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Presence and Colocalization of Type-1 Cannabinoid Receptors with Acetylcholine Receptors in the Motor End-Plate of Twitch Skeletal Muscle Fibers in the Frog

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Abstract Using polyclonal and monoclonal antibodies to visualize under a confocal microscope type-1 cannabinoid receptors (CB_1) and acetylcholine (ACh) receptors, respectively, or α-bungarotoxin conjugated to Alexa-Fluor 555 for Ach receptors, we found that they colocalize on twitch muscle fibers in the frog (*Rana pipiens*). We show that both the CB_1 and ACh receptors are present on the fast skeletal muscle motor end-plate. The CB_1 receptor is present along the entire membrane of the muscle fiber, whereas the ACh receptor is expressed primarily at the motor end-plate. Analysis of the colocalization produced a cross-correlation coefficient of 0.519 ± 0.021 ($n = 9$) for both receptors at the muscle motor end-plate. This study suggests a close proximity between these two types of receptor proteins and that they could interact. CB_1 could function at some stage of excitation–contraction coupling in these muscle fibers. However, further investigation is needed in order to clarify these issues.

Keywords Cannabinoid receptor type-1 · Acetylcholine receptor · Motor end-plate · Skeletal muscle · Colocalization · Twitch skeletal muscle fiber

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Introduction

Skeletal muscles possess two main types of muscle fibers: fast (or twitch) and slow (or tonic). Twitch fibers are mainly mono-innervated with large-diameter motor axons, produce propagated action potentials, and generate a transient contracture with high K^+ solutions and spontaneous relaxation. In contrast, tonic fibers are polyneuronally innervated with small-diameter motor axons, which do not produce propagated action potentials, and their contracture is prolonged with high K^+ or acetylcholine (ACh)-containing solutions (Kuffler and Vaughan-Williams 1953; Gilly and Hui 1980; Huerta et al. 1986; Katina and Nasledov 2008; Trujillo et al. 2014).

Cannabinoids, the active compounds in marijuana (*Cannabis sativa*), cause psychoactive and motor effects when the plant is consumed. These effects are produced by the interaction of these compounds with the cannabinoid membrane receptors CB_1 and CB_2 (Howlett et al. 2002). These receptors function through G-protein activation (Soderstrom et al. 2000). To date, only the orthologous CB_1 receptors have been identified in amphibians (Soderstrom et al. 2000; McPartland et al. 2006). Expression of the CB_1 transcript has been reported in the two types of muscle fibers (Sánchez-Pastoret al. 2004; Cavuoto et al. 2007; Huerta et al. 2009). Moreover, the activation of CB_1 by synthetic anandamide (ACPA) (Hillard et al. 1999) reduces the amplitude of miniature end-plate potentials (MEPPs) and caffeine contractures in twitch fibers (Huerta et al. 2009; Sánchez-Pastor et al. 2007).

The primary aim of the present study was to investigate the presence and localization of CB_1 on skeletal muscle fibers in the frog. Here, we report the colocalization of CB_1 and ACh receptors.

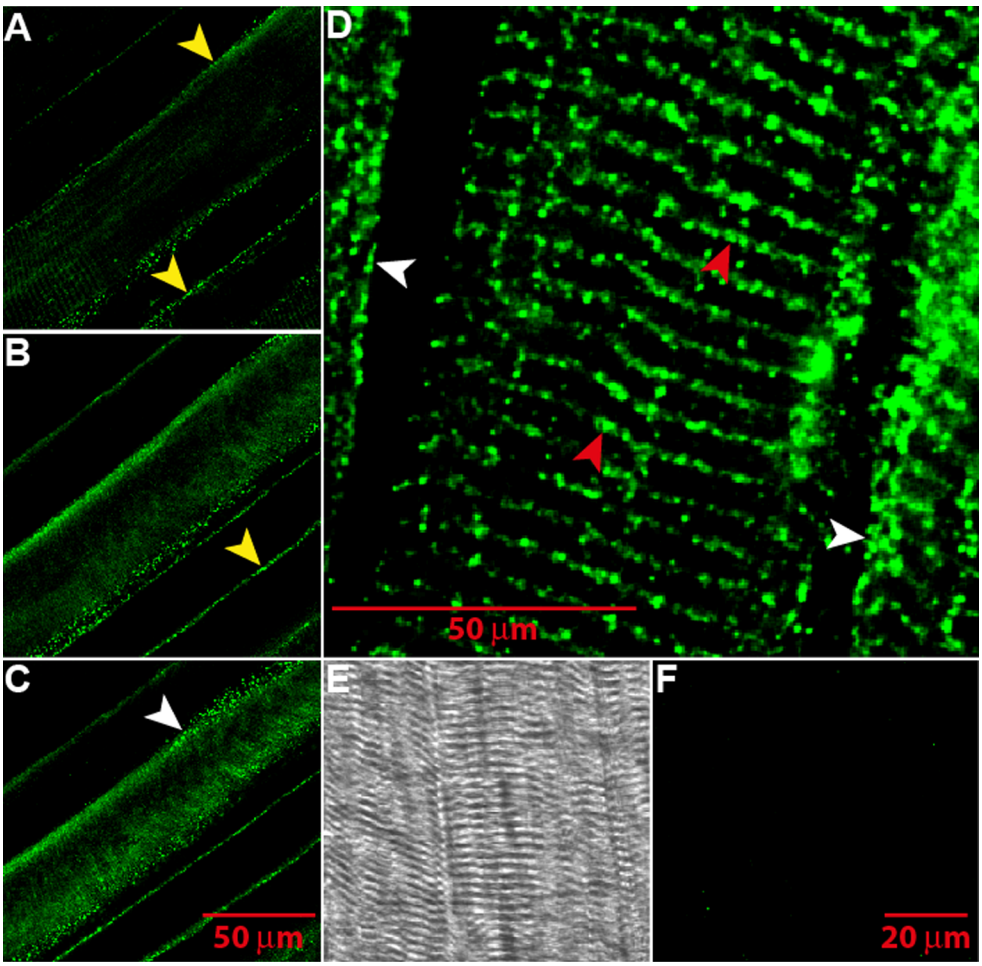

Fig. 1 Membrane localization of CB_1 receptors on cutaneous pectoris muscle fibers. **a** Muscle fibers labeled with CB_1-specific antibody. *Yellow arrowheads* indicate CB_1 receptor clusters, and *red arrowhead* points striations. **b** A different plane showing CB_1 receptors at the muscle fiber periphery (*yellow arrowhead*). **c** Different plane indicating with a *white arrowhead* the receptor clusters at the top of the muscle fiber cell. **d** High magnification of fiber surface showing CB_1 receptors on striations. *Red arrowheads* indicate muscle fiber striations on the surface. The *white arrowhead* indicates CB_1 clusters on the muscle fiber membrane. **e** Muscle fibers scanned using a photomultiplier for transmitted light so that the striations along the muscle fibers can be seen. **f** The same fibers as **e** labeled using the pre-absorbed CB_1 antibody (negative control). $n = 8$ animals

Materials and Methods

Animals

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

Antibody Staining and Confocal Microscopy

We isolated the cutaneous pectoris muscle from the frog and removed all vasculature and connective tissue under a dissection microscope. The muscles were pre-fixed with 4 % paraformaldehyde (without picric acid) for 25 min and washed three times with PBS. Polyclonal antibodies against CB_1 (rabbit, 1:500; Abcam, Cat. No. 3559 and Abcam Cat. No. 23703) and monoclonal antibodies against the ACh receptor (mouse monoclonal, 1:500; Abcam Cat. No. 24631) were used for immunohistochemistry. The specificity of the antibodies was checked by performing experiments with no first antibody or by pre-absorbing the CB_1 antibody with its immunogenic peptide. The muscle fiber preparations were incubated with 10 % bovine serum albumin (BSA) for 30 min at room temperature for background blocking followed by overnight incubation with the primary antibodies in PBS with 0.2 % Triton X-100 and 1 % BSA at 4 °C. At the next day, the muscles were washed in PBS with 0.2 % Triton X-100 and 1 % BSA and incubated with the secondary antibodies, FITC-conjugated

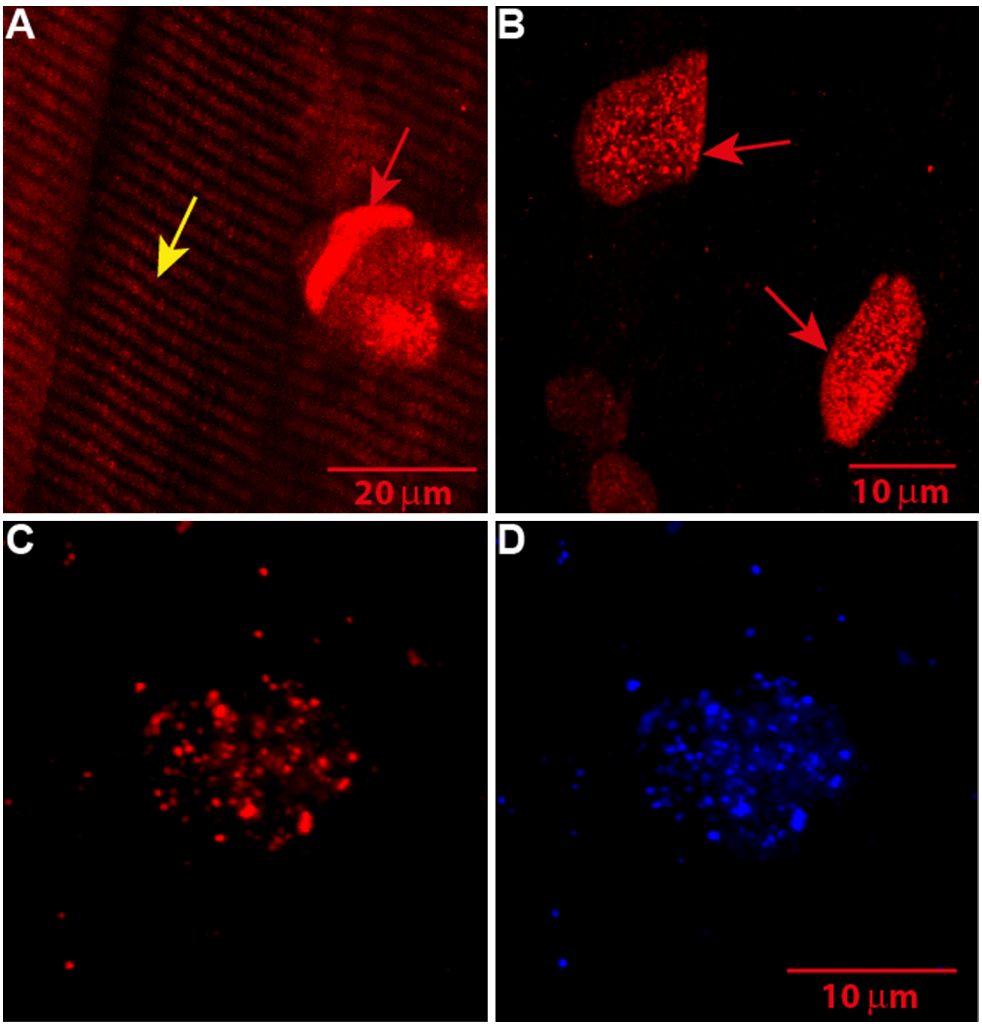

Fig. 2 Localization of ACh receptors at the membrane of cutaneous pectoris muscle fibers. **a** The *red arrow* indicates the saturated intensity in the region corresponding to the motor end-plate of a muscle fiber. The *yellow arrow* indicates the striations in the muscle fiber. **b** Two different motor end-plates are shown using non-saturating laser intensity (*red arrows*). These two motor end-plates correspond to different cells. Striations could not be seen under non-saturating settings. **c** ACh receptors labeled with specific antibody. **d** ACh receptors tagged using α-bungarotoxin. $n = 8$ animals

anti-rabbit IgG (1:250; Abcam), and Alexa fluor-568-conjugated anti-mouse IgG (1:250; Molecular Probes, Eugene, OR), for 2 h in the dark. Finally, the muscles were washed three times in PBS with 0.2 % Triton X-100 and 1 % BSA and two times in PBS for 5 min each. Additional experiments using α-bungarotoxin conjugated to Alexa-Fluor 555 were performed to label ACh receptors. The α-bungarotoxin (2 μg/ml) was applied for 15 min at 24 °C prior to slide mounting (Lindgren et al. 2013). The muscles were mounted on microscope slides using Prolong Antifade (Molecular Probes) and coverslips. Confocal images were taken in 1 μm increments using a LSM700 Zeiss confocal microscope with a 40× plan-apochromatic objective at a resolution of 0.078 m/pixel. Images were 3D blind deconvolved using Autoquant X3 (Media Cibernetics Inc., Rockville, MD, USA) and analyzed using ZEN 2009 (Zeiss).

Colocalization Analysis

The colocalization cross-correlation was analyzed using Protein Proximity Analyzer software in accordance with the published methodology (Wu et al. 2012; Zinchuk et al. 2011). This quantification is capable of determining the fraction of proteins in one channel that colocalized with the proteins in the other channel. In this kind of analysis, a sharp peak indicates a high degree of colocalization, which may suggest an interaction between the analyzed proteins.

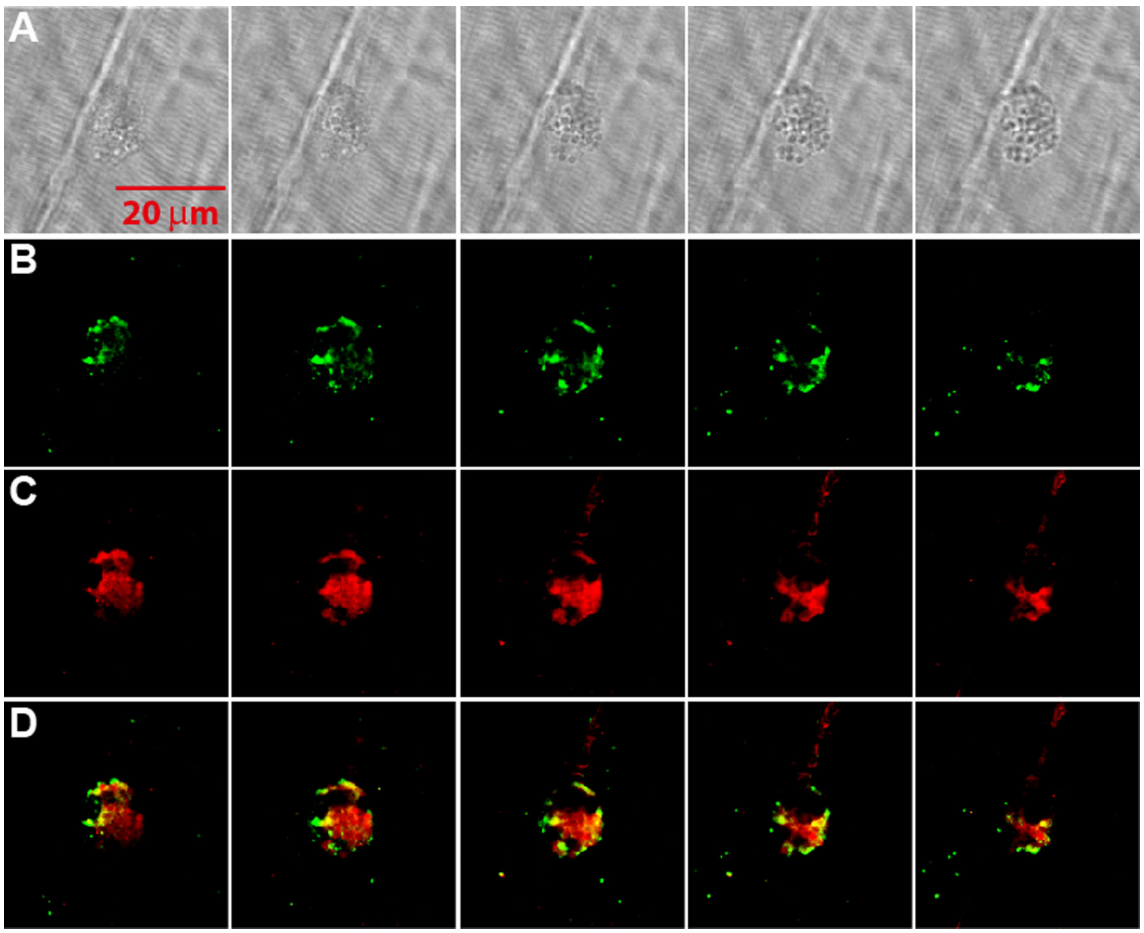

Fig. 3 Sectioning of muscle fibers co-labeled for CB_1 and ACh receptors. Both receptors are highly expressed at the neuromuscular end-plate. **a** Muscle fiber surface visualized by transmitted light. **b** CB_1 receptors. **c** ACh receptors. **d** Overlay of CB_1 and ACh receptors. $n = 7$ animals

Results

Confocal Images of CB_1 in Skeletal Muscle Fibers

The representative images in Fig. 1a–d show the distribution of CB_1 receptors on the surface of cutaneous pectoris cells on different planes. These cells were single-labeled with the CB_1 antibody. Yellow arrowheads indicate the receptor in the membranes of different fibers. The white arrow in Fig. 1c points CB_1 receptor clusters on the membrane. Negative controls were performed by incubating the muscle fibers with only the secondary antibody without the CB_1 antibody, which revealed the background signal in the absence of CB_1 antibody (not shown). Similar results were obtained when labeling four muscles with another CB_1 antibody (Abcam, Cat. No. 23703) that recognizes a different region within the receptor (Fig. 1d). Figure 1d shows a magnified image pointing the striations (red arrowheads) on the surface of the muscle fiber and some clusters at the membrane of the fibers (white arrowheads). The specificity of the labeling was tested by pre-absorbing the antibody overnight at 4 °C with its immunogenic peptide (100 μg/ml; Abcam, Cat. No. 50542) prior to incubation on the samples, which revealed the background signal (Fig. 1f). The fibers labeled with the pre-absorbed CB_1 antibody are shown in Fig. 1e.

Confocal Images of Acetylcholine Receptors in Skeletal Muscle Fibers

Figure 2 shows the localization of ACh receptors in muscle fibers of the frog. By using high laser intensity, we were able to establish the ACh receptor distribution along the entire muscle fiber membrane. Localization of these receptors on the striations of the muscle fibers is clearly shown in the first image (Fig. 2a). The characteristic striations on the surface are indicated by a yellow arrow, showing the existence of ACh receptors on the complete fiber surface. An expanded image is shown in Fig. 2b to appreciate the high density of ACh receptors at the motor end-plates in two different muscle fibers. ACh receptor expression was much higher at the motor end-plate than

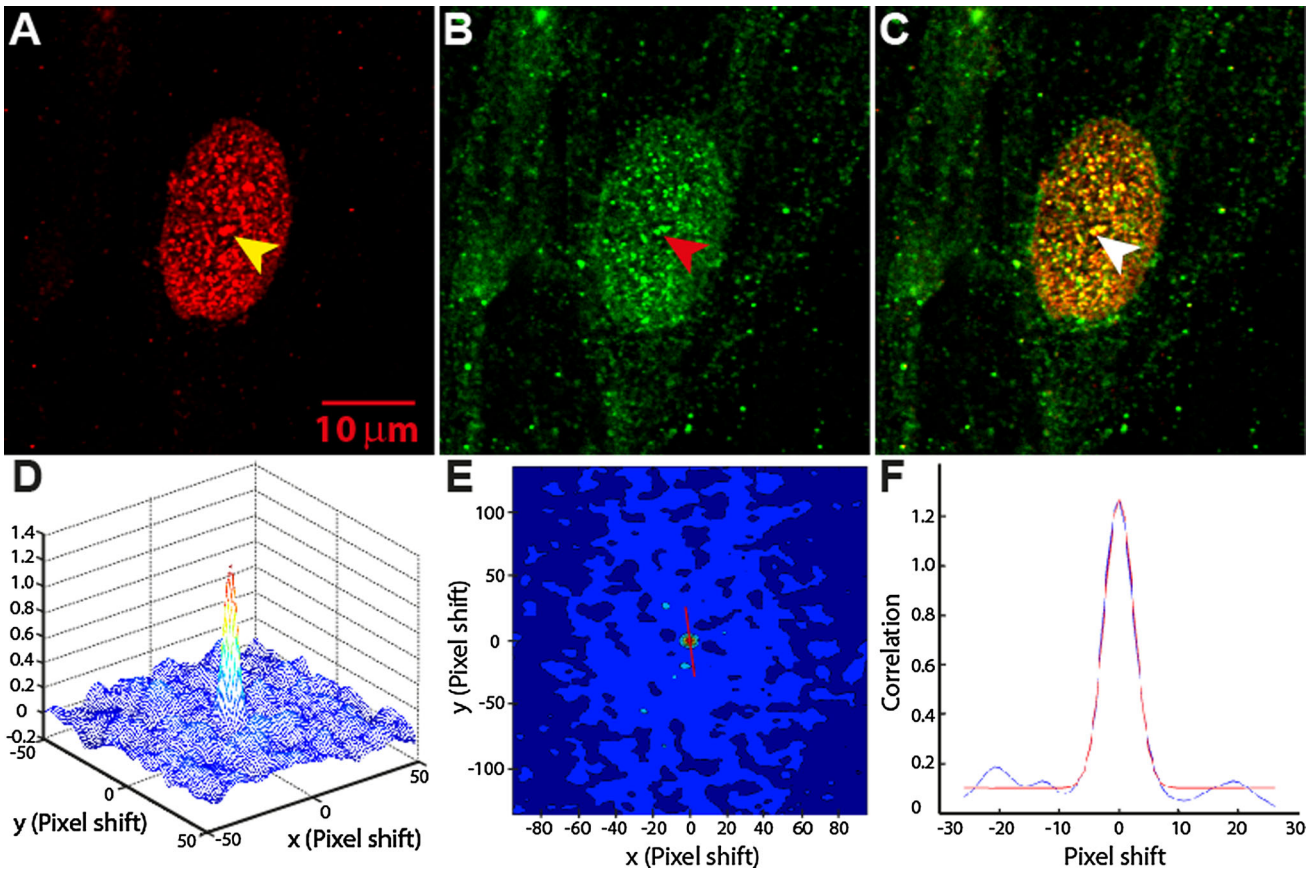

Fig. 4 CB_1 co-localizes with ACh receptors at the motor end-plate on the membranes of muscle fibers in the frog. **a** ACh receptor expression, labeled with its specific antibody and Alexa-fluor 568 (*Red*), is much higher at the motor end-plate than along the rest of the muscle fiber membrane (*yellow arrowhead*). **b** CB_1 receptors, labeled with antibody and FITC (*Green*), are expressed mainly at the motor end-plate (*red arrowheads*). **c** Overlaying the images produces a *yellow color* where both receptors co-localize (*white arrowheads*). (**d**–**f**) Colocalization analysis showing the cross-correlation by pixel-shift. **d** Three-dimensional mesh plot of the cross-correlation function. The sharp peak corresponds to colocalization. **e** Contour plot of the cross-correlation function. The *straight line* corresponds to the region used for fitting (**f**). **f** The *red line* indicates the non-linear fit of the indicated region in (**e**) to a single Gaussian function

along the rest of the muscle membrane (Fig. 2b, red arrows). The labeling of ACh receptors by α-bungarotoxin is shown in Fig. 2d; the labeling pattern coincided with the labeling observed when ACh receptor antibody was used (Fig. 2c) in the same end-plate, which supports the specificity of the antibody used in this study.

CB_1 Colocalizes with ACh Receptor at the Motor End-plate in the Frog

In order to determine whether CB_1 colocalizes with the ACh receptor at the motor end-plate, we co-labeled muscle fibers with both CB_1 receptor antibody and α-bungarotoxin conjugated to Alexa-Fluor 555 labeling ACh receptors (Fig. 3) or Ach antibody (Fig. 4). Several sections were acquired at high magnification in order to show ACh receptor clusters colocalized with CB_1 receptor clusters on muscle fiber end-plates. Figure 3a shows several planes of the muscle fiber membrane acquired with transmitted light where striations can be seen and many invaginations at the motor end-plate. CB_1 clusters are localized at the motor end-plate (Fig. 3b) which coincides with the expression of Ach receptors as well as in the motor end-plate (Fig. 3c–d).

Further analysis was performed in order to determine whether CB_1 colocalizes with the ACh receptor at the motor end-plate. At higher magnification, the expression of ACh receptors(labeled with Ach receptor antibody and Alexa-fluor 568) was elevated at the motor end-plate (Fig. 4a). We also observed an increase in CB_1 receptor expression at the motor end-plate (Fig. 4b). The overlay of the images is shown in Fig. 4c. The expression of both receptors was much higher at the motor end-plate than on the whole surface, and CB_1 exhibited a high degree of colocalization (yellow, red, and white arrowheads) with the ACh receptor.

Finally, we performed protein proximity analysis to quantify the colocalization of the receptors (Fig. 4d–f). The mesh plot for the cross-correlation function is shown in Fig. 4d. The straight line in the contour plot (Fig. 4e)

indicates the region where the fitting was conducted using a double Gaussian function (Fig. 4f). The protein proximity index (PPI) values for these images were 0.523 for ACh receptors and 0.481 for CB_1 receptors, and the cross-correlation coefficient was 0.502. This analysis was performed for nine end-plates on images taken every 0.5 μm in the *Z*-axis, which were analyzed from the muscles of seven different frogs. The average PPI was 0.573 ± 0.034 for ACh receptors, 0.473 ± 0.021 for CB_1 receptors, and 0.519 ± 0.021 for the cross-correlation coefficient.

Discussion

A previous study (Sánchez-Pastor et al. 2004; Huerta et al. 2009) demonstrated that the transcript for the cannabinoid receptor, CB_1, is present in the twitch and slow skeletal muscle fibers of the frog. The present study shows the first direct evidence of the presence of the cannabinoid receptor on the membranes of these muscle fibers. We previously reported that the synthetic cannabinoids, WIN212-2 (WIN) and ACPA, diminish the amplitude of the MEPP (Sánchez-Pastor et al. 2007). However, pertussis toxin, a G-protein inhibitor, and AM281, a CB_1 antagonist, inhibit the ACPA effect on MEPP amplitude. These effects suggest the presence of post-synaptic CB_1 receptors.

Using polyclonal anti-CB_1 antibodies, we have shown that CB_1 is present on the entire membrane of fast muscle fibers in the frog. The specificity of the antibody used in these experiments was demonstrated previously by showing the typical staining pattern in the human cerebellum compared to a lack of antibody labeling in CB_1-knockout mice (Chung et al. 2009), and we also made some controls using the immunogenic peptide. This result is consistent with the finding that cannabinoids reduce the tension of caffeine-evoked contractures of slow and fast muscle fibers (Huerta et al. 2009). This effect does not occur when the fiber is treated with pertussis toxin or AM281, suggesting that cannabinoids decrease tension via the activation of cannabinoid receptors, functioning at some step of excitation–contraction coupling in frog skeletal muscle fibers.

Furthermore, by co-labeling with a monoclonal antibody against the ACh receptor or α-bungarotoxin, we detected the presence of both CB_1 and ACh receptors on the skeletal muscle motor end-plate. Based on the cross-correlation coefficient, the present study shows that these two receptors are very close in the motor end-plate of these fast skeletal muscle fibers and suggests a close interaction between these two proteins, possibly through modulation of the activity of the ACh receptor by G proteins (Butt and Pitman 2002). Further experiments are necessary to determine the functional interaction between the CB_1 and ACh receptors.

Conclusions

The present study shows the first direct evidence for the presence of the cannabinoid receptor on the membrane of these muscle fibers. CB_1 is normally present in the membranes of skeletal muscle fibers and is expressed at higher levels in the end-plate, where it colocalizes with the ACh receptor, which is primarily expressed at the end-plate. Further investigation is necessary to clarify the functional interaction between these receptors.

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Conflict of interest The authors declare no conflicts of interest.

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