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Effects of Cannabinoids on Tension Induced by Acetylcholine and Choline in Slow Skeletal Muscle Fibers of the Frog

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Abstract We investigated the effects of cannabinoids on acetylcholine (ACh) or choline contractures in slow skeletal muscle fibers from *Rana pipiens*. Bundles of cruralis muscle fibers were incubated with the cannabinoid receptor 1 (CB_1) agonist, arachidonylcyclopropylamide (ACPA), which diminished the maximum isometric tension by 10 % and the total tension by 5 % of the ACh contracture, and 40 and 22 % of the choline contracture, respectively. Preincubation with the CB_1 antagonist, AM281, or with pertussis toxin (PTX) completely blocked the effect of ACPA on the ACh contracture. On the other hand, the decrease in choline contracture by ACPA was only partially blocked by AM281 (~16 % decrease), PTX (20 %), or by dantrolene (~46 %). Our results show that ACPA modulates ACh and choline contractures, and suggest that this effect involves the participation of CB_1, the ACh receptor, and −RyR in ACh contractures. For choline contractures, ACPA may also be acting through cannabinoid receptor-independent mechanisms.

Keywords Cannabinoid receptor type 1 · Acetylcholine receptor · Acetylcholine contracture · Choline contracture · Skeletal muscle · Slow skeletal muscle fiber

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Introduction

Skeletal muscles possess two main types of muscle fibers: twitch or fast, and slow or tonic. Twitch fibers are mainly mono-innervated by a large-diameter motor axon, produce propagated action potentials, and generate a transient contracture of high K^+ solutions with spontaneous relaxation. In contrast, tonic fibers are polyneuronally innervated by small-diameter motor axons, do not produce propagated action potentials, and generate a prolonged contracture of high K^+, acetylcholine (ACh), or choline solutions (Kuffler and Vaughan-Williams 1953a, b; Gilly and Hui 1980; Huerta et al. 1986; Katina and Nasledov 2008).

Cannabinoid reduces motor activity in mammals, and they might be therapeutically useful for muscle relaxation, spasticity, muscle stiffness, and tremor (Dewey 1986; Sañudo-Peña et al. 2000; Zajicek et al. 2003; Grotenhermen 2004). They reduce motor activity at the level of the central nervous system, so we expected to elucidate whether part of the effect of the cannabinoids occur directly at the level of skeletal muscle. Cannabinoids cause motor effects by interacting with membrane receptors in skeletal muscle. The cannabinoid receptor, CB_1, is expressed in both twitch and slow skeletal muscle fibers (Huerta et al. 2009) besides, the activation of CB_1 on these muscle fibers causes a decrease in caffeine-evoked muscle tension (Huerta et al. 2009).

Our primary aim was to examine the effects of a synthetic analog of anandamide, arachidonylcyclopropylamide (ACPA) (Hillard et al. 1999), a cannabinoid agonist, on

ACh- and choline-evoked tension in slow skeletal muscle fibers of the frog.

Materials and Methods

Animals

Frogs (*Rana pipiens*) were used in accordance with Alworth (2007) and Institute for Laboratory Animal Research's Guide for the Care and Use of Laboratory Animals (1996). All other general aspects of the methods were as described by Huerta et al. (1986).

Isometric Tension Measurements

Isometric tension was recorded from bundles of a few fibers (0.5 mm diameter) that were dissected from the tonic bundle of the cruralis muscle of *R. pipiens* as previously described (Gilly 1975; Huerta et al. 1986; Huerta et al. 2009), using an isometric mechanoelectrical transducer (400A; Cambridge Technology, Lexington, MA, USA). The bundles were equilibrated for 3–5 min with a continuous flow (30 mL/min) of the testing solution before applying 10^{-4} M Ach or 115 mM choline, which were each added for 4 min duration, and contractures were recorded in the presence or absence of the cannabinoid receptor agonist or antagonist (1 μM ACPA or 1 μM AM281; Tocris Bioscience, Ellisville, MO, USA). 150 μM dantrolene, 100 μM d-tubocurarine, and 2 μg/mL pertussis toxin (PTX) (Sigma-Aldrich, St. Louis, MO, USA), were also used. In some experiments, the bundles were curarized. Experiments were performed at room temperature (20–22 °C). Data were acquired and processed using CybmerAmp and Digidata 1200 (Axon Instruments, Foster City, CA, USA), respectively.

Solutions

The normal saline solution in which all working solutions were prepared was 117.5 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$. The pH was adjusted to 7.4 using imidazole chloride. The cannabinoid, ACPA, and the antagonist, AM281 (Tocris Cookson Inc., Ellisville, MO, USA), were prepared as a 10 mM stock solution in dimethyl sulfoxide (the diluent concentration was 0.01 %; Sigma-Aldrich, St. Louis, MO, USA). The experiments using ACPA were performed in the dark.

Data Analysis

Tension analysis was performed using the Clampfit subroutine (pClamp 9.0 software, Axon Instruments). The analyzed parameters of the contracture were maximum tension (peak tension) and tension–time integral (total tension). The experimental results are presented as mean ± standard error. Mean data were subjected to Student's *t* test; differences were considered significant at $p < 0.05$.

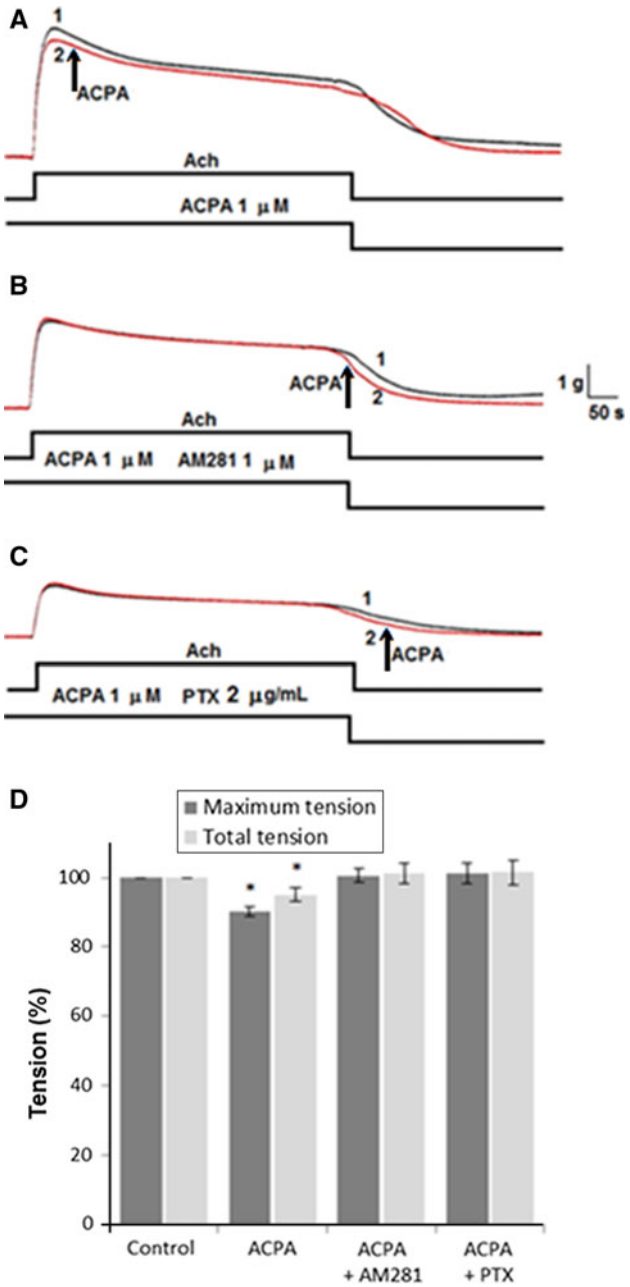

Fig. 1 **a** Acetylcholine contracture from tonic bundles of the cruralis muscle of *Rana pipiens* in (*1*) normal saline, and (*2*) 1 μM ACPA, or **b** in (*1*) normal saline, and (*2*) 1 μM ACPA in the presence of AM281 1 μM, or **c** in (*1*) normal saline, and (*2*) 1 μM ACPA, after the bundle was incubated with 2 μg/mL of pertussis toxin (PTX) for 22–24 h. Different bundles were used for each comparison. Time of exposure to ACh is indicated. Insertions are *bar graphs*. Data are presented as mean percentage of change ± standard error; $^*p < 0.05$, compared with the control condition

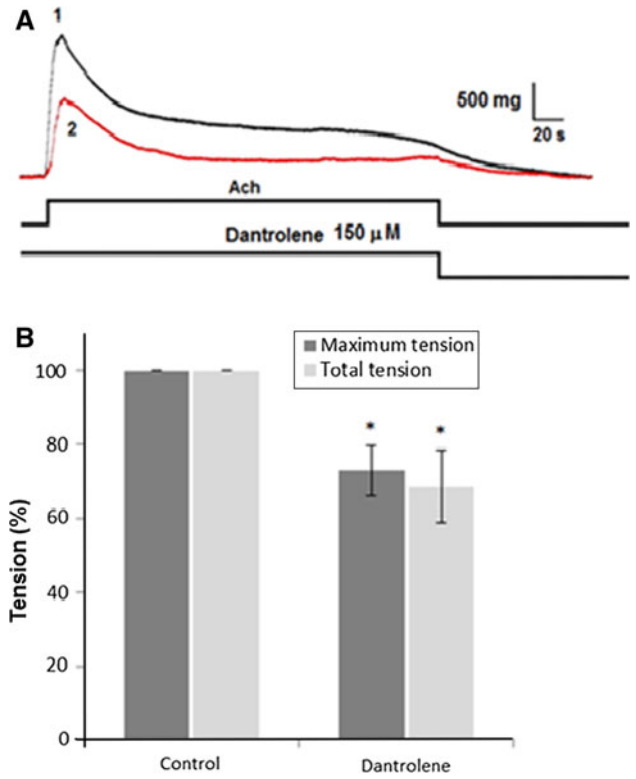

Fig. 2 Acetylcholine contractures of tonic muscle fibers in (*1*) normal saline, and (*2*) 150 µM dantrolene. Time of exposure to ACh is indicated. For illustration, we use an experimental record where the effect was maximal. Insertions are *bar graphs*. Data are presented as mean percentage of change ± standard error; $*p < 0.05$, compared with the control condition

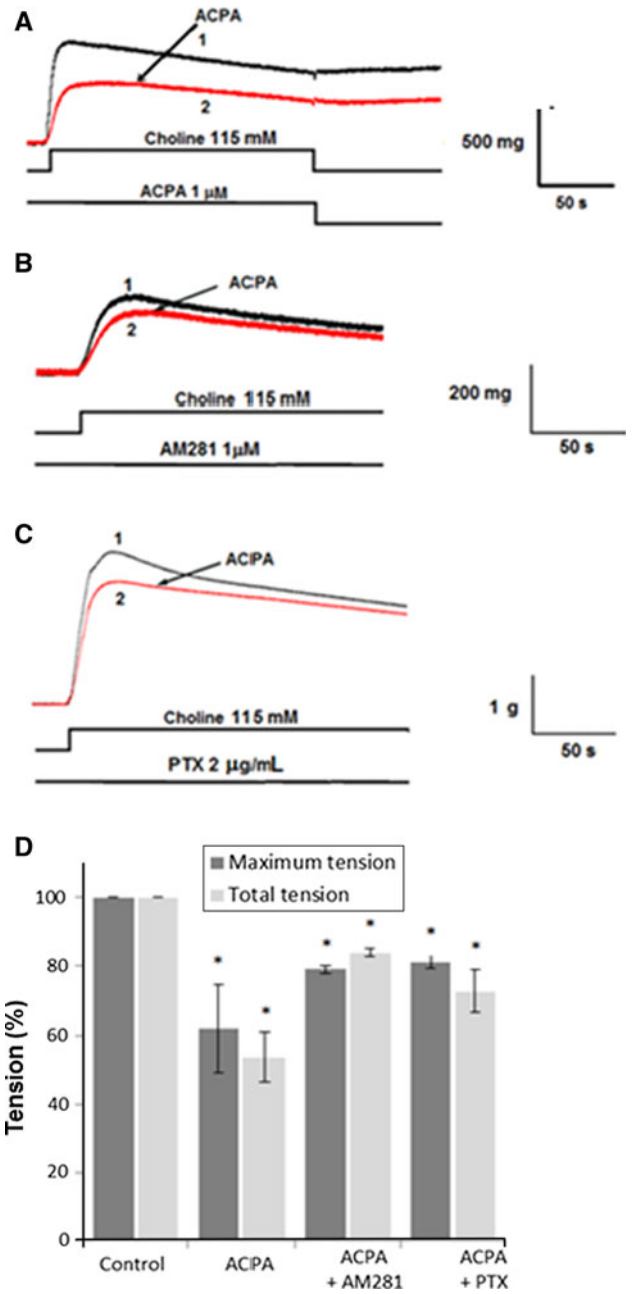

Fig. 3 Choline contracture of tonic muscle fibers in **a** normal saline (*1*) and (*2*) 1 µM ACPA, or **b** in (*1*) normal saline, and (*2*) 1 µM AM281, or (**C**) in (*1*) normal saline, and (*2*) in ACPA after incubation with PTX for 22–24 h. Different bundles were used for each comparison. Time of exposure to choline is indicated (labeled and indicated with *arrows*). Insertions are *bar graphs*. Data are presented as mean percentage of change ± standard error; $*p < 0.05$, compared with the control condition

Results

Effects of Cannabinoids on ACh Contractures

Figure 1 shows the contractile response of a slow bundle to 10^{-4} M ACh. The bundles generated tension while ACh was present in the bath solution, and relaxed when they were returned to the normal saline solution (Fig. 1a, ACh control). To examine whether cannabinoids modulate tension induced by ACh in slow skeletal muscle fibers of the frog, we used the synthetic cannabinoid, ACPA (analogous of the endogenous cannabinoid, anandamide). ACPA selectively binds to the cannabinoid receptor, CB_1. 1 µM ACPA reduced the amplitude of the maximum tension by about 10 % (90.16 ± 1.38 %; $p = 0.04$, $n = 6$), and total tension by about 5 % (95.03 ± 1.96 %; $p < 0.05$, $n = 6$) (Fig. 1a, 2), with respect to control in all six bundles tested ($p < 0.05$) (Fig. 1a, 2). This effect was blocked by preincubation with the CB_1 antagonist, AM281 (1 µM), resulting in no change in maximum tension (100.57 ± 1.82; $n = 6$), or total tension (101.14 ± 2.60; $n = 6$) (Fig. 1b, 2).

To test whether the effects of ACPA on tension are mediated through G proteins, we used PTX, which inhibits certain types of G proteins (G_i and G_o) (Reisine and Law 1992). The bundles were incubated for 22–24 h with 2 g/mL PTX, which prevented the tension reduction by ACPA (Fig. 1c, 1, 2). These results suggest that ACPA reduces tension in slow skeletal muscle fibers of the frog through a G protein-coupled receptor mechanism.

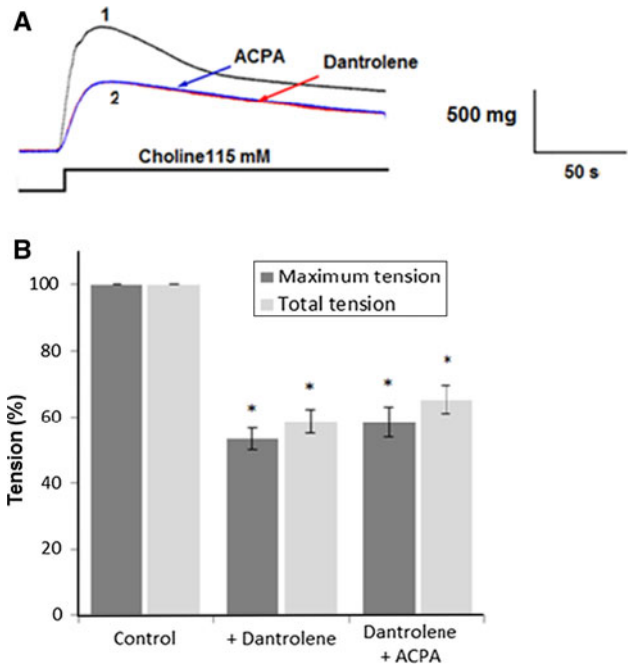

Fig. 4 Choline contractures of tonic muscle fibers in (*1*) normal saline, and (*2*) 1 μM ACPA after dantrolene treatment (labeled and indicated with *arrows*). Insertions are *bar graphs*. Data are presented as mean percentage of change ± standard error. *$p < 0.05$ compared with the control condition

Effect of Dantrolene on Acetylcholine Contractures

Blocking the isoform of the ryanodine receptor (−RyR) with 150 μM dantrolene caused a reduction of the ACh contracture in maximum tension (72.89 ± 6.78 %; $n = 5$, $p = 0.007$) and total tension (68.55 ± 9.49 %; $n = 5$, $p = 0.019$) (Fig. 2, 2).

Effects of Cannabinoids on Choline Contractures

Choline induces sustained contractures with a slow relaxation phase. Similar to recent reports for other slow skeletal muscles in the frog, we found that the choline-evoked contracture depends on the interaction with the ACh receptor (AChR), as demonstrated by its nearly complete inhibition (6.51 ± 0.37 %, $n = 4$) in the presence of 100 μM tubocurarine, an AChR inhibitor (data not shown). To test the effects of cannabinoids on choline contracture, we incubated the frog muscle fibers with 1 μM ACPA, which diminished the choline contracture by ∼40 % in maximum tension (62.14 ± 13 % $p = 0.028$; $n = 5$), and in total tension (78.86 ± 1.13 %, $p < 0.05$) (Fig. 3a, 2). This effect was partially blocked by preincubation with the CB_1 antagonist, AM281 (1 μM), decreasing total tension to 83.61 ± 1.21 % ($p = 0.001$, $n = 5$) (Fig. 3b, 2). Pretreating the bundles with pertussis toxin (2 μg/mL) also caused a partial block of the ACPA effect (∼20 % in maximum tension, 81.10 ± 1.86 %; $p < 0.05$) (Fig. 3c, 2). These results suggest that ACPA reduces choline-evoked muscle contraction partially through a mechanism involving the activation of CB_1 receptors, but also through CB_1 receptor-independent mechanisms. On the other hand, blocking −RyR with dantrolene (150 μM) causes a reduction of the choline contracture by approximately 46 % (53.08 ± 1.27; $n = 4$, $p = 0.001$) (Fig. 4, 2). Once the −RyR was blocked, ACPA did not cause any further decrease in tension (Fig. 4, 2), suggesting the involvement of −RyR in the effect caused by ACPA on the choline contractures.

Discussion

The results of the present study show that the cannabinoid, ACPA, decreased the tension of ACh contractures in the slow muscle fibers of the frog. ACPA activates cannabinoid receptors interacting with G_i/G_o proteins. Thus, it was no surprise that these effects of ACPA were blocked by pertussis toxin (Bokoch et al. 1983), indicating that ACPA acts through G protein-coupled receptors. The effects of ACPA were also blocked by the CB_1 antagonist AM281 (Howlett et al. 2002), indicating that ACPA diminishes tension of ACh contractures in the frog skeletal muscle fibers by activating CB_1 receptors. Consistent with this finding, a previous study (Huerta et al. 2009) showed that the cannabinoid receptor CB_1 transcript is expressed in both fast and slow skeletal muscle fibers of the frog.

It is generally accepted that the main excitation–contraction coupling mechanism in skeletal muscles is the release of Ca^{2+} from the sarcoplasmic reticulum (SR) through ryanodine receptors (RyRs) (Franzini-Armstrong and Protasi 1997; Zucchi and Ronca-Testoni 1997). We used dantrolene, an inhibitor of RyRs, to explore the role of this ion channel in the development of the contractile responses induced by ACh and choline. Our results with dantrolene demonstrate that the SR plays an important role in mechanisms mainly involved in choline contractures. We first considered that this effect of choline was primarily through a choline-mimetic effect on n-AChRs (Tsuneki et al. 2003) because both contractures were inhibited by d-tubocurarine. However, the effects of dantrolene were different between the ACh and choline contractures; the reduction in ACh contractures caused by dantrolene was greater than by ACPA (30 vs. 10 %), which suggests different mechanism of action, a reason to not use these drugs together; whereas, for choline contractures, the reduction caused by ACPA was similar than by dantrolene.

ACPA reduced choline contractures significantly more than ACh contractures, as measured in both maximum and total tension. This effect of ACPA on choline contractures was partially blocked by AM281 and PTX. These results

suggest that ACPA decreases choline-induced tension only partially through CB_1 receptors and G proteins, inhibiting ryanodine-sensitive channels or blocking Ca^{2+} channels in a similar manner as K^+ channels on nerve endings (Sánchez-Pastor et al. 2007; Vásquez et al. 2003). ACPA must also decrease choline contracture through cannabinoid receptor-independent mechanisms, possibly through the activation of vanilloid receptors localized on the SR (Xin et al. 2005). Support for this hypothesis comes from the evidence that dantrolene reduced the tension in the choline-evoked contractures, with no additional effect by ACPA, suggesting that ACPA acts, at least in part, through the same mechanism as dantrolene.

Renkin (1961) has shown that choline penetrates into the fiber of the skeletal muscle of the frog. Therefore, the accumulation of choline in the myoplasm could have an action on the RyR system (Hasselbach and Migala 1998; Du et al. 1998; Franzini-Armstrong and Protasi 1997; Meissner et al. 1997). Replacing sodium ions (100 %) with choline caused tonic fibers to contract. We cannot rule out an effect of ACPA on Na^+–Ca^{2+} transporters to reduce contractile activity of tonic fibers (Huerta et al. 1991; Muñiz et al. 1991; Même and Léoty 1999). Cannabinoids have different effects on ACh- and choline-evoked contractures, with a greater effect on the latter. The effect of dantrolene suggests that the ryanodine receptor system is the target of choline.

Our results show that ACPA modulates ACh and choline contractures and suggest that these effects mainly involve CB_1, AChR, and −RyR. ACPA is likely acting through cannabinoid receptor-independent mechanisms to exert an effect on choline contractures, a possibility is that they act through vanilloid receptors (Zygmunt et al. 1999). In conclusion, the functional significance of the cannabinoid receptors in muscle could be the modulation of tension. They might be therapeutically useful.

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Conflict of interest The authors declare no conflict of interest.

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