Phosphocreatine, an intracellular high-energy compound, is found in the extracellular fluid of the seminal vesicles in mice and rats

(sperm function/³¹P NMR/exocytosis/ectokinase)

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Communicated by Mildred Cohn, June 20, 1988

ABSTRACT High levels of phosphocreatine, a compound known to serve as an intracellular energy reserve, were found in the fluid contained in seminal vesicle glands. The concentrations of phosphocreatine in the extracellular fluid in the mouse and rat were found to be 5.6 ± 1.6 and 2.2 ± 0.8 $\mu\text{mol/g}$, respectively, which are higher than the intracellular levels reported for smooth muscles. The creatine concentrations in the seminal vesicular fluid from these two species were 22.8 ± 3.1 and 13.0 ± 5.3 $\mu\text{mol/g}$, respectively. These creatine levels are approximately 100 and 65 times higher than the creatine levels in mammalian blood. Smaller amounts of ATP (phosphocreatine/ATP ratio of 20–40) and traces of ADP were also found. Comparison of the pattern of distribution of macromolecules (proteins and DNA) with the distribution of phosphocreatine between the cells and the fluid of the seminal vesicle indicates that cell lysis did not account for the phosphocreatine in the seminal vesicle fluid. Rather, the available evidence strongly suggests that this high-energy compound is actively secreted. We found that in the testes, the sperm are exposed to the highest known creatine concentration in any mammalian tissue studied. Based on these results and other recent reports, we propose that the extracellular phosphocreatine, ATP, and creatine are involved in sperm metabolism.

Phosphocreatine (PCr), a guanidinophosphate, was first discovered in skeletal muscle (1, 2). It is believed to serve as an energy reserve by virtue of its ability to phosphorylate ADP, leading to the production of ATP and creatine (Cr). This reversible phosphoryl transfer, mediated by creatine kinase (CK; ATP:creatine N-phosphotransferase, EC 2.7.3.2), can be represented as follows.

CK/PCr-mediated energy modulator systems have since been demonstrated in several other cell types, including brain (3, 4), smooth muscle (5–7), mammalian preimplantation embryo (8), and spermatozoa (9, 10), and in the mitotic spindle of proliferating animal cells (11). In addition to an ATP-buffering role, reaction 1 has been suggested to act as an intracellular energy-transport system (12). Strong experimental evidence for such an energy-channeling role for PCr in sea urchin sperm has been produced (10). PCr and Cr in higher organisms are considered to be dead-end metabolites in that the CK-mediated reversible transfer of phosphate groups is the only known enzyme reaction in which PCr and Cr participate. However, a small portion of the total body creatine (PCr + Cr) is converted by a nonenzymatic reaction to the anhydride, creatinine, which can readily cross cell membranes and is the excretory product of PCr and Cr (13).

It has been shown (14) that PCr in muscle is in equilibrium with phosphocreatinine and that phosphocreatinine is an intermediate in the nonenzymatic production of creatinine. Phosphocreatinine and several other compounds have been implicated in the formation of creatinine from PCr (15). No evidence exists for the export of PCr out of intact cells or for its use in any extracellular function. This is not surprising in view of the imperviousness of cell membranes to PCr. We have found that the seminal vesicles in the mouse[¶] and rat contained extremely high levels of Cr and PCr. Unexpectedly, most of the PCr was found in the fluid secreted by the seminal vesicle. In this report we present our results to support the view that this high-energy phosphate is a secretory product of the vesicular cells. Also summarized is the evidence that suggests possible roles for this extracellular PCr and Cr in the metabolic regulation of sperm.

MATERIALS AND METHODS

Seminal Vesicles and Vesicular Fluid. Seminal vesicles were obtained from Swiss Webster mice (8 weeks old) or from Sprague–Dawley rats (10–12 weeks old). The animals were acclimated to a standard laboratory chow and to a 12-hr light/dark cycle for 5–7 days. They were killed by inhalation of CO₂ in a large desiccator. The seminal vesicles were rapidly removed, frozen in liquid N₂, and stored at –70°C. In experiments where seminal fluids were separated from the vesicles, the fresh vesicles were immersed in ice-cold phosphate-buffered saline (150 mM NaCl/20 mM phosphate, pH 7.2) for 1 min to aid the coagulation of the seminal fluid. The coagulate (SVF) was then gently extruded from the gland (SV) into an ice-cold container. The separated SV and SVF were then immediately frozen and stored at –70°C.

Testes and Testicular Preparations. The testes used for analysis of the high-energy phosphates were rapidly frozen (liquid-N₂ Wollenberger tongs) *in situ* via a ventral incision while the animal was under pentobarbital anesthesia. This approach was found to be essential to the preservation of PCr in this tissue. After removal the testes were immersed in liquid N₂ and stored as described above. In experiments to determine the localization of Cr, fresh unfrozen testes were immersed in ice-cold phosphate-buffered saline. Pieces of tissue, cut into small cubes (≈ 2 mm³), were obtained with a razor blade. The slices (60–100 mg) were transferred to 10 volumes (0.6–1.0 ml) of ice-cold phosphate-buffered saline in plastic centrifuge tubes and gently shaken for 5 min at 0°C. The slices were sedimented by centrifugation at $3000 \times g$ for 5 min and the supernatant (S1) was decanted. The process

Abbreviations: PCr, phosphocreatine; Cr, creatine; CK, creatine kinase; SVF, fluid removed from the seminal vesicle; SV, seminal vesicle with fluid removed.

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¶A preliminary report of this research has been presented by Lee, H. J. & Iyengar, M. R. at the 78th Annual Meeting of the American Society of Biological Chemists, June 7–11, 1987, Philadelphia.

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was repeated to generate a second collection of supernatant (S2). The residual slices (P) and supernatants S1 and S2 were frozen and stored as indicated above.

Tissue Extracts. The labile phosphate compounds were extracted from the tissues and fluids by the perchloric acid/KOH procedure of Lowry and Passonneau (16). In brief, the liquid N₂-frozen sample was pulverized with 5 volumes of liquid N₂-frozen 0.5 M HClO₄ in a liquid N₂-cooled stainless steel centrifuge tube. The extract was thawed in ice water and centrifuged (17,000 × g, 5 min, 0°C), and the supernatant was removed, neutralized to pH 7.0 with ice-cold 0.5 M KOH, and then centrifuged to remove the precipitate. The supernatant was frozen and stored for later analysis as described for the tissues.

Assay of PCr, ATP, and ADP. The amounts of PCr, ATP, and ADP in the neutralized extracts were determined by the direct enzymatic method of Lowry and Passonneau (16). ATP and PCr were determined sequentially in the same sample by adding CK after the ATP assay without the removal of the NADPH equivalent of ATP (16). All reactions were carried out in 1-ml cuvettes in a Turner model III fluorometer (Turner Associates, Palo Alto, CA) at 25°C.

³¹P NMR Spectroscopy. Extracts of SVF were pooled from 6–8 mice or 4–5 rats and lyophilized. The resultant powder was dissolved in 1.0 ml of ice-cold water containing 15% ²H₂O. In experiments directed toward further characterization of the putative PCr by reaction with ADP and CK, the lyophilized powder was dissolved in 0.1 M triethanolamine buffer (pH 7.8). One milliliter of the solution was treated with 10 μl of 1.6 M Mg(OAc)₂/0.2 M ADP/1.0 M triethanolamine, pH 7.8, and 10 μl of 0.1 M triethanolamine buffer containing 100 μg of rabbit muscle CK. The mixture was incubated at 25°C for 30 min before the NMR spectra were obtained. ³¹P NMR spectra were recorded in a Bruker WH-360 spectrometer (spectrometer frequency, 145.7 MHz; sweep width, 5 KHz; pulse width, 25 μsec; angle, 56°; 2000–5000 scans; 8000 data points). Chemical shifts were determined with PCr set at 0 ppm.

Cr Determination. Cr was routinely measured by a modification of the colorimetric procedure of Eggleton *et al.* (17). Extracts of SV, SVF, and testicle were also analyzed for Cr by enzymatic reactions with CK and ATP. The colorimetric reaction volume was 1.5 ml. A₅₂₅ was read in a uv/160 spectrophotometer (Shimadzu, Columbia, MD) after 30 min of incubation at room temperature. For the enzymatic assay of putative Cr, samples containing about 65 μg of Cr, as indicated by colorimetry, were incubated with CK in a total volume of 1.0 ml. The reaction mixtures, each containing 0.1 M triethanolamine (pH 7.8), 0.5 mM MgCl₂, 0.5 mM ATP, and 50 μg of rabbit muscle CK, were incubated at 37°C for 30 min. The residual Cr at the end of incubation was again determined colorimetrically, while the PCr produced was quantified by reversed-phase HPLC (14).

NaDodSO₄/PAGE. NaDodSO₄/PAGE of the proteins in SV and SVF was performed essentially by the method of Laemmli (18) on fresh tissue samples. Separation was accomplished in a vertical slab apparatus (Idea Scientific, Corvallis, OR) with 4% stacking and 12.5% running gels and 0.025 M Tris/0.19 M glycine/0.1% NaDodSO₄, pH 8.3, as the running buffer. A starting current of 12 mA was applied for 5 min; this was followed by 4 hr of separation at 25 mA (120 V). Densitometry of the Coomassie blue R250-stained bands was obtained with an UltroScan XL laser densitometer (Pharmacia-LKB).

Hydrolysis of PCr by Prostate Acid Phosphatase. PCr (2.5 mM) in 1.0 ml of 0.1 M triethanolamine buffer (pH 7.8) was incubated at 37°C with 8 units of human prostate acid phosphatase [phosphoric monoester hydrolase (acid optimum), EC 3.1.3.2; Calbiochem] at pH values discussed in

Results and Discussion. Samples (50 μl) were withdrawn at 5-min intervals and Cr was determined by colorimetry (17).

RESULTS AND DISCUSSION

PCr, Cr, and ATP Levels in SVF and SV. SV and SVF from mouse and rat contain PCr (Table 1). The high concentration of PCr in the secretory fluid is particularly impressive. The PCr concentration in mouse SVF is comparable to that found in cardiac muscle (19, 20) and brain (4, 20). The levels of extracellular PCr in both species are higher than those found inside smooth muscle cells (5–7). The major portion of the PCr content of the gland is in the SVF (Table 1). The Cr concentrations in mouse and rat SVF are 100 and 65 times greater than the concentration of Cr in blood. The concentrations of Cr retained by the cells in the two species correspond to about 70 and 25 times the blood level. These results demonstrate the ability of the cells of the seminal vesicles to accumulate extraordinarily high levels of Cr against unfavorable concentration gradients as well as the capacity to release a major fraction into the vesicular fluid in the lumen of the gland. More surprising, PCr, formed by the intracellular phosphorylation of Cr by ATP, is also released into the secreted fluid. To our knowledge, synthesis and export of a high-energy compound at concentrations matching and even exceeding the intracellular concentration has not been reported for other tissues. The concentration of ATP in SVF is much lower than that of PCr. The PCr/ATP ratios are about 40 and 20 in the mouse and rat, respectively. Only traces of ADP were detected in the SVF.

³¹P NMR Spectra of the Soluble Fractions of SVF. The ³¹P NMR spectra support the results of the biochemical analysis and provide a more complete picture of all major phosphorus compounds in the SVF. Profiles of the phosphorus compounds detectable by ³¹P NMR spectroscopy in the perchloric acid extracts of SVF from mouse and rat (Fig. 1 A and B, respectively) demonstrate that PCr is a major component of the SVF in both species. In fact, PCr is by far the most abundant soluble phosphorus compound in mouse SVF. Other compounds, tentatively identified by their chemical shifts (4), include glycerophosphocholine, glycerophosphoethanolamine, P_i, phosphocholine, glycerol 2,3-bisphosphate, fructose 1,6-bisphosphate, and glycerol 1-phosphate. The presence of glycerophosphocholine and phosphocholine, but not of PCr, in the secretions of the reproductive system of several species has been reported (21, 22). Except in SVF to which exogenous P_i was added as a marker (as in Fig. 1C), the intensity of the P_i peak is low relative to that of PCr. The absence of resonance peaks corresponding to ATP (and ADP) confirms the results of the fluorometric analyses (Table 1), which showed the much lower concentration of ATP. Stable levels of ATP after reaction with CK and ADP (Fig. 1 C and D) establish that ATP and ADP are not destroyed by unknown factors (e.g., nucleotidases) in the SVF preparation. Thus the low value of ATP relative to PCr (Table 1) represents the *in situ* profile of these two high-energy phosphates. The low intensity of the P_i peaks in the SVF shows that Cr in the SVF does not result from the hydrolysis of PCr. In terms of the compounds involved in energy metabolism, the SVF in mouse and rat is characterized by exceptionally high concentrations of PCr and Cr, a modest level of ATP as well as of P_i, and only traces of ADP.

NaDodSO₄/PAGE of Proteins in the SVF and SV. The NaDodSO₄/PAGE pattern of mouse SV (Fig. 2A) shows the presence of numerous protein bands distributed over the entire molecular mass range as would be expected for total cell proteins. The electropherogram of the SVF (Fig. 2B) shows six prominent bands (70, 42, 40, 16, 15, and 13 kDa) and a minor band (8–9 kDa). Quantitation by densitometry demonstrated that the seven bands accounted for 95% of the

Table 1. PCr, Cr, ATP, ADP in SVF and SV of mice and rats

Source	PCr			Cr			ATP			ADP, nmol/mg	Wet weight, mg
	Conc.,* nmol/mg	Total nmol	% of total	Conc.,* nmol/mg	Total nmol	% of total	Conc.,* nmol/mg	Total nmol	% of total		
Mouse											
SVF	5.64 ± 1.38	384.1	90.4	22.79 ± 3.06	1552.0	74.9	0.13 ± 0.04	8.9	52.7	<0.07	68.1 ± 17.2
SV	1.59 ± 0.75	40.8	9.6	14.78 ± 4.25	453.9	25.1	0.23 ± 0.06	7.1	47.3	0.30 ± 0.03	30.7 ± 2.7
Rat											
SVF	2.24 ± 0.14	453.4	77.6	12.96 ± 1.51	2623.1	74.0	0.12 ± 0.02	24.3	43.6	<0.07	202.4 ± 54.0
SV	0.76 ± 0.09	130.9	22.4	5.34 ± 1.04	920.0	26.0	0.18 ± 0.02	31.0	56.4	0.19 ± 0.03	172.3 ± 47.3

SVF was prepared from seminal vesicles immediately after removal of the gland and frozen in liquid N₂. Perchloric acid extraction and neutralization with KOH were done as described (14). PCr, ATP, and ADP were determined by the direct fluorometric method (14). Cr was assayed colorimetrically (17).

*Mean ± SD, *n* = 15.

total protein content of SVF. Previous studies (23, 24) established that the high protein content (30%, wt/vol) of SVF in mouse and rat is composed of 6–8 proteins actively secreted by the SV epithelium. NaDodSO₄/PAGE of our rat SVF preparations revealed five major bands. The absence of significant amounts of intracellular proteins and the abundance of secretory proteins in the SVF preparation argue against cytolysis being a major source of the metabolite profile in this fluid. This was supported by DNA measurements (results not shown), which demonstrated that >85% of the total DNA was retained in the SV preparation. We have found that PCr in the secreted fluid varies in parallel with

changes in the known androgen-dependent secretory proteins of the epithelium (unpublished results). Thus it appears that the PCr in SVF is a product of secretion by the epithelial cells of the gland.

PCr and Cr Levels in the Testes. The levels of PCr and Cr found in the rat and mouse testes are shown in Table 2. Sea urchin sperm have been shown to contain PCr (9, 10). We have found that sperm released from the mouse caudal epididymis contain 30–80 nmol of PCr per 10⁸ cells (unpublished results). It is likely that the epithelial cells of the seminiferous tubules and smooth muscle cells in the testes may account for some of this PCr. The most striking feature of the results in Table 2 are the concentrations of free Cr,

FIG. 1. ³¹P NMR spectra of SVF. (A) Spectrum of perchloric acid extract (pH 9.0) of mouse SVF. (B) Spectrum of perchloric acid extract of rat SVF. (C) SVF pooled from five mouse glands, diluted with 0.1 M triethanolamine (pH 7.8) to simulate the concentration of PCr in A (2.2 mM), was incubated with Mg(OAc)₂ (16 mM), ADP (2 mM), and rabbit muscle CK (100 μg/ml) at 25°C for 30 min. Na₂HPO₄ was added as a marker before scanning. (D) A neutralized extract of rat SVF treated with Mg(OAc)₂, ADP and CK as for C. Peaks were identified on the basis of chemical shifts (ppm, scale at bottom of each panel): 1, PCr (set at 0.00); 2, glycerophosphocholine (2.99); 3, glycerophosphoethanolamine (3.80); 4, P_i (5.75); 5, phosphocholine (6.44); 6, glycerol 2,3-bisphosphate (7.03); 7, fructose 1,6-bisphosphate (7.22); 8, glycerol 1-phosphate (7.41). Peaks due to the α and β (and γ) phosphates of ADP (ATP) are indicated.

FIG. 2. NaDodSO₄/PAGE of proteins solubilized from mouse SV (A) and SVF (B). Samples containing 8 μg of protein were loaded. Densitometer tracings were obtained by laser scanning at 633 nm.

Table 2. PCr and Cr in mouse and rat testes

Species	$\mu\text{mol/g}$ wet weight	
	PCr	Cr
Mouse	1.99 ± 0.2 ($n = 10$)	12.5 ± 0.8 ($n = 10$)
Rat	0.79 ± 2.0 ($n = 5$)	11.2 ± 2.9 ($n = 12$)

PCr was determined by direct fluorometric assay (14) of perchloric acid/KOH extracts of frozen testes. Cr was assayed by colorimetry (17). Values are means $\pm$ SD.

which are higher than those of Cr in skeletal muscles (17, 25, 26). A previous study (27) reported that testes from albino rats and mice (of unspecified age and strain) contained 250 and 275 mg of Cr per 100 g wet weight, which corresponds to 19–21 $\mu\text{mol/g}$. The data for free Cr in Table 2 were obtained by the colorimetric method (17). To avoid possible artifacts of nonspecificity of the 1-naphthol/2,3-butanedione reaction, the more specific CK reaction was carried out on rat testis (Table 3). After treatment with Mg-ATP and CK, an average of 45 μg out of 65 μg of the 1-naphthol-positive compound had disappeared. This decrease was accompanied by a corresponding increase in PCr as determined by HPLC (65.2 ± 6.1 μg of PCr was recovered in the six experiments shown in Table 3, representing a yield of 94%). These results confirm the exceptionally high values of Cr in the testes. Similar results were obtained with neutralized extracts from SV and SVF.

Cr in the Testes Is Extracellular. The separation of the testicular fluids from the cellular components was accomplished under mild conditions. After two washes of the tissue slices with ice-cold phosphate-buffered saline, >90% of the total Cr was found in the soluble fraction (Fig. 3). The two soluble fractions (S1 and S2) were monitored for CK activity by spectrophotometric measurement of the reverse reaction as described (8). No significant CK activity was found in either of the two soluble fractions, S1 and S2, indicating that there was no leakage of the enzyme from damage of the tissue-slice membranes. The presence of 90% or more of the total testicular Cr in the soluble fractions suggests that most of the Cr in testes is in the extracellular fluid.

Rates of Hydrolysis of PCr by Prostate Acid Phosphatase. Under physiological conditions SVF mixes to varying degrees with secretions from the prostate and other accessory sex glands before becoming part of the seminal plasma-sperm system. Since prostatic fluid is particularly rich in acid phosphatase, we studied the action of a partially purified human enzyme on PCr. The initial rates of hydrolysis of PCr, under our experimental conditions, were 2.4×10^{-2} , 2.7×10^{-2} , and 7.9×10^{-2} nmol/min at pH 7.5, 7.0, and 6.0, respectively. The pH range (7.5–6.0) is probably similar to the *in vivo* pH, since the nearly neutral SVF progressively mixes with the slightly acidic prostatic secretion and distinctly acid vaginal fluid. A previous study (28) has shown

Table 3. Enzymatic determination of putative Cr in rat testis extracts

Sample	Cr, $\mu\text{g/ml}$	
	– CK	+ CK
1	64.6	18.9
2	58.0	19.6
3	62.5	20.7
4	67.7	19.1
5	61.3	22.5
6	67.3	21.7
Mean $\pm$ SD	63.5 ± 3.7	20.4 ± 1.5

Samples of testis extracts containing ≈ 65 μg of Cr were treated with 10-fold excess Mg-ATP and incubated for 30 min at 25°C with or without CK. Cr was assayed by colorimetry (17).

FIG. 3. Distribution of Cr in rat testes. Tissue slices (≈ 2 mm³) were obtained from fresh unfrozen rat testes and extracted with ice-cold phosphate-buffered saline. Two successive supernatants (S1 and S2) were obtained by centrifugation. The residue (P) after the second wash was extracted with perchloric acid and neutralized with KOH. Cr was determined by colorimetry (17).

that acid phosphatases can catalyze the transfer of the phosphoryl group in PCr to water or to an acceptor like glucose. The rates of hydrolysis of the N~P bond under our experimental conditions, however, are <1% of the rates reported for the X-O-P class of substrates (29).

Sperm as Target Cells for Extracellular PCr and Cr. The secretion of large amounts of a high-energy phosphate by an accessory sex gland is intriguing. In view of the fact that SVF constitutes 60–70% of the seminal plasma (22) and that the mating cycle of the rodent is governed by circadian rhythms, the frequent loss of substantial PCr is a major drain on the epithelial-cell energy metabolism. No role for extracellular PCr and related compounds is known. We suggest that the target cells for the extracellular phosphagen are the sperm. Available evidence leads to two possible functions in support of this view.

In sea urchin sperm, PCr has been shown to play a key role in transporting the metabolic energy generated by the mitochondria to the dynein ATPase sites in the distal regions of the tail, where ATP is regenerated by a CK-mediated reaction (10). Increased motility and velocity of human sperm in response to added PCr by unspecified mechanisms have been claimed (30). This present study has demonstrated that the developing sperm in the testis are surrounded by high levels of creatine. During the subsequent passage of the sperm through the epididymis, most of the testicular fluid is reabsorbed (see ref. 31 for review). The secretion from the seminal vesicle, which constitutes the major portion of the fluid component of semen, can essentially restore the milieu of high Cr to the sperm by virtue of its high Cr content and by slow release of Cr by the action of prostatic phosphatase on PCr. The maintenance of a high Cr gradient across the sperm membrane could ensure an adequate supply of Cr. Increases in the rate of uptake and in the intracellular level of Cr in response to high extracellular Cr have been shown in ascites tumor cells (32) and in muscle (25, 33).

A second possible function for the extracellular PCr is as an energy source for cell-surface phosphorylations of proteins in the sperm membrane. Extracellular protein kinases ("ectokinases") have been found in rat, ram, and human sperm (34–36). Such kinases have been reported to utilize ATP secreted by neurons, chromaffin cells of the adrenal medulla, and platelets (37). CK, known to be present in the

seminal plasma (38), can catalyze the regeneration of ATP for sperm surface phosphorylations.

We thank Professors Samuel K. Chacko, Vincent J. Cristofalo, Robert E. Davies, and Bayard K. Storey for helpful comments in preparing the manuscript and Su Wu for excellent technical assistance. This research was supported by Grant BRSG S07 RR05464 awarded by the Biomedical Research Program of the National Institutes of Health, by Grant ME-4525 from the Pennsylvania Department of Agriculture, and by National Institutes of Health Grant DK37027.

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