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Peripheral inflammation upregulates P2X receptor expression in satellite glial cells of mouse trigeminal ganglia: A calcium imaging study

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ABSTRACT

Satellite glial cells (SGCs) in sensory ganglia are altered structurally and biochemically as a result of nerve injury. Whereas there is ample evidence that P2 purinergic receptors in central glial cells are altered after injury, there is very little information on similar changes in SGCs, although it is well established that SGCs are endowed with P2 receptors. Using calcium imaging, we characterized changes in P2 receptors in SGCs from mouse trigeminal ganglia in short-term cultures. Seven days after the induction of submandibular inflammation with complete Freund's adjuvant, there was a marked increase in the sensitivity of SGCs to ATP, with the threshold of activation decreasing from 5 μM to 10 nM. A similar observation was made in the intact trigeminal ganglion after infra-orbital nerve axotomy. Using pharmacological tools, we investigated the receptor mechanisms underlying these changes in cultured SGCs. We found that in control tissues response to ATP was mediated by P2Y (metabotropic) receptors, whereas after inflammation the response was mediated predominantly by P2X (ionotropic) receptors. As the contribution of P2X1,3,6 receptors was excluded, and the sensitivity to a P2X7 agonist did not change after inflammation, it appears that after inflammation the responses to ATP are largely due to P2X2 and/or 5 receptors, with a possible contribution of P2X4 receptors. We conclude that inflammation induced a large increase in the sensitivity of SGCs to ATP, which involved a switch from P2Y to P2X receptors. We propose that the over 100-fold augmented sensitivity of SGCs to ATP after injury may contribute to chronic pain states.

1. Introduction

Receptors for ATP and related nucleotides were identified in central and peripheral neurons, and there is ample evidence that signaling via nucleotide P2 receptors (P2Rs) is an important mode of neuronal communication (for reviews see Illes and Ribeiro, 2004; North and Verkhratsky, 2006; Burnstock, 2007; Abbracchio et al., 2009). Two main receptor types mediate the effects of P2R agonists: P2X receptors (P2XRs), which are ligand-gated ion channels, and P2YRs, which are G protein-coupled (Abbracchio et al., 2009). Each of these receptor types has several subtypes with a variety of actions and distributions in the nervous system (Burnstock, 2007). P2Rs play a role in synaptic transmission (Halassa et al., 2009) and in mediating trophic actions of nucleotides on glial cells and neurons (Neary and Zimmermann, 2009). In recent years there has been a growing interest in P2Rs in glial cells, and it appears that ATP that is released from neurons and also from glia may act on P2Rs in glial cells (for review see Fields and Burnstock, 2006). Activation of these receptors may increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_{in}$) in glia, and contribute to Ca^{2+} wave generation, which in turn can alter neuronal excitability (Scemes and Giaume, 2006). ATP is thus established as a signaling molecule among glial cells and between neurons and glial cells.

It is becoming clear that glial cells participate in various pathological changes in the nervous system, and one aspect that attracted considerable attention is that microglia in the spinal cord are closely involved in mechanisms of chronic pain. Tsuda et al. (2003) have found that P2X4Rs in microglia are upregulated following axotomy and that this change is essential for the generation and maintenance of chronic pain. It thus appears that glial activation (gliosis) in the CNS is part of the changes underlying chronic pain. Further studies largely supported these observations (Guo et al., 2005), for reviews see Inoue and Tsuda (2009) and Jarvis (2010).

In recent years there has been an increasing interest in glial cells in sensory ganglia, in particular those that form an envelope around sensory neurons. These cells, termed 'satellite glial cells' (SGCs) are greatly altered by peripheral damage, and undergo changes consistent with activation, as observed in the CNS glia (for reviews

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see Hanani, 2005; Ohara et al., 2009; Takeda et al., 2009). There is evidence that these changes in SGCs may contribute to pain (Ohara et al., 2008; Huang et al., 2010). SGCs are endowed with several receptors for hormones and neurotransmitters (for reviews see Hanani, 2005; Takeda et al., 2009), but the functional significance of most of these receptors is not known. Some information is available on P2Rs in these cells. Using calcium imaging, Weick et al. (2003) found that activation of P2YRs in SGC in intact mouse trigeminal ganglia (TG) leads to an increase in $[Ca^{2+}]_{in}$. Ceruti et al. (2008) studied SGCs from TG in culture and also identified P2YRs in SGCs. They also found that *in vitro* exposure to bradykinin increased SGC sensitivity to UDP, indicating upregulation of P2YRs. In the present work we asked whether *in vivo* inflammation alters P2Rs in SGC in the mouse TG. To address this question we used Ca^{2+} imaging in short-term cultures and in intact ganglia. Some of the results have been described in meeting proceedings (Hanani et al., 2007).

2. Materials and methods

2.1. Animals

Mice (Balb/c, 2–4 months old, M/F ratio 1:1) were used. The procedures were approved by the Animal Care and Use Committee of the Hebrew University-Hadassah Medical School and adhered to the guidelines of the International Association for the Study of Pain and conform to the NIH standards for the care and use of laboratory animals.

2.2. Pain models

A model of oro-facial inflammation was obtained by injecting 50 μl of 1:1 complete Freund's adjuvant (CFA):saline under the submandibular skin of mice anesthetized with xylazine (3 mg/kg) and ketamine (135 mg/kg). Controls were injected with the same volume of saline. At 1–28 days after treatment the animals were killed by CO_2 inhalation, and both TGs removed. Skin inflammation was assessed by staining for myeloperoxidase in whole mounts (Huang and Hanani, 2005) and hematoxylin-eosin (H&E) staining of 5 μm thick sections.

Infraorbital nerve axotomy was performed as described by Cherkas et al. (2004). Animals were anesthetized with chloral hydrate 200 mg/kg. The right infra-orbital nerve was exposed and sectioned. Six to eight days after the operation the animals were killed by CO_2 inhalation and the ipsilateral ganglia were removed and studied *in vitro*. Non-treated animals were used for control.

2.3. Primary culture of TG cells

The TG were cleaned from connective tissue and blood vessels and transferred to a 1.5 ml vial containing 1 mg/ml collagenase type 1A in Krebs solution. The vial was placed on the tilting stage (with a rate of 50 min^{-1}) for 45 min at 37 °C. Following that the collagenase solution was replaced with MEM-alpha growth medium containing 10% fetal bovine serum, 4.5 g glucose/l, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin. The ganglia were then triturated by repeated pipetting until complete tissue homogenization, and placed on MatTek glass-bottomed dishes (MatTek Corp., Ashland, MA, USA). The dishes were placed in a humidified 5% CO_2 incubator at 37 °C. Calcium recordings were performed after 18–24 h of incubation.

2.4. Ca^{2+} microfluorimetry of SGCs

For Ca^{2+} microfluorimetry in cultured SGCs, cells were loaded with the Ca^{2+} indicator Fluo-3 AM (1 μM) in MEM-alpha medium for 45 min in an incubator. The dye is preferentially taken up by SGCs (Weick et al., 2003). Cultures were placed in a recording chamber that was mounted on the stage of an inverted microscope and superfused at 5.5 ml/min with Krebs solution saturated with 95% O_2/5% CO_2. Test substances were applied by rapidly changing the bath solution. Images were acquired with cooled CCD camera (Cool Snap HQ, Photometrics, www.photomet.com). Fluorescence was excited at 506 nm, and emitted fluorescence (at 526 nm) increased by elevated $[Ca^{2+}]_{in}$. Images were recorded at 0.3 Hz. The fluorescence ratio F/F_0, where F_0 is the baseline, was used to describe relative changes in $[Ca^{2+}]_{in}$. As the field of view contained 10–40 SGCs in the focal plane, recordings were made from several cells simultaneously.

SGCs in intact ganglia were loaded with Fura-2 AM (1 μM) in Krebs solution bubbled with 95 % O_2/5 % CO_2 for 45 min at 37 °C, followed by 15 min wash of excess dye. Next, ganglia were placed in a recording chamber and were perfused at 5 ml/min with Krebs solution. Glial cells could be identified by their size and morphology (see Weick et al., 2003). Test substances were applied by rapidly changing the bath solution. Images were acquired with SensiCam camera (PCO, Kelheim, Germany), using Imaging Workbench 5.(www.imagingworkbench.com) mounted on an upright microscope. Fluorescence above 510 nm was excited at 340 nm (F_{340}) and 380 nm (F_{380}), and images were recorded at 0.3 Hz. The fluorescence ratio F_{340}/F_{380} (delta R) was used to describe relative changes in Ca^{2+} concentration. In most cases we did not administer more than one concentration of an agonist, and usually only one application was carried out to avoid desensitization. In the small number of cases where more than one concentration was used, we started with the lower one.

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): Adenosine 5′-triphosphate (ATP), αβ-methyleneadenosine 5′-triphosphate (αβ-meATP), benzoylbenzoyl adenosine 5′-triphosphate (BzATP), brilliant blue G (BBG), complete Freund's adjuvant (CFA), collagenase type 1A, methyltioadenosine 5′-triphosphate (2MeSATP), 6-N,N-diethyl-β-γ-dibromomethylene-D-adenosine-5′-triphosphate (ARL67156), pyridoxalphosphate-6-azophenyl-2′4′-disulfonic acid (PPADS), uridine 5′-triphosphate (UTP). The calcium indicators were purchased from Invitrogen (www.invitrogen.com).

2.5. Statistics

One-way ANOVA or *t*-test were used to analyze data obtained in the imaging experiments; $P < 0.05$ was considered as statistically significant.

3. Results

We asked whether peripheral inflammation alters responses of SGCs to neurotransmitters and inflammatory mediators, and used a model in mice, in which CFA was injected into the submandibular region. We verified that this treatment was associated with local inflammation and pain. Histological examination of sections of the submandibular skin showed clear infiltration of inflammatory cells from day 3 to day 14 after CFA injection. Lowered threshold in response to tactile stimulation of the submandibular skin was observed from day 3 to day 53 after CFA injection; for full details see Hanstein et al. (2010).

3.1. Preliminary pharmacological observations on cultured SGCs

Cultured cells were identified as described previously (Ceruti et al., 2008; Suadicani et al., 2010). As the cultures were young (18–24 h), the original organization of the SGCs around individual neurons was maintained. With phase optics it was easy to identify the neurons on the basis of their round shape and large size, compared to that of SGCs (Fig. 1A). The identity of SGCs was verified by immunostaining for the glial marker glutamine synthetase (Fig. 1B). Incubation of the cultures in the Ca^{2+} indicator Fluo-3 AM resulted in the labeling of SGCs, but not of neurons (Fig. 1C). The neurons were viable, as evidenced by the firing of action potentials (Suadicani et al., 2010) and by growing fine processes.

Using Ca^{2+} imaging, we examined responses of SGCs to several neurotransmitters and inflammatory mediators in control tissues and in tissues from CFA-treated mice (see Table 1). Substance P and glutamate were tested in treated tissues only because we have found previously that they had no effect on SGCs in normal TG (Weick et al., 2003). It appears from Table 1 that adrenergic agents as well as capsaicin, glutamate, bradykinin, and substance P did not evoke an increase in $[Ca^{2+}]_{in}$ in cultured SGCs. In contrast, as described below, ATP elicited responses clearly and reproducibly at concentrations in the μM range in control tissues, and in the nM range in tissues from treated mice. We therefore focused on this important neurotransmitter and pain mediator.

3.2. Reponses of SGCs to ATP

In control cultures, SGCs responded to ATP with a rise in $[Ca^{2+}]_{in}$, with threshold concentration of about 5 μM (see Fig. 1). Threshold response was defined as ATP concentration that elicits responses in 20% of the cells. We found that using the percentage of responding cells was more reliable and consistent measure of response than the