Development of a chemogenetic approach to manipulate intracellular pH

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ABSTRACT: Chemogenetic Operation of iNTRacellular prOton Levels (pH-Control) is a novel substrate-based enzymatic method that enables precise spatio-temporal control of ultra-local acidification in cultured cell lines and primary neurons. The genetically encoded biosensor SypHer3s showed that pH-Control effectively acidifies cytosolic, mitochondrial, and nuclear pH exclusively in the presence of β-Chloro-D-alanine in living cells in a concentration-dependent manner. The pH-Control approach is promising for investigating the ultra-local pH imbalance associated with many diseases.

Intracellular pH levels are tightly regulated[1]. Gene expression, cell motility, and metabolic processes are a few examples of the many cellular processes under the control of local pH fluctuations[2]. Hence, multiple disorders, such as cancer[3], cardiovascular diseases[4], and neurological diseases[5], may be associated with the dysregulation of pH. The ability to monitor[6] and manipulate[7] intracellular pH levels directly inside a single cell has enormous ramifications for understanding subcellular and sub-organelle processes, disease diagnosis, and developing novel therapeutic strategies[8,9]. Several technologies have been advanced to investigate the role of pH at single cell level; however, conventional methods such as the application of micropipettes[10], genetic or chemical manipulation of proton pumps[11], optogenetic approaches[12], and small chemical inhibitors[13] have off-target effects or are less practical (Supporting Information Figure 1S). Therefore, the lack of tractable experimental tools permitting manipulating pH levels with high spatio-temporal resolution in the acidic range undermines studying the relationship between pH imbalance and cell function in health and disease.

We present pH-Control, an acronym for **C**hemogenetic **O**peration of i**NTR**acellular pr**O**ton **L**evels, as a novel chemogenetic approach that we have combined with the genetically encoded biosensor SypHer3s[14] for simultaneous visualization of ultra-local acidification in living cells (Figure 1a). Substrate-based chemogenetic tools are silent recombinant proteins until their biochemical stimulus - typically an unnatural amino acid is provided[15]. Combined with genetically encodable biosensors, these experimental systems have opened up new lines of investigation, allowing the analysis of intracellular pathways that modulate physiological and pathological cell responses[16].

pH-Control is a chimera of a red fluorescent protein variant (DsRed) and a Salmonella Typhimurium-derived enzyme termed D-Cysteine Desulfhydrase (stDCyD)[17]. stDCyD converts the unnatural amino acid β-chloro-D-alanine (βCDA) to its corresponding α-ketoacid and generates the byproducts hydrochloric acid (HCl), ammonium (NH_4^+),

and pyruvate in the presence of the cofactor pyridoxal 5' phosphate (PLP). βCDA is a well-established antibacterial agent and cannot be metabolized by human cells and tissues[18].

The stDCyD enzyme is differentially targetable to subcellular locales where it remains quiescent until its substrate (βCDA) is provided to generate HCl. Theoretical calculations and experimental approaches showed that the amount of generated byproducts is neglectable (Supporting Information Table S1 and Figure S2). At the same time, the change in [H^+] equals a 900% increase upon a pH change of one order of magnitude during the enzymatic activity of stDCyD (Supporting Information Table S1).

Overexpressing pH-Control with SypHer in cultured cells (HEK293T) did not show any visible toxicity (Figure 1b) even if differentially targeted to the cytosol, mitochondria, or cell nucleus (Supporting Information Figure S3). Administration of high concentrations of βCDA to cells expressing pH-Control yielded robust intracellular acidification as documented by the pH-sensitive biosensor SypHer3s. In contrast, wild-type cells without the enzyme showed marginal response to the same treatment (Figure 1c). A single mutation at position Y287F in the stDCyD enzyme yielded a dysfunctional control construct incapable of acidifying cells upon provision of βCDA (Figure 1d and Supporting Information Figure S4).

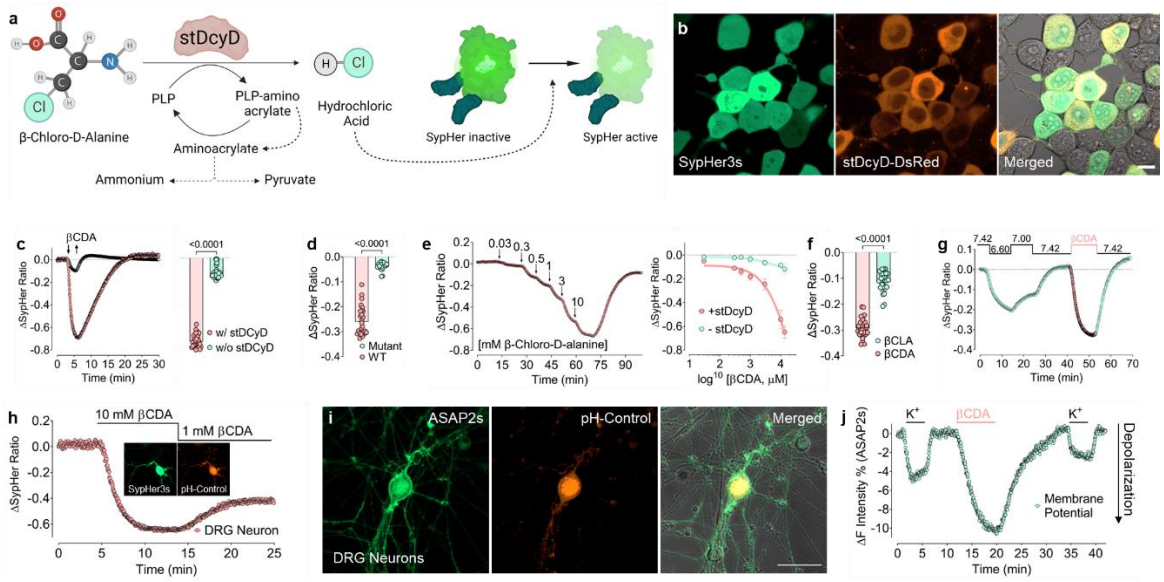

Figure 1. Characterization of pH-Control. (a) Schematic representation of the pH-Control pathway and simultaneous visualization with the pH-sensitive biosensor SypHer3s. (b) Representative confocal images of HEK293T cells co-expressing DsRed-stDCyD. Scale bar= 20 μm. (c) Real-time SypHer3s traces of cytosolic pH in WT cells (n=3/39) or cells expressing DsRed-stDCyD (n=3/32) in response to 13.4 mM βCDA. (d) Bars show SypHer3s biosensor responses in cells expressing the WT DsRed-stDCyD (n=4/21) and mutated and nonfunctional DsRed-stDCyD (n=4/18) upon administration of 1 mM βCDA. (e) The left panel shows a representative curve of SypHer3s in response to various concentrations of βCDA, as indicated in the figure. The right panel shows a concentration-response curve in HEK293T cells without enzyme (green curve) or expressing stDCyD (red curve) upon administration of indicated concentrations of βCDA. N=3 for all experiments and n=8-49 individual cells. (f) Bars show the selectivity test of cells treated with βCDA (n=3/29) or βCLA (n=3/29). (g) Representative real-time traces of HEK293T cells co-expressing SypHer3s and pH-Control in response imaging medium with different pH levels and 1 mM βCDA as indicated (n=3/17). (h) Representative real-time traces of SypHer signals in DRG neurons expressing pH-Control in response to 10 or 1 mM βCDA. The inset shows representative confocal images of DRG neurons co-expressing SypHer3s and pH-Control. (i) Representative confocal images of dorsal root ganglion neurons co-expressing ASAP2s and pH-Control eight days after viral infection. Scale bar= 50 μm. (j) Representative real-time curve shows signals of the voltage sensor ASAP2s in DRG neurons co-expressing pH-Control in response to high potassium (50 mM) and 10 mM βCDA as indicated (similar results were obtained from 4 different experiments and eleven individual cells). Student's t-test was applied.

Constitutive administration of different levels of βCDA to cells expressing pH-Control showed a concentration-dependent and fully reversible SypHer response (Figure 1e, left panel). At the same time, cells only expressing SypHer remained unresponsive to the same treatment (Figure 1e, right panel). Selectivity tests unveiled that the enzyme remained agnostic to D-alanine (data not shown) and showed marginal responses to β-chloro-L-alanine (βCLA) in comparison to βCDA (Figure 1f).

Figure 1g demonstrates that even low pH levels of the extracellular imaging medium are insufficient to acidify cytosolic pH to the degree to which the administration of low concentrations (1 mM) of βCDA achieved. To estimate the acidification levels using pH-Control, we calibrated the SypHer biosensor. We found the dynamic range of the pH biosensor between pH 7.5 and 5.5 (Supporting Information Figure S5). Our results imply that pH-Control allows manipulation of intracellular pH in one order of magnitude, typically from pH 7.5 to 6.5 in the cytosol. Another critical observation was that after the withdrawal of βCDA, the biosensor's signal overshot the baseline after recovery, indicating a cellular alkalization, which is in line with a recent report[19] (Figure 1c, g and Supporting Information Figure S3a). To tackle this issue further, we visualized the overcorrection in cells in the presence and absence of monensin and nigericin to disentangle controlled proton transport from H^+ channels (Supporting Information Figure S6). Intracellular pH overcorrection was diminished when cells were permeabilized with monensin and nigericin.

We next attempted to use the pH-Control method in mouse primary dorsal root ganglion (DRG) neurons. Cells displayed high expression levels of both constructs, pH-Control, and SypHer3s, eight days following viral transduction (Figure 1h inset). Administration of different concentrations of βCDA yielded robust SypHer3s signals in DRG neurons (Figure 1h). We next sought to test whether pH-Control mediated acidification is sufficient to manipulate intact primary neurons. We used the voltage sensor ASAP2s[20], a GFP-based biosensor targeted to the outer cell membrane, assuming that H^+ generation would cause membrane potential depolarization (Figure 1i and Supporting Information Figure S7). Provision of βCDA depolarized primary neurons even stronger than high extracellular potassium (Figure 1j and Supporting Information Figure S8). Overall, our results show that the pH-Control method is effective for cytosolic acidification of both cell lines and primary cells with functional consequences.

In conclusion, we developed pH-Control, a novel substrate-based chemogenetic method that enables temporal and precise manipulation of intracellular pH levels. Even in complex cell systems like neurons, pH-Control is easily combinable with any suitable biosensor for simultaneous imaging of intracellular acidification. We anticipate that the introduction of our new method to transgenic animal model systems in the future will make it possible to dynamically modify pH balance in various cells and tissues alongside the ability to identify new therapeutic targets implicated in pathological acidification and physiological pathways.

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Author Contributions

AGZ conceived the idea, performed experiments, analyzed data, and wrote the manuscript. MM performed cloning experiments and generated differentially targeted SypHer and pH-Control constructs. TAC performed DRG isolation and confocal imaging experiments. ENY and MŞA generated viral particles and managed cell culture. GÖ coordinated and interpreted experiments with neurons. EE coordinated and designed the study, performed experiments, analyzed data, and wrote the manuscript.

Conflict of interest

The authors AGZ, MM, GÖ, and EE, have filed a patent application (patent application number 2023/000206, patent pending) describing parts of this manuscript's research, which does not alter the authors' adherence to the policies on sharing data and materials presented in this study. The remaining authors declare no competing financial interests.

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