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Capsaicin and *N*-Arachidonoyl-dopamine (NADA) Decrease Tension by Activating Both Cannabinoid and Vanilloid Receptors in Fast Skeletal Muscle Fibers of the Frog

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Abstract Previous studies have indicated that vanilloid receptor (VR1) mRNA is expressed in muscle fibers. In this study, we evaluated the functional effects of VR1 activation. We measured caffeine-induced contractions in bundles of the extensor *digitorum longus* muscle of *Rana pipiens*. Isometric tension measurements showed that two VR1 agonists, capsaicin (CAP) and N-arachidonoyl-dopamine (NADA), reduced muscle peak tension to 57 ± 4 % and 71 ± 3 % of control, respectively. The effect of CAP was partially blocked by a VR1 blocker, capsazepine (CPZ), but the effect of NADA was not changed by CPZ. Because NADA is able to act on cannabinoid receptors, which are also present in muscle fibers, we tested the cannabinoid antagonist AM281. We found that AM281 antagonized both CAP and NADA effects. AM281 alone reduced peak tension to 80 ± 6 % of control. With both antagonists, the CAP effect was completely blocked, and the NADA effect was partially blocked. These results provide pharmacological evidence of the functional presence of the VR1 receptor in fast skeletal muscle fibers of the frog and suggest that capsaicin and NADA reduce tension by activating both cannabinoid and vanilloid receptors.

Keywords Vanilloid receptor · Cannabinoids receptor · Capsaicin · NADA · Skeletal muscle · Caffeine contracture · Frog

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Introduction

The vanilloid (capsaicin) receptor, VR1, has been described in rodent skeletal muscle; it is localized mainly in the sarcoplasmic reticulum (SR) (Xin et al. 2005; Cavuoto et al. 2007; Lotteau et al. 2013). One agonist of VR1 is capsaicin, which is a vanilloid present in chili peppers (Szallasi and Blumberg, 1999; Vriens et al. 2009). VR1 is a non-selective cation channel; its activation by capsaicin enhances the deposition of Ca^{2+} onto the relaxed skeletal muscle (Xin et al. 2005; Cavuoto et al. 2007). In smooth muscle, activation of VR1 inhibits L-type voltage-dependent Ca^{2+} channels and activates the delayed rectifier K^+ channel (Yeon et al. 2001; Hopps et al. 2012). In neurons, VR1 activation also blocks voltage-activated calcium channels (Bleakman et al. 1990; Wu et al. 2005). In addition, endocannabinoids, such as anandamide, which activates the type 1 cannabinoid receptor (CB_1 receptor), have been shown to stimulate VR1 in neurons (Zygmunt

et al. 1999; Starowicz et al. 2007). NADA is an endogenous compound that activates both cannabinoid receptors and VR1 (also known as TRPV1; Bisogno et al. 2000; Huang et al. 2002; Tóth et al. 2005; Huang and Walker 2006; Starowicz et al. 2007). NADA activates VR1 in the nervous system, but at relatively high concentrations (3–10 μM) (Marinelli et al. 2007).

Frog skeletal muscle comprises fast and slow types of muscle fibers (Kuffler and Vaughan-Williams, 1953; Gilly and Hui, 1980; Huerta et al. 1986). Evidence indicates that CB_1 receptors are present in frog skeletal muscle fibers (Huerta et al. 2009, 2011; Trujillo et al. 2014b). The CB_1 receptor agonist, arachidonylcyclopropylamide (ACPA) (Hillard et al. 1999), diminished the tension evoked by caffeine in skeletal muscle fibers. This effect was only partially reversed by the cannabinoid receptor antagonist, AM281 (Huerta et al. 2009). That result suggested that more than one pathway must participate in cannabinoid action on caffeine-evoked tension in fast skeletal muscle fibers (Huerta et al. 2009). On the other hand, capsaicin causes vasorelaxation in smooth muscle (Hopps et al. 2012).

In this work, we studied the effects of capsaicin (a VR1 agonist), NADA, capsazepine (a VR1 antagonist), and AM281 (a CB_1 antagonist) separately and in combination on caffeine-evoked tension in skeletal muscle fibers. We also detected VR1 mRNA expression in slow and fast skeletal muscle fibers of the frog, rat, and chicken.

Methods

Muscle Preparation

The study was performed on extensor *digitorum longus digiti* IV bundles (0.3–0.5 mm diameter) from *Rana pipiens* using isometric tension recordings. Frogs were used in accordance with the Institute for Laboratory Animal Research's Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research 1996) and Alworth and Harvey (2007). They were kept at room temperature (22–24 °C), and fed with a mixture of chicken liver and cod liver oil. To minimize pain and distress, under cold anesthesia frogs were killed and demedullated to extract the muscle.

Tension Recording

A continuous-perfusion recording chamber with an adjustable width and a central channel of 3 cm length was used to record tension in the bundles. The proximal end of each bundle was fixed to the wall of the chamber, while the distal end was connected to the lever of an FT03 force displacement transducer (Grass Technologies, Warwick, RI, USA) or a 400A force transducer (Aurora Scientific Inc.). Isometric tension recording was performed. Mechanical responses were evoked using caffeine solution (see "Solutions" section), both in the control trials and after pre-incubation for 4 min with the VR1 agonist (capsaicin), NADA, the VR1 antagonist (capsazepine), or the CB_1 receptor antagonist (AM281). A data-acquisition system (CyberAmp 320; Axon Instruments, Foster City, CA) and a Digidata 1200 (Axon Instruments) were used to process and store the data on a computer. The experimental results are presented as mean ± standard error. Mean data were subjected to Student's *t* test; differences were considered significant at a threshold of $p < 0.05$.

VR1 Receptor Expression in Fast and Slow Muscle Fibers

To assess the expression levels of VR1 in fast and slow skeletal muscle fibers, we performed real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of the extensor *digitorum longus* IV (fast) or bundles of the *cruralis* (slow) muscles from frog, *soleus* (slow) and *plantaris* (fast) from rat, and anterior (slow) and posterior *latissimus dorsi* (fast) from chicken. Total RNA was isolated from 10 mg of muscles using Trizol™ Reagent (15596-018; Invitrogen, Carlsbad, CA). For each sample, 800 ng of RNA were reverse transcribed using the SuperScript III First Strand kit (18080-093; Invitrogen, Carlsbad, CA), employing oligo dT as the primer. Real-time PCR reactions were performed using 40 ng of cDNA on the LightCycler 1.5 system (Roche, Nutley, NJ) with the Light Cycler DNA master SYBR Green I kit (12015099001; Roche, Nutley, NJ) and the following set of intron-spanning primers for *Trpv1*: Trpv1F-CTTTATGACC TGTCCTGCAT and Trpv1R-CACCACAGCTGTGGAA AATC. Constitutive glyceraldhyde-3-phosphate dehydrogenase (*GAPDH*) expression was used to normalize data and was amplified using the following set of primers: GAPDHF-C TTCACCACCATGGAGAAGG, GAPDHR-GTTGTCATG GATGACCTTGGC. The specificity of primers was verified using Blast resource; the annealing temperature was 57 °C, and an amplicon of 648 bp (rat) or 651 bp (chicken and frog) was obtained. Both of the pair of initiators was designed using shared sequences observed in an alignment of nucleotides of the genes for VR1 and GAPDH from rat and chicken. To characterize amplification specificity, a melting curve analysis was performed at the end of the 40th cycle of amplification for each sample. To determine reaction efficiencies, standard curves were elaborated for both genes (using serial dilutions of cDNA from rat muscle in the range of 1:10, 1:50, 1:100, and 1:250); such values were 99.5 and 99.8 % for VR1 and GAPDH, respectively. Threshold cycle (Ct) values were then used to obtain the relative expression in the fast muscle versus slow muscle using the $2^{-\Delta\Delta Ct}$ method, where

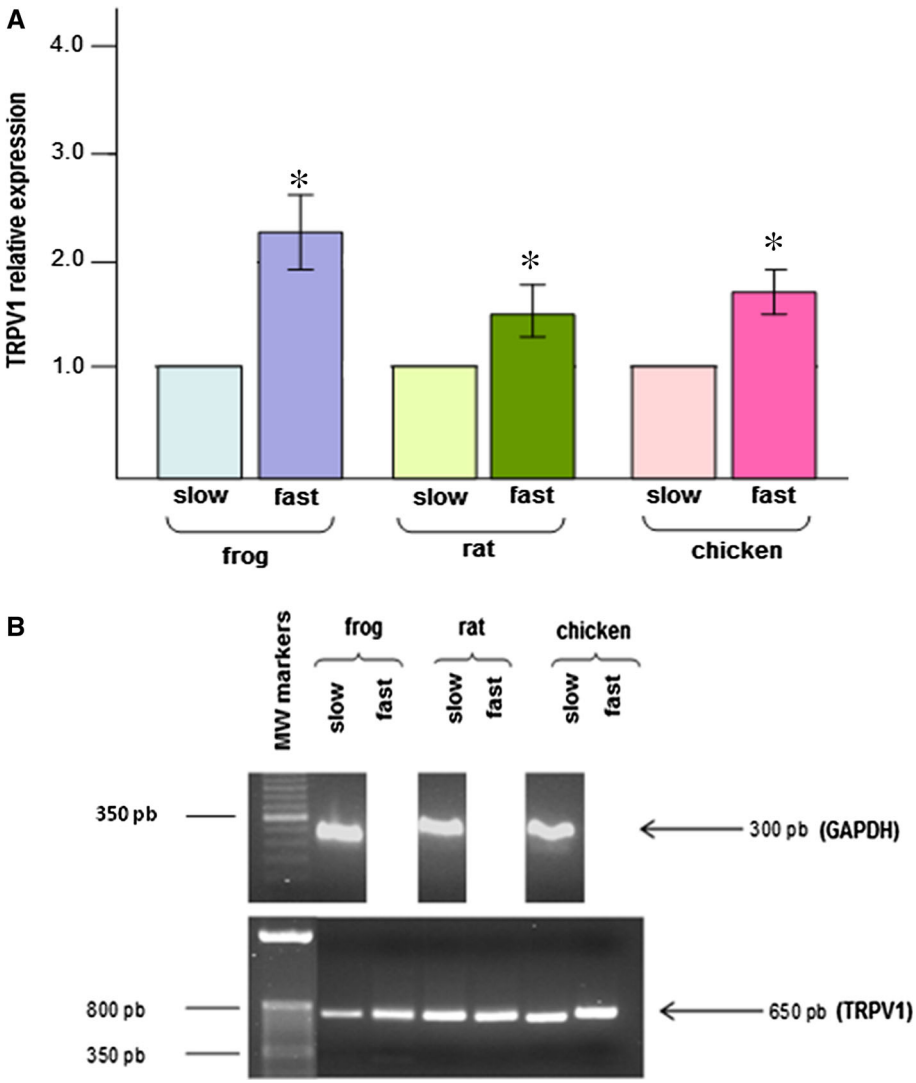

Fig. 1 **a** Relative expression of the VR1 gene in slow and fast skeletal muscles of the frog, rat, and chicken. The graph depicts the fold difference of VR1 expression in fast skeletal muscle relative to slow skeletal muscle for each animal type. Data are shown as mean ± standard error of three independent samples. VR1 expression was normalized against the constitutively expressed *GAPDH* gene. *$P < 0.05$, Student's *t* test. **b** Gel electrophoresis (2 % agarose) of VR1and GAPDH products obtained by RT-PCR from slow and fast muscles from rat, frog, and chicken

$\Delta\Delta Ct = \Delta Ct_{(\text{fast muscle})} - \Delta Ct_{(\text{slow muscle})}$, and $\Delta Ct = Ct_{(TRPV1)} - Ct_{(GAPDH)}$.

Solutions

Normal saline solution contained (in mM) NaCl 117.5, KCl 2.5, and CaCl2 1.8. The pH was adjusted to 7.4 with imidazole Cl. Contractions were induced using caffeine solution (normal saline solution plus 6 mM caffeine). In some mechanical experiments, the bundle was curarized with 50 μM d-tubocurarine (Sigma, St. Louis, MO). Respective stock solutions of AM281, (E)-capsaicin, NADA, capsazepine, and AM281 (Tocris Cookson, Ballwin, MO), were made up in dimethyl sulfoxide (DMSO) or ethanol (Sigma, St. Louis, Mo.). The final concentration of DMSO in the bath solution was <0.01 %, which has no significant effect on caffeine (Velasco et al. 2003). Experiments were performed at room temperature (22–24 °C).

Results

Presence of VR1 Receptor mRNA in the Skeletal Muscle Fibers of the Frog

In neurons, the TRPV1 receptors are localized in the plasma membrane and in the SR (Olah et al. 2001); in rat skeletal muscle, VR1 is expressed mainly in the SR (Xin et al. 2005; Cavuoto et al. 2007; Lotteau et al. 2013). In these studies, the presence of VR1 mRNA was assessed in fast and slow skeletal muscle fibers from chicken, frog, and rat.

To further confirm the presence of VR1 mRNA in frog, chicken, and rat skeletal muscle fibers, we conducted a quantitative analysis of VR1 mRNA levels using real-time RT-PCR. The expression levels of VR1 receptor mRNA were assessed by the intensity of fluorescence produced by SYBR green (Fig. 1b). As with conventional RT-PCR, the

expression levels were lower in slow muscle fibers than in fast muscle fibers (Fig. 1a). The concentration levels for VR1 mRNA corroborate the approximate 2:1 relationship between fast and slow muscle fibers. To determine the *TRPV1* mRNA concentrations, we used a sample with known mRNA concentration as a control. The mean values of fluorescence intensity obtained were 2.5 ± 0.3 for frog muscles, 1.5 ± 0.2 for rat, and 1.6 ± 0.2 for chicken.

Capsaicin Effects on Caffeine Contractures in the Presence and Absence of Vanilloid and Cannabinoid Receptor Antagonists

We explored the effect of the vanilloid receptor agonist capsaicin on the caffeine-evoked contractures of fast skeletal muscle fibers. Figure 2 depicts a caffeine contracture in normal solution (control) and after pre-incubation of fibers with capsaicin (10 μM) in the bathing solution for 4 min, which decreases the muscle tension compared with control by 57.3 ± 4 % ($p < 0.05$, $n = 7$). Thus, these results with capsaicin demonstrate that capsaicin reduces caffeine-evoked tension (Fig. 5a, bar 2). Next, we explored whether the capsaicin antagonist capsazepine inhibits the effect of capsaicin. Figure 3 shows that capsazepine (1 μM) partially inhibits the capsaicin effect (71.3 ± 3 %) compared with control ($p = 0.03$, $n = 5$) (Fig. 5a, bar 3). To test whether the vanilloid receptor is completely blocked, we used higher concentrations of capsazepine (5 and 10 μM), which resulted in respective reductions of muscle tension of 66.2 ± 6 % ($n = 5$) and 62.6 ± 4 % ($n = 4$) (Fig. 5a, bar 4). The effects of capsazepine 5 μM and 10 μM did not differ significantly ($p = 0.59$). These results show that all vanilloid receptors were blocked. Next, we tested whether the CB_1 receptor antagonist AM281 (Howlett et al. 2002) could block the effect of capsaicin, we pretreated muscle fibers with AM281 plus capsaicin, then added caffeine to evoke a contraction. The CB_1 antagonist AM281 also partially inhibited the effect of capsaicin, resulting in a significantly greater maximum peak tension than capsaicin alone (79.1 ± 6 % of control level, $p < 0.05$, $n = 5$) (Figs. 4 and 5a, bar 5). These results suggest that cannabinoid receptors mediate the effect of capsaicin in the fast muscle fibers of the frog. However, when capsazepine and AM281 were applied together, the maximum tension recovered to approximately 95.2 ± 1 % of the control level ($p = 0.91$, $n = 5$) (Fig. 5a, bar 6 and Fig. 5b). These findings provide pharmacological evidence that capsaicin activates both CB_1 and VR1 receptors in the fast skeletal muscle fibers of the frog. Pretreatment with capsaicin for 10 min did not significantly changes its effect on the contracture evoked by caffeine ($p = 0.85$; $n = 4$). When capsazepine or AM281 was applied alone, they did not alter the baseline or the evoked caffeine contracture.

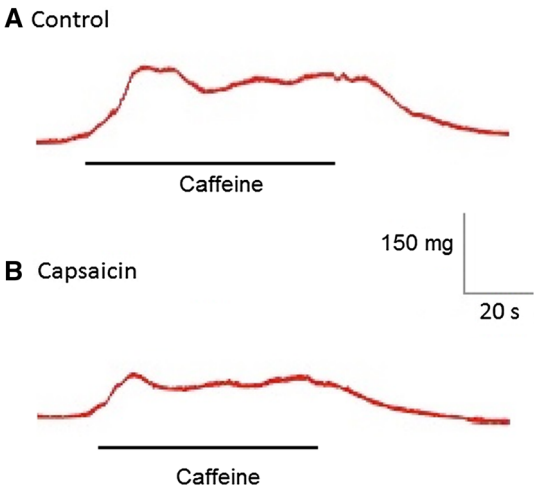

Fig. 2 Effects of capsaicin on caffeine contracture in fast muscle fibers. Experiments were performed using the exterior *digitorum longus* IV muscle of frog. Control caffeine (6 mM) (**a**). On (**b**), tension evoked by caffeine is significantly reduced in the presence of capsaicin (10 μM)

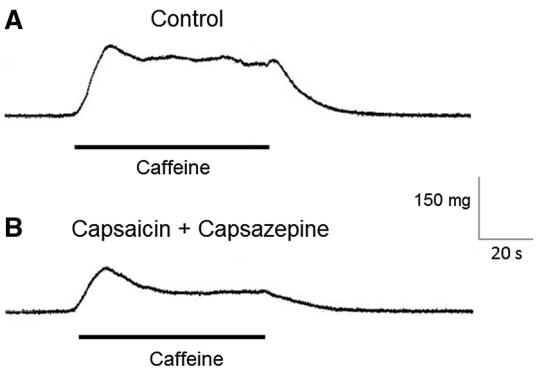

Fig. 3 Effects of capsazepine (1 μM) on tension evoked by caffeine in fast skeletal muscle fibers. **a**, the control contracture of caffeine (6 mM, **b**) compared with the caffeine contracture in the presence of capsaicin plus the vanilloid receptor antagonists (1 μM)

Effects of NADA on Caffeine Contractures in the Presence and Absence of Vanilloid and Cannabinoid Receptor Antagonists

We explored the effect of the endovanilloid/endocannabinoid NADA on the tension of caffeine-evoked contractures in fast skeletal muscle fibers. We intended to determine whether NADA, which stimulates both receptors, is able to produce a decrement in tension similar to capsaicin. In Fig. 6, we show a caffeine control contracture; after pre-incubation for 4 min with NADA (5 μM), muscle contraction was reduced by approximately 63.5 ± 4 % compared with control ($p < 0.05$, $n = 5$; Figs. 6 and 7, bar 2).

This effect was not inhibited by the addition of 5 μM capsazepine, which increased the maximum peak tension compared with 5 μM NADA alone (69.1 ± 5 % of control

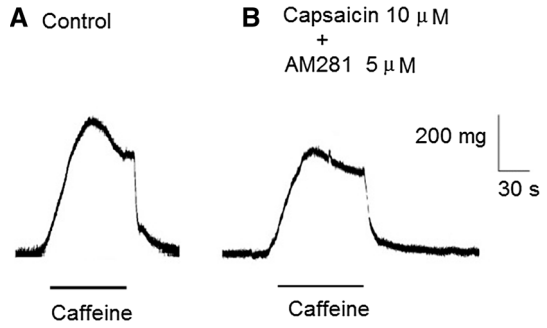

Fig. 4 Effects of AM281 (5 μM) on tension evoked by caffeine. In (**a**), the control caffeine contracture (6 mM). In (**b**), the caffeine contracture in the presence of the cannabinoid antagonist AM281, diminishes tension significantly

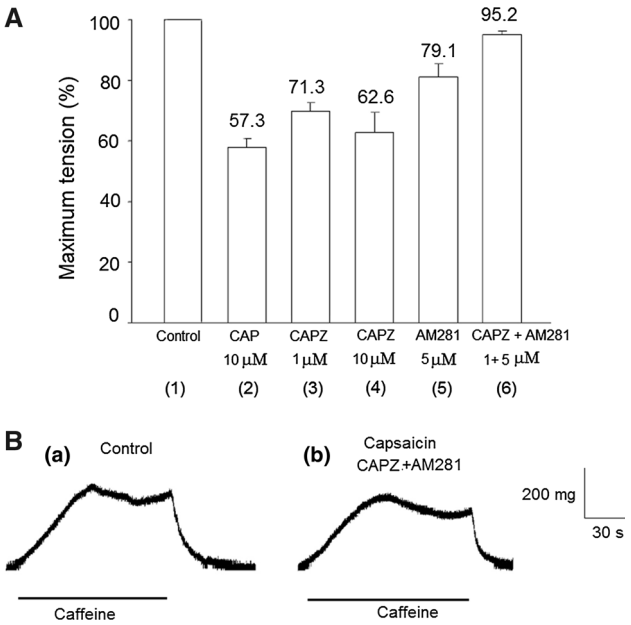

Fig. 5 **a** Effects of capsaicin on caffeine contractures. Data are presented as the mean percent ± standard error. In **b**-(*a*), the caffeine control contracture (6 mM). In **b**-(*b*), it shows the caffeine contracture in the presence of capsaicin (10 μM), plus the vanilloid receptor antagonist capsazepine (1 μM), and the cannabinoid receptor antagonist AM281 (5 μM). Note that both contractures are similar

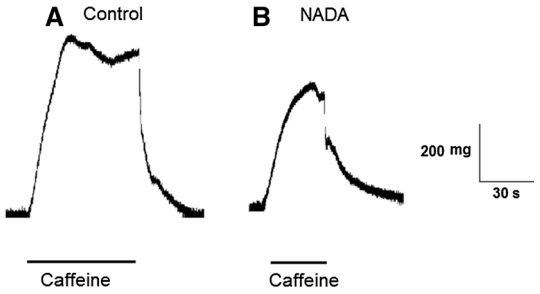

Fig. 6 Effects of NADA on caffeine contractures in fast muscle fibers. **a** The control contracture. **b** The caffeine contracture in the presence of NADA. It decreased 37 %. Note that the tension evoked by caffeine is significantly reduced in the presence of NADA (5 μM)

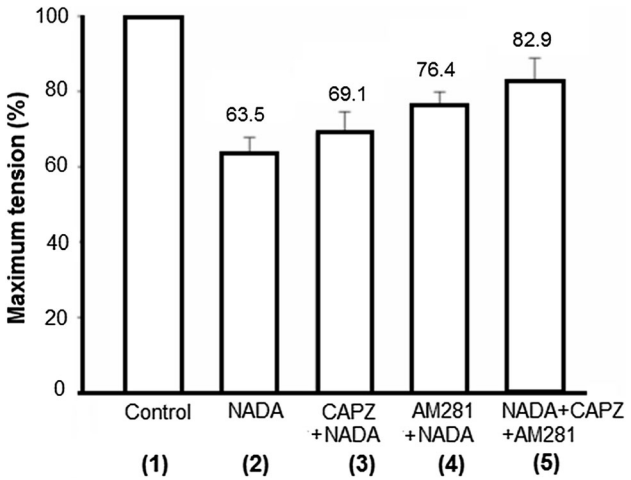

Fig. 7 Effects of NADA on tension evoked by caffeine (6 mM) in fast skeletal muscle fibers. Data are presented as mean percentage of change ± standard error. NADA (5 μM), capsazepine (CAPZ, 1 μM), AM281 (5 μM)

level, $n = 5$; $p = 0.45$ vs. NADA alone; Fig. 7, bar 3). The addition of 5 μM AM281 partially inhibited the effect of NADA, resulting in a greater maximum peak tension than 5 μM NADA alone (76.4 ± 3 % of control level, Fig. 7, bar 4, $p < 0.05$ vs. control, $n = 5$). The effect of AM281 10 μM was smaller than the effect with AM281 5 μM ($n = 3$). However, when both receptor antagonists were applied, the maximum peak tension recovered to approximately 82.9 ± 6 % of the control level, and differ significantly from the control level ($p = 0.07$ vs. control, $n = 5$; Fig. 7, bar 5). These results show CB_1 and VR1 receptors are involved in the decrement of tension by NADA in the fast skeletal muscle fibers of the frog, similar to capsaicin.

To explore the possibility that capsaicin and NADA interact, we pretreated the fiber bundle for 4 min with NADA (5 μM), and then recorded the caffeine-evoked tension in the presence of capsaicin. Although there was a tendency toward increased tension, this increase was not statistically significant (67.5 ± 9 %, $p = 0.38$, $n = 3$) (compared with capsaicin, Fig. 5, bar 2). Pretreatment with NADA did not evoke contractile muscular response.

Discussion

These results indicate that *TRPV1* mRNA is present in the fast and slow skeletal muscle fibers of the frog. We used thin bundles that were carefully cleaned by removing vessels and nerves using glass dissectors to minimize the vessels as a

possible source of mRNA. Although we cannot rule out that part of the VR1 receptor mRNA that we observed was at the level of the nerve terminal. This study has shown that the VR1 receptor mRNA is present in the frog's fast and slow skeletal muscle fibers. The slow muscle fibers that we used were dissected from the tonic bundle of the *cruralis* muscle, while the fast muscle fibers were obtained from the extensor *digitorum longus digiti* IV. It is known that fast muscle fibers are normally mono-innervated, in contrast to slow fibers, which are multi-innervated.

We found that the level of *TRPV1* mRNA expression was lower in slow skeletal muscle fibers than in the fast muscle fibers of the frog. Regarding rat muscle fibers, we have also demonstrated the presence of *TRPV1* mRNA. In the chicken, we observed that there was approximately 1.5-fold as much expression in fast skeletal muscle (the posterior *latissiumus dorsi*, which is comprised exclusively of fast fibers) as in slow muscle (the anterior *latissimus dorsi*, which is comprised exclusively of slow muscle fibers). The vanilloid receptor in avian neurons is reportedly a different form from that found in frog or rat (Jordt and Julius, 2002).

In this study, we also attempted to separate the vanilloid and cannabinoid receptor effects evoked by capsaicin and NADA by blocking the respective activities of vanilloid and cannabinoid receptors, separately and in combination. Our results regarding the fast muscle fibers demonstrated that 10 μM capsaicin diminished the amplitude of the maximum tension of the evoked caffeine contracture. This effect was partially reversed by capsazepine, suggesting that the action of capsaicin on tension modulation involves VR1 vanilloid receptors. Thus, the lessening of tension by capsaicin in the fast skeletal muscle fibers might be explained by activating the vanilloid receptor, and possibly also by a direct effect on the activation of cannabinoid receptors, which alter cellular activity by activating G proteins of the $G_{i/o}$ subtype (Soderstrom et al. 2000). Our experimental data demonstrated that when capsaicin was used in the combined presence of two vanilloid and cannabinoid receptor antagonists, the peak tension nearly returned to the control peak tension level. The mechanism by which this phenomenon occurs remains unknown; however, there are several mechanisms that may participate. One is the inhibition of the L-type Ca^{2+} channel, as occurs in smooth muscle (Lo et al. 1995; Hopps et al. 2012); capsaicin abolishes the contraction of porcine coronary arteries induced by the L-type calcium activator Bay-K8644. A second possibility is that capsaicin-induced relaxation in smooth muscle may be due to the activation of delayed rectifier K channels; when these channels are blocked, the relaxation does not succeed (Yeon et al. 2001). A third possibility is capsaicin pretreatments, which activate TRPV1, may deplete Ca^{2+} stores (Lotteau et al. 2013) resulting in reduced caffeine release and a consequent decline of contraction tension. In these experiments, pretreatment with capsaicin for 4 or 10 min does not significantly modify capsaicin's effects. However, additional studies with longer periods of pretreatment with capsaicin or NADA are required. Finally, in slow muscle fiber from frog skeletal muscle, some authors suggest that the RyR channel is involved in the effect of ACPA on the choline contractures (Trujillo et al. 2014a). One or several of these mechanisms might participate in capsaicin's effect on tension. However, additional research is needed to elucidate these mechanisms. Regarding the action of the vanilloid receptor on skeletal muscle, Xin et al. (2005) have suggested that the VR1 receptor-channel participates mainly in the calcium leak from the SR during the resting condition or in the dysfunction of muscle SR Ca^{2+} release (Lotteau et al. 2013). In this study, the entire signaling machinery of the cannabinoid and vanilloid receptors is present in this intact adult frog fast skeletal muscle.

Experiments employing NADA confirm this hypothesis, because NADA is known to activate, cannabinoid, and vanilloid receptors (Starowicz et al. 2007, 2008, 2012), obtaining similar results in the presence of capsaicin. However, there is a small proportion of tension inhibition that might be caused by another different mechanism, which must be investigated.

On the other hand, the reduction of tension by NADA, which activates both vanilloid and cannabinoid receptors, then it could use the same mechanisms used by capsaicin. Our results suggest a possible interaction between these two systems.

In conclusion, we have reported the effects on tension reduction caused by both capsaicin and NADA in fast skeletal muscle fibers. The effects of reduced tension in these fibers are caused by a direct effect on muscle, because they occur in the presence of 50 μM *d*-tubocurarine. Thus, cannabinoid and capsaicin receptors are present in fast skeletal muscle fibers, and their activation changes muscle activity. On the other hand, activated CB_1 receptors reduce motor activity at the central level in humans and/or in mammalian skeletal muscle (Dewey, 1986; Sañudo-Peña et al. 2000; Zajicek et al. 2003). TRPV channels have been reported at motor nerve endings in the mouse (Thyagarajan et al. 2009), and there is pharmacological evidence of its presence at motor nerve endings in the frog (Silveira et al. 2010). Cannabinoids (1 μM) decrease neurotransmitter release in the frog neuromuscular junction by interacting with CB_1 receptors (Sánchez-Pastor et al. 2007), and capsaicin (at 0.4 μM) reportedly increases the transmitter release (Silveira et al. 2010). To our knowledge, this work is the first to show direct evidence of the diminishment of tension by capsaicin and NADA on intact frog fast muscle fibers. These effects of capsaicin and NADA on contraction and transmitter release in fast skeletal frog muscle might

have a future therapeutic use. More studies are needed to clarify this mechanism.

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