BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **244,** 275–279 (1998)
ARTICLE NO. RC988188

Negative Chronotropic Effect of Botulinum Toxin on Neonatal Rat Cardiac Myocytes

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Received January 20, 1998

We demonstrated that botulinum neurotoxin attenuated the spontaneous beating rate of cultured cardiac myocytes. Primary cultured cardiac myocytes were prepared from the ventricles of neonatal Wistar rats (1–3 days old). On 7 days after cell seeding, botulinum toxin type A incorporated into liposomes was added to the culture medium. At a final concentration of 5.0 μg/ml, botulinum toxin markedly attenuated the beating rate of cardiac myocytes within 2–4 hours. These results demonstrated the effect of SNARE-complex proteins on the spontaneous beating of cardiac myocytes.

Key Words: **Clostridium botulinum; cardiac myocytes; botulinum toxin; chronotropic effect; SNARE; SNAP-25.**

Botulinum toxins, whichare the most potent neurotoxinsproduced by a bacterium of *Clostridium botulinum,* can cause death of suffered animals by asphyxia by paralyzing the respiratory musculature. The botulinum toxins are classified into seven groups (A to G) based on their antigenicity. The toxins characterized to date are proteins with molecular weights of about 150 k, synthesized as single polypeptide chains. They are cleaved by endogenous proteases or trypsin to yield two subunits, a heavy chain (molecular weight about 100,000) and a light chain (molecular weight about 50,000) that contains a zinc-binding motif characteristic of metalloendoproteases. When the toxins are produced, they are associated with hemagglutinin components and a nontoxic-nonhemagglutinin component to produce large molecular complexes of about 30 to 90 kDa, which are M, L, or LL toxin (1). The toxins specifically inhibit neurotransmitter release (2-5) by cleaving SNARE family of proteins designated SNAP-25 (synaptosomal-associated protein 25), synaptobrevin (vesicle-associated membrane protein; VAMP) and syntaxin, which are important for neurotransmitter release (6-12). It has been reported that cells of many organs contain the SNARE proteins (13-15). For myocytes, it has been suggested that these proteins are participating in muscle contraction (16). We predicted that the botulinum type A toxin, which cleaves SNAP-25 specifically, would affect the contraction of the cardiac myocytes.

MATERIALS AND METHODS

Preparation of cultured cardiac myocytes. Cardiac myocytes were prepared from the ventricles of neonatal Wistar rats (1 to 3 days old) by collagenase digestion (200 U/ml), as described previously (17). Briefly, 15 to 20 neonatal rats were decapitated and their hearts were removed and the atria were trimmed, and the ventricles were bisected and added to a tissue bath perfused with Ca^{2+} - and Mg^{2+} - free Hanks' balanced salt solution (pH 7.4) buffered with 10 mM HEPES. After preincubation for 30 min at 37°C, the ventricle fragments were minced and digested with collagenase. To limit the amount of nonmyocardial cells, the isolated cells were added to 60 × 15 mm culture dishes in a CO_2 incubator. After 45 min, unattached cells, which were enriched with cardiac myocytes, were seeded on 35 × 15 mm culture dishes coated with type I collagen (rat tail collagen) at a density of 1.5×10^6 cells/dish in 1.5 ml of Dulbecco's modified Eagle's medium (DMEM) buffered with 5 mM of HEPES, which contained 10% fetal calf serum (FCS) and antibiotics (5,000 I.U./ml penicillin and 5,000 MCG/ml streptomycin). The cells were cultured in a humidified atmosphere of 95% air and 5% CO_2. The cultured cells

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0006-291X/98 $25.00

were fed fresh medium every 2 days. All of the experiments were performed using cardiac myocytes from day 7 of culture.

Monitoring of myocyte beating using a Fotonic Sensor™. Spontaneously beating cultured cardiac myocytes were monitored using a Fotonic Sensor, a fiber-optic displacement measurement instrument (MTI 1000, MTI Co., Latham, USA) which has been described previously (17,18). It detects changes in the distance between the probes and 150 to 200 of the myocytes vertically extruded by synchronized beating. The probe and cultured cardiac myocytes were placed on a horizontally rotating shaker in a CO_2 incubator. The analog output from the Fotonic Sensor was collected on a personal computer through a preamplifier (Bioelectric Amplifier AB620G, Nippon Koden Co., Tokyo). This method was used to determine the effect of drugs such as norepinephrine (17,19), prazosin (alpha 1-adrenoreceptor antagonist) (20), heptanol (21), carbachol, atropine, isoproterenol and propranolol (22) on cardiac myocytes.

Botulinum toxin and anti-botulinum neurotoxin antibodies. Type A Botulinum L toxin was purified from cultures of *C. botulinum* strain 62A using a previously described method (23). Two monoclonal antibodies, EL161-38 and A107, were used in this study; EL161-38 recognizes the light chain of the botulinum toxin but does not neutralize the lethal activity of the toxin (24), and A107 recognizes the heavy chain of the toxin and can neutralize the lethal activity (25).

Introduction of botulinum toxin into the cardiac myocytes. The culture medium of the cardiac myocytes were replaced with 1.8 ml of DMEM which included 5 mM of HEPES, 10% FCS, but not antibiotics in 35 × 15 mm culture dishes. The dishes were placed in a CO_2 incubator for 30 min for stabilization.

To produce efficient incorporation of the toxin into cardiac myocytes, a liposome forming method was employed using LIPOFECTAMINE (GIBCO BRL, New York, USA). Botulinum toxin type A diluted with DMEM was incubated for 45 min at room temperature with an equal volume of LIPOFECTAMINE diluted 1:6 with DMEM. After incubation, 200 μl aliquots were added to culture dishes, which were mixed by shaking for 15 sec at 60 rpm horizontally. As previously reported (17), the application of vehicle alone and shaking did not affect the beating of the cells. The beating of the myocytes were investigated sequentially.

Neutralization of toxin activity. Aliquots of 30 μg of toxin were incubated with 8 μg of monoclonal antibodies for 30 minutes at room temperature before incubation with LIPOFECTAMINE, and added to the culture medium. Bovine serum albumin (BSA) was used as the control.

RESULTS

The cardiac myocytes on day 3 of culture produced spontaneous but irregular contraction. From days 3 to 7 of culture, the beating rate increased and synchronized contraction was observed. On day 7 of culture, the cells produced spontaneous and synchronized contraction (Fig. 1-A) with a relatively constant rate (120.0 ± 16.0 beats/min) as previously reported (17). The cells were analyzed on day 7 of culture. The beating rate was maintained for 6 hours during the experiments.

At a final concentration of 5.0 μg/ml, botulinum toxin pretreated with LIPOFECTAMINE markedly depressed the beating rate of the cardiac myocytes within 2-4 hours (Fig. 1-B,2). No chronotropic effect occurred when the toxin was added without incubation with LIPOFECTAMINE (Fig. 1-B,2). LIPOFECTAMINE alone had no chronotropic effect on the cells (Fig. 1-B,2).

The neutralizing monoclonal antibody A107 markedly inhibited the chronotropic effect of botulinum toxin on the cells. In contrast, BSA, used as a nonspecific control protein, had an insignificant affect on the chronotropic effect of the toxin. The nonneutralizing monoclonal antibody EL161-38 inhibited the effect of the toxin on the cells, but did not block the effect (Fig. 3).

DISCUSSION

The target of botulinum toxin is SNARE proteins which promote neurotransmitter release. Although these proteins occur in cells of nonneuronal organs (14), the botulinum toxin selectively effects the nerve system in vivo, because of toxin receptors on the cells. For in vitro experiments, botulinum toxin is introduced into permeabilized cells, that are not nerve cells, to determine the function of SNARE proteins in the cells, because the toxin specifically cleaves and inactivates SNARE proteins. It has been reported that botulinum toxin inhibits glucose transport in adipocytes(26,27). It has also been reported that the release of insulin and catecholamines from pancreatic cells and chromaffin cells, respectively, can be inhibited by botulinum toxin (28-31).

Previous studies have determined that muscle contractions induce trafficking of glucose transporter 4 (GLUT-4) containing vesicles by a mechanism, that is similar to neurotransmitter release(16,32). It is possible that botulinum toxin would affect the contraction of muscle cells. We have developed a cultured cardiac myocyte method to determine the contraction and the beating rate of the myocytes (17,18). In previous study, which analyzed SNARE proteins by botulinum toxin, the toxin was introduced into nonneuronal cells by permeabilization of cells. However, permeabilization of cardiac myocytes rapidly blocked the spontaneous beating of the cells (data not shown). The liposome making method was less cytotoxic and originally developed for DNA transfection, but the liposome could introduce negatively charged proteins into cells in a similar manner to DNA. In this study, the incubation of the cardiac myocytes with botulinum toxin, which was incorporated into liposomes, markedly depressed the beating rate of the myocytes. On the other hand, liposome alone and the toxin alone did not affect the beating rate. The results demonstrated the efficacy of liposomes for the introduction of proteins into cells, and that botulinum toxin affects the spontaneous beating of cardiac myocytes.

It might be suggested that nerve cells would contaminate in the cultured cardiac myocyte system, and that botulinum toxin could affect the cardiac myocytes by inhibiting neurotransmitter release from the contaminated nerve cells. Botulinum toxin can directly affected nerve cells without liposome (33,34), however in this study, the toxin could not affect the cardiac myocytes

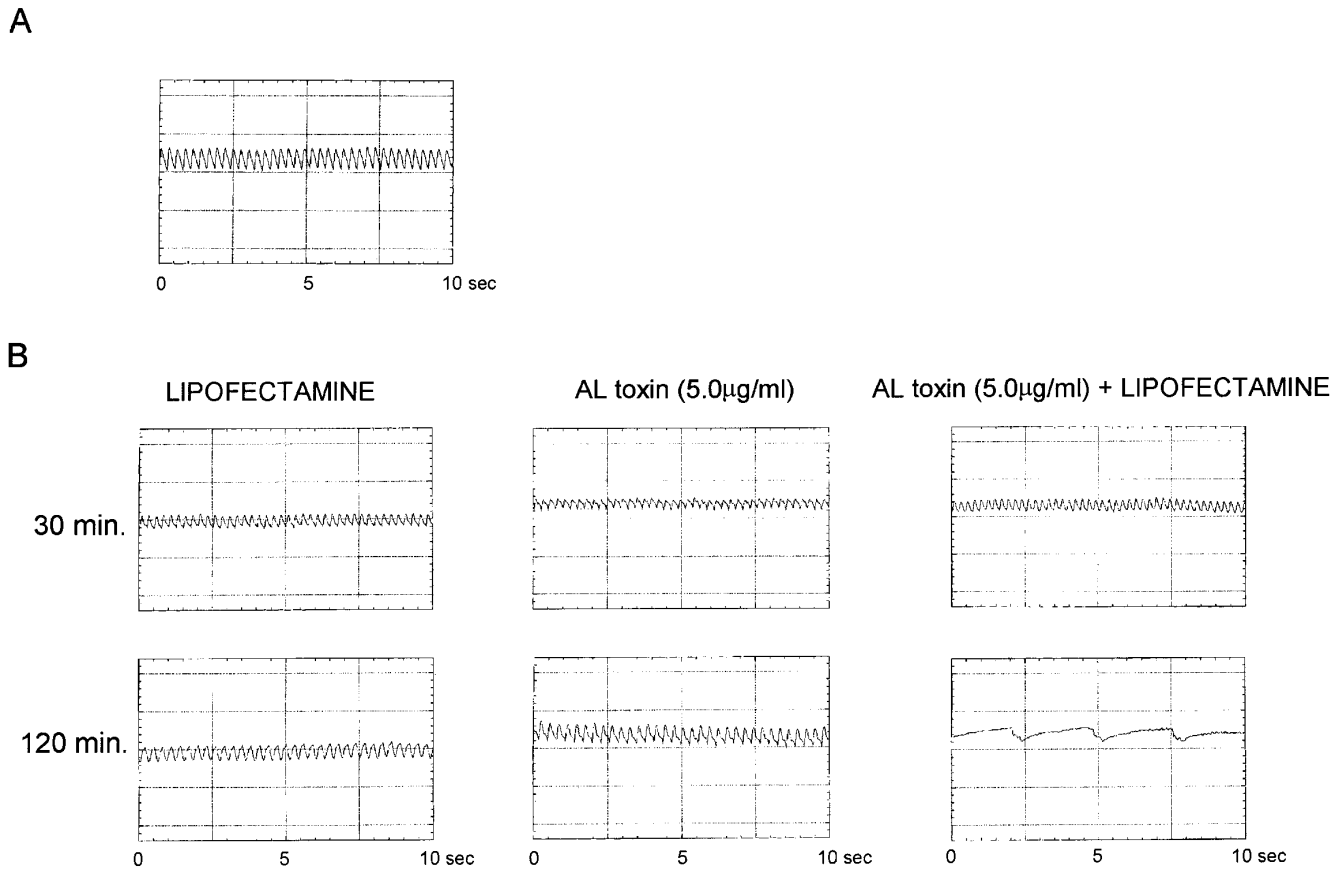

FIG. 1. Monitoring of the beating of neonatal rat cardiac myocytes. Panel A, on day 7 before the addition of the toxin; panel B, 30 min (top) or 120 min (bottom) after the addition of the LIPOFECTAMINE, the toxin, and the toxin in LIPOFECTAMINE.

without liposome. In addition, it was demonstrated that neurotransmission was not responsible for contraction and beating rate of cultured cardiac myocytes in our system (19). Therefore, participation of nerve cells in the negative chronotropic effect of botulinum toxin on the cardiac myocytes was denied.

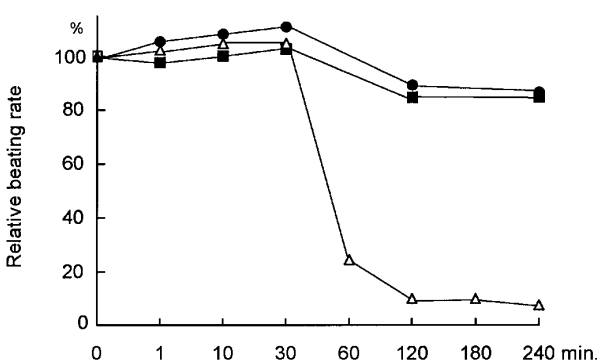

FIG. 2. Efficiency of the effect of the botulinum toxin on the synchronous beating rate of cardiac myocytes. ●, botulinum toxin (5.0 μg/ml); ■, LIPOFECTAMINE; △, botulinum toxin (5.0 μg/ml) incorporated into LIPOFECTAMINE.

Formation of ion channel is another suggestion to explain the effect of botulinum toxin on the cardiac myocytes. The formation of ion channels was identified

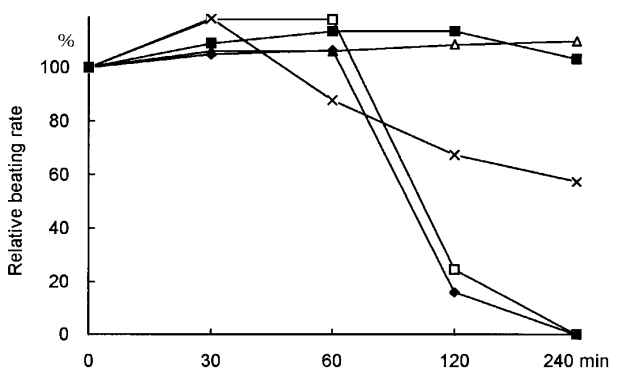

FIG. 3. Inhibition of the negative chronotropic effect of botulinum toxin by anti-botulinum neurotoxin monoclonal antibodies. The toxin (30 μg) was preincubated with (◆) or without (□) 8 μg of BSA, or with 8 μg of monoclonal anti-botulinum toxin monoclonal antibodies (△, A107; ×, EL161-38). Subsequently the solutions were incubated with LIPOFECTAMINE. ■, 38 μg of BSA incubated with LIPOFECTAMINE as a control.

using botulinum toxin or synthesized oligopeptides of putative transmembrane segments of the toxin (35,36). The importance of ion channels for activity of cardiac myocytes is well known, and it is possible that channel formation by the toxin depressed the beating rate of cardiac myocytes. However, ion channel formation by botulinum toxin was reported only in the artificial bilayer system. (35,36). Further, materials incorporated in liposomes are taken up into cells by endocytosis, and then transported to endosomes (37-39). So, it is unlikely that botulinum toxin incorporated into liposomes remained in cell membrane and could make ion channels.

The monoclonal antibody A107 neutralized lethal activity of the toxin and inhibited the negative chronotropic effect of the toxin, but the nonneutralizing monoclonal antibody EL161-38 could not block the negative chronotropic effect. The effect of the monoclonal antibodies on lethal activity and the chronotropic effect of the toxin was similar, which suggested a similar mechanism in spontaneous muscle beating as in neurotransmitter release. One may point out that the monoclonal antibody EL161-38 can not neutralized the lethal activity of botulinum toxin, but could attenuated the toxin activity on the cardiac myocytes. Neutralization activity of antibodies against botulinum toxin is determined by inhibition of lethal activity of the toxin in vivo using mice. So, many factors would affect the neutralizing effect of antibodies. In this study, the toxin-antibody complex was directly introduced into the cells, and therefore, could avoid influence of factors which affected neutralizing activity of the antibody. It is possible that the antibody EL161-38 could moderately suppress cleavage of SNAP-25 by botulinum toxin, however in vivo, some factors would interfere the antibody inhibition activity.

Because botulinum type A toxin selectively cleaved SNAP-25 (40), these results suggested that SNAP-25 concerned in the spontaneous beating of cardiac myocytes, but the mechanism remains unknown.

ACKNOWLEDGMENT

This study was supported by a grant from the Akiyama Foundation.

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