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Effects of Fluoride and Caffeine on the Metabolism and Motility of Ejaculated Bovine Spermatozoa[1]

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ABSTRACT

Treatment of washed, ejaculated bovine sperm with 30 mM sodium fluoride immoblized the cells in a characteristically rigid form. In cells metabolizing endogenous substrates, fluoride decreased respiration by about 60%, but did not inhibit the cells' ability to produce adenosine-5'-triphosphate (ATP) via oxidative phosphorylation and did not block access to endogenous substrates. Fluoride-immobilized sperm maintained maximal ATP titers for at least 60 min, but oligomycin treatment rapidly depleted ATP, indicating that ATP synthesis and metabolism was occurring in immobilized sperm. The putative phosphodiesterase inhibitor caffeine (2.5 mM) restored motility and increased respiration in fluoride-treated sperm, but 8-bromo-adenosine-3',5'-monophosphate (8-bromo-cAMP) did not, even though 8-bromo-cAMP stimulated respiration in control (untreated) sperm. Carboxyfluorescein analysis of the intracellular pH of untreated sperm indicated a normal pH of 6.3. Fluoride addition decreased the apparent intracellular pH slightly, but this effect was attributable to dilution. Caffeine did not change internal pH in untreated or fluoride-immobilized sperm. Fluoride did not appear to affect cAMP metabolism, but caffeine increased intracellular cAMP titers by about 35% in both untreated and fluoride-inhibited sperm. However, caffeine treatment did not mimic 8-bromo-cAMP, as analyzed by electrophoresis and autoradiography of sperm proteins labeled with ^{32}P from endogenously generated [^{32}P]ATP. Clearly, caffeine is not stimulating motility in fluoride-treated sperm by affecting the cyclic AMP system. Fluoride also inhibited motility in digitonin-permeabilized sperm by a mechanism that may have involved magnesium depletion, but caffeine had no stimulatory effect on either untreated or fluoride-immobilized, permeabilized sperm.

INTRODUCTION

Soon after the cyclic adenosine-3',5'-monophosphate (cAMP)-mediated second messenger system was described, Garbers et al. (1971) determined that cAMP was involved in the stimulation of sperm motility and metabolism. Phosphodiesterase inhibitors, such as the methylxanthines, caffeine, and theophylline, stimulated sperm motility, fructolysis, and respiration and also increased cAMP titers (Garbers et al., 1973; Hoskins et al., 1974). Cyclic AMP has since been shown to be essential for the initiation and maintenance of dynein-generated sperm motility (Ishiguro et al., 1982; Lindemann et al., 1983). Elevated intracellular cAMP (Cascieri et al., 1976) and changes in cAMP-stimulated protein phosphorylation (Hoskins et al., 1974; Brandt and Hoskins, 1980;) have also been associated with development of the capacity for motility in immature sperm (Hoskins et al., 1974). However, although most of the components of the second messenger system have been identified in mammalian sperm (for review, see Garbers and Kopf, 1980), the metabolic sequence whereby methylxanthine-induced elevations of cAMP titers result in stimulated sperm motility has not been detailed.

Fluoride ion is another inhibitor that has been extensively used in sperm metabolic studies. Lardy and Phillips (1943, a,b) established that fluoride inhibited bull sperm respiration, motility, and glycolysis, and that while pyruvate restored glycolysis, it would not restore motility. However, motility was restored in fluoride-treated sperm by washing and transfer into fresh fluoride-free medium (Lardy and Philips, 1943a; Mann and Lutwak-Mann, 1955). Fluoride acts in most cases by forming an insoluble complex with magnesium and phosphate and thus

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affects many enzymes and metabolic pathways that involve phosphate transfer. Of these, the inhibition of the glycolytic enzyme enolase has been best characterized, but many other enzymes, such as acid, alkaline, and phosphoprotein phosphatase are also inhibited by fluoride (Jain, 1982), making it a useful tool to prevent the degradation of phosphorylated products. Fluoride has recently been used in such a manner to preserve the phosphorylated state of endogenous (Brandt and Hoskins, 1980) and exogenous (Babcock et al., 1983) protein kinase substrates of mammalian sperm. While studying fluoride's effects on sperm, we observed that fluoride-inhibited motility could be restored by caffeine and other methylxanthines in the absence of added substrates. Since caffeine and fluoride appeared to counteract each other, we have investigated several possible inhibitory mechanisms to resolve the mechanism of normal sperm motility.

MATERIALS AND METHODS

Cells. Fresh semen was provided by American Breeders Service (DeForest, WI) and sperm were isolated by centrifugation at 600 × *g* at 20°C for 20 min, followed by two cycles of resuspension in NKM (NaCl, 120 mM; KCl, 14 mM; $MgCl_2$, 1 mM; 3-[N-morpholino] propanesulfonic acid [MOPS], 10 mM, pH 7.4) and centrifugation at 600 × *g* for 15 min. Sperm were finally resuspended in NKM at 3–5 × 10^9 cells/ml and kept at ambient temperature (20°C).

Respiration. Oxygen uptake was determined polarographically in a Gilson Oxygraph (Gilson Medical Electronics, Inc., Middleton, WI) fitted with a Clark-type electrode. All measurements were taken with sperm at 1.76 × 10^8 cells/ml in NKM at 30°C. Substrates, stimulators, or inhibitors were added either before the sperm or during the linear stage of oxygen consumption, which was determined in parallel assays with control cells.

Motility. Due to the difficulty in accurately characterizing motility with regard to the percentage of motile cells and their vigor, we have restricted our description of motility to its presence or absence. However, during the course of this and other studies, we have established a range of normal motility observed in samples of washed sperm. We have also determined typical responses to fluoride and caffeine. All washed sperm samples were therefore observed under normal, inhibited, and stimulated conditions, and any batches that responded abnormally (2% of all samples) were not used.

Fluoride treatment. Fluoride inhibited bull sperm motility by a mechanism that was dependent on both the drug and cell concentrations. With sperm concentrations between 1.0 and 3.5 × 10^8 cells/ml, sodium fluoride (NaF) at 5–10 mM slowly inhibited motility such that all movement had stopped after about 10 min. At higher cell concentrations, this concentration of NaF did not totally inhibit motility. In the experiments presented in this study, 30 mM NaF completely immobilized sperm at the above concentration within 2 min at 30°C.

Digitonin treatment. Sperm at 2 × 10^8 cells/ml were incubated with digitonin (Sigma Chemical Co., St. Louis, MO; recrystallized twice from ethanol) at 0.043 mg/10^8 cells for 5 min in NKM at 30°C. These nonmotile, permeabilized sperm were either reactivated directly or centrifuged through percoll (1.09 g/ml), resuspended in NKM at 2 × 10^8 cells/ml at 20°C, and then reactivated. Reactivation was accomplished by adding (final concentrations) adenosine-5′-triphosphate (ATP), 0.5 mM; cAMP, 0.025 mM; $MgCl_2$, 3.0 mM; and inosine-5′-triphosphate (ITP), 3.0 mM (ITP was added as a substrate for sperm phosphatases, which otherwise rapidly depleted added ATP. ITP did not support motility. Schoff, unpublished observations). The percentage of motile cells in reactivated preparations appeared to be comparable to that in intact preparations, but the vigor was generally reduced. The duration of motility varied from 5 to 15 min and could be extended by adding fresh ATP.

ATP determinations. Sperm (1.5 × 10^8 cells/ml) were incubated in NKM at 30°C with stimulators or inhibitors and, at the indicated times, 0.45-ml aliquants were removed and placed in 0.25 ml of cold 2 N perchloric acid, incubated for 10 min on ice, and centrifuged. The supernatant fraction was removed, neutralized with 2 N KOH, and centrifuged. The supernatant fraction was assayed immediately for ATP by an enzymatic technique employing hexokinase and glucose-6-phosphate dehydrogenase (Bergmeyer, 1974). Fluoride and caffeine had no effect on the assay.

6-Carboxyfluorescein analysis. Sperm internal pH (pH_i) was analyzed by using a modification of a technique previously described (Babcock, 1983). Sperm were incubated in NKM containing 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.7, without added substrates with 4 μM 6-carboxyfluorescein diacetate for 20 min at 20°C, then centri-

fuged for 5 min at 600 × *g* and resuspended in NKM. Carboxyfluorescein absorbance was monitored in suspensions of 2×10^7 cells/ml. Sperm at this concentration were poorly motile, but normal levels of motility could be attained in dilute suspensions by centrifuging semen (0.25 ml) through 1.5 ml percoll (1.09 g/ml) at 700 × *g* for 15 min, then resuspending sperm in NKM. Percoll-washed sperm were used without further washing. These cells retained motility at low concentrations and responded to stimulators and inhibitors like concentrated suspensions did. Their pH_i also matched that of sperm not treated with percoll.

Cyclic AMP assays. Sperm cAMP titers were analyzed by a radioactive binding assay (Diagnostic Products Corp., Los Angeles, CA). After incubation at 30°C, sperm cytosol was extracted as reported above. cAMP was assayed by following the protocol included with the assay kit.

[^{32}P] Orthophosphate incorporation and endogenous phosphorylation. Sperm (3.0×10^8 cells/ml) were incubated in NKM containing fructose, 10 mM; acetoacetate, 10 mM; succinate, 10 mM; KH_2PO_4, 0.01 mM; and [^{32}P] orthophosphate at 15 μCi/nmole PO_4. After 60 min at 20°C, sperm were centrifuged at 600 × *g* for 15 min at 20°C, then resuspended to 3×10^9 cells/ml in NKM containing succinate, 10 mM; KH_2PO_4, 0.01 mM; and [^{32}P] orthophosphate at 15 μCi/nmole PO_4. To deplete the cells of ATP stores, the cell suspension was overlayed with argon, sealed, and incubated for 30 min at 20°C. After 30 min, aliquots of the concentrated cell suspension were added to incubation tubes containing NKM at 30°C with KH_2PO_4, 1.0 mM, and other additions as noted. At the indicated times, aliquants were removed and added to solubilizing media as reported previously (Schoff et al., 1982). Electrophoresis, gel processing, and autoradiography were performed as in Schoff et al. (1982), except that the gels consisted of 8.5–12.5% acrylamide gradients.

RESULTS

Effects of Sodium Fluoride on Sperm Metabolism and Motility

Sperm treated with 30 mM fluoride became immobile within 2 min and their flagella assumed a linear, rodlike conformation (Fig. 1A) that was distinctly different from sperm immobilized by rotenone (Fig. 1B). Respiration of cells treated with

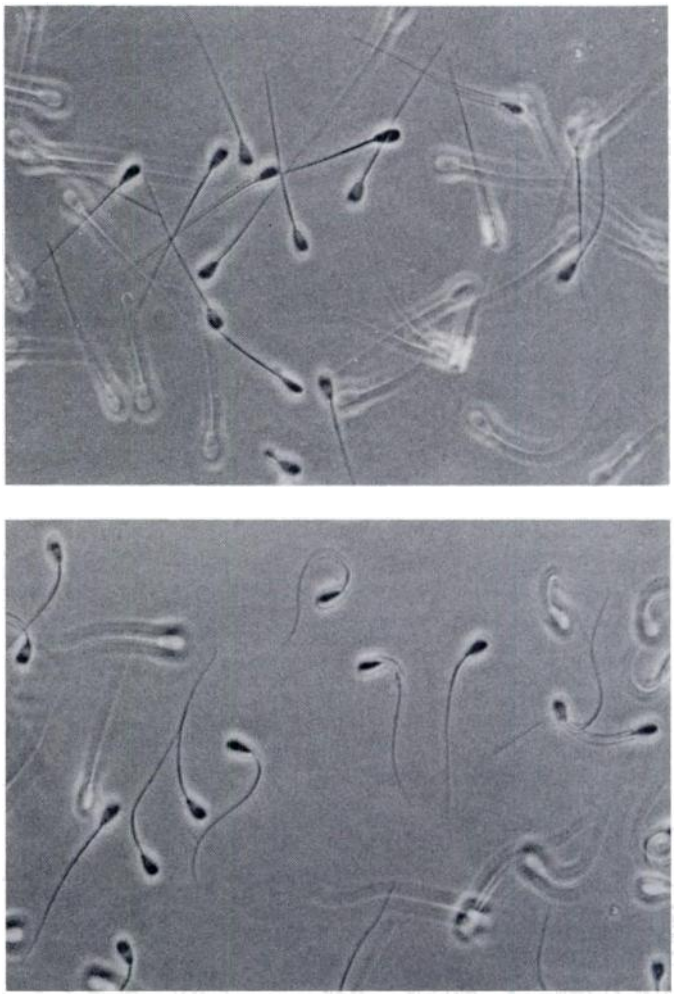

FIG. 1. Ejaculated bovine sperm immobilized by (*a*) fluoride and (*b*) rotenone. Sperm treated with 30 mM NaF became immobile over about 1 min at 20°C. Flagellar motion decreased to a quiver and finally stopped altogether with the tail in a rigid form.

fluoride decreased over several minutes to about 40% of the control rate (without added substrate) (Table 1). This decrease probably reflected the decreased energy demand of nonmotile cells rather than a specific inhibition of respiratory enzymes, since uncoupled respiration rates of fluoride-treated cells (Table 1) indicated that endogenous substrates were available and could be metabolized via oxidative phosphorylation. Although fluoride blocked the production of lactate from added glucose (not shown), it had no apparent effect on ATP production from endogenous substrates (Fig. 2). As indicated by the initial assay points in Figure 2, dense sperm suspensions ($3.0–5.0 \times 10^9$ cells/ml) incubated without added substrates contained very low ATP titers. When these sperm were diluted into NKM

TABLE 1. Respiration by sperm treated with fluoride and caffeine.

Treatment[a]	Respiration: μg atoms O_2/10^8 cells/min[b]	Respiration: % of control	Motility[d]
No addition	7.7 ± 1.7 (3)[c]	100	+
+ CCCP	31.1 ± 4.5	423 ± 114	
NaF	3.7 ± 0.6	49 ± 5	−
+ CCCP	25.7 ± 3.7	345 ± 60	
No addition	11.9 ± 5.9 (7)	100	+
+ caffeine	28.4 ± 11.4	248 ± 49	+++
NaF	5.4 ± 1.1	51 ± 15	−
+ caffeine	11.9 ± 4.4	105 ± 25	+
No addition	6.0 ± 1.5 (3)	100	+
+ 8-bromo-cAMP	15.2 ± 1.4	263 ± 43	++
NaF	3.3 ± 0.5	43 ± 4	−
+ 8-bromo-cAMP	3.4 ± 0.5	46 ± 12	−

[a]All perturbants were added by syringe after a linear respiration rate was established (1–3 min).

[b]All measurements were taken by a Clark-type oxygen electrode in the upper 2/3 of the saturation curve to assure adequate O_2 tension for cell viability. Carbonyl cyanide-*m*-chlorophenylhydrozone (CCCP) = 2.5 μM in dimethyl sulfoxide (DMSO); NaF = 30 mM; caffeine, when used alone = 2.5 mM, with NaF = 7.5 mM; 8-bromo-cAMP = 0.05 mM. DMSO had no effect on sperm respiration.

[c]Each group of measurements represents a compilation of average rates from separate batches of sperm (in parentheses); thus, differences appear between control, inhibited, and stimulated rates compiled from different batches of sperm.

[d]Motility was observed at approximately 1.5 min after addition of sperm (controls) or after addition of inhibitors or stimulators. Sperm were removed from the oxygraph chamber via a 50-μl capillary inserted through the entry port. (−) = No motility; (+) = normal motility; (++) = stimulated above normal motility; (+++) = maximally stimulated motility.

(1.0–3.0 × 10^8 cells/ml) at 30°C, their ATP content rapidly increased to about 15 nmoles/10^8 cells. This synthesis was apparently oxygen-dependent because oligomycin blocked the burst of ATP production in diluted sperm (Fig. 2). Sperm kept densely concentrated (essentially anaerobic) that had been treated for 15–30 min with 30 mM fluoride were completely immotile upon dilution, but the normal burst of ATP synthesis still took place. Sperm treated with fluoride before or after dilution remained immobile and retained high ATP titers over 15–60 min (Fig. 2), while untreated sperm became more vigorous after about 15 min of incubation. The ATP titers in these sperm decreased concomitantly with increased motility (Fig. 2). Even though fluoride-treated sperm were immobile, they remained alive and metabolized ATP over 60 min, as indicated by the ATP-depleting effects of oligomycin (Fig. 3).

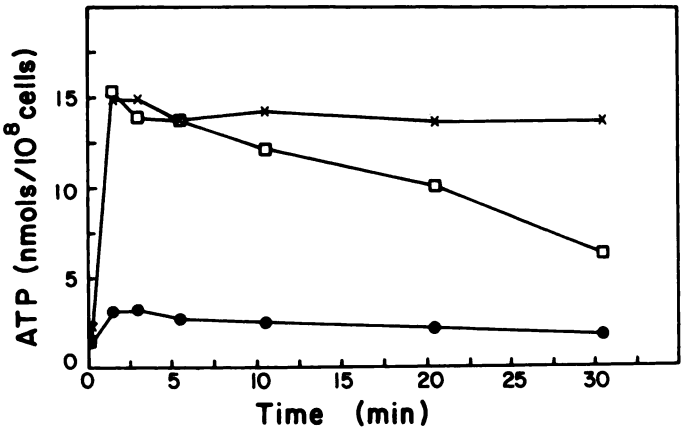

FIG. 2. Production of adenosine-5′-triphosphate (ATP) after dilution of concentrated cells. Washed sperm were incubated at 5.75 × 10^9 cells/ml for 1 h at 20°C, then diluted to 1.5 × 10^8 cells/ml into NKM alone (□) or with 30 mM NaF (×) or with 30 mM NaF and 1.5 μM oligomycin (●) and assayed for ATP content, as described in *Materials and Methods*. The initial assay portion was taken at 10 s. ATP content at that time depended on the amount of stirring or agitation of the concentrated stock, i.e. agitation produced higher initial ATP titers. Flushing the stock container with argon during the experiment eliminated this problem.

Reactivation of Fluoride Immobilized Cells

Several agents that stimulated motility and metabolism in untreated sperm, including NH_4Cl, $NaHCO_3$, 8-bromo-cAMP, and caffeine, were used in attempts to reactivate motility in fluoride-immobilized sperm. Of these, only caffeine stimulated motility and respiration (Table 1). If caffeine was added at the

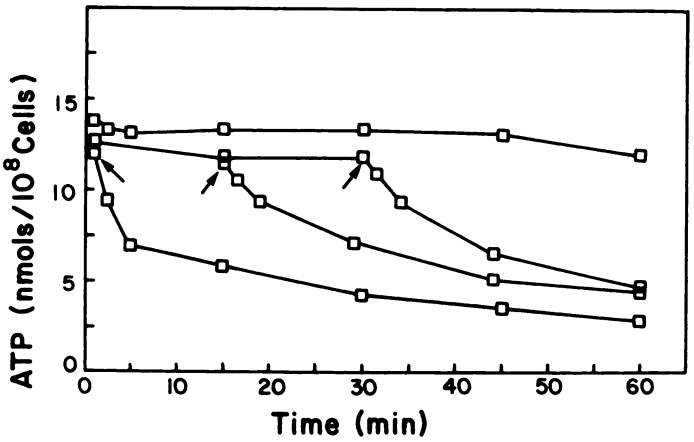

FIG. 3. Effects of oligomycin on the adenosine-5′-triphosphate (ATP) content of fluoride-immobilized sperm. ATP content was determined in fluoride-treated sperm over 60 min. Concentrated sperm (6.5 × 10^9 cells/ml) were diluted to 1.3 × 10^8 cells/ml into NKM containing 30 mM NaF. Oligomycin (5 μM) was added at the times indicated by *arrows*. All sperm were immotile throughout the experiment.

same time as fluoride or within 5 min after fluoride application, motility was immediately stimulated. However, the stimulatory action of caffeine diminished as the fluoride incubation period increased.

Internal pH

The effects of fluoride and caffeine on sperm pH_i were analyzed. Fluoride inhibition of sperm motility was apparently not caused by intracellular acidification. Analysis of sperm pH_i with 6-carboxyfluorescein diacetate (Table 2) indicated a value of 6.3 when sperm were incubated in NKM at 30°C without added substrates. Treatment with fluoride caused an acidification of approximately 0.1 pH units. Although lower sperm pH_i generally corresponds to reduced motility, alterations in pH_i alone could not account for the fluoride effect. When the weak base NH_4Cl was added to fluoride-inhibited sperm, the pH_i shifted upward, but this was not accompanied by the metabolic stimulation that was observed in sperm not exposed to fluoride. Conversely, $NaHCO_3$, which lowered pH_i and stimulated motility in control sperm, also lowered pH_i in fluoride-inhibited sperm, but did not stimulate motility.

As indicated in Table 2, caffeine did not act as a permeant weak base (Babcock et al., 1983) in either untreated or fluoride-inhibited cells. In fact, caffeine produced a slight drop in apparent pH_i, which was accounted for by dilution. These data eliminate major changes in pH_i as the mechanism for the effects of fluoride and caffeine in intact sperm. However, limited or local pH_i changes might not be distinguished by this method and remain a possibility.

Intracellular cAMP

cAMP titers increased about 35% after caffeine treatment in both control and fluoride-treated sperm (Fig. 4). Comparable increases were produced in cells that were incubated with fluoride for 5 min before caffeine was added. Thus, cAMP might be responsible for the stimulatory effect of caffeine on fluoride-immobilized sperm. However, it should be noted that cAMP titers in fluoride-immobilized cells did not differ from controls, indicating that, since control sperm were motile, lack of cAMP was not the proximate cause of fluoride-inhibition. Also, even though cAMP titers increased after caffeine stimulation of fluoride-immobilized cells, 8-bromo-cAMP did not stimulate motility in fluoride-treated sperm. These results indicate that increased cAMP titers may not be the only effect of caffeine in sperm cells.

TABLE 2. Effects of dilution, fluoride, and motility stimulators on apparent intracellular pH and motility.[a]

Treatment[b]	Apparent pH change[c]	Motility[d]
NKM	−0.15 0.06	+
NaF	−0.19 0.06	−
+caffeine	−0.26 0.12	+
$+NaHCO_3$	−0.27 0.04	−
$+NH_4CL$	+0.13 0.004	−

[a]Sperm were loaded with 6-carboxyfluorescein diacetate, as described in Materials and Methods. Intracellular pH was determined in stirred cuvettes at 30°C by null-point analysis. All further analysis was carried out in NKM at pH 7.4. Internal pH (pH_i) of sperm was unaffected by external pH between 6.2 and 6.9.

[b]NaF = 30 mM; caffeine = 7.5 mM; $NaHCO_3$ = 30 mM; NH_4Cl = 25 mM. Caffeine and other drugs were added to the cuvette after a baseline of fluoride-treated sperm was established.

[c]Apparent pH_i of untreated wasehd sperm = 6.32 ± 0.04 (N = 9).

[d]Motility was observed after 2.5 min.

Fluoride Inhibition of Motility in Permeabilized Sperm

Fluoride also inhibited the reactivated motility of permeabilized sperm preparations. The sperm were treated with digitonin, which permeabilized the sperm membranes and caused complete immobiliza-

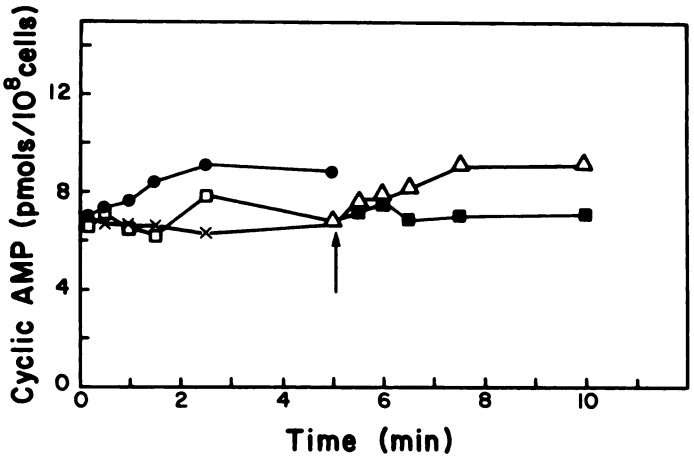

FIG. 4. Analysis of cyclic adenosine-3′,5′-monophosphate (cAMP) content of sperm treated with fluoride and caffeine. Sperm were diluted to 3.25 X 10^8 cells/ml into NKM at 30°C, and the aliquants were extracted by perchloric acid (PCA) precipitation at the indicated times. The acid extracts were neutralized (as described in *Materials and Methods*) and their cAMP content was analyzed by a binding protein assay. Sperm were diluted into NKM alone (□) or with 30 mM NaF (X), or with 2.5 mM caffeine (●). After 5 min (*arrow*), 30 mM NaF was added to the sperm in NKM alone (■), and 7.5 mM caffeine was added to the sperm in fluoride (Δ).

TABLE 3. Motility of permeabilized sperm.

Treatment[a]	Motility[b]
Digitonin permeabilization	
ATP	–
ATP + cAMP	+
ATP + NaF	–
ATP + NaF + caffeine	–
Fluoride pretreatment[c]	
ATP	–
ATP + cAMP	–
Caffeine pretreatment	
ATP	+
ATP + cAMP	+
Caffeine pretreatment + centrifugation	
ATP	–
ATP + cAMP	+

[a]Sperm at 5.0–6.0 × 10^9 cells/ml were diluted to 2.0 × 10^8 cells/ml in NKM containing digitonin and incubated at 30°C for 5 min before adding nucleotides. Adenosine-5′-triphosphate (ATP), 0.5 mM; cyclic adenosine-3′,5′-monophosphate (cAMP), 0.025 mM; NaF, 30 mM; caffeine, 2.5, mM.

[b]Motility was observed 1 min after addition of nucleotides.

[c]Pretreatment protocols: Concentrated sperm were diluted into NKM containing NaF, 30 mM, or caffeine, 2.5 mM, for 5 min at 30°C before being treated with digitonin. Caffeine pretreatment and digitonin. Caffeine pretreatment and digitonin permeabilization were followed by centrifugation of the sperm through percoli at 600 × *g* for 10 min at 20°C. The pelleted sperm were resuspended in NKM and treated with nucleotides.

tion; then, motility was stimulated by MgATP and cAMP (Table 3) added directly to the medium or after centrifuging the sperm through percoll and resuspending them in fresh NKM. Permeabilization was verified first by the ability of ATP and cAMP to restimulate motility, and second by the ability of the impermeant compound sodium ortho vanadate at 5 μM to immobilize the reactivated cells (Gibbons et al., 1978). Fluoride, when applied after reactivation, immobilized the sperm within several minutes (Table 3). As with intact cells, fluoride concentrations below 30 mM also blocked motility, but required a longer exposure for complete inhibition. Sperm immobilized by fluoride before digitonin treatment could not be restimulated by MgATP and cAMP; but, as with intact cells, fluoride inhibition was relieved by washing, and motility could be restimulated by adding MgATP and cAMP. When the sperm are permeabilized, centrifuged through percoll, and treated with fluoride for 5 min before MgATP and cAMP, motility was never present. Adding excess cAMP or 8-bromo-cAMP did not stimulate motility in fluoride-inhibited cells or protect reactivated cells from fluoride inhibition. Additions of 15 mM $MgSO_4$, or 1 mM MgATP, MnATP, or CaATP before or after fluoride did not restore motility. However, if the sperm were permeabilized in NKM that contained 1 mM $MnCl_2$ instead of $MgCl_2$, and reactivated with MnATP instead of MgATP, the addition of 30 mM NaF reduced but did not eliminate motility.

Caffeine Effects on Permeabilized Sperm

Caffeine has no apparent effect on motility in digitonin-permeabilized cells (Table 3). It did not stimulate motility in the absence or presence of ATP and cAMP, and it did not prevent the fluoride-induced inhibition of reactivated motility, nor did it reinitiate motility in reactivated, fluoride-immobilized cells. We also tested whether the increased cAMP titers produced by caffeine treatment prior to digitonin permeabilization would produce a cell that would not require cAMP for reactivation. When sperm were incubated for 5 min with caffeine before digitonin treatment, they did not require cAMP for restimulation, but became motile with MgATP alone (Table 3). Thus, the cAMP resulting from caffeine treatment appears to be available for cellular metabolism.

Protein Phosphorulation in Cells Treated with Fluoride and Caffeine

Patterns of endogenous protein phosphorylation (Fig. 5) also show little change with fluoride or with caffeine. Intact cells loaded with [^{32}P] orthophosphate phosphorylated several proteins in the absence of cAMP and phosphorylated one additional protein at about M_r 60,000 in response to cAMP. However, there was no qualitative difference in the labeling pattern of cells stimulated with caffeine. Also, other than a slight reduction of the intensity of radioactive labeling, there appears to be no difference in the labeling pattern of fluoride-treated cells with or without caffeine. The cAMP-dependent phosphorylated protein also appears in fluoride-treated cells.

Protein Phosphorylation in Digitonin-Permeabilized Sperm

After permeabilization with digitonin, sperm were treated with [^{32}P] ATP, with or without cAMP, and protein phosphorylation was monitored by gel electrophoresis and autoradiography (Fig. 6). A number of major proteins were labeled with ^{32}P in the absence of cAMP, and several more appeared when cAMP was added. Although fluoride treatment after permeabilization reduced the quantity of

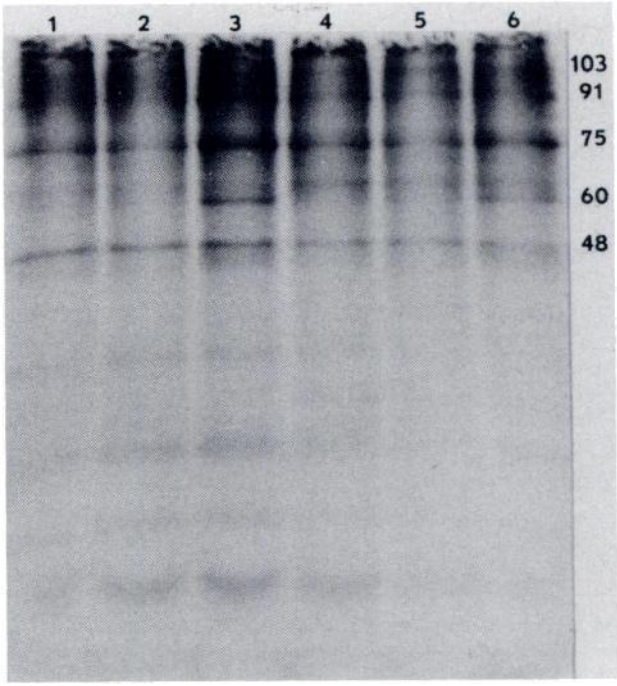

FIG. 5. Autoradiograph of sperm proteins from cells incubated with [^{32}P] orthophosphate. Sperm were incubated with [^{32}P] orthophosphate (as described in *Materials and Methods*), then diluted into NKM at 30°C. After 5 min, the sperm were solubilized and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 10–12.5% linear gradient gel. *Lane 1*, sperm diluted into NKM alone; *Lane 2*, caffeine, 2.5 mM; *Lane 3*, 8-bromo-cAMP, 0.050 mM; *Lane 4*, NaF, 30 mM; *Lane 5*, NaF, 30 mM and caffeine, 7.5 mM; *Lane 6*, NaF, 30 mM and 8-bromo-cAMP, 0.025 mM. The approximate molecular weights (X 10^{-3}, right) of the most prominant phosphorylated proteins were calculated from standards separated on the same gel. Motility was observed in samples from a nonradioactive assay conducted concomitantly: *Lane 1*, control = 60% motile; *Lane 2*, caffeine = motile vigor greatly stimulated; *Lane 3*, 8-bromo-cAMP = motility obviously stimulated but not to caffeine level; *Lane 4*, NaF = 0 motility; *Lane 5*, NaF plus caffeine = stimulated motility; *Lane 6*, NaF plus 8-bromo-cAMP = 0 motility.

phosphorylation, it did not appear to change the number or position of labeled proteins. This was the case in cells treated with fluoride either before or after cAMP and ATP. Similarly, phosphorylation profiles of sperm treated with fluoride prior to permeabilization did not differ from controls (Fig. 6). Oddly, caffeine applied prior to digitonin permeabilization, which obviated the need for cAMP for the stimulation of motility, did not change the phosphorylation profile (Fig. 6). These data may indicate that the putative critical phosphorylation occurs in a protein too minor to be detected by these methods.

DISCUSSION

Sperm that were metabolizing endogenous, nonglycolytic substrates were immobilized in a distinctive, seemingly rigid form by 30 mM fluoride. Several observations support the hypothesis that fluoride acted directly on the motile apparatus without substantially affecting other metabolic systems. First, fluoride did not block energy generation via oxidative phosphorylation, as demonstrated by the ability of fluoride-immobilized sperm to form ATP in oxygenated solutions. Second, fluoride did not appear to restrict the metabolic utilzation of endogenous substrates, since the uncoupled respiration rates of fluoride-inhibited and control sperm were nearly equal. Third, oligomycin decreased ATP titers in fluoride-immobilized sperm, indicating that they were using (and thus also producing) ATP throughout the experiment. Finally, permeabilized sperm, in which metabolic systems other than the flagellar mechanism had been bypassed, were also sensitive to fluoride. In contrast to other reports (Tash and Mann, 1973), we found that cAMP titers of fluoride-treated sperm did not differ from controls. Moreover, caffeine, presumably acting as a phosphodiesterase inhibitor, increased cAMP titers in fluoride-treated sperm, indicating that both phosphodiesterase and adenylate cyclase must have been functioning.

Caffeine, which stimulated motility and metabolism in untreated sperm, restored motility in fluoride-treated sperm. However, other stimulatory agents, such as NH_4Cl, $NaHCO_3$, and 8-bromo-cAMP, were unable to counteract the fluoride-induced immobilization. The failure of 8-bromo-cAMP to mimic the effects of caffeine in fluoride-treated sperm was especially significant because the ability of methylxanthines to stimulate sperm metabolism has usually been attributed to the elevated cAMP titers produced by phosphodiesterase inhibition. We attempted to correlate the effects of fluoride, caffeine, and 8-bromo-cAMP on motility and metabolism with the phosphorylation of sperm proteins using endogenously generated [^{32}P] ATP. In general, the results of these experiments can be summarized as follows: The autoradiograph of untreated sperm (motile, with average cAMP titers) revealed several major and minor phosphorylated proteins. Addition of 8-bromo-cAMP, which stimulated motility and metabolism, produced qualitative and quantitative increases in protein phosphorylation, most notably in the addition of a heavily labeled protein at about M_r 60,000. However, caffeine, which stimulated motility and metabolism and also increased cAMP titers, produced no distinguishable difference in protein phosphorylation compared with untreated cells. Fluoride treatment

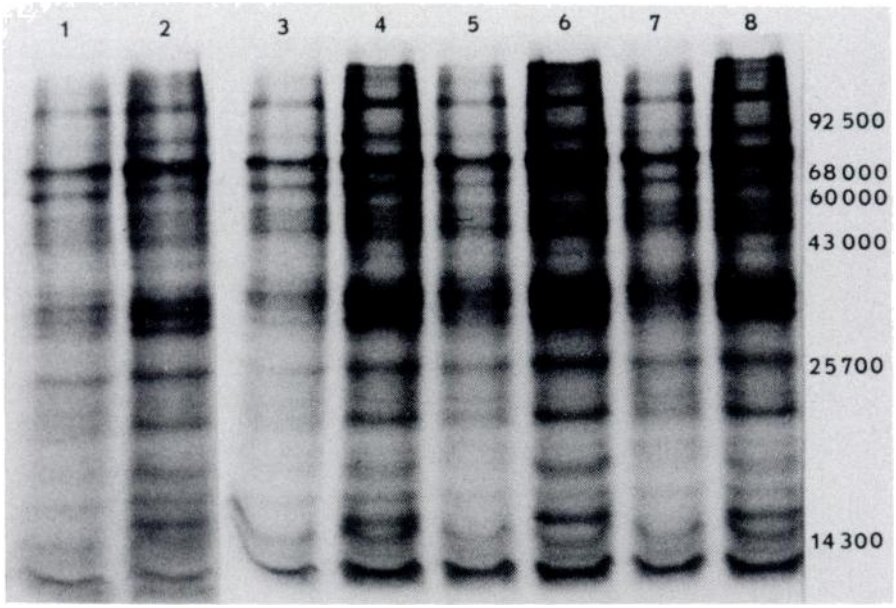

FIG. 6. Autoradiographs of ^{32}P-labeled proteins from permeabilized sperm reactivated with [γ-^{32}P] adenosine-5′-triphosphate (ATP). Sperm were permeabilized with digitonin, as described in *Materials and Methods*, then motility was reactivated in the same solution using 0.5 mM ATP containing 0.25 μCi/nmole [γ-^{32}P] ATP in the absence or presence of 0.025 mM cAMP. After incubation with nucleotides at 30°C for 2.5 min, the sperm were solubilized, and 100 μg (protein) aliquants were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis in an 8.5–12.5% linear gradient gel. This figure represents results from the same experiment analyzed on two gels. *Lanes 1* and *2*: concentrated sperm (4.5×10^9 cells/ml) diluted to 2.0×10^8 cells/ml into NKM containing digitonin, incubated for 5 min at 30°C, then treated with [γ-^{32}P] ATP alone (1) or with cAMP (2). *Lanes 3* and *4*: concentrated sperm diluted into 30°C NKM for 5 min before digitonin was added, then treated as above; (3) [γ-^{32}P] ATP alone, (4) with cAMP. *Lanes 5* and *6*: concentrated sperm diluted into NKM containing 30 mM NaF for 5 min, then treated with digitonin as above; (5) [γ-^{32}P] ATP alone, (6) with cAMP. *Lanes 7* and *8*: concentrated sperm diluted into NKM containing 2.5 mM caffeine for 5 min, then treated with digitonin as above; (7) [γ-^{32}P] ATP alone, (8) with cAMP. Motility analysis was performed at 2.5 min in nonradioactive assays run concurrently. Lane 1 not diluted before permeabilization; ATP = near 0% motility, i.e. quivering motion was seen in a few (<10%) flagella. *Lane 2*, No dilution; ATP and cAMP = 60% motile, full coordinated motility. *Lane 3* diluted into NKM; ATP = 10%. *Lane 4* diluted into NKM; ATP plus cAMP = 60%. *Lane 5* diluted into NaF; ATP = 0%, no motion in any flagella. *Lane 6* diluted into NaF; ATP plus cAMP = 0%, no motion. *Lane 7* diluted into caffeine, ATP = 50%. *Lane 8* diluted into caffeine; ATP plus cAMP = 60%.

reduced the phosphorylation of exogenous protein substrates (Schoff, unpublished data) but did not change the phosphorylation pattern of endogenous sperm proteins. Caffeine, which stimulated fluoride-immobilized sperm and increased their cAMP content, had no effect on the phosphorylation profile. However, in fluoride-immobilized sperm, 8-bromo-cAMP did stimulate the phosphorylation of the M_r 60,000 protein, albeit at a reduced level, but did not stimulate motility. Thus it appears that caffeine and cAMP have disparate effects. Both stimulate motility in otherwise untreated sperm; however, only 8-bromo-cAMP increases protein phosphorylation, whereas in fluoride-immobilized sperm, caffeine stimulates motility without additional protein phosphorylation. While it is evident from this and other studies (Garbers et al., 1971; Lindemann, 1978; Lindemann et al., 1983; Ishiguro et al., 1982; Opresko and Brokaw, 1983) that cAMP is a stimulator of sperm motility, it seems arguable that the mechanism of caffeine stimulation of fluoride-immobilized sperm is mediated by cAMP. Other recent reports (Levin et al., 1981; Turner and Giles, 1982; Kopf et al., 1984) have challenged the hypothesis that methylxanthine stimulation of sperm metabolism is primarily due to phosphodiesterase inhibition and questioned whether changes in cAMP titers correlate with changes in motility (Hammerstedt and Hay, 1980).

The failure to detect changes in protein phosphorylation that correlate with changes in motility may be explained several ways. It is possible that the essential proteins are phosphorylated at levels too low to be detected by single dimension electrophoresis, or the critical phosphorylated motility protein is rapidly dephosphorylated and never accumulates to a detectable level. Fluoride has been used (Brandt and Hoskins, 1980) to block protein phosphatases and thereby "freeze" the phosphorylation state of proteins

in sperm homogenates; in the present case, however, adding fluoride with caffeine or 8-bromo-cAMP did not change the phosphorylation profiles.

Several alternative mechanisms for the actions of fluoride and caffeine also exist. For example, they could operate as opposing pH_i effectors. Recent reports indicate that pH_i changes accompany and are perhaps responsible for metabolic stimulation in invertebrate (Lee et al., 1981) and mammalian (Babcock et al., 1983) sperm. Although we found that fluoride and caffeine caused minimal change in sperm pH_i, localized pH changes might not be detected by the methods used here, and conceivably could account for the observed inhibition and stimulation of motility. Caffeine is also known to disrupt calcium transport mechanisms (Clusin, 1983; Hahn et al., 1979), which could affect sperm motility and protein phosphorylation (Tash and Means, 1982). Recently, another methyl-xanthine, 1-methyl 3-isobutyl xanthine, was found to mobilize calcium from storage organelles to cytosol in liver cells (Gabbay and Lardy, 1986).

Fluoride may act by sequestering magnesium and thus inactivate processes requiring MgATP. Although fluoride did not prevent MgATP use by sperm protein kinases, the observation that sperm in manganese-containing buffers reactivated with MnATP were resistant to fluoride immobilization might indicate that magnesium titers have been affected by fluoride treatment. Meyerhof and Schultz (1938) demonstrated that manganese was much more soluble than magnesium in the presence of fluoride and phosphate, and thus, reactions using MnATP might operate in fluoride-treated cells where magnesium is complexed with fluoride. Finally, fluoride could directly inhibit the dynein ATPase in sperm, as has recently been reported in cilia (Blum and Hayes, 1984).

Considering that 8-bromo-cAMP stimulates sperm metabolism but did not restore motility in fluoride-inhibited sperm, it seems probable that caffeine stimulates motility by multiple mechanisms: one involving the production of cAMP and stimulation of protein kinases, and another operating on the mechanism that is affected by fluoride. Studies on the mechanism of fluoride inhibition of sperm motility and of caffeine's ability to relieve that inhibition should help elucidate the biochemical basis of sperm motility.

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