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Effect of some Cardiac and Respiratory Drugs on Succinate-Cytochrome c Reductase

MAHMOUD BALBAA*, JEHAN AL-MEER and AMINA AL-KHAL

Department of Chemistry, College of Science, Qatar University, P.O. Box 2713, Doha, Qatar

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Succinate-cytochrome c reductase was inhibited *in vitro* and *in vivo* by phenobarbitone, aminophylline and neostigmine using both 2,6-dichlorophenolindophenol (DCIP) and cytochrome c (cyt c) as substrates. The enzyme was also activated by gallamine towards both substrates. *In vitro*, phenobarbitone and aminophylline inhibited the enzyme with respect to the reduction of DCIP and cyt c in a non-competitive manner with K_i values of 1.5×10^{-5} and 5.7×10^{-5} M, respectively. Moreover, neostigmine competitively inhibited the enzyme towards both substrates with K_i values of 1.36×10^{-5} and 1.50×10^{-5} M, respectively.

Keywords: Inhibition; Cytochrome c reductase; Kinetic parameters

INTRODUCTION

The main respiratory chain in mitochondria consists of dehydrogenases, including succinate-cytochrome c reductase (SCR), linked to flavoproteins and the cytochrome system.[1] This enzyme catalyzes the oxidation of succinate by cytochrome c (cyt c) or 2,6-dichlorophenolindophenol (DCIP) under optimal conditions.[1] The reduction of DCIP by succinate was found to be highly dependent upon DCIP concentration.[2] This enzyme complex is described as reconstituted active succinate dehydrogenase and possesses the ability to reconstitute with hydroquinone[3] or with soluble cytochromes b-c1 complex[4] and uses ubiquinone as an electron acceptor.[5]

Several drugs have been implicated as having various respiratory and cardiac effects. For example, phenobarbitone (Figure 1) is a central nervous system sedative and acts as a short acting barbiturate in the sedation of angina pectoris[6,7] and is also an anticonvulsant.[8] Neostigmine, which is a stimulant of the parasympathetic nervous system, acts as a reversible anti-cholinesterase[9] for the treatment myasthenia gravis and certain types of glaucoma[9] and is implicated in the development of post-operative nausea.[10] Aminophylline (75% theophylline (Figure 1) and 25% ethylenediamine) is a vasodilator,[11] which improves the cardiac index and pulmonary vascular resistance.[12] Gallamine is considered as a muscle relaxant that has a cardiovascular effect.[13–15] These drugs were selected for the present study because of their clinical application without enough knowledge of their specific action on the enzymes of the respiratory chain. Here, the effect of these drugs on the intact SCR is described.

MATERIALS AND METHODS

Chemicals and Reagents

Crystalline Oxidized horse cyt c type III was purchased from Sigma-Aldrich (Chemie Steinheim, Germany). Its concentration was determined spectrophotometrically using the millimolar extinction coefficient of 18.5 at 550 nm (difference spectrum of reduced minus oxidized forms).[16] DCIP and phenobarbitone were obtained from BDH (Alexandria, Egypt). Gallamine and aminophylline were a kind gift from Alexandria Company for pharmaceuticals, Egypt. Neostigmine was purchased from Amriya Pharmaceutical Industries, Alexandria, Egypt. Other chemicals were of the highest purity commercially available.

*Corresponding author. Address: On working leave from Department of Biochemistry, Faculty of Science, Alexandria University, Egypt. Fax: +974-485-1049. E-mail: m_balbaa@hotmail.com

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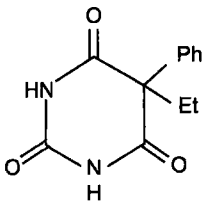
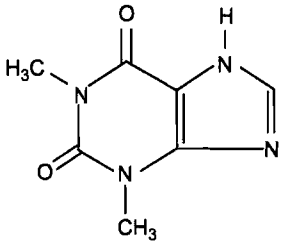


Phenobarbitone



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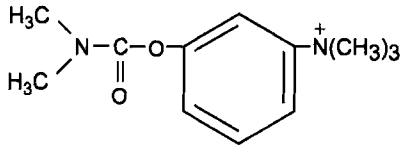
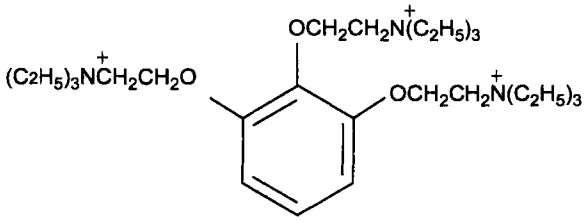


Neostigmine



Gallamine

FIGURE 1 The drugs tested for their effect on SCR.

Animals and Treatments

Seven to eight weeks old Swiss albino mice (24 g) were obtained from the animal house of the Medical Research Institute, Alexandria University, Egypt. They were usually kept in cages under convenient conditions of temperature and climate, with food supplement of milk and bread, carrots and tap water *ad libitum*. Vitamins and minerals were taken into consideration. The mice were treated with different doses (5–80 μM) of the above mentioned drugs in saline by *i.m.* or *s.c.* injections. The animals were separated into groups (4–7 animals per group) and each group received a different dose of the drug. All dose injections occurred for only 24 h interval and triplicate treatment was done for each dose. Control mice were treated with only the same volume of saline.

Isolation of SCR

Isolation of SCR was performed as described previously.[17] Briefly, liver removed from the sacrificed animals was minced, washed 6 times with double distilled water and stirred for 20 min. The mince was further mixed with 0.1 M sodium phosphate buffer, pH 7.4 and then stirred and washed with deionized water. The mince was ground in a mortar with 0.02 M sodium phosphate buffer, pH 7.4, containing 0.5% sodium cholate and 1 mM EDTA, diluted with the same buffer and centrifuged at 800 × g for 30 min. The supernatant was cooled to 2°C with crushed ice, brought to pH 5.5 with 1 M acetic acid and then centrifuged at 4,000–5,000 × g for 15 min at 4°C. The pellet from the centrifugation was re-washed with 0.01 M KH_2PO_4 and centrifuged for 15 min at 4,000–5,000 × g at 4°C. The residue obtained was suspended in borate–phosphate buffer, pH 7.4, containing 0.5% sodium cholate using a potter homogenizer and then centrifuged at 4,000–5,000 × g for 30 min at 4°C. The enzyme was then prepared from the supernatant according to the previously reported method.[2] Under the experimental conditions, SCR activity was determined using DCIP or cyt c as a substrate.

Kinetic Assay of SCR

The assay of SCR was performed for the reduction of either cyt c[18] or DCIP.[1] The reaction mixture (3 ml) contained 20 mM succinate, 5–50 μM oxidized cyt c or DCIP, 1 mM sodium azide and 0.2 mM EDTA. All enzyme assays were carried out at 25°C in 0.1 M phosphate buffer, pH 7.4, at wavelengths of 550 nm and 600 nm for the reduction of cyt c and DCIP, respectively. The decrease of absorbance per second (ΔA/s) was measured using a Pharmacia-Biotech spectrophotometer (Ultrospec 1000). The enzyme concentrations were chosen to give initial rates in the first 1–2 min and the reaction was started by the addition of either cyt c or DCIP.[1] For *in vitro* studies, all activities were measured in the presence or absence of each of the test compounds of (5–50 μM), unless otherwise indicated. The maximum velocity is expressed as ΔA/s protein of SCR in catalyzing the reduction of both cyt c and DCIP. The *in vivo* and the *in vitro* investigations were performed as described previously.[19] All assays were carried out in triplicate and mean ± S.E values were calculated.

Determination of the Kinetic Parameters of SCR

Initial velocities of SCR as ΔA/s were determined from the time courses of the enzyme reactions using cyt c or DCIP. The kinetic parameters were determined from Lineweaver–Burk plots,[20] whereas the constants were calculated and measured statistically by Harvard graphics (HGW4).

Protein Assay

The Folin–Lowry method[21] was used for determining protein in the homogenates and enzyme preparations with bovine serum albumin as standard.

RESULTS

Inhibition of SCR by Different Drugs

Four different drugs were tested for their *in vitro* and *in vivo* effects on SCR. The kinetic parameters K_m and V_{max} of the enzyme were determined by Lineweaver–Burk plots by measuring the value of the initial rate of the enzyme reaction at different concentrations of either DCIP or cyt c. As shown in Table I, *in vitro* phenobabitone and aminophylline inhibited the reduction of DCIP and cyt c by SCR, the V_{max} (DCIP) being decreased from $0.090 \pm 0.006\,\Delta A/s/mg$ protein (control) to $0.06 \pm 0.002\,\Delta A/s/mg$ protein by 20 μM phenobabitone i.e. 33% inhibition. Similarly, V_{max} (cyt c) decreased from $0.15 \pm 0.012\,\Delta A/s/mg$ protein (control) to $0.07 \pm 0.003\,\Delta A/s/mg$ protein, corresponding to 55% inhibition. With 20 μM of aminophylline the activity of SCR towards DCIP and cyt c was inhibited by 33 and 63%, respectively (Table I). When the concentrations of phenobarbitone was increased gradually to 50 μM (0–50 μM), the Lineweaver–Burk plots showed non-competitive inhibition (Figure 2A). The observed K_i values were $(5.68 \pm 0.46) \times 10^{-5}$ and $(2.55 \pm 0.20) \times 10^{-5}$ M for DCIP and cyt c, respectively (Table II). A similar pattern of inhibition was obtained with aminophylline (Figure 2B) with K_i values of $(4.46 \pm 0.25) \times 10^{-5}$ and $(1.46 \pm 0.20) \times 10^{-5}$ M for DCIP and cyt c, respectively.

Neostigmine, a reversible anti-cholinesterase, inhibited the reduction of DCIP catalyzed by the SCR and Lineweaver–Burk plots using variable concentrations of neostigmine (0–50 μM) showed competitive inhibition with a K_i value of $1.36 \pm 0.10 \times 10^{-5}$ M. Furthermore, neostigmine similarly inhibited the reduction of cyt c in a similar with a K_i value of $1.50 \pm 0.14 \times 10^{-5}$ M (Table II).

The inhibition of the enzyme by these compounds was reversible. Dialysis recovered the enzyme activity after its preincubation with the tested compounds (data not shown). Also, very slight variations in the K_m values were observed (Table I) as a result of the enzyme inhibition by phenobarbitone and aminophylline. The measured K_m values of SCR towards DCIP and cyt c in absence of drugs are 0.028 ± 0.007 and 0.015 ± 0.003 mM, respectively. This is consistent with what has been reported previously.[19] As a result of the competitive inhibition of the enzyme by neostigmine, these values are increased to 0.20 ± 0.009 and 0.13 ± 0.005 mM, respectively (Table I).

The effect of the tested compounds on SCR showed more or less a similar effect on enzyme activity *in vivo* as they acted *in vitro* on the reduction of DCIP and/or of Cyt c reduction. Phenobarbitone and aminophylline inhibited the enzyme in a non-competitive manner and neostigmine in a competitive manner. The *in vivo* effect of the drugs on the enzyme activity is summarized in Table III.

Activation of SCR by Gallamine

As shown in Table I, when SCR catalyzed the oxidation of succinate in the presence of 20 μM of gallamine, the reduction of DCIP was activated *in vitro* by 91% more than the control. All reactions followed the pattern of Michaelis-Menten Kinetics (Figure 3). On studying the Lineweaver–Burk analysis of the intact enzyme, activation was very high when increasing concentrations of gallamine (0–50 μM) were employed. The reduction of cyt c also followed Michaelis-Menten kinetics with 49% activation compared with the control. On the other hand, *in vivo* treatment showed a slight change in the K_m values and 46% and 35% activation of the enzyme V_{max} towards DCIP and cyt c, respectively, compared with the control (Table III).

DISCUSSION

In the present study, different drugs with diverse physiological actions have been investigated for their effect on SCR, an enzyme complex involved in

TABLE I *In vitro* effect of drugs on the kinetic parameters of SCR catalyzing the oxidation of either DCIP or cyt c. The reactions were performed at 25°C and the kinetic parameters were determined by Lineweaver–Burk plots. The measured V_{max} values are calculated in the absence and presence of 20 μM drug. Data are shown as mean ± S.E (n = 5)

	DCIP		Cyt c	
Drug	V_{max} [a]	K_m [b]	V_{max} [a]	K_m [b]
Control	0.09 ± 0.006	0.028 ± 0.007	0.15 ± 0.01	0.015 ± 0.003
Phenobarbitone	0.06 ± 0.002	0.028 ± 0.004	0.07 ± 0.003	0.015 ± 0.005
Aminophylline	0.06 ± 0.004	0.027 ± 0.002	0.05 ± 0.005	0.018 ± 0.004
Neostigmine	0.09 ± 0.005	0.200 ± 0.009	0.15 ± 0.005	0.130 ± 0.005
Gallamine	0.18 ± 0.007	0.025 ± 0.005	0.22 ± 0.006	0.027 ± 0.0004

[a] V_{max} is expressed as ΔA/s /mg protein. [b] K_m is expressed as mM.

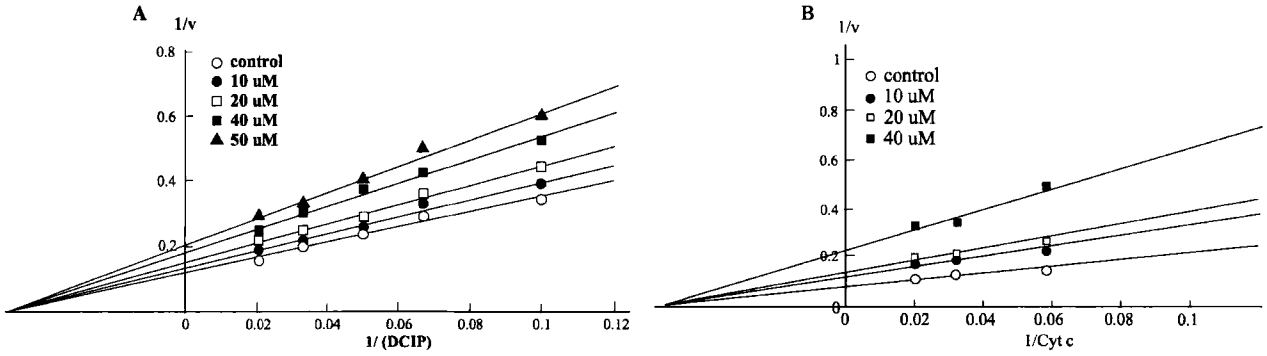


FIGURE 2 (A) Lineweaver–Burk plot of the *in vitro* effect of phenobarbitone (0–50 uM) on SCR using DCIP and 20 mM of potasium succinate. (B) Lineweaver–Burk plots of the *in vitro* effect of aminophylline (0–50 uM) on SCR using cyt c and 20 mM of potassium succinate.

the mitochondrial electron transport system.[1] The results show that phenobarbitone had an inhibitory effect on SCR with a K_i value of the order of 10^{-5} (Table II). The keto group at C_6 of phenobarbitone (Figure 1) has a tendency to form a hydrazone derivative which may contribute to the inhibitory effect. Alternatively, this inhibitory effect may be attributed to a hydrophobic interaction of the phenyl group. It has been reported that a macrocyclic complex of a benzoquinoid structure completely inhibited SCR.[19] Also, it has been described previously that a large number of drug-and steroid-metabolizing enzymes were induced by phenobarbitone through their liver-specific genes.[22,23] Recently, it was reported that a phenobarbitone derivative has both *in vitro* and *in vivo* Inhibition of hepatic aspartate trans-carbamylase.[24]

Gallamine and neostigmine have a contradictory effect on SCR activity; the first is an activator and the latter is an inhibitor (Tables I & III). This contradictory effect may be attributed to the difference in their structures. Gallamine has two quaternary N^+ atoms whereas neostigmine has only one.

In the present study, the compounds had a reversible inhibitory effect on SCR with enzymatic activity completely recovered after dialysis showing, formation of an enzyme inhibitor complex which is readily reversible[25] unlike, some inhibitors which have been reported to dissociate from enzyme-inhibitor complexes very slowly e.g. trehazlin with trehalase,[26] constanospermine with sucrase,[27] swainsonine with α-mannosidase,[28] substrate analog inhibitor with HIV protease[29] and 6-azasteroids with 5-α-reductases.[30]

SCR is composed of succinate-ubiquinone reductase and ubiquinol-cyt c reductase[31] to further clarify the mechanism of action of the tested compounds on SCR, subsequent work should focus on the separated enzyme instead of the intact one to study both components. In conclusion, SCR which catalyzes the electron transfer from succinate to cyt c is activated by gallamine and inhibited by phenobarbitone, aminophylline and neostigmine both *in vitro* and *in vivo* in a different patterns reading to the conclusion that completely different

TABLE II The inhibition constant (K_i) values of *in vitro* inhibition of SCR catalyzing the oxidation of either DCIP or cyt c. The reactions were performed at 25°C and K_i values were determined by Lineweaver–Burk plots and expressed as M × 10^{-5}

Drug	DCIP	Cyt c
Phenobarbitone	5.68 ± 0.46*	2.55 ± 0.20
Aminophylline	4.46 ± 0.25	1.46 ± 0.20
Neostigmine	1.36 ± 0.10	1.50 ± 0.14

*Mean ± S.E (n = 5).

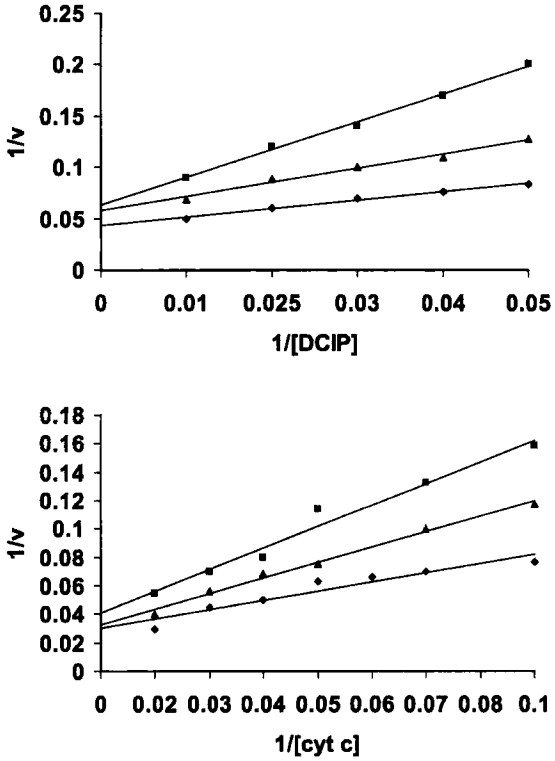


FIGURE 3 Lineweaver–Burk plots of the *in vitro* effect of gallamine (■, control; ▲, 10 μM; ◆, 20 μM) on SCR using DCIP (upper panel) or cyt c (lower panel) at 20 mM of potassium succinate.

TABLE III *In vivo* effect of drugs on the Kinetic parameters of SCR catalyzing the oxidation of either DCIP or cyt c. The reactions were performed at 25°C and the kinetic parameters were determined by Lineweaver–Burk plots. The measured V_{max} values are calculated after 20 μM treatment of each drug compared to control. Data are shown as mean ± S.E (n = 5)

	DCIP		Cyt c	
Drug	V_{max} [a]	K_m [b]	V_{max} [a]	K_m [b]
Control	5.3 ± 0.40	0.028 ± 0.010	4.8 ± 0.7	0.020 ± 0.004
Phenobarbitone	3.2 ± 0.20	0.030 ± 0.003	2.0 ± 0.3	0.022 ± 0.005
Aminophylline	1.5 ± 0.01	0.033 ± 0.005	2.2 ± 0.2	0.020 ± 0.007
Neostigmine	5.0 ± 0.40	0.300 ± 0.007	4.5 ± 0.5	0.025 ± 0.005
Gallamine	7.8 ± 0.40	0.015 ± 0.003	6.4 ± 0.6	0.012 ± 0.005

[a] V_{max} is expressed as ΔA/s /mg protein. [b] K_m is expressed as mM.

mechanisms are operative through the effect of these drugs on the enzyme.

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References

[1] Takemori, S. and King, T.E. (1964) *J. Biol. Chem.* **239**, 3456.
[2] Yu, L. and Yu, C.-A. (1987) *J. Biol. Chem.* **257**, 2016.
[3] Chang, A.-Y. and Linda, Y. (1980) *Biochemistry* **19**, 3579.
[4] Chang, A.-Y., Linda, Y. and Tsoo, E.K. (1974) *J. Biol. Chem.* **249**, 4905.
[5] Linda, Y., John, P. and Chang, A.-Y. (1987) *Biochim. Biophys. Acta.* **893**, 75.
[6] Butler, T.C. (1950) *J. Pharmacol. Exp. Ther.* **98**, 121.
[7] Kraatz, C.P. and Gluckman, M.L. (1953) *J. Pharmacol. Exp. Ther.* **107**, 437.
[8] Boonstra, H., Duran, V. and Weaver, K. (1999) *IPPF Med. Bull.* **33**, 1.
[9] Columbia Encyclopedia, 6th ed., (2003) www. encyclopedia.com, Alacritude, LLC.
[10] Lovstad, R.Z., Thagaard, K.S., Berner, N.S. and Raeder, J.C. (2001) *Acta Anaesthesiol. Scand.* **45**, 495.
[11] Gibert, N.C., Fenn, G.K. and Nalefski, L.A. (1949) *J. Am. Med. Assoc.* **141**, 892.
[12] Spalding, M. (2001) *Academic Dissertation* (Oulu University, Finland).
[13] Hoppe, J.A. (1955) *Anesthesiology* **16**, 91.
[14] Burgen, A.S.V. and Spero, L. (1968) *Br. J. Pharmacol.* **34**, 99.
[15] Davies, P. (1983) In: Maueux, R. and Rosen, W.C., eds, *Advances in Neurology* (Raven Press, New York), pp 75–86.
[16] Yu, C.-A., Yu, L. and King, T.E. (1972) *J. Biol. Chem.* **247**, 1012.
[17] King, T.E. (1961) *J. Biol. Chem.* **236**, 2342.
[18] King, T.E. and Takemori, S. (1964) *J. Biol. Chem.* **239**, 3559.
[19] Balbaa, M., Khalifa, M., El-Sabaway, M. and Kandeel, K. (2001) *J. Enz. Inhib.* **16**, 381.
[20] Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658.
[21] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265.
[22] Conney, A.H. (1967) *Pharmacol. Rev.* **19**, 317.
[23] Waxman, D.J. and Azaroff, L. (1992) *Biochem. J.* **281**, 577.
[24] Balbaa, M., Yacout, G., Ghonaim, T. and Othman, D. (2001) *J. Enz. Inhib.* **16**, 259.
[25] Morrision, J.F. and Walsh, C.T. (1988) *Adv. Enzymol.* **61**, 201.
[26] Anodo, O., Nakajima, M., Kifune, M., Fang, H. and Tanzama, K. (1995) *Biochim. Biophys. Acta.* **1244**, 295.
[27] Danzin, C. and Ehrard, A. (1987) *Arch. Biochem. Biophys.* **257**, 472.
[28] Kang, M.S. and Elbetin, A.D. (1983) *Plant Physiol.* **71**, 551.
[29] Peranteau, A.G., Kuzmic, P., Angell, Y., Garcia-Echeverria, C. and Rich, D.H. (1995) *Anal. Biochem.* **227**, 242.
[30] Moss, M.L., Kuzmic, P., Stuart, J.D., Tian, G., Peranteau, A.G., Frye, S.V., Kadwell, S.H., Kost, T.A., Overton, L.K. and Patel, I.R. (1996) *Biochemistry* **35**, 3457.
[31] Yu, C.-A. and Yu, L. (1980) *Biochim. Biophys. Acta.* **591**, 409.