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INITIAL EVIDENCE FOR THE MODIFICATION OF HAMSTER SPERM Na^+, K^+-ATPase ACTIVITY BY CYCLIC NUCLEOTIDE-MEDIATED PROCESSES

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Na^+, K^+-ATPase activity of homogenates prepared from cauda epididymal golden hamster sperm increased after the addition of cGMP (50μM), monobutyryl cGMP (0.5μM) or cGMP-dependent protein kinase (0.94μg/ml). Addition of monobutyryl cAMP (0.5μM) or purified catalytic subunit of cAMP-dependent protein kinase (1.26μg/ml) inhibited the activity of the Na^+, K^+-ATPase. Preincubation with a partially purified preparation of cAMP-dependent protein kinase inhibitor (75μg/ml) stimulated the activity of the Na^+, K^+-ATPase, and this stimulation was decreased by the addition of 5μM monobutyryl cAMP. It is not yet known whether direct and/or indirect mechanisms are involved, but these results are the first to describe such opposing effects by cyclic nucleotide-mediated processes on a Na^+, K^+-ATPase activity.

Na^+, K^+-ATPase (EC 6.3.1.3) is arguably the most thoroughly studied of the membrane-bound enzymes and represents a major pathway in eukaryotic cells for the transport of Na^+ and K^+ across the plasma membrane (1). It has been suggested that *in vivo* regulation (both inhibitory and stimulatory) of this enzyme occurs through cyclic nucleotides (2-4). Na^+, K^+-ATPase has been demonstrated in sperm of a variety of mammalian species (5). Its activity is required for maintenance of the bull sperm membrane potential and thereby flagellar motility (6) and is essential for hamster sperm capacitation and/or the acrosome reaction (7), those changes in the mammalian sperm head which are required for fertilization (8). The addition of analogues of cAMP or GMP or phosphodiesterase (PDE) inhibitors (compounds which presumably raise cyclic nucleotide levels in the cell) have been reported to affect motility, capacitation and/or

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the acrosome reaction in sperm of a variety of mammalian species (9) including hamster (10). In the present report, preliminary evidence is presented which suggests that both stimulation and inhibition of hamster sperm Na^+, K^+-ATPase activity can occur through cyclic nucleotide-dependent processes.

MATERIALS AND METHODS

Chemicals and Preparations: The following were purchased: Tris base and sucrose (each ultrapure grade) from Schwartz-Mann; Omnifluor from New England Nuclear; (γ-^{32}p) ATP from ICN; and cGMP (Na salt) BtcGMP (N^2-monobutyryl cGMP, Na salt), cAMP (Na salt), BtcAMP (N^6-monobutyryl cAMP, Na salt), ATP ("vanadium-free" Na salt), GMP (Na salt), guanosine (crystalline), ouabain octahydrate, sodium dodecyl sulfate, benzamidine, Ribonuclease A (type III-A, bovine pancreas) and protein kinase inhibitor (type II, bovine heart) from Sigma; other chemicals obtained were of highest grade commercially available. The following were gifts: the catalytic subunit of cAMP-dependent protein kinase from bovine heart muscle, prepared essentially as described (11), from Drs. Susan Whitehouse and Donal Walsh; and the cGMP-dependent protein kinase from bovine lung, prepared as described (12), from Drs. Gordon Gill and Claude Monken.

Preparation of Sperm Homogenate: Cauda epididymal sperm were obtained from mature golden hamsters and washed as previously described (7). Pelleted sperm were disrupted in 2.5ml of a 0.32M sucrose, 1.0mM EDTA, 1.0mM benzamidine (SEB) solution at pH 7.0 (4°C) using a Potter-Elvehjem homogenizer. The homogenization was repeated and the combined supernatants which contained no detectable sperm heads or tails, were combined and kept at 4°C (no change in activity was detected after 24 hours) until assayed. Based on the number of sperm heads in the pellet following the second homogenization, the crude membrane preparation was diluted with SEB to produce a stock such that further dilutions by the assay procedures produced a final value equivalent to 1.75 x 10^6 sperm or approximately 7mg of protein per assay tube. Protein concentration of the crude membrane preparation was determined following detergent solubilization as described (13).

Enzyme Assay Condition: A 225µl aliquot of diluted sperm homogenate was added to an equal volume of assay buffer (210mM NaCl, 50mM Tris, 5mM $MgCl_2$, pH = 7.4 measured at 37°C) containing either KCl (final conc. of 10mM) or ouabain (final conc. of 1.0mM) and preincubated for 10 minutes at 25°C. The assay buffer and conditions had been determined in preliminary experiments to be maximal for the demonstration of the ouabain inhibitable ATPase activity. The concentration of ouabain used under assay conditions (1.0mM) was ten times that found to maximally inhibit the enzyme of this hamster sperm preparation. Compounds to be tested were added five minutes after the addition of KCl or ouabain. Additions of carrier buffers were made in each case to serve as controls. The assay was initiated by the addition of a stock ATP solution to produce a final concentration of 1.0mM (γ - ^{32}P) ATP 0.5mCi/mmole). The final volume of each tube was always 500µl and the tubes were incubated at 37°C. Assays were linear for 45 minutes. In the routine 15 minute incubations used for all treatments tested, less than 1.0% of substrate ATP was hydrolyzed, and total ATPase activity was similar in all experiments (data not shown). The assay was terminated by the addition of 100µl of a 6% sodium dodecyl sulfate solution. Released inorganic phosphate was separated from ATP by extraction of a phosphomolybdate complex into an organic phase and quantitated as described (14). It should be noted that an ATPase activity of several somatic tissues from golden hamster is stimulated by ouabain in the absence of K^+ (15). However, in the present experiments no difference in hamster sperm ATPase activity was observed in the presence or absence of K^+ when a saturating concentration of ouabain was included. The presence of ouabain

also ruled out the possibility of K^+-independent active transport of Na^+ by Na^+, K^+-ATPase in our determination of enzyme activity.

Electron Microscopy: A freshly prepared sperm homogenate was centrifuged at 100,000 X g for 18 hrs. at 4°C. A sample of disrupted head and tail fragments was obtained from the 17,000 X g pellet following preparation of the sperm homogenate. Standard procedures of fixation, staining and examination were used (17).

RESULTS AND DISCUSSION

The pellet obtained by ultracentrifugation of the assay preparation was comprised of unilaminar vesicles devoid of head and tail fragments (Fig. 1A). Analysis of the disrupted sperm head and tail fragments showed no remaining plasma membrane while mitochondrial and inner acrosomal membranes appeared intact (Fig. 1B). Therefore, the preparations used for these Na^+, K^+-ATPase assays were comprised of both head and tail plasma membrane, outer acrosomal membrane, and soluble acrosomal and cytoplasmic components. Therefore, our

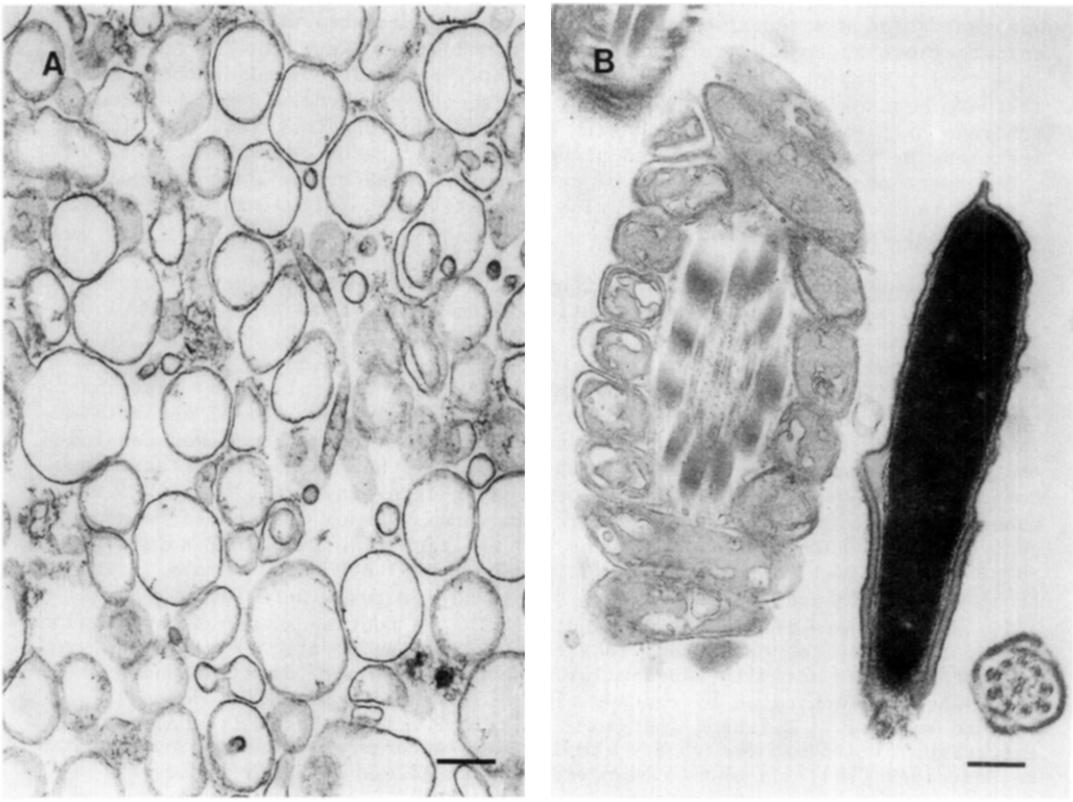

Figure 1: Transmission electron micrographs of thin sections from A) pelleted vesicles of the hamster sperm homogenate and B) pelleted remains of disrupted sperm heads and tails. Bars, 0.2μm; X 50,000.

Table 1: Effect of cyclic nucleotides, cyclic nucleotide-dependent protein kinases and protein kinase inhibitor on the Na^+, K^+-ATPase activity of the hamster sperm assay preparation.

Treatment	(n)	Experimental Activity	Control Activity	Percentage[1] of control
cGMP				
50 µM	7	3.17 ± 0.53	1.94 ± 0.36	163
5 µM	3	1.80 ± 0.66	1.86 ± 1.06	96
0.5 µM	4	1.99 ± 0.42	1.95 ± 0.60	101
GMP				
50 µM	4	1.20 ± 0.20	1.17 ± 0.43	103
cAMP				
50 µM	7	1.79 ± 0.33	1.94 ± 0.36	92
5 µM	3	1.74 ± 0.24	1.86 ± 1.06	93
0.5 µM	3	1.92 ± 0.39	1.86 ± 1.06	103
BtcGMP				
0.5 µM	3	2.22 ± 0.46	1.60 ± 0.41	138
BtcAMP				
0.5 µM	3	1.08 ± 0.38	1.06 ± 0.41	68
Butyrate				
5 µM	3	1.76 ± 0.59	1.76 ± 0.52	100
cGMP-dependent Protein kinase[2]				
0.94 µg/ml	3	2.12 ± 0.45	1.54 ± 0.51	137
Heat treated[2,3]	2	1.69 ± 0.06	1.99 ± 0.42	85
Catalytic Subunit cAMP-dependent Protein Kinase[4]				
1.26 µg/ml	2	0.66 ± 0.10	1.21 ± 0.12	55
Ribonuclease A[4]				
1.26 µg/ml	2	1.62 ± 0.35	1.69 ± 0.25	95
Protein kinase Inhibitor				
75 µg/ml	4	2.20 ± 0.20	1.25 ± 0.12	177
plus 5 µM BtcAMP	2	1.57 ± 0.49	1.20 ± 0.11	133

Homogenates containing soluble components and membrane vesicles prepared from washed cauda epididymal hamster sperm were incubated, extracted and assayed as described under "Materials and Methods." Activity is expressed as a mean ±SEM in nmoles P_i released/10^6 sperm/hour.

[1]Comparisons were made to control values obtained in the same assay.

[2]Carrier buffer was 10mM potassium phosphate, 1mM EDTA, 1mM dithiothreitol and 20% gycerol, pH=6.8.

[3]Preparation was heated at 80°C for 20 minutes.

[4]Carrier buffer was 20mM potassium phosphate, 15mM 2-mercaptoethanol, 0.2mM EDTA and 50% glycerol, pH=6.7.

experiments could not distinguish whether a treatment affected the enzyme activity from the plasma membrane of all regions of the sperm (head or flagellum) in a uniform manner.

The results presented demonstrate that cyclic nucleotides can modify hamster sperm Na^+, K^+-ATPase activity in an homogenate containing both soluble components and membrane vesicles (Table 1). Enzyme activity was stimulated

by BtcGMP or high concentration of cGMP or by preincubation with purified cGMP-dependent protein kinase. Incubation of the homogenate with BtcAMP or catalytic subunit of cAMP-dependent protein kinase inhibited Na^+, K^+-ATPase. BtcAMP, capable of activating cAMP-dependent protein kinase function (18) also reduced the stimulation of Na^+, K^+-ATPase activity produced by preincubation with protein kinase inhibitor. Since the initial state (i.e. the extent of activation or inhibition) of each Na^+, K^+-ATPase preparation was unknown, the effects produced by these treatments may or may not have been maximal.

The present experiments do not indicate whether the increase in Na^+, K^+-ATPase activity by cGMP-dependent protein kinase occurred through a direct phosphorylation of the Na^+, K^+-ATPase. Preferential phosphorylation of several membrane proteins by cGMP has been documented (reviewed in 19). The bovine lung cGMP-dependent protein kinase preparation used in these experiments appeared fully activated (no further stimulation occurred with cGMP addition, data not shown) and contained one to two moles of cGMP per mole protein kinase (G. Gill, personnal communication). Stimulation of the Na^+, K^+-ATPase by the kinase could not have been caused by kinase associated cGMP since, at most, 10nM cGMP was present in the assay mixture due to the presence of the kinase. This concentration is much lower than that required (50μM) to elicit a stimulation.

The inability of low concentration of cGMP and all cAMP concentrations tested to effect Na^+, K^+-ATPase activity was probably due to the presence of PDE activity in the assay preparation, since mammalian sperm contain multiple forms of PDE capable of both cAMP and cGMP hydrolysis (9). Addition of BtcAMP or BtcGMP, which are more resistant to hydrolysis by PDE (18), were capable of affecting Na^+, K^+-ATPase activity (Table 1). Both cAMP and cGMP can be present in sperm (9), but the endogenous levels of these cyclic nucleotides were not measured in these assay preparations. Differences in the endogenous cyclic nucleotide levels and the resulting state of the Na^+, K^+-ATPase in different preparations might explain the variability observed in some control and experimental treatments (Table 1).

Although bovine lung cGMP-dependent protein kinase stimulated the sperm preparation of Na^+, K^+-ATPase, as yet no cGMP-dependent protein kinase has been demonstrated biochemically in mammalian sperm (9). However, immunocytochemical localization of a "perinuclear" rat sperm cGMP-dependent kinase has been reported (20). Cyclic AMP-dependent protein kinase has been found in both the head and tail of mammalian sperm (21) and may comprise as much as 30% of the sperm soluble proteins (22,23). Although cGMP-dependent protein kinase affected hamster sperm Na^+, K^+-ATPase activity, stimulation by added cGMP or BtcGMP may or may not have been completely mediated through a kinase. Several cGMP binding proteins with no apparent phosphotransferase activity have been observed in other cells (24), and cGMP can stimulate a PDE which preferentially hydrolyzes cAMP (25)

Somatic cell Na^+, K^+-ATPases have been reported to be inhibited (2) or stimulated (3) by cAMP-dependent protein kinase and stimulated by cGMP (4). The present results suggest that cAMP-mediated and cGMP-mediated processes act in an opposing manner on hamster sperm Na^+, K^+-ATPase, an enzyme activity which is essential for motility (6) and for capacitation and/or the acrosome reaction of mammalian sperm (7). Whatever the function of this control by cyclic nucleotides and whether the control is direct or indirect, to our knowledge the present report is the first to demonstrate opposing effects of cAMP and cGMP or cAMP-dependent and cGMP-dependent protein kinases on a Na^+, K^+-ATPase activity.

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REFERENCES

1. Schwartz A., Lindenmayer, G.E. and Allen, J.C. (1975) Pharmacological Reviews 27, 3-134.
2 Braughler, J. M. and Corder, C.N. (1978) Biochem. Biophys. Acta 524, 455-465.
3. Scheid, C.R., Honeyman, T.W. and Fay, F.S. (1979) Nature 277, 32-36.
4. Shi, M.M., Stewart, D. J. and Sen, A.K. (1980) Can. J. Biochem. 58, 1223-1229.

5. Uesugi, S. and Yamazoe, S. (1966) Nature 209, 403.
6. McGrady, A. (1979) J. Reprod. Fert. 56, 549-553.
7. Mrsny, R. J. and Meizel, S. (1981) J. Cell. Biol. 91, 77-8
8. Meizel, S. (1978) in Development in Mammals (Johnson, M.E., ed), Vol. 3, pp. 1-64, Elsevier, Amsterdam.
9. Garbers, D. L. and Kopf, G.S. (1980) in Adv. in Cyclic Nucleotide Res. (P. Greengard and G.A. Robinson, eds.) Vol. 13, pp. 251-306, Raven Press, New York.
10. Mrsny, R. J. and Meizel, S. (1980) J. Exp. Zool. 211, 153-157.
11. Rubin, C.S., Erlichman, J., and Rosen, O.M. (1974) in Methods in Enzymology (Hardman, J.G. and O'Malley, B.O., eds.) Vol. 36, pp. 308-315, Academic Press, New York.
12. Monken, C.E. and Gill, G.N. (1980) J. Biol. Chem. 255, 7067-7070.
13. Hess, H.H., Lees, M.B. and Derr, J.E. (1978) Anal. Biochem. 85, 295-300.
14. Seals, J.R., McDonald, J.M., Bruns, D., and Jarett, L. (1978) Anal. Biochem. 90, 785-795.
15. Teisinger, J., Vyskocil, F. and Dlouha, H. (1981) Experentia 37, 383-384.
16. Forgac, M. and Chin, G. (1981) J. Biol. Chem. 256, 3645-3646.
17. Kelly, L.K., King, B.F.. Johnson, L.W. and Smith, C.H. (1979) Exp. Cell Res. 123, 157-176.
18. Castagna, M., Palmer, W.K. and Walsh, D.A. (1977) Arch. Biochem. Biophys. 181, 46-60.
19. Glass, D.B. and Krebs, E.G. (1980) Ann. Rev. Pharmacol. Toxicol. 20, 363-388.
20. Spruill, W.A., Koide, Y., Huang, H-L., Levine, S.N., Ong, S-H., Steiner, A.L. and Beavo, J.A. (1981) Endocrinology 109, 2239-2248.
21. Tash, J.S. and Means, A.R. (1982) Biol. Reprod. 26, 745-763.
22. Hoskins, D.D., Casillas, E.R. and Stephens, D.T. (1972) Biochem. Biophys. Res. Commun. 48, 1331-1338.
23. Garbers, D.L., First, N.L. and Lardy, H.A. (1973) J. Biol. Chem. 248, 875-879.
24. Lincoln, T.M., Hall, C.L., Park, C.R., and Corbin, J.D. (1976), Proc. Natl. Acad. Sci. USA 73, 2559-2563.
25. Martins, T.J., Mumby, M.C. and Beavo, J.A. (1982) J. Biol. Chem. 257, 1973-1979.