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REPLY

Reply to "TRPA1-dependent calcium transients and CGRP release in DRG neurons require extracellular calcium"

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In this issue, Gebhardt et al. (2020. *J. Cell Biol.* https://doi.org/10.1083/jcb.201702151) express interest in our recently published work (Shang et al. 2016. *J. Cell Biol.* https://doi.org/10.1083/jcb.201603081). Here, we would like to address their concerns regarding the lysosomal TRPA1-mediated intracellular calcium transients in dorsal root ganglion neurons.

Gebhardt et al. (2020) first questioned our conclusion that TRPA1 mediates both Ca^{2+} influx through plasma membrane and Ca^{2+} release from lysosome-related internal Ca^{2+} store in dorsal root ganglion (DRG) neurons (Shang et al., 2016). The reason for raising this doubt is that although they were able to reproduce our result that the TRPA1 agonist allyl isothiocyanate (AITC) triggers a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of DRG neurons bathed in the Ca^{2+}-free extracellular solution containing 1 mM EGTA, this effect was largely abolished with the solution that contained 10 mM EGTA. Based on this observation, Gebhardt et al. (2020) concluded that 1 mM EGTA must not be enough to buffer the residual Ca^{2+} present in the nominally Ca^{2+}-free physiological solution made using commercial American Chemical Society grade constituents.

Gebhardt et al. (2020) are correct in citing the reference from Patton et al. (2004) that the Ca^{2+} concentration in nominally Ca^{2+}-free solutions can be as high as 12 µM, a fact that the majority of researchers in the Ca^{2+} field have been fully aware of for decades. This is why Ca^{2+} chelators, such as EGTA, HEDTA, or BAPTA, are often included in the Ca^{2+}-free solutions. In their paper, Patton et al. measured the free Ca^{2+} concentration in the absence of any Ca^{2+} chelator (Patton et al., 2004). Thus, with the addition of 1 mM EGTA in such a solution, the free Ca^{2+} concentration would be ∼3.6 nM based on the following equation: Kd = [Ca][EGTA] / [EGTA*Ca], where [Ca], [EGTA], and [EGTA*Ca] are concentrations of the free and bound species and Kd (300 nM) is the dissociation constant (Patton et al., 2004; Whitten et al., 1992). The conversion of the formula gives $[Ca^{2+}]_e \approx Kd / ([EGTA]_{total} / [Ca^{2+}]_{total} - 1) \approx 3.6$ nM, when $[EGTA]_{total}$ and $[Ca^{2+}]_{total}$ are 1,000 and 12 µM, respectively. Thus, even if the buffer error of 1 mM EGTA is 12.2% produced by the 12 µM Ca^{2+} contamination, the $[Ca^{2+}]_e$ would only increase to 3.36 nM. This concentration is more than 10 times lower than the basal $[Ca^{2+}]_i$ (∼50 nM) in DRG neurons (Wang et al., 2016) and it is theoretically insufficient to drive Ca^{2+} inflow, especially under conditions of TRPA1 activation when the membrane is depolarized. Indeed, no inward Ca^{2+} currents or $[Ca^{2+}]_i$ rise through plasma membrane Ca^{2+} permeable channels, such as voltage-gated Ca^{2+} channels, could be detected in the Ca^{2+}-free bath that contained 1 mM EGTA (Zhang and Zhou, 2002).

Gebhardt et al. (2020) also assumed that there would be some "loosely attached calcium ions" that bind to "the anionic extracellular moieties of membrane proteins" and that they would become free in the 1 mM EGTA solution and contribute to Ca^{2+} entry through the plasma membrane TRPA1 channels. We find this assumption to be without any evidence to support it. First, it is not known if the DRG neurons express a large number of membrane proteins with the extracellular Ca^{2+} binding capability. Second, even if the proteins did bind to Ca^{2+} and they did release the ions to the solution, the total amount of the released Ca^{2+} would be insignificant compared with the large volume of the 1 mM EGTA solution applied to the culture of low-density DRG neurons. Third, many bound calcium ions would probably remain bound to the proteins unless the protein's Ca^{2+} binding affinity was much lower than that of EGTA. Again, in our experimental conditions using the 1 mM EGTA-buffered Ca^{2+}-free

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solution, we detected $[Ca^{2+}]_i$ rise in response only to AITC but not to strong (+20 mV for 0.5 s) depolarization, which activates voltage-gated Ca^{2+} channels in DRG neurons (see Fig. 1 of Huang et al. [2019]).

Importantly, Gebhardt et al. (2020) actually confirmed our results that AITC induces $[Ca^{2+}]_i$ rise in DRG neurons bathed in the 1 mM EGTA-buffered Ca^{2+}-free solution (Figs. 2 and 3 of their paper). They also detected AITC-evoked $[Ca^{2+}]_i$ increase in neurons bathed in the Ca^{2+}-free solution buffered by 2 mM BAPTA. The most likely reason for the loss of this phenomenon in the 10 mM EGTA-buffered solution is that although EGTA is considered membrane impermeable, it is taken up into the cell through fluid-phase endocytosis, a mechanism commonly used to load membrane impermeable dyes and substrates into endolysosomes (Lin et al., 2015). While a large fraction of the endocytosed substances will remain in the endolysosomes, some will also end up in the cytosol (Zhou et al., 1998). Although the endocytosed EGTA is a weak Ca^{2+} chelator in the lysosomal lumen at low pH in absence of AITC, the chelating effect should recover as Ca^{2+} releases from the lysosome, which is known to also cause proton release (Morgan and Galione, 2007). In addition, proton efflux may occur through the AITC-activated lysosomal TRPA1 channels. Here, no matter if EGTA was retained in the endolysosomes or delivered to the cytosol, it would interfere with the detection of TRPA1-mediated Ca^{2+} release from the lysosome-related Ca^{2+} stores, as chelating Ca^{2+} in the lumen prevents the release and buffering Ca^{2+} in the cytoplasm disrupts the detection of the Ca^{2+} signal.

Not surprisingly, such interference was dependent on not only the concentration of the chelator, but the loading time as well. When Gebhardt et al. (2020) pretreated the neurons with 10 mM EGTA for just 1 min, they indeed detected weak, but not none, $[Ca^{2+}]_i$ increases in response to AITC in some DRG neurons (Fig. 3 D of their paper). Moreover, because the concentration of EGTA in the cytoplasm tends to be low, it likely has more impact on the detection of more moderate $[Ca^{2+}]_i$ increases (close to the affinities of EGTA and Fura-2) than the higher increases that tend to saturate the Fura-2 signal (i.e., evoked $[Ca^{2+}]_i$ is nonlinearly related to Ca^{2+} chelator; see our previous work, Schneggenburger et al. [1993]). This explains why the decay phase of the KCl-evoked responses appears to be faster in cells that were pretreated with 10 mM than 1 mM EGTA (compare Fig. 3, C and D of their paper). Furthermore, it cannot be ruled out that EGTA, under Ca^{2+}-free conditions, also enters the cell through the open TRPA1 channels. Evidence exists that highly Ca^{2+}-permeable cation channels, including TRPA1, TRPV1, and P2X7, permeate large molecules, such as Yo-Pro (mol wt. 376 D; Chen et al., 2009; Li et al., 2011), Lucifer Yellow (mol wt. 457 D; Duan et al., 2003) and ATP (mol wt. 507 D; Xiong et al., 2018). EGTA (mol wt. 380 D) is about the same size as Yo-Pro, which is readily taken up by TRPA1 (Chen et al., 2009). In our hands, following the standard protocol of AITC treatment in the Ca^{2+}-free buffer containing either 1 or 10 mM EGTA (Fig. 1 of Shang et al. [2016]; Fig. 3 of Gebhardt et al. [2020]), a brief (3-s) pulse with KCl in the presence of 2 mM extracellular Ca^{2+} elicited ~10 times larger $[Ca^{2+}]_i$ rise in DRG neurons pretreated with 1 mM EGTA than those treated with 10 mM EGTA, supporting that 10 mM extracellular EGTA leads to stronger intracellular Ca^{2+} buffering than 1 mM EGTA.

It is important to point out that the apparently attenuated (but not eliminated) amplitude of AITC-induced $[Ca^{2+}]_i$ rise in the 10 mM EGTA solution, as compared with that in 1 mM EGTA, likely resulted from the strong negative impact of the intracellular Ca^{2+} buffer (EGTA and Fura-2) on the amplitude of $[Ca^{2+}]_i$. As clearly demonstrated in our previous work, Ca^{2+} buffer (Fura-2) strongly impacts the $[Ca^{2+}]_i$ amplitude, such that a 10-times increase in [Fura-2] also reduces the $[Ca^{2+}]_i$ amplitude by >10 times (see Fig. 1 of Schneggenburger et al. [1993]). Therefore, the change in Fura-2 ratio (F_{358}/F_{391}), which indicates $[Ca^{2+}]_i$ amplitude, is not linearly correlated with the net Ca^{2+} entry into the cytoplasm, either through influx or release from internal stores. Instead, the fluorescence Ca^{2+} signal, $Fd = F_{358} - F_{391}$ can provide a linear assay for the amount of Ca^{2+} influx/release (see Zhou and Bers [2000]). Using Fd, we showed that ~40% of the total Ca^{2+} that entered the cytoplasm in response to AITC stimulation in the 2.5 mM Ca^{2+} bath arose from the lysosome-related Ca^{2+} store (Shang et al., 2016).

Therefore, all new data shown by Gebhardt et al. (2020) can be explained by the increased loading of EGTA to the lysosomal store and the cytosol when cells were exposed to 10 mM, as compared with 1 mM, of the Ca^{2+} chelator. Particularly, even with respect to the lack of calcitonin gene related peptide (CGRP) release from tracheal nerve endings in the EGTA buffered conditions (both 1 and 10 mM), as shown in Fig. 1 of Gebhardt et al. (2020), the explanation may lie in the fact that the nerve terminals have much larger surface/volume ratios than the cell body. Thus, the same uptake mechanism will lead to higher cytosolic (or even lysosomal) EGTA concentrations at the terminals than at the soma, resulting in stronger Ca^{2+} buffering to suppress $[Ca^{2+}]_i$ rise in the nerve endings. Therefore, with 1 mM EGTA-buffered Ca^{2+}-free solution, we were able to detect AITC-induced CGRP release from cultured DRG neurons (Shang et al., 2016), whereas Gebhardt et al. (2020) could not detect the same activity from the nerve terminals. Moreover, the long preincubation period (10 min) of the tracheal tissue in the Ca^{2+}-free solution used by Gebhardt et al. (2020) might have also contributed to their failure. On the other hand, Quallo and colleagues indeed observed CGRP release from dorsal spinal cord terminals in the Ca^{2+}-free solution (Quallo et al., 2015).

The overemphasis on the lack of effect of AITC in the 10 mM EGTA-buffered solution should not overshadow the series of strong evidence about the contribution of lysosomal TRPA1 in AITC-induced Ca^{2+} signals and cellular function in nociceptors (Shang et al., 2016). These include: (1) In 1 mM EGTA-buffered Ca^{2+}-free solution, only TRPA1 (17% of fractional Ca^{2+} current (Karashima et al., 2010) but not voltage-gated Ca^{2+} channel (100% fractional Ca^{2+} current) produces Ca^{2+} raise in the cytosol (see Fig. 1 of Huang et al. [2019]), excluding the possible contamination of Ca^{2+} influx through plasma membrane; (2) 1 mM EGTA or 2 mM BAPTA in the extracellular solution failed to block the AITC-induced Ca^{2+} transients, while intracellular loading of BAPTA completely inhibited the Ca^{2+} response; (3) the internal store contributes to ~40% of the overall AITC-induced Ca^{2+} signal in 2.5 mM Ca^{2+} extracellular solution; (4) in the same 2.5 mM Ca^{2+} solution, membrane-impermeable TRPA1 antagonist, ruthenium red, decreased the AITC-induced Ca^{2+} signal by ~60%, while membrane permeable TRPA1 antagonists, HC-030031

and A-967079 completely blocked the AITC-induced Ca^{2+} transients; (5) the AITC-induced $[Ca^{2+}]_i$ increase in the Ca^{2+}-free solution was blocked by pre-depleting the lysosome store with bafilomycin A1; (6–8) immunostaining, structured illumination microscopy imaging, and immunogold EM imaging all showed TRPA1 localization on both plasma membrane and lysosomes; (9) functionally, Gly-Phe-β-naphthylamide (GPN), which induces lysosome lysis, abolished AITC-induced Ca^{2+} signal in the Ca^{2+}-free buffer and partially reduced the nocifensive responses and thermal hyperalgesia induced by the topical injection of AITC or acrolein to mouse hind paws; (10) by line-scan confocal imaging, we directly visualize AITC-induced Ca^{2+} release from where lysosomes were found; (11) in the Ca^{2+}-free solution, AITC induced exocytosis and neuropeptide release from DRG neurons. These multiple pieces of hard evidence indicate that not only does TRPA1 activation, in addition to Ca^{2+} influx, mobilize Ca^{2+} from lysosome-related stores, but also that the internally generated Ca^{2+} signals are able to support several known physiological functions of this channel (Shang et al., 2016).

Lastly, Gebhardt et al. (2020) questioned the GPN effect. They assumed TRPA1 to be permeable to protons by citing Ye et al. (2018). However, this is a misunderstanding, because Ye et al. showed that TRPA1 can be regulated by pH (Ye et al., 2018). Based on the assumption that the lysosomal TRPA1 should mediate proton release, which has no hard evidence to support it, Gebhardt compared the effect of GPN and AITC lysosomal pH, using Lysotracker as a readout. They saw increases in the fluorescence intensity of Lysotracker in both GPN- and AITC-treated cells (Fig. 3, G and H of Gebhardt et al. [2020]). Only after ∼2 min, the intensity started to decrease in the continued presence of GPN. However, for AITC, the treatment was <30 s; therefore, it is not known how the signal would change in the continued presence of AITC. The interpretation of these results remains a mystery at best.

Collectively, we would like to offer an alternative explanation to Gebhardt et al. (2020) for their results obtained from using solutions containing 10 mM EGTA. Under the same experimental conditions, their data are in fact consistent with our earlier finding that intracellular lysosome-related stores contribute to a significant portion of the Ca^{2+} signal induced by TRPA1 activation in sensory neurons. We hope that our clarifications are helpful.

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