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SNAP-8 EFFICACY STUDIES IN VITRO

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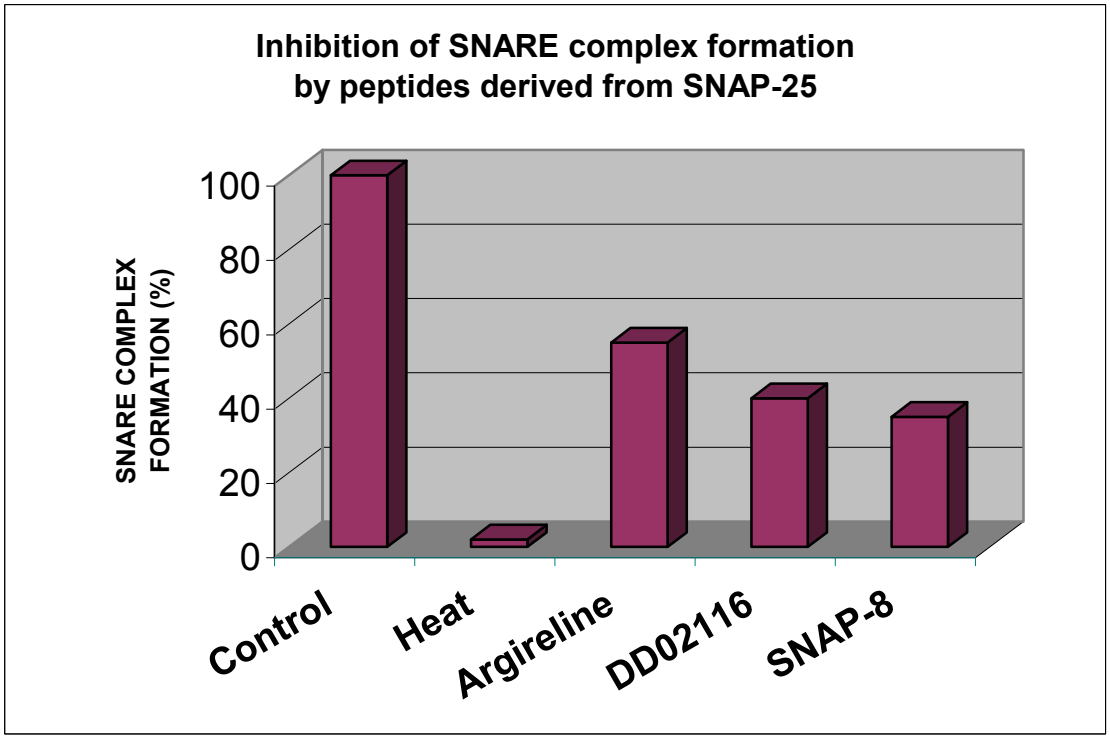
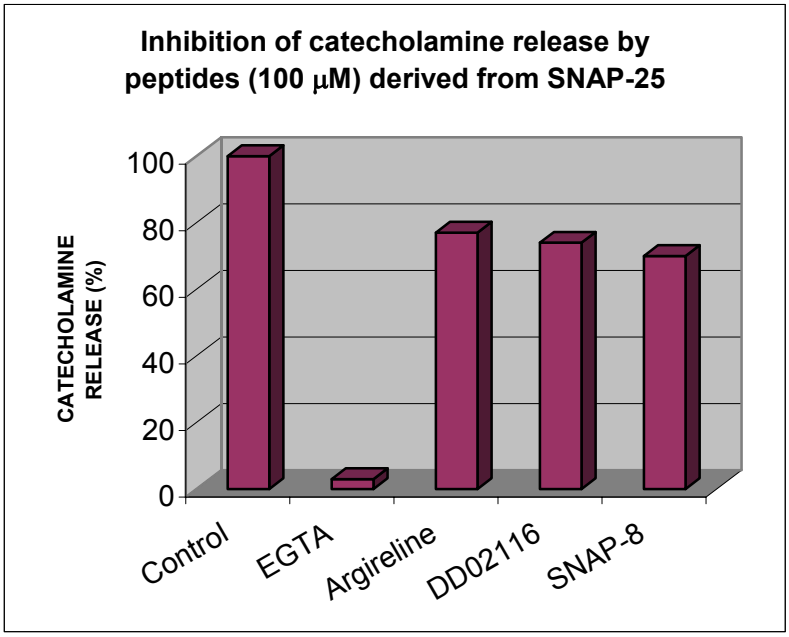
INHIBITION OF SNARE COMPLEX FORMATION

To assay the efficacy of small peptides on the stability of the SNARE complex we develop an in vitro method that readily allows us to follow the formation and thermal stability of the reconstituted protein complex. The rational of the method evaluates the antagonistic competitive efficacy of small peptides patterned after the SNAP-25 N-terminal domain with the wild type protein on its capacity to assemble with syntaxin and synaptobrevin forming the SNARE complex.



INHIBITION OF CATECHOLAMINE RELEASE

To test the cellular activity of the peptides, we measured the inhibitory activity of this peptide on Ca2+-evoked neurotransmitter release from digitonin-permeabilised chromaffin cells. This is a reliable assay that allows a rapid assessment of the biological activities of toxins and peptides.



ANTIWRINKLE ACTIVITY UNITS

Dose-response curves obtained from the catecholamine release tests yield IC50 values for the different peptides. We can therefore quantify and compare the exocytosis-blocking activity, which is directly related to the antiwrinkle power. This facilitates the definition of an Antiwrinkle Activity Unit (AAU), as described in *Int.Journal Cosm Sci*, 2002, 24, 303 – 310.

$$AAU_{SAMPLE} = [IC_{50}]_{ESUP\ E} / [IC_{50}]_{SAMPLE}$$

COMPOUND	**IC_{50}**	**AAU**
BoNT A	~0.0260 µM	12
ESUP E	0.310 µM	1 (by definition)
SNAP-8	55 µM	0.0056

PROTOCOLS

Method for SNARE complex destabilization by peptides.

To assay the efficacy of small peptides on the stability of the SNARE complex we develop an in vitro method that readily allows us to follow the formation and thermal stability of the reconstituted protein complex. The rational of the method evaluates the antagonistic competitive efficacy of small peptides patterned after the SNAP-25 N-terminal domain with the wild type protein on its capacity to assemble with syntaxin and synaptobrevin forming the SNARE complex. For this purpose, the concentration of SNAP25 is limiting to allow efficient peptide-SNAP-25 competition. A significant improvement of the method has been to use in vitro translated [^{35}S]SNAP25, that has remarkably increased the sensitivity level of the assay. The specific details of the *in vitro* reconstitution and modulation of SNARE complex are as follow:

1. *Expression and purification of recombinant syntaxin and synaptobrevin proteins.* Recombinant syntaxin and synaptobrevin were expressed in the E. coli strain BL21DE3 (Blanes-Mira et al. 2001). Protein expression was induced with 1 mM IPTG for 5 h at 30ºC. Bacterial cultures were pelleted, washed with lysis buffer (10 mM phosphate pH 7.4, 136 mM NaCl, 2,7 mM KCl), digested with 0.1 mg/ml lysozyme for 10 min at 22ºC in lysis buffer, supplemented with 2 mM PMSF, 5 mM iodoacetamide, 5 mM EDTA, and sonicated (3x45 s) in a Branson 250 sonifier at 4ºC. Lysates were solubilized with 1% Triton X-100 for 20 min at 4ºC and cleared by centrifugation at 20,000 x g for 30 min at 4ºC. GST-syntaxin and GST-synaptobrevin fusion proteins were present primarily as soluble fractions in the supernatant. Recombinant proteins were purified by affinity chromatography on glutathione agarose (Pharmacia) following manufacturer´s instructions. Purification of both proteins was performed in 20 mM Hepes pH 7.4, 100 mM NaCl, 0,05% n-octyl-β-D-glucopyranoside (OG), 5 mM DTT. Resin-bound fusion proteins were released by digestion with thrombin protease (Pharmacia) for 5 h at 23ºC. Recombinant proteins were dialyzed into 20 mM Hepes pH 7.4, 80 mM KCl, 20 mM NaCl, 0.1% OG buffer. Proteins concentration was assayed with the BCA kit (Pierce), and purity verified by SDS-PAGE analysis.

2. *In vitro* translation of [^{35}S]-SNAP-25. In vitro transcription-translation of the cDNA clone coding for SNAP-25 in the presence of [^{35}S]methionine was done by the couple reticulocyte lysate system from Promega (Ferrer-Montiel et al. 1996). Components were mixed and the reaction proceeded for 90 min at 30ºC. Translation was stopped with 5 μM cicloheximide. [^{35}S]-SNAP-25 integrity was analysed by SDS-PAGE and fluorography.

3. *In vitro SNARE complex assemby and modulation by peptides.* Equimolar amounts of recombinant syntaxin and synaptobrevin were incubated in the absence or presence of peptides at 4ºC for 2 h. Thereafter, 4 μl of [^{35}S]-SNAP-25 were added and mixture further incubated at 4ºC for 12 h. SNARE complex assembly was analysed by SDS-PAGE on 12% gels, followed by fluorographic detection on Kodak X-OMAT AR x-ray films. Temperature-dependent disassembly of SNARE complex was used to identify recombinant SNARE. Small peptides were tested at 1 and/or 2 mM.

Method for inhibition of catecholamine release.

1. *Chromaffin cell cultures.* Chromaffin cell culture were prepared from bovine adrenal glands by collagenase digestion and further separated from debris and erythrocytes by centrifugation on Percoll gradients as described (Gutierrez et al. 1997 and Ferrer-Montiel et al. 1998). Cells were maintained in monolayer cultures at a density of 625,000 cells/cm^2 and were used between the third and sixth day after plating. All the experiments were performed at 37 ^oC.

2. *Determination of catecholamine release from detergent-permeabilized chromaffin cells*. Secreted [^{3}H]noradrenaline was determined in digitonin-permeabilized cells as described (Gutierrez et al. 1997 and Ferrer-Montiel et al. 1998). Briefly, cells were incubated with [^{3}H]noradrenaline (1 µCi/ml) in DMEM supplemented with 0.56 mM ascorbic acid during 4 h. Thereafter, monolayers were washed 4 times with a Krebs/HEPES basal solution: 15 mM Hepes pH 7.4 with 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.56 mM ascorbic acid and 11 mM glucose. Cell permeabilization was accomplished with 20 µM digitonin in 20 mM Pipes, pH 6.8 with 140 mM monosodium glutamate, 2 mM $MgCl_2$, 2 mM Mg-ATP, and 5 mM EGTA. This incubation was carried out in the absence or presence of peptides or dithiothreitol (DTT)-reduced BoNT E as indicated. BoNT E was reduced with 10 mM DTT for 30 min at 37^{o}C. Following permeabilization, media were discarded and cells were incubated for 10 min in digitonin-free medium in presence or absence of peptides. Basal secretion was measured in 5 mM EGTA, whereas stimulated secretion was measured in a medium containing 10 µM buffered Ca^{2+} solution. Media were collected and released catecholamines as well as the total cell content were determined by liquid scintillation counting. The CPM released from control cells under basal conditions were ~3,000, and they were increased to ~11,000, when stimulated with 10 µM Ca^{2+}. The total number of counts obtained from detergent-permeabilized cells was ~110,000. Thus, the normalized basal release represents the 3.5% of the total secretion, and the Ca^{2+}-evoked ~10%. Statistical significance was calculated using Student *t*-test with data from 4 or more independent experiments.

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