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RESEARCH PAPER

Myosin light chain kinase-independent inhibition by ML-9 of murine TRPC6 channels expressed in HEK293 cells

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Background and purpose: Myosin light chain kinase (MLCK) plays a pivotal role in regulation of cellular functions, the evidence often relying on the effects of extracelluarly administered drugs such as ML-9. Here we report that this compound exerts non-specific inhibitory actions on the TRPC6 channel, a transient receptor potential (TRP) protein.
Experimental approach: Macroscopic and single channel currents were recorded from transfected HEK293 cells by patch-clamp techniques.
Key results: Cationic currents elicited by carbachol (CCh; 100 μM) in HEK293 cells overexpressing murine TRPC6 (I_{TRPC6}) were dose-dependently inhibited by externally applied ML-9 ($IC_{50} = 7.8\,\mu$M). This inhibition was voltage-dependent and occurred as fast as external Na^+ removal. Another MLCK inhibitor, wortmannin (3 μM), and MLCK inhibitory peptides MLCK-$IP_{11\text{-}19}$ (10 μM) and -$IP_{480\text{-}501}$ (1 μM) showed little effects on I_{TRPC6} density and the inhibitory efficacy of ML-9. The extent of the inhibition also unchanged with co-expression of wild-type or a dominant negative mutant of MLCK. Inhibitory effects of ML-9 on I_{TRPC6} remained unaffected whether TRPC6 was activated constitutively or by a diacylglycerol analogue OAG (100 μM). Similar rapid inhibition was also observed with a ML-9 relative, ML-7. Intracellular perfusion of ML-9 via patch pipette, dose-dependently suppressed I_{TRPC6}. In inside-out patch configuration, bath application of ML-9 (and ML-7) rapidly diminished $\sim$35pS single TRPC6 channel activities. Contrarily, currents due to TRPC7 expression were rapidly enhanced by externally applied ML-9 and ML-7, which was not prevented by MLCK inhibitory peptides.
Conclusion and implications: These results strongly suggest that ML compounds inhibit TRPC6 channels via a mechanism independent of inhibition of MLCK activity.
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Keywords: transient receptor potential; receptor-operated Ca^{2+}-permeable cation channel; TRPC6; TRPC7; myosin light chain kinase; naphthalene sulphonamide derivative; ML-9; dominant-negative MLCK

Abbreviations: CaM, calmodulin; CaMKII, calmodulin-dependent kinase II; ML-9, [1-(5-chloronaphthalene-1-sulphonyl) homopiperazine, HCl]; ML-7, [1-(5-iodonaphthalene-1-sulphonyl)homopiperazine, HCl]; MLCK, myosin light chain kinase; MLCK-IP, myosin light chain kinase inhibitory peptide; mut-MLCK, mutant myosin light chain kinase; NMDG, *N*-methyl D-glucamine; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; TRP, transient receptor potential protein; TRPC6, TRP canonical subfamily isoform 6; TRPC7, TRP canonical subfamily isoform 7; wt-MLCK, wild-type myosin light chain kinase

Introduction

The transient receptor potential (TRP) superfamily is a newly emerging channel gene family consisting of approximately 30 mammalian homologues, which encode, except for two isoforms, a Ca^{2+}-permeable non-selective cation channel (NSCC). Among this superfamily, members of the TRPC family (TRPC1–7) show the highest sequence similarity to the archetypal *Drosophila* dTRP or dTRPL, and because of their activation via phospholipase Cβ or -γ (PLC_β or PLC_γ)-linked receptors, are proposed to be the most promising candidates for store-operated and receptor-operated (and store-independent) Ca^{2+} channels (hereafter

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abbreviated as SOCCs and ROCCs, respectively) identified in various types of native cells (for review see Minke and Cook, 2002; Clapham, 2003; Montell, 2005).

TRPC6 belongs to the TRPC3/6/7 subfamily of the TRPC family. Heterologously expressed TRPC6 channels are activated by PLC-linked receptors via generation of a lipid mediator diacylglycerol, although a different mode of activation by activated inositol 1,4,5-triphosphate receptor or modulatory role of phosphatidylinositol (PI) and 4,5-bisphosphate (PIP_2)/PI 3,4,5-trisphosphate (PIP_3) have been suggested (Hardie, 2007; Kwon *et al*., 2007). The mRNA or protein of TRPC6 is ubiquitously expressed in vascular smooth muscle tissues (Beech *et al*., 2004; Dietrich *et al*., 2005; Inoue *et al*., 2006). Recent studies have suggested that this protein is an essential component of vascular α_1-adrenoceptor NSCC activated during increased sympathetic activity (Inoue *et al*., 2001), and may also be activated by vasopressor hormones such as vasopressin, endothelin and angiotensin II (Jung *et al*., 2002; Soboloff *et al*., 2005; Maruyama *et al*., 2006). In cerebral resistance arteries, the generation of myogenic tone has been ascribed to TRPC6 (Welsh *et al*., 2002) as a mechanosensitive NSCC (Spassova *et al*., 2006), and in pulmonary artery, TRPC6 has been implicated as a SOCC in platelet-derived growth factor-stimulated or pathologically enhanced smooth muscle cell proliferation in idiopathic pulmonary arterial hypertension (Yu *et al*., 2003, 2004). Thus, TRPC6 is likely to play a pivotal role in both acute and long-term controls of vascular smooth muscle functions (Inoue *et al*., 2006).

It has been shown that heterologously expressed TRPC6 channels are subject to complex regulation by Ca^{2+} from both extracellular and intracellular sides and, most notably, its receptor-mediated activation depends critically on phosphorylation by calmodulin (CaM)-dependent kinase II, although sole activation of this enzyme by elevated Ca^{2+} concentration does not suffice to activate the channel (Shi *et al*., 2004). An analogous dependence on Ca^{2+}/CaM-dependent kinase has been reported for the native vascular counterpart of TRPC6, α_1-adrenoceptor NSCC, whose activation appears to involve Ca^{2+}/CaM-mediated activation of myosin light chain kinase (MLCK) as an indispensable step (Aromolaran *et al*., 2000). A similar crucial role of MLCK in the regulatory process has also been reported for another TRPC member, TRPC5, based on siRNA silencing of endogenous MLCK (Kim *et al*., 2006) or co-expression of a dominant-negative mutant of MLCK (Shimizu *et al*., 2006). It is well accepted that MLCK has central importance in the generation of muscle contraction as well as regulation of other cellular activities tightly associated with cytoskeletal organization in non-muscle cells via phosphorylation of myosin light chain (MLC), including cytokinesis, integrin clustering at focal adhesions, stress fibre formation and regulation/trafficking of ion channels, transporters and exchangers (Kamm and Stull, 2001; Pfitzer, 2001).

Notably, such biological significance of MLCK has been demonstrated based on the effectiveness of agents that are thought to specifically inhibit the MLCK activity. A naphthalene sulphonamide derivative, [1-(5-chloronaphthalene-1-sulphonyl)homopiperazine, HCl] (ML-9), is a membrane permeable, organic MLCK inhibitor frequently used in various functional studies. Especially, in experiments where intracellular administration of MLCK-specific peptides is not feasible, the effectiveness of this compound has often been regarded as an important indication of the involvement of MLCK activity (Saitoh *et al*., 1987). ML-9 and its congener [1-(5-iodonaphthalene-1-sulphonyl)homopiperazine, HCl] (ML-7) have been reported to inhibit ROCC and SOCC activities or Ca^{2+} influx evoked by receptor stimulation (Kim *et al*., 1997; Wingard and Murphy, 1999; Aromolaran *et al*., 2000; Kuroiwa-Matsumoto *et al*., 2000), and several other membrane ion-transporting proteins including a TRPC member TRPC5 (Kim *et al*, 2006; Shimizu *et al*., 2006), voltage-dependent K^+ channels, an Na^+, K^+, Cl^- co-transporter and Na^+/H^+ exchanger (Wu *et al*., 1998; Szaszi *et al*., 2000; Akar *et al*., 2001). In addition, in our preliminary experiments, we have found that other TRPC channels, such as TRPC6 and -7, are also subject to modulation by this compound. In considering such a broad target spectrum, a question inevitably arises as to whether the observed inhibitory effects of ML-9 genuinely reflect the specific inhibition of MLCK activity or may involve some non-specific actions. If the latter is true, the interpretation of studies based solely on the effects of ML-9 and its relative compounds could be misleading and thus may require re-evaluation. To clarify this point, in this study, we examined in detail the effects of ML-9 on heterologously expressed TRPC6 channels, in comparison with its closest relative TRPC7 (Okada *et al*., 1999). We have found that ML-9 (and ML-7 as well) exerted rapid inhibition of the TRPC6 channel and enhancement of the TRPC7 channel, neither of which seemed to involve the inhibition of MLCK activity.

Methods

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and maintained in 60 mm culture dishes. For transfection, HEK293 cells were re-seeded in a 35 mm culture dish and were allowed to grow to about 50% confluency. Cells were then transfected with a mixture of 2 μg plasmid vector (pCI-neo) incorporated with the whole cDNA sequence of murine TRPC6 (Inoue *et al*., 2001; Shi *et al*., 2004) or TRPC7 (Okada *et al*., 1999) and 0.4 μg pCI-neo-πH3-CD8 (cDNA of the T-cell antigen CD8) with the aid of 20 μl of a transfection reagent SuperFect (Qiagen, Germany). Polystyrene bead-conjugated anti-CD8 antibody (Dynabeads M-450 CD8, Dynal Biotech, Oslo, Norway) was used to select cells successfully expressing TRPC proteins, as performed previously (Shi *et al*., 2004). With this method, more than 90% of selected cells showed large inward currents in response to muscarinic receptor stimulation. After incubating for about 24 h, the cells were trypsinized and re-seeded onto coverslips pre-coated with 100 μg ml^{-1} poly-L-lysine. Electrophysiological measurements were performed within 24–48 h thereafter.

In some experiments where wild-type MLCK (wt-MLCK) cloned from HEK293 cells or its dominant-negative mutant

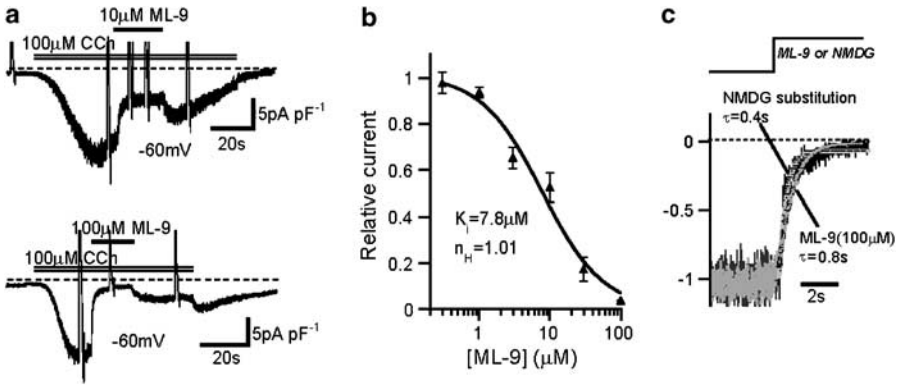

Figure 1 Inhibitory effects of ML-9 on TRPC6 current (I_{TRPC6}). Nystatin-perforated recording in normal external solution with a holding potential: −60 mV. (**a**) Typical traces for the inhibitory effects of ML-9 of 10 μM (upper) and 100 μM (lower) on CCh (100 μM)-evoked I_{TRPC6}. (**b**) Relationship between ML-9 concentration and relative I_{TRPC6} amplitude after inhibition. Relative amplitude is calculated as the ratio of current amplitudes 5 s after, to just before, application of ML-9. Data points are fitted by the Hill equation with non-linear least-square routine ($IC_{50} = 7.8\ \mu$M, $n_H = 1.0$, $n = 4$–6; see the Methods). (**c**) Expanded time courses of the effects of Na^+ removal (NMDG substitution) and 100 μM ML-9 on I_{TRPC6}. Representative of separate six experiments. Each current trace is normalized to its amplitude just before drug application and then superimposed. The time courses of the currents are fitted by an exponential function: $I = I_0 \exp(-(t-t_0)/\tau) + C$ (smooth curves), where I_0, t_0, τ and C denote the amplitude of drug-inhibited component, the onset of drug application, time constant of drug's effect and the steady level reached after drug application, respectively, and $I_0 + C$ is set to be −1. τ Values (in seconds) in the figure indicate the results of best fit by non-linear least-square routine. CCh, carbachol; ML-9, [1-(5-chloronaphthalene-1-sulphonyl)homopiperazine, HCl]; NMDG, *N*-methyl D-glucamine.

lacking Lys^{561} (mut-MLCK) (Shimizu *et al*., 2006) was co-expressed with TRPC6 (Figure 6), we used a fivefold higher amount of plasmid DNA for MLCK (10 μg) than TRPC6 (2 μg) to carry out the transfection. Although it was not possible to exactly evaluate the rate of successful co-expression of both gene products in the same cell, we anticipated that this would be high enough, based on the results of CD8 co-expression (>90%; see above).

Electrophysiology

Two modes of the patch-clamp technique were employed in the present study, that is nystatin-perforated and conventional whole-cell recordings. For both recordings, microglass pipettes with a resistance of 2.5–4 MΩ (when filled with internal solution) were used. Voltage generation and current signal acquisition were implemented with a high-impedance, low-noise, patch-clamp amplifier (EPC9; HEKA Electronics, Germany). Sampled data were low-passed filtered at 1 kHz and digitized at 5 kHz (for example, Figure 2a, current–voltage relationship: *I–V* curve). Longer time-frame recordings (for example, whole-cell current traces in Figure 1) were performed in conjunction with an A/D, D/A converter, PowerLab/400 (AD Instruments, Australia; sampling rate: 100 Hz), and data evaluation was made by using the accessory software, Chart v3.6. The extent of inhibition or enhancement of TRPC6 and -7 currents were calculated as the ratio of their amplitudes after, to just before, application of the ML compounds. Because of progressive desensitization, which was most evident with carbachol (CCh) as an agonist, it was difficult to specify the time point at which the effects of drugs reached the maximum. We therefore empirically chose the time point of 5 s after the onset of drug application. Although this would introduce some errors, with the same method, we could get similar results with 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) to activate these currents, which showed much slower desensitization. Concentration–response relationships were constructed by fitting data points with a non-linear least-square routine using the Hill equation:

$$I = 1.0/[1 + (C/IC_{50})^{n_H}]$$

where I, C, IC_{50} and n_H denote the normalized current amplitude (the ratio of current amplitudes after, to before, drug application), drug concentration, the half-maximal inhibitory concentration and a cooperative factor, respectively.

For single-channel recordings, electrodes having a resistance of 4–5 MΩ (with the pipette solution described below) were used for the inside-out (I/O) configuration. Sampled data were low-pass filtered at 1 kHz and stored on a computer hard disc after digitization at 10 kHz. Single channel analysis was made using the software 'Clampfit' v.9.2 (Axon Instruments, Foster City, CA, USA). To evaluate the unitary current amplitude, all-points-in-open-state amplitude histograms were constructed. The extent of single-channel current inhibition by ML compounds was calculated as the ratio of mean currents (averaged for 2 s) 5 s after, to just before, application of the drugs. Determination of the levels of baseline and channel openings and construction of all-points-in-open-state amplitude histograms were performed by using the 'event detection' analysis of Clampfit v.9.2.

Drugs were rapidly applied onto cells or excised patch membrane by using a fast solution change device called 'Y-tube' as performed previously (Inoue *et al*., 2001; Shi *et al*., 2004).

All experiments were performed at room temperature (22–26°C).

Solutions

Solutions with the following compositions were used. Pipette solution for nystatin-perforated recording (in mM):

140 CsCl, 2 $MgCl_2$, 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10 glucose (adjusted to pH 7.2 with Tris base); pipette solution for conventional whole-cell recording (in mM): 120 CsOH, 120 aspartate, 20 CsCl, 2 $MgCl_2$, 1 EGTA (ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid), 0.4 $CaCl_2$, 10 HEPES, 2 ATP, 0.1 GTP, 10 glucose (adjusted to pH 7.2 with Tris base). Various concentrations of ML-9 or MLCK inhibitory peptides (see below) were included in the latter internal solution where necessary. The normal external solution contained (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 1 EGTA, 10 HEPES, 10 glucose (pH 7.4, adjusted with Tris base). For Ca^{2+}-free external solution, Ca^{2+} was omitted from normal external solution. Pipette solution for I/O recording contained (in mM): 140 NaCl, 5 tetraethylammonium-Cl, 0.1 DIDS [4,4-diisothiocyanostilbene-2,2-disulphonic acid, 2Na], 1.2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 glucose, 0.1 CCh (pH 7.4, adjusted with Tris base). Bathing solution for I/O recording (in mM): 120 CsOH, 120 aspartate, 20 CsCl, 2 $MgSO_4$, 2 EGTA, 0.4 Ca, 10 HEPES, 2 ATP, 0.1 GTP (pH 7.2, adjusted with Tris base).

Statistics

All data are expressed as means ± s.e.m. Paired and unpaired Student's *t*-test was used to evaluate statistical significance with a criterion of $P<0.05$. For multiple comparisons, statistical significance was evaluated by analysis of variance followed by Bonferroni's *t*-test.

Chemicals

ML-9, ML-7, wortmannin, DIDS, OAG and MLCK inhibitory peptide MLCK-$IP_{480-501}$ were purchased from Calbiochem (La Jolla, CA, USA), MLCK-IP_{11-19} from Alexis (Nottingham, UK); Carbachol (CCh) and HEPES from Sigma (St Louis, MO, USA); ATP, GTP, and EGTA from Dojindo (Kumamoto, Japan). Stock solutions for ML-9, ML-7, DIDS and OAG were made by dissolving them in dimethylsulphoxide (DMSO) and then diluting more than 1000 times for use. We confirmed that, up to 0.1%, DMSO produced no detectable changes in basal currents or those activated by CCh or OAG.

Results

Features of heterologously expressed TRPC6 current

Under the nystatin-perforated voltage-clamp (holding potential: −60 mV), in non-transfected HEK293 cells, little discernible current was induced by external application of 100 μM CCh (current density at −60 mV, 0.32 ± 0.15 pA pF^{-1}; $n=12$). In contrast, in nearly all HEK cells transfected with TRPC6 plasmids and positive to anti-CD8 beads selection, the density of basal current was increased to 1.0 ± 0.5 pA pF^{-1} ($n=25$) and that of CCh-induced current at −60 mV was as large as 41.7 ± 8.4 pA pF^{-1} ($n=25$). Replacement of external Na^+ by a large impermeant cation, *N*-methyl D-glucamine (NMDG), reversibly abolished the CCh-induced current (Cl^- substitution with benzene sulphonate was ineffective; data not shown). The reversal potential of CCh-induced current was near zero under normal ionic conditions (Figure 2a). These results together indicate the non-selective permeation of cations. The current–voltage (*I–V*) relationship of CCh-induced current showed an S-shape double rectification (Figure 2a), and application of La^{3+} (100 μM) or pretreatment with the phospholipase C inhibitor U73122 (10 μM) strongly inhibited the current (data not shown). These features match those reported previously (Inoue *et al.*, 2001), indicating that CCh-induced current observed in this study reflect the activation of TRPC6 channels via stimulation of muscarinic receptors endogenous to HEK293 cells (hereafter designated as I_{TRPC6}).

Acute actions of ML-9 on I_{TRPC6}

External application of ML-9 caused a dose-dependent inhibition of I_{TRPC6} (Figure 1). The inhibition was detectable at 1 μM or lower concentrations and almost complete at 100 μM (lower trace in Figure 1a). The Hill analysis of ML-9 concentration–I_{TRPC6} amplitude relationship gives the IC_{50} of 7.8 μM and cooperative factor of 1.0 (Figure 1b). The

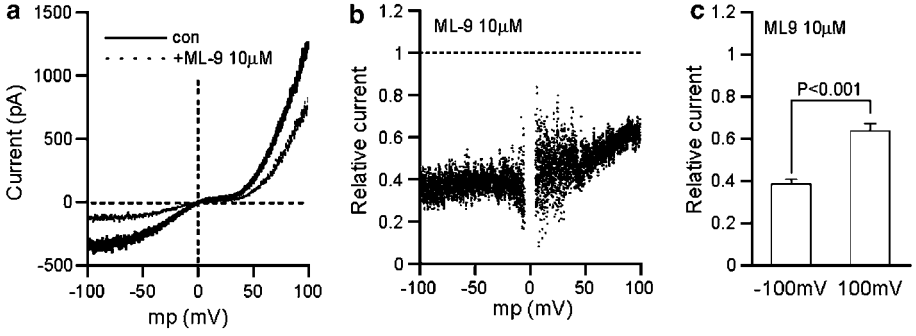

Figure 2 Voltage-dependency of ML-9's effects on I_{TRPC6}. (**a**) Current–voltage (*I–V*) relationships of I_{TRPC6} before (bold curve) and during (dotted curve) application of 10 μM ML-9. These curves were constructed by applying a 2 s rising ramp voltage, which can be seen as short vertical deflections in Figure 1a. (**b**) Voltage-dependent effects of ML-9. The fraction of I_{TRPC6} remaining in the presence of 10 μM ML-9 is calculated from the *I–V* curves in (**a**), and plotted against the membrane potential. Values near the reversal potential are omitted because of large scattering due to 'division by zero'. (**c**) The fractions of I_{TRPC6} remaining after ML-9 inhibition at −100 and 100 mV averaged from seven different experiments. Paired *t*-test ($n=7$). I_{TRPC6}, TRPC6 current; ML-9, [1-(5-chloronaphthalene-1-sulphonyl)homopiperazine, HCl].

inhibition of I_{TRPC6} by ML-9 occurred rapidly, the rate of onset being comparable to that of external Na^+ substitution with NMDG (time constant τ; 0.61 ± 0.09 s vs 0.46 ± 0.06 s, for 100 μM ML-9 and NMDG, respectively, $n=6$, $P<0.01$; Figure 1c). The extent of the inhibition was relieved by membrane depolarization (Figures 2a and b). The ratio of I_{TRPC6} amplitude after, to before, application of 10 μM ML-9, which was calculated from the *I–V* relationship constructed by a 2 s rising ramp voltage, was different at −100 or 100 mV ($P<0.001$ with paired *t*-test, $n=7$; Figure 2c). These results indicate that there is a moderate voltage-dependency present in the inhibitory actions of ML-9 on I_{TRPC6}.

Other MLCK inhibitors are ineffective on I_{TRPC6}

The rapidity and voltage-dependence of I_{TRPC6} inhibition by ML-9 makes us suspect that the inhibition may arise from a mechanism not involving MLCK activity. To clarify this point, we employed other manoeuvres known to affect the MLCK activity.

Wortmannin is another frequently used organic MLCK inhibitor structurally unrelated to ML-9 (IC_{50} *in vitro*, 200 nM; Yano *et al*., 1993), although non-specifically inhibiting the phophatidylinositol-3-, -4- and MAP-kinases. This compound was however, entirely ineffective on I_{TRPC6} at the concentration of 3 μM which has been reported to effectively inhibit MLCK activity in smooth muscle (Burdyga and Wray, 1998; Longbottom *et al*., 2000; Ito *et al*., 2004), whether applied acutely following full I_{TRPC6} activation by CCh or by pretreatment for 5–10 min before application of CCh (Figures 3a and b).

In the next step, we examined the effects of two MLCK-specific inhibitory peptides (MLCK-IP_{11-19} and MLCK-$IP_{480-501}$). It has been reported that the IC_{50} value for MLCK-$IP_{480-501}$ is about 0.25 μM (Smith *et al*., 1990) and 5 μM is high enough for MLCK-IP_{11-19} to inhibit the smooth muscle MLCK activity (Aromolaran *et al*., 2000). As demonstrated in Figure 4a, 10 μM MLCK-IP_{11-19} or 1 μM MLCK-$IP_{480-501}$, which was included in the patch pipette, failed to

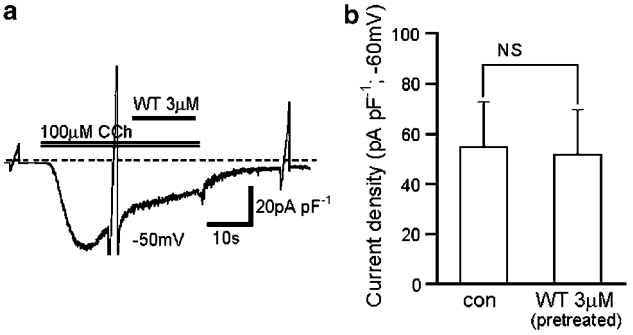

Figure 3 Wortmannin was ineffective on I_{TRPC6}. Nystatin-perforated recording at −60 mV. (**a**) Actual trace of I_{TRPC6}. Wortmannin (WT) (3 μM) was introduced into the bath 5–10 min before application of CCh. In this experiment, WT was further applied after activation of I_{TRPC6} by CCh (100 μM), but no discernible change was observed. (**b**) Current density of I_{TRPC6} with and without pretreatment with 3 μM WT. $P>0.05$ with unpaired *t*-test ($n=6$). CCh, carbachol; I_{TRPC6}, TRPC6 current.

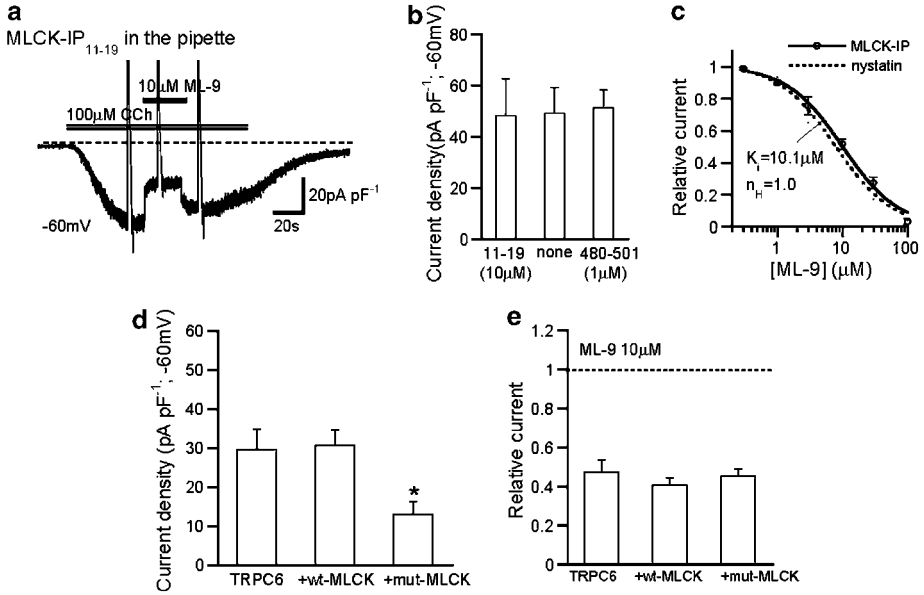

Figure 4 Intracellular perfusion of MLCK-inhibitory peptides and dominant negative inhibition of MLCK did not affect the inhibitory efficacy of ML-9 on I_{TRPC6}. Conventional whole-cell recording at −60 mV. To reduce the degree of desensitization, Ca^{2+}-free rather than normal external solution was used as the bathing solution. (**a**) Representative trace for the extracellular ML-9 (10 μM) effects on I_{TRPC6} in the presence of 10 μM MLCK IP_{11-19} peptide in the pipette. (**b**) Summary of the effects of 10 μM MLCK IP_{11-19} and 1 μM MLCK $IP_{480-501}$ on I_{TRPC6} density ($n=7$–11). (**c**) ML-9 concentration–inhibition curves for I_{TRPC6} with (solid) and without (nystatin-perforated recording; dotted; taken from Figure 1b) inclusion of MLCK inhibitory peptides (10 μM MLCK IP_{11-19} or 1 μM MLCK $IP_{480-501}$). The solid curve is drawn according to the best fit of the data points with Hill equation (see Methods). $IC_{50}=10.1$ μM; $n_H=1.0$ ($n=4$–6). There was no statistically significant difference between two curves with ANOVA. (**d** and **e**) Influence of overexpression of wild type (wt-MLCK) or a dominant-negative mutant of MLCK (mut-MLCK) on current density of I_{TRPC6} (**d**; $n=15$–18) and inhibitory efficacy of ML-9 for I_{TRPC6} (**e**; $n=8$–11). (**d**) *$P<0.05$ from other columns with ANOVA and Bonferroni's *t*-test. ANOVA, analysis of variance; IP, inhibitory peptides; ML-9, [1-(5-chloronaphthalene-1-sulphonyl)homopiperazine, HCl]; MLCK, myosin light chain kinase.

reduce the density of I_{TRPC6} (Figure 4b). Moreover, the efficacy of ML-9 to inhibit I_{TRPC6} remained almost unaffected even after intracellular application of these MLCK inhibitory peptides (Figure 4c vs Figure 1b). The ML-9 concentration I_{TRPC6} amplitude curves obtained in the presence and absence of MLCK peptides nearly superimpose, giving similar IC_{50} values (10.1 vs 7.8 μM).

Taken together, these results strongly suggest that MLCK plays, if any, only a minor role in the regulation of TRPC6 channels overexpressed in HEK293 cells.

Overexpression of wild type or dominant-negative MLCK does not affect the inhibitory actions of ML-9

To validate further the above conclusion and also explore possible long-term consequences of MLCK inhibition, we carried out the dominant-negative suppression of MLCK by co-expressing a non-functional form of MLCK (mut-MLCK; see Methods; Shimizu *et al*., 2006) with TRPC6 protein. As graphically summarized in Figure 4, expression of mut-MLCK did not affect the extent of fast inhibition by ML-9 (10 μM; Figure 4e), although it moderately decreased the density of I_{TRPC6} (Figure 4d). On the other hand, overexpression of wt-MLCK induced no significant changes in either the density or extent of the inhibition of CCh-induced I_{TRPC6} (Figures 4d and e), or in basal current in the absence of receptor stimulation (data not shown). These results further reinforce the idea that MLCK is not a major target of the fast inhibitory effects of ML-9 on I_{TRPC6}.

I_{TRPC6} inhibition by ML-9 and ML-7 does not depend on the mode of activation

The rapid and MLCK-independent actions of ML-9 tempt us to speculate that the site of actions of ML-9 may be close to or on the TRPC6 channel itself. To gain more insight into this possibility, we examined the effects of ML-9 on TRPC6 channels, which were activated in different ways. As demonstrated and summarized in Figure 5, even when I_{TRPC6} was induced by a membrane-permeable analog of diacylglycerol OAG (100 μM) by bypassing the receptor/G-protein/phospholiapse C pathway to activate TRPC6 channels more directly, ML-9 exerted a similar extent of inhibition to that seen with I_{TRPC6} activated with CCh (Figures 5a and c). Similarly, the extent of inhibition of small basal currents, which probably reflect constitutive activation of expressed TRPC6 channels, did not change from those obtained with CCh and OAG (Figures 5a and b). These results strongly support the site of action of ML-9 being in close proximity to the TRPC6 channel.

In addition, we tested another ML compound, ML-7, and found that, except for its slightly weaker efficacy, the inhibitory effects of this compound did not essentially differ from those of ML-9 being independent of MLCK (Figures 5b–d). We also found that another naphthalene sulphonamide derivative W-7 (50 μM) caused an acute inhibition of I_{TRPC6}, but the time course of this inhibition appeared much slower than ML-9 or ML-7 (data not shown) and was not further pursued in the present study.

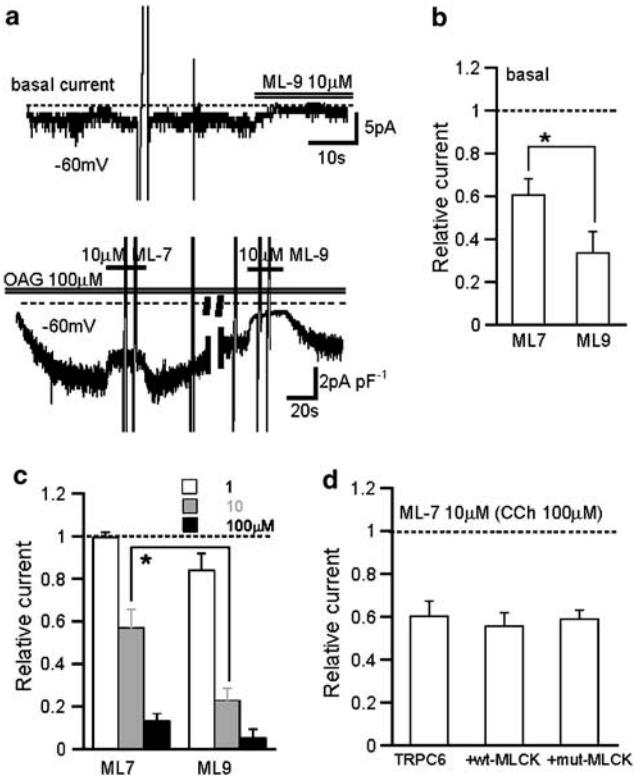

Figure 5 Activation mode of TRPC6 did not affect the efficacy of ML-9 and ML-7. Conventional whole-cell recording at −60 mV with Ca^{2+}-free external solution. (**a**) Representative traces showing the effects of ML-9 and ML-7 on basal (upper) and OAG (100 μM; lower)-induced I_{TRPC6}. (**b** and **c**) Summary of the effects of ML-9 and ML-7 on basal I_{TRPC6} at 10 μM (**b**) and on OAG-induced I_{TRPC6} at three different concentrations (1, 10, 100 μM) (**c**). $n=6$–7. (**d**) Inhibitory efficacy of ML-7 for I_{TRPC6} with overexpression of wild type (wt-MLCK) or a dominant negative mutant of MLCK (mut-MLCK). $n=$ 8–11. *$P<0.05$. ML-7, [1-(5-iodonaphthalene-1-sulphonyl)homopiperazine, HCl]; ML-9, [1-(5-chloronaphthalene-1-sulphonyl)homopiperazine, HCl]; MLCK, myosin light chain kinase.

ML-9 and ML-7 can rapidly act on TRPC6 channel from the inside of the membrane

In the final stage of the present study, we questioned if there was 'membrane sidedness' for the rapid action of ML-9. As shown in Figures 6a and b, after intracellular perfusion of ML-9, the density of I_{TRPC6} activated by CCh was reduced dose-dependently; more than half and almost complete inhibition occurred at 10 and 30 μM, respectively. Importantly, after equilibration of internal perfusion with ML-9, its external application at the same concentration failed to produce further inhibition (Figure 6a). These results suggest that ML-9 may access almost equally, from the outside or the inside of the cell membrane, a site(s) associated with TRPC6 channel inhibition. Consistent with this speculation, bath application of ML-9 as well as its congener ML-7, rapidly diminished ~35 pS single TRPC6 channel activities in cell-free, inside-out patch configuration (Figure 7). It was, however, difficult to determine unequivocally whether the inhibition occurred due to reduced unitary amplitude, reduced open probability or both, because of infrequent openings of the channel after application of these drugs. In any case, this finding further corroborates the conclusion

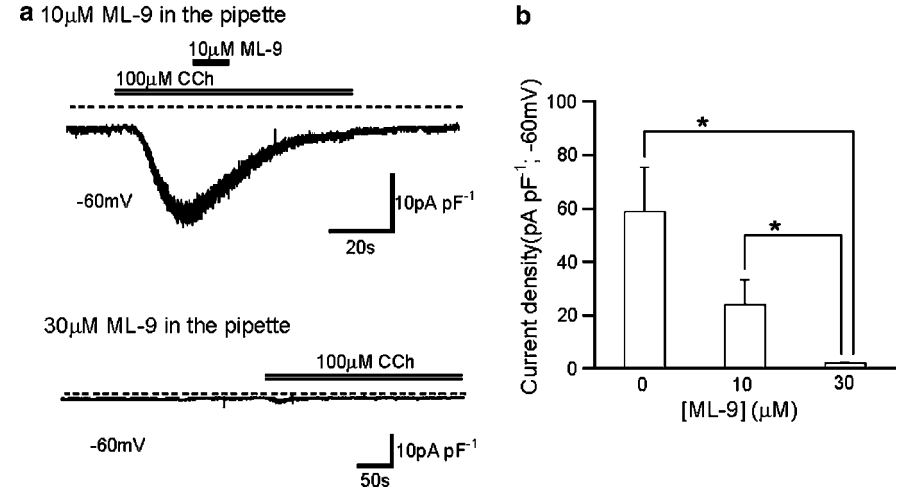

Figure 6 Concentration-dependent inhibition of I_{TRPC6} by intracellularly applied ML-9. ML-9 was included in the pipette under conventional whole-cell clamp (−60 mV), with bath containing Ca^{2+}-free external solution. (**a**) Typical traces of I_{TRPC6} with intrapipette inclusion of 10 μM ML-9, which abolished the inhibitory effect of extracellularly applied ML-9 at the same concentration (upper), and of 30 μM ML-9, which strongly inhibited I_{TRPC6} (lower). (**b**) Summary of the effects of intracellularly applied ML-9 on I_{TRPC6} density. *$P<0.05$ with ANOVA and Bonferroni's *t*-test. $n=5$–8. ANOVA, analysis of variance; ML-9, [1-(5-chloronaphthalene-1-sulphonyl)homopiperazine, HCl].

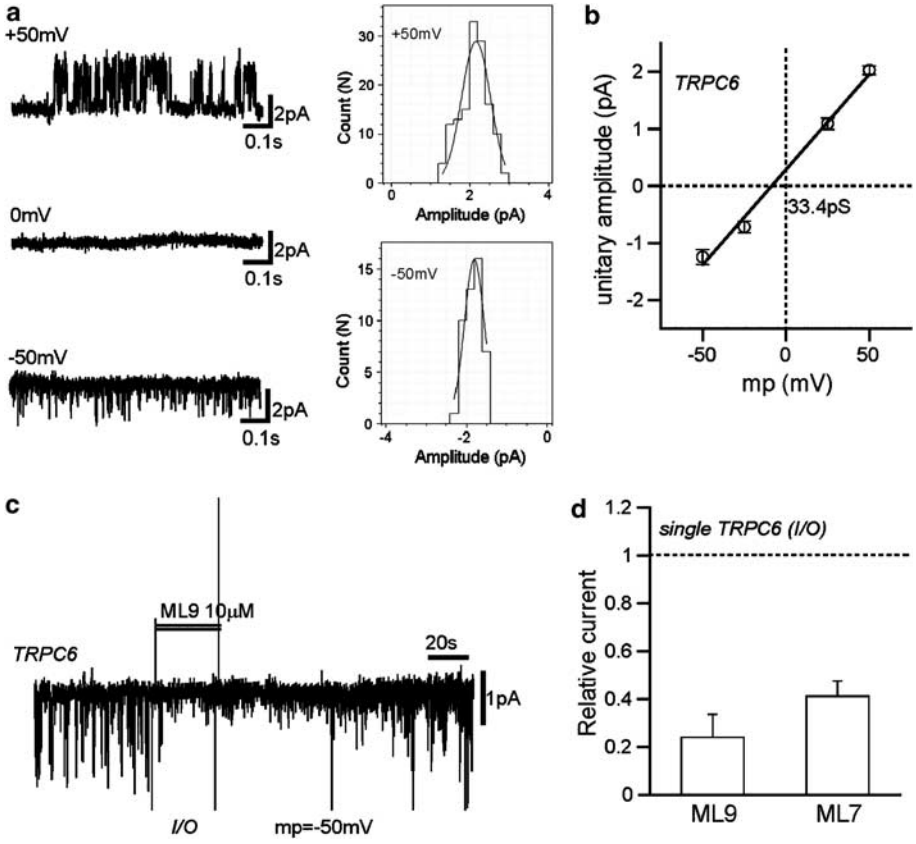

Figure 7 Effects of ML-9 and ML-7 on single TRPC6 channels. Inside-out (I/O) patch recording with CCh (100 μM) in the pipette. To suppress K^+ and Cl^- channels, 5 mM tetraethylammonium and 100 μM DIDS were also added in the pipette. (**a**) Actual traces at three different potentials (−50, 0 and 50 mV). Boxes on the right indicate all-points-in-open-state amplitude histograms with the results of Gaussian fit (smooth curves). (**b**) *I–V* relationship for single TRPC6 channel. Linear regression of data points between −50 and 50 mV gives a unitary conductance of 33.4 pS ($n=5$–8), which is in good accordance with the value obtained previously (Shi *et al*., 2004). (**c**) Inhibition of single TRPC6 channel activity by 10 μM ML-9, which was applied rapidly into the bath at the bar. Two large vertical deflections indicate the electrical artefacts of a solenoid valve. (**d**) Summary of the inhibitory effects of 10 μM ML-9 and ML-7 on single TRPC6 channels. The extent of single-channel current inhibition by ML compounds was calculated as the ratio of mean currents (averaged for 2 s) 5 s after, to just before, application of the drugs. $n=8$–11. CCh, carbachol; DIDS, [4,4-diisothiocyanostilbene-2,2-disulphonic acid, 2Na]; ML-7, [1-(5-iodonaphthalene-1-sulphonyl)homopiperazine, HCl]; ML-9, [1-(5-chloronaphthalene-1-sulphonyl)homopiperazine, HCl].

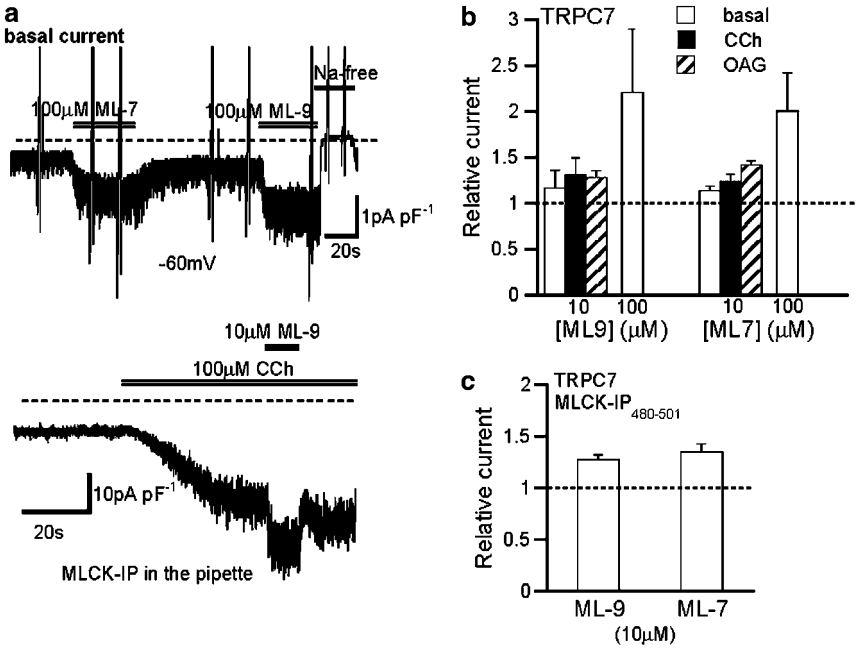

Figure 8 Enhancing actions of ML-compounds on macroscopic TRPC7 current (I_{TRPC7}). Conventional whole-cell recording at −60 mV with Ca^{2+}-free external solution. (**a**) Representative records of basal (upper) and CCh-induced (lower) I_{TRPC7} demonstrating the enhancing effect of ML-9 and ML-7. (**b**) Fractional change in I_{TRPC7} amplitude after rapid application of ML-9 and ML-7. $n=6$–8. (**c**) Ineffectiveness of MLCK inhibitory peptide (MLCK-$IP_{480-501}=1$ μM) on the effects of extracellular ML-9 and ML-7 on CCh (100 μM)-induced I_{TRPC7}. $n=5$–6. CCh, carbachol; IP, inhibitory peptides; ML-7, [1-(5-iodonaphthalene-1-sulphonyl)homopiperazine, HCl]; ML-9, [1-(5-chloronaphthalene-1-sulphonyl)homopiperazine, HCl]; MLCK, myosin light chain kinase.

above that the inhibitory effects of ML-9 (and ML-7) were not mediated by the inhibition of MLCK activity but were, rather, direct actions on the TRPC6 channel.

TRPC7 channels show the opposite sensitivity to ML compounds

TRPC7 possesses a high degree of similarity to TRPC6 but shows distinct properties with respect to the sensitivity to Ca^{2+} and some organic compounds (Inoue *et al*., 2001; Shi *et al*., 2004). We were thus intrigued to see how ML compounds affect this TRPC isoform. Somewhat surprisingly, both ML-9 and ML-7 caused a rapid and reversible enhancement rather than inhibition of currents arising from TRPC7 expression, whether they were constitutively activated (basal) or activated via muscarinic receptor stimulation (CCh) or more directly by OAG (Figures 8a and b). As in the case of TRPC6, these actions do not seem to involve the inhibition of MLCK activity, since prior intracellular application of MLCK-$IP_{480-501}$ could not prevent this enhancement (Figure 8c).

Discussion

The unexpected new finding of the present study is that ML-9 (and its congener ML-7 as well), which has frequently been used to suppress MLCK activities, exerts rapid reversible inhibitory actions on TRPC6 channels via a mechanism independent of MLCK. Although enhancing rather than inhibitory, similarly rapid effects of ML-9 were also observed on TRPC7, a closest homologue of TRPC6. This conclusion is supported by several different lines of evidence.

Firstly, neither wortmannin, a structurally different MLCK inhibitor (Davies *et al*., 2000; Bain *et al*., 2003) nor MLCK-specific inhibitory peptides (MLCK-IP_{11-19} and MLCK-$IP_{480-501}$) targeted to the catalytic and ATP binding domains of MLCK (Ikebe, 1990; Kemp *et al*., 1991), affected the extent of channel activation (that is, current density) or of inhibition by ML-9. Ineffectiveness of these peptide inhibitors is unlikely due to their insufficiently high concentrations (cf Smith *et al*., 1990; Aromolaran *et al*., 2000) or incomplete intracellular perfusion, since we employed patch pipettes whose input and resultant series resistances had been low enough to attain almost complete inhibition of calmodulin-dependent kinase II activity by its inhibitory peptides (2.5–4 and <10 MΩ, respectively; see Shi *et al*., 2004). Secondly, the fast inhibitory actions of ML-9 on TRPC6 current were not appreciably affected by overexpression of wild-type or a dominant-negative mutant MLCK, despite that this procedure was anticipated to alter the MLCK activity and, in turn, the phosphorylated status of target proteins. Thirdly, a similar extent of inhibitory actions by ML-9 was still observed on single TRPC6 channel activities ($\sim$35 pS) recorded in cell-free, inside-out patch configuration, where a complete set of intracellular constituents/enzymes requisite for phosphorylation/dephosphorylation is unlikely to be retained. Finally, the onset of ML-9 action was extremely fast (less than 1 s), being comparable to those of Na^+ substitution by NMDG (Figure 1b), and the extent of TRPC6 inhibition was significantly relieved by membrane depolarization even during a short ramp potential (2 s; Figure 2b). Such rapid actions cannot be accounted for by a series of biochemical reactions, exemplified by facilitated internalization of channel protein from the cell surface

following MLCK inhibition, which has recently been demonstrated for a different TRPC subfamily member TRPC5 (Shimizu *et al*., 2006). This internalization process has been shown to result from reduced MLC phosphorylation and consequent cytoskeletal rearrangements, the whole process requiring minutes to become overt.

Thus, the most straightforward interpretation of the above observations is that ML-9 acts on a site directly or closely associated with the gating or ion permeation of TRPC6 channels to exert its inhibition, although its longer application might also reduce the cell membrane expression of the channel protein via a MLCK-dependent mechanism (see also the following paragraph). Interestingly, it has been reported that flufenamate, a general NSCC blocker, and external Ca^{2+}, can also exert similar rapid but contrary effects on TRPC6 and -7 channels (potentiation and inhibition, respectively; Inoue *et al*., 2001; Shi *et al*., 2004). In contrast, no such reciprocal effects were observed on the two TRPC isoforms by a widely used NSCC channel blocker SK&F96365, which caused only inhibition (Shi and Inoue, unpublished observation). Furthermore, W7, another naphthalene sulphonamide derivative capable of blocking MLCK, also inhibited TRPC6 current but only with a much slower time course than the ML compounds (see above). At present, structural basis for these pharmacological differences remain entirely unclear. Future mutagenic scanning of amino-acid residues near the putative pore region (and if available, those responsible for gating) of TRPC6 and -7 channels may help clarify this point.

Despite that distinct Ca^{2+}/CaM-dependent kinases are involved in the activation process, both overexpressed TRPC6 channel and its likely native counterpart α_1-adrenoceptor NSCC in rabbit portal vein myocytes (Aromolaran *et al*., 2000) are susceptible to micromolar concentrations of ML-9. However, careful comparison of the results of Hill analysis reveals that the IC_{50} value is significantly lower for α_1-adrenoceptor NSCCs (2.0 μM) than that for expressed TRPC6 channels (7.8 μM), and the former appears even lower than that required to inhibit MLCK activity *in vitro* (3.8 μM; Saitoh *et al*., 1987). More importantly, the cooperativity factor (n_H) for α_1-adrenoceptor NSCC (2.5) is more than twice that of overexpressed TRPC6 channel which is equal to unity (1.0) (Aromolaran *et al*., 2000; present study). These stoichiometric data suggest that ML-9 may exert at least two distinct effects, that is inhibition of MLCK activity and more direct inhibition of NSCC. Both effects seem to be present in the α_1-adrenoceptor NSCC, whereas only the latter may be operative in TRPC6 channels expressed in HEK293 cells where, as compared with calmodulin-dependent kinase II, the physiological impact of MLCK activity on NSCC activation would be much weaker than in muscle tissues. Indeed, this may have partly been manifested as reduced availability of TRPC6 channel with overexpresion of mutant MLCK (Figure 4d), but we did not further pursue this issue in the present study. On the other hand, ML-7, another naphthalene sulphonamide derivative, is more than 30-fold more potent in inhibiting MLCK ($IC_{50} = 300$ nM; Saitoh *et al*., 1987) than the TRPC6 channel (IC_{50} appears slightly larger than 10 μM; see Figure 5c). This means that even though this compound's MLCK-independent effect on TRPC6 channel would be operative in the micromolar range, this would already be masked by its submicromolar inhibitory effect on MLCK, thus the former effect would not be clearly observable. This actually seems to occur in rabbit portal vein α_1-adrenoceptor NSCC, which ML-7 ($IC_{50} = 0.8\,\mu$M) inhibits more effectively than ML-9 but the value of n_H is nearly equal to unity (Aromolaran *et al*., 2000). In this regard, to separate the effects on MLCK and TRPC6 channel, ML-7 may be more advantageous than ML-9, when it is used at low micromolar concentrations.

The non-specific inhibitory effects of ML-9 on Ca^{2+} transporting activities have been documented in the literature. For instance, in porcine aortic endothelial cells, one study reported that bradykinin or thapsigargin-induced Ca^{2+} entry was suppressed by both ML-9 and wortmannin with a parallel inhibition of MLC phosphorylation (Watanabe *et al*., 1998). However, in the same preparation, another study provided a conflicting evidence that only ML-9 but not wortmannin-inhibited thapsigargin-induced Ca^{2+} entry (Kuroiwa-Matsumoto *et al*., 2000). In guinea pig tracheal smooth muscle, while ML-9 was found to be able to reduce Ca^{2+} entry and concomitant contraction induced by high K^+, methacholine or thapsigargin, wortmannin only inhibited the contraction without affecting $[Ca^{2+}]_i$ (Ito *et al*., 2004). All these findings appear to be compatible with the idea that ML-9 may act as a direct inhibitor of several different types of Ca^{2+} entry channels rather than via its effects on MLCK activity. TRPC6 is known to be a major TRPC isoform expressed in vascular smooth muscle cells (Inoue *et al*., 2006), but its relatively abundant expression has also been detected in airway smooth muscle cells and endothelial cells (Ong *et al*., 2003; Yip *et al*., 2004). It is thus possible that part of the inhibitory actions of ML-9 observed in the above studies may also reflect the non-specific effects of ML-9 on Ca^{2+} entry channels *per se*, as observed in the present study.

In summary, the present results clearly show that ML-9 effectively inhibited TRPC6 channel in a manner independent of MLCK inhibition. This inhibition is likely to occur through an interaction with a site(s) closely associated with the TRPC6 channel protein, and may rely on subtle structural differences between different TRPC isoforms, since ML-9 exhibits the opposite, enhancing, effects on TRPC7 channels which shows a high degree of amino-acid sequence identity over the six transmembrane domains. This non-specificity could mislead the conclusion of studies investigating the functional significance of MLCK activity on Ca^{2+} transporting activity, if evaluation is made based solely on ML-9's effects. This potential problem would become particularly serious when smooth muscle tissues are of interest, where MLCK functions as the dominant effector of Ca^{2+}/CaM-dependent pathway and expression of TRPC6 protein may also be ubiquitous.

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Conflict of interest

The authors state no conflict of interest.

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