

Nitric oxide inhibits capacitative Ca^{2+} entry by suppression of mitochondrial Ca^{2+} handling

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1 Nitric oxide (NO) is a key modulator of cellular Ca^{2+} signalling and a determinant of mitochondrial function. Here, we demonstrate that NO governs capacitative Ca^{2+} entry (CCE) into HEK293 cells by impairment of mitochondrial Ca^{2+} handling.

2 Authentic NO as well as the NO donors 1-[2-(carboxylato)pyrrolidin-1-yl]diazem-1-ium-1,2-diolate (ProliNO) and 2-(N,N-diethylamino)-diazem-2-oxide (DEANO) suppressed CCE activated by thapsigargin (TG)-induced store depletion. Threshold concentrations for inhibition of CCE by ProliNO and DEANO were 0.3 and 1 μM , respectively.

3 NO-induced inhibition of CCE was not mimicked by peroxynitrite (100 μM), the peroxynitrite donor 3-morpholino-sydnonimine (SIN-1, 100 μM) or 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP, 1 mM). In addition, the guanylyl cyclase inhibitor 1H-[1,2,4] oxadiazole[4,3-a] quinoxalin-1-one (ODQ, 30 μM) failed to antagonize the inhibitory action of NO on CCE.

4 DEANO (1–10 μM) suppressed mitochondrial respiration as evident from inhibition of cellular oxygen consumption. Experiments using fluorescent dyes to monitor mitochondrial membrane potential and mitochondrial Ca^{2+} levels, respectively, indicated that DEANO (10 μM) depolarized mitochondria and suppressed mitochondrial Ca^{2+} sequestration. The inhibitory effect of DEANO on Ca^{2+} uptake into mitochondria was confirmed by recording mitochondrial Ca^{2+} during agonist stimulation in HEK293 cells expressing ratiometric-pericam in mitochondria.

5 DEANO (10 μM) failed to inhibit Ba^{2+} entry into TG-stimulated cells when extracellular Ca^{2+} was buffered below 1 μM , while clear inhibition of Ba^{2+} entry into store depleted cells was observed when extracellular Ca^{2+} levels were above 10 μM . Moreover, buffering of intracellular Ca^{2+} by use of N,N'-[1,2-ethanediy]bis(oxy-2,1-phenylene)] bis [N-[25-[(acetyloxy) methoxy]-2-oxoethyl]-, bis[(acetyloxy)methyl] ester (BAPTA/AM) eliminated inhibition of CCE by NO, indicating that the observed inhibitory effects are based on an intracellular, Ca^{2+} dependent-regulatory process.

6 Our data demonstrate that NO effectively inhibits CCE cells by cGMP-independent suppression of mitochondrial function. We suggest disruption of local Ca^{2+} handling by mitochondria as a key mechanism of NO induced suppression of CCE.

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Abbreviations: BAPTA/AM, N,N'-[1,2-ethanediy]bis(oxy-2,1-phenylene)] bis [N-[25-[(acetyloxy) methoxy]-2-oxoethyl]-, bis[(acetyloxy)methyl] ester; 8-Br-cGMP, 8-bromoguanosine 3',5'-cyclic monophosphate; $[\text{Ca}^{2+}]_e$, extracellular Ca^{2+} concentration; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; $[\text{Ca}^{2+}]_m$, mitochondrial Ca^{2+} concentration; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; CCE, capacitative Ca^{2+} entry; CCE_C, capacitative Ca^{2+} entry channels; DEANO, 2-(N,N-diethylamino)-diazem-2-oxide sodium salt; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; ER, endoplasmic reticulum; FCCP, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone; fura-2/AM, 5-Oxazolecarboxylic acid, 2-[6-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-[2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]-2-benzofuranyl]-, (acetyloxy)methyl ester; IP₃, inositol 1,4,5-trisphosphate; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl-carbocyanide iodine; M, mitochondria; NO, nitric oxide; ODQ, 1H-[1,2,4] oxadiazole[4,3-a] quinoxalin-1-one; ProliNO, 1-[2-(carboxylato)-pyrrolidin-1-yl]diazem-1-ium-1,2-diolate; rhod-2/AM, Xanthylium, 9-[4-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-3-[2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]-amino]phenoxy]-ethoxy]phenyl]-3,6-bis(dimethylamino)- bromide; SERCA, sarco (endo)plasmic reticulum Ca^{2+} ATP-ase; SIN-1, 3-morpholino-sydnonimine; TG, thapsigargin

Introduction

Depletion of Ca^{2+} stores by either inositol 1,4,5-trisphosphate (IP₃)-dependent or IP₃-independent pathways results

in activation of a Ca^{2+} influx pathway that is termed store-operated Ca^{2+} entry or 'capacitative' Ca^{2+} entry (CCE; Putney, 1986; 1990). The molecular nature of this phenomenon is still elusive. As to the signalling pathways that link CCE channels to intracellular stores, a diffusible Ca^{2+} influx factor (Randriamampita & Tsien, 1993) as well

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as a direct physical coupling between CCE channels and components of the endoplasmic reticulum (ER) membrane (conformational coupling; Putney, 1999) or the fusion of CCE channel-containing membrane vesicles with the plasma membrane (Fasolato *et al.*, 1993) have been proposed. In addition, changes in the sub-plasmalemmal Ca²⁺ concentration may play a critical role in the process of activation (Krause *et al.*, 1999; Putney *et al.*, 2001). Recently, evidence has been accumulated for the regulation of CCE by an intracellular Ca²⁺ store other than the ER, i.e. mitochondria. Mitochondria have been proposed to control Ca²⁺ channel gating in T lymphocytes (Hoth *et al.*, 2000) and in Jurkat T cells (Makowska *et al.*, 2000). In Jurkat cells, capacitative Ca²⁺ entry channels were reported to remain inactive during depletion of Ca²⁺ stores when the mitochondrial protonomotive force is collapsed by carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or myxothiazol (Makowska *et al.*, 2000). The effects of mitochondrial uncoupling on CCE of Jurkat cells were not related to changes in cellular ATP/ADP ratio. Mitochondria are likely to sequester Ca²⁺ from sub-plasmalemmal compartments, thereby blunting the elevation of sub-plasmalemmal Ca²⁺ during Ca²⁺ entry and consequently Ca²⁺-mediated negative feedback inhibition of CCE channels (Lawrie *et al.*, 1996; Rizzuto *et al.*, 1993; 1994; Rutter *et al.*, 1993). Impairment of mitochondrial Ca²⁺ handling such as blockade of mitochondrial Ca²⁺ uptake leads to severe alterations in intracellular Ca²⁺ homeostasis (Werth & Thayer, 1994; Herrington *et al.*, 1996) and was shown to suppress sustained Ca²⁺ influx into T-lymphocytes (Hoth *et al.*, 1997).

Nitric oxide (NO) is a mediator which governs Ca²⁺ homeostasis in a highly complex and cell specific manner (Clementi & Meldolesi, 1997; Clementi, 1998). NO was reported to potentiate CCE in pancreatic acinar cells and colonic epithelial cells (Bischof *et al.*, 1995) but inhibits CCE in platelets (Trepakova *et al.*, 1999) and smooth muscle cells (Wayman *et al.*, 1996; Cohen *et al.*, 1999). NO has been proposed to control Ca²⁺ transport via cGMP-dependent (Gukovskaya & Pandol, 1994) as well as cGMP-independent pathways (Watson *et al.*, 1999). Promotion of SERCA-dependent refilling of intracellular Ca²⁺ stores has been postulated as the mechanism by which NO causes inhibition of Ca²⁺ entry signals (Trepakova *et al.*, 1999). Since mitochondrial function has been recognized as an important modulator of cellular Ca²⁺ signalling, it appeared of interest to test for a possible role of mitochondria as a cellular switchboard linking NO and Ca²⁺ signals. With the present study, we demonstrate that NO is able to inhibit capacitative Ca²⁺ entry into HEK 293 cells by a mechanism that is independent of cGMP or peroxynitrite formation, but involves uncoupling of mitochondrial Ca²⁺ handling.

Methods

Cell culture

HEK293 (human embryonic kidney) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum and used for the experiments.

Measurement of intracellular Ca²⁺

Intracellular Ca²⁺ was measured with 5-Oxazole-carboxylic acid, 2-[6-bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-[2-[2-bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]-2-benzofuranyl-, (acetyloxy)methyl ester (fura-2/AM). Fura-2/AM was initially dissolved in DMSO at 2 mM and used at a final concentration of 2 μ M. Confluent cells were harvested by enzymatic digestion (0.25% trypsin) and suspended in 5 ml DMEM without serum, and loaded with fura-2/AM for 60 min at 37°C and 5% CO₂. Thereafter, cells were washed once with Ca²⁺-containing Tris buffer (mM: NaCl 100, KCl 5, Tris 50, CaCl₂ 2.5, MgCl₂ 1, pH 7.4), incubated for 20 min and washed in Tris buffer without Ca²⁺. Fluorescence measurements were carried out with a dual wavelength spectrofluorimeter (Hitachi F2000). Cells were maintained at 37°C, and emission was collected at 510 nm at excitation of 340 nm and 380 nm. For store depletion 100 nM thapsigargin (TG) was added, and extracellular Ca²⁺ was elevated subsequently by adding 1 mM Ca²⁺ to induce Ca²⁺ entry. [Ca²⁺]_i was determined from the fluorescence ratio F₃₄₀/F₃₈₀ according to Grynkiewicz *et al.* (1985). The fluorescence after sequential addition of 0.1% Triton X-100 and 50 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) to the cells provided the maximum fluorescence ratio (R_{max}) and the minimum fluorescence ratio (R_{min}) respectively. [Ca²⁺]_i was calculated using the equation

$$[\text{Ca}^{2+}]_i = (\text{R} - \text{R}_{\text{min}}) / (\text{R}_{\text{max}} - \text{R}) \times \beta \times K_D$$

where β is the ratio of the emission fluorescence at 380 nm excitation in the presence of Triton X-100 and Triton X-100 plus EGTA, respectively.

Measurement of cGMP accumulation

HEK293 cells were subcultured on 24-well plates. Cells were incubated with NO containing solutions or NO donors for 14 min (corresponding to the incubation time in experiments measuring Ca²⁺ entry) and the incubation was stopped by removal of medium and addition of 0.01 M HCl. Guanosine 3',5'-cyclic monophosphate (cGMP) was measured in the samples by radioimmunoassay as described in Schmidt *et al.* (1989).

Measurement of Ba²⁺ entry

Ba²⁺ entry into TG-stimulated HEK293 cells was measured as described above by recording of the fluorescence ratio (F₃₄₀/F₃₈₀) from Fura-2-loaded cells. The experiments were conducted either in nominally Ca²⁺-free or in extracellular Ca²⁺-chelated condition as specified. Ba²⁺ entry was initiated by adding 3 mM Ba²⁺, and control experiments were performed with vehicle only. Ba²⁺ entry was quantified as the initial slope of fluorescence ratio calculated from the changes in fluorescence observed within 1 min after Ba²⁺ addition. TG stimulated Ba²⁺ entry about 2.5 fold over basal values which were not subtracted in the analysis.

Measurement of cellular oxygen consumption

HEK293 cells from one petri dish were harvested and suspended in 1.8 ml of 50 mM Tris buffer, pH 7.4, containing (mM): NaCl 100, KCl 5, MgCl₂ 1, and CaCl₂ 3. Oxygen consumption was measured with a Clark-type electrode (ISO₂, World Precision Instruments, Mauer, Germany) in a temperature controlled glass vial sealed with a rubber septum. After equilibration (~1 min), 5 µl of a stock solution of the NO donor or solvent was injected through the septum, and O₂ consumption was monitored over 10–15 min under constant stirring. Two-point calibration of the sensor was performed in air-saturated H₂O at 37°C (6.9 p.p.m.; 0.216 mM O₂) and argon atmosphere (zero O₂).

Measurement of mitochondrial membrane potential with JC-1

Cells were loaded at room temperature with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanide iodine (JC-1, 5 µM) for 10 min in the dark. Prior to the experiments, cells were washed twice and mounted in a customized superfusion chamber. To monitor mitochondrial membrane potential, cells were illuminated for 200 ms alternatively at 485 nm (485DF15; Omega Optical, Brattleboro, VT, U.S.A.) and 575 nm (575DF25; Omega Optical, Brattleboro, VT, U.S.A.). Emission was detected at 528 and 633 nm (528-633DBEN, XF53; Omega Optical, Brattleboro, VT, U.S.A.).

Measurement of mitochondrial Ca²⁺ with rhod-2/AM

For measurements of mitochondrial Ca²⁺, cells were seeded on poly-L-lysine-coated cover slips (6 × 6 mm) and loaded with Xanthylum, 9-[4-[bis[2-(acetyloxy)methoxy]-2-oxoethyl]amino]-3-[2-[2-[bis[2-(acetyloxy)methoxy]-2-oxoethyl]amino]phenoxy]ethoxy]phenyl]-3,6-bis(dimethylamino)- bromide (rhod-2/AM) in DMEM without fetal calf serum, at a concentration of 2 µM for 45 min at 37°C. Cells were illuminated using a monochromator (Polychrome II, Till Photonics, Gräfelfing, Germany) at 540 nm and fluorescence emission was collected at 605 nm. Fluorescence was monitored on a Nikon Diaphot 300 microscope equipped with a CCD camera system (Sensicam, PCO, Kelheim, Germany) and analysed using the Axon Imaging Workbench 2.1 software (Axon Instruments, Foster City, U.S.A.).

Measurement of mitochondrial Ca²⁺ using heterologous expression of ratiometric-pericam that is specifically targeted to mitochondria

Mitochondrial free Ca²⁺ concentration was monitored in single, adherent HEK293 cells expressing ratiometric-pericam-mt which was kindly provided by Dr Atsushi Miyawaki, RIKEN, Saitama, Japan. Cells were grown up to 50% of confluence and were transiently transfected with cDNA encoding the ratiometric-pericam-mt (Nagai *et al.*, 2001), using SuperFect™ (Qiagen, BioTrade, Vienna, Austria), in the standard transfection protocol described for this transfection reagent. Between 24–36 h after transfection, cells were mounted in a customized perfusion chamber that allowed

superfusion with buffer (flow rate 1–2 ml/min). Experiments were performed at room temperature (20–23°C). For measuring the mitochondrial free Ca²⁺ concentration ([Ca²⁺]_m), an imaging microscope was used (Frieden *et al.*, 2002; Graier *et al.*, 1998; Paltauf-Doburzynska *et al.*, 2000). Briefly, a Nikon inverted microscope (Eclipse 300TE, Nikon, Vienna) was equipped with CFI Plan Fluor ×40 oil immersion objective (NA 1.3, Nikon, Vienna, Austria), an epifluorescence system (150 W XBO; Optiquip, Highland Mills, NY, U.S.A.) and a computer-controlled filter wheel (Ludl, Electronic Products, Hawthorne, NY, U.S.A.). To monitor [Ca²⁺]_m, cells were illuminated at room temperature for 500 ms alternatively at 410 nm (410DF20; Omega Optical, Brattleboro, VT, U.S.A.) and 480 nm (480AF30; Omega Optical, Brattleboro, VT, U.S.A.). Emission was monitored at 535 nm (535AF26 with dichroic 455DRVP; Omega Optical, Brattleboro, VT, U.S.A.) using a liquid-cooled CCD camera (–30°C; Quantix KAF 1400G2, Roper Scientific, Acton, MA, U.S.A.). All devices were controlled by Metafluor 4.0 (Visitron Systems, Puchheim, Germany). Mitochondrial Ca²⁺ concentration is presented as the fluorescence ratio (F480/410) at 535 nm emission.

Chemicals

Chemicals were purchased from the following suppliers: Tissue culture medium was from Gibco BRL (Vienna, Austria); fura-2/AM, rhod-2/AM, JC-1 and BAPTA/AM were from Molecular Probes (Leiden, Netherlands); DEANO was from Alexis (Switzerland) and all other chemicals were purchased from Sigma Chemical Co. (Vienna, Austria). NO solutions were prepared as described in Kukovetz & Holzmann (1989).

Statistical analysis

Time courses of fluorescence changes are illustrated as mean values obtained from the indicated number of experiments. For clarity, error bars were omitted in time course illustrations. Averaged data given for specific time points are expressed as mean values ± s.e.mean. Differences were considered statistically significant at *P* < 0.05 using Student's *t*-test for unpaired values.

Results

Inhibition of capacitative Ca²⁺ entry into HEK293 cells by NO and NO donors

Exposure of HEK293 cells to NO or NO donors (DEANO, ProliNO) inhibited the capacitative Ca²⁺ entry (CCE) induced by thapsigargin (TG) in a classical Ca²⁺ re-addition protocol. Typical effects of 1 µM and 10 µM DEANO are shown in Figure 1A,B, respectively. Intracellular Ca²⁺ levels of 713 ± 28 nM (*n* = 12) were measured 3 min after Ca²⁺ re-addition in the absence of DEANO and 492 ± 36 nM (*n* = 8) and 323 ± 18 nM (*n* = 8) in the presence of 1 µM and 10 µM DEANO, respectively. DEANO did not affect the intracellular Ca²⁺ signal evoked by TG-induced store depletion in the absence of extracellular Ca²⁺, but by itself, elevated cytoplasmic Ca²⁺ at higher concentrations (10 µM, Figure

1B). At 10 μM , the NO donor produced a change in the time course of the Ca²⁺ entry signal in that elevation of intracellular Ca²⁺ became essentially slow. Figure 1C shows the inhibitory effect obtained when an NO solution was administered (nominally 100 μM). In the presence of NO, intracellular Ca²⁺ levels were $281 \pm 22 \text{ nM}$ ($n=8$) at 3 min after Ca²⁺ re-addition. The inhibitory effect of NO was not mimicked by 8-Br-cGMP (1 mM, Figure 1D), the peroxynitrite donor SIN-1 (100 μM ; Figure 1E) or by authentic peroxynitrite (100 μM ; Figure 1F). Moreover, decomposed DEANO (10 μM) and ProlINO (10 μM) did not inhibit TG-induced CCE in HEK 293 cells (not shown, $n=3$). These results suggested inhibition of CCE by NO *via* a cGMP and peroxynitrite-independent mechanism. To further test this concept we studied the relation between inhibition of CCE and cellular cGMP levels for the different NO donors and performed experiments with the guanylyl cyclase inhibitor ODQ.

ProlINO is a NONOate that decomposes more rapidly than DEANO. Thus, the actual peak NO concentration obtained with prolINO is expected to be close to the nominal concentration of the NO donor and higher than that obtained with DEANO. The threshold concentration for ProlINO-induced inhibition of CCE, as quantified by changes in cytosolic Ca²⁺ at 3 min after Ca²⁺ re-addition to TG-

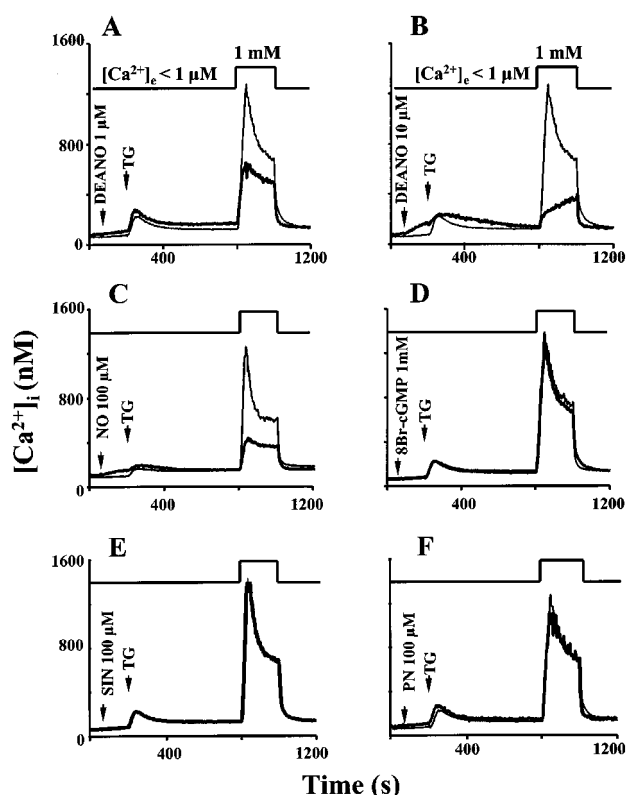


Figure 1 Time courses of $[\text{Ca}^{2+}]_i$ determined from fura-2 fluorescence. Cells were stimulated with thapsigargin (TG) in Ca²⁺-free solution, and extracellular Ca²⁺ (1 mM) was added to induce Ca²⁺ entry as indicated. Thin lines denote control experiments performed in the absence of drugs, thick lines represent experiments performed in the presence of the indicated compounds (A) DEANO, 1 μM ; (B) DEANO, 10 μM ; (C) NO in solution, 100 μM ; (D) 8-Br-cGMP, 1 mM; (E) SIN-1, 100 μM ; (F) peroxynitrite, PN, 100 μM). Data points are mean values derived from 6–15 experiments.

stimulated cells, was slightly lower than that of DEANO (0.3 μM versus 1 μM). At 0.3 μM , ProlINO inhibited CCE significantly but failed to elicit any detectable change in cellular cGMP levels (Figure 2). A similar situation was found with NO in solution at its threshold to produce significant inhibition of CCE (1 μM). By contrast, 100 μM SIN-1 produced a significant elevation of cellular cGMP without any change in CCE. Higher concentrations of ProlINO (1 μM) and NO (nominally 10 μM) induced concomitant changes in CCE and cGMP levels. Preincubation of cells with 30 μM ODQ was sufficient to prevent elevation of cGMP but failed to suppress inhibition of CCE by 1 μM ProlINO and 10 μM NO, respectively (Figure 2).

NO reduces oxygen consumption of HEK293 cells

A substantial effect of NO on energy metabolism was evident from experiments measuring oxygen consumption of HEK293 cells. DEANO (1–10 μM) reduced oxygen consumption in a concentration-dependent manner, while SIN-1 (100 μM) did not exert any effect (Figure 3). These results indicate that NO impairs mitochondrial respiration in HEK293 cells by a mechanism independent of peroxynitrite and/or cGMP generation.

NO depolarizes mitochondria and impairs mitochondrial Ca²⁺ handling

To test whether NO affects mitochondrial function in HEK293 cells, we first studied the effects of DEANO on mitochondrial membrane potential using JC-1 as a reporter dye. As illustrated in Figure 4, mitochondrial membrane potential recorded as JC-1 fluorescence was significantly reduced by 10 μM DEANO *versus* control. The effect of 10 μM DEANO was less pronounced than that of the protonophore carbonyl cyanide p-(trifluoromethoxy) phenyl-

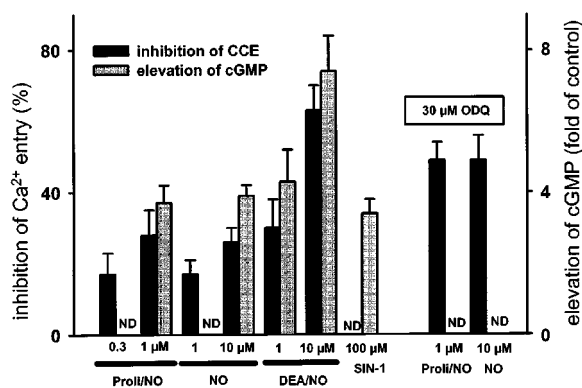


Figure 2 Comparison of inhibition of CCE (black columns) and elevation of cGMP levels (grey columns) by NO and NO donors (ProlINO, DEANO and SIN-1). Values obtained with 1 μM ProlINO and 10 μM NO in the presence of the guanylyl cyclase inhibitor ODQ are shown in the right panel. Significant elevation of cGMP was not detectable (ND) with 0.3 μM ProlINO, 1 μM NO and in the presence of ODQ. Inhibition of CCE was not detectable (ND) with 100 μM SIN-1. Inhibition of Ca²⁺ entry was determined from $[\text{Ca}]_i$ levels measured 3 min after Ca²⁺ re-addition. Mean values \pm s.e. mean derived from 4–8 experiments are given, all effects are statistically significant.

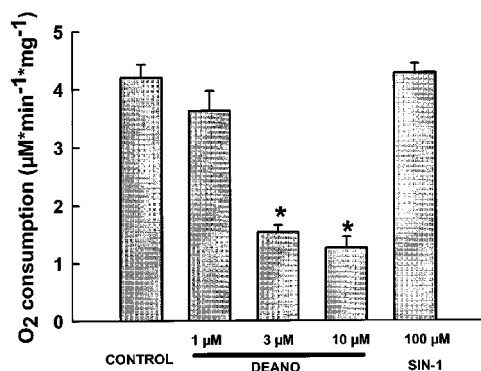


Figure 3 Effects of DEANO (1–10 μM) and SIN-1 (100 μM) on oxygen consumption of HEK293 cells. Mean values \pm s.e. mean derived from 4–6 experiments are given. *Denotes statistically significant difference at $P < 0.05$ versus control.

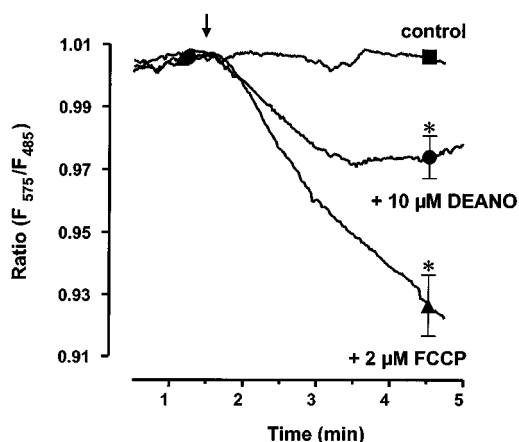


Figure 4 Time courses of mitochondrial membrane potential measured as JC-1 fluorescence during exposure of cells to 10 μM DEANO, 1 μM FCCP or vehicle (control). Mean values of JC-1 fluorescence \pm s.e. mean at 3 min after administration of drugs or vehicle are given. *Denotes statistically significant difference at $P < 0.05$ versus control.

hydrazine (FCCP, 1 μM), a classical uncoupler of mitochondrial function.

To monitor the mitochondrial Ca²⁺ concentration, cells were loaded with the Ca²⁺ sensitive dye rhod-2 which accumulates in mitochondria. Rhod-2 fluorescence was localized in discrete spots within the cells. As shown in Figure 5A, 3 μM DEANO caused a rapid decline in rhod-2 fluorescence, an effect that was mimicked by 300 nM of the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP), indicating that the NO donor and the protonophore affect mitochondrial Ca²⁺ handling to a similar extent at these concentrations. Figure 6A,B compares the effects of 3 μM DEANO and 300 nM CCCP on TG-induced Ca²⁺ signals. Both agents elicited a slight elevation of basal Ca²⁺ when administered in Ca²⁺-free solution and exerted comparable inhibitory effects on TG-induced CCE. To further test for NO-induced suppression of mitochondrial Ca²⁺ handling we employed an additional approach based on expression of ratiometric-pericam that is targeted specifically to mitochondria (ratiometric-pericam-mt). HEK293 cells expressing ratiometric-pericam-mt were challenged with

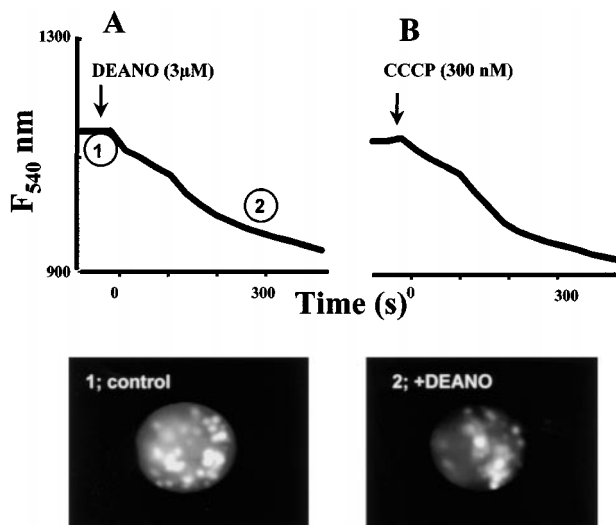


Figure 5 Time courses of mitochondrial rhod-2 fluorescence in single HEK293 cells during administration of DEANO (A, 3 μM) and the mitochondrial uncoupler CCCP (B, 300 nM). Data points represent the mean derived from 32 and 40 cells, respectively. Lower panel shows typical images corresponding to time points indicated in A.

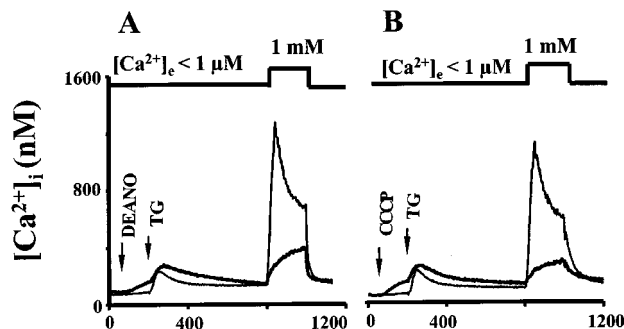


Figure 6 Time courses of $[\text{Ca}^{2+}]_i$ determined from fura-2 fluorescence. Cells were stimulated with thapsigargin (TG) in Ca²⁺-free solution, and extracellular Ca²⁺ (1 mM) was added to induce Ca²⁺ entry as indicated. Thin lines denote control experiments performed in the absence of drugs, thick lines represent experiments performed in the presence of DEANO (A) 3 μM ; and CCCP (B) 300 nM. Data points are mean values derived from eight experiments.

carbachol to elicit profound Ca²⁺ loading of mitochondria, which was detectable by a rise in the Ca²⁺-sensitive fluorescence ratio as shown in Figure 7. This carbachol-induced Ca²⁺ loading of mitochondria was significantly reduced in the presence of 10 μM DEANO (Figure 7).

Inhibition of capacitative Ca²⁺ entry channels by NO requires the presence of extracellular Ca²⁺ and is eliminated by intracellular Ca²⁺ buffering

To test the concept that NO inhibits CCE due to impairment of local Ca²⁺ handling and promotion of Ca²⁺-mediated autoregulation, we performed experiments with Ba²⁺ ions which are known to permeate through many Ca²⁺ entry channels but barely activate negative feedback mechanisms. Neither DEANO (10 μM) nor CCCP (300 nM) inhibited Ba²⁺ entry when extracellular free Ca²⁺ was buffered below 1 μM , as predicted for a Ca²⁺-mediated mechanism (Figure 8A,C).

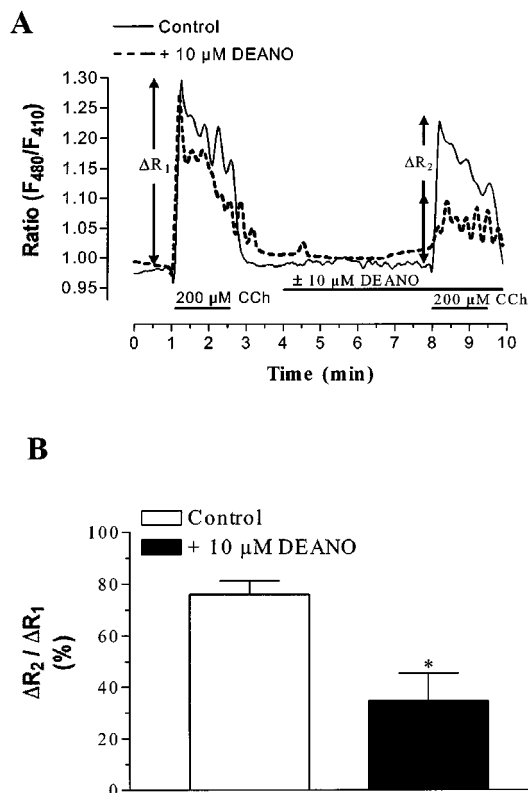


Figure 7 Effects of DEANO (10 μM) on Ca²⁺ loading of mitochondria during carbachol (CCh; 200 μM) stimulation of HEK293 cells. Mitochondrial Ca²⁺ was monitored by expression of a ratiometric-pericam that is specifically targeted to mitochondria. (A) Representative time courses of Ca²⁺ sensitive fluorescence in individual cells with ΔR_1 and ΔR_2 taken as a measure of the increase in mitochondrial Ca²⁺ before and after administration of DEANO or vehicle. (B) Effect of 10 μM DEANO on mitochondrial Ca²⁺ uptake calculated as the relation between ΔR_1 and ΔR_2 for DEANO and vehicle (control), respectively. Mean values \pm s.e. mean derived from six experiments. *Denotes statistically significant difference at $P < 0.05$ versus control.

Moreover, the inhibitory action of both agents was regained when extracellular Ca²⁺ was elevated above 10 μM (Figure 8B,D). Thus, the inhibitory action of both CCCP and the NO donor was Ca²⁺-dependent. Mean values of the inhibitory effects of both compounds at different extracellular free Ca²⁺ concentrations are depicted in Figure 8E. Consistent with the hypothesis that both NO and CCCP inhibit CCE due to promotion of an intracellular Ca²⁺-dependent mechanism, the inhibitory action of both agents was eliminated by preincubation of cells with the cell-permeant Ca²⁺ chelator BAPTA/AM. Loading of the cells with BAPTA/AM (10 μM) substantially enhances the intracellular Ca²⁺ buffer capacity whilst still allowing for moderate elevation of fura-2 fluorescence during Ca²⁺ mobilization and entry. Figure 9 illustrates that in BAPTA/AM-loaded cells, both DEANO and CCCP failed to inhibit TG-induced CCE in HEK293 cells.

Discussion

With the present study we provide evidence for a key role of mitochondria in NO-mediated control of capacitative Ca²⁺

entry and thus in the coordination of cellular NO and Ca²⁺ signals. Our results demonstrate for the first time that two previously recognized actions of NO, i.e. modulation of mitochondrial function and inhibition of CCE, are tightly coupled.

NO-induced inhibition of CCE in HEK293 cells is independent of cGMP and peroxynitrite formation

The classical target of NO is soluble guanylyl cyclase (Dierks & Burstyn, 1996), and a variety of biological actions of NO involve cGMP/cGMP-kinase-mediated signal transduction (Looms *et al.*, 2001). Besides this classical signal transduction pathway, NO is known to directly affect other target proteins such as components of the respiratory chain (Brown, 1999) and to exert biological effects due to modification of proteins by S-nitrosation or tyrosine nitration (Hanafy *et al.*, 2001; Poteser *et al.*, 2001). Here, we report that NO as well as NO donors inhibit TG-induced (capacitative) Ca²⁺ entry (CCE) in HEK293 cells. The rapidly decomposing NO donor ProliNO, which generates peak NO levels close to the donor concentration, exerted significant inhibitory effects at sub-micromolar concentrations, indicating that these rather physiological NO levels are able to affect store operated Ca²⁺ signaling. High concentrations of NO donors as well as mitochondrial uncouplers gave rise to a moderate elevation of basal intracellular Ca²⁺ levels and a change in the time course of intracellular Ca²⁺ during CCE. It remains unclear whether the change in kinetics of the intracellular Ca²⁺ signals is due to suppression of CCE channel activity below a certain critical level or reflects an additional inhibitory principle of the NO donor.

The inhibitory actions of NO were neither mimicked by 8-Br-cGMP nor by SIN-1, a NO donor that decomposes to produce peroxynitrite resulting in elevated cGMP levels and enhanced protein tyrosine phosphorylation (Takehara *et al.*, 1996). Inhibitory effects of NO and NO donors were not correlated with intracellular cGMP levels. NO and ProliNO inhibited CCE without any detectable increase in cellular cGMP when administered at threshold concentrations or when higher concentrations were administered in the presence of the guanylyl cyclase inhibitor ODQ. Moreover, SIN-1 produced a substantial elevation of cellular cGMP without affecting CCE. These results suggest that NO governs CCE in HEK293 cells by a mechanism independent of cGMP or peroxynitrite formation. The lack of mediator function of peroxynitrite was corroborated by experiments with authentic peroxynitrite. Both NO itself as well as peroxynitrite are known to affect mitochondrial function, albeit in a different manner. NO at physiological levels appears to interact predominantly with cytochrome *c* oxidase, leading to reduced mitochondrial oxygen uptake and consequently in rather profound alterations in mitochondrial function. By contrast, peroxynitrite is able to cause irreversible oxidative damage of components of the respiratory chain, depending on the effectiveness of mitochondrial scavenging systems, which protect against modification of mitochondrial proteins by peroxynitrite (Radi *et al.*, 2002). The lack of effect of peroxynitrite in the present study indicates a relatively high resistance of mitochondria in HEK293 cells against oxidative modification.

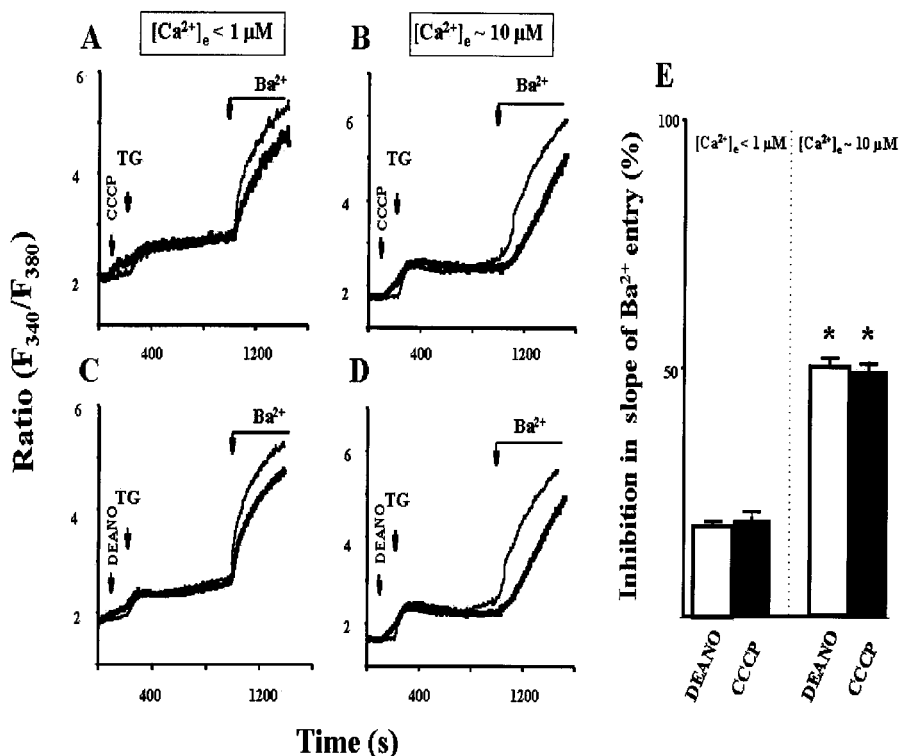


Figure 8 Inhibition of TG-induced Ba²⁺ entry by DEANO (3 μM) and CCCP (300 nM) at different extracellular Ca²⁺ concentrations (<1 μM: A&C; 10 μM: B&D). Time courses of fura-2 fluorescence ratio (F₃₄₀/F₃₈₀) during stimulation of cells with thapsigargin (TG) and addition of 3 mM Ba²⁺. Thin lines denote control experiments performed in the absence of drugs, thick lines represent experiments performed in the presence of DEANO (A, 3 μM); and CCCP (B, 300 nM). Data points are mean values derived from 10 experiments (E). Inhibition of the initial rate of Ba²⁺ entry (calculated from the increase in fluorescence ratio recorded within 1 min upon Ba²⁺ addition) by DEANO (white columns) and CCCP (black columns). Mean values ± s.e.mean derived from 6–8 experiments. *Denotes statistically significant difference at *P* < 0.05 versus control.

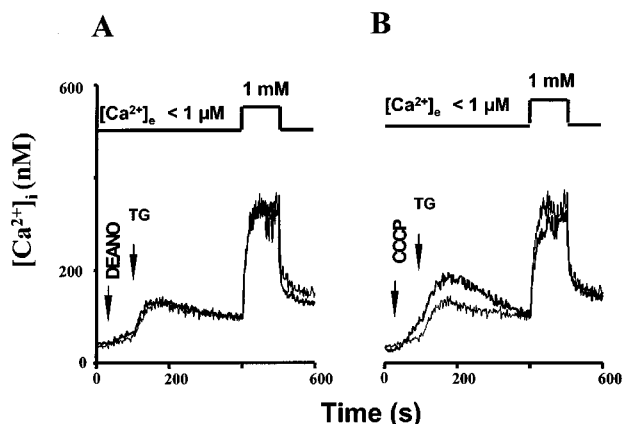


Figure 9 Time courses of [Ca²⁺]_i determined from fura-2 fluorescence. Cells were loaded with BAPTA/AM (10 μM) and stimulated with thapsigargin (TG) in Ca²⁺-free solution, and extracellular Ca²⁺ (1 mM) was added to induce Ca²⁺ entry as indicated. Thin lines denote control experiments performed in the absence of drugs, thick lines represent experiments performed in the presence of DEANO (A; 10 μM) and CCCP (B; 300 nM). Data points are mean values derived from 6–18 experiments. Mean values ± s.e.mean are given for *n* = 6–18. *Denotes statistically significant differences at *P* < 0.05 versus control.

Since a key role of mitochondria in Ca²⁺ signalling is well established (Werth & Thayer, 1994; Herrington *et al.*, 1996), we were prompted to hypothesize that NO exerts its effect on CCE by modulation of mitochondrial Ca²⁺ handling.

NO modifies mitochondrial Ca²⁺ handling in HEK293 cells

An inhibitory effect of DEANO on mitochondrial function was evident from the observed suppression of oxygen consumption. Cellular respiration was suppressed by DEANO but not by SIN-1, indicating that the effect on oxygen consumption was, as that on CCE, not mediated by cGMP or peroxynitrite. Experiments with the fluorescent dyes JC-1 and rhod-2, which report mitochondrial membrane potential and Ca²⁺ levels, respectively, confirmed the substantial impairment of mitochondrial function by NO. NO reduced mitochondrial membrane potential as evident from a significant reduction of JC-1 fluorescence, and NO-induced reduction of rhod-2 fluorescence indicated suppression of mitochondrial Ca²⁺ accumulation. Since rhod-2 fluorescence may be affected to some extent by changes in mitochondrial membrane potential, we set out to test for impairment of mitochondrial Ca²⁺ sequestration by use of an additional, highly specific method for recording of mitochondrial Ca²⁺. Cells were transfected to express a ratiometric-pericam that is targeted to mitochondria. Measurements of Ca²⁺ uptake into mitochondria by this approach revealed a clear inhibitory effect of NO on mitochondrial Ca²⁺ sequestration. These results are in agreement with previous reports suggesting the ability of NO to decrease mitochondrial Ca²⁺ levels in hepatocytes and the β-cell lines, INS-1 (Richter *et al.*, 1994; Laffranchi *et al.*, 1995). In principle, the effects of

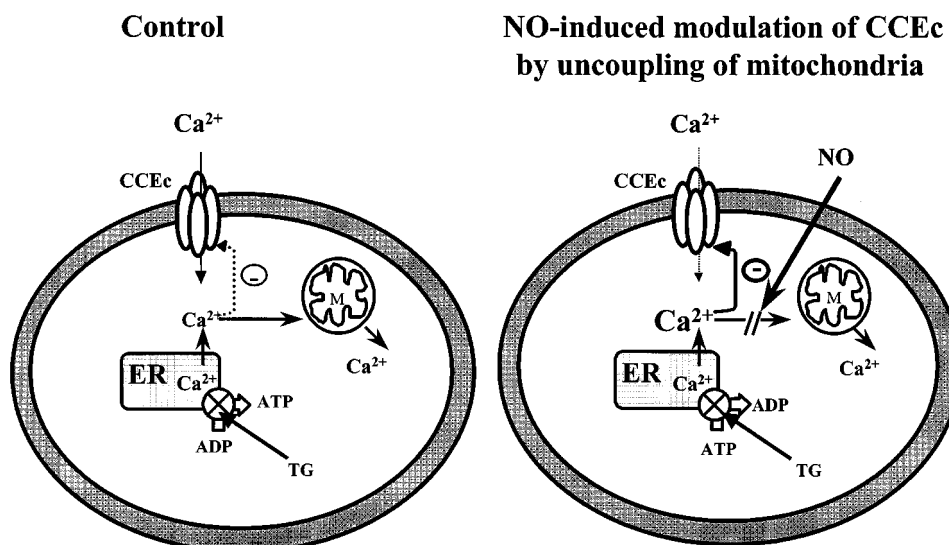


Figure 10 Mitochondria are proposed to function as a sink that reduces the Ca^{2+} concentration close to cytoplasmic face of CCE_c . NO as well as CCCP inhibit mitochondrial Ca^{2+} uptake and increase Ca^{2+} concentrations in regions close to cytoplasmic face of CCE_c . The resulting accumulation of local Ca^{2+} promotes Ca^{2+} -induced negative feedback regulation of CCE_c .

NO on mitochondrial Ca^{2+} handling may be the consequence of mitochondrial depolarization (Richter *et al.*, 1994) due to inhibition of cytochrome c oxidase (Kushnareva *et al.*, 2001). Consistently, the classical mitochondrial uncoupler CCCP, which dissipates mitochondrial H^+ gradients and membrane potential mimicked the effects of NO on CCE.

To further analyse the mechanisms that link changes in mitochondrial function to inhibition of CCE, we tested whether a Ca^{2+} -dependent negative feedback regulation of CCE channel is involved.

Impairment of mitochondrial function by NO promotes Ca^{2+} -mediated negative feedback regulation of CCE

Functional mitochondria appear to maintain CCE after depletion of intracellular Ca^{2+} stores by effective sequestration of subplasmalemmal Ca^{2+} and by the consequent attenuation of Ca^{2+} -induced inactivation of CCE channels (Hoth *et al.*, 1997). The possible role of Ca^{2+} -mediated inactivation of CCE channels in the inhibitory action of NO was studied by variation of the divalent cations permeating through the CCE channels. Ba^{2+} is a divalent which is transported well by many Ca^{2+} entry channels but lacks classical negative feedback effects on the involved transport systems e.g. the voltage-gated L-type channel (Hofer *et al.*, 1997) or capacitance Ca^{2+} entry channels (Zweifach & Lewis, 1995). Ba^{2+} entry into TG-stimulated HEK293 cells was completely insensitive to NO. Thus, the regulation of CCE by NO was clearly dependent on the permeant divalent. Addition of Ca^{2+} ions at micromolar concentrations was sufficient to regain significant inhibitory effects of NO in experiments with Ba^{2+} as the main extracellular cation. Moreover, attenuation of intracellular Ca^{2+} rises by use of BAPTA/AM eliminated the inhibitory effects of NO. Although elevation of basal Ca^{2+} at the inner mouth of CCE channels may not be prevented completely by BAPTA/AM, sub-plasmalemmal Ca^{2+} gradients are most likely blunted. Modulation of intracellular Ca^{2+} gradients by use of Ba^{2+} as substrate for divalent cation entry or BAPTA/

AM as intracellular Ca^{2+} buffer is expected to affect a variety of cellular signalling processes. Thus, the results from these experiments need to be interpreted with caution. Nonetheless, our results strongly suggest Ca^{2+} dependence of CCE inhibition by NO and indicate an involvement of Ca^{2+} -dependent feedback regulation of CCE channels. Based on this hypothesis we propose a novel concept (Figure 10) in which NO-induced impairment of mitochondrial functions causes a substantial promotion of Ca^{2+} -mediated feedback inhibition of CCE. This feedback regulation is unlikely to involve facilitated refilling of the regulatory Ca^{2+} store since refilling of stores was effectively prevented in our experiments by thapsigargin. The role of mitochondria in NO-induced modulation of Ca^{2+} signals which are initiated by phospholipase C-dependent depletion of intracellular Ca^{2+} stores remains to be clarified. Since mitochondrial Ca^{2+} uptake has been recognized as a key determinant of the spatiotemporal organization of IP_3 -induced cytosolic Ca^{2+} responses (Hajnoczky *et al.*, 2000), it is tempting to speculate that impairment of mitochondrial function by NO represents an effective mechanism for the control of phospholipase C-dependent Ca^{2+} signalling. NO-induced suppression of Ca^{2+} uptake into mitochondria during phospholipase C stimulation may affect agonist-induced Ca^{2+} entry not only by promotion of Ca^{2+} -dependent feedback regulation of CCE channels but also by incomplete discharge and/or enhanced filling of the stores.

With the present study we provide evidence for a role of mitochondria as a link between NO and Ca^{2+} signalling. Since capacitance Ca^{2+} entry is a rather ubiquitous Ca^{2+} signalling mechanism which appears of importance for both excitable as well as non-excitable cells (Philipp *et al.*, 1998; Freichel *et al.*, 1999) it is tempting to speculate that the proposed mitochondrial-based crosstalk between NO and CCE is a general regulatory principle. We propose the spatial and functional relation between mitochondria and Ca^{2+} entry channels as a key determinant of the sensitivity of capacitance Ca^{2+} entry to NO.

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