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Title

IL-1 β augments H₂S-induced increase in intracellular Ca²⁺ through polysulfides
generated from H₂S/NO interaction

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Abstract

H₂S has excitatory and inhibitory effects on Ca²⁺ signals via transient receptor potential ankyrin 1 (TRPA1) and ATP-sensitive K⁺ channels, respectively. H₂S converts intracellularly to polysulfides, which are more potent agonists for TRPA1 than H₂S. Under inflammatory conditions, changes in the expression and activity of these H₂S target channels and/or the conversion of H₂S to polysulfides may modulate H₂S effects. Effects of proinflammatory cytokines on H₂S-induced Ca²⁺ signals and polysulfide production in RIN14B cells were examined using fluorescence imaging with fura-2 and SSP4, respectively. Na₂S, a H₂S donor, induced 1) the inhibition of spontaneous Ca²⁺ signals, 2) inhibition followed by [Ca²⁺]_i increase, and 3) rapid [Ca²⁺]_i increase without inhibition in 50% (23/46), 22% (10/46), and 17% (8/46) of cells tested, respectively. IL-1β augmented H₂S-induced [Ca²⁺]_i increases, which were inhibited by TRPA1 and voltage-dependent L-type Ca²⁺ channel blockers. However, IL-1β treatment did not affect [Ca²⁺]_i increases evoked by a TRPA1 agonist or high concentration of KCl. Na₂S increased intracellular polysulfide levels, which were enhanced by IL-1β treatment. A NOS inhibitor suppressed the increased polysulfide production and [Ca²⁺]_i increase in IL-1β-treated cells. These results suggest that IL-1β augments H₂S-induced [Ca²⁺]_i increases via the conversion of H₂S to polysulfides through NO synthesis, but not via changes in the activity and expression of target channels. Polysulfides may play an important role in the effects of H₂S during inflammation.

Keywords: Hydrogen sulfide, polysulfide, TRPA1, K_{ATP} channels, IL-1β, NO

1. Introduction

Hydrogen sulfide (H₂S) is a gasotransmitter that is synthesized from cysteine through several enzymatic pathways in mammals (Kamoun, 2004; Miyamoto et al., 2014). H₂S plays important roles in physiological functions such as the modulation of neuronal activity (Abe and Kimura, 1996), the relaxation of smooth muscle (Hosoki et al., 1997; Zhao et al., 2001), and the secretion of hormone and autacoid (Yang et al., 2005; Kaneko et al., 2006, Delgermurun et al., 2016). We previously reported excitatory and inhibitory effects of H₂S on Ca²⁺ signals, i.e., H₂S induces Ca²⁺ influx through transient receptor potential ankyrin 1 (TRPA1) channels, while inhibiting spontaneous Ca²⁺ oscillations through ATP-sensitive K⁺ (K_{ATP}) channels, in RIN14B cells, a cell line derived from rat pancreatic δ cells (Ujike et al., 2015). H₂S also increases the intracellular Ca²⁺ concentration ([Ca²⁺]_i) through TRPA1 activation in TRPA1-expressing CHO cells, HEK293 cells and sensory neurons (Streng et al., 2008; Miyamoto et al., 2011; Ogawa et al., 2012). Moreover, H₂S has inhibitory effects on insulin release and smooth muscle contraction through K_{ATP} channels (Zhao et al., 2001; Yang et al., 2005). TRPA1 and K_{ATP} channels are thought to be important for bidirectional modulation of cellular functions by H₂S.

H₂S also acts as a pathological signaling molecule. The amount of H₂S and expression of H₂S-producing enzymes increases under inflammatory conditions (Li et al., 2005; Wallace et al., 2009; Flannigan et al., 2011). Increased H₂S may be involved in exacerbation or resolution of inflammation (Li et al., 2006; Wallace et al., 2012; Linden, 2014). In addition to H₂S production, TRPA1 channels expression also changes in inflammation. IL-1 α and TNF- α , proinflammatory cytokines, upregulate TRPA1

expression and enhance Ca^{2+} responses in synoviocytes and odontoblast-like cells (Hatano et al., 2012; El Karim et al., 2015). $\text{TNF-}\alpha$ also promotes transportation of TRPA1 to the plasma membrane in trigeminal ganglion neurons (Meng et al., 2016). In addition to the changes in TRPA1, K_{ATP} channel expression increased in experimental colitis (Mathias and Weid, 2013). These changes in H_2S target channels could affect the action of H_2S to control Ca^{2+} signals. Moreover, inflammatory factors, such as neutrophil oxidants and nitric oxide (NO), convert H_2S to polysulfides (Nagy and Winterbourn, 2010; Cortese-Krott et al., 2015; Miyamoto et al., 2017), which possess sulfane sulfurs and a higher potency against TRPA1 than H_2S (Kimura et al., 2013; Hatakeyama et al., 2015). The effects of H_2S via TRPA1 may predominate during inflammation. Therefore, it is worth examining the effects of H_2S under inflammatory conditions in RIN14B cells expressing TRPA1 and K_{ATP} channels to reveal the influence of the changes in target channels and the conversion of H_2S described above.

In the present study, we investigated the effects of proinflammatory cytokines on H_2S -induced Ca^{2+} signals in RIN14B cells. We used Na_2S as a H_2S donor and measured $[\text{Ca}^{2+}]_{\text{i}}$ and intracellular polysulfide levels using fluorescence imaging with fura-2, a fluorescent Ca^{2+} indicator, and SSP4, a fluorescent sulfane sulfur probe, respectively. The involvement of NO in polysulfide production under inflammatory conditions was also examined.

2. Materials and methods

2.1. Cell culture

RIN14B cells were purchased from DS Pharma Biomedical (Osaka, Japan) and

cultured in RPMI1640 medium (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Gibco/Thermo Fisher Scientific) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Cells were placed on coverslips coated with poly-D-lysine (4–5 µg/cm², Sigma-Aldrich/Merck, Darmstadt, Germany) for experiments and cultured for 24 h. Cells were then treated with recombinant rat IL-1β, incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 24–48 h, and designated as IL-1β-treated cells.

2.2. *Ca²⁺ imaging*

Cells on coverslips were incubated with 10 µM fura-2 acetoxymethyl ester (Dojindo, Kumamoto, Japan) and 0.002% cremophor EL (Sigma-Aldrich/Merck) in normal solution (140 mM NaCl, 3.3 mM KH₂PO₄, 0.8 mM K₂HPO₄, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM glucose, and 20 mM HEPES, pH adjusted to 7.4 with NaOH) for 1 h at room temperature (22–25°C). Fura-2 fluorescence was measured using an inverted microscope (Eclipse Ti-U, Nikon, Tokyo, Japan) with a fluorescence ratio imaging system, a laser source (C7773, Hamamatsu Photonics, Shizuoka, Japan), and a charge-coupled device (CCD) camera (C6790, Hamamatsu Photonics). Cells were continuously superfused with normal solution and illuminated by excitation at 340 and 380 nm for 92 ms at 5 s intervals. The respective fluorescence signals (F₃₄₀ and F₃₈₀) were detected at 510 nm. The ratio of F₃₄₀ to F₃₈₀ (R) was analyzed using Aqua Cosmos software (Hamamatsu Photonics). [Ca²⁺]_i was calculated using the following equation: [Ca²⁺]_i = K_Dβ × (R - R_{min}) / (R_{max} - R) (Grynkiewicz et al., 1985). The dissociation constant of fura-2 and Ca²⁺ (K_D), the ratio of F₃₈₀ at saturating Ca²⁺ to F₃₈₀ at zero Ca²⁺ (β), and

the minimum ($R_{\min}$) and maximum ($R_{\max}$) of the fluorescence ratio (F_{340}/F_{380}) were calculated using a Calcium Calibration Buffer Kit (Invitrogen/Thermo Fisher Scientific). All experiments were performed at room temperature.

2.3. Intracellular polysulfide imaging

Real-time polysulfide imaging in living cells using SSP4 (Dojindo), a sulfane sulfur probe, was performed as per Moustafa and Habara's method (Mustafa and Habara, 2016). Cells on coverslips were washed once with serum-free RPMI1640 medium and incubated with SSP4 (50 μ M) and 0.003% cremophor EL, to load SSP4 into living cells, in serum-free RPMI1640 medium for 20 min at 37°C. After washing 2–3 times with serum-free medium, SSP4 fluorescence was measured using an inverted microscope (Diaphot 300, Nikon) with a fluorescence ratio imaging system, a laser source (C6979, Hamamatsu Photonics), and a CCD camera (ORCA-ER, Hamamatsu Photonics). Cells were continuously superfused with normal solution and illuminated at 482 nm for 183 ms at 5 s intervals at room temperature. Fluorescence signals were detected at 515 nm and analyzed using Aqua Cosmos software. SSP4 fluorescence was normalized to the value before stimulant application.

2.4. Real-time PCR

Total RNAs was extracted from RIN14B cells using TRI Reagent (Sigma-Aldrich), according to the manufacturer's instructions, and treated with DNase I (Invitrogen/Thermo Fisher Scientific). RNA samples were reverse-transcribed using ReverTra Ace with Oligo dT primers (TOYOBO, Osaka, Japan). Real-time PCR reactions were performed with Thunderbird SYBR qPCR Mix (TOYOBO). The

following primers designed to detect TRPA1 and GAPDH were used:
5'-TGCTGAGATCGACGGGAGTG-3' (forward) and
5'-GGGTATGCCAACTCATTCTGAAC-3' (reverse) for *Trpa1* (product size: 193 bp),
5'-GTCGGTGTGAACGGATTG-3' and 5'-TGGAAGATGGTGATGGGTTT-3' for
Gapdh (218 bp). Samples were incubated for 1 min at 95°C (for initial denaturation)
followed by 40 cycles of denaturation (15 s, 95°C) and annealing (30 s, 60°C) using an
Eco Real-Time PCR System (Illumina, San Diego, CA, USA). Relative mRNA
expression of TRPA1 was calculated by the $\Delta\Delta C_t$ method. The C_t of TRPA1 was
normalized to that of GAPDH as a reference gene and that of TRPA1 in non-treated
cells as a calibration sample.

2.5. Data analyses

Results are expressed as means $\pm$ S.E.M. (n = number of independent measurements).
Statistical comparisons between two groups were made using Student's *t*-test. For
multiple comparisons, ANOVA followed by Dunnett's test was used. A *P*-value of less
than 0.05 was considered significant. The AUC for $[Ca^{2+}]_i$ was calculated with Origin
software (OriginLab, Northampton, MA, USA).

2.6. Materials

Na₂S and diethylamine (DEA) NONOate were purchased from
Sigma-Aldrich/Merck. HC030031 and SB366791 were from Tocris (Bristol, UK).
Diltiazem hydrochloride, allyl isothiocyanate (mustard oil), diazoxide,
N^G-monomethyl-L-arginine (L-NMMA) and ruthenium red were from Wako Pure
Chemical Industries (Osaka, Japan). Na₂S₃ was from Dojindo (Kumamoto, Japan).

Recombinant rat IL-1 β was from Pepro Tech (Rocky Hill, NJ, USA). For the high concentration KCl solution, the 20 mM NaCl in normal solution was replaced with 20 mM KCl. For the Ca-free solution, the CaCl₂ in normal solution was removed and 1 mM EGTA (Dojindo) was added. Na₂S was prepared at time of use. Na₂S was dissolved at 0.1 M in distilled water saturated with N₂, and diluted with normal solution just before the application.

3. Results

3.1. IL-1 β augments H₂S-induced [Ca²⁺]_i increases

The effects of Na₂S, a H₂S donor, on [Ca²⁺]_i in RIN14B cells were examined. RIN14B cells showed spontaneous Ca²⁺ signals under resting conditions (Fig. 1A). Na₂S induced several patterns of [Ca²⁺]_i response. Application of Na₂S (100 μ M for 10 min) either inhibited the spontaneous Ca²⁺ signals (in 23 of 46 cells, 50%) (Inhibition, Fig. 1B) or increased basal [Ca²⁺]_i (in 8 of 46 cells, 17%) (Increase, Fig. 1D). In 10 of 46 cells (22%), Na₂S initially inhibited the spontaneous Ca²⁺ signals but later increased basal [Ca²⁺]_i (Inhibition + Increase, Fig. 1C). IL-1 β treatment (1–10 ng/ml for 24 h) increased the percentage of cells showing [Ca²⁺]_i increase, as shown in Fig. 1E. In response to Na₂S, [Ca²⁺]_i signals were inhibited in 12% or increased in 60% of IL-1 β -treated (3 ng/ml for 24 h) cells (Fig. 1F). The increment in [Ca²⁺]_i responses was also augmented by IL-1 β treatment (Fig. 1H). Although we examined the effect of TNF- α , it caused cytotoxic effect in RIN-14B cells (data not shown).

We also confirmed the expression of H₂S-producing enzymes in RIN-14B cells (Suppl. Fig. 1). Cystathionine β -synthase (CBS), but not cystathionine γ -lyase (CSE),

was expressed in RIN-14B cells. IL-1 β treatment (1–10 ng/ml for 24 h) did not affect the expression of CBS and CSE.

3.2. TRPA1 and L-type Ca²⁺ channels are involved in H₂S-induced Ca²⁺ increases in IL-1 β -treated cells

The contribution of Ca²⁺-permeable channels in IL-1 β -treated cells was next investigated (Fig. 2). We previously reported that H₂S activates TRPA1 and thus increases [Ca²⁺]_i in RIN14B cells (Ujike et al., 2015). Na₂S (100 μ M)-induced [Ca²⁺]_i increases were abolished by HC030031 (30 μ M), a selective TRPA1 antagonist, in both non-treated and IL-1 β -treated cells. By contrast, diltiazem (50 μ M), a voltage-dependent L-type Ca²⁺ channel blocker, did not affect Na₂S-induced [Ca²⁺]_i increases in non-treated cells. However, the blocker significantly, but partly, inhibited Na₂S-induced [Ca²⁺]_i increases in IL-1 β -treated cells. These results indicate that TRPA1 is essential for [Ca²⁺]_i responses to H₂S in both normal and inflammatory conditions. In addition, L-type Ca²⁺ channels may participate in the response to H₂S under the IL- β -treated condition because the facilitation of TRPA1 activity produces depolarization sufficient to activate Ca²⁺ channels.

Using mustard oil (10 μ M), a selective TRPA1 agonist, and a high concentration of KCl (20 mM), to activate voltage-dependent Ca²⁺ channels, we examined whether IL-1 β treatment directly sensitizes the activity of TRPA1 and L-type Ca²⁺ channels. IL-1 β treatment did not affect mustard oil- and KCl-induced [Ca²⁺]_i increases (Fig. 3A-D). Proinflammatory cytokines upregulate TRPA1 expression in synoviocytes (Hatano et al., 2012). However, there were no significant differences in TRPA1 expression between non-treated and IL-1 β -treated RIN14B cells (Fig. 3E).

H₂S inhibits spontaneous Ca²⁺ signals through K_{ATP} channels (Ujike et al., 2015). Attenuation of K_{ATP} channel activity could contribute to the excitatory effect of H₂S. Thus, the effect of IL-1β treatment on K_{ATP} channel activity was examined. Diazoxide (100 μM), a K_{ATP} channel opener, significantly inhibited spontaneous Ca²⁺ signals in non-treated and IL-1β-treated cells to the same extent (Fig. 3F, G), suggesting that IL-1β did not affect K_{ATP} channel activity.

These results indicate that IL-1β did not affect the activity of TRPA1, K_{ATP}, and L-type Ca²⁺ channels, and TRPA1 expression is not involved in the increased Ca²⁺ response to H₂S in IL-1β-treated cells.

3.3. IL-1β increases polysulfide production from H₂S

Polysulfides are substances that have a varying number of sulfane sulfurs. They can be generated from H₂S and act as signal molecules (Nagy and Winterbourn, 2010; Toohey, 2011; Greiner et al., 2013). Furthermore, they are more potent activators of TRPA1 than H₂S (Kimura et al., 2013; Hatakeyama et al., 2015). To investigate the involvement of polysulfides in the effects of Na₂S, changes in intracellular polysulfide levels were measured using SSP4, a fluorescence probe sensitive to sulfane sulfur (Fig. 4). Na₂S (100 μM) application increased intracellular polysulfide levels, indicating that H₂S was intracellularly converted to polysulfides. The fluorescence intensity of SSP4 rapidly increased within approximately 2 min after Na₂S application and was maintained up to 10 min (Fig. 4A). IL-1β (3 ng/ml, 24 h) significantly augmented the Na₂S-induced increase in intracellular polysulfide levels (Fig. 4B). These results suggest that IL-1β facilitates polysulfide production from H₂S.

The chemical reaction of H₂S and NO results in production of polysulfides

(Cortese-Krott et al., 2015), and IL-1 β increases NO production (Corbett et al., 1992; Larsen et al., 1998; Cardozo et al., 2001). We therefore examined whether NO is involved in the effects of IL-1 β . In the presence of L-NMMA (100 μ M), a non-selective NO synthase (NOS) inhibitor, IL-1 β failed to enhance the Na₂S-induced increase in polysulfide levels (Fig. 4A, B). Na₂S-induced [Ca²⁺]_i increases were also not enhanced by IL-1 β in the presence of L-NMMA (Fig. 4C). We also evaluated the inducible NO (iNOS) expression, but IL-1 β did not affect the protein level of iNOS in RIN-14B cells (Supple. Fig. 2).

Polysulfides increase [Ca²⁺]_i through TRPA1 in astrocytes, sensory neurons, and RIN14B cells (Kimura et al., 2013; Hatakeyama et al., 2015). We also examined the effects of exogenous polysulfides on RIN14B cells using Na₂S₃, and found that, like Na₂S, Na₂S₃ showed both inhibitory and excitatory effects on Ca²⁺ signals (Fig. 5A). Na₂S₃ (1 nM) induced three patterns of [Ca²⁺]_i responses, “Inhibition”, “Inhibition + Increase” and “Increase” in 31%, 8% and 46% of cells, respectively (Fig. 5B). Higher concentrations of Na₂S₃ decreased the percentage of cells showing the inhibitory effect of Na₂S₃ and increased the percentage of cells showing the excitatory effect. Na₂S₃ increased [Ca²⁺]_i in a concentration-dependent manner, with higher potency than Na₂S (Fig. 5C). Na₂S₃ (1 μ M)-induced [Ca²⁺]_i increases were inhibited by the Ca-free solution, ruthenium red (10 μ M), a non-selective TRP antagonist, and HC030031 (30 μ M), but not by SB366791 (10 μ M), a selective TRPV1 antagonist, and diltiazem (3 μ M) (Fig. 5D, E), suggesting that polysulfides increase [Ca²⁺]_i through TRPA1 similar to H₂S. Next, the effects of IL-1 β on the actions of exogenous polysulfide were examined. IL-1 β (3 ng/ml, 24 h) failed to increase the percentage of cells showing the excitatory effect of Na₂S₃ or the increment in [Ca²⁺]_i responses to Na₂S₃ (Fig. 5F, G).

Finally, we examined whether NO application augmented the effects of H₂S (Fig. 6). Simultaneous application of DEA NONOate (30 μM), a NO donor, markedly enhanced the Na₂S-induced (3 μM) increase in [Ca²⁺]_i (Fig. 6A). In the presence of DEA NONOate, lower concentrations of Na₂S (0.1–3 μM) increased [Ca²⁺]_i in a concentration-dependent manner (Fig. 6C). Ca-free solution and a TRPA1 antagonist inhibited the [Ca²⁺]_i increases by Na₂S and DEA NONOate (Fig. 6E). The effects of polysulfide Na₂S₃ (1–100 nM) on [Ca²⁺]_i were not increased but rather inhibited by DEA NONOate (Fig. 6B, D). A single application of Na₂S (3 μM) or DEA NONOate (30 μM) did not increase intracellular polysulfide levels, while simultaneous application of Na₂S and DEA NONOate greatly increased intracellular polysulfide levels to the same extent as Na₂S₃ (1 μM) application (Fig. 6F, G). These results indicate that the combination of H₂S and NO induces polysulfide production and increases [Ca²⁺]_i through TRPA1.

4. Discussion

This study demonstrates that IL-1β augments H₂S-induced [Ca²⁺]_i increases that are mediated through TRPA1 and voltage-dependent L-type Ca²⁺ channels. This effect of IL-1β may be due to increased polysulfide production by interactions between H₂S and NO.

H₂S showed inhibitory and excitatory effects on [Ca²⁺]_i in RIN14B cells, and IL-1β decreased the percentage of cells showing inhibition of spontaneous Ca²⁺ signals by H₂S and enhanced H₂S-induced [Ca²⁺]_i increases. Although the particular feature of the individual cells that is correlated with these different responses to H₂S is unclear, our

1 previous study showed that H₂S inhibits and increases Ca²⁺ signals through K_{ATP} and
2 TRPA1 channels, respectively (Ujike et al., 2015). In this study, the enhanced [Ca²⁺]_i
3 responses to H₂S in IL-1β-treated cells were inhibited by TRPA1 and L-type Ca²⁺
4 channel blockers. IL-1β activates a variety of signaling pathways, such as NF-κB and
5 p38/MAPK, through the IL-1 receptor, and regulates gene transcription of inflammatory
6 factors such as IL-6 and IL-8 (Weber et al., 2010). NF-κB can induce TRPA1 expression
7 through hypoxia-induced factor-1α in human synoviocytes (Hatano et al., 2012). TRPA1
8 is also upregulated via the p38/MAPK pathway (Obata et al., 2005). However, in this
9 study, IL-1β did not affect TRPA1 expression or [Ca²⁺]_i responses to TRPA1 and K_{ATP}
10 channel agonists and a high K⁺ solution. The enhancement by IL-1β likely occurs
11 through mechanisms other than changes in the activity and expression of these H₂S
12 target channels.

13 Another possibility is the involvement of polysulfides, molecules generated from the
14 oxidation of H₂S (Nagy and Winterbourn, 2010; Toohey, 2011). TRPA1 is activated
15 through covalent modification of cysteine residues in the intracellular N-terminal
16 domain by electrophiles such as mustard oil (Hinman et al., 2006; Macpherson et al.,
17 2007). H₂S is also thought to activate TRPA1 via this mechanism to increase [Ca²⁺]_i
18 (Miyamoto et al., 2011; Ogawa et al., 2012; Ujike et al., 2015). Polysulfides may
19 mediate H₂S-induced cysteine modification of target proteins (Toohey, 2011; Greiner et
20 al., 2013). Furthermore, polysulfides have a greater potency against TRPA1 than H₂S
21 and increase [Ca²⁺]_i in rat astrocytes, mouse sensory neurons, and RIN14B cells
22 (Kimura et al., 2013, Hatakeyama et al., 2015). The present study showed that
23 application of H₂S increased intracellular polysulfide levels, which was enhanced by
24 IL-1β. Although application of polysulfide itself also increased [Ca²⁺]_i via TRPA1,

1 IL-1 β did not enhance the [Ca²⁺]_i increases. These results suggest that IL-1 β promotes
2 the production of polysulfide from H₂S and enhances the activation of TRPA1.

3 Like H₂S, polysulfides also showed an inhibitory effect on Ca²⁺ signals, indicating
4 their ability to activate K_{ATP} channels. It has been reported that cysteine modification in
5 the extracellular N-terminal of SUR1 mediates H₂S-induced K_{ATP} channel opening
6 (Jiang et al., 2010). Polysulfides are thought to open K_{ATP} channels through cysteine
7 modification. Higher concentrations of Na₂S₃ showed a marked excitatory effect. The
8 efficacy of polysulfides may be higher against TRPA1 than against K_{ATP} channels.
9 Higher concentrations of polysulfides effectively activate TRPA1 and increase [Ca²⁺]_i,
10 which might mask the inhibitory effect of polysulfides through K_{ATP} channels.

11 Our results showed that L-type Ca²⁺ channels were also involved in H₂S-induced
12 [Ca²⁺]_i increases in IL-1 β -treated cells, but not in non-treated cells. It is likely that the
13 increased TRPA1 activation by H₂S in IL-1 β -treated cells induced depolarization
14 sufficient to activate voltage-dependent L-type Ca²⁺ channels. By contrast, in
15 non-treated cells, H₂S opened K_{ATP} channels, so that the hyperpolarizing cell membrane
16 may have prevented activation of L-type Ca²⁺ channels. Although diltiazem did not
17 inhibit Na₂S₃-induced [Ca²⁺]_i increases, the effect of diltiazem at the same concentration
18 and time of application should be examined. Since H₂S are reported to inhibit L-type
19 Ca²⁺ channels (Sun et al., 2008; Tang et al., 2013), there is a possibility that high
20 concentrations of exogenous polysulfides inhibit L-type Ca²⁺ channels.

21 L-NMMA, a NOS inhibitor, inhibited polysulfide production and [Ca²⁺]_i increases
22 by H₂S in IL-1 β -treated cells. Furthermore, NO application augmented the effects of
23 H₂S on intracellular polysulfide levels and [Ca²⁺]_i increases. The chemical reaction
24 between NO and H₂S can produce polysulfides (Cortese-Krott et al., 2015; Miyamoto et

al., 2017). Taken together, it is likely that the increased NO by IL-1 β treatment reacts with H₂S and produces polysulfides, resulting in [Ca²⁺]_i increases through TRPA1. IL-1 β induces NOS expression and increases endogenous NO as an inflammatory factor (Corbett et al., 1992; Larsen et al., 1998; Cardozo et al., 2001). However, in this study, IL-1 β failed to increase iNOS expression in RIN-14B cells (Supple. Fig. 2). IL-1 β increases NO production by iNOS-independent mechanisms such as the enhancement of L-arginine uptake (Simmoun et al., 1996; Shimomura et al., 20017). Further research is needed to reveal the effects of IL-1 β on NO production in RIN-14B cells. On the other hand, NO decreased the effect of Na₂S₃. The oxidation increases the number of sulfurs of polysulfides until the number reaches 8, at which point it cyclizes and separates as colloidal elemental sulfur S₈ (Toohey, 2011). It is thus likely that the further oxidation of polysulfide produces the insoluble S₈, resulting in a reduction in its potency.

In addition to polysulfides, other chemicals, such as HNO, may, at least partly, contribute to the augmentation of H₂S-induced [Ca²⁺]_i increases by IL-1 β since it was reported that HNO is also produced by the interaction of H₂S with NO, and activates TRPA1 (Eberhardt et al., 2014; Dux et al., 2016). On the other hand, Miyamoto et al. (2017) recently reported that the mixture of H₂S and NO activates TRPA1 mainly via polysulfides, but not via HNO. Further research is needed to explore this issue.

The present study reveals that promotion of intracellular polysulfide production plays a key role in the effects of H₂S under inflammatory conditions. H₂S production increases in inflammation, and H₂S promotes the resolution of inflammation (Wallace et al., 2009; Flannigan et al., 2011; Wallace et al., 2012). Since polysulfides can mediate the cytoprotective effects of H₂S (Koike et al., 2013; 2016), polysulfides may be involved in the anti-inflammatory effects of H₂S. It is also possible that increased

polysulfide levels are involved in the pathogenesis of inflammation through the modulation of Ca^{2+} signals.

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Conflicts of interest: none.

References

- Abe K., Kimura H., 1996. The possible role of hydrogen sulfide as an endogenous neuromodulator. *J. Neurosci.* 16, 1066-1071.
- Cardozo A.K., Heimberg H., Heremans Y., Leeman R., Kutlu B., Kruhøffer M., Ørntoft T., Eizirik D.L., 2001. A comprehensive analysis of cytokine-induced and nuclear factor- κ B-dependent genes in primary rat pancreatic β -cells. *J. Biol. Chem.* 276, 48879-48886.
- Corbett J.A., Wang J.L., Sweetland M.A., Lancaster J.R. Jr, McDaniel M.L., 1992. Interleukin 1 β induces the formation of nitric oxide by β -cells purified from rodent islets of Langerhans. Evidence for the β -cell as a source and site of action of nitric oxide. *J. Clin. Invest.* 90, 2384-2391.

Cortese-Krott M.M., Kuhnle G.G., Dyson A., Fernandez B.O., Grman M., DuMond J.F.,
 Barrow M.P., McLeod G., Nakagawa H., Ondrias K., Nagy P., King S.B., Saavedra J.E.,
 Keefer L.K., Singer M., Kelm M., Butler A.R., Feelisch M., 2015. Key bioactive
 reaction products of the NO/H₂S interaction are S/N-hybrid species, polysulfides, and
 nitroxyl. *Proc. Natl. Acad. Sci. USA* 112, E4651-E4660.

Delgermurun D., Yamaguchi S., Ichii O., Kon Y., Ito S., Otsuguro K., 2016. Hydrogen
 sulfide activates TRPA1 and releases 5-HT from epithelioid cells of the chicken thoracic
 aorta. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 187, 43-49.

Dux M., Will C., Vogler B., Filipovic M.R., Messlinger K., 2016. Meningeal blood flow
 is controlled by H₂S-NO crosstalk activating a HNO-TRPA1-CGRP signalling pathway.
Br. J. Pharmacol. 173, 431-445.

Eberhardt M., Dux M., Namer B., Miljkovic J., Cordasic N., Will C., Kichko T.I., de la
 Roche J., Fischer M., Suárez S.A., Bikiel D., Dorsch K., Leffler A., Babes A., Lampert
 A., Lennerz J.K., Jacobi J., Martí M.A., Doctorovich F., Högestätt E.D., Zygmunt P.M.,
 Ivanovic-Burmazovic I., Messlinger K., Reeh P., Filipovic M.R., 2014. H₂S and NO
 cooperatively regulate vascular tone by activating a neuroendocrine
 HNO-TRPA1-CGRP signalling pathway. *Nat. Commun.* 5, 4381.

El Karim I., McCrudden M.T., Linden G.J., Abdullah H., Curtis T.M., McGahon M.,
 About I., Irwin C., Lundy F.T., 2015. TNF- α -induced p38MAPK activation regulates
 TRPA1 and TRPV4 activity in odontoblast-like cells. *Am. J. Pathol.* 185, 2994-3002.

1

2 Flannigan K.L., McCoy K.D., Wallace J.L., 2011. Eukaryotic and prokaryotic

3 contributions to colonic hydrogen sulfide synthesis. *Am. J. Physiol. Gastrointest. Liver*

4 *Physiol.* 301, G188-G193.

5

6 Greiner R., Pálincás Z., Bäsell K., Becher D., Antelmann H., Nagy P., Dick T.P., 2013.

7 Polysulfides link H₂S to protein thiol oxidation. *Antioxid. Redox Signal.* 19, 1749-1765.

8

9 Grynkiewicz G., Poenie M., Tsien R.Y., 1985. A new generation of Ca²⁺ indicators with

10 greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440-3450.

11

12 Hatakeyama Y., Takahashi K., Tominaga M., Kimura H., Ohta T., 2015. Polysulfide

13 evokes acute pain through the activation of nociceptive TRPA1 in mouse sensory

14 neurons. *Mol. Pain* 11, 24.

15

16 Hatano N., Itoh Y., Suzuki H., Muraki Y., Hayashi H., Onozaki K., Wood I.C., Beech

17 D.J., Muraki K., 2012. Hypoxia-inducible factor-1 α (HIF1 α) switches on transient

18 receptor potential ankyrin repeat 1 (TRPA1) gene expression via a hypoxia response

19 element-like motif to modulate cytokine release. *J. Biol. Chem.* 287, 31962-31972.

20

21 Hinman A., Chuang H.H., Bautista D.M., Julius D., 2006. TRP channel activation by

22 reversible covalent modification. *Proc. Natl. Acad. Sci. USA* 103, 19564-19568.

23

24 Hosoki R., Matsuki N., Kimura H., 1997. The possible role of hydrogen sulfide as an

1 endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem. Biophys.*
2 *Res. Commun.* 237, 527-531.

3

4 Jiang B., Tang G., Cao K., Wu L., Wang R., 2010. Molecular mechanism for
5 H₂S-induced activation of K_{ATP} channels. *Antioxid. Redox Signal.* 12, 1167-1178.

6

7 Kamoun P., 2004. Endogenous production of hydrogen sulfide in mammals. *Amino*
8 *Acids* 26, 243-254.

9

10 Kaneko Y., Kimura Y., Kimura H., Niki I., 2006. L-cysteine inhibits insulin release from
11 the pancreatic β -cell: possible involvement of metabolic production of hydrogen sulfide,
12 a novel gasotransmitter. *Diabetes* 55, 1391-1397.

13

14 Kimura Y., Mikami Y., Osumi K., Tsugane M., Oka J., Kimura H., 2013. Polysulfides
15 are possible H₂S-derived signaling molecules in rat brain. *FASEB J.* 27, 2451-2457.

16

17 Koike S., Ogasawara Y., Shibuya N., Kimura H., Ishii K., 2013. Polysulfide exerts a
18 protective effect against cytotoxicity caused by t-buthylhydroperoxide through Nrf2
19 signaling in neuroblastoma cells. *FEBS Lett.* 587, 3548-3555.

20

21 Koike S., Kayama T., Yamamoto S., Komine D., Tanaka R., Nishimoto S., Suzuki T.,
22 Kishida A., Ogasawara Y., 2016. Polysulfides protect SH-SY5Y cells from
23 methylglyoxal-induced toxicity by suppressing protein carbonylation: A possible
24 physiological scavenger for carbonyl stress in the brain. *Neurotoxicology* 55, 13-19.

1
 2 Larsen C.M., Wadt K.A., Juhl L.F., Andersen H.U., Karlsen A.E., Su M.S., Seedorf K.,
 3 Shapiro L., Dinarello C.A., Mandrup-Poulsen T., 1998. Interleukin-1 β -induced rat
 4 pancreatic islet nitric oxide synthesis requires both the p38 and extracellular
 5 signal-regulated kinase 1/2 mitogen-activated protein kinases. *J. Biol. Chem.* 273,
 6 15294-15300.
 7
 8 Shimomura T., Nakano T., Goto K., Wakabayashi I., 2017. R59949, a diacylglycerol
 9 kinase inhibitor, inhibits inducible nitric oxide production through decreasing
 10 transplasmalemmal L-arginine uptake in vascular smooth muscle cells. *Naunyn*
 11 *Schmiedeberg's Arch. Pharmacol.* 390, 207-214.
 12
 13 Simmons W.W., Closs E.I., Cunningham J.M., Smith T.W., Kelly R.A., 1996. Cytokines
 14 and insulin induce cationic amino acid transporter (CAT) expression in cardiac
 15 myocytes. Regulation of L-arginine transport and no production by CAT-1, CAT-2A,
 16 and CAT-2B. *J. Biol. Chem.* 271, 11694-11702.
 17
 18 Li L., Bhatia M., Zhu Y.Z., Zhu Y.C., Ramnath R.D., Wang Z.J., Anuar F.B., Whiteman
 19 M., Salto-Tellez M., Moore P.K., 2005. Hydrogen sulfide is a novel mediator of
 20 lipopolysaccharide-induced inflammation in the mouse. *FASEB J.* 19, 1196-1198.
 21
 22 Li L., Bhatia M., Moore P.K., 2006. Hydrogen sulphide - a novel mediator of
 23 inflammation? *Curr. Opin. Pharmacol.* 6, 125-129.
 24

Linden D.R., 2014. Hydrogen sulfide signaling in the gastrointestinal tract. *Antioxid. Redox Signal.* 20, 818-830.

Macpherson L.J., Dubin A.E., Evans M.J., Marr F., Schultz P.G., Cravatt B.F., Patapoutian A., 2007. Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature* 445, 541-545.

Mathias R., von der Weid P.Y. (2013). Involvement of the NO-cGMP-K_{ATP} channel pathway in the mesenteric lymphatic pump dysfunction observed in the guinea pig model of TNBS-induced ileitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 304, G623-G634.

Meng J., Wang J., Steinhoff M., Dolly J.O., 2016. TNF α induces co-trafficking of TRPV1/TRPA1 in VAMP1-containing vesicles to the plasmalemma via Munc18-1/syntaxin1/SNAP-25 mediated fusion. *Sci. Rep.* 6, 21226.

Miyamoto R., Otsuguro K., Ito S., 2011. Time- and concentration-dependent activation of TRPA1 by hydrogen sulfide in rat DRG neurons. *Neurosci. Lett.* 499, 137-142.

Miyamoto R., Otsuguro K., Yamaguchi S., Ito S., 2014. Contribution of cysteine aminotransferase and mercaptopyruvate sulfurtransferase to hydrogen sulfide production in peripheral neurons. *J Neurochem* 130, 29-40.

Miyamoto R., Koike S., Takano Y., Shibuya N., Kimura Y., Hanaoka K., Urano Y.,

Ogasawara Y., Kimura H., 2017. Polysulfides (H_2S_n) produced from the interaction of hydrogen sulfide (H_2S) and nitric oxide (NO) activate TRPA1 channels. *Sci Rep* 7, 45995.

Moustafa A., Habara Y., 2016. Cross talk between polysulfide and nitric oxide in rat peritoneal mast cells. *Am. J. Physiol. Cell Physiol.* 310, C894-C902.

Nagy P., Winterbourn C.C., 2010. Rapid reaction of hydrogen sulfide with the neutrophil oxidant hypochlorous acid to generate polysulfides. *Chem. Res. Toxicol.* 23, 1541-1543.

Ogawa H., Takahashi K., Miura S., Imagawa T., Saito S., Tominaga M., Ohta T., 2012. H_2S functions as a nociceptive messenger through transient receptor potential ankyrin 1 (TRPA1) activation. *Neuroscience* 218, 335-343.

Obata K., Katsura H., Mizushima T., Yamanaka H., Kobayashi K., Dai Y., Fukuoka T., Tominaga A., Tominaga M., Noguchi K., 2005. TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. *J. Clin. Invest.* 115, 2393-2401.

Streng T., Axelsson H.E., Hedlund P., Andersson D.A., Jordt S.E., Bevan S., Andersson K.E., Högestätt E.D., Zygmunt P.M., 2008. Distribution and function of the hydrogen sulfide-sensitive TRPA1 ion channel in rat urinary bladder. *Eur. Urol.* 53, 391-399.

1 Sun Y.G., Cao Y.X., Wang W.W., Ma S.F., Yao T., Zhu Y.C., 2008. Hydrogen sulphide is
 2 an inhibitor of L-type calcium channels and mechanical contraction in rat
 3 cardiomyocytes. *Cardiovasc. Res.* 79, 632-641.
 4
 5 Tang G., Zhang L., Yang G., Wu L., Wang R., 2013. Hydrogen sulfide-induced
 6 inhibition of L-type Ca^{2+} channels and insulin secretion in mouse pancreatic beta cells.
 7 *Diabetologia* 56, 533-541.
 8
 9 Toohey J.I., 2011. Sulfur signaling: is the agent sulfide or sulfane? *Anal. Biochem.* 413,
 10 1-7.
 11
 12 Ujike A., Otsuguro K., Miyamoto R., Yamaguchi S., Ito S., 2015. Bidirectional effects
 13 of hydrogen sulfide via ATP-sensitive K^{+} channels and transient receptor potential A1
 14 channels in RIN14B cells. *Eur. J. Pharmacol.* 764, 463-470.
 15
 16 Wallace J.L., Vong L., McKnight W., Dicay M., Martin G.R., 2009. Endogenous and
 17 exogenous hydrogen sulfide promotes resolution of colitis in rats. *Gastroenterology* 137,
 18 569-578.
 19
 20 Wallace J.L., Ferraz J.G., Muscara M.N., 2012. Hydrogen sulfide: an endogenous
 21 mediator of resolution of inflammation and injury. *Antioxid. Redox Signal.* 17, 58-67.
 22
 23 Weber A., Wasiliew P., Kracht M., 2010. Interleukin-1 (IL-1) pathway. *Sci. Signal* 3,
 24 cm1.

1

2 Yang W., Yang G., Jia X., Wu L., Wang R., 2005. Activation of K_{ATP} channels by H₂S in
3 rat insulin-secreting cells and the underlying mechanisms. J. Physiol. 569, 519-531.

4

5 Zhao W., Zhang J., Lu Y., Wang R., 2001. The vasorelaxant effect of H₂S as a novel
6 endogenous gaseous K_{ATP} channel opener. EMBO J. 20, 6008-6016.

Figure Legends

Fig. 1 Effects of IL-1 β on [Ca²⁺]_i responses to H₂S

(A-E) Representative [Ca²⁺]_i signals with spontaneous Ca²⁺ oscillations under the resting condition (A) and in the presence of Na₂S (100 μ M for 10 min, B-E). (B) Spontaneous Ca²⁺ oscillations were inhibited (Inhibition). (C) Inhibition was followed by increased [Ca²⁺]_i (Inhibition + Increase). (D) [Ca²⁺]_i immediately increased without inhibition (Increase). (E) [Ca²⁺]_i increased in IL-1 β (3 ng/ml, 24 h)-treated cells. (F) The percentage of cells showing each [Ca²⁺]_i response to Na₂S (100 μ M) in IL-1 β (1–10 ng/ml, 24 h, n = 32–57, F). (G) The AUC of [Ca²⁺]_i responses to Na₂S in IL-1 β -treated cells. **P* < 0.05 vs. non-treated cells (Dunnett's test).

Fig. 2 Effects of TRPA1 and L-type Ca²⁺ channel blockers on H₂S-induced [Ca²⁺]_i increases in IL-1 β -treated cells

(A, B) Representative traces of [Ca²⁺]_i responses to Na₂S (100 μ M) in non-treated (A) and IL-1 β (3 ng/ml, 24 h)-treated cells (B) in the presence of HC030031 (30 μ M) or diltiazem (50 μ M), or their absence (Control). (C) The AUC of [Ca²⁺]_i responses to Na₂S in the presence or absence of the blockers (n = 7–21). **P* < 0.05, ***P* < 0.01 vs. Control (Dunnett's test).

Fig. 3 Effects of TRPA1, L-type Ca²⁺, and K_{ATP} channel agonists and TRPA1 expression in IL-1 β -treated cells

(A, B) Representative traces of [Ca²⁺]_i responses to mustard oil (10 μ M for 5 min, A) and summarized data of Δ [Ca²⁺]_i (B, n = 27–31) in non-treated or IL-1 β (3 ng·ml⁻¹, 24

h)-treated cells. (C, D) Representative traces of $[Ca^{2+}]_i$ responses to a high concentration of KCl (20 mM for 1 min, C) and summarized data of $\Delta[Ca^{2+}]_i$ (D, $n = 26-31$) in non-treated or IL-1 β -treated cells. (E) Relative expression of TRPA1 in IL-1 β (1–10 ng/ml, 24 h)-treated cells ($n = 3$). (F, G) Representative traces of $[Ca^{2+}]_i$ responses to diazoxide (100 μ M for 10 min, F) and summarized data of $\Delta[Ca^{2+}]_i$ (G, $n = 12-19$) in non-treated or IL-1 β -treated cells. DMSO (0.1%) was used as a vehicle for diazoxide. $**P < 0.01$ (unpaired Student's *t*-test).

Fig. 4 Intracellular polysulfide in IL-1 β -treated cells

(A) Representative traces of the change in SSP4 fluorescence in response to Na₂S (100 μ M) in non-treated or IL-1 β (3 ng/ml, 24 h)-treated cells. Cells were pre-treated with L-NMMA (100 μ M) for 25 min. SSP4 fluorescence intensity was normalized to the value before Na₂S application. (B) Normalized SSP4 fluorescence at 10 min after Na₂S application in the absence or presence of L-NMMA in non-treated ($n = 157-421$) or IL-1 β -treated cells ($n = 159-403$). (C) The AUC of $[Ca^{2+}]_i$ responses to Na₂S in the absence or presence of L-NMMA in non-treated ($n = 14-23$) or IL-1 β -treated cells ($n = 14-23$). $**P < 0.01$, $*P < 0.05$ (unpaired Student's *t*-test).

Fig. 5 Effects of exogenous polysulfide on $[Ca^{2+}]_i$

(A) Representative traces of $[Ca^{2+}]_i$ responses to Na₂S₃ (1 nM for 10 min). (B) The percentage of cells showing each $[Ca^{2+}]_i$ response to Na₂S₃ (1 nM–3 μ M, $n = 22-53$). (C) Concentration-response relationships for Na₂S₃ (1 nM–3 μ M, $n = 96-124$) and Na₂S (1–300 μ M, $n = 54-84$). (D) Representative traces of $[Ca^{2+}]_i$ responses to Na₂S₃ (1 μ M) in the presence of HC030031 (HC, 30 μ M) or diltiazem (Dilti, 3 μ M). (E) The AUC of

[Ca²⁺]_i responses to Na₂S₃ (1 μM) in the presence of ruthenium red (RR, 10 μM), HC030031 (30 μM), SB366791 (SB, 10 μM) or diltiazem (3 μM), or absence of extracellular Ca²⁺ (Ca-free, containing 1 mM EGTA) (n = 84–113). **P* < 0.05 vs. control (Dunnett's test). (F) The percentage of cells showing [Ca²⁺]_i responses (Increase or Inhibition) to Na₂S₃ (1 nM–100 μM) in non-treated (n = 22–53) or IL-1β (3 ng/ml, 24 h)-treated (n = 28–66) cells. Cells showing both responses (Inhibition + Increase) were included in both categories. (G) The AUC of [Ca²⁺]_i responses to Na₂S₃ (1 nM–100 μM for 5 min) in non-treated (n = 22–53) or IL-1β (3 ng/ml, 24 h, n = 20–40)-treated cells.

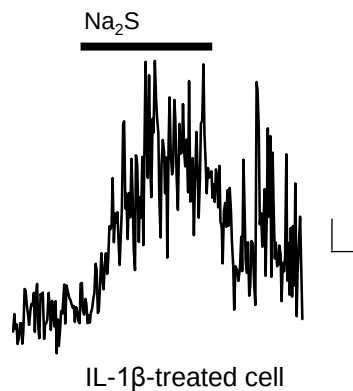
Fig. 6 Effects of simultaneous application of H₂S and NO

(A, B) Representative traces of [Ca²⁺]_i responses to Na₂S (3 μM for 5 min, A) or Na₂S₃ (0.1 μM for 5 min, B) with DEA NONOate (DEA/NO, 30 μM). (C, D) The AUC of [Ca²⁺]_i responses to Na₂S (0.1–3 μM for 5 min, n = 82–105, C) or Na₂S₃ (1–100 nM for 5 min, n = 99–113, D) in the presence or absence of DEA NONOate. ***P* < 0.01 (paired Student's *t*-test). (E) The AUC of [Ca²⁺]_i responses to Na₂S (3 μM) with DEA NONOate (30 μM) in the presence of HC030031 (HC, 30 μM) or diltiazem (Dilti, 3 μM), or absence of extracellular Ca²⁺ (Ca-free, containing 1 mM EGTA) (n = 90–124). **P* < 0.05 vs. Na₂S+DEA/NO (Dunnett's test). (F) Representative traces of the change in SSP4 fluorescence in response to Na₂S (3 μM for 10 min), DEA NONOate (30 μM), combination of Na₂S and DEA NONOate or Na₂S₃ (1 μM). SSP4 fluorescence intensity was normalized to the value before application of stimulants. (G) Normalized SSP4 fluorescence at the maximum by application of Na₂S, DEA NONOate, the combination of Na₂S and DEA NONOate or Na₂S₃ (n = 61–65). **P* < 0.05 vs. Na₂S (Dunnett's test).

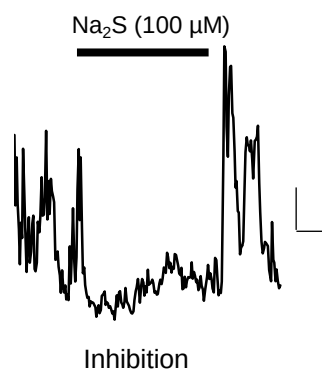
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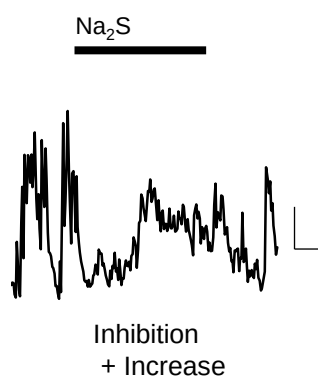
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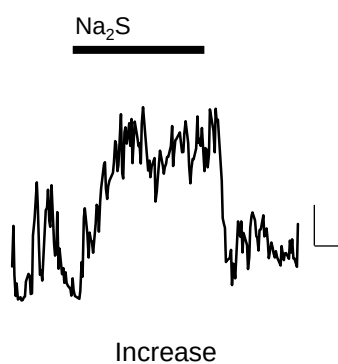
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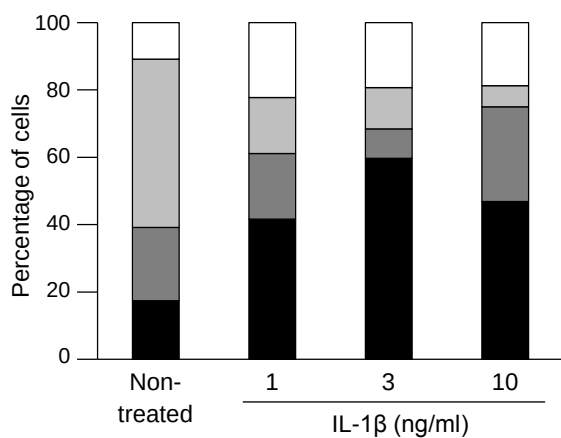
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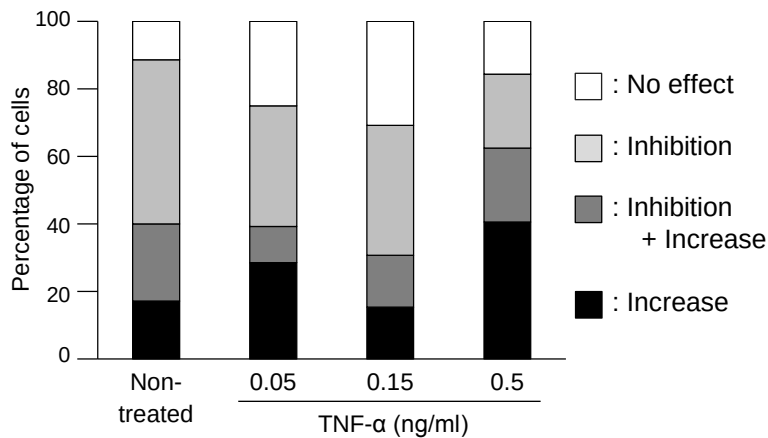
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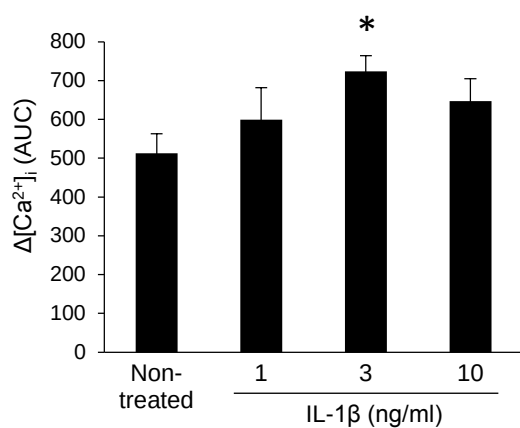
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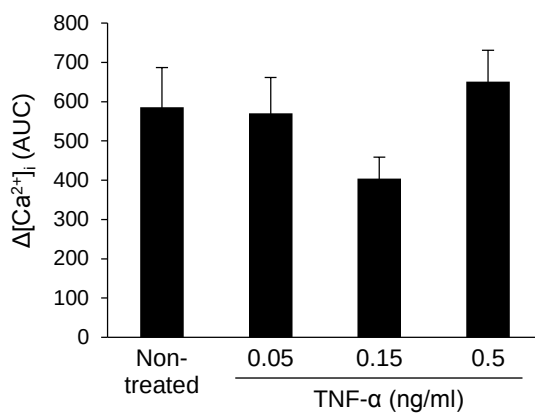
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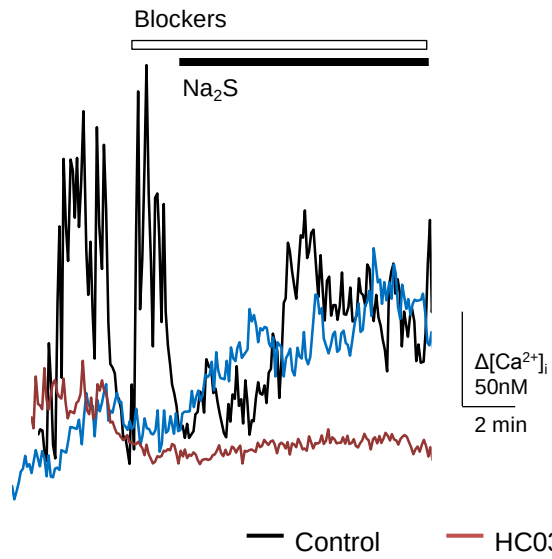
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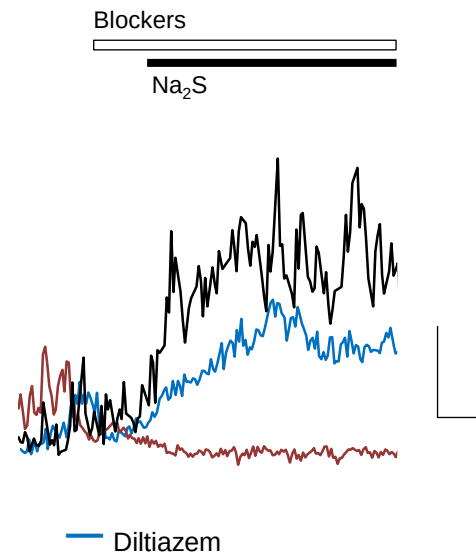
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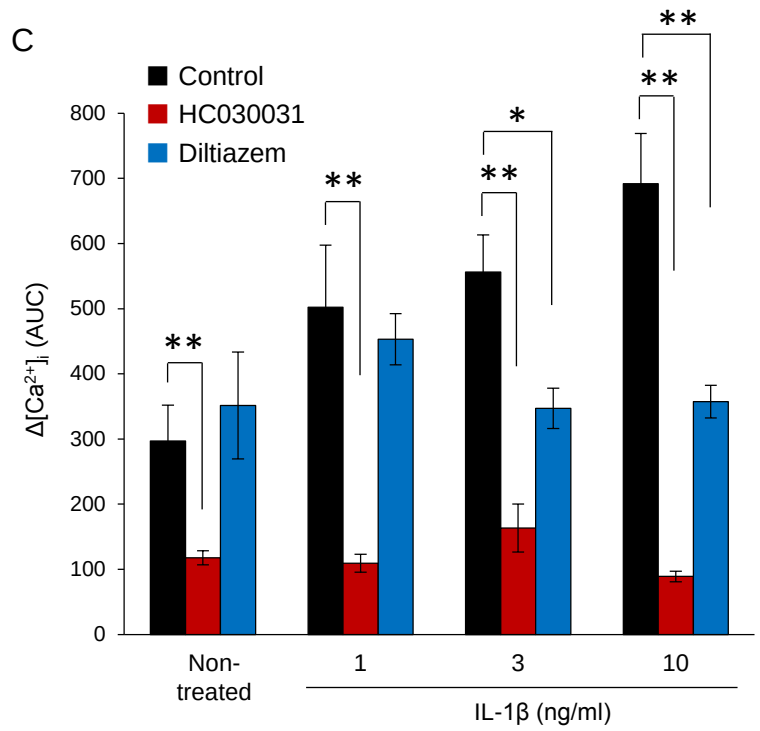
A Non-treated cells

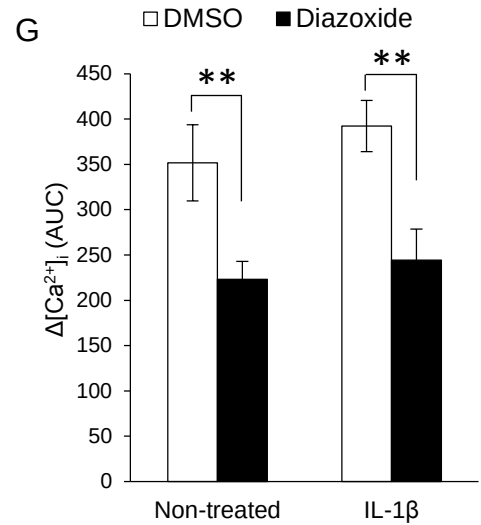
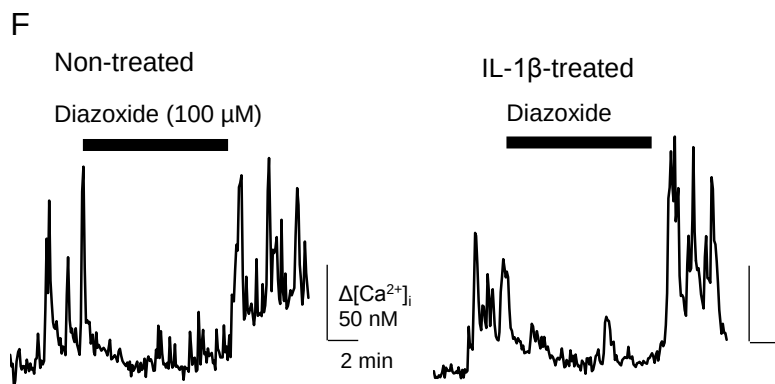
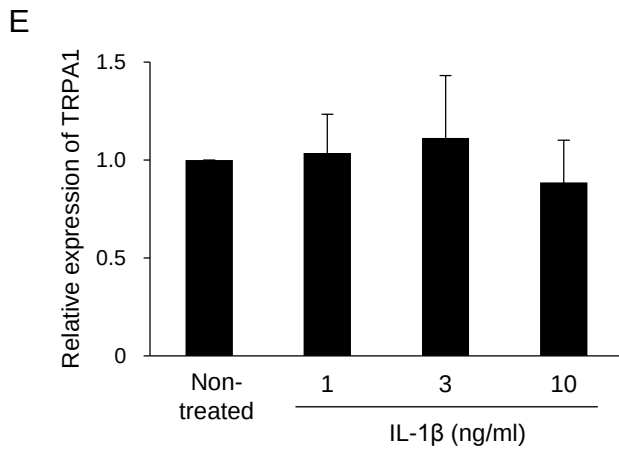
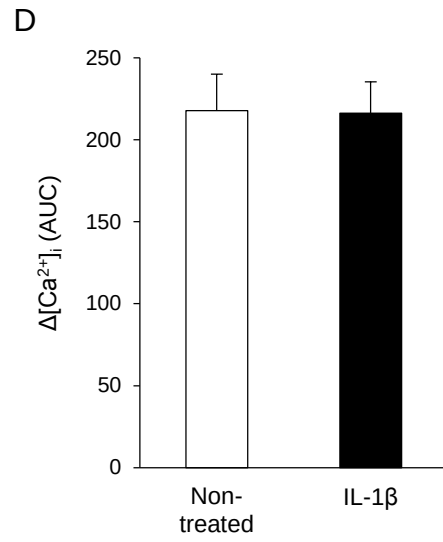
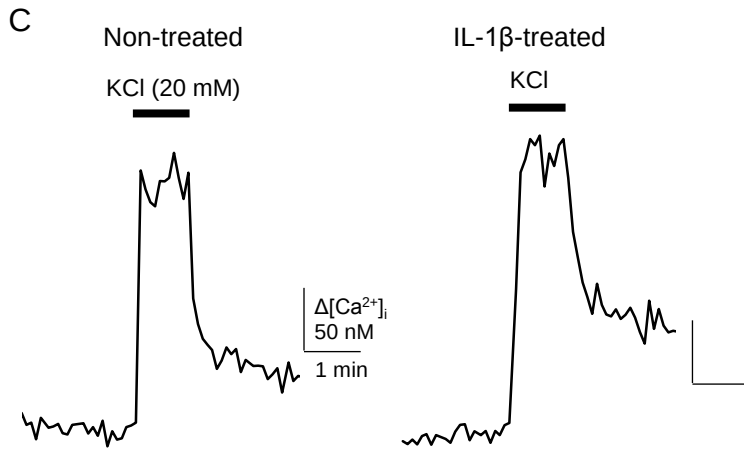
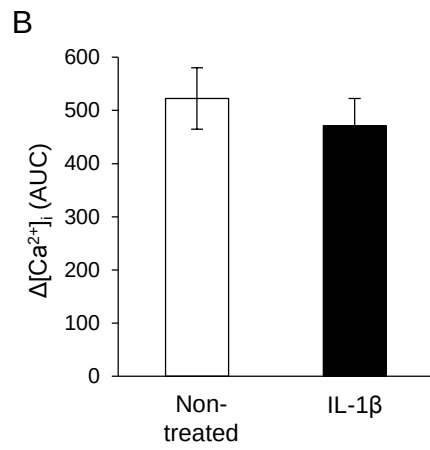
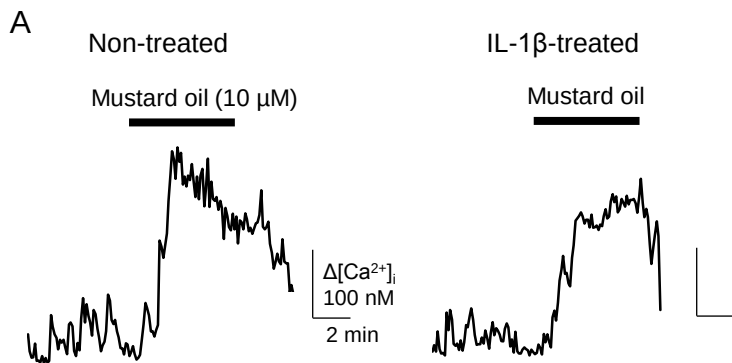


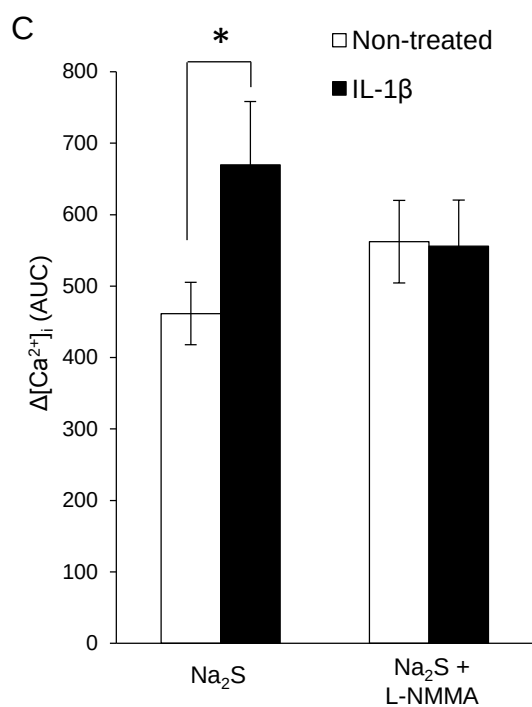
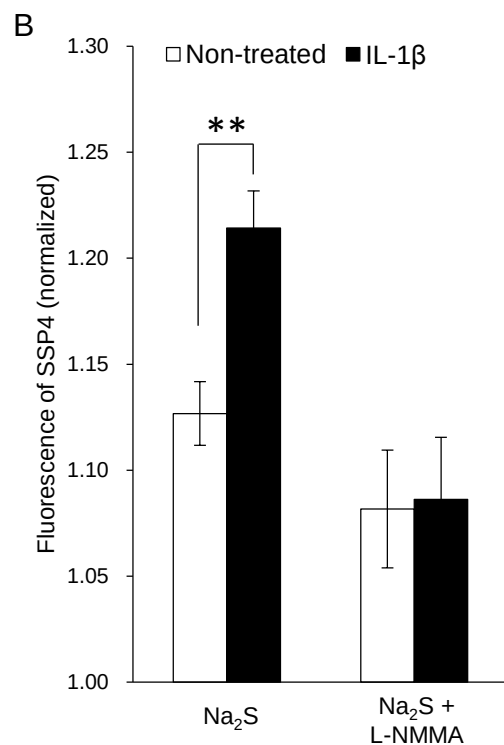
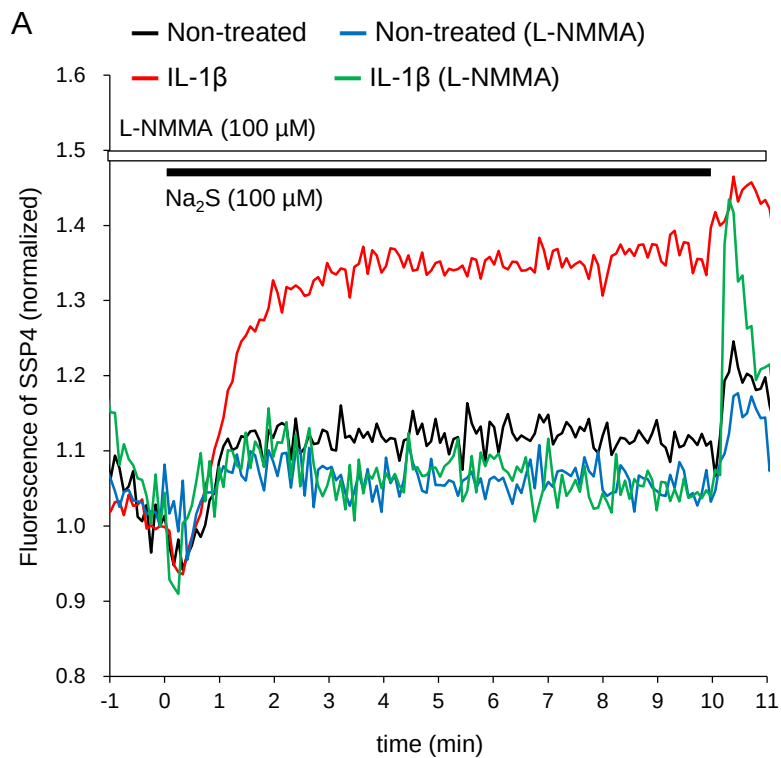
B IL-1 β -treated cells



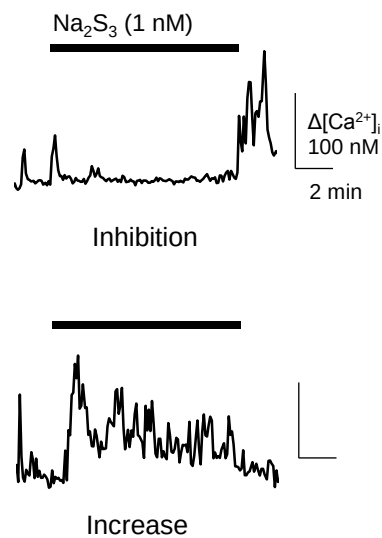
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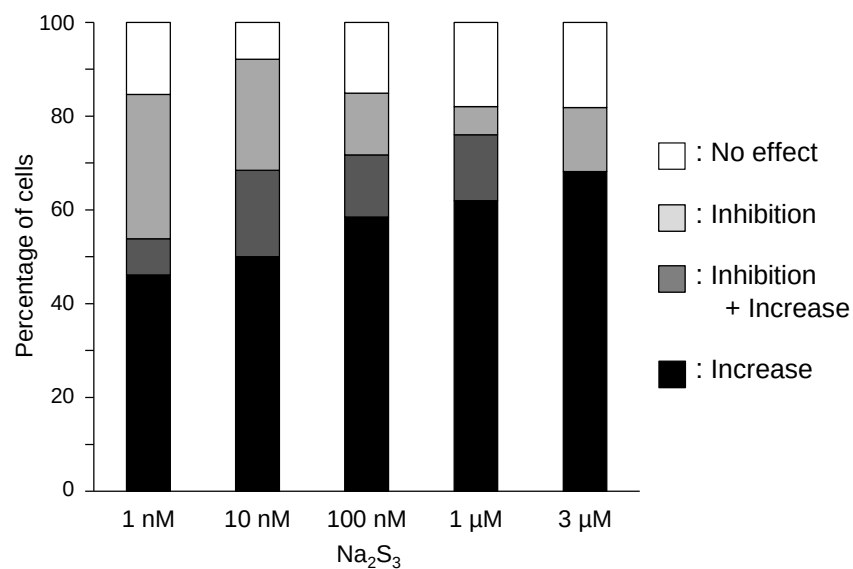




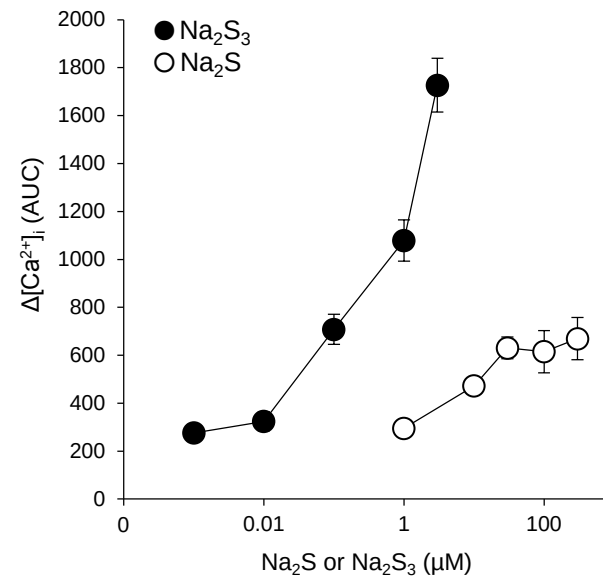
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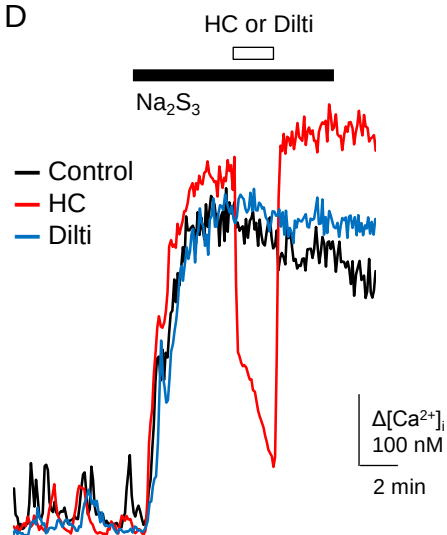
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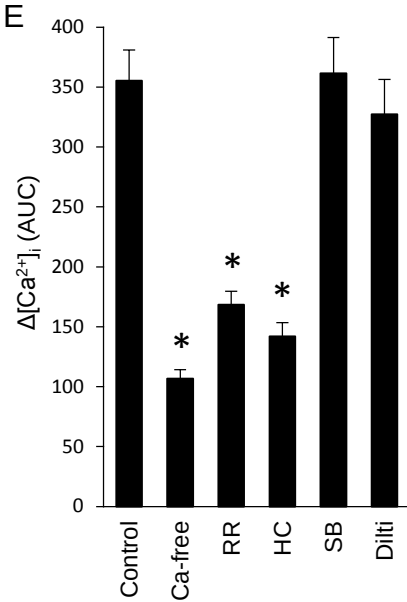
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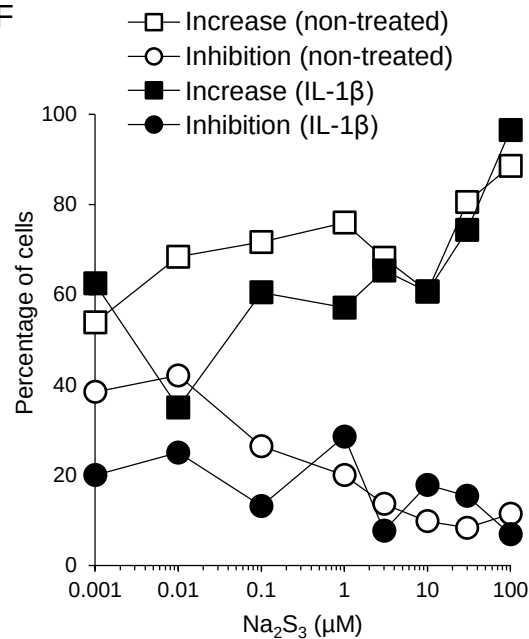
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