Trypsin-induced increase in cyclic AMP concentration in rat thymocytes

An effect independent of calcium and calmodulin

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Trypsin produces a dose-related increase in cellular cyclic AMP concentration in rat thymocytes [Shneyour, Patt & Trainin (1976) *J. Immunol.* **117**, 2143–2149; Segal & Ingbar (1983) *Clin. Res.* **31**, 277A]. In the present study, I examined whether this effect of trypsin requires Ca^{2+} and whether it is modified by calmodulin. In fresh thymocytes suspended in standard medium (containing 1 mM- Ca^{2+}), trypsin produced a concentration-dependent increase in cytoplasmic free Ca^{2+} concentration, which was evident at a concentration of 50 μg of trypsin/ml and reached maximal values at about 1 mg/ml. This effect of trypsin was very prompt in onset, almost immediate, and reached maximal values within 2–3 min. But in cells suspended in essentially Ca^{2+} -free medium (6 nM free Ca^{2+}), trypsin had no effect on cytoplasmic free Ca^{2+} concentration, which indicates that trypsin acted by increasing Ca^{2+} uptake rather than Ca^{2+} release from an intracellular pool. However, the increase in thymocyte cyclic AMP concentration produced by trypsin was independent of extracellular Ca^{2+} and was not influenced by calmodulin, because it was the same in the presence or absence of Ca^{2+} and was not changed by the calmodulin inhibitor trifluoperazine. I therefore suggest that in rat thymocytes the trypsin-induced increase in cyclic AMP concentration does not require Ca^{2+} and is not influenced by calmodulin.

INTRODUCTION

Trypsin increases cellular cyclic AMP concentration in rat thymocytes (Segal & Ingbar, 1983; Shneyour *et al.*, 1976). However, the mechanism through which trypsin exerts this action is not known. It has been suggested that trypsin acts to increase adenylate cyclase activity (Friedman *et al.*, 1983; Guiraud-Simpson & Colobert, 1977; Johnson *et al.*, 1985; Richter *et al.*, 1978; Ryan *et al.*, 1975), which could result from the inactivation, by tryptic hydrolysis, of a plasma-membrane inhibitor of adenylate cyclase (Johnson *et al.*, 1985; Pinkett & Anderson, 1980; Stiles & Lefkowitz, 1982). But the possibility that trypsin increases cellular cyclic AMP concentration by inhibiting phosphodiesterase activity has also been suggested (Russell & Pastan, 1973). Calmodulin, a Ca^{2+} -dependent regulatory protein (Cheung, 1980; Means *et al.*, 1982), has been shown to stimulate adenylate cyclase activity in various tissues (Amiranoff *et al.*, 1983; Brostrom *et al.*, 1976, 1978; Cheung *et al.*, 1978; Hyne & Garbers, 1979; LeDonne & Coffee, 1980; Ofulue & Nijjar, 1981; Pinkus *et al.*, 1983; Sharp *et al.*, 1980), including rat thymocytes (Segal *et al.*, 1986) and trypsin has been demonstrated to influence Ca^{2+} metabolism in various tissues (Rossi & Schatzmann, 1982; Sorensen *et al.*, 1980; St. Louis & Sulakhe, 1978) and to increase Ca^{2+} uptake and cytoplasmic free Ca^{2+} concentration in human lymphocytes and L_8 muscle cells (Klip *et al.*, 1984; Parker, 1974). Therefore it is conceivable that the trypsin-induced increase in cellular cyclic AMP concentration could be influenced by Ca^{2+} and calmodulin. To test this possibility, I have examined,

in freshly isolated rat thymocytes, the effect of trypsin on Ca^{2+} metabolism and the possible involvement of Ca^{2+} and calmodulin in its stimulatory effect on cyclic AMP concentration. In the rat thymocyte, trypsin increased free Ca^{2+} concentration, but its stimulatory effect on cyclic AMP concentration did not require extracellular Ca^{2+} and was independent of calmodulin.

EXPERIMENTAL

Animals used were 27-day-old female rats of the Sprague–Dawley strain [CrI:CD(SD)BR; Charles River Laboratories, Wilmington, MA, U.S.A.]. Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW publication no. (NIH) 78–23, revised 1978]. Thymocytes were isolated by a procedure previously described (Segal & Ingbar, 1979). Unless stated otherwise, freshly isolated thymocytes were suspended in a Krebs–Ringer–Tris buffer, pH 7.4 (standard buffer), consisting of 20 mM-Tris/HCl, 5 mM-Tris base, 120 mM-NaCl, 5 mM-KCl, 1 mM- CaCl_2 , 2.5 mM- MgCl_2 , 1.5 mM- NaH_2PO_4 and 15 mM-Hepes.

Measurement of cytoplasmic free Ca^{2+} concentration

Thymocyte cytoplasmic free Ca^{2+} concentration was measured by the quin 2 technique (Tsien *et al.*, 1982). Thymocytes were suspended in the standard medium

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lacking Hepes. In some experiments, 5 mM-EGTA was added to the medium, decreasing free Ca^{2+} concentration there to 6 nM. Samples (2 ml; 10^8 cells/ml) were transferred into plastic tubes and were incubated with 50 μM -quin 2/AM (esterified quin 2; Calbiochem-Behring, San Diego, CA, U.S.A.) at 37 °C in the dark for 20 min. Then the extracellular indicator was removed by centrifugation and washing, and cells were resuspended with the buffer and kept at room temperature. Apparently, quin 2 leaks out from the quin 2-loaded thymocytes at a rate of about 9% and 12% at room temperature and at 37 °C respectively. Before measurements, a sample of cells (4×10^7 /ml) was centrifuged at 10000 g for 4 s, the supernatant was aspirated and the cells were resuspended with 2 ml of warm (37 °C) buffer and transferred into a cuvette placed in a spectrofluorimeter. During the entire measurement period, cells were kept at 37 °C with continuous mixing. Quin 2 fluorescence was measured with the SF-1 Baird spectrofluorimeter with the following settings: excitation, at 339 nm with a 1.5 nm slit; emission, at 492 nm with a 6 nm slit. After 3 min of equilibration, trypsin (211 units/mg; Worthington, Freehold, NJ, U.S.A.) at various concentrations was added to cells, and the change in the fluorescence intensity was monitored continuously. Cytoplasmic free Ca^{2+} concentration was calculated by a procedure described by Tsien *et al.* (1982); in thymocytes, it was thus found to be 131 nM.

Measurement of ^{45}Ca accumulation

Cells were suspended in the standard buffer, and samples (3 ml; 45×10^6 cells/ml) were transferred to 25 ml Erlenmeyer flasks. Cells were allowed to equilibrate in a shaking water bath for 30 min at 37 °C. Then trypsin in various concentrations was added, and after various periods of time 3 μCi of ^{45}Ca (sp. radioactivity 15.7 mCi/mg; New England Nuclear, Boston, MA, U.S.A.)/ml was added, and 1 min later the reaction was stopped and ^{45}Ca accumulation in the cells was measured as previously described (Segal & Ingbar, 1984).

Measurement of cellular cyclic AMP concentration

Thymocytes (3 ml; 45×10^6 cells/ml) were suspended in the standard medium and transferred into 25 ml Erlenmeyer flasks. In some experiments, cells were suspended in Ca^{2+} -free buffer in the presence or absence of 0.1 mM-EGTA. [Ca^{2+} -free medium was the same as the standard medium, but without CaCl_2 ; this medium contains 5 μM - Ca^{2+} , measured by flame spectrophotometry (Perkin-Elmer spectrophotometer model 303), owing to calcium contaminant in the buffer salts. Addition of 0.1 mM-EGTA to the Ca^{2+} -free buffer decreased free Ca^{2+} concentration to approx. 1 nM.] Cells were preincubated with or without trifluoperazine (Sigma, St. Louis, MO, U.S.A.) in various concentrations for 20 min at 37 °C. Then trypsin in various concentrations was added and 2 min later the incubation was stopped and cellular cyclic AMP concentration was measured as previously described (Segal & Ingbar, 1981).

Measurement of adenylate cyclase activity

Adenylate cyclase activity was measured in freshly isolated plasma-membrane preparations as previously described (Segal *et al.*, 1985). Membranes were allowed to equilibrate in the absence or presence of various

concentrations of trifluoperazine for 10 min at 37 °C. The reaction started on addition of ATP and 5 min later was stopped with ice-cold trichloroacetic acid (final concn. 6%, v/v) and the cyclic AMP produced was measured.

Cell viability

Cell viability was measured by the Trypan Blue technique (Segal & Ingbar, 1979), in which a viable cell is one which excludes the dye. The viability of thymocytes suspended in the standard buffer (containing 1 mM- Ca^{2+}) was not changed by quin 2 (and quin 2/AM), by trypsin at concentrations of 5 mg/ml and less, or by trifluoperazine at a concentration of 200 μM or less. However, cells suspended in essentially Ca^{2+} -free medium [standard medium containing 5 mM-EGTA (6 nM free Ca^{2+}), or Ca^{2+} -free medium (5 μM - Ca^{2+}) alone or with 0.1 mM-EGTA (1 nM free Ca^{2+})] were much more sensitive to lysis by trypsin; cell viability decreased progressively with trypsin concentrations of 25 μM and higher. Quin 2-loaded cells suspended in standard medium containing 5 mM-EGTA were more resistant to trypsin, so that a decrease in their viability became evident only at trypsin concentrations of 200 μM and higher. The effect of trypsin together with trifluoperazine on the viability of thymocytes suspended in the standard medium is shown in Table 1.

Statistical analysis

Where appropriate, statistical analysis of the differences between experimental groups and a single control was performed by the Dunnett's test (Zar, 1974).

RESULTS

Ca^{2+} metabolism

(a) **Cytoplasmic free Ca^{2+} concentration.** The effect of trypsin on thymocyte cytoplasmic free Ca^{2+} concentration was measured by the quin 2 technique as described in the Experimental section. Trypsin in various concentrations was added to the quin 2-loaded cells (suspended in standard medium, containing 1 mM- Ca^{2+}) and cytoplasmic free Ca^{2+} concentration was measured. Trypsin produced a very rapid, almost immediate, increase in cytoplasmic free Ca^{2+} concentration, reaching maximal values within 2–3 min. This effect of trypsin was dose-related, being evident at a concentration of 50 μg of trypsin/ml and reaching maximal values at about 1 mg/ml (Fig. 1). But trypsin had no effect on quin 2-loaded cells suspended in standard medium containing 5 mM-EGTA (6 nM free Ca^{2+}) (Fig. 1). In the latter medium, trypsin, at concentrations of 25 μg /ml and higher, produced a progressive decline in cell viability.

(b) **^{45}Ca accumulation.** Thymocytes suspended in standard medium were incubated with various concentrations of trypsin for various periods of time and their 1 min accumulation of ^{45}Ca was then measured. In all the incubation periods studied, trypsin produced a marked concentration-related decrease in the accumulation of ^{45}Ca in the cells (Fig. 2). This most probably resulted from the tryptic hydrolysis of Ca^{2+} -binding proteins on the outer surface of the plasma membrane (Kawaguchi & Konishi, 1980), which, collectively, are said to have a large binding capacity for Ca^{2+} (McDonald & Jarett,

Fig. 1. Effect of trypsin on cytoplasmic free Ca^{2+} concentration in rat thymocytes

Quin 2-loaded thymocytes were suspended in standard medium (1 mM- Ca^{2+} ; ●) or in standard medium containing 5 mM-EGTA (6 nM free Ca^{2+} ; ○). Trypsin in various concentrations was added to cells and its effect on their cytoplasmic free Ca^{2+} concentration was measured. Results shown are means $\pm$ S.D. from three separate experiments: * and ** significantly different from values for trypsin-free thymocytes suspended in standard medium ($P < 0.05$ and $P < 0.01$ respectively).

1980; McDonald *et al.*, 1976; Mikkelsen & Freerksen, 1980; Porzig & Stoffel, 1978; Reinhart *et al.*, 1984; Tolone *et al.*, 1980; Yamagami & Terayama, 1979).

Cyclic AMP concentration

Trypsin in various concentrations was incubated with thymocytes for 2 min, and its effect on cellular cyclic AMP concentration was measured. In cells suspended in standard medium, trypsin produced a dose-related increase in cyclic AMP, evident at 10 $\mu\text{g}/\text{ml}$ (+48.9% above control) (Fig. 3) and reaching maximal values at about 1 mg/ml (+310% above control; results not shown). As indicated in the Experimental section, cells suspended in Ca^{2+} -free medium, in the presence or absence of 0.1 mM-EGTA, or in standard medium containing 5 mM-EGTA (6 nM free Ca^{2+}), were much more sensitive to tryptic hydrolysis than were cells suspended in standard medium, and suffered progressive decrease in viability at trypsin concentrations of 25 $\mu\text{g}/\text{ml}$ and higher. But in these cells, trypsin at concentrations of 5–20 $\mu\text{g}/\text{ml}$ produced a dose-related increase in cyclic AMP concentration, similar to that in cells suspended in the standard medium (Fig. 3).

Calmodulin

We have previously demonstrated in rat thymocyte plasma membranes that adenylate cyclase activity is modified by calmodulin (Segal *et al.*, 1986). In these

Fig. 2. Effect of trypsin on accumulation of ^{45}Ca in rat thymocytes

Thymocytes, suspended in standard medium, were incubated with various concentrations of trypsin for various periods of time: ●, 1 min; ○, 5 min; ▲, 15 min. Then 3 μCi of $^{45}\text{Ca}/\text{ml}$ was added and its 1 min accumulation by the cells was measured. Results shown are the means from two separate experiments.

Fig. 3. Effect of trypsin on cyclic AMP concentration in rat thymocytes

Thymocytes were suspended in standard medium (1 mM- Ca^{2+} ; ●), Ca^{2+} -free medium (5 μM - Ca^{2+} ; ▲), or Ca^{2+} -free medium containing 0.1 mM-EGTA (1 nM- Ca^{2+} ; △). Trypsin in various concentrations was added to cells, and 2 min later the reaction was stopped and cellular cyclic AMP concentration was measured. Results shown are means (S.D. values were 6–21% of the corresponding means) of those obtained in three separate experiments: * significantly greater than the corresponding trypsin-free group ($P < 0.01$).

Fig. 4. Effect of trifluoperazine on adenylate cyclase activity and cellular cyclic AMP concentration in rat thymocytes

The effect of various concentrations of trifluoperazine on adenylate cyclase activity (plasma membranes) or cellular cyclic AMP concentration (45×10^6 cells/ml suspended in standard medium) was measured as described in the Experimental section. Values shown are the means obtained from two separate experiments.

studies, three different calmodulin antagonists were employed, trifluoperazine, calmidazolium, and specific IgG antibodies against calmodulin. All produced a concentration-related decrease in adenylate cyclase (guanine nucleotide-mediated) activity, which was reversed completely by the addition of exogenous calmodulin. From these results, we have concluded that the inhibitory effect of these three agents on thymocyte adenylate cyclase activity is ascribed to their action as calmodulin antagonists.

The calmodulin antagonist trifluoperazine, incubated with the plasma membranes or with intact cells, produced a dose-dependent decrease in adenylate cyclase activity or in cellular cyclic AMP concentration respectively (Fig. 4). [Previous studies in rat thymocytes, with the crude plasma-membrane preparation, have shown that, as in the intact cell, EGTA did not change adenylate cyclase activity (Segal *et al.*, 1985), indicating that sufficient tightly bound Ca^{2+} is present within the EGTA-exposed membranes (EGTA-non-releasable,

Table 1. Effect of trypsin and trifluoperazine on rat thymocyte viability

Thymocytes, suspended in standard medium, were incubated with various concentrations and combinations of trypsin and trifluoperazine for 2 min, and their viability was measured as described in the Experimental section.

Trypsin (μg/ml)	Trifluoperazine (μM)	Cell viability (%)
—	—	90–95
10	—	90–95
10	50	90–95
10	100	45–55
20	—	90–95
20	25	90–95
20	50	85–90
20	100	35–45
50	—	90–95
50	25	90–95
50	50	45–60
100	—	90–95
100	12.5	90–95
100	25	85–90
100	50	10–25

trichloroacetic acid-extractable: about 5.0 nmol of Ca^{2+} /50 μg of plasma-membrane protein).]

To examine whether the stimulatory effect of trypsin on cellular cyclic AMP concentration is influenced by calmodulin, the cyclic AMP response to trypsin, in the presence or absence of trifluoperazine, was measured. When incubated together, only at certain concentrations and combinations did cell viability remain unchanged (Table 1). In such combinations, the proportionate effect of trypsin on cyclic AMP concentration was not changed by trifluoperazine (Fig. 5).

DISCUSSION

In the present study, I have examined whether Ca^{2+} and calmodulin influence the stimulatory effect of trypsin on cyclic AMP concentration in the rat thymocyte. Trypsin, incubated with thymocytes suspended in standard medium (containing 1 mM- Ca^{2+}), produced a rapid and dose-related increase in cytoplasmic free Ca^{2+} concentrations. This is consonant with the observations of Klip *et al.* (1984) in L_6 muscle cells. But trypsin had no effect in cells suspended in buffer essentially free of Ca^{2+} . This indicates that in the rat thymocyte trypsin increased cytoplasmic free Ca^{2+} concentration by increasing extracellular Ca^{2+} uptake rather than releasing Ca^{2+} from an intracellular pool.

However, the trypsin-induced increase in thymocyte cyclic AMP concentration was the same in the presence or absence of extracellular Ca^{2+} , and was not inhibited by the calmodulin antagonist trifluoperazine.

This demonstrates that the two functions of trypsin in rat thymocytes, on Ca^{2+} uptake and cytoplasmic free Ca^{2+} concentration, and on cyclic AMP concentration, are not related and that Ca^{2+} and calmodulin are not essential for the stimulatory effect of trypsin on cellular cyclic AMP concentration.

In the rat thymocyte, adrenaline, like trypsin, but probably through a different mechanism, increases both cytoplasmic free Ca^{2+} concentration (J. Segal, unpub-

Fig. 5. Effect of trypsin in the presence or absence of trifluoperazine on cyclic AMP concentration in rat thymocytes

Thymocytes, suspended in standard medium, were pre-incubated with or without various concentrations of trifluoperazine for 20 min, then trypsin in various concentrations was added and 2 min later the incubation was stopped and cellular cyclic AMP concentration was measured. Results shown are from a single experiment and are similar to those obtained in another experiment.

lished work) and cellular cyclic AMP concentration, and, like trypsin, its effect on cellular cyclic AMP concentration does not require extracellular Ca^{2+} (Segal & Ingbar, 1981).

The studies described here, together with observations described elsewhere in the same and other tissues, demonstrate that an increase in Ca^{2+} uptake and cytoplasmic free Ca^{2+} concentration in itself does not always result in a change in a metabolic function that has been demonstrated to be modulated by a different agent through a Ca^{2+} -dependent process. Thus, in rat thymocytes, La^{3+} produces a marked increase in both ^{45}Ca accumulation and cytoplasmic free Ca^{2+} concentration, but fails to change sugar uptake and cyclic AMP concentration (Segal & Ingbar, 1984), and in L_6 muscle cells trypsin increases cytoplasmic free Ca^{2+} concentration but has no effect on sugar uptake (Klip *et al.*, 1984).

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