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Evaluation of phenylthiourea derivatives as inhibitors of transglutaminase-catalyzed reaction in Chinese hamster ovary cells

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1-(5-Aminopentyl)-3-phenylthiourea (PPTU), a recently developed inhibitor of the transglutaminase-catalyzed reaction (K.N. Lee, L. Fesus, S.T. Yancey, J.E. Girard, and S.I. Chung, (1985) J. Biol. Chem. 260, 14689–14694) was evaluated as a possible probe to examine the physiological role of transglutaminase (EC 2.3.2.13) in Chinese hamster ovary (CHO) cells. The [^{14}C]PPTU in cell culture was readily taken up by CHO cells and was found to be covalently attached to high-molecular-weight proteins which are associated with the particulate fractions. Incubating cell homogenates, in the presence of Ca^{2+}, incorporated the labeled PPTU exclusively into high-molecular-weight proteins that were also undergoing polymerization. PPTU at 0.1 mM, a concentration close to the K_i value reported for inhibition of tissue transglutaminase-catalyzed amine incorporation into the B chain of oxidized insulin, decreased high-molecular-weight protein polymerization, whereas PPTU at the same concentrations showed no effect on CHO cell proliferation or on in vitro calmodulin activation of cyclic nucleotide phosphodiesterase. These results suggest that transglutaminase may not be a constitutive enzyme in cell proliferation.

Introduction

The members of the transglutaminase (EC 2.3.2.13, glutaminylpeptide : amine γ-glutamyltransferase) isozyme family catalyze a calcium-dependent acyl transfer reaction between peptide-bound glutamine residues and primary amines including the ε-amino group of lysine residues in the appropriate peptides (for details see Refs 1 and 2). The substrate and function of some transglutaminases are well recognized; i.e., blood coagulation factor XIII in the cross-linking of fibrin and α_2-plasmin fibrin inhibitor forming a proteinase-resistant stabilized clot [1,3], epidermal transglutaminase in the formation of cornified envelopes by cross-linking involucrin during the terminal differentiation of epidermal cells [4,5] and prostate transglutaminase in the formation of vaginal plug postejaculation in rodents [6]. However, the substrate or function of the most abundant cellular or 'liver type' transglutaminase is still an enigma. Recently, we synthesized a homologous series of compounds, phenylthiourea-$(CH_2)_n$-NH_2, where $n = 2,3,4,5$ and 6, as inhibi-

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Abbreviations: PPTU, 1-(5-aminopentyl)-3-phenylthiourea; EPTU, 1-(2-aminoethyl)-3-phenylthiourea; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

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tors, and we found that PPTU ($n = 5$) was the most effective (K_i value; $4.9 \cdot 10^{-5}$ M) and EPTU ($n = 2$) had no activity for the inhibition of guinea pig liver transglutaminase-catalyzed amine incorporation into B chain of oxidized insulin [7].

CHO cells were chosen for our studies because of their reportedly high transglutaminase activity [8]. The transglutaminase in CHO cells is the cellular ('tissue' or 'liver') type enzyme. It has been observed that transglutaminase activity is increased several-fold in CHO cells at maximum density [8]. Using an ELISA technique [9] it could be demonstrated that the increased activity is the result of higher level of transglutaminase protein in the confluent state of CHO cells as compared to proliferating cultures.

In this report, we have evaluated PPTU and EPTU as inhibitors of the transglutaminase-catalyzed reaction in CHO cells and we have used PPTU as a probe to examine a possible role of transglutaminase in association with other vital cellular functions.

Materials and Methods

Materials. Monodansylcadaverine hydrochloride, bovine brain calmodulin, bovine brain 3′,5′-cyclic adenosine monophosphate phosphodiesterase, adenosine 5′-monophosphate (AMP), chymostatin, and 3′,5′-cyclic adenosine monophosphate (cAMP) were purchased from Sigma; [2,8-^{3}H]cAMP (33.5 Ci/mmol) and [8-^{14}C]AMP (56 Ci/mmol) were from Amersham; α-modified Eagle's medium, fetal calf serum, L-glutamine, penicillin and Dulbecco-Vogt's phosphate-buffered saline were from Gibco. PPTU, EPTU, [^{14}C]PPTU and [^{14}C]EPTU were synthesized according to published procedures [7].

Cell culture. CHO cells were grown in monolayer culture in α-modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 μg/ml streptomycin and 50 units/ml penicillin. The cells were incubated at 37° C in an atmosphere containing 5% CO_2, and routinely passaged at 3 day intervals.

Cellular uptake of [^{14}C]PPTU and [^{14}C]EPTU. 5 ml of α-modified Eagle's medium containing 0.25 μCi of [^{14}C]PPTU (0.52 μCi/μmol) or [^{14}C]EPTU (0.52 μCi/μmol) were added to several dishes of preconfluent CHO cells. The cells were incubated in culture for the appropriate time intervals. The uptake was terminated by placing the plates on ice and the cultures were washed four times with 10 ml portions of ice-cold PBS (Dulbecco-Vogt's phosphate buffered saline, pH 7.4). The cells were harvested in Tris-buffered saline containing EDTA; (TBS-EDTA: 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4) and were counted using a hemacytometer. DNA contents of the cells were also measured by the Burton's diphenylamine method [10]. The cells were collected by centrifugation for 3 min at 1000 × *g* in a Beckman clinical centrifuge, and resuspended in a hypotonic buffer containing 5 mM Tris-HCl (pH 7.2), 5 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 0.1 mM chymostatin (buffer 1). The suspended cells were lysed by quick freezing and thawing (three times) and proteins were precipitated by addition of cold 10% trichloroacetic acid (TCA). The precipitate collected by centrifugation was resuspended in 1.0 ml aliquots of cold 5% TCA solution containing 1 mM PPTU or EPTU and the precipitate was recollected by centrifugation. This washing process of the TCA precipitate was repeated two more times. The bound TCA in the precipitate was extracted with diethyl ether.

Fractionation of cell homogenates. 5 ml of α-modified Eagle's medium containing 0.61 μCi of [^{14}C]PPTU (0.52 μCi/μmol) or [^{14}C]EPTU (0.52 μCi/μmol) were added to each dish of preconfluent CHO cells. After 12 h incubation under culture conditions, cell homogenates were prepared as described by Courtneidge et al. [11]. Five dishes, each containing $6 \cdot 10^6$ cells, were washed with cold PBS four times and the cells suspended in TBS-EDTA were transferred into microfuge tube and pelleted at 1000 × *g*. The collected cells were suspended in buffer 1 ($2 \cdot 10^7$ cells/ml) for 15 min and lysed in a tight-fitting Dounce homogenizer by stroking 30 times with a pestle. Homogenates were first separated into 'nuclear pellet' and supernatant by centrifuging the homogenates for 2 min at 1000 × *g*. The 'nuclear pellet' containing some whole cells was suspended in 1.5 ml of buffer 1 containing 0.25 M sucrose, rehomogenized in a similar manner, and recentrifuged for 2

min at 1000 × *g*. To obtain 'membrane (P 150 K)' and 'cytosol (S 150 K)' fractions, the pooled supernatants were centrifuged in thick-walled polyallomer tubes at 40000 rpm in a Beckman SW 50.1 rotor (150000 × *g*) for 1 h at 4°C. The radioactivity of each fraction was measured for the percent distribution of [^{14}C]PPTU or [^{14}C]EPTU. All three fractions; the nuclear (nuclear pellet), the membrane (P 150 K), and the cytosol (S 150 K), were analyzed for protein [12] and DNA [10] content as well as for marker enzymes, 5′-nucleotidase [13] and lactate dehydrogenase [14]. In addition, the distribution of transglutaminase activity [15] in subcellular fractions was determined.

Incorporation of [^{14}C]PPTU into CHO cellular proteins. Confluent cells washed with ice-cold TBS were harvested and lyzed in buffer 1 as described above. Portions of the cell homogenates (400–500 μg protein) were incubated at 37°C for various time intervals in a total volume of 100 μl of buffer 1 containing various concentrations of [^{14}C]PPTU (0.4 μCi/μmol) and 3 mM $CaCl_2$. Particulate and cytosol fractions were then separated by centrifugation for 30 s in a Beckman microfuge. The reaction was stopped by addition of an equal volume of cold 20% TCA and the precipitates were exhaustively washed with cold 5% TCA containing 1 mM PPTU by repeated suspension and centrifugation. The protein-bound radioactivity was determined by a liquid scintillation spectrometer and protein concentrations were determined according to Bradford [12] using bovine gamma globulin as standard.

Inhibition studies of cell proliferation. CHO cells were plated to obtain a cell density of $2.0 \cdot 10^6$ cells per dish. PPTU or EPTU in varying concentrations was added. 24 h later, cell number and DNA content of each dish was determined.

Assay of calmodulin-stimulated cyclic nucleotide phosphodiesterase. Phosphodiesterase activity was assayed by measurement of 5′-AMP formation [16] separating 3′,5′-cAMP and 5′-AMP on an ion-exchange column (AG-50WX8, 200–400 mesh, H^+ form). The reaction mixture (0.1 ml) contained 40 mM Tris-HCl (pH 8.0), 0.05 mM $CaCl_2$, 3 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 1.0 mM [^{3}H]cAMP (48562 cpm), 0.001 mM [^{14}C]AMP (1000 cpm), 1.5 nM bovine brain calmodulin and various concentrations of inhibitors (monodansylcadaverine, PPTU or EPTU).

SDS-polyacrylamide gel electrophoresis. One-dimensional electrophoresis was carried out according to Laemmli [17]. Proteins in cell extracts were denatured and then subjected to electrophoresis through a 10% polyacrylamide separating and 5% stacking gel.

Results

Cellular uptake and subcellular distribution of PPTU and EPTU

The cellular uptake of PPTU and EPTU had a similar pattern reaching a plateau within 2 h and the level did not significantly change during a 12-h incubation period (Fig. 1). The relative amount of labeled PPTU incorporated into TCA-precipitable material of CHO cells during the 12-h incubation period, however, was higher than that of EPTU. Metabolic change of [^{14}C]PPTU and EPTU was not observed in CHO cells as tested by a thin-layer chromatography method described previously [7].

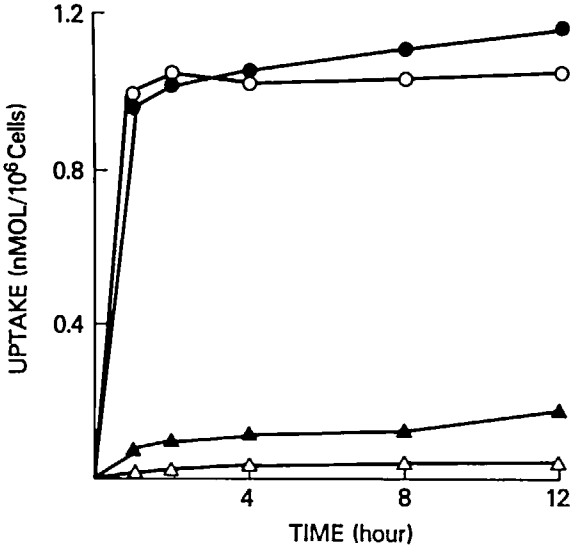

Fig. 1. Time-dependent uptake of [^{14}C]PPTU and [^{14}C]EPTU by CHO cells: total count for [^{14}C]PPTU (●) and [^{14}C]EPTU (○); TCA-precipitable counts for [^{14}C]PPTU (▲) and for [^{14}C]EPTU (△). Cells were grown in the presence of [^{14}C]PPTU or [^{14}C]EPTU at a final concentration of 0.1 mM. At various times during a 12-h period (preconfluent cultures) $(6–10) \cdot 10^6$ cells were removed and the radioactivity in TCA-soluble and -insoluble material was determined.

TABLE I

DISTRIBUTION OF [^{14}C]PPTU AND [^{14}C]EPTU IN SUBCELLULAR FRACTION OF CELL HOMOGENATE

Fraction	Percentage of total recovered				
	PPTU	5′-nucleotidase	lactate dehydrogenase	protein	transglutaminase [a]
Nuclear pellet	52	18	13	50	21
P 150 K	7	77	2	16	10
S 150 K	41	5	85	34	69
	EPTU	5′-nucleotidase	lactate dehydrogenase	protein	
Nuclear pellet	38	21	23	40	
P 150 K	7	61	3	15	
S 150 K	55	18	74	45	

[a] The distribution of transglutaminase activity was determined in a separate experiment. The P 150 K, particular fraction; S 150 K, soluble fraction.

In order to determine whether the PPTU concentration in cells reaches a concentration comparable to that present in media, the intracellular PPTU concentration was calculated assuming that the volume of $1 \cdot 10^6$ packed cells (4.2 μl) from high-gravity-force centrifugation reflects total cell volume. When CHO cells were grown in the presence of 0.01, 0.1 and 1 mM PPTU, calculations gave concentrations of PPTU in cells similar to those in the media.

To examine the distribution of PPTU and EPTU among subcellular fractions of CHO cells, [^{14}C]PPTU and [^{14}C]EPTU-labeled cells were homogenized in a low-ionic-strength buffer. Nuclear, particulate (P 150 K) and soluble (S 150 K) fractions were then prepared (Table I). The particulate fraction is enriched with plasma membrane, intracellular membranes such as endoplasmic reticulum and Golgi bodies, organelles such as lysosomes and mitochondria [18,19] and 5′-nucleotidase, a plasma membrane marker enzyme. The soluble fractions (S 150 K) show the highest activity of cytosol enzyme, lactate dehydrogenase. Transglutaminase was mainly associated with the cytosol fraction though nuclear and membrane fractions also contained some activity. The amount of [^{14}C]PPTU or [^{14}C]EPTU was quantitated by counting the radioactivity in each fraction. Both [^{14}C]PPTU and [^{14}C]EPTU were associated with the cytosol, nuclear and membrane fractions (Table I). Adding labeled PPTU and EPTU to the cell lysates following homogenization did not result in the same distribution: all the radioactivity was found in the cytosol fractions (data not shown).

PPTU as substrate and inhibitor of CHO cell transglutaminase

Cell homogenates were incubated with [^{14}C] PPTU or [^{14}C]EPTU at 37 °C during 1–60 min in the presence of Ca^{2+}. PPTU was incorporated rapidly into cellular proteins, and the incorporation approached a maximum with 10–60 min, while EPTU did not show any significant incorporation during the incubation period (Fig. 2). The incubation mixtures were separated into particulate and cytosol fractions and subjected to SDS-polyacrylamide gel electrophoresis. Autoradiography of the gels were performed. [^{14}C]PPTU-labeled proteins were found only in particulate fractions (Fig. 2) and remained at the top of the stacking gel. Fig. 3 shows that [^{14}C]PPTU is incorporated into proteins of CHO cellular homogenates in a concentration-dependent manner also, while [^{14}C]EPTU is not. Again, most of the protein-bound PPTU is sedimented into the particulate fractions and associated with polymers not entering the stacking gel. These results imply that PPTU is a substrate for the cellular transglutaminase and glutamine-containing protein substrates exist in CHO cells. As seen on the gel pattern, the formation of high-molecular-weight polymers was not inhibited, even at high con-

centrations of PPTU. We noticed, however, that in the presence of Ca^{2+} (necessary for transglutaminase activity) visible precipitates are formed in total cell homogenate making a substantial amount of proteins, in addition to nuclear and membrane proteins, sedimentable into the particulate fraction. PPTU, however, could interfere with this process suggesting that it may bind to some cellular components in the presence of Ca^{2+} or may compete with protein-bound lysine residues, and thereby may not be completely available as a substrate for transglutaminase. When the particulate fraction was separated before the addition of PPTU and Ca^{2+}, the above phenomenon was not observed. Consequently, the capability of PPTU to inhibit protein polymerization was

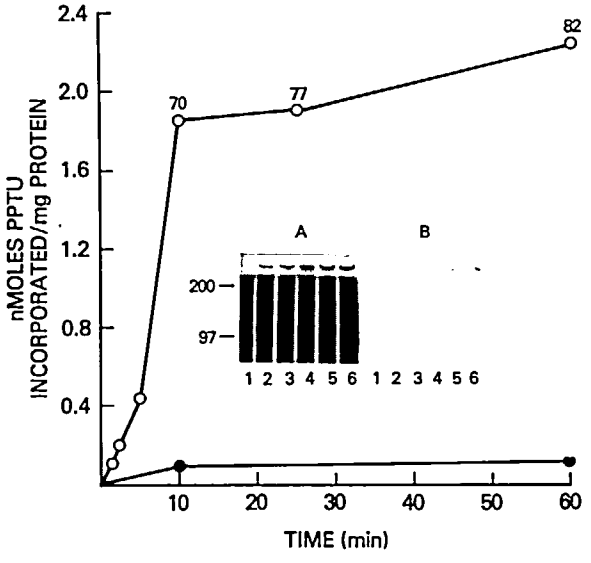

Fig. 2. Time-dependent incorporation of [^{14}C]PPTU into CHO cell proteins. Cell homogenates prepared from cells of preconfluent cultures and each containing 0.5 mg of protein, were incubated with 4.0 mM [^{14}C]PPTU (0.4 μCi/μmol) or [^{14}C]EPTU (0.40 μCi/mmol) at 37° C for the indicated times in the presence of 3 mM Ca^{2+}. Particulate and cytosol fractions were then separated by centrifugation at 20000 × g then TCA-precipitable radioactivity was quantitated in both. For the value of total incorporation, at each time point, radioactivity in both particulate and cytosol fractions was summed; PPTU (○), EPTU (●). The values above the points at 10, 25 and 60 min, represent the percent incorporation of [^{14}C]PPTU into particulate fractions. The inset shows the top portion of Coomassie blue-stained SDS-gel of particulate fractions (A) and autoradiography of the same portion of the gel (B); Lanes: 1, 1 min; 2, 2.5 min; 3, 5 min; 4, 10 min; 5, 25 min; 6, 60 min.

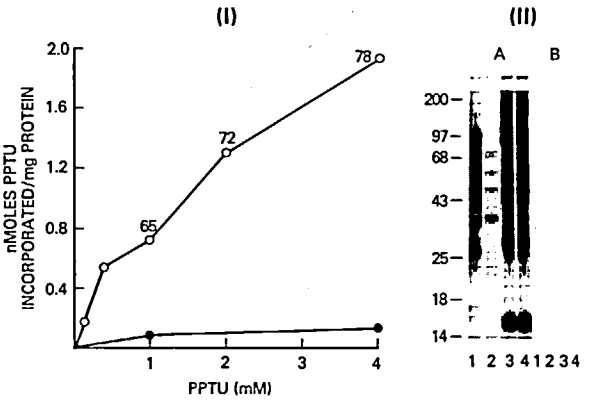

Fig. 3. Concentration-dependent incorporation of [^{14}C]PPTU into CHO cellular proteins. Cell homogenates prepared from cells of preconfluent cultures were incubated with various concentrations of [^{14}C]PPTU (0.4 μCi/μmol) or [^{14}C]EPTU (0.40 μCi/μmol) at 37° C for 10 min. TCA-precipitable radioactivity of the particulate and cytosol fractions was quantitated. (I) Total incorporation of [^{14}C]PPTU (○) or [^{14}C]EPTU (●); values indicated at 1, 2 and 4 mM represent percent incorporation of [^{14}C]PPTU into particulate fractions. (II) Coomassie blue-stained SDS-gel of the particulate and cytosol fractions (A) and autoradiography of the same gel (B); Lanes: 1 , 4 mM PPTU and cytosol fraction; 2, 0.1 mM and cytosol fraction; 3, 4 mM and particulate fraction; 4, 0.1 mM and particulate fraction.

tested also in the cytosol fraction. When this was incubated with 3 mM $CaCl_2$ in the presence of 0.05 mM PPTU, a band of macromolecular proteins appeared at the top of the stacking gel (Fig. 4, A, 2). The band completely disappeared when PPTU was added at the final concentration of 4.0 mM (A, 1), while 4 mM caused no effect (A,4). A final concentration of 0.4 mM PPTU could partially inhibit the marcomolecular protein formation (A,3). Although labeled PPTU and EPTU were used in these experiments, radioactive protein bands could not be detected, except the high-molecular-weight protein fraction at the top of the stacking gel at concentrations of 0.05 and 0.4 mM PPTU.

In order to examine whether PPTU or EPTU is incorporated into proteins of intact cells, preconfluent CHO cells were grown in medium containing 0.1 mM [^{14}C]PPTU or [^{14}C]EPTU for 12 h. Cells were homogenized then separated into cytosol and particulate fractions. As is shown in Fig. 5, PPTU was incorporated into proteins of intact cells and the particulate fraction contained

twice as much protein-bound PPTU as the cytosol fraction did. The TCA-precipitable count of PPTU was much higher than that of EPTU in both fractions. In an effort to find protein substrates in CHO cells, each sample was subjected to electrophoresis and then autoradiography. In the particulate fraction a [^{14}C]PPTU-labeled protein band (larger than 200 kDa) was found at the top of the separating gel (Fig. 5, B, 4). However, a larger radiolabeled band which was detected following the incubation of cytosol with labeled PPTU (see Fig. 4) was not visible at the top of the stacking gel. To increase the chance of detection of more radiolabeled proteins, the experiment was repeated at higher concentration (0.5 mM) of labeled PPTU with the same specific activity and again

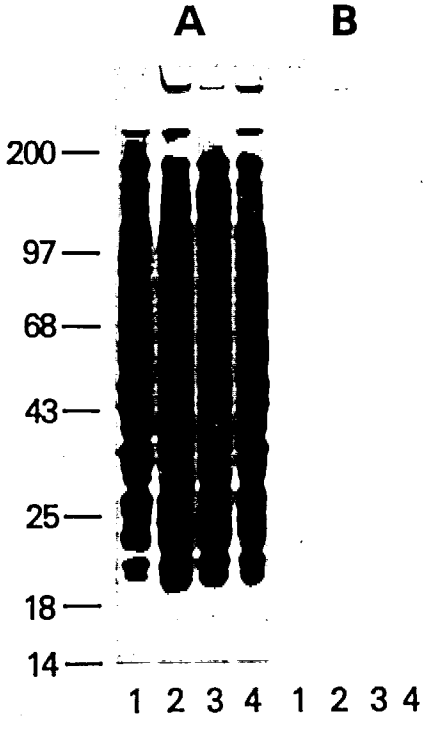

Fig. 4. Effects of PPTU and EPTU on macromolecular protein formation during incubation of the cytosol fraction with Ca^{2+}. CHO cell cytosol containing about 100 μg protein was incubated for 10 min with [^{14}C]PPTU (0.52 μCi/μmol) at different final concentrations then processed for SDS-gel electrophoresis. Coomassie blue-stained SDS gel (A) and autoradiography of the same gel (B); Lanes: 1, 4 mM [^{14}C]PPTU; 2, 0.05 mM [^{14}C]PPTU; 3, 0.4 mM [^{14}C]PPTU; 4, 4 mM [^{14}C]EPTU.

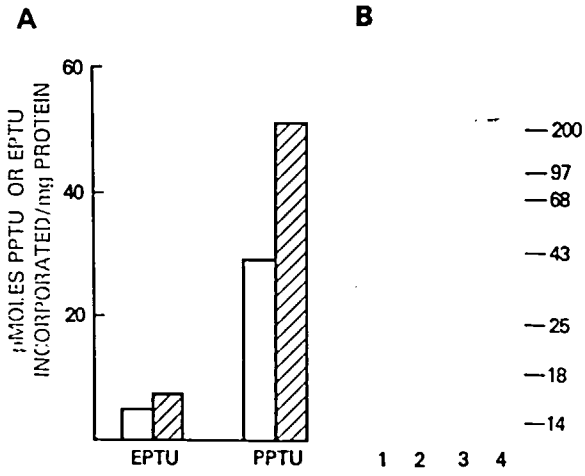

Fig. 5. Incorporation of [^{14}C]PPTU and [^{14}C]EPTU into proteins of growing CHO cells. (A) Preconfluent CHO cells were incubated in α-modified Eagle's medium containing [^{14}C]PPTU or [^{14}C]EPTU at a final concentration of 0.1 mM (0.52 μCi/μmol for both compounds) at 37 °C for 12 h. Cells were then homogenized and the homogenates were separated into cytosol and particulate fractions. TCA-precipitable radioactivity of the cytosol (white bar) and particulate (hatched bar) fractions was then quantitated. (B) The cytosol and particulate fractions, each containing 0.4 mg of protein, were subjected to gel electrophoresis, and autoradiography was performed on the same gel. Lanes: 1, cytosol fraction with [^{14}C]EPTU; 2, particulate fraction with [^{14}C]EPTU; 3, cytosol fraction with [^{14}C]PPTU, 4, particulate fraction with [^{14}C]PPTU.

only the high-molecular-weight polymers were seen (results not shown). In addition, we repeated the experiments using proliferating (logarithmic phase) as well as confluent cells and the same results were observed (data not shown).

Effects of PPTU and EPTU on cell proliferation

PPTU or EPTU was added to the medium to give the final concentrations indicated on Fig. 6. After 24 h of culture, cells in each dish were harvested and counted. The number of cells in a dish containing medium without inhibitor was 5.3 $\cdot 10^6$ cells. This number was used as control (Fig. 6B). As shown in Fig. 6B, both PPTU and EPTU inhibit the proliferation of CHO cells in a concentration-dependent manner. It should be noted, however, that PPTU at its K_i concentration (0.049 mM for cellular transglutaminase [7]) did not affect CHO cell proliferation and the cells were still growing when 5 mM PPTU was used which was a 100-fold higher concentration than its K_i value

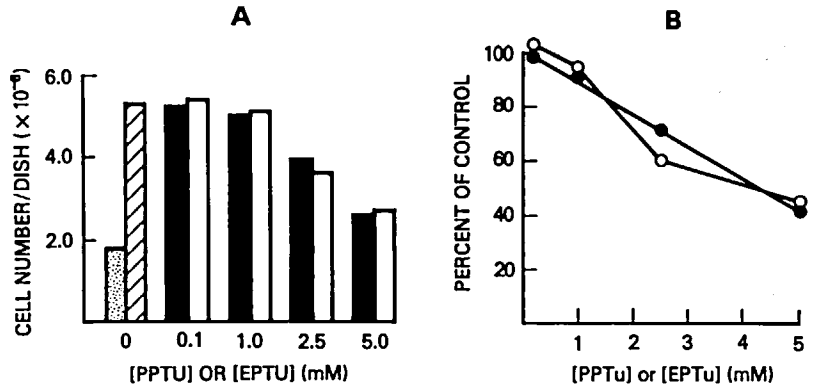

Fig. 6. Effects of PPTU and EPTU on the proliferation of CHO cells. (A) Initial (stippled bar) and final (hatched bar) number of cells per dish without PPTU and EPTU; final number of cells with PPTU (black bar) or EPTU (white bar). (B) Data shown as percent of control: PPTU (●), EPTU (○).

for cellular transglutaminase (Fig. 6A). Similar results were obtained when the DNA contents of the cells was determined (data not shown) instead of cell number.

Effects of PPTU and EPTU on the activities of calmodulin-stimulated cyclic nucleotide phosphodiesterase

It has been reported that a commercially available *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), which has a structure similar to that of monodansylcadaverine, inhibits cell proliferation of human carcinoma cell lines [20], and that monodansylcadaverine inhibits calmodulin activated phosphodiesterase activity [21]. We compared the inhibitory effect of PPTU, EPTU, and monodansylcadaverine on a calmodulin-dependent reaction. When calmodulin was added to a cell-free assay for 3′,5′-cyclic nucleotide phosphodiesterase activity, the rate of the enzyme reaction increased approx. 5-fold in the presence of 1.5 mM calmodulin; i.e., total activity was 5-times higher than basal activity in this assay system. When PPTU or EPTU was added, both inhibited the calmodulin-activated phosphodiesterase activity but neither compound inhibited the basal activity (Fig. 7). PPTU and EPTU showed similar inhibitory capacity for the inhibition of calmodulin-dependent phosphodiesterase activation. Both inhibited the activity by 50% at a concentration of 0.4 mM, which is 10-fold higher than its K_i value for cellular transglutaminase [7], whereas monodansylcadaverine reduced the process by 50% at a concentration of 0.15 mM. It has been reported that W-7 is more potent than monodansylcadaverine [21]. Therefore, PPTU is the least effective inhibitor of the three for this particular calmodulin-dependent reaction.

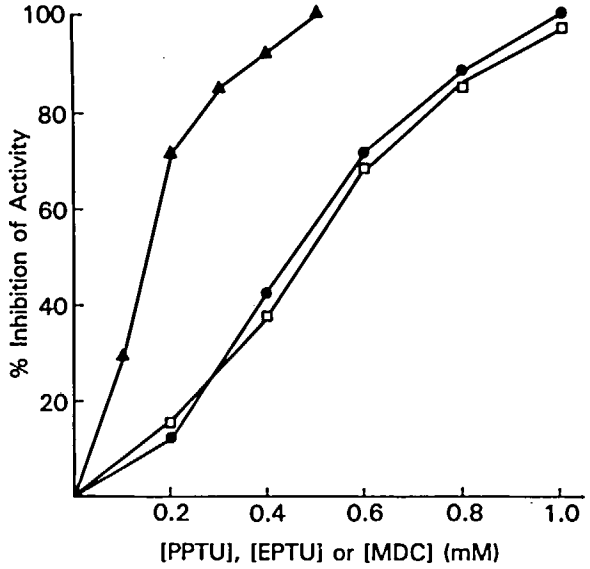

Fig. 7. Effects of PPTU (●), EPTU (□) and monodansylcadaverine (MDC) (▲) on the activity of calmodulin-activated cyclic nucleotide phosphodiesterase.

Discussion

Using competitive amine inhibitors with radioactive tracer to study transglutaminases in cells and body fluids can, in theory, provide three kinds

of information: one can (a) prove the presence of a catalytically functioning enzyme; (b) identify and characterize substrate proteins with transglutaminase sensitive glutamine residues; (c) achieve inhibition of a presumed transglutaminase-dependent biological phenomenon. This approach was successfully utilized in several instances, such as studies of blood coagulation factor XIII [3,22] or identification of involucrin, a major substrate of epidermal transglutaminase [4,23]. However, such studies have limitations, mostly arising from the nature of the amines available and experiments involving intact cells. Several amine substrates of transglutaminase, like polyamines or histamine, are biologically important molecules with specific functions and metabolic pathways inside cells. While the possibility of transglutaminase-catalyzed incorporation of these amines into proteins of regulatory significance at physiological amine concentration is still an open question [24,25], their application in concentrations sufficiently high to inhibit cellular transglutaminase is not conceivable. Other amine substrates like hydroxylamine, methylamine, ethylamine, etc., are either toxic to the cells or exert several effects unrelated to transglutaminase inhibition (increase of lysosomal pH, selective release of lysosomal enzymes, slowing of receptor recycling). Dansylcadaverine, the most potent competitive amine substrate inhibitor of transglutaminases [26] accumulates in the cell membrane, not even entering into cells in significant quantities [27]. Using the above compounds in studies aimed at supporting the notion that cellular transglutaminase plays an important role in receptor-mediated endocytosis has resulted in controversial findings not confirmed in subsequent experiments [28–30]. We used a new compound, PPTU, which is almost as potent as dansylcadaverine to inhibit the transglutaminase-catalyzed reaction [7] and enters the cells and becomes distributed in the main cell compartments (Fig. 1, Table I), and can be studied in a controlled manner by comparing its effect to that elicited by its close analogue, EPTU. EPTU has shown properties very similar to PPTU in cell uptake studies, in its distribution in subcellular compartments, and in its inhibition of the activity of calmodulin-dependent phosphodiesterase. However, it was not incorporated into cellular proteins when either cell extracts or intact CHO cells were studied.

The incorporation of PPTU into proteins of intact CHO cells clearly shows that cellular transglutaminase is catalytically active in these cells. Previous studies did not provide conclusive information on this aspect. It was reported that protein-bound ε(γ-glutamyl)lysine, the product of transglutaminase action, is present in a wide variety of cells and tissues [31]. However, it is now known that protein bound ε(γ-glutamyl)lysine as well as γ-glutamyl amines are formed non-enzymatically through highly reactive intramolecular γ-thioesters of certain proteins [32,33], a possibility which could not be addressed in previous studies. The formation of protein-bound γ-glutamyl polyamines in lectin-stimulated lymphocytes [24] or γ-glutamylhistamine during degranulation of mast cells stimulated immunologically [25] has also been reported. However, these products have not been observed in cells not stimulated by mitogen or antigens. In addition, the presence of a certain percentage of dead cells in the lymphocyte culture or the drastic change in the cell structure, during degranulation of mast cells, make these observations quite different from those reported here. In our studies, CHO cells were grown under regular cell culture conditions, and cell viability was practically 100% (dead cells are removed by extensive washing of the cell layer). There are several arguments against the possibility that the observed incorporation of PPTU into CHO cell proteins is a result of the reaction of PPTU with proteins containing thioester bonds either in CHO cells or in the media (in the latter case, the PPTU-containing proteins might have been taken up by the cells subsequent to their formation). First, EPTU is just as capable for this type of chemical reaction as PPTU. However, formation of EPTU-protein conjugates have not been observed under any circumstances. Second, PPTU-protein conjugates were not found when CHO cell homogenates and PPTU were incubated in the absence of Ca^{2+} (incorporation of amines into proteins through thioester is not Ca^{2+} dependent). Third, none of the known thioester containing proteins have a molecular weight similar to the high-molecular-weight polymer which was found to be labeled by [^{14}C]PPTU in CHO cells.

In all of our approaches to identify the protein substrate(s) of CHO cell transglutaminase by means of incorporation of [^{14}C]PPTU, only the high-molecular-weight protein polymers, which do not enter the stacking gel or the separating gel during electrophoresis in the presence of SDS, were labeled. On adding Ca^{2+} to cell homogenate in the presence of PPTU and observing the time course of events, a time-dependent formation of these polymers was found and, coordinately, the incorporation of PPTU into these polymers. Increasing the concentration of PPTU could decrease but not prevent the formation of the high-molecular-weight material in cell extracts, probably because of its interaction with some particulate structures or of its competition with ϵ-amino groups of protein-bound lysine residues, thereby resulting in a lower PPTU concentration available for transglutaminase action. Using only the cytosol fraction of cell homogenates, the inhibition of polymer formation could be accomplished in a concentration-dependent manner (partial inhibition at 0.4 mM, complete inhibition at 4 mM concentration), similar to our data obtained when the inhibition of fibrin polymerization by Factor XIIIa was studied in a purified system [7]. In intact cells, PPTU was also incorporated into a high-molecular-weight polymer which was smaller than that observed in cell homogenates. The formation of this high-molecular-weight polymers in intact cells could not be completely prevented even with high concentration of PPTU; there was always label present in the high-molecular-weight polymer. The possible explanation of this finding might be that the free PPTU concentration available for transglutaminase does not correspond to the actual intracellular concentration (which is a phenomena similar to that observed with total cell homogenate in our in vitro experiments with cell extracts also existing in living cells); protein polymerization is preferable to amine incorporation under intracellular conditions because of the structural organization of participating proteins. It has been suggested recently [34] that the function of transglutaminase in cells is to form a covalently cross-linked matrix which may be the basis of a so called super-thin (2–3 nm) filament structure. The formation of such a matrix may proceed continuously in cells in such a way that even high concentrations of amine substrates like PPTU can only partially interfere with it. When cells are homogenized and a cytosol fraction is prepared, the transglutaminase-catalyzed polymer formation can be prevented. Under these circumstances, it was possible to attempt the identification of the precursor protein(s) of the polymer by using labeled PPTU.

Unfortunately, we were not able to detect these proteins, possibly because there are more than one or two such proteins and the specific activity of PPTU was not high enough to make the detection of protein-bound PPTU (presumably distributed among several proteins) possible. It has been reported recently that in keratinocyte there are at least six proteins involved in the transglutaminase-catalyzed formation of high-molecular-weight polymers when the cells are permeabilized for Ca^{2+} [35].

The possibility that the so far unrecognized function of cellular transglutaminase is coupled to the regulation of cell proliferation has been raised from several aspects (for a review see Ref. 36). Based on a series of studies with human fibroblasts in culture, it has been suggested that low transglutaminase activity and cross-linking level make cell cycling possible and that resting cells are in a highly cross-linked state with a high level of transglutaminase activity [37]. On the other hand, transglutaminases have been also implicated in growth stimulatory pathways: increased transglutaminase activity was found in lymphocytes stimulated by mitogens [38], a transient increase of transglutaminase activity and quantity was observed in resting fibroblasts following their stimulation by platelet-derived growth factor (Fesus, L. and Grotendorst, G.R., unpublished data), and in regenerating liver and mitogen-stimulated lymphocytes an increased portion of the enzyme was found to be associated with the cell nuclei [39]. It has been also reported that the activation of transglutaminase occurs during the cell cycle in CHO cells, having high activity during the G_2 phase and decreasing after its release from mitosis [40]. Our present results do not support the idea of the direct involvement of transglutaminase in either a negative or positive growth control mechanism. The presence of PPTU at concentrations of up to 1 mM (a concentration sufficient to inhibit

transglutaminase-catalyzed reactions) in the culture media and inside the cells has not altered the rate of cell proliferation. At higher concentrations of PPTU, a concentration-dependent inhibition was observed. However, cell proliferation was inhibited by EPTU as well as showing the same pattern of concentration dependence as PPTU. Since the pattern of the inhibition of calmodulin-dependent phosphodiesterase activity by PPTU and EPTU is also very similar, and the importance of calmodulin-dependent reactions in the regulation of cell proliferation is well recognized, it can be assumed that the decrease in the rate of cell proliferation at PPTU and EPTU concentrations higher than 1 mM may be the consequence of the inhibition of calmodulin-dependent reactions inside the cells and may not be related to interference with the function of cellular transglutaminase. It has also been reported that effects of retinoic acid on transglutaminase activity have no correlation with the cell growth of neoplastic human keratinocyte lines [41]. We obtained similar results when studying the effect of these two amines on several other cell lines in culture including malignant ones. Interestingly, when malignant cells were studied as solid tumors growing in mice (Lewis lung carcinoma, B16 melanoma), the systemic administration of PPTU resulted in a significant reduction of the growth rate with a concomitant increase of the median survival time and a decrease rate of metastasis [36]. EPTU, at the same dose and blood level as PPTU, did not show these effects, suggesting the existence of a transglutaminase function present under pathological conditions. The nature of this transglutaminase-related phenomenon, which could be inhibited by PPTU and seems to be operational in vivo (at least during the in vivo growth of certain tumor cells), but not under cell culture condition, is not known. It may involve other types of transglutaminase-like blood coagulation factor XIII [42] or may be related to changes of the transglutaminase level during tumor growth [36,43].

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