On-line Figure Legends

Fig. 1. *Assessment of cardiomyocyte preparation and expression of renin-angiotensin components in cardiomyocytes*. **A**, The purity of isolated cardiomyocytes was assessed by Western blot, with aliquots (40 μg each) of proteins isolated from cardiomyocytes, fibroblasts, smooth muscle cells, endothelial cells and whole-brain. Proteins were separated by SDS-PAGE and transferred onto PVDF. After blocking, membranes were incubated with antisera specific for Van Willebrand's Factor (VWF), neuron-specific enolase (NSE), discoidin domain receptor 2 (DDR2), α-smooth muscle actin (αSMA), myosin heavy chain (MyHC) and GAPDH. Immunoreactive bands were visualized by ECL (examples of Western blots are illustrated, we performed 4 separate experiments/antibody/fraction) **B**, The expression of renin-angiotensin components were confirmed with RNA isolated from the cytosol of cardiac myocytes, by qRT-PCR with primers for angiotensinogen (AGT), angiotensin converting enzyme (ACE), cathepsin and renin, data are presented as mRNA expression relative to an average of three housekeeping genes (N=8/primer)

Fig. 2. *Immunoreactivity of angiotensin II receptor subtypes 1 and 2 in purified cardiac nuclei.* **A**, Rat hearts were fractionated into four fractions: total extract (A), supernatant (B), crude nuclear (C) and enriched nuclear (D) fractions. Aliquots (30 μg) of each fraction were separated by SDS-PAGE and transferred onto PVDF. After blocking, membranes were incubated with antisera specific for Calpactin I, GRP78, Nup62, GM130, AT1 or AT2 (examples of Western blots are illustrated, N=6/antibody/per fraction). Immunoreactive bands were visualized by ECL. **B**, Mean±SEM expression levels for GRP78, GM130, Nup62, AT1, AT2 normalized to Calpactin I. *P<0.05, **P<0.01 vs. respective fraction in B. **C**, Representative images of a purified nuclear preparation. **Left**: DNA-staining with Hoechst 33342; **Right**: Corresponding phase-contrast image. **D**, Mean±SEM DNA content (μg/ml) of cytoplasmic or nuclear fractions (N=10/group). ***P<0.001 vs. cytoplasmic.

Fig. 3. *Endocytosis and intracellular trafficking of Ang-II*. **A**, FITC-Ang-II (1 nM) was applied extracellularly to the bathing medium. Images are shown immediately after Ang-II application (0 min) and then 30 and 60 min later. Merged images are superimposed fluorescent images. **B,** cells were preincubated with valsartan (10 μM, for 25 min) prior to administration of Ang-II. Hoechst 33342 was used as a nuclear marker. Z-stacks were acquired every 5 min using a Zeiss LSM-510 confocal microscope. (N=6/group)

Fig. 4. *AT1R and AT2R in nuclear membranes regulate transcription initiation*. **A**, De novo RNA synthesis. Isolated nuclear preparations were stimulated with Ang-II, L 162,313 (a non-peptide AT1R-selective agonist), CGP 42112A (an AT2R-selective agonist), candesartan plus Ang-II, or PD123177 plus Ang-II (all at 10-μM), as indicated. Following the addition of antagonists candesartan (Cand) and PD123177, nuclei were preincubated for 30 min prior to the addition of Ang-II. ***P<0.001 vs. control; $^{++}$P<0.01 vs. Ang-II. **B**, Nuclei were treated with PTX 5 μg/ml for 2 hours and then stimulated with Ang-II. Data represent mean ± SEM of at least five separate experiments performed in triplicate and normalized to control. ***P<0.001 vs. control; N.S. = non-significant vs. control. **C**, representative 2% agarose gel electrophoresis of RNA extracted from purified nuclei stained with ethidium bromide (GTP and CTP not omitted) under the following conditions: non-stimulated (lane 1); stimulated with Ang-II (10 μM, lane 2); stimulated with Ang-II in the presence of valsartan (10 μM, lane 3); stimulated with Ang-II in the presence of PD123177 (10 μM, lane 4). 1 μg of each sample was loaded. Bottom panel shows mean±SEM results as arbitrary optical density (O.D.) units from 6 different experiments/condition. **P<0.01 vs. control, $^{++}$P<0.01 vs. Ang-II.

Fig. 5. *Regulation of nuclear NFκB mRNA expression by nuclear ATRs*. Isolated nuclei were treated for 2 hours with Ang-II at different concentrations. NFκB mRNA was quantified by qPCR. **A**, Data are presented as NFκB mRNA expression relative to an average of three housekeeping genes (N=6/concentration/group). ***P<0.001 vs. intact cells, N.S. = non-significant. **B**, Valsartan (10 μM) or

PD123177 (10 μM) significantly reduced the Ang-II (10 μM) induced NFκB activity, while pretreatment of nuclei with both AT1 and AT2 antagonists completely abolished the NFκB gene expression-response. Results are expressed as mean±SEM *P<0.05 vs. control, $^{+}$P<0.01 vs. Ang-II, $^{\#}$P<0.01 vs. valsartan, N.S: non significant vs. control

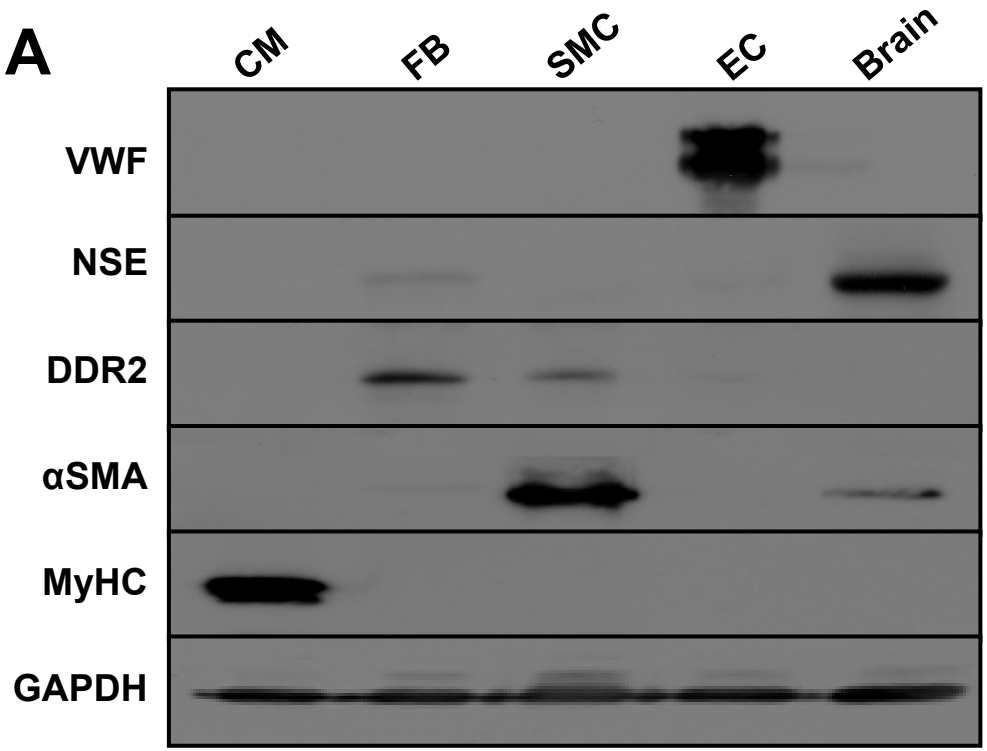
Fig. 6. *Vehicle control for calcium transients.* Typical recordings of intracellular Ca^{2+} are shown, with recordings from isolated nuclei preparations shown in insets, after administration of DMSO

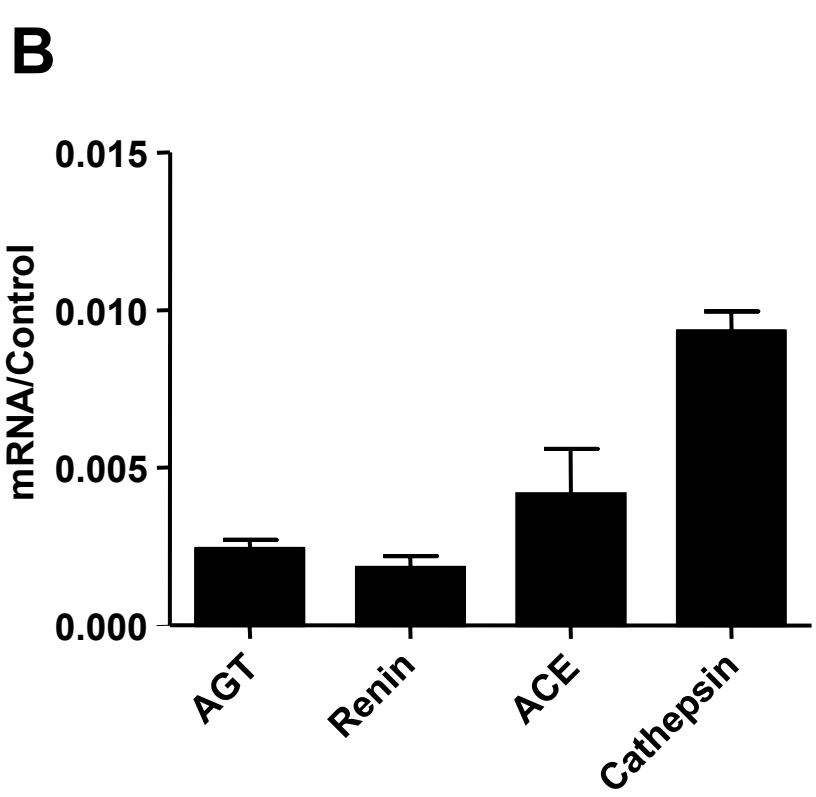
Fig. 7. *Coupling of ATRs to Ca^{2+} entry in nuclear enriched preparations*. FURA-2/AM-loaded freshly isolated cardiac nuclei were seeded onto glass slides and nuclear Ca^{2+} concentration [Ca^{2+}] was measured with an IonOptix microspectrofluorimeter (λex = 340 and 380 nm; λem = 509 nm). Typical recordings of intracellular Ca^{2+} are shown, with recordings from each preparation studied shown in insets, after administration of: **A**, Ang-II (10 μM); **B**, L-162,313 (10 μM); **C**, candesartan (10 μM) plus Ang-II (10 μM); **D**, CGP 42112A (10 μM); **E**, PD123177 (10 μM) plus Ang-II (10 μM). **F**, Summary of mean±SEM [Ca^{2+}] transient amplitudes (N=6/group/condition) *P<0.05 vs. control, N.S. = non-significant vs. control; arrows in **A**-**E** indicate the time of drug application.

Fig. 8. *IP_3-dependent Ca^{2+} signals from isolated nuclei.* Isolated nuclei were exposed to **A**, IP_3 (10 μM). **B**, the IP_3R blocker 2-APB (10 μM) followed by exposure to IP_3. **C**, the IP_3R blocker 2-APB (10 μM) followed by exposure to Ang-II (10 μM). **D**, summary of [Ca^{2+}] transient amplitudes, mean±SEM (N=5/group/condition) ***P<0.001; arrows indicate the time of drug application in **A**-**C**.

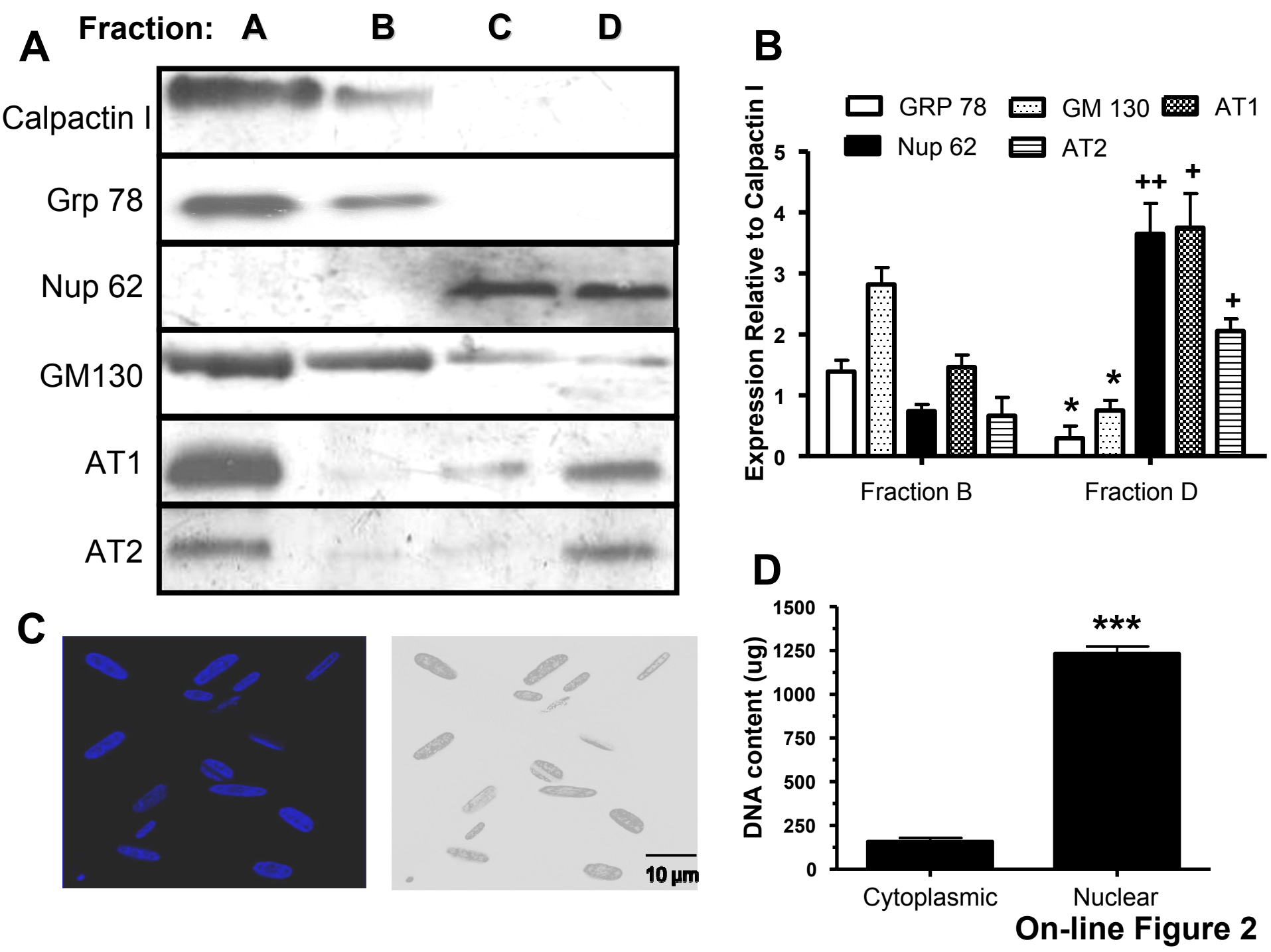
Fig. 9. *Role of IP_3R-signaling for AT1R-mediated transcription initiation*. Isolated nuclei were pre-treated with various concentrations of 2-APB for 30 minutes and incubated with: **A,** Ang-II **B,** L-162,313 or **C,** CGP42112A (all at 10 μM) and de novo RNA synthesis was measured by ^{32}P-UTP incorporation.

***P<0.001 or **P<0.01 vs. agonist alone (Ang-II or L-162,313); $^{\#\#}$P<0.01 or $^{\#}$P<0.05 vs. DMSO or 2-APB alone (15 μM); N.S: non-significant.



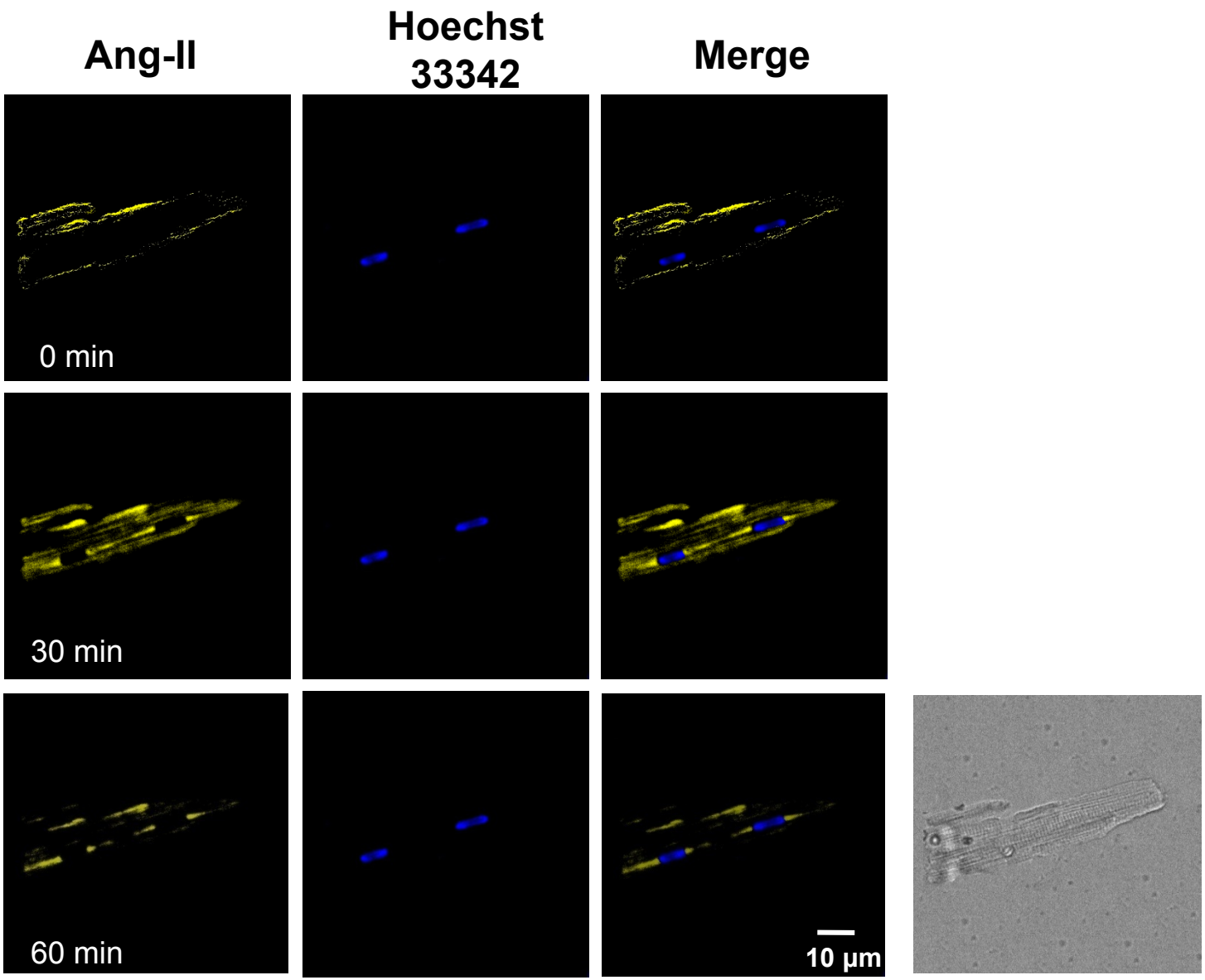


On-line Figure 1



On-line Figure 2

A



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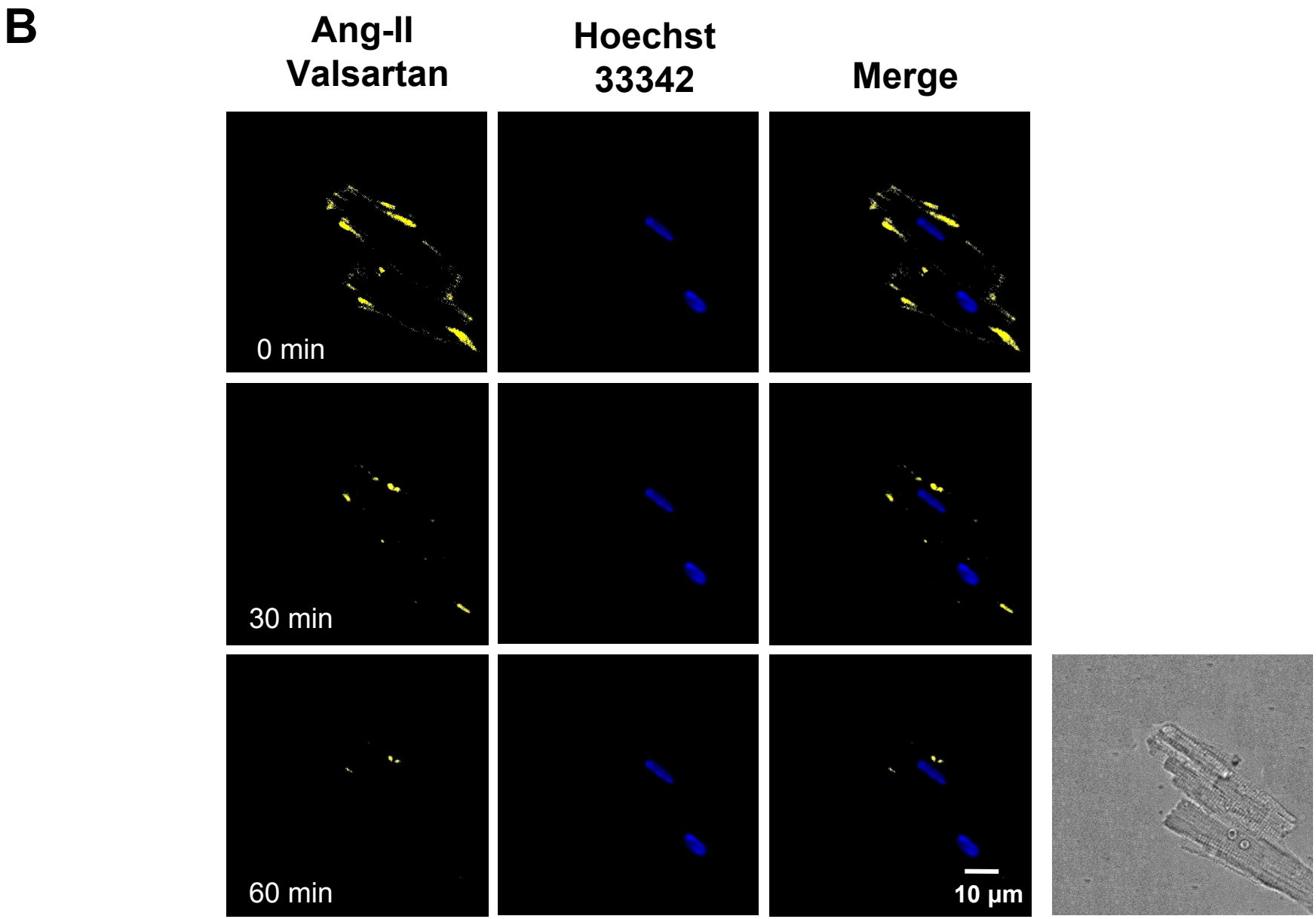

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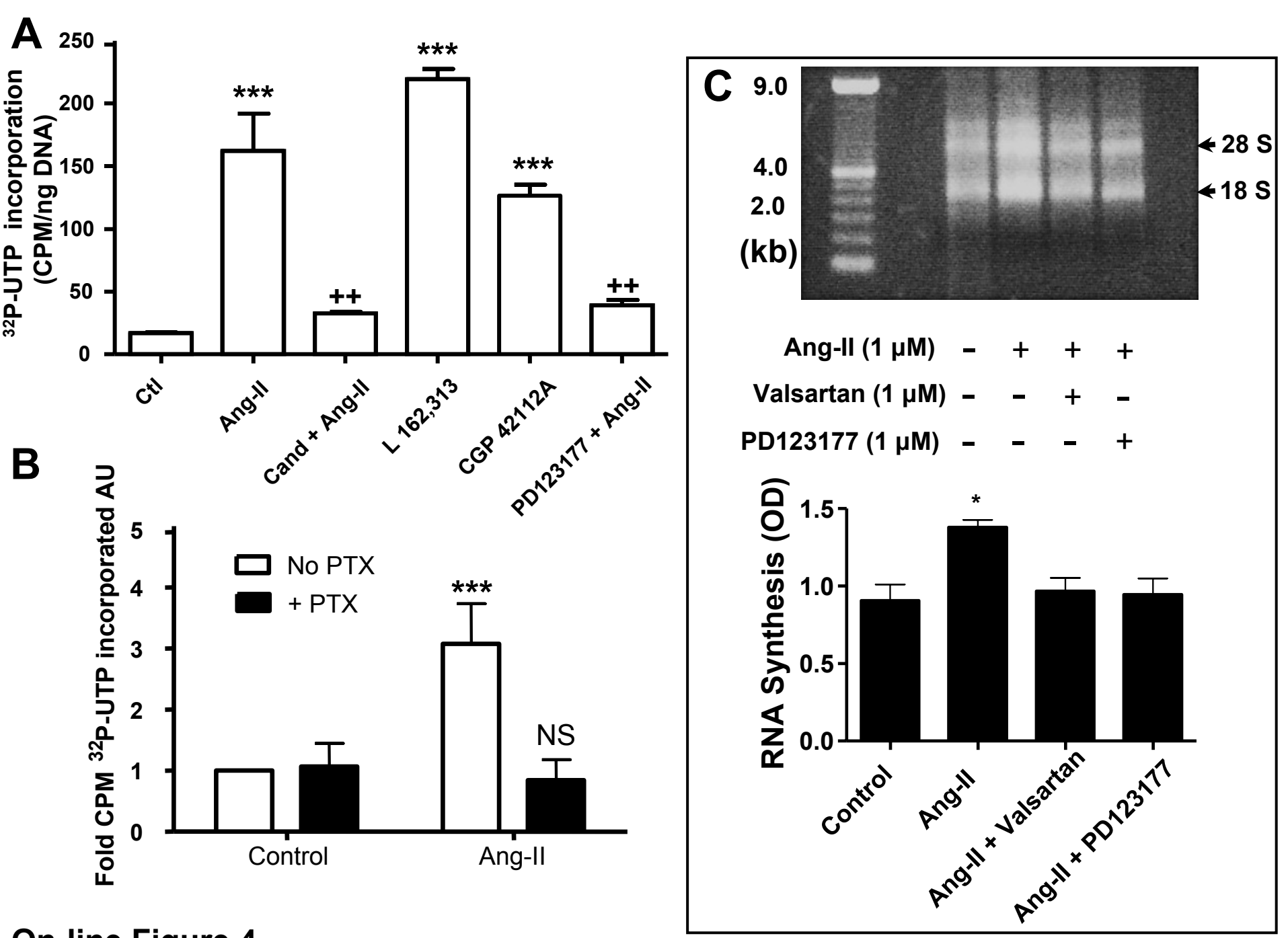

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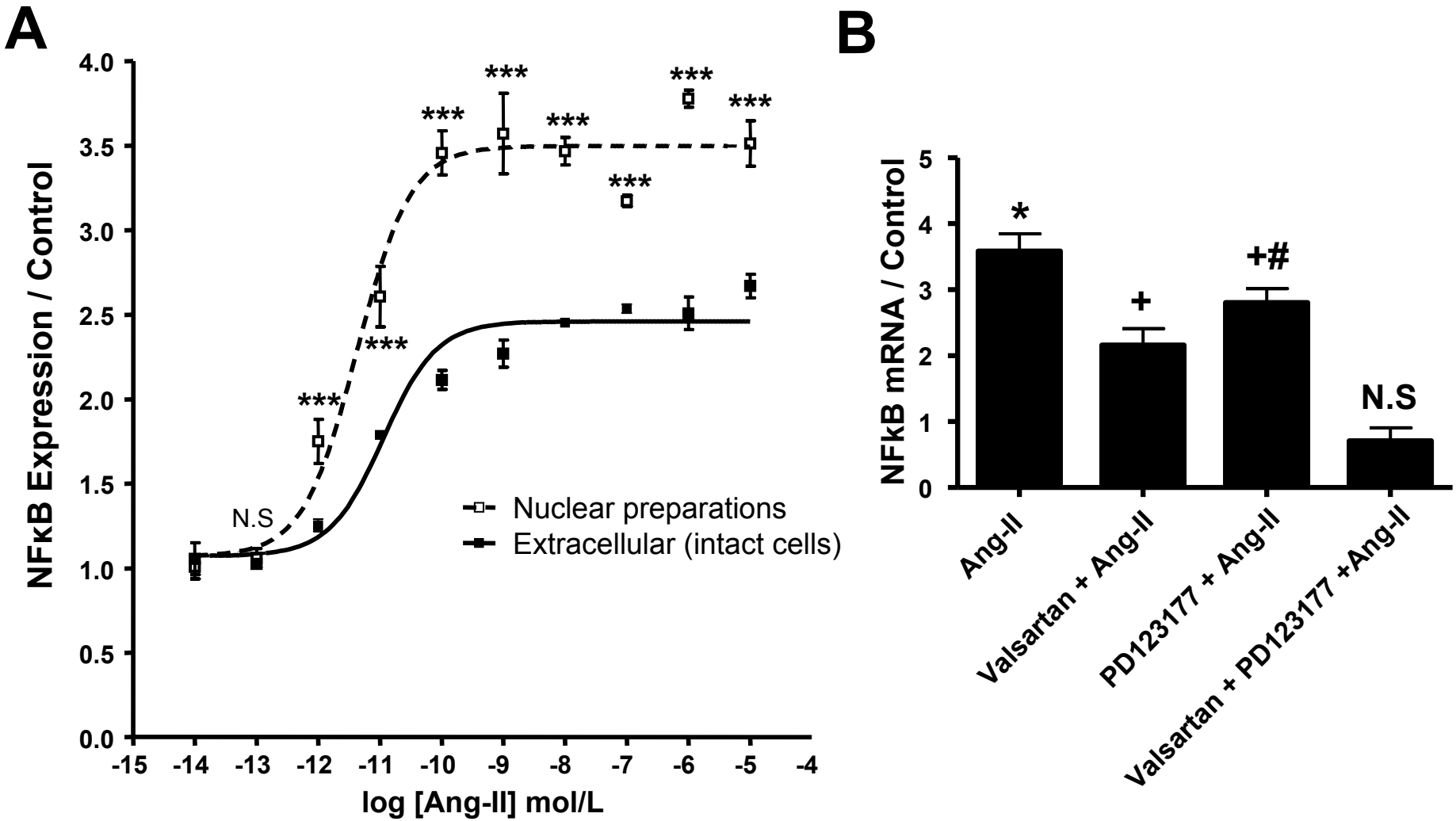

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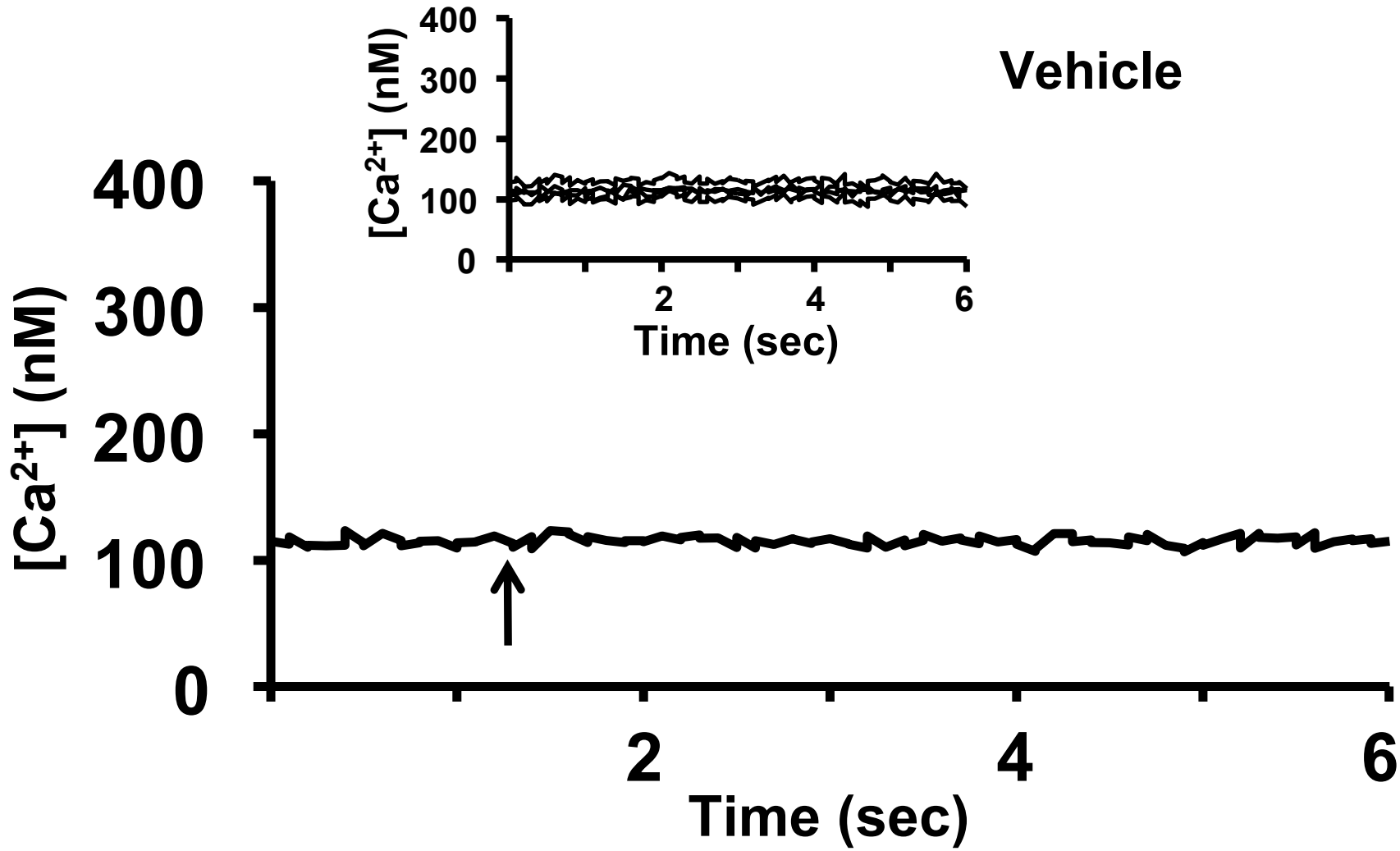

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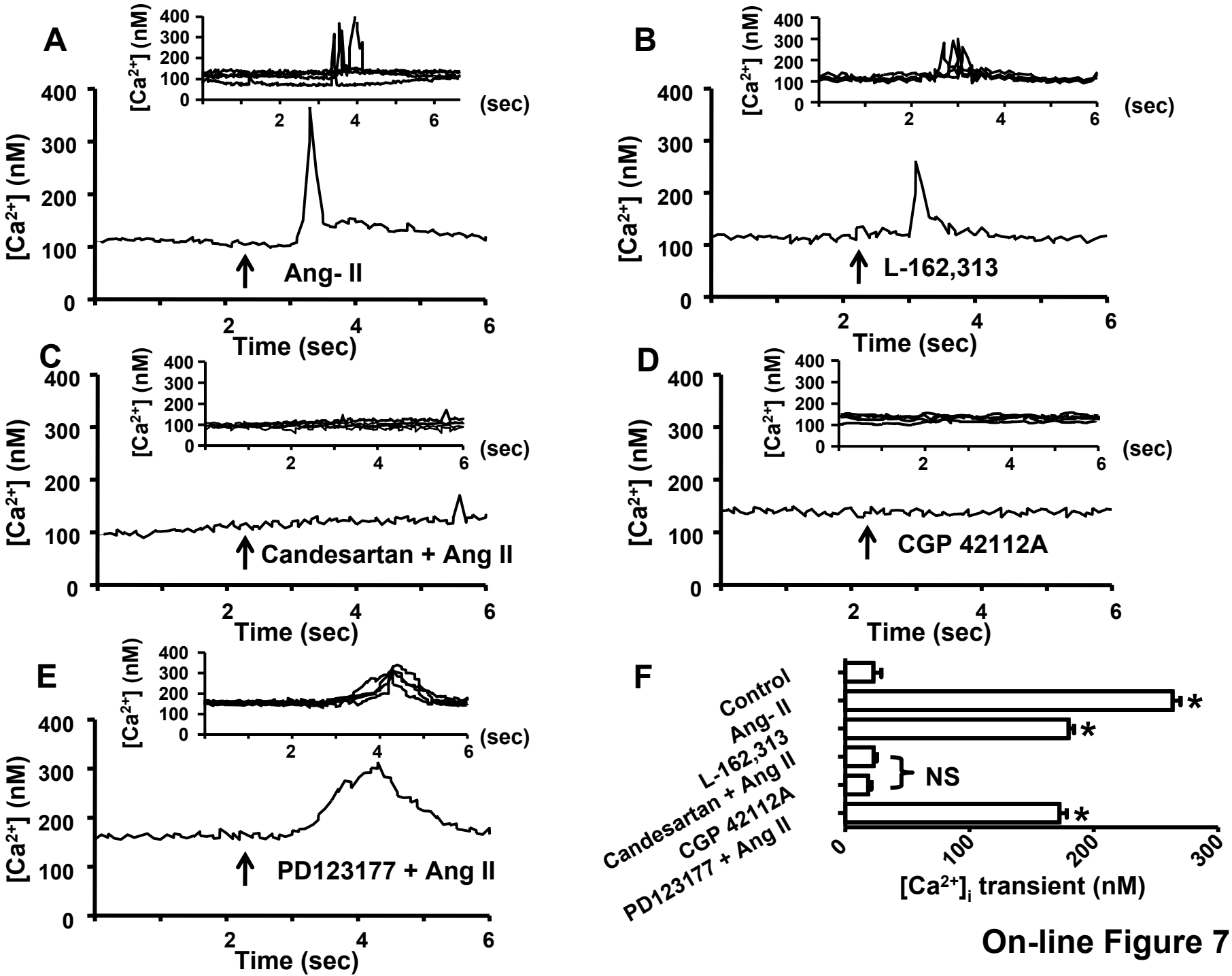

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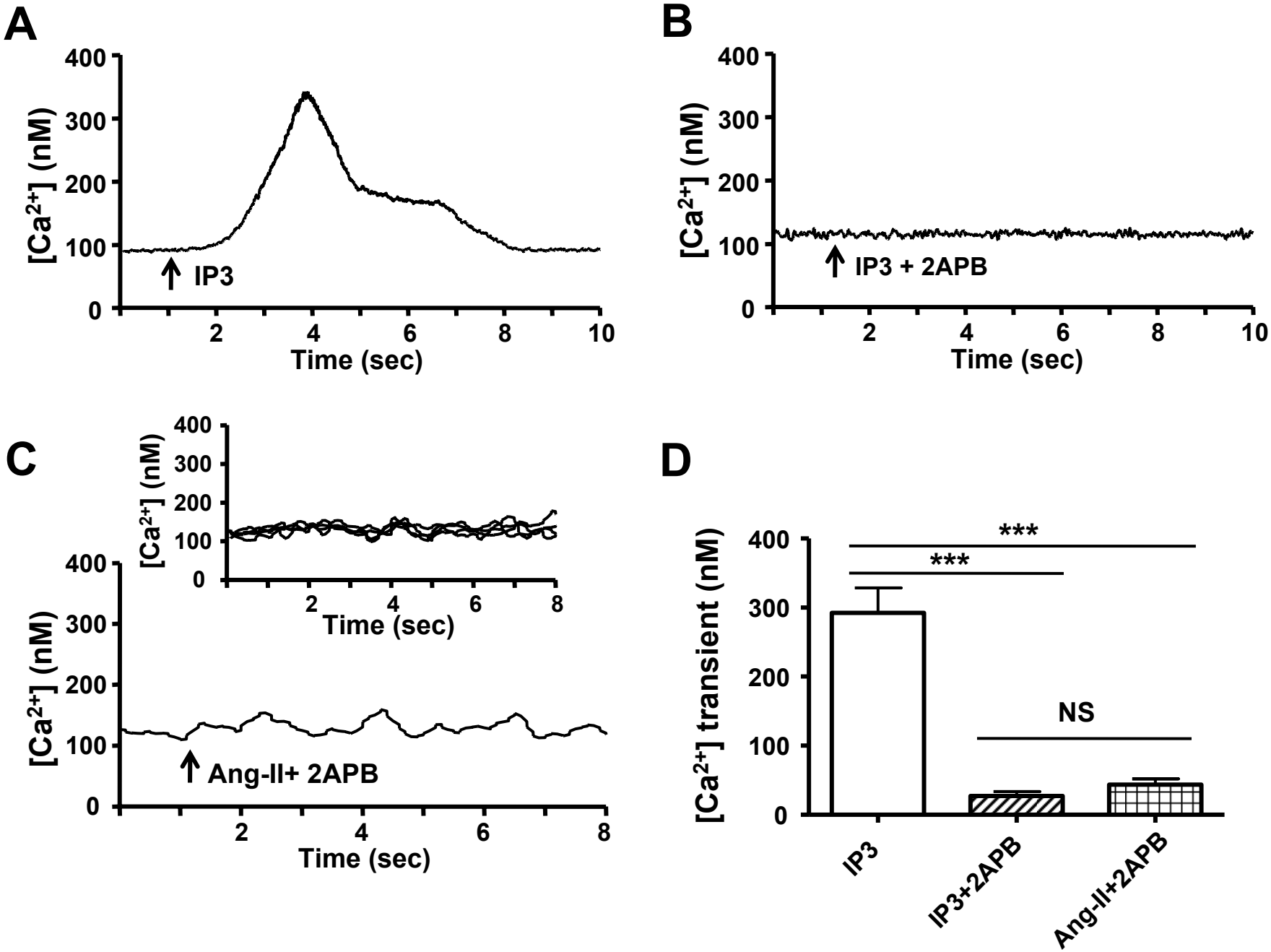

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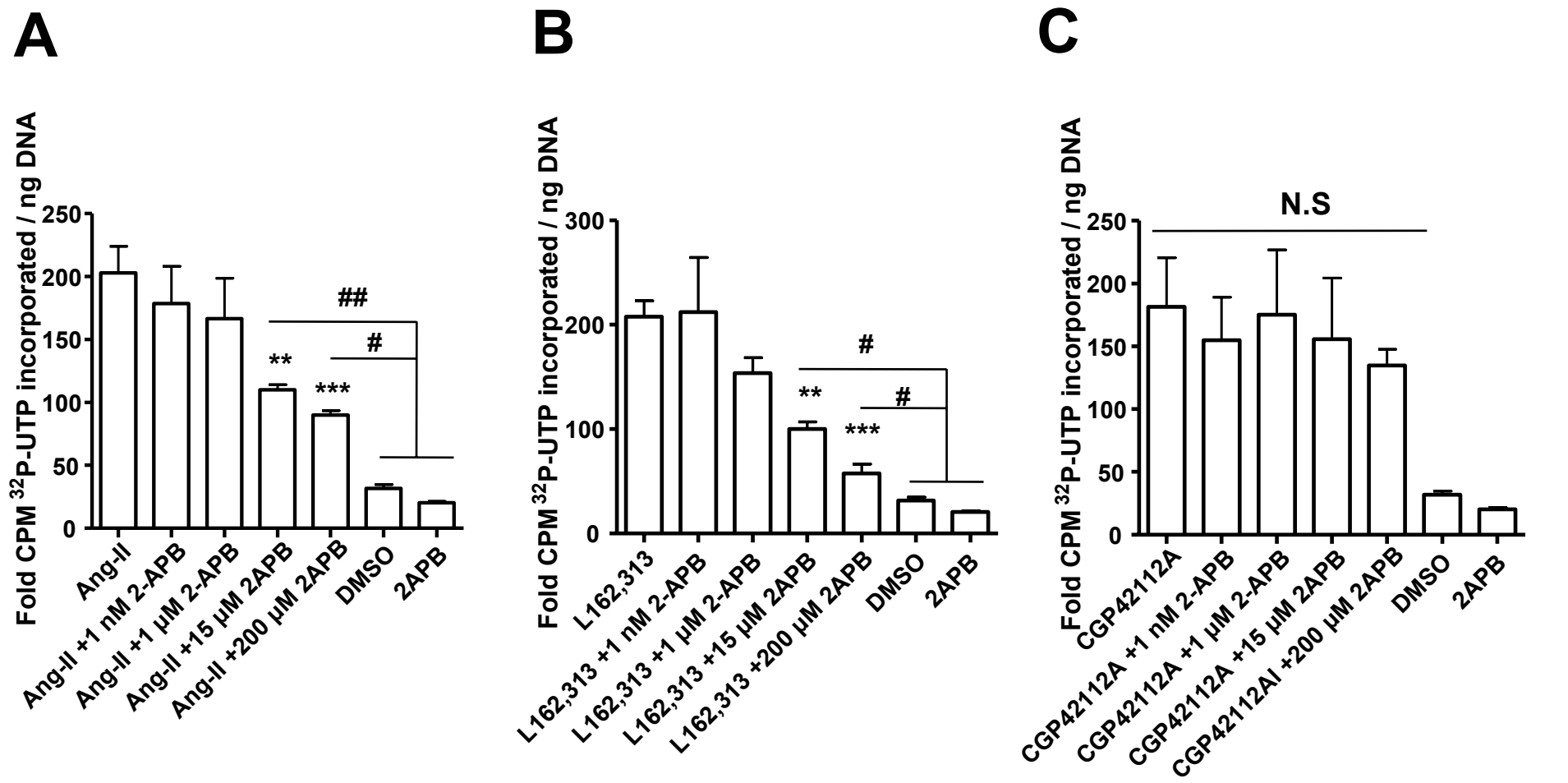

On-line Figure 9

On-Line Methods

Isolation of heart nuclei. Nuclear isolation was performed according to a modified version of a previously-described method (13). Briefly, rat hearts were manually ground into a powder under liquid nitrogen, resuspended in cold phosphate-buffered saline (PBS) (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl with a final pH of 7.4, NaOH) and homogenized with a Polytron. The total extract was labelled Fraction A. The subsequent steps were carried out on ice or in a cooling cabinet at 4°C. Homogenates were centrifuged at 1400×g for 15 min and the supernatant, denoted Fraction B, was diluted 1:1 with buffer A (10 mM K-HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4 with a final pH of 7.4, NaOH), incubated for 10 min on ice and re-centrifuged at a higher speed (3500xg) for 15 min. The resulting supernatant was discarded and the pellet, containing crude nuclei (designated Fraction C) was resuspended in buffer B (0.3 M K-HEPES, 1.5 M KCl, 0.03 M $MgCl_2$, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4 with a final pH of 7.4, NaOH), incubated on ice for 10 min, and centrifuged for 30 min at 5000×g. The pellet, comprising a nuclear-enriched fraction (designated Fraction D), was resuspended in buffer C (20 mM Na-HEPES, 25% (volume/volume) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4 with a final pH of 7.4, NaOH) or 1× transcription buffer (see below) and either used freshly or aliquoted, snap-frozen with liquid nitrogen, and stored at -80°C. Protein concentrations were determined by Bradford assay using a NanoDrop ND-1000 Spectrophotometer (Wilmington, DE).

1. Xiao, L., Coutu, P., Villeneuve, L.R., Tadevosyan, A., Maguy, A., Le Bouter, S., Allen, B.G., and Nattel, S. (2008) Circ Res **103**(7), 733-742

On-line Table I

Primary Antibody Information

Protein	*Isotype*	*Company*	*Catalogue Number*	*Dilution*
AT1 (43kDa)	Rabbit-polyclonal	Alomone labs	ZZR-011-AN01	1:400
AT1-c18	Goat-polyclonal	Santa Cruz	Sc-31181	1:500
AT2(47kDa)	Rabbit-polyclonal	Alomone labs	AAR-012-AN01	1:400
AT2-c18	Goat-polyclonal	Santa Cruz	Sc-7420	1:500
Calpactin-I (36kDa)	Mouse-monoclonal	BD Biosciences	610068	1:2000
DDR2 (98kDa)	Rabbit-polyclonal	Abcam	Ab5520	1:200
GM-130 (130kDa)	Mouse-monoclonal	BD Biosciences	610823	1:250
GRP78 (75kDa)	Rabbit-polyclonal	Abcam	Ab21685	1:1000
Histone 3 (17kDa)	Rabbit-polyclonal	Cell Signaling	#9715	1:1000
MyHC (223kDa)	Mouse-monoclonal	Abcam	Ab15	1:1000
NSE (47kDa)	Rabbit-polyclonal	Abcam	Ab16873	1:2000
Nup-62 (62kDa)	Mouse-monoclonal	BD Biosciences	610498	1:1000
VWF (130, 190kDa)	Goat-polyclonal	Santa Cruz	Sc-8068	1:100
αSMA (42kDa)	Mouse-monoclonal	Sigma	A5228	1:2000

Secondary Antibody Information

Antibody Description	*Company*	*Catalogue Number*	*Dilution*
Donkey anti-rabbit IgG	Jackson	711-005-152	1:10000
Donkey anti-mouse IgG	Jackson	711-005-150	1:15000
Donkey anti-goat IgG	Jackson	711-005-003	1:10000

On-line Table II

Primers Information

Primers	***Description***	***Sequences***	***RefSeq mRNA***
Ace	Angiotensin-converting enzyme	F: CCTGATCAACCAGGAGTTTGCAGAG R: GCCAGCCTTCCCAGGCAAACAGCAC	NM_012544.1
Agt	Angiotensinogen	F: GACCGCGTATACATCCACCCCTTTCATCTC R: GTCCACCCAGAACTCATGGAGCCCAGTCAG	NM_134432.2
B2m	Beta-2 microglobulin	F: GAATTCAGTGTGAGCCAGGATG R: CAAGTGTACTCTCGCCATCCAC	NM_012512.1
Cts	Cathepsin	F: GGGGGAAATCTACAAAAATG R: AAAGACTCCTATCTGCCTCACT	NM_022597.2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: TACATGTTCCAGTATGACTC R: TGTGAGGGAGATGCTCAGTG	NM_017008
Hspcb	Heat shock protein 90kDa alpha, class B member 1	F: TGTTTCTTCACCACCTCCTTGA R: CCTACCTGGTGGCAGAGAAAGT	NM_001004082
NFκB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	F: CTGCGATACCTTAATGACAGCG R: AATTTGGCTTCCTTTCTTGGCT	XM_342346.3
Ren	Renin	F: CTGGGAGGCAGTGACCCTCAACATTACCAG R: GAGAGCCAGTATGCACAGGTCATCGTI'CCT	NM_012642.4