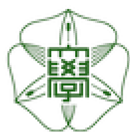
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Title	IL-1 beta augments H2S-induced increase in intracellular Ca2+ through polysulfides generated from H2S/NO interaction
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1 **Title**

2 IL-1β augments H_2S-induced increase in intracellular Ca^{2+} through polysulfides

3 generated from H_2S/NO interaction

4

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15

16

Abstract

H_2S has excitatory and inhibitory effects on Ca^{2+} signals via transient receptor potential ankyrin 1 (TRPA1) and ATP-sensitive K^+ channels, respectively. H_2S converts intracellularly to polysulfides, which are more potent agonists for TRPA1 than H_2S. Under inflammatory conditions, changes in the expression and activity of these H_2S target channels and/or the conversion of H_2S to polysulfides may modulate H_2S effects. Effects of proinflammatory cytokines on H_2S-induced Ca^{2+} signals and polysulfide production in RIN14B cells were examined using fluorescence imaging with fura-2 and SSP4, respectively. Na_2S, a H_2S donor, induced 1) the inhibition of spontaneous Ca^{2+} signals, 2) inhibition followed by $[Ca^{2+}]_i$ increase, and 3) rapid $[Ca^{2+}]_i$ increase without inhibition in 50% (23/46), 22% (10/46), and 17% (8/46) of cells tested, respectively. IL-1β augmented H_2S-induced $[Ca^{2+}]_i$ increases, which were inhibited by TRPA1 and voltage-dependent L-type Ca^{2+} channel blockers. However, IL-1β treatment did not affect $[Ca^{2+}]_i$ increases evoked by a TRPA1 agonist or high concentration of KCl. Na_2S increased intracellular polysulfide levels, which were enhanced by IL-1β treatment. A NOS inhibitor suppressed the increased polysulfide production and $[Ca^{2+}]_i$ increase in IL-1β-treated cells. These results suggest that IL-1β augments H_2S-induced $[Ca^{2+}]_i$ increases via the conversion of H_2S 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 H_2S during inflammation.

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

1 **1. Introduction**

2

3 Hydrogen sulfide (H_2S) is a gasotransmitter that is synthesized from cysteine
4 through several enzymatic pathways in mammals (Kamoun, 2004; Miyamoto et al.,
5 2014). H_2S plays important roles in physiological functions such as the modulation of
6 neuronal activity (Abe and Kimura, 1996), the relaxation of smooth muscle (Hosoki et
7 al., 1997; Zhao et al., 2001), and the secretion of hormone and autacoid (Yang et al.,
8 2005; Kaneko et al., 2006, Delgermurun et al., 2016). We previously reported excitatory
9 and inhibitory effects of H_2S on Ca^{2+} signals, i.e., H_2S induces Ca^{2+} influx through
10 transient receptor potential ankyrin 1 (TRPA1) channels, while inhibiting spontaneous
11 Ca^{2+} oscillations through ATP-sensitive K^+ (K_{ATP}) channels, in RIN14B cells, a cell line
12 derived from rat pancreatic δ cells (Ujike et al., 2015). H_2S also increases the
13 intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through TRPA1 activation in
14 TRPA1-expressing CHO cells, HEK293 cells and sensory neurons (Streng et al., 2008;
15 Miyamoto et al., 2011; Ogawa et al., 2012). Moreover, H_2S has inhibitory effects on
16 insulin release and smooth muscle contraction through K_{ATP} channels (Zhao et al., 2001;
17 Yang et al., 2005). TRPA1 and K_{ATP} channels are thought to be important for
18 bidirectional modulation of cellular functions by H_2S.
19 H_2S also acts as a pathological signaling molecule. The amount of H_2S and
20 expression of H_2S-producing enzymes increases under inflammatory conditions (Li et
21 al., 2005; Wallace et al., 2009; Flannigan et al., 2011). Increased H_2S may be involved
22 in exacerbation or resolution of inflammation (Li et al., 2006; Wallace et al., 2012;
23 Linden, 2014). In addition to H_2S production, TRPA1 channels expression also changes
24 in inflammation. IL-1α 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). TNF-α 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 $[Ca^{2+}]_i$ 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% CO_2 and 95% air.

Cells were placed on coverslips coated with poly-D-lysine (4–5 $\mu g/cm^2$, 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% CO_2 and 95% air for 24–48 h, and designated as IL-1β-treated cells.

2.2. Ca^{2+} 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 KH_2PO_4, 0.8 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 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 (F_{340} and F_{380}) were detected at 510 nm. The ratio of F_{340} to F_{380} (R) was analyzed using Aqua Cosmos software (Hamamatsu Photonics). $[Ca^{2+}]_i$ was calculated using the following equation: $[Ca^{2+}]_i = K_D\beta \times (R-R_{min})/(R_{max}-R)$ (Grynkiewicz et al., 1985). The dissociation constant of fura-2 and Ca^{2+} (K_D), the ratio of F_{380} at saturating Ca^{2+} to F_{380} at zero Ca^{2+} (β), and

1 the minimum (R_{min}) and maximum (R_{max}) of the fluorescence ratio (F_{340}/F_{380}) were
2 calculated using a Calcium Calibration Buffer Kit (Invitrogen/Thermo Fisher Scientific).
3 All experiments were performed at room temperature.
4
5 *2.3. Intracellular polysulfide imaging*
6 Real-time polysulfide imaging in living cells using SSP4 (Dojindo), a sulfane
7 sulfur probe, was performed as per Moustafa and Habara's method (Mustafa and Habara,
8 2016). Cells on coverslips were washed once with serum-free RPMI1640 medium and
9 incubated with SSP4 (50 μM) and 0.003% cremophor EL, to load SSP4 into living cells,
10 in serum-free RPMI1640 medium for 20 min at 37°C. After washing 2–3 times with
11 serum-free medium, SSP4 fluorescence was measured using an inverted microscope
12 (Diaphot 300, Nikon) with a fluorescence ratio imaging system, a laser source (C6979,
13 Hamamatsu Photonics), and a CCD camera (ORCA-ER, Hamamatsu Photonics). Cells
14 were continuously superfused with normal solution and illuminated at 482 nm for 183
15 ms at 5 s intervals at room temperature. Fluorescence signals were detected at 515 nm
16 and analyzed using Aqua Cosmos software. SSP4 fluorescence was normalized to the
17 value before stimulant application.
18
19 *2.4. Real-time PCR*
20 Total RNAs was extracted from RIN14B cells using TRI Reagent (Sigma-Aldrich),
21 according to the manufacturer's instructions, and treated with DNase I
22 (Invitrogen/Thermo Fisher Scientific). RNA samples were reverse-transcribed using
23 ReverTra Ace with Oligo dT primers (TOYOBO, Osaka, Japan). Real-time PCR
24 reactions were performed with Thunderbird SYBR qPCR Mix (TOYOBO). The

1 following primers designed to detect TRPA1 and GAPDH were used:
2 5′-TGCTGAGATCGACGGGAGTG-3′ (forward) and
3 5′-GGGTATGCCAACTCATTCCTGAAC-3′ (reverse) for *Trpa1* (product size: 193 bp),
4 5′-GTCGGTGTGAACGGATTTG-3′ and 5′-TGGAAGATGGTGATGGGTTT-3′ for
5 *Gapdh* (218 bp). Samples were incubated for 1 min at 95°C (for initial denaturation)
6 followed by 40 cycles of denaturation (15 s, 95°C) and annealing (30 s, 60°C) using an
7 Eco Real-Time PCR System (Illumina, San Diego, CA, USA). Relative mRNA
8 expression of TRPA1 was calculated by the ΔΔCt method. The Ct of TRPA1 was
9 normalized to that of GAPDH as a reference gene and that of TRPA1 in non-treated
10 cells as a calibration sample.
11
12 *2.5. Data analyses*
13 Results are expressed as means ± S.E.M. (n = number of independent measurements).
14 Statistical comparisons between two groups were made using Student's *t*-test. For
15 multiple comparisons, ANOVA followed by Dunnett's test was used. A *P*-value of less
16 than 0.05 was considered significant. The AUC for $[Ca^{2+}]_i$ was calculated with Origin
17 software (OriginLab, Northampton, MA, USA).
18
19 *2.6. Materials*
20 Na_2S and diethylamine (DEA) NONOate were purchased from
21 Sigma-Aldrich/Merck. HC030031 and SB366791 were from Tocris (Bristol, UK).
22 Diltiazem hydrochloride, allyl isothiocyanate (mustard oil), diazoxide,
23 N^G-monomethyl-L-arginine (L-NMMA) and ruthenium red were from Wako Pure
24 Chemical Industries (Osaka, Japan). Na_2S_3 was from Dojindo (Kumamoto, Japan).

Recombinant rat IL-1β 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 $CaCl_2$ in normal solution was removed and 1 mM EGTA (Dojindo) was added. Na_2S was prepared at time of use. Na_2S was dissolved at 0.1 M in distilled water saturated with N_2, and diluted with normal solution just before the application.

3. Results

3.1. IL-1β augments H_2S-induced $[Ca^{2+}]_i$ increases

The effects of Na_2S, a H_2S donor, on $[Ca^{2+}]_i$ in RIN14B cells were examined. RIN14B cells showed spontaneous Ca^{2+} signals under resting conditions (Fig. 1A). Na_2S induced several patterns of $[Ca^{2+}]_i$ response. Application of Na_2S (100 μM for 10 min) either inhibited the spontaneous Ca^{2+} signals (in 23 of 46 cells, 50%) (Inhibition, Fig. 1B) or increased basal $[Ca^{2+}]_i$ (in 8 of 46 cells, 17%) (Increase, Fig. 1D). In 10 of 46 cells (22%), Na_2S initially inhibited the spontaneous Ca^{2+} signals but later increased basal $[Ca^{2+}]_i$ (Inhibition + Increase, Fig. 1C). IL-1β treatment (1–10 ng/ml for 24 h) increased the percentage of cells showing $[Ca^{2+}]_i$ increase, as shown in Fig. 1E. In response to Na_2S, $[Ca^{2+}]_i$ signals were inhibited in 12% or increased in 60% of IL-1β-treated (3 ng/ml for 24 h) cells (Fig. 1F). The increment in $[Ca^{2+}]_i$ responses was also augmented by IL-1β 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 H_2S-producing enzymes in RIN-14B cells (Suppl. Fig. 1). Cystathionine β-synthase (CBS), but not cystathionine γ-lyase (CSE),

1 was expressed in RIN-14B cells. IL-1β treatment (1–10 ng/ml for 24 h) did not affect
2 the expression of CBS and CSE.
3
4 *3.2. TRPA1 and L-type Ca^{2+} channels are involved in H_2S-induced Ca^{2+} increases in*
5 *IL-1β-treated cells*
6 The contribution of Ca^{2+}-permeable channels in IL-1β-treated cells was next
7 investigated (Fig. 2). We previously reported that H_2S activates TRPA1 and thus
8 increases $[Ca^{2+}]_i$ in RIN14B cells (Ujike et al., 2015). Na_2S (100 μM)-induced $[Ca^{2+}]_i$
9 increases were abolished by HC030031 (30 μM), a selective TRPA1 antagonist, in both
10 non-treated and IL-1β-treated cells. By contrast, diltiazem (50 μM), a voltage-dependent
11 L-type Ca^{2+} channel blocker, did not affect Na_2S-induced $[Ca^{2+}]_i$ increases in
12 non-treated cells. However, the blocker significantly, but partly, inhibited Na_2S-induced
13 $[Ca^{2+}]_i$ increases in IL-1β-treated cells. These results indicate that TRPA1 is essential for
14 $[Ca^{2+}]_i$ responses to H_2S in both normal and inflammatory conditions. In addition,
15 L-type Ca^{2+} channels may participate in the response to H_2S under the IL-β-treated
16 condition because the facilitation of TRPA1 activity produces depolarization sufficient
17 to activate Ca^{2+} channels.
18 Using mustard oil (10 μM), a selective TRPA1 agonist, and a high concentration of
19 KCl (20 mM), to activate voltage-dependent Ca^{2+} channels, we examined whether IL-1β
20 treatment directly sensitizes the activity of TRPA1 and L-type Ca^{2+} channels. IL-1β
21 treatment did not affect mustard oil- and KCl-induced $[Ca^{2+}]_i$ increases (Fig. 3A-D).
22 Proinflammatory cytokines upregulate TRPA1 expression in synoviocytes (Hatano et al.,
23 2012). However, there were no significant differences in TRPA1 expression between
24 non-treated and IL-1β-treated RIN14B cells (Fig. 3E).

1 H_2S inhibits spontaneous Ca^{2+} signals through K_{ATP} channels (Ujike et al., 2015).
2 Attenuation of K_{ATP} channel activity could contribute to the excitatory effect of H_2S.
3 Thus, the effect of IL-1β treatment on K_{ATP} channel activity was examined. Diazoxide
4 (100 μM), a K_{ATP} channel opener, significantly inhibited spontaneous Ca^{2+} signals in
5 non-treated and IL-1β-treated cells to the same extent (Fig. 3F, G), suggesting that IL-1β
6 did not affect K_{ATP} channel activity.
7 These results indicate that IL-1β did not affect the activity of TRPA1, K_{ATP}, and
8 L-type Ca^{2+} channels, and TRPA1 expression is not involved in the increased Ca^{2+}
9 response to H_2S in IL-1β-treated cells.
10
11 *3.3. IL-1β increases polysulfide production from H_2S*
12 Polysulfides are substances that have a varying number of sulfane sulfurs. They can
13 be generated from H_2S and act as signal molecules (Nagy and Winterbourn, 2010;
14 Toohey, 2011; Greiner et al., 2013). Furthermore, they are more potent activators of
15 TRPA1 than H_2S (Kimura et al., 2013; Hatakeyama et al., 2015). To investigate the
16 involvement of polysulfides in the effects of Na_2S, changes in intracellular polysulfide
17 levels were measured using SSP4, a fluorescence probe sensitive to sulfane sulfur (Fig.
18 4). Na_2S (100 μM) application increased intracellular polysulfide levels, indicating that
19 H_2S was intracellularly converted to polysulfides. The fluorescence intensity of SSP4
20 rapidly increased within approximately 2 min after Na_2S application and was
21 maintained up to 10 min (Fig. 4A). IL-1β (3 ng/ml, 24 h) significantly augmented the
22 Na_2S-induced increase in intracellular polysulfide levels (Fig. 4B). These results suggest
23 that IL-1β facilitates polysulfide production from H_2S.
24 The chemical reaction of H_2S and NO results in production of polysulfides

1 (Cortese-Krott et al., 2015), and IL-1β increases NO production (Corbett et al., 1992;
2 Larsen et al., 1998; Cardozo et al., 2001). We therefore examined whether NO is
3 involved in the effects of IL-1β. In the presence of L-NMMA (100 μM), a non-selective
4 NO synthase (NOS) inhibitor, IL-1β failed to enhance the Na_2S-induced increase in
5 polysulfide levels (Fig. 4A, B). Na_2S-induced $[Ca^{2+}]_i$ increases were also not enhanced
6 by IL-1β in the presence of L-NMMA (Fig. 4C). We also evaluated the inducible NO
7 (iNOS) expression, but IL-1β did not affect the protein level of iNOS in RIN-14B cells
8 (Supple. Fig. 2).

9 Polysulfides increase $[Ca^{2+}]_i$ through TRPA1 in astrocytes, sensory neurons, and
10 RIN14B cells (Kimura et al., 2013; Hatakeyama et al., 2015). We also examined the
11 effects of exogenous polysulfides on RIN14B cells using Na_2S_3, and found that, like
12 Na_2S, Na_2S_3 showed both inhibitory and excitatory effects on Ca^{2+} signals (Fig. 5A).
13 Na_2S_3 (1 nM) induced three patterns of $[Ca^{2+}]_i$ responses, "Inhibition", "Inhibition +
14 Increase" and "Increase" in 31%, 8% and 46% of cells, respectively (Fig. 5B). Higher
15 concentrations of Na_2S_3 decreased the percentage of cells showing the inhibitory effect
16 of Na_2S_3 and increased the percentage of cells showing the excitatory effect. Na_2S_3
17 increased $[Ca^{2+}]_i$ in a concentration-dependent manner, with higher potency than Na_2S
18 (Fig. 5C). Na_2S_3 (1 μM)-induced $[Ca^{2+}]_i$ increases were inhibited by the Ca-free
19 solution, ruthenium red (10 μM), a non-selective TRP antagonist, and HC030031 (30
20 μM), but not by SB366791 (10 μM), a selective TRPV1 antagonist, and diltiazem (3
21 μM) (Fig. 5D, E), suggesting that polysulfides increase $[Ca^{2+}]_i$ through TRPA1 similar
22 to H_2S. Next, the effects of IL-1β on the actions of exogenous polysulfide were
23 examined. IL-1β (3 ng/ml, 24 h) failed to increase the percentage of cells showing the
24 excitatory effect of Na_2S_3 or the increment in $[Ca^{2+}]_i$ responses to Na_2S_3 (Fig. 5F, G).

Finally, we examined whether NO application augmented the effects of H_2S (Fig. 6). Simultaneous application of DEA NONOate (30 μM), a NO donor, markedly enhanced the Na_2S-induced (3 μM) increase in $[Ca^{2+}]_i$ (Fig. 6A). In the presence of DEA NONOate, lower concentrations of Na_2S (0.1–3 μM) increased $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 6C). Ca-free solution and a TRPA1 antagonist inhibited the $[Ca^{2+}]_i$ increases by Na_2S and DEA NONOate (Fig. 6E). The effects of polysulfide Na_2S_3 (1–100 nM) on $[Ca^{2+}]_i$ were not increased but rather inhibited by DEA NONOate (Fig. 6B, D). A single application of Na_2S (3 μM) or DEA NONOate (30 μM) did not increase intracellular polysulfide levels, while simultaneous application of Na_2S and DEA NONOate greatly increased intracellular polysulfide levels to the same extent as Na_2S_3 (1 μM) application (Fig. 6F, G). These results indicate that the combination of H_2S and NO induces polysulfide production and increases $[Ca^{2+}]_i$ through TRPA1.

4. Discussion

This study demonstrates that IL-1β augments H_2S-induced $[Ca^{2+}]_i$ increases that are mediated through TRPA1 and voltage-dependent L-type Ca^{2+} channels. This effect of IL-1β may be due to increased polysulfide production by interactions between H_2S and NO.

H_2S showed inhibitory and excitatory effects on $[Ca^{2+}]_i$ in RIN14B cells, and IL-1β decreased the percentage of cells showing inhibition of spontaneous Ca^{2+} signals by H_2S and enhanced H_2S-induced $[Ca^{2+}]_i$ increases. Although the particular feature of the individual cells that is correlated with these different responses to H_2S is unclear, our

1 previous study showed that H_2S inhibits and increases Ca^{2+} signals through K_{ATP} and
2 TRPA1 channels, respectively (Ujike et al., 2015). In this study, the enhanced $[Ca^{2+}]_i$
3 responses to H_2S in IL-1β-treated cells were inhibited by TRPA1 and L-type Ca^{2+}
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 $[Ca^{2+}]_i$ 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 H_2S
12 target channels.
13 Another possibility is the involvement of polysulfides, molecules generated from the
14 oxidation of H_2S (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). H_2S is also thought to activate TRPA1 via this mechanism to increase $[Ca^{2+}]_i$
18 (Miyamoto et al., 2011; Ogawa et al., 2012; Ujike et al., 2015). Polysulfides may
19 mediate H_2S-induced cysteine modification of target proteins (Toohey, 2011; Greiner et
20 al., 2013). Furthermore, polysulfides have a greater potency against TRPA1 than H_2S
21 and increase $[Ca^{2+}]_i$ 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 H_2S increased intracellular polysulfide levels, which was enhanced by
24 IL-1β. Although application of polysulfide itself also increased $[Ca^{2+}]_i$ via TRPA1,

1 IL-1β did not enhance the $[Ca^{2+}]_i$ increases. These results suggest that IL-1β promotes
2 the production of polysulfide from H_2S and enhances the activation of TRPA1.
3 Like H_2S, polysulfides also showed an inhibitory effect on Ca^{2+} 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 H_2S-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 Na_2S_3 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 $[Ca^{2+}]_i$,
10 which might mask the inhibitory effect of polysulfides through K_{ATP} channels.
11 Our results showed that L-type Ca^{2+} channels were also involved in H_2S-induced
12 $[Ca^{2+}]_i$ increases in IL-1β-treated cells, but not in non-treated cells. It is likely that the
13 increased TRPA1 activation by H_2S in IL-1β-treated cells induced depolarization
14 sufficient to activate voltage-dependent L-type Ca^{2+} channels. By contrast, in
15 non-treated cells, H_2S opened K_{ATP} channels, so that the hyperpolarizing cell membrane
16 may have prevented activation of L-type Ca^{2+} channels. Although diltiazem did not
17 inhibit Na_2S_3-induced $[Ca^{2+}]_i$ increases, the effect of diltiazem at the same concentration
18 and time of application should be examined. Since H_2S are reported to inhibit L-type
19 Ca^{2+} channels (Sun et al., 2008; Tang et al., 2013), there is a possibility that high
20 concentrations of exogenous polysulfides inhibit L-type Ca^{2+} channels.
21 L-NMMA, a NOS inhibitor, inhibited polysulfide production and $[Ca^{2+}]_i$ increases
22 by H_2S in IL-1β-treated cells. Furthermore, NO application augmented the effects of
23 H_2S on intracellular polysulfide levels and $[Ca^{2+}]_i$ increases. The chemical reaction
24 between NO and H_2S can produce polysulfides (Cortese-Krott et al., 2015; Miyamoto et

1 al., 2017). Taken together, it is likely that the increased NO by IL-1β treatment reacts
2 with H_2S and produces polysulfides, resulting in $[Ca^{2+}]_i$ increases through TRPA1.
3 IL-1β induces NOS expression and increases endogenous NO as an inflammatory factor
4 (Corbett et al., 1992; Larsen et al., 1998; Cardozo et al., 2001). However, in this study,
5 IL-1β failed to increase iNOS expression in RIN-14B cells (Supple. Fig. 2). IL-1β
6 increases NO production by iNOS-independent mechanisms such as the enhancement of
7 L-arginine uptake (Simmoun et al., 1996; Shimomura et al., 20017). Further research is
8 needed to reveal the effects of IL-1β on NO production in RIN-14B cells. On the other
9 hand, NO decreased the effect of Na_2S_3. The oxidation increases the number of sulfurs
10 of polysulfides until the number reaches 8, at which point it cyclizes and separates as
11 colloidal elemental sulfur S_8 (Toohey, 2011). It is thus likely that the further oxidation of
12 polysulfide produces the insoluble S_8, resulting in a reduction in its potency.
13 In addition to polysulfides, other chemicals, such as HNO, may, at least partly,
14 contribute to the augmentation of H_2S-induced $[Ca^{2+}]_i$ increases by IL-1β since it was
15 reported that HNO is also produced by the interaction of H_2S with NO, and activates
16 TRPA1 (Eberhardt et al., 2014; Dux et al., 2016). On the other hand, Miyamoto et al.
17 (2017) recently reported that the mixture of H_2S and NO activates TRPA1 mainly via
18 polysulfides, but not via HNO. Further research is needed to explore this issue.
19 The present study reveals that promotion of intracellular polysulfide production
20 plays a key role in the effects of H_2S under inflammatory conditions. H_2S production
21 increases in inflammation, and H_2S promotes the resolution of inflammation (Wallace et
22 al., 2009; Flannigan et al., 2011; Wallace et al., 2012). Since polysulfides can mediate
23 the cytoprotective effects of H_2S (Koike et al., 2013; 2016), polysulfides may be
24 involved in the anti-inflammatory effects of H_2S. 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.

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1 **Figure Legends**

2

3 Fig. 1 Effects of IL-1β on $[Ca^{2+}]_i$ responses to H_2S

4 (A-E) Representative $[Ca^{2+}]_i$ signals with spontaneous Ca^{2+} oscillations under the

5 resting condition (A) and in the presence of Na_2S (100 μM for 10 min, B-E). (B)

6 Spontaneous Ca^{2+} oscillations were inhibited (Inhibition). (C) Inhibition was followed

7 by increased $[Ca^{2+}]_i$ (Inhibition + Increase). (D) $[Ca^{2+}]_i$ immediately increased without

8 inhibition (Increase). (E) $[Ca^{2+}]_i$ increased in IL-1β (3 ng/ml, 24 h)-treated cells. (F) The

9 percentage of cells showing each $[Ca^{2+}]_i$ response to Na_2S (100 μM) in IL-1β (1–10

10 ng/ml, 24 h, n = 32–57, F). (G) The AUC of $[Ca^{2+}]_i$ responses to Na_2S in IL-1β-treated

11 cells. **P* < 0.05 vs. non-treated cells (Dunnett's test).

12

13 Fig. 2 Effects of TRPA1 and L-type Ca^{2+} channel blockers on H_2S-induced $[Ca^{2+}]_i$

14 increases in IL-1β-treated cells

15 (A, B) Representative traces of $[Ca^{2+}]_i$ responses to Na_2S (100 μM) in non-treated (A)

16 and IL-1β (3 ng/ml, 24 h)-treated cells (B) in the presence of HC030031 (30 μM) or

17 diltiazem (50 μM), or their absence (Control). (C) The AUC of $[Ca^{2+}]_i$ responses to

18 Na_2S in the presence or absence of the blockers (n = 7–21). **P* < 0.05, ***P* < 0.01 vs.

19 Control (Dunnett's test).

20

21 Fig. 3 Effects of TRPA1, L-type Ca^{2+}, and K_{ATP} channel agonists and TRPA1 expression

22 in IL-1β-treated cells

23 (A, B) Representative traces of $[Ca^{2+}]_i$ responses to mustard oil (10 μM for 5 min, A)

24 and summarized data of $\Delta[Ca^{2+}]_i$ (B, n = 27–31) in non-treated or IL-1β (3 $ng \cdot ml^{-1}$, 24

1 h)-treated cells. (C, D) Representative traces of $[Ca^{2+}]_i$ responses to a high concentration

2 of KCl (20 mM for 1 min, C) and summarized data of $\Delta[Ca^{2+}]_i$ (D, n = 26–31) in

3 non-treated or IL-1β-treated cells. (E) Relative expression of TRPA1 in IL-1β (1–10 ng/

4 ml, 24 h)-treated cells (n = 3). (F, G) Representative traces of $[Ca^{2+}]_i$ responses to

5 diazoxide (100 μM for 10 min, F) and summarized data of $\Delta[Ca^{2+}]_i$ (G, n = 12–19) in

6 non-treated or IL-1β-treated cells. DMSO (0.1%) was used as a vehicle for diazoxide.

7 $^{**}P < 0.01$ (unpaired Student's *t*-test).

8

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

10 (A) Representative traces of the change in SSP4 fluorescence in response to Na_2S (100

11 μM) in non-treated or IL-1β (3 ng/ml, 24 h)-treated cells. Cells were pre-treated with

12 L-NMMA (100 μM) for 25 min. SSP4 fluorescence intensity was normalized to the

13 value before Na_2S application. (B) Normalized SSP4 fluorescence at 10 min after Na_2S

14 application in the absence or presence of L-NMMA in non-treated (n = 157–421) or

15 IL-1β-treated cells (n = 159–403). (C) The AUC of $[Ca^{2+}]_i$ responses to Na_2S in the

16 absence or presence of L-NMMA in non-treated (n = 14–23) or IL-1β-treated cells (n =

17 14–23). $^{**}P < 0.01$, $^{*}P < 0.05$ (unpaired Student's *t*-test).

18

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

20 (A) Representative traces of $[Ca^{2+}]_i$ responses to Na_2S_3 (1 nM for 10 min). (B) The

21 percentage of cells showing each $[Ca^{2+}]_i$ response to Na_2S_3 (1 nM–3 μM, n = 22–53).

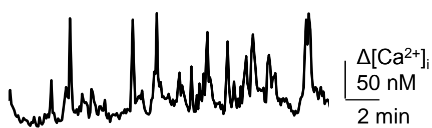
22 (C) Concentration-response relationships for Na_2S_3 (1 nM–3 μM, n = 96–124) and Na_2S

23 (1–300 μM, n = 54–84). (D) Representative traces of $[Ca^{2+}]_i$ responses to Na_2S_3 (1 μM)

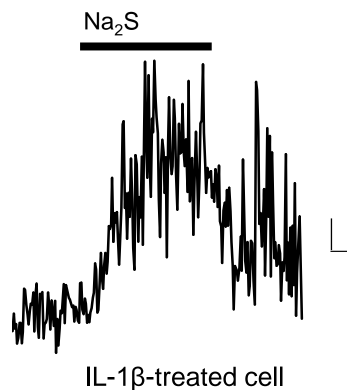
24 in the presence of HC030031 (HC, 30 μM) or diltiazem (Dilti, 3 μM). (E) The AUC of

1 $[Ca^{2+}]_i$ responses to Na_2S_3 (1 μM) in the presence of ruthenium red (RR, 10 μM),
2 HC030031 (30 μM), SB366791 (SB, 10 μM) or diltiazem (3 μM), or absence of
3 extracellular Ca^{2+} (Ca-free, containing 1 mM EGTA) (n = 84–113). *$P < 0.05$ vs.
4 control (Dunnett's test). (F) The percentage of cells showing $[Ca^{2+}]_i$ responses (Increase
5 or Inhibition) to Na_2S_3 (1 nM–100 μM) in non-treated (n = 22–53) or IL-1β (3 ng/ml, 24
6 h)-treated (n = 28–66) cells. Cells showing both responses (Inhibition + Increase) were
7 included in both categories. (G) The AUC of $[Ca^{2+}]_i$ responses to Na_2S_3 (1 nM–100 μM
8 for 5 min) in non-treated (n = 22–53) or IL-1β (3 ng/ml, 24 h, n = 20–40)-treated cells.
9

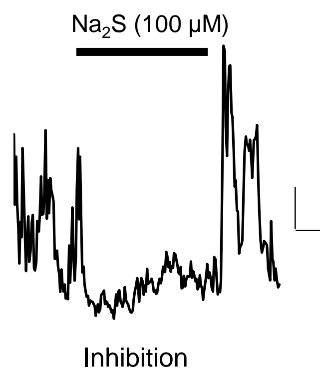
10 Fig. 6 Effects of simultaneous application of H_2S and NO
11 (A, B) Representative traces of $[Ca^{2+}]_i$ responses to Na_2S (3 μM for 5 min, A) or Na_2S_3
12 (0.1 μM for 5 min, B) with DEA NONOate (DEA/NO, 30 μM). (C, D) The AUC of
13 $[Ca^{2+}]_i$ responses to Na_2S (0.1–3 μM for 5 min, n = 82–105, C) or Na_2S_3 (1–100 nM for
14 5 min, n = 99–113, D) in the presence or absence of DEA NONOate. **$P < 0.01$ (paired
15 Student's *t*-test). (E) The AUC of $[Ca^{2+}]_i$ responses to Na_2S (3 μM) with DEA NONOate
16 (30 μM) in the presence of HC030031 (HC, 30 μM) or diltiazem (Dilti, 3 μM), or
17 absence of extracellular Ca^{2+} (Ca-free, containing 1 mM EGTA) (n = 90–124). *$P <$
18 0.05 vs. Na_2S+DEA/NO (Dunnett's test). (F) Representative traces of the change in
19 SSP4 fluorescence in response to Na_2S (3 μM for 10 min), DEA NONOate (30 μM),
20 combination of Na_2S and DEA NONOate or Na_2S_3 (1 μM). SSP4 fluorescence intensity
21 was normalized to the value before application of stimulants. (G) Normalized SSP4
22 fluorescence at the maximum by application of Na_2S, DEA NONOate, the combination
23 of Na_2S and DEA NONOate or Na_2S_3 (n = 61–65). *$P < 0.05$ vs. Na_2S (Dunnett's test).



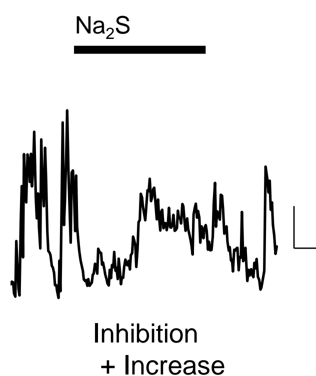
A
Δ[Ca2+]i
50 nM
2 min



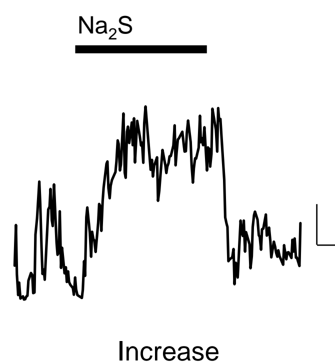
E
Na2S
IL-1β-treated cell



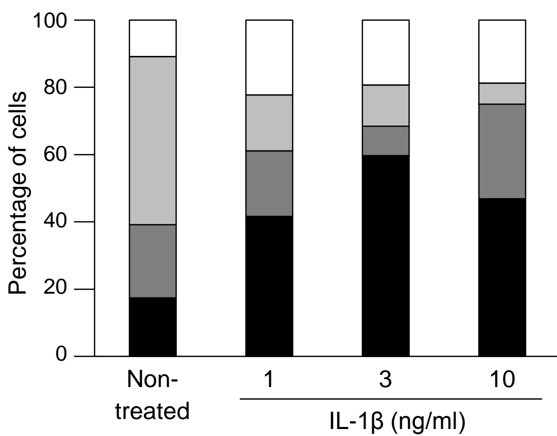
B
Na2S (100 μM)
Inhibition



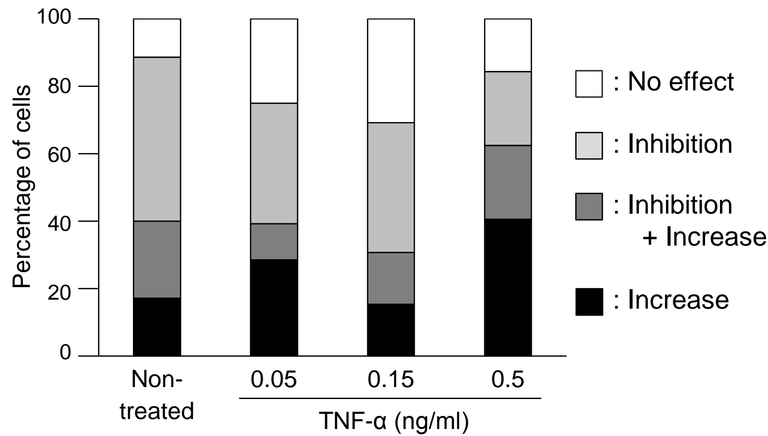
C
Na2S
Inhibition
+ Increase



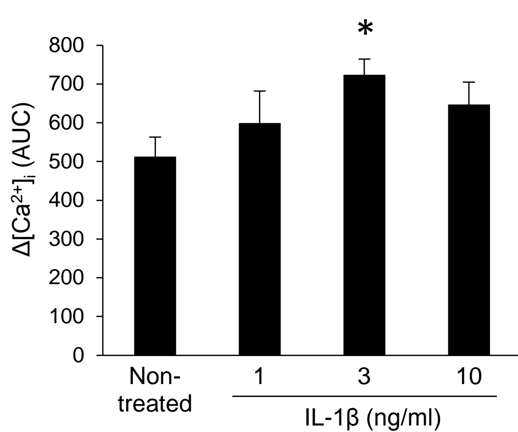
D
Na2S
Increase



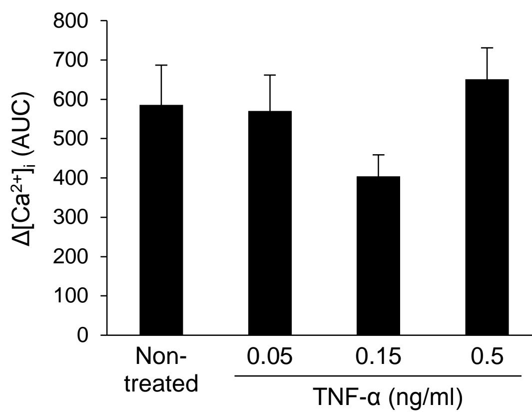
F
Percentage of cells
0
20
40
60
80
100
Non-
treated
1
3
10
IL-1β (ng/ml)



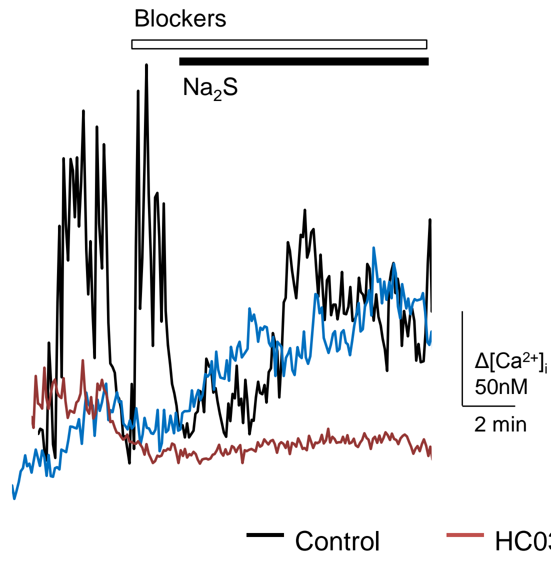
G
Percentage of cells
0
20
40
60
80
100
Non-
treated
0.05
0.15
0.5
TNF-α (ng/ml)
: No effect
: Inhibition
: Inhibition
+ Increase
: Increase



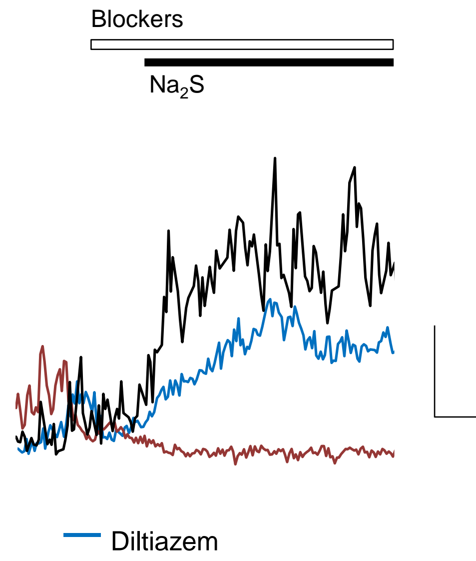
H
Δ[Ca2+]i (AUC)
0
100
200
300
400
500
600
700
800
*
Non-
treated
1
3
10
IL-1β (ng/ml)



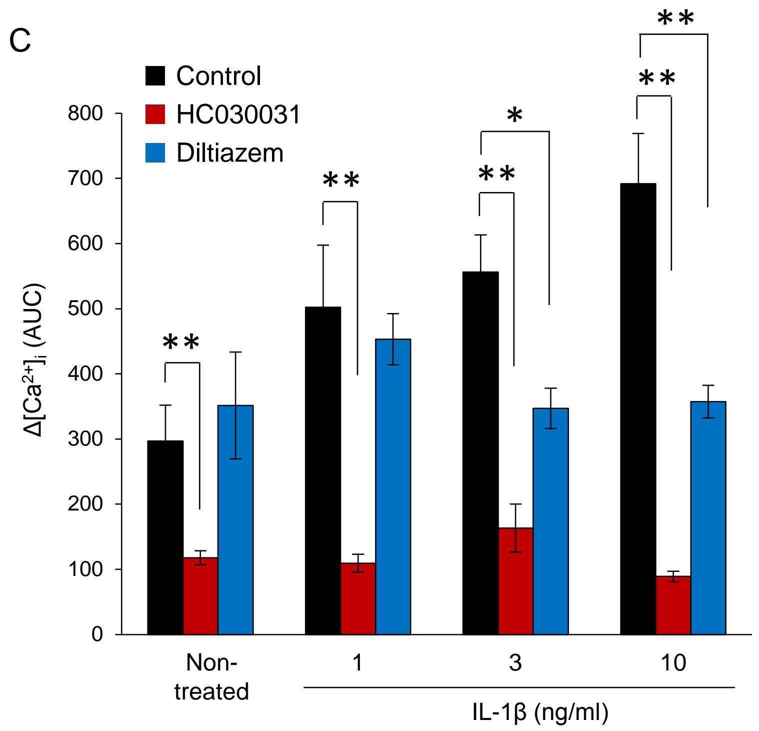
I
Δ[Ca2+]i (AUC)
0
100
200
300
400
500
600
700
800
Non-
treated
0.05
0.15
0.5
TNF-α (ng/ml)



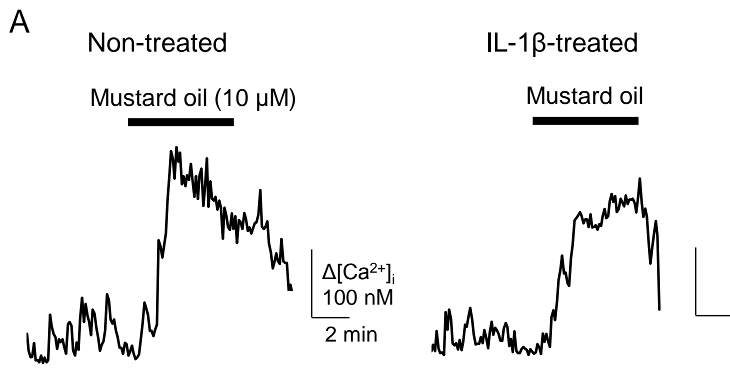
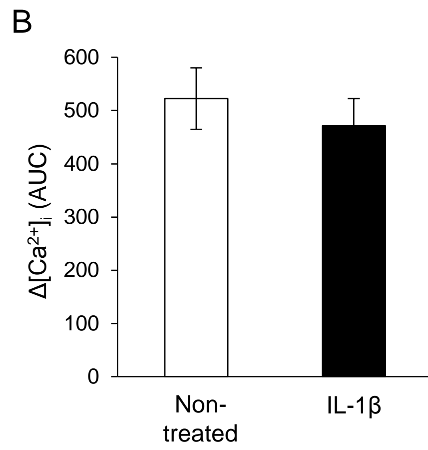
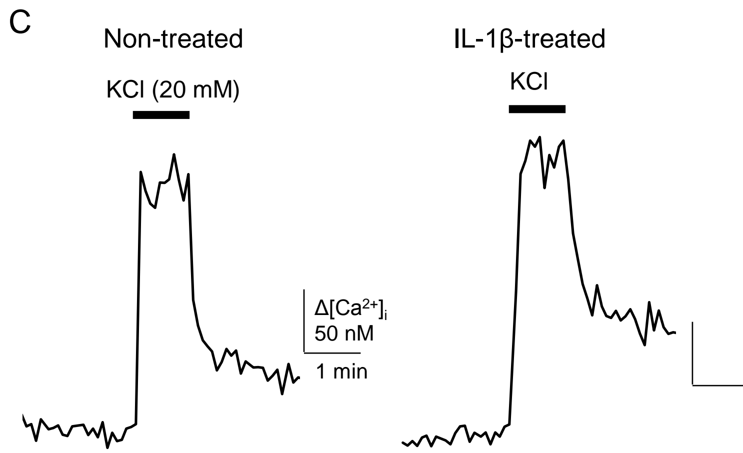
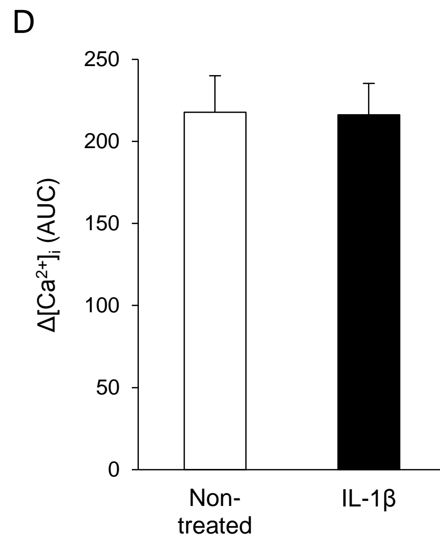
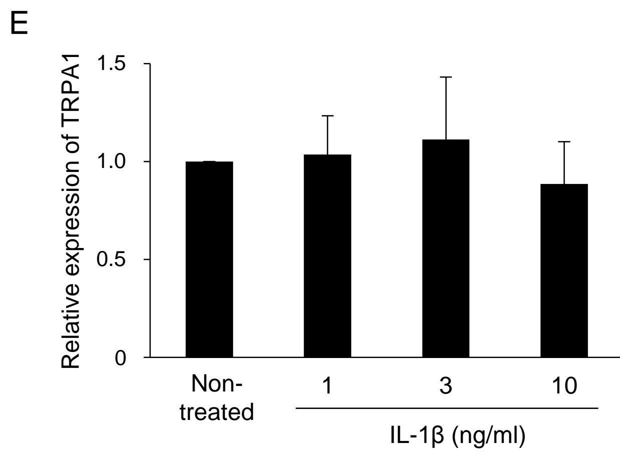
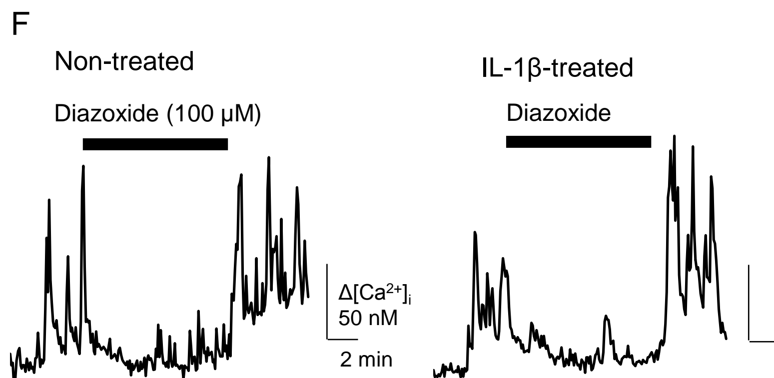
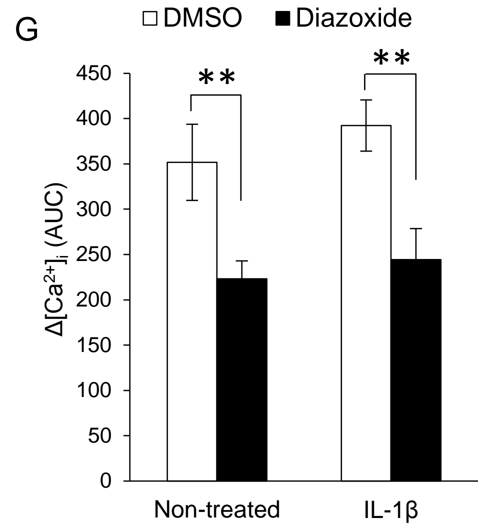
A
Non-treated cells
Blockers
Na_2S
Δ[Ca^{2+}]$_i$
50nM
2 min

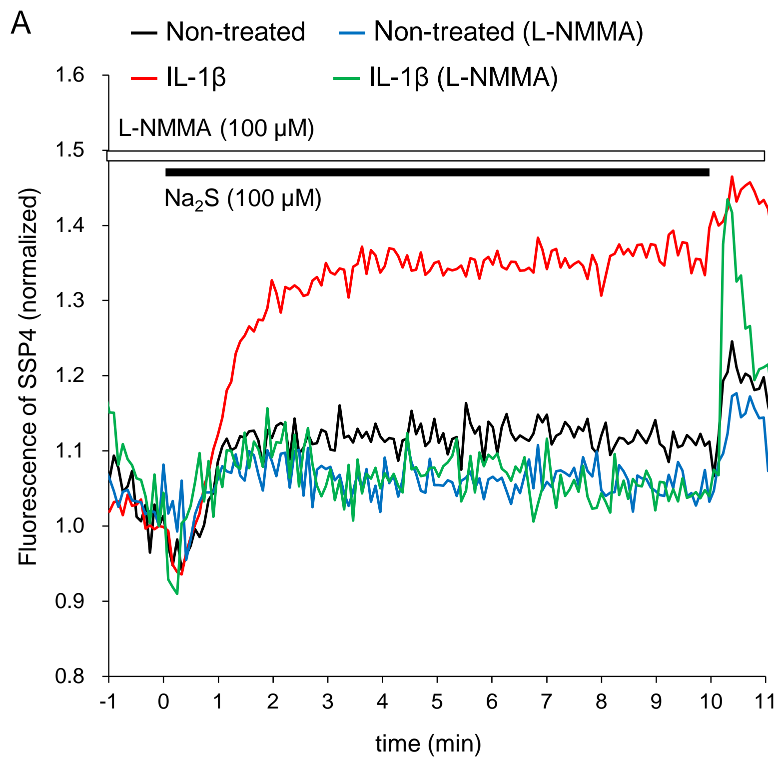


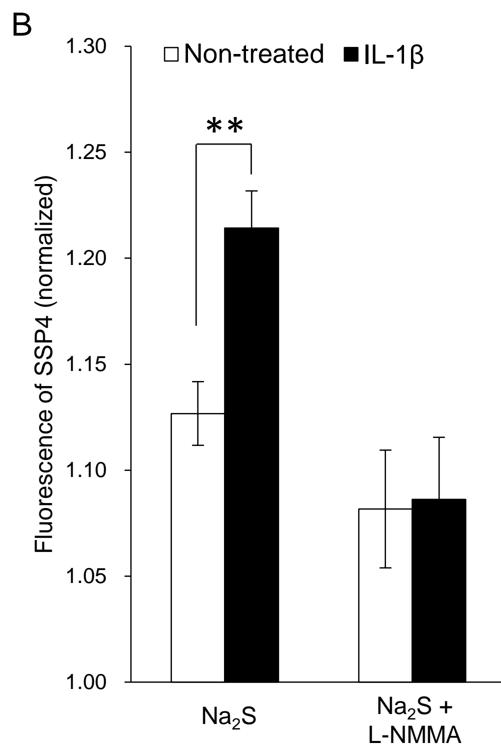
B
IL-1β-treated cells
Blockers
Na_2S
Control
HC030031
Diltiazem

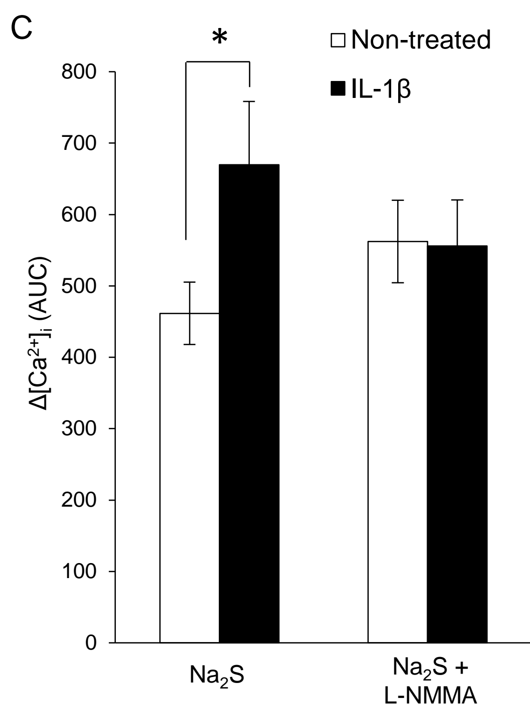


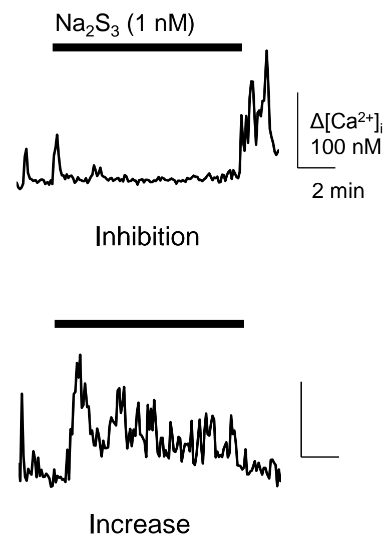
C
Control
HC030031
Diltiazem
Δ[Ca^{2+}]$_i$ (AUC)
800
700
600
500
400
300
200
100
0
Non-treated
1
3
10
IL-1β (ng/ml)
**
**
**
*
**
**

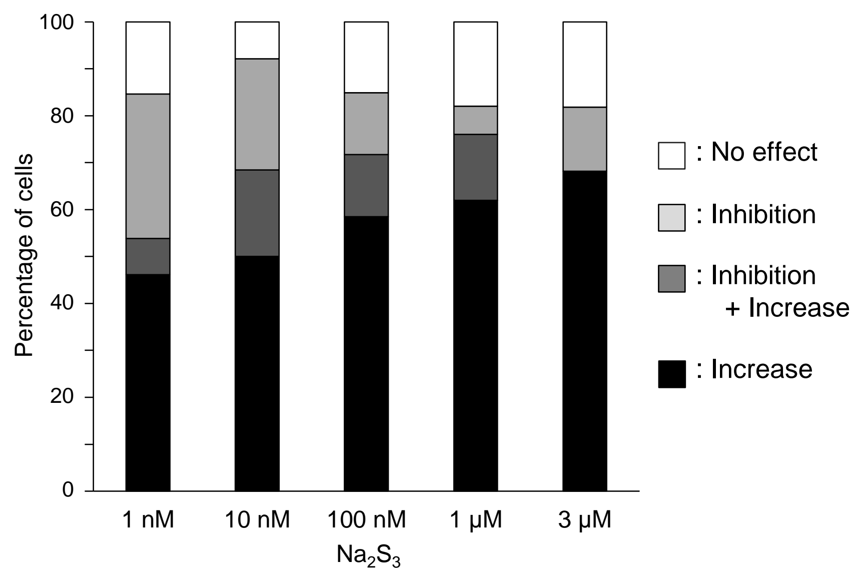

A
Non-treated
IL-1β-treated
Mustard oil (10 μM)
Mustard oil
Δ[Ca2+]i
100 nM
2 min

B
Δ[Ca2+]i (AUC)
600
500
400
300
200
100
0
Non-treated
IL-1β

C
Non-treated
IL-1β-treated
KCl (20 mM)
KCl
Δ[Ca2+]i
50 nM
1 min

D
Δ[Ca2+]i (AUC)
250
200
150
100
50
0
Non-treated
IL-1β

E
Relative expression of TRPA1
1.5
1.0
0.5
0
Non-treated
1
3
10
IL-1β (ng/ml)

F
Non-treated
IL-1β-treated
Diazoxide (100 μM)
Diazoxide
Δ[Ca2+]i
50 nM
2 min

G
DMSO
Diazoxide
Δ[Ca2+]i (AUC)
450
400
350
300
250
200
150
100
50
0
**
**
Non-treated
IL-1β

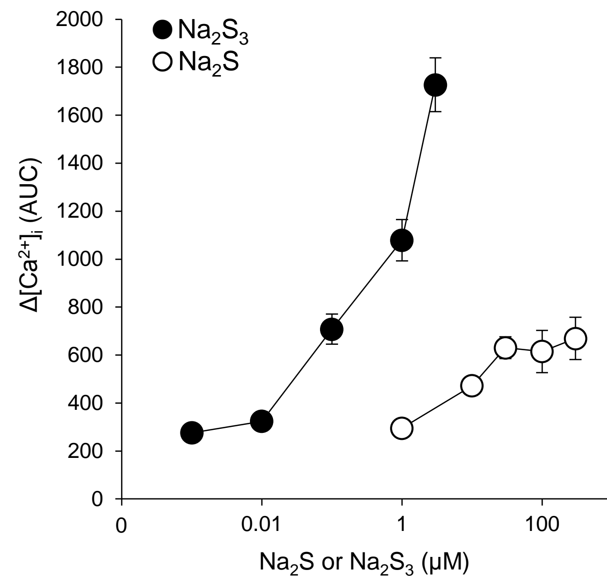

A
Non-treated
Non-treated (L-NMMA)
IL-1β
IL-1β (L-NMMA)
L-NMMA (100 μM)
Na2S (100 μM)
Fluorescence of SSP4 (normalized)
time (min)
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
-1
0
1
2
3
4
5
6
7
8
9
10
11

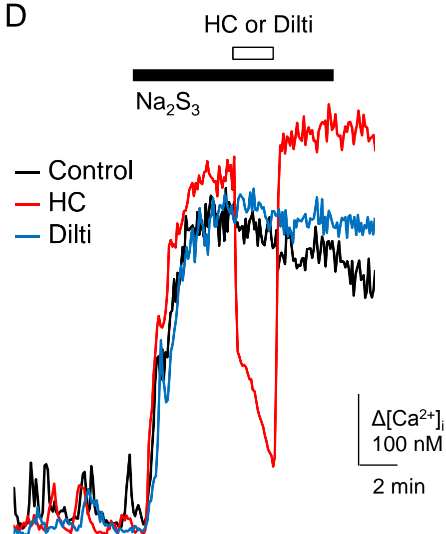

B
Non-treated
IL-1β
**
Fluorescence of SSP4 (normalized)
1.30
1.25
1.20
1.15
1.10
1.05
1.00
Na2S
Na2S +
L-NMMA

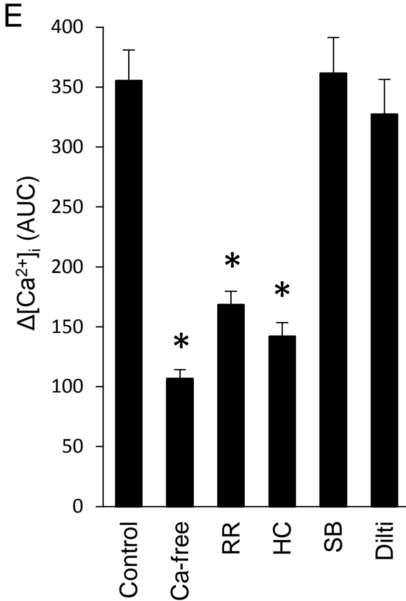

C
*
Non-treated
IL-1β
Δ[Ca2+]i (AUC)
800
700
600
500
400
300
200
100
0
Na2S
Na2S +
L-NMMA

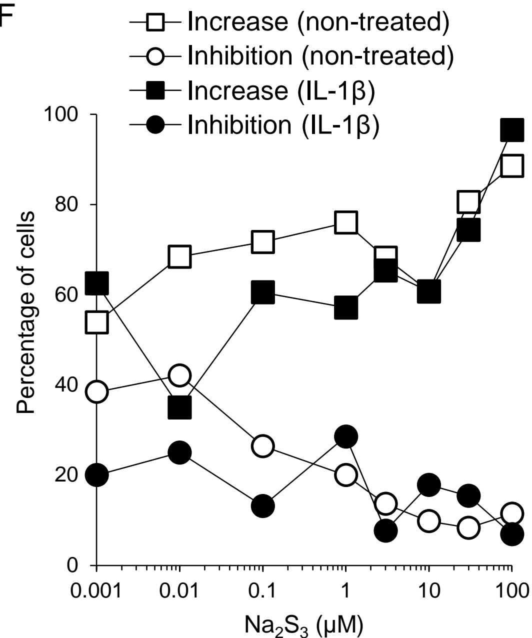

A
Na2S3 (1 nM)
Δ[Ca2+]i
100 nM
2 min
Inhibition
Increase

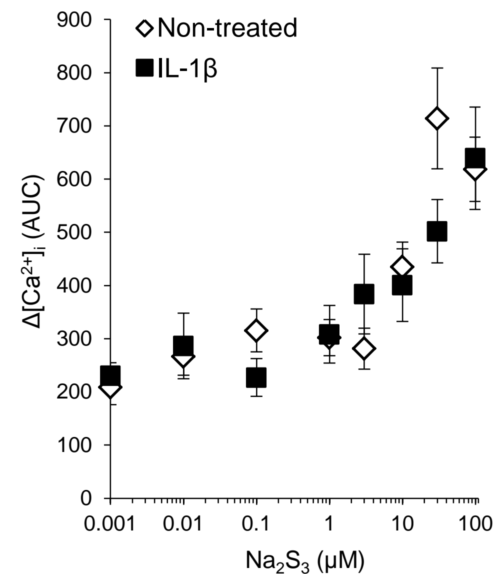

B
Percentage of cells
100
80
60
40
20
0
1 nM
10 nM
100 nM
1 μM
3 μM
Na2S3
: No effect
: Inhibition
: Inhibition
+ Increase
: Increase

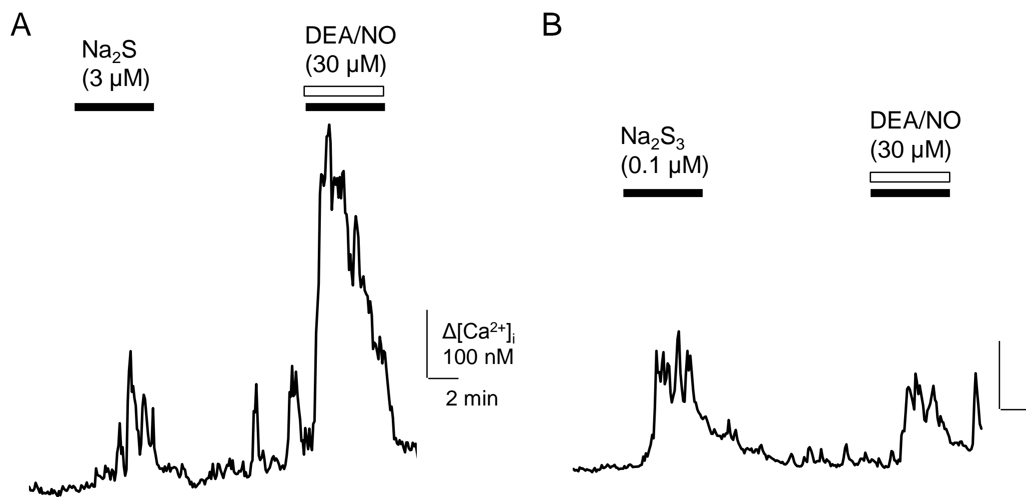

C
Na2S3
Na2S
Δ[Ca2+]i (AUC)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
0.01
1
100
Na2S or Na2S3 (μM)

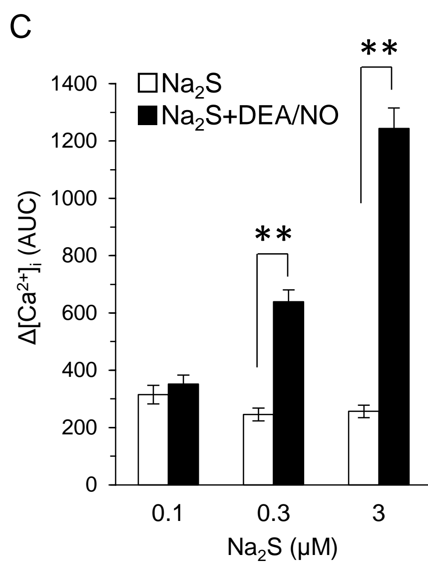

D
HC or Dilti
Na2S3
Control
HC
Dilti
Δ[Ca2+]i
100 nM
2 min

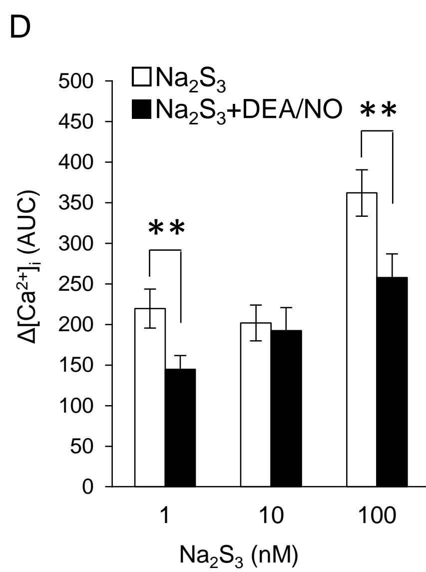

E
Δ[Ca2+]i (AUC)
400
350
300
250
200
150
100
50
0
Control
Ca-free
RR
HC
SB
Dilti

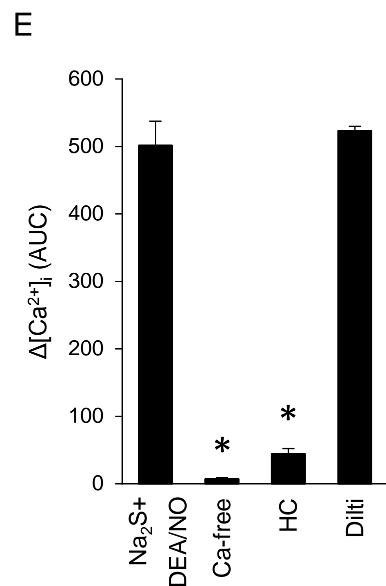

F
Increase (non-treated)
Inhibition (non-treated)
Increase (IL-1β)
Inhibition (IL-1β)
Percentage of cells
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
Na2S3 (μM)

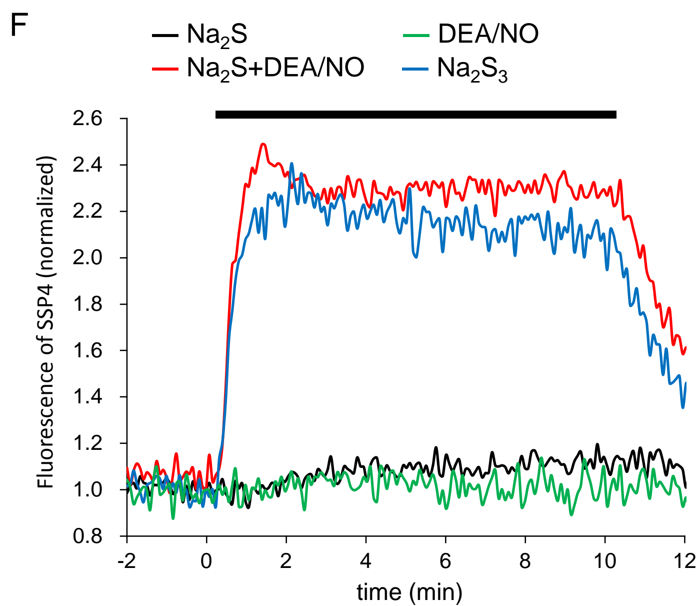

G
Non-treated
IL-1β
Δ[Ca2+]i (AUC)
900
800
700
600
500
400
300
200
100
0
0.001
0.01
0.1
1
10
100
Na2S3 (μM)

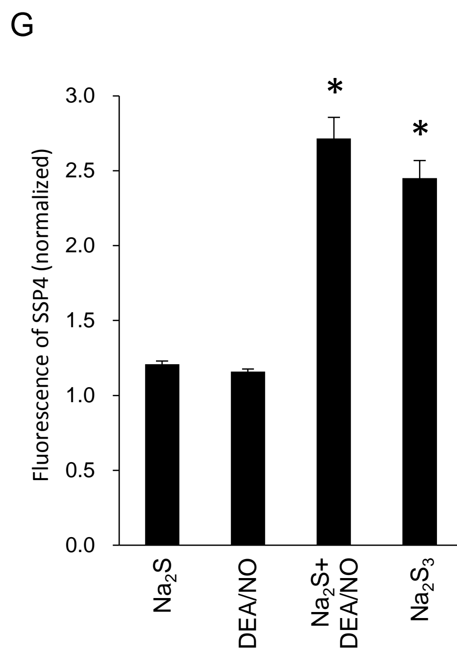

A
Na_2S
(3 μM)
DEA/NO
(30 μM)
Δ$[Ca^{2+}]_i$
100 nM
2 min
B
Na_2S_3
(0.1 μM)
DEA/NO
(30 μM)


C
Na_2S
Na_2S+DEA/NO
Δ$[Ca^{2+}]_i$ (AUC)
0
200
400
600
800
1000
1200
1400
**
**
0.1
0.3
3
Na_2S (μM)


D
Na_2S_3
Na_2S_3+DEA/NO
Δ$[Ca^{2+}]_i$ (AUC)
0
50
100
150
200
250
300
350
400
450
500
**
**
1
10
100
Na_2S_3 (nM)


E
Δ$[Ca^{2+}]_i$ (AUC)
0
100
200
300
400
500
600
*
*
Na_2S+
DEA/NO
Ca-free
HC
Dilti


F
Na_2S
Na_2S+DEA/NO
DEA/NO
Na_2S_3
Fluorescence of SSP4 (normalized)
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
-2
0
2
4
6
8
10
12
time (min)


G
Fluorescence of SSP4 (normalized)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
*
*
Na_2S
DEA/NO
Na_2S+
DEA/NO
Na_2S_3