MITOCHONDRIAL DYSFUNCTION INDUCED BY A CYTOTOXIC ADENINE DINUCLEOTIDE PRODUCED BY ADP-RIBOSYL CYCLASES FROM cADPR

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Running title: Mitochondrial effects of P24

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ADP-ribosyl cyclases were previously shown to produce three new adenine dinucleotides, P1,P2 diadenosine 5'-diphosphate (Ap2A) and two isomers thereof (P18 and P24), from cyclic ADP-ribose (cADPR) and adenine (Basile, G. et al. Proc. Natl. Acad. Sci. USA. 102, 14509-14, 2005). The Ap2A isomer P24, containing an unusual C1'-N3 N-glycosidic bond, is shown here to affect mitochondrial function through, i) opening of the permeability transition pore complex (and consequent proton gradient dissipation), and ii) inhibition of Complex I of the respiratory chain. While proton gradient dissipation is dependent upon the extracellular Ca\(^{2+}\) influx triggered by P24, the effect on oxygen consumption is Ca\(^{2+}\)-independent. The proton gradient dissipation induces apoptosis in HeLa cells and thus appears to be responsible for the already described potent cytotoxic effect of P24 on several human cell types. The other products of ADP-ribosyl cyclase activity, Ap2A and cADPR, antagonize P24-induced proton gradient dissipation and cytotoxicity, suggesting that the relative concentration of P24, cADPR and Ap2A in cyclase-positive cells may affect the balance between cell life and death.

ADP-ribosyl cyclases (ADPRCs)\(^1\) are a family of multifunctional enzymes, present from protists to mammals and higher plants, that generate a number of products affecting the intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)), arguably the most influential regulatory signal in cell physiology (1, 2). Consequently, ADPRCs are involved in the regulation of several Ca\(^{2+}\)-controlled cell functions of increasing complexity from protists (3) to lower Metazoa, mammals (4) and plants (5). The Ca\(^{2+}\)-active products of ADPRCs known so far include cyclic ADP-ribose (cADPR), ADP-ribose (ADPR), nicotinic acid adenine dinucleotide phosphate (NAADP\(^+\)) and the ADP-ribose dimer (APDR\(_D\)). cADPR is produced from NAD\(^+\) by means of the reaction typical of the ADPRC family, which removes the nicotinamide moiety and creates an unusual C1'-N1 bond that cyclises the ADPR molecule (4, 6). cADPR binds to specific receptor channels on the endoplasmic reticulum, the ryanodine receptors (RyR), releasing Ca\(^{2+}\) (4, 7, 8). All ADPRCs also display to a variable degree a cADPR-degrading activity, which introduces water into the C1'-N1 bond, generating ADPR (4, 6, 7). ADPR has been recognised as an intracellular agonist gating some members of the transient receptor potential (TRP) family of non-selective, Ca\(^{2+}\)-permeable plasmamembrane channels, notably TRPM2, with consequent influx of extracellular Ca\(^{2+}\) (9-12). NAADP\(^+\) is produced by all ADPRCs from NADP\(^+\) and nicotinic acid (NA) (13). NAADP\(^+\) binds to specific Ca\(^{2+}\) channels, tentatively identified on lysosomal membranes in selected cell types (14). Finally, ADPR, which is produced by ADPRC from NAD\(^+\) in the presence of ADPR, has been reported to synergize with cADPR in releasing Ca\(^{2+}\) from sea urchin egg microsomes (15).

Recently, ADPRCs from Porifera, molluscs and mammals have been shown to catalyse an additional reaction on cADPR, introducing adenine into the C1'-N1 bond of the cyclic nucleotide and generating three new adenine homodinucleotides, called P18, P24 and P31 from their HPLC retention times (16). P31 proved to be P1,P2 diadenosine 5'-diphosphate (Ap2A). Though presence of Ap2A had been already reported in ADPRC\(^+\) platelets and cardiac myocytes (17, 18), the enzyme responsible for its synthesis was unknown. P18 and P24 are two isomers of Ap2A, each containing an unusual N-glycosidic bond between the newly introduced adenine and one ribose: C1'-N1 in P18 and C1'-N3 in P24 (16). They are the first dinucleotides featuring a non-canonical N-glycosidic bond demonstrated in (ADPRC\(^+\)) animal cells (16, 19).
Through different mechanisms, Ap2A and its isomers all affect the $[\text{Ca}^{2+}]$, when applied extracellularly to intact cells at micromolar (1 µM) concentrations: P18 induces a decrease of the $[\text{Ca}^{2+}]$, P24 conversely induces an increase of the $[\text{Ca}^{2+}]$, through extracellular calcium influx, and Ap2A synergizes with cADPR in releasing $\Delta \Psi$ from RyR (16). Not unexpectedly, all three dinucleotides affect cell proliferation (a typically $\Delta \Psi$-controlled cell function) in human cell lines and in hemopoietic progenitors (HP). Specifically, P18 and P24 both show a potent cytotoxic effect on cell lines and an even more severe growth-inhibitory effect on HP colony growth (with IC$_{50}$ values of 1.0 and 0.18 µM, respectively). Ap2A conversely stimulates HP colony growth and synergizes with cADPR, recently demonstrated to behave as a hemopoietic growth factor (20), at sub-optimal concentrations of both nucleotides (16).

These results prompted us to investigate the mechanisms underlying the cytotoxic effect of P18 and of P24, also in view of their potential use as novel antileukemic pharmaceuticals in clinical hematology. Results obtained demonstrate that mitochondria are a major target of P24 toxicity, with micromolar concentrations of the dinucleotide inducing, i) dissipation of the mitochondrial proton gradient ($\Delta \Psi$) in intact cells through opening of the permeability transition pore (PTP), and, ii) inhibition of the respiratory chain in isolated mitochondria, acting on Complex I. Conversely, P18 and Ap2A do not show significant effects on any of these functions, indicating the absolute requirement for a C1'-N3 bond in the adenine dinucleotide to exert its mitochondrial effects. While dissipation of $\Delta \Psi$ is dependent upon the $[\text{Ca}^{2+}]$, increase, inhibition of Complex I is $\text{Ca}^{2+}$-independent.

These results identify P24 as a novel endogenous regulator of mitochondrial function in mammalian cells, thus extending the physiological functions of the family of ADPRC products to include control of cell respiration. Interestingly, cADPR and Ap2A partially antagonize P24-induced cytotoxicity and proton gradient dissipation, suggesting that the relative intracellular concentrations of these ADPRC products may affect cell life and death.

**Experimental Procedures**

*Materials* - P18 and P24 were produced and purified as described in (16). Ap2A was synthesized from AMP and HPLC-purified as described in (21). Cyclosporin A (CsA), carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP), Rotenone, tetramethylrhodamine methyl ester (TMRM), propidium iodide (PI) and Hoechst 33258 were purchased from Sigma (Milan, Italy). Cyclosporin H (CsH) was obtained from Axxora (San Diego, CA).

**Microscopical evaluation of necrosis and apoptosis - HeLa cells** (obtained from ATCC, Manassas, VA) were grown in Dulbecco's modified medium, supplemented with 2 mM glutamine and 10% fetal calf serum plus 50 units/ml penicillin and 50 µg/ml streptomycin in a humidified, 5% CO$_2$ atmosphere at 37°C. Cells (2 x 10$^4$/well) were seeded into 24-well plates and exposed to either P18 or P24 at the concentrations and for the times indicated in the legend to Fig. 1. Cells were then incubated with 10 µM Hoechst 33258 and 1 µM PI for 5 min to estimate the percentage of apoptotic and necrotic cells, respectively, as described in (22). After washing with Hank's balanced salt solution (HBSS), stained cells were identified under a fluorescence microscope using excitation/emission wavelengths of 340/440 ± 25 nm and 568/585 ± 25 nm for Hoechst 33258 and PI, respectively. Three randomly selected fields were acquired from each treatment. The corresponding bright field images were also acquired, and the three channels were overlaid using the appropriate function of the Metamorph software. At least 100 cells were counted for each treatment and the percentage of apoptotic or necrotic cells was determined.

**Swelling and oxygen consumption of isolated mitochondria - Rat liver mitochondria** were isolated from albino Wistar rats by standard centrifugation techniques, as described in (23). Mitochondria (0.5 mg protein/ml) were suspended in standard medium containing 250 mM sucrose, 1 mM KH$_2$PO$_4$, 20 µM EGTA, 10 mM MOPS, pH 7.4, and 5/2.5 mM glutamate/malate or 5 mM succinate as substrates. Mitochondrial swelling was followed as the change of 90 degrees light scattering of the mitochondrial suspension at 540 nm in a Perkin-Elmer 650-40 fluorescence spectrophotometer (24). Oxygen consumption was determined polarographically using a Clark oxygen electrode (24). All assays were performed at 25°C on instruments equipped with thermostatic control and magnetic stirring.

**Mitochondrial membrane potential in intact cells** - For measurements of mitochondrial membrane potential, cells were washed and incubated in HBSS and 10 mM HEPES in the...
presence of 10 nM TMRM and 1.6 µM CsH at 37°C for the time indicated in Fig. 2. Treatment of control cells with CsH is necessary because the extent of cell and hence of mitochondrial loading with potentiometric probes is affected by the activity of the plasma membrane multidrug resistance P-glycoprotein, which is inhibited by CsH (25). Cell fluorescence images were acquired on a Leica TCS SL confocal microscope, equipped with a HCX PL APO CS 63.0x1.40 oil objective, at 1 min intervals. Cell calcium measurements - For cytosolic Ca²⁺ measurements, adherent HeLa cells (2x10⁴/well in 96 well-plates) were incubated in complete medium with Fluo 3-AM (10 µM) for 45 min at 37°C. Cells were then washed twice in HBSS and cell fluorescence was monitored at 1 min intervals on a Fluostar Optima microplate reader (BMG Labtechnologies, Offenburg, Germany) at λex 485 nm and λem 520 nm after addition of the various dinucleotides, as indicated in the legend to Fig. 5A. For mitochondrial Ca²⁺ measurements, adherent HeLa cells in complete medium (10³/well, in LabTek chambers, Nalge Nunc Int. Corp., Naperville, IL, USA) were co-incubated with the fluorescent Ca²⁺ probe Rho 2-AM (4.5 µM) and the mitochondrial tracer Mitofluor green (20 nM) for 30 min at 22°C. Cells were washed twice in HBSS and images of the regions of probe co-localization (4-5/cell, ≥10 cells/field) were acquired at 1 min intervals on a Leica TCS SL confocal microscope. Cytotoxicity assays - HeLa cells were seeded in triplicate (10⁴/well) in 96 well-plates and exposed to the various treatments described in the legends to Figs. 4 and 6. After 24 h culture, cells were washed twice in HBSS and incubated for 20 min in the presence of calcine green (2.5 mg/ml). A standard curve of cells, freshly prepared and stained in parallel. Cells were then washed three times in HBSS and the fluorescence was measured with a FluoStar Optima microplate reader at λex 485 nm and λem 520 nm. The number of viable cells was estimated by comparison of sample fluorescence with the linear standard curve.

RESULTS

P24 reduces cell viability by inducing apoptosis. Previous work had shown that both P18 and P24 are cytotoxic on a number of cell lines, including HeLa cells (16). Indeed, HeLa cell viability was severely reduced by P24 in a time- and concentration-dependent manner: the IC₅₀ of P24 was 10 µM after 24 h incubation, similar to that already reported (16). At the same concentrations and for the same duration of exposure, P18 was ineffective, in agreement with the reported IC₅₀ value of P18 in HeLa cells, which is 1 log higher than that of P24 (16). The occurrence of necrosis and/or apoptosis was investigated by assessing the staining of nuclei with PI and Hoechst 33258, respectively: Fig. 1A shows representative images of HeLa cells incubated in the absence (control) or in the presence of 10 µM P24 for 24 h. The P24-treated cultures showed a marked decrease of the cell number and an increase of the proportion of blue, i.e. apoptotic, cells. The percentage of necrotic and apoptotic cells in P24- and P18-treated cultures was determined and results are shown in Fig. 1B and C, respectively. An incubation time shorter than 24 h was chosen to avoid loss of adherent cells due to cell death. The decrease of cell viability in P24-incubated cultures occurred mostly because of apoptosis, although at higher P24 concentrations a significant proportion of cell death was due to necrosis. Conversely, at the same concentrations, P18 did not significantly modify the percentage of necrotic and apoptotic cells as compared to untreated controls.

P24 dissipates the mitochondrial proton gradient in intact cells. Since mitochondrial alterations are known to play a major role in determining cell death, we investigated the effect of P24 on mitochondrial functions to elucidate the mechanisms underlying its cytotoxicity. Thus, intact cells were incubated in the presence of P24 and ΔΨm was measured with the fluorescent probe TMRM. As shown in Fig. 2A (blue trace) and 2C, P24 induced a progressive decrease in TMRM fluorescence, reflecting a fall of ΔΨm. The slight decrease in fluorescence obtained upon addition of the uncoupler FCCP indicated that ΔΨm was already almost completely abolished after incubation of the cells for 30 minutes with 20 µM P24. The effect of P24 on ΔΨm was concentration-dependent, with as low as 50 nM P24 dissipating approximately 30% of the TMRM fluorescence after 2 h incubation of the cells (not shown).

Under physiological conditions, ΔΨm is maintained by the proton pumping activity of the respiratory chain complexes. However, when the electron flow is hampered, ΔΨm maintenance is allowed by the proton pumping activity of the F₁F₀-ATPase, powered by ATP hydrolysis. The contribution of respiratory chain or ATP hydrolysis to ΔΨm maintenance
can be assessed by using selective inhibitors. Addition of oligomycin, an inhibitor of \( \text{F}_0\text{F}_1\)-ATPase, did not affect \( \Delta \Psi \)m (Fig. 2A, black trace, and 2C), indicating that, in the absence of any impairment of the respiratory chain, ATP hydrolysis does not contribute to the generation of mitochondrial membrane potential, as expected. Conversely, the addition of oligomycin to P24-treated cells caused an abrupt fall of \( \Delta \Psi \)m (Fig. 2A, red trace, and 2C). On the one hand, this result suggests an inhibitory effect of P24 on the respiratory chain. On the other hand, the effect elicited by oligomycin rules out the possibility that adenylate translocase or \( \text{F}_0\text{F}_1\)-ATPase are also severely affected by P24, as oligomycin would have been without further effect if ATP uptake or hydrolysis were already inhibited. The addition of rotenone alone, an inhibitor of Complex I, resulted in only a slight decrease of the \( \Delta \Psi \)m (Fig. 2A, purple trace, and 2C), due to the progressive consumption of ATP for proton pumping by the \( \text{F}_0\text{F}_1\)-ATPase. The addition of rotenone to P24-treated cells, however, induced a faster and greater decrease of \( \Delta \Psi \)m (Fig. 2A, green trace, and 2C) than either that of P24 (blue trace) or of rotenone (purple trace) alone, suggesting that a mechanism other than respiratory chain inhibition is primarily responsible for \( \Delta \Psi \)m dissipation triggered by P24.

P24 has been shown to induce an increase of the \([\text{Ca}^{2+}]_i\) through extracellular \( \text{Ca}^{2+} \) influx (16). Pre-incubation of the cells for 1 h with the intracellular \( \text{Ca}^{2+} \)-chelator EGTA-AM (0.5 mM) prior to exposure to P24 prevented \( \Delta \Psi \)m dissipation (Fig. 2B, red trace, and 2C), as did presence of EDTA (not shown), demonstrating a causal role of the \([\text{Ca}^{2+}]_i\) increase in mediating the effect of P24 on \( \Delta \Psi \)m. P18, cADPR and Ap2A, at concentrations ranging between 1.0 and 10 \( \mu \)M, did not significantly affect \( \Delta \Psi \)m (not shown).

**P24 affects oxygen consumption of isolated rat liver mitochondria.** To verify an inhibitory effect of P24 on the respiratory chain and to identify the site of inhibition, experiments were carried out on isolated rat liver mitochondria. Oxygen consumption was measured in the presence of Cyclosporin A (CsA) to avoid possible interference of the mitochondrial permeability transition pore (PTP). In fact, PTP opening can affect oxygen consumption due to its uncoupling-like effect and to the release of pyridine nucleotides (26, 27).

In the presence of the NAD\(^+\)-dependent substrates glutamate/malate, the increase over basal values of the oxygen consumption stimulated by the uncoupler FCCP was reduced by 86\% after preincubation of mitochondria with 10 \( \mu \)M P24 for 10 min (Table 1). Conversely, P24 did not affect the utilization of succinate, in the presence of rotenone (Table 1). Therefore, P24 inhibits electron transfer at the level of Complex I, but not of Complex II. P24 also inhibited ADP-stimulated respiration (State 3) in the presence of the Complex I substrates glutamate/malate (Table 1), nearly as efficiently as it inhibited the uncoupler-induced respiration (88\% vs. 86\%). The fact that P24 failed to inhibit the ADP-stimulated oxygen consumption in the presence of the Complex II substrate succinate unequivocally demonstrates that P24 does not affect \( \text{F}_0\text{F}_1\)-ATPase or adenine nucleotide translocase as their inhibition would have affected also the activity of Complex II.

Basal oxygen consumption (State 4) was significantly higher in P24-treated mitochondria, compared to controls, in the presence of substrates of either Complex I (20\% increase) or Complex II (37\% increase) (Table 1). Thus, P24 behaves as a mild uncoupler, in line with the decrease of the \( \Delta \Psi \)m described in the previous paragraph. The effect of P24 on uncoupled respiration displayed a dose-dependency, since oxygen consumption was inhibited by 20\% and 40\% in the presence of 0.1 and 1.0 \( \mu \)M P24, respectively.

Similar results as those described above were obtained in the presence of EGTA, indicating that the effects of P24 on respiration were not \( \text{Ca}^{2+} \)-dependent.

**P24 induces mitochondrial swelling by opening the permeability transition pore.** The fact that P24 and rotenone together induced a larger dissipation of \( \Delta \Psi \)m than that caused by either compound alone (Fig. 2A, C) suggested presence of an additional mechanism of \( \Delta \Psi \)m dissipation triggered by P24, beside inhibition of the respiratory chain. Since neither \( \text{F}_0\text{F}_1\)-ATPase nor adenylate translocase were inhibited by P24, we investigated whether P24 induced the opening of the mitochondrial permeability transition pore (PTP), which could account for the observed decrease of \( \Delta \Psi \)m (27).

Swelling of the mitochondrial matrix was monitored as decrease in absorbance at 540 nm, reflecting a decrease in light scattering of the mitochondrial suspension. Intact mitochondria were suspended in the presence of the highest
Ca\(^{2+}\) concentration insufficient per se to trigger mitochondrial swelling, yet necessary to ensure PTP opening in response to specific stimuli (28). Indeed, P24 at 5 µM caused mitochondrial swelling (Fig. 3), which could be attributed to PTP opening based on the significant (75%) inhibition exerted by CsA (29). The fact that CsA also significantly reduced (by approximately 70%) the drop of TMRM fluorescence by P24 (Fig. 2B, green trace, and 2C) indicates a causal role of PTP opening in ΔΨ\(_{m}\) dissipation. Thus, the larger drop in ΔΨ\(_{m}\) caused by the concomitant addition of rotenone and P24 to HeLa cells, as compared to the effect of either rotenone or P24 alone (Fig. 2A, C), was likely the consequence of both respiratory chain inhibition and PTP opening. Indeed, the simultaneous occurrence of these two alterations hampers the ability of F\(_{0}\)F\(_{1}\)-ATPase to maintain ΔΨ\(_{m}\) (27).

P18, cADPR and Ap2A, at concentrations ranging between 1.0 and 10 µM, did not induce mitochondrial swelling (not shown).

**P24 cytotoxicity is causally related to the dissipation of ΔΨ\(_{m}\).** The proton gradient dissipation and the inhibition of Complex I exerted by P24 at concentrations (5-20 µM) in the same range as those inducing cytotoxic effects on HeLa cells (Fig. 1B) suggested that the mitochondrial effects of the dinucleotide could be responsible for cell death. Indeed, CsA at 1 µM remarkably reduced P24 cytotoxicity (Fig. 4). Moreover, extracellular Ca\(^{2+}\) chelation during exposure of the cells to P24 completely prevented cytotoxicity (Fig. 4), demonstrating the fundamental role of the [Ca\(^{2+}\)]\(_{i}\) increase in mediating the cytotoxic effect of P24. This experiment also highlights the fact that a brief (2 h) exposure of HeLa cells to P24 is sufficient for the dinucleotide to exert its cytotoxic effect, similarly to what observed on colony forming cells (16). As P24-induced ΔΨ\(_{m}\) dissipation is Ca\(^{2+}\)-dependent (Fig. 2B, red trace, and 2C), while Complex I inhibition is not (see above), the protective effect of EDTA and of CsA against P24 cytotoxicity indicate that PTP opening and ΔΨ\(_{m}\) dissipation play a major role in inducing cell death. In line with this conclusion, the Complex I inhibitor rotenone, at concentrations (1-2 µM) which induced respiratory chain inhibition, proved to be devoid of toxicity on HeLa cells over a 24 h incubation time (not shown).

The effects of P24 on the [Ca\(^{2+}\)]\(_{i}\), on ΔΨ\(_{m}\) and on cell viability are partly antagonized by cADPR and Ap2A. Given the causal role of the [Ca\(^{2+}\)]\(_{i}\) increase in mediating the adverse effects of P24 on both maintenance of ΔΨ\(_{m}\) and on cell viability, we investigated the effect of cADPR, Ap2A and P18, all affecting the [Ca\(^{2+}\)]\(_{i}\), on the [Ca\(^{2+}\)] increase induced by P24.

At a five-fold higher concentration than P24 (i.e. 100 µM), P18, but not Ap2A, almost completely prevented the P24-induced increase of the [Ca\(^{2+}\)]\(_{m}\), which is due to extracellular Ca\(^{2+}\) influx, being abrogated by EDTA (ref. 16 and Fig. 5A). cADPR also prevented the P24-induced [Ca\(^{2+}\)]\(_{m}\) rise (Fig. 5A), as the slight increase of the [Ca\(^{2+}\)]\(_{m}\) observed in the presence of cADPR and P24 together was similar to that produced by 100 µM alone (Fig. 5A, inset). A sustained cytosolic [Ca\(^{2+}\)] increase is known to result also in a mitochondrial [Ca\(^{2+}\)] rise (30). In fact, P24 also induced a sustained [Ca\(^{2+}\)]\(_{m}\) increase (Fig. 5B), as determined by confocal microscopy on HeLa cells loaded with the mitochondria-targeted fluorescent Ca\(^{2+}\) probe Rho-2 AM (Fig. 5C). In line with their above-described effects on the [Ca\(^{2+}\)]\(_{m}\), cADPR, but not Ap2A, almost completely reduced the P24-induced increase of the [Ca\(^{2+}\)]\(_{m}\) (Fig. 5B).

Interestingly, P18, which antagonized the increase of the [Ca\(^{2+}\)]\(_{m}\) triggered by P24, synergized with P24 in inducing an even higher rise of the [Ca\(^{2+}\)]\(_{m}\) than that observed with P24 alone (Fig. 5B). In fact, P18 alone also induced an increase of the [Ca\(^{2+}\)]\(_{m}\) similar to that observed with P24 alone (not shown).

In line with its inhibition of the P24-induced [Ca\(^{2+}\)]\(_{m}\) rise, and with the causal role of the [Ca\(^{2+}\)]\(_{m}\) increase in determining ΔΨ\(_{m}\) dissipation, cADPR also prevented the drop of TMRM fluorescence induced by P24 (Fig. 2B, black trace, and 2C). Interestingly, Ap2A showed a similar protective effect as cADPR on P24-induced ΔΨ\(_{m}\) dissipation (Fig. 2B), although it did not affect either the cytosolic or the mitochondrial [Ca\(^{2+}\)] increase induced by P24. This result suggests that Ap2A antagonizes a Ca\(^{2+}\)-independent effect of P24 on the PTP, which is necessary, together with the [Ca\(^{2+}\)]\(_{m}\) increase, for PTP opening. That both Ca\(^{2+}\) and P24 are required to ensure PTP opening is also confirmed by the following observations: i) in the absence of P24, the addition of 10 µM Ca\(^{2+}\) was not sufficient for PTP opening (Fig. 3), and, ii) cADPR and Ap2A both induce a similar [Ca\(^{2+}\)]\(_{m}\) increase as P24 (16), but they do not have any effect on the ΔΨ\(_{m}\) (not shown).

At a five-fold higher concentration than P24, both cADPR and Ap2A significantly reduced P24 cytotoxicity on HeLa cells, in line with their protective effects on the ΔΨ\(_{m}\) dissipation: the IC\(_{50}\) value of P24 increased from 10 µM to 16...
shown to cross the plasmamembrane of intact
Extracellularly added P24 and Ap2A have been
between these dinucleotides and ADP.
statistical similarity
nucleotide-binding site is present on the PTP
affecting the $[\text{Ca}^{2+}]$ rise, suggesting a competition
between Ap2A and P24 for direct binding to the
PTP. In this respect, a regulatory, adenylic
nucleotide-binding site is present on the PTP
protein complex (34), which might be targeted
by P24/Ap2A, given the structural similarity
between these dinucleotides and ADP.
Extracellularly added P24 and Ap2A have been
shown to cross the plasmamembrane of intact
HeLa cells, allowing access of these
dinucleotides to the mitochondria (16).
Dissipation of $\Delta \Psi_m$ and inhibition of Complex I
by P24 appear to be two independent effects.
PTP opening and $\Delta \Psi_m$ dissipation by P24 are
not secondary to inhibition of Complex I, as
rotenone does not induce a significant loss of
$\Delta \Psi_m$ (Fig. 2A, C). Inhibition of Complex I by
P24 is not caused by dissipation of $\Delta \Psi_m$, as it
occurs also in the presence of EDTA and CsA
(Table 1 and Results), i.e. under conditions
where PTP opening and $\Delta \Psi_m$ dissipation are
largely prevented.
The cytotoxic effect of P24 appears to be
causally related to the dissipation of $\Delta \Psi_m$
through PTP opening, rather than to inhibition
of Complex I. In fact, the Complex I inhibitor
rotenone did not show any cytotoxic effect on
HeLa cells. Conversely, PTP opening is
recognized as a pivotal process in cell death,
both in apoptosis and in necrosis (35). Indeed,
P24 cytotoxicity was significantly reduced by
CsA and completely prevented by EDTA (Fig. 4).

The effects of P24 on the PTP and on Complex
I appear to be strictly related to the peculiar
chemical structure of this dinucleotide, as
neither of its isomers P18 and Ap2A shows any
effect on $\Delta \Psi_m$ or on mitochondrial respiration.
Interestingly, however, P18, Ap2A and also
cADPR affect P24 cytotoxicity: P18 decreases
the IC$_{50}$ of P24 from 10 $\mu$M to 6 $\mu$M, Ap2A and
cADPR instead increase the IC$_{50}$ value to 16
$\mu$M and 23 $\mu$M, respectively (Fig. 6). The
additive effect of P18 and P24 on cytotoxicity
confirms the conclusion, drawn from the above-
described experiments on mitochondrial
function, that P18 and P24 have different
targets of action. Ap2A and cADPR both
prevent the P24-induced $\Delta \Psi_m$ dissipation (Fig.
2B), which is the principal cause for cell death,
but through different mechanisms: cADPR
prevents the $[\text{Ca}^{2+}]$ rise triggered by P24 (Figs.
5 and 7), which is necessary for PTP opening,
while Ap2A does not affect the P24-induced
$[\text{Ca}^{2+}]$ increase (Fig. 5), thus apparently
interfering with a $[\text{Ca}^{2+}]$-independent effect of
P24 on the PTP (Fig. 7).
Among the important issues that remain to be
elucidated at a molecular level, the channel
responsible for $\text{Ca}^{2+}$ entry triggered by P24 (and
antagonized by cADPR and P18) and the site of
action of P24 on the PTP deserve priority. The
extracellular $\text{Ca}^{2+}$ influx triggered by P24 is not
dependent on Complex I inhibition and consequent possible $\text{ATP}$ shortage to
plasmamembrane $\text{Ca}^{2+}$-extruding pumps.
because rotenone does not induce any $[\text{Ca}^{2+}]$, increase in HeLa cells (not shown).

In conclusion, results presented here identify the ADPRC product P24 as a novel endogenous regulator of mitochondrial function. The fact that Ap2A and cADPR antagonize P24-induced $\Delta\Psi_m$ dissipation and cytotoxicity suggests that the relative concentration of these ADPRC products may affect the delicate balance between survival and death in ADPRC$^+$ cells.

REFERENCES

1. Berridge, M.J., Bootman, M.D., and Roderick, H.L. (2003) Nat. Rev. Mol. Cell Biol. 4, 517-29
2. Carafoli, E. (2005) FEBS J. 272, 1073-1089
3. Masuda, W., Takenaka, S., Inageda, K., Nishina, H., Takahashi, K., Katada, T., Tsuyama, S., Inui, H., Miyatake, K., and Nakano, Y. (1997) FEBS Lett. 405, 104-106
4. Lee, H. C. (2002) in Cyclic ADP-Ribose and NAADP: Structures, Metabolism and Functions, Kluwer Academic Publishers, Norwell, MA
5. Wu, Y., Kuzma, J., Marechal, E., Graeff, R., Lee, H.C., Foster, R., and Chua, N.H. (1997) Science 278, 2126-2130
6. Schuber, F., and Lund, F.E. (2004) Curr. Mol. Med. 4, 249-261
7. Lee, H.C., Walseth, T.F., Bratt, G.T., Hayes, R.N. and Clapper, D.L. (1989) J. Biol. Chem. 264, 1608-1615
8. Guse, A.H. (2005) FEBS J. 272, 4590-4597
9. Perraud, A.L., Fleig, A., Dunn, C.A., Bagley, L.A., Launay, P., Schmitz, C., Stokes, A.J., Zhu, Q., Bessman, M.J., Penner, R., Kinet, J.P., and Scharenberg, A.M. (2001) Nature 411, 595-599
10. Kolisik, M., Beck, A., Fleig, A., and Penner, R. (2005) Mol. Cell 18, 61-69
11. Heiner, I., Eisfeld, J., Warmstedt, M., Radukina, N., Jungling, E., and Luckoff, A. (2006) Biochem. J. 398, 225-232
12. Gasser, A., Glassmeier, G., Flieger, R., Langhorst, M.F., Meinke, S., Hein, D., Kruger, S., Weber, K., Heiner, I., Oppenheimer, N., Schwarz, J.R., and Guse A.H. (2006) J. Biol. Chem. 281, 2489-2496
13. Lee, H.C. (2005) J. Biol. Chem. 280, 33693-33696
14. Churchill, G.C., Okada, Y., Thomas, J.M., Genazzani, A.A., Patel, S., and Galione, A. (2002) Cell 111, 703-708
15. De Flora, A., Guida, L., Franco, L., Zocchi, E., Bruzzone, S., Benatti, U., Damonte, G., and Lee, H.C. (1997) J. Biol. Chem. 272, 12945-12951
16. Basile, G., Tagliatela-Scafati, O., Damonte, G., Armiriotti, A., Bruzzone, S., Guida, L., Franco, L., Usai, C., Fattoruso, E., De Flora, A., and Zocchi, E. (2005) Proc. Natl. Acad. Sci. USA 102, 14509-14514
17. Jankowski, J., Hagemann, J., Tepel, M., van Der Giet, M., Stephan, N., Henning, L., Gouni-Berthold, I., Sachinidis, A., Zidek, W., and Schluter, H. (2001) J. Biol. Chem. 276, 8904-8909
18. Luo, J., Jankowski, J., Knobloch, M., Van der Giet, M., Gardanis, K., Russ, T., Vahlensieck, U., Neumann, J., Schmitz, W., Tepel, M., Deng, M.C., Zidek, W., and Schluter, H. (1999) FASEB J. 13, 695-705
19. Walseth, T.F. (2005) Proc. Natl. Acad. Sci. USA 102, 14941-14942
20. Podestà, M., Benvenuto, F., Pitto, A., Figari, O., Bacigalupo, A., Bruzzone, S., Guida, L., Franco, L., Paleari, L., Bodrato, N., Usai, C., De Flora, A., and Zocchi, E. (2005) J. Biol. Chem. 280, 5343-5349
21. Rossi, L., Brandi, G, Schiavano, G.F., Balestra, E., Millo, E., Scarfi, S., Damonte, G., Gasparini, A., Magnani, M., Perno, C.F., Benatti, U., and De Flora, A. (1998) AIDS Res. and Hum.
Retroviruses 14, 435-444

22. Ormerod, M.G., Sun, X.M., Brown, D., Snowden, R.T., and Cohen, G.M. (1993) Acta Oncol. 32, 417–424

23. Costantini, P., Petronilli, V., Colonna, R., and Bernardi, P. (1993) Toxicology 99, 77-88

24. Scorrano, L., Petronilli, V., Di Lisa, F., and Bernardi, P. (1999) J. Biol. Chem. 274, 22581-22585

25. Ryffel, B., Woerly, G., Rodriguez, C. and Foxwell, B.M. (1991) J. Recept. Res. 11, 675-686

26. Di Lisa, F., Menabò, R., Canton, M., Barile, M., and Bernardi, P. (2001) J. Biol. Chem. 276, 2571-2575

27. Di Lisa, F., and Bernardi, P. (2006) Cardiovasc. Res. 70, 191-199

28. Bernardi, P. (1999) Physiol. Rev. 79, 1127-1155

29. Halestrap, A.P., Connern, C.P., Griffiths, E.J., and Kerr, P.M. (1997) Mol. Cell Biochem. 174, 167-172

30. Szabadkai, G., Simoni, A. M., and Rizzuto, R. (2003) J. Biol. Chem. 278, 15153-15161

31. Chinopoulos, C., Starkov, A.A., and Fiskum, G. (2003) J. Biol. Chem. 278, 27382-27389

32. Almofti, M.R., Ichikawa, T, Yamashita, K., Terada, H., and Shinozhara, Y. (2003) J. Biochem. 134, 43-49

33. Malkevitch, N.V., Dedukhova, V.I., Simonian, R.A., Skulachev, V.P., and Starkov, A.A. (1997) FEBS Lett. 412, 173-178

34. Halestrap, A.P., and Brennerb, C. (2003) Curr. Med. Chem. 16, 1507-1525

35. Brenner, G., and Grimm, S. (2006) Oncogene 25, 4744-4756

FOOTNOTES

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1The abbreviations used are: ADPRC, ADP-ribosyl cyclase; [Ca^{2+}], intracellular calcium concentration; cADPR, cyclic ADP-ribose; ADPR, ADP-ribose; NAADP\(^+\), nicotinic acid adenine dinucleotide; RyR, ryanodine receptors; Ap2A, P1,P2 diadenosine 5’-diphosphate; HP, hemopoietic progenitors; ΔΨ\(_{m}\), mitochondrial proton gradient; PTP, permeability transition pore; CsA, Cyclosporin A; FCCP, carbonyl cyanide 4-trifluoro-methoxyphenylhydrazone; TMRM, tetramethylrhodamine methyl ester; PI, propidium iodide; CsH, Cyclosporin H; HBSS, Hank’s balanced salt solution.

FIGURE LEGENDS

Fig. 1. P24 induces cell death mainly through apoptosis. A. Hela cells were incubated in the absence (control, left panel) or in the presence of 10 µM P24 (right panel) for 24 h at 37°C and then stained with PI (red staining) and with Hoechst 33258 (blue staining), to identify necrotic and apoptotic cells, respectively.

B, C. Hela cells were incubated for 16 h at 37°C in the absence (control, CT) or in the presence of increasing concentrations of P24 (5, 10 and 20 µM, panel B) or of P18 (5, 10 and 20 µM, panel C). Necrosis (grey bars) and apoptosis (white bars) were assessed by staining of cells with PI and Hoechst 33258, respectively. Results are expressed as percentage of stained cells and are the mean ± SD from 4 experiments.

Fig. 2. Loss of ΔΨ\(_{m}\) induced by P24. Hela cells were loaded with TMRM, as described...
under Experimental Procedures. Images were acquired continuously on a Leica confocal microscope. Addition of the uncoupler FCCP (2 µM) is marked by the abrupt fall of the fluorescence signals, at the end of the recordings.

A. The arrows indicate the addition of a single compound (first arrow), or the sequential addition of two compounds (first and second arrow) during the fluorescence recordings. Additions were as follows: 2 µM oligomycin (black); 20 µM P24 and oligomycin (red); 20 µM P24 and rotenone (green); 20 µM P24 (blue); 2 µM rotenone (purple).

B. HeLa cells were incubated without (control, blue) or with 100 µM cADPR (black), 1 µM CsA (green) or 0.5 mM EGTA-AM (red) during TMRM loading. At the end of the incubation, cells were washed and the various compounds were added again during fluorescence recordings, which were started by the addition of 20 µM P24. Recordings from Ap2A-treated cells were superimposable with those from cADPR-treated cells (trace not shown for the sake of clarity).

In A and B, each trace is the mean of the fluorescence values recorded in ≥ 20 different cells and is from one out of four experiments, giving comparable results.

C. Summary of all data obtained from experiments described in panels A and B. TMRM fluorescence was measured after 30 min from the addition of the first compound (for experiments shown in panel A) or from the addition of P24 (for experiments shown in panel B) and expressed as percentage of the value at time=zero. Results are the mean±SD from 4 experiments.

Fig. 3. Effect of P24 on mitochondrial swelling. Mitochondrial swelling was monitored as the decrease in light scattering at 540 nm (see Experimental Procedures). Rat liver mitochondria (0.5 mg protein/ml) were incubated in the presence of 10 µM Ca²⁺ and 5/2.5 mM glutamate/malate as the respiratory substrates. Sequential additions are marked by the arrows. Trace 1, 10 µM Ca²⁺; trace 2, 1 µM P24; trace 3, 5 µM P24 and 1 µM CsA; trace 4, 5 µM P24. Representative traces from one out of four different experiments are shown.

Fig. 4. Cyclosporin A and EDTA protect against P24 cytotoxicity. HeLa cells were incubated for 2 h at 37°C in the absence (control) or in the presence of the indicated concentrations of P24, without (white bars) or with 1 µM CsA (grey bars) or 0.1 mM EDTA (black bars). Thereafter, cells were washed and further cultured for 22 h in absence of any addition. The number of live cells was assessed by staining with calcein green (see Experimental Procedures for details). Results are expressed as percentage of growth inhibition relative to untreated controls. Mean values ± SD from four experiments are shown.

Fig. 5. Effect of P24 and of other ADPRC products on the [Ca²⁺]_{cyt} and on the [Ca²⁺]_{mit}. HeLa cells were loaded with Fluo 3-AM (for measurement of the [Ca²⁺]_{cyt}, panel A) or with Rho-2 AM (for measurement of the [Ca²⁺]_{mit}, panel B) and fluorescence was detected as described under Experimental Procedures. Panel C shows the fluorescence signals in HeLa cells of, from left to right, Rho-2AM, Mitofluor Green and the co-localization of the two signals, where the regions of interest were chosen for measurement of the [Ca²⁺]_{mit}. P24 at 20 µM was added alone (black squares), or together with a five-fold excess of Ap2A (white squares), or P18 (white triangles) or cADPR (black triangles), or with 0.2 mM EDTA (black rhombus). Inset to panel A: the effect of 100 µM cADPR alone (white circles) is compared with that of 100 µM cADPR together with 20 µM P24 (black triangles). Representative traces from one out of four different experiments are shown.

Fig. 6. Effect of P18, cADPR and Ap2A on P24 cytotoxicity. HeLa cells were incubated for 24 h at 37°C in the presence of P24 at increasing concentrations (5-20 µM), without (rhombus) or with the addition of a five-fold excess of P18 (square), or cADPR (triangle) or Ap2A (circle). The number of viable cells was estimated with calcein green (see Experimental Procedures). Results are expressed as percentage of growth inhibition relative to untreated cultures and are the mean from 4 different experiments.
Fig. 7. Schematic representation of the effects of P24 on mitochondria. The extracellular Ca\(^{2+}\) influx triggered by P24 (1-10 µM), which is antagonized by EDTA, cADPR and P18 but not by Ap2A, leads to an increase of cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)\textsubscript{cyt}]). This in turn induces a rise of the mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)\textsubscript{mit}]), synergested by P18 and antagonized by EDTA, EGTA-AM and by cADPR, but not by Ap2A. The increase of the [Ca\(^{2+}\)\textsubscript{mit}] and P24 synergize in opening the permeability transition pore complex (PTP), thus triggering proton gradient dissipation (ΔΨ\textsubscript{m}). P24 induces PTP opening also through a Ca\(^{2+}\)-independent mechanism (possibly direct binding to the PTP complex), which is antagonized by Ap2A. Proton gradient dissipation is the principal cause of cell death, which occurs mainly through apoptosis and can be prevented or significantly reduced by co-incubation of cells with EDTA, EGTA-AM, the PTP desensitizer Cyclosporin A (CsA), cADPR or Ap2A together with P24. P24 also inhibits cell respiration at the level of Complex I (I), an effect which appears to be unrelated to ΔΨ\textsubscript{m} dissipation, as it is not affected by either EDTA or CsA, and not responsible for induction of apoptosis. Indeed, rotenone is not cytotoxic on HeLa cells. P18 synergizes with P24 in inducing a higher rise of the [Ca\(^{2+}\)\textsubscript{mit}] and an increase of the cytotoxic effect.
TABLE 1. Effect of P24 on oxygen consumption of isolated mitochondria

Purified rat liver mitochondria were incubated without (control) or with 10 µM P24 for 10 min in the presence of 1 µM CsA to prevent PTP opening. Rates of oxygen consumption were measured in the presence of different substrates, to allow activity of the respiratory chain to start either from Complex 1 (glutamate/malate) or from Complex 2 (succinate, in the presence of Complex 1 inhibitor rotenone). State 4: basal \( O_2 \) consumption; State 3: ADP-stimulated \( O_2 \) consumption. FCCP: \( O_2 \) consumption in the presence of the uncoupler FCCP. Results are the mean from 5 different experiments for glutamate/malate and from 4 different experiments for succinate/rotenone. \(*p<0.05; **p<0.01\), comparing P24-treated samples with the corresponding controls.

| Substrates       | State 4 O\(_2\) consumption (nanoatoms O\(_2\)/min/mg) | State 3 O\(_2\) consumption (nanoatoms O\(_2\)/min/mg) | FCCP O\(_2\) consumption (nanoatoms O\(_2\)/min/mg) |
|------------------|------------------------------------------------------|------------------------------------------------------|------------------------------------------------------|
| Glutamate/Malate | Control 8.90±1.03                                    | 18.21±2.21                                          | 24.57±3.68                                           |
|                  | P24 10.73±1.29\*                                     | 11.80±1.93\*                                        | 12.90±1.82\*                                         |
| Succinate/Rotenone | Control 24.14±2.78                                   | 73.76±8.11                                          | 96.11±8.65                                           |
|                  | P24 33.21±4.04\*                                     | 76.46±9.18                                          | 100.49±12.06                                         |
Fig. 1
Fig. 2
Fig. 3

Fig. 4
Fig. 5
Fig. 6
Fig. 7
Mitochondrial dysfunction induced by a cytotoxic adenine dinucleotide produced by ADP-ribosyl cyclases from cADPR
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