The effect of trypsin on sugar uptake in rat thymocytes

Modulation of cellular cyclic AMP concentration and the sugar-transport system

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I have shown that cyclic AMP stimulates sugar uptake in rat thymocytes. However, trypsin treatment, which increases rat thymocyte cyclic AMP concentration, fails to increase sugar uptake. The purpose of the present study is to examine this seeming inconsistency, and to evaluate further the function of trypsin. Mild trypsin treatment of rat thymocytes produced a dose-related increase in cellular cyclic AMP concentration. Trypsin produced the same proportionate increase in cyclic AMP concentration in the presence or absence of optimal concentrations of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, which suggests that trypsin acts to increase thymocyte cyclic AMP concentration by stimulating adenylate cyclase activity. Trypsin at concentrations of 0.3 mg/ml and less had no effect on the uptake of the glucose analogue 2-deoxy-D-glucose (2-DG), whereas at concentrations of 1 mg/ml and higher trypsin produced a small, dose-related, decrease in basal 2-DG uptake, becoming significantly lower than control values only at 5 mg/ml (−22.7%, P < 0.05). Thymocyte sugar transporters, characterized by means of cytochalasin B binding, consist of a single class of sites with an apparent KD of 0.15 μM and maximum binding capacity of 2.73 pmol/20 x 10^4 cells (8.4 x 10^4 sites/thymocyte). Trypsin produced a dose-related decrease in the sugar-displaceable binding of cytochalasin B, so that at 5 mg of trypsin/ml the number of sugar transporters was decreased by approx. 50%. Thus trypsin treatment of rat thymocytes on the one hand increases cellular cyclic AMP concentration, which itself potentiates 2-DG uptake, and on the other hand decreases the number of sugar transporters, which itself decreases cellular sugar uptake, indicating that the apparent effect of trypsin on thymocyte 2-DG uptake is the result of the balance of its effects on these two systems.

INTRODUCTION

We have demonstrated in rat thymocytes that an increase in cellular cyclic AMP concentration results in an increase in the uptake of the glucose analogue 2-deoxy-D-glucose (2-DG) by the cell [1,2]. We have further shown that mild trypsin treatment of freshly isolated rat thymocytes, which does not damage the cell [3], produces a progressive dose-related increase in cellular cyclic AMP concentration, which is independent of Ca^2+ and calmodulin [3,4]. This effect of trypsin, however, is not accompanied by an increase in 2-DG uptake, but 5 mg of trypsin/ml, the highest concentration used, produced a small decrease in thymocyte 2-DG uptake [3]. This seems to be inconsistent with our hypothesis, that in rat thymocytes an increase in cellular cyclic AMP concentration is associated with an increase in 2-DG uptake. The purpose of the present study is to clarify this dilemma, and to elucidate further the effect of trypsin on the related cellular events.

In rat thymocytes, the analogue dibutyl cyclic GMP decreases basal 2-DG uptake [2], and trypsin treatment results in some changes in the plasma membrane, but not in its permeability, as evident from the decrease in the binding of 3,3',5-tri-iodo-l-thyronine to its plasma-membrane receptors [3]. Hence, it is conceivable that the failure of trypsin to enhance sugar uptake in rat thymocytes results from a concomitant increase in cellular cyclic GMP, a decrease in the number of functional sugar transporters (which reside in the plasma membrane and are thought to be the principal vehicle through which D-glucose is transported into the cell [1]), or both. To this end, I have examined here the effect of trypsin on cellular cyclic GMP concentration and on the number of sugar transporters.

Trypsin treatment did not change cellular cyclic GMP concentration, but it produced a progressive, dose-related, decrease in the number of functional sugar transporters in the thymocyte. From this I conclude that trypsin fails to increase 2-DG uptake in the rat thymocyte because its increase of cellular cyclic AMP concentration is accompanied by a decrease in the number of sugar transporters, responses that counterbalance one another.

EXPERIMENTAL

Materials

The materials used were purchased from the following sources: dibutyl cyclic AMP, dibutyl cyclic GMP and dimethyl sulfoxide from Sigma Chemical Co., St. Louis, MO, U.S.A.; cytochalasin B (Cyt B) and 3-isobutyl-1-methylxanthine (MIX) from Aldrich Chemi-

Abbreviations used: 2-DG, 2-deoxy-D-glucose; Cyt B, cytochalasin B; MIX, 3-isobutyl-1-methylxanthine; SU1, sugar uptake index.

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cal Co., Milwaukee, WI, U.S.A.; trypsin from Worthington Biochemical Co., Freehold, NJ, U.S.A.; soya-bean trypsin inhibitor from Calbiochem–Behring, San Diego, CA, U.S.A.; [3H]cytochalasin B (sp. radioactivity 1 mCi/9.6 μg), cyclic [3H]AMP (sp. radioactivity 36.4 Ci/mmol), [3H]mannitol (sp. radioactivity 17.0 Ci/mmol), [3H]2-DG (sp. radioactivity 5 Ci/mmol) and cyclic GMP radioimmunoassay kit from New England Nuclear, Boston, MA, U.S.A.; cyclic AMP radioimmunoassay kit from Becton Dickinson Immuno-diagnostics, Orangeburg, NY, U.S.A.

Isolation of thymocytes

Animals used were 25–28-day-old female rats of the Sprague–Dawley strain [Crl:CD(SD)BR; Charles River Laboratories, Wilmington, MA, U.S.A.]. Animals used 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 as previously described [5]. In short, animals were killed by cervical dislocation, exsanguinated, and thymus glands were quickly removed, washed, and placed in ice-cold Krebs–Ringer–Tris buffer (20 mM-Tris/HCl, 5 mM-Tris base, 120 mM-NaCl, 5 mM-KCl, 1 mM-CaCl₂, 2.5 mM-MgCl₂, 1.5 mM-NaH₂PO₄, 15 mM-Hepes), pH 7.4. Glands were then teased with forceps and the freed cells were filtered through a nylon mesh, centrifuged at 300 g for 15 min, and resuspended in the buffer to give the desired concentration.

Trypsin treatment of thymocytes

The suspended cells were transferred to a shaking water bath and were allowed to equilibrate for 15 min at 37 °C in air. Then trypsin in various concentrations, ranging from 0.01 to 5 mg/ml, was added, and 15 min later the trypsin hydrolysis was stopped by the addition of soya-bean trypsin inhibitor (0.1–5 mg/ml). Soya-bean trypsin inhibitor added before trypsin abolished the effect of trypsin. Soya-bean trypsin inhibitor alone had no effect on the various cellular functions measured. This trypsin treatment does not produce gross damage to the cell, because it does not change thymocyte binding of concanavalin A or their ability to exclude Trypan Blue and [3H]mannitol, but it does change the properties of the thymocyte plasma membrane, as evident from the decrease in the binding of tri-[14C]lidothyrinone by the plasma membrane. At higher concentrations (10 and 20 mM), trypsin produces a marked decrease in cell viability. A 15 min period of incubation with trypsin was chosen because, over a period of 60 min, trypsin was found to produce a small, but significant, inhibition of basal 2-DG uptake; this inhibitory effect of trypsin was time-dependent and reached a maximum at 15 min.

Measurement of [3H]2-DG uptake

Thymocytes (45 × 10⁶ cells/ml) were first incubated with or without various concentrations of trypsin for 15 min. Trypsin treatment was terminated with the soya-bean inhibitor, and incubation continued for an additional 65 min. Then 1 μCi of [3H]2-DG/ml, in the presence or absence of various concentrations of unlabelled 2-DG, was added, and 15 min later its uptake by the cells was measured as previously described [5]. The experimental procedure for the measurement of [3H]2-DG uptake is the same as that used in previous studies, in which the effect of trypsin on thymocyte [3H]2-DG uptake was examined [3]. Under these conditions, [3H]2-DG uptake rate remains the same during the first 60 min of incubation; thus the 15 min incubation period with [3H]2-DG measures initial rates of sugar transport [5]. In measurements of the effect of Cyt B on thymocyte 2-DG uptake, cells were first incubated with various concentrations of Cyt B, and 30 min later 1 μCi of [3H]2-DG/ml was added and its 15 min uptake was measured.

In comparable specimens, the extracellular marker [3H]mannitol (1 μCi/ml) rather than [3H]2-DG was added, and its content in the 10000 g/30 s cell pellet was measured 5 min later as previously described [5]. Both trypsin and Cyt B did not change [3H]mannitol content in the cell pellet. Values of the extracellular volume were subtracted from total values to give the net uptake of [3H]2-DG by the cells.

Measurement of cytochalasin B binding

A stock solution of 20 mM-Cyt B in dimethyl sulfoxide was stored at 4 °C; when needed, it was diluted with buffer to give the desired Cyt B concentration. Dimethyl sulfoxide at concentrations of 0.1%, and less was without effect. After trypsin treatment of thymocytes (20 × 10⁶ cells/ml in plastic tubes), 0.1 μCi of [3H]Cyt B, in the presence or absence of various concentrations of unlabelled Cyt B, was added, and incubation at 37 °C was continued for additional 30 min. Then triplicate 0.25 ml samples were transferred on to GF/A filters (Whatman) under low vacuum (25 mmHg). Filters and cells were then washed with 3 × 4 ml of ice-cold Krebs–Ringer–Tris buffer, pH 7.4, dried at 40 °C for 10 min, and transferred into vials containing liquid scintillation cocktail (Liquiscint; National Diagnostics, Manville, NJ, U.S.A.), and their content of 3H was counted in a liquid-scintillation counter. Non-specific binding was defined as the 3H radioactivity (c.p.m.) measured in the presence of 10 μM-Cyt B, and was subtracted from total 3H radioactivity to give the specific binding of Cyt B. Binding of [3H]Cyt B to the GF/A filters was practically nil.

Cell viability

This was assessed by the Trypan Blue technique [5], in which a viable cell is one which excludes the dye. In
general, cell viability in the different experimental groups was not different from that in the control group, in the range 90–95%.

**Statistical analysis**

Where appropriate, statistical analysis of differences between the experimental groups was assessed by analysis of variance, followed by the Newman–Keuls multiple range test for comparisons among multiple samples [6].

**RESULTS**

**Effect of trypsin treatment on cellular cyclic AMP and cyclic GMP concentrations**

Cells were treated with trypsin (concentrations 0.01–5 mg/ml) in the absence or presence of 5 mM-MIX. MIX produced a dose-dependent increase in both cyclic AMP and cyclic GMP concentration (results not shown). Maximal increase was seen at 5 mM-MIX: 8.8±0.9-fold for cyclic AMP and 5.6±0.6-fold for cyclic GMP. Trypsin, in the presence or absence of MIX, produced a dose-related increase in thymocyte cyclic AMP concentration (Fig. 1), and essentially the same proportionate increase in cyclic AMP was produced by trypsin in the presence or absence of the phosphodiesterase inhibitor. But trypsin, in the presence or absence of MIX, had no effect on thymocyte cyclic GMP concentration; cellular cyclic GMP concentration (pmol/10^9 cells; mean±s.d.) with and without 5 mM-MIX was 3.2±0.4 or 0.57±0.06 respectively.

**Effect of trypsin treatment on 2-deoxyglucose uptake**

Under the experimental conditions used, transport is the rate-limiting step in 2-DG uptake by the rat thymocyte [5]. Trypsin in concentrations of 0.01–1 mg/ml had no significant effect on the uptake of tracer concentration of [3H]2-DG by rat thymocytes, and only at a concentration of 5 mg/ml did it produce a small, but significant, decrease in the sugar uptake (77±11% of control, P < 0.05, n = 6; also see ref. [3]). The effect of trypsin treatment on the kinetic parameters of 2-DG uptake in the rat thymocyte was then measured. Cells were first incubated with or without 1 or 5 mg of trypsin/ml for 15 min. Trypsin inhibitor was then added, and 65 min later the 15 minute uptake of 2-DG was measured. 2-DG uptake by rat thymocytes is a saturable process (Fig. 2; ref. [5]), and again trypsin produced a small decrease in 2-DG uptake (Fig. 2). Eadie–Hofstee plot analysis of these data revealed that trypsin produced a significant decrease in the $V_{\text{max}}$ values and increased $K_m$ values, although not significantly (Table 1).

The uptake rate of sugar by the cell is a function of both the $V_{\text{max}}$ and $K_m$ of the sugar-uptake system, and the sugar uptake index (SUI) can be defined as follows:

$$\text{SUI} = \frac{V_{\text{max}}}{K_m}$$

![Fig. 1. Effect of trypsin on cyclic AMP concentration in rat thymocytes](image1)

Cells were incubated with various concentrations of trypsin in the absence (●) or presence (○) of 5 mM-MIX; 15 min later trypsin treatment was stopped with soya-bean trypsin inhibitor, and 5 min later cellular cyclic AMP concentration was measured. Results are expressed as percentages of the corresponding trypsin-free control. Values shown are the means from two separate experiments.

![Fig. 2. Effect of trypsin on 2-deoxyglucose uptake by rat thymocytes](image2)

Cells were first incubated without (●) or with trypsin (○, 1 mg/ml; ▲, 5 mg/ml), and 60 min later their uptake of [3H]2-DG in the presence or absence of various concentrations of unlabelled 2-DG was measured. Results shown are from a single experiment, and similar results were observed in two other experiments. Eadie–Hofstee plots are shown in the inset.

**Table 1. Effect of trypsin treatment on the kinetic parameters of 2-deoxyglucose uptake in rat thymocytes**

<table>
<thead>
<tr>
<th>Trypsin (mg/ml)</th>
<th>$K_m$ (mm)</th>
<th>$V_{\text{max}}$ (pmol/15 min per 10^9 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.53±0.26</td>
<td>2.40±0.12</td>
</tr>
<tr>
<td>1</td>
<td>2.61±0.20</td>
<td>2.24±0.09</td>
</tr>
<tr>
<td>5</td>
<td>2.86±0.31</td>
<td>2.14±0.07*</td>
</tr>
</tbody>
</table>

The kinetic parameters of 2-DG uptake (mean±s.d) were calculated from Fig. 2. *P < 0.05 (Student’s $t$ test).
Fig. 3. Effect of cytochalasin B on 2-deoxyglucose uptake by rat thymocytes

Cells were incubated with various concentrations of Cyt B, and 30 min later their uptake of [3H]2-DG was measured. Values shown are means ± s.d. obtained from three separate experiments.

Here, an excellent correlation between trypsin-induced changes in SUI and sugar uptake was obtained, where 5 mg of trypsin/ml decreased thymocyte 2-DG uptake by 22.7% and SUI by 21.1%.

Effect of cytochalasin B on 2-deoxyglucose uptake

Cyt B has been shown to inhibit D-glucose uptake in various tissues, presumably by competing with the sugar on the sugar-binding sites (sugar transporters) located in the plasma membrane [7–12]. Here, in the rat thymocyte, Cyt B produced a marked dose-related decrease in [3H]2-DG uptake, so that at 10 μM-Cyt B [3H]2-DG uptake was virtually nil (Fig. 3).

[3H]cytochalasin B binding

Binding of [3H]Cyt B by rat thymocytes was very rapid and time-dependent; it reached 45% of maximal values within the first 30 s of incubation and maximal values at 10 min, and remained at about maximum during the next 20 min (Fig. 4). At 60 min of incubation, Cyt B binding was smaller than maximal values. In ensuing experiments [3H]Cyt B was incubated for 30 min.

Effect of sugars on [3H]cytochalasin B binding

Cellular binding of Cyt B, which is associated with a decrease in cellular sugar uptake, is reversible and is blocked by D-glucose [7–12]. [3H]Cyt B (0.1 μCi/ml) was incubated in the presence or absence of various concentrations of D-glucose, 2-DG, and L-glucose, and its binding by thymocytes was measured. Binding of Cyt B by thymocytes was inhibited by D-glucose and 2-DG, but not by L-glucose (Fig 5). D-Glucose and 2-DG produced a comparable, dose-related, inhibition of [3H]Cyt B binding; maximal decrease in Cyt B binding, to 56% of glucose-free values, was produced by 0.5 μM-2-DG. This indicates that in the rat thymocyte 44% of total Cyt B binding is confined to the sugar-displaceable sites (sugar transporters).

Fig. 4. Binding of [3H]cytochalasin B by rat thymocytes

[3H]Cyt B was added to thymocytes, and at various periods of time thereafter its binding by the cells was measured. Non-specific binding of [3H]Cyt B was measured in the presence of 10 μM unlabelled Cyt B: ○, total binding; ○, non-specific binding; △, specific binding. Results shown are from a single experiment. Similar results were observed in another experiment.

Fig. 5. Effect of sugar on the binding of [3H]cytochalasin B by rat thymocytes

Cells were incubated with [3H]Cyt B in the presence or absence of various concentrations of D-glucose (●), L-glucose (▲) or 2-DG (○), and 30 min later the binding of [3H]Cyt B by the cell was measured. Results shown are from a single experiment, and similar results were observed in another experiment. Values shown are expressed as the percentages of the sugar-free-group values.

Effect of trypsin treatment on [3H]cytochalasin B binding

Cells were first incubated with various concentrations of trypsin, and their binding of [3H]Cyt B, in the absence or presence of 0.5 μM-2-DG, was measured. In both control and trypsin-treated cells, 2-DG produced a similar proportionate decrease in thymocyte binding of [3H]Cyt B, to about 45% of total binding (Fig. 6). Trypsin decreased, in a dose-related fashion, the binding
Trypsin treatment and sugar uptake in rat thymocytes

Fig. 6. Effect of trypsin treatment on the binding of \[^3H\]cytochalasin B by rat thymocytes

Cells were first treated with various concentrations of trypsin for 15 min. Then \[^3H\]Cyt B, with or without 0.5 m-2-DG, was added, and 30 min later Cyt B binding by the cells was assessed. ●, Total binding of \[^3H\]Cyt B; ○, \[^3H\]Cyt B binding in the presence of 0.5 m-2-DG; △, 2-DG-displaceable binding of \[^3H\]Cyt B, calculated by subtracting the values of Cyt B binding in the presence of 0.5 m-2-DG from total Cyt B binding. Values shown are means ± S.D. obtained from three separate experiments.

of \[^3H\]Cyt B by the sugar-displaceable sites, so that at 0.3 mg of trypsin/ml, a concentration that produced almost maximal increase in cyclic AMP concentration, binding of Cyt B by the sugar-displaceable sites, i.e. the number of functional sugar transporters, was decreased by approx. 40%, and was further decreased, to about 50%, at 5 mg of trypsin/ml.

Experiments were then conducted to assess the kinetic parameters of the binding of Cyt B by the sugar-displaceable sites in the rat thymocyte and the effect of trypsin thereupon. Binding of Cyt B by the sugar-displaceable sites was saturable, and again was decreased by trypsin (Fig. 7). Scatchard-plot analysis of these data revealed the presence of a single class of binding sites for Cyt B; in the control cells, an apparent $K_D$ of 0.15 $\mu$M and maximum binding capacity of 2.73 pmol/20 x $10^6$ cells (8.4 x $10^4$ sites/thymocyte). Hill plots demonstrated no co-operativity in the binding of Cyt B by these sites ($h = 0.97$).

Trypsin (5 mg/ml) produced about 50% decrease in the binding of Cyt B by the sugar-displaceable sites, owing to a decrease in the number of these binding sites (sugar transporters), without essentially changing their affinity $[B_{\text{max}} = 1.82 \text{ pmol}/20 \times 10^6 \text{ cells}; K_D = 0.16 \mu\text{M}; h \text{ (Hill plot)} = 0.98]$.

**DISCUSSION**

We have demonstrated that in the rat thymocyte an increase in cellular cyclic AMP concentration results in an increase in sugar uptake [1,2]. Additional studies have indicated that this observation is true provided that the cellular cyclic GMP concentration remains unchanged, because an increase in cyclic GMP concentration results in a decrease in thymocyte sugar uptake [2]. We have also demonstrated that trypsin increases thymocyte cyclic AMP concentration, but has no effect on 2-DG uptake [3,4], an observation which seemingly is inconsistent with our postulate. However, this could be explained as a result of a concomitant increase in cellular cyclic GMP concentration, or a proteolytic degradation of, and thereby a decrease in, the number of the plasma-membrane sugar transporters of the sugar-uptake system, or both (under the experimental conditions used, transport is the rate-limiting step in 2-DG uptake by the rat thymocyte [5]). This is the subject of the present study.

Trypsin produced a dose-related increase in cellular cyclic AMP concentration, but failed to change cyclic GMP concentration in the rat thymocyte. Further, trypsin produced the same proportionate increase in cellular cyclic AMP concentration in the presence or absence of a maximally effective concentration of the phosphodiesterase inhibitor MIX, indicating that trypsin acted by increasing adenylate cyclase activity. This is consonant with other studies in the same and different tissues, in which trypsin has been suggested to increase adenylate cyclase activity [13–15].

Trypsin failed to increase thymocyte 2-DG uptake, but at a concentration of 5 mg/ml it produced a small, significant, decrease in 2-DG uptake (−23%; $P < 0.05$). Kinetic analysis of this effect of trypsin revealed that trypsin decreased both the $V_{\text{max}}$ and the affinity of the sugar-uptake system; 5 mg/ml of trypsin/ml decreased the SUI values by 21%, which is consistent with its inhibition of 2-DG uptake.

As in many other systems [7–12], Cyt B was found to be a useful means in the characterization of sugar transporters in the rat thymocyte. Binding of Cyt B by
the rat thymocyte sugar-displaceable sites (sugar transporters) was saturable, having a single class of binding sites with an apparent \(K_D\) of 0.15 \(\mu M\) and a maximum binding capacity of 2.73 pmol/20 \(\times 10^6\) cells, which is consonant with the binding parameters of Cyt B by the sugar sites reported in other tissues [7,11,16–18].

In the rat thymocyte, trypsin produced a concentration-dependent decrease in the binding of Cyt B by the functional sugar sites, owing to a decrease in the number of sugar transporters with no significant change in their affinity, so that a concentration of 0.3 mg of trypsin/ml, which produced about maximal increase in thymocyte cyclic AMP concentration, decreased the number of sugar transporters by about 40\% (a further decrease was seen with 5 mg of trypsin/ml). Of interest are our previous studies in rat thymocytes [1] which show that dibutyl cyclic AMP produces a dose-related increase in thymocyte \(^{3H}\)2-DG uptake, having a maximal effect of about 40\%.

This points to a good quantitative correlation between these two functions; thus 0.3 mg of trypsin/ml on the one hand produced a maximal increase in cellular cyclic AMP concentration, itself increasing 2-DG uptake by about 40\%, whereas on the other hand it decreased the number of sugar transporters by about 40\%, which accounts for the failure of trypsin (0.3 mg/ml) to change 2-DG uptake by the cell. The additional decrease in the number of the sugar transporters produced by 5 mg of trypsin/ml resulted in a decrease in 2-DG uptake.

The mechanism through which trypsin acts to decrease the number of functional sugar transporters located at the plasma membrane is not clear. Trypsin may do so by hydrolysing the functional plasma membrane sugar transporters, or by enhancing the internalization of the transporters, or a combination of the two. Our studies in the rat thymocytes, which show that trypsin decreases the number of plasma membrane receptors for tri-iodothyronine, adrenaline and prostaglandin \(E_1\) [3], favours the former, although the contribution of the latter cannot be excluded.

Hence, in freshly isolated rat thymocytes, trypsin produced an increase in cellular cyclic AMP concentration, with no effect on cyclic GMP concentration, that was accompanied by a decrease in the number of sugar transporters. Consequently, I suggest that the overall effect of trypsin on sugar uptake in the cell is the sum of the individual changes in cellular cyclic AMP concentration (positive) and in the number of functional sugar transporters (negative), which results in the failure of trypsin to increase sugar uptake in the rat thymocyte.

This work was supported in part by Grant No. AM-18416 from the National Institute on Arthritis, Metabolism, and Digestive Diseases, N.I.H., Bethesda, MD, U.S.A., and by a Grant from the Center of Absorption in Science, Ministry of Absorption, Israel.

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Received 2 January 1987/26 March 1987; accepted 19 May 1987