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TRPM4 regulates migration of mast cells in mice

Takahiro Shimizu^{a,c,1}, Grzegorz Owsianik^{a,1}, Marc Freichel^b, Veit Flockerzi^b,
Bernd Nilius^{a,*}, Rudi Vennekens^a

^a Department of Molecular Cell Biology, Laboratory of Ion Channel Research, KU Leuven, Campus Gasthuisberg, Herestraat 49, bus 802, B-3000 Leuven, Belgium

^b Experimentelle und Klinische Pharmakologie und Toxikologie, Universität des Saarlandes, D-66421 Homburg, Germany

^c Department of Cell Physiology, National Institute for Physiological Sciences, 444-8585 Okazaki, Japan

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ABSTRACT

We demonstrate here that the transient receptor potential melastatin subfamily channel, TRPM4, controls migration of bone marrow-derived mast cells (BMMCs), triggered by dinitrophenylated human serum albumin (DNP-HSA) or stem cell factor (SCF). Wild-type BMMCs migrate after stimulation with DNP-HSA or SCF whereas both stimuli do not induce migration in BMMCs derived from TRPM4 knockout mice (*trpm4*^{−/−}). Mast cell migration is a Ca²⁺-dependent process, and TRPM4 likely controls this process by setting the intracellular Ca²⁺ level upon cell stimulation. Cell migration depends on filamentous actin (F-actin) rearrangement, since pretreatment with cytochalasin B, an inhibitor of F-actin formation, prevented both DNP-HSA- and SCF-induced migration in wild-type BMMC. Immunocytochemical experiments using fluorescence-conjugated phalloidin demonstrate a reduced level of F-actin formation in DNP-HSA-stimulated BMMCs from *trpm4*^{−/−} mice. Thus, our results suggest that TRPM4 is critically involved in migration of BMMCs by regulation of Ca²⁺-dependent actin cytoskeleton rearrangements.

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1. Introduction

Mast cells have a pivotal role in inflammation reactions, defense against pathogens such as bacteria and virus, and autoimmune diseases [1–3]. They express a number of high-affinity receptor (Fc ϵ RI) for immunoglobulin E (IgE) on their plasma membrane to trigger protective functions in response to multivalent antigens. The cross-linking of antigen to the antigen-specific IgE-coupled receptor causes aggregation of the receptor to initiate multiple signal cascades, followed by cellular activation and release of chemical mediators like histamine and *de novo* synthesis of pro-inflammatory lipid mediators such as cytokines and chemokines [1]. Mast cells accumulate at local inflammatory tissues. Some reports demonstrated that the migration of mast cells is directed not only by chemotactic factors [4–7] but also by antigens [8]. The molecular mechanism of antigen-induced migration is not yet understood. Nevertheless, it has been shown that sphingosine-1-phosphate (S1P) is one of the important signals in the antigen-induced migration [9,10]. In the presence of antigen, IgE-mediated degranulation of mast cells relies on Ca²⁺ influx through Ca²⁺ release-activated Ca²⁺ (CRAC) channels [11,12] and

changes of membrane potential, which are modulated by ion channels that control the driving force for Ca²⁺ [13,14]. We have recently demonstrated that in mast cells the transient receptor potential melastatin 4 channel (TRPM4) regulates Ca²⁺ influx by modulating the membrane potential [14].

In the present study, we examined antigen-triggered migration of sensitized mast cells, and a possible role of TRPM4 in this process. We demonstrate that TRPM4 is directly involved in migration of mast cells in the presence of antigen, likely through controlling the intracellular Ca²⁺ level upon cell stimulation. Mast cell migration is Ca²⁺-dependent and possibly relies on TRPM4-dependent induction of filamentous actin (F-actin) formation. Taken together, our results demonstrate that TRPM4 is a regulator of antigen-induced migration of activated mast cells.

2. Materials and methods

2.1. Isolation and sensitization of mast cells

Bone marrow-derived mast cells (BMMC) were isolated from wild-type and *trpm4*^{−/−} mice as described in previous papers [14,15]. Then BMMC were maintained in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 μ M β -mercaptoethanol, 10 U/ml of each penicillin and streptomycin, and 2 ng/ml recombinant murine IL-3 (R&D Systems, Minneapolis, MN)

* Corresponding author. Tel.: +32 16 345 937; fax: +32 16 345 991.

E-mail address: bernd.nilius@med.kuleuven.be (B. Nilius).

¹ These authors equally contributed to this work.

under a 37 °C and 5% CO₂ condition. To sensitize BMMC to dinitrophenylated human serum albumin (DNP-HSA; Sigma, St. Louis, MO), BMMC were preincubated overnight with 300 ng/ml anti-DNP-HSA immunoglobulin E (IgE) antibody (Sigma) in IMDM.

2.2. Migration assays

BMMC migration was measured in 12-well chemotaxis chambers (Neuro probe, Gaithersburg, MD) using polycarbonate filters with 8 μm pore size (Neuro probe). Briefly, sensitized BMMC with the anti-DNP IgE antibody were suspended in RPMI-1640 medium (Invitrogen) without phenol red, supplemented with 1% FCS after washing them in phosphate buffered saline (PBS) and then placed in the upper wells at density of 60,000 cells/well. The bottom wells were filled with 1% FCS-containing RPMI medium. Depending on experiments, DNP-HSA was applied into upper and/or bottom wells to stimulate BMMC migration. After the chambers were incubated for 1–3 h at 37 °C in 5% CO₂ atmosphere, migrated BMMC into bottom wells were counted by using a hemacytometer. In some experiments, we used K-rich RPMI medium containing 108.7 mM KCl, 5.6 mM Na₂HPO₄·2H₂O, 23.8 mM NaHCO₃, 0.4 mM MgSO₄·7H₂O, 0.4 mM Ca(NO₃)₂·4H₂O, 11.1 mM D-glucose, and an appropriate amount of RPMI-1640 amino acids solution (Sigma). As a control for K-rich experiments, 103.4 mM KCl in K-rich solutions was replaced with an equal amount of NaCl. Stem cell factor (SCF; Millipore, Temecura, CA) was used as a chemoattractant. SCF-induced chemotaxis was observed in non-sensitized BMMC. SCF was added into upper and/or bottom wells. After 1.5 h, migrated BMMC were measured in the same way.

In some experiments, BMMC were pretreated with 50 μM 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) for 20 min or 10 μM cytochalasin B for 15 min. 100 μM flufenamate was added in upper wells and 10 mM BAPTA was applied in both wells. To induce cell depolarization by a high K⁺ concentration, NaCl in the RPMI-1640 medium was replaced by KCl.

2.3. Actin staining

To observe rearrangement of globular or filamentous actin, we performed immunohistochemistry using monoclonal mouse anti-β-actin antibody (Sigma) and staining with the Alexa488-conjugated phalloidin probe (Invitrogen). Briefly, sensitized BMMCs were placed on cover glasses coated with poly-L-lysine and stimulated with 1 ng/ml DNP-HSA for 10 min at 37 °C in 5% CO₂ conditions. The cells were fixed with 3.7% formaldehyde for 10 min, permeabilized with 0.2% TritonX-100 for 10 min, and blocked with 3% BSA for 3 h. For β-actin detection, cells were incubated overnight with anti-β-actin antibodies and then for 1 h with Alexa594-conjugated secondary anti-mouse IgG antibodies (triple washing with PBS supplemented with 0.1% Tween 20 between each step). To visualize F-actin, permeabilized cells were probed with Alexa488-conjugated phalloidin. Images were taken using a confocal microscope imaging system (Zeiss).

2.4. Ca²⁺ Imaging

Cytosolic Ca²⁺ levels in BMMC were measured with Fura-2, as described before [14].

2.5. Statistics

Data are presented as means ± S.E.M with *n* denoting the number of cells tested. Statistics were performed using unpaired Student's *t*-test. *p* < 0.05 was considered significant.

3. Results

3.1. DNP-HSA has a direct effect on the migration of IgE-specific antigen sensitized BMMC

To study BMMC migration we used a modified Boyden-chamber assay. In this assay, two chambers are placed on top of each other, separated by a porous membrane. A population of mast cells is placed in the upper chamber, and migration towards the bottom chamber is monitored. As previously described, sensitized BMMC actively migrate through the membrane toward the bottom chamber in the presence of DNP-HSA in that compartment ([8] and see Fig. 1A). Migration is time- and dose-dependent as shown in Fig. 1A. In all further experiments we used 1 h induction with 1 ng/ml DNP-HSA. The lower concentration was used to avoid massive degranulation of mast cells.

To examine whether the migration of mast cells is due to chemotaxis toward the antigen, we compared the effects when DNP-HSA was only present in the bottom chamber with those when DNP-HSA was present in both reservoirs of the Boyden-chamber. Sensitized BMMC directly stimulated with the antigen also migrated into the bottom reservoir (Fig. 1B). Moreover, a similar level of mast cell migration was observed when the antigen was present in both reservoirs (Fig. 1B). Thus, DNP-HSA directly induced migration of sensitized BMMC cells independently of the antigen gradient.

Fig. 1. Antigen induced BMMC migration (A) concentration and time dependence of BMMC migration after stimulation with DNP; (B) DNP-HSA-induced migration was independent of the antigen gradient. DNP-HSA was included at a concentration of 1 ng/ml into upper well or as indicated in the bottom or in both wells. **p* < 0.05, when compared to non-treated control. †*p* < 0.05, when compared to data in the presence of 1 ng/ml DNP-HSA.

Fig. 2. The TRPM4 channel is an essential modulator of the antigen-stimulated BMMC migration. (A) Flufenamate inhibited DNP-HSA-triggered migration. (B) TRPM4-deficient mast cells failed to migrate after DNP-HSA application. (C) and (D) Intracellular (C) and extracellular (D) Ca^{2+} are required for DNP-HSA-stimulated migration. (E) Antigen-induced BMMC migration is absent in depolarized cells (media with high K^{+} concentration). * $p < 0.05$, indicates the comparison between non-treated and DNP-treated WT cells, † $p < 0.05$, between DNP-treated WT and *trpm4*^{-/-} cells under various conditions.

3.2. The lack of TRPM4 activity inhibits antigen-induced BMMC migration

An application of 100 μM flufenamic acid, a potential blocker of TRPM4 [16], in the upper reservoir abolished migration of DNP-HSA-stimulated BMMC (Fig. 2A). To determine whether the loss of migration by flufenamic acid was indeed due to the block of TRPM4 channel activity, we performed migration assays with BMMC isolated from *trpm4*^{-/-} mice. DNP-HSA-stimulated BMMC lacking TRPM4 channels did not display a significant migration independently of DNP-HSA-application in any side of the migration chamber (Fig. 2B, and data not shown).

3.3. Ca^{2+} influx is required for antigen-induced BMMC migration

TRPM4 functions as a Ca^{2+} -activated non-selective cation channel in mast cells, and regulates the intracellular Ca^{2+} level upon mast cell stimulation with DNP [14]. The intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is an important factor for antigen-stimulated migration of BMMC. Indeed, when we loaded mast cells with BAPTA-AM to chelate $[\text{Ca}^{2+}]_i$, application of DNP-HSA did not evoke

migration of the cells (Fig. 2C). Similar results were obtained when extracellular Ca^{2+} was chelated by addition of 10 mM BAPTA into the extracellular medium (Fig. 2D), indicating that Ca^{2+} influx is essential for antigen-induced migration of mast cells.

Since the membrane potential determines the driving force for Ca^{2+} influx in mast cells [14], we performed migration assays in medium with a high K^{+} concentration, which prevents membrane hyperpolarization after antigen stimulation. Under high K^{+} conditions, sensitized BMMC failed to migrate even in the presence of DNP-HSA (Fig. 2E). This result confirms that a sufficient driving force for the Ca^{2+} influx is required to cause antigen-stimulated BMMC migration.

3.4. TRPM4 function is important for actin cytoskeleton (re)organization

A dynamic F-actin rearrangement (polymerization/depolymerization) plays a crucial role in cell migration [17,18]. Using a fluorescent phalloidin probe, we examined whether the loss of TRPM4 function affects actin cytoskeleton rearrangements in sensitized BMMC. In the absence of DNP-HSA,

Fig. 3. TRPM4 channels regulate actin cytoskeleton polymerization in BMMC after stimulation with antigen. (A) Cortical distribution of F-actin in sensitized wild-type and *trpm4*^{-/-} mast cells. Typical images are shown with (right) or without (left) 10 min application with 1 ng/ml DNP-HSA. (B) F-actin layer size in DNP-HSA-stimulated WT and TRPM4-deficient BMMC. Note the decreased F-actin contents in the *trpm4*^{-/-} cells. (C) Distribution of globular actin (red staining) in sensitized wild-type and *trpm4*^{-/-} mast cells. Typical images are shown with (right) or without (left) 10 min application with 1 ng/ml DNP-HSA. The DAPI staining (blue) visualizes nuclei. (D and E) Quantifications of globular actin signals in sensitized wild-type (D) and *trpm4*^{-/-} BMMCs (E) are represented as histograms of absolute pixel frequencies. (F) Effects of cytochalasin B on DNP-HSA-induced migration. **p* < 0.05, indicates the comparison between non-treated and DNP-treated WT cells, †*p* < 0.05, between DNP-treated WT and *trpm4*^{-/-} cells with or without cytochalasin B.

wild-type and *trpm4*^{-/-} BMMC displayed a similar level of F-actin level per cell (Fig. 3A and B). A 10 min application of DNP-HSA to wild-type BMMC induced no change of F-actin pattern when compared to non-stimulated cells. In contrast, *trpm4*^{-/-} BMMC showed significant decrease of F-actin formation (~20%) upon stimulation with DNP-HSA (Fig. 3A and B). This observation was related to elevated levels of globular actin in *trpm4*^{-/-} BMMC (Fig. 3C–E).

To confirm the importance of F-actin rearrangements on BMMC migration, we used cytochalasin B, an inhibitor of F-actin polymerization [19]. A 15 min pretreatment with 10 μM cytochalasin B resulted in significant decrease of migration of wild-type BMMC after stimulation with DNP-HSA (Fig. 3F).

3.5. TRPM4 contributes to SCF-induced BMMC migration

We have shown that antigen stimulation induced a direct effect on BMMC migration, which does not depend on a concentration gradient of the stimulating agent as would be required for chemotaxis. In contrast, stem cell factor (SCF) is a chemotactic factor of mast cell migration [4,20]. Application of SCF directly to BMMC cells results in a slow increase of $[Ca^{2+}]_i$, which is significantly higher in *trpm4*^{-/-} compared to WT mast cells (Fig. 4A). Wild-type BMMC displayed concentration-dependent chemotaxis when SCF was applied in bottom reservoir of the Boyden-chamber (Fig. 4B and C). Conversely, *trpm4*^{-/-} BMMC showed an impaired chemotaxis

Fig. 4. SCF induced chemotaxis in BMMC. (A) Mean traces for the time-dependent change in $[Ca^{2+}]_{cyt}$ in Fura-2-loaded BMMCs after the application of SCF (100 ng/ml; at arrowhead) to WT ($n = 103$) and *trpm4*^{-/-} BMMCs ($n = 130$). $p < 0.01$. (B) SCF-induced migration is dependent on the gradient. SCF (100 ng/ml) was present in the upper and/or the bottom wells as indicated. (C) Concentration dependence of the SCF-induced chemotaxis. (D) TRPM4-deficient mast cells failed to migrate after SCF stimulation (black column: untreated; striped column: 100 ng/ml SCF). (E) Intracellular Ca^{2+} dependence of the SCF-stimulated chemotaxis (black column: untreated; striped column: 100 ng/ml SCF). (F) Effects of cytochalasin B on BMMC chemotaxis induced by SCF (black column: untreated; striped column: 100 ng/ml SCF). * $p < 0.05$, and † $p < 0.05$ as explained in Fig. 2.

triggered by SCF in BMMC (Fig. 4D). Similar inhibiting effects on the chemotaxis towards SCF was caused by application of 100 μ M flufenamic acid (not shown), by pretreatment with 50 μ M BAPTA-AM for 20 min or by 15 min preincubation with 10 μ M cytochalasin B (Fig. 4F and G, respectively). These results convincingly show the involvement of TRPM4 in chemotactic migration of non-sensitized BMMC with SCF.

4. Discussion

It is generally established that mast cells play a critical and multifaceted role in IgE-dependent hypersensitivity reactions, inflammation and innate immune defense against pathogens. In

addition to the production and release of various chemoattractants and pro-inflammatory mediators, mast cells have been shown to increase in numbers (mastocytosis) during inflammatory conditions such as airway hyper-reactivity [21,22], interstitial cystitis [23], and wound healing [24]. In some of these cases, it has been shown that this increase is not due to a proliferation of local mast cells but is, in fact, a product of mast cell recruitment from other body sites. The exact mechanisms by which this recruitment occurs remain unclear [25].

We show here that both antigen-induced migration and SCF-mediated chemotaxis are deficient in *trpm4*^{-/-} mast cells. Both processes are highly Ca^{2+} -dependent. When intracellular and extracellular Ca^{2+} are sequestered, mast cells do not migrate. But

apparently there also exists an upper Ca^{2+} level for this process, which is set by TRPM4. We have shown before that in *trpm4*^{−/−} mast cells, antigen stimulation leads to significantly increased Ca^{2+} influx compared to WT mast cells, due to a lack of control on the driving force for Ca^{2+} influx, leading to increased degranulation of mast cells and increased cutaneous anaphylaxis response in KO mice. Furthermore, we now show that also SCF stimulation of mast cells induces a Ca^{2+} rise, which is significantly higher in KO compared to WT mast cells. Thus, we hypothesize that intracellular Ca^{2+} levels in a mast cell must be constrained within a specific concentration window to allow proper migration in an antigen- or SCF-directed fashion. Importantly, the lack of migration in our experiments is likely not due to increased degranulation or even cell death because of the very low antigen concentrations used for stimulating migration, and since we observe similar effects between SCF- and DNP-stimulated migration.

The cellular events downstream of intracellular Ca^{2+} leading to mast cell migration are currently unclear, but involve the actin cytoskeleton. In this context, the rearrangement of actin cytoskeleton is obviously important. Cell migration is critically dependent on actin cytoskeleton dynamics [26]. Our results show indeed that antigen stimulation of sensitized *trpm4*^{−/−} BMMC displayed a significant (~20%) decrease in F-actin content. This observation was related to elevated levels of globular actin in *trpm4*^{−/−} BMMC. In line with these results pretreatment of sensitized BMMC with cytochalasin B, an actin polymerization inhibitor, resulted in strong impairment of BMMC migration after stimulation with antigen. Previous reports have demonstrated that a rise in cytosolic Ca^{2+} concentration is associated with actin depolymerization in mast cells [27,28]. Further details on the mechanism of this are, however, lacking.

Mast cells derive from pluripotent hemopoietic cells in bone marrow. Partially differentiated, they enter the circulation and complete their differentiation in peripheral tissues. We have shown before that mast cell development and the number of mast cells in *in vivo* tissue is not different between WT and *trpm4*^{−/−} mast cells [14]. Thus, the above-described migratory defect does not influence mast cell maturation and differentiation.

The *trpm4*^{−/−} mouse is not the first genetic model with a deficient mast cell migration phenotype. Previously, it was shown that mast cells deficient of fyn or syk kinase, several PKC isoforms or the fyn kinase regulatory protein PTPα have similar defects in SCF-mediated chemotaxis [29–31]. However, we have previously shown that downstream activity of MAP-kinases (i.e. p38 and Akt) and PLCγ1 and γ2 upon FcεRI is not different between WT and *trpm4*^{−/−} mice [14]. This indicates that fyn and syk kinase activity is normal in *trpm4*^{−/−} mast cells, and suggests that the intracellular Ca^{2+} level is the major malefactor in *trpm4*^{−/−} mast cell migration. It might be tempting to speculate that faulty regulation of PKC isoforms by Ca^{2+} in *trpm4*^{−/−} mast cells is causing the migration defect [31].

It will be intriguing to check whether migration of other cell types is similarly affected in *trpm4*^{−/−} mice. Interesting in this regard is a recent publication showing an essential role for TRPM4 in migration, but not maturation of dendritic cells, much in the same way as for mast cells [32]. Migration is a somewhat underappreciated aspect of mast cell physiology. However, SCF-mediated mast cell infiltration and activation aggravates inflammation and immunosuppression in a tumor microenvironment and promotes tumor growth [33]. Also, mast cell migration is stimulated by angiogenic factors such as vascular endothelial cell growth factor and platelet-derived growth factor [34]. Thus, it might be interesting to study tumor growth in *trpm4*^{−/−} mice, to assess whether inhibition of mast cell migration could serve as therapeutic strategy for cancer.

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