Na^+ H^+ exchanger regulatory factor 2 (NHERF-2) is a scaffold for the plasma membrane Ca^{2+} ATPase (PMCA)

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Resting cytosolic Ca^{2+} levels are maintained at nanomolar levels by the sequestration of Ca^{2+} into intracellular stores or the extrusion of Ca^{2+} across the plasma membrane by the PMCA. Despite the ubiquitous distribution of PMCA and its pivotal role in Ca^{2+} signalling, little is known about how PMCA activity is regulated during G protein coupled receptor signalling. There are 4 isoforms of PMCA (1-4) and many splice variants of all isoforms have been identified and all PMCA-b splice variants have a consensus class 1 PSD-95/Dlg/Zo-1 (PDZ) binding motif (Strehler *et al.*, 2001). Protein-protein interactions mediated by PDZ modules are now recognized as playing a key role in spatially constraining many ion channels and transporters into signalling complexes in membrane microdomains (Pawson *et al.*, 1997). Previously, PMCA 2b has been reported to interact with NHERF-2 in a heterologous expression system (DeMarco *et al.*, 2003). This study investigated whether PMCA interacts with NHERF-2 in a native epithelial cell and the physiological significance of this interaction in terms of G-protein mediated Ca^{2+} signalling via the muscarinic M3 receptor.

This study used the polarised epithelial HT29 cell line which expresses only the M3 isoform of the muscarinic receptor. RT-PCR and Western blotting were used to confirm the presence of both PMCA and NHERF-2 in these cells. Cell surface biotinylations were performed to investigate the changes in levels of PMCA at the plasma membrane following activation of M3 receptor by acetylcholine (ACh). NHERF-2 contains 3 binding domains, PDZ-1, PDZ-2 and the C-terminus. We used GST-fusions of these domains as well as full length NHERF-2 to characterise the interaction between NHERF-2 and PMCA. These interactions were validated *in vivo* using co-immunoprecipitation with a polyclonal NHERF-2 antibody and subsequent Western blotting with a pan-PMCA antibody. To examine the functional role of NHERF-2, endogenous protein was knocked down by transfecting siRNA plasmids. Changes in intracellular Ca^{2+} were measured using FURA-2 in a microplate assay.

RT-PCR and Western blots confirmed that HT29 cells expressed both NHERF-2 and PMCA isoform 1 and 4 (n = 3). Importantly, we found that the levels of PMCA at the plasma membrane increased by 62 ± 12% (n = 3) within 1 min of exposure to ACh and returned to control levels within 3 min. GST-pulldown experiments in HT29 cell lysates clearly showed that PMCA interacted with the second PDZ module of NHERF-2 (n=4). Co-immunoprecipitation experiments using HT29 cell lysates confirmed the interaction between NHERF-2 and PMCA occurred under *in vivo* conditions (n=3). Silencing of NHERF-2 reduced the levels of endogenous NHERF-2 by a 68 ± 10% (n = 3). When we examined the Ca^{2+} response to ACh in the cells where NHERF-2 had been silenced we observed that the rate of recovery from the peak Ca^{2+} transient was 50 ± 10% (n = 3; $P < 0.05$) faster than in control cells.

These data reveal for the first time that the increase in intracellular Ca^{2+} in response to M3 receptor activation is accompanied by a rapid increase in PMCA at the plasma membrane, presumably due to translocation from subplasmalemmal stores. The functional interaction between NHERF-2 and PMCA may underlie the changes in recovery rate of Ca^{2+} following exposure to ACh. Further studies will provide new insights into how scaffold proteins may confer specificity in terms of G-protein mediated Ca^{2+} signalling.

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