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*J Immunol* 2011; 186:3686-3692; Prepublished online 14 February 2011; doi: 10.4049/jimmunol.1001346

http://www.jimmunol.org/content/186/6/3686
Acid-Sensing Ion Channels Contribute to the Effect of Acidosis on the Function of Dendritic Cells

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As an H⁺-gated subgroup of the degenerin/epithelial Na⁺ channel family, acid-sensing ion channels (ASICs) were reported to be involved in various physiological and pathological processes in neurons. However, little is known about the role of ASICs in the function of dendritic cells (DCs). In this study, we investigated the expression of ASICs in mouse bone marrow-derived DCs and their possible role in the function of DCs. We found that ASIC1, ASIC2, and ASIC3 are expressed in DCs at the mRNA and protein levels, and extracellular acid can evoke ASIC-like currents in DCs. We also demonstrated that acidosis upregulated the expression of CD11c, MHC class II, CD80, and CD86 and enhanced the Ag-presenting ability of DCs via ASICs. Moreover, the effect of acidosis on DCs can be abolished by the nonsteroidal anti-inflammatory drugs ibuprofen and diclofenac. These results suggest that ASICs are involved in the acidosis-mediated effect on DC function. The Journal of Immunology, 2011, 186: 3686–3692.

Materials and Methods

Animals

Female C57BL/6 and BALB/c mice, 6–8 wk old, were obtained from the Institute of Organ Transplantation of Tongji Hospital, Huazhong University of Science and Technology. Neonatal Sprague-Dawley rats (days 0–3) were obtained from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology. The animals were housed under specific pathogen-free conditions. All studies were performed according to the guidelines of the Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology.

Cell culture

DCs were generated as previously described (21, 22), with minor modifications. In brief, bone marrow cells were obtained from the femurs and tibias of C57BL/6 mice, suspended at 1 × 10⁶ cells/ml in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 mg/ml), t-glutamine (2 mM), and 2-ME (50 mM); and then seeded in 100-mm dishes at 10 ml/dish. Cultures were supplemented...

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with 10 ng/ml GM-CSF and 5 ng/ml IL-4 (both from PeproTech). The cells were fed on days 3 and 5, by aspirating 75% of the medium and adding back fresh medium with GM-CSF and IL-4 at appropriate concentrations, and cultured for 7 d. By day 7, >85% of the harvested cells expressed CD11c. Immature DCs (iDCs) used in this study were cultured for 7 d. To obtain mature DCs (mDCs), iDCs were activated with 1 µg/ml LPS (Sigma-Aldrich) for 24 h.

Primary rat cortical neurons were isolated as described in our previous study (23, 24), with some modifications. Briefly, the cortex of newborn Sprague-Dawley rats were dissected and rinsed in ice-cold Dulbecco’s PBS. The dissected tissues were treated with 0.125% trypsin in HBSS for 25 min at 37°C and mechanically dissociated using fire-polished Pasteur pipettes. Cells were collected by centrifugation and resuspended in DMEM/F12 (1:1) with 10% FBS. For whole-cell patch-clamp recording, cells (20,000–40,000) were seeded on poly-L-lysine-coated coverslips and kept at 37°C in a 5% CO2 incubator. After 24 h, the culture medium was changed with DMEM medium supplemented with 2% B27, and the cortical neurons were fed with fresh medium twice a week. Microscopically, glial cells were not apparent by using this protocol. The neurons were maintained for 7–10 d in primary culture until used.

RT-PCR

Total RNA was isolated from cells using TRizol reagent (Invitrogen), according to the manufacturer’s instructions. cDNA synthesis was performed using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio Technologies). PCR amplifications of cDNA were performed by standard methods. The following specific primers (forward, reverse) were used: ASIC1, 5′-CAG ATG GTC CTA TCC TTC TTC-3′, 5′-AAG TGC ACC TGC TCT CGC TCT-3′; ASIC2, 5′-TGA CAT TGG TCA AAT GGT-3′, 5′-ATC ATG GGT CCC TCT CTC TCT-3′; ASIC3, 5′-AGG AAG AAC TCC AAG ACC ATC-3′, 5′-GAC ACT CCA TCA CCA GGA AAT-3′ (25); TRPV1, 5′-TCT ACC TGG TGT CTC TCT GG-3′, 5′-TGGAG GGA GTT CTT GCT TTC TGC-3′ (26); and GAPDH, 5′-GAT ACC TCC ACC ATG GAG AAC GC-3′, 5′-GTC TCT ACC TGG GTG CAT GA-3′ (27). GAPDH was used for normalization. PCR products were analyzed by 2% agarose gel electrophoresis.

Western blot

Cells were lysed in lysis buffer (50 mM Tris [pH 8], 150 mM NaCl containing 1% Nonidet P-40, 0.02% NaN3, 0.5 mM PMSF, 1 µg/ml aprotinin, and 1 µg/ml leupeptin). Whole-cell lysate was mixed with 5× SDS loading buffer and boiled for 5 min. The proteins were separated by electrophoresis on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% (w/v) nonfat milk in TBST for 2 h at 37°C and then incubated with rabbit anti-ASIC1, rabbit anti-ASIC2, or rabbit anti-ASIC3 Abs (all from Alomone Laboratories), as well as mouse anti-actin Ab-5 Ab (BD Biosciences), which served as a loading control, diluted in 5% (w/v) nonfat milk in 0.1% TBST overnight at 4°C. Membranes incubated with Abs that were preincubated with respective antigenic peptides were used to test specificity of Abs. After washing three times (10 min each) in 0.1% TBST, the membranes were incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary Ab (both from Pierce) for 2 h at 37°C. The membrane was washed three times in 0.1% TBST, and the proteins were detected using Peroxide Solution (Millipore) with a Kodak rabbit or goat anti-mouse secondary Ab (both from Pierce) for 2 h at room temperature. After extensive washing, coverslips were mounted on glass slides with 50% glycerol. Staining was visualized with an Olympus FV500 confocal microscope.

Electron microscopy

DCs were fixed with a solution of 4% paraformaldehyde and 1.25% glutaral for 30 min. After washing three times in PBS, cells were permeabilized with 0.5% Triton X-100 and 25 µg/ml protease A for 20 min, in sequence. After extensive washing, cells were blocked with normal goat serum for 2 h at room temperature. Cells were incubated with rabbit anti-ASIC1, rabbit anti-ASIC2, or rabbit anti-ASIC3 primary Ab diluted in PBS overnight at 4°C. Then, cells were washed with PBS three times before staining with anti-rabbit IgG (whole molecule)-Gold (Sigma-Aldrich) for 30 min. Cells were postfixed with 1% OsO4 for 1 h and were dehydrated, embedded, made into ultrathin sections, and stained with uranyl acetate, using conventional methods. Images were taken with an FEI-Tecnai G2 12 transmission electron microscope (FEI).

Whole-cell patch-clamp recordings

The procedure for whole-cell patch-clamp recording was performed as described in our previous studies (28, 29), with minor modification. The bath solution for recording ASIC currents contained NaCl 150 mM, KCl 5 mM, MgCl2, 1 mM, CaCl2, 2 mM, HEPES 10 mM, and glucose 10 mM; pH was adjusted to 7.4 with NaOH. Glass pipettes were pipettes were made using an EPC-10 amplifier, driven by Pulse/PulseFit software (both from HEKA Elektronik). Drug actions were measured only after steady-state conditions were reached, which were judged by the amplitudes and time courses of currents remaining constant. All recordings were made at room temperature (20°C–22°C). All experiments were repeated three times using different batches of cells, and at least three or four dishes of cells were used for recording in different batches of cells.

Flow cytometry

Cells (1 × 10⁶) were stained with PE-conjugated Abs diluted in PBS containing 1% BSA (FACS buffer). Before staining, cells were incubated with purified anti-mouse CD16/32 (Biotrend) for 10 min on ice to block FcR. The following PE-conjugated Abs (all from eBioscience) were used: anti-CD11, anti-i-FcR (MHC class II), anti-CD80, and anti-CD86. After incubating with Abs for 30 min at 4°C, cells were washed twice with FACS buffer and analyzed by a LSR II flow cytometer (BD Bioscience). The results are shown as mean fluorescence intensity (MFI).

MLR

Splenic T cells from normal BALB/c mice were stained with CFSE and used as responders. A total of 2 × 10⁶/ml T cells was cultured with 2 × 10⁶/ml DCs treated with mitomycin C (30 µg/ml) and incubated at 37°C in 5% CO2 for 5 d. After three washes with PBS, the cells were harvested and analyzed for T cell proliferation using a LSR II flow cytometer (BD Bioscience).

Statistical analysis

Data are presented as mean ± SEM. The Student t test with paired comparisons was used to evaluate differences; p < 0.05 was considered statistically significant.

Results

ASICS are expressed in iDCs

To determine whether ASIC1, ASIC2, and ASIC3 are expressed in iDCs, we performed RT-PCR to detect the expression of mRNA for ASICS. Cortex neurons served as positive control. The sizes of the expected PCR products for ASIC1, ASIC2, and ASIC3 were 140, 139, and 107 bp, respectively. As a result, mRNAs for all three ASICS were detected in iDCs (Fig. 1A). Then, we confirmed the expression of ASIC proteins by Western blotting. Abs for ASIC1, ASIC2, and ASIC3 were preincubated with the corresponding Ag peptide as specific control (Fig. 1B). Furthermore, we used immunocytochemistry to determine cellular and subcellular distributions of ASICS in iDCs. As shown in Fig. 1C, ASIC1 and ASIC3 proteins were predominantly expressed in cytoplasm. Most ASIC2 is expressed on the plasma membrane. We further observed, by electron microscopy, that ASIC1 was expressed in

The Journal of Immunology 3687

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endoplasmic reticulum and perinuclear regions, whereas ASIC3 was found in mitochondria (Fig. 1D).

Electrophysiological characteristics of ASIC-like currents in iDCs

A whole-cell patch-clamp recording was performed to determine whether the expression of ASIC proteins in iDCs has a function. Cells were held at −60 mV and then a rapid decrease in extracellular pH, from 7.4, was applied to the bath. In 85% (n = 36/42) of the recorded iDCs, the rapid decrease in extracellular pH elicited inward ASIC-like currents, indicating the presence of functional ASIC channels. Usually, this transient inward current contained two phases: early fast and late slow inactivation (Fig. 2A). The mean amplitude of ASIC-like currents induced by pH 6 was 329.63 ± 52.36 pA. Similar to the proton-gated currents in sensory and central neurons, the ASIC-like currents in iDCs were reversibly inhibited by amiloride (100 μM), a blocker of ASICs (n = 6, p < 0.05; Fig. 2A). As shown in Fig. 2B, the response of iDCs to acidosis was pH dependent. The threshold extracellular pH to elicit the inward current was ~7, and the maximum response appeared at pH 5. The extracellular pH producing 50% effect was 6.08 ± 0.05, and the Hill coefficient was 1.03 ± 0.07 (n = 6) (Fig. 2C).

Transient receptor potential vanilloid-1 is not functionally expressed in iDCs

As a nonselective cation channel, transient receptor potential vanilloid-1 (TRPV1) also can evoke membrane currents at low extracellular pH (<6). Thereby, we examined the effect of capsaicin (Cap), a selective agonist of TRPV1, on iDCs with the whole-cell patch-clamp technique. As shown in Fig. 3A, 10 μM Cap significantly evoked an inward current in cortex neurons. However, 10 or 100 μM Cap failed to induce any inward currents in iDCs, indicating that the inward currents induced by a decrease in extracellular pH may not be mediated by TRPV1 channels in iDCs. To further confirm this ratiocination, RT-PCR was performed to investigate whether TRPV1 is expressed in iDCs, with cortex neurons as positive control. We did not detect TRPV1 mRNA expression in iDCs (Fig. 3B). These results suggested that TRPV1 is not involved in the elicitation of inward currents induced by low extracellular pH in iDCs.
The characteristics of ASICs in mDCs

After demonstrating the characteristics of ASIC expression in iDCs, we further investigated that in mDCs. As shown in Fig. 4A and 4B, the distribution and expression of ASICs were similar in iDCs and mDCs. However, the amplitude of ASIC currents induced by extracellular pH 6 was increased significantly in mDCs (Fig. 4C), suggesting an enhanced function of ASICs in mDCs. ASICs are involved in acidosis-induced DC maturation characterized by upregulation of CD11c, MHC, CD80, and CD86

It was reported that exposure to pH 6.5 for 4 h resulted in a significant maturation of DCs, characterized by a significant increase in CD11c, MHC class II, and CD86 (16). In addition to those molecules, CD80 on DCs was upregulated after exposure to pH 6.5 for 4 h (Fig. 5); however, it is still unknown how acidosis induces DC maturation. Because ASICs are proton-gated cation channels and can be activated by extracellular H⁺, we hypothesized that the effect of acidosis on DCs is mediated via ASICs. To examine this hypothesis, DCs were incubated at pH 7.3 for 30 min at 37°C in the presence of amiloride (100 μM), a blocker of ASICs, before being exposed to pH 6.5 for 4 h at 37°C. Amloride had no effect on CD11c, MHC class II, CD80, or CD86 at pH 7.3 (Supplemental Fig. 1); however, upregulation of CD11c, MHC class II, CD80, and CD86 stimulated by acidosis was significantly inhibited by amiloride (Fig. 5), suggesting that ASICs are involved in DC maturation induced by acidosis.

Nonsteroidal anti-inflammatory drugs suppress inward ASIC currents in DCs

Previous studies demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs) significantly inhibited ASIC currents in hippocampus and sensory neurons (30, 31). To investigate the effect of NSAIDs on ASICs in DCs, the responses of ASIC currents to NSAIDs (ibuprofen or diclofenac) were tested. Extracellular application of 200 μM ibuprofen significantly reduced ASIC currents from 364.17 ± 57.15 pA to 176.07 ± 28.77 pA (Fig. 6A, 6B). The inhibitory percentage was 51.49 ± 3.75%, and the inhibition could be reversed by extensive washing with bath solution. Similarly, diclofenac (200 μM) significantly reduced ASIC currents from 353.97 ± 51.58 pA to 166.7 ± 18.57 pA (Fig. 6C, 6D). The inhibition was also reversible, and the inhibitory percentage was 51.67 ± 8.3%. These data suggested that ASIC currents in iDCs are also sensitive to NSAIDs. Therefore, ASICs in DCs are possible targets for NSAIDs in inflammation.

NSAIDs inhibit DC maturation induced by acidosis

After determining the inhibitory effect of NSAIDs on acid-sensing channels in DCs, we examined the effect of NSAIDs on DC maturation induced by acidosis. DCs were preincubated with ibuprofen or diclofenac at pH 7.3 for 30 min at 37°C and were exposed to pH 6.5 for 4 h. Ibuprofen and diclofenac had no effect on the expression of CD11c, MHC II, CD80, or CD86 at pH 7.3 (Supplemental Fig. 2), but they significantly abrogated the upregulation of CD11c, MHC class II, CD80, and CD86 molecules induced by acidosis (Fig. 7), indicating that NSAIDs can inhibit the maturation of DCs stimulated by acidosis.

Acidosis increases the Ag-presenting ability of DCs via ASICs

The data above demonstrated that ASICs are involved in the up-regulation of cell-surface molecules related to Ag presentation induced by acidosis in DCs. MLR was used to examine the effect of acidosis on the Ag-presenting ability of DCs and the role of ASICs. DCs were incubated in pH 7.3 medium for 30 min in the presence or absence of amiloride (100 μM), ibuprofen (200 μM), or diclofenac (200 μM) before being exposed to pH 6.5 for 4 h; DCs incubated at pH 7.3 for 4.5 h served as controls. After washing, DCs were pretreated with mitomycin C and cultured with T cells stained with CFSE at pH 7.3 for 5 d, and T cell proliferation was detected by flow cytometry. As expected, pH 6.5 increased the Ag-presenting ability of DCs, whereas amiloride and NSAIDs (ibuprofen or diclofenac) abrogated this effect (Fig. 8), suggesting that ASICs play a critical role in the regulation of the Ag-presenting ability of DCs by acidosis.

FIGURE 3. TRPV1 is not functionally expressed in iDCs. A, Cap (10 or 100 μM) did not elicit current in iDCs. Currents evoked by Cap (10 μM) served as positive control. B, No TRPV1 expression at the mRNA level in iDCs was detected by RT-PCR. Cortex neurons were used as positive control.

FIGURE 4. ASIC1, ASIC2, and ASIC3 are functionally expressed in mDCs. A, Localization of ASICs in mDCs was detected by double-staining immunofluorescence (original magnification ×400). Nuclei were counterstained with Hoechst33342 (blue). Green, ASICs; red, CD11c. B, Expression of ASICs in mDCs. iDCs served as control and β-actin was used for normalization. C, ASIC-like currents elicited by pH 6 in iDCs and mDCs (n = 6 for each group). *p < 0.05, significant difference between the two groups.
The results demonstrated that ASICs are expressed in DCs and are involved in the maturation of DCs induced by acidosis. As the most potent APC, DCs are specialized to capture and process Ags and present them to T cells to induce immune responses. Therefore, DCs are critical for the induction of immune responses triggered by microorganisms, tumors, and autoantigens. Recently, there is increasing evidence to suggest that inflammation plays an important role in various diseases, such as tumors and autoimmune diseases (32–35). Inflammation is always accompanied by tissue acidification, which means that the microenvironment of pathological sites is acidic compared with normal sites; however, the effect of an acidic microenvironment on the physiological functions of DCs is less well known. Although a few studies have focused on the role of acidosis in DC function, the mechanism underlying the effect remains unclear (16, 36).

Because ASICs are H+-gated cation channels and are activated by extracellular protons, and the expression of ASICs in T cells, B cells, and macrophages of mice was reported recently (37), we proposed that ASICs serve as sensors for extracellular acidosis and mediate the responses of DCs to acidosis.

To test this hypothesis, the expression of ASICs in mouse iDCs and mDCs was investigated. As expected, iDCs expressed ASIC1, ASIC2, and ASIC3 at the mRNA and protein levels (Fig. 1A, 1B). Then, immunofluorescence confocal microscopy and electron microscopy were used to examine their localization in iDCs. Unlike the distribution in neurons, ASIC1 and ASIC3 are found mainly in cytoplasm rather than on the cell membrane, whereas ASIC2 is located exclusively on the cell membrane (Fig. 1C, 1D).

Consistently, the distribution and expression of ASICs in mDCs were similar to those in iDCs (Fig. 4A, 4B).

We further examined the electrophysiological characteristics of ASICs in DCs. We found that the inward ASIC-like currents in DCs were reversibly inhibited by a blocker of ASICs, amiloride (Fig. 2), suggesting that ASICs are functionally expressed in DCs. Interestingly, the amplitude of ASIC currents was larger in mDCs than in iDCs (Fig. 4C), indicating that the function of ASICs is enhanced in mDCs.

TRPV1 is a nonselective cation channel with high permeability for Ca2+. It was reported to be responsive to a variety of stimuli, such as noxious heat, Cap, endovanilloids, and acid in neurons (38). A recent study suggested that TRPV1 is expressed in human
iDCs and plays a role in the differentiation and activation of iDCs (39). However, previous studies reported conflicting data on the existence and function of TRPV1 in mouse iDCs (26, 40). Using Cap, a specific agonist for TRPV1, we tried to elicit currents in iDCs but were unsuccessful (Fig. 3A). Furthermore, we could not detect TRPV1 expression in iDCs using RT-PCR (Fig. 3B). These results suggested that currents evoked by acid in iDCs are not via TRPV1.

In periphery tissues, DCs are in an immature stage and have phagocytic abilities. After encountering pathogens, they undergo a process of maturation to become efficient APCs and migrate from periphery tissues into the draining lymph nodes to induce primary immune responses. One important feature of mDCs is the increased expression of MHC molecules and costimulatory molecules on the cell membrane. Vermeulen et al. (16) reported that extracellular acidosis upregulates the expression of cell-surface proteins that are involved in Ag presentation of DCs, indicating that acidosis has an important role in the maturation of DCs. Consistent with the data of Vermeulen et al., we found that the surface expression of CD11c, MHC class II, and CD86 on DCs was upregulated in an acidic environment (Fig. 5). In addition to those molecules, the expression of costimulatory molecule CD80 was increased at pH 6.5 (Fig. 5). The upregulations were inhibited by amiloride, a blocker for ASICs (Fig. 6), suggesting that ASICs mediate the maturation of DCs induced by acidosis. Enhanced migration is another characteristic of DC maturation. However, the results suggested that extracellular acidosis does not influence the expression of CCR7 on DCs (Supplemental Fig. 2).

It was suggested that NSAIDs inhibit ASIC currents in various neurons and may protect neurons from damage associated with acidosis. Although Dorofeeva et al. (30) demonstrated that NSAIDs reduced the maximal response to a decrease in pH but did not induce a significant decrease in ASICs’ sensitivity to protons in hippocampal interneurons, the mechanism of the NSAID action is still not clear. Similar to previous reports, the results showed that ibuprofen and diclofenac significantly inhibited ASIC currents in DCs (Fig. 6). Moreover, we found that the maturation of DCs induced by acidosis was also inhibited by ibuprofen and diclofenac (Fig. 7), indicating that the inhibitory effect of NSAIDs on acidosis-induced maturation is mediated by ASICs. However, the mechanism by which NSAIDs inhibit ASICs of DCs needs to be investigated further. Additionally, acidosis increased the ability of DCs to stimulate the proliferation of allogeneic T cells; this effect was significantly inhibited by amiloride, ibuprofen, and diclofenac (Fig. 8), indicating that ASICs mediate the maturation of DCs induced by acidosis and that ASICs in DCs may be a novel target for NSAIDs in the anti-inflammation reaction.

In conclusion, the results showed that ASICs play an important role in the physiologic function of mouse bone marrow-derived DCs as receptors for extracellular acidosis. Functional ASICs...
are expressed in DCs and mediate the upregulation of CD11c, MHC class II, CD80, and CD86 induced by acidosis. ASICs also mediate the Ag-presenting ability of DCs induced by acidosis. ASICs may be a new therapeutic target for regulating the function of DCs in inflammation, tumors, and autoimmune diseases. However, further efforts will be made to clarify the precise mechanism by which ASICs transduce the signal of extracellular acidosis to DCs and induce their maturation.

Acknowledgments
We thank Dr. Jixin Zhong, Center for Biotechnology and Genomic Medicine, Medical College of Georgia, for assistance with preparing the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References

3692 EXTRACELLULAR ACIDOSIS REGULATES DC FUNCTION VIA ASICs
Figure S1. Amiloride and NSAIDs (ibuprofen and diclofenac) do not have any effect on the expression of surface molecules CD11c, MHC II, CD80 and CD86 in DCs cultured at pH 7.3.

DCs were incubated with or without amiloride (100 µM), ibuprofen (200 µM) or diclofenac (200 µM) at pH 7.3 for 30 min. After washing, DCs were cultured for another 4 h at pH 7.3 and the MFI values of CD11c, MHC II, CD80 and CD86 were analyzed by Flow cytometry. The data represent mean ± SEM of six independent experiments.

Figure S2. pH 6.5 does not have effect on the expression of CCR7 on DCs.

DCs were cultured at pH 7.3 or pH 6.5 for 4 h and the MFI values of CCR7 were analyzed by Flow cytometry. The data represent mean ± SEM of six independent experiments.