The Mitochondrial Citrate Carrier (CIC) Is Present and Regulates Insulin Secretion by Human Male Gamete

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The mechanisms through which sperm manage their energy metabolism are poorly understood. The present study provides biochemical and morphological evidence that mitochondrial citrate carrier (CIC) is present in ejaculated human sperm and is restricted to the midpiece. The inhibition of CIC with the specific substrate analog 1,2,3-benzenetricarboxylate resulted in the reduction of cholesterol efflux, protein tyrosine phosphorylation, phospho-AKT, phospho-p60src, hyperactivated motility and acrosome reaction, suggesting a role for this mitochondrial carrier in sperm physiology. Furthermore, inhibition of CIC by 1,2,3-benzenetricarboxylate resulted in a reduction of glucose-stimulated insulin secretion and autocrine insulin secretion by sperm. Remarkably, blocking CIC also reduced glucose-6-phosphate dehydrogenase activity, probably in accordance with its regulation on insulin secretion. Capacitation and glucose metabolism were stimulated by glucose as well as citrate, the specific substrate of CIC, implying a similar action because glucose and citrate both induced insulin secretion by sperm. In the present finding, we discovered a new site of action for CIC in the regulation of metabolism, and it may be assumed that CIC works with other factors in the regulation of sperm energy metabolism to sustain capacitation process and acrosome reaction. **(*Endocrinology* 153: 1743–1754, 2012)**

A lack of knowledge exists on how mammalian spermatozoa manage their energy status, a vital point for the understanding the unique physiology of sperm and the mechanisms implied in mammalian sperm acquisition of capacitation, a prerequisite of egg fertilization. The spermatozoon leaves the testis and moves through the female genital tract in the unfamiliar body of the opposite gender, toward the incognito. Once arrived, the sperm needs to change its physiological status to have just one opportunity to fertilize the oocyte. On the basis of these observations, mammalian spermatozoa must have a sophisticated metabolic system that allows them to survive in two different milieus and to sustain the switch from uncapacitated to capacitated status to achieve the specific changes required to accomplish fertilization. The signaling events associated with the changes in sperm energy metabolism are poorly understood.

Sperm glucose metabolism can proceed through glycolysis, mitochondrial oxidative phosphorylation, or the pentose phosphate pathway (PPP) (1). The sperm energy requirement and its ability to remain motile in glucose-free media (2) are supported by endogenous glucose obtained from glycogen stores, gluconeogenesis, and by the use of energy substrates other than monosaccharides (3, 4). In fact, several studies have reported that sperm obtain energy, not only from glucose and fructose (5, 6) but also

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Abbreviations: Ab, Antibody; BTA, benzenetricarboxylate; CHOD, cholesterol oxidase; CIC, citrate/isocitrate carrier; DMSO, dimethylsulfoxide; DNase, deoxyribonuclease; FITC, fluorescein isothiocyanate; G6PDH, glucose-6-phosphate dehydrogenase; GSIS, glucose-stimulated insulin secretion; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Nif, nifedipine; p-, phospho-; PI3K, phosphatidylinositol 3-kinase; PNA, peanut agglutinin; POD, peroxidase; PPP, pentose phosphate pathway.

from nonhexose compounds like citrate and lactate to increase their survival rate in conditions where monosaccharide supply is limited (7–9). Furthermore, although glycolysis is important for sperm functions, this metabolic pathway does not appear to be responsible for successful gamete fusion (10, 11). Instead, the beneficial effect of glucose on the acquisition of fertilizing ability as well as on gamete fusion is mediated by glucose metabolism through the PPP and the concomitant generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH). A functional PPP has been reported in spermatozoa from different species (12–14), and sperm need to produce NADPH via the PPP to be able to achieve fertilization. Nevertheless the observation that NADPH is detectable despite the absence of glucose suggested that other pathways provide NADPH in spermatozoa (14).

In somatic cells, NADPH can be produced via one of three pyruvate cycling pathways, the pyruvate/malate, the pyruvate/citrate, or the pyruvate/isocitrate pathways, via cytosolic $NADP^+$-dependent isoforms of malic enzyme (used in the pyruvate/malate and pyruvate/citrate pathways) or cytosolic, $NADP^+$-dependent isocitrate dehydrogenase (used in the pyruvate/isocitrate cycle) (15, 16). Operation of pyruvate cycles from these pathways requires efficient export of tri- and dicarboxylic acids from the mitochondria to the cytosol. Two proteins that mediate these activities are the dicarboxylate carrier (17), which primarily transports malate, and the tricarboxylate or citrate/isocitrate carrier (CIC). The CIC primary function is to catalyze an electroneutral exchange of a tricarboxylate for another tricarboxylate, a dicarboxylate (L-malate), or phosphoenolpyruvate across the mitochondrial inner membrane (18–23). This carrier protein plays a central role in intermediary metabolism because the citrate efflux from mitochondria provides the carbon source and $NADPH + H^+$ for a number of process such as fatty acid synthesis, sterol biosyntheses, insulin secretion, and histone acetylation (16, 24, 25). The CIC contribution to NADPH production is correlated to its role as a key component of the pyruvate/isocitrate and the pyruvate/citrate pathways (16). Moreover, a recent report indicates that CIC-mediated export of citrate and/or isocitrate from the mitochondria to the cytosol is an important step in control of glucose-stimulated insulin secretion (GSIS) in pancreatic β-cells (25).

In the present study, we showed that CIC is present in human ejaculated spermatozoa, providing evidence for a physiological role of this protein in capacitation, acrosome reaction, and sperm metabolism.

Materials and Methods

Chemicals

Total RNA isolation system kit, enzymes, buffers, and nucleotides 100-bp ladder used for RT-PCR were purchased from Promega (Milan, Italy). Moloney murine leukemia virus was purchased from Gibco-BRL Life Technologies Italia (Milan, Italy). Oligonucleotide primers were synthesized by Invitrogen (Milan, Italy). BSA protein standard, Laemmli sample buffer, prestained molecular weight markers, Percoll (colloidal polyvinylpyrrolidone-coated silica for cell separation), sodium bicarbonate, sodium lactate, sodium pyruvate, dimethylsulfoxide (DMSO), Earle's balanced salt solution, and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100 and eosin Y were from Farmitalia Carlo Erba (Milan, Italy). Gel band purification kit, ECL Plus Western blotting detection system, Hybond ECL, and HEPES sodium salt were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Cholesterol oxidase (CHOD)-peroxidase (POD) and enzymatic colorimetric kit were from Inter-Medical (Biogemina Srl, Catania, Italy). Goat polyclonal phospho- (p-)p60src antibody (Ab), rabbit polyclonal p60src Ab, rabbit polyclonal antiphosphotyrosine Ab (PY99), antirabbit IgG fluorescein isothiocyanate (FITC)-conjugated, rabbit anti-p-Akt1/Akt2/Akt3 S473 Ab, rabbit anti-Akt1/Akt2/Akt3 Ab, and POD-coupled antimouse, antirabbit, and antigoat IgG secondary Abs were from Santa Cruz Biotechnology (Heidelberg, Germany). CIC was detected with a rabbit Ab against anti-C-terminal CIC (26). CHOD-POD and glucose-6-phosphate dehydrogenase (G6PDH) activity enzymatic colorimetric and insulin RIA kits were from Biogemina Italia Srl. The nifedipine (Nif), fibronectin, FITC-conjugated peanut agglutinin (PNA) and the specific substrate analogs of CIC 1,2,3-benzenetricarboxylate (1,2,3-BTA) and 1,3,5-BTA were from Sigma (Milan, Italy). Nif was dissolved in DMSO (0.02% final concentration in culture) and used as solvent controls, which did not induce any positive result in all *in vitro* assays (data not shown).

Semen samples and spermatozoa preparations

Human semen was collected according to the World Health Organization (WHO) Laboratory Manual (27) recommended procedure from healthy volunteer donors of proven fertility. Spermatozoa preparations were performed as previously described (4). Briefly, semen samples with normal parameters of volume, sperm count, motility, vitality, and morphology, according to the WHO Laboratory Manual, were included in this study. Each experiment conducted in our study was performed on sperm cells processed by pooling the ejaculates of three normozoospermic samples. Varicocele samples of patients with a diagnosed varicocele of grade III on the left testis visible without palpation were also analyzed in our study. Cryptozoospermia was classified when spermatozoa were not detected in the counting chamber with a detection limit of 0.1×10^6/ml, but found in the pellet obtained by centrifugation of 1 ml ejaculate at $1200 \times g$ for 15 min.

The study has been approved by the local medical-ethical committee of the University of Calabria, and all participants gave their informed consent.

Processing of ejaculated sperm

After liquefaction, normal semen samples were centrifuged (800 × *g*), washed in Earle's medium by centrifugation, and subjected on a discontinuous Percoll density gradient (80:40% vol/vol) (4). The 80% Percoll fraction was examined using an optical microscope equipped with a ×100 oil objective to ensure that a pure sample of sperm was obtained. An independent observer, who checked several fields for each slide, inspected the cells. Percoll-purified sperm were washed with unsupplemented Earle's medium (uncapacitating medium) and were incubated for 30 min at 37 C and 5% CO_2 without (control) or with the treatments (experimental).

Treatments containing 16.7 mM glucose, 3.3 nM insulin, and 20 and 50 mM citrate were incubated for 30 min. When the cells were treated with Nif or with the specific inhibitors of CIC 1,2,3-BTA or 1,3,5-BTA (19, 21, 25, 28–31) all alone or each combined with glucose or with citrate, a pretreatment of 15 min was performed. DMSO (0.01% final concentration in culture) and EtOH (0.02% final concentration in culture) were used as solvent controls and did not induce any different result with respect to the control in all *in vitro* assays. Because by using 1,2,3-BTA and 1,3,5-BTA we obtained similar results, in the manuscript, we presented the results obtained with 1,2,3-BTA.

Evaluation of sperm viability

Viability was assessed by using eosin Y method. Spermatozoa were washed in uncapacitating medium and centrifuged at 800 × *g* for 20 min. To test the effects of 0.5 mM 1,2,3-BTA and 25 μM Nif on sperm viability, spermatozoa and 10 ml eosin Y were mixed with an equal volume of sperm sample on a microscope slide. The stained dead cells and live cells that excluded the dye were scored among a total of 200 cells by an independent observer. Sperm viability was calculated as percentage of total motile sperm to exclude some toxic effects of the treatments.

RNA isolation and RT-PCR

Total RNA was isolated from human ejaculated spermatozoa as previously described (32). Before RT-PCR, RNA was incubated with ribonuclease-free deoxyribonuclease (DNase) I in single-strength reaction buffer at 37 C for 15 min followed by heat inactivation of DNase I at 65 C for 10 min. Two micrograms of DNase-treated RNA samples were reverse transcribed by 200 IU Moloney murine leukemia virus reverse transcriptase in a reaction volume of 20 μl (0.4 μg oligo-deoxythymidine, 0.5 mM deoxynucleotide triphosphate, and 24 IU RNasin) for 30 min at 37 C, followed by heat denaturation for 5 min at 95 C. The fragment corresponding to the of CIC was amplified by 30 cycles of PCR in a final volume of 50 μl containing 200 nM of both forward and reverse primers. As a control, a 534-bp *GAPDH* fragment was amplified with the same conditions described above. The specific primers for CIC amplified fragment were forward, 5′-GAAGGCCAAGCTGACGCAC-3′, and reverse, 5′-GGGAGGTCTGGTCGTGGATG-3′, whereas for *GAPDH*, fragment primers were forward, 5′-ACCCCTTCATTGACCTCAACTACAT-3′, and reverse, 5′-CCAGTAGAGGCAGGGATGATGTT-3′. The PCR-amplified products were analyzed as previously reported (33).

Western blot analysis of sperm proteins

Sperm samples, washed twice with Earle's medium (uncapacitating medium), were incubated with or without the indicated treatments, and then centrifuged for 5 min at 5000 × *g*. The pellet was resuspended in lysis buffer as previously described (32). Equal amounts of protein (70 μg) were boiled for 5 min, separated by 10% polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets, and probed with an appropriate dilution of the indicated Ab. The binding of the secondary Ab was detected with the ECL Plus Western blotting detection system according to the manufacturer's instructions. As an internal control, all membranes were subsequently stripped (0.2 M glycine, pH 2.6, for 30 min at room temperature) of the first Ab and reprobed with anti-β-actin Ab.

Immunofluorescence assay

Sperm cells, recovered from Percoll gradient, were rinsed three times with 0.5 mM Tris-HCl buffer (pH 7.5) and fixed with absolute methanol for 7 min at −20 C. The staining was carried out after blocking with normal horse serum (10%), using a rabbit polyclonal Ab raised against mitochondrial citrate carrier (26) or mitochondrial oxoglutarate carrier (34) or mitochondrial phosphate carrier (35, 36) as the primary Ab and an antirabbit FITC-conjugated IgG (1:100) as the secondary Ab. Sperm cells incubated with normal rabbit serum instead of the primary Ab were used as the negative control. The slides were examined under a fluorescence microscope (Olympus BX41, Milan, Italy), and a minimum of 200 spermatozoa per slide were scored.

Measurement of cholesterol efflux

Cholesterol was measured in duplicate by a CHOD-POD enzymatic colorimetric method according to manufacturer's instructions in the incubation medium from human spermatozoa as previously described (37, 38). Percoll-purified sperm samples, washed twice with uncapacitating medium were incubated in the absence (control) or in the presence of 16.7 mM glucose or 3.3 nM insulin. Other samples were incubated with 16.7 mM glucose plus increasing concentrations of 1,2,3-BTA (from 0.5–2 mM) for 30 min at 37 C and 5% CO_2. In addition, the sperm samples were incubated with increasing concentrations of citrate (from 20–50 mM) alone or combined with 25 μM Nif. At the end of the sperm incubation, the culture media were recovered by centrifugation, lyophilized, and subsequently dissolved in 1 ml reaction buffer. The samples were incubated for 10 min at room temperature, and then the cholesterol content was measured spectrophotometrically at 505 nm. The cholesterol standard was used at 200 mg/dl. The limit of sensitivity for the assay was 0.05 mg/dl. Inter- and intraassay variations were 0.02 and 0.04%, respectively. Cholesterol results are presented as milligrams per 10×10^6 spermatozoa.

Assessment of sperm-hyperactivated motility

As previously published (39), we identified hyperactivated sperm using a chemotaxis assay because sperm acquire their chemotactic responsiveness as part of a capacitation process and lose it when the capacitated state is terminated (40). Changes in the motility of sperm were assessed by their ability to migrate through a polycarbonate membrane containing 8-mm pores. The lower wells of the 24-well chamber were filled with a preparation of human sperm (10×10^6). Sperm were prepared in unsupplemented Earle's medium (noncapacitating medium) in the absence (control) or presence of the above-mentioned treatments (experimental)

(41). The chambers were incubated for 1 h at 37 C, and the sperm accumulating in the upper chamber were assessed by direct counting using an optical microscope equipped with a ×100 oil objective.

Simultaneously in another set of experiments with the same above-mentioned treatments, hyperactivated sperm, which are characterized by pronounced flagellar movements, marked lateral excursion of the sperm head, and a nonlinear trajectory, were estimated by direct counting using an optical microscope equipped with a ×100 oil objective. A minimum of 100 sperm were examined per slide, and the percentage of hyperactivated sperm was estimated. No differences with respect to the above-described method were obtained (data not shown). Sperm motility was assessed independently by two individuals.

Acrosome reaction

The evaluation of the acrosome reaction was performed by using FITC-PNA. At the end of incubation, sperm cells were washed three times with 0.5 mmol/liter Tris-HCl buffer (pH 7.5) and were allowed to settle onto slides. Smears, dried in air, were dipped in absolute methanol for 15 min and left at room temperature. The samples were then incubated with a solution of FITC-PNA in a humid chamber at room temperature. After 30 min, the slides were washed with PBS to remove the excess label. Scoring of the staining was immediately assessed, by an epifluorescence microscope (Olympus BX41) according to a published scoring system (40). A minimum of 200 live sperm were examined for each treatment, and they were classified into two main categories on the basis of the FITC-PNA staining. 1) Complete fusion of the plasma membrane and the outer acrosomal membrane upon completion of the process of the acrosome reaction will result in the complete loss of the acrosomal cap. Consequently, spermatozoa demonstrate fluorescence that is limited only to the equatorial segment (acrosome reacted). 2) Bright fluorescence over the acrosomal cap indicates the presence of the outer acrosomal membrane and can be used to identify acrosome-intact sperm. Values are expressed as percentage of acrosome-reacted cells.

Measurement of insulin secreted by human spermatozoa

A competitive RIA was applied to measure insulin in the sperm culture medium (31). Purified spermatozoa were washed twice with unsupplemented Earle's medium and incubated in the same medium for 30 min at 37 C in 5% CO_2. A final concentration of 10×10^6 sperm/500 ml was used. At the end of the sperm incubations, the culture media were recovered by centrifugation. Human insulin concentrations were determined in duplicate using an insulin RIA kit according to manufacturer's instructions. Insulin standards ranged from 0–300 μIU/ml. The limit of sensitivity for the assay was 0.01 μIU/ml. Inter- and intraassay variations were 3.9 and 4.3%, respectively. Insulin results are presented as the original concentrations of the supernatants and are expressed as micro-international units per milliliter.

Assay of the G6PDH activity

The conversion of $NADP^+$ to NADPH + H^+, catalyzed by G6PDH, was measured by the increase of absorbance at 340 nm (31). Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37 C and 5% CO_2. Other samples were incubated in the presence of the indicated treatments. After incubation, 50 μl of sperm extracts were loaded into individual cuvettes containing buffer [100 mM triethanolamine, 100 mM $MgCl_2$, 10 mg/ml glucose-6-phosphate, 10 mg/ml $NADP^+$ (pH 7.6)] for spectrophotometric determination. The absorbance of samples was read at 340 nm every 20 sec for 1.5 min. Data are expressed in nanomoles per minute per 10^6 sperm. The enzymatic activity was determined with three control media: one without glucose-6-phosphate as substrate, one without the coenzyme ($NADP^+$), and the third without either substrate or coenzyme (data not shown).

Statistical analysis

Three independent RT-PCR experiments were performed, whereas Western blot analysis was performed in at least four independent experiments.

The data obtained from the cholesterol assay, G6PDH activity measurements, and insulin assay (10 replicate experiments using duplicate determinations) were presented as the mean ± SEM. The differences in mean values were calculated using ANOVA with a significance level of $P \leq 0.05$.

Results

CIC protein and mRNA are present in human sperm

The presence of CIC in normal human ejaculated sperm was investigated by Western blot analysis using an Ab

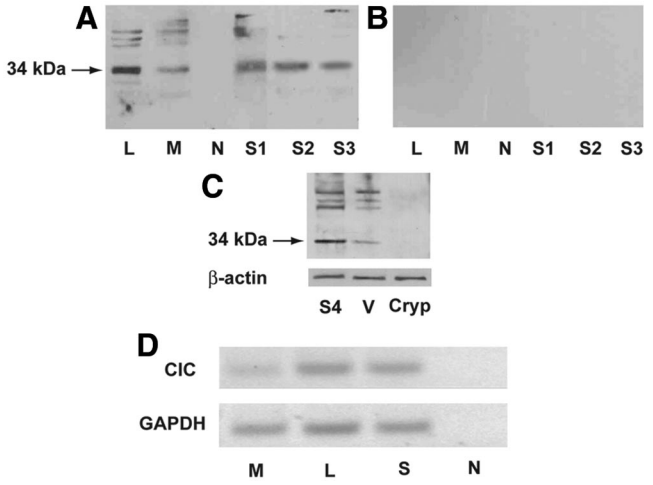

FIG. 1. CIC is present in human ejaculated spermatozoa. A and B, Extracts from pooled purified ejaculated spermatozoa were subjected to electrophoresis on 10% SDS-PAGE, blotted onto nitrocellulose membranes, and probed with rabbit polyclonal Ab to anti-C-terminal CIC (A) or with normal rabbit serum (B). LNCap (L) and MCF-7 (M) extracts were used as positive controls. N, Negative control. S1, S2, and S3 represent three samples of ejaculated spermatozoa from normal men. The Western blot experiments were repeated at least four times, and the autoradiographs of the figure show the results of one representative experiment. C, Western blot analysis of normoospermic (S4), varicocele (V), and cryptozoospermic (Cryp) patients. The Western blot experiments were repeated at least four times, and the autoradiographs of the figure show the results of one representative experiment. D, Ethidium bromide staining of the RT-PCR products obtained using specific primers for human CIC transcript on cDNA from MCF-7 (M), LNCap (L), and human spermatozoa (S). N indicates the negative control. GAPDH was amplified as a control for RNA integrity. The pictures shown are representative examples of experiments that were performed at least three times with reproducible results.

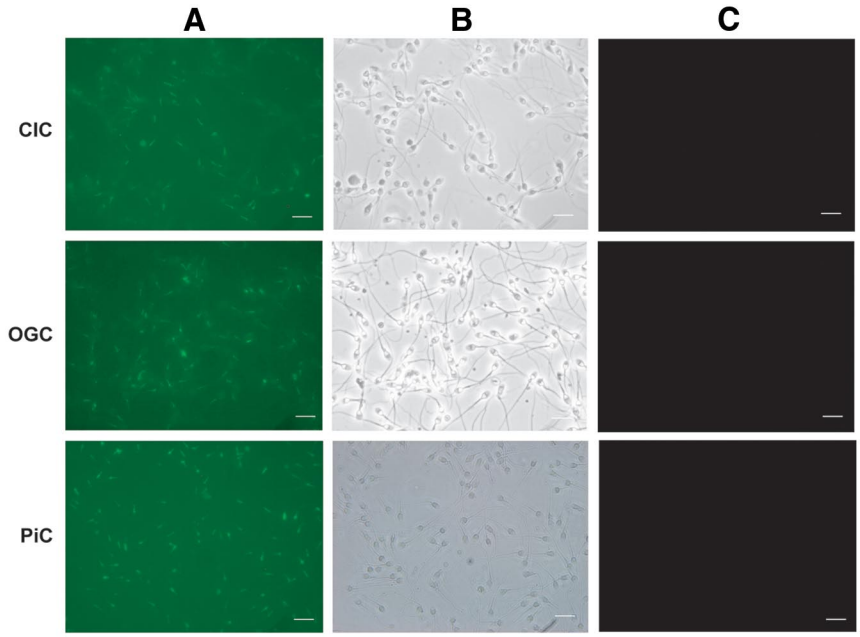

FIG. 2. Immunolocalization of different mitochondrial carriers in human ejaculated spermatozoa. Spermatozoa were extensively washed and incubated in the unsupplemented Earle's medium for 30 min at 37 C and 5% CO_2. Spermatozoa were then fixed and analyzed by immunostaining as detailed in *Materials and Methods*. A, Mitochondrial carriers immunolocalization; B, transmitted light pictures of sperm cells; C, sperm cells incubated with the Ab against the different mitochondrial carriers replaced by normal rabbit serum were used as negative control. The primary Ab used in the mitochondrial carrier immunolocalization were raised against CIC, oxoglutarate carrier (OGC), and phosphate carrier (PiC). *Scale bars*, 5 μm. The pictures shown are representative examples of experiments that were performed at least three times with reproducible results.

raised against the carboxy terminus of the mature CIC protein (Fig. 1A). Our Ab revealed mainly one immunoreactive band at 34 kDa, corresponding to the human mitochondrial citrate carrier, in prostate cancer cells LNCaP (Fig. 1A, lane L) and human breast cancer cells MCF-7 (Fig. 1A, lane M) and in the lysates from uncapacitated normal sperm samples (Fig. 1A, lanes S1, S2, and S3); the negative control (Fig. 1A, lane N) was performed using a sperm lysate, where CIC was previously removed by preincubation with the respective Ab and subsequently immunoprecipitated with protein A/G-agarose. Any immunoreactive bands were detected when normal rabbit serum was used, indicating that the band is specific for CIC and confirming the specificity of the Ab binding (Fig. 1B). Furthermore, the presence of CIC was reduced in varicocele sample (Fig. 1C, lane V), although it was undetectable in a cryptozoospermic sample (Fig. 1C, lane Cryp). To determine whether mRNA for CIC is also present in human ejaculated spermatozoa, RNA isolated from Percoll-separated sperm of normal men (Fig. 1D, lane S) was analyzed by RT-PCR. As positive controls, MCF-7 and LNCaP cells were also tested (Fig. 1D, lanes M and L, respectively). The RT-PCR amplifications revealed the expected PCR product size of 415 bp of the coding region of the human CIC (Fig. 1D). These products were sequenced and found to correspond to the classical human CIC. No band of expression was found in the negative control performed without mRNA, eliminating the possibility of DNA contamination (Fig. 1D, lane N). A control RT-PCR was carried out using specific primers for GAPDH (Fig. 1D). The RT-PCR products were not a result of any genomic DNA contamination because the RNA extraction was subjected to DNase treatment.

CIC is located in the midpiece

Immunofluorescence, using the same Ab that was used for Western blotting, showed a positive signal for CIC in human spermatozoa. In all the samples examined, immunoreactivity was clearly compartmentalized to the midpiece, where mitochondria are restricted, whereas the heads and tails did not contain fluorescence (Fig. 2, lane A). The same cellular distribution was found for known mitochondrial carriers such as the oxoglutarate and the phosphate. No fluorescent signal was obtained when the normal rabbit serum was used instead of the primary Ab (Fig. 2, lane C), thus confirming the specificity of the Ab binding.

CIC affects both cholesterol efflux and protein tyrosine phosphorylation in sperm

We investigated whether the presence of CIC in ejaculated human sperm influences the acquisition of fertilizing capacity by examining two representative parameters of capacitation: cholesterol efflux and protein tyrosine phosphorylation.

Washed pooled sperm from normal samples were treated as indicated in *Materials and Methods* and centrifuged. The upper phase of the sample was used to determinate the cholesterol levels, whereas the sperm were lysed to evaluate protein tyrosine phosphorylation.

Our results showed a significant increase in cholesterol efflux upon glucose and insulin treatment, as previously reported (38), whereas the addition of increasing concentration of 1,2,3-BTA (from 0.5–2 mM), a known specific inhibitor of CIC (19, 21, 25, 28, 29), clearly reverted the increased cholesterol efflux. Furthermore, a dose-dependent increase of cholesterol efflux was observed in presence of citrate, the specific substrate of CIC. In particular,

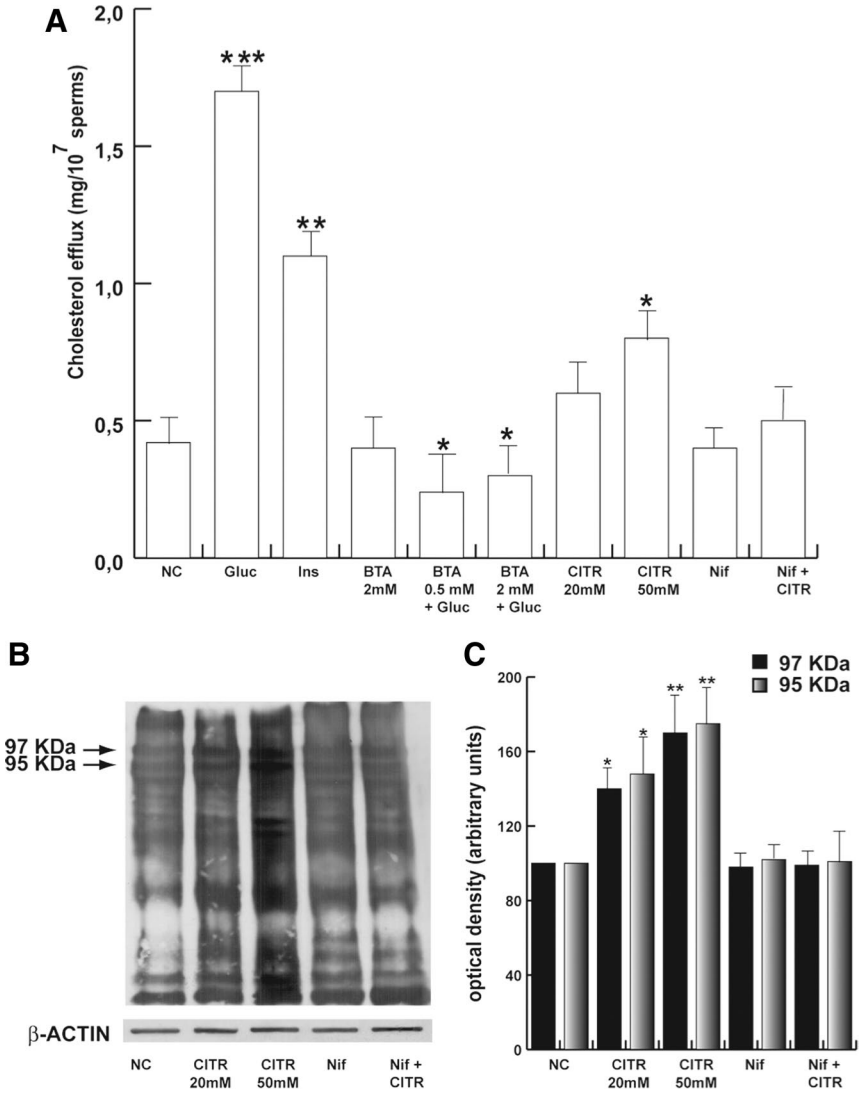

FIG. 3. CIC inhibition reduced cholesterol efflux and protein tyrosine phosphorylation in human sperm. Washed spermatozoa were incubated under uncapacitating conditions for 30 min at 37 C and 5% CO_2, in the absence [negative control (NC)] or in the presence of 16.7 mM glucose (Gluc) or 3.3 nM insulin (Ins) each alone. Other samples were incubated with 16.7 mM glucose plus increasing concentrations of 1,2,3- BTA (0.5 and 2 mM) or with increasing concentration of citrate (CITR) (20 and 50 mM) each alone or 50 mM citrate combined with 25 μM Nif. A, Cholesterol in culture medium from human ejaculated spermatozoa was measured by enzymatic colorimetric assay. Columns are mean ± SEM of six independent experiments performed in duplicate. *, $P < 0.05$ *vs.* control; **, $P < 0.01$ *vs.* control; ***, $P < 0.001$ *vs.* control. B, Sperm lysates (70 μg) were used for Western blot analysis of protein tyrosine phosphorylation. C, Quantitative representation after densitometry of the bands. *, $P < 0.05$ *vs.* control; **, $P < 0.01$ *vs.* control.

50 mM citrate was able to sustain the greatest augmentation, which was reversed after treatment with 25 μM Nif (Fig. 3A). None of the aforementioned effects of 1,2,3-BTA and Nif could be ascribed to cytotoxicity, because treatment of sperm for 1 h with 2 mM 1,2,3-BTA or 25 μM Nif had no effect on cell viability, as assessed by the eosin Y method (data not shown).

To better define the role of citrate in capacitation, we tested its effect on sperm protein tyrosine phosphorylations. Incubation with 20 and 50 mM citrate induced a noticeable increase in the intensity of all bands, especially, the 95- to 97-kDa bands. Furthermore, the effect of 50 mM citrate was reduced by 25 μM Nif (Fig. 3, B and C).

CIC effects on p-p60src and p-Akt in human spermatozoa

The tyrosine kinase p-p60src is a key player in the signal transduction cascade involved in the regulation of tyrosine phosphorylation during sperm capacitation. In this study, we evaluated the influence of citrate on p-p60src activity and on phosphorylation of p-Akt, the major metabolic intermediate downstream of the phosphatidylinositol 3-kinase (PI3K), the key pathway in insulin signaling. PI3K was examined in our previous studies, revealing an important role in both sperm survival and metabolism (4, 42).

The results obtained here showed that citrate significantly induced p-p60src activity in a dose-dependent manner, and the addition of 25 μM Nif attenuated the effect (Fig. 4, A and B). A similar pattern of response was observed for the p-Akt phosphorylation. These data suggest that citrate is involved in the induction of p-p60src and p-Akt activities.

CIC blockage reduced sperm motility hyperactivation and acrosome reaction

Capacitation encompasses initiation of hyperactivated motility and the acquisition of competence for acrosomal exocytosis in response to an appropriate physiological stimulus.

Glucose and insulin treatments were able to produce an enhancement of the sperm motility/migration as result of capacitation occurring in the chemotactic assay (Fig. 5A). Instead, the addition of increasing concentrations of 1,2,3-BTA (from 0.5–2 mM) reduced the effect. Furthermore, a dose-dependent increase of hyperactivated motility was observed in presence of citrate, the specific substrate of CIC. In particular, 50 mM citrate was able to sustain the greatest augmentation, which was reversed after treatment with 25 μM Nif (Fig. 5A).

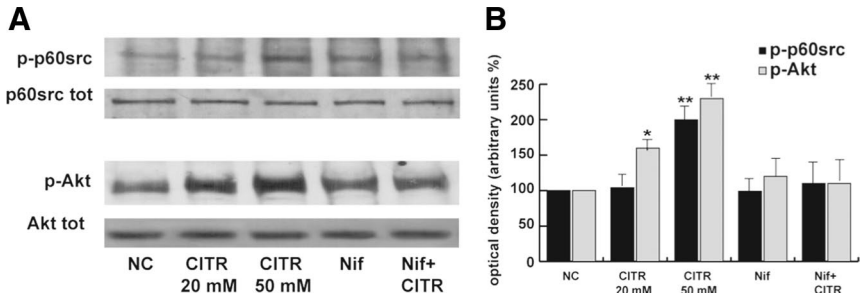

FIG. 4. CIC action on p-p60src and p-Akt in human ejaculated spermatozoa. Washed spermatozoa were incubated in the unsupplemented Earle's medium for 1 h at 37 C and 5% CO_2 in the absence [negative control (NC)] or in the presence of increasing citrate (CITR) (20 and 50 mM) each alone or 50 mM citrate (CITR) combined with 25 μM Nif. A, A total of 70 μg sperm lysates was used for Western blot analysis of p-60src and p-Akt; B, quantitative representation after densitometry of the bands. Total p-60src and p-Akt were used as loading control. *, $P < 0.05$ *vs.* control; **, $P < 0.01$ *vs.* control.

The FITC-PNA staining revealed a significant difference between the treatments. A highly significant increase in reacted sperm cells was seen in sperm incubated with glucose, insulin, and 50 mM citrate with respect to the basal samples. The incidence of reacted sperm was reduced in samples treated with glucose plus increasing concentrations of 1,2,3-BTA with respect to glucose alone and in samples treated with 25 μM Nif plus 50 mM citrate with respect to 50 mM citrate alone, whereas either 2 mM 1,2,3-BTA or 25 μM Nif alone was similar to the control (Fig. 5B).

CIC is involved in the regulation of GSIS and insulin secretion in sperm

Our previous data have demonstrated that insulin is present in and secreted from human ejaculated spermatozoa (31). Glucose is the main physiological secretagogue of insulin in pancreatic cells (30), and sperm was found to be also responsive to glucose (31). In particular, glucose induced a dose-dependent stimulation of insulin secretion; the largest increase was observed in response to 16.7 mM glucose (31). A recent study reported that CIC is involved in the regulation of GSIS in rat insulinoma cells (robustly glucose-responsive INS-1-derived 832/13 cells) or in primary rat islets (25).

To evaluate whether CIC plays a regulatory role in GSIS in human ejaculated spermatozoa, we measured the insulin secretion from sperm by using glucose alone or in the presence of increasing concentrations of 1,2,3-BTA (from 0.5–2 mM). Interestingly, our results showed that the effect of the CIC inhibitor is dose dependent; specifically, the addition of 16.7 mM glucose plus 2.0 mM 1,2,3-BTA to sperm significantly inhibited GSIS (Fig. 6A). None of the aforementioned effects of 1,2,3-BTA could be ascribed to cytotoxicity, because treatment of sperm for 1 h with 2 mM 1,2,3-BTA alone had no effect on cell viability, as assessed by the eosin Y method (data not shown).

As expected, increasing citrate concentrations (from 20–50 mM) induced insulin secretion, although to a lesser extent than 16.7 mM glucose (Fig. 6B). In addition, 50 mM citrate plus 25 μM Nif diminished the effect confirming that an autocrine regulation of insulin secretion exists in sperm. Altogether, these data may indicate that CIC regulates not only GSIS but also insulin secretion in sperm and therefore does not occur in a strictly glucose-dependent manner.

CIC effects on G6PDH activity

The effect of glucose on the fertilizing ability of sperm appears to be mediated by the production of NADPH from glucose-6-phosphate by G6PDH, the key rate-limiting enzyme in the PPP (14, 43). In human spermatozoa, it has been shown that NADPH increased significantly when the glucose concentration was raised from 0.6 to 16.7 mM in the incubation medium (31).

To assess the role of CIC on G6PDH activity in human sperm, we stimulated sperm with 3.3 nM insulin and 16.7 mM glucose alone or in combination with 0.5 mM or 2 mM 1,2,3-BTA. The CIC inhibitor significantly decreased G6PDH activity in sperm compared with sperm incubated with 16.7 mM glucose (Fig. 7A). Moreover, treatment of human sperm with citrate increased the G6PDH activity in a dose-dependent manner and was reduced by adding 25 μM Nif (Fig. 7B).

Discussion

Sperm energy management influences its ability to perform its overall function; however, the mechanisms through which the regulation of sperm metabolism occur are not clear. In this work, we have demonstrated the presence of CIC in human ejaculated spermatozoa by measuring mRNA expression, protein expression, and immunolocalization. Furthermore, we have demonstrated that inhibition of CIC reduced the activation of pathways that are important for sperm acquisition of fertilizing ability as well as the GSIS, insulin secretion, acrosome reaction, and G6PDH activity.

First, we showed that CIC is present in human sperm by Western blotting and RT-PCR. CIC is a protein, 298 amino acids long, that spans the inner mitochondrial membrane six times with both the N and C termini protruding toward the cytosol (26). CIC mRNA and/or CIC protein levels are high in human liver, pancreas, and kidney but low or absent in heart, skeletal muscle, placenta,

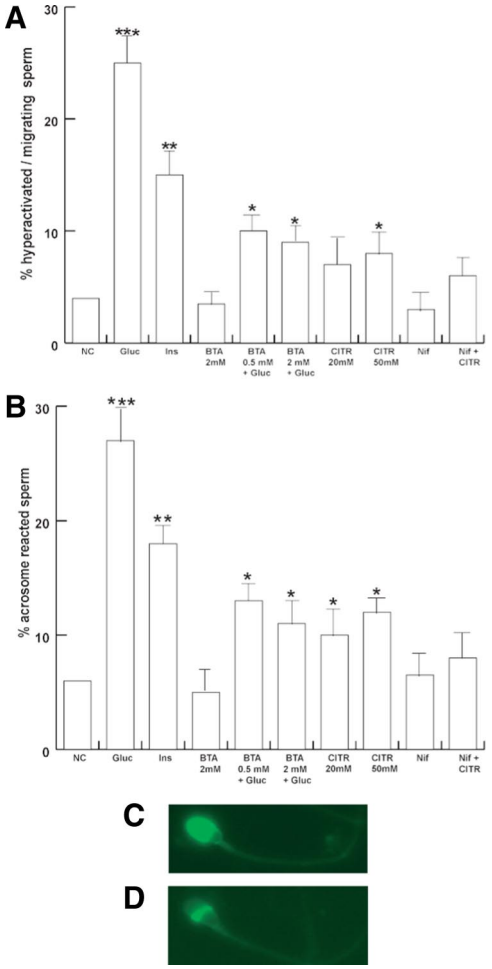

FIG. 5. CIC inhibition reduces hyperactivated sperm motility and acrosome reaction. Washed spermatozoa were incubated under uncapacitating conditions for 30 min at 37 C and 5% CO_2 in the absence [negative control (NC)] or in the presence of either 16.7 mM glucose (Gluc) or 3.3 nM insulin (Ins) alone. Other samples were incubated with 16.7 mM glucose plus increasing concentrations of 1,2,3-BTA (0.5 and 2 mM) or with increasing concentration of either citrate (CITR) (20 and 50 mM) alone or 50 mM citrate combined with 25 μM Nif. A, Hyperactivated sperm motility was determined as described in *Materials and Methods*, and the values are expressed as percentage of hyperactivated sperm. Columns represent mean ± SEM of four independent experiments, each done in duplicate. *, $P < 0.05$, **, $P < 0.02$; ***, $P < 0.01$ *vs.* control. B, Acrosome reaction was determined as described in *Materials and Methods*, and the values are expressed as percentage of acrosome-reacted cells. Columns represent mean ± SEM of four independent experiments, each done in duplicate. *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$ *vs.* control. C, Sperm with acrosome intact. D, Acrosome-reacted sperm.

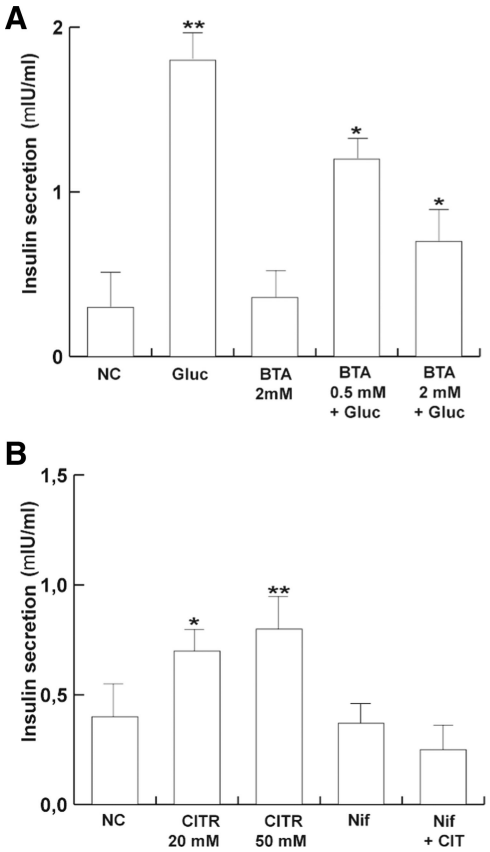

FIG. 6. CIC regulates insulin secretion from human ejaculated spermatozoa. Insulin secretion in culture medium from human ejaculated spermatozoa was measured by RIA. A, Washed sperm were incubated in unsupplemented Earle's balanced salt solution for 1 h at 37 C in 5% CO_2 in the absence [negative control (NC)] or in the presence of 16.7 mM glucose (Gluc) or 2 mM 1,2,3-BTA. Other samples were incubated with 16.7 mM glucose combined with increasing concentrations of 1,2,3-BTA (0.5 and 2 mM). B, The sperm samples, under the same conditions described above, were incubated in the absence (NC) or in the presence of increasing concentrations of citrate (CITR) (20 and 50 mM) or 50 mM citrate combined with 25 μM Nif. Values are the mean ± SEM of 10 replicates. *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

brain, and lungs (44). From our data, CIC has the same molecular weight as was found in LNCap cells and MCF-7 cells, thus revealing a new site of expression of this protein. It is noteworthy that the reduced level of CIC in varicocele sperm as well as its absence in sperm from cryptozoospermic patients support a physiological role for this protein in human male gametes. On the basis of recent research showing that mutation in the mitochondrial citrate carrier gene produces DNA instability that in turn induces frequent chromosome breaks (24), it may be supposed that unbalanced CIC levels may be involved in the reported increase in DNA fragmentation in sperm from varicocele and cryptozoospermic patients (45).

Mammalian spermatozoa are highly differentiated cells that display extreme polarization of architecture and function. Briefly, the mature sperm cell has three highly specialized regions: the sperm head, which is involved in sperm-oocyte interaction; the midpiece with mitochondria, which is involved in energy production; and the flagellum, which is involved in metabolism and motility. In regard to the sperm polarization, it was hypothesized that sperm possess compartmentalized metabolic and signaling pathways in the regions where they are needed (11). Our immunohistochemical assays demonstrated that CIC is compartmentalized in the midpiece where mitochondria are restricted; this result is supported by the colocalization

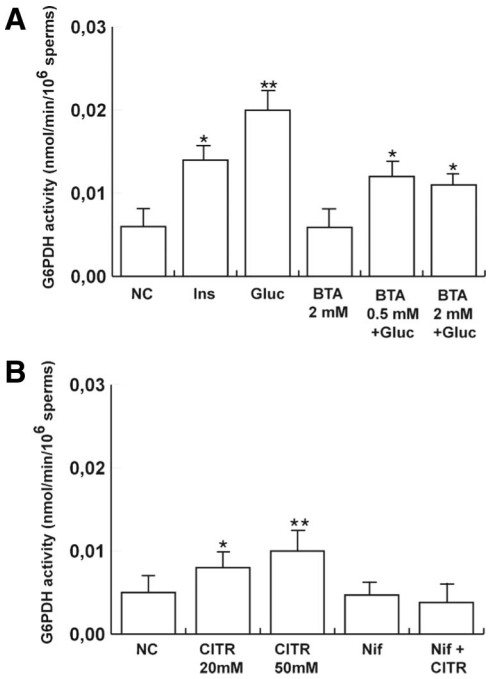

FIG. 7. Action of CIC on G6PDH activity in human ejaculated spermatozoa. A, Washed spermatozoa were incubated for 1 h at 37 C under uncapacitating conditions in the absence [negative control (NC)] or in the presence of 3.3 nM insulin (Ins) or 16.7 mM glucose (Gluc) or 2 mM 1,2,3-BTA. Other samples were incubated with 16.7 mM glucose combined with increasing concentrations of 1,2,3-BTA (0.5 and 2 mM). B, The sperm samples, under the same conditions described above, were incubated in the absence (NC) or in the presence of increasing concentrations of citrate (CITR) (20 and 50 mM) or 50 mM citrate combined with 25 μM Nif. Data are expressed as nanomoles per minute per 10^6 sperm. Values are the mean ± SEM of 10 replicates. *, $P < 0.05$ *vs.* control; **, $P < 0.01$ *vs.* control.

with two other mitochondrial markers and is in agreement with the reported CIC location in other cell types (44, 46).

Successful sperm maturation depends on sequential steps in both the male and the female reproductive tracts. After ejaculation, the mammalian male gamete must undergo the capacitation process, a prerequisite for egg fertilization (47). Despite the importance of this process, the biochemical and molecular events of this phenomenon are poorly understood, and the identities of all the factors that trigger gamete activation are unknown. To evaluate whether CIC and its substrate citrate participate in capacitation, we have considered two representative parameters of capacitation: cholesterol efflux and protein tyrosine phosphorylation. The efflux of cholesterol is historically known to be one of the first steps of capacitation (37, 48, 49), and it initiates the signaling events leading to tyrosine phosphorylation of sperm proteins. Many studies have established a correlation between capacitation and phosphorylation on tyrosine residues of multiple proteins in human (48–56) spermatozoa. In fact, capacitation has been shown to be correlated with changes in sperm plasma membrane fluidity (57) that arise from a reduction in the cholesterol to phospholipid ratio as a consequence of the efflux of cholesterol from the plasma membrane (57–60). Moreover, it was demonstrated that insulin is an endogenous factor involved in the autocrine induction of the process, and the initial massive insulin secretion may be considered a feature of this event (31). In pancreatic β-cells, there is a great deal of evidence supporting the idea that the alteration of lipid content and the reorganization of phospholipids in the plasma membrane also serve as a long-term signal to promote insulin exocytosis. Insulin secretion is triggered by ATP generated during glucose metabolism; however, numerous studies showed that glucose retains the ability to stimulate insulin secretion even when ATP-sensitive K^+ channels are rendered unresponsive, strongly implying that others metabolic signals are important for GSIS (61, 62). Several byproducts of mitochondrial glucose metabolism have been invoked as candidates for mediating these ancillary signaling pathways, including citrate (25). It was reported that INS-1 cells stimulated with a high concentration of glucose exhibited an increased efflux of citrate from mitochondria to the cytoplasm (25). In this regard, CIC in sperm could play a pivotal role in capacitation process by connecting carbohydrate catabolism and lipogenesis. In fact, it exports citrate from mitochondria that could be cleaved by ATP-citrate lyase to oxaloacetate and acetyl-coenzyme A, the latter used for fatty acid and cholesterol syntheses (8).

In our study, 16.7 mM glucose significantly increased cholesterol efflux and insulin secretion, which in turn were reduced by 1,2,3-BTA blockage, suggesting that CIC modulation might be important in the early phases (initiation) of capacitation. Furthermore, 50 mM citrate significantly increased cholesterol efflux and insulin secretion that were reduced by the blockage of insulin secretion through Nif. Concerning the tyrosine phosphorylation of sperm proteins, it is well known that both glucose and insulin are able to induce this feature of the capacitation process; from our data, it also emerges that citrate increased in a dose-dependent manner protein tyrosine phosphorylation, and the combination with Nif reduced the effect. Besides, citrate produced an activation of p60src and Akt that play an important role in capacitation and survival (63, 64). Interestingly, CIC inhibition reduced sperm hyperactivated motility as well as acrosome reaction, and citrate induced these key sperm activities, further supporting a physiological role for CIC in sperm physiology. Altogether, these results indicate that citrate had similar effects to that of glucose in inducing insulin secretion, which in turn through an autocrine short loop regulates the recruitment of energetic substrates according to sperm energy needs. It should be noted that capacitation involves numerous physiological changes including an increased metabolic

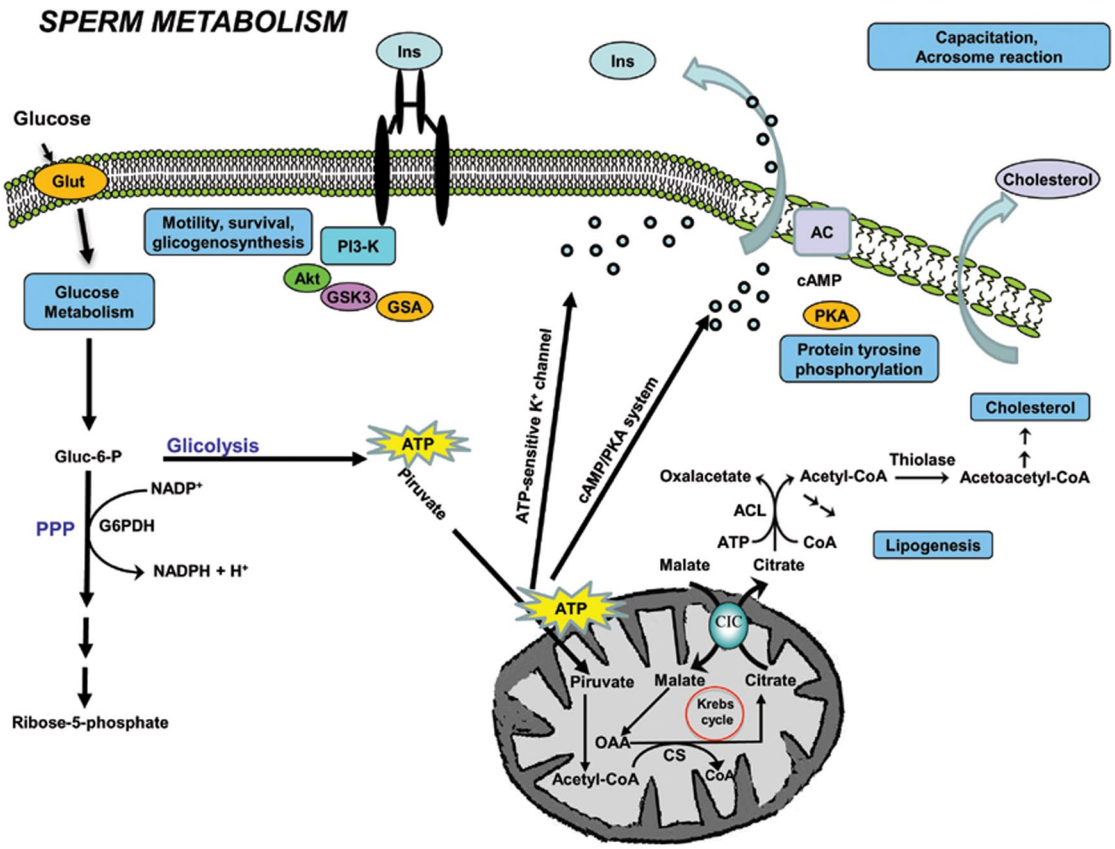

FIG. 8. Role of CIC in sperm metabolism. Glucose enters into sperm via glucose transporter (Glut) and it is phosphorylated to glucose-6-phosphate (Gluc-6-P) to be used in the PPP and in glycolysis producing NADPH and ATP, respectively. The augmentation of ATP obtained via glycolysis and via Krebs cycle induces insulin secretion via the ATP-sensitive K^+ channel or via the cAMP/protein kinase A (PKA) system. In uncapacitated sperm, insulin secretion may act in an autocrine fashion to induce glycogen synthesis through the PI3K/AKT/glycogen synthase kinase 3 (GSK3) pathway activating the glycogen synthase (GSA). The glucose destined for lipogenesis and cholesterol synthesis exits from the mitochondria as citrate. ATP-citrate lyase (ACL) metabolizes citrate to acetyl-coenzyme A (CoA), which is converted to acetoacetyl-CoA by thiolase. The acetoacetyl-CoA through numerous enzymatic steps leads to cholesterol synthesis. During the capacitation process, an augmentation of membrane fluidity occurs, and both cholesterol and insulin escape from the cell. Cholesterol efflux and the activation of PKA induce tyrosine phosphorylation of sperm proteins. AC, Adenylate cyclase; CS, citrate synthase; Ins, insulin; OAA, oxaloacetate.

rate and overall energy expenditure to accomplish all the necessary changes. Our recent data suggest that sperm has the ability to modulate itself based upon the availability of the energetic substrate and its energy needs independently of the systemic regulation (31). Different metabolic substrates can be used by sperm to maintain their energy requirements. In fact, several authors have reported that sperm obtains energy from not only glucose and fructose but also from nonhexose compounds like citrate and lactate in conditions where a monosaccharide supply is limited (7–9). Sperm metabolization of nonglucidic substrates is a functional characteristic of these cells. For example, whereas the acrosome reaction requires lactate or pyruvate for ATP production, gamete fusion requires glucose to produce NADPH by the PPP, sperm motility appears to be supported by relatively low ATP levels, but achievement of high ATP levels is essential for tyrosine phosphorylation linked to motility hyperactivation (Fig. 8). In our study, both glucose and citrate were able to induce G6PDH activity that was reverted by 1,2,3-BTA and Nif, respectively, suggesting their involvement in the regulation of energy metabolism to sustain capacitation process.

Altogether, our data demonstrate that CIC contributes to the acquisition of sperm fertilizing ability through the control of insulin secretion and that this mechanism seems to be modulated by the CIC-specific substrate citrate (Fig. 8). The present findings support the idea of a new site of action for CIC in the regulation of metabolism, opening a new area of study in male reproduction.

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