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Control of Choline Oxidation in Rat Kidney Mitochondria

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Key words: choline, betaine, mitochondria, osmolyte, kidney, transporter

Abbreviations: EGTA, ethylenebis(oxethylenenitrilo)tetraacetic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhyrazone; HEPES, 4-(2-hydroxyethyl)-1-peiperazine-ethanesulfonic acid; MOPS, (3-[N}-morphilino)propane sulphonic acid; TPMP, methyltriphenylphosphonium

ABSTRACT

Choline is a quaternary amino cationic organic alcohol that is oxidized to betaine in liver and kidney mitochondria. Betaine acts as an intracellular organic osmolyte in the medulla of the kidney. Evidence is provided that kidney mitochondria have a choline transporter in their inner membrane. The transporter has a Km of 173±64μM and a Vmax of 0.4±0.1nmol/min/mg mitochondrial protein (at 10^0C). Uptake of choline is not coupled to betaine efflux. Transporter activity demonstrates a dependence on membrane potential and choline transport is inhibited by hemicholinium-3. Steady-state oxygen consumption due to choline oxidation in kidney mitochondria was measureable at 37^0C (125±6 pmolO_2/min/mg mitochondrial protein), in the absence of other mitochondrial electron transport chain substrates and the choline transporter was shown to be the major site of control (96±4%) over choline oxidation flux in isolated kidney mitochondria. We conclude that the choline transporter in rat kidney mitochondria is the major site of control over the production of the organic osmolyte, betaine.

1. Introduction

Choline (*N,N,N*-trimethyl–β-hydroxyethanolamine) is a quaternary amino cationic organic alcohol. Choline is termed an essential dietary component [1-4] although it can also be derived indirectly in vivo in liver from sequential methylation of phospatidylethanolamine to form phosphatidylamine in endoplasmic reticulum and mitochondria-associated membranes [5]. Choline itself is a precursor for the synthesis of the neurotransmitter acetylcholine and membrane phospholipids such as phosphatidylcholine and sphingomyelin [1].

The oxidation of choline occurs in kidney [6] and liver [7] mitochondria, where an FAD-linked membrane bound choline dehydrogenase (EC 1.1.99.1) converts choline to betaine aldehyde, and a matrix NAD-linked betaine aldehyde dehydrogenase oxidizes betaine aldehyde to betaine (*N,N,N*-trimethyl glycine aka glycine betaine). A transporter for choline across the inner membrane of rat liver mitochondria has been characterized [8] and has been shown to be the major site of control over choline oxidation flux in isolated liver mitochondria [9]. However the fate of betaine lies outside the mitochondria. Thus far no inner membrane transporter for betaine has been described, however diffusion of betaine from liver mitochondria has been described [10,11]. Betaine can act as a methyl donor to homocysteine, generating methionine via betaine homocysteine methyltransferase (EC 2.1.1.5) in the cytoplasm of liver and kidney of humans and pigs, but only the liver of rats [12]. However betaine also plays a crucial role as an osmolyte in the kidney [13]. The renal medulla is the only tissue in mammals where the extracellular sodium chloride concentration exceeds that in the blood. The

concentrating mechanism in the kidney causes an osmotic gradient in the extracellular fluid of the medulla particularly towards the tip of the papilla [14]. The cells of the renal papilla become surrounded by osmolytes such as sodium chloride and urea. Thus the intracellular osmolarity has to increase to balance that of the extracellular fluid in order to maintain normal cell structure, and this is achieved through accumulation of organic osmolytes such as betaine [15]. Betaine also has a protective effect on proteins and enzymes against the denaturing effect of urea [16]. The source of the betaine can be extracellular through plasma membrane betaine transporters [15] or intracellularly through choline oxidation [6]. Interestingly, a family of plasma membrane choline transporters have been characterized, splice variants of the SLC44A1 gene [17] and antibodies to the solute carrier (SLC44A1) have detected a 72kDa choline transporter predominantly in the mitochondrial outer membrane of liver and heart mitochondria [18].

In this study we describe, for the first time, the existence of a choline transporter in rat kidney mitochondria and we show that the choline transporter is the main site of control over choline oxidation to betaine in isolated kidney mitochondria.

2. MATERIALS AND METHODS

2.1 Material

[^{14}C-methyl]-choline chloride, [^{3}H-methyl]-choline chloride and [^{3}H]-methyltriphenylphosphonium (TPMP+) (iodide salt) were purchased from Amersham International, UK. Perchloric acid was from British Drug Houses,

Dorset, UK. Sucrose, KCl and $CuSO_4$, $MgCl_2$ were from Merck Damstadt, Germany. Ammonia, Methanol were from (Fluka) Sigma-Aldrich Ireland. All other Chemicals were from Sigma-Aldrich Ireland.

2.2 Animals

Female Wistar rats (200-250g) were obtained from the BioResources Unit at Trinity College Dublin. All animals were fed ad libitum on a standard Harlan 2018 Teklad Global 18% Protein Rodent Diet and had free access to water.

2.3 Isolation of Mitochondria

Rat liver and kidney mitochondria were isolated by differential centrifugation according to the procedure of Chappell and Hansford [19].

2.4. Protein determination

Tissue and mitochondrial protein concentration were determined by the reduction of Folins-Ciocalteau phosphomolybdic-phosphotungstic reagent according to the method of Markwell et al. [20].

2.5 Uptake of choline

Rat kidney mitochondria (3mg/ml) were incubated in 120mM KCl, 5mM HEPES-KOH, pH 7.2, 5mM succinate (potassium salt), 1mM EGTA, 5μM rotenone, oligomycin (1μg/ml), nigericin (200pmoles/mg), 1μM TPMP+ (iodide salt). Uptake of choline was initiated by addition of cold choline (containing either methyl-[^{14}C]-choline or methyl-[^{3}H]-choline chloride. Choline not

accumulated into the mitochondrial matrix was corrected for using [^{14}C]-or [^{3}H]sucrose, as sucrose is unable to cross the inner membrane. Matrix volume was measured in parallel from the differential mitochondrial space occupied by [^{3}H]$_2$O and [^{14}C]-sucrose. Samples (500μl) of incubating mitochondria were taken at various time points or between 1min and 3.5min intervals for rate of uptake determination. Mitochondria were pelleted and lysed with perchloric acid. The accumulation ratio of choline was determined as follows: (dpm due to methyl-[^{14}C]-choline in the pellet) ÷ (dpm due to methyl-[^{14}C]-choline in the supernatant) x (supernatant volume) minus (dpm due to [^{3}H]sucrose in the pellet) ÷ (dpm due to [^{3}H]sucrose in the supernatant) x (supernatant volume)[8].

2.6 Determining mitochondrial membrane potential

Rat kidney mitochondria (3mg/ml) were incubated in 120mM KCl, 5mM HEPES-KOH, pH 7.2, 5mM succinate (potassium salt), 1mM EGTA, 5μM rotenone (1μg/ml), nigericin (200pmoles/mg), 1μM methyltriphenylphosphonium (TPMP+) (iodide salt) and [^{3}H]-TPMP+ (final specific activity of 1.2mCi/mmol) and at various choline concentrations. Membrane potential was varied using 0-5mM malonate and 40nM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). Samples (500μl) of incubating mitochondria were taken. Mitochondria were pelleted and lysed with perchloric acid and the accumulation ratio of choline determined as described previously [8].

2.7 Thin layer chromatographic separation of choline and betaine. Thin layer chromatography was performed on pelleted mitochondria lysed with perchloric acid on Polygram Sil G, thin layer chromatography plastic coated silica sheets. The solvent system was 1M NaCl: methanol: 14M NH_3 (10:10:1, v/v/v). $1cm^2$ samples of silica were scraped into vials containing 4ml scintillant. Rf values for radiolabelled compounds corresponded with those obtained for unradiolabelled standards detected using Dragendorfs reagent. Rf values for radiolabelled choline and radiolabelled betaine were 0.55 and 0.75, respectively. No radiolabelled betaine aldehyde was detected.

2.8 Loading mitochondria with radiolabelled betaine

Rat kidney mitochondria (5mg/ml) were incubated for 8 min in the absence of rotenone in a single vessel containing 175mM sucrose, 10mM D-glucose, 5mM MOPS, 5mM KH_2PO_4, 1mM EGTA, 5mM ADP, 0.2mM $MgCl_2$ and 0.5mM choline (containing methyl-[^{14}C]-choline to give a final specific activity of 1.2mCi/mmol) adjusted to pH 7 with Tris free base.

2.9 Determination of flux control coefficient for the choline transporter over choline oxidation in rat kidney mitochondria.

Rat kidney mitochondria (3mg/ml) were incubated in 120mM KCl, 5mM 100μM ADP and 5mM $MgCl_2$, at 37^0C. Choline (50μM final concentration) was injected into the chamber of the Oxygraph Respirometer consumption rate was measured, 5μM additions of hemicholinium-3 were titrated into the chamber until choline oxidation was inhibited. The flux control coefficient (C)

was calculated from the following equation, $C = -T.I_{max}/J_O$, derived by Kacser and Burns [21], where T is the slope of the tangent to the curve at zero hemicholinium-3 concentration, I_{max} is the extrapolated maximal inhibitor concentration and J_O is the choline oxidation flux at zero hemicholinium-3 concentration. The application of this equation was based on the assumptions of Lettlier et al [22] and on of the observation that steady-state choline oxidation was a linear function of hemicholinium-3 concentration, despite hemicholinium-3 most probably being a competitive inhibitor of the choline transporter in kidney mitochondria, as it was shown to be in liver mitochondria [8]. Furthermore, uptake of choline by diffusion was minimized by not facilitating the generation of the substantial membrane potentials that are achieved using electron transport chain substrates such as succinate.

2.9 Statistical analysis

Rectangular hyperbola were fitted to data in fig. 4 using Enzfitter. All data are represented as mean ± standard error of the mean for (n) number of separate experiments, measured in at least triplicate

3. RESULTS

Conditions for separation of choline and betaine on Polygram Sil G thin layer chromatography plastic coated silica sheets were optimized using commercially purchased choline and betaine and the use of Dragendorf's reagent which detects quaternary amino groups (results not shown). However, figure 1 shows an example of (a) our methodology to separate choline (Rf value 0.55) and betaine (Rf value 0.75) using this thin layer

chromatography, (b) our ability to prevent oxidation of choline to betaine by addition of rotenone and (c) our ability to allow oxidation of all choline to betaine by not adding rotenone. It is interesting to note that we did not detect any betaine aldehyde which has an Rf value in between that of choline and betaine.

Figure 2 demonstrates the relative degree of accumulation of choline into rat kidney, liver and skeletal muscle mitochondria. The accumulation of choline into rat kidney mitochondria, in the presence of succinate, is 30-fold within the first 10min at 10^0C, which is ~5-6 times that of liver mitochondria and ~10 times that of skeletal muscle mitochondria.

Figure 3 demonstrates that maximal choline accumulation (30-fold) into rat kidney mitochondria is inhibited (to 5-fold accumulation) by the known choline transporter inhibitor, hemicholinium-3, and is sensitive to uncoupling of mitochondria with FCCP (only 4-fold accumulation).

Figure 4 demonstrates that uptake of choline into rat kidney mitochondria is a saturable process, indicative of a transporter. The experiments were performed at 10^0C in order to slow down the transport process, so that the rate of uptake of choline could be determined. The curve was fitted to a rectangular hyperbola for Michaelis-Menten kinetics and a Km of 173±64μM and a Vmax of 0.4±0.1nmol/min/mg were calculated.

Figure 5 demonstrates that the choline uptake rate, in the presence of the electron transport chain substrate succinate, is a function of mitochondrial membrane potential. Membrane potential was titrated with malonate, a competitive inhibitor of succinate dehydrogenase and FCCP was added to reduce membrane potential further.

Using radiolabelled betaine loaded mitochondria (~40nmol/mg), the experiment in figure 6 demonstrates that the betaine efflux rate or the extent of betaine efflux is similar in the presence and absence of externally added choline.

Figure 7 demonstrates that choline oxidation by rat kidney mitochondria, in ionic medium, could be measured using an Oxygraph Respirometer at 37^0C (125 ±6 (3) pmolO_2/min/mg mitochondrial protein (mean ± sem (3)). No discernable oxygen consumption could be measured when choline was given to rat liver mitochondria (results not shown). We then used this indirect measure of choline oxidation flux to determine how much control the choline transporter had over steady-state choline oxidation flux by titrating the choline transporter with hemicholinium-3. We know hemicholinium-3 cannot be inhibiting choline dehydrogenase directly, as to do it would have to cross the mitochondrial inner membrane and we have been able to demonstrate that [^{3}H]-hemicholinium-3 is not accumulated by mitochondria (results not shown). We could demonstrate that choline oxidation decreased more-or-less linearly with increasing concentration of hemicholinium-3 and we were able to estimate an I_{max} value of ~41μM based on extrapolation of the curve to the x-axis. The flux control coefficient (C) was calculated from the following equation $C = -T.I_{max}/J_O$, where T is the slope of the tangent to the curve at zero hemicholinium-3 concentration, I_{max} is the extrapolated maximal inhibitor concentration and J_O is the choline oxidation flux at zero hemicholinium-3 concentration [21, 22]. The flux control coefficient for the choline transporter (CT) over choline oxidation (COX) was calculated to be 0.96±0.04 (7). Data are from 7 experiments performed in triplicate (mean ± sem (n)).

4. DISCUSSION

The ability to inhibit oxidation of choline with rotenone (Fig. 1) enabled us to investigate choline transport into mitochondria as a single step (Figs. 2, 3, 4 and 5) without the added complication of apparent uptake of radiolabel being due to oxidation of choline. In addition, we observed that when rotenone was not present, all choline given externally to rat kidney mitochondria was oxidized to betaine within the mitochondria (Fig. 1). This enabled us to load rat kidney mitochondria with radiolabelled betaine as exemplified in fig. 6 and reassured us that all oxygen consumption we were measuring in fig. 7 was due to oxidation of choline to betaine. Our choline oxidation data for rat kidney mitochondria differ slightly to those for rat liver mitochondria [11] in that we never detected betaine aldehyde in the course of measuring choline oxidation in rat kidney mitochondria.

The degree of accumulation of choline by rat kidney mitochondria is markedly (~5-6 times) more extensive than that for liver mitochondria which has a choline transport and oxidation system [1,8]. In addition, it is shown here that there is a 30-fold accumulation of choline into rat kidney mitochondria within 10min, whereas, there is a 5-fold accumulation of choline into rat liver mitochondria for the same time frame (Fig. 2). These data demonstrate that the apparent rate of choline uptake into rat kidney mitochondria is far greater than that into rat liver mitochondria. The 10-fold lower accumulation of choline into skeletal muscle mitochondria, compared to that of kidney mitochondria, probably reflects electrophoretic diffusion of choline into skeletal

muscle mitochondria, as we known that skeletal muscle mitochondria do not possess a mitochondrial choline transporter or choline oxidation system [1].

The fact that choline uptake into rat kidney mitochondria is inhibited by hemicholinium-3 (Fig. 3) is strong evidence for a specific transporter for choline. We have previously demonstrated that the choline transporter in rat liver mitochondria is inhibited competitively by hemicholinium-3 (Ki ~17 μM)[8]. Any accumulation of choline observed in the presence of 500μM hemicholinium-3 into rat kidney mitochondria is probably due to electrophoretic diffusion. The inhibition of uptake of choline in the presence of the uncoupler, FCCP, demonstrates that choline uptake into rat kidney mitochondria is driven by the electrochemical gradient across the inner membrane. Any accumulation observed in the presence of this uncoupler is probably due solely to facilitated diffusion of choline (Fig. 3). Similar energy dependence for choline transport was observed for rat liver mitochondria [8].

Further evidence for there being a transporter for choline in the inner membrane of rat kidney mitochondria, comes from the observation that choline uptake rates show saturable kinetics (Fig.4). The Km of 173μM and Vmax of 0.4nmol/min/mg mitochondrial protein at 10^0C are similar to those observed for the choline transporter in rat liver mitochondria (Km, 220μM; Vmax 0.4nmol/min/mg) but in the latter case measured at 25^0C [8]. As is evident in figures 2 and 3, apparent choline uptake rates are 5-6 times greater in kidney mitochondria when compared to liver mitochondria.

Building upon the observation that choline accumulation into rat kidney mitochondria is energy dependent (Fig. 3), we looked at the dependence of choline uptake rate as a function of (proton) electrochemical gradient across

the inner membrane (Fig. 5). The data suggest a dependence of choline uptake on membrane potential. However, it is difficult to interpret whether the dependence is linear or non-ohmic. A non-ohmic membrane potential dependence is usually associated with a diffusion process or a voltage-gated transporter, whereas a linear dependence on membrane potential has been reported for metabolite transp[orters [8,11]. A linear dependence was observed for the choline transporter in rat liver mitochondria [8]. Clearly further work is required to elucidate the nature of the relationship between membrane potential and choline transporter activity in kidney mitochondria.

The fact that the product of choline oxidation, betaine, is used in the cytoplasm in vivo and is predominantly neutral at pH 7, led to the possibility that the transport of cationic choline might not be via a uniporter but may occur in exchange for betaine. Therefore we loaded mitochondria with betaine and measured it efflux rate with and without externally added choline. However, we did not observe any increase in the rate/amount of radiolabelled betaine efflux from rat kidney mitochondria when cold choline was added externally (Fig. 6). We conclude that betaine efflux from rat kidney mitochondria is not coupled to choline uptake and therefore the choline transporter is probably a uniporter.

The higher rate of choline uptake in rat kidney mitochondria compared to that in liver mitochondria led us to consider using oxygen consumption as a means to determine choline oxidation rates. We were able to measure oxygen consumption by rat kidney mitochondria respiring on choline at 37^0C (Fig. 7). By titrating the choline transporter activity with hemicholinium-3 during steady-state choline oxidation, we were able to determine that the choline transporter

had 96% of the control over choline oxidation flux under these conditions. These results for rat kidney mitochondria mirror those determined for the choline transporter in rat liver mitochondria (95% control) [9], however, in the case of rat liver mitochondria, choline oxidation to betaine had to be determined using thin layer chromatography and not oxygen consumption rates, as has been achieved for rat kidney mitochondria.

We conclude that rat kidney mitochondria possess a choline transporter in their inner membrane. The transporter has a Km of 173±64μM and Vmax of 0.4±0.1nmol/min/mg mitochondrial protein (determined at 10^0C). The transport rate is estimated to be ~5-6 times that of the choline transporter in rat liver mitochondria. Uptake of choline in kidney mitochondria is not coupled to betaine efflux. Choline transporter activity demonstrates a dependence on membrane potential and is inhibited by hemicholinium-3. Steady-state choline oxidation in kidney mitochondria was measurable using oxygen consumption rates and the choline transporter was shown to be the major site of control (96±4%) over choline oxidation flux in isolated kidney mitochondria. By extension to the whole cell, the choline transporter is probably the major site of control of the supply of the osmolyte betaine in the mammalian kidney.

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References

[1] S.H. Zeisel, Dietary choline: Biochemistry, physiology, and pharmacology, Ann. Rev Nutr. (1981) 95-121.

[2] S.H. Zeisel, J.K. Blusztajn, Choline and Human Nutrition, Annu. Rev. Nutr. 14 (1994) 269-296.

[3] S.H. Zeisel, Choline: Ann essential nutrient for humans, Nutrition 16 (2000) 669-671

[4] S.H. Zeisel, Choline: Critical role during fetal development and dietary requirements in adult, Annu. Rev Nutr. 26 (2006) 29-250

[5] D.E. Vance, C.J. Walkey, Roles for the methylation of phosphatidylethanolamine, Curr. Opin. Lipidol. 9 (1998) 125-130.

[6] E.B. Grossman, S.C. Herbert, Renal inner medullary choline dehydrogenase activity: characterization and modulation, Am. J. Physiol. 256 (1989) F107-F112.

[7] D.R. Haubrich and N.H. Gerber, Choline dehydrogenase. Assay, properties and inhibitors, Biochem.Pharmacol. 30 (1981) 2993-3000.

[8] R.K. Porter, J.M. Scott, M.D. Brand, Choline transport into rat liver mitochondria, J. Biol. Chem. 267 (1992) 14637-14646.

[9] C.P Kaplan, R.K. Porter, M.D. Brand, The choline transporter is the major site of control of choline oxidation in isolated rat liver mitochondria, FEBS Letts., 321 (1993) 24-26.

[10] J.J.M. De Ridder and K. van Dam, The efflux of betaine from rat-liver mitochondria, a possible regulating step in choline oxidation, Biochim. Biophys. Acta 291 (1973) 557-563.

[11] R.K. Porter, J.M. Scott, M.D. Brand, Characterization of betaine efflux from rat liver mitochondria, Biochim. Biophys. Acta 1141 (1993) 269-274.

[12] M.P. McKeever, D.G. Weir, A. Molloy, J.M. Scott, Betaine-homocysteine methyltransferase: organ distribution in man, pig and rat and subcellular distribution in the rat, Clin. Sci., 81 (1991) 551-556.

[13] M.B. Burg, J.D. Ferraris, Intracellular organic osmolytes: Function and regulation, J. Biol. Chem. 283 (2008) 7309-7313.

[14] B. Schmidt-Neilsen, B. Graves, J. Roth, Water removal and solute additions determining increases in renal medullary osmolality, Am. J. Physiol. 244 (1983) F472-F482.

[15] A. Yamauchi, S Uchida, H.M. Kwon, A.S. Preston, R.B. Robey, A Garzia-Perez, M.B. Burg, J.S. Handler, Cloning of a Na(+)- and Cl(-)-dependent betaine transporter that is regulated by hypertonicity, J. Biol. Chem 267 (1992) 649-652.

[16] M.B. Burg, E.D. Kwon, E.M. Peters, Glycerophosphocholine and betaine counteract the effect of urea on pyruvate kinase, Kidney Int. Suppl. 57 (1996) S100-S104.

[17] E. Traiffort, M. Ruat, S. O'Regan, F.M. Maunier, Molecular charterization of the family of choline transporter-like proteins and their splice variants, J. Neurochem. 92 (2005) 1116-1125.

[18] V. Michel, M. Bakovic, The solute carrier 44A1 is a mitochondrial protein and mediates choline transport, FASEB J. 23 (2009) Epub ahead of print

[19] J.B. Chappell, R.G. Hansford, in Subcellular components: Preparation and Fractionation (G.D. Birnie ed.) 2nd edn. (1972) p77-91, Butterworths, London.

[20] M.A. Markwell, S.M. Haas, L.L. Bieber, N.E. Tolbert, A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples, Anal. Biochem. 87 (1978) 206-210.

[21] H, Kacser, J.A. Burns, in: Rate control of biological processes (D.D. Davies ed.) (1973) pp65-104, Cambridge University Press

[22] T. Lettelier, M. Malgat, J-P, Mazat, Control of oxidative phosphorylation in rat muscle mitochondria: implications for mitochondrial myopathies, Biochim. Biophys. Acta 1141 (1993) 58-64.

FIGURE LEGENDS

Figure 1. Thin layer chromatographic separation of choline and betaine. Rat kidney mitochondria (5mg/ml) were incubated for 8min in the presence (O) and absence (●) of rotenone (5μM) in a single vessel containing 175mM sucrose, 10mM D-glucose, 5mM MOPS, 5mM KH_2PO_4, 1mM EGTA, 5mM ADP, 0.2mM $MgCl_2$ and 0.5mM choline (containing methyl-[^{14}C]-choline to give a final specific activity of 1.2mCi/mmol) adjusted to pH 7 with Tris free base. Thin layer chromatography was performed on pelleted mitochondria lysed with perchloric acid on Polygram Sil G, thin layer chromatography plastic coated silica sheets. The solvent system was 1M NaCl: methanol: 14M NH_3 (10:10:1, v/v/v). 1cm^2 samples of silica were scraped into vials containing 4ml scintillant. Rf values for radiolabelled compounds corresponded with those obtained for unradiolabelled standards detected using Dragendorfs

reagent. Rf values for radiolabelled choline and radiolabelled betaine were 0.55 and 0.75, respectively. No radiolabelled betaine aldehyde was detected.

Figure 2. Time Course of Choline Accumulation by Mitochondria from Kidney, Liver and Skeletal Muscle of Rat.

Rat kidney (●), liver (O) and skeletal muscle (□) mitochondria (3mg/ml) were incubated in 120mM KCl, 5mM HEPES-KOH, pH 7.2, 5mM succinate (potassium salt), 1mM EGTA, 5μM rotenone, oligomycin (1μg/ml) at 10^0C. Uptake of choline was initiated by addition of 75μM choline (containing methyl-[^{3}H]-choline chloride to a final specific activity of 0.2mCi/mmol). Samples (500μl) of incubation medium were taken at 1min, 5min and 5min intervals. Mitochondria were pelleted and lysed with perchloric acid and the accumulation ratio of choline determined. Data are from 3 separate experiments performed in triplicate (mean ± sem (3)).

Figure 3. Time Course of Choline Accumulation by Mitochondria from Kidney, in the presence of excess FCCP and hemicholinium-3.

Rat kidney mitochondria (3mg/ml) were incubated in 120mM KCl, 5mM HEPES-KOH, pH 7.2, 5mM succinate (potassium salt), 1mM EGTA, 5μM rotenone, oligomycin (1μg/ml) at 10^0C, alone (●), plus 40nM FCCP (▲) and 500μM hemicholinium-3 (■). Uptake of choline was initiated by addition of 75μM choline (containing methyl-[^{3}H]-choline chloride to a final specific activity of 0.2mCi/mmol). Samples (500μl) of incubation medium were taken at 1min, 5min and 5min intervals. Mitochondria were pelleted and lysed with

perchloric acid and the accumulation ratio of choline determined. Data are from 3 separate experiments performed in triplicate (mean ± sem (3)).

Figure 4. Saturation Kinetics of Choline Uptake by Rat Kidney Mitochondria.

Rat kidney mitochondria (3mg/ml) were incubated in 120mM KCl, 5mM HEPES-KOH, pH 7.2, 5mM succinate (potassium salt), 1mM EGTA, 5μM rotenone, oligomycin (1μg/ml), nigericin (200pmoles/mg) at 10^0C. Uptake of choline was initiated by addition of 7.5-300μM choline (containing methyl-[^{3}H]-choline chloride to a final specific activity of 0.05-0.2mCi/mmol). Samples (500μl) of incubation medium were taken at 1min and 3.5min intervals and the rate of uptake determined. Mitochondria were pelleted and lysed with perchloric acid and the accumulation ratio of choline determined. A Km of 173±64μM and a Vmax of 0.4±0.1nmol/min/mg mitochondrial protein at 10^0C were calculated from 3 separate plots of three separate experiments performed in triplicate (mean ± sem (3)).

Figure 5. Effect of Varying Mitochondrial Membrane Potential on the Rate of Choline Uptake by Rat Kidney Mitochondria.

Rat kidney mitochondria (3mg/ml) were incubated in 120mM KCl, 5mM HEPES-KOH, pH 7.2, 5mM succinate (potassium salt), 1mM EGTA, 5μM rotenone, oligomycin (1μg/ml), nigericin (200pmoles/mg), 1μM TPMP+ (iodide salt) and [^{3}H]-TPMP+ (final specific activity of 1.2mCi/mmol) at 25^0C. Uptake of choline was initiated by addition of 80μM choline (containing methyl-[^{14}C]-choline chloride to a final specific activity of 1.2mCi/mmol). Matrix volume was measured in parallel using [^{3}H]$_2$O) and [^{14}C]-sucrose. Membrane potential

was varied using 0-5mM malonate (●) and 40nM FCCP (O). Samples (500μl) of incubation medium were taken at 1min and 3.5min intervals and the rate of uptake determined. Mitochondria were pelleted and lysed with perchloric acid and the accumulation ratio of choline determined.

Figure 6. Effect of Externally Added Choline on Efflux of Radiolabelled Betaine from Rat Kidney Mitochondria

Radiolabelled betaine loaded (~40nmol betaine/mg mitochondrial protein) rat kidney mitochondria (5mg/ml) were incubated in 120mM KCl, 5mM HEPES-KOH, pH 7.2, in the presence (●) and absence (O) of externally added cold choline (500μM). Samples (500μl) of incubation medium containing were taken at 0,2,4,6,8,10 min. Mitochondria were pelleted and lysed with perchloric acid and the concentration of betaine in the matrix was determined. The rate of betaine efflux in the presence and absence of externally added choline was approximately 2nmol/min/mg protein. Data are from 3 experiments performed in triplicate (mean ± sem (3)).

Figure 7. Determination of Control of the Choline Transporter over Steady-state Choline Oxidation Flux in rat Kidney Mitochondria.

Rat kidney mitochondria (3mg/ml) were incubated in 120mM KCl, 5mM KH_2PO_4, 20mM D-glucose, 3mM HEPES pH 7.2, with KOH, 1mM EGTA, 100μM ADP and 5mM $MgCl_2$, at 37^0C. Choline (50μM final concentration) was injected into the chamber and when a steady-state oxygen consumption rate was measured, 5μM additions of hemicholinium-3 were titrated in to the chamber until choline oxidation was inhibited. Maximal choline oxidation flux

equated to 125 ±6 (3) pmolO_2/min/mg mitochondrial protein (mean ± sem (3)).The flux control coefficient (C) was calculated from the following equation $C = -T.I_{max}/J_O$, where T is the slope of the tangent to the curve at zero hemicholinium-3 concentration, I_{max} is the extrapolated maximal inhibitor concentration and J_O is the choline oxidation flux at zero hemicholinium-3 concentration. The flux control coefficient for the choline transporter (CT) over choline oxidation (COX) was calculated to be 0.96±0.04 (7). Data are from 7 experiments performed in triplicate (mean ± sem (7)).

Figure 1

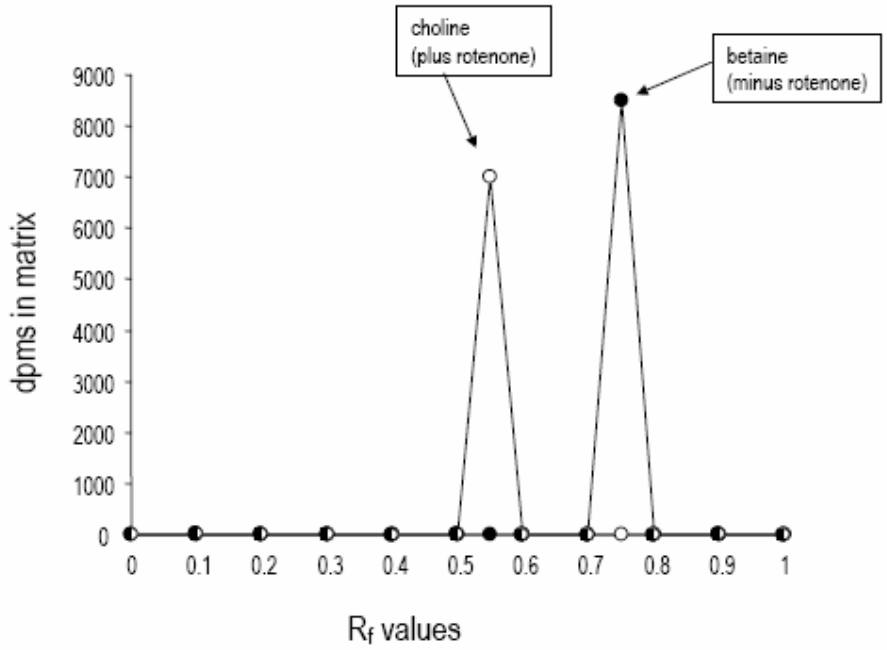

Figure 2

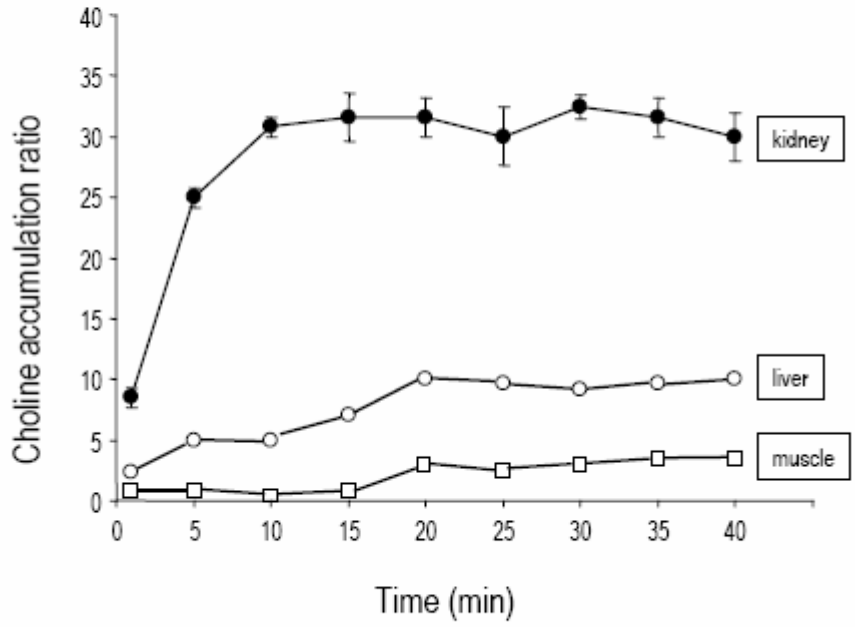

Figure 3

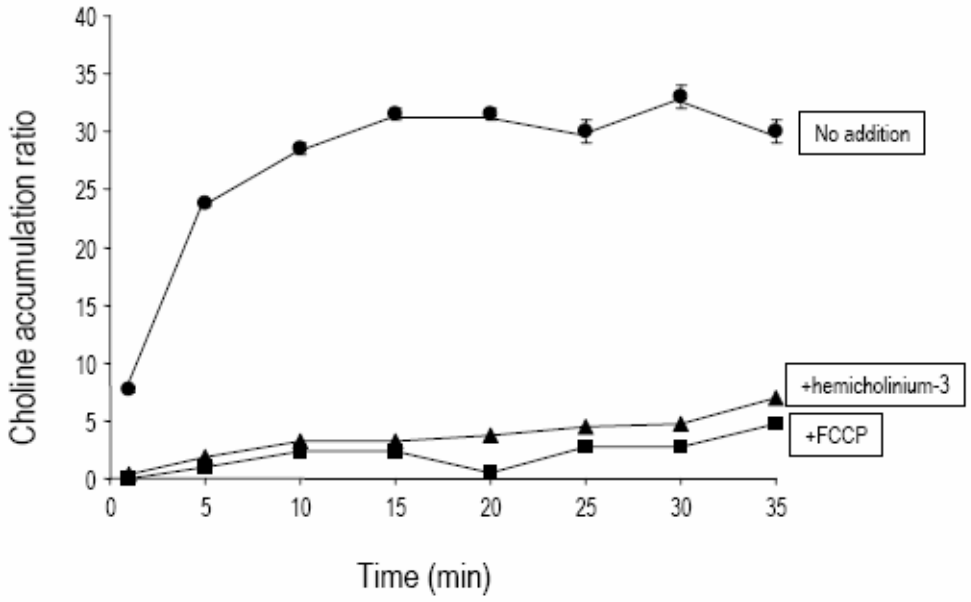

Figure 4

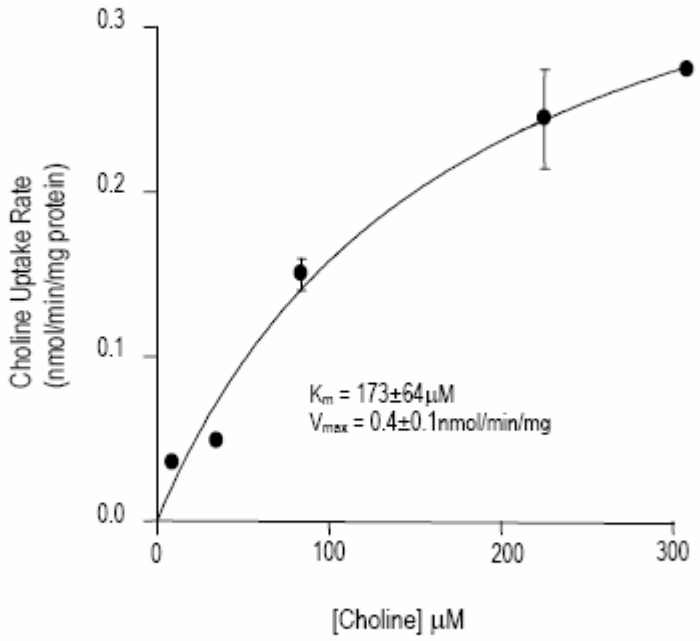

Figure 5

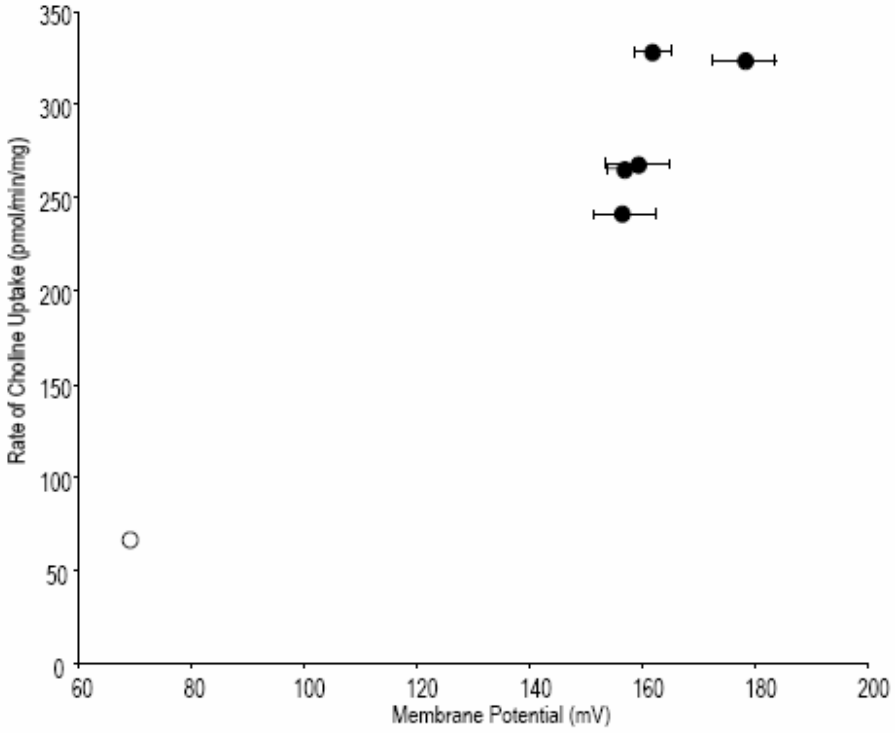

Figure 6

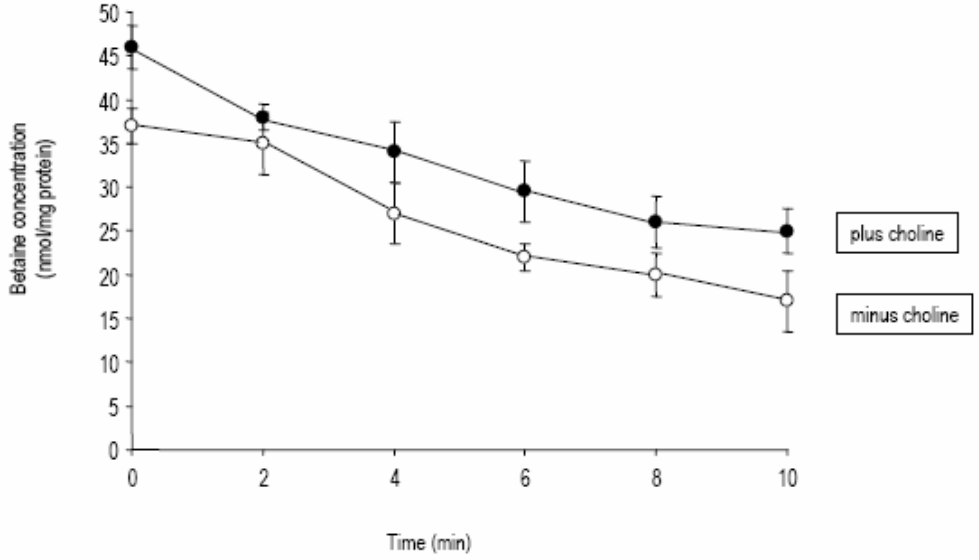

Figure 7