The Role of Adenine Nucleotides and the Effect of Caffeine and Dibutyryl Cyclic AMP on the Metabolism of Guinea Pig Epididymal Spermatozoa (37604)

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Spermatozoa taken from various segments of the guinea pig epididymis have been shown to differ markedly in the rates of *in vitro* energy metabolism (1). Glycolysis is low in caput sperm but increases progressively in more distal segments with the sharpest rise occurring in sperm taken from the cauda section. *In vitro* motility also increases progressively and in parallel with changes in metabolism. In view of work from other laboratories suggesting that sperm metabolism in several mammalian species is under nucleotide control (2) and that sperm motility is linked to adenyl cyclase activity (3–6), we have attempted to assess the role of intracellular adenine nucleotides and cyclic nucleotides on these progressive changes in guinea pig epididymal sperm metabolism and motility. Our results show that large changes in nucleotide levels in sperm occur during epididymal transit but that these changes only partially account for the magnitude of the metabolic changes observed. We also conclude that the effect of cyclic nuleotide analogs and phosphodiesterase inhibitors on sperm are not restricted to a stimulation of motile mechanisms alone but also involve a direct stimulation of metabolism.

Methods and Materials. Adult guinea pigs (Carworth, New City, NY), weighing approximately 1 kg, were used in all the experiments.

The treatment and isolation of sperm from different segments of the epididymis was described elsewhere (1). A Tris-based salts medium, buffered at pH 7.4, was used in all experiments (7).

Experiments were performed in stoppered 25-ml Erlenmeyer flasks. Incubations were carried out with shaking in a thermostated Dubnoff bath at 37°. Drugs and glucose were added to the incubated suspensions at concentrations described in the Results section. Small portions of the suspensions were taken at various times to determined motility. Aliquots of the sperm suspensions were removed from the flasks at selected intervals, deproteinized immediately by boiling for 10 min, and centrifuged and the clear solution was frozen until assay.

Lactic acid was determined fluorometrically in a Turner Model 111 (8). ATP, ADP and AMP were also determined by standard fluorometric procedures (9). Standard ATP, ADP and AMP were run concurrently with each assay.

Energy charge (EC) as defined by Atkinson and Walton (10) was calculated according to the equation:

$$EC = \frac{ATP + \frac{1}{2} ADP}{ATP + ADP + AMP}.$$

Materials. Chemicals used were either Sigma Grade or Baker reagent grade. Purified enzymes were purchased from the Boehringer-Mannheim Co.

Results. The intracellular levels of adenine nucleotides isolated at 0° in sperm taken from various segments of the epididymis and vas deferens of the guinea pig are shown in Fig. 1. The highest levels were found in caput sperm where the nucleotide concentration averaged a massive 160 nmoles/10^8 sperm. Levels in corpus and cauda sperm were about 25 and 15% of the caput level, respectively. ATP also decreased sharply in sperm taken from segments increasingly dis-

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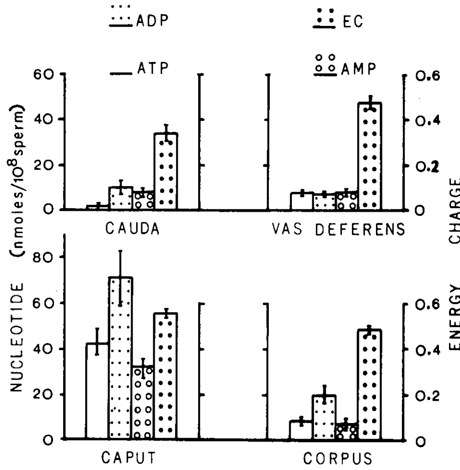

FIG. 1. The concentrations of ATP, ADP, AMP and the calculated energy charge of sperm isolated at 0° from different parts of the epididymis. Values are mean ± SE of 5 estimations.

tal to the caput epididymis. Energy charge, which is a measure of the fraction of the adenylate pool containing high energy phosphate bonds (10), decreased in parallel, but less markedly than either the total nucleotide or ATP concentrations. A slight increase in energy charge occurred in sperm taken from the vas deferens.

In order to determine whether there was any relation between these nucleotide changes, particularly ATP, and changes in the glycolytic rate, sperm from the various segments were incubated in the absence of glucose for periods up to 6 hr in order to reduce nucleotide levels. The rate of glycolysis was then determined after each hour of glucose deprivation. The results of these experiments are shown in Fig. 2. A dependence of glycolysis on cellular ATP concentration is most apparent in caput sperm. During the 6 hr of incubation in the absence of glucose, ATP levels fell nearly tenfold. Glycolysis increased with the fall in ATP and after 4 hr, sperm were capable of metabolizing glucose at almost four times the initial rate. Several hours of substrate deprivation generally induced increases in glycolysis in sperm taken from the other segments, but the relation between ATP concentration and glycolysis was not as striking. Extending the period in which cells were deprived of glucose for more than 3 hr, abolished glycolysis in cauda and vasa sperm. Sperm from other segments, with higher initial levels of ATP, were able to tolerate longer periods of substrate deprivation. Apparently as the intracellular level of ATP approached 5–10 nmoles/10^8 sperm (as the ATP curves in all four segments indicate), the difference between the ATP requirement for hexose phosphorylation and the inhibitory effects of this compound become critically small. Cell death may occur if ATP levels fall below this level, while higher levels increasingly inhibit the ability of cells to metabolize glucose at high rates. This appears to be the metabolic state of sperm just prior to ejaculation.

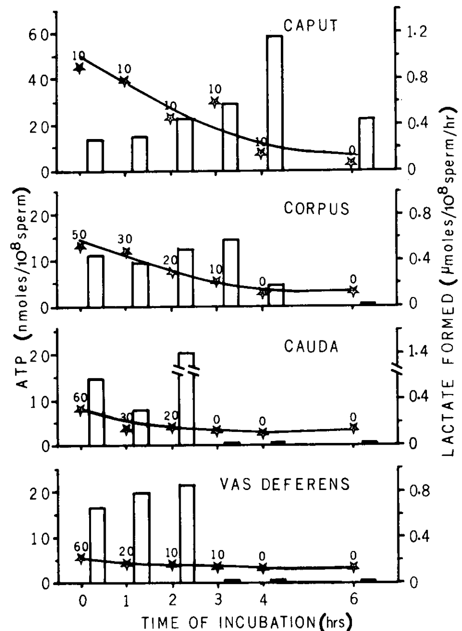

FIG. 2. Effect of glucose deprivation (0 to 6 hr) on the levels of ATP and the glycolytic capacity of sperm from different segments of the epididymis. Glycolysis was induced after each period of preincubation by adding glucose (10 mM). The sperm suspensions were then incubated for an additional 2 hr. Sperm concentration per flask were as follows: caput, 30 × 10^6 sperm/cc; corpus, 89 × 10^6 sperm/cc; cauda, 90 × 10^6 sperm/cc and vas deferens, 45 × 10^6 sperm/cc. ★-★ ATP concentration at the end of each period of preincubation in the absence of substrate. The numbers indicated at each point represent percentage motile sperm. Histograms: lactic acid production.

Additional observations in these experiments, also shown in Fig. 2, are that no apparent relation exists between changes in the glycolytic rate and the motility of sperm taken from any particular segment. Despite the marked increases in glycolysis at the lower ATP levels in caput sperm, for example, motility remained low and did not increase above 10%.

The initial motility of sperm taken from other segments was higher than that of caput sperm but motility declined progressively in the absence of substrate and did not increase above the initial level regardless of an increase in the glycolytic rate.

Motility and glycolysis, however, were enhanced in all segments by caffeine or dibutyryl cyclic AMP (dbcAMP). Caffeine was slightly more effective than the cyclic AMP analog in inducing these changes but maximum changes were obtained when the two compounds were added together to cell suspensions. The effects of these compounds on several aspects of energy metabolism in guinea pig epididymal sperm are detailed in Table I. In order to obtain the maximum effect of these compounds on metabolism and motility, sperm were incubated for 1 hr in the presence of caffeine and dbcAMP prior to the addition of glucose. Glycolytic capacity and ATP levels were not further changed by extending the preincubation period beyond this time. The important results in Table I are seen by comparing, vertically, changes in the various parameters in sperm taken from the various segments treated in the same manner, and by comparing, horizontally, changes in sperm taken from the same segment but given either glucose or glucose, caffeine and dbcAMP. First, comparisons among segments for both the control and treated cells recapitulates the relation between ATP, motility and glycolysis in each segment of the epididymis. This comparison also shows that, in both control and treated cells, there was a correlation between the initial energy charge (i.e., the energy charge prior to the addition of glucose) and the glycolytic rate. Glycolysis tended to be higher at lower initial values of the energy charge. The addition of glucose restored the energy charge in most instances to values between 0.6 and 0.8. Hoskins (2) has found a similar correlation in bovine epididymal sperm

Second, comparisons between control and treated cells show that glycolysis and motility were markedly increased by caffeine and dbcAMP in sperm taken from all segments of the epididymis. Glycolysis increased nearly fourfold in caput sperm; motility increased sixfold and was comparable to the maximum percentage motility observed in sperm from other segments. Similar increases in glycolysis occurred in sperm taken from the other epididymal segments, while changes in vasa sperm were less marked.

Further inspection of Table I reveals additional information. Consider the energy charge that was generated in sperm after the addition of glucose in control and treated cells. Comparisons of this parameter in sperm from all segments of the epididymis shows that there was either no change or an increase in energy charge in cell induced to high rates of glycolysis by caffeine and dbcAMP. This suggests that the increased rate of glycolysis in treated cells was not solely a secondary event induced by increased motility and any concomitant lowering of energy charge, but was also a result of the direct stimulation of the glycolytic apparatus.

Finally, it should be observed that although glycolysis is increased nearly fourfold by caffeine and dbcAMP in sperm taken from all segments of the epididymis, the capacity for glycolysis was still progressively higher in sperm taken from segments increasingly distal to the caput segment. This is true despite the fact that internal levels of ATP and energy charge were similar in treated cells after the addition of glucose. This indicates that other factors, in addition to intracellular nucleotide levels, are involved in the development of epididymal sperm metabolism.

Discussion. The results of these experiments support the idea that intracellular nucleotide levels play an important role in the control of the metabolism and motility of epididymal spermatozoa. The massive nucleotide concentrations in caput sperm may serve as a store of chemical energy that ensures sperm survival during the 10–15 day period of epididymal transit. Metabolism

TABLE I. Effect of a Combination of Caffeine (10 m*M*) and dbcAMP (2 m*M*) on the Levels of ATP, Energy Charge, Motility and Glycolysis of Sperm from the Segments of the Epididymis.[f]

		Buffer					+ Caffeine + $(Bt)_2$-c-AMP				
		n	ATP (nmoles/10^8 sperm)	Energy charge	Motility (%)	ZL	*n*	ATP (nmoles/10^8 sperm)	Energy charge	Motility (%)	ZL
Caput	Initial value	7	106.9 ± 20.2[e]	0.78 ± 0.03	10		5	142.0 ± 10.7	0.85 ± 0.02	30	
	1 hr preincubation	5	28.8 ± 8.4	0.54 ± 0.07	10		4	24.1 ± 9.6	0.32 ± 0.09	30	
	2 hr glycolysis	5	54.7 ± 19.7	0.69 ± 0.08	10	0.460 ± 0.036	4	91.3 ± 10.0	0.64 ± 0.06	60	1.66 ± 0.291[b]
Corpus	Initial value	7	46.2 ± 10.9	0.66 ± 0.08	50		5	57.8 ± 8.2	0.75 ± 0.06	60	
	1 hr preincubation	5	18.4 ± 6.8	0.48 ± 0.09	40		4	12.8 ± 2.4	0.36 ± 0.06	70	
	2 hr glycolysis	5	39.1 ± 14.8	0.70 ± 0.09	30	0.590 ± 0.057	4	70.9 ± 18.3	0.81 ± 0.03	70	2.54 ± 0.192[a]
Cauda	Initial value	7	36.4 ± 9.8	0.46 ± 0.07	50		5	38.6 ± 11.2	0.46 ± 0.07	60	
	1 hr preincubation	5	4.7 ± 1.7	0.16 ± 0.03	30		4	3.8 ± 0.7	0.13 ± 0.03	25	
	2 hr glycolysis	5	61.2 ± 30.1	0.63 ± 0.11	50	1.18 ± 0.24	4	105.5 ± 25.5	0.80 ± 0.07	70	5.02 ± 1.07[b]
Vas deferens	Initial value	7	9.2 ± 1.4	0.36 ± 0.04	50		4	9.9 ± 0.4	0.40 ± 0.02	50	
	1 hr preincubation	5	4.5 ± 0.9	0.22 ± 0.04	20		3	1.5 ± 0.02[c]	0.06 ± 0.01[d]	10	
	2 hr glycolysis	5	15.2 ± 0.5	0.52 ± 0.11	30	1.050 ± 0.117	2	31.0 ± 1.0[a]	0.58 ± 0.08	60	1.89 ± 0.16

[a] Significantly higher from the buffer level by $p < 0.001$.
[b] Significantly lower from the buffer level by: $p < 0.01$; [c] $p < 0.05$; [d] $p < 0.001$.
[e] m ± SE.
[f] The sperm suspensions were prepared at room temperature and then incubated for 1 hr in the presence of caffeine and dbcAMP. Glycolysis was then induced by adding glucose (10 m*M*). ZL = lactic acid production (μmoles/10^8 sperm/hr). *n* = no. of estimations.

probably occurs at a very slow rate, if at all, *in situ* in view of the absence of appreciable quantities of utilizable carbohydrate (11) and since the diffusion of oxygen into the densely packed cells of the epididymal duct is probably also restricted. High intracellular levels of ATP would obviate the need for any extensive metabolic activity. Events occurring during epididymal passage reduce sperm nucleotides to near critical levels in the cauda epididymis and vas deferens. These low nucleotide levels appear to raise the cells potential for a maximum glycolytic response to the carbohydrates that become available in seminal plasma at ejaculation. However, survival of these cells in the absence of ejaculation is not likely to be long since further depletion of the already low nucleotide reserves abolishes the ability of these cells to respond to the glycolyzable substrates. This may account for the generally poor motility and metabolic capacity of vasa sperm as compared to sperm taken from the cauda epididymis.

The motility of guinea pig epididymal sperm does not appear to entirely depend on the glycolytic rate of developing epididymal sperm since inducing high rates of glycolysis by lowering nucleotide levels (in the absence of caffeine or dbcAMP) did not stimulate motility. However, since motility in caput sperm could be stimulated almost maximally by increasing the intracellular levels of cyclic AMP, it is probable that the basic apparatus responsible for sperm motility is fully developed in caput sperm. Further increases in the spontaneous motility of epididymal sperm in other segments may, therefore, simply reflect an increased activity of adenyl cyclase in these cells. If this is true, increasingly higher levels of cyclic AMP should be found in sperm as they approach the cauda segment. This is currently being tested.

The role of adenine nucleotides in bovine epididymal sperm metabolism has been discussed in a recent report by Hoskins (3). Based on a correlation between the glycolytic rate and the energy charge in these cells prior to the addition of glucose, Hoskins suggested that glycolysis was predominantly under nucleotide control. High energy charge (i.e., a high portion of the adenylate pool contains phosphoanhydride bonds) slows glycolysis since nucleotide inhibitors of key glycolytic enzymes are present in high concentration (ATP, ADP) and activators (AMP) are present in low concentration. The stimulation of glycolysis by cyclic AMP analogs and phosphodiesterase inhibitors was considered to be due to a lowering of the energy charge that resulted from a direct stimulation of motility and the associated increase in ATPase activity.

However, our experiments on the effect of caffeine and dbcAMP on guinea pig epididymal sperm suggest that increased levels of cyclic AMP have a direct effect on both metabolism and motility. Our argument is based on the observation that cells from the same segment of the epididymis develop approximately the same energy charge and total nucleotide concentration in the presence of glucose whether they have been exposed to caffeine and dbcAMP or not. The glycolytic rate in treated cells, however, is almost fourfold greater than the rate of untreated cells. It is known that cyclic AMP is an activator of phosphofructokinase (12) and that phosphofructokinase is involved in the control of glycolysis of several species of mammalian sperm (2, 13). This may be the site stimulated by cyclic AMP in guinea pig serum.

As already mentioned, the results reported here strongly suggest that adenyl cyclase is involved in the changes in *in vitro* sperm metabolism that occur during epididymal passage. It is apparent, however, that other mechanisms are also involved. We noted that even when epididymal sperm were induced to maximum motility and glycolysis by caffeine and dbcAMP, the rate of cauda sperm glycolysis was still some three times greater than the rate of glycolysis in caput sperm. This could not be attributed to differences in nucleotide levels or energy charge. These differences may be due to other factors such as changes in the catalytic capacity of key glycolytic enzymes during epididymal passage or to a change in the kinetic responsiveness of these enzymes to controlling ligands. The nature of these other factors is currently being investigated.

Summary. High levels of adenine nucleo-

tides are present in sperm taken from the caput segment of the guinea pig epididymis. This level declines progressively in the corpus and cauda segments where there is also a progressive increase in *in vitro* glycolysis. Nucleotide control of glycolysis is suggested by the observation that the depletion of nucleotide pools by incubation in the absence of substrate leads to increased glycolytic rates. This is particularly pronounced in caput sperm. Sperm motility in different segments, however, is not enhanced by lowering nucleotide levels in this manner.

Sperm motility and metabolism are markedly increased in all segments of the epididymis and vas deferens by caffeine and dibutyryl cyclic AMP. High rates of glycolysis in treated cells occur, however, without any significant differences from controls in energy charge and ATP levels, suggesting that the glycolytic apparatus as well as the mechanisms involved in motility are stimulated. Although metabolism in sperm from all segments increased about fourfold in treated cells, the glycolytic rate of sperm taken from segments increasingly distal to the caput segment shows the same pattern of increasing glycolytic capacity as observed in controls. This suggests that other mechanisms, in addition to changes in adenyl cyclase activity, are apparently involved in the changes in sperm metabolism that occur during epididymal transit.

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