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Accumulation of lipophilic dications by mitochondria and cells

Meredith F. ROSS*[2], Tatiana DA ROS†[2], Frances H. BLAIKIE‡, Tracy A. PRIME*, Carolyn M. PORTEOUS§, Inna I. SEVERINA¶, Vladimir P. SKULACHEV¶, Henrik G. KJAERGAARD‡, Robin A. J. SMITH‡ and Michael P. MURPHY*[1]

*Medical Research Council Dunn Human Nutrition Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, UK, §Pharmaceutical Science Department, Trieste University, 34127 Trieste, Italy, ‡Department of Chemistry or §Department of Biochemistry, University of Otago, Box 56, Dunedin, New Zealand, and ¶A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119992, Russia.

[1]To whom correspondence should be addressed: Dr Michael P. Murphy, Medical Research Council Dunn Human Nutrition Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, UK. Phone: +44 1223 252 900; Fax: +44 1223 252 905; E-mail: mpm@mrc-dunn.cam.ac.uk.

[2]Joint first authors

Running title: Mitochondrial accumulation of lipophilic dications

Abbreviations used: ACR, accumulation ratio; BLM, bilayer planar phospholipid membrane; DTPA, diethylenetriaminepentaacetic acid; $\Delta\psi$, mitochondrial membrane potential; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; PC_nP, bistriphenylphosphonium cation linked by an *n* carbon methylene bridge; ROS, reactive oxygen species; TMA, tetramethylammonium hydroxide; TPB, tetraphenyl borate anion; TPMP, methyltriphenylphosphonium cation; TPP, triphenylphosphonium cation.

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Abstract

Lipophilic monocations can pass through phospholipid bilayers and accumulate in negatively charged compartments such as the mitochondrial matrix, driven by the membrane potential. This property is used to visualise mitochondria, to deliver therapeutic molecules to mitochondria and to measure the membrane potential. In theory, lipophilic dications have a number of advantages over monocations for these tasks as the double charge should lead to a far greater and more selective uptake by mitochondria, increasing their therapeutic potential. However, the double charge might also limit the movement of lipophilic dications through phospholipid bilayers and little is known about their interaction with mitochondria. To see if lipophilic dications could be taken up by mitochondria and cells, we made a series of bistriphenylphosphonium (bisTPP) cations comprising two TPP moieties linked by a 2-, 4-, 5-, 6-, or 10-carbon methylene bridge. The 5-, 6-, and 10-carbon dications were taken up by energised mitochondria, while the 2- and 4-carbon dications were not. The accumulation of the dication was greater than that of the monocation methyltriphenylphosphonium (TPMP). However the uptake of dications was only described by the Nernst equation at low levels of accumulation and beyond a threshold membrane potential of 90–100 mV there was negligible increase in dication uptake. Interestingly, the 5- and 6-carbon dications were not accumulated by cells, due to lack of permeation through the plasma membrane. These findings indicate that conjugating compounds to dications offers only a minor increase over monocations in delivery to mitochondria. Instead, this suggests that it may be possible to form dications within mitochondria that then remain within the cell.

Key words: lipophilic cations, mitochondria, dications, membrane potential, membrane transport, mitochondrial targeting.

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INTRODUCTION

Lipophilic cations, such as methyltriphenylphosphonium (TPMP), easily cross phospholipid bilayers and accumulate within the negatively-charged mitochondrial matrix [1, 2]. This ability to pass through biological membranes is due to the distribution of charge over a large, hydrophobic surface area [3]. The extent of uptake of these cations in response to the membrane potential (Δψ) is described by the Nernst equation, where z is the charge on the cation [3].

$$\Delta\psi(mV) = \frac{2.303RT}{zF}\log_{10}\frac{[cation]_{in}}{[cation]_{out}}$$

At 37˚C the factor before the logarithmic term for monocations is 61 mV, therefore as the mitochondrial membrane potential *in vivo* is ~120–180 mV [4], lipophilic monocations accumulate 10^2-10^3 fold within mitochondria [3]. The mitochondrial accumulation of lipophilic monocations is used extensively to measure membrane potential and to visualise mitochondria in cells [5, 6].

We have conjugated triphenylphosphonium (TPP) cations with antioxidants and other probes to direct them selectively to mitochondria within cells and *in vivo* and one such compound is now in phase II trials for Parkinson's disease [4, 7–11]. As well as promising potential therapies, these compounds are useful tools to explore mitochondrial oxidative damage [12-14]. We therefore sought to improve the extent and selectivity of mitochondrial delivery by using lipophilic dications, which offer the theoretical prospect of being dramatically more effective at delivering antioxidants to mitochondria *in vivo*. This is because the Nernst equation indicates that a dication should accumulate 100-fold within mitochondria for every 60 mV increase in membrane potential (in contrast to 10-fold for a monocation [15]), giving a 10^2-10^3–fold increase in uptake over a monocation. Such an increase would be a significant improvement in designing mitochondria-targeted therapies.

Little is known about the interaction of lipophilic dications with mitochondria, and there is therefore a need to explore their mitochondrial uptake. Furthermore, the limits to the Nernstian accumulation of these cations by mitochondria have not been tested and dications offer the possibility of exploring these limits. Only a few studies have been carried out on the interaction of lipophilic dications with mitochondria [16–20], and in none of these investigations was the dependence of dication uptake on

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membrane potential determined, so it is unclear whether dication accumulation is described by the Nernst equation.

It also remains unclear whether the rate of uptake of a dication across the mitochondrial inner membrane will be limiting. Lipophilic cations pass readily across membranes because their interaction with the hydrophobic core of the membrane has a favourably high neutral energy (proportional to the hydrophobic surface area of the cation) and a favourably low Born energy (proportional to the square of the dication charge, and inversely proportional to the cation radius [3, 21, 22]). However, the Born energy for a dication will be four times greater than that of a monocation of the same radius, so a dication will have to be more hydrophobic than a related monocation to ensure a comparable rate of uptake.

To determine whether lipophilic dications were accumulated to a greater extent than monocations we used bisTPP dications comprising two TPP cations separated by alkyl linkers of various lengths. We hypothesised that uptake would depend on the length of the alkyl link because separating the cations sufficiently might facilitate their independent interaction with the membrane, so that the Born energy would be two, rather than four, times greater than that of a comparable monocation. Furthermore, the greater hydrophobicity as the alkyl chain is lengthened should lower the activation energy for transport. Therefore a dication series of two TPP moieties attached by 2-, 4-, 5-, 6- and 10-carbon methylene bridges was constructed. We then determined whether these molecules were taken up by energised mitochondria in accordance with the Nernst equation, and whether they were accumulated by mitochondria within cells.

EXPERIMENTAL

Chemical syntheses

Known bis(triphenylphosphonium)alkyl dications were prepared by refluxing dihalides with triphenylphosphine in acetonitrile for 24 h followed by evaporation and trituration with diethyl ether unless otherwise stated. Detailed descriptions of the syntheses are given in the online Supplementary Material.

Partition coefficients and solubility

Octan-1-ol/phosphate buffered saline (PBS) partition coefficients were measured as described previously [8]. The partition coefficient of [^{3}H]PC$_5$P was determined by

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adding 1 μM compound to 1 ml PBS and 1 ml octan-1-ol, followed by incubation at room temperature for 30 min. After centrifugation (1000 x g for 4 min), the amount of compound in each phase was determined by scintillation counting. To assess solubility, 10 nmol [^{3}H]PC$_5$P in ethanol was added to Eppendorf tubes with 0, 0.5 or 5 nmol sodium TPB. The ethanol was removed under vacuum, 200 μl cyclohexane was added, and after vortexing and incubation at 37°C for 30 min, the tubes were centrifuged and the [^{3}H]PC$_5$P content of the cyclohexane (100 μl) was determined by scintillation counting.

Preparation and incubation of mitochondria

Rat liver mitochondria were prepared at 4°C in 250 mM sucrose, 5 mM Tris, and 1 mM EGTA, pH 7.4 [23], or in 250 mM sucrose, 5 mM HEPES, and 1 mM EGTA, pH 7.2, neutralised with tetramethylammonium hydroxide (TMA-OH) for the ^{86}Rb$^+$ uptake experiments. The protein concentration was determined by the biuret assay using BSA as a standard [24]. To establish the concentrations at which bisTPP dications disrupted mitochondria, we measured their effects on respiration rate and membrane potential. To measure respiration mitochondria (2 mg protein.ml^{-1}) were suspended in 120 mM KCl, 10 mM HEPES, pH 7.2, 1 mM EGTA, 10 mM KP$_i$ supplemented with rotenone (4 μg.ml^{-1}) at 25°C in the 3 ml chamber of an oxygen electrode (Rank Brothers, Bottisham, Cambridge). BisTPP dications or DMSO carrier were added and coupled respiration on succinate (10 mM) was measured followed by respiration in the presence of ADP (200 μM). There was negligible effect of the dications on respiration at concentrations below 1 μM. Slight inhibition of State III respiration began at 2–5 μM for the PC$_6$P and PC$_{10}$P dications, and at 5–10 μM for the other dications. To measure membrane potential mitochondria (2 mg protein.ml^{-1}) were suspended in 250 μl KCl medium as above supplemented with 500 nM TPMP and [^{3}H]TPMP (50 nCi.ml^{-1}). After 5 min respiring on succinate mitochondria were pelleted, and 200 μl supernatant was removed to a scintillation vial. The remaining medium was aspirated off and the pellet was dried with a tissue and resuspended in 40 μl of 20% (v/v) Triton X-100 and transferred to a scintillation vial. Scintillant (3 ml) was added and the radioactivity was quantified. The membrane potential was estimated using the Nernst equation, assuming a mitochondrial volume of 0.5 μl.mg protein^{-1} and that 60% of intramitochondrial TPMP is membrane-bound [25]. Minor effects of PC$_6$P and PC$_{10}$P were noticeable at 2.5–5 μM, while concentrations of 5–10

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μM were required for disruption by the other compounds. Yeast mitochondria were prepared from lactate-grown yeast as described [26] and stored at –80°C in 0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4, supplemented with 10 mg.ml^{-1} BSA. On thawing, these mitochondria maintain the same membrane potential as freshly isolated mitochondria, as measured by TPMP accumulation by mitochondria respiring on ethanol.

Measurement of mitochondrial uptake of bisTPP dications using an ion-selective electrode

A series of TPP ion-selective electrodes was constructed from a PVC membrane incorporating a TPB ion-exchanger and filled with an aqueous solution of the cation (10 mM for TPMP and PC_2P, 1 mM for other dications) [27, 28]. The electrode and a Ag/AgCl reference electrode were inserted through the air-tight Perspex lid of a stirred incubation chamber thermostatted at 30°C and containing 3 ml buffer comprising 120 mM KCl, 1 mM EGTA, 10 mM HEPES, pH 7.2 which was supplemented with nigericin (500 nM) and rotenone (4 μg.ml^{-1}). Log_{10}[dication or cation] versus voltage was linear (R^2 0.992–0.999). For TPMP, the electrode response gave a slope of 62.9 mV/decade. For the PC_4P, PC_5P and PC_6P dications the slopes were 27.6–34.9 mV/decade, while PC_2P and $PC_{10}P$ gave slopes of 22.2 mV/decade and 37.6 mV/decade, respectively.

Uptake of [^{3}H]TPMP, [^{3}H]PC_5P and [^{3}H]PC_6P by isolated mitochondria

To investigate mitochondrial dication uptake, mitochondria (2 mg protein.ml^{-1}) were added to 250 μl of 120 mM KCl, 10 mM HEPES, 1 mM EGTA, 100 μM DTPA, pH 7.2 with 4 μg.ml^{-1} rotenone, 300 nM nigericin and 1 μM [^{3}H]PC_5P, [^{3}H]PC_6P or [^{3}H]TPMP (7, 48 and 100 nCi.ml^{-1} respectively) at 30°C. After 1 min, 10 mM succinate was added and at various times mitochondria were pelleted by centrifugation (1 min at 16,000 x g) and the [^{3}H] content of the supernatant (200 μl) and the mitochondrial pellet determined by scintillation counting. The accumulation of cation within mitochondria was expressed as the apparent pellet space (μl.mg protein^{-1}) using the equation: apparent pellet space = 400 x [^{3}H]DPM_{pellet}/[^{3}H]$DPM_{supernatant}$. These values were not corrected for non-mitochondrial pellet [^{3}H] which is negligible at high cation accumulation [5].

To measure the simultaneous accumulation of $^{86}Rb^+$ and [^{3}H]cations mitochondria (2 mg protein.ml^{-1}) were incubated in 250 μl of 250 mM sucrose, 5 mM

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HEPES, 1 mM EGTA, pH 7.2 (TMA-OH) supplemented with 4 μg.ml^{-1} rotenone, 0.2 nmol valinomycin.mg protein^{-1}, 10 mM succinate (TMA salt), ^{86}RbCl (5 nCi.ml^{-1}) and 1 μM [^{3}H]PC$_5$P, [^{3}H]PC$_6$P or [^{3}H]TPMP (16, 16 and 20 nCi.ml^{-1} respectively; experiments using 200 nM and 40 nM [^{3}H]PC$_5$P were carried out at 3.2 and 0.64 nCi.ml^{-1} respectively). The membrane potential was varied by KCl (0–20 mM), or FCCP (0.5 μM) ± 20 mM KCl. After 5 min mitochondria were pelleted and processed for scintillation counting as above. The ^{86}Rb$^+$ and [^{3}H] DPM contents of the supernatant and pellets were then determined by dual label scintillation counting. The amount of [^{3}H] compound taken up by mitochondria was calculated from the specific activity of the incubation mixture. In parallel experiments the non-mitochondrial space in the pellets was determined by incubation with [^{14}C]sucrose (50 nCi.ml^{-1}) over the range of KCl concentrations [5]. The sucrose pellet space was 8.1 ± 0.9 μl.mg protein^{-1} (mean ± SD), did not vary significantly with [KCl], and was used to correct the apparent pellet spaces for non-mitochondrial ^{86}Rb$^+$ or [^{3}H]. These values were then divided by the mitochondrial volume under these conditions (0.6 μl.mg protein^{-1}: M. P. Murphy and M. D. Brand, unpublished) to give the accumulation ratio (ACR) [5].

$$ACR = \frac{\left[\left(\frac{400 \cdot DPM_{pellet}}{DPM_{supernatant}}\right) - sucrose \quad space \quad \left(\mu l \cdot mg \quad protein^{-1}\right)\right]}{mitochondrial \quad volume \quad \left(\mu l \cdot mg \quad protein^{-1}\right)}$$

Yeast mitochondria were washed in buffer (0.6 M mannitol, 0.5 mM EDTA, 10 mM Tris-maleate, 5 mM P$_i$, pH 6.8) and suspended at 1 mg protein.ml^{-1} at 30°C in 250 μL of the same buffer supplemented with ethanol (5.7 mM), valinomycin (0.2 nmol.mg protein^{-1}), [^{3}H]PC$_5$P (1 μM; 16 nCi.ml^{-1}) and ^{86}Rb$^+$ (5 nCi.ml^{-1}) and after 3 min incubation the pellet and supernatant were assessed as above. Accumulation ratios were estimated by correcting for the non-mitochondrial pellet space using [^{14}C]sucrose data from liver mitochondria and assuming a yeast mitochondrial volume of 1.8 μl.mg protein^{-1} (referred to in [29]). The data were qualitatively unchanged if the correction for non-mitochondrial pellet volume was omitted.

Cell culture and incubations

To measure dication toxicity, Jurkat cells were seeded in duplicate at 1 x 10^5 cells per well in 2 ml RPMI medium containing 10% foetal calf serum (FCS) and compounds were added from DMSO stock solutions and incubated for 24 h. Cell death was

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assessed by propidium iodide uptake by flow cytometry using the Calbiochem Annexin V-FITC Apoptosis Kit. Neither PC_5P, PC_6P nor TPMP up to 5 μM were toxic and showed similar toxicity up to 100 μM. To measure the uptake of [^{3}H]cations, Jurkat cells (10^6 or 10^7 cells.ml^{-1}) were incubated as above in medium supplemented with 1 μM [^{3}H]TPMP, [^{3}H]PC_5P or [^{3}H]PC_6P (15, 6 or 10 nCi.ml^{-1}, respectively). At various times 1 ml samples were taken, cells were pelleted by centrifugation (16,000 x g for 1 min) and the amount of [^{3}H] compound in the pellet measured by scintillation counting as described above. To correct for cell growth, the cell densities of parallel incubations with unlabelled cations were determined at each time. Over 23 h the cell density increased by ~100% for 1 x 10^6 cells.ml^{-1} or ~40% for 1 x 10^7 cells.ml^{-1}. To measure $PC_{10}P$ uptake, cell-associated dication was extracted into 90% acetonitrile/0.1% TFA, analysed by reverse phase HPLC (C18) on an acetonitrile gradient and detected at 220 nm. $PC_{10}P$ was quantified by comparison with standards of known concentration.

Human fibroblasts were grown overnight in DMEM medium containing 10% FCS, then incubated with fresh medium supplemented with 1 μM TPMP (20 nCi.ml^{-1}) or 1 μM PC_5P (16 nCi.ml^{-1}) for 5 h at 37°C. The medium was removed, cells were rapidly washed with 500 μl PBS and then resuspended by scraping in 200 μl 1 % Triton X-100. The [^{3}H] content of the cells was determined by scintillation counting.

Preparation of bilayer planar phospholipid membranes (BLM)

BLM were formed on the 1 mm aperture of a Teflon septum bisecting the experimental chamber. To form BLM, *Escherichia coli* phospholipids (57% phosphatidylethanolamine, 15% phosphatidylglycerol, 10% cardiolipin, 18% others) were dissolved in decane (20 mg.ml^{-1}). Measurements were carried out with AgCl electrodes and a VA-J-5 electrometer, and analyzed using a personal computer with the help of L-card L-154 and homemade software [30]. To measure the generation of a membrane potential by lipophilic cations, both compartments contained 100 nM NaTPB in 50 mM Tris HCl, pH 7.4 at 22ºC. The concentration of cation in one compartment was fixed at 1 μM while that in the other compartment was varied from 1 μM to 100 μM and the voltage determined. Under these conditions the tetraphenylphosphonium cation gave a slope of 60 mV/decade when the voltage was plotted against the Log_{10}[cation], as predicted by the Nernst equation.

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RESULTS AND DISCUSSION

Synthesis and partition coefficients of bisTPP dications

The series of bisTPP dications synthesised and their octanol-PBS partition coefficients are shown in Figure 1. The partition coefficients for PC_2P to PC_6P ranged from 0.41 to 0.84, marginally more hydrophobic then the simple TPP monocation methyltriphenylphosphonium (TPMP, partition coefficient = 0.35; [7]). Even the most hydrophobic dication, $PC_{10}P$, has a partition coefficient of only 12.8, despite the 10-carbon chain linking the two cations. This contrasts with the TPP monocation, decylTPP, which has a partition coefficient of 5000 [9]. Therefore bisTPP dications are significantly less hydrophobic than related TPP monocations.

Chain length dependence of bisTPP dication uptake by energised mitochondria

To see if bisTPP dications were accumulated by energised mitochondria we used ion-selective electrodes specific for the TPP moiety [27, 31]. In the first set of experiments, mitochondria were added to the incubation chamber in the presence of the respiratory inhibitor rotenone to prevent membrane potential formation, as shown by no uptake of TPMP in its presence (data not shown). The electrode response was then calibrated by 5 x 1 μM additions of dication (Fig 2, panels A–E). The respiratory substrate succinate was then added to generate a membrane potential, with a decrease in dication concentration indicating uptake into mitochondria. Energisation led to rapid mitochondrial accumulation of PC_5P, PC_6P and $PC_{10}P$ (Fig 2, panels C–E). The subsequent addition of the uncoupler FCCP abolished the membrane potential, releasing the dications from the mitochondria. In contrast, neither PC_2P nor PC_4P (Fig 2, panels A and B) were accumulated by energised mitochondria.

In the next set of experiments, dications were incubated with the lipophilic anion tetraphenyl borate (TPB), which facilitates the movement of lipophilic cations through biological membranes [31, 32]. The PC_4P dication was internalised rapidly by energised mitochondria when substoichiometric amounts of TPB were added, while the PC_2P dication was not (Fig 2, panels F and G). This suggests that TPB lowers the activation energy sufficiently to allow passage of the PC_4P dication through the membrane. In summary, there is a clear dependence of uptake by energised mitochondria on the length of the alkyl chain connecting the two TPP moieties.

Quantitation of TPMP, PC_5P and PC_6P accumulation by energised mitochondria

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To quantify uptake, we prepared [^{3}H]PC$_5$P and [^{3}H]PC$_6$P and compared their uptake by energised mitochondria with that of the monocation [^{3}H]TPMP (Fig 3A, B). This was done by measuring the apparent pellet space (the amount of compound per mg protein in the mitochondrial pellet divided by the amount of compound per μl of supernatant; expressed in μl.mg protein^{-1}). Dividing the apparent pellet space by the mitochondrial volume per mg of protein gives the accumulation ratio, the ratio of the compound concentrations in the mitochondria and the supernatant, which is used in the Nernst equation. Incubating [^{3}H]PC$_5$P or [^{3}H]PC$_6$P with energised mitochondria led to their accumulation over 2–3 min, at which point they came to similar steady state apparent pellet spaces of ~8000–9000 μl.mg protein^{-1}. The uptake of the bisTPP dications was prevented, and reversed, by FCCP (Fig 3A, C), confirming the extensive membrane potential-dependent dication uptake seen in electrode traces (Fig 2). The uptake of the monocation [^{3}H]TPMP was also determined and in Figure 3A this is shown on the same scale as the dications, and on an expanded scale in Figure 3B. The effect of FCCP added 5 min after TPMP addition is also shown (Fig 3D). From these experiments it is clear that the uptake of the bisTPP dications is significantly greater than that of TPMP: the maximum apparent pellet space for TPMP was ~600 μl.mg protein^{-1}, while the maximum apparent pellet space for the dications was 15-fold greater at ~ 9000 μl.mg protein^{-1}.

The increase in apparent pellet spaces for the dications over that for the monocation is far less than expected from the Nernst equation, which predicts that the dication accumulation ratio should be the square of that for the monocation, giving an apparent pellet space of ~360,000 μl.mg protein^{-1}. The measured apparent pellet spaces for the dications are ~40-fold less than this. However, comparisons of TPMP and the bisTPP dication uptake from separate experiments are not definitive as there may be differences in membrane potential and intramitochondrial binding. To overcome these difficulties we determined the mitochondrial accumulation ratio of [^{3}H]PC$_5$P, [^{3}H]PC$_6$P or [^{3}H]TPMP simultaneously with that of rubidium-86 (^{86}Rb$^+$) (Fig 4). This procedure was chosen because ^{86}Rb$^+$ is not thought to bind within mitochondria and therefore its uptake in the presence of the ionophore valinomycin is solely determined by the membrane potential [5]. Dual isotope counting enables the apparent pellet space of ^{86}Rb$^+$ and of [^{3}H]cations to be determined in the same incubation and this measurement can be repeated over a range of membrane potentials

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set by various amounts of KCl [25, 33, 34]. Parallel measurement of the non-mitochondrial space in the pellet from the distribution of [^{14}C]sucrose enables non-mitochondrial ^{3}H and $^{86}Rb^{+}$ in the pellet to be corrected for, and knowledge of the mitochondrial volume enables the true accumulation ratios for [^{3}H]cations to be calculated over a range of known membrane potentials.

In Figure 4A the accumulation ratio of [^{3}H]TPMP is plotted against that of $^{86}Rb^{+}$ over a range of membrane potentials, giving the expected straight line [25]. The uptake of TPMP is nernstian up to the highest $^{8}Rb^{+}$ accumulation ratio of ~700, which corresponds to a membrane potential of ~170 mV. The slope of 2.34 indicates that about 57% of intramitochondrial TPMP is membrane-bound in agreement with previous reports [25]. These experiments were repeated for [^{3}H]PC_5P and [^{3}H]PC_6P, and the dication accumulation ratios were plotted against [$^{86}Rb^{+}$ accumulation ratio]^{2}, which should give a straight line if the dications accumulate in accordance with the Nernst equation (Fig 4 B, C). For both [^{3}H]PC_5P and [^{3}H]PC_6P the dication accumulation ratio rises steeply at first with [$^{86}Rb^{+}$ accumulation ratio]^{2}, but there is a clear discontinuity at about 95–100 mV (corresponding to a [Rb^{+} accumulation ratio]^{2} of 1500–2200), above which the relative increases in the dication accumulation ratios are far less. The insets to Figures 4B and C show that at membrane potentials below about 100 mV, the dication accumulation ratios are linear functions of the square of the [$^{86}Rb^{+}$ accumulation ratio]^{2}, therefore below the discontinuity dication uptake is nernstian. The slopes of the nernstian regions of uptake indicate that within mitochondria about 80% of the accumulated dications are membrane bound, compared to ~57% bound for TPMP, which is consistent with their relative hydrophobicities.

The uptake of [^{3}H]TPMP increases exponentially with membrane potential, in accordance with its nernstian uptake characteristics (Fig 4D). In contrast, [^{3}H]PC_5P uptake increases dramatically at low values of membrane potential but above about 90–100 mV the further increase in uptake with membrane potential is small. At low membrane potentials dication accumulation is about five times greater than that of the monocation, but this drops to less than two-fold greater at higher membrane potential.

Why is dication accumulation non-nernstian at high membrane potentials?

These unexpected findings raise the question of why dication accumulation becomes non-nernstian above about 100 mV. This discontinuity is not due to any effect of

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dications on the membrane potential as the simultaneous measurement of the $^{86}Rb^+$ accumulation ratio shows that the membrane potential is unaffected. One possible limitation to dication uptake is that their extensive adsorption to the inner membrane may saturate all available binding sites so that further dications taken up cannot be membrane-bound and remain free in solution. If this were the case then the slope of the dication accumulation ratio against the $[^{86}Rb^+$ accumulation ratio$]^2$ would become 1 upon saturation. However, this does not occur and instead above the discontinuity the slopes are 0.01–0.02 (Fig 4).

The high dication concentration within mitochondria may inhibit subsequent uptake, perhaps due to excessive dication adsorption on the membrane affecting further uptake or to the limited dication solubility in the matrix. The former possibility was appealing as uptake requires the cation to jump from adsorption sites on the outside of the membrane to similar sites on the inside of the membrane, which if saturated inhibit transport [22]. If either of these possibilities led to the discontinuity then lowering or raising the dication concentration should shift the discontinuity to a higher or lower membrane potential. However, when the $[^3H]PC_5P$ accumulation ratio against $[^{86}Rb^+$ accumulation ratio$]^2$ was measured over a range of $[^3H]PC_5P$ concentrations from 40 nM to 5 μM, all gave similar plots and for all concentrations the discontinuity occurred between 96–103 mV (Fig 5A, B). Furthermore, below the discontinuity the plots were linear and had similar slopes for all concentrations, indicating that the proportion of accumulated dication bound to the inner membrane was concentration independent (Fig 5B). In addition, we measured the solubility of the PC_5P dication in PBS and water, giving values of ~100 mM and ~500 mM respectively at room temperature. The shift to non-nernstian behaviour is occurring well below these concentrations, since at the highest concentration of PC_5P used (5 μM) the maximum possible matrix concentration was 4.2 mM under our experimental conditions. This makes it unlikely that insolubility of the dications in the matrix is contributing to the observed discontinuity.

To explore further the possibility that dication binding to the membrane affected subsequent cation uptake, we measured the effect of 5 μM unlabelled PC_5P on $[^3H]$TPMP uptake (Fig 5C). The effect of PC_5P on the relationship between the $^{86}Rb^+$ and $[^3H]$TPMP accumulation ratios was negligible. As both TPMP and PC_5P are expected to bind to the same region of the membrane, this suggests that PC_5P does not

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saturate lipophilic cation binding sites on the matrix surface of the mitochondrial inner membrane. We next determined whether dication accumulation somehow increased the activation energy sufficiently that above the discontinuity further uptake was inhibited. TPB increases lipophilic cation uptake by lowering the activation energy for transport. However, the presence of TPB slightly lowered [^{3}H]PC$_5$P uptake and the discontinuity occurred at the same membrane potential (Fig 5D).

An alternative explanation for the discontinuity is that high dication accumulation induces a specific efflux pathway, so that uptake beyond a certain level is prevented. Energetic considerations suggest that such a process would have to be either driven directly by ATP hydrolysis, or occur electroneutrally. Furthermore, this putative pathway would have to be activated at the same membrane potential for a wide range of dication concentrations, so it is unlikely to be simply a carrier with a high K_m for the dication. Even so, we investigated whether a putative dication efflux pathway might exist by measuring the effect of a series of mitochondrial transport inhibitors on the relationship between the [^{3}H]PC$_5$P accumulation ratio and [$^{86}Rb^+$ accumulation ratio]^2 (Fig 6A). Inhibitors were chosen against a range of mitochondrial transport processes: calcium uniporter (ruthenium red), Na^+/H^+ exchanger (amiloride), ornithine carrier (ornithine), Ca^{2+}/Na^+ exchanger (CGP-37157), permeability transition (cyclosporin A) and the adenine nucleotide carrier (bongkrekic acid and carboxyatractyloside). N-ethylmaleimide (NEM) inhibits many mitochondrial transport activities and finally, inhibition of intramitochondrial ATP synthesis with oligomycin was used to decrease ATP-dependent export. If an efflux pathway existed then its inhibition should increase the [^{3}H]PC$_5$P accumulation ratio dramatically at high membrane potentials. None of the inhibitors increased the [^{3}H]PC$_5$P accumulation ratio (Fig 6A), and the data conformed to the line used to describe the dependence of the [^{3}H]PC$_5$P accumulation ratio on [$^{86}Rb^+$ accumulation ratio]^2 from Figure 4B, indicating that any inhibitor effects were solely due to changes in the membrane potential. This finding makes it unlikely that the discontinuity is due to induction of a catalytic dication efflux pathway.

Finally, to see if the discontinuity in dication uptake was a general property of mitochondria, we repeated this experiment in yeast mitochondria. Yeast mitochondria accumulated [^{3}H]PC$_5$P in a similar fashion to liver mitochondria, with uptake increasing sharply with membrane potential at low potentials, before plateauing at

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about 100 mV (Fig 6B). Although there were quantitative differences between dication uptake by yeast and mammalian mitochondria, this result suggests that the discontinuity in lipophilic dication uptake is a general property of mitochondria.

In summary, we have eliminated a number of possible explanations for the non-nernstian accumulation of lipophilic dications at high membrane potentials. To see if this discontinuity was a general property of phospholipid bilayers, we measured the diffusion potential established by varying the dication concentration gradient across a bilayer planar phospholipid membrane (BLM). The diffusion potential was nernstian (~ 30 mV for every 10-fold transmembrane concentration difference) up to a concentration gradient of ~ 50–60-fold, but beyond this point there was only a limited increase in diffusion potential as the concentration gradient increased up to 400-fold (data not shown). This suggests that non-nernstian distribution of dications at higher membrane potentials may be a general property of phospholipid bilayers.

Uptake of TPMP, PC_5P and PC_6P by cells

We next investigated whether there was greater uptake of PC_5P and PC_6P over TPMP by mitochondria within Jurkat cells [35]. [^{3}H]TPMP was taken up into cells and this uptake was largely abolished by FCCP, myxothiazol or gramicidin (data not shown). We next compared the uptake of [^{3}H]TPMP with that of [^{3}H]PC_5P and [^{3}H]PC_6P over 23 h, and found that there was extensive uptake of [^{3}H]TPMP, but not of [^{3}H]PC_5P or [^{3}H]PC_6P (Fig 7A, B). The lack of even minor dication uptake was confirmed by addition of FCCP, which did not affect the amount of dication associated with the cell pellets (Fig 7A, B). [^{3}H]TPMP was also taken up by human fibroblasts in an FCCP-dependent fashion, but [^{3}H]PC_5P was not (data not shown). Therefore neither [^{3}H]PC_5P nor [^{3}H]PC_6P are taken up into cells.

To see if TPB could facilitate dication uptake into cells, we incubated cells with 500 nM TPB for 5 h and found that TPB did not increase uptake of [^{3}H]PC_5P or [^{3}H]PC_6P (Fig 7C). However, a large excess of TPB (10 μM) did lead to the FCCP-sensitive uptake of [^{3}H]PC_5P and [^{3}H]PC_6P by cells, indicating some uptake into mitochondria. This suggests that the PC_5P and PC_6P dications are not accumulated into cells because they cannot cross the plasma membrane. To test the possibility that the hydrophobicity of the PC_5P and PC_6P dications was too low to enable cellular uptake, we also compared the uptake of PC_5P and the $PC_{10}P$ dication into Jurkat cells. In accordance with previous data (Fig 7A,B), only a very small amount of PC_5P (~ 3

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pmol/10^6 cells) was cell-associated, and this association was not FCCP-sensitive. In contrast, $PC_{10}P$ was taken up into cells (12 ± 2 pmol/10^6 cells; mean±range) over 4 h with little further increase over 24 h, and about 50% of this uptake was $\Delta\psi_m$-dependent. These findings imply that the plasma membrane is a greater activation energy barrier to dication transport than the mitochondrial inner membrane, possibly because the dielectric constant is higher within the mitochondrial inner membrane due to its greater protein content [3, 36]. However, cellular uptake of $PC_{10}P$, and that of PC_5P and PC_6P in the presence of TPB, only reached levels similar to that of TPMP, implying that even when the plasma membrane barrier was overcome, dication uptake by cells was not greater than that of the monocation. This lack of enhancement of cellular uptake suggests that lipophilic dications are unlikely to be more useful than monocations as mitochondrial delivery vectors *in vivo*.

Conclusions

BisTPP dication uptake into energised mitochondria showed a clear chain length dependence, with only dications linked by a 5-carbon chain or longer being taken up. A plausible explanation for this is the differing surface electrostatic potentials of the dications, which are shown in Supplementary Figure 1. For PC_2P the double charge is distributed over the whole surface of the molecule, while as the chain length increases the surface potentials separate into two discrete areas, resulting in a lower Born energy. In addition, as the chain length increases the overall dication surface potential decreases, increasing hydrophobicity (Fig 1). Together these will decrease the activation energy for transport. Therefore lipophilic dications can be accumulated by energised mitochondria; this is favoured by separating the charges and the dications need to be more hydrophobic than a comparable monocation.

Dication uptake was only adequately modelled by the Nernst equation at membrane potentials up to about 90–100 mV. This finding limits the utility of lipophilic dications as vectors for the delivery of compounds such as antioxidants to mitochondria. Even so, dications are accumulated to a significantly greater extent than monocations and as this differential is particularly large at low membrane potentials, lipophilic dications could be used to deliver compounds to, and measure membrane potential in, isolated mitochondria at low membrane potentials.

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There was no uptake of the PC_5P and PC_6P dications into cells, and even when the plasma membrane barrier was overcome by addition of TPB, or by increasing dication hydrophobicity, the uptake was no greater than that of TPMP. These findings suggest that it may be possible to form dications inside mitochondria *in vivo* that then remain within the cell. Since mitochondria *in vivo* rapidly accumulate orally-administered lipophilic monocations [10], simultaneous administration of two monocations containing functionalities that enable them to link together within mitochondria should lead to formation of a dicationic compound that would then be locked within the cell. This approach may prove to be a useful way to make probes for mitochondrial function *in vivo* or to prevent efflux of drugs or toxins from cells.

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Figure legends

Figure 1 Structures and octanol-saline partition coefficients of bisTPP dications

The partition coefficients are means ± SEM of three determinations.

Figure 2 Mitochondrial uptake of bisTPP dications measured using an ion-selective electrode

An electrode selective for the TPP moiety was used to measure dication concentration. TPB (500 nM) was present for the lower panels (F–J). Mitochondria (2 mg protein.ml^{-1}) were present from the start of the incubation. The electrode response was calibrated with 5 x 1 μM additions of the bisTPP dication. Succinate (10 mM) and FCCP (500 nM) were added where indicated. Data are typical traces, all of which were repeated at least three times. Voltage scales were different for each trace and have been normalised for clarity.

Figure 3 Accumulation of [^{3}H]TPMP, [^{3}H]PC$_5$P and [^{3}H]PC$_6$P by energised mitochondria

Mitochondria (2 mg protein.ml^{-1}) were added to buffer containing 1 μM [^{3}H]TPMP, [^{3}H]PC$_5$P or [^{3}H]PC$_6$P and energised with succinate. At various times mitochondria were pelleted, the [^{3}H] content of the supernatant and pellet determined by scintillation counting and used to calculate the apparent pellet space (μl.mg protein^{-1}). Panel A, grey symbols show the apparent pellet space versus time for TPMP, PC$_5$P and PC$_6$P. White symbols are for incubations in the presence of 1 μM FCCP. Data are means ± SEM from at least three experiments. Panel B shows the TPMP data from panel A on an expanded y-axis to facilitate comparison. Panel C shows the effect of addition of FCCP after PC$_5$P had accumulated within mitochondria for 5 min. PC$_6$P behaved in an identical fashion (data not shown). Panel D shows the effect of FCCP addition on the mitochondrial TPMP content after a 5-minute accumulation period. Data are means ± SEM from at least three experiments.

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Figure 4 Simultaneous accumulation of $^{86}Rb^+$ and [^{3}H]TPMP, [^{3}H]PC$_5$P or [^{3}H]PC$_6$P by energised mitochondria

Panels A – C. Mitochondria energised with succinate were incubated with $^{86}Rb^+$ (5 nCi.ml^{-1}) and either [^{3}H]TPMP (Panel A), [^{3}H]PC$_5$P (Panel B) or [^{3}H]PC$_6$P (Panel C) (all at 1 μM; 10–20 nCi.ml^{-1}) ±FCCP (0.5 μM) over a range of membrane potentials set by different KCl concentrations (0–20 mM) and the accumulation ratios for $^{86}Rb^+$ and the [^{3}H]-labelled compound measured. Data for each plot are combined from two independent experiments, each of which was done in duplicate. For Panel A the [^{3}H]TPMP accumulation ratio (ACR) is plotted as a function of the $^{86}Rb^+$ accumulation ratio, while in Panels B and C the accumulation ratio for [^{3}H]PC$_5$P or [^{3}H]PC$_6$P are plotted against [$^{86}Rb^+$ accumulation ratio]^2. The equations for the regression line that fit all the data (Panel A) or the data above the discontinuity (Panels B and C) are shown: R^2 = 0.96, 0.80 and 0.59 for A, B and C respectively. In the insets to Panels B and C the data below the discontinuities are replotted on an expanded scale and the equations for the regression lines are shown: R^2 = 0.8 and 0.6 for insets to B and C respectively. Calculation of the intercepts of the regression lines for data above and below the discontinuities enabled the $^{86}Rb^+$ accumulation ratio and thus the membrane potential at the discontinuities to be calculated, and they were 96 and 99 mV for B and C respectively. Panel D. For the experiments shown in Panels A and B the concentration (pmol.mg protein^{-1}) of [^{3}H]TPMP (circles) and [^{3}H]PC$_5$P (triangles) accumulated into mitochondria were determined and are plotted against the mitochondrial membrane potential ($\Delta\psi_m$; calculated from the $^{86}Rb^+$ accumulation ratio). In Panels A–C, lines of best fit were computed in Excel; in Panel D, trendlines were fitted by eye.

Figure 5 Effect of lipophilic dication concentration and TPB on [^{3}H]PC$_5$P accumulation ratio

In Panel A, mitochondria were incubated as in Figure 4B except that a range of [^{3}H]PC$_5$P concentrations from 40 nM to 5 μM were used. The accumulation ratio (ACR) of [^{3}H]PC$_5$P is plotted against [$^{86}Rb^+$ accumulation ratio]^2. Data are combined from two independent experiments. For all [^{3}H]PC$_5$P concentrations, the slopes of the lines before and after the discontinuity were determined and used to calculate the

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membrane potential at which the discontinuity occurred (Panel B). In Panel C the mitochondria were incubated with 1 μM [^{3}H]TPMP and with $^{86}Rb^+$ over a range of membrane potentials as in Figure 4A, but in the presence or absence of 5 μM unlabelled PC_5P. The [^{3}H]TPMP accumulation ratio is plotted as a function of the $^{86}Rb^+$ accumulation ratio in the presence (triangles) or absence (circles) of PC_5P. Data are from a single experiment that was carried out in duplicate. In Panel D, the mitochondria were incubated with 1 μM [^{3}H]PC_5P and with $^{86}Rb^+$ over a range of membrane potentials as in Figure 4B, but in the presence (squares) or absence (circles) of 500 nM TPB. Data are from two experiments that were carried out in duplicate. Trendlines were fitted by eye.

Figure 6 Effect of transport inhibitors and different types of mitochondria on [^{3}H]PC_5P uptake

In Panel A, mitochondria were incubated with 1 μM [^{3}H]PC_5P and with $^{86}Rb^+$ over a range of membrane potentials as in Figure 4B, but in the presence of a range of inhibitors of mitochondrial metabolite and ion transport at the concentrations shown in the key. The line is the regression line for the incubation of 1 μM [^{3}H]PC_5P at a range of membrane potentials shown in Figure 4B. In Panel B, *Saccharomyces cerevisiae* mitochondria energised with ethanol (5.7 mM) were incubated in the presence of $^{86}Rb^+$ (5 nCi.ml^{-1}) and [^{3}H]PC_5P (1 μM; 16 nCi.ml^{-1}) ± FCCP (0.5 μM), and the membrane potential was varied using a range of KCl concentrations (0–20 mM) as described for mammalian mitochondria. Data is combined from three separate experiments, each of which was done in duplicate. Trendlines were fitted by eye. ACR, accumulation ratio; NEM, N-ethylmaleimide.

Figure 7 Accumulation of [^{3}H]TPMP, [^{3}H]PC_5P and [^{3}H]PC_6P by cells

Jurkat cells (10^6 or 10^7 cells.ml^{-1}) were incubated with 1 μM [^{3}H]TPMP, [^{3}H]PC_5P or [^{3}H]PC_6P for the indicated times in the presence or absence of FCCP (10 μM) or TPB (500 nM or 10 μM). The cells were then pelleted by centrifugation and the amount of radioactivity in the cell pellet used to calculate the amount of cation taken up by the cells, and the cell density corrected for by parallel measurements of cell density under these conditions. Filled symbols are without FCCP; open symbols are in the presence of FCCP. Panel A. Uptake of [^{3}H]TPMP, [^{3}H]PC_5P or [^{3}H]PC_6P by 10^6 cells.ml^{-1}.

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Data are means ± range of a single experiment (two experiments for TPMP data), which was determined in triplicate. Panel B. Uptake of [^{3}H]TPMP, [^{3}H]PC_5P or [^{3}H]PC_6P by 10^7 cells.ml^{-1}. Data are individual data points from a single experiment. Panel C. Effect of 500 nM TPB on uptake of [^{3}H]TPMP, [^{3}H]PC_5P or [^{3}H]PC_6P by 10^7 cells.ml^{-1} over a 5 h incubation. Data are means ± range of two experiments, each of which was determined in triplicate. Panel D. Effect of 10 μM TPB on uptake of [^{3}H]TPMP, [^{3}H]PC_5P or [^{3}H]PC_6P by 10^7 cells.ml^{-1} over a 5 h incubation. Data are means ± range of two experiments, each of which was determined in duplicate.

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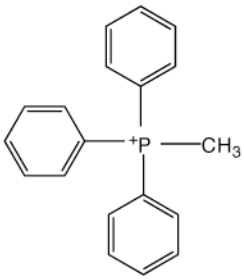
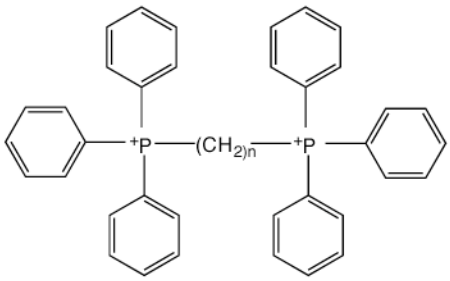
Figure 1





	n	Partition coefficient
TPMP		0.34 ± 0.01
PC_2P	2	0.41 ± 0.01
PC_4P	4	0.45 ± 0.01
PC_5P	5	0.81 ± 0.04
PC_6P	6	0.84 ± 0.02
$PC_{10}P$	10	12.8 ± 1.7

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Figure 2

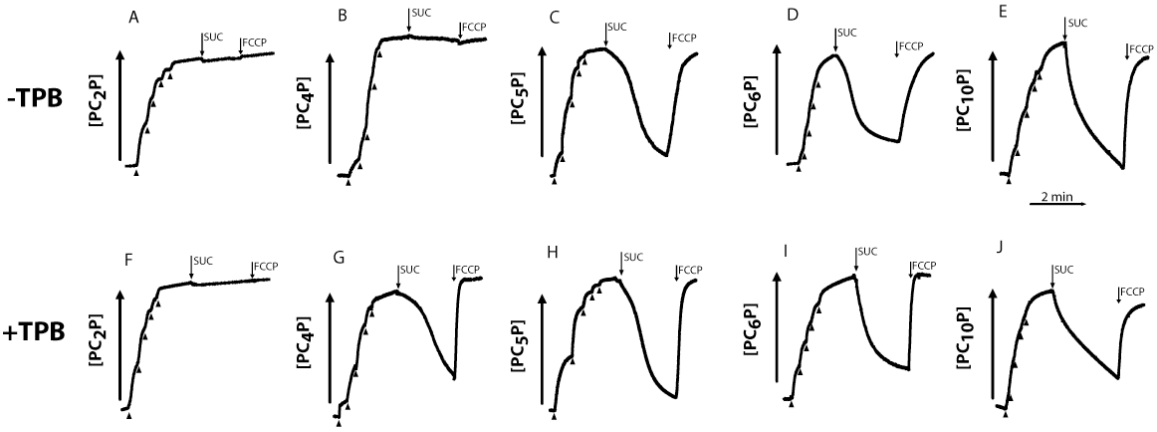

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Figure 3

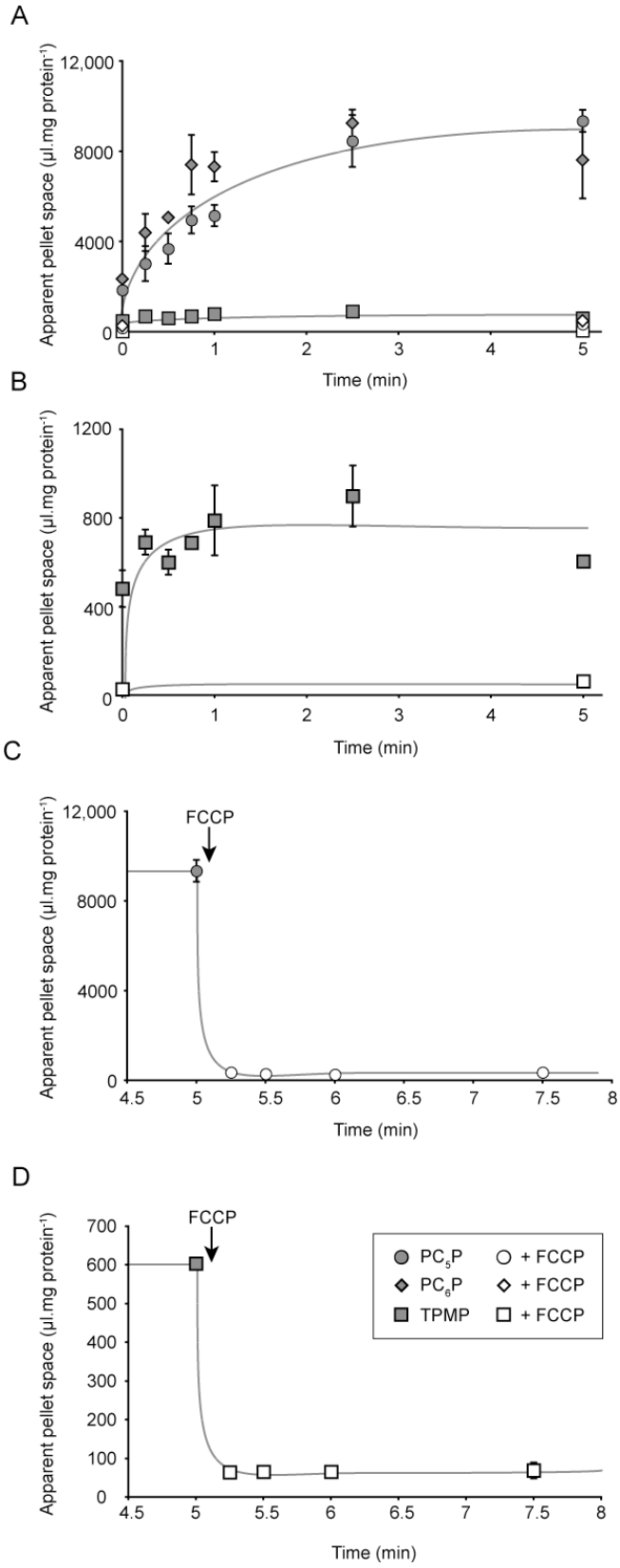

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Figure 4

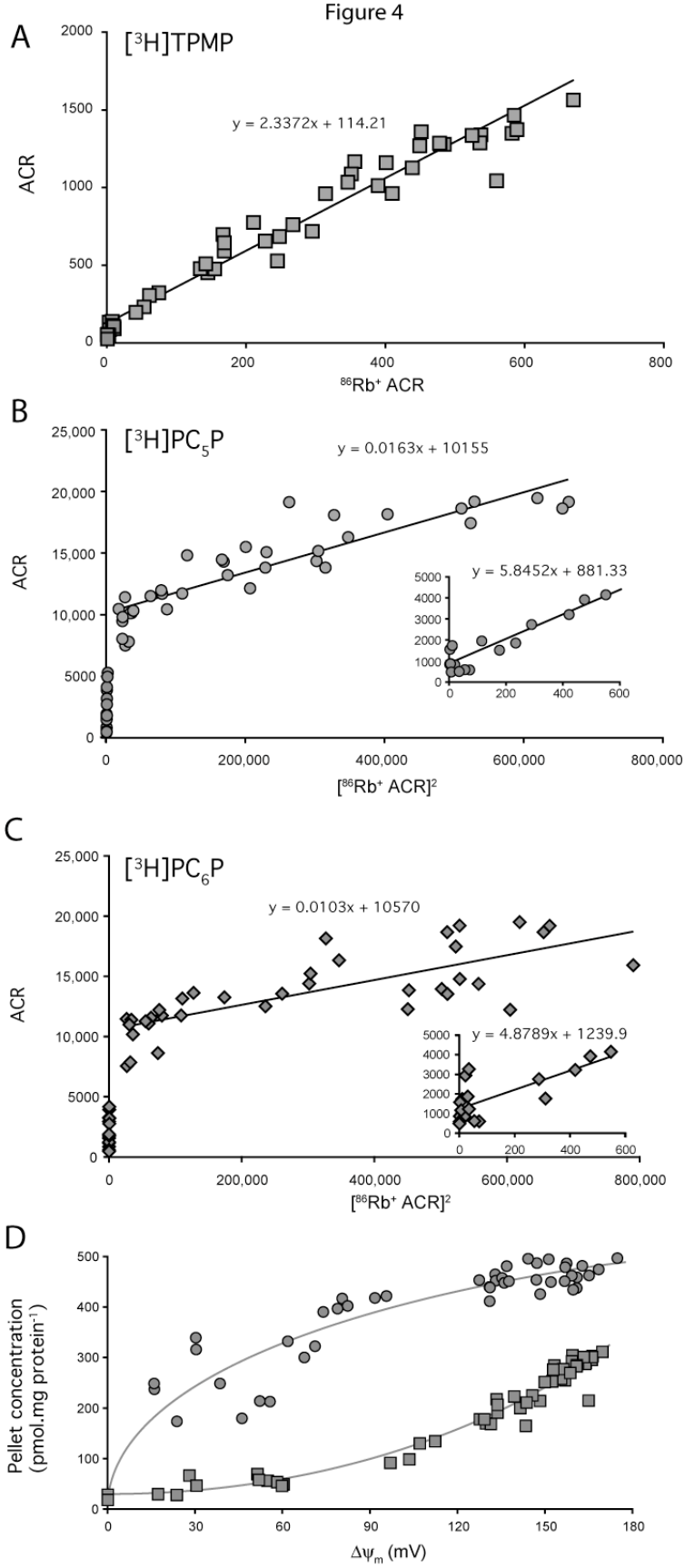

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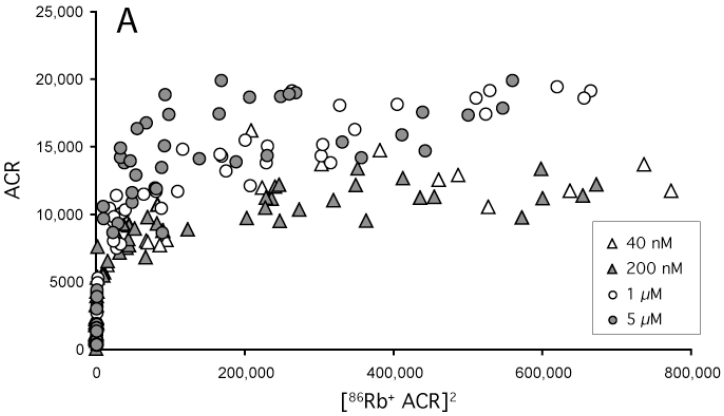
Figure 5



B

[PC$_s$P] (μM)	Slope of best fit: before discontinuity	Slope of best fit: after discontinuity	Δψ at intercept
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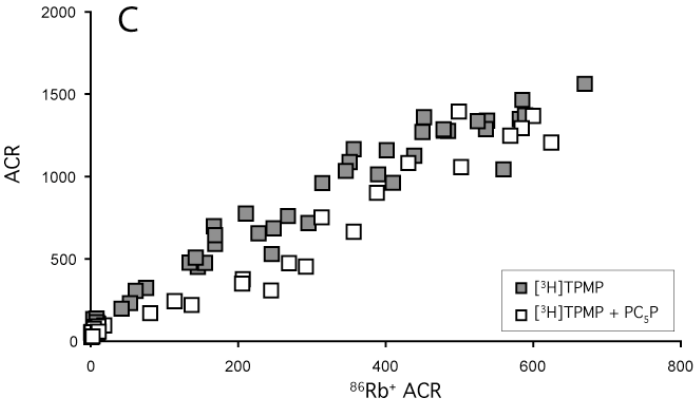
0.2	2.7	0.007	103
1	5.8	0.016	96
5	6.7	0.011	98



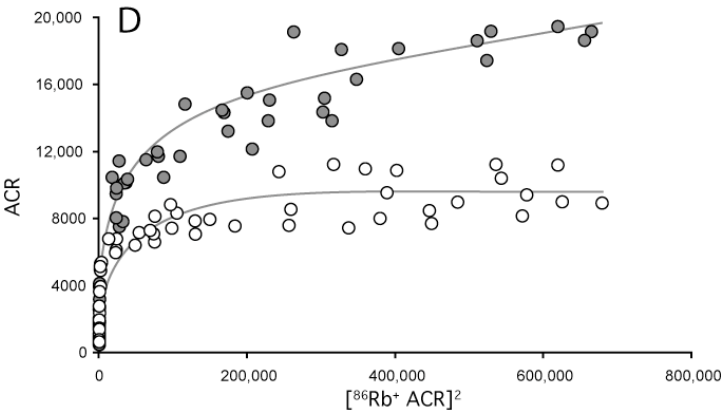


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Figure 6

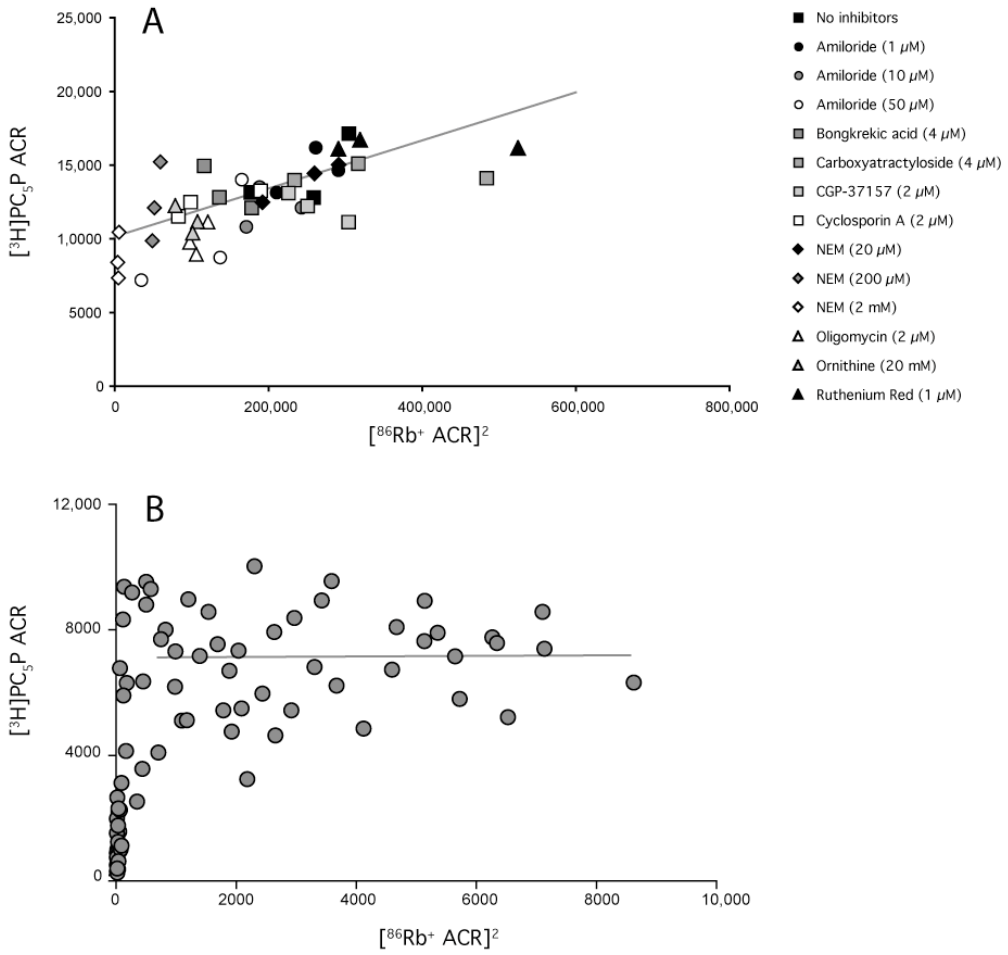

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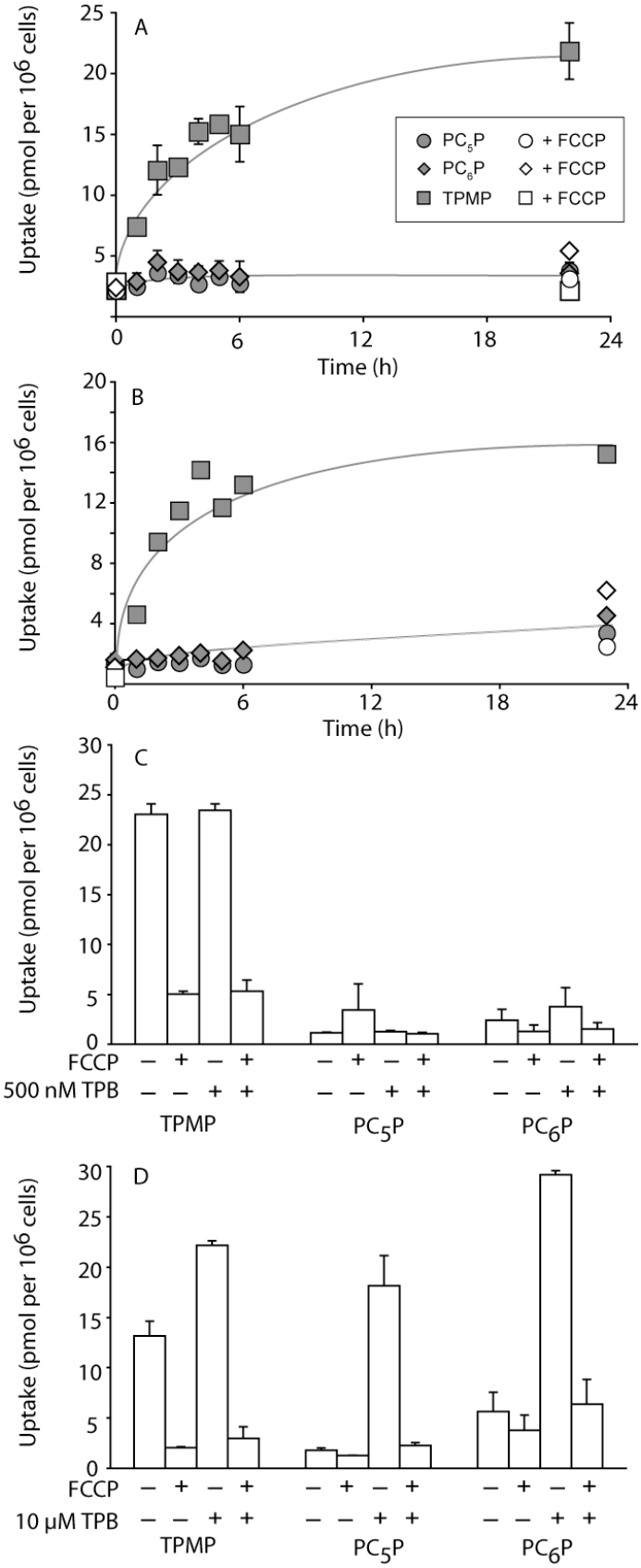
Figure 7



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