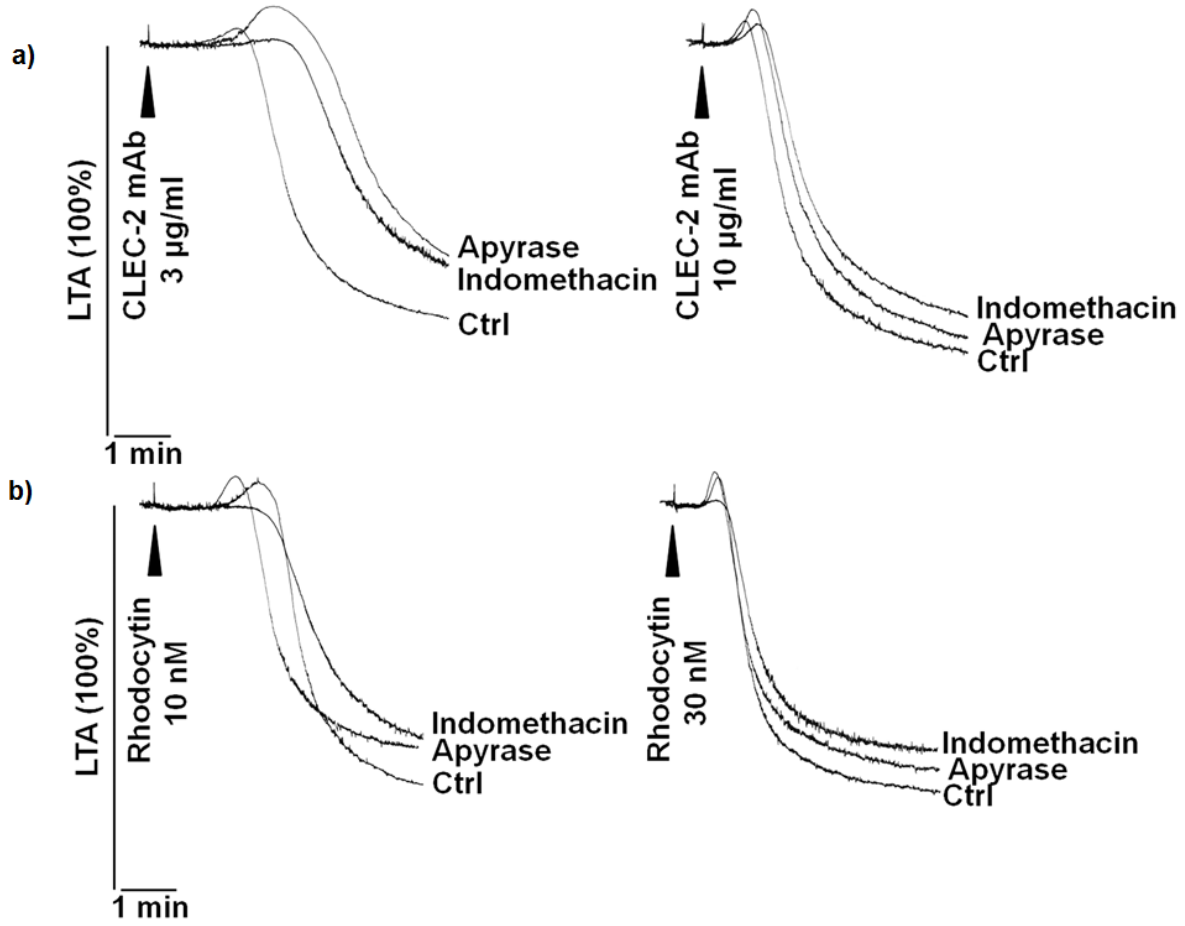
SUPPLEMENTARY TABLE 1.

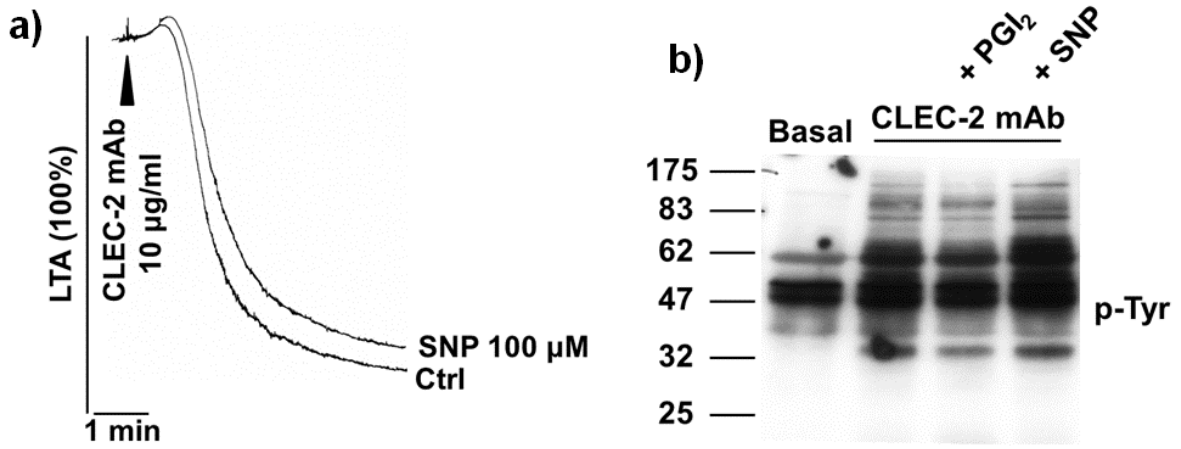
Antibody	Source	Address
Rat anti-mouse CLEC-2	previously described sources [1, 17]	
Anti-Syk	previously described sources [1, 17]	
Anti-PLCγ2	previously described sources [1, 17]	
Anti-phospho-tyrosine (clone 4G10)	Millipore	Bucks, UK
Anti VASPp-ser239	Cell Signaling Technology	Herts, UK
Anti VASPp-ser157	Cell Signaling Technology	Herts, UK
Anti podoplanin-PE conjugated antidody (clone NZ-1.3)	eBioscience	Hatfield, UK
Mouse anti CD62P antibody (clone Psel.KO.2.7)	Novus Biologicals	Littleton, CO, USA
Rat anti-mouse CD41 antibody (clone MWReg30)	BD Pharmingen	Oxford, UK
Goat anti rat IgG-Alexa 647	Invitrogen	Paisley, UK
Goat anti-mouse IgG-Alexa 488	Invitrogen	Paisley, UK
Anti-mouse HRP-conjugated secondary antibody	GE Healthcare	Bucks, UK
Anti-rabbit HRP-conjugated secondary antibody	GE Healthcare	Bucks, UK
Anti-rat HRP-conjugated	Santa Cruz Biotechnology	Heidelberg, Germany

SUPPLEMENTARY FIGURE 1.



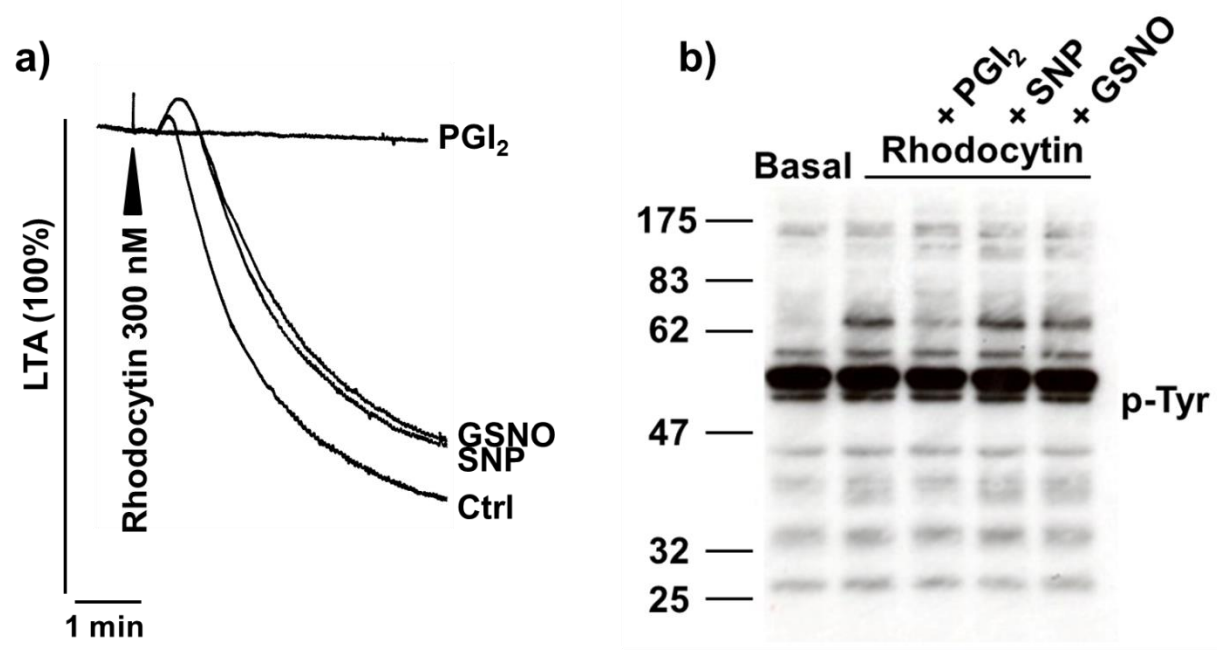
Marginal effect of apyrase or indomethacin on CLEC-2-dependent platelet aggregation- The effect of inhibition of the feedback agonists ADP and TxA_2 on CLEC-2-dependent platelet activation was assessed by light transmission aggregometry (LTA) (a, b). Washed platelets ($2x10^8$/ml) were stimulated with CLEC-2 mAb (a) or rhodocytin (b) in the presence and in the absence of 2 U/ml apyrase or 10 μM indomethacin and allowed to aggregate. Representative traces with 3-10 μg/ml CLEC-2 mAb and 10-30 nM rhodocytin are shown.

SUPPLEMENTARY FIGURE 2.



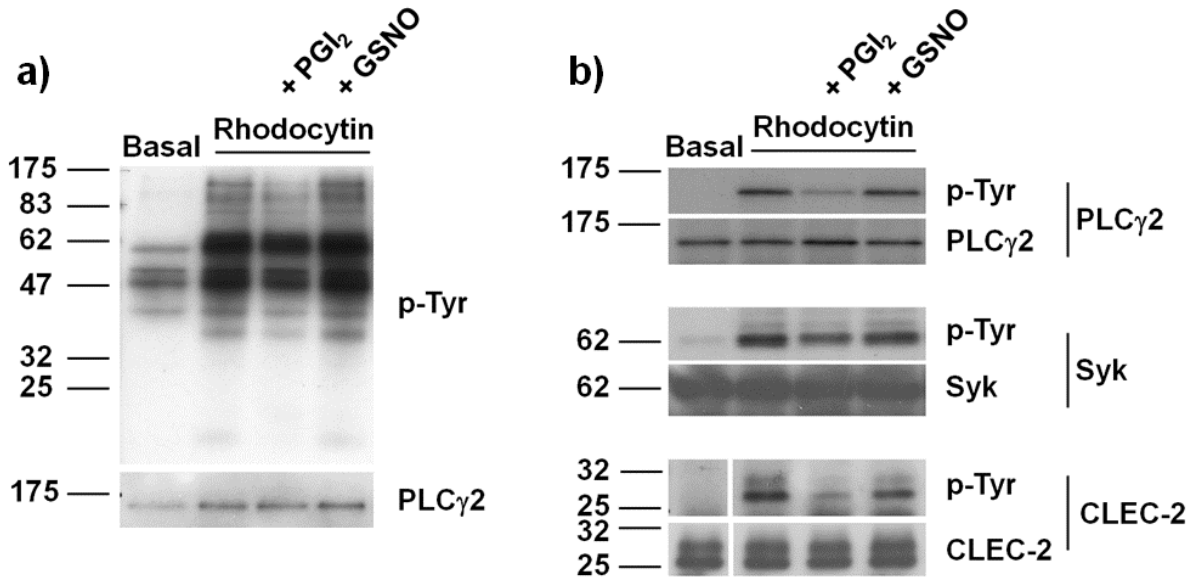
Lack of effect of SNP on CLEC-2-dependent platelet aggregation and tyrosine phosphorylation- The effect of SNP (100 μM) on CLEC-2 mAb-dependent platelet aggregation (a) and tyrosine phosphorylation (b) was studied. Washed platelets ($2x10^8$/ml) were treated as indicated for 3 minutes prior to activation with mAb and allowed to aggregate (a). Washed platelets ($4x10^8$/ml) treated with of apyrase (2 U/ml), indomethacin (10 μM) and lotrafiban (10 μM) were added with SNP (100 μM) in comparison with PGI_2 (1 μM) and stimulated with 10 μg/ml CLEC-2 mAb for 3 minutes prior to lysis. Aliquots were analysed by SDS-PAGE and blots were probed with anti phospho-tyrosine monoclonal antibody (clone 4G10) (b).

SUPPLEMENTARY FIGURE 3.



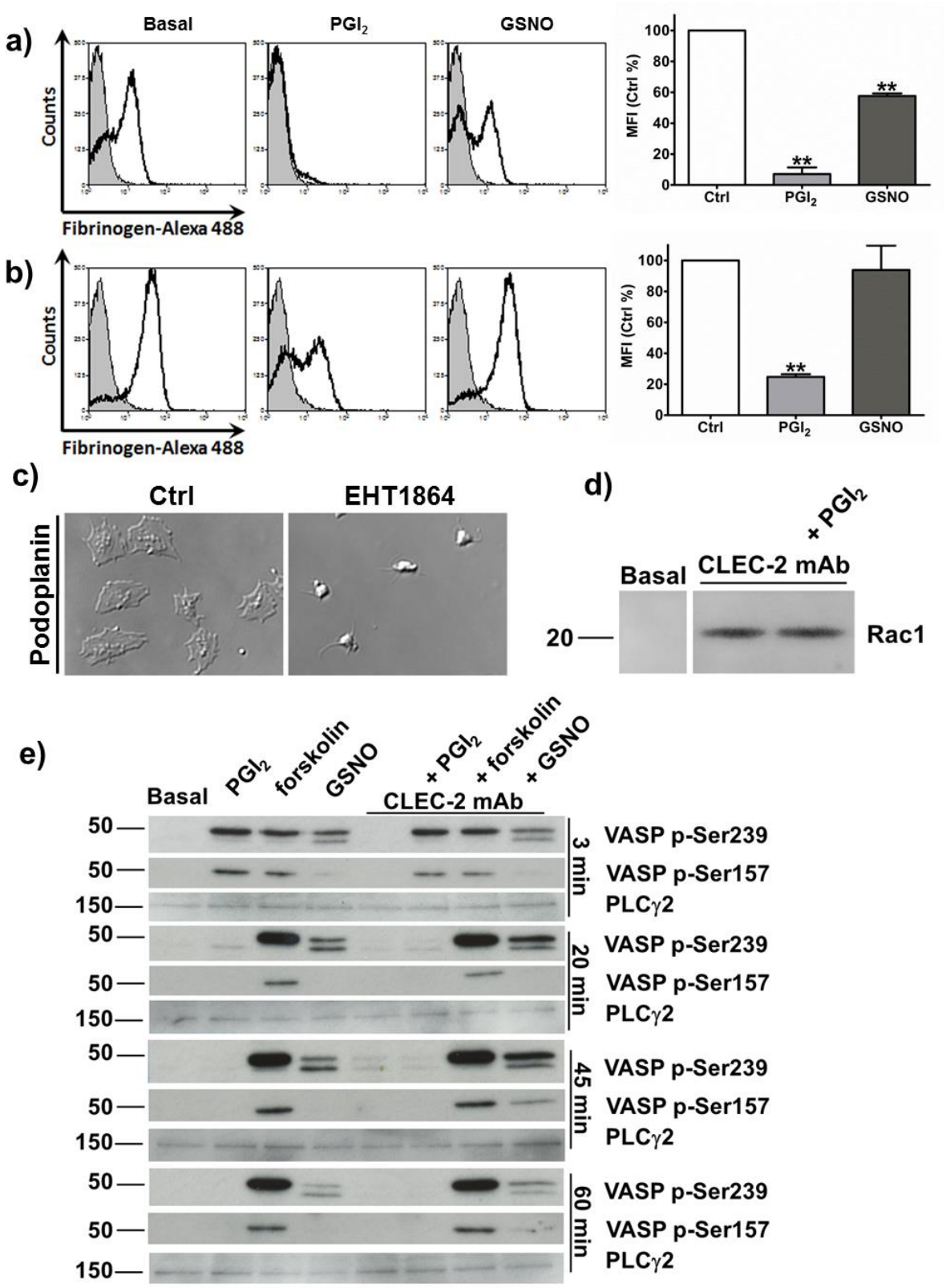
Effect of cyclic nucleotide-elevation on CLEC-2-dependent platelet aggregation and tyrosine phosphorylation in human platelets- The effect of PGI2 (0.1 μM), GSNO and SNP (100 μM) on rhodocytin-induced platelet aggregation (a) and tyrosine phosphorylation (b) was studied. Washed platelets ($2x10^8$/ml) were treated as indicated for 3 minutes prior to activation with mAb and allowed to aggregate (a). Washed platelets ($5x10^8$/ml) treated with apyrase (2 U/ml), indomethacin (10 μM) and lotrafiban (10 μM) were treated with PGI_2 (1 μM), GSNO or SNP (100 μM) and stimulated with 300nM rhodocytin for 3 minutes prior to lysis. Aliquots were analysed by SDS-PAGE and blots were probed with anti phospho-tyrosine monoclonal antibody (clone 4G10) (b).

SUPPLEMENTARY FIGURE 4.



Rhodocytin-dependent tyrosine phosphorylation is partially reduced by cyclic nucleotide-elevation- The effect of cyclic nucleotide-elevation on rhodocytin-dependent tyrosine phosphorylation was assessed by use of PGI_2 (2 μM) and GSNO (100 μM) in the presence of apyrase (2 U/ml), indomethacin (10 μM) and lotrafiban (10 μM). Washed platelets ($4x10^8$/ml) were stimulated with 30 nM rhodocytin for 3 minutes and lysed with NP40 detergent. (a) Aliquots were analysed by SDS-PAGE and (b) the remaining was used to immunoprecipitate PLCγ2, Syk and CLEC-2. Results are representative of 3 experiments. Blots were probed with anti phospho-tyrosine monoclonal antibody (clone 4G10) (a&b) and reprobed for equal loading control. The total CLEC-2 blot was obtained by running in parallel 25% of the sample.

SUPPLEMENTARY FIGURE 5.



cAMP and cGMP have differential effects on different platelet pathways - Washed platelets (2×10^7/ml) were pre-incubated for 3 min with the stated concentrations of PGI_2, GSNO or vehicle and stimulated with 10 μg/ml CLEC-2 mAb for 3 min (a) and 45 min (b) at 37°C in the presence of 60 μg/ml Alexa-Fluor-488–labelled fibrinogen. Incubations were fixed by addition of ice cold 1% formaldehyde and analysed in a FACScalibur flow cytometer using Cell-Quest software (BD Biosciences, Oxford, UK). Representative traces are shown. Mean fluorescence intensity values were plotted after subtraction of the unstimulated control levels and plotted as mean ± SEM (n=3). Statistical difference was evaluated by one way ANOVA test and Dunnet's post test (**$p<0.01$).

$2x10^7$/ml washed platelets were treated with EHT1864 (50μM), before spreading on podoplanin-coated coverslips (10μg/ml). Coverslips were fixed, mounted and imaged as described in Figure 4 (c). A suspention of platelets (10^9/ml) was activated by CLEC-2 mAb (10μg/ml) for 45 min in the presence or absence of 1μM PGI_2 and Rac activation was studied by GTP-Rac pull down assay using a commercially available kit according to manufacturer's instructions. A positive control for Rac activation was prepared by incubating platelets in parallel with GTPγS as specified in the kit manual (not shown). Pulled down beads were analyzed by SDS-PAGE (10%) and blots were probed for Rac1(d) (n=3).

Washed platelets ($4x10^8$/ml) were activated with 10 μg/ml CLEC-2 mAb in the presence or absence of PGI_2 (1μM) or GSNO (1mM) and lysed after 3, 20, 45 or 60 min with 5x Laemmli sample buffer. Aliquots were analysed by SDS-PAGE prior to probing with anti VASP p-Ser239. The antibody was then stripped and the membranes reprobed for VASP p-Ser157 and PLCγ2 for loading control (n=2).