Volume 219, number 1, 219–223 FEB 04903 July 1987

Botulinum neurotoxin inhibits depolarization-stimulated protein phosphorylation in pure cholinergic synaptosomes

Xavier Guitart, Gustau Egea, Carles Solsona and Jordi Marsal

Departament de Biologia Cellular i Anatomia Patològica, Facultat de Medicina, Hospital de Bellvitge, Universitat de Barcelona, Casanova, 143, E-08036 Barcelona, Spain

Received 20 May 1987

Botulinum neurotoxin, a strong blocker of acetylcholine release at peripheral cholinergic synapses, inhibits depolarization-stimulated protein phosphorylation in pure cholinergic synaptosomes isolated from the electric organ of *Torpedo marmorata*. Moreover, tetrodotoxin has the same effect on protein phosphorylation when cholinergic synaptosomes are depolarized by veratridine. Correlation between presynaptic protein phosphorylation and acetylcholine release is suggested by the fact that botulinum neurotoxin blocks specifically neurotransmitter release without affecting membrane depolarization and calcium fluxes in our synaptosomal preparation.

Botulinum neurotoxin; Protein phosphorylation; Acetylcholine release; Presynaptic membrane; (*Torpedo* cholinergic synaptosome)

1. INTRODUCTION

Cholinergic synaptosomes isolated from the electric organ of *Torpedo* are a useful model for studying the mechanisms of ACh release, since the synthesis of neurotransmitter, uptake of its precursors [1], induced release of ACh [2–4] and calcium fluxes [5,6] have been thoroughly characterized in these nerve endings.

Protein phosphorylation has been implicated in the molecular mechanisms of neurotransmitter release [7,8], since depolarization of nerve endings results in the phosphorylation of specific proteins in rat brain synaptosomes [9,10] and *Torpedo* cholinergic synaptosomes [11].

Correspondence address: J. Marsal, Departament de Biologia Cellular i Anatomia Patològica, Facultat de Medicina, Hospital de Bellvitge, Universitat de Barcelona, Casanova, 143, E-08036 Barcelona, Spain

Abbreviations: ACh, acetylcholine; BoNTx, botulinum neurotoxin; TTX, tetrodotoxin

ACh release can be induced by several depolarizing agents such as veratridine which increases the sodium membrane conductivity and triggers ACh release. This effect can be prevented by TTX, a known blocker of the sodium current.

On the other hand, BoNTx is a strong and specific blocker of ACh release at the neuromuscular junction (review [12]), at the electric organ [13] and in synaptosomes isolated from the electric organ of *T. marmorata* [14]. We have described in the latter preparation that BoNTx affects neither the membrane potential nor the calcium uptake, under either resting or depolarizing conditions, whereas the ACh release is blocked [14]. Therefore, BoNTx can be a tool for investigating whether protein phosphorylation is linked to the ACh release mechanisms. Moreover, it has been suggested [15] that BoNTx may act on protein kinases or synapsin I, which are presumably involved in neurotransmitter release [16].

Using pure cholinergic synaptosomes from the electric organ of *Torpedo*, we report here the effect

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00145793/87/$3.50

of depolarizing agents, such as a high extracellular potassium concentration or veratridine, on the rate of phosphorylation of synaptosomal proteins during ACh release. In order to relate protein phosphorylation and ACh release we have investigated the effects of BoNTx (an acetylcholine release blocker) and TTX (a depolarization blocker) on synaptosomal protein phosphorylation.

2. MATERIALS AND METHODS

2.1. *Materials*

Veratridine was from Sigma, TTX from Boehringer Mannheim and the Ca^{2+} ionophore A23187 from Calbiochem. $^{32}P_i$, as orthophosphate, was from The Radiochemical Centre (Amersham, England). Acrylamide, *N,N'*-methylenebisacrylamide, SDS, *N,N,N',N'*-tetramethylethylenediamine and ammonium persulfate were from BioRad. Crystalline toxin preparations were obtained from a culture of *Clostridium botulinum* type A (NCTC 2916) [17,18] and hemagglutinin was removed from the toxin complex by affinity and DEAE-Sephacel chromatography, as described [19,20]. The LD_{50} of the purified neurotoxin amounts to 0.66 ng/kg in mice. All other reagents used were analytical grade.

2.2. *Cholinergic synaptosomes*

Fragments of electric organ excised from living specimens of *T. marmorata* were kept in a saline solution containing (in mM): 280 NaCl, 3 KCl, 1.8 $MgCl_2$, 3.4 $CaCl_2$, 100 sucrose, 300 urea, 5.5 glucose, 3.6 Hepes/NaOH buffer (pH 6.8) and 5 $NaHCO_3$ to give a pH of 7.0. Pure cholinergic synaptosomes were isolated from these fragments as in [21]. The synaptosomal fraction (0.2 mg protein/ml) was concentrated in the presence of a calcium-free saline solution containing 2 mM EGTA and then resuspended (2 mg protein/ml) in an oxygenated calcium-free medium containing 0.1 mM EGTA. All steps were carried out at 4°C.

2.3. *^{32}P labeling of synaptosomes and depolarization-induced phosphorylation*

The synaptosomal suspension, equilibrated for 15 min at room temperature, was incubated in the presence of carrier-free $^{32}P_i$ (0.250 mCi/ml final concentration) for 45 min at room temperature with constant shaking. When the effects of TTX and BoNTx were tested, these toxins were added during the last 10 min of the labeling period, at a final concentration of 40 μg/ml and 1.25 nM, respectively, and aliquots of these intoxicated synaptosomes were then stimulated. To analyze the effects of depolarization on phosphorylation patterns, aliquots of $^{32}P_i$-labeled synaptosomes (50–60 μg synaptosomal protein) were added to reaction tubes containing the same volume of saline solution supplemented with 50 mM KCl (substituting for NaCl), or 100 μM veratridine or 10 μM A23187 plus 10 mM $CaCl_2$ (final concentrations). All reactions were stopped after 30 s by adding 30 μl of a stopping solution containing SDS and 2-mercaptoethanol [22].

2.4. *Polyacrylamide gel electrophoresis and protein phosphorylation measurement*

Phosphorylated samples were loaded on SDS-polyacrylamide slab gels consisting of a 4% stacking gel and a 6–17% separating gel. After drying, gels were exposed to Kodak X-Omat RP films at −80°C for 3–5 days, using intensifying screens. Developed autoradiograms were scanned and the ^{32}P-labeled polypeptide bands quantified by measuring the areas under the peaks [9]. The area of these peaks was assumed to be proportional to the amount of ^{32}P incorporated into different proteins, the results being expressed in arbitrary units and as experimental/control peak ratios.

3. RESULTS AND DISCUSSION

As demonstrated by Michaelson and Avissar [11], the incubation of cholinergic synaptosomes of *Torpedo* with $^{32}P_i$ results in the incorporation of phosphate into several proteins. In addition, the depolarization-induced Ca^{2+} uptake linked to ACh release has been correlated with the state of phosphorylation of a specific protein band of approx. 100 kDa [11]. These results do not demonstrate a causal relationship between ACh release and protein phosphorylation [11], since the increase in protein phosphorylation could at least be due to either the calcium uptake process or the ACh release mechanism.

Our results show that the state of phosphorylation of some synaptosomal proteins can be modified by potassium-induced depolarization of

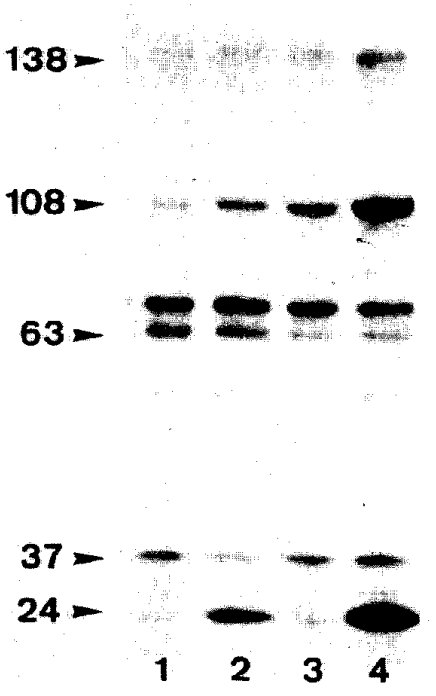

Fig.1. Autoradiogram showing the effect of potassium-induced depolarization on synaptosomal protein phosphorylation in botulinized and non-botulinized cholinergic synaptosomes. All samples contained calcium (3.4 mM). Lanes: (1) BoNTx, 3 mM KCl; (2) BoNTx, 50 mM KCl; (3) 3 mM KCl; (4) 50 mM KCl. Apparent molecular masses (in kDa) of selected proteins are indicated by arrowheads.

cholinergic synaptosomes in the presence of calcium; more specifically, we have observed an increase in the state of phosphorylation of five protein bands with molecular masses of 138, 108, 63, 37 and 24 kDa (fig.1 and table 1). The same changes in phosphorylation pattern are obtained using other stimulating agents such as veratridine (table 2) or the Ca^{2+} ionophore A23187 (table 3). Partially similar results have been reported previously [11].

We have also investigated whether phosphorylation of synaptosomal proteins is linked to ACh release by using BoNTx, since this toxin inhibits potassium-induced ACh release without affecting Ca^{2+} uptake and membrane potential in cholinergic synaptosomes of *Torpedo* electric

Table 1

Effect of botulinum neurotoxin on protein phosphorylation

Protein band (kDa)	Phosphorylation ratios (experimental/control)		
	K^+ (50 mM)	BoNTx	BoNTx + K^+ (50 mM)
138	1.89 ± 0.14	0.77 ± 0.14	0.84 ± 0.05
108	3.07 ± 0.34	0.88 ± 0.08	0.94 ± 0.08
63	1.54 ± 0.11	2.87 ± 0.23	2.52 ± 0.67
37	1.35 ± 0.12	1.21 ± 0.15	0.48 ± 0.06
24	5.54 ± 1.35	0.79 ± 0.08	1.35 ± 0.22

After prelabeling with ^{32}P, the synaptosomes were intoxicated or not with BoNTx as described in section 2 and then depolarised. The synaptosomal proteins were then run in polyacrylamide gels and the autoradiograms scanned. The areas under the peaks provide a measure of protein phosphorylation. The results, expressed as ratios between experimental and control peaks, are from 4 different experiments (means ± SE)

Table 2

Partial reversion by A23187 of BoNTx action on synaptosomal protein phosphorylation

Protein band (kDa)	Phosphorylation ratios (experimental/control)	
	A23187	A23187 + BoNTx
138	1.98 ± 0.21	0.89 ± 0.05
108	3.28 ± 0.63	2.08 ± 0.14
63	2.11 ± 0.41	3.06 ± 0.21
37	2.12 ± 0.52	1.28 ± 0.09
24	5.81 ± 0.73	2.11 ± 0.39

After prelabeling with $^{32}P_i$, botulinized and non-botulinized synaptosomes were stimulated with A23187 for 30 s. Protein phosphorylation was measured as described in section 2. Results are from three separate experiments and express means ± SE of the ratio between experimental and control peak conditions. Partial reversion is observed if we compare these results with those of table 1

organ [14]. Under non-depolarizing conditions, BoNTx causes an increase in phosphorylation state of a 63 kDa protein band. Furthermore, botulinization of synaptosomes prior to

Table 3

Effect of veratridine and TTX on protein phosphorylation

Protein band (kDa)	Phosphorylation ratios (experimental/control)		
	VT	TTX	VT+TTX
138	2.35 ± 0.35	1.18 ± 0.36	1.15 ± 0.41
108	2.18 ± 0.02	1.28 ± 0.09	1.16 ± 0.14
63	1.53 ± 0.11	3.68 ± 0.85	2.90 ± 0.15
37	2.08 ± 0.23	1.12 ± 0.14	2.15 ± 0.19
24	6.56 ± 1.17	1.38 ± 0.17	1.16 ± 0.11

After prelabeling, synaptosomes were depolarized by veratridine in the absence or presence of tetrodotoxin. The synaptosomal proteins were then run in polyacrylamide gels and the autoradiograms obtained from these scanned. The areas under the peaks provide a measure of protein phosphorylation. The results, expressed as ratios between experimental and control peaks, are from 4 different experiments (means ± SE)

depolarization by potassium prevents the increase in phosphorylation of the 138, 108 and 24 kDa protein bands (fig.1 and table 1) and even induces dephosphorylation of the 37 kDa protein band.

A protein fraction (fraction 2), involved in the release of ACh when incorporated into artificial membranes, has recently been isolated from the presynaptic membrane of pure cholinergic synaptosomes [23]. Four major bands are always found in this fraction, one of them being a 36 kDa protein, that could correspond to the 37 kDa protein described here.

It has been reported that the Ca^{2+} ionophore A23187 can partially revert the ACh release blockade induced by BoNTx in the presence of a high calcium level [14]. In our preparation, BoNTx also inhibits protein phosphorylation stimulated by A23187 in the 138, 108, 37 and 24 kDa bands (table 2), but to a lesser extent than in the case of potassium-induced depolarization (table 1), which can perhaps be taken as a partial reversion by the ionophore of the depolarization-stimulated phosphorylation. This reversion is more apparent in the 108 kDa protein band (table 2 and fig.2). This fact also suggests that these proteins could be related to the ACh release mechanism, since there is a simultaneous partial restitution of ACh release and protein phosphorylation.

Since protein phosphorylation seems to be related to ACh release, we have tested the effect of TTX, which binds to the sodium channel and prevents the depolarizing effect of veratridine, and thus depolarization and ACh release. Veratridine is also able to stimulate (table 3), in a calcium-dependent manner, phosphorylation of the same proteins that are phosphorylated by increasing the extracellular potassium concentration (table 1). Under non-depolarizing conditions, TTX does not change the pattern of protein phosphorylation except in the case of the 63 kDa peptide, which presents a large increase in incorporation of $^{32}P_i$ (table 3). When synaptosomes treated with TTX are depolarized by veratridine we observe a phosphorylation rate of the 138, 108 and 24 kDa proteins similar to that under resting conditions (table 3).

When the effects of BoNTx and TTX are compared, it is interesting to point out that TTX does not prevent the increase in phosphorylation of the 37 kDa protein induced by veratridine (table 3) and, in contrast, BoNTx dephosphorylates this protein (table 1). On the other hand, the 63 kDa protein shows a similar phosphorylation rate irrespective of the agent used to inhibit ACh release. A protein of 63 kDa has been found in the *Torpedo* electric organ [24], suggesting that it could be a subunit of *Torpedo* neurofilaments. This

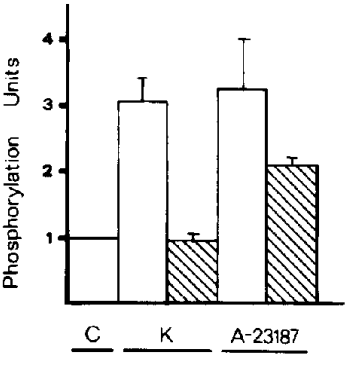

Fig.2. Effect of the Ca^{2+} ionophore A23187, in the presence of a high calcium concentration (10 mM), on phosphorylation of the 108 kDa synaptosomal protein in the presence or absence of botulinum toxin. (C) Control; (K) 50 mM KCl. (A23187) 10 μM A23187 plus 10 mM $CaCl_2$. Dashed bars indicate the presence of botulinum toxin.

cytoskeletal protein, a minor constituent of the electric organ could be homologous to the L-polypeptide of the mammalian neurofilament triplet. We can speculate that this protein exists in several forms, more or less phosphorylated, and that their degree of phosphorylation is related to the ACh release mechanism. Altogether, our results suggest that phosphorylation of synaptosomal proteins can be a regulatory step in the ACh release mechanism, since, during synaptic activity, ACh release and protein phosphorylation are both stimulated by depolarization and inhibited (with the exception of the 63 kDa band) by the same set of drugs. Further experiments are necessary to clarify the precise correlation between protein phosphorylation and neurotransmitter release.

ACKNOWLEDGEMENTS

We thank Dr P. Arté (Institut d'Investigaciones Pesqueres, CSIC, Barcelona, Spain) for providing specimens of *T. marmorata*. We are indebted to Dra A. Casanova for the preparation of *C. botulinum* culture and to the Departament de Genètica Molecular, CID, CSIC, Barcelona, for technical facilities. This work was supported by CAICYT grant 84/2154 and CIRIT grant AR82/2-73. G.E. is a fellow from FPI (MEC).

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