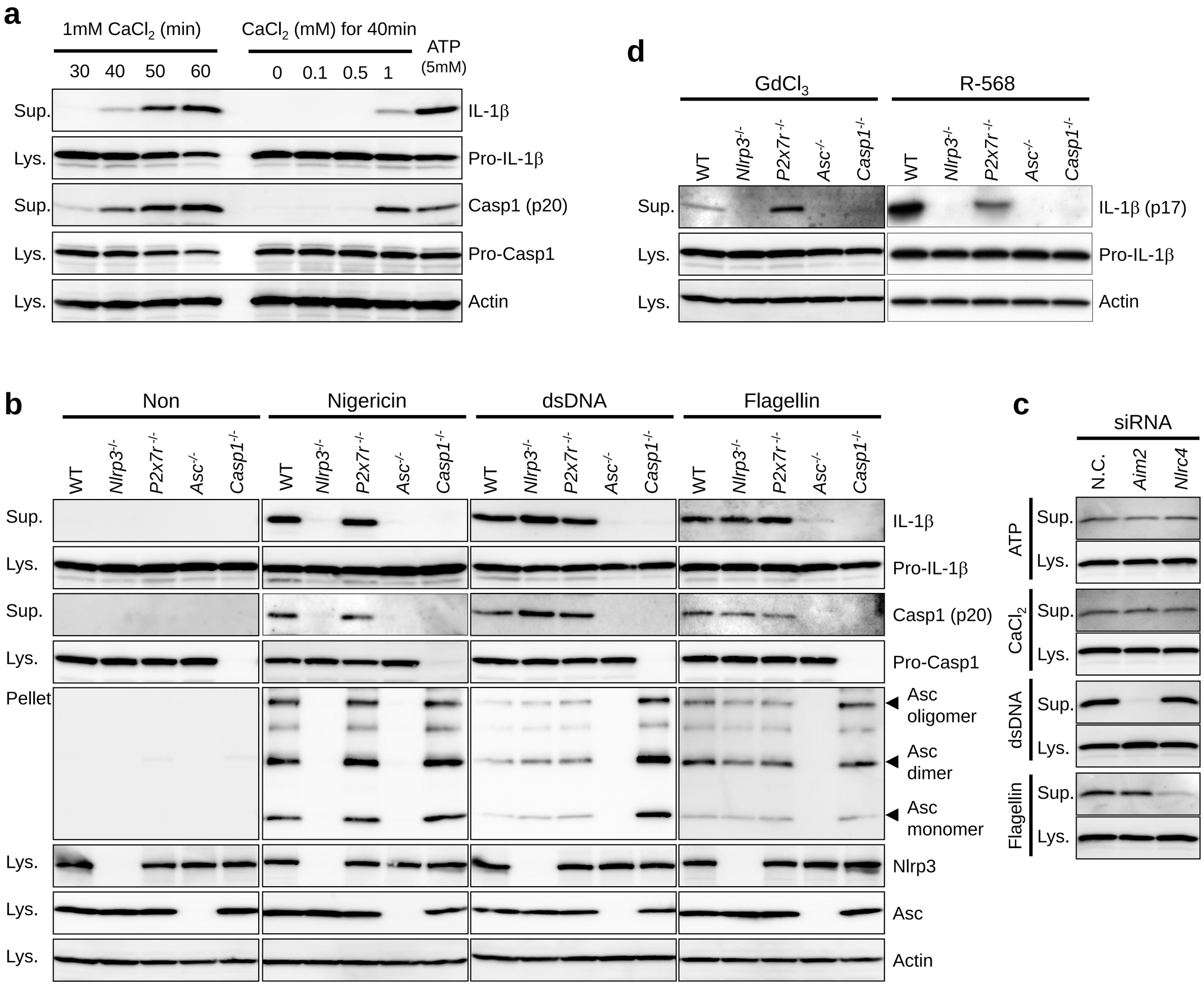
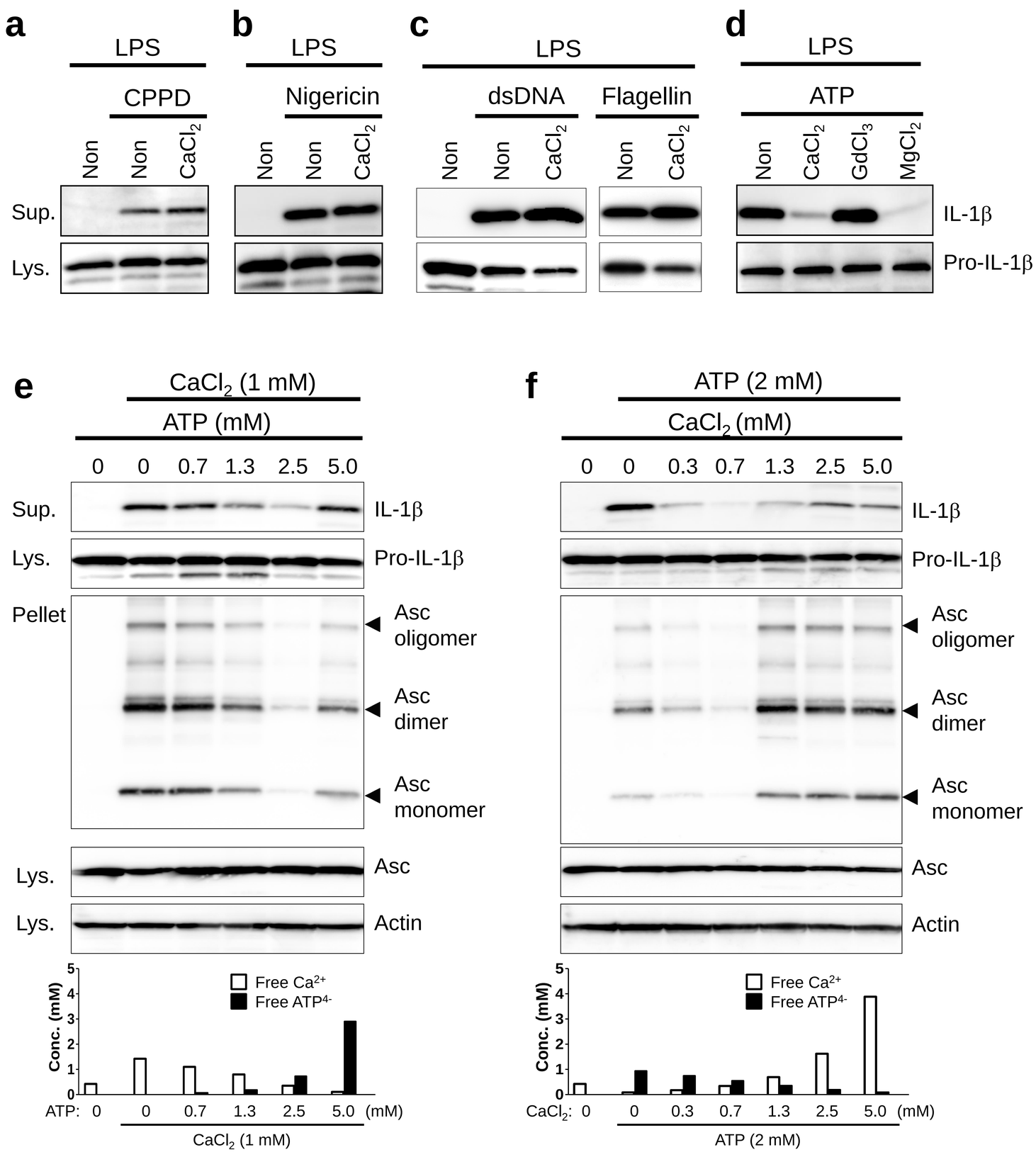


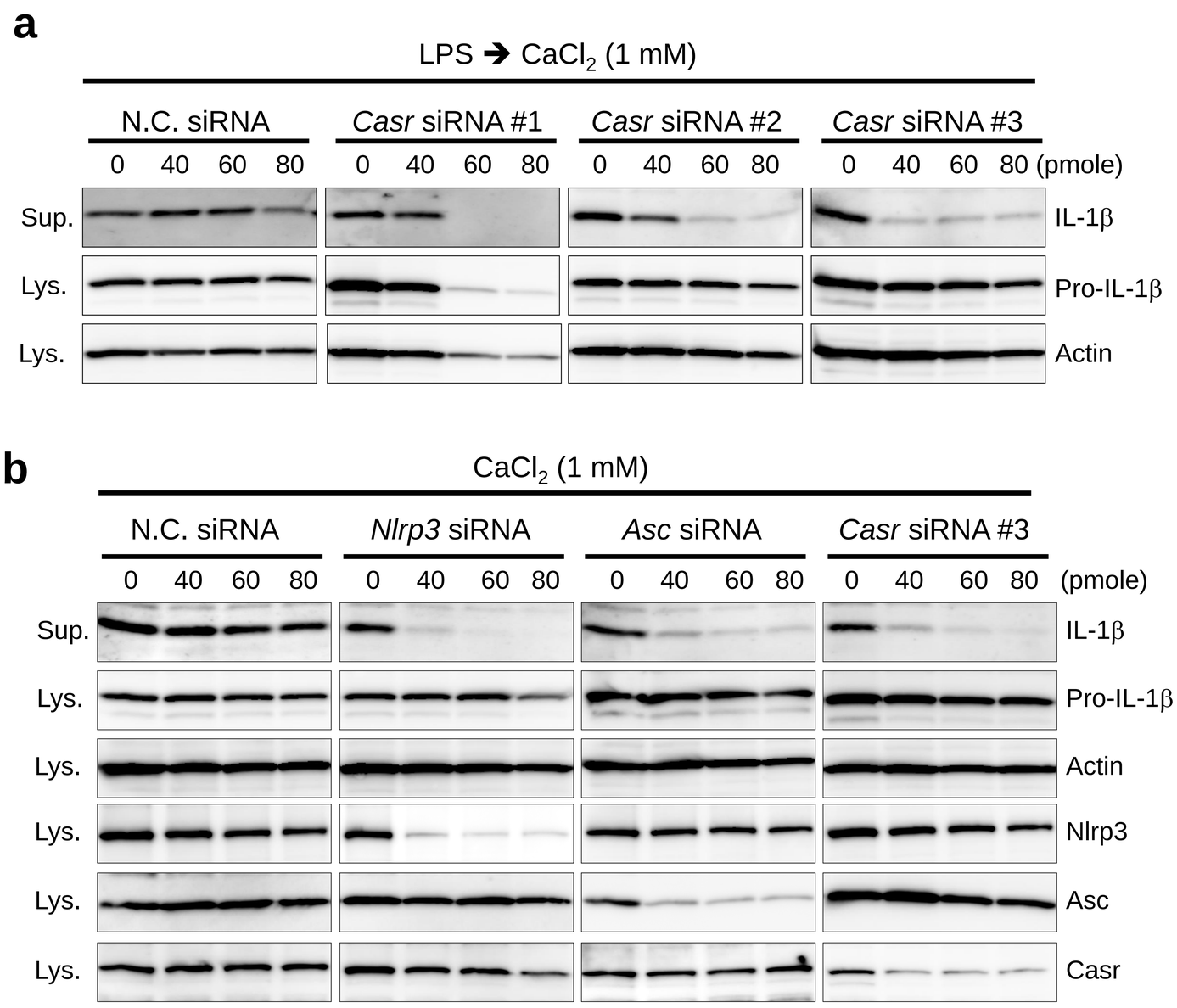
Supplementary Figure 1. Extracellular calcium induces IL-1β secretion independent of the AIM2 or NLRC4 inflammasomes.

a, LPS (1 μg ml^{-1} for 3 h)-primed BMDMs were treated with $CaCl_2$ (1 mM) for the indicated time duration (left) or with the indicated concentrations (right) of $CaCl_2$ for 40 min. **b**, LPS-primed BMDMs from WT, *Nlrp3*, *P2x7r*, *Asc*, or *Caspase-1* (*Casp1*)-deficient mice were cultured without additional treatment (Non) or treated with nigericin (20 μM) for 30 min, dsDNA (1 μg ml^{-1} dsDNA with 2.5 μl ml^{-1} Lipofectamine 2000) for 30 min, or flagellin (0.5 μg ml^{-1} flagellin with 25 μl ml^{-1} DOTAP) for 40 min. For detection of Asc oligomers, pellets from whole-cell lysates were cross-linked with disuccinimidyl suberate (DSS) and analyzed by immunoblot for Asc. **c**, BMDMs transiently transfected with scrambled (negative control, N.C.), *Aim2*, or *Nlrc4* siRNAs were primed with LPS and then treated with ATP (5 mM) for 40 min, $CaCl_2$ (1 mM) for 50 min, dsDNA for 40min, or flagellin for 50 min. **d**, LPS-primed BMDMs were treated with $GdCl_3$ (1 mM, agonist of the CaSR) or R-568 (10 μM, allosteric agonist of the CaSR) for 50 min. Cell culture supernatants (Sup.) and cell lysates (Lys.) were analyzed by immunoblotting as indicated. All immunoblot data shown are representative of more than three independent experiments.



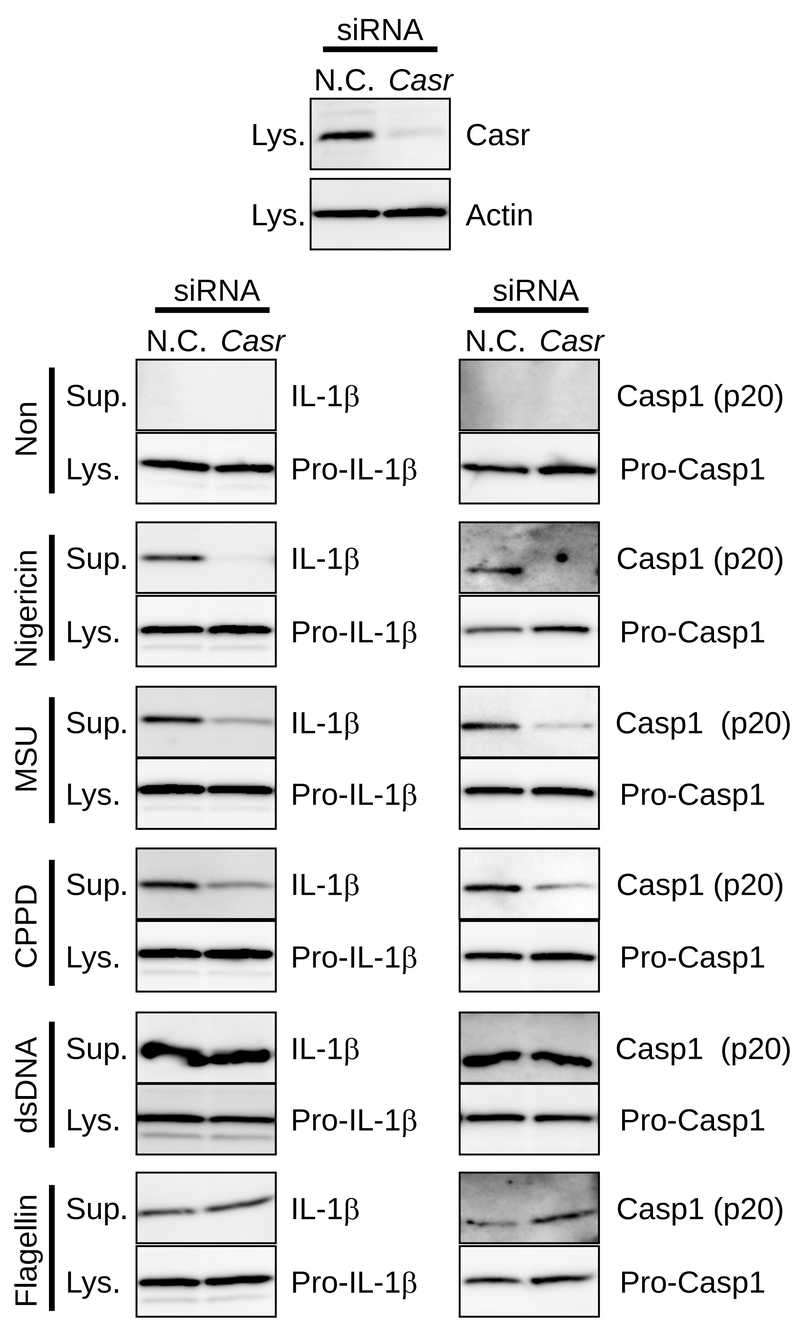
Supplementary Figure 2. Extracellular calcium inhibits ATP-driven NLRP3 inflammasome activation by formation of Ca^{2+}-ATP complexes, but does not inhibit CPPD or nigericin-induced NLRP3 inflammasome activation or AIM2 or NLRC4 inflammasome activation.

a, LPS-primed BMDMs were treated without or with CPPD (200 μg/ml) in the absence or presence of added $CaCl_2$ (1 mM). **b**, LPS-primed BMDMs were treated without or with nigericin (20 μM) in the absence or presence of added $CaCl_2$ (1 mM). **c.** LPS-primed BMDMs were treated with dsDNA (1 μg ml^{-1} dsDNA with 2.5 μl ml^{-1} Lipofectamine 2000) or flagellin (0.5 μg ml^{-1} flagellin with 25 μl ml^{-1} DOTAP) in the absence or presence of added $CaCl_2$ (1 mM) as indicated. **d**, LPS-primed BMDMs were treated with ATP (2 mM) in the absence or presence of added $CaCl_2$ (1 mM), $GdCl_3$ (1 mM), or $MgCl_2$ (1 mM). **e**, LPS-primed BMDMs were treated with 1 mM $CaCl_2$ in the presence of various concentrations (0 to 5 mM) of ATP. **f**, LPS-primed BMDMs were treated with 2 mM ATP in the presence of various concentrations (0 to 5 mM) of $CaCl_2$. Cell culture supernatants (Sup.) and cell lysates (Lys.) were analyzed by immunoblotting for IL-1β and Asc oligomerization. Estimated concentration of free Ca^{2+} and ATP^{4-} (bottom) were calculated by Ca/Mg/ATP/EGTA Calculator v1 constants from Theo Schoenmakers' Chelator (http://www.stanford.edu/~cpatton/CaMgATPEGTA-TS.htm), based on RPMI (pH and concentrations of $MgCl_2$ and monovalent ions) at 37°C with the standard 0.42 mM Ca^{2+} before added $CaCl_2$.



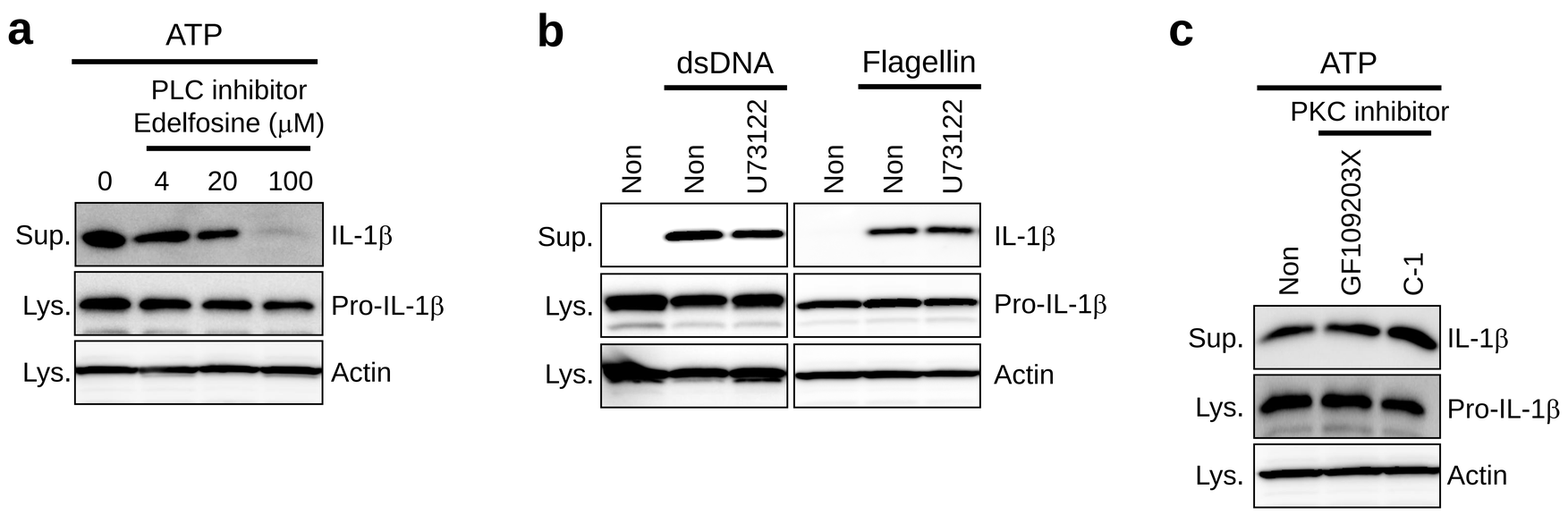
Supplementary Figure 3. Optimization of experimental conditions for gene knockdowns.

a, Three siRNA oligonucleotides were tested for *Casr* knockdown experiments. BMDMs were transiently transfected with indicated amount of scrambled (negative control, N.C.) or three independent *Casr* siRNAs. Then cells were stimulated with $CaCl_2$ (1 mM) for 50 min after priming with LPS. Cell culture supernatants (Sup.) and cell lysates (Lys.) were analyzed by immunoblotting as indicated. *Casr* si RNA #3 was effective at inhibiting IL-1β release into the supernatant with minimal effects on pro-IL-1β and actin. **b**, BMDMs were transiently transfected with the indicated amount of scrambled (negative control, N.C.) or selected siRNAs for knockdown of *Nlrp3*, *Asc*, or *Casr*. Then cells were stimulated with $CaCl_2$ (1 mM) for 50 min after priming with LPS. Cell culture supernatants (Sup.) and cell lysates (Lys.) were analyzed by immunoblotting as indicated.



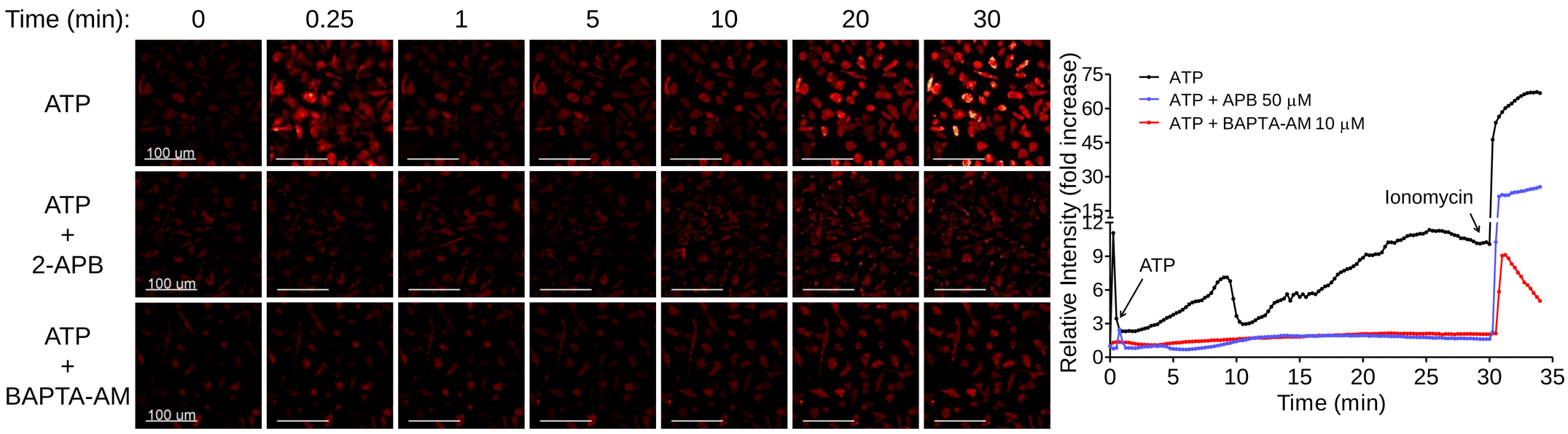
Supplementary Figure 4. The CaSR is essential for the activation of the NLRP3 inflammasome but not the AIM2 or NLRC4 inflammasomes.

BMDMs were transiently transfected with scrambled siRNA (negative control, N.C.) or *Casr* siRNA and then either not subjected to further treatment (Non), or treated with nigericin (20 μM for 30 min), MSU (200 μg ml^{-1} for 1h), CPPD (200 μg ml^{-1} for 1h), dsDNA (1 μg ml^{-1} dsDNA with 2.5 μl ml^{-1} Lipofectamine 2000 for 30 min), or flagellin (0.5 μg ml^{-1} flagellin with 25 μl ml^{-1} DOTAP for 40 min) after priming with LPS. Cell culture supernatants and cell lysates were analyzed by immunoblotting for IL-1β and caspase-1.



Supplementary Figure 5. PLC-IP_3 mediated intracellular calcium accumulation triggers NLRP3 but not AIM2 or NLRC4 inflammasome activation.
a, LPS (1 μg ml^{-1} for 3 h)-primed BMDMs were co-treated with ATP (5 mM) and a PLC inhibitor (edelfosine), and IL-1β secretion was analyzed. **b**, LPS-primed BMDMs were treated with dsDNA (1 μg ml^{-1} dsDNA with 2.5 μl ml^{-1} Lipofectamine 2000 for 30 min), or flagellin (0.5 μg ml^{-1} flagellin with 25 μl ml^{-1} DOTAP for 40 min) in the presence of U73122 (PLC inhibitor, 10 μM). Cell culture supernatants and cell lysates were analyzed by immunoblotting for IL-1β. **c**, LPS-primed BMDMs were co-treated with ATP (5 mM) and PKC inhibitors (50 μM GF109203X and 50 μM C-1), and IL-1β secretion was analyzed by immunoblot.

a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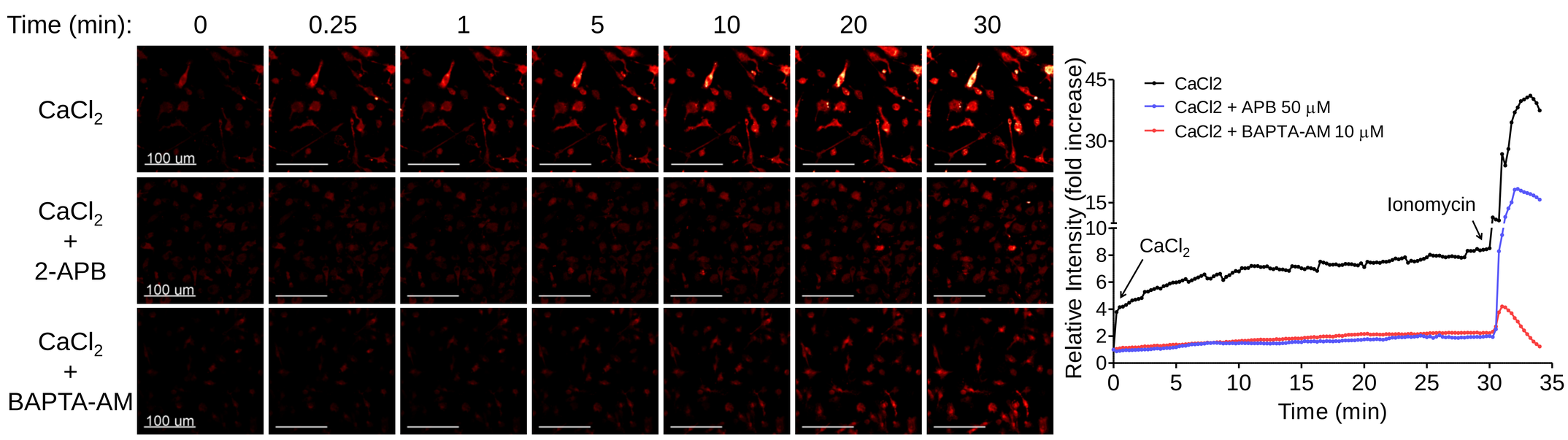
ATP + 2-APB
ATP + BAPTA-AM
100 um
Relative Intensity (fold increase)
ATP
ATP + APB 50 μM
ATP + BAPTA-AM 10 μM
ATP
Ionomycin
Time (min)

b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CaCl2 + BAPTA-AM
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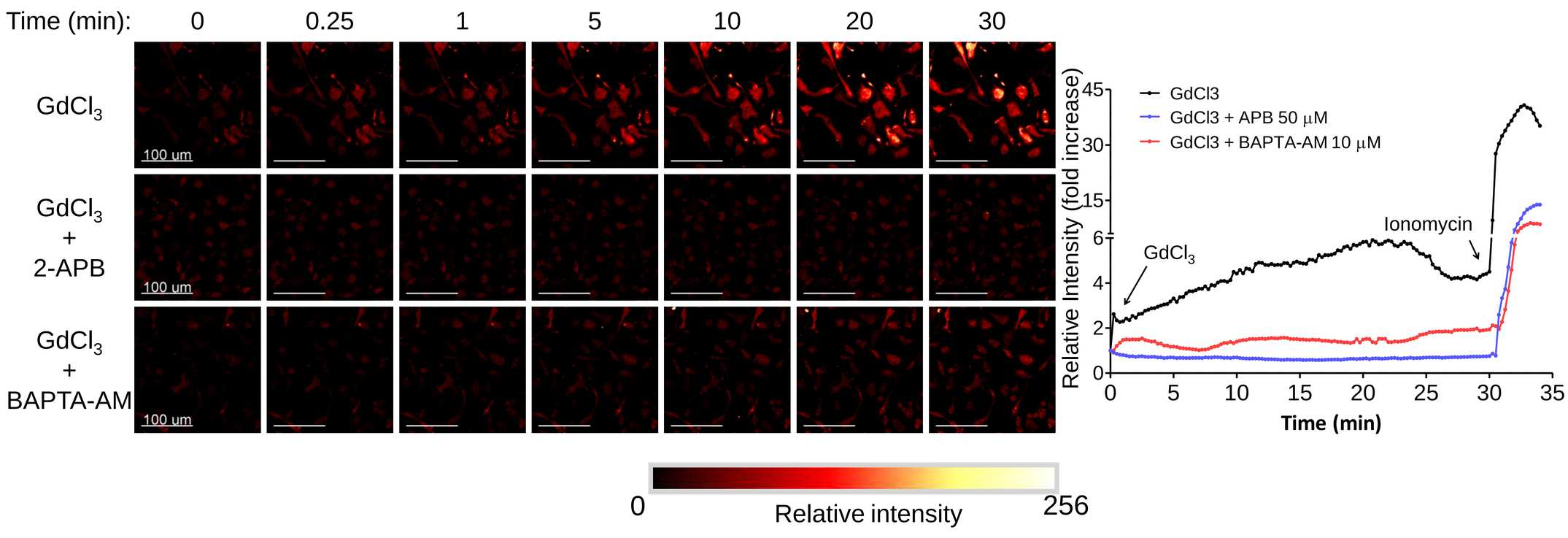
Relative Intensity (fold increase)
CaCl2
CaCl2 + APB 50 μM
CaCl2 + BAPTA-AM 10 μM
CaCl2
Ionomycin
Time (min)

c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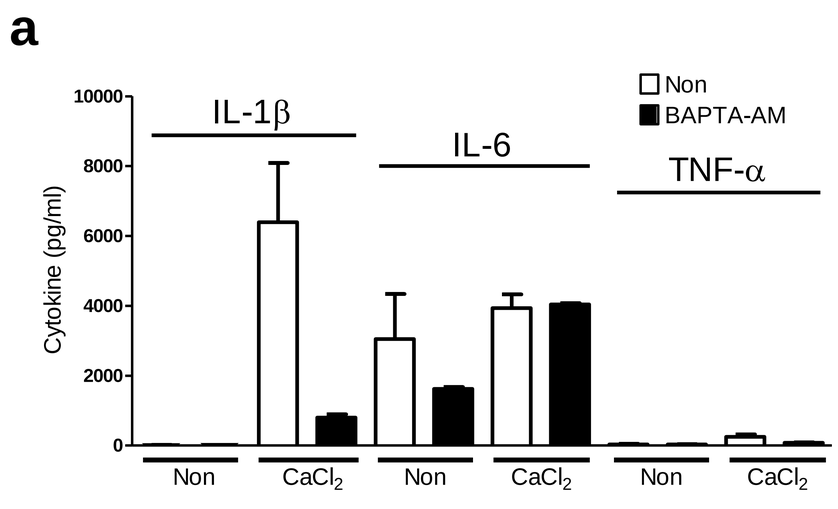
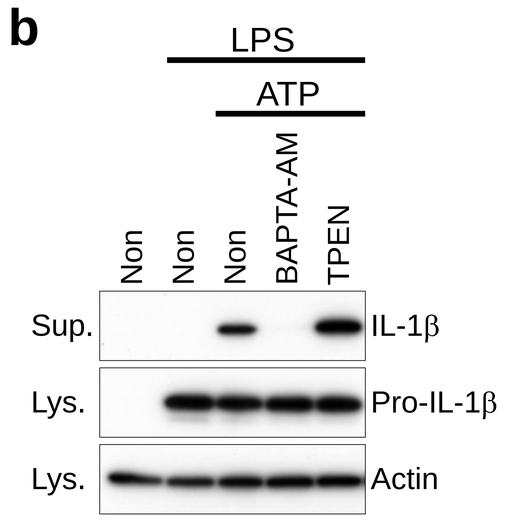
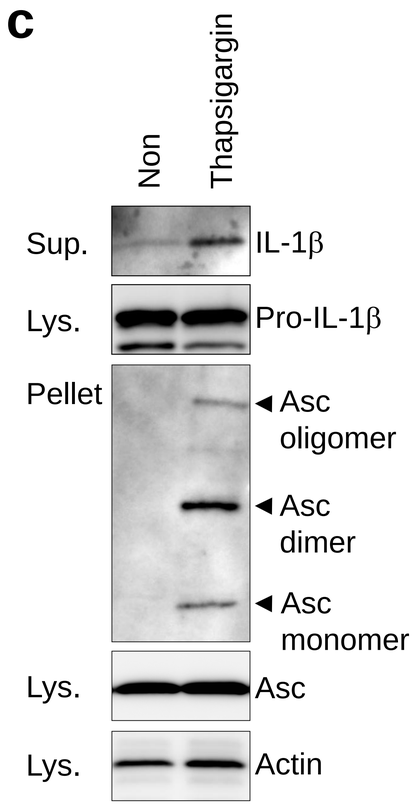
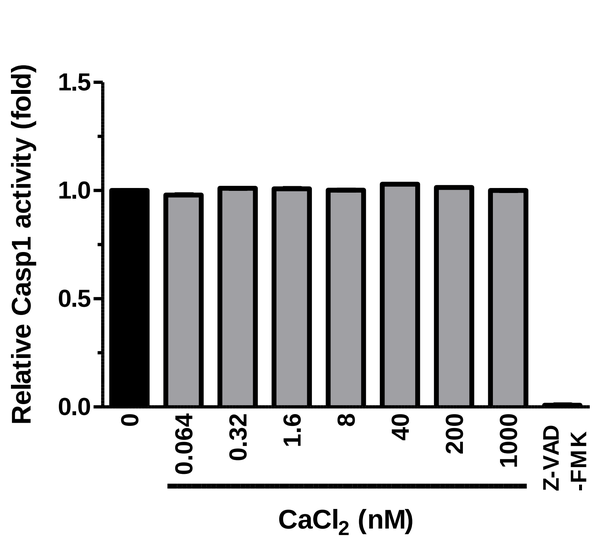
GdCl3 + BAPTA-AM
100 um
Relative Intensity (fold increase)
GdCl3
GdCl3 + APB 50 μM
GdCl3 + BAPTA-AM 10 μM
GdCl3
Ionomycin
Time (min)
0
Relative intensity
256

Supplementary Figure 6. ATP and CaSR agonists induce intracellular calcium influx. BMDMs were plated on 4-chambered coverglass dishes and stained with fluo-4/AM. Cells were treated with or without the IP_3 receptor blocker 2-APB (50 μM) or intracellular calcium chelator BAPTA-AM (10 μM), followed by treatment with 1 mM ATP (**a**), 1 mM $CaCl_2$ (**b**) or 1 mM $GdCl_3$ (**c**). The estimated free $[Ca^{2+}]_o$ in (**a)** is 0.22 mM, when adjusted for Ca^{2+}-ATP complexes. Images of untreated cells were acquired (t=0), then ATP, $CaCl_2$, or $GdCl_3$ were added and cells were imaged for 30 min with acquisition at 15 sec intervals. Sustained Ca^{2+} traces in **(a)** are likely due to the availability of extracellular Ca^{2+} through the P2X7 receptor, which is necessary for ATP-induced (but not $CaCl_2$-induced) NLRP3 inflammasome activation (Fig. 1b). After 30 min, ionomycin was added to the medium to a final concentration of 5 μM. Images were acquired on a Leica SP5 Confocal Imaging System, and analyzed using Imaris 7.3.1 software. Representative images from three independent experiments are shown in left panels, and the fold increase in absolute intensity of all cells in a field relative to time '0' (20-25 cells per field) is shown in right panels. For all comparisons (ATP vs ATP + 2-APB, ATP vs ATP + BAPTA-AM, $GdCl_3$ vs $GdCl_3$ + 2-APB, $GdCl_3$ vs $GdCl_3$ + BAPTA-AM , $CaCl_2$ vs $CaCl_2$ + 2-APB, $CaCl_2$ vs $CaCl_2$ + BAPTA-AM), p-values from an unpaired t-test were < 0.0001.



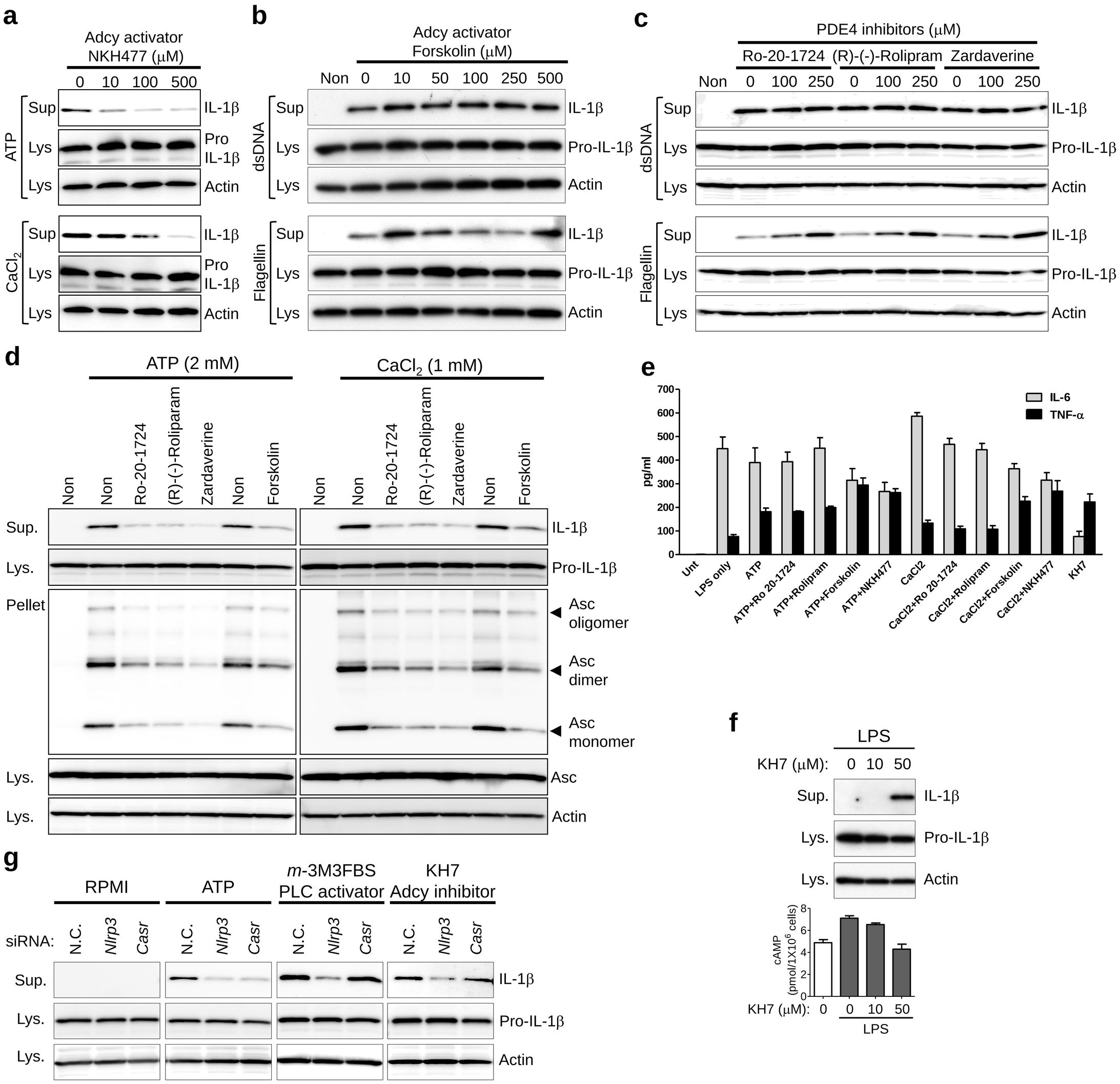






Supplementary Figure 7. Increase of intracellular calcium triggers NLRP3 inflammasome activation, but not IL-6 or TNF-α production.

a, LPS-primed BMDMs were treated with $CaCl_2$ (1 mM) alone or in the presence of BAPTA-AM (150 μM). Cell culture media were collected and IL-1β, IL-6, and TNF-α levels were measured by cytokine multiplex assay. **b,** LPS-primed BMDMs were treated with ATP (2 mM) in the presence of BAPTA-AM (10 μM) or TPEN (10 μM). **c,** LPS-primed BMDMs were treated with or without thapsigargin (50 nM) for 30 min, and were analyzed for IL-1β secretion and Asc pyroptosome by immunoblot. **d,** The activity of recombinant human active caspase-1 was measured in the presence of the indicated concentration of $CaCl_2$ using a colorimetric caspase-1 activity assay. Data represent the mean ± s.e.m from three or four independent experiments.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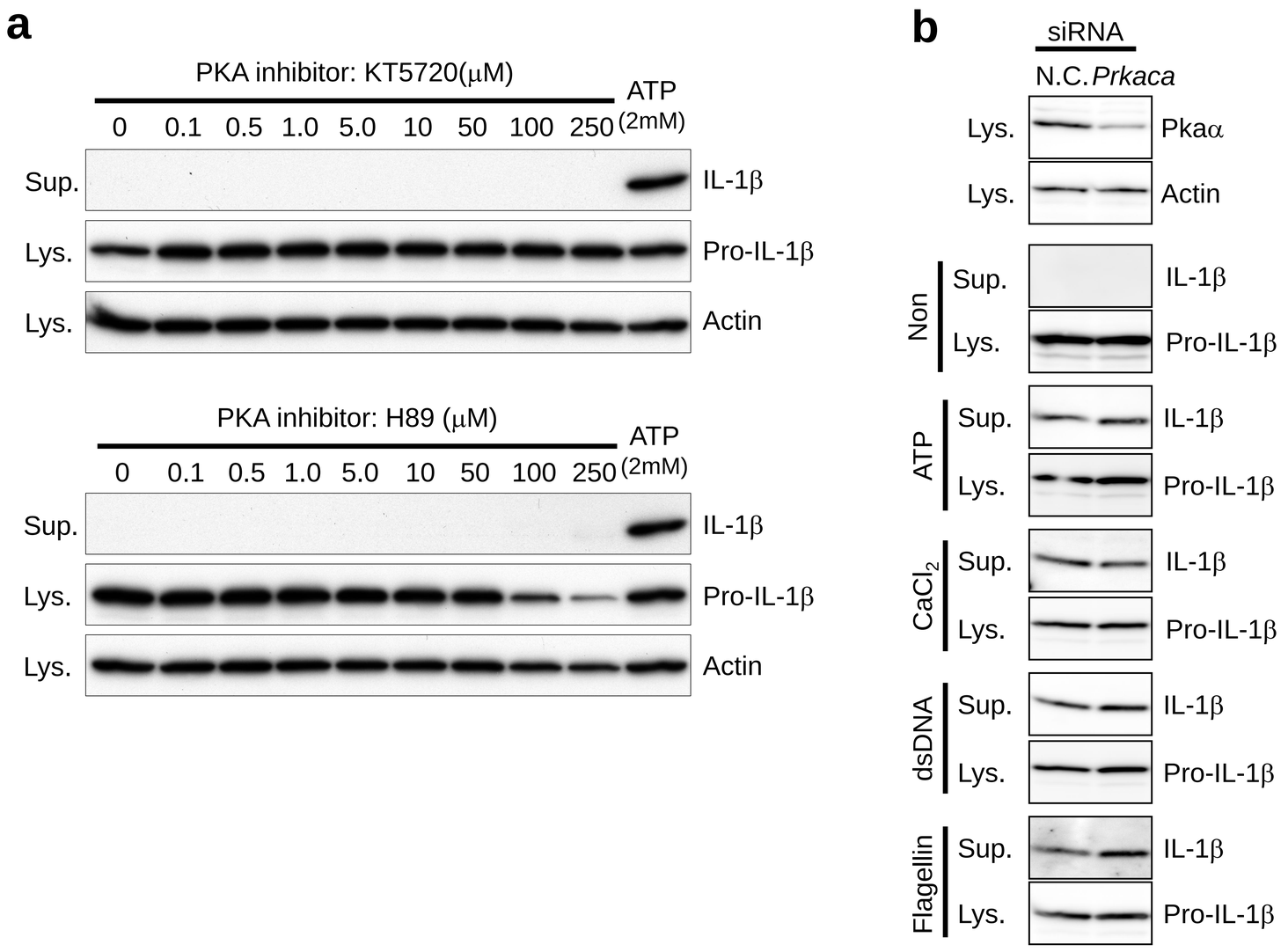
ATP
CaCl2
Sup
Lys
IL-1β
Pro IL-1β
Actin
b
Adcy activator
Forskolin (μM)
Non 0 10 50 100 250 500
dsDNA
Flagellin
Pro-IL-1β
c
PDE4 inhibitors (μM)
Ro-20-1724
(R)-(-)-Rolipram
Zardaverine
Non 0 100 250 0 100 250 0 100 250
d
ATP (2 mM)
CaCl2 (1 mM)
Non
Ro-20-1724
(R)-(-)-Roliparam
Zardaverine
Forskolin
Sup.
Lys.
Pellet
Asc oligomer
Asc dimer
Asc monomer
Asc
Actin
e
pg/ml
IL-6
TNF-α
Unt
LPS only
ATP
ATP+Ro 20-1724
ATP+Rolipram
ATP+Forskolin
ATP+NKH477
CaCl2
CaCl2+Ro 20-1724
CaCl2+Rolipram
CaCl2+Forskolin
CaCl2+NKH477
KH7
f
LPS
KH7 (μM): 0 10 50
cAMP (pmol/1X10^6 cells)
g
RPMI
ATP
m-3M3FBS
PLC activator
KH7
Adcy inhibitor
siRNA:
N.C.
Nlrp3
Casr
h
Relative gene expression
(x-fold vs. Adcy3 gene)
N.D.
Adcy suptypes
i
Pull-down:
Bead only
Pull-down: ATP-bead
ATP (mM)
0 1.0 3.0 5.0 7.5 10
Lysate
WB:
Nlrp3
cAMP (mM)

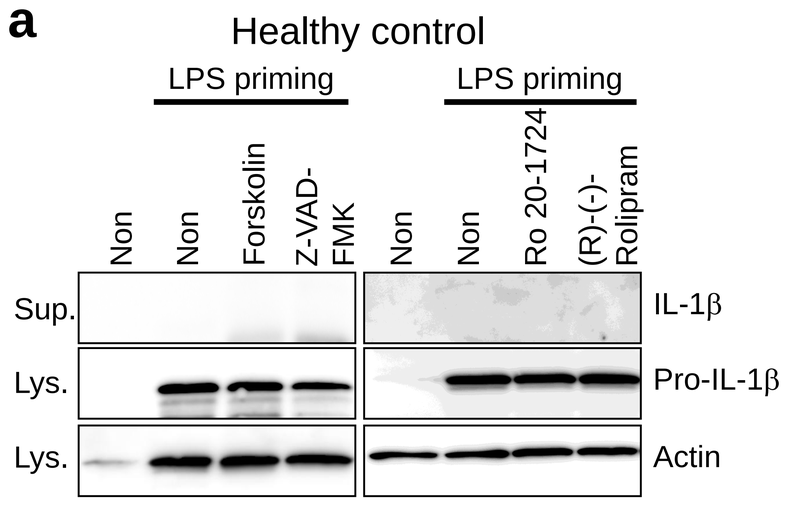
Supplementary Figure 8. cAMP directly suppresses NLRP3 inflammasome activation. **a**, LPS (1 μg ml^{-1} for 3 h)-primed BMDMs were treated with the indicated doses of NKH477 (Adcy activator), in the presence of ATP (5 mM) or $CaCl_2$ (1 mM), and then were analyzed for IL-1β secretion by immunoblots. **b**, LPS-primed BMDMs were treated with the indicated doses of forskolin in the presence of dsDNA (1 μg ml^{-1} dsDNA with 2.5 μl ml^{-1} Lipofectamine 2000 for 30 min) or flagellin (0.5 μg ml^{-1} flagellin with 25 μl ml^{-1} DOTAP for 40 min). Cell culture supernatants and cell lysates were analyzed by immunoblotting for IL-1β. **c**, LPS-primed BMDMs were treated with the indicated doses of PDE4 inhibitors (Ro 20-1724, (R)-(-)-Rolipram, or zardaverine) in the presence of dsDNA (1 μg ml^{-1} dsDNA with 2.5 μl ml^{-1} Lipofectamine 2000 for 30 min) or flagellin (0.5 μg ml^{-1} flagellin with 25 μl ml^{-1} DOTAP for 40 min). Cell culture supernatants and cell lysates were analyzed by immunoblotting for IL-1β. **d**, LPS-primed BMDMs were co-treated with ATP (2 mM) for 30 min or $CaCl_2$ (1 mM) for 40 min either alone, or in the presence of Ro 20-1724, (R)-(-)-Rolipram, zardaverine, or forskolin. Cell culture supernatants and lysates were analyzed for IL-1β secretion and Asc oligomerization. **e**, LPS-primed BMDMs were treated with ATP (2 mM) or $CaCl_2$ (1 mM) alone or in the presence of PDE4 inhibitors, an Adcy activator, or an Adcy inhibitor. Cell culture media were collected and IL-6 and TNF-α levels were measured by ELISA. Data represent the mean ± s.e.m from three independent experiments. **f**, LPS-primed BMDMs were treated with the indicated doses of KH7, an inhibitor of soluble Adcy, and analyzed for IL-1β secretion. **g**, The function of Nlrp3 is unaffected by *Casr* knockdown. BMDMs were transiently transfected with scrambled (negative control, N.C.), *Nlrp3*, or *Casr* siRNAs. After priming with LPS, cells were treated with nothing (RPMI), ATP, *m*-3M3FBS (PLC activator), or KH7 (Adcy inhibitor). Cell culture supernatants (Sup.) and cell lysates (Lys.) were analyzed by immunoblotting as indicated. **h**, Relative gene expression profile of Adcy subtypes in BMDMs assayed by RT-QPCR. **i,** Cyclic AMP has no effect on the interaction of NLRP3 with ATP. The lysates of LPS-primed BMDMs were incubated with ATP-conjugated beads or unconjugated beads in the presence of ATP or cAMP (with various concentration as indicated) for 30 min at RT. After washing, bound proteins were eluted from the beads and analyzed by immunoblot for Nlrp3. The gel running and Western blots for the two assays were done simultaneously, and chemiluminescent detection was done on the same X-ray film from which both immunoblot images were taken.



Supplementary Figure 9. NLRP3 inflammasome activation is not suppressed by protein kinase A (PKA).

a, LPS-primed BMDMs were treated with the indicated doses of PKA inhibitors, KT5720 or H89 for 40 min, and then analyzed for IL-1β secretion. **b**, BMDMs were transiently transfected with scrambled siRNA (N.C.) or *Prkaca* siRNA and then treated without (Non) or with ATP (5 mM) for 40 min, or $CaCl_2$ (1 mM) for 50 min, dsDNA for 30 min, or flagellin for 50 min after priming with LPS. IL-1β secretion was analyzed by immunoblotting.

a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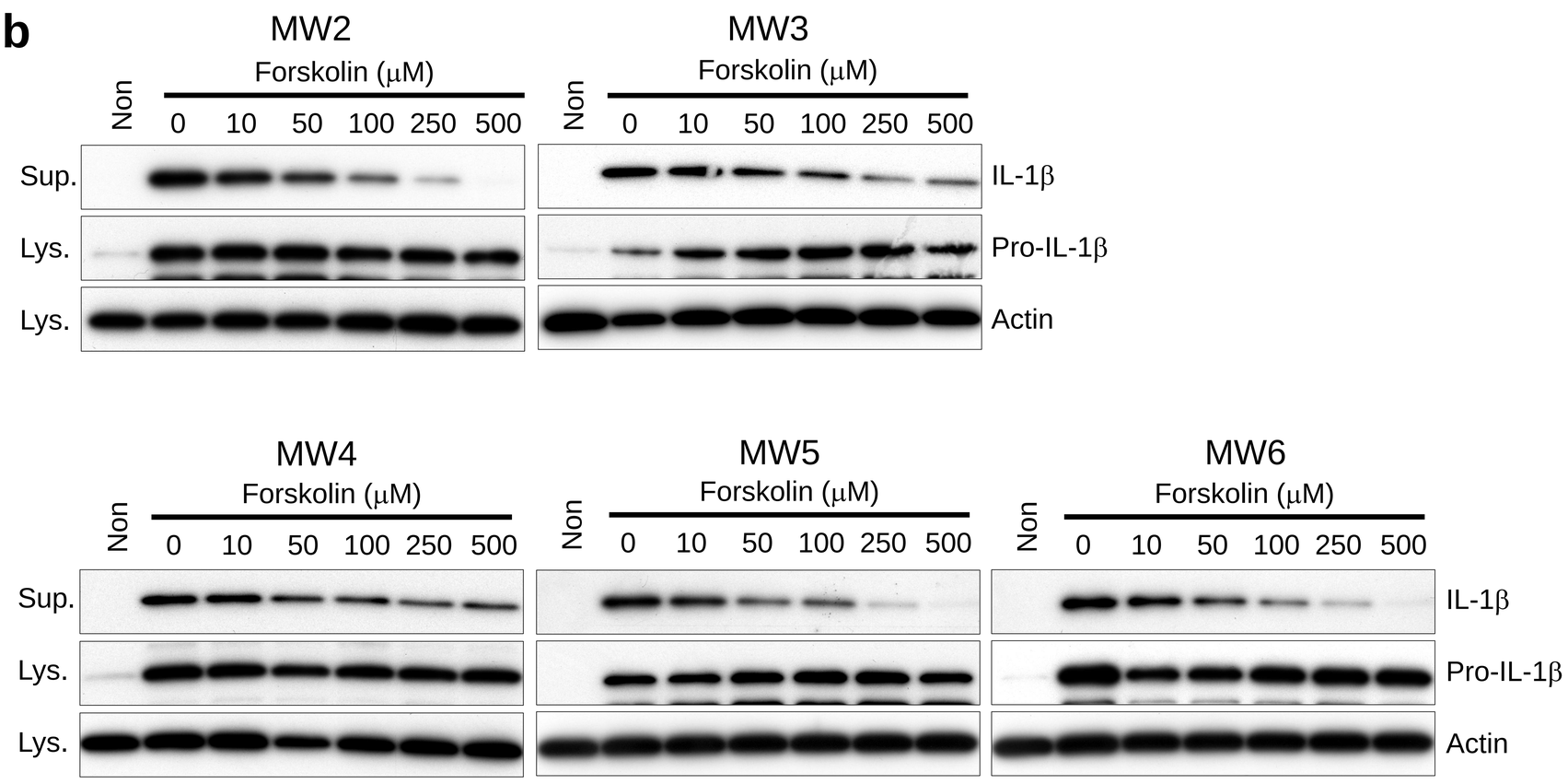
LPS priming
LPS priming
Non
Non
Forskolin
Z-VAD-FMK
Non
Non
Ro 20-1724
(R)-(-)-Rolipram
Sup.
Lys.
Lys.
IL-1β
Pro-IL-1β
Actin

b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MW3
MW4
MW5
MW6
Forskolin (μM)
Non
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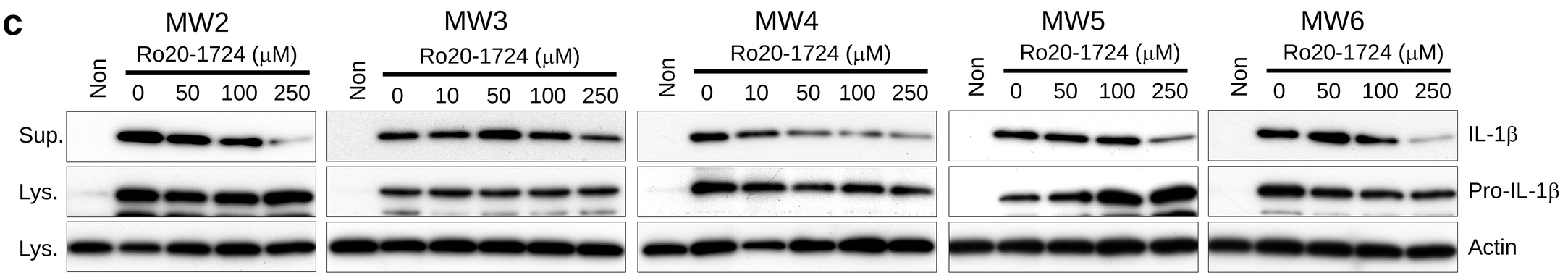
Sup.
Lys.
Lys.
IL-1β
Pro-IL-1β
Actin

c


MW2
MW3
MW4
MW5
MW6
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Non
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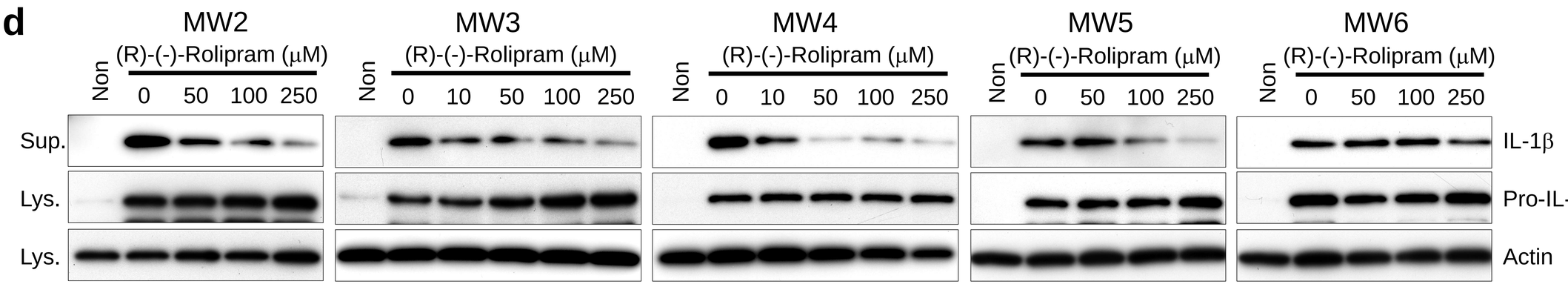
0 10 50 100 250
Sup.
Lys.
Lys.
IL-1β
Pro-IL-1β
Actin

d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MW3
MW4
MW5
MW6
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0 10 50 100 250
Sup.
Lys.
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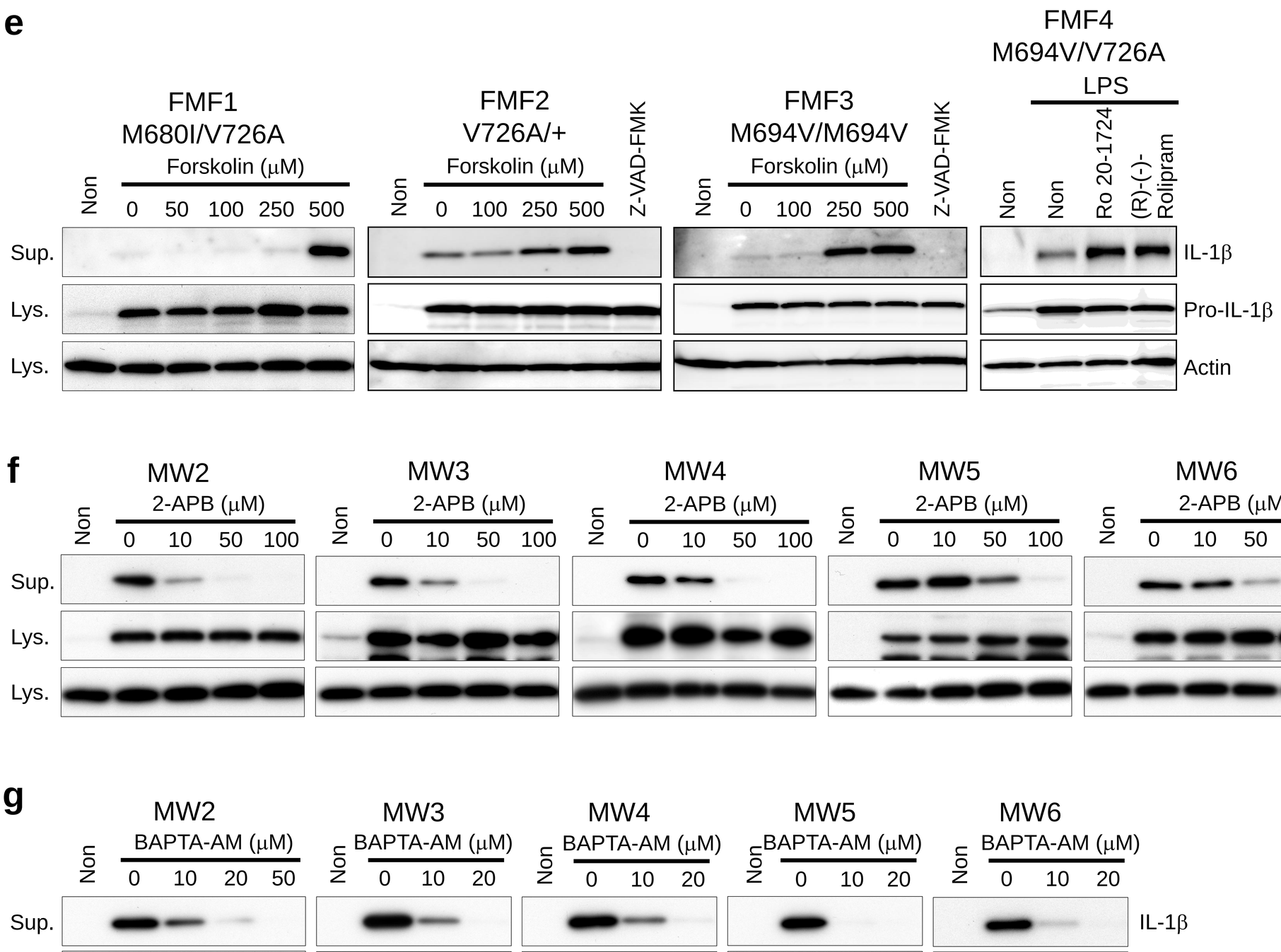
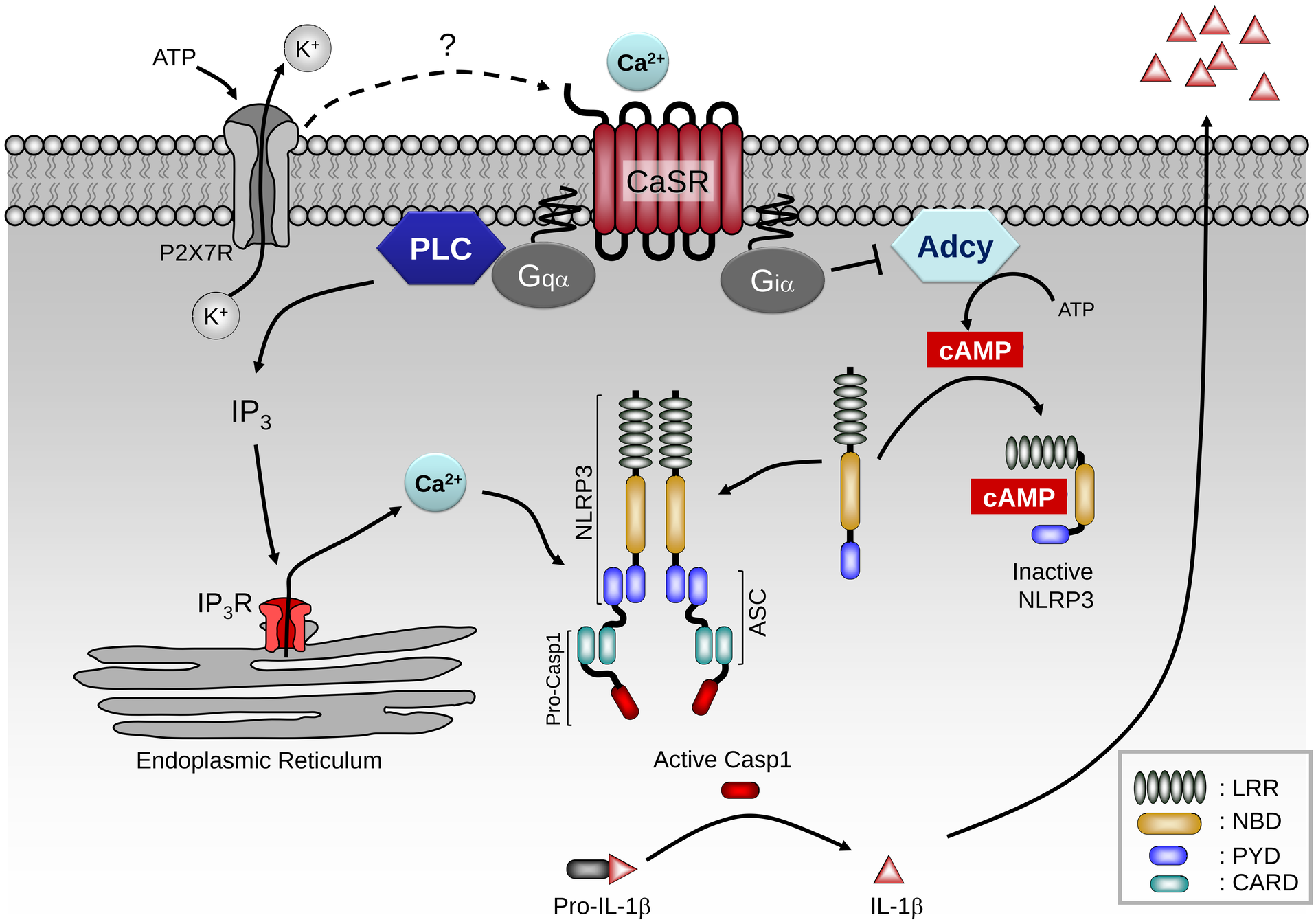
Actin

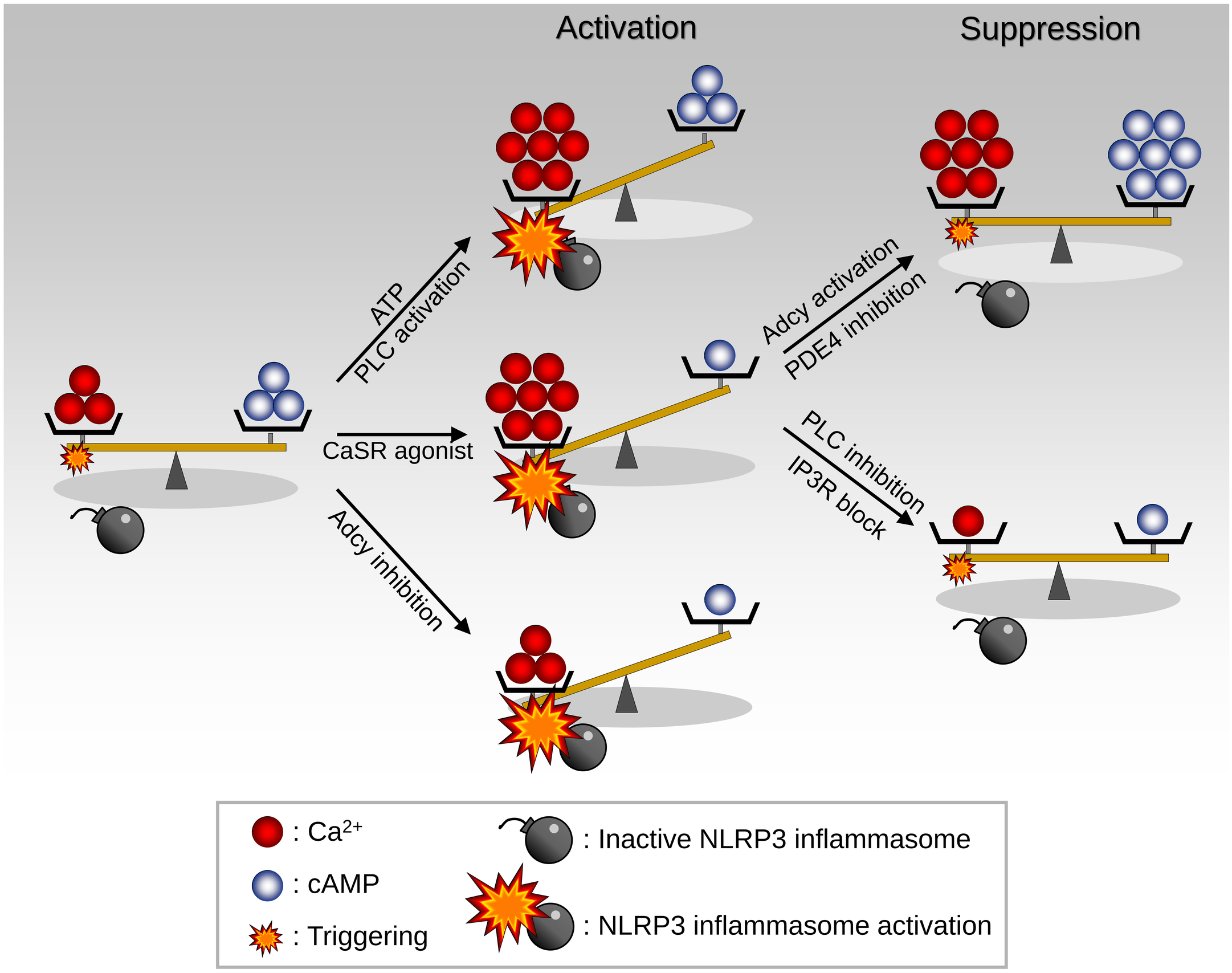


Supplementary Figure 10. The role of cAMP and calcium signaling in the pathogenesis of CAPS.

a, Healthy control PBMCs were non-primed or primed with LPS (1 μg ml^{-1}) for 3h and then treated with forskolin (500 μM), Z-VAD-FMK (20 μg ml^{-1}), Ro 20-1724 (250 μM), or (R)-(-)-Rolipram (250 μM) for 40 min. Cell lysates and cell culture supernatants were analyzed for IL-1β secretion by immunoblot. **b,** PBMCs from five MWS patients with a F523C mutation in NLRP3 were non-primed or primed with LPS (1 μg ml^{-1}) for 3h and then treated with the indicated dose of forskolin. Cell lysates and cell culture supernatants were analyzed for IL-1β secretion by immunoblot. **c,d**, Non-primed or LPS-primed PBMCs from five MWS patients were treated with the indicated dose of Ro 20-1724 (**c**) or (R)-(-)-Rolipram (**d**) Cell lysates and cell culture supernatants were analyzed for IL-1β secretion by immunoblot. **e**, Non-primed or LPS-primed PBMCs from FMF patients (with *MEFV* mutations as indicated) were treated with the indicated dose of forskolin (first three panels) or treated with 250 μM Ro 20-1724 or 250 mM (R)-(-)-Rolipram for 40 min (fourth panel). Cell lysates and cell culture supernatants were analyzed for IL-1β secretion by immunoblot. **f**,**g**, Non-primed or LPS-primed PBMCs from five MWS patients were treated with the indicated dose of 2-APB (**f**) or BAPTA-AM (**g**). Cell lysates and cell culture supernatants were analyzed for IL-1β secretion by immunoblot.



Supplementary Figure 11. Proposed molecular mechanism of NLRP3 inflammasome activation mediated by the CaSR.
In macrophages stimulated by PAMPs, such as LPS, the NLRP3 inflammasome is activated in response to extracellular danger signals, ATP, or Ca^{2+}, through CaSR-mediated signal transduction pathways. The CaSR, a G protein-coupled receptor, activates PLC, which induces intracellular Ca^{2+} release from the ER following the interaction of IP_3 with its receptor. The CaSR also inhibits adenylate cyclase and subsequently reduces cAMP levels. An increase in intracellular Ca^{2+} and/or decrease in cAMP activates the NLRP3 inflammasome, which may be regulated by a coordination of intracellular Ca^{2+} and cAMP levels.



Supplementary Figure 12. Proposed model of NLRP3 inflammasome activation regulated by the balance of Ca^{2+} and cAMP.
In the unstimulated state, the NLRP3 inflammasome is not activated due to a balance between the levels of intracellular Ca^{2+} and cAMP (left). Upon stimulation of LPS-primed macrophages with CaSR agonists, increased intracellular Ca^{2+} and decreased cAMP coordinately alter the balance to activate the NLRP3 inflammasome (middle). This activation can be blocked by deliberately increasing cAMP with an Adcy activator or PDE4 inhibitor (right, top) or alternatively by decreasing intracellular Ca^{2+} with a PLC inhibitor or IP_3R blocker (right, bottom). In addition, the NLRP3 inflammasome can be activated by increasing intracellular Ca^{2+} without changing cAMP (middle, top) or decreasing cAMP without changing intracellular Ca^{2+} (middle, bottom) in the absence of known NLRP3 inflammasome stimuli. Autoinflammatory disease mutations of NLRP3 reduce its binding affinity for cAMP, pushing the balance toward inflammasome activation.