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The NALCN ion channel is activated by M3 muscarinic receptors in a pancreatic β -cell line

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A previously uncharacterized putative ion channel, NALCN (sodium leak channel, non-selective), has been recently shown to be responsible for the tetrodotoxin (TTX)-resistant sodium leak current implicated in the regulation of neuronal excitability. Here, we show that NALCN encodes a current that is activated by M3 muscarinic receptors (M3R) in a pancreatic β -cell line. This current is primarily permeant to sodium ions, independent of intracellular calcium stores and G proteins but dependent on Src activation, and resistant to TTX. The current is recapitulated by co-expression of NALCN and M3R in human embryonic kidney-293 cells and in *Xenopus* oocytes. We also show that NALCN and M3R belong to the same protein complex, involving the intracellular I–II loop of NALCN and the intracellular i3 loop of M3R. Taken together, our data show the molecular basis of a muscarinic-activated inward sodium current that is independent of G-protein activation, and provide new insights into the properties of NALCN channels.

Keywords: ion channel; G proteins; M3 muscarinic receptor; NALCN; Src

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INTRODUCTION

Ion channels are crucial components for cell excitability. They are involved in numerous physiological processes, as well as in pathologies referred to as channelopathies, and are drug targets of interest (Ashcroft, 2006; Kaczorowski *et al*, 2008). In 1999, Lee *et al* (1999) reported the cloning of a new putative ion channel mainly expressed in the brain and, to a lesser extent, in the heart and pancreas (supplementary Fig S1A,B online). The corresponding protein, NALCN (sodium leak channel, non-selective), is highly homologous to the voltage-gated sodium channel- α and calcium channel- α_1 pore-forming subunits (Snutch & Monteil, 2007). Recently, Lu *et al* (2007) provided evidence that NALCN is responsible for a tetrodotoxin (TTX)-resistant sodium leak current implicated in neuronal excitability by generating knockout mice and overexpressing the corresponding complementary DNA (cDNA) in human embryonic kidney (HEK)-293 cells. In addition, the same group also reported a modulation of NALCN by substance P and neurotensin through a G-protein-independent and Src family of tyrosine kinases (SFKs)-dependent pathway (Lu *et al*, 2009).

We independently set out to characterize NALCN channel activity. In this study, we used both overexpression and knock-down approaches in the MIN6 pancreatic β -cell line, which endogenously expresses NALCN. After initial experiments, we did not detect any evidence of leak activity mediated by NALCN in this cellular model (supplementary Fig S2 online). We then investigated the possibility that NALCN could encode another type of current in these cells. Here, we report that in MIN6 cells, NALCN encodes an inward cationic channel activated by acetylcholine through the activation of M3 muscarinic receptors (M3R). Similar to the leak current observed by Lu *et al* (2007), the M3R-activated NALCN current observed in MIN6 cells is primarily mediated by sodium ions, is resistant to TTX and is sensitive to gadolinium ions. This current was recapitulated in the

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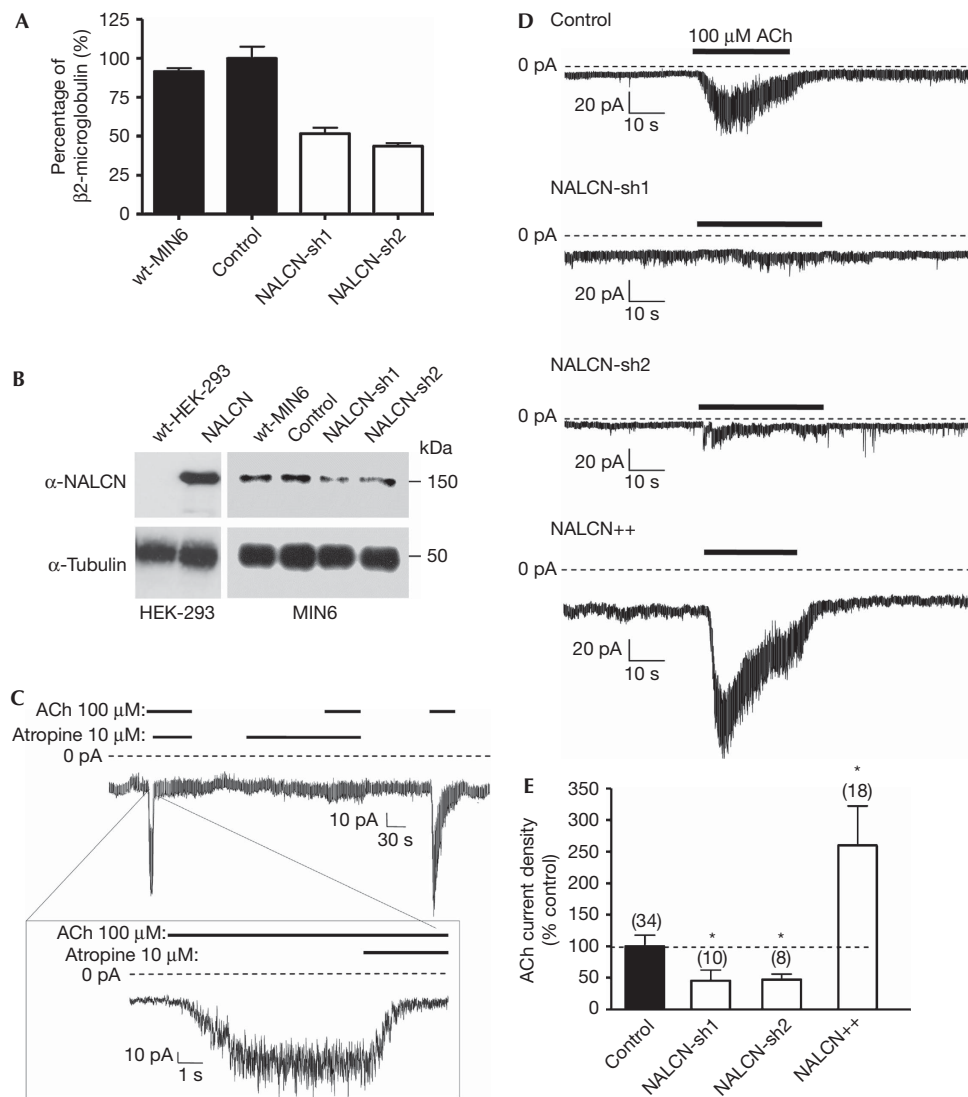


Fig 1 | Knockdown and overexpression of NALCN in MIN6 cells alter an atropine-sensitive acetylcholine-activated current. (A) Endogenous NALCN expression in MIN6 cells assessed by RT-qPCR (data are presented as a percentage of expression compared with the $\beta 2$ M level) and (B) western blot analysis in the absence and presence of shRNAs. (C) Representative sample of acetylcholine (ACh) activated current on a MIN6 pancreatic β -cell held at -80 mV in a standard whole-cell voltage-clamp configuration. (D) This current is substantially reduced with shRNA targeted against NALCN and increased with NALCN overexpression. (E) Summary of the effects of NALCN shRNA knockdown and overexpression on acetylcholine current density, expressed as a percentage of mean control-cell current. NALCN, sodium leak channel, non-selective; RT-qPCR, reverse transcription quantitative PCR; shRNA, short hairpin RNA.

HEK-293 cell line as well as in *Xenopus* oocytes, and involves the inclusion of both NALCN and M3R in a protein complex. Thus, this study provides new insights into the complexity of NALCN gating properties.

RESULTS

Knockdown and overexpression of NALCN in MIN6 cells

To facilitate the study of NALCN, we screened pancreatic β -cell lines for its expression using reverse transcription quantitative PCR (RT-qPCR) and western blot analysis because expression of the corresponding messenger RNA (mRNA) was detected in islets of Langerhans (supplementary Fig S1B online). We observed robust

expression in the MIN6 pancreatic β -cell line (Fig 1A,B). Owing to NALCN's apparent voltage independence and the lack of any leak activity in our experimental conditions, we decided to investigate 'orphan' currents—that is, a current of unknown molecular identity—activated by means other than changes in membrane potential in pancreatic β -cells and related cell lines. Primary pancreatic β -cells have been reported to have an inward sodium conductance of unknown molecular origin activated by muscarinic acetylcholine receptors (MRs), without G-protein involvement (Rolland *et al*, 2002). In keeping with these observations, application of acetylcholine (100 μ M) to MIN6 pancreatic β -cells held at -80 mV in a standard whole-cell voltage-clamp

configuration activated an atropine-sensitive inward current with a mean current density of -7.7 ± 0.9 pA/pF ($N=34$; Fig 1C). This current was found to show some desensitization during acetylcholine application (Fig 1C,D). We therefore used both NALCN knockdown and overexpression strategies to investigate the hypothesis that NALCN encodes the observed β -cell MR-activated sodium conductance. Using two different short hairpin RNA (shRNA) sequences, we were able to substantially reduce the expression of endogenous NALCN, as confirmed by RT-qPCR and western blotting ($\sim 50\%$ at the mRNA level, with a transduction rate of $\sim 80\%$; Fig 1A,B). Acetylcholine-activated current density in MIN6 cells was significantly reduced by NALCN-shRNA knockdown (NALCN-sh1: $44.8 \pm 17.1\%$ of control, $N=10$, $P=0.03$; NALCN-sh2: $43.1 \pm 9.3\%$ of control, $N=8$, $P=0.02$; Fig 1D,E), and was increased by NALCN overexpression ($260.2 \pm 62.1\%$ of control, $N=18$, $P=0.02$; Fig 1D,E). Taken together, these results suggest that application of acetylcholine induces an inward cationic current through activation of NALCN.

Permeation and gating properties in MIN6 cells

We then examined some of the ion permeation and gating properties of the acetylcholine-activated current in MIN6 cells. Substitution of sodium ions by *N*-methyl-D-glucamine or lithium in the extracellular solution had a robust inhibitory effect, suggesting that sodium is the main ion species contributing to the NALCN inward conductance ($21.4 \pm 9.4\%$ of control, $N=5$, $P=0.001$ and $53.2 \pm 9.7\%$ of control, $N=5$, $P=0.008$, respectively; Fig 2A). The current was also insensitive to TTX ($2 \mu\text{M}$; $103.3 \pm 9.8\%$ of control, $N=7$; Fig 2A), but was partly blocked by gadolinium ($10 \mu\text{M}$; $51.4 \pm 6.1\%$ of control, $N=9$, $P<0.0001$; Fig 2A). Addition of heparin (1 mg/ml) to the patch pipette did not affect the current density, indicating that the current was independent of intracellular calcium stores mobilized through activation of IP3 receptors ($97.1 \pm 22.2\%$ of control, $N=12$, $P=0.89$; Fig 2A). Replacement of GTP with either GDP- β -S (1 mM) or GTP- γ -S (1 mM) in the pipette solution did not significantly change the current density ($72.3 \pm 18.7\%$ of control, $N=9$, $P=0.178$, and $86 \pm 23.8\%$ of control, $N=7$, $P=0.579$, respectively; Fig 2A). Even the use of a pipette solution with no GTP did not have any effect on the current (data not shown). It has been shown that some G-protein-coupled receptors (GPCRs) can signal directly through SFKs (McGarrigle & Huang, 2007). Thus, we also examined the possibility that NALCN could be activated through an SFK-dependent pathway by using the SFK inhibitor 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1) (Fig 2A,B). Bath application of PP1 ($20 \mu\text{M}$) almost fully and reversibly abolished the acetylcholine-activated current ($6.1 \pm 2.5\%$ of control, $N=5$, $P<0.0001$; Fig 2A,B). A strong inhibition was also observed with the SFK inhibitor SU6656 ($20.1 \pm 11.9\%$ of control, $N=5$, $P=0.001$; Fig 2A and supplementary Fig S3 online). The current-voltage (*I*-*V*) relationship of the acetylcholine-activated current was determined by using a voltage ramp protocol in the absence and presence of acetylcholine ($N=8$; Fig 2C). We found the relationship to be linear in the range of -110 to -20 mV, with a slope conductance of 76.8 ± 2.5 pS/pF, and a reversal potential E_{rev} of approximately -23 mV. As the activation of the current by acetylcholine was associated with an increase in membrane noise, we also carried out a noise analysis to estimate the single-channel amplitude and

found a value of 1.6 ± 0.1 pA at -80 mV with an estimated single-channel conductance of ~ 27 pS (supplementary Fig S4 online). To determine further which MRs were involved in the modulation of NALCN, we first carried out an analysis by using RT-qPCR in MIN6 cells (Fig 2D). Our results indicated that M3R and M4R are the muscarinic receptor subtypes present in MIN6 cells (Fig 2D). We therefore used specific antagonists of M3R ($7 \mu\text{M}$ 4-diphenylacetoxy-*N*-methylpiperidine methiodide) and M4R ($10 \mu\text{M}$ tropicamide) to determine the MR subtype responsible for current activation (Fig 2E,F). Our results suggest that M3R was responsible for current activation (4-diphenylacetoxy-*N*-methylpiperidine methiodide: $20.9 \pm 2.6\%$ of control, $N=5$, $P=0.0002$; tropicamide: $94.8 \pm 18.3\%$ of control, $N=9$; Fig 2F). These data suggest that M3R activation induces an inward, primarily sodium, conductance through NALCN channels that is independent of intracellular calcium stores and G proteins, dependent on SFK activation, resistant to TTX and sensitive to gadolinium inhibition.

Heterologous co-expression of NALCN and M3R

Next we sought to address whether NALCN and M3R are sufficient to reconstitute an acetylcholine-activated current in two commonly used heterologous expression systems. Expression of NALCN in HEK-293 cells stably expressing M3R, in which NALCN is not endogenously expressed (Fig 1B and supplementary Fig S6A online), resulted in a significant acetylcholine-activated current with a similar profile to but of a smaller density than that observed in MIN6 cells (NALCN + M3R -2.7 ± 0.3 pA/pF, $N=17$, $P=0.001$; M3R -1.2 ± 0.1 pA/pF, $N=16$; Fig 3A,B). This result shows that M3R and NALCN are two components necessary for recapitulating the current of interest, and suggests that additional proteins probably modulate the channel activity and/or the channel level at the plasma membrane in native systems. We next examined whether the current could be recapitulated even in *Xenopus* oocytes by expressing NALCN and an M3R mutant (M3R*) lacking a stretch of 10 amino acids (YKETEKRTKE) in the i3 loop involved in G-protein activation in this cellular model (Lechleiter et al, 1990). As expected, agonist stimulation of M3R* did not result in the activation of a strong endogenous Ca^{2+} -activated Cl^- current as did M3R (Fig 3C,D). When NALCN and M3R* were co-expressed, carbachol ($100 \mu\text{M}$) application resulted in the activation of an inward current (M3R* + NALCN -42.3 ± 9.9 nA, $N=8$, $P=0.008$; Fig 3C,D), as this current was abolished by the substitution of sodium ions using *N*-methyl-D-glucamine (supplementary Fig S5 online). Interestingly, contrary to our data for MIN6 cells, the NALCN-generated current did not show any desensitization in HEK-293 cells or *Xenopus* oocytes. In our study, the desensitization process was not found to be agonist dependent (data not shown), suggesting that it is likely to be cell-type dependent. We did not observe any significant effect resulting from the expression of NALCN on the leak activity in HEK-293 cells (NALCN + M3R -2.1 ± 0.7 pA/pF, $N=17$; M3R -2.5 ± 0.7 pA/pF, $N=16$; Fig 3E) or in *Xenopus* oocytes (control: -97.7 ± 18.3 nA, $N=11$; NALCN: -72.3 ± 15.6 nA, $N=3$; M3R: -178.6 ± 55.9 nA, $N=5$; M3R*: -264.9 ± 72 nA, $N=17$; and M3R* + NALCN: -287.5 ± 60.6 nA, $N=8$; Fig 3F). Thus, we conclude that, under our experimental conditions, NALCN did not encode for a leak channel but for an inward cationic channel activated by acetylcholine through M3R.

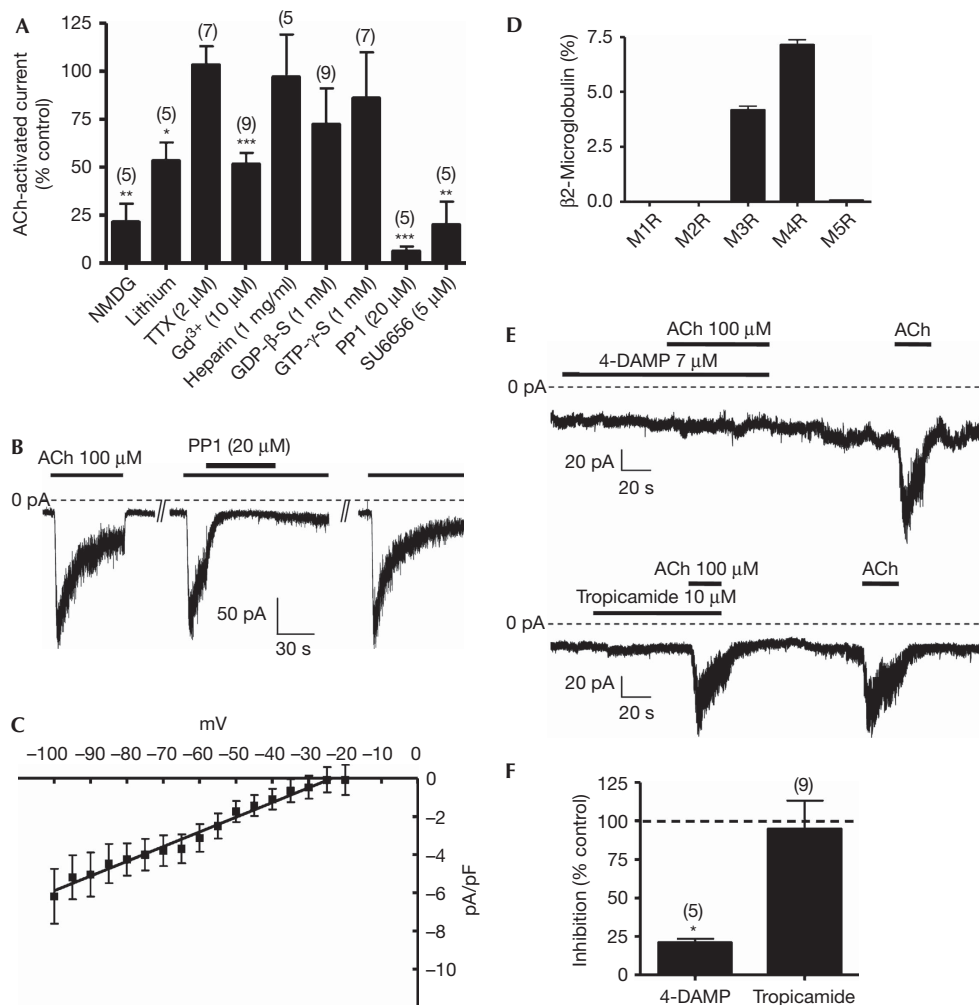


Fig 2 | Permeation and gating properties of the acetylcholine-activated current in MIN6 cells. (A) Replacement of sodium ions by NMDG or lithium in the extracellular solution greatly diminishes the acetylcholine (ACh)-induced inward current. The current is also resistant to 2 μ M TTX and was partly blocked by 10 μ M Gd³⁺. Inclusion of heparin (1 mg/ml), GTP- γ -S (1 mM) or GDP- β -S (1 mM) in the pipette solution does not significantly affect the current, whereas the SFK inhibitors PP1 (20 μ M) and SU6656 (5 μ M) have a strong inhibitory effect. (B) Representative traces showing the effects of an SFK inhibitor (PP1, 20 μ M) on the acetylcholine-activated inward current in MIN6 cells. (C) A voltage-ramp protocol (–100 to 100 mV over 0.2 s) was used to determine the current–voltage relationship of the acetylcholine-activated current. Values obtained in the absence of acetylcholine were subtracted from values obtained in the presence of acetylcholine to determine the *I*–*V* curve attributable to acetylcholine. Currents obtained at voltages greater than –20 mV were too variable for meaningful analysis and were therefore excluded. (D) RT–qPCR analysis shows that only mRNA from M3R and M4R can be detected in MIN6 cells. (E) Representative traces showing the effects of an M3R-specific antagonist (4-DAMP, 7 μ M) and an M4R-specific antagonist (tropicamide, 10 μ M) on the acetylcholine-activated inward current in MIN6 cells. (F) Summary of the effects of 4-DAMP and tropicamide on the acetylcholine-activated current, expressed as a percentage of control current. 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; Gd³⁺, gadolinium; MR, muscarinic receptor; NMDG, *N*-methyl-*D*-glucamine; mRNA, messenger RNA; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; RT–qPCR, reverse transcription quantitative PCR; SFK, Src family of tyrosine kinase; TTX, tetrodotoxin.

NALCN and M3R belong to the same protein complex

Considering that NALCN was activated by M3R but not by M4R, whereas it has been suggested that both muscarinic receptors can activate SFKs (Rosenblum *et al*, 2000), we next investigated the hypothesis that NALCN could associate with M3R in the same complex, thus involving a spatially localized signalling event. Owing to the lack of available antibodies to perform immunoprecipitation experiments from native systems, we carried out

co-immunoprecipitation experiments with haemagglutinin-tagged NALCN and Flag-tagged M3R that were heterologously expressed in HEK-293 cells (Fig 4A). Our results indicated that NALCN and M3R associate with one another, whereas this is not the case with M4R and with the β 2-adrenergic receptor used as controls (Fig 4A). It remains to be determined whether this is through a direct interaction or through co-inclusion in a larger protein complex. Intracellular portions of ion channels and GPCRs are

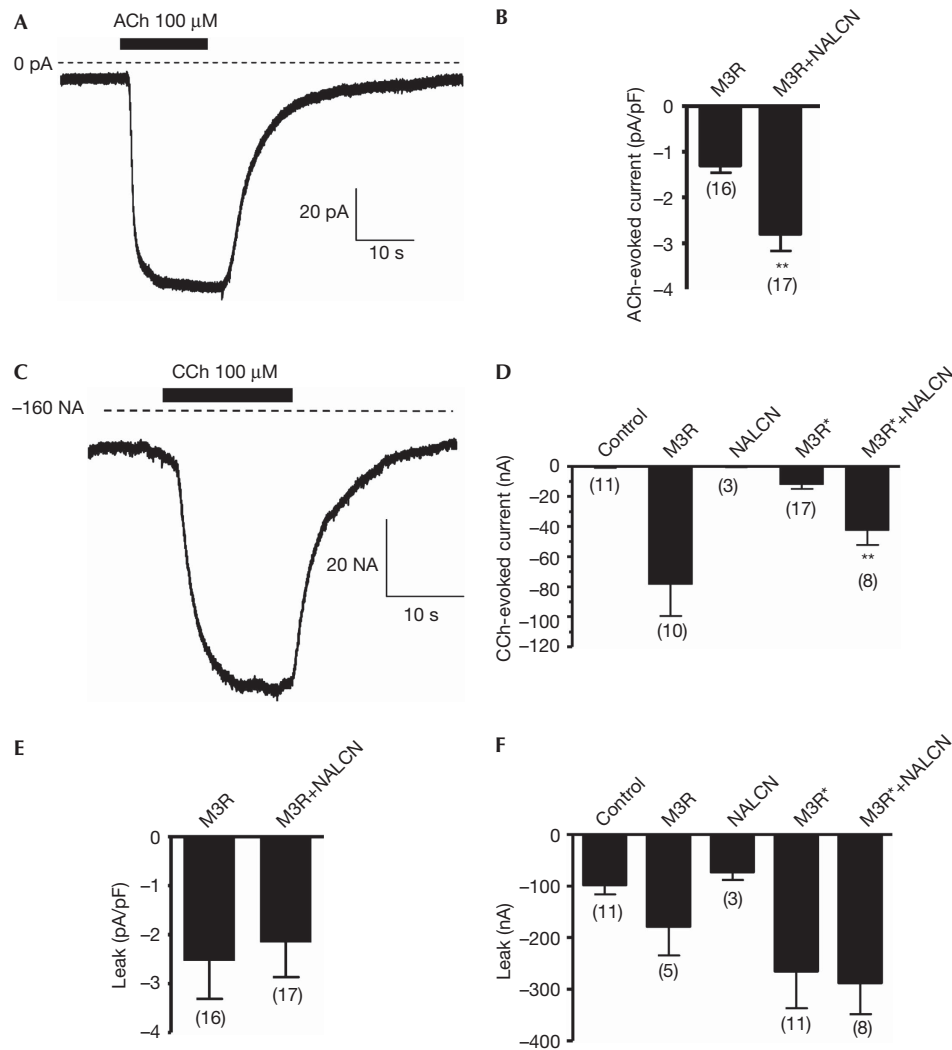


Fig 3 | Co-expression of NALCN and M3R in the HEK-293 cell line and in *Xenopus* oocytes results in the expression of an M3R agonist-activated current. (A) Co-expression of NALCN and M3R cDNAs in HEK-293 cells results in the expression of an acetylcholine (ACh)-activated inward current with the same profile as in the MIN6 cells. (B) Mean acetylcholine-activated current density in HEK-293 cells expressing NALCN and M3R. (C) Co-expression of NALCN and an M3R deletion mutant (M3R*) that is deficient in the activation of G proteins in *Xenopus* oocytes results in the expression of a carbachol (CCh)-activated inward cationic current. (D) Mean carbachol-activated current density in *Xenopus* oocytes, mock-injected or expressing M3R, NALCN, M3R* or NALCN + M3R*. (E) Leak activity in HEK-293 cells and (F) in *Xenopus* oocytes is not significantly altered by NALCN overexpression. cDNA, complementary DNA; HEK, human embryonic kidney; M3R, M3 muscarinic receptor; NALCN, sodium leak channel, non-selective.

known to be involved in protein–protein interactions in some protein complexes (Bockaert et al, 2004; Levitan, 2006). Thus, we next examined whether intracellular fragments of NALCN and M3R were involved in their interaction by using GFP fusion proteins (Fig 4B,C). We found that M3R co-immunoprecipitates with the I–II loop of NALCN and that NALCN co-immunoprecipitates with the i3 loop and the carboxy-terminal portion of M3R (Fig 4B,C). Overexpression of these intracellular fragments in MIN6 cells showed that the I–II loop of NALCN and the i3 loop of M3R significantly inhibited the native acetylcholine-activated inward current, probably through uncoupling of NALCN and M3R (loop I–II, $7.8 \pm 3.2\%$ of control, $N=13$, $P<0.01$; loop i3,

$17.3 \pm 4.6\%$ of control, $N=9$, $P<0.01$; Fig 4D,E). These data suggest that NALCN and M3R coexist in the same protein complex and that this requires molecular determinants present in the I–II loop of NALCN and the i3 loop of M3R.

DISCUSSION

Our data show that NALCN channels can be activated by M3R receptors without the involvement of G proteins but through an SFK-dependent pathway, and involves the inclusion of the two molecules in the same protein complex. We have shown that the resulting current is endogenous to the MIN6 pancreatic β -cell line,

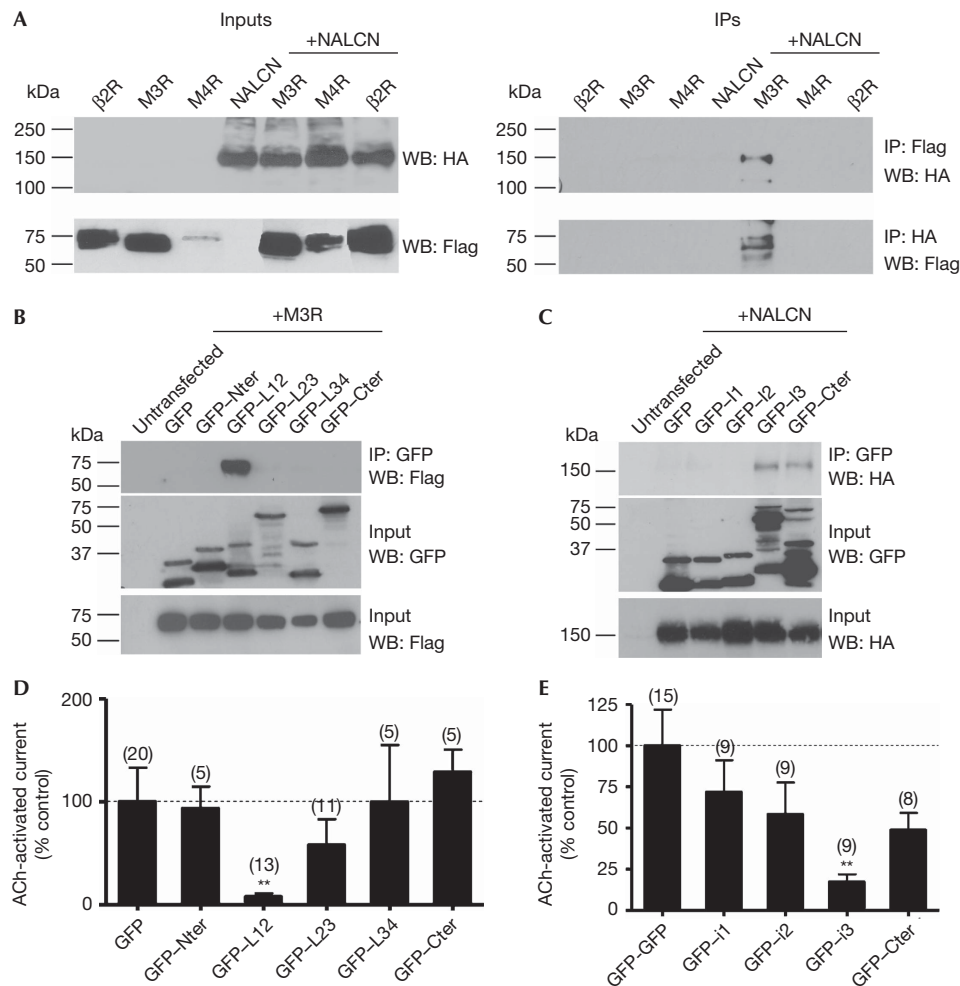


Fig 4 | NALCN and M3R belong to the same protein complex in HEK-293 cells. (A) NALCN and M3R co-immunoprecipitate (IP) when co-expressed in the HEK-293 cell line. (B) M3R co-immunoprecipitates with the NALCN's GFP-tagged intracellular I–II loop. (C) NALCN co-immunoprecipitates with the M3R's GFP-tagged intracellular segment i3 and the carboxy-terminus (Cter) part. (D) Overexpression of the NALCN's GFP-tagged intracellular loop I–II and (E) M3R's GFP-tagged intracellular segment i3 in the MIN6 cell line results in a strong inhibition of the acetylcholine (ACh)-activated inward current. GFP, green fluorescent protein; HA, haemagglutinin; HEK, human embryonic kidney; M3R, M3 muscarinic receptors; NALCN, sodium leak channel, non-selective; WB, western blot.

and can be reconstituted in recombinant systems. The M3R-mediated activation of the NALCN current possibly occurs through a direct interaction between these proteins or through interactions resulting from their co-assembly into a larger protein complex. Activation of inward cationic currents similar to those observed in this study and that do not require G-protein activation has been previously described in native pancreatic β-cells (Rolland *et al*, 2002), CA3 pyramidal neurons (Guerineau *et al*, 1995) and cardiac myocytes (Shirayama *et al*, 1993). Further studies are needed to determine the physiological roles of NALCN in these cell types.

Our results show that M3R and NALCN are necessary for recapitulating the native current in recombinant systems, but also suggest that further proteins probably modulate channel activity and/or the level of channel expression at the plasma membrane in native systems. With respect to this idea, it is known that most ion channels exist as heteromultimeric protein complexes in which binding partners modulate the transport and the biophysical

properties of the pore-forming subunit (Levitan, 2006). Further work is needed to determine the proteins that might be included in any complex together with NALCN and M3R. One clue about such proteins is provided by genetic evidence suggesting a functional interaction between NALCN orthologues in invertebrates and two proteins named UNC-79 and UNC-80 (Humphrey *et al*, 2007; Jospin *et al*, 2007; Yeh *et al*, 2008). The corresponding mRNAs are not expressed in HEK-293 cells but are detected in MIN6 cells and in the islets of Langerhans (supplementary Fig S5A,B online). In addition, while this paper was under consideration, a report by Lu *et al* showed that UNC-80 is required to observe a strong modulation of NALCN activity by substance P through neurokinin 1 receptors in HEK-293 cells (Lu *et al*, 2009). Whether UNC-80 and/or UNC-79 is required in the NALCN–M3R complex remains to be established.

It should be noted that under our experimental conditions, we have been unable to detect any NALCN-induced leak activity in

MIN6 cells, as well as in recombinant expression systems such as HEK-293 cells and *Xenopus* oocytes. Instead, we observed that NALCN is activated by M3R in our cellular models. It is known that GPCRs can show constitutive activity that is modulated by factors such as binding partners (Ango *et al*, 2001; Milligan, 2003). Thus, one hypothesis to explain the leak activity described by Lu *et al* (2007) in neurons is that it results from constitutive activation of NALCN by one or more GPCRs. Lu *et al* (2007) were also able to record a leak current after expression of NALCN in HEK-293 cells. We, and others, have been unable to observe any leak activity in this cell type (M. Biel, personal communication). However, this cell line is known to show a variation in the repertoire of expressed genes depending on the culture procedure, passage number and sub-clone types (Thomas & Smart, 2005), thus providing a possible explanation for this discrepancy.

In conclusion, our study indicates that NALCN is activated by M3Rs in the MIN6 cell line and might behave as a leak channel depending on the cellular environment. Our study provides new insights into the properties of NALCN channels.

METHODS

Reagents. Human complementary DNAs (cDNAs) encoding wild-type M3R, Flag-tagged M3R and Flag-tagged β 2-adrenergic receptor, as well as the pLVTHM vector were provided by Drs E. Lutz, M. Hosey and D. Trono, respectively. All drugs were purchased from Sigma (Saint-Quentin, Fallavier, France), with the exception of PPI and SU6656 (Calbiochem, Nottingham, UK).

Cloning of human NALCN and antibody generation. Human NALCN cDNA was amplified from human brain RNA using RT-PCR (Clontech, Palo Alto, CA, USA) and cloned into pcDNA5FRT (Invitrogen, Cergy Pontoise, France). The NALCN rabbit polyclonal antibody was generated with a glutathione-S-transferase fusion protein of the last 288 amino acids of NALCN and affinity-purified.

Other constructs. Haemagglutinin-tagged human NALCN, Flag-tagged M4R (amino terminus) and M3R* were generated by PCR and cloned into pcDNA5FRT (Invitrogen).

Lentivirus-mediated RNA interference. The shRNAs used in this study target sequences encoding for the III–IV linker (no alternative splicing events detected; shRNA-1: AAGAU CGCAGCCUCUUC AU; shRNA-2: AAUGUAUGACAUAACCCAGC; and control: GCUCAGUACGAUCAUACUCAC). Corresponding pairs of oligonucleotides were annealed and cloned in the pLVTHM lentiviral vector. Cells were transduced at a multiplicity of infection of 10.

Quantitative RT-PCR. DNase-treated (Ambion, Austin, TX, USA) RNAs were reverse-transcribed using random hexameric oligonucleotides and MoMuLV-RT (Invitrogen). Real-time PCR was carried out in duplicate (7500 System; Applied Biosystems, Courtaboeuf, France), according to the manufacturer's instructions. Target RNA expression level was normalized to β 2-microglobulin, according to the $2^{-\Delta C_t}$ method ($2^{-(C_t(X)-C_t(B2M))}$), where C_t is the threshold cycle for the target RNA ($C_t(X)$) and β 2-microglobulin ($C_t(B2M)$).

Electrophysiology. Patch-clamp measurements were carried out in whole-cell voltage-clamp configuration (Axopatch 200B patch clamp amplifier, Clampex9.2 software (Axon Instruments, Molecular Devices, Wokingham, UK)). Pipettes were pulled from borosilicate glass to give 4–6 M Ω resistance. Acetylcholine-activated currents were measured in cells held at –80 mV at

room temperature (20–25 °C). The extracellular solution consisted of NaCl 119 mM, KCl 4 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, CaCl₂ 2.5 mM, Na₂CO₃ 0.42 g/l, HEPES 20 mM and glucose 10 mM (pH 7.2 with NaOH; osmolarity approximately 290 mOsm). The pipette solution consisted of K-aspartate 100 mM, KCl 25 mM, NaCl 10 mM, Mg-ATP 2 mM, Na-GTP 0.1 mM, creatine phosphate 5 mM, HEPES 5 mM, CaCl₂ 0.04 mM and MgCl₂ 1 mM (pH 7.2 with KOH; osmolarity ~290 mOsm). Alterations to solutions are noted in the text and figure legends wherever appropriate. Current amplitude was defined as peak-current value during acetylcholine application compared with baseline. Nuclear injection and recording of macroscopic currents in *Xenopus* oocytes were carried out as reported previously (Altier *et al*, 2001).

Data analysis. Data are presented as mean \pm s.e.m. Statistical significance was evaluated using Student's *t*-test for paired or unpaired data (significance level 0.05). Multiple comparisons were carried out by analysis of variance followed by Tukey's multiple comparison test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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