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TRPC6 Regulates CXCR2-Mediated Chemotaxis of Murine Neutrophils

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Unraveling the mechanisms involved in chemotactic navigation of immune cells is of particular interest for the development of new immunoregulatory therapies. It is generally agreed upon that members of the classical transient receptor potential channel family (TRPC) are involved in chemotaxis. However, the regulatory role of TRPC channels in chemoattractant receptor-mediated signaling has not yet been clarified in detail. In this study, we demonstrate that the TRPC6 channels play a pronounced role in CXCR2-mediated intermediary chemotaxis, whereas N-formyl-methionine-leucine-phenylalanine receptor–mediated end-target chemotaxis is TRPC6 independent. The knockout of TRPC6 channels in murine neutrophils led to a strongly impaired intermediary chemotaxis after CXCR2 activation which is not further reinforced by CXCR2, PI3K, or p38 MAPK inhibition. Furthermore, CXCR2-mediated Ca\(^{2+}\) influx but not Ca\(^{2+}\) store release was attenuated in TRPC6–/– neutrophils. We demonstrate that the TRPC6 deficiency affected phosphorylation of AKT and MAPK downstream of CXCR2 receptor activation and led to altered remodeling of actin. The relevance of this TRPC6–depending defect in neutrophil chemotaxis is underscored by our in vivo findings. A nonseptic peritoneal inflammation revealed an attenuated recruitment of neutrophils in the peritoneal cavity of TRPC6–/– mice. In summary, this paper defines a specific role of TRPC6 channels in CXCR2-induced intermediary chemotaxis. In particular, TRPC6–mediated supply of calcium appears to be critical for activation of downstream signaling components. The Journal of Immunology, 2013, 190: 5496–5505.
subfamily (for review see Ref. 15). Store-operated calcium entry, however, is mediated by STIM and Orai proteins via inositol 1,4,5-trisphosphate–mediated depletion of intracellular Ca2+ stores probably in concert with TRPC channels (for review see Ref. 16). TRPC6 predominantly regulates ROCE-mediated Ca2+ entry (17, 18). There has been increasing evidence in the past years that TRPC channels are involved in directed cell migration (19–23). TRPC6 channels have been shown to be related to regulation of actin dynamics during podocyte migration (24), cross-regulation of TRPC5 in migration of endothelial cells (25) and MIP-2–induced migration and actin dynamics of murine neutrophils (21). However, so far it is not known whether TRPC6 channels are involved in neutrophil chemotaxis and whether TRPC6 channels can be assigned to end-target or intermediary chemoattractant signaling.

Using wild-type (WT) and TRPC6-deficient (TRPC6−/−) neutrophils, we show that TRPC6 channels play a crucial role in CXCR2-mediated intermediary chemotaxis, whereas they are not required for iMLF-mediated end-target chemotaxis. Consequently, less neutrophils are recruited into the peritoneal cavity of TRPC6−/− mice in a model of a nonseptic peritonitis. Collectively, our data indicate that TRPC6 channels may be a therapeutic target for inflammatory diseases such as asthma that are driven by intermediary chemoattractants.

Materials and Methods

Animals

For all experiments neutrophils from 8- to 10-wk-old male/female 129Sv/C57BL/6d WT and TRPC6−/− (26) mice were used. Only cells from 129Sv/C57BL/6d littermates were analyzed. Experimental protocols were approved by the local committee for animal care.

Reagents Abs and inhibitors

Fibronectin, fMLF, casein, Histopaque 1077, and Histopaque 1119 were purchased from Sigma-Aldrich (Steinheim, Germany), and the anti–CXCR2 Ab from Novus Biologicals (Hamburg, Germany). The anti-GAPDH Ab from Dianova (Hamburg, Germany), and the anti–phospho-Akt Ab as well as the anti-p38 MAPK Ab from Invitrogen (Darmstadt, Germany), and HBSS as well as PBS were purchased from Biochrom (Hamburg, Germany), and the anti-CXCR2 Ab from Novus Biologicals (Hamburg, Germany) was purchased from Sigma-Aldrich, the anti-GAPDH Ab from Dianova (Hamburg, Germany), and DMEM was purchased from Sigma-Aldrich (Steinheim, Germany) RPMI 1640 medium supplemented with 10% WEHI-3B–conditioned medium, 10% 100 U/ml penicillin, and 100 µg/ml streptomycin in a 37°C humidified atmosphere of 5% CO2.

Nonseptic peritonitis assays

A nonseptic peritonitis was induced by injecting 1 ml of a 9% casein (Sigma-Aldrich, Steinheim, Germany) solution in PBS into the peritoneal cavity of 10- to 12-wk-old male C57BL/6 mice. After 5 h, 5 ml sterile PBS was injected into the peritoneal cavity. The peritoneal exudates were collected through a short cut and cell count was quantified with a CASY TT cell counter (Roche, Mannheim, Germany). Moreover, the percentage of leukocytes in the peritoneal exudates was determined via Hemacolor staining (Merck, Darmstadt, Germany).

Preparation of chemokine mixture

A nonseptic peritonitis was induced in mice as described above. Peritoneal exudates were collected after 5 h and cells were pelleted (1500 rpm, 10 min) and discarded. The protein concentration of the peritonitis supernatant was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and adjusted to 4.5 mg/ml with HBSS. Aliquots of this chemokine-mixture were frozen and stored for later experiments.

In vitro three-dimensional chemotaxis assays

Neutrophils from overnight suspension cultures were pelleted (4°C, 1000 rpm, 10 min), resuspended in RPMI 1640 containing 25 mM HEPES and stored at 4°C until use. Chemotaxis experiments were performed in fibronectin-coated (1 µg/cm2, Sigma-Aldrich, Steinheim, Germany) chemotaxis chambers (ibidi, Martinsried, Germany). Cells were seeded in a three-dimensional collagen I (2.1 mg/ml [pH 7.4]; BD Biosciences, Heidelberg, Germany) matrix in HBSS. A chemokine gradient was established by supplementing HBSS with chemokine-mixture (final concentration 4.5 mg/ml) plus 100 µg/ml patentblue (Chroma, Münster, Germany), KC (final concentration 0.38 µM) plus 100 µg/ml patentblue or with fMLF (final concentration 1 µM) plus 100 µg/ml patentblue. When applicable the experimental solutions were supplemented with the following blockers (final concentrations in parenthesis): CXCR2 blocker SB225002 (500 nM), PAFR blocker ABT-491 (50 µM), P3K inhibitor LY 294002 (10 µM), p38 MAPK inhibitor SB203580 (1 µM). Notably, the concentration of the blockers was uniform in the chemotaxis chambers.

Cell migration was recorded in 5-s intervals for 30 min at 37°C using video cameras (models XC-ST70CE and XC-77CE, Hamamatsu, Tokyo, Japan) and PCI-vision frame grabber boards (Hamamatsu, Herrsching, Germany). Acquisition of image stacks was controlled by Hamamatsu’s Mercury Communication Systems, Carlsbad, CA). The tracking allowed the calculation of parameters such as migratory velocity (micrometers per minute) and translocation (micrometers) by using self-made JAVA programs and the National Institutes of Health ImageJ software (http://rsb.info.nih.gov/ij) (29). Migration was defined as the movement of the cell center per time unit, the velocity was calculated applying a three point difference quotient. Translocation was determined as the distance between the cells’ positions at the start and the end of the experiment. The chemotaxis index was defined as a quotient of the translocation in the direction of the chemokine gradient and the total distance covered during the course of the experiment.

Intracellular calcium measurements

Neutrophils from overnight suspension cultures were pelleted (4°C, 1000 rpm, 10 min), resuspended in RPMI 1640 containing 25 mM HEPES and stored at 4°C until use. Ca2+ measurements were performed in fibronectin coated (1 µg/cm2) µ-slide 1 chambers (ibidi, Martinsried, Germany). Cells were preincubated for 20 min with RPMI 1640 containing 25 mM HEPES and 3 µM of the Ca2+ dye Fura-2-AM (Calbiochem) at room temperature. After dye loading cells were seeded into µ-slide 1 slides for 10 min at 37°C and washed blindly with 1 ml Ringer solution (122.5 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl2, 0.8 mM MgCl2, 5.5 mM Na-glucose, and 10.0 mM HEPES [pH 7.4]) or Ca2+ free Ringer solution (122.5 mM 5.4 mM NaCl, KCl, 5 mM EGTA, 0.8 mM MgCl2, 5.5 mM Na-glucose, and 10.0 mM HEPES [pH 7.4]). Experiments were carried out at room temperature. Excitation wavelength alternated between 340 and 380 nm, and the fluorescence emission was recorded at 500 nm. Images were acquired in 5-s intervals. Motility was determined, camera and data acquisition were controlled by MetaFlour software (Visitron Systems, Puchheim, Germany). Fluorescence intensity was measured over the whole cell area and was corrected for background fluorescence. During measurements, a 5-min control period with Ringer
solution or Ca\(^{2+}\) free Ringer solution was followed by 10 min stimulation with 1 μM IMLF, 0.13 μM KC, or 1.125 mg/ml chemokine mixture. At the end of each experiment, the measurements were calibrated by applying 2 μM ionomycin (MP Biomedicals, Solon, OH) containing Ringer solution with 5 mM EGTA or 5 mM Ca\(^{2+}\). Cytosolic Ca\(^{2+}\) concentration [Ca\(^{2+}\)] was calculated as described before (30) and area under the curve was calculated as integral calculated for the time period following the application of the agonist.

The manganese (Mn\(^{2+}\)) quenching method was used for indirectly measuring chemotactant-induced Ca\(^{2+}\) influx (23, 31). Mn\(^{2+}\) enters the cell via Ca\(^{2+}\) channels, binds to Fura-2 with higher affinity than Ca\(^{2+}\) and thereby decreases the Ca\(^{2+}\)-induced fluorescence intensity. The decrease of Ca\(^{2+}\)-bound Fura-2 fluorescence intensity is used as an indirect measure of Ca\(^{2+}\) influx. Fura-2 was excited at its isobestic point at 365 nm, where the emitted fluorescence at 500 nm is independent of changes in [Ca\(^{2+}\)]. Fura-2 loaded neutrophils were seeded into ibidi slides for 10 min at 37°C and washed gently with 1 mM Mn\(^{2+}\) Ringer solution (122.5 mM NaCl, 5.4 mM KCl, 250 mM NaCl, 0.8 mM MgCl\(_2\), 5.5 mM Cl\(_2\), and 10.0 mM HEPES [pH 7.4]). Experiments were carried out at room temperature and images were acquired in 5-s intervals. During measurements, a 3-min control period with Mn\(^{2+}\) Ringer solution was followed, in the continued presence of Mn\(^{2+}\), by 15 min stimulation with 1 μM IMLF or 0.13 μM KC. Fluorescence intensity was measured over the whole-cell area and was corrected for background fluorescence. Regression analysis of fluorescence intensity over time was performed to determine the change in fluorescence quenching Δ m (%/h) before and after chemokinetic stimulation.

**Western blotting**

Neutrophils were seeded in fibronectin-coated (1 μg/cm\(^2\)) cell culture dishes and stimulated with 1 μM for 1 min with the respective chemotactant. Afterwards cells were lysed with radioimmunoassay lysis buffer containing: 25 mM Tris HC1 (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, protease inhibitor (Complete Mini; Roche), and phosphatase inhibitor (Phos Stop; Roche). The protein concentration of the lysates was determined with the Pierce Bicinchoninic Acid Protein Assay Kit (Thermo Scientific). Equal amount of proteins were transferred on nitrocellulose membranes (PROTRAN; Schleichter Schuell, Dassel, Germany). After blocking unspecific binding sites (5% fat-free milk and 0.5% Tween in PBS) proteins of interest were detected overnight using the following primary Abs in the described blocking solution: anti-CXCR2 Ab (1:100), anti–phospho-Akt Ab (1:500) and anti–phalloidin for 45 min, and samples were covered with mounting medium (Dako, Carpinteria, CA). Images were taken under widefield fluorescence microscopy. The protein amount was corrected by the amount of housekeeping proteins, membrane or fMLF-induced chemotaxis of WT or TRPC6\(^{-/-}\) neutrophils. Cells were seeded in chemotaxis slides in a three-dimensional collagen I matrix (see in vitro three-dimensional chemotaxis assays).

**Results**

**Loss of TRPC6 channels decreases immune response in vivo**

To confirm the physiological relevance of the loss of TRPC6 channels, we investigated neutrophil recruitment during an inflammation in vivo. We induced a nonseptic peritonitis by injecting a 9% casein solution into the peritoneum of WT and TRPC6\(^{-/-}\) deficient mice. After 5 h, the total number of invaded cells and the percentage of neutrophils were quantified. TRPC6\(^{-/-}\) mice showed a reduced number of invaded neutrophils (Fig. 1). The total number of invaded cells was diminished by 33% from 25.3 × 10\(^6\) ± 4.1 (WT) to 17.0 × 10\(^6\) ± 2.8 (TRPC6\(^{-/-}\)). The number of invaded neutrophils was reduced by 38% from 13.6 × 10\(^6\) ± 1.8 (WT) to 8.46 × 10\(^6\) ± 1.3 (TRPC6\(^{-/-}\)). Thus, on the basis of the number of invaded neutrophils, TRPC6\(^{-/-}\) mice develop a diminished peritoneal inflammation after casein treatment.

**Intermediary chemotaxis depends on TRPC6 channels**

For chemotaxis experiments, neutrophils were seeded in a three-dimensional collagen gel into ibidi chemotaxis slides. To mimic the physiological conditions encountered by neutrophils in vivo, we used a mixture of intermediary chemotacticants. For this purpose, we induced a nonseptic peritonitis with casein and isolated the cell-free supernatant of the peritoneal exudates from WT mice. Chemotaxis toward this intermediary chemokine mixture was strongly impaired in TRPC6\(^{-/-}\) neutrophils (Fig. 2A, Supplemental Videos 1, 2). Although the velocity of TRPC6\(^{-/-}\) neutrophils was almost identical as that of WT cells (10.3 ± 0.3 versus 11.6 ± 0.3 μm/min), the translocation and chemotaxis index was strongly reduced. Translocation decreased from 170.7 ± 7.0 μm (WT) to 106 ± 6.2 μm (TRPC6\(^{-/-}\)), and the chemotaxis index was reduced by 50% from 0.44 ± 0.01 (WT) to 0.22 ± 0.02 (TRPC6\(^{-/-}\)). These findings indicate that TRPC6 channels are predominantly involved in the “compass mechanism” of neutrophils in a gradient of intermediary chemokines. In contrast, their impact on the “migration engine” appears only marginal.

**F-actin staining**

Neutrophils were seeded on fibronectin-coated coverslips (1 μg/cm\(^2\)) for 10 min. After 40 s or 60 s stimulation with 1 μM IMLF or 0.13 μM KC, cells were fixed with 3.5% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in TBS. Unspecific binding sites were blocked with 3% BSA in PBS. Cells were incubated with Oregon Green 488–conjugated phalloidin for 45 min, and samples were covered with mounting medium (Dako, Carpinteria, CA). Images were taken under widefield fluorescence conditions using an inverted microscope (Axiovert 200; Zeiss, Jena, Germany) with a 100 × 1.45 objective lens and a digital camera (model 9.0, RT-SE-Spot; Visirion Systems, Puchheim, Germany). Image aggregation was controlled by MetaVue software (Visirion Systems). Fluorescence intensity was measured over the whole-cell area while values were corrected for background fluorescence. Fluorescence intensity was calculated in percentage of the mean intensity of unstimulated WT or TRPC6\(^{-/-}\) cells.

**Statistical analysis**

All experiments were repeated at least three times. Values are reported as mean values ± SEM. All data were tested for normality before performing any statistical analysis. For normally distributed data, the Student t test was used; otherwise, the Kruskal–Wallis or Mann–Whitney U test was used for statistical analyses. Differences between experimental groups reaching p ≤ 0.05 were considered significant.
To analyze which chemokine receptors could be involved in the signaling events mediated by the ex vivo chemokine mixture, we inhibited two major intermediary chemokine receptors pharmacologically. CXCR2 binds a wide range of chemokines like KC, MIP-2, and LIX (32–35) and is classified as the most important receptor for CXC-motif chemokines in mice. To inhibit CXCR2-mediated chemotaxis, we used SB225002 (36, 37). Furthermore, ABT-491 was used to block PAFR-mediated chemotaxis (38, 39). The CXCR2 inhibitor SB225002 reduced chemotaxis of WT neutrophils to the same extent as the lack of TRPC6 channels (Fig. 2A). When WT neutrophils were treated with SB225002, velocity was decreased by 16% to 9.7 ± 0.3 μm/min (Fig. 2B). Translocation and chemotaxis index were reduced by 45% to 94.2 ± 6.8 μm and 58% to 0.18 ± 0.03, respectively (Fig. 2C, 2D). Notably, CXCR2 blockade elicited absolutely no additional effect in TRPC6−/− neutrophils. Blocking the PAFR with ABT-491 produced similar results. PAFR blockade had a strong effect on WT neutrophils, whereas TRPC6−/− neutrophils were hardly affected. Velocity of WT cells was reduced by 21% to 9.4 ± 0.3 μm/min, translocation was decreased by 31% to 117.9 ± 9.8 μm, and chemotaxis index was diminished by 31% to 0.33 ± 0.04. In TRPC6−/− neutrophils, ABT-491 only slightly reduced the velocity by 11% to 9.2 ± 0.4 μm/min, whereas translocation and chemotaxis index were not affected.

These data indicate that ligands for both chemoattractant receptors, the CXCR2 and PAFR, are important components of the ex vivo chemokine mixture used in our experiments. Moreover, these findings suggest that TRPC6 channels are crucial for both CXCR2- and PAFR-mediated chemotaxis.

CXCR2-mediated chemotaxis requires TRPC6 channels
To further substantiate our hypothesis that TRPC6 channels are required for CXCR2-mediated chemotaxis, we substituted the chemokine mixture with murine KC, a ligand of CXCR2. KC is supposed to be the murine equivalent of the human Gro-α (CXCL1) (32). For three-dimensional chemotaxis experiments, we used 0.38 μM KC. As shown in Fig. 3, KC is a very powerful chemoattractant for WT neutrophils. Importantly, chemotaxis of TRPC6−/− neutrophils toward KC was even more strongly impaired than chemotaxis toward the chemokine mixture (reduction of the chemotaxis index by 80% from 0.45 ± 0.02 in WT cells to 0.09 ± 0.03 in TRPC6−/− cells) (Fig. 3D). As in the previous experiments, velocity was hardly affected (reduction of 8% from 15.8 ± 0.4 to 14.5 ± 0.4 μm/min), whereas translocation was also markedly diminished (reduction by 42% from 235.7 ± 14.4 to 136.9 ± 10.7 μm). The dramatic phenotype of TRPC6−/− neutrophils was mimicked by inhibiting CXCR2 with SB225002. In the presence of SB225002, chemotaxis of WT cells toward KC was as inefficient as that of TRPC6−/− neutrophils without inhibition of CXCR2 (reduction by 72% from 0.45 ± 0.02 to 0.12 ± 0.03) (Fig. 3D). Moreover, SB225002 reduced the velocity of WT cells by 20% to 12.6 ± 0.4 μm/min and translocation by 57% to 102.0 ± 9.0 μm. In TRPC6−/− neutrophils, CXCR2 blockade with SB225002 elicited only small additional effects. Blockade of CXCR2 in TRPC6−/− cells reduced the chemotaxis index by 91% from 0.45 ± 0.02 (WT) to 0.04 ± 0.02 (SB225002 treated TRPC6−/−) with TRPC6 knockout accounting for 80% of this inhibition (see above). Moreover, velocity was reduced by 33%
from 15.8 ± 0.4 μm/min (WT) to 10.6 ± 0.3 (SB225002-treated TRPC6−/−), and transmigration was diminished by 72% from 235.7 ± 14.4 μm (WT) to 66.5 ± 5.9 μm (SB225002-treated TRPC6−/−). Thus, the importance of TRPC6 channels in CXCR2-mediated chemotaxis toward the intermediary chemokine KC is further underlined by the observation that CXCR2 inhibition of TRPC6−/− cells elicited hardly any additive effect. 

\[ fMLF-mediated end-target chemotaxis is TRPC6 independent \]

To analyze the chemotaxis toward an end-target chemoattractant, we used 1 μM of the bacterially derived iMLF. In three-dimensional chemotaxis experiments both WT and TRPC6−/− neutrophils migrated in a directed fashion within the iMLF gradients. No obvious difference between WT and TRPC6−/− neutrophils could be seen in velocity, translocation, and chemotaxis index (Fig. 4, Supplemental Videos 3, 4). Fig. 4A shows individual trajectories of WT and TRPC6−/− neutrophils in iMLF gradients with similar patterns for both cell types. Fig. 4B–D display statistical evaluations. WT and TRPC6−/− neutrophils did not differ; they covered ∼150 μm within 30 min, migrated at a rate ∼13 μm/min, and their chemotaxis index was ∼0.32.

**Loss of TRPC6 effects CXCR2-mediated PI3K and p38 MAPK activation**

To clarify the question of how the loss of TRPC6 channels influences CXCR2-mediated signaling, we applied inhibitors for two important downstream targets of CXCR2: PI3K and p38 MAPK. LY 294002 was used for PI3K inhibition, and SKF-86002 was applied for blockade of p38 MAPK. Inhibition of PI3K had a distinct effect on KC-mediated chemotaxis and translocation of WT neutrophils (Fig. 5A, 5C, 5D). Chemotaxis was reduced by 51% from 0.45 ± 0.02 to 0.22 ± 0.02, and translocation was diminished by 45% from 235.7 ± 14.4 to 128.7 ± 8.3. Velocity of WT neutrophils was only hardly affected by PI3K inhibition (reduction by 14% 15.8 ± 0.4 to 13.6 ± 0.5 μm/min) (Fig. 5B). In TRPC6−/− neutrophils, inhibition of PI3K with LY 294002 caused only minor additional effects. Blockade of PI3K in TRPC6−/− cells induced no significant additional effect on chemotaxis. Chemotaxis index of TRPC6−/− cells was reduced by 64% from 0.45 ± 0.02 (WT) to 0.16 ± 0.02 (LY 294002–treated TRPC6−/−). Furthermore, translocation was diminished by 52% from 235.7 ± 14.4 μm (WT) to 111.1 ± 8.3 μm (LY 294002–treated TRPC6−/−), and velocity was reduced by 20% from 15.8 ± 0.4 μm/min (WT) to 12.6 ± 0.6 (LY 294002–treated TRPC6−/−).

Blockade of p38 MAPK via SKF-86002 in WT and TRPC6−/− neutrophils caused very similar effects on chemotaxis as inhibition of PI3K (Fig. 5 A–D). In WT cells, chemotaxis was reduced by 49% from 0.45 ± 0.02 to 0.23 ± 0.03, and translocation was diminished by 40% from 235.7 ± 14.4 to 141.4 ± 11.4. Velocity of WT cells was only minimally reduced by p38 MAPK inhibition (18% reduction from 15.8 ± 0.4 to 13.0 ± 0.5 μm/min). In TRPC6−/− cells, p38 MAPK inhibition was as ineffective as PI3K blockade in inducing any additional impairment of chemotaxis. The inhibition of p38 MAPK elicited a 71% reduction of chemotaxis from 0.45 ± 0.02 (WT) to 0.13 ± 0.03 (SKF-86002–treated TRPC6−/−). Moreover, translocation was diminished by 52% from 235.7 ± 14.4 μm (WT) to 113.1 ± 7.8 μm (SKF-86002–treated TRPC6−/−), and velocity was diminished by 22% from 15.8 ± 0.4 μm/min (WT) to 12.4 ± 0.5 (SKF-86002–treated TRPC6−/−). These data indicate that TRPC6 channels are required for the proper function of two components of the CXCR2
signaling pathway, PI3K and p38 MAPK, which have a direct impact on chemotaxis of neutrophils. This conclusion was confirmed with biochemical experiments. To this end, we compared phosphorylation of Akt and p38 MAPK via immunoblot. They are key elements of PI3K and MAPK pathways, respectively. CXCR2-mediated phosphorylation of Akt was reduced in TRPC6−/− cells after stimulation with chemokine mixture or KC (Fig. 5E, 5F). Phosphorylation of p38 MAPK was decreased in TRPC6−/− neutrophils after stimulation with chemokine mixture and increased in TRPC6−/− cells when activated with KC. Finally, we analyzed whether the attenuated response of TRPC6−/− neutrophils toward CXCR2 ligands is secondary to a reduced CXCR2 expression in TRPC6−/− cells. CXCR2 was expressed at identical levels in both WT and TRPC6−/− neutrophils (Fig. 5E).

CXCR2-mediated Ca2+ influx is reduced in TRPC6−/− neutrophils

In the next set of experiments, we determined whether TRPC6 channels were involved in chemoattractant-mediated Ca2+ signaling. To this end, we measured the cytosolic Ca2+ concentration in WT and TRPC6−/− neutrophils in response to 1 μM fMLF, the chemokine mixture, or 0.13 μM KC. Application of all chemoattractants led to a transient elevation of [Ca2+]i with a fast rise and a slow decay toward baseline. These experiments are depicted in Fig. 5. The left panel displays mean [Ca2+]i over time, and the right panel represents the respective integrals calculated for the time period following the application of the agonist.

The chemokine mixture and KC elicited a transient rise of [Ca2+]i in WT and TRPC6−/− neutrophils (Fig. 6Ai, 6Aii, 6Bi, 6Bii). However, in TRPC6−/− neutrophils, the elevation of [Ca2+]i was attenuated. As expected from the behavior in chemotaxis experiments, 1 μM fMLF elicited the same response in WT and TRPC6−/− neutrophils (Fig. 6Ci, 6Cii). Thus, the defect of TRPC6−/− neutrophils in intermediary chemotaxis is accompanied by a depression of intracellular Ca2+ signaling after CXCR2 activation by its specific agonists.

To identify the source of the mobilized Ca2+, we measured the [Ca2+]i in response to 1 μM fMLF or 0.13 μM KC without Ca2+ in the extracellular medium or by adding manganese to the extracellular medium. By omitting Ca2+ from the extracellular medium, the mobilization of Ca2+ from internal stores can be analyzed.
Mn2+ is an indirect measure of Ca2+ influx. Fig. 7Ci and 7Di depict the decrease of the Fura-2 fluorescence following the addition of an attenuated Ca2+ mobilization from intracellular stores. The TRPC6 integrals of [Ca2+]i are depicted in (Ai). KC elicited a higher quenching rate of the chemotactic receptor activation, and Fig. 7Cii and Dii describe the percentage decrease of the fura-2 fluorescence intensity after attractants induced a similar response in WT and TRPC6 neutrophils (Fig. 7Ai,7 Bi). Thus, the depression in CXCR2-mediated Ca2+ signaling in TRPC6 neutrophils is not based on an attenuated Ca2+ mobilization from intracellular stores.

Using the manganese-quenching method, we determined the role of TRPC6 in chemoattractant receptor mediated Ca2+ influx. The decrease of the Fura-2 fluorescence following the addition of Mn2+ is an indirect measure of Ca2+ influx. Fig. 7Cc and 7Dc depict the percentage decrease of the fura-2 fluorescence intensity after chemoattractant receptor activation, and Fig. 7Ciii and Diii describe the Δ of slope Δ m (m1 − m2) directly before (m1) and after agonist application (m2). KC elicited a higher quenching rate of the fluorescence in WT compared with TRPC6 neutrophils. In contrast, application of fMLF leads to the same depression of Fura-2 fluorescence in WT and TRPC6 neutrophils.
Discussion

Chemotaxis is based on the translation of an extracellular chemoattractant signal into intracellular signaling cascades. On a cellular level, this involves the activation of G protein–coupled receptors, which lead to the activation of PI3K, MAPK, PLC, as well as Rho-GTPases and induces the reorganization of the actin cytoskeleton (for review see Refs. 40 and 41). It is generally accepted that the activation of chemoattractant receptors leads to a rise of cytosolic Ca\(^{2+}\) (13, 42, 43). However, the contribution of molecularly identified Ca\(^{2+}\) transport proteins to chemotactic signaling and their physiological significance have not yet been clarified in detail. Evans and Falke (10) showed that Ca\(^{2+}\) influx is essential for actin polymerization, and PI3K activity in chemokinetically stimulated RAW macrophages and speculated about a contribution of TRP channels. In recent years, several groups verified the influence of different members of the TRPC channel family in chemotaxis. We could demonstrate the involvement of TRPC1 in fibroblast growth factor 2–induced chemotaxis of epithelial cells and Damann et al. (21) revealed a role of TRPC6 in neutrophil chemokinesis (23). On our way to identify the physiological role of molecularly identified Ca\(^{2+}\) channels in chemoattractant receptor induced Ca\(^{2+}\) signaling, we focused on TRPC6 channels in neutrophil chemotaxis.

In this study, we demonstrated that TRPC6 channels are essential signaling components in neutrophil chemotaxis triggered by CXCR2 activation. This conclusion is based on the following observations: 1) chemotaxis of TRPC6\(^{-/-}\) neutrophils in a gradient of an ex vivo chemokine mixture is strongly impaired; 2) this defect was not further reinforced by CXCR2 blockade; 3) the CXCR2 activator KC almost completely failed to induce chemotaxis in TRPC6\(^{-/-}\) neutrophils; 4) Ca\(^{2+}\) influx in response to CXCR2 ligands is attenuated in TRPC6\(^{-/-}\) cells; and 5) TRPC6 knockout also impacts on the phosphorylation of intracellular signaling modules of CXCR2 and CXCR2-mediated actin polymerization. The physiological significance of these findings is underscored by our in vivo experiments. Using a nonseptic peritonitis model, we could show that less neutrophils emigrated into the peritoneal cavity of TRPC6\(^{-/-}\) mice. This is in line with the observations made in an allergic airway model, which revealed an attenuated recruitment of eosinophils into the alveolar space (44). Our data indicate that TRPC6 channels are not only required for CXCR2-mediated chemotaxis but also for PAFR-mediated chemotaxis. A PAFR-mediated activation of TRPC6 was shown before in human neutrophils (45). Thus, TRPC6 channel blockade could constitute a new anti-inflammatory therapeutic approach.

Importantly, our experiments clearly show a specificity of TRPC6 channels in chemotaxis. First, TRPC6 channels are not involved in signaling of all chemoattractants. They play no role at all in chemotaxis toward the end-target chemotractant fMLF. Moreover, TRPC6 channels almost exclusively affect the “compass mechanism” of chemotactating neutrophils. The “engine mechanism” appears to be unaffected because TRPC6\(^{-/-}\) neutrophils migrate almost with the identical speed as WT cells.

TRPC6 deficiency leads to impaired Ca\(^{2+}\) signaling after stimulation of the neutrophils with the chemokine mixture or single CXCR2 activation with KC. The Ca\(^{2+}\) measurements in the absence of extracellular Ca\(^{2+}\) and the Mn\(^{2+}\) quench experiments indicate that the remaining increase in [Ca\(^{2+}\)]\(_i\) in TRPC6-deficient cells may be induced by Ca\(^{2+}\) release from intracellular stores and by store-operated calcium entry through recently described STIM and Orai proteins in PMNs (46, 47), whereas ROCE through TRPC6 is lost. Expectedly, the rise of the [Ca\(^{2+}\)]\(_i\) was not altered in TRPC6\(^{-/-}\) neutrophils after stimulation with fMLF and in the absence of extracellular Ca\(^{2+}\). The question remains how TRPC6 channels exclusively control the compass-mechanism in CXCR2-mediated chemotaxis. A hypothetical organization of all signal transduction components in large signaling complexes remains an intriguing possibility. DAG is the known endogenous activator of TRPC6 (17, 48). DAG is a product of PLC-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate with PLC being activated by G protein–coupled receptors like CXCR2 (49, 50). Thus, CXCR2-mediated ROCE via TRPC6 channels is likely to be induced via DAG generated by PLC (Fig. 9). PI3K/Akt signaling is reported to play a central role in chemotaxis toward intermediate chemoattractants, whereas p38 MAPK signaling plays a subordinate role therein (4). We could support this hypothesis with our chemotaxis data. Blocking of PI3K or p38 MAPK affected chemotaxis of WT neutrophils, whereas there was no additive effect on chemotaxis in TRPC6\(^{-/-}\) cells. The TRPC6\(^{-/-}\)-dependent chemotaxis defect was accompanied by impaired Ca\(^{2+}\) signaling, reduced Akt phosphorylation, and diminished F-actin formation. This would be consistent with observations made in LPS-stimulated RAW macrophages. In these cells, Evans and Falke (10) described a Ca\(^{2+}\) dependence for maintaining PI3K activity at the leading edge. The loss of Ca\(^{2+}\) influx led to reduced F-actin dynamics in migrating cells (10, 21), which is supported by our observation that the loss of TRPC6 leads to a reduced F-actin formation after CXCR2 stimulation. Although the PI3K signaling element Akt is known to regulate actin dynamics via cofilin and girdin (51), no direct Ca\(^{2+}\) dependence is known for activation of PI3K. Thus, other regulators of actin dynamics such as Rac and Cdc42 are likely to be involved (52). Both are known to be tightly regulated by guanine-nucleotide-exchange factors, which are activated by PI3K-mediated phosphatidylinositol (3,4,5)-trisphosphate production and via Ca\(^{2+}\) (53–55). Furthermore, Rac activation and actin polymerization stimulates PI3K-mediated phosphatidylinositol (3,4,5)-trisphosphate production, which loops PI3K and Rac activation in a positive way (56–59). Thus, a reduced Rac activation based on diminished Ca\(^{2+}\) mobilization after CXCR2 stimulation in TRPC6\(^{-/-}\) neutrophils could disrupt the positive feedback loop involving PI3K and Rac. This would result in an incomplete amplification of chemoattractant receptor–mediated signaling cascades and thereby cause a defect in chemotaxis.

**Figure 9.** CXCR2-mediated TRPC6 activation. Binding of ligands to CXCR2 leads to activation of PLC. Inositol 1,4,5-trisphosphate mobilizes Ca\(^{2+}\) from internal stores, whereas DAG activates TRPC6. The following rise of cytosolic Ca\(^{2+}\) promotes the phosphorylation of Akt.


