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Arachidonic acid closes innexin/pannexin channels and thereby inhibits microglia cell movement to a nerve injury

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Abstract

Pannexons are membrane channels formed by pannexins and are permeable to ATP. They have been implicated in various physiological and pathophysiological processes. Innexins, the invertebrate homologues of the pannexins, form innexons. Nerve injury induces calcium waves in glial cells, releasing ATP through glial pannexon/innexon channels. The ATP then activates microglia. More slowly, injury releases arachidonic acid (ArA). The present experiments show that ArA itself reduced the macroscopic membrane currents of innexin- and of pannexin-injected oocytes; ArA also blocked K⁺-induced release of ATP. In leeches, whose large glial cells have been favorable for studying control of microglia migration, ArA blocked glial dye-release and, evidently, ATP-release. A physiological consequence in the leech was block of microglial migration to nerve injuries. Exogenous ATP (100 μ M) reversed the effect, for ATP causes activation and movement of microglia after nerve injury, but nitric oxide directs microglia to the lesion. It was not excluded that metabolites of ArA may also inhibit the channels. But for all these effects, ArA and its non-metabolizable analogue eicosatetraynoic acid (ETYA) were indistinguishable. Therefore, ArA itself is an endogenous regulator of pannexons and innexons. ArA thus blocks release of ATP from glia after nerve injury and thereby, at least in leeches, stops microglia at lesions.

Keywords

neuroglia; microglia; pannexin/innexin; adenosine triphosphate; nerve injury; arachidonic acid

Introduction

Arachidonic acid (ArA) is enzymatically freed from membrane-bound phospholipids by phospholipase A2. This action occurs in response to a variety of physiological and pathophysiological conditions, such as increases in intracellular calcium (Sun et al., 2005). ArA and its products, via the cyclooxygenase (COX) and lipoxygenase (LOX) signaling pathways, have a well documented presence at sites of neuronal and brain injury (Sun et al., 2004; Gabryel et al., 2007; Lopez-Vales et al., 2011). They are thought to be involved in diverse processes including vasodilatation, recruitment of immune cells, release of cytokines, and platelet aggregation (Minghetti and Levi, 1998; Minghetti, 2004; Phillis et al., 2006). Invertebrates are also able to produce ArA (Watts and Browse, 2002) and process

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it by COX and LOX pathways (De Petrocellis and Di, V, 1994; Jarving et al., 2004), and the products may control membrane processes, including in leeches (Scuri et al., 2005). In this study, we investigated a novel role for ArA as an inhibitor of pannexon/innexon channels. We also examined consequences of this inhibition for responses of microglial cells in the leech. Leeches are favorable for *in situ* study of control of microglial cell migration to nerve lesions (von Bernhardt and Muller, 1995; Duan et al., 2005).

ArA has long been recognized as a potent inhibitor of gap junctions in a variety of tissues (Giaume et al., 1989; Fluri et al., 1990; Miyachi et al., 1994; Boger et al., 1999; Martinez and Saez, 1999). However, its effect on gap junction “hemichannels” (see Sosinsky et al., 2011, for nomenclature) has not been assessed. Pannexin 1 (Pnx1) is a member of a recently discovered mammalian gap junction family homologous to the invertebrate gap junction family, the innexins. Pnx1 forms exclusively non-junctional membrane channels, which have the related name pannexons, on cells including neurons and glia. Recently, Pnx1 has been implicated in the activation of the inflammasome (Kanneganti et al., 2007; Pelegrin et al., 2008; Silverman et al., 2009).

The overlapping pharmacology of pannexons and connexons, the latter forming the mammalian gap junction channels, has made it difficult to distinguish the two functionally. Most invertebrates, including the leech, do not have connexins but only pannexin-like proteins, the innexins, making them useful for the study of these channels. We previously found that *Hmnx2*, a leech pannexin homologue present in glia (Dykes and Macagno, 2006) and important for selective formation of gap junctions (Firme, III et al., 2012; Yazdani et al., 2013), forms ATP permeable membrane channels (innexons) that when expressed in oocytes are activated by intracellular calcium and inhibited by carbenoxolone (CBX, ~50% inhibition at 1 μ M) (Bao et al., 2007). In the same study, loss of 6-carboxyfluorescein and Lucifer Yellow dyes from glial cells decreased under conditions that closed innexon channels, including acidification or CBX (10 μ M). Moreover, RNA knockdown of *Hmnx2* showed that that innexin was specifically involved (Samuels et al., 2010).

Glial cells are intermingled in the nervous system with an unrelated class of cells, the microglia, which are immune cells of the central nervous system (CNS) (Aguzzi et al., 2013). Extracellular ATP is a well recognized activator of microglia (Honda et al., 2001; Inoue, 2002; Davalos et al., 2005; Samuels et al., 2010). There is evidence that glia are the cellular source of this extracellular ATP released at a distance after injury (Salter and Hicks, 1995; Guthrie et al., 1999; Verderio and Matteoli, 2001; Schipke et al., 2002; Bianco et al., 2005; Scemes and Giaume, 2006). Moreover, in vertebrates the molecular mechanism of ATP release from the glial cells is apparently through pannexon channels in vertebrates (Dahl and Locovei, 2006; Iglesias et al., 2009).

Leech microglia resemble mammalian microglia in their cytology, physiology, histochemical staining, and phagocytic ability (Coggeshall and Fawcett, 1964; Kuffler and Nicholls, 1966; Morgese et al., 1983; von Bernhardt and Muller, 1995). Because of this and their ready identification in the living nerve cord, they have been useful for understanding migration of microglia to nerve lesions (Morgese et al., 1983; McGlade-McCulloh et al., 1989; Duan et al., 2005; Ngu et al., 2007). Experiments closing or specifically knocking down innexons formed by *Hmnx2* have shown that ATP release from glia via the innexon channels is required for the migration of microglia to nerve injuries (Samuels et al., 2010). But no endogenous regulator of innexon or pannexon channels, other than intracellular calcium, low pH and extracellular ATP itself (Qiu and Dahl, 2009), has been identified. In particular, it is of interest to know what might inhibit the channels and thereby reduce the concentration of extracellular ATP, thus slowing or stopping microglia when they reach lesions.

The present study aimed to determine whether ArA affects microglial movement and whether pannexons/innexons are involved in this process. Moreover, it was important to know whether any effects were produced directly by ArA, as determined by whether its non-metabolizable analogue eicosatetraenoic acid (ETYA) could also produce them. First, we tested whether ArA reduces currents of pannexons and glial innexons expressed in oocytes. Since the migration of microglia to injury sites in the leech CNS is dependent on functional innexon channels releasing ATP, a second aim was to determine if ArA and ETYA reduce or block microglia migration to lesions in the leech.

Methods

DRUGS AND mRNA-INJECTED OOCYTES

Solutions—Oocyte Ringer's solution (OR2) in mM: 82.5 NaCl, 2.5 KCl, 1.0 MgCl₂, 1.0 CaCl₂, 1.0 Na₂HPO₄, 5.0 HEPES, antibiotics (penicillin, 10,000 units/ml; streptomycin, 10 mg/ml), pH7.5). Patch pipette and extracellular KGlu solution: 140 mM potassium gluconate, 10 mM KCl, 5.0 mM TES, pH 7.5. Drugs were administered by bath application at their final concentrations. ArA (all-cis 5,8,11,14-eicosatetraenoic acid) and ETYA (5,8,11,14-eicosatetraenoic) were dissolved in ethanol (final concentration 0.1%). All drugs were from Sigma-Aldrich (St. Louis, MO).

mRNA injected oocytes—*Xenopus laevis* were anaesthetized, decapitated, and oocytes from 5 animals were isolated by incubating small pieces of ovary in 2 mg ml⁻¹ collagenase in calcium-free OR2 and stirring at 1 Hz for 3 hours at room temperature. After being thoroughly washed with regular OR2, oocytes devoid of follicle cells and having uniform pigmentation at stage VI were selected and stored in OR2 at 18 °C.

Hirudo innexin 2 (*Hminx2*) had been cloned into the cloning vector pCR-BluntII-TOPO (gift of Drs. M. Baker and E. Macagno). mRNA was transcribed by SP6 (*Hminx2*) RNA polymerase from 10 µg of *Xba*I- (*Hminx2*) linearized plasmid using the mMessage mMachine kit (Ambion), which was also used for preparation of Panx1 mRNA as described (Bao et al., 2004). mRNA was quantified by absorbance (260nm), and the proportion of full-length transcripts was checked by agarose gel electrophoresis. 20 nl of mRNA (50 ng/µl) was injected into oocytes. The injected oocytes were then transferred into fresh OR2 medium with elevated Ca²⁺ (5 mM) and incubated at 18 °C for 24-48 hours. For electrophysiological recordings, oocytes were transferred to regular OR2.

Voltage clamp—Voltage clamp recording was performed with two intracellular microelectrodes, one for monitoring intracellular voltage and the other for delivering current to maintain the clamped potential across the cell membrane. Oocytes were held at constant voltages and given 5 second depolarizing pulses at 0.1 Hz of magnitude 5 mV to 100 mV, depending on the experiment. From Ohm's Law, changes in currents required to produce fixed voltage pulses give a measure of conductance changes due to opening or closing of the pannexin or innexin channels. In some experiments the oocytes were bathed in a solution of modified OR2 in which 50% NaCl was replaced with potassium gluconate (50% KGlu), which has been shown to open the innexon channels (Bao et al., 2007; Samuels et al., 2010).

ATP release from oocytes—As in previous work, only oocytes at stage VI were used, which ensures that ATP measurements were from a homogeneous population of single cells. Oocytes injected individually with mRNA were incubated 4 days and pannexin/innexin expression and cell viability in the batches were confirmed electrophysiologically using a standard approach (Qiu and Dahl, 2009). Luciferin and luciferase solutions (Sigma-Aldrich; St. Louis) were mixed with supernatants collected from Panx1- or *Hminx2*-injected and

uninjected cells treated with OR2 or potassium gluconate (KGlu) in the presence of 1, 10 or 100 μM ArA or ETYA. Cells, pre-treated for 10 minutes with ArA or ETYA where applicable, were isolated for 10 minutes in 150 μL of the experimental solutions. 100 μL supernatant was obtained for each condition. Each condition was done in quintuplicate. A Victor 1420 multi-label counter (Perkin-Elmer) on a 96 well culture plate was used to measure luminance, which other experiments had shown was linear with ATP concentration over the range observed (Qiu et al., 2011).

LEECH NERVE CORD AND MICROGLIA

Preparation—Adult leeches (*Hirudo sp.*, Leeches USA, Westbury, NY), 3-4g, were maintained in artificial pond water (Forty Fathoms, 0.5 g/L H_2O ; Marine Enterprises, Towson, MD) at 15°C. Nerve cords were dissected from a total of 30 leeches anaesthetized on ice. Cords were pinned in a physiological saline solution (Ringer's (Kuffler and Potter, 1964)) in 35 mm Petri dishes with 1 mL Sylgard 184 (Dow Corning, Midland, MI) silicone rubber on the bottom. Nerve cords were maintained up to several days in Leibowitz-15 culture medium supplemented with 2% fetal bovine serum, 1% glutamine, 0.06% glucose, and 5 $\mu\text{g}/\text{mL}$ gentamicin (L-15).

Nerve cords were prepared as previously described (Samuels et al., 2010). Connectives were crushed for 8-12 seconds with fine forceps (Dumont #5, ground to have parallel edges) and incubated in leech Ringer's solution at room temperature for 4 hours with or without drugs; controls were not crushed. The tissue was then fixed overnight at 4°C in PBS with 4% paraformaldehyde (pH 7.2), washed in PBS, and mounted on a microscope slide in 85% glycerol with Hoechst 33258 nuclear stain (10 $\mu\text{g}/\text{mL}$). Mounted preparations were photographed through a 20X objective, NA 0.7, with a Spot-RT camera (Diagnostic Instruments, Sterling Heights, MI) mounted on a Leica DM RZA2 fluorescence microscope. To identify the location of the crush without bias, an image of the connective's autofluorescence was made using a fluorescein filter. Cell nuclei were imaged using a UV filter set. To measure microglia accumulation in each preparation, a series of 10 optical sections was photographed at 2 μm intervals through a combined depth of 18 μm . Each stack of images was combined and collapsed using the Metamorph® program (Molecular Devices, Downingtown, PA), and the microglia were counted in a 100 μm^2 region at the edge of each crush. The counts were statistically analyzed with an ANOVA and post-hoc Fisher Test (Statistica®, StatSoft Inc., Tulsa, OK). Although a few nuclei in each stack of images were of sheath cells, including in the “distal” counts in Figure 3, only microglia moved (McGlade-McCulloh et al., 1989) and only microglia accumulated at the lesion.

Low light video microscopy—Nerve cords dissected as described above, incubated for 30 minutes in Hoechst 33258 nuclear dye (0.001%), and rinsed 3 times for 5 minutes were kept overnight in L-15 medium at 15°C. The following day the tissue was crushed, or not crushed for controls. It was then treated as for microglia accumulation studies described above, and imaged on a Zeiss WL epifluorescence microscope (40X water immersion objective, NA 0.75) equipped with a 12V, 100 W tungsten-halogen lamp operated at 8-10V and a Zeiss 02 (UV) filter set. Metamorph® imaging software controlled a shutter and CCD camera (Hamamatsu XC-77, Hamamatsu City, Japan) to acquire time-lapse images (averages of 256 frames at 35 frames/sec, 512×512 pixels in size, collected at 2.5 minute intervals for up to 4 hours). In the region adjacent to the crush (approximately 100-500 μm away), the number of microglia nuclei moving more than 50 μm in two hours was counted. Microglia movement during the time-lapse recording was measured double-blind by assistants who tracked individual microglia nuclei, noted their direction of movement, and counted the number moving more than 30 or 50 μm , depending on the experiment. The

counts were statistically analyzed with an ANOVA and post-hoc Fischer test (Statistica, StatSoft Inc., Tulsa, OK).

Dye release from living nerve cord—A 1 mm piece of leech nerve cord was dissected in physiological saline, pinned to a Sylgard-lined dish, and incubated overnight in L-15 medium (Samuels et al., 2010). The following day the nerve cord was washed in Ringer's solution and the connective glial cell was iontophoretically injected through a microelectrode filled with 6-carboxyfluorescein (6-CF, MW 376.32, 5 μ M, -2 nA for 15 minutes) that impaled the cell through a cut end. The dye was allowed to diffuse throughout the cell for 20 minutes required for equilibration. Images were then collected every 5 minutes on a Leica fluorescence microscope with a 10X objective (0.3 NA) equipped with a Spot-RT camera (Diagnostic Instruments, Sterling Heights, MI) operating in a linear range. Drugs were bath applied at their final concentrations. Washes consisted of briefly removing the tissue from the microscope and rinsing the tissue three times with leech Ringer's before returning it to the microscope in the same location, determined optically from micrographs.

Fluorescence intensities of the paired connectives were measured with Metamorph® (Molecular Devices, Sunnyvale, CA) using a region analysis tool. Pixel intensities were averaged to obtain an average intensity. The percent dye lost during treatment was calculated by dividing the intensity of each sequential image by the previous image's intensity. A repeated measures ANOVA followed by a post-hoc Fischer test was used for comparison and statistical analysis of the dye loss.

Results

ArA EFFECTS ON CURRENTS THROUGH INNEXIN AND PANNEXIN CHANNELS

Mouse Panx1 or leech glial-associated innexin (*Hminx2*) mRNA was injected into *Xenopus* oocytes and 2 to 4 days later, oocytes were penetrated with a pair of microelectrodes electrodes for voltage clamp experiments. The cells were held at depolarized or positive potentials to open the pannexons and innexons on the membrane surface, as indicated by the increased currents during 5 sec pulses at 0.1 Hz. Such currents are not present in uninjected cells or before there has been adequate time for expression.

The conductance associated with *Hminx2* was quickly and reversibly inhibited by 100 μ M ETYA and by 100 μ M ArA (Figure 1a, b). Similar inhibition occurred for innexon channels that had been opened by treating the cells with oocyte saline in which the NaCl was replaced with half the molar amount of potassium gluconate (50% KGlu) while the membrane potential was clamped at the resting potential (Figure 1b,c). Figure 1d shows an alternative pulse protocol (from -30 mV to +60 mV), which was also known to open innexons (Bao et al., 2007). Again, ArA blocked the innexons. The current measured during the intracellular acidification of the cell in panel on the right was a positive control for the closing of the innexon channels.

To determine ArA effects on the vertebrate pannexons, ArA and ETYA were applied to oocytes expressing mouse Panx1. As shown in the example in Figure 1e, the pannexon currents were significantly and reversibly decreased in either 100 μ M ArA (by approximately 15-20%) or 100 μ M ETYA (by approximately 45-50%). Thus, ArA itself causes innexon and pannexon channels to close.

To determine if the reduction in pannexon current by ArA corresponded to a significant reduction in ATP release, ATP release was measured from individual oocytes expressing Panx1. As background, channel blockers may produce a small reduction of current through pannexons expressed in *Xenopus* oocytes when those same drugs cause a large reduction in

permeability to dye or ATP (Qiu and Dahl, 2009). Generally, agents or conditions known to close innexon channels have also been found to block ATP passage through pannexons expressed in *Xenopus* oocytes. Therefore, the effects of ArA and ETYA on ATP release from *Xenopus* oocytes injected 4 days earlier with mouse Panx1 mRNA and known to express functional channels were measured. As shown in Figure 2, potassium gluconate (KGlu) solution (see Methods) used to open the pannexons caused release of ATP, as measured by increased luminance in the luciferin-luciferase assay. The ATP release was stopped by 100 μ M ArA and by 100 μ M ETYA ($p < 0.001$ below the KGlu control) and reduced by 10 μ M ArA ($p < 0.02$). Thus, both ArA and ETYA reduced release of ATP through pannexons in a dose-dependent manner.

ArA EFFECT ON MICROGLIAL ACCUMULATION AND MIGRATION THAT NORMALLY OCCURS IN RESPONSE TO INJURY

CBX is a pannexon and innexon channel blocker that inhibits microglial migration to lesions in the leech CNS by blocking the release of ATP through innexon channels expressed in glial cells (Samuels et al., 2010). It was therefore of interest to know whether ArA and ETYA behave similarly to CBX and can prevent the accumulation of microglia to a crush injury when applied exogenously. Microglia accumulation assays were performed with varying doses of both ArA and ETYA; both compounds applied at 100 μ M concentration significantly reduced accumulation (Figure 3a, b). The “distal” microglia were those always present in the nerve cord and were counted at a distance of at least 1 mm from the lesion. Therefore, those that accumulated in addition at the lesion was the total number at the lesion minus the distal number, which typically was about a third of the total, as we have described (Samuels et al., 2010). This number was the same in the presence of 0.1% ethanol.

The inhibitory effect of ArA on leech microglia movement and accumulation was prevented if the tissue was treated simultaneously with 100 μ M ATP (Figure 3c). This was a dose of ATP that also reversed the inhibition of microglia accumulation by CBX, which inhibits both ATP release and dye release from glia (Samuels et al., 2010). Microglia in both the “ATP” alone and “ATP + ArA” conditions accumulated at the lesion as did microglia in the “saline” control nerve cord crushed in saline alone (Figure 3c). In time-lapse studies of microglia in crushed connectives in 100 μ M ArA, the microglia did not move, but in 100 μ M ArA with 100 μ M ATP the microglia moved directly toward the lesion (Figure 3d). This indicates that ArA inhibition of microglial accumulation occurred because of an overall decrease in microglial movement, and not because of abnormal directional movement, as reported for drugs that reduce accumulation by interfering with the production or action of nitric oxide (Duan et al., 2009). When ATP was added to the ArA treatment, the overall movement of the microglia increased. Moreover, the movement was directed toward the crush, as when ATP was added in the presence of CBX, which blocks innexons and pannexons (Samuels et al., 2010). Because ETYA, the non-metabolizable homologue of ArA, yielded the same results as ArA, it can be concluded that ArA directly inhibited the migration of microglia to an injury.

ArA EFFECT ON 6-CF DYE LOSS FROM THE CONNECTIVE GLIAL CELL

ArA and ETYA resembled CBX in their inhibition of microglia migration. CBX, like CO₂, closes glial innexons and prevents the release of ATP and of dyes 6-carboxyfluorescein (6-CF) and Lucifer Yellow from the connective glial cell (Bao et al., 2007; Samuels et al., 2010). It was, therefore, of interest to know if the physiological compound ArA and its non-metabolizable analogue ETYA could also inhibit dye release from glial cells. ArA significantly reduced 6-CF loss from the glial cell as compared to its vector (ethanol) control, although there appeared to be a delay in the effect (Figure 4a). Therefore, the normalized rate of loss was higher in the first 30 minutes than in the second 30 minutes. A

similar assay with the non-metabolizable form of ArA, ETYA, demonstrated a more rapid reduction in dye loss that persisted for about half an hour after the ETYA was removed and then also recovered (Figure 4b). The basis for the delay in onset with ArA compared to ETYA is unknown, but unlike ETYA, ArA was likely catabolized by tissue, which could delay its reaching peak levels along the involuted membrane of the glial cell (Coggeshall and Fawcett, 1964; Elliott and Muller, 1981). These studies demonstrated that ArA itself directly blocked dye loss from the connective glial cell in a manner similar to CBX, the pannexon/innexon channel blocker.

Discussion

Now in addition to ATP itself, calcium and voltage, ArA can be considered to be an endogenous regulator of glial innexon and pannexon channels, and it may play an important role in modulating the microglial response to nerve injury. It is not known whether ArA inhibits the channels only extracellularly, as is evidently true for voltage-dependent sodium channels (Fang et al., 2011), whether it acts intracellularly, as for arachidonate-regulated Ca^{2+} -selective (ARC) channels (Shuttleworth, 2009), or whether it acts within the membrane as for K^{+} -channels (Börjesson and Elinder, 2011). If it passes out of cells through channels in the membrane, such as through unpaired innexon or pannexon channels, it may also regulate its own release. There is evidence for such passage of ArA through channels (Jiang et al., 2007) that are exclusively pannexons, in light of what is now known about glibenclamide's block of pannexons (Qiu et al., 2011).

Other agents that reduce pannexon conductance by only 15 to 20% also can block uptake or release of ATP and dye. This is because pannexons and innexons have multiple conductance states (Bao et al., 2004), with different dye permeabilities for different states. Thus, even a small reduction in total conductance in shifting from one state to another may completely block the ability of dyes and ATP to pass through the channel. This phenomenon was observed for the effects of different drugs on current and flux of ATP or dyes through Panx1 channels, including BBG (Qiu and Dahl, 2009; Wang and Dahl, 2010), bongkreikic acid and artemisinin [Qiu and Dahl, unpublished]. Taken together, these experiments provide evidence for a novel function of ArA as an inhibitor of the innexon and pannexon family of channels, and as an inhibitor of the microglial response to injury in the leech CNS. The reversibility of the microglial response when ATP is added to ArA is consistent with earlier results with the blocker CBX (Duan et al., 2009; Samuels et al., 2010). This indicates that ArA inhibits microglial migration by blocking glial innexons rather than by directly affecting microglia. It is not known if it affects other innexons that might be important for other functions. The similar effectiveness of ETYA and ArA in all assays is evidence that ArA itself and not its downstream signaling products, such as produced through the COX and LOX pathways, is responsible for the actions of ArA. Our data do not exclude the possibility that ArA metabolites also inhibit the pannexons and innexons, but they indicate that ArA may act as an endogenous regulator of pannexon and innexon channels, reducing their open probability or changing the single channel conductance.

The expected rise in ArA after nerve injury may have additional physiological effects that were not examined in the present study. For example, there is evidence for a direct effect of ArA on stretch-activated potassium channels (Maingret et al., 1999; Monaghan et al., 2011). And ArA may inhibit on astrocytes a large conductance channel, termed a maxi-anion channel, that is permeable to ATP in response to oxygen-glucose deprivation, but is not blocked by classic blockers of pannexons such as carbenoxolone (Liu et al., 2008).

Mass spectroscopy has indicated that levels of ArA and its metabolites increase in the CNS after injury (Westcott et al., 1987; Miller et al., 2009). In the leech CNS, ArA may act as an

endogenous inhibitor and regulator of the innexon channels with injury. High local levels of ArA may occur close to the site of the crush, closing innexons, reducing the release of ATP, and slowing the movement of microglia approaching the injury so that they accumulate. The immediate rise in glial $[Ca^{2+}]_i$ and production of nitric oxide at the injury (Kumar et al., 2001; Samuels et al., 2010) may cause a rise in cytosolic phospholipase A2 (PLA2) activity (Xu et al., 2008).

In mammals, there are many downstream products of ArA that are involved in inflammation, and many of the COX and LOX enzymes are induced upon injury and activation of PLA2 (Gronert, 2008). Pannexon channel activation is known to be part of a cascade of inflammatory signals in neurons and glia, including activation of the inflammasome, a multiprotein complex involved in innate immunity (Silverman et al., 2009; MacVicar and Thompson, 2010). The early activation of PLA2 by calcium synthesizes high levels of ArA that will be present immediately upon injury. These high levels of ArA would initially inhibit the activity of the inflammasome while the inducible COX2 would increase its expression. When large amounts of COX2 are made as a result of the injury, the balance would shift in favor of the ArA metabolites, as ArA would be metabolized as soon as it is made. This would release the inhibition on the innexons or pannexons, which could allow for the simultaneous actions of the prostaglandins and inflammasomes. The initially high levels of ArA that inhibit the pannexon channels may act as an endogenous checkpoint for determining whether the cells will have a full inflammatory response. ArA's inducible catabolic enzymes may overcome this ArA inhibition, which could be the trigger for the cells to have a strong response. However, in milder injuries that produce less COX2, ArA would inhibit the pannexon channels, and the cells would only have a mild inflammatory response to injury. The ArA effect on pannexons may therefore be a type of molecular switch that determines the level and quality of the inflammatory response by damaged cells.

In the leech, from the present results and those that have been previously published, the following model emerges for control of microglia migration to lesions. Within seconds of nerve injury a calcium wave culminating in a maintained calcium gradient is generated in the ensheathing glial cell (Samuels et al., 2010), contributing to the opening of innexons and release of ATP from the glia (Bao et al., 2007), which activates microglia up to a millimeter from the lesion (Duan et al., 2005). Activated microglia migrate toward the lesion, directed by NO (Duan et al., 2009) generated by the gradient of intracellular calcium that activates a constitutive nitric oxide synthase (Shafer et al., 1998; Chen et al., 2000). The higher levels of NO at the lesion itself may slow or stop the microglia (Chen et al., 2000; Kumar et al., 2001). ArA generated as a consequence of the injury would inhibit the release of ATP, helping to account for the progressive decline in numbers of migrating microglia and their accumulation at the lesion (McGlade-McCulloh et al., 1989).

It remains to be determined whether ArA controls mammalian microglia as it can in leeches, but the similar responses and roles reported for microglia across species suggest that ArA may be an intrinsic signal that slows and stops microglia by reducing release of ATP locally from glial cells.

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Abbreviations used in this paper

ArA	arachidonic acid, or all-cis 5,8,11,14-eicosatetraenoic acid
CBX	carbenoxolone
6-CF	6-carboxyfluorescein
COX	cyclooxygenase
ETYA	eicosatetraenoic acid
Hminx2	<i>Hirudo m.</i> innexin 2
KGlu	potassium gluconate
L-15	Leibowitz-15 tissue culture medium
LOX	lipoxygenase
OR2	oocyte Ringer's solution
PLA2	phospholipase A2

References

- Aguzzi A, Barres BA, Bennett ML. Microglia: scapegoat, saboteur, or something else? *Science*. 2013; 339:156–161. [PubMed: 23307732]
- Bao L, Locovei S, Dahl G. Pannexin membrane channels are mechanosensitive conduits for ATP. *FEBS Lett*. 2004; 572:65–68. [PubMed: 15304325]
- Bao L, Samuels S, Locovei S, Macagno ER, Muller KJ, Dahl G. Innexins form two types of channels. *FEBS Lett*. 2007; 581:5703–5708. [PubMed: 18035059]
- Bianco F, Pravettoni E, Colombo A, Schenk U, Moller T, Matteoli M, Verderio C. Astrocyte-derived ATP induces vesicle shedding and IL-1 beta release from microglia. *J Immunol*. 2005; 174:7268–7277. [PubMed: 15905573]
- Boger DL, Sato H, Lerner AE, Guan X, Gilula NB. Arachidonic acid amide inhibitors of gap junction cell-cell communication. *Bioorg Med Chem Lett*. 1999; 9:1151–1154. [PubMed: 10328303]
- Börjesson SI, Elinder F. An electrostatic potassium channel opener targeting the final voltage sensor transition. *J Gen Physiol*. 2011; 137:563–577. [PubMed: 21624947]
- Chen A, Kumar SM, Sahley CL, Muller KJ. Nitric oxide influences injury-induced microglial migration and accumulation in the leech CNS. *J Neurosci*. 2000; 20:1036–1043. [PubMed: 10648709]
- Coggeshall RE, Fawcett DW. The fine structure of the central nervous system of the leech, *Hirudo medicinalis*. *J Neurophysiol*. 1964; 27:229–289. [PubMed: 14129772]
- Dahl G, Locovei S. Pannexin: to gap or not to gap, is that a question? *IUBMB Life*. 2006; 58:409–419. [PubMed: 16801216]
- Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB. ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci*. 2005; 8:752–758. [PubMed: 15895084]
- De Petrocellis L, Di M, V. Aquatic invertebrates open up new perspectives in eicosanoid research: biosynthesis and bioactivity. *Prostaglandins Leukot Essent Fatty Acids*. 1994; 51:215–229. [PubMed: 7846088]
- Duan Y, Panoff J, Burrell BD, Sahley CL, Muller KJ. Repair and regeneration of functional synaptic connections: Cellular and molecular interactions in the leech. *Cell Mol Neurobiol*. 2005; 25:441–450. [PubMed: 16047551]
- Duan Y, Sahley CL, Muller KJ. ATP and NO dually control migration of microglia to nerve lesions. *Dev Neurobiol*. 2009; 69:60–72. [PubMed: 19025930]
- Dykes IM, Macagno ER. Molecular characterization and embryonic expression of innexins in the leech *Hirudo medicinalis*. *Dev Genes Evol*. 2006; 216:185–197. [PubMed: 16440200]

- Elliott EJ, Muller KJ. Long-term survival of glial segments during nerve regeneration in the leech. *Brain Res.* 1981; 218:99–113. [PubMed: 7023608]
- Fang YJ, Zhou MH, Gao XF, Gu H, Mei YA. Arachidonic acid modulates Na⁺ currents by non-metabolic and metabolic pathways in rat cerebellar granule cells. *Biochem J.* 2011; 438:203–215. [PubMed: 21564022]
- Firme CP III, Natan RG, Yazdani N, Macagno ER, Baker MW. Ectopic expression of select innexins in individual central neurons couples them to preexisting neuronal or glial networks that express the same innexin. *J Neurosci.* 2012; 32:14265–14270. [PubMed: 23055495]
- Fluri GS, Rudisuli A, Willi M, Rohr S, Weingart R. Effects of arachidonic acid on the gap junctions of neonatal rat heart cells. *Pflügers Arch.* 1990; 417:149–156. [PubMed: 1707515]
- Gabryel B, Chalimoniuk M, Stolecka A, Langfort J. Activation of cPLA2 and sPLA2 in astrocytes exposed to simulated ischemia in vitro. *Cell Biol Int.* 2007; 31:958–965. [PubMed: 17462919]
- Giaume C, Randriamampita C, Trautmann A. Arachidonic acid closes gap junction channels in rat lacrimal glands. *Pflügers Arch.* 1989; 413:273–279. [PubMed: 2717374]
- Gronert K. Lipid autacoids in inflammation and injury responses: a matter of privilege. *Mol Interv.* 2008; 8:28–35. [PubMed: 18332482]
- Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB. ATP released from astrocytes mediates glial calcium waves. *J Neurosci.* 1999; 19:520–528. [PubMed: 9880572]
- Honda S, Sasaki Y, Ohsawa K, Imai Y, Nakamura Y, Inoue K, Kohsaka S. Extracellular ATP or ADP induce chemotaxis of cultured microglia through Gi/o-coupled P2Y receptors. *J Neurosci.* 2001; 21:1975–1982. [PubMed: 11245682]
- Iglesias R, Dahl G, Qiu F, Spray DC, Scemes E. Pannexin 1: the molecular substrate of astrocyte “hemichannels”. *J Neurosci.* 2009; 29:7092–7097. [PubMed: 19474335]
- Inoue K. Microglial activation by purines and pyrimidines. *Glia.* 2002; 40:156–163. [PubMed: 12379903]
- Jarving R, Jarving I, Kurg R, Brash AR, Samel N. On the evolutionary origin of cyclooxygenase (COX) isozymes: characterization of marine invertebrate COX genes points to independent duplication events in vertebrate and invertebrate lineages. *J Biol Chem.* 2004; 279:13624–13633. [PubMed: 14732711]
- Jiang H, Zhu AG, Mamczur M, Falck JR, Lerea KM, McGiff JC. Stimulation of rat erythrocyte P2X7 receptor induces the release of epoxyeicosatrienoic acids. *Br J Pharmacol.* 2007; 151:1033–1040. [PubMed: 17558440]
- Kanneganti TD, Lamkanfi M, Kim YG, Chen G, Park JH, Franchi L, Vandenabeele P, Nunez G. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity.* 2007; 26:433–443. [PubMed: 17433728]
- Kuffler SW, Nicholls JG. The physiology of neuroglial cells. *Ergebn Physiol.* 1966; 57:1–90. [PubMed: 5330861]
- Kuffler SW, Potter DD. Glia in the leech central nervous system: physiological properties and neuron-glia relationship. *J Neurophysiol.* 1964; 27:290–320. [PubMed: 14129773]
- Kumar SM, Porterfield DM, Muller KJ, Smith PJ, Sahley CL. Nerve injury induces a rapid efflux of nitric oxide (NO) detected with a novel NO microsensor. *J Neurosci.* 2001; 21:215–220. [PubMed: 11150338]
- Liu HT, Sabirov RZ, Okada Y. Oxygen-glucose deprivation induces ATP release via maxi-anion channels in astrocytes. *Purinergic Signal.* 2008; 4:147–154. [PubMed: 18368522]
- Lopez-Vales R, Ghasemlou N, Redensek A, Kerr BJ, Barbayianni E, Antonopoulou G, Baskakis C, Rathore KI, Constantinou-Kokotou V, Stephens D, Shimizu T, Dennis EA, Kokotos G, David S. Phospholipase A2 superfamily members play divergent roles after spinal cord injury. *FASEB J.* 2011; 25:4240–4252. [PubMed: 21868473]
- MacVicar BA, Thompson RJ. Non-junction functions of pannexin-1 channels. *Trends Neurosci.* 2010; 33:93–102. [PubMed: 20022389]
- Maingret F, Patel AJ, Lesage F, Lazdunski M, Honore E. Mechano- or acid stimulation, two interactive modes of activation of the TREK-1 potassium channel. *J Biol Chem.* 1999; 274:26691–26696. [PubMed: 10480871]

- Martinez AD, Saez JC. Arachidonic acid-induced dye uncoupling in rat cortical astrocytes is mediated by arachidonic acid byproducts. *Brain Res.* 1999; 816:411–423. [PubMed: 9878857]
- McGlade-McCulloh E, Morrissey AM, Norona F, Muller KJ. Individual microglia move rapidly and directly to nerve lesions in the leech central nervous system. *Proc Natl Acad Sci USA.* 1989; 86:1093–1097. [PubMed: 2915975]
- Miller TM, Donnelly MK, Crago EA, Roman DM, Sherwood PR, Horowitz MB, Poloyac SM. Rapid, simultaneous quantitation of mono and dioxygenated metabolites of arachidonic acid in human CSF and rat brain. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2009; 877:3991–4000.
- Minghetti L. Cyclooxygenase-2 (COX-2) in inflammatory and degenerative brain diseases. *J Neuropathol Exp Neurol.* 2004; 63:901–910. [PubMed: 15453089]
- Minghetti L, Levi G. Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. *Prog Neurobiol.* 1998; 54:99–125. [PubMed: 9460796]
- Miyachi E, Kato C, Nakaki T. Arachidonic acid blocks gap junctions between retinal horizontal cells. *Neuroreport.* 1994; 5:485–488. [PubMed: 7911687]
- Monaghan K, Baker SA, Dwyer L, Hatton WC, Sik PK, Sanders KM, Koh SD. The stretch-dependent potassium channel TREK-1 and its function in murine myometrium. *J Physiol.* 2011; 589:1221–1233. [PubMed: 21224218]
- Morgese VJ, Elliott EJ, Muller KJ. Microglial movement to sites of nerve lesion in the leech CNS. *Brain Res.* 1983; 272:166–170. [PubMed: 6616194]
- Ngu EM, Sahley CL, Muller KJ. Reduced axon sprouting after treatment that diminishes microglia accumulation at lesions in the leech CNS. *J Comp Neurol.* 2007; 503:101–109. [PubMed: 17480028]
- Pelegri P, Barroso-Gutierrez C, Surprenant A. P2X7 receptor differentially couples to distinct release pathways for IL-1 β in mouse macrophage. *J Immunol.* 2008; 180:7147–7157. [PubMed: 18490713]
- Phillips JW, Horrocks LA, Farooqui AA. Cyclooxygenases, lipoxygenases, and epoxygenases in CNS: their role and involvement in neurological disorders. *Brain Res Rev.* 2006; 52:201–243. [PubMed: 16647138]
- Qiu F, Dahl G. A permeant regulating its permeation pore: inhibition of pannexin 1 channels by ATP. *Am J Physiol Cell Physiol.* 2009; 296:C250–C255. [PubMed: 18945939]
- Qiu F, Wang J, Spray DC, Scemes E, Dahl G. Two non-vesicular ATP release pathways in the mouse erythrocyte membrane. *FEBS Lett.* 2011; 585:3430–3435. [PubMed: 21983290]
- Salter MW, Hicks JL. ATP causes release of intracellular Ca²⁺ via the phospholipase C β /IP3 pathway in astrocytes from the dorsal spinal cord. *J Neurosci.* 1995; 15:2961–2971. [PubMed: 7722640]
- Samuels SE, Lipitz JB, Dahl G, Muller KJ. Neuroglial ATP release through innexin channels controls microglial cell movement to a nerve injury. *J Gen Physiol.* 2010; 136:425–442. [PubMed: 20876360]
- Scemes E, Giaume C. Astrocyte calcium waves: what they are and what they do. *Glia.* 2006; 54:716–725. [PubMed: 17006900]
- Schipke CG, Boucsein C, Ohlemeyer C, Kirchhoff F, Kettenmann H. Astrocyte Ca²⁺ waves trigger responses in microglial cells in brain slices. *FASEB J.* 2002; 16:255–257. [PubMed: 11772946]
- Scuri R, Mozzachiodi R, Brunelli M. Role for calcium signaling and arachidonic acid metabolites in the activity-dependent increase of AHP amplitude in leech T sensory neurons. *J Neurophysiol.* 2005; 94:1066–1073. [PubMed: 15872070]
- Shafer OT, Chen A, Kumar SM, Muller KJ, Sahley CL. Injury-induced expression of endothelial nitric oxide synthase by glial and microglial cells in the leech central nervous system within minutes after injury. *Proc Biol Sci.* 1998; 265:2171–2175. [PubMed: 9872006]
- Shuttleworth TJ. Arachidonic acid, ARC channels, and Orai proteins. *Cell Calcium.* 2009; 45:602–610. [PubMed: 19278724]
- Silverman WR, Rivero Vaccari JP, Locovei S, Qiu F, Carlsson SK, Scemes E, Keane RW, Dahl G. The pannexin 1 channel activates the inflammasome in neurons and astrocytes. *J Biol Chem.* 2009

- Sosinsky GE, Boassa D, Dermietzel R, Duffy HS, Laird DW, Macvicar B, Naus CC, Penuela S, Scemes E, Spray DC, Thompson RJ, Zhao HB, Dahl G. Pannexin channels are not gap junction hemichannels. *Channels (Austin)*. 2011; 5:193–197. [PubMed: 21532340]
- Sun GY, Xu J, Jensen MD, Simonyi A. Phospholipase A2 in the central nervous system: implications for neurodegenerative diseases. *J Lipid Res*. 2004; 45:205–213. [PubMed: 14657205]
- Sun GY, Xu J, Jensen MD, Yu S, Wood WG, Gonzalez FA, Simonyi A, Sun AY, Weisman GA. Phospholipase A2 in astrocytes: responses to oxidative stress, inflammation, and G protein-coupled receptor agonists. *Mol Neurobiol*. 2005; 31:27–41. [PubMed: 15953810]
- Verderio C, Matteoli M. ATP mediates calcium signaling between astrocytes and microglial cells: modulation by IFN-gamma. *J Immunol*. 2001; 166:6383–6391. [PubMed: 11342663]
- von Bernhardi R, Muller KJ. Repair of the central nervous system: lessons from lesions in leeches. *J Neurobiol*. 1995; 27:353–366. [PubMed: 7673894]
- Wang J, Dahl G. SCAM analysis of Panx1 suggests a peculiar pore structure. *J Gen Physiol*. 2010; 136:515–527. [PubMed: 20937692]
- Watts JL, Browse J. Genetic dissection of polyunsaturated fatty acid synthesis in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A*. 2002; 99:5854–5859. [PubMed: 11972048]
- Westcott JY, Murphy RC, Stenmark K. Eicosanoids in human ventricular cerebrospinal fluid following severe brain injury. *Prostaglandins*. 1987; 34:877–887. [PubMed: 2835791]
- Xu L, Han C, Lim K, Wu T. Activation of cytosolic phospholipase A2alpha through nitric oxide-induced S-nitrosylation. Involvement of inducible nitric-oxide synthase and cyclooxygenase-2. *J Biol Chem*. 2008; 283:3077–3087. [PubMed: 18029351]
- Yazdani N, Firme CP III, Macagno ER, Baker MW. Expression of a dominant negative mutant innexin in identified neurons and glial cells reveals selective interactions among gap junctional proteins. *Dev Neurobiol*. 2013

Figure 1.

Hminx2 and mouse Panx1 channels expressed by oocytes were closed by ArA and ETYA. (a) *Hminx2* channels were closed by ArA (100 μM) and ETYA (100 μM). The membrane potential of an *Hminx2* expressing oocyte was shifted to positive potentials, as indicated in the top trace which also shows +15 mV voltage pulses applied at a rate of 0.1 Hz. The increased membrane currents during pulses, reflecting the opening of the innexons, were attenuated by applying ETYA or ArA in the bath. As a control, 0.1% ethanol, the vector in which both ArA and ETYA were dissolved, was applied before each drug as indicated by a horizontal line. Same scales for (a), (b) and (c). (b) *Hminx2* channels, opened with KGlu, closed in response to ArA. Oocytes expressing *Hminx2* were voltage clamped at -30 mV and treated with a modified OR2 solution in which 50% of NaCl was replaced with potassium gluconate (50% KGlu) to open the innexons. The increased conductance of the oocyte with 50% KGlu, shown by increased current during 15 mV pulses, was inhibited by treatment with ArA (50 μM). As a control, 0.1% ethanol was applied before ArA. (c) *Hminx2* channels, opened with KGlu, closed in response to ETYA and CBX. Oocytes expressing *Hminx2* were voltage clamped at -30 mV and, to open innexons, treated with 50% KGlu. The increased conductance of the oocyte in response to the 50% KGlu was blocked with a treatment of ETYA (50 μM), the non-metabolizable ArA analogue. As a control, 0.1% ethanol was applied before ETYA. 100 μM CBX also reduced the 50% KGlu-induced increase in conductance. (d) Oocytes injected with *Hminx2* mRNA were voltage clamped and the membrane potential was shifted between -30 and +60 mV, a technique known to open pannexon and innexon channels. The large conductance of this oocyte was nearly blocked by ArA. The current was also measured during the intracellular acidification of this cell with CO₂, in the panel on the right, as a positive control for the closing of the innexon channels. (e) ArA inhibited mouse pannexin channels expressed in oocytes. Mouse Panx1 expressing oocytes were voltage clamped and the membrane potential shifted between -50 and +50 mV at 0.1 Hz to open the pannexon channels. ArA (100 μM) caused a

decrease in conductance (relative to CBX) of approximately 10%, and its non-metabolizable analogue ETYA (100 μ M) caused a decrease in the cellular conductance of approximately 50% (n=4). As controls, the vector 0.1% ethanol was applied before each drug.

Figure 2.

ArA and ETYA inhibited the release of ATP from oocytes expressing mouse pannexons opened by treatment with potassium gluconate solution (KGlu). For each bar, 4 oocytes were injected with *Panx1* mRNA, treated as indicated, and the luciferinluciferase assay of the supernatant, averaged for each condition, gave ATP release as luminescence on a linear, arbitrary scale. Saline was a control, with significance of decreases of ATP release compared to KGlu alone indicated by * for $p < 0.02$ and ** for $p < 0.001$.

Figure 3.

The inhibition of microglia migration and accumulation by ArA and ETYA was reversed by exogenous ATP. Microglia accumulation was measured in connectives fixed 4 hours after crushing and bathing in indicated solutions using standard methods in which tissues were stained with Hoechst 33258 dye, photographed and cell nuclei counted (see Figure 2A in reference Samuels et al., 2010). Bars represent mean numbers of cells at the lesions in a $100 \times 100 \times 18 \mu\text{m}^3$ volume. “Distal” was a measure of microglia in the same volume in an uncrushed region, approximately 1 mm from any injury; it was used as a baseline for the number of cells distributed throughout the tissue as a background level. Control accumulations were in 0.1% ethanol. The difference between the “ethanol” and “distal” values was the accumulation of microglia, the only cells that moved. **(a)** ArA reduced the accumulation of microglia at lesions in a dose dependent manner ($N=3$, $p<0.01$). **(b)** The ArA non-hydrolysable analogue eicosatetraynoic acid, ETYA, reduced the accumulation of microglia at lesions in a dose dependent manner ($N=3$, $p<0.01$). **(c)** Microglia accumulation at sites of injury, inhibited by ArA, occurred in the combined presence of ArA (100 μM) and ATP (100 μM) ($N=3$, $p<0.01$). **(d)** ATP reversed the ArA inhibition of microglia movement toward a crush. Time lapse images of crushed connectives with Hoechst-stained nuclei, treated with either ArA alone (100 μM) or ArA and ATP together (100 μM for each), were collected and made into a movie. About 3 times more microglia moved when extracellular ATP was added, but the directional movement was approximately the same

($N=3$, $p<0.05$). Therefore, decreased movement rather than misdirection caused decreased accumulation at lesions.

Figure 4.

ArA and ETYA reduced 6-CF dye loss from a dye-injected glial cell. **(a)** The graph shows the rate of normalized dye loss per 5 minutes from the glial cell, averaged during the first 30 minutes and the second 30 minutes of ArA treatment. As indicated by the asterisk, the inhibited rate of dye loss in the ArA treated tissue was significantly different from the ethanol-treated control only in the second 30 minutes ($n=4$, $p<0.05$). **(b)** ETYA, a non-metabolizable analogue of ArA, significantly inhibited 6-CF dye loss from the connective glial cell throughout the period of measurement. Graph shows normalized rate of dye loss, measured as in (a), from a 1 mm piece of connective glial cell every 5 minutes. In saline alone and in saline with 0.1% ethanol vector there was a substantial loss of dye (~12%), which was significantly reduced during the first half hour after ETYA was added ("ETYA", $n=4$, $p<0.05$). This effect had reached a peak by the first half hour of wash ("ethanol wash, 0-30"), with a significant reduction compared to controls throughout the ethanol wash at $p<0.001$ (double asterisk shown only for the first time point, $n=4$). During the wash, the rates of dye loss at the first and last time points were significantly different from each other at $p<0.05$.