Cytotoxic necrotizing factor 1 (CNF-1) is an exotoxin of Escherichia coli that constitutively activates the GTPases Rho, Rac, and CDC42. Stimulation of Rho was shown to enhance myosin light-chain (MLC) phosphorylation via Rho kinase-mediated inhibition of MLC phosphatase in endothelial cells. Here we report that 3 h after CNF stimulation of endothelial cells, RhoA was activated and MLC phosphorylation was increased in a Rho/Rho-kinase-dependent manner, but no decrease in MLC phosphatase activity could be detected. Despite continuous RhoA activation, MLC phosphatase activity was doubled after 24 h of CNF stimulation, and this coincided with decreased MLC phosphorylation and cell spreading. Rac was also activated at 3 to 24 h but did not contribute to MLC phosphorylation, and its amount gradually decreased in the CNF-stimulated cells. CDC42Hs was not activated above control values by CNF. These results suggest that CNF can induce specific decoupling (Rho kinase from MLC phosphatase) and deactivation events in Rho GTPase signaling, potentially reflecting cellular protection mechanisms against permanently active Rho GTPases.