The platelet cytoskeletal-membrane interface as a target for annexin V

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Platelets circulate as flat oval discs which, upon stimulation with a variety of agents including ADP, thrombin, collagen and glass, transform into compact spheres from which extend spikes (filopodia) and veils [1].

These changes in platelet morphology result from reorganization of the cytoskeleton. In addition to generating shape changes and tension, the cytoskeleton plays an important role in signalling events, stabilizes the plasma membrane and may have a role in regulating channels and pumps [2].

Many changes in the cytoskeletal organization of platelets require calcium; it is therefore likely that calcium-binding proteins play a role in the regulation of cytoskeletal function. One group of calcium-binding proteins which may fulfill such a regulatory role are the annexins. Annexins are a family of at least 13 proteins that contain repeats of a highly conserved seventy amino acid sequence and have been shown to copurify with cytoskeletal proteins, suggesting that they may play a role in cytoskeletal function [3].

It has previously been shown that, following platelet activation, annexin V relocates from the cytosol and binds to membranes in one of two ways: firstly, in a manner where it binds at high [Ca\(^{2+}\)] (i.e. 8.8\(\mu\)M) and can be extracted by the calcium chelator EGTA, and secondly in a manner where it binds at lower [Ca\(^{2+}\)] (i.e. 0.8\(\mu\)M) and requires the non-ionic detergent Triton X-100 for its solubilization [4]. Here we have used immunofluorescence microscopy to investigate the association of annexin V with platelet membranes and cytoskeleton at varying [Ca\(^{2+}\)].

Figure 1 demonstrates the calcium-dependency of the association of annexin V with the platelet cytoskeleton; it is apparent that 0.8\(\mu\)M Ca\(^{2+}\) does not induce annexin V to bind to the cytoskeleton (fig.1b), whereas 8.8\(\mu\)M Ca\(^{2+}\) causes relocation to the cytoskeleton.

It was previously reported that, following platelet activation, annexin V relocates from the cytosol and binds to membranes and that at 0.8\(\mu\)M Ca\(^{2+}\), annexin V reaches a maximal level in the membrane fraction [4]. Experiments were therefore performed to investigate which membranes annexin V binds to upon stimulation. When cytochalasin E, which blocks addition of actin monomers to actin filament barbed ends, was used to inhibit both the morphological alterations due to actin assembly in platelets spreading on glass, platelets did not spread, remaining discoid or spherical [6].

Figure 2 shows that annexin V is concentrated at the periphery of ionophore-treated cells, whereas in cells fixed in suspension without stimulation, annexin V is found homogeneously throughout the cell. This finding suggests that annexin V relocates and binds to the plasma membrane of the platelet upon stimulation, rather than to intracellular membranes.

In conclusion, annexin V can associate with the cytoskeleton only at [Ca\(^{2+}\)] which are observed in the cytosol of platelets following physiological activation. Thus, following platelet activation, annexin V relocates from the cytosol to the plasma membrane and to the cytoskeleton.

Annexin V therefore seems to play a role in coupling increases in calcium that follow activation to the regulation of platelet function at the membrane-cytoskeletal interface.

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**Fig. 1. Annexin V associates with the platelet cytoskeleton at 8.8\(\mu\)M Ca\(^{2+}\)**

*Immunostaining of glass-activated platelets with (a) rhodamine-phalloidin to label F-actin and (b) antiseraum against annexin V (x 4200).*

Washed platelets were allowed to spread on glass coverslips for 5min at 37° C. Platelets were fixed with 0.25% glutaraldehyde after permeabilisation with 0.5% Triton X-100 in a cytoskeleton stabilising buffer containing CaCl\(_2\)/EGTA buffers to give 0.8\(\mu\)M or 8.8\(\mu\)M free Ca\(^{2+}\). Primary antibody incubations were carried out overnight at a dilution of 1:100 in PBS containing 5% goat serum. FITC-labelled secondary antibodies were used for visualization.

**Fig. 2 Immunolocalization of annexin V in cytochalasin E - treated platelets**

Washed and cytochalasin E-treated platelets were fixed after incubation with 1mM Ca\(^{2+}\) (a) in the presence of the calcium ionophore A23187 (5\(\mu\)M ) or (b) in suspension without stimulation and stained for annexin V. (x 4200).