Inhibition by Gossypol of Cyclic Nucleotide-independent Phosvitin Kinase from Pig Testis

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ABSTRACT. Gossypol, a male anti-fertility agent, was found to be an inhibitor of cyclic nucleotide-independent phosvitin kinase from pig testis. The IC_{50} value (concentration causing 50% inhibition) was 42 μ M using phosvitin as substrate. Kinetic analysis indicated that the compound inhibited the enzyme noncompetitively with respect to ATP ($K_i=17$ μ M) and competitively with respect to phosvitin ($K_i=11$ μ M). The gossypol inhibition was reversed by the addition of polyamines. The compound also inhibited phosphorylation of high mobility group (HMG) proteins HMG 14 and 17 (the endogenous substrates in the testis located in nucleosome), with an IC_{50} value of 48 μ M.—**KEY WORDS:** Gossypol, phosvitin, pig testis.

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Gossypol [1, 1', 6, 6', 7, 7'-hexahydroxy-5, 5'-diisopropyl-3, 3'-dimethyl-(2, 2'-binaphthalene)-8, 8'-dicarboxylaldehyde] is an inhibitor of spermatogenesis and is known to be an active male contraceptive agent in mammals [15]. The compound has been shown to effectively inhibit Ca^{2+} -ATPase in sperm [8], lactate dehydrogenase isozyme X from both testis [16] and *Trypanosoma cruzi* [14], adenylate cyclase from testis [17], 5- and 12-lipoxygenase from rat basophilic leukemia cells [4], protein kinase X from brain [19], and protein kinase C from testis [12].

Cyclic nucleotide-independent phosvitin kinase is one of the major protein kinase in the testis and has been purified from pig testis [9]. The enzyme is classified into the group of casein kinase II [6], because phosvitin kinase preferentially phosphorylates acidic proteins such as phosvitin and casein as exogenous substrates, and is inhibited by heparin and stimulated by

polyamines [10]. The enzyme is present in nuclei as well as in cytosol, and phosphorylates chromatin-associated non-histone proteins, particularly high mobility group (HMG) proteins HMG 14 and 17 [10]. HMG 14 and 17 are located in the nucleosome core [20] and phosphorylation of the HMG proteins by casein kinase II is thought to be involved in the regulation of RNA synthesis [23, 24].

Since gossypol inhibits cell proliferation and protein synthesis [21, 22] and phosvitin kinase is suggested to be involved in the regulation of RNA synthesis in the testis, it is of interest to ascertain the effect of the compound on the enzyme activity. In the present paper, we report that gossypol is an inhibitor of phosvitin kinase using HMG proteins as well as phosvitin as substrates.

MATERIALS AND METHODS

Gossypol acetic acid, phosvitin, spermine, spermidine and putrescine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [^{32}P] phosphate was from Japan Atomic Energy Research Insti-

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tute (Tokaimura, Japan). Pig testis was obtained from a local slaughter house.

Phosvitin kinase from pig testis was purified to apparent homogeneity by DEAE-cellulose, Sephacryl S-300 chromatography, followed by subsequent second DEAE-cellulose and phosvitin-Sepharose 4B affinity chromatography, and assayed as described previously [9]. Specific activity of the purified enzyme was 0.3–0.4 $\mu\text{mol}/\text{min}/\text{mg}$ protein, using phosvitin as substrate. High mobility group proteins were prepared from pig testis by the method of Goodwin *et al.* [3].

Gossypol solution was prepared by dissolving gossypol acetic acid in 10 mM sodium carbonate buffer (pH 8.3), according to the method of Olgiate and Toscano [16]. Phosvitin kinase was not affected by the addition of 1 mM final concentration of sodium carbonate buffer (pH 8.3) in the reaction mixture. Protein was determined by the method of Bradford [1] using ovalbumin as the standard. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was prepared according to the method of Post and Sen [18]. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ synthesized was sepa-

rated from $[\text{}^{32}\text{P}]$ phosphate by AG1-X8 ion exchange column (1×4 cm) with a linear gradient elution of Tris/Cl buffer (pH 2.5) (10–300 mM; total 100 ml).

RESULTS

Gossypol inhibited phosvitin kinase in a concentration dependent manner using phosvitin as substrate as shown in Fig. 1. The IC_{50} value (concentration causing 50% inhibition) was estimated to be 42 μM . Kinetic studies were carried out to elucidate how the gossypol inhibition is related to the enzyme substrates (ATP and phosvitin). Gossypol inhibited the enzyme noncompetitively with respect to ATP, with average K_i value of 17 μM (Fig. 2). In comparison, the gossypol inhibition with respect to phosvitin was shown to be competitive, with an average K_i of 11 μM (Fig. 3).

Since polyamines are stimulators of phosvitin kinase and reverse the inhibition of the enzyme activity by heparin [10], we examined the effect of polyamines on gossypol inhibition. As shown in Fig. 4, polyamines restored the enzyme activity which had been inhibited by gossypol. Of the polyamines used, spermine and spermidine were more

Fig. 1. Inhibition by gossypol of phosvitin kinase. The reaction mixture in a final volume of 0.2 ml contained 25 mM Tris/Cl (pH 7.5), 10 mM MgCl_2 , 5mM 2-mercaptoethanol, 0.25 mM ethylene glycol bis (β -aminoethyl)-N, N, N', N'-tetraacetic acid (EGTA), 100 μg phosvitin, 0.16 μg phosvitin kinase, 100 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and various concentrations of gossypol as indicated. The reaction was started by the addition of ATP and carried out for 10 min at 30°C.

Fig. 2. Double reciprocal plots showing inhibition by gossypol of phosvitin kinase as a function of ATP concentrations. The enzyme was incubated without ($\circ$) or with 20 μM (Δ) and 40 μM gossypol ($\blacktriangle$). Other conditions were as described in Fig. 1. Unit of v was pmol/min.

Fig. 3. Double reciprocal plots showing inhibition by gossypol of phosvitin kinase as a function of phosvitin concentrations. The enzyme was incubated without (○) or with 20 μ M (△) and 40 μ M gossypol (▲). Other conditions were as described in Figs. 1 and 2.

effective than putrescine in reversing the inhibition.

We have previously shown that HMG proteins HMG 14 and 17 are phosphorylated by phosvitin kinase [10]. Indeed, as shown in Fig 5, a significant phosvitin kinase activity (37 pmol/min, ○ on the ordinate in the figure) could be detected using HMG proteins (unfractionated) as substrate, and the activity was comparable to that using phosvitin as substrate (56 pmol/min in Fig. 1). We therefore tried whether gossypol inhibited the phosphorylation of HMG proteins by phosvitin kinase. Gossypol inhibited the enzyme activity (Fig. 5). The IC_{50} value for the HMG protein phosphorylation was calculated to be 48 μ M, and the value was similar to that obtained using phosvitin as substrate.

DISCUSSION

Present studies have shown that gossypol is an inhibitor of cyclic nucleotide-independent phosvitin kinase from pig testis. Gossypol is also an inhibitor of protein kinase C (phospholipid/ Ca^{2+} -dependent protein kinase) from pig testis [12]. The mode of inhibition of phosvitin kinase is different from that of protein kinase C. The

Fig. 4. Effects of polyamines on the gossypol inhibition of phosvitin kinase. The enzyme was assayed in the presence of 40 μ M gossypol and various concentrations of polyamines as indicated. Mg^{2+} concentration used in this experiment was 1 mM, because polyamines were not effective in stimulating the enzyme at higher Mg^{2+} concentrations such as 10 mM [10]. ○, no polyamine; ●, spermine; △, spermidine; ▲, putrescine. ○ on the ordinate indicated by arrow shows enzyme activity in the absence of gossypol.

Fig. 5. Inhibition by gossypol of phosphorylation of HMG proteins by phosvitin kinase. The enzyme was assayed in the presence of HMG proteins (unfractionated, 40 μ g/0.2 ml) instead of phosvitin. Other conditions were as described in Fig. 1.

inhibition of protein kinase C is competitive with respect to the cofactors (phosphatidylserine and Ca^{2+}), but noncompetitive with respect to the protein substrate (histone). This difference may be due to the different substrate specificities of the two enzymes; *i.e.*, phosvitin kinase phosphorylates acidic substrates but not basic substrates such as

histone [9], whereas protein kinase C preferentially uses histones as exogenous substrates.

A number of inhibitors of casein kinase II have been identified including heparin [5], 2, 3-bisphosphoglycerate [7], inositol hexasulfate [6], and quercetin [2]. These inhibitors, except for quercetin, are competitive with respect to protein substrates. Among these inhibitors, the inhibitory mechanism has been most exhaustively studied in heparin [5, 6]. Since inhibition by heparin of casein kinase II is reversed by polyamines [13] and competed by the protein substrate, the mechanism of gossypol inhibition seems to be similar to that of heparin inhibition.

Phosvitin kinase is suggested to be involved in the regulation of RNA synthesis in the testis because of distribution in nuclei, phosphorylation of HMG 14 and 17 and stimulation by polyamines. Therefore, it is conceivable that one of the actions of gossypol on spermatogenesis is the inhibition of RNA synthesis through the inhibition of phosvitin kinase. HMG 1, the other high mobility group protein, is phosphorylated by protein kinase C [11], and the phosphorylation is also inhibited by gossypol [12]. The IC_{50} value for isolated HMG 1 phosphorylation by protein kinase C is 88 μ M [12]. This value is comparable to that obtained in the protein phosphorylation by phosvitin kinase in the present study, although we have not yet determined IC_{50} values for isolated HMG 14 and 17 because of very labile nature of the two proteins during the purification procedures [3]. At present, data on the actual concentration of gossypol in the testis which inhibit spermatogenesis have not yet been accumulated. However, it is of interest and suggestive that testicular phosvitin kinase and protein kinase C are inhibited by the similar concentrations of gossypol. Sequential and simultaneous phosphorylation of HMG proteins by the two protein kinases may be important

in spermatogenesis, thereby assuming inhibition by gossypol of the protein kinase cascade.

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要 約

豚精巢のサイクリックヌクレオチド非依存性ホスビチンキナーゼのゴシポールによる阻害：加藤憲夫*・木村和弘（北海道大学獣医学部生化学教室，*現：農林水産省家畜衛生試験場北海道支場）——男性用避妊薬であるゴシポールは，豚精巢から精製したサイクリックヌクレオチド非依存性ホスビチンキナーゼを阻害した。IC₅₀はホスビチンを基質にして測定したところ，42μMであった。阻害様式はATPに対しては非拮抗（K_i=17μM）であったが，ホスビチンに対しては拮抗阻害（K_i=11μM）を示した。ホスビチンの代りにMMGタンパク質（本酵素の内在基質でヌクレオソームに局在する）を基質に用いた場合も，ゴシポールにより阻害された。その時のIC₅₀は48μMであった。