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No evidence for calpain I involvement in fodrin rearrangements linked to regulated secretion

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Stimulation of secretion in chromaffin and parotid acinar cells is associated with dramatic rearrangements of the subplasmalemmal cytoskeleton, notably of fodrin and F-actin. It has been proposed that a proteolytic cleavage of fodrin resulting from an activation of the neutral calcium activated protease (calpain) could be responsible for these changes. Using an affinity-purified anti-α-fodrin antibody, several cleavage products of fodrin could clearly be detected following incubation of total cell homogenates from chromaffin and parotid acinar cells with purified calpain I. On the other hand, maximum stimulation of secretion of chromaffin cells by nicotine, and of parotid acinar cells by carbachol plus isoproterenol, was not associated with an increased appearence of cleavage products of fodrin. This result is not compatible with the 'proteolytic cleavage' hypothesis.

Fodrin; Brain spectrin; Secretory cell; Calpain I

1. INTRODUCTION

Cytoskeletal rearrangements correlated with secretion have been reported in several cell types, chromaffin cells of the adrenal medulla [1,2], mast cells [3,4], parotid acinar cells [5] and parietal cells of the stomach [6]. In the case of the actin-reticulating protein, fodrin, it can be observed in resting cells as a continuous ring under the plasma membrane, while upon stimulus-induced secretion it transiently redistributes either as patches [1], or it disappears from the subplasmalemmal area [5]. The molecular mechanisms underlying this phenomenon are unknown. A physical redistribution of fodrin from the plasma membrane to the cytoplasm and to vesicles has been reported in the adrenal medulla following insulin stress [7].

Fodrin is a good substrate of the calcium-dependent protease I (calpain I), and fodrin degradation has been reported during platelet activation [8], neutrophil degranulation [9], long-term potentiation [10] and glutamate-induced neurotoxicity [11]. Accordingly, a degradation of fodrin via calpain has been postulated by several authors [12–14] as a putative mechanism accounting for the fodrin rearrangements observed upon stimulation in chromaffin cells.

Therefore, we looked for fodrin degradation products in secreting chromaffin and parotid acinar cells, using as a tool affinity-purified anti-α-fodrin antibodies and the immunoblotting technique.

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2. MATERIALS AND METHODS

A fraction enriched in bovine brain fodrin was prepared by low salt extraction, followed by ion-exchange chromatography and gel filtration [15].

Antibodies directed against the α-subunit of bovine brain fodrin were raised in rabbit, characterized, and affinity purified [5].

Calpain I was purified according to [16].

Primary cultures of guinea-pig salivary gland parotid acinar cells were performed as described in detail in [5]. Primary cultures of chromaffin cells of bovine adrenal medulla were performed according to [1]. Cells were plated in 24 well plates (Nunc, Kamstrup, Denmark) at a density of 1,106 cells per well. Amylase secretion was determined according to [17], and catecholamine secretion by direct fluorimetry [18].

After stimulation, the cells were extracted for 5 min on ice with Triton extraction buffer (1% Triton X-100, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 2 mM PMSF (phenylmethylsulfonylfluoride), 10 mM Tris-HCl, pH 7.0). The insoluble proteins were resuspended in hot sample buffer, and scraped from the culture dish with a rubber policeman. Both Triton-insoluble and -soluble fractions were submitted to SDS-PAGE [19] on a 5% polyacrylamide gel and blotted onto nitrocellulose according to [20]. Blots were incubated in the presence of affinity-purified anti-α-fodrin antibody diluted 1:100 for 2 h, followed by an incubation for 1 h with a peroxidase-coupled antibody against rabbit IgG, raised in goat (Dianova, Hamburg, Germany) and diluted 1:1,000. All incubations were performed at 37°C in the presence of 3% (w/v) dry milk and 10% (v/v) fetal calf serum in PBS (phosphate-buffered salt).

Total cell homogenates were prepared as follows: cells were rinsed with ice-cold PBS, scraped from the culture dish with a rubber policeman and homogenized by hand in a glass Potter homogenizer.

3. RESULTS AND DISCUSSION

3.1. *The anti-α-fodrin antibody recognizes fodrin degradation products*

A fraction enriched in bovine brain fodrin was incubated for 1 h at 37°C in the presence of calpain I and calcium, submitted to SDS-PAGE, blotted onto nitro-

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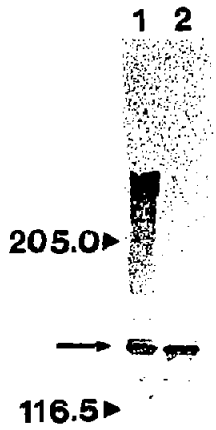

Fig. 1. Detection of calpain-induced fodrin proteolytic degradation products by immunoblotting. (Lane 1) Control, 5 μg of purified bovine brain fodrin; (lane 2) 5 μg of bovine brain fodrin after incubation with 0.2 μg calpain I for 1 h at 37°C. Apparent molecular masses are indicated in kDa (arrowheads). Note the disappearance of the 240 kDa α-fodrin band and the increase in the amount of degradation products around 150 kDa (arrow).

cellulose and the material cross-reacting with the anti-α-fodrin antibody was detected by the indirect immunoperoxidase method (Fig. 1). The undigested (lane 1) fraction presents a main band at 240 kDa corresponding to the native α-subunit of fodrin, a band around 150 kDa corresponding to a typical fodrin degradation product, and some minor bands of lower molecular weight. In contrast, upon incubation with calpain I (lane 2) one can observe the complete disappearence of the 240 kDa band, an increase in the amount of material banding around 150 kDa and the appearance of immunoreactive material of lower apparent molecular weights. These results allow us to confidently use our anti-fodrin antibody for the detection of putative fodrin degradation products in stimulated secretory cells.

3.2. *Research of calpain I-induced digestion fragments of fodrin in secreting chromaffin and parotid acinar cells in culture*

Parotid acinar cells in primary culture were stimulated with a mixture of 10 μM carbachol and 10 μM isoproterenol, which induced the release of 50.9 ± 1.2% of the total amylase content of the cells. Similarly, chromaffin cells in culture were challenged with 20 μM nicotine, leading to the release of 30.1 ± 1.1% of the total cellular catecholamines.

Following stimulation, the cells were submitted to an extraction with Triton; Triton-soluble and -insoluble fractions were submitted to SDS-PAGE, and fodrin immunoreactive material was detected by immunoblotting. As a control, both the chromaffin and parotid acinar total-cell extracts were incubated in the presence of exogenous calpain I. In both cases fodrin degradation products were detected (Figs. 2 and 3). Interestingly, fodrin from chromaffin cells seemed to be more sensitive to calpain than their parotid counterpart. In contrast, in unstimulated chromaffin (Fig. 2) and parotid (Fig. 3) cells, a main band at 240 kDa was detected only in the Triton-insoluble fraction. Upon stimulation, degradation products could not be detected immunologically either in the Triton-soluble- or in the Triton-insoluble fraction. Moreover, in stimulated cells, fodrin remained associated with the Triton-insoluble fraction.

We did not detect any enhancement of fodrin degradation in secreting chromaffin and parotid acinar cells under conditions where a maximum rearrangement of fodrin had been shown [1,5], in spite of the sensitivity of our antibody, and in spite of the fact that fodrin in chromaffin cells was particularly sensitive to proteolytic degradation.

Our results therefore rule out a massive degradation of fodrin upon stimulation. If any proteolytic breakdown of fodrin had occurred during stimulated secretion, it would have to have been very discrete. Such a discrete process could by no means explain the massive cytoskeletal rearrangements observed in stimulated chromaffin or parotid acinar cells. Furthermore, the hypothesis that the proteolytic degradation of fodrin accounts for the observed rearrangement of fodrin associated with secretion does not take into account the fact that the fodrin redistribution is reversible upon removal

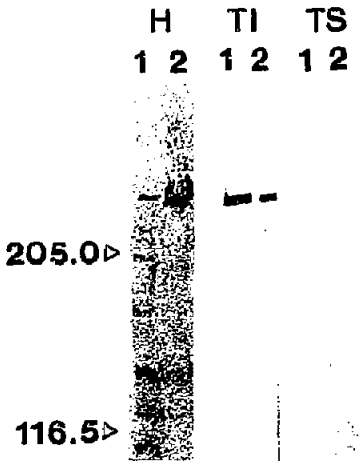

Fig. 2. Detection of fodrin-immunoreactive material in stimulated vs. unstimulated chromaffin cells by immunoblotting. 1,106 cells were used per assay. H, total chromaffin cell homogenate; (lane 1) the total chromaffin cell homogenate was incubated for 1 h in the presence of 0.2 μg of calpain I at 37°C; (lane 2) undigested control. Note the strong decrease in immunoreactivity at 240 kDa and the appearance of various smaller immunopositive degradation products. TI, Triton-insoluble fraction; TS, Triton-soluble fraction. Chromaffin cells in culture were stimulated for 30 min in the presence (lane 1) or absence (lane 2) of 20 μM nicotine, and subsequently incubated on ice with the Triton extraction buffer. H, TI and TS fractions were submitted to SDS-PAGE, blotting onto nitrocellulose and immunodetection. Apparent molecular masses of marker proteins are given in kDa (open arrowheads).

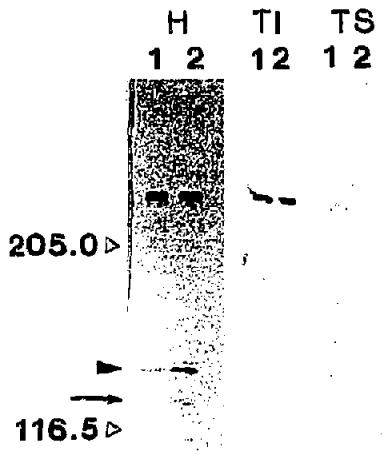

Fig. 3. Comparison of fodrin-immunoreactive material in stimulated vs. unstimulated parotid acinar cells by immunoblotting. 1,106 cells were used per assay. H, total cell homogenate. (lane 1) undigested parotid acinar cells total homogenate. (lane 2) parotid acinar cells total homogenate incubated for 1 h at 37°C in presence of 0.2 μg calpain I at 37°C. Note that in this system fodrin is much more resistant to calpain I than in chromaffin cells. Nevertheless, immunoreactive degradation products are either augmented (filled arrowhead), or appear anew (arrow). TI, Triton-insoluble fraction; TS, Triton-soluble fraction. Parotid acinar cells in culture were stimulated for 30 min in the presence (lane 1) or absence (lane 2) of 10 μM isoproterenol plus 20 μM carbachol and subsequently incubated on ice with the Triton-extraction buffer. H, TI and TS fractions were submitted to SDS-PAGE, blotting onto nitrocellulose and immunodetection. Apparent molecular masses of marker proteins are given in kDa (open arrowheads).

of the stimulus, with fodrin labeling returning to normal 20–30 min after removal of the stimulus [1,5].

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