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Supplementary Materials for

Regulation of P2X2 Receptors by the Neuronal Calcium Sensor VILIP1

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Supplementary Materials and Methods

Molecular biology, antibodies and plasmids:

Wild-type and XFP-tagged P2X plasmids were all available from previous work(1). NCS1 and VILIP3 plasmids were from Prof. R Burgoyne (University of Liverpool, UK) and Prof K.-H Braunewell (Department of Biochemistry and Molecular Biology, Southern Research Institute, Birmingham AL, USA). Mutants were made using the Quick Change Site Directed mutagenesis kit (Stratagene), and verified by sequencing. Standard molecular biology methods were used to propagate the plasmids and to purify DNA. Polyclonal rabbit anti-VILIP1 was a gift from D. Ladant (Institut Pasteur, Paris), polyclonal guinea pig anti-P2X2 was from Neuromics (GP14106), and monoclonal mouse anti-FLAGM2 was from Sigma (F3165). pGEX-3X vector for bacterial production of GST fusion protein was engineered to introduce a TEV protease cleaving site at the 3'end of the GST open reading frame, in front of the multi-cloning sites. Briefly, a DNA linker coding for the consensus TEV cleaving amino acid sequence (KLEKLYFQGIQ) was introduced between the *EcoRI* and the *BamH1* sites of pGEX-3X, in the open reading frame of the GST protein, (vector pGEX-TEV). Because the GST-$P2X_2$ fusion protein encoding the C-terminal tail of full length P2X2 receptor produced in bacteria was poorly soluble and mostly present in inclusion bodies we used the C-terminal tail sequence of the splice variant P2X2b receptor, in which a 49 residue alternative proline-rich exon is lacking. Sequence encoding the residues 350–403 of P2X2b was subcloned into pGEX-TEV vector, resulting in an open reading frame encoding GST-TEV-P2X2b tail fusion protein. GST-TEV-P2X2b fusion protein was soluble and could be purified without re-folding steps. All engineered plasmids were verified by sequencing. P2X2-FLAG plasmid has been described (2). VILIP1 and VILIP1 EF-hand mutant plasmids were gifts from F. Coussen (Institut F. Magendie, Bordeaux).

Co immunoprecipitation methods for Fig S1:

HEK cells were co transfected with P2X2-FLAG and either VLIP1 or the VILIP1 EF hand mutant. Cell lysates and washes were prepared essentially as described in the Material and Methods section of the main manuscript. Two different cell lysis buffers were used: Calcium-free buffer, which contained 5 mM EDTA, or calcium buffer with no EDTA and 500µM calcium. All washes were performed in one or the other of these cell lysis buffers. In some experiments, cells were incubated with 100 µM ATP or with 2 µM ionomycin in culture medium for 5 minutes at 37°C prior to cell lysis. Immunoprecipitations were performed with anti-FLAG-conjugated agarose beads as described in the main Material and Methods section, except that proteins were eluted by competition with FLAG peptide (200µg/ml in lysis buffer, 2 x 1 hour at 4°C on a rotating wheel). Eluted proteins were denaturated in Laemmli sample buffer and separated by SDS PAGE prior western blotting.

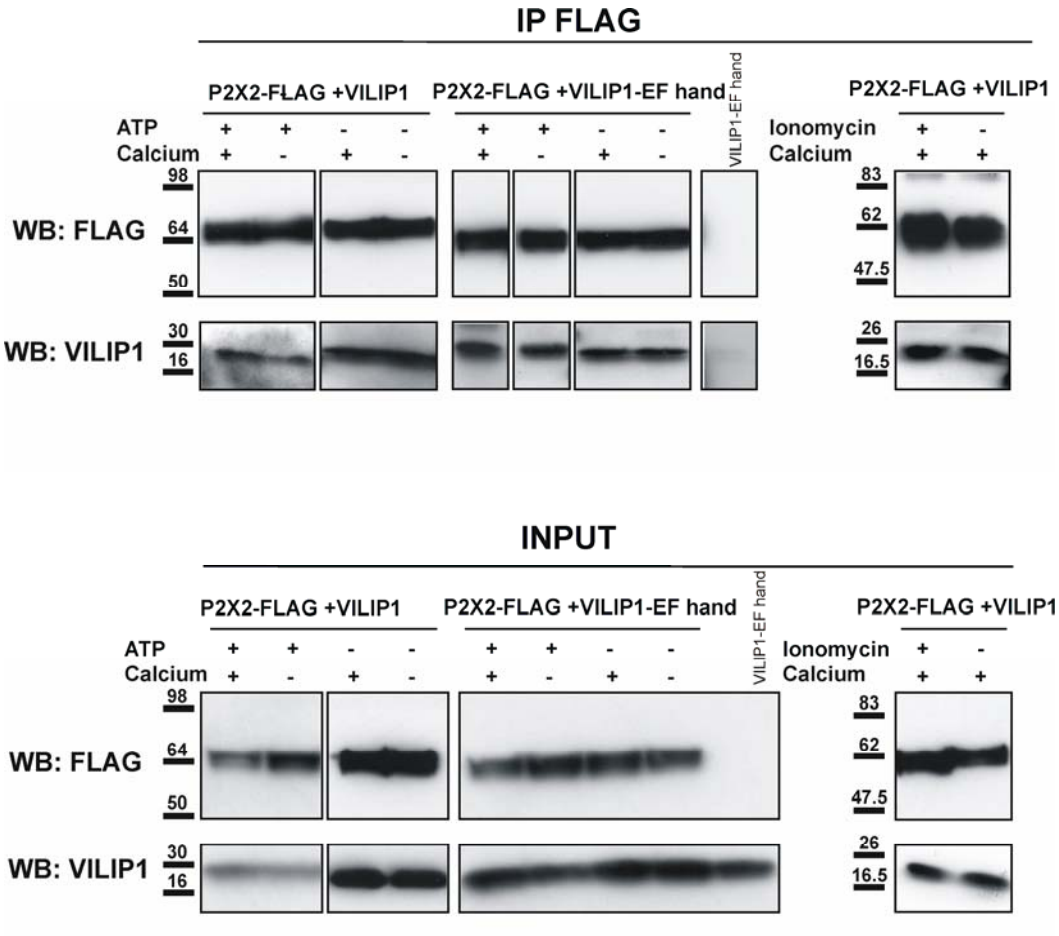

Fig S1. The constitutive interaction between P2X2 receptors and VILIP1 is calcium independent.

Calcium dependence of the interaction between P2X2 receptors and VILIP1 was assessed by co-immunoprecipitation from HEK cells expressing P2X2-FLAG receptors and either VILIP1 or VILIP1 EF-hand mutants. The effect of calcium on the interaction was tested before cell lysis by increasing intracellular calcium with ATP application or ionomycin treatment, and also during the immunoprecipitation steps. In the latter case, experiments were carried in the absence of calcium and in the presence of 5 mM EDTA or in the absence of EDTA and in the presence of 500µM calcium. P2X2 receptors were immunoprecipitated using a FLAG antibody conjugated to agarose beads. Bound proteins were eluted by competition using a FLAG peptide. As shown in the top panel, no difference in P2X2-FLAG-VILIP1 interaction was observed in the presence or the absence of calcium in the immunoprecipitation buffer. In addition, stimulation of transfected cells with ATP (100µM, 5 minutes) or with ionomycin (2µM, 5 minutes) prior to lysis did not affect the interaction between the two proteins. A similar observation was made when the VILIP1 EF hand mutant was co-immunoprecipitated. No immunoprecipitation was observed when VILIP1 was transfected alone. The bottom panel shows the total protein input used for immunoprecipitation.

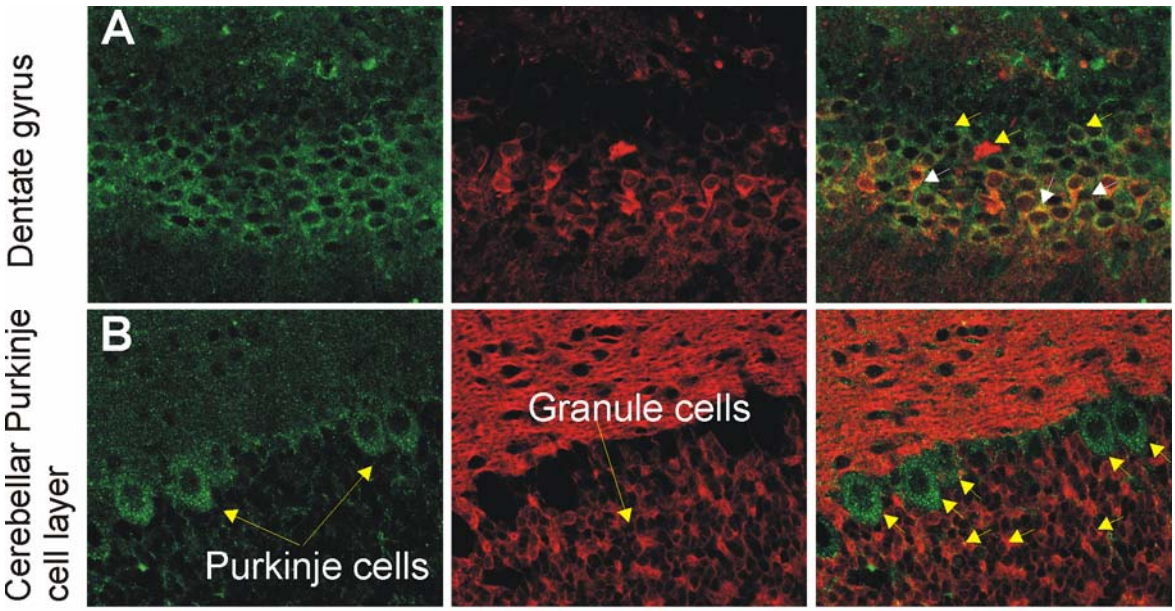

Fig S2. Colocalization between P2X2 receptors and VILIP1.
We determined that P2X2 receptors and VILIP1 colocalized in neurons using immunocytochemistry (ICC). P2X2 receptors and VILIP1 colocalized in all neurons of the deep cerebellar nuclei (Fig 1D); colocalization per neuron was 47±6% (n=12), in other words ~47% of pixels that showed signal for P2X2 also showed equal signal for VILIP1. This is because VILIP1 was more diffusely expressed than P2X2 receptors, and all areas of the neurons that expressed P2X2 receptors colocalize with VILIP1. We saw no colocalization in Purkinje and granule cells of the cerebellum (**b**). These neurons expressed signal for either P2X2 receptors or VILIP1, but not both. The neuron-specific labelling indicates that the signals are specific. We also found partial colocalization in granule cells in the dentate gyrus of the hippocmapus (**a**). The staining we report is consistent with previous work for both P2X2 receptors and VILIP1(3-5). These experiments were repeated on ~50 μm sections from 3 brains from P15 rats. We saw no signal in sections where the secondary antibody had been excluded, in sections with no primary antibody, or when the antibody had been pre-absorbed with blocking peptide. White arrows point to strong colocalization and yellow arrows point to no colocalization.

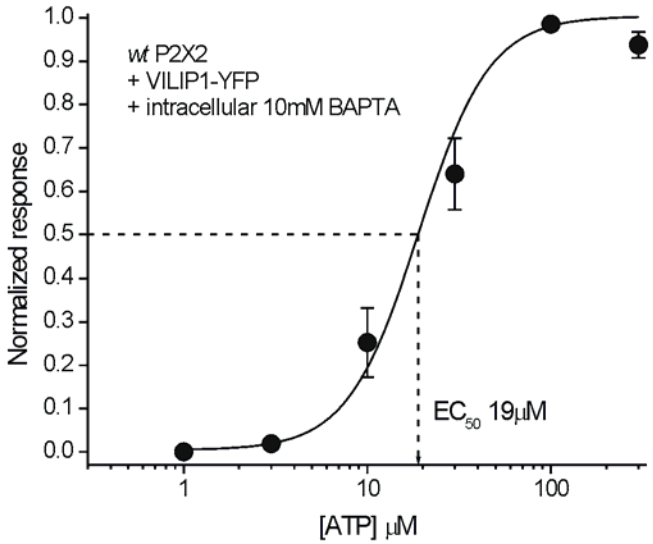

Fig S3. ATP concentration-response curve for HEK cells expressing P2X2 receptors and VILIP1-YFP, and dialyzed with BAPTA.

In these experiments, cells were dialysed with 10 mM BAPTA. The concentration-response curve is almost identical to the one shown in Fig. 2A for these constructs in the absence of intracellular BAPTA and with 1mM calcium in the recording buffer. We could not use 0 mM Ca^{2+} in the extracellular recording buffer because this made it problematic to maintain the whole-cell configuration long enough to construct full concentration-response curves. In addition, calcium directly modulates P2X2 receptors from the outside (6-8) making comparisons between 0 and 1 mM extracellular calcium concentrations problematic.

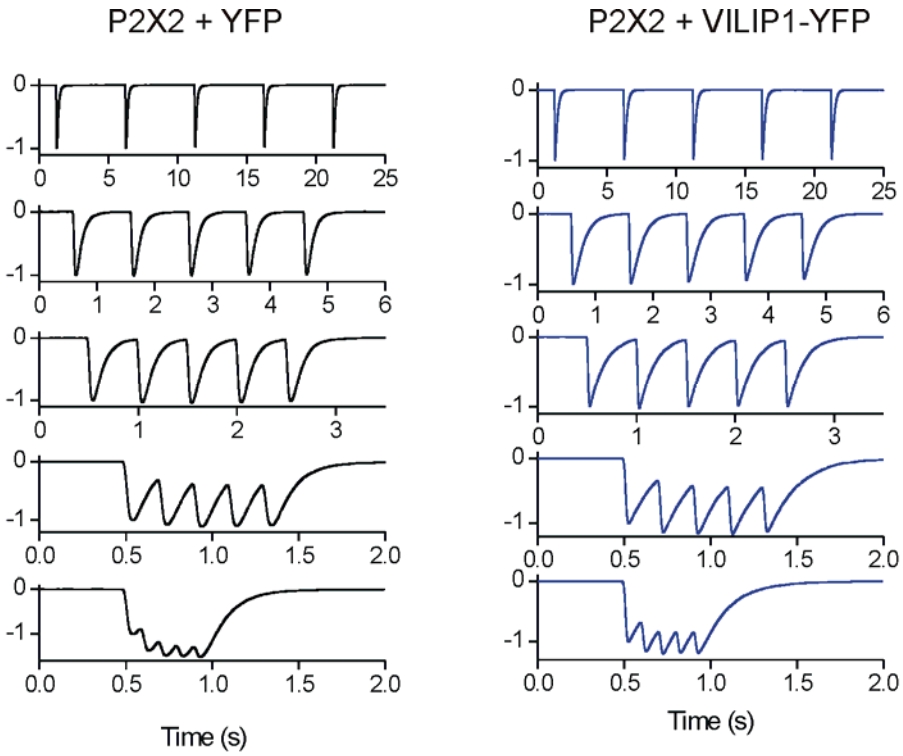

Fig S4. Responses evoked by repetitive ATP application.
Representative traces for cells expressing P2X2 receptors and VILIP1-YFP (right hand panels) and P2X2 receptors and YFP (left hand panels). We applied 100 μM ATP (for ~30ms) five times with interpulse intervals of 5, 1, 0.5, 0.2 and 0.1 s (top to bottom in the graphs). We recorded robust ATP-evoked responses, shown normalized to the first response in each case. We found no difference between cells expressing P2X2 receptors and VILIP1-YFP and those expressing P2X2 receptors and YFP as shown in the representative traces. Similar data were gathered in six other cells for each expression pair. We conclude that, under these conditions, VILIP1 does not alter P2X2 receptor responses to repetitive ATP applications.

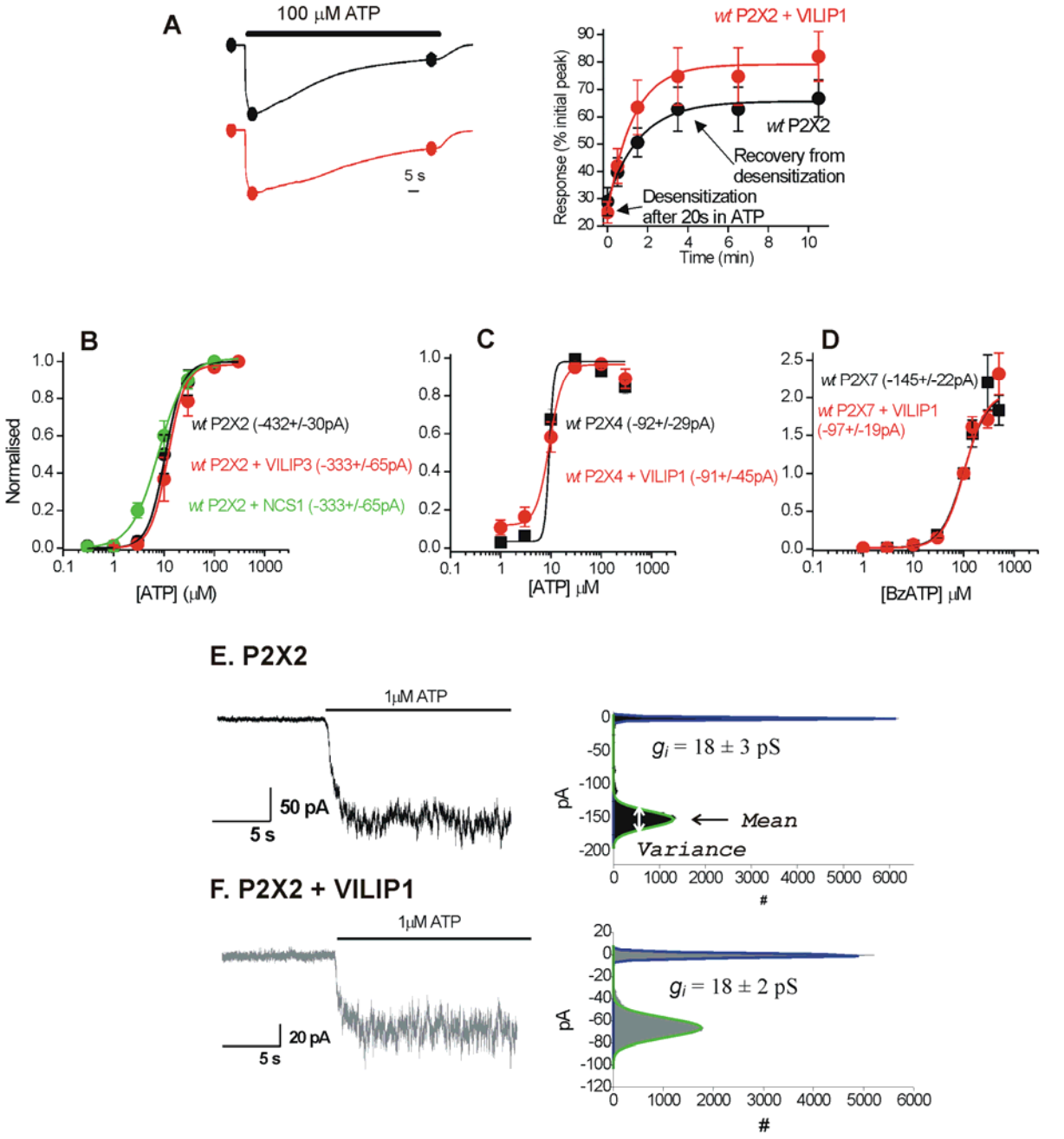

Fig S5. Effects of VILIP1 on P2X2 receptor function.
A. Similar desensitization (left panel; normalised traces) and recovery from desensitization (right panel) for P2X2 receptors with and without VILIP1; n>5 for each experiment. There was no difference in the recovery curves by students *t* test for each point (p>0.05). **B-D.** Controls for VILIP1-P2X2 interactions. Dose-response curves for P2X receptors with and without VILIP3 and NCS1 (B), for P2X4 receptors (C), and P2X7 receptors (D) with and without VILIP1. There were no differences by Students *t* test or ANOVA for either the EC_{50} or peak currents (n>6; *p*>0.05). **E.** Whole-cell current activated by 1 μM ATP from a HEK 293 cell expressing P2X2 receptors. The open channel noise was used to calculate single channel conductance. **F.** As in E but for cells expressing P2X2 receptors and VILIP1. In both E and F the single channel conductance was the same.

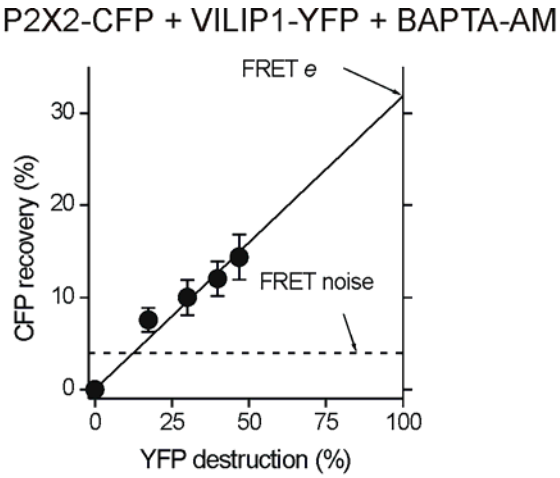

Fig S6. FRET efficiency determination between P2X2-CFP receptors and VILIP1-YFP in the absence of calcium.

As expected from Fig 3 the FRET value (n=16) was greater than FRET noise, which was ~3% for these experiments. There was a trend for the FRET efficiency estimate for these experiments to be greater than that observed in the presence of calcium in Fig 3. Cells were loaded with 30μM BAPTA-AM (30 min followed by 30 min cleavage) and bathed in 0.1 mM calcium recording buffer.

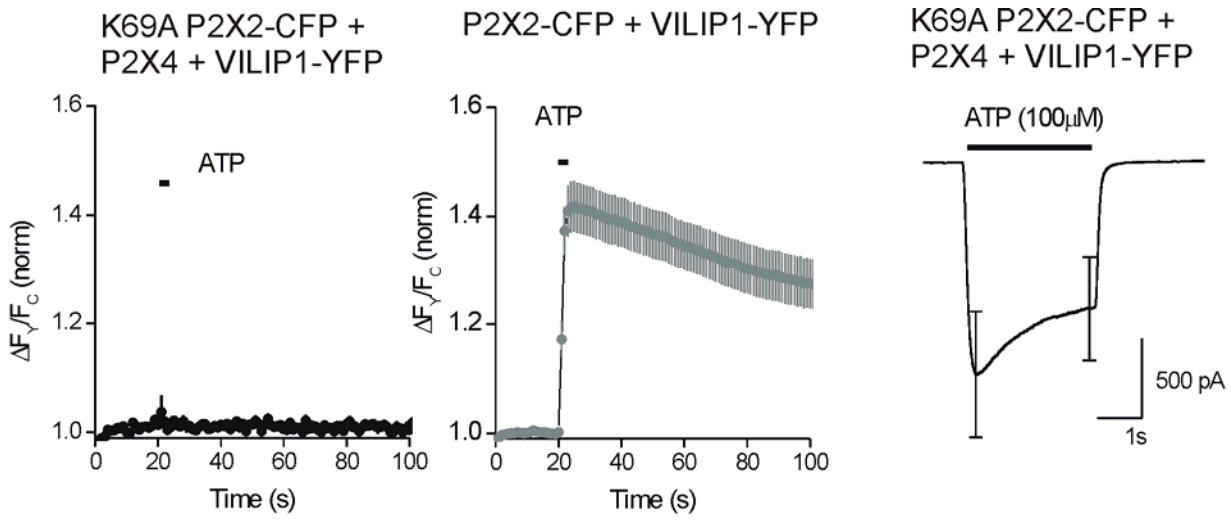

Fig S7. Calcium entry independently of P2X2 receptors does not lead to elevated FRET between P2X2-CFP and VILIP1-YFP.

We carried out a set of experiments to determine if calcium entering into cells independently of P2X2-CFP receptors could evoke enhanced FRET with VILIP1-YFP. To this end we used K69A P2X2-CFP receptors in conjunction with *wt* P2X4 receptors. K69A P2X2-CFP receptors do not bind ATP (9-11) and therefore ATP application does not cause calcium entry through them. P2X4 receptors express robustly in HEK cells (10, 11), have the highest calcium fluxes of the P2X receptors (12) (14%; *versus* 6% for P2X2), and do not form heteromers with P2X2 receptors (13). Thus, co-expressing K69A P2X2-CFP receptors with *wt* P2X4 allows us to determine if extracellular Ca^{2+} entry through independent P2X4 receptors can cause FRET changes for K69A P2X2-CFP receptors and VILIP1-YFP. ATP applications (100μM for 3s) to cells co-expressing K69A P2X2-CFP, VILIP1-YFP and P2X4 receptors failed to cause significant increases in FRET (n=5; see left panel). For comparison, the middle panel shows the result for cells expressing P2X2-CFP and VILIP1-YFP. We have shown that P2X4 receptors cause large increases in intracellular calcium concentration, and in the present experiments P2X4 receptors were strongly expressed as evidenced by the large ATP-evoked inward currents (right hand traces; n=5). Taken together these data indicate that the P2X2-CFP and VILIP1-YFP FRET signals are not simply the result of global calcium changes.

Supplementary Table 1. Proteomic analysis of proteins interacting with the carboxyl-terminal cytoplasmic region of the P2X2 receptor (see Methods and Results).

Position in gel	Protein I.D.	Swiss-Prot Accession #	Number of identified peptides	Coverage %
1	HSP90ß	Q9GKX8	17	25
2	VILIP1	Q4W4C9	8	41
3	Synapsin IIb	Q64332	13	42
4	V-ATPase subunit α	P50516	15	25
5	Tubulin α1	P02551	22	56
6	Glutamine synthase	Q91VC6	22	57
7	Vesicular amine transporter 1	Q8R3G0	14	45
8	Vesicular-fusion protein NSF	P46460	38	53
9	Glutamate decarboxylase 65 kDa isoform	P48320	19	42

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