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Mitochondrial inhibitors activate influx of external Ca^{2+} in sea urchin sperm

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ABSTRACT

Sea urchin sperm have a single mitochondrion which, aside from its main ATP generating function, may regulate motility, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and possibly the acrosome reaction (AR). We have found that acute application of agents that inhibit mitochondrial function via differing mechanisms (CCCP, a proton gradient uncoupler, antimycin, a respiratory chain inhibitor, oligomycin, a mitochondrial ATPase inhibitor and CGP37157, a Na^+/Ca^{2+} exchange inhibitor) increases $[Ca^{2+}]_i$ with at least two differing profiles. These increases depend on the presence of extracellular Ca^{2+}, which indicates they involve Ca^{2+} uptake and not only mitochondrial Ca^{2+} release. The plasma membrane permeation pathways activated by the mitochondrial inhibitors are permeable to Mn^{2+}. Store-operated Ca^{2+} channel (SOC) blockers (Ni^{2+}, SKF96365 and Gd^{2+}) and internal-store ATPase inhibitors (thapsigargin and bisphenol) antagonize Ca^{2+} influx induced by the mitochondrial inhibitors. The results indicate that the functional status of the sea urchin sperm mitochondrion regulates Ca^{2+} entry through SOCs. As neither CCCP nor dicycloexyl carbodiimide (DCCD), another mitochondrial ATPase inhibitor, eliminate the oligomycin induced increase in $[Ca^{2+}]_i$, apparently oligomycin also has an extra mitochondrial target.

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1. Introduction

The main function of mitochondria is the production of high-energy ATP through the electron transport chain and lipid oxidation. In addition, this organelle is important in the regulation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]$cyt). For many years it had been thought that mitochondria had the potential to accumulate and release Ca^{2+} to the cytosol only when cells were damaged and $[Ca^{2+}]$cyt elevated to >10 μM. Now it is generally accepted that agonist stimulation, which in many healthy cell types increases $[Ca^{2+}]$cyt, leads to mitochondrial Ca^{2+} accumulation. Depending on the cell type, the increase in mitochondrial Ca^{2+} concentration ($[Ca^{2+}]$mt) varies between 1 and 20 μM [1]. Mitochondria can locally alter the spatio-temporal pattern of Ca^{2+} changes following Ca^{2+}-influx and Ca^{2+}-release by buffering this divalent from cytoplasmic microdomains [2]. Therefore, this organelle can modulate site-specific, Ca^{2+}-dependent ion channel activation and inactivation. The sub-cellular localization of mitochondria in conjunction with cell-specific channel expression is fundamental for Ca^{2+} homeostasis [2]. Furthermore, changes in $[Ca^{2+}]$mt regulate the metabolic state of this organelle thus coupling ion fluxes to metabolic requirements [3].

It is accepted that Ca^{2+} enters the mitochondria via an electrogenic Ca^{2+} uniporter (CaUP; $EC_{50}\sim$10–20 μM; [4]) driven by the electrical potential difference across the inner mitochondrial membrane. Subsequently, mitochondrial Ca^{2+} can be released either by Na^+/Ca^{2+} or H^+/Ca^{2+} exchangers [5]. At elevated $[Ca^{2+}]$mt and upon mitochondrial depolarization, the permeability transition pore of the mitochondria (mPTP) opens, producing a massive Ca^{2+}-release [2,6,7]. The molecular identity, regulation and pharmacology of the mPTP are still not fully understood [6]. Modulation of $[Ca^{2+}]_i$ dynamics by mitochondria has been demonstrated in many forms of Ca^{2+} signaling [8–11]. The increase in $[Ca^{2+}]$mt that results from intracellular Ca^{2+} signaling regulates ATP synthesis to compensate for increased energy demands [10,12–15].

In sea urchin sperm, the sole mitochondrion is localized to the head (at its intersection with the flagellum) and is responsible for ATP production which is crucial for motility regulation. These cells lack glycogen stores and do not synthesize lactate dehydrogenase [16]. Hence, ATP production results entirely from fatty acid oxidation [17,18]. A creatine kinase/phosphocreatine shuttle transports the high-

Abbreviations: ASW, artificial sea water; BF, bisphenol; $[Ca^{2+}]$cyt, cytosolic Ca^{2+} concentration; $[Ca^{2+}]$mt, mitochondrial Ca^{2+} concentration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CGP37157, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one; DCCD, dicycloexyl carbodiimide; DMSO, dimethylsulfoxide; ER, endoplasmic reticulum; PM, plasma membrane; SKF96365, (1-[b-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride); SOCC, store operated calcium channels; TG, thapsigargin

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energy phosphate of ATP along the flagellum to the dyneins in the axoneme [19].

Here we examined the role of mitochondria in the regulation of $[Ca^{2+}]_i$ in sea urchin sperm physiology. For this purpose we used different agents that alter normal mitochondrial function to determine how they affect $[Ca^{2+}]_i$. Sea urchin sperm suspensions loaded with Ca^{2+} sensitive fluorescent indicators fluo-4 or fura-2, were exposed to i) CCCP, a protonophore uncoupler (it also increases plasma membrane H^+ permeability) ii) antimycin A, a respiratory chain inhibitor iii) CGP37157 (CGP), an inhibitor of the mitochondrial Na^{2+}/Ca^{2+} exchanger [20] or to iv) oligomycin, an inhibitor of the F_1F_0-ATPase [21], to determine if and how these inhibitors affect $[Ca^{2+}]_i$. Addition of all mitochondrial inhibitors to sperm suspensions induced increases of $[Ca^{2+}]_i$ with different characteristics, but all involving plasma membrane Ca^{2+} influx. The permeability pathways activated by the mitochondrial inhibitors are permeable to Mn^{2+} whose uptake in turn is blocked by Ni^{2+}. The oligomycin induced $[Ca^{2+}]_i$ increase was also inhibited by Ni^{2+} (IC_{50} of ~10 μM). Furthermore, SKF96365 and Gd^{3+}, blockers of capacitative Ca^{2+} uptake and of TRP channels respectively [22,23], inhibited this Ca^{2+} influx by ~80% and ~50% respectively. The pharmacological profiles of the other mitochondrial inhibitors were also explored. The results suggest that Ca^{2+} uptake, possibly through SOC-type channels, in the sea urchin sperm plasma membrane can be regulated by the functional status of its mitochondrion.

2. Materials and methods

2.1. Gametes and reagents

S. purpuratus sea urchins were obtained from Marinus (Long Beach, CA, USA) and from Pamanes (Ensenada, Baja California, Mexico). Spawning was induced by intracelomic injection of 0.5 M KCl. Dry sperm were collected and kept on ice until used. Fura-2-AM and fluo-4-AM were obtained from Invitrogen-Molecular Probes (Eugene, OR, USA). SKF96365 (1-[b-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride) was from Calbiochem (La Jolla, CA). The rest of the reagents used were of the highest quality available. Artificial sea water (ASW) was prepared with the following composition (in mM): 475 NaCl, 26 $MgCl_2$, 10 KCl, 30 $MgSO_4$, 10 $CaCl_2$, 2.5 $NaHCO_3$ and 0.1 EDTA; the pH was adjusted to 8.0 with NaOH. Ca^{2+} free or 1 mM Ca^{2+} ASWs were the same, with the exception that the former also contained 0.2 mM EGTA and the latter was pH 7.0. In all cases the osmolarity was 950–1000 mOsm.

2.2. $[Ca^{2+}]_i$ and Mn^{2+} quenching measurements

Fluorometric determinations were performed in a SLM 8000 Aminco spectrofluorimeter with a temperature-controlled cell holder (15 °C), equipped with a magnetic stirrer. Stock solutions (1 mM) of fura-2 AM, and fluo-4 AM were made in DMSO. Sperm were loaded in the dark at 15 °C, by diluting dry sperm (~25–50 µl) 1:4 in 1 mM Ca^{2+} ASW, containing 20 µM fura-2 AM/0.6% Pluronic F-127, or 10 µM fluo-4/0.6% Pluronic F-127 [24]. After loading, 10 ml of 1 mM Ca^{2+} ASW, was added and celomocyte cells and spines were removed from the sperm suspension by centrifugation at 121 *g* for 7 min at 4 °C. The pellet was discarded and the dye remaining in the media was removed by centrifugation (1000 *g* for 8 min at 4 °C). The sperm pellet was resuspended in the original volume of 1 mM Ca^{2+} ASW and kept on ice until used. A 10–20 µl aliquot of this suspension was added to a round cuvette containing 800 µl ASW or the indicated modified ASW, at 15 °C. The suspension was stirred constantly for 30 s and thereafter the different mitochondrial inhibitors in DMSO were added (1.5–2 µl) never exceeding a concentration of 0.4% v/v. Fluo-4 was excited at 495 nm and its fluorescence emission monitored at 515 nm. Mn^{2+} entry was determined by recording the loss of fura-2 fluorescence at its isosbestic wavelength for Ca^{2+} (excitation at 358 nm, emission 500 nm) in ASW containing 3 mM $MnCl_2$, pH 8.0. Loss of the fura-2 signal by Mn^{2+} was expressed as the percentage of fluorescence decrease induced by the mitochondrial inhibitors. All measurements were taken every 0.2–0.5 s. Resting levels of $[Ca^{2+}]_i$ in *S. purpuratus* sea urchin sperm populations measured with fura-2 vary between 65 and

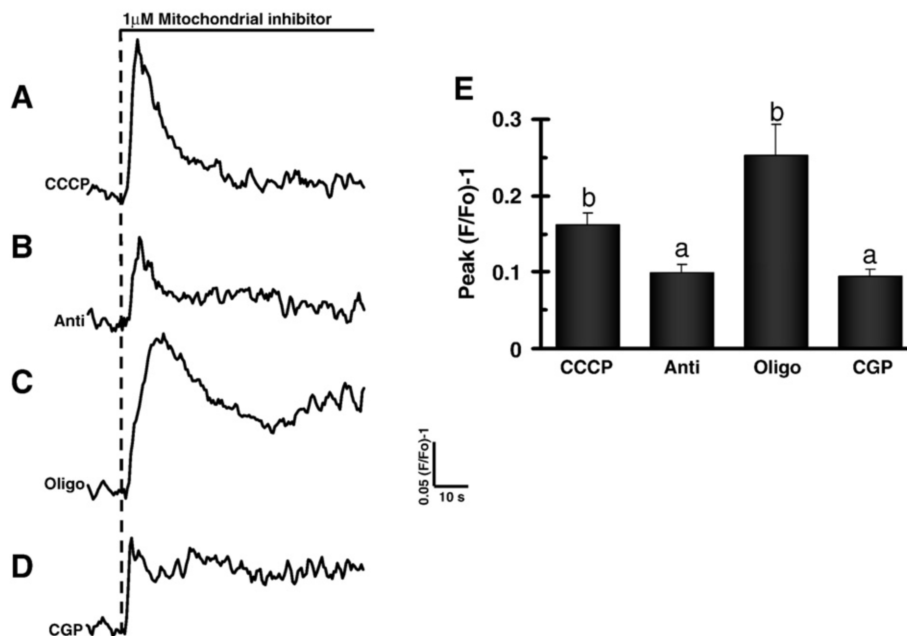

Fig. 1. Mitochondrial inhibitors (1 µM) produce transient $[Ca^{2+}]_i$ rises in sea urchin sperm. In all figures $[Ca^{2+}]_i$ responses to the addition of mitochondrial inhibitors, indicated by the vertical broken line, were measured using fluo-4 loaded cells, as indicated in Materials and methods. (A) The addition of CCCP, (B) Antimycin, (C) Oligomycin or (D) CGP, caused transient increases in $[Ca^{2+}]_i$ with at least two different response patterns. These representative patterns are shown along with the percentage of sperm lots displaying such a response. Control experiments using the vehicle elicited no response (data not shown). (E) Peak increases for CCCP and oligomycin (b) were significantly higher ($P<0.01$) than those of antimycin and CGP (a). However, the difference between CCCP and oligomycin or antimycin and CGP (Average±SEM, n=26, 28, 37 and 13 respectively for CCCP, antimycin, oligomycin and CGP) were not significantly different.

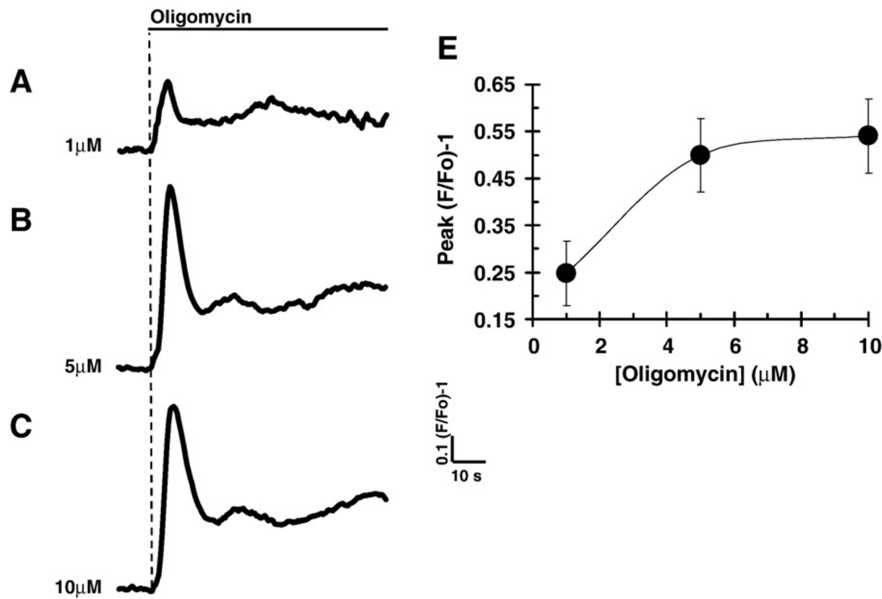

Fig. 2. Oligomycin induces dose-dependent $[Ca^{2+}]_i$ transient increases in sea urchin sperm. Representative responses to (A) 1, (B) 5 or (C) 10 μM oligomycin. (E) Maximal response was reached at 5 μM oligomycin and peak $[Ca^{2+}]_i$ increases differed significantly ($P<0.01$) between 1 and 5 μM but not between 5 and 10 μM oligomycin (Average±SEM of three independent experiments, each repeated in triplicate).

200 nM [24–27] (and this paper). Considering the fura-2 measurements, the largest response to a mitochondrial inhibitor (5 μM oligomycin) increases $[Ca^{2+}]_i$ to 150–500 nM. These values are relative as they assume a 700 nM Kd of fura-2 for Ca^{2+} which is difficult to determine precisely.

2.3. ATP determinations

ATP levels were determined as described [28] in a Bio Orbit luminometer using a kit from Invitrogen Molecular Probes (A22066). The experimental conditions were exactly those used in the sperm

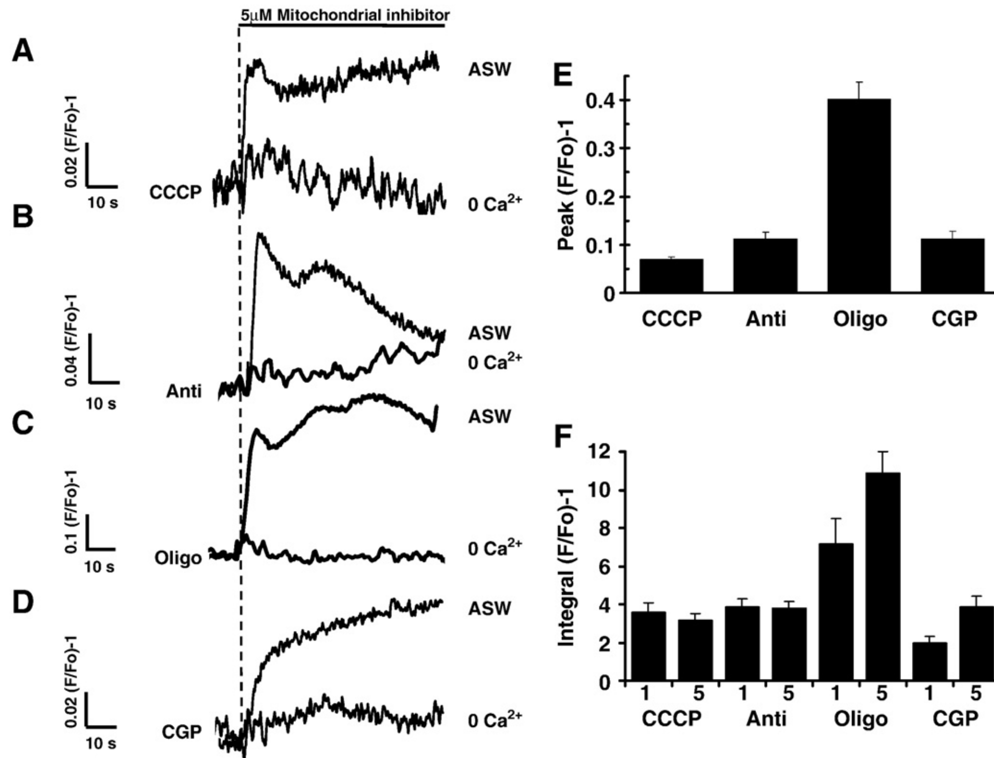

Fig. 3. The transient increase in sea urchin sperm $[Ca^{2+}]_i$ induced by 5 μM CCCP (A), antimycin (B), oligomycin (C) and CGP (D) is dependent on external Ca^{2+}. No responses were observed when sperm were suspended in Ca^{2+}-free ASW. Representative records are shown, along with the percentage of sperm lots displaying such response pattern. E and F summarize results in ASW which contains 10 mM $CaCl_2$. (E) The peak $[Ca^{2+}]_i$ increase for oligomycin was significantly higher ($P<0.01$) than that elicited by the other three mitochondrial inhibitors. The use of CCCP induced the lowest increase in $[Ca^{2+}]_i$. There were no differences between the $[Ca^{2+}]_i$ increases provoked by antimycin and CGP (Average±SEM, n=50, 51, 82 and 36 respectively for CCCP, antimycin, oligomycin and CGP). (F) Integral (50 s) of the $[Ca^{2+}]_i$ responses induced by 1 and 5 μM (1=1 μM; 5=5 μM) CCCP, antimycin, oligomycin and CGP (values are Averages±SEM; n=>25, >11, >34, >11, respectively).

$[Ca^{2+}]_i$ determinations. Data were normalized considering the ATP concentration of control sperm (without mitochondrial inhibitor) at the indicated times. After 10 s in the fluorometer cell, the sperm suspension contained 3.06±0.76 pMol ATP/μg protein, $n=8$, a value similar to previous reports [32].

2.4. Statistics

Peak amplitudes were calculated using Igor software (Igor 4.01 WaveMetrics, Inc, Oregon). The values given in bar plots are the means±S. E. M. with the number of experiments indicated. Student's t test or ANOVA were used for statistical evaluations. Non parametric results were analyzed with Excel Analyze-it and Kruskal-Wallis Anova. Differences were considered significant when $p<0.05$).

3. Results

3.1. Mitochondrial inhibitors elevate sperm $[Ca^{2+}]_i$

We first examined the influence of various mitochondrial inhibitors on the resting $[Ca^{2+}]_i$ of sea urchin sperm populations loaded with fluo-4. Addition of 1 μM CCCP, antimycin, oligomycin or CGP to sperm suspensions produced rapid increases in $[Ca^{2+}]_i$ (Fig. 1A–D). Though all inhibitors displayed a transitory component, the kinetics and amount of sustained $[Ca^{2+}]_i$ elevation differed for each one. The figure shows examples of representative $[Ca^{2+}]_i$ responses obtained for each inhibitor. It is worth mentioning that the responses vary within sperm batches. In addition, as the season through which the urchins have mature sperm progresses, the responses tend to become smaller. The experiments with all the mitochondrial inhibitors reported here span at least 2 seasons.

Oligomycin induced the largest peak response and frequently displayed thereafter one, or more, smaller responses. Pooled data of the initial peak responses from at least 5 individual sea urchin sperm batches made with all the mitochondrial inhibitors, with a minimum of two repetitions, are summarized in Fig. 1E. Fig. 2 shows a dose-response curve for oligomycin obtained from three independent experiments in triplicate. The peak amplitude of the initial oligomycin response nearly saturated at 5 μM.

3.2. $[Ca^{2+}]_i$ increases induced by mitochondrial inhibitors depend on external Ca^{2+}

To determine if the increase in $[Ca^{2+}]_i$ induced by mitochondrial inhibitors results from Ca^{2+} release from i) mitochondria or other intracellular stores ii) Ca^{2+} entry from the outside or iii) both sources, $[Ca^{2+}]_i$ was recorded in sperm suspended in Ca^{2+} free ASW (+0.2 mM EGTA). Fig. 3A–D display representative responses to 5 μM of the mitochondrial inhibitors in normal ASW and their absence in Ca^{2+}-free ASW. These results, summarized in Fig. 3E, indicate that $[Ca^{2+}]_i$ increases induced by these inhibitors are strongly dependent on external Ca^{2+}. In Ca^{2+}-free ASW we were unable to detect Ca^{2+} release from the sperm mitochondrion or from other internal stores.

Washing and suspending sea urchin sperm in Ca^{2+} free ASW could deplete Ca^{2+} inside mitochondria or other internal stores, thus explaining the inability of the mitochondrial inhibitors to increase $[Ca^{2+}]_i$. To minimize this problem, we removed external Ca^{2+} from normal ASW by adding 15 mM EGTA shortly before exposing sperm to the mitochondrial inhibitors. Under these conditions, where Ca^{2+} leakage from mitochondria and other internal stores should be minimal, mitochondrial inhibitors were still unable to elevate $[Ca^{2+}]_i$ (results not shown).

As the responses to the mitochondrial inhibitors are complex, displaying an initial peak and then a sustained increase in many cases, we calculated the integral over time of the responses during 50 s at 1 and 5 μM of the inhibitors (Fig. 3F). Oligomycin displayed dose dependence for both the peak (Fig. 2) and the integrated response; this was also the case for the integrated response to CGP. In contrast, the integrated responses to CCCP and antimycin saturated at 1 μM.

Further characterization of the sperm plasma membrane Ca^{2+} influx pathway stimulated by the mitochondrial inhibitors was carried out using Mn^{2+} under the assumption that this divalent ion permeates through Ca^{2+} entry pathways [29]. All mitochondrial inhibitors induced Mn^{2+} entry, monitored as the loss of fura-2 fluorescence at its isosbestic wavelength for Ca^{2+} (359 nm excitation, see Materials and methods) (Fig. 4). As anticipated, oligomycin caused the largest response followed by CCCP, antimycin and CGP. Altogether, these findings indicate that one of the effects of the mitochondrial inhibitors is the opening of a Ca^{2+} influx pathway in the sperm plasma membrane.

3.3. Ni^{2+}, SKF96365 and Gd^{3+} differentially affect Ca^{2+} influx induced by mitochondrial inhibitors

To pharmacologically characterize the Ca^{2+} influx pathway activated by mitochondrial inhibitors, we first tested Ni^{2+} (Fig. 5A and B). In the sea urchin sperm, low concentrations of this divalent cation (IC_{50} 10 μM) block channels activated by the emptying of Ca^{2+} stores (store-operated Ca^{2+} channels, SOCs) and the “second Ca^{2+} channel” involved in the AR [27]. Higher Ni^{2+} concentrations (~300 μM) inhibit voltage-dependent Ca^{2+} channels also present in

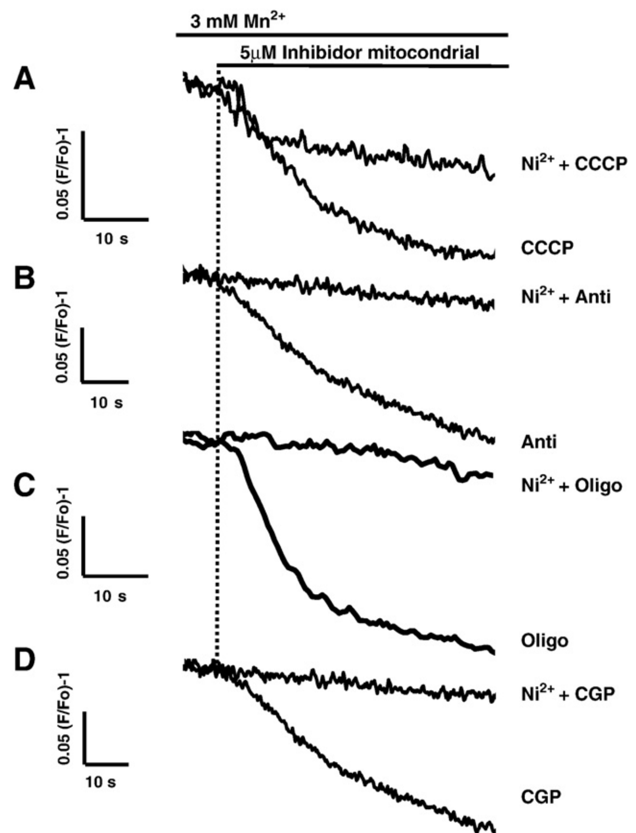

Fig. 4. Addition of 5 μM CCCP (A), antimycin (B), oligomycin (C) and CGP (D) induce Mn^{2+} influx in sea urchin sperm populations. Mn^{2+} uptake was determined measuring fura-2 fluorescence quenching at the isosbestic wavelength for Ca^{2+} (359 nm excitation) in ASW containing 3 mM Mn^{2+} (see Materials and methods for full explanation). Ni^{2+} (300 μM) inhibited the response to all mitochondrial inhibitors. Representative records are shown ($n=4$).

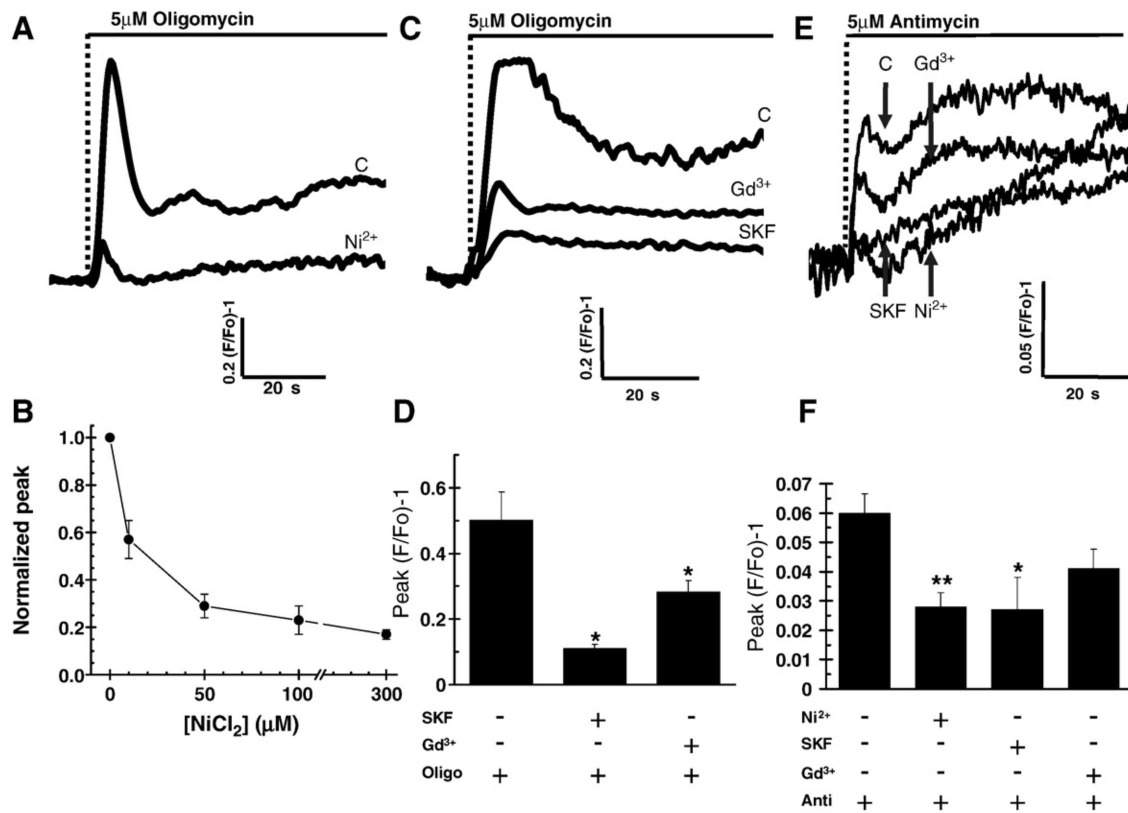

Fig. 5. Store-operated Ca^{2+} channel blockers inhibit the increase in $[Ca^{2+}]_i$ induced by oligomycin and antimycin. Ni^{2+} inhibited the increase in $[Ca^{2+}]_i$ induced by oligomycin in a dose dependent manner. (A) Representative records of control (C) and maximal Ni^{2+} blockade are shown. (B) The average of three independent experiments with duplicates shows that half maximal Ni^{2+} inhibition occurs at ~10 μM. (C) Oligomycin-induced increases in $[Ca^{2+}]$i are inhibited by 10 μM SKF and 5 μM Gd^{3+} respectively (representative traces). (D) The average of three independent experiments as described in C. (E) Representative responses to antimycin alone (C) or in the presence of 300 μM Ni^{2+}, 10 μM SKF and 5 μM Gd^{3+}. (F) Average±SEM of at least 7 experiments (*: $P<0.05$, **: $P<0.01$).

these cells [30,31]. Fig. 4 illustrates how 300 μM Ni^{2+} interferes with the mitochondrial inhibitor induced Mn^{2+} entry. This Ni^{2+} concentration also blocks >75% of the Ca^{2+} influx induced by oligomycin (Fig. 5A). A half-maximal inhibition of 10 μM is obtained from the Ni^{2+} inhibition dose response curve of the oligomycin peak (Fig. 5B). Two well known blockers of SOC channels, SKF96365 and Gd^{3+}, also inhibited oligomycin-induced Ca^{2+} influx. Representative experiments are shown in Fig. 5C and the results are summarized in Fig. 5D. SKF96365 and Gd^{3+} inhibited by ~80% and ~50%, respectively the $[Ca^{2+}]_i$ rise induced by oligomycin (*$p<0.05$).

Fig. 5E and F illustrate that the pharmacological profile of the response to antimycin is similar to that of oligomycin, though blockade by Gd^{3+} is smaller and not statistically significant. These observations altogether indicate that these two inhibitors may induce the opening of Ca^{2+} entry pathways with pharmacological properties that resemble the SOCs which participate in the AR. Though their mode of action is different, antimycin and oligomycin must lead to a common mechanism capable of activating such a channel in sea urchin sperm. On the other hand, the results obtained for CCCP and CGP (not shown) indicate that the mechanism is somewhat different since only SKF96365 produced a statistically significant inhibition of the Ca^{2+} influx.

3.4. CCCP inhibits the $[Ca^{2+}]_i$ elevation induced by antimycin and by CGP, but not by oligomycin

Data from the literature [32,33] and our own experiments performed with membrane potential sensitive dyes indicate that CCCP and antimycin cause a mitochondrial depolarization in sea urchin sperm (data not shown). As a consequence, these two inhibitors could decrease Ca^{2+} uptake into mitochondria by perturbing the steady state [34]. On the other hand, oligomycin was shown to elevate mitochondrial potential in these cells, as measured with radioactive TPP^+ [33]. In contrast, CGP, by blocking the mitochondrial Na^+/Ca^{2+} exchanger (believed to be electrogenic (three Na^+/H^+ are exchanged for one Ca^{2+}; [3])), would hyperpolarize mitochondria and promote a $[Ca^{2+}]$mt increase. To evaluate the contribution of mitochondrial depolarization to the $[Ca^{2+}]_i$ increase induced by antimycin, oligomycin and CGP, we pre-treated sperm with CCCP. This drug significantly inhibited the response to antimycin by 71% and to CGP (35%). In contrast, the oligomycin response was not inhibited by CCCP, though its profile was changed and its peak amplitude was slightly enhanced (but not statistically significant, Fig. 6). These observations suggest that the $[Ca^{2+}]_i$ increase elicited by CCCP and antimycin requires mitochondrial depolarization while the responses caused by oligomycin and CGP may involve a different mechanism.

3.5. Emptying of intracellular stores prevents the mitochondrial inhibitor-induced $[Ca^{2+}]_i$ increases

As the oligomycin response does not primordially depend on mitochondrial potential and may involve SOCs, we examined if emptying Ca^{2+} from internal stores would affect it. Recently it was reported that sea urchin sperm lack a SERCA-type Ca^{2+} ATPase, but instead contain a secretory pathway Ca^{2+} ATPase (SPCA) in their mitochondrion [35,36]. This enzyme is inhibited by higher thapsigargin (TG) concentrations (~10 μM) than the SERCA-type Ca^{2+} ATPase, and more specifically by bis(2-hydroxy-3-tert-butyl-5-methylphenyl) methane (bisphenol or BP) [37]. Both compounds are

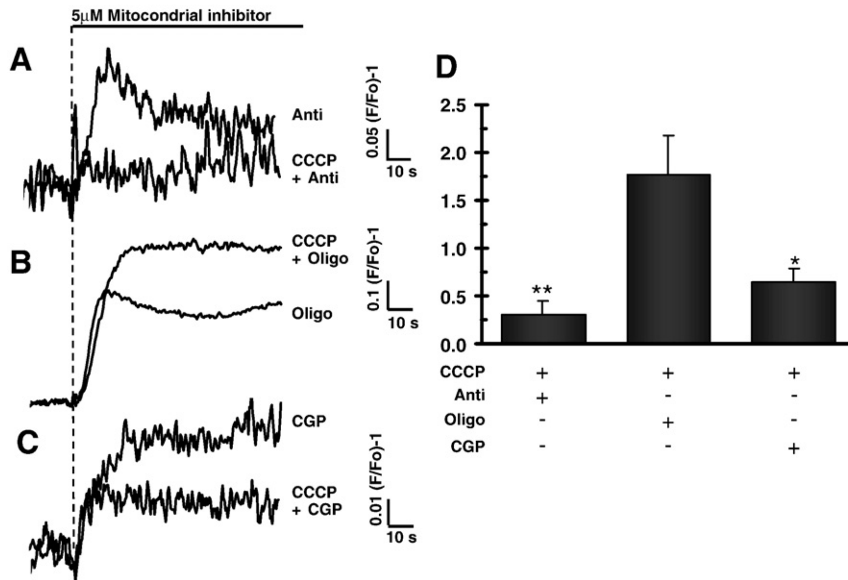

Fig. 6. Pre-treating sea urchin sperm populations with 1 μM CCCP inhibited the $[Ca^{2+}]_i$ increases caused by antimycin (A) and CGP (C) but not by oligomycin (B). Representative records are shown. (D) Normalized results considering the CCCP responses as 1 (Average±SEM of at least 6 experiments (* $P<0.05$, ** $P<0.01$)).

known to induce Ca^{2+} uptake by activating SOCs in sea urchin sperm [27,37].

On the other hand, as the profile of the oligomycin-induced $[Ca^{2+}]_i$ changes is complex, more than one target could be involved. To further explore this possibility we employed dicyclohexyl carbodiimide (DCCD), another inhibitor of the mitochondrial ATPase [38]. Fig. 7 shows that TG (10 μM) and BP (30 μM) alone increase sperm $[Ca^{2+}]_i$ as anticipated. DCCD, which can affect other proteins besides the mitochondrial ATPase [38], also increased $[Ca^{2+}]_i$. Remarkably, TG and BP inhibited the oligomycin response while DCCD did not. This result suggests that oligomycin affects another target which is responsible for increasing $[Ca^{2+}]_i$. BP also inhibited the CCCP, antimycin

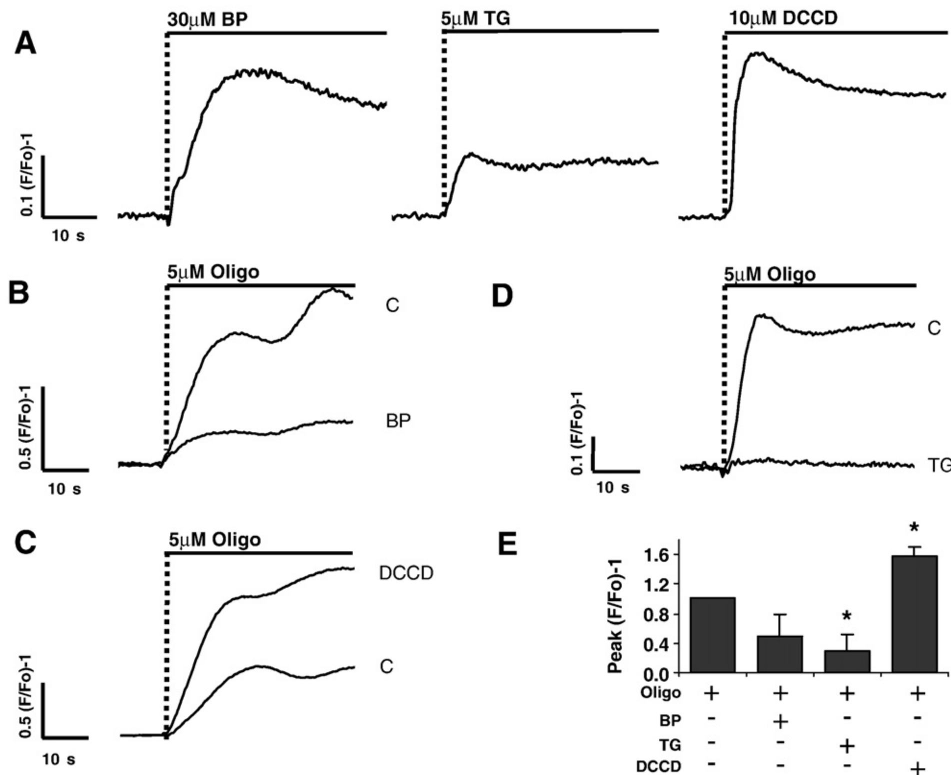

Fig. 7. Emptying internal stores inhibits the oligomycin response while DCCD, another mitochondrial ATPase inhibitor does not. (A) $[Ca^{2+}]_i$ increases elicited by bisphenol (BP) and 10 μM thapsigargin (TG), both inhibitors of SPCA under these conditions. DCCD at 10 μM also causes an $[Ca^{2+}]_i$ increase. Representative traces of at least 9 experiments are shown. (B) BP inhibits the $[Ca^{2+}]_i$ increase induced by oligomycin (C: control). (C) DCCD potentiates the $[Ca^{2+}]_i$ peak rise induced by oligomycin. (D) TG inhibits the $[Ca^{2+}]_i$ increase elicited by oligomycin. (E) Average±SEM of at least 3 experiments (BP: 3, TG: 4, DCCD: 8). Representative records are shown.

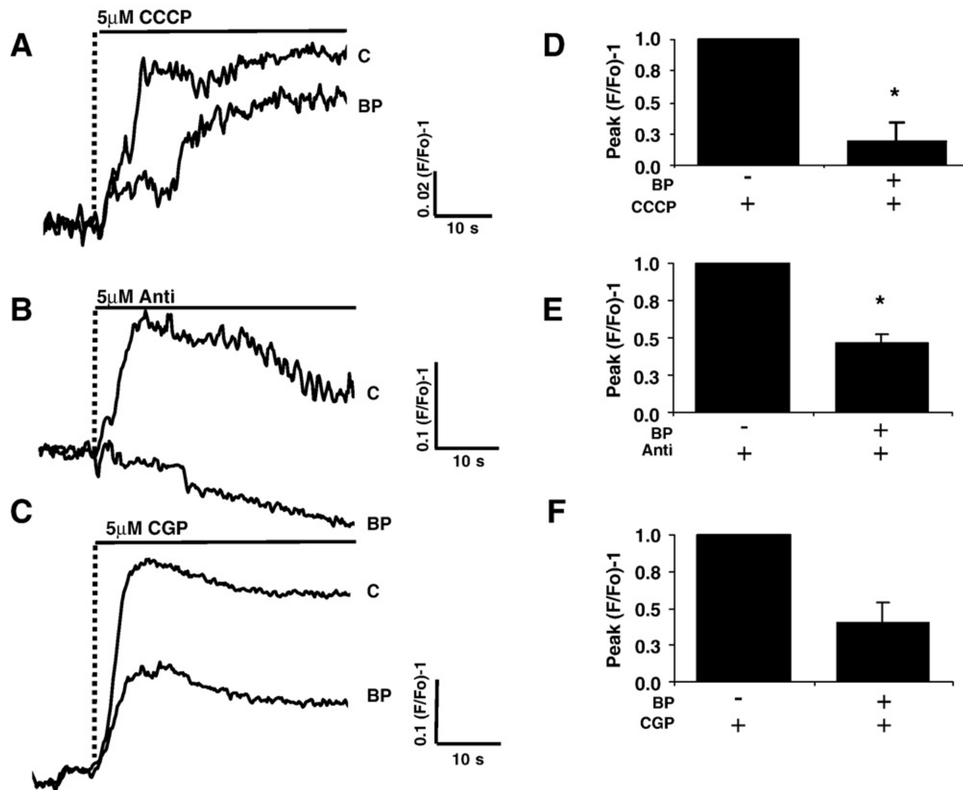

Fig. 8. Bisphenol inhibits the transient $[Ca^{2+}]_i$ increases induced by CCCP (A), Antimycin (B) and CGP (C). Representative records of $[Ca^{2+}]_i$ increases induced by each mitochondrial inhibitor without (C) and with BP (BP). (D–F) Average ± SEM of 3 (antimycin) or 4 (CCCP, CGP) experiments.

and less significantly the CGP induced increases in $[Ca^{2+}]_i$ (Fig. 8). Together, these results are consistent with the involvement of SOC type Ca^{2+} uptake in response to the mitochondrial inhibitors.

4. Discussion

Mitochondria can modulate Ca^{2+} signals by taking up and releasing Ca^{2+} and play a central role in some forms of cell death [34]. They can also respond to these Ca^{2+} fluxes by elevating ATP production to support increased energy demands [3]. There are few cell types as the sea urchin sperm, which display such extensive areas of close apposition between mitochondria and plasma membrane [3,39]. In this sense, sea urchin sperm constitute an excellent system to study the role of its only mitochondrion [39] in the regulation of resting $[Ca^{2+}]$cyt, sperm motility, and the AR [27,40]. The basic findings presented here show that mitochondrial inhibitors elicit increases in $[Ca^{2+}]_i$ in sea urchin sperm which depend on external Ca^{2+}. Mitochondrial inhibitors produce an imbalance in the relative rates of mitochondrial Ca^{2+} uptake and extrusion. Depending on the mitochondrial Ca^{2+} load, a net Ca^{2+} efflux (and therefore an elevation of $[Ca^{2+}]$cyt) is expected when the rate of Ca^{2+} extrusion by the mitochondrial Na^+ or H^+/Ca^{2+} exchangers exceeds the rate of Ca^{2+} uptake by the uniporter. Antimycin A and (to a greater extent) CCCP reduce the electrochemical driving force required for Ca^{2+} uptake, suppressing Ca^{2+} accumulation and promoting mitochondrial Ca^{2+} release. In addition, they inhibit ATP production [32,33,41] which in these cells depends entirely on the mitochondrion [17]. Conversely, CGP, a specific inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger inhibits mitochondrial Ca^{2+} efflux, and therefore can promote Ca^{2+} accumulation and either enhance or not significantly affect ATP production [20,42]. On the other hand, oligomycin, by inhibiting the F_1F_0-ATPase [21], rapidly decreases the sperm ATP levels, mildly increases mitochondrial potential [32], and would not thus lead to Ca^{2+} release from this organelle.

4.1. Acute addition of mitochondrial inhibitors causes $[Ca^{2+}]_i$ elevations in sea urchin sperm which depend on external Ca^{2+}

As shown in Figs. 1–3, all mitochondrial inhibitors raise $[Ca^{2+}]_i$ in sea urchin sperm populations. However, the profile of the $[Ca^{2+}]_i$ changes depends on the particular inhibitor. All the tested inhibitors initially elevate $[Ca^{2+}]_i$. The initial increase may relax to a variable plateau and then increase again. At 1 μM, this initial elevation is larger for oligomycin followed by CCCP, then by antimycin and CGP which produce responses of similar magnitude. When added at 5 μM, the CCCP response becomes the smallest while those to CGP and oligomycin increase. A similar pattern is observed when the integration over time of $[Ca^{2+}]_i$ responses (50 s) are considered (Fig. 3). Though all the inhibitors can induce more than one peak, oligomycin stands out in this regard.

Interestingly, our results suggest that the $[Ca^{2+}]_i$ increases are not mainly due to mitochondrial Ca^{2+} release, but involve the opening of Ca^{2+}-permeable pathways in the sperm plasma membrane. The responses to all mitochondrial inhibitors tested here were obliterated in Ca^{2+}-free ASW. In addition, these inhibitors induce Mn^{2+} influx which, in turn, is blocked by Ni^{2+}. Our findings suggest that even though these mitochondrial inhibitors act in quite different ways, they all result in stimulation of sperm Ca^{2+} uptake.

The initial increase in $[Ca^{2+}]_i$ caused by antimycin, oligomycin and CGP is also sensitive to Ni^{2+}. In particular the Ni^{2+} concentration dependence of the oligomycin response is reminiscent of a Ca^{2+} pathway that has been shown to operate during the AR. In sea urchin sperm the AR (a necessary step for fertilization), is associated to the sequential opening of two different Ca^{2+} channels [43]. The first channel is dihydropyridine-sensitive and Ca^{2+} selective, and the second Ca^{2+} channel is pH_i dependent, permeable to Mn^{2+} and blocked by Ni^{2+}. Although sea urchin sperm lack endoplasmic reticulum, relatively high concentrations of inhibitors of the Ca^{2+} ATPase of the sarco-endoplasmic reticulum (SERCA) (~10 μM),

thapsigargin or ciclopiazonic acid can induce a SOC-type Ca^{2+} influx. It has been suggested that the second channel involved in the AR is a SOC channel since the Ca^{2+} influx pathway induced by thapsigargin and the second channel have a similar sensitivity to inhibition by Ni^{2+} (blocking IC_{50} ~8 μM; [27]). Sea urchin sperm have been shown to posses at least three mechanisms to maintain $[Ca^{2+}]_i$ low (~100 nM): 1) a K^+ dependent Na^+/Ca^{2+} exchanger (NCKX) [44], 2) a plasma membrane Ca^{2+} ATPase (PMCA) [37] and 3) a secretory-pathway ATPase (SPCA); but apparently not a SERCA [45]. Interestingly, SPCA was immunolocalized to the mitochondrion and its specific inhibitor, bisphenol, caused a $[Ca^{2+}]_i$ increase displaying a major external-Ca^{2+} dependent component.

The $[Ca^{2+}]_i$ increases triggered by the mitochondrial inhibitors tested here are also blocked by other SOC inhibitors such as SKF96365 and Gd^{2+} [46], and are not significantly affected by voltage-dependent Ca^{2+} channel blockers such as verapamil and nimodipine (not shown). Indeed, the presence of SKF6992-sensitive SOC-type channels in sea urchin sperm has been confirmed [47]. Unfortunately, the molecular identity of SOCs is still a matter of debate and may depend on the cell type. Orai1 and TRPC1 have been proposed as candidates and recently were reported to interact with each other and with STIM1, the putative ER-Ca^{2+} sensor protein [48,49]. We have preliminary results indicating that transcripts for TRPC3 and TRPC6 are present in *S. purpuratus* testis, as well as the proteins they code for in mature sperm (Granados-González, G., Mendoza-Lujambio, I, Rodríguez, E., Galindo, B. E., Beltrán, C. and Darszon, A., unpublished data).

While this work suggests the involvement of SOC type channels in the Ca^{2+} influx induced by the mitochondrial inhibitors, it neither establishes their identity nor the signal that conveys information of the functional status of the mitochondria to the sea urchin sperm plasma membrane to activate Ca^{2+} influx.

It is known that mitochondria have the ability to modulate SOC channels and thus, transmembrane Ca^{2+} entry [11,50–53]. In most cells, the impairment of mitochondrial Ca^{2+} homeostasis by CCCP, antimycin or oligomycin, results in inhibition of SOC channel activity induced by thapsigargin (e.g. see [54]). This inhibition being greater at pH 7.2 than at pH 7.8 [55]. However, these studies do not examine the direct effects of the mitochondrial inhibitors on $[Ca^{2+}]_i$ in the presence of external Ca^{2+} and thus cannot be directly compared with our results. In any case, as the pH of both sea water and cytoplasm of sea urchin sperm is quite alkaline, pH 8.0 and ~7.4 respectively, the reported inhibitory effects of mitochondrial antagonists on thapsigargin-induced SOC activation might be minimized in these cells. Furthermore as indicated earlier, activation of the SOC channel involved in the AR requires an increase in pH_i [43]. It is thus possible that mitochondrial inhibition, and the subsequent collapse of its pH_i gradient, could increase pH_i in the vicinity of this channel and lead to an elevation of $[Ca^{2+}]$cyt. It has been reported that CatSper, a new class of sperm-specific Ca^{2+} channel required for hyperactivated motility and fertility in mouse, is activated by pH_i increases, and is present in the *S. purpuratus* genome [56].

Another possibility to consider is that mitochondria can release diffusible factors that modulate the activity of SOC channels. In pancreatic β-cells mitochondrial activation generates glutamate, which directly triggers insulin exocytosis [57]. In skeletal muscle, an unidentified messenger is generated by mitochondria that diffuses to the plasma membrane and opens Na^+ channels [58]. It is conceivable that SOC channels in sperm are similarly activated by a diffusible factor released upon mitochondrial inhibition. Alternatively, though most attention has focused on the link between the endoplasmic reticulum and SOC channels in the plasma membrane, mitochondria are essential for the activation of macroscopic store operated Ca^{2+} currents under physiological conditions of weak intracellular Ca^{2+} buffering [59]. Our findings showing that mitochondrial inhibitors increase $[Ca^{2+}]_i$ by opening Ca^{2+} influx pathways in the plasma membrane are consistent with a form of intimate communication between the plasma membrane and the mitochondria.

Notably, TG and bisphenol, which inhibit internal store Ca^{2+}-ATPases, as expected cause a $[Ca^{2+}]_i$ increase which depends on external Ca^{2+}, and both inhibit the $[Ca^{2+}]_i$ elevation induced by oligomycin and the other mitochondrial inhibitors. These findings imply that somehow these compounds end up stimulating SOCs. DCCD, a different mitochondrial ATPase inhibitor, is unable to eliminate the response to oligomycin, indicating it is acting at an alternate site. In other cell types oligomycin alone, at concentrations as high as 10 μM, does not affect basal $[Ca^{2+}]_i$, or the magnitude of Ca^{2+} in mitochondria [60]. Higher oligomycin concentrations can also inhibit the Na^+/K^+ATPase [61]. Nevertheless ouabain, a specific inhibitor of this ATPase, did not mimic the oligomycin responses obtained in sea urchin sperm (data not shown). On the other hand, in Jurkat-T and CHO cells, oligomycin in the 1–10 μM was shown to inhibit Icrac and a volume regulated Cl^- channel [54]. Thus, it is not unlikely that the direct action of this macrolide from *Streptomyces* on a sperm ion channel may lead to $[Ca^{2+}]_i$ increases, for instance by affecting the Cl^- balance and increasing pH_i. Further experiments are required to determine the nature of the communication between the mitochondrion and the plasma membrane, and the molecular identity of the plasma membrane Ca^{2+} influx pathway(s) activated as a result of mitochondrial inhibition.

4.2. Why do mitochondrial inhibitors that have different modes of action induce similar $[Ca^{2+}]_i$ responses in sea urchin sperm?

It is conceivable that all the inhibitors used in this work lead to mitochondrial Ca^{2+} depletion due to mitochondrial depolarization and/or decreased ATP levels. As suggested above, the emptying of mitochondrial Ca^{2+} somehow activates plasma membrane SOCs. With the exception of CGP, all mitochondrial inhibitors used in this work diminish mitochondrial ATP production and/or depolarize this organelle. It is likely that ATP depletion is neither severe nor rapid, since sperm continue to swim for minutes in the presence of these mitochondrial inhibitors (data not shown). To corroborate this we examined the ATP content of sperm under the exact conditions where $[Ca^{2+}]_i$ was measured (see Materials and methods). The results (supplementary Fig. 1) show that the inhibitors used in this work, with the exception of oligomycin, did not cause a statistically significant ($P<0.05$) decrease in ATP content, with respect to untreated controls, at the times (10–20 s) when they increased $[Ca^{2+}]_i$. Oligomycin decreased ATP levels by ~20% in the first 10 s, however, since its effect was insensitive to preincubation with CCCP or DCCD, its main action does not involve ATP depletion. These findings altogether indicate that the external Ca^{2+} dependent increases in $[Ca^{2+}]_i$ induced by the mitochondrial inhibitors are not mainly due to an ATP concentration decrease. Furthermore, the stimulation of Mn^{2+} uptake caused by the mitochondrial inhibitors cannot be explained by decreased ATP levels. Therefore, our observations are more consistent with decreased mitochondrial Ca^{2+} content which stimulates SOCs.

Regarding the results obtained with CGP, one of the transport systems present in mitochondria is the permeability transition pore (mPTP). The mPTP has a diameter that allows molecules up to a molecular weight of 1500 D to equilibrate across the mitochondrial membrane. Its activation leads to a non-specific increase in inner mitochondrial membrane permeability resulting in efflux of both small molecules (Ca^{2+} and NAD^+/NADH) and small proteins, loss of mitochondrial membrane potential, possible rupture of the outer membrane, and severe mitochondrial swelling [62,63]. mPTP can open in response to mitochondrial Ca^{2+} overload during excitotoxicity or anoxia/ischemia. As CGP is likely to cause mitochondrial Ca^{2+} accumulation, its action in sea urchin sperm could lead to mitochondrial depolarization and thereafter to Ca^{2+} loss [7].

Finally, it is important to mention that the respiratory chain of mitochondria is one of the most productive ROS generating systems in

sperm. Mitochondrial ROS destruction is important for cells as they can induce nuclear DNA fragmentation, lipid peroxidation, protein–protein cross linking and ion channel regulation [64–66]. Though preliminary results using vitamin E and POBN did not significantly inhibit the $[Ca^{2+}]_i$ induced by the mitochondrial inhibitors used here (data not shown), ROS effects on mitochondrial ion transport must be further characterized in sea urchin sperm.

4.3. Concluding remarks

Our findings taken altogether suggest that Ca^{2+} entry and its homeostasis in sea urchin sperm are influenced by the functional status of their mitochondrion. A direct link between the mitochondrial membrane and the head plasma membrane may play an important role in this process. Oligomycin, aside from its action on the mitochondrial ATPase, induces remarkable $[Ca^{2+}]_i$ fluctuation whose origin deserves future analysis and could involve direct interactions with sperm plasma membrane ion channels.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2008.10.003.

References

[1] M.R. Duchen, Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death, J. Physiol. 516 (Pt 1) (1999) 1–17.
[2] D. Poburko, C.H. Lee, C. van Breemen, Vascular smooth muscle mitochondria at the cross roads of Ca(2+) regulation, Cell Calcium 35 (2004) 509–521.
[3] R. Rizzuto, T. Pozzan, Microdomains of intracellular Ca2+: molecular determinants and functional consequences, Physiol. Rev. 86 (2006) 369–408.
[4] K.K. Gunter, T.E. Gunter, Transport of calcium by mitochondria, J. Bioenerg. Biomembranes 26 (1994) 471–485.
[5] T. Pozzan, R. Rizzuto, The renaissance of mitochondrial calcium transport, Eur. J. Biochem. 267 (2000) 5269–5273.
[6] D.R. Pfeiffer, T.E. Gunter, R. Eliseev, K.M. Broekemeier, K.K. Gunter, Release of Ca2+ from mitochondria via the saturable mechanisms and the permeability transition, IUBMB Life 52 (2001) 205–212.
[7] P. Bernardi, A. Krauskopf, E. Basso, V. Petronilli, E. Blachly-Dyson, F. Di Lisa, M.A. Forte, The mitochondrial permeability transition from in vitro artifact to disease target, FEBS J. 273 (2006) 2077–2099.
[8] S.L. Budd, D.G. Nicholls, A reevaluation of the role of mitochondria in neuronal Ca2+ homeostasis, J. Neurochem. 66 (1996) 403–411.
[9] G. David, J.N. Barrett, E.F. Barrett, Evidence that mitochondria buffer physiological Ca2+loads in lizard motor nerve terminals, J. Physiol. 509 (Pt 1) (1998) 59–65.
[10] G. Hajnoczky, L.D. Robb-Gaspers, M.B. Seitz, A.P. Thomas, Decoding of cytosolic calcium oscillations in the mitochondria, Cell 82 (1995) 415–424.
[11] M. Hoth, C.M. Fanger, R.S. Lewis, Mitochondrial regulation of store-operated calcium signaling in T lymphocytes, J. Cell Biol. 137 (1997) 633–648.
[12] T.E. Gunter, D.R. Pfeiffer, Mechanisms by which mitochondria transport calcium, Am. J. Physiol. 258 (1990) C755–786.
[13] J.G. McCormack, R.M. Denton, Mitochondrial Ca2+ transport and the role of intramitochondrial Ca2+in the regulation of energy metabolism, Dev. Neurosci. 15 (1993) 165–173.
[14] J.G. McCormack, A.P. Halestrap, R.M. Denton, Role of calcium ions in regulation of mammalian intramitochondrial metabolism, Physiol. Rev. 70 (1990) 391–425.
[15] L.D. Robb-Gaspers, P. Burnett, G.A. Rutter, R.M. Denton, R. Rizzuto, A.P. Thomas, Integrating cytosolic calcium signals into mitochondrial metabolic responses, EMBO J. 17 (1998) 4987–5000.
[16] B. Baccetti, V. Pallini, A.G. Burrini, Localization and catalytic properties of lactate dehydrogenase in different sperm models, Exp. Cell Res. 90 (1975) 183–190.
[17] M. Mita, I. Yasumasu, Regulation of the tricarboxylic acid cycle in sea urchin eggs and embryos, J. Exp. Zool. 228 (1983) 71–77.
[18] A.F. Quest, B.M. Shapiro, Membrane association of flagellar creatine kinase in the sperm phosphocreatine shuttle, J. Biol. Chem. 266 (1991) 19803–19811.
[19] R.M. Tombes, A. Farr, B.M. Shapiro, Sea urchin sperm creatine kinase: the flagellar isozyme is a microtubule-associated protein, Exp. Cell Res. 178 (1988) 307–317.
[20] D.A. Cox, L. Conforti, N. Sperelakis, M.A. Matlib, Selectivity of inhibition of Na(+)-Ca2+ exchange of heart mitochondria by benzothiazepine CGP-37157, J. Cardiovasc. Pharmacol. 21 (1993) 595–599.
[21] I.R. Collinson, M.J. van Raaij, M.J. Runswick, I.M. Fearnley, J.M. Skehel, G.L. Orriss, B. Miroux, J.E. Walker, ATP synthase from bovine heart mitochondria. In vitro assembly of a stalk complex in the presence of F1-ATPase and in its absence, J. Mol. Biol. 242 (1994) 408–421.
[22] Y.V. Bobkov, B.W. Ache, Pharmacological properties and functional role of a TRP-related ion channel in lobster olfactory receptor neurons, J. Neurophysiol. 93 (2005) 1372–1380.
[23] H.T. Ma, R.L. Patterson, D.B. van Rossum, L. Birnbaumer, K. Mikoshiba, D.L. Gill, Requirement of the inositol trisphosphate receptor for activation of store-operated Ca2+ channels, Science 287 (2000) 1647–1651.
[24] E. Rodriguez, A. Darszon, Intracellular sodium changes during the speract response and the acrosome reaction in sea urchin sperm, J. Physiol. 546 (2003) 89–100.
[25] R.W. Schackmann, P.B. Chock, Alteration of intracellular [Ca2+] in sea urchin sperm by the egg peptide speract. Evidence that increased intracellular Ca2+ is coupled to Na+ entry and increased intracellular pH, J. Biol. Chem. 261 (1986) 8719–8728.
[26] S.P. Cook, D.F. Babcock, Selective modulation by cGMP of the K+ channel activated by speract, J. Biol. Chem. 268 (1993) 22402–22407.
[27] M.T. Gonzalez-Martinez, B.E. Galindo, L. de De La Torre, O. Zapata, E. Rodriguez, H.M. Florman, A. Darszon, A sustained increase in intracellular Ca(2+) is required for the acrosome reaction in sea urchin sperm, Dev. Biol. 236 (2001) 220–229.
[28] L. Massieu, M.L. Haces, T. Montiel, K. Hernandez-Fonseca, Acetoacetate protects hippocampal neurons against glutamate-mediated neuronal damage during glycolysis inhibition, Neuroscience 120 (2003) 365–378.
[29] R.W. Tsien, P. Hess, E.W. McCleskey, R.L. Rosenberg, Calcium channels: mechanisms of selectivity, permeation, and block, Annu. Rev. Biophys. Biophys. Chem. 16 (1987) 265–290.
[30] C.D. Wood, A. Darszon, M. Whitaker, Speract induces calcium oscillations in the sperm tail, J. Cell Biol. 161 (2003) 89–101.
[31] G. Granados-Gonzalez, I. Mendoza-Lujambio, E. Rodriguez, B.E. Galindo, C. Beltran, A. Darszon, Identification of voltage-dependent Ca(2+) channels in sea urchin sperm, FEBS Lett. 579 (2005) 6667–6672.
[32] R. Christen, R.W. Schackmann, B.M. Shapiro, Metabolism of sea urchin sperm. Interrelationships between intracellular pH, ATPase activity, and mitochondrial respiration, J. Biol. Chem. 258 (1983) 5392–5399.
[33] R.W. Schackmann, R. Christen, B.M. Shapiro, Measurement of plasma membrane and mitochondrial potentials in sea urchin sperm. Changes upon activation and induction of the acrosome reaction, J. Biol. Chem. 259 (1984) 13914–13922.
[34] M. Giacomello, I. Drago, P. Pizzo, T. Pozzan, Mitochondrial Ca2+as a key regulator of cell life and death, Cell Death Differ. 14 (2007) 1267–1274.
[35] L.L. Wootton, C.C. Argent, M. Wheatley, F. Michelangeli, The expression, activity and localisation of the secretory pathway Ca2+-ATPase (SPCA1) in different mammalian tissues, Biochim. Biophys. Acta 1664 (2004) 189–197.
[36] C. Harper, L. Wootton, F. Michelangeli, L. Lefievre, C. Barratt, S. Publicover, Secretory pathway Ca(2+)-ATPase (SPCA1) Ca(2)+ pumps, not SERCAs, regulate complex [Ca(2+)](i) signals in human spermatozoa, J. Cell Sci. 118 (2005) 1673–1685.
[37] H.J. Gunaratne, A.T. Neill, V.D. Vacquier, Plasma membrane calcium ATPase is concentrated in the head of sea urchin spermatozoa, J. Cell Physiol. 207 (2006) 413–419.
[38] A. Azzi, R.P. Casey, M.J. Nalecz, The effect of N,N′-dicyclohexylcarbodiimide on enzymes of bioenergetic relevance, Biochim. Biophys. Acta 768 (1984) 209–226.
[39] F.J. Longo, E. Anderson, Sperm differentiation in the sea urchins *Arbacia punctulata* and *Strongylocentrotus purpuratus*, J. Ultrastruct. Res. 27 (1969) 486–509.
[40] A. Darszon, J.J. Acevedo, B.E. Galindo, E.O. Hernandez-Gonzalez, T. Nishigaki, C.L. Trevino, C. Wood, C. Beltran, Sperm channel diversity and functional multiplicity, Reproduction 131 (2006) 977–988.
[41] M. Kazama, K. Asami, A. Hino, Fertilization induced changes in sea urchin sperm: mitochondrial deformation and phosphatidylserine exposure, Mol. Reprod. Dev. 73 (2006) 1303–1311.
[42] E. Hernandez-SanMiguel, L. Vay, J. Santo-Domingo, C.D. Lobaton, A. Moreno, M. Montero, J. Alvarez, The mitochondrial Na+/Ca2+ exchanger plays a key role in the control of cytosolic Ca2+ oscillations, Cell Calcium 40 (2006) 53–61.
[43] A. Guerrero, A. Darszon, Evidence for the activation of two different Ca2+channels during the egg jelly-induced acrosome reaction of sea urchin sperm, J. Biol. Chem. 264 (1989) 19593–19599.
[44] Y.H. Su, V.D. Vacquier, A flagellar K(+)-dependent Na(+)/Ca(2+) exchanger keeps Ca(2+) low in sea urchin spermatozoa, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 6743–6748.
[45] H.J. Gunaratne, V.D. Vacquier, Evidence for a secretory pathway Ca2+-ATPase in sea urchin spermatozoa, FEBS Lett. 580 (2006) 3900–3904.

[46] A.B. Parekh, J.W. Putney Jr., Store-operated calcium channels, Physiol. Rev. 85 (2005) 757–810.
[47] N. Hirohashi, V.D. Vacquier, Store-operated calcium channels trigger exocytosis of the sea urchin sperm acrosomal vesicle, Biochem. Biophys. Res. Commun. 304 (2003) 285–292.
[48] I.S. Ambudkar, H.L. Ong, X. Liu, B. Bandyopadhyay, K.T. Cheng, TRPC1: the link between functionally distinct store-operated calcium channels, Cell Calcium 42 (2007) 213–223.
[49] R.S. Lewis, The molecular choreography of a store-operated calcium channel, Nature 446 (2007) 284–287.
[50] K.C. Fernando, R.B. Gregory, G.J. Barritt, Protein kinase A regulates the disposition of Ca2+ which enters the cytoplasmic space through store-activated Ca2+ channels in rat hepatocytes by diverting inflowing Ca2+ to mitochondria, Biochem. J. 330 (Pt 3) (1998) 1179–1187.
[51] A. Gamberucci, B. Innocenti, R. Fulceri, G. Banhegyi, R. Giunti, T. Pozzan, A. Benedetti, Modulation of Ca2+ influx dependent on store depletion by intracellular adenine-guanine nucleotide levels, J. Biol. Chem. 269 (1994) 23597–23602.
[52] A. Makowska, K. Zablocki, J. Duszynski, The role of mitochondria in the regulation of calcium influx into Jurkat cells, Eur. J. Biochem. 267 (2000) 877–884.
[53] J. Duszynski, R. Koziel, W. Brutkowski, J. Szczepanowska, K. Zablocki, The regulatory role of mitochondria in capacitative calcium entry, Biochim. Biophys. Acta 1757 (2006) 380–387.
[54] J.H. Cho, M. Balasubramanyam, G. Chernaya, J.P. Gardner, A. Aviv, J.P. Reeves, P.G. Dargis, E.P. Christian, Oligomycin inhibits store-operated channels by a mechanism independent of its effects on mitochondrial ATP, Biochem. J. 324 (Pt 3) (1997) 971–980.
[55] K. Zablocki, J. Szczepanowska, J. Duszynski, Extracellular pH modifies mitochondrial control of capacitative calcium entry in Jurkat cells, J. Biol. Chem. 280 (2005) 3516–3521.
[56] J. Xia, D. Reigada, C.H. Mitchell, D. Ren, CATSPER channel-mediated Ca2+ entry into mouse sperm triggers a tail-to-head propagation, Biol. Reprod. 77 (2007) 551–559.
[57] P. Maechler, C.B. Wollheim, Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis, Nature 402 (1999) 685–689.
[58] N. Ortenblad, D.G. Stephenson, A novel signalling pathway originating in mitochondria modulates rat skeletal muscle membrane excitability, J. Physiol. 548 (2003) 139–145.
[59] J.A. Gilabert, A.B. Parekh, Respiring mitochondria determine the pattern of activation and inactivation of the store-operated Ca(2+) current I(CRAC), EMBO J. 19 (2000) 6401–6407.
[60] T. Szikra, D. Krizaj, Intracellular organelles and calcium homeostasis in rods and cones, Vis. Neurosci. 24 (2007) 733–743.
[61] T. Arato-Oshima, H. Matsui, A. Wakizaka, H. Homareda, Mechanism responsible for oligomycin-induced occlusion of Na+ within Na/K-ATPase, J. Biol. Chem. 271 (1996) 25604–25610.
[62] A.P. Halestrap, G.P. McStay, S.J. Clarke, The permeability transition pore complex: another view, Biochimie 84 (2002) 153–166.
[63] K.A. Foster, F. Galeffi, F.J. Gerich, D.A. Turner, M. Muller, Optical and pharmacological tools to investigate the role of mitochondria during oxidative stress and neurodegeneration, Prog. Neurobiol. 79 (2006) 136–171.
[64] L. Annunziato, A. Pannaccione, M. Cataldi, A. Secondo, P. Castaldo, G. Di Renzo, M. Taglialatela, Modulation of ion channels by reactive oxygen and nitrogen species: a pathophysiological role in brain aging? Neurobiol. Aging 23 (2002) 819–834.
[65] X.D. Tang, L.C. Santarelli, S.H. Heinemann, T. Hoshi, Metabolic regulation of potassium channels, Annu. Rev. Physiol. 66 (2004) 131–159.
[66] C. Camello-Almaraz, P.J. Gomez-Pinilla, M.J. Pozo, P.J. Camello, Mitochondrial reactive oxygen species and Ca2+ signaling, Am. J. Physiol. Cell Physiol. 291 (2006) C1082–1088.
[67] B.O. Fanger, Adaptation of the Bradford protein assay to membrane-bound proteins by solubilizing in glucopyranoside detergents, Anal. Biochem. 162 (1987) 11–17.