Phosphorylation of Membrane-bound Guanylate Cyclase of Sea Urchin Spermatozoa

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Abstract. When *Arbacia punctulata* spermatozoa are incubated in seawater containing ammonium hydroxide (pH 8.8), the sperm plasma membrane-bound guanylate cyclase is dephosphorylated, its electrophoretic mobility increases (from an apparent molecular mass of 160 to 150 kD), and its enzymatic activity decreases 3.5-fold. Transfer of these cells into ammonium-free seawater (pH 7.4) results in the rephosphorylation of the cyclase, its reconversion to 160 kD, and recovery of the enzymatic activity lost upon dephosphorylation. This is the first direct demonstration that the activity of membrane-bound guanylate cyclase can be regulated by phosphorylation. A plasma membrane preparation is described that specifically supports the in vitro phosphorylation of the guanylate cyclase. This preparation will be useful in more detailed studies on the relationship between phosphorylation state and enzymatic activity of membrane-bound guanylate cyclase.

S EA urchin eggs are surrounded by a gelatinous matrix known as the egg jelly layer, through which spermatozoa must pass before contacting the egg surface. The major macromolecular component of egg jelly is a fucose sulfate-rich glycoconjugate (10, 28, 29) that induces the exocytotic acrosome reaction of spermatozoa by mechanisms involving altered ion fluxes (25–27, 36). Egg jelly also contains small peptides that affect sperm respiration and motility. The best characterized of these peptides is "speract," a peptide of 10 amino acids isolated from the egg jelly of *Strongylocentrotus purpuratus* and *Hemicentrotus pulcherrimus* (16, 33). The sequence of speract has been determined (11, 33), analogues have been synthesized (11, 21), and a 77-kD speract receptor in the sperm plasma membrane has been identified (4). A different peptide of 14 amino acids named "resact" has been isolated from egg jelly of the sea urchin *Arbacia punctulata*, and its sequence determined (32, 34). Resact has recently been shown to be a potent chemoattractant for *A. punctulata* spermatozoa (42).

When *A. punctulata* spermatozoa are exposed to solubilized egg jelly the electrophoretic mobility (on SDS gels) of a major integral membrane protein changes from an apparent molecular mass of 160 to 150 kD (39). This protein has been purified to homogeneity and identified as guanylate cyclase (40). Sea urchin spermatozoa contain extremely high levels of this enzyme (7, 15), which is localized in the plasma membrane of the flagellum (14, 24, 39). Correlated with the egg jelly–induced mobility shift of the cyclase is a large decrease in its enzymatic activity (22, 40). Resact has been identified as the component of egg jelly responsible for inducing the 160- to 150-kD mobility shift of the cyclase (34).

Previous evidence has suggested that the resact-induced electrophoretic mobility shift of the cyclase results from dephosphorylation of the 160-kD (phosphorylated) form of the enzyme. When spermatozoa are incubated in seawater containing $^{32}P_i$, label is incorporated into the 160-kD cyclase, in the form of [^{32}P]phosphoserine (39). Upon exposure of the labeled spermatozoa to egg jelly (or resact), all radiolabel is rapidly (within 3 s) lost from the 160-kD band and the mobility shift to 150 kD occurs (34, 39). A similar shift in apparent molecular mass and loss of radiolabel from the cyclase occurs in vitro when sperm extracts are treated with calf intestinal alkaline phosphatase (39). While these and other (41) data suggest a dephosphorylation mechanism, the definitive demonstration would be the rephosphorylation of the 150-kD form of the cyclase and its reconversion to the 160-kD form. Here we describe in vivo conditions under which rephosphorylation and reconversion to 160 kD will occur. Rephosphorylation of the cyclase recovers the enzymatic activity lost upon dephosphorylation. The preliminary characterization of a sperm plasma membrane preparation supporting the in vitro phosphorylation of sperm guanylate cyclase is also presented.

Materials and Methods

Gametes and Reagents

A. punctulata were spawned by intracoelomic injection of 0.3 ml of 0.5 M KCl. Gametes were collected from the gonopores with a pipette, spermatozoa were stored at 0°C as undiluted semen, and egg jelly was prepared as described (39). SDS (70% lauryl sulfate; catalog No. L-5750) was from Sigma Chemical Co. (St. Louis, MO) and sodium orthovanadate was from

Fisher Scientific (Tustin, CA). [γ-^{32}P]ATP, 4,000 Ci/mmol, [γ-^{32}P]GTP, 3,000 Ci/mmol, and [8-^{3}H]GTP, 10–20 Ci/mmol, were from ICN Radiochemicals Inc. (Irvine, CA). All other reagents were from Sigma Chemical Co.

Induction of Reversible Dephosphorylation of Guanylate Cyclase

Semen (200 μl) was incubated 3 h at 21°C with 200 μCi ^{32}P$_i$ and then diluted into 40 ml Millipore-filtered natural seawater (MFSW) that contained 10 mM Tris-HCl and had been titrated to pH 8.8 with concentrated NH$_4$OH (final NH$_4$OH, 18.6 mM). Aliquots were removed at 1-min intervals for 6 min and diluted with 4 vol MFSW containing 20 mM Tris-HCl (unadjusted pH, 7.05). The diluted suspensions (final pH, 7.36) were incubated at 21°C for up to 5 min. At various times aliquots were removed, precipitated with 10% wt/vol TCA, and analyzed by SDS-PAGE.

Determination of Guanylate Cyclase Activity

Semen (∼0.3 ml) was diluted into 40 vol MFSW (2°C) and centrifuged 12 min at 175 g to sediment pigment cells. Spermatozoa were pelleted from the supernatant by 10-min centrifugation at 2,500 g. The pellet was resuspended to 2.5 × 10^8 cells/ml in either MFSW (pH 7.9, control) or MFSW containing 10 mM Tris-HCl and 18.6 mM NH$_4$OH (pH 8.8, 21°C). After 5 min at 23°C, 1.0-ml aliquots of control and ammonium-treated cells were pelleted (15 s at 12,000 g), solubilized (2°C) in 1.0 ml Buffer A (0.25% vol/vol Triton X-100, 20 mM 2-(N-morpholino)ethanesulfonic acid, 2 mM NaF (pH 6.5), and stored on ice. The remaining ammonium-treated cells were divided into two lots. One lot (1.0 ml) was added to 0.5 ml MFSW, supplemented with 10 mm Hepes (pH 7.9) and egg jelly at 2 μg fucose/ml, and incubated 30 s before pelleting the cells and solubilization in 1.0 ml Buffer A. The other lot was added to 4 vol MFSW containing 20 mM Tris-HCl (pH unadjusted). After 5 min at 21°C, an aliquot (1.0 ml) was removed, and the cells pelleted and solubilized in 0.2 ml Buffer A. The remaining cells were treated 30 s with egg jelly (1 μg fucose/ml) before being pelleted and solubilized in Buffer A.

Spermatozoa solubilized in Buffer A were assayed for guanylate cyclase

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1. *Abbreviations used in this paper*: CMV, cavitated sperm plasma membrane vesicles; MFSW, Millipore-filtered natural seawater, pH 7.9.

activity as described (8, 40). Final assay conditions were: 0.06% vol/vol Triton X-100, 25 mM Tris-HCl, 5 mM 2-(N-morpholino)ethanesulfonic acid, 1 mM 3-isobutyl-1-methylxanthine, 3 mM MnCl$_2$, 8 mM NaN$_3$, 20 mM NaF, 200 μM Na orthovanadate, 4 mM dithiothreitol, 1 mM cGMP (2), and 0.5 mM GTP (including [^{3}H]GTP at 10^6 cpm/200-μl assay tube) (pH 6.5). Product formation was linear with both protein concentration (37) and time.

Preparation of a Sperm Plasma Membrane Fraction Supporting the Phosphorylation of Guanylate Cyclase

Spermatozoa were subjected to nitrogen cavitation and cavitated plasma membrane vesicles (CMV) prepared as described (40), except that ATP was omitted from the cavitation buffer (480 mM NaCl, 10 mM MgCl$_2$, 10 mM KCl, 20 mM benzamidine-HCl, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM 2-(N-morpholino)ethanesulfonic acid [pH 5.8]). This method yielded CMV with the guanylate cyclase in the 160-kD form. Preparations of CMV made at pH 7.5 (buffered with 10 mM Hepes in place of 2-(N-morpholino)ethanesulfonic acid) yielded the cyclase in its 150 kD form. The standard in vitro phosphorylation procedure was as follows. CMV were resuspended in cavitation buffer to 0.2 mg protein/ml and then lysed (0–4°C) in 9 vol 10 mM Pipes (pH 7.0) containing 50 μM ATP (including 50–500 mCi/mmol [γ-^{32}P] ATP). Buffer B (1.2 M NaCl, 600 mM KCl, 40 mM MgCl$_2$, and 80 mM Pipes [pH 7.0]) was added to bring the final concentrations of NaCl to 300 mM, KCl to 150 mM, and MgCl$_2$ to 10 mM. The reaction mixture was warmed to 23°C and at various times aliquots were removed, precipitated with 10% wt/vol TCA, and analyzed by SDS PAGE.

SDS PAGE and Peptide Mapping

SDS PAGE and autoradiography were as described (39, 40). Silver-stained gels (18) were densitometrically scanned using the gel scanner accessory on a Techtron 635 spectrophotometer (Varian Associates, Palo Alto, CA).

Partial proteolytic digestion with chymotrypsin and *Staphylococcus aureus* V8 protease was as described (3), and the fragments were resolved by 20% SDS PAGE (39). Cyanogen bromide cleavage fragments were prepared and resolved as described (20, 39).

To determine ^{32}P-labeling stoichiometry, the 160-kD band was sliced from a gel and the amount of protein in the gel slice measured by extraction
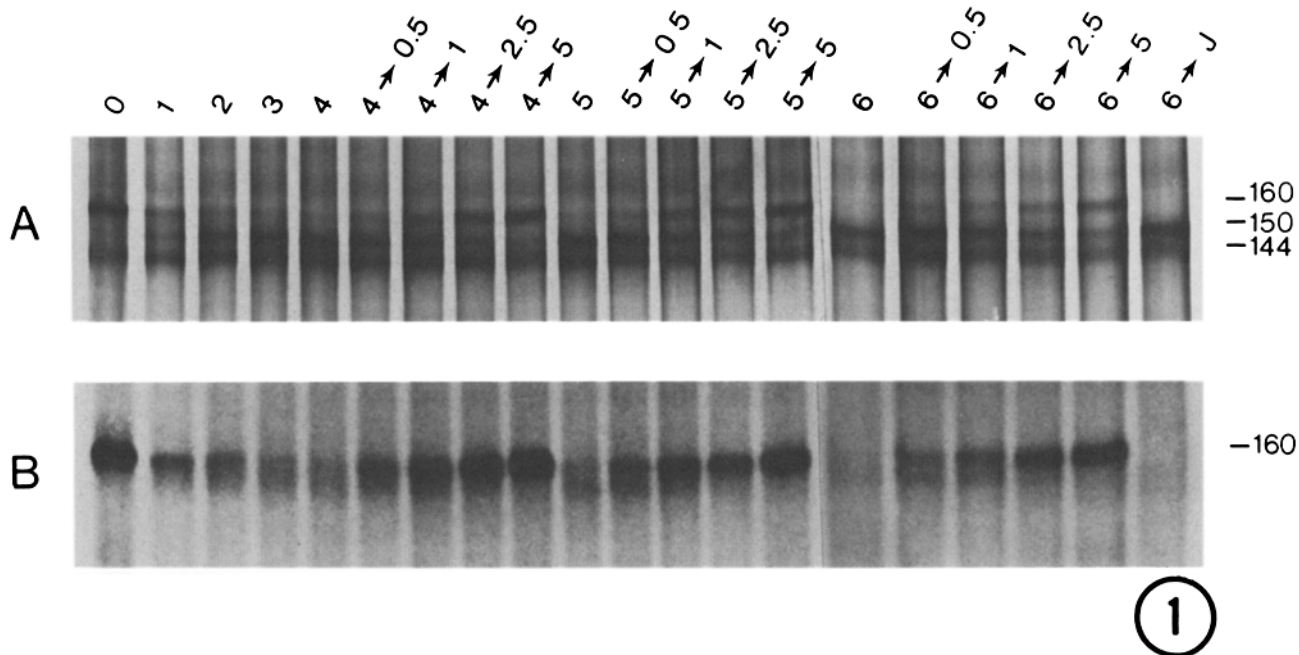
Figure 1. Dephosphorylation and rephosphorylation of sperm guanylate cyclase in intact cells. (*A*) High molecular weight region of silver-stained gel. (The electrophoretic profile of total sperm protein is shown in reference 39.) 5 μg of protein was loaded per lane; numbers on right are molecular mass in kilodaltons. (*B*) Autoradiogram of lanes in *A*. ^{32}P$_i$-labeled spermatozoa were exposed to ammoniated seawater (pH 8.8) for 0–6 min. During this time samples were taken for SDS PAGE (lanes 0–6). At 4, 5, and 6 min, samples were diluted into 4 vol 20 mM Tris-HCl seawater and incubated for 0.5, 1, 2.5, and 5 min before precipitation with TCA and processing for SDS PAGE. Residual label associated with the ammonium-induced 150-kD protein (*B*, lane 6) was removed when these cells were treated with egg jelly (*B*, lane 6→J).

Results

In Vivo Rephosphorylation of Guanylate Cyclase

Ammonium, monensin, and high external pH (9.2) increase the intracellular pH of *A. punctulata* spermatozoa and induce the 160- to 150-kD mobility shift of the guanylate cyclase in the absence of resact (34, 41). The combination of high external pH (pH 8.8) and a weak penetrating base (18.6 mM NH_4OH) was found to be particularly effective in causing the increase in electrophoretic mobility (Fig. 1 *A*) and loss of radiophosphate (Fig. 1 *B*) from the cyclase. When spermatozoa were treated for 5 min with pH 8.8 ammoniated seawater and then diluted into 4 vol of ammonium-free seawater containing 20 mM Tris-HCl, the 150-kD form of the cyclase was rephosphorylated and its electrophoretic mobility shifted back to 160 kD. The optimum length of pH 8.8 treatment before diluting the cells into Tris-HCl seawater was 5 min; pretreatment for 4 min converted less of the 160-kD form to 150 kD, and after 6 min in the pH 8.8 seawater less of the 150-kD form could be rephosphorylated (Fig. 1). After a 5-min pretreatment at pH 8.8, the extent of 150- to 160-kD reconversion (determined by densitometric scans of silver-stained gels) ranged in four separate experiments from 53 to 86% (average, 70.2% ± 13% SEM). The phosphopeptide profile of the rephosphorylated protein (generated by cleavage with cyanogen bromide) was identical to the phosphopeptide profile of the original ^{32}P-labeled 160-kD protein (data not shown). A small amount of radiophosphate remained on the cyclase after its conversion to 150 kD by ammonium (Fig. 1 *B*, lanes *4–6*). This residual label was removed from the protein by subsequent exposure to egg jelly (Fig. 1 *B*, lane *6→J*). Although the dephosphorylation of the cyclase by ammonium was reversible under conditions of low external pH, the dephosphorylation induced by soluble egg jelly (or the ethanol soluble fraction of egg jelly containing resact) could not be reversed under these or a variety of other conditions.

Rephosphorylation of the Guanylate Cyclase and Enzymatic Activity

A large decrease in guanylate cyclase activity accompanies the dephosphorylation of the enzyme (22, 40). The activity
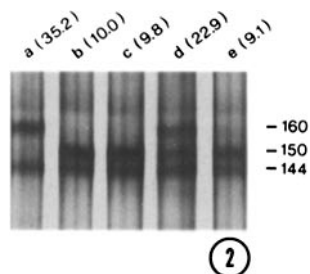
Figure 2. Rephosphorylation of the guanylate cyclase restores the enzymatic activity lost upon dephosphorylation. Silver-stained gel (high molecular weight region); 5 μg protein/lane. Numbers in parentheses are guanylate cyclase specific activity in nanomoles cGMP formed per minute per milligram protein. Lane *a*, control cells; *b*, cells exposed to pH 8.8 ammoniated seawater for 5 min; *c*, the cells in *b* after treatment with egg jelly; *d*, the cells in *b* 5 min after transfer to 4 vol of 20 mM Tris-HCl seawater; and *e*, the cells in *d* after treatment with egg jelly.
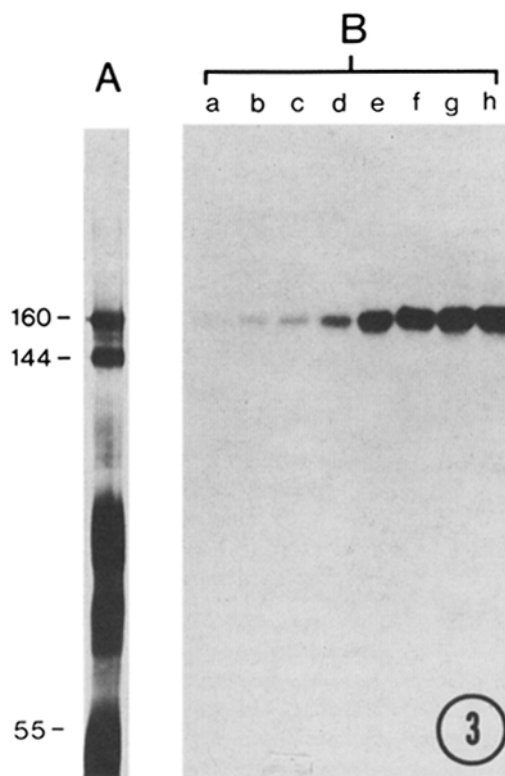

Figure 3. Time course of guanylate cyclase phosphorylation in vitro in the CMV preparation. CMV were prepared at pH 5.8 and incubated at 23°C under the standard in vitro phosphorylation conditions (see Materials and Methods). Samples were precipitated with 10% wt/vol TCA for SDS-PAGE at (*a*) 4 s, (*b*) 30 s, (*c*) 60 s, (*d*) 2 min, (*e*) 5 min, (*f*) 10 min, (*g*) 20 min, and (*h*) 30 min. (*A*) Silver-stained gel of total CMV protein (5 μg) from the reaction mixture. (*B*) Autoradiogram of the preparation. Numbers on left are molecular mass in kilodaltons.

lost upon dephosphorylation is recovered upon rephosphorylation and reconversion to the 160-kD form (Fig. 2). In the experiment shown, the 160-kD guanylate cyclase of control spermatozoa (lane *a*) possessed an activity of 35.2 nmol cGMP formed/min/mg. After a 5-min exposure of the spermatozoa to ammoniated seawater at pH 8.8, the cyclase was converted to its 150-kD form (lane *b*) and its activity decreased to 10.0 nmol/min/mg. An aliquot of the ammonium-treated cells was removed and treated with egg jelly, which did not cause a significant further decrease in activity (lane *c*; 9.8 nmol/min/mg). The remainder of the cells was then diluted with 4 vol 20 mM Tris-HCl seawater. After 5 min in the low pH seawater, a significant fraction of the 150-kD form reconverted to 160 kD (lane *d*), with a corresponding increase in enzymatic activity (to 22.9 nmol/min/mg). In four separate experiments, reconversion of 37% (±2% SEM) of the cyclase from 150 to 160 kD resulted in the recovery of 36% (±8% SEM) of the enzymatic activity lost upon dephosphorylation. The extent of reconversion was consistently less in these experiments than under the conditions used in Fig. 1 because of the extra manipulations involved in preparing the cells for the guanylate cyclase assay (see Materials and Methods). The rephosphorylated cyclase (Fig. 2, lane *d*) could be dephosphorylated a second time by treating the cells with egg jelly (lane *e*), and a decrease in enzymatic activity (to 9.1 nmol/min/mg) was again observed.

These results demonstrate that phosphorylation and dephosphorylation of the cyclase result in changes in its enzymatic activity.

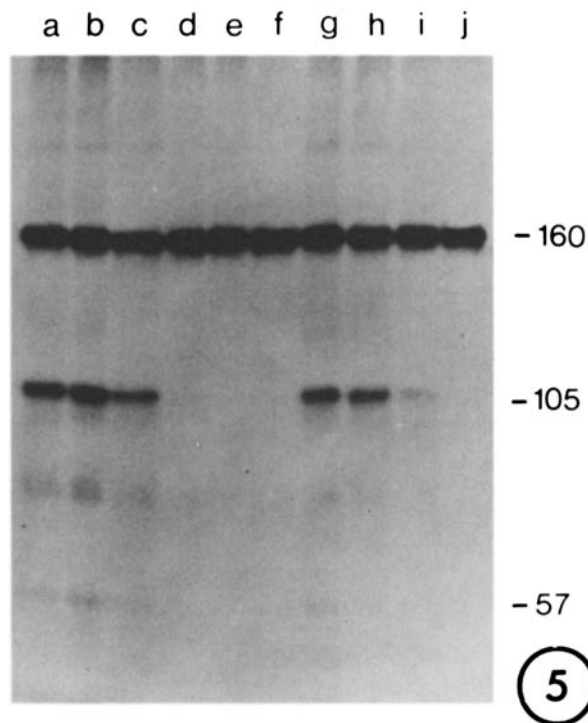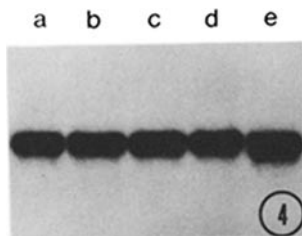## A Plasma Membrane Preparation Supporting the In Vitro Phosphorylation of Sperm Guanylate Cyclase

Adding [γ-^{32}P] ATP to a preparation of sperm plasma membrane vesicles produced by nitrogen cavitation (CMV; reference 40) resulted in the very specific labeling of the guanylate cyclase (Fig. 3). At 21°C, label was incorporated maximally by ~20 min (Fig. 3, lane g). At 2°C, maximal labeling of the 160-kD band occurred in 10 h. At 35°C, labeling of the 160-kD form was rapid for 5 min, but after 5–10 min the 160-kD form spontaneously converted to the 150-kD form. Decreasing the pH below 7.0 decreased the rate of phosphorylation (at pH 6.0 and 21°C label was barely detectable by 12 min). At pHs above 7.5, the 160-kD form spontaneously converted to 150 kD.

The intensity of in vitro labeling of the 160-kD band increased in Triton X-100, 0.01% vol/vol, giving maximal incorporation. The phosphorylation was supported by 10 mM Mg^{2+} (but not 10 mM Mn^{2+}), and ATP (but not GTP) served as the phosphate donor. When the total amount of ATP added to the reaction mixture was varied (while keeping the specific activity of the [γ-^{32}P]ATP constant), the intensity of the 160-kD labeling increased steadily to a maximum at 50 μM ATP. The in vitro phosphorylation of the 160-kD cyclase was independent of calcium; the extent and specificity of labeling was identical in media containing 1 mM Ca^{2+} and in Ca^{2+}-free medium containing 1 mM EGTA (Fig. 4).

Adding cAMP to the reaction mixture induced the phosphorylation of several CMV proteins, in particular one at 105 kD, but had no effect on phosphorylation of the 160-kD band (Fig. 5, lanes a–f). Addition of high concentrations of cGMP (>10 μM) yielded the same result seen for cAMP (Fig. 5, lanes g–j). Sea urchin spermatozoa contain no detectable cGMP-dependent protein kinase activity (7) and effects at 1–10 μM cGMP, such as those seen here, may reflect activation of the cAMP-dependent protein kinase by cGMP (44).

The in vitro labeling of the 160-kD cyclase was blocked by 13.5 mM EDTA (Fig. 6 B, lanes b and c), by 2 mM zinc acetate plus 2 mM sodium fluoride (Fig. 6 C, lanes b and c), or by dilution of the specific activity of the [γ-^{32}P]ATP 25-fold (Fig. 6 D, lanes b and c). The addition of EDTA, zinc

Figure 5. In vitro phosphorylation of guanylate cyclase is cyclic nucleotide-independent. The standard reaction mixture contained 1 mM 3-isobutyl-1-methylxanthine as a phosphodiesterase inhibitor and 0.01% vol/vol Triton X-100. cAMP was added to (a) 100 μM, (b) 10 μM, (c) 1 μM, (d) 100 nM, (e) 10 nM, and (f) 1 nM. cGMP was added to (g) 1 mM, (h) 100 μM, (i) 10 μM, and (j) 1 μM. The samples in lanes g–j contained 50 mM MnCl$_2$. 5 μg protein/lane. Shorter autoradiographic exposure times yielded qualitatively similar results. Numbers on right side of autoradiogram denote apparent molecular mass in kilodaltons. From previous determinations of the cyclic nucleotide content of intact sea urchin spermatozoa (7), we estimate that after preparation of the CMV and dilution in the phosphorylation medium, endogenous cAMP is present at <0.5 nM, and endogenous cGMP is present at <0.1 nM.

Figure 4. CMV-supported phosphorylation of guanylate cyclase is Ca^{2+}-independent. Reaction mixtures contained 0.01% Triton X-100 and were incubated for 20 min at 23°C. The final Ca^{2+} concentrations were (a) 1 mM, (b) 100 μM, (c) 25 μM, (d) 0 Ca^{2+}, (e) 0 Ca^{2+} plus 1 mM EGTA. 5 μg protein was loaded per lane; only the 160-kD region of the autoradiogram is shown. Shorter autoradiographic exposure times yielded qualitatively similar results.

plus fluoride, or unlabeled ATP to a phosphorylation already in progress blocked further labeling, but label already incorporated was not chased off (Fig. 6, B–D, lanes b* and c*).

When CMV containing the guanylate cyclase in its 150-kD form (CMV prepared at pH 7.5 rather than pH 5.8) were incubated under the standard in vitro phosphorylation conditions, labeling of the 150-kD band was observed. Approximately 10% of the 150-kD form could be reconverted to the 160-kD form under these conditions (as judged by densitometry of silver-stained gels).

Phosphopeptide Mapping of Guanylate Cyclase

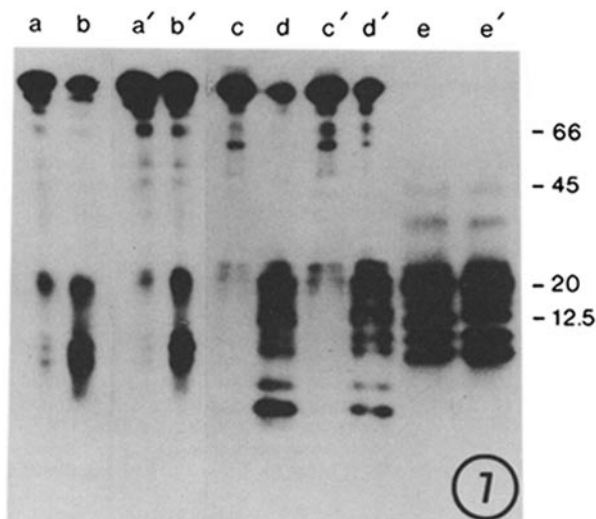One-dimensional phosphopeptide mapping showed that the 160-kD guanylate cyclase was labeled in vitro (in the CMV preparation) and in vivo (in cells incubated in ^{32}P$_i$; reference 39) on the same cyanogen bromide- and protease-derived fragments (Fig. 7). In contrast, the phosphopeptide map of the in vitro-labeled 150-kD cyclase was strikingly different from that of the 160-kD form (Fig. 8).
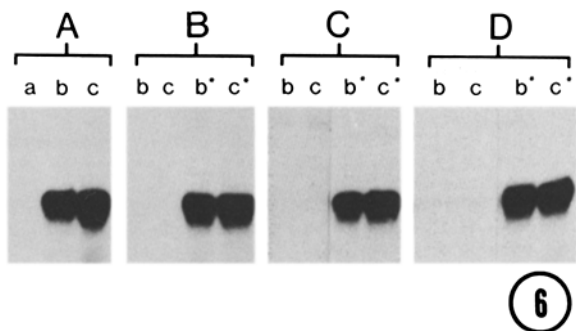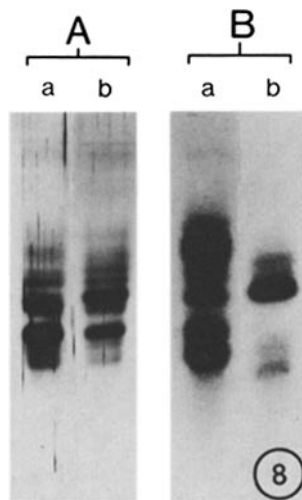
Figure 6. Inhibition of the in vitro phosphorylation reaction. The reaction mixture contained 0.01% Triton X-100 and was incubated at 21°C for (*a*) 0 min, (*b* and *b**) 12 min, or (*c* and *c**) 24 min. (*A*) No additions; (*B*) 13.5 mM EDTA added at 0 time (*b* and *c*), or at 12 min (*b** and *c**); (*C*) 2 mM NaF plus 2 mM zinc acetate added at 0 time (*b* and *c*), or at 12 min (*b** and *c**); (*D*) 1.25 mM ATP added at 0 time (*b* and *c*), or at 12 min (*b** and *c**). 5 μg of protein/lane; the 160-kD region of the autoradiogram is shown.

Discussion

We have previously suggested (39, 41) that the egg jelly–induced 160- to 150-kD electrophoretic mobility shift of sperm guanylate cyclase results from dephosphorylation. The apparent irreversibility (34, 39) of the egg jelly- (or resact) induced mobility shift has therefore been of concern. Here we describe in vivo conditions under which the 150-kD form of the cyclase (generated by pH 8.8 and ammonia) can be rephosphorylated and reconverted to the 160-kD form. A change in phosphorylation state is known to influence the electrophoretic mobility of many proteins (e.g., phospho-

Figure 7. One-dimensional ^{32}P-phosphopeptide maps of in vivo (*a–e*) and in vitro (*a'–e'*) labeled 160-kD guanylate cyclase. The 160-kD cyclase was ^{32}P-labeled in vivo (39) or in vitro (Fig. 3) and resolved by SDS-PAGE. The 160-kD band was sliced from the gel and digested with 50 ng V8 protease (*a* and *a'*), 2.5 μg V8 protease (*b* and *b'*), 1 μg chymotrypsin (*c* and *c'*), 4 μg chymotrypsin (*d* and *d'*), or 275 mg cyanogen bromide (*e* and *e'*; 5 h, 23°C). The cleavage products were resolved by SDS-PAGE; the autoradiogram is shown here. The positions of 66-, 45-, 20-, and 12-kD molecular mass standards are indicated.

Figure 8. Phosphopeptide map of the in vitro-labeled 150-kD guanylate cyclase. CMV prepared at pH 5.8 to yield the cyclase in its 160-kD form (*a*), or at pH 7.5 to yield the 150-kD form (*b*), were incubated for 20 min under the standard in vitro phosphorylation conditions. Gels of both preparations were run and the labeled 160- and 150-kD bands sliced out and digested with cyanogen bromide. The fragments were resolved by SDS PAGE, silver stained (*A*), and autoradiographed (*B*).

lamban [43], the transforming protein of PRC II avian sarcoma virus [1], and phenylalanine hydroxylase [31]). The absolute amount of phosphate that is removed from the 160-kD form of the cyclase in response to resact is not yet known; determination of phosphorylation stoichiometry will only be possible once a procedure for isolating the 160-kD form of the enzyme has been developed (34, 40).

The 160- to 150-kD mobility shift induced in vivo by pH 8.8 ammoniated seawater is reversible (Figs. 1 and 2), but it appears to be irreversible if induced by egg jelly or resact. There probably are differences in the mechanisms by which resact (the natural inducer) and ammonium (the artificial inducer) cause the 160- to 150-kD shift (41). Perhaps the mechanism underlying the effect of ammonium is reversible (e.g., a pH-dependent shift in the kinase/phosphatase equilibrium), whereas the mechanism underlying the effect of resact is irreversible (e.g., proteolytic inactivation of the kinase). Alternatively, once resact has bound its sperm surface receptor (and activated the dephosphorylation mechanism), it may not readily dissociate. Speract is known to bind to its receptor with relatively high affinity (30). It should also be noted that ammonium treatment removes most of the radiophosphate from the cyclase, but in contrast to resact, it does not remove all of the label (Fig. 1). Perhaps the dephosphorylation of these remaining sites by resact irreversibly changes the conformation of the protein such that it cannot be rephosphorylated.

The in vivo rephosphorylation of the cyclase with recovery of enzymatic activity (Fig. 2) demonstrates for the first time in any cell that the enzymatic activity of membrane-bound guanylate cyclase can be regulated by phosphorylation. Given the widespread occurrence of this enzyme (12, 13, 19) and the fact that its activity changes in response to physiological agents in many cells and tissues (5, 12, 13, 19, 38), the results reported here may prove to be of general significance. The soluble form of guanylate cyclase can be phosphorylated in vitro by cAMP-dependent protein kinase (45) or protein kinase C (46); in both cases phosphorylation results in increased enzymatic activity.

Adding [γ^{32}-P]ATP to isolated sperm plasma membranes (CMV) results in the specific phosphorylation of the 160-kD form of the cyclase. This result appears paradoxical; how can

the 160-kD form be labeled if in its 160-kD form it is already phosphorylated? The possibility that under in vitro conditions the 160-kD form is phosphorylated at sites different from those phosphorylated in vivo can probably be excluded, since the phosphopeptide maps of the in vivo and in vitro labeled 160-kD cyclase are identical (Fig. 7). A second possibility is that labeling of the 160-kD form results from phosphate turnover rather than incorporation at unphosphorylated serines. If this was true it should be possible to chase radiophosphate off the in vitro labeled 160-kD band. In an attempt to do so, various agents were added into a phosphorylation reaction already in progress (Fig. 6): EDTA (to inhibit kinase activity through the removal of free Mg^{2+}); zinc and fluoride (to inhibit phosphatase activity, reference 35); or a large excess of unlabeled ATP. In each case, further labeling of the 160-kD band was blocked, but label already incorporated was not chased off. A third possible explanation is that in vitro labeling of the 160-kD form may result from the rephosphorylation (and reconversion to 160 kD) of trace amounts of the 150-kD form. If this was true, the stoichiometry of labeling should be low. Our best estimate of labeling stoichiometry, based on the elution of Coomassie Blue (6) from gel slices of the 160-kD band, is that after 20-min labeling in the in vitro CMV preparation (21°C) 0.10 (±0.02 SEM) mol phosphate have been incorporated/mol 160-kD cyclase. When the 150-kD form is incubated in vitro in the CMV preparation, ∼10% reconverts to the 160-kD form. Thus the stoichiometry of labeling in vitro is low and may indeed result from reconversion of small amounts of the 150- to the 160-kD form.

The phosphopeptide maps of in vitro labeled 150- and 160-kD forms of the cyclase are quite different (Fig. 8). Fewer fragments are labeled in the 150-kD form. The sites phosphorylated on the 160-kD form, but not phosphorylated on the 150-kD form, could be the sites responsible for the 160- to 150-kD mobility shift and decrease in enzymatic activity. Further characterization of these differences may indicate which phosphorylation sites are involved in changes in guanylate cyclase activity.

The only protein kinase activity previously identified in sea urchin spermatozoa is the cAMP-dependent protein kinase (7, 9, 17). The results presented here demonstrate that the kinase(s) that acts on the guanylate cyclase is independent of both cAMP and cGMP. Phosphorylation of the cyclase is seen only when ATP (not GTP) is the phosphate donor, which is a characteristic shared by many, but not all, serine kinases (23, 44). The phosphorylation is also independent of calcium, although this result must be interpreted with caution since several calcium-dependent protein kinases are known to lose Ca^{2+}-dependency upon limited proteolysis (23).

The in vitro phosphorylation system described here will be useful for the further characterization and isolation of the kinase and phosphatase activities that regulate the phosphorylation state (and hence the enzymatic activity) of sperm membrane-bound guanylate cyclase.

References

1. Adkins, B., and T. Hunter. 1982. Two structurally and functionally different forms of the transforming protein of PRC II avian sarcoma virus. Mol. Cell. Biol. 2:890–896.
2. Böhme, E., and G. Schultz. 1974. Separation of cyclic nucleotides by thin-layer chromatography on polyethyleneimine cellulose. Methods Enzymol. 38:27–38.
3. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252:1102–1106.
4. Dangott, L. J., and D. L. Garbers. 1984. Identification and partial characterization of the receptor for speract. J. Biol. Chem. 259:13712–13716.
5. de Azeredo, F. A. M., W. D. Lust, and J. V. Passonneau. 1981. Light-induced changes in energy metabolites, guanine nucleotides, and guanylate cyclase within frog retinal layers. J. Biol. Chem. 256:2731–2735.
6. Fenner, C., R. R. Traut, D. T. Mason, and J. Wikman-Coffelt. 1975. Quantification of Coomassie blue stained proteins in polyacrylamide gels based on analyses of eluted dye. Anal. Biochem. 63:595–602.
7. Garbers, D. L., and G. S. Kopf. 1980. The regulation of spermatozoa by calcium and cyclic nucleotides. Adv. Cyclic Nucleotide Res. 13:251–306.
8. Garbers, D. L., and F. Murad. 1979. Guanylate cyclase assay methods. Adv. Cyclic Nucleotide Res. 10:57–67.
9. Garbers, D. L., D. J. Tubb, and G. S. Kopf. 1980. Regulation of sea urchin sperm cyclic AMP-dependent protein kinases by an egg-associated factor. Biol. Reprod. 22:526–532.
10. Garbers, D. L., G. S. Kopf, D. J. Tubb, and G. Olson. 1983. Elevation of sperm adenosine 3':5'-monophosphate concentrations by a fucose-sulfate-rich complex associated with eggs. I. Structural characterization. Biol. Reprod. 29:1211–1220.
11. Garbers, D. L., H. D. Watkins, J. R. Hansbrough, A. Smith, and K. S. Misono. 1982. The amino acid sequence and chemical synthesis of speract and of speract analogues. J. Biol. Chem. 257:2734–2737.
12. Garbers, D. L., J. K. Bentley, L. J. Dangott, C. S. Ramarao, H. Shimomura, N. Suzuki, and D. Thorpe. 1986. Peptides associated with eggs: mechanisms of interaction with spermatozoa. In Molecular and Cellular Biology of Fertilization. J. Hedrick, editor. Plenum Publishing Corp., New York. In press.
13. Goldberg, N. D., and M. K. Haddox. 1977. Cyclic GMP metabolism and involvement in biological regulation. Annu. Rev. Biochem. 46:823–896.
14. Gray, J. P., and G. I. Drummond. 1976. Guanylate cyclase of sea urchin sperm: subcellular localization. Arch. Biochem. Biophys. 172:31–38.
15. Gray, J. P., G. I. Drummond, D. W. T. Luk, J. G. Hardman, and E. W. Sutherland. 1976. Enzymes of cyclic nucleotide metabolism in invertebrate and vertebrate sperm. Arch. Biochem. Biophys. 172:20–30.
16. Hansbrough, J. R., and D. L. Garbers. 1981. Speract. Purification and characterization of a peptide associated with eggs that activates spermatozoa. J. Biol. Chem. 256:1447–1452.
17. Lee, M. Y. W., and R. M. Iverson. 1976. An adenosine 3':5' monophosphate dependent protein kinase from sea urchin spermatozoa. Biochim. Biophys. Acta. 429:123–136.
18. Morrissey, J. H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. 117:307–310.
19. Murad, F., W. P. Arnold, C. K. Mittal, and J. M. Braughler. 1979. Properties and regulation of guanylate cyclase and some proposed functions for cyclic GMP. Adv. Cyclic Nucleotide Res. 11:175–204.
20. Nikodem, V., and J. R. Fresco. 1979. Protein fingerprinting by SDS-gel electrophoresis after partial fragmentation with CNBr. Anal. Biochem. 97:382–386.
21. Nomura, K., and S. Isaka. 1985. Synthetic study on the structure-activity relationship of sperm activating peptides from the jelly coat of sea urchin eggs. Biochem. Biophys. Res. Commun. 126:974–982.
22. Ramarao, C. S., and D. L. Garbers. 1985. Receptor-mediated regulation of guanylate cyclase activity in spermatozoa. J. Biol. Chem. 260:8390–8396.
23. Roach, P. J. 1984. Protein kinases. Methods Enzymol. 107:81–101.
24. Sano, M. 1976. Subcellular localizations of guanylate cyclase and 3':5'-cyclic nucleotide phosphodiesterase in sea urchin sperm. Biochim. Biophys. Acta. 428:525–531.
25. Schackmann, R. W., and B. M. Shapiro. 1981. A partial sequence of ionic changes associated with the acrosome reaction of Strongylocentrotus purpuratus. Dev. Biol. 81:145–154.
26. Schackmann, R. W., R. Christen, and B. M. Shapiro. 1981. Membrane potential depolarization and increased intracellular pH accompany the acrosome reaction of sea urchin sperm. Proc. Natl. Acad. Sci. USA. 78:6066–6070.
27. Schackmann, R. W., E. M. Eddy, and B. M. Shapiro. 1978. The acrosome reaction of Strongylocentrotus purpuratus sperm: ion requirements and movements. Dev. Biol. 65:483–495.
28. SeGall, G. K., and W. J. Lennarz. 1979. Chemical characterization of the component of the jelly coat from sea urchin eggs responsible for induction of the acrosome reaction. Dev. Biol. 71:33–48.
29. SeGall, G. K., and W. J. Lennarz. 1981. Jelly coat and induction of the acrosome reaction in echinoid sperm. Dev. Biol. 86:87–93.
30. Smith, A. C., and D. L. Garbers. 1983. The binding of an ^{125}I-speract analogue to spermatozoa. In Biochemistry of Metabolic Processes. D. L. F.

Lennon, F. W. Stratman, and R. N. Zahlten, editors. Elsevier Science Publishing Co., Inc., New York. 15–28.

31. Smith, S. C., B. E. Kemp, W. J. McAdam, J. F. B. Mercer, and R. G. H. Cotton. 1984. Two apparent molecular weight forms of human and monkey phenylalanine hydroxylase are due to phosphorylation. *J. Biol. Chem.* 259:11284–11289.

32. Suzuki, N., and D. L. Garbers. 1984. Stimulation of sperm respiration rates by speract and resact at alkaline extracellular pH. *Biol. Reprod.* 30:1167–1174.

33. Suzuki, N., K. Nomura, H. Ohtake, and S. Isaka. 1981. Purification and the primary structure of sperm-activating peptides from the jelly coat of sea urchin eggs. *Biochem. Biophys. Res. Commun.* 99:1238–1244.

34. Suzuki, N., H. Shimomura, E. W. Radany, C. S. Ramarao, G. E. Ward, J. K. Bentley, and D. L. Garbers. 1984. A peptide associated with eggs causes a mobility shift in a major plasma membrane protein of sea urchin spermatozoa. *J. Biol. Chem.* 259:14874–14879.

35. Swarup, G., and D. L. Garbers. 1982. Phosphoprotein phosphatase activity of sea urchin spermatozoa. *Biol. Reprod.* 26:953–960.

36. Tilney, L. G., D. P. Kiehart, C. Sardet, and M. Tilney. 1978. Polymerization of actin. IV. Role of Ca^{2+} and H^+ in the assembly of actin and in membrane fusion in the acrosomal reaction of echinoderm sperm. *J. Cell Biol.* 77:536–550.

37. Undenfriend, S., S. Stein, P. Böhlen, W. Dairman, W. Leimgruber, and M. Weigele. 1972. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science (Wash. DC).* 178:871–872.

38. Waldman, S. A., R. M. Rapoport, and F. Murad. 1984. Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissues. *J. Biol. Chem.* 259:14332–14334.

39. Ward, G. E., and V. D. Vacquier. 1983. Dephosphorylation of a major sperm membrane protein is induced by egg jelly during sea urchin fertilization. *Proc. Natl. Acad. Sci. USA.* 80:5578–5582.

40. Ward, G. E., D. L. Garbers, and V. D. Vacquier. 1985. Effects of extracellular egg factors on sperm guanylate cyclase. *Science (Wash. DC).* 227:768–770.

41. Ward, G. E., G. W. Moy, and V. D. Vacquier. Dephosphorylation of sperm guanylate cyclase during sea urchin fertilization. 1986. *In* Molecular and Cellular Biology of Fertilization. J. Hedrick, editor. Plenum Publishing Corp., New York. In press.

42. Ward, G. E., C. J. Brokaw, D. L. Garbers, and V. D. Vacquier. 1985. Chemotaxis of *Arbacia punctulata* spermatozoa to resact, a peptide from the egg jelly layer. *J. Cell Biol.* 101:2324–2329.

43. Wegenar, A. D., and L. R. Jones. 1984. Phosphorylation-induced mobility shift in phospholamban in sodium dodecyl sulfate-polyacrylamide gels. *J. Biol. Chem.* 259:1834–1841.

44. Weller, M. 1979. Protein Phosphorylation. The Nature, Function, and Metabolism of Proteins Which Contain Covalently Bound Phosphorus. Pion Ltd., London. 557 pp.

45. Zwiller, J., M.-O. Revel, and P. Basset. 1981. Evidence for the phosphorylation of rat brain guanylate cyclase by cAMP-dependent protein kinase. *Biochem. Biophys. Res. Commun.* 101:1381–1387.

46. Zwiller, J., M.-O. Revel, and A. N. Malviya. 1985. Protein kinase C catalyzes the phosphorylation of guanylate cyclase *in vitro*. *J. Biol. Chem.* 260:1350–1353.