

Copper Ions Disrupt Dopamine Metabolism *via* Inhibition of V-H⁺-ATPase: A Possible Contributing Factor to Neurotoxicity

D. Shyamali Wimalasena[‡], Thomas J. Wiese[†] and Kandatege Wimalasena^{‡*}
[‡]Department of Chemistry, Wichita State University, Wichita, KS 67260-0051 and
[†]Department of Chemistry, Fort Hays State University, Hays, KS 67601-4009

**Address correspondence to* Kandatege Wimalasena
 Department of Chemistry, Wichita State University, Wichita, KS 67260-0051
Tel: 316-978-7386; Fax: 316-978-3431 E-mail: kandatege.wimalasena@wichita.edu

Footnotes

¹The abbreviations used: A β ₁₋₄₂, β -amyloid precursor protein peptide 1-42; APP, β -amyloid precursor protein; Asc, ascorbic acid; Asc_{out}, extragranular ascorbic acid; CNS, central nervous system; Cu_{in}, intragranular copper; Cu_{out}, extragranular copper; DA, dopamine; D β M, dopamine β -monooxygenase; DMEM, Dulbecco's Modified Eagles Medium; DOPAC, 3,4-dihydroxyphenyl acetic acid; E, epinephrine; EC, electrochemical detection; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; HVA, Homovanillic acid; KRB, Krebs-Ringer Buffer; Neocuproine; 2,9-dimethyl-1,10-phenanthroline; NE, norepinephrine; Trien; Triethylenetetramine; Tris, tris (hydroxymethyl)-aminomethane, V-H⁺-ATPase; vesicular hydrogen translocating ATPase; VMAT, vesicular monoamine transporter.

²We note that the extent of inhibition observed in this series of experiments is somewhat higher than the data shown in figure 1B with a 5 μ M copper concentration, which could probably be due to the small amount of ghost protein used in these experiments. The addition of micromolar concentrations of A β peptide to the standard ghost preparations under standard experimental conditions produced a turbid mixture resulting in the determination of pH gradient by the acridine orange method unreliable. Therefore, 1/2-1/3 of ghost proteins was used in this series of experiments to avoid the uncertainty. Furthermore, although similar experiments were carried out under conversion conditions, the results were not highly reproducible most likely due to the ill-behavior of A β ₁₋₄₂ in the presence of Cu²⁺ and Asc_{out} even in the presence of catalase (for example *see ref.* (Dikalov et al. 2004)).

³The apparent more potent inhibition of the pH gradient generation, in comparison to H⁺-ATPase inhibition, at similar Cu_{out} concentrations could be due to the use of fixed 1 mM ATP concentration in these experiments.

Abstract

The involvement of copper in the pathophysiology of neurodegeneration has been well documented but is not fully understood. Commonly, the effects are attributed to increased reactive oxygen species (ROS) production due to inherent redox properties of copper ions. Here we show copper can have physiological effects distinct from direct ROS production. First, we show that extragranular free copper inhibits the vesicular H⁺-ATPase of resealed chromaffin granule ghosts. Extragranular ascorbate potentiates this inhibition. The inhibition is mixed type with $K_{is} = 6.8 \pm 2.8 \mu\text{M}$ and $K_{ii} = 3.8 \pm 0.6 \mu\text{M}$, with respect to ATP. Second, extracellular copper causes an inhibition of the generation of a pH-gradient and rapid dissipation of pre-generated pH and catecholamine gradients. Copper chelators, and the β -amyloid peptide 1-42 were found to effectively prevent the inhibition. The inhibition is reversible and time independent suggesting the effects of extracellular copper on H⁺-ATPase is direct, and not due to reactive oxygen species. The physiological significance of these observations was shown by the demonstration that extracellular copper causes a dramatic perturbation of dopamine metabolism in SH-SY5Y cells. Thus, we propose that the direct inhibition of the vesicular H⁺-ATPase may also contribute to the neurotoxic effects of copper.

Keywords: Vesicular H⁺-ATPase, Copper, Neurodegenerative Diseases, Chromaffin Granules, Catecholamines, and SH-SY5Y cells

Running title: Copper Disrupts Dopamine Metabolism

Cytoplasmic neurotransmitters, especially monoamines and acetylcholine, are actively transported from the cytosol into storage vesicles through closely related vesicular neurotransmitter transporters to maintain high intravesicular concentrations (Schuldiner et al. 1995; Henry et al. 1998; Gasnier 2000; Parsons 2000). The free energy for these processes is provided by large pH gradients (~2 pH units) and membrane potentials generated through a transmembrane proton pump, vesicular H⁺-ATPase (H⁺-ATPase)¹. The functional coupling between the H⁺-ATPase-mediated generation of the pH gradient and the vesicular neurotransmitter transporters mediated transport of monoamines and acetylcholine have been studied in detail and, both the pH-gradient and membrane potential have been shown to be mandatory for the intragranular accumulation and storage of neurotransmitters (Apps et al. 1980; Knoth et al. 1980; Salama et al. 1980). In addition, an H⁺-ATPase-generated pH-gradient is also vital in maintaining both an acidic environment and high levels of intragranular ascorbic acid (Asc_{in}). This is accomplished through the cytochrome b₅₆₁-mediated Asc-regenerating system in catecholaminergic neurons (Njus et al. 1986; Wakefield et al. 1986; Njus et al. 1987) which is vital to protect catecholamines from autooxidation. Recent studies suggest that the vesicular H⁺-ATPase mediated acidification may also play a vital role in sorting and packing of various granule proteins, suggesting that the normal synthesis of neurotransmitter storage vesicles may also be dependent on the transmembrane pH gradient (Taupenot et al. 2005). Therefore, vesicular H⁺-ATPase plays a critical role in the uptake, biosynthetic transformation, storage, and release of monoamines and acetylcholine neurotransmitters as well as some peptide hormones.

The effects of copper on the functions of the CNS are multiple, complex and are not completely understood (Harris 2000; Gaetke and Chow 2003; Prohaska and Gybina 2004). Copper is required for normal synaptic transmission, and several studies indicate that as much as

15 μM copper is continuously released from the nerve endings upon stimulation (Kardos et al. 1989; Hopt et al. 2003). Copper is an essential cofactor for several important enzymes in the brain, including dopamine- β -monooxygenase (DBM), peptidyl- α -amidating monooxygenase, Cu/Zn superoxide dismutase, cytochrome C oxidase, and lysine oxidase. On the other hand, the imbalance of copper homeostasis in the brain has been shown to be closely associated with the pathophysiology of neurodegenerative disorders, including Parkinson's, Alzheimer's, Wilson's, and Prion diseases, and amyotrophic lateral sclerosis (Sayre et al. 2000; Strausak et al. 2001). For example, a markedly increased level of copper (70 μM) is found in Alzheimer's disease affected brains (Deibel et al. 1996; Lovell et al. 1998). Increased oxidative stress caused by the age related accumulation of copper in the CNS has been proposed to be the primary reason for the adverse effects of copper (Perry et al. 2003; Barnham et al. 2004). Although the specific proteins closely associated with neurodegenerative disorders, including the prion protein (Stockel et al. 1998; Viles et al. 1999), β -amyloid protein (Hesse et al. 1994; Multhaup et al. 1996), α -synuclein (Paik et al. 1999) and S100B (Nishikawa et al. 1997) have been shown to have specific and high affinity for copper, neither the significance of the copper binding properties nor the physiological functions of these proteins are fully understood.

Our laboratory has studied catecholamine metabolism using chromaffin granule ghosts for many years. Recently, we made the fortuitous discovery that copper added to incubation mixtures resulted in a complete blockage of uptake and conversion of DA to NE. Further, studies have revealed that this is due to the inhibition of the vesicular H^+ -ATPase by extragranular free copper (Cu_{out}). The inhibition was more pronounced in the presence of extragranular Asc (Asc_{out}) in the medium and was reversible, time independent, and mixed-type with respect to extragranular ATP. The effects of Cu_{out} on the vesicular H^+ -ATPase are direct,

and not due to the reactive oxygen species generated by copper ions in the medium. Inhibition of H^+ -ATPase by Cu_{out} is also associated with the inhibition of the ATP mediated generation of the transmembrane pH-gradient and dissipation of the pre-generated pH and neurotransmitter concentration gradients in resealed chromaffin granule ghosts. High affinity copper chelators such as Trien and neocuproine, as well as the copper-binding domain of the β -amyloid precursor protein (APP), peptide 1-42 ($A\beta_{1-42}$), effectively prevent the inhibition when added together with Cu_{out} . The physiological relevance of these observations was shown by the demonstration that low levels of extracellular copper ($>25 \mu M$) causes a dramatic perturbation of dopamine (DA) metabolism in SH-SY5Y cells, a widely used neuronal cell line. Thus, we propose that the direct inhibition of the vesicular H^+ -ATPase may also be a contributing factor to the neurotoxicity of copper.

Materials and methods

Materials

Standard chemicals and reagents were purchased from Sigma-Aldrich or Fisher Scientific unless otherwise noted. Protein assay reagent was obtained from Biorad. Catecholamine standards were obtained from ESA. Ficoll was from Amersham Biosciences and catalase was purchased from Boehringer Mannheim. Human $A\beta_{1-42}$ was obtained from Calbiochem. Centrifugations were performed using Beckman Coulter J2MC and Optima LE-80K refrigerated centrifuges. HPLC-EC analyses were performed using ESA Model 582 solvent delivery module and Coulochem-II electrochemical detector with ESA 501 chromatographic software. HPLC-UV analyses were performed using a Spectra System P4000 gradient pump equipped with a SCM 1000 vacuum degasser coupled to an LDC Analytical SM 4000 UV detector. All the solutions were prepared in MilliQ-purified water (Millipore, Billerica, MA). Copper sulfate was used as

the source of Cu(II) in all experiments. Protein was determined by the method of Bradford (Bradford 1976) using BSA as the standard.

Preparation of Chromaffin Granule Ghosts

Chromaffin granules and lysed granule membranes were prepared from fresh bovine adrenal medullae as previously described (Wimalasena and Wimalasena 1995; Wimalasena and Wimalasena 2004). Granule membranes were washed and resealed to contain 7.5 units/mL ascorbate oxidase with no ascorbate (uptake conditions), pH 7.0 or 20 mM Tris-phosphate, 100 mM KCl, 150 mM sucrose, 10 mM sodium fumarate, 4.0 μ M copper, 100 μ g/mL catalase and 20 mM Asc (conversion conditions). The resealed ghosts were purified by a 15% Ficoll, 0.3 M sucrose, 10 mM HEPES, pH 7.0 discontinuous density gradient as previously described (Wimalasena and Wimalasena 1995; Wimalasena and Wimalasena 2004). The resealed chromaffin granules prepared as above were shown to actively take-up DA and efficiently convert NE in the presence of intra- and extra-granular Asc [(conversion conditions), mimic physiological conditions]. On the other hand, exclusion of Asc from both media and inclusion of high concentrations of ascorbate oxidase to remove contaminating Asc reduces the DA to NE conversion drastically without affecting the DA uptake (uptake conditions). A high concentration of catalase is routinely included in the extra- and intra-granular media of resealed granule ghosts to minimize the oxidative damage caused by H₂O₂ and related oxidants to the granular proteins, especially in the presence of high concentrations of Asc and catecholamines (Wimalasena and Wimalasena 1995; Wimalasena and Wimalasena 2004).

Steady State Kinetics of the H⁺-ATPase Activity of Chromaffin Granule Ghosts: (a) the Colorimetric Molybdate Assay for Inorganic Phosphate

The ghosts prepared as described above were suspended in a medium containing 0.3 M sucrose, 10 mM HEPES, pH 7.0, 5 mM MgSO₄, 100 µg/mL catalase, 5 mM Asc in a total volume of 0.2 mL. These mixtures were pre-incubated for 10 min at 30 °C and the reactions were initiated by the addition of the desired concentration of ATP as detailed in the figure legend. After incubating for 6 min at 30 °C, 150 µL aliquots were withdrawn and the samples were quenched with 150 µL of a solution containing 12% SDS in distilled deionized water. Then 300 µL of a solution containing 12% ascorbic acid in 1 M HCl and 2% ammonium molybdate (1:1 v/v) were added to each sample and mixed well. After 3 min (and before 7 min) 450 µL of a solution containing 2% sodium citrate and 2% sodium meta-arsenite in 2% acetic acid were added and the absorbance readings were taken after 20 min at 850 nm to quantify the inorganic phosphate (Gonzalez-Romo et al. 1992).

(b) The reversed phase HPLC-UV Quantification of ADP

The ghosts prepared as described above were suspended in a medium containing 0.3 M sucrose, 10 mM HEPES, pH 7.0, 5 mM MgSO₄, 100 µg/mL catalase, 5 mM Asc in a total volume of 0.5 mL. These mixtures were pre-incubated for 10 min at 30 °C and the reactions were initiated by the addition of the desired concentration of ATP as detailed in the corresponding figure legends. After incubating for 6 min at 30 °C, 400 µL aliquots were withdrawn and the samples were quenched with 40 µL of 6 M H₂SO₄, diluted with 440 µL of 0.3 M sucrose, 10 mM HEPES, pH 7.0 and the pH was adjusted to about 7.0 with saturated Tris-base. These samples were centrifuged at 10,000 × g for 10 min at 4 °C and 2 µL of the supernatants were analyzed for ADP and ATP by reversed phase HPLC on a Supelco Discovery C₁₈ column (250 × 4.6 mm; 5 µM particle size) with 75% 20 mM sodium phosphate, 5 mM tetra *n*-butyl ammonium phosphate, pH

7.0, 25% CH₃OH as the mobile phase (Samizo et al. 2001; Ushimaru and Fukushima 2003) with UV detection at 259 nm.

Steady State Kinetics of Inhibition of the H⁺-ATPase Activity in Chromaffin Granule Ghosts by Cu_{out}

The ghosts prepared as described above were suspended in a medium containing 0.3 M sucrose, 10 mM HEPES, pH 7.0, 5 mM MgSO₄, 100 µg/mL catalase, 5 mM Asc in a total volume of 0.2 mL. These mixtures were preincubated for 10 min at 30 °C, and the desired concentrations of copper (CuSO₄) were added (see corresponding figure legends for further details) and incubated further for 2 min. The reactions were initiated by the addition of the desired concentration of ATP. After incubating for 6 min at 30 °C, H⁺-ATPase activity was determined either (a) by quantifying inorganic phosphate by the colorimetric molybdate method or (b) by quantifying ADP by reversed phase HPLC according to the procedures described above.

Effect of Pre-incubation of Resealed Granule Ghosts with Cu_{out} on the H⁺-ATPase Activity

The ghosts prepared as described above were suspended in a medium containing 0.3 M sucrose, 10 mM HEPES, pH 7.0, 5 mM MgSO₄, 100 µg/mL catalase, 5 mM Asc in a total volume of 0.7 mL. This mixture was pre-incubated for 10 min at 30 °C, and then incubated with 7 µM extragranular copper or without copper (control) under conversion conditions. At the indicated time intervals, 100 µL aliquots were withdrawn and diluted into 0.5 mL of a standard H⁺-ATPase assay solution under conversion conditions and the ATPase activity in each sample was determined by the HPLC-UV method as described above.

Effect of Cu_{out} on the Transmembrane pH-Gradient of Chromaffin Granule Ghosts

The formation and dissipation of the pH-gradient across the granule membrane was measured by the previously described acridine orange method (Cidon et al. 1983). Granule ghosts prepared as above were pre-incubated with 1 mM $MgSO_4$, 100 $\mu g/mL$ catalase and 7.5 units/mL ascorbate oxidase (uptake conditions) or 4 mM Asc (conversion conditions) in the absence or presence of varying concentrations of copper in a total volume of 1 mL at 30 °C for 10 min in two cuvettes. Then, acridine orange (3 μM final concentration) was added to the sample cuvette and scanned against the blank every 30 sec, for 5 min in the wave-length range 480–550 nm, to obtain a stable baseline. The reaction was initiated by the addition of 1 mM Mg^{2+} -ATP to the sample cuvette. The scanning was continued for 15 more minutes (for further details *see* corresponding figure legends). In pH-gradient dissipation experiments, the desired concentration of copper was added to both cuvettes after the pH-gradient was fully generated and scanned further for 10 min to follow the effects of Cu_{out} on the pre-generated pH-gradient. The $\Delta A_{(492-540\text{ nm})}$ was plotted against time to determine the formation or dissipation of the pH gradient under various experimental conditions. The decrease in $\Delta A_{(492-540\text{ nm})}$ has been shown to be proportional to the ΔpH (Cidon et al. 1983).

Effect of the Copper Chelators, Trien, and Neocuproine on the Cu_{out} -Mediated Effects on the Transmembrane pH-Gradient of Chromaffin Granule Ghosts

Ghosts were prepared as above and pre-incubated with 1 mM $MgSO_4$, 100 $\mu g/mL$ catalase, 4 mM Asc, desired concentrations of Trien or neocuproine, and 5-10 μM copper at 30 °C for 10 min and the formation or the inhibition of the pH-gradient was monitored by the acridine orange method as described above (for further details *see* corresponding figure legends). Although

numerous attempts were made to determine whether copper (I) or (II) is responsible for the effect on H⁺-ATPase using several copper (I) salts directly, these experiments were not successful due to the instability, low solubility and other technical difficulties associated with handling of copper(I) under the experimental conditions.

Effect of A β ₁₋₄₂ on the Inhibition of pH Gradient by Cu_{out} under Uptake Conditions

Ghosts were prepared as detailed above with 7.5 units/mL internal ascorbate oxidase and preincubated with (a) 1 mM MgSO₄, 100 μ g/mL catalase, 7.5 units/mL ascorbate oxidase; (b) 1 mM MgSO₄, 100 μ g/mL catalase, 7.5 units/mL ascorbate oxidase, and 5 μ M copper; (c) 1 mM MgSO₄, 100 μ g/mL catalase, 7.5 units/mL ascorbate oxidase, 5 μ M copper and 3 μ M A β ₁₋₄₂; (d) 1 mM MgSO₄, 100 μ g/mL catalase, 7.5 units/mL ascorbate oxidase, 5 μ M copper and 5 μ M A β ₁₋₄₂ at 30 °C for 10 min. The formation or the inhibition of the pH-gradient was monitored as described above (for further details *see* corresponding figure legends).

Effect of Cu_{out} on the DA Uptake and Intragranular Conversion to NE in Chromaffin Granule Ghosts

Aliquots of ghosts prepared as described above were suspended in a medium containing 0.3 M sucrose, 10 mM HEPES, pH 7.0, 5 mM Mg²⁺-ATP, 5 mM MgSO₄, 100 μ g/mL catalase and 20 mM Asc (conversion conditions) or 7.5 units/mL ascorbate oxidase (uptake conditions) in a total volume of 0.5 mL. This mixture was preincubated for 10 min at 30 °C, after which the desired concentrations of copper were added (*see* corresponding figure legends for further details) and incubated further for 2 min. The reactions were initiated by adding 200 μ M DA. After incubating at 30 °C for 6 min, 400 μ L of the incubate was withdrawn and diluted into 5.0 mL of ice-cold 0.4 M sucrose, 10 mM HEPES, pH 7.0 and stored at 0 °C until the incubation was

completed. These samples were then centrifuged at $36,000 \times g$ for 25 min at 4 °C and the supernatants were removed to re-isolate the ghosts (Wimalasena and Wimalasena 1995; Wimalasena and Wimalasena 2004). The pellets were gently washed three times with 0.4 M sucrose, 10 mM HEPES, pH 7.0, and the tubes swabbed dry. Then, 500 μ L of 0.1 M HClO₄ was added, the pellets homogenized and the extraction was allowed to proceed for 20 min at room temperature. After low speed centrifugation, 20 μ L of the acidic extracts were analyzed for catecholamines, by reversed phase HPLC-EC as previously described (Wimalasena and Wimalasena 1995; Wimalasena and Wimalasena 2004).

Cell Culture

Neuroblastoma cell line SH-SY5Y (Biedler et al. 1978) was obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were grown in high glucose Dulbecco's Modified Eagles Medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μ g/mL streptomycin. Cells were fed three times weekly and passaged by incubation in Trypsin-Versene and plated at 1×10^6 cells. For experimental conditions where serum-free conditions were needed, cells were placed in Krebs-Ringer buffer (KRB) supplemented with glucose (125 mM NaCl, 2 mM KCl, 1.4 mM MgSO₄, 1.2 mM CaCl₂, 25 mM glucose, and 20 mM HEPES, pH 7.4).

Cellular Dopamine Transport and Metabolism

Cells were plated at 1×10^6 cells/well in Falcon 6-well plates and used as they neared confluence. Medium was aspirated and 1 mL of KRB per well was added. Stock solutions of DA and Asc were added to achieve the desired final concentrations depending on the experiment

(normally 50 μM each for DA and Asc). After a 10 min incubation (uptake period), this solution was aspirated and replaced with KRB supplemented only with Asc and incubated an additional 60 min to allow for conversion to NE (conversion period). Following the 60 min incubation, the solution was aspirated and the cells washed 3 \times with ice-cold PBS (137 mM NaCl, 13 mM KCl, 7 mM NaH_2PO_4 , 3 mM K_2HPO_4 , pH 7.4). Cells were physically detached and transferred to a microcentrifuge tube. Duplicate aliquots were removed for protein determination and the remainder pelleted at 1000 \times g for 1 min. Cells were lysed with 100 μL of 0.1 M HClO_4 and the catecholamines were separated and quantified by HPLC-EC as described above for resealed granule ghosts (Wimalasena and Wimalasena 1995; Wimalasena and Wimalasena 2004). In experiments where the effect of copper on DA uptake and conversion was investigated, the cells were pre-incubated with appropriate amount of extracellular copper, typically for 30 min, and intracellular DA and NE levels were measured as described above for control experiments. In a second set of experiments, cells were first loaded with DA for 10 min and, then treated with copper to determine whether the effects of copper on intra-granular catecholamine levels is due to the inhibition of cellular DA uptake through the plasma membrane monoamine transporter. All the experiments were done at least three times in duplicate or triplicate.

Atomic Absorption Spectroscopy

The portion of PCA-digested cells not used for DA metabolite analysis was dissolved in 5% nitric acid and diluted to a known final volume. Copper content was determined with a Varian Atomic Absorption 55 Spectrometer at 324.8 nm by comparison to a standard curve of 0-10 μM copper.

Data analysis

For H⁺-ATPase activity determination, a calibration curve of authentic standards of known concentrations of ADP or inorganic phosphate were used to determine nmol product, and protein assay results from each time point are used to normalize the measurement to nmol/min-mg. For granule HPLC-EC measurements, a calibration curve of authentic standards of DA and NE were used to integrate peak area to nmol DA or its metabolites, and normalized to protein content. Initial rate kinetic data were analyzed by Cleland's kinetic programs (Cleland 1979). Calibration curves based on peak height was used in the analysis of cellular catecholamines. Peak height was normalized to protein content and reported directly or converted to a percent of control cells not exposed to copper. All granule experiments were carried out with 3-4 different preparations and results presented are representative of those experiments. The error bars in the figures related to granule experiments were omitted to improve the clarity, since many they contain large number of points.

Results

Steady State Kinetics of H⁺-ATPase Activity in Chromaffin Granule Ghosts

Preliminary experiments revealed that the H⁺-ATPase activity of resealed chromaffin granule ghosts could be conveniently determined by quantifying the inorganic phosphate released by the hydrolysis of extragranular ATP using the colorimetric molybdate assay (Gonzalez-Romo et al. 1992) or by separating and quantifying the extragranular ADP produced, by HPLC-UV (Samizo et al. 2001; Ushimaru and Fukushima 2003). These experiments have shown that the H⁺-ATPase activity of resealed granule preparations is linear up to 10 min and the initial rate of ATP hydrolysis could be accurately determined even with 20 μM extragranular ATP concentrations under the experimental conditions (data not shown). The initial rates of the V-H⁺-ATPase

catalyzed ATP hydrolysis (measured for the initial 6 min incubation period by the molybdate assay for inorganic phosphates) obeys Michaelis Menten kinetics (data not shown), and yield an apparent K_m and V_{max} of $399 \pm 23 \mu\text{M}$ and $116.5 \pm 2.6 \text{ nmoles/mg}\cdot\text{min}$ respectively, under the experimental conditions. Reducing the incubation time from 6 min to 2 min (to minimize the depletion of ATP at low concentrations) had no significant effect on the above kinetic parameters. Similarly, the steady state kinetic parameters determined by HPLC separation and quantification of ADP were very similar to the above (data not shown), suggesting that both assays were reliable in determining the kinetics of H^+ -ATPase activity in resealed granule ghosts. In addition, the kinetic parameters of H^+ -ATPase for ATP were not significantly affected by excluding Asc_{out} and/or Asc_{in} (i.e. uptake conditions).

Kinetics of Inhibition of H^+ -ATPase Activity in Chromaffin Granule Ghosts by Cu_{out}

The data presented in Fig. 1A show the effect of increasing concentrations of Cu_{out} (0 to $7 \mu\text{M}$) on the H^+ -ATPase activity of resealed chromaffin granule ghosts under the conversion conditions as determined by the molybdate assay for inorganic phosphates. The direct fit of the initial velocity data to various reversible inhibition kinetic schemes using Cleland's COMP, NONCOMP, and UNCOMP programs (Cleland 1979) revealed that the inhibition could be best described as mixed-type with $K_{is} = 6.8 \pm 2.8 \mu\text{M}$ and $K_{ii} = 3.8 \pm 0.6 \mu\text{M}$, with respect to ATP. This fitting routine also yielded apparent K_m and V_{max} parameters of $377 \pm 58.4 \mu\text{M}$ and $117.6 \pm 6.8 \text{ nmoles/mg}\cdot\text{min}$, respectively which were in agreement with the directly determined K_m and V_{max} parameters under similar experimental conditions, further validating the kinetic analysis. Similar kinetic experiments in which the activity of H^+ -ATPase was determined by HPLC separation and quantification of ADP, yielded K_{is} and K_{ii} of $7.9 \pm 1.8 \mu\text{M}$ and $4.6 \pm 0.9 \mu\text{M}$

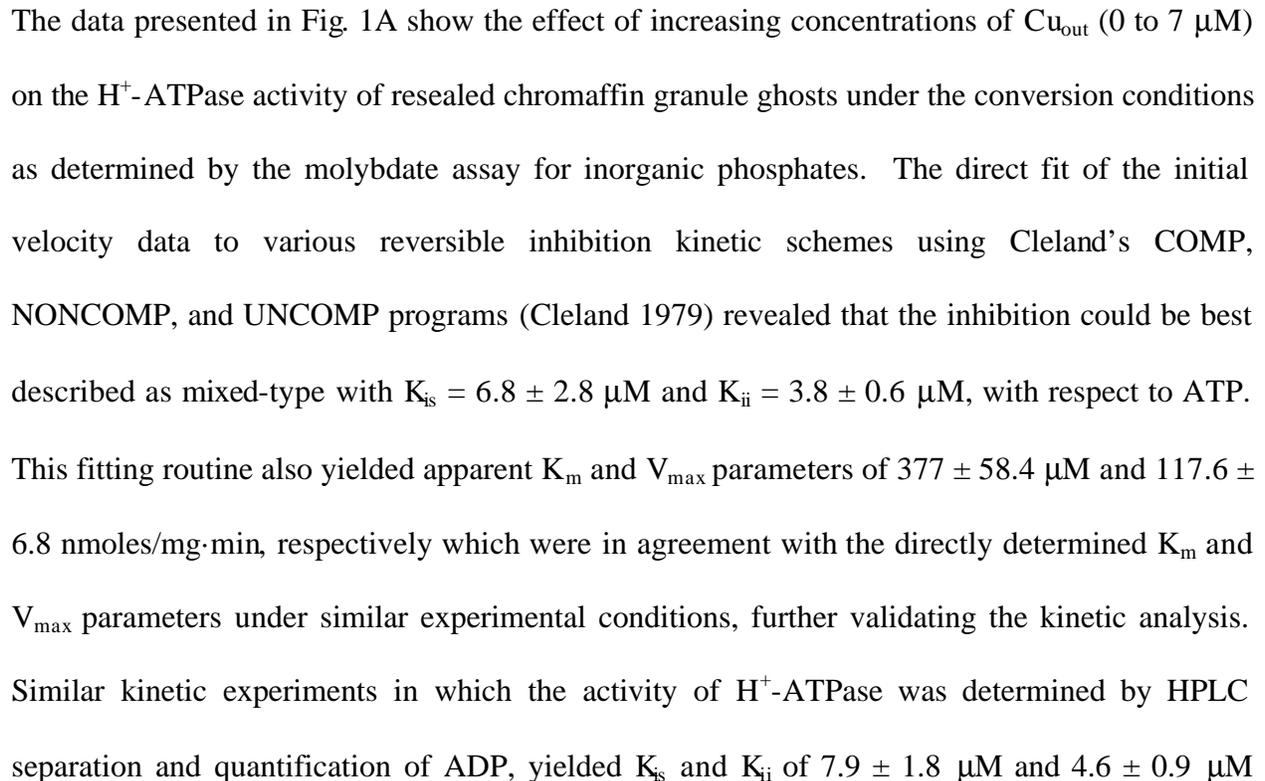


Fig. 1

respectively, with respect to ATP (Fig. 1B). In addition, as shown from the data in Fig. 1C, the inhibition under uptake conditions was also mixed-type with K_{is} and K_{ii} of $24 \pm 10 \mu\text{M}$ and $11.5 \pm 3.8 \mu\text{M}$, respectively (determined by the HPLC-UV, ADP quantification method). These results show that the inhibition of H^+ -ATPase under uptake conditions, i.e. in the absence of extra- or intra-granular Asc is significantly weaker than that of under conversion conditions. Experiments with K_2ATP and externally added Mg^{2+} (0-5 mM) showed that Mg^{2+} has no significant effect on the inhibition of H^+ -ATPase by copper (data not shown).

Reversibility and Time Independence of the Inhibition of H^+ -ATPase Activity in Chromaffin Granule Ghosts by Cu_{out}

In order to determine whether the effect of Cu_{out} on the H^+ -ATPase activity of granule ghosts was time dependent or irreversible, ghosts were pre-incubated with $7 \mu\text{M}$ Cu_{out} , under conversion conditions and assayed for the remaining H^+ -ATPase activity as a function of time, for a period of 35 min, by diluting the aliquots of the incubate into a standard assay solution, similar to the standard dilution assay for irreversible inhibitors. In these experiments, the final copper concentrations in the diluted incubates were $1.1 \mu\text{M}$ which were below the concentrations required for significant inhibition of the of H^+ -ATPase activity. These data show that the ATPase activity of the Cu_{out} pre-incubated sample was not significantly different from a parallel control without copper pre-incubation, for the entire 35 min time course of the experiment, demonstrating that the effect of Cu_{out} on the H^+ -ATPase is time independent and reversible (Fig. 2).

Fig. 2

Effects of Cu_{out} on the Transmembrane pH-Gradient of Chromaffin Granule Ghosts

The data presented in Fig. 3A show that 1-5 μM of Cu_{out} at 1.0 mM ATP concentration inhibit the H^+ -ATPase-mediated generation of the pH-gradient in a concentration dependent manner, with a complete inhibition at 5 μM Cu_{out} , as determined by the well defined acridine orange method (Cidon et al. 1983). The data also indicate that Cu_{out} affects both the initial rate of the generation of the pH gradient as well as the magnitude of the final gradient. Parallel to the H^+ -ATPase inhibition results, the pH gradient generation inhibition by Cu_{out} under uptake conditions is less pronounced with $27 \pm 2\%$ inhibition at 5 μM copper. But, essentially complete inhibition was observed at 10 μM concentrations at an ATP concentration of 1.0 mM (Fig. 3B). In addition, a fully pre-generated pH-gradient (by pre-incubating the ghosts with 1.0 mM extragranular Mg^{2+} -ATP) is also rapidly dissipated upon the addition of 10 μM Cu_{out} under both sets of conditions (Fig. 4). Interestingly, exclusion of intragranular Asc from the conversion conditions or inclusion of intragranular copper up to 30 μM under uptake or conversion conditions had no significant effect on Cu_{out} -mediated effects on the pH-gradient (data not shown). Similarly, exclusion of catalase from intra- and extra-granular media did not significantly alter the outcome of the above experiments. Furthermore, similar concentrations of Zn^{2+} had only minor effects on the pH-gradient under similar experimental conditions (data not shown).

Effects of Copper Chelators, on the Cu_{out} -mediated Effects on the pH-gradient of Chromaffin Granule Ghosts

The details of the inhibition of H^+ -ATPase by Cu_{out} was further examined by using the well-characterized copper chelators Triethylenetetramine [Trien; (Golub et al. 1995)] and 2,9-dimethyl-1,10-phenanthroline [neocuproine; (Lei and Anson 1995)]. As shown in Fig. 5,

Fig. 3

Fig. 4

Fig. 5

inclusion of Trien in the incubation medium prior to Cu_{out} treatment effectively abolishes the inhibition of the pH-gradient generation in a concentration dependent manner under both uptake (data not shown) and conversion conditions (Fig. 5). Neocuproine was also effective in preventing the inhibition, under both uptake and conversion conditions; however, somewhat less effective in comparison to similar concentrations of Trien (data not shown). Interestingly, as shown in Fig. 6, the high affinity copper binding domain of APP, $\text{A}\beta_{1-42}$, also prevents the inhibition of pH gradient generation by Cu_{out} . For example, while 5 μM Cu_{out} inhibit the H^+ -ATPase mediated generation of the pH gradient by $73.0 \pm 0.6\%$ under uptake conditions², with the inclusion of 3 μM and 5 μM $\text{A}\beta_{1-42}$ with 5 μM copper, the inhibition was reduced to $29.0 \pm 0.6\%$ and essentially zero, respectively. These results demonstrate that $\text{A}\beta_{1-42}$ is also effective in protecting H^+ -ATPase from the inhibition by Cu_{out} .

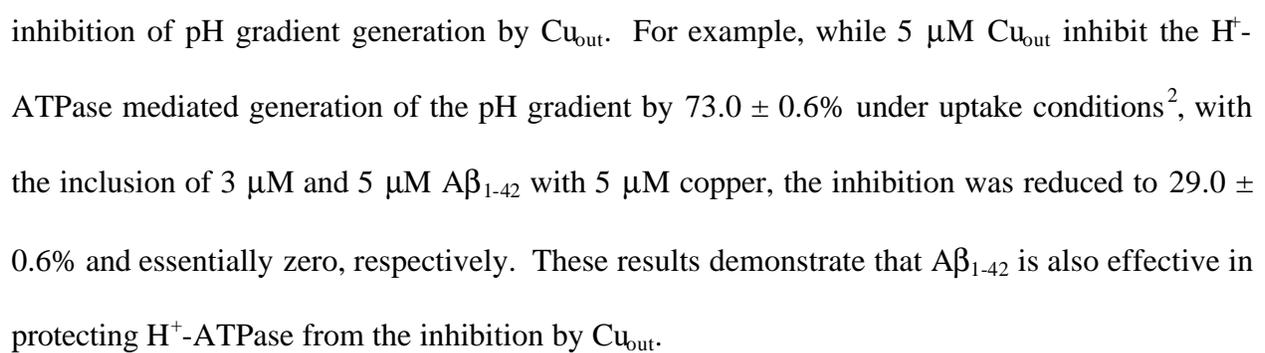


Fig. 6

Effect of Cu_{out} on the DA Uptake and Intragranular Conversion to NE in Chromaffin Granule Ghosts

As shown in Fig. 7A, under conversion conditions, Cu_{out} (0-20 μM) inhibits the intragranular DA accumulation, in a concentration dependent manner (50% inhibition at ~3-4 μM extragranular copper), with respect to a standard control without added Cu_{out} . Intragranular E, NE as well as total catecholamine levels were also decreased with increasing Cu_{out} under these conditions with respect to the control, although they are less pronounced in comparison to the effects on DA accumulation (Figs. 7B, C & D). The effects of Cu_{out} on the intragranular catecholamine levels under uptake conditions were parallel to the results under conversion conditions, but significantly less pronounced (Fig. 7) as expected. Control experiments revealed that the exclusion of catalase from intra- or extra-granular media or 10 min pre-incubation of ghosts with

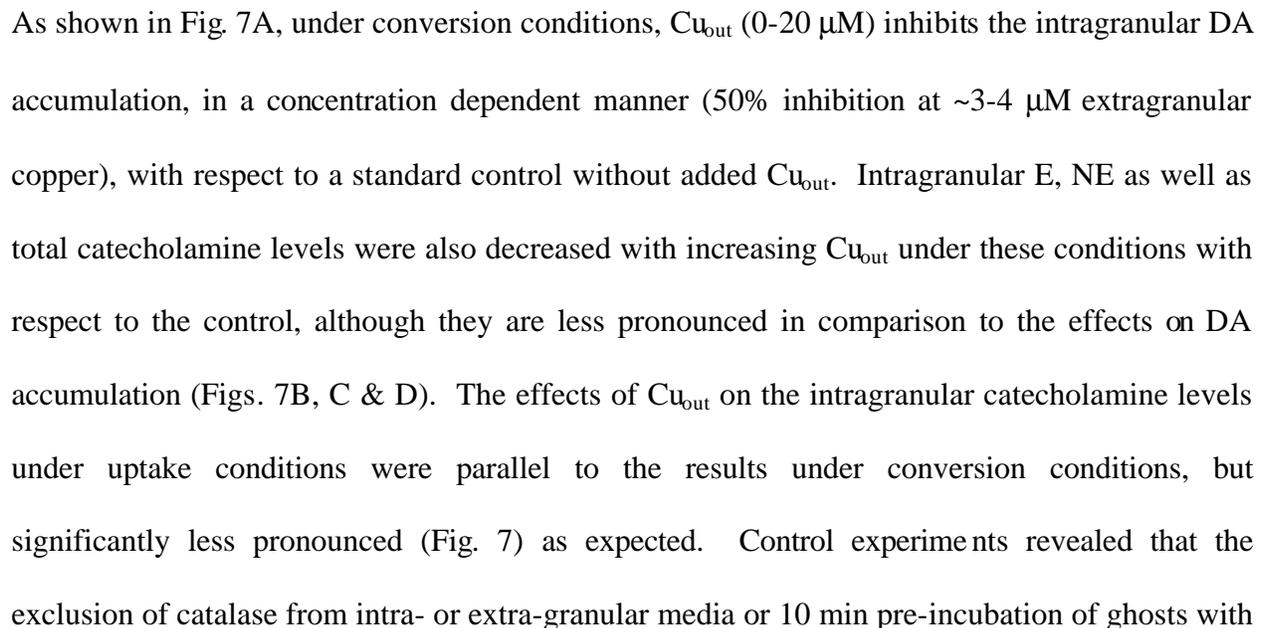


Fig. 7

Cu_{out} had no significant effect on the results of the above experiments under either condition (data not shown) suggesting neither H_2O_2 in the medium nor time dependent irreversible oxidative processes are responsible for the observed perturbation of DA uptake and conversion to NE by copper. Similarly, intragranular copper up to 30 μM had no significant effect on the DA accumulation or intragranular NE or E levels under uptake or conversion conditions or attenuating effect on the Cu_{out} -mediated effects (data not shown). In addition, the effects of Cu_{out} on the intragranular E and NE levels were not significantly affected by the replacement of DA with the non-redox active vesicular monoamine transporter/D β M substrate, tyramine.

Effect of Copper on Cellular DA Metabolism

In order to determine the physiological significance of the findings with granule ghosts at the cellular level, a series of experiments were carried with undifferentiated SH-SY5Y cells. Initial studies revealed that undifferentiated SH-SY5Y cells contain very low levels of DA and NE, and extracellular DA is efficiently taken up and converted to NE in a dose- and time-dependent manner (data not shown; manuscript in preparation). We also established that DA uptake is fully saturated at 50 μM and linear at least up to 10 min under our experimental conditions (data not shown). In these experiments, the intracellular origin of DA was confirmed by including DHBA as an external standard at the end of the experiment, prior to the washing steps (see Materials and methods). Incubations with copper(II) were carried out in the absence of ascorbate in KRB using a two stage reaction procedure in order to minimize the extracellular oxidative damage. Thus, the cells were initially incubated with the desired concentration of copper for 30 min, washed and immediately used for the DA uptake and conversion in the presence of ascorbate as

described in the experimental section. We note that catecholamine metabolites DOPAC and HVA are very low and insignificant in all these experiments (data not shown).

Reversed phase HPLC-EC separation and quantification of intracellular catecholamines allowed the accurate determination both cellular uptake and conversion of DA under various experimental conditions. As shown in Fig. 8, SH-SY5Y cells that were pre-incubated with extracellular copper for 30 min caused a dramatic decrease in the intracellular levels of both DA (panel A) and NE (panel B) with respect to copper untreated controls. The effects were clearly apparent at 25 μ M and maximal at 75 μ M at 30 min exposure. On the other hand, copper treated cells appeared normal throughout the incubations, as assessed by phase contrast microscopy and, no loss of number of cells were apparent as determined by total protein measurements suggesting that the low levels of catecholamine in copper treated cells could not be due to the loss of cell viability. The decrease of DA uptake and conversion was proportional to the intracellular copper level as assessed by atomic absorption spectroscopy (data not shown).

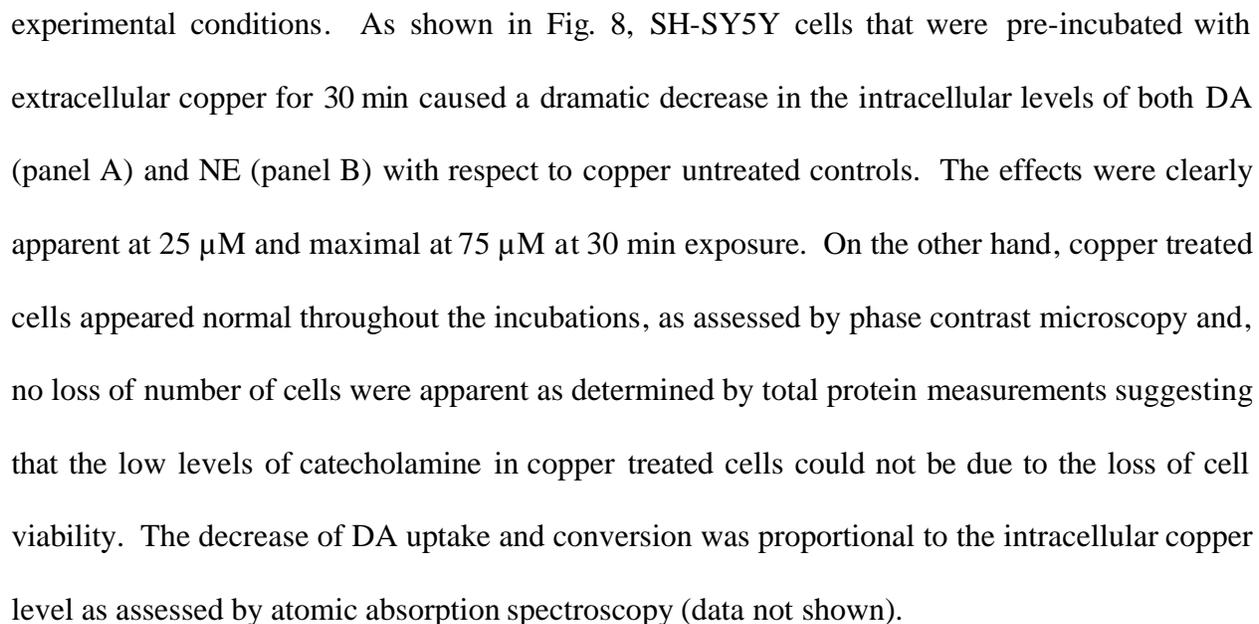


Fig. 8

In order to determine whether the depletion of catecholamines in copper pre-treated cells was due to the inhibition of the cellular uptake of DA by extracellular copper, a second series of experiments were carried out using an altered protocol in which cells were first treated with 50 μ M DA and 50 μ M Asc for 10 min as usual, and 50 μ M copper was included during the 60 min second incubation period. As shown from the data in Fig. 9, the level of both NE and DA were decreased to a similar extent, as in the copper pre-treated experiments above, ruling out the possibility that the depletion of DA and NE by copper is due to the inhibition of cellular DA uptake. In order to further confirm that the catecholamine depleting effects of copper is associated with the perturbation of the granulation of cytosolic DA, the effects of copper on intracellular catecholamine levels is compared with that of reserpine which is known to deplete

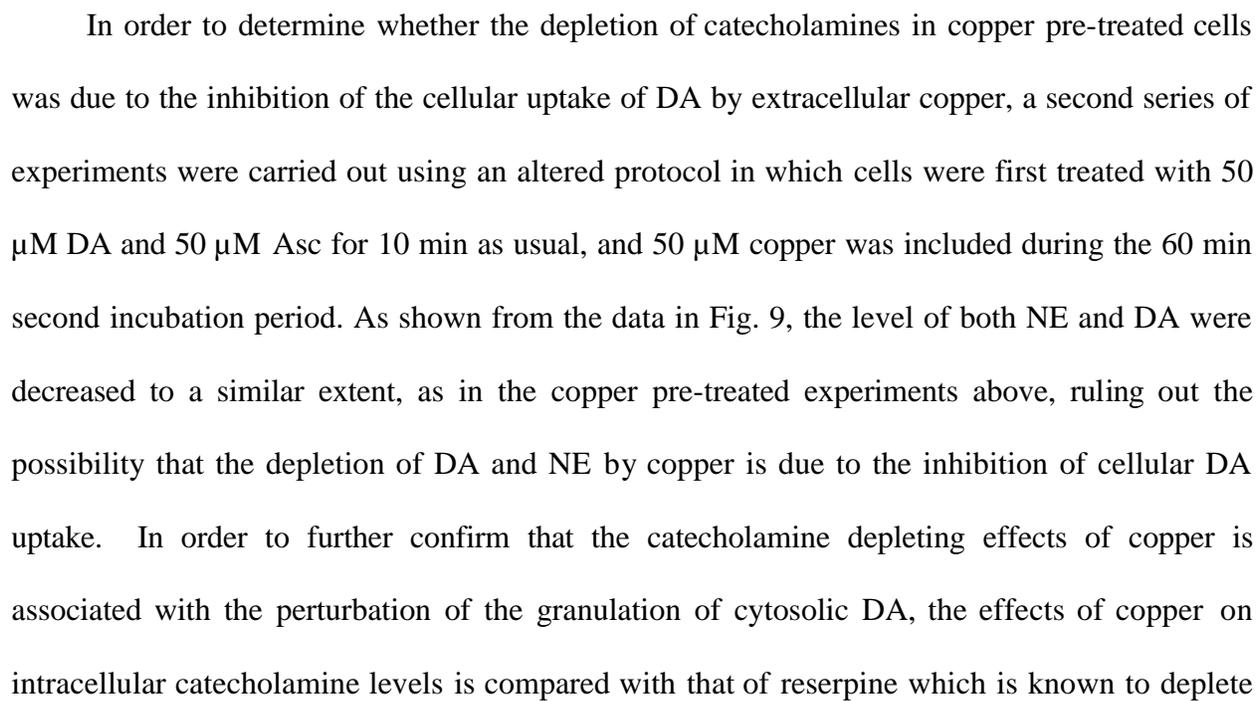


Fig. 9

cellular catecholamines through VMAT inhibition (Dixon et al. 1975). In these experiments, SH-SY5Y cells were initially incubated with 50 μ M DA and 50 μ M Asc for 10 min and 100 nM reserpine was included during the 60 min second period. As shown from the data in Fig. 9, the level of both NE and DA were decreased to a similar extent in both copper and reserpine treated cells confirming that the effect of copper on intracellular catecholamine is similar to that of reserpine.

Discussion

Bovine adrenal chromaffin granule ghosts are a convenient model to study the coordinated functions of vital granule membrane proteins with respect to uptake, biosynthetic transformation, and storage of monoamine neurotransmitters (Wimalasena and Wimalasena 1995; Wimalasena and Wimalasena 2004). We and others have previously shown that chromaffin granule ghosts resealed to contain Asc actively accumulate extragranular DA and efficiently converts it to NE, in the presence of extragranular ATP and Asc (conversion conditions), and could be used to study the kinetics and mechanistic details of DA uptake and conversion to NE. On the other hand, ghosts prepared to contain no intragranular Asc, but a high concentration of extra- and intra-granular ascorbate oxidase (uptake conditions), could be used to study DA uptake, since only a small intragranular DA to NE conversion is observed under these conditions (Wimalasena and Wimalasena 1995; Wimalasena and Wimalasena 2004). In the present study, highly purified resealed chromaffin granule ghosts have been used to characterize the inhibition of H⁺-ATPase by Cu_{out}. The physiological relevance of the findings of granule ghost experiments was tested using the catecholaminergic cell line, SH-SY5Y, under conditions.

The apparent steady state kinetic parameters, K_m and V_{max} , for the H⁺ATPase activity of resealed granule ghosts were determined using two separate assays under multiple assay

conditions. The apparent V_{\max} determined in the presence of intra- and extra-granular Asc was similar to the value reported previously for the membrane fragment H^+ -ATPase activity (Johnson et al. 1982). However, the apparent K_m determined in the present study was significantly higher than the previously reported value [$\sim 400 \mu\text{M}$ vs. $69 \mu\text{M}$ (Johnson et al. 1982)]. However, additional experiments revealed that the K_m for ATP was independent of the assay conditions used. Therefore, we conclude that the difference of apparent K_m with respect to ATP must primarily be due to the differences in the use of membrane fragments in the previous studies and intact resealed granule ghosts in the present study.

Steady state kinetic studies show that low micromolar concentrations of Cu_{out} effectively inhibit the H^+ -ATPase activity of resealed chromaffin granule ghosts, under conversion conditions (conversion conditions closely mimic the physiological conditions). The inhibition was mixed-type with respect to ATP, suggesting that Cu_{out} interacts with both ATP bound and unbound forms of H^+ -ATPase with similar affinities. Experiments with K_2ATP and externally added Mg^{2+} (0-5 mM) showed no significant effect on the inhibition kinetics, suggesting that Cu_{out} does not interact with the Mg^{2+} binding site of H^+ -ATPase. The inhibition was less pronounced in the absence of Asc_{out} , even in the presence of intragranular Asc, but retained the same mixed-type of inhibition pattern. Since intragranular Asc has no significant effect on the inhibition potency, the difference in the inhibition potencies in the presence and absence of Asc_{out} could be due to the preferential reduction of Cu_{out} by Asc_{out} , and that copper(I) is a better inhibitor for H^+ -ATPase than copper(II). On the other hand, if copper(II) is not an effective inhibitor, then the weaker inhibition in the absence of Asc_{out} could be due to the weak or partial reduction of copper(II) by small amounts of contaminating Asc/catechols in ghost preparations or other reducing agents in the incubation medium. We have routinely observed that granule

preparations contain small contaminations of Asc, which could not be removed, even with high concentrations of ascorbate oxidase without losing the functional integrity of granules (unpublished observations).

We are aware that H_2O_2 is closely associated with the Asc/copper-mediated generation of reactive oxygen species [see (Milton 2004) and references therein]. In addition, Wang and Floor (Wang and Floor 1998) have reported that H^+ -ATPase in synaptic vesicle preparations is irreversibly inactivated by 50-100 μM concentrations of H_2O_2 in a time- and concentration-dependent manner. However, these authors have shown that inclusion of extragranular catalase fully protects the H^+ -ATPase from inhibition by H_2O_2 . Since a high concentration of catalase (6500 U/mL) is included in the extra- and intra-granular media in all the granule experiments reported in this study any H_2O_2 generated in the reaction medium by Asc/copper pair should be efficiently scavenged. Thus, the inhibition of H^+ -ATPase by Cu_{out} must primarily be due to the direct and reversible interaction of Cu_{out} with H^+ -ATPase, since the production of reactive oxygen species through Fenton or other chemistry under these conditions should be minimal. This view is also strongly supported by the following observations. First, dilution assay experiments have shown that the resealed granule ghosts pre-incubated with 7 μM Cu_{out} in the presence of Asc_{out} retain at least 95% of the initial H^+ -ATPase activity for 35 min incubation period (Fig. 2). Second, exclusion of extra- or intra-granular catalase had no effect on the kinetics of the inhibition of H^+ -ATPase by Cu_{out} , suggesting that H_2O_2 plays no significant role in the inhibition of H^+ -ATPase under the experimental conditions.

The inhibition of H^+ -ATPase by Cu_{out} is also associated with the inhibition of the transmembrane pH-gradient generation, as expected (Figs. 3A & B). Parallel to H^+ -ATPase inhibition, pH gradient generation inhibition was also more pronounced in the presence of Asc_{out} .

Cu_{out} also causes the instant and rapid dissipation of the pre-generated pH gradient under both uptake and conversion conditions suggesting that the inhibition of the H^+ -ATPase activity lead to rapid dissipation of the transmembrane pH gradient in resealed granule ghosts (Fig. 4). On the other hand, Cu_{out} may also interact with the granule membrane to dissipate the pre-generated pH gradient, independent of H^+ -ATPase inhibition. However, this possibility is unlikely since the kinetics and concentration dependency of H^+ -ATPase and pH gradient generation inhibitions by Cu_{out} were comparable (Figs. 1A & B & 2A)³ and the two processes appear to be related.

The effective synthetic copper chelators, Trien and neocuproine, abolish the inhibition of H^+ -ATPase by Cu_{out} , in the presence or absence of extra-granular Asc, when added prior to the addition of copper to the incubation medium, demonstrating only the solution free copper is capable of interacting with H^+ -ATPase (Fig. 5). However, the delayed addition of copper chelators, even 20-30 sec after the addition of copper, does not reverse the effects of Cu_{out} on the pH gradient completely. These results appear to indicate a fast and apparent irreversible effect of Cu_{out} on H^+ -ATPase. However, as argued above, dilution assay and other experimental evidence strongly suggest that the effect of Cu_{out} on the H^+ -ATPase is time independent and reversible (Fig. 2). Therefore, this behavior could be due to the complex chemistry of copper(II) in the presence of Asc in the incubation medium. For example, copper(II) in the medium could be effectively reduced to copper(I) by Asc_{out} (slowly by contaminating Asc/catechols in the absence of added Asc_{out}) under the experimental conditions and the chelators may not effectively remove copper(I) from the solution, due to weak copper(I) chelation properties and/or the low solubility of copper(I) in the incubation medium. On the other hand, the copper-binding site of H^+ -ATPase may have a higher affinity for Cu_{out} than the chelator, under the experimental conditions especially if copper(I) interacts with the protein. We note that the effective solution copper(I)

concentrations under the experimental conditions could be much lower than the externally added Cu_{out} concentrations; thus, effective inhibition constants could also be much lower than the above reported values, if copper(I) is responsible for the inhibition. Although numerous attempts were made to better define these possibilities by direct examination of the effect of copper(I) on H^+ -ATPase, these experiments were not successful due to the instability, low solubility and other technical difficulties associated with handling of copper(I) under the experimental conditions. Regardless of these apparent complexities, the above results show that the free Cu_{out} , most probably copper(I) in the solution, specifically and reversibly inhibit the H^+ -ATPase activity in the granule membrane causing the inhibition of pH gradient generation.

Cu_{out} also has a significant effect on the ATP dependent DA uptake and intragranular DA to NE conversion and total catecholamine levels in granule ghosts (Fig. 7) which is consistent with the H^+ -ATPase inhibition. DA uptake was the most affected with 50% inhibition at $\sim 3\text{-}4\ \mu\text{M}$ Cu_{out} under the conversion conditions. In addition, Cu_{out} also dissipates the intragranular E levels in granule ghosts (50% depletion at $\sim 7\text{-}8\ \mu\text{M}$ extragranular copper), suggesting that the dissipation of the transmembrane pH gradient may also cause the dissipation of the intragranular catecholamine gradients (note that 5-10 nmoles/mg protein of E is retained in standard resealed granule ghost preparations, although NE is not converted to E in chromaffin granules). A parallel behavior was also observed with the intragranular NE levels, but somewhat less pronounced than the effects on E, probably due to the slow conversion of some intragranular DA to NE under the experimental conditions. The effects of Cu_{out} on the intragranular catecholamine levels under uptake conditions were less pronounced than under conversion conditions (Fig. 7), following a trend similar to that was observed with the H^+ -ATPase inhibition patterns. In contrast to Cu_{out} , intragranular copper had no significant effect on the intragranular

catecholamine levels even at 30 μM concentration, in comparison to control experiments, under uptake or conversion conditions, suggesting that Cu_{out} interferes with the DA uptake and catecholamine gradients through interaction with the cytosolic phase of the granule membrane. In addition, the effects of Cu_{out} on the intragranular E and NE levels were not significantly affected by the replacement of DA with the non-redox active vesicular monoamine transporter/D β M substrate (Wimalasena and Wimalasena 1995), tyramine, indicating that the redox chemistry between dopamine/copper is not responsible for the effects of Cu_{out} on intragranular catecholamine levels. These findings together with the parallel kinetics observed for the inhibition of H^+ -ATPase activity, pH gradient generation, and DA uptake, with respect to Cu_{out} suggests that these three processes are interrelated and primarily dependent on the H^+ -ATPase inhibition.

The physiological significance of the above findings with granule ghosts is apparent from the SH-SY5Y cell experiments. These experiments show that the effects of extracellular copper on cellular catecholamine levels are similar to that of granule ghosts. Pre-incubation of SH-SY5Y cell with 25 μM extracellular copper reduces the cellular uptake of DA and its conversion to NE by $31 \pm 4\%$ and at 50 μM copper these levels were reduced by about $80 \pm 2\%$ with respect to a copper untreated control (Fig. 8). Similarly pre-loaded catecholamines are also depleted by extracellular copper (Fig. 9) suggesting that the depletion of catecholamine levels could not be due to the inhibition of cellular DA uptake by extra-cellular copper. Although one can argue that these results are due to the cellular damage or oxidative destruction of catecholamines by intracellular copper, several lines of evidence argue against such a possibility. (1) copper and DA/Asc incubations were carried out in two stages to minimize the interaction of copper with Asc and/or DA; (2) copper pre-incubations were carried out for a short periods of times, typically

30 min with low copper concentrations; (3) the order of incubation i.e. copper followed by DA/Asc *versus* DA/Asc followed by copper, does not significantly alter the outcome of the experiment; (4) effects of copper on NE levels could not be due to direct interaction of NE with copper, since NE is synthesized inside the granules and cytosolic copper does not get into the granules; (5) no significant loss of cell viability was observed under the experimental conditions. Therefore, we conclude that the depletion of the intra-cellular catecholamine levels by copper is due to the rapid dissipation of the granular catecholamine gradient as a consequence of the inhibition of H⁺-ATPase, similar to what was observed in the granule ghost model, resulting in the cellular efflux through the plasma membrane monoamine transporter. Side by side comparison of the effects of reserpine, which is known to deplete cellular catecholamines (Dixon et al. 1975) through VMAT inhibition, showed effects similar to that of copper treatment (Fig. 9) further supporting the above conclusions.

The efficient uptake and storage of monoamines and acetylcholine in storage vesicles is vital for the proper functioning of monoaminergic and cholinergic neurons, respectively. As mentioned above, the free energy for these processes is provided by the vesicular H⁺-ATPase generated transmembrane pH gradient (Apps et al. 1980; Knoth et al. 1980; Salama et al. 1980). Similarly, H⁺-ATPase generated pH gradient is also vital for the b₅₆₁-mediated Asc regenerating system in catecholaminergic neurones, since the interior acidic pH will shift the reduction potential of the Asc/semidehydro ascorbic acid couple from +70 mV outside (pH 7.0) to +220 mV inside (pH 5.5), facilitating the preferential electron flux from cytosol to the interior of the granule through, cytochrome b₅₆₁, to regenerate the ascorbic acid consumed by the DβM monooxygenation reaction (Njus et al. 1986; Wakefield et al. 1986; Njus et al. 1987). In addition, a high concentration of Asc, low pH, and low levels of redox active agents in granule

matrix assures a reductive environment which is critical in preserving catecholamines from autooxidation. Consequently, perturbation of intra-granular accumulation could lead to cytosolic accumulation and cellular efflux of catecholamines causing increased autooxidation, high oxidative stress, and consequent neurodegeneration (for example *see* Obata 2002 and references therein). Thus, the inhibition of vesicular H⁺-ATPase activity by cytosolic copper could exacerbate the generally high oxidative stress in catecholaminergic neurons.

Numerous previous studies suggest that the adverse effects of copper on the CNS are primarily mediated by the reactive oxygen species generated by the redox active copper, especially in the presence of strong reducing agents such as Asc (Perry et al. 2003; Barnham et al. 2004). However, the above findings suggest that the inhibition of the H⁺-ATPase activity by cytosolic copper could further contribute to the adverse effects of copper. Although intracellular free copper levels were assumed to be very low in most cells, based on the experiments with yeast cells (Rae et al. 1999; O'Halloran and Culotta 2000), the free copper levels in neuronal cells have never been determined or reported to our knowledge. On the other hand, significant levels of copper have been shown to continuously release from the storage vesicles during synaptic transmission reaching synaptic concentrations as high as 15 μ M under normal physiological conditions (Kardos et al. 1989; Hopt et al. 2003). Especially, adrenergic, noradrenergic, and amidated peptide containing neurons release high concentrations of the copper proteins, soluble-D β M and soluble-peptidyl- α -amidating monooxygenase respectively, during synaptic transmission. Thus, in overactive, stressed or disease conditions, the intra- and extra-cellular copper levels in the CNS could increase significantly, especially in the areas which are rich in adrenergic, noradrenergic, and/or peptidergic neurons. In agreement with this proposal, a markedly increased level of copper (70 μ M) is found in Alzheimer's disease affected

brains (Deibel et al. 1996; Lovell et al. 1998). Thus, as mentioned above, in addition to the ROS mediated effects, the inhibition of H⁺-ATPase by cytosolic free copper could also contribute to the neurotoxicity of copper.

Based on the above conclusions, it is tempting to speculate that the specific copper binding proteins in the CNS could be a part of an intricate mechanism in place for the regulation of Cu_{out} in neuronal cells especially under certain stress, overactive or disease conditions. While some of these proteins including copper-ATPase (Wilson's disease protein) may assist in the transport of copper through the cell and/or granule membranes [for example *see* (Brown and Harris 2003; Bellingham et al. 2004)], others may specifically bind solution copper to buffer high levels of cytosolic free copper (Sayre et al. 2000; Strausak et al. 2001; Barnham et al. 2004). The possibility that the deposition of copper-containing plaques in Alzheimer's disease may be to protect from free copper induced neurotoxicity has not been ruled out. Further work is needed to explore the significance of these findings and the physiological role of the CNS copper binding proteins with respect to various neurodegenerative diseases.

Acknowledgement

This work was supported by a grant from the National Institutes of Health NS 39423 (to K. W.) and RSEC fellowship (to T. J. W) from the National Science Foundation.

Figure Legends

Fig. 1. Steady State Kinetics of Inhibition of the H⁺-ATPase Activity in Chromaffin Granule Ghosts by Cu_{out} under Conversion (Panels A & B) and Uptake Conditions (Panel C). Resealed ghosts were incubated with desired concentrations of Cu_{out} and the reactions were initiated by the addition of the appropriate amount of ATP. The mixtures were incubated for 6 min and aliquots were withdrawn and H⁺-ATPase activity was determined either by quantifying inorganic phosphate by the colorimetric molybdate assay (panel A) or ADP by HPLC-UV at 259 nm (panels B & C) as detailed in Materials and methods. Initial rate kinetic data were analyzed by Cleland's COMP, UNCOMP, and NON-COMP programs (Cleland 1979) to determine the inhibition patterns and respective kinetic parameters. Cu_{out} concentrations were *Panel A:* (?) 0 μM, (?) 3 μM, (?) 4 μM, (?) 5 μM, and (?) 7 μM; *Panel B:* (?) 0 μM, (?) 3 μM, (?) 5 μM, (?) 7 μM, and (?) 10 μM; *Panel C:* (?) 0 μM, (?) 10 μM, (?) 15 μM, (?) 20 μM. Solid lines show the fit of the data to straight lines at each copper concentration.

Figure 2. Effect of Pre-incubation of Resealed Granule Ghosts with Extragranular Copper on the H⁺-ATPase Activity: Resealed chromaffin granule ghosts were incubated with 7 μM extragranular copper under conversion conditions. At the indicated time intervals, 100 μL aliquots were withdrawn and diluted into a standard H⁺-ATPase assay solution and the ATPase activity in each sample was determined by HPLC assay as described in Figure 1. A parallel identical control experiment was also carried out except that the 7 μM extragranular copper was omitted from the incubation medium. (---) remaining % H⁺-ATPase activity of the control without extragranular copper, (???) remaining % H⁺-ATPase activity of the test with 7 μM extragranular copper.

Fig. 3. Effect of Cu_{out} on the Transmembrane pH-Gradient of Chromaffin Granule Ghosts under Conversion (Panel A) and Uptake (Panel B) Conditions. The vesicular H⁺-ATPase

mediated generation of the transmembrane pH-gradient was monitored by acridine orange method (Cidon et al. 1983) following the decrease of $\Delta A_{(492-540)}$ nm as a function of time as described under Materials and methods in the presence of various concentrations of Cu_{out} and 1.0 mM ATP under conversion (*Panel A*) and uptake (*Panel B*) conditions. The copper concentrations were (?) 0, (?) 1, (?) 2, (?) 3, (?) 5, and (?) 10 μM .

Fig. 4. Dissipation of the Pre-generated Transmembrane pH-Gradient by Cu_{out} . The H^+ -ATPase mediated generation of the transmembrane pH-gradient was monitored as detailed in Fig. 3. After full generation of the pH-gradient (14 min), 10 μM copper was added and the dissipation of the pH-gradient was followed for an additional 10 min. (?) $\Delta A_{(492-540)}$ under uptake conditions; (?) $\Delta A_{(492-540)}$ under conversion conditions.

Fig. 5. Effect of Copper Chelator, Trien, on the Inhibition of the Transmembrane pH-Gradient Generation in Chromaffin Granule Ghosts by Cu_{out} . Chromaffin granule ghosts were preincubated with various concentrations of Trien under conversion conditions and 10 μM Cu_{out} and 1.0 mM ATP were added and the H^+ -ATPase mediated generation of the transmembrane pH gradient was monitored as detailed in Fig. 3. Trien concentrations were (?) 0, (?) 10, (?) 25, & (?) 200 μM .

Fig. 6. Effect of Ab_{1-42} on the inhibition of the Transmembrane pH-Gradient Generation in Chromaffin Granule Ghosts by Cu_{out} . Chromaffin granule ghosts were preincubated with various amounts of Ab_{1-42} under uptake conditions and desired amount of Cu_{out} and 1.0 mM ATP were added and the H^+ -ATPase mediated generation of the transmembrane pH gradient was monitored as detailed in Fig. 3. (?) 0 μM Cu_{out} , (?) 5 μM Cu_{out} , (?) 5 μM Cu_{out} and 3 μM Ab_{1-42} , (?) 5 μM Cu_{out} and 5 μM Ab_{1-42} .

Fig. 7. Effect of Cu_{out} on DA, NE, E, and Total Catecholamine Levels in Chromaffin Granule Ghosts. Resealed chromaffin granule ghosts were incubated with varying concentrations of Cu_{out} (0-30 μ M) under uptake and conversion conditions as described in Materials and methods. At zero time, the reactions were initiated with 200 μ M DA and intragranular catecholamine levels were determined after a 6 min incubation period. The fractions of various catecholamines were calculated with respect to the catecholamine levels of the zero Cu_{out} control. The baseline levels of DA, NE and E were in the range of 0, 5-8 and 4-7 nmoles/mg of protein, respectively, depending on the granule preparation. Intragranular DA (*panel A*), NE (*panel B*), E (*panel C*), and total catecholamine levels (*panel D*) under uptake (?) and (?) conversion conditions.

Fig. 8. Effect of Copper on DA Uptake and Conversion in SH-SY5Y Cells. SH-SY5Y cells were pre-treated with 0-100 μ M copper for 30 min, and subsequently allowed to accumulate DA (50 μ M) for 10 min, and convert to NE for 60 min as detailed in the experimental section. DA (*panel A*) and NE (*panel B*) were separated and quantified by HPLC-EC and normalized to protein content. Data are representative of experiments performed five times in duplicate or triplicate.

Fig. 9. Comparison of Effects of Reserpine and Copper on Pre-accumulated DA and NE in SH-SY5Y cells. SH-SY5Y cells were incubated with 50 μ M DA and 50 μ M ascorbate for 10 min. This solution was replaced with buffer or buffer containing 100 nM reserpine or 50 μ M copper and incubated 60 min. NE, DOPAC, and DA were separated and quantified by HPLC-EC and normalized to protein. Data are from a single experiment repeated four times in duplicate.

FIGURE 1

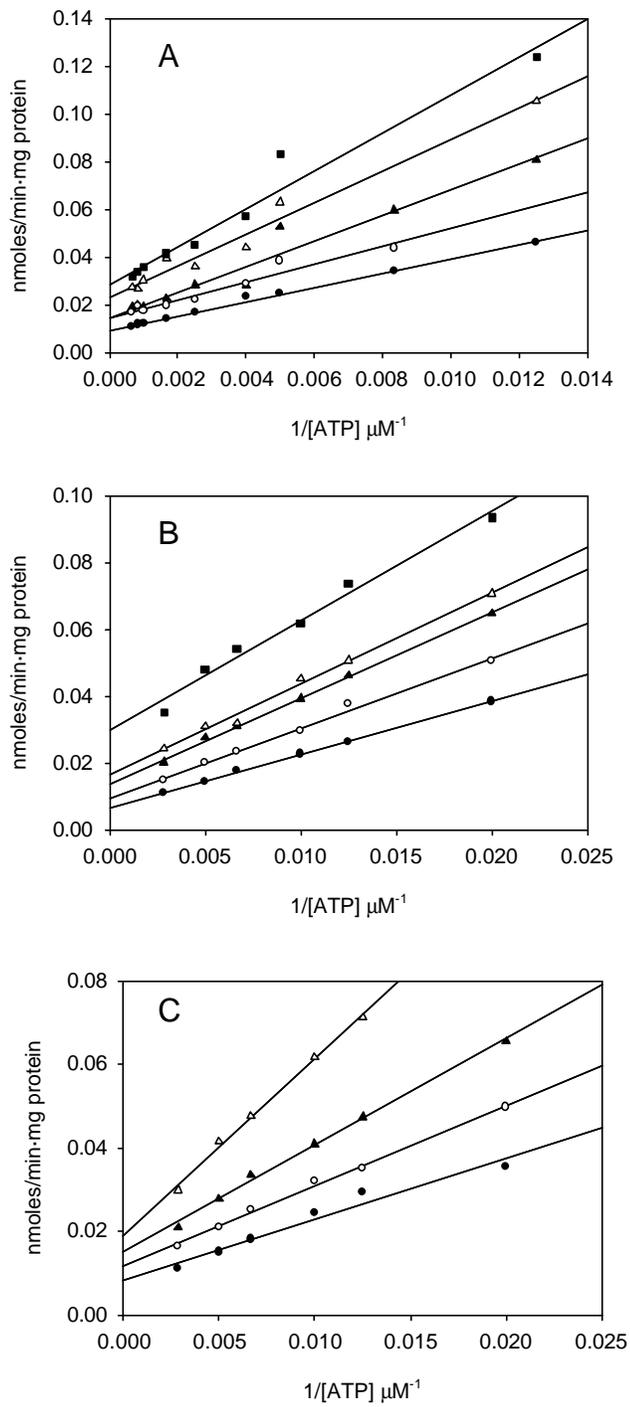


FIGURE 2

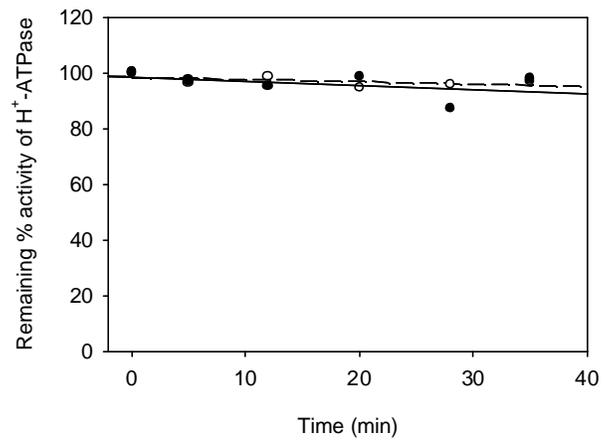


FIGURE 3

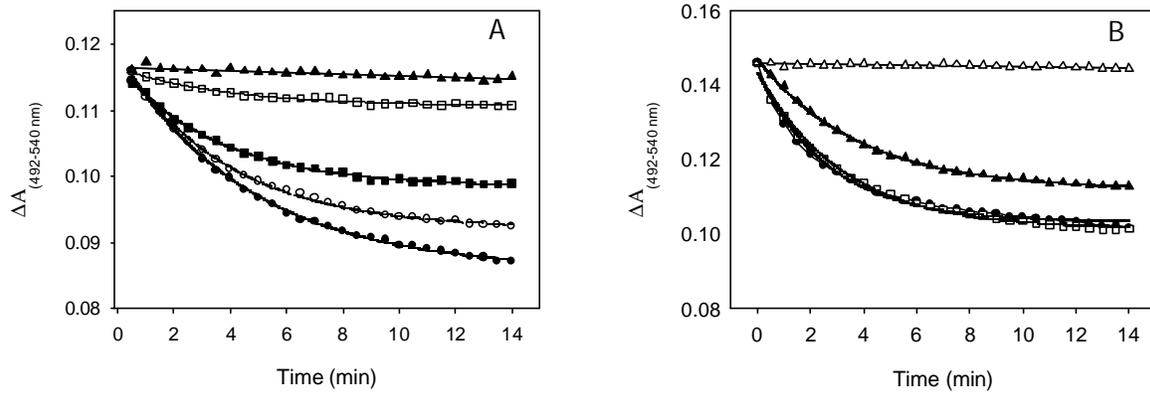


FIGURE 4

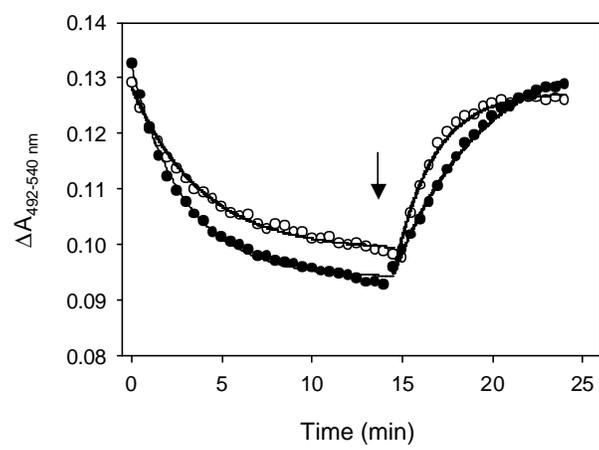


FIGURE 5

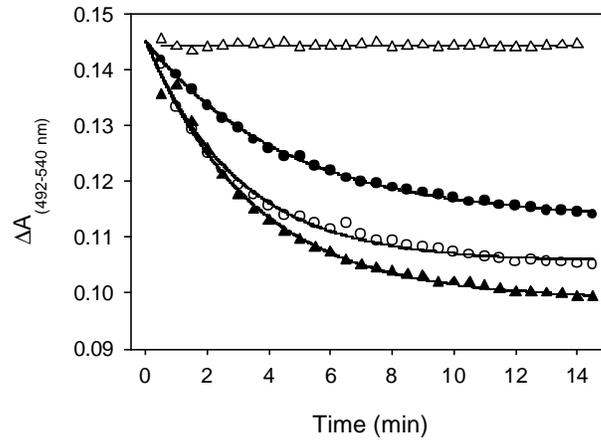


FIGURE 6

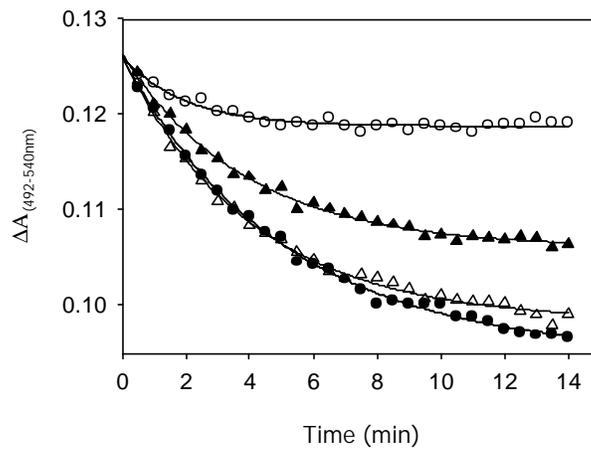


FIGURE 7

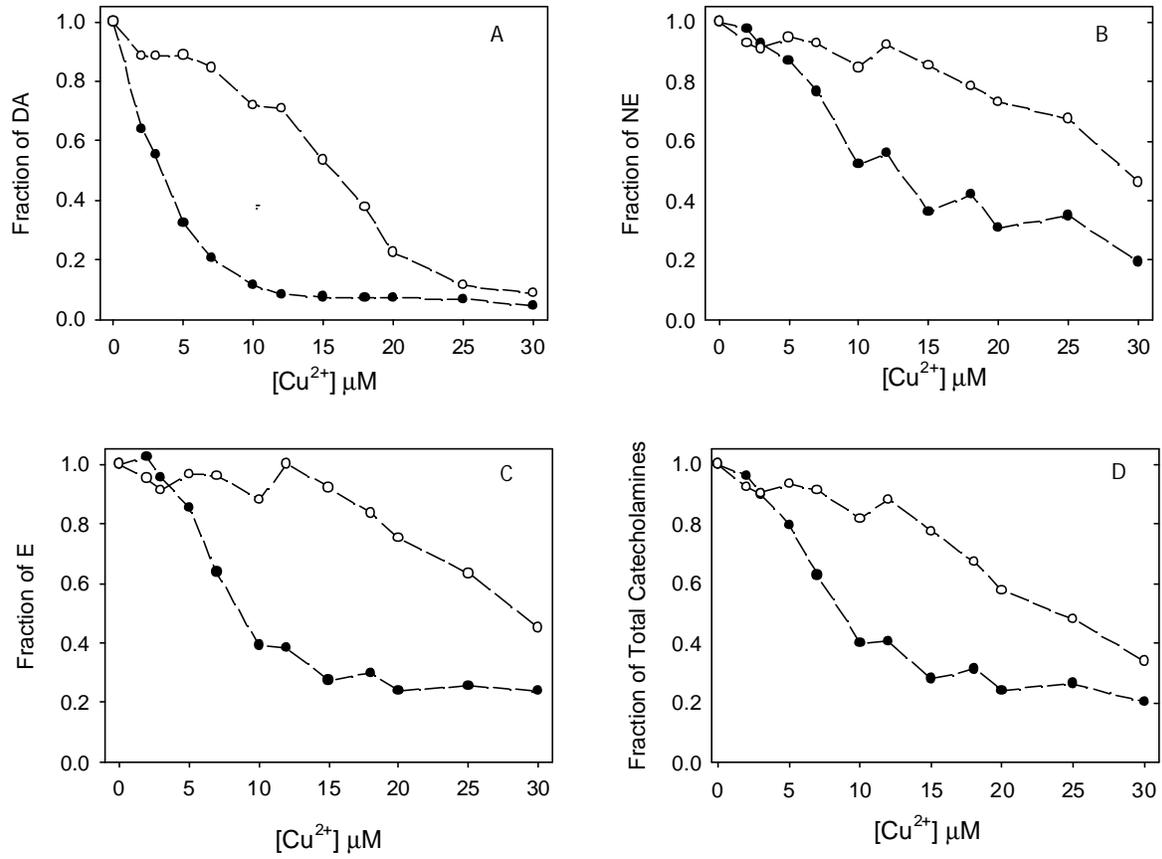


Fig. 8

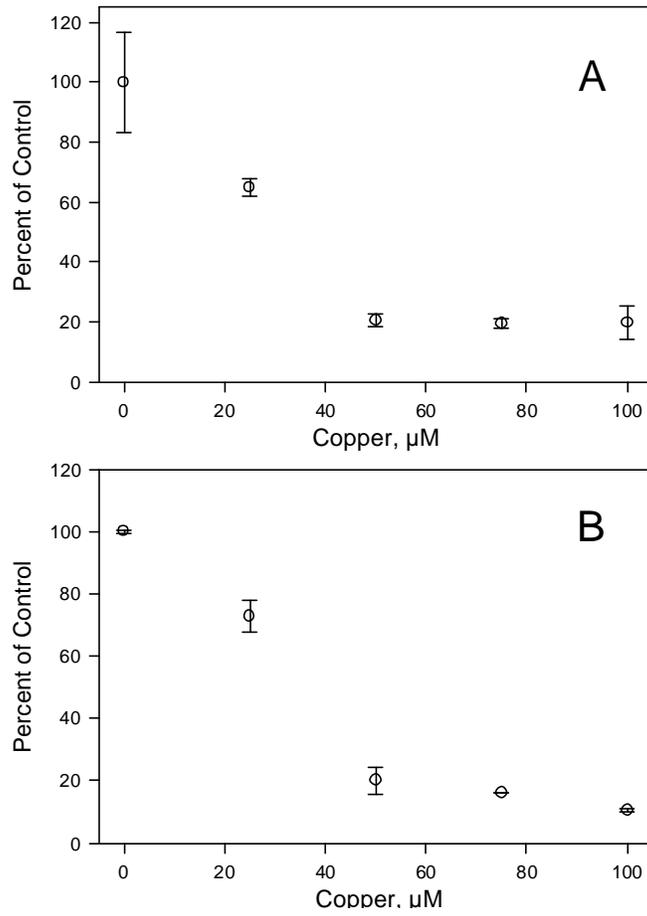
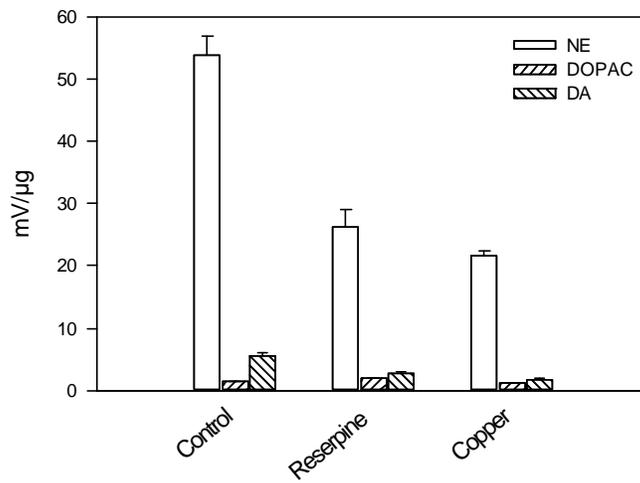


Fig. 9



References

- Apps D. K., Pryde J. G. and Phillips J. H. (1980) Both the transmembrane pH gradient and the membrane potential are important in the accumulation of amines by resealed chromaffin-granule 'ghosts'. *FEBS Letters* **111**, 386-390.
- Barnham K. J., Masters C. L. and Bush A. I. (2004) Neurodegenerative diseases and oxidative stress. *Nat.Rev.Drug Discov.* **3**, 205-214.
- Bellingham S. A., Lahiri D. K., Maloney B., La F. S., Multhaup G. and Camakaris J. (2004) Copper depletion down-regulates expression of the Alzheimer's disease amyloid-beta precursor protein gene. *Journal of Biological Chemistry* **279**, 20378-20386.
- Biedler J. L., Roffler-Tarlov S., Schachner M. and Freedman L. S. (1978) Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Res.* **38**, 3751-3757.
- Bradford M. M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry* **72**, 248-254.
- Brown L. R. and Harris D. A. (2003) Copper and zinc cause delivery of the prion protein from the plasma membrane to a subset of early endosomes and the Golgi. *J.Neurochem.* **87**, 353-363.
- Cidon S., Ben-David H. and Nelson N. (1983) ATP-driven proton fluxes across membranes of secretory organelles. *Journal of Biological Chemistry* **258**, 11684-11688.
- Cleland W. W. (1979) Statistical analysis of enzyme kinetic data. *Methods Enzymol.* **63**, 103-138.
- Deibel M. A., Ehmann W. D. and Markesbery W. R. (1996) Copper, iron, and zinc imbalances in severely degenerated brain regions in Alzheimer's disease: possible relation to oxidative stress. *J.Neurol.Sci.* **143**, 137-142.
- Dikalov S. I., Vitek M. P. and Mason R. P. (2004) Cupric-amyloid beta peptide complex stimulates oxidation of ascorbate and generation of hydroxyl radical. *Free Radic.Biol.Med.* **36**, 340-347.
- Dixon W. R., Garcia A. G. and Kirpekar S. M. (1975) Release of catecholamines and dopamine beta-hydroxylase from the perfused adrenal gland of the cat. *J.Physiol* **244**, 805-824.
- Gaetke L. M. and Chow C. K. (2003) Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology* **189**, 147-163.

- Gasnier B. (2000) The loading of neurotransmitters into synaptic vesicles. *Biochimie* **82**, 327-337.
- Golub G., Cohen H., Paoletti P., Bencini A., Messori L., Bertini L. and Meyerstein D. (1995) Use of Hydrophobic Ligands for the Stabilization of Low-Valent Transition Metal Complexes. 1. The Effect of N-Methylation of Linear Tetraazaalkane Ligands on the Properties of Their Copper Complexes. *J.Am.Chem.Soc.* **117**, 8353-8361.
- Gonzalez-Romo P., Sanchez-Nieto S. and Gavilanes-Ruiz M. (1992) A modified colorimetric method for the determination of orthophosphate in the presence of high ATP concentrations. *Analytical Biochemistry* **200**, 235-238.
- Harris E. D. (2000) Cellular copper transport and metabolism. *Annu.Rev.Nutr.* **20**, 291-310.
- Henry J. P., Sagne C., Bedet C. and Gasnier B. (1998) The vesicular monoamine transporter: from chromaffin granule to brain. *Neurochem.Int.* **32**, 227-246.
- Hesse L., Beher D., Masters C. L. and Multhaup G. (1994) The beta A4 amyloid precursor protein binding to copper. *FEBS Letters* **349**, 109-116.
- Hopt A., Korte S., Fink H., Panne U., Niessner R., Jahn R., Kretschmar H. and Herms J. (2003) Methods for studying synaptosomal copper release. *J.Neurosci.Methods* **128**, 159-172.
- Johnson R. G., Beers M. F. and Scarpa A. (1982) H⁺ ATPase of chromaffin granules. Kinetics, regulation, and stoichiometry. *Journal of Biological Chemistry* **257**, 10701-10707.
- Kardos J., Kovacs I., Hajos F., Kalman M. and Simonyi M. (1989) Nerve endings from rat brain tissue release copper upon depolarization. A possible role in regulating neuronal excitability. *Neurosci.Lett.* **103**, 139-144.
- Knoth J., Handloser K. and Njus D. (1980) Electrogenic epinephrine transport in chromaffin granule ghosts. *Biochemistry* **19**, 2938-2942.
- Lei Y. and Anson F. C. (1995) Dynamics of the Coordination Equilibria in Solutions Containing Copper(II), Copper(I), and 2,9-Dimethyl-1,10-phenanthroline and Their Effect on the Reduction of O₂ by Cu(I). *Inorg.Chem.* **34**, 1083-1089.
- Lovell M. A., Robertson J. D., Teesdale W. J., Campbell J. L. and Markesbery W. R. (1998) Copper, iron and zinc in Alzheimer's disease senile plaques. *J.Neurol.Sci.* **158**, 47-52.
- Milton N. G. (2004) Role of hydrogen peroxide in the aetiology of Alzheimer's disease: implications for treatment. *Drugs Aging* **21**, 81-100.

- Multhaup G., Schlicksupp A., Hesse L., Beher D., Ruppert T., Masters C. L. and Beyreuther K. (1996) The amyloid precursor protein of Alzheimer's disease in the reduction of copper(II) to copper(I). *Science* **271**, 1406-1409.
- Nishikawa T., Lee I. S., Shiraishi N., Ishikawa T., Ohta Y. and Nishikimi M. (1997) Identification of S100b protein as copper-binding protein and its suppression of copper-induced cell damage. *Journal of Biological Chemistry* **272**, 23037-23041.
- Njus D., Kelley P. M. and Harnadek G. J. (1986) Bioenergetics of secretory vesicles. *Biochim.Biophys.Acta* **853**, 237-265.
- Njus D., Kelley P. M., Harnadek G. J. and Pacquing Y. V. (1987) Mechanism of ascorbic acid regeneration mediated by cytochrome b561. *Ann.N.Y.Acad.Sci.* **493**, 108-119.
- Obata T. (2002) Dopamine efflux by MPTP and hydroxyl radical generation. *J. Neural Transm.* **109**, 1159-1180.
- O'Halloran T. V. and Culotta V. C. (2000) Metallochaperones, an intracellular shuttle service for metal ions. *Journal of Biological Chemistry* **275**, 25057-25060.
- Paik S. R., Shin H. J., Lee J. H., Chang C. S. and Kim J. (1999) Copper(II)-induced self-oligomerization of alpha-synuclein. *Biochem.J.* **340 (Pt 3)**, 821-828.
- Parsons S. M. (2000) Transport mechanisms in acetylcholine and monoamine storage. *FASEB J.* **14**, 2423-2434.
- Perry G., Taddeo M. A., Petersen R. B., Castellani R. J., Harris P. L., Siedlak S. L., Cash A. D., Liu Q., Nunomura A., Atwood C. S. and Smith M. A. (2003) Adventitiously-bound redox active iron and copper are at the center of oxidative damage in Alzheimer disease. *Biometals* **16**, 77-81.
- Prohaska J. R. and Gybina A. A. (2004) Intracellular Copper Transport in Mammals. *J.Nutr.* **134**, 1003-1006.
- Rae T. D., Schmidt P. J., Pufahl R. A., Culotta V. C. and O'Halloran T. V. (1999) Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science* **284**, 805-808.
- Salama G., Johnson R. G. and Scarpa A. (1980) Spectrophotometric measurements of transmembrane potential and pH gradients in chromaffin granules. *J.Gen.Physiol* **75**, 109-140.
- Samizo K., Ishikawa R., Nakamura A. and Kohama K. (2001) A highly sensitive method for measurement of myosin ATPase activity by reversed-phase high-performance liquid chromatography. *Analytical Biochemistry* **293**, 212-215.

- Sayre L. M., Perry G., Atwood C. S. and Smith M. A. (2000) The role of metals in neurodegenerative diseases. *Cell Mol.Biol.(Noisy.-le-grand)* **46**, 731-741.
- Schuldiner S., Shirvan A. and Linial M. (1995) Vesicular neurotransmitter transporters: from bacteria to humans. *Physiol Rev.* **75**, 369-392.
- Stockel J., Safar J., Wallace A. C., Cohen F. E. and Prusiner S. B. (1998) Prion protein selectively binds copper(II) ions. *Biochemistry* **37**, 7185-7193.
- Strausak D., Mercer J. F., Dieter H. H., Stremmel W. and Multhaup G. (2001) Copper in disorders with neurological symptoms: Alzheimer's, Menkes, and Wilson diseases. *Brain Res.Bull.* **55**, 175-185.
- Taupenot L., Harper K. L. and O'Connor D. T. (2005) Role of H⁺-ATPase-mediated acidification in sorting and release of the regulated secretory protein chromogranin A: evidence for a vesiculogenic function. *Journal of Biological Chemistry* **280**, 3885-3897.
- Ushimaru M. and Fukushima Y. (2003) Complete separation of adenine nucleotides for ATPase activity assay by ion-pair reversed-phase high-performance liquid chromatography. *Analytical Biochemistry* **313**, 173-175.
- Viles J. H., Cohen F. E., Prusiner S. B., Goodin D. B., Wright P. E. and Dyson H. J. (1999) Copper binding to the prion protein: structural implications of four identical cooperative binding sites. *Proc.Natl.Acad.Sci.U.S.A* **96**, 2042-2047.
- Wakefield L. M., Cass A. E. and Radda G. K. (1986) Functional coupling between enzymes of the chromaffin granule membrane. *Journal of Biological Chemistry* **261**, 9739-9745.
- Wang Y. and Floor E. (1998) Hydrogen peroxide inhibits the vacuolar H⁺-ATPase in brain synaptic vesicles at micromolar concentrations. *J.Neurochem.* **70**, 646-652.
- Wimalasena D. S. and Wimalasena K. (2004) Kinetic evidence for channeling of dopamine between monoamine transporter and membranous dopamine-beta-monooxygenase in chromaffin granule ghosts. *Journal of Biological Chemistry* **279**, 15298-15304.
- Wimalasena K. and Wimalasena D. S. (1995) The reduction of membrane-bound dopamine beta-monooxygenase in resealed chromaffin granule ghosts. Is intragranular ascorbic acid a mediator for extragranular reducing equivalents? *Journal of Biological Chemistry* **270**, 27516-27524.