The role of protease-activated receptor type 2 in nociceptive signaling and pain

Petra Mrozkova [1,2] Jiri Palecek[1] Diana Spicarova[1]

[1] Department of Functional Morphology, Institute of Physiology v.v.i., Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic

[2] Department of Physiology, Faculty of Science, Charles University in Prague, Viničná 7, 128 44 Praha 2

Corresponding author:
Diana Spicarova MSc, PhD
Department of Functional Morphology, Institute of Physiology v.v.i., Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic
e-mail: dianaspicarova@biomed.cas.cz
office: (+420) 24106 2540
lab: (+420) 24106 -2664, -2621, -2531

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Abstract

Protease-activated receptors (PARs) belong to the G-protein-coupled receptor family, that are expressed in many body tissues especially in different epithelial cells, mast cells and also in neurons and astrocytes. PARs play different physiological roles according to the location of their expression. Increased evidence supports the importance of PARs activation during nociceptive signaling and in the development of chronic pain states. This short review focuses on the role of PAR2 receptors in nociceptive transmission with the emphasis on the modulation at the spinal cord level. PAR2 are cleaved and subsequently activated by endogenous proteases such as tryptase and trypsin. In vivo, peripheral and intrathecal administration of PAR2 agonists induces thermal and mechanical hypersensitivity that is thought to be mediated by PAR2-induced release of pronociceptive neuropeptides and modulation of different receptors. PAR2 activation leads also to sensitization of transient receptor potential channels (TRP) that are crucial for nociceptive signaling and modulation. PAR2 receptors may play an important modulatory role in the development and maintenance of different pathological pain states and could represent a potential target for new analgesic treatments.

Introduction

Extracellular proteases such as thrombin, trypsin, serin protease 1 and coagulation factors VII and Xa can regulate target cells by cleaving and activating receptors that belong to a family of G-protein-coupled protease-activated receptors (PARs) (Brass and Molino 1997, Corvera *et al.* 1997, Molino *et al.* 1997, Camerer *et al.* 2000, Vergnolle 2000). This receptor family has at least four members (PAR1–4). Mechanism of receptor triggering involves proteolytic unmasking of a cryptic N-terminal sequence on the extracellular membrane that acts as a tethered receptor-activating ligand. In general, several proteases can activate a single PAR by cleaving it at specific site and exposing the tethered ligand domain. For example, trypsin, tryptase, coagulation factors VIIa and Xa and certain membrane-anchored proteases can all cleave and activate PAR2, although with varying potencies (Cenac *et al.* 2003, Chen *et al.* 2011, Rothmeier and Ruf 2012).

There are short specific synthetic peptides, based on the tethered ligand sequences, designed as PARs 1,2 and 4 agonists (Brass and Molino 1997). Some authors consider PAR3 as a co-factor for the activation of other PARs and it has no selective activating peptide (Noorbakhsh et al. 2003, Zhao et al. 2014). These synthetic peptides have been shown to activate the specific receptors and mimic the effects of the activating proteases for example SLIGKV-NH$_2$ and 2-Furoyl-LIGRLO-NH$_2$ are frequently used for PAR2 activation. Due to these selective peptides, the physiological consequences of activating different PARs may be studied and distinguished accurately (Hollenberg et al. 1997, Kawabata et al. 1999). For the purpose of research there were also developed compounds which are able to act as antagonist, e.g. FSLLRY-NH$_2$ and GB83 inhibit PAR2.

PARs are present in most body tissues, with the highest expression in the epithelium (lungs, liver, digestive tract, skin, blood vessels) and they are also present in the peripheral

and central nervous system (Dery et al. 1998, Vergnolle et al. 2001b, Hollenberg and Compton 2002). The physiological effect of their activation varies in different tissues.

PAR2 receptors are known to play important roles in the organism response to tissue injury, notably in the process of inflammation and repair (Dery *et al.* 1998). In particular, agonists of PAR2, tryptase and trypsin released from different cell types including mast cells, have widespread proinflammatory effects in part via a neurogenic mechanism (Saifeddine *et al.* 1996, Vergnolle *et al.* 1999, Steinhoff *et al.* 2000, Seeliger *et al.* 2003). PAR2 are expressed on a subset of primary sensory neurons and PAR2 agonists stimulate release of substance P (SP) and calcitonin gene-related peptide (CGRP) in peripheral tissues via activation of peripheral nerve endings (Steinhoff *et al.* 2000). It was also reported that PAR2 activation can sensitize adult rat dorsal root ganglion (DRG) neurons in vitro (Steinhoff *et al.* 2000). Intraplantar injection of subinflammatory doses of PAR2 agonists in rat and mice induced thermal and mechanical hyperalgesia and elevated Fos protein expression in the spinal cord (Vergnolle *et al.* 2001a). All these changes indicate an important role for PAR2 in nociceptive transmission.

This review focuses on the function of PAR2 in the nervous system, particularly in connection with transmission and modulation of nociceptive stimuli at the spinal cord level and with pain.

Mechanisms of PAR2 activation and intracellular signal transduction

PARs are G-protein-coupled receptors (GPCRs), a family of receptors with large seven-transmembrane helical domain protein that sense molecules outside the cell and activate intracellular signaling pathways (Nystedt *et al.* 1994, Hoogerwerf *et al.* 2001, Macfarlane *et al.* 2001). Thrombin and trypsin are usually regarded as the main activators of PARs. PAR2 is a target for trypsin and other serine proteases, such as mast-cell tryptase, but it

is not activated by thrombin (Nystedt *et al.* 1995, Nystedt *et al.* 1996, Molino *et al.* 1997). PAR2 lacks a hirudin-like thrombin-binding domain, but trypsin cleavage site was detected in its extracellular N terminus domain (Nystedt *et al.* 1995). PAR2 is activated by proteolytic cleavage of its extracellular amino terminus (Hollenberg *et al.* 1997, Steinhoff *et al.* 2000). These receptors can be also activated by exogenous proteases, as well as non-proteolytically by exogenous peptide sequences that mimic the final amino acids of the tethered ligand (Hollenberg *et al.* 1997, Al-Ani *et al.* 1999).

The signaling pathways that are triggered by PAR2 activation through the binding to activation molecules on G-proteins (Gq/11, Gi/o) may activate several signaling pathways including phospholipases, Jun N-terminal kinase and p44/42 mitogen-activated protein kinase (MAPK), ERK1/2, phosphatidylinositol 3-kinase (PI3K), adenylate cyclase (AC), protein kinase C (PKC), protein kinase A (PKA) and members of the SRC-family of tyrosine kinases (Kanke *et al.* 2001, Suen *et al.* 2010, Chen *et al.* 2011, Suen *et al.* 2014, Bao *et al.* 2015). These signaling pathways can affect various cellular activities – proliferation, gene transcription, morphological changes, motility and survival (Macfarlane *et al.* 2001, Kawabata *et al.* 2004). PAR2 activation was also reported to result in up/down-regulation of about 2500 genes that are important mainly for cell metabolism (around 1000), cell cycle, complement and MAPK pathway, sirtuin enzymes, histone deacetylases and inflammatory cytokines (Suen *et al.* 2010).

Expression of PAR2 in the nervous system

High density expression of PAR2 receptors was documented in neurons of hippocampus, cortex, amygdala, thalamus, hypothalamus, striatum and in DRG neurons in rats (Striggow *et al.* 2001). All four PARs are also present in astrocytes culture from rat brain (Wang *et al.* 2002). PAR2 are expressed on neurons and astrocytes also in human CNS

(D'Andrea *et al.* 1998, Noorbakhsh *et al.* 2006). Localization on guinea-pig myenteric and submucosal neurons was demonstrated earlier (Corvera *et al.* 1999). For the presence of PAR2 in the spinal cord dorsal horn exist mainly functional electrophysiological evidence (Alier *et al.* 2008, Fujita *et al.* 2009, Huang *et al.* 2011), while recently PAR2 were detected also using western blot analysis in the rat spinal cord tissue (Chen *et al.* 2015). PAR2 were immunohistochemically detected in many small-sized and some medium- to large-sized DRG neurons (Steinhoff *et al.* 2000, Dai *et al.* 2004). Significant population of small DRG neurons expressing TRPV1 receptors, also showed expression of PAR2 (Amadesi *et al.* 2004, Dai *et al.* 2004), suggesting possible interactions between them.

Activation of PAR2 in neuronal tissue

Trypsin and tryptase are known as the main PAR2-activating proteases. Potentially, the main source of tryptase to activate neural PAR2 are mast cells that have been found in the choroid plexus, in the parenchymal and perivascular areas in the CNS and in close contact with peripheral nerves (Stead *et al.* 1987). Mast cell tryptase, although being less potent than trypsin, can regulate neuronal activity by cleaving PAR2 (Steinhoff *et al.* 2000, Reed *et al.* 2003) and other receptors. Selective PAR2 antagonist (FSLLRY-NH$_2$) was able to block paclitaxel induced neuropathic pain that was accompanied by mast cell tryptase activity in spinal cord and DRG in mice (Chen *et al.* 2011). Tryptase is thus a strong candidate for neuronal PAR2 activation. There is a number of other possible PAR2 activators, such as P22, another trypsin-like serine protease with PAR2-activating capacity that has been detected in rat brain (Sawada *et al.* 2000). Precursors of trypsin (trypsinogen-IV, trypsinogen-III) and other PAR2 activators (factor X) are also expressed in brain and human neural cell lines (Wiegand *et al.* 1993, Shikamoto and Morita 1999).

The function of PAR2 in the nervous system

All four PARs are expressed throughout the peripheral and central nervous system and were suggested to play many different roles in neurogenic inflammation, pain perception, pruritus sensation, nerve regeneration, secretory functions and Ca^{2+} mobilization (Corvera *et al.* 1999, de Garavilla *et al.* 2001, Linden *et al.* 2001, Noorbakhsh *et al.* 2003). The role of PAR2 on the peripheral terminals of nociceptive DRG neurons is relatively well known. They have been implicated in the activation and/or modulation of nociceptor function (Vergnolle *et al.* 2001a, Fiorucci and Distrutti 2002, Cenac and Vergnolle 2005). The peripheral nerve endings may be activated by proteases which are generated and released during tissue trauma and inflammation. Increased PAR2 immunoreactivity has been reported on primary afferent nerve fibers in the skin of patients with atopic dermatitis. Intracutaneous injection of a PAR2 agonist provoked an itch response in these patients (Steinhoff *et al.* 2003).

PAR2 agonists induced and prolonged hyperexcitability of guinea-pig submucosal neurons (Reed *et al.* 2003). PAR2 activation rapidly increased cytosolic concentration of Ca^{2+} from intracellular stores, whereas sustained Ca^{2+} elevation was dependent on the influx from extracellular space in adult rat DRG neurons in vitro (Steinhoff *et al.* 2000). Over 60% of DRG neurons coexpressed PAR2 with SP and CGRP, and PAR2 agonists stimulated the release of these peptides in peripheral tissues and in the spinal cord (Steinhoff *et al.* 2000). The release of these neuropeptides is important for nociceptive transmission. Intraplantar administration of subinflammatory doses of PAR2 agonists enhanced and prolonged hyperalgesia and induced activation of second-order nociceptive neurons at the spinal cord level in rodents. Also experiments in PAR2-deficient mice proved that PAR2 activation significantly contributes to inflammatory hyperalgesia (Vergnolle *et al.* 2001a).

Activation of PAR2 receptors triggers several intracellular signaling cascades, one of them is associated with G-protein and PLC/Ca^{2+}/PKC signaling pathway (Bohm *et al.* 1996,

Seatter *et al.* 2004) and another one is associated with β-arrestin including MAPK (ERK1/2) signaling (DeFea *et al.* 2000, Rothmeier and Ruf 2012). Activation of PKC may lead to the activation and nuclear translocation of NF-κB in neurons (Lilienbaum and Israel 2003). Activation of NF-κB - mediated signaling increased the histone acetylation and facilitated the expression of BDNF in the central neurons (Peng *et al.* 2011). Some groups observed that activation of PAR2 signaling was required for the inflammation induced BDNF release from microglia (Yuan *et al.* 2010, Fan *et al.* 2014).

The role of PAR2 at the spinal cord level

Although PAR2 are known to be expressed on neurons and astrocytes in rodent and human CNS (Noorbakhsh *et al.* 2006), their expression in the spinal cord was not proved by immunohistochemical methods (Alier *et al.* 2008). However, functional studies on spinal cord slices suggested PAR2 presence on the central terminals of primary afferent nerve fibers and in dorsal horn neurons (Fujita *et al.* 2009, Huang *et al.* 2011). Very recently PAR2 were detected by western blot analysis in the superficial dorsal horn tissue (Chen *et al.* 2015).

PAR2 are colocalized with proinflammatory neuropeptides, such as substance P and CGRP, in DRG sensory neurons. Trypsin, tryptase and selective agonists of PAR2 stimulate the release of both CGRP and SP from C-fibers in peripheral tissues and in the spinal cord (Steinhoff *et al.* 2000). Peripheral administration of trypsin, tryptase and PAR2-activating peptide caused SP and CGRP release from sensory nerves. It caused massive edema that could be prevented by NK1R and CGRP1 receptor inhibition (Steinhoff *et al.* 2000).

Further studies at spinal cord level demonstrated that intrathecal (i.t.) application of PAR2 agonist induced mechanical allodynia and thermal hyperalgesia in healthy animals and augmented increased sensitivity present in a peripheral inflammatory pain model (Alier *et al.* 2008, Huang *et al.* 2011). PAR2-induced hypersensitivity was

mediated by PGE2 release via COX activation in the spinal cord (Koetzner *et al.* 2004). In a paclitaxel-induced neuropathic pain model, i.t. application of PAR2 antagonist reversed mechanical allodynia and heat hyperalgesia (Chen *et al.* 2011).

Electrophysiological study with whole-cell recordings from young rat substantia gelatinosa neurons, showed no increase in spontaneous excitatory postsynaptic current (sEPSC) frequency or neuronal excitability following application of synthetic PAR2 agonists or trypsin. Moreover, trypsin slightly decreased sEPSC frequency in the dorsal horn (Alier *et al.* 2008). In comparison, another study showed that PAR2 agonist significantly enhanced frequency of sEPSC in a similar preparations from adult rats, suggesting involvement of PAR2 receptors in modulation of nociceptive synaptic transmission at the spinal cord level (Fujita *et al.* 2009). Direct activation of PAR2 in the spinal cord was suggested also in a study demonstrating decrease of spontaneous inhibitory postsynaptic currents frequency and amplitude in substantia gelatinosa neurons, implicating that PAR2 activation in the spinal cord may potentiate nociception by affecting the inhibitory rather than the excitatory transmission in the spinal dorsal horn neurons (Huang *et al.* 2011).

Altogether, this published evidence suggests that PAR2 play an important role in the process of nociceptive transmission and in neurogenic inflammatory mechanisms both in the periphery and at the spinal cord level. The precise role and exact importance for spinal nociceptive modulation is not completely understood and needs further research.

PAR2-induced activation of downstream protein kinases and other enzymes in neural tissue

PAR2 are expressed in nociceptive DRG neurons (Dai *et al.* 2004) and colocalizes with PKC and PKA, which are activated downstream of PAR2 receptors (Amadesi, Cottrell et al. 2006). The PKC activation cascade involves PAR2-induced activation of PLC leading to

production of diacylglycerol and inositol 1,4,5-trisphosphate, which increases intracellular concentration of Ca^{2+} and activates PKC (Mule *et al.* 2002). The PKA activation cascade involves PAR2-induced mobilization of cAMP (Amadesi, Cottrell et al. 2006).

A selective PAR2 agonist can induce phosphorylation and activation of PKD in cultured rat DRG neurons (Amadesi *et al.* 2009). DRG neurons express all three isoforms of PKD, PKD1 (also known as PKCμ), PKD2 and PKD3. PKDs are downstream targets of diacylglycerol (DAG) and PKCs, which may be induced by PAR2 activation (Amadesi *et al.* 2009). PKD1 directly interacts with TRPV1 in cell lines and primary sensory neurons, suggesting that PKD may regulate TRPV1 activity (Wang *et al.* 2004).

PAR2 activation leads to increased PLC activity and generation of IP3 and mobilization of Ca^{2+} in neurons and astrocytes (Rothmeier and Ruf 2012), which can result in PLA2 activation and increased production and release of arachidonic acid (AA), which is the substrate for cyclooxygenase (COX) (Poole *et al.* 2013). An increase in AA production leads also to increased synthesis and release of PGE that through stimulation of AC may increase intracellular cAMP. Subsequently these steps lead to neuronal hyperexcitability and hyperalgesia (Koetzner *et al.* 2004).

PAR2 agonists lead to activation of ERK1/2 MAP kinases in multiple ways. It is known that the ERK/MAPK activation can contribute to increased nociceptive responses in the dorsal horn and DRG neurons following inflammation or nerve injury (Ji *et al.* 2009). Activation of PAR2 induces assembly of a MAPK signaling cascade by a mechanism that depends on β-arrestins (DeFea *et al.* 2000). Beta-arrestin dependent endocytosis of PAR2 is required for intracellular targeting of activated ERK1/2, which is retained in the cytosol instead to be translocated to the nucleus. The concentration of ERK1/2 in cytosol depends on the formation of a signaling complex that includes internalized PAR2, β-arrestin 1, Raf-1 and pERK1/2 (DeFea *et al.* 2000).

Transient receptor potential channels sensitization

PAR2 is highly co-expressed with TRPV1 receptors in the DRG neurons (Amadesi *et al.* 2004, Hoogerwerf *et al.* 2004). PAR2 activation leads to TRPV1 sensitization via PLC (Amadesi *et al.* 2004) and via two key kinases PKCε and PKA that phosporylate TRPV1 receptor (Amadesi *et al.* 2006, Spicarova and Palecek 2008). Sensitized TRPV1 receptor could be subsequently activated by endogenous agonists (Spicarova and Palecek 2009, Spicarova *et al.* 2014b). PAR2 activation enhanced TRPV1 agonist capsaicin stimulated neuropeptides (SP, CGRP) release within the spinal cord dorsal horn (Amadesi *et al.* 2004). In vivo, intraplantar injection of PAR2 agonist induced thermal hyperalgesia dependent on TRPV1 receptor activation (Amadesi *et al.* 2004). TRPV1 mediated DRG and spinal neurons activation in paclitaxel inducted neuropathic pain (Li *et al.* 2015). It was also shown that spinal TRPV1 activation mediates chemokine CCL2- or plantar incision-induced hyperalgesia (Spicarova *et al.* 2014a, Uchytilova *et al.* 2014). Recently it was demonstrated that blocking of spinal PAR2 and TRPV1 receptors attenuated oxaliplatin-induced neuropathic pain and this effect was mediated by decreased release of SP and CGRP in the superficial dorsal horn of the spinal cord (Chen *et al.* 2015).

PAR2 co-expression in rat DRG neurons with TRPV4 receptors and neuropeptides SP and CGRP was also demonstrated (Grant *et al.* 2007, Poole *et al.* 2013). Activation of PAR2 sensitized TRPV4 receptors to agonist application due to PLC-β, PIP2, IP3, PLA, PKA, PKC and AA activation (Grant *et al.* 2007, Poole *et al.* 2013). These authors also showed that TRPV4 activation promoted SP and CGRP release from afferent nerves in the spinal cord and this process was enhanced by PAR2 agonist pretreatment. In vivo, intraplantar injection of PAR2 agonist resulted in mechanical hyperalgesia that was prevented in TRPV4 knock-out

mice. The same PAR2 agonist treatment robustly enhanced hyperalgesia induced by the injection of TRPV4 agonist (Grant *et al.* 2007).

PAR2 is co-expressed with TRPA1 in small DRG neurons (Dai et al. 2007). TRPA1 receptors could be activated by chemical compounds occurring in mustard, wasabi, garlic, onion or cinnamon, they are potential sensors for noxious cold and the mechanosensitivity is also suggested (Hill and Schaefer 2007, Laursen et al. 2014). Electrophysiological studies have shown that PAR2 sensitizes TRPA1, and this effect can be blocked by a PLC inhibitor (Dai et al. 2007). Similarly it was shown that PAR2 activation of PKA and PLC also participate in TRPA1 mediated sensitization in nociceptive transmission (Wang et al. 2008). In vivo it has been demonstrated that PAR2 mediates paclitaxel-induced mechanical, heat and cold hypersensitivity through the activation of TRPA1 (Chen et al. 2011).

Role of PAR2 in inflammatory, neuropathic and cancer pain

Critical involvement of PAR2 in the pathogenesis of several types of inflammatory or neuropathic pain was demonstrated previously (Bao *et al.* 2014). PAR2 signaling is involved in the sensitization of peripheral nociceptors and dorsal horn neurons in several pathological pain states (Dai *et al.* 2007, Grant *et al.* 2007, Chen *et al.* 2011).

PAR2 are present in many cells involved in inflammation, their activation provokes the release of numerous inflammatory mediators, such as prostaglandins, SP, CGRP or cytokines (interleukine-1 and TNF-α), that in turn may induce or modulate pain perception (Steinhoff *et al.* 2000, Vergnolle *et al.* 2001a, Cenac *et al.* 2002). PAR2-deficient mice developed significantly less pronounced inflammatory hyperalgesia in response to intraplantar injection of formalin or the mast cell degranulator compound 48/80 (Vergnolle *et al.* 2001a).

PAR2 agonist-mediated hyperalgesia was also dependent on a mechanism involving central activation of neurokinin-1 receptors, and release of prostaglandins (Vergnolle *et al.* 2001a).

Neuropathic pain often develops after peripheral and central nervous system injuries, local inflammation, diabetic neuropathy, viral infection, major surgeries, stroke and after chemotherapy application (Woolf and Mannion 1999, Ji and Strichartz 2004). In a chemotherapy (paclitaxel) induced model of neuropathic pain, increased mast cell tryptase activity on the periphery, DRG as well as in spinal cord was demonstrated in mice (Chen *et al.* 2011). Intrathecal administration of PAR2 antagonist prevented paclitaxel-induced mechanical allodynia and thermal hyperalgesia and attenuated cold hypersensitivity, while it was also selectively blocked by systemic administration of TRPV1, TRPV4 and TRPA1 receptors antagonists. All these TRP receptors were also shown to be sensitized after PAR2 activation, probably due to the activation of PKC and PKA pathways (Chen *et al.* 2011). It was shown that PAR2 activation may mediate increased cAMP and PKA activity and cAMP-dependent neuronal hyperexcitability in a model of prolonged compression of DRG. The application of PAR2 antagonist resulted in blocking activation of the cAMP-PKA pathway, which contributes to the hyperalgesia (Huang *et al.* 2012).

Proteases and their receptors may be also involved in cancer pain. Since carcinomas and associated inflammatory cells (mast cells) produce and secrete proteases during carcinogenesis. PAR2 activation was identified as a novel mechanism of cancer-dependent allodynia which was abolished by serine protease inhibition, diminished by mast cell depletion and it was absent in PAR2-deficient mice (Lam *et al.* 2012). Further the proportion of neurons that exhibited PAR2-immunoreactivity was also increased in this model (Lam *et al.* 2012). It was suggested that serine protease inhibitors and PAR2 antagonists may be useful for treatment of cancer pain (Lam and Schmidt 2010).

In the model of bone cancer pain, induction and persistence of pain behaviour correlated with tumor cell implantation and induced up-regulation of PAR2 in sciatic nerve and DRG. PAR2 knock-out or intrathecal administration of PAR2 antagonist prevented and in same case reversed the bone cancer-related pain behavior and related neurochemical changes in the DRG and spinal cord dorsal horn (Liu *et al.* 2013). PAR2 was suggested to be an important mediator for peripheral sensitization of bone cancer pain and inhibiting PAR2 activation as a new therapeutic target in bone cancer pain development and therapy (Liu *et al.* 2013).

Summary

Pain is a complex, subjective phenomenon that involves participation of numerous receptors in different cells, tissues and organs. Protease-activated receptors type 2 are localized in many tissues and significant expression of these receptors was demonstrated in different parts of the peripheral and central nervous system. Recent evidence clearly demonstrated that PAR2 play an important role in the process of pain development, modulation and transmission. Endogenous activators, proteases such as trypsin and tryptase are considered as the major signaling molecules, which can regulate nociceptive transmission by PAR2 activation. PAR2 signaling was shown to have a significant impact on the induction and maintenance of persistent pain, especially in inflammatory, neuropathic and cancer pain. All this available information about the role of PAR2 in nociception and pain suggests that PAR2 may be an attractive target for the development of new options for pain treatment.

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