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The Stimulation of Bovine Epididymal Sperm Metabolism by Cyclic Nucleotide Phosphodiesterase Inhibitors[1]

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Bovine epididymal spermatozoan motility and respiration are enhanced by cyclic nucleotide phosphodiesterase inhibitors such as caffeine (Garbers *et al.*, 1971). This report deals with additional aspects of the relationship of these cyclic nucleotide phosphodiesterase inhibitors and bovine epididymal sperm metabolism. Caffeine induced elevated cyclic-3′,5′-adenosine monophosphate (cyclic AMP) concentrations within 1 min after addition, with either endogenous substrate or 10 m*M* fructose as substrate. Although the stimulation of motility was rapid, the maximal respiratory rate with either pyruvate or glucose as substrate did not occur until 1 to 4 min. After incubation of sperm with caffeine for a period of time, no respiratory lag occurred on addition of pyruvate. With fructose as substrate, ATP concentrations declined significantly between 3 to 5 min after addition of caffeine, whereas ATP concentrations were significantly reduced by 2 min in the absence of exogenous substrate. ADP and AMP concentrations increased as ATP decreased, resulting in a marked decline in the phosphate potential. Bongkrekic acid (BKA), an inhibitor of mitochondrial adenine nucleotide translocase, blocked sperm motility and respiration with acetate or pyruvate as substrate, whereas it had no effect on motility in the presence of glucose. Caffeine was capable of stimulating motility in the presence of glucose and BKA. Caffeine also increased glucose utilization rates, and lactate accumulation from glucose, in the absence or presence of BKA. Pyruvate utilization was either not affected or was reduced by caffeine, whereas lactate accumulation from pyruvate was consistently reduced.

These results suggest a general stimulation of sperm metabolism by cyclic nucleotide phosphodiesterase inhibitors. The increase in sperm motility and cyclic AMP is rapid and precedes any significant decline in ATP concentrations or increases in respiratory rate. The data suggest that the fall in ATP concentrations, and therefore the decline in "phosphate potential," is a causal factor responsible for the glycolytic and respiratory rate enhancement observed in sperm treated with cyclic nucleotide phosphodiesterase inhibitors.

INTRODUCTION

Various cyclic nucleotide phosphodiesterase inhibitors, such as caffeine, markedly stimulate bovine epididymal sperm motility (Garbers *et al.*, 1971a; Drevius, 1972) and respiration (Garbers *et al.*, 1971a) and

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bring about a decreased cellular concentration of ATP. We later showed that ejaculated bovine sperm motility was both enhanced and maintained by these phosphodiesterase inhibitors (Garbers *et al.*, 1971b). This article describes other metabolic effects of the phosphodiesterase inhibitors and suggests an order to these various responses.

MATERIALS AND METHODS

Bovine epididymides were obtained from Oscar Mayer and Company, Madison, Wisconsin and were flushed of sperm by techniques previously described (Garbers *et al.*, 1971a). The buffer used in these experiments was a modified Krebs–Ringer buffer (120 m*M* NaCl, 5 m*M* KCl, 10 m*M* KH_2PO_4, 5 m*M* $MgSO_4$, and 10 m*M* Tris(Cl) at pH 7.2). The sperm were washed twice in this buffer by centrifugation at 500*g* for 4 to 5 min. All operations were at 0°–2°C.

In experiments using the Gilson oxygraph, collodion-covered vibrating platinum electrodes were used, with a final sperm concentration of approximately 2.5×10^8 cells/ml in a total volume of 2 ml. The incubations were at 30°C.

Pyruvate and glucose utilization and lactate production were studied in 15-ml Erlenmeyer flasks shaken in a water bath at 37°C. Sperm concentrations ranged from $(4–5) \times 10^8$/ml. Pyruvate and lactate were measured in perchloric acid extracts neutralized to pH 7, according to methods by Bergmeyer (1963) using lactate dehydrogenase. Glucose was measured using the glucose oxidase method described by Boehringer–Mannheim.

We obtained ^{3}H cyclic AMP from New England Nuclear. The various enzymes used in the assays were obtained from Boehringer–Mannheim and the substrates from Sigma or Cal-biochem. Purified cyclic nucleotide phosphodiesterase from beef heart was a gift from Dr. W. D. Lust.

NUCLEOTIDE ASSAYS

Spermatozoa (between 10^8 to 10^9 sperm/ml) were incubated in the modified Krebs–Ringer buffer in a 1-ml total volume at 30°C on a shaking water bath in 15-ml Erlenmeyer flasks. The reactions were stopped by addition of either 0.5 or 1.0 ml of 20% TCA and the flask's contents transferred to a centrifuge tube and chilled on ice. The nucleotide extractions were then completed as described previously using cold, H_2O-saturated ether (2°C) to extract the TCA (Garbers *et al.*, 1971a).

ATP, ADP, and AMP were determined on the neutralized TCA extracts by the methods described by Bergmeyer (1963) using a spectrophotometer at 340 nm. Hexokinase and glucose 6-phosphate dehydrogenase were used for measurement of ATP, while lactate dehydrogenase, pyruvate kinase, and myokinase were used to measure ADP and AMP.

Cyclic AMP was assayed by modifications of the Walton and Garren (1970) and Gilman (1970) protein-binding assays. The assay procedure we developed is described in greater detail in the Ph.D. dissertation from which this paper is derived (Garbers, 1972), but part of the procedure will be presented in this paper.

To collect the cyclic AMP binding protein, epididymal sperm were collected and washed as usual. The final pellet was resuspended in 50 m*M* Tris(Cl), 1 m*M* dithiothreitol (DTT) at pH 7, then sonicated at full amperage on a Branson Sonifier, Model LS-75, for 0.5–1.0 min at three different intervals. Centrifugation at 15,000*g* for 30 min followed by centrifugation of the supernatant fluid at 85,500*g* for 1 h resulted in a clear supernatant fluid containing the crude soluble binding protein. This supernatant fluid was dialyzed against 50 m*M* Tris(Cl), 1 m*M* DTT at pH 7 for approximately 12 h and applied to a DEAE-Sephadex (A-50) column (2.5×38 cm), which had been equilibrated with the same buffer. After washing the column with 50 to 100 ml of the buffer, a linear gradient of 250 ml of 50 m*M* Tris(Cl), 1 m*M* DTT at pH 7 in the mixing chamber, and 250 ml of 50 m*M* Tris(Cl), 1 m*M* DTT, and 0.5 *M* KCl at pH 7 in the opposite chamber was started. Two peaks of binding activity were consistently eluted (Fig. 1). The fractions comprising each peak were pooled separately and used in the binding assay.

The assay was run at pH 6. Although 1 m*M* free ATP did not seriously interfere with cyclic AMP binding as reported by

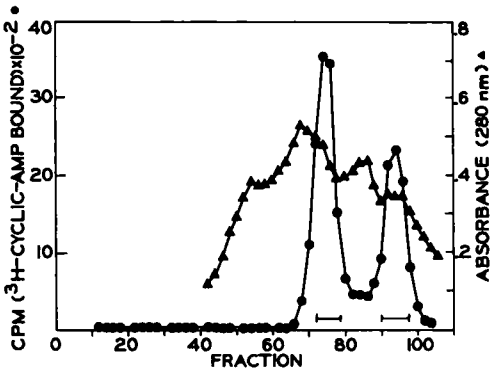

FIG. 1. Elution of the cyclic AMP binding proteins from DEAE-Sephadex. Fractions of 4.5 ml were collected from the column as described under nucleotide assays. In the assay for cyclic AMP binding, 15 μl of column effluent, 0.21 ml of 200 m*M* EDTA, 50 m*M* acetate buffer at pH 6, and 32.7 pmoles of ^{3}H cyclic AMP (14,500 cpm) in a volume of 25 μl were incubated on ice for about 1 h. These samples were then washed over millipore filters with 10 ml of 200 m*M* EDTA, 50 m*M* acetate at pH 6.

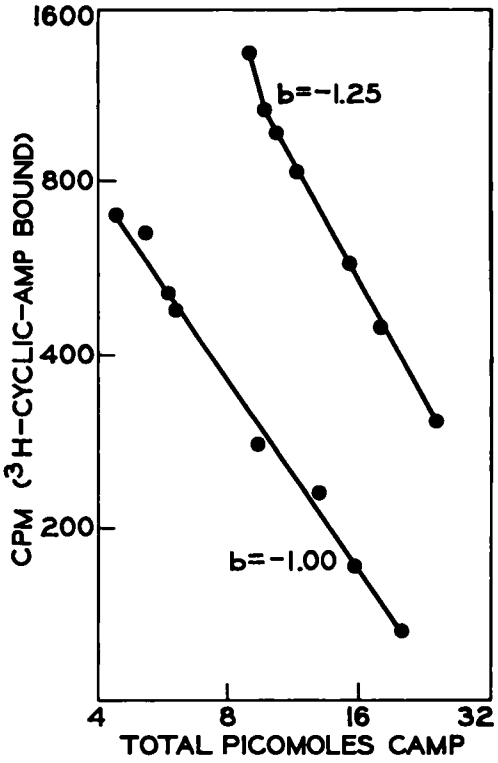

FIG. 2. Standard curves representing the means of standard curves run over a 6-month period. The top curve represents the line obtained when using 9.08 pmoles of ^{3}H cyclic AMP, and the bottom curve when using 4.54 pmoles ^{3}H cyclic AMP per assay tube. The assay was run as described in the text. The "b" values represent the slopes of the experimental curves.

Gilman (1970), ATP (Mg^{2+}) did interfere. EDTA (168 m*M*) eliminated interference by ATP (Mg^{2+}) without affecting cyclic AMP binding.

Figure 2 presents the standard curves obtained by taking the means of standard curves run over a 6-month period. The theoretical curve should have a slope of —1.0. The standard curve obtained by using ^{3}H cyclic AMP at a content of 9.08 pmoles/tube showed consistent nonlinearity at the initial part of the curve and also deviated from the theoretical slope. The standard curve using 4 54 pmoles ^{3}H cyclic AMP/tube was linear and had a slope of —1.0. Assays were run under both circumstances, and both conditions resulted in similar estimates of cyclic AMP concentration. Cyclic GMP did not interfere with cyclic AMP binding until the concentration was at least tenfold greater than that of cyclic AMP. Caffeine, theophylline, ADP, and AMP at 1 m*M* concentrations did not interfere with cyclic AMP binding.

To test further for binding specificity, purified cyclic nucleotide phosphodiesterase was added to aliquots of six different extracted samples (Table 1). The sample extracts decreased ^{3}H cyclic AMP binding, indicating that cyclic AMP was present. After incubating the samples with phosphodiesterase for 30 min at 37°C, no displacement of ^{3}H cyclic AMP occurred, suggesting that the assay was specific for cyclic nucleotides. Addition of known aliquots of unlabeled cyclic AMP to the sample after incubation with phosphodiesterase resulted in displacement of ^{3}H cyclic AMP paralleling the displacement observed in the standard curve. It does remain possible, however, that the cyclic nucleotide phosphodiesterase removes agents

TABLE 1

THE SPECIFICITY OF SPERM CYCLIC NUCLEOTIDE BINDING PROTEIN[a]

Treatment	Counts/min	Experiments
Standard (9.08 pmoles ^{3}H cyclic AMP)	1480 (83)	4
Standard (^{3}H cyclic AMP) + 3.16 pmoles unlabeled cyclic AMP	790 (15)	4
Standard (^{3}H cyclic AMP) + 9.48 pmoles unlabeled cyclic AMP	430 (27)	4
Samples	930 (18)	6
Samples + phosphodiesterase (PDE)	1580 (34)	6
Samples + PDE—then 3.16 pmoles unlabeled cyclic AMP	820 (16)	6
Samples + PDE—then 9.48 pmoles unlabeled cyclic AMP	430 (13)	6

[a] The phosphodiesterase incubation and assay methods are described in methods. None of the mean values of the standards differed from the mean of the respective samples after treatment with phosphodiesterase and readdition of unlabeled cyclic AMP. The value in parenthesis represents the SEM.

other than cyclic AMP, which might compete with ^{3}H cyclic AMP.

The concentrations of cyclic AMP agreed reasonably well with concentrations measured in bovine epididymal sperm using an enzymatic cycling assay (Garbers *et al.*, 1971a), with concentrations measured in ejaculated monkey sperm using a protein kinase dependent on cyclic AMP (Casillas and Hoskins, 1971), and with concentrations in bovine ejaculated sperm, also assayed using an enzymatic cycling technique (Gray, 1971).

For the actual assay of cyclic AMP, a total volume of 250 μl was used consisting of 25 μl sperm extract, 10 μl (9.08 or 4.54 pmoles) cyclic AMP labeled with ^{3}H, 5 μl (20μg) cyclic AMP binding protein, and 210 μl of 200 m*M* EDTA, 50 m*M* acetate, pH 6. The standard curves were obtained by adding unlabeled cyclic AMP (1 to 25 μl) instead of the sperm extract. The samples were then placed on ice for 60 min and rinsed with 10 ml of 200 m*M* EDTA, 50 m*M* acetate, pH 6 buffer on to millipore filters (0.3 μm, 25 mm diameter). The filters were dried and counted in 10 ml of a solution containing 0.1 g of dimethyl-1,4-bis[2-(5-phenyloxazolyl)] benzene and 4 g of diphenyloxazole per liter of toluene in a scintillation counter.

The recovery of ^{3}H cyclic AMP after TCA extraction was greater than 95%, and therefore corrections for losses were ignored.

RESULTS

Earlier experiments showed that caffeine stimulated sperm motility rapidly; we therefore determined the time interval between caffeine addition and the increased respiratory response (Fig. 3). A lag of 1 to 4 minutes between addition of caffeine and maximum respiration with either pyruvate or glucose as substrate was consistently observed. No respiratory lag occurred when 2,4-dinitrophenol was used as an inducer of increased respiratory rate. If sperm were incubated with caffeine for a period of time, the addition of pyruvate resulted in an immediate acceleration of respiration. Therefore, caffeine induces a change in the cell metabolic state in the absence or presence of exogenous substrate, and a certain period of time is required to achieve this altered state.

Bovine epididymal sperm have a rate of glycolysis sufficient to maintain motility under anaerobic conditions (Lardy and Phillips, 1941; Mann, 1964). The addition of glucose to sperm oxidizing pyruvate in the presence of caffeine reduced the respiratory rate (Fig. 3, curve D). These results suggested that glycolysis could provide at least a part of the additional energy needed to support the motility induced by the phosphodiesterase inhibitor. Glucose utilization and lactate and pyruvate accumulation from glucose were measured on sperm samples incubated in the presence or absence of caffeine. To simulate

anaerobic conditions, in which no ATP is supplied by mitochondria bongkrekic acid (BKA), a mitochondrial adenine nucleotide translocase inhibitor, was used (Henderson and Lardy, 1970). Bongkrekic acid allowed only a basal respiratory rate in sperm incubated with acetate or fructose as substrate (Table 2). Glucose supported motility in the presence of BKA, whereas acetate and endogenous substrate did not. Sperm treated with caffeine and BKA respired slightly faster than BKA-treated sperm. With glucose as substrate, motility was stimulated by caffeine in BKA-treated sperm but not in sperm using acetate as substrate.

Glucose utilization and lactate accumulation from glucose were increased by caffeine in the absence or presence of BKA (Table 3). BKA, itself, increased glucose utilization. If all the glucose utilized not appearing as pyruvate or lactate were oxidized completely, the theoretical μg atoms O/10^8 cells/30 min would be 1.88 for the controls and 2.76 for the caffeine-treated cells. The actual values are about 0.6 and 1.3 μg atoms O/10^8 cells/30 min, respectively. Therefore glucose metabolic products must accumulate at certain steps in the catabolic routes.

The theoretical yield of ATP can be calculated from these data. The only assumption in this calculation is that 2 ATP are produced for each glucose utilized, and 6 ATP are produced for each molecule of O_2 consumed (Morton and Lardy, 1967). The prediction of ATP production for the glucose, glucose + caffeine, glucose + BKA, and glucose + caffeine + BKA treatments would be, respectively, 2.3, 4.6, 1.2, and 2.6 μmoles ATP produced/10^8 cells/30 min. This would equal a production of 0.6 to 2.6 nmoles ATP/10^8 cells/second. A similar calculation of theoretical ATP production from acetate oxidation gives values of about 0.8 for controls and 3.6 nmoles ATP/10^8 cells/sec for caffeine-treated cells Although these estimates of ATP production are only approximate, the fact that we find

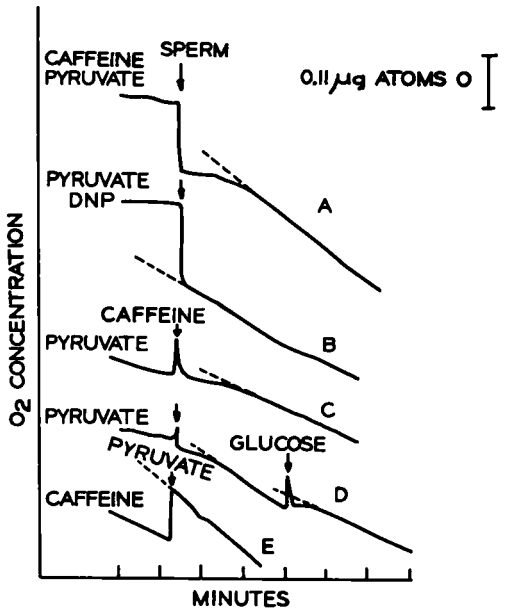

FIG. 3. The effects of various treatments on the rate of oxygen consumption of bovine epididymal sperm. (A) The effect of adding 2.5×10^8 sperm/ml to a reaction mixture containing 10 m*M* caffeine and 10 m*M* pyruvate. (B) 2.5×10^8 sperm/ml added (at arrow) to a reaction mixture containing 1.5×10^{-4} *M* 2,4-dinitrophenol (DNP) and 10 m*M* pyruvate. (C) The delay in respiratory response of sperm oxidizing pyruvate after 10 m*M* caffeine addition. (D) The delay in respiratory response of sperm oxidizing pyruvate after 10 m*M* caffeine addition (at arrow) and the inhibition of this respiratory rate by addition of 10 m*M* glucose (Crabtree Effect). (E) Sperm were incubated 10 to 15 min with 10 m*M* caffeine without exogenous substrate. No respiratory lag now occurred on addition of 10 m*M* pyruvate. All incubations were at 30°C.

20 to 30 nmoles ATP/10^8 cells (presented in later sections of this paper) indicates the relationship between ATP turnover rate and the steady-state concentration of ATP. A following paper (Garbers *et al.*, 1972) estimates similar ATP production rates in porcine ejaculated sperm.

Pyruvate utilization was only slightly affected by caffeine treatment, and lactate production from pyruvate was reduced in some experiments (Table 4).

Concentrations of ATP began to decline by 1 min after adding caffeine to sperm

TABLE 2

THE EFFECT OF 10^{-4} *M* BONGKREKIC ACID (BKA) AND 2 m*M* CAFFEINE ON THE RESPIRATORY RATE AND MOTILITY OF BOVINE EPIDIDYMAL SPERM

Substrate	Additions			
	None	2 m*M* Caffeine	BKA	BKA + Caffeine
	μg Atoms oxygen/10^8 cells/h			
None	0.72 (30)[a]	0.53 (40)	0.23 (0)	—
Acetate	1.03 (35)	4.30 (75)	0.25 (0)	0.65 (0)
Fructose	1.25 (30)	2.52 (75)	0.24 (30)	0.76 (60)

[a] Values in parenthesis represent percent of progressively motile sperm after a 1-hr incubation.

TABLE 3

THE EFFECT OF CAFFEINE ON GLUCOSE UTILIZATION AND LACTATE AND PYRUVATE ACCUMULATION IN THE PRESENCE OR ABSENCE OF BONGKREKIC ACID IN BOVINE EPIDIDYMAL SPERM[a]

	10 m*M* Caffeine	Glucose utilized	Lactate produced	Pyruvate produced
		μmoles/10^8 cells/30 min		
Control	−	0.22 (0.006)	0.12 (0.037)	0.004 (0.0009)
	+	0.43 (0.021)	0.40 (0.052)	0.006 (0.0013)
BKA[b]	−	0.40 (0.021)	0.54 (0.010)	0.020 (0.0006)
	+	0.75 (0.009)	1.15 (0.021)	0.009 (0.0012)

[a] The means represent the values from four experiments. The numbers in parenthesis are the SEM.
[b] BKA is bongkrekic acid (final concentration, 5×10^{-5} *M*).

TABLE 4

THE EFFECT OF 10 m*M* CAFFEINE ON PYRUVATE UTILIZATION AND LACTATE ACCUMULATION IN BOVINE EPIDIDYMAL SPERM. SPERM CONCENTRATIONS RANGED FROM (5–12) × 10^8 SPERM/ML. INCUBATIONS WERE FOR 30 MIN AT 37°C

Pyruvate utilization	Experiment number				
	I	II	III	IV	V
	μmoles/10^8 cells/30 min				
Control	0.48	0.57	1.44	1.10	0.86
Caffeine	0.48	0.51	1.40	1.06	0.91
Lactate accumulation					
Control	0.24	0.24	0.76	0.52	0.42
Caffeine	0.23	0.23	0.40	0.40	0.34

oxidizing only endogenous substrate (Fig. 4). The initial rate of decline was approximately 0.033 nmoles ATP/10^8 cells/sec. Since caffeine does not influence the sperm respiratory rate when exogenous substrate is not added, the value of 0.033 represents the increased rate of ATP utilization by sperm oxidizing endogenous substrate in the presence of caffeine. This interpretation can be assumed since sperm maintain a steady-state concentration of ATP under aerobic conditions when oxidizing endogenous substrate. For the control sperm oxidizing only endogenous substrate the actual concentration of ATP at zero time was 23.7 ± 2.5 and 23.1 ± 2.5 after 10 min of incubation. Sperm motility is enhanced by caffeine in cells using only endogenous substrate, despite the failure of respiratory activation. This enhanced motility is not maintained, however, and it gradually returns to control levels. Cyclic AMP concen-

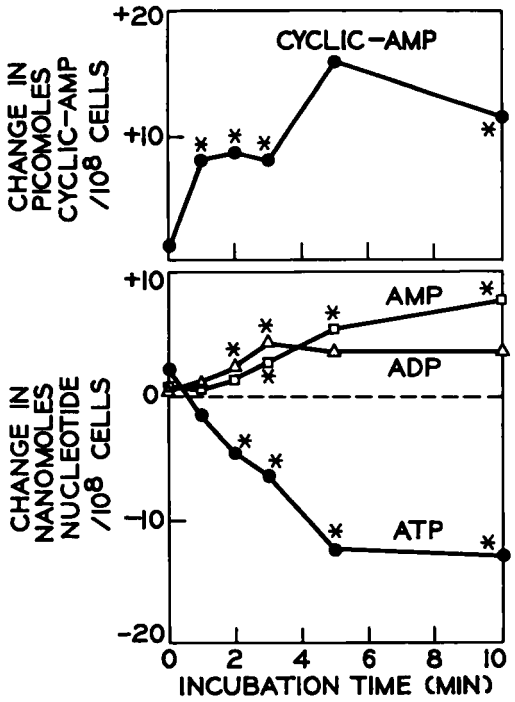

FIG. 4. The effect of 10 m*M* caffeine on cyclic AMP, ATP, ADP, and AMP concentrations in bovine epididymal sperm. Sperm were incubated at 30°C in a water bath in the presence or absence of caffeine. No exogenous substrate was added. At the indicated times, the reactions were halted by adding 10 or 20% TCA. The values, which are the means of 4–6 experiments, represent the differences between the controls and caffeine-treated samples at each incubation time. The control samples mean concentrations of ATP, ADP, and AMP were 24.8, 2.86, and 1.77 nmoles/10^8 cells, respectively; cyclic AMP was 35.5 pmoles/10^8 cells. Asterisk indicates statistical difference ($P < 0.05$) using the paired t-test.

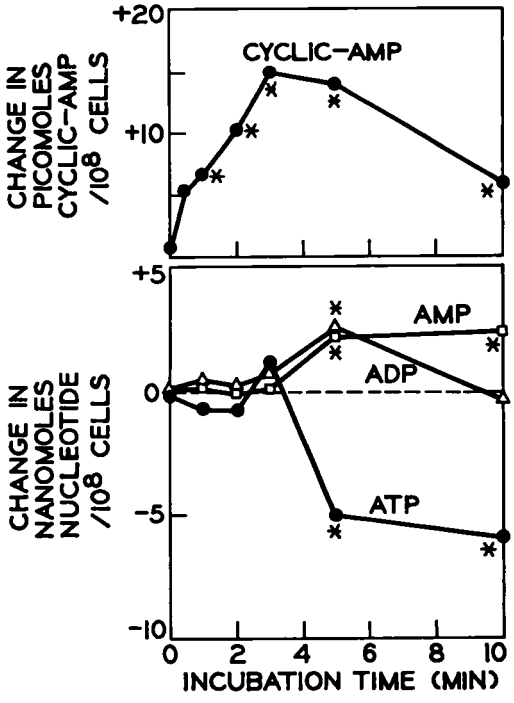

FIG. 5. The effect of 10 m*M* caffeine on ATP, ADP, AMP, and cyclic AMP concentraitons in bovine epididymal sperm in the presence of 10 m*M* fructose. In control samples the mean concentrations of ATP, ADP, and AMP were 25.5, 3.24, and 1.26 nmoles/10^8 cells, respectively; cyclic AMP was 37.0 pmoles/10^8 cells. Asterisk indicates statistical difference ($P < 0.05$) using the paired t-test.

trations were elevated by 1 min after caffeine addition to the spermatozoa. Concentrations of ADP were increased by caffeine within 2 min, but were not significantly greater than the control concentrations after 5 min of incubation. The AMP concentration increased significantly by 3 min and continued to increase throughout the incubation.

Concentrations of ATP did not decline with fructose as substrate until between 3 to 5 min after incubation with caffeine (Fig. 5). Note that the scale of Fig. 4 is twice that of Fig. 5 and that the presence of fructose helped maintain the ATP reserve. ADP increased, then returned toward normal concentrations, whereas AMP reached greater concentrations than controls by 5 min after addition of caffeine. Cyclic AMP was significantly increased by 1 min after addition of caffeine and tended ($P < 0.1$) to be increased by 30 sec.

Plots of energy charge (ATP + ½ ADP/ATP + ADP + AMP) showed that the ratio markedly decreased in caffeine-treated sperm with either endogenous substrate or with fructose as substrate (Fig. 6). Although the decreased ATP concentrations induced by caffeine make the reduction in energy charge intuitive, the low variation observed in this parameter was

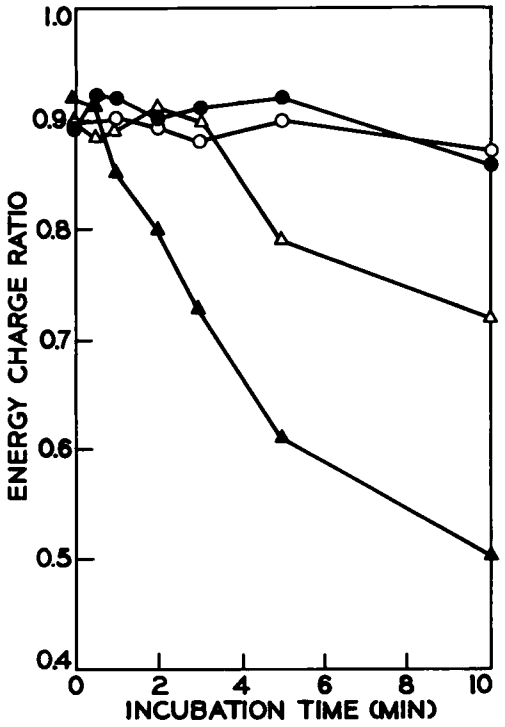

FIG. 6 The effect of 10 m*M* caffeine on the energy charge ratio (ATP + ½ ADP/ATP + ADP + AMP) of bovine epididymal sperm. —●—endogenous substrate; —▲—endogenous substrate + caffeine; —○—10 m*M* fructose; —△— 10 m*M* fructose + caffeine. The values represent the means of four different experiments.

not necessarily expected. Values of 0.9 were consistently found in bovine epididymal sperm prior to treatment with cyclic nucleotide phosphodiesterase inhibitors. Caffeine reduced the energy charge ratio to values of 0.5 to 0.75 within 10 min.

DISCUSSION

These experiments demonstrate an order, with respect to time, of various events occurring in bovine epididymal spermatozoa after the addition of cyclic nucleotide phosphodiesterase inhibitors. Cyclic AMP concentrations of motility are increased prior to ATP reduction or respiratory activation. This observation suggests that the main effect of cyclic nucleotides is on motility, with resultant secondary effects on ATP concentrations and respiration. The observation that when cells oxidizing endogenous substrate are incubated with caffeine, subsequent addition of exogenous substrate results in immediate respiratory stimulation, supports our concept that the respiratory stimulation is a secondary effect.

Williamson (1966) studied the time sequence of events after administration of epinephrine to the isolated perfused rat heart. Cyclic AMP concentrations were increased within a few seconds and reached peak concentrations at 10 sec. Concentrations of ATP decreased but returned to normal in 2 min. His work suggested that the increased contractile force of the heart in response to epinephrine was caused by the increased cyclic AMP concentrations. This work also ruled out the possibility that increased concentrations of hexose phosphates, phosphofructokinase activity, or high energy phosphate compounds were involved in the increased muscle contraction induced by epinephrine.

Since sperm oxidizing endogenous substrate do not show an altered respiratory rate, despite dramatic reduction in cellular ATP concentrations, it appears that the rate of endogenous lipid catabolism in sperm does not depend on the cellular ATP/ADP or energy charge ratio. The rate of lipid oxidation is also apparently independent of cyclic AMP concentration. These observations are especially interesting, since ejaculated sperm respire at a rate of about 1.7 μg atoms $O/10^8$ cells/h, whereas epididymal sperm respire at a rate of about 0.7 μg atoms $O/10^8$ cells/h when using endogenous lipid (Lardy *et al.*, 1945). It was postulated (Lardy *et al.*, 1949) that a metabolic regulator, which is bound by epididymal sperm, might be released on ejaculation and enhance sperm cell respiration. Since motility appears equal in bovine epididymal and ejaculated sperm, Lardy *et al.* (1949) suggested that the metabolic regulator acted to uncouple oxidative phosphorylation. Using various uncouplers of oxidative phosphorylation, Morton (1965), in this laboratory, could

not stimulate endogenous respiration of bovine epididymal cells to rates found in ejaculated cells. He did, however, report that valinomycin and nonactin, two uncouplers of oxidative phosphorylation by virtue of effects on ion transport, stimulated epididymal sperm respiration to rates seen in ejaculated sperm. Morton (1965) then suggested that the metabolic regulator might act by causing breakdown of a high-energy intermediate formed in the biosynthesis of ATP. Why a decreased concentration of this intermediate would increase lipid oxidation rates is not immediately clear, but certainly the ATP/ADP or energy charge ratio do not appear to control endogenous respiratory rate in sperm. Earlier work from our laboratory (Garbers *et al.*, 1971a) did demonstrate positive effects of caffeine and cyclic GMP in combination on endogenous respiratory rate. These observations have not been extended.

Our studies also showed that when a glycolyzable sugar was present, the motility response of bovine epididymal sperm could be divorced from respiration. This was shown by the use of BKA. These results were expected, since it was earlier reported (Lardy *et al.*, 1949) that epididymal sperm were capable of rapid glycolytic rates with Meyerhof oxidation quotients near 13. Aalbers *et al.* (1961), however, demonstrated equal and low glycolytic rates in porcine-ejaculated sperm under either aerobic or anaerobic conditions. Using porcine-ejaculated sperm, we have shown caffeine to have no effect on glucose utilization (Garbers *et al.*, 1972).

The caffeine-induced reduction of lactate accumulation, with pyruvate as substrate, is probably due to preferential shunting of NADH through oxidative pathways. Lactate dehydrogenase (LDH) isoenzyme X is probably a mitochondrial enzyme in sperm (Clausen, 1969; Machado *et al.*, 1970), and therefore direct competition for mitochondrial NADH between the oxidative pathway and LDH-X would occur.

From a physiological viewpoint, we do not as yet know what, if anything, activates the adenylate and/or guanylate cyclases of sperm. Casillas and Hoskins (1971) tested FSH, LH, GH, testosterone, dihydrotestosterone, androstenedione, progesterone, estradiol, epinephrine, and glucagon as activators of ejaculated monkey sperm adenylate cyclase and found no effects. They found thyroxine and T_3 to increase adenylate cyclase activity 1.5 to 1.9 times normal, but intracellular cyclic AMP concentrations were not altered. Epinephrine, norepinephrine, isoproterenol, FSH, HCG, LH, PGE_1, and insulin had no effect on human sperm adenylate cyclase (Gray, 1971).

A recent abstract by Carlson and Robbins (1972) reported positive effects of PGE_2 and dibutyryl cyclic AMP on the motility of frog esophagus cilia. PGA_1, PGB_1, PGF_2, thyroxine, T_3, cortisol, progesterone, estradiol, testosterone, epinephrine, serotonin, dopamine, isoproterenol, or tyramine had no effect.

Sperm cells, and possibly ciliated cells in general, are unique in that they show no response to hormones known to stimulate adenylate cyclase in other cells. The only exception to this at the moment is the positive response of frog esophagus cilia to PGE_2. It has been postulated on the basis of considerable evidence that adenylate cyclase and the β-adrenergic receptor are either closely associated or the same (Robison *et al.*, 1971). The sperm adenylate cyclase appears to be an exception. The sperm enzyme is also not stimulated by fluoride (Gray *et al.*, 1970).

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