Inhibition of rat liver transglutaminase by nucleotides

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Received 30 August 1990; accepted 10 January 1991

Summary. Tissue-type transglutaminase (TGase) was purified from rat liver, and the effects of nucleotides on its activity were examined. The enzyme activity is inhibited by ATP in a concentration-dependent way, with complete inhibition by 3 mM ATP. Partially-purified TGase from human brain was inhibited by ATP in a manner similar to that observed with the rat liver enzyme. This suggests that the inhibition is a common phenomenon for tissue-type TGase in all species and tissues. The inhibition is reversible since full activity is restored by lowering the ATP concentration. CTP has a TGase-inhibitory potency equivalent to that of ATP, whereas GTP and UTP possess about 50% of the inhibitory activity of ATP. ADP inhibits TGase activity to the same extent as ATP, but AMP causes much less inhibition, and there is no inhibition by adenosine or adenine. The inhibition by ATP is insensitive to ionic strength and is non-competitive with the substrate putrescine. Since ATP levels in cells are of mM order, these results suggest that TGase activity is controlled by ATP in vivo.

Key words. Transglutaminase; nucleotide.

Transglutaminases (TGase; protein-glutamine γ-glutamyl-transferase, EC 2.3.2.13) are calcium-dependent thiol enzymes that catalyze the formation of γ-glutamyl-ε-lysine isopeptides between the side chains of glutamine and lysine residues in proteins, resulting in protein cross-linking[1]. They play important roles in a number of biological systems. Well-characterized cases include fibrin stabilization by plasma factor XIIIa[2], cross-link formation by hair-follicle TGase[3], formation of insoluble, cross-linked keratinocyte envelopes by epidermal TGase[4,5], and vaginal plug formation by prostate TGase[6]. Besides these specialized TGases, a TGase called tissue-type TGase exists ubiquitously in a variety of tissues[1]. The exact function of tissue-type TGase has not been clarified yet, but it has been postulated that it is involved in receptor-dependent endocytosis[7,8], membrane protein cross-linking[9,10], cell growth and differentiation[11,12].

TGase activity is controlled by the Ca^{2+} concentration within the cell, and it is also possible that various kinds of amines modulate the activity by acting as competitive inhibitors. In a search for other substances involved in the regulation of TGase activity, I examined the effects of nucleotides on the activity of TGase purified from rat liver. This was done because previous workers have shown that the formation of γ-glutamyl-ε-lysine cross-links in extracts of tissue-cultured embryonic chick heart myofibrils is accelerated by the addition of ATP[13,14], whereas the incorporation of amines into platelet and muscle actin catalyzed by factor XIIIa, an extracellular TGase, is inhibited by ATP, ADP, GTP, and CTP[15].

Materials and methods

Materials. Resins for column chromatography were obtained as follows: DE-52 from Whatman, Ultrogel AcA 34 from LKB, hydroxyapatite from Daiichi Kagaku, and DEAE-Sephacel from Pharmacia. Other chemicals were purchased as follows: [1,4-^{14}C]putrescine dihydrochloride (118 mCi/mmol) from Amersham, *N,N*-dimethylcasein from Calbiochem, and ATP, ADP, AMP, GTP, CTP, UTP, adenosine, and adenine from Sigma.

Purification of rat liver transglutaminase. Wistar rat livers (30 g) were homogenized in 3 vol. (v/w) of 20 mM Tris-HCl (pH 7.5)-5 mM EDTA (buffer A) in a Waring blender at 0 °C and centrifuged at 15,000 × g for 20 min. The supernatant was loaded onto a DE-52 column (2.0 × 60 cm) and the proteins were eluted by increasing the NaCl concentration first to 0.15 M and then to 0.40 M. TGase, which eluted at 0.40 M NaCl, was precipitated by the addition of solid ammonium sulfate to 70% saturation at 4 °C. The precipitate was collected by centrifugation, dissolved in 40 ml of 0.1 M NaCl-buffer A, and loaded onto an Ultrogel AcA 34 column (5 × 130 cm) pre-equilibrated with the same buffer. Active fractions were collected, loaded onto a hydroxyapatite column (1.5 × 8 cm) and developed with a linear gradient of sodium phosphate buffer (pH 7.0) from 0.0 M to 0.4 M. TGase, which eluted at 0.25 M phosphate, was then purified on a DEAE-Sephacel column (1.0 × 27 cm), by eluting it with a linear gradient of NaCl from 0.15 M to 0.40 M in buffer A. The purified rat liver TGase gave a single band on SDS-polyacrylamide gel electrophoresis[16], with an apparent molecular weight of 80,000.

Determination of TGase activity. The procedure was essentially that of Lorand et al.[17]. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 24 mM 2-mercaptoethanol, 1.1 mg/ml dimethylcasein, and 0.12 mM [^{14}C]-putrescine in a total volume of 100 µl. The reaction was started by the addition of enzyme and allowed to proceed for 60 min at 37 °C. An 80-µl aliquot was then withdrawn, spotted onto a small piece of filter paper, and immersed in cold 10% TCA.

The filters were washed successively with 5% TCA, ethanol/acetone (50:50, v/v), and acetone, and then dried. The radioactivity was determined in toluene scintillator.

Results

When ATP was included in the activity assay mixture, TGase activity was inhibited in a manner dependent on ATP concentration, with more than 90% inhibition at 2.0 mM ATP (fig. 1). CTP had a similar inhibitory potency, but with UTP and GTP much higher concentrations were required for inhibition. Thus, although all these nucleotide triphosphates are potent inhibitors of TGase, there is some base-dependency. The inhibition of TGase activity by ATP is not a phenomenon restricted to the rat liver enzyme, since partially purified TGase from human brain was also inhibited by ATP (fig. 2).

The inhibition by ATP is reversible. When TGase was incubated with 2.6 mM ATP in the presence of $CaCl_2$ at 37 °C for 1 h and then added to an ATP-free assay mixture (final ATP concentration, 0.125 mM), about 90% of the initial activity was detected. This result suggests that covalent modifications of TGase by ATP, for example phosphorylation, are not involved in the inhibition process.

Next, a series of adenine derivatives were assessed for their inhibitory activities against TGase. TGase activity was inhibited by ADP to the same extent as by ATP, but much less by AMP, and not at all by adenosine or adenine (fig. 3). This indicates that the phosphate groups are critically important for the inhibitory activity of ATP, ADP, and AMP.

Since the phosphate groups of ATP are necessary for its inhibitory activity, and putresecine, the TGase substrate used here, is a very basic compound, it is possible that the TGase activity was inhibited through substrate depletion caused by complex formation between putrescine and ATP. To exclude this possibility, inhibition was tested in the presence of high concentrations of salt. If inhibition arises from ionic interactions between putrescine and

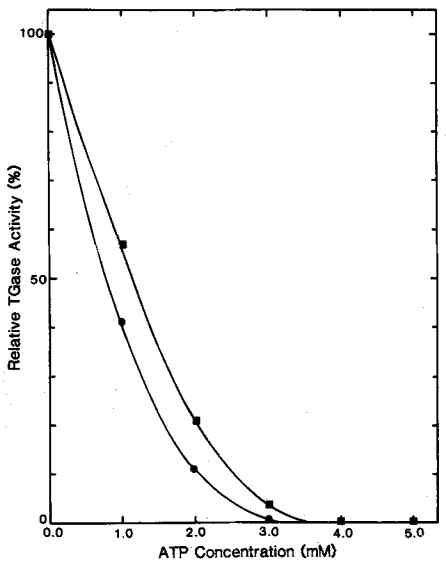

Figure 2. Inhibition of partially purified human brain TGase by ATP. Purified rat liver TGase (125 ng, -●—●-) and the same amount of activity of partially purified human brain TGase (-■—■-) were assayed in the presence of various concentrations of ATP. The activities in the absence of ATP, which catalyzed incorporation of 9000 dpm of ^{14}C-putrescine, were taken as 100%.

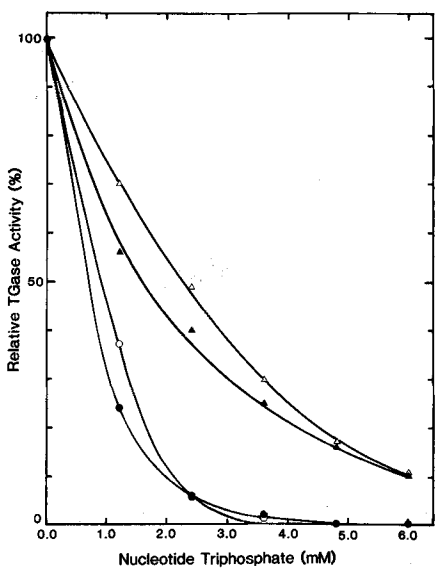

Figure 1. Inhibition of rat liver TGase by nucleotide triphosphates. Purified rat liver TGase (125 ng) was assayed for activity in the presence of various concentrations of ATP (-●—●-), CTP (-○—○-), UTP (-▲—▲-), and GTP (-△—△-). The activity in the absence of nucleotide triphosphate, which catalyzed incorporation of 9000 dpm ^{14}C-putrescine, was taken as 100%.

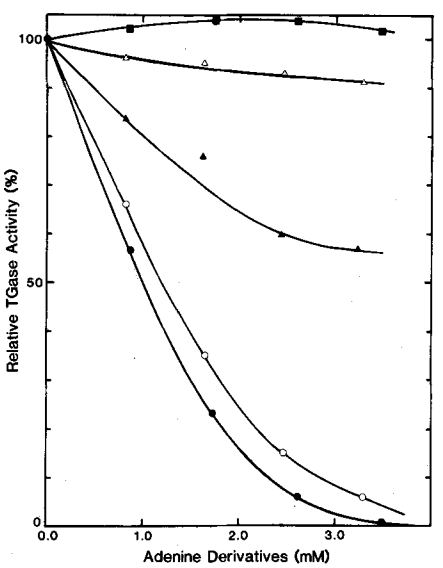

Figure 3. Inhibition of rat liver TGase by adenine derivatives. Purified rat liver TGase (125 ng) was assayed for activity in the presence of various concentrations of ATP (-●—●-), ADP (-○—○-), AMP (-▲—▲-), adenosine (-△—△-), and adenine (-■—■-). The activity in the absence of adenine derivatives, which catalyzed incorporation of 9000 dpm ^{14}C-putrescine, was taken as 100%.

ATP, these should be dissociated under high ionic strength conditions and no TGase inhibition should occur. As shown in figure 4, TGase activity itself was partially inhibited under high salt concentrations, but the ATP-induced inhibition was never reversed.

Finally, the mode of inhibition was examined (fig. 5). ATP did not change the K_m value for putrescine (K_m = 26 μM); only V_{max} changed according to ATP concentration. Thus ATP has no effect on the putrescine binding site of TGase. Instead, it seems likely that ATP binds to an allosteric site on the TGase molecule.

Discussion

The effect of ATP on TGase was first reported by Brenner and Wold[18]. They found that human erythrocyte TGase activity was stabilized when ATP was included during purification of the enzyme. It is a rather common phenomenon that an inhibitor acts as a stabilizer, so their result is not inconsistent with my finding that ATP inhibits TGase activity. A similar result was obtained by Cohen et al.[15] who showed that the cross-linking of actin by factor XIIIa is inhibited by ATP, ADP, GTP, and CTP, but not by AMP or cAMP. This specificity is almost the same as that for tissue-type TGase as described above. Although the molecular properties of factor XIIIa and tissue-type TGase are different in some respects, it seems likely that they share common features in their sensitivity to nucleotides. On the other hand, Loewy and Matacic, using slime molds and cultured chick embryos, demonstrated that ATP-Mg^{2+} acts in different ways, depending on the presence of Ca^{2+}; cross-linking was decreased by ATP-Mg^{2+} in the absence of Ca^{2+}, but increased in the presence of Ca^{2+}[13,14]. Their hypothesis that ATP-Mg^{2+} has dual functions depending on the presence or absence of Ca^{2+}, i.e. the activation of glutamic acid side chains to acyl phosphates and the cleavage of cross-links by phosphorylation, is very attractive. But these reactions require other enzymes and co-factors and might not be due directly to the action of ATP on TGase. More recently it was reported that GTP inhibits erythrocyte membrane TGase[19] and guinea pig liver TGase[20,21] activities. These authors claimed that the inhibition is specific for GTP, GDP, and GMP, and that ATP, CTP, and UTP are ineffective even at concentrations of 0.5 mM. This is in conflict with my results, which show that the inhibition is only slightly affected by the base of the nucleotide triphosphates. One possible explanation for this descrepancy is a difference in the Ca^{2+} concentrations used in the experiments, because the inhibitory effect of GTP was found to diminish with increasing Ca^{2+} concentrations.

TGase is postulated to be involved in cell proliferation since non-mitotic cells contain more cross-linked products and higher TGase activities than proliferating cells, and inhibitors of TGase activity, such as primary amines, inhibit cell proliferation[21-24]. But most enzymes are provided with control mechanisms other than enzyme concentration alone. In the case of TGase, Ca^{2+} is an

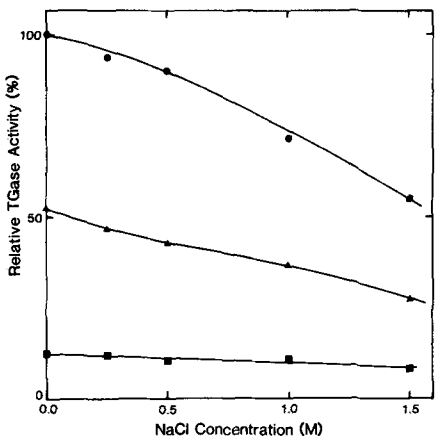

Figure 4. Effect of NaCl on the inhibition of rat liver TGase by ATP. Purified rat liver TGase (125 ng) was assayed for activity in the presence of various concentrations of ATP and/or NaCl: -●—●-, 0.0 mM ATP; -▲—▲-, 1.0 mM ATP; -■—■-, 2.0 mM ATP. The activity in the absence of ATP and NaCl, which catalyzed incorporation of 9000 dpm ^{14}C-putrescine, was taken as 100%.

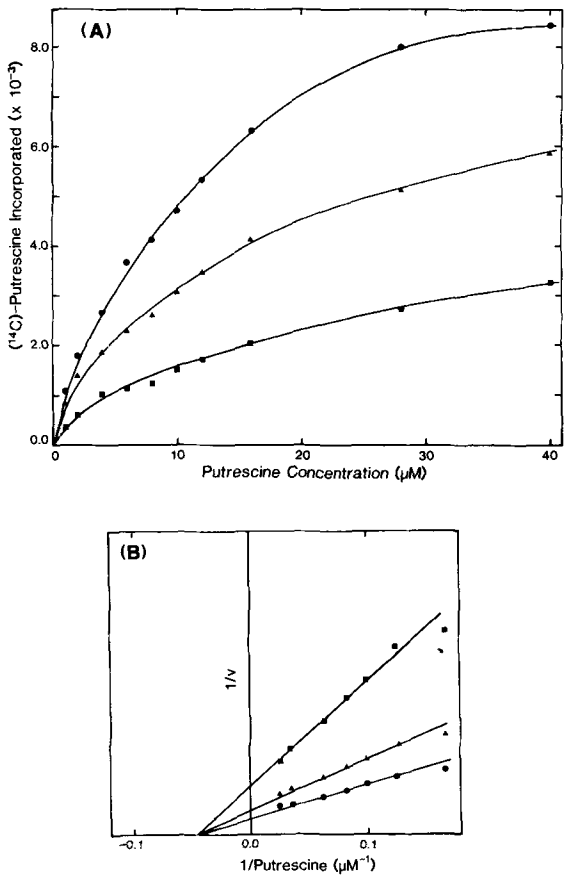

Figure 5. Putrescine-dependency of rat liver TGase activity in the presence of ATP. Purified rat liver TGase (125 ng) was assayed for activity in the absence (-●—●-) or the presence of 0.75 mM ATP (-▲—▲-) or 1.50 mM ATP (-■—■-), at putrescine concentrations from 0 μM to 40 μM. *A* Incorporation of putrescine into methylcasein. *B* Double reciprocal plot.

activator. Therefore, there should be inhibitory factors to control over-expression of TGase activity. ATP is a candidate for such a function, since it inhibits TGase activity at physiological concentrations. Moreover, intracellular ATP concentration is related to the physiological state of cells.

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0014-4754/91/070709-04$1.50 + 0.20/0

Changes in olfactory perception during the menstrual cycle

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Received 10 December 1990; accepted 9 January 1991

Summary. The aim of the study was to find correlations between changes in olfactory sensitivity and the menstrual cycle. 14 young, healthy volunteers participated in the experiments. Subjects menstruated regularly and did not use oral contraceptives. Three odorants were investigated: phenylethyl alcohol, androstenone, and nicotine. Dilution series of the odorants were prepared, and presented to the subjects in order to determine the detection thresholds (triple forced choice). Additionally, the subjects' hedonic estimates of the odorants were measured, and mood states as well as hormonal levels of LH and estrogen were determined. Before the actual experiments started, subjects participated in three training sessions.

One experiment was subdivided into 5 phases (two pre- and two postovulatory phases; one ovulatory phase). Only with regard to androstenone did trend analyses reveal a significant quadratic relationship between hedonic estimates and phases of the menstrual cycle, peaking at ovulation. Olfactory sensitivity was not significantly influenced by the menstrual cycle.

Key words. Menstrual cycle; olfaction; threshold; hormone; hedonic estimates; mood; intensity estimates.

Changes in olfactory sensitivity in the course of the menstrual cycle have been reported in several studies[1–3]. It was observed that the highest degree of olfactory sensitivity coincided with ovulation. These findings, however, were not received without dissent. Amoore et al.[4], Kloek[5] and Henkin (cited from Vierling and Rock[3]) did not find changes in olfactory sensitivity during the menstrual cycle. The reason for this discrepancy might be the fact that these groups did not determine the serum levels of hormones, the establishing of which would have fixed the exact time of ovulation. Moreover, of all these teams only Vierling and Rock[3] and Amoore et al.[4] submitted their data to statistical analyses.

Recently, Doty et al.[6] were able to confirm the findings of Le Magnen[1]. Within the scope of a signal-detection-paradigm, they statistically determined two peaks of ol-