In order to maintain an adequate blood clotting response, the enzyme α-thrombin needs to be generated in a timely manner. The formation of α-thrombin is both a membrane-and $Ca^{++}$-dependent process in which prothrombin is proteolytically cleaved at $Arg^{271}$ and $Arg^{320}$ by the macromolecular prothrombinase complex, composed of the serine protease factor Xa and its non-enzymatic cofactor factor Va, bound in a 1:1 stoichiometric complex. Work from model systems using purified proteins and phospholipid vesicles of defined content suggests that initial cleavage occurs at $Arg^{320}$ to form the active intermediate meizothrombin and is followed by cleavage at $Arg^{271}$ to produce α-thrombin; however, substantial data indicate that the prothrombinase complex assembled on phospholipid vesicles does not mimic that assembled on the physiologically-relevant platelet surface. In this study, we assessed the activity of the prothrombinase complex assembled on platelets activated with well-known physiological activators or their mimetics. These included thrombin, collagen, or Par 1/4 peptides. Further, since physiologically, factor Va can be provided by two distinct compartments, a plasma and platelet-derived pool, experiments were performed to determine if they contributed to this process equivalently. Radioactively-labeled prothrombin was used to follow the activation process. Our results indicated that initial cleavage of prothrombin by the prothrombinase complex on the activated-platelet surface was at $Arg^{271}$ to form the inactive intermediate prothrombin-2. This was rapidly followed by a second cleavage at $Arg^{320}$ to form α-thrombin. Further, the order of cleavage was independent of the source of factor Va. These studies are the first to define the pathway of prothrombin cleavage on the activated platelet surface and suggest that

the membrane surface dictates the order of cleavage. To determine if the activation pathway is cell type-specific, future studies will investigate prothrombin activation on other cell membranes, including those of endothelial cells, monocytes, and megakaryocytes.