Protease-activated receptor mediated calcium signaling and cytokine production in cultured c2c12 skeletal muscle cells

A.J. Bakker, K.A. Burlinson, N. Asokananthan, G.A. Stewart and G.J. Pinniger, School of Biomedical, Biomolecular & Chemical Sciences, University of Western Australia, Crawley, WA 6009, Australia.

Protease-activated receptors (PARs) are activated by proteases such as thrombin and tryptase, *via* proteolytic cleavage. In many cells, PAR activation results in a rise in intracellular Ca^{2+} through activation of G_q and phospholipase C, and store-mediated Ca^{2+} entry. This Ca^{2+} signal is thought to trigger the appropriate cellular response. PARs are thought to play a significant role in the inflammatory process, by triggering the release of cytokines from activated cells (Asokananthan *et al.*, 2002). Skeletal muscle myotubes express PAR receptors, and increased intracellular Ca^{2+} has recently been shown to increase the production of inflammatory cytokines such as IL-6 in muscle cells (Keller *et al.*, 2006). In this study, we examined the nature of PAR-mediated Ca^{2+} signaling in cultured skeletal muscle myotubes and determined whether PAR activation resulted in the release of the cytokine IL-6 from these cells.

C_2C_{12} myoblasts were grown in Dulbecco's modified Eagles medium with 20% foetal calf serum and equilibrated with 5% CO_2, 95% air at 37°C. Differentiation of myoblasts into myotubes was induced by lowering the serum concentration to 2%. PARs were activated with thrombin (activates PAR-1, 3 & 4 isoforms) and trysin (activates PAR-2 isoform. The intracellular Ca^{2+} concentration was measured using the fluorescent Ca^{2+} indicator fura-2. Measurements were undertaken using a Cairn Spectrophotometer. Experiments were undertaken at 22°C and IL-6 levels were detected by ELISA.

Exposure of the myotubes to thrombin resulted in a significant increase in intracellular Ca^{2+} (mean amplitude; 0.38 ± 0.03 μM, n = 13) that was larger in peak than electrically-induced Ca^{2+} transients elicited in similar C_2C_{12} myotubes (mean amplitude; 0.20 ± 0.07 μM, n = 11). Pre-exposure to thrombin (10 U/ml) for one hour, 12 hours before experimentation resulted in 93% of myotubes responding to thrombin with a Ca^{2+} response. Experiments with specific PAR-activating peptides indicated that thrombin was activating Ca^{2+} signalling *via* PAR-1. In myotubes exposed to thrombin under Ca^{2+} free conditions, only 17% (n = 6) of myotubes displayed Ca^{2+} influx upon return of extracellular Ca^{2+}. In addition, only 12% of cells (n = 17) responded with Ca^{2+} influx after the store was depleted with the sarcoplasmic reticulum Ca^{2+} pump inhibitor 2,5-di-(tert-butyl)-1,4-benzohydroquinone, suggesting that in most myotubes, store-operated Ca^{2+} entry was absent. Exposure of myotubes to thrombin (5 U/ml) for 24 hours increased IL-6 levels to 140% of control levels (p = 0.01). Pre-exposure to thrombin (10 U/ml) for one hour, followed by a 24 hour exposure to thrombin (5 U/ml) 12 hours later, increased IL-6 levels to 188% of control levels (p = 0.01).

These results suggest that the Ca^{2+} signal associated withevoked by PAR-1 activation is predominantly associated with intracellular Ca^{2+} release, and that thrombin exposure results in the release of the IL-6, which may play a role in the muscle response to injury and inflammation.

Asokananthan N., Graham PT, Fink J, Knight DA, Bakker AJ, McWilliam AS, Thompson PJ & Stewart GA. (2002) *Journal of Immunology*, **168:** 3577-85.

Keller C., Hellsten Y., Steensberg A. & Pedersen BK. (2006) *Cytokine*, **36:** 141-7.