**Proteolytic regulation of TRP channels: implications for pain and neurogenic inflammation**

*N.W. Bunnett, Monash Institute of Pharmacological Sciences, 318 Royal Parade, Parkville, Victoria 3052, Australia.*

Proteases are major regulators of neuronal activity. Injury and inflammation trigger the activation of multiple proteases that derive from the circulation and immune, neuronal and epithelial cells. Activated proteases regulate neurons by generating or degrading bioactive peptides and by cleaving ion channels and receptors at plasma membrane. Certain proteases activate nociceptive neurons by cleaving protease-activated receptors (PARs), notably PAR2. Activated PAR2 stimulates the release of neuropeptides in peripheral tissues to cause neurogenic inflammation. In the dorsal horn of the spinal cord, this results in pain transmission. PAR2 also sensitizes or activates TRPV1, TRPV4 and TRPA1 ion channels, which amplify proteases signalling. However, the identity and localization of proteases that are activated during pain states, and the mechanisms by which they regulate nociceptors are not fully understood.

Since proteases are regulated by post-translational control of activity rather than through altered expression, approaches are required to detect activated proteases. Activity-based probes, comprised of a warhead protease inhibitor group and a near-infrared tag for detection, covalently bind only to activated proteases. We administered probes for cysteine cathepsins and serine proteases to mice with inflammatory diseases and identified tissues and cells with activated proteases by using non-invasive fluorescence molecular tomography imaging and cellular confocal imaging. We identified proteases that are activated in macrophages and spinal microglial cells during injury, inflammation and pain states, including cathepsins B, L and S, and trypsin IV. Studies of mice lacking PARs and TRP channels indicate that these proteases cause neurogenic inflammation and pain that requires expression of PAR1, PAR2 and TRPV1, TRPV4 and TRPA1. We examined the signalling mechanisms by which proteases control activity of PARs and TRPs using model cell lines (HEK) and primary nociceptive neurons. Some proteases (*e.g.* trypsin, tryptase) cleave PAR2 at the canonical activation site to induce Gq-mediated calcium signalling, receptor interaction with β-arrestins, and β-arrestin-mediated receptor endocytosis and MAP kinase signalling. Other proteases (*e.g.* elastase, cathepsin S) cleave PAR2 at distinct sites and act as biased agonists that induce distinct mechanisms of receptor signalling and trafficking. However, both mechanisms of PAR2 activation can amplify responses to TRP channel agonists, indicative of divergent mechanisms of TRP sensitization, depending on the site of PAR2 hydrolysis. Activated PAR2 promotes influx of $Ca^{2+}$ ions in HEK cells and neurons that requires expression of TRPV4, suggesting that PAR2 couples to this channel. This mechanism depends in part on PAR2-induced generation of TRPV4 agonists such as 5′6′-EET, and also requires TRPV4 phosphorylation (Y110) by unidentified tyrosine kinases. Although proteases and PAR2 agonists sensitize and activate TRP channels, activated TRP channels can desensitize PAR2, representing a mechanism of bidirectional communication that terminates protease signalling. β-arrestins interact with agonist-occupied receptors at the cell-surface to mediate receptor desensitization and endocytosis. Analysis of PAR2/β-arrestin/TRP channel interactions by using bioluminescence resonance energy transfer (BRET) and co-immunoprecipitation approaches indicates that PAR2 or TRP channel activation triggers the assembly of a multiprotein complex comprising PAR2, β-arrestins and TRP channels. This complex promotes efficient PAR2 and TRP channel coupling, and also facilitate TRP channel-dependent desensitization of PAR2.

These results show that inflammation and injury trigger the activation of diverse proteases that regulate the activity of nociceptors by assembling multiprotein complexes of activated proteases, PARs, β-arrestins and TRP channels. These complexes may provide a matrix for the efficient bi-directional communication between PARs and TRP channels, by which activate PARs couple to TRPs, and activated TRPs desensitize PARs. Unravelling the complexity and functions of these multiprotein complexes may provide new insights into protease regulation of neuronal functions, with therapeutic implications.

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