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ATP release from pure cholinergic synaptosomes is not blocked by tetanus toxin

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Tetanus toxin (TeTx) is a neurotransmission impairing toxin that acts on several neurotransmitter systems. TeTx also inhibits the K^+-induced release of acetylcholine (ACh) from synaptosomes isolated from the electric organ of *Torpedo*. Neither the membrane potential and depolarization, nor the depolarization-induced calcium uptake into cholinergic nerve terminals is modified after TeTx poisoning. On the other hand, it is known that, when cholinergic nerve terminals are stimulated, there is a release of ATP associated with the release of ACh. We have explored the action of TeTx on this co-release, and have found that there is no action of TeTx on the nucleotide release. Thus, TeTx blocks ACh release without modifying ATP release.

Tetanus toxin; ATP release; Acetylcholine release; Ca^{2+} uptake; Membrane potential; Cholinergic synaptosome

1. INTRODUCTION

As it is known [1], ATP is stored within synaptic vesicles, and, when cholinergic nerve terminals are depolarised, ATP is co-released with ACh [2,3]. Both, ATP and ACh release are calcium dependent processes [4]. TeTx, an exotoxin produced by *Clostridium tetani*, impairs neurotransmitter release from several different neurotransmitter systems (review [5]) and, also, like botulinum toxin type A (BoTx), another clostridial toxin, blocks neuromuscular transmission by blocking ACh release [6,7]. The electric organ of *Torpedo marmorata* is a modified neuromuscular system, in which presynaptic nerve terminals are highly multiplied [8]. From this tissue, a fraction of pure cholinergic nerve endings can be obtained [9], which are a suitable model to study ACh and ATP co-release. Using this fraction of pure cholinergic synaptosomes, we have observed that BoTx inhibits ACh release without modifying ATP release [10]. Since there are many similarities between the actions of TeTx and BoTx, it was interesting to explore the actions of TeTx on the co-release of ACh and ATP from the nerve terminals of the electric organ. Thus, we have re-examined the co-release of ACh and ATP by investigating the action of TeTx on the K^+-induced release of ACh and ATP from isolated nerve endings of the *Torpedo* electric organ.

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Abbreviations: ACh, acetylcholine; BoTx, botulinum toxin; TeTx, tetanus toxin

2. MATERIALS AND METHODS

2.1. *Materials*

T. marmorata specimens were caught from the Catalan coast of the Mediterranean sea, and maintained in seawater. Tetanus toxin was purified by

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one of us (B.B.) as described [11]. The fluorescent dye 3,3′-di-ethyl-thiadicarbocyanine iodide was a gift from Dr F.M. Meunier (CNRS, Gif-sur-Yvette, France). Choline oxidase, horseradish peroxidase, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), D-luciferin and luciferase were purchased from Sigma (USA), and $^{45}CaCl_2$ (27.1 mCi/mg) from Amersham (England). All other chemicals used were of reagent grade.

2.2. *Isolation of cholinergic synaptosomes*

Excised fragments of electric organ 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 $NaHCO_3$ to give a pH of 7.0. Pure cholinergic synaptosomes were isolated as described elsewhere [12].

2.3. *ACh and ATP release measurement*

ACh and ATP release were continuously monitored by chemiluminescent methods. ACh release was detected according to Israel and Lesbats [13] with the luminol-peroxidase reaction. ATP release was measured with the luciferin-luciferase technique [4]. In both cases, release was triggered by the addition of KCl (100 mM, final concentration). TeTx was applied at a concentration of 12.5 nM in the medium during 10 min, before the addition of the luminescent mixtures.

2.4. *Calcium uptake determination*

^{45}Ca uptake by the cholinergic synaptosomes was measured by Millipore filtration as described [14]. Synaptosomes were incubated with $^{45}CaCl_2$ (2 μCi/ml), either at rest or during a KCl (100 mM, final concentration) depolarization, for 30 s. TeTx was applied to the external medium 10 min before the addition of the labelled Ca^{2+}.

2.5. *Membrane potential measurement*

The membrane potential of the cholinergic synaptosomes was monitored with a fluorescent dye as described by Meunier [15]. The dye, 3,3′-di-ethyl-thiadicarbocyanine iodide was added to the synaptosomal suspension (4 μM, final concentration). Synaptosomes were depolarized by high external potassium concentration (100 mM).

3. RESULTS

3.1. *Calcium uptake*

Since ACh and ATP release are calcium dependent in potassium depolarized synaptosomes [4], we have investigated calcium uptake by the nerve terminals during the action of TeTx either, in resting conditions, or during a K^+ depolarization. After depolarization, calcium uptake is increased about 2-fold with respect to the resting condition (table 1). Our results show that calcium uptake is not modified by TeTx in any condition.

3.2. *Membrane potential*

The relationship between the changes in the fluorescent intensity, and the logarithm of the potassium concentration in the medium, in presence of valinomycin, is linear [15]. This finding allows us to calibrate in mV the fluorescent signals, using the Nernst equation. Using this technique, we have seen that TeTx does not modify

Table 1

Action of tetanus toxin on the cholinergic synaptosomes

	Control	Tetanus toxin
Calcium uptake (nmol/mg protein per min) (n = 3)		
Resting conditions	10.9 ± 2.5	9.5 ± 2.4
After K^+ depolarization	21.6 ± 1.5	22.1 ± 2.1
Membrane potential (mV) (n = 3)		
Resting conditions	−50.3 ± 2.2	−51.7 ± 1.9
After K^+ depolarization	−22.5 ± 1.4	−20.4 ± 2.1
ACh release (pmol/g_t tissue) (n = 4)	2187 ± 352	638 ± 213[a]
ATP release (pmol/g_t tissue) (n = 3)	142 ± 13	153 ± 17

Calcium uptake and membrane depolarization of cholinergic synaptosomes poisoned with TeTx were induced by stimulation with KCl (100 mM, final concentration). Results were compared to non-poisoned synaptosomes. K^+ (100 mM)-induced release of ACh and ATP (given in pmol per g of initial tissue) were chemiluminescently monitored and quantified.

Statistical significance, [a] $p < 0.01$

the resting potential of cholinergic synaptosomes, nor the decrease of membrane potential reached after 100 mM K^+ depolarization (see table 1).

3.3. *ACh release*

As we have mentioned, TeTx inhibits ACh release from neuromuscular junction [6]. Here we have assayed the ability of this toxin to inhibit ACh release from pure cholinergic synaptosomes isolated from the electric organ of *Torpedo*. When we have continuously measured the K^+-induced ACh release by the chemiluminescent technique, we have found that TeTx has an inhibitory effect on ACh release, up to 70% (table 1).

3.4. *ATP release*

Since ATP is co-stored with ACh in the cholinergic synaptic vesicles, and co-released with the neurotransmitter when cholinergic nerve terminals are depolarized [3], we have also explored the action of TeTx on the release of ATP. Our results (table 1) show that the K^+-induced release of ATP, continuously monitored by the luciferin-luciferase reaction, is not affected by the toxin.

4. DISCUSSION

Isolated nerve terminals from the electric organ of *T. marmorata* are a pure subcellular fraction [9] that is an efficient tool to study ACh and ATP release from the cholinergic synapse. ACh release from the neuromuscular junction is blocked by TeTx [6,7] and, as it has been shown in this paper, the K^+-induced release of ACh from isolated cholinergic synaptosomes is also inhibited by TeTx. BoTx, another neurotoxin produced by related microorganisms (*C. botulinum*), that acts specifically at the cholinergic synapse (see [16] as a review), also reduces ACh release from the isolated cholinergic synaptosomes [10]. It has been reported [17] that both toxins do not modify the ionic fluxes through the presynaptic membrane. Furthermore, since calcium entry is the determinant for the release of the neurotransmitter, we have studied the action of TeTx on the calcium uptake by the synaptosomes. Like BoTx [10], TeTx does not modify calcium uptake either at rest or after K^+ depolarization.

It is known that ATP is co-stored with ACh in cholinergic synaptic vesicles isolated from the electric organ of *T. marmorata* [1]. During the K^+ depolarization of isolated *Torpedo* electric organ synaptosomes, there is a simultaneous release of ACh and ATP, which exhibits similar release kinetics [3,18]. But, as it has been demonstrated, BoTx inhibits ACh release from isolated cholinergic nerve endings without modifying ATP release. Moreover, here we have explored the action of TeTx on the nucleotide release, and our results show that TeTx, like BoTx, does not alter the K^+-induced release of ATP. Thus, it seems that ACh release and ATP release are two separate processes. Different possibilities arise from this result. Are there two kinds of synaptic vesicle populations? Are ATP and ACh released by the same mechanism? Recently, it has been suggested that ACh could be released non-exocytotically from the cytoplasmic pool of neurotransmitter (review [19,20]), since proteoliposomes which contain presynaptic membrane proteins are able to promote an induced release of ACh [21,22]. Further work is needed to understand the mechanisms of ACh and ATP release from cholinergic nerve endings.

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