Figures and figure supplements

A Gs-coupled purinergic receptor boosts Ca\(^{2+}\) influx and vascular contractility during diabetic hyperglycemia

**Maria Paz Prada et al**
Figure 1. Extracellular nucleotides promote vasoconstriction, Ca^2+\textsubscript{1.2} activity and Ser^{1928} phosphorylation in response to 20 mM D-glucose in murine arterial myocytes. (A) Representative diameter recordings and summary

Figure 1 continued on next page

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arterial tone data from pressurized (60 mmHg) wt mouse cerebral arteries before and after application of 20 mM D-glucose in the absence (n = 6 arteries from six mice) and presence (n = 6 arteries from six mice) of apyrase (apy; 0.32 U/ml; *p<0.05, Wilcoxon matched pairs test; Figure 1—source data 1). (B) Characteristic \( I_{Na} \) recordings from the same cell and summary \( I_{Na} \) data from wt mouse cerebral arterial myocytes evoked by step depolarizations from −70 to +10 mV before and after application of 20 mM D-glucose in the absence (n = 11 cells from five mice) and presence of apyrase (n = 9 cells from five mice) (*p<0.05, paired t test; Figure 1—source data 2). (C) Representative immunoblot detection of phosphorylated Ser\(_{1928}\) (pSer\(_{1928}\)) and total Ca\(_V_{1.2}\) from wt mouse cerebral and mesenteric arteries after 10 min incubation with 10 mM or 20 mM D-glucose in the absence and presence of apyrase (n = 10 arterial lysates per condition), and quantification of pSer\(_{1928}\) (AU = arbitrary units) (*p<0.05, Kruskal-Wallis with Dunn’s multiple comparisons; Figure 1—source data 3).

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Figure 1—figure supplement 1. K⁺-induced arterial constriction in the absence and presence of apyrase, no changes in arterial tone, I_{Ba} and pSer^{1928} in response to 20 mM mannitol, and full-length blots corresponding to Figure 1C. (A) Bar plot of % constriction in response to high K⁺ (60 mM) from wt Figure 1—figure supplement 1 continued on next page
mouse cerebral arteries exposed to 10 mM D-glucose in the absence and presence of apyrase (apy; 0.32 U/ml; n = 6 arteries from six mice per condition; Figure 1—figure supplement 1—source data 1). Response to high K+ (60 mM) was obtained in pressurized arteries at 20 mmHg. (B) Representative diameter recording and summary arterial tone data from pressurized (60 mmHg) wt mouse cerebral arteries before and after application of 20 mM mannitol (n = 6 arteries from six mice; Figure 1—figure supplement 1—source data 2). (C) Exemplary I_{Ba} recording from the same cell and summary I_{Ba} data from wt mouse cerebral arterial myocytes evoked by step depolarizations from −70 to +10 mV before and after application of 20 mM mannitol (n = 7 cells from three mice; Figure 1—figure supplement 1—source data 3). (D) Complete scan of representative phosphorylated Ser^{1928} (pSer^{1928}) and total Ca_{V1.2} blots for mouse arteries incubated with 10 mM D-glucose, 20 mM D-glucose and 20 mM D-glucose + apyrase. Red boxes indicate the crop region displayed in the main Figure 1C. (E) Representative immunoblot detection of phosphorylated Ser^{1928} (pSer^{1928}) and α-tubulin (loading control) from wt mouse arteries after 10 min incubation in either 10 mM D-glucose or 20 mM mannitol (n = 5 arterial lysates from five mice per condition) and quantification of pSer^{1928} (AU = arbitrary units) (*p=0.3835, unpaired t-test; Figure 1—figure supplement 1—source data 4). DOI: https://doi.org/10.7554/eLife.42214.003
Figure 1—figure supplement 2. Enhanced $I_{Ba}$ in response to elevated glucose is prevented by continuous bath perfusion. (A) Representative $I_{Ba}$ recordings from the same cell and (B) summary $I_{Ba}$ data from wt mouse cerebral arterial myocytes induced by step depolarizations from $-70$ to $+10$ mV during exposure to $10$ mM D-glucose and in response to $20$ mM D-glucose with constant bath perfusion (flow) and after stopping perfusion (e.g. static) ($n = 9$ cells from three mice; *p<0.05, Friedman Test with Dunn’s multiple comparisons; Figure 1—figure supplement 2—source data 1). For these experiments, cells were patched in a bath solution containing 10 mM D-glucose at a perfusion rate of 2.1 mL/min. After establishment of a stable gigaseal for at least 5 min, $I_{Ba}$ were recorded in the presence of 10 mM D-glucose under continuous flow. Cells were then perfused with a bath solution containing 20 mM D-glucose under continuous flow for at least 5 min before recording of $I_{Ba}$ again. Subsequently, the bath perfusion was stopped, and cells were bathed in the 20 mM D-glucose solution under static bath condition for five more minutes before recording $I_{Ba}$ one more time. If the three experimental conditions could not be performed in the same cells, the results were discarded.

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**Figure 2.** P2Y\(_{11}\) protein and distribution in arterial myocytes. (A) Representative blot of immunoreactive bands of expected molecular weight for endogenous P2Y\(_{11}\) (~40 kDa), overexpressed P2Y\(_{11}\)-GFP (~70 kDa), and β-actin (~43 kDa) in untransfected, vehicle-treated (empty transfection) and

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Figure 2 continued

P2Y11-GFP transfected tsA-201 cells (n = 3 lysates per condition). Note that tsA-201 cells endogenously express P2Y11 (Dreisig and Kornum, 2016). (B) Representative blot of immunoreactive bands of expected molecular weight for endogenous P2Y11 (~40 kDa), overexpressed P2Y11-GFP (~70 kDa), and β-actin (~43 kDa) in tsA-cells transfected with P2Y11-GFP as well as corresponding P2Y11 sense (SNS) or antisense (ANS) ODNs (64% reduction in endogenous P2Y11 expression in cells treated with ANS; 62% reduction in P2Y11-GFP expression in P2Y11-GFP-transfected cells treated with ANS; n = 3 lysates per condition, Figure 2—source data 1). (C) Representative immunoblot detection of P2Y11 (~40 kDa) in lysates from human and wt mouse arteries (n = 3 arterial lysates per sample). (D) Representative confocal images of P2Y11-associated fluorescence (green), wheat germ agglutinin (WGA, red) and merged channels in human (n = 11 cells from three humans) and wt mouse (n = 14 cells from three mice) arterial myocytes. (E) Line profile of the P2Y11- and WGA-associated fluorescence from the area highlighted by the dotted lines in the representative human and mouse arterial myocytes in D.

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Figure 2—figure supplement 1. Full-length blot for Figure 2C, knock down of P2Y11 in arterial lysates, P2Y11 immunoreactivity in isolated mouse arterial lysates, negative controls for immunofluorescence experiments in Figure 2D and P2Y11 antibody control. (A) Full-length blot corresponding to Figure 2—figure supplement 1 continued on next page.
Figure 2—figure supplement 1 continued

**Figure 2C.** Red box indicates the crop region displayed in main figure. (B) Representative blots of immunoreactive bands of expected molecular weight for P2Y₁₁ (~40 kDa) in human (n = 5 arterial lysates per condition) and mouse (n = 5 arterial lysates per condition) arterial lysates treated with P2Y₁₁ sense (SNS) and antisense (ANS) ODNs (*p<0.05, Wilcoxon matched pairs test; Figure 2—figure supplement 1—source data 1). (C) Representative confocal images of human (left; n = 10 cells from two humans) and mouse (right; n = 10 cells from two mice) arterial myocytes in which the primary antibody for P2Y₁₁ (no 1° Ab) was excluded from the preparation (e.g. negative control). Wheat germ agglutinin (WGA, red) was used to label the plasma membrane. (D) Representative blots of immunoreactive bands of expected molecular weight for P2Y₁₁ (~40 kDa) and total protein in isolated mouse arterial myocyte lysates (n = 2 lysates). (E) Exemplary confocal images of P2Y₁₁-associated fluorescence (left) and wheat germ agglutinin (WGA, right) in wt mouse arterial myocytes stained with unboiled (n = 7 cells from six mice) or boiled (n = 7 cells from six mice) P2Y₁₁ primary antibody.

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Figure 3. Nanometer organization of P2Y\(_{11}\) with Ca\(_{v}1.2\) and PKA\(_{\text{cat}}\) in human arterial myocytes. Representative conventional TIRF images (top) and corresponding GSD reconstruction maps (bottom) from human arterial myocytes labeled for (A) P2Y\(_{11}\) and Ca\(_{v}1.2\) and (B) P2Y\(_{11}\) and PKA\(_{\text{cat}}\). Lower
panels display enhanced magnifications of areas shown in yellow boxes (scale bar, 400 nm). (C) Histograms of the area of P2Y₁₁, Caᵥ₁.₂ and PKA₉ clusters in isolated human arterial myocytes (1621 ± 29, 1209 ± 16 and 1322 ± 20 nm², respectively; Figure 3—source data 1). (D) Bar plot of cluster density for P2Y₁₁, Caᵥ₁.₂ and PKA₉ (38 ± 2, 29 ± 4, and 25 ± 3 clusters/µm², respectively; Figure 3—source data 2). Enlarged merged image (left) and associated x-y fluorescence intensity profile (right) from area highlighted by the dotted lines of sites of close proximity between (E) P2Y₁₁ (red) and Caᵥ₁.₂ (green) and (F) P2Y₁₁ (red) and PKA₉ (green) (scale bar, 200 nm). Histograms of the lowest intermolecular distance to P2Y₁₁ centroids for (G) Caᵥ₁.₂ (n = 19,611 particles from 6 cells; Figure 3—source data 3) and (H) PKA₉ (n = 22,425 particles from 6 cells; Figure 3—source data 4) fluorescence particles. Data were fit with a sum of two Gaussian functions with depicted R² and centroids. (I) Bar plot of % overlap of P2Y₁₁ with Caᵥ₁.₂ or PKA₉ for experimental (Caᵥ₁.₂: n = 36 segments from 12 cells; PKA₉: n = 22 segments from 11 cells) and randomized simulations images (Caᵥ₁.₂: n = 6 segments from 6 cells; PKA₉: n = 6 segments from 6 cells) (*p<0.05, unpaired t test with Welch’s correction; Figure 3—source data 5).

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Figure 3—figure supplement 1. Validation for PKAcat primary antibody. Representative confocal images of PKAcat-associated fluorescence (top) and differential interference contrast (DIC, bottom) in wt mouse arterial myocytes stained with an anti-PKAcat antibody (- PKAcat blocking peptide on left side; n = 7 cells) and an anti-PKAcat antibody preabsorbed with a PKAcat blocking peptide (+PKAcat blocking peptide on right side; n = 8 cells). DOI: https://doi.org/10.7554/eLife.42214.018
Figure 3—figure supplement 2. Nanometer organization of P2Y\(_{11}\), Ca\(_{V}\)1.2 and PKA\(_{\text{cat}}\) in murine arterial myocytes. Representative TIRF images (top) and corresponding GSD reconstruction maps (bottom) from murine arterial myocytes labeled for (A) P2Y\(_{11}\) and Ca\(_{V}\)1.2 and (B) P2Y\(_{11}\) and PKA\(_{\text{cat}}\). Lower
panels show enhanced magnifications of areas highlighted in yellow boxes (scale bar, 400 nm). (C) Histograms of the area of P2Y₁₁, Caᵥ₁.₂ and PKA₅ cat clusters in arterial myocytes (180 ± 22, 211 ± 68, and 1836 ± 28 nm², respectively, Figure 3—figure supplement 2—source data 1). (D) Bar plot of cluster density for P2Y₁₁, Caᵥ₁.₂ and PKA₅ cat (37 ± 2, 24 ± 2, and 23 ± 3 clusters/µm², respectively, Figure 3—figure supplement 2—source data 2).

Enlarged merged image (left) and associated x-y fluorescence intensity profile (right) from area highlighted by the dotted lines of sites of close proximity between (E) P2Y₁₁ (red) and Caᵥ₁.₂ (green) and (F) P2Y₁₁ (red) and PKA₅ cat (green) (scale bar, 200 nm). Histograms of the lowest intermolecular distance to P2Y₁₁ centroids for (G) Caᵥ₁.₂ (n = 23,722 particles from 6 cells; Figure 3—figure supplement 2—source data 3) and (H) PKA₅ cat (n = 19,219 particles from 5 cells; Figure 3—figure supplement 2—source data 4) fluorescence particles. Data were fit with a sum of two Gaussian functions with depicted R² and centroids.

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Figure 3—figure supplement 3. Negative controls for GSD images in human and murine arterial myocytes, and experimental and randomized reconstruction maps. Representative TIRF images (top) and corresponding GSD reconstruction maps (bottom) from freshly dissociated (A) human (n = 6 cells from four humans) and (B) mouse (n = 6 cells from six mice) arterial myocytes labeled with secondary antibodies only (no 1° Ab, Alexa 647 or no 1° Ab, Alexa 568). (C) Representative binarized sub-image area of experimental GSD super-resolution localization maps (top) and randomized simulation distribution images (bottom) for P2Y$_{11}$ with Ca$_{v}$1.2 (left panels; experimental, n = 38 sub-image area from 12 cells; randomized, n = 6 sub-image area from 6 cells) and P2Y$_{11}$ with PKA$_{cat}$ (right panels; experimental, n = 24 sub-image area from 11 cells; randomized, n = 6 sub-image area from 6 cells) in arterial myocytes. A smoothing filter was applied to the P2Y$_{11}$, Ca$_{v}$1.2 and PKA$_{cat}$ images for presentation. Note that randomization data were repeated six times from six different experimental GSD super-resolution localization maps. The overlap images for both experimental and simulated data were generated by multiplying the P2Y$_{11}$ image by the respective Ca$_{v}$1.2 or PKA$_{cat}$ image, thus revealing overlapping objects.

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Figure 4. P2Y_{11} associates with $\text{Ca}_{v}1.2$ and $\text{PKA}_{\text{cat}}$ in human and murine arterial myocytes. (A) Exemplary fluorescence PLA (red)/DAPI (blue) and differential interference contrast (right) images of human arterial myocytes labeled for P2Y_{11} + $\text{Ca}_{v}1.2$ and P2Y_{11} + $\text{PKA}_{\text{cat}}$. (B) Quantification of PLA puncta/µm².

**Figure 4 continued on next page**
fluorescent puncta per cell area (puncta/μm²) for human arterial myocytes labeled for P2Y₁₁ (n = 26 cells from three human samples), Caᵥ₁.2 (n = 20 cells from three humans), PKAₑᵥ (n = 17 cells from three humans), P2Y₁₁ + Caᵥ₁.2 (n = 23 cells from three humans), and P2Y₁₁ + PKAₑᵥ (n = 20 cells from three humans) (*p<0.05, Kruskal-Wallis with Dunn’s multiple comparisons; Figure 4—source data 1). (C) Representative fluorescence PLA (red)/ DAPI (blue) and differential interference contrast (right) images of mouse arterial myocytes labeled for P2Y₁₁ + Caᵥ₁.2 and P2Y₁₁ + PKAₑᵥ. (D) Quantification of PLA fluorescent puncta per μm² cell area for mouse arterial myocytes labeled for P2Y₁₁ (n = 44 cells from six mice), Caᵥ₁.2 (n = 15 cells from six mice), P2Y₁₁ + Caᵥ₁.2 (n = 25 cells from six mice), and P2Y₁₁ + PKAₑᵥ (n = 29 cells from six mice) (*p<0.05, Kruskal-Wallis with Dunn’s multiple comparisons; Figure 4—source data 2).

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Figure 4—figure supplement 1. Negative controls for PLA in human and murine arterial myocytes. Fluorescence PLA (red)/DAPI (blue) (left) and differential interference contrast (right) images of negative controls for human (A) and murine (B) arterial myocytes labeled with one 1° antibody (CaV1.2: n = 20 cells from three humans, n = 15 cells from six mice; P2Y11: n = 26 cells from three humans, n = 44 cells from six mice; PKAcat: n = 17 cells from three humans, n = 19 cells from six mice).

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Figure 5. Glucose and the P2Y<sub>11</sub> agonist NF546 increase sarcolemmal cAMP synthesis in arterial myocytes, and these effects are prevented by the P2Y<sub>11</sub> antagonist NF340. (A) Time course of YFP/CFP (donor/acceptor) FRET ratios (normalized to resting levels before treatment) in human arterial cell. (B) Maximum FRET response (YFP/CFP) in human arterial cell. (C) Time course of YFP/CFP (donor/acceptor) FRET ratios (normalized to resting levels before treatment) in mouse arterial cell. (D) Maximum FRET response (YFP/CFP) in mouse arterial cell.
myocytes expressing the ICUE3-PM biosensor in response to 15 mM D-glucose (black; n = 19 cells from two humans), 500 nM NF546 (green; n = 18 cells from two humans) and 15 mM D-glucose + 500 nM NF546 (red; n = 14 cells from two humans) before and after application of broad adenylyl cyclase agonist forskolin (1 μM). In a set of experiments, cells were first pre-treated with the P2Y11 antagonist NF340 (10 μM) for at least 15–20 min before treatment with 500 nM NF546 (orange; n = 15 cells from two humans) and 15 mM D-glucose + 500 nM NF546 (blue; n = 13 cells from two humans). Horizontal bars indicate treatment. Increases in cAMP production are represented by decreases in YFP/CFP ratio due to binding of cAMP to the biosensor. (B) Bar plot of maximum FRET responses (YFP/CFP) for human arterial myocytes in response to the indicated treatment (*p<0.05, Kruskal-Wallis with Dunn’s multiple comparisons; Figure 5—source data 1. Statistical differences were compared between 15 mM D-glucose vs NF546, 15 mM D-glucose vs 15 mM D-glucose + NF546, 15 mM D-glucose vs NF546 + NF340, 15 mM D-glucose + NF546 vs NF546 + NF340, NF546 vs 15 mM D-glucose + NF546 + NF340, 15 mM D-glucose vs 15 mM D-glucose + NF546 + NF340, 15 mM D-glucose + NF546 vs 15 mM D-glucose + NF546 + NF340). (C) Time course of YFP/CFP (donor/acceptor) FRET ratios (normalized to resting levels before treatment) in mouse arterial myocytes expressing the ICUE3-PM biosensor in response to 20 mM D-glucose (black; n = 83 cells from three mice), 500 nM NF546 (green; n = 13 cells from three mice) and 20 mM D-glucose + 500 nM NF546 (red; n = 13 cells from three mice). Horizontal bars indicate treatment. In a set of experiments, cells were first pre-treated with the P2Y11 antagonist NF340 (10 μM) for at least 15–20 min before treatment with 500 nM NF546 (orange; n = 17 cells from three mice) and 20 mM D-glucose + 500 nM NF546 (blue; n = 18 cells from three mice). Increases in cAMP production are represented by decreases in YFP/CFP ratio due to binding of cAMP to the biosensor. (D) Bar plot of maximum FRET responses (YFP/CFP) for mouse arterial myocytes in response to the indicated treatment (*p<0.05, Kruskal-Wallis with Dunn’s multiple comparisons; Figure 5—source data 2. Statistical differences were compared between all datasets). DOI: https://doi.org/10.7554/eLife.42214.034
Figure 5—figure supplement 1. The P2Y₁₁ agonist NF546 increases cAMP and this is blocked in the presence of the P2Y₁₁ inhibitor NF340 but not with the P2Y₁ inhibitor MRS2179 or P2Y₆ inhibitor MRS2578 in tsA-201 cells, and no increases in cAMP in arterial myocytes with mannitol. (A) Time course of YFP/CFP (donor/acceptor) FRET ratios (normalized to resting levels before treatment) in tsA-201 cells expressing the ICUE3-PM biosensor in response to 500 nM NF546 (black; n = 19 cells) and 500 nM NF546 in the presence of the P2Y₆ inhibitor MRS2179 (10 μM; red; n = 18 cells) or the P2Y₁₁ inhibitor NF340 (10 μM; orange; n = 20 cells) before and after application of the broad adenylyl cyclase agonist forskolin (1 μM). Horizontal bars indicate treatment. All inhibitors were applied to the preparation 15–20 min before treatment. Increases in cAMP production are represented by decreases in YFP/CFP ratio due to binding of cAMP to the biosensor. Bar plot of maximum FRET responses (YFP/CFP) for tsA-201 cells in response to the indicated treatment (*p<0.05, Kruskal-Wallis with Dunn’s multiple comparisons; Figure 5—figure supplement 1—source data 1). (B) Time course of YFP/CFP (donor/acceptor) FRET ratios (normalized to resting levels before treatment) in human arterial myocytes expressing the ICUE3-PM biosensor in response to 15 mM D-glucose (black; n = 19 cells from two humans), 500 nM NF546 (green; n = 18 cells from two humans), and 15 mM mannitol (purple; n = 20 cells from two humans) before and after application of forskolin (1 μM). Bar plot of maximum FRET responses (YFP/CFP) for human arterial myocytes in response to indicated treatment. Data for 15 mM D-glucose and NF546 are from Figure 5A (*p<0.05, Kruskal-Wallis with Dunn’s multiple comparisons; Figure 5—figure supplement 1—source data 2). (C) Time course of YFP/CFP (donor/acceptor) FRET ratios (normalized to resting levels before treatment) in mouse arterial myocytes expressing the ICUE3-PM biosensor in response to 20 mM D-glucose (black; n = 83 cells from three mice), 500 nM NF546 (green; n = 13 cells from three mice), and 20 mM mannitol (purple; n = 23 cells from three mice).
Figure 5—figure supplement 1 continued

mice). Bar plot of maximum FRET responses (YFP/CFP) for mouse arterial myocytes in response to indicated treatment. Data for 20 mM D-glucose and NF546 is from Figure 5B (*p<0.05, Kruskal-Wallis with Dunn’s multiple comparisons; Figure 5—figure supplement 1—source data 3).

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Figure 6. The P2Y<sub>11</sub> inhibitor NF340 prevents glucose-induced elevations in Ser<sup>1928</sup> phosphorylation, LTCC activity, and vasoconstriction in human and murine arterial myocytes. (A) Representative I<sub>Ca</sub> recordings from the same cell and (B) summary current density data obtained from freshly dissociated human arterial myocytes in response to 5 mM and 15 mM D-glucose. (C) Representative I<sub>Ca</sub> recordings from the same cell and (D) summary current density data obtained from freshly dissociated murine arterial myocytes in response to 10 mM and 20 mM D-glucose.

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human arterial myocytes before and after increasing extracellular D-glucose from 5 mM to 15 mM in the absence (n = 12 cells from six humans) and presence (n = 10 cells from five humans) of NF340 (10 µM) (*p<0.05, paired t test; Figure 6—source data 1). (C) Exemplary $I_{Na}$ traces from the same cell and (D) amalgamated current density data recorded from mouse cerebral arterial myocytes treated with 10 µM NF340 (n = 8 cells from three mice) or 100 nM MRS2578 (n = 7 cells from five mice) before and after increasing extracellular D-glucose from 10 mM to 20 mM (*p<0.05, Kruskal-Wallis with Dunn’s multiple comparisons; Figure 6—source data 2). Representative immunoreactive bands for phosphorylated Ser$^{1928}$/Ca$\text{V}_{1.2}$ and densitometry for pSer$^{1928}$/Ca$\text{V}_{1.2}$ ratio in lysates from (E) human arteries exposed to 5 mM and 15 mM D-glucose in the absence and presence of 10 µM NF340 (n = 6 arterial lysates per condition; *p<0.05, Kruskal-Wallis with Dunn’s multiple comparisons; Figure 6—source data 3) and (F) mouse cerebral and mesenteric arteries exposed to 10 mM or 20 mM D-glucose in the presence of 10 µM NF340 or 100 nM MRS2578 (n = 4 arterial lysates per condition; *p<0.05 Kruskal-Wallis with Dunn’s multiple comparisons; Figure 6—source data 4). Representative diameter recordings and summary data from pressurized (60 mmHg) mouse cerebral arteries exposed to (G–H) 10 µM NF340 (n = 4 arteries from four mice; Friedman with Dunn’s multiple comparisons; Figure 6—source data 5) or (I–J) 100 nM MRS2578 (n = 7 arteries from seven mice; *p<0.05, Wilcoxon matched pair test; Figure 6—source data 6) before and after application of 20 mM D-glucose.

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Figure 6—figure supplement 1. Full-length blots for Figure 6E and F, high K⁺ induced constriction in arteries pretreated with NF340 and MRS2578 corresponding to data in Figure 6, and glucose-induced vasoconstriction is not prevented or inhibited by a selective P2Y₁ antagonist. Representative Figure 6—figure supplement 1 continued on next page.
Figure 6—figure supplement 1 continued

full-length blots corresponding to (A) Figure 6E and (B) Figure 6F. Red boxes indicate the crop region displayed in the main figure. (C) Bar plot of % constriction in response to 60 mM K+ from mouse arteries in control (-) condition (same control as in Figure 1—figure supplement 1A) and arteries pretreated with 10 μM NF340 (n = 4 arteries from four mice), 100 nM MRS2578 (n = 7 arteries from seven mice) or 10 μM MRS2179 (n = 7 arteries from four mice) (Figure 6—figure supplement 1—source data 1). (D) Representative diameter recording and summary data from pressurized (60 mmHg) mouse cerebral arteries exposed to 10 μM MRS2179 before and after application of 20 mM D-glucose in the absence and presence of 500 nM NF546 (n = 7 arteries from four mice; *p<0.05, Friedman test with Dunn’s multiple comparisons for datasets in the presence of MRS2179. Mann-Whitney test was performed between 10 mM D-glu and 10 mM D-glu +MRS2179; Figure 6—figure supplement 1—source data 2). (E) Representative diameter recording and summary data from pressurized (60 mmHg) mouse cerebral arteries exposed to 20 mM D-glucose before and after application of 10 μM MRS2179 (n = 6 arteries from six mice; *p<0.05, Friedman test with Dunn’s multiple comparisons; Figure 6—figure supplement 1—source data 3).

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Figure 7. The P2Y₁₁ agonist NF546 increases Ser¹⁹²⁸ phosphorylation, LTCC activity, and induces vasoconstriction. (A) Representative I_{Ba} recordings from the same cell (top) and summary I_{Ba} data (bottom) from freshly dissociated human arterial myocytes in response to step depolarizations from −70
Representative $I_{\mathrm{Ba}}$ recordings from the same cell (left) triggered by step depolarization from −70 mV to voltages ranging from −60 to +60 mV before and after application of 500 nM NF546 in mouse cerebral arterial myocytes and corresponding $I_{\mathrm{Ba}}$-voltage relationship (right) (n = 8 cells from five mice) (*p<0.05, paired t test; Figure 7—source data 2). (C) Exemplary immunoblot detection of phosphorylated Ser$^{1928}$ (pSer$^{1928}$) and total Ca$_{\text{V}1.2}$ from human (left) and mouse cerebral and mesenteric arteries (right) incubated with 500 nM NF546 and respective densitometry quantification of pSer$^{1928}$/Ca$_{\text{V}1.2}$ ratio (n = 6 arterial lysates per condition for humans; n = 6 arterial lysates per condition for mice) (*p<0.05, Wilcoxon matched pairs test; Figure 7—source data 3). (D) Representative $I_{\mathrm{Ba}}$ recordings from the same cell (top) and summary $I_{\mathrm{Ba}}$ data (bottom) from mouse arterial myocytes evoked by step depolarizations from −70 to +10 mV before and after application of 500 nM NF546 in the absence (n = 9 cells, four mice) and presence of 100 nM PKI (n = 9 cells, five mice) or 10 μM rpcAMP (n = 9 cells, four mice) (*p<0.05, paired t test; Figure 7—source data 4). (E) Representative diameter recording and summary arterial tone data from pressurized (60 mmHg) mouse cerebral arteries exposed to 500 nM NF546 (n = 6 arteries, six mice) (*p<0.05, Wilcoxon matched pairs test; Figure 7—source data 5). (F) Representative diameter recordings and summary arterial tone data from pressurized (60 mmHg) wt mouse cerebral arteries exposed to 20 mM D-glucose before and after application of 500 nM NF546 (n = 6 arteries, six mice, left; *p<0.05, Friedman with Dunn’s multiple comparisons; Figure 7—source data 6) and S9128A mouse cerebral arteries after NF546 application (n = 4 from three mice, right; Figure 7—source data 6). DOI: https://doi.org/10.7554/eLife.42214.052
Figure 7—figure supplement 1. D-glucose and NF546 elicit changes in $I_{Ba}$ of similar magnitude in mouse arterial myocytes, ATPγS increases $I_{Ba}$ in arterial myocytes, full-length blots corresponding to data in Figure 7C, and high K⁺-induced constriction in wt and S1928A arteries treated with NF546 corresponding to data in Figure 7. (A) Summary data showing changes in $I_{Ba}$ in response to elevating D-glucose from 10 mM to 20 mM ($−1.9 ± 0.2$ pA/pF; $n = 11$ cells from five mice) or after application of the P2Y₁₁ agonist NF546 (500 nM; $−2.8 ± 0.6$ pA/pF; $n = 9$ cells from four mice) ($*p=0.1326$, unpaired t test; Figure 7—figure supplement 1—source data 1). (B) Representative $I_{Ba}$ recordings from the same cell (left) and summary $I_{Ba}$ data (right) from mouse arterial myocytes evoked by step depolarizations from $−70$ to $+10$ mV before and after application of 1 μM ATPγS ($n = 10$ cells from four mice) ($*p<0.05$, paired t test; Figure 7—figure supplement 1—source data 2). (C) Full-length blots corresponding to Figure 7C. Red boxes Figure 7—figure supplement 1 continued on next page
Figure 7—figure supplement 1 continued

indicate the crop regions displayed in the main figure. (D) Bar plot of % constriction in response to 60 mM K⁺ from wt mouse arteries treated with 500 nM NF546 (n = 6 arteries, six mice) and 20 mM D-glucose +NF546 (n = 6 arteries, six mice) and S1928A mouse arteries treated with NF546 (n = 4 from three mice) (Figure 7—figure supplement 1—source data 3).

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Figure 8. Augmented LTCC activity and Ser<sup>1928</sup> phosphorylation in response to chronic extracellular glucose elevations are prevented in the presence of the P2Y<sub>11</sub> antagonist NF340. (A) Representative single LTCC recordings obtained during a 2 s step depolarization from −80 to −30 mV and (B) bar graph showing LTCC nPo in response to 10 mM D-glucose (black), 20 mM mannitol (purple), and 20 mM D-glucose + NF340 (red). (C) Western blot analysis of pSer<sup>1928</sup>/Cav1.2 relative density (AU) in cells treated with 10 mM D-glucose (black), 20 mM D-glucose (red), and 20 mM D-glucose + NF340 (purple). (D) Schematic overview of the signaling pathway involving elevated glucose (hyperglycemia), NUC, P2Y<sub>11</sub>, G<sub>s</sub>, AC, cAMP, PKA, Ca<sup>2+</sup>, and vasoconstriction.
plot of LTCC nP<sub>o</sub> in arterial myocytes isolated from mouse cerebral arteries incubated for 48 hr in 10 mM D-glucose (n = 10 cells from three mice), 20 mM mannitol (n = 13 cells from four mice), 20 mM D-glucose (n = 10 cells from four mice) and 20 mM D-glucose +10 μM NF340 (n = 10 cells from four mice). Channel openings (o) are represented by downward deflections from baseline (c) (*p<0.05, one-way ANOVA with Tukey post hoc test; Figure 8—source data 1). (C) Representative immunoblot detection of phosphorylated Ser<sup>1928</sup> (pSer<sup>1928</sup>) and total Ca<sub>V</sub>1.2 from mouse cerebral and mesenteric arteries incubated for 48 hr in 10 mM D-glucose, 20 mM D-glucose and 20 mM D-glucose +10 μM NF340 and densitometry quantification of pSer<sup>1928</sup>/Ca<sub>V</sub>1.2 ratio (n = 7 arterial lysates per condition) (*p<0.05, Kruskal-Wallis with Dunn’s multiple comparisons; Figure 8—source data 2). (D) Proposed model for the role of P2Y<sub>11</sub> in PKA-dependent stimulation of LTCC activity and vasoconstriction during diabetic hyperglycemia (NUC = nucleotides).

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**Figure 8—figure supplement 1.** Full-length blots corresponding to data in Figure 8C and unchanged LTCC nPo in response to chronic elevations in extracellular glucose in S1928A arterial myocytes. (A) Full-length blots corresponding to Figure 8C. Red boxes indicate the crop region displayed in the main figure. (B) Representative single LTCC recordings obtained during a 2 s step depolarization from −80 to −30 mV and bar plot of LTCC nPo in arterial myocytes isolated from S1928A mouse cerebral arteries incubated for 48 hr in 10 mM D-glucose (n = 9 cells from three mice) and 20 mM D-glucose (n = 8 cells from three mice) ([Figure 8—figure supplement 1—source data 1](https://doi.org/10.7554/eLife.42214.064)). Channel openings (o) are represented by downward deflections from baseline (c). Titles for source data files.

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