INTACT SPERM PROTEIN PHOSPHORYLATION LEVELS ARE ALTERED BY MODULATORS OF SPERM FUNCTION

by

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ABSTRACT

To accomplish the task of fertilization, spermatozoa need to locate and penetrate the egg. Physiological events necessary for the sperm to complete this task include: motility, capacitation, the acrosome reaction, and sperm-egg fusion. Many reports have described the effects of second messengers such as cAMP and Ca^{2+} on sperm physiology, but few details are known about the molecular mechanisms or biochemical pathways affected by these compounds in sperm. The goal of my research is to determine if modulators of sperm function alter sperm protein phosphorylation levels, and to identify phosphoproteins that may play a role in regulating sperm behavior.

Our previous work focused on the regulation of bovine sperm motility by pH. Bovine sperm are stored in the epididymis in a quiescent state. Upon ejaculation, sperm are diluted with seminal vesicle fluid and vigorous motility initiates. We found that either of two manipulations is sufficient for initiation of motility: 1) elevation of the epididymal fluid pH from 5.8 to 7.0 or 2) a 10-fold dilution of epididymal fluid into buffer at pH 5.8. As bovine epididymal fluid contains a high concentration of a permeant weak acid (8 mM lactate) which is capable of shuttling protons across a membrane, we hypothesized that both pH elevation and dilution were regulating motility by elevating sperm intracellular pH (pHi). To test this hypothesis, the pHi of intact sperm was measured by loading sperm with the fluorescent pH probe, carboxyfluorescein. Sperm pHi increases by approximately 0.4 units in response to both these motility-initiating manipulations.

To determine if this stimulation of motility was accompanied by changes in protein phosphorylation, intact sperm were preloaded with $^{32}PO_4$ and motility was

regulated by changing extracellular pH in the presence or absence of lactate. One phosphoprotein, Mr = 255,000 (pp255), reversibly changes its level of phosphorylation in response to pHi manipulation. However, its level of phosphorylation decreases as motility increases. Three other membrane-associated proteins also change their phosphorylation levels in response to pHi changes, but the changes are minor and can only be detected after subcellular fractionation.

Two other well-known modulators of sperm function, isobutyl-3-methylxanthine (MIX) and Ca^{2+}, also selectively alter the phosphorylation level of pp255. MIX stimulates motility and increase phosphorylation of pp255, while Ca^{2+} inhibits motility and decreases phosphorylation of many proteins, but especially pp255. Ca^{2+} is also involved in the regulation of capacitation and the acrosome reaction. Upon subcellular fractionation, pp255 co-sediments with the sperm heads. The unique sensitivity of pp255 to these agents suggests that phosphorylation of this protein plays a central role in the regulation of sperm behavior. However, based on the inverse relationship between phosphorylation of pp255 and pHi regulated motility, and on localization of pp255 to the sperm head, it seem unlikely that pp255 is an intermediate in the regulation of sperm motility.

An antibody against rat brain microtubule-associated protein 2 (MAP-2) reacts strongly with pp255 on western blots. The shift in electrophoretic mobility of pp255 in response to treatment with MIX or Ca^{2+} are mimicked by mobility changes in the MAP-2 antibody staining pattern. Other evidence suggesting that pp255 is a sperm MAP-2 includes: a) the size of pp255, b) the high degree of phosphorylation per molecule, and c) phosphorylation on a serine residue, all of which are appropriate for MAP-2 as described in other tissues and species. The identification

of MAP-2 in sperm, coupled with details on the regulation of phosphorylation of MAP-2 by intracellular messengers, may provide insight into the biochemical mechanisms of sperm functions as well as contribute to a better understanding of the role of MAP-2 in cell behavior.

Any mechanism that regulates sperm motility must, directly or indirectly, exert an effect on the dynein/microtubule apparatus. The activity of dynein, isolated from bovine sperm, is not significantly affected by the addition of cAMP, MIX, Ca^{2+} or calmodulin to the assay mixture. The catalytic subunit of protein kinase A does phosphorylate several subunits of dynein, but this phosphorylation does not result in increased ATPase activity. We conclude that if a phosphorylation reaction is involved in the regulation of motility, it probably does not work directly on the dynein ATPase activity.

INTRODUCTION

The spermatozoon. Sperm cells are highly differentiated, motile gametes. They are the end product of the process of spermatogenesis, which occurs in the testis. This process involves a series of mitotic and meiotic divisions by spermatocytes and the eventual release of the free cell into the lumen of the seminiferous tubule (1). Sperm cells were first described by Leeuwenhoek in 1677. They are typically one of the smallest cells in an organism and are highly specialized for the task of delivering their haploid DNA to the egg. To accomplish this, they are equipped with a strong flagellum but are unencumbered by cytoplasmic organelles such as ribosomes, endoplasmic reticulum or Golgi apparatus. Mature sperm no longer synthesis proteins other than mitochondrial proteins (2). The DNA in the nucleus is inactive and highly condensed, so that its volume and vulnerability are minimized for transport (3).

Sperm anatomy. Sperm are usually divided into two morphologically and functionally distinct regions, the head and the tail or flagellum (Fig. 1). The head contains the nucleus, the acrosome and lesser amounts of cytoskeletal structures and cytoplasm. The acrosome is a large secretory vesicle that surrounds the anterior end of the nucleus and contains hydrolytic enzymes, which help the sperm to penetrate the egg's outer coat (4). The sperm tail contains a central axoneme, which emanates from a basal body situated slightly posterior to the nucleus. The axoneme consists of two central singlet microtubules surrounded by nine evenly spaced microtubule

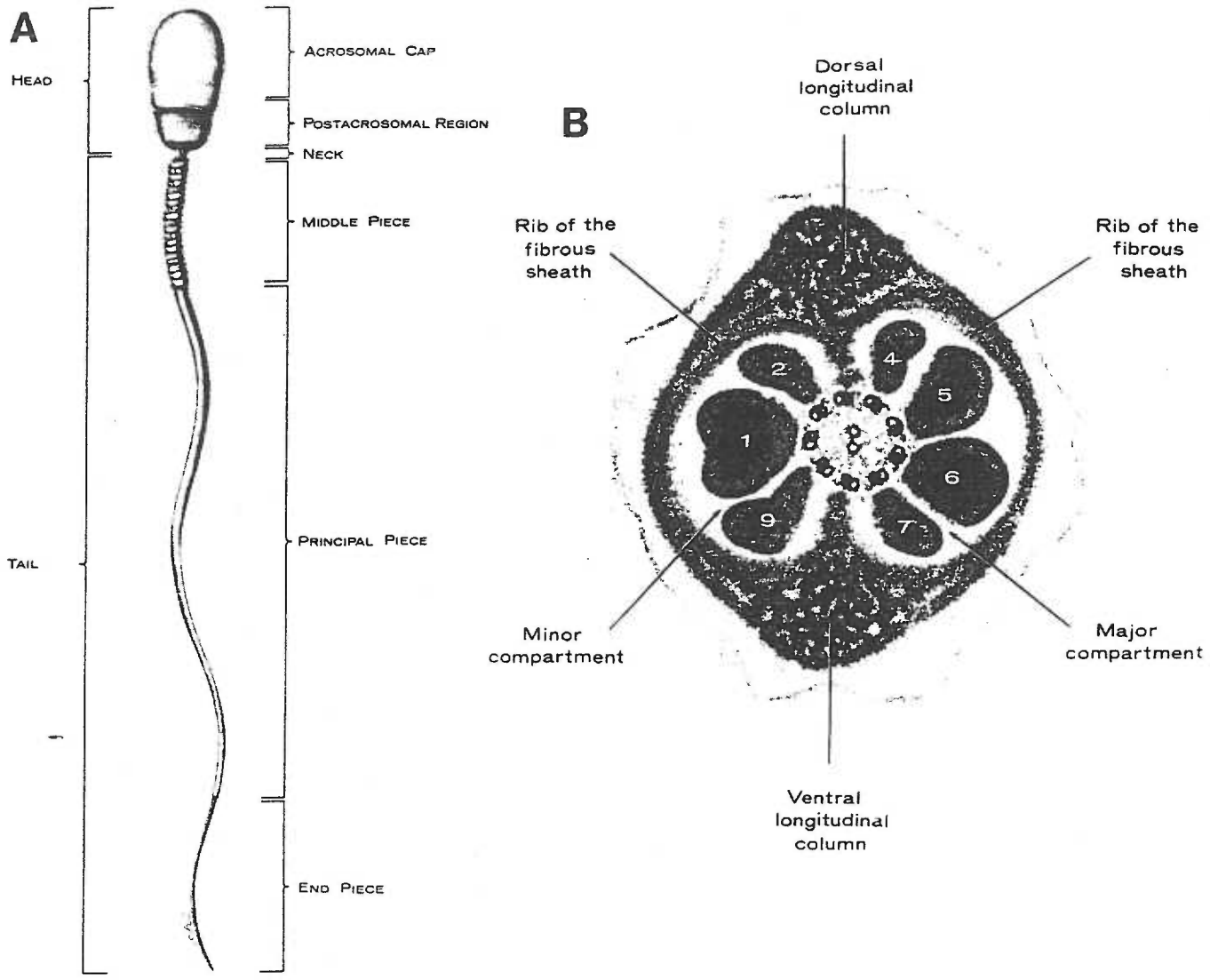

Fig. 1. Drawing of a mammalian spermatozoon as seen with the light microscope (Panel A). Transverse section through the principal piece (Panel B). The axoneme is located in the center of the flagellum and contains 9 outer doublet microtubules and two singlet inner microtubules. The cross section is asymmetrical, with a major compartment containing four outer dense fibers and a minor compartment containing three. The doublet microtubule doublets are numbered the same as the adjacent outer dense fibers. From Fawcett, D.W. (1975) Devel. Biol. 44:394-436.

doublets. The flagellum of mammalian sperm differ from cilia or other flagella in that the axoneme is surrounded by nine outer dense fibers extending from the head to near the posterior end of the axoneme (1). The posterior part of the tail contains the fibrous sheath which surrounds the dense fibers. The dense fibers and the fibrous sheath make up the cytoskeleton of the flagellum, but their role in motility is still unknown. The anterior part of the tail (called the midpiece) contains the mitochondria, which are wrapped in a tight helix around the dense fibers (5). These mitochondria provides the ATP to power flagellar movement.

The sperm plasma membrane tightly surrounds both the head and the tail leaving only a thin layer of cytoplasm (1). The plasma membrane is subdivided into sharply delineated regional domains that differ in composition and function (6). The main regions of the head are the anterior acrosome (acrosomal cap), the equatorial segment and the postacrosomal region covering the portion of the head posterior to the acrosome. These domains are relatively stable, but do undergo changes in organization and composition during the life of the cell (1).

The region connecting the tail to the head is called the neck or connecting piece. The neck actually inserts into the head similar to a ball-and-socket joint (7). The main cytoskeletal components of the neck include two relatively large laminated fibers, called implantation plates, and five smaller laminated fibers, which enter the base of the head (3). These plates merge into a structure that conforms to the implantation socket in the head and is called the capitulum. The implantation socket is formed by a specialized region of the nuclear envelope and a dense plaque of material on the cytoplasmic surface of the nuclear envelope, the basal plate (1).

The space between the two membranes of the nuclear envelope in this region is traversed by regular periodic structures 6 nm wide and 6 nm apart (3). Freeze-fracture studies indicated that the membrane of the nuclear envelope, which lines the implantation socket, contains a dense population of large, regularly-spaced, intramembranous particles surrounding a particle-free region of membrane (8). Fine filaments traverse the narrow region between capitulum and basal plate; presumably, they are responsible for attaching the capitulum of the flagellum to the basal plate of the head (9,10). The basal plate and capitulum are composed of proteins that are soluble in ionic detergent containing a disulfide-bond reducing agent (11,12).

The connecting piece also contains a pair of centrioles. The distal centriole is continuous with the axoneme and is thought to be the template upon which the axonemal microtubules are formed (3). In many species, the distal centriole disintegrates late in spermatogenesis (5). The proximal centriole lies transversely or obliquely between the longitudinally oriented distal centriole and a depression in the capitulum (3). It also disintegrates during the latter part of spermiogenesis in some species.

Sperm physiology To accomplish their biological mission, spermatozoa must move from the male reproductive tract to the egg in the female tract. Once at the egg, the sperm binds to and penetrates the outer layers of the egg, enters the egg by fusing with the egg membrane, activates the egg to block additional sperm penetration, and finally, the sperm DNA must decondense and combine with the DNA from the egg (4). The physiological events necessary for the sperm to

complete all of the above activities include a complex program of motility, capacitation, the acrosome reaction, and membrane fusion with the egg. The detailed molecular events which produce these physiological processes are not well understood.

Capacitation Mammalian spermatozoa, matured in the epididymis and ejaculated, are still not able to fertilize eggs. They must reside in the female genital tract for a period of time and must undergo several biochemical changes before they can fertilize an egg (2). These changes, which render the sperm capable of the acrosome reaction and subsequently fertilization, are collectively call *capacitation* (4).

The events that occur during capacitation are still poorly understood and some investigators still cast doubt on the necessity of sperm capacitation. Epididymal or freshly-ejaculated spermatozoa, which have never been exposed to the female genital tract, are capable of fertilizing eggs, but there is always a time delay before fertilization occurs (4). It seems probable that exposure to the artificial medium used for inseminating eggs mimics the sperm-capacitating action of the female tract. Capacitation is considered to be complete when the sperm are able to undergo the acrosome reaction. The acrosome reaction is an almost universal phenomenon in the animal kingdom, while capacitation is found almost exclusively in mammals (13).

Capacitation is a multistep process, but it seems certain that the removal or alteration of substances which were added to the sperm surface, in the male reproductive tract, constitutes an important part of this process (4,14). Changes in

the plasma membrane during capacitation are reflected by changes in antibody binding, reduction in negative surface charge, modification in lectin-binding ability, changes in membrane fluidity, changes in lipid and protein composition, and release of constraints forming some domain boundaries (1). Other changes that occur during capacitation include: increased metabolism, *hyperactivated* motility, changes in intracellular ions (sodium, potassium and calcium) and changes in the structure of the acrosome and nucleus (2).

Very little is known about the conditions or factors directly controlling capacitation of spermatozoa within the female genital tract. Apparently, no specific substance from the female reproductive tract is required to induce capacitation. Even the systematic deletion of all the components from an artificial medium (usually a modified Krebs-Ringer's solution) produces no one component that is absolutely necessary for capacitation (4). Sperm adenylate cyclase activity increases during capacitation (15-18), and cAMP stimulation of protein kinase may increase sperm plasma membrane protein phosphorylation (19-21) causing changes in membrane properties. Little is known about the role of intracellular Ca^{2+} during capacitation, although some researchers claim that capacitation is dependent on Ca^{2+} availability (82).

Acrosome Reaction The acrosome is a cap-like structure beneath the plasma membrane that sits over the nucleus in the anterior portion of the sperm head and is enclosed by an inner and outer acrosomal membrane. The acrosome is believed to be analogous to a lysosome in that it contains several hydrolytic enzymes (5).

These enzymes are necessary for sperm penetration of and fusion with the plasma membrane of the egg during fertilization (2).

The acrosome reaction involves multiple fusions between the outer acrosomal membrane and the overlying plasma membrane. This enables the contents of the acrosome to escape through the membrane fenestrations (Fig. 2). Natural substances capable of inducing the acrosome reaction include a fucose-sulfate-rich polysaccharide that surrounds sea urchin eggs and compounds in the vitelline layers of the sea urchin egg. In mammals, one of three glycoproteins that makes up the zona pellucida, ZP3, can induce the acrosome reaction (22). However, sperm can undergo the acrosome reaction in vitro, in the absence of any egg-associated material (4). Conditions or agents which, directly or indirectly, alter plasma membrane permeability to ions (e.g. Ca^{2+} and Na^{+}), trigger the acrosome reaction in capacitated sperm (4).

Ca^{2+} and pH are thought to be the primary intracellular triggers controlling the acrosome reaction. According to a working model presented by Yanagimachi (4), when capacitated mammalian sperm come into contact with the zona pellucida, the glycoproteins of the zona bind to and activate putative sperm receptors. These receptors are Ca^{2+}-transport proteins which promote a massive influx of Ca^{2+}. The high Ca^{2+} concentration inactivates a membrane Na^{+}-K^{+} ATPase, resulting in a rapid increase in intracellular Na^{+}. This initiates an efflux of H^{+} (through Na^{+}/H^{+} antiporter), resulting in a rise in intracellular pH. The increased Ca^{2+} facilitates the fusion of the plasma membrane and the outer acrosomal membrane by binding to anionic phospholipids and inducing a phase separation of membrane phospholipids.

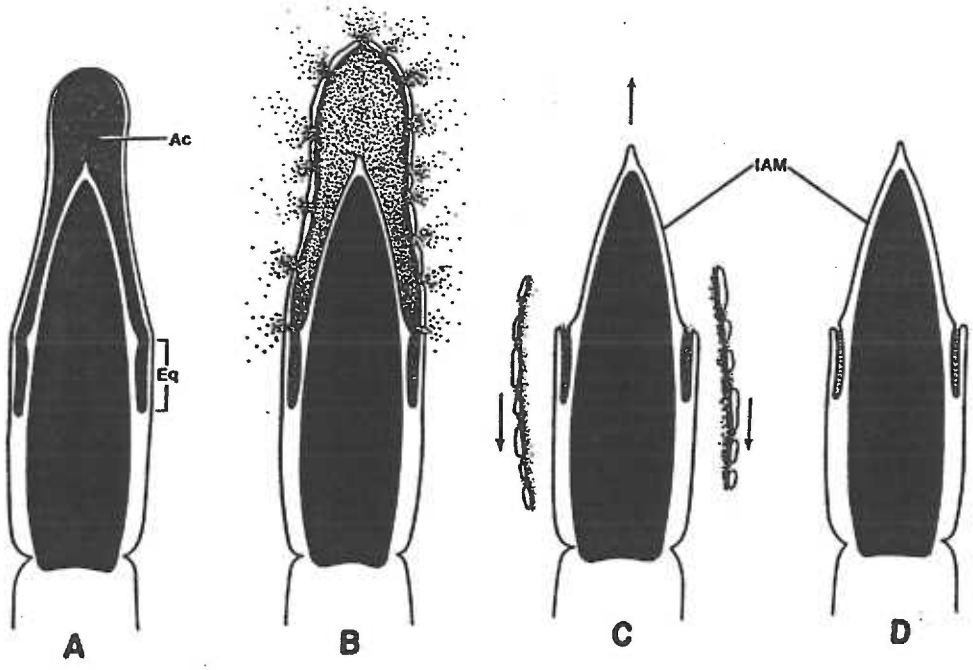

Fig. 2. Sequence of the acrosome reaction of mammalian sperm. Acrosome (Ac), Equatorial Segment (Eq), Inner Acrosomal Membrane (IAM). From Yanagimachi, R. (1988) The Physiology of Reproduction p. 148.

When the membranes are about to fuse or have fused, Ca^{2+} enters and H^{+} leaves the acrosome matrix, initiating the conversion of proacrosin to enzymatically active acrosin, which disperses the acrosomal matrix containing other enzymes. Although intracellular cAMP concentration increases dramatically in sea urchin sperm under the conditions that initiate the acrosome reaction (23), its physiologic significance is unclear. It has also been shown that cAMP affects the rate of the acrosome reaction (24-26).

Sperm-egg Interaction Sperm bind tightly to the zone pellucida while penetrating into it. Sperm membranes have proteins or glycoproteins with strong affinities for zona pellucida proteins (ZP2 and ZP3) (27,28). In the mouse, the sperm's receptors for ZP3 are located on the plasma membrane over the acrosomal cap. When this membrane disappears, during the acrosomal reaction, a new set of receptors are unmasked on the inner acrosomal membrane that bind to ZP2 (29). The structural nature of these sperm receptors is still unknown. However, Yanagimachi (4) has postulated that an insoluble, "sticky" component of the mammalian acrosomal matrix, which supports the fenestrated membranes during the acrosome reaction, may also play a role analogous to the "bindin" protein of sea urchin spermatozoa. Bindin is the sea urchin sperm protein which sticks to the vitelline coat of the sea urchin egg (30).

After passing through the zona pellucida, the sperm head attaches to and fuses with the egg plasma membrane. Careful examination of spermatozoa in the act of fertilizing zona-intact eggs has revealed that it is the plasma membrane above

the equatorial segment of the sperm that fuses first with the egg plasma membrane (31). Only sperm which have undergone the acrosomal reaction are capable of fusing with the egg (32).

Motility Sperm movement (motility) results from the propagation of a bending wave down the flagella. The flagellar wave moves in a plane perpendicular to the central pair of microtubules of the axoneme, and passes through doublet 1 and between doublets 5 and 6, with the active stroke being toward doublet 1 (33). The force for this bending is produced by the hydrolysis of ATP, which powers the cross-bridge cycles between dynein ATPases and the microtubule doublets (34).

Mammalian spermatozoa leaving the testis cannot fertilize eggs (4). They gain this ability while making the slow passage through the epididymis (epididymal maturation). The epididymis is a long convoluted tubule, divided into three regions, the caput, the corpus and the cauda. Sperm leaving the testis are moved by peristalsis through the caput and corpus to be stored in the cauda epididymis and the vas deferens. One of the most prominent changes in sperm, during this maturation process, is the development of the capacity for motility (2). Sperm removed from the bovine caput epididymis are motionless or weakly motile (35, 37, 44). Fully mature sperm, isolated from the cauda epididymis, initiate progressive motility upon dilution into physiological salt solutions (35). Most species apparently store sperm in a non-motile, quiescent state in the caudal epididymis to conserve energy (2, 68). Motility is initiated at ejaculation by dilution into accessory gland fluid. Dilution of caudal sperm into physiological buffers mimics this motility

initiation. Bovine sperm quiescence is apparently maintained by acidification of internal pH (36). This lowered intracellular pH (pHi) is due to an acidic external pH (caudal epididymal fluid pH=5.8) and the presence of a permeant weak acid. Caudal epididymal fluid contains 8 mM lactate, which can cross the membrane and contribute protons to the sperm cytoplasm. Dilution of the sperm into physiological buffer raises the external pH and also dilutes the concentration of permeable weak acid.

Structural changes in spermatozoa undergoing maturation include migration of the cytoplasmic droplet (a cytoplasmic remnant which has been severed from the residual body of the spermatid at the time of spermiation), reduction in the amount of cytoplasm, and alteration in the size, shape and contents of the plasma membrane and other organelles such as the acrosome and the midpiece. Changes have been reported in surface charge, lectin binding, intramembranous particle distribution, membrane fluidity, lipid composition, protein composition, and antibody binding (1). Transfer of several substances from the epididymal fluid to the spermatozoa, such as "forward motility protein" (37-39), carnitine (40) and glycerophosphocholine (41), is believed to be important for the development of sperm's ability to move. Tail components, e.g. the coarse outer fibers and fibrous sheath, and the chromatin are stabilized by disulfide bonds which develop during maturation (11).

Metabolic changes also appear to be important for the acquisition of the capacity for motility. The cAMP levels in sperm from a number of species (42,43) increase during epididymal transit. In addition, cAMP phosphodiesterase inhibitors, which elevate levels of cAMP in sperm, can induce motility in caput sperm (44).

Although a variety of factors appear to be involved in the initiation and regulation of sperm motility (e.g. Ca^{2+}, cAMP, pHi, adenosine, and various proteins), a unifying hypothesis for how these factors interact to accomplish this regulation is lacking (1). The slow progress in this area is partially due to the lack of a clearly identifiable extracellular messenger (1st messenger) that regulates sperm motility (45). Another problem is the complexity of motility regulation itself. It is postulated that at least three levels of control are necessary to account for the beating motion of an axoneme (46). Simultaneous sliding of all 9 outer doublets would produce no bending or movement, so there must exist a control system or switch that activate only one side of the axoneme at a time. Control systems are also necessary to explain the several different types of wave forms observed in cilia and flagella, including variations in amplitude, direction and beat frequency. The third control mechanism is necessary for the initiation and perhaps the maintenance of motility. This last mechanism is the one which most likely is regulated by a protein phosphorylation reaction (47).

Evidence supporting a stimulatory role for cAMP in regulation of sperm motility include: a) cAMP levels increase in sperm during epididymal transit (42,43); b) a number of phosphodiesterase inhibitors, which elevate levels of cAMP in sperm, stimulate motility (35,48,49); c) several compounds, such as forskolin and adenosine, stimulate both cAMP and motility (80-81); and d) the addition of cAMP to Triton X-100-demembranated sperm, reactivated with Mg-ATP, stimulates motility (50). As in other cells, the major role of cAMP in sperm is probably to mediate cAMP-dependent protein phosphorylation(s), which initiate and/or maintain motility (45).

In demembranated sperm models, addition of exogenous protein kinase stimulates motility; inhibitors of protein kinase inhibit cAMP stimulated motility (51).

Attempts to identify a protein substrate for this kinase reaction have mostly been conducted in demembranated sperm models. Much attention has been given to a 56,000 Dalton phosphoprotein thought to be involved in motility regulation (51-53). This protein was named axokinin and shown to be required and sufficient for motility in demembranated dog sperm (51,53). In subsequent studies, axokinin was identified by other investigators as the regulatory subunit of protein kinase II (54,55), and its role in motility regulation is controversial. In trout sperm, a protein (Mr = 15,000) that is phosphorylated on a tyrosine residue by a cAMP-dependent kinase appears to be required for initiation of axonemal movement (56). A phosphorylated soluble sea urchin protein factor of unknown size appears to be necessary for reactivation of demembranated sea urchin sperm (57). Treatment of sea urchin sperm with egg jelly induces the phosphorylation of histone H1 by a cAMP-dependent protein kinase but the biological function of this phosphorylation is not yet known (58).

The role of Ca^{2+} in the regulation of motility appears to be more complex and often in opposition to that of cAMP. Depending on the concentration of Ca^{2+} and the sperm species, responses to Ca^{2+} are numerous and varied (59). Calcium modifies flagellar and ciliary waveforms at free cation concentrations between 0.1 and 1 μM, and inhibits motility completely at higher concentrations (60-62). Swimming parameters (e.g. beat frequency or curvature of bending wave) for rat (63,64) and dog (61,62) sperm models are modified by Ca^{2+}. The identification of

a calmodulin-dependent phosphoprotein phosphatase (calcineurin) in dog, pig and sea urchin sperm (61) may explain the Ca^{2+} inhibition of motility.

The involvement of pHi in motility regulation is probably best understood in sea urchin sperm. Upon release into sea water, exposure to increased extracellular sodium ions activates a Na^+/H^+ exchange pump, which increases pHi and stimulates motility (65-67). The pHi shift is thought to have a direct effect on dynein ATPase activity (66). In mammals, a change in pHi at the time of ejaculation has been hypothesized to regulate the initiation of sperm motility (36,68).

Although these intracellular regulators (cAMP, Ca^{2+}, and pHi) can separately regulate motility, several reports suggests a synergism exists between two or more of these modulators. The addition of Ca^{2+} causes a 25-fold increase in cAMP in guinea pig sperm, but only in the presence of bicarbonate (a known regulator of intracellular pH)(69); the elevation of sea urchin cAMP by egg factors requires the presence of Ca^{2+} (70); and the activation of motility by cAMP is pH dependent (71,72).

Dynein Flagellar motion is dependent on dynein, a mechanochemical enzyme that interacts with microtubules to generate force. Inner and outer dynein arms are attached to the A tubule of the microtubule doublet at regular 24 nm intervals (Fig. 3). Other structures in the axoneme include the nexin links, which connect the microtubule doublets and are thought to constrain microtubule sliding; and the radial spokes, which connect the doublets to the central pair of single microtubules surrounded by an inner sheath. Mutants lacking radial spokes and the central

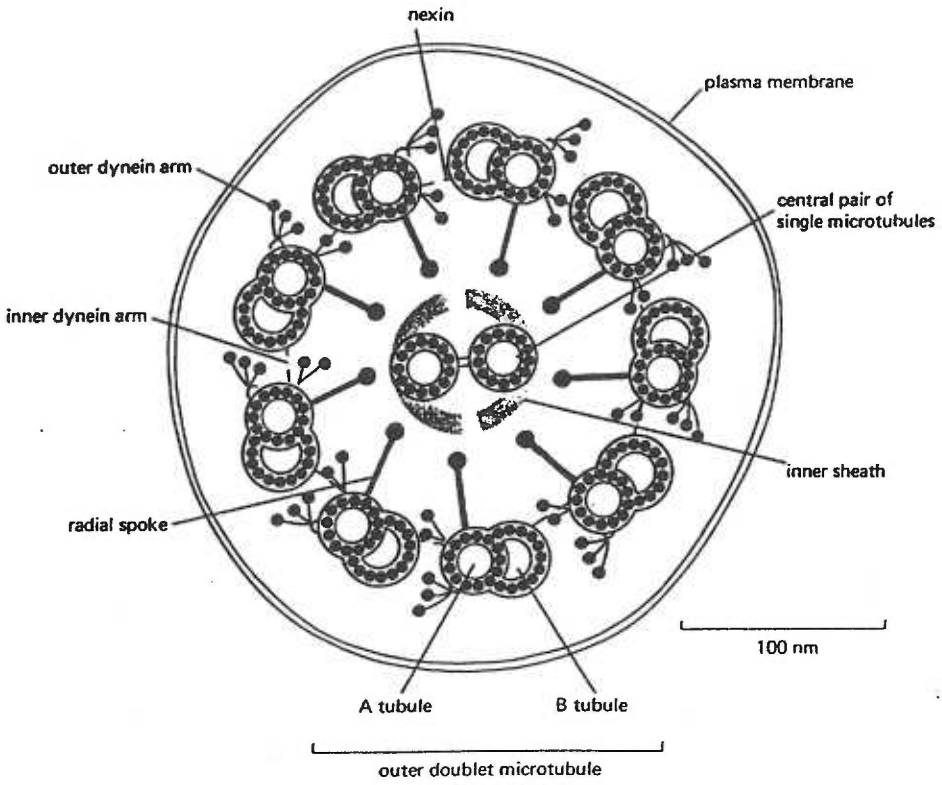

Fig. 3. Schematic diagram of a cilium shown in cross-section. The various projections from the microtubules occur at regular intervals along the cilium. From Alberts, B. et al. (1989) Molecular Biology of the Cell p. 647.

microtubule pairs still beat, suggesting that they are not essential for motility (73).

The bending force is produced by the coordinated sliding of microtubules. Mild tryptic digestion of isolated axonemes disrupts both the nexin links and the radial spokes and causes the nine doublet microtubules to actively slide or telescope out of the tip of the tail, when reactivated by MgATP (74). In the intact structure, this sliding is converted to bending.

Each dynein arm consist of a large protein complex containing two or three globular heads linked to a common root by thin flexible strands. Each dynein complex contains two to three "heavy chains", (Mr > 300 kDa) one for each globular domain, and several chains of intermediate (40-120 kDa) and low (15-25 kDa) molecular masses. The large globular domain contains the ATPase activity and also an ATP-sensitive microtubule binding site (47). This binding site is thought to be involved in the transient association that occurs between the dynein arm and the B tubule of the outer doublet (75). The opposite end of dynein is attached to the A tubule in an ATP-insensitive manner.

Little has been published concerning the regulation of dynein ATPase activity. Microtubules can stimulate its activity about 6 fold (76), but this does not account for the initiation of motility or the changes in waveform which are seen with the addition of Ca^{2+}. Regulation of dynein activity by phosphorylation has been postulated, and several reports demonstrate that dynein can be phosphorylated in intact sperm and in vitro (77-79), but there has been no report describing the effect of in vitro phosphorylation on dynein ATPase activity.

Thesis Rationale The focus of my research is to examine the involvement of protein phosphorylation in the regulation of sperm functions. This includes studies on the effects of known modulators, such as pHi, Ca^{2+} and MIX, on intact sperm protein phosphorylation; the identification of phosphoproteins that may be important in sperm functions; and the effects of phosphorylation on sperm enzymes.

Previous work has shown that the initiation of motility of bovine sperm upon ejaculation is regulated by pH. Many reports also suggest that a protein phosphorylation reaction plays a role in regulating the initiation of motility, capacitation and the acrosome reaction. Compounds that regulate motility, such as methyl xanthines and Ca^{2+}, also change cAMP concentrations. Calcium is also thought to be the primary trigger for the acrosome reactions and may be required for capacitation. Although a phosphorylation event is thought to be involved in these functions, a physiologically important substrate for a protein kinase reaction has yet to be identified. My thesis work has focused on trying to identify which sperm phosphoproteins may play an important role in regulation of sperm functions. In pursuit of these phosphoproteins, I have taken both an upstream and a downstream approach. To effect sperm motility, one eventually has to exert an effect on the motility motor or dynein ATPase. To determine if phosphorylation effects dynein directly, I have isolated and characterized dynein from bovine sperm and compared its ATPase activity before and after phosphorylation.

The downstream approach involves using physiological regulators such as pH, calcium and methyl xanthine to determine which phosphoproteins are altered by their addition. I found one phosphoprotein which was particularly sensitive to

regulation by all these agents, subsequently I localized, characterized and immunologically identified it as MAP-2.

REFERENCES

1. Eddy, E.M. (1988) in *The physiology of reproduction* (Knobil, E., and Neill, J., eds) pp. 27-68, Raven Press, Ltd., New York

2. Zaneveld, L.J.D., and Chatterton, R.T. (1982) *Biochemistry of mammalian reproduction*, John Wiley and Sons, Inc., New York

3. Fawcett, D.W. (1975) The mammalian spermatozoon. *Dev. Biol.* **44**, 394-436

4. Yanagimachi, R. (1988) in *The physiology of reproduction* (Knobil, E., and Neill, J., eds) pp. 135-185, Raven Press, Ltd., New York

5. Mann, T., and Lutwak-Mann, C. (1981) *Male Reproductive Function and Semen*, Springer-Verlag, Berlin

6. Holt, W.V. (1984) in *International Review of Cytology, Vol. 87* (Bourne, G.H., and Danielli, J.F., eds) pp. 159-194, Academic Press, New York

7. Saacke, R.G., and Almquist, J.O. (1963) Ultrastructure of bovine spermatozoa. II. The neck and tail of normal, ejaculated sperm. *Am. J. Anat.* **115**, 163-184

8. Friend, D.S., and Fawcett, D.W. (1974) Membrane differentiations in freeze-fractured mammalian sperm. *J. Cell Biol.* **63**, 641-664

9. Wooding, F.B.P. (1973) The effect of Triton X-100 on the ultrastructure of ejaculated bovine sperm. *J. Ultrastruct. Res.* **42**, 502-516

10. Pedersen, H. (1972) Further observations on the fine structure of the human spermatozoon. *Z. Zellforsch.* **123**, 305-315

11. Bedford, J.M., and Calvin, H.I. (1974) Changes in the -S-S- linked structures of the sperm tail during epididymal maturation with comparative observations in

sub-mammalian species. *J. Exp. Zool.* **187**, 181-204

12. Bellve, A.R., Anderson, E., and Hanley-Bowdoin, L. (1975) Synthesis and amino acid composition of basic proteins in mammalian sperm nuclei. *Dev. Biol.* **47**, 349-365

13. Bedford, J.M. (1983) Significance of the need for sperm capacitation before fertilization in eutherian mammals. *Biol. Reprod.* **28**, 108-120

14. Oliphant, G., Reynolds, A.B., and Thomas, T.S. (1985) Sperm surface components involved in the control of the acrosome reaction. *Am. J. Anat.* **174**, 269-283

15. Berger, T., and Clegg, E.D. (1983) Adenylate cyclase activity in porcine sperm in response to female reproductive tract secretions. *Gamete. Res.* **7**, 169-177

16. Morton, B.E., and Albagli, L. (1973) Modification of hamster sperm adenyl cyclase by capacitation in vitro. *Biochem. Biophys. Res. Commun.* **50**, 695-703

17. Stein, D.M., and Fraser, L.R. (1984) Cyclic nucleotide metabolism in mouse epididymal spermatozoa during capacitation in vitro. *Gamete. Res.* **10**, 283-299

18. Stein, D.M., Fraser, L.R., and Monks, N.J. (1986) Adenosine and Gpp(NH)p modulate mouse sperm adenylate cyclase. *Gamete. Res.* **13**, 151-158

19. Huacuja, L., Delgado, N.M., Merchant, H., Pancardo, R.M., and Rosado, A. (1977) Cyclic AMP induced incorporation of 33Pi into human sperm membrane components. *Biol. Reprod.* **17**, 89-96

20. Hyne, R.V., and Edwards, K.P. (1985) Influence of 2-deoxyl-D-glucose and energy substrates on guinea-pig sperm capacitation and acrosome reaction. *J. Reprod. Fertil.* **73**, 59-69

21. Noland, T.D., Olson, G.E., and Garbers, D.L. (1984) Protein phosphorylation of plasma membranes from bovine epididymal spermatozoa. *Biol. Reprod.* **31**, 185-194

22. Wassarman, P.M. (1987) The biology and chemistry of fertilization. *Science* **235**, 553-560

23. Garbers, D.L. (1981) The elevation of cyclic AMP concentrations in flagella-less sea urchin sperm heads. *J. Biol. Chem.* **256**, 620-624

24. Hyne, R.V., and Garbers, D.L. (1979) Calcium-dependent increase in adenosine 3',5'-monophosphate and induction of the acrosome reaction in guinea pig spermatozoa. *Proc. Natl. Acad. Sci. USA* **76**, 5699-5703

25. Hyne, R.V., and Garbers, D.L. (1979) Regulation of guinea pig sperm adenylate cyclase by calcium. *Biol. Reprod.* **21**, 1135-1142

26. Mrsny, R.J., and Meizel, S. (1980) Evidence suggesting a role for cyclic nucleotides in acrosome reactions of hamster sperm in vitro. *J. Exp. Zool.* **211**, 153-157

27. O'Rand, M.G., Matthews, J.E., Welch, J.E., and Fisher, S.J. (1985) Identification of zona binding proteins of rabbit, pig, human and mouse spermatozoa on nitrocellulose blots. *J. Exp. Zool.* **235**, 423-428

28. Sullivan, R., and Bleau, G. (1985) Interaction of isolated components from mammalian sperm and egg. *Gamete. Res.* **12**, 101-116

29. Bleil, J.D., and Wassarman, P.M. (1986) Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm. *J. Cell Biol.* **102**, 1363-1371

30. Yanagimachi, R., and Phillips, D.M. (1984) The status of acrosomal caps of hamster spermatozoa immediately before fertilization in vitro. *Gamete. Res.* **9**, 1-19

31. Bedford, J.M., Moore, H.D.M., and Franklin, L.E. (1979) Significance of the equatorial segment of the acrosome of the spermatozoa in eutherian mammals. *Exp. Cell Res.* **119**, 119-126

32. Yanagimachi, R., and Noda, Y.D. (1970) Physiological changes in the post-nuclear cap region of mammalian spermatozoa: A necessary preliminary to the membrane fusion between sperm and egg cells. *J. Ultrastruct. Res.* **31**, 486-493

33. Wassarman, P.M., Florman, H.M., and Greve, J.M. (1985) in *Fertilization, Vol. 2* (Metz, C.B., and Monroy, A., eds) pp. 341-360, Academic Press, New York

34. Bell, C.W., Fraser, C., Sale, W.S., Tang, W.J., and Gibbons, I.R. (1982) Preparation and purification of dynein. *Methods. Cell Biol.* **24**, 373-397

35. Carr, D.W., and Acott, T.S. (1984) Inhibition of bovine spermatozoa by caudal epididymal fluid: I. Studies of a sperm motility quiescence factor. *Biol. Reprod.* **30**, 913-925

36. Acott, T.S., and Carr, D.W. (1984) Inhibition of bovine spermatozoa by caudal epididymal fluid: II Interaction of pH and a quiescence factor. *Biol. Reprod.* **30**, 926-935

37. Acott, T.S., and Hoskins, D.D. (1978) Bovine sperm forward motility protein. Partial purification and characterization. *J. Biol. Chem.* **253**, 6744-6750

38. Acott, T.S., and Hoskins, D.D. (1981) Bovine sperm forward motility protein: binding to epididymal spermatozoa. *Biol. Reprod.* **24**, 234-240

39. Acott, T.S., Katz, D.F., and Hoskins, D.D. (1983) Movement characteristics of bovine epididymal spermatozoa: effects of forward motility protein and epididymal maturation. *Biol. Reprod.* **29**, 389-399

40. Casillas, E.R. (1973) Accumulation of carnitin by bovine spermatozoa during maturation in the epididymis. *J. Biol. Chem.* **248**, 8227-8232

41. Infante, J.P., and Huszagh, V.A. (1985) Synthesis of highly unsaturated phosphatidylcholine in the development of sperm motility: A role for epididymal glycerol-3-phosphorylcholine. *Mol. Cell. Biochem.* **69**, 3-9

42. Amann, R.P., Hay, S.R., and Hammerstedt, R.H. (1982) Yield, characteristics, motility and cAMP content of sperm isolated from seven regions of ram epididymis. *Biol. Reprod.* **27**, 723-733

43. Hoskins, D.D., Stephens, D.T., and Hall, M.L. (1974) Cyclic adenosine 3'5'-monophosphate and protein kinase levels in developing bovine spermatozoa. *J. Reprod. Fertil.* **37**, 131-133

44. Hoskins, D.D., Hall, M.L., and Munsterman, D. (1975) Induction of motility in immature bovine spermatozoa by cyclic AMP phosphodiesterase inhibitors. *Biol. Reprod.* **13**, 168-176

45. Garbers, D.L., and Kopf, G.S. (1980) The regulation of spermatozoa by calcium cyclic nucleotides. *Adv. Cyclic. Nucleotide. Res.* **13**, 251-306

46. Gibbons, I.R. (1989) in *Cell Movement: Vol. 1. The Dynein ATPases* (Warner, F.D., Satir, P., and Gibbons, I.R., eds) pp. 3-22, Alan R. Liss, Inc., New York

47. Johnson, K.A. (1985) Pathway of the microtubule-dynein ATPase and the structure of dynein: a comparison with actomyosin. *Annu. Rev. Biophys. Biophys. CHEM.* **14**, 161-188

48. Garbers, D.L., First, N.L., Sullivan, J.J., and Lardy, H.A. (1971) Stimulation and maintenance of ejaculated bovine spermatozoan respiration and motility by caffeine.

Biol. Reprod. **5**, 336-339

49. Garbers, D.L., Lust, W.D., First, N.L., and Lardy, H.A. (1971) Effects of phosphodiesterase inhibitors and cyclic nucleotides on sperm respiration and motility. *Biochemistry.* **10**, 1825-1831

50. Lindemann, C.B. (1978) A cAMP-induced increase in the motility of demembranated bull sperm models. *Cell* **13**, 9-18

51. Tash, J.S., Kakar, S.S., and Means, A.R. (1984) Flagellar motility requires the cAMP-dependent phosphorylation of a heat-stable NP-40-soluble 56 kd protein, axokinin. *Cell* **38**, 551-559

52. Brandt, H., and Hoskins, D.D. (1980) A cAMP-dependent phosphorylated motility protein in bovine epididymal sperm. *J. Biol. Chem.* **255**, 982-987

53. Tash, J.S., Hidaka, H., and Means, A.R. (1986) Axokinin phosphorylation by cAMP-dependent protein kinase is sufficient for activation of sperm flagellar motility. *J. Cell Biol.* **103**, 649-655

54. Noland, T.D., Abumrad, N.A., Beth, A.H., and Garbers, D.L. (1987) Protein phosphorylation in intact bovine epididymal spermatozoa: identification of the type II regulatory subunit of cyclic adenosine 3',5'-monophosphate-dependent protein kinase as an endogenous phosphoprotein. *Biol. Reprod.* **37**, 171-180

55. Paupard, M.C., MacLeod, J., Wasco, W., and Orr, G.A. (1988) Major 56,000-dalton, soluble phosphoprotein present in bovine sperm is the regulatory subunit of a type II cAMP-dependent protein kinase. *J. Cell Biochem.* **37**, 161-175

56. Hayashi, H., Yamamoto, K., Yonekawa, H., and Morisawa, M. (1987) Involvement of tyrosine protein kinase in the initiation of flagellar movement in

rainbow trout spermatozoa. *J. Biol. Chem.* **262**, 16692-16698

57. Ishiguro, K., Murofushi, H., and Sakai, H. (1982) Evidence that cAMP-dependent protein kinase and a protein factor are involved in reactivation of triton X-100 models of sea urchin and starfish spermatozoa. *J. Cell Biol.* **92**, 777-782

58. Porter, D.C., Moy, G.W., and Vacquier, V.D. (1988) CAMP-dependent protein kinase of sea urchin sperm phosphorylates sperm histone H1 on a single site. *J. Biol. Chem.* **263**, 2750-2755

59. Otter, T. (1989) in *Cell movement, Vol. 1: The dynein ATPases* (Warner, F.D., Satir, P., and Gibbons, I.R., eds) pp. 281-298, Alan R. Liss, Inc., New York

60. Brokaw, C.J. (1987) Regulation of sperm flagellar motility by calcium and cAMP-dependent phosphorylation. *J. Cell Biochem.* **35**, 175-184

61. Tash, J.S., Krinks, M., Patel, J., Means, R.L., Klee, C.B., and Means, A.R. (1988) Identification, characterization, and functional correlation of calmodulin-dependent protein phosphatase in sperm. *J. Cell Biol.* **106**, 1625-1633

62. Tash, J.S., and Means, A.R. (1982) Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. *Biol. Reprod.* **26**, 745-763

63. Lindemann, C.B., and Goltz, J.S. (1988) Calcium regulation of flagellar curvature and swimming pattern in triton X-100--extracted rat sperm. *Cell Motil. Cytoskeleton.* **10**, 420-431

64. Lindemann, C.B., Goltz, J.S., and Kanous, K.S. (1987) Regulation of activation state and flagellar wave form in epididymal rat sperm: evidence for the involvement of both Ca2+ and cAMP. *Cell Motil. Cytoskeleton.* **8**, 324-332

65. Christen, R., Schackmann, R.W., and Shapiro, B.M. (1982) Elevation of the intracellular pH activates respiration and motility of sperm of the sea urchin, Strongylocentrotus purpuratus. *J. Biol. Chem.* **257**, 14881-14890

66. Christen, R., Schackmann, R.W., and Shapiro, B.M. (1983) Metabolism of sea urchin sperm. Interrelationships between intracellular pH, ATPase activity, and mitochondrial respiration. *J. Biol. Chem.* **258**, 5392-5399

67. Lee, H.C., Johnson, C., and Epel, D. (1983) Changes in internal pH associated with initiation of motility and acrosome reaction of sea urchin sperm. *Dev. Biol.* **95**, 31-45

68. Carr, D.W., Usselman, M.C., and Acott, T.S. (1985) Effects of pH, lactate, and viscoelastic drag on sperm motility: a species comparison. *Biol. Reprod.* **33**, 588-595

69. Garbers, D.L., Tubb, D.J., and Hyne, R.V. (1982) A requirement of bicarbonate for Ca2+-induced elevations of cyclic AMP in guinea pig spermatozoa. *J. Biol. Chem.* **257**, 8980-8984

70. Tubb, D.J., Kopf, G.S., and Garbers, D.L. (1978) The elevation of sperm adenosine 3',5'-monophosphate concentrations by factors released from eggs requires calcium. *Biol. Reprod.* **18**, 181-185

71. Vijayaraghavan, S., Critchlow, L.M., and Hoskins, D.D. (1985) Evidence for a role for cellular alkalinization in the cyclic adenosine 3',5'-monophosphate-mediated initiation of motility in bovine caput spermatozoa. *Biol. Reprod.* **32**, 489-500

72. Goltz, J.S., Gardner, T.K., Kanous, K.S., and Lindemann, C.B. (1988) The interaction of pH and cyclic adenosine 3',5'-monophosphate on activation of motility in Triton X-100 extracted bull sperm. *Biol. Reprod.* **39**, 1129-1136

73. Brokaw, C.J., and Luck, D.J.L. (1985) Bending patterns of Chlamydomonas flagella. III. A radial spoke head deficient mutant and a central pair deficient mutant. *Cell Motil.* **5**, 195-208

74. Bell, C.W., and Gibbons, I.R. (1983) Preparation and properties of dynein ATPase. *Muscle and Nonmuscle Motil.* **2**, 1-36

75. McIntosh, J.R., and Porter, M.E. (1989) Enzymes for microtubule-dependent motility. *J. Biol. Chem.* **264**, 6001-6004

76. Omoto, C.K., and Johnson, K.A. (1986) Activation of the dynein adenosinetriphosphatase by microtubules. *Biochemistry.* **25**, 419-427

77. Piperno, G., and Luck, D.J. (1981) Inner arm dyneins from flagella of Chlamydomonas reinhardtii. *Cell* **27**, 331-340

78. Nakamura, K.I., and Masuyama, E. (1977) Studies on the initial phase of dynein ATPase activity. *Biochim. Biophys. Acta* **481**, 660-666

79. Travis, S.M., and Nelson, D.L. (1988) Regulation of axonemal Mg2+-ATPase from Paramecium cilia: effects of Ca2+ and cyclic nucleotides. *Biochim. Biophys. Acta* **966**, 84-93

80. Vijayaraghavan, S. and Hoskins, D.D. (1985) Forskolin stimulates bovine epididymal sperm motility and cyclic AMP levels. *J. Cyclic Nucleo. Prot. Phos. Res.* **10**, 499-510

81. Vijayaraghavan, S. and Hoskins, D.D. (1986) Regulation of bovine sperm motility and cyclic adenosine 3',5'-monophosphate by adenosine and its analogues. *Biol. Reprod.* **34**, 468-477

82. Lindemann, C.B. and Kanous, K.S. Regulation of mammalian sperm motility. *Arch. Androl.* **23**, 1-22

MANUSCRIPT 1

Intracellular pH Regulates Bovine Sperm Motility and Protein Phosphorylation[1]

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ABSTRACT

Bovine sperm, suspended in neat caudal epididymal fluid, become motile in response to either pH elevation or dilution of the fluid with no change in pH. Buffers, containing permeant weak acids at physiologic concentrations, are able to mimic the epididymal fluid environment. These observations led to the hypothesis that a pH-dependent epididymal fluid quiescence factor regulates bovine sperm motility by modulating sperm intracellular pH (pH_i). Here, we report that sperm pH_i, measured with the fluorescent pH probe carboxyfluorescein, increases by ≈0.4 units in response to either of these motility-initiating manipulations.

At least 26 discrete phosphoprotein bands are distinguishable by SDS-PAGE after incubation of intact caudal sperm with $^{32}PO_4$. A prominent phosphoprotein, with Mr ≈ 255,000 (pp255) and a relatively high specific radioactivity, is reversibly dephosphorylated in response to elevations of pH_i that initiate sperm motility. Unlike most of the sperm phosphoproteins, the extraction of pp255 requires reducing agents. This phosphoprotein cosediments with the sperm heads but not the tail, midpiece, soluble, or plasma membrane fractions. No other pH_i-dependent phosphorylation changes are apparent in gels of whole sperm extracts. However, subcellular fractionation allows the detection of increased phosphorylation of two plasma membrane phosphoproteins (Mr ≈ 105,000 and 97,000) and decreased phosphorylation of another plasma membrane phosphoprotein (Mr ≈ 120,000) in response to increasing pH_i. This is the first report describing changes in endogenous phosphoproteins from intact motile and nonmotile bovine sperm that are regulated by pH_i.

INTRODUCTION

Both cAMP and intracellular pH (pH_i) have been implicated as regulators of sperm motility and some investigators have speculated that these two regulators are interrelated (Goltz et al., 1988). Although a physiological first messenger remains elusive, other compounds (e.g. caffeine or forskolin) that stimulate motility also increase levels of sperm cAMP (for reviews see: Garbers and Kopf, 1980; Tash and Means, 1982; Brokaw, 1987). Sperm intracellular pH also regulates motility. The events involved in the motility initiation of the sea urchin sperm are perhaps the best understood. Upon release into sea water, an increase in sodium ions activates a Na^+/H^+ pump which increases pHi and stimulates motility (Christen et al., 1982, 1983; Lee et al., 1983). Although the elevated pHi is thought to directly activate dynein ATPase (Christen et al., 1983), other investigators still postulate a cAMP-dependent phosphorylation is also involved in the regulation of sea urchin motility (Brokaw, 1987). In other species, the cAMP activation of motility has been shown to be pH dependent (Brokaw, 1987; Goltz et al., 1988).

A change in pH_i, at the time of ejaculation, has been hypothesized to regulate the initiation of bovine sperm motility (Acott and Carr, 1984). Mature bovine sperm are stored in the cauda epididymidis prior to ejaculation and, when examined in undiluted caudal fluid, exhibit minimal motility. The pH of this fluid is 5.8; if the pH is elevated to approximately 7.0 (without dilution) or the fluid is diluted approximately 1 to 10 (with no change in pH), the sperm become vigorously motile (Acott and Carr, 1984; Carr and Acott, 1984). Both of these manipulations (dilution at same pH and an elevation of fluid pH) would be expected to increase internal

pH, if there were permeant weak acids in the fluid (Babcock et al. 1983). Both dilution and an elevation of pH occur during ejaculation; either is sufficient to produce reversible motility initiation. These observations lead to the conclusion that bovine sperm are quiescent due to a pH-dependent motility inhibitor in cauda epididymal fluid. Lactate, at concentrations measured in bovine caudal fluid (8-10 mM), will mimic the concentration-dependent and pH-dependent effects of the motility quiescence factor (Carr et al., 1985) and appears to be the quiescence factor. Babcock et al. (1983) have also reported changes in bovine sperm motility and respiration under conditions that produce changes in pH_i. During epididymal maturation, bovine sperm develop the capacity for progressive motility (Acott and Hoskins, 1978; Hoskins et al., 1978), and the pH_i of washed caudal sperm is higher than that of washed caput sperm (Vijayaraghavan et al., 1985). However, changes in bovine sperm pH_i have not been reported under conditions appropriate to the initiation of motility at ejaculation. To address this question, a pH dependent chromophore was used to monitor pH_i under conditions where motility was initiated either by increasing external pH or by dilution, using buffered lactate at physiologic levels.

There is an abundance of evidence indicating that protein phosphorylation is involved in motility regulation (Garbers and Kopf, 1980; Tash and Means, 1982; Brokaw, 1987), but the mechanism by which a phosphoprotein affects motility has yet to be determined. An obstacle to the study of protein phosphorylation in mammalian spermatozoa has been the inability to incorporate sufficient amounts of $^{32}PO_4$ into cellular ATP (Babcock et al., 1975). Recently, methods for increased

$^{32}PO_4$ uptake into the nucleotide pool of bovine sperm have been described (Noland et al., 1987; Schoff and Lardy, 1987), facilitating intact sperm protein phosphorylation studies. To evaluate the relationship between sperm pH_i, motility, and protein phosphorylation, we have conducted studies of changes in protein phosphorylation in intact bovine sperm in response to manipulation of sperm pH_i.

MATERIALS AND METHODS

Materials

Bovine epididymides were obtained from local slaughterhouses. Carboxyfluorescein (CF) and carboxyfluoresceindiacetate (CFDA) were from Molecular Probes, Inc. (Junction City, OR); bovine serum albumin (BSA, type V), digitonin, and lactate were from Sigma Chemical Co. (St. Louis, MO); carrier free [^{32}P]-orthophosphate ($^{32}PO_4$) was from Amersham (Arlington Heights, IL).

Sperm Collection and Motility Measurements

Mature bovine sperm were extruded from caudal epididymides by retrograde flush of the vas deferens and were washed once before loading with either CFDA or $^{32}PO_4$. Motility units, defined as the percentage of motile sperm plus their flagellar intensity from 0 to 100 divided by 2, were measured as previously described (Carr and Acott, 1984).

Measurement of pH_i

Sperm pH_i was measured essentially as previously described (Babcock, 1983; Babcock et al., 1983). Briefly, sperm were loaded with CF by incubating for 20 min at room temperature in a buffer containing 2 μM CFDA, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM fructose and 10 mM (2-[N-morpholino]ethanesulfonic acid) (MES) (pH=6.2). Free CFDA was removed by centrifugation for 3 min at 2000 xg and sperm were resuspended with or without BSA (5 mg/ml) at the indicated pH in a buffer containing 105 mM NaCl, 45 mM KCl and 20 mM MES. The relative fluorescence intensities were measured at an emission wavelength of 515 nm with excitation at 495 nm with a Farrand Mark I spectrofluorometer and a strip chart

recorder. Excitation and emission slits were 10 nm pregrating and 5 nm postgrating. Samples were placed in 3 ml cuvettes and relative fluorescence intensities were measured at room temperature with continuous, gentle stirring. Intracellular pH values were estimated by null-point curve analysis (Babcock, 1983), using digitonin (50 μM, final concentration) to equilibrate the extra- and intracellular spaces. To correct for small differences in CF concentration between tubes, the pH was elevated to 10 (driving all of the chromophore into the high-pH form), after the final readings were made, and the fluorescence was measured again. BSA interacts with CF after permeabilization with digitonin and affects fluorescence intensity in a pH-dependent manner. When necessary, corrections were made for this effect by using the BSA quench curves plotted in Figs. 3B and 3C. To control for quenching artifacts due to dye-dye interactions within the cell at high CF concentrations, fluorescence measurements were made with sperm that were loaded for different times and at different concentrations of extracellular CFDA. All of the studies presented were made at concentrations below those at which this artifact becomes significant. Solution pH values were determined with a glass combination microelectrode (Micro-electrodes, Inc., Londonderry, NH) inserted directly into the cuvette using an Ion 85 pH meter (Radiometer, Copenhagen, Denmark).

Protein Phosphorylation Studies

Intact sperm were loaded with $^{32}PO_4$ by a modification of the method of Noland et al. (1987). Washed sperm were resuspended at 1 x 10^9 sperm/ml in loading buffer containing 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 20 mM (3-[N-morpholine]propanesulfonic acid) (MOPS) (pH=7.4) and 1 mCi/ml

$^{32}PO_4$. Except as indicated, these sperm were incubated for 2 hrs at 37°C under aerobic conditions. The free $^{32}PO_4$ was then removed by diluting the sperm 10-fold in buffer without $^{32}PO_4$ and centrifuging for 5 mins at 1100 xg; the sperm were then resuspended in buffer at the pH values indicated in the text. The phosphorylated sperm proteins were prepared for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis by adding 75 μl of sperm to 200 μl of boiling 2 % SDS, 50 mM CHES (pH=9.5), and 5 % β-mercaptoethanol (SDS/CHES/β-ME). The samples were then spun at 13,000 xg to sediment the DNA. To solubilize under non-reducing conditions, the sperm were boiled in buffer containing 100 mM Tris-HCl (pH=6.8), 3% SDS, and 10% glycerol and spun at 13,000 for 10 min. The supernatant was removed, mercaptoethanol (5%) was added, and the samples were boiled again before being electrophoresed.

$^{32}PO_4$ Uptake and Incorporation

Washed sperm were incubated with $^{32}PO_4$ for various times in loading buffer (MOPS, pH=7.4, same as above) or buffer in which 10 mM sodium bicarbonate was substituted for MOPS. Sperm uptake and macromolecular incorporation of $^{32}PO_4$ were determined by layering an aliquot (100 μl) of this sperm suspension on a step gradient of 100 μl glycerol and 100 μl of silicone oil in a microfuge tube. The sperm were centrifuged for 5 min at 13,000 xg and the interface between the silicone oil and the glycerol (which contained the sperm) was cut out and counted (Cerenkov) to determine uptake or added to 0.5 ml of 10% trichloroacetic acid (TCA), centrifuged, washed three times by resuspension in TCA and counted to determine macromolecular incorporation.

Electrophoresis and Data Analysis

SDS-PAGE was conducted on 4-12 % linear gradient acrylamide slab gels by the method of Laemmli (1970). Dried, Coomassie Brilliant Blue-stained gels were autoradiographed using X-Omat AR5 or OMC film (Kodak, Rochester, NY). Autoradiographs and Coomassie Brilliant Blue-stained gels were scanned and analyzed with a Hoefer densitometer and GS365 software. Individual peak areas were determined by Gaussian integration of densitometric scans and were corrected for the relative amount of protein applied to each lane, as determined by integration of the whole lane. The ratio of ^{32}P-labeled phosphoprotein per Coomassie Brilliant Blue-stained protein (relative specific radioactivity) was determined by dividing the digitized autoradiography scan by the stained protein scan of the same lane.

Subcellular Fractionation

Sperm were loaded with $^{32}PO_4$ for 2 hr at pH 7.4 then resuspended for 30 min at pH 7.4 or 5.5 with 10 mM lactate added. Subcellular fractionation was carried out using two different procedures. The first was a method for separation of heads and tails after sonication as described by Calvin (1976) and the second was a method for isolation of sperm plasma membrane after nitrogen cavitation as described by Noland et al. (1983). In both cases, just before cellular disruption, the sperm were spun down and resuspended in buffer designed to freeze the phosphorylation state of the proteins and inhibit proteases. The buffer contained 100 mM NaF, 15 mM EDTA, 1 mM EGTA, 50 mM NaCl, 10 mM benzamidine-HCl, 50 mM MOPS (pH=7.4). The samples were also kept at 4°C after cellular disruption until they were solubilized in boiling SDS/CHES/β-ME. For the sonication method, the

sperm were resuspended in 3 ml of buffer, sonicated for 20 secs, layered on a sucrose step gradient (3 ml each of 1.6 M, 2.05 M and 2.2 M sucrose), and spun for 1 hr at 91,000 xg. The cytosolic fraction remained at the top of the tube (the top 2 mls were collected and concentrated 20-to-1 before use), membranes were collected from the first interface, tails and midpieces were collected from the 1.6/2.05 M interface and the heads were in the pellet. The membranes and tails were collected with a 18 g needle attached to a syringe, diluted 1:5 in buffer without sucrose and centrifuged at 27,000 xg. The fractions were resuspended in SDS/CHES/β-ME and boiled for 3 min. The nitrogen-bomb method for isolating the plasma membranes was essentially the same as that of Noland et al. (1983). The membrane fractions shown in Fig. 7 are equivalent to their band 2. For both separation methods, microscopic analysis of fractions was used to determine contents and purity.

RESULTS

Changes in pH_i and Motility with Changes in Extracellular pH

The motility of bovine caudal sperm, resuspended in either undiluted bovine cauda epididymal fluid or 10 mM lactate in buffer, have identical pH dependencies (Acott and Carr, 1984; Carr and Acott, 1984; Carr et al., 1985). The pH dependence of sperm motility in the presence and absence of 10 mM lactate are quite different (Fig. 1, Panel A). The pH-dependent inhibition of motility in the presence of lactate is fully reversible (Acott and Carr, 1984). This differential pH dependence of motility in the presence and absence of lactate was correlated with the lowering of the pH_i of sperm, which was reflected in the lowering of the relative fluorescence intensity of carboxyfluorescein loaded sperm (Fig. 1B). In the absence of lactate, the sperm pH_i and motility were nearly independent of fluid pH. However, in the presence of lactate, both motility and pH_i were reduced at the lower pH.

Changes in Motility and pH_i with Fluid Dilution

A 1:10 dilution of nonmotile sperm suspended in either cauda epididymal fluid or pH 5.8 buffer containing 10 mM lactate into pH 5.8 buffer without lactate allows full motility initiation (Carr and Acott, 1984; Carr et al., 1985). To determine if dilution of the lactate was effecting pH_i, sperm loaded with CF were suspended in pH 5.8 buffer containing 10 mM lactate. At Time 0, the sperm were diluted 1:10 into the same buffer or into pH 5.8 buffer without lactate (Fig. 2). Both motility (Panel A) and relative fluorescence intensity of the CF (Panel B) were monitored from 0 to 360 s. Dilution of the sperm in the presence of lactate produced no change in either motility or pH_i. Dilution into buffer without lactate was followed

by an increase in motility and a significant intracellular alkalinization.

Effects of BSA and Determination of Absolute pH_i Values

Adding BSA (5 mg/ml) to sperm loaded with CF did not affect the relative fluorescence intensity (pH_i) but it did significantly change sperm motility (Fig. 3A). BSA is known to bind fluorescein (Andersson et al., 1971) and would therefore alter the measurements used in determining the pH_i by the null-point method. Prior to digitonin treatment, BSA did not change intracellular CF fluorescence intensity. However, after the membranes were permeabilized, BSA interacted with and quenched the fluorescence of the extracellular chromophore. Previous studies of sperm pH_i have been conducted in the absence of BSA (Babcock, 1983; Babcock et al., 1983, Vijayaraghavan et al., 1985) to avoid its effects on absolute pH_i measurements. However, the presence of BSA is desireable for motility measurements, partly because it keeps the sperm from sticking to the glass slide and coverslip. Therefore, we quantified the pH dependent effect of BSA on the fluorescence intensity of CF (Fig. 3B). These curves were used to correct for the quenching by BSA. The percent of quenching caused by 5 mg/ml BSA at various pHs (Fig. 3C) was used to determine the null point values plotted in Fig. 3D. The pH_i value of 6.44 determined by this method was not significantly different from values we obtained using the null point method without BSA present and was similar, although slightly lower, than values reported for bovine sperm pH_i (6.5 to 6.6, Babcock, 1983; Smith et al., 1985). If the null point curve had not been corrected for BSA quenching, the apparent pH_i value would have been $\approx$ 6.9. From null point determinations, corrected for BSA, the change in pH_i with motility

initiation (Figs. 1 or 2) was 0.40 ± 0.05 pH units.

Equilibration of $^{32}PO_4$ into Phosphoproteins

Noland et al. (1987) suggested that bicarbonate facilitates the uptake of $^{32}PO_4$ by intact sperm. To evaluate this suggestion, we measured the uptake of $^{32}PO_4$ by intact sperm in the presence and absence of bicarbonate (Fig. 4A). In the absence of bicarbonate, uptake continued to increase up to at least 3 h, in the presence of bicarbonate, uptake leveled off after 1 h. The addition of 10 mM glucose increased $^{32}PO_4$ uptake both in the presence and absence of bicarbonate, but 25 mM glucose was less effective because it caused a lowering of the pH (data not shown). Incorporation of radiolabel into trichloroacetic acid-precipitable macromolecules also increased for at least 3 h (Fig. 4B), but was slightly less efficient in the presence of bicarbonate than in its absence. After 3 h of continuous exposure, radiolabel incorporation into macromolecules was ≈ 8% of total uptake.

Sperm were incubated for various times in $^{32}PO_4$ and then were solubilized and analyzed by SDS-PAGE. Lanes 1-9 of the Coomassie Brilliant Blue-stained gel (Fig. 4D) correspond to equivalent lanes in the autoradiograph (Fig. 4C). Sperm, continuously exposed to radiolabel, showed increasing incorporation of radiolabel into phosphoproteins for at least 5 h (Fig. 4C, Lanes 1-5). If sperm are loaded with $^{32}PO_4$ for 2 h, washed and resuspended in buffer without radiolabel for 5, 15, 30 or 60 min, and then analyzed, there was little or no change in the level of protein phosphorylation (Fig. 4C, Lanes 6-9). The following studies used this protocol to achieve radiolabel equilibration of the phosphoproteins.

Protein Phosphorylation Patterns

Analysis of autoradiographs (Fig. 5A) and the associated Coomassie Brilliant Blue-stained SDS-PAGE slab gels (Fig. 5B), allowed the identification of at lease 26 discrete and reproducible phosphoprotein bands; different exposure times for the autoradiographs were necessary to optimize identification of all 26 bands. Phosphoproteins were numbered from the top of the gel, as bands 1 through 26 (see numbering of major bands in Fig. 5A) for reference purposes here and later. A large band of small radiolabeled molecules, phospholipids and free ^{32}P-phosphate was observed below the dye front (not shown).

Densitometric scans of the lanes of a typical autoradiograph (Fig. 5A) and of the same lane of the dried, Coomassie Brilliant Blue-stained gel (Fig. 5B) show the level of phosphorylation and staining of each protein band. A relative specific radioactivity profile (Fig. 5C), was obtained by dividing the digitized densitometric scan of the autoradiograph by the scan of the Coomassie Brilliant Blue-stained gel. This allowed comparisons (within one autoradiograph) of the phosphoprotein bands in lanes to which slightly different amounts of protein may have been added. Note the high relative specific radioactivity of band 5 (Fig. 5C).

Effect of Sperm pH_i on Protein Phosphorylation

To determine if the phosphoprotein profile of motile sperm was the same or different from that of nonmotile sperm, the sperm were loaded with $^{32}PO_4$ and then resuspended in various buffers designed to modulate their motility by changing their pH_i. When sperm were resuspended at either pH 7.4 or 5.5 in the absence of lactate there was little difference in either pH_i or motility (Fig. 6C, Lanes 1 and 2 respectively), or in phosphoprotein profiles (Fig. 6A, Lanes 1 and 2, respectively);

a small difference in band 5 (between asterisks) was detectable. If sperm were resuspended at either pH 7.4 or 5.5 in the presence of lactate, pH_i was much lower at pH 5.5 and motility was reduced at pH 5.5 (Fig. 6C, Lanes 3 and 4, respectively), and the phosphorylation of band 5 was increased at pH 5.5 (Fig. 6A, Lanes 3 and 4, respectively).

These differences were all reversible. When the sperm initially at high pH_i with lactate (Lane 3) were resuspended at low pH_i with lactate (Lane 6), band 5 phosphorylation increases. When sperm initially at low pH_i with lactate (Lane 4) were resuspended at high pH_i with lactate (Lane 5), band 5 phosphorylation was decreased. Motility also was reversed (Fig. 6C, lanes 5 and 6) as was sperm pH_i (data not shown). The slightly higher levels of motility observed in this experiment (Fig. 6C), compared to earlier studies (Fig. 3), was due to the different temperatures (37°C compared to room temperature).

A comparison of the autoradiograph densities of band 5 from Lanes 1-6 are shown in Fig. 6B. In the absence of lactate, resuspension at pH 5.5 increased phosphorylation slightly compared to resuspension at pH 7.4, but in the presence of lactate the increase was more than 60%. This phosphorylation difference was reversible (Fig. 6C) by reversing the pH of the buffer. The values in Panel 6B were normalized to correct for small differences in the total amount of protein applied to each lane. The total protein per lane was determined by integrating the densitometric scan of the entire Coomassie Brilliant Blue-stained lane (Fig. 6A, Lanes 7-12). An inverse correlation between phosphorylation of band 5 (pp255) and sperm motility was apparent when Fig. 6B and 6C were compared. No other

significant differences in phosphorylation were detectable under these conditions.

Subcellular Localization of pp255 and other Phosphoproteins

To determine where pp255 was located, sperm were loaded with $^{32}PO_4$ and then resuspended under motile (Fig. 7; pH 7.4, odd numbered lanes) or nonmotile (Fig. 7; pH 5.5 with 10 mM lactate, even numbered lanes) conditions, followed by sonication and fractionated by sucrose density gradient centrifugation. Panel A shows the autoradiograph of the corresponding proteins in Panel B. The fractions were collected as described in Materials and Methods: whole sperm (Lanes 1 and 2), supernatants from high-speed centrifugation (Lanes 5 and 6), plasma membranes (Lanes 7 and 8), tail fragments and midpieces (Lanes 11 and 12), and heads (Lanes 13 and 14). The pp255 (large asterisks) was found only in the fraction containing the sperm heads.

Fractionation of these phosphoproteins prior to electrophoresis allowed the identification of other pH_i-dependent phosphorylation changes, that were masked in the whole sperm extraction gels. The membrane fractions, obtained by either the sonication method (Fig. 7, lanes 7 and 8) or by the nitrogen bomb method (lanes 9 and 10) contained two phosphoproteins (Mr $\approx$ 105,000 and 97,000, bottom two asterisks next to lane 8) that were more heavily phosphorylated at high pH_i and one phosphoprotein (Mr $\approx$ 120,000, top asterisk next to lane 8) that was less heavily phosphorylated at high pH_i. Although other minor pH_i-dependent changes were detectable, these were the only significant differences observed after correction for protein concentration and that showed consistent changes in three separate experiments.

During differential extraction experiments, we observed that pp255 only was extracted in the presence of reducing agents. When whole sperm were extracted with (Fig. 7, lanes 1 and 2) or without (lanes 3 and 4) β-mercaptoethanol, this difference was apparent (large asterisks). Although almost all of the other phosphoproteins were solubilized in the absence of reducing agents, pp255 was totally absent under these conditions.

DISCUSSION

The first objective of these studies was to monitor sperm pH_i during motility initiation under conditions previously shown to mimic the initiation of mammalian sperm motility that occurs at ejaculation (Acott and Carr, 1984; Carr and Acott, 1984; Carr et al., 1985). Although motility/pH_i relationships have been reported for bovine sperm (Babcock et al., 1983; Vijayaraghavan et al., 1985), the conditions used in earlier studies were not selected to address this specific question. The results presented in Figs. 1 and 2 provide support for our hypothesis that the initiation of bovine sperm motility at the time of ejaculation is mediated by cytoplasmic alkalinization. The quiescence of sperm prior to ejaculation is due to their acidic pH_i, which in turn is caused by the low pH of the surrounding caudal fluid and a quiescence factor (probably lactate). The motility of some invertebrate sperm, before and after release, also appears to be regulated via changes in pH_i, although the extracellular modulation appears to be different (Christen et al., 1982, 1983; Lee et al., 1983).

Macromolecular changes associated with pH_i-mediated alterations in motility include changes in phosphorylation levels of at least 4 proteins. Two show a positive correlation with motility (105,000 and 97,000) and two show a negative correlation (120,000 and 255,000). These changes in phosphorylation are probably not due to changes in cAMP levels, since we have previously shown that increasing pH_i does not increase cAMP levels in bovine sperm (Carr and Acott, 1984), and Babcock et al. (1983) have shown that increasing sperm pH_i does not change the activation state of cAMP-dependent protein kinase. Since the pH-dependence of sperm protein

kinase(s) and phosphoprotein phosphatase(s) with endogenous sperm substrates has not been determined, these activities are likely candidates for the point of action of the pH_i change. This difference in phosphorylation could be due to the pH-dependence of either enzyme's activity or to pH-induced changes in the charge or conformation of the protein substrates.

Although the co-sedimentation of pp255 with the head fraction may suggest that it is not involved in motility regulation, this role has not been ruled out yet. It is possible that the protein is located at the base of the head or in the neck region and thus would be in a position to exert control over axonemal movement. Also, co-sedimentation studies do not provide positive proof that the protein is located only in the head. We are currently pursuing more detailed localization and characterization of pp255.

A reciprocal relationship between motility and phosphorylation is not surprising for a protein that regulates a cyclical event like flagellar beating. It's possible that phosphorylation of pp255 initiates each flagellar wave and that pp255 is dephosphorylation between beats (~20 beats/sec for mature caudal sperm, Acott et al., 1983). Lowering pH_i could reduce motility by inhibiting a phosphoprotein phosphatase, which would cause build-up of phosphates on pp255, but phosphate turnover would be inhibited. Other agents which stimulate motility by activating a protein kinase would also be expected to produce a net increase in phosphorylation of pp255, but in this case, the phosphate turnover rate would increase. Our method of analysis provides no information on phosphorylation turnover.

In addition to pp255, three membrane-associated phosphoproteins change with pH_i modulation of motility. Because the plasma membrane fraction that we isolated (by either method) is from the whole sperm, one or more of these phosphoproteins may be on the neck, midpiece, or tail. Subsequently, any or all of these phosphoproteins may be involved in the regulation of sperm motility by pH_i. Recently, the pH dependence of cAMP modulation of motility in modeled, demembranated bovine sperm was reported (Goltz et al., 1988); their permeabilization may or may not have removed the membrane phosphoproteins that we have identified.

Much attention has been given to phosphoproteins at Mr ≈ 56,000 that were reported to be involved in motility regulation (Brandt and Hoskins, 1980; Tash and Means, 1982; Tash et al., 1984, 1986; Noland et al., 1987; Paupard et al., 1988). In our system, we can detect a phosphoprotein band at this molecular weight, although it is not a major band. It localizes mostly to the soluble fraction and its phosphorylation state is not significantly changed with modulation of motility by pH_i.

In summary, pH_i is a strong candidate for the intracellular regulator of bovine sperm motility initiation at ejaculation. We find 4 proteins (pp97, pp105, pp130 and pp255) that show phosphorylation changes with pH_i modulation and suggest that any of these may be causally involved in regulating the bovine motility apparatus. Additional studies are necessary to define these relationships in detail.

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REFERENCES

Acott TS and Carr DW, 1984. Inhibition of bovine spermatozoa by caudal epididymal fluid: II. Interaction of pH and a quiescence factor. Biol Reprod 30:926-35

Acott TS and Hoskins DD, 1978. Bovine sperm forward motility protein: Partial purification and characterization. J Biol Chem 253:6744-50

Acott TS, Katz DF and Hoskins DD, 1983. Motility characteristics of bovine epididymal spermatozoa: Effects of forward motility protein and epididymal maturation. Biol Reprod 29:389-99

Andersson L-O, Rehnstrom A and Eaker DL, 1971. Studies on "nonspecific binding. The nature of the binding of fluorescein to bovine serum albumin. Eur J Biochem 20:371-80

Babcock DF, 1983. Examination of the intracellular ionic environment and of ionophore action by null point measurements employing the fluorescein chromophore. J Biol Chem 258:6380-9

Babcock DF, First NL, Lardy HL, 1975. Transport mechanism for succinate and phosphate localized in the plasma membrane of bovine spermatozoa. J Biol Chem 250:6488-95.

Babcock DF, Rufo GA Jr and Lardy HA, 1983. Potassium-dependent increases in cytosolic pH stimulate metabolism and motility of mammalian sperm. Proc Natl Acad Sci 80:1327-31

Bevington, PR, 1969. Data Reduction and Error Analysis for the Physical Sciences. New York: McGraw-Hill

Bloom W and Fawcett DW, 1975. A Textbook of Histology. Philadelphia: W.B. Saunders

Brandt H and Hoskins DD, 1980. A cAMP-dependent phosphorylated motility protein in bovine epididymal sperm. J Biol Chem 255:982-7

Brokaw CJ, 1987. Regulation of sperm flagellar motility by calcium and cAMP-dependent phosphorylation. J Cell Biochem 35:175-84

Calvin, H.I., 1976. Isolation and Subfractionation of Mammalian Sperm Heads and Tails. Methods in Cell Biol. Vol 13. New York: Academic Press, pp 85-104

Carr DW and Acott TS, 1984. Inhibition of bovine spermatozoa by caudal epididymal fluid: I. Studies of a sperm motility quiescence factor. Biol Reprod 30:913-25

Carr DW, Usselman MC and Acott TS, 1985. Effect of pH, lactate, and viscoelastic drag on sperm motility: A species comparison. Biol Reprod 33:588-95

Christen R, Schackmann RW and Shapiro BM, 1982. Elevation of the intracellular pH activates respiration and motility of sperm of the sea urchin, Strongylocentrotus purpuratus. J Biol Chem 257:14881-90

Christen R, Schackmann RW and Shapiro BM, 1983. Metabolism of sea urchin sperm: Interrelationships between intracellular pH, ATPase activity, and mitochondrial respiration. J Biol Chem 258:5392-9

Garbers DL and Kopf GS, 1980. The regulation of spermatozoa by calcium and cyclic nucleotides. In: Advances in Cyclic Nucleotide Research vol. 13

(Greengard, P. and Robison, G. A. eds) NY, Raven Press, pp 251-306

Goltz JS, Gardner TK, Kanous KS and Lindemann CB, 1988. The interaction of pH and cyclic adenosine 3'-5' monophosphate on activation of motility in Triton X-100 extracted bull sperm. Biol Reprod, 39:1129-36

Hoskins DD, Brandt H and Acott TS, 1978. Initiation of sperm motility in the mammalian epididymis. Fed Proc 37:2534-42

Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-5

Lee HC, Johnson C and Epel D, 1983. Changes in internal pH associated with initiation of motility and acrosome reaction of sea urchin sperm. Devel Biol 95:31-45

Noland TD, Abumrad NA, Beth AH and Garbers DL, 1987. Protein phosphorylation in intact bovine epididymal spermatozoa: Identification of the type II regulatory subunit of cyclic adenosine 3',5'-monophosphate-dependent protein kinase as an endogenous phosphoprotein. Biol Reprod 37:171-80

Noland TD, Olson GE, and Garbers DL, 1983. Purification and partial characterization of plasma membranes from bovine epididymal spermatozoa. Biol Reprod 29:987-98

Paupard M-C, MacLeod J, Wasco W and Orr GA, 1988. Major 56,000-Dalton, soluble phosphoprotein present in bovine sperm is the regulatory subunit of a type II cAMP-dependent protein kinase. J Cellular Biochem 37:161-75

Saacke RG and Almquist JO, 1964. Ultrastructure of Bovine Spermatozoa. II. The neck and tail of normal, ejaculated sperm. Am J Anat 115:163-84

Salisbury JL, Aebig KW and Coling DE, 1986. Isolation of the calcium-modulated contractile protein of striated flagellar roots. Meth Enzymol 134:408-14

Salisbury JL and Floyd GL, 1978. Calcium-induced contraction of the Rhizoplast of a Quadriflagellate Green Alga. Science 202:975-77

Schoff PK and Lardy HA, 1987. Effects of fluoride and caffeine on the metabolism and motility of ejaculated bovine spermatozoa. Biol Reprod 37:1037-4627.

Smith MB, Babcock DF and Lardy, HA, 1985. A ^{31}P NMR study of the epididymis and epididymal sperm of the bull and hamster. Biol Reprod 33:1029-40

Tash JS, Hidaka H and Means AR, 1986. Axokinin phosphorylation by cAMP-dependent protein kinase is sufficient for activation of sperm flagellar motility. J Cell Biol 103:649-55

Tash JS, Kakar SS and Means AR, 1984. Flagellar motility requires the cAMP-dependent phosphorylation of a heat-stable NP-40-soluble 56kd protein, axokinin. Cell 38:551-9

Tash JS and Means AR, 1982. Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. Biol Reprod 26:745-623

Vijayaraghavan S, Critchlow LM and Hoskins DD, 1985. Evidence for a role for cellular alkalinization in the cyclic adenosine 3',5'-monophosphate-mediated initiation of motility in bovine caput spermatozoa. Biol Reprod 32:489-500

FIG. 1. Dependence of sperm motility and pH_i on extracellular pH with and without lactate present. Sperm motility (A) and relative fluorescence intensity of carboxyfluorescein (B) were measured at the indicated extracellular pH values in the absence (dashed lines) and presence (solid lines) of 10 mM lactate. All samples contained BSA (5 mg/ml) and were preincubated for 10 mins before measurements were taken. Relative fluorescence intensities were normalized to a common control value.

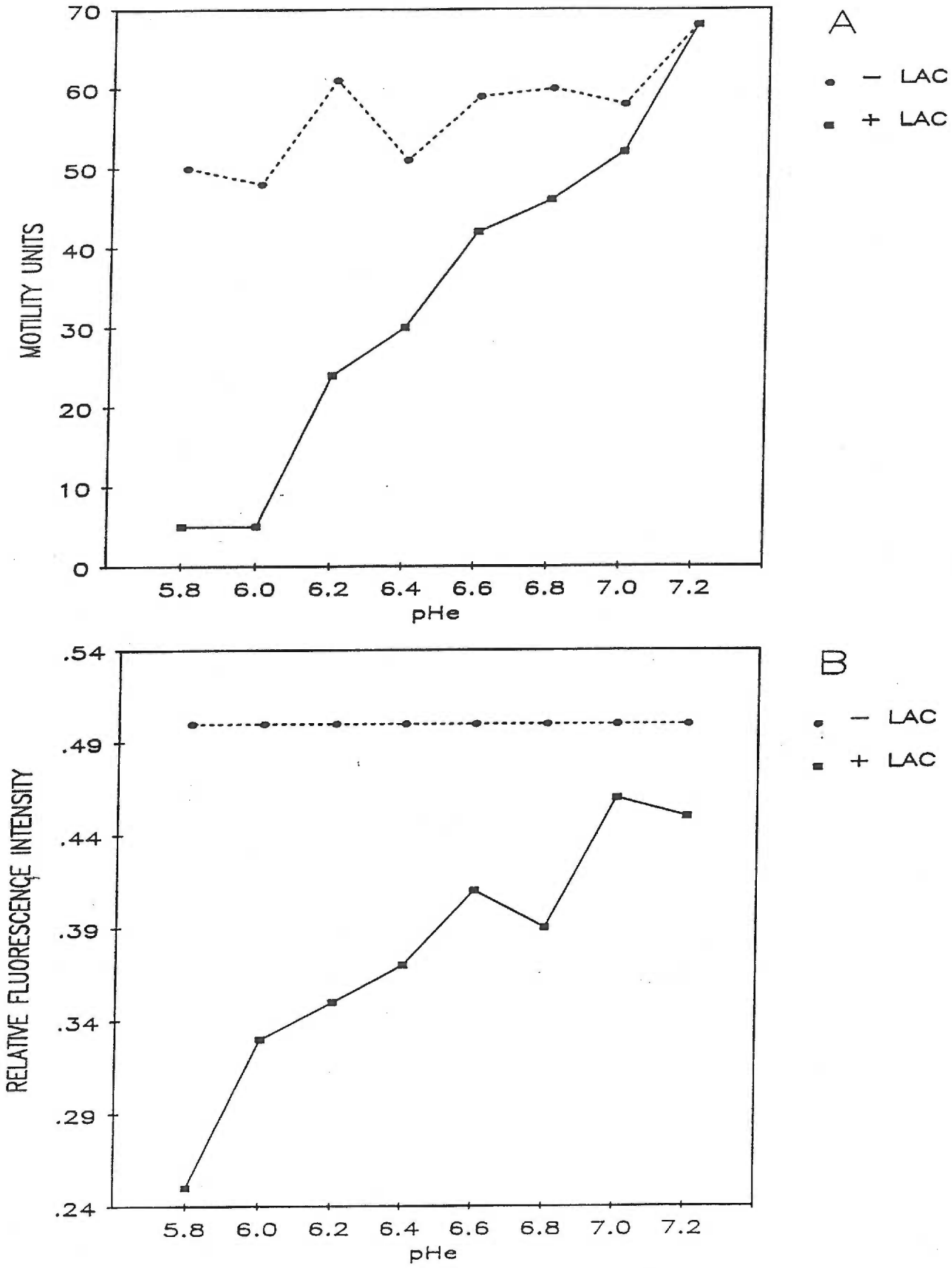
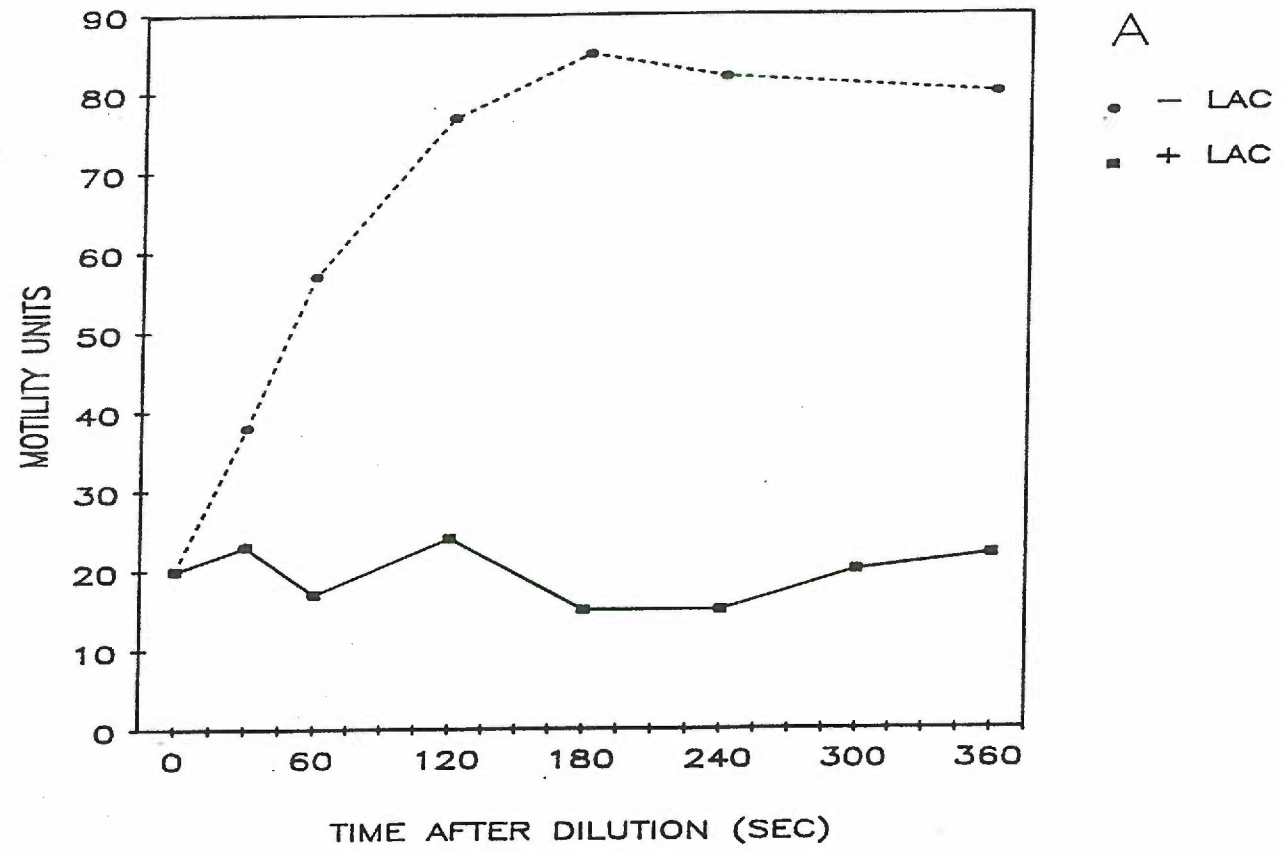

A
− LAC
+ LAC
MOTILITY UNITS
70
60
50
40
30
20
10
0
5.8
6.0
6.2
6.4
6.6
6.8
7.0
7.2
pHe
B
− LAC
+ LAC
RELATIVE FLUORESCENCE INTENSITY
.54
.49
.44
.39
.34
.29
.24
5.8
6.0
6.2
6.4
6.6
6.8
7.0
7.2
pHe

FIG. 2. Time course of sperm pH_i and motility changes following dilution. Sperm in buffer containing 10 mM lactate and BSA (5 mg/ml) at pH 5.8 were diluted 1:10 into the same buffer with (solid line) or without (dashed line) 10 mM lactate. Motility A) and relative fluorescence intensity (B) were determined at the times indicated.


A
– LAC
+ LAC
MOTILITY UNITS
90
80
70
60
50
40
30
20
10
0
0
60
120
180
240
300
360
TIME AFTER DILUTION (SEC)

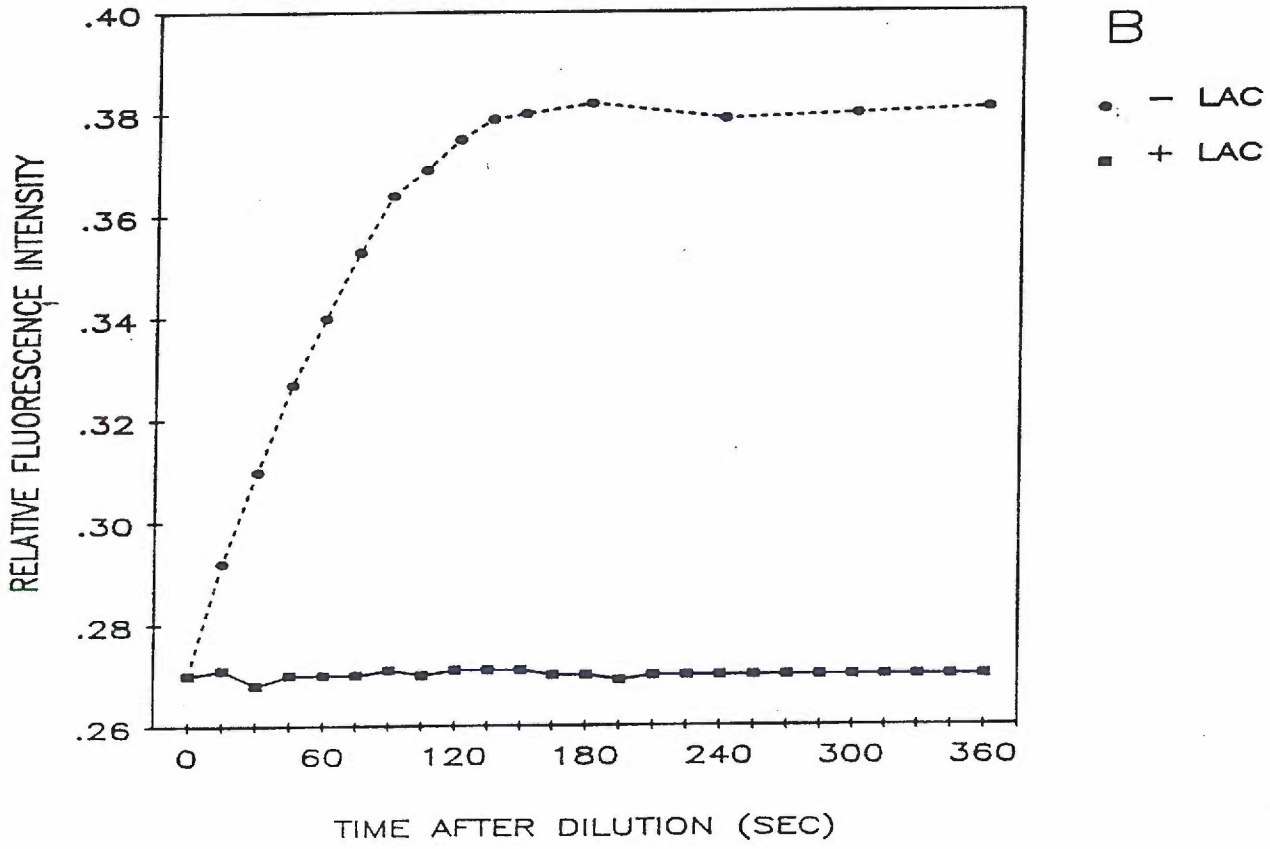

B
– LAC
+ LAC
RELATIVE FLUORESCENCE INTENSITY
.40
.38
.36
.34
.32
.30
.28
.26
0
60
120
180
240
300
360
TIME AFTER DILUTION (SEC)

FIG. 3. Effects of BSA on motility and upon absolute pH_i measurements. A: Relative fluorescence intensity and motility were measured at pH 6.1 and 7.2 in the absence and presence of BSA (5 mg/ml) in buffer containing 10 mM lactate. Values are means ± SEM. B: Relative fluorescence intensity was determined for buffered solutions containing 0.1 μM CF and the indicated concentrations of BSA over the pH range indicated. C: Correction curve for BSA effects on absolute pH_i measurements at 5 mg BSA / ml. The percent quenching of the relative fluorescence intensity of CF by 5 mg/ml BSA, when compared to the relative fluorescence intensity measured in the absence of BSA, is shown at the pH values indicated. D: Typical null-point curve showing the change in relative fluorescence intensity of CF-loaded sperm that is produced by the permeabilization of the membranes by digitonin (50 μM), thereby allowing equilibration of the intra- and extracellular compartments.

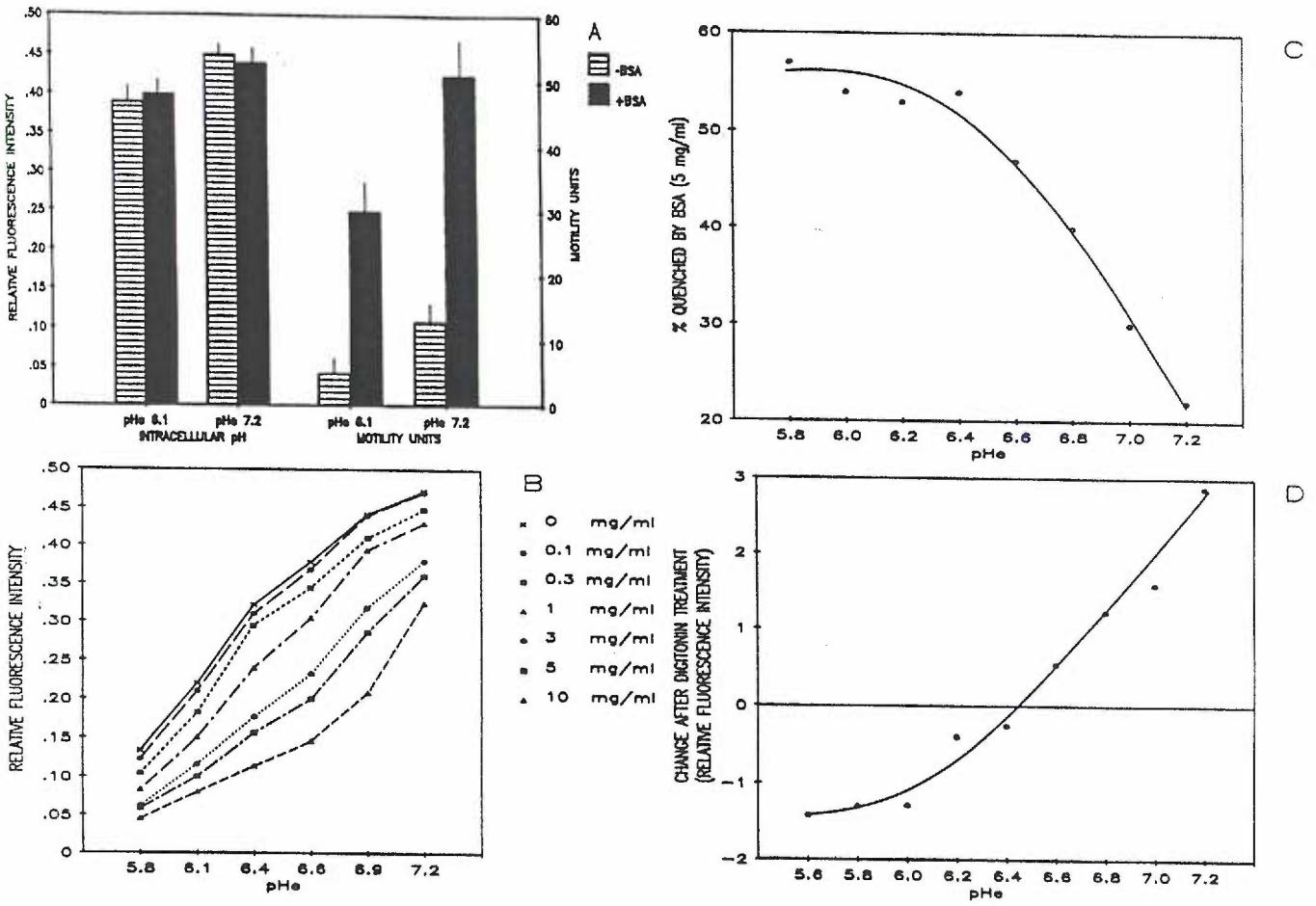
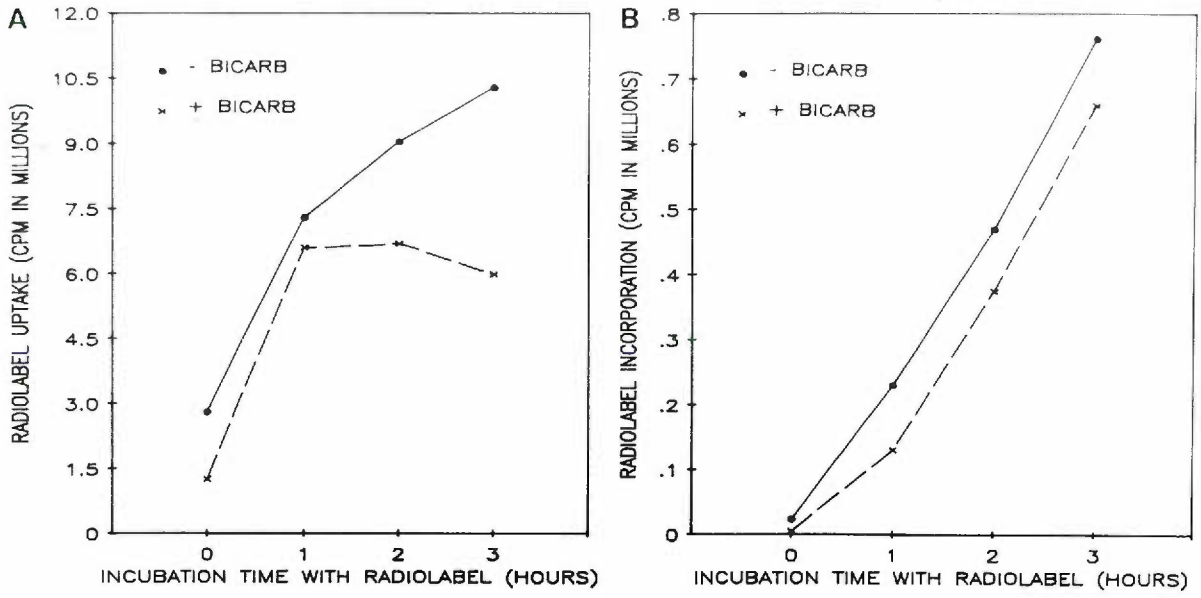

A
-BSA
+BSA
RELATIVE FLUORESCENCE INTENSITY
MOTILITY UNITS
pHe 6.1
pHe 7.2
pHe 6.1
pHe 7.2
INTRACELLULAR pH
MOTILITY UNITS
B
0 mg/ml
0.1 mg/ml
0.3 mg/ml
1 mg/ml
3 mg/ml
5 mg/ml
10 mg/ml
RELATIVE FLUORESCENCE INTENSITY
pHe
C
% QUENCHED BY BSA (5 mg/ml)
pHe
D
CHANGE AFTER DIGITONIN TREATMENT
(RELATIVE FLUORESCENCE INTENSITY)
pHe

FIG. 4. Time courses for ^{32}P uptake by sperm and for incorporation into sperm macromolecules. Sperm were incubated in either MOP's buffer (pH=7.4) or 10 mM bicarbonate buffer (pH=7.4) for the times indicated. The sperm were then washed and the amount of total radiolabel taken up by the sperm (A) or the amount of total radiolabel incorporated into TCA-precipitable material (B) was determined. Panel C is an autoradiograph of an SDS-PAGE gel (D, coomassie blue stained). Intact sperm were incubated 1 mCi/ml $^{32}PO_4$ for 1, 2, 3, 4, and 5 hours (lanes 1-5 respectively) before being solubilized in boiling SDS/CHES/β-ME and applied to the gel. Sperm exposed to radiolabel for 2 hours were washed and resuspended in the same buffer without radiolabel and then aliquots were solubilized after 5, 15, 30 or 60 minutes (lanes 6-9, respectively). The unlabeled left lane in D contains molecular weight markers; Mr = 200,000, 116,000, 97,000, 66,000 and 43,000 from top to bottom; phospholipids and nucleotides which migrate beyond the marker dye are not shown.


A
12.0
10.5
9.0
7.5
6.0
4.5
3.0
1.5
0
- BICARB
+ BICARB
RADIOLABEL UPTAKE (CPM IN MILLIONS)
0
1
2
3
INCUBATION TIME WITH RADIOLABEL (HOURS)
B
.8
.7
.6
.5
.4
.3
.2
.1
0
- BICARB
+ BICARB
RADIOLABEL INCORPORATION (CPM IN MILLIONS)
0
1
2
3
INCUBATION TIME WITH RADIOLABEL (HOURS)

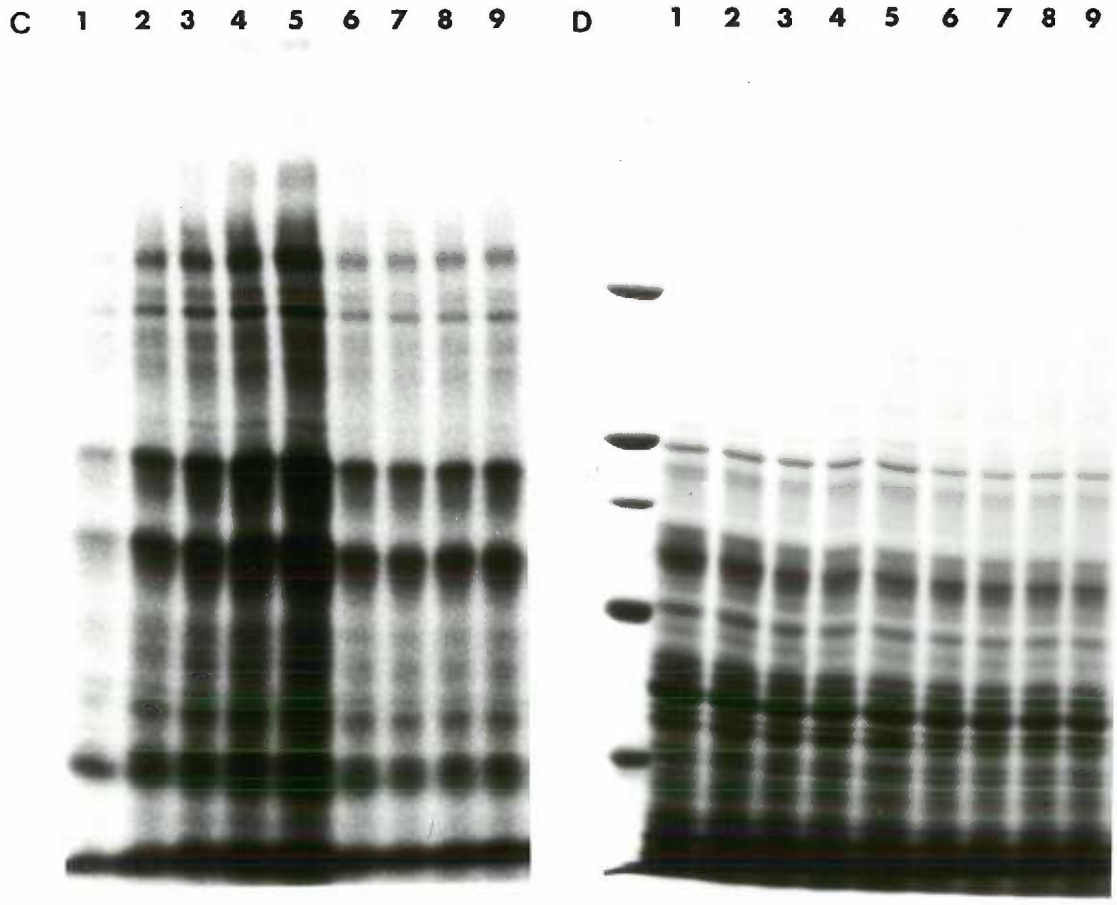

C 1 2 3 4 5 6 7 8 9
D 1 2 3 4 5 6 7 8 9

FIG. 5. Identification of discrete phosphoprotein bands and determination of relative specific radioactivity of each band. An extract of whole sperm, loaded for two hours with radiolabel, was applied to a 4-12% linear gradient SDS-polyacrylamide gel. Panel A is the autoradiograph (Autorad) of the coomassie blue stained proteins in panel B. The densitometric scans are shown underneath the lanes (units are relative absorbance). The top of the gel is on the left side of the panel. The digitized scan of the autoradiograph was divided by the scan of the protein to produce the ratio of $^{32}PO_4$ per stain protein (C). Approximately 26 discrete phosphoprotein bands were consistently identifiable (using several autoradiographic exposure times) and were labeled 1-26 from the top to the bottom of the gel. The numbers associated with the 5 major phosphoprotein bands are shown (A). The M_r's for bands 5, 7, 12, 13, and 21 are 255,000, 190,000, 105,000, 82,000 and 43,000 respectively.

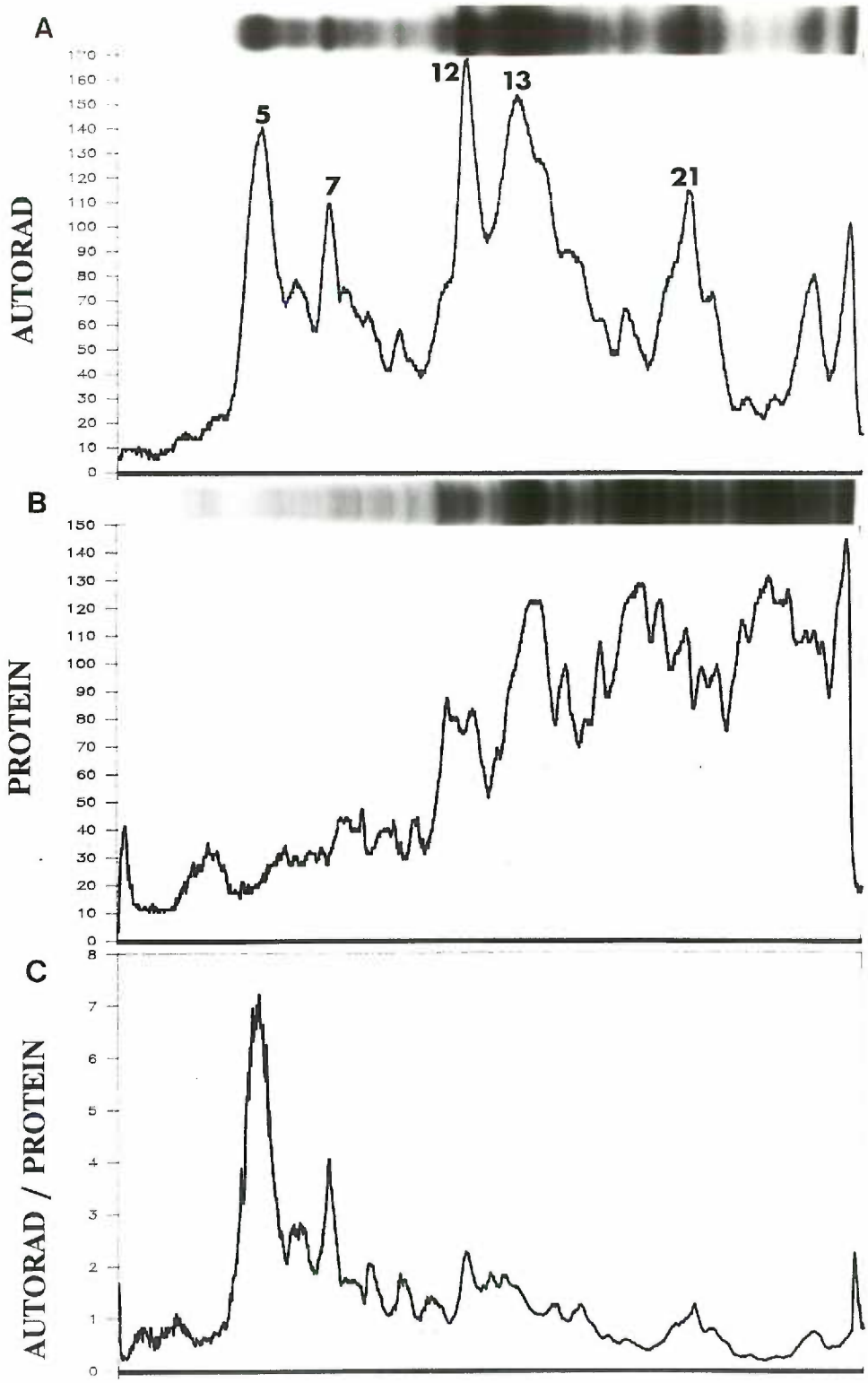

A
AUTORAD
5
7
12
13
21
B
PROTEIN
C
AUTORAD / PROTEIN

FIG. 6. Reversible effects of changes in sperm pH_i on phosphoprotein profile and sperm motility. Washed sperm, which had been incubated with $^{32}PO_4$ (1 mCi/ml) for 2 hours, were resuspended for 15 min in buffer without radiolabel in the absence of lactate at pH 7.4 (lane 1) or pH 5.5 (lane 2); or in the presence of lactate pH 7.4 (lane 3) or pH 5.5 (lane 4). Sperm from lanes 3 and 4 were then centrifuged and resuspended with lactate at the other pH to test for reversibility of the changes, i.e. lane 3 sperm were resuspended at pH 5.5 (lane 6) and lane 4 sperm were resuspended at pH 7.4 (lane 5). Lanes 7-12 are the coomassie blue stained proteins which correspond to the autoradiographs in lanes 1-6. Band 5, pp255, is seen in A (between the asterisks); the arrows indicate the positions of molecular weight standards with Mr = 200,000, 116,000, 97,000, 66,000 and 43,000, respectively from top to bottom. The area under the peak of band 5 (255,000 Daltons) was calculated for each lane by Gaussian integration and corrected for protein concentration (panel B). Motility measurements were performed using aliquots of the same sperm samples that were applied to the various lanes. All incubations were for 15 min at 37° C without BSA.

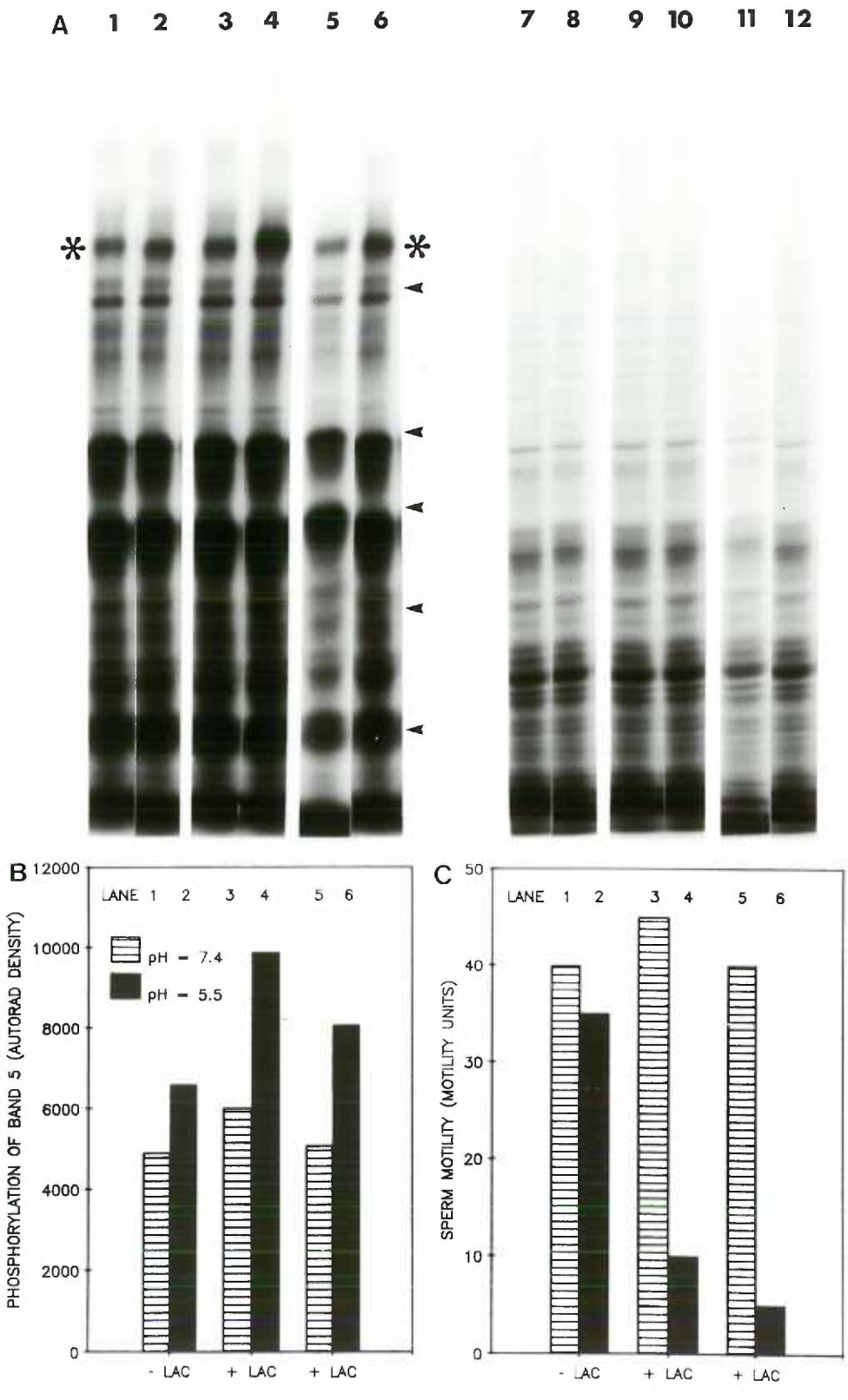
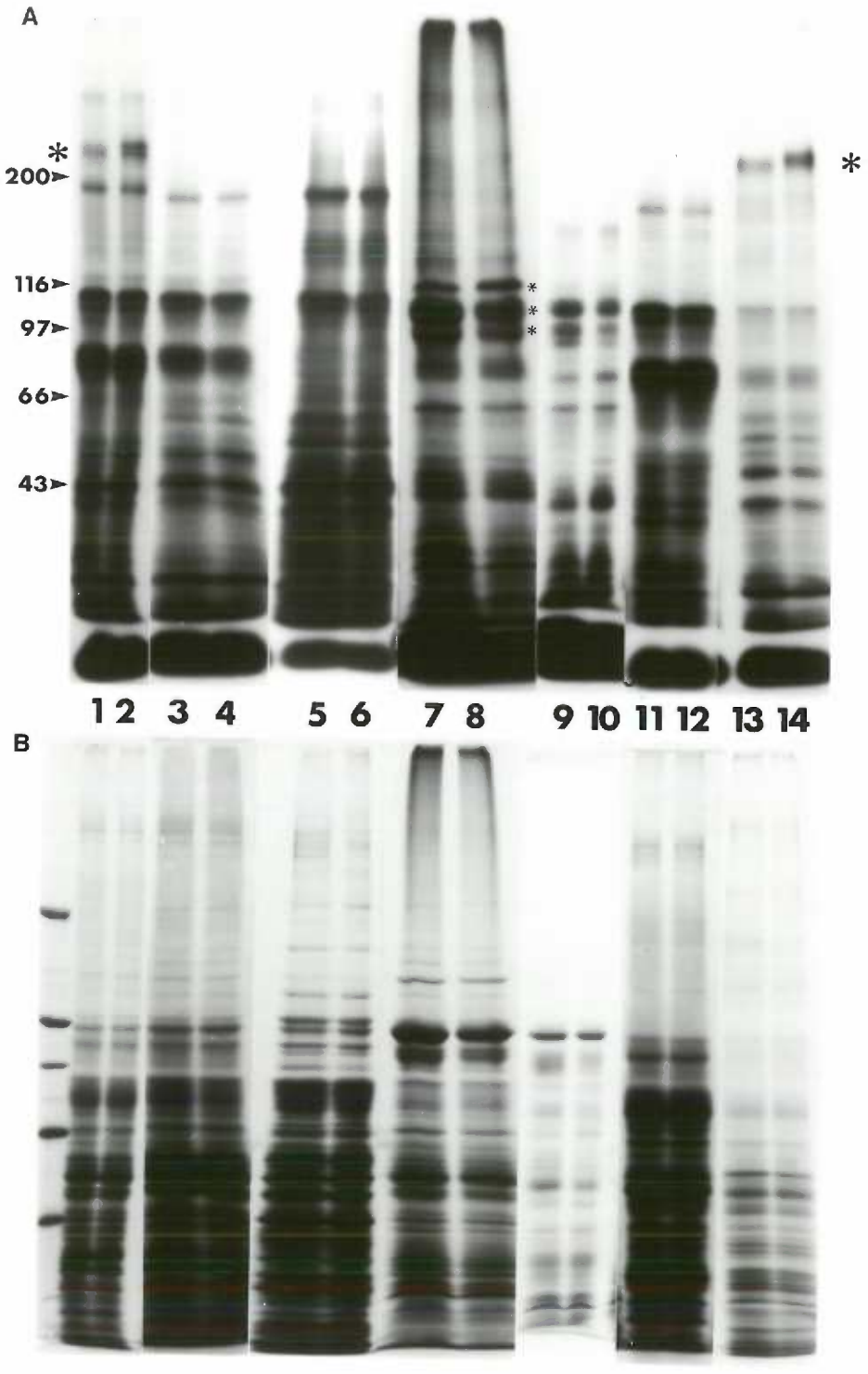

A
1 2 3 4 5 6
7 8 9 10 11 12
B
LANE 1 2 3 4 5 6
pH = 7.4
pH = 5.5
PHOSPHORYLATION OF BAND 5 (AUTORAD DENSITY)
12000
10000
8000
6000
4000
2000
0
- LAC
+ LAC
+ LAC
C
LANE 1 2 3 4 5 6
SPERM MOTILITY (MOTILITY UNITS)
50
40
30
20
10
0
- LAC
+ LAC
+ LAC

FIG. 7. Subcellular localization of sperm proteins and phosphoproteins. Radiolabel-loaded sperm were incubated with 10 mM lactate at pH 7.4 (odd numbered lanes) or pH 5.5 (even numbered lanes) prior to direct extraction or subcellular fractionation (see methods). An autoradiograph (A) was taken of the coomassie blue stained gel (B); exposure times were optimized individually for each pair of lanes. Whole sperm were extracted in the presence of mercaptoethanol (lanes 1 and 2), or in the absence of reducing agents (lanes 3 and 4). The rest of the lanes are subcellular fractions: high speed supernatant from sonication method (lanes 5 and 6), plasma membranes from sonication method (lanes 7 and 8), plasma membranes from nitrogen bomb method (lanes 9 and 10), tails and midpieces from the sonication method (lanes 11 and 12), and heads from the sonication method (lanes 13 and 14). The large asterisks indicate the location of band 5, (255 kD) and the three smaller asterisks next to lane 8 indicate three plasma membrane bands which change phosphorylation levels in response to changes in pH_i.


A
*
200
116
97
66
43
*
*
*
*
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B

MANUSCRIPT 2

Regulation of Sperm Protein Phosphorylation by Isobutyl-3-Methylxanthine and Calcium: Immunological Identification of pp255 as Microtubule-Associated Protein 2*

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SUMMARY

Protein phosphorylation may be involved in the regulation of several critical processes necessary for sperm to fertilize an egg. Reduction of intracellular pH or treatment with isobutyl-3-methylxanthine dramatically increases the phosphorylation of one bovine sperm protein, pp255. The addition of 0.1 mM free Ca^{2+}, plus the ionophore A23187, to intact bovine sperm, removes all detectable phosphates from pp255. Ca^{2+} also stimulates the dephosphorylation of many other sperm phosphoproteins, although not as dramatically as pp255. The protein kinase inhibitor H-8 decreases, and the phosphoprotein phosphatase inhibitor fluoride increases pp255 phosphorylation with only small effects on other sperm phosphoproteins. This unique sensitivity of pp255 phosphorylation to these modulators suggests a critical role for pp255 in the regulation of bovine sperm behavior.

Pp255 is phosphorylated on serine residues and after sonication, fractionates with the sperm heads. On immunoblots, a polyclonal antibody to brain microtubule-associated protein 2 (MAP-2) reacts strongly with pp255. Other antibodies, including those to spectrin, filamin, ankyrin and the sodium channel do not react with pp255. We conclude that the phosphorylation of sperm MAP-2, or an immunologically-related protein, plays an important role in the regulation of sperm function.

INTRODUCTION

Mature sperm are highly specialized cells with only one function, to deliver DNA to the cytoplasm of the egg. To transit the female reproductive tract and penetrate the egg, mammalian sperm require a complex and coordinated program of motility patterns and the ability to release hydrolytic enzymes that facilitate penetration of the egg's outer envelopes. The regulation of this program, timed and coordinated by signals external to the sperm, appears to involve intracellular messengers (e.g. Ca^{2+}, cAMP and pHi[1]) that are common to other cellular processes. Stimulation of motility has been correlated with increases in cAMP concentration and the elevation of pHi, while Ca^{2+} seems to have a variety of effects on flagellar beating, depending on concentration and species (1-5). Capacitation, a series of physiological changes that render the spermatozoa capable of undergoing the acrosome reaction, involves an increase in adenylate cyclase activity and, presumably, an increase in cAMP (6-8). The acrosome reaction, a fusion of the sperm plasma membrane and the outer acrosomal membrane allowing the release of hydrolytic enzymes, is triggered by an influx of Ca^{2+} and an elevation of pHi (9). The timing of the acrosome reaction is affected by cAMP (10-12). The molecular mechanisms by which these intracellular messengers exert control over sperm function is still

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[1] The abbreviations used are: pHi, intracellular pH; pp255, a phosphoprotein with Mr≈255,000; MAP, microtubule-associated protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MIX, isobutyl-3-methylxanthine; EGTA, [Ethylenebis(oxyethylenenitrilo)]tetraacetic acid; H-8, N-[2-(methylamino)ethyl]-isoquinolinesol-fonamide dihydrochloride; MOPS, 3-(N-morpholino)propanesulfonic acid; CHES, 2[N-cyclohexylamino]ethanesulfonic acid; TTBS, 20 mM tris-HCl pH=7.5, 0.1% Tween-20, 0.5 M NaCl, and 2% NaN_3; MU, sperm motility units.

poorly understood, but it is logical to assume that protein phosphorylation is involved. Several attempts have been made to identify phosphoproteins that regulate motility (13,14). In mammals, the involvement of a 56,000 Da protein in motility initiation has been described (15-17), but its identity and function are currently controversial (18,19).

In previous studies, using intact sperm loaded with $^{32}PO_4$, we identified one protein (Mr = 255,000) whose phosphorylation state is uniquely sensitive to changes in sperm pHi (20). Elevation of pHi causes the initiation of motility in bovine cauda epididymal sperm (21-23). In this report we examine the effects of Ca^{2+} and MIX (a potent stimulator of motility) on intact bovine sperm protein phosphorylation. We find that these modulators also produce dramatic and relatively specific effects on pp255 phosphorylation. Studies of the subcellular localization, characterization and immunologic reactivity of pp255 with an antibody to the cytoskeletal protein, MAP-2, are also reported.

EXPERIMENTAL PROCEDURES

Materials

Bovine epididymides were obtained from local slaughterhouses and kept at 4 °C until sperm were removed (usually 16 hrs after killing). Carrier free [^{32}P]-orthophosphate ($^{32}PO_4$) was from Amersham. MIX, A23187, EGTA, phosphoamino acids, anti-chicken spectrin polyclonal antibody, anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody, and Extravidin-alkaline phosphatase-conjugate were from Sigma; the alkaline phosphatase-conjugate substrate kit was from Bio-Rad. H-8 was from Seikagaku America. Biotinylated Protein A was from Vector; ampholytes were from Hoefer; anti-rat brain MAP-2 polyclonal antibody was from ICN. A polyclonal antibody to rat brain sodium channel, peptide SP19 (24) was a gift of Dr. W. A. Catterall, University of Washington.

Phosphorylation and Regulator Studies.

Bovine epididymal sperm were collected and loaded with $^{32}PO_4$ as previously described (20). Loaded sperm were then washed and resuspended in buffer containing 20 mM MOPS (pH=7.4, except as indicated), 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 10 mM glucose and incubated for 30 min at 37 °C. Other agents, i.e. MIX, H-8 and NaF, were added to this buffer where indicated. Where Ca^{2+} was the agent to be added, the resuspension buffer contained 50 mM MOPS (pH=7.4), 120 mM NaCl, 5 mM KCl, 10 mM glucose, and 0.1 mM EGTA and was prepared with Chelex (Bio-Rad) pretreated water and was stored in polyethelene bottles. The concentrations of free Ca^{2+} in the presence of EGTA were calculated using MAX Chelator version 2.5 (written by Chris Patton, Hopkins Marine Station, Stanford

University). Sperm were then solubilized by adding 75 μl of sperm to 200 μl of boiling 2% SDS, 50 mM CHES (pH=9.5), and 5% ß-mercaptoethanol. After boiling for 5 min, the samples were centrifuged at 13,000 xg for 15 min to sediment DNA. The supernatant was then analyzed by electrophoresis as described below. Sperm motility was measured as previously described and is presented in motility units (MU), which reflect equal contributions from the percentage of sperm which were motile and the relative vigor of their motility (22).

One- and Two-Dimensional Electrophoresis

One-dimensional electrophoresis was conducted on 4-12 % acrylamide gradient SDS-PAGE gels, followed by coomassie staining and autoradiography as previously described (20). Two-dimensional electrophoresis was perform as previously described (25) with slight modifications. Sperm were solubilized in SDS as described above and Nonidet P-40 was added (10%). Samples were subjected to isoelectric focusing on 1.5 mm x 12 cm polyacrylamide tube gels containing 2% ampholytes (20% pH range 3.5 to 10 and 80% pH range 4 to 8) for 11,000 volt/hr. These gels were then equilibrated for 30 min at room temperature in a solution of 2% SDS, 5% β-mercaptoethanol and 62 mM Tris-HCl (pH=6.8) prior to SDS-PAGE on 4-12% linear gradients with a 3% stacking gel. For determination of molecular weights, one lane of high molecular weight standards (Bio-Rad) was added to the edge of the second-dimension. Silver staining was performed using the Rapid Silver Stain Kit (ICN).

Subcellular Localization

Sperm were decapitated and demembranated by sonication and subcellular fractionation was performed as previously described (20) with the slight modification that the sonication buffer, which was designed to limit changes in the phosphorylation state of proteins, also contained 1 mM dithiotreitol and 0.1 mM phenylmethylsulfonyl fluoride.

Identification of Phosphorylated Amino Acids in pp255.

$^{32}PO_4$ loaded sperm were resuspended in 1 mM MIX to fully phosphorylate pp255, followed by solubilization and separation of the proteins by one dimensional gel electrophoresis as described above. The pp255 band was located by autoradiography and cut from the gel. The protein was electroeluted from the gel and hydrolyzed in 6 N HCl for 2 hr at 110°C. The hydrolysate, mixed with phosphorylated amino acid standards (Ser, Thr and Tyr), was analyzed by thin-layer electrophoresis at pH=1.9 in the first dimension and by thin-layer chromatography with isobutyric acid/ammonium hydroxide in the second dimension (26). The phosphorylated amino acid standards were stained with ninhydrin and the thin-layer sheet was analyzed by autoradiography.

Immunoblot Analysis of Sperm and Rat Brain Proteins.

Sperm, $^{32}PO_4$-loaded and incubated with MIX, Ca^{2+} or control buffer, were extracted and proteins separated on 4-12% SDS-PAGE gels (as described earlier). Gels were incubated in 192 mM tris and 25 mM glycine (pH=8.3) for 30 min before proteins were electrophoretically transferred (6 hrs at 50 volts) to nitrocellulose (0.45 μm) in the same buffer. Two different immunoblot methods were used: Method A was a low-stringency procedure for detecting any possible immunoreactivity with

pp255, method B was a high-stringency procedure to detect only high-affinity binding. In method A, the nitrocellulose sheets were blocked with 3% gelatin (melted at 37°C) in 20 mM tris-HCl (pH=7.5), 0.1% Tween-20, 0.5 M NaCl and 2% NaN_3 (TTBS), washed 3 times for 5 mins in TTBS, incubated for 2 hr with the primary antibodies (dilutions = anti-chicken spectrin 1:200, anti-Na channel 1:50, anti-MAP-2 1:200) in TTBS plus 0.1% BSA, washed 3 more times, incubated with biotinylated-protein A for 45 min, washed 3 times, incubated with alkaline phosphatase-conjugated Extravidin for 45 min, washed 3 times and the blots were developed using an alkaline phosphatase substrate kit. In method B, the blots were blocked with 5% non-fat dry milk in TTBS for 2 hr, washed 3 times, incubated for 1.5 hr with primary antibody (MAP-2 1:1000) in TTBS plus 0.1% BSA, washed 4 times, incubated for 1 hr in secondary antibody (1:1000) conjugated to alkaline phosphatase, washed 4 times and developed as above. Blots were photographed and then analyzed by autoradiography.

RESULTS

Regulation of pp255 Phosphorylation Levels.

Treatment of $^{32}PO_4$-loaded intact sperm with the phosphodiesterase inhibitor, MIX, increases the phosphorylation of pp255, while no other major changes in protein phosphorylation levels are apparent (Fig. 1A). Sperm motility is also stimulated by treatment with MIX (22); under these conditions, motility increases from 40 to approximately 80 motility units (MU). In previous studies, we found that the addition of 10 mM lactate to sperm, suspended in buffer at pH 5.5, lowered the internal pH of the sperm and inhibited motility while selectively increasing the phosphorylation of pp255 (20). MIX (1 mM) was able to overcome this motility inhibition without increasing pHi. Comparison of the effects of MIX, lactate, and MIX plus lactate at pH=5.5 on sperm phosphoproteins reveals that both lactate and MIX increase phosphorylation of pp255 and the addition of both results in the greatest increase (Fig. 1B). Minimal changes in the phosphorylation levels of other sperm proteins are observed. Although MIX and lactate have opposite effects on motility, they have very similar effects on protein phosphorylation.

Treatment of sperm with 0.1 μM Ca^{2+} in the presence of the ionophore, A23187, causes a significant (25% as measured by scanning densitometry) and selective reduction in the phosphorylation level of pp255 (Fig. 2). The addition of the ionophore by itself produced no effect on protein phosphorylation (data not shown). At higher concentrations of Ca^{2+}, many bands show reduced phosphorylation (Fig. 2). After 8 minutes at 100 μM Ca^{2+}, most bands are reduced by approximately 30%, while a few bands show larger reductions, such as the bands

at Mr=350,000, 190,000, and 63,000. However, pp255 is the only phosphoprotein which is virtually undetectable at this concentration of Ca^{2+}. Motility is not significantly affected by low levels of Ca^{2+}, but at 0.1 mM Ca^{2+}, motility is reduced from 70 MU (control) to 30 MU.

The ionophore A23178 is an antiporter which promotes the exchange of two protons for each divalent cation. To investigate the possibility that the proton efflux, instead of the Ca^{2+} influx, was responsible for the change in phosphorylation, 10 mM Mg^{2+} was added instead of Ca^{2+}. This should produce a maximum efflux of protons. However, the addition of Mg^{2+} significantly increased the phosphorylation of pp255 (Fig. 2), suggesting that the phosphorylation change was not due to a proton efflux.

Presumably, a modulator of protein phosphorylation would act on either a protein kinase or a phosphoprotein phosphatase (or possibly the substrate itself). To gain insight into mechanism by which MIX and Ca^{2+} were regulating phosphorylation of pp255, we added them to sperm in combination with NaF and H-8. NaF is an inhibitor of bovine sperm motility, but this inhibition can be overcome by the addition of the methylxanthine analog, caffeine (27). Fluoride inhibits several enzymes involved in phosphate transfers, including phosphoprotein phosphatases (28). H-8 is a protein kinase inhibitor with a high affinity for cyclic nucleotide-dependent kinases. The addition of 50 mM NaF increases pp255 phosphorylation with little effect on other sperm phosphoproteins (Fig. 3A). This effect is dramatically accentuated by the addition of MIX. However, Ca^{2+} (0.2 mM), even in the presence of 50 mM NaF, is still able to stimulate the removal of phosphates from proteins, but not as effectively as in the absence of NaF (Fig. 3B).

H-8 diminishes the phosphorylation of pp255 and other sperm protein. It also blocks the increase in phosphorylation of pp255 after treatment with MIX. H-8 and Ca^{2+} together produce even greater reductions in protein phosphorylation than either alone. Although other phosphoproteins are affected by these manipulations, the magnitude of the effects is greatest on pp255. Sperm motility is totally inhibited by fluoride and this is not reversed by the addition of MIX. H-8 has no effect on motility by itself, but H-8 plus Ca^{2+} (15 MU) inhibits more than Ca^{2+} alone (40 MU). MIX by itself or in the presence of H-8 stimulates motility (80 MU) compared to control or control plus H-8 (50 MU).

Subcellular Localization and Extraction of pp255.

Because of the discrete compartmental structure of sperm, localization of pp255 should provide information on its potential function. We have previously shown that the pHi-dependent phosphorylated form of pp255 localizes with the sperm head during subcellular fractionation (20). To eliminate the possibility that MIX is increasing the phosphorylation of a different population of pp255 molecules that are found in another sperm compartment or that a phosphorylation-dependent translocation might be occurring, subcellular fractionation studies were conducted with sperm incubated in the presence and absence of MIX. As can be seen (Fig. 4), pp255 fractionates with the sperm heads, with or without MIX stimulation of phosphorylation.

Identification of Phosphorylated Amino Acid(s) in pp255.

Two-dimensional thin-layer electrophoresis and chromatography of acid-hydrolysed pp255, which had been cut from an SDS-PAGE gel, shows that virtually

all of the detectable phosphorylation is on serine (Fig. 5). Careful examination of the autoradiogram shows that threonine is phosphorylated slightly, but not at levels above a few percent of the total ^{32}P incorporation into pp255. No tyrosine phosphorylation was detected.

Comparison of Sperm Phosphoproteins by 2-D Electrophoresis After Treatment with MIX or Ca^{2+}.

Autoradiographs of 2-dimensional SDS-PAGE gels containing sperm extracts after control, MIX or Ca^{2+} treatment are shown in Fig. 6A, B and C, respectively. The addition of 1 mM MIX dramatically increases pp255 (series of spots between arrows) but has little or no effect on other phosphoproteins. The addition of 0.2 mM Ca^{2+} removes all detectable phosphates from pp255 and also reduces the phosphorylation level of many, but not all, other sperm proteins. In both panels A and B, several spots are apparent in the Mr=255,000 range. Each spot probably represents different degrees of phosphorylation of a single protein species (the more phosphates per molecule of protein, the farther it will migrate during isoelectric focusing). The pp255 dot at the far left (the top of the IEF gel) shows equivalent or higher phosphorylation in the control when compared to the MIX-treated sperm. This makes sense because the control would be expected to contain more of the slower migrating protein molecules.

Identification of pp255 by Western analysis.

In an attempt to identify pp255, several antibodies that had been produced against protein antigens similar in size to pp255, were used to immunoblot "Western" transfers from SDS-PAGE gels. For each blot, (Fig. 7, panels A-F), three lanes

were loaded with solubilized proteins from $^{32}PO_4$ loaded sperm that had been treated with Ca^{2+}, (lane 2), with MIX, (lane 3) or no addition (lane 4). Treatment with Ca^{2+} and MIX affects the phosphorylation level of pp255 and, consequently, it's migration rate in a SDS-polyacrylamide gel. As seen in the autoradiograph of the blot (panel E), the addition of MIX slows the mobility of pp255 compared to control. The pp255 band is also more spread out after MIX treatment, because each protein molecule can contain various numbers of phosphates. The addition of Ca^{2+} removes most of the phosphate from pp255, so the band should migrate farther and be tighter. An antibody that recognizes pp255 should mimic the movements of pp255 caused by these agents. Each blot also contains non-radioactive proteins from a fresh rat brain homogenate (lane 1).

Antibodies against filamin, spectrin, ankyrin and the sodium channel did not react with bovine sperm pp255 (Fig. 7, filamin and ankyrin data not shown). The sodium channel antibody does react with a 260 kDa protein from the rat brain homogenate which was run as a positive control (Fig. 7C, lane 1). Antibodies made against rat brain MAP-2 intensely labeled rat brain proteins (2 bands approximately 300 kDa)(Fig. 7D, lane 1) and sperm proteins (approximately 255 kDa)(Fig. 7D, lanes 2-4). The shift in migration of pp255 on the autoradiograph (Fig. 7E) matches that observed on the immunoblot probed with the MAP-2 antibody (Fig. 7D). Additional bands are stained using the MAP-2 antibody, particularly bands at 45 kDa and 55 kDa. These could either be proteolytic products of MAP-2 or forms of Tau, a smaller MAP (reported Mr's from 38,000 to 65,000) that shares tubulin-binding domains with MAP-2 (29).

The immunoblots in panels A-D were analyzed using a biotinylated protein A detection system. Several proteins are stained even in the absence of any primary antibody or biotinylated protein A (Fig. 7A). The simplest explanation for this is that there are naturally occurring biotinylated proteins in both sperm and rat brain. A similar immunoblot was probed with the same MAP-2 antibody (diluted 1:1000 instead of 1:200) but using a secondary antibody conjugated to alkaline phosphatase and different incubation times and blocking conditions (see method B in experimental procedures)(Fig. 7F). Under these conditions, pp255 is the only dominant band identified in the sperm protein lanes. The lane containing the rat brain homogenate shows another immunoreactive band at approximately 70 kDa. This might be MAP-2C, a protein known to share antigenic determinants with the higher molecular weight MAP-2A and MAP-2B. Probing with pre-immune rat serum under similar conditions produced no detectable bands (data not shown).

DISCUSSION

When intact bovine sperm are exposed to several agents known to modulate sperm function, the phosphorylation state of one protein, pp255, is dramatically changed. Other protein phosphorylation changes which do occur are less pronounced, and are not affected by all agents. MIX and NaF increase pp255 phosphorylation, while Ca^{2+}, H-8 and elevated pHi decrease its phosphorylation. Because of the sensitivity of sperm function to modulation by these agents (5), it is probable that pp255 phosphorylation is of central importance in sperm behavior. We initially expected the phosphorylation of pp255 to be an intermediary in the regulation of sperm motility. The positive correlation between phosphorylation of pp255 and motility after treatment with MIX or Ca^{2+} supports this hypothesis. However, the involvement of pp255 in motility regulation now seems unlikely based on several observation: 1) the localization of pp255 to the sperm head; 2) the inverse relationship between pp255 phosphorylation and sperm motility as regulated by pHi; 3) the stimulation of motility by MIX in the presence of H-8, with no concomitant increase in pp255 phosphorylation; and d) the decrease in pp255 phosphorylation at low concentrations of Ca^{2+} (0.1 μM), which do not inhibit motility. Additional studies will be necessary to absolutely exclude a role for pp255 in motility, as well as to determine its role in other sperm processes.

Determining the molecular site of action of these modulators on the sperm phosphorylation machinery will require extensive studies, partially because none of these agents are absolutely specific. However, some conclusions are possible. The dephosphorylation of most sperm proteins in response to treatment with Ca^{2+}

suggests that Ca^{2+}s primary action is the stimulation of the Ca^{2+}/calmodulin-dependent phosphoprotein phosphatase, calcineurin, which has recently been identified in sperm (30). Ca^{2+} is also known to stimulate a Ca^{2+}/calmodulin-dependent cyclic nucleotide phosphodiesterase (31) and to modulate a Ca^{2+}-inhibitable protein kinase (32). Both of these enzymes could contribute to the dephosphorylation of sperm proteins, but the fact that the Ca^{2+} effect was not mitigated at all by the addition of H-8, while the addition of NaF did partially block the Ca^{2+} effect, suggests that the phosphatase may be the primary site of action.

The ability of H-8, a protein kinase inhibitor, to totally block the stimulatory effect of MIX on pp255 phosphorylation, suggests that MIX is directly or indirectly stimulating a kinase. The fact that MIX was able to stimulate motility in the presence of H-8 suggests that the effect of MIX on motility may not require a phosphorylation reaction. Other investigators have shown that caffeine is able to stimulate motility without changing sperm phosphoproteins (27). They concluded that caffeine can stimulate motility by multiple mechanism, one involving the production of cAMP and stimulation of protein kinase and one independent of cAMP. Our data also suggests that methyl xanthines can stimulate motility independently from the cyclic AMP system.

An antibody against rat brain MAP-2 reacts strongly with pp255. The changes in electrophoretic mobility of pp255 in response to treatment with MIX or Ca^{2+} are mimicked by the changes in the MAP-2 antibody staining pattern. This provides rigorous evidence that this antibody is recognizing pp255 and not another sperm protein of similar size. Other evidence suggesting that pp255 is a sperm MAP-2

include: a) the Mr of pp255, which is within the range from 250 to 300 kDa reported for MAP-2 by other investigators (33,34); b) both pp255 and MAP-2 are heavily phosphorylated, pp255 has the highest specific radioactivity (ratio of $^{32}PO_4$ incorporation per coomassie blue staining intensity) of any sperm phosphoprotein (20) and MAP-2 is reported to have up to 46 moles of phosphate per mole of protein (35); and c) both pp255 and MAP-2 are phosphorylated primarily on serine residues. Rat brain MAP-2 is seen as a doublet protein and is larger than bovine sperm MAP-2, but tissue and species heterogeneity is commonly observed with high Mr MAPs (33). Peptide mapping or sequence information will be necessary to extend our apparent identification of pp255 as a sperm MAP-2. Our data only establish that pp255 and rat brain MAP-2 share one or more common epitopes.

Although sperm are known to be a rich source for microtubules, to our knowledge this is the first report of MAP-2 or a MAP-2 like protein in sperm. In other tissues, MAP-2 has been shown to modulate microtubule assembly, and to form crossbridges between microtubules and other cytoskeletal elements (34,36). MAP-2 phosphorylation reduces its ability to promote tubulin polymerization and to cross-link microtubules and microfilaments (37-42). The ability of phosphorylated MAPs to form crosslinks is pH dependent and peaks sharply between pH 6.2 and 6.3 (43). This pH range is very similar to the internal pH reported for bovine sperm, pH 6.4 to 6.6 (20,44,45), suggesting that sperm would be very sensitive to regulation by phosphorylated MAP-2.

Several different kinases are capable of phosphorylating MAP-2 <u>in vitro</u> (32,46,47), however, comparison of <u>in vivo</u> with <u>in vitro</u> phosphorylation sites suggest

that they are different (48). Our studies with intact sperm suggest that pHi, Ca^{2+}, and a MIX stimulated protein kinase (probably protein kinase A), all may be involved in regulating the phosphorylation of MAP-2. A recent study has identified a Ca^{2+}-inhibitable protein kinase that phosphorylates MAP-2 (32). This enzyme showed 50% inhibition by 0.5 μM free Ca^{2+} and was almost totally inhibited by 2 μM free Ca^{2+}. If a similar kinase exists in sperm, this could explain the unique sensitivity of pp255 phosphorylation to modulation by Ca^{2+} and perhaps the other modulators that we studied. Since RII, the regulatory subunit of cAMP-dependent kinase, binds with high affinity to the projection domain of MAP-2 (35,49), and RII is known to be important in sperm function (17,18,50-53), it will be interesting to determine if RII is associated with MAP-2 in sperm.

Several sperm cytoskeletal structures have been identified that are potential sites of MAP-2 involvement in the regulation of sperm structure and function. The basal striations are located in the postnuclear sheath near the base of the head and contain structures which resemble microtubules and which require reducing agents for extraction (54); SDS extraction of pp255 also requires a reducing agent (20). A macromolecular complex is also associated with the outer acrosomal membrane, which contains phosphoproteins with Mrs similar to that of pp255 (55). If pp255 is a sperm form of MAP-2, it may be involved in the development and maintenance of the unique pattern of sperm structural domains (2). It is perhaps more likely, based upon pp255's unusual sensitivity to phosphorylation regulation, that it is involved in regulating capacitation, the acrosome reaction or some aspect of sperm-egg interaction.

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REFERENCES

1. Lindemann, C.B., and Kanous, K.S. (1989) *Arch. Androl.* **23**, 1-22

2. Eddy, E.M. (1988) in *The physiology of reproduction* (Knobil, E., and Neill, J., eds) pp. 27-68, Raven Press, Ltd., New York

3. Brokaw, C.J. (1987) *J. Cell Biochem.* **35**, 175-184

4. Tash, J.S., and Means, A.R. (1983) *Biol. Reprod.* **28**, 75-104

5. Garbers, D.L., and Kopf, G.S. (1980) *Adv. Cyclic. Nucleotide. Res.* **13**, 251-306

6. Stein, D.M., Fraser, L.R., and Monks, N.J. (1986) *Gamete. Res.* **13**, 151-158

7. Stein, D.M., and Fraser, L.R. (1984) *Gamete. Res.* **10**, 283-299

8. Berger, T., and Clegg, E.D. (1983) *Gamete. Res.* **7**, 169-177

9. Yanagimachi, R. (1988) in *The physiology of reproduction* (Knobil, E., and Neill, J., eds) pp. 135-185, Raven Press, Ltd., New York

10. Hyne, R.V., and Garbers, D.L. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5699-5703

11. Hyne, R.V., and Garbers, D.L. (1979) *Biol. Reprod.* **21**, 1135-1142

12. Mrsny, R.J., and Meizel, S. (1980) *J. Exp. Zool.* **211**, 153-157

13. Hayashi, H., Yamamoto, K., Yonekawa, H., and Morisawa, M. (1987) *J. Biol. Chem.* **262**, 16692-16698

14. Ishiguro, K., Murofushi, H., and Sakai, H. (1982) *J. Cell Biol.* **92**, 777-782

15. Brandt, H., and Hoskins, D.D. (1980) *J. Biol. Chem.* **255**, 982-987

16. Tash, J.S., Kakar, S.S., and Means, A.R. (1984) *Cell* **38**, 551-559

17. Tash, J.S., Hidaka, H., and Means, A.R. (1986) *J. Cell Biol.* **103**, 649-655

18. Noland, T.D., Abumrad, N.A., Beth, A.H., and Garbers, D.L. (1987) *Biol. Reprod.* **37**, 171-180

19. Paupard, M.C., MacLeod, J., Wasco, W., and Orr, G.A. (1988) *J. Cell Biochem.* **37**, 161-175

20. Carr, D.W., and Acott, T.S. (1989) *Biol. Reprod.* (in press)

21. Acott, T.S., and Carr, D.W. (1984) *Biol. Reprod.* **30**, 926-935

22. Carr, D.W., and Acott, T.S. (1984) *Biol. Reprod.* **30**, 913-925

23. Babcock, D.F., Rufo GA, J.r., and Lardy, H.A. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 1327-1331

24. Gordon, D., Merrick, D., Wollner, D.A., and Catterall, W.A. (1988) *Biochemistry.* **27**, 7032-7038

25. Dunbar, B.S. (1986) *Handbook for two-dimensional polyacrylamide gel electrophoresis and immunological techniques for basic and clinical research*, Plenum, New York

26. Cooper, J.A., Sefton, B.M., and Hunter, T. (1983) *Methods. Enzymol.* **99**, 387-402

27. Schoff, P.K., and Lardy, H.A. (1987) *Biol. Reprod.* **37**, 1037-1046

28. Jain, M.K. (1982) *Handbook of Enzyme Inhibitors (1965-1977)*, John Wiley & Sons, New York

29. Lewis, S.A., Wang, D.H., and Cowan, N.J. (1988) *Science* **242**, 936-939

30. Tash, J.S., Krinks, M., Patel, J., Means, R.L., Klee, C.B., and Means, A.R. (1988) *J. Cell Biol.* **106**, 1625-1633

31. Wasco, W.M., Kincaid, R.L., and Orr, G.A. (1989) *J. Biol. Chem.* **264**, 5104-5111

32. Hoshi, M., Nishida, E., and Sakai, H. (1988) *J. Biol. Chem.* **263**, 5396-5401

33. Wiche, G. (1989) *Biochem. J.* **259**, 1-12

34. Olmsted, J.B. (1986) *Annu. Rev. Cell Biol.* **2**, 421-457

35. Tsuyama, S., Terayama, Y., and Matsuyama, S. (1987) *J. Biol. Chem.* **262**, 10886-10892

36. Wiche, G. (1985) *Trends Biochem. Sci.* **10**, 67-70

37. Murthy, A., and Flavin, M. (1983) *Eur. J. Biochem.* **137**, 37-46

38. Yamamoto, H., Fukunaga, K., Goto, S., Tanaka, E., and Miyamoto, E. (1985) *J. Neurochem.* **44**, 759-768

39. Akiyama, T., Nishida, E., Ishida, J., Saji, N., Ogawara, H., Hoshi, M., Miyata, Y., and Sakai, H. (1986) *J. Biol. Chem.* **261**, 15648-15651

40. Jameson, L., and Caplow, M. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 3413-3417

41. Nishida, E., Hoshi, M., Miyata, Y., Sakai, H., Kadowaki, T., Kasuga, M., Saijo, S., Ogawara, H., and Akiyama, T. (1987) *J. Biol. Chem.* **262**, 16200-16204

42. Selden, S.C., and Pollard, T.D. (1983) *J. Biol. Chem.* **258**, 7064-7071

43. Nishida, E., Kuwaki, T., and Sakai, H. (1981) *J. Biochem. (Tokyo.)* **90**, 575-578

44. Smith, M.B., Babcock, D.F., and Lardy, H.A. (1985) *Biol. Reprod.* **33**, 1029-1040

45. Babcock, D.F. (1983) *J. Biol. Chem.* **258**, 6380-6389

46. Hoshi, M., Akiyama, T., Shinohara, Y., Miyata, Y., Ogawara, H., Nishida, E., and Sakai, H. (1988) *Eur. J. Biochem.* **174**, 225-230

47. Goldenring, J.R., Vallano, M.L., and DeLorenzo, R.J. (1985) *J. Neurochem.* **45**, 900-905

48. Murthy, A.S.N., Bramblett, G.T., and Flavin, M. (1985) *J. Biol. Chem.* **260**, 4364-4370

49. Vallee, R.B., DiBartolomeis, M.J., and Theurkauf, W.E. (1981) *J. Cell Biol.* **90**, 568-576

50. Horowitz, J.A., Voulalas, P., Wasco, W., MacLeod, J., Paupard, M.C., and Orr, G.A. (1989) *Arch. Biochem. Biophys.* **270**, 411-418

51. Horowitz, J.A., Toeg, H., and Orr, G.A. (1984) *J. Biol. Chem.* **259**, 832-838

52. Horowitz, J.A., Wasco, W., Leiser, M., and Orr, G.A. (1988) *J. Biol. Chem.* **263**, 2098-2104

53. Pariset, C., Feinberg, J., Dacheux, J-L., Oyen, O., Jahnsen, T., and Weinman, S. (1989) *J. Cell Biol.* **109**, 1195-1205

54. Koehler, J.K. (1973) *J. Ultrastruct. Res.* **44**, 355-368

55. Olson, G.E., Winfrey, V.P., Garbers, D.L., and Noland, T.D. (1985) *Biol. Reprod.* **33**, 761-779

Fig. 1. Effects of MIX and pHi on sperm protein phosphorylation levels. Autoradiographs (A, lanes 1 and 2; B, lanes 1-4) and coomassie blue staining patterns (A, lanes 3 and 4; B, lanes 5-8) of SDS-PAGE gels are shown. A) Whole sperm were loaded with $^{32}PO_4$ for 2 hours, centrifuged for 5 min and then resuspended for 30 min in buffer at pH = 7.4 with no additions (lanes 1 and 3) or with 1 mM MIX (lanes 2 and 4) prior to extraction and electrophoresis (see Methods). B) Conditions were identical except that sperm were resuspended in buffer at pH = 5.5 with no additions (lanes 1 and 5), with 1 mM MIX (lanes 2 and 6), with 10 mM lactate (lanes 3 and 7), or with both MIX and lactate (lanes 4 and 8) for 30 min. Lanes labeled "S" are Mr standards, with scales as indicated; arrows indicate the position of pp255.

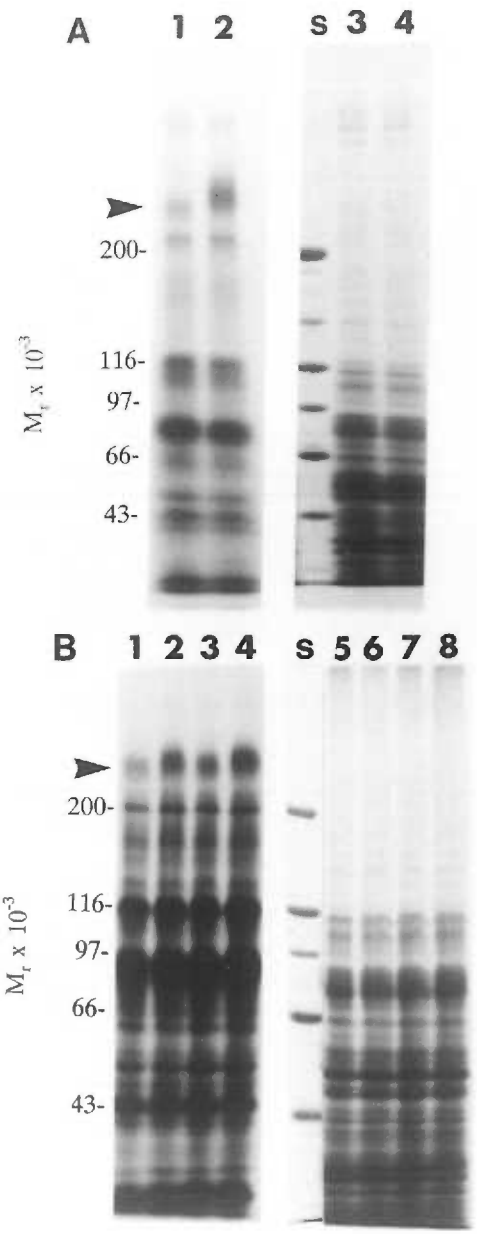

A
1 2
S 3 4
200-
116-
97-
66-
43-
M_r x 10^{-3}
B
1 2 3 4
S 5 6 7 8
200-
116-
97-
66-
43-
M_r x 10^{-3}

Fig. 2. Effects of Ca^{2+} concentration on sperm protein phosphorylation levels. Autoradiograph (A) and coomassie blue staining pattern (B) of sperm loaded with $^{32}PO_4$, which were extracted for electrophoresis after 8 min (lanes 1-6) or 20 min (lanes 7-12) resuspension in buffer at pH = 7.4 with 0.1 mM EGTA and 30 μM A23187 (Ca^{2+} ionophore) in all tubes. Treatments are: no other additions (cont); 10^{-7} M Ca^{2+} (pCa 7); 10^{-6} M Ca^{2+} (pCa 6); 10^{-5} M Ca^{2+} (pCa 5); 10^{-4} M Ca^{2+} (pCa 4); or 10^{-2} M Mg^{2+} (pMg 2). Mr standards are apparent in B) and scale is shown in A); arrows indicate the position of pp255.

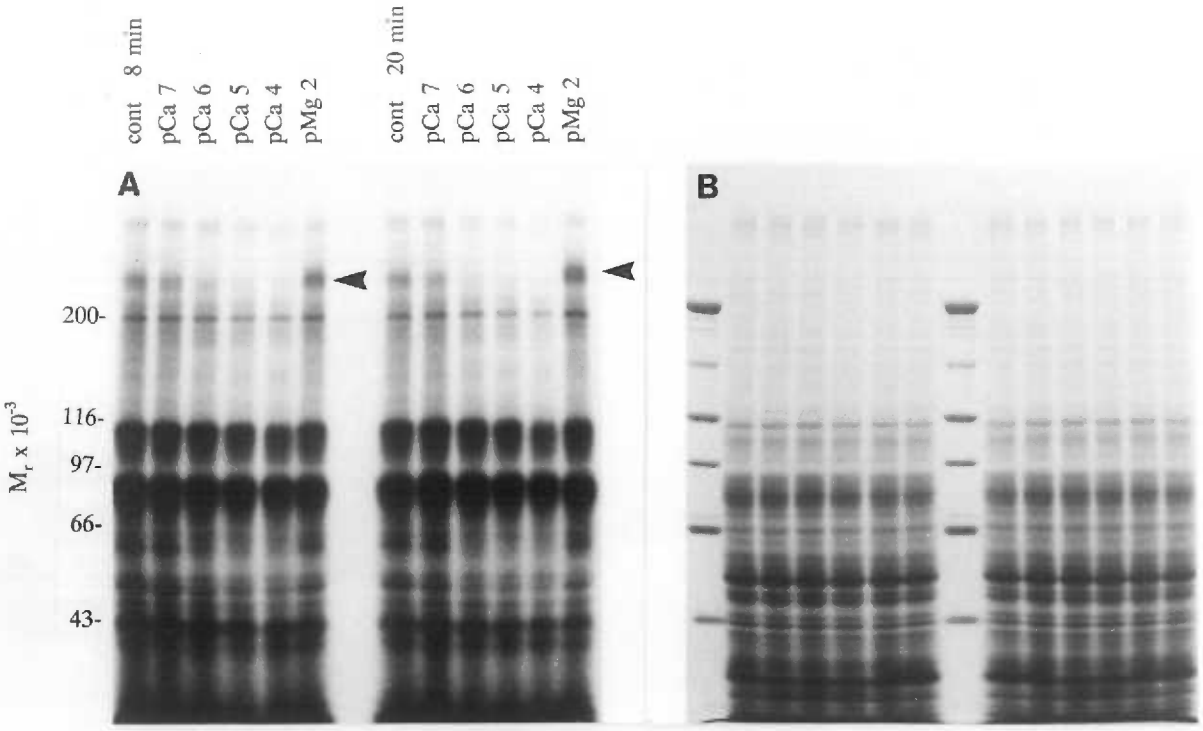

cont 8 min
pCa 7
pCa 6
pCa 5
pCa 4
pMg 2
cont 20 min
pCa 7
pCa 6
pCa 5
pCa 4
pMg 2
A
B
200-
116-
97-
66-
43-
M_r x 10^{-3}

Fig. 3. Effects of fluoride and H-8 on sperm phosphoprotein levels in the presence and absence of Ca^{2+} and MIX. Autoradiograph of sperm extract which had been treated with various combinations of 1 mM MIX, 50 mM NaF, 2 mM H-8 or 0.2 mM free Ca^{2+} (see methods for details of treatment conditions); in addition to these indicated additives, all lanes in panel B also contained 0.1 mM EGTA and 30 μM ionophore (A-23187). Scale shows apparent Mr of standards and arrows indicates the position of pp255.

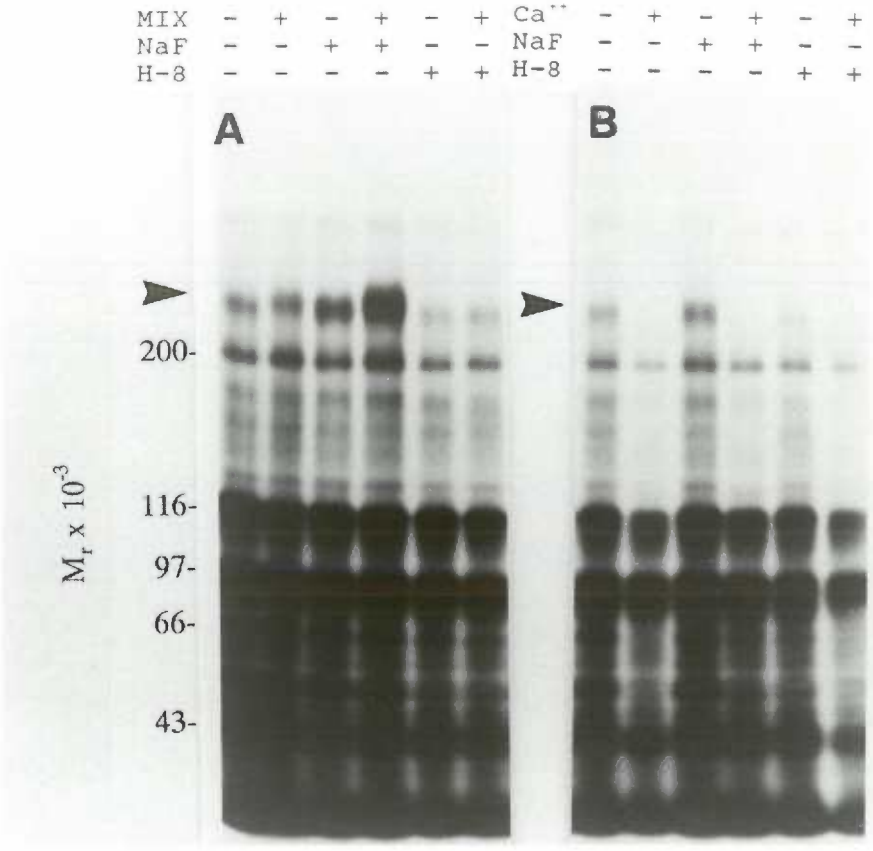

MIX - + - + - +
NaF - - + + - -
H-8 - - - - + +
Ca++ - + - + - +
NaF - - + + - -
H-8 - - - - + +
A
B
200-
116-
97-
66-
43-
$M_r \times 10^{-3}$

Fig. 4. Subcellular fractionation of sperm with and without MIX treatment. Sperm, which had been loaded with $^{32}PO_4$ and resuspended in buffer for 20 min at pH 7.4 without (- MIX, below lanes) or with 1 mM MIX (+ MIX, below lanes) added. Sperm were then sonicated, fractionated, extracted and electrophoresed (see Methods). The lanes in this autoradiograph correspond to: the sperm supernatant (S), the membrane fractions (M), the tails and midpieces (T) and the heads (H). A scale for the Mr standards is shown; arrow indicate the position of pp255.

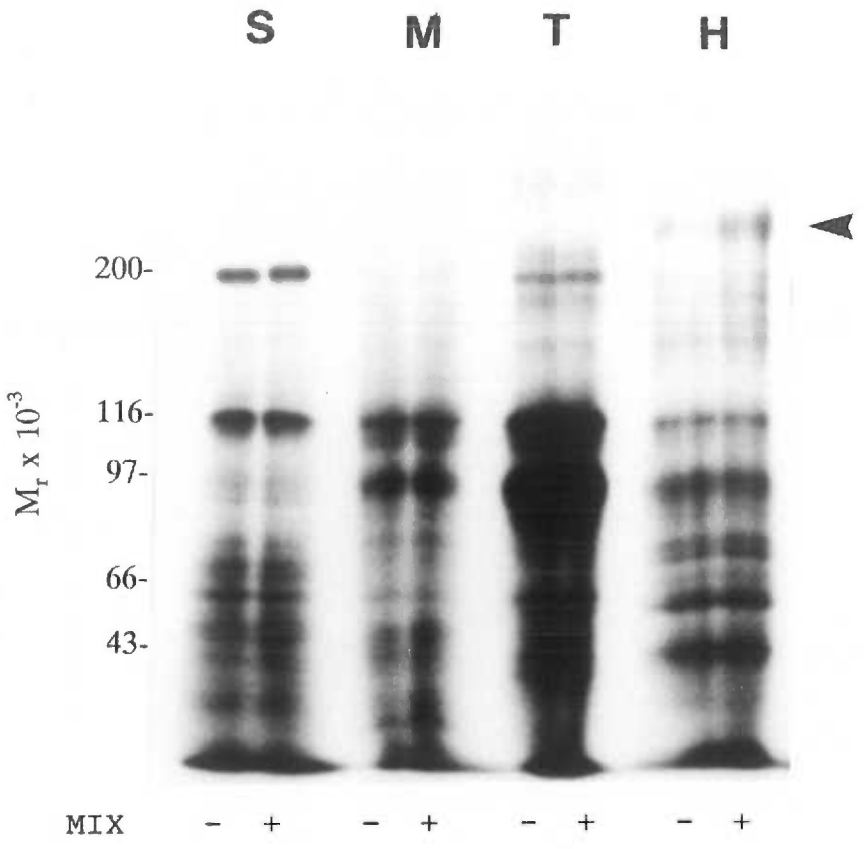

S
M
T
H
200-
116-
97-
66-
43-
M_r x 10^{-3}
MIX
- + - + - + - +

Fig. 5. Identification of the amino acid(s) phosphorylated in pp255. Sperm pp255 was phosphorylated in ^{32}P-loaded sperm in the presence of MIX, electroeluted from bands cut from an SDS-PAGE gel, hydrolyzed with acid and subjected to 2-dimensional thin-layer electrophoresis/chromatography (see Methods). Phosphorylated amino acid standards (Ser, Thr and Tyr) were added prior to separations. A) Shows the positions of the ninhydrin-stained phosphoamino acids standards; and B) shows the autoradiograph of the same sheet. Arrows indicate the direction of electrophoresis in the first dimension and chromatography in the second dimension.

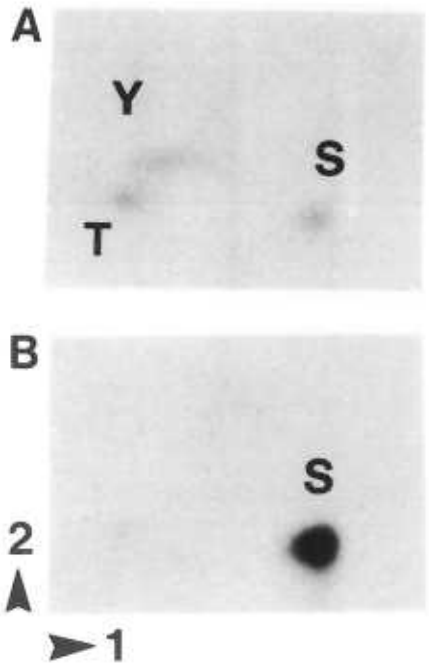

A
Y
S
T
B
S
2
1

Fig. 6. Two-dimensional electrophoresis of sperm phosphoproteins after treatment with MIX and Ca^{2+}. Sperm, treated with no additions (A), with 1 mM MIX (B) or with 0.2 mM free Ca^{2+}, 0.1 mM EGTA and 30 μM A23187 (C), were extracted and samples were subjected to 2-dimensional gel electrophoresis and autoradiography (see Methods). Scale shows Mr of standards and arrows indicate migration of pp255 in the second dimension. The plus and minus indicate the polarity of the IEF electrodes.

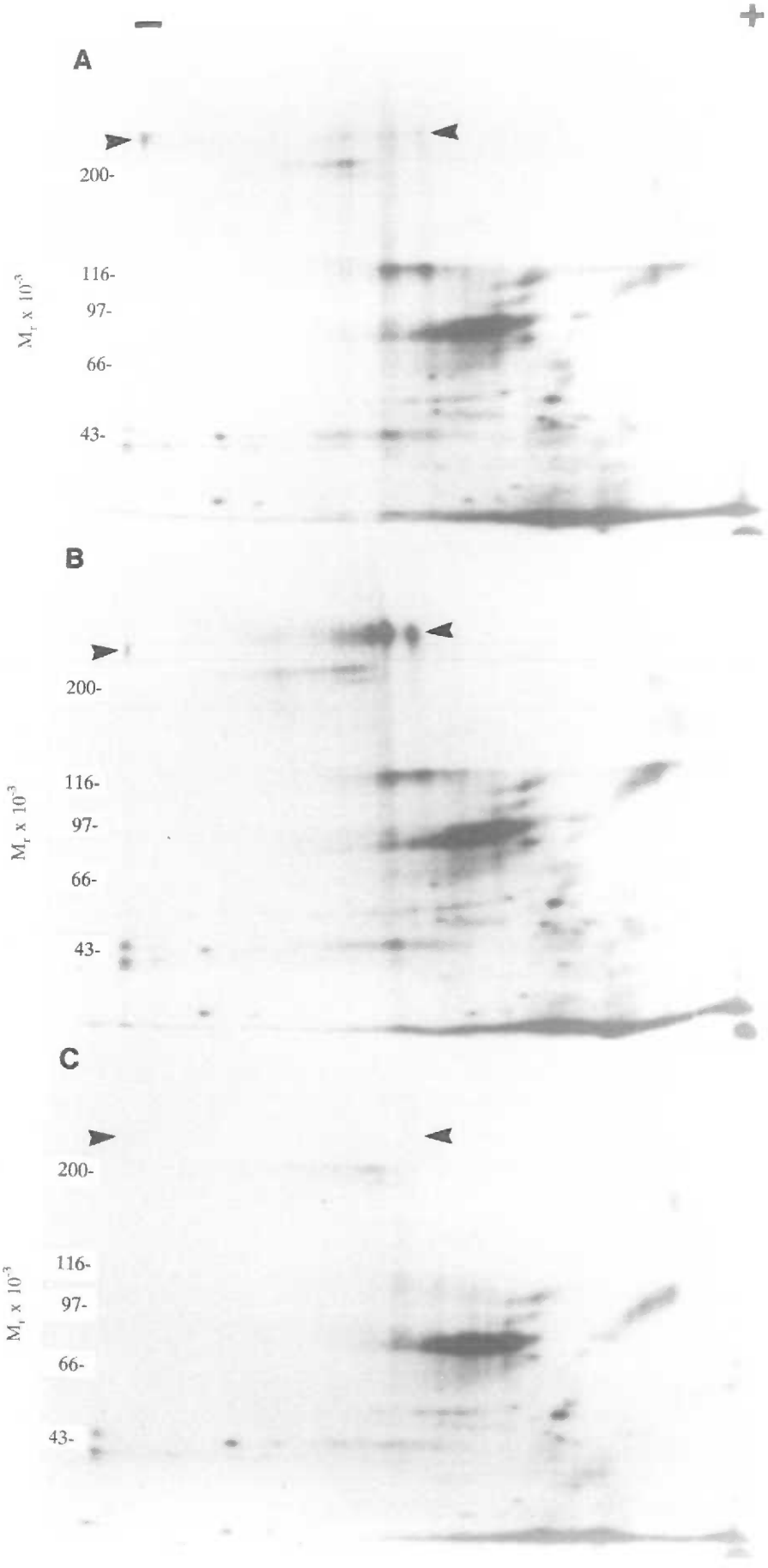
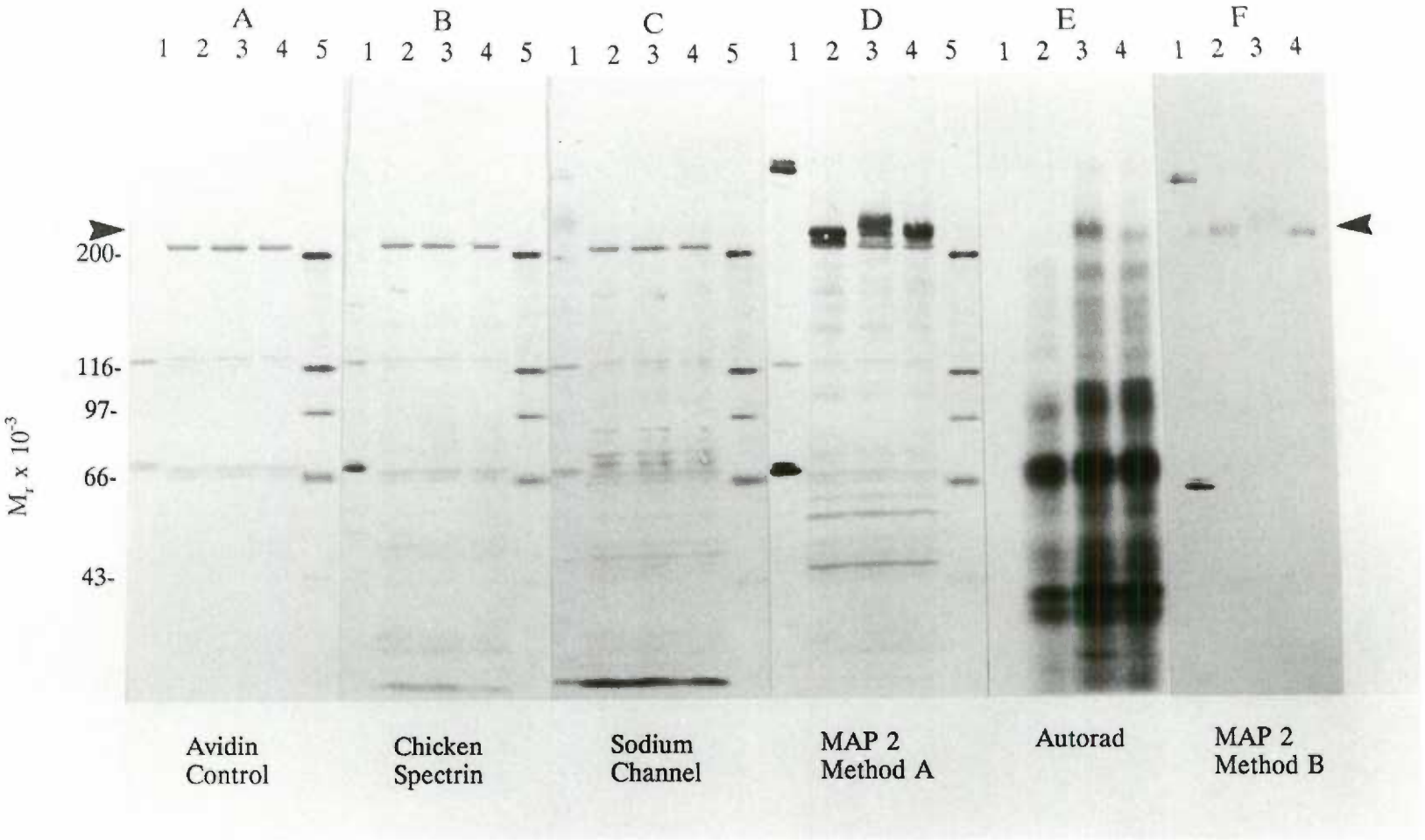

−
+
A
200-
116-
97-
66-
43-
$M_r \times 10^{-3}$
B
200-
116-
97-
66-
43-
$M_r \times 10^{-3}$
C
200-
116-
97-
66-
43-
$M_r \times 10^{-3}$

Fig. 7. Immunoblots and autoradiographs of rat brain homogenate and sperm extracts after treatment with MIX or Ca^{2+}.

Immunoblots were developed using a biotinylated protein A system (panels A-D)(see method A in experimental procedures) or using a secondary antibody system (panel F)(see method B in experimental procedures). The samples for all panels are rat brain homogenate (lane 1), or $^{32}PO_4$-loaded sperm treated with 0.2 mM Ca^{2+} (lane 2), 1 mM MIX (lane 3), or control (lane 4)(except panel F which has the Ca^{2+} and control lanes reversed). Biotinylated molecular weight markers are in lane 5. Panel E is a representative autoradiograph of the blots (all the blots produce identical autoradiographs). The blots were incubated with avidin only (no antibody or biotinylated protein A)(panel A), anti-chicken spectrin (panel B), anti-sodium channel (panel C), anti-MAP-2 diluted 1:200 (panel D) or anti-MAP-2 diluted 1:1000 (panel F). Arrows mark the position of pp255.


A
B
C
D
E
F
1 2 3 4 5
200-
116-
97-
66-
43-
M_r x 10^{-3}
Avidin Control
Chicken Spectrin
Sodium Channel
MAP 2 Method A
Autorad
MAP 2 Method B

MANUSCRIPT 3

Regulation of Bovine Sperm Dynein ATPase by Phosphorylation[1]

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Running page heading: Regulation of Dynein ATPase by Phosphorylation

Key words: Bovine, Spermatozoa, Dynein, Phosphorylation, cAMP, Calcium, Protein Kinase

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ABSTRACT

Any mechanism that regulates sperm motility must directly or indirectly affect the dynein/microtubule apparatus. Since regulators of sperm motility such as cAMP, Ca^{2+} and isobutyl-3-methylxanthine (MIX) often mediate changes in protein phosphorylation, we investigated: 1) the direct effects of these regulators on purified dynein ATPase activity; 2) the effect of in vitro phosphorylation of dynein on ATPase activity; and 3) the ability of MIX to modify dynein phosphorylation in intact bovine sperm.

Dynein ATPase activity was isolated by high salt extraction of bovine epididymal sperm and purified by sucrose density gradient centrifugation. A major peak of ATPase activity was observed at 12S. The ATPase activity was linear with time for 90 min, linear with protein concentration to 20% conversion of substrate to product, and showed minimal pH dependence from pH 6 to 9. The ATPase activity was inhibited by vanadate and erythro-9-[3-(2-hydroxynonyl)]adenine (EHNA), but not by ouabain, oligomycin or azide, indicating the dynein ATPase assay was not contaminated by other forms of ATPases. Triton (0.05%) inhibited the ATPase activity approximately 20%, while reducing agents increased activity by 40%. This suggests that bovine sperm dynein is significantly different than the latent form of dynein observed in sea urchin sperm.

MIX, cAMP, and calmodulin had no significant effect on the purified dynein ATPase activity. CaATP, substituted for MgATP as substrate, significantly increased activity but only by 15%. W7, a calmodulin antagonist, did inhibit activity by approximately 35%, however, the inhibition is not reversed by the addition of

calmodulin. The addition of the catalytic subunit of protein kinase A to dynein, at various stages of purification, reveals that several dynein subunits can be phosphorylated. Phosphorylation of dynein resulted in an inhibition of ATPase activity ranging from 0 to 40%.

Several bands with Mr similar to dynein heavy chains (300 to 400 kDa) are phosphorylated in intact sperm, which had been preloaded with $^{32}PO_4$. The addition of 1 mM MIX stimulates sperm motility and increases the phosphorylation of a protein with Mr=255,000, however, no change in phosphorylation of dynein heavy chains are observed. We conclude that the motility modulators cAMP, MIX and Ca^{2+} do not modify dynein ATPase directly nor do they alter dynein heavy chain phosphorylation via a protein kinase.

INTRODUCTION

The beat of a cilia or flagella requires the coordinated interaction of the dynein heads with the adjacent axonemal microtubules. At least three levels of regulation have been postulated (2) to account for axoneme beating: 1) Since simultaneous sliding of all 9 outer doublets would be non-productive, one level of control must restrict activation to one side of the axoneme at a time; 2) a second level of control is necessary to modulate the various types of wave forms observed, including variations in amplitude, shape, direction and beat frequency; and 3) a third control mechanism is required to initiate and to maintain motility. This third mechanism appears to involve the phosphorylation of one or more axonemal proteins (3-5). Chemical modulators that stimulate or inhibit motility must either directly or indirectly regulate the dynein ATPase activity. Known regulators of sperm motility include MIX, cAMP, Ca^{2+}, and pHi (3,6-10). A testable hypothesis is that these modulators stimulate dynein ATPase activity directly or stimulate a protein kinase which phosphorylates dynein, resulting in altered ATPase activity.

Methylxanthines, such as MIX, caffeine and theophylline, stimulate sperm motility (11-13). These compounds are phosphodiesterase inhibitors and cAMP is well established as a regulator of sperm motility (4-5). However, methylxanthines have been shown to affect sperm motility by pathways that do not involve changes in cAMP levels (14). As in other cells, cAMP activates a sperm protein kinase A (15), and inhibitors of protein kinase A, such as the heat-stable protein kinase inhibitor (PKI) or the isoquinolinesulfonamide derivative H-8, block the effect of cAMP on motility (16).

The involvement of Ca^{2+} in the regulation of motility is complex (17). Low concentrations of Ca^{2+} (0.1 to 1 μM) alter flagellar and ciliary waveforms, while higher concentrations inhibit beating completely (3,18,19). Ca^{2+} also changes the direction of the effective stroke in cilia, increases beat frequency, produces asymmetric bending waves and causes differential activity of two flagella in one cell (for review see (17)). It is reasonable to assume that some of these responses are mediated by calmodulin, which has been identified in both the sperm head and the tail (20,21). A Ca^{2+}/calmodulin-dependent phosphodiesterase (22) and phosphoprotein phosphatase (18) have been identified in sperm (18). The existence of these enzymes suggests that regulation of at least some sperm functions by Ca^{2+} involves a protein phosphorylation mechanism.

Dynein can be phosphorylated in intact sperm or after isolation (23-25), but the effect of phosphorylation on ATPase activity is unknown. To determine if MIX, cAMP, Ca^{2+} or calmodulin regulate dynein directly, we have examined the effects of these agents on bovine sperm dynein ATPase activity. In addition, we have phosphorylated isolated dynein and measured the effects on its ATPase activity. Using intact sperm, we have also compared the phosphorylation levels of dynein heavy chains from control and motility-stimulated sperm.

MATERIALS AND METHODS

Materials

Fresh bovine epididymides were obtained from a local slaughterhouse. Erythro-9-[3-(2-hydroxynonyl)]adenine (EHNA) was from Burroughs Wellcome (Research Triangle Park, NC). Triton X-100, dithiothreitol (DTT), sodium orthovanadate, ouabain, oligomycin B, sodium azide, cAMP, isobutyl-3-methylxanthine, calmodulin, protein kinase catalytic and regulatory subunits, PMSF, Benzamidine, and DEAE-sephacel were from Sigma. ATP was from Boehringer Mannheim, ^{32}P-ATP was from New England Nuclear, and $^{32}PO_4$ was from Amersham. W7 was from Seikagaku America.

Isolation of bovine sperm

Spermatozoa were collected from bovine caudal epididymides by retrograde flush of the vas deferens with 7% sucrose (26). Undiluted sperm were centrifuged at 2000xg for 10 min, the supernatant was removed, and the sperm were washed 2 times in buffer (20 mM HEPES (pH 7.4), 5 mM KCl, 120 mM NaCl, 10 mM $NaHCO_3$, 1 mM $MgCl_2$ and 10 mM glucose), and resuspended as indicated below.

Extraction of dynein ATPase

Washed sperm were demembranated by sonication on ice for 40 sec in 5 volumes of buffer containing: 20 mM tris-HCl (pH 8.0), 1 mM [Ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 10 mM Benzamidine-HCl, 0.055 TIU/ml aprotinin and 1 mM PMSF. The sperm were maintained at 4°C for all subsequent procedures, except as indicated. Demembranated sperm were

centrifuged for 10 min at 9000xg, and the pellet resuspend in extraction buffer containing: 0.6 M NaCl, 20 mM tris-HCl (pH 8.0), 4 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 10 mM Benzamidine-HCl, 0.055 TIU/ml aprotinin and 1 mM PMSF. This solution was vortexed, allowed to sit for 10 min, centrifuged for 20 min at 27,000xg, and the supernatant, containing partially purified dynein, was collected and stored at 4°C.

Purification of dynein

Dynein, partially purified as detailed above, was concentrated approximately 10-fold using Amicon Centriprep concentrators. It was then layered on a 12 ml isokinetic (top concentration = 10%) sucrose gradient in buffer containing: 300 mM NaCl, 8 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA, and 40 mM tris-HCl (pH 8.0). After centrifugation for 16 hr at 40,000 rpm in a Beckman SW-40 rotor at 5°C, 0.5 ml fractions were collected by puncturing the bottoms of the tubes and pumping in 60% sucrose. The elution was monitored spectrophotometrically at 280 nm. Values for S were determined in parallel tubes using catalase and α-macroglobulin as standards. In some cases, as indicated in the text, dynein was purified by DEAE-Sephacel column chromatography (27), prior to sucrose gradient centrifugation.

ATPase activity determination

Enzyme assays were initiated by adding crude or purified dynein to a reaction mixture (final volume=0.4 ml) containing 20 mM tris-HCl (pH 8.0), 150 mM KCl, 2 mM $MgCl_2$, 4 mM ATP and ^{32}P-ATP (100,000 cpm per tube). Where indicated, Mg^{2+} was replaced with Ca^{2+} and for inhibition experiments, the ATP concentration was reduced to 0.1 mM. Incubations were for 60 min at 37°C and the reaction was

terminated by adding 3.5 ml of ice cold 2% charcoal suspended in 0.1 N HCl, followed by vortexing and filtration through a 0.45 μm filter. The tube was washed 2 times with 0.1 N HCl and the contents were also poured over the filter. The filtrate was analyzed for Cerenkov radiation in a liquid scintillation counter. Blanks, without enzyme added, were less than 1% of the total counts and were subtracted from all experiments. Error bars represent standard error of the means and significance was determined by t-tests.

Combination ATPase and protein kinase assays

Combination assays were performed in a final volume of 100 μl containing 50 mM MOPS (pH=7.0), 10 mM $MgCl_2$, 10 μM cAMP, 1 mM vanadate, 0.2 mM ATP and ^{32}P-ATP (2.5 x 10^6 cpm/assay tube). The catalytic subunit of protein kinase A (22 μg) was dissolved in 90 μl of 50 mg/ml DTT. Where indicated, either 10 μl of protein kinase (final concentration equal 80 picomolar Units/assay tube) or a DTT control was added to the reaction mixture. The regulatory subunit of type II protein kinase (1 μg/assay tube) was added as a positive control for the kinase assay. The assays were initiated by the addition of dynein or protein kinase immediately followed by dynein and incubations were for 30 min at 37° C. Assays were terminated by adding 40 μl aliquots to 40 μl of 2% SDS, 50 mM CHES (pH 9.5) and 5% ß-mercaptoethanol, samples were boiled for 3 min and analyzed for protein kinase activity by electrophoresis on a 4-12% acrylamide gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels followed by autoradiography. The remaining 60 μl of reaction mixture was analyzed for ATPase activity by adding 3.5 ml of cold 0.1 N HCl containing charcoal and processing as

described for the ATPase assay.

Gel electrophoresis

SDS-PAGE was conducted on a 4-12% linear acrylamide gradient (Fig. 6) or 4% acrylamide (Fig. 7) slab gels according to the method of Laemmli (28). After electrophoresis, the gels were stained, dried and then autoradiographed using Kodak X-Omat AR5 or OMC film.

Incorporation of $^{32}PO_4$ into intact sperm

Sperm were prepared and loaded with $^{32}PO_4$ and the proteins extracted as previously described (10).

RESULTS

Characterization of bovine sperm dynein ATPase activity

The ATPase activity of high-salt extracts from bovine sperm was determined at various times, enzyme concentrations, temperatures and pHs (Fig. 1). The activity was linear with time for at least 90 minutes at the enzymes and substrate concentrations used (Fig. 1A). This differs from previous reports in which the activity had plateaued by 60 minutes (29). The addition of proteinase inhibitors in our assay buffers might account for this difference. All subsequent assays were incubated for 60 minutes. ATPase activity was linear with increasing enzyme concentration until approximately 20% of the substrate had been converted to product, after which activity declined slightly (Fig. 1B). In subsequent experiments, concentrations of dynein were chosen such that only 5 to 10% of the substrate was hydrolyzed in 60 min. ATPase activity increases with increasing temperature up to 40°C (Fig. 1C). All subsequent experiments were performed at the physiological temperature of 37°C.

Dynein ATPase activity exhibits a broad pH-optimum ranging from approximately pH 7 to at least pH 9 (Fig. 1D). The activity at pH 5.5 is 40% less than optimal activity. Three buffers were used to control the pH over this range. These results are in agreement with Young and Smithwick (30), but Belles-Isles et al. (31), reported a sharp decline in dynein activity below pH 7. Their activity at pH 5.5 was 10% of that observed at pH 7. Comparison of their extraction procedures and assay conditions with ours reveals many differences, but none that would obviously reconcile the different results. All subsequent assays were performed at

pH 8.0.

Effects of Ca^{2+} and Triton X-100 on ATPase activity

Dynein ATPase activity was increased approximately 15% ($p<0.01$) when CaATP was substituted for MgATP (Fig. 2). The addition of 0.05% triton, which stimulates latent dynein activity from many species (32), inhibited the activity of bovine dynein ATPase by approximately 25% ($p<0.01$) using either Ca^{2+} or Mg^{2+}. Since Mg^{2+} is thought to be the physiological substrate, all subsequent assays used Mg^{2+} as the divalent cation.

Effect of Dithiothreitol

Sulfhydryl reagents also stimulate the latent forms of dynein obtained from other species (33). DTT stimulates bovine dynein ATPase activity in a dose-dependent manner (Fig. 3). At 10 mM DTT, the ATPase activity is increased by approximately 60%.

Sucrose Density Gradient Centrifugation

Salt-extracted dynein ATPase was further purified by sucrose density gradient centrifugation (SDGC). The vast majority of ATPase activity co-sedimented with less than 1% of the total protein at 12 S (Fig. 4). Smaller amounts of activity were also found in the 19 to 21 S range.

Effects of ATPase Inhibitors

Penningroth (34) has suggested specific criteria for distinguishing dynein from other ATPase activities. Included in his criteria were dose-dependent inhibition by vanadate (0.001-0.5 mM) and EHNA (0.05-1.0 mM); and insensitivity to inhibition by ouabain (0.1 mM), oligomycin (5 ug/ml), and azide (0.1 mM). The SDGC-

purified ATPase activity was appropriately sensitive or insensitive to all of the above, suggesting the ATPase being measured was a dynein ATPase with little or no contamination from other types of ATPases (Fig. 5).

Effects of cAMP, MIX and Calmodulin

Addition of 10 μM cAMP, 0.5 mM MIX or 0.01 mg/ml calmodulin has no significant effect on dynein ATPase activity (Fig. 6). Addition of 0.5 mM W7, an antagonist of calmodulin, inhibited dynein ATPase by approximately 40% and was not reversed by the addition of calmodulin. The effect of W7 was dose-dependent (0.1 mM inhibited $\approx$ 10% and 1 mM inhibited $\approx$ 65%) and produced the same inhibitory effect whether MgATP or CaATP was used as the substrate (data not shown).

Effect of phosphorylation

Dynein ATPase assays were conducted in the presence and absence of the catalytic subunit of protein kinase A. To determine if dynein was being phosphorylated under these conditions, an aliquot of the reaction mixture was solubilized by boiling in 2% SDS buffer, followed by SDS-PAGE and autoradiography. This allowed determination of both endogenous and exogenous protein kinase activity. The regulatory subunit of protein kinase A (R_{II}), a known substrate for protein kinase A, was added to alternating samples as a positive control for the kinase reaction. Crude dynein contained significant amounts of endogenous kinase activity and preferentially phosphorylated bands at approximately 40,000 and 20,000 Daltons (Fig. 7, lanes 5 and 6). The addition of exogenous kinase slightly enhanced this same pattern of bands (Fig. 7, lanes 7 and 8). R_{II} is phosphorylated