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NMDA receptors and associated signaling pathways: a role in knee joint blood flow regulation

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Abstract

Blood flow changes in response to *N*-methyl-D-aspartate (NMDA) receptor activation were assessed using a laser Doppler flowmeter. Treatment of the joint with NMDA (1 mM; 0.1 ml) resulted in a significant increase in blood flow while the control phosphate buffer (PB) injection (0.1 M; pH 7.4) had no effect. Blocking NMDA receptors with the antagonist MK 801 (0.1 mM) prevented the increase in blood flow observed following NMDA injection, suggesting specificity of action. The NMDA-evoked vasodilation has been shown to be mediated through activation of several intracellular signaling transduction molecules, namely nitric oxide, release of calcitonin gene-related peptide (CGRP) and CAM kinase II. Blocking actions of these molecules with L-NAME (10 mg/ml), $CGRP_{8-37}$ (0.01 mM) and KN-93 (1 μM), respectively, prevented the increase in blood flow induced by NMDA in the present study. These results provide new evidence implicating NMDA receptors in knee joint inflammatory responses.

Keywords: NMDA; Inflammation; Joint; Nitric oxide; CGRP; CAM kinase

1. Introduction

Joint inflammation is associated with a variety of cellular, physiological and molecular changes in the affected tissue. Acute arthritis is characterized by pain, redness, heat, swelling and behavioral signs of hyperalgesia. Several initiating factors and chemical mediators originating from different sources within joint tissue have been identified as being responsible for producing peripheral sensitization of articular afferents (Woolf and Dieppe, 1987; Levine et al., 1988; Grubb et al., 1991; Herbert and Schmidt, 1992; Schepelmann et al., 1992; Millan, 1999), plasma extravasation, and vasodilation (Richardson and Vasko, 2002; Schaible et al., 2002). A number of potential mediators have been associated specifically with arthritis, both clinically and in animals with experimentally induced arthritis (Levine et al., 1988; Scott et al., 1994; Kunkel et al., 1996). Some of these substances, such as substance P, calcitonin gene-related peptide (CGRP), nitric oxide and bradykinin are released from neural stores or synthesized and released during the events that follow tissue injury (Schepelmann et al., 1992; Green et al., 1993; Bileviciute et al., 1994; 1997; Cambridge and Brain, 1995). These have a profound impact on articular afferent fiber activity and on the production of inflammation.

In addition to neuropeptides, a new initiator of inflammation in the knee joint has recently been identified (Lawand et al., 2000). Glutamate, an excitatory amino acid, was found to be importantly involved in the peripheral inflammatory process. It was shown that acute inflammation is associated with the release of glutamate into the knee joint. The glutamate was presumed to be of neural origin since intra-articular injection of lidocaine or unilateral dorsal rhizotomies blocked the increase in glutamate in joint fluid after inflammation.

Previous behavioral and electrophysiological studies have shown that glutamate contributes to peripheral sensitization during joint inflammation. Behaviorally, the

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injection into the knee joint of EAAs acting specifically on ionotropic *N*-methyl-D-aspartic acid (NMDA) and non-NMDA receptors produces secondary heat hyperalgesia and mechanical allodynia in normal animals, while injection of the specific antagonists reverses these behaviors (Lawand et al., 1997a). Electrophysiologically, it was shown that an injection of EAAs into the knee joint significantly increases the discharge rate of primary articular afferents and leads to their sensitization (Lawand, 2000). Altogether, these results suggest a critical role for glutamate and its receptors in the development and maintenance of inflammation and peripheral sensitization.

Having a role as nociceptive mediators in peripheral tissues, it was conceivable that excitatory amino acids and their receptors are also important participants in the inflammatory process, in particular in dilation of blood vessels to cause tissue warming and redness, two important features of inflammation. So, in the present study, we attempted to investigate this hypothesis by looking at the role of a specific subtype of glutamate receptor, the NMDA receptor, in modulating blood flow to the knee joint.

Several research studies have demonstrated that activation of NMDA receptors triggers a cascade of intracellular events that can generate different physiological responses. For instance, activation of NMDA glutamate receptors can lead to the release of nitric oxide (NO), a potent vasodilator (endothelium-derived relaxing factor) present in the endothelial cells lining the blood vessels, in neuronal tissues and in the synoviocytes (Grabowski et al., 1997). The NO causes vasodilation by relaxing vascular smooth muscle by stimulation of guanylate cyclase (Ortega and Amaya, 2000). Previously, we have demonstrated that blockade of nitric oxide synthesis in the periphery decreases the edema formation associated with acute arthritis and prevents the development of heat hyperalgesia associated with inflammation (Lawand et al, 1997b). Therefore, in this study, we also examined whether the blood flow increase in the knee joint produced by activation of NMDA receptors is mediated through the release of NO.

Another mechanism by which glutamate receptors may exert an effect on blood vessels is by activation of calmodulin kinase II (CaMKII). CAMKII is present in primary sensory neurons and may play a critical role in inflammation (Carlton, 2002; Carlton and Hargett, 2002). Another possible candidate substance to mediate the blood flow changes produced by glutamate receptor activation is calcitonin gene-related peptide (CGRP). CGRP is known to be present in sensory neurons and has been shown to contribute significantly to the development of joint inflammation by causing vasodilation (Brain et al., 1992; Cambridge and Brain, 1992; Kilo et al., 1997; Lam and Ferrell, 1993; McMurdo et al., 1997). However, the signaling pathway mediating the release of CGRP from peripheral nerve fibers is still under study (Jackson and Hargreaves, 1999). Therefore, experiments were designed to determine the signal transduction pathways that mediate the changes observed in regional blood flow following activation of peripheral NMDA receptors.

2. Methods

2.1. Experimental procedures

All experimental protocols were approved by the Institutional Animal Care and Use Committee and were in accordance with the guidelines of the National Institutes of Health and the International Association for the Study of Pain.

Experiments were conducted on adult male Sprague–Dawley rats (250–350 g) anesthetized with sodium pentobarbital (40 mg/kg; i.p.). Deep anesthesia was maintained throughout the experiment as judged by the absence of a flexor withdrawal reflex response to pinch applied to the hindlimb.

Rats were divided into six experimental groups. The first group received an injection of 1 mM NMDA directly into the knee joint. To control for the injection, a second group of animals was injected with the vehicle (phosphate buffer; pH 7.4). To examine whether the changes observed in the blood flow to the knee joint are mediated specifically by NMDA receptors, a solution of an NMDA receptor antagonist (MK-801; 0.1 mM) mixed with NMDA (1:1) was injected intra-articularly prior to the administration of the agonist. Another group of rats was injected with phosphate buffer plus the agonist and this served as a control group.

The roles of endothelial and neuronal NO in mediating NMDA-induced blood flow changes in the joint were tested by injecting two groups of animals with either an endothelial (L-NAME; 10 mg/ml) or a neuronal specific (7-NINA; 1 mM) nitric oxide synthase (NOS) inhibitor directly into the knee joint.

Since the changes in blood flow induced by NMDA could result from an indirect activation of CGRP receptors present on sensory neurons or in the surrounding tissue, a solution that consisted of a combination (1:1) of NMDA and a CGRP receptor antagonist ($CGRP_{8-37}$; 0.01 mM) was injected directly into the knee joint of a different group of rats. To examine whether the changes observed in the blood flow to the joint are mediated by CaM Kinase II, a solution of a CaM Kinase II inhibitor (KN-93; 1μM) mixed with NMDA (1:1) was injected into the joint cavity.

All drugs were dissolved in a phosphate buffer solution (pH 7.4). Agonists and antagonists were administered as a bolus injection into the knee joint in a volume of 0.1 ml.

2.2. Blood flow measurement

Blood flow measurements were made using a laser Doppler flow meter (Moore Instruments). Anesthetized rats were placed in a prone position, a small incision was made

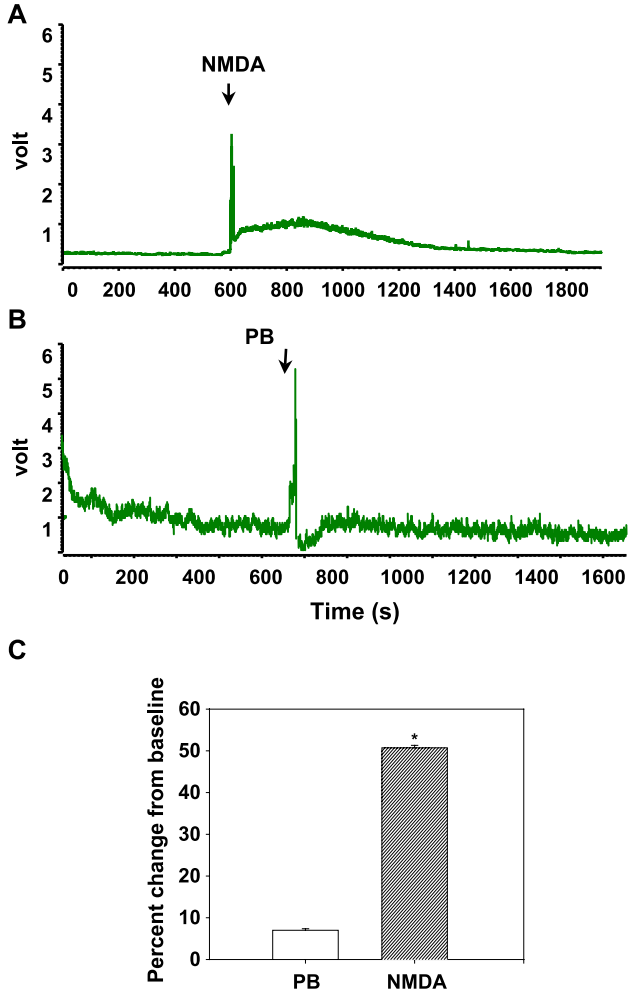

Fig. 1. (A) Raw data illustrating the increase in blood flow to the knee joint over baseline after local injection of NMDA (1 mM) into the knee joint. (B) Raw data illustrating the lack of change in the blood flow after intraarticular injection of phosphate buffer. (C) Average percent change in blood flow over baseline in PB(*n=11*) and NMDA(*n=15*)-treated rats. NMDA produced a significant increase in regional blood flow when compared to the control PB group. The arrows denote the time of injection. * indicates statistically significant difference from the PB-treated animals, ($p<0.05$).

in the skin to expose the joint and the probe discs were inserted subdermally on the anteromedial aspect of the knee joint. The probes were secured in position using masking tape. Baseline measurements were taken for at least 10 min prior to any treatment. Blood flow was then recorded for up to 1 h after injection. Average responses in control and treated animals were expressed as percentage change from baseline flow.

2.3. Data analysis

Statistical analysis of the data was performed using STATISTICA software. Assuming a normal distribution, comparisons of mean values *within* groups before and after administration of the testing substance was done using ANOVA or student's *t*-test where appropriate. Means± standard error of the mean (S.E.M.) were used throughout and all quoted *P* values were two-tailed. A *P* value <0.05 was considered significant.

3. Results

In the present study, activation of specific NMDA receptors with NMDA (1 mM) in the knee joint yielded a significant increase in regional blood flow when compared to baseline values (Fig. 1A), whereas injection of phosphate buffer produced just mechanical artifacts (Fig. 1B). This increase in blood flow by NMDA was blocked with MK-801, a selective NMDA receptor antagonist, indicating specificity of effect (Fig. 2A). No significant changes in the blood flow were seen following an intra-articular injection of the vehicle, phosphate buffer (PB) solution (Fig. 2B) or a lower concentration of NMDA (0.1 mM). It is noteworthy to mention here that none of the antagonists tested in this study have modified the blood flow when injected alone (data not

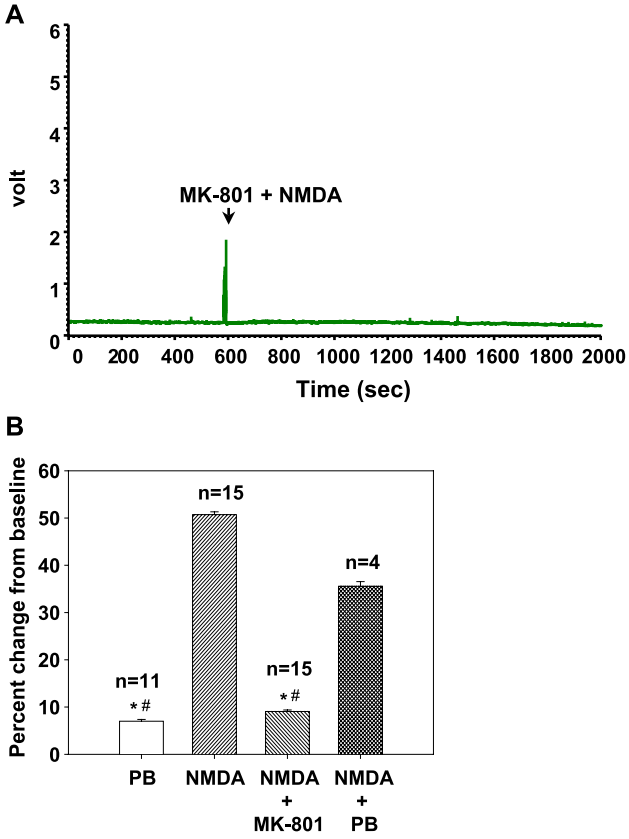

Fig. 2. (A) Raw data demonstrating the effect of MK-801+NMDA on blood flow changes in the knee joint. An intraarticular injection of 0.1 ml of a mixture of MK-801 (0.1 mM) and NMDA (1 mM) prevented the increase in blood flow typically seen after NMDA injection. The arrow denotes the time of the injection. (B) Average percent change over baseline in vehicle-treated and three experimental groups of rats. Blood flow changes were significantly lower in animals treated with PB or MK-801+NMDA when compared to animals injected with either NMDA with or without phosphate buffer. * indicates statistically significant difference from the NMDA-treated group ($p<0.05$).

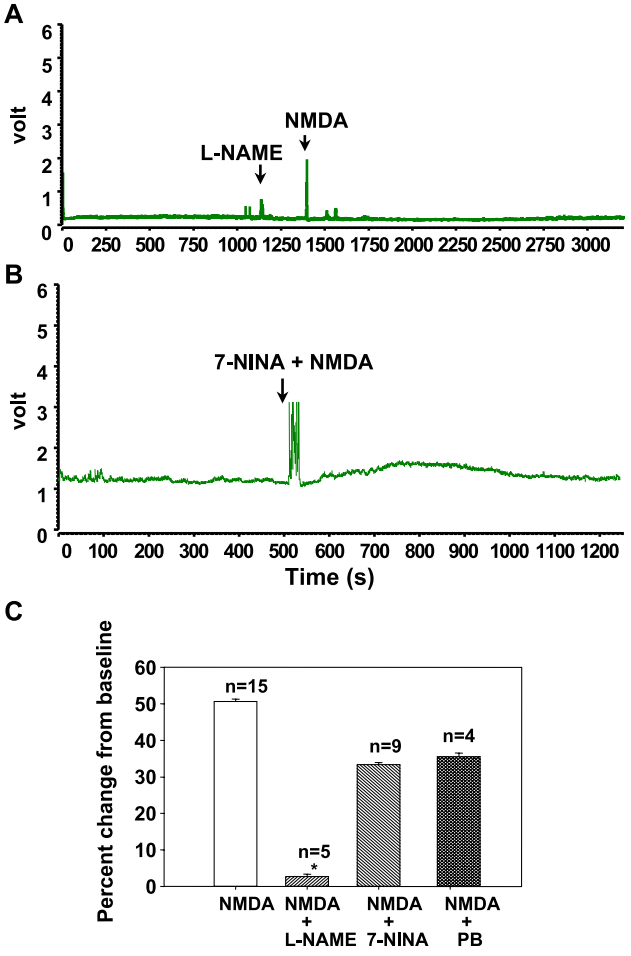

Fig. 3. (A) Raw data showing the effect of L-NAME on regional blood flow changes induced by local administration of NMDA. Prior administration of L-NAME (37 mM) prevented the increase in blood flow produced by NMDA (1 mM) injection into the knee joint. The arrows denote the times of the injections. (B) Raw data illustrating the effect of a neuronal nitric oxide inhibitor (7-NINA; 1 mM; 0.1 ml) on regional blood flow changes induced by NMDA injection into the knee joint. 7-NINA alone was shown to have no effect (data not presented). The arrow denotes the time of the injection. (C) Average percent change over baseline in three experimental groups of NMDA-treated rats. Blood flow changes were significantly lower in animals treated with L-NAME+NMDA as compared to the NMDA+PB. The 7-NINA injected with NMDA did not significantly decrease the blood flow. * Indicates statistically significant difference from the NMDA-treated group ($p<0.05$).

shown). Data analysis revealed a 50% increase in the blood flow over baseline following NMDA injection (Fig. 1A), suggesting that NMDA receptors play an important role in the development of inflammation.

Since activation of NMDA receptors leads to release of nitric oxide, an important vasodilator, the effects of specific endothelial (L-NAME) and neuronal (7-NINA) nitric oxide synthase (NOS) inhibitors were examined in this study. The results shown in Fig. 3A–C indicate that the non-selective NOS inhibitor, L-NAME, was more effective in diminishing the blood flow induced by NMDA receptor activation than 7-NINA. Intra-articular injection of L-NAME produced a significant reduction in the blood flow when compared to injection of a mixture of NMDA and PB into the joint. These results demonstrate that the effect of NMDA in enhancing blood flow in joint tissue is at least in part mediated through release of NO, a key player in inflammatory events.

Another important inflammatory substance known to be involved in knee joint inflammation is the neuropeptide, CGRP. When the CGRP receptor antagonist ($CGRP_{8-37}$) was injected in combination with NMDA locally into the joint, the typical blood flow increase obtained with NMDA injection was significantly diminished (Fig. 4A). No significant decrease in blood flow was seen with the combination of NMDA and PB. Therefore, it is possible that activation of NMDA receptors in the joint triggers the release of CGRP to act on specific CGRP receptors and cause vasodilation.

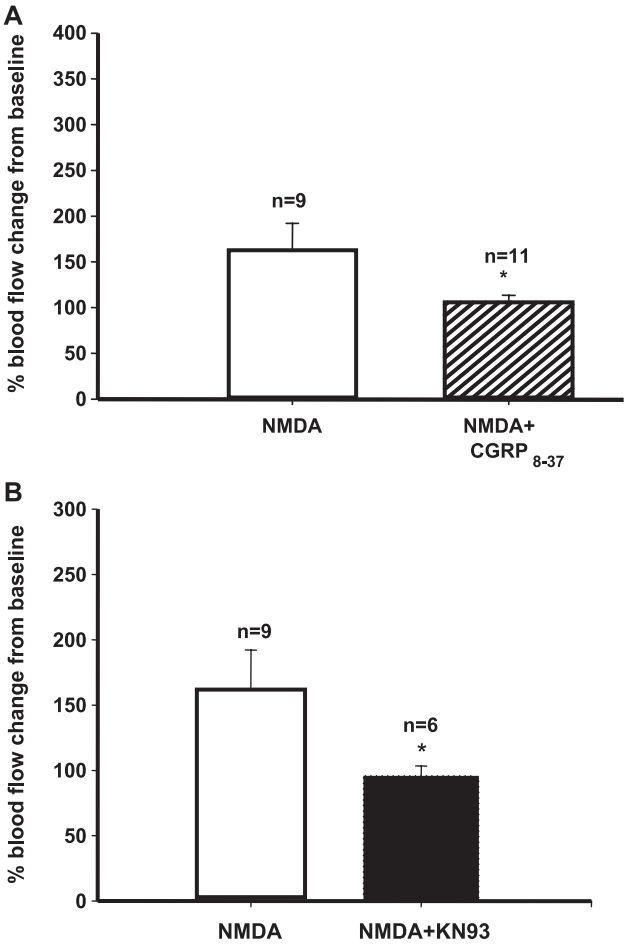

Fig. 4. (A) Average percent change over baseline in three experimental groups treated with NMDA. Blood flow changes are significantly reduced in animals treated with CGRP8-37+NMDA as compared to the NMDA+PB or to NMDA alone. * Indicates statistically significant difference from the NMDA-treated group ($p<0.05$). (B) The effect of CAM kinase II inhibitor on blood flow changes. This graph illustrates the average percent change over baseline in rats treated with either NMDA or NMDA+KN93. Blood flow changes are significantly lower in animals treated with KN93 as compared to the NMDA+PB or to NMDA alone. * Indicates statistically significant difference from the NMDA-treated group ($p<0.05$).

Calcium/calmodulin kinase II (CAMKII) is one of the enzymes in signal transduction pathways coupled with NMDA receptor activation. To test for a role of CAMKII in inflammation, a CAMKII inhibitor, KN93, was injected into the knee joint in combination with NMDA. Fig. 4B indicates a significant decrease in regional blood flow in the group of animals receiving a combination of NMDA/KN-93 compared to the control groups. These results provide new evidence to indicate a possible involvement of CAMKII in vasodilatation.

4. Discussion

The results of the current study indicate for the first time that activation of NMDA receptors in joint tissue causes vasodilation, an important feature of inflammation. Vasodilation, as measured by an increase in blood flow, is the physiological mechanism underlying the development of redness and heat in inflamed tissues. Previous studies have implicated several neurotransmitters and modulators in this phenomenon. However, a role for glutamate receptors as a contributor to these physiological changes has been overlooked. Very recently, our studies (Lawand, 2000) in this laboratory (Lawand et al., 1998) have first demonstrated that co-activation of ionotropic and metabotropic glutamate receptors in the knee joint of rats increases the firing rate of primary articular afferents, leading to their sensitization and consequently to the development of behavioral signs of heat hyperalgesia and mechanical allodynia (Lawand et al., 1997a). Additionally, it was shown that rats injected with ACPD (a metabotropic glutamate receptor agonist) and ASP (an NMDA receptor agonist) developed joint swelling as a result of plasma extravasation (Lawand, 2000). Consistent with our study is that of Beirith et al. (2002), who showed that glutamate injection into the mouse paw causes edema and nociception, providing further evidence to implicate glutamate receptors in inflammatory processes.

Moreover, our previous work demonstrated an increase in the glutamate concentration in knee joint dialysate after induction of inflammation with an intra-articular injection of kaolin and carrageenan in rats (Lawand et al., 2000), and in patients with rheumatoid arthritis (McNearney et al., 2000) suggesting that glutamate may be transported in the peripheral axons and released from peripheral nerve terminals in response to noxious stimuli.

In this study, we conducted experiments to corroborate this notion and to investigate not only the involvement of glutamate receptors but also the signal transduction pathways coupled with these receptors that mediate the changes seen in joint tissues. Our results have shown that injection of NMDA directly into the knee joint of rats produces a significant increase in regional blood flow when compared to injection of the control vehicle solution. This increase in blood flow could be prevented by co-injection of the NMDA receptor antagonist, MK801, indicating that NMDA acted specifically on NMDA receptors present either on blood vessels or in surrounding tissues, including peripheral terminals of primary afferents. Activation of NMDA receptors in these tissues can also trigger an intracellular cascade of events leading to activation of second messenger pathways. Among these pathways, NO is considered a critical substance involved importantly in the generation of inflammatory responses (Najafipour and Ferrell, 1993). NO released in the periphery either from the endothelial cells (Wahl et al., 2003) or from nervous tissue (Pozza et al., 1998; Thippeswamy and Morris, 2002) can act directly on blood vessels to cause their dilation, thereby increasing the blood flow to the affected area. Our results have demonstrated that the endothelial NOS inhibitor (L-NAME) was able to reduce, in a significant manner, the blood flow increase seen following NMDA injection. However, the neuronal NOS inhibitor 7-NINA was not very effective, suggesting that the source of NO release subsequent to NMDA activation is from the endothelial cells lining the blood vessels, rather than from neuronal tissue. It should be noted here that the concentrations of L-NAME and 7-NINA used in this study were chosen based on our previous behavioral data. These results are consistent with our previous findings that intra-articular injection of L-NAME, but not 7-NINA, blocks the nociceptive behaviors evoked by stimulation of the joint and significantly reduces edema formation in the inflamed joint (Lawand et al., 1997b).

Another mechanism by which activation of NMDA receptors may have produced vasodilation was through stimulation of CGRP receptors by CGRP, since administration of $CGRP_{8-37}$ into the knee joint was able to block the increase in blood flow produced by NMDA. Recent immunohistochemical studies have shown that CGRP-positive primary sensory neurons contain the NR2B subunit of the NMDA receptors, suggesting that NMDA receptors play an important role in the modulation of neuropeptide release (Ma and Hargreaves, 2000). The major source of CGRP release in joint tissues is presumed to be from peptidergic articular afferents. CGRP can be released into the knee joint in response to various stimulants (Bileviciute et al., 1994; 1997; Larsson et al., 1989; 1991). When CGRP is released in peripheral tissues, it acts on specific CGRP receptors and causes vasodilation (Cambridge and Brain, 1992). Other sources of CGRP, such as macrophages, blood vessels and synoviocytes, cannot be excluded. Many studies have provided evidence for a role of CGRP and its receptors in vasodilation (Lam and Ferrell, 1993; McMurdo et al., 1997). These investigators have shown that CGRP applied topically to the joint capsule produces a dose-dependent increase in regional blood flow that is more significant than that produced by SP, while application of a CGRP receptor antagonist elicits vasoconstriction in the rat synovium. These findings indicate that CGRP acts as a potent vasodilator in the knee joint. However, in a study by Jackson and Hargreaves (1999), activation of AMPA and KA receptors but not NMDA receptors in the dental pulp

was shown to release CGRP. The difference in NMDA action could be due to site differences.

Another signaling pathway that could be involved in the NMDA-evoked vasodilation is the CAMKII pathway. This protein kinase has been shown to be involved in long-term potentiation and in central sensitization of nociceptive neurons (Fang et al., 2002; see review by Willis, 2002). It has been demonstrated that CaMKII phosphorylates the NR2B subunit of the NMDA receptors and enhances excitatory amino acid responses (Kolaj et al., 1994). This interaction was found to be an integral component of synaptic plasticity (Omkumar et al., 1996). Nothing is yet known about the functions of CAMKII in primary articular afferents during the course of inflammation. However, recent findings by Carlton et al. have demonstrated that CaMKII alpha is present in small diameter cutaneous primary sensory neurons and that it is transported into the peripheral and central terminals of these cells (Carlton, 2002; Carlton and Hargett, 2002). During inflammation, CaMKII has also been shown to be upregulated. Altogether, these findings suggest an important role for CaMKII in the processing of sensory information and in the initiation of peripheral inflammatory events.

In summary, our findings suggest that NMDA receptors present in the knee joint are not only involved in pain transduction but are key players in inflammatory events. Activation of NMDA receptors causes the release of many inflammatory mediators, including NO and CGRP possibly secondary to activation of CaMKII. It is presumed that these substances would in turn contribute either directly or indirectly to the regulation of inflammation.

Acknowledgments

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