Brief communication

Plasma membrane electron transport in frog blood vessels

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In an attempt to see if frog blood vessels possess a plasma membrane electron transport system, the postcaval vein and aorta isolated from *Rana tigrina* were tested for their ability to reduce ferricyanide, methylene blue, and 2,6-dichloroindophenol. While the dyes remained unchanged, ferricyanide was reduced to ferrocyanide. This reduction was resistant to inhibition by cyanide and azide. Heptane extraction or formalin fixation of the tissues markedly reduced the capability to reduce ferricyanide. Denuded aortas retained only 30% of the activity of intact tissue. Our results indicate that the amphibian postcaval vein and aorta exhibit plasma membrane electron transport.

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1. Introduction

It is well known that mitochondria are the subcellular organelles responsible for biological oxidation orchestrated through a clearly defined electron transport system. Evidence is accumulating of a similar mechanism operating in the plasma membranes of various cells and tissues in health and disease. Cell membranes of bacteria (Ertl *et al.* 2000), fungi (Pőnitz and Roos 1994), protozoans (Bera *et al.* 2006), parasites (Biswas *et al.* 2008), red blood cells (May *et al.* 2004) and cultured melanoma cells (Brar *et al.* 2001) have been shown to transport electrons from the inside to the outside. Recently, such activity was observed in mouse intestinal tissue (Pountney *et al.* 1996) and in cultured pulmonary artery endothelial cells (Merker *et al.* 1997). We tested the frog postcaval vein and aorta to see if amphibian vasculature exhibits such an electron transport system.

2. Materials and methods

2.1 *Isolation of the postcaval vein and aorta*

Frogs (*Rana tigrina*) obtained from the local supplier were used for the study. They were double pithed as per the guidelines of the institutional animal ethics committee (Kasturba Medical College, Manipal, India). An expert zoologist identified the species. The aorta and postcaval vein of the frog were identified as per the description provided by Parker and Haswell (1960) and blood vessels measuring 3–5 mm were secured by ligatures and isolated.

2.2 *Assay of ferricyanide reduction*

Before the isolated 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). They were then incubated in frog Ringer containing potassium ferricyanide (1 mM) in a test-tube with constant stirring at room temperature (28°C). The incubation mixture was centrifuged at 3000 rpm to pellet out the tissue and the clear supernatant was used for the assay. Aliquots of supernatant (100 μl) were collected at 30 s, 60 s and thereafter at 10 min intervals for 2 h. 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

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presence of tissue. Hence, it was not taken into consideration during later calculations. The amount of ferrocyanide generated was measured by the *ortho*-phenanthroline method of Avron and Shavit (1963). The absorbance was measured at 510 nm in a spectrophotometer (Genesys UV10). Supernatants from incubation mixtures containing Ringer 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 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 frog Ringer of the same concentration with ferricyanide in the absence of tissue. Reduction of ferricyanide in the paired control (frog Ringer without tissue) was found to be less than 0.5% when compared with the test (frog Ringer with tissue) and the appropriate correction was made. A pre-determined absorption coefficient of 21.6 mM^{-1} cm^{-1} was used to calculate the ferrocyanide formed.

2.3 *Dye measurements*

The ability of the tissues to reduce methylene blue and 2,6, dichloroindophenol was assessed by incubating tissue in 200 μl of 20 μM dye solutions prepared in frog Ringer. The concentrations of the oxidized forms of methylene blue and 2,6 dichloroindophenol were measured by their absorbance changes at 666 nm and 600 nm, respectively, at similar time intervals. Parallel controls without tissue showed no reduction of the dyes. The sampling procedure was similar to the one described above.

2.4 *Influence of known inhibitors*

The effect of potassium cyanide or sodium azide on ferricyanide reduction by tissue was determined by including these compounds in the incubation medium at a concentration of 1 mM. The isolated tissues were incubated with 2% formalin for 1 h at room temperature or with heptane for 4 h at 60°C prior to testing to determine their effects. Aortas were denuded of endothelium by gentle scratching with a matchstick and tested for the generation of ferrocyanide. The postcaval vein was too small to be denuded.

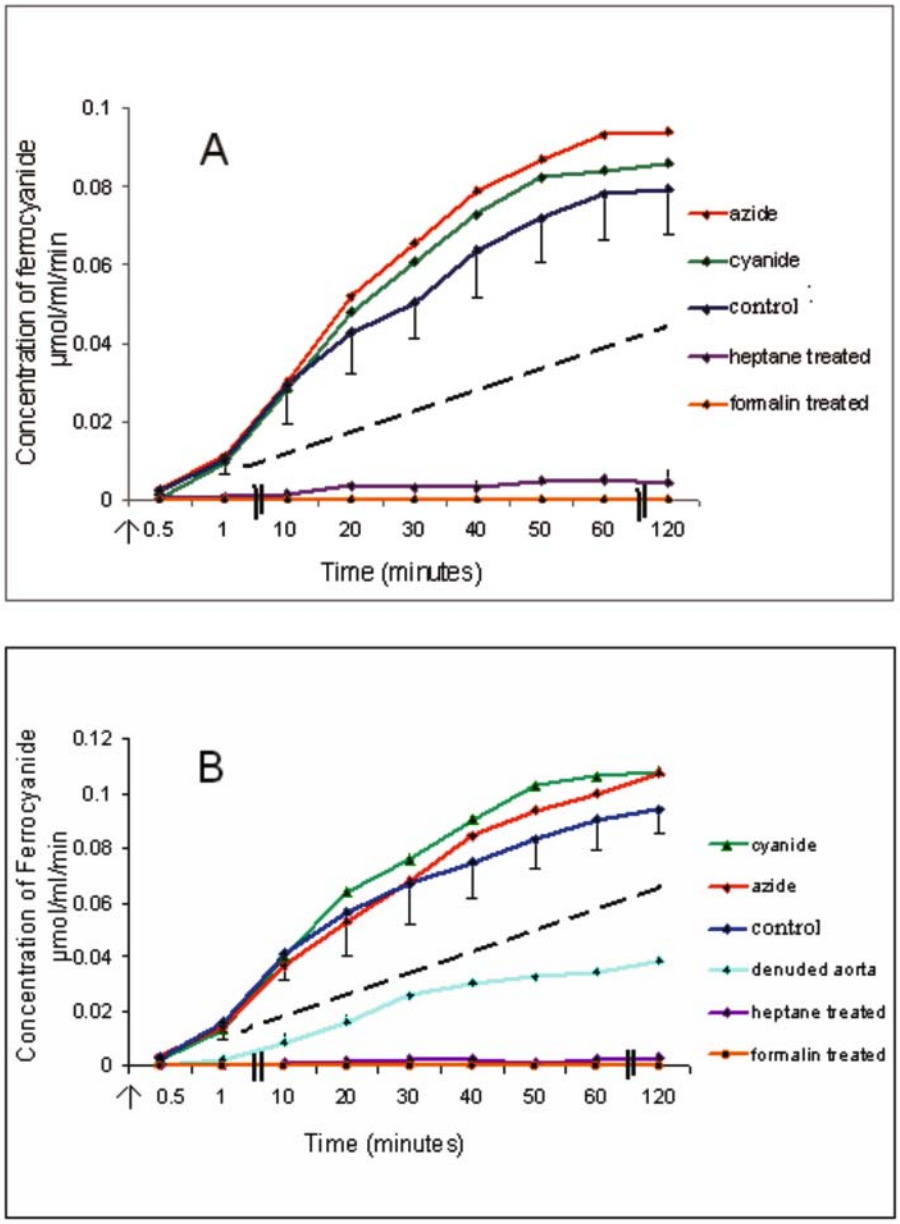
3. Results

As shown in figure 1, both the tissues had reduced ferricyanide. The aliquots of supernatant were estimated for the generation of ferrocyanide at intervals of 30 s, 60 s, and thereafter at 10 min intervals for 2 h. After an initial rapid phase, the generation of ferrocyanide in the control (ferricyanide reduction by tissues in the absence of any known inhibitor) remained fairly steady for 60 min. The generation reached a plateau between 60 and 120 min in both the tissues. Neither methylene blue nor 2,6 dichloroindophenol was reduced by the tissues. Incorporation of azide and cyanide failed to affect ferricyanide reduction by either the postcaval vein or aorta. However, the presence of formalin and heptane prevented ferrocyanide generation in both the tissues. Denuded aortas showed only 30% of activity when compared with intact blood vessels.

4. Discussion

Since neither ferricyanide nor ferrocyanide penetrates the cell membrane, reduction of the former by a tissue is considered as evidence of the existence of a plasma membrane electron transport system in the tissue. Many studies have employed this method to demonstrate transplasma membrane electron transport (Pountney *et al.* 1996; Wright and Kuhn 2002; May *et al.* 2004). The lack of inhibition found in our study by known inhibitors of mitochondrial electron transport is in agreement with previous studies reporting such resistance in other tissues (Bera *et al.* 2006; Crane *et al.* 1991). Extraction by heptane and fixation by formaldehyde, which affect the structural integrity of the membrane, markedly diminished the capacity of the tissues to reduce ferricyanide. The former showed a similar effect in an earlier study (Villalba *et al.* 1995). Dyes such as methylene blue and 2,6 dichloroindophenol have been employed in certain studies to demonstrate plasma membrane electron transport (May *et al.* 2004; Wright and Kuhn 2002). However, in our study, frog blood vessels failed to reduce these dyes. This may be due to a lack of specific reductases capable of reducing these dyes.

It is generally believed that, along with electrons, protons are also transported out of the cells, which are accepted by oxygen to form water (Crane *et al.* 1991). Protons along with electrons are required for dye reduction exhibited by certain cells (May *et al.* 2004; Merker *et al.* 1997). It is not clear whether protons accompany electrons during their extrusion by frog blood vessels. A marked decrease in the ability of denuded aortas to reduce ferricyanide indicates that the endothelium is responsible for the reduction of ferricyanide. A number of functions have been attributed to the plasma membrane electron transport system – regulation of cell growth, phosphorylation of membrane proteins, and induction of expression of proto-oncogenes (Sun *et al.* 1992). While our findings indicate the existence of a plasma membrane electron transport system in frog blood vessels, further studies are needed to elucidate its function.



Figures 1. Reduction of ferricyanide by frog (**A**) postcaval vein or (**B**) aorta plotted as the amount of ferrocyanide in the supernatant at various time intervals. The arrow indicates the time of addition of ferricyanide. Each point on a curve is the mean of 3–4 experiments. For the sake of clarity, standard error bars are shown for only two curves. Points on the curves above the arbitrary interrupted line are significantly different from corresponding points of the curves below the line. Student *t*-test was used and $P<0.05$ was taken as significant.

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