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Receptor-mediated suppression of potassium currents requires co-localization within lipid rafts.

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Running Title: localized signalling within lipid rafts

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Abbreviations:

PIP_2 - phosphatidylinositol 4,5-bisphosphate

DRG - dorsal root ganglion

MES - N-morpholino)ethanesulfonic acid

IP_3 – inositol 3,4,5 trisphosphate

GPCR – G protein coupled receptor

5-HT - 5-hydroxytryptamine

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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 PIP_2. Expression of Kv7.2/7.3 channels in HEK293 cells produced a non-inactivating 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 co-expression 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 pre-treatment 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 pre-treatment 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 co-localized both muscarinic receptors and channel subunits to enable receptor-mediated suppression of channel activity, a spatial co-localization that enables specificity of coupling between receptor and ion channel.

Introduction

The M-current is a non-inactivating potassium (K^+) 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, with 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 (Suh and Hille, 2009; Zhang et al., 2003).

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^oC and displaying low buoyant density (Chamberlain, 2004). Many different types of proteins have been found to be associated with lipid rafts, such as many G-protein coupled receptors (GPCRs), G-proteins and various enzymes. For example, β1 and 2 adrenoceptors,

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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 signalling 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 CHO and HEK293 cells (e.g. Selyanko et al., 2000; Li et al., 2005; Jensen et al., 2009). These studies have co-expressed 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 non-transfected 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 over-expression 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 pre-treatment of cells with methyl-β-cyclodextrin. Finally, suppression of

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M-current by muscarinic receptor activation in DRG neurons was also lost when dissociated cells were pre-treated with methyl-β-cyclodextrin. These data suggested that the co-localization of native and over-expressed 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 previously published (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 3xHA-tagged constructs (DNA.org). Channels and/or receptors were transiently expressed in HEK293 cells. Cells were maintained in modified essential medium (DMEM) (Gibco, Invitrogen), supplemented with 10% foetal calf serum (Gibco, Invitrogen) and 100 U/ml penicillin/100 μg/ml streptomycin at 37°C. Cells were plated onto 35 mm dishes (Falcon, Primaria) 48 hrs before transfection for electrophysiology. Transient transfections of HEK293 cells were made using polyethylenimine (PEI) (Alfa Aesar, Inc) with 1 μg of each construct encoding channel subunits and/or receptors. Proteins were co-transfected with the plasmid pEGFP-C2 (Clontech) 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, being harvested 48 hours later. Lipid rafts were dissipated by pre-treating cells with the cholesterol-depleting agent methyl-β-cyclodextrin (10 mM) in unsupplemented media for 1 hour immediately before electrophysiology or biochemistry.

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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 F12 medium. Ganglia were subjected to 0.25% collagenase P for 1 hour at 37°C, washed in PBS, and treated enzymatically with trypsin-EDTA for 10 minutes 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 re-suspended in F12 medium supplemented with 5% horse serum, 1 mM glutamine, and 10 ng/ml gentamycin, 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 composition (mM): NaCl 144; KCl 2.5; HEPES(Na) 10; D-glucose 10; $MgCl_2$ 1.2; $CaCl_2$ 2.5, pH adjusted to 7.4 with NaOH. Whole-cell electrodes were fabricated from KG-33 glass (Friedrich and Dimmock, CT, USA) and filled with a solution of composition (mM): KAspartate 130; KCl 20; HEPES 10; ATP(Na_2) 3; EGTA 0.1; $MgCl_2$ 3; $CaCl_2$ 6 (to give 50 nM free Ca^{2+}); NaOH 3 (adjusted to pH 7.4 with KOH) to give resistances of 2-5 MΩ. The liquid junction potential was calculated to be approximately 8 mV, which was not corrected for. All standard chemicals were purchased from Sigma, except $CaCl_2$ and $MgCl_2$ which were purchased from Fluka.

Recording

Whole-cell currents were recorded with an Axopatch 200A (Molecular Devices). Capacitance and series resistance compensation (95%) was used throughout. Currents were low-pass filtered at 1 kHz (8-pole Bessel, Frequency Devices) and acquired at 20 kHz using Pulse (HEKA Elektronic). All recordings were performed at room temperature (20-25^{o}C). Non-inactivating 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 depolarising 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. 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 minutes at 37°C in a standard HEPES-buffered salt solution (HBSS) supplemented with Pluronic acid 0.02% w/v. The HBSS consisted of: (mM) NaCl, 135; KCl, 5; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 2; D-glucose, 30; pH 7.3 (with NaOH). Coverslips were then washed and maintained in HBSS at room temperature for up to 150 minutes, 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). A CCD

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camera (Hamamatsu Orca 12AG) was used to collect fluorescence images (emission wavelength ~ 515 nm) from a 20X objective. Pair-wise 200 ms exposures to 340 nm and 380 nm excitation light were provided at 0.2 Hz by a Sutter DG4 illumination source. 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 prior to generating a 340:380 ratio channel. Analysis of $[Ca^{2+}]_i$ in individual cells was made following their selection as regions of interest (ROIs). For experiments on transiently transfected cells, $[Ca^{2+}]_i$ was only analysed 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). Briefly, cells from a 100 mm dish were washed twice in ice-cold phosphate-buffered saline, scraped into 1 ml ice-cold MBS (25 mM MES, 150 mM NaCl) containing 1% Triton X-100, 4 μg each leupeptin. Antipain, aprotinin and 0.5 mM benzamidine and homogenized with 10 strokes in a hand-held Dounce homogenizer. 1 ml of homogenate was mixed with an equal volume of 40% sucrose, over-layered with 5 ml 35% sucrose in MBS and then with 4 ml 5% sucrose in MBS. Gradients were subject to overnight centrifugation at 34000xg in an AH641 rotor.

Western Blotting

Aliquots from gradient fractions were subjected to SDS-polyacrylamide gel electrophoresis and proteins transferred onto PVDF membrane using a semi-dry blotter. Blots were probed for

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channel subunits, m1 muscarinic receptor and the raft markers, flotillin-2. A rabbit antibody against Kv7.2 was from Santa Cruz and monoclonal anti-flotillin-2 from BD Biosciences. Over-expressed, HA-tagged m1 receptor was detected using an anti-HA monoclonal antibody (Covance). Unfortunately, the poor quality of commercial antibodies directed against the Kv7.3 subunit (Alomone) or m3 muscarinic receptor (Santa Cruz) prevented presentable distribution of proteins within membrane fractions. Following subsequent incubation with Horseradish peroxidase-labelled secondary antibody (GE Healthcare), 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 Western blotting of membrane fractions from non-transfected 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 (Figure 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. (Figure 1A). This was confirmed using the muscarinic agonists muscarine (10 µM) and oxotremorine-M (10 µM), which both produced rises of $[Ca^{2+}]_I$ that were antagonized by atropine (1 µM) (Figure 1B). Application of the M1-selective agonist 77-LH-28-1 (1 µM) (Langmead et al. 2008) failed to produce a response in non-transfected HEK293 cells, although these same cells were responsive to carbachol (Figure 1C). The lack of a response to 77-LH-28-1 was found in multiple experiments using two different batches of compound, with both compound stocks successfully producing

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depolarization of hippocampal neurones in a pirenzepine-sensitive manner (data not shown). These data suggested that HEK293 cells do not possess an endogenous functional m1 muscarinic receptor, but possessed an endogenous functional m3 receptor that coupled to hydrolysis of PIP_2 and a subsequent IP_3-mediated rise of $[Ca^{2+}]_I$,

Activation of endogenous m3 receptors coupled to $G\alpha_q$-mediated signalling 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, with 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 (Figure 2A 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 (Figure 2B and C). The data illustrated in Figures 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 co-expressed 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 utilized co-expression of m1 muscarinic receptors (e.g. Selyanko et al., 2000; Li et al., 2005; Jensen et al.,

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2009). We observed robust suppression of expressed Kv7.2/7.3 current in cells where either m1 or m3 muscarinic receptors were co-expressed. Muscarine (10 µM) evoked a 65.1 ± 7.1 % (n=8) reversible suppression of Kv7.2/7.3 current in cells co-expressing HA-tagged m1 muscarinic receptors (Figure 3A and C). Expressed current was also suppressed by the m1-selective agonist 77-HL-146 (1 µM) (54.6 ± 15.6 %, n=3), confirming that the effect of muscarine was mediated by activation of expressed m1 receptors (Figure 3C). Application of muscarine (10 µM) to cells co-expressing m3 muscarinic receptors and Kv7.2/7.3 channels evoked a 58.3 ± 10.4 % (n=6) suppression of current, with 77-HL-146 having no significant effect (5.3 ± 3.0 %, n=5) (Figure 3B and C). Finally, 77-HL-146 (1 µM) evoked a rise of $[Ca^{2+}]_I$ in cells expressing m1 muscarinic receptors (Figure 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 a co-expressed 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 (Allen et al., 2007; Cooper et al., 2000). 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 taken from HEK293 cells expressing m1 muscarinic receptors with anti-flotillin 2 showed immunoreactivity in fractions 3-5, indicating that these fractions contained lipid rafts (Figure 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 (Figure 4B). Kv7.2 subunits were located in the same membrane fractions that were immunoreactive for flotillin 2 and m1 muscarinic

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receptors (fractions 3-5), indicating that these channel subunits were also predominantly located within lipid rafts (Figure 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 & 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 co-localize 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 pre-treated with methyl-β-cyclodextrin (10 mM) for one hour immediately prior to 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; Martens et al., 2001). The time course of Kv7.2/7.3 current deactivation was hastened in cells pre-treated with methyl-β-cyclodextrin (Figure 5A). In contrast, the voltage dependence of the time course of current deactivation (τ_{deact}) was not significantly affected by pre-treatment with methyl-β-cyclodextrin, with τ_{deact} changing e-fold in 23.6 ± 1.1 mV for control (n=17) and 26.2 ± 1.5 mV (n=14) (p=0.14) for cells pre-treated with methyl-β-cyclodextrin (Figure 5A). The lack of an effect on the voltage dependence of τ_{deact} suggested that the voltage dependence of activation would also not be

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affected by pre-treatment with methyl-β-cyclodextrin. Figure 5B shows that this was the case, with the mid-point 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 pre-treated 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).

Pre-treatment 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 (10 μM) to cells expressing m3 receptors that were pre-treated with methyl-β-cyclodextrin only suppressed Kv7.2/7.3 current by 16.6 ± 8.0 % (n=8) (Figure 6A and C). In contrast, application of Ba^{2+} (1 mM) to the same cells blocked 51.1 ± 4.7 % of expressed current (Figure 6B and C). Similarly, muscarine only suppressed 5.6 ± 2.5 % (n=7) of Kv7.2/7.3 current in cells expressing m1 muscarinic receptors after pre-treatment with methyl-β-cyclodextrin (Figure 7A and C). The lack of suppression was not the result of poor access of applied agonist to voltage-clamped cells, as application of Ba^{2+} blocked 56.7 ± 2.0 % (Figure 7B and C). Pre-treatment of cells with methyl-β-cyclodextrin also abolished the rise of $[Ca^{2+}]_i$ evoked by 77-HL-146 that was previously observed after expression of m1 receptors (Figures 7D and 3D). A rise of $[Ca^{2+}]_i$ however, was observed with the non-selective muscarinic receptor agonist, oxotremorine-M (10 μM), demonstrating that pre-treatment of cells with methyl-β-cyclodextrin did not affect signalling by endogenous m3 muscarinic receptors (Figure 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 (Figure 7E). The presence of both flotillin 2 and m1 receptors in lipid rafts was lost in membrane fractions from cells pre-treated with methyl-β-cyclodextrin (Figure 7E).

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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) (Figure 8A). This suppression was greatly reduced in DRG neurons pre-treated with methyl-β-cyclodextrin, with application of oxotremorine-M (10 µM) suppressing M-current by only 15.6 ± 1.8 % (n=4) ($p<0.05$)(Figure 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 signalling molecules have been found to be associated with rafts, leading to the suggestion that they may provide an environment for localized signalling (Simon and Toomre, 2000; Allen et al., 2007). There has been some precedence for this proposal. For example, the signalling 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, signalling 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 signalling, where signalling 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 utilized by GPCR signalling cascades (Wang et al., 2008). It was reported that cholesterol depletion attenuated both neuronal 5-HT_{1A} receptor and cardiac β_2 adrenoceptor signalling, but in neither case was it attempted to

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determine whether receptors were located within lipid rafts (Sjögren et al., 2008; Calaghan 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. It is possible that 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 is seems more likely that bilayer fluidity would be important, allowing the channel to more faster between gating states but 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 over-expression. The localization of Kv7.2/7.3 channel subunits within lipid rafts is in agreement with their localization in CNS neurons (Cooper et al., 2000). In contrast, the presence of m1 muscarinic receptors within rafts has not been reported previously. 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

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and channels. These data suggested that lipid rafts were utilized by m1 and m3 muscarinic receptors to enable localized signalling. 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 pre-treatment of methyl-β-cyclodextrin abolished the increase of $[Ca^{2+}]_i$ evoked by activation of expressed m1 receptors. In contrast, pre-treatment 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 co-localized 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 signalling 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 suggest

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that lipid rafts containing muscarinic receptors and Kv7.2/7.3 channels do not recruit additional proteins to enable signalling to occur.

Deficits in muscarinic signalling are an important facet of the early stages of Alzheimer's disease (AD), with 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). CNS 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 man 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). Notably, 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 individuals with an ApoE4 genotype results in less 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 co-localization of a GPCR with signalling molecules to cause suppression of ion channel function.

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Legends for Figures

Figure 1. Activation of functional endogenous m3 muscarinic receptors evoked a rise in $[Ca^{2+}]_i$.

A. Traces illustrating the ratio of emission from 340 and 380 nm excitation for Fura-2-loaded non-transfected 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 non-transfected 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-146 (1 µM) failed to increase $[Ca^{2+}]_i$. This indicated that non-transfected cells lacked functional m1 receptors.

Figure 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 mV to -50 mV. Current represents deactivation of non-inactivating 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

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zero current level. **C.** Plot showing mean ± S.E.M % inhibition of expressed Kv7.2/7.3 current by applied muscarine or Ba^{2+}.

<u>Figure 3.</u> 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 co-expressing 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 co-expressing m3 muscarinic receptors. Dashed lines represent the zero current level. **C.** Plot showing the mean ± S.E.M. % inhibition of expressed Kv7.2/7.3 current by applied muscarine, Ba^{2+}, or the m1 selective agonist 77-HL-146 in cells either co-expressing m1 or m3 muscarinic receptors. **D.** Application of the 77-HL-146 (1 µM) to cells co-expressed 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).

<u>Figure 4.</u> Co-expressed 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 3-5 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 3-5, indicating that these channel subunits were also located within lipid rafts.

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Figure 5. **Pre-treatment of HEK293 cells expressing Kv7.2/7.3 channel subunits with methyl-β-cyclodextrin hastened current deactivation.**

A. Upper: Deactivation current relaxations evoked by 10 mV incremental voltage steps from -40 to -100 mV (holding potential -20 mV) in cells co-transfected with the m1 muscarinic receptor under control conditions control or pre-treated with methyl-β-cyclodextrin (10 mM) for 1 hour prior to recording. Dashed lines represent the zero current level. **Lower:** Plot showing that deactivation was hastened at all potentials by pre-treatment of cells co-expressing m1 (○) or m3 (□) muscarinic receptors when compared with control cells co-expressing m1 (●) or m3 (■) receptors. Values shown are mean ± S.E.M. (see text for details). **B. Upper:** 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 pre-treated with methyl-β-cyclodextrin (○), showing that pre-treatment did not significantly affect the voltage dependence of activation.

Figure 6. **Pre-treatment 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 pre-treated 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) when compared with block by Ba^{2+}.

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Figure 7. Pre-treatment 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) when compared with block by Ba^{2+} (1 mM). **D.** Plot of 340:380 ratio showing that pre-treatment of cells with methyl-β-cyclodextrin abolished the increase of $[Ca^{2+}]_i$ previously evoked by 77-HL-146 (1 µM). In contrast, application of the non-selective muscarinic agonist oxotremorine-M (10 µM) evoked an increase of $[Ca^{2+}]_i$ after pre-treatment 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 pre-treated 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 pre-treatment 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.

Figure 8. Muscarinic receptor-mediated suppression of M-current native to mouse DRG neurons was abolished by pre-treatment of cells with methyl-β-cyclodextrin.

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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 pre-treated with methyl-β-cyclodextrin. Dashed lines represent the zero current level.

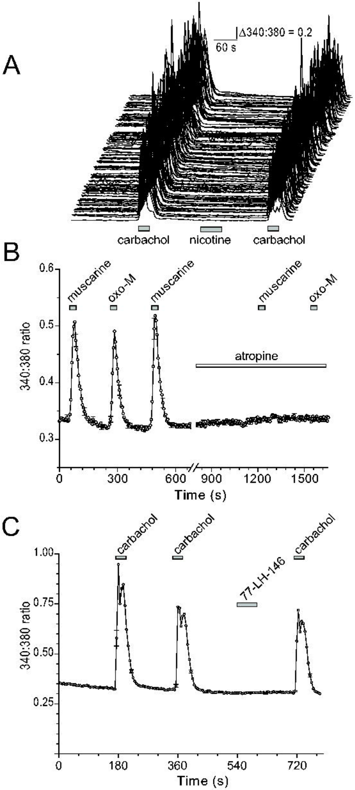
A
Δ340:380 = 0.2
60 s
carbachol
nicotine
carbachol
B
muscarine
oxo-M
muscarine
muscarine
oxo-M
atropine
340:380 ratio
0.6
0.5
0.4
0.3
0
300
600
900
1200
1500
Time (s)
C
carbachol
carbachol
carbachol
77-LH-146
340:380 ratio
1.00
0.75
0.50
0.25
0
180
360
540
720
Time (s)

Figure 1

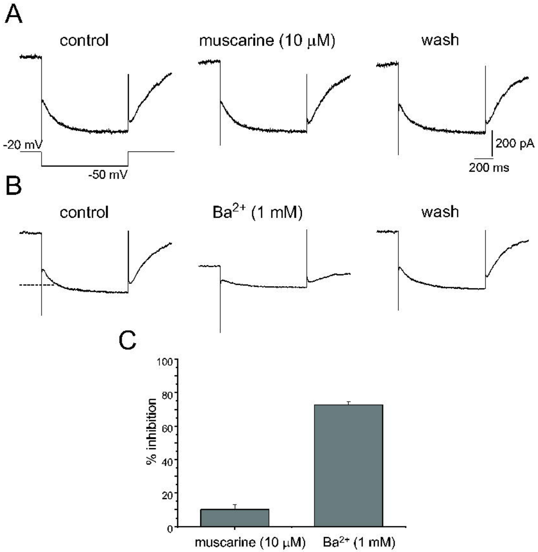
A
control
muscarine (10 μM)
wash
-20 mV
-50 mV
200 pA
200 ms
B
control
Ba2+ (1 mM)
wash
C
% inhibition
100
80
60
40
20
0
muscarine (10 μM)
Ba2+ (1 mM)

Figure 2

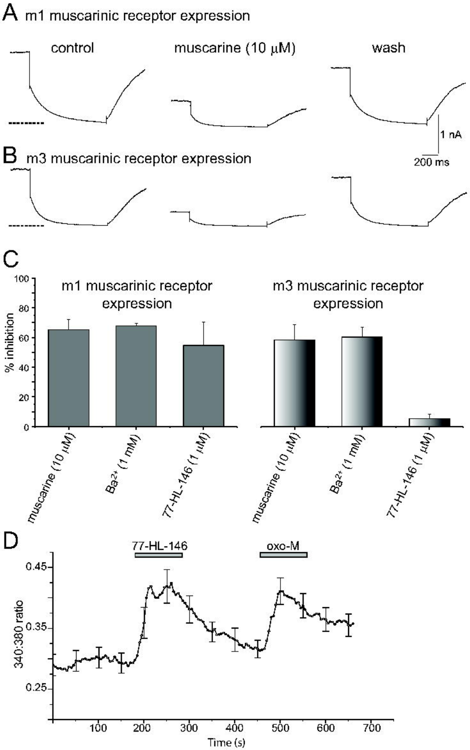
A m1 muscarinic receptor expression
control
muscarine (10 μM)
wash
1 nA
200 ms
B m3 muscarinic receptor expression
C
m1 muscarinic receptor expression
m3 muscarinic receptor expression
% inhibition
0
20
40
60
80
100
muscarine (10 μM)
Ba2+ (1 mM)
77-HL-146 (1 μM)
D
77-HL-146
oxo-M
340:380 ratio
0.25
0.35
0.45
100
200
300
400
500
600
700
Time (s)

Figure 3

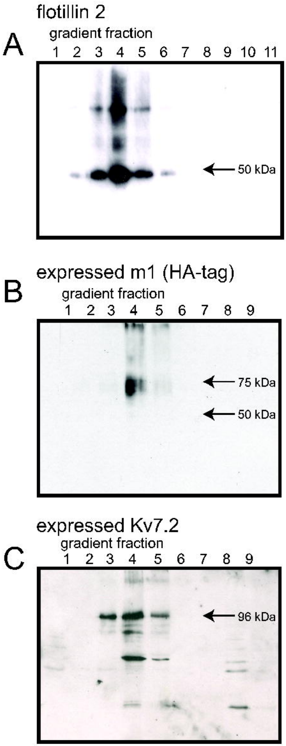
flotillin 2
A
gradient fraction
1 2 3 4 5 6 7 8 9 10 11
50 kDa
expressed m1 (HA-tag)
B
gradient fraction
1 2 3 4 5 6 7 8 9
75 kDa
50 kDa
expressed Kv7.2
C
gradient fraction
1 2 3 4 5 6 7 8 9
96 kDa

Figure 4

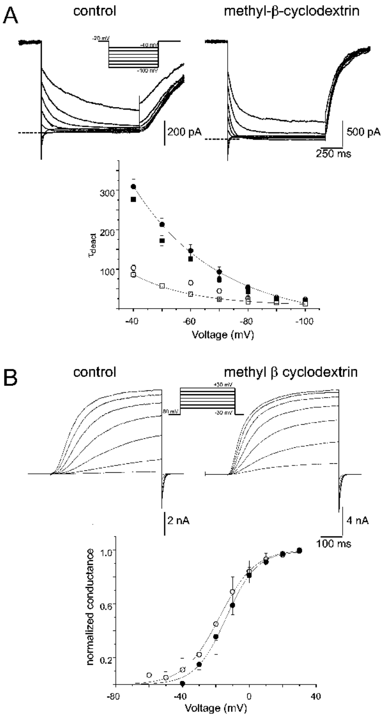
A
control
methyl-β-cyclodextrin
-20 mV
-40 mV
-100 mV
200 pA
500 pA
250 ms
300
200
100
τdeact
-40
-60
-80
-100
Voltage (mV)
B
control
methyl β cyclodextrin
+30 mV
-80 mV
-30 mV
2 nA
4 nA
100 ms
1.0
0.6
0.2
normalized conductance
-80
-40
0
40
Voltage (mV)

Figure 5

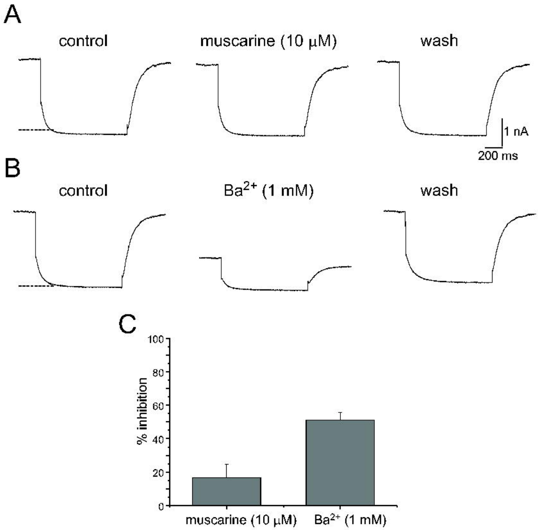
A
control
muscarine (10 μM)
wash
1 nA
200 ms
B
control
Ba2+ (1 mM)
wash
C
100
80
60
40
20
0
% inhibition
muscarine (10 μM)
Ba2+ (1 mM)

Figure 6

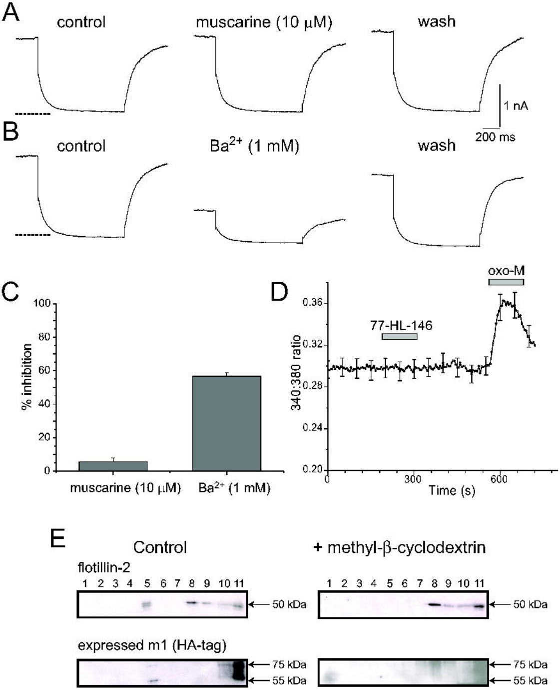
A
control
muscarine (10 μM)
wash
1 nA
200 ms
B
control
Ba2+ (1 mM)
wash
C
% inhibition
100
80
60
40
20
0
muscarine (10 μM)
Ba2+ (1 mM)
D
oxo-M
77-HL-146
340:380 ratio
0.36
0.32
0.28
0.24
0.20
0
300
600
Time (s)
E
Control
+ methyl-β-cyclodextrin
flotillin-2
1 2 3 4 5 6 7 8 9 10 11
50 kDa
expressed m1 (HA-tag)
75 kDa
55 kDa

Figure 7

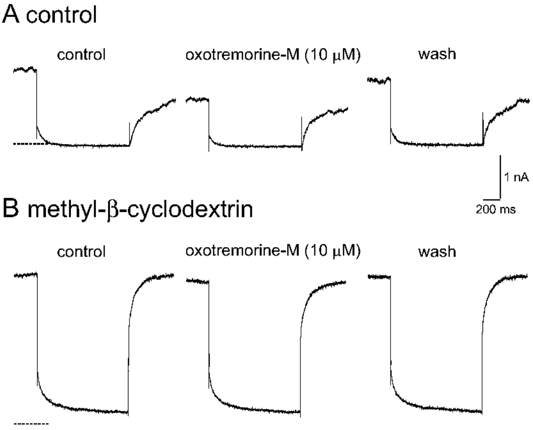
A control
control
oxotremorine-M (10 μM)
wash
1 nA
200 ms
B methyl-β-cyclodextrin
control
oxotremorine-M (10 μM)
wash

Figure 8