PLASMA MEMBRANE CA^{2+}-ATPASE SULFHYDRYL MODIFICATIONS: IMPLICATION FOR OXIDIZED RED CELL

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Abstract. A common perturbation found in cells under oxidative stress is alteration in cellular Ca^{2+} homeostasis. In order to understand the effects of such oxidative damage, human red cell plasma membrane Ca^{2+}-ATPase (PMCA) was studied by measuring PMCA activity, both in the presence and absence of calmodulin (CaM), following treatment with sulfhydryl agents, N-ethylmaleimide, iodoacetate and diamide. PMCA activity of washed red cell membrane was measured by coupling with pyruvate kinase, using phosphoenolpyruvate as substrate, and lactate dehydrogenase to convert pyruvate to lactate employing β-NADH as co-factor. All treatments inhibited basal and CaM-stimulated activity in a dose-dependent manner (0.01-1 mM), but at low concentrations, basal Ca^{2+}-ATPase activity was inhibited whereas CaM-stimulated activity was unaffected. Inhibition by diamide, a disulfide-forming agent, was reversed with dithiotreitol (DTT). Treatment with calpain, a calcium-dependent protease, elevated basal PMCA activity to CaM-stimulated level, but abolished response to CaM. Further treatment with diamide inhibited PMCA activity, which could be restored by DTT, but only to basal and not CaM-stimulated level. These studies indicated that it is necessary to protect against both sulfhydryl and proteolytic damages to red cell PMCA if perturbation to Ca^{2+} homeostasis is to be minimized. This has implications for membranes under oxidative stress, such as in the hereditary anemia, thalassemia, where membrane-bound unmatched hemoglobin chains cause oxidative damage to red blood cells.

Keywords: Ca^{2+}-ATPase, calmodulin, PMCA, sulfhydryl agent, thalassemia

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INTRODUCTION

Normal physiological and pathological events in cells are accompanied by intracellular ionic changes, in particular that of calcium ions (Ca^{2+}), and thus, regulation of the intracellular concentration of this cation is of importance. Calcium

homeostasis is maintained through a balance between cell membrane permeability and energy-dependent transport of calcium ions by Ca^{2+}-ATPase located at the plasma and sarcoplasmic/endoplasmic reticulum membranes (as well as by other Ca^{2+}-transporters). Plasma membrane Ca^{2+}-ATPase (PMCA) pump is stimulated by calmodulin (CaM), a ubiquitous intracellular Ca^{2+} sensor, in the presence of free calcium (Leva *et al*, 2008). CaM binds to a domain near the C-terminus of PMCA, relieving its auto inhibitory function.

Generation of reactive oxygen and reactive nitrogen species is a normal process in oxidative cellular metabolism. However, levels of these oxidants can be significantly elevated in a variety of pathophysiological disorders, such as atherosclerosis, post-ischemic tissue injury, Alzheimer's disease, Parkinson's disease and thalassemia (Halliwell and Gutteridge, 1989). Thalassemia, a hereditary anemia caused by mutations in the globin gene cluster producing an imbalance in globin synthesis, is commonly found in regions where there is or has been malaria transmission, including Thailand (Fucharoen and Winichagoon, 1992). It can be divided into two major classes: α-thalassemia due to deletions of the α-globin genes and β-thalassemia caused by point mutations in the β-globin gene. The marked severity of anemia in β-thalassemia subjects compared to those with α-thalassemia is due to the presence of unmatched α-hemoglobin on the cytoplasmic surface of membranes, causing oxidative damage to both lipid and protein components and leading to ineffective erythropoiesis and reduced life span of circulating red blood cells (Schrier, 2002). A common perturbation found in cells under oxidative stress is the significant alteration in cellular Ca^{2+} homeostasis (Berridge *et al*, 2000; Sastry and Rao, 2000; Bootman *et al*, 2002). Elevated levels of Ca^{2+} have been reported also in β-thalassemic red blood cells (Shalev *et al*, 1984; Suthipark *et al*, 1991).

Previous study of oxidative damage to PMCA has been limited to that of hydrogen peroxide (H_2O_2) (Zaidi *et al*, 2003). In this study, so as to provide an *in vitro* model of oxidized red blood cell membrane, a number of sulfhydryl modifying agents (diamide, iodoacetate and N-ethylmaleimide (NEM)) were used to treat CaM-depleted normal human red cell membrane preparation and PMCA activity was measured in the presence and absence of CaM.

MATERIALS AND METHODS

Chemicals and reagents

CaM (bovine brain), calpain (rabbit muscle, 25 U/ml), lactate dehydrogenase (1,000 U/ml), leupeptin, β-nicotinamide adenine dinucleotide reduced (β-NADH), phenylmethylsulfonyl fluoride (PMSF), phosphoenolpyruvate, and pyruvate kinase (700 U/ml) were purchased from Sigma Chemical (St Louis, MO). All other reagents were of analytical grade.

Preparation of calmodulin-depleted red blood cell membrane

Washed human red blood cells were lysed with 10 volumes of hypotonic buffer (10 mM Tris-HCl, pH 7.4, containing 1 mM ethylene diamine tetraacetic acid). CaM-depleted ghost membrane was obtained by washing 3 times with vigorous shaking in hypotonic buffer containing 0.1 mM PMSF, and kept at -80°C in storage buffer consisting of 10 mM HEPES, pH 7.4, containing 130 mM NaCl, 0.5 mM $MgCl_2$, and 0.05 mM $CaCl_2$ until assay. Membrane was considered CaM-depleted when basal

Ca^{2+}-ATPase activity could be stimulated 2.5 folds upon addition of CaM.

Assay of human red blood cell membrane Ca^{2+}-ATPase activity

Assay utilized coupling of Ca^{2+}-ATPase activity with pyruvate kinase, using phosphoenolpyruvate as substrate, and lactate dehydrogenase to convert pyruvate to lactate employing β-NADH as co-factor as previously described (Sarkadi *et al*, 1986). Reaction was followed spectrophotometrically at 340 nm at 37°C (Shimadzu UV-2501PC spectrophotometer). CaM-stimulated activity was measured in the presence of 0.1 mM CaM. Protein concentration was determined by Bradford method, with bovine serum albumin as standard (Bradford, 1976).

Treatment of human red blood cell membrane preparation

CaM-depleted human red blood cell membrane preparations were treated with oxidants [diamide, iodoacetate, or N-ethylmaleimide (NEM)] at the designated concentrations for 30 minutes at 25°C and then immediately washed with storage buffer before being assayed. Treatment with calpain (0.04 U/ml) was conducted for 15 minutes at 25°C in the presence of 5 mM $CaCl_2$, and the reaction was terminated with addition of 15 mM leupeptin.

Statistical analysis

At least 3 independent experiments were performed, each conducted in triplicate. Results are presented as mean ± S.E.M. Statistical difference between control and treated sample was determined by one-way ANOVA, with $p < 0.05$ considered significant.

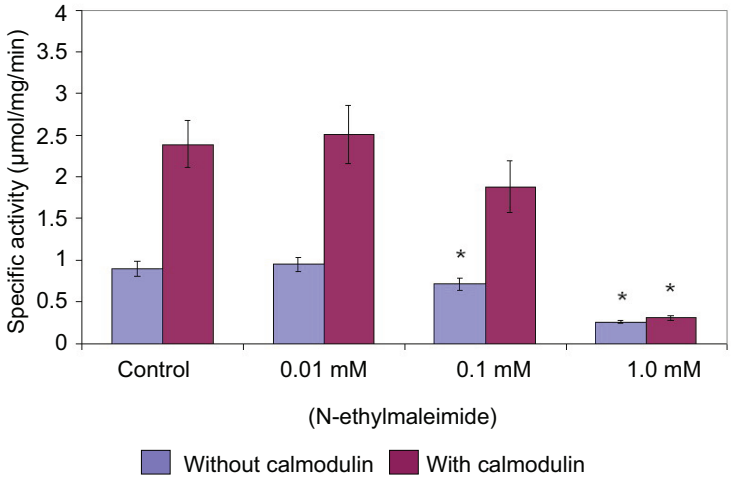

Fig 1–Inhibition of PMCA activity by N-ethyl-maleimide. Ca^{2+}-ATPase activity of calmodulin-depleted human red blood cell membrane was measured spectrophotometrically at 340 nm at 37°C using an enzyme coupled (pyruvate kinase/lactate dehydrogenase) system, with and without addition of 0.1mM calmodulin (bovine brain). *, $p < 0.05$ relative to control.

RESULTS

Treatment of human red blood cell membrane with the sulfhydryl reagent NEM (0.01-1 mM) resulted in a dose-dependent loss of both basal and CaM-stimulated Ca^{2+}-ATPase activity (Fig 1). However, at low NEM concentrations, basal Ca^{2+}-ATPase activity was inhibited whereas CaM-stimulated activity remained unchanged from control. Similar phenomena were observed with iodoacetate (0.1-500 mM) and the sulfhydryl crosslinking agent diamide (0.01-0.5 mM) (data not shown).

When human red blood cell membrane was treated with diamide (up to 0.5 mM) but not with NEM or iodoacetate, inhibition of basal and CaM-stimulated

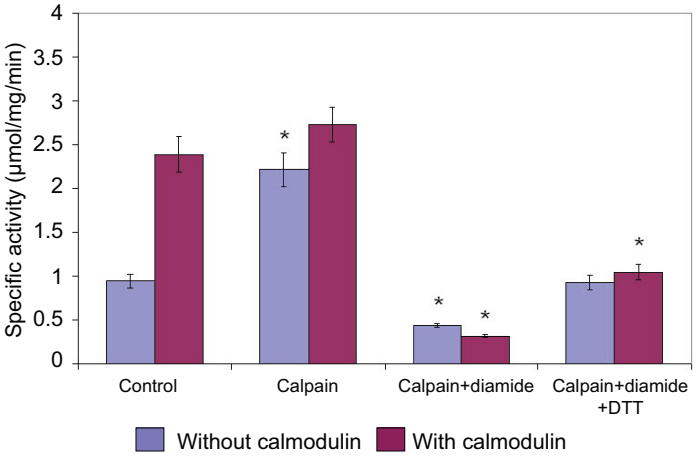

Fig 2-Effect of combination of calpain, diamide and DTT on PMCA activity. Ca^{2+}-ATPase activity of calmodulin-depleted human red blood cell membrane was measured as described in legend to Fig 1. Membrane was sequentially treated with calpain (rabbit muscle) (0.04 U/ml), diamide (0.5 mM) and DTT (5 mM). *, $p < 0.05$ relative to control.

activity was able to be reversed completely to their original value (from 0.53 ± 0.06 to 0.85 ± 0.09 µmol ATP hydrolyzed/mg/min and 0.71 ± 0.08 to 2.4 ± 0.3 pmol/ATP hydrolyzed/mg/min, respectively) by incubating with 5 mM DTT for 15 minutes at 30°C.

Elevated intracellular Ca^{2+} level can activate calpain, a Ca^{2+}-dependent cysteine protease ubiquitously present in most eukaryotic cells (Saez *et al*, 2006). Two isozymes exist, namely, µ-calpain, stimulated by micromolar Ca^{2+}, and m-calpain, activated by millimolar Ca^{2+}. Digestion with rabbit m-calpain increased basal Ca^{2+}-ATPase activity to that of CaM-stimulated level, but this activity could not be stimulated further by addition of CaM (Fig 2). This has been previously explained by a cleavage from PMCA of a CaM-binding C-terminal domain, thereby exposing the enzyme ATP-binding site (Wang *et al*, 1988). However, when calpain-digested red cell membrane preparation was further treated with diamide (0.5 mM), this resulted in reduction of PMCA activity, which could be restored with DTT (5 mM), but only to the basal level and could not be stimulated further by CaM (Fig 2). Moreover, treatment of red blood cell membrane with diamide, followed by calpain and then DTT, produced a similar result, namely, PMCA with basal activity that no longer responded to CaM-stimulation (data not shown).

DISCUSSION

Zaidi *et al* (2003) have shown that oxidation by H_2O_2 (25-100 µM) of PMCA purified from human erythrocyte membrane results in inhibition of basal and CaM-stimulated Ca^{2+}-ATPase activity, and leads to formation of aggregates, which is partially reversible by DTT but not to full recovery of activity. The authors suggested that disulfide bond formation produces conformational changes that are not fully reversed when such bonds are reduced. However, the ability of DTT in our studies to fully reverse the inhibitory effect of the disulfide forming agent, diamide, suggests that the irreversible inhibition of PMCA activity by H_2O_2 previously observed is not related to disulfide bond formation, but probably is due to other kinds of covalent modification of critical sulfhydryl (and other) group(s), as demonstrated by treatment with NEM and iodoacetate in this study.

Thalassemic red blood cells, particularly those in β-thalassemia, are under oxidative stress (Schrier, 2002) and show

elevation of intracellular Ca^{2+} level (Shalev *et al*, 1984; Suthipark *et al*, 1991) although this has been questioned (Bookchin *et al*, 1988). Surprisingly, no reduction in PMCA activity of β-thalassemic red cells was reported (Shalev *et al*, 1984). Results of the present studies indicated that if oxidative damage to red blood cell membrane sulfhydryls could be prevented (by appropriate antioxidant measures), then normal PMCA activity can be maintained, minimizing perturbation to calcium homeostasis, and possibly leading to an amelioration of anemia in thalassemia patients. Once intracellular calcium is elevated, activation of calpain(s) may lead to cleavage of CaM-binding domain from PMCA, which should lead to a beneficial elevation of Ca^{2+}-ATPase activity, accounting for the absence of the decrease in PMCA activity reported in thalassemic red cells (Shalev *et al*, 1984); but as shown in this study, if there is concomitant oxidation of the truncated PMCA, this converts it to a non-physiological form, *ie* one with basal Ca^{2+}-ATPase activity but unable to respond to changes in intracellular Ca^{2+} concentration (CaM), even if oxidative damage (formation of disulfide bond) is corrected. This is reminiscent of the H_2O_2-treatment (Zaidi *et al*, 2003), implying that (diamide-) induced disulfide formation of the truncated PMCA locks the enzyme in a stable conformation that is not reversed upon reduction of disulfide bond(s), and this conformation allows limited access to ATP (possibly forming the compact auto-inhibited form recently observed by Mangialavori *et al*, 2009), and as expected, without the CaM-binding domain, it can no longer be activated by CaM.

Although previous attempts with supplementation of high dosage of the anti-oxidant vitamin E failed to improve anemia of thalassemic patients (Tesoriere *et al*, 2001; Dissayabutra *et al*, 2005), future studies with a cocktail of oxidants that also affords protection to membrane proteins hold better promise (Rund and Rachmilewitz, 2005).

In conclusion this study has shown that, upon proteolytic cleavage by a Ca^{2+}-dependent protease (calpain), although PMCA activity is elevated due to loss of CaM inhibition, oxidation (via disulfide formation) of the truncated PMCA form results in irreversible inhibition of activity. These findings are pertinent to anti-oxidant therapies of diseases due to oxidative stress.

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REFERENCES

Berridge M J, Lipp P, Bootman MD. The versatility and universality of calcium signaling. *Nature Rev Mol Cell Biol* 2000; 1: 11-21.

Bookchin RM, Ortiz OE, Shalev O, *et al*. Calcium transport and ultrastructure of red cells in β-thalassemia intermedia. *Blood* 1988; 72: 1602-7.

Bootman MD, Berridge MJ, Roderick HL. Calcium signaling: more messengers, more channels, more complexity. *Curr Biol* 2002; 12: R563-5.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-54.

Dissayabutra T, Tosukhowong P, Seksan P. The benefits of vitamin C and vitamin E in children with beta-thalassemia with high oxidative stress. *J Med Assoc Thai* 2005; 88: S317-21.

Fucharoen S, Winichagoon P. Thalassemia in Southeast Asia: problems and strategy for prevention and control. *Southeast Asian J Trop Med Public Health* 1992; 23: 647-55.

Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3rd ed. Oxford: Oxford University Press, 1989.

Leva FD, Domi T, Fedrizzi L, Lim D, Carafoli E. The plasma membrane Ca^{2+} -ATPase of animal cells: structure, function and regulation. *Arch Biochem Biophys* 2008; 476: 65-74.

Mangialavori I, Giraldo AMV, Buslje CM, Gomes MF, Caride AJ, Rossi JPFC. A new conformation in sarcoplasmic reticulum calcium pump and plasma membrane Ca^{2+} pumps revealed by a photoactivatable phospholipidic probe. *J Biol Chem* 2009; 284: 4823-8.

Rund D, Rachmilewitz E. β-Thalassemia. *N Eng J Med* 2005; 353: 1135-46.

Saez ME, Ramirez-Lorca R, Moron FJ, Ruiz A. The therapeutic potential of the calpain family: new aspects. *Drug Discov Today* 2006; 11: 917-23.

Sarkadi B, Enyedi A, Foldes-Papp Z, Gardos G. Molecular characterization of the in situ red cell membrane calcium pump by limited proteolysis. *J Biol Chem* 1986; 261: 9552-7.

Sastry PS, Rao KS. Apoptosis and the nervous system. *J Neurochem* 2000; 74: 1-20.

Schrier SL. Pathophysiology of thalassemia. *Curr Opin Hematol* 2002; 9: 123-6.

Shalev O, Mogilner S, Shinar E, Rachmilewitz EA, Schrier SL. Impaired erythrocyte calcium homeostasis in β-thalassemia. *Blood* 1984; 64: 564-6.

Suthipark KU, Likidlilid A, Fucharoen S, *et al*. Red cell and plasma calcium, copper and zinc in beta-thalassemia/hemoglobin E. *Southeast Asian J Trop Med Public Health* 1991; 22: 171-5.

Tesoriere L, D'Arpa D, Butera D, *et al*. Oral supplements of vitamin E improve measures of oxidative stress in plasma and reduce oxidative damage to LDL and erythrocytes in beta-thalassemia intermedia patients. *Free Radical Res* 2001; 34: 529-40.

Wang KK, Roufogalis BD, Villalobo A. Further characterization of calpain-mediated proteolysis of the human erythrocyte plasma membrane Ca^{2+}-ATPase. *Arch Biochem Biophys* 1988; 267: 317-27.

Zaidi A, Barron L, Sharov VS, Schoneich C, Michaelis EK, Michaelis ML. Oxidative inactivation of purified plasma membrane Ca^{2+}-ATPase by hydrogen peroxide and protection by calmodulin. *Biochemistry* 2003; 42: 12001-10.