Inorganic Polyphosphate (poly P) is a polymer made from as few as ten to several hundred phosphate molecules linked by phosphoanhydride bonds similar to ATP. Poly P is ubiquitous in all mammalian organisms, where it plays multiple physiological roles. The metabolism of poly P in mammalian organisms is not well understood. We have examined the mechanism of poly P production and the role of this polymer in cell energy metabolism. Poly P levels in mitochondria and in intact cells were estimated using a fluorescent molecular probe, DAPI. Poly P levels were dependent on the metabolic state of the mitochondria. Poly P levels were increased by substrates of respiration and in turn were reduced by mitochondrial inhibitor (rotenone) or an uncoupler (FCCP). Oligomycin – an inhibitor of mitochondrial ATP-synthase, blocked the production of poly P. Enzymatic depletion of poly P from cells significantly altered the rate of ATP metabolism. We propose the existence of a feedback mechanism where poly P production and cell energy metabolism regulate each other.

INTRODUCTION

Poly P is found in all living organisms ranging from bacteria to mammals (1). Poly P performs multiple physiological functions, which are distinct and dependent on the type of organism and the subcellular localization of the polymer. In microorganisms, poly P primarily plays a role in transcription. Additionally, poly P serves as an energy store (2) and as a reserve pool of inorganic phosphates (3). However, in mammalian organisms, poly P plays predominantly a regulatory role (4) and has been implicated in the regulation of enzyme activity in cancer.
cells (5), stimulation of blood coagulation (6),
regulation of mitochondrial ion transport (7),
and regulation of respiratory chain activity (8).

Although a specific enzyme(s)
responsible for poly P production in mammals
is currently not known (1), poly P synthesis
has been detected in intact mammalian cells.
Lysis of mammalian cells leads to loss of poly
P synthesis activity, suggesting that poly P
synthesis in mammalian cells is likely an
energy-dependent process linked to membrane
transport and integrity (1;9). Taking into
account that membrane potential generated at
the mitochondrial inner membrane is a major
energy source for cellular metabolism, we
hypothesized that mitochondria may be the
likely source of poly P production in
mammalian cells.

Poly P is found in mammalian cells at
significantly lower levels when compared with
microorganisms (9); therefore, it is very
difficult to adapt poly P measurement methods
developed for bacterial studies for the study of
mammalian cells. Recently we developed a
protocol, which we optimized for suitability
for measuring low amounts of poly P using the
fluorescent probe DAPI (10). In our previous
study we used this method to confirm poly P
hydrolyzing activity of yeast polyphosphatase
expressed in mitochondria of mammalian
cultured cells (8). Here we take advantage of
this tool to examine real time changes in poly
P amounts in mammalian cultured cells, as
well as in isolated mitochondria. The aim of
this study was to establish key pathways for
poly P metabolism in mammalian cells and to
elucidate how production of mitochondrial
poly P is related to the energy metabolism and
energy consumption. Another study attempted
to assess poly P changes in mammalian
mitochondria using isolated rat liver
mitochondria in early 1960’s, but lead to
inconclusive results, presumably due to
insufficient sensitivity and reliability of assay
methods available at that time ((11) see also
discussion in (12)). Thus, to our knowledge
this is the first study that addresses the issue of
poly P metabolism in mammalian
mitochondria.

EXPERIMENTAL PROCEDURES

Cell culture and mitochondria isolation

Isolated cortical astrocytes were
prepared as previously described (13). Cerebra
taken from 2-5 day old Sprague-Dawley rats
(UCL breeding colony) or 4 day old mice were
used. The cerebra were chopped and triturated
until homogenous, passed through a 297 μm
mesh and trypsinised (50000 U/ml porcine
pancreas, Sigma, Gillingham, UK) with 336
U/ml DNAse 1 (bovine pancreas, Sigma,
Gillingham, UK), and collagenase 1.033 U/ml
(Sigma, Gillingham, UK) at 37°C for 15
minutes. After the addition of fetal bovine
serum (10% of final volume) and filtering
through 140 μm mesh, the tissue was
centrifuged through 0.4 M sucrose (400 g, 10
minutes), and the resulting pellet transferred to
Minimal Essential Medium (MEM), which
was supplemented with 5% fetal calf serum, 2
mM glutamine, and 1 mM malate in tissue
culture flasks pre-coated with 0.01% poly-D-
lysine. The cells reached confluency at 12-14
DIV and were harvested and reseeded onto
glass coverslips precoated with 0.01% poly-D-
lysine for fluorescence measurements and used
during 2-4 days.

Mitochondria were isolated by
differential centrifugation as previously
described (7). Liver from one Sprague-
Dawley rat was homogenized using Teflon-
glass homogenizer and resuspended in 50 ml
of the isolation buffer, which contained 70
mM sucrose, 230 mM mannitol, 1 mM EGTA,
and 5 mM Hepes-KOH, pH = 7.4. Unbroken
cells were centrifuged at 600X g for 15
minutes. The supernatant was collected and
spun at 8500X g for 20 min. The resulting
pellet was re-suspended in 10 ml of the
isolation buffer without EGTA. Functional
activity of mitochondrial preparations were
tested by measuring the oxygen consumption
following activation of the respiratory chain
by addition of succinate (5 mM) in the
presence of rotenone (5μM) in a buffer
solution containing 150 mM KCl, 20 mM
Hepes-KOH, pH=7.0 using Clark oxygen
electrode (Rank Brothers, USA).

Measurements of fluorescence.

DAPI (4',6-diamidino-2-
phenylindole) fluorescence was measured
using a DeltaRAM V fluorimeter (Photon
Technology International, Lawrenceville, N.J.,
USA). All recordings for isolated
mitochondria were done in plastic cuvette with
2ml of buffer solution containing 150 mM
KCl, 20 mM Hepes-KOH, pH=7.0. A silicone
stirrer was used to continuously mix the recording solution during fluorescence measurements. DAPI stock solution was prepared in water to the final concentration of DAPI 20 mM. Stock solutions of poly P standards (sodium salt with poly P content of 60% (provided by Dr. T. Shiba Regenetiss, Inc, Japan) were prepared in recording buffer to final concentrations of 0.5 mg/ml or 50 µg/ml. DAPI and poly P additions were made directly to the cuvette containing the recording buffer. For all results presented here, the total amount of poly P added to the cuvette is reported. For fluorescence spectra 1 µg of poly P corresponds to the 6 µM concentration expressed in orthophosphates. In our control experiments, similar fluorescent changes were detected using mitochondria amounts ranging from 0.1 to 0.8 mg*protein/ml, which suggest that observed effects are not caused by changes in fluorescence due to spectroscopic artifacts of added mitochondria. For the DAPI experiments presented in this study, mitochondria were added to the recording solution at a concentration defined by a protein content of 0.5 mg/ml.

Alternatively, spectra were collected with a Fluorolog-3 with a dual excitation monochrometer to alleviate scattering artifacts (Jobin-Yvon Horriba, N.J.). For imaging of poly P, [Mg$^{2+}$], and $\Delta\psi_m$ cells were loaded for 30 min at room temperature with 0.5 µM DAPI (Molecular Probes, Eugene, OR) or MagFura-2 AM (with 0.005% Pluronic) in a HEPES-buffered salt solution (HBSS) containing (in mM units): 156 NaCl, 3 KCl, 2 MgSO$_4$, 1.25 KH$_2$PO$_4$, 2 CaCl$_2$, 10 glucose, and 10 HEPES, pH adjusted to 7.35 with NaOH. For measurement of $\Delta\psi_m$, TMRM (20 nM, Molecular Probes, Eugene, OR) was added into the cultures 40 minutes prior to the experiment.

Fluorescence measurements of MagFura were obtained using an Olympus epifluorescence inverted microscope with a 20x fluorite objective. Excitation light from a Xenon arc lamp was selected using a monochromator at 340 and 380 nm (Cairn Research, Faversham, UK). Emitted light passed through a long-pass filter to a cooled CCD camera (Retiga, QImaging, Canada) and was digitized to a 12 bit resolution (Digital Pixel Ltd, UK). All imaging data were collected and analyzed using software from Andor (Belfast, UK). Cells were protected from phototoxicity by interposing a shutter in the light path to limit exposure between acquisition of successive images.

Confocal images were obtained using a Zeiss 510 CLSM equipped with a META detection system and a 40x oil immersion objective. A 543 nm laser line and a 560 nm long pass filter were used to obtain TMRM measurements. DAPI emission spectra were measured using 405 nm laser for excitation. The specific DAPI-poly P emission signal was measured above 550 nm, using the Zeiss ‘META’ system and were presented as arbitrary units (a.u.). Illumination intensity was kept to a minimum (at 0.1% of laser output) to avoid phototoxicity and the pinhole was set to give an optical slice of ~2 µm. All data presented were obtained from at least 5 coverslips and from 2-3 different cell preparations. Ca$^{2+}$ uptake by isolated canine cardiac SR vesicles was monitored using Fura-2 as previously described (14). 100 µg of SR vesicles were continuously stirred in a cuvette with 2 ml of uptake buffer containing: 100 mM KCl, 4 mM MgCl$_2$, 10 mM oxalate, 20 mM HEPES, pH 7.0 in the presence of 2.9 µM Fura-2. Uptake was initiated by the addition of 1.5 mM Na-ATP, 1.5 mM creatine phosphate, and 0.4 U/ml of creatine phosphokinase. Fura-2 fluorescence signals (alternating 340/380 nm excitation and 510 nm emission) were measured at 1 second intervals.

**Luminescence Measurements.**

Cell luminescence was measured in a home built luminometer. Cells (400,000–600,000 per coverslip) were constantly perfused with a modified Krebs–Ringer buffer containing: 125 mM NaCl, 5 mM KCl, 1 mM Na$_3$PO$_4$, 1 mM MgSO$_4$, 1 mM CaCl$_2$, 20 µM luciferin, and 20 mM HEPES (pH 7.4 at 37°C) supplemented with either 5.5 mM glucose or 0.1 mM pyruvate/1 mM lactate. Under these conditions, the light output of a coverslip of transfected cells was in the range of 500 cps (counts per second) for each measurement vs. a background lower than 5 cps. Luminescence was entirely dependent on the presence of luciferin and was proportional to the perfused luciferin concentration ~50 µM.

**Extraction and assay of poly P.**
Acid-soluble and salt-soluble poly P fractions were extracted at 4 °C with 0.5-N HClO₄ and a saturated solution of NaClO₄ in 1-N HClO₄, respectively. The remaining biomass was treated with 0.5-N HClO₄ for 30 minutes at 90 °C, and the level of acid-insoluble poly P fraction was measured by the amount of released Pi (15). Nucleotides were removed from the acid-soluble fraction by adsorption to Norit A charcoal (16). The level of poly P in the acid-soluble and salt-soluble poly P fractions was calculated as a difference in the Pi amount before and after hydrolysis of samples in the presence of 1-N HCl for 10 minutes at 100 °C (labile phosphorus). The DE81 filter-paper treatment was used in parallel to estimate the amount of poly P (17). Protein was determined as in (18) using bovine serum albumin as a standard.

**Poly P electrophoresis.**

Extracted poly P was subjected to electrophoresis in 20% polyacrylamide gel in the presence of 7-M urea. The gel was stained with toluidine blue (9). Poly P with a chain length of P₁₄, ₆₀, ₁₃₀ (Regenetiss Inc., Japan) were used as standards.

**Statistical analysis.**

Statistical analysis and exponential curve fitting were performed using Origin 8 software (Microcal Software Inc., Northampton, MA). Results were expressed as means ± standard error of the mean (S.E.M.). **Curve fitting.** Curve fitting was performed using Sigma Plot software with the following functions: \( y=a*x+b \) and \( y=Y_0+a*exp(-b*x) \) for analysis of reaction kinetics, and \( y=a*exp(-0.5*((x-X_0)/b)^2) \) for spectral analysis.

**RESULTS**

**Measurement of poly P changes in live mammalian cells.**

To investigate the role of poly P in cell energy metabolism, we utilized the modified DAPI fluorescence measurement of poly P, and we monitored the changes in poly P levels in response to activators and inhibitors of cellular metabolism. Application of 1μM FCCP (p-trifluoromethoxy carbonyl cyanide phenyl hydrazone – a mitochondrial uncoupler which dissipates mitochondrial membrane potential) to the astrocytes induced a decrease of 58.7±4.1% in DAPI-poly P fluorescence in the mitochondrial area (n=47; p<0.001; Figure 1A). FCCP also reduced the DAPI fluorescence signal in the cytosolic area (data not shown), suggesting an actual decrease in the amount of poly P by the protonophore, rather than a redistribution of poly P from the mitochondria to other cellular compartments. FCCP caused a complete depolarization of the mitochondrial membrane, which resulted in induction of the maximal rate of respiration and inhibited production of ATP by oxidative phosphorylation. Furthermore, FCCP stimulated ATP consumption by the mitochondrial F₁Fₒ-ATPase and the release of Ca²⁺ from the mitochondria into the cytosol (19). The observed decrease in the amount of poly P after FCCP addition suggests that poly P metabolism in astrocytes is linked to the ability of the cells to produce ATP and/or the possibility that poly P concentration is linked to the ability of the mitochondria to maintain their membrane potential.

The addition of 20 μM Iodo Acetic Acid (IAA) - an inhibitor of glycolysis - significantly reduced DAPI-poly P fluorescence in both mitochondrial (by 43.5±3.7%; Figure 1 B) and cytosolic (by 42.4 ± 2.7%; data not shown) areas of cortical astrocytes (n=56; p<0.05). Since glycolysis provides substrates for mitochondrial respiratory complexes; it is possible that the level of poly P in these cells depends on the activity of the respiratory chain. Application of 5 mM glutamate or pyruvate - a substrates for complex I –increased fluorescence of DAPI-poly P by 23.1 ± 1.7%, n=47 (21.5±0.9%, for pyruvate). Subsequent inhibition of complex I with 10 μM rotenone decreased the DAPI-poly P signal by 34.6 ± 3.2% as compared to control. Activation of respiration with addition of 5 mM of methyl succinate (mesuccinate) – a membrane permeable analogue of succinate and a substrate for complex II - increased the poly P level by 27.4 ± 1.9% (Figure 1C). The observed effects of FCCP, IAA, mitochondrial substrates, and rotenone support the idea that the rate of production and consumption of poly P depends on the activity of the oxidative phosphorylation machinery.

We also observed that the rate of changes in DAPI-poly P fluorescence was dependent on mitochondrial substrates, inhibitors of mitochondrial respiration, oxidative phosphorylation, and glycolysis. We should note here that fluorescent
measurements do not allow us to quantitatively estimate the amount of poly P present in mitochondria. However, in vitro experiments indicate that the DAPI fluorescence responds linearly to the amount of poly P present (10). Thus, the kinetics of DAPI fluorescence change reflect the kinetics of relative poly P change and can be used for calculations of rates of poly P flux in levels. The rate of changes in DAPI-poly P fluorescence under application of substrates for complex I was higher (61.4±5.9 a.u./min for pyruvate; 60.6±5.6 a.u/min for glutamate comparing to 51.2±4.1 a.u./min for m-succinate; Figure 1D), although this difference is not considered significant. Note that IAA and rotenone induced a faster decrease of poly P level in comparison to FCCP and oligomycin (figure 1D).

The effect of the F_{1}F_{0}-ATP synthetase inhibitor oligomycin on the level of poly P was dependent on cell type. Application of oligomycin (2μg/ml) to HEK cells induced a large increase in the DAPI-poly P fluorescence (n=44; Fig. 2A). Interestingly, when the same inhibitor was added to primary astrocytes, the DAPI-poly P signal decreased by 54.6±3.6% (n=71, p<0.001; Fig 2B; see also Fig 1C). We further investigated the differences seen between these two types of cells in response to the addition of oligomycin by measuring the effects of oligomycin on the mitochondrial membrane potential (Δψ_{m}) using the potential sensitive dye TMRM. Addition of oligomycin to cortical astrocytes resulted in “classical” hyperpolarization of the mitochondrial membrane (n=39, Figure 2D). On the other hand, oligomycin addition to HEK cells induces a depolarization of the mitochondrial membrane (n=41; Figure 2C). In cells with normal oxidative phosphorylation, the Δψ_{m} is maintained by the proton pumping activity of the respiratory chain. However, if respiration is impaired, hydrolysis of ATP by the F_{1}F_{0}-ATPase (complex V) may take over, pumping protons across the inner membrane and thus maintaining Δψ_{m} (20). Our data suggest that in HEK cells, oligomycin inhibits ATP consumption, which leads to an increase in the poly P level. Conversely, in cortical astrocytes, oligomycin inhibits ATP production, which results in a decrease in the level of poly P. These observations further support the notion that the level of poly P in mammalian cells is directly dependent on the level of ATP.

Calculation of rate constants for poly P hydrolysis.

To obtain further insight into the kinetics of poly P metabolism, we attempted to measure rate constants of chemical reactions responsible for these changes. Taking into account that current work deals with the whole organelle in an intact cell rather than purified enzyme(s), it is impossible to tell at this level how many enzymes are involved in the process, and which substrates are potentially used for the synthesis. For this reason, results of rate constant measurements should be taken with care, as we cannot tell how many reactions are potentially involved in the process and what the order of these reactions are. In spite of these difficulties, we were able to find significant differences in the kinetics of poly P hydrolysis depending on the applied conditions of study. In our analysis the kinetics of poly P hydrolysis we tested two possibilities: 1) 0-order reaction, which will take place if polyP polymer binds to the enzyme and is not released until the whole molecule is hydrolyzed – in this case enzymatic cleavage of poly P will be the rate limiting factor and decrease of fluorescence should follow 0-order linear kinetics; 2) 1st-order reaction that would take place if poly P is used as a substrate for some other reaction and becomes bound and released after cleavage of a single orthophosphate. We have found that kinetics of poly P hydrolysis induced by addition of FCCP (Fig. 1A) or IAA (Fig. 1B) follows a 1st-order reaction and can be well fitted with a single exponential, but not with a linear function. With this model reaction rate constants were 0.25±0.04 min^{-1} for FCCP and 0.66±0.04 min^{-1} for IAA. To the contrary, application of oligomycin stimulated poly P hydrolysis with kinetics, which were fit best with linear rather than exponential function. This suggested 0-order kinetics of this reaction with rate constant of 12 ± 1 min^{-1} for experiments shown at figure 1C and 9.9±0.4 min^{-1} for the experiment shown at figure 2B.

Overall, these results allow us to suggest that the mechanism of poly P hydrolysis in the presence of F_{1}F_{0}-ATPase inhibitor is fundamentally different. These
results support the idea of direct involvement of F$_{1}$F$_{0}$-ATPase in the process of poly P metabolism. Additionally, they contribute significantly to the notion that poly P hydrolysis under the condition of respiratory chain is uncoupled or inhibited.

**Measurement of poly P in isolated mitochondria.**

To ensure that our observations were not simply due to changes in DAPI fluorescence in response to changes in ATP levels, we determined how the presence of ATP as well as ADP might affect the fluorescence of DAPI-poly P. The DAPI fluorescence spectrum was measured *in vitro* using a fluorimeter in the presence of ATP and poly P. For these experiments, the excitation wavelength was set at 405 nm, which corresponds to the excitation wavelength used in the confocal fluorescence experiments.

The intensity of DAPI fluorescence can be modulated by a variety of compounds, but only DAPI bound to poly P demonstrates a specific shift in both the excitation and emission spectra (21). This property of DAPI is determined by the unique, high density distribution of negative charges found only in poly P. In our experimental conditions, changes in the DAPI fluorescence induced by non-poly P compounds are most prominent at 475 nm whereas fluorescence changes induced by poly P binding to DAPI are further red shifted and thus best detectable at 550 nm. As shown in Figure 3, addition of ATP (1.25 mM) increased the fluorescence intensity at both 475 nm and 550 nm fluorescence emissions. However, subsequent addition of 1 µg (6 µM) of poly P induces a large increase in the 550 nm fluorescence intensity, and a small decrease in the 475 nm fluorescence, consistent with a poly P induced shift of DAPI fluorescence. Addition of the enzyme hexokinase, which converts ATP to ADP, decreased the fluorescence intensity at 475 nm, whereas the signal at 550 nm remained virtually unchanged, indicating that the fluorescence intensity at 550 nm is independent of the ratio between ATP and ADP. These experiments demonstrate that the fluorescence intensity changes at 475 nm provide reliable estimates of the fluorescence change induced by non specific DAPI interactions, whereas fluorescence changes at 550 nm reflect specific change in DAPI fluorescence due to poly P interaction with DAPI.

We further confirmed the results we obtained in intact cells by using isolated mitochondria and monitoring the DAPI fluorescence simultaneously at both 475 nm and 550 nm with excitation at 415 nm. Stimulation of mitochondrial respiration with substrates for respiratory complex II (succinate, 5 mM) + phosphate (1mM) in the presence of rotenone (5 µM) significantly increased poly P-dependent DAPI fluorescence (550nm) with no changes in the 475nm emission fluorescence (Figure 4A; n=5). These results confirmed the dependence of poly P synthesis on activity of the mitochondrial respiration.

The relative contribution of mitochondrial complexes and respiration in general to poly P homeostasis can be estimated by the effects of added substrates or the presence of respiratory chain inhibitors on DAPI fluorescence. Application of 5 mM glutamate – substrate for complex I – to mitochondria increased the level of DAPI-poly P by 12±4% (n=5, p<0.01; figure 4B). Addition of rotenone (5µM) decreased the DAPI fluorescence but, interestingly, not to basal levels. Subsequent addition of substrate for complex II (succinate, 5 mM) not only restored, but also increased the level of poly P in the mitochondria (26±10%, n=7; p<0.001). Addition of a mitochondrial uncoupler – CCCP (1µM), reduced the DAPI-poly P fluorescence by 13±4% (n=4; p<0.01) but, again, not to basal levels (Figure 4B). The experiments described in this section were done in the presence of phosphate and in the absence of ADP. It is established that under these conditions the mitochondrial respiratory chain is active, but its activity is not coupled to ATP production (22;23). In our control experiments conducted in the absence of ADP, addition of succinate induced 2.5±1 fold (n=3) increase of the rate of respiration. This rate was 34±8% (n=3) of the maximal rate of mitochondrial respiration achieved in the presence of CCCP. Thus, the level of poly P in mitochondria is dependent on the activity of mitochondrial respiration and not on the production of ATP. These results suggest that mitochondria do not use ATP as a substrate for poly P synthesis.
Figure 4C shows the application of rotenone and succinate to mitochondria in the presence of oligomycin. Under these conditions the effect of substrates of respiration on DAPI fluorescence in mitochondria was completely abolished. This suggests that in mammalian mitochondria poly P production is closely related to the activity of the oligomycin-dependent F1F0-ATP synthase.

To confirm that DAPI fluorescence changes reflect changes in poly P amount, rather than its complex formation with other molecules, we compared the total amount of poly P in succinate stimulated mitochondria in the presence and absence of CCCP using a biochemical assay. It is important to note that it was not possible to do similar experiments using tissue culture cells due to the requirement of large amounts of material and sample differences arising from mitochondrial isolation from cell culture. For these experiments poly P was extracted from large preparations (50 mg/protein) of pretreated mitochondria and their amounts were measured using biochemical assay as described in materials and methods. We have found that in the presence of CCCP the level of poly P in mitochondria was reduced to 35±5 pmol/mg from 75±6 pmol/mg in the absence of CCCP. Interestingly, addition of CCCP not only decreased the total concentration of poly P, but also induced a significant decrease in the poly P chain length (figure 4D).

Finally, we further confirmed that fluorescent changes seen in our experiments reflect changes in DAPI/poly P fluorescence by analyzing the full spectrum of mitochondrial fluorescence before and after stimulation with succinate in the presence of rotenone. As can be seen from figure 5A, stimulation of mitochondria results in an increase of fluorescence in the red edge, but not on the blue side of the spectrum. Shape of the spectra was analyzed by performing Gaussian fits. We have found that upon stimulation, both fluorescence intensity, and maximum wavelength, changed. Fluorescence intensity increased from 110200±200 to 119000±200 CPM, whereas the peak of the spectrum shifted towards longer wavelength’s ranging from 511.8±0.1 to 515.6 ±0.1 nm. Similar effect was observed when low (50 to 300 ng) amounts of synthetic poly P were added to unstimulated mitochondria (Figure 5B). Difference in fluorescence spectra obtained by subtraction of stimulated mitochondria spectrum from control mitochondria spectrum, and then compared to the spectrum obtained by subtraction of mitochondrial spectrum in the presence and absence of added synthetic poly P was virtually indistinguishable. Figure 5C shows the overlay of these two normalized spectra along with Gaussian fits, in turn illustrating the similarity in both cases. In the case of stimulated mitochondria, the emission maximum was at 544.9±0.6 nm compared to 544.3±0.9 nm in the case of added synthetic poly P. We should note that fitting results indicated some difference in spectral width 43.4±0.7 vs. 39.7±0.9, which might be explained by the difference in microenvironment of poly P (intra- vs extra-mitochondrial). Thus, supporting the idea that the full spectrum represents fluorescence from both non-specific DAPI binding, and DAPI/poly P fluorescence, changes caused by stimulation of mitochondria, can still be observed in the red edge that corresponds to changes in poly P concentration.

To date the enzymes responsible for poly P synthesis and consumption have not been identified in mammalian mitochondria. Previously we demonstrated that the level of poly P in mitochondria of intact cells can be reduced to a minimum level by over expression of the mitochondrial-targeted yeast polyphosphatase (MTS-GFP-PPX)(8). To further investigate the role of poly P in the regulation of ATP production, we co-transfected MTS-GFP-PPX and mitochondrial-targeted luciferase in C2C12 cells. We have found that the rate of ATP production in cells with mitochondrial PPX in response to substrates for complexes I and II (Figure 6A) is slower when compared to the control. This is consistent with alteration of the redox index and Δψm in MTS-GFP-PPX cells (8), confirming the role of poly P in oxidative phosphorylation.

Since Mg2+ is released from MgATP upon hydrolysis (24), we measured cellular free [Mg2+]c as a measure of ATP consumption...
using Mg\(^{2+}\) sensitive fluorescent probe MagFura-2. Application of inhibitors of glycolysis (IAA, 20μM) and F\(_{1}F_{0}\)-ATP synthase (oligomycin, 2mg/ml) completely blocks ATP production in cells, which eventually leads to an increase in the MagFura-2 fluorescence due to ATP depletion and consequent Mg\(^{2+}\) release. In addition to binding Mg\(^{2+}\), MagFura-2 is also used as a low affinity Ca\(^{2+}\) indicator. Thus, the energy capacity of the cell can be estimated using Mag-Fura-2 by measuring the time between application of inhibitors of glycolysis, ATP-synthase, and energetic collapse when cells have depleted all the ATP needed for maintenance of Ca\(^{2+}\) homeostasis (25). As shown in Figure 6B, there was no difference in the rate of ATP consumption between the control and MTS-PPX C2C12 cells (compare 0.021±0.001 Magfura-2 ratio/min in control C2C12 cells, n=99 to 0.022±0.001 in MTS-GFP-PPX, n=67). However, addition of 2 μg/ml of oligomycin + 5 mM IAA induced cell collapse at a faster rate in C2C12 cells with no poly P in mitochondria compared to control (48.4±3.2 min compared to 67.2±5.1 min in control, p<0.05). These results suggest that poly P may be used by the cells as a source of energy, prolonging the time required for complete energetic collapse.

In light of these findings, we explored the ability of sarcolemmal-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) to use poly P as an alternate source of energy to ATP by measuring the rate of Ca\(^{2+}\) uptake into isolated SR vesicles. SERCA did not demonstrate any measurable activity in the absence of ATP and in the presence of up to 1 mM of either short (poly P-14) or long (poly P-130) chain poly P. Moreover, short chain poly P and to lesser degree long chain poly P, decreased the maximal rate of Ca\(^{2+}\) uptake from 0.16 μmol/min-mg in control, to 0.06 μmol/min-mg in the presence of short chain poly P and 0.12 μmol/min-mg in the presence of long chain poly P (figure 6C). These results demonstrate that poly P cannot be used directly by SERCA as an energy source, but do not rule out the possibility that other enzymes in the cell could use poly P to meet the energy demand of the cell.

**DISCUSSION**

The results presented in this study demonstrate that the level of poly P in eukaryotic cells is very dynamic and rapidly changes in response to various inhibitors and activators of mitochondrial respiration. In agreement with previous reports (9) we propose that poly P is constantly produced and consumed by mammalian cells. A high rate of turnover of poly P in eukaryotic cells is consistent with a regulatory role of poly P in cell physiology. We have found that production of poly P is directly linked to mitochondrial respiration and oxidative phosphorylation. Inhibition of poly P synthesis by oligomycin and reduction of the poly P levels in response to mitochondrial inhibitors suggests that the production of this polymer might occur through a mechanism that involves the F\(_{1}F_{0}\)-ATP synthase using a proton gradient by mechanisms similar to ATP synthesis. Considering the opposing effects of oligomycin in cells with F\(_{1}F_{0}\)-ATPase working in reverse mode (Figure 2), we propose that there are at least two enzymes which produce poly P in mammalian cells. Inhibition of glycolysis by IAA also decreased the level of poly P in the cells, although this observation may not be due to a direct effect of glycolysis on production of poly P. This could be related to the interaction of glycolysis and mitochondrial respiration.

Recently it was demonstrated that activity of the F\(_{1}F_{0}\)-ATPase can be regulated by Cyclophilin D – a key protein involved in regulation of mitochondrial Permeability Transition Pore (mPTP) (26). Our previous reports indicate that poly P plays an essential role in activation and/or formation of mPTP (7;8). We would also like to suggest that a link exists between F\(_{1}F_{0}\)-ATPase regulation of poly P metabolism and activation of mPTP.

The enzyme responsible for poly P synthesis in mammalian cells remains elusive. DNA database search shows no obvious homology between any of the bacterial poly P kinases and the mammalian genome, although it has been suggested that a homologous kinase might still exist in mammals (27) and mammals have DdPPK2 homologs. On the other hand, it is possible that mammals lack a dedicated poly P kinase, rather, poly P production may occur in these cells as a byproduct of multiple enzymes complexes. Supporting this idea, the plasma membrane Ca\(^{2+}\) ATPase in human erythrocytes, in addition to transporting Ca\(^{2+}\), can also be
involved as a polyphosphate kinase, i.e. it exhibits ATP-poly Phosphate transferase and poly Phosphate-ADP transferase activities (28). Interestingly, bacteria with double knock-out poly P kinases still retain the ability to produce significant levels of poly P (1), further emphasizing the notion that poly P may be produced by a variety of enzymes.

In conclusion, we have shown that: 1) mammalian mitochondria poly P production is directly linked to their energetic state; 2) poly P synthesis is closely linked to the activity of ATP synthase but does not require ATP as a substrate; 3) the level of poly P regulates the level of cellular ATP.

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REFERENCES


FIGURE LEGENDS

**Figure 1. Inhibition of activation of mitochondrial metabolism changes the level of DAPI-poly P in the cells.** Mitochondrial uncoupler FCCP (1 µM, A) and an inhibitor of glycolysis, IAA (20µM, B) decrease poly P-DAPI fluorescence in mitochondrial and cytosolic areas of cortical astrocytes. C - Effect of substrates for respiratory complexes I (pyruvate, 5mM) and II (me-succinate, 5mM), inhibitor of complex I (rotenone, 5µM) and F1F0-ATP synthase inhibitor (oligomycin, 2µg/ml) on the level of poly P of cortical astrocytes. D – the rate of changes in poly P level in response to mitochondrial substrates and inhibitor and inhibitors of glycolysis and oxidative phosphorylation in cortical astrocytes.

**Figure 2. Oligomycin causes opposite effect on DAPI-Poly P fluorescence in HEK cells and in astrocytes.** Inhibitor of the F1F0-ATP synthase, oligomycin (2µg/ml) increases the level of poly P in HEK cells (A) and decrease DAPI-poly P fluorescence in cortical astrocytes (B). Note that oligomycin induced mitochondrial depolarization in HEK cells (mitochondrial membrane potential was measured as TMRM fluorescence – C), but hyperpolarized mitochondria in cortical astrocytes (D). Uncoupler FCCP (1µM) indicates complete mitochondrial depolarization.

**Figure 3. DAPI fluorescence in the presence of purified ATP and poly P.** Fluorescence was excited at 405 nm and the emission was monitored simultaneously at 475 and 550 nm. Arrows indicate points of addition of 6 µM of poly P and 1.25 mM of ATP. The change in DAPI fluorescence in response to ATP addition is larger when emission is measured 475 nm as compared to
550 nm. Subsequent addition of poly P substantially increased the 550 nm emission fluorescence while reducing the 475 nm emission fluorescence, consistent with a shift in DAPI-poly P emission spectrum. Addition of hexokinase to convert all the ATP in the cuvette to ADP reduced the fluorescence emission at 475 nm without any measurable effect on the 550 nm emission.

Figure 4. Changes in DAPI fluorescence in isolated rat liver mitochondria in response to activation and inhibition of the respiratory chain. (A) The emission fluorescence of DAPI was monitored at 475 nm and 550 nm after addition of succinate (5mM) and phosphate (1 mM) in the presence of rotenone. The increase in emission fluorescence at 550 nm with no change in 475 nm confirmed that the measured changes in DAPI fluorescence were due to direct changes in poly P levels. (B) Changes in DAPI fluorescence measured at 550 nm emission in response to substrates (glutamate, 5mM and succinate, 5 mM); inhibitor (rotenone, 5µM) and uncoupler (CCCP, 1µM) of respiratory chain. (C) Presence of oligomycin (2µg/ml) to block mitochondrial ATP synthase prevented the increase in DAPI fluorescence (grey trace) observed with activation of mitochondrial respiration after addition of rotenone (5 µM) and succinate (5mM; black trace). (D) change in poly P length in mitochondria treated with succinate (5mM) in the absence (s) and presence of CCCP. Average length of poly P standards is as follows: Short: 14; Middle: 60; Long 130.

Figure 5. Fluorescence spectrum of DAPI in the presence of mitochondria. (A) Emission spectrum of DAPI was first collected after addition of rat liver mitochondria and again from the same sample following stimulation by 5 mM of succinate in the presence of 5 µM of rotenone. (B) Emission spectrum of DAPI was first collected after addition of mitochondria and again following the addition of various amounts of synthetic poly P. (C) Normalized fluorescence along with results of Gaussian fits obtained by subtraction of spectrum shown on panel (A) stimulated mitochondria minus control mitochondria (gray lines) and (B) mitochondria in the presence of 150 ng of poly P minus control mitochondria (black lines). Samples were excited at 415 nm wavelength

Figure 6. Role of poly P in ATP metabolism. Panel (A) reports representative normalized traces of C2C12 cells expressing mitochondrially targeted luciferase (mt-Luc) and mt-GFP as control (black trace) or MTS-PPX (gray trace) perfused with 50µM luciferin and challenged in sequence with 5mM pyruvate, 10µM rotenone and 5mM me-succinate (n=3 different transfections). (B) Measurements of [Mg^{2+}], which increases as ATP is hydrolysed (see text) in control and MTS-PPX C2C12 cells in response to inhibitors of ATP production IAA (20µM) and oligomycin (2µg/ml). (C) Effect of poly P on the SERCA activity estimated as the rate of Ca^{2+} uptake into isolated canine cardiac SR vesicles.
Figure 1

A. FCCP

B. IAA

C. Pyruvate and Me-Succinate

D. Bar Graph

Rate of DAPI-poly P fluorescence, a.u/min

- oligomycin
- me-succinate
- rotenone
- glutamate
- pyruvate
- IAA
- FCCP
Figure 2

A

Oligomycin

HEK

Time (min)

0 2 4 6 8 10 12

DAPI-poly P fluorescence (a.u.)

550 600 650 700 750 800 850 900

B

Oligomycin

Astrocytes

Time (min)

0 2 4 6 8 10

DAPI-poly P fluorescence (a.u.)

500 750 1000 1250 1500 1750 2000 2250 2500

C

Oligomycin FCCP

HEK

Time (sec)

0 50 100 150 200 250 300 350

TMRM fluorescence (a.u.)

260 280 300 320 340

D

Oligomycin FCCP

Astrocytes

Time (sec)

0 50 100 150 200 250 300 350

TMRM fluorescence (a.u.)

2500 2250 2000 1750 1500 1250 1000 750 500

Figure 2
Figure 5

A

B

C
Figure 6

A. Luciferin 50μM

B. IAA + oligomycin

C. Extravesicular Ca\(^{2+}\) (μM)

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