The cardiac sodium/calcium exchanger (NCX1) regulates intracellular Ca in cardiac muscle. Inappropriate NCX1 function contributes to cardiac contraction abnormalities and heart failure: reduced NCX1 activity reduces Ca removal therefore impairing relaxation. Overactive NCX1 unloads intracellular Ca stores and impairs systole. Although the structure-function relationship of NCX1 is well characterised, regulation of NCX1 function by post-translational modifications is controversial. We investigated palmitoylation of NCX1 from isolated adult rat ventricular myocytes (ARVM), using site-specific resin assisted capture (Acyl Rac). Acyl Rac purified 100% of the constitutively palmitoylated protein caveolin 3 from ARVM lysates, and 60% of NCX1, indicating a biologically meaningful fraction of NCX1 is palmitoylated in ventricular muscle. Mutagenesis of selected cysteines revealed that C739, which is located in the large intracellular domain, is the principal palmitoylation site in NCX1. Palmitoylation is required to anchor this domain to membranes, and occurs in the secretory pathway. We measured NCX1 cell surface localization using membrane impermeable biotinylation reagents. The palmitoyl acyl transferase inhibitor 2-bromopalmitate (2-BP) reduced NCX1 palmitoylation, with no effect on cell surface localisation. We investigated the effect of palmitoylation on NCX1 function in voltage clamped stably transfected BHK cells. Application of 2-BP decreased inward current density from 1.9±0.2 pA/pF to 1.1±0.1 pA/pF, but was without effect on outward current density. In conclusion, palmitoylation of NCX1 influences its transport function and occurs in the secretory pathway.