current with a similar characteristic to I_{CRAC} was also recorded in other cell types, including endothelial cells. In this cell type, the current was of significantly smaller amplitude than on the "prototypical" rat basophil leukemia (RBL) cells [8] and less Ca^{2+}-selective than I_{CRAC} (review in [34]). Recently, a paper reported a very small I_{CRAC} current on endothelial cells supported by Orai1 and STIM1 [1]. The tiny current amplitude and a peculiar kinetic of activation raised some controversies about the exact nature of this CRAC current in endothelial cells [4, 57], while the implication of STIM1 and Orai1 as SOCE components in endothelial cells was firmly established by this study.

As already raised up [4, 33], the experimental conditions used to record I_{CRAC} might prevent other events to take place and thus preclude the recordings of current(s) different from I_{CRAC}. A major issue is the presence of a high concentration of Ca^{2+} chelator inside the patch pipette that impedes Ca^{2+}-activated current(s) to be stimulated. In the present study, we wanted to determine what kind of current involved in Ca^{2+} entry is/are getting activated upon agonist stimulation and whether this/these current(s) are distinguishable from I_{CRAC} on a human endothelial cell line. For this purpose, we used the perforated variant of the whole-cell recordings and applied either TG to fully deplete stores and activate SOCE or histamine to record other types of current (RACE). We show that great caution is necessary in order to prevent unexpected store depletion upon histamine stimulation due to experimental conditions rather than to physiological process. Hence, we demonstrated that histamine activated a current that shares pharmacological and biophysical properties with the Na^{+}/Ca^{2+} exchanger (NCX) and that is implicated in the Ca^{2+} entry process in this particular cell type.

Materials and methods

Material

Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were obtained from Invitrogen. Fetal calf serum (FCS) was from PPA Laboratories (Linz, Austria). Histamine and thapsigargin were obtained from Sigma. KB-R7943 was from Tocris. Acetoxymethyl ester form of fura-2 (fura-2/AM) was from Invitrogen. The ER-targeted cameleon probe ($D1_{ER}$) was kindly provided by Drs Amy Palmer and Roger Tsien.

Cell culture and transfection

Experiments were performed on the human umbilical vein endothelial cells derived cell line EA.hy926 at passages >45. Cells were grown in DMEM containing 10% FCS, 1% HAT (5 mM hypoxanthin, 20 µM aminopterin, 0.8 mM thimidine), 50 U/ml penicillin, and 50 µg/ml streptomycin and were

maintained at 37°C in 5% CO_2 atmosphere. For experiments, cells were plated on 30 mm glass cover slips 2–4 days before use. For the $D1_{ER}$ experiments, cells were grown until 70–80% confluence and were transiently transfected with 2 μg cDNA encoding the $D1_{ER}$ construct, using TransFectin™ (BioRad).

Electrophysiological recordings

Cells were plated 1 day before the experiments to avoid cell–cell contact. We performed whole-cell recording using the perforated patch approach ([18]). Nystatin was included in the patch pipette at a concentration of 350 μg/ml (300–400 μg/ml). Borosilicate glass pipettes (Harvard Apparatus) were pulled with a Sutter puller, fired polished, and had a resistance between 3 and 6 MΩ. Patch-clamp recordings were made using an Axopatch 200B amplifier, a Digidata 1440A, and a pClamp 8 software (Molecular Devices). Voltage ramps (−120 to +80 mV, 500 ms) were repeatedly applied in order to obtain the current–voltage relationships. Cell capacitance (25–50 pF) was obtained for each cell measured, and the current was normalized to the cell capacitance. Average currents obtained before the cell stimulation were subtracted from the activated current. Pipette solution contained (in mM) 140 CsAsp, 10 NaCl, 1 $MgCl_2$, 10 Hepes, pH 7.2 with CsOH. Bath solutions contained (in mM) 125 NaCl, 10 $BaCl_2$, 2 $CaCl_2$, 1 $MgCl_2$, 5 KCl, 10 Hepes, pH 7.45 with NaOH (referred as 10 mM Ba^{2+}/2 mM Ca^{2+}), or 125 NaCl, 10 $CaCl_2$, 1 $MgCl_2$, 5 KCl, 10 Hepes pH 7.45 with NaOH (referred as 10 mM Ca^{2+}). For some experiments, NaCl was substitute for *N*-methyl-D-glucamine (NMDG). The junction potential of around 15 mV between the pipette and the bath solution was corrected off-line.

Ca^{2+} measurements

Cells were loaded with 2 μM fura-2 AM for 40 min in the dark at room temperature in a medium containing (in mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes acid, 2.6 $NaHCO_3$, 0.44 KH_2PO_4, 10 glucose with 0.1% vitamins and 0.2% amino acids, pH adjusted to 7.45 with NaOH. After washing the cells, 10–15 min was needed to allow de-esterification. For cytosolic Ca^{2+} measurements, cells were alternatively illuminated at 340 nm (340AF15; Omega Optical) and 380 nm (380AF15; Omega Optical) using a Lambda DG4 illumination system (Sutter Instrument Company, Novato, CA, USA), through a 415DCLP dichroic mirror, and emission was collected through a 510WB40 filter (Omega Optical). Fluorescence images were collected using a cooled, 12-bit CCD camera (CoolSnap HQ, Ropper Scientific, Trenton, NJ, USA) operated by the Metafluor 6.3 software (Universal Imaging, West Chester, PA, USA). To measure the ER Ca^{2+}, cells were illuminated at 440 nm (440AF21; Omega Optical), and emission was collected through a 455DRLP dichroic mirror, alternatively at 480 nm (480AF30; Omega Optical) and 535 nm (535AF26; Omega Optical). For cytosolic and ER Ca^{2+} measurements, the experiments were performed in Hepes-buffered solution containing (in mM) 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Hepes, 10 Glucose, pH adjusted at 7.45 with NaOH. In solutions with various Na^+ concentrations, Na^+ was replaced by NMDG. All data are expressed as R/Ro, Ro being the initial ratio value obtained before cell stimulation.

Statistics

Experimental data are expressed as mean ± SEM. Student's t test was used to compare results, with $p<0.05$ taken as the level of significance.

Results

TG and histamine both activated a CRAC-like current in the presence of extracellular Ba^{2+}

In order to electrophysiologically characterize the currents activated by TG and histamine in endothelial cells, we used the perforated patch variant of the whole-cell configuration. Using this approach, we expected to minimally alter the cell machinery, especially upon agonist stimulation, and thus to increase the chance to differentiate SOCE currents from agonist-activated currents. In the first series of experiments, 10 mM $BaCl_2$ was added in the extracellular medium, as many Ca^{2+} channels, including CRAC channels, conduct Ba^{2+} better than Ca^{2+}, at least at negative membrane potentials [19, 42]. We previously showed that in the absence of extracellular Ca^{2+}, histamine leads to strong ER Ca^{2+} depletion ([22]); hence, 2 mM Ca^{2+} was also added in the bath solution to minimize store depletion. In these conditions (10 mM Ba^{2+}/2 mM Ca^{2+}), TG and histamine activated inward rectifying currents, reaching a maximal amplitude (measured at −115 mV) of $-0.59 \pm 0.04\,\mathrm{pA/pF}$ $(n = 30)$ for histamine and $-0.48 \pm 0.07\,\mathrm{pA/pF}(n = 11)$ for TG (Fig. 1a, b). The activation kinetic was similar for both stimuli, and the current remained stable over time. As shown in Fig. 1c, d, 10 μM La^{3+} inhibited both TG- and histamine-activated currents. The reversal potential, E_{rev}, was estimated around −11 mV for TG and +7 mV for histamine, but due to the pronounced inward rectification, an accurate measure of E_{rev} was problematic. The strong inward rectification, the very small size of the current, and the block with lanthanide are three characteristics of I_{CRAC}. To confirm that TG and histamine activated I_{CRAC} or a CRAC-like

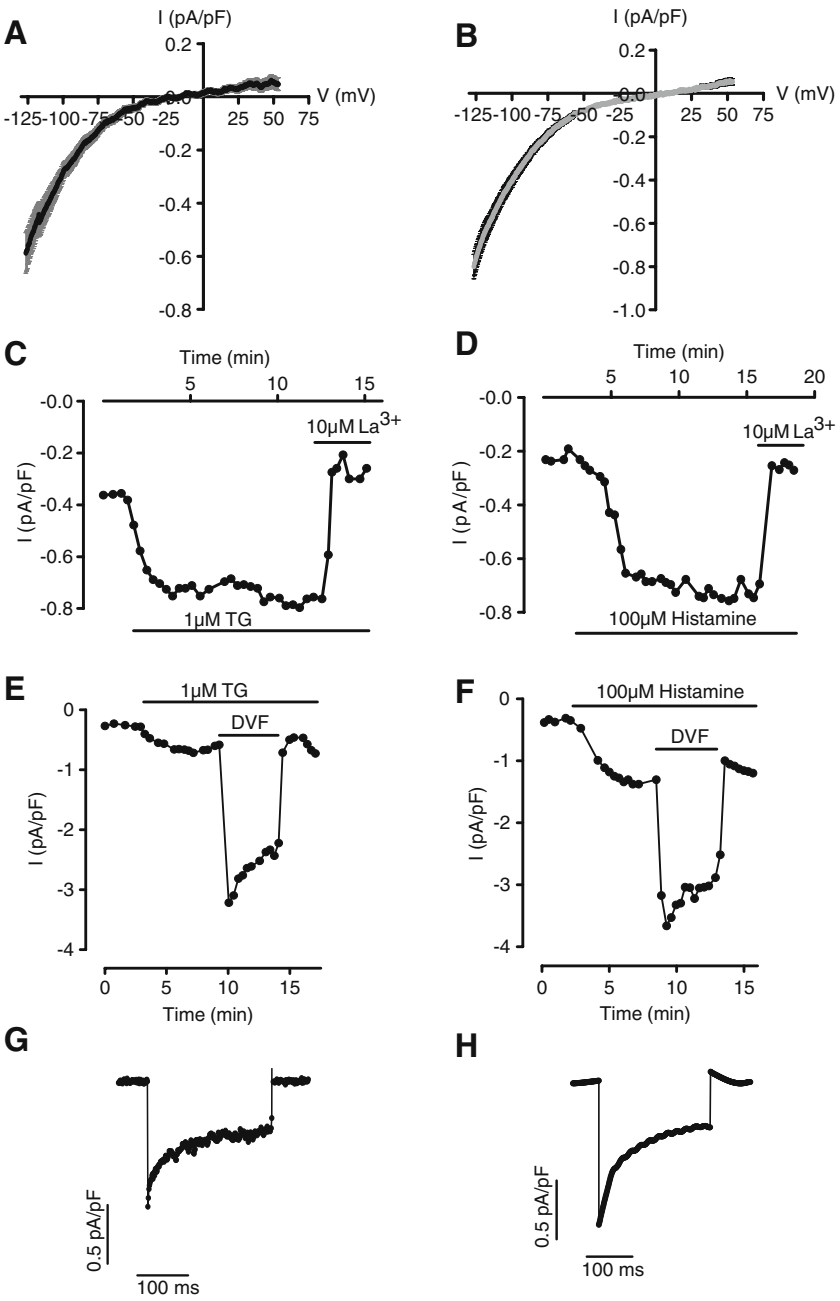

Fig. 1 TG and histamine both activated a CRAC-like current in presence of 10 mM Ba^{2+}/2 mM Ca^{2+}. Whole-cell recordings (perforated patch) of EA.hy926 activated by 100 μM histamine or 1 μM TG. **a**, **b** Average current–voltage relationships obtained at the maximal stimulation after addition of TG (**a**; n=16) or histamine (**b**; n=30) in the presence of 10 mM Ba^{2+} and 2 mM Ca^{2+} in the bath. Traces are mean ± SEM. **c**, **d** Time course of the current activated by TG (**c**) or histamine (**d**), followed by the inhibition by 10 μM La^{3+}. Current was measured at −115 mV over time. **e**, **f** Time course of the current activated by TG (**e**) or by histamine (**f**) in the presence of extracellular divalent ions, followed by the switch in divalent-free (DVF) condition. Current was measured at −115 mV over time. **g**, **h** Representative traces of the current during a step of voltage from 0 to −120 mV after TG (**g**) or histamine (**h**) activation

current, we tested the behavior of the current in divalent-free (DVF) extracellular medium. Removing the extracellular divalent cations after stimulation with TG (Fig. 1e) or histamine (Fig. 1f) led to a large Na^+ current due to the removal of Ca^{2+} block. This large Na^+ current exhibited a rapid depotentiation characteristic of I_{CRAC} ([52]). Because of the slow solution exchange of our system, we probably missed the initial increase in the current upon divalent removal, and the current amplitude increased about three times in DVF medium. Another known characteristic of I_{CRAC} is the rapid inactivation, which is due to a negative feedback of Ca^{2+} entering through the CRAC channel itself [65]. To test this characteristic, a step of voltage from 0 to −120 mV was applied once the currents activated by histamine or TG were fully developed. As shown in Fig. 1g, h, a fast inactivation was clearly observed. Even though the current shared common properties with the I_{CRAC}, the relatively negative E_{rev} points to a reduced Ca^{2+} selectivity compared to the I_{CRAC}. In any instance, the current activated by TG or histamine could not be differentiated in these experimental conditions, with 10 mM Ba^{2+}/2 mM Ca^{2+} in the bath.

TG and histamine activated different currents in presence of extracellular Ca^{2+}

In the second series of experiments, we added 10 mM Ca^{2+} instead of 10 mM Ba^{2+} in the extracellular solution (10 mM Ca^{2+}). Ba^{2+} (100 μM) was added to the medium to prevent the activation of the inward rectifying K^+ channels (K_{ir}), which were occasionally present on these cells (data not shown). In these conditions, the current activated after passive depletion of the ER Ca^{2+} stores with TG was inward rectifying with a reversed potential of +7.5±6.5 mV, (n=13) and an amplitude of -0.34 ± 0.04 pA/pF($n = 13$) at −115 mV (Fig. 2a). Surprisingly, histamine evoked an outward rectifying current that reversed at -37.5 ± 1.9 mV($n = 12$) (Fig. 2b). The two currents can be sequentially activated on the same cell: the addition of histamine activated the outward rectifying current, and the addition of TG on top of histamine activated the inward rectifying current (Fig. 2c, d). Both currents were inhibited by 10 μM La^{3+} (Fig. 2c).

The difference between the histamine-activated currents in the presence of 10 mM Ba^{2+}/2 mM Ca^{2+} versus 10 mM Ca^{2+} suggested a block of the outward rectifying current by Ba^{2+}. To test this hypothesis, the cells were first activated with histamine in the presence of 10 mM Ca^{2+}, and then the medium was switched to 10 mM Ba^{2+}/2 mM Ca^{2+}. The outwardly rectified current activated by histamine was rapidly inhibited in the presence of Ba^{2+}, and after a certain

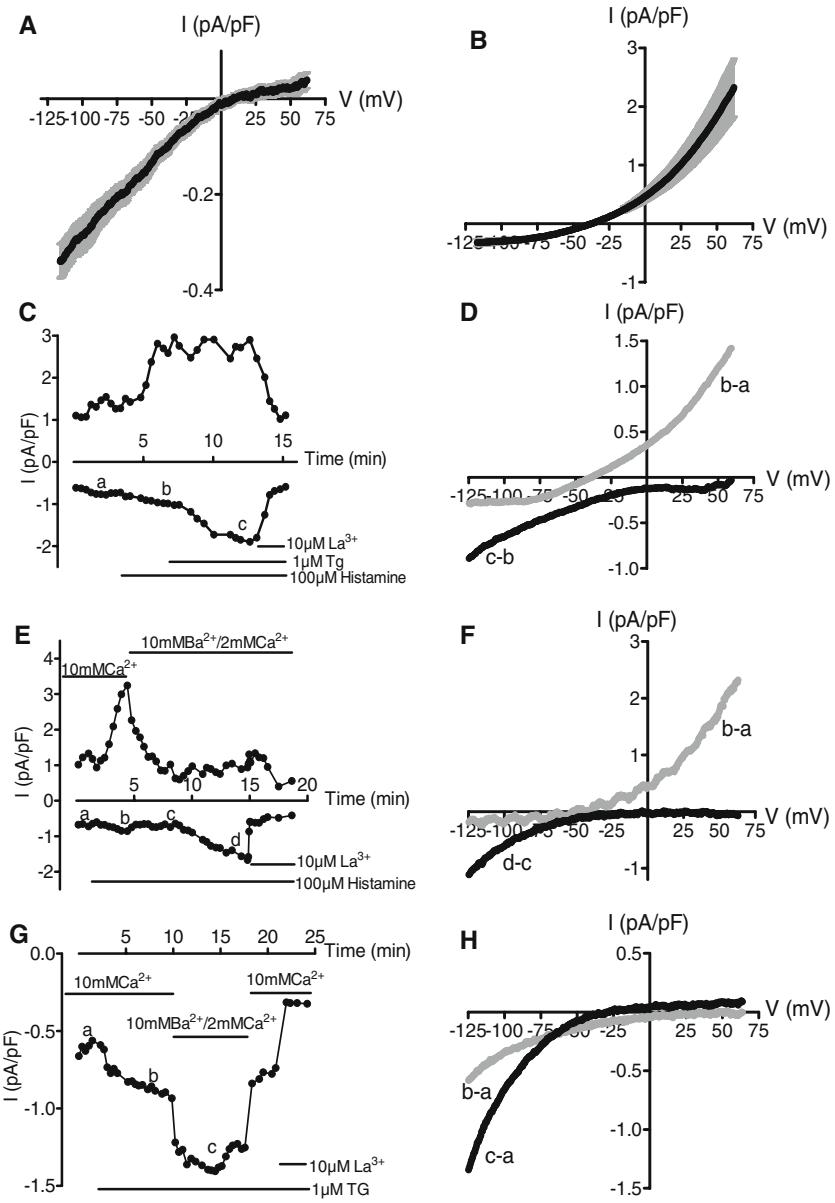

Fig. 2 Currents activated by TG or histamine in the presence of 10 mM Ca^{2+}. **a**, **b** Current–voltage relationships obtained at the maximal stimulation after addition of 1 μM TG (**a**; n=12) or 100 μM histamine (**b**; n=13) in the presence of 10 mM Ca^{2+}. Traces are mean ± SEM. **c** Time course of the current upon sequential activation with 100 μM histamine and 1 μM TG in the presence of 10 mM Ca^{2+}, followed by the inhibition with 10 μM La^{3+}. **d** Current–voltage relationships of the histamine activation (*b–a*; *gray dots*) and the TG activation (*c–b*; *black dots*). **e**, **g** Time course of the current activated by histamine (**e**) or TG (**g**) in 10 mM Ca^{2+} followed by a switch in 10 mM Ba^{2+}/2 mM Ca^{2+}. Currents were then inhibited by 10 μM La^{3+}. **f**, **h** Current–voltage relationships after histamine (**f**) or TG (**h**) activation in 10 mM Ca^{2+} (*b–a*; *gray dots*) or in 10 mM Ba^{2+}/2 mM Ca^{2+} (*d–c* for **f** or *c–a* for **h**; *black dots*). For all the time courses shown, the current value from each ramp was taken at −115 mV or/and at +60 mV

delay, an inward rectifying current developed (Fig. 2e, f). The data confirm our assumptions that Ba^{2+} prevents the histamine-activated outward rectifying current, while under these conditions, a distinct current substitutes for Ca^{2+} entry (the CRAC-like current). The same experiment was performed during cell stimulation with TG. In that case, the substitution of Ca^{2+} by Ba^{2+} increased the current amplitude at negative potentials (Fig. 2g, h), in line with previous I_{CRAC} recordings obtained on T lymphocytes [2].

ER Ca^{2+} levels upon cell stimulation measured during voltage clamp experiments

The inhibition of histamine-activated outward rectifying current by Ba^{2+} (Fig. 2e) explains that we could not observe this current in 10 mM Ba^{2+}/2 mM Ca^{2+} condition, but it does not explain the activation of a CRAC-like current in Ba^{2+} medium. For SOCE to get activated, a substantial ER Ca^{2+} depletion has to take place, which normally is not the case upon histamine stimulation due to a sufficient ER Ca^{2+} replenishment. In our electrophysiological recording condition, however, the ER might get more depleted than normal, as we clamped the potential at 0 mV between each ramp of voltages, decreasing the driving force for Ca^{2+} entry. Hence, the presence of Ba^{2+} and the clamp of voltage at 0 mV might possibly impact on the filling status of Ca^{2+} stores. To assess the effect of Ba^{2+} on ER Ca^{2+} levels during cell stimulation with histamine, we transiently transfected the cells with a cameleon-based Ca^{2+} probe targeted to the ER, $D1_{ER}$ [21, 31, 40]. The level of Ca^{2+} within the ER was then measured on cells clamped at 0 mV under the same experimental conditions as used for Figs. 1 and 2. Cells were first stimulated with 100 μM histamine in the presence of 10 mM Ca^{2+} or 10 mM Ba^{2+}/2 mM Ca^{2+}, followed by 1 μM TG to maximally empty the stores. The results presented in Fig. 3 clearly showed that, upon histamine stimulation, the ER Ca^{2+} level decreased more in the presence of high Ba^{2+} than in the presence of high Ca^{2+}. In 10 mM Ca^{2+}, histamine depleted the ER by $23.52 \pm 4.67\%(n = 6)$ of the depletion caused by TG, while in 10 mM Ba^{2+}/2 mM Ca^{2+}, the level of depletion reached $58.34 \pm 9.31\%(n = 6)$ of the response due to TG. Hence, even though 2 mM Ca^{2+} was present in the high Ba^{2+} solution during whole-cell recordings, the Ca^{2+} level within the ER decreased to a level likely sufficient to activate SOCE and might explain the activation of a CRAC-like current upon histamine stimulation.

Pharmacological profile of histamine-activated Ca^{2+} entry

We suspected that the NCX could be involved in the RACE mainly based on two points: the similarity between the shape of the IV curve of histamine-induced current compared to

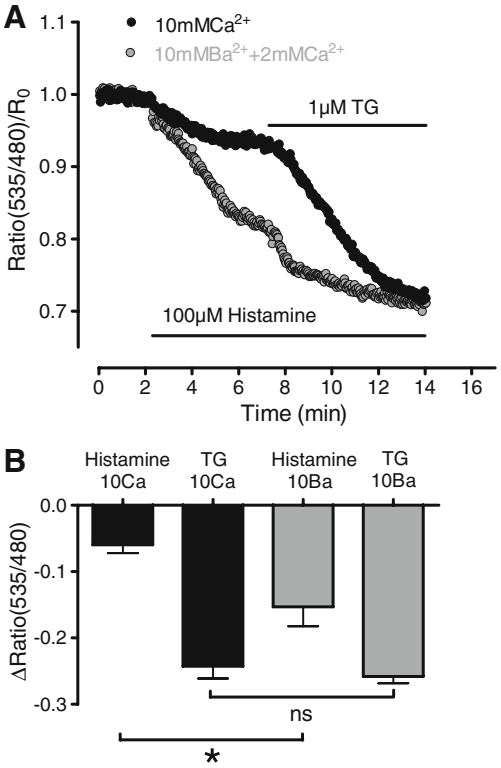

Fig. 3 Measurements of the ER Ca^{2+} level during whole-cell recordings. Cells were clamped at 0 mV under the same experimental conditions as presented in Figs. 1 and 2. The cells were transiently transfected with the ER-targeted cameleon probe $D1_{ER}$. **a** Histamine (100 μM) was applied in the presence of 10 mM Ca^{2+} (*black dots*) or 10 mM Ba^{2+} /2 mM Ca^{2+} (*gray dots*). After approximately 4 min of histamine stimulation, 1 μM TG was added to obtain a total depletion of the ER. **b** Statistics of the level of ER depletion after histamine and TG addition in the two different bath solutions. *Bars* are mean ± SEM (n=6 in 10 mM Ca^{2+}; n=6 in 10 mM Ba^{2+}/2 mM Ca^{2+}), *p<0.05

previous reports on NCX current [12, 30, 38] and reports demonstrating the implication of the NCX in Ca^{2+} entry [43, 48, 54]. Indeed, under certain conditions, the NCX can work in the reverse mode and allow Ca^{2+} to enter the cell. We thus wanted to determine whether part of the RACE was due to the reverse mode of the NCX on EA.hy926 cells. As a first approach, we stimulated the cells with histamine and added Ni^{2+}, a well-accepted blocker of the NCX (review [6]), on top of the response. As shown in Fig. 4a, adding Ni^{2+} initially completely blocked the response, and then about 50% of the cells showed some level of recovery. On the contrary, Ni^{2+} minimally affected TG-induced Ca^{2+} entry (Fig. 4b). We also tried another known inhibitor of the NCX, KB-R7943 [20]. At 10 μM or below, KB-R7943 was reported to inhibit preferentially the reverse mode of the NCX. However, it was also reported to have some off-targets; in particular, KB-R7943 activates BK_{Ca} channels [28]. As these channels are present on EA.hy926 cells and are activated by histamine [11], we used 5 mM tetraethylammonium (TEA) to prevent their activations. Adding 10 μM KB-R7943 during the

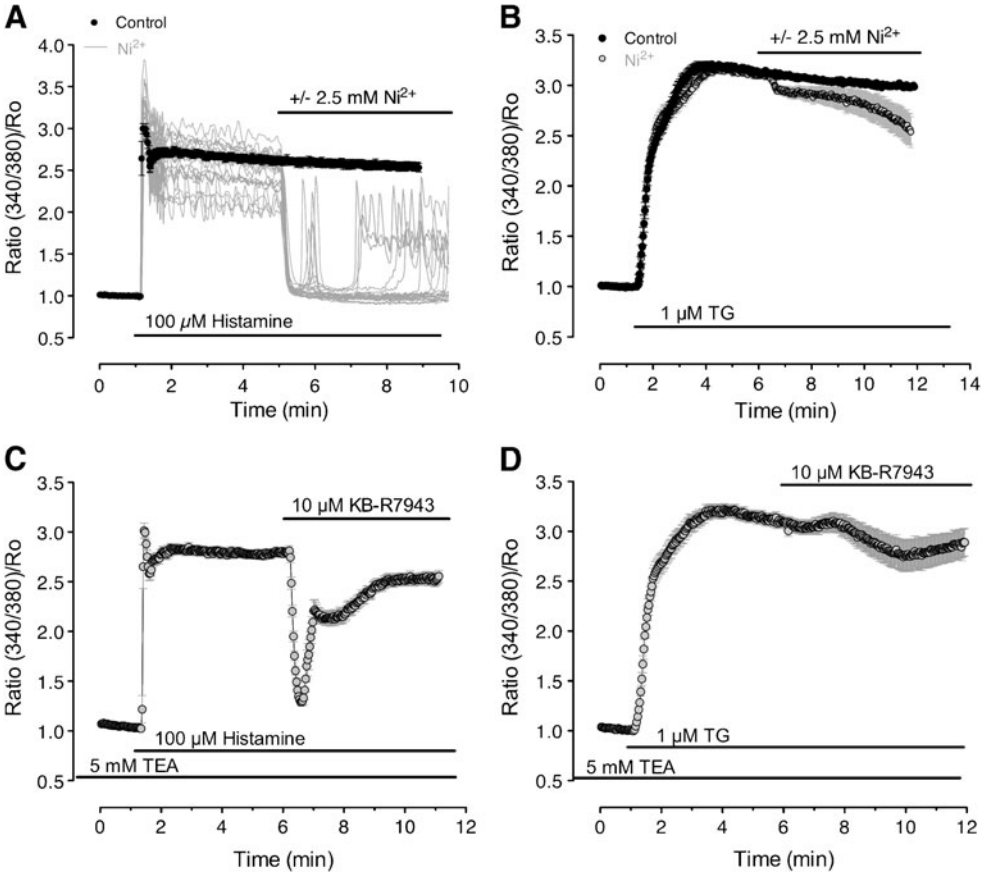

Fig. 4 Pharmacological profile of histamine-activated Ca^{2+} entry. EA.hy926 cells were loaded with 2 μM fura-2 to monitor cytosolic Ca^{2+} changes. Each trace is the mean of a representative cover slip out of three to four experiments. **a**, **b** Cells were stimulated with 100 μM histamine (**a**) or TG (**b**) in medium containing 2 mM Ca^{2+}, and 2.5 mM Ni^{2+} was added directly on the top of the response. Traces are mean ± SEM (histamine: n=11 control; TG: n=23 control; n=20 Ni^{2+}), except for **a** in the presence of Ni^{2+}, where all traces from a cover slip are graphed. **c**, **d** Cells were stimulated with 100 μM histamine (**a**) or TG (**b**) in medium containing 2 mM Ca^{2+}, and 10 μM KB-R7943 was added on during the plateau phase of the response. Traces are mean ± SEM (n=13 histamine; n=15 TG)

plateau phase of histamine response severely decreased Ca^{2+} entry, but the effect was transient and followed by a recovery phase (Fig. 4c). On the contrary, the response upon TG stimulation was mildly affected by the addition of KB-R7943 (Fig. 4d). The inhibition by Ni^{2+} and KB-R7943 thus suggested that the NCX is involved in histamine-induced Ca^{2+} entry.

Pharmacological profile of histamine-activated current

First, we wanted to ensure that the current activated by histamine was dependent on the signaling pathway activated by the agonist. For this purpose, we applied U73122, an inhibitor of the PLC (phospholipase C) during histamine-induced outward current, that fully blocked the response (Fig. 5a). The inactive analogue of the PLC blocker, U73343, did not inhibit the current activated by histamine (data not shown). We then used the same inhibitors as those tested on the cytosolic Ca^{2+} response to confirm the role played by the NCX. As shown on Fig. 5b, Ni^{2+} significantly inhibited the current activated by histamine by $61 \pm 13\%(n = 4)$. Similarly, 10 μM KB-R7943 also reduced by $80 \pm 10\%(n = 4)$ the current activated by histamine (Fig. 5c). In addition, neither Ni^{2+} nor KB-R7943 affected the current activated by TG, showing that the NCX is not implicated in SOCE (data not shown). Besides using inhibitors, we also investigated the effect of Na^+ removal on the histamine-induced outward current. As shown in Fig. 5d, in the absence of extracellular Na^+, the outward current strongly increased, which is in line with an increased activity of the NCX working in the reverse mode. Furthermore, the current recorded in 0 mM Na^+ was blocked by the addition of KB-R7943, as shown on the time course presented in Fig. 5e, again pointing to a current supported by the NCX. We also performed experiments under conditions where the intracellular Na^+ was increased to 50 mM, instead of 10 mM. In these conditions, an outward rectifying current was already present before the stimulation, which is in line with a basal stimulation of the NCX due to the high $[Na^+]_i$ conditions (Fig. 5f). Upon histamine stimulation, both the outward and the inward currents increased. This increase in the inward current (Fig. 5f), similar to what we observed in the absence of extracellular Na^+ (Fig. 5d), is not compatible with a current through the NCX, as the $E_{NCX}(E_{NCX} = 3E_{Na} - 2E_{Ca})$ under these ionic conditions is very negative and should lead exclusively to the outward current. This suggests that the inward current, at least in these experimental conditions, was unlikely due to NCX activation.

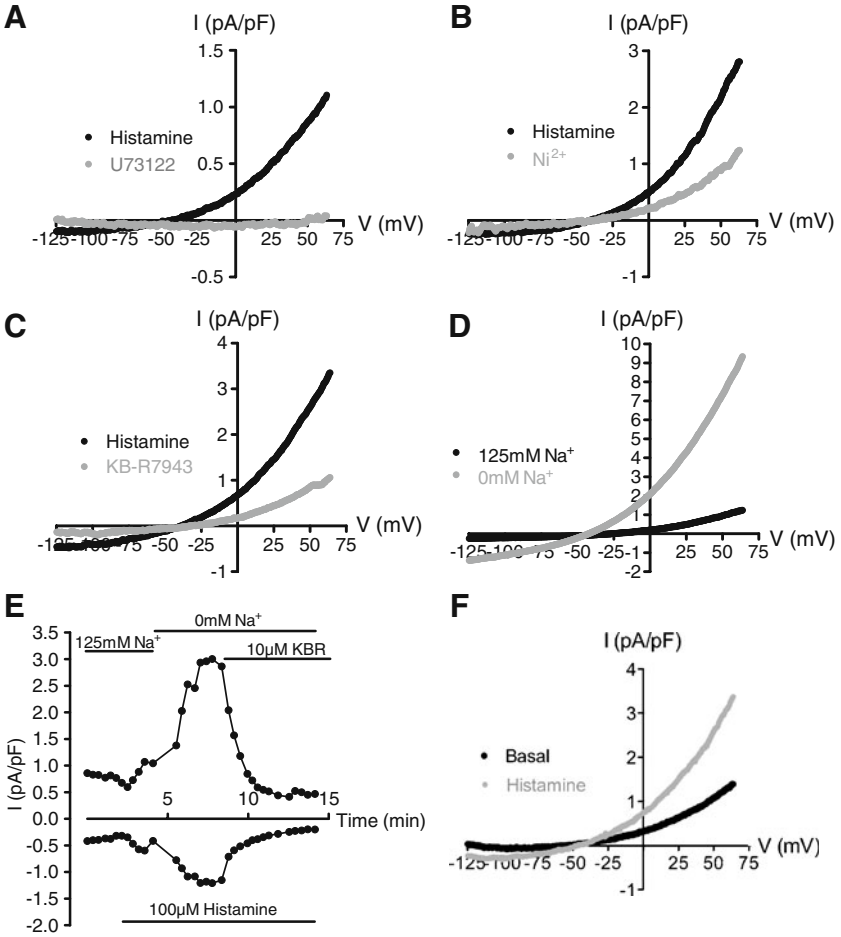

Fig. 5 Pharmacological profile of histamine-activated Ca^{2+} current. Whole-cell recordings (perforated patch) of EA.hy926 cells activated by 100 μM histamine. **a**–**c** Representative current–voltage relationships at the maximal stimulation after activation with histamine (*black dots*) and after addition of blockers (*gray dots*) in the presence of 10 mM Ca^{2+} in the bath. The blockers were added directly on the top of the histamine-activated current: 2 μM U731222 (**a**), 2.5 mM Ni^{2+} (**b**), and 10 μM KB-R7943 (**c**). **d** Representative current–voltage relationships obtained after stimulation with histamine in the presence of 125 mM Na^+ (*black dots*) and after a switch in a 0 mM Na^+ solution (*gray dots*). **e** Time course of the current after stimulation by 100 μM histamine in the presence of 125 mM Na^+ in the bath. After an initial increase in the current, the extracellular Na^+ was removed, leading to an increase in the current amplitude, which was subsequently inhibited by the addition of 10 μM KB-R7943. The current value from each ramp was taken at −115 mV and at +60 mV. **f** Representative current–voltage relationships obtained in basal condition with 50 mM Na^+ inside the patch pipette in basal condition (*black dots*), and after histamine stimulation (*gray dots*)

Discussion

The aim of the present study was to investigate whether it is possible, using an electrophysiological approach, to distinguish between current activated by passive store depletion from current activated by the inositol 1,4,5-trisphosphate (IP_3)-generating agonist histamine on endothelial cells. In other words, we intend to elucidate whether histamine-induced Ca^{2+} entry (the RACE) has an electrophysiological signature different from the SOCE, which would imply the activation of different channels. One of our main concerns was to avoid important ER Ca^{2+} depletion upon histamine stimulation. Indeed, in our previous papers [22, 31], we showed that in Ca^{2+}-containing medium, histamine stimulation did not induce an important ER Ca^{2+} store depletion, leading us to postulate that Ca^{2+} entry upon agonist stimulation does not essentially rely on SOCE but rather on RACE. For this reason, a minimum of 2 mM Ca^{2+} was present in all electrophysiological experiments. The other point was to let the intracellular signaling machinery as close as possible to what is taking place in an intact cell, thereby increasing the chance to record current different from the SOCE. Hence, we were using the perforated patch configuration to prevent dialysis of cytosolic compounds. We took these precautions to avoid uncontrolled activation of the SOCE in particular during experiments using an agonist to stimulate Ca^{2+} entry.

The CRAC-like current we recorded in 10 mM Ba^{2+}/2 mM Ca^{2+} extracellular medium shared many common features with the prototypical I_{CRAC} measured in RBL cells. For instance, it was strongly inward rectifying, was blocked by micromoles of lanthanides, and displayed a fast Ca^{2+}-dependent inactivation. It also permeates monovalent ions better when divalent ions are removed from the bath, a phenomenon called anomalous mole fraction. The current recorded in high Ba^{2+} showed a marked increased in amplitude at potentials below −40/−50 mV, a phenomenon already reported when using Ba^{2+} as a charge carrier in RBL cells [2, 19]. However, in Jurkat T cells, the Ba^{2+} permeability was reported to be lower than the Ca^{2+} permeability at all potentials [19]. A recent paper strengthened that the relative abundance of STIM1 compared to Orai1 affected the Ba^{2+} permeability of the I_{CRAC} [49], possibly explaining the discrepancy regarding the permeability of Ba^{2+} versus Ca^{2+}. A peculiar aspect of the CRAC-like current present on EA.hy926 cells was the relative negative E_{rev}, both in Ba^{2+} as well as in Ca^{2+} medium. This points to the fact that the CRAC-like current on EA.hy926 cells is less Ca^{2+} selective than the "classical" I_{CRAC}. Such a feature was often reported for SOCE currents in endothelial cells [34]. On EA.hy926 cells, we do not know whether it reflects a peculiar characteristic of SOCE or whether the current is actually the sum of different conductances, one being very similar to I_{CRAC}, with a high Ca^{2+} selectivity that displays the anomalous mole fraction and the fast Ca^{2+}-dependent inactivation, and the other being a nonselective cation current of unknown nature. A complete characterization of the current(s) activated by store depletion would require additional investigation, but this was beyond the scope of the present study. Recently, a CRAC-like current with a similar electrophysiological signature was reported on endothelial cells from human umbilical vein mediated by STIM1 and Orai1 proteins [1]. Hence, in our cellular system, we could not distinguish between the current activated by histamine from the current activated by TG, both sharing characteristics of the I_{CRAC}. This rather unexpected result prompted us to measure the Ca^{2+} level within the ER during electrophysiological recordings, and we found out that in fact histamine induced important Ca^{2+} store depletion, even though caution was taken to prevent it. The level of store depletion upon histamine stimulation was smaller than upon TG addition (about 60%; Fig. 3), but it was certainly strong enough to activate the SOCE. The depletion of the ER even in the presence of 2 mM Ca^{2+} can be explained by the following reasons: first, the presence of Ba^{2+} that blocked the outward rectifying current activated by histamine (see below) and, second, the reduced driving force for Ca^{2+} entry as the cells were clamped at 0 mV between the voltage ramps. This observation stressed the fact that ER Ca^{2+} depletion occurs easily and that great caution has to be taken in order to avoid it and thus allow the recording of store-independent agonist-induced current.

When Ba^{2+} was absent from the bath solution and replaced by 10 mM Ca^{2+}, the current activated by TG was very similar to that in Ba^{2+} containing medium except the E_{rev} that was slightly more positive. On the contrary, the current activated by histamine had a completely different shape from the one recorded in 10 mM Ba^{2+}/2 mM Ca^{2+}. In 10 mM Ca^{2+}, the current was outward rectifying with an E_{rev} around −40 mV. One should mention that this current was activated in about 70–80% of the cells, whereas in 10 mM Ba^{2+}/2 mM Ca^{2+}, almost 100% of the cells showed a CRAC-like current. In the remaining patches, either no detectable current was activated, or a current with a double rectification could be recorded. Due to the very low frequency of the later one, we could not further characterize this particular current.

In order to determine what type of current was activated by histamine, we used two different approaches: the addition of blockers and the ion substitution. The results we obtained all pointed to a current supported mainly by the NCX. The NCX can operate in both directions, meaning transporting Ca^{2+} out of the cell (forward mode) or into the cell (reverse mode). The direction of the ion exchange depends on the concentration gradients of both Na^{+} and Ca^{2+} and on the membrane potential (review in [6]). The reverse mode of the NCX was reported to take place in, e.g., smooth muscle cells [26, 27], NG2 glial cells [55], and also in endothelial cells [41, 48, 54]. An important condition leading to the reverse mode of the NCX is a local Na^{+} loading, a process that was suggested to significantly contribute to Ca^{2+} entry in endothelial cells [14, 41]. This was shown to happen as a consequence of nonselective cation channel opening that allows substantial Na^{+} entry, which in turn reverses the mode of the NCX. Functional link between TRPC (canonical transient receptor potential) channel activation and Ca^{2+} entry through the NCX was recently reported in different cell types [16, 27, 43, 47], and thus it is tempting to speculate that the same mechanism is responsible for the Na^{+} loading in EA.hy926 cells. We have to notice that we could not separate a current responsible for the Na^{+} loading from the current due to the exchanger. In addition, we cannot exclude that histamine activates the turnover rates of the NCX, favoring both the forward and the reverse modes. To drive the reverse mode activity of the exchanger, however, the intracellular Na^{+} concentration has to rise sufficiently to counteract the intracellular Ca^{2+} elevation that occurs during cell stimulation, and thus the system very likely requires a Na^{+} loading process. To assess the role of the NCX in our cellular system, we used Ni^{2+} and KB-R7943, which are both well-accepted blockers of the NCX even though they are not

considered as fully specific (review [6]). Indeed, KB-R7943 was shown to inhibit at least three different TRPC channels [25], and Ni^{2+} was described blocking SOCE [5, 13] as well as TRPC1 [53]. In our cellular system, both blockers reduced the current by about 70%, which is in favor of a role played by the NCX, while we cannot rule out that other conductances are also part of the current activated by histamine. Additionally, adding Ba^{2+} to the bath fully blocked the current, explaining why we did not record the outward rectifying current in 10 mM Ba^{2+}/2 mM Ca^{2+} medium. Previous studies that have investigated the ability of the NCX to transport ions other than Na^+ and Ca^{2+} have shown that indeed the transport efficiency of the exchanger was strongly reduced in the presence of Ba^{2+} [24, 50, 56], which is in line with our findings. Other reports suggested on the contrary that Ba^{2+} can be transported by the NCX, in particular extruded by the exchanger [7, 60]. The reasons for the contradictory results regarding the transport of Ba^{2+} by the exchanger remain to be clarified. We also showed that La^{3+} fully blocked the current, which was reported in previous studies [6, 24]. In the case of La^{3+}, it is more than likely that this compound not only inhibits the NCX but also other conductances, like TRPC channels that would be responsible for the initial Na^+ loading process. Hence, and even though no specific blockers exist for the NCX, all that we tested strongly inhibited histamine-induced outward current. The other approach we used was to change the extracellular Na^+ concentration. Removing Na^+ significantly increased the current amplitude, a behavior expected if the outward current was due to NCX. The concomitant increase in the inward current that we also observed when intracellular Na^+ was elevated (Fig. 5f) cannot be due to the NCX, as the E_{NCX} is very negative under these experimental conditions. The inward current could be due to TRPC channel activation, but the exact characterization of this current remains to be determined. Regarding the outward current, and as K^+ currents were prevented by the presence of Cs^+ in the patch pipette, the remaining possibility to explain the histamine-induced current was chloride. A large implication of a Cl^- current appears very unlikely for several reasons: in endothelial cells, the size of the Cl^- current activated by an agonist was in general much larger with a faster activation kinetic than the one activated on EA.hy926 cells [36, 37, 39]. The inhibitory effect of La^{3+}, Ba^{2+}, Ni^{2+}, and KB-R7943 that we observed on the outward rectifying current was, to our knowledge, never reported for Cl^- currents (review [35]). Finally, the increase in current amplitude upon Na^+ removal (Fig. 5d) was not compatible with a current due to Cl^- ions. Altogether, our experimental evidence point to a current supported for a large part by the NCX.

Our Ca^{2+} imaging experiments further pointed to an involvement of the NCX in the overall Ca^{2+} entry upon histamine stimulation. The addition of 2.5 mM Ni^{2+} strongly reduced the Ca^{2+} entry (Fig. 4a), an inhibition that was followed by a partial recovery of about half of the cells. Using 1 mM Ni^{2+} similarly blocked the response but with a more pronounced recovery phase (data not shown). Using KB-R7943 to reveal the role of the NCX was more problematic. Indeed, KB-R7943 alone hardly affected the plateau phase of Ca^{2+} entry (data not shown). Even though KB-R7943 is a potent blocker of the exchanger, it has also a rather long list of side effects. Among them, it was reported to activate BK_{Ca} channels [28], an effect that would increase Ca^{2+} entry as it hyperpolarizes the cells. Thus, to better reveal the action of KB-R7943 on the NCX, we blocked the BK_{Ca} channels with TEA. In these experimental conditions, KB-R7943 induced a transient drop of the Ca^{2+} plateau followed by a recovery. This pattern of inhibition with a recovery phase is a strong indication that agonist-induced Ca^{2+} influx is the result of several processes simultaneously engaged. Blocking only one of these players (like the NCX) is compensated possibly by other channels involved in the RACE or by channels supporting the SOCE pathway. Considering that EA.hy926 cells have a redundant system to allow Ca^{2+} entry, it is surprising that we could not consistently measure other types of currents on endothelial cells. We do not have so far a clear explanation for that, but we can propose three hypotheses: one, which is the most likely, is that the current activated by histamine was not purely supported by the NCX but that another conductance was also activated. Another explanation would be that the other currents supporting Ca^{2+} entry are of very small amplitude that makes their recording very challenging. Finally, the experimental conditions we were using might preclude the recording of additional currents. As a consequence, the current supported by the NCX is so far the only conductance involved in Ca^{2+} entry besides the SOCE current that we could characterize on EA.hy926 cells. The NCX working in the reverse mode accounts for a significant part of the Ca^{2+} entry, but certainly other players are also involved that would fully explain the RACE phenomenon.

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