CAP modulates acetylcholine release at the myoneural junction

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Abstract

Transient receptor potential (TRP) proteins are non-selective cation channel proteins that are expressed throughout the body. Previous studies demonstrated the expression of TRP Vanilloid 1 (TRPV1), capsaicin (CAP) receptor, in sensory neurons. Recently, we reported TRPV1 expression in mouse motor nerve terminals [MNTs; (Thyagarajan et al., 2009)], where we observed that CAP protected MNTs from botulinum neurotoxin A (BoNT/A). Phrenic nerve diaphragm nerve muscle preparations (NMP) isolated from isoflurane anesthetized adult mice were analyzed for twitch tension, spontaneous (mEPCs) and nerve stimulus evoked (EPCs) acetylcholine release. When acutely applied to isolated NMP, CAP produced a concentration-dependent decline of twitch tension and produced a significant decline in the amplitude of EPCs and quantal content without any effect on the mEPCs. The suppression of nerve stimulus evoked acetylcholine release by CAP was antagonized by capsazepine (CPZ), a TRPV1 antagonist. CAP did not suppress phrenic nerve stimulus evoked acetylcholine release in TRPV1 knockout mice. Also, CAP treatment, *in vitro*, interfered with the localization of adapter protein 2 in cholinergic Neuro 2a cells. Wortmannin, (WMN; non-selective phosphoinositol kinase inhibitor), mimicked the effects of CAP by inhibiting the acetylcholine exocytosis. Our data suggest that TRPV1 proteins expressed at the MNT are coupled to the exo-endocytic mechanisms to regulate neuromuscular functions.

Keywords

CAP; capsazepine; TRPV1; motor nerve terminal; acetylcholine release; twitch tension; end plate currents; spontaneous release; clathrin; PIP2; endocytosis; exocytosis

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Conflict of interests
None.

1.0 Introduction

Transient receptor potential vanilloid 1 (TRPV1), also known as CAP receptor, is a member of the TRP superfamily. TRPV1 is activated by various exogenous (capsaicin, resiniferatoxin, etc) and endogenous (pH, temperature, endovanilloids, etc) ligands (Caterina et al., 1997). Although reported to be exclusively expressed in the dorsal root ganglion neurons, recent studies have shown a widespread expression of TRPV1 in brain (Toth et al., 2005) spinal cord and in peripheral nociceptive neurons (Cui et al., 2006). At the peripheral terminals of primary nociceptors, TRPV1-mediated Ca^{2+} influx triggers the release of neuropeptides, which contribute to the development of neurogenic inflammation (Caterina and Julius, 2001). Capsaicin (CAP), a pungent principle from hot peppers of the capsicum family and resiniferatoxin are naturally occurring agonists that activate TRPV1 (Holzer, 1991; Szallasi and Blumberg, 1991). CAP activates the TRPV1 ion channel resulting in depolarization and excitation (Marsh et al., 1987) and the effects of CAP are blocked competitively by the antagonist capsazepine [CPZ; (Bevan et al., 1992)].

As early as in 1982, the presence of CAP-sensitive afferent fibers in the skeletal muscles was recognized and it was shown that stimulation of these group IV afferents (nonmyelinated C fibers) by CAP caused reflex cardiovascular effects (Kaufman et al., 1982). At the frog neuromuscular junction, it was shown that cannabinoid and vanilloid receptor agonist arachidonyl-2-chloroethylamide (ACEA) and CAP increased the quantal content of stimulus evoked acetylcholine release and this effect was blocked by CPZ (Silveira et al., 2010). In a recent study, expression of TRPV1 receptor was demonstrated for the mouse MNT and that the receptor was sensitive to CAP (Thyagarajan et al., 2009). Although the existence of TRPV1 receptors in the mammalian motor nerve terminal (MNT) has been shown, the role it plays in the physiology of acetylcholine release has not been investigated. This work fills this knowledge gap by investigating the effect of CAP on the mouse MNT and analyzes the role of TRPV1 activation in the modulation of acetylcholine release at the MNT.

2.0. Material and methods

2.1. Animals

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee. Wild type C57BL/6 and TRPV1$^{-/-}$ mice were obtained from Jackson Laboratories, CT, USA. Animals were housed in the research animal facility located in the School of Pharmacy and were given food and water *ad libitum*.

2.2. Muscle twitch tension

Diaphragm nerve muscle preparations (NMP) were isolated from adult male wild type or TRPV1 knockout (TRPV1$^{-/-}$) mice anesthetized with isoflurane. After assuring full anesthesia, the mice were killed by cervical dislocation. The procedure for removal of diaphragm muscle with the phrenic nerve, mounting in the tissue bath and recording nerve-elicited muscle twitches was according to that described earlier (Thyagarajan et al., 2009; Thyagarajan et al., 2010). The diaphragm with attached phrenic nerve was mounted in a

glass chamber (Rodnoti Glass Technology, Inc., USA) filled with oxygenated (95% O_2 - 5% CO_2) normal Ringer solution (physiological solution; pH 7.4, 37 °C) containing (mM) NaCl (135), KCl (5), $MgCl_2$ (1), $CaCl_2$ (2), Na_2HPO_4 (1), $NaHCO_3$ (15) and glucose (5.5). The phrenic nerve was drawn into a suction electrode for indirect activation (1 Hz) of muscle twitches. One tendon of the muscle was tied to a Grass force transducer connected to a Digidata 1440A (Molecular Devices Inc., USA) for acquisition of the mechanical response (grams of tension developed) in response to the nerve stimulation. Muscles were stretched to optimal length for force generation and equilibrated for 30 min prior to nerve stimulation at 1 Hz for data acquisition. CAP and CPZ were added to the physiological solution and concentrations were expressed as final bath concentration (µM). Data collected at 120 min. period following the addition of different cumulative concentrations CAP was analyzed with pCLAMP 10 software. In experiment where CAP dose response was studied, no more than two concentrations of CAP were studied in one experiment (n = 4 diaphragms (8 hemidiaphragms) obtained from 4 mice for each experiment).

2.3. EPC and mEPC measurement using Two Electrode Voltage Clamp

Diaphragm NMP were dissected from isoflurane-anesthetized mice after cervical dislocation as described above. The muscles were pinned to a Sylgard-lined plexiglass chamber, and bathed in HEPES Ringer Solution (HRS; 22–25 °C) containing (mM): NaCl (135), KCl (5), $MgCl_2$ (1), $CaCl_2$ (2), Na_2HPO_4 (1), HEPES (10, pH 7.4) and glucose (5.5). Spontaneous transmitter release [miniature endplate currents (mEPCs)] and nerve stimulus evoked endplate currents (EPCs) were recorded from the endplate region (−75 mV holding potential) using two-electrode voltage clamp technique as described previously (Thyagarajan et al., 2009; Thyagarajan et al., 2010). Two electrodes were inserted into the endplate membrane at interelectrode distance of approximately 50 µm. One electrode filled with 4 M potassium acetate served to deliver current while the other electrode filled with 3 M KCl recorded membrane voltage. The phrenic nerve was drawn into a suction electrode and stimulated supramaximally at 1 Hz or 20 Hz. The recordings of mEPCs and EPCs were obtained using Axoclamp 900A (Molecular Devices, Sunnyvale, CA) and analyzed offline for the EPC amplitude and mEPC amplitude and frequency. Prior to EPC recordings, 0.75 µM Conotoxin GIIIB was added to the HRS solution to block muscle contractions during the EPC recordings. Control recordings were made before application of CAP or CPZ. Collected data were analyzed with pCLAMP 10 software. Quantal content was calculated as ratio of the mean EPC amplitude to mean mEPC amplitude and represented as mean ± S.E.M.

2.4 Cell Culture and transfection

Mouse cholinergic neuroblastoma (Neuro 2a) cells were cultured in Dulbecco's modified Eagle's F12 medium, pH 7.4, supplemented with 10% fetal bovine serum and antibiotics. Human embryonic kidney 293 (HEK293) cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. The rat TRPV1 tagged with the *myc* epitope on the N terminus in pCDNA3 vector was transfected using the Effectene reagent (Qiagen, Chatsworth, CA) in HEK293 cells or Neuro 2a cells. Transfected cells were exposed to Dulbecco's modified Eagle's medium (DMEM) containing 1 mg/ml G418 on day 3 and stable clones were obtained on day 21. Cells that

stably expressed TRPV1 were maintained in G410 (250 μg/ml) and the medium was changed to normal DMEM (no G418) 48 hours before the experiments.

2.5 Confocal microscopy and immunoblotting

In order to detect if TRPV1 is expressed at the mouse MNT, immunoblotting was performed on the phrenic nerve diaphragm NMP. HEK293 cells expressing TRPV1 channel protein and wild type HEK293 cells served as positive and negative controls, respectively. Diaphragm NMPs were used for immunoblotting experiments to detect the extent of TRPV1 expression. Endplate-rich regions from the muscle NMP were stained for acetylcholine esterase (AChE) by a previously described method (Koelle and Horn, 1968) and modified by Gautron (Gautron, 1982). Acetylthiocholine was the substrate for the staining reaction. AChE labeling was performed at room temperature. After a 30-min reaction period, NMP were washed with HRS. Endplate regions were cut under a stereo zoom microscope and flash frozen in liquid nitrogen. Tissues were homogenized in phosphate-buffered saline (PBS) containing 1% Nonidet 40 and complete protease inhibitor cocktail (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The homogenate was then centrifuged at 20817 g for 10 min. at 4 °C, The crude lysate (100 μg) was resolved via SDS-polyacrylamide gel electrophoresis after boiling in 1 X Laemmli. HEK293 cells that stably express TRPV1 were homogenized and resolved by SDS-PAGE similarly.

To demonstrate that CAP pretreatment modifies the subcellular distribution of AP-2 (adapter protein-2), TRPV1 stably expressing Neuro 2a cells were used. Two groups of TRPV1 expressing Neuro 2a cells were seeded on poly (L-lysine)-coated cover slips. The experimental group was incubated (at 37 °C) in HRS containing 1 μM CAP (See Fig. 6 legend) for 60 min and washed. The control group was incubated at 37 °C with vehicle only. After washing the coverslips with HEPES, cells were immunostained with AP-2μ-1 antibody (Santa Cruz Biotech, Inc, USA) followed by FITC (secondary antibody) and mounted with Vectashield on a slide and kept frozen at −20°C before imaging with an LSM 710 confocal microscope. Images were saved and represented as TIFF files and analyzed with Image J software (National Institutes of Health, Bethesda, MD).

2.6 Chemicals and drugs

μ-Conotoxin GIIIB was obtained from Alamone Labs, Israel. CAP, capsazepine, wortmannin, and all other chemicals and drugs were obtained from Sigma, USA.

2.7 Data analyses

Data are expressed as mean ± S.E.M. The number of mice used for each experiment and the number of muscles/endplates from which recordings were made are shown in the figure legends. Student's *t* test and ANOVA evaluated the statistical significance of population means. Figures from analyzed data were plotted using Microcal Origin 6.0 software.

3.0 Results

3.1. Expression of TRPV1 (CAP receptor) in mouse MNT

We first wanted to confirm the presence of TRPV1 receptor at the mouse MNT. The results of western blot showing TRPV1 in lysates of mouse diaphragm NMP and HEK293 cells transfected with TRPV1 are shown in Fig.1A. TRPV1 expression (band at ~100 kDa) was seen in the diaphragm NMP in both lane 1 and 2 (n = 6) as well as in HEK 293 cell transfected with TRPV1 (n = 6). The expression of this protein was undetectable in wild type HEK293 cells. Fig.1B illustrates the western blotting for TRPV1 in the diaphragm NMP of the wild type and TRPV1$^{-/-}$ mice. These findings confirmed previous reports showing the expression of TRPV1 in the mouse MNT (Thyagarajan et al., 2009) and in frog neuromuscular junctions (Silveira et al., 2010).

3.2. Effects of CAP on twitch tensions of diaphragm

Since CAP activates TRPV1 and CPZ is a competitive antagonist of CAP, we decided to study the effect of these drugs on the mouse NMJ *in vitro*. The time course of twitch tensions recorded in response to 1 Hz phrenic nerve stimulation and the mean twitch tensions before (30 min equilibration period), soon after CAP (1 μM) or 30 min. pretreatment of CPZ (10 μM) and at the end of 250 min are shown in Fig. 2A and B respectively. In control preparations exposed to only physiological solution, there was approximately 10–15 % decline in the twitch tension by 250 min (2487 ± 122 mg at 0 time to 2188 ± 109 mg at 250 min; Fig. 2B). This decline was within the expected limits for muscles perfused *in vitro* for 4 hr. duration since *in situ* conditions cannot be duplicated *in vitro* CAP produced a progressive decline of the twitch tension over 250 min. (Fig.2A and 2B). To study the effect of TRPV1 antagonist CPZ, the muscles were pretreated with CPZ, 30 min prior to CAP addition, and both compounds were present throughout the duration of experiment. At 250 min. the combination of two drugs produced only about 40% drop in twitch tension (Fig. 2B; 1466 ± 44 mg with CAP + CPZ *versus* 258 ± 14 mg in CAP only, P<0.001). CPZ alone did not cause any inhibition of stimulus twitch tension as shown in Fig. 2A (orange trace) and B.

A concentration- response study with CAP in concentrations ranging from 1 nM to 800 μM was performed. The data demonstrated that as little as 10 nM produced a decrease in the normalized muscle tension significantly (Fig. 3; 5 ± 2 %). IC50 for the inhibition of twitch tension by CAP was calculated using the concentration-response curve. The theoretical IC50 for stimulus evoked twitch tension by CAP was calculated to be 1.77 μM. For the electrophysiology studies, we have used 1 μM CAP, which is lower than IC50 dose but was found to be sufficient to block neuromuscular transmission. Also, 1 μM CAP significantly activates TRPV1 and we used this concentration of CAP for the entire study.

3.3. Effects of CAP on EPCs and mEPCs

We characterized the nature of nerve stimulus evoked endplate currents (EPCs) and spontaneous transmitter (miniature; mEPCs) following in vitro application of CAP (1 μM). Muscle contractions were eliminated with μ-conotoxin GIIIB (100 nM; for 60 min). As shown in Fig. 4A EPCs were almost completely inhibited at 120 min after 1 μM CAP and

this inhibition was prevented by CPZ (10 μM; Fig. 4A), which was added to the bath 30 min. before the application of CAP and was present for the entire duration of the experiment. CPZ alone had neither an activating nor an inhibiting effect on acetylcholine release (Fig. 4A d). During 120 min. exposure to both drugs, alone or in combination, the mEPC amplitude and frequency (Fig. 4C and D) were unaltered indicating that the effect of CAP was primarily on the stimulus evoked acetylcholine release. The data for EPC amplitude and quantal content (Fig. 4 B and E) showed that CAP drastically reduced EPC amplitude and quantal content after 120 min exposure (mean EPC amplitude of 12 ± 5 nA with CAP vs 72 ± 9 in control, $P<0.001$ and quantal content of 10 ± 3 with CAP *versus* 42 ± 6 in control, $P<0.001$). CPZ (10 μM) prevented CAP–induced inhibition of EPCs (quantal content for NMP treated with CPZ + CAP was 38 ± 4; Fig. 4E). These data clearly indicate that CAP blocked nerve stimulus evoked acetylcholine release at the mouse MNT, an effect that was prevented by CPZ and secondly CAP did not alter spontaneous acetylcholine release.

3.4. Lack of effect of CAP on EPCs in diaphragm NMP of TRPV1$^{-/-}$ mice

If CAP activation of TRPV1 at the MNT causes a decline in EPC amplitude over the course of 120 min, similar experiments performed in the mouse NMP removed from mice lacking TRPV1 (TRPV1$^{-/-}$) should show EPC amplitude comparable to that exhibited in controls. The data from EPC recordings in MNTs of TRPV1$^{-/-}$ mice are shown in Fig. 5. Representative EPC and mEPC amplitudes for control and CAP treated diaphragm NMP obtained from TRPV1$^{-/-}$ mice are shown in Fig. 5A. Fig. 5B shows the mean EPC amplitude in diaphragm MNTs from wild type mice was significantly decreased by 120 min. of 1 μM CAP exposure ($P<0.001$; 13 ± 3 nA and 73 ± 8 nA with CAP and control respectively). In contrast, no significant difference in amplitude was seen in EPC amplitude for TRPV1$^{-/-}$ NMPs exposed to CAP as compared to EPCs recorded from NMPs exposed to CAP in WT mice. Also, there was no significant difference between control EPC amplitudes (before the addition of CAP) recorded from endplates of diaphragm NMP of wild type and TRPV1$^{-/-}$ mice indicating that the neuromuscular physiology in TRPV1$^{-/-}$ MNTs was comparable to that shown in wild type mice. Consistent to this, CAP did not have effects on diaphragm NMPs of TRPV1$^{-/-}$ at 20 Hz stimulation, while significantly inhibited the EPCs of wild type NMPs (Fig. 5C).

3.5. Interference with the expression/localization of adapter protein 2 (AP2 -μ1) in Neuro 2a cells by CAP pretreatment

In order to determine if CAP interfered with the endocytic mechanisms that couple to exocytosis, experiments were conducted with TRPV1 expressing Neuro 2a cholinergic cells. The cells were preincubated with CAP (1 μM) for 60 min. and immunostained with *AP2 -μ1* antibody followed by FITC labeled secondary antibody (see methods for details). As shown in Fig. 6, *AP2 -μ1* was localized near the plasma membrane (periphery of the cell) in the control condition. CAP treatment delocalized *AP2 -μ1* in TRPV1 expressing Neuro 2a cells. Conversely, CAP treatment did not affect AP-2 in wild type Neuro 2a cells that did not express TRPV1 (Fig. 6; upper and lower panels on right; four independent experiments; n = 288 for *AP2 -μ1*).

3.6. Effect of wortmannin on EPCs and mEPCs

Membrane bound phosphatidyl inositol (4,5) bisphosphate (PIP2) is an important regulator of exo-endocytosis coupling at the synapses and is modulated by neuronal activity (Koch and Holt, 2012; Micheva et al., 2001). Furthermore, previous research demonstrates that CAP-induced Ca^{2+} influx via TRPV1 causes a decrease in membrane PIP2 via PLC dependent mechanisms (Lukacs et al., 2007; Rohacs et al., 2008; Rosenbaum and Simon, 2007). Next, we tested if wortmannin (WMN), a PI-3 and PI-4 Kinase inhibitor (Hong and Chang, 1999; Winks et al., 2005), which decreases PIP2 synthesis (Halet et al., 2002), will inhibit EPCs and mEPCs at the mouse MNT. The logic for the use of WMN was to examine a TRPV1-independent mechanism, which would decrease PIP2 levels in the nerve terminal by blocking its synthesis. WMN suppresses PIP2 by inhibiting PI Kinases (Halet et al., 2002; Winks et al., 2005). It is known that clathrin coated pit (CCP) mediated endocytosis requires PIP2 (Nakano-Kobayashi et al., 2007) and WMN sensitive PI kinases are involved in clathrin-dependent endocytosis (Haucke, 2005; Nakano-Kobayashi et al., 2007; Wenk et al., 2001). Since, a tight balance between synaptic vesicle exocytosis and endocytosis is important for maintaining synaptic structure and function, inhibiting either of the component will affect this balance and suppress the other. That is, inhibiting endocytic process will have an inhibiting effect on exocytosis. Previous research work demonstrates that inhibition of PI3 kinase slows down endocytosis (Richards et al., 2004). Further, WMN treatment has been shown to inhibit clathrin-dependent endocytosis of neurotoxin by delocalizing endocytic proteins (Potian et al., 2010). If the endocytic mechanisms couple to exocytosis, then WMN treatment should suppress exocytosis. These background literatures provide a rationale for testing the effects of WMN (20 μM) on isolated NMPs. If WMN decreases membrane PIP2 dependent exocytosis, then inhibiting the synthesis of PIP2 by WMN will suppress stimulus evoked acetylcholine release. Consistent to this notion, WMN treatment for 120 min. significantly suppressed the EPCs (Fig. 7A). Also, washing of NMPs even up to 240 min. did not restore the suppressive effect of WMN. The mean EPC amplitude recorded from several NMP showed a significant decrease from the controls (71 ± 6 in control *versus* 9 ± 1.1 with WMN; P<0.01; Fig. 7B). The quantal content decreased from 38 ± 2 in controls to 4 ± 0.9 with WMN (Fig. 7E). However, WMN did not alter mEPC amplitude while slightly increased the mEPC the frequency (P< 0.05; Fig. 7 C and D).

3.7. WMN inhibits EPCs in TRPV1$^{-/-}$ mouse MNT

We tested WMN on the EPCs recorded from diaphragm MNTs in TRPV1$^{-/-}$ mice. As seen in Fig. 8, CAP did not change EPC amplitudes whereas a 120 min. exposure of diaphragm NMP to 20 μM WMN inhibited the EPC amplitude significantly (P<0.01) indicating that the effects of WMN were independent of TRPV1.

4.0. Discussion

The molecular machinery underlying nerve stimulus evoked and spontaneous acetylcholine release at the mammalian MNT has been intensely studied in recent years. Although the role of voltage gated Ca^{2+} channels and intracellular Ca^{2+} sensors that regulate the fusion and release of acetylcholine containing vesicles at the presynaptic nerve terminal have been recognized for number of years, the precise role of Ca^{2+}-dependent mechanism/s involved in

the exo-endocytic processes at the myoneural junction is still under investigation. Also, our understanding of how endocytosis at the motor nerve endings couples to acetylcholine exocytosis still remains unclear.

Nonselective cation channels, including TRP proteins are expressed in the motor nerve endings (Silveira et al., 2010; Thyagarajan et al., 2009). Understanding the role of TRPV1 in MNT may provide new understanding of the relation between exo-endocytotic processes. In the present study, we have used CAP, *in vitro*, as a tool to elucidate the molecular events involved in endocytosis and its connection to the exocytosis of acetylcholine release. Using immunoblotting technique, we have shown that the CAP receptor, TRPV1 is expressed at the mouse MNT (Fig. 1). However, the precise role of TRPV1 in the endoexocytic processes at the neuromuscular junction has not been studied until now. Recently, we have shown that CAP pretreatment prevented botulinum neurotoxin A (BoNT/A) uptake at the MNT. and accelerated recovery of the muscles from the toxin-induced paralysis (Thyagarajan et al., 2009). The interference of CAP in BoNT/A uptake suggests that CAP affects endocytic mechanisms. Our data demonstrate that activation of TRPV1 by CAP leads to suppression of acetylcholine release at the MNT.

CAP produced a concentration-dependent decline of twitch tension in response to the phrenic nerve stimulation (Fig. 2). The effect of CAP was indeed due to activation of TRPV1 channels because CPZ (a TRPV1 antagonist) was able to antagonize CAP - induced depression of twitch response. Furthermore, results from intracellular recordings of EPCs and mEPCs from MNTs revealed that 1 μM CAP inhibited EPCs (reduced amplitude and quantal content) within 120 min and the inhibitory effect was antagonized by CPZ. Washing CAP did not reverse its suppressing effect on acetylcholine release. However, CAP did not alter the amplitude or frequency of mEPCs in MNTs of diaphragms from WT mice (Fig. 4). Further, CAP did not depress EPCs recorded from MNTs of TRPV1$^{-/-}$ mouse diaphragms (Fig. 4 and 5). Taken together, these findings demonstrate that at the mouse MNT, *in vitro* application of CAP preferentially causes inhibition of neuromuscular transmission by decreasing the quantal content without any change in the spontaneous (mEPC) release of acetylcholine vesicles.

Previously, we have shown that a local injection of CAP near the innervations of mouse extensor digitorum longus (EDL) muscle (3 μl of 1 mM stock solution) did not cause any overt neuromuscular changes in the EDL muscle 24 hr. after injection. The measurements included compound muscle action potential, twitch tension, and EPC amplitude (Thyagarajan et al., 2009). The same investigation also showed that preinjection of CAP significantly protected mouse EDL from the neuroparalytic effects of BoNT/A. This was a seminal finding that also indicated that CAP prevented the endocytosis of the neurotoxin into MNT. The lack of inhibitory effects of CAP injection, near the EDL region *in vivo*, on the neuromuscular functions, is suggestive of a disconnection between the *in vitro* and *in vivo* effects of CAP.

Experiments performed with Neuro 2a cells that stably express TRPV1 demonstrate that CAP caused delocalization of adapter protein-2 μ1 (AP2-μ1). We used cholinergic Neuro 2a cells for these experiments since the spatial and tempora resolution of MNT is not readily

amenable to immunohistochemical manipulations to analyze the subcellular disposition of *AP2* -µ1. As shown in Fig. 6, CAP treatment significantly perturbed the subcellular distribution of *AP2*-µ1 in TRPV1 expressing Neuro cells. Furthermore, in wild type Neuro 2a cells (that lack TRPV1), CAP treatment did not alter the cellular localization of *AP2* -µ1 (Fig. 6, right panel top and bottom). These data suggest that CAP treatment interferes with an endocytic process, in vitro, which is dependent on TRPV1 channel protein. Connecting our observations on the suppressive effects of CAP on acetylcholine in wild type NMP, delocalizing effects of CAP on AP2-µ1 in TRPV1 expressing Neuro 2a cells and the lack of effects of CAP on TRPV1$^{-/-}$ NMP and wild type Neuro 2a cells, it is reasonable to hypothesize that CAP downregulates acetylcholine release by disrupting endocytosis-dependent exocytosis mechanism.

One of the important regulator of endocytosis is membrane bound PIP2. Increasing evidence indicates that membrane bound PIP2 is an important regulator of endocytic mechanisms. PIP2 is concentrated at the synaptic membrane and has been shown to interact with the components of the endocytotic machinery during clathrin coated pit-mediated endocytosis (Haucke, 2005). Also, PIP2 depletion has been shown to affect clathin coat formation (Di Paolo et al., 2002; Zoncu et al., 2007). Moreover, disruption of PI3Kinase activity inhibits clathrin-dependent as well as -independent endocytic processes (Anas et al., 2009; Gommerman et al., 1997; Sato et al., 1996). These data demonstrate that interfering with phosphinositides synthesis have profound regulatory effects on exo-endocytic mechanisms and that at the synapses, PIP2 plays a pivotal role in exo-endocytosis coupling (Koch and Holt, 2012).

At the presynaptic nerve terminals, Ca^{2+} channels have also been shown to couple exocytosis to endocytosis (Xue et al., 2012). Though such a coupling was shown previously through voltage- gated Ca^{2+} channels, the role of TRPV1 in this process cannot be ignored. If Ca^{2+} influx via TRPV1 can alter membrane PIP2 levels, then it would also affect PIP2-dependent endo-exocytic coupling. Consistent to this, previous study demonstrate that CAP -stimulated Ca^{2+} influx via TRPV1 channels caused hydrolysis of PIP2 to form inositol trisphosphate (IP3) and diacylglycerol (DAG) *via* catalytic action of phospholipase C [PLC; (Lukacs et al., 2007; Prescott and Julius, 2003). Therefore, CAP treatment can cause similar effects at the MNT to decrease membrane PIP2 level, which will affect endocytosis and exocytosis. Our data presented in Fig. 2, 3, 4, 5, and 6 support this notion. The delocalizing effect of CAP on *AP2*-µ1 in TRPV1 expressing Neuro 2a cells (Fig. 6) reinforces our hypothesis that endocytic process was indeed perturbed by CAP due to its ability to decrease membrane PIP2 levels via TRPV1-PLC dependent mechanisms. Our observation that CAP did not suppress acetylcholine release immediately following its application but had a time lag of about 120 min. is suggestive of a signaling mechanism that precedes the inhibition of acetylcholine release. One possibility is that the increase of presynaptic Ca^{2+} caused by activation of TRPV1 by CAP could stimulate PLC-dependent PIP2 hydrolysis. This could interfere with the subcellular disposition of *AP2* -µ1, which in turn reduces endocytosis and thereby suppresses endocytosis-dependent exocytosis of acetylcholine.

Although we did not perform direct measurements of PIP-dependent vesicle recycling, we evaluated the effects of wortmannin (WMN), which cause a decrease in membrane PIP2

levels by a TRPV1-independent (PI3K dependent; WMN) mechanism. WMN suppresses PIP2 by inhibiting PI Kinase enzyme activity. WMN treatment caused delocalization of clathrin heavy chain ((Potian et al., 2010) and suppressed the EPC and quantal content of diaphragm NMP (Fig. 7). These data support our hypothesis that perturbation of membrane bound PIP2 levels affects endocytosis as well as exocytosis.

The results of this study raise the following question: Does *AP2*-µ1-dependent endocytosis couple to acetylcholine exocytosis at the MNT? Answering this question would provide an insight into exo-endocytic coupling at the NMJ. Future research work should address this directly and evaluate the role of TRPV1 channel proteins in PIP2-dependent endo-exocytic coupling at the myoneural junction.

Our study suggests that TRPV1 channels are coupled to acetylcholine release. However, the mechanism whereby TRPV1 regulates exocytosis *via* Ca^{2+}-dependent mechanisms still remains to be investigated. Our observation that CAP fails to suppress EPC in NMP of $TRPV1^{-/-}$ animals provide compelling evidence for the role of TRPV1 in exocytosis. The inhibitory effects of WMN on EPC of $TRPV1^{-/-}$ NMP illustrate the effects of WMN that are independent of TRPV1. Nonetheless, these data suggest an equivocal role of PIP2 in exocytosis. That is, even in the absence of TRPV1, WMN can inhibit membrane PIP2 levels and inhibit EPC.

5.0. Conclusions

Collectively, our data demonstrate a regulatory role of TRPV1 activation in vesicular acetylcholine release. CAP significantly decreased acetylcholine release in the wild type but not in $TRPV1^{-/-}$ NMP. Further, CAP treatment interfered with the subcellular disposition of endocytic adapter protein. We hypothesize that the reduction of endocytosis and exocytosis occurred *via* intracellular Ca^{2+} dependent mechanisms. Moreover, WMN inhibited acetylcholine release and mimicked the effects of CAP. These data support our hypothesis that membrane PIP2 is a determinant of both endocytosis and exocytosis and that perturbation to PIP2 affects the exo-endocytic processes *in vitro*. Fig. 9 illustrates the possible mechanism involved in the delocalizing effect of CAP or WMN on endocytic protein machinery coupled to exocytosis. CAP may affect exo-endocytic processes by decreasing membrane PIP2 levels. The primary findings that we present in this study warrants future research to specifically address the regulatory role of TRPV1 in the mechanisms by which endocytosis couples to exocytosis at the synapses.

Acknowledgment

Abbreviations

AP-2   adapter protein 2

TRP	transient receptor potential
TRPV1	transient receptor potential vanilloid 1
TRPV1$^{-/-}$	TRPV1 knockout
MNT	motor nerve terminal
BoNT/A	botulinum neurotoxin type A
HRS	HEPES Ringer Solution
mEPC	miniature endplate currents
EPC	endplate current
CAP	capsaicin
CPZ	capsazepine
EDL	extensor digitorum longus
NMP	nerve muscle preparation
WMN	wortmannin

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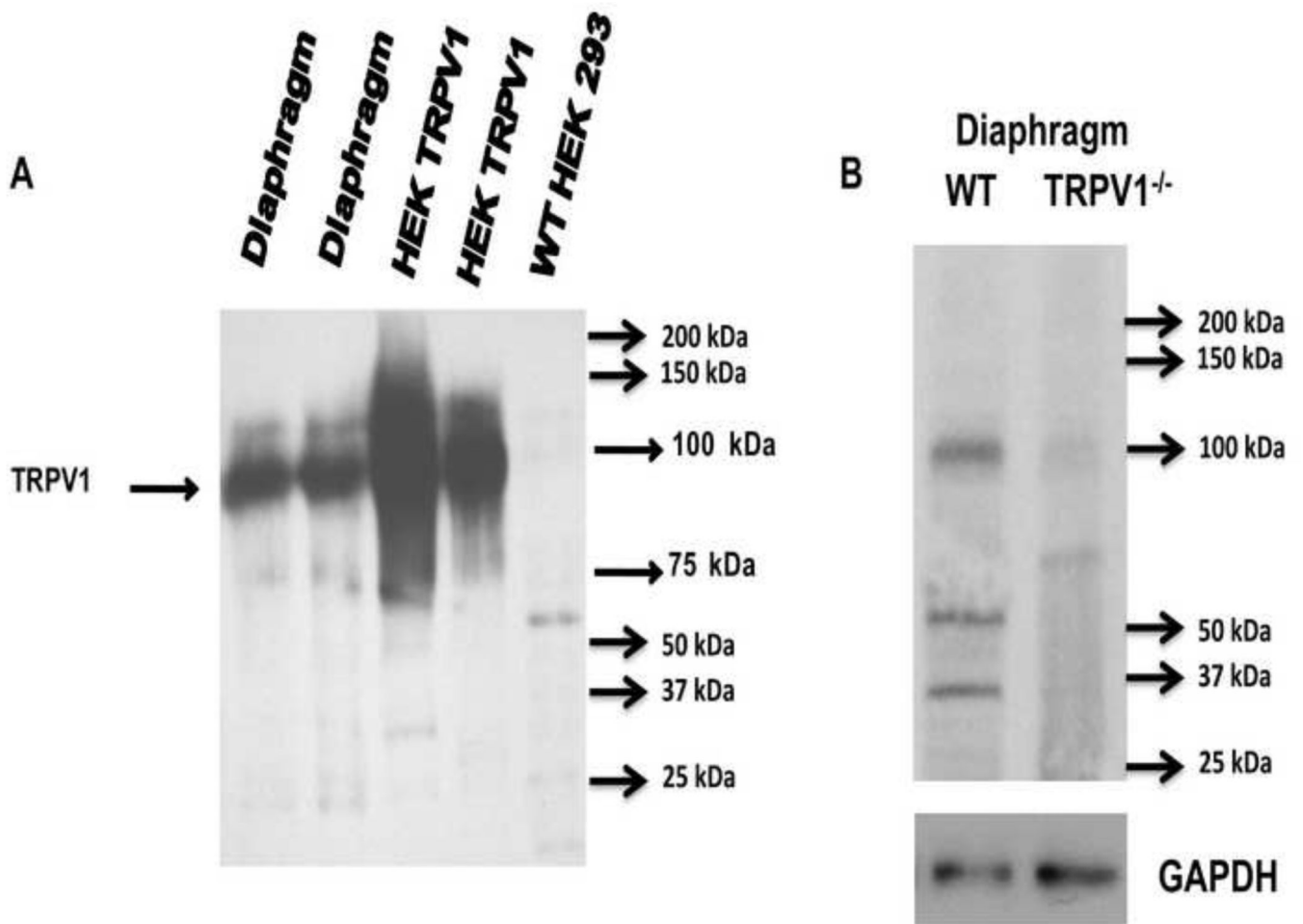
Figure 1. Mouse NMJ expresses TRPV1
A. Western blot showing TRPV1 in lysates of mouse diaphragm and HEK293 cells transfected with TRPV1. TRPV1 expression is undetectable in wild type HEK293 cells. B. Representative western blot (n = 3 independent experiments) for TRPV1 in wild type and TRPV1−/− diaphragms. GAPDH was used as loading control.
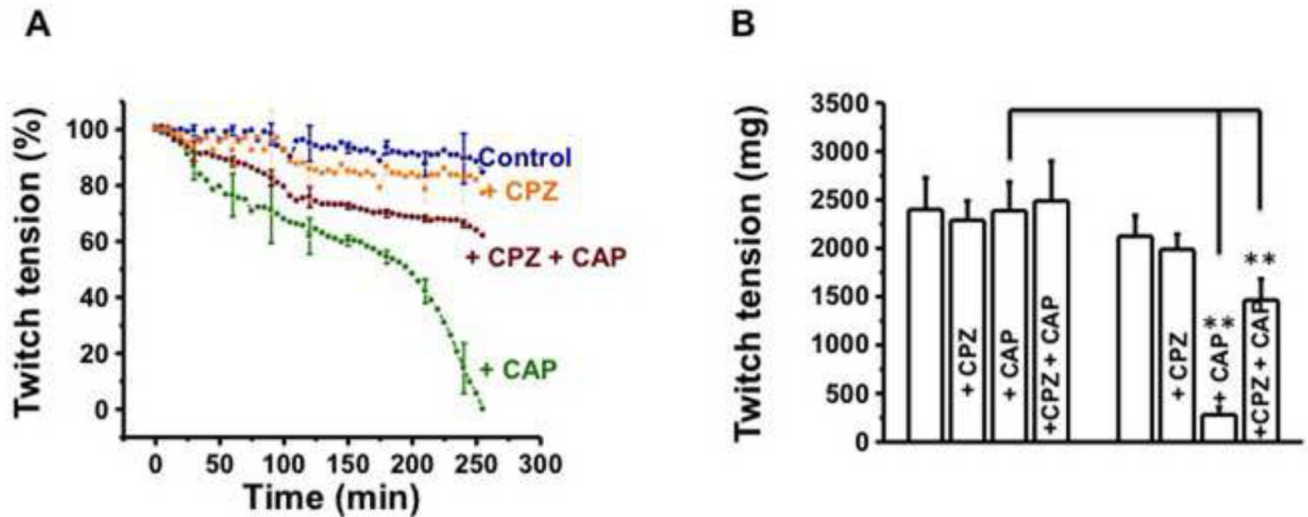
Figure 2. CAP (CAP) inhibits stimulus evoked twitch tension of hemidiaphragm nerve muscle preparations and capsazepine (CPZ) antagonizes the effects of CAP

A. Time course of stimulus evoked twitch tension (%) of control, + CAP (1 μM) and + CPZ (10 μM) + CAP treated hemidiaphragm nerve muscle preparations. B. Representative twitch tensions of control, + CAP and + CPZ + CAP (1 μM) preparations at 0 min (before any treatment) and at 250 min. after treatment with CAP alone or + CPZ + CAP. The data are from 8–12 nerve-muscle preparations obtained from 12 mice. ** represents statistical significance for $P<0.001$.
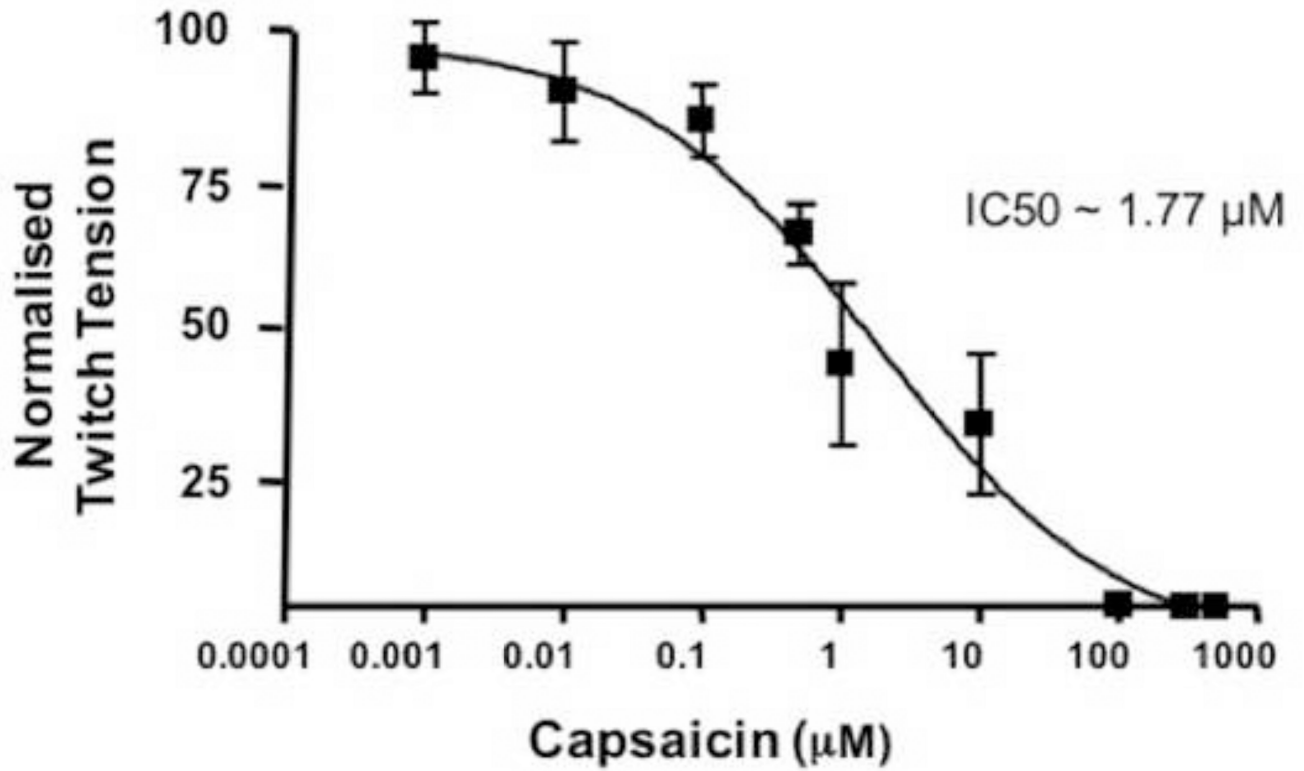
Figure 3. CAP concentration-response curve *in vitro*
A. IC50 of CAP determined by measuring the concentration-dependent inhibitory effects of CAP on nerve stimulus evoked twitch tensions of diaphragm NMP *in vitro*. Data collected at 90 min. period following the addition of different concentrations CAP were shown. No more than two concentrations of CAP were studied in one experiment.
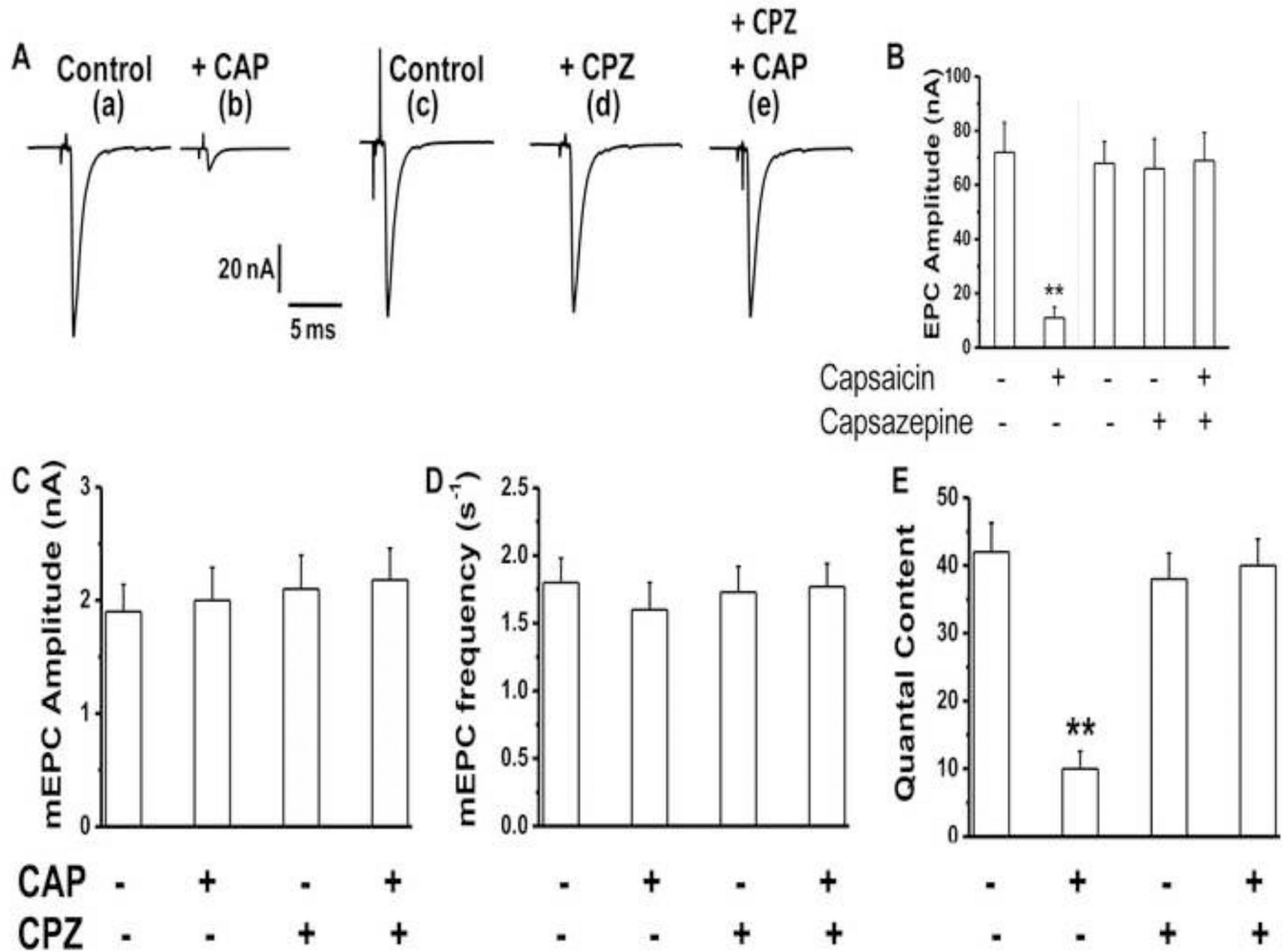
Figure 4. CAP inhibits stimulus evoked neurotransmitter release and capsazepine (CPZ; 10 μM) antagonizes the effects of CAP

A. Representative traces of 1 Hz stimulus evoked end plate currents (EPC) of control (a and c), + CAP (b), + CPZ (d), and + CPZ + CAP (e) treated nerve muscle preparations (n = 4–6, 16–22) at the end of 120 min. after the application of either vehicle alone or the drug. CPZ was pretreated and was present throughout the experiments (d and e). B. EPC amplitudes ± S.E.M of control (a and c), + CAP (b), + CPZ (d), and + CPZ + CAP (e) treated nerve muscle preparations (n = 4–6, 16–22). C, D and E. Represent the spontaneous miniature end plate current (mEPC) amplitude, frequency and quantal content of control, + CAP, + CPZ + CAP, and + CPZ treated preparations. Numbers in parentheses represent the numbers of nerve muscle preparations and fibers respectively obtained from six mice for each condition. (Quantal content = ratio of EPC amplitude to mEPC amplitude). ** represents statistical significance for P<0.001.
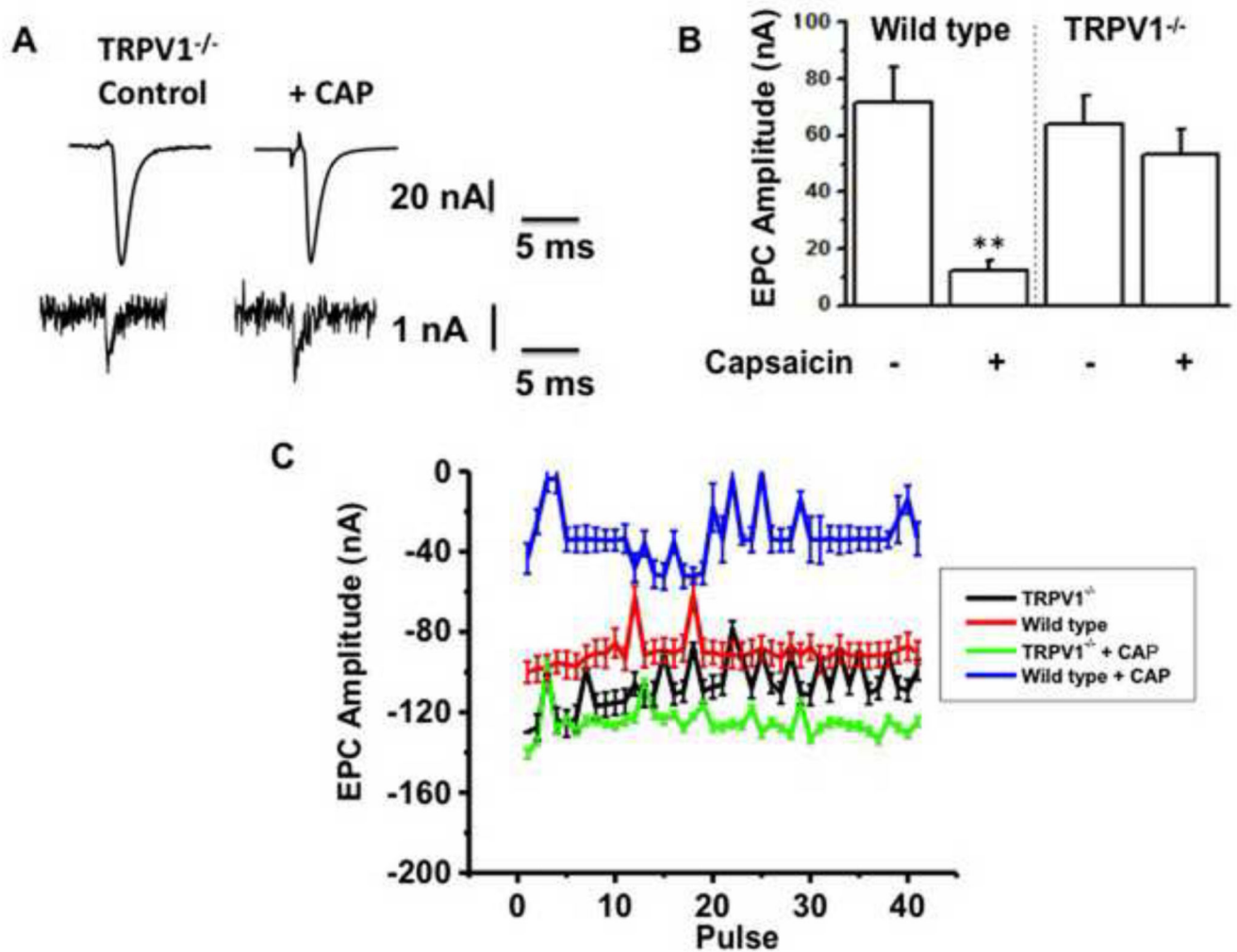
Figure 5. CAP inhibits EPC of wild type but not TRPV1$^{-/-}$

A. Representative EPC (top) and mEPC (bottom) traces for control and CAP treated diaphragm from TRPV1$^{-/-}$ mice. B. Bar graph represent the mean EPC ± S.E.M. for the diaphragm obtained from wild type and TRPV1$^{-/-}$ animals (n = 6–8 animals, 18–24 endplates). C. EPC amplitudes for control and CAP-treated wild type and TRPV1$^{-/-}$ diaphragm at 120 min. vehicle or CAP application. ** represents statistical significance for P<0.001.
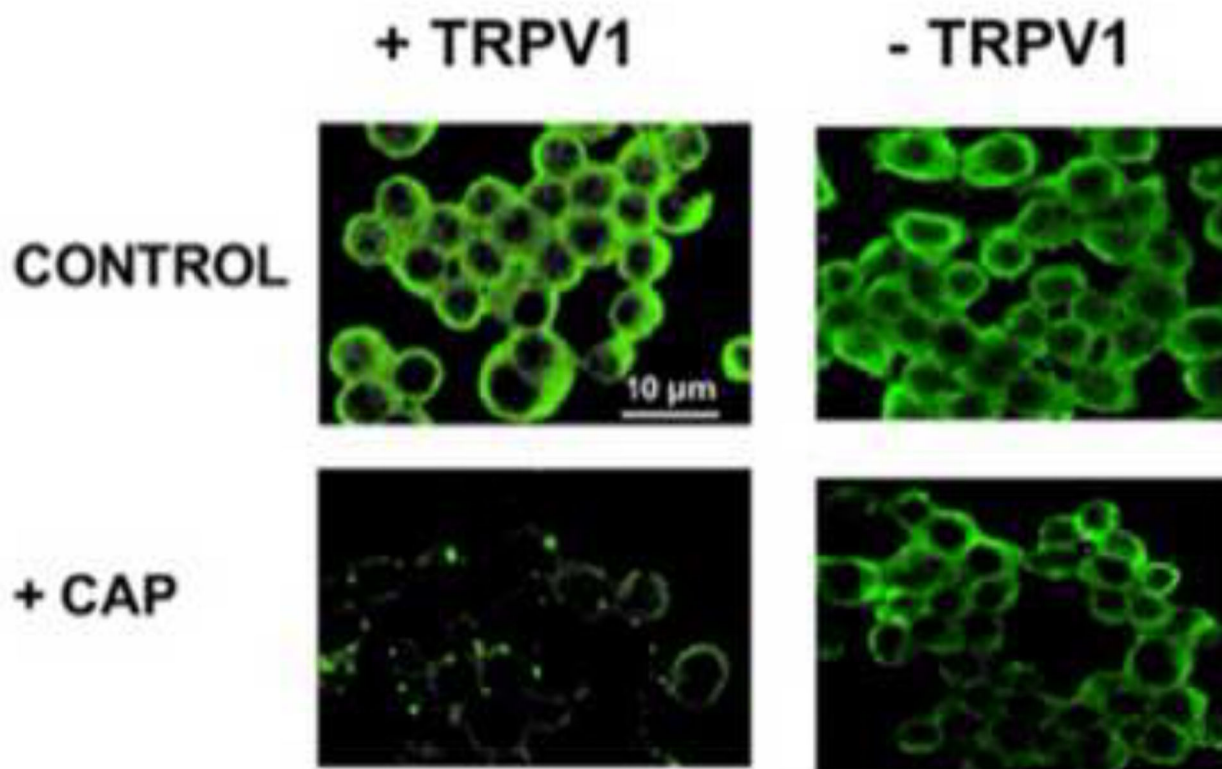
Figure 6. CAP pretreatment alters the subcellular disposition of AP-2 in Neuro 2a cells
Confocal micrographs represent the immunostaining for AP-2 µ1 in control and CAP treated wild type Neuro 2a cells and TRPV1 expressing Neuro 2a cells.
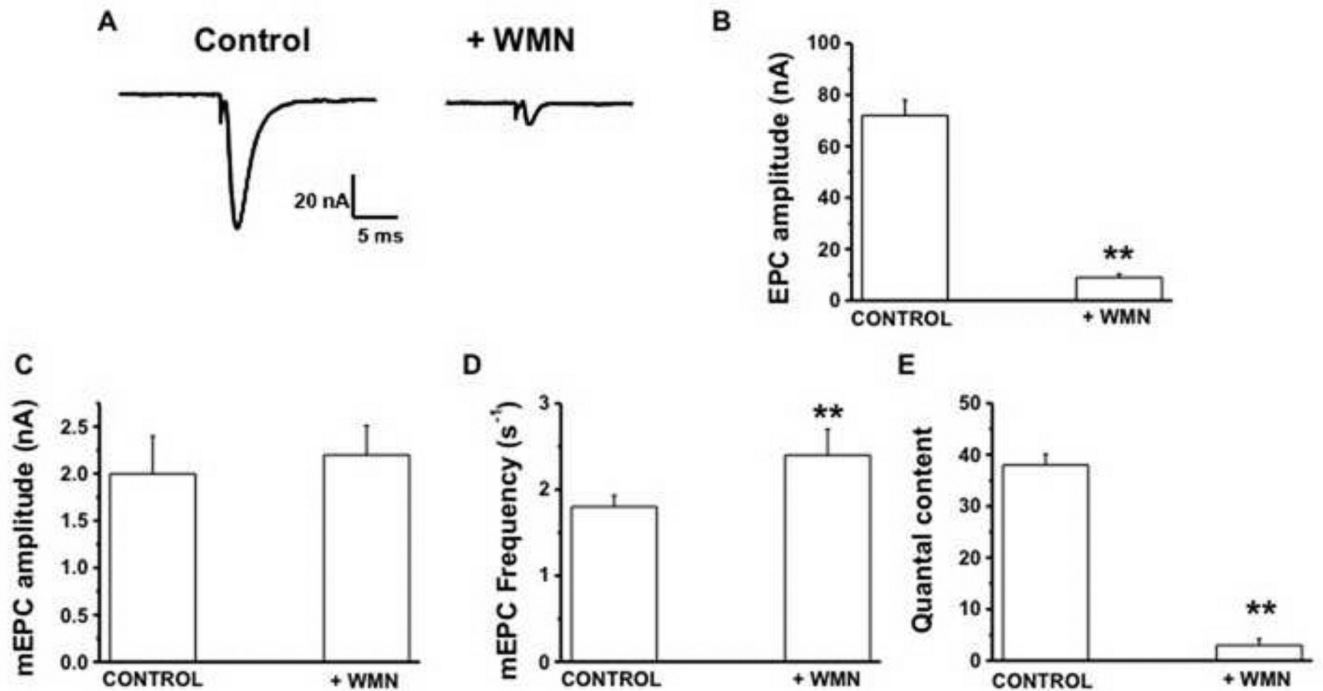
Figure 7. Wortmannin (WMN; 20 μM) inhibits stimulus evoked neurotransmitter release
A. Representative traces of 1 Hz stimulus evoked end plate currents (EPC) of control and WMN treated nerve muscle preparations (n = 4–6 muscles and 16–22 neuromuscular junctions) at the end of 120 min. after the application of either vehicle alone or WMN (120 min. treatment). B. EPC amplitudes ± S.E.M of control and WMN treated NMP (n = 3–5, 12–26). C, D and E. Represent the spontaneous miniature end plate current (mEPC) amplitude, frequency, and quantal content of control and WMN treated preparations. Numbers in parentheses represent the numbers of nerve muscle preparations and fibers respectively. ** represents statistical significance for P<0.001.
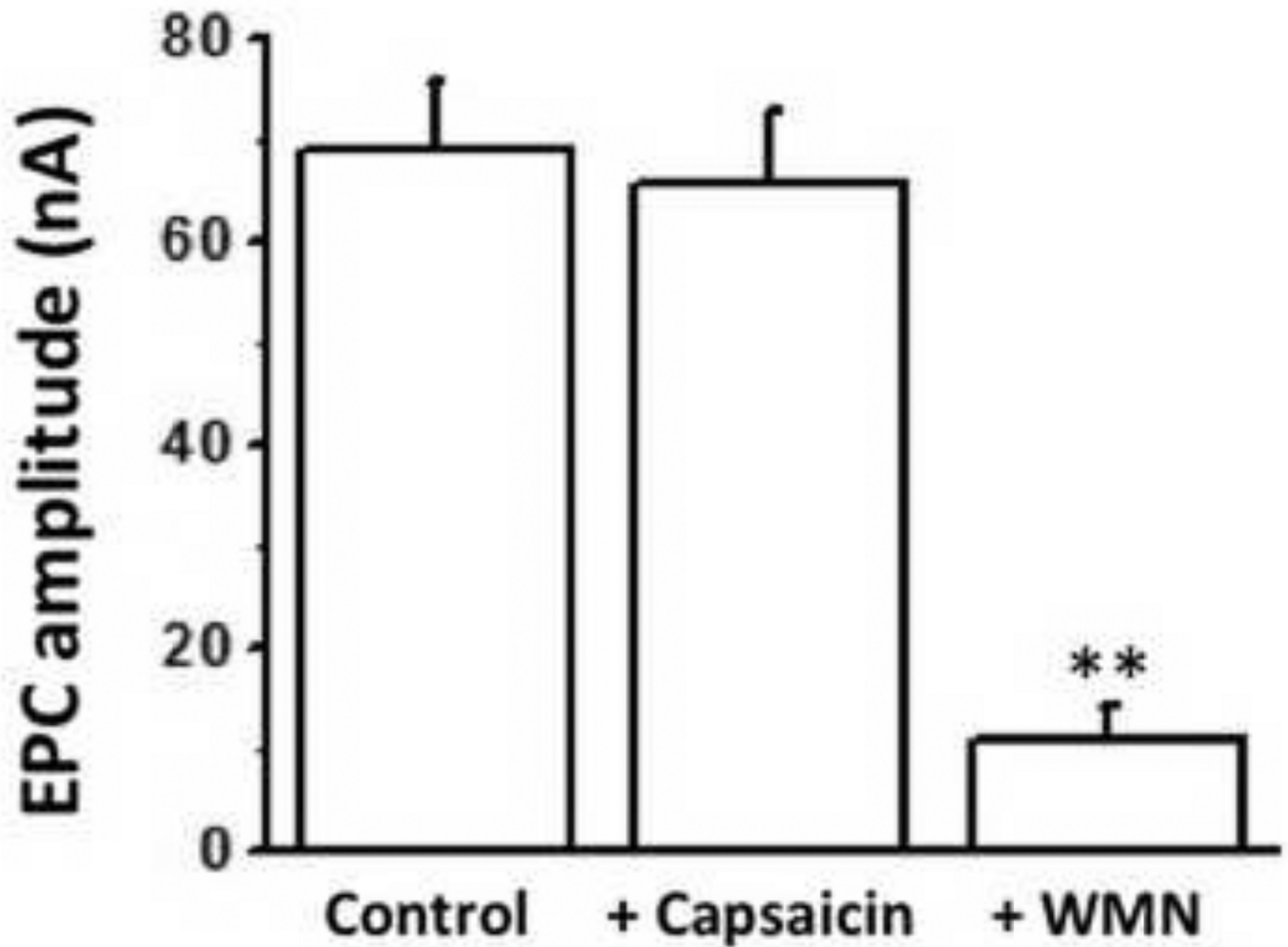
Figure 8. WMN but not CAP inhibits stimulus evoked neurotransmitter release in TRPV1$^{-/-}$ NMP

A. Mean amplitudes (nA) in response to 1 Hz stimulus evoked end plate currents (EPC) of control, CAP (1 μM) or WMN (20 μM) treated NMP (n = 4–6 muscles and 14–20 neuromuscular junction, from 6 mice) at the end of 120 min. after the application of either vehicle alone (first bar) or CAP or WMN (second and third bars respectively). ** represents statistical significance for P<0.001.
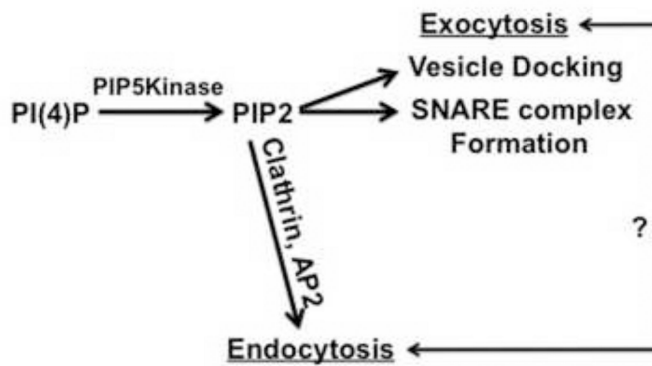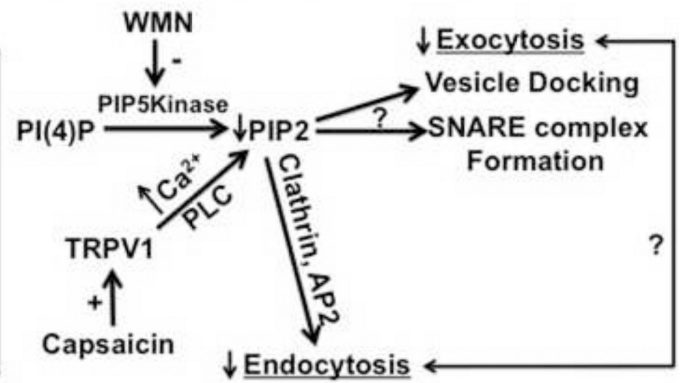
Figure 9. Model describing the effect of CAP or WMN on PIP2 dependent exo-endocytic mechanism

PIP2 regulates exocytosis by facilitating SNARE complex formation and synaptic vesicle docking, fusion and release. PIP2 also participates in the formation and nucleation of endocytic machinery. The mechanism of how PIP2 regulation of endocytosis couples to exocytosis still remains unclear. CAP activates TRPV1 and causes decrease in PIP2 via Ca^{2+}-PLC dependent mechanisms. WMN inhibits PI Kinase and decreases PIP2 levels. The reduction in PIP2 level down-regulates endocytosis as well as exocytosis. + sign denotes activation. ? Denotes unknown mechanism by which endocytosis and exocytosis couple with each other. Upward and downward arrows represent increase and decrease respectively.