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THE EFFECTS OF CELL-RELEASED PROTEASE NEXIN ON THE MEASUREMENT OF THROMBIN BINDING TO MOUSE CELLS

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We previously showed that fibroblast-like cells release protease nexin into their growth medium. Protease nexin links to thrombin and mediates the cellular binding of thrombin via the protease nexin part of the complex to a site different from that for unlinked thrombin (1,2). To determine the effect that cell-released protease nexin had on the measurement of total cell-bound thrombin, we separately measured the cellular binding of both ^{125}I-thrombin and ^{125}I-thrombin-protease nexin complexes. Scatchard analysis of our binding data indicates that the cellular binding affinity of linked ^{125}I-thrombin is about 19-fold higher than that of unlinked ^{125}I-thrombin. We show that protease nexin acts to increase the apparent affinity of ^{125}I-thrombin for cellular binding sites.

Introduction

Thrombin is a potent mitogen for a variety of cells in culture including mouse embryo cells (3,4). These cells have high affinity thrombin binding sites on their cell surface (5,6,7) and also have cell surface binding sites for protease nexin (2). Protease nexin is released by cells and forms covalent linkages with thrombin and other serine proteases (2,8). Thrombin-protease nexin complexes, formed in the medium, bind to cells via the protease nexin part of the complex to a site different from that which binds unlinked thrombin (2). Here we separately measure the cellular binding of both unlinked thrombin and thrombin-protease nexin complexes and show that protease nexin acts to increase the apparent affinity of ^{125}I-thrombin for cellular binding sites.

Materials and Methods

Highly purified human thrombin (about 3100 National Institutes of Health units/mg) was generously supplied by Dr. John W. Fenton II (9). Ovalbumin (B grade) was from Calbiochem, PhAsO was from Aldrich, and lactoperoxidase (320 un/mg) was from Worthington. We purchased DV medium from Flow Laboratories; antibiotics were from Gibco and calf serum was from Irvine Scientific (Santa Ana, California). Na ^{125}I was obtained from Amersham and Iodogen was from Pierce.

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Secondary mouse embryo fibroblast-like cells were cultured as described (4). Binding studies were performed as described (2,7) except where noted. Thrombin was iodinated using a scaled-down lactoperoxidase procedure (9) and retained full activity in a fibrinogen clotting assay at less than 0.4 mole I/mole thrombin (7×10^5 - 1×10^6 cpm/pmol) (7). Hirudin was iodinated using Iodogen and the hirudin assay for measuring cell-bound thrombin was performed as described (7).

Levels of ^{125}I-thrombin and ^{125}I-thrombin-protease nexin in the medium were determined using Weber-Osborne sodium dodecyl sulfate-polyacrylamide gels (11). Samples were analyzed using 10% polyacrylamide as described except that they were not heated prior to electrophoresis. Cell-bound ^{125}I-thrombin and ^{125}I-thrombin-protease nexin were measured using a bis-tris sample buffer (2) and mono-tris/bis-tris sodium dodecyl sulfate polyacrylamide slab gels as described (12). ^{125}I-thrombin-protease nexin, formed in the medium or bound to cells, was stable under the conditions of analysis described above. After electrophoresis, quantitation of radioactivity in gel slices (2 mm each) was carried out as described (13). Lines were drawn through data points in Figures 1 and 2 using least squares analysis.

Results

To measure the cellular binding of linked and unlinked thrombin, increasing amounts of ^{125}I-thrombin were added to mouse cells and ^{125}I-thrombin and ^{125}I-thrombin-protease nexin complexes were separated and quantitated using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Since our results indicated that most cell-bound ^{125}I-thrombin-protease nexin was formed via binding of complexes in the medium to cells (2), the amount of free ligand used in the determination of cellular binding of complexes was taken to be the amount of ^{125}I-thrombin-protease nexin in the medium at each ^{125}I-thrombin concentration[1]. Figure 1 shows that the apparent affinity of ^{125}I-thrombin-protease nexin for its binding sites using cells in fresh medium was about 19-fold higher than that of ^{125}I-thrombin for its binding sites. Importantly, analysis of total thrombin binding (linked plus unlinked thrombin) using cells in fresh medium shows that cellular binding of ^{125}I-thrombin-protease nexin increased the apparent affinity of ^{125}I-thrombin for cells by about 1.5-fold (Fig. 1, lower panel).

A potential rate limiting step in the formation of cell-bound ^{125}I-thrombin-protease nexin complexes is the formation of complexes in the medium; this step is dependent on levels of protease nexin in the medium. Since re-

[1]We have found that the amount of cell-bound ^{125}I-thrombin-protease nexin is proportional to levels of ^{125}I-thrombin-protease nexin but not to levels of ^{125}I-thrombin in the medium. Also, our results indicate that blockage of formation of ^{125}I-thrombin-protease nexin complexes in the medium almost completely inhibits the formation of cell-bound complexes (not shown).

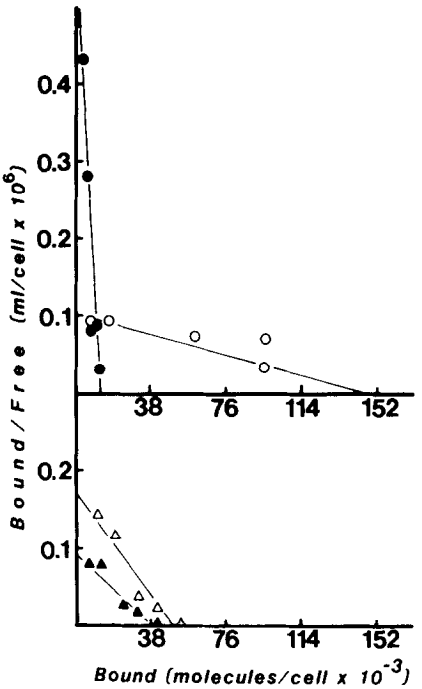

Figure 1. Scatchard analysis of the binding of ^{125}I-thrombin and ^{125}I-thrombin-protease nexin to mouse cells. Mouse cells (9.0 x 10^5 cells/35 mm dish) were washed and incubated in serum-free Dulbecco-Vogt modified Eagle's medium containing ovalbumin (50 µg/ml). After five days medium from some of the dishes was removed and replaced with fresh medium. Increasing amounts of ^{125}I-thrombin were then added to cultures, with and without the addition of unlabeled thrombin (280 nM). After 1 hr at 37°C, the amount of ^{125}I-thrombin and ^{125}I-thrombin-protease nexin, both in the medium and cellbound, was determined as described in Materials and Methods. The amount of radioactivity in each sample was normalized based on the fraction: (total radioactivity recovered on gel/total radioactivity in sample) which was about 0.8. The amount of nonspecific binding (binding in the presence of 280 nM thrombin) was subtracted to yield the amount of specifically-bound ^{125}I-thrombin and ^{125}I-thrombin-protease nexin at each ^{125}I-thrombin concentration. The amount of ^{125}I-thrombin-protease nexin in the medium was used as the "free" concentration in the Scatchard transformation (14) of binding data shown in the upper panel. (●——●), ^{125}I-thrombin-protease nexin using fresh medium, K_d= 2.9 x 10^{-11} M; (○——○), ^{125}I-thrombin-protease nexin using conditioned medium, K_d = 2 x 10^{-9} M; (▲——▲), ^{125}I-thrombin using fresh medium K_d = 6.2 x 10^{-10} M; (△——△), total thrombin binding (^{125}I-thrombin plus ^{125}I-thrombin-protease nexin) using fresh medium, K_d = 4.1 x 10^{-10} M.

lease of protease nexin is gradual (not shown), we performed binding incubation in conditioned medium, which contains substantial amounts of protease nexin (2). At maximal ^{125}I-thrombin levels (14 nM) there were about 8-fold as many ^{125}I-thrombin-protease nexin complexes formed in conditioned versus fresh medium (not shown). As shown in Figure 1 (upper panel), using conditioned medium the total number of ^{125}I-thrombin-protease nexin binding sites measured increased by about 12-fold. However, the apparent cellular affin-

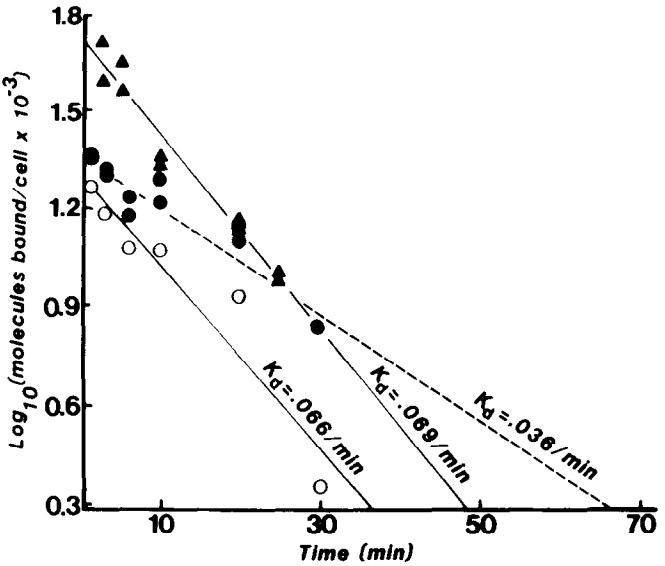

Figure 2. Dissociation of ^{125}I-thrombin and thrombin from mouse cells. Mouse cells (6.5 x 10^5 cells/35 mm dish) received thrombin (2.8 nM) or ^{125}I-thrombin (2.8 nM), with and without the addition of thrombin (280 nM), in 2 ml of serum-free Dulbecco-Vogt modified Eagle's medium containing Hepes (10 mM) and ovalbumin (0.1%). After a 30 min incubation at 37°C, cells were rinsed twice using the serum-free medium described above and 2 ml of this medium was added to each dish. At increasing time intervals after this second incubation, cells were cooled to 4°C and were rinsed 3 times in ice-cold Dulbecco's phosphate buffered saline (D-PBS) and once in D-PBS containing phenylarsine oxide (0.1 mM). After a final rinse in D-PBS the amount of specifically bound ^{125}I-thrombin (linked plus unlinked) remaining on one set of cell cultures was determined using sodium hydroxide solubilization as described (1,5). Also, the fraction of specifically-bound ^{125}I-thrombin that was linked to protease nexin was determined immediately following the first 30 min incubation as described in Figure 1 and was equal to 0.20. The amount of unlabeled thrombin remaining on dishes was determined using the hirudin assay as described (5). (●——●), total cell-bound ^{125}I-thrombin (^{125}I-thrombin plus ^{125}I-thrombin-protease nexin); (○——○), cell-bound ^{125}I-thrombin (after subtraction of the amount of cell-bound ^{125}I-thrombin-protease nexin); (▲——▲), cell-bound thrombin.

ity of complexes in conditioned medium was reduced by about 70-fold compared to incubations in fresh medium. These results showed that both the apparent number of cellular binding sites and the apparent cellular affinity of ^{125}I-thrombin-protease nexin were dependent on the conditions used for binding incubations.

We previously developed a method called the hirudin assay for measuring picogram amounts of cell-bound thrombin (7). Figure 2 shows that the dissociation rate of thrombin from the cell surface (measured with the hirudin assay) was about twice that for ^{125}I-thrombin. However, at the thrombin concentration used in this experiment about 20% of specifically cell-bound thrombin was

linked to protease nexin (binding data used for Fig. 1). We showed previously that only about 16% of total cell-bound ^{125}I-thrombin-protease nexin was cell-dissociated after an incubation at 37°C for 3 h (7). Therefore, we subtracted the amount of cell-bound ^{125}I-thrombin-protease nexin from each point in the ^{125}I-thrombin dissociation curve. As shown in Figure 2, after correction for undissociable ^{125}I-thrombin-protease nexin, the dissociation rate for unlinked ^{125}I-thrombin (.066/min) was about the same as that for thrombin (.069/min).

Discussion

We recently showed that a large fraction of the molecules in ^{125}I-thrombin preparations contained diodotyrosine residues (7). ^{125}I-thrombin containing diiodotyrosine was impaired in its ability to bind to the cell surface binding site for thrombin. Additionally, the binding affinity of ^{125}I-thrombin containing monoiodotyrosine appeared to be about twice that for thrombin, measured using the hirudin assay. In the latter experiment total cell-bound ^{125}I-thrombin was measured. As we show here (Figure 1), protease nexin mediated the binding of a significant fraction of ^{125}I-thrombin at low ^{125}I-thrombin concentrations and significantly increased the apparent affinity of binding sites for ^{125}I-thrombin. Previously we underestimated the amount of thrombin linked to protease nexin because the complexes were unstable on the alkaline, Tris-containing Laemmli gels used to analyze complex formation (1). Because the hirudin assay appears to measure unlinked but not linked thrombin (7), much of the apparent difference in the binding affinities of ^{125}I-thrombin containing monoiodotyrosine and thrombin appears to be the result of the different methods of measurement of thrombin binding.

If the above hypothesis is correct, then the dissociation rate of ^{125}I-thrombin and thrombin should be similar. In Figure 2 we show that after subtracting the undissociable fraction of ^{125}I-thrombin linked to protease nexin, the dissociation rates of ^{125}I-thrombin and thrombin are almost equal. Because the cellular dissociation rate of unlinked but not linked thrombin is measured by the hirudin assay (7), measurement of thrombin dissociation from cells is accurate.

The reduced apparent binding affinity of ^{125}I-thrombin-protease nexin in conditioned versus fresh medium could be due to the presence of 2 or more binding sites for complexes. Since lower levels of ^{125}I-thrombin-protease nexin are formed in fresh medium than in conditioned medium, the high affinity binding site would preferentially be measured. Alternatively, much of the protease nexin in conditioned medium might be linked to cell-released proteases, thus lowering the apparent cellular affinity of ^{125}I-thrombin-protease nexin via competitive binding. This phenomenon would occur to a lesser extent in fresh medium since the fraction of protease nexin linked to ^{125}I-thrombin versus that linked to cell-released proteases would be increased due to the presence of ^{125}I-thrombin during the time that cellular release of protease nexin occurred.

In summary, results presented here indicate that the cellular binding affinity of ^{125}I-thrombin containing monoiodotyrosine (7) is similar to that of thrombin. Our results show that cellular binding of linked and unlinked thrombin must be discriminated in order to accurately determine the interaction of thrombin with cell surface binding sites.

Acknowledgments

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