Volume 318, number 1, 88–90 FEBS 12137 February 1993

Inhibition of protein prenylation by patulin

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Received 25 September 1992

The antibiotic patulin was found to inhibit protein prenylation in mouse FM3A cells. Thus, the agent reduced incorporation of [^{3}H]mevalonate into proteins by 50% at a concentration of 7 μM. In a cell-free assay, patulin inhibited rat brain farnesyl:protein transferase, one of the enzymes responsible for protein prenylation. The inhibition was 50% at a concentration of 290 μM.

Patulin; Protein prenylation; Mevalonate; Farnesyl:protein transferase; FM3A cell

1. INTRODUCTION

Recent studies have demonstrated that eukaryotic cells contain proteins that are post-translationally modified by thioether-linked prenyl (polyisoprene) groups [1]. Two types of modifying prenyl groups have been identified so far: the 15-carbon farnesyl group and 20-carbon geranylgeranyl group. The modification occurs on a cysteine residue near the COOH terminus and a set of protein modifications follows this reaction. These modifications promote binding of the protein to the cell membrane and/or influence their function. A dramatic example are the Ras proteins [2], since variant forms of these proteins encoded by oncogenes are unable to transform cells unless they are prenylated.

We have searched for microbial metabolites that affect protein prenylation and an active compound was isolated from a strain of *Byssochlamys nivea*. This compound was identified as the mycotoxin patulin. Patulin was first isolated in the early 1940's as an antibiotic [3], while it was later classified as a mycotoxin by its toxicity and carcinogenicity. Several cellular functions including protein and RNA synthesis are shown to be affected by patulin [4,5]. However, its effect on protein prenylation have not been studied. The present communication describes the inhibitory effect of patulin on prenylation of proteins.

2. MATERIALS AND METHODS

2.1. *Materials*

RS-[5-^{3}H]mevalonolactone (40.0 Ci/mmol) and [1-^{3}H]farnesyl pyrophosphate (15.0 Ci/mmol) were obtained from DuPont NEN. [U-^{14}C]Leucine (184 mCi/mmol) was obtained from ICN. ES medium was purchased from Nissui Seiyaku Co., Japan. The synthetic heptapeptide KTSCVIM was obtained from Takara Biomedicals, Japan. Patulin was isolated from a culture of *Byssochlamys nivea* IFO 8972.

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2.2. *Incorporation of [^{3}H]mevalonate into proteins and non-saponifiable lipids, and of [^{14}C]leucine into proteins in FM3A cells*

FM3A cells (2.3×10^6 cells) were suspended in 1 ml of ES medium supplemented with 5% bovine serum and incubated with 0.104 μM [^{3}H]mevalonate (40.0 Ci/mmol), 0.272 μM [^{14}C]leucine (184 mCi/mmol) and 30 μM compactin at 37°C for the indicated periods in the presence of the indicated concentrations of patulin, cycloheximide or roridin E. After collecting cells by centrifugation and washing with phosphate-buffered saline, protein was precipitated by the addition of ice-cold 10% trichloroacetic acid. The resultant precipitate was collected, washed with methanol, solubilized in 0.2 N NaOH and then counted for both ^{3}H and ^{14}C in a scintillation liquid (ACS II, Amersham).

[^{3}H]Mevalonate incorporation into non-saponifiable lipids was measured in the same mixture but containing no [^{14}C]leucine. After the indicated periods, the mixture received 1 ml of 20% (w/v) KOH in ethanol and saponified at 75°C for 60 min. Non-saponifiable lipids were extracted with petroleum ether and counted for radioactivity in a toluene-based scintillation liquid.

2.3 *Farnesyl:protein transferase assay*

Rat brain farnesyl:protein transferase was partially purified by ammonium sulfate precipitation, ion-exchange chromatography and gel filtration as described by Reiss et al. [6]. The reaction mixture (10 μl) contained 50 mM potassium phosphate (pH 6.5), 0.2 mM $ZnCl_2$, 4 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 3.4 μM synthetic heptapeptide KTSCVIM, 0.2 μM [^{3}H]farnesyl pyrophosphate (15.0 Ci/mmol) and 35 μg/ml of the enzyme. After incubation at 37°C for 1 h, the reaction was stopped by the addition of 10 μl of solvent A (*n*-propanol/ammonia/water, 6:3:1). A portion of the mixture was spotted onto silica gel plate and developed in solvent A. Spots of farnesylated peptide were visualized by a BIOSCAN system 200 imaging scanner and counted for radioactivity.

In experiments testing for time dependence of the patulin action, farnesyl·protein transferase (3.5 μg) was preincubated in a mixture (5 μl) containing 50 mM potassium phosphate (pH 6.5), 0.2 mM $ZnCl_2$, 4 mM $MgCl_2$, 50 mM KCl and 1 mM dithiothreitol in the absence or presence of 0.5 mM or 1 mM patulin. After incubation for 0, 5, 10, 20, or 30 min at 37°C, the reaction was started by the addition of 5 μl mixture containing 50 mM potassium phosphate (pH 6.5), 0.2 mM $ZnCl_2$, 4 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 6.8 μM synthetic heptapeptide KTSCVIM and 0.4 μM [^{3}H]farnesyl pyrophosphate. The reaction was continued for 5 min at 37°C and the radioactivity of farnesylated peptide was counted as described above.

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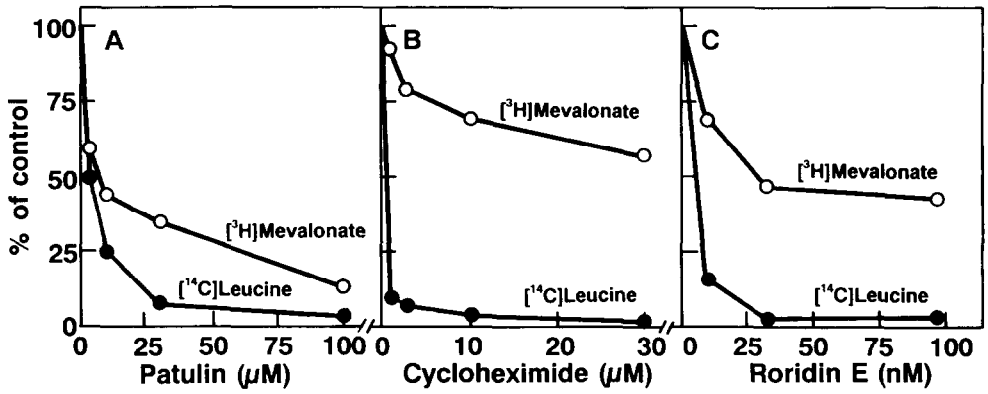

Fig. 1. Effect of patulin (A), cycloheximide (B) and roridin E (C) on the incorporation of [^{3}H]mevalonate and [^{14}C]leucine into proteins in FM3A cells. Incorporation of [^{3}H]mevalonate (○) and [^{14}C]leucine (●) into proteins in FM3A cells was determined as described in section 2 with an incubation period of 3 h. Each value represents the average of duplicate determinations. The mean control values (pmol/h/mg protein) were 0.131 for [^{3}H]mevalonate incorporation and 16.7 for [^{14}C]leucine incorporation.

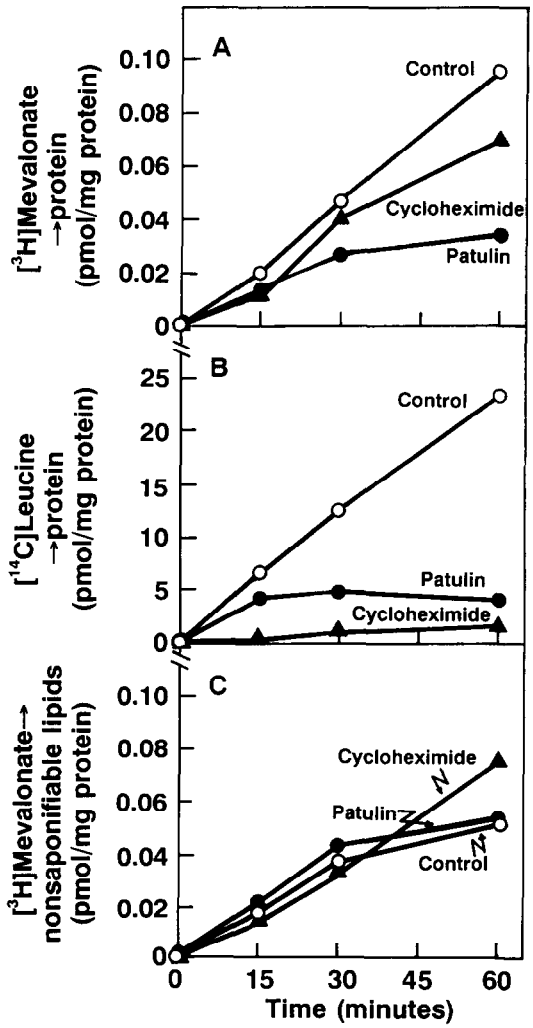

Fig. 2. The time course for the effects of patulin and cycloheximide on the incorporation of [^{3}H]mevalonate into proteins (A) and non-saponifiable lipids (C) and of [^{14}C]leucine into proteins (B). FM3A cells were incubated at 37°C with [^{3}H]mevalonate and [^{14}C]leucine in the absence (○) or presence of either 10 μM patulin (●) or 3.5 μM cycloheximide (▲). After the indicated periods, radioactivity incorporated into proteins and non-saponifiable lipids was determined. Each value represents the average of duplicate determinations.

3. RESULTS AND DISCUSSION

The prenylation of cell proteins was measured for 3 h as the incorporation of [^{3}H]mevalonate into proteins in FM3A cells. Patulin inhibited 50% this activity at 7 μM and 80% at 100 μM (Fig. 1A). Protein synthesis, as measured by the incorporation of [^{14}C]leucine, was also inhibited by patulin. The inhibition was 50% at 3 μM and >90% at 30 μM (Fig. 1A). To distinguish the patulin effects on protein synthesis and prenylation, the effects of two other compounds which inhibit protein synthesis were studied. Cycloheximide and roridin E inhibited [^{14}C]leucine incorporation by >90% at 4 μM and 25 nM, respectively (Fig. 1B and C). Under these conditions both agents reduced [^{3}H]mevalonate incorporation by 25–50%, and the reduction was 40–60% even at higher concentrations which caused nearly complete inhibition of protein synthesis (Fig. 1B and C).

The difference between the inhibitory activity of patulin and cycloheximide was more pronounced in experiments with a short incubation period. Thus, inhibition of [^{3}H]mevalonate incorporation into proteins by patulin (10 μM) was 40% after incubation for 30 min and 70% at 60 min. However, cycloheximide (3.5 μM) caused a slight reduction in protein prenylation during incubation for 15–60 min (Fig. 2A). Under these conditions, [^{14}C]leucine incorporation was inhibited by 30–80% and >90% by patulin and cycloheximide, respectively (Fig. 2B).

Prenyl groups of proteins are formed via the cholesterol synthetic pathway. Thus, the effect of patulin on this pathway was examined by measuring the incorporation of [^{3}H]mevalonate into non-saponifiable lipids. As shown in Fig. 2C, patulin did not affect this activity under the conditions where the agent inhibited incorporation of both [^{3}H]mevalonate and [^{14}C]leucine into proteins. Similarly, non-saponifiable lipid synthesis was not affected by cycloheximide. From these results, it was suggested that patulin inhibits protein prenylation by a

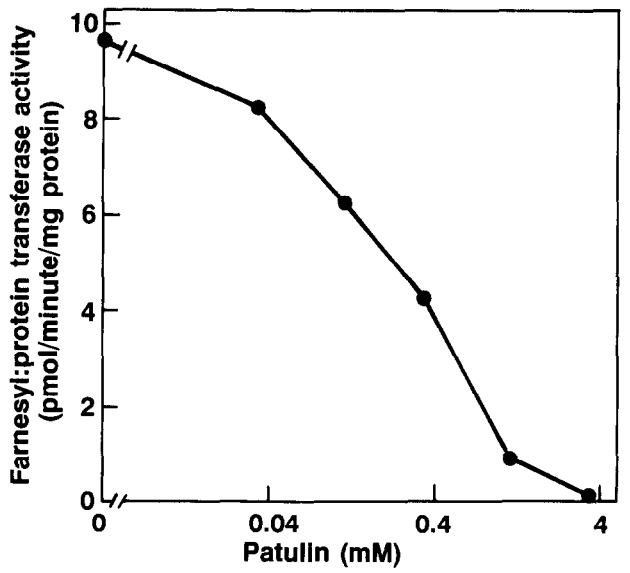

Fig. 3. Inhibition of rat brain farnesyl:protein transferase by patulin in a cell-free system. Experimental conditions are shown in section 2. Each value represents the average of duplicate determinations.

mechanism distinct from the inhibition of the synthesis of proteins or sterols. Inhibition of prenylation by cycloheximide and roridin E in a long-term incubation may be a result of deprivation of proteins that accept prenyl group [7].

To confirm the possibility that patulin inhibits the protein prenylation reaction, farnesyl:protein transferase was partially purified from rat brain and assayed for patulin inhibition in a cell free system. Results demonstrated that patulin inhibited farnesyl:protein transferase activity 50% at a concentration of 290 μM (Fig. 3). This inhibition was time-dependent and pseudo-first order inactivation was observed after preincubation of the enzyme with patulin (Fig. 4).

In summary, the present experiments demonstrate that patulin inhibits farnesyl:protein transferase in a cell-free system. The observed reduction by patulin of [^{3}H]mevalonate incorporation into proteins in FM3A cells can be explained, at least in part, by this inhibition.

Acknowledgements: The authors thank Ms. Y. Sasaki, Mr. M. Nishimura and Mr. Y. Takayasu for their technical assistance.

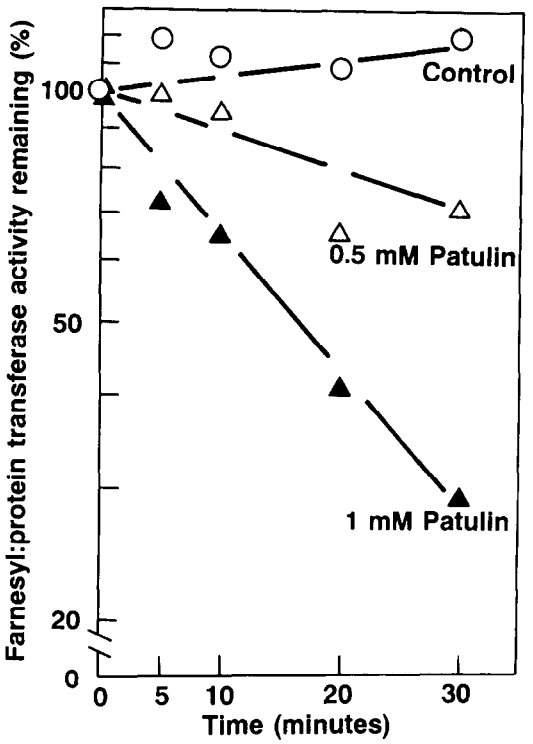

Fig. 4. Inactivation of rat brain farnesyl:protein transferase by patulin. Experimental conditions are shown in section 2. Each value represents the average of duplicate determinations. Initial (time 0) enzyme activities (pmol/min/mg protein) were 6.47 for control (in the absence of patulin) (○), 5.94 for 0.5 mM patulin (△) and 5.69 for 1 mM patulin (▲).

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