Brief Communications

The Scaffold Protein NHERF2 Determines the Coupling of P2Y1 Nucleotide and mGluR5 Glutamate Receptor to Different Ion Channels in Neurons

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Expressed metabotropic group 1 glutamate mGluR5 receptors and nucleotide P2Y1 receptors (P2Y1Rs) show promiscuous ion channel coupling in sympathetic neurons: their stimulation inhibits M-type [Kv7, K(M)] potassium currents and N-type ($Ca_V2.2$) calcium currents (Kammermeier and Ikeda, 1999; Brown et al., 2000). These effects are mediated by G_q and $G_{i/o}$ G-proteins, respectively. Via their C-terminal tetrapeptide, these receptors also bind to the PDZ domain of the scaffold protein NHERF2, which enhances their coupling to G_q-mediated Ca^{2+} signaling (Fam et al., 2005; Paquet et al., 2006b). We investigated whether NHERF2 could modulate coupling to neuronal ion channels. We find that coexpression of NHERF2 in sympathetic neurons (by intranuclear cDNA injections) does not affect the extent of M-type potassium current inhibition produced by either receptor but strongly reduced $Ca_V2.2$ inhibition by both P2Y1R and mGluR5 activation. NHERF2 expression had no significant effect on $Ca_V2.2$ inhibition by norepinephrine (via α_2-adrenoceptors, which do not bind NHERF2), nor on $Ca_V2.2$ inhibition produced by an expressed P2Y1R lacking the NHERF2-binding DTSL motif. Thus, NHERF2 selectively restricts downstream coupling of mGluR5 and P2Y1Rs in neurons to G_q-mediated responses such as M-current inhibition. Differential distribution of NHERF2 in neurons may therefore determine coupling of mGluR5 receptors and P2Y1 receptors to calcium channels.

Introduction

The different subtypes of metabotropic receptor for common neurotransmitters usually show a relatively selective interaction with one or other of the main classes of resident G-proteins in neurons. This exerts some constraint on the particular species of ion channel that are affected when the receptors are activated, and hence on the overall response of the neuron to a transmitter. For example, the much-studied neurons of the rat superior cervical sympathetic ganglion possess four subtypes (M1 through M4) (Brown et al., 1995) of the muscarinic acetylcholine receptor (mAChR), but each shows a restricted spectrum of ion channel effects when activated. Thus, the M1-mAChR couples preferentially to G_q to induce hydrolysis of membrane phosphatidylinositol-4,5-bisphosphate (PIP_2) and so reduce membrane PIP_2 levels. This causes the closure of ion channels that require high levels of PIP_2 for their activation, such as M-type (Kv7.2/7.3) potassium channels and N-type ($Ca_V2.2$) calcium channels (for review, see Gamper and Shapiro, 2007). In contrast, the M4-mAChR selectively couples to G_o (Caulfield et al., 1994) and also inhibits the $Ca_V2.2$ channel through an action of the $G_o\beta\gamma$ subunits (Delmas et al., 1998), but does not affect the M channel. This action is shared by the endogenous α_2 adrenergic receptor (Herlitze et al., 1996; Ikeda, 1996). Finally, the M2-mAChR does not inhibit the $Ca_V2.2$ channel (Fernandez-Fernandez et al., 1999) but instead preferentially couples to G_i and activates inwardly rectifying Kir3.1/3.2 channels, which the M4-mAChR does not (Fernández-Fernández et al., 2001).

In contrast, we found that the P2Y1 nucleotide receptor (P2Y1R), when expressed in these neurons, coupled with equal facility to G-proteins of both $G_{q/11}$ and $G_{i/o}$ classes. Hence, when activated, it reproduced the effects of stimulating all of the muscarinic receptor types in sympathetic neurons—inhibition of M and $Ca_V2.2$ currents, and sequential activation and inhibition of Kir currents (Brown et al., 2000; Filippov et al., 2000, 2004). This is surprising because in its other responses P2Y1R acts as a $G_{q/11}$-selective receptor (Abbracchio et al., 2006).

Responses to metabotropic receptor activation are frequently controlled by ancillary "scaffold" proteins (Kreienkamp, 2002; Bockaert et al., 2010). One such scaffold protein that binds to the P2Y1R is the type-2 Na^+/H^+ exchange regulatory factor (NHERF2): this augments the G_q-mediated responses to P2Y1R stimulation when coexpressed in cell lines (Fam et al., 2005). We therefore wondered whether NHERF2 might also restrict P2Y1R coupling to G_q in neurons. We show that it does so—not by enhancing G_q-mediated M-current inhibition, but by suppressing G_o-mediated $Ca_V2.2$ inhibition. We also show that it has a

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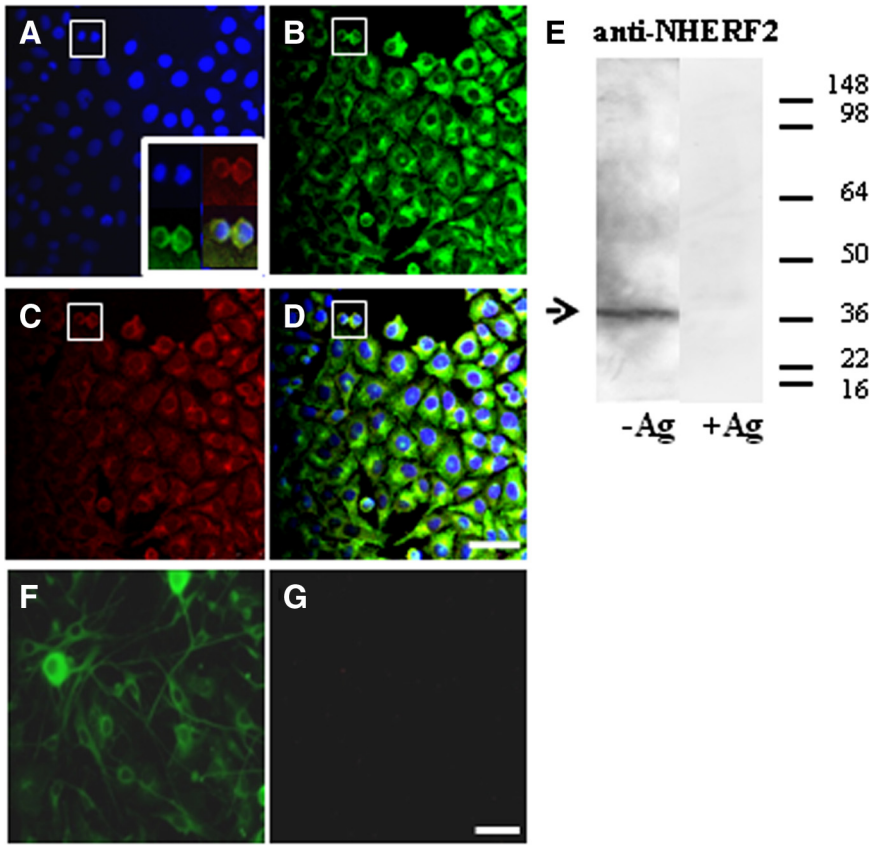

Figure 1. NHERF2 is endogenous in HEK cells but not in sympathetic neurons. ***A–D***, HEK293 cells stably transfected with hP2Y1R cDNA. The same field is stained with (***A***) DAPI (1:1000), to show cell nuclei; (***B***) anti-P2Y1 antibody (1:200), (***C***) anti-NHERF2 antibody (1:100). ***D***, Merged image of the 2 antibody stains, showing strong overlap (yellow). Inset: 2 representative cells in the boxes marked in ***A–D***, at higher power, showing the colocalization. Each antibody staining could be fully blocked by its peptide antigen (Ag): for the anti-NHERF2, as shown in Western blots of rat brain membranes (***E***) showing (unblocked) a single band of the predicted size (37 kDa, see arrow), and in anti-P2Y1R immunostaining [Filippov et al. (2006), their Fig. 2*D*]. ***F***, ***G***, Rat sympathetic neurons cultured *in vitro* for 1 week express endogenous P2Y1R immunoreactivity to varying degrees (***F***), but no detectable NHERF2 immunoreactivity (***G***). Scale bars, 50 μm.

similar effect on responses of the mGluR5 receptor, another NHERF2-binding partner (Paquet et al., 2006b).

Materials and Methods

Patch-clamp experiments were performed on neurons dissociated from superior cervical sympathetic ganglia (SCGs) isolated from 17- to 19-d-old rats and cultured for 24–48 h (Filippov et al., 1998). The disrupted patch was used for whole-cell Ca^{2+} current recordings; an amphotericin-perforated patch was used for M-type potassium current recordings. Currents were recorded with an Axoclamp 2B amplifier and pClamp10 software (Molecular Devices) and analyzed with Origin 5 software (Microcal Software). Ca^{2+}-currents were evoked using 50 ms pulses from −90 to 0 mV applied every 20 s and currents measured 7 ms after beginning the pulse (near to peak current in control). Residual currents in the presence of the Ca^{2+} channel blocker $CdCl_2$ (100 μM), added at the end of experiment, were subtracted. M-current deactivation relaxations were recorded during 1 s steps from −20 to −40 mV applied every 20 s, and initial amplitudes were estimated from double-exponential fitting. For dose-response curves, M-current was measured as holding current at −20 mV minus the current remaining in the presence of the M-channel blocker XE991, 3 μM, applied at the end of experiment. Curves were fitted to the data using the Hill equation, $y = y_{max} \times x^{nH}/(x^{nH} + K^{nH})$, where y = observed percentage inhibition, y_{max} = extrapolated maximal percentage inhibition, x = nucleotide concentration (in nM), $K = IC_{50}$ (in nM), and n_H = Hill coefficient. Data are presented as means ± SEM. Student's t test was applied to determine statistical significance. The difference was considered significant if $p \leq 0.05$.

Patch pipettes were filled with the following (in mM): 90 $KOOCCH_3$, 20 KCl, 3 $MgCl_2$, 40 HEPES, 0.1 BAPTA, and 0.125 mg/ml amphotericin B (adjusted to pH 7.4 by KOH) for M current, and with CsCl 110, $MgCl_2$ 3, HEPES 40, EGTA 3, Na_2ATP 2, and Na_2GTP 0.5 (pH adjusted to 7.4 with CsOH) for Ca^{2+} current. For M-current recordings, cells were perfused at 15–20 ml/min with a solution containing the following (in mM): NaCl 120, KCl 3, $MgCl_2$ 1.5, $CaCl_2$ 2.5, HEPES 10, glucose 11.1, tetrodotoxin 0.0005, and adjusted to pH 7.4 with NaOH. For Ca^{2+} current recordings, tetraethylammonium Cl was substituted for NaCl and Ca^{2+} replaced by 5 mM Ba^{2+}.

Proteins were expressed in neurons by intranuclear cDNA injection performed 5 h after plating the cells, and recordings were made after overnight incubation (Filippov et al., 2000). EGFP-C1 cDNA was coinjected as a marker of expression when necessary. NHERF2-EGFP-C2 cDNA plasmid was a gift from Dr. R. Hall (Emory University, Atlanta, GA), and mGluR5a cDNA was a gift from Dr. S. R. Ikeda (National Institutes of Health/National Institute on Alcohol Abuse and Alcoholism/Division of Intramural Clinical and Biological Research, Bethesda, Maryland). Mutant (-DTSL) P2Y1R cDNA was previously prepared in our Cambridge laboratory (Choi et al., 2008). cDNAs were injected at the following concentrations (in ng/μl): NHERF2 100; mGluR5a 10, P2Y1R as indicated in the text, mutant P2Y1R 1, and EGFP-C1 25.

Immunocytochemistry. Western blotting of rat brain membranes, stained with a NHERF2-specific antibody (ab40825, Abcam) was as described by Choi et al. (2008). Immunostaining of cell cultures was as described for hippocampal neurons (Filippov et al., 2006), but using also (with identical results) a custom-made antibody to an extracellular epitope of rat P2Y1R (J. Simon and E. Barnard, unpublished observations). All images were captured in identical conditions by a Leica confocal microscope (SP 5) with excitation (Ex) 405 nm/emission (Em) 420–470 (blue); Ex 488 nm/Em 500–535 nm (green) and Ex 543 nm/Em 560–615 nm (red).

Chemicals. MRS2365, (S)-3,5-dihydroxyphenylglycine (DHPG) and oxotremorine M (OxoM) were from Tocris Bioscience; 2MeSADP and norepinephrine from Sigma.

Results

The scaffold protein NHERF2 is not present in sympathetic neurons

We tested for the presence of NHERF2 in sympathetic neurons using a NHERF2-specific antibody. This antibody gave a single band of predicted NHERF2 size (37 kDa) in brain Western blots (Fig. 1*E*) and did not stain the closely related PDZ scaffold protein, NHERF1 (data not shown). HEK293 cells showed clear native NHERF2 immunoreactivity, colocalizing with expressed P2Y1R immunoreactivity (Fig. 1*A–D*). In contrast, no NHERF2 immunoreactivity was detectable in rat SCG neurons (Fig. 1*F*, *G*).

Dual coupling of P2Y1 receptors to Ca^{2+}- and M-type K^+ channels in SCG neurons is not due to receptor overexpression

To check whether dual coupling of expressed P2Y1Rs in sympathetic neurons resulted from receptor overexpression, we varied the P2Y1 cDNA concentration used for intranuclear injections.

We found that parallel P2Y1R-mediated inhibitions of both Ca^{2+}- and M-currents were maintained over a >1000-fold cDNA dilution (Fig. 2*A*). Moreover, activation of the low level of endogenous P2Y1Rs (Fig. 1*F*) in the absence of P2Y1R-cDNA injection still produced a small inhibition of both Ca^{2+}- and M-currents. Thus, dual ion channel coupling of P2Y1Rs in sympathetic neurons did not depend on receptor overexpression.

NHERF2 prevents coupling of P2Y1 receptors to Ca^{2+} channels but not to M channels

Coexpression of NHERF2 scaffold protein with P2Y1 receptors prevented P2Y1R-mediated inhibition of Ca^{2+} current but did not change the inhibition of M current (Figs. 2*B*, 3). Thus, dose-response curves showed complete suppression by NHERF2 of the Ca^{2+}-current inhibition by the specific P2Y1R agonist MRS2365 (Fig. 3). In contrast, there was no significant difference between the IC_{50} values for M-current inhibition, or the maximal current inhibition, with or without NHERF2 expression. Mean values of Hill-plot constants (see Materials and Methods) were as follows: for Ca^{2+}-current inhibition without NHERF2 ($n = 3$): $y_{max} = 34.5 \pm 1.3\%$; $K = 1.5 \pm 0.2$ nM; $n_H = 1.2 \pm 0.2$; with NHERF2 ($n = 4$): no inhibition; for M-current inhibition without NHERF2 ($n = 3$): $y_{max} = 33.6 \pm 0.8\%$; $K = 0.8 \pm 0.1$ nM; $n_H = 1.0 \pm 0.1$; and with NHERF2 ($n = 3$): $y_{max} = 32.0 \pm 0.8\%$; $K = 0.5 \pm 0.1$ nM; $n_H = 0.9 \pm 0.1$.

The DTSL motif at the C terminus of P2Y1R is critical for P2Y1R binding to NHERF2 (Fam et al., 2005). Hence, we checked whether NHERF2 could modulate Ca^{2+}-channel coupling of a mutated P2Y1R receptor in which the DTSL binding motif was deleted. Ca^{2+}-current inhibition by this mutant receptor was unaffected by NHERF2 (Fig. 2*B*).

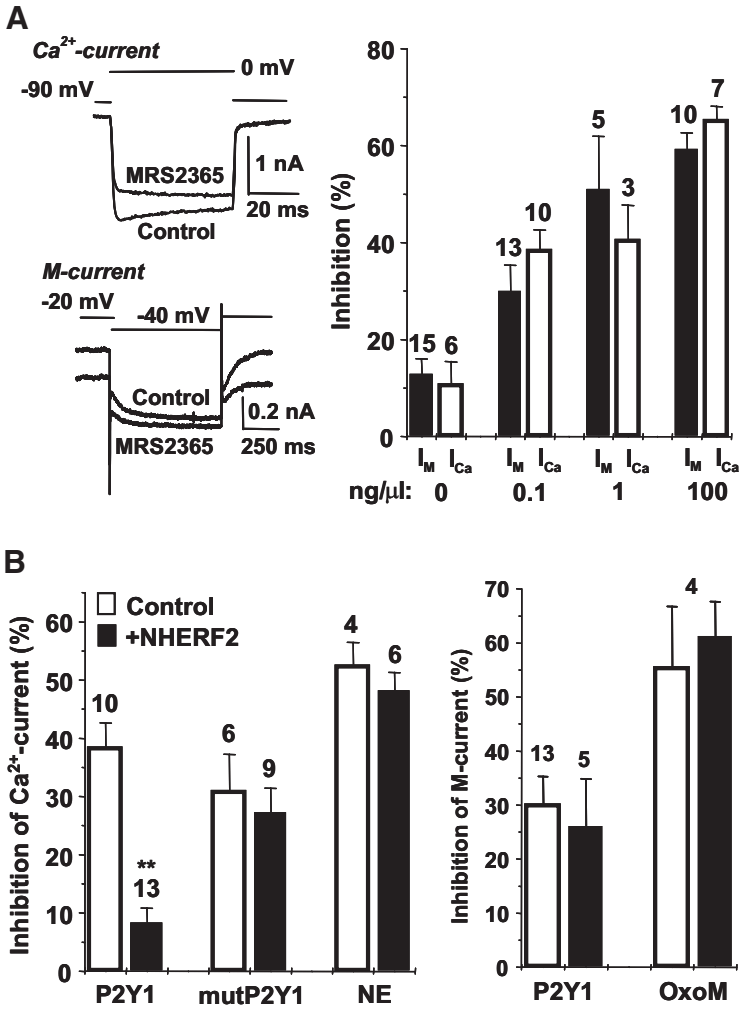

Figure 2. ***A***, Activation of endogenous and expressed P2Y1 receptors produces parallel inhibition of Ca^{2+} current and M current in rat sympathetic neurons. Records (left panel) show representative traces of Ca^{2+} current (I_{Ca}) and M current (I_M) inhibition, respectively, by the P2Y1-specific agonist MRS2365 (10 nM). The voltage protocol is shown above the traces. P2Y1 cDNA 0.1 ng/μl. Bar chart (right panel) shows mean ± SEM inhibition of I_M (black) and I_{Ca} (white) by MRS2365 or 2-MeSADP (1 μM) in sympathetic neurons injected with different concentrations of P2Y1R cDNA. No difference was seen using these two agonists; MRS2365 was used to exclude possible effects of stimulating endogenous P2Y12 receptors (Lechner et al., 2004) on Ca^{2+}-currents (Simon et al., 2002). Numbers over bars = number of cells. ***B***, Scaffold protein NHERF2 inhibits coupling of P2Y1 receptors to Ca^{2+} channels but not to M channels. Bar chart (left) shows inhibition of Ca^{2+}-channel current by the P2Y1R-specific agonist, MRS2365 (10 nM) in sympathetic neurons injected with 0.1 ng/μl P2Y1R cDNA or mutant (-DTSL) P2Y1R cDNA (mutP2Y1) alone or together with NHERF2 cDNA. NHERF2 prevents inhibition of Ca^{2+}-channel current via the wild-type P2Y1 receptor but not via the mutant receptor that does not bind NHERF2. In contrast NHERF2 does not prevent adrenergic inhibition of Ca^{2+}-channel current by norepinephrine (NE, 10 μM) or P2Y1R-mediated inhibition of M current (right panel bar chart) by 1 μM 2MeSADP, or muscarinic suppression of M current by OxoM, 10 μM. Numbers over bars = number of cells. $^{**}p < 0.001$ (difference from control).

NHERF2 had no effect on adrenergic receptor suppression of Ca^{2+} current by norepinephrine (Fig. 2*B*). It also had no effect on muscarinic receptor suppression of M current by oxotremorine-M (Fig. 2*B*). Hence, its site of action was not at the Ca^{2+} channel, nor its modulation by G_o, but was specific to the P2Y1R.

NHERF2 prevents coupling of mGluR5 receptors to Ca^{2+} channels but not to M channels

As with the nucleotide P2Y1Rs, expressed glutamate mGluR5 receptors also show promiscuous ion channel coupling in sympathetic neurons: their stimulation inhibits M-type [Kv7, K(M)] potassium currents and N-type ($Ca_V2.2$) calcium currents, and these effects are mediated by G_q and $G_{i/o}$ G-proteins, respectively (Kammermeier and Ikeda, 1999). Also like P2Y1 receptors, mGluR5 receptors bind to NHERF2, and this augments G_q-mediated Ca^{2+} signaling in cell lines (Paquet et al., 2006b). Hence, we tested whether NHERF2 can modulate mGluR5 coupling to Ca^{2+} channels and to M-type K^+ channels. We found that expression of NHERF2 selectively suppressed mGluR5-mediated Ca^{2+}-current inhibition without effect on M-current inhibition (Fig. 4), just like its effect on P2Y1R signaling.

Discussion

The main conclusion from these experiments is that the scaffold protein NHERF2 is capable of selectively directing the coupling of the NHERF2-binding receptors P2Y1R and mGluR5 to G_q-coupled ion channels in effects such as inhibition of M current, at the expense of G_o-dependent effects such as inhibition of $Ca_V2.2$ channels. This is not due to an enhancement of the G_q/M-

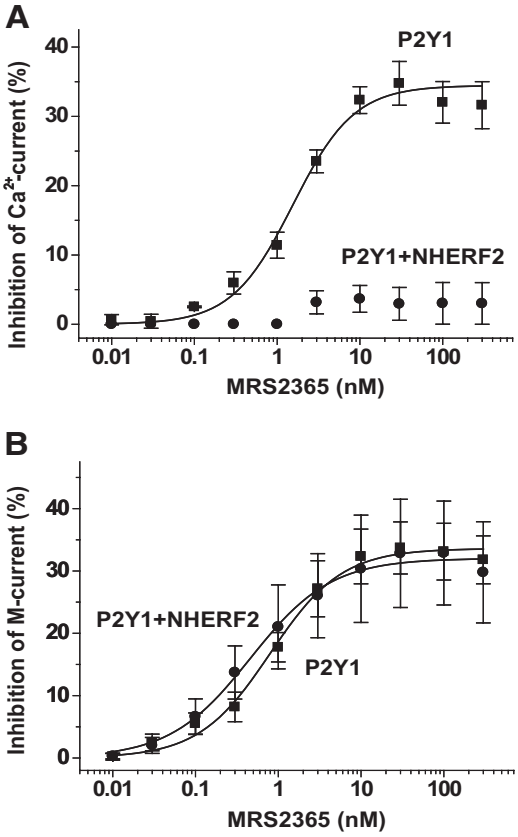

Figure 3. NHERF2 completely blocks P2Y1R-mediated inhibition of Ca^{2+} current without change of M-current inhibition. ***A***, ***B***, Dose-response curves for Ca^{2+}-current inhibition (***A***) and M-current inhibition (***B***) by MRS2365. Curves were fitted to the means (shown, with SEM bars) using the Hill equation (see Materials and Methods). SCG neurons were injected one night before with 1 ng/μl P2Y1 cDNA without (squares) or with (circles) NHERF2 cDNA.

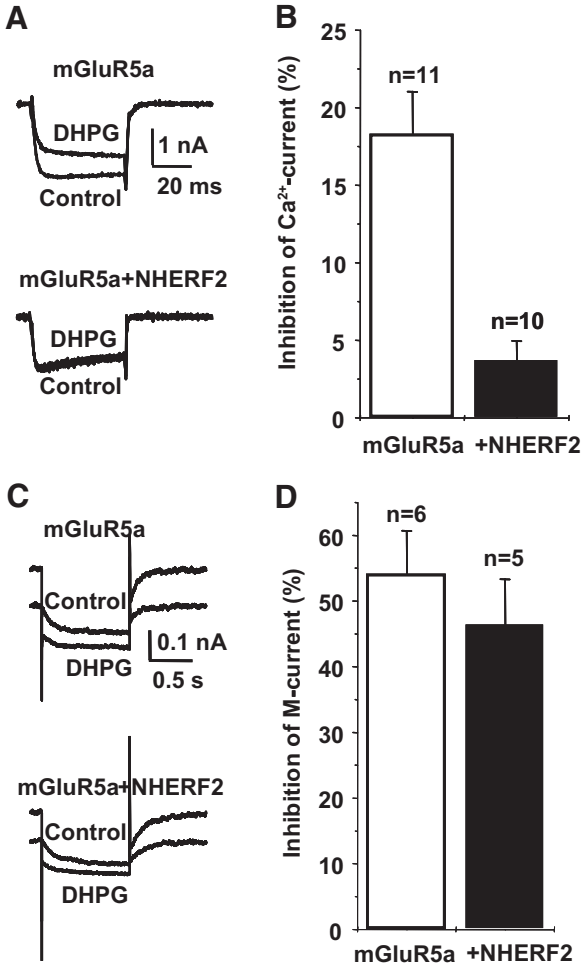

Figure 4. NHERF2 selectively prevents mGluR5a-mediated inhibition of Ca^{2+} current but not that of M current. ***A***, ***C***, Records show representative traces of Ca^{2+}-current (***A***) and M-current (***C***) inhibition, respectively, by the mGluR5 agonist DHPG (10 μM) in SCG neurons injected with mGluR5a cDNA without or with NHERF2 cDNA. The voltage protocol is as for Figure 2. ***B***, ***D***, Bar charts show mean inhibition of Ca^{2+} current (***B***) and M current (***D***) by DHPG in SCG neurons without or with NHERF2. n = number of cells.

channel effect (as might have been predicted from previous experiments on G_q-mediated responses in non-neural cells) (Fam et al., 2005; Paquet et al., 2006b), since M-channel inhibition was quantitatively unchanged by NHERF2. The reasons for this are probably that M-channel inhibition by muscarinic agonists in these neurons results from the fall in membrane PIP_2 following PIP_2 hydrolysis, not from the action of products of PIP_2 hydrolysis (Winks et al., 2005), and that the limiting factor is the supply of phospholipase C rather than the extent of G_q activation (Jensen et al., 2009). Instead, it could be attributed to a virtually complete suppression of G_o-mediated Ca^{2+}-current inhibition.

This suppression appears to be directed at the first stage on the signaling pathway—the interaction of the receptor with the G-protein—since the equivalent response to norepinephrine was unchanged. In the case of the P2Y1R, it required binding of a PDZ domain of NHERF2 to the previously reported DTSL motif on the receptor C terminus (Fam et al., 2005), since the Ca^{2+}-channel coupling of a mutant P2Y1R devoid of this sequence was unaffected by NHERF2. It might well involve the equivalent domain (SSSL) in the mGluR5 receptor, although we have not yet tested this. Two other sites within the total 46-residue tail have been found to be involved in P2Y1R G_q coupling (Fam et al., 2003; Ding et al., 2005), but both effects are independent of the presence of the terminal DTSL. Directional control of G-protein coupling by cognate receptors may be a general property of NHERF2 since it has previously been reported to switch the coupling of the parathyroid hormone receptor PTHR from G_s to $G_{i/o}$ (Mahon et al., 2002).

Metabotropic glutamate receptors have previously been noted to link to other scaffold proteins. Thus, group 1 mGluRs receptors (including mGluR5) can also bind to Homer to form macromolecular signaling complexes (for review, see Enz, 2007), Homer proteins have been reported to regulate coupling of group1 mGluRs to neuronal Ca^{2+}- and M-type K^+ channels; however, in this case, expression of the long-form Homer protein reduces inhibition of both channels, rather than selectively directing coupling to one or the other (Kammermeier et al., 2000).

Scaffold proteins are known to play an important role in G-protein-coupled signaling in the brain. For example, uncoupling of the PDZ-interacting protein PICK1 from mGluR7 prevents mGluR7-mediated inhibition of Ca^{2+} current in cerebellar granule cells and causes absence epilepsy in rodents (Bertaso et al., 2008). NHERF2 is present in the brain (Lee et al., 2007), where it is associated with both glia and neurons (Paquet et al., 2006a); preliminary immunocytochemical information indicates that it is strongly expressed in hippocampal pyramidal neurons (E. A. Barnard and J. Simon, unpublished observations). If it exerted comparable effects in central neurons to those we have observed in sympathetic neurons, it could (for example) serve to suppress G_o-mediated inhibitory effects of P2Y1Rs and mGluR5s on Ca^{2+}

channels (and hence on transmitter release) while preserving their postsynaptic G_q-mediated excitatory effects (Mannaioni et al., 2001; Filippov et al., 2006).

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