# Improving Sensitivity of the NociOcular Assay to Evaluate Ion Channel Activation by Acidic Cosmetic Ingredients

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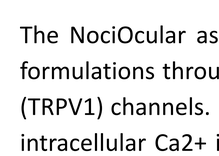
## ABSTRACT

The NociOcular assay is designed to predict the eye stinging potential of surfactant formulations through the activation of transient receptor potential vanilloid subtype 1 (TRPV1) channels. TRPV1 channel activation is determined via measurements of acute intracellular Ca2+ influx in TPRV1-overexpressing neuroblastoma cells exposed to test substances. Specificity of the response is confirmed by addition of capsazepine, a TRPV1 channel inhibitor. Traditionally, the assay buffer, Krebs-Ringer-Hepes (KRH) salt solution, is prepared to have a 25 mM concentration of the organic chemical buffering agent, Hepes. In this study, TRPV1-overexpressing SH-SY5Y cells were exposed to a variety of acids and the concentration of Hepes in the KRH buffer was adjusted to varying concentrations up to 25 mM. It was found that when the buffering capability of KRH was reduced, the tested acids were capable of eliciting a $Ca^{2+}$ influx at lower concentrations. Additionally, it is known that both TRPV1 and acid-sensing ion channels (ASICs) are involved in nociception. Both channels have been shown to be directly activated by protons and TRPV1 activation is also mediated by noxious heat, acidic conditions, and a variety of chemical compounds. To investigate the possibility of ASICs contributing to Ca2+ influx, naïve SH-SY5Y cells were exposed to several acids as well as the nonspecific ASIC inhibitor, amiloride (AM). The study revealed that lactic acid and benzoic acid were able to induce $Ca^{2+}$ influx that was blocked by amiloride. Alternatively, capsazepine (CZ) significantly decreased $Ca^{2+}$ influx, in TRPV1-overexpressing SH-SY5Y cells, induced by citric and pidolic acid, but not by lactic acid, glycolic acid, and benzoic acid. In conclusion, the study has shown that it is possible to improve the sensitivity of the NociOcular assay, when acidic solutions are tested, by adjusting the buffering capacity in the incubation medium and that acid-induced nociception in the assay may involve ion channels other than TRPV1 channels.

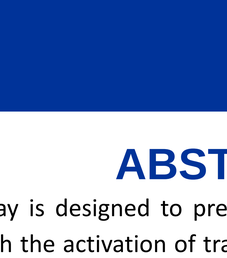
## NOCIOCULAR *IN VITRO* ASSAY

Cell Seeding



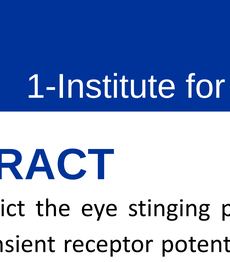
Step 1: SH-SY5Y cells are seeded in 96-well plates and incubated until an appropriate confluency is achieved.

Addition of Calcium Indicator/Rinsing



Step 2: The cells are treated with a calcium dye indicator and rinsed twice prior to the addition of assay buffer. Half of the wells receive buffer with a TRPV1 antagonist or ASICs inhibitor.

Test Article Preparation



Step 3: A dilution of the test article is prepared and added to a 96-well compound plate that is later used for dosing. The compound plate also contains the solvent control (assay buffer) and the positive control (TRPV1 agonist) for comparison.

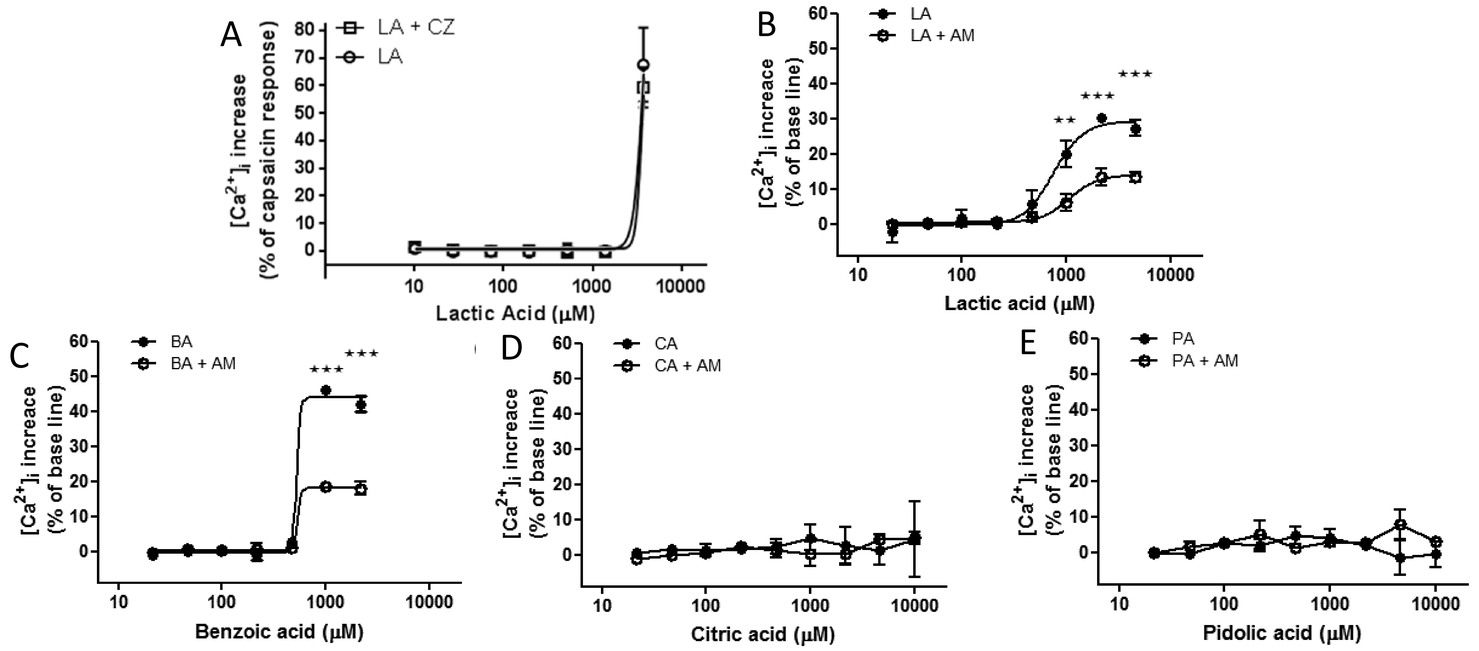
Dose and Read Plate

Step 4: A cell plate, compound plate, and tips are loaded into the FlexStation. The cell plate is systematically dosed and the fluorescence intensity is recorded using SoftMax Pro software. The data is saved and analyzed using SoftMax Pro, Microsoft Excel, and Prism software.

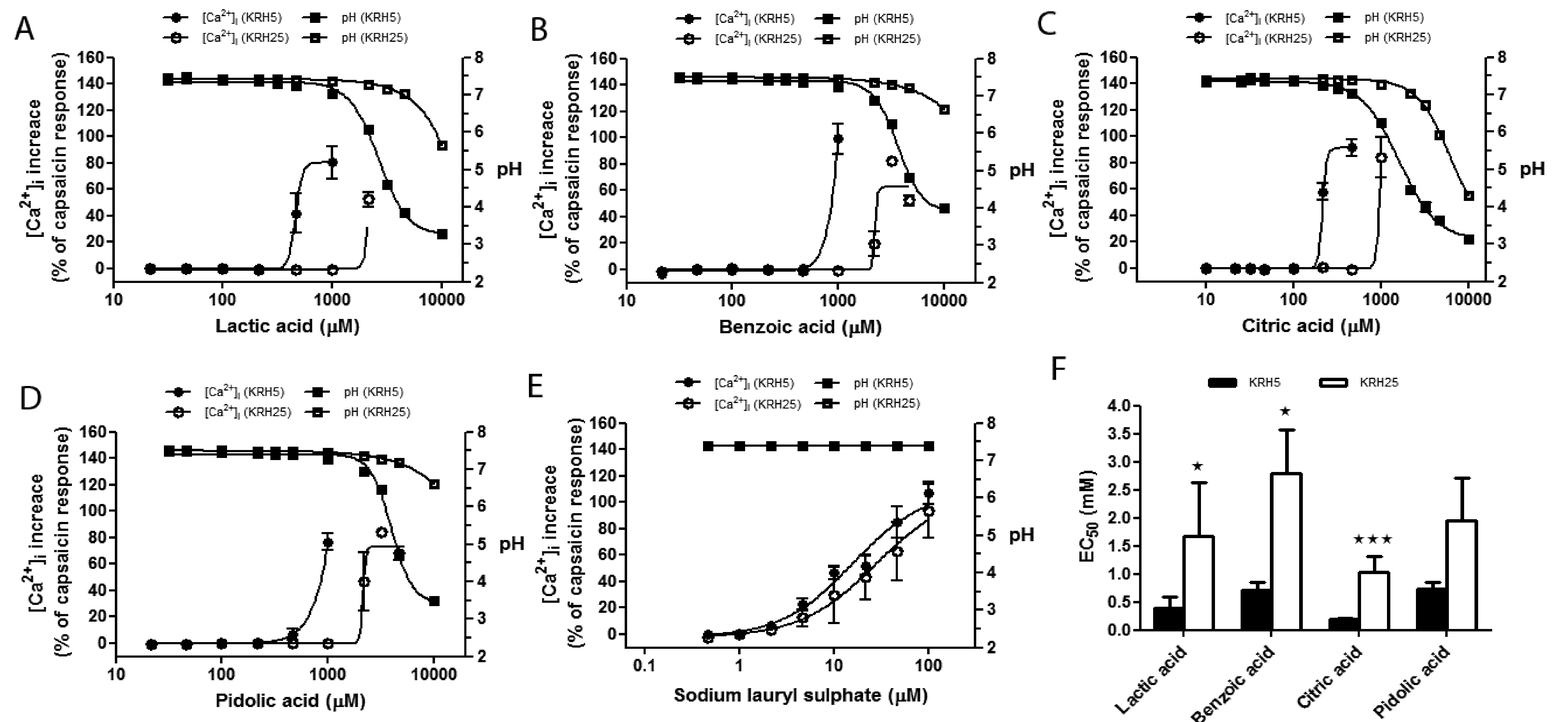
## CONCLUSIONS

Previously, it was assumed that $Ca^{2+}$ influx observed during the NociOcular assay was due to the activation of TRPV1 channels. However, through the use of CZ and AM, it was possible to demonstrate that $Ca^{2+}$ influx in the NociOcular assay is mediated by TRPV1 channel activation as well as other ion channels such as ASICs. The assay was originally designed to test anionic surfactants' nociceptive effects; this study was able to show that the acidic additives found in many cosmetic products also contribute to the $Ca^{2+}$ influx observed in both naïve and TRPV1 overexpressing SH-SY5Y cells due to the involvement of other ion channels. Additionally, this study has shown that, when testing acidic solutions, an adjustment of the buffering capacity of the incubation and dilution mediums may allow for improved sensitivity for that class of products.

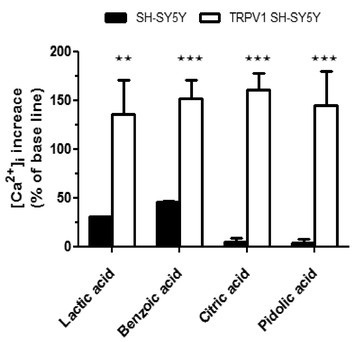
## RESULTS



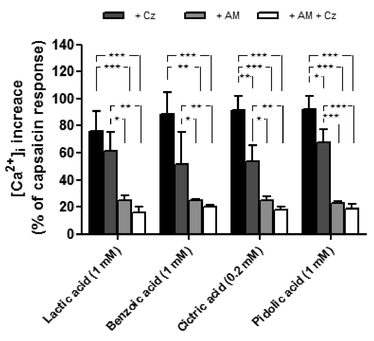

**Lactic acid and benzoic acid activate acid-sensing ion channels (ASICs) in native SH-SY5Y cells.** The dose response curve (A) illustrates that lactic acid does not exhibit a TRPV1 specific response in SH-SY5Y cells overexpressing TRPV1. The remaining dose response curves illustrate an increase in $Ca^{2+}$ influx above baseline for (B) lactic acid (C) benzoic acid, (D) citric acid, (E) and pidolic acid in the presence or absence of amiloride. **P<0.01 and *** P<0.001 significantly different from amiloride treated cells unless otherwise indicated.




**Acid-induced $Ca^{2+}$ influx in NociOcular assay is dependent on the buffering capacity of the incubation medium.** Dose response curves illustrate $Ca^{2+}$ influx and pH values for (A) lactic acid (B) benzoic acid (C) citric acid, (D) pidolic acid and (E) sodium lauryl sulphate. (F) $EC_{50}$ values for citric acid, lactic acid, benzoic acid and pidolic acid in KRH5 (KRH with 5mM Hepes) and KRH25 (KRH with 25mM Hepes). *P<0.05 and ***P<0.001 significantly different from KRH5.




**Indication of involvement of ion channels other than TRPV1, in acid-induced $Ca^{2+}$ influx.** Maximum $Ca^{2+}$ influx in native and TRPV1 overexpressing SH-SY5Y cells at non-cytotoxic concentrations of citric acid (0.5 mM), lactic acid (1.2 mM), benzoic acid (1 mM) or pidolic acid (1 mM). **P<0.01 and *** P<0.001 significantly different from native SH-SY5Y cells.




**Acid-induced $Ca^{2+}$ influx in the NociOcular assay involves ASICs and TRPV1 channels.** TRPV1-SH-SY5Y cells were treated with the indicated acid alone, in presence of a specific TRPV1 inhibitor (CZ), unspecific ASIC inhibitor (AM), or a combination of CZ and AM . *P<0.05, **P<0.01 and *** P<0.001 significantly different from indicated treatment.

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