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Disulfide Linkage of Biotin Identifies a 106-kDa Ca^{2+} Release Channel in Sarcoplasmic Reticulum*

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Reactive disulfide reagents (RDSs) with a biotin moiety have been synthesized and found to cause Ca^{2+} release from sarcoplasmic reticulum (SR) vesicles. The RDSs oxidize SH sites on SR proteins via a thiol-disulfide exchange, with the formation of mixed disulfide bonds between SR proteins and biotin. Biotinylated RDSs identified a 106-kDa protein which was purified by biotin-avidin chromatography. Disulfide reducing agents, like dithiothreitol, reverse the effect of RDSs and thus promoted active re-uptake of Ca^{2+} and dissociated biotin from the labeled protein indicating that biotin was covalently linked to the 106-kDa protein via a disulfide bond. Several lines of evidence indicate that this protein is not Ca^{2+},Mg^{2+}-ATPase and is not a proteolytic fragment or a subunit of the 400-kDa Ca^{2+}-ryanodine receptor complex (RRC). Monoclonal antibodies against the ATPase did not cross-react with the 106-kDa protein, and polyclonal antibodies against the 106-kDa did not cross-react with either the ATPase or the 400-kDa RRC. RDSs did not label the 400-kDa RRC with biotin. Linear sucrose gradients used to purify the RRC show that the 106-kDa protein migrated throughout 5–20% linear sucrose gradients, including the high sucrose density protein fractions containing 400-kDa RRC. Protease inhibitors diisopropylfluorophosphate used to prevent proteolysis of 400-kDa proteins did not alter the migration of 106-kDa in sucrose gradients nor the patterns of biotin labeling of the 106-kDa protein. Incorporation of highly purified 106-kDa protein (free of RRC) in planar bilayers revealed cationic channels with large Na^+ (g_{Na^+} = 375 ± 15 pS) and Ca^{2+} ($g_{Ca^{2+}}$ = 107.7 ± 12 pS) conductances which were activated by micromolar $[Ca^{2+}]_{free}$ or millimolar [ATP] and blocked by micromolar ruthenium red or millimolar $[Mg^{2+}]$. Thus, the SR contains a sulfhydryl-activated 106-kDa Ca^{2+} channel with apparently similar characteristics to the 400-kDa "feet" proteins.

In the previous article, we have described a class of "reactive" disulfide (RDS)[1] compounds (*i.e.* dithiopyridines) that cause Ca^{2+} release from sarcoplasmic reticulum (SR) vesicles (1). The RDSs caused release by oxidizing critical sulfhydryl groups on SR proteins through a thiol-disulfide exchange reaction and the formation of mixed disulfides between the SR protein(s) and the RDS compounds. The oxidation reaction opened a Ca^{2+} channel pathway which was reversed by reducing the mixed disulfide bond with GSH or DTT, resulting in active Ca^{2+} re-uptake by SR Ca^{2+} pumps (1). Among the dithiopyridines that were tested, SPDP (*N*-succinimidyl 3-(2-pyridyldithio)propionate) is a heterobifunctional reagent for the thiolation and production of intermolecular conjugates. It provides an elegant method to covalently link proteins with an easily identifiable probe, facilitating isolation and purification of proteins involved in sulfhydryl-activated Ca^{2+} release.

In the present study, two methods were used to synthesize SPDP-biotin conjugates: PDP-biocytin and PDP-biotin hydrazide. Both were effective at low concentrations (10–20 μM) in causing SR Ca^{2+} release, with characteristics similar to those described for RDSs (1). The RDS-biotin conjugates labeled an SR protein which was identified by biotin-avidin peroxidase reaction. The biotinylated protein isolated and purified by biotin-avidin chromatography had an apparent molecular mass of 106,000 daltons, did not cross-react with monoclonal antibodies to the Ca^{2+},Mg^{2+}-ATPase, and comprised about 0.3% of total SR protein. Immunological evidence indicated that the 106-kDa is neither a fragment nor a subunit of the 400-kDa Ca^{2+} ryanodine receptor complex (RRC) (2–6). Incorporation of purified 106-kDa protein in lipid bilayers revealed a cationic channel with a large Na^+ conductance and exhibited three additional subconductance states. Like the RCC, the sulfhydryl-activated 106-kDa channel was activated by micromolar $[Ca^{2+}]$ or millimolar [ATP] and inhibited by micromolar ruthenium red or millimolar $[Mg^{2+}]_{free}$. Preliminary reports of these studies have been presented (7, 8).

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[1] The abbreviations used are: RDS, reactive disulfide; SR, sarcoplasmic reticulum; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MOPS, morpholinopropanesulfonic acid; PIPES, 1,4-piperazinediethanolsulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; AP III, antipyrylazo III; DIFP, diisopropylfluorophosphate; DTT, dithiothreitol; SPDP, *N*-succinimidyl 3-(2-pyridyl)dithiopropionate; RRC, ryanodine receptor complex; PDP, 2-pyridyldithiolpropionate; PAGE, polyacrylamide gel electrophoresis; IAM, iodoacetamide.