

# PHOSPHOLIPASE A<sub>2</sub> (PLA<sub>2</sub>) REGULATES NEUROEXOCYTOSIS TO COUNTERACT BOTULINUM TOXIN A (BoNT/A) POISONING

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## ABSTRACT

In this study, BoNT/A is one of the most serious biological threats faced by the U. S. military and allied forces. Previously we reported that in nerve growth factor (NGF)-differentiated PC12 cells, arachidonic acid (AA) release is associated with acetylcholine (ACh) release and BoNT/A inhibits both. We report the effect of PLA<sub>2</sub> over expression on inhibition of ACh exocytosis due to BoNT/A light chain (LC) in PC12 cells. Over expression of PLA<sub>2</sub> alone augmented the stimulated release of ACh and AA. PLA<sub>2</sub> over expression also effectively prevented the inhibition of stimulated ACh and AA release due to BoNT/A LC.

## INTRODUCTION

Botulinum toxin type A (BoNT/A) acts by blocking Ca<sup>2+</sup>-dependent ACh release (neuroexocytosis) at peripheral neuromuscular junctions, sometimes causing fatal neuromuscular paralysis. The clonal pheochromocytoma PC12 cell line was used to study the biochemical mechanisms of action of BoNT/A. To explain the mechanism of action of BoNT/A, we previously reported that when NGF differentiated PC12 cells are stimulated with high K<sup>+</sup> (80 mM), AA and ACh are co-released and BoNT/A inhibits both release processes. (Ray, P. *et al.*, 1993, J. Biol. Chem., 268, 11057-11064). Stimulus-induced ACh release and AA release are inhibited in the presence of Ca<sup>2+</sup>-dependent PLA<sub>2</sub> inhibitors (AACOCF<sub>3</sub>, DEDA). On the contrary, PLA<sub>2</sub> activators such as mastoparan can cause ACh release in cells (Ray, P. *et al.*, 1997, NeuroReport, 8, 2271-2274).

PLA<sub>2</sub> acts on membrane phospholipids to generate AA and lysophosphatidic acid (LPA). AA acts as a fusogen to induce exocytosis. LPA activates Rho GTPases, which have been implicated in mechanisms correlating actin cytoskeletal organization and exocytosis. In this study, we show that PLA<sub>2</sub> over expression prevented the inhibition of stimulated ACh and AA release due to BoNT/A LC.

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## METHODS

### CELL CULTURE

PC12 cells (CLONTECH) were grown in 75-cm<sup>2</sup> tissue culture flasks in DMEM (GIBCO) containing 5 % (v/v) FBS 10 % (v/v) donor horse serum (CLONTECH), streptomycin 100 µg/ml and 100 units/ml penicillin G sodium (Sigma). The cells ( $1.5 \times 10^6$ ) were incubated at 37°C in a humidified atmosphere of 90% air 10% CO<sub>2</sub>. After 3 days the cells were differentiated with nerve growth factor (NGF, 50 ng/ml growth medium) for 4 days.

### sPLA<sub>2</sub> OVER EXPRESSION IN PC12 CELLS

The levels of sPLA<sub>2</sub> in differentiated PC12 cells after transfection with pTracer-PLA<sub>2</sub> was analysed by immunoprecipitation and Western blotting analysis using rabbit antiserum against sPLA<sub>2</sub> (Cayman Chemical ).

### BoNT/A LC OVER EXPRESSION IN PC12 CELLS

Expression of BoNT/A LC after transfection was analysed by Western blotting with use of GFP monoclonal antibody (CLONTECH).

### SNAP-25 DETECTION

SNAP-25 was visualized by SNAP-25 antibody and TRITC conjugated IgG.

### ARACHIDONIC ACID (AA) RELEASE ASSAY

Arachidonic acid release assay was performed by modification of the method of Ray.P et al. as described (J. Biol. Chem., 268, 11057-11064).

### ACETYLCHOLINE (ACH) RELEASE ASSAY

Acetylcholine release assay was performed by modification of method of Ray.P et al. as described (J. Biol. Chem., 268, 11057-11064).

## RESULTS

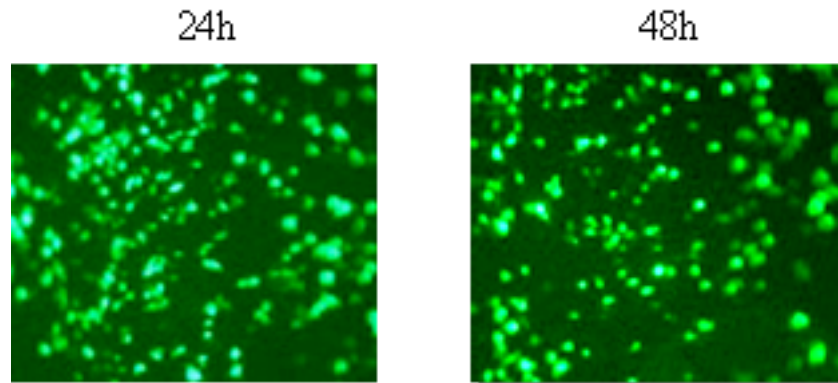


Figure 1. Transfection efficiency of PC 12 cells by pEGFP-BoNT/A LC

The transfection efficiency (>70%) was calculated twenty-four hours and forty-eight hours after transfection with pEGFP-BoNT/A LC by fluorescence microscope. Images were taken twenty-four hours and forty-eight hours after transfection

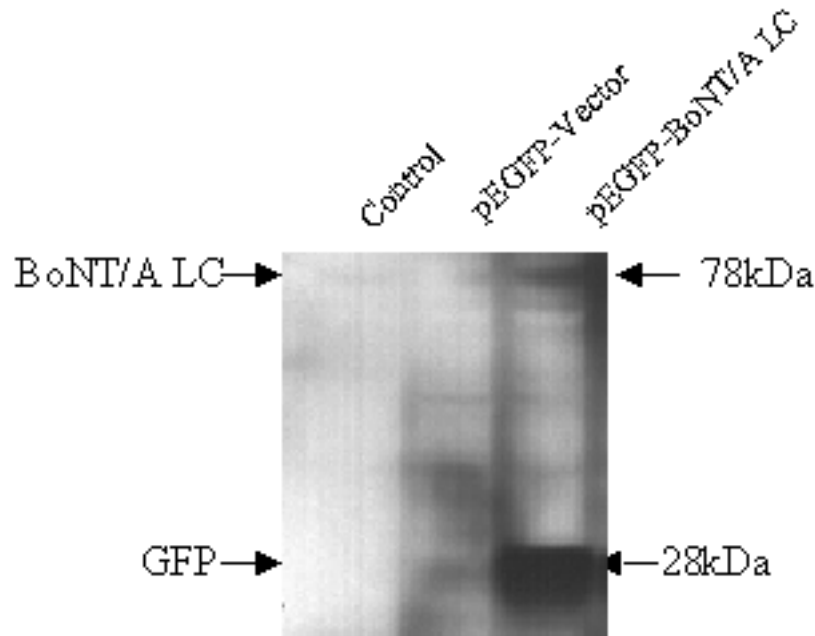


Figure 2. BoNT/A LC over expression in PC12 cells

Twenty-four hours after transfection of differentiated PC 12 cells with pEGFP-BoNT/A LC, the expression level of LC was confirmed by Western blotting using of GFP antibody. Control: No GFP was expressed; Transfection with pEGFP vector alone: GFP (28 kDa) was expressed; Transfection with pEGFP-BoNT/A LC: GFP-fused LC (78 kDa) was expressed.

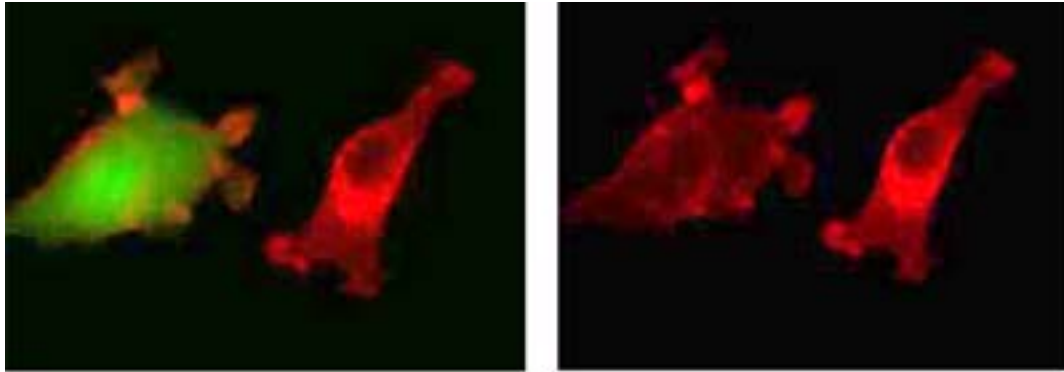


Figure 3. Effect of transfection of BoNT/A LC on SNAP-25 degradation

Twenty-four hours after transfection of differentiated PC12 cells with pEGFP-BoNT/A LC, SNAP-25 was visualized by SNAP-25 antibody and TRITC conjugated IgG. There was a marked degradation of SNAP-25 (red) in LC expressing cells (green), but no degradation was seen in untransfected cells.



Figure 4. sPLA<sub>2</sub> over expression

Immunoprecipitation followed by Western blotting analysis with sPLA<sub>2</sub> antibody, showed that transfection with sPLA<sub>2</sub> over expressed sPLA<sub>2</sub> (14 kDa). However, control cell (no transfection) or vector alone transfection did not express detectable amount of the protein.

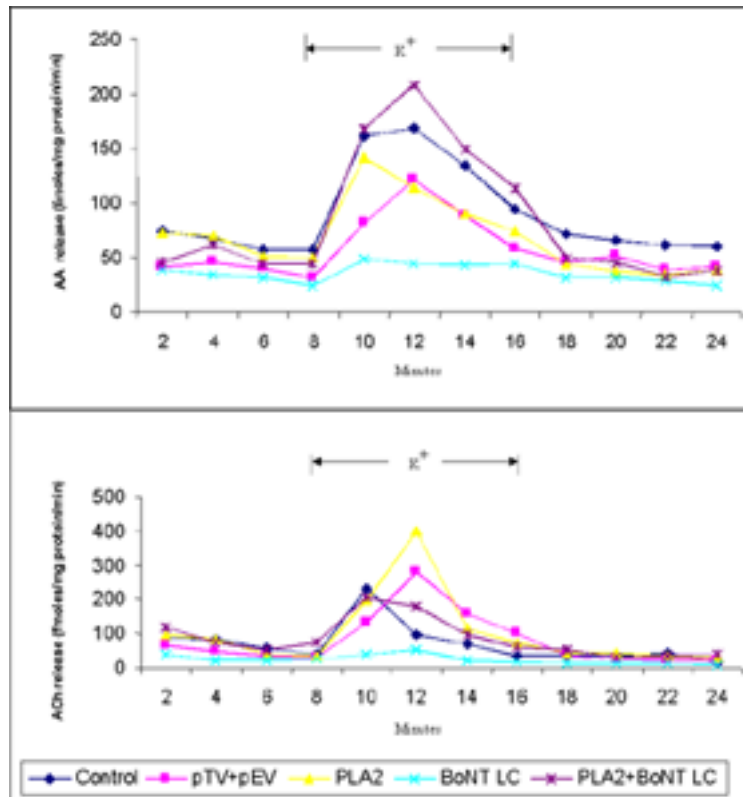


Figure 5. Effect of cotransfection of BoNT/A LC and PLA<sub>2</sub> on AA and ACh release

NGF-differentiated PC12 cells were transiently transfected with either pEGFP (pEV)-BoNT/A LC or cotransfected with pTracer (pTV)-sPLA<sub>2</sub>. Over expression of PLA<sub>2</sub> in these BoNT/A LC transfected cells effectively prevented the inhibition of stimulated (by high K<sup>+</sup>) ACh and AA release due to BoNT/A LC.

## CONCLUSION

- PLA<sub>2</sub> mediated mechanisms may regulate presynaptic ACh exocytosis.
- BoNT/A inhibits neuroexocytosis by targeting these mechanisms.
- Manipulating PLA<sub>2</sub> mechanisms in neuroexocytosis may serve as prospective strategies to counteract BoNT/A poisoning.

## REFERENCES

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