The Ca²⁺ release-activated Ca²⁺ current (I_{CRAC}) mediates store-operated Ca²⁺ entry in rat microglia

Lily Ohana,1,† Evan W. Newell,1,2,‡ Elise F. Stanley1,2 and Lyanne C. Schlichter1,2,*

1Toronto Western Research Institute; University Health Network; 2Department of Physiology; University of Toronto; Toronto, ON CA
‡Current address: Department of Microbiology and Immunology; Stanford University; Stanford, CA USA
†These authors contributed equally to this work.

Ca²⁺ signaling plays a central role in microglial activation, and several studies have demonstrated a store-operated Ca²⁺ entry (SOCE) pathway to supply this ion. Due to the rapid pace of discovery of novel Ca²⁺ permeable channels, and limited electrophysiological analyses of Ca²⁺ currents in microglia, characterization of the SOCE channels remains incomplete. At present, the prime candidates are ‘transient receptor potential’ (TRP) channels and the recently cloned Orai1, which produces a Ca²⁺-release-activated Ca²⁺ (CRAC) current. We used cultured rat microglia and real-time RT-PCR to compare expression levels of Orai1, Orai2, Orai3, TRPM2, TRPM7, TRPC1, TRPC2, TRPC3, TRPC4, TRPC5, TRPC6 and TRPC7 channel genes. Next, we used Fura-2 imaging to identify a store-operated Ca²⁺ entry pathway that was reduced by depolarization and blocked by Gd³⁺, SKF-96365, DES, diethylstilbestrol (DES), and a high concentration of 2-aminoethoxydiphenyl borate (50 μM 2-APB). The Fura-2 signal was increased by hyperpolarization, and by a low concentration of 2-APB (5 μM), and exhibited Ca²⁺-dependent potentiation. These properties are entirely consistent with Orai1/CRAC, rather than any known TRP channel and this conclusion was supported by patch-clamp electrophysiological analysis. We identified a store-operated Ca²⁺ current with the same properties, including high selectivity for Ca²⁺ over monovalent cations, pronounced inward rectification and a very positive reversal potential, Ca²⁺-dependent current potentiation, and block by SKF-96365, DES and 50 μM 2-APB. Determining the contribution of Orai1/CRAC in different cell types is crucial to future mechanistic and therapeutic studies; this comprehensive multi-strategy analysis demonstrates that Orai1/CRAC channels are responsible for SOCE in primary microglia.

Introduction

In response to central nervous system (CNS) damage or disease, microglia undergo complex responses, often collectively called ‘activation’. This can result in upregulation of functions that involve Ca²⁺ signaling: proliferation, migration, phagocytosis, and production of nitric oxide, interleukins, cytokines and chemokines (reviewed in refs. 1–7). More than 20 receptor/ligand interactions have been reported to elevate Ca²⁺ in microglia, and Ca²⁺ entry can be mediated by ionotropic purinergic receptors, reversed Na⁺/Ca²⁺ exchange,8 and store-operated Ca²⁺ entry (SOCE) (reviewed in refs. 9–11). SOCE serves as a central pathway for Ca²⁺ signaling in non-excitable cells12 and, in microglia, numerous receptor-mediated responses evoke Ca²⁺ release from intracellular stores, followed by SOCE. The resulting Ca²⁺ rise that lasts for minutes to hours can immediately affect activity of Ca²⁺-dependent proteins, such as calmodulin and Ca²⁺-activated K⁺ channels,13 and help replenish intracellular Ca²⁺ stores, but it can also have long-term effects on gene expression and cell cycle regulation (reviewed in refs. 10 and 12).

There is little electrophysiological information about the identity of the underlying Ca²⁺-permeable channel(s) in microglia, despite numerous studies using Ca²⁺-sensitive dyes to investigate receptor-mediated signals. The literature on other cell types suggests that SOCE can be conferred by multiple channel types, collectively called store-operated channels (SOCs), but whose molecular identities are contentious (see Discussion). A subtype
ICRAC mediates SOCE in microglia

Channels 2009; Vol. 3 Issue 2

...mediates SOCE in microglia. The molecular identity of CRAC also remained elusive for many years, until the cloning of Orai1/CRACM16,17 and evidence it contributes to the pore-forming unit.18,19 Importantly, with recent molecular, biophysical and pharmacological fingerprinting, the CRAC current has been better defined, and a clearer distinction made from other SOC currents. Three patch clamp studies have addressed store-operated and CRAC currents in microglia20-22 and each has provided valuable information. The earlier studies were done before several fundamental properties of Orai1/CRAC had been elucidated and, as addressed in the Discussion, it is crucial to consolidate and extend these studies using the latest criteria for distinguishing Orai/CRAC from other co-existing current in microglia. Hence, we have combined Ca2+ imaging, patch-clamp recordings and pharmacological approaches to study SOCE and the underlying currents in primary cultures of rat microglia.

Results

Expression of putative Ca2+-permeable channels in rat microglia. Quantitative real-time RT-PCR (qRT-PCR) was used to compare mRNA expression levels of Ca2+-permeable channels in cultured microglia (Fig. 1A); including the main candidate genes for store operated Ca2+ channels (Fig. 1B). Transcripts for several transient receptor potential (TRP) genes were detected. Quantitative comparisons of mRNA expression showed that TRPM7 > TRPC6 > TRPM2 > TRPC1 > TRPC3 > TRPC4 > TRPC5 > TRPC2, where ‘>’ denotes a significant difference (p < 0.05) from the preceding gene, and ‘≥’ indicates a non-significant difference (one way ANOVA, followed by Tukey’s test for multiple comparisons). Note that mRNA expression for TRPM7 and for the microglial marker, complement receptor 3 (CR3), was high and comparable to the housekeeping gene, HPRT-1. Relatively high mRNA levels were seen for the recently cloned Orai genes (Orai3 ≥ Orai1 > Orai2), which were more abundant than TRPC genes, except for TRPC6. Orai expression levels were similar to the most commonly studied ion channel in microglia, voltage gated delayed rectifier potassium channel, Kv1.3, which contributes to microglial proliferation and activation.23,34

Properties of store-operated Ca2+ entry (SOCE) in rat microglia. Having detected mRNA expression for several candidates for Ca2+ release activated Ca2+ (CRAC/Orai) channels and other SOCs; we next examined store-operated Ca2+ entry. In microglia loaded with Fura-2AM in the standard bath solution containing 1 mM Ca2+, intracellular Ca2+ (Ca2+i) was low (23 ± 2 nM; n = 99 cells), and there was little change upon removal of extracellular Ca2+ (nominally Ca2+-free, 0 Ca) (Fig. 2A). Depleting the stores with thapsigargin (1 μM) evoked a small Ca2+ transient that varied in size and kinetics (Figures show average responses from several cells). Then, when external Ca2+ (Ca2+o) was restored to 1 mM, Ca2+ rebound increased Ca2+i within seconds, reaching 170 ± 9 nM after one minute. A similar response was seen in all cells examined; i.e., >500 cells from 12 separate cell cultures, clearly demonstrating SOCE. In control recordings without drugs or solution changes (not shown), the Ca2+i signal spontaneously declined to a plateau in the continued presence of external Ca2+; decreasing to 50% from the peak value in 54 ± 26 sec (n = 58 cells from three separate cultures).

As a first step toward determining which of the expressed channels is involved in the SOCE (Orai/CRAC or another SOC), we examined its pharmacological properties (Fig. 2), using the well known SOCE inhibitors, SKF-96365 and Gd3+,12,15 as well as diethylstilbestrol (DES)35 and 2-aminoethoxydiphenyl borate (2-APB).12,36 When thapsigargin was added in a Ca2+-free bath solution and SOCE was evoked by restoring external Ca2+ (Fig. 2A), 10 μM DES nearly abolished the Ca2+i rise. Similarly, when SOCE was triggered by restoring Ca2+ after pre-incubation with thapsigargin in a Ca2+-free bath solution (Fig. 2B); it was decreased by SKF-96365 and Gd3+. An important pharmacological discrimination was then based on 2-APB, which blocks some TRP
channels, but exerts a dual effect on CRAC—increasing it at low concentrations (1–5 μM) and blocking it at higher concentrations (e.g., 40–50 μM). After evoking SOCE as in panel B, perfusing in 5 μM 2-APB (Fig. 2C) evoked a substantial increase in Ca\(^{2+}\) (as expected for CRAC), and this was reversed upon drug washout. The higher 2-APB concentration (50 μM; Fig. 2D) evoked a small increase just as the drug began to enter the bath, and then the Ca\(^{2+}\) rise was essentially abolished. Block by these four compounds and the dual effect of 2-APB provides evidence that CRAC channels mediate this SOCE in microglia.

Further characterization was based on the effect of membrane potential on SOCE. We have previously shown that both K\(^{+}\) and Cl\(^{-}\) channels contribute to the membrane potential (V\(_m\)) of rat microglia, and that 55 mM external K\(^{+}\) depolarizes the cells to about -15 mV. After evoking SOCE as in panel B, perfusing in 1 mM Sr\(^{2+}\) (Fig. 4A) evoked a substantial increase in Ca\(^{2+}\) (as expected for CRAC), and this was reversed upon drug washout. The Sr\(^{2+}\) response was compared in each microscope field between several intact microglia and a single patch-clamped cell whose membrane potential was held at -50 mV to reflect the normal resting potential, which we previously measured with voltage-sensitive dyes. SOCE was decreased by depolarizing V\(_m\) to -10 mV and increased by hyperpolarizing to -90 mV (Fig. 3B). Similar results were seen in eight different fields of microglial cells, consistent with depolarization reducing the driving force for Ca\(^{2+}\) entry. Cells whose V\(_m\) was not controlled by patch clamping (dashed curve) showed the typical monotonic Ca\(^{2+}\) rise that occurs when external Ca\(^{2+}\) is restored to store-depleted cells.

Ca\(^{2+}\)-dependent potentiation of SOCE as evidence for CRAC channels. A biophysical property that helps distinguish Orai/CRAC from TRP channels is that the CRAC current displays Ca\(^{2+}\)-dependent potentiation; i.e., an increase in inward current as Ca\(^{2+}\) enters. Ca\(^{2+}\)-dependent potentiation is more pronounced with hyper-polarization (less Ca\(^{2+}\) enters at depolarized potentials) and it is not supported by Ba\(^{2+}\) or Sr\(^{2+}\). Such ion substitutions are useful because several TRPC channels (TRPC1, C4, C5, C6) are permeable to Ba\(^{2+}\), but do not display Ca\(^{2+}\)-dependent potentiation. Ba\(^{2+}\) and Sr\(^{2+}\) influx can be detected by Fura-2 imaging, thus, we compared Fura-2 signals when Ca\(^{2+}\) was replaced with either of these divalent cations. In microglia that were exposed to a Ca\(^{2+}\)-free bath solution (without thapsigargin), there was no Ca\(^{2+}\) rebound when 1 mM Ca\(^{2+}\) was restored, nor was there a response to a brief exposure to 1 mM Sr\(^{2+}\) (Fig. 4A). When thapsigargin was added, Ca\(^{2+}\) release evoked only a transient Fura-2 signal that returned to baseline as the stores were depleted (see Materials and Methods for Fura-2 Kd’s and calculations). Re-exposure to 1 mM Sr\(^{2+}\) did not elicit a Fura-2 signal; however, thapsigargin had activated the SOCE, as seen by the large Fura-2 signal evoked by restoring Ca\(^{2+}\). This signal immediately decreased when Ca\(^{2+}\) was again replaced by Sr\(^{2+}\). This result is consistent with Sr\(^{2+}\) neither evoking nor sustaining a previously activated SOCE. An alternative explanation, that the Sr\(^{2+}\) permeability is too low, is
ICRAC mediates SOCE in microglia

Channels 2009; Vol. 3 Issue 2

solution, cytoplasmic Ca²⁺ was 20.2 nM, as calculated from the initial Fura-2 ratio (0.29 ± 0.002; n = 53 cells). Depletion of intracellular Ca²⁺ is expected to prevent the Ca²⁺-dependent potentiation of Orai/CRAC channels, if they are active. The first exposure to 1 mM Ba²⁺ (Fig. 4B) produced a small increase in the Fura-2 signal (in 53/60 cells), corresponding with 44 nM cytoplasmic Ba²⁺ at 60 s (Fura-2 ratio, 0.31 ± 0.004; see Materials and Methods for Fura-2 K_d's and calculations). Then, restoring external Ca²⁺ evoked a substantial Fura-2 signal that increased over time to reach 133 nM Ca²⁺ at 60 s (Fura-2 ratio, 0.51 ± 0.01; n = 38 cells). The Fura-2 signal decreased substantially when Ba²⁺ was substituted for Ca²⁺. Overall, these responses to Ba²⁺ are consistent with a lower Fura-2 response to Ba²⁺ (i.e., higher K_d, see Materials and Methods) and with reversal of CRAC potentiation in the absence of Ca²⁺ influx. As a further control, we confirmed that Fura-2 responds to Ba²⁺ influx. That is, activation of the Ca²⁺- and Ba²⁺-permeable ionotropic purinergic P2X receptors by 100 μM ATP produced a large Fura-2 signal with Ba²⁺ as the permeant ion, which corresponded with ~890 nM final Ba²⁺. As expected, the Ba²⁺ signal remained elevated after its removal from the bath because the Ca²⁺ efflux pumps do not transport Ba²⁺. After correcting for differences in the K_d of Fura-2 for binding Ca²⁺ (236 nM) versus Ba²⁺ (780 nM), the intracellular Ca²⁺ rise at 60 s was ~4 times higher than the Ba²⁺ rise. This is a conservative estimate because the reduced ability of cells to extrude Ba²⁺ compared to Ca²⁺ will underestimate the steady-state flux ratio. Our result is consistent with the 3–4 fold Ca²⁺-dependent potentiation of CRAC current expected at -50 mV in 1 mM external Ca²⁺.46 In addition, the relatively small Fura-2 signals with Sr²⁺ or Ba²⁺ are similar to CRAC-mediated Sr²⁺ and Ba²⁺ signals described previously, rather than the larger signals produced by TRPC channels.44

Whole-cell recordings of the store-operated Ca²⁺ current in microglia. The pharmacological profile, divalent cation permeability and Ca²⁺-dependent potentiation strongly support the notion
ICRAC mediates SOCE in microglia

that the SOCE in microglia is mediated by Orai/CRAC channels. Thus, we used whole-cell patch-clamp recordings to isolate and characterize the store-operated Ca²⁺ currents. Key to the success of these experiments was finding ionic conditions that eliminated several potentially contaminating currents, as follows. All recordings were begun in standard bath solution, before any external ions were substituted. Then, Ca²⁺ currents were recorded using K⁺ free external (and internal) solutions to avoid the K⁺ currents we have observed in rat microglia under these culturing conditions; i.e., Kv1.3, inward-rectifier K⁺, Kir2.1, rat ether-a-go-go related gene (rErg) and Ca²⁺-activated K⁺, KCa3.1.13,23,24,34,47-49 A high intracellular Mg²⁺ concentration (8 mM) was used to inhibit the TRPM7 current, which spontaneously activates within minutes after establishing whole cell recordings in rat microglia.50 Inward anion currents e.g., swelling activated26,47 were minimized by eliminating transmembrane osmotic gradients and by replacing almost all the internal Cl⁻ with the poorly permeant anion, aspartate.

To activate store-operated Ca²⁺ currents, we used low Ca²⁺ pipette solutions with strong Ca²⁺ buffering (10 mM BAPTA; 20 nM free Ca²⁺) and then evoked depletion of the intracellular stores by bath applying 1 μM thapsigargin. After 5–7 min the standard bath solution was replaced with a K⁺ free bath solution containing elevated Ca²⁺ (10 mM); the store-activated Ca²⁺ current was isolated by subtraction of the component blocked by 20 μM SKF-96365 (Fig. 5A and B). The example in Figure 5A shows total current in pA, while the other examples are presented as current density (pA/pF). The blocker-sensitive current component was inwardly rectifying and averaged 0.32 ± 0.03 pA/pF at -110 mV (n = 3). As expected, owing to lack of Cs⁺ permeability...
current. Variable levels of the background current produced a variable reversal potential of the total current in panels A, C and E.

The Ca²⁺ selectivity of the store-operated current was confirmed in cells in which the intracellular stores had been depleted using thapsigargin and strong intracellular Ca²⁺ buffering (Fig. 6). When Na⁺, K⁺ and Ca²⁺ in the bath were replaced with the bulky cation, NMDG⁺, the small remaining current reversed at a very negative membrane potential (about -100 mV; Fig. 6A). Then, with 20 mM Ca²⁺ in the bath solution, the inward current increased and the reversal potential became less negative (about -75 mV). The difference current obtained from point-by-point subtraction was an inwardly rectifying current (Fig. 6B), with an average amplitude at -110 mV of 0.69 ± 0.06 pA/pF (n = 3).

Further evidence for a CRAC current: Ca²⁺-dependent potentiation and pharmacology. Results from experiments illustrated in Figures 5 and 6 show an inwardly rectifying Ca²⁺ current activated after depletion of intracellular Ca²⁺ stores, and blocked by three known SOC channel inhibitors. However, because Orai1/CRAC and TRPC channels conduct Ca²⁺, further experiments were needed to determine which channel most likely conducts the current. A hallmark of the CRAC current is the Ca²⁺-dependent potentiation,¹² that was shown above using Fura-2 imaging of the SOCE. In whole-cell recordings, potentiation is usually demonstrated as a time-dependent decrease in current in Ca²⁺ free solution, superimposed on a tonic increase in monovalent cation current.⁵³ Hence, the stereotypical pattern upon removal of external divalent cations is a larger inward current with a rapid relaxation.¹⁴,⁵⁴

Evidence for Ca²⁺-dependent potentiation of the store-operated current in microglia is presented in Figure 7. In panel A, the stores were depleted by thapsigargin in the standard bath solution containing 1 mM Ca²⁺. Then, the bath was exchanged for a divalent-free (DVF) and K⁺-free solution with EDTA and HEDTA to eliminate any residual divalent cations (see Table 2).

After substituting DVF and K⁺-free bath solution there was a large inward current carried by Na⁺, which rapidly decayed as expected for CRAC. The inward current reached a plateau level, and then was nearly abolished after perfusing in the standard divalent-containing bath solution, as expected for channels with a very low conductance for Ca²⁺. Adding 50 μM 2-APB greatly reduced the monovalent (Na⁺) current in DVF solution, which then decayed to the same plateau level. The current-versus-voltage relations (Fig. 7B; recorded at the times indicated in panel A) and the 2-APB-sensitive component (difference current in Fig. 7C) show inward rectification, which was slightly less than with Ca²⁺ as the permeant ion, and no current reversal was seen. This is identical behavior to Orai1/CRAC channels under similar ionic conditions, owing to their low permeability to Ca²⁺.⁵⁴ Lack of Cs⁺ efflux also distinguishes Orai1/CRAC from TRPC channels, which are permeable to a wide range of monovalent cations.⁵⁵,⁵⁶

We exploited the large monovalent inward current carried by Na⁺ (as in Fig. 7A–C) to examine the sensitivity of the store-operated current to three SOC inhibitors. The amplitude of the 2-APB-sensitive monovalent current at -110 mV was 4.4 ± 1.3 pA/pF (n = 6; 50 μM 2-APB), which was 55.3 ± 2.4% of the total monovalent current. The DES-sensitive component was 4.9 ± 1.9 pA/pF (n = 5; 10 μM DES), which was 56.1 ± 5.2% of the current. The SKF-96365-sensitive component was 4.4 ± 1.5 pA/pF (n = 10; 20 μM SKF-96365), 57.4 ± 5.6% of the current. The similarity in the amplitude and percent of current blocked by each of these SOC blockers provides evidence that the same channel underlies the SOCE seen with Fura-2 imaging and the store-operated current in whole-cell recordings in microglia.

The final experiments concerned the monovalent current that remained in the presence of SOC blockers. It is important to note that neither rundown nor hysteresis following exposure to DVF solution reduced the current, and thus overestimated the block by 2-APB in Figure 7A. The amplitude of the remaining 2-APB insensitive monovalent current was the same (3.6 ± 1.1 pA/pF at -110 mV; n = 3) as in cells treated with 2-APB before the first exposure to DVF solution (i.e., 3.3 ± 1.4 pA/pF; n = 8; p = 0.7558). To verify that store depletion was necessary in order to elicit the 2-APB-sensitive current carried by Na⁺ (Fig. 7C), we examined separate cells lacking depletion; i.e., without thapsigargin treatment and with an internal solution containing 100 nM free Ca²⁺ (see Materials and Methods). Under these conditions, the monovalent current in DVF solution (Fig. 7D) was quite variable (i.e., 3.0 ± 2.1 pA/pF; n = 7) but it did not exhibit a decay (unlike panel A) and was not sensitive to 50 μM 2-APB (i.e., it was reduced by 0.5 ± 0.3 pA/pF, n = 3). Thus, the remaining current was not the store-operated current described in Figure 7A. We next addressed the possibility that an inward-rectifying K⁺ current could have produced the monovalent current in DVF bath
solutions. The rationale was that, under these culturing conditions, rat microglia have a prominent Kir, which is thought to be Kir2.1.24,47 Both this Kir current and cloned Kir2.1 channels are blocked by external divalent cations;24,47,58 thus, DVF solution might have reversed the channel block. Because Kir2.1 can be blocked by external Cs+,59 we repeated the experiment from Figure 7A, but with 10 mM Cs+ added to the DVF solution. Exposure to DVF solution (Fig. 7E) evoked a large inward Na+ current, which rapidly decayed and was blocked by 10 μM DES; the remaining current was 0.5 ± 0.2 pA/pF (n = 3; Fig. 7F). We could not find reports of Na+ permeability of Kir2.1 under the K+- and divalent-free conditions used here, but under normal conditions external Na+ blocks K+ influx at very negative potentials, causing a current relaxation. While our data support the possibility that Kir mediates the blocker-insensitive Na+ current, we cannot rule out an unidentified Cs+-sensitive current. We could not systematically test the SOC blockers on the remaining current because 10 mM external Cs+ reduced the recording stability.

Discussion

The broad importance of store-operated Ca2+ entry (SOCE) has generated considerable interest in identifying the underlying current. In microglia, SOCE occurs in response to activation of IP3-linked metabotropic receptors: purinergic P2Y,60-62 C3a and C5a complement,63 and platelet-activating factor.64 Intracellular Ca2+ can also increase in response to chemokines, lipopolysaccharide (LPS), β-amyloid peptide, tumor necrosis factor-α, interleukin-1β, interferon-γ, thrombin and lysophosphatidic acid.7,10,11,60,65 There are several candidates for the channel underlying SOCE, but insufficient pharmacological, biophysical and molecular information to distinguish among them. The cloning of TRP and Orai genes and evidence that Orai1 encodes the CRAC channel has provided useful new information that we have applied in this study. Thus, we quantified expression of several TRP and Orai genes in primary rat microglia, and then used patch-clamp and Fura-2 imaging methods to characterize the biophysical and pharmacological properties of a CRAC current that appears to account for the SOCE.

While numerous studies have addressed Ca2+ signaling in microglia (see Introduction), very few have attempted to identify the underlying currents. It can be difficult to separate the multiple Ca2+ currents that appear to be store dependent, and lack of consensus about which channel properties best discriminate among them has contributed to the confusion (reviewed in refs. 12 and 15). This is true for microglia as well, where TRPM7 was previously mistaken for Orai1/CRAC.51,54 Distinguishing Orai1/CRAC from some TRP channels requires extensive biophysical and pharmacological fingerprinting. Orai1/CRAC has an extremely small single-channel conductance (~0.2 pS), high selectivity for Ca2+ over monovalent cations (Pca/PNa > 1,000), pronounced inward rectification with a very positive reversal potential, Ca2+-dependent current potentiation, and is blocked by SKF-96365, DES35 and relatively high concentrations of 2-APB, but increased by low 2-APB concentrations.12,36 TRPM7 has a much larger conductance (~40 pS with Na+ as the permeant ion), is not store-dependent, and is pharmacologically distinct, with block by spermine and intracellular Mg2+, low sensitivity to SKF-96365 and 2-APB, and no block by DES (reviewed in refs. 12 and 15). Thus, the 42 pS current previously attributed to CRAC in rat microglia22 is likely mediated by TRPM7, which we characterized biophysically and pharmacologically in these cells.50 TRPC1, TRPC3, TRPC6 and TRPC7 have been proposed as store-operated (reviewed in ref. 12) but they are less Ca2+ selective than Orai1/CRAC, with Pca/PNa ratios of 1.6 for TRPC3,66 5 for TRPC6,67 and <0.4 for TRPC1.68 Thus, one crucial test is to compare Ca2+-containing and divalent-free solutions.

Figure 7. Ca2+-dependent potentiation and pharmacology of the store-operated current. As in Figure 5, microglia were incubated with 1 μM thapsigargin (A–C, E and F); 5–7 min, standard bath solution) to deplete intracellular Ca2+ stores, and then exposed to K+ free solution. Current-versus-voltage (I-V) relations were continuously monitored by applying voltage ramps from -110 to +25 mV every 1 s from a holding potential of 0 mV. (A) Time course of the current amplitude monitored at -110 mV. (B) I-V relations taken from the cell in (A) at the numbered times; i.e., #1, K+-free bath solution with 1 mM Ca2+; #2, 2-APB bath solution; #3, 2-APB bath solution with 50 μM 2-APB. (C) Isolation of the 2-APB-sensitive monovalent current using point-by-point subtraction of the current in trace #3 (2-APB bath solution) from the current in trace #2 (DFV solution alone). (D) The same protocol and bath solutions as in (A), but applied to a microglial cell without store depletion (see text for conditions). (E) Two different microglial cells exposed to the same protocol as in (A), but using a DFV bath solution with 10 mM Cs+ alone (D) or in the presence of 10 μM diethylstilbestrol (DES; E).
Norenberg et al. first showed that IP$_3$ in the pipette evoked an inwardly rectifying (‘CRAC-like’) Ca$^{2+}$ current in rat microglia, which showed no reversal up to +100 mV. Other properties of the current were not examined and it was not related to store-operated Ca$^{2+}$ entry; hence, it was important for us to extend that study using the most recent information about Orai1/CRAC. First, we identified a SOCE pathway that was entirely consistent with CRAC; i.e., inhibited by depolarization, and pharmacologically by Gd$^{3+}$, SKF-96365, DES and 50 μM 2-APB (a relatively high concentration). Conversely, it was increased by hyper-polarization, by 5 μM 2-APB (a relatively low concentration), and exhibited Ca$^{2+}$-dependent potentiation, which was not supported by Sr$^{2+}$ or Ba$^{2+}$. Then, we isolated a store-operated Ca$^{2+}$ current and showed that its biophysical and pharmacological properties are entirely consistent with the currently known properties of cloned Orai1/CRAC channels; including activation by store depletion, high selectivity for Ca$^{2+}$ over monovalent cations, pronounced inward rectification and a very positive reversal potential, Ca$^{2+}$-dependent current potentiation, block by SKF-96365, DES or by 50 μM 2-APB. By assessing the properties of the store-operated Ca$^{2+}$ entry and the Ca$^{2+}$ current in the same cell type, this study strongly supports the contention that Orai1/CRAC channels produce the SOCE in microglia.

Physiological implications. In principle, SOCE and underlying Orai1/CRAC channels in microglia could affect numerous Ca$^{2+}$-dependent molecules, including KCa channels, adenylyl cyclase, which raises cAMP, phospholipase C, calmodulin and ionized calcium-binding adaptor molecule 1 (Iba-1). Studies are beginning to address whether the microglial activation state regulates SOCE and the expression induction of the underlying currents. While it is anticipated that activity of Orai/CRAC channels will play a central role in microglial activation, there is very little information directly linking SOCE and CRAC currents. The biophysical and pharmacological evidence that SOCE can occur through an Orai/CRAC channel in microglia will be useful for future studies of Ca$^{2+}$ signaling, and in discriminating its roles from other channels. One contribution of the present study was to establish conditions necessary to isolate and identify the small Orai/CRAC current carried by Ca$^{2+}$ (~0.5 pA/pF) and the larger monovalent current carried by Ca$^{2+}$ in the absence of external Ca$^{2+}$. The earliest studies of the currents lacked the necessary information to separate and identify CRAC currents, and would benefit from further investigation. For instance, the ability of LPS to activate SOCE in microglia and evoke a chronic Ca$^{2+}$ rise suggests a sustained current activation, but an IP$_3$-activated ‘CRAC’ current was reportedly downregulated by LPS in murine microglia. Of note, in rat microglia, the current amplitude was an order of magnitude larger in LPS-activated cells than in the unstimulated cells used in the present study.

In moving forward, it will be important to keep in mind that other non-selective cation currents have been seen in microglia. A non-rectifying current in rat microglia and human C13 cells can be carried by Ca$^{2+}$ (P$_{Ca}$/P$_{Na}$ = 0.71) and shares some properties with cloned TRPM2 channels. It was activated by intracellular ADP-ribose, and by extracellular hydrogen peroxide, but only after LPS treatment in the rat microglia. In murine microglia, a non-rectifying ‘TRPM4-like’ cation current (called ‘ICAN’) was activated by micromolar levels of intracellular Ca$^{2+}$, but was not affected by LPS. There might be species-dependent differences. We have not observed a Ca$^{2+}$-activated ICAN in rat microglia, despite testing a wide range of intracellular (pipette) Ca$^{2+}$ concentrations (16 nM–40 μM, using both perforated- and conventional-patch clamp recordings), and assessing microglia in various activation states; i.e., ex vivo or cultured with serum-free medium, astrocyte-conditioned medium, LPS or phorbol ester. Our present finding that TRPC1-TRPC7 are expressed in rat microglia raises the possibility of further Ca$^{2+}$-permeable channels that might be dependent or independent of store depletion. Further channel complexity should be considered because of reports that TRPC and Orai1/CRAC channels co-assemble. However, most recent studies conclude that Orai1 forms homomultimers that co-assemble with the stromal interaction molecule 1 (STIM-1) protein, and that TRPC1 and Orai1 produce separate currents.

We have demonstrated the conditions necessary for isolating the CRAC current from the numerous other currents that exist in rat microglia, and for comparing it with Ca$^{2+}$-sensitive dye measurements of SOCE. This degree of characterization will be essential for clearly separating its contributions from identified TRP channels and for future studies of regulation of CRAC expression and activity. It is important to note that establishing roles for the CRAC current in microglia will require the development of a selective inhibitor. Microglia, like macrophages, are extremely resistant to siRNA-mediated knockdown, transfection and viral-mediated infection (we have carried out an exhaustive search for a molecular approach using >20 reagents/viral constructs). Thus, the only means to parse out Orai1/CRAC function is a pharmacological approach. Because the best available inhibitors, including SKF-96365, 2-APB and diethylstilbestrol, block other channels in addition to CRAC, it is not yet possible to assess its roles in assays of microglia function in vitro or in vivo.

Materials and Methods

Microglial cultures. All procedures were approved by the University Health Network animal care committee, in accordance with guidelines established by the Canadian Council on Animal Care. Microglia were isolated from brains of 1–2 day-old Wistar rats that had been killed by cervical dislocation. Cell cultures were prepared, as we have previously described. After carefully removing the meninges, whole brain tissue was mashed through a stainless steel sieve (100 mesh; Tissue Grinder Kit #CD-1; Sigma; Oakville, Canada), and then centrifuged (10 min, 1,000 g), re-suspended and seeded into flasks with MEM containing 10% fetal bovine serum (FBS) and 100 μg/mL gentamycin (all from Invitrogen, Burlington, Canada). Two days later, cellular debris, non-adherent cells, and supernatant were removed and fresh medium was added to the flask. The mixed cultures were allowed to grow for 7–10 days and then shaken for 3 h on an orbital shaker at 8–10 Hz in a standard tissue culture incubator. The supernatant containing detached microglial cells was centrifuged (10 min, 1,000 g) and the cell pellet was re-suspended. Cells were

136 Channels 2009; Vol. 3 Issue 2
counted and plated at 3.5 x 10^4 cells per 15 mm diameter glass cover slip for electrophysiology or imaging. Before experiments, the plated microglia were cultured for 1–5 days in MEM with 100 μM gentamycin, and a reduced serum concentration (2% FBS) to maintain a more resting state. This procedure yielded highly purified cultures of microglia (99–100%; see Fig. 1A), as judged by labeling with FITC-conjugated isolectin B4 or tomato lectin (both from Sigma, St. Louis, MO) or by immunofluorescence using the OX-42 monoclonal antibody (Serotec, Raleigh, NC), which recognizes complement receptor 3. We previously used quantitative real-time RT-PCR to demonstrate ~100% purity.

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). Gene transcript levels were monitored in primary microglia (≥99% pure) using qRT-PCR, as we recently described. Gene-specific primers (Table 1) were designed using the ‘Primer3Output’ program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). RNeasy mini kits (Qiagen, Mississauga, ON) were used to isolate RNA after degrading any contaminating DNA with DNaseI (0.1 U/ml, 15 min, 37°C; Amersham Biosciences, PQ). A two-step reaction was performed according to the manufacturer’s instructions (Invitrogen). In brief, total RNA (1 μg) was reverse transcribed in 20 μl volume using 200 U of SuperScriptII RNase H-reverse transcriptase, with 0.5 mM dNTPs (Invitrogen) and 0.5 μM oligo dT (Sigma). Amplification was performed on an ABI PRISM 7700 Sequence Detection System (PE Biosystems, Foster City, CA) at 95°C for 40 cycles at 95°C for 15 s, 56°C for 15 s and 72°C for 30 s. ‘No-template’ and ‘no-amplification’ controls were included for each gene, and melt curves showed a single peak, confirming specific amplification. The threshold cycle (C_T) for each gene was determined and normalized against the housekeeping gene, hypoxanthine guanine phosphoribosyl transferase (HPRT1). The amplification efficiency was 90–95% for all the primers used.

Chemicals. Unless otherwise indicated, all chemicals including channel blockers were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Single-cell fluorescence imaging. Microglia on cover slips were mounted in a perfusion chamber (Model RC-25, Warner Instruments, Hamden, CT) and the tissue culture medium was replaced with a bath solution containing (in mM): 135 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 5 glucose and 10 HEPES, adjusted to pH 7.4 (with NaOH) and to ~300 mOsm with sucrose. For ion-substitution experiments, NaCl was partly or completely replaced with KCl, N-methyl-D-glucamine-chloride (NMDG-Cl) or LiCl, as indicated. For the nominally Ca²⁺-free solutions, CaCl₂ was omitted without adding EGTA, because we previously found that chelating all extracellular Ca²⁺ can evoke spontaneous Ca²⁺ depletion from immune cells. This depletion compromises calibrating microglial Ca²⁺ levels from dye measurements. Images were acquired at room temperature using a Nikon Diaphot inverted microscope, Retiga-EX camera (Q-Imaging, Burnaby, BC, Canada), DG-4 arc lamp with excitation wavelength changer (Sutter Instruments, Novato, CA), and Northern Eclipse image acquisition software (Empix Imaging, Mississauga, ON, Canada).

Images were acquired every 2–3 s, and the excitation shutter was closed between acquisitions to prevent photobleaching.

To measure intracellular Ca²⁺, microglia were loaded (~45 min, room temperature) with 3.5 μg/ml Fura-2-AM (Invitrogen, Burlington, ON, Canada) made in the indicated bath solution. Images were acquired at 340 and 380 nm excitation wavelengths, and ratios obtained using a 505 nm dichroic mirror and a 510 nm emission filter. In the representative figures, Fura-2 data are presented as the 340:380 nm ratio, and the summarized data in the text are presented as intracellular Ca²⁺ and Ba²⁺ levels, calibrated as previously described using the values we determined for Rₘᵢₙ (0.25 ± 0.002) and Rₘₐₓ (2.3 ± 0.097). Since we used 1 mM external Ca²⁺, while 2 mM is often used in studies on microglia, we compared the effects of 1 versus 2 mM external Ca²⁺. Resting intracellular Ca²⁺ was 70 ± 5 mM (56 cells) with 1 mM Ca²⁺ in the bath, 79 ± 5 mM after switching to 2 mM, and 85 ± 8 mM after returning to 1 mM (p > 0.2 for all comparisons). Hence, intracellular Ca²⁺ was well buffered against moderate changes in external Ca²⁺. For comparing Ca²⁺ and Ba²⁺ signals, the Kᵋ values for

Table 1

| Primer       | Accession # | Sequence                  |
|--------------|-------------|---------------------------|
| HPRT1        | NM_012583.2 | F: CAGTACAGCGCCCCAAATGGT R: CAAAGGCAATATCCAAACACA |
| TRPC1        | NM_053558  | F: GTGGCTGGCGCTTGAGAT R: TGCCCATGCTGGGGGAAAC |
| TRPC2        | NM_022638  | F: ACCGGACATTTACCATCCTGTC R: GAGGCGAACATCCCTCACT |
| TRPC3        | NM_021771  | F: AGAGACACGGGGCCAAAGG R: GTGGCAGTGGGGTGAAG |
| TRPC4        | NM_053434  | F: CGTGCAGGCTGCACTATC R: CTGAAAGCGGTGAGAA |
| TRPC5        | NM_080898  | F: CAGAGGCAAAGGATGGAGAG R: GTACAGCGAAAGGAGT |
| TRPC6        | AB051214   | F: GCAGCAAGATGGGGAAGA R: GAGCAGCCAGGAAAGAT |
| TRPC7        | XM_225159  | F: TCTCAGGCTTACGCAACAAC R: ACOGACATCAATAAGG |
| TRPM2        | NM_001011559.1 | F: CCCCTAAGACGAAAAAGTGT R: GGCGAAGAAGGAGT |
| TRPM7        | XM_001056331 | F: AGGGCAGTGTGTGCTGT R: CAGGGCCCAAAACATGT |
| Orai1        | NM_001013982.1 | F: GACTGGATGCAGGGAGGTGT R: GAGAGCAGAGGGAGGT |
| Orai2        | XM_222288.4 | F: CCGTGAGCAACATCCACA R: CAGCCAGGAAGGAGA |
| Orai3        | NM_001014024.1 | F: CCACACCAGTACCAACCAAC R: CCAGGCCCCAAACAAACAC |
| CR3          | NM_012711  | F: TGCTGAGACTGGAGGGCACAC R: CTCGCCACGCTTTGTTT |
| Kᵥ1.3        | M30312     | F: GCTCTCCGCGCTCCTAAGG R: TCGTCTGCCACAGAAAGT |
intracellular Fura-2 binding were taken as 236 nM for Ca\(^{2+}\),\(^{31,32}\) and 780 nM for Ba\(^{2+}\).\(^{32}\)

**Patch clamp recordings.** Conventional whole-cell recordings were made at room temperature with pipettes (2–5 MΩ resistance) pulled from borosilicate glass (WPI, Sarasota, FL). Recordings were made with either an Axon multiclamp 700A or an Axopatch 200 amplifier (Molecular Devices, Sunnyvale, CA), compensated on-line for capacitance and series resistance, and filtered at 5 kHz. Patch-clamp data were acquired and digitized using a Digidata 1322A board with pCLAMP software (version 9.2, Molecular Devices), and analyzed using Origin ver7.0 software (OriginLabs, Northampton, MA). Liquid-liquid junction potentials were calculated using the utility in pCLAMP, confirmed using a 3 M KCl electrode,\(^5\) and subtracted before data analysis. Standard intracellular (pipette) solution contained (in mM): 100 K aspartate, 40 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 10 EGTA (20 nM free Ca\(^{2+}\)), 10 HEPES, 2 MgATP. The pH of all pipette solutions was adjusted to 7.2 with KOH. The high buffering-capacity pipette solution used for isolating the CRAC current contained (in mM) 125 Cs-aspartate, 8 MgCl\(_2\), 10 BAPTA, 10 HEPES. The low buffering-capacity pipette solution contained (in mM) 125 Cs-aspartate, 8 MgCl\(_2\), 0.475 CaCl\(_2\), 1 BAPTA (100 nM free Ca\(^{2+}\)), 10 HEPES. Table 2 shows the composition of all extracellular solutions, which were adjusted to pH 7.4 with NaOH. The osmolarity of all recording solutions was adjusted to 290–310 mOsm by adding sucrose.

In a subset of recordings, the voltage dependence of Ca\(^{2+}\) entry was assessed by combining Ca\(^{2+}\) imaging with perforated patch-clamp recordings. In this case, microglial cells were labeled with Fura-2-AM as described above, and the pipette solution contained 200 μM amphotericin (Sigma) diluted from a stock solution made in DMSO. After obtaining a giga-ohm seal, amphotericin caused a gradual decrease in series resistance, and when it reached <100 MΩ, experiments were begun.

Unless otherwise stated, all quantitative data are presented as the mean ± SD. For clarity, statistical tests are described with each result.

**Acknowledgements**

Part of this work was published as an abstract; Evan W. Newell and Lyanne C. Schlichter, Abstract 845.17 Society for Neuroscience Annual Meeting, Washington, Nov. 2005. We thank Xiaoping Zhu for conducting the real-time RT-PCR analysis and Guillaume Ducharme for helpful discussions. This work was supported by grants to Lyanne C. Schlichter from the Canadian Institutes for Health Research (CIHR; #MT-13657) and the Heart and Stroke Foundation, Ontario chapter (#T4670), and a grant to Elise F. Stanley (CIHR; MOP-86643). Evan W. Newell was supported by a Ruth L. Kirschstein National Research Service Award pre-doctoral scholarship from the National Institutes of Neurological Diseases and Stroke (#F31NS049742).

### References

1. Inoue K, Nakajima K, Morimoto T, Kikuchi Y, Koizumi S, Illes P, Kohsaka S. ATP stimulation of Ca\(^{2+}\)-dependent plasminogen release from cultured microglia. Br J Pharmacol 1998; 123:1304-10.
2. McLarnon JG, Wang X, Bae JH, Kim SU. Endothelin-induced changes in intracellular calcium in human microglia. Neurosci Lett 1999; 263:9-12.
3. Moriwaki K, Quan M, Murakami M, Yamashita M, Fukuda Y, P2 Purinoceptor expression and functional changes of hypoxia-activated cultured rat retinal microglia. Neurosci Lett 2000; 282:153-6.
4. Moller T, Contos JJ, Musante DR, Chun J, Ransom BR. Expression and function of lysophosphatidic acid receptors in cultured rodent microglial cells. J Biol Chem 2001; 276:25946-52.
5. D’Aversa TG, Yu KO, Berman JW. Expression of chemokines by human fetal microglia after treatment with the human immunodeficiency virus type 1 protein Tat. J Neurovirol 2004; 10:86-97.
6. Moller T, Kann O, Verkhratsky A, Kettenmann H. Activation of mouse microglial cells affects P2 receptor signaling. Brain Res 2003; 985:49-59.
7. Farber K, Kettenmann H. Functional role of calcium signals for microglial function. Glia 2006; 54:656-65.
8. Newell EW, Stanley EF, Schlüchter LC. Reversed Na+/Ca\(^{2+}\) exchange contributes to Ca\(^{2+}\) influx and respiratory burst in microglia. Channels (Austin) 2007; 1:366-76.
9. Eder C. Regulation of microglial behavior by ion channel activity. J Neurosci Res 2005; 81:314-21.
10. Moller T. Calcium signaling in microglial cells. Glia 2002; 40:184-94.
11. Farber K, Kettenmann H. Purinergic signaling and microglia. Pflogers Arch 2006; 452:615-21.
12. Parekh AB, Putney JW Jr. Store-operated calcium channels. Physiol Rev 2005; 85:757-810.
13. Kaushal V, Koehberle PD, Wang Y, Schlüchter LC. The Ca\(^{2+}\)-activated K\(^+\) channel KCNN4/KCa3.1 contributes to microglia activation and nitric oxide-dependent neurodegeneration. J Neurosci 2007; 27:234-44.
14. Hoth M, Penner R. Calcium release-activated calcium current in rat mast cells. J Physiol 1993; 465:359-86.
15. Prakriya M, Lewis RS. CRAC channels: activation, permeation, and the search for a molecular identity. Cell Calcium 2003; 33:311-21.
16. Feske S, Gwack Y, Prakriya M, Srikant S, Poppel SH, Tanasa B, et al. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. Nature 2006; 441:179-85.
17. Vig M, Peinelt C, Beck A, Koosmoa DL, Rabah D, Koblan-Huberson M, et al. CRACM1 is a plasma membrane protein essential for store-operated Ca\(^{2+}\) entry. Science 2006; 312:1220-3.

### Table 2 Ionic composition of the bath solutions

| Solution | NaCl | KCl | MgCl\(_2\) | CaCl\(_2\) | HEPES | D-glucose | EDTA | HEDTA\(^1\) | NMDG-Cl | CsCl |
|----------|------|-----|-----------|-----------|-------|-----------|-------|-----------|--------|------|
| 1. Standard | 130  | 5   | 1         | 1         | 10    | 5         |       | 10        | 5      | 5    |
| 2. K free | 135  |     |           |           |       |           |       |           | 5      | 5    |
| 3. K free + Ca\(^{2+}\) | 135  |     | 1         | 10        | 10    | 5         |       |           | 5      | 5    |
| 4. DVF | 135  |     |           |           |       |           |       |           | 10     | 10   |
| 5. DVF + Cs\(^+\) | 115  | 5   |           |           | 10    | 5         |       |           | 10     |      |
| 6. NMDG\(^+\) | 3    |     |           |           | 10    | 5         |       | 135       |        |      |
| 7. NMDG\(^+\) + Ca\(^{2+}\) | 1    | 20  | 10        | 10        | 10    | 5         |       | 108       |        |      |

\(^{1}\)N-(2-Hydroxyethyl)ethylenediamine-N,N',N'-triacetic acid.
18. Yeromin AV, Zhang SL, Jiang W, Yu Y, Safrina O, Cahalan MD. Molecular identification of the IC RAC channel by altered ion selectivity in a mutant of Ori4. Nature 2006; 443:226-9.

19. Prakriya M, Feske S, Gwack Y, Sirlinath S, Rao A, Hogan PG. Ori4 is an essential subunit of the CRAC channel. Nature 2006; 443:230-3.

20. Newell EW, Schlichter LC. Integration of K+ and Cl- currents regulate steady-state and dynamic membrane potentials in cultured rat microglia. J Physiol 2005; 567:869-90.

21. Burkhardt P, Penner R, Fleig A. Lipopolysaccharide-induced downregulation of Ca2+-release-activated Ca2+ currents (I CRAC) but not Ca2+-activated TRPM4-like currents (I CRAC) in cultured mouse microglial cells. J Physiol 2008; 586:427-39.

22. Hahn J, Jung W, Kim N, Uhm DY, Chung S. Characterization and regulation of rat microglial Ca2+ release-activated Ca2+ (CRAC) channel by protein kinases. J Biol Chem 2006; 281:118-24.

23. Fordyce CB, Jaggars A, Zhu X, Schlichter LC. Microglia K+1,3 channels contribute to their ability to kill neurons. J Neurosci 2005; 25:7195-49.

24. Newell EW, Schlichter LC. Integration of K+ and Ca2+ currents regulate steady-state and dynamic membrane potentials in cultured rat microglia. J Physiol 2005; 567:869-90.

25. Bastin SA, Nolan T. Pinfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech 2004; 15:155-66.

26. Ducharme G, Newell EW, Pinto C, Schlichter LC. Small-conductance Ca2+ channels contribute to volume regulation and phagocytosis in microglia. Eur J Neurosci 2007; 26:2119-30.

27. Wasserman JK, Zhu X, Schlichter LC. Evolution of the inflammatory response in the brains of intracerebral hemorrhage and effects of delayed minocycline treatment. Brain Res 2007; 1180:140-54.

28. Wasserman JK, Schlichter LC. Neuron death and inflammation in a rat model of intracerebral hemorrhage: effects of delayed minocycline treatment. Brain Res 2007; 1136:208-18.

29. Schlichter LC, Sakellaropoulos G. Intracellular Ca2+ signaling induced by osmotic shock in human T lymphocytes. Exp Cell Res 1999; 245:211-22.

30. Grynkieicz G, Poenie M, Tien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985; 260:3440-50.

31. Fangher CM, Neben AL, Cahalan MD. Differential Ca2+ influx, K+ channel activity, and Ca2+ clearance distinguish Th1 and Th2 lymphocytes. J Immunol 2000; 164:1155-60.

32. Schilling WP, Rajan L, Strotb-Jager E. Characterization of the bradykinin-stimulated calcium influx pathway of cultured vascular endothelial cells. Saturability, selectivity and kinetics. J Biol Chem 1989; 264:12838-48.

33. Barry PH, Lynch JW. Liquid junction potentials and small cell effects in patch-clamp analysis. The Journal of membrane biology 1991; 121:101-17.

34. Kotrecha SA, Schlichter LC. A K+1,5 to K+1,3 switch in endogenously hippocampal microglia and a role in proliferation. J Neurosci 1999; 19:10680-93.

35. Zakharov SI, Smani T, Dobrydenova Y, Monge F, Fichandler C, Blackmore PF, Bolotina VM. Diethylthylstibestrol is a potent inhibitor of store-operated channels and capacitative Ca2+ influx. Mol Pharmacol 2004; 66:702-7.

36. Peinelt C, Liu A, Beck A, Fleig A, Penner R. 2-Aminoethoxydiphenyl borate directly activates CRAC channels in Jurkat T cells. J Biol Chem 1999; 274:14445-51.

37. Christian EP, Spence KT, Toggi JA, Dargis PG, Patel J. Calcium-dependent enhancement of TRPC3-mediated T cell calcium influx not related to store depletion. J Gen Physiol 2002; 119:487-50.

38. Choi HB, Hong SH, Ryu JK, Kim SU, McLarnon JG. Differential activation of subtype purinergic receptors modulates Ca2+ mobilization and COX-2 in human microglia. J Neurosci 2003; 23:95-103.

39. Schilling WP, Rajan L, Strotb-Jager E. Characterization of the bradykinin-stimulated calcium influx pathway of cultured vascular endothelial cells. Saturability, selectivity and kinetics. J Biol Chem 1989; 264:12838-48.

40. Barry PH, Lynch JW. Liquid junction potentials and small cell effects in patch-clamp analysis. The Journal of membrane biology 1991; 121:101-17.

41. Zhang L, Saffen D. Muscarinic acetylcholine receptor regulation of TRP6 Ca2+ channels in human T lymphocytes. J Cell Biol 2007; 178:1163-76.

42. Balsam A, Penner R, Fleig A. Lipopolysaccharide-induced downregulation of Ca2+-release-activated Ca2+ currents (I CRAC) but not Ca2+-activated TRPM4-like currents (I CRAC) in cultured mouse microglial cells. J Physiol 2008; 586:427-39.

43. Hahn J, Jung W, Kim N, Uhm DY, Chung S. Characterization and regulation of rat microglial Ca2+ release-activated Ca2+ (CRAC) channel by protein kinases. J Biol Chem 2006; 281:118-24.

44. Grimaldi M, Maratos M, Verma A. Transient receptor potential channel activation causes a novel form of [Ca2+]i oscillations and is not involved in capacitative Ca2+ entry in glial cells. J Neurosci 2003; 23:10797-80.

45. North RA. Molecular physiology of P2X receptors. Physiol Rev 2002; 82:1013-67.

46. Zweifach A, Lewis RS. Calcium-dependent potentiation of store-operated calcium channels in human T lymphocytes. J Physiol 1999; 518:345-58.

47. Hofmann T, Kasum A, Ohlemeyer C, Hanisch UK, Kettenmann H. Elevation of basal intracellular calcium as a central element in the activation of brain macrophages (microglia): suppression of receptor-evoked calcium signaling and control of release function. J Neurosci 2003; 23:4410-9.

48. Lewis RS. The molecular choreography of a store-operated calcium channel. Nature 2003; 427:226-9.

49. Sinkins WG, Estracion M, Schilling WP. Functional expression of TRPC1 channels in the human homologue of the Drosophila C1r/C1s protein. Biochem J 1998; 331:331-9.

50. Zhang L, Saffen D. Muscarinic acetylcholine receptor regulation of TRPC6 Ca2+ channel isoforms. Molecular structures and functional characterization. J Biol Chem 2001; 276:13319-9.

51. Großner K. Coassembly of TRP1 and TRP3 proteins generates diacylglycerol- and Ca2+-sensitive cation channels. J Cell Biol 2002; 155:37-49.