

# P2Y<sub>1</sub> receptor signaling is controlled by interaction with the PDZ scaffold NHERF-2

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**P2Y<sub>1</sub> purinergic receptors (P2Y<sub>1</sub>Rs) mediate rises in intracellular Ca<sup>2+</sup> in response to ATP, but the duration and characteristics of this Ca<sup>2+</sup> response are known to vary markedly in distinct cell types. We screened the P2Y<sub>1</sub>R carboxyl terminus against a recently created proteomic array of PDZ (PSD-95/*Drosophila* Discs large/ZO-1 homology) domains and identified a previously unrecognized, specific interaction with the second PDZ domain of the scaffold NHERF-2 (Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor type 2). Furthermore, we found that P2Y<sub>1</sub>R and NHERF-2 associate in cells, allowing NHERF-2-mediated tethering of P2Y<sub>1</sub>R to key downstream effectors such as phospholipase Cβ. Finally, we found that coexpression of P2Y<sub>1</sub>R with NHERF-2 in glial cells prolongs P2Y<sub>1</sub>R-mediated Ca<sup>2+</sup> signaling, whereas disruption of the P2Y<sub>1</sub>R–NHERF-2 interaction by point mutations attenuates the duration of P2Y<sub>1</sub>R-mediated Ca<sup>2+</sup> responses. These findings reveal that NHERF-2 is a key regulator of the cellular activity of P2Y<sub>1</sub>R and may therefore determine cell-specific differences in P2Y<sub>1</sub>R-mediated signaling.**

G protein-coupled receptor | purinergic | ATP | proteomic array

Adenine-based nucleotides such as ATP and ADP are prominent extracellular signaling molecules that mediate a wide variety of physiological actions in tissues throughout the body. Many of the physiological effects evoked by ATP and ADP are mediated by metabotropic P2Y receptors (P2YRs) (1), which are members of the G protein-coupled receptor (GPCR) superfamily. To date, seven distinct mammalian P2YR subtypes have been cloned: P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub>. Most P2YRs are coupled to G<sub>α<sub>i</sub></sub> proteins and thus to the activation of phospholipase Cβ (PLCβ) and generation of diacylglycerol and inositol-3,4,5-trisphosphate, ultimately leading to the activation of PKC and release of Ca<sup>2+</sup> from internal stores (1). Some P2YRs, including P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub>, are also known to couple to G<sub>α<sub>i</sub></sub> and the inhibition of adenylyl cyclase (2–5). The purinergic P2YR type 1 (P2Y<sub>1</sub>R) subtype is abundantly expressed in a number of tissues, including the CNS (6, 7), where it plays a key role in the transmission of astrocytic Ca<sup>2+</sup> waves (8), activation of mitogenic responses in astrocytes to brain trauma (9), inhibition of neuronal N-type voltage-activated Ca<sup>2+</sup> channels (10), and embryonic brain development (11). P2Y<sub>1</sub>R also plays critical roles in the cardiovascular system, including the regulation of coronary vasodilation (12) and platelet aggregation (13).

Signaling by P2Y<sub>1</sub>Rs is known to be heavily dependent on cellular context. For example, stimulation of P2Y<sub>1</sub>Rs in some cell types is known to strongly promote cell proliferation (14), whereas P2Y<sub>1</sub>R stimulation in other cell types is known to induce apoptosis (15). Furthermore, P2Y<sub>1</sub>Rs can exert cellular effects that are quite different from those exerted by other P2YRs expressed in the same cell type and that couple to similar G proteins. For example, in astrocytes, both P2Y<sub>1</sub>Rs and P2Y<sub>2</sub>Rs can couple by means of G<sub>q</sub> to the release of Ca<sup>2+</sup> from a common intracellular pool (16). Yet, P2Y<sub>1</sub>Rs and P2Y<sub>2</sub>Rs exhibit striking temporal differences in their Ca<sup>2+</sup> response patterns and susceptibility to regulation by PKC (17). At present, it is not clear why P2Y<sub>1</sub>R activity is so dependent

on cellular context or why P2Y<sub>1</sub>Rs can exert physiological effects that are so different from related P2YR subtypes.

GPCR signaling can be regulated by various types of receptor-associated scaffold proteins (18). Many of the known interactions between GPCR scaffold proteins and their cognate GPCRs are mediated by the binding of PSD-95/*Drosophila* Discs large/ZO-1 homology (PDZ) domains within the scaffold proteins to specialized motifs at the carboxyl terminus (CT) of the GPCRs (18, 19). Among the >440 PDZ domains in the human genome, approximately one-quarter are believed to be class I PDZ domains, which are characterized by preferential binding to the carboxyl-terminal motif S/T-x-§ (where § represents a hydrophobic residue at the terminal position). Interestingly, P2Y<sub>1</sub>R-CT terminates in the motif D-T-S-L, suggesting that P2Y<sub>1</sub>R might associate with class I PDZ scaffold proteins. In this study, we used a recently created proteomic array of 96 distinct PDZ domains derived from a variety of cytosolic proteins to identify proteins that might selectively associate with the CT of P2Y<sub>1</sub>R. Using this technique, we found a previously unrecognized high-affinity association of P2Y<sub>1</sub>R-CT with the PDZ scaffold protein NHERF-2 (Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor type 2). We also observed that NHERF-2 and P2Y<sub>1</sub>R exhibit similar patterns of expression in native brain tissue and that NHERF-2 can exert substantial control over P2Y<sub>1</sub>R functional activity.

## Materials and Methods

**Construction of the PDZ Domain Proteomic Array.** PDZ domains were expressed as His- and S-tagged fusion proteins by using the vector pET30A (Novagen) and were purified by using ProBond nickel resin (Invitrogen). We thank the large number of colleagues who sent us cDNA constructs encoding various PDZ proteins (listed in the supporting information, which is published on the PNAS web site). These cDNAs were used as templates to amplify by means of PCR the regions encoding various PDZ domains to subclone them into pET30A for fusion protein expression.

**Overlays, Fusion Protein Pull-Downs, and Plate-Binding Assays.** To assess the binding of receptor carboxyl-terminal GST fusion protein to the PDZ domain array, the purified PDZ domain fusion proteins were spotted at 1 μg per bin onto Nytran SuperCharge 96-grid nylon membranes (Schleicher & Schuell). The membranes were allowed to dry overnight and then blocked in “blot buffer” (2% nonfat dry milk/0.1% Tween 20/50 mM NaCl/10 mM Hepes, pH 7.4) for 1 h at room temperature. Overlays with P2Y<sub>1</sub>R-CT fusion protein (100 nM in blot buffer) were then performed by using an

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Abbreviations: P2YR, purinergic P2Y receptor; P2Y<sub>1</sub>R, P2YR subtype 1; GPCR, G protein-coupled receptor; NHERF-2, Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor type 2; PDZ, PSD-95/*Drosophila* Discs large/ZO-1 homology; PDZ2, second PDZ domain; PLCβ, phospholipase Cβ; CT, carboxyl terminus; HA, hemagglutinin; 2-MeSADP, 2-methylthio-ADP.

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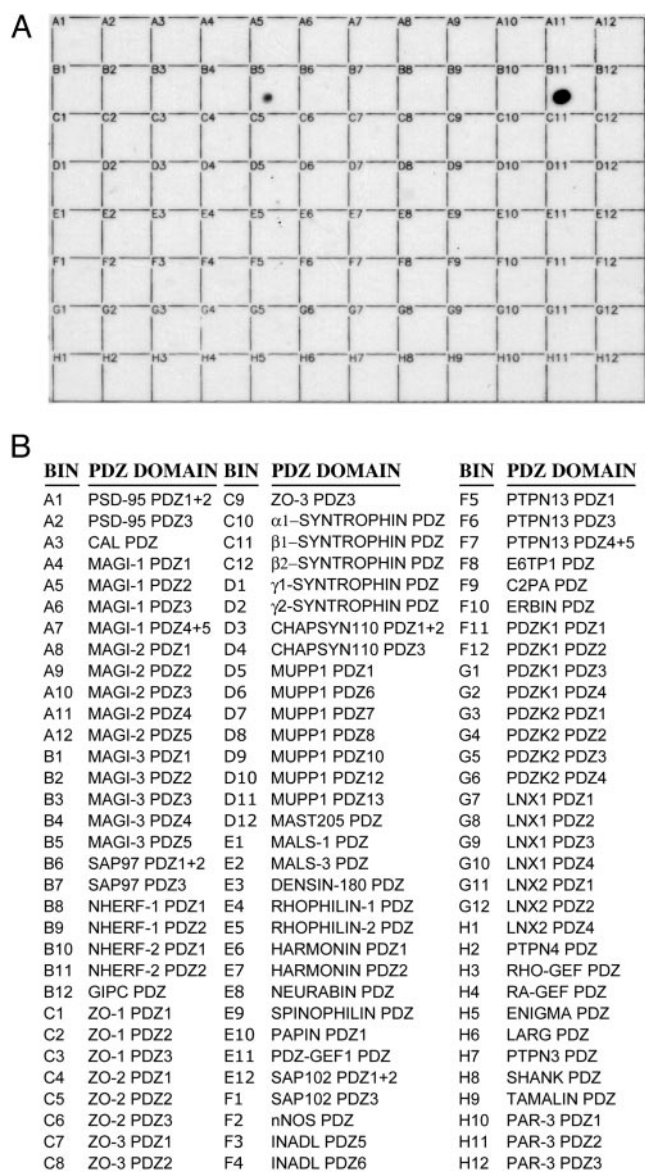
overlay technique that is described in refs. 20 and 21. Interactions were confirmed by means of fusion protein pull-downs, and affinity estimates were generated in saturation binding assays by using a plate-binding assay described in ref. 20.

**Cell Culture and Transfection.** HEK-293 and 1321N1 cells were regularly maintained in DMEM (GIBCO) supplemented with 10% FBS and 1% penicillin/streptomycin in a 37°C/5% CO<sub>2</sub> incubator. C6 glioma cells were maintained in DMEM (GIBCO) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were split every 3–4 days. For transfections, cells were split onto 100-mm plates, grown to 90–100% confluency, and transfected with the appropriate cDNA by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. cDNAs used in transfections were P2Y<sub>1</sub>R-pcDNA3, P2Y<sub>1</sub>R/L373A-pcDNA3, FLAG-tagged NHERF-2-pcDNA3, and hemagglutinin (HA)-tagged NHERF-2-pcDNA3.

**Immunoprecipitation, Surface Expression Assay, and Western Blotting.** Coimmunoprecipitation of full-length proteins from HEK-293 cells was performed by using appropriate primary antibodies and methods described in ref. 21. Surface expression of P2Y<sub>1</sub>R was verified by using a luminometer-based surface expression assay as described in ref. 21; no significant differences in surface expression between the WT P2Y<sub>1</sub>R and L373A mutant receptor were observed (data not shown). Purified proteins, cell extracts, and/or immunoprecipitated samples were separated by SDS/PAGE and blotted onto nitrocellulose as described in refs. 20 and 21. Monoclonal anti-HA 12CA5 antibody (Roche Applied Science, Indianapolis) and monoclonal anti-Flag M2 antibody (Sigma) were the primary antibodies used to detect and immunoprecipitate epitope-tagged proteins. To detect endogenous and/or recombinant P2Y<sub>1</sub>R, PLCβ<sub>1</sub>, and NHERF-2, we used polyclonal anti-P2Y<sub>1</sub>R antibody (Zymed), anti-PLCβ<sub>1</sub> (Santa Cruz Biotechnology), and anti-NHERF-2 (22).

**Immunocytochemistry and Electron Microscopy Analysis.** To visualize immunostaining for P2Y<sub>1</sub>R and NHERF-2 in native brain tissue, C57BL/6 mice were killed, and brain sections were prepared and processed for the preembedding immunoperoxidase labeling as described in ref. 23 with the following modifications: 2% milk was used as a blocking agent, and rabbit anti-P2Y<sub>1</sub>R and anti-NHERF-2 antibodies were used at 1:1,000 and 1:7,000 dilutions, respectively. A random sampling of 33–62 micrographs of small capillaries were taken from ultrathin sections of the cerebral cortex with a charge-coupled device camera (DualView 300W, Gatan, Pleasanton, CA) controlled by DIGITALMICROGRAPH 3.6.5 software (Gatan). Digitally acquired micrographs were adjusted for brightness and contrast; the image resolution was kept constant. All processes (147–218) in apposition with endothelial cells were analyzed for the presence or absence of amorphous diaminebenzidine deposit corresponding to immunoreactivity for P2Y<sub>1</sub>R or NHERF-2.

**Calcium Imaging.** The Ca<sup>2+</sup>-sensitive fluorophore fura-2 (Molecular Probes) was used for ratiometric Ca<sup>2+</sup> imaging in C6 glioma and 132N1 human astrocytoma cells. All fluorescence measurements were made from subconfluent areas of the dishes so that individual cells could be readily identified. After transfection in 100-mm plates, cells were split onto coverslips immersed in 0.5 ml of media in 24-well plates and grown for 1–2 days. Before imaging, coverslips were incubated at room temperature for 30 min in extracellular recording solution (ECS) composed of 150 mM NaCl/10 mM Hepes/3 mM KCl/2 mM CaCl<sub>2</sub>/2 mM MgCl<sub>2</sub>/5.5 mM glucose, pH 7.3, 325 milliosmolar. ECS was supplemented with pluronic acid (0.001%) and fura-2 AM (2 μM). Subsequently, coverslips were thoroughly rinsed with extracellular solution lacking fura-2 AM and BSA and mounted onto the microscope stage for imaging. Intensity images of 510-nm emission wavelengths were taken at 340- and

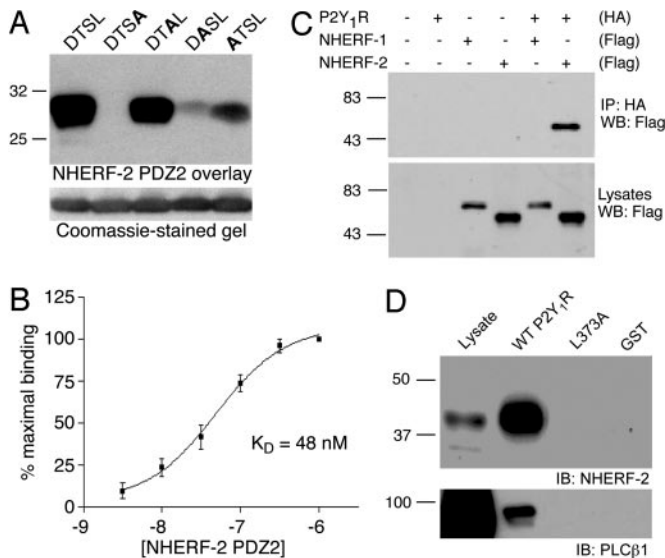


**Fig. 1.** P2Y<sub>1</sub>R-CT binds selectively to NHERF-2 PDZ2. The CT of P2Y<sub>1</sub>R was prepared as a GST fusion protein and overlaid onto a proteomic array containing 96 putative class I PDZ domains. (A) The results of the overlay. (B) A list of the PDZ domains that were spotted in each bin of the array. P2Y<sub>1</sub>R-CT exhibited strong binding to bin B11, the PDZ2 of NHERF-2, and weaker binding to bin B5, the fifth PDZ domain of MAGI-3. Much longer exposures (not shown) revealed faint binding of P2Y<sub>1</sub>R-CT to bin B8 (NHERF-1 PDZ1) and bin A12 (MAGI-2 PDZ5). These results are representative of five independent experiments.

380-nm excitation wavelengths, and the two resulting images were taken from individual cells for ratio calculations. IMAGING WORKBENCH 2.2.1 (Axon Instruments, Union City, CA) was used for acquisition of intensity images and conversion to ratios. The observed calcium responses were mainly due to liberation of calcium from intracellular stores (~70%), with a minor component attributable to influx of extracellular calcium (~30%), as assessed in experiments comparing responses in the presence versus absence of extracellular calcium (*n* = 95 cells; data not shown).

## Results

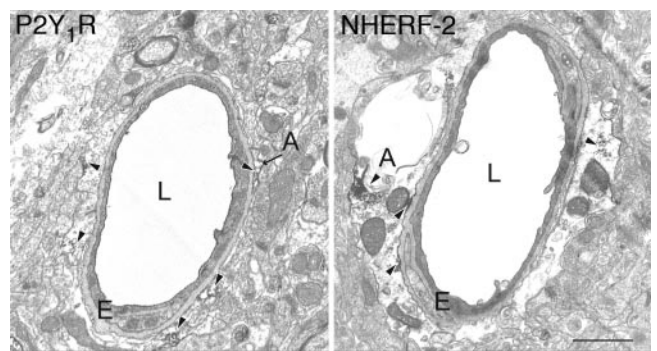
**Screening of a PDZ Domain Proteomic Array Reveals Strong Association of P2Y<sub>1</sub>R-CT with the Second PDZ Domain (PDZ2) of NHERF-2.** To identify PDZ domain-containing proteins that might associate with



**Fig. 2.** Structural determinants of the P2Y<sub>1</sub>R–NHERF-2 PDZ2 interaction *in vitro* and in cells. (A) The last four amino acids of P2Y<sub>1</sub>R-CT (D-T-S-L) were sequentially mutated to alanine, and WT P2Y<sub>1</sub>R-CT as well as the four mutants were expressed as GST fusion proteins. These fusion proteins were loaded equally (2 μg) onto SDS/PAGE gels (Lower), transferred to nitrocellulose, and overlaid with 100 nM His-tagged NHERF-2 PDZ2 fusion protein (Upper). The data shown are representative of three independent experiments. (B) Affinity of the P2Y<sub>1</sub>R-CT–NHERF-2 PDZ2 interaction. The wells of a 96-well plate were coated with P2Y<sub>1</sub>R-CT–GST and overlaid with six different concentrations of His-tagged NHERF-2 PDZ2 (3 nM to 1 μM). Binding was expressed as a percentage of the binding observed at 1 μM NHERF-2 PDZ2. The binding was saturable and yielded an estimated dissociation constant of 48 nM (*n* = 3). Points and error bars represent mean ± SEM. (C) Coimmunoprecipitation of P2Y<sub>1</sub>R and NHERF-2 from cells. HA-tagged P2Y<sub>1</sub>R was expressed in HEK-293 cells in the absence and presence of Flag-tagged NHERF-1 and NHERF-2. P2Y<sub>1</sub>R was immunoprecipitated by using anti-HA antibodies, and the immunoprecipitates were probed with anti-Flag antibodies (Upper). The total expression of Flag-tagged NHERF-1 and NHERF-2 in the cell lysates is shown by anti-Flag Western blot (Lower). The data shown are representative of four independent experiments. (D) NHERF-2 physically links P2Y<sub>1</sub>R-CT to PLCβ1. Lysates from untransfected HEK-293 cells were incubated with fusion proteins corresponding to GST alone, GST–P2Y<sub>1</sub>R-CT, or L373A P2Y<sub>1</sub>R-CT. Pull-down samples were probed with antibodies against NHERF-2 (Upper) or PLCβ1 (Lower). These data are representative of four independent experiments.

P2Y<sub>1</sub>R, we generated a GST fusion protein containing the last 50 aa of P2Y<sub>1</sub>R-CT, including the terminal D-T-S-L PDZ-domain binding motif. This P2Y<sub>1</sub>R-CT–GST fusion protein was used to screen a recently created proteomic array of various class I PDZ domains derived from multiple PDZ proteins. The PDZ domains were expressed as His- and S-tagged fusion proteins, purified, and spotted in equal amounts onto individual bins within gridded nylon membranes. P2Y<sub>1</sub>R-CT did not detectably associate with the vast majority of the 96 PDZ domains on the array. However, a robust association was found between P2Y<sub>1</sub>R-CT and PDZ2 of NHERF-2 (Fig. 1). A somewhat weaker interaction was also detected with the fifth PDZ domain of the multi-PDZ scaffold protein MAGI-3.

**Structural Determinants of the P2Y<sub>1</sub>R–NHERF-2 Interaction.** Because the D-T-S-L motif at the P2Y<sub>1</sub>R-CT is a canonical class I PDZ-domain binding motif, we generated a series of mutant P2Y<sub>1</sub>R-CT fusion proteins with sequential substitutions of the various residues in this motif. Overlay studies revealed a robust association of the NHERF-2 PDZ2 fusion protein with the WT P2Y<sub>1</sub>R-CT as well as with the mutant P2Y<sub>1</sub>R-CT in which the serine at the –1 position was replaced by alanine, suggesting that this residue is not obligatory for the interaction (Fig. 2A). Mutations of the –2 and –3 positions of P2Y<sub>1</sub>R-CT, however, resulted in markedly reduced



**Fig. 3.** P2Y<sub>1</sub>R and NHERF-2 exhibit similar patterns of expression in native brain tissue. Examples of P2Y<sub>1</sub>R (Left) and NHERF-2 (Right) immunopositive astrocytic processes in mouse cerebral cortex. Several of the most prominent immunoperoxidase deposits in each frame are indicated with arrowheads. A, astrocytic process; E, endothelial cell; L, lumen of capillary. (Scale bar, which applies to Left and Right: 1 μm.)

binding of NHERF-2 PDZ2, suggesting that the threonine and aspartic acid residues at these positions are involved in the P2Y<sub>1</sub>R–NHERF-2 association. Most striking, however, was the lack of any detectable association of NHERF-2 PDZ2 with the P2Y<sub>1</sub>R-CT fusion protein in which the terminal leucine residue was mutated to alanine. Taken together, these results reveal that three of the last four residues of P2Y<sub>1</sub>R are key determinants of the interaction with NHERF-2 PDZ2.

To more quantitatively assess the association of NHERF-2 PDZ2 with P2Y<sub>1</sub>R-CT, we performed *in vitro* binding assays overlaying NHERF-2 PDZ2 fusion proteins at various concentrations onto 96-well plates coated with P2Y<sub>1</sub>R-CT fusion protein. As shown in Fig. 2B, binding of NHERF-2 PDZ2 was saturable within the range of concentrations tested, yielding an estimated dissociation constant (*K<sub>d</sub>*) of 48 nM. This estimated affinity is within the same range of other PDZ–receptor interactions that are known to occur in cells and have physiological relevance for receptor function (19).

**Association of Full-Length P2Y<sub>1</sub>R with NHERF-2 in Cells.** Next, we determined whether association of NHERF-2 with P2Y<sub>1</sub>R occurs between full-length forms of the proteins in living cells. Full-length HA-tagged P2Y<sub>1</sub>R was expressed in HEK-293 cells either alone or in combination with Flag-tagged NHERF-2 or a FLAG-tagged version of a closely related protein, NHERF-1. When HA–P2Y<sub>1</sub>R was immunoprecipitated from the lysates of cells cotransfected with HA–P2Y<sub>1</sub>R and FLAG–NHERF-2, robust coimmunoprecipitation of FLAG–NHERF-2 was observed (Fig. 2C). No detectable coimmunoprecipitation, however, was observed from lysates of cells in which HA–P2Y<sub>1</sub>R was cotransfected with FLAG–NHERF-1. Similarly, we failed to detect coimmunoprecipitation of NHERF-2 from lysates of HEK-293 cells cotransfected with FLAG–NHERF-2 and a full-length mutant P2Y<sub>1</sub>R in which the terminal leucine was changed to alanine (P2Y<sub>1</sub>R L373A). These results demonstrate that NHERF-2 strongly associates with P2Y<sub>1</sub>R in a cellular context and that this interaction depends on the same motif identified in the fusion protein studies.

**P2Y<sub>1</sub>R, PLCβ, and NHERF-2 Form a Multiprotein Complex.** P2Y<sub>1</sub>R exerts effects on cellular physiology mainly through coupling to Gα<sub>q</sub>. Interestingly, NHERF family proteins are known to interact with a number of proteins implicated in Gα<sub>q</sub>-mediated signal transduction, including Gα<sub>q</sub> itself (24), PKCα (25), and subtypes of PLCβ (26). NHERF-2 might therefore be able to strongly influence P2Y<sub>1</sub>R-mediated signaling efficacy by clustering these key signaling components in the vicinity of the receptor. To determine whether NHERF-2 might serve as a scaffold to physically link P2Y<sub>1</sub>R and PLCβ in cells, we pulled down protein



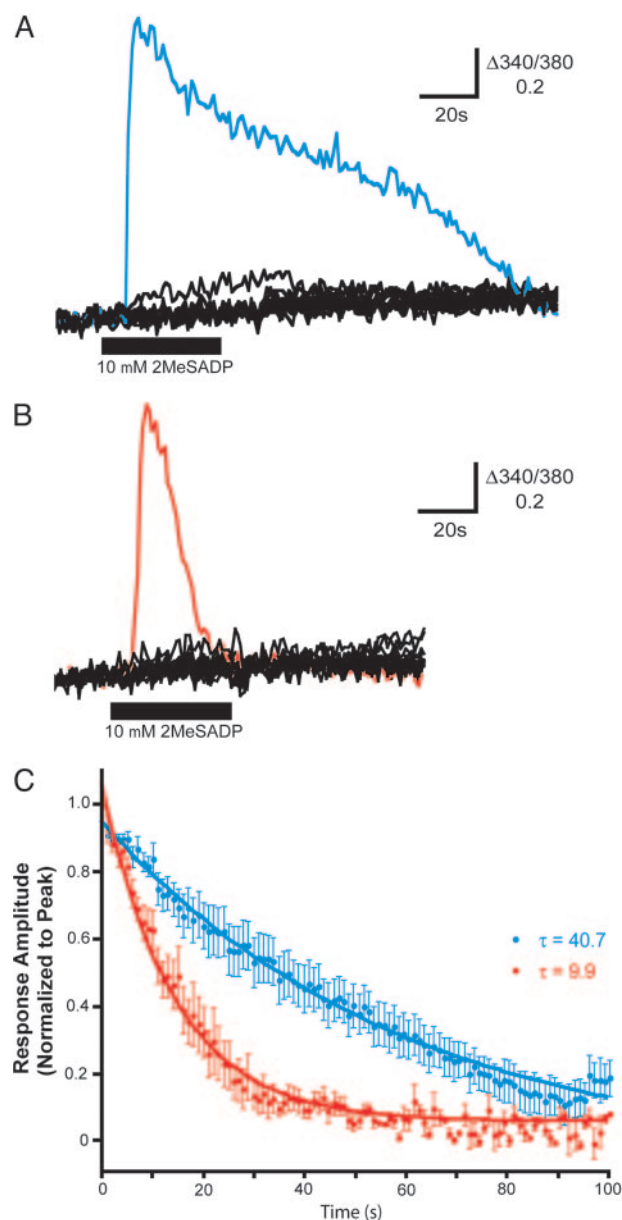
Because NHERF-2 can interact with and regulate the activity of various  $G_q$  signaling components (24–26), the effects of NHERF-2 on  $P2Y_1R$ -mediated  $Ca^{2+}$  signaling observed in the experiments with the C6 glioma cells might reflect general effects of NHERF-2 on the efficiency of  $G_q$  signaling (or on  $Ca^{2+}$  signaling in general) rather than specific effects on  $P2Y_1R$ -mediated signaling due to the NHERF-2 interaction with  $P2Y_1R$ . Thus, we performed parallel experiments in human 1321N1 astrocytoma cells that lack endogenous  $P2Y_1R$  (8) but express significant levels of endogenous NHERF-2 (data not shown). These glial cells lacking endogenous  $P2Y_1R$  allowed for direct comparisons between WT  $P2Y_1R$ s and L373A mutant receptors that are unable to bind to the endogenous NHERF-2 expressed in these cells. As expected, 1321N1 cells transfected with either WT  $P2Y_1R$  or L373A mutant  $P2Y_1R$  exhibited transient  $Ca^{2+}$  responses after application of 2-MeSADP (Fig. 5*A* and *B*). Comparisons of the  $Ca^{2+}$  responses evoked in WT versus L373A-expressing cells revealed no significant differences in either the response magnitude ( $0.56 \pm 0.10$ ,  $n = 5$  versus  $0.92 \pm 0.12$ ,  $n = 7$ ;  $P = 0.068$ , Student's *t* test) or the latency ( $11.4 \pm 1.1$  s,  $n = 8$  versus  $16.0 \pm 2.6$  s,  $n = 5$ ;  $P = 0.0876$ , Student's *t* test). However, there was a dramatic difference in the duration of  $Ca^{2+}$  responses evoked by WT versus L373A mutant  $P2Y_1R$ s, with the WT  $P2Y_1R$ -mediated responses persisting >3-fold longer than those mediated by activation of L373A mutant receptors (Fig. 5*C*). This difference between the WT and L373A mutant  $P2Y_1R$ s observed in 1321N1 cells expressing endogenous NHERF-2, considered together with a similar difference observed for endogenous  $P2Y_1R$ s in C6 glioma cells in the presence versus absence of transfected NHERF-2, provides strong evidence that NHERF-2 association with  $P2Y_1R$  controls receptor signaling by prolonging the duration of  $P2Y_1R$ -mediated responses.

## Discussion

The interaction between  $P2Y_1R$  and NHERF-2 described in this study was found by screening the  $P2Y_1R$ -CT against a recently created proteomic array of PDZ domains. Potential PDZ interaction motifs are found at the carboxyl termini of many receptors, ion channels, enzymes, and other signaling proteins (19). For such proteins with predicted PDZ interaction motifs, the focused screening of a specific proteomic array such as the PDZ domain array described here can be a useful alternative to yeast two-hybrid library screening and other unbiased approaches for finding protein–protein interactions. The  $P2Y_1R$ –NHERF-2 interaction identified in the screens of the PDZ domain array was found to be quite specific; the vast majority of PDZ domains on the array did not detectably associate with the  $P2Y_1R$ -CT.

The key structural determinants of the  $P2Y_1R$ –NHERF-2 interaction are PDZ2 of NHERF-2 and the D-T-S-L motif at the CT of  $P2Y_1R$ . Interestingly, it has previously been shown that there is a very weak interaction between the  $P2Y_1R$ -CT and NHERF-1 (29). This interaction, however, is too weak to be detected in cells and is therefore probably not of physiological significance (29). Indeed, in the present study, extended overexposures of the  $P2Y_1R$ -CT-GST overlays of the PDZ domain array revealed weak binding of  $P2Y_1R$ -CT to NHERF-1 PDZ1 and a handful of other PDZ domains, but these interactions were all determined to be at least 10- to 50-fold lower in affinity than the  $P2Y_1R$ –NHERF-2 interaction (data not shown). The strong and specific binding observed between  $P2Y_1R$  and NHERF-2, as well as the similar patterns of expression of these two proteins in native tissues and the striking functional effects of NHERF-2 on  $P2Y_1R$  signaling, suggest that NHERF-2 is a primary cellular partner of  $P2Y_1R$ .

We observed that NHERF-2 dramatically prolongs the time course of  $P2Y_1R$ -mediated  $Ca^{2+}$  signaling. Overexpression of NHERF-2 in C6 glioma cells, which do not detectably express endogenous NHERF-2 according to our Western blots, resulted in a 3-fold increase in the duration of  $Ca^{2+}$  responses evoked by activation of endogenous  $P2Y_1R$ . Similarly, in 1321N1 cells, which



**Fig. 5.** WT  $P2Y_1R$  exhibits  $Ca^{2+}$  responses of longer duration than mutant L373A  $P2Y_1R$ . (*A* and *B*) Individual traces show records of  $\Delta 340/380$  fura-2 fluorescence in a field of 1321N1 cells transfected with WT (*A*) or mutant L373A (*B*)  $P2Y_1R$ . 2-MeSADP ( $10 \mu M$ ) was applied as indicated by the black bar. Representative traces of responsive cells in each field are shown in blue (WT) and red (L373A), respectively. (*C*) The mean  $\pm$  SEM amplitudes of  $Ca^{2+}$  responses in 1321N1 cells expressing WT (blue;  $n = 8$ ) or mutant L373A (red;  $n = 5$ )  $P2Y_1R$  evoked by applying 2-MeSADP ( $10 \mu M$ ) plotted as a function of time elapsed since the onset of peak amplitude. Response amplitudes are normalized to the peak amplitude of each response. Comparable differences between WT  $P2Y_1R$  and the L373A mutant receptor were also observed at lower doses of agonist (1 nM and 20 nM 2-MeSADP; data not shown). The lines represent the best single exponential fit approximated for the decay phase of each data set and are defined by the equations  $y = 1.002e^{-0.017030x}$  (WT  $P2Y_1R$  cells) and  $y = 1.001e^{-0.02822x}$  (L373A  $P2Y_1R$  cells).

express substantial levels of endogenous NHERF-2 according to our Western blots, activation of WT  $P2Y_1R$  evoked  $Ca^{2+}$  responses that persisted for >3-fold longer than those evoked by activation of L373A  $P2Y_1R$ . Thus, both sets of experiments revealed that association of  $P2Y_1R$  with NHERF-2 allows the receptor to couple to sustained increases in intracellular  $Ca^{2+}$ . Based on these obser-

variations, one might predict that cells expressing endogenous NHERF-2 should exhibit much more sustained P2Y<sub>1</sub>R-mediated Ca<sup>2+</sup> responses than cells lacking endogenous NHERF-2. Indeed, we observed that the duration of Ca<sup>2+</sup> signaling of WT P2Y<sub>1</sub>Rs in 1321N1 versus C6 cells was, in fact, profoundly different, with the Ca<sup>2+</sup> responses lasting markedly longer in cells expressing endogenous NHERF-2 (the 1321N1 cells) than in cells lacking endogenous NHERF-2 (the C6 cells). It is well known that the activity levels of various calcium-dependent signaling cascades depend heavily on the localization, magnitude, frequency, and duration of the Ca<sup>2+</sup> response (30, 31). By prolonging the ability of P2Y<sub>1</sub>R to mobilize intracellular Ca<sup>2+</sup>, NHERF-2 may determine which signaling pathways are ultimately engaged after P2Y<sub>1</sub>R stimulation in different cell types.

NHERF family proteins are known to associate with many of the intermediates involved in Gα<sub>q</sub>-mediated signaling, including Gα<sub>q</sub> itself (24), PKCα (25), and PLCβ (26). Thus, NHERF-2 may control P2Y<sub>1</sub>R activity by acting as a scaffold to link these key signaling proteins in the vicinity of the receptor, allowing for a more sustained signaling response. This model is analogous to the model proposed for the action of the PDZ domain-containing protein InaD in regulating rhodopsin-mediated visual signaling in *Drosophila* (32) and the action of the non-PDZ protein Homer in regulating metabotropic glutamate receptor-mediated calcium signaling in mammalian neurons (33). However, InaD, Homer, and NHERF-2 have quite distinct effects on the dynamics of the calcium responses induced by their associated receptors, presumably tailored to meet the unique requirements of the cell types in which these receptor/scaffold combinations are expressed. Beyond the interaction with P2Y<sub>1</sub>R described here, NHERF-2 is also known to associate with and regulate the activity of at least one other GPCR, the parathyroid hormone receptor 1 (PTH<sub>1</sub>R), with the primary demonstrated action of NHERF-2 on PTH<sub>1</sub>R being an effect on the receptor's G protein-coupling specificity (34). Interestingly, PTH<sub>1</sub>R and P2Y<sub>1</sub>R are known to exhibit substantial cross-talk and synergistic signaling (35), which might be related to the fact that both of these receptors can associate with NHERF-2 in a functionally relevant manner.

In addition to linking P2Y<sub>1</sub>R to downstream effectors and enhancing P2Y<sub>1</sub>R signaling, the P2Y<sub>1</sub>R–NHERF-2 interaction may have other physiologically important consequences. First, NHERF-2 is known to interact with actin-associated ezrin-radixin-moesin proteins (36) and thus might serve as a link between P2Y<sub>1</sub>R

and the actin cytoskeleton. Second, the ability of NHERF proteins to interact with PKC (25) might facilitate the tethering of PKC in the vicinity of P2Y<sub>1</sub>R and thereby modulate the ability of PKC to phosphorylate and regulate the activity of P2Y<sub>1</sub>R (17). Third, NHERF-2 is known to regulate the activity of Na<sup>+</sup>/H<sup>+</sup> exchangers (37) and therefore may play a role in linking P2Y<sub>1</sub>R to modulation of Na<sup>+</sup>/H<sup>+</sup> exchange (38). Finally, it is interesting to note that NHERF-2 has been shown to form a high-affinity complex with the cystic fibrosis transmembrane regulator (CFTR) in A6 cells (39). It has also recently been shown that CFTR activity in A6 cells is regulated by P2Y<sub>1</sub>R in a manner that is blocked by overexpression of a dominant-negative NHERF-2 construct (40). These unusual data, considered together with our description of a direct interaction between P2Y<sub>1</sub>R and NHERF-2, suggest the possibility that P2Y<sub>1</sub>R, NHERF-2, and CFTR form a cellular triple complex that is critical for purinergic regulation of CFTR channel activity in certain cell types.

P2YRs are major targets for commonly prescribed antithrombotic drugs (41) and are also targets for pharmaceuticals that might be useful in the future treatment of epilepsy, chronic pain, brain tumors, heart disease, atherosclerosis, cystic fibrosis, and other diseases (42). To fully exploit the enormous therapeutic potential of the P2YR family, however, it is essential to understand how the various P2YR subtypes are different from one another and how their activity might be differentially regulated in distinct tissues. We have found that the PDZ scaffold protein NHERF-2 associates with a specific motif on the P2Y<sub>1</sub>R CT and profoundly regulates P2Y<sub>1</sub>R functional activity in a cell-specific manner. These data provide a striking example of GPCR regulation by a receptor-associated scaffold protein and also represent an important step toward understanding the molecular mechanisms underlying subtype-specific and cell-specific regulation of P2YRs.

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