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The excitation and modulation of TRPV1-, TRPA1- and TRPM8-expressing sensory neurons by the pruritogen chloroquine

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*Running title: *Multiple actions of chloroquine on sensory neurons*

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Background: Chloroquine evokes the sensation of itch by exciting peripheral sensory neurons.
Results: Chloroquine not only directly excites a diverse population of sensory neurons, but also strongly modulates ion channels involved in pain and itch transduction.
Conclusion: Chloroquine exerts widespread actions on peripheral sensory neurons.
Significance: Our results increase our understanding of the action of chloroquine on sensory neurons.

SUMMARY

The sensations of pain, itch and cold often interact with each other. Pain inhibits itch, whereas cold inhibits both pain and itch. TRPV1 and TRPA1 channels transduce pain and itch, whereas TRPM8 transduces cold. The pruritogen chloroquine (CQ) was reported to excite TRPA1, leading to the sensation of itch. It is unclear how CQ excites and modulates TRPA1$^+$, TRPV1$^+$ and TRPM8$^+$ neurons and thus affects the sensations of pain, itch and cold. Here, we show that only 43% of CQ-excited dorsal root ganglion (DRG) neurons express TRPA1; as expected, the responses of these neurons were completely prevented by the TRPA1 antagonist HC-030031. The remaining 57% of CQ-excited neurons did not express TRPA1, and excitation was not prevented by either a TRPA1 or a TRPV1 antagonist, but was prevented by the general TRPC channel blocker BTP2 and the selective TRPC3 inhibitor Pyr3. Furthermore, CQ caused potent sensitization of TRPV1 in 51.9% of TRPV1$^+$ neurons, and concomitant inhibition of TRPM8 in 48.8% of TRPM8$^+$ DRG neurons. Sensitization of TRPV1 is mainly caused by activation of the PLC-PKC pathway following activation of the CQ receptor MrgprA3. By contrast, inhibition of TRPM8 is caused by a direct action of activated Gα_q independently of the PLC pathway. Our data suggest the involvement of TRPC3 channel acting together with TRPA1 to mediate CQ-induced itch. CQ not only elicits itch by directly exciting itch-encoding neurons, but also exerts previously unappreciated widespread actions on pain-, itch- and cold-sensing neurons, leading to enhanced pain and itch.

Pain is a distressing somatic sensation closely related to itch. Temperature-activated transient receptor potential (TRP) ion channels have important roles in the sensation of pain. For example, TRPV1 and TRPA1 channels mediate heat hyperalgesia and cold pain, respectively (1-5). By contrast, activation of the cold-activated TRPM8 channel by innocuous cooling inhibits pain (6,7). Under pathological conditions such as inflammation the activities of these ion channels are either sensitized (e.g. TRPV1 and TRPA1) or inhibited (e.g.TRPM8) through different signaling mechanisms, leading to a more painful outcome. Sensitization of TRPV1 and TRPA1 by inflammatory mediators that activate Gα_q-coupled G protein-coupled receptors (GPCRs) is mainly caused by activation of the PLC signaling pathway (8,9). However, inhibition of TRPM8 by inflammatory mediators is largely independent of the PLC pathway; activated Gα_q protein instead directly inhibits TRPM8(10).

In contrast to pain, the transduction of itch (pruritus) is less well understood. There are two different types of itch: histamine-dependent (histaminergic) and histamine-independent itch (non-histaminergic), though the majority of pruritus is non-histaminergic and cannot be treated with anti-histamines (11). Non-histaminergic itch is normally caused by exogenous pruritogens such as cowhage and chloroquine (CQ) (12-14).

Pruritogens cause itching by exciting sensory neurons largely through activation of $G\alpha_q$-coupled GPCRs, which then couple to downstream excitable ion channels, predominantly TRP ion channels. For example, histamine excites sensory neurons by activating TRPV1 through a signaling mechanism involving both PLCβ3 and PLA2-lipoxygenase pathway (15-17). However, the non-histaminergic pruritogens serotonin and endothelin-1 induce itching without the involvement of TRPV1 although TRPV1-expressing neurons are required (16). CQ, on the other hand, excites DRG neurons and elicits itching by activating a Mas-related GPCR, MrgprA3, which then causes the opening of TRPA1 through a signaling mechanism involving Gβγ but not PLCβ (18,14,16). Therefore, different pruritogens seem to activate differential signaling mechanisms to excite DRG neurons.

Collectively, TRPV1 and TRPA1 are involved in the transduction of both pain and itch. Interestingly, activation of TRPM8 by cooling or by the cooling compound menthol inhibits pain and itch through both peripheral and central mechanisms (6,19-21). It is thus not a surprise that there is a complex and antagonistic relationship among pain, itch and cold pathways (20,22-24). However, it is unclear how itching stimuli such as CQ affect pain and cold pathways. In this study, we investigated how the non-histaminergic pruritogen CQ could excite and modulate the activity of $TRPV1^+$, $TRPA1^+$ and $TRPM8^+$ DRG neurons, which are involved in transducing pain, itch and cold.

EXPERIMENTAL PROCEDURES

Culture of DRG neurons. DRG neurons were isolated from neonatal C57BL/6 mice as described previously (25). Briefly, mice of either sex were sacrificed by cervical dislocation followed by decapitation. DRGs were collected from all cervical, thoracic and lumbar segments, and were then incubated with calcium- and magnesium- free Hank's balanced salt solution (Invitrogen) containing 2.5mg/ml type IV collgenase (Worthington Biochemicals) at 37°C for 1 hour. After incubation, DRGs were washed in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) followed by trituration with 25G needles to dissociate neurons. The dissociated neurons were plated onto coverslips pre-coated with poly-l-lysine (Sigma) and laminin (BD Biosciences) and cultured in DMEM medium containing 10% fetal bovine serum (Invitrogen), 2mM L-glutamine, 100 IU/ml penicillin and 100μg/ml streptomycin supplemented with 5μM cytosine β-D-arabinofuranoside (Sigma) and 1×N2 supplement (Life Technologies). Cultured neurons were used for experiments within 24 hours after plating. DRG neurons were also isolated from adult mice (22 days after birth) and cultured in a similar manner as neonatal DRG neurons. NGF and GDNF were not used for DRG cultures to avoid inducing the expression of TRP channels.

Culture and transfection of cell lines. The HEK293 cell line and mice embryonic fibroblast (MEF) cells lacking endogenous $G\alpha_{q/11}$(26) were maintained as described previously (10). Briefly, cells were cultured in DMEM medium containing 10% fetal bovine serum, 2mM L-Glutamine, 100 IU/ml penicillin and 100μg/ml streptomycin. Cells were transfected with cDNAs encoding rTRPM8 and MrpgrA3 by using polyfect transfection reagent (Qiagen). Briefly, 4.0μg cDNAs were incubated with 20μl polyfect reagent in 150μl serum-free DMEM medium for 10 minutes. After incubation, the solution was added to the cells and incubated for 24 hours. Transfected cells were then replated onto small dishes for electrophysiological recording.

MEF cells were transfected by using cell line nucleofection kits (Lonza). Briefly, cell pellets were resuspended in 100μl of transfection solution containing 4.0 μg cDNAs and were electroporated by using an Amaxa device. After transfection, cells were plated onto small dishes and used for electrophysiology recordings in 24 hours.

Fluorescence imaging. Calcium imaging was performed at room temperature as described previously (10,25). Briefly, DRG neurons plated onto a coverslip were incubated with 7μM Fluo-4-AM (Invitrogen) for 40 minutes at 37°C. After loading, cells-containing coverslips were

transferred to a custom-made chamber and were continuously perfused with normal Hanks solution containing (in mM) 140 NaCl, 4 KCl, 10 HEPES, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 glucose, pH 7.4 with NaOH. Cell images were collected every 3s using a Bio-Rad confocal microscope. Bradykinin (BK, 1μM), histamine (100μM) and chloroquine (CQ, 1mM) were pulsed onto cells for 2 minutes, whereas pulses of menthol (100μM), AITC (100μM) and capsaicin (500nM) were applied for 15 seconds. All chemicals were applied every 4 minutes sequentially as appropriate to the individual experiment. To study the effect of PKC, cells were pre-treated with the PKC inhibitor Bisindolylmaleimide (BIM) I (Calbiochem) as described previously(10). The TRPC inhibitor YM58483 (BTP2) (Tocris), the TRPC3 inhibitor Pyr3 (Tocris), the TRPA1 inhibitor HC-030031(Tocris) and ruthenium red (Sigma) were applied as indicated in individual experiments. Ionomycin was applied at the end of experiment to allow maximal calcium influx. Neurons unresponsive to ionomycin were excluded for further analysis. We considered that a chemical induced a calcium response in DRG neurons if the difference between the elicited peak calcium response and the baseline fluorescence (ΔF/Fmax) was larger than 0.06. We also performed similar calcium imaging experiments in calcium-free Hanks solution. Under these conditions no significant calcium responses (0 out of 694 neurons) were observed for all chemicals excluding the possibility of the contribution of intracellular calcium stores to excited calcium responses.

To study the modulation of TRPV1 and TRPM8 by CQ, the effect of CQ was quantified as a response ratio by dividing the 5^{th} by the 4^{th} peak response amplitude. In control experiments on cells not exposed to CQ, the distribution of response ratios was found to be well fitted by a normal distribution (Fig.3E), from which a threshold ratio was derived at a 95% confidence level and used to determine cells significantly sensitized by CQ.

Tubby-R332H-cYFP (kindly provided by Dr. Gerald Hammond) translocation was determined by live-scanning using a Leica confocal microscopy. Images of MEF cells transfected with the fluorescence probe and MrgprA3 were collected every 0.75s. The translocation of Tubby-R332H-cYFP was quantified by calculating the ratio of membrane fluorescence to that of cytosol using Image J software.

Single-cell reverse transcription PCR(RT-PCR). Neurons were first examined by calcium imaging. CQ-sensitive neurons under each category were then aspirated into a glass electrode and transferred into a PCR tube. The total RNA was isolated from collected neurons by using RNeasy micro kit (Qiagen) followed by reverse transcription with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The products of reverse transcription were then amplified by PCR with primers listed in Table 1. Cycling conditions: 95°C 2min and 50 cycles of 50 sec at 95°C, 45 sec at 60°C and 46 sec at 72°C, followed by 5 min at 72°C. The PCR products were then analysed with 1% agarose gel.

Electrophysiology. Whole-cell patch recordings were performed largely as described previously (9,10). Briefly, patch electrodes were filled with internal solution of the following composition (in mM): 140 KCl, 2.0 $MgCl_2$, 5.0 EGTA, 10 HEPES, PH 7.4 with KOH. Standard Hanks solution was used as the external solution. TRPV1 inward currents were recorded at a holding potential of -60mV. For recordings of the TRPM8 current, we used calcium-free solution in which 1.8mM $CaCl_2$ was replaced with 5mM EGTA to prevent the interference of calcium-dependent desensitization. TRPM8 inward and outward currents were measured at holding potentials of -60mV and +60mV, respectively. Cells were pre-treated with 1mM CQ for 1minute before break-in to the whole cell to measure TRPM8 currents activated by menthol. Series resistance was routinely ≤10.0MΩ and was compensated by>70%. All recordings were made at room temperature (24°C) with an Axopatch 200B patch clamp amplifier (Axon) in conjunction with pClampex 10.2 version software (Molecular Devices). Signals were analog filtered using a 1 kHz low-pass Bessel filter.

Co-immunoprecipitation. It was performed as described previously with some modifications (10). Briefly, HEK293 cells expressing TRPM8-V5 and MrgprA3 were solubilised, and TRPM8 was precipitated by using anti-V5 antibody (Invitrogen) and protein-A agarose (Santa Cruz). The co-precipitates were then analysed in 10% SDS-PAGE gel and transferred to a blot. $G\alpha_q$ protein was then detected with polyconal $G\alpha_q$ antibody (Santa Cruz). The V5 tag does not

affect the binding of $G\alpha_q$ to TRPM8, because $G\alpha_q$ can also be similarly precipitated by using a TRPM8 polyclonal antibody (10).

Statistics. All data are means ± s.e.m. Differences between groups were assessed by one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test. Results were considered significant at $P<0.05$.

RESULTS

CQ excites a subpopulation of sensory neurons expressing TRPV1, TRPA1 and TRPM8- CQ is traditionally used to treat malaria. One of the common side effects associated with CQ therapy is the production of serious itching. CQ-induced itching has recently been found to be caused by the excitation of the TRPA1 ion channel in DRG neurons after activation of the CQ receptor MrgprA3(18,14). However, it is unclear which populations of DRG neurons are excited by CQ, and how they relate to $TRPV1^+$, $TRPA1^+$ and $TRPM8^+$ neurons, which mediate the sensations of pain, itch and cold.

To investigate this question, we assessed the responses of neonatal DRG neurons after exposure sequentially to CQ, the TRPM8 agonist menthol, the TRPA1 agonist AITC and the TRPV1 agonist capsaicin by monitoring intracellular calcium rises. Two minutes of CQ treatment rapidly induced calcium increases in a subpopulation of neurons (Fig. 1A-1D). About 12.8% (344 out of 2693 total neurons) of DRG neurons were found to be excited by CQ (Fig. 1F and 1H). Strikingly, only 43.3% (149 out of 344) of these neurons also responded to AITC and thus co-express TRPA1 (Fig. 1B, 1F and 1H), some of these neurons (99 out of 149) responded to both AITC and capsaicin and thus co-express both TRPA1 and TRPV1 (Fig. 1D and 1F). Notably, 49.7% (171 out of 344) of CQ-responsive neurons did not co-express TRPA1 but solely expressed TRPV1, and the remaining 7% (24 out of 344) of CQ-sensitive neurons expressed neither TRPV1 nor TRPA1(Fig. 1C, 1F and 1H). Consistent with the pharmacological data, the single-cell RT-PCR revealed that MrgprA3 mRNA was expressed in all CQ-responsive neurons (Fig. 1E). Some of them co-expressed TRPA1 and/or TRPV1 mRNA, whereas others co-expressed neither TRPV1 nor TRPA1 mRNA (Fig. 1E). Fig. 1F shows a summary of percentages of the individual population of CQ-excited neurons described above related to a total of 2693 neurons isolated from neonatal mice. Similar percentages of CQ-excited DRG neurons for these individual populations were also obtained from a total of 1450 neurons isolated from adult mice (Fig. 1G). Hence, the properties of CQ-excited neurons do not change significantly after the postnatal development.

We also found that a small proportion (14.2%, 49 out of 344) of CQ-excited neurons was also excited by menthol, and thus co-express TRPM8 (Fig. 1H). Most of these neurons (76%) also co-express TRPV1 and/or TRPA1(Fig. 1H), and are thus contained within $TRPV1^+$ and $TRPA1^+$ populations. Taken together, CQ excited three main populations of neurons co-expressing either TRPA1 or TRPV1 or neither channel.

CQ excites sensory neurons through both TRPA1-dependent and TRPC3-dependent mechanisms- To examine whether co-expressed TRPA1 or TRPV1 in DRG neurons are *bone fide* ion channels responsible for mediating responses caused by CQ, we treated CQ-responsive neurons with the specific TRPA1 blocker HC-030031, the TRPV1 blocker capsazepine or the general TRP channel blocker ruthenium red. We found that CQ-excited responses were completely blocked by the specific TRPA1 antagonist HC-030031 in all neurons that were also responsive to AITC (Fig. 2A and 2F). This experiment shows that TRPA1 is the sole molecule mediating the excitation of this population of DRG neurons evoked by CQ, consistent with the proposed role of TRPA1 in CQ-induced itch (18). However, HC-030031 had no effect on the responses excited by CQ in neurons that were unresponsive to AITC (Fig. 2B and 2F). Since most of this population of neurons co-express TRPV1, we then investigated whether TRPV1 might mediate the excitation of this population of neurons by treating neurons with the specific TRPV1 antagonist capsazepine. Fig. 2F shows that capsazepine failed to prevent CQ-induced responses in neurons that were responsive to the TRPV1 agonist capsaicin, suggesting that ion channels other than TRPV1 mediate the excitation of $TRPV1^+$ neurons elicited by CQ. Calcium responses evoked by CQ are not likely to be caused by the release of intracellular calcium store, because no calcium responses were observed when similar experiments were performed in a calcium-free solution (Fig.2C). The CQ-induced calcium responses were,

however, prevented by the general TRP channel blocker ruthenium red in all neurons, whether AITC-sensitive or not (Fig. 2D and 2F), further supporting the notion that the calcium responses evoked by CQ are caused by extracellular calcium influx through Ca^{2+} permeable TRP ion channels in the plasma membrane (14). These results demonstrate that CQ-induced excitation of sensory neurons is mediated by TRPA1 only in neurons co-expressing TRPA1 or TRPA1/TRPV1, which account for 43.3% of the total CQ-excited neurons. The responses of the remaining populations of neurons (56.7%) to CQ are not mediated by either TRPA1, or TRPV1, but instead by an as-yet unidentified TRP ion channel.

Transient receptor potential canonical (TRPC) channels are Ca^{2+}-permeable non-selective cation channels. Several subtypes are abundantly expressed in DRG neurons (27). To determine the role of TRPC channels in TRPA1-independent excitation of DRG neurons caused by CQ, DRG neurons were treated with the general TRPC channel inhibitor BTP2. The CQ-induced responses in $TRPA1^-$ neurons (insensitive to AITC) were greatly reduced when neurons were pre-treated with BTP2 (Fig. 2E and 2F). TRPC3 has recently been found to be involved in the excitation of sensory neurons induced by IgG immune complex (28). We then examined the possible contribution of TRPC3 to CQ-excited responses by treating neurons with the selective TRPC3 inhibitor Pyr3. Fig.2F shows that Pyr3 prevented CQ-elicited responses in TRPA1-negative neurons to a similar extent as BTP2. The single-cell RT-PCR further revealed that this population of DRG neurons co-expressed TRPC3 mRNA (Fig. 1E). These data demonstrate that the excitation of DRG neurons in the $TRPA1^-$ DRG neurons caused by CQ is mediated by TRPC3.

CQ sensitizes TRPV1 in DRG neurons- Since most of the CQ-excited neurons (78.5%) co-express TRPV1 (Fig.1H), and yet TRPV1 is not a mediator for the direct excitation of $TRPV1^+$ neurons by CQ, we wondered whether CQ might be involved in the modulation of TRPV1 function instead, thereby affecting the sensations of pain and itch. To test this hypothesis, we monitored TRPV1-mediated calcium responses evoked by capsaicin in DRG neurons. A short treatment (2mins) with CQ caused a robust enhancement of TRPV1-mediated calcium responses (Fig. 3A). However, CQ treatment itself did not generate calcium signals. To exclude the possible effect of repeated capsaicin stimulation on the CQ-excited calcium response, neurons were also stimulated first with CQ and then with capsaicin. The second pulse of CQ similarly enhanced the TRPV1-mediated calcium increase despite the fact that CQ did not evoke a calcium response by itself (Fig. 3B). In fact, 93% of the neurons that were sensitized by CQ did not show a CQ-excited response. TRPV1 is thus unlikely to be a downstream effector ion channel underlying CQ-initiated excitation of neurons, in agreement with our findings (above) and the previous observations of others (18). The absence of CQ-excited calcium responses in these sensitized neurons may be caused by the lack of a downstream effector ion channel such as TRPA1. Indeed most of these neurons were not co-excited by AITC (Fig. 3A and 3B); however, in some neurons (7%), CQ not only sensitized TRPV1, but also excited a calcium response itself despite the lack of expression of TRPA1(Fig. 3C). Surprisingly, CQ did not cause the sensitization of TRPV1 in some neurons (25 out of 581 $TRPV1^+$ neurons) that were also excited by CQ (Fig. 3D). Presumably, CQ excites these neurons through an MrgprA3-independent mechanism.

Interestingly, although CQ excited only 24.5% (270 out of 1100) of $TRPV1^+$ neurons (Fig. 1H), CQ caused sensitization in 51.9% of $TRPV1^+$ neurons (Fig. 3E and 3F), comparable to the sensitization induced by bradykinin (BK) (9). These data suggest that the CQ receptor is expressed in at least twice as many sensory neurons than what we have estimated above by monitoring CQ-excited calcium responses, and that the CQ receptor is widely distributed across diverse populations of DRG neurons and thus may have much more widespread actions on DRG neurons than previously appreciated.

CQ-induced sensitization of TRPV1 involves the PLC-PKC pathway- CQ acts on the CQ receptor MrgprA3, which belongs to a family of orphan GPCRs, known as mrgs. Two other members, MrgA1 and MrgC11, are coupled to the $G\alpha_{q/11}$ pathway (29). To test whether MrgprA3 is also coupled to $G\alpha_{q/11}$, we used Tubby-R332H-cYFP, a sensitive reporter for the membrane lipid PIP_2, to monitor whether activation of MrgprA3 causes PIP_2 depletion. Fig. 4A shows that CQ stimulated a rapid translocation of tubby fluorescence away from the plasma membrane, indicating depletion of PIP_2. Moreover, CQ caused tubby translocation

with similar kinetics and to a similar extent as BK (10). These experiments demonstrate that MrgprA3 is a $G\alpha_{q/11}$-coupled receptor, and that activation of MrgprA3 has a similar ability to activate PLCβ and thereby hydrolyze PIP_2 as activation of the BK receptor B2R.

CQ excites the TRPA1 channel through Gβγ, which is released after activation of the CQ receptor MrgprA3(18). We were therefore interested in investigating the underlying signaling mechanisms for CQ-induced sensitization of TRPV1. We recorded TRPV1 currents in HEK293 cells expressing both TRPV1 and MrgprA3. Consistent with CQ-induced sensitization of TRPV1 in DRG neurons, CQ potently sensitized the TRPV1 inward current to a similar extent as BK (Fig. 4C) (9). The sensitization of TRPV1 by CQ was completely inhibited by the PLC inhibitor U73122, and also completely abolished by co-expression of a C-terminal domain of PLCβ (PLCβ-ct) (Fig. 4D), which is used to sequester endogenous $G\alpha_q$ and thus block the PLC pathway (30). CQ-induced sensitization of TRPV1 was also prevented by the specific PKC inhibitor BIM in both DRG neurons and the exogenous expression system (Fig. 3F and 4E). Mutation of two PKC phosphorylation sites on TRPV1 similarly prevented the sensitization of TRPV1 by CQ (Fig. 4E). These experiments suggest that the PLC-PKC pathway plays a key role in the sensitization of TRPV1 caused by CQ.

CQ inhibits TRPM8 in a subpopulation of DRG neurons- In contrast to TRPV1 and TRPA1 which mediate the sensations of pain and itch, activation of TRPM8 by moderate cooling or menthol inhibits pain and itch (20). Since CQ-excited neurons were also found to overlap partly with $TRPM8^+$ neurons (Fig.1H), we were interested in determining whether CQ could also modulate TRPM8 function in DRG neurons. To this end, we monitored TRPM8-mediated calcium responses elicited by menthol. Importantly, menthol was also reported to activate TRPA1 channel(31). To identify specifically $TRPM8^+$ neurons, therefore, we also applied AITC and selected neurons that were both menthol-sensitive and AITC-insensitive. In fact, only 3.2% of AITC-sensitive neurons were also menthol-sensitive (Fig.1H), suggesting that menthol largely activates $TRPM8^+$ neurons. Fig. 5A shows that 2min of exposure to CQ significantly reduced the TRPM8-mediated calcium increase, and that CQ itself also induced a transient calcium response. In some neurons CQ still inhibited TRPM8 even if CQ did not elicit a calcium response (Fig. 5B), these neurons probably expressed the CQ receptor MrgprA3 but lacked downstream effector ion channels for excitation. Overall, CQ inhibited 48.8% (21 out of 43) of $TRPM8^+$ neurons. The mean ratio of calcium responses after and before CQ treatment was significantly reduced (Fig. 5C and 5D).

We have previously found that TRPM8 can also be inhibited by BK in DRG neurons (10), Compared to BK-elicited inhibition of TRPM8, the TRPM8-mediated calcium response ratio after and before CQ treatment was significantly larger than that caused by BK (Fig. 5E), showing that CQ inhibits TRPM8 to a lesser extent than BK.

CQ-mediated inhibition of TRPM8 involves direct actions of activated $G\alpha_{q/11}$ on TRPM8- We then investigated the mechanisms underlying CQ-elicited inhibition of TRPM8. In contrast to PKC-mediated sensitization of TRPV1 caused by CQ, CQ-induced inhibition of TRPM8 was not prevented by treatment with the specific PKC inhibitor BIM (Fig. 5D), suggesting that inhibition of TRPM8 by CQ is not caused by PKC.

To further investigate possible signaling mechanisms, TRPM8 inward and outward currents were recorded from HEK293 cells expressing both TRPM8 and MrgprA3. Fig. 6A shows that CQ stimulation inhibited TRPM8 inward currents but not outward currents. It is noteworthy that although MrgprA3 and the BK receptor B2R are both $G\alpha_q$-coupled receptors and can initiate PIP_2 depletion to a similar extent (Fig. 4A and 4B) (10), CQ caused TRPM8 inhibition to a much lesser extent than BK (Fig. 6B)(10), consistent with the above findings in DRG neurons. CQ-induced inhibition of TRPM8 was not reversed by either the PLC inhibitor U73122 or co-expression of PLC-ct (Fig. 6A and 6B), both of which completely inhibited CQ-induced sensitization of TRPV1 (Fig. 4E). These data suggest that inhibition of TRPM8 by CQ takes place through a mechanism independent of the PLC-PKC pathway.

We have previously found that BK and histamine inhibit TRPM8 via a direct action of activated $G\alpha_q$ on TRPM8 (10). To test whether this mechanism also applies to CQ-induced inhibition of TRPM8, we performed similar

experiments in MEF cells lacking endogenous $G\alpha_{q/11}$. As expected, inhibition of TRPM8 was absent after CQ treatment in MEF cells (Fig. 6C). However, co-expression of the $3G\alpha_{qiq}$ chimera, which is unable to couple to PLCβ (10), rescued inhibition of TRPM8 caused by CQ (Fig.6C and 6D). Furthermore, TRPM8 interacted with $G\alpha_q$ as indicated by the co-precipitation of $G\alpha_q$ with TRPM8 (Fig. 6E). However, CQ did not induce significant additional binding of $G\alpha_q$ to TRPM8 (Fig. 6E), suggesting that a conformation change in activated $G\alpha_q$ is sufficient to inhibit TRPM8. These experiments indicate that inhibition of TRPM8 evoked by CQ is caused by a direct action of activated $G\alpha_q$ on TRPM8 independent of downstream signaling pathways.

DISCUSSION

CQ causes itching by activating the CQ receptor MrgprA3, which then couples to the excitation of TRPA1 channel (14,18). Hence, both MrgprA3 and TRPA1 are essential for mediating CQ-induced itch. In this study we found that CQ excited a diverse population of DRG neurons, and that TRPA1 mediated responses in only 43.3% of CQ-excited DRG neurons. The responses in the remaining 56.7% of CQ-excited neurons are not mediated either by TRPA1 or by TRPV1, despite the fact that CQ-excited neurons (78.5%) co-express TRPV1. All CQ-excited responses were, however, abolished by the general TRP channel blocker ruthenium red. The CQ-induced responses in a subpopulation of $TRPA1^-$ DRG neurons were also inhibited by both a general TRPC channel blocker and a selective TRPC3 inhibitor. These results suggest that TRPC3 channel may also be involved in CQ-induced itch.

Very recently, $MrgprA3^+$ neurons were reported as itch-specific DRG neurons dedicated to the transduction of itch, and it was shown that activation of TRPV1 in $MrgprA3^+$ neurons caused itch, but not pain behaviour (32). TRPV1 was also reported to mediate histamine-induced itch (15). Interestingly, we found that TRPV1 was robustly sensitized by CQ, though TRPV1 did not mediate CQ-induced excitation of DRG neurons directly. This finding suggests that indirect sensitization of TRPV1 within $MrgprA3^+$ itch-specific neurons could lead to the sensitization of both histaminergic and non-histaminergic itching responses. Hence, CQ not only causes itching through direct excitation of itch-transducing neurons via TRPA1, but also enhances itching through facilitation of activation of TRPV1.

Additionally, we found that activation of MrgprA3 by CQ inhibited the cold-sensitive ion channel TRPM8. Activation of TRPM8 by cooling was reported to inhibit pain and itch (20); thus concomitant inhibition of TRPM8 could also lead indirectly to enhanced sensations of pain and itch.

CQ acts on multiple TRP ion channels; it excites TRPA1, sensitizes TRPV1 and inhibits TRPM8. Strikingly, CQ produces these actions through completely different signaling mechanisms. The CQ receptor MrgprA3 is a $G\alpha_q$-coupled GPCR. Activation of TRPA1 by CQ is believed to be mediated by released $G\beta\gamma$ after activation of MrgprA3. On the other hand, sensitization of TRPV1 elicited by CQ is mainly mediated by activation of the PLC-PKC pathway. However, CQ-evoked inhibition of TRPM8 is independent of either of these two pathways; instead a direct action of activated $G\alpha_q$ on the TRPM8 channel seems to be responsible (Fig.7).

Both the CQ receptor MrgprA3 and the BK receptor B2R are $G\alpha_{q/11}$-coupled receptors. Activation of these two receptors depletes PIP_2 to a similar extent as shown in Fig. 4A and 4B and in a similar experiment with BK reported previously(10). Furthermore, CQ caused sensitization of TRPV1 to a similar extent as BK in both DRG neurons and transfected cells (Fig. 4E) (9), suggesting that a similar degree of activation of downstream PKC has been triggered by CQ and BK. Despite these similarities between CQ- and BK-initiated signaling, CQ inhibits TRPM8 to a much lesser extent than BK, suggesting that PIP_2 cleavage caused by the activation of PLCβ may not be the major mediator of the modulation of TRPM8 by activated $G\alpha_{q/11}$. We have recently also found that activated $G\alpha_{11}$ inhibits TRPM8 to a much smaller extent than activated $G\alpha_q$ although these proteins are indistinguishable in activating PLC and hydrolyzing PIP_2 (33). The weaker inhibition of TRPM8 caused by CQ could thus be due to differential expression of $G\alpha_{11}$ in $MrgprA3^+$ neurons. It could also be caused by the differential binding of $G\alpha_{q/11}$ to TRPM8 in $MrgprA3^+$ neurons, leading to different extents of TRPM8 inhibition.

Taken together, our data suggest that CQ produces a much broader action than previously appreciated by acting on multiple TRP ion channels, including TRPV1, TRPA1, TRPC3 and TRPM8 across diverse populations of DRG neurons, leading to enhanced pain and itch. TRP ion channels thus represent attractive targets for the treatment of both pain and itch.

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FIGURE LEGENDS:

FIGURE 1. Direct excitation of DRG neurons by CQ. *A,* Fluorescence images of DRG neurons loaded with Fluo-4-AM under basal conditions, and after stimulation with CQ (1mM, 2mins) and AITC (100μM, 15s). On the right is a bright field image of the neurons. Arrows indicate neurons responding to CQ. Scale bars are 10 microns. ***B, C, D,*** Representative traces from DRG neurons as in A activated by chloroquine (CQ, 1mM), menthol (Men, 100μM), AITC (100μM) and capsaicin (Cap, 500nM) sequentially. Ionomycin (Iono, 10μM) was added at the end. CQ-responsive neurons either co-express TRPA1(B) or TRPV1 (C) or both TRPV1 and TRPA1 (D). ***E,*** The expression of TRP channels mRNA in individual DRG neurons amplified by RT-PCR from different subpopulations categorized by calcium imaging as shown in A-D. "+" indicates neurons sensitive to CQ, or menthol (Men), or AITC, or capsaicin (Cap), "-", insensitive. The last lane is the amplification from the control bath solution. ***F, G,*** Summary of percentages of CQ-responsive, capsaicin(Cap)-sensitive and AITC-responsive neurons related to total 2693 neurons isolated from neonatal mice (F) or 1450 neurons isolated from adult mice (G) in experiments similar to those in A, B and C. ***H,*** The Venn diagram illustrates the mutual relationship of $TRPV1^+$ and $TRPA1^+$, $TRPM8(M8)^+$ neurons with chloroquine-(CQ) responsive neurons. The number of neurons is shown for each population and is obtained from 2693 neonatal neurons in total.

FIGURE 2. CQ excites DRG neurons through both TRPA1- and TRPC3-dependent mechanisms. *A, B,* Representative calcium responses from three DRG neurons activated by chloroquine (CQ, 1mM) and AITC (100μM). The TRPA1 antagonist HC-030031(10μM) was added together with the 2nd CQ application, as indicated. ***C, D, E,*** Typical calcium responses from DRG neurons excited by CQ followed by exposure to AITC (100μM) and capsaicin (Cap, 500nM). The calcium free solution (C), ruthenium red (RR, 10μM) (D) and BTP2 (10μM) were perfused together with the 2nd CQ addition, as indicated. ***F,*** Summary of ratio percentages between the 2nd CQ-excited response and the 1st CQ-induced response obtained from neonatal DRG neurons in experiments similar to those in A-E. Chloroquine(CQ)-sensitive (CQ), AITC-sensitive and capsaicin(Cap)-sensitive neurons are indicated with "+", insensitive neurons indicated by "-". The TRPA1 antagonist HC-030031 (10μM), the TRPC channel blocker BTP2 (10μM), the TRPC3 channel inhibitor Pyr3 (10μM) and the TRPV1 antagonist capsazepine(CSZ, 10μM) and ruthenium red were added, as indicated. The numbers of neurons are given above each bar. Data are means±s.e.m. ***P<0.001; NS, not significant. All results are compared to the first bar.

FIGURE 3. CQ sensitizes TRPV1 in DRG neurons. *A, B,* The calcium response elicited by capsaicin (Cap, 100nM) was enhanced by CQ (1mM) in a DRG neuron; however, the neuron did not respond to 100μM AITC, and CQ did not elicit a calcium increase, irrespective of whether CQ was applied before (B) or after capsaicin (A). ***C, D,*** A representative calcium response trace from a DRG neuron in a similar experiment to that in A, but CQ induced a transient calcium increase. Note that the application sequence of AITC in D is different from that of C. AITC was added after the 2nd application of CQ in D. ***E,*** Distribution of TRPV1-dependent calcium response ratios after (5th response) and before CQ (4th response) in DRG neurons from experiments similar to those in A. n_{cell}=137 for control, n_{cell}=581 for CQ treated. ***F,*** Summary of experiments similar to those in E. Control, n_{exp}=5; 1mM CQ treated, n_{exp}=11; BIM pretreated, n_{exp}=7, n_{cell}=108. Data are mean±s.e.m. ***P<0.001 compared to control; ###P<0.001 compared to bar 2.

FIGURE 4. The PLC-PKC pathway is involved in the sensitization of TRPV1 induced by CQ. *A,* Translocation of Tubby-R332H-cYFP caused by CQ in HEK293 cells expressing MrgprA3. CQ (1mM) was added at 70s. Scale bar, 10 microns. ***B,*** Quantification of relative membrane Tubby fluorescence signal as a function of time in A. This experiment was repeated at least four times with similar results. ***C,*** CQ sensitized TRPV1 inward current activated by 50nM capsaicin (Cap, 5s) recorded from a HEK293 cell expressing MrgprA3 and TRPV1. The dotted line is zero current. ***D,*** CQ did not cause sensitization of the TRPV1 inward current recorded from a HEK293 cell expressing MrgprA3, TRPV1 and PLCβ-ct. The dotted line represents zero current. ***E***, Summary of results

similar to those in C and D. Sensitization of TRPV1 is blocked by 2.5μM U73122, or co-expression with PLCβ-ct, or 1μM BIM or by double mutant (DM) TRPV1 S502A/S801A . All error bars are means±s.e.m. The number of experiments is given above each bar. ***P<0.001 compared to control; ##P<0.01; ###P<0.001 compared to bar 2.

FIGURE 5. CQ inhibits TRPM8 in DRG neurons. ***A, B,*** CQ inhibited calcium increase elicited by the TRPM8 agonist menthol (Men, 100μM) in a DRG neuron. 100μM AITC and capsaicin (Cap, 500nM) were added as indicated. ***C,*** The histogram distribution of calcium response ratios after 5th response) and before (4th response) CQ treatment. Control, n_{cell}=30; CQ treated, n_{cell}=43. ***D,*** Summary of results similar to those in A after pre-treatment with 1μM BIM (n_{cell}=38). All data are means±s.e.m. ***P<0.001 compared to bar 1. ***E,*** Summary of TRPM8-mediated calcium response ratios between after and before bradykinin (BK) or CQ treatment in experiments similar to those in A and D and those obtained previously(10), but only inhibited neurons were included. The numbers of inhibited neurons are given above each bar. All data are means±s.e.m. ***P<0.001 compared to bar 1.

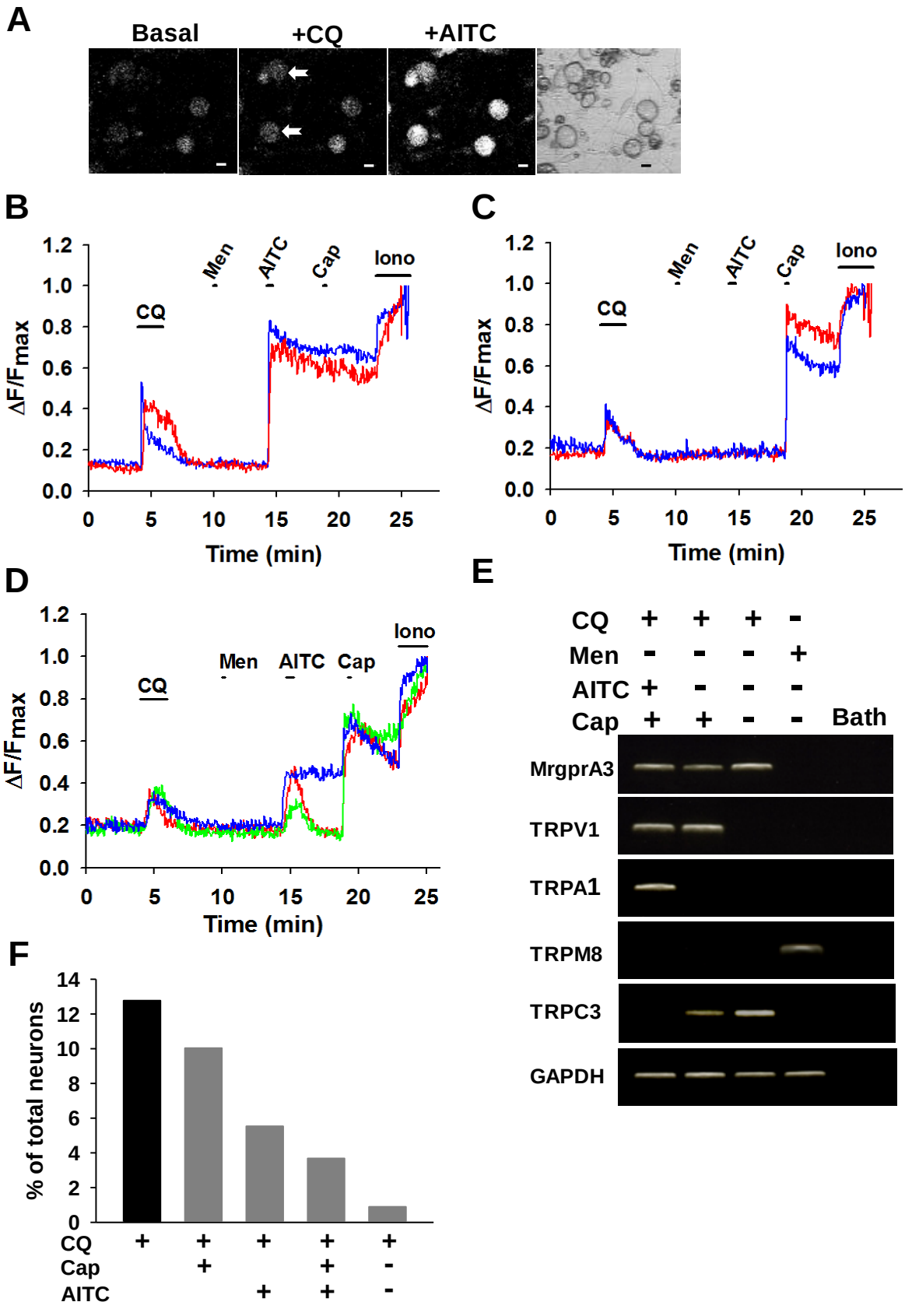
FIGURE 6. CQ-induced TRPM8 inhibition is caused by a direct action of activated $G\alpha_q$. ***A,*** Inward and outward currents (at -60mV and +60mV), activated by menthol (200μM, 5s) in HEK293 cells expressing TRPM8 and MrgprA3 were inhibited by pre-treatment with CQ (1mM, 1min) applied alone, or together with U73122 (2.5μM), or after co-expression with PLCβ-ct. The dotted line represents zero current. ***B,*** Summary of peak currents in experiments similar to those in A. The number of experiments is shown above each bar. ***C,*** Representative traces of TRPM8 inward and outward currents (at -60mV and +60mV) activated by menthol (200μM, 5s) in $G\alpha_{q/11}^{-/-}$ MEF cells transfected with MrgprA3 and TRPM8. Currents are shown before and after CQ (1mM, 1min) treatment. The dotted line represents zero current. ***D,*** Summary of results similar to those in C. The number of experiments is shown above each bar. All data are means±s.e.m. **P<0.01; ***P<0.001; NS, not significant. ***E,*** HEK293 cells expressing TRPM8-V5 and MrgprA3 were stimulated with 1mM CQ for 5 minutes. TRPM8 was then immune-precipitated with anti-V5, and co-precipitated $G\alpha_q$ was detected by anti-$G\alpha_q$. The same blot was stripped and redetected with anti-V5. TCl, total cell lysate. Moleclular weight is shown on the right.

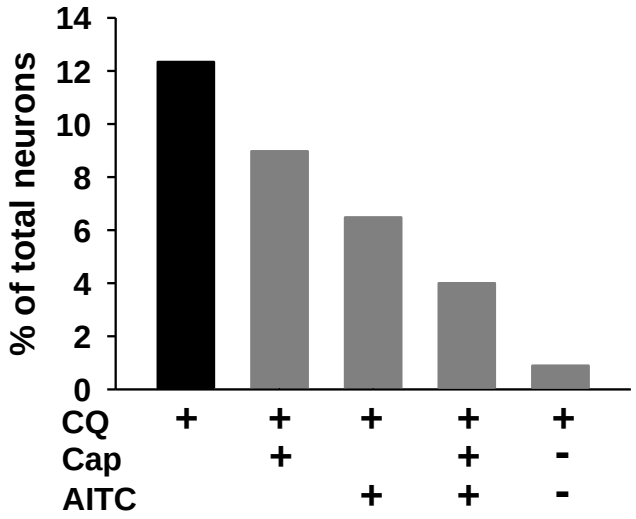
FIGURE 7. CQ excites TRPA1 and TRPC3, sensitizes TRPV1 and inhibits TRPM8 through different signaling mechanisms. Binding of CQ to the CQ receptor MrgprA3 causes activation of $G\alpha_q$ and release of $G\beta\gamma$. The released $G\beta\gamma$ then activates the TRPA1 channel, leading to opening of the channel and calcium influx. Meanwhile, activated $G\alpha_q$ activates PLCβ leading to the excitation of the TRPC3 channel and calcium influx. Activated $G\alpha_q$ also stimulates PKC and increases phosphorylation of TRPV, leading to the sensitization of TRPV1and subsequently enhanced itch. The activated $G\alpha_q$ also directly inhibits TRPM8 by binding to TRPM8 without the need to involve the PLC pathway. Inhibition of TRPM8 probably also contributes indirectly to enhanced pain and itch.

Table 1. Primer sequences for single-cell RT-PCR

Genes	Primer sequences 5'-3'	Product length (bp)	Accession no.
MrgprA3	CGACAATGACACCCACAACAA GGAAGCCAAGGAGCCAGAAC	150	NM_153067.2
TRPV1	CTGGAGCTGTTCAAGTTCACC TTGGTGTTCCAGGTAGTCCAG	362	NM_001001445.1
TRPA1	CCAAGATGCCTTCAGCACCC GGGTGGCTAATAGAACAATGTGTTTTAGTC	684	AY231177
TRPM8	GTGGGAGCAACTGTCTGGAGC GTGTCCATAACGTTCCATAGGTCG	458	NM_134252.
TRPC3	GGAACTGGGCATGGGTAACTC CACTGGGGTTCAGTTTCTCACTG	317	NM_019510.2
GAPDH	CATCCATGACAACTTTGGCA CCTGCTTCACCACCTTCTTG	302	NM_008084

Figure 1





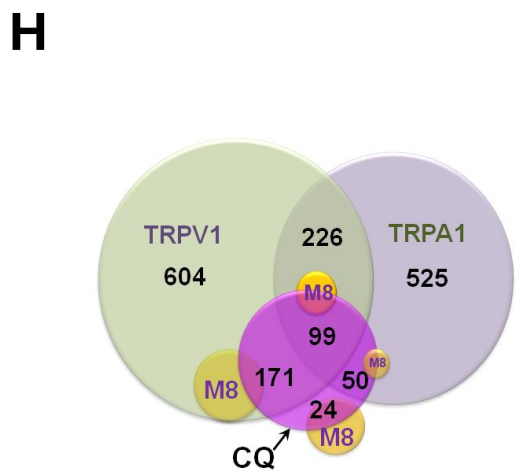


Figure 2

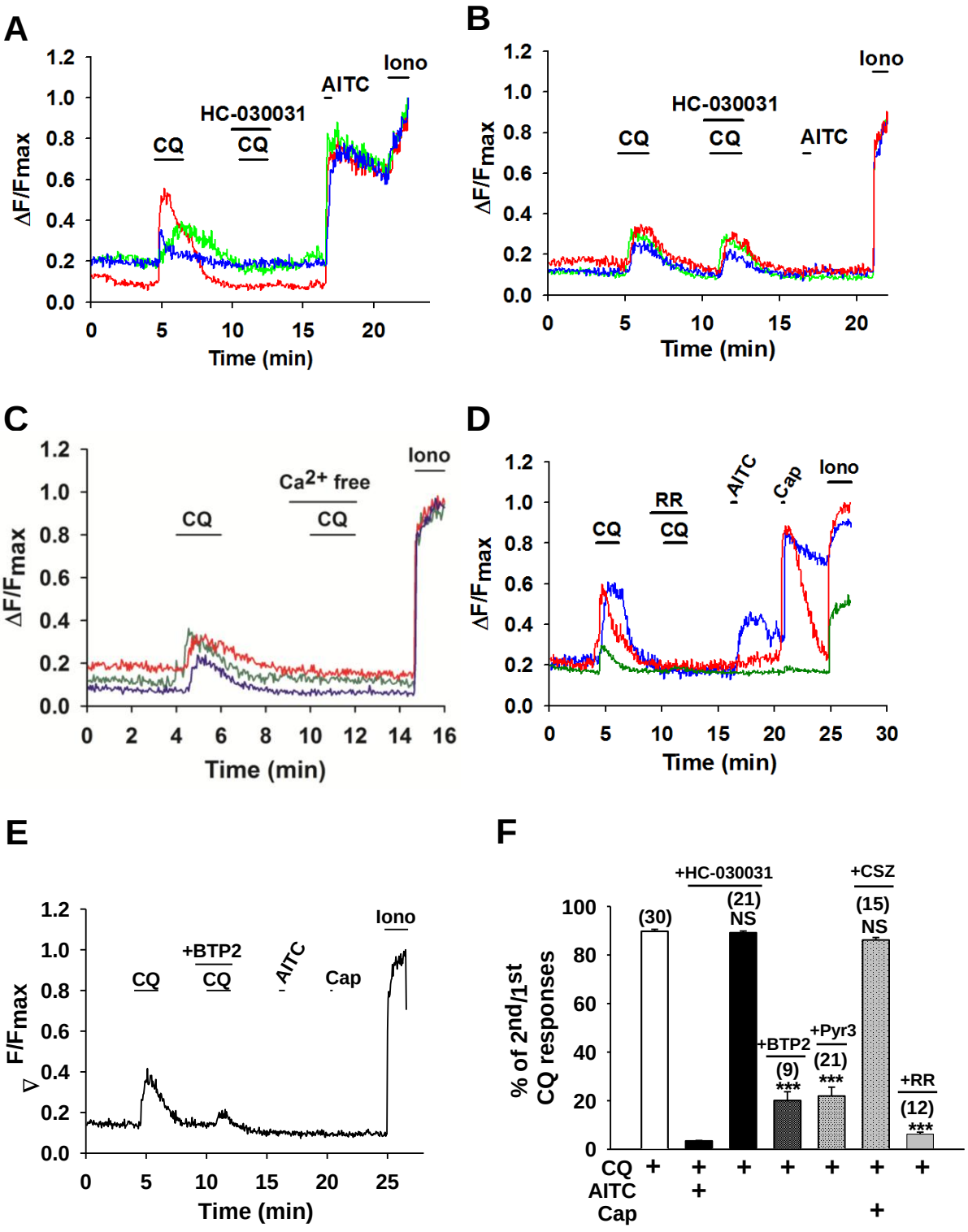


Figure 3

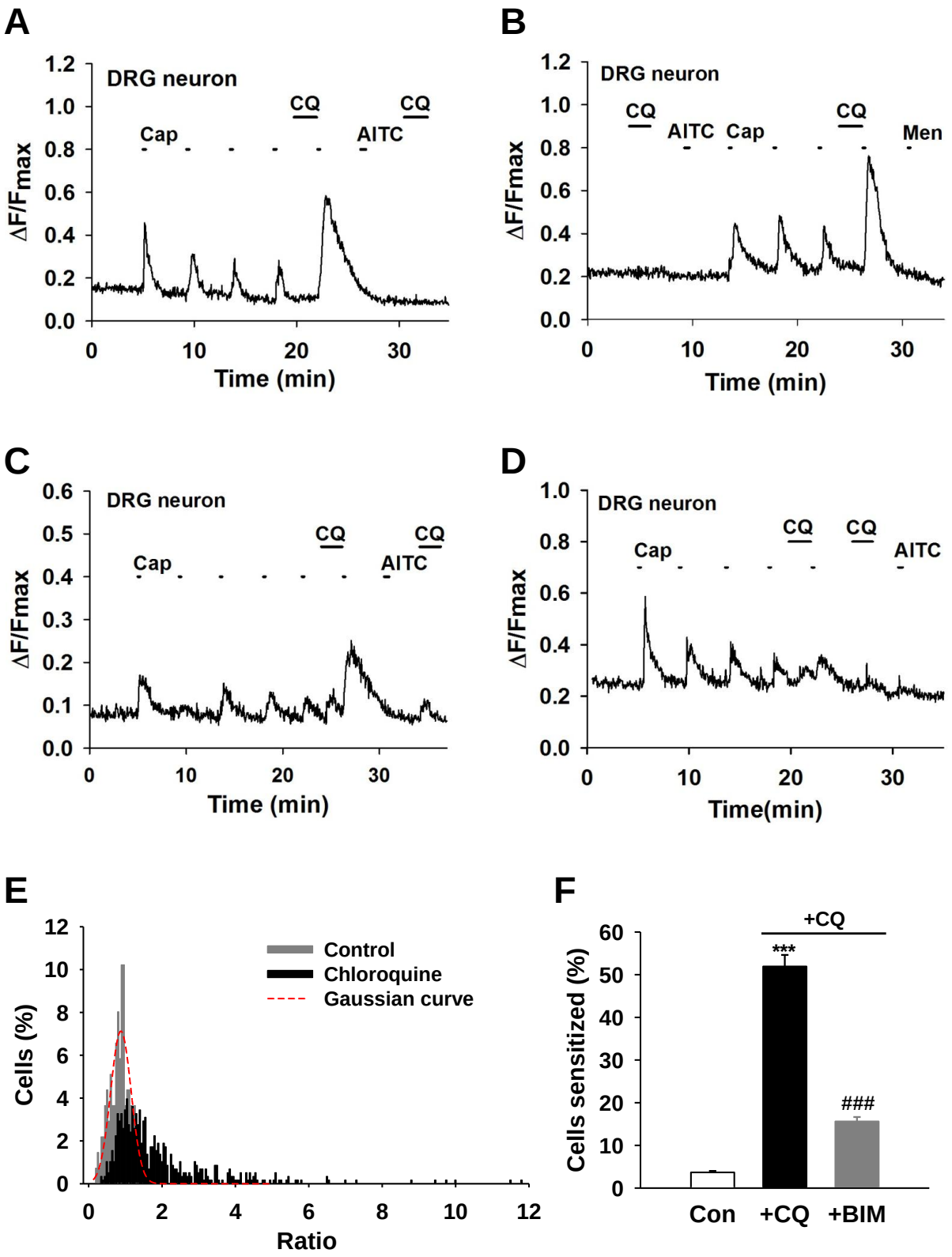


Figure 4

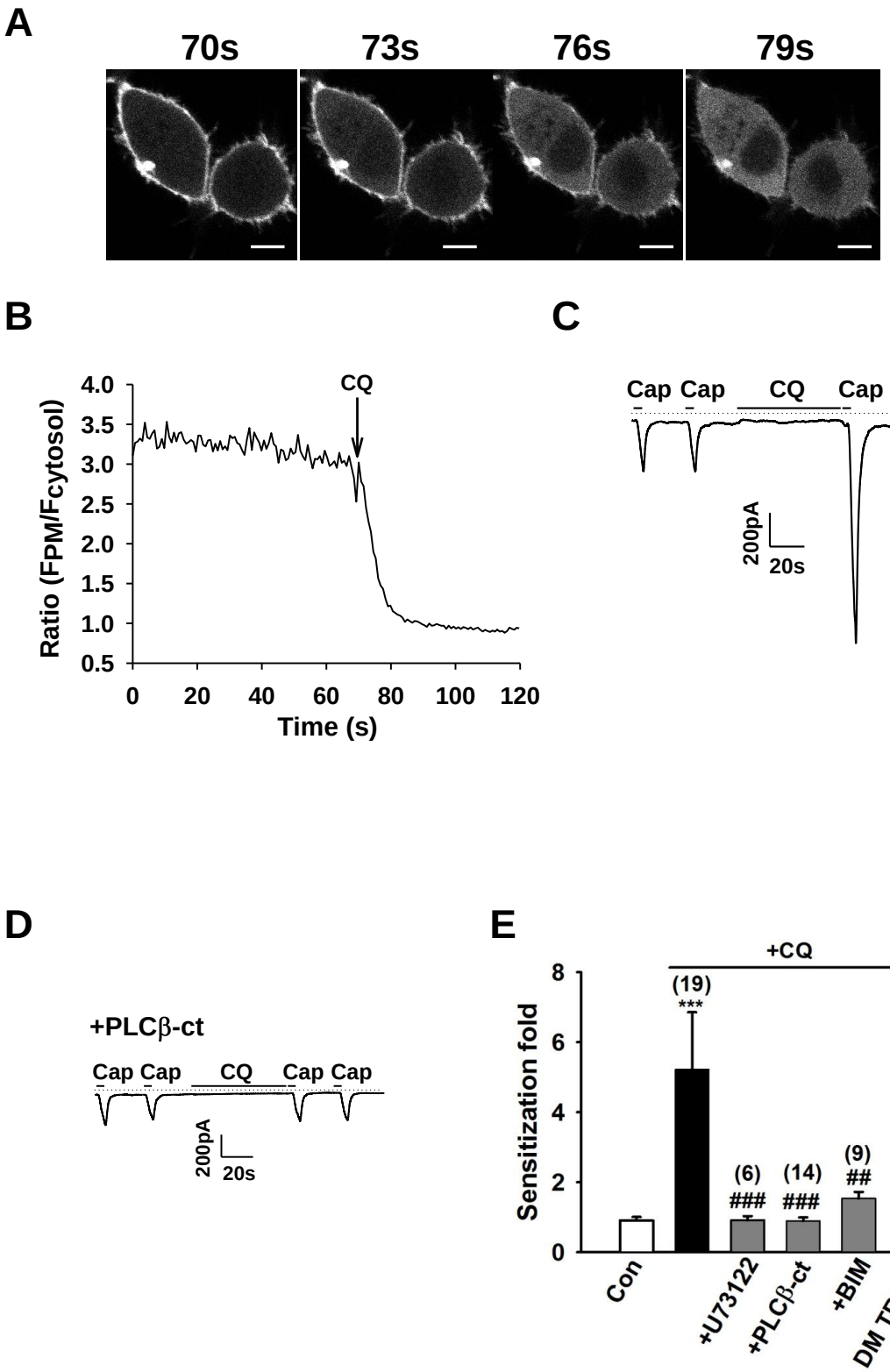


Figure 5

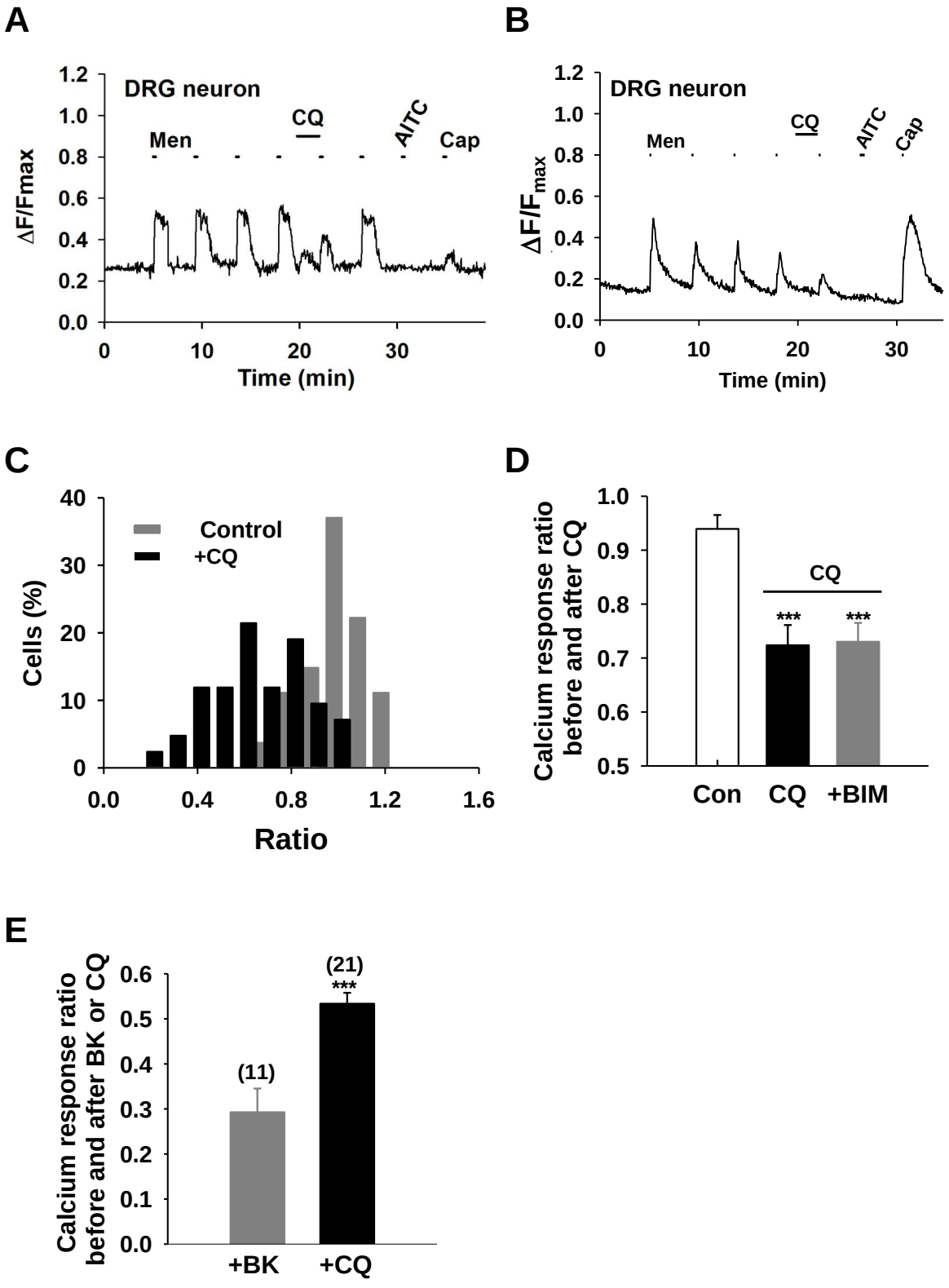


Figure 6

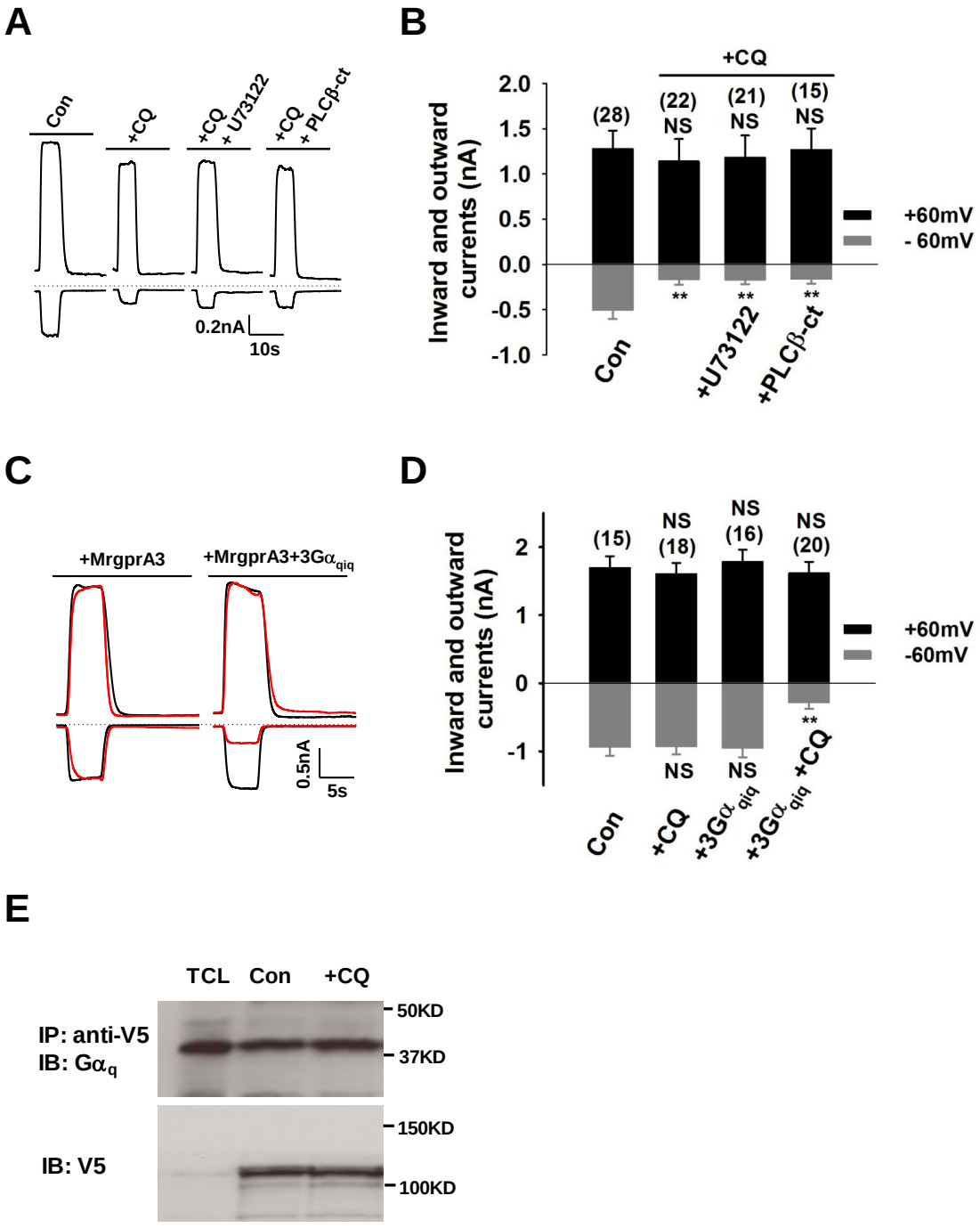


Figure 7