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RESEARCH PAPER

The plasma membrane Na^+/Ca^{2+} exchange inhibitor KB-R7943 is also a potent inhibitor of the mitochondrial Ca^{2+} uniporter

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Background and purpose: The thiourea derivative KB-R7943, originally developed as inhibitor of the plasma membrane Na^+/Ca^{2+} exchanger, has been shown to protect against myocardial ischemia-reperfusion injury. We have studied here its effects on mitochondrial Ca^{2+} fluxes.
Experimental approach. $[Ca^{2+}]$ in cytosol, mitochondria and endoplasmic reticulum (ER), and mitochondrial membrane potential were monitored using both luminescent (targeted aequorins) and fluorescent (fura-2, tetramethylrhodamine ethyl ester) probes in HeLa cells.
Key results: KB-R7943 was also a potent inhibitor of the mitochondrial Ca^{2+} uniporter (MCU). In permeabilized HeLa cells, KB-R7943 inhibited mitochondrial Ca^{2+} uptake with a Ki of 5.5 ± 1.3 μM (mean ± S.D.). In intact cells, 10μM KB-R7943 reduced by 80% the mitochondrial $[Ca^{2+}]$ peak induced by histamine. KB-R7943 did not modify the mitochondrial membrane potential and had no effect on the mitochondrial Na^+/Ca^{2+} exchanger. KB-R7943 inhibited histamine-induced ER-Ca^{2+} release in intact cells, but not in cells loaded with a Ca^{2+}-chelator to damp cytosolic $[Ca^{2+}]$ changes. Therefore, inhibition of ER-Ca^{2+}-release by KB-R7943 was probably due to the increased feedback Ca^{2+}-inhibition of inositol 1,4,5-trisphosphate receptors after MCU block. This mechanism also explains why KB-R7943 reversibly blocked histamine-induced cytosolic $[Ca^{2+}]$ oscillations in the same range of concentrations required to inhibit MCU.
Conclusions and Implications: Inhibition of MCU by KB-R7943 may contribute to its cardioprotective activity by preventing mitochondrial Ca^{2+}-overload during ischemia-reperfusion. In addition, the effects of KB-R7943 on Ca^{2+} homeostasis provide new evidence for the role of mitochondria modulating Ca^{2+}-release and regenerative Ca^{2+}-oscillations. Search for permeable and selective MCU inhibitors may yield useful pharmacological tools in the future.
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Keywords: Ca^{2+} signalling; mitochondria; endoplasmic reticulum; KB-R7943; Ca^{2+} uniporter; inositol 1,4,5-trisphosphate receptor

Abbreviations: AM, acetoxymethyl ester; $[Ca^{2+}]_c$, cytosolic $[Ca^{2+}]$; $[Ca^{2+}]_M$, mitochondrial $[Ca^{2+}]$; $[Ca^{2+}]_{ER}$, $[Ca^{2+}]$ in endoplasmic reticulum; ER, endoplasmic reticulum; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; HEDTA, *N*-(2-hydroxyethyl)ethylenediamine-*N*,*N*′,*N*′-triacetic acid; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea; MCU, mitochondrial Ca^{2+} uniporter; TMRE, tetramethylrhodamine ethyl ester

Introduction

During cell activation, cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) rises and activates the mitochondrial Ca^{2+} uniporter (MCU). This is a selective Ca^{2+} channel, which transports and accumulates Ca^{2+} in mitochondria, driven by the large electrical potential difference between the cytosol and the mitochondrial matrix. MCU is a highly elusive channel from the molecular point of view, as it has neither been cloned nor isolated yet, and its activity has only been measured by monitoring Ca^{2+} transport into mitochondria (Rizzuto *et al.*, 1994; Bernardi, 1999) or more recently by patch-clamping of mitoplasts (Kirichok *et al.*, 2004). The activity of MCU is important, first, to determine the rate of Ca^{2+} entry into mitochondria and thus the mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_M$).

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It has been shown that the increase in $[Ca^{2+}]_M$ activates mitochondrial oxidative processes leading to increased NADH and ATP production (Jouaville *et al*., 1999; Rutter and Rizzuto, 2000). On the other hand, mitochondrial Ca^{2+} overload may lead to opening of the permeability transition pore and induce necrosis or apoptosis (Bernardi *et al*., 2001; Hajnoczky *et al*., 2003; Rizzuto *et al*., 2003), a process which has important pathological implications. There is evidence, for example, that this process occurs after heart or brain ischemia and reperfusion and is a major mediator of the subsequent cellular injury and death (for reviews see Halestrap, 2006; Di Lisa and Bernardi, 2006; Vercesi *et al*., 2006).

In addition, in the last decade, increasing evidence has pointed to the role of mitochondria as a modulator of cytosolic Ca^{2+} signalling (Babcock *et al*., 1997; Giovannucci *et al*., 1999; Duchen, 2000; Montero *et al*., 2000; Rizzuto *et al*., 2000). This role is fulfilled mainly through the activity of MCU for Ca^{2+} uptake into mitochondria, and the mitochondrial Na^+/Ca^{2+} exchange (NCX) for Ca^{2+} exit from mitochondria (see Bernardi, 1999), although the permeability transition pore may also play a role under certain conditions (Ichas *et al*., 1997). MCU is closed under resting conditions and becomes activated when $[Ca^{2+}]_c$ rises to the micromolar range. This low-Ca^{2+} affinity implies that mitochondrial Ca^{2+} uptake is effective in modulating the local high-Ca^{2+} microdomains that trigger most of the physiological effects of Ca^{2+} signalling (Berridge *et al*., 2003). For example, mitochondria have been shown to modulate catecholamine secretion in chromaffin cells (Giovannucci *et al*., 1999; Montero *et al*., 2000), the Ca^{2+}-dependence of voltage-dependent Ca^{2+} channels (Hernández-Guijo *et al*., 2001) and capacitative Ca^{2+} channels (Hoth *et al*., 2000), the rate of cytosolic Ca^{2+} waves (Boitier *et al*., 1999), and the dynamics of $[Ca^{2+}]_c$ oscillations (Collins *et al*., 2000; Hernández-SanMiguel *et al*., 2006; Vay *et al*., 2007).

KB-R7943 was developed 10 years ago as a selective plasma membrane NCX inhibitor (Iwamoto *et al*., 1996), and was the starting compound of a family of NCX inhibitors, which have been shown to protect against myocardial ischemia–reperfusion injury (Matsuda *et al*., 2001; Iwamoto, 2004; Iwamoto and Kita, 2004; Hagihara *et al*., 2005; Matsunaga *et al*., 2005). We show here that KB-R7943 is also a potent MCU inhibitor, an effect which could contribute to its cardioprotective activity. In addition, given that HeLa cells lack any detectable plasma membrane NCX activity (Furman *et al*., 1993; Low *et al*., 1993), KB-R7943 could be considered a specific inhibitor of MCU in these cells. We use here this new property of KB-R7943 to show that MCU block inhibits $InsP_3$-mediated Ca^{2+} release and $[Ca^{2+}]$ oscillations in intact HeLa cells. This provides new evidence for the role of mitochondria modulating $[Ca^{2+}]_c$ homeostasis and opens the way for the search of more specific and permeable MCU blockers.

Methods

Cell culture and targeted aequorin expression

HeLa cells were grown in Dulbecco's-modified Eagle's medium supplemented with 10% fetal calf serum, 50 IU ml^{-1} penicillin and 50 IU ml^{-1} streptomycin. The HeLa cell clone MM5, which stably expresses mitochondrially targeted mutated aequorin has been described previously (Montero *et al*., 2002). The constructs for aequorin targeted to the cytosol and mutated aequorin targeted to either the endoplasmic reticulum (ER) or the mitochondria have been also described previously (Montero *et al*., 1995, 2000). Transfections were carried out using Metafectene (Biontex, Munich, Germany).

$[Ca^{2+}]_M$, $[Ca^{2+}]_c$ and $[Ca^{2+}]_{ER}$ measurements with aequorin

The HeLa cell clone MM5 expressing mitochondrially targeted mutated aequorin was used for most of the $[Ca^{2+}]_M$ measurements, and similar results were obtained using transiently transfected HeLa cells. $[Ca^{2+}]_c$ measurements in cell populations were carried out using HeLa cells transiently transfected with the plasmid for cytosolic aequorin. Cells were plated onto 13 mm round coverslips. For aequorin reconstitution in experiments using intact cells, HeLa cells expressing either cytosolic or mitochondrially targeted aequorin were incubated for 1–2 h at room temperature in standard medium (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose and 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), pH 7.4) with 1 μM of wild-type coelenterazine. Cells were then placed in the perfusion chamber of a purpose-built luminometer maintained at 37°C. For experiments with permeabilized cells, MM5 cells expressing mitochondrially targeted mutated aequorin were reconstituted with 1 μM coelenterazine n for 1–2 h and then placed in the luminometer as above. Then, standard medium containing 0.5 mM ethylene glycol tetraacetic acid (EGTA) instead of Ca^{2+} was perfused for 1 min, followed by 1 min of intracellular medium (130 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM K_3PO_4, 0.5 mM EGTA, 1 mM ATP, 20 μM ADP, 2 mM succinate, 20 mM HEPES and pH 7) containing 100 μM digitonin. Then, intracellular medium without digitonin was perfused for 5–10 min, followed by buffers of known $[Ca^{2+}]$ prepared in intracellular medium using *N*-(2-hydroxyethyl)ethylenediamine-*N*,*N*′,*N*′-triacetic acid (HEDTA)/Ca^{2+}/Mg^{2+} mixtures. Measurements of $[Ca^{2+}]$ in endoplasmic reticulum ($[Ca^{2+}]_{ER}$) were carried out using HeLa cells transiently transfected with the plasmid for ER-targeted aequorin. Cells were plated onto 13 mm round coverslips. Before reconstituting aequorin, $[Ca^{2+}]_{ER}$ was reduced by incubating the cells for 10 min at 37°C with the sarcoplasmic and ER Ca^{2+}-ATPase inhibitor 2,5-di-tert-buthyl-benzohydroquinone 10 μM in standard external medium containing (in mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; glucose, 10; HEPES, 10, pH 7.4, supplemented with 0.5 mM EGTA. Cells were then washed and incubated for 1 h at room temperature in the same medium with 1 μM coelenterazine n. In some experiments, 10 μM EGTA-acetoxymethyl ester (EGTA-AM) was also added during this period to load the cells with a Ca^{2+} chelator. Then, the coverslip was placed in the perfusion chamber of a purpose-built luminometer (see above), and standard medium containing 0.5 mM EGTA was perfused for 5 min before the experiment. Calibration of the luminescence data into $[Ca^{2+}]$ was made using an algorithm as described previously (Montero *et al*., 2002).

Measurement of mitochondrial membrane potential
Mitochondrial membrane potential was monitored using the fluorescent indicator tetramethylrhodamine ethyl ester (TMRE). HeLa cells were placed in the cell chamber, permeabilized as described above and then perfused with intracellular medium containing 20nM TMRE until a steady-state fluorescence was reached (usually about 5 min). Single cell fluorescence was excited at 540 nm using a Cairn monochromator (200 ms excitation every 2 s) and images of the fluorescence emitted between 570 and 630 nm obtained with a $\times 40$ Fluar objective were recorded by a Hamamatsu ORCA-ER camera. Single cell fluorescence records were analyzed off-line using the Metafluor program (Universal Imaging, Photometrics UK, Marlow, Buckinghamshire, UK). Experiments were performed at 37°C using an on-line heater (Harvard Apparatus, Holliston, MA, USA).

Single cell $[Ca^{2+}]_c$ measurements
Fura-2 loaded HeLa cells were used for these measurements. Although this dye may distribute heterogeneously in different intracellular compartments, including ER and mitochondria, it is known to reflect mainly the behavior of the $[Ca^{2+}]$ in the cytosolic compartment. In HeLa cells in particular, both fura-2 and cytosolic aequorin have been shown to report similar histamine-induced $[Ca^{2+}]$ peaks (Brini *et al*., 1995), and abolition of mitochondrial Ca^{2+} uptake with a protonophore produced little changes in the histamine-induced $[Ca^{2+}]$ peak monitored with fura-2 (Rizzuto *et al*., 1994).

HeLa cells were loaded with fura-2 by incubation in standard medium containing 2 μM fura-2-AM for 45 min at room temperature. Cells were then washed with standard medium for 45 min at room temperature and mounted in a cell chamber on the stage of a Zeiss Axiovert 200 microscope (Carl Zeiss Inc., Thornwood, NY, USA) under continuous perfusion. Single cell fluorescence was excited at 340 and 380 nm using a Cairn monochromator (100 ms excitation at each wavelength every 2 s, 10 nm bandwidth) and images of the emitted fluorescence obtained with a $\times 40$ Fluar objective were collected using a 400DCLP dichroic mirror and a D510/80 emission filter (both from Chroma Technology Corp., Rockingham, VT, USA) and recorded by a Hamamatsu ORCA-ER camera (Hamamatsu Photonics KK, Hamamatsu City, Shizuoka Pref., Japan). Single cell fluorescence records were ratioed and calibrated into $[Ca^{2+}]$ values off-line as described before (Grynkiewicz *et al*., 1985) using the Metafluor program (Universal Imaging). Experiments were performed at 37°C using an on-line heater (Harvard Apparatus).

Data analysis
Statistical data are given as mean $\pm$ s.e.m., unless otherwise stated. IC_{50} values were calculated using the four parameter ($logEC_{50}$) fitting routine of the BioDataFit program (Chang Bioscience Inc., Castro Valley, CA, USA). Differences between means were evaluated with analysis of variance (ANOVA) and significance when $P<0.05$

Materials
Wild-type coelenterazine, coelenterazine n, fura-2-AM and TMRE were obtained (Molecular Probes, OR, USA). KB-R7943 (Tocris, Bristol, UK). Other reagents were (Sigma, Madrid or Merck, Darmstadt).

Results

Inhibition by KB-R7943 of the MCU
In Figure 1, we show the effects of KB-R7943 on mitochondrial Ca^{2+} uptake in permeabilized HeLa cells perfused with a controlled Ca^{2+} buffer. A HeLa cell clone expressing the Ca^{2+}-dependent photoluminescent protein aequorin targeted to mitochondria was used for these experiments, so that we could monitor directly $[Ca^{2+}]$ changes inside mitochondria from the light emission generated. The rate of increase in $[Ca^{2+}]_M$ in the presence of the $[Ca^{2+}]$ buffer is proportional to the activity of the MCU. In the upper panel, KB-R7943 dose-dependently inhibited Ca^{2+} uptake into mitochondria. The lower panel shows the sigmoidal dose–effect curve obtained from a series of experiments similar to those in the upper panel. The IC_{50} was 5.5 ± 1.3 μM (mean $\pm$ s.d., four parameter fitting).

The inhibition of mitochondrial Ca^{2+} uptake by KB-R7943 was not owing to mitochondrial depolarization. Figure 2 shows that 20 μM KB-R7943, a concentration well above the IC_{50}, did not induce any depolarization of the mitochondrial membrane potential in permeabilized HeLa cells. In contrast, subsequent addition of the protonophore FCCP rapidly

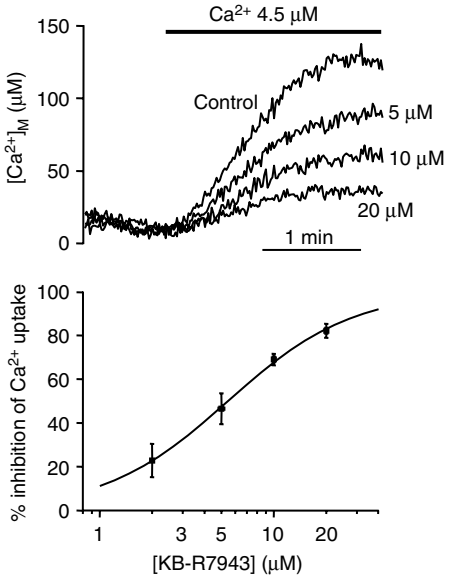

Figure 1 Effect of KB-R7943 on mitochondrial Ca^{2+} uptake in permeabilized cells. In the upper panel, HeLa cells expressing mitochondrially targeted mutated aequorin reconstituted with coelenterazine n were permeabilized as described in Methods and then a 4.5 μM $[Ca^{2+}]$ buffer was perfused either in control cells or in the presence of different concentrations of KB-R7943, as indicated in the figure. The inhibitor was added 5 min before perfusion of the $[Ca^{2+}]$ buffer. The lower panel shows the dose-dependence of the inhibition obtained from 4–8 results obtained at each concentration.

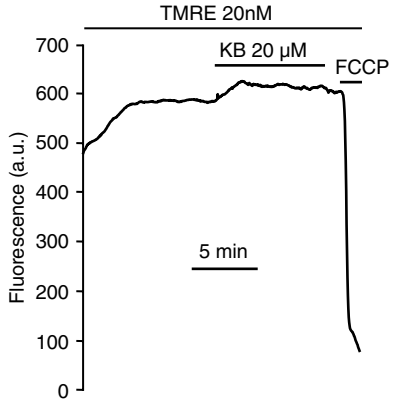

Figure 2 Effect of KB-R7943 on mitochondrial membrane potential in permeabilized HeLa cells. Cells were permeabilized as described in Methods and then 20 nM TMRE was perfused until a steady-state fluorescence was reached. Then, either 20 μM KB-R7943 or 2 μM FCCP were added as indicated in the same intracellular medium containing 20 nM TMRE. The trace shown is the mean response of 19 cells present in the microscope field in this experiment and is representative of the results obtained in three similar experiments.

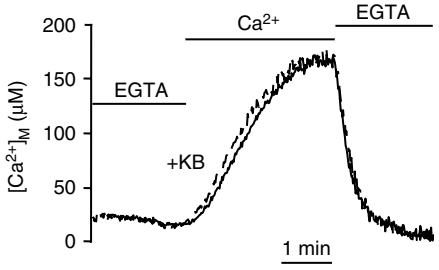

Figure 3 Effect of KB-R7943 on mitochondrial Ca^{2+} release in permeabilized cells. HeLa cells expressing mitochondrially targeted mutated aequorin reconstituted with coelenterazine n were permeabilized as described in Methods. Then, a $[Ca^{2+}]$ buffer was perfused both in control cells (continuous line) or in the presence of 20 μM KB-R7943 (dashed line), as indicated in the figure. The $[Ca^{2+}]$ in that buffer was adjusted to obtain similar rates of Ca^{2+} uptake (4.5 μM in the control and 5.5 μM in the presence of KB-R7943). Finally, the $[Ca^{2+}]$ buffer was substituted by medium containing 0.5 mM EGTA with or without 20 μM KB-R7943, to trigger Ca^{2+} release from mitochondria. The traces shown are the mean of three similar experiments and are representative of 16 similar ones of each kind.

collapsed membrane potential. On the other hand, as KB-R7943 is known to inhibit the plasma membrane NCX, we have also tested if the mitochondrial NCX system is sensitive to this inhibitor. Figure 3 shows that 20 μM KB-R7943 did not modify Ca^{2+} release from mitochondria when the $[Ca^{2+}]$ buffer was substituted by EGTA. The rate of Ca^{2+} uptake in the presence of KB-R7943 was adjusted to be similar to that in the control by increasing the $[Ca^{2+}]$ in the perfusion buffer. Then, when the $[Ca^{2+}]$ buffer was substituted by EGTA, the rate of Ca^{2+} release from mitochondria was the same both in the presence and in the absence of this drug. Therefore, as KB-R7943 does not inhibit the mitochondrial NCX and HeLa cells do not possess plasma membrane NCX

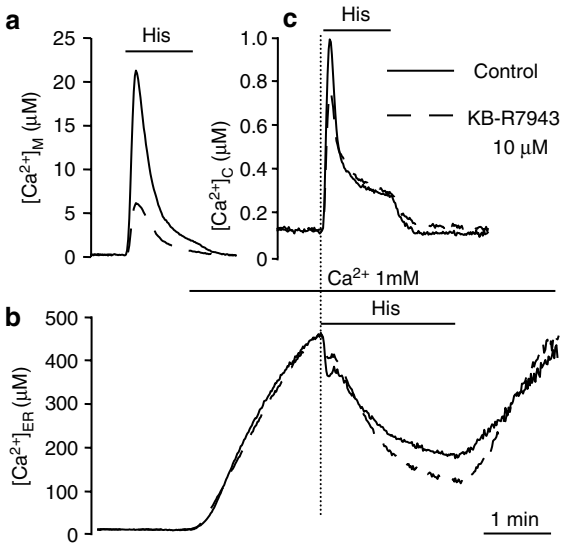

Figure 4 Effect of KB-R7943 on mitochondrial, ER and $[Ca^{2+}]_c$ transients induced by histamine. HeLa cells expressing aequorin targeted either to mitochondria, to the ER or to the cytosol were stimulated with 100 μM histamine either in the absence or in the presence of 10 μM KB-R7943. The inhibitor was added in all the cases 5 min before the stimulation with histamine. All panels use the same time scale and a dotted line marks the addition of histamine in (**b** and **c**), to facilitate comparison of $[Ca^{2+}]_c$ and $[Ca^{2+}]_{ER}$ dynamics. In (**a**), the traces shown are the mean of six different experiments of each type. In (**b**), the traces shown are the mean of four different experiments of each type. In (**c**), the traces shown are the mean of nine different experiments of each type.

(Furman *et al*., 1993; Low *et al*., 1993), this compound can be considered in principle a selective inhibitor of MCU in this cell type.

Effect of MCU inhibition with KB-R7943 on ER-Ca^{2+} release and $[Ca^{2+}]_c$ peak

We have taken advantage of this property of KB-R7943 to study the effect of a selective inhibition of MCU on ER-Ca^{2+} release and cytosolic Ca^{2+} signaling. Figure 4a shows that the $[Ca^{2+}]_M$ peak induced by histamine was substantially reduced in the presence of 10 μM KB-R7943. In six similar experiments of each type, the $[Ca^{2+}]_M$ peak was (mean $\pm$ s.e.) 21.3 $\pm$ 2.5 μM in the controls and 6.2 $\pm$ 1.0 μM in the presence of 10 μM KB-R7943. This phenomenon could be directly attributed to the inhibition of MCU. However, that mechanism was only partially responsible for the dramatic decrease in the $[Ca^{2+}]_M$ peak. Figure 4b shows that the release of Ca^{2+} from the ER induced by histamine was also modified in the presence of KB-R7943. As we have reported previously (Montero *et al*., 1997), histamine induces a biphasic Ca^{2+} release from the ER, an initial fast drop of about 100 μM $[Ca^{2+}]$ in less than 10 s followed by a slower phase of Ca^{2+} release leading to ER-$[Ca^{2+}]$ values of around half the initial ones and stable while histamine is present. Washing out histamine then allows the ER to recover the initial steady-state ER-$[Ca^{2+}]$ values around 500 μM. The presence of KB-R7943 reduced the initial drop of $[Ca^{2+}]_{ER}$ to half the control values (decrease by 114 $\pm$ 6 μM in the controls and by only

$52 \pm 6\,\mu$M in the presence of $10\,\mu$M KB-R7943, mean $\pm$ s.e., $n=8$; $P<0.0005$, ANOVA test), but increased the rate of Ca^{2+}-release during the subsequent slow phase, as well as the total Ca^{2+} release (in the presence of $10\,\mu$M KB-R7943, the $[Ca^{2+}]_{ER}$ obtained 2 min after histamine addition was $185 \pm 12\,\mu$M in the controls and $128 \pm 6\,\mu$M in the presence of $10\,\mu$M KB-R7943, mean $\pm$ s.e., $n=8$; $P<0.005$, ANOVA test). These effects of KB-R7943 on Ca^{2+} release from the ER are directly mirrored by the $[Ca^{2+}]_c$ peak (Figure 4c). The $[Ca^{2+}]_c$ peak induced by histamine was reduced from $1.02 \pm 0.02\,\mu$M in the controls to 0.78 ± 0.03 in the presence of KB-R7943 (mean $\pm$ s.e., $n=9$ in both cases; $P<0.0005$, ANOVA test), but the subsequent shoulder was higher in the presence of KB-R7943, consistent with the faster rate of Ca^{2+} release at that point. The figure shows in the same timescale both ER-Ca^{2+} release and the $[Ca^{2+}]_c$ peak to allow comparison.

The inhibition of the fast phase of Ca^{2+} release through $InsP_3R$ by KB-R7943 could be an indirect consequence of its effect on MCU and the resulting interference with the mitochondria–ER-Ca^{2+} interplay, but could also be just a direct effect of this compound on $InsP_3R$. To exclude this possibility, we measured the rate of histamine-induced Ca^{2+} release from the ER in cells loaded with a Ca^{2+} chelator. As we have shown previously (Montero *et al.*, 1997), loading a Ca^{2+} chelator removes feedback Ca^{2+} inhibition of $InsP_3R$ and largely prolongs the fast phase of ER-Ca^{2+} release. The presence of a high-capacity and high-affinity Ca^{2+} chelator in the cytosol also damps $[Ca^{2+}]_c$ increase and avoids Ca^{2+} uptake by mitochondria, which are no longer able to regulate ER-Ca^{2+} release. Under these conditions, KB-R7943 did not modify histamine-induced Ca^{2+} release (Figure 5), showing that its effects are not due to a direct inhibition of $InsP_3R$.

Effect of MCU inhibition with KB-R7943 on $[Ca^{2+}]_c$ oscillations

The data of Figures 4 and 5 suggested that inhibition of MCU leads to a significant reduction in the fast initial drop of ER-Ca^{2+} release induced by histamine in intact cells. This is consistent with the previously reported inhibition of ER-Ca^{2+} release in HeLa cells in the presence of protonophores (Collins *et al.*, 2000) and shows that a selective inhibition of MCU, in the absence of mitochondrial depolarization, produces the same results. Collins *et al.* (2000) also showed that protonophores reversibly abolish histamine-induced cytosolic Ca^{2+} oscillations. We then decided to study if selective inhibition of MCU could also produce the same results. That was the case, as shown in Figure 6a where $10\,\mu$M KB-R7943 completely and reversibly blocked histamine-induced $[Ca^{2+}]_c$ oscillations. This effect

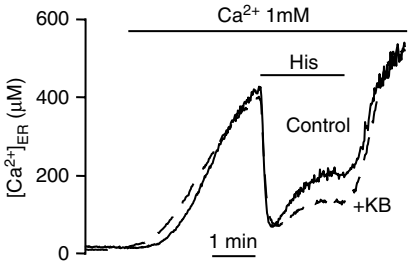

Figure 5 Effect of KB-R7943 on histamine-induced Ca^{2+} release in cells loaded with a Ca^{2+} chelator. HeLa cells expressing aequorin targeted to the ER were loaded with EGTA by incubation with $10\,\mu$M EGTA-AM for 1 h during aequorin reconstitution with coelenterazine n. Then, after refilling the ER with Ca^{2+}, they were stimulated with $100\,\mu$M histamine either in the absence or in the presence of $10\,\mu$M KB-R7943. The inhibitor was present 5 min before stimulation with histamine. The traces shown are the mean of three different experiments of each type and are representative of eight similar experiments of each type.

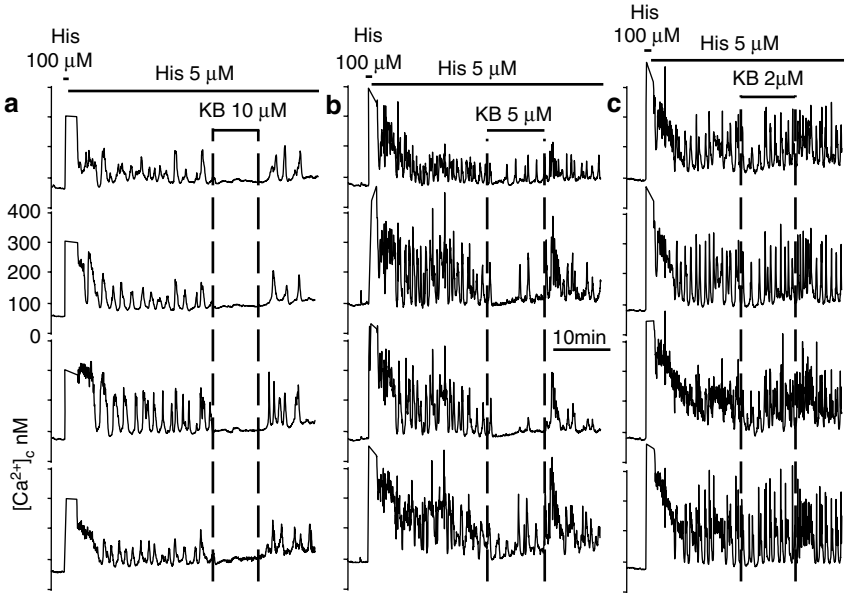

Figure 6 Effect of KB-R7943 on histamine-induced $[Ca^{2+}]$ oscillations. Fura-2-loaded HeLa cells were stimulated with histamine and treated with either 10, 5 or $2\,\mu$M KB-R7943, as indicated in the figure. Traces of four representative cells present in the same microscope field in one experiment of each type are shown. The initial histamine-stimulated $[Ca^{2+}]_c$ peaks are truncated for convenience. Data are representative of 306 (**a**), 326 (**b**) and 53 (**c**) analyzed cells in several experiments similar to those shown in the figures.

of KB-R7943 on $[Ca^{2+}]_c$ oscillations had the same dose-dependency as the inhibition of MCU shown in Figure 1. Figure 6b shows that 5 μM KB-R7943 (concentration producing half-maximal inhibition of MCU) only partially blocked oscillations. In a series of 326 analyzed cells, 5 μM KB-R7943 completely blocked oscillations in 132 (40%), whereas 10 μM KB-R7943 completely abolished oscillations in 261 of 306 analyzed cells treated with this concentration of the inhibitor (85%). In these cells, the oscillatory behavior remained blocked for as long as the inhibitor was present (up to 20 min in some experiments, data not shown) and reappeared rapidly when it was washed out. The rest of the analyzed cells treated with 5 or 10 μM KB-R7943 showed in any case a clear decrease in the oscillatory frequency. Interestingly, KB-R7943 concentrations below 5 μM did not block oscillations but still produced some subtle effects on the oscillatory pattern. Figure 6c shows that 2 μM KB-R-7943 induced a transient decrease in the frequency of the oscillations immediately after addition of the compound. Then, after a couple of minutes, the oscillatory frequency recovered even though KB-R7943 was still present. This recovery was probably an adaptation of the oscillatory mechanism to the new situation because, when the compound was washed out, there was an immediate burst of activity before turning back to the original pattern.

Discussion

We describe in this paper a new target for KB-R7943 in the MCU. This compound was originally developed as an inhibitor of the plasma membrane NCX (Iwamoto *et al*., 1996), and was in fact the starting compound of a family of inhibitors of this exchange system which are being explored for their possible cardioprotective efficacy in cardiac ischemia–reperfusion injury (Matsuda *et al*., 2001; Iwamoto, 2004; Iwamoto and Kita, 2004; Hagihara *et al*., 2005; Matsunaga *et al*., 2005). We show here that KB-R7943 is also a potent inhibitor of the MCU at concentrations similar to those required for the inhibition of the plasma membrane NCX. This new target may be relevant in interpreting its effects in cardioprotection and ischemia–reperfusion injury. In fact, ischemia–reperfusion injury has been widely shown to involve mitochondrial Ca^{2+} overload and opening of the permeability transition pore (for reviews see Halestrap, 2006; Di Lisa and Bernardi, 2006; Vercesi *et al*., 2006). Thus, inhibition by KB-R7943 of the MCU may block the increase in $[Ca^{2+}]_M$ and the subsequent opening of the permeability transition pore.

The inhibitory effect of KB-R7943 on mitochondrial Ca^{2+} uptake could also be useful in exploring the role of mitochondrial Ca^{2+} uptake in global cell Ca^{2+} homeostasis. However, KB-R7943 is not very specific. In addition to its effects on the plasma membrane NCX, KB-R7943 inhibits other plasma membrane channels and transporters, such as NMDA, K^+, Na^+ and Ca^{2+} channels (Sobolevsky and Khodorov, 1999; Matsuda *et al*., 2001; Tanaka *et al*., 2002) and the nicotinic acetylcholine receptor (Pintado *et al*., 2000). Most of these channels and transporters, however, are only present in excitable cells, so that the only remaining target in many non-excitable cells (such as the HeLa cells used in this paper) appears to be the MCU. In addition, KB-R7943 is the first available inhibitor of the MCU, which is freely permeable through the plasma membrane. Up to now, the only MCU inhibitors known were Ruthenium Red and Ru360 (Bernardi, 1999), two compounds with very low permeability through the plasma membrane. Thus, the only way to investigate the effects of mitochondrial Ca^{2+} uptake on Ca^{2+} dynamics in intact cells was to use protonophores or inhibitors of the electron-transport chain. KB-R7943 now provides a possibility of investigating in non-excitable cells, the effect of inhibiting mitochondrial Ca^{2+} uptake in the absence of mitochondrial depolarization and other non-specific effects of those drugs.

We have used this property here to study the effect of inhibiting MCU on ER-Ca^{2+} release and histamine-induced $[Ca^{2+}]$ oscillations in HeLa cells. It has been shown previously that treatment with protonophores of HeLa cells inhibits Ca^{2+} release through $InsP_3R$ and blocks $[Ca^{2+}]$ oscillations (Collins *et al*., 2000). We show here that the same findings can be obtained simply by inhibiting MCU with KB-R7943. The conclusion is that MCU is a key controller of $InsP_3$-induced Ca^{2+} release.

The mechanism of the effect of MCU inhibition on Ca^{2+} release probably relies in the feedback inhibition by Ca^{2+} of $InsP_3R$. It has been known for many years that $InsP_3R$ are regulated by the $[Ca^{2+}]_c$ levels in the cytosolic mouth of the channel in a bell-shaped way (Bezprozvanny *et al*., 1991; Kaftan *et al*., 1997). This means that local $[Ca^{2+}]_c$ is a necessary co-agonist of the channel but an increase of $[Ca^{2+}]_c$ above the micromolar range becomes inhibitory. Inhibition of mitochondrial Ca^{2+} uptake will tend to increase the local $[Ca^{2+}]_c$ levels close to MCU, which probably may mean also close to $InsP_3R$. Physical close contacts between ER and mitochondria have been observed and mitochondria take up Ca^{2+} much more effectively after $InsP_3$-induced Ca^{2+} release than after global homogeneous Ca^{2+} increases (Rizzuto *et al*., 1998; Csordas *et al*., 1999). Thus, assuming that MCU and $InsP_3R$ partially co-localize, inhibition of MCU should lead to an increase in the levels of $[Ca^{2+}]_c$ around $InsP_3R$. Because of the biphasic dependence of Ca^{2+} release on the local $[Ca^{2+}]_c$ levels, a local $[Ca^{2+}]_c$ increase may produce either stimulation or inhibition of Ca^{2+} release, depending of the magnitude of the local $[Ca^{2+}]_c$ microdomain or of the Ca^{2+} sensitivity to stimulation or inhibition by Ca^{2+} of the particular $InsP_3R$ isoform involved (Mak *et al*., 2001). In hepatocytes, inhibition of mitochondrial Ca^{2+} uptake with protonophores increased Ca^{2+} release, suggesting that the increase in local $[Ca^{2+}]$ obtained by that maneuver was just enough to activate $InsP_3R$ in these cells (Hajnoczky *et al*., 1999). In HeLa cells, in contrast, we have shown that feedback Ca^{2+} inhibition is a major factor responsible for blocking histamine-induced Ca^{2+} release (Montero *et al*., 1997). This may explain why in these cells blocking MCU leads to a faster block of Ca^{2+} release. It is interesting to also note that MCU block led to a prolonged (at least 10–20 min) and rapidly reversible inhibition of cytosolic Ca^{2+} oscillations induced by histamine. This effect can be explained as a rapid feedback Ca^{2+} inhibition of local $InsP_3R$ (due to the absence of mitochon-

drial Ca^{2+} buffering) blocking the propagation of the Ca^{2+} wave required to initiate a global Ca^{2+} spike. Under these conditions, $InsP_3R$ may be periodically generating Ca^{2+} 'puffs,' but too small to propagate into a global Ca^{2+} signal. Then, when the inhibitor is washed out, these local Ca^{2+} signals would be immediately enhanced, thus restoring the oscillatory activity. The overshoot of oscillatory activity observed in many cells at that moment could be due to the higher Ca^{2+} content of the ER, which would be full because of the absence of previous releasing activity. Then, when periodic oscillations are again established, the mean Ca^{2+} content of the ER is reduced (Ishii *et al.*, 2006; Vay *et al.*, 2007) and cells return to the original activity.

In summary, our results show that the interplay between mitochondria and ER controlling the activation of $InsP_3R$ is a key factor responsible for the generation of Ca^{2+} oscillations. Inhibition of MCU rapidly and reversibly blocked oscillations, and even a very small degree of MCU inhibition was able to change the pattern of $[Ca^{2+}]_c$ oscillations. This effect is consistent with our recent reports of the stimulation of ER-Ca^{2+} release and $[Ca^{2+}]_c$ oscillations both by CGP37157, an inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger (Hernández-SanMiguel *et al.*, 2006), and by several activators of MCU recently developed (Montero *et al.*, 2004; Lobatón *et al.*, 2006; Vay *et al.*, 2007). As could be expected, inhibition of mitochondrial Ca^{2+} uptake (with KB-R7943, this paper) produced effects opposite to those of either activation of mitochondrial Ca^{2+} uptake or inhibition of mitochondrial Ca^{2+} exit. The conclusion is that mitochondrial Ca^{2+} fluxes, both influx and efflux, tightly control ER-Ca^{2+} release and finely tune associated dynamic Ca^{2+} phenomena such as oscillations. In addition, as mentioned above, mitochondrial Ca^{2+} fluxes are also key factors in controlling the $[Ca^{2+}]_M$ level, the rate of energy production and the opening of the permeability transition pore. Development of specific drugs targeted to these fluxes may be potentially useful as new pharmacological tools in the future.

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Conflict of interest

The authors state no conflict of interest.

References

Babcock DF, Herrington J, Park Y-B, Hille B (1997). Mitochondrial participation in the intracellular Ca^{2+} network. *J Cell Biol* **136**: 833–843.

Bernardi P (1999). Mitochondrial transport of cations: channels, exchangers and permeability transition. *Physiol Rev* **79**: 1127–1155.

Bernardi P, Petronilli V, Di Lisa F, Forte M (2001). A mitochondrial perspective on cell death. *Trends Biochem Sci* **26**: 112–117.

Berridge MJ, Bootman MD, Roderick HL (2003). Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* **4**: 517–529.

Bezprozvanny I, Watras J, Ehrlich BE (1991). Bell-shaped calcium-response curves of $Ins(1,4,5)P_3$- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature* **351**: 751–754.

Boitier E, Rea R, Duchen MR (1999). Mitochondria exert a negative feedback on the propagation of intracellular Ca^{2+} waves in rat cortical astrocytes. *J Cell Biol* **145**: 795–808.

Brini M, Marsault R, Bastianutto C, Alvarez J, Pozzan T, Rizzuto R (1995). Transfected aequorin in the measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). A critical evaluation. *J Biol Chem* **270**: 9896–9903.

Collins TJ, Lipp P, Berridge MJ, Li W, Bootman MD (2000). Inositol 1,4,5-trisphosphate-induced Ca^{2+} release is inhibited by mitochondrial depolarization. *Biochem J* **347**: 593–600.

Csordas G, Thomas AP, Hajnoczky G (1999). Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria. *EMBO J* **18**: 96–108.

Di Lisa F, Bernardi P (2006). Mitochondria and ischemia–reperfusion injury of the heart: fixing a hole. *Cardiovasc Res* **70**: 191–199.

Duchen MR (2000). Mitochondria and calcium: from cell signalling to cell death. *J Physiol* **529**: 57–68.

Furman I, Cook O, Kasir J, Rahamimoff H (1993). Cloning of two isoforms of the rat brain Na^+-Ca^{2+} exchanger gene and their functional expression in HeLa cells. *FEBS Lett* **319**: 105–109.

Giovannucci DR, Hlubek MD, Stuenkel EL (1999). Mitochondria regulate the Ca^{2+}-exocytosis relationship of bovine adrenal chromaffin cells. *J Neurosci* **19**: 9261–9270.

Grynkiewicz G, Poenie M, Tsien RY (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**: 3440–3450.

Hagihara H, Yoshikawa Y, Ohga Y, Takenaka C, Murata KY, Taniguchi S *et al.* (2005). Na^+/Ca^{2+} exchange inhibition protects the rat heart from ischemia–reperfusion injury by blocking energy-wasting processes. *Am J Physiol Heart Circ Physiol* **288**: H1699–H1707.

Hajnoczky G, Davies E, Madesh M (2003). Calcium signaling and apoptosis. *Biochem Biophys Res Commun* **304**: 445–454.

Hajnoczky G, Hager R, Thomas AP (1999). Mitochondria suppress local feedback activation of inositol 1,4,5-trisphosphate receptors by Ca^{2+}. *J Biol Chem* **274**: 14157–14162.

Halestrap AP (2006). Calcium, mitochondria and reperfusion injury: a pore way to die. *Biochem Soc Trans* **34**: 232–237.

Hernández-Guijo JM, Maneu-Flores VE, Ruiz-Nuño A, Villarroya M, Garcia AG, Gandia L (2001). Calcium-dependent inhibition of L, N, and P/Q Ca^{2+} channels in chromaffin cells: role of mitochondria. *J Neurosci* **21**: 2553–2560.

Hernández-SanMiguel E, Vay L, Santo-Domingo J, Lobaton CD, Moreno A, Montero M *et al.* (2006). The mitochondrial Na^+/Ca^{2+} exchanger plays a key role in the control of cytosolic Ca^{2+} oscillations. *Cell Calcium* **40**: 53–61.

Hoth M, Button DC, Lewis RS (2000). Mitochondrial control of calcium-channel gating: a mechanism for sustained signaling and transcriptional activation in T lymphocytes. *Proc Natl Acad Sci USA* **97**: 10607–10612.

Ichas F, Jouaville LS, Mazat J-P (1997). Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals. *Cell* **89**: 1145–1153.

Ishii K, Hirose K, Iino M (2006). Ca^{2+} shuttling between endoplasmic reticulum and mitochondria underlying Ca^{2+} oscillations. *EMBO Rep* **7**: 390–396.

Iwamoto T, Kita S (2004). Development and application of Na^+/Ca^{2+} exchange inhibitors. *Mol Cell Biochem* **259**: 157–161.

Iwamoto T, Watano T, Shigekawa M (1996). A novel isothiourea derivative selectively inhibits the reverse mode of Na^+/Ca^{2+} exchange in cells expressing NCX1. *J Biol Chem* **271**: 22391–22397.

Iwamoto T (2004). Forefront of Na^+/Ca^{2+} exchanger studies: molecular pharmacology of Na^+/Ca^{2+} exchange inhibitors. *J Pharmacol Sci* **96**: 27–32.

Jouaville LS, Pinton P, Bastianutto C, Rutter GA, Rizzuto R (1999). Regulation of mitochondrial ATP synthesis by calcium: evidence for a long-term metabolic priming. *Proc Natl Acad Sci USA* **96**: 13807–13812.

Kaftan EJ, Ehrlich BE, Watras J (1997). Inositol 1,4,5-trisphosphate ($InsP_3$) and calcium interact to increase the dynamic range of $InsP_3$ receptor-dependent calcium signalling. *J Gen Physiol* **110**: 529–538.

Kirichok Y, Krapivinsky G, Clapham DE (2004). The mitochondrial calcium uniporter is a highly selective ion channel. *Nature* **427**: 360–364.

Lobatón CD, Vay L, Hernandez-SanMiguel E, SantoDomingo J, Moreno A, Montero M *et al*. (2005). Modulation of mitochondrial Ca^{2+} uptake by estrogen receptor agonists and antagonists. *Br J Pharmacol* **145**: 862–871.

Low W, Kasir J, Rahamimoff H (1993). Cloning of the rat heart Na^+–Ca^{2+} exchanger and its functional expression in HeLa cells. *FEBS Lett* **316**: 63–67.

Mak DO, Mcbride S, Foskett JK (2001). Regulation by Ca^{2+} and inositol 1,4,5-trisphosphate ($InsP_3$) of single recombinant type 3 $InsP_3$ receptor channels. Ca^{2+} activation uniquely distinguishes types 1 and 3 $InsP_3$ receptors. *J Gen Physiol* **117**: 435–446.

Matsuda T, Arakawa N, Takuma K, Kishida Y, Kawasaki Y, Sakaue M *et al*. (2001). SEA0400, a novel and selective inhibitor of the Na^+-Ca^{2+} exchanger, attenuates reperfusion injury in the *in vitro* and *in vivo* cerebral ischemic models. *J Pharmacol Exp Ther* **298**: 249–256.

Matsunaga M, Saotome M, Satoh H, Katoh H, Terada H, Hayashi H (2005). Different actions of cardioprotective agents on mitochondrial Ca^{2+} regulation in a Ca^{2+} paradox-induced Ca^{2+} overload. *Circ J* **69**: 1132–1140.

Montero M, Alonso MT, Carnicero E, Cuchillo-Ibañez I, Albillos A, Garcia AG *et al*. (2000). Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca^{2+} transients that modulate secretion. *Nature Cell Biol* **2**: 57–61.

Montero M, Barrero MJ, Alvarez J (1997). [Ca^{2+}] microdomains control agonist-induced Ca^{2+} release in intact HeLa cells. *FASEB J* **11**: 881–885.

Montero M, Brini M, Marsault R, Alvarez J, Sitia R, Pozzan T *et al*. (1995). Monitoring dynamic changes in free Ca^{2+} concentration in the endoplasmic reticulum of intact cells. *EMBO J* **14**: 5467–5475.

Montero M, Lobatón CD, Hernández-SanMiguel E, SantoDomingo J, Vay L, Moreno A *et al*. (2004). Direct activation of the mitochondrial calcium uniporter by natural plant flavonoids. *Biochem J* **384**: 19–24.

Montero M, Lobaton CD, Moreno A, Alvarez J (2002). A novel regulatory mechanism of the mitochondrial Ca^{2+} uniporter revealed by the p38 mitogen-activated protein kinase inhibitor SB202190. *FASEB J* **16**: 1955–1957.

Pintado AJ, Herrero CJ, Garcia AG, Montiel C (2000). The novel Na^+/Ca^{2+} exchange inhibitor KB-R7943 also blocks native and expressed neuronal nicotinic receptors. *Br J Pharmacol* **130**: 1893–1902.

Rizzuto R, Bastianutto C, Brini M, Murgia M, Pozzan T (1994). Mitochondrial Ca^{2+} homeostasis in intact cells. *J Cell Biol* **126**: 1183–1194.

Rizzuto R, Bernardi P, Pozzan T (2000). Mitochondria as all-round players of the calcium game. *J Physiol* **529**: 37–47.

Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM *et al*. (1998). Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. *Science* **280**: 1763–1766.

Rizzuto R, Pinton P, Ferrari D, Chami M, Szabadkai G, Magalhaes PJ *et al*. (2003). Calcium and apoptosis: facts and hypotheses. *Oncogene* **22**: 8619–8627.

Rutter GA, Rizzuto R (2000). Regulation of mitochondrial metabolism by ER Ca^{2+} release: an intimate connection. *Trends Biochem Sci* **25**: 215–221.

Sobolevsky AI, Khodorov BI (1999). Blockade of NMDA channels in acutely isolated rat hippocampal neurons by the Na^+/Ca^{2+} exchange inhibitor KB-R7943. *Neuropharmacology* **38**: 1235–1242.

Tanaka H, Nishimaru K, Aikawa T, Hirayama W, Tanaka Y, Shigenobu K (2002). Effect of SEA0400, a novel inhibitor of sodium–calcium exchanger, on myocardial ionic currents. *Br J Pharmacol* **135**: 1096–1100.

Vay L, Hernandez-SanMiguel E, Santo-Domingo J, Lobaton CD, Moreno A, Montero M *et al*. (2007). Modulation of Ca^{2+}-release and Ca^{2+} oscillations by mitochondrial Ca^{2+} uniporter stimulation. *J Physiol* **580**: 39–49.

Vercesi AE, Kowaltowski AI, Oliveira HC, Castilho RF (2006). Mitochondrial Ca^{2+} transport, permeability transition and oxidative stress in cell death: implications in cardiotoxicity, neurodegeneration and dyslipidemias. *Front Biosci* **11**: 2554–2564.