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Effect of protein synthesis inhibitors on synexin levels and secretory response in bovine adrenal medullary chromaffin cells

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Abstract

The effects of the protein synthesis inhibitors actinomycin D and cycloheximide on the cellular content of the calcium binding protein synexin, and on the secretory response of cultured bovine adrenal medullary chromaffin cells were determined. Both protein synthesis inhibitors produced a slow decrease in the cellular synexin content. The synexin level was reduced by 50% after 133 h of incubation in the presence of 2 μg/ml actinomycin D or 5 μg/ml cycloheximide. However, this was partly due to an artefactual stabilization of synexin, since metabolic labelling of synexin with [^{35}S]methionine showed that the half-time of degradation was only 40 h. The secretory response of chromaffin cells was quickly diminished in the presence of protein synthesis inhibitors. Catecholamine secretion induced by membrane depolarization or barium stimulation of intact cells, or by calcium stimulation of digitonin-permeabilized cells was decreased by 77–82% after 24 h of incubation in the presence of 5 μg/ml cycloheximide. These results suggest that, in addition to synexin, at least one or more proteins with a shorter half-time of degradation than synexin are involved in the secretory response of adrenal chromaffin cells.

Keywords: Chromaffin; Synexin; Protein synthesis; Secretion; Cycloheximide; Actinomycin D

1. Introduction

Membrane fusion is a process which occurs during exocytotic secretion of enzymes, hormones and neurotransmitters. In various cell types, exocytosis is preceded by a profound increase in the intracellular Ca^{2+} concentration [1]. This observation has promoted a great deal of attention to the search for intracellular proteins that could act as Ca^{2+} receptor-effector molecules.

Biochemical studies of membrane fusion reactions have revealed proteins which may be important for membrane docking and/or fusion during intracellular trafficking. These include *N*-ethylmaleimide-sensitive factor (NSF), αSNAP (soluble NSF attachment protein), synaptobrevin, syntaxin and SNAP-25, which have been proposed to associate into a high molecular weight (20S) 'fusion complex' [2]. Also identified in this complex is the synaptic protein synaptotagmin, which binds to syntaxin [3]. Synaptotagmin is considered to be one of the Ca^{2+} sensors that triggers neurotransmitter release [4]. However, the deletion of synaptotagmin from rat PC12 cells, *Caenorhabditis elegans* and *Drosophila melanogaster* leaves exocytosis unaffected [5–7].

Synexin (annexin VII) is a Ca^{2+}-dependent phospholipid membrane binding protein that is present in a wide variety of cells and tissues [8–11]. It has been shown that upon activation by Ca^{2+} synexin induces both aggregation of chromaffin granules and pancreatic zymogen granules, as well as phospholipid vesicle fusion [12–14], suggesting that this protein might play a role in promoting membrane interactions during exocytosis. It has recently been proposed that the Ca^{2+} sensor for exocytosis has a low-affinity for Ca^{2+}, in the range of 50–200 μM, and that it is likely to be located at or near the site of exocytosis [15–18]. The concentration of Ca^{2+} needed for half-maximal aggregation induced by synexin is 200 μM and membrane fusion by synexin occurs in just 4 μs [8]. Furthermore, electronmicroscopic immunocytochemistry studies have revealed synexin to be localized to chromaffin granules and plasma membranes, and that upon nicotinic stimulation of chromaffin cells the reactivity of synexin

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with a specific monoclonal antibody is rapidly changed [19]. These data thus lend more support to the hypothesis that synexin may play a role in exocytotic secretion.

To further test this hypothesis we have used an indirect approach to study the relationship between the cellular synexin level and the secretory response in bovine adrenal chromaffin cells. We were led to use this approach because our attempts to reduce synexin levels in rat, human and mouse cells, using antisense phosphodiester and phosphorothioate oligonucleotides were unsuccessful. Therefore, we blocked the synthesis of synexin in chromaffin cells using protein synthesis inhibitors, and determined the effect of this treatment on the stimulated secretion of catecholamines. Our results suggest that synexin may be involved in the membrane fusion events that occur during secretion, but they also indicate that other proteins with shorter half-times also are likely to play a role in this process.

2. Methods

2.1. Cell cultures

Adrenal medullary tissue was dissociated from bovine adrenal glands by perfusion digestion with 0.2% collagenase (2×10 min at 37° C). The medullary tissue was removed, minced and filtered through nylon gauze, and chromaffin cells were purified on a self-generating Percoll gradient as described by Kuijpers et al. [20]. The cells were suspended in a Dulbecco's modified Eagle's medium (DMEM/F-12 mixture, 1:1) (Sigma), supplemented with 10% fetal calf serum (FCS), 100 μg/ml streptomycin and 100 U/ml penicillin. Cells were plated at a density of $0.5 \cdot 10^6$/ml or $1.0 \cdot 10^6$/ml culture medium in either 24-well culture plates (Costar) or bacterial dishes (100×15 mm) (Falcon), and incubated at 37° C in a humidified CO_2 incubator. Cells cultured in the bacterial dishes did not attach to the dishes, but remained in suspension.

Rat PC 12 cells were obtained from Dr. G. Guroff (NIH, Bethesda, MD, USA) and mouse GT1-7 cells (immortalized hypothalamic neurons) [21] were obtained from Dr. D.J. Spergel (NIH, Bethesda, MD, USA). PC-12 cells were cultured in DMEM supplemented with 7% horse serum, 7% FCS, 100 μg/ml streptomycin and 100 U/ml penicillin. GT1-7 cells were cultured in DMEM/Fl2 mixture (1:1) supplemented with 0.1 mg/ml gentamycin and 10% heat inactivated FCS.

2.2. Oligonucleotides synthesis

Phospohodiester oligonucleotides (18-mer) were synthesized on an Applied Biosystems DNA synthesizer and phosphorothioate oligonucleotides were synthesized by Bioserve Biotechnologies. Oligonucleotides were purified by polyacrylamide gel electrophoresis (PAGE).

2.3. Oligonucleotide treatment

Oligonucleotide treatments of PC 12 or GT1-7 cells grown on 6-well culture plates were started at 30–40% confluence. Phosphodiester oligonucleotides (5–30 μM) were added twice a day in serum-free medium, and after 1 h of incubation at 37° C with oligonucleotides, serum was returned to the medium. Phosphorothioate oligonucleotides (2–10 μM) were added once a day in medium containing serum. These protocols were repeated for 5–7 days. Oligonucleotides were also administered with lipofectin (Gibco-BRL) as a carrier in order to increase their cellular uptake [22]; a mixture of 5 μM oligonucleotides and 10 μg/ml lipofectin was added in serum free-medium to cells for 4 h at 37° C. After 4 h, serum was returned to the medium. This protocol was repeated for 3 days.

2.4. Incorporation of [^{14}C]leucine and [^{14}C]uridine

Chromaffin cells were incubated in 24-well culture plates in DMEM, containing 1 μCi/ml [^{14}C]leucine (310 mCi/mmol) or 1 μCi/ml [^{14}C]uridine (503 mCi/mmol) and different drug concentrations. After incubation, the culture plates were put on ice for 30 min, and cellular proteins were precipitated with cold 10% trichloroacetic acid. The precipitates were collected and washed on O.45 μm Millipore filters, and their radioactivity was determined by liquid scintillation counting.

2.5. Immunodetection of synexin

Chromaffin cells cultured in bacterial dishes or PC12 and GT1-7 cells cultured in 6-well culture plates were lysed in 1% Triton, 50 mM Tris (pH 8), 5 mM EGTA, 5 mM $MgCl_2$, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 10 μg/ml Pepstatin, 2 mM phenylethanesulfonyl fluoride and 400 μg/ml DNase I (lysis buffer), mixed with SDS-sample buffer (50 mM Tris-HCl (pH 6.8), 10% glycerol, 2% sodium dodecylsulfate (SDS), 100 mM dithiothreitol and 0.1% Bromophenol blue) and heated for 2 min in a boiling water bath. Total cell proteins were separated by SDS-PAGE in 10% polyacrylamide and electrophoretically transferred (overnight, 32 V) to nitrocellulose 0.45 μm membranes (Novex) in a buffer containing 20 mM Tris, 150 mM glycine, 20% methanol and 0.02% SDS (pH 8.3). The blots were initially blocked with 5% nonfat dry milk in 0.01 M phosphate-buffered saline (PBS) for 1 h and then incubated with a mouse monoclonal anti-(bovine)-synexin antibody (lOE7) diluted in PBS (1:1000) for 1.5 h at room temperature. After washing, the blots were incubated with anti-mouse [^{125}I]IgG (0.2 μCi/ml) for 1.5 h. Then, blots were washed with PBS and exposed to Kodak X-OMAT-AR at −70° C overnight. After autoradiography, bands were excised from the nitrocellulose paper and the relative bound [^{125}I]IgG was determined by counting the ^{125}I-radioactivity of each band.

2.6. Metabolic labelling and immunoprecipitation of synexin

Chromaffin cells in suspension were collected by centrifugation, washed in methionine-free medium (DMEM, Biofluids), suspended in 5 ml of methionine-free medium containing 20 μCi of [^{35}S]methionine/ml, and incubated at 37° C for 24 h. The radioactive precursor was then removed from the medium, and the cells were incubated in DMEM. At different times after the pulse, the cells were washed with Locke's solution containing 154 mM NaCl, 5.6 mM KCl, 5.0 mM $NaHCO_3$, 5.6 mM glucose and 5.0 mM Na-Hepes (pH 7.35), and lysed in lysis buffer. Synexin was immunoprecipitated from 300 μl of lysate with 50 μl of a goat polyclonal anti-(bovine)-synexin antibody (RII) and 10 μl protein G-Sepharose 4B (Zymed). Proteins from this immunoprecipitation step were separated by 10% SDS-PAGE, and the radiolabelled proteins were detected by autoradiography. After autoradiography, the synexin band was excised from the gel and the ^{35}S-radioactivity of the band was counted.

2.7. Secretion of catecholamines

Chromaffin cells cultured in 24-well culture plates were washed in a balanced salt solution containing 150 mM NaCl, 5.6 mM KCl, 1.0 mM $MgCl_2$, 2.2 mM $CaCl_2$, 10 mM Hepes, 10 mM glucose and 0.5% bovine serum albumin (pH 7.35), at room temperature. After 10 min the medium was replaced with a medium in which 56 mM NaCl was replaced by 56 mM KCl, or to which 2.0 mM $BaCl_2$ was added. After 10 min of incubation, the media were collected for the determination of released catecholamines, and the cells were lysed in 10% acetic acid and a freeze-thaw cycle for the determination of cellular catecholamine content.

2.8. Permeabilization of chromaffin cells

Cells cultured in 24-well culture plates were permeabilized in 250 μl of 140 mM potassium aspartate, 5 mM $MgCl_2$, 5 mM ATP, 5 mM glucose, 0.5 mM EGTA and 20 mM Pipes (pH 6.8), containing 10 μM digitonin. After 10 min, the buffer was removed and the cells were incubated for 10 min in permeabilization buffer containing 20 μM free calcium, as calculated according to Fabiato and Fabiato [23]. The cells were lysed in 10% acetic acid and the lysate was used for the determination of catecholamines.

2.9. Determination of catecholamines

Catecholamine content of incubation media and cell lysates was determined by the trihydroxyindole method, as modified by Kelner et al. [24], using epinephrine as a standard. The results on catecholamine release were expressed as percentage of control release from cells that were incubated in the absence of protein synthesis inhibitor.

2.10. Protein content and cell survival

Protein content of the samples was measured using the MicroBCA Protein Assay Reagent (Pierce). Cell survival was determined by the trypan blue exclusion test.

2.11. Statistical analyses

Results are expressed as the means ± S.E. Significance of differences was calculated with one-way analysis of variance (ANOVA).

3. Results and discussion

Antisense oligonucleotides have been used to block the expression of many different proteins [25], and we anticipated that this approach might also be useful in depleting synexin from secretory cells. We used two secretory cell lines, PC12 rat pheochromocytoma cells and GT1-7 mouse hypothalamic neurons, which were incubated with different concentrations (2–30 μM) of antisense phosphodiester or phosphorothioate oligonucleotides (5'-ATAGCCTGG-GTATGACAT-3'), complementary to the first 18 nucleotides of the coding region of mouse synexin MRNA. Two pairs of oligomers were used as controls: a sense

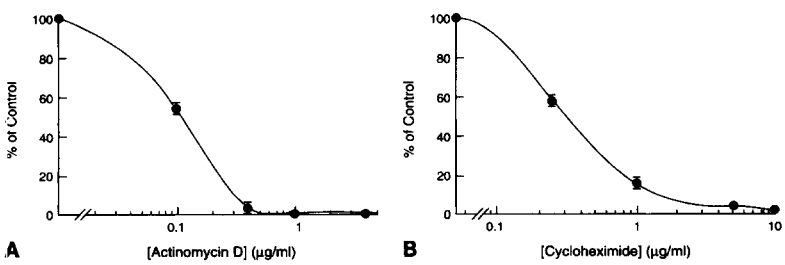

Fig. 1. Inhibition of [^{14}C]uridine incorporation by actinomycin D (A) and [14C]leucine incorporation by cycloheximide (B). Cultured bovine chromaffin cells were incubated in the presence of the radioactive precursor (1 μCi/ml) and different concentration of the protein synthesis inhibitor for 48 h. After incubation, radioisotope incorporation was determined as described in Methods. Each point represents the average of three determinations ± S.E.

oligonucleotide (5'-ATGTCATACCCAGGCTAT-3') and a misantisense oligonucleotide (5'-ATCGCATGGT-TATGACAG-3'), which has the same sequence as the antisense except for the presence of four mismatched bases. In spite of the long treatment with the antisense oligonucleotides (3–7 days), in the absence or presence of lipofectin, antisense phosphodiester or phosphorothioate oligonucleotides did not cause a decrease in synexin levels in PC12 and GT1-7 cells as determined by Western Blot. Attempts to lower synexin levels in human colon carcinoma cells, HT29 and T84, as well as pancreatic adenocarcinoma CFPAC-1 cells, were also unsuccessful.

Two different protein synthesis inhibitors were used to block synexin synthesis and the secretory response in chromaffin cells, since this is a good model of secretory cells. These included actinomycin D, which blocks the activity of RNA polymerase, and so prevents RNA and protein synthesis, and cycloheximide, which blocks the translocation reaction in ribosomes, and so prevents protein synthesis. Our initial problem was to determine the concentrations of actinomycin D and cycloheximide needed to block RNA and protein synthesis, respectively, in cultured chromaffin cells. Fig. 1 shows the effect of actinomycin D on [^{14}C]uridine incorporation (Fig. 1A) and cycloheximide on [^{14}C]leucine incorporation (Fig. 1B) in cells incubated for 48 h in the presence of the radioactive precursor. Maximal inhibitions (100%) were achieved with 1 μg/ml actinomycin D and 10 μg/ml cycloheximide, respectively. In subsequent experiments, we used 2 μg/ml actinomycin D and 5 μg/ml cycloheximide. After 120 h of treatment with 2 μg/ml actinomycin D and 5 μg/ml cycloheximide, [^{14}C]uridine and [^{14}C]leucine incorporation was blocked by 100% and 94%, respectively. Under those conditions, the respective cell survivals were 70% and 80% of the control values for actinomycin D and cycloheximide.

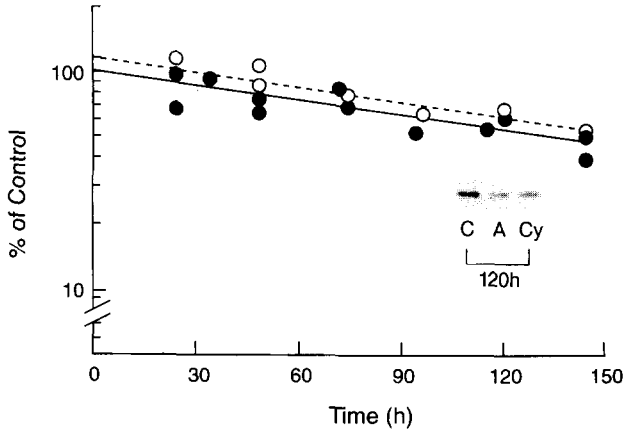

Fig. 2. Effect of actinomycin D and cycloheximide on synexin levels in bovine chromaffin cells. Chromaffin cells were incubated for varying periods of time with 2 μg/ml actinomycin D (●–●) or 5 μg/ml cycloheximide (○- - -○). After incubations, cells were collected, lysed in SDS-sample buffer, proteins were separated by SDS-PAGE and synexin was detected by Western blot. Controls are cells incubated in the absence of drugs. Inset: Western blot of synexin (47 kDa) after 120 h of incubation in the absence (control, C) or in the presence of 2 μg/ml Actinomycin D (A) or 5 μg/ml cycloheximide (Cy).

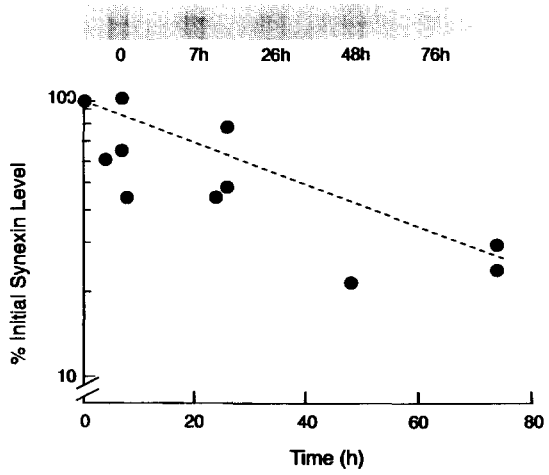

Fig. 3. Time-course of synexin degradation. Chromaffin cells were incubated with 20 μCi/ml [^{35}S]methionine. After 24 h, the radioactive precursor was removed from the medium, and cells were incubated with a normal medium (DMEM). At different time points after the [^{35}S]methionine pulse, samples were lysed and the antigen purified by immunoprecipitation and electrophoresis. The radioactivity of each band was determined as described under Methods. Data are from three experiments. Inset: SDS-PAGE of radiolabelled synexin (47 kDa) purified by immunoprecipitation at various incubation times after [^{35}S]methionine pulse.

The effects of actinomycin D and cycloheximide on synexin levels in chromaffin cells were determined by immunodetection using a monoclonal anti-(bovine)-synexin antibody (1OE7). Fig. 2 shows the time-course of the cellular synexin content in the presence of 2 μg/ml actinomycin D and 5 μg/ml cycloheximide. The basal synexin content of chromaffin cells was 70 ng/100 μg protein, as calculated using purified bovine lung synexin as a standard. Degradation constant (K_d) and half-time of degradation ($t_{1/2}$) in the presence of either synthesis inhibitor were approximately identical, and were $5.2 \cdot 10^{-3}$ h^{-1} and 133 h, respectively.

It has been demonstrated that cycloheximide and other synthesis inhibitors can prevent the degradation of certain cellular proteins [26–28], and can stabilize mRNAs [29–31]. This effect has been attributed to the blockage of cellular proteinases [28]. Therefore, to determine if actinomycin D and cycloheximide could delay synexin degradation, we measured synexin turnover in cells in which synexin was metabolically labelled with [^{35}S]methionine. Fig. 3 shows the time course of the loss of cellular [^{35}S]synexin content. The K_d and $t_{1/2}$ values of synexin degradation were 0.017 h^{-1} and 40 h, respectively. Thus, the $t_{1/2}$ obtained with this experimental approach was much smaller than the one obtained upon incubation with actinomycin D or cycloheximide. These data indicate that protein synthesis inhibitors cause an artefactual stabilization of synexin in chromaffin cells.

Table 1
Effect of cycloheximide on the secretory response from bovine chromaffin cells

Time	56 mM KCl		2 mM $BaCl_2$		20 μM $CaCl_2$	
	control response (%) [a]	inhibition (%) [b]	control response (%) [a]	inhibition (%) [b]	control response (%) [a]	inhibition (%) [b]
24 h	4.8 ± 0.9	77 ± 11	20.0 ± 1.8	82 ± 4	11.0 ± 1.2	82 ± 11
48 h	5.0 ± 0.8	71 ± 4	18.0 ± 0.9	84 ± 4 [$]	12.0 ± 1.1	87 ± 9 [&]
72 h	4.5 ± 1.0	72 ± 5 [*]	16.0 ± 1.2	90 ± 2 [*$]	8.0 ± 1.4	100 [*&]

Cultured bovine chromaffin cells were incubated in the presence of 5 μg/ml cycloheximide for 24, 48 and 72 h. After incubation, the secretory response induced by 56 mM KCl or 2 mM $BaCl_2$ in intact cells, or by 20 μM $CaCl_2$ in digitonin-permeabilized cells was evaluated as described in Methods. Data shown are control responses, expressed as percentage of total cellular catecholamine content [a] after 24, 48 and 72 h of incubation, and percentages inhibition of these responses by 5 μg/ml cycloheximide [b]. Values are means ± S.E. of three determinations.
[*] Difference among group means is significant with $P = 0.0003$.
[$] Difference between group means is significant ($P < 0.0003$).
[&] Difference between group means is significant ($P < 0.001$) (ANOVA).

We next proceeded to evaluate the effects of protein synthesis inhibitors on the response of intact and permeabilized chromaffin cells to different secretagogues. Catecholamine release in intact cells was induced by depolarization with high potassium or $BaCl_2$. Barium ions enter chromaffin cells via voltage-sensitive calcium channels, although they act by a different mechanism than calcium [32]. Catecholamine secretion in digitonin-permeabilized cells was induced by $CaCl_2$. In this case Ca^{2+} has direct access to the intracellular proteins responsible for the secretory events.

When cultured cells were incubated in the presence of 5 μg/ml cycloheximide for 24, 48 and 72 h, the secretory response induced by high extracellular potassium or $BaCl_2$ in intact cells, or by calcium in digitonin-permeabilized cells, was strongly reduced. After 24 h of incubation, the responses induced by the different stimulatory treatments appeared to be inhibited by 77%, 82% and 82%, respectively (Table 1). After this time, the cellular synexin content in the presence of cycloheximide was reduced by only 12%, as calculated using the determined K_d value. After 48 h, secretion was not changed significantly as compared to 24 h, in all cases. After 72 h, the inhibition of secretion induced by $BaCl_2$ in intact cells and $CaCl_2$ in permeabilized cells was further reduced significantly, while the response to high potassium was the same after 72 h as after 48 or 24 h of incubation with cycloheximide. At 48 and 72 h of incubation the cellular synexin content was decreased by 22% and 31%, respectively.

The extents of catecholamine secretion induced by the three different treatments in the presence of cycloheximide were not significantly different from each other after 24 h and after 48 h of incubation with the protein synthesis inhibitor. The lack of a significant difference between these values suggests that the same protein machinery is responsible for these secretory responses. However, a significant difference between the secretory responses was observed after 72 h of incubation with cycloheximide. Catecholamine release induced by high potassium was inhibited by 72% at this time, while release caused by $BaCl_2$ in intact cells or by $CaCl_2$ in permeabilized cells was inhibited by 90% and 100%, respectively. These data suggest that crucial proteins involved in the high potassium induced secretory response have a somewhat slower rate of degradation than the ones involved in the other two types of secretory responses.

On the basis of these findings, we can not reject the hypothesis that synexin participates in the secretion event, since a partial decrease in the cellular synexin content is accompanied by a decrease in the cells' secretory capacity. However, because the values of these two parameters, on a percentage basis, differ considerably, the results also suggest that other proteins with a shorter half-time of degradation participate in the fusion complex, and are thus also involved in the secretory process in chromaffin cells.

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