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Dopamine Oxidation Products Inhibit Na^+, K^+-ATPase Activity in Crude Synaptosomal–mitochondrial Fraction from Rat Brain

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The diverse damaging effects of dopamine (DA) oxidation products on brain subcellular components including mitochondrial electron transport chain have been implicated in dopaminergic neuronal death in Parkinson's disease. It has been shown in this study that DA (50–200 μM) causes dose-dependent inhibition of Na^+, K^+-ATPase activity of rat brain crude synaptosomal–mitochondrial fraction during *in vitro* incubation up to 2 h. The enzyme inactivation is prevented by catalase and the metal-chelator (diethylenetriamine penta-acetic acid) but not by superoxide dismutase or hydroxyl-radical scavengers like mannitol and dimethylsulphoxide (DMSO). Further, reduced glutathione and cysteine, markedly prevent DA-mediated inactivation of Na^+, K^+-ATPase. Under similar conditions of incubation, DA (200 μM) leads to the formation of quinoprotein adducts (protein-cysteinyl catechol) with synaptosomal–mitochondrial proteins and the phenomenon is also prevented by glutathione (5 mM) or cysteine (5 mM).

The available data imply that the inactivation of Na^+, K^+-ATPase in this system involves both H_2O_2 and metal ions. The reactive quinones by forming adducts with protein thiols also probably contribute to the process, since reduced glutathione and cysteine which scavenge quinones from the system protect Na^+, K^+-ATPase from DA-mediated damage. The inactivation of neuronal Na^+, K^+-ATPase by DA may give rise to various toxic sequelae with potential implications for dopaminergic cell death in Parkinson's disease.

Keywords: Dopamine; Quinoprotein; Na^+, K^+-ATPase; Oxygen free radicals; Cytochrome c; Quinones

INTRODUCTION

Oxidative stress resulting from enzymatic or autoxidation of dopamine (DA) has been implicated as a major mechanism of dopaminergic neuronal death in brain in Parkinson's disease.[1,2] The products of DA oxidation include both oxygen free-radicals and reactive quinones. While the damaging effects of oxygen radicals in etiopathogenesis of Parkinson's disease have been highlighted by many investigators, there is increasing evidence that reactive quinones are equally deleterious for the cells especially in causing mitochondrial dysfunction or inhibition of important enzymes.[3,4] We have recently reported that in rat brain crude synaptosomal–mitochondrial fraction exposed to autoxidizing DA, quinoprotein adduct (protein-cysteinyl catechol) formation and protein cross-linking occur and the phenomena are apparently mediated by quinone products and not by toxic oxygen free-radicals.[5] In the present study, it has been demonstrated that in the same brain membrane fractions under similar conditions of incubation, DA oxidation products cause inactivation of Na^+, K^+-ATPase.

The membrane-bound Na^+, K^+-ATPase plays a key role in the maintenance of Na^+ and K^+ gradients across the cell membrane and this in turn affects the resting membrane potential and electrical excitability in cells like neurons.[6,7] The inactivation of

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neuronal Na^+, K^+-ATPase has been suggested to give rise to partial membrane depolarization, cell-swelling and other toxic consequences.[8–10] Our results demonstrating the inhibition of brain Na^+, K^+-ATPase activity by DA oxidation products may provide important clues to the understanding of neuronal death in Parkinson's disease.

MATERIALS AND METHODS

Animals

Adult albino rats of Charles-Foster strain kept on laboratory chow and water *ad libitum* were used for this study.

Reagents and Chemicals

Catalase (from bovine liver, 10,000–25,000 units/mg protein), phenylmethyl sulphonyl fluoride, superoxide dismutase (from bovine erythrocytes, lyophilized powder), diethylenetriamine pentaacetic acid (DTPA), ouabain, mannitol and ATP were purchased from Sigma Chemical Co. (USA). Reduced glutathione, nitrobluetetrazolium (NBT), L-cysteine and dimethylsulphoxide (DMSO) were obtained from Sisco Research Laboratory (India). All other chemicals used were of analytical grade.

Preparation of Synaptosomal-Mitochondrial Fraction (P_2 Pellet)

The animals were killed by cervical dislocation, decapitated and brains removed. The brain tissue was homogenized in 9 volumes of 320 mM sucrose and 5 mM HEPES at pH 7.4 containing 0.1 mM phenylmethyl sulphonyl fluoride. The crude synaptosomal–mitochondrial fraction (P_2 pellet) was prepared by the method of Gray and Whittaker.[11]

In Vitro Incubation of P_2 Pellet Fraction with DA

The synaptosomal–mitochondrial fraction (protein content 1.0–2.2 mg/ml) was incubated for varying periods up to 2 h at 37°C in presence or absence of DA (0.01–0.2 mM) with or without other additions like mannitol (20 mM) or DMSO (20 mM) or catalase (50 μg/ml) or reduced glutathione (5 mM) or cysteine (5 mM) or DTPA (0.1 mM) or SOD (50 μg/ml) in 75 mM Tris, pH 7.4. At the end of the incubation, the membranes were pelleted by centrifugation at 4°C and washed again with 75 mM Tris, pH 7.4. The membranes in each tube were finally resuspended in 25 mM Tris, pH 7.4 in a total volume of 500 μl and used for Na^+, K^+-ATPase assay.

Measurement of Na^+, K^+-ATPase Activity

An aliquot (100 μl) of resuspended P_2 pellet membranes containing 100–200 μg of protein was used for the assay of Na^+, K^+-ATPase activity in a reaction mixture containing 100 mM NaCl, 10 mM KCl, 6 mM $MgCl_2$ and 3 mM ATP in 25 mM Tris, pH 7.4 in the presence or absence of 2 mM ouabain as adapted from the method of Mallick *et al.*[12] The activity of the enzyme was expressed as μmoles of inorganic phosphate liberated/mg protein/h. Ouabain sensitive ATPase activity was taken as the measure of Na^+, K^+-ATPase.

Measurement of Quinoprotein Adducts (Protein-Cysteinyl Catechol) in P_2 Pellet Fraction

For these experiments, an aliquot of P_2 pellet suspension (250 μg protein) was incubated with DA (0.2 mM) for 2 h at 37°C in 50 mM phosphate buffer, pH 7.4 with or without other additions followed by precipitation and delipidation of membrane proteins and measurement of quinoprotein adduct (protein-cysteinyl catechol) formation by NBT/glycinate assay as described earlier.[5]

Protein Estimation

The protein was estimated by the method of Lowry *et al.*[13] after solubilizing the membrane in 1% sodium dodecylsulphate (SDS).

RESULTS

In crude synaptosomal–mitochondrial fraction pre-incubated with DA for 2 h, Na^+, K^+-ATPase was inhibited by the latter in a dose-dependent manner [Fig. 1(a)]. The degree of inhibition after 2 h of incubation with 0.2 mM DA varied from 50 to 77% in different sets of experiments [Figs. 1(a),(b) and 2]. The time-course of the enzyme inactivation shows that the inhibition occurred slowly and a significant loss of activity was apparent after 1 h of incubation with DA [Fig. 1(b)]. Reduced glutathione (5 mM) prevented the inactivation of Na^+, K^+-ATPase by DA throughout the period of incubation [Fig. 1(b)]. Cysteine (5 mM) also protected the enzyme from inactivation by DA to the same extent as seen in case of reduced glutathione (data not shown).

Results presented in Fig. 2 show that the inactivation of Na^+, K^+-ATPase of crude synaptosomal–mitochondrial fraction by DA was not prevented by hydroxyl radical scavengers like mannitol (20 mM) or DMSO (20 mM) or by the anti-oxidant enzyme SOD (50 μg/ml). Neither mannitol nor DMSO in the concentration used had any effect *per se* on Na^+, K^+-ATPase activity of control P_2 pellet

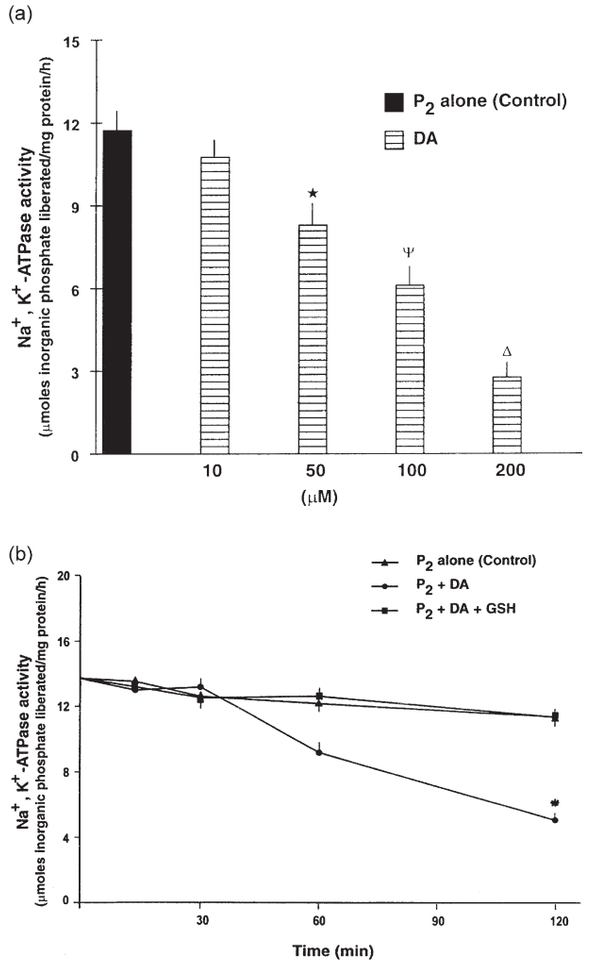

FIGURE 1 Effect of DA on Na^+, K^+-ATPase activity of rat brain synaptosomal–mitochondrial (P_2) fraction. P_2 fraction from rat brain was incubated in the absence (control) or presence of DA with or without other additions up to 2 h followed by the measurement of Na^+, K^+-ATPase activity as described in the text. (a) Dose-dependent inhibition of Na^+, K^+-ATPase by DA after incubation for 2 h. Results are mean ± SE. of five observations. Statistical significance was calculated by Student's "*t*" test, paired. ★, Ψ, Δ indicate $p < 0.01$, compared to control. (b) Time-course of inactivation of Na^+, K^+-ATPase by DA (0.2 mM) and protection by GSH (5 mM). Values are mean ± S.E. of four observations. The bars indicating S.E. are projected either upwards or downwards and removed from 0 and 15 min, points for the sake of clarity of the drawing. (★) Indicates statistical significance, $p < 0.01$ compared to control (Student's "*t*" test, paired).

membranes incubated without DA (results not presented). However, catalase (50 μg/ml) or DTPA (0.1 mM) markedly protected the enzyme from inactivation by DA indicating the involvement of H_2O_2 and metal ions in this phenomenon (Fig. 2). Further, a significant formation of quino-protein adducts was noticed when synaptosomal–mitochondrial fraction from rat brain was incubated with DA (0.2 mM) for 2 h and this phenomenon was significantly inhibited by GSH or cysteine at a concentration of 5 mM (Table I).

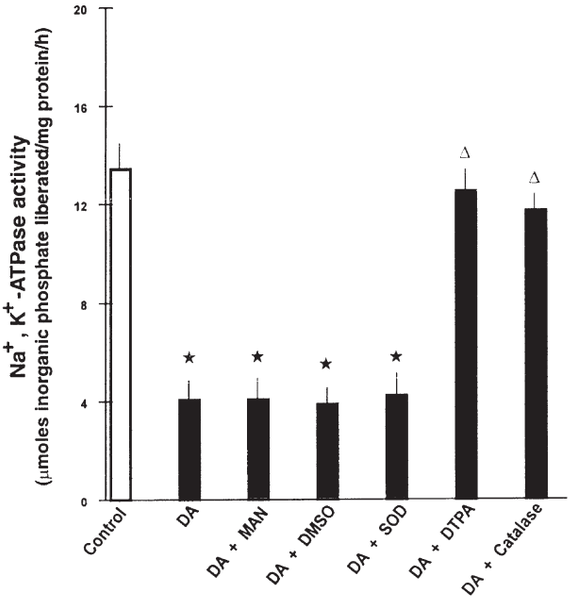

FIGURE 2 Na^+, K^+-ATPase activity of rat brain P_2 pellet fraction incubated without (control) or with DA (0.2 mM) in the presence or absence of other additions for 2 h. Incubation and measurement of enzyme activity were carried out as given in the text. Values are mean ± S.E. of four observations. Statistical comparisons were made by Student's "*t*" test paired. ★$p < 0.001$, when compared to control. Δ$p < 0.001$, when compared to DA treated samples.

DISCUSSION

The autoxidation of DA produces H_2O_2 which with the help of transition metals like iron can generate reactive $^\bullet$OH radicals through Fenton's reaction.[1,2] The latter mechanism is further promoted in the system by DA presumably because of its reducing property.[14] The reactive free-radicals such as the $^\bullet$OH radicals can cause various forms of protein damage including inactivation of enzymes.[15,16] The protein damage is often mediated by site-specifically generated $^\bullet$OH radicals by the interaction of H_2O_2 with bound metals ions on the target protein.[17–19] In our system, DA-induced inactivation of Na^+, K^+-ATPase activity is prevented by catalase and DTPA indicating the involvement of H_2O_2 and metal ions

TABLE I Quino-protein adduct formation in P_2 pellet membranes

Incubation mixture	Absorbance at 530 nm
P_2 pellet incubated alone (control)	0.098 ± 0.006
P_2 + DA	0.280 ± 0.005*
P_2 + DA + GSH	0.106 ± 0.010$^\blacktriangledown$
P_2 + DA + cysteine	0.090 ± 0.005$^\blacktriangledown$

Rat brain P_2 pellet membranes were incubated at 37° for 2 h with or without DA (0.2 mM) in the presence or absence of GSH (5 mM) or cysteine (5 mM) followed by the measurement of quinoprotein formation by NBT/glycinate assay as described in the text. Results are mean ± S.E. of 5 observations. *Statistically significant with respect to control, $P < 0.001$, Student's "t" test paired; $^\blacktriangledown P < 0.001$ with respect to P_2 + DA, Student's "*t*" test paired.

and possibly of Fenton's chemistry. However, $^{\bullet}OH$ radicals may not be the actual damaging species in our experimental system, since the radical scavengers like mannitol (20 mM) and DMSO (20 mM) have failed to protect the enzyme from inactivation (Fig. 2). An involvement of superoxide radicals is also excluded since SOD failed to prevent DA-mediated damage to Na^+, K^+-ATPase. On the other hand, thiol containing agents like reduced glutathione (5 mM) and cysteine (5 mM) which are known to form conjugates with reactive quinones prevent DA-mediated inactivation of Na^+, K^+-ATPase as also the formation of quinoprotein adducts (Table I and Fig. 1(b)). It implies that the reactive quinones, free or conjugated to protein thiols (protein-cysteinyl catechol), are playing a key role in the inactivation of Na^+, K^+-ATPase by DA under our experimental conditions. It has been reported earlier that reactive quinones can form cysteinyl adducts with several enzymes leading to their inactivation.[20] In this study, however, it is not clear from the available data how H_2O_2, metal ion and quinones interact among themselves in the inactivation mechanism of Na^+, K^+-ATPase during exposure to DA. In rat brain membranes, oxidative stress induced by iron and ascorbate or Fe^{2+}-EDTA or Fe^{2+}-EDTA plus H_2O_2 causes inhibition of Na^+, K^+-ATPase activity which has been attributed largely to associated membrane lipid peroxidation.[21,22] However, DA does not promote lipid peroxidation but rather acts as a potent inhibitor of iron or iron and ascorbate stimulated lipid peroxidation.[1,5] The possibility of Na^+, K^+-ATPase inactivation by lipid peroxidation in our system can, therefore, be excluded.

In acutely dissociated rat neostriatal neurons, which contain many dopaminoceptive cells, an isoform-specific and receptor-mediated (D*1* and D*2* receptor) inhibition of Na^+, K^+-ATPase has been reported following a short exposure (5–10 min) to a low concentration (10 μm) of DA.[6,7] This action of DA on Na^+, K^+-ATPase has significance in relation to its neurotransmitter function on post-synaptic neurons. In contrast, in our study, we have exposed whole brain synaptosomes dispersed in a hypotonic medium to a relatively high concentration of DA (50–200 μM) for a prolonged period (up to 2 h) in order to identify the various forms of DA-mediated damage to synaptosomal components including Na^+, K^+-ATPase. The rationale of this study stems from the fact that in dopaminergic neurons the storage, secretion and uptake of DA take place at the presynaptic endings and, thus, the protein and lipid components of synaptosomes will be early targets of DA-mediated damage which, in turn, may trigger other deleterious intra-cellular events.

Although the precise mechanism of DA-induced damage has not been established, the results of this study appear to be important since the inhibition of Na^+, K^+-ATPase activity in neurons may lead to partial membrane depolarization, cell swelling, decreased uptake of neurotransmitters and even activation of voltage-dependent *N*-methyl-D-aspartate (NMDA) receptors.[8–10,23] The well-documented mitochondrial dysfunction in Parkinson's disease may lead to energy depletion and consequent reduced activity of Na^+, K^+-ATPase. Any further inhibition of the enzyme by DA oxidation products will aggravate the cellular damage and may have potential implications in the context of dopaminergic neuronal death in Parkinson's disease.

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