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Receptor-Mediated Suppression of Potassium Currents Requires Colocalization within Lipid Rafts

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ABSTRACT

Expression of KCNQ2/3 (Kv7.2 and -7.3) heteromers underlies the neuronal M current, a current that is suppressed by activation of a variety of receptors that couple to the hydrolysis of phosphatidylinositol 4,5-bisphosphate. Expression of Kv7.2/7.3 channels in human embryonic kidney (HEK) 293 cells produced a noninactivating potassium current characteristic of M current. Muscarinic receptors endogenous to HEK293 cells were identified as being M3 by pharmacology and Western blotting, producing a rise of intracellular calcium ($[Ca^{2+}]_i$) upon activation. Activation of these endogenous muscarinic receptors however, failed to suppress expressed Kv7.2/7.3 current. Current suppression was reconstituted by coexpression of HA-tagged muscarinic m1 or m3 receptors. Examination of membrane fractions showed that both expressed receptors and Kv7.2 and -7.3 channel subunits resided within lipid rafts. Disruption of lipid rafts by pretreatment of cells expressing either m1 or m3 muscarinic receptors with methyl-β-cyclodextrin produced a loss of localization of proteins within lipid raft membrane fractions. This pretreatment also abolished both the increase of $[Ca^{2+}]_i$ and suppression of expressed Kv7.2/7.3 current evoked by activation of expressed m1 or m3 muscarinic receptors. A similar loss of muscarinic receptor-mediated suppression of M current native to rat dorsal root ganglion neurons was observed after incubating dissociated cells with methyl-β-cyclodextrin. These data suggested that lipid rafts colocalized both muscarinic receptors and channel subunits to enable receptor-mediated suppression of channel activity, a spatial colocalization that enables specificity of coupling between receptor and ion channel.

The M current is a noninactivating potassium current present in a number of cell types. It has a dominant effect on cell excitability by being the only sustained K^+ current active in the voltage range of action potential initiation (Marrion, 1997). The current was first discovered in sympathetic ganglia and was so-named because it was suppressed by activation of muscarinic receptors (Brown and Adams, 1980). The sustained K^+ current is encoded by Kv7 subunits, heteromers composed of Kv7.2 and -7.3 subunits dominating in sympathetic and dorsal root ganglion neurons (Wang et al., 1998; Passmore et al., 2003; Miceli et al., 2008).

Muscarinic suppression of the native M current in sympathetic ganglia is mediated by M1 receptors (Marrion et al., 1989; Bernheim et al., 1992), which couple via $G\alpha_q$ to the activation of phospholipase C (PLC) and the hydrolysis of PIP_2. Subsequently, it has been documented that any receptor that couples to this pathway will suppress the M current (Marrion, 1997). Recovery of M-/Kv7.2/7.3-current suppressed by activation of muscarinic receptors required PIP_2 synthesis (Suh and Hille, 2002, 2009), leading to the hypothesis that local depletion of membrane PIP_2 mediates suppression of channel activity (Zhang et al., 2003; Suh and Hille, 2009).

It has been proposed that PIP_2 is enriched in detergent-insoluble fractions (Pike and Casey, 1996; Klopfenstein et al., 2002; Hur et al., 2004), a subtype of which are termed lipid rafts (Allen et al., 2007). Lipid rafts are membrane microdomains that are rich in glycosphingolipid and cholesterol, giving them a gel-like liquid-ordered state that is thought to limit diffusion. They are characterized biochemically as being resistant to solubilization in detergents such as Triton X-100 at 4°C and displaying low buoyant density (Chamberlain, 2004). Many different types of proteins have been found to be

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ABBREVIATIONS: PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; HEK, human embryonic kidney; GPCR, G protein-coupled receptor; HA, hemagglutinin; EGFP, enhanced green fluorescent protein; DRG, dorsal root ganglion; HBSS, HEPES-buffered salt solution; MBS, MES-buffered saline; MES, *N*-morpholino)ethanesulfonic acid; 77-LH-28-1, 1-(3-(4-butyl-1-piperidinyl)propyl)-3,4-dihydro-2(1*H*)-quinolinone; AD, Alzheimer's disease; ApoE, apolipoprotein E.

associated with lipid rafts, such as many G-protein coupled receptors (GPCRs), G proteins, and various enzymes. For example, β1 and β2 adrenoceptors, muscarinic M2 receptors, and the metabotropic glutamate receptor mGluR1a are reported to be localized to rafts. In addition, both $G\alpha_q$ and $G\alpha_s$ and PLC are also associated with rafts (for review, see Allen et al., 2007). Finally, different voltage-dependent ion channel subunits have also been demonstrated to be associated with lipid rafts, such as Cav2.1 (Davies et al., 2006), Kv2.1 (Martens et al., 2000), Kv1.5 (Martens et al., 2001), and Kv7.2 and 7.3 (Cooper et al., 2000). This has led to the proposal that lipid rafts are membrane domains that localize components to provide localized signaling cascades (Simons and Toomre, 2000; Allen et al., 2007).

Resolution of the mechanism(s) underlying GPCR-mediated suppression of Kv7.2/7.3 currents has been often accomplished using cell lines, such as Chinese hamster ovary and HEK293 cells (e.g., Selyanko et al., 2000; Li et al., 2005; Jensen et al., 2009). These studies have coexpressed both channel subunits and a PLC-coupled GPCR, usually the muscarinic m1 receptor, despite cells endogenously expressing functional muscarinic receptors coupled to PLC. Activation of these receptors produced accumulation of inositol phosphate, demonstrating the hydrolysis of PIP_2 (Mundell and Benovic, 2000). mRNA for the m3 muscarinic receptor in HEK293 cells had been reported (Ancellin et al., 1999), which has been substantiated by functional pharmacology (Kurian et al., 2009). Therefore, it would be expected that activation of endogenous receptors would evoke suppression of expressed Kv7.2/7.3 currents. Contrary to expectations, activation of endogenous muscarinic receptors failed to suppress expressed Kv7.2/7.3 currents. Receptors were demonstrated to be functional by showing that nontransfected HEK293 cells displayed a rise of intracellular calcium ($[Ca^{2+}]_i$) evoked by application of a muscarinic receptor agonist. Coupling between receptor activation and suppression of Kv7.2/7.3 currents was reconstituted by overexpression of m1 or m3 muscarinic receptors. Both expressed Kv7.2/7.3 channel subunits and m1 receptors were located in lipid rafts and coupling was lost when lipid rafts were dispersed by pretreatment of cells with methyl-β-cyclodextrin. Finally, suppression of M current by muscarinic receptor activation in DRG neurons was also lost when dissociated cells were pretreated with methyl-β-cyclodextrin. These data suggest that the colocalization of native and overexpressed receptors and Kv7.2/7.3 channels within lipid rafts was required to permit coupling.

Materials and Methods

Cell Culture, Expression of Channels

Rat Kv7.2 (GenBank accession no. AF087453) and rat Kv7.3 (GenBank accession no. AF091247) constructs have been published previously (Prole et al., 2003). Plasmids encoding human m1 (GenBank accession no. AF498915) and m3 (GenBank accession no. AF498917) muscarinic receptors were commercially obtained as N-terminal 3×HA-tagged constructs (Missouri S&T cDNA Resource Center, Rolla, MO). Channels and/or receptors were transiently expressed in HEK293 cells. Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal calf serum (Invitrogen) and 100 U/ml penicillin & 100 μg/ml streptomycin at 37°C. Cells were plated onto 35-mm dishes (Falcon Primaria; BD Biosciences Discovery Labware, Bedford, MA) 48 h before transfection for electrophysiology. Transient transfections of HEK293 cells were made with the use of polyethylenimine (Alfa Aesar, Inc., Ward Hill, MA) and 1 μg of each construct encoding channel subunits and/or receptors. Proteins were cotransfected with the plasmid pEGFP-C2 (Clontech, Mountain View, CA) that encoded enhanced green fluorescent protein (EGFP) (0.5 μg/35-mm dish), which was used as a marker for transfection. For biochemical experiments, cells were grown on 100-mm dishes and transfected with channel/receptor constructs using Lipofectamine and harvested 48 h later. Lipid rafts were dissipated by pretreating cells with the cholesterol-depleting agent methyl-β-cyclodextrin (10 mM) in unsupplemented media for 1 h immediately before electrophysiology or biochemistry.

Dorsal Root Ganglion Cells

Cultures were performed as described previously (Holmes et al., 2000). In brief, adult mice were killed by cervical dislocation, and DRGs from the lumbar, thoracic, and cervical regions were removed aseptically, trimmed of connective tissue and nerve roots, and pooled in Ham's F12 medium. Ganglia were subjected to 0.25% collagenase P for 1 h at 37°C, washed in phosphate-buffered saline, and treated enzymatically with trypsin-EDTA for 10 min at 37°C. Ganglia were washed in medium containing trypsin inhibitor and then mechanically dissociated by trituration using a flame-narrowed Pasteur pipette. After centrifugation, cells were resuspended in Ham's F12 medium supplemented with 5% horse serum, 1 mM glutamine, and 10 ng/ml gentamicin, plated on coverslips treated with 0.5 mg/ml polyornithine and 5 μg/ml laminin and used after being maintained overnight at 37°C in a humidified incubator.

Electrophysiology

Solutions. Expressed macroscopic currents were resolved using whole-cell recording. Cells were superfused with an external solution of the following composition 144 mM NaCl, 2.5 mM KCl, 10 mM HEPES(Na), 10 mM D-glucose, 1.2 mM $MgCl_2$, and 2.5 mM $CaCl_2$, pH adjusted to 7.4 with NaOH. Whole-cell electrodes were fabricated from KG-33 glass (Friedrich and Dimmock, Millville, NJ) and filled with a solution of the following composition: 130 mM potassium aspartate, 20 mM KCl, 10 mM HEPES, 3 mM ATP(Na_2), 0.1 mM EGTA, 3 mM $MgCl_2$, and 6 mM $CaCl_2$ (to give 50 nM free Ca^{2+}); 3 mM NaOH (adjusted to pH 7.4 with KOH) to give resistances of 2 to 5 MΩ. The liquid junction potential was calculated to be approximately 8 mV, which was not corrected for. All standard chemicals were purchased from Sigma (St. Louis, MO), except $CaCl_2$ and $MgCl_2$, which were purchased from Fluka Chemical Corp. (Ronkonkoma, NY).

Recording. Whole-cell currents were recorded with an Axopatch 200A (Molecular Devices, Sunnyvale, CA). Capacitance and series resistance compensation (95%) was used throughout. Currents were low-pass filtered at 1 kHz (eight-pole Bessel; Frequency Devices, Haverhill, MA) and acquired at 20 kHz using Pulse (HEKA, Lambrecht/Pfalz, Germany). All recordings were performed at room temperature (20–25°C). Noninactivating Kv7.2/7.3 currents were evoked by a holding potential of −20 mV and revealed by their deactivation from 1-s duration hyperpolarizing voltage steps (−100 to −30 mV). The suppression by receptor activation or block by application of extracellular barium (Ba^{2+}) was quantified by the effect on the deactivation current, evoked by a 1-s duration hyperpolarizing voltage step to −50 mV. Activation curves were derived from leak-subtracted (P/4) Kv7.2/7.3 currents evoked by depolarizing voltage steps (−60 to +30 mV) from a holding potential of −80 mV. Currents were normalized to peak conductance and fitted with a standard Boltzmann distribution. The time course of Kv7.2/7.3 current deactivation was determined by the fitting of a single exponential function to currents revealed by hyperpolarizing voltage steps from −40 to −100 mV. The change of measured tau with voltage is expressed as e(2.719)-fold/mV. Data were tested for significance using the unpaired t test, with means ± S.E.M. shown.

Imaging. Ratiometric determination of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in individual HEK293 cells was performed using Fura-2-based microfluorimetry. Methods were similar to those described recently by Dunlop et al. (2009). In brief, HEK293 cells were plated on 13-mm round glass coverslips and loaded with Fura-2AM (2 μM) by incubation for 30 min at 37°C in a standard HEPES-buffered salt solution (HBSS) supplemented with Pluronic acid 0.02% (w/v). The HBSS consisted of 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 30 mM D-glucose, pH 7.3 (with NaOH). Coverslips were then washed and maintained in HBSS at room temperature for up to 150 min before being transferred to a constantly perfused low-volume chamber mounted on the stage of an inverted microscope (Nikon TE2000). Standard ratiometric fluorescence imaging was performed under control of Volocity 4.0 imaging software (Improvision, Coventry, UK). A charge-coupled device camera (Orca 12AG; Hamamatsu Corporation, Bridgewater, NJ) was used to collect fluorescence images (emission wavelength, ~515 nm) from a 20× objective. Pair-wise 200-ms exposures to 340 nm and 380 nm excitation light were provided at 0.2 Hz by an illumination source (DG4; Sutter Instrument Company, Novato, CA). Drugs were applied by addition to the standard perfusing HBSS. All experiments were performed at room temperature.

For analysis, individual frames were background-subtracted and a threshold was set before generating a 340:380 ratio channel. Analysis of $[Ca^{2+}]_i$ in individual cells was made after their selection as regions of interest. For experiments on transiently transfected cells, $[Ca^{2+}]_i$ was analyzed only for cells positive for the GFP transfection marker. All data are presented as the change in 340:380 ratio.

Biochemistry

Sucrose Density Gradients. Lipid rafts were isolated by discontinuous sucrose gradient centrifugation based on the method of Silva et al. (1999). In brief, cells from a 100-mm dish were washed twice in ice-cold phosphate-buffered saline, scraped into 1 ml of ice-cold MBS (25 mM MES and 150 mM NaCl) containing 1% Triton X-100, 4 μg each leupeptin, antipain, and aprotinin, and 0.5 mM benzamidine, and homogenized with 10 strokes in a hand-held Dounce homogenizer. Homogenate (1 ml) was mixed with an equal volume of 40% sucrose, overlaid with 5 ml of 35% sucrose in MBS and then with 4 mlof 5% sucrose in MBS. Gradients were subject to overnight centrifugation at 34,000*g* in an AH641 rotor (Sorvall; Thermo Fisher Scientific, Basingstoke, UK).

Western Blotting. Aliquots from gradient fractions were subjected to SDS-polyacrylamide gel electrophoresis, and proteins transferred onto polyvinylidene difluoride membrane using a semidry blotter. Blots were probed for channel subunits, m1 muscarinic receptor, and the raft marker flotillin-2. A rabbit antibody against Kv7.2 was from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal anti-flotillin-2 was from BD Biosciences (San Jose, CA). Overexpressed, HA-tagged m1 receptor was detected using an anti-HA monoclonal antibody (Covance Research Products, Princeton, NJ). Unfortunately, the poor quality of commercial antibodies directed against the Kv7.3 subunit (Alomone Labs, Jerusalem, Israel) or m3 muscarinic receptor (Santa Cruz Biotechnology) prevented presentable distribution of proteins within membrane fractions. After subsequent incubation with horseradish peroxidase-labeled secondary antibody (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), bands were visualized using ECL or ECL+ kits (GE Healthcare) as appropriate for the sensitivity required.

Results

Activation of Functional Endogenous Muscarinic Receptors Did Not Suppress Expressed Kv7.2/7.3 Current. HEK293 cells endogenously express functional m3 muscarinic receptors that couple to hydrolysis of PIP_2 (Kurian et al., 2009). We confirmed their presence by West-

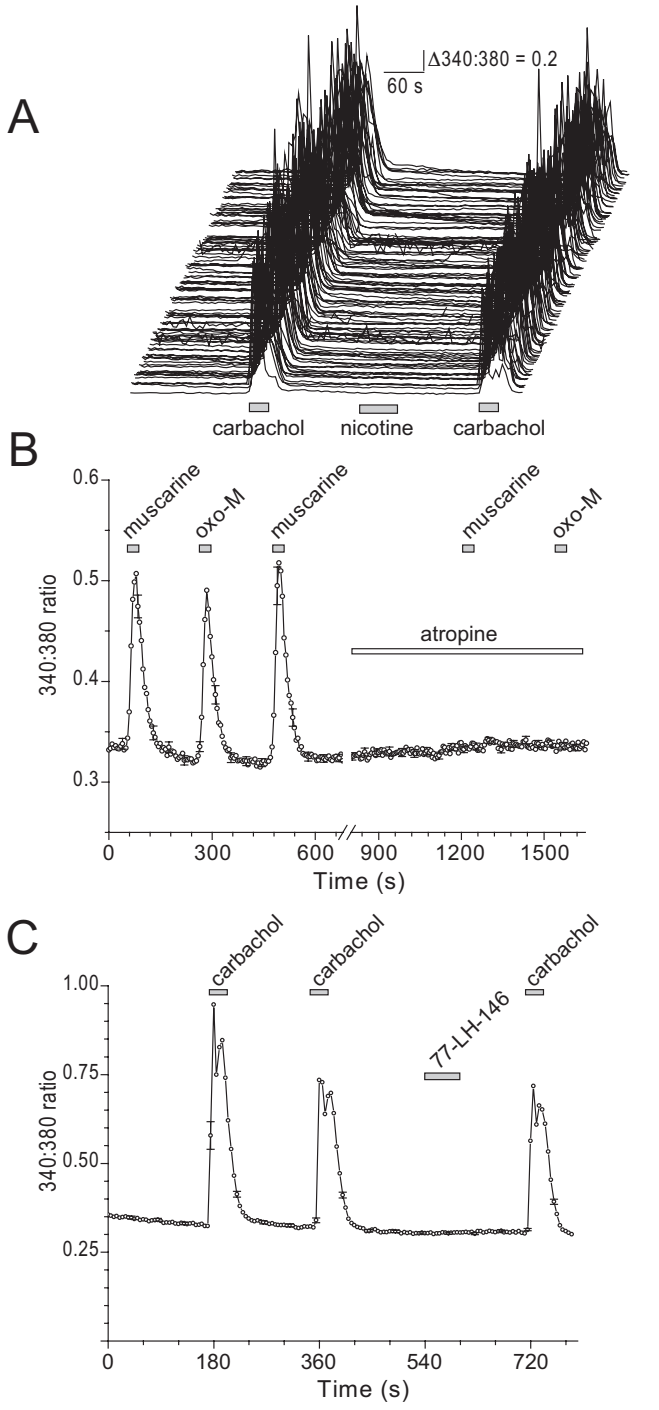

Fig. 1. Activation of functional endogenous m3 muscarinic receptors evoked a rise in $[Ca^{2+}]_i$. A, traces illustrating the ratio of emission from excitation at 340 and 380 nm for Fura-2-loaded nontransfected HEK293 cells. Application of carbachol (5 μM) evoked an increase in the 340:380 ratio corresponding to an increase of $[Ca^{2+}]_i$. Subsequent application of nicotine (300 μM) failed to increase $[Ca^{2+}]_i$. In contrast, a second application of carbachol (5 μM) evoked an increase of $[Ca^{2+}]_i$ that was slightly smaller than seen with the first application. B, plot of the 340:380 nm ratio from nontransfected cells, showing repeated increases of $[Ca^{2+}]_i$ evoked by successive applications of the muscarinic receptor agonists muscarine (10 μM) and oxotremorine-M (10 μM). Incubation with atropine (1 μM) antagonized responses to both agonists, confirming that responses were mediated by activation of muscarinic receptors. C, repeated applications of carbachol (5 μM) evoked increases of $[Ca^{2+}]_i$. In contrast, application of the m1-selective agonist 77-LH-28-1 (1 μM) failed to increase $[Ca^{2+}]_i$. This indicated that nontransfected cells lacked functional m1 receptors.

ern blotting of membrane fractions from nontransfected HEK293 (data not shown). Application of the mixed cholinergic agonist carbachol (5 μM) produced a rise of $[Ca^{2+}]_i$ in >99% of HEK293 cells (Fig. 1A). An increase of $[Ca^{2+}]_i$ was not generated by a high concentration of nicotine (300 μM), indicating that carbachol was acting via muscarinic receptors. (Fig. 1A). This was confirmed using the muscarinic agonists muscarine (10 μM) and oxotremorine-M (10 μM), both of which produced rises of $[Ca^{2+}]_i$ that were antagonized by atropine (1 μM) (Fig. 1B). Application of the M1-selective agonist 77-LH-28-1 (1 μM) (Langmead et al., 2008) failed to produce a response in nontransfected HEK293 cells, although these same cells were responsive to carbachol (Fig. 1C). The lack of a response to 77-LH-28-1 was found in multiple experiments using two different batches of compound, both compound stocks successfully producing depolarization of hippocampal neurones in a pirenzepine-sensitive manner (data not shown). These data suggest that HEK293 cells do not possess an endogenous functional m1 muscarinic receptor, but possess an endogenous functional m3 receptor that couples to hydrolysis of PIP_2 and a subsequent inositol 1,4,5-trisphosphate-mediated rise of $[Ca^{2+}]_i$.

Activation of endogenous m3 receptors coupled to $G\alpha_q$-mediated signaling cascades would be expected to suppress expressed Kv7.2/7.3 currents. Expressed Kv7.2/7.3 currents were revealed by their deactivation from the positive holding potential of −20 mV, an instantaneous current reflecting current through open Kv7.2/7.3 channels preceding the time-dependent inward current resulting from channel closure (Brown and Adams, 1980). Application of muscarine (10 μM) did not suppress Kv7.2/7.3 currents significantly, with only a 10.2 ± 2.8% (n = 5) reduction in the amplitude of Kv7.2/7.3 current deactivation observed (Fig. 2, A and C). Block of Kv7.2/7.3 current by Ba^{2+} was assessed, to ensure that the lack of effect of applied muscarine was not the result of a problem of access of agonist to the voltage-clamped cell. Application of Ba^{2+} (1 mM) reversibly blocked Kv7.2/7.3 current (72.7 ± 1.8%, n = 5), producing a net inward current resulting from block of the sustained outward Kv7.2/7.3 current and a corresponding reduction in the deactivation current relaxation (Fig. 2, B and C). The data illustrated in Figs. 1 and 2 indicated that endogenous m3 muscarinic receptors in HEK293 cells were coupled to hydrolysis of PIP_2 and the subsequent release of intracellular Ca^{2+}. However, activation of these endogenous receptors did not couple to suppression of heterologously expressed Kv7.2/7.3 channel current.

Activation of Coexpressed m1 or m3 Muscarinic Receptors Suppressed Kv7.2/7.3 Current. Previous reports concerning modulation of Kv7.2/7.3 current in cell lines have used coexpression of m1 muscarinic receptors (e.g., Selyanko

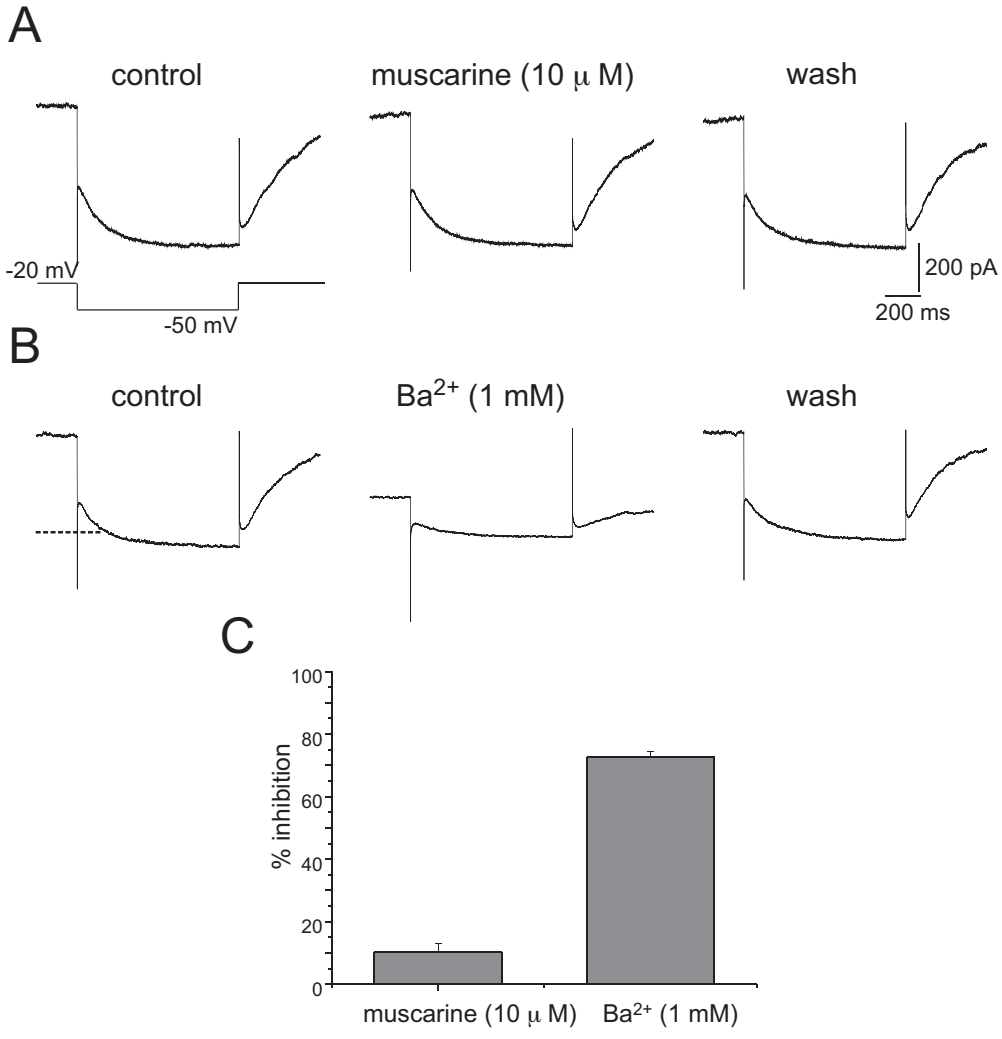

Fig. 2. Activation of functional endogenous muscarinic receptors did not suppress expressed Kv7.2/7.3 channel current. A, membrane current in response to a hyperpolarizing voltage step from a holding potential of −20 to −50 mV. Current represents deactivation of noninactivating Kv7.2/7.3 current active at −20 mV. Application of muscarine (10 μM) had little effect on sustained Kv7.2/7/3 current. B, in contrast, application of Ba^{2+} (1 mM) suppressed the outward Kv7.2/7.3 current, producing a net inward current and a reduction of the deactivation current relaxation. Dashed lines represent the zero current level. C, plot showing mean ± S.E.M. percentage inhibition of expressed Kv7.2/7.3 current by applied muscarine or Ba^{2+}.

et al., 2000; Li et al., 2005; Jensen et al., 2009). We observed robust suppression of expressed Kv7.2/7.3 current in cells in which either m1 or m3 muscarinic receptors were coexpressed. Muscarine (10 μM) evoked a 65.1 $\pm$ 7.1% (n = 8) reversible suppression of Kv7.2/7.3 current in cells coexpressing HA-tagged m1 muscarinic receptors (Fig. 3, A and C). Expressed current was also suppressed by the m1-selective agonist 77-LH-28-1 (1 μM; 54.6 $\pm$ 15.6%, n = 3), confirming that the effect of muscarine was mediated by activation of expressed m1 receptors (Fig. 3C). Application of muscarine (10 μM) to cells coexpressing m3 muscarinic receptors and Kv7.2/7.3 channels evoked a 58.3 $\pm$ 10.4% (n = 6) suppression of current, 77-LH-28-1 having no significant effect (5.3 $\pm$ 3.0%, n = 5) (Fig. 3, B and C). Finally, 77-LH-28-1 (1 μM) evoked a rise of $[Ca^{2+}]_i$ in cells expressing m1 muscarinic receptors (Fig. 3D). These data confirmed that expressed m1 and m3 muscarinic receptors were functional and able to evoke an increase of $[Ca^{2+}]_i$, demonstrating that activation of

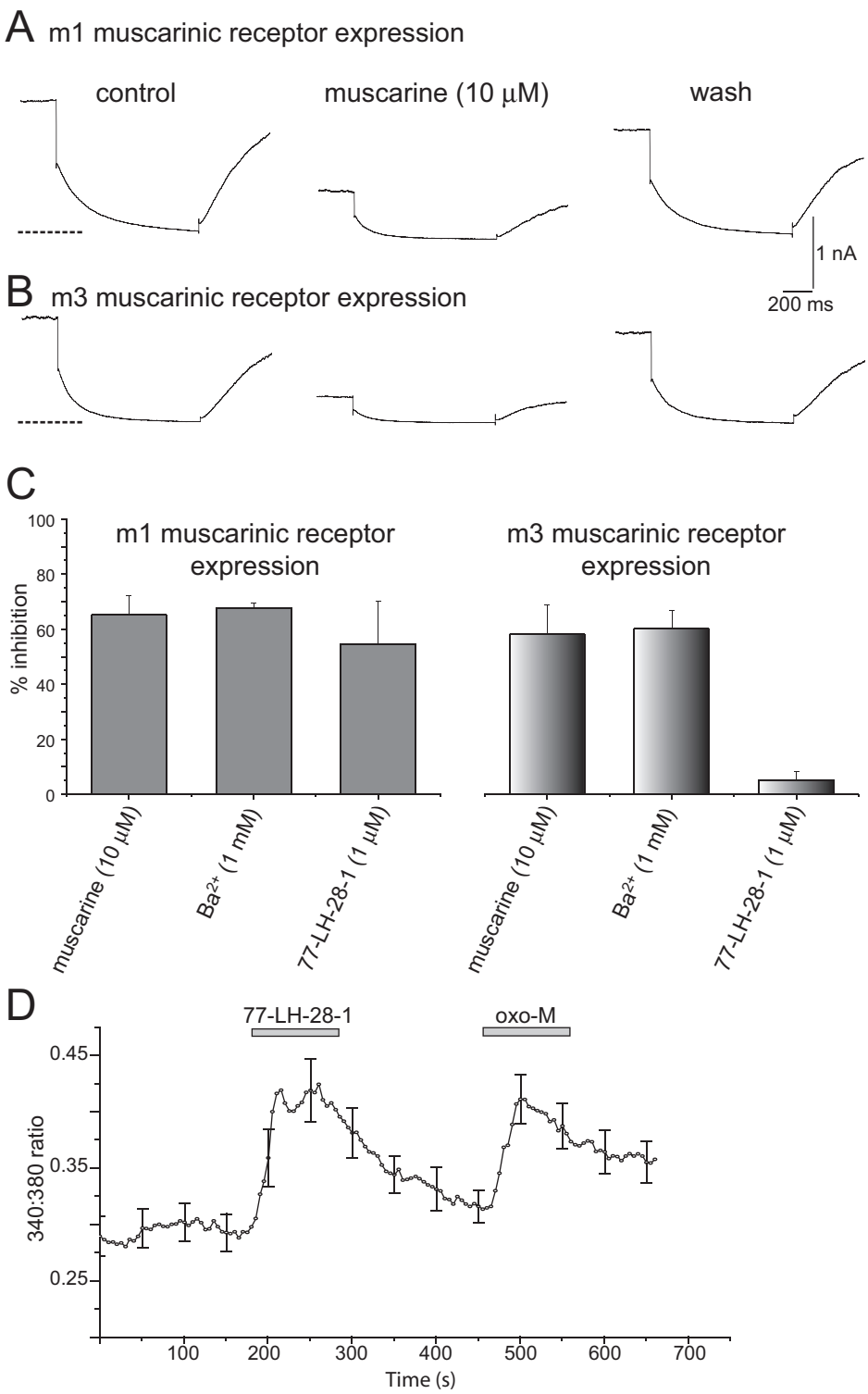

Fig. 3. Activation of expressed m1 or m3 muscarinic receptors did suppress expressed Kv7.2/7.3 channel current. A, deactivation Kv7.2/7.3 current relaxations in cells coexpressing m1 muscarinic receptors. Application of muscarine (10 μM) reversibly suppressed expressed Kv current. B, application of muscarine (10 μM) reversibly suppressed expressed Kv7.2/7.3 current in cells coexpressing m3 muscarinic receptors. Dashed lines represent the zero current level. C, plot showing the mean $\pm$ S.E.M. percentage inhibition of expressed Kv7.2/7.3 current by applied muscarine, Ba^{2+}, or the m1 selective agonist 77-LH-28-1 in cells either coexpressing m1 or m3 muscarinic receptors. D, application of the 77-LH-28-1 (1 μM) to cells coexpressing with m1 muscarinic receptors produced an increase of $[Ca^{2+}]_i$, demonstrating the presence of functional receptors (340:380 ratio was taken from 33 cells expressing EGFP).

a coexpressed receptor was able to suppress expressed Kv7.2/7.3 current.

Receptors and Channel Subunits Were Located within Lipid Rafts. It has been reported that m2 muscarinic receptors and Kv7.2 subunits are localized to lipid rafts (Cooper et al., 2000; Allen et al., 2007). Rafts can be characterized by association with particular proteins such as caveolin 1 and flotillin 2 (Munro, 2003; Allen et al., 2007). This enables rafts to be isolated by sucrose gradient centrifugation and identified using antibodies directed against a marker protein. For example, immunoblotting membrane fractions

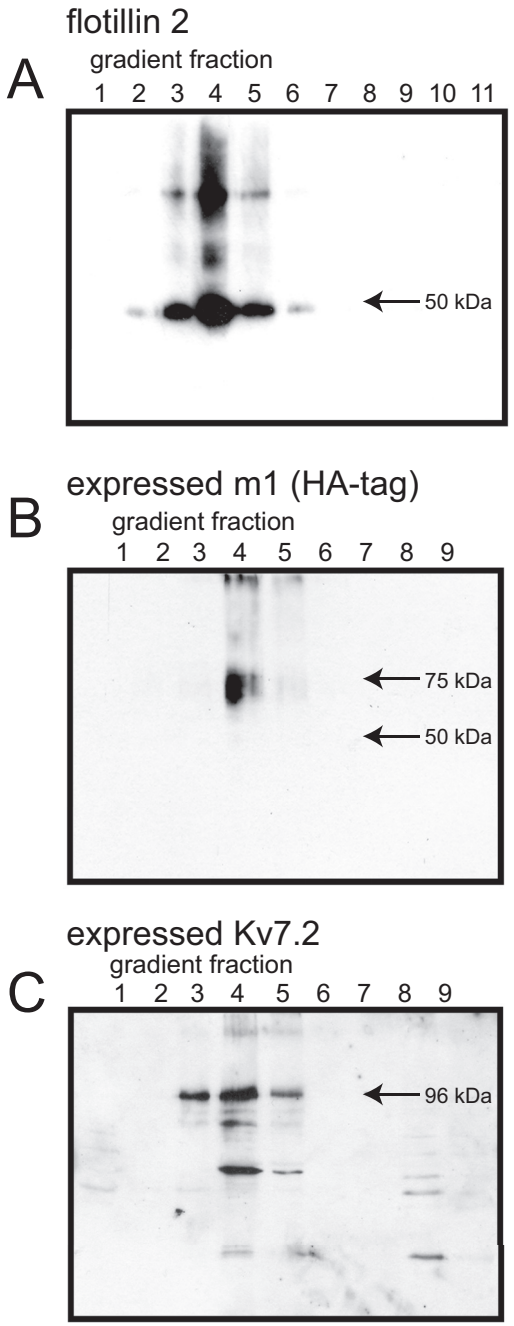

Fig. 4. Coexpressed Kv7.2/7.3 channel subunits and m1 receptors resided within lipid rafts. A, Western blot analysis of the presence of the raft marker flotillin 2 showed that lipid rafts were located in membrane fractions 3 to 5 derived from sucrose gradient centrifugation. B, expressed m1 muscarinic receptors were visualized to be present principally in membrane fractions 4 (by using anti-HA), indicating that the expressed receptors were predominantly located in lipid rafts. C, expressed Kv7.2 subunits were found to be present in membrane fractions 3 to 5, indicating that these channel subunits were also located within lipid rafts.

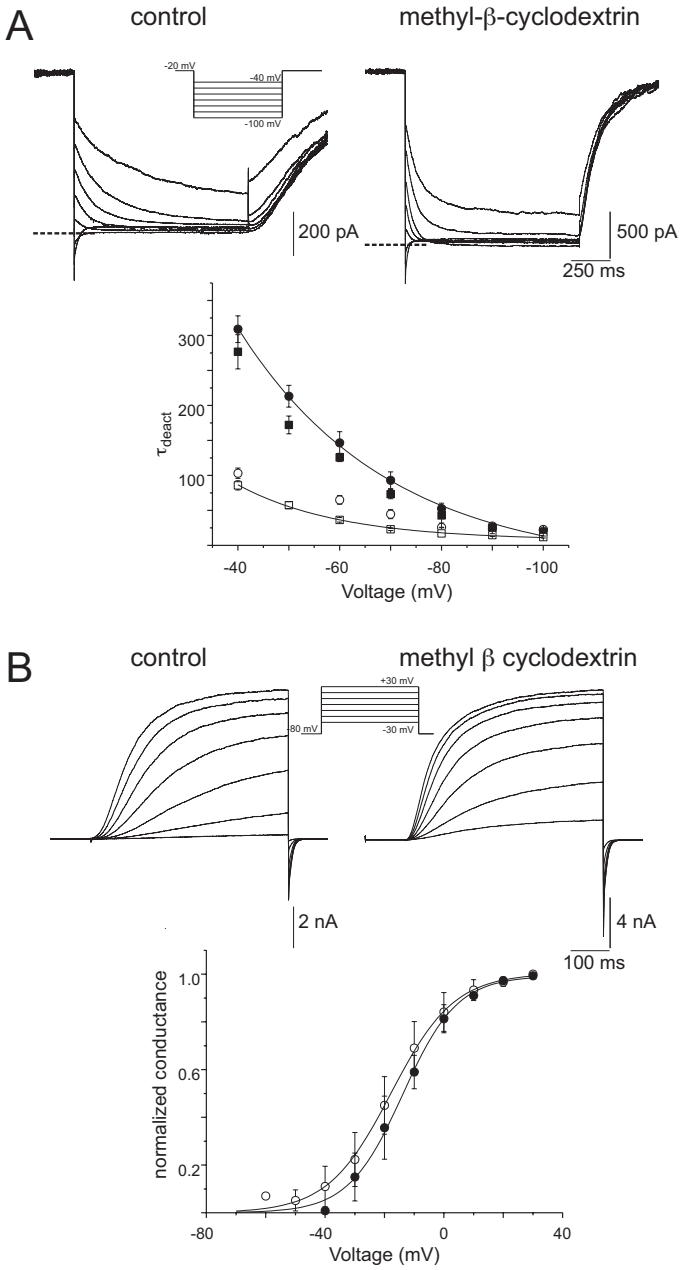

Fig. 5. Pretreatment of HEK293 cells expressing Kv7.2/7.3 channel subunits with methyl-β-cyclodextrin hastened current deactivation. A, top, deactivation current relaxations evoked by 10 mV incremental voltage steps from −40 to −100 mV (holding potential, −20 mV) in cells cotransfected with the m1 muscarinic receptor under control conditions or pretreated with methyl-β-cyclodextrin (10 mM) for 1 h before recording. Dashed lines represent the zero current level. Bottom, plot showing that deactivation was hastened at all potentials by pretreatment of cells coexpressing m1 (○) or m3 (□) muscarinic receptors compared with control cells coexpressing m1 (●) or m3 (■) receptors. Values shown are mean ± S.E.M. (see *Disruption of Lipid Rafts Abolishes Receptor-Mediated Suppression of Kv7.2/7.3 Current* under *Results* for details). B, top, leak-subtracted activation current relaxations evoked by depolarizing voltage steps from a holding potential of −80 mV using the protocol template shown in the inset. Lower, activation curves for Kv7.2/7.3 current in control (●) and cells pretreated with methyl-β-cyclodextrin (○), showing that pretreatment did not significantly affect the voltage dependence of activation.

taken from HEK293 cells expressing m1 muscarinic receptors with anti-flotillin 2 showed immunoreactivity in fractions 3 to 5, indicating that these fractions contained lipid rafts (Fig. 4A). Immunoblotting with an antibody directed against the N-terminal HA-tag on expressed m1 muscarinic receptors showed immunoreactivity in the same membrane fractions (fraction 4), indicating that m1 receptors are predominantly located within lipid rafts (Fig. 4B). Kv7.2 subunits were located in the same membrane fractions that were immunoreactive for flotillin 2 and m1 muscarinic receptors (fractions 3–5), indicating that these channel subunits were also predominantly located within lipid rafts (Fig. 4C). The antibody against Kv7.3 was poor, but a band of the expected molecular weight for Kv7.3 (97 kDa) was seen in the raft fractions only (data not shown).

Disruption of Lipid Rafts Abolishes Receptor-Mediated Suppression of Kv7.2/7.3 Current. A distinguishing characteristic of lipid rafts is their dependence upon cholesterol and disruption by extraction of cholesterol using methyl-β-cyclodextrin, redistributing associated proteins (Simons and Toomre, 2000; van Rheenen et al., 2005). The finding that both m1 muscarinic receptors and Kv7 subunits resided within lipid raft membrane fractions suggested that cells used this lipid microenvironment to colocalize and enable coupling between receptors and channels. It would be predicted that disruption of lipid rafts by cholesterol extraction would abolish receptor-mediated suppression of Kv7.2/7.3 current. Cells expressing Kv7.2/7.3 channel subunits and either m1 or m3 muscarinic receptors were pretreated with methyl-β-cyclodextrin (10 mM) for 1 h immediately before either electrophysiology or separation of membrane fractions by sucrose gradient centrifugation.

Depletion of membrane cholesterol by methyl-β-cyclodextrin affected voltage-dependent gating of some Kv channel subtypes (Martens et al., 2000, 2001). The time course of Kv7.2/7.3 current deactivation was hastened in cells pretreated with methyl-β-cyclodextrin (Fig. 5A). In contrast, the voltage dependence of the time course of current deactivation (τ_{deact}) was not significantly affected by pretreatment with methyl-β-cyclodextrin, τ_{deact} changing e-fold in 23.6 $\pm$ 1.1 mV for control (n = 17) and 26.2 $\pm$ 1.5 mV (n = 14; p = 0.14) for cells pretreated with methyl-β-cyclodextrin (Fig. 5A). The lack of an effect on the voltage dependence of τ_{deact} suggested that the voltage dependence of activation would also not be affected by pretreatment with methyl-β-cyclodextrin. Figure 5B shows that this was the case, the midpoint of activation ($V_{0.5}$) being -14.7 ± 4.6 mV for control (n = 4) and -17.8 ± 5.4 mV (n = 4) for cells pretreated with methyl-β-cyclodextrin. Therefore, the effect of methyl-β-cyclodextrin on current deactivation did not result from a change in the channel sensing the transmembrane field (see *Discussion*).

Pretreatment with methyl-β-cyclodextrin abolished the coupling between both m1 or m3 muscarinic receptors and Kv7.2/7.3 current. For example, application of muscarine

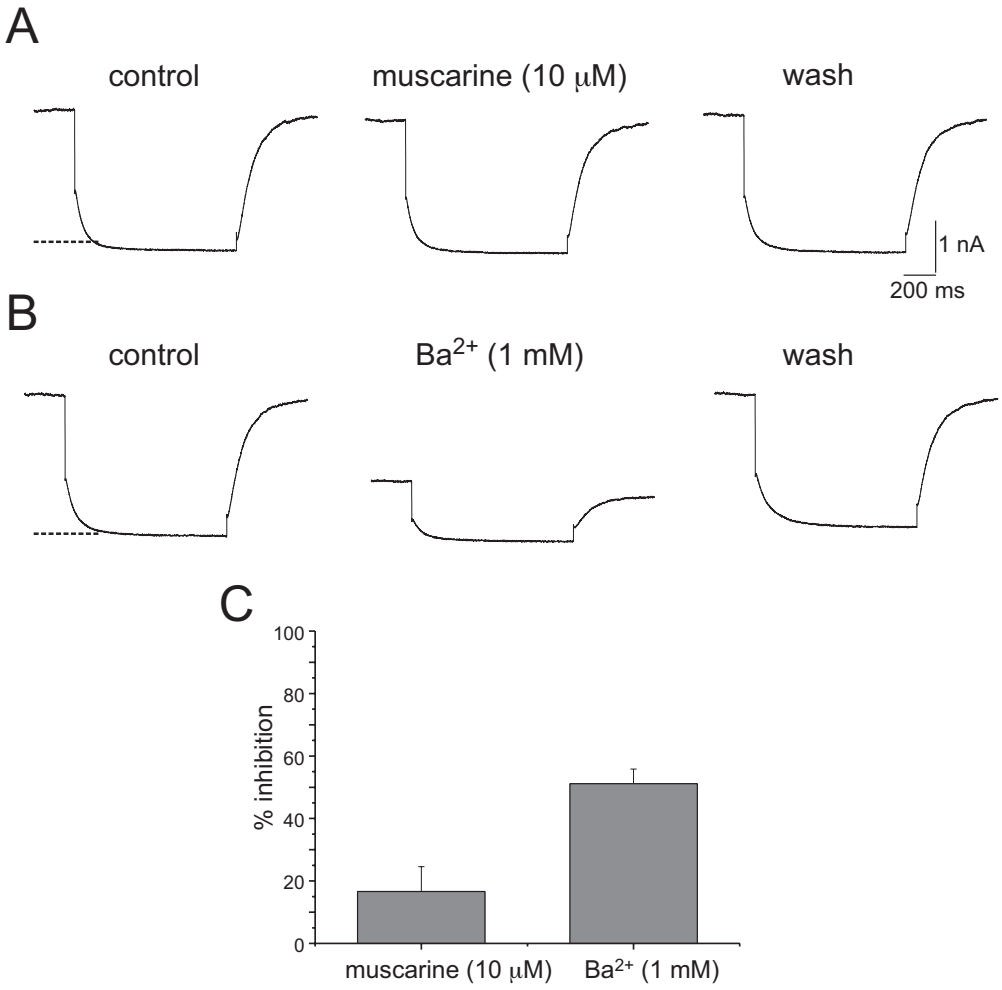

Fig. 6. Pretreatment of HEK293 cells with methyl-β-cyclodextrin reduced m3 muscarinic receptor-mediated suppression of Kv7.2/7.3 current. A, deactivation current relaxations evoked by a hyperpolarizing voltage step to -50 mV from a holding potential of -20 mV. Application of muscarine (10 μM) did not significantly affect Kv7.2/7.3 current in cells pretreated with methyl-β-cyclodextrin. B, application of Ba^{2+} to the cell in A, reversibly blocked Kv7.2/7.3 current. Dashed lines represent the zero current level. C, plot showing the greatly reduced suppression of Kv7.2/7.3 current by muscarine (10 μM) compared with block by Ba^{2+}.

(10 μM) to cells expressing m3 receptors that were pretreated with methyl-β-cyclodextrin only suppressed Kv7.2/7.3 current by 16.6 ± 8.0% (n = 8) (Fig. 6, A and C). In contrast, application of Ba^{2+} (1 mM) to the same cells blocked 51.1 ± 4.7% of expressed current (Fig. 6, B and C). Likewise, muscarine suppressed only 5.6 ± 2.5% (n = 7) of Kv7.2/7.3 current in cells expressing m1 muscarinic receptors after pretreatment with methyl-β-cyclodextrin (Fig. 7, A and C). The lack of suppression was not the result of poor access of applied agonist to voltage-clamped cells, because

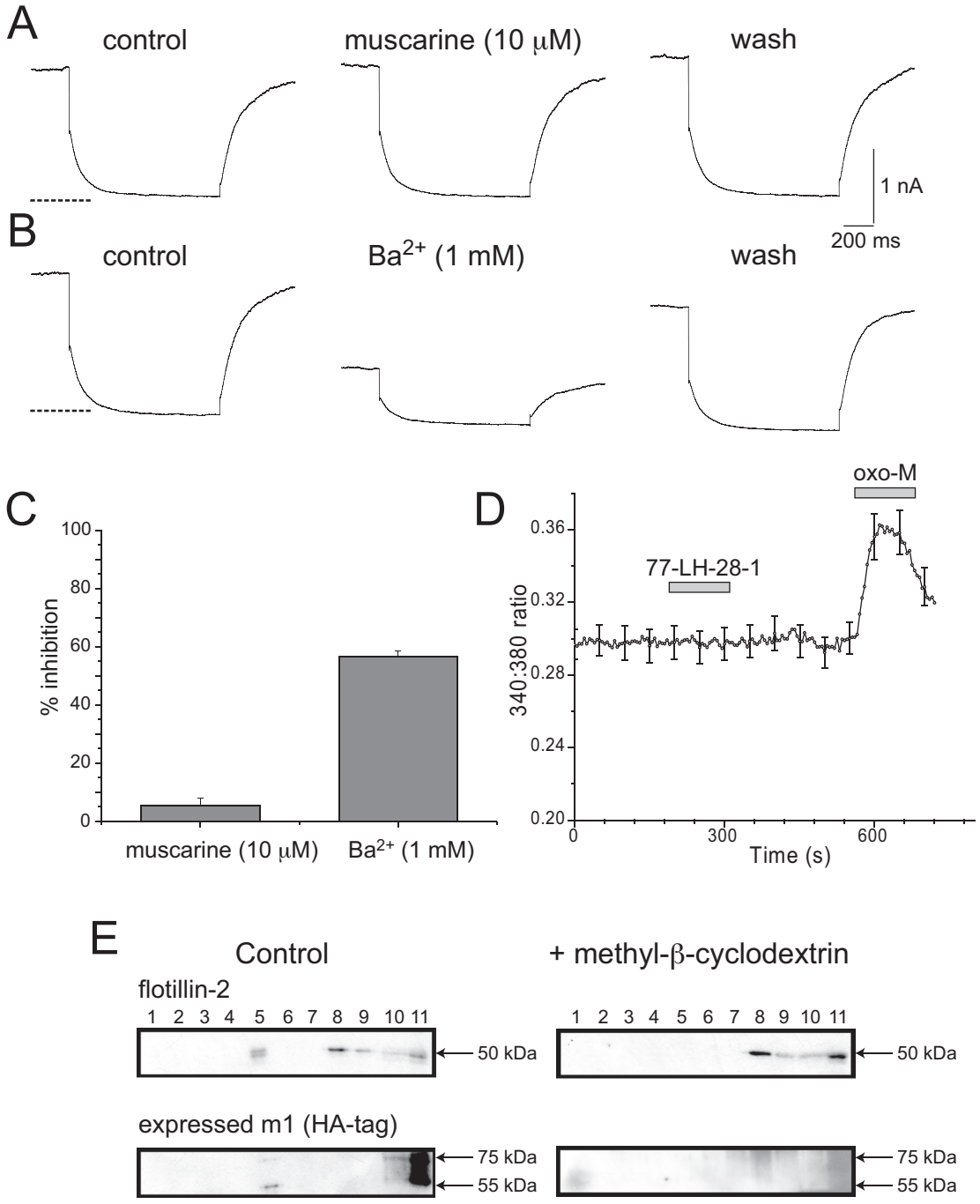

Fig. 7. Pretreatment of HEK293 cells with methyl-β-cyclodextrin abolished m1 muscarinic receptor-mediated suppression of Kv7.2/7.3 current and residence within lipid rafts. A, deactivation current relaxations evoked by a step hyperpolarization to −50 mV from a holding potential of −20 mV. Application of muscarine (10 μM) did not significantly affect Kv7.2/7.3 current. B, application of Ba^{2+} to the cell in A reversibly blocked Kv7.2/7.3 current. Dashed lines represent the zero current level. C, plot showing the lack of m1 muscarinic receptor-mediated suppression of Kv7.2/7.3 current by muscarine (10 μM) compared with block by Ba^{2+} (1 mM). D, plot of 340:380 ratio showing that pretreatment of cells with methyl-β-cyclodextrin abolished the increase of $[Ca^{2+}]_i$ previously evoked by 77-LH-28-1 (1 μM). In contrast, application of the nonselective muscarinic agonist oxotremorine-M (10 μM) evoked an increase of $[Ca^{2+}]_i$ after pretreatment with methyl-β-cyclodextrin (340:380 ratio taken from 70 EGFP-expressing cells). E, Western blots of membrane fractions taken from control cells or those pretreated with methyl-β-cyclodextrin. Immunoblotting with antibodies directed against the raft marker flotillin 2, or the HA-tag of m1 muscarinic receptors showed immunoreactivity for both proteins in fraction 5, indicating the presence of m1 receptors within lipid rafts. Depleting cells of membrane cholesterol by pretreatment with methyl-β-cyclodextrin displaced immunoreactivity for both flotillin 2 and m1 muscarinic receptors into heavier membrane fractions, indicating that depletion of cholesterol disrupted lipid rafts.

application of Ba^{2+} blocked by 56.7 ± 2.0% (Fig. 7, B and C). Pretreatment of cells with methyl-β-cyclodextrin also abolished the rise of $[Ca^{2+}]_i$ evoked by 77-LH-28-1 that was previously observed after expression of m1 receptors (Figs. 3D and 7D). A rise of $[Ca^{2+}]_i$, however, was observed with the nonselective muscarinic receptor agonist oxotremorine-M (10 μM), demonstrating that pretreatment of cells with methyl-β-cyclodextrin did not affect signaling by endogenous m3 muscarinic receptors (Fig. 7D). Probing immunoblots of membrane fractions from control cells expressing m1 receptors with anti-HA showed that these muscarinic receptors resided in the same fraction as the lipid raft marker flotillin 2 (Fig. 7E). The presence of both flotillin 2 and m1 receptors in lipid rafts was lost in membrane fractions from cells pretreated with methyl-β-cyclodextrin (Fig. 7E).

DRG neurons endogenously express m3 muscarinic receptors that couple to an increase of $[Ca^{2+}]_i$ (Takizuka et al., 2007). Activation of these receptors by application of oxotremorine-M (10 μM) suppressed M current native to DRG neurons by 49.2 ± 12.1% ($n = 5$) (Fig. 8A). This suppression was greatly reduced in DRG neurons pretreated with methyl-β-cyclodextrin, application of oxotremorine-M (10 μM) suppressing M current by only 15.6 ± 1.8% ($n = 4$) ($p < 0.05$)(Fig. 8B). These data demonstrated that receptor-mediated suppression of both native and heterologously expressed Kv7.2/7.3 M current required both receptor and channel subunits to be localized within lipid rafts.

Discussion

There has been considerable discussion concerning the possible role(s) of lipid rafts. Many signaling molecules have been found to be associated with rafts, leading to the suggestion that they may provide an environment for localized signaling (Simons and Toomre, 2000; Allen et al., 2007). There has been some precedence for this proposal. For example, the signaling initiated by IgE binding to the FceRI receptors in mast cells and basophils has been shown to involve lipid rafts (Sheets et al., 1999a). Crucially, signaling was abolished if membrane cholesterol was depleted by methyl-β-cyclodextrin (Sheets et al., 1999b). A similar dependence on membrane cholesterol was found for T-cell antigen signaling, where signaling was lost after cholesterol depletion by methyl-β-cyclodextrin (Saeki et al., 2009). A number of different GPCRs are localized to lipid rafts, together with G proteins and enzymes (for review, see Allen et al., 2007). There is some evidence that lipid rafts are used by GPCR signaling cascades (Wang et al., 2008). It has been reported that cholesterol depletion attenuated both neuronal 5-hydroxytryptamine$_{1A}$ receptor and cardiac β_2 adrenoceptor signaling, but in neither case did they attempt to determine whether receptors were located within lipid rafts (Calaghan et al., 2008; Sjögren et al., 2008).

Several Kv channel subtypes, including Kv2.1 (Martens et al., 2000) and Kv1.5 (Martens et al., 2001), are associated with lipid rafts. Depletion of membrane cholesterol by methyl-β-cyclodextrin caused large shifts in the voltage dependence of inactivation of Kv2.1 current (Martens et al., 2000) and more modest effects on activation and inactivation of Kv1.5 current (Martens et al., 2001). Disruption of lipid rafts by methyl-β-cyclodextrin caused a significant increase in rate of Kv7.2/7.3 current deactivation. However, contrary to the effects on other Kv channels, the voltage-dependence of deactivation and activation were not affected by the depletion of membrane cholesterol. Rafts might have unique biophysical properties that could affect channel function, such as bilayer fluidity, thickness, and surface charge (Tillman and Cascio, 2003). The lack of effect on the voltage-dependence of gating transitions of Kv7.2/7.3 channels suggests that effects on surface charge do not play a role. It seems more likely that bilayer fluidity would be important, allowing the channel to

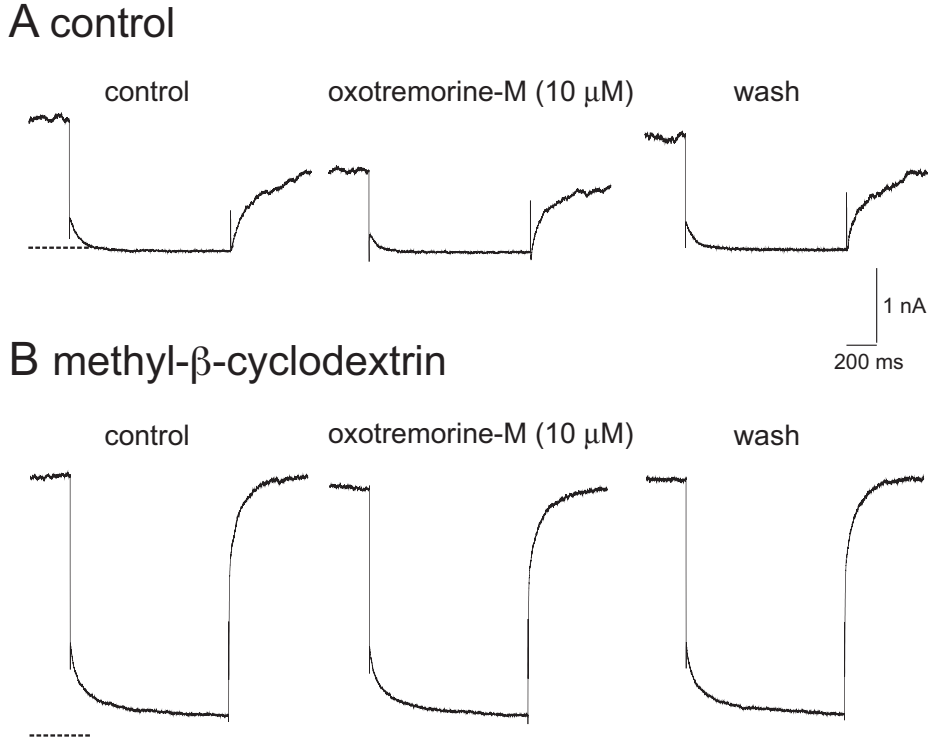

Fig. 8. Muscarinic receptor-mediated suppression of M current native to mouse DRG neurons was abolished by pretreatment of cells with methyl-β-cyclodextrin. A, deactivation current relaxations from a dissociated DRG neuron, evoked by a 30-mV hyperpolarizing voltage step from a holding potential of −20 mV. Application of oxotremorine-M reversibly (10 μM) suppressed M current, producing a net inward current and reduction in the amplitude of deactivation current relaxations. B, deactivation current relaxations evoked by a hyperpolarizing voltage step to −50 mV from a holding potential of −20 mV. Application of oxotremorine-M (10 μM) did not significantly affect M current in cells pretreated with methyl-β-cyclodextrin. Dashed lines represent the zero current level.

transition more quickly between gating states with no effect on the sensing of voltage that dictates gating.

We have demonstrated that expressed Kv7.2/7.3 channel current was not suppressed by activation of functional muscarinic receptors endogenous to HEK293 cells. However, coupling between receptor and channel was reconstituted when both were predominantly localized within lipid rafts after overexpression. The localization of Kv7.2/7.3 channel subunits within lipid rafts is in agreement with their localization in central nervous system neurons (Cooper et al., 2000). In contrast, the presence of m1 muscarinic receptors within rafts has not previously been reported. Receptor-mediated suppression was abolished when lipid rafts were dispersed by depletion of membrane cholesterol by methyl-β-cyclodextrin, for both native and heterologously expressed receptors and channels. These data suggest that lipid rafts were used by m1 and m3 muscarinic receptors to enable localized signaling. This arrangement would provide an environment where local changes in membrane PIP_2 levels would be used to suppress Kv7.2/7.3 channel current, without depleting the entire cell membrane of the lipid.

Depletion of membrane cholesterol by pretreatment with methyl-β-cyclodextrin abolished the increase of $[Ca^{2+}]_i$ evoked by activation of expressed m1 receptors. In contrast, pretreatment with methyl-β-cyclodextrin did not affect the ability of endogenous m3 receptors to evoke an increase of $[Ca^{2+}]_i$. This finding has important implications regarding receptor function. Endogenous m3 muscarinic receptors must be able to evoke hydrolysis of PIP_2 without localization of receptor, $G\alpha_q$, and PLC within lipid rafts. In contrast, expressed m1 receptors did require localization with effector molecules within lipid rafts to evoke hydrolysis of PIP_2. It is not known why expressed m1 muscarinic receptors could not couple to hydrolysis of PIP_2 after loss of lipid rafts. It is possible that endogenous receptors are colocalized with effector molecules using a mechanism(s) different from lipid rafts. If so, it suggests that GPCRs might always use appropriate mechanisms to localize the receptor in close association with G proteins and enzymes and/or channels.

It is possible that rafts could be used to recruit required enzymes to the localized environment to enable signaling to take place. It has recently been determined which steps in the proposed cascade to suppress Kv7.2/7.3 channel current determine the slow rate of response. Expressed Kv7.2/7.3 channels are suppressed after a delay of approximately 500 ms and with a rate of approximately 5 s^{-1}, a time course ascribed to PIP_2 hydrolysis (Jensen et al., 2009). These data are in agreement with the findings that lipid rafts can already contain $G\alpha_q$ and PLC, and they suggest that lipid rafts containing muscarinic receptors and Kv7.2/7.3 channels do not recruit additional proteins to enable signaling to occur.

Deficits in muscarinic signaling are an important facet of the early stages of Alzheimer's disease (AD), clinical management involving agents that boost cholinergic function through cholinesterase inhibition (Pepeu and Giovannini 2009). Drugs that suppress Kv7 channels, and thus mimic muscarinic receptor actions, are cognitive enhancers (Fontana et al., 1994). Central nervous system cholesterol homeostasis also plays a key role in the pathophysiology of AD. Apolipoprotein E (ApoE) is the major transport molecule for cholesterol in the brain and in humans occurs in three major isoforms (ApoE2, -3, and -4). The presence of the ApoE4 variant is the major genetic determinant of increased risk of AD, as well as being a risk factor for frontal temporal dementia, stroke, Parkinson's disease, and a number of other neurological and psychiatric conditions (Raber et al., 2004, Riddell et al., 2008). It is noteworthy that ApoE4 is a less effective cholesterol transport molecule producing impaired cholesterol secretion from astrocytes (Riddell et al., 2008). It is possible that impaired cholesterol transport in persons with an ApoE4 genotype results in fewer lipid rafts and consequent reduction of muscarinic coupling to suppression of Kv7-mediated current in neurons. This would be predicted to impair cognitive performance, especially against a background of declining cholinergic inputs, and places the function of lipid rafts in important perspective. The presented data have demonstrated that lipid rafts can provide an environment for the colocalization of a GPCR with signaling molecules to cause suppression of ion channel function.

References

Allen JA, Halverson-Tamboli RA, and Rasenick MM (2007) Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci* **8:**128–140.

Ancellin N, Preisser L, Le Maout S, Barbado M, Créminon C, Corman B, and Morel A (1999) Homologous and heterologous phosphorylation of the vasopressin V1a receptor. *Cell Signal* **11:**743–751.

Bernheim L, Mathie A, and Hille B (1992) Characterization of muscarinic receptor subtypes inhibiting Ca^{2+} current and M current in rat sympathetic neurons. *Proc Natl Acad Sci U S A* **89:**9544–9548.

Brown DA and Adams PR (1980) Muscarinic suppression of a novel voltage-sensitive K^+ current in a vertebrate neurone. *Nature* **283:**673–676.

Calaghan S, Kozera L, and White E (2008) Compartmentalisation of cAMP-dependent signalling by caveolae in the adult cardiac myocyte. *J Mol Cell Cardiol* **45:**88–92.

Chamberlain LH (2004) Detergents as tools for the purification and classification of lipid rafts. *FEBS Lett* **559:**1–5.

Cooper EC, Aldape KD, Abosch A, Barbaro NM, Berger MS, Peacock WS, Jan YN, and Jan LY (2000) Colocalization and coassembly of two human brain M-type potassium channel subunits that are mutated in epilepsy. *Proc Natl Acad Sci U S A* **97:**4914–4919.

Davies A, Douglas L, Hendrich J, Wratten J, Tran Van Minh A, Foucault I, Koch D, Pratt WS, Saibil HR, and Dolphin AC (2006) The calcium channel alpha2delta-2 subunit partitions with CaV2.1 into lipid rafts in cerebellum: implications for localization and function. *J Neurosci* **26:**8748–8757.

Dunlop J, Lock T, Jow B, Sitzia F, Grauer S, Jow F, Kramer A, Bowlby MR, Randall A, Kowal D, et al. (2009) Old and new pharmacology: positive allosteric modulation of the α7 nicotinic acetylcholine receptor by the 5-hydroxytryptamine(2B/C) receptor antagonist SB-206553 (3,5-dihydro-5-methyl-*N*-3-pyridinylbenzo[1,2-*b*:4,5-*b*′]di pyrrole-1(2*H*)-carboxamide). *J Pharmacol Exp Ther* **328:**766–776.

Fontana DJ, Inouye GT, and Johnson RM (1994) Linopirdine (DuP 996) improves performance in several tests of learning and memory by modulation of cholinergic neurotransmission. *Pharmacol Biochem Behav* **49:**1075–1082.

Holmes FE, Mahoney S, King VR, Bacon A, Kerr NC, Pachnis V, Curtis R, Priestley JV, and Wynick D (2000) Targeted disruption of the galanin gene reduces the number of sensory neurons and their regenerative capacity. *Proc Natl Acad Sci U S A* **97:**11563–11568.

Hur EM, Park YS, Lee BD, Jang IH, Kim HS, Kim TD, Suh PG, Ryu SH, and Kim KT (2004) Sensitization of epidermal growth factor-induced signaling by bradykinin is mediated by c-Src. Implications for a role of lipid microdomains. *J Biol Chem* **279:**5852–5860.

Jensen JB, Lyssand JS, Hague C, and Hille B (2009) Fluorescence changes reveal kinetic steps of muscarinic receptor-mediated modulation of phosphoinositides and Kv7.2/7.3 K^+ channels. *J Gen Physiol* **133:**347–359.

Kurian N, Hall CJ, Wilkinson GF, Sullivan M, Tobin AB, and Willars GB (2009) Full and partial agonists of muscarinic M_3 receptors reveal single and oscillatory Ca^{2+} responses by β_2-adrenoceptors. *J Pharmacol Exp Ther.* doi: 10.1124/jpet.109.153619.

Klopfenstein DR, Tomishige M, Stuurman N, and Vale RD (2002) Role of phosphatidylinositol(4,5)bisphosphate organization in membrane transport by the Unc104 kinesin motor. *Cell* **109:**347–358.

Langmead CJ, Austin NE, Branch CL, Brown JT, Buchanan KA, Davies CH, Forbes IT, Fry VA, Hagan JJ, Herdon HJ, et al. (2008) Characterization of a CNS penetrant, selective M1 muscarinic receptor agonist, 77-LH-28-1. *Br J Pharmacol* **154:**1104–1115.

Li Y, Gamper N, Hilgemann DW, and Shapiro MS (2005) Regulation of Kv7 (KCNQ) K^+ channel open probability by phosphatidylinositol 4,5-bisphosphate. *J Neurosci* **25:**9825–9835.

Marrion NV, Smart TG, Marsh SJ, and Brown DA (1989) Muscarinic suppression of the M-current in the rat sympathetic ganglion is mediated by receptors of the M1-subtype. *Br J Pharmacol* **98:**557–573.

Marrion NV (1997) Control of M-current. *Annu Rev Physiol* **59:**483–504.

Martens JR, Navarro-Polanco R, Coppock EA, Nishiyama A, Parshley L, Grobaski TD, and Tamkun MM (2000) Differential targeting of Shaker-like potassium channels to lipid rafts. *J Biol Chem* **275:**7443–7446.

Martens JR, Sakamoto N, Sullivan SA, Grobaski TD, and Tamkun MM (2001) Isoform-specific localization of voltage-gated K^+ channels to distinct lipid raft populations. Targeting of Kv1.5 to caveolae. *J Biol Chem* **276:**8409–8414.
Miceli F, Soldovieri MV, Martire M, and Taglialatela M (2008) Molecular pharmacology and therapeutic potential of neuronal Kv7-modulating drugs. *Curr Opin Pharmacol* **8:**65–74.
Mundell SJ and Benovic JL (2000) Selective regulation of endogenous G protein-coupled receptors by arrestins in HEK293 cells. *J Biol Chem* **275:**12900–12908.
Munro S (2003) Lipid rafts: elusive or illusive? *Cell* **115:**377–388.
Passmore GM, Selyanko AA, Mistry M, Al-Qatari M, Marsh SJ, Matthews EA, Dickenson AH, Brown TA, Burbidge SA, Main M, et al. (2003) KCNQ/M currents in sensory neurons: significance for pain therapy. *J Neurosci* **23:**7227–7236.
Pepeu G and Giovannini MG (2009) Cholinesterase inhibitors and beyond. *Curr Alzheimer Res* **6:**86–96.
Pike LJ and Casey L (1996) Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains. *J Biol Chem* **271:**26453–26456.
Prole DL, Lima PA, and Marrion NV (2003) Mechanisms underlying modulation of neuronal KCNQ2/KCNQ3 potassium channels by extracellular protons. *J Gen Physiol* **122:**775–793.
Raber J, Huang Y, and Ashford JW (2004) ApoE genotype accounts for the vast majority of AD risk and AD pathology. *Neurobiol Aging* **25:**641–650.
Riddell DR, Zhou H, Atchison K, Warwick HK, Atkinson PJ, Jefferson J, Xu L, Aschmies S, Kirksey Y, Hu Y, et al. (2008) Impact of apolipoprotein E (ApoE) polymorphism on brain ApoE levels. *J Neurosci* **28:**11445–11453.
van Rheenen J, Achame EM, Janssen H, Calafat J, and Jalink K (2005) PIP_2 signaling in lipid domains: a critical re-evaluation. *EMBO J* **24:**1664–1673.
Saeki K, Fukuyama S, Ayada T, Nakaya M, Aki D, Takaesu G, Hanada T, Matsumura Y, Kobayashi T, Nakagawa R, et al. (2009) A major lipid raft protein raftlin modulates T cell receptor signaling and enhances th17-mediated autoimmune responses. *J Immunol* **182:**5929–5937.
Selyanko AA, Hadley JK, Wood IC, Abogadie FC, Jentsch TJ, and Brown DA (2000) Inhibition of KCNQ1–4 potassium channels expressed in mammalian cells via M1 muscarinic acetylcholine receptors. *J Physiol* **522:**349–355.
Sheets ED, Holowka D, and Baird B (1999a) Membrane organization in immunoglobulin E receptor signaling. *Curr Opin Chem Biol* **3:**95–99.
Sheets ED, Holowka D, and Baird B (1999b) Critical role for cholesterol in Lyn-mediated tyrosine phosphorylation of FcepsilonRI and their association with detergent-resistant membranes. *J Cell Biol* **145:**877–887.
Silva WI, Maldonado HM, Lisanti MP, Devellis J, Chompré G, Mayol N, Ortiz M, Velázquez G, Maldonado A, and Montalvo J (1999) Identification of caveolae and caveolin in C6 glioma cells. *Int J Dev Neurosci* **17:**705–714.
Simons K and Toomre D (2000) Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1:**31–39.
Sjögren B, Csöregh L, and Svenningsson P (2008) Cholesterol reduction attenuates 5-HT1A receptor-mediated signaling in human primary neuronal cultures. *Naunyn Schmiedebergs Arch Pharmacol* **378:**441–446.
Suh BC and Hille B (2002) Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. *Neuron* **35:**507–520.
Suh BC and Hille B (2009) PIP_2 is a necessary cofactor for ion channel function: how and why? *Annu Rev Biophys* **37:**175–195.
Takizuka A, Minami K, Uezono Y, Horishita T, Yokoyama T, Shiraishi M, Sakurai T, Shigematsu A, and Ueta Y (2007) Dexmedetomidine inhibits muscarinic type 3 receptors expressed in *Xenopus* oocytes and muscarine-induced intracellular Ca^{2+} elevation in cultured rat dorsal root ganglia cells. *Naunyn Schmiedebergs Arch Pharmacol* **375:**293–301.
Tillman TS and Cascio M (2003) Effects of membrane lipids on ion channel structure and function. *Cell Biochem Biophys* **38:**161–190.
Wang HS, Pan Z, Shi W, Brown BS, Wymore RS, Cohen IS, Dixon JE, and McKinnon D (1998) KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel. *Science* **282:**1890–1893.
Wang D, Wang W, Duan Y, Sun Y, Wang Y, and Huang P (2008) Functional coupling of Gs and CFTR is independent of their association with lipid rafts in epithelial cells. *Pflugers Arch* **456:**929–938.
Zhang H, Craciun LC, Mirshahi T, Rohács T, Lopes CM, Jin T, and Logothetis DE (2003) PIP(2) activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. *Neuron* **37:**963–975.

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