Calcium Store Depletion Induced by Mitochondrial Uncoupling in Prostatic Cells

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Abstract. The effects of mitochondrial uncoupling on the calcium homeostasis of prostatic cells were investigated using the prostatic cancer cell line LNCaP and indo-1 spectrofluorimetry. Carbonyl cyanide m-chloro-phenylhydrazone (CCCP) was used as uncoupler. Resting LNCaP cells responded to CCCP by a biphasic increase in $[Ca^{2+}]_i$. The first phase of increase which corresponded to the release of a mitochondrial CCCP-sensitive Ca^{2+} store was followed by a second increase phase consisting of Ca^{2+} influx through the plasma membrane. The relationship between the CCCP- and the $InsP_3$-sensitive stores was investigated using thapsigargin (TG). The release part of the Ca^{2+} response to TG was reduced in a time-dependent manner by previous exposure of the cells to CCCP, suggesting that CCCP also acts on non-mitochondrial stores. Our results show that CCCP releases Ca^{2+} from both mitochondrial and non-mitochondrial stores in prostatic cells. The possible mechanisms of these effects are discussed.

Key words: Mitochondria — Calcium — Carbonyl cyanide m-chloro-phenylhydrazone — Thapsigargine-sensitive pool — Calcium influx.

Introduction

Intracellular calcium concentration ($[Ca^{2+}]_i$) is a key regulator of a number of cell functions including proliferation, differentiation, apoptosis, etc. (Lu and Means 1993; Berridge 1995). Studies in different cell types have shown that calcium homeostasis is achieved by a number of overlapping mechanisms which interact in a complex manner.

Until recently, the role of mitochondria in intracellular Ca^{2+} homeostasis was controversial. Mitochondria were first recognized as the major source of cellular ATP and were thus involved in metabolic regulation (Nicholls and Ferguson 1992). The mitochondria possess an elaborate system for transporting Ca^{2+} across their inner membrane. Calcium entry into the mitochondria is mediated by a uniporter

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located in the inner membrane (Gunter and Pfeiffer 1990), driven by a large negative potential (up to −180 mV). Export is achieved by two principal mechanisms, an electroneutral Na^+/Ca^{2+} exchanger and an Na^+-independent mechanism, depicted as an active $Ca^{2+}/2H^+$ exchanger (Gunter et al. 1994) driven by pH gradients. Two physiological functions of mitochondrial Ca^{2+} have so far been recognized: protection of the cytosol against damage due to high Ca^{2+} concentrations (Farber 1981) and control of various Ca^{2+}-sensitive metabolic processes, including activation of dehydrogenases (Hansford 1985; Denton and Mc Cormack 1990). However, the precise role of mitochondrial Ca^{2+} in cell Ca^{2+} homeostasis is not completely understood.

Recent studies have attributed mitochondria a role in the rapid uptake of Ca^{2+} during high cytosolic Ca^{2+} loads, followed by slow release of the sequestered Ca^{2+} back into the cytosol in neurons (Thayer and Miller 1990; Friel and Tsien 1994; White and Reynolds 1995; Budd and Nicholls 1996), rabbit cardiac myocytes (Chacon et al. 1996), and chromaffin cells (Park et al. 1996; Herrington et al. 1996; Babcock et al. 1997). Mitochondria are able to buffer intracellular Ca^{2+} according to a high-capacity, low-affinity mechanism, which has been found to operate at cytosolic Ca^{2+} concentrations above 0.5 μmol/l (Herrington et al. 1996). Mitochondrial Ca^{2+} participates in IP_3-triggered calcium waves in *Xenopus* ovocytes (Jouaville et al. 1995) as well as in Ca^{2+} oscillations triggered by GnRH in rat gonadotrophs (Hehl et al. 1996). These observations suggest that the importance of mitochondria in regulating Ca^{2+} signals varies with cell types.

Prostate epithelial cells are non-excitable cells (Skryma et al. 1997) which have been shown to undergo apoptosis upon depletion of their IP_3-sensitive Ca^{2+} stores by thapsigargin (TG) (Furuya et al. 1994). To our knowledge, the involvement of mitochondria in Ca^{2+} homeostasis has not yet been studied in this cell type. The aim of the present study was to investigate the role of the mitochondrial Ca^{2+} pool in the Ca^{2+} homeostasis of prostatic cells and its putative relationship with the TG-sensitive pool, using the prostatic cancer cell line LNCaP as a cell model. To this end, we used the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), which dissipates the mitochondrial membrane potential (Werth and Thayer 1994), allowing the mitochondria to release accumulated Ca^{2+} and preventing its further accumulation in these organelles. We showed by spectrofluorimetry that mitochondrial uncoupling by CCCP in LNCaP cells induces the release of a CCCP-sensitive Ca^{2+} pool and activates Ca^{2+} influx from the extracellular medium. We also found CCCP to reduce non-mitochondrial Ca^{2+} stores. The possible mechanisms of these effects are discussed.

Materials and Methods

Cell culture

LNCaP cells were maintained in RPMI 1640 medium (BioWhittaker, Strasbourg, France) supplemented with 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate and

10 % fetal bovine serum (Eurobio, Les Ullis, France). Cells were routinely grown in 50 ml flasks (Nunc, Poly-Labo, Strasbourg, France) and kept at 37℃ in a humidified incubator in an air/CO_2 (95%/5%) atmosphere. They were passaged every 8 days and fed with fresh medium every 2 or 3 days.

Cells were subcultured on 30 mm diameter round glass coverslips precoated with polyornithine (5 μg/ml) for single cell microspectrofluorimetric measurements, and on poly-ornithine precoated 10 mm diameter glass coverslips for population spectrofluorimetric measurements. Cells were used after 6 days of culture.

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements

Cell population measurements. Experiments were performed using the fluorescent dye indo-1, as already described (Dufy-Barbe et al. 1992). The cells were loaded for 30 min at 37℃ in HBSS (in mmol/l): 142.6 NaCl; 5.6 KCl; 2 $CaCl_2$; 0.8 $MgCl_2$; 5 glucose and 10 HEPES; pH 7.25, adjusted with NaOH; osmolality was 306 mosmol) containing 5 μmol/l indo-1 acetoxy-methylester (indo-1 AM, Sigma) and 0.02 % Pluronic F127 (Molecular Probes, Eugene, OR). The cells were then rinsed three times and the glass coverslip placed in a plastic holder in a quartz cuvette. Indo-1 was excited at 355 nm and the emitted fluorescence measured at 405 and 480 nm in a Hitachi F 2000 spectrofluorimeter. Cell autofluorescence was subtracted from each measurement before ratio determination. $[Ca^{2+}]_i$ was calculated using the 405/480 nm ratio as described by Grynkiewicz et al. (1985). The dissociation constant for the indo-1 Ca^{2+} complex was taken as 406 nmol/l. Values for R max and R min were calculated from measurements with 1 μmol/l ionomycin and 3 mmol/l EGTA in the bath medium, respectively.

Single cell measurements. $[Ca^{2+}]_i$ was recorded in single cells using an inverted microscope (Nikon, Paris, France) equipped for microspectrofluorimetry. The fluorescence ratio (F 405/F 480) was obtained on-line using a laboratory-built analog divider.

Test substances were applied to the cells by low pressure ejection from micropipettes (tip diameter 3–5 μm) positioned approximately 20 μm from the cell membrane.

Most experiments were performed in complete HBSS. In some cases, CaCl2 was omitted from HBSS, the solution will be referred to as no Ca^{2+}-HBSS.

ATP measurements

The cells were seeded on a 24 well plate at 5.10^4 cells/well. After 2 days, the culture medium was discarded and the wells rinsed with 1 ml HBSS. The cells were equilibrated in 1 ml HBSS for 30 min at 37℃. CCCP was added to the experimental wells at a final concentration of 1 μmol/l. At 2 and 10 min, 50 μl of 65 % cold $HClO_4$ were added to the experimental wells. The cells were then scraped and the well content centrifuged for 5 min at $3500 \times g$ at 4℃. The supernatant was neutralized with 2N KOH / 0.3 mol/l MOPS (morpholinopropane sulfonic acid) to pH 6.2–6.8. After centrifugation again, the supernatant was taken for ATP assay.

ATP was measured with a commercial luciferin-luciferase kit (ATP-monitoring kit, Labsystems, Helsinki, Finland), using an LKB Wallac luminometer as described by Ezzahid et al. (1986).

Chemicals

All the chemicals were purchased from Sigma (St Louis, USA) unless otherwise specified. Carbonyl cyanide m-chloro-phenylhydrazone (CCCP), oligomycin, and antimycin were diluted in ethanol as stock solutions at 10, 50 and 100 mmol/l, respectively. For Ca^{2+} measurements in the cell population configuration, CCCP was only used at a final concentration of 1 μmol/l, as higher concentrations significantly interfered with the indo-1 fluorescence. This concentration was sufficient to produce mitochondrial Ca^{2+} mobilization. In the single cell configuration, CCCP could be used at 1, 10, and 25 μmol/l. Cells were challenged using an intermediate concentration of 5 μmol/l. Thapsigargin (TG) was diluted as 1 mmol/l stock solution in dimethylsulfoxide (DMSO). SKF 96365 (1-[3-(4-methoxyphenyl) propoxyl-1-(4-methoxyphenyl)-ethyl-1H-imidazole HCl), a capacitative Ca^{2+} entry inhibitor, was purchased from Calbiochem (La Jolla, CA) and diluted as a 50 mmol/l stock solution in water.

Statistical analysis

Results were expressed as $[Ca^{2+}]_i \pm$ SEM or as percentage increase $\pm$ SEM. The independent, non parametric Mann-Whitney test was used for statistical comparisons; $p < 0.05$ was taken as the level of significance.

Results

Effect of CCCP on $[Ca^{2+}]_i$

Single cell results. $[Ca^{2+}]_i$ was very stable in LNCaP cells and no spontaneous variations were observed. The resting $[Ca^{2+}]_i$ of individual cells was 188 ± 8 nmol/l ($n = 31$), as measured by single-cell microspectrofluorimetry. A brief (10 s) application of 1 μmol/l CCCP induced a gradual increase in $[Ca^{2+}]_i$ which reached a maximum of 418 ± 45 nmol/l (122 ± 32 % over control) after 122 ± 8 s ($n = 9$), followed by a plateau (Fig. 1). 10 and 25 μmol/l CCCP induced an $[Ca^{2+}]_i$ increase of similar magnitude but the latency of the peak was significantly shortened, as compared to 1 μmol/l (Fig. 1, Table 1). In this experimental configuration, the maximal duration of recordings could not exceed 10–12 min, due to photobleaching of the indo-1 probe. Incubation of cells for 30 to 45 min in 1 or 5 μmol/l CCCP induced a $[Ca^{2+}]_i$ increase of approximately 120 % over control values (Table 2).

When the cells were placed in no-Ca^{2+} HBSS containing 3 mmol/l EGTA, a highly significant drop ($p < 0.0001$) in basal $[Ca^{2+}]_i$ was observed after 10–20 min, from 188 ± 8 nmol/l to 96 ± 5 nmol/l ($n = 16$). Under these conditions, 10 μmol/l CCCP induced a transient $[Ca^{2+}]$i increase, which peaked at 204 ± 19 nmol/l, corresponding to a relative increase of 112 ± 16 % over pre-CCCP values

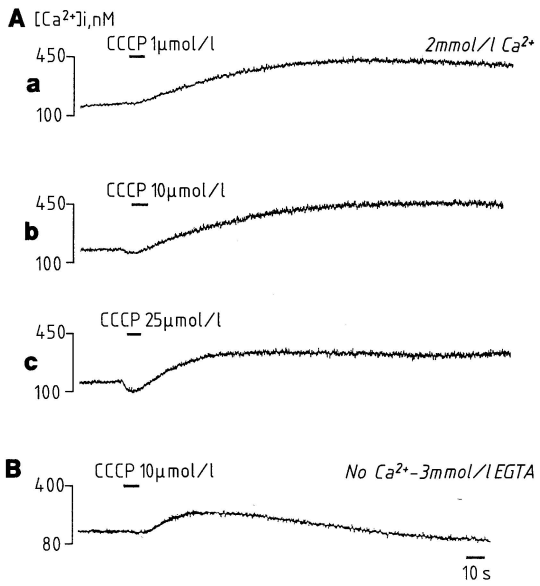

Figure 1. Effects of CCCP on $[Ca^{2+}]_i$ of LNCaP cells as measured by the single-cell method. **A:** Effects of three concentrations of CCCP on the $[Ca^{2+}]_i$ of single cells placed in HBSS containing 2 mmol/l $CaCl_2$. **B**: Effects of 10 μmol/l CCCP on $[Ca^{2+}]_i$ of a cell placed in Ca^{2+}-free HBSS. Individual examples of responses are shown. The transient decrease observed after the addition of 10 and 25 μmol/l is an optical artifact corresponding to the interference of CCCP with the emitted light.

Table 1. Acute CCCP effects on $[Ca^{2+}]_i$

	$[Ca^{2+}]_i$ nmol/l	% $[Ca^{2+}]_i$ increase	Peak latency (s)
Control	188 ± 8 ($n = 31$)		
CCCP 1 μmol/l (10 s)	*418 ± 45 ($n = 9$)	122 ± 32 ($n = 9$)	122 ± 8 ($n = 6$)
CCCP 10 μmol/l (10 s)	*455 ± 24 ($n = 16$)	132 ± 14 ($n = 16$)	**90 ± 6.5 ($n = 16$)
CCCP 25 μmol/l (10 s)	*431 ± 47 ($n = 20$)	129 ± 11 ($n = 20$)	**87 ± 5 ($n = 16$)

* $p < 0.05$ vs control; ** $p < 0.05$ vs 1 μmol/l

Table 2. Effect of incubation in CCCP on $[Ca^{2+}]_i$

	Control	CCCP 1 μmol/l 30 min	CCCP 5 μmol/l 30 min	CCCP 5 μmol/l 45 min
$[Ca^{2+}]_i$ nmol/l	188 ± 8 ($n = 31$)	*417 ± 60 ($n = 9$)	*417 ± 56 ($n = 9$)	*395 ± 81 ($n = 7$)
% $[Ca^{2+}]_i$ increase		122 ± 32 ($n = 9$)	122 ± 30 ($n = 9$)	110 ± 43 ($n = 7$)

* $p < 0.05$ vs control

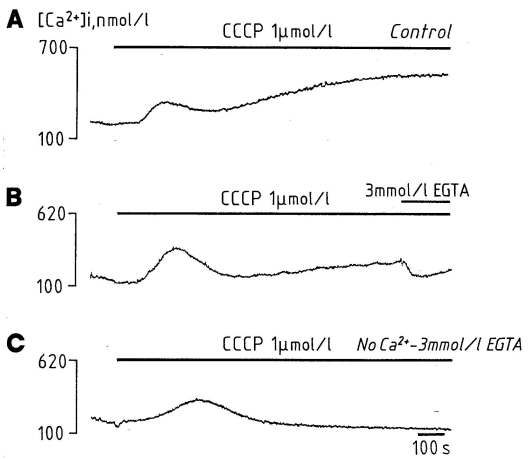

Figure 2. Effects of CCCP on $[Ca^{2+}]_i$ of LNCaP cells as measured by the cell-population method. **A** : Effects of 1 μmol/l CCCP in a recording medium containing 2 mmol/l $CaCl_2$. One of the 9 performed experiments is represented. **B** : Inhibition by 3 mmol/l EGTA of the second phase of the response to CCCP. **C**: Effects of 1 μmol/l CCCP in no-Ca^{2+} HBSS containing 3 mmol/l EGTA.

($n = 8$). This peak value was reached within 74 $\pm$ 5 s and returned toward basal levels after 223 $\pm$ 14 s ($n = 8$) (Fig. 1B).

The response to CCCP seen in Fig. 1A is thus composed of an initial increase phase independent of extracellular Ca^{2+}, which probably results from the release of an intracellular Ca^{2+} store (s), followed by a phase of sustained elevation relying on the entry of extracellular Ca^{2+}.

Cell population results. In this configuration, the test substances were added to the bath medium at the indicated final concentrations and the cells were exposed to these concentrations for the duration of the recording period. Longer recordings were possible with this configuration, as indo-1 bleaching was considerably attenuated as compared to the single cell method.

In complete HBSS containing 2 mmol/l Ca^{2+}, 1 μmol/l CCCP induced a biphasic increase in $[Ca^{2+}]_i$ (Fig. 2A). CCCP triggered an initial $[Ca^{2+}]_i$ increase (first phase) which peaked after 168 $\pm$ 24 s ($n = 9$ experiments), followed by a slight, transient decrease. The initial increase phase, which peaked at 372 $\pm$ 18 nmol/l, corresponding to a 110 $\pm$ 9 % increase over baseline $[Ca^{2+}]_i$ levels, lasted for 349 $\pm$ 29 s ($n = 9$). It was followed by a gradual, long-lasting $[Ca^{2+}]_i$ increase (second phase), which persisted for the whole duration of the recording period (up to 20 min). This second, persistent $[Ca^{2+}]_i$ rise, observed in complete HBSS, was interrupted by the addition of 3 mmol/l EGTA to the bath medium (Fig. 2B). It was completely suppressed in no-Ca^{2+} HBSS containing 3 mmol/l EGTA (Fig. 2C).

The second phase of the CCCP response was completely inhibited by 4 mmol/l $NiCl_2$ (Fig. 3A), a general Ca^{2+}-entry blocker. SKF 96365 (50 μmol/l), a selective "capacitative-entry" blocker (Fig. 3B), caused a 38% inhibition of Ca^{2+} entry. Potassium chloride (KCl, 50 mmol/l), which reduces the electrochemical gradient for Ca^{2+} entry, induced a 50% inhibition (Fig. 3C) while similar concentrations of NaCl had no effect (not shown).

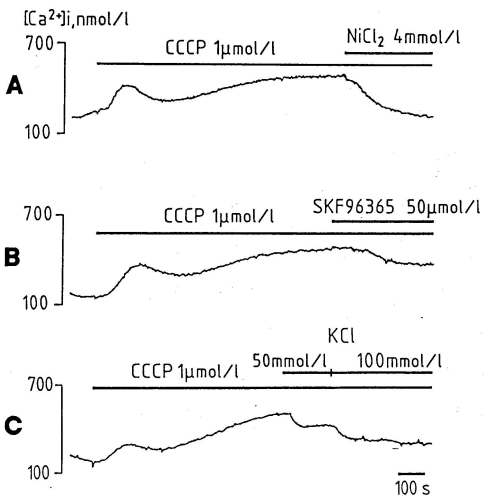

Figure 3. Inhibition of the second phase of the response to CCCP by 4 mmol/l $NiCl_2$ (**A**), 50 μmol/l SKF 96365 (**B**), and 50 and 100 mmol/l KCl (**C**). Cell-population recordings were performed in HBSS containing 2 mmol/l $CaCl_2$. Examples of responses are represented.

The results obtained using the two experimental configurations show that the Ca^{2+} response to CCCP consists of two phases, corresponding to the release of an intracellular CCCP-sensitive Ca^{2+} store (first phase) and Ca^{2+} entry from the extracellular medium (second phase), respectively.

Specificity of the effects of CCCP

To study the specificity of the effects of CCCP, we used thapsigargin (TG) to deplete the $InsP_3$-sensitive intracellular Ca^{2+} stores, independently of $InsP_3$ formation.

Under control conditions (Fig. 4A), the addition of 1 μmol/l TG to the bath medium of an LNCaP cell population caused a significant increase in $[Ca^{2+}]_i$, which peaked at 864 $\pm$ 30 nmol/l, corresponding to a 372 $\pm$ 17 % increase over control. This peak occurred after 401 $\pm$ 35 s ($n = 14$). It was most often followed by a slight decrease towards a plateau. This response could be divided into two parts: a first part, which persisted in no-Ca^{2+} HBSS (not shown) and a second part, which was interrupted by chelating extracellular Ca^{2+} from complete HBSS with 3 mmol/l EGTA (Fig. 4B). These two parts thus correspond to the release of an intracellular Ca^{2+} pool and to Ca^{2+} influx from the extracellular medium, respectively.

When 1 μmol/l TG was applied 750 s after 1 μmol/l CCCP, a response to TG was still observed, as shown in the cell population illustrated in Fig. 5Aa. Under these conditions, the cumulative increases induced by CCCP and TG corresponded to a 313 $\pm$ 28 % ($n = 4$ experiments) over baseline $[Ca^{2+}]_i$. TG specifically induced a 106 $\pm$ 25 % increase over post-CCCP $[Ca^{2+}]_i$ levels. As shown in Fig. 5Ab, a similar result was obtained when TG was applied 220 s after CCCP. These experiments show that CCCP reduces the amplitude of the calcium release induced by TG. This was also the case when the cells were preincubated for 15 to 80 min

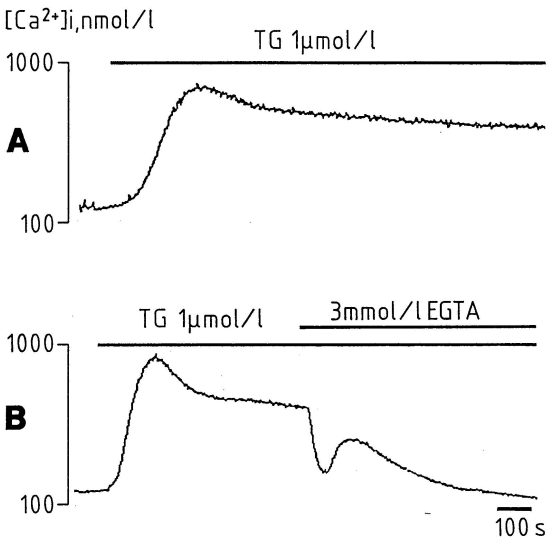

Figure 4. A: Effects of 1 μmol/l thapsigargin (TG) on $[Ca^{2+}]_i$ of a cell population recorded in HBSS containing 2 mmol/l $CaCl_2$. **B**: The addition of 3 mmol/l EGTA to the recording medium during the plateau phase completely inhibited the response.

in CCCP before being challenged with TG (Fig. 5B). Previous exposure to CCCP gradually inhibited the amplitude of the release part of the response to TG, in a time-dependent manner (Fig. 5B). A 30 % inhibition was observed at 15 min, 62 % at 45 min and 81 % at 80 min ($n = 3$).

One may suspect that the decrease of TG-induced calcium release in cells preincubated with CCCP might be due to lowering of intracellular ATP. It has been shown that the effects of some mitochondrial poisons may be reversed by stimulation of glycolysis, which increases ATP synthesis. This can be achieved by increasing the glucose concentration in the extracellular medium. We found that increasing to 20 mmol/l the glucose concentration in the bath medium was without effect on the inhibition of the release part of the TG response induced by preincubating the cells in CCCP (not shown).

Two possibilities thus remain: intracellular ATP may not be involved in the response, or intracellular ATP remains high enough in the presence of CCCP. We have followed the variations of the ATP content in cells incubated with CCCP. We found 1 μmol/l CCCP to significantly decrease the ATP cell content at 2 min (-20 %) as compared to controls. This decrease was almost reversed at 10 min (Fig. 6).

To further assess the specificity of CCCP towards mitochondrial Ca^{2+} stores, we also used oligomycin and antimycin, two agents known to alter mitochondrial function. The addition to the bath medium of oligomycin (50 μmol/l), an ATP-synthase inhibitor, did not modify $[Ca^{2+}]_i$ (Fig. 7A). Antimycin (10 μmol/l), an inhibitor of electron transport through the respiratory chain, induced a $[Ca^{2+}]_i$ increase of 55 $\pm$ 3 % over control ($n = 8$) (Fig. 7B). Incubation of cells in both antimycin (10 μmol/l) and oligomycin (50 μmol/l) for 30 min induced a $[Ca^{2+}]_i$

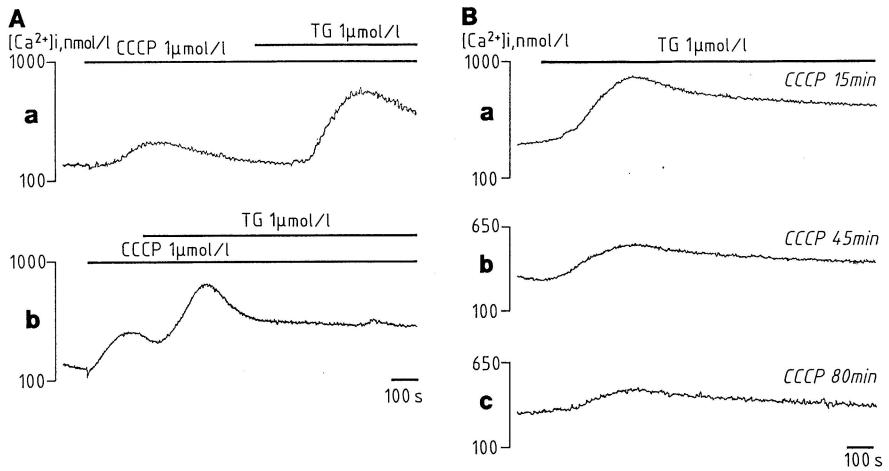

Figure 5. A: Cumulative effect on Ca^{2+} of CCCP and TG. CCCP was first applied to a cell population in control HBSS. TG was then added to the bath 750 s (5Aa) or 200 s (5Ab) after CCCP. **B**: Effects of preincubation of the cells in CCCP (1 μmol/l) on the response to TG. Increasing the preincubation time from 15 to 80 min in 1 μmol/l CCCP resulted in the gradual inhibition of the first part of the response to TG and completely abolished the entry phase.

increase of 68 $\pm$ 8 % over control, with a maximum of approximately 330 nmol/l Ca^{2+}, and completely abolished the response to an acute application of CCCP (1 μmol/l) ($n = 6$) (Fig. 7C).

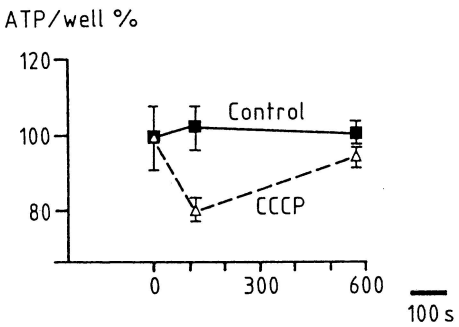

Figure 6. Effect of 1 μmol/l CCCP on the ATP cell content of LNCaP cells. The cells were seeded at 5×10^4 cells per well. After 2 days, the culture medium was replaced by HBSS. 1 μmol/l CCCP was added to the experimental wells. The cells were fixed at 2 and 10 min in cold $HClO_4$. The cells were then processed for ATP assay as described in Materials and Methods. Results are expressed as % of controls.

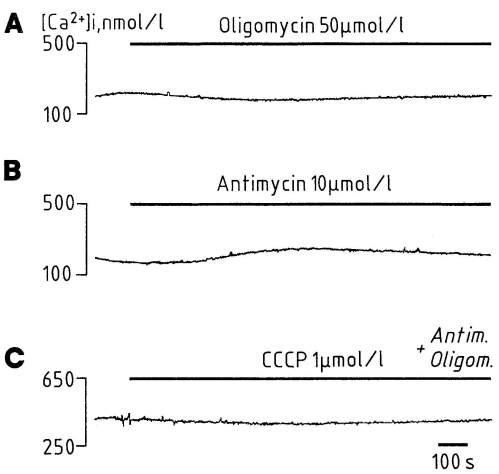

Figure 7. Effects of oligomycin and antimycin on $[Ca^{2+}]_i$ of LNCaP cell populations. Oligomycin (50 μmol/l) had no effect (**A**) whereas antimycin (10 μmol/l) increased $[Ca^{2+}]_i$ slightly (**B**). Incubation for 30 min with the two substances completely suppressed the response to CCCP (**C**). Examples of responses obtained in the different situations are shown.

Discussion

We report here for the first time that the mitochondrial uncoupler carbonyl cyanide m-choro-phenylhydrazone (CCCP) causes the release of a CCCP-sensitive Ca^{2+} store in resting prostatic cells. This first phase of release is followed by a second phase of gradual increase in cytosolic Ca^{2+}, corresponding to a Ca^{2+} influx from extracellular sources.

CCCP is a protonophore known to inhibit Ca^{2+} uptake through the mitochondrial uniporter by collapsing the mitochondrial proton gradient and dissipating the potential across the inner membrane which provides the driving force for Ca^{2+} import (Thayer and Miller 1990; Friel and Tsien 1994; Babcock et al. 1997). CCCP not only stops Ca^{2+} accumulation but also allows the mitochondria to release accumulated Ca^{2+}. In addition, CCCP uncouples mitochondrial respiration from mitochondrial ATP synthesis.

In LNCaP cells, CCCP induced an initial increase in $[Ca^{2+}]_i$ which was independent of extracellular sources, and thus corresponded to the release of an intracellular Ca^{2+} store. This result suggests that resting prostatic cells possess a CCCP-releasable Ca^{2+} store which is probably contained in the mitochondria. A confirmation that CCCP acted on mitochondrial Ca^{2+} was provided by the use of two other agents targeted at mitochondrial function, antimycin and oligomycin. Antimycin inhibits electron transport through the respiratory chain at the level of complex III, preventing oxidative reactions from creating a proton electrochemical gradient accross the inner mitochondrial membrane. Oligomycin blocks mitochondrial ATP production by direct inhibition of ATP synthase. A 30 min incubation of cells in both oligomycin and antimycin significantly increased cytosolic Ca^{2+} levels, probably by releasing mitochondrial stores, and completely suppressed the response to CCCP, showing that Ca^{2+} uptake in the CCCP-sensitive Ca^{2+} pool was inhibited by the combination of the two substances.

By depolarizing the inner mitochondrial membrane, uncouplers induce the formation of the permeability transition pore (PTP) through their protonophoretic action (for a review, see Zoratti and Szabo 1995). It is thus possible that the Ca^{2+} release from the mitochondria observed in our experiments occurs via PTP induction by CCCP.

To investigate the relationship between the CCCP-sensitive and other intracellular Ca^{2+} stores, we used thapsigargin (TG), a Ca^{2+}-ATPase inhibitor, which depletes the Ca^{2+} pools of the endoplasmic reticulum (ER) and more generally $InsP_3$-sensitive Ca^{2+}-pools (Thastrup et al. 1990). We found that, although TG was still capable of inducing Ca^{2+} release following acute application of CCCP, the amplitude of this response was reduced in a time-dependent manner by preexposing the cells to CCCP.

The release of the CCCP-sensitive Ca^{2+} store was followed by a secondary increase in cytosolic Ca^{2+} which started 4-6 min after CCCP application and persisted throughout the duration of the recording. This second phase of Ca^{2+} increase was inhibited by $NiCl_2$, a blocker of non-specific Ca^{2+} channels, and reduced by SKF 96365, which has been described as a "capacitative Ca^{2+} entry" blocker (Sage et al. 1989; Merritt et al. 1990). This phase was also reduced by KCl, which depolarizes the cell membrane and reduces the driving force for Ca^{2+} entry. This second phase of the response to CCCP thus consists of an influx of extracellular Ca^{2+} through the plasma membrane.

The mechanism of this Ca^{2+} influx triggered by CCCP in prostatic cells is not clear. The plasma membrane permeability of non-excitable cells is regulated in part by the degree of filling of intracellular storage compartments. It is possible that depletion of mitochondrial Ca^{2+} directly activates Ca^{2+} channels at the plasma membrane level by a mechanism similar to that involved in the capacitative Ca^{2+} entry triggered by depletion of the IP_3-TG-sensitive stores (Putney 1990; Penner et al. 1993; Clapham 1995). Alternatively, inhibition of mitochondrial ATP synthesis by CCCP which reduces total ATP cell content may impair several energy dependent cell processes, including the filling of the stores of the ER by the

Ca^{2+} ATPases (Marriot and Mason 1995; Gamberucci et al. 1998). We found the ATP cell content to become only slightly (−20 %) and transiently reduced at 2 min after CCCP addition. Moreover, this inhibitory effect of CCCP on ATP cell content was reversed at 10 min, probably due to increased ATP supply from the glycolysis. It is not known whether this transient reduction of ATP was capable of altering the intraluminal Ca^{2+} concentration in the ER of LNCaP cells. However, as the affinity of the Ca^{2+}-ATPases of the ER for the nucleotide is in the micromolar range, the slight reduction in ATP observed at 2 min is unlikely to induce an ER pool depletion sufficient to provoke the long lasting Ca^{2+} entry observed in our experiments. Conversely, we propose that the neo synthesis of ATP which follows the transient decrease participates in the "capacitative-like" Ca^{2+} entry elicited to replenish the intracellular stores according to a mechanism described in other cell models (Gamberucci et al. 1994).

The mechanism(s) by which preincubation for more than 30 min in CCCP dramatically reduced the TG-sensitive Ca^{2+} stores of LNCaP cells is/are unknown. We found that increasing glucose concentration in the medium, which is known to activate glycolytic synthesis of ATP (Mohr and Fewtrell 1990; Budd and Nicholls 1996) did not reverse this inhibiting effect of CCCP on the TG-sensitive pool. This negative result suggests that in prostatic cells, depletion of the TG-sensitive pool is not directly related to bulk energy supply. Alternatively, CCCP may act, in this case, on non-mitochondrial stores. It has been shown that FCCP, another mitochondrial uncoupler, released Ca^{2+} from ER vesicles isolated from the pancreas and decreased the sequestration into non-mitochondrial Ca^{2+} stores of permeabilized pancreatic cells (Wakasugi et al. 1982). These data were interpreted to support the proposal that a proton gradient accross the ER is necessary for Ca^{2+} retention in these stores (Galvan and Lucas 1987).

It was recently reported (Makowska et al. 2000) that in Jurkat cells, another non-excitable cell type, CCCP did not alter the TG-sensitive Ca^{2+} pool but inhibited the Ca^{2+} influx induced by store depletion. These results differ from those reported here. Although the reasons for this discrepancy are not known, they might be related to differences in $[Ca^{2+}]$i regulatory mechanisms in the two cell models. It should be mentioned that in both cases, the effects of CCCP are not due to a decrease in intracellular ATP content.

In summary, CCCP has complex effects on the Ca^{2+} homeostasis of prostatic cells. CCCP triggers the release of Ca^{2+} presumably from the mitochondria, and activates Ca^{2+} influx through the plasma membrane. It is not known whether the activation of Ca^{2+} entry by CCCP is specific to prostatic cells or whether it also operates in other non-excitable cell models. It may not be observed in excitable cells such as rat pheochromocytoma cells, where the increase in cytosolic Ca^{2+} caused by CCCP was found to be exclusively of intracellular origin by Luo et al. (1997). In addition to its mitochondrial effects, CCCP also induces the depletion of the non-mitochondrial, IP_3-TG-sensitive stores, stressing the importance of mitochondrial function in the Ca^{2+} homeostasis of this particular cell type.

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