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The role of calcium-activated potassium channels and store-operated calcium channels in human macrophages

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1. Introduction

1.1 Macrophages

1.1.1 Biology of macrophages

Origin and tissue distribution of macrophages

Macrophages belong to the mononuclear phagocytic system. During the hematopoiesis in the bone marrow, granulocyte-monocyte progenitor cells differentiate into promonocytes, which leave the bone marrow and enter the blood, where they differentiate into mature monocytes. Circulating monocytes in the bloodstream give rise to a variety of tissue-resident macrophages throughout the body, including alveolar macrophages in the lung, histiocytes in connective tissue, Kupffer cells in the liver, mesangial cells in the kidney, microglial cells in the brain and osteoclasts in bone. Using monoclonal antibodies, macrophages have been found to be highly heterogeneous; this heterogeneity reflects the specialization of function that is adopted by macrophages in different anatomical locations (Gordon and Taylor, 2005).

Activation of macrophages

Although macrophages normally are in a resting state, a variety of stimuli in the process of immune responses can activate macrophages. Various pathways of macrophages activation resulting from microbial, cellular and cytokine interaction have been described. A classical activation is interferon-γ (IFN-γ)-dependent activation. IFN-γ primes macrophages for activation but cannot activate macrophages alone. Tumor necrosis factor (TNF) acts as a second signal for activation of macrophages (Mosser, 2003). Exposure of macrophages to microbes or microbial products such as bacterial lipopolysaccharide (LPS) induces endogenous TNF production by T-helper 1 (Th1) type response. Classical activation is associated with high microbicidal activity, pro-inflammatory cytokine production and cellular immunity. Alternative activation results from culture of macrophages with IL-4 or

IL-13. These cells act as regulatory macrophages and play diverse biological roles different from the classically activated cells (Mosser, 2003). They are associated with tissue repair and humoral immunity. Innate activation is induced by microbial stimuli that are recognized by pattern-recognition receptors such as Toll-like receptors (TLR) and CD14 (the macrophage receptor for LPS). These stimuli induce the production of pro-inflammatory cytokines, such as interferon-α/β, reactive oxygen species (ROS) and nitric oxide (NO), which are associated with microbicidal activity (Gordon, 2002; Gordon and Taylor, 2005; Mosser, 2003). The humoral activation mediated by ligation of some Fc receptors or complements receptors on macrophages is associated with cytotoxic activity and production of pro-and/or anti-inflammatory cytokines, such as IL-12, IL-10 (Mosser, 2003). Deactivation of macrophages is induced by culture together with IL-10 and transforming growth factor (TGF)-β, or by ligation of inhibitory receptors such as CD200 or CD172a, and is associated with anti-inflammatory cytokine production and reduced MHC-II expression (Gordon and Taylor, 2005; Mosser, 2003).

Functional roles of macrophages

Macrophages have the most central and essential functions in the innate immunity, and have multiple roles in host defense (Gordon and Taylor, 2005). Upon encounter with infectious agents, macrophages are capable of initiating an effective innate immune response against microbes by recognizing pathogen-associated molecular patterns (PAMPS) through pattern-recognition receptors (PRPs) (Taylor et al., 2005). Following phagocytosis and endocytosis, macrophages destroy most microbes. By processing and presenting antigen to T cells, macrophages regulate the adaptive immune response (Van Ginderachter et al., 2006). Activated macrophages can secrete an array of cytokines and chemokines (IL-1β, IL-6, IL-12, IL-18, TNF-α and IL-10) and phagocytose necrotic and apoptotic cells (Gordon, 2004). These cytokines have important local and systemic effects that contribute to both innate and adaptive immunity (Janeway et al., 2004). As key regulators of specific as well as innate immune response, macrophages boost as well as limit induction and effector mechanisms of the specific immune response by positive and negative feedback

(Gordon, 2004). Macrophages play an important role in wound healing and inflammatory diseases (Goldsby et al., 2002) as well as tumor immunity (Van Ginderachter et al., 2006).

1.1.2 Ca^{2+} and macrophages

Change in cytosolic free Ca^{2+} of macrophages controls phagocytosis and secretion of cytokines, which will be discussed in detail in the following sections. Ca^{2+} also changes gene expression of macrophages, such as IL-6 (Hanley et al., 2004) and inducible nitric oxide synthase (iNOS) (Denlinger et al., 1996).

Phagocytosis

Most of the studies focused on the role of Ca^{2+} in phagocytosis of macrophages. Phagocytosis is mediated by Fc receptors on macrophages (Gordon, 2002), but the role of Ca^{2+} in phagocytosis is still controversial. Ligation of Fcγ receptors triggers transient increase in $[Ca^{2+}]_i$ in mouse J774 and peritoneal macrophages (Young et al., 1984; Di Virgilio et al., 1988), but other studies showed that during the ligation of FcγR with IgG coated erythrocytes, no rise in intracellular Ca^{2+} was observed (McNeil et al., 1986). This variation may be due to different cell lines cultured in different conditions. For example, thioglycollate-elicited peritoneal macrophages (Thio-macrophages) exhibited an increase in $[Ca^{2+}]$i only in suspension (Di Virgilio et al., 1988). Many lines of evidence indicate that Ca^{2+} is not required for phagocytosis. For example, lowering the cytosolic Ca^{2+} concentration does not alter the FcR mediated phagocytosis (Di Virgilio et al., 1988; McNeil et al., 1986; Greenberg et al., 1991). F-actin is a key cytoskeletal element of pseudopodia; its polymerization is a very important cytoskeletal alteration that accompanies phagocytosis. The assembly of actin is Ca^{2+}-independent (Greenberg et al., 1991), which is consistent with the statement that Ca^{2+} is not required for phagocytosis. FcR isoforms may result in different Ca^{2+} responses and requirement in phagocytosis. In human monocytes, phagocytosis mediated by human Fcγ receptors IIa is $[Ca^{2+}]$i dependent, whereas phagocytosis by human Fcγ receptors Ia is $[Ca^{2+}]$i independent (Edberg et al., 1995). The abilities of these two receptors to induce activation of NADPH oxidase and O_2 generation in guinea-pig macrophages are also different, and consistent with their

ability to induce an increase in $[Ca^{2+}]i$ (Imamichi et al., 1990), which means that Ca^{2+} mobilization is essential for FcR induced oxygen burst (Macintyre et al.,1988) and enhances the antimicrobial activity of macrophages. In human alveolar macrophages, inhibition of the increase of $[Ca^{2+}]i$ by the Ca^{2+} chelator BAPTA abrogated *Klebsiella pneumoniae* phagocytosis and killing (Hickman-Davis et al., 2002).

Cytokine secretion

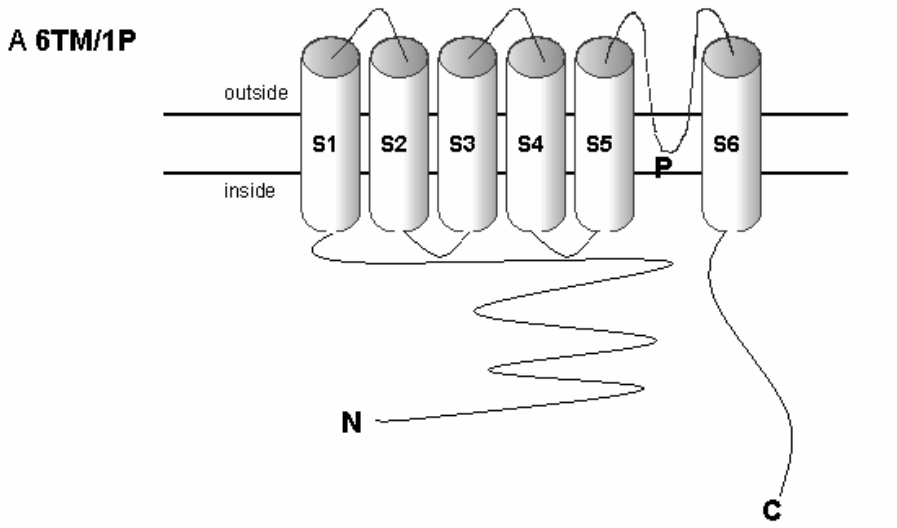
The cytokine interleukin-1 (IL-1) is a proinflammatory mediator produced by activated monocytes and macrophages. IL-1 exists as two distinct isoforms (IL-1α and IL-1β), which contribute to IL-1 biological activity. IL-1α and IL-1β both are produced as a 31-kD procytokines, IL-1α and its 17-kD cleavage product display equivalent signaling activity. Treatment of macrophages with bacterial LPS results in the production of high levels of pro-IL-1β that accumulate in lysosomal structures. Pro-IL-1β is not biologically active, and must be cleaved to its mature active 17-kD form by caspase-1 (Brough et al., 2003). A second signal provided by activation of P2X7 receptors with ATP accelerates the rate of processing and release of IL-1β. P2X7 receptors act as a non-selective cation channel, which allows Ca^{2+} and Na^{+} influx into the cells and K^{+} efflux from the cells. Many studies have focused on the role of Ca^{2+} in ATP induced IL-1β release of macrophages.

Studies on murine macrophages indicated that the membrane permeable Ca^{2+} chelator BAPTA-AM dose-dependently inhibited ATP stimulated IL-1β release, and also inhibited intracellular processing of pro-IL-1β to mature IL-1β (Brough et al., 2003), which is consistent with another study (Gudipaty et al., 2003). Without activation of P2X7 receptors, increasing intracellular Ca^{2+} with Ca^{2+} ionophore ionomycin increased release of pro-IL-1β, but not IL-1β. This increased release of pro-IL-1β may contribute to cell death (Brough et al., 2003). Another Ca^{2+} ionophore A-23187 gave similar results in mouse Bac1 macrophages (Gudipaty et al., 2003). Further studies showed that prior depletion of ER Ca^{2+} store with the SERCA inhibitor thapsigargin inhibited ATP and nigericin-induced IL-1β release (Brough et al., 2003). Taken together, these data imply that Ca^{2+} released from ER store is necessary for ATP-induced release of IL-1β, but not sufficient to stimulate the release; and other

concomitant factors, which most likely include cell volume decrease evoked by K^+ efflux through P2X7 or by K^+ ionophore nigericin (Perregaux et al., 1994), are necessary for this process.

The processing and release of IL-1α is also Ca^{2+}- dependent. A previous study showed that processing of pro-IL-1α depends on Ca^{2+}-dependent calpain enzyme (Kavita et al., 1995); ATP and ionomycin both induced release of por-IL-1α and mature IL-1α from murine macrophages (Brough et al., 2003). The presence of EGTA in the extracellular medium inhibits this process, indicating that the source of Ca^{2+} required for calpain activation and IL-1α release should be extracellular (Watanabe et al., 1994).

The exact mechanism by which Ca^{2+} regulates the processing and release of cytokines is still unknown. In human monocytes, ATP induced IL-1β release is Ca^{2+}-dependent. ATP stimulates the activation of phosphatidylcholine-specific phospholipase C (PC-PLC) and rise in $[Ca^{2+}]i$, which in turn activates cytosolic phospholipase A2 (cPLA2). Activated cPLA2 leads to membrane fusion of lysosome with plasma membrane, which results in release of IL-1β contained in lysosome (Andrei et al., 2004). The role of this PC-LPC, Ca^{2+} and cPLA2 pathway in cytokine release of human macrophages is still unknown.



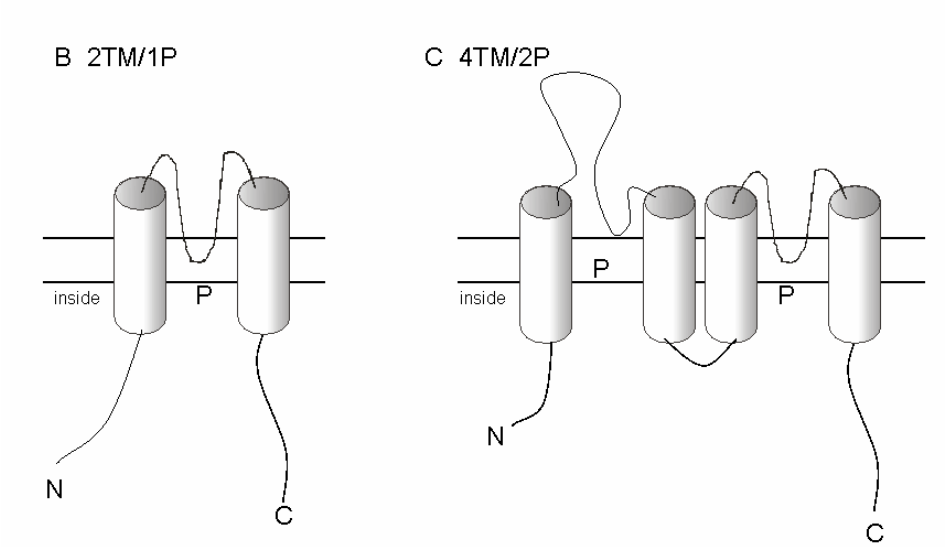


Figure1: Schematic diagram of K^+ channel structures. (A) Subunit of Kv channels with 6 transmembrane segments and 1 pore domain; (B) Subunit of Kir channels with 2 transmembrane segments and 1 pore domain; (C) Subunit of K2P channels with 4 transmembrane segments and 2 pore domains. The pore domain of all K^+ channels has conserved GY (F) G motif. S4 segment of Kv channels contains 4-8 positive charged residues and acts as voltage sensor.

1.2 Potassium channels

Ion channels are a large superfamily of membrane proteins that form selective ion pores. K^+ channels are the most numerous and diverse family of channels known. They play important roles in both excitable cells such as neurons and cardiac muscle and non-excitable cells such as endothelial cells and macrophages.

1.2.1 General properties of potassium channels

So far, more than 70 mammalian K^+ channels have been cloned. Based on molecular structures, K^+ channels are classified into three different groups (Fig. 1): voltage-dependent K^+ channels (Kv) with 6 transmembrane segments (TM) and 1 pore domain (P) (6TM/1P); 2TM/1P inwardly rectifying K^+ channels (Kir); and tandem pore background K^+ channels with 4TM/2P (K_2P). Each group is further divided into multiple families based on sequence similarity.

K^+ channel are involved in maintenance of resting membrane potential of cells , which

is a function of the differential distribution of the most abundant common ions (Na^+, K^+ and Cl^-) between the inside and the outside of the cell (Yost et al.,1999). In most excitable cells, the resting membrane potential is set near the equilibrium potential of potassium (E_K). E_K is the balance point for the concentration and electrical forces, where no net movement of K^+ occurs in either direction through open K^+ selective ion channels.

Many factors cause K^+ channels to open. These factors include changes in voltage across the membrane, increases of intracellular Ca^{2+}, G protein-coupling either directly or indirectly (through a change in intracellular second messenger) and changes in intracellular ATP concentration (Yost et al., 1999). The opening of background K2P channels is regulated by diverse factors such as free fatty acids, pH, membrane tension, hypoxia, heat, volatile anesthetics and G protein coupled receptor agonists (Kim, 2005).

An important feature of voltage-gated K^+ channels is inactivation. There are two types of inactivation: a rapid N type inactivation and a slow C type inactivation. N type inactivation occurs through the interaction between the N terminal of channel protein and the ion-conducting pore. The C type inactivation involves protein rearrangement, leading to narrowing of the inner mouth of the pore (Yost et al., 1999). The inward rectifying of Kir channels is due to block of outward current by intracellular Mg^{2+} and polyamines (Kim, 2005).

K^+ channels play an essential role both in excitable cells and in non-excitable cells. In excitable cells such as neurons and heart muscle cells, it contributes to determining the duration and frequency of action potential or spiking. Human disease caused by alterations in K^+ channel function is channelopathy. For example, mutation of HERG (human ether-a go-go-related gene) results in long Q-T syndrome (Kass et al., 2005). In non-excitable cells, it also plays diverse roles ranged from smooth muscle contraction, epithelium transport, cell apoptosis and proliferation. ATP-sensitive K^+ channels (K_{ATP}) expressed in pancreatic β cells are involved in insulin secretion and serve as the target for sulfonylurea drugs used to treat type 2 diabetes; mutations in K_{ATP} channels result in congenital hyperinsulinemia and neonatal diabetes (Ashcroft, 2005).

1.2.2 Patch-clamp technique

Patch-clamp technique is a refinement of voltage-clamp technique and was developed by Neher E and Sakman B in 1976. It provides a large array of different applications to asses the function of ion channels.

Briefly, a freshly made glass pipette with a tip diameter of only a few micrometers is pressed gently on the cell membrane to form an ionically tight, high-resistance seal (Gigaseal). Then different recording configurations can be carried out according to the study purpose, these configurations include (Fig. 2):

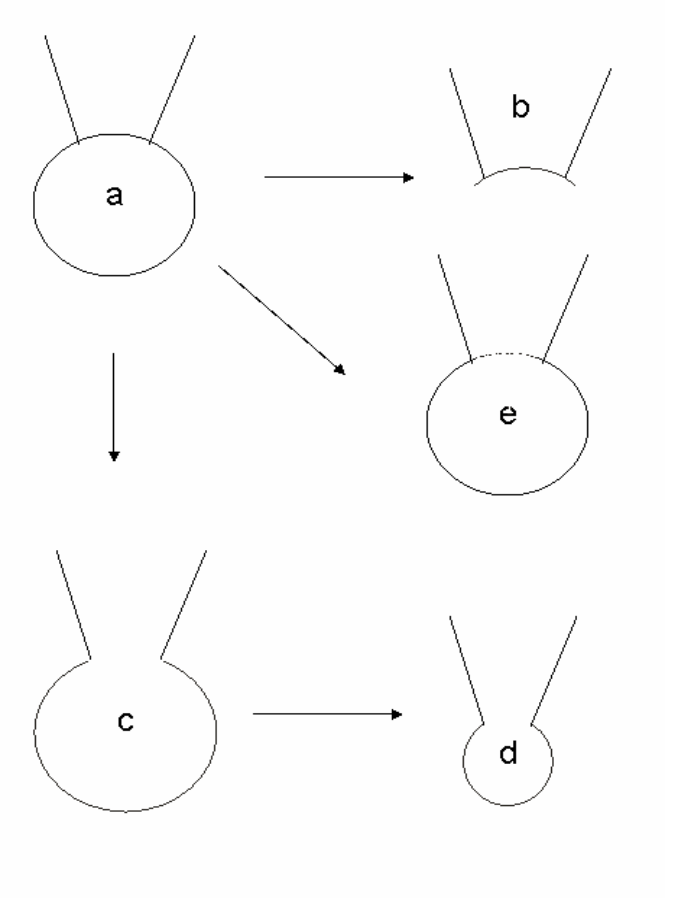


Figure 2: Different recording configurations of patch-clamp technique. (a) Cell-attached single channel recording; (b) Inside-out single channel recording; (c) Traditional whole- cell recording; (d) Outside-out single channel recording; (e) Perforated patch whole-cell recording.

1) Cell-attached recording (on cell)

This recording configuration allows the recording of any current flowing exclusively through the membrane of the patch. The cell remains intact under this configuration.

2) Inside-out recording

Once the on cell configuration is obtained, withdrawing the pipette will excise a patch with the internal membrane surface facing the bath solution (and the external surface facing the pipette solution) called an inside-out patch. The intracellular side of the membrane patch is exposed to the bath. This allows the testing of various intracellular channel modulators, such as calcium or ATP.

3) Whole-cell recording

When suction is applied to the pipette, the membrane breaks and the cytoplasm and pipette solution start to mix. After a short while, this mixing is complete and the ionic environment in the cell is similar to the saline filling solution used in the pipette. Thus, whole-cell configuration allows the control of the components of solutions on both sides of the cell membrane. In whole-cell configuration, two different electrical parameters can be measured: membrane potential at a given current (Vm) measured under the current-clamp mode and the current across the membrane at given voltage (I) under voltage-clamp mode.

Once the whole-cell mode is established, the patch pipette and cell form a complex circuitry, which is schematically demonstrated in figure 3.

4) Outside-out recording

Once the whole cell mode is established, pulling the pipette away from the cell will excise a patch with the extracellular side of the membrane facing the bath solution (and intracellular side facing the pipette filling solution). This configuration allows the single channel current to be recorded, but from an opposite direction of inside-out configuration.

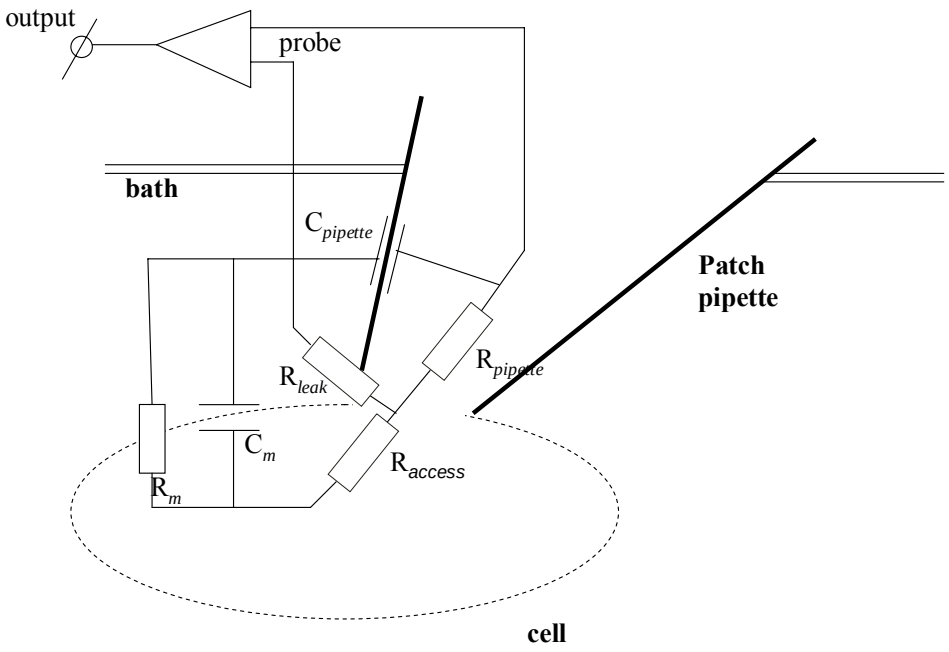


Figure 3: Equivalent circuitry for the whole-cell configuration. After disrupting the patch of membrane, the resistance of patch becomes very low and then is renamed access resistance (R_{access}). The series circuitry consists of the pipette resistance $R_{pipette}$, the R_{access} and the membrane resistance R_m. Rm is the largest resistor, so this configuration allows the observation of currents through R_m. Parallel to the circuitry is the leak resistance R_{leak}, which should be as high as possible to minimize short–circuiting of the membrane current. The membrane capacitance Cm forms an RC circuit with R_{series} and $R_{pipette.}$ (Revised from Molleman, 2002).

5) Perforated whole-cell recording

This is a variation of whole-cell configuration. Addition of polyene antimycotics such as nystatin or amphothericin B to the pipette solution introduces small, nonselective pores into the membrane while the physical characteristics of lipid bilayer remain intact. It may be used to minimize dialysis and avoid the loss of large cytoplasmic molecules into the pipette.

1.2.3 Ca^{2+}-activated potassium channels

Ca^{2+}-activated K^+ channels (K_{Ca}) are members of six/seven transmembrane K^+-selective channels (Wei et al., 2005). According to single channel conductance, these channels are classified as large (BK_{Ca}), intermediate (IK_{Ca}) and small (SK_{Ca}) conductance channels.

BK_{Ca} is a highly potassium selective channel with a $P_K/P_{Na}>50$. It has a single channel conductance of 100-250 pS in symmetrical K^+ solutions. It can be activated by elevation of intracellular Ca^{2+} as well as membrane depolarization. Scorpion toxin iberiotoxin, charybdotoxin, mycotoxin, paxilline and non-selective K^+ channel blocker TEA block BK_{Ca} channels. BK_{Ca} channels are widely expressed in excitable and non-excitable cells ranged from smooth muscle cells, neurons to blood cells, but not in plasma membrane of heart muscle cells. These channels are composed of α and β subunits. The α subunit is encoded by *Slo* gene located on chrosome 10q 22.3, has seven transmembrane segments (S0-S6) with extracellular N-terminal and cytoplasmic C terminal. S1-S6 domains fold similarly to that in voltage-gated K^+ channels, which have a pore domain between S5 and S6, and a voltage sensor in S4 (Meera et al., 1997). A series of negative charged amino acids on the tail of C terminal act as a Ca^{2+} sensor and confer the channel Ca^{2+} sensitivity (Wei et al., 1994). An extra transmembrane domain S0 confers the channel regulation by β subunits, which are two transmembrane region proteins encoded by KCNMB1-4 and act as modulators of channels to enhance the Ca^{2+} sensitivity as well as the toxin binding properties (Tseng-Crank et al., 1996; Wei et al., 2005).

SK_{Ca} channel has a single channel conductance of 4-14 pS. It is highly sensitive to Ca^{2+} with a Kd of 0.6-0.7 μM and is insensitive to membrane potential (Wei et al., 2005). SK_{Ca} chanenels are abundantly expressed in central nervous system where they modulate the firing pattern and give rise to an after-hyperpolarization, and in some peripheral tissue including T lymphocytes (Kohler et al., 1996). SK_{Ca} family has three members, which are encoded by KCNN1-3 (SK1-3) and have 70-80 % amino acids sequence identity to each other. They have six transmembrane domains with intracellular N and C terminus. Calmodulin is tightly bound to C terminal of SK_{Ca} channels, which confer these channels Ca^{2+} sensitivity (Xia et al., 1998). These

channels are blocked by bee venom peptide apamin and scorpion peptide scyllatoxin (Wei et al., 2005).

IK_{Ca} channel has a single channel conductance of 11-40 pS, is very sensitive to intracellular Ca^{2+} with a Kd of 0.1-0.3 μM, and are voltage-independent. KCNN4 gene encodes IK_{Ca} (IK_{Ca} is also known as IK1, hSK4, $K_{Ca}4$, $K_{Ca}3.1$) (Wei et al., 2005). Charybdotoxin, clotrimazole and TRAM-34 block IK_{Ca} channel whereas 1-EBIO and DCEBIO activate the channel. Ba^{2+} blocks IK_{Ca} channels only at hyperpolarized potential (Gallin, 1989).

1.2.4 Intermediate conductance Ca^{2+} activated K^+ channels (IK_{Ca})

Expression pattern of IK_{Ca}

Human IK_{Ca} shows a widespread tissue expression with the highest levels in salivary gland, placenta, trachea and lung; it is apparently absent in excitable tissue but present in various blood cells, including T cells, erythrocytes and monocytes (Jensen et al., 1998). It is noticeable that expression of IK_{Ca} is associated with functional roles of cells, for example, expression of IK_{Ca} is upregulated during T cell activation (Ghanshani et al., 2000). In rat aorta smooth muscle cells, IK_{Ca} expression is enhanced during the phenotype shift from a contractile phenotype to a de-differentiated or immature SMC, which may play a role in smooth muscle proliferation (Neylon et al., 1999).

Regulation of IK_{Ca}

1) Ca^{2+} and calmodulin

BK_{Ca}, SK_{Ca} and IK_{Ca} channels show different Ca^{2+} sensitivities. The Ca^{2+} sensor of BK_{Ca} resides in a negatively charged Ca^{2+} bowl domain in the C tail of α subunits (Wei et al., 1994). In contrast, SK1-3 and IK1 do not contain any EF-hand or Ca^{2+} bowl motifs in their amino acids, which indicates that Ca^{2+} does not directly regulate channel activity although these channel have higher Ca^{2+} sensitivity than BK_{Ca}. Calmodulin (CAM) acts as an accessory protein in the Ca^{2+}-dependent gating of IK_{Ca} (Fnager et al., 1999). CAM constitutively interacts with C terminus of IK1 and the binding of CAM to IK1 is independent of Ca^{2+} (Fanger et al., 1999). This is proven by the evidences that mutation of CAM (Fanger et al., 1999) or deletion of C terminus of IK1 (Khanna et al.,

1999) abolished the currents, although the blockers of CAM have contradictory effects on Ca^{2+} dependent gating of IK_{Ca} (Fanger et al., 1999; Khanna et al., 1999). The proximal C terminus of IK1 (Ct1) is the binding sites of CAM. Deletion of the Ct1 abolished the CAM binding as well as the channel activity; over-expression of Ct1 domain with IK1 inhibited the trafficking of IK1 channel to the plasma membrane and reduced the channel activity; co-expression CAM abrogates this effect of Ct1 (Khanna et al., 1999). CAM regulates trafficking of IK1 by affecting the multimerization and the assembly of the channels (Joiner et al., 2001). In a summary, CAM regulates the activity of IK1 channels in different mechanisms, which include Ca^{2+} dependent gating as well as channel trafficking.

2) Phosphatidylinositol-3-phosphate (PI (3) P)

IK_{Ca} requires PI (3) P for its activity. The PI (3) P phosphotase myotubularin related protein 6 (MTMR6) inhibited the activity of IK_{Ca} by dephosphorylating the D3 position in PI (3) P (Srivastava et al., 2005 and 2006a). PI (3) P indirectly regulates IK_{Ca} because addition of PI (3) P to isolated inside-out patches did not affect IK_{Ca} channel activity (Srivastava, 2006a). Further studies using chimeric channels between $K_{Ca}3.1$ and the related SK channel K_{Ca} 2.3 that dose not require PI (3) P for channel activity identified a stretch of 14 amino acids in the C terminus of K_{Ca} 3.1 that mediated regulation of IK_{Ca} by PI (3) P (Srivastava, 2006a). These 14 amino acids act to recruit nucleoside diphosphate kinase (NDPK) B to $K_{Ca}3.1$, which then activates IK_{Ca} by phosphating a histidine residue (H358) contained in these same 14 amino acids of C terminus of $K_{Ca}3.1$. NDPKB together with PI (3) P leads to the activation of IK_{Ca} (Srivastava, 2006c). PI (3) P and NDPKB both are required for activation of $CD4^+$ T cells (Srivastava, 2006 b and c).

3) Arachidonic acid

Arachidonic acid (AA) is an important second messenger in a variety of cellular processes. AA modulates many kinds of ion channels including K^+, Na^+, Ca^{2+} and Cl^- channels. AA has an inhibitory effect on IK_{Ca} channel activity, with an IC_{50} of 0.42 μM in endogenous expressed channels (Devor et al.,1998) and (1.4 ± 0.7) μM in hIK_{Ca} expressed in HEK293 cells (Hamilton et al., 2003). Increasing Ca^{2+} concentration after AA inhibition fails to recover the channel activity, indicating AA does not displace

Ca^{2+} from its binding site to reduce the channel NPo (Devor et al., 1998). AA interacts with two pore-lining amino acids, Thr 250 and Val 275, in hIK_{Ca}, and directly inhibits channel activity. These two amino acids also confer IK_{Ca} the sensitivity to clotrimazole; mutation of these two amino acids diminishes the inhibitory effects of AA as well as clotrimazole, indicating AA and clotrimazole share the same binding sites on IK_{Ca} (Hamilton et al., 2003).

4) ATP and PKA, PKC

In the existence of cytosolic Ca^{2+}, ATP stimulates the activity of IK_{Ca} (Gerlach et al., 2000). The effect of ATP on stimulation the activity of IK_{Ca} requires the existence of Mg^{2+} and can be reversed by alkaline or acid phosphatase and the cAMP-dependent kinase (PKA) inhibitor PKI_{5-24} (Gerlach et al., 2000). In rat submandibular acinar cells, presence of ATP/Mg^{2+} in the pipette solution reduces run-down of endogenous IK_{Ca}; PKA inhibitor Rp-cAMPs reverses this effect. In addition, cAMP and adenylyl cyclase activator forskolin also increase rat IK_{Ca} currents (Hayashi et al., 2004). Taken together, these data suggest that ATP activates IK1 partially by activation of PKA, which then phosphorylates channel protein or a protein interacting with IK_{Ca} and increases channel open probability.

Another study showed that C- terminal domain of IK_{Ca} channels mediates the effect of ATP on IK_{Ca} (Gerlach et al., 2001). ATP does not activate rat SK2 channel (rSK2), but it could activate IK1/rSK2 chimeras containing the hIK1 C-terminal amino acids His299 – Lys427. Substitution of 14 C-terminal amino acids Arg^{355}-Met^{368} of hIK1 into rSK2 resulted in ATP-dependent activation, which was ~50% of that of hIK1. These results indicate that these 14 amino acids confer the sensitivity to ATP (Gerlach et al., 2001). This appears to contradict the studies mentioned above since the last 14 amino acids of IK1 do not contain a putative PKA phosphorylation site. Further studies are required to resolve this controversy.

In addition to PKA, protein kinase C (PKC) also plays an important role in ATP dependent activation (Wulf, 2002). ATP and ATPγS increased the activity of heterogeneously expressed canine IK1 (cIK1) channels in the presence of 100 nM cytosolic Ca^{2+}, this effect is blocked by PKC inhibitor calphostin C and mimicked by PKC activator phorbol 12-myristate 13-acetate (PMA). PKC also mediates the

activation effect of angiotensin II on IK_{Ca} channel in rat aorta smooth muscle cells (Hayabuchii et al., 2006). Studies on rIK1 expressed in Xenopus laevis occytes yielded contradictory results, which showed that PKC activator and inhibitor have no effect on channel activity (von Hahn et al., 2001). Similar to that of PKA, sole or simultaneous mutation of three putative PKC phosphorylation sites showed no changes of sensitivity to ATP and calphostin C (Gerlach et al., 2000).

5) Intracellular pH, volatile anesthetics and cell swelling

Decrease in intracellular pH ([pH]i) also inhibits IK_{Ca} channel activity by reducing open probability of the channel but not single channel conductance. Increase of [pH]i has no effect on channel activity. Increase of intracellular Ca^{2+} does not diminish this inhibitory effect of [pH]i, which indicates a direct effect of [pH]i on IK_{Ca} rather than on the Ca^{2+}-binding activity of calmodulin (Pedersen et al., 2000).

Cell swelling activates hIK_{Ca} channel expressed in HEK293 cells, and this effect is not dependent on Ca^{2+} but on the intact F-actin cytoskeleton, which indicates a direct or indirect interaction between IK_{Ca} and F-actin (Jorgensen et al., 2003; Grunnet et al., 2002).

Volatile anesthetics modulate several kinds of ion channels including Ca^{2+}, Na^{+} and K^{+} channels. Volatile anesthetics like halothane, isoflurane and sevofurane can inhibit IK_{Ca} channels. Increasing cytosolic Ca^{2+} concentration does not affect the effect of volatile anesthetics, suggesting that Ca^{2+} gating mechanism is not involved (Namba et al., 2000).

Physiological roles of $K_{Ca}3.1$ channels

1) Cell migration

Cell migration plays an important role in wound healing, immune defense, tumor metastasis and some allergic responses including asthma. Many migrating cells including leukocytes, microglial cells, fibroblasts and melanoma cells express $K_{Ca}3.1$ channels (Schwab et al., 2006). These data implied that $K_{Ca}3.1$ might be involved in the process of cell migration. MDCK cells transfected with human $K_{Ca}3.1$ showed an increased migration distance compared with wild type. These channels are concentrated at the front of cells or leading edge of lamellipodium, but most of the

channels are inactive because adding 1-EBIO to the leading edge of lamellipodium decreased cell migration (Schwab et al., 2006). The underlying mechanism of IK_{Ca} on migration is still not clear. Blocking IK_{Ca} with CHTX or TRAM-34 also inhibits migration of human lung mast cells induced by chemokine CXCL10, or by the supernatant from TNF-α stimulated asthmatic airway smooth muscle cells (ASM), suggesting that IK_{Ca} is involved in infiltration of mast cells in ASM in asthma subjects (Cruse et al., 2006).

2) Regulation of microvascular function

IK_{Ca} plays an important role in acetylcholine (ACh) induced hyperpolarizaton and dilation of a variety of blood vessels (Crane et al., 2003; Coleman et al., 2004; Jiang et al., 2006) via the endothelium-derived hyperpolarization factor (EDHF). Spreading of hyperpolarization induced by IK_{Ca} and/or SK_{Ca} from endothelium to underneath smooth muscle via myoendothelial gap junctions contributes to the EDHF induced vascular relaxation (Sandow et al., 2002). $K_{Ca}3.1$ knock out mice showed not only elevated blood pressure but also partially impaired ACh-induced hyperpolarization and dilation (Si et al., 2006). These studies implicate the crucial role of IK_{Ca} in EDHF mediated vascular tone regulation. VDCC blocker dihydropyridines (DHP) also inhibit the hyperpolarization and dilation of artery by blocking IK_{Ca}, the clinical relevance of the effect of DHP on IK_{Ca} is still unknown (Jiang et al., 2007).

3) Cell volume regulation and epithelial transport

Besides hematopoietic system, IK_{Ca} channels are also expressed in cells of colon, lung and salivary gland which are involved in salt and fluid transport (Jensen et al.,1998), indicating that IK_{Ca} channel may play a role in fluid secretion and cell volume regulation.

Cells respond to volume perturbations by activating volume regulatory mechanisms. The processes by which swollen and shrunk cells return to normal volume are collectively termed regulatory volume decrease (RVD) and regulatory volume increase (RVI) (Strange, 2004).

IK_{Ca} is the so-called Gardos channel responsible for dehydration of erythrocytes in sickle anemia disease (Hoffman et al., 2003). Erythrocyte volume decreases due to KCl efflux after $[Ca^{2+}]_i$ elevation is reduced in KCNN4 knock-out mice (Begenisisch et al.,

2004); blocking IK_{Ca} channels with clotrimazole prevents erythrocytes dehydration in patients with sickle cell disease (Brugnara et al.,1996). All these data confirmed the importance of IK_{Ca} in cell volume regulation and in the pathogenesis of sickle cell disease.

IK_{Ca} seems not to play a role in regulatory volume decrease (RVD). RVD of parotid cell from KCNN4 knock out mice is not different from that of wild type mice; the saliva flow and content are also same in these two groups (Begenisisch et al., 2004).

IK_{Ca} also mediates K^+ secretion in the rat proximal colon. During dietary K^+ depletion, the transcription of the rSK4 channels down regulated to prevent K^+ loss (Joiner et al., 2003). Apoptotic cell volume decrease (AVD) is an early event of apoptosis. In T lymphocytes, knock-out or blockage of IK_{Ca} with clotrimazole completely inhibits AVD and cell death (Elliott et al., 2003; Begenisisch et al., 2004), implicating the important role of IK_{Ca} in cell shrinkage prior to apoptosis.

4) Cell cycle regulation

The effect of IK_{Ca} on cell growth was first described in fibroblast cells (Pena et al, 1999), which showed that IK_{Ca} blocker CHTX and *Stichodactyla* toxin (StK) dose-dependently inhibited bFGF stimulated 10T1/2-MRF cells growth through the ras/ERK signaling pathway (Pena et al., 1999). In HaCaT keratinocytes, down-regulation of hIK1 accompanied with a loss of mitogenic activity and a strong increase in cell size (Koegel et al., 2003). Other studies showed that IK_{Ca} also indirectly modulates T cell proliferation by influencing the Ca^{2+} influx through SOC channels (Srivastava et al., 2006 b and c).

IK_{Ca} channels are also cell cycle dependently expressed in tumor cell ranged from melanoma cell line IGR1 (Tajima et al., 2006), human breast cancer cell line MCF-7 (Ouadid-Ahidouch et al., 2004) to human prostate cancer cells (Pariahr et al., 2004). An increase in K^+ channel activity results in the hyperpolarization of the membrane potential, increased Ca^{2+} influx, and increased intracellular free Ca^{2+}, which then regulates the cell proliferation and cell cycle (Ouadid-Ahidouch et al., 2004). IK_{Ca} induced membrane hyperpolarization may contribute to cell mitogenesis. Blocking of IK_{Ca} more or less inhibits the proliferation of human prostate cells, which may be a new strategy for cancer treatment (Pariahr et al., 2004). Additionally, in rat model of

balloon catheter injury, expression of IK_{Ca} is upregulated in neointimal vascular smooth muscle cells (VSMC); blocking of IK_{Ca} suppresses EGF stimulated VSMC proliferation. *In vivo* administration of IK_{Ca} blocker reduces intimal hyperplasia without changes in the rate of apoptosis and collagen content. These data suggest that IK_{Ca} could be a new therapeutic target to prevent restenosis after angioplasty (Kohler et al., 2003).

1.2.5 K^+ channels in macrophages

BKCa

Previous patch-clamp studies on human macrophages revealed the presence of a voltage- and calcium-activated K^+ channel with a conductance of 130 pS in 5 mM external K^+ and 240 pS in symmetrical K^+ (Gallin et al., 1984). Excised patch and cell-attached single channel data showed that this 240 pS K_{Ca} channels were present in >85% of patches from macrophages cultured longer than 7 days but absent in freshly isolated monocytes (Gallin et al., 1988). The mRNA and protein assay confirmed the observation that expression of BK_{Ca} in cells of myelo-monocytic origin is restricted to macrophages (Blunck et al., 2001; Papavlassopoulos et al., 2006). *In vitro* differentiation of monocytes with M-CSF induced transcription of BK_{Ca}, which was enhanced over time and reached peak at day 7 (Blunck et al., 2001). *In vivo* differentiated human alveolar macrophages also transcribe BK_{Ca} mRNA (Papavlassopoulos et al., 2006). Our previous work also confirmed the expression of BK_{Ca} in macrophage differentiated from human peripheral blood monocytes (Hanley et al., 2004). Bacterial endotoxin LPS activates BK_{Ca} activity in outside-out patch clamp measurements (Scheel, et al., 2006); this effect is not due to the rise of intracellular Ca^{2+} concentration $[Ca^{2+}]i$ (Haslberger et al., 1992).

BK_{Ca} in macrophages may play a role in LPS-stimulated production of cytokines such as TNF-α and IL-8 (Papavlassopoulos et al., 2006; Maruyama et al., 1994; Haslberger et al., 1992); nuclear factor κB (NF-κB) signaling cascade may mediate this effect (Papavlassopoulos et al., 2006).

IK_{Ca}

IK_{Ca} in human macrophages was initially identified by patch-clamp measurement

(Gallin, 1989) and later RT-PCR experiment confirmed the expression of KCNN4 in macrophages (Hnaley et al., 2004). It has a single channel conductance of 28 pS and 37 pS in physiological and symmetrical K^+ solution respectively; activation of the channel by increasing intracellular Ca^{2+} is always associated with a hyperpolarization of macrophages (Gallin, 1989).

Functionally, IK_{Ca} is involved in intracellular Ca^{2+} and membrane potential oscillation of macrophages induced by extracellular nucleotide such as UTP or ATP (Hanley et al., 2004). It also mediates reactive oxygen intermediates production induced by UTP or ATP (Schmid- Antomarchi et al., 1997).

Voltage-gated K^+ (Kv) channels

Electrophysioloical and RT-PCR assay confirmed the expression of voltage-dependent K^+ channels in macrophages (DeCoursey et al., 1996). Kv1.3 and Kv1.5 functionally co-localized on the membrane of mouse bone marrow-derived macrophage (BMDM). The activity of Kv1.3 is required for the proliferation of monocytes. Kv1.3 mRNA and delayed-rectified K^+ currents were found in undifferentiated THP-1 monocytes but not in differentiated THP-1 macrophages (Decoursey et al., 1996). Macrophage colony-stimulating factor (M-CSF) induces differentiation and proliferation of moncytes and leads to upregulation of Kv currents and Kv1.3 expression in mouse BMDM (Vicente et al., 2006). Activation by LPS and TNF-α increase the expression of Kv1.3 (Vicente et al., 2003 and 2006) but not Kv1.5 (Vicente et al., 2006) in macrophages. RT-PCR and electrophysiological data showed that human alveolar macrophages only express Kv1.3 (Mackenzie et al., 2003). In another study, Vicente et al.(2005) found that the major Kv beta subunits expressed by macrophages are Kvβ1.1, Kvβ1.2, Kvβ1.3, Kvβ2.1. TNF–α, M-CSF and LPS differently regulate the expression of Kvβ subunits, which results in changed biophysical properties of the channels. This regulation may render more flexibility of the immune responses.

Kv channels are associated with macrophages functions such as migration, proliferation, activation and cytokine production (Eder, 1998). Kv blocker margatoxin (MgTx) inhibits M-CSF induced BMDM proliferation as well as LPS and TNF-α induced increase in iNOS expression (Vicente et al., 2003), but has no effects on P2X7 receptor evoked cytokine IL-1β release (Mackenzie et al., 2003).

Inwardly rectifying potassium (Kir) channels

In human and mouse macrophages, patch-clamp studies also revealed inwardly rectifying K^+ currents with a single channel conductance of 28 pS and 29 pS respectively (Gallin and Mckinney,1988; McKinney and Gallin,1988). RT-PCR revealed that the molecular basis of this conductance is Kir2.1 (Vicente et al., 2003).

Many factors influence the expression and activity of Kir channels in macrophages, e.g. adherence of macrophages leads to the increase of whole-cell Kir current and the membrane hayperpolarization (McKinney and Gallin, 1990); LPS reversely decreased whole-cell Kir current (McKinney and Gallin, 1990; Vicente et al., 2003). Incubation with TNF-α also inhibits Kir currents and mRNA expression in Mouse BMDM (Vicente et al., 2003). Differentiation from monocytes to macrophages dramatically increased Kir channel expression (DeCoursey et al., 1996). In mouse BMDM, incubation with M-CSF significantly increased whole-cell Kir currents as well as mRNA. Blocking Kir channels with 1 mM Ba^{2+} inhibits mouse BMDM growth and proliferation, suggesting the requirement of Kir for the growth of macrophages (Vicente et al., 2003).

1.3 Store-operated Ca^{2+} channels (SOC) and Ca^{2+}- release-activated Ca^{2+} currents (I_{CRAC})

1.3.1 Introduction

Ca^{2+} is an important second messenger and is involved in many types of cellular functions. A rise in intracellular Ca^{2+} concentration initiates diverse responses of cells, including neurotransmitter release; muscle contraction; cell metabolism; cell growth, proliferation and death; changes in gene expression.

Cells increase intracellular Ca^{2+} concentration in two ways: release of Ca^{2+} from ER Ca^{2+} store or Ca^{2+} influx across the cell membrane. Because of the limited capacitance of ER Ca^{2+} store, Ca^{2+} influx across the membrane to the cytoplasm is essential to these cellular responses. In excitable cells, voltage-dependent Ca^{2+} channels (VDCC) is the major Ca^{2+} entry pathway. In many types of non-excitable cells lacking VDCCs, store-operated Ca^{2+} entry (SOCE) is the major Ca^{2+} influx pathway (Parekh and

Putney, 2005).

Depletion of Ca^{2+} from the endoplasmic reticulum (ER) activates Ca^{2+} entry across the plasma membrane in a variety of cell types, a process known as SOCE. By using the patch-clamp technique, the SOC current was first recorded in Jurkat T lymphocytes (zweifach and Lewis, 1993) and mast cells (Hoth and Penner, 1992), which is called Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}). The key characteristics of I_{CRAC} include high selectivity for Ca^{2+} over other monovalent cations, an extremely low unitary conductance for Ca^{2+}, voltage-independent gating, inward rectification and inactivation by Ca^{2+} (zweifach and Lewis, 1993; Prakria and Lewis, 2003).

Physiologically, store emptying can be induceded by an increase in levels of inositol 1, 4, 5- triphosphate (IP3) or some other Ca^{2+} releasing second messengers including Ca^{2+}, cyclic ADP ribose and nicotinic acid adenine dinucleotide phospahete (NADDP). Other methods used to deplete Ca^{2+} store include: application of Ca^{2+} ionophore ionomycin to permeabilize the ER membrane, dialyzing the cytoplasm with high concentration of Ca^{2+} chelators EGTA or BAPTA, which chelate Ca^{2+} leaked from the stores and hence prevent store refilling, exposure to sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitors such as thapsigargin or cyclopiazine acid. Although these above described second messengers and methods may differ in their mechanisms to empty Ca^{2+} store, but the net effect is activation of SOCs (Parekh and Putney, 2005).

Agonists induced receptor-operated Ca^{2+} channels (ROC) is not equal to SOCs. ROCs may include several different Ca^{2+} entry pathway including SOCs, Ca^{2+} entry through TRP channels due to activation of PLC-DAG pathway and also the arachidonic acid regulated Ca^{2+} entry (ARC) by low concentration agonists (Shuttleworth et al., 2004).

The major roles of SOCE in Ca^{2+} signaling include replenishing cellular Ca^{2+} store, prolonging the elevation of Ca^{2+} concentration and sustaining Ca^{2+} signaling required by some physiological processes as well as an important role in Ca^{2+} oscillation (Putney, 2001).

1.3.2 Molecular identity of SOC channels

According to the Ca^{2+} selectivity, SOC channels are classified into two types: The highly Ca^{2+}-selective CRAC channels and the non-Ca^{2+}-selective SOCs. Very recent studies using RNAi screen have found stromal interaction molecule 1 (STIM1) as the possible Ca^{2+} sensor, and CRACM1 (Orai1) may be the pore-forming protein of CRAC. Transient receptor potential (TRP) channels, especially TRPC, may be the molecular candidates of the non-Ca^{2+}-selective SOCs.

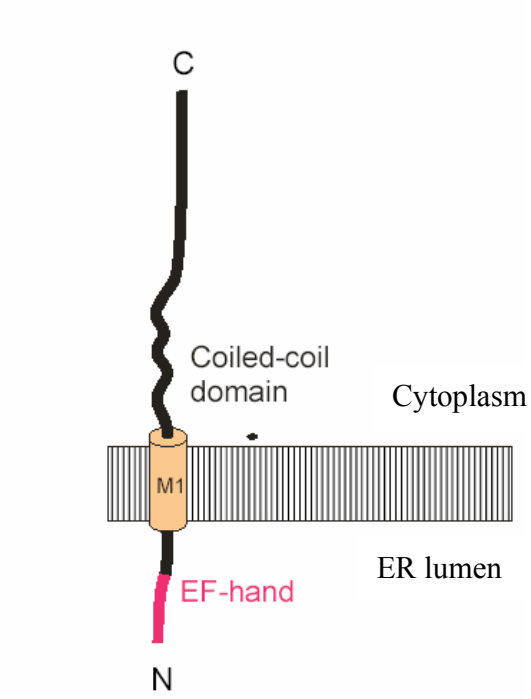


Figure 4: Schematic structure of STIM1. STIM1 is an ER protein with a luminal domain containing an EF-hand, a single transmembrane segment and a cytoplasmic coiled-coil structure.

Ca^{2+} Sensors

Recent studies using RNA interference screen found that STIM1 is the Ca^{2+} sensor that detects the fall of Ca^{2+} concentration in Ca^{2+} store and transmit this signal to plasma membrane (Roos et al., 2005; Zhang et al., 2005). STIM1 is initially characterized as an adhesion molecule of bone marrow stromal cells and as a putative tumor growth suppressor. STIM1 is a transmembrane protein containing a putative EF-hand Ca^{2+}

binding site in the lumen of the ER (Fig. 4) (Liou et al., 2005; Zhang et al., 2005). Mutations in EF hand transformed the SOC channel to a constitutively opened and Ca^{2+} store independent mode (Zhang et al., 2005; Spassova et al., 2005), indicating that STIM1 has a plasma membrane role. Depletion of Ca^{2+} stores causes the formation of STIM1 puncta in the ER region underneath plasma membrane without detectable insertion of STIM1 into the PM (Wu et al., 2006; Xu et al., 2006), although another study using small molecular tag found that STIM1 does insert into plasma membrane after store depletion (Hauser and Tsien, 2007). The CRAC channel activity was only detected in the immediate vicinity of STIM1 puncta (Luik et al., 2006). N-terminal Flag-tagged STIM1 could not be detected in plasma membrane but still fully supported store-operated Ca^{2+} entry (Baba et al., 2006). The C- terminus of STIM1 is sufficient to activate SOC (Huang et al., 2006). Taken together, these data indicate that STIM1 is the long sought Ca^{2+} sensor, which translocates to ER puncta near plasma membrane and activates SOC channels after store depletion.

Molecular candidates of SOC channel pore components

1) Orai proteins

RNA interference screen also found CRACM1 (CRAC modulator) as a modulator of CRAC currents (Vig et al., 2006a). This is a plasma membrane resident protein with 4 transmembrane domains and cytosolic N- and C-terminal (Fig. 5). Another group also independently identified the same gene, which they named as Orai1 (Feske et al., 2006). Mutation of Orai1 causes severe combined immunodeficiency disease due to lacking functional CRAC current, which is restored by expression wild type Orai1. Over-expression either STIM1 or Orai1 alone does not increase I_{CRAC} and store-operated Ca^{2+} entry (SOCE); however, co-expression STIM1 and Orai1 results in a massive increase of I_{CRAC} and SOCE (Peinelt et al., 2006; Soboloff et al., 2006), which indicates Orai1 may be a channel component. Point mutations of conserved amino acids in helix 1 and 3 reduces the selectivity of the channel to Ca^{2+} and also CRAC currents (Yeromin et al., 2006; Prakriya et al., 2006; Vig et al., 2006b). Furthermore, these mutated Orai1 act as dominant-negative protein to inhibit the CRAC currents, suggesting that Orai1 multimers might form the Ca^{2+}-selective SOC

channels (Vig et al., 2006b). Because of lacking typical pore-forming loop or the characteristic selectivity filter of Ca^{2+}-selective channels in Orai1, and the difference in permeability to Ba^{2+} and Sr^{2+} between reconstructed I_{CRAC} by STIM1 and Orai1 and native I_{CRAC} (Peinelt et al., 2006), further studies are needed to identify the pore domain or other subunits of channels.

Two other members of Orai family, Orai2 and Orai3 are also store-operated Ca^{2+} channels when co-expressed with STIM1 (Lis et al., 2007). Orai2 conducts smaller current than Orai1 but with similar properties (Mercer et al., 2006; Lis et al., 2007). Experiments with murine Orai2 splice variants got similar results (Gross et al., 2007).

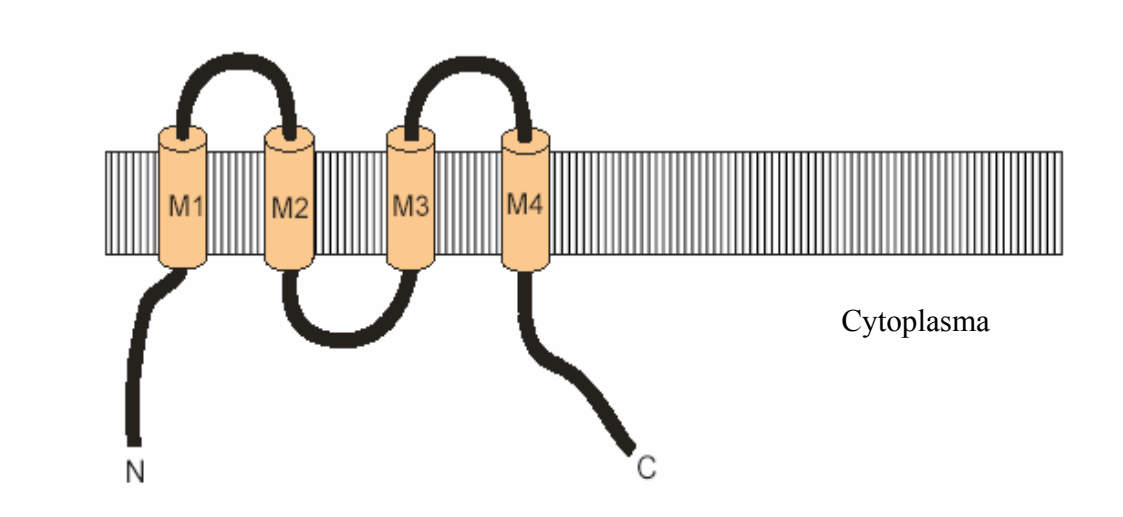


Figure 5: Schematic structure of human Orai1protein. Orai1 is an intrinsic plasma membrane protein with four transmembrane segments and intracellular N- and C- termini.

Orai3 conducts even smaller current below the limits of detection; however, it rescued the knockdown of Orai1 in HEK293 cells (Mercer et al., 2006). Orai3-dependent currents can also be recorded when Na^{+} carries the current, and is somewhat resistant to depotentiation by extracellular Ca^{2+} (DeHaven et al., 2007). A non-conducting mutation of Orai1 (E106Q) acts as a dominant negative for all three Orai homolog, suggesting that they can form heteromultimeric channel complexes (Lis et al., 2007).

2) TRP channels

Another molecular candidate of SOC channels is TRP channel, whose role in

mediating SOC is still controversial. TRPC channels are non-selective Ca^{2+} permeable cation channels that are activated by stimulation of G protein-coupled and tyrosine phosphorylated receptors (Worley et al., 2007). Emptying Ca^{2+} store with SERCA inhibitor thapsigargin (TPG) or ionophore could activate TRPC overexpressed in HEK-293 cells; knockdown of endogenous expressed TRPC led to the same conclusion. The conclusion that TRPC1 and TRPC3 are components of SOC channels is based on the data that co-expression of TRPC1 and TRPC3 in COS cells resulted in Ca^{2+} entry after stimulation with agonists (Zhu et al.,1996). The later studies by same group found that addition of agonists to thapsigargin-treated TRPC3 cells resulted in a further increase in the entry of Ca^{2+} (Zhu et al., 1998); this result is similar to numerous reports indicating that TRPC channels are activated by PLC coupled receptors but not by store depletion with TPG or ionomycin (Venkatachalam et al. 2002). Other studies showed that TRPC3 and TRPC7 behave as SOCs at low-level expression but function as store-independent channels when expressed at high level, which indicated that altered TRPC channels behavior based on their expression level may account for some of the variable results (Worley et al., 2007).

Recent studies have found that STIM1 and Orai1-3 protein may interact with TRPC channels and function as regulatory subunits of TRPC channels. STIM1 binds TRPC1, 4, 5 but not TRPC3, 6; STIM1 is essential for TRPC1 activity after store depletion (Huang et al., 2006). STIM1 directly regulates TRPC1, 4, 5, and indirectly regulates TRPC3, 6 by STIM1-dependent heteromultimerization of TRPC3 with TRPC1 and TRPC6 with TRPC4. STIM1 is obligatory for activation of TRPC channels by agonists, but is not necessary for channel function (Yuan et al., 2007). Another study showed that TRPC1, STIM1 and Orai1 form a ternary complex and function as SOC channels (Ong et al., 2007). Interestingly, Orai1-3 proteins interact with TRPC3 and TRPC6 and confer TRPC channels sensitivity to store depletion (Liao et al., 2007). All these data suggest that TRPC channels may form non-Ca^{2+}-selective SOC channels and the sensitivity to Ca^{2+} store depletion depends on the interaction with STIM1 or Orai proteins.

1.3.3 Electrophysiology and pharmacology of I_{CRAC}

I_{CRAC} shows a characteristic inwardly rectifying current-voltage relationship with a positive reversal potential (>+60mV) when Ca^{2+} is the charge carrier (Hoth and Penner, 1992), which indicates that CRAC channes are highly Ca^{2+}-selective. Like voltage-operated Ca^{2+} channels and TRPV5/6 channels, CRAC channels lose their selectivity in divalent-free (DVF) solution and are permeable to Na^{+}. Thus the whole-cell currents developed in DVF solution have similar time course but five to eightfold amplitude of the corresponding Ca^{2+} currents. In DVF solution, the unitary conductance of CRAC is less than 0.2 pS in the presence of intracellular Mg^{2+} (Prakriya et al., 2002). CRAC channels require external Ca^{2+} to maintain their maximal activity, a process called Ca^{2+}-dependent potentiation. Although CRAC channels are voltage-independently gated, they still exhibits slow voltage dependence in RBL-1 cells in that hyperpolarizing holding potentials reduce the size of I_{CRAC} (Parekh et al., 2005).

I_{CRAC} can be blocked by trivalent cations such as La^{3+} or Gd^{3+}, as well as a relatively selective blocker 2-APB. At low concentration (1-5 μM), 2-APB potentiates I_{CRAC} up to fivefold whereas at higher concentration (>10 μM), 2-APB demonstrates a biphasic effects on $I_{CRAC,}$ i.e. initial activation followed by dominant inhibitory effect (Parekh et al., 2005). The biphasic response to 2-APB has been regarded as an important characteristic of I_{CRAC} and is used to identify I_{CRAC}.

1.3.4 Activation mechanisms

Although patch-clamp experiments have identified the biophysical characteristic of I_{CRAC}, the mechanisms that link ER store depletion to activation of SOCs are still unknown. Several mechanisms proposed for signaling of SOCs are: 1) activation by Ca^{2+} influx factor; 2) exocytosis model. Depletion of stores causes fusion of vesicles containing CRAC channels with the plasma membrane; 3) Ca^{2+} regulation model. Ca^{2+} discharged from a repleted Ca^{2+} pool keeps the channels in an inhibited state. Discharge of the stores removes the source of this inhibitory Ca^{2+} and relieves the inhibition; 4) the conformational coupling model. Discharge of Ca^{2+} stores leads to a conformational change in the Ins (1, 4, 5) P_3 receptor, which is transmitted to plasma

membrane Ca^{2+} channels by a direct protein-protein interaction (Putney, 2001). None of these hypotheses can explain all the characteristics of CRAC channels. Recently STIM1 and Orai1 have been identified as Ca^{2+} sensor and pore-forming subunits of CRAC channels, thus a new model for activation of SOCs was developed (Wu et al., 2007). Ca^{2+}-bound STIM1 and Orai1 distributes throughout the ER and plasma membrane in the resting cells with high free Ca^{2+} concentration in ER. Store depletion causes STIM1 redistribution and forms punctuate accumulation underneath plasma membrane, increases the functional coupling of STIM1 and Orai1. Orai1 proteins also accumulate at sites closed to STIM1. The parallel accumulation of both proteins allows STIM1 and Orai1 to interact and causes the local activation of CRAC channels at individual junctions.

1.3.5 Modulation of SOCs and I_{CRAC}

Ca^{2+}-dependent inactivation

Ca^{2+}-dependent inactivation is a common feature to many Ca^{2+} channels. For CRAC channels, Ca^{2+} feedback occurs through three different mechanisms: rapid inactivation, store refilling induced deactivation and slow inactivation (Parekh and Putney, 2005). After entering the cells, Ca^{2+} accumulates near the inner mouth of the pore and elicits rapid inactivation over several tens milliseconds (Zweifach and Lewis, 1995a). The inactivation is Ca^{2+} dependent; chelating Ca^{2+} with fast Ca^{2+} chelator BAPTA or using Ba^{2+} as the charge carrier reduces the inactivation speed. Facilitating Ca^{2+} influx by enhancing the hyperpolarization also increases the inactivation speed.

Deduced from its opening by Ca^{2+} store depletion, store refilling switches the SOC channel off. SERCA pump inhibitor thapsigargin (TPG) partially reverses the decline of I_{CRAC}; omission of ATP from pipette solution or clamping Ca^{2+} at very low concentration inhibits this effect of TPG, indicating that store refilling contributes to deactivation (Zweifach and Lewis,1995b; Parekh and Putney, 2005).

Slow inactivation of I_{CRAC} happens after a global rise in intracellular Ca^{2+} with a time constant of tens of seconds. This inactivation is also Ca^{2+}-dependent because slow Ca^{2+} chelator EGTA partially suppresses the inactivation (Zweifach and Lewis, 1995 b). The mechanism underlying this inactivation is not clear. Ca^{2+}-dependent inactivation is

an important autoregulatory mechanism that controls the duration and amplitude of Ca^{2+} influx.

Protein kinases

It has been reported that PKC inhibits Ca^{2+} influx through CRAC channels in HL-60 cells (Song et al., 1998), microglial cells (Hahn et al., 2000) and rat basophilic leukemia cells (Parekh and Penner, 1995). Since DAG produced after stimulation of receptors engaged in the phosphoinositide pathways would activate PKC, the inhibition by PKC may be an important negative feedback regulation on CRAC channels. In contrast to PKC, PKA activates Ca^{2+} influx through CRAC channels in HL-60 cells (Song et al., 1998) and microglial cells (Hahn et al., 2000). The effect of cGMP-dependent protein kinase on CRAC channels is likely cell type specific. It has no effect on CRAC channels in microglial cells (Hahn et al., 2000), but inactivates SOCE in A7r5 vascular smooth muscle cells (Moneer et al., 2003).

Arachidonic Acids

Arachidonic acid (AA) inhibits I_{CRAC} in rat liver cells (Rychkov et al., 2005) and A7r5 smooth muscle cells (Moneer et al., 2003). In rat liver cells, endogenous AA released from membrane phospholipids by activation PLA2 has the same effect as exogenous AA (Rychkov et al., 2005). The mechanism of the action of AA *on* I_{CRAC} is still not clear. A possible role of NO has been described in A7r5 smooth muscle cells (Moneer et al., 2003). AA stimulates NO synthase III and leads to the production of NO, which then stimulates guanylyl cyclase and production of cGMP, which then inhibits CRAC channels.

Mitochondria

Mitochondria are involved in the control of CRAC channels activity and Ca^{2+} signals in T-cells (Hoth et al., 1997 and 2000). By importing Ca^{2+} in the immediate vicinity of CRAC entry sites, mitochondria act as Ca^{2+} buffer and are able to reduce Ca^{2+}-dependent inactivation of CRAC channels, thereby increases CRAC activity and the amplitude of Ca^{2+} signals (Hoth et al., 2000). This buffering role of mitochondria is critically dependent on energy status. Respiring mitochondria are essential for activation of SOCs under physiological conditions of weak Ca^{2+} buffering (Gilabert et al., 2000); dissipation of mitochondrial membrane potential unmasks Ca^{2+}-dependent

inactivation of I_{CRAC} (Hoth et al., 2000).

Ca^{2+} influx through CRAC channels causes translocation of mitochondria to compartment near plasma membrane and reduces the distance between mitochondria and plasma membrane, thus enhances the function of mitochondria as a Ca^{2+} buffer to take up more Ca^{2+} near CRAC, prevents Ca^{2+} dependent I_{CRAC} inactivation and sustains Ca^{2+} signals (Quintana et al., 2006). CRAC-induced Ca^{2+} signaling is involved in proinflammatory signal leukotriene C4 (LTC4) release. Mitochondrial depolarization suppresses the generation of arachidonic acid and LTC4 secretion in mast cell line RBL-1 (Chang et al., 2004). Ca^{2+} is a very important mediator in the malignant growth of tumor cells. Inhibition of the function of mitochondria with diazoxide resulted in fast inactivation of I_{CRAC} and reduced Ca^{2+} influx into tumor cells, which led to proliferation arrest of the tumor cells (Holmuhamedov et al., 2002).

Sphingosine

Sphingosine, sphigomyelinase and ceramide all can inhibit Ca^{2+} influx through CRAC channels in human Jukat T cells. Blocking of CRAC by these sphingomyelinase metabolites partially mediates the inhibitory effect of the CD95 receptor on T cell activation (Lepple-Wienhues et al., 1999).

Extracellular pH

In human macrophages, external acidification reversely inhibits I_{CRAC} with a pKa at pH 8.2. Changes in extracellular pH alone failed to induce current activation. Thus, changes in external pH, as would be encountered by macrophages at sites of inflammation, could change the time course and magnitude of the [Ca]i transient associated with receptor activation by regulating the influx of Ca^{2+} ions (Malayev et al., 1995).

Membrane potential as a driving force for Ca^{2+} influx

Depolarization of the membrane inhibits Ca^{2+} entry through SOCs channels; whereas hyperpolarization of membrane potential provides the driving force for Ca^{2+} influx through SOC although hyperpolarization does not directly open channel. Any ion channel that is involved in setting the membrane potential may play a role in Ca^{2+} entry. Activation of IK_{Ca} and SK_{Ca} channels has been show to be important for Ca^{2+} influx through SOCs in T lymphocytes (Srivastava et al., 2006 b, c; Fanger et al., 2001) and

in mast cells (Mark Duffy et al., 2004). Role of Kir channel in facilitating Ca^{2+} entry has been confirmed in microglial cells, macrophages resident in brain (Franchini et al., 2004). Chloride channels also provide driving force for Ca^{2+} influx through SOCs in T cells (Wang et al., 2006). Depolarization of membrane with high K^+ solution decreases the Ca^{2+} entry through SOCs in microglia (Franchini et al., 2004).

1.3.6 Physiological and Pathophysiological roles of SOCE

Besides its general roles in refilling Ca^{2+} store, sustaining cytoplasmic Ca^{2+} elevation and involvement in Ca^{2+} oscillation, SOCE still has some short-term and long-term physiological and pathophysiological effects (Parekh and Putney, 2005).

Short-term responses to SOCE

These responses include regulation in exocytosis, enzyme activity, muscle contraction, sperm chemotaxis and acrosome reaction.

In a varity of non-excitable cells such as RBL and mast cells (Mahmoud and Fewtrell, 2001), exocytosis is triggered by a rise in cytoplasmic Ca^{2+} influxed through SOC channels. In cytotoxic T cells, contacting with target cells activates capacitative Ca^{2+} entry and then granule exocytosis (Lyubchenko et al., 2001).

Ca^{2+} entry through SOCs also affects the activity of enzymes such as adenylyl cyclase, NO synthase, PLC-δ and plasma membrane Ca^{2+}-ATPase (PMCA) (Parekh and Putney, 2005). PLC-δ activated by Ca^{2+} may represent a positive feedback mechanism that prolongs agonist evoked Ca^{2+} entry (Kim et al., 1999). In T cells, PMCA is a major target of Ca^{2+} influx through CRAC channels. Elevation of $[Ca^{2+}]i$ slowly modulates PMCA activity to ensure the stability and enhance the dynamic nature of Ca^{2+} signals, which is therefore important for preventing Ca^{2+} overload (Bautista et al., 2004).

I_{CRAC} have been observed in smooth muscle cells from different tissues (Albert et al., 2003). CPA and TPG induced contraction of rat pulmonary smooth muscles, which was inhibited by SOC blockers but not by VDCC blockers, whereas CPA did not evoke contraction of rabbit portal vein myocytes. These data suggest that SOCE induced contraction depends on tissue heterogeneity.

SOCE also plays a role in sperm chemotaxis and acrosome reaction (Fukami et al., 2003).

Long-term response of SOCE

SOCE is also involved in long-term cellular responses like transcription regulation, cell proliferation and apoptosis.

The effects of SOCE on gene transcription have been extensively investigated in T cells. Ca^{2+} influx through SOCs results in a rise of $[Ca^{2+}]i$, leads to the formation of Ca^{2+}-calmodulin complex, which then activates the phosphatase calcineurin. Activated calcineurin enters the nuclear, dephosphorylates nuclear factors like NFAT, NF-κB and OctA and initiates transcription and translation of IL-2 gene and its receptor (Lewis, 2003).

SOCE is also an important regulator of cell cycle in many different cells. Reduction of Ca^{2+} influx through SOCs leads to a decrease in T cell proliferation (Srivastava et al., 2006 b and c). In addition, I_{CRAC} also appears to be cell cycle dependent. It is upregulated in periods of preparation for and during chromatin duplication but strongly inhibited during mitosis of RBL-2H3 mast cells (Tani et al., 2007).

Although Ca^{2+} plays a central role in apoptosis, the role of SOCE in apoptosis is still controversial (Parekh and Putney, 2005). TPG could induce apoptosis in androgen-sensitive human prostate cancer (Skryma et al., 2000) and human colon cancer cell line (He et al., 2002). Many studies found that it is Ca^{2+} store depletion but not Ca^{2+} entry through SOCs that triggers apoptosis. This is supported by the report that oncogene Bcl-2 protects against TPG-induced apoptosis by diminishing the extent of ER Ca^{2+} store depletion (He et al., 2002).Another study showed that inhibition of Ca^{2+} entry even further stimulates apoptosis (Skryma et al., 2000), this effect may be contributed to the diminished Ca^{2+} refilling. Decreasing extracellular free Ca^{2+} or adding Ni^{2+} enhanced TPG-induced apoptosis of human prostate cancer cells; the ability of TPG to induce apoptosis was not reduced by loading the cells with intracellular Ca^{2+} chelator (BAPTA) (Skryma et al., 2000). These results indicate that the depletion of intracellular Ca^{2+} stores may trigger apoptosis but there is no requirement for the activation of SOCE in induction and development of apoptosis.

Pathophysiological roles of SOCs

Functional deficiency of SOCs is associated with severe combined immunodeficiency (SCID) (Feske et al., 2005). Orai1 mutation leads to a completely absence of I_{CRAC} in T

cells; expression of wild type Orai1 recoveres I_{CRAC} in T cells from SCID (Feske et al., 2006). Diminished Ca^{2+} entry through SOCs may also contribute to pathogenesis of Alzheimer's disease (Putney, 2000). On the other hand, sustained or prolonged Ca^{2+} entry through SOCs is also associated with pathogenesis of some diseases. This is well demonstrated by acute pancreatitis (Raraty et al., 2000) that a sustained rise in $[Ca^{2+}]i$ induced by cholecystokinin results in premature intracellular activation of trypsin. Removal of external Ca^{2+} or using Ca^{2+} chelator BAPTA prevents this process.

1.4 P2X and P2Y receptors

Nucleotides are ubiquitous extracellular signaling molecules that induce a wide spectrum of biological effects. The appearance of nucleotides in extracellular fluids results from cell lysis, excytosis of nucleotide-concentrating granules or efflux through membrane transport proteins (Communi et al., 2000). The plasma membrane receptors for extracellular nucleotide are termed P2 receptors. According to the molecular structures, they are classified into 2 subfamilies: G protein coupled P2Y receptors and ligand-gated ion channels, the P2X receptors. To date, seven human P2X receptors and eight human P2Y receptors have been cloned and characterized (Di Virgilio et al., 2001; Abbracchio, et al., 2006). P2 receptors are distinguishable from P1 (adenosine) receptors in that they bind adenine and/or Uralic nucleotide triphosphates or diphosphates depending on the subtype (Burnstock, 2006).

1.4.1 P2X receptors

Ionotropic P2X receptors show following features: two transmembrane spanning regions (TM1 and TM2), TM1 involved in channel gating and TM2 lining the pore; intracellular N- and C- termini; large extracellular loop with an ATP binding site (Burnstock, 2006). P2X receptors coassemble with other subunits to form heterotrimers or heterohexamers except P2X7, which has distinct properties in comparison with other P2X receptors (Torres et al., 1999). All P2X receptors are ATP-gated ion channels. The EC50 for ATP is 1- to 10 μM ranges for all recombinant P2X receptors, except for P2X7, which has an EC50 of approximately 100 μM.

Functionally, P2X receptors are associated with vascular tone regulation, initiation of

pain, cell growth, differentiation and apoptosis (Burnstock et al., 2006). P2X7 receptors expressed in monocytes and macrophages are important for cytokine secretion such as IL-1β (Solle et al., 2001; Gudipaty et al., 2003).

1.4.2 P2Y receptors

Metabotropic P2Y receptors are characterized by: 1) seven-membrane-spanning regions; 2) an external N-terminal and a cytoplasmic C-terminal; 3) a high level of sequence homology between some transmembrane spanning regions; 4) structure diversity of intracellular loops and C- terminal among P2Y subtypes, thus influencing the degree of coupling with $G_{q/11}$, G_s and G_i protein (Burnstock et al., 2006). The responses to nucleotide and the coupled G protein of each P2Y receptors are summarized in Table1. In response to nucleotide activation, P2Y receptors either activate phospholipase C and release intracellular Ca^{2+} or affect adenylyl cyclase and alter cAMP levels.

Table 1 Human P2Y receptors

group	receptor	chromosome	agonist	G-protein	Phenotype of knock-out mice
A	$P2Y_1$	3q24-25	ADP	Gq	Inhibition of platelet aggregation Increased bleeding time Resistance to thromboembolism
	$P2Y_2$	11q13.5	ATP=UTP	Gq(+Gi)	Abolition of chloride secretary response to ATP/UTP in airways
	$P2Y_4$	Xq13	UTP	Gq(+Gi)	Abolition of chloride secretary response to ATP/UTP in jejunum and colon
	$P2Y_6$	11q13.5	UDP	Gq	No knock-out mice are available
	$P2Y_{11}$	19p31	ATP	Gq+Gs	No murine P2Y11 gene
B	$P2Y_{12}$	3q21-25	ADP	Gi	Inhibition of platelet aggregation Increased bleeding time Resistance to thromboembolism
	$P2Y_{13}$	3q24-25	ADP	Gi	Available , no phenotype yet
	$P2Y_{14}$	3q24-25	UDP-glucose	Gi	No knock-out mice are available

(Revised from Abbracchio et al., 2006, and Boeynaems et al., 2005)

P2Y receptors are involved in many kinds of cellular function (Burnstock et al., 2006). P2Y2 and P2Y4 receptors expressed in airway epithelia may stimulate Cl^- transport and are important for mucociliary clearance and preventing airway infection. P2Y2 activation increases salt, water and mucus excretion and is potential treatment for dry eyes. P2Y1 and P2Y12 receptors are important for platelet aggregation; antagonists to these two receptors have been clinically used to reduce the risk of recurrent strokes and heart attack. Activation of P2Y1 and P2Y2 on vascular endothelial cells results in release of EDHF or NO and then vasodilatation. In addition, P2Y receptors also play a role in cell cycle progress and growth as well as apoptosis. P2Y1 and P2Y2 receptors located on inflammatory and immune cells play a pivotal role in inflammation and immune modulation. P2Y2 and adenosine receptor 3 (A3) mediates neutrophil chemotaxis induced by ATP (Chen et al., 2006).

1.4.3 P2Y receptors in macrophages

Macrophages express several subtypes of P2Y receptors. The expression pattern of P2Y receptors is correlated to differentiation stages and tissue distribution. Human macrophages derived from PBMCs express P2Y1, P2Y2 and P2Y11 receptors (Hanley et al., 2004) whereas human monocytes express P2Y1, P2Y2, P2Y4, P2Y11 and P1Y13 receptors (Kaufmann et al., 2005). Among human macrophage cell lines, THP-1 and U937 cells express P2Y2, P2Y4 and P2Y6 receptors. P2Y2 mRNA declines during maturation of monocytes to macrophages as demonstrated in THP-1 cell line differentiation induced by Phorbol-12-myristate-13-acetate (PMA) (Di Virgilio, 2001). Interferon-γ and LPS have the same effects as PMA on P2Y2 expression in THP-1 cells (Martin et al., 1997). The expression pattern of P2Y receptors is also different in tissue resident macrophages. Rabbit osteoclasts express P2Y1, P2Y2 and P2Y6 receptors (Korcok, et al 2005). In rat microglia (the brain resident macrophages) P2Y receptors 1, 2, 12 are expressed (Haynes et al., 2007). Rat alveolar macrophages express P2Y receptors 1, 2, 4 and 12 (Bowler, et al., 2003). P2Y1, P2Y2 and P2Y6 have been detected by PCR analysis in mouse peritoneal macrophages; $P2Y_2$ is the only G protein-coupled receptor linking extracellular UTP and ATP to phospholipase C-β and Ca^{2+} release (Del Rey et al., 2006). The variations

of the expression of P2Y receptors in different macrophages suggest that these P2Y receptors may be involved in development and differentiation of macrophages.

P2Y receptors play diverse roles in biological functions of macrophages. The Gi-protein coupled P2Y12 receptors are necessary and sufficient for ATP induced chemotaxis of microglia (Haynes et al., 2007). Gq-coupled P2Y6 receptors induce a transient increase of $[Ca^{2+}]i$ and translocation of transcription factor NF-kB to nucleus, thus increase the survival of osteoclasts (Korcok et al., 2005). UTP acts through P2Y6 receptors to potentiate LPS-induced NF-kB activation and phosphorylation of NF-kB inhibitor IkB (Chen and Lin, 2001), which may influence the expression of some inflammatory mediators. In fact, activation of P2Y receptors inhibits LPS induced iNOS expression in mouse macrophages (Denlinger et al., 1996). Our previous data also showed that stimulation of P2Y2 receptors with ATP enhances the expression of IL-6, which may also play a role in inflammatory responses (Hanley et al., 2004).

1.5 Objective of this study

The present study aims to clarify the role of IK_{Ca} in regulation of SOCE in macrophages. IK_{Ca} is expressed in the membrane of human macrophages and plays a role in macrophage membrane potential oscillation (Hanley et al., 2004). SOCE is the major Ca^{2+} influx pathways in non-excitable cells including macrophages, which lack Ca^{2+} entry through voltage-dependent Ca^{2+} channels. Therefore, the regulation of Ca^{2+} influx through SOC is very important for Ca^{2+}-dependent physiological and pathophysiological processes of macrophages. We hypothesized that Ca^{2+} entry through SOC channels could activate IK_{Ca} in human macrophages and hyperpolarize the macrophages. K^+ efflux through IK_{Ca} may provide the counter ions for Ca^{2+} influx through SOCs and may thus prevent membrane depolarization. In this way, IK_{Ca} may help to maintain driving force for Ca^{2+} entry through SOCs in human macrophages.

In this study, we describe the electrophysiological properties of IK_{Ca} in human macrophages and the membrane potential changes after Ca^{2+} influx through SOC. In addition, we will check the changes of store-operated Ca^{2+} influx after blocking IK_{Ca} by Ca^{2+} fluorescence measurements. We will also determine the possible molecular candidates of SOC channels in human macrophages by RT-PCR.

I did all the work of monocytes isolation, culture and differentiation; investigated the electrophysiological properties of human macrophages. Dr. Peter J Hanley contributed to the Ca^{2+} fluorescence measurements. Susanne Rinné contributed to the RT-PCR expression assay of STIM 1, 2, Orai 1-3 and TRP channels in macrophages. Marylou Zuzarte helped with the immunohistochemistry assay of CD14.

2. Materials and Methods

2.1 Isolation of monocytes and culture of macrophages

Macrophages were prepared from human peripheral blood mononuclear cells (PBMCs) isolated from whole blood donated by healthy volunteers following receipt of informed consent. Monocyte isolation was performed using the following protocol reported previously (Hanley et al., 2004):

- Add 20 ml of Biocoll separating solution (Biochrom AG, Berlin, Germany) into each of 3 sterile 50 ml conical centrifuge tubes. Let the Biocoll centrifuge solution warm up to room temperature.
- Collect 60 ml vein blood in heparinized syringe, layer 20 ml of blood on top of each Biocoll layer. Keep the centrifuge tube at a 45 degree angle and let sample run down along the wall of the tube. Be careful not to mix these two layers.
- Centrifuge at 400 g for 30 min at room temperature (about 22 ^{o}C) without brake. The cells will be separated into three layers as demonstrated in figure 6.

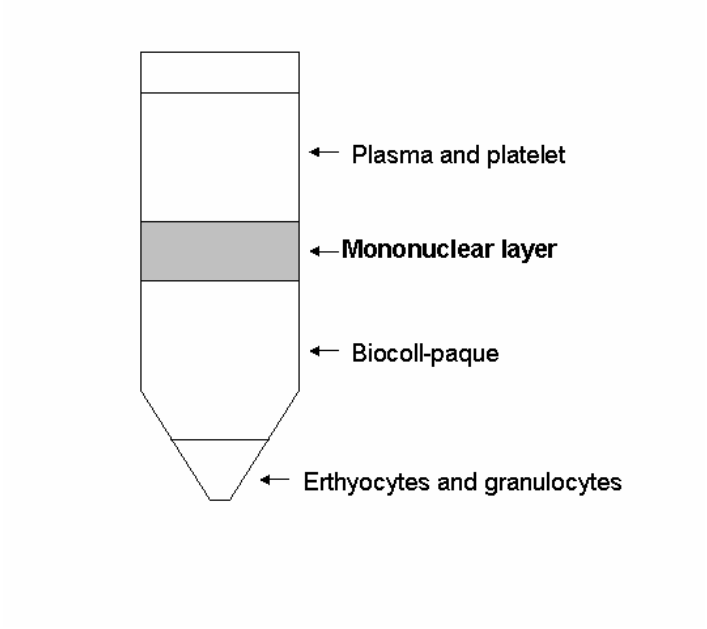


Figure 6: Different blood layers after density-gradient configuration.

- Aspirate off the upper layer of plasma down to about 0.5-1 cm above Buffy coat (mononuclear layer), and then use another sterile pipette to transfer the mononuclear layer to a 50 ml conical tube.
- Wash the cells by adding PBS (Biochrom AG, Berlin, Germany) to the tube to reach a total volume of 50 ml, mix them gently and the centrifuge at 250 g for 10 min at room temperature.
- Remove supernatant and resuspend the pellet in 50 ml at 4 ^{o}C in PBS by careful titration, centrifuge at 250 g for 10 min at 4 ^{o}C.
- Aspirate off the supernatant as before and resuspend the pellet in 30 ml RMPI 1640 (Biochrom AG, Berlin, Germany) medium and disperse the cells by gentle titration. Centrifuge at 250 g for 10 min at 4 ^{o}C.
- Remove the supernatant as before and resuspend the pellet in 10 ml of very low endotoxin RPMI 1640 medium complemented with 10% heat-inactivated fetal bovine serum (FBS, Biowest, France), 10 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (all from Biochrom) as before.
- Count the cell number using a hematocytometer. Add 10 µl of this cell suspension and 90 µl medium to a 0.5 ml Eppendorf tube to get a 1:10 dilution. Add 10 µl of this solution to slide; and using the 10×microscope lens count the entire square area with cross-hatched grid to obtain cell number n. The total number of cells is (n/4) $\times 10^6$/ml.
- Dilute the cells with complement medium at concentration of 2.5×10^6 cells/ml. Put 2 ml of this cell suspension into each 3.5 cm cell culture Petri dishes and incubate at 37 ^{o}C, 5% CO_2 overnight.
- Remove the non-adherent cells and wash once with complete RMPI 1640 medium. Culture the cells in the presence of 5 ng/ml recombinant human macrophage-colony- stimulating factor (Pepro Tech, Rocky hill, USA) at 37 ^{o}C in an incubator with 5% CO_2/95% air for 7-14 days. Every 4-5 days feed cells with fresh complete RMPI 1640 medium plus MCSF.

The typical macrophages with fried-egg-like shape were chosed for patch-clamp experiments.

2.2 Immunofluorescence assay of macrophages

Monocytes were grown in RPMI 1640 medium in glass bottom dishes (Well Co. Amsterdam, Netherlands). M-CSF was used to induce differentiation as described in Section 2.1. At the 14th day, macrophages were washed three times with PBS and fixed with 4 % paraformaldehyde for 15 min at 37 ^{o}C. After washing three times with PBS, the cells were permeablized with 0.2% Triton-X 100 for 5 min. Then the cells were washed with PBS and blocked with 1 % BSA for 10 min. After washing three times with PBS, CD14 antibody was added to the dishes. The anti-CD14 antibody (Santa-Cruz, California, USA) was diluted to 1:1000. The macrophages were incubated with CD14 antibody at room temperature for 30 min and then washed three times with PBS. The donkey anti-mouse Fluor 594 (Molecular Probes) was used as second antibody at a dilution of 1:100. After incubation with the second antibody at room temperature for 5 min, the cells were washes three times with PBS. Then the F-actin of macrophages was stained with phalloidin (Molecular Probes) at dilution of 1:50 and incubated at room temperature for 5 min. After washing with PBS, the nuclei of the macrophages were stained with Hoechst 33258 (Molecular Probes) at a dilution of 1:500 for 2 min at room temperature. After washing three times with PBS, the glass bottom of dishes were taken out and embedded onto glass slides using a drop of Mowiol mounting medium-containing anti-fade agent N-propyl-gallate. The slides with samples were dried and used for imaging.

Microscopy and imaging were performed on Olympus IX71 microscope with a 60 × N.A. 1.3 PL APO objective or a 100 × N.A. 1. 4 PL APO objective (Olympus), standard EGFP/ Texas Red filter sets and a cooled 12-bit CCD camera (SensiCam QE; PCO, Kehlheim, Germany). The images were processed using Image-Pro® Plus 4.5 (Media Cybernetics, Maryland; USA).

2.3 Whole-cell recording on macrophages

All patch-clamp experiments were performed at room temperature using a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Forster city, USA). Petri dishes (Cell Star, Frickenhausen, Germany) with 10-14 days old macrophages were placed on the stage of an IX 50 inverted microscope (Olympus, Japan). Macrophages were

superfused with bath solution in a recording chamber with an inner diameter of 1.5 mm. Glass pipettes were fabricated using a horizontal puller (DMZ-Universal-Puller, Zeitz Instruments, Augsburg, Germany) from borosilicon glass (GB-150F-8P, 0.86×1.50×80 mm, Science Products GmbH, Hofheim, Germany). After back filling with pipette solution, the glass pipettes were placed on a holder and moved toward the selected cell with a 3-D micromanipulator (Narishige MHW-103, Narishige, Tokyo, Japan). After the formation of a Gigaseal, the patch membrane was disrupted by application of negative pressure and the whole-cell configuration was established. Current clamp and voltage clamp modes were used to record the membrane potential and current, respectively. The data were recorded and analyzed with the software PC.DAQ1.1.

1) Membrane capacitance recording

The cell membrane and the intracellular and extracellular media form a capacitor. The amount of charge stored (Q) can be calculated from the equation Q= Em×C, where Em is the potential difference across the membrane (in Volt, V) and C is the membrane capacitance (in Farad, F). Measurement of capacitance provides a good estimation of the membrane surface area under investigation. Most biological membranes have a specific capacitance of 1 $\mu F/cm^2$. For measurement of macrophage membrane capacitance a ramp voltage was imposed to the membrane and the current change was recorded. The membrane capacitance was calculated according to the equation C= dI/(dU/dt).

2) The current-clamp mode

In the current-clamp mode, when the current is clamped at 0 pA, the membrane potential (Vm) can be recorded. Vm equals the inside potential minus the outside potential. The out side of the cell is considered to be at ground potential (0 mV). Normally the resting membrane potential of a cell is negative. It is referred to as a depolarization when Vm becomes less negative; and Vm becomes more negative is defined as hyperpolarization. For continuous recording the Vm of macrophages, the sampling rate was 2 kHz. The acquired data were filtered with a low-pass Bessel filter at 1 kHz; the output gain was 2.

3) The voltage-clamp mode

The voltage-clamp mode is a procedure used during study of ion channels to keep the

membrane potential constant. It allows direct measurement of ionic current across a membrane. In our study, the membrane potential of macrophages was clamped at -70 mV and the current was elicited with two different stimulation modes: voltage steps and voltage ramps. The sampling rate was 5 kHz and a low-pass Bessel filter with a corner frequency of 2 kHz was used, the output gain was 2. Current flowing from inside to outside of membrane is referred to as outward current, and current flowing into the cell is referred to as inward current. The reversal potential indicates at which Vm the current changes its directions. When the conductance of ions through the channel is voltage dependent in such a way that more current flows in one direction than in the other direction, this is called rectification.

4) Solutions and drugs

Solutions used for the measurement of macrophage membrane potential and IK_{Ca} current:

- Ca^{2+}-free bath solution (in mM): NaCl 140, KCl 4.5, $MgCl_2$ 1.13, HEPES 10, Glucose 10, EGTA 0.5. pH was adjusted to 7.4 with 3N NaOH.
- 2 mM Ca^{2+} bath solution (in mM): NaCl 140, KCl 4.5, $MgCl_2$ 1.13, HEPES 10, Glucose 10, $CaCl_2$ 2. pH adjusted to 7.4 with 3N NaOH.
- 100 nM Ca^{2+} pipette solution (in mM): K^+aspartate 100, KCl 40, EGTA 0.1, $MgCl_2$ 1, HEPES 10, the calculated free Ca^{2+} concentration was about 100 nM at 22 ^oC (according to MAXCHELATOR, http://www.stanford.edu/~cpatton/maxc.html). pH was adjusted to 7.2 with 3 N KOH.
- 3 nM Ca^{2+} pipette solution (in mM): K^+aspartate 100, KCl 40, EGTA 10, $MgCl_2$ 1, HEPES 10, $CaCl_2$ 0.2. pH 7.2.
- 1 μM Ca^{2+} pipette solution (in mM): K^+aspartate 100, KCl 40, EGTA 5, $MgCl_2$ 1, HEPES 10, $CaCl_2$ 4.4. pH 7.2.

Solution for measurement of I_{CRAC} in macrophages (modulated from Prakriya 2002):

- Ca^{2+}-free bath solution (in mM): NaCl 150, KCl 4.5, $MgCl_2$ 2, glucose 10, HEPES 5, EGTA1, CsCl 10. The pH was adjusted to 7.4 with 3 N NaOH.
- 20 mM Ca^{2+} bath solution (in mM): NaCl 150, KCl 4.5, $MgCl_2$ 2, glucose 10, HEPES 5, $CaCl_2$ 20, CsCl 10. The pH was adjusted to 7.4 with 3 N NaOH.

• Pipette solution (in mM): Cesium methanesulfonate 100, CsCl 50, BAPTA 10, $MgCl_2$ 5, MgATP 2, HEPES 10, and Na^+ methanesulfonate 8. To record UTP and thapsigargin induced I_{CRAC}, BAPTA was replaced by 10 mM EGTA. The pH was adjusted to 7.2 with 1 N CsOH.

Drugs:

If not stated otherwise, salts and other chemicals were purchased from Sigma (St Louis, USA). Recombinant charybdotoxin and UTP were obtained from Sigma and directly dissolved in water to reach a concentration of 100 μM and 10 mM, respectively. The stock solutions weer stored at -20°C. Shortly before the measurement, CHTX and UTP were diluted in bath solution to reach a final concentration of 100 nM and 100 μM respectively. Clotrimazole (CLT), thapsigargin (TPG) and 2-APB were dissolved in DMSO to make up a stock solution of 1 mM, 0.5 mM and 10 mM respectively. The stock solutions were added to the bath solution to reach a final concentration of 1 μM for CLT, 0.5 μM for TPG and 50 μM for 2-APB, respectively. The final concentration of DMSO had no electrophysiological effects on channels studied.

2.4 Ca^{2+} fluorescence measurements

Macrophages were grown on glass coverslips as described at chapter 2.1. A coverslip with macrophages was sealed onto the bottom of a Perspex bath mounted on the stage of an inverted microscope (Nikon Diaphot 300, Japan). Cells were superfused with physiological salt solution containing 5% BSA and (in mM): NaCl 140, KCl 5.4, $MgCl_2$ 1, NaH_2PO_4 0.33, HEPES 5, $CaCl_2$ 1 and Glucose 10 (pH7.4). Fluo-3/AM (Molecular Probes) was first dissolved in DMSO (Sigma-Aldrich, Germany) containing 20% Pluronic F-127. The macrophages were loaded with fluo-3 by incubation with 10 μM fluo-3/AM in physiological salt solution (final concentration of DMSO was 0.1%) at room temperature for 20 min. A single macrophage with diameter of 15-25 μm was selected and excited at 488 nm by means of a monochromator and fluorescence was detected at 530±15 nm.

Only one cell per coverslip was used for measurements. The fluorescence signals were normalized with respect to the resting fluorescence intensity (F_0) and expressed as F/F_0.

2.5 RT-PCR analysis of messenger RNA

1) RNA extraction

Total RNA was extracted from cultured human macrophages by using high pure RNA isolation kit (Roche, Mannheim, Germany). Macrophages of maximal purity for RT-PCR were isolated by positive selection with anti-CD14-MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in SuperMACS, following the manufacturer's protocol.

2) Reverse transcription

cDNA was transcribed from 1µg total RNA using Supercript II reverse transcriptase (Invitrogen) and a random hexanucleotide (Applied Biosystems). For negative controls, Supercript II was omitted.

RNA was denaturized at 70 ^{o}C for 10minutes. RT reaction was carried out at 42 ^{o}C for 50 min, and Superscript II reverse transcriptase was inactivated by heating to 75 ^{o}C for10 min.

RT reaction mixture (25 µl)	
RNA (1 µg)	10 µl
5× RT buffer	5 µl
10× hexanucleotide	0.5 µl
dNTP (2 mM)	6 µl
DTT (0.1µM)	2.5 µl
Reverse transcriptase, Superscript II (200 U/µl)	1 µl

3) PCR reaction

RT-PCR expression assays were performed as follows in a GenAmp PCR system 9600 (Applied Biosystem):

PCR reaction (20 µl)	
10× PCR buffer	2 µl
2 mM dNTPs	2 µl
Forward primer (25 µM)	0.8 µl
Reverse primer (25 µM)	0.8 µl
DMSO	0.8 µl
Taq Gold DNA polymerase(5 U/µl)	0.12 µl
cDNA	0.8 µl
RNase-DNase free water	12.68 µl

Samples were cycled under the following conditions:

	Cycle	Temperature	Time
hot start	1	96 ^{o}C	5 min
denaturation	35	96 ^{o}C	30 sec
annealing		55 ^{o}C	30 sec
extension		72 ^{o}C	1.5 min
final extension	1	72 ^{o}C	10 min
hold		4 ^{o}C	

The PCR product was size fractionated by gel electrophoresis. The house keeping gene GADPH was used as a positive control. The following primers were used:

Genes	Primers	Size(bp)
hGAPDH	For 5′-CATCACCATCTTCCAGGAGCGA-3′	343
	Rev 5′-GTCTTCTGGGTGGCAGTGATGG-3′	
hOrai1	For 5′-AATCTCAACTCGGTCAAGGAGTC-3′	343
	Rev 5′-ACTGTCGGTCAGTCTTATGGCTA-3′	
hOrai2	For 5′-CGCAGTACCAGTACCCGCGGCCG-3′	327
	Rev 5′-AGCCCGTGTGACTCCCAGGGCCA-3′	
hOrai3	For 5′-GCGAGCAGGCCCCGCTGAAC-3′	348
	Rev 5′-CTTCAATGTGGGGCAGCAGACAC-3′	
hSTIM1	For 5′-GTTTGCCTATATCCAGAACCGTTA-3′	342
	Rev 5′-TACCATGAGCTGTGAGATTCTAGC-3′	
hSTIM2	For 5′-AGATGGTGGAATTGAAGTAGAGG-3′	344
	Rev 5′-CTTGAGCTGAAGTTTTTGTCTGTG-3′	
hTRPC1	For 5′-CTGCAGCTTCTTTTGGACTACGG-3′	295
	Rev 5′-ATTGCCGGGCTAGTTCCTCATAAT-3′	
hTRPC3	For 5′-CAGTAAAGTGACACTCCCACCAGA-3′	300
	Rev 5′-GCAGCATTAACTTTAGCCCCAAGG-3′	
hTRPC4	For 5′-TGGATGATATTACCGTGGGTCCTG-3′	238
	Rev 5′-TAGCAAATAAAGCCTCTGCCACCA-3′	
hTRPC5	For 5′-CTCCCTGGTAGTGCTGCTGAACAT-3′	266
	Rev 5′-TGAAAACTTCTCAAGTTGCGCCTTC-3′	
hTRPC6	For 5′-CTGCCAACAGCAACTTCTCTCCAT-3′	256
	Rev 5′- GCCTTCAAATCTGTCAGCTGCATT-3′	
hTRPC7	For 5′-CCGAGCAAAACTCTGGCTGTCTTA-3′	266
	Rev 5′-TGGTGGGCTTGCTCAAAGTGTTAT-3′	
hTRPV6	For 5′-CTGCTCATGCTCAACCTCCTCATT-3′	220
	Rev 5′-GGTTGAGATCTTGCCTGTCTTCCA-3′	
hTRPM2	For 5′-GACCTTCTCATTTGGGCCATTGTC-3′	249
	Rev 5′-GGTGAGCAGTTTCTGGGCTCTCTC-3′	
hTRPM7	For 5′-ATTTGAGCTTCACCCACGAATCAA-3′	200
	Rev 5′-TTTCAATCACTCCCCATGGAGCTA-3′	

2.6 Statistics

All the data are summarized and expressed as means ± standard deviation. *n* represents the number of experiments. Statistical significance was estimated by Student's *t*-test and the data were considered to be significantly different for $P<0.05$.

3. Results

3.1 Morphology and immunohistology of macrophages

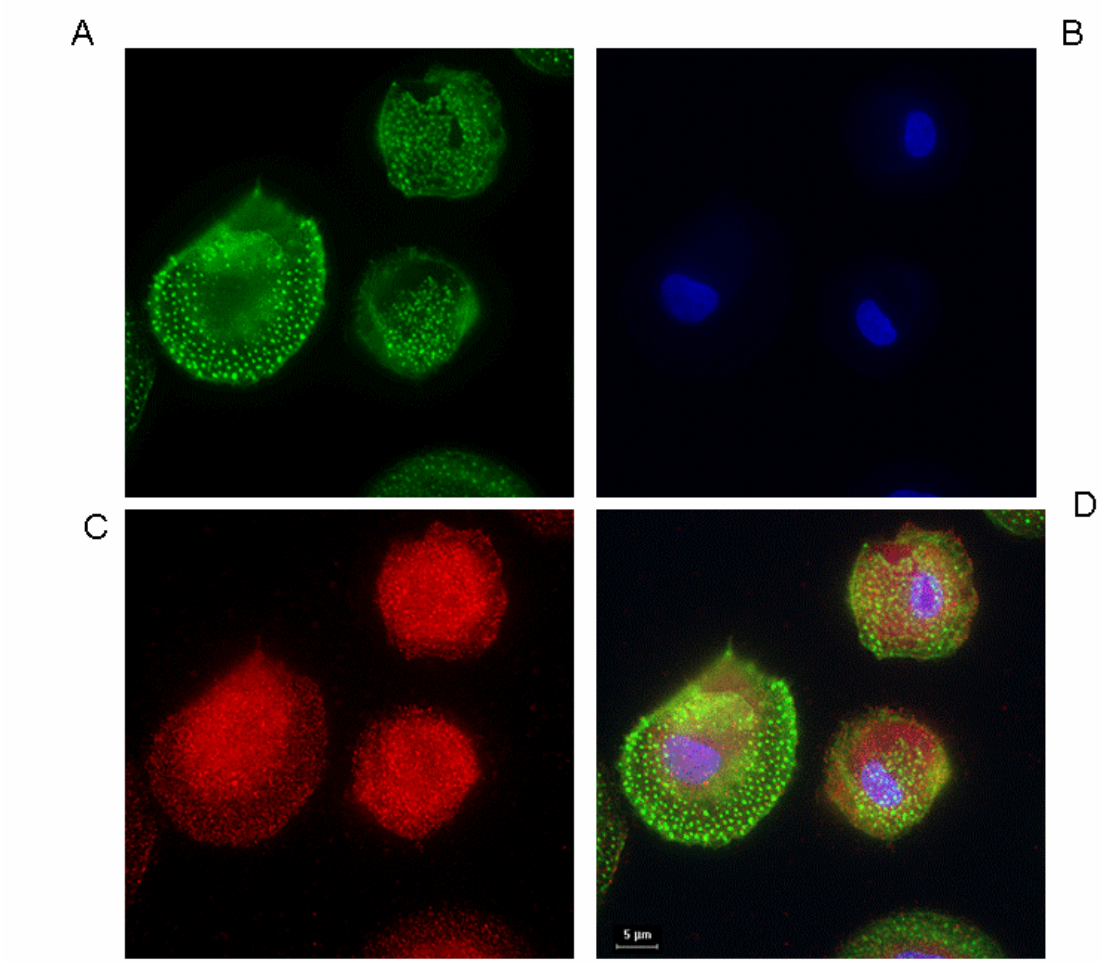


Figure 7: Macrophages differentiated from human PBMCs after 10 days in culture. (A)Fluorescence image of filamentous actin. (B) Fluorescence image of nuclei stained with Hoechst 33258. (C) Immunofluorescence of CD14. (D) Overlay of fluorescence images shown in A, B and C.

After 3-4 days of culture in RPMI1640 medium at the presence of 10ng/ml M-CSF, the adherent mononuclear cells began to expand. They showed three different morphologies: elongated or spindle-shaped cells, round cells with fried-egg shape and

irregular-shape cells. Immunofluoresence experiment showed that most of these cells were positive for the macrophage molecular marker CD14 (Fig.7).

3.2 General electrophysiological features of macrophages

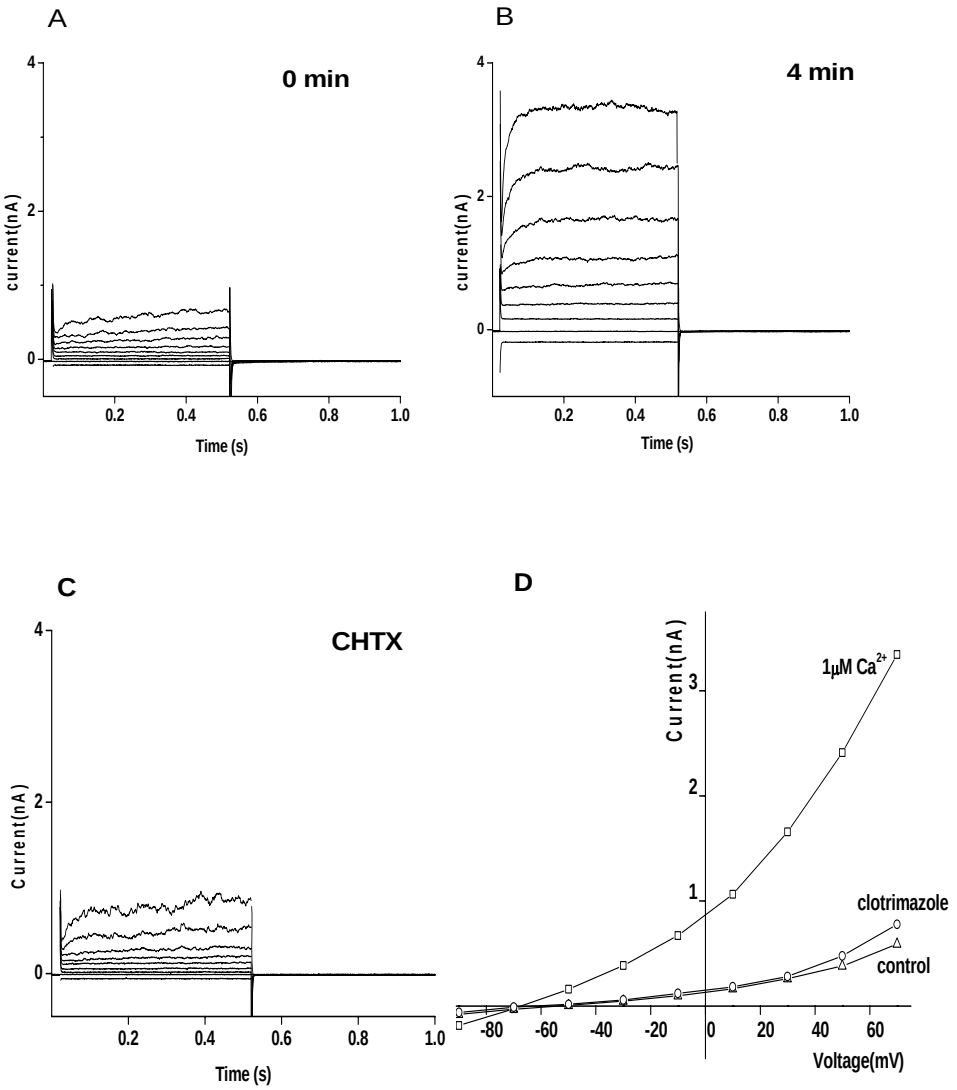
The average membrane capacitance of macrophages was 97.1± 22.5 pF (*n*=130), which is very similar to that reported by Musset (Musset, 2004) and Nelson et.al (1990). The resting membrane potential of macrophages was recorded immediately after membrane rupture in the current clamp mode in Ca^{2+}-free bath solution. Under these conditions, the average resting membrane potential was -21.2 ± 8.3 mV (*n*=141), which is lower than that in 2 mM Ca^{2+} bath solution (Nelson et al., 1990).

3.3 IK_{Ca} current in macrophages

Our previous study has shown that *KCNN4* (SK4/K_{Ca}3.1) channels are expressed in human macrophages (Hanley et al., 2004). Whole-cell patch clamp recordings in macrophages confirm the functional expression of IK_{Ca} in human macrophages. To activate IK_{Ca} sufficiently, we perfused macrophages with pipette solution containing free Ca^{2+} buffered to about 1 μM and bath solution containing 4.5 mM K^+. The currents recorded immediately after the disruption of membrane were used as control (Fig. 8A). After 2 min perfusion with 1 μM Ca^{2+} pipette solution, both the outward currents and the inward currents increased significantly (Fig. 8B), which could be inhibited by 1 μM clotrimazole, a blocker of SK_{Ca} and IK_{Ca} channels (Fig. 8C). The IV curve reversed at about -70 mV, which is close to equilibrium potential of K^+ (E_K).

In another experiment we also demonstrated that the whole-cell currentS at 1 μM pipette Ca^{2+} could be partially blocked by 100 nM charybdotoxin (CHTX), a blocker of IK_{Ca} and BK_{Ca} (Fig. 8 E and F). Our group (Hanley et al., 2004) and other studies (Blunck et al., 2001; Papavlass-opoulos et al., 2006) have demonstrated the functional expression of BK_{Ca} in macrophages. Elevation of cytosolic Ca^{2+} and membrane depolarization activate BK_{Ca}. The IV curve of BK_{Ca} is outwardly rectifying. In our experiments, we found that in some cells IK_{Ca} were the dominant K_{Ca} currents, as shown in Fig.8E, whereas in others cells, BK_{Ca} were the dominant K_{Ca} currents, as shown in Fig. 8F. In Fig. 8E, the Ca^{2+} sensitive current was almost linear, which

indicates that the main component of this current is IK_{Ca}, whereas in Fig. 8F, the Ca^{2+} sensitive current is linear at potentials more negative than +20 mV (which may represent an IK_{Ca} component) and became outwardly rectifying at >+20 mV, which may represent the BK_{Ca} component. At +70 mV, the CHTX-sensitive current at 1 μM $[Ca^{2+}]_i$ was 35.3±4.7 % of total current (n=5) in BK_{Ca}-dominant cells, and 63.6±11.2 % of total current (n=4) in IK_{Ca}-dominant cells.



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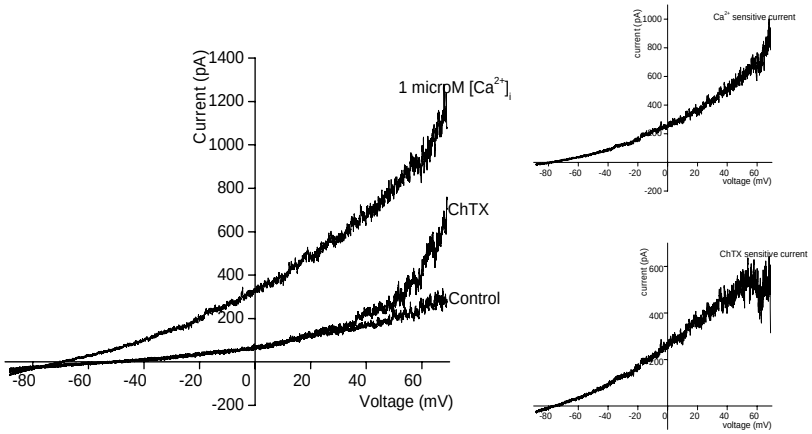

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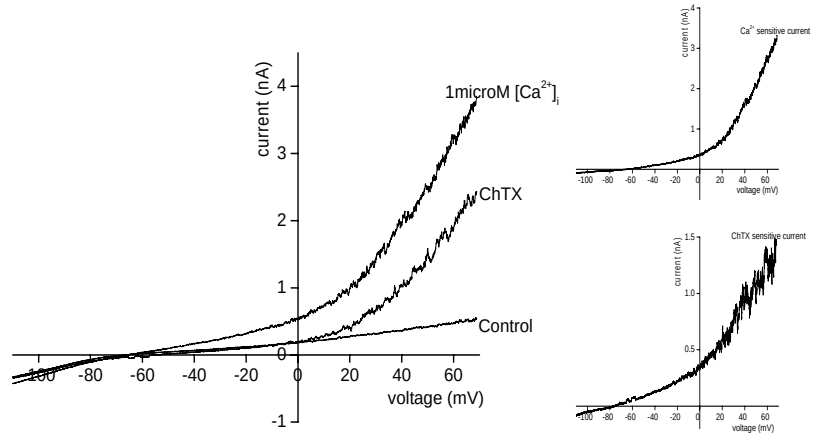


Figure 8: IK_{Ca} currents in macrophages. Representative recording of whole-cell current (A) Immediately after rupture of membrane; (B) after 4 min superfusion with 1 μM Ca^{2+} pipette solution; and (C), after blockade with 1 μM clotrimazole. (D) the IV curves of whole-cell current immediately after rupture of membrane (Δ), 4 min after perfusion with 1 μM Ca^{2+} (□) and blocked with 1 μM clotrimazole (○). The cells were clamped at -70 mV and stimulated with voltage steps from -90 mV ~ +70 mV. (E and F) Representative recording of whole-cell ramp currents of macrophage immediately after rupture of membrane (control), after 2 min perfusion with 1 μM Ca^{2+} (1 μM Ca^{2+}) and after blocking with 100 nM charybdotoxin (CHTX). Insert: Difference current of Ca^{2+}-induced current and CHTX-sensitive currents.

DCEBIO is a derivate of the IK_{Ca} channel activator 1-EBIO but with an almost 100 fold enhanced bioactivity compared to that of 1-EBIO (Singh, et al., 2001). We tested the effects of DCEBIO on whole-cell current of macrophages with a pipette solution containing weakly buffered Ca^{2+} (the free Ca^{2+} concentration was ~100 nM). After superfusd with 100 nM Ca^{2+} pipette solution for 2 min, stimulation of the macrophages with a voltage step elicited a current with some degree of outwardly rectification (Fig. 9A); treatment of the cell with 10 µM DCEBIO significantly increased the current (Fig. 9B), resulting in an almost linear IV-curve (Fig. 9C), which is typical for IK_{Ca} currents. The additional current induced by DCEBIO reversed near E_K (Fig. 9C). All these data demonstrate the functional expression of IK_{Ca} channels in human macrophages and are consistent with other studies (Hanley et al., 2004).

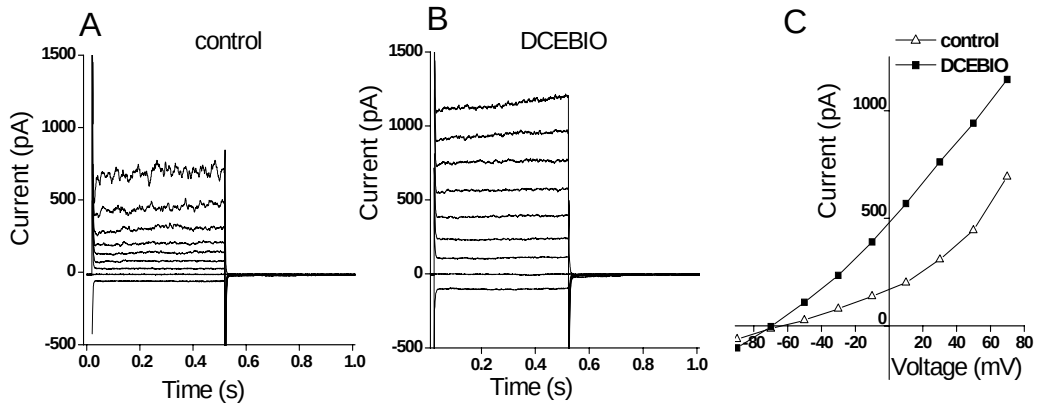
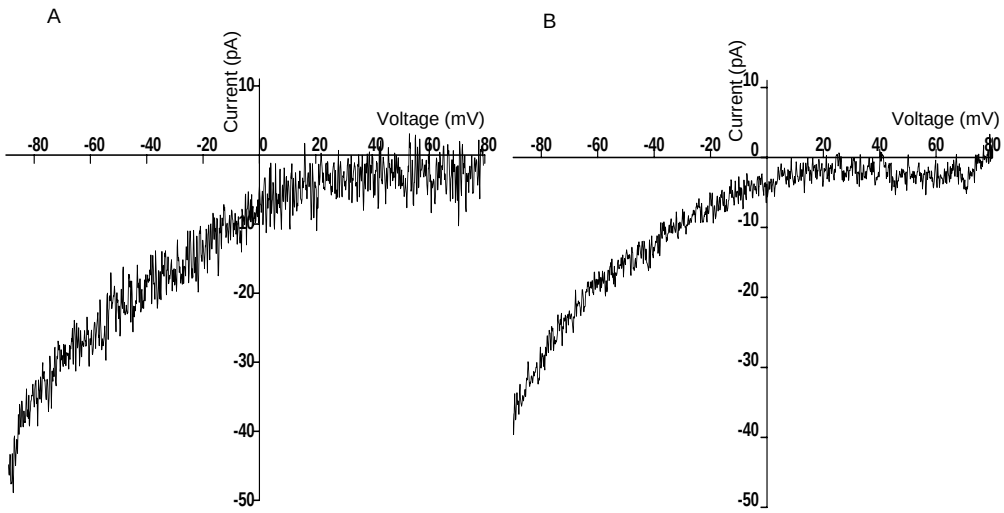


Figure 9: DCEBIO activated IK_{Ca} currents of macrophages. (A) representative recording of whole-cell currents with weak Ca^{2+} buffer in pipette solution (100 nM free Ca^{2+}). (B) 10 µM DCEBIO activated the whole-cell currents. (C) IV curves of the whole-cell currents shown in A (control, Δ) and B (DCEBIO, ■).

3.4 I_{CRAC} in macrophages

In order to demonstrate that depletion of the ER Ca^{2+} store with different methods could activate Ca^{2+} influx, we recorded I_{CRAC} in human macrophages using the whole-cell patch clamp technique. We showed that in 20 mM Ca^{2+} bath solution, passively emptying the ER Ca^{2+} store with 10 mM Ca^{2+} chelator EGTA slowly activated an inwardly rectifying current with a reversal potential >+ 60 mV (Fig. 10 A). Emptying the ER Ca^{2+} store with 500 nM SERCA inhibitor thapsigargin also activated an inward current after re-addition of 20 mM Ca^{2+} into bath solution (Fig.10 B). The P2Y receptor agonist UTP induced Ca^{2+} oscillations at lower concentration (Hanley et al., 2004), whereas at higher concentration (100 μM), it emptied the ER store through the PLC-IP3 pathway (Communi et al., 2000). UTP at 100 μM also induced an inward current in the bath solution containing 20 mM Ca^{2+}. This current was similar to that induced by EGTA and thapsigargin (Fig. 10C) and was completely inhibited by 50 μM 2-APB, a relatively specific blocker of SOC channels (Fig.10 C and D). These data demonstrate that no matter how the Ca^{2+} store was emptied, the inward currents possessed all the features of I_{CRAC}. These currents were very similar to that reported by Malayev et al. (1995) in human macrophages.



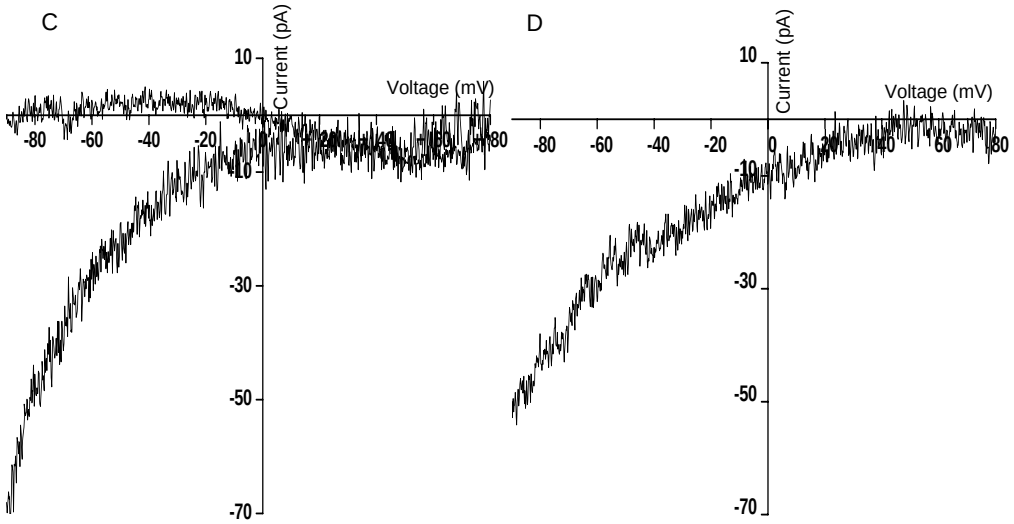
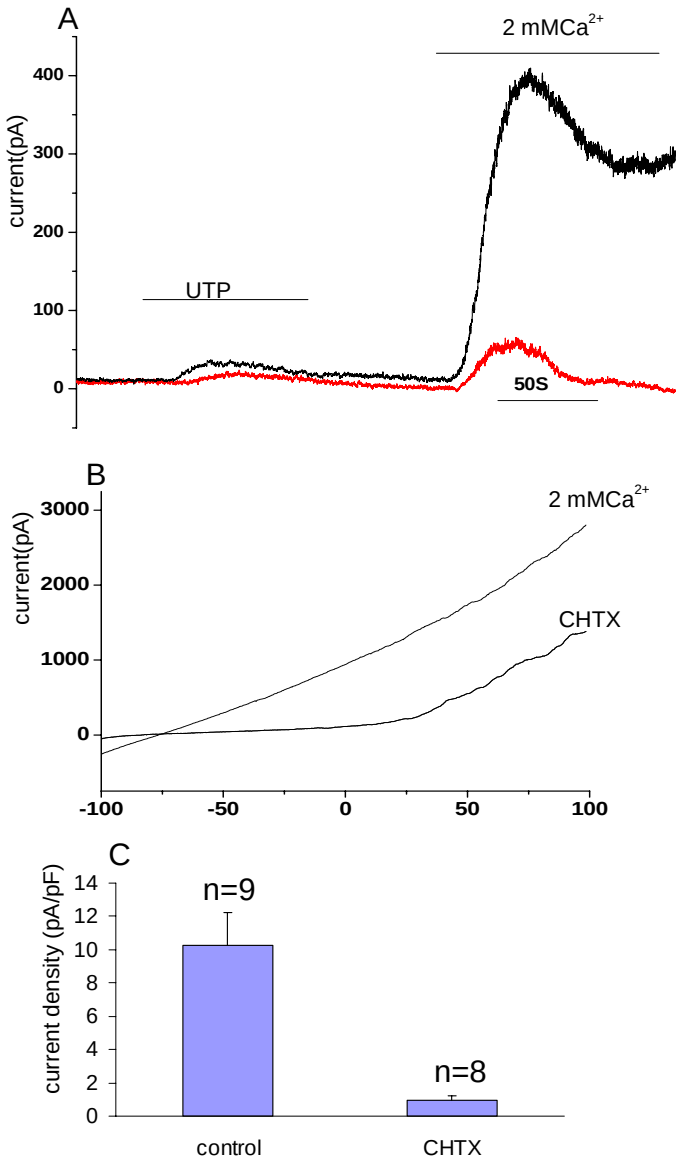


Figure 10: I_{CRAC} in human macrophages. The macrophages were bathed in a solution containing 20 mM Ca^{2+}. The ramp current immediately after membrane rupture was used as control; the currents shown here were the difference-currents obtained by subtraction from the control currents. (A) Representative I_{CRAC} induced by emptying Ca^{2+} store with 10 mM EGTA. (B) Representative I_{CRAC} induced with 500 nM thapsigargin. (C) I_{CRAC} induced by emptying Ca^{2+} store with 100 μM UTP and blocked with 50 μM 2-APB. (D) 2-APB-sensitive current.

3.5 Store-operated Ca^{2+} entry induced an outward current

Ca^{2+} influx through SOC channels will increase intracellular Ca^{2+} concentration, which may activate Ca^{2+}-activated K^+ channels. To confirm this hypothesis, we continuously recorded whole-cell current at 0 mV. Our results showed that 100 μm UTP induced a small outward current whereas re-addition of Ca^{2+} resulted in a robust outward current with a current density of 10.3 ±1.9 pA/pF (n=9). The mean current density of this outward current reduced to 0.9±0.3 pA/pF (n=8; P<0.001) when the macrophages were pre-incubated with the IK_{Ca} blocker charybdotoxin (CHTX) (Fig. 11A, C). The current induced by re-addition of Ca^{2+} had a typical linear IV relationship and was blocked by 100 nM CHTX. The CHTX-sensitive current component reversed at about –75 mV, which was close to E_K (Fig.11B). Furthermore, SOCs blocker 2-APB (50 μm)

significantly reduced the current density of the outward current from 22.1±7.6 pA/pF (n=6) to 4.2±2.0 pA/pF (n= 5; P<0.01) (Fig, 11 D-F). All these data demonstrate that in human macrophages Ca^{2+} influx through SOCs activates IK_{Ca} channels.



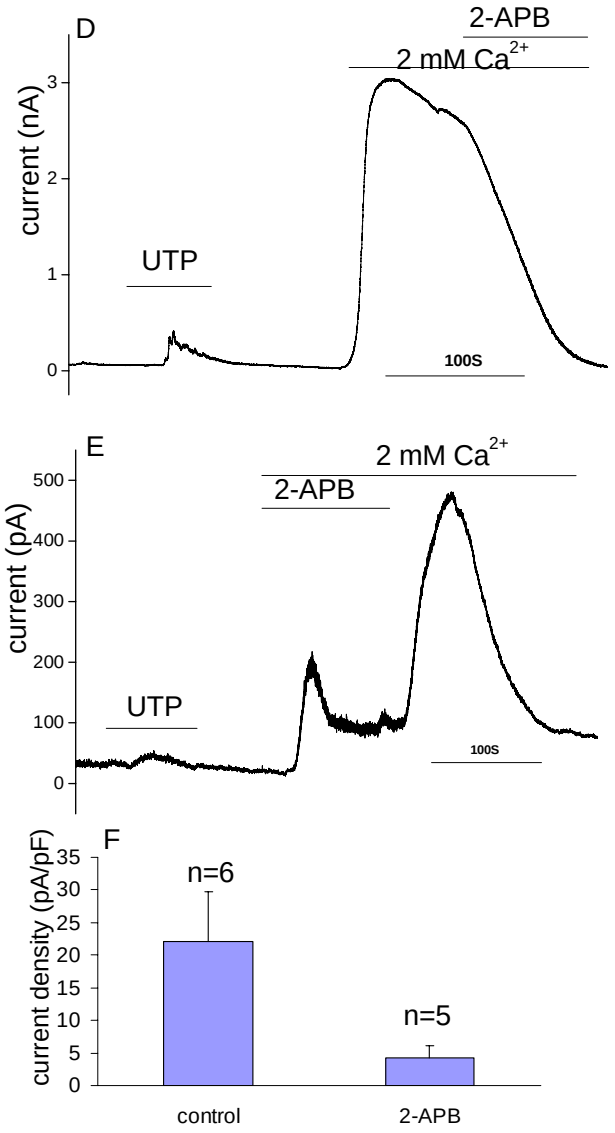


Figure 11: IK_{Ca} currents activated by Ca^{2+} influx through SOCs. (A) Ca^{2+} influx through SOC by emptying Ca^{2+} store with UTP induced an outward current at 0 mV, which was significantly reduced at presence of 100 nM CHTX (red). Cells were bathed in Ca^{2+} free solution. (B) CHTX blocked the current elicited by UTP-induce Ca^{2+} entry through SOCs. (C) Peak current density at 0 mV induced by re-addition of Ca^{2+} in the absence (control) and presence of 100 nM CHTX (CHTX).(**P*<0.001). (D) The outward current at 0 mV induced by Ca^{2+} influx was blocked by 50 μM 2-APB. (E) Pretreatment with 50 μM 2-APB reduced the outward current induced by Ca^{2+} influx, which was partially recovered by washing out of 2-APB. (F) Peak current density in the absence (control, as in D) and presence of 50 μM 2-APB (2-APB, as in E) (**P*<0.01).

3.6 Membrane hyperpolarization induced by Ca^{2+} influx through SOCs

Ca^{2+} influx through SOCs will depolarize the membrane. At the same time, influxed Ca^{2+} will activate IK_{Ca} channels and induce K^+ efflux, thus hyperpolarize the membrane. To elucidate the net membrane potential changes occurred during Ca^{2+} entry, we continuously recorded membrane potential of human macrophages at 0 pA.

1) Store-dependence of membrane hyperpolarization induced by Ca^{2+} influx.

With weak Ca^{2+}-buffer (100 nM free Ca^{2+}) in pipette solution, macrophages had a mean membrane potential of -21.3 ± 8.3 mV (*n*=141) in the Ca^{2+}-free bath solution. As shown in Fig. 12A, 10 μM UTP induced a membrane potential oscillation in Ca^{2+}-free solution, which is similar to that reported previously (Hanley et al., 2004). Re-addition of 2 mM Ca^{2+} into the bath solution had no effects on the membrane potential (Fig. 12A), indicating that there was no Ca^{2+} influx through SOCs. In contrast, 100 μM UTP induced a transient and single membrane hyperpolarization with no obvious oscillations. Unexpectedly, the ensuing re-addition of 2 mM Ca^{2+} to bath solution caused a large membrane hyperpolarization (Fig. 12B). In some cases, 100 μM UTP induced only a Ca^{2+} oscillation but not a transient Ca^{2+} release.This resulted in diminished Ca^{2+} influx after re-addition of Ca^{2+} into bath solution (Fig.12C), indicating the Ca^{2+} influx is Ca^{2+} store dependent. The maximal membrane potential evoked by Ca^{2+} entry was -66.2 ± 6.0 mV (*n*=16) (Fig. 13C). These data suggest that Ca^{2+} influx has an important role in regulating membrane potential.

2) Ca^{2+} influx through SOC is inhibited by CHTX

IK_{Ca} channels are very sensitive to elevation of intracellular Ca^{2+} and may be involved in Ca^{2+} entry-induced hyperpolarization. To confirm this, we further investigated the effect of the IK_{Ca} channel blocker CHTX on membrane potential. CHTX at 100 nM blocked membrane hyperpolarization induced by Ca^{2+} entry evoked by emptying Ca^{2+} store with UTP (Fig. 13A). Pretreatment of the macrophage with 100 nM CHTX also significantly reduced Ca^{2+} entry-induced hyperpolarization to -22.8±3.4 mV (*n*=6; Fig. 13 B, C). To clarify the underlying Ca^{2+} entry pathways, we tested the effect of 2-APB on membrane hyperpolarization. 2-APB is a relatively specific blocker of SOCE. 50μM 2-APB reversibly inhibited Ca^{2+} entry induced membrane hyperpolarization to

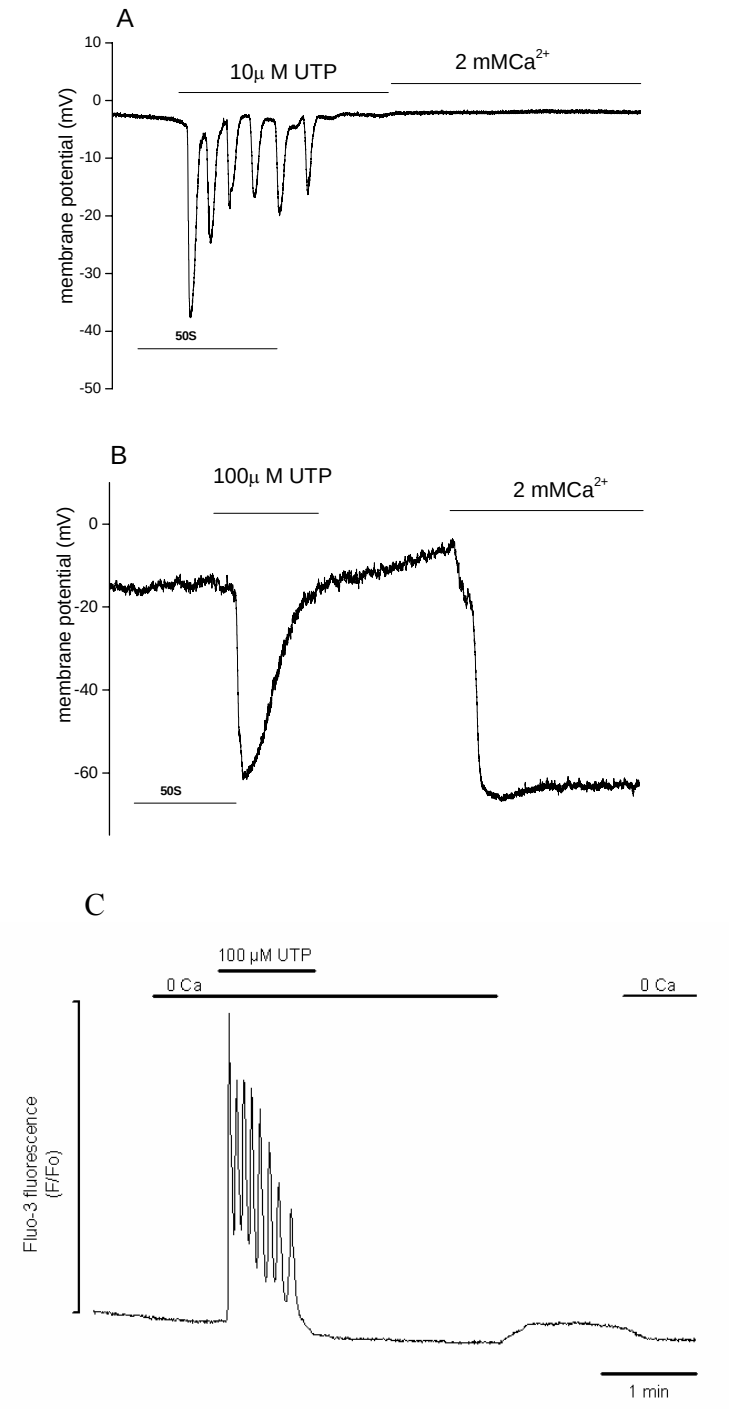


Figure 12: Store-dependent Ca^{2+} entry (SOCE). (A) Membrane potential continuously recorded in the current-clamp mode. In Ca^{2+}-free solution, 10 μM UTP induced membrane potential oscillations; re-addition of Ca^{2+} to the bath solution did not cause any change in membrane potential. (B) 100 μm UTP induced a transient hyperpolarization; re-addition of Ca^{2+} caused a strong hyperpolarization.

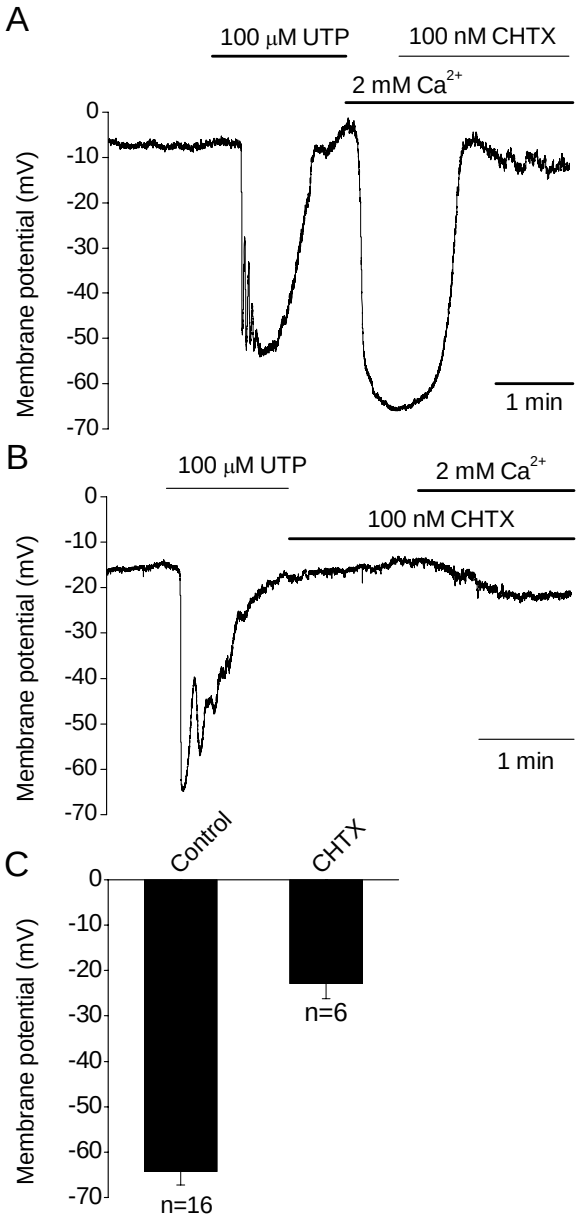


Figure 13: Hyperpolarization induced by Ca^{2+} influx after depletion Ca^{2+} store with UTP. Cells were bathed in Ca^{2+} free solution and membrane potentials were continuously recorded in the current-clamp mode. (A) 100 nM CHTX inhibited hyperpolarization induced by re-addition of extracellular Ca^{2+}. (B) Presence of CHTX reduced the hyperpolarization following re-addition of 2mM Ca^{2+}. (C) Peak membrane potential at absence and presence of CHTX (**P*<0.001).

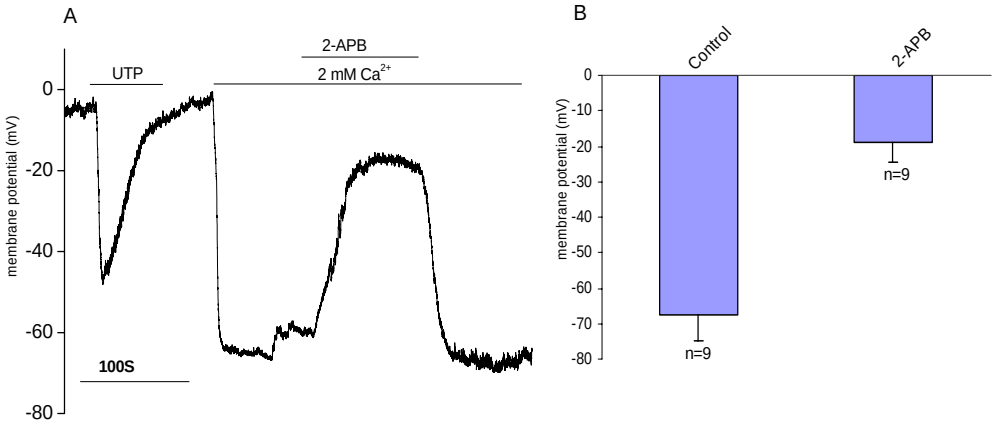


Figure 14: 2-APB inhibited hyperpolarization induced by Ca^{2+} influx. Cells were bathed in Ca^{2+}-free solution and membrane potentials were continuously recorded at 0 pA. (A) After depletion of Ca^{2+} store with UTP, re-addition of Ca^{2+} into bath solution caused a hyperpolarization, which was inhibited by 50 µM 2-APB.The hyperpolarization recovered after washout of 2-APB. (B) Peak membrane potential before and after treatment with 2-APB (P<0.001).

-19.1± 5.3 mV (n=9) (Fig.14 A, B). Taken together, these data suggest that Ca^{2+} entry through SOCs can induce membrane hyperpolarization by activation of IK_{Ca}. The hyperpolarization induced by activation of IK_{Ca} is dominant and masks the depolarization caused by Ca^{2+}entry itself.

We have shown that TPG can induce I_{CRAC} in macrophages. Membrane potential measurement proved that TPG also induced membrane hyperpolarization. After treatment macrophages with 500 nM TPG for 1.5 min, re-addition of 2 mM Ca^{2+} into the bath solution induced membrane hyperpolarization with an amplitude similar to that with UTP. Subsequent removal of the extracelluar Ca^{2+} abolished the hyperpolarization. This process could be repeated for several times by repeated addition and removal of Ca^{2+} from the bath solution (Fig. 15A). CHTX at 100 nM could partially inhibit the hyperpolarization (Fig. 15B).

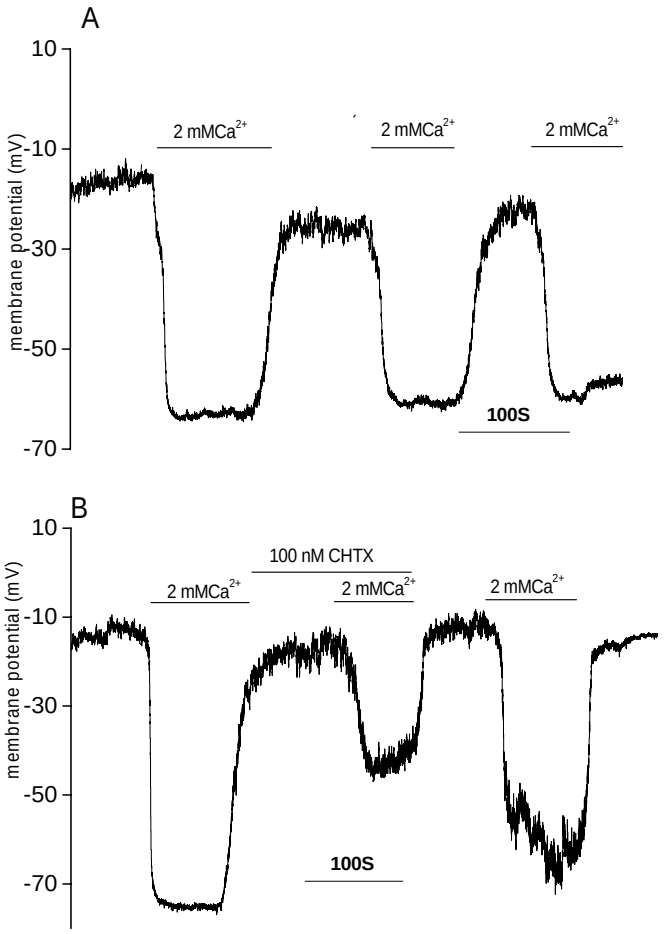


Figure 15: Hyperpolarization induced by Ca^{2+} influx after TPG treatment. Macrophages were treated with 500 nM TPG for 1.5 min to completely empty the Ca^{2+} store before the continuous recording of membrane potential in the current-clamp mode. (A) Addition of 2 mM Ca^{2+} to bath solution induced membrane hyperpolarization. (B) CHTX inhibited membrane hyperpolarization induced by addition of Ca^{2+}.

3.7 IK_{Ca} regulates store-operated Ca^{2+} entry

We then tested the effect of IK_{Ca} channel blocker CHTX on cytoplasmic Ca^{2+} using Fluo-3/AM as the Ca^{2+} indicator. As shown in Fig.16 A and B, TPG induced transient increase of intracellular free Ca^{2+} ($[Ca^{2+}]i$). Re-addition of 1.3 mM Ca^{2+} into the bath solution induced dramatic increase of $[Ca^{2+}]i$, which decayed slowly. In the presence of 100 nM CHTX, the peak level of Ca^{2+} fluorescence ($F/F_0 = 4.1\pm0.5$) (*n*=11) was not significantly different from that measured under control conditions ($F/F_0 = 4.1 \pm 0.6$) (*n*=7). However, CHTX accelerated the decay of $[Ca^{2+}]_i$. Under control conditions $[Ca^{2+}]i$ decayed to 80.3 ± 5.3 % of the peak level (*n*=7) within 4 min; at presence of CHTX, $[Ca^{2+}]_i$ decayed to 52.8 ± 6.4% of the peak level within 4 min (Fig. 16C; *n*=11). After re-addition of 0.3 mM extracellular Ca^{2+}, $[Ca^{2+}]$ i decayed to 69.6 ± 5 % of peak level at 4 min.

In symmetrical K^+ solution, the membrane potential is almost "clamped" at 0mV. We then compared Ca^{2+} influx properties in symmetrical K^+ and in physiological K^+ solution. As shown in Fig.17A and B, TPG induced Ca^{2+} influx could be repeated for several times by addition and removal of Ca^{2+} from the bath solution, although the peak level tended to decay slowly. Switching of the bath solution to symmetrical K^+ had little effects on peak levels of Ca^{2+} fluorescence but slowed the initial rates of Ca^{2+} influx. In contrast, changing the bath solution from symmetrical K^+ solution to physiological solution not only accelerated the Ca^{2+} influx rate, but also increased the peak levels. As shown in Fig.17C, time to 50% peak levels of Ca^{2+} fluorescence was 7.0±1.4 s, 17.3±3 s and 7.4±0.8 s respectively in physiological K^+ (control) solution, symmetrical K^+ (high K^+) solution and in the presence of 100 nM CHTX.

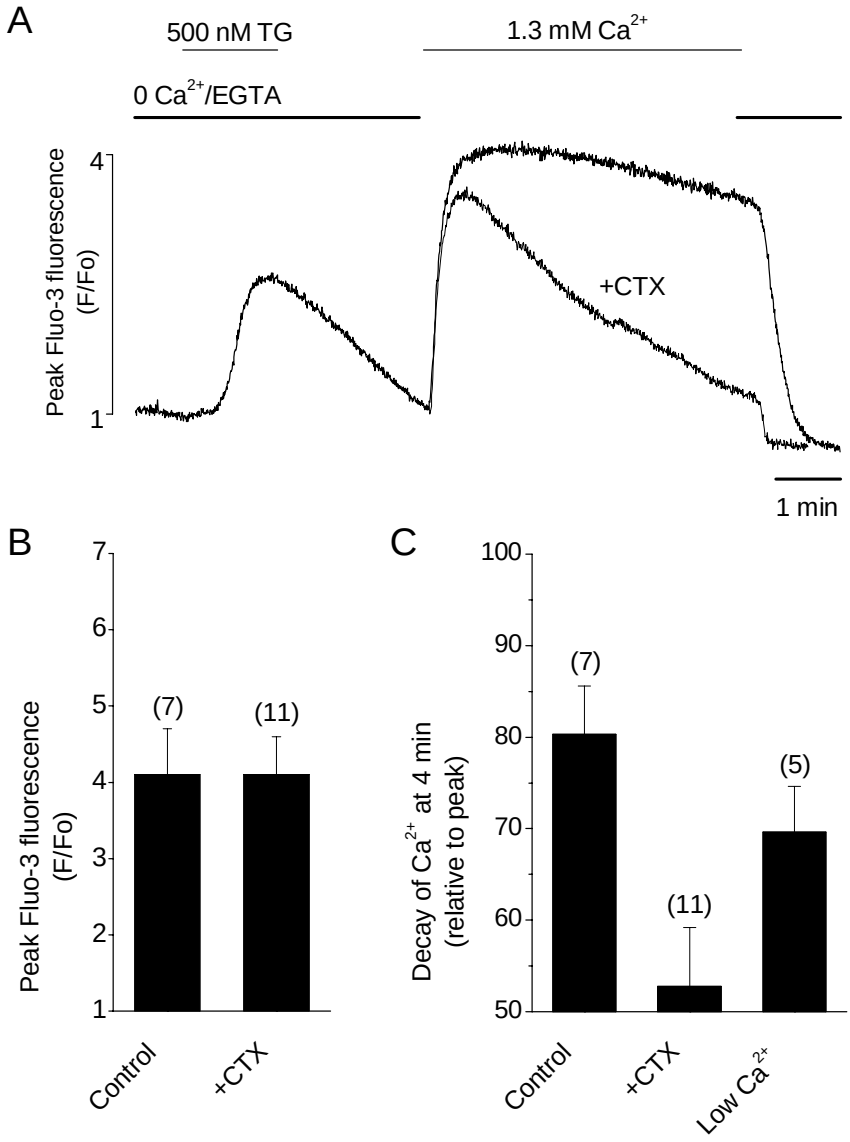


Figure 16: Effects of CHTX on SOCE in human macrophages. (A) Ca^{2+} fluorescence measurement of Ca^{2+} influx induced by TPG in the presence and absence of CHTX. (B) Peak Fluo-3 fluorescence in the absence and presence of CHTX. (C) The decay of Ca^{2+} fluorescence relative to value of the peak levels after 4 min.

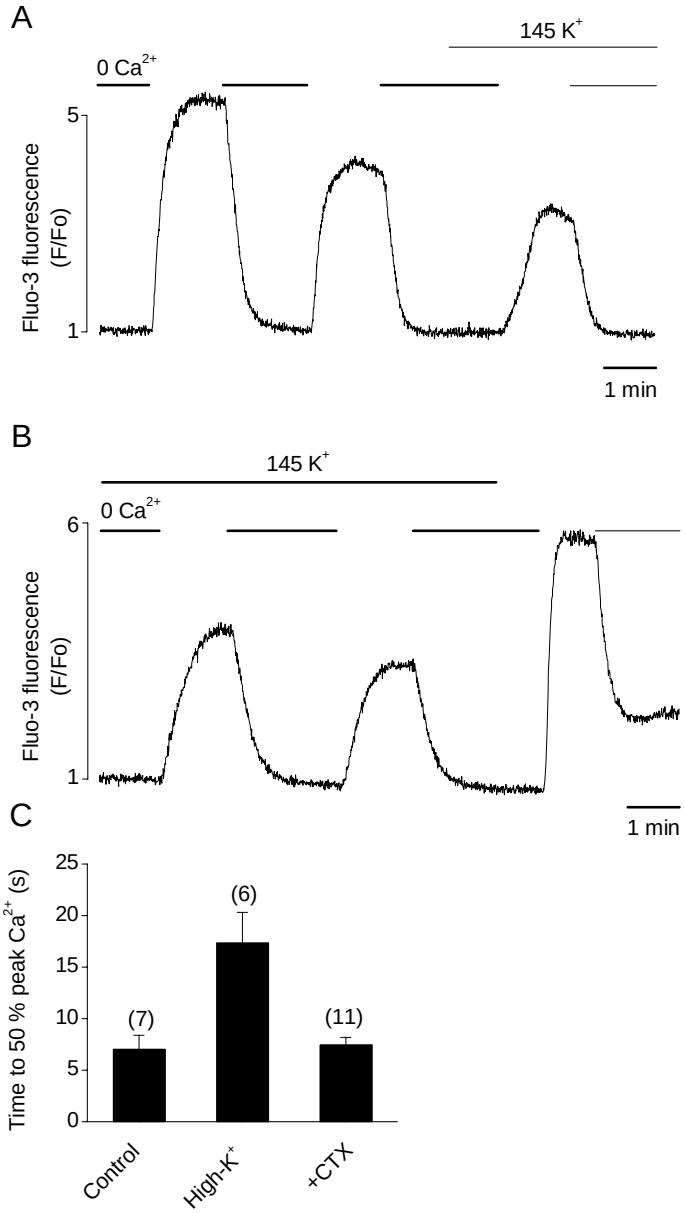


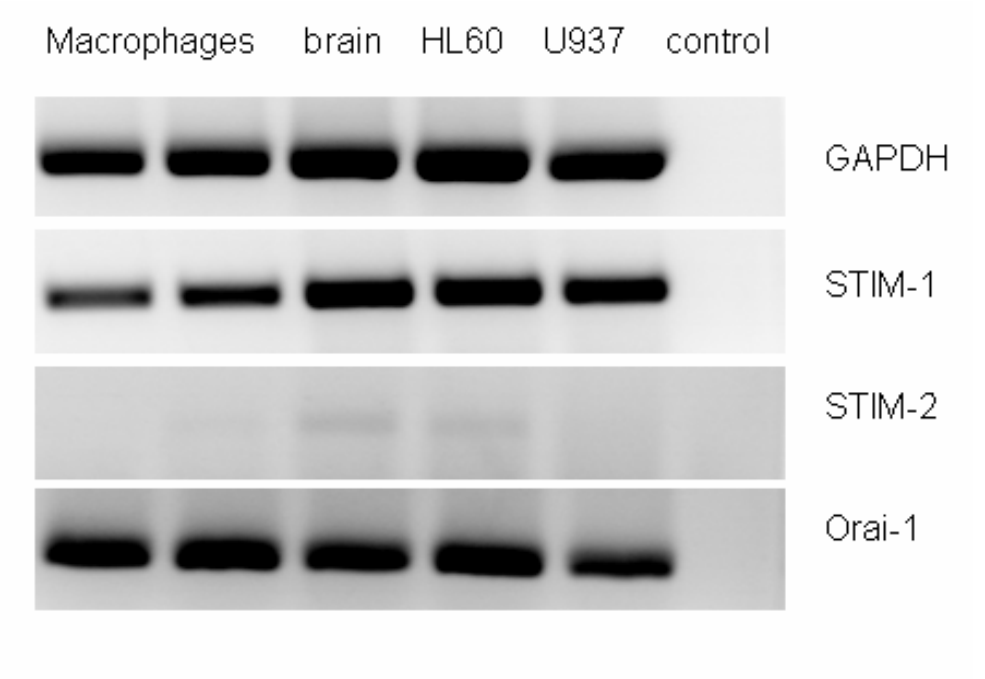
Figure 17: SOCE in physiological and symmetrical K^+ solutions. Intracellular Ca^{2+} stores were depleted by application of 500 nM TPG. (A) SOCE was measured in physiological K^+ solution and then in 145 K^+ solution. (B) SOCE was measured in 145 K^+ solution and then in physiological K^+ solution. (C) The time to 50% peak Ca^{2+} levels was used to demonstrate the initial rates of Ca^{2+} influx in control (physiological K^+) , high K^+ solution (145 mM K^+) and in the presence of 100 nM CHTX.

3.8 Molecular candidates of store-operated Ca^{2+} channels

Up to date, the molecular identity of SOC channels that mediates Ca^{2+} entry in response to Ca^{2+} store depletion and the mechanisms by which Ca^{2+} store depletion is communicated to the SOCs at the cell surface are still not clear. Recent studies using RNA interference techniques have found two important proteins that may be the long sought Ca^{2+} sensors and SOC channels. STIM1 was identified as a primary candidate of the Ca^{2+} sensor that couples Ca^{2+} store depletion to SOC activation (Roos et al., 2005; Zhang et al., 2005), whereas membrane protein Orai1 was found to be essential for the function of SOC channels (Feske et al., 2006). In addition, previous studies also suggested that all members of TRPC channel family except TRPC2, which is pseudogene in human, as well as TRPV6, TRPM2 and TRPM7 might be molecular candidates for SOC channels (Parekh, 2005). Latest studies found that STIM1 interacts with TRPC channels and this interaction may be crucial for the formation of SOC channels (Yuan et al., 2007). We therefore investigated the expression pattern of all these possible molecular candidates of SOCs in human macrophages derived from PBMCs and some other tissues.

Our RT-PCR assay demonstrated that STIM1 and Orai1 intensively expressed in human brain, whereas STIM2 showed weak expression (Fig.18). TRPC1, TRPC3-6, TRPM2 and TRPV6 also expressed in human brain with different intensity (Fig 19). In human macrophages derived from PBMC at day 14 and CD14 selected, transcription of TRPM2 and TRPM7 were detected (Fig. 19). All the three members of Orai protein family Orai1, Orai2 and Orai3 abundantly expressed in human macrophages. STIM1 also strongly expressed in human macrophages. In contrast, STIM2 only weakly expressed at the mRNA level in human macrophages (Fig.18). U937 is a human promonocytic cell line that can differentiate to macrophages by PMA; the cell line HL60 can differentiate to neutrophil-like cells by the chemoattractant formyl-methionyl-leucyl-phenylalanine (fMLP). At the mRNA level, we found strong expression of STIM1 and Orai1 as well as weak expression of STIM2 in these two cell lines (Fig.18).

A



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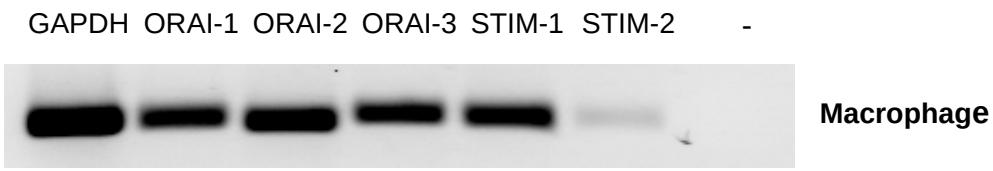

Figure 18: Expression pattern of STIM1, STIM2 and Orai1. (A) First band, constitutively expressed GAPDH was used as a measure of amount of input RNA. Second band, STIM1 strongly expressed in human brain, macrophages, and leukemia cell line HL60 and U937, whereas STIM2 only showed a weakly expression in these types of cells. Bottom band, Orai1 strongly expressed in all the tissues checked. As a control, cDNA was omitted from the reactions in the last lane. (B) In human macrophages, all three members of Orai proteins strongly expressed; the expression pattern of STIM1 and STIM2 was the same as in A.

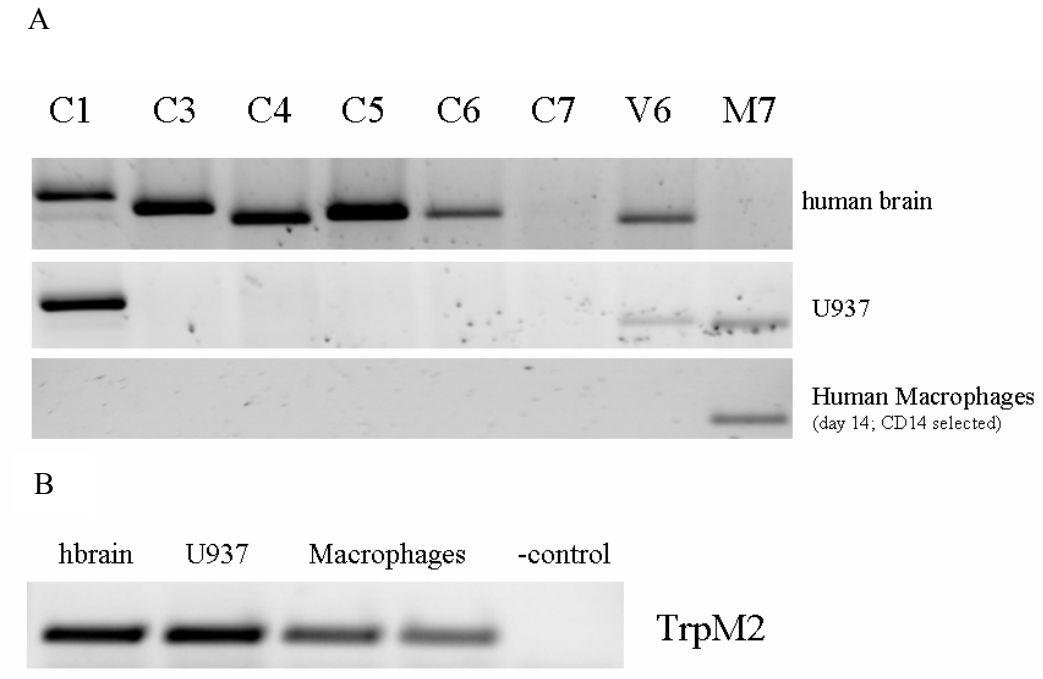

Figure 19: TRP channels in human tissues. (A) Analysis of the expression of TRPC 1-6, TRPV6 in human brain (top row), in U937 cells (middle row) and in human macrophages (bottom row). TRPC1, TRPV6 and TRM7 expressed in U937 cells. In human macrophages, only TRPM7 was detected by RT-PCR. (B) Expression of TRPM2 in human brain, U937 cells and human macrophages.

4. Discussion

4.1 Ca^{2+}-activated K^+ channel in human macrophages

Our experiments have clearly demonstrated the existence of outward currents flowing through intermediate-conductance Ca^{2+}-activated potassium channels (IK_{Ca}) in human macrophages.These IK_{Ca} currents could be activated by perfusion macrophages with 1 μM Ca^{2+} pipette solution, by IK_{Ca} activator DCEBIO and by Ca^{2+} entry. This is consistent with previous study which showed the expression of KCNN4 ($IK_{Ca}1$) and KCNMA1 (BK_{Ca}) mRNA on human macrophages (Hanley et al., 2004). All the currents elicited by these methods had almost linear IV curve reversed at E_K and could be blocked by clotrimazole or CHTX, which are consistent with features of cloned IK_{Ca} channels (Joiner et al., 1997) and other studies on human macrophages (Gallin, 1989). In addition, our previous single channel recording (Hanley et al., 2004) showed that BK_{Ca} channels are also functionally expressed in human macrophages but play a minor role in macrophage membrane potential oscillation. In this study, we found that at 100 nM pipette Ca^{2+}, treatment the macrophages with IK_{Ca} activator DCEBIO gave rise to a current with linear IV curve (Fig. 9). Furthermore, most of the current induced by Ca^{2+} entry was blocked by CHTX (Fig. 8E). Considering that Kv channels are also expressed in human macrophages, the remaining small outward current may come from Kv channel and/or BK_{Ca} channels. The hyperpolarization evoked by Ca^{2+} entry was dramatically reduced by CHTX, suggesting that IK_{Ca} channels mediate changes of membrane potential. This is not consistent with the study in rat pinealocytes, which showed that BK_{Ca} is responsible for membrane hyperpolarization induced by store-operated Ca^{2+} entry (SOCE) (Lee et al., 2006). In human macrophages, we did not find any expression of SK_{Ca} channels, this is not consistent with the result in rat alveolar macrophage, which showed that the outward K^+ current induced by ATP or UTP is an apamin-sensitive SK_{Ca} current (Bowler et al., 2003). These results suggest that the expression of K_{Ca} channels in macrophages may vary between species.

4.2 UTP induced Ca^{2+} release

Hanley et al. (2004) previously demonstrated that low concentration of UTP (10 μM) induces membrane potential and $[Ca^{2+}]i$ oscillation due to the Ca^{2+} recycling between cytoplasm and ER Ca^{2+} store under the effects of IP3R and Ca^{2+}-ATPase. In the present study, we showed that high concentration (100 μM) UTP did not induce oscillation of membrane potential and $[Ca^{2+}]i$ but transient and single increase of $[Ca^{2+}]i$ and membrane hyperpolarization in Ca^{2+}-free solution. Re-addition of Ca^{2+} (2 mM) induced sustained strong hyperpolarization. UTP increased peak $[Ca^{2+}]i$ with an EC_{50} of 0.7 μM in mice peritoneal macrophages (Del Rey et al., 2006), with an EC50 of 4 μM in rat alveolar macrophages (Bowler et al., 2003) and with an EC50 of 0.44 μM in promonocytic U937 cells (Santiago-perez et al., 2001). These data suggested that UTP-induced Ca^{2+} responses saturated at concentration of 100 μM. Therefore, we conclude that high concentration of UTP depletes Ca^{2+} store adequately and thus it can be used as a tool to induce the opening of SOC channels.

Our previous study found P2Y1, P2Y2 and P2Y11 as well as P2X1, P2X4 and P2X7 receptors are expressed in CD14 selected human macrophages (Hanley et al., 2004). The UTP-recognizing P2Y receptors include only P2Y2 and P2Y4 (Abbracchio et al., 2006). Therefore, P2Y2 receptors mainly mediate the effects of UTP on Ca^{2+} release. UTP failed to induce Ca^{2+} response in P2Y2 knockout mouse peritoneal macrophages, also indicating that P2Y2 is the sole receptor for UTP in macrophages (Del Rey et al., 2006). In rat alveolar macrophages (Bowler et al., 2003) and intracardiac neurons (Liu et al., 2000), P2Y signaling could be inhibited by the PLC inhibitor U73122. Stimulation of non-P2Y2 Gq-coupled receptors with complement factor C5a in mice macrophages gave rise to a similar Ca^{2+} response (Del Rey et al., 2006). These data indicate that activation of P2Y2 receptors by UTP leads to activation of PLC, resulting in the production of IP3, which then binds to the IP3 receptor on the ER membrane and releases Ca^{2+} from the intracellular stores (Fig. 20). The evidence that IP3 included in pipette solution could also induce I_{CRAC} (Malayev et al., 1996) supports the idea that IP3 mediates UTP-induced Ca^{2+} release. Ca^{2+}-induced-Ca^{2+}-release (CICR) via ryanodine receptors could not play a role in UTP-induced Ca^{2+} release because our

previous study showed that caffeine had no effects on Ca^{2+} oscillation induced by ATP or UTP (Hanley et al., 2004).

The Ca^{2+} release induced by UTP was transient and deactivated in a few minutes. This is due to Ca^{2+}-and/or ligand-dependent inactivation of the release channels (IP3R) themselves, clearance of Ca^{2+} from the cytosol by resequestration into other organelles (notably ER and mitochondria) as well as extrusion from the cell by Na^{+}/Ca^{2+} exchanger and Ca^{2+}-ATPases in the plasma membrane (Parekh and Putney, 2005). For some cellular processes of macrophages, a sustained $[Ca^{2+}]_i$ elevation is required and accomplished by Ca^{2+} entry.

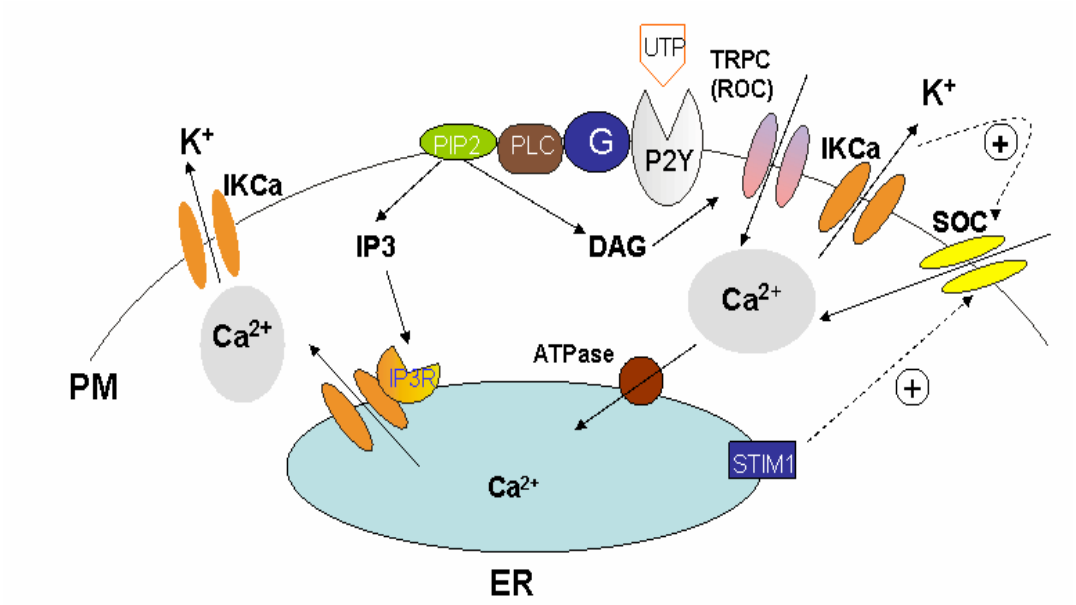

Figure 20: Schematic diagram the signaling pathways of UTP induced Ca^{2+} entry and the positive feedback between IK_{Ca} activation and Ca^{2+} entry. UTP binds to P2Y receptors, which are coupled to PLC by G_q protein. PLC hydrolyses PIP2 to DAG and IP3. IP3 binds to IP3 receptors on the ER membrane. IP3 receptors act as Ca2+ channels and mediate the release of Ca^{2+} from ER. ER Ca^{2+} store depletion evokes the translocation of STIM1 to ER membrane underneath plasma membrane (PM) where STIM1 interacts with SOC channels (Orai) and activates SOC. Ca^{2+} entry through SOC activates IK_{Ca} channels and induces membrane hyperpolarization, which will enhance driving force for Ca^{2+} entry. Thus, Ca^{2+} entry and IK_{Ca} compose a Ca^{2+} entry positive feedback loop. In some cases, DAG produced from PIP2 activates TRPC channels on plasma membrane, which is the receptor-operated Ca^{2+} entry pathway. Ca^{2+} released from ER Ca^{2+} store or entered from outside are sequestered into ER Ca^{2+} by SERCA ATPase, or PMCA and mitochondria (not shown).

4.3 Ca^{2+} store dependence of Ca^{2+} influx

The most important feature of SOCE is that it is strictly dependent on the filling state of ER store, no matter how the Ca^{2+} stores are emptied. Consistently, we showed that I_{CRAC} could be activated by perfusion macrophages with the Ca^{2+} chelator EGTA at high concentration, by the P2Y2 receptor agonist UTP and the SERCA inhibitor TPG. There was no significant difference in the current density of I_{CRAC} induced in these distinct ways. Furthermore, both UTP and TPG could activate an outward current and induce membrane hyperpolarization after re-addition of Ca^{2+}; the amplitude of the outward currents and membrane potentials were also comparable. These results provide strong evidence in support of the fundamental concept of SOCE that Ca^{2+} content of intracellular Ca^{2+} pool is the primary determinant of the rate of Ca^{2+} entry (Takemura, 1989). The SOCE activated by Ca^{2+}-mobilizing agonists such as UTP or ATP can also be equally triggered by depletion of intracellular Ca^{2+} store with TPG. After inhibition of the SERCA with TPG, repeated addition and removal of extracellular Ca^{2+} elicited repeated membrane hyperpolarization (Fig.15) and Ca^{2+} influx (Fig.17). In contrast, UTP usually induced only single Ca^{2+} release and entry response. We speculate that the desensitization of P2Y receptor induced by UTP (Del Rey et al., 2006; Santiago-Perez et al., 2001) underlies the difference. Another factor that contributes to the difference between the effects of UTP and TPG may be that SERCA inhibition by TPG delays Ca^{2+} store refilling.

As discussed before, we showed that high concentration of UTP induces Ca^{2+} entry whereas low concentration of UTP more likely induces Ca^{2+} oscillations (Hanley et al., 2004). This is consistent with the observation that low concentration of IP3 can partially empty Ca^{2+} store yet fails to activate Ca^{2+} influx (Parekh et al., 1997). In a few macrophages, as shown in Fig.12, 100 μM UTP only induced Ca^{2+} and membrane potential oscillation without subsequent Ca^{2+} influx and membrane hyperpolarization. The model proposed by Ferrio and Parekh (Fierro and Parekh, 2000) that a threshold exists within IP3-sensitive Ca^{2+} store, below which intraluminal Ca^{2+} needs to fall before I_{CRAC} activates, could explain this result. Taken together, these data demonstrate the Ca^{2+} store dependence of Ca^{2+} influx although in a nonlinear fashion. Therefore, the

cellular processes that require only Ca^{2+} release can be activated separately from those require Ca^{2+} entry (Fierro and Parekh, 2000).

Our experiments showed that both the membrane hyperpolarization and the outward current induced by Ca^{2+} influx could be inhibited by 50 μM 2-APB, indicating the both changes are mediated by SOCE in human macrophages. 2-APB is a compound commonly used to inhibit store-operated Ca^{2+} channels and IP3 receptors. Besides its effects on SOC channels, 2-APB also activates TRPV1, TRPV2 and TRPV3 channels (Hu et al., 2004) and inhibits the activity of TRPC6, TRPM8 (Hu et al., 2004); TRPC3 (Ma et al., 2000); TRPC5, TRPC6 and TRPM3 (Xu et al., 2005). TRPM2 is not sensitive to 2-APB (Xu et al., 2005). Another study showed that 2-APB has the potential to inhibit the SERCA Ca^{2+} pump; and this effect was dependent on isoforms of SERCA, with SERCA 2B being more sensitive than SERCA 1A (Bilmen et al., 2002). Although TRPM2 and TRPM7 are expressed in human macrophages, they are not sensitive to 2-APB. In our study, 2-APB will not influence the action of IP3 on Ca^{2+} store because it was added to bath solution after treatment the macrophages with UTP. In addition, we also showed that 50 μM 2-APB completely blocked the inward I_{CRAC} evoked by UTP. These data suggest that in the present study the inhibitory effects of 2-APB on membrane potential and outward current are contributed to its effects on SOC channels.

4.4 Localized $[Ca^{2+}]_i$ elevation coupled to K^+ channels

We showed that 100 μM UTP induced a transient outward current and membrane hyperpolarization in human macrophages at Ca^{2+}-free solution. Similar outward currents induced by UTP could also be shown in rat intracardiac neurons (Liu et al., 2000) and rat alveolar macrophages (Bowler et al., 2003) with the same Ca^{2+} mobilizing mechanism as in human macrophages. Activation of endothelial IK_{Ca} channels has been demonstrated to contribute to cerebral arteries dilatation induced by UTP (Marreli et al., 2003). Thus, IK_{Ca} activated by Ca^{2+} released from ER store contributes to the outward current and membrane hyperpolarization. SOCE also evoked an outward current and membrane hyperpolarization by activation of IK_{Ca} channels. These data suggest that IK_{Ca} channels are functionally coupled to $[Ca^{2+}]i$

elevation. In fact, Ca^{2+} released from ER or entered from outside is not equally dispersed in cytoplasm but is initially localized at sites near IP3 receptors or near the inner mouth of SOC channels to form Ca^{2+} microdomain (Berridge, 2006). Thus, the rise in local $[Ca^{2+}]_i$ could sufficiently activate K_{Ca} channels in the vicinity of Ca^{2+} release or entry channels. The coupling between SOC Ca^{2+} entry and (the much less Ca^{2+}-sensitive) BK_{Ca} channels has been shown in rat pinealocytes (Lee et al., 2006) and mice colonic myocytes (Bayguinov et al, 2001). SOCE also activated IK_{Ca} and SK_{Ca} in T cells (Verheugen et al., 1995; Fanger et al., 2001). A very recent study showed that TRPC3 channels mediated Ca^{2+} entry was also coupled to IK_{Ca} channels in cochlear outer hair cells (Raybould *et al*. 2007). In addition, BK_{Ca} and voltage-dependent Ca^{2+} channels (CaV) (Berkefeld et al., 2006), SK_{Ca} and CaV (Wolfart et al., 2002) in central nervous system also form a functional complexes that mediate rapid and localized K_{Ca} channels signaling. Thus localized $[Ca^{2+}]_i$ elevation could induce a global membrane potential changes mediated by high Ca^{2+} affinitive IK_{Ca} channels, and may thus enhance the sensitivity of macrophages to diverse agonists.

4.5 I_{CRAC} in human macrophages

Store-operated Ca^{2+} entry (SOCE) was first electrophysiologically characterized as CRAC current (I_{CRAC}） by Hoth and Penner (1992) in mast cells. Subsequently, I_{CRAC} have been recorded in other types of blood cells or cell lines including T cells (Zweifach and Lewis, 1993), HL-60 cells (Song et al., 1998), rat basophilic leukemia cells (Feriio and Parekh, 2000), pro-monocytic U937 cells (Floto, et al., 1996) and human macrophages (Malayev et al., 1996). In macrophages, I_{CRAC} induced by depletion Ca^{2+} store with IP3, TPG and EGTA (Malayev et al., 1996) have similar current size and properties to that in our recording. Similar I_{CRAC} have also been described in rat microglia, the brain resident macrophages (Hahn et al., 2000). We are the first group to demonstrate that purinergic agonist UTP could also induce I_{CRAC} in human macrophages via activation of P2Y receptors. The purinergic agonist ATP also induced an inward Ca^{2+} current in rat macrophages (Naumov et al., 1995), which is distinct from UTP induced I_{CRAC} in that: 1) ATP-induced current was Mg^{2+}-sensitive; 2) it could not be activated by UTP; 3) it could also be recorded at outside-out

configuration. P2X channels expressed in the membrane of macrophages may underlie the ATP-induced current. In U937 cells, I_{CRAC} is correlated to differentiation and the size of I_{CRAC} is cell-to-cell variable, the underlying mechanisms of these differences are not clear (Floto, et al., 1996). In contrast, macrophages derived from PBMC showed much similar size of I_{CRAC} in spite of the heterogeneity of macrophages (Gordon and Taylor, 2005). The strong inward rectification of I_{CRAC} in human macrophages also provides evidence to support our hypothesis that hyperpolarization of membrane will facilitate Ca^{2+} influx. In addition, the extracellular pH also inhibits I_{CRAC} in human macrophages although the mechanism is still not clear (Malayev et al,. 1996). It may be an intrinsic protective mechanism by which Ca^{2+} influx into inflammatory cells is inhibited in acidic environment characteristic of tumor and abscess.

Recently, the arachidonic acid regulated Ca^{2+} channel (ARC) has been described as a novel receptor-activated Ca^{2+} entry pathway (Shuttleworth et al., 2004). The currents through ARC are much similar to I_{CRAC} in that it displays marked inward rectification, a reversal potential greater than + 40 mV; high selectivity for Ca^{2+} and inhibition by La^{3+}. Unlike I_{CRAC}, ARC currents do not show any fast inactivation and inhibition by 2-APB and reduction in extracellular pH. Based on these differences, we concluded that 100 μM UTP induced Ca^{2+} current is mediated by CRAC but not ARC. Because stimulation with low concentration agonists specially activates ARC (Shuttleworth et al., 20004), it appears possible that ARC will play a role in low concentration UTP induced Ca^{2+} oscillations, although the existence of ARC in human macrophages has not been verified.

4.6 Voltage dependence of Ca^{2+} entry through SOC

Our experiments showed that Ca^{2+} influx through SOC in macrophages was reduced at high extracellular K^+ (which causes a depolarization) or in the presence of the IK_{Ca} channel blocker CHTX, indicating that IK_{Ca} channels play a role in facilitation of Ca^{2+} influx through SOC. This result is consistent with studies in other cell types including T cells (Fanger et al., 2001; Srivastava et al, 2006 b, c) and mast cells (Mark Duffy et al., 2004), which also showed that IK_{Ca} channels sustain the Ca^{2+} influx. Studies in T lymphocytes demonstrated that membrane hyperpolarization is followed by an

increase in $[Ca^{2+}]_i$, whereas membrane depolarization results in a decrease of $[Ca^{2+}]_i$ (Verheugen et al., 1995). The CRAC channels have a voltage-independent gating and the IV curve is inwardly rectifying (Hoth and Penner, 1992). This means that once the CRAC channels are activated, the electrochemical driving force for Ca^{2+} will determine the magnitude of the inward currents. The Ca^{2+} influx evoked by depletion of the intracellular Ca^{2+} stores will depolarize the membrane potential. In the absence of counterbalancing cation efflux, this depolarization will decrease further Ca^{2+} entry due to the diminished electrochemical driving force for Ca^{2+}. In macrophages, an elevation of $[Ca^{2+}]_i$ will cause IK_{Ca} activation and result in hyperpolarization, which may facilitate Ca^{2+} influx in two ways. Firstly, it will enhance Ca^{2+} entry by increasing the driving force; secondly, the strong inward rectification of I_{CRAC} indicates that Ca^{2+} influx increases at hyperpolarizaed membrane potential although the gating of the CRAC channels is voltage-independent. Thus, we propose here for the first time a positive feedback loop existed between membrane potential and Ca^{2+} entry in human macrophage mediated by IK_{Ca} channels, which will sustain the Ca^{2+} signaling needed for the regulation of biological role of macrophages. The Ca^{2+}-dependent inactivation of I_{CRAC} channels (Parekh and Putney, 2005) then terminates the SOCE.

The high sensitivity of IK_{Ca} channels to elevation in $[Ca^{2+}]_i$ indicates that the membrane potential of macrophages is primarily set by IK_{Ca} when the $[Ca^{2+}]_i$ is increased. Thus, IK_{Ca} channel is the molecular basis for this positive feedback loop. Macrophage membrane also functionally expresses another Ca^{2+}- activated K^+ channel, large conductance K_{Ca} channel (BK_{Ca}). BK_{Ca} is important in TNF-α and IL-8 secretion of macrophages stimulated by LPS (Papavlassopoulos et al, 2006) and is coupled to norepinephrine activated SOC Ca^{2+} entry in rat pinealocytes (Lee et al, 2006). Even though, BK_{Ca} plays a minor role in facilitating Ca^{2+} entry due to the low Ca^{2+} sensitivity, only at higher elevation of $[Ca^{2+}]_i$. The hyperpolarization provided by IK_{Ca} will reach a steady state set by attenuation of Ca^{2+} influx due to Ca^{2+}-dependent inactivation of I_{CRAC} and Ca^{2+} extrusion out of macrophages by PMCA.

High extracellular K^+ and K^+ channel blockers can attenuate the membrane hyperpolarization. Symmetrical K^+ will set the membrane potential close at $E_K \approx 0$ mV. In this condition, the feedback between membrane potential and Ca^{2+} entry is no longer

present. Our experiments showed that blocking IK_{Ca} channels with CHTX depolarized the membrane and reduced the driving force for Ca^{2+} entry but to a lesser extent than symmetrical K^+ solution. Two factors may contribute to this difference. Firstly, some other K^+ channels, e.g. Kv and Kir channels (Decoursey et al, 1996; Vincent et al., 2006), presented on the membrane of macrophages might be involved in providing driving force for Ca^{2+} influx. Kir channels play a major role in setting membrane potential in many cell types. Role of Kir channel in Ca^{2+} entry has been confirmed in microglial cells, macrophages resident in brain (Franchini et al., 2004). Secondly, the presence of Ca^{2+} activated Cl^- channel on human macrophage has been demonstrated by electrophysiological measurements (Holevinsky et al., 1994). In the absence of K^+ conductance, elevation of $[Ca^{2+}]i$ will cause the activation of chloride channels and set the membrane potential near Cl^- equilibrium potential (about -30 mV). In fact, our experiments showed that blocking IK_{Ca} with CHTX reduced the Ca^{2+} entry-induced hyperpolarization to about -20 mV, which is close to E_{Cl} calculated according to our bath and pipette solution (-33 mV). Thus Ca^{2+}-activated chloride channels may provide driving force for Ca^{2+} entry when IK_{Ca} channels are blocked. The importance of chloride channel in Ca^{2+} influx has been proven by studies in T cells (Wang et al., 2006), which showed that blocking Cl^- channels with DIDS reduced ConA evoked Ca^{2+} entry.

4.7 The molecular basis of SOCE in human macrophages

Recent studies using RNAi technology identified two proteins, STIM1 and Oria1, as components of SOC channels and I_{CRAC} (Vig et al., 2006). Co-expression of Orai1 and Stim1 give rise to typical I_{CRAC} (Peinelt et al., 2006). Besides Orai1, Orai2 and Orai3 are also been proved to conduct I_{CRAC} when co-expressed with STIM1, although the currents are smaller than that of Orai1 (Lis et al., 2007; DeHaven et al., 2007). Our RT-PCR results showed that STIM1, Orai1, Orai2 and Orai3 were expressed in human macrophages. We also recorded typical I_{CRAC} in human macrophages. Taken together, we speculate that STIM1 and one or more members of Orai proteins are most likely the molecular components of SOCs human in macrophages. STIM1 may function as the

ER Ca^{2+} sensor, whereas the role of each Orai protein is still to be elucidated. Further studies are required to resolve the molecular arrangements and interactions of these proteins. It is also necessary to examine the cellular location of STIM1 in human macrophages.

Very recent studies also suggest that TRPC channels may constitute non-Ca^{2+}-selective store-operated channels when co-expressed with STIM1 (Yuan et al., 2007). We did not find any expression of TRPC channels in human macrophages, therefore TRPC channels could not be the molecular candidates of SOCE in human macrophages.

Additionally, members of TRPM and TRPV channel family have also been proposed as components of SOC channels (Venkatachalam et al., 2002). We found that TRPM2 and TRPM7 strongly expressed on human macrophages at mRNA levels. TRPM7 channels cannot be components of CRAC channels in human macrophages because the biophysical properties of TRPM7 are distinct from that of I_{CRAC}. TRPM7 is a relatively nonselective cation channel with kinase activity at C terminus (Kozak et al., 2002). It is not store-operated, with a chord conductance about 80 pS at 100 mV, strongly outwardly rectifying, and inhibited by intracellular Mg^{2+}. It is not potentiated by low doses of 2-APB and is less sensitive to block by high doses of 2-APB. All these features are different from CRAC, which is an inwardly rectifying Ca^{2+}-selective channel with an estimated single channel conductance of 0.2 pS in DVF and a biphasic response to 2-APB (Prakiya et al., 2002; Kozak et al., 2002).

TRPM2 forms nonselective Ca^{2+} permeable cation channel. Intracellular ADP ribose induces the opening of this channel whereas elevation of cytosolic Ca^{2+} enhances the channel activity (Perraud et al., 2001). IV curve of TRPM2 is linear and reverses at 0 mV. In contrast, CRAC channel is permeable to Ca^{2+} in divalent cation-containing bath solution and permeable to Na^{+} in DVF solution (Prakiya et al., 2002). I_{CRAC} is inactivated by elevation of intracellular Ca^{2+} and is strongly inwardly rectifying with a reversal potential > +60 mV. The obvious differences between CRAC and TRPM2, TRPM7 indicate that these two TRP channels are unlikely to represent components of SOCE in human macrophages.

Our experiments found that in addition to STIM1 and Orai1, TRPC1 also expressed in monocytic U937 cells that can differentiate to macrophages. TRPC1 has been shown to

be required for activities of SOCE in different cell types (Smyth et al., 2006). Most recent studies demonstrated that TRPC1 is colocalized with Orai1 and STIM1 in human salivary gland cells; Orai1 and STIM1 are required for TRPC1 mediated SOC channels. Ca^{2+} store depletion induced a complex formation by TRPC1/STIM1/Orai1 (Ong et al, 2007). These data indicate that STIM1 and/or Orai1 may confer TRPC channels sensitivity to Ca^{2+} store depletion. It is not clear whether TRPC1 in U937 cells also forms a complex with Orai1 and STIM1. The different expression pattern of these proteins in U937 cells and macrophages suggests that molecular components of SOCE may vary in different cell types and differentiation states and may explain our observation that SOCE in monocytes is stronger than that in differentiated macrophages. Further studies are required to elucidate the relationship between the different activity and molecular candidates of SOCE in monocytes and macrophages.

4.8 Conclusions

In the present study, we found that depleting the ER Ca^{2+} store with UTP or thapsigargin can activate store-operated calcium entry in the plasma membrane of human macrophages; SOCE activates IK_{Ca} channels and leads to membrane hyperpolarization. Blockage of IK_{Ca} channels accelerates the decay of Ca^{2+} fluorescence, indicating that IK_{Ca} induced membrane hyperpolarization provides a higher driving force for Ca^{2+} influx. We also found that STIM1 and Orai1 are expressed in human macrophages and may be the molecular basis of I_{CRAC} recorded in human macrophages. Orai2 and Orai3 are also expressed in human macrophages and the functional role of these two proteins is not clear. Our results suggest the existence of a positive feedback loop composed of SOC channels and IK_{Ca} channels in human macrophages. This may sustain Ca^{2+} signaling in immune cells and play an important role in the innate immune system. The signaling pathway evoked by UTP is summarized in Fig. 20.

5. Summary

Intracellular Ca^{2+} is an important regulator of diverse functions of macrophages. Store-operated Ca^{2+} entry (SOCE) is the major Ca^{2+} influx pathway of human macrophages. Ca^{2+} activated potassium channels of the $K_{Ca}3.1$ subtype (IK_{Ca} channels) are expressed in human macrophages. We hypothesized that IK_{Ca} may be activated by store-operated Ca^{2+} entry and the resulting hyperpolarization may serve to sustain the Ca^{2+} influx through store-operated channels (SOCs).

Human macrophages were differentiated from peripheral blood mononuclear cells, had the typical morphological properties, and expressed the macrophage marker CD14. The calcium stores were depleted in Ca^{2+}-free solution by activation of P2Y purinergic receptors with UTP or by application of the calcium pump inhibitor thapsigargin. Current-clamp experiments showed that re-addition of Ca^{2+} to the bath solution resulted in membrane hyperpolarization. This hyperpolarization was inhibited by IK_{Ca} blocker charybdotoxin (CHTX) and by the SOC blocker 2-APB. Whole-cell patch clamp at 0 mV showed that SOCE induced an outward current which was also blocked by CHTX and 2-APB. These data indicate that IK_{Ca} channels are the dominant K_{Ca} channels in human macrophages and can be activated by SOCE, which results in hyperpolarization of macrophages. By using cesium-based pipette solution, we recorded the inward current carried by calcium release-activated Ca^{2+} channels (CRAC) after depletion of the intracellular calcium stores with the calcium buffer EGTA, with the purinergic agonist UTP or the Ca^{2+}-pump blocker thapsigargin. CRAC current (I_{CRAC}) had a reversal potential >+50 mV and could be blocked by 2-APB.

Fluorometric measurements of intracellular free Ca^{2+} with fluo-3 showed that UTP or thapsigargin induced a transient increase of intracellular Ca^{2+} in Ca^{2+}-free solution, which was followed by a sustained Ca^{2+} influx after re-addition of Ca^{2+} to bath solution. Blockage of IK_{Ca} with CHTX resulted in accelerated decay of Ca^{2+} fluorescence but had no effects on initial rate of Ca^{2+} influx. These findings suggest that Ca^{2+} influx activates IK_{Ca} and hyperpolarizes the membrane potential, which will maintain the

driving force for Ca^{2+} influx by providing counter ions for Ca^{2+} influx through store-operated channels..

Very recent studies have shown that protein Orai1, 2, 3 may be the molecular candidates of CRAC and that the protein STIM1 may represent the sensor of ER Ca^{2+} content. Using RT-PCR, we found that Orai1, Orai 2, Orai3 and STIM1 were expressed by human macrophages. These results suggest that one or more members of Orai protein family may form the store-operated Ca^{2+} entry pathway in human macrophages and that STIM1 may act as a Ca^{2+} sensor.

Our data indicate that IK_{Ca} channels and SOCE may provide a feedback loop for Ca^{2+} influx. The sustained Ca^{2+} influx is important for proper function of immune system.

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7. Abbreviations

1-EBIO	1-ethyl-2-benzimidazolinone
2-APB	2-aminoethoxydiphenyl borate
AA	arachidonic acid
ACh	acetylcholine
ATP	adenosine 5'-triphosphate
BAPTA	2-bis(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid
BK_{Ca}	large conductance K_{Ca} channels
BMDM	mouse bone marrow derived macrophage
cAMP	adenosine 3':5'-cyclic monophospahte
CAM	calmodulin
CHTX	charybdotoxin
CPA	cyclopiazonic acid
CRAC	Ca^{2+}-release-actiavated Ca^{2+} channels
DAG	diacyglycerol
DCEBIO	6-dichloro-1-ethyl-2-benzimidazolinone
EDHF	endothelia-dependent hyperpolarization factor
ER	endoplasmic reticulum
EGTA	Ethylene glycol-bis(2-aminoethyl)-N.N.N'.N'-tetra-acetic acid
FBS	fetal bovine serum
FcγR	Fc gamma receptor
Gi	GTP binding protein that inhibits adenylate cyclase
Gs	GTP-binding protein that activates adenylate cyclase
HEK	human embryonic kidney cells
HEPES	N- [2-hydrooxyethyl] piperazine-N'-[2-ethanesulfonic acid]
I_{CRAC}	Ca^{2+}-release actiavated Ca^{2+} currents
IK_{Ca}	intermediate conductance K_{Ca} channels
iNOS	inducible nitric oxide synthase
IP3	inositol 1, 4, 5-triphospahte

Kir	inward rectifying K^+ channels
Kv	voltage-gated K^+ channels
LPS	lipopolysaccharide
M-CSF	macrophage colony stimulating factor
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor κB
NPo	open probability
NO	nitric oxide
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PC-PLC	phosphotidylcholine-specific PLC
PKA	protein kianse A
PKC	protein kinase C
PLA2	phosphoalipase A2
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMCA	plasma membrane Ca^{2+}-ATPase
ROC	receptor-operated Ca^{2+} entry
RT-PCR	reverse transcription polymerase chain reaction
SERCA	sarco-endoplasma reticulum Ca^{2+}-ATPase
siRNA	short interfering RNA
SK_{Ca}	small conductance K_{Ca}
SOC	store-operated Ca^{2+} channel
SOCE	store-operated Ca^{2+} entry
TNF-α	tumor necrosis factor- α
TPG	thapsigargin
TRP	transient receptor potential channels
UTP	uridine 5'-triphosphate
VDCC	voltage-dependent Ca^{2+} channels

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Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel "The role of calcium-activated potassium channels and store-operated calcium channels in human macrophages" im Institut für Physiologie und Pathophysiologie der Philipps-Universität Marburg unter Leitung von Prof. Dr. Dr. Jürgen Daut ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Marburg, den 01. 08. 2007

(Yadong Gao)