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# Formyl-peptide receptor like 1: A potent mediator of the $Ca^{2+}$ release-activated $Ca^{2+}$ current $I_{CRAC}$ ☆

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ABSTRACT

In electrically non-excitable cells, one major source of $Ca^{2+}$ influx is through the store-operated (or $Ca^{2+}$ release-activated $Ca^{2+}$) channel by which the process of emptying the intracellular $Ca^{2+}$ stores results in the activation of $Ca^{2+}$ channels in the plasma membrane. Using both whole-cell patch-clamp and $Ca^{2+}$ imaging technique, we describe the electrophysiology mechanism underlying formyl-peptide receptor like 1 (FPRL1) linked to intracellular $Ca^{2+}$ mobilization. The FPRL1 agonists induced $Ca^{2+}$ release from the endoplasmic reticulum and subsequently evoked $I_{CRAC}$-like currents displaying fast inactivation in K562 erythroleukemia cells which expresses FPRL1, but had almost no effect in K562 cells treated with FPRL1 RNA-interference and HEK293 cells which showed no FPRL1 expression. The currents were impaired after either complete store depletion by the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase inhibitor thapsigargin, or after inhibition of PLC by U73122. Our results present the first evidence that FPRL1 is a potent mediator in the activation of CRAC channels.



Intracellular free $Ca^{2+}$ concentration is a ubiquitous signal that regulates a broad spectrum of kinetically disparate processes ranging from exocytosis to cell growth, inflammation, proliferation, and apoptosis [1,2]. In non-excitable cells, depletion of internal $Ca^{2+}$ stores opens store-operated $Ca^{2+}$ channels (SOC)[1] in the plasma membrane, a process called capacitative or store-operated $Ca^{2+}$ entry. In many cases, this elicits an inwardly rectifying, non-voltage-gated $Ca^{2+}$ current called $I_{CRAC}$, which is a major $Ca^{2+}$ current of these non-excitable cell types [3–5].

Formyl-peptide receptor like 1 (FPRL1), which was initially cloned as a formyl-peptide receptor (FPR) homologue and also known as lipoxin $A_4$ receptor ($LXA_4R$) or aspirin-triggered lipoxin receptor (ALXR), is one of the classic chemoattractant receptors, i.e., a G protein-coupled seven transmembrane receptor [6–8]. Previous reports suggested FPRL1 expressed in blood and brain [9–12], played an important role in signaling cell rolling, adhesion, migration, and homing to injured and inflamed tissue for repair [13,14]. Its most potent and selective agonists are lipoxins (LXs) such as lipoxin $A_4$ ($LXA_4$), lipoxin $B_4$ ($LXB_4$) and the aspirin-triggered 15-epi-$LXA_4$ (ATL), which are generated from arachidonic acid via sequential actions of lipoxygenases and play the "stop signals" role in inflammation, regulating inflammatory processes and their resolution [15–18]. Recently, several FPRL1 agonists such as endogenous lipid mediators BML-111 and annexin A1 (ANXA1) have been identified to play similar roles in inflammation [19–21].

Arachidonic acid (AA), the precursor of lipoxins whereas does not play its function by activating FPRL1, has been confirmed to evoke a non-capacitative $Ca^{2+}$ entry current (NCCE) namely arachidonate-regulated calcium current ($I_{ARC}$) [22–24] and inhibits capacitative $Ca^{2+}$ entry [25]. Although several studies have shown that lipooxygenase blockers reduced both the amount of $Ca^{2+}$ that is released from intracellular store in $Ca^{2+}$-free solution and the $Ca^{2+}$ influx component that occurred when external $Ca^{2+}$ was re-admitted [26–28], and that $LXA_4$ stimulated a $Ca^{2+}$ increase in many cell types [29–31], the effect of eicosanoids on the properties of $Ca^{2+}$ current is still vague. The exact mechanisms of FPRL1 agonists on mobilizing the cytosolic $Ca^{2+}$ have not been elucidated.

The present study is the first report of FPRL1 effects on the activation of store-operated $Ca^{2+}$ current $I_{CRAC}$. We focused on K562 erythroleukemia wild type (K562-wt) and HEK293 wild type (HEK293-wt) cell lines, representing models with different expression level of FPRL1: K562-wt cells express FPRL1 whereas HEK293-wt cells do not and K562 cells treated with a lentivirus short hairpin RNA

☆ FPRL1 mediates $I_{CRAC}$.

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[1] *Abbreviations used:* SOC, store-operated $Ca^{2+}$ channels; FPRL1, formyl-peptide receptor like 1; $LXA_4R$, lipoxin $A_4$ receptor; ALXR, aspirin-triggered lipoxin receptor; LXs, lipoxins; $LXA_4$, lipoxin $A_4$; $LXB_4$, lipoxin $B_4$; AA, arachidonic acid.

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(LVshRNA) vector interference against the FPRL1 receptor (K562–FPRL1–RNAi) express low levels of the receptor. Using whole-cell patch-clamp and $Ca^{2+}$ image technology, we found that the FPRL1 agonists ($LXA_4$, BML-111, and ANXA1) nonlinearly elicited $I_{CRAC}$-like currents in K562-wt cells. In contrast, activation of the $Ca^{2+}$ channels was negligible in cells with no FPRL1 (HEK293-wt) or low FPRL1 levels (K562–FPRL1–RNAi). In addition, $Ca^{2+}$ entry evoked by the agonists in K562-wt was via a phospholipase C (PLC) and store-dependent mechanism. Thus our results demonstrate that FPRL1 has an essential role in mediation of $Ca^{2+}$ release-activated $Ca^{2+}$ entry pathway in human erythroleukemia cells.

## Materials and methods

### *Cell culture*

Human erythroleukemia K562 cell line and human embryonic kidney cell line HEK293 were obtained from the American Type Cell Culture (ATCC) and were cultured in a 5% $CO_2$ incubator at 37 °C. K562 were cultured in glass flasks in RPMI-1640 containing 10% fetal bovine serum and antibiotics (100 mg $ml^{-1}$ streptomycin and 100 U $ml^{-1}$ penicillin) and were plated on coverslips (0.4 × 0.4 cm) 12–48 h before an electrophysiology experiment [32]. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and antibiotics were plated at least 4 h before patch-clamp experimentation [33].

### *Chemicals*

Arachidonic acid (AA) and lipoxin $A_4$ ($LXA_4$) were purchased from Cayman. BML-111 was obtained from Biomol. U73122 was bought from Alexis. SYBR Green I Nucleic Acid Gel Stain was from Cambrex. 2-Aminoethoxydiphenyl borate (2-APB), annexin A1 (ANXA1), Fluo-3/AM, Fura-2 acetoxymethyl ester (Fura-2/AM) and thapsigargin (TG) were bought from Sigma. $LXA_4$, BML-111, and ANXA1 were dissolved in ethanol as a 0.28, 1, and 1 mM stock, respectively. 2-APB was dissolved in DMSO (50 mM stock solutions) and freshly prepared every time. Final DMSO and ethanol concentration were <0.05%. $LXA_4$ was stored at −80 °C, the other stocks were stored at −20 °C and new aliquots were used for each coverslip.

### *RNA knockdown experiments*

For all transfection studies, K562 cell lines were seeded in 6-well plates until 80% confluent. Lentivirus short hairpin RNA vector of FPRL1 (LVshFPRL1) sequences were designed, synthesized, and labeled with RFP (GeneChem, Inc., Shanghai) and the latter were utilized to specifically knockdown FPRL1 expression by K562-wt cell line. For each of the LVshRNA constructs, the K562 cells were transfected using Lipofectamine-2000 reagent, after which these cells (K562–FPRL1–RNAi) were utilized for the functional studies 24–48 h later.

### *Quantitative real-time PCR*

Total cellular RNA was isolated from K562-wt, K562–FPRL1–RNAi, and HEK293-wt cells by the guanidium thiocyanate/phenol/chloroform method using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). The concentrations of total RNA were measured using an UV spectrophotometer (UV-1201, Shimadzu Corp., Kyoto, Japan). Four micrograms of total RNA was used in a reverse transcription reaction using 200 U of M-MLV Reverse Transcriptase in a 25 μl reaction mixture. First strand cDNAs were amplified using a real-time PCR thermal cycler (Rotor-Gene 3000A, Corbett, Australia). Expression levels of receptors were determined by reverse transcriptase polymerase chain reaction (RT-PCR). Real-time quantitative polymerase chain reaction (PCR) was performed to compare the amounts of $LXA_4$ receptor in the cells. The primers used for quantitative real-time PCR were as follows: 5′-AACCCC ATGCTTTACGTCTTTGTG-3′ (sense: nt 1045–1068) and 5′-ATTGG CAGCCGTGTCATTAGTTG-3′ (antisense: nt 1145–1167) for human $LXA_4$ receptor (GenBank Accession No. X63819), 5′-ACCAGCCCC AGCAAGAGCACAAG-3′ (sense: nt 1081–1103) and 5′-TTCAA GGGGTCTACATGGCAACTG-3′ (antisense: nt 1180–1203) for human GAPDH (GenBank Accession No. M33197). Both sizes of amplified DNA for human $LXA_4$ receptor, human GAPDH were 123. Optimal reaction conditions were 40 cycles of a two stage PCR (denaturation at 95 °C for 15 s, annealing at 53 °C for 1 min) after an initial denaturation step (95 °C for 10 min) [34]. For the relative comparison of each gene, the data of real-time PCR were analyzed with $\Delta\Delta C_t$ method [35]. Normalization the amount of sample cDNA added to each reaction, the $C_t$ value of the endogenous control (GAPDH) was subtracted from the $C_t$ value of each target gene.

### *Patch-clamp recordings*

Whole-cell patch-clamp experiments were conducted in the tight-seal whole-cell configuration at room temperature (20–25 °C) [28]. Sylgard-coated, fire-polished pipettes had DC resistances of 3–5 MΩ when filled with standard internal solution that contained (mM): CsCl 140, $MgCl_2$ 1, Mg–ATP 2, EGTA 10 ($[Ca^{2+}]_i$ clamped to about 140 nM by a Ca–EGTA/EGTA mixture of 4.6/5.4 mM so that stores would not be depleted passively by the $Ca^{2+}$ chelator), Hepes 10, adjusted to pH 7.2 with CsOH. Extracellular solution contained (mM): NaCl 145, KCl 2.8, $CaCl_2$ 10, $MgCl_2$ 2, CsCl 10, glucose 10, Hepes 10, adjusted to pH 7.2 with NaOH. Divalent ion-free external solution contained (mM): NaCl 155, KCl 2.8, CsCl 10, EDTA 2, glucose 10, Hepes 10, adjusted to pH 7.4 with NaOH. High resolution current recordings were acquired by a computer-based patch-clamp amplifier system (EPC-10; HEKA, Lambrecht, Germany). Holding potential was usually 0 mV. After acquisition of a stable current baseline (meaning to make sure that no inward currents develops within 90 s or so), drugs were applied to detect their effects on the activation of $Ca^{2+}$ currents. The development of $I_{CRAC}$ was assessed by measuring the current amplitudes at a potential of −80 mV, taken from high resolution currents in response to voltage ramps spanning the voltage range between −100 to +100 mV over a period of 150 ms, and delivered at a rate of 0.5–1 Hz. All voltages were corrected for a liquid-junction potential of 10 mV between external and internal solutions. Currents were filtered at 2.3 kHz and digitized at 100 μs intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp using the automatic capacitance compensation of the EPC-10.

### *$Ca^{2+}$ imaging*

Intracellular $Ca^{2+}$ was monitored using the fluorescent $Ca^{2+}$ indicator fura-2/AM as description in our previous study [36]. Briefly, the cells grown on coverslips with poly-D-lysine were loaded in extracellular solution (for composition see below) with 1 μM fura-2/AM for 30 min at 37 °C. Coverslips were then transferred to a chamber (about 1 ml) mounted on a microscope stage where they were superfused for at least 10 min with extracellular solution at a rate of 4 ml/min to wash away the extracellular dye. Measurements of $[Ca^{2+}]_i$ of single cells were performed using an inverted fluorescence microscope (IX70, Olympus, Tokyo, Japan) equipped with a dual excitation fluorometric imaging system (T.I.L.L. Photonics GmbH, Germany). The illumination was generated by a 75 W Xenon bulb. The excitation wavelength was alter-