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A novel protein phosphatase inhibitor, tautomycin

Effect on smooth muscle

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The antibiotic, tautomycin, was found to be a potent inhibitor of protein phosphatases and equally effective for the type-1 and type-2A enzymes. For the catalytic subunits of the type-1 and type-2A phosphatases the IC_{50} value was 22 to 32 nM. For the phosphatase activity present in chicken gizzard actomyosin the IC_{50} value was 6 nM. Tautomycin had no effect on myosin light chain kinase activity. Tautomycin induced a Ca^{2+}-independent contraction of intact and permeabilized smooth muscle fibers and this was accompanied by an increase in the level of myosin phosphorylation. Thus, tautomycin by virtue of its ability to inhibit phosphatase activity is a valuable addition for studying the role of protein phosphorylation.

Tautomycin; Protein phosphatase inhibitor; Smooth muscle contraction

1. INTRODUCTION

Tautomycin is an antibiotic isolated from *Streptomyces spiroverticillatus* [1–3] that was found to inhibit the endogenous protein phosphatases of mouse brain [4]. Our objectives in the experiments described here were to examine the effects of tautomycin on defined protein phosphatases, specifically type-1 and type-2A and also to evaluate its action on the contraction of smooth muscle fibers. It was found that tautomycin is a potent inhibitor of type-1 and type-2A phosphatases and induces contraction of smooth muscle under Ca^{2+}-free conditions. Thus, tautomycin is similar in its actions to calyculin-A and to a lesser extent okadaic acid [5] and is an additional and valuable tool for evaluating roles of protein phosphorylation in various systems.

2. MATERIALS AND METHODS

2.1. *Protein phosphatases*

Type-1 phosphatase was partially purified from turkey gizzard native actomyosin as described previously [5] with additional steps of ammonium sulfate fractionation between 30 and 60% saturation, followed by chromatography on Sephacryl S-300, DEAE-Sepharose Fast Flow and finally heparin-agarose. The catalytic subunit of type-1 phosphatase from skeletal muscle was kindly provided by Drs D. Brautigan and B. Martin (Brown University) and the catalytic subunit of type-2A phosphatase from bovine cardiac muscle by Dr M.C. Mumby (University of Texas). Phosphatase assays were carried out at 30°C using the ^{32}P-labeled 20 kDa myosin light chain (MLC) obtained from turkey gizzard [6]. Phosphatase activity was calculated from initial linear rates [5]. Activity of chicken gizzard endogenous phosphatase was determined from the rate of dephosphorylation of phosphorylated MLC on removal of Ca^{2+} and ATP [7].

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2.2. *Cytosolic Ca^{2+} level and muscle tension*

Cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) was measured simultaneously with muscle contraction as reported previously [8]. Strips of rat aorta (5–10 mg wet weight) were loaded with 5 μM acetoxy methyl ester of fura-2 for 3 h at 23–25°C and then placed in a tissue bath at 37°C. Each muscle strip was illuminated alternatively (48 Hz) with 340 and 380 nm light, and emission was detected at 500 nm. The ratio of the 500 nm fluorescence induced by 340 nm and 380 nm excitation was used as an indicator of $[Ca^{2+}]_i$ with values normalized to 0% in resting and 100% in high K^+-stimulated muscle, respectively.

2.3. *Contraction of skinned smooth muscle*

Skinned (permeabilized) smooth muscle was made by freezing the strips of guinea-pig taenia caeci or rabbit mesenteric artery (1–2 mg wet-weight) in liquid nitrogen followed by freeze-drying. Before use, the dried muscle strips were treated with a relaxing solution containing 10% glycerol for 15 min. Other experimental procedures are similar to those described previously [7].

2.4. *MLC phosphorylation and MLC kinase*

Native actomyosin was prepared from chicken gizzard [7]. MLC phosphorylation was determined by monitoring the shift in electrophoretic motility of MLC in a urea polyacrylamide gel [9]. Protein bands were visualized by means of the silver stain [7]. MLC kinase was isolated from frozen turkey gizzard as described by Ikebe et al. [10].

2.5. *Drugs and chemicals*

The following drugs and chemicals were used: norepinephrine, verapamil (Sigma Chemical Co., St. Louis, MO, USA) and acetoxymethyl ester of fura-2 (Dojindo Lab., Kumamoto, Japan). Tautomycin (donated by Dr Kiyoshi Isono, Riken: The Institute of Physical and Chemical Research), okadaic acid (donated by Dr

Table I

Inhibition of protein phosphatases by tautomycin, okadaic acid and calyculin-A

Phosphatase	Tautomycin	Okadaic acid	Calyculin A
	IC_{50} (nM)		
Type-2A	32	10	3
Type-1 (smooth muscle)	32	224	5
Type-1 (skeletal muscle)	22	237	–
Smooth muscle endogenous	6	70	1

– : not determined

Daisuke Uemura, Shizuoka University) and calyculin-A (donated by Dr Nobuhiro Fusetani, The University of Tokyo) were dissolved in 100% ethanol to a 10 mM stock.

3. RESULTS

As shown in Fig. 1 and Table I, tautomycin inhibited the type-1 and type-2A phosphatases with IC_{50} values of 22–32 nM and the smooth muscle endogenous phosphatase with an IC_{50} of 6 nM. Okadaic acid and calyculin A are known inhibitors of these phosphatases [5,11] and for comparison the effects of these compounds are included in Table I. Tautomycin, up to 80 μM, did not influence the activity or calmodulin-dependence of MLC kinase (data not shown).

As shown in Fig. 2, 10 μM tautomycin induced a sustained contraction of rat aorta with only a small increase in $[Ca^{2+}]_i$. A Ca^{2+} channel blocker, 10 μM verapamil, decreased the tautomycin-induced increase in $[Ca^{2+}]_i$ to resting levels. Subsequent addition of 4 mM EGTA further decreased $[Ca^{2+}]_i$. However, neither verapamil nor EGTA inhibited the tautomycin-induced contraction. As shown in Fig. 3, cumulative addition of tautomycin induced a graded contraction in rat aorta with an EC_{50} of 2 μM. The contractile effect of tautomycin was not affected by removing external Ca^{2+} with EGTA.

In rat aorta, high K^+ (65.4 mM) or 1 μM norepinephrine induced sustained contraction. Addition of tautomycin or calyculin-A during these contractions induced additional increase in muscle tension (data not shown). By contrast, addition of okadaic acid inhibited the contraction at 0.1–1 μM whereas higher concentrations of okadaic acid ($\geq$ 10 μM) induced additional increase in muscle tension, as reported previously [12].

Tautomycin induced contraction in the absence of Ca^{2+} ($pCa^{2+} < 1$ nM) in skinned taenia and mesenteric artery (Fig. 3). The IC_{50} value of tautomycin for skinned muscle fiber (14–17 nM) was approx. 100 times lower than those for intact smooth muscle. Similar observations were made previously with okadaic acid and calyculin A [7]. The reason for the different potencies of these compounds in skinned compared to intact fibers is due probably to limited diffusion across the plasma membrane or compartmentalization in the intact fiber.

MLC was phosphorylated almost completely in the presence of 100 μM Ca^{2+} and only 5% in the absence of Ca^{2+}, as reported previously [7]. In the absence of Ca^{2+}, 10 nM and 100 nM tautomycin increased the phosphorylation to 36% and 40%, respectively. Okadaic acid (100 nM) and calyculin-A (100 nM) also increased the phosphorylation to 32% and 42%, respectively, as reported previously [7].

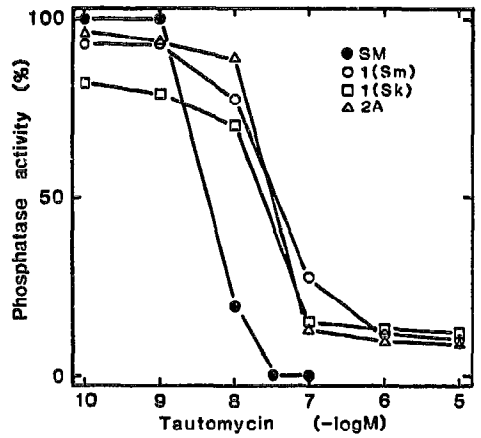

Fig. 1. Inhibitory effect of tautomycin on protein phosphatases. Conditions see section 2. Catalytic subunits of type-1 phosphatase from smooth muscle (○) and skeletal muscle (□). Endogenous phosphatase from smooth muscle (●). Catalytic subunit of type-2A phosphatase (△).

4. DISCUSSION

In the studies described above it was shown that tautomycin is an effective inhibitor of type-1 and type-2A phosphatases. The IC_{50} values for each phosphatase are similar and in this respect tautomycin resembles calyculin-A rather than okadaic acid since the latter is more effective with the type-2A enzyme [5]. Tautomycin appears to be slightly less potent than calyculin-A by a factor of about ten-fold, but this must be regarded as tentative until K_i values are determined.

Phosphorylation of MLC by MLC kinase is accepted as a dominant mechanism in the regulation of contractile activity in smooth muscle [13,14]. Activation of MLC kinase occurs following an increase in $[Ca^{2+}]_i$ and the formation of the ternary complex, Ca^{2+}, calmodulin, MLC kinase. However, in these studies it was shown that tautomycin induces contraction of intact and skinned smooth muscle fibers in the absence of increased $[Ca^{2+}]_i$. The concentrations of tautomycin needed for contraction (IC_{50}: 14–17 nM) and elevated phosphorylation (30 nM) in skinned fibers were similar to those concentrations required to inhibit protein phosphatase activities. In addition, there appears to be

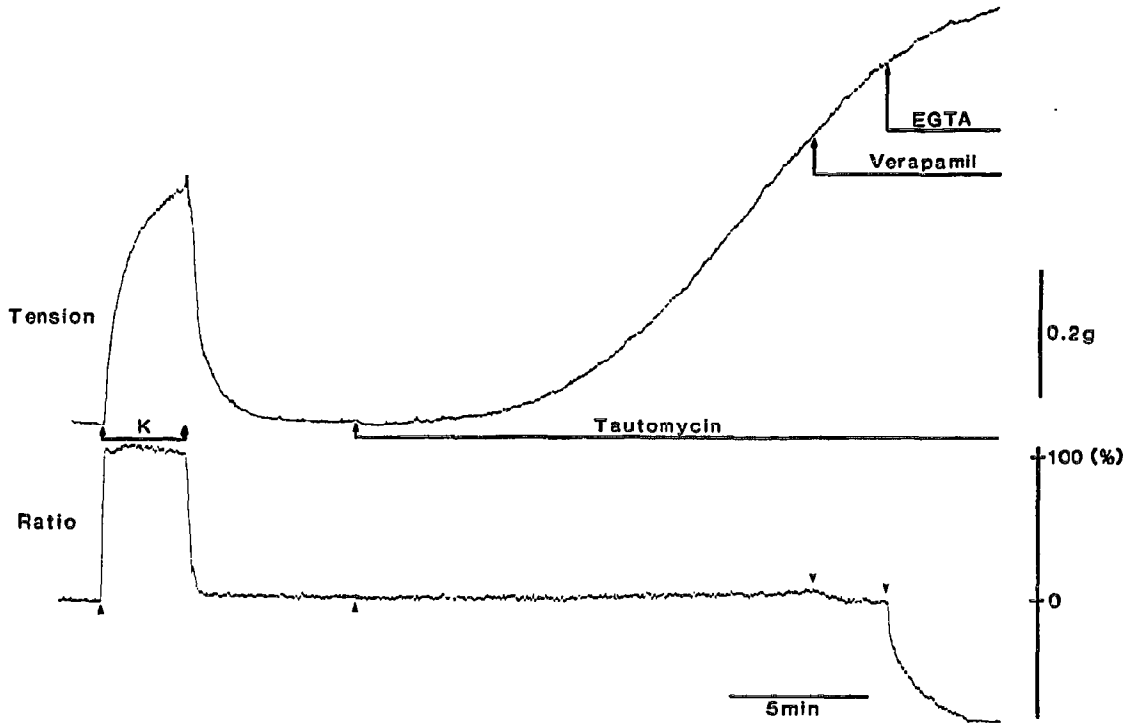

Fig. 2. Effect of tautomycin on $[Ca^{2+}]_i$ and contractile tension in rat aorta. Muscle was first contracted in 72.7 mM KCl (made by replacing NaCl with KCl) and then with 10 μM tautomycin. Verapamil (10 μM) and 4 mM EGTA were sequentially added in the presence of tautomycin as indicated.

no direct effect of tautomycin on MLC kinase activity. These results, therefore, support the hypothesis that the tautomycin-induced and Ca^{2+}-independent contraction is due to the 'unmasking' of significant MLC kinase activity via phosphatase inhibition. A similar conclusion was reached earlier for calyculin-A and okadaic acid [5,7]. It is surprising that the level of MLC kinase activity in resting cell is sufficient to account for this and the possibility that other kinases might be involved should be considered. In this regard it is interesting that the type II Ca^{2+}/calmodulin-dependent protein kinase was recently shown to be capable of phosphorylating smooth muscle myosin [15].

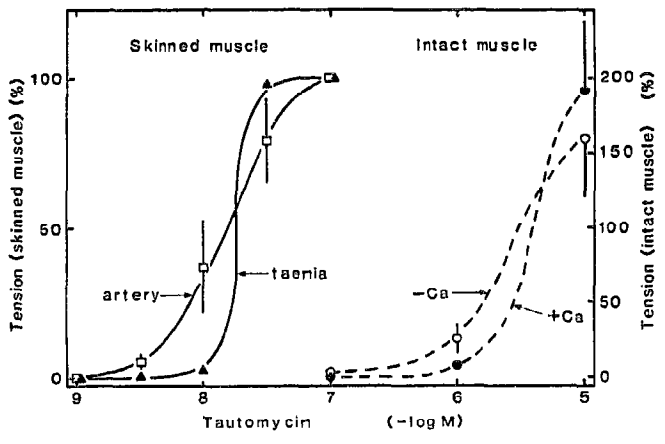

Fig. 3. Concentration–response curves for contractile effect of tautomycin on intact rat aorta in the presence (+Ca) or absence of Ca^{2+} (−Ca), and on skinned smooth muscle of guinea-pig taenia and rabbit mesenteric artery. Values are mean ± SEM of 4 muscle strips. 100% for intact muscle (right ordinate) represents the contractile tension induced by 72.7 mM KCl and 100% for skinned muscle (left ordinate) represents the contractile tension induced by 100 nM tautomycin.

Okadaic acid has a dual effect on smooth muscle mechanics; at low concentrations it relaxes a contracted muscle strip and at higher concentrations it induces contraction [12,16]. In the present experiment, it was found that neither tautomycin nor calyculin-A behaved similarly and neither inhibited contraction induced by high K^+ or norepinephrine. Tentatively the distinctive behavior of okadaic acid may reflect its preferential inhibition of type-2A phosphatase and the involvement of this enzyme in processes other than myosin phosphorylation. It follows therefore that the type-1 phosphatase is more important in regulating phosphorylation of the myosin light chain.

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