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1 **NAADP/SERCA3-dependent Ca²⁺ stores pathway specifically controls early autocrine ADP secretion**
2 **potentiating platelet activation.**

3
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11
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17
18 **Short title:** NAADP and SERCA3-dependent activation pathway.

19
20 **Subject codes:** Cell Signaling/Signal Transduction, Platelets

21
22

1 **Abstract**

2
3 **Rationale:**

4 Ca²⁺ signaling is a key and ubiquitous actor of cell organization and its modulation controls many cellular responses.
5 Sarco-endoplasmic reticulum Ca²⁺-ATPases (SERCAs) pump Ca²⁺ into internal stores that play a major role in the
6 cytosolic Ca²⁺ concentration rise upon cell activation. Platelets exhibit 2 types of SERCAs, SERCA2b and
7 SERCA3, which may exert specific roles, yet ill-defined. We have recently shown that Ca²⁺ mobilization from
8 SERCA3-dependent stores was required for full platelet activation in weak stimulation conditions.
9

10 **Objective:**

11 To uncover the signaling mechanisms associated with Ca²⁺ mobilization from SERCA3-dependent stores leading
12 to ADP secretion.
13

14 **Methods and Results:**

15 Using platelets from wild-type or SERCA3-deficient mice, we demonstrated that an early (within 5 to 10 seconds
16 following stimulation) secretion of ADP specifically dependent on SERCA3 stored Ca²⁺ is exclusively mobilized
17 by nicotinic acid adenosine dinucleotide phosphate (NAADP): both Ca²⁺ mobilization from SERCA3-dependent
18 stores and primary ADP secretion are blocked by the NAADP receptor antagonist Ned-19, and reciprocally both
19 are stimulated by permeant NAADP. In contrast, Ca²⁺ mobilization from SERCA3-dependent stores and primary
20 ADP secretion were unaffected by inhibition of the production of inositol-1,4,5-trisphosphate (IP3) by
21 phospholipase-C, and accordingly were not stimulated by permeant IP3.
22

23 **Conclusions:**

24 Upon activation a NAADP/SERCA3 Ca²⁺ mobilization pathway initiates an early ADP secretion, potentiating
25 platelet activation, and a secondary wave of ADP secretion driven by both an IP3/SERCA2b-dependent Ca²⁺ stores
26 pathway and the NAADP/SERCA3 pathway. This does not exclude that Ca²⁺ mobilized from SERCA3 stores may
27 also enhance platelet global reactivity to agonists. Because of its modulating effect on platelet activation, this
28 NAADP-SERCA3 pathway may be a relevant target for anti-thrombotic therapy.
29

30 **Keywords**

31 SERCA3, calcium, NAADP, platelet, secretion
32

| | | |
|----|---|---|
| 1 | Non-standard Abbreviation and Acronyms | |
| 2 | | |
| 3 | ADP | adenosine 5'-diphosphate |
| 4 | cCa ²⁺ | Cytosolic Ca ²⁺ |
| 5 | ER | Endoplasmic Reticulum |
| 6 | FITC | Fluorescein isothiocyanate |
| 7 | Fura-2-NM | Fura-2-NearMembrane-AM probe |
| 8 | IP3 | inositol-1,4,5-trisphosphate |
| 9 | NAADP | nicotinic acid adenosine dinucleotide phosphate |
| 10 | OAG | 1-Oleoyl-2-acetyl-sn-glycerol |
| 11 | PLC | phospholipase C |
| 12 | PMA | phorbol 12-myristate 13-acetate |
| 13 | PMCA _s | Plasma Membrane Ca ²⁺ ATPases |
| 14 | PRP | Platelet-rich plasma () |
| 15 | PPACK | d-Phe-Pro-Arg chloromethylketone dihydrochloride |
| 16 | SERCA _s | Sarco-endoplasmic reticulum Ca ²⁺ -ATPases |
| 17 | Serca3 ^{-/-} | SERCA3 knockout mice |
| 18 | Tg | thapsigargin |
| 19 | tBHQ | 2,5-dinucleotide-(tert-butyl)-1,4-benzohydroquinone |
| 20 | TxA ₂ | Thromboxane A ₂ |
| 21 | WT | wild-type mice |
| 22 | | |

1 Introduction

2 Cytosolic Ca^{2+} (cCa^{2+}) is central in cell signaling. The regulatory mechanisms of cCa^{2+} concentration involve entry
3 from the extra cellular medium through several channels including, TRPC, or Orai-1 coupled to signaling (SOCE,
4 Store Operated Ca^{2+} Entry).¹⁻³ The other cCa^{2+} sources are mitochondria and Ca^{2+} intracellular stores. cCa^{2+} is down-
5 regulated by Na/Ca exchangers and Plasma Membrane Ca^{2+} ATPases (PMCA) that export Ca^{2+} to the outside, by
6 uniporters that recapture Ca^{2+} into mitochondria (above a high threshold)⁴ and most importantly by Sarco-
7 Endoplasmic Reticulum Ca^{2+} ATPases (SERCAs) that stock Ca^{2+} into intracellular stores.⁵ Platelets have long been
8 used as a model for studying the regulation of cCa^{2+} , because these anucleate cells recapitulate all regulatory and
9 signaling Ca^{2+} pathways, and express SERCAs in quantitative amounts, including SERCA3. Among these pathways,
10 mobilization of Ca^{2+} , from SERCA-dependent internal stores, is described as an early event upstream of all other
11 signaling pathways. Ca^{2+} mobilization is highly relevant since involved in platelet activation and engagement of
12 integrin $\alpha_{\text{IIb}}\beta_3$ in aggregation.⁶ Platelet SERCAs have been particularly well characterized. SERCAs are encoded
13 by 3 genes, *ATP2A1*, *ATP2A2* and *ATP2A3*, which produce several alternate transcripts and protein isoforms:
14 SERCA1a/b, SERCA2a-c, and SERCA3a-f. They are found in multiple tissues but platelets exhibit only SERCA2b
15 and SERCA3 isoforms.⁷⁻¹⁰ SERCAs maintain a Ca^{2+} concentration gradient between the cytosol (100 nmol/L) and
16 the Endoplasmic Reticulum (ER) (1 mmol/L).¹¹ However, several questions remain to be answered, among which:
17 does the expression of multi-SERCA isoforms correlate with corresponding distinct effects and Ca^{2+} signaling
18 organization in a given cell, here platelets?

19 SERCA enzymes share similar structures with distinct intrinsic activities: Ca^{2+} affinity higher for SERCA2b than
20 for SERCA3 ($K_{1/2} \sim 0.27 \mu\text{mol/L}$ versus $1 \mu\text{mol/L}$) but Ca^{2+} uptake lower (7 nmol/min/mg of protein versus 21
21 nmol/min/mg, respectively)^{12, 13} allowing both SERCAs to maintain cCa^{2+} at low levels in the resting cells.

22 Pathologies and mouse models provide insight into SERCA2b and SERCA3 functions. Mutations in the human
23 *ATP2A2* gene affecting SERCA2, lead to the Darier's syndrome in humans, a dermatological syndrome.^{14, 15}
24 SERCA2 and SERCA3 mutations are associated with some cancers¹⁶⁻¹⁸ suggesting involvement in cell
25 differentiation.^{19, 20} SERCA3 human mutations also seem associated with type II diabetes.²¹ Mouse SERCA2
26 knockouts are not viable at the homozygous state, but heterozygotes exhibit SERCA2a- (defect in heart contractility
27 and relaxation)²² and SERCA2b-type defects, evocative of the Darier's syndrome.²³ Mouse SERCA3 knockouts
28 (*Serca3^{-/-}*) exhibit no phenotypic alterations,²⁴ except for an altered gustatory nerve response.²⁵ *In vitro*, impaired
29 relaxation of *Serca3^{-/-}* aorta rings was reported, with defective relaxation of vascular smooth muscle cells, altered
30 Ca^{2+} signaling and low NO production.²⁴ *In vitro* also, low insulin secretion and altered Ca^{2+} oscillations were
31 reported.²⁶⁻²⁸ Altogether these results point to a potential specific role for SERCA3 in Ca^{2+} signal modulation.

32 In platelets, among other differences, immuno-electron microscopy localization studies suggested distinct
33 topologies for SERCAs: peripheral for SERCA3, and more central for SERCA2b.^{29, 30} Functional differentiation
34 based on pharmacological studies, suggested association of SERCA3 with acidic Ca^{2+} stores,³¹ as well as with
35 STIM1, a Ca^{2+} sensor of store operated Ca^{2+} entry (SOCE) participating in Ca^{2+} stores replenishing by SERCA3.³²
36 We have recently reported that Ca^{2+} mobilization from Ca^{2+} stores dependent on SERCA3 but not on SERCA2b is
37 involved in a secretion pathway important for weak agonist platelet stimulation.³³ Here, we show that this secretion
38 of both ADP and ATP is an early secretory pathway within seconds following activation, and controlled by NAADP
39 mobilizing Ca^{2+} from SERCA3-dependent stores (heretofore designated as SERCA3 stores) and independently
40 from inositol-3-phosphate (IP3), which in turn triggers the bulk of secretion following Ca^{2+} mobilization from
41 SERCA2b- dependent Ca^{2+} stores (heretofore designated as SERCA2b stores).

42

1 **Methods**

2 The authors declare that all supporting data are available within the article [and its online supplementary files], and
3 available from the corresponding author on reasonable request.
4

5 Online Data Supplement contains detailed description of methods.
6

7 **Results**

8 **SERCA3-dependent ADP secretion occurs early and precedes SERCA2b-dependent secretion**

9 We recently showed that a secretion, specifically triggered by Ca^{2+} mobilization from SERCA3 stores, amplifies
10 low agonist platelet stimulation by thrombin (through the PAR4 receptor in mouse platelets), or collagen.³³ This
11 pathway most likely involved ADP secretion, since ADP (but not ATP) addition to thrombin rescued the defect in
12 platelets from SERCA3 deficient (*Serca3^{-/-}*) mice, while its suppression by apyrase scavenging reduced activation
13 of WT platelets to the level observed in *Serca3^{-/-}* platelets, unaffected by apyrase.³³ However, direct evidence for
14 SERCA3-dependent ADP release was missing, since only ATP secretion was measured. Here, we directly assessed
15 ADP secreted from platelets as well as its kinetic, comparatively to ATP. 3 minutes after thrombin stimulation (40
16 μM), ADP was detected in the supernatant of *Serca3^{-/-}* mouse platelets to roughly half the level of WT platelets
17 (Figure 1A), a ratio comparable to ATP (Figure 1B). Importantly, when secretion kinetics were assessed by
18 thrombin inhibition by PPACK at different time points (Figure 1C; Online Figure IIA), ADP secretion was
19 detectable in WT platelets as early as 5 seconds after thrombin stimulation, while not detected at all in *Serca3^{-/-}*
20 platelets. At 10 seconds, ADP secretion augmented in WT platelets and started rising in *Serca3^{-/-}* platelets. After 3
21 minutes, ADP secretion rose to its maximal level in WT platelets, reaching only half that level in *Serca3^{-/-}* platelets.
22 A similar kinetics was seen for ATP (Figure 1D; Online Figure IIB), showing co-secretion of both molecules.
23 Finally, confirming functional involvement of SERCA3 in early secretion, platelet pretreatment for 5 minutes with
24 tBHQ (10 μM), a SERCA3-specific pharmacological inhibitor,³³ suppressed early secretion (as assessed by
25 ATP, Figure 1D) at 5 seconds in WT platelets, mimicking *Serca3^{-/-}* platelets, expectedly unaffected by SERCA3
26 inhibition. Both followed the same ATP secretion kinetics up to 180 seconds. We concluded that within the first 5
27 seconds following platelet stimulation by thrombin, ADP and ATP secretions were exclusively dependent on
28 SERCA3-controlled signaling, while SERCA3-independent secretions set off after 10 seconds, likely dependent on
29 Ca^{2+} from SERCA2b stores, both pathways adding up or the SERCA3-dependent pathway potentiating the
30 SERCA2b-dependent pathway.
31

32 **Specific detection of Ca^{2+} mobilization from SERCA3 stores by the intracellular plasma membrane- 33 associated probe Fura-2-NearMembrane**

34 Since early ADP secretion may be essentially dependent on SERCA3, we postulated that Ca^{2+} mobilization from
35 SERCA3 stores was the consequence of platelet activation and was independent of ADP, secreted ADP being then
36 involved in reinforcement of Ca^{2+} mobilization from SERCA2b stores. To compare both mobilizations, we searched
37 for Ca^{2+} -probes that might be specific for one SERCA and not the other. Considering that SERCA3 may be localized
38 near the plasma membrane and SERCA2b more central (^{29, 31} and unpublished results), we compared Ca^{2+}
39 measurements obtained with the Fura-2-AM and with the Fura-2-NearMembrane-AM probe (Fura-2-NM), a
40 derivative of the FFP18 probe modified to localize to the inner leaflet of membranes and to selectively detect
41 intracellular Ca^{2+} concentration proximal to membranes.³⁴ Washed platelets, pre-loaded with either probe, were
42 sedimented onto poly-D-lysine-coated coverslips and cytosolic Ca^{2+} concentration was assessed by
43 videomicroscopy as described in Methods. WT or *Serca3^{-/-}* platelets loaded with Fura-2 exhibited Ca^{2+} mobilization
44 (in presence of extracellular EGTA) upon thrombin challenge (Figure 2A), visually slightly less intense for *Serca3^{-/-}*
45 platelets. In contrast, using Fura-2-NM, thrombin triggered a positive, though moderate, fluorescence signal in
46 WT platelets and no fluorescence increase in *Serca3^{-/-}* platelets (Figure 2B). Image quantification confirmed a
47 stronger signal with Fura-2 than with Fura-2-NM in WT platelets (Figure 2C and 2E and Figure 2I and 2K).
48 Interestingly, *Serca3^{-/-}* platelets under Fura-2 exhibited reduced Ca^{2+} mobilization compared to WT platelets (Figure
49 2A, 2C and 3A), confirming our earlier results using flow cytometry.³³ The absence of detection of Ca^{2+}
50 mobilization in *Serca3^{-/-}* platelets loaded with Fura-2-NM (Figure 2E and 2K), suggests that Fura-2-NM does not
51 detect Ca^{2+} mobilization from SERCA2b stores (the only Ca^{2+} stores left in *Serca3^{-/-}* platelets). Furthermore,
52 SERCA3 pharmacological inhibition with tBHQ resulted in the well-documented Ca^{2+} emptying of SERCA3 stores
53 in resting WT platelets yielding a moderate mobilization signal (corresponding in fact to a Ca^{2+} leak usually
54 compensated for by constitutive SERCA3 activity, pumping cytosolic Ca^{2+} back into stores) whether visualized
55 with Fura-2 or Fura-2-NM (Figure 2D and 2F). In contrast and as expected, tBHQ did not induce any Ca^{2+} leak in
56 *Serca3^{-/-}* platelets prior to thrombin stimulation (Figure 2D and 2F), thus confirming absence or undetectable non-
57 selective inhibition of SERCA2b at the concentration used (10 μM). Note that unsolicited activation or signaling

1 desensitization by tBHQ-induced Ca^{2+} leakage from SERCA3-dependent stores in WT platelets was avoided by the
2 3 to 5 minutes preincubation time, to allow stabilization of cytosolic Ca^{2+} . In Fura-2-loaded platelets, tBHQ
3 pretreatment lowered Ca^{2+} mobilization in response to thrombin in WT platelets to the level of Serca3^{-/-} platelets
4 (due to Ca^{2+} mobilization from the sole SERCA2b stores left) (Figure 2D), while no mobilization in response to
5 thrombin was detected by Fura-2-NM (Figure 2F). In addition, the Ca^{2+} response to thrombin after tBHQ
6 pretreatment of WT and Serca3^{-/-} platelets, as visualized by Fura-2, was consistent with SERCA2b store release.
7 The fact that Fura-2-NM only allowed visualization of tBHQ-induced calcium leakage from SERCA3 stores in WT
8 platelets but no further mobilization in response to thrombin or in Serca3^{-/-} platelets, suggested that Fura-2-NM is
9 specific for Ca^{2+} exit from SERCA3 stores. As expected, inhibition of SERCA2b (the only SERCA left in Serca3^{-/-}
10 platelets) by thapsigargin (200nmol/L, conditions specific for SERCA2b, not affecting SERCA3 function)³³
11 abolished thrombin-induced Ca^{2+} mobilization in Serca3^{-/-} but not in WT platelets (because of the SERCA3 stores
12 which remain intact after thapsigargin), as visualized by both Fura-2 and Fura-2-NM (Figure 2G and 2H,
13 respectively). Altogether, these results 1) indicate that the Fura-2-NM probe can be used as a specific probe for
14 Ca^{2+} mobilization from SERCA3 stores, and 2) confirm a peripheral localization for Ca^{2+} mobilized from SERCA3
15 stores, consistent with its possible role in early secretion.

16 **Ca^{2+} mobilization from SERCA3 stores is independent of ADP.**

17 The specific monitoring, with Fura-2-NM, of Ca^{2+} mobilization from SERCA3 stores being established, we next
18 analyzed the relationship between Ca^{2+} mobilization from SERCA3 stores and ADP. Control experiments showing
19 Ca^{2+} mobilization in response to thrombin as detected with Fura-2 and Fura-2-NM are displayed respectively in
20 Figure 2I and 2K. When observed with Fura-2, ADP scavenging by apyrase diminishes the overall Ca^{2+}
21 mobilization in WT platelets down to the level of Serca3^{-/-} platelets (Figure 2J), confirming our previous results by
22 flow cytometry on platelets in suspension.³³ In contrast, Ca^{2+} mobilization from SERCA3 stores detected by Fura-
23 2-NM in WT platelets was not affected by apyrase (Figure 2L). Conversely, exogenous ADP added to thrombin,
24 while stimulating Ca^{2+} mobilization in Serca3^{-/-} platelets up to the same level than WT platelets under Fura-2 (Figure
25 2M), did not modify Ca^{2+} mobilization in WT nor in Serca3^{-/-} platelets as visualized by Fura-2-NM (Figure 2N).
26 These results show that Ca^{2+} mobilization from SERCA3 stores is independent of ADP, consistent with the primary
27 Ca^{2+} mobilization from SERCA3 stores taking place upstream of the initial ADP release.

28 **Ca^{2+} mobilization from SERCA3 stores is independent of the second messenger inositol-1,4,5 tris-phosphate**

29 We next checked whether differential Ca^{2+} mobilization between SERCA3 and SERCA2b stores, and sensitivity to
30 the Ca^{2+} fluorophore Fura-2-NM, may extend to regulation by distinct signaling pathways. Inositol 1,4,5-tris
31 phosphate (IP3) is the prominent second messenger driving Ca^{2+} mobilization from ER,³⁵ the recognized SERCA2b
32 storing organelle.¹² To determine whether IP3 mobilized SERCA2b and/or SERCA3 stores, we analyzed Ca^{2+}
33 mobilization in response to thrombin of platelets pre-treated with U73122, a well-characterized inhibitor of
34 phospholipase C (PLC), the main IP3 source.³⁶ After assessing dose-dependent effects on SERCA2b-dependent
35 Ca^{2+} mobilization using Serca3^{-/-} mice as controls (Online Figure IIIA through IIID), we found, as visualized with
36 Fura-2, that U73122 (0.75 $\mu\text{mol/L}$) completely suppressed Ca^{2+} mobilization in response to thrombin in Serca3^{-/-}
37 platelets (Figure 3A and Online Figure IIIC, IVA), indicating that IP3 mobilized Ca^{2+} essentially from SERCA2b
38 stores (the only functional stores in Serca3^{-/-} platelets). Ca^{2+} mobilization in WT platelets was affected by U73122,
39 but only partially, consistent with SERCA3 stores mobilization independent from IP3. The fact that 1 $\mu\text{mol/L}$
40 U73122 resulted in complete inhibition of Ca^{2+} mobilization in WT platelets (Online Figure IIID) suggested some
41 off-target effect at high concentration (such as was shown for PKC).³⁷ Interestingly, when visualized with Fura-2-
42 NM, U73122 pretreatment did not affect Ca^{2+} mobilization from SERCA3 stores in WT platelets, while in Serca3^{-/-}
43 platelets, Ca^{2+} mobilization remained undetected (Figure 3B and Online Figure IVD). In Fura-2 loaded WT
44 platelets, while pretreatment with both U73122 and Tg reduced Ca^{2+} mobilization in response to thrombin,
45 pretreatment with both U73122 and tBHQ completely abolished it, further suggesting that IP3 mobilized calcium
46 from SERCA2b stores and not from SERCA3 stores (Online Figure IIIE and IIIF, respectively).

47 Interestingly, addition of exogenous ADP to thrombin in the presence of U73122, did not restore mobilization in
48 both WT and Serca3^{-/-} platelets when visualized by Fura-2 (Figure 3A and Online Figure IVE), and did not affect
49 it when visualized by Fura-2-NM (Figure 3B and Online Figure IVF). Altogether, these results are consistent with
50 the paradigm of a Ca^{2+} mobilization from SERCA3 stores being independent of IP3. They also suggest that IP3 acts
51 downstream of early ADP secretion and that this early ADP potentiates the PLC pathway, since blocked by U73122.
52 Confirming that IP3 is not involved in Ca^{2+} mobilization from SERCA3 stores, exogenously added IP3-AM induced
53 a similar Ca^{2+} mobilization in WT and Serca3^{-/-} platelets under Fura-2 monitoring (Figure 3C), not detected using
54 Fura-2-NM (Figure 3D). IP3-AM-induced Ca^{2+} mobilization was abolished in Fura-2 loaded platelets after
55 treatment with Tg (depleting SERCA2b stores) but not with tBHQ (depleting SERCA3 stores) (Figure 3E, 3F and
56 3G).

3G), confirming that SERCA3 stores are not mobilized by IP3. The slight decrease in Ca²⁺ mobilization induced by IP3-AM after tBHQ pre-treatment compared to IP3-AM alone for both WT and Serca3^{-/-} platelets may be due to a limited non-selective effect of tBHQ, on SERCA2b,³⁸ or to a slight interference of tBHQ with IP3-AM.

Ca²⁺ mobilization from SERCA3 stores is triggered by nicotinic acid adenosine dinucleotide-phosphate.

Two other second messengers, Nicotinic Acid Adenosine Dinucleotide-Phosphate (NAADP) and cyclic Adenosine Dinucleotide-Phosphate ribose (cADPr), are also known to elicit Ca²⁺ mobilization,³⁹ though the presence and the role of cADPr in platelets is controversial.⁴⁰ Interestingly, NAADP has been shown to act as second messenger specific for mobilization of Ca²⁺ from acidic stores in a wide range of cell types.⁴¹ SERCA3 has been found associated with acidic stores in platelets,⁴² and based on pharmacological studies, NAADP has been suggested to be a second messenger for platelet SERCA3 stores.³¹ Here, we used the Serca3^{-/-} mouse model to confirm and extend these observations. In Figure 4A and Online Figure VA and VC, as visualized with Fura-2, pre-incubation with Ned-19 (0.5 μmol/L) for 3 minutes, a well-characterized specific antagonist of NAADP,⁴³ diminished global Ca²⁺ mobilization in response to thrombin in WT platelets to the level of Serca3^{-/-} platelets, themselves unaffected by Ned-19 pretreatment. Similar results were obtained with BAPTA-Oregon green-loaded platelets analyzed by flow cytometry (Online Figure VIIA and VIIB). Moreover, addition of exogenous ADP simultaneously to thrombin bypassed Ned-19 inhibition and restored Ca²⁺ mobilization in both WT and Serca3^{-/-} platelets as visualized by Fura-2 (Figure 4A and Online Figure VE). This restored Ca²⁺ mobilization appeared to be completely dependent on PLC activity since it was totally suppressed in both WT and Serca3^{-/-} platelets when U73122 was added to Ned-19 (Figure 4A and Online Figure VG and VH), consistent with ADP triggering IP3-dependent Ca²⁺ mobilization from SERCA2b stores. Of note, when visualized with Fura-2-NM, addition of Ned-19 suppressed thrombin Ca²⁺ mobilization from SERCA3 stores in WT platelets (Figure 4B and Online Figure VB and VD), and as expected, this effect was not reversed by exogenous ADP (Figure 4B and Online Figure VF). These results thus strongly suggest that NAADP is the main second messenger mobilizing Ca²⁺ from SERCA3 stores.

Consistent with involvement of NAADP in the mobilization of SERCA3 stores, NAADP-AM, in absence of thrombin, while exhibiting mobilization in WT platelets, did not mobilize Ca²⁺ in Serca3^{-/-} platelets visualized with both Fura-2 and Fura-2-NM (Figure 4C and 4D, and Online Figure VIA through VIC). Accordingly, pre-incubation with Ned-19 prevented NAADP-AM mediated Ca²⁺ mobilization (Figure 4E and 4F, and Online Figure VIB and VIC). Depleting SERCA3 stores with the SERCA3 inhibitor tBHQ also prevented subsequent Ca²⁺ mobilization by NAADP-AM in WT platelets (Figure 4G and 4H, and Online Figure VIB and VIC), while the SERCA2b-specific inhibitor thapsigargin (Tg, 200 nmol/L) was without effect and did not block Ca²⁺ mobilization by NAADP-AM in WT platelets (Online Figure IXA, IXB). Similar observations were obtained with BAPTA-Oregon green-loaded platelets analyzed by flow cytometry (Online Figure VIID through F). Additionally, NAADP-AM induced Ca²⁺ mobilization in WT platelets was not increased by ADP addition which, as expected, mobilized Ca²⁺ in Serca3^{-/-} platelets (Online Figure VIIIA through D).

Altogether, these results are consistent with a Ca²⁺ mobilization from SERCA3 stores being specifically dependent on the second messenger NAADP, but not IP3.

SERCA3-dependent primary secretion is mediated by NAADP.

We next assessed whether the NAADP pathway was involved in early secretion. In Figure 5A, NAADP pathway inhibition by Ned-19 reduced secretion after thrombin stimulation in WT platelets to the level of Serca3^{-/-} platelets, unaffected throughout secretion kinetics (5 seconds, 10 seconds and 3 minutes). Consistent with absence of effect on SERCA3 store mobilization, U73122 did not inhibit early secretion at 5 seconds in WT platelets, while blocking further secretion at later time points (Figure 5A), confirming that the primary secretion in WT platelets is independent of IP3. Likewise, addition of exogenous ADP to thrombin bypassed Ned-19 inhibition, restoring full secretion in both WT and Serca3^{-/-} platelets (Figure 5B). These results confirm NAADP as second messenger specific for Ca²⁺ mobilization from SERCA3 stores leading to early secretion. Interestingly, NAADP-AM, by itself, was unable to trigger secretion (Figure 5C), most likely because secretion requires not only Ca²⁺ but also PKCs activation by diacylglycerol from PLC or/and PLD-mediated phospholipids break down.⁴⁴ We tested this possibility by addition of the PKC activator PMA (phorbol-myristyl-acetate, 10 nmol/L) (Figure 5C), after determination of the optimal concentration (Online Figure Xa). NAADP-AM in the presence of PMA triggered secretion in WT, but not in Serca3^{-/-} platelets. Interestingly the level of secretion reached was comparable to that of thrombin 40 mU/ml within 5 to 10 seconds of stimulation (Figure 5A). The other PKC activator, OAG (1-Oleoyl-2-acetyl-*sn*-glycerol)⁴⁵ yielded similar results (Online Figure XB). IP3-AM in the presence of PMA induced secretion to comparable levels in Serca3^{-/-} platelets and WT platelets, consistent with SERCA2b-dependent secretion (Figure 5D). Moreover, NAADP-AM in combination with PMA and IP3 amplified secretion of WT platelets (comparable to thrombin), but, as expected, not of Serca3^{-/-} platelets (Figure 5D). Of note, no secretion was observed in presence of ADP alone

1 and the level of secretion induced in presence of PMA with 10 $\mu\text{mol/L}$ ADP was similar in WT and Serca3^{-/-}
2 platelets (not shown). In WT platelets, ADP+PMA-induced secretion was not significantly different from that
3 obtained with NAADP and PMA, and was markedly lower than the secretion obtained with thrombin in WT
4 platelets (Online Figure VIII E).

5 Finally, we wished to verify the role of the NAADP pathway in platelet aggregation induced by low concentration
6 of thrombin (40 mU/mL). Ned-19 demonstrated a partial inhibitory effect on aggregation of WT and not of Serca3^{-/-}
7 platelets (Figure 6A and 6B). Aggregation of both WT and Serca3^{-/-} platelets in the presence of Ned-19 was
8 completely restored by exogenous ADP (Figure 6C), consistent with the contention that ADP bypasses the
9 SERCA3/NAADP-dependent pathway. U73122 inhibited aggregation to low thrombin concentration, partially for
10 WT platelets, and completely for Serca3^{-/-} platelets (Figure 6D). Moreover, Ned-19 exhibited an inhibitory effect
11 additive to U73122 on WT platelets (Figure 6E), consistent with involvement of both the NAADP-SERCA3 and
12 the IP3-SERCA2b pathways in aggregation.

13 **Thromboxane A₂ is involved in the potentiation of thrombin signaling by the Serca3/NAADP pathway**

14 Thromboxane A₂ (TxA₂) is an important autocrine agonist in the amplification step of platelet activation.⁴⁶ To
15 determine whether it was involved in the SERCA3/NAADP pathway, we have assessed the response to thrombin
16 of indomethacin-pretreated (5 $\mu\text{mol/L}$, the most efficient concentration tested) platelets for 10 minutes.
17 Indomethacin partially inhibited Ca²⁺ mobilization in WT, but not in Serca3^{-/-} platelets (Figure 7A through C). The
18 inhibitory effect of indomethacin on Ca²⁺ mobilization was not affected by Ned-19, nor rescued by ADP (Figure
19 7C). Interestingly indomethacin did not affect early secretion at 5 or 10 seconds of WT platelets, and only partially
20 (about 25%) at 180 seconds (Figure 7D and 7E) in WT platelets. However, it completely inhibited secretion of
21 Serca3^{-/-} platelets at 180 seconds (Figure 7E), consistent with the known dependence of IP3 (and the Serca2b
22 pathway) on TxA₂. Accordingly, indomethacin prevented restoration of secretion by ADP of Serca3^{-/-} platelets.
23 These results argue against a role for TxA₂ in the SERCA3/NAADP pathway itself. Since it does not affect
24 mobilization nor secretion in Serca3^{-/-} platelets, this indicates also that TxA₂ does not play a role in Serca2b-
25 dependent Ca²⁺ store mobilization or secretion either. The partial inhibition of Ca²⁺ mobilization and secretion in
26 WT platelets is thus best explained by a role for TxA₂ at the level of the potentiation of thrombin signaling by ADP
27 secreted by the SERCA3/NAADP pathway. Similar results were also observed in human platelets (Figure 8).
28

29 **Human platelets exhibit also a SERCA3/NAADP pathway controlling an early ADP secretion potentiating activation.**

30 Because we previously demonstrated that human platelets also exhibited a SERCA3-dependent Ca²⁺ mobilization
31 controlling secretion,³⁵ we verified that, like for mouse platelets, this pathway was dependent on NAADP. Ca²⁺
32 mobilization in response to thrombin was strongly decreased when human platelets were pretreated with either
33 tBHQ or Ned-19 (Figure 8A and 8B). As expected, inhibition of thromboxane A₂ production with indomethacin,
34 also strongly decreased Ca²⁺ mobilization in response to thrombin, but ADP addition to thrombin did not restore
35 Ca²⁺ mobilization, contrasting with Ned-19 (Figure 8C). Furthermore, as expected, tBHQ and Ned-19 pretreatment
36 blocked early ATP secretion measured at 5 seconds, but secretion induced by thrombin in indomethacin-pretreated
37 platelets was similar with control (Figure 8D through F). Finally, at 180 seconds, while both Ned-19 and
38 indomethacin pretreatment resulted in a significant decrease in secretion (Figure 8G and 8H), like in mouse platelets,
39 only Ned-19 inhibition was by-passed in presence of extracellular ADP, suggesting that both human and mouse
40 platelets share a SERCA3/NAADP pathway triggering platelet activation upstream of the TxA₂ pathway.
41

42 Altogether these results are consistent with two distinct platelet Ca²⁺ stores controlling secretion differentially: the
43 first Ca²⁺ store, sensitive to tBHQ and Ned-19, is dependent on SERCA3, independent from ADP and rapidly
44 mobilized by NAADP to elicit a quick initial ADP secretion; the second, sensitive to thapsigargin and U73122,
45 dependent on SERCA2b is dependent in part on ADP secreted by the SERCA/NAADP pathway and mobilized by
46 IP3, and triggers together with SERCA3 stores, the bulk of secretion (Online Figure XI).
47

48 **Discussion**

49 We have recently reported that a specific secretion triggered by Ca²⁺ mobilization of SERCA3 stores, was necessary
50 for complete platelet activation in conditions of low concentration of agonists.³³ Here, we provide evidence that
51 SERCA3-dependent Ca²⁺ stores drive early phase secretion, though they are also involved throughout secretion.
52 Furthermore, we demonstrate that both Ca²⁺ stores are strictly mobilized by distinct second messengers: NAADP
53 for SERCA3 stores, and IP3 for SERCA2b stores. Differential mobilization of SERCA3 and SERCA2b Ca²⁺ has
54 already been suggested by earlier studies,^{31, 39, 47} but these were based only on pharmacological evidence, and
55
56
57

1 therefore lacked more direct proofs. The present study definitively establishes that SERCA3 and SERCA2b stores
2 are differentially regulated, and more importantly link these mobilizations to distinct pathways of ADP secretion.
3 We demonstrate a functional connection between Ca²⁺ mobilization from SERCA3 stores and rapid initial ADP
4 secretion, that exhibits an autocrine function particularly relevant in conditions of platelet activation with low
5 agonist concentrations. This pathway is triggered by NAADP, while the other pathway, taking place secondarily,
6 is dependent on SERCA2b stores mobilized exclusively by IP3 (Online Figure XI).
7 The topological difference between SERCAs, SERCA3 being located at the periphery of the platelet membrane ²⁹.
8 ³⁰ (and unpublished) where it participates in the regulation of the Store Operated Calcium Entry (SOCE) through
9 the plasma membrane,³² is in agreement with the fact that Ca²⁺ mobilization visualized with the Fura-2-NM-AM
10 probe, designed to associate with the inner leaflet of the membrane,^{34,48} originated only from SERCA3 stores, but
11 not from SERCA2b stores. This suggests that SERCA3 stores mobilization occurs preferentially near the plasma
12 membrane, consistent with early secretion.

13 Of note, our observation shows that in addition to participating to early secretion, SERCA3 stores contribute to
14 secretion throughout, since late secretion (3 min) is also defective in Serca3^{-/-} platelets upon thrombin stimulation
15 (Figure 1). Interestingly defective SERCA3-dependent secretion is not due to a global defect in cytosolic Ca²⁺ rise,
16 since high thrombin concentration (0.1 μM/mL) which restores normal Ca²⁺ levels in Serca3^{-/-} platelets, does not
17 rescue defective secretion (data not shown, and ref 33, Figure 4). This thus argues against a simple control of
18 cytosolic Ca²⁺ levels by SERCA3 stores, together with SERCA2b stores, in the regulation of secretion, and rather,
19 points to a more specific role, such as control of peripheral Ca²⁺. However, we do not exclude a role for SERCA3
20 in enhancing platelet reactivity to agonists, acting together with SERCA2b-dependent Ca²⁺ stores.

21 Our recording systems could not discriminate between SERCA3/NAADP and SERCA2b/IP3-dependent Ca²⁺
22 mobilization kinetics. It is possible that both mobilizations initiated within the same time frame. But our findings
23 show that the PLC/IP3 pathway is amplified following Ca²⁺ mobilization from SERCA3 stores and ADP secretion.
24 This means that SERCA3 stores might be mobilized first and suggests the existence of an NAADP store or the
25 rapid generation of NAADP prior to IP3 production. A study in platelets on the role of CD38, a membrane enzyme
26 that catalyzes the exchange of the nicotinamide group of NADP⁺ with the nicotinic acid to synthesize NAADP,⁴⁹
27 suggested that CD38 products were involved in platelet functions. Others observed a decreased Ca²⁺ signal in a
28 CD38^{-/-} mouse model, or upon inhibition of NAADP-sensitive stores.³⁹ These authors also suggested that NAADP
29 synthesis was secondary to IP3 synthesis, since NAADP production was reduced (not blocked) by the PLC inhibitor
30 U73122.³⁹ Our results are clearly in contradiction with this observation since in our hands, NAADP-dependent
31 mobilization takes place within the 5 first seconds of activation and is not sensitive to PLC inhibition by U73122.
32 Different experimental conditions may explain these opposite results: these authors used high concentrations of
33 thrombin, 500 μM/mL, more than 10 times higher than 40 μM/mL in our study. Also, platelets were subjected to
34 aggregation which adds up activation signals as well as signaling downstream integrin α_{IIb}β₃, while we focused on
35 initial activation and used unstirred conditions to prevent α_{IIb}β₃ engagement and subsequent secretion.

36 One of our most relevant findings is that early ADP secretion appeared detectable within seconds of agonist
37 stimulation of platelets and in a manner fully dependent on the SERCA3-NAADP pathway, while a secondary ADP
38 secretion pathway follows, dependent on SERCA2b and IP3. The delay between SERCA3/NAADP-dependent and
39 SERCA2b/IP3-dependent secretions could be due to differential signaling and/or secretory machinery: for example,
40 SERCA3 stores which are peripheral will mobilize peripheral granules, thus inducing a quick exit of ADP, while
41 SERCA2b stores, more central, will mobilize granules further away from the membrane and their exocytosis will
42 take longer. The major consequence is that both pathways regulate secretion sequentially, at least initially. This
43 temporal organization, with NAADP-dependent Ca²⁺ mobilization ahead of other mobilization pathways has
44 already been observed in a number of other cell types, including sea urchin sperm,⁵⁰ pancreatic cells,⁵¹ or T-
45 lymphocytes.^{52,53} Though dense granule secretion (or equivalent, such as lysosomes) has not been documented in
46 these cell types and correlated with Ca²⁺ mobilization differential pathways, our observation may be a general
47 mechanism by which an NAADP-SERCA3 Ca²⁺ mobilization pathway regulates an early secretion of functional
48 relevance to trigger full activation of cells. The fact that Ca²⁺ mobilization from SERCA3 stores whether induced
49 directly by NAADP-AM or by thrombin in presence of apyrase did not trigger a secondary Ca²⁺ mobilization
50 suggests that, at least, in platelets, NAADP-dependent Ca²⁺ mobilization leads to secondary Ca²⁺ mobilization (from
51 SERCA2b stores) not directly via Calcium Induced Calcium Release (CICR) but indirectly via ADP release and
52 signaling. Furthermore, we observed that direct mobilization of Ca²⁺ using NAADP-AM was unable to induce
53 primary secretion nor platelet aggregation, both requiring an additional stimulus. Only when NAADP-AM was
54 associated to PMA or OAG-AM, both PKC activators, we observed an ADP secretion of the same level than that
55 observed at 5 seconds of thrombin stimulation. This result suggests that Ca²⁺ mobilized from SERCA3 stores and
56 diacyl-glycerol (DAG, from phospholipids breakdown by phospholipases) are needed to induce initial ADP
57 secretion. It is possible that in physiological conditions most of the DAG is produced by PLC. However an

1 alternative possibility is that U73122 did not block initial ADP secretion because of another source of DAG,
2 U73122-insensitive: potential candidates are phosphatidic acid phosphatases,⁵⁴ phospholipid transferases,
3 monoglycerol acyl transferases, triacylglycerol lipases.⁵⁵ This possibility is currently under investigation.
4 One issue raised by this study is related to the nature of the first wave of ADP (and ATP) release upon
5 NAADP/SERCA3 Ca²⁺ store mobilization: since only a fraction of the total content of dense granules is released,
6 this brings up the possibility of 2 separate dense granule populations, each associated with one type of SERCA Ca²⁺
7 stores signaling pathway: according to this hypothesis, one subtype of dense granules would be physically and/or
8 functionally associated with SERCA3 stores, and would be the only granules able to release their content upon Ca²⁺
9 mobilization from SERCA3 stores. The remaining granules would then enter the secretory pathway upon Ca²⁺
10 mobilization from the SERCA2b stores. Alternatively, the dense granule population would be homogeneous but
11 granules close to SERCA3 stores (possibly near the plasma membrane) would secrete their content upon Ca²⁺
12 mobilization from these stores. Distinguishing between these two possibilities will require further investigations.
13 Finally, we recently observed in platelets from patients with severe obesity, a pathological condition associated
14 with increased circulating pro-thrombotic factors, an acquired decrease in SERCA3 expression associated with
15 lower ability of platelets to get activated.⁵⁶ This observation suggests that targeting the NAADP/SERCA3 signaling
16 pathway could be a way to limit spontaneous platelet activation, representing a potential future anti-thrombotic drug
17 therapy.

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11 Disclosure of conflicts-of-interest

12 The authors declare no conflict-of-interest.

13 Supplemental materials

14 Online Materials and method details

15 Online Figures I-XI

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34

1 Figure legends

2 **Figure 1. SERCA3-dependent ADP secretion occurs early and precedes SERCA2b-dependent secretion.**

3
4 WT (open squares) and Serca3^{-/-} (gray circles) washed platelets in suspension (9x10⁷ in 300 μL) were stimulated
5 with thrombin (40 mU/mL) for 3 min and released ADP (A) or ATP (B) in the supernatants (10 μL) were assessed
6 (see Methods). Data are expressed as dot plots and means ± SEM using 2-way ANOVA, followed by Tukey's post-
7 test, n=3. The kinetics of secretion of ADP (C) and ATP (D) were assessed in the same conditions, but thrombin
8 stimulation was stopped at indicated time points by addition of the thrombin inhibitor D-Phe-Pro-Arg-
9 chloromethylketone (PPACK, 100 μM) and immediate chilling of platelets on ice and pelleting at 4°C. To
10 demonstrate functional involvement of SERCA3 during secretion, tBHQ (10 μM) was preincubated with platelets
11 for 5 min prior to thrombin stimulation (40 mU/mL) and ATP secretion measured in the supernatant of WT (open
12 triangles) or Serca3^{-/-} (gray triangles) platelets at 0, 5, 10 and 180 seconds. Data are expressed as dot plots and
13 means ± SEM, 2-way ANOVA, followed by Tukey's multiple comparison test, n=3 (C) and dot plots and means ±
14 SEM, 3-way ANOVA, followed by Tukey's multiple comparison test, n = 4 (D).
15

16 **Figure 2. Ca²⁺ mobilization from SERCA3 stores is specifically detected by the intracellular plasma** 17 **membrane-associated probe Fura-2-NearMembrane and is independent of ADP.**

18 Washed platelets immobilized on glass coverslips coated with poly-D-lysine, were loaded with Fura-2-AM (Fura-
19 2, left panel & tracings) or Fura-2-NearMembrane-AM (Fura-2-NM, right panel & tracings), and activated with
20 thrombin (50 mU/mL). Fluorescence intensity images were recorded at 340 nm and 380 nm using videomicroscopy.
21 As described in methods, cCa²⁺ concentration ([Ca²⁺]) in WT and Serca3^{-/-} platelets was visualized by pseudocolor
22 imaging generated from the ratio of fluorescence intensities before and after thrombin stimulation with Fura-2 (A)
23 or Fura-2-NM (B). Scale bar = 5 μm. The kinetics of [Ca²⁺] intensity were assessed in Arbitrary Units (AU) using
24 the 340/380 nm excitation fluorescence ratio, the value 1 corresponding to the basal level. WT (black tracings), and
25 Serca3^{-/-} (gray tracings) platelets were stimulated with thrombin alone, and visualized with Fura-2 (C) or Fura-2-
26 NM (E). To assess SERCA3 functional involvement, platelets labeled with Fura-2 (D, G) or Fura-2-NM (F, H)
27 were incubated with the SERCA3 inhibitor tBHQ (10 μM) (D, F), or the SERCA2b inhibitor thapsigargin (Tg, 200
28 nM) (G, H), and challenged with thrombin (50 mU/mL). Recordings shown are representative of 3 independent
29 experiments.

30 To assess the role of ADP, platelets were activated with thrombin (Thr) alone (50 mU/mL) (I, K, or in the presence
31 of the nucleotide phosphate scavenger apyrase (APY, 10 U/mL) (J, L), or of ADP (10 μM) (M, N). Data presented
32 are representative of 3 independent experiments. Scale bar = 1 min.
33

34 **Figure 3. Ca²⁺ mobilization from SERCA3 stores is independent of IP3.**

35 Washed WT or Serca3^{-/-} platelets preloaded with Fura-2 (A) or Fura-2-NM (B) were immobilized on poly-D-lysine
36 glass coverslips and fluorescence intensity at 340 & 380nm was recorded using videomicroscopy. Platelets were
37 preincubated for 1 minute with DMF (N, N-dimethylformamide) as control or with 0.75 μM U73122 (in order to
38 block IP3 production) before stimulation by thrombin (Thr) alone (50 mU/mL) or Thr in the presence of ADP (10
39 μM). Ca²⁺ mobilization visualized under Fura-2-AM (Fura-2) or Fura-2-NM (Fura-2-NM) was quantitated as area
40 under curves (tracings shown in Supplemental Figures S4) for a time lapse of 2 min, relative to thrombin in WT
41 platelets taken as 100%: open squares, WT and gray circles, Serca3^{-/-}; absence (-) or presence (+) of U73122 or
42 ADP are indicated below the graph. Data are presented as dot plots and means ± SEM, using the 2-way ANOVA
43 followed by Tukey's multiple comparison test (a) n=4 (b) n=3. WT (black tracings) and Serca3^{-/-} (gray tracings)
44 platelets, preloaded with Fura-2 (C) or with Fura-2-NM (D), and immobilized on poly-D-lysine glass coverslips,
45 were stimulated with IP3-AM (IP3) (10 μM). The images taken before and after IP3-AM stimulation are presented
46 in pseudocolors corresponding to the 340/380 ratio of fluorescence intensity (scale on the right) as visualized with
47 Fura-2 (C) or with Fura-2-NM (D) and corresponding curves are presented below the images. Scale bar = 5 μm. In
48 (E) and (F) Fura-2 loaded platelets were pretreated with either Tg (200 nM) for 8 min or with tBHQ (10 μM) for 5
49 min, respectively, before stimulation with IP3-AM (10 μM). (G): Quantitation of Ca²⁺ mobilization in WT or
50 Serca3^{-/-} platelets under Fura-2 was assessed by areas under curves for a time lapse of 2 min. WT (open squares)
51 and Serca3^{-/-} (gray circles); absence (-) or presence (+) of IP3, Tg (n=5) or tBHQ (n=6) are indicated below the
52 graphs. Data are expressed as dot plots and means ± SEM, using the 2-way ANOVA followed by Tukey's multiple
53 comparison test.
54

1 **Figure 4. Ca²⁺ mobilization of SERCA3 stores is blocked by the NAADP antagonist Ned-19 and directly**
2 **stimulated with NAADP.**

3 Washed platelets preloaded with Fura-2 (A, C, E, G) or Fura-2-NM (B, D, F, H) were immobilized on poly-D-
4 lysine glass coverslips and fluorescence intensity at 340 & 380 nm was recorded using videomicroscopy. (A, B)
5 Platelets were pre-incubated with either DMSO, U73122 (0.75 μM) or the NAADP receptor inhibitor Ned-19 (0.5
6 μM) and stimulated with thrombin (50 mU/mL) or thrombin and ADP (10 μM). Areas under curves (tracings shown
7 in Online Figure V) for 2 min were quantitated and expressed as % of the thrombin response of WT platelets. WT
8 platelets, open squares; Serca3^{-/-} platelets, gray circles; absence (-) or presence (+) of thrombin (Thr), Ned-19, ADP
9 or U73122 is indicated below the graphs. Data are presented as dot plots and means ± SEM using the 2-way
10 ANOVA followed by Tukey's multiple comparison test (A) n=4, (B) n=3.

11 Platelets were then challenged with NAADP-AM (NAADP) (1 μM) alone (C, D), or after preincubation with either
12 Ned-19 (0.5 μM) (E, F), or tBHQ (10 μM) (G, H). [Ca²⁺] was calculated as indicated in Methods as relative
13 fluorescent ratios to basal level. Scale bar = 1 min. Areas under curves for 2 min, visualized with Fura-2 or Fura-2-
14 NM, are displayed in Online Figure VI. Data are representative of 3 to 4 experiments.

15
16 **Figure 5. Role of SERCA3, IP3 and NAADP in secretion.**

17 Washed WT (open squares) or Serca3^{-/-} (gray circles) platelets were activated with thrombin (40 mU/mL), then the
18 reaction was stopped by addition of PPACK (100 μM) at different time points (5, 10 or 180 sec), and platelet
19 chilling on ice before centrifugation. ATP was assessed (in pmoles) in supernatants as described in Methods. (A)
20 As indicated by (+), platelets were pretreated with Ned-19 (0.5 μM) or U73122 (0.75 μM). Data are presented as
21 dot plots and means ± SEM, n=4, using the 2-way ANOVA followed by Tukey's multiple comparison test. (B)
22 Restoration of ATP secretion by ADP (10 μM) added to thrombin (Thr, 40 mU/mL) was assessed after 180 sec in
23 the supernatant of WT (open squares) or Serca3^{-/-} (gray circles) platelets pretreated (+) or not (-) with Ned-19. Data
24 are presented as dot plots and means ± SEM, n=3, using the 2-way ANOVA followed by Tukey's multiple
25 comparison test. (C) Secretion induced by NAADP-AM (NAADP, 1 μM) for 180 sec was assessed in absence (-)
26 or presence (+) of 10 nM PMA. Data are presented as dot plots and means ± SEM, n=4, using the the 2-way ANOVA
27 followed by Bonferroni's multiple comparison test. (D) Comparative secretion at 180 sec as induced by NAADP (1
28 μM), PMA (10 nM) or IP3-AM (IP3, 10μM) alone or in combination for 180 sec. Thrombin (Thr, 40 mU/mL, 180
29 sec) is used as positive control. (-) absence, (+) presence of agonist. Data are presented as dot plots and means ±
30 SEM, n=8 or n=3 (for combination of NAADP, IP3 and PMA), using the mixed-effects analysis (REML) followed
31 by Tukey's multiple comparison test.

32
33 **Figure 6. Role of SERCA3, IP3 and NAADP in aggregation.**

34 Washed WT (black tracings) or Serca3^{-/-} (gray tracings) platelets were subjected to aggregation in a Chronolog
35 Lumiaggregometer (Chrono-Log Corporation, USA) after activation with thrombin (40 mU/mL) (A), in the
36 presence of Ned-19 (0.5 μM) (B), Ned-19 and ADP (10 μM) (C), U73122 (0.75 μM) (D) or U73122 and Ned-19
37 (E). Arrows indicate the time point of agonist addition. Scale bar = 1 min. Aggregation recordings are representative
38 of at least 3 independent experiments.

39
40 **Figure 7. The SERCA3/NAADP pathway is independent of thromboxane A₂.**

41 BAPTA-Oregon green loaded platelets from WT (black tracings) or Serca3^{-/-} (gray tracings) animals, pretreated or
42 not (A, C) with indomethacin (B, C) for 10 min alone (n=6) or in combination with Ned-19 (0.5 μM) (n=3) for 3
43 min (c) were analyzed for Ca²⁺ mobilization in response to thrombin 40mU/mL (Thr) using flow cytometry.
44 Restoration of Ca²⁺ mobilization by ADP (10μM) added to thrombin in indomethacin pretreated platelets was
45 assessed (n=3). Areas under curves for 2 min were quantitated and expressed as % of the thrombin response of WT
46 platelets taken as 100%. WT platelets, open squares; Serca3^{-/-} platelets, gray circles; absence (-) or presence (+) of
47 Ned-19, Indomethacin (Indo) or ADP is indicated below the graphs. Data are expressed as dot plots and means ±
48 SEM, using the mixed effects analysis (REML) followed by Bonferroni's multiple comparison test. (D) Washed
49 WT (open squares) or Serca3^{-/-} (gray circles) platelets, pretreated for 10 min with indomethacin (5 μM) (open
50 triangles and gray triangles for WT and Serca3^{-/-} platelets respectively) were activated with thrombin (40 mU/mL),
51 then the reaction was stopped by addition of PPACK (100 μM) and platelet chilling and centrifuging at different
52 time points of 0 sec, 5 sec, 10 sec or 180 sec, and ATP was assessed (in pmoles) in supernatants as described in
53 Methods. Data are expressed as dot plots (n=6) and means ± SEM, using the 3-way ANOVA followed by Tukey's
54 multiple comparison test. (E) WT (open squares) or Serca3^{-/-} (gray circles) platelets pretreated for 10 min with (+)
55 or not (-) with indomethacin (5 μM) (n=8) and/or Ned-19 (0.5μM) (n=3) for 10 and 3 min, respectively, prior
56 activation with thrombin for 180 sec. Restoration of ATP secretion by ADP (10 μM) added (n=3) to thrombin (Thr,

1 40 mU/mL) in indomethacin pretreated platelets was assessed. Thrombin (Thr, 40 mU/mL, 180 sec) is used as
2 positive control. Data are presented as dot plots and means \pm SEM, using the mixed-effects analysis (REML)
3 followed by Bonferroni's multiple comparison test.

4
5 **Figure 8. Human platelets display early ADP secretion dependent on a SERCA3/NAADP pathway.** (A) For
6 Ca^{2+} mobilization experiments, human platelets were loaded with BAPTA-Oregon green 488 and pretreated with
7 10 μM tBQH for 5 min (gray tracing), 0.5 μM Ned-19 for 3 min (dot tracing) or 5 μM indomethacin for 10 min
8 (thin black tracing) or solvent (thick black tracing) before flow cytometry analysis of Ca^{2+} mobilization in response
9 to thrombin (Thr, 40 mU/mL). (B) Areas under curves for 2 min were quantitated and expressed as % of the
10 thrombin response taken as 100% (black squares). The presence of tBHQ (open squares), Ned-19 (grey squares) or
11 Indomethacin (Indo, black & white squares) is indicated below the graphs. Data are presented as dot plots with
12 means \pm SEM, n = 4, 1-way ANOVA followed by Tukey's multiple comparison test. (C) human platelets pretreated
13 with 0.5 μM Ned-19 for 3 min (circles) or 5 μM indomethacin for 10 min (triangles) were stimulated with thrombin
14 (50 mU/mL) (open) or thrombin and ADP (10 μM) (grey). Areas under curves for 2 min were quantitated and
15 expressed as % of the thrombin response in untreated platelets (black squares) taken as 100%. Data are
16 representative of 3 experiments, and expressed as dot plots with means \pm SEM, 1-way ANOVA followed by Tukey's
17 multiple comparison test.

18 For secretion experiments, washed platelets were activated with thrombin (40 mU/mL), then the reaction was
19 stopped by the PPACK/ice method at 5 sec (D), 10 sec (E) or 180 sec (F), and ATP was assessed (in pmoles) in
20 supernatants as described in Methods. As indicated, platelets were pretreated with 10 μM tBHQ for 5 min (open
21 squares), 0.5 μM Ned-19 for 3 min (grey squares) or 5 μM indomethacin for 10 min (black & white squares) or
22 solvent (black squares). Data are expressed as dot plots with means \pm SEM, n=3, 1-way ANOVA followed by
23 Tukey's multiple comparison test. (G and H) Washed platelets were pretreated with 0.5 μM Ned-19 for 3min or 5
24 μM indomethacin for 10 min and were then activated with thrombin (50 mU/mL) (open squares) or thrombin and
25 ADP (10 μM) (grey squares). Data are presented as dot plots with means \pm SEM, n=4, 2-way ANOVA followed
26 by Tukey's multiple comparison test.

27

Figure 1

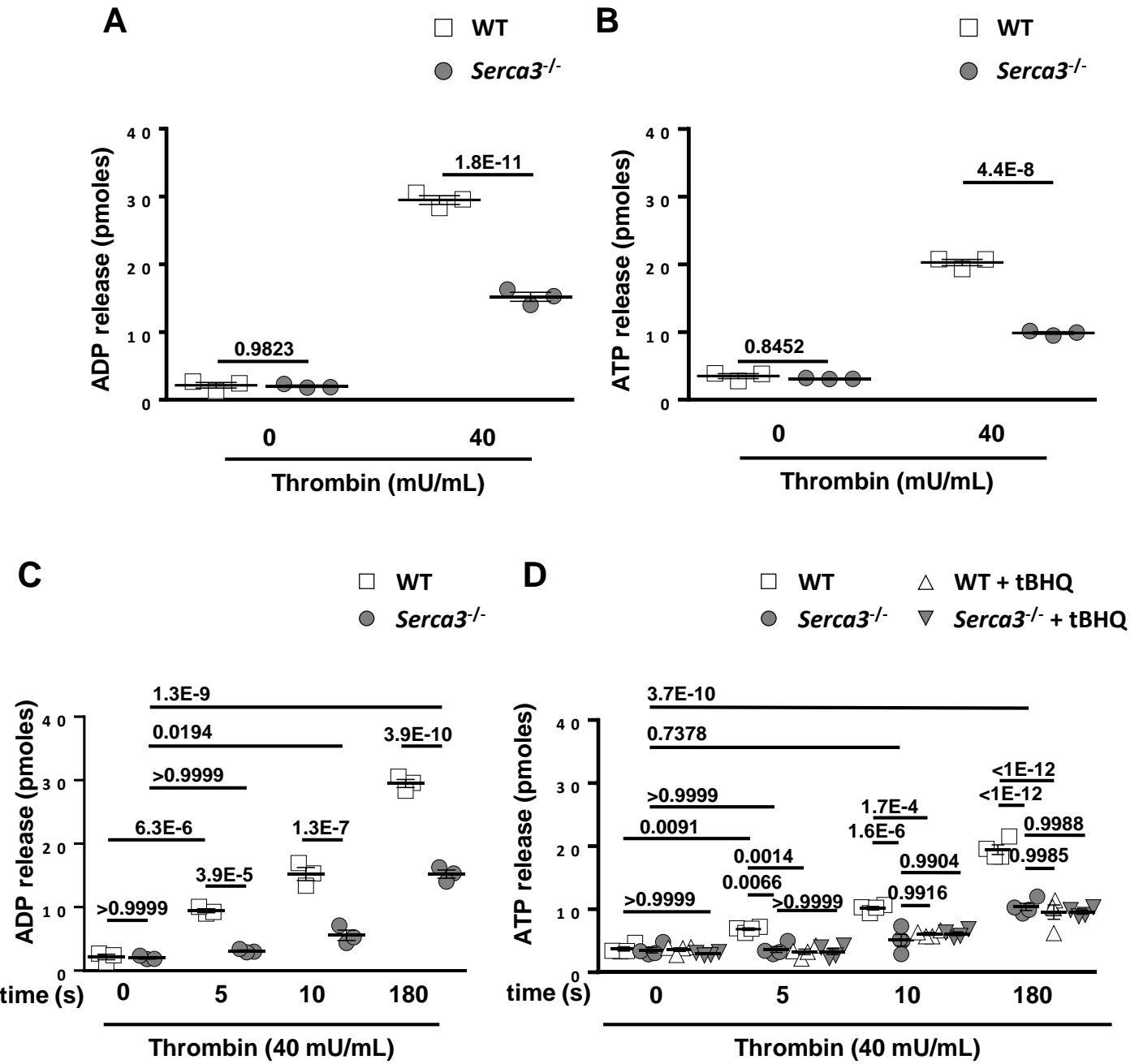
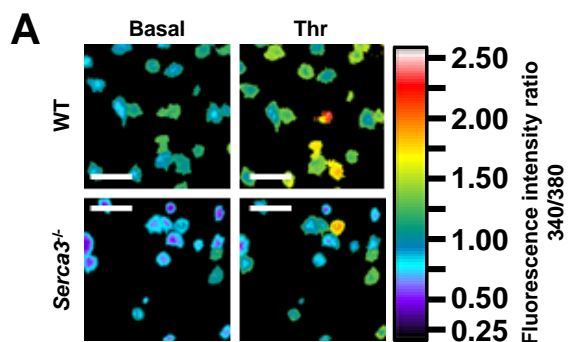
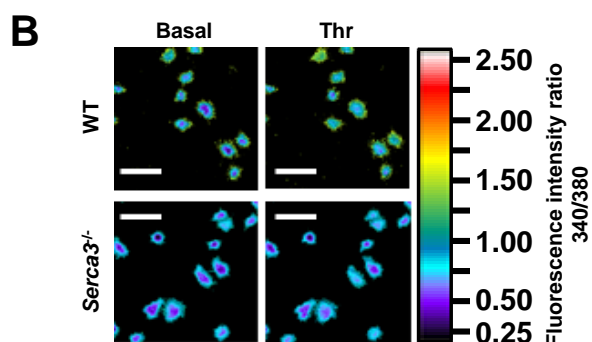


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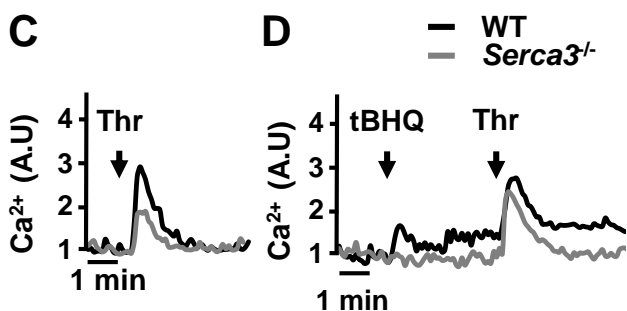
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Fura-2-NM



Fura-2



Fura-2-NM

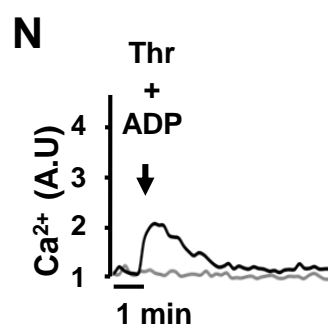
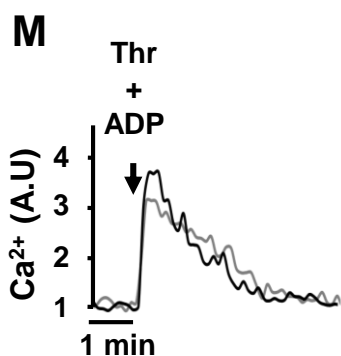
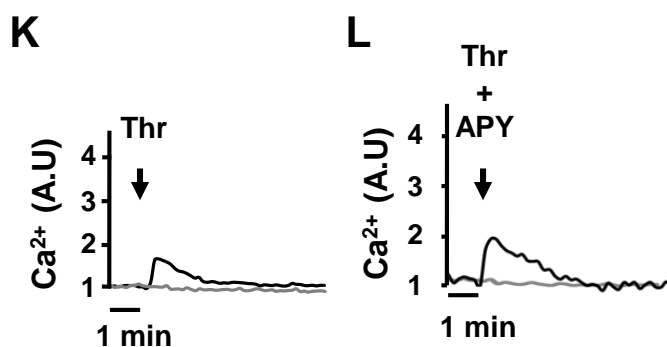
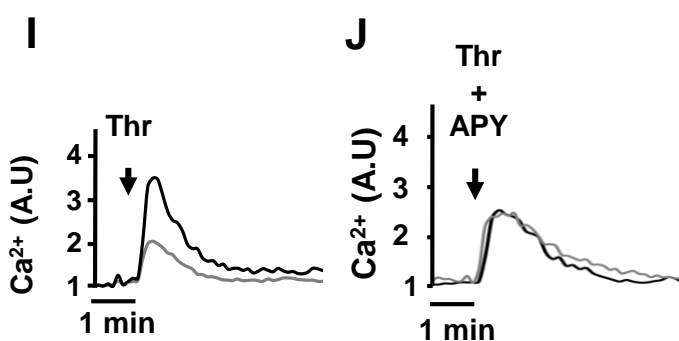
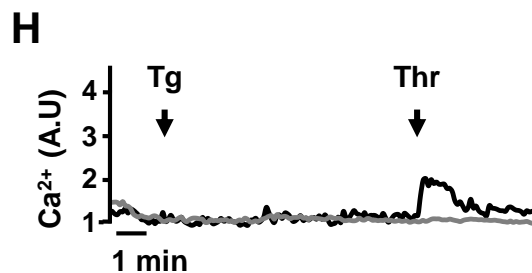
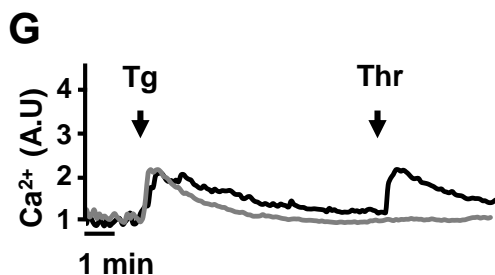
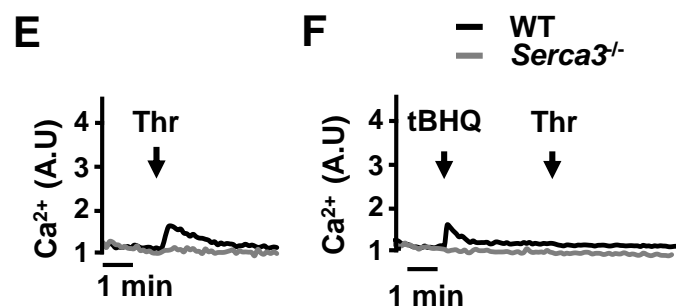


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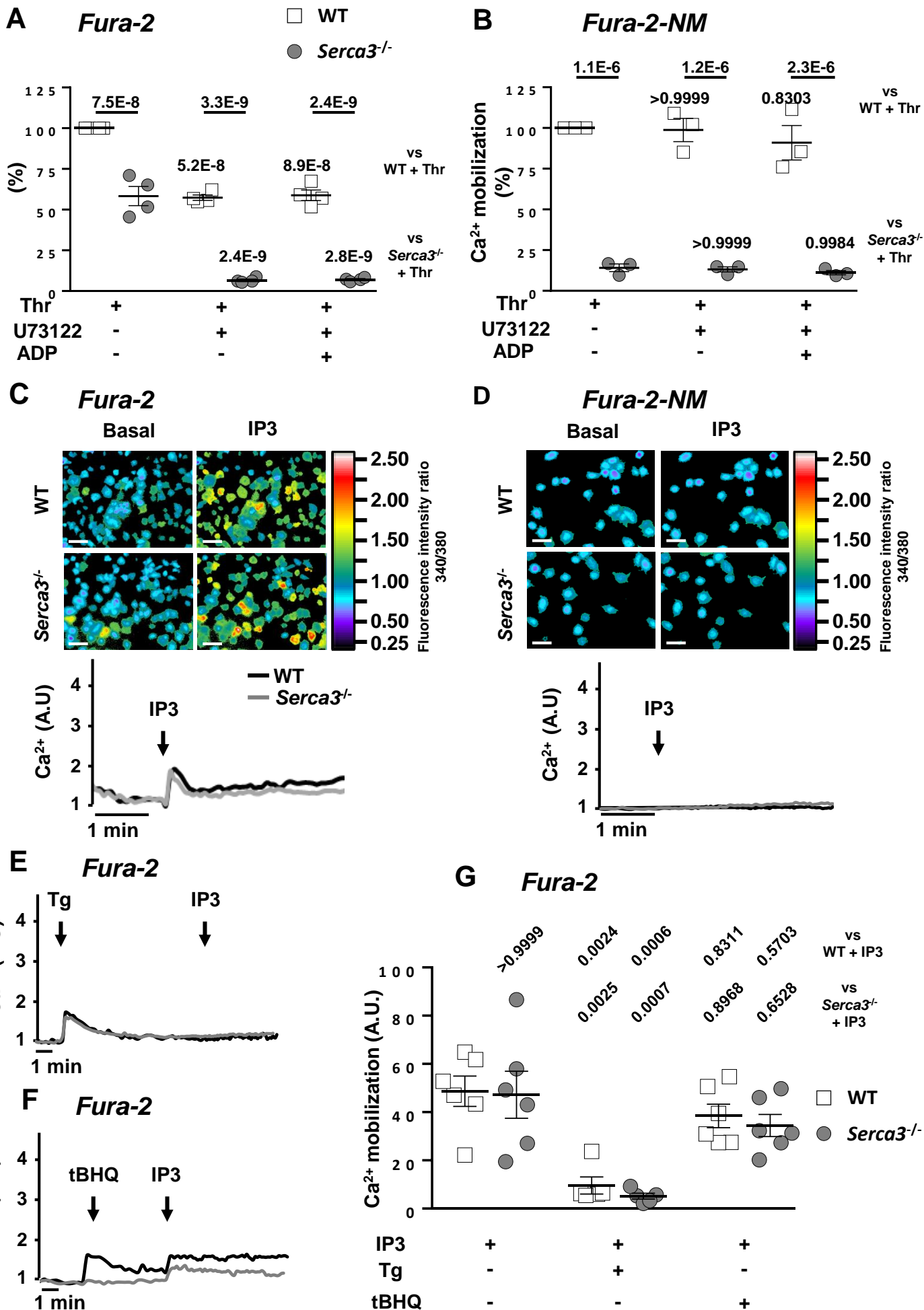


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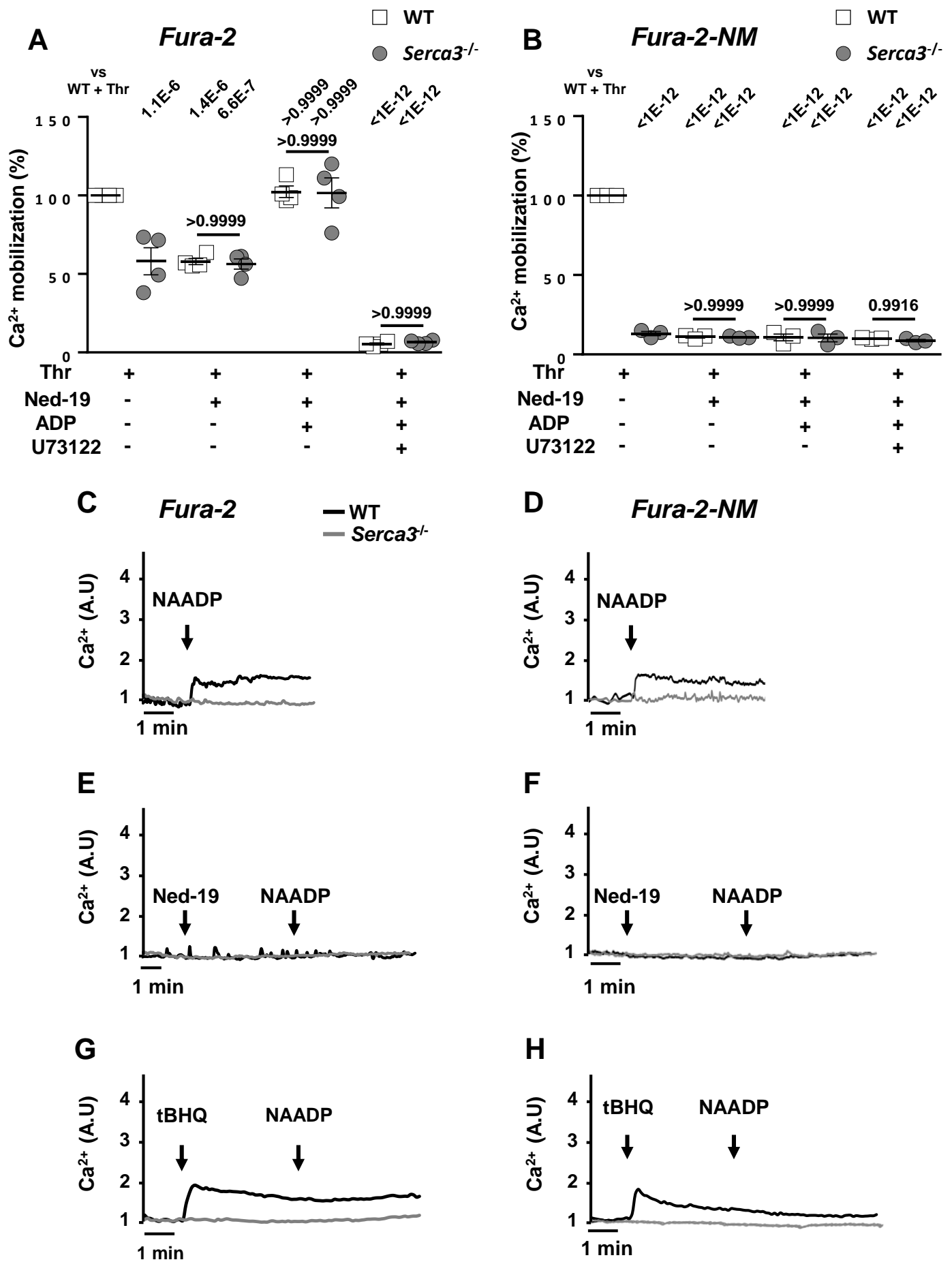


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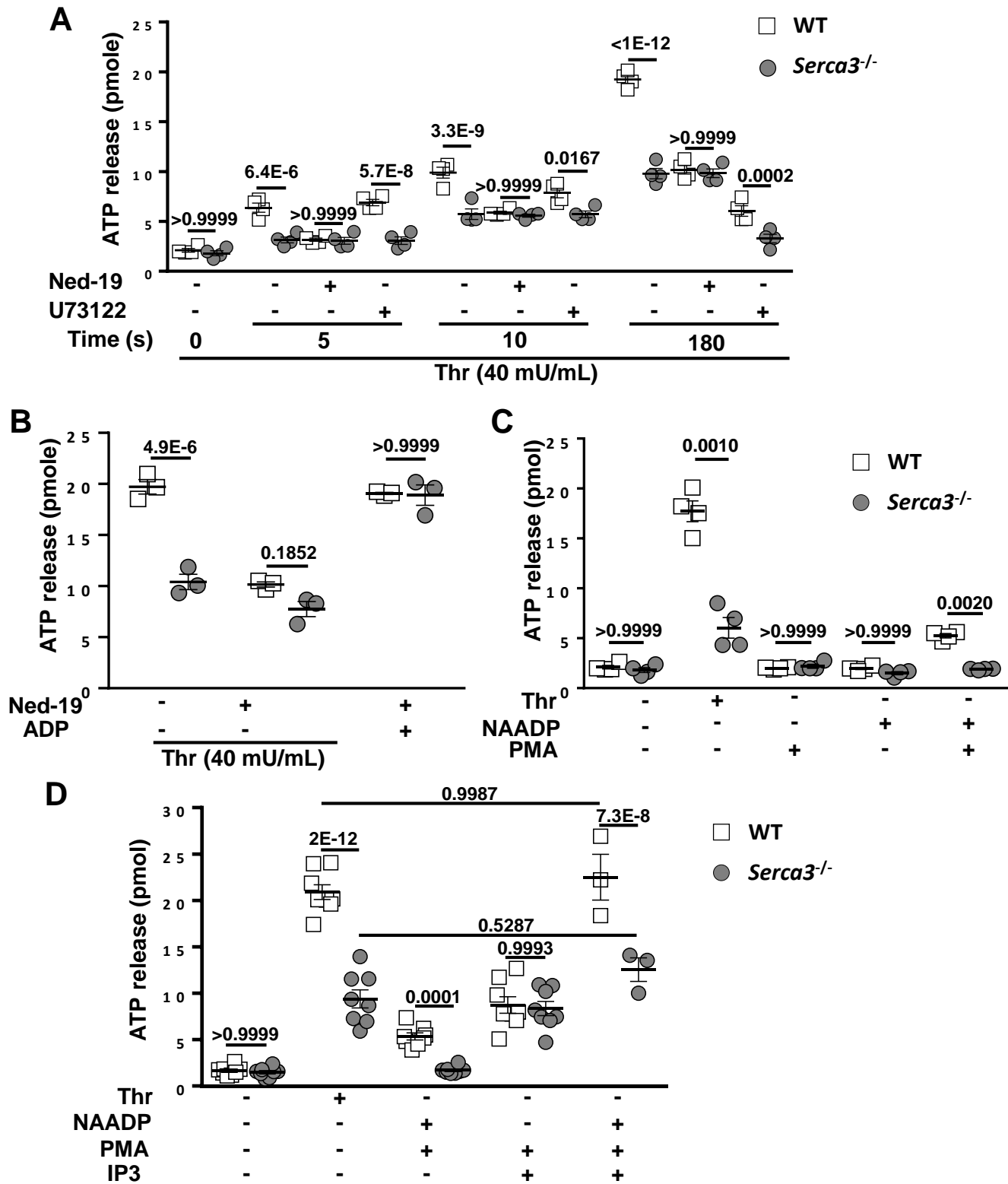


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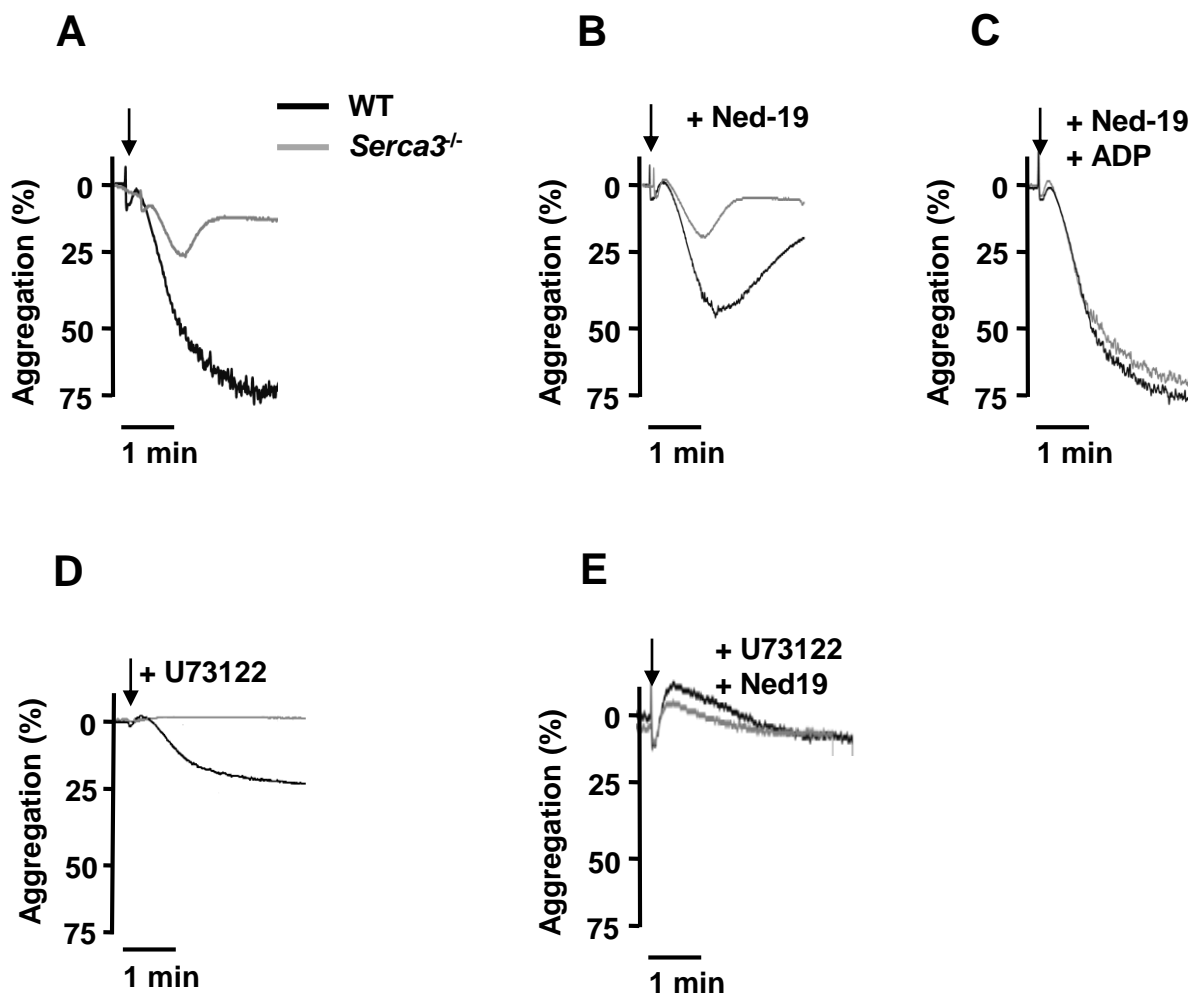


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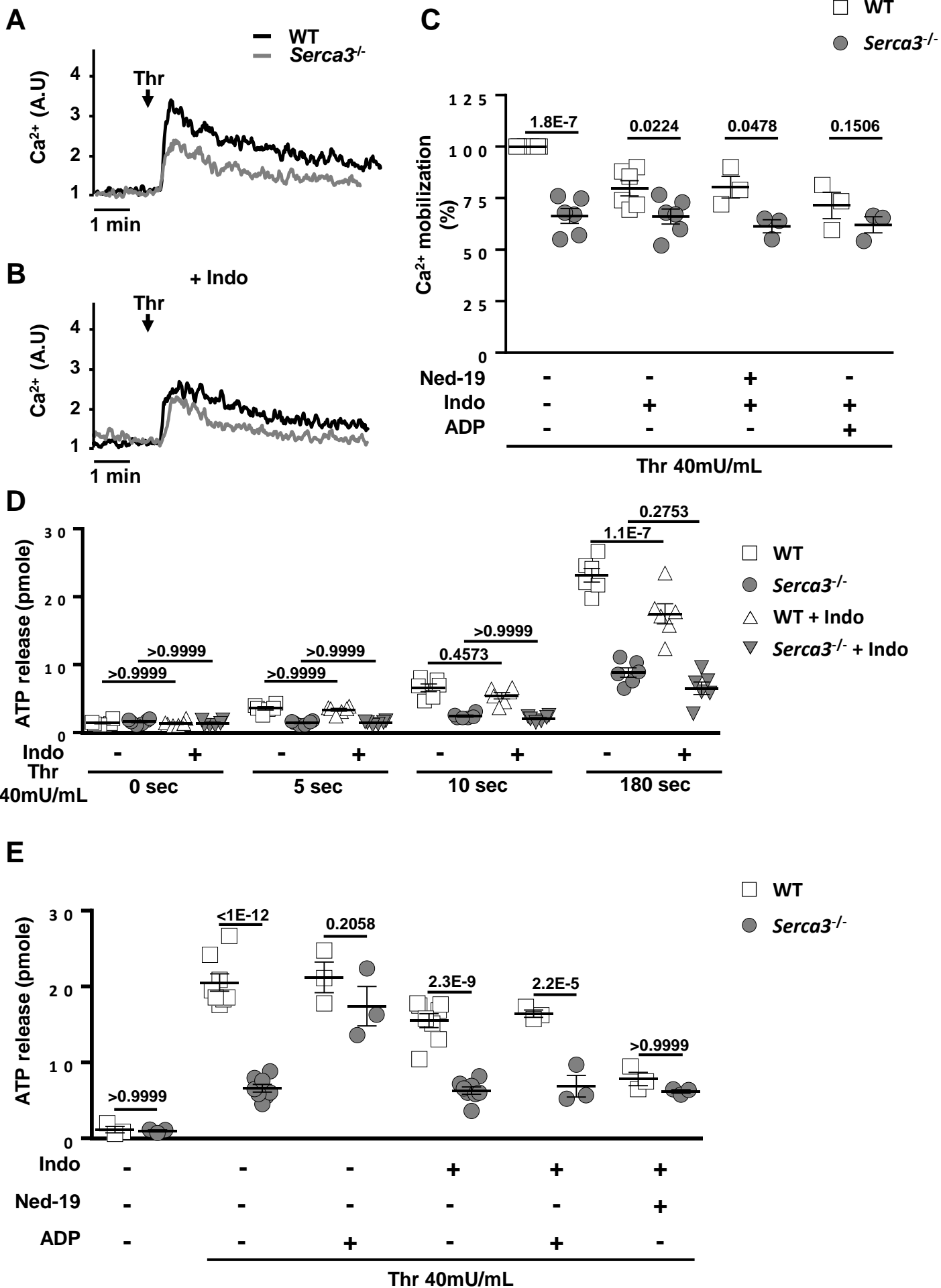


Figure 8

