Modulation of testicular galactolipid sulphotransferase activity by phosphorylation

Stimulation of enzyme activity *in vitro* by an endogenous kinase

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Evidence is presented for a testicular protein kinase activity capable of stimulating the activity *in vitro* of a partially purified preparation of the testicular galactolipid sulphotransferase. This enzyme is responsible for the synthesis of the major mammalian testicular glycolipid, sulphogalactosylglycerol, and is an early marker of differentiation during spermatogenesis. This stimulatory activity has been separated by affinity chromatography, using 3',5'-ADP-agarose, from both the detergent-solubilized microsomes (microsomal fractions) and the soluble fraction of the testicular homogenate. The stimulator was eluted from the affinity matrix by either a salt, or, more selectively, a cyclic AMP gradient. Thus this matrix can function as an analogue of 3',5'-cyclic AMP. The activity of the sulphotransferase stimulator was ATP-dependent and coincident with protein kinase activity. Sulphotransferase activity was also stimulated in the presence of commercial preparations of cyclic AMP-dependent protein kinase and stimulation was prevented in the presence of kinase inhibitors. Our results suggest that sulphogalactolipid biosynthesis is regulated by a phosphorylation process during spermatogenesis. In addition, our results suggest that affinity chromatography on 3',5'-ADP-agarose may provide a general method for the purification of cyclic AMP-dependent kinases.

INTRODUCTION

Sulphogalactosylalkylglycerol (SGG) [1] is synthesized at the zygotene and early-pachytene spermatocyte stages of rat spermatogenesis [2], about 20 days after birth. Once synthesized, SGG is a major membrane component and is stable throughout the remainder of spermatogenesis and sperm maturation [3]. Although SGG is the major product *in vivo*, galactosylceramide is preferentially sulphated *in vitro* [4]. The galactolipid sulphotransferase is a Golgi membrane enzyme [5] that is regulated during the later stages of spermatogenesis by the appearance of a selective inhibitor at the mid-pachytene stage [2,4]. Mutant rats lacking this inhibitory activity were found to contain elevated testicular sulphotransferase activity and were sterile [6]. In addition, some evidence was presented to suggest the presence of a soluble stimulator of sulphotransferase activity at early stages of spermatogenesis [4]. We have shown that the enzyme *in vitro* is inactivated at 37 °C in the absence of ATP and have concluded that this is due to an endogenous ATP-inhibited protein phosphatase [7]. We now present evidence of an endogenous protein kinase activity in the early stages of spermatogenesis capable of substrate-selective stimulation of galactolipid sulphotransferase activity *in vitro*.

MATERIALS AND METHODS

Materials

[³⁵S]PAPS (0.9–5.1 Ci/mmol) and [³²P]ATP (9–12 Ci/mol) were from New England Nuclear. SGG and galactosylalkylglycerolipid (GG) were prepared as previously described [4]. Galactosylceramide (GC), protein kinase inhibitor, histone type III and rabbit muscle and bovine heart cyclic AMP-dependent protein kinases were purchased from Sigma. 3',5'-ADP-agarose was from Pharmacia.

Enzyme preparation

The testes were removed from 20-day-old Wistar rats after CO₂ asphyxiation. The tunicae were removed and the tubules homogenized in 0.32 M-sucrose/1 mM-EDTA. The homogenate was centrifuged at 8000 g for 5 min. The pellet was then extracted in 10 mM-Tris/HCl, pH 7.4, containing 25 % (v/v) glycerol and 0.5 % Triton X-100 at 4 °C for 1 h. The 100 000 g supernatant, containing ~ 8 mg of protein/ml, was used as the solubilized sulphotransferase preparation. Protein concentrations were determined by a procedure involving the use of the BCA protein-assay reagent (Pierce, Rockford, IL, U.S.A.).

Abbreviations used: SGG, sulphogalactosylalkylglycerolipid; GG, galactosylalkylglycerolipid; SGC, sulphogalactosylceramide; GC, galactosylceramide; PAPS, 3'-phosphoadenosine 5'-phosphosulphate; EB, equilibration buffer.

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Sulphotransferase assay

A 25 nmol portion of galactolipid substrate was air-dried together with 1 mg of Triton X-100. The residue was dissolved in 0.1 ml of 100 mM-Tris/HCl, pH 8.0, containing 2.5 mM-MgCl₂, 0.8 mM-K₂SO₄, 5 mM-KCl, 5 mM-ATP, 2 μ M-[³⁵S]PAPS and 25 μ l of enzyme fraction [4]. In stimulation assays, 25 μ l of stimulator fraction was also included within the same incubation volume and expressed relative to the sulphotransferase activity of the enzyme fraction in the absence of stimulator. The solution was incubated at 37 °C for 2 h and the reaction terminated by the addition of 4 vol. of chloroform/methanol (2:1, v/v) and 4 vol. of 0.88 % KCl. The mixture was vortex-mixed vigorously and, after centrifugation (1000 g, 10 min), the upper phase (containing no radiolabelled product) was discarded and the lower phase dried and the glycolipids separated by t.l.c. (chloroform/methanol/water, 65:25:4, by vol.). Radiolabelled sulphoglycolipids were detected by autoradiography and compared with unlabelled standards revealed with an orcinol spray. The corresponding bands were eluted and counted for radioactivity in a liquid-scintillation spectrometer.

3',5'-ADP affinity chromatography

Aliquots of solubilized sulphotransferase or homogenate supernatant (1 ml) were applied to a 3 ml 3',5'-ADP-agarose column at 4 °C. In the case of commercial protein kinase, 1 ml samples containing 4 mg of protein were applied. The column was then washed with 5 column vol. of equilibration buffer (EB) (10 mM-Tris/HCl, pH 8.0, containing 2.5 mM-MgCl₂ and 0.05 % Triton X-100). The column was eluted with either a 20 ml linear KCl gradient (0.01–3 M) or a cyclic AMP gradient (0.05–5 μ M). The eluted fractions were dialysed against EB and assayed for protein, sulphotransferase activity, sulphotransferase stimulation and protein kinase activity.

Ion-exchange chromatography

A 2 ml portion of the solubilized sulphotransferase was applied to a 40 ml column of DEAE-cellulose previously equilibrated with EB as previously described [7]. The column was washed with EB and eluted with a linear NaCl gradient in EB. The unbound sulphotransferase fraction, which is active in the absence of ATP [7], was used in this study.

Fig. 1. Separation of testicular stimulator of galactolipid sulphotransferase by affinity chromatography

A solubilized testicular galactolipid sulphotransferase preparation was applied to a 3',5'-ADP-agarose column. The column was washed with EB and eluted with either a linear KCl (*a, b*) or a cyclic AMP gradient (*c, d*) as described in the Materials and methods section. The eluted fractions were dialysed and fractions were assayed for sulphotransferase activity (using GC as substrate), protein (*a, c*), protein kinase and stimulatory activity (*b, d*). Stimulation was measured by the relative ability of aliquots (25 μ l) of the eluted fractions to increase the sulphotransferase activity of an equal volume of the unbound sulphotransferase fraction.

Fig. 2. Demonstration of galactolipid sulphotransferase stimulator in the testicular-homogenate 8000 g supernatant

The testicular supernatant from 20-day-old rats was separated by 3',5'-ADP-agarose affinity chromatography as described in Fig. 1. (a) and (b), Sulphotransferase-stimulatory activity, assayed by using unbound sulphotransferase fractions from Fig. 1, was eluted with a KCl gradient (a, b). In (c) and (d), fractions were assayed after dialysis against EB as described in the Materials and methods section, but were eluted with a cyclic AMP gradient. (a, c) Sulphotransferase activity and protein concentration; (b, d) protein kinase and stimulatory activity.

Protein kinase assay

Kinase activity was assayed by a modification of the method of Wise *et al.* [8]. The phosphorylation incubation mixture contained 10 mM-[32 P]ATP (10^6 d.p.m.), 20 mM-Tris/HCl, pH 7.4, 2 mM-EGTA, 10 mM-MgCl₂, 50 μ g of histone type III and 25 μ l of kinase preparation in a final volume of 250 μ l. The reaction was terminated after incubation at 37 °C for 10 min by addition of 3 ml of 10% (w/v) trichloroacetic acid and the precipitate was filtered (Whatman GF/B filters), washed with 3 $\times$ 3 ml of 10% trichloroacetic acid and the filters were counted for radioactivity in a liquid-scintillation spectrometer.

RESULTS

3',5'-ADP affinity chromatography

The Triton-solubilized testicular galactolipid sulphotransferase was applied to a 3',5'-ADP affinity column (Fig. 1). Approx. 50% of the enzyme activity was recovered in the wash fractions. No sulphotransferase activity could be eluted from the column in the presence of high salt (Fig. 1a). However, addition of the KCl-eluted fractions to the unbound sulphotransferase fractions from the column (Fig. 1a, fractions 6–8) resulted

in the marked stimulation of the sulphotransferase activity within the latter (Fig. 1b). Maximal stimulation was observed for eluted fractions containing protein kinase activity (Fig. 1b). This kinase activity was not phospholipid- or Ca²⁺-dependent (results not shown). This experiment was repeated with a cyclic AMP gradient to elute the stimulatory activity (Figs. 1c and 1d).

Sulphotransferase-stimulatory activity was also obtained after application of an aliquot of the testicular-homogenate 8000 g supernatant to the 3',5'-ADP column. Stimulator was eluted with either KCl (Figs. 2a and 2b) or cyclic AMP (Figs. 2c and 2d) as for the detergent extract (cf. Fig. 1). After dialysis, stimulatory activity in these fractions was assayed on sulphotransferase eluted from the 3',5'-ADP column in the washing buffer in the previous experiment (Fig. 1a, fractions 7–9 pooled).

ATP-dependence of sulphotransferase stimulation

Although ATP is an absolute requirement for the crude sulphotransferase preparation, a partially purified fraction prepared by DEAE-cellulose ion-exchange chromatography does not require ATP for sulphation *in vitro* [7]. Stimulation of the activity of this enzyme

Fig. 3. ATP-dependence of sulphotransferase stimulation

3',5'-ADP-agarose KCl-eluted stimulatory activity from three separate experiments as in Fig. 1(b) was assayed for stimulation of a partially purified sulphotransferase preparation in the absence (lanes A–D) or presence (lanes E–H) of 10 mM-ATP, using GC as exogenous substrate (endogenous GG sulphated also). Radiolabelled products were separated by t.l.c. and revealed by autoradiography. Lanes A and E, sulphotransferase alone; lanes B–D and F–H, sulphotransferase plus 3',5'-ADP-agarose KCl eluate. Arrows indicate the positions of standard SGG (above) and SGC.

preparation was not observed in the absence of ATP (Fig. 3).

Effect of commercial cyclic AMP-dependent protein kinase

The stimulatory effect of a standard cyclic AMP-dependent protein kinase from rabbit muscle on the sulphotransferase activity of the DEAE-cellulose-purified enzyme [7] is shown in Fig. 4. This stimulation was deleted in the presence of protein kinase inhibitor (Fig. 4, lane C). Cyclic AMP alone had no effect on the sulphotransferase and no sulphation was detected for the protein kinase preparation alone (results not shown).

Heat inactivation

The susceptibility of the stimulatory activity to heat was investigated (Table 1). The stimulator was resistant to 10 min at 50 °C. Sulphotransferase activity was completely inactivated at 45 °C. Stimulatory activity was lost after treatment at 70 °C for 10 min.

Fig. 4. Sulphotransferase stimulation by rabbit muscle cyclic AMP-dependent protein kinase

DEAE-cellulose-purified sulphotransferase ('DEAE enzyme') was assayed for activity in the presence of muscle kinase (1 mg/ml) + 2 mM-cyclic AMP, using GC as substrate. Radiolabelled SGC was separated by t.l.c. and detected by autoradiography. Lane A, DEAE enzyme alone; lane B, DEAE enzyme plus muscle kinase; lane C, as B, plus protein kinase inhibitor (1 mg/ml). The arrow indicates position of SGC standard.

Galactolipid substrate specificity

Stimulation was monitored at different galactolipid concentrations (Fig. 5 and Table 2). Up to 10-fold stimulation was observed at appropriate galactolipid concentrations (Table 2). Within the range 120–3000 μ M, stimulations of GC and GG sulphation were similar. At 60 μ M, only stimulation of GG sulphation was observed. Indeed at 60 μ M-GC stimulation of SGG (not SGC)

Table 1. Thermal stability of (a) stimulator and (b) sulphotransferase**(a) Stimulator**

Temperature (°C)*	Stimulation remaining (%)
Control	100.00
50	70.01
70	7.96
100	8.62

(b) Sulphotransferase

Temperature (°C)	Activity remaining (%)
Control	100.0
40	19.8
45	2.8
50	0.6
75	0.3
100	0.2

* Samples of the KCl-eluted stimulator (see Fig. 1) were heated for 10 min at the temperature indicated.

biosynthesis (from endogenous GG) was observed (Fig. 5, lanes A and B).

Isolation of other cyclic AMP-dependent kinases

We wondered whether this 3',5'-ADP column might prove a general method for the purification of cyclic

Table 2. Substrate selectivity of stimulator (stim)*

Substrate	Concn. (μM)	Sulphotransferase activity (pmol/h per mg of protein)†		Fold increase
		– stim	+ stim	
GC (→ SGC)	60	113	80.2‡	0
	120	59	524	8.9
	480	222	408	1.8
	960	203	604	3.0
GG (→ SGG)	60	91.1	246	2.7
	120	38.5	295	7.7
	600	6.4	75.0	11.7
	3000	14.7	32.1	2.2

* Quantification of data in Fig. 5.

† Calculated excluding stimulator-protein content.

‡ At this GC concentration stimulation of endogenous GG sulphation is observed.

AMP-dependent protein kinases and therefore applied a sample of bovine heart cyclic AMP-dependent protein kinase (Fig. 6). We found that all the kinase activity was bound to the 3',5'-ADP column and considerable protein was removed in the wash fraction. Protein kinase activity was subsequently eluted with a KCl gradient (Fig. 6) as for the sulphotransferase stimulator. Eluted kinase activity was, however, no longer cyclic AMP-dependent. Similar results (not shown) were obtained for the commercial rabbit muscle cyclic AMP-dependent protein kinase.

DISCUSSION

The testicular galactolipid sulphotransferase is a membrane-bound Golgi-located enzyme [5,9] that

Fig. 5. Comparison of stimulation of biosynthesis of SGG and SGC *in vitro*

Increasing concentrations of GC (lanes A–H) or GG (lanes I–P) were incubated with aliquots of the DEAE-cellulose purified sulphotransferase in the absence (lanes A, C, E, G, I, K, M and O) or presence of the KCl-eluted sulphotransferase stimulator. Galactolipid substrate concentrations: lanes A, B, I and J, 60 μM; C, D, K and L, 120 μM; E and F, 480 μM; G and H, 960 μM; M and N, 600 μM; O and P, 3000 μM. Arrows indicate position of standard SGG (above) and SGC. The data are quantified in Table 2.

Fig. 6. 3',5'-ADP affinity chromatography of bovine heart cyclic AMP-dependent protein kinase

The kinase was applied in EB and eluted with KCl as described in Fig. 1(a), followed by 3 M-KCNS. The eluted fractions were dialysed before assay for kinase activity. Fractions were assayed for kinase activity in the presence and absence of 2 μM-cyclic AMP. Data obtained under the latter conditions are shown.

catalyses the transfer of sulphate from PAPS to the 3' position of the galactose moiety of galactosylglycerol. Although GC is a better substrate *in vitro* [4], SGG is the major product *in vivo* [10]. For some time we have been attempting to purify this enzyme by a variety of techniques, including affinity chromatography. 3',5'-ADP is the co-product of the transferase reaction and is itself a potent inhibitor of the reaction. Immobilized 3',5'-ADP is commercially available, and attempts were made to use this matrix to separate the sulphotransferase. Despite recovering only 50% of the applied enzyme activity in the unbound wash fractions, we were never able to elute any of the presumed bound activity from the column. Re-application of the unbound fraction resulted in no further loss in activity and we therefore concluded that the enzyme was heterogeneous and that one form of the enzyme bound very tightly to 3',5'-ADP.

During the course of these studies, the inactivation of the enzyme at 37 °C (in the absence of ATP) was investigated [7] and the presence of an ATP-inhibited testicular phosphatase which can inactivate the sulphotransferase was inferred. In the light of these experiments and the previous detection of a soluble sulphotransferase stimulator during the early stages of spermatogenesis [4], it was reasonable to suppose that the activity of the sulphotransferase could be enhanced by phosphorylation. Since 3',5'-ADP is also a reasonably close analogue of 3',5'-cyclic AMP, it was possible that such a protein kinase activity might also bind to a 3',5'-ADP matrix. The KCl-eluted fractions from the 3',5'-ADP column were therefore assayed, after dialysis, for protein kinase activity. These fractions were also recombined with portions of the unbound sulphotransferase and the stimulation of sulphotransferase activity measured. We found that the eluted fractions were able to stimulate the sulphotransferase activity of the unbound fractions by up to 4-fold, even though they contained no sulphotransferase activity themselves. Our finding that maximum stimulatory activity corresponded to protein kinase activity (Fig. 1b) suggested, but did not prove, that the stimulation might be mediated via phosphorylation. The kinase activity was inhibited in the presence of phosphatidylserine, diacylglycerol and Ca^{2+} (results not shown). The stimulatory 'protein kinase' activity could be more selectively eluted by using a gradient of cyclic AMP (Figs. 1c and 1d). Recombination of the eluted and unbound fractions resulted in the full recovery of the sulphotransferase activity originally applied on the column. Thus, in the unfractionated enzyme preparation, the sulphotransferase assay *in vitro* represents a balance between phosphorylating and dephosphorylating mechanisms. Removal of the phosphorylating component by chromatography on 3',5'-ADP-agarose results, under the standard assay conditions, in a 50% loss in sulphotransferase activity.

In a previous study of the regulation of the activity of this enzyme during spermatogenesis we had found a sulphotransferase-stimulatory activity in the supernatant of testicular homogenates of rats at the early primary spermatocyte stage [4], although an inhibitor was detected at later stages [4]. In order to determine whether such stimulatory activity might also be due to a protein kinase, we applied this same supernatant to a 3',5'-ADP column (Fig. 2) and tested the KCl-eluted (Fig. 2b) or cyclic AMP-eluted (Fig. 2d) material for kinase activity and the ability to stimulate the 3',5'-ADP-unbound

sulphotransferase from the experiment shown in Fig. 1. As for the detergent-solubilized sulphotransferase preparation (Fig. 1), we found a peak of sulphotransferase-stimulatory activity in the eluted material which contained protein kinase activity (Fig. 2). Thus a soluble kinase activity is also present in the immature testis (before the appearance of maximum sulphotransferase activity) that can elevate sulphotransferase activity *in vitro*. Not all fractions containing kinase activity (as monitored by phosphorylation of histone) were able to stimulate sulphotransferase activity, indicating that a specific kinase was responsible for stimulation. These results suggest that the initial induction of sulphotransferase activity during spermatogenesis might be via a phosphorylation mechanism. Further, the finding that, at low substrate concentrations, the stimulator has a selective effect on the sulphation of GG (Fig. 5 and Table 2) suggests that the substrate specificity of the sulphotransferase may also be modulated by such phosphorylation. Indeed this may in part rationalize why GC is a better sulphotransferase substrate than galactosylglycerol *in vitro* when no, or very little, SGC is made *in vivo* [4,10]. At low (physiological?) galactolipid concentration the stimulator would facilitate only the synthesis of SGG. Although substrate inhibition is observed for GG at higher levels (Fig. 4 and Table 2), stimulation is still effective under these conditions (Table 2).

The fact that the stimulatory activity can be eluted from the 3',5'-ADP matrix in the presence of cyclic AMP (Figs. 1d and 2d) further indicates the stimulator to be a cyclic AMP-regulated protein kinase and that the column is functioning as a cyclic AMP analogue. The samples contain additional kinases which are not retained on the 3',5'-ADP column (Figs. 1b and 2d). Presumably these are not cyclic AMP-dependent activities.

The heat-sensitivity of the stimulator was investigated to exclude trivial explanations for stimulation. The stimulatory activity was not affected by heating at 50 °C for 10 min, but was completely inactivated at 70 °C (Table 1). In comparison, testicular sulphotransferase activity was completely inactivated at 45 °C. Thus the stimulation cannot be due to additional sulphotransferase, to some heat-stable chemical cofactor for the sulphotransferase reaction or to addition of extra galactolipid substrate. Moreover, stimulation was ATP-dependent (Fig. 3), which strongly supports a role for phosphorylation in this process.

The effect of a commercial cyclic AMP-dependent kinase preparation from rabbit muscle convincingly demonstrates that the testicular galactolipid sulphotransferase can be stimulated by phosphorylation (Fig. 4) and further supports our contention that the 3',5'-ADP-purified stimulator is an endogenous kinase.

Our subsequent studies further verified that the 3',5'-ADP matrix functions as an effective cyclic AMP analogue. Cyclic AMP-dependent bovine heart protein kinase was found to bind to the 3',5'-ADP matrix, and kinase activity could be eluted with KCl (Fig. 6). Eluted kinase activity was now cyclic AMP-independent, suggesting that the 3',5'-ADP column acts, in addition, to separate the regulatory and catalytic subunits of the kinase in a manner similar to cyclic AMP. Thus 3',5'-ADP affinity chromatography may prove a general method for the purification of the catalytic subunit of cyclic AMP-dependent protein kinases. Cyclic AMP-

independent protein kinase C activity from brain did not bind to this matrix (results not shown).

Although the kinase activity which we propose to be responsible for the stimulation of galactolipid sulphotransferase activity is neither cyclic AMP-, phospholipid- nor Ca^{2+} -dependent, we have found that purified preparations of protein kinase C from rat or human brain can stimulate rat testicular sulphotransferase activity *in vitro* (C. A. Lingwood, unpublished work). It has also been reported that the phospholipid- and Ca^{2+} -dependency of protein kinase C varies according to the kinase substrate [13,14]. Protein kinase C has been detected in the rat testis [14,15] and, interestingly, sulphogalactolipids have been shown to activate this enzyme in a similar manner to diacylglycerol [16]. However, since we found that brain protein kinase C is not bound by 3',5'-ADP, our results suggest that this enzyme is not responsible for the sulphotransferase stimulation we observe in the present studies. Stimulation is not Ca^{2+} -dependent, and although exogenous cyclic AMP is also unnecessary, we have found that *bona fide* cyclic AMP-dependent protein kinase activity is no longer cyclic-AMP dependent after elution from 3',5'-ADP-agarose.

In conclusion, our studies lead us to propose that the synthesis of SGG during spermatogenesis is regulated by (cyclic AMP-dependent?) phosphorylation of the galactolipid sulphotransferase. A balance between phosphorylation/activation and dephosphorylation/inactivation [7] is achieved. Such modulation may regulate the galactolipid-specificity of the sulphotransferase. Phosphorylation predominates during the period of highest specific enzyme activity. These studies represent the first example of direct regulation of carbohydrate sulphotransferase activity by phosphorylation.

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