Brief communication

Evidence for functional interaction of plasma membrane electron transport, voltage-dependent anion channel and volume-regulated anion channel in frog aorta

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Frog aortic tissue exhibits plasma membrane electron transport (PMET) owing to its ability to reduce ferricyanide even in the presence of mitochondrial poisons, such as cyanide and azide. Exposure to hypotonic solution (108 mOsmol/kg H_2O) enhanced the reduction of ferricyanide in excised aortic tissue of frog. Increment in ferricyanide reductase activity was also brought about by the presence of homocysteine (100 μM dissolved in isotonic frog Ringer solution), a redox active compound and a potent modulator of PMET. Two plasma-membrane-bound channels, the volume-regulated anion channel (VRAC) and the voltage-dependent anion channel (VDAC), are involved in the response to hypotonic stress. The presence of VRAC and VDAC antagonists–tamoxifen, glibenclamide, fluoxetine and verapamil, and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), respectively–inhibited this enhanced activity brought about by either hypotonic stress or homocysteine. The blockers do not affect the ferricyanide reductase activity under isotonic conditions. Taken together, these findings indicate a functional interaction of the three plasma membrane proteins, namely, ferricyanide reductase (PMET), VDAC and VRAC.

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1. Introduction

On exposure to a hypotonic environment, cells swell rapidly because of a redistribution of osmolytes and water (Hoffmann *et al*. 2009). The cell volume is challenged thus during a variety of pathophysiological conditions. Some examples of these are hypoxia/ischaemia, hyponatremia, (when hormonal and renal functions are impaired), and hypothermia (inhibits the Na+, K+-ATPase, and increases extracellular K+ concentration) and intracellular acidosis/diabetic ketoacidosis (Hoffmann *et al*. 2009). It is well known that the plasma-membrane-bound, voltage-dependent anion channel (pl-VDAC) and the volume-regulated anion channel (VRAC) are involved in the regulation of intracellular osmolality in the face of hypotonic challenge (Elinder *et al*. 2005; Harvey *et al*. 2009). Pl-VDAC has been shown to contain ferricyanide reductase activity and to take part in plasma membrane electron transport (PMET) (Baker *et al*. 2004; Elinder *et al*. 2005). Thus, these reports on various cell and tissue types indicate that hypotonic stress induces activity in each of these plasma-membrane-bound proteins, i.e. ferricyanide reductase (PMET), VDAC and VRAC. The present investigation was undertaken to examine if a hypotonic challenge could affect the activity of these three plasma-membrane-bound proteins and to reveal the functional interaction between them. In addition, the effect of homocysteine, a redox active compound that is a potent stimulator of the endothelial plasma membrane redox system

Keywords. Ferricyanide reductase; homocysteine; hypotonic stress; *Rana tigrina*

Abbreviations used: DIDS, 4,4′-diisothiocyanatostilbene 2,2′-disulfonic acid; PMET, plasma membrane electron transport; RVD, regulatory volume decrease; VDAC, voltage-dependent anion channel; VRAC, volume-regulated anion channel.

in bovine aortic endothelial cells (BAEC) (Rodríguez-Alonso *et al*. 2008), was also studied in this system. The study was carried out on excised frog aortic tissue, which is known to exhibit PMET because of its ability to reduce ferricyanide even in the presence of mitochondrial poisons such as cyanide and azide (Rao *et al*. 2009).

2. Materials and methods

2.1 *Chemicals*

4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), fluoxetine, glibenclamide, *p*-chloromercuribenzoyl sulphonate (*p*CMBS), 1,10-phenanthroline, tamoxifen, quinacrine and verapamil were purchased from Sigma-Aldrich (Bengaluru, India). Doxorubicin and cisplatin were a gift from Dr Narendra Bhat, Shridi Sai Baba Cancer Research Hospital, Manipal, India. All other chemicals used were of analytical grade and purchased from Sigma-Aldrich (Bengaluru, India).

2.2 *Isolation of frog aortic tissue*

Sixty six frogs (*Rana tigrina*) obtained from a local supplier were used in this study. They were double-pithed as per the guidelines of the institutional animal ethics committee (Kasturba Medical College, Manipal, India). An expert zoologist performed the species identification. The ascending excised aortic tissue of frog was identified (Parker and Haswell 1960), and the proximal excised aortic tissue, measuring 3–5 mm, was secured, isolated, washed and kept in frog Ringer solution containing NaCl (117 mM), KCl (3 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), Na_2HPO_4 (0.8 mM) and NaH_2PO_4 (0.2 mM) at pH 7.3.

2.3 *Assay of ferricyanide reduction*

Before the isolated frog aortic tissues were tested for their ability to reduce ferricyanide to ferrocyanide, they were washed with frog Ringer solution (117 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.8 mM Na_2HPO_4 and 0.2 mM NaH_2PO_4 at pH 7.3). The reaction was started by the addition of 5.6 ml of 1 mM potassium ferricyanide in Ringer solution to each tissue sample in a test tube and incubated at room temperature (28°C). On completion of microfuging the tissue samples in cold at 3000 rpm for 5 min, the amount of ferrocyanide generated in the supernatant was measured using the *ortho*-phenanthroline method of Avron and Shavit (1963). At the indicated times (0, 3, 6, 9, 12, 15, 18 and 21 min), 0.1 ml supernatant was removed and mixed with 0.15 ml (v/v/v = 2:2:1) of a pre-mix containing 3 M sodium acetate (pH 6.5), 0.2 M citric acid and 3.3 mM ferric chloride (in 0.1 M acetic acid). Finally, 0.05 ml of 1% (w/v in ethanol) 1,10-phenanthroline was added and the samples incubated at room temperature for 5 min. The absorbance was measured in a final volume of 1 ml in 1-cm-pathlength quartz cuvettes at 510 nm in a spectrophotometer (Genesys UV10). A predetermined absorption coefficient of 21.6/mM/cm was used to calculate the ferrocyanide formed.

In order to monitor the background scattering due to slow tissue degradation or secretion of light-absorbing materials during the time course of incubation, which was observed in mouse duodenal tissue (Pountney *et al*. 1996), absorbance changes over a series of wavelengths ranging from 400 nm to 600 nm were studied. In our study, background absorbance noted in the absence of ferricyanide was not found to change over time in the presence of tissue. Hence, this was not taken into consideration during later calculations. Supernatants from incubation mixtures containing Ringer solution without ferricyanide showed only negligible activity. Supernatants from the frog Ringer solution (not containing ferricyanide) in which the tissues had been incubated were not able to reduce ferricyanide. This indicates that there was no leakage, secretion or disintegration of the tissue as found in the study of Pountney *et al*. (1996). Corrections were made as required in a paired sample for the absorbance generated by the frog Ringer solution of the same concentration with ferricyanide in the absence of tissue. Reduction of ferricyanide in the paired control (frog Ringer solution without tissue) was found to be less than 0.5% when compared with the test sample (frog Ringer solution with tissue), and the appropriate correction was made. Ferricyanide reductase activity was calculated as μmol ferrocyanide generated per min per mg tissue protein.

2.4 *Influence of modulators*

Once the control values of reduction of ferricyanide for 10 min by the excised aortic tissues of frog were obtained as described above, the samples were incubated in isotonic solution (216 mOsmol/kg H_2O) containing quinacrine (1 mM) or doxorubicin (10 μM) or *p*-chloromercuribenzoyl sulphonate (*p*CMBS, 1 mM) for 5 min. Ferricyanide (1 mM) was then added and its reduction was determined after 10 min as described above. The effects of VRAC and VDAC blockers in the presence of isotonic or hypotonic frog Ringer solution were evaluated. Blockers were dissolved in 700 μl isotonic (216 mOsmol/kg H_2O) or hypotonic (108 mOsmol/kg H_2O) frog Ringer solution containing 1 mM ferricyanide, and the incubation was carried out for an additional period of 20 min. The ferrocyanide generated was assayed as described earlier. Tamoxifen (10 μM), verapamil (10 μM), fluoxetine (1 μM), glibenclamide (100 μM) and DIDS (100 μM) were added to the isotonic or hypotonic buffer from stock solutions in dimethyl sulphoxide (DMSO; final

concentration <1% v/v). The effect of homocysteine (10 μM, 50 μM, 100 μM) on the reductase activity was investigated in a similar manner. Homocysteine was dissolved in 700 μl isotonic (216 mOsmol/kg H_2O) frog Ringer solution.

The effects of VRAC and VDAC blockers on the ferricyanide reductase activity in the presence of 100 μM homocysteine were evaluated.

2.5 *Protein estimation*

Upon completion of the assay, the tissue fragments were gently blotted, rinsed twice in frog Ringer solution and homogenized in 1 ml of a solution containing 0.1% Triton-X 100, 2.7 mM ethylenediaminetetraacetic acid (EDTA) (neutralized) and 5.0% v/v β-mercaptoethanol prepared in frog Ringer buffer (pH 7.3). The concentration of protein was determined by the method of Bradford with bovine serum albumin (BSA) as the standard (Bradford 1976). The protein reagent comprised 0.01% (w/v) Coomassie brilliant blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid. To 0.1 ml tissue protein homogenate, 5 ml protein reagent was added and mixed. The absorbance at 595 nm was measured after 2 min in 1 ml cuvettes against a reagent blank prepared from 0.1 ml of the frog Ringer buffer (pH 7.3) and 5 ml of protein reagent in a spectrophotometer (Genesys UV10).

2.6 *Statistical analysis*

All values are expressed as mean ± SD of measurements made on tissues taken from a minimum of three frogs. Statistical significance was determined using Student's *t*-test. $P < 0.05$ was taken as significant.

3. Results

The concentration of ferrocyanide after 20 min of incubation of frog aortic tissue in isotonic solution was 0.023 ± 0.007 μmol/mg tissue protein in the supernatant; this increase was linear and time bound (figure 1). Exposure to hypotonic Ringer solution and solutions containing homocysteine (100 μM) enhanced the generation of ferrocyanide significantly, i.e. from 0.023 ± 0.007 μmol/mg tissue protein to 0.040 ± 0.008 μmol/mg tissue protein (P<0.01) and 0.049 ± 0.008 μmol/mg tissue protein (P<0.001), respectively, as shown in figure 1. Figure 2 shows restoration of enzyme activity under hypotonic stress to basal level in the presence of VDAC and VRAC inhibitors.

Ferrocyanide concentration generated in the supernatants after 20 min of incubation in 10 μM and 50 μM homocysteine solutions was 0.022 ± 0.01 and 0.024 ± 0.001 μmol/mg tissue protein, respectively. Figure 3 shows that

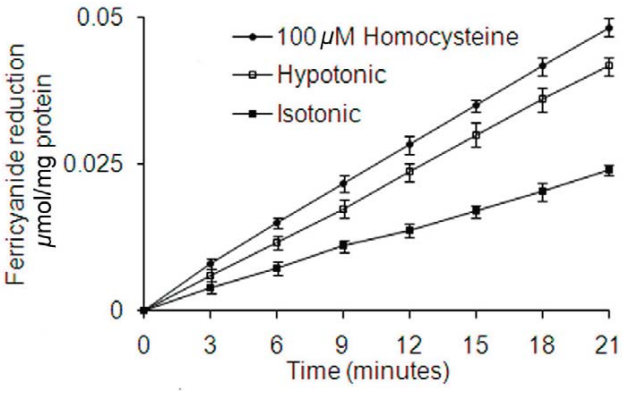

Figure 1. Time course of frog aortic tissue ferricyanide reductase activity. Closed squares indicate the reduction of ferricyanide in isotonic condition (216 mOsmol/kg H_2O). Open squares indicate the reduction in hypotonic condition (108 mOsmol/kg H_2O). Closed circles represent the reduction in the presence of homocysteine (100 μM), dissolved in isotonic solution (216 mOsmol/kg H_2O). Ferricyanide reductase activity was calculated as μmol ferrocyanide generated/mg tissue protein. Data represent mean ± SD of determinations from three individual frog aortic tissues. All the values except the initial 3 min value, obtained under isotonic conditions are significantly less than those obtained under hypotonic condition or in the presence of homocysteine. $P \leq 0.01$ is highly significant as observed for the ferrocyanide generated after 9 min interval in the presence of hypotonic solution (108 mOsmol/kg H_2O). $P \leq 0.001$ is very highly significant as observed for the ferrocyanide generated after 6 min interval in the presence of 100 μM homocysteine.

the effect of homocysteine (100 μM) dissolved in isotonic Ringer solution on frog aortic tissue reductase activity was significantly reduced in the presence of VDAC and VRAC inhibitors. The various established inhibitors that were used for PMET (quinacrine, 1 mM; doxorubicin, 10 μM; and *p*CMBS, 1 mM), VDAC (DIDS; 100 μM) and VRAC (tamoxifen, 10 μM; verapamil, 10 μM; fluoxetine, 1 μM; and glibenclamide, 100 μM) were observed to exert only minimal inhibition by themselves on the reductase activity under isotonic conditions (216 mOsmol/kg H_2O) (table 1).

4. Discussion

4.1 *Effect of PMET inhibitors on frog aortic tissue*

Using the indirect method of determining the reduced form of ferricyanide (Avron and Shavit 1963), which is sensitive enough to be used successfully on frog aortic tissue (Rao *et al*. 2009), the effect of a few selected inhibitors of plasma redox system was studied on the reduction of ferricyanide in this tissue (table 1).

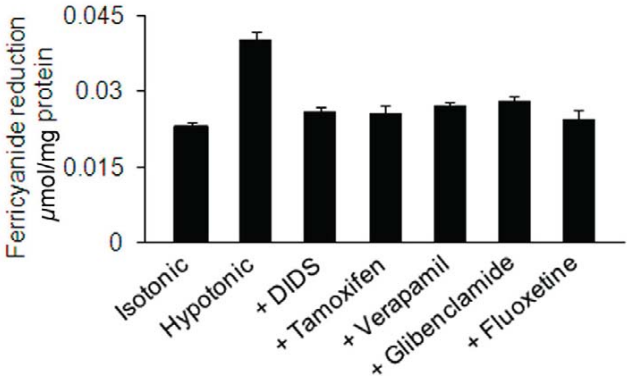

Figure 2. Effect of VDAC and VRAC inhibitors on hypotonic-stress-induced (108 mOsmol/kg H_2O) frog aortic tissue ferricyanide reductase activity. Incubations were carried out as described in Materials and methods. Ferricyanide reductase activity was calculated as μmol ferrocyanide generated/mg tissue protein. Data represent mean ± SD of determinations from three individual frog aortic tissues. Ferricyanide reduction rate observed in hypotonic frog Ringer solution (108 mOsmol/kg H_2O) is greater than the activity exhibited by tissue treated with isotonic frog Ringer solution (216 mOsmol/kg H_2O) (highly significant: $P \leq 0.01$). Ferrocyanide generation enhanced in the presence of hypotonic frog Ringer solution (108 mOsmol/kg H_2O) was brought back to the basal levels when VDAC inhibitor (DIDS; 100 μM) and VRAC inhibitors (tamoxifen, 10 μM; verapamil, 10 μM; glibenclamide, 100 μM; and fluoxetine, 1 μM) were incorporated in the incubation medium in a significant manner ($P \leq 0.01$). The "+" sign indicates the addition of the anion channel inhibitors to the hypotonic frog Ringer solution (108 mOsmol/kg H_2O).

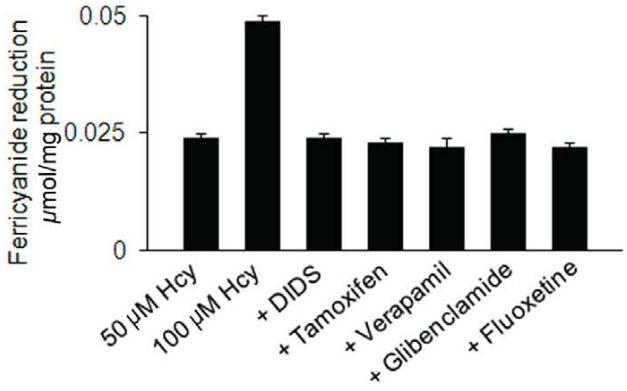

Figure 3. Effect of VDAC and VRAC inhibitors on homocysteine-induced frog aortic tissue ferricyanide reductase activity. Incubations were carried out as described in Materials and methods. Ferricyanide reductase activity was calculated as μmol ferrocyanide generated/mg tissue protein. Data represent mean ± SD of determinations from three individual frog aortic tissues. The 50 μM and 100 μM homocysteine used in this experiment were dissolved in isotonic frog Ringer solution (216 mOsmol/kg H_2O). Ferricyanide reductase activity at 100 μM homocysteine is greater than the activity exhibited by tissue treated with 50 μM homocysteine (very highly significant: $P \leq 0.001$). Ferrocyanide generation enhanced by the presence of 100 μM homocysteine was brought back to the basal levels when VDAC inhibitor (DIDS; 100 μM) and VRAC inhibitors (tamoxifen, 10 μM; verapamil, 10 μM; glibenclamide, 100 μM; and fluoxetine, 1 μM) were also present in the incubation medium in a significant manner ($P \leq 0.01$). The "+" sign indicates the addition of the anion channel inhibitors to the 100 μM homocysteine.

Of these, quinacrine, a flavin analog, which is known to inhibit the respiratory burst O_2 reduction by neutrophils (Cross 1990), had no effect on the aortic tissue ferricyanide reducing activity. Nor did doxorubicin, which has been shown to be particularly effective in inhibiting ferricyanide reduction by cancer cells (Kim *et al*. 2002), exert an effect when it was added to the Ringer solution containing excised frog aortic tissue. However, the addition of *p*-chloromercuribenzoyl sulphonate (*p*CMBS), a thiol-binding membrane-impermeant sulfhydryl reagent and a well-established PMET inhibitor (Baker *et al*. 2004), to the Ringer solution in which the frog aortic tissue was suspended inhibited ferricyanide reductase activity by 99% (table 1), indicating that the cysteine residues on the extracellular surface of the channel could play a key role in ferricyanide reduction.

4.2 *Ferricyanide reductase and VDAC-cored VRAC*

DIDS, a large electronegative compound that binds to positively charged lysine residues and a well-known inhibitor of VDAC conductance (Baker *et al*. 2004), was unable to inhibit the ferricyanide reductase activity in aortic tissue under isotonic conditions (table 1), implying that the lysine residues targeted by the inhibitor are not necessary for redox activity of VDAC. However, the enhanced ferricyanide reductase activity, observed when the aortic tissue was exposed to a hypotonic solution or to homocysteine in isotonic frog Ringer solution, was inhibited in the presence of DIDS (figures 1 and 2). This suggests that this enhanced activity takes place because of the opening of VDAC and the subsequent exposure of the hidden cysteine residues that may lie in the tunnel of the channel. Although the present investigation does not provide any evidence, it is likely that the presence of DIDS causes closure of the anion channel by interacting with lysine residues and preventing the exposure of the hidden sulfhydryl groups to the extracellular compartment. Tamoxifen, verapamil, glibenclamide and fluoxetine, the other established VRAC inhibitors (Hisadome *et al*. 2002), failed to affect the reductase activity in the aortic tissue under isotonic conditions (table 1). However, the enhanced ferricyanide

Table 1. The effect of various inhibitors on frog aortic tissue ferricyanide reductase activity

Compound	Concentration	Percentage of inhibition
Quinacrine	1 mM	0
Doxorubicin	10 μM	2.2 ± 2
*p*CMBS	1 mM	99 ± 0.3
VDAC inhibitor		
DIDS	100 μM	5.2 ± 1.5
VRAC inhibitors		
Tamoxifen	10 μM	9.8 ± 4.3
Verapamil	10 μM	9.4 ± 5.1
Fluoxetine	1 μM	6.5 ± 3
Glibenclamide	100 μM	7 ± 2.2

After the normal ferricyanide reductase activity of the excised frog aortic tissue was monitored for 10 min, the tissue was washed in Ringer solution thrice. The compounds indicated, dissolved in isotonic frog Ringer solution (216 mOsmol/kg H_2O), were incubated with the excised frog aortic tissue for 5 min. Potassium ferricyanide (1 mM) was then added to the aortic tissue, and the ferrocyanide generated was measured after 10 min as described in Materials and methods.

reductase activity observed when the aortic tissue was exposed to hypotonic solutions or to homocysteine dissolved in isotonic frog Ringer solution was brought back to basal level in the presence of VRAC inhibitors (figures 1 and 2). These results indicate that the ferricyanide reductase activity observed in frog aortic tissue is associated with plasma-membrane-bound VDAC and belongs to the fourth category in the classification of reductases given by Baker *et al*. (2004). In a recent study, pl-VDAC was reported to be present at the core of VRAC and was envisaged to play a role in regulatory volume decrease (RVD) responses (Elinder *et al*. 2005; Thinnes 2009). If VRAC is assumed to be present at the core of VDAC, then VRAC inhibitors may not affect the ferricyanide reductase activity in VDAC. However, if VDAC were present at the core of VRAC, then closure of VRAC by the VRAC inhibitors may impede the access of cysteine residues of VDAC to the extracellular environment, thereby bringing the ferricyanide reductase activity to its basal level. Such an idea finds support in the assumptions made by Elinder *et al*. (2005) and Thinnes (2009) and offers an explanation for our findings.

4.3 *Possible mechanism of modulation of reductase activity by homocysteine*

Homocysteine at a concentration of 10 μM and 50 μM in isotonic frog Ringer solution was ineffective in altering the ferricyanide reductase activity in frog aortic tissue. However, at a higher concentration (100 μM), it was able to enhance the reductase activity. It was possible to reduce the enhanced activity to basal levels in the presence of VDAC and VRAC inhibitors (figure 3). This suggests that homocysteine at higher concentrations can modulate the PMET through the activation of VDAC and VRAC. Finally, it is suggested that since VDAC has been conserved functionally over a long evolutionary time frame (Komarov *et al*. 2004), it is likely that similar responses and functional interactions of the three plasma membrane proteins also exist in humans.

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