CMLS, Cell. Mol. Life Sci. 61 (2004) 2991–2997
1420-682X/04/232991-07
DOI 10.1007/s00018-004-4371-x
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CMLS Cellular and Molecular Life Sciences

Research Article

Morphine but not fentanyl and methadone affects mitochondrial membrane potential by inducing nitric oxide release in glioma cells

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Received 19 August 2004; received after revision 16 September 2004; accepted 7 October 2004

Abstract. We have observed that treatment of human glioma cells with morphine in the nanomolar range of concentration affects the mitochondrial membrane potential. The effect is specific to morphine and is mediated by naloxone-sensitive receptors, and is thus better observed on glioma cells treated with desipramine; moreover, the mitochondrial impairment is not inducible by fentanyl or methadone treatment and is prevented by the nitric oxide (NO) synthase inhibitor L-NAME. We conclude that in cultured glioma cells, the morphine-induced NO release decreases the mitochondrial membrane potential, as one might expect based on the rapid inhibition of the respiratory chain by NO. The identification of new intracellular pathways involved in the mechanism of action of morphine opens additional hypotheses, providing a novel rationale relevant to the therapy and toxicology of opioids.

Key words. Cytochrome oxidase; nitrosative stress; opioid responsiveness; bioenergetics; antinociceptive drug; radicals.

Nitric oxide (NO) is a signalling molecule of paramount importance, with functions in the cardiovascular, immune and nervous systems [1–4]. Recently, conclusive evidence has been collected on the reactivity of NO with mitochondrial complexes [5–7]; in particular, NO inhibits mitochondrial complex IV, i.e. cytochrome c oxidase (CcOX), reversibly and on a time scale compatible with physiology, thereby controlling cell bioenergetics [8]. Endogenous NO is produced by three distinct NO synthases isoforms (NOSs) differently expressed in cells and tissues [9 and references therein]. Further supporting the hypothesis of a bioenergetic role for NO, experiments aimed at revealing the existence of a specific mitochondrial mtNOS [10, 11] showed that the NOS extracted from mitochondria is a membrane-bound isoform of the constitutive neuronal nNOS-α [12]. NO is produced by mitochondria isolated to a high degree of purity from brain, heart and other organs [13] and once released inhibits respiration, the inhibition being prevented by L-nitroso-arginine-methyl-ester (L-NAME) and other NOS inhibitors [14].

The activation of the N-methyl-D-aspartate receptor of neuroblastoma SH-SY5Y cells induces a transient Ca^{2+} flux with the activation of the constitutive Ca^{2+}-dependent NOS [15], an observation relevant to the study presented here. The concomitant increase in the endogenous NO level caused by the Ca^{2+} transient induces, in fact,

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a decrease in the ability of mitochondria to import electrophoretically cationic fluorescence probes, similar to the inhibition caused by incubation of cells with exogenous NO [14].

Morphine activation of opioid receptors was also shown to trigger Ca^{2+} fluxes in human [16] and in cloned mouse neuroblastoma [17] cell lines. Morphine binding is coupled to NO release [18], possibly by activating the N-methyl-D-aspartate receptor [19], an effect that may participate in the mechanism by which morphine produces tolerance and hyperalgesia during cancer pain treatment [20, 21].

In this work, we studied the effect of morphine, methadone and fentanyl on the mitochondrial membrane potential ($\Delta\psi$) of glioma cells. We examined the ability of cell mitochondria to import the fluorescent probe JC_1 and to form J-aggregates, a property closely related to variations in $\Delta\psi$ in the high (≥ 200 mV) potential value region [22]. Experiments were aimed at investigating both acute (incubation for tens of minutes) and chronic (incubation for tens of hours) effects and proved that morphine, in contrast to methadone or fentanyl, and particularly following chronic treatment induces a significant decrease in the mitochondrial membrane potential, i.e. by approximately 30% ($n = 15$, $p \leq 0.01$). These findings add information on the molecular mechanism(s) underlying the biological peculiarities of opioids.

Materials and methods

Cell culture

Human glioma cells were grown in RPMI 1640 medium with 10% FBS, supplemented with 2 mM glutamine and 100 units/ml penicillin-0.1 mg/ml streptomycin. All cultures were maintained in humidified incubators at 37 °C, and under 5% CO_2 and 95% air. When necessary, before morphine/opioid treatment, cells were grown for 24 h in the presence of 20 μM desipramine [23]. Cells for fluorescence bulk measurements were grown in 25-cm^2 flasks (one flask for each condition).

Nitrite/nitrate determination

Nitrite accumulation in the culture medium was measured after (typically) 24 h morphine treatment. Nitrite levels were determined spectrophotometrically on triplicate samples using the Griess reaction (Nitric Oxide Synthase Assay Kit-Colorimetric; Calbiochem). Absorbance was measured using a microplate photometer reader (Spectracount; Packard Instrument Co.) equipped with a 540-nm filter. Absorbance of samples was compared with that of standard sodium nitrite solutions. The nitrite concentration was expressed as percentage of the concentration measured in control cells, and equal to $[NO_2^-] = 43 \pm 3\ \mu M \times mg^{-1}$ protein ($n = 9$); the protein concentration was determined according to Bradford's procedure [24]. Because of the relatively high level of nitrite present in RPMI medium, incubations of cells for nitrite measurements were performed in serum-free D-MEM.

Morphine, methadone and fentanyl treatment

Cells pre-treated for 24 h with 20 μM desipramine and incubated in the presence of desipramine with 20 nM morphine, methadone or fentanyl for a further 24 h were used for measurements; cells incubated in parallel with desipramine were used as controls. The number of different cell preparations used for independent experiments was 15 for morphine and never less than 3 for all other conditions (as specified in the figure legends). Each assay was carried out at least in duplicate. Where indicated, levels of statistical significance were determined using Student's t test. A p value below 0.05 was considered significant.

Mitochondrial membrane potential measurement

The mitochondrial membrane potential, negative on the matrix side, is responsible for the accumulation of positive charged molecules, such as rhodamines and carbocyanines like JC_1 [22]. The fluorescence emission of JC_1 depends both on its concentration and on the excitation wavelength. Upon exciting at 490 nm, JC_1 monomers display a cytoplasmic fluorescence emission centered at 537 nm (green band). Beyond a critical concentration, better reached at high mitochondrial membrane potential values ($\Delta\psi \geq 200$ mV) [22], JC_1 aggregates are formed in the mitochondria, characterised by an intense emission band centered at 590 nm (red band).

Fluorescence emission spectra of JC_1-loaded cells were recorded between 500 and 620 nm with a single-photon counting spectrofluorometer (Fluoromax, Jobin-Yvon) equipped with a magnetic stirrer and thermostated cuvette of 1-cm light path. The excitation wavelength was 490 nm when the whole spectrum was recorded, or 575 nm when the kinetics of formation of the red JC_1 aggregates was followed. When necessary, the NOS inhibitor L-NAME was added, at 1 mM concentration together with morphine and maintained in the medium throughout the incubation. The fluorescence measurements were carried out in culture medium containing 137 mM KCl and 3.6 mM NaCl, and in the presence of 2 μM ouabain in order to dissipate the plasma membrane potential, thus abolishing any aspecific cytoplasmic fluorescence [25]. Accumulation of the JC_1 aggregates was started by adding to the cells, pre-mixed with the dye, 0.6 μM nigericin; this ionophore converts the ΔpH component of $\Delta\mu_{H^+}$ into the electrical membrane potential gradient $\Delta\psi$, allowing full expression of mitochondrial $\Delta\mu_{H^+}$ as $\Delta\psi$ [26]. The concentration of JC_1 was 0.4 μM.

For time-based experiments, cells were gently transferred into a fluorometer cuvette, containing 3 ml of high-K^+

buffer (see above), 2 µM ouabain and 0.4 µM JC_1. Upon addition of nigericin, the red peak signal rises, due to formation of J-aggregates [22]. The excitation and emission wavelengths were 575 and 590 nm, respectively.

Cytotoxicity assay

Cell viability and cytotoxicity were measured according to a standard procedure using lactate dehydrogenase release [27]. Lysed cells and fresh medium were used as 100 and 0% cytotoxicity, respectively. Treatments with morphine, methadone or fentanyl, as well as with naloxone under all conditions used had no significant, or very little (≤ 5%) effect on cell viability, at least over the time scale of the experiments (24–48 h). RPMI 1640 medium, Dulbecco's modified Eagle's medium, and foetal bovine serum were from Invitrogen Life Technologies; all other reagents were from Sigma. Stock solutions of NO (Air Liquide) were prepared by equilibrating degassed water with the pure gas suitably purged of higher NOx, at 1 atm and 20 °C. The stock NO concentration was independently evaluated as [NO] = 2.1 ± 0.1 mM.

Results

Glioma cells incubated overnight with 20 nM morphine, displayed a detective mitochondrial accumulation of rhodamine (RD_{123}) by approximately 50%, with respect to untreated cells. This is shown in figure 1, where the same cells incubated with excess N-methyl-D-aspartate to stimulate the production of endogenous NO displayed a mitochondrial fluorescence level lower than controls (fig. 1, panel 3).

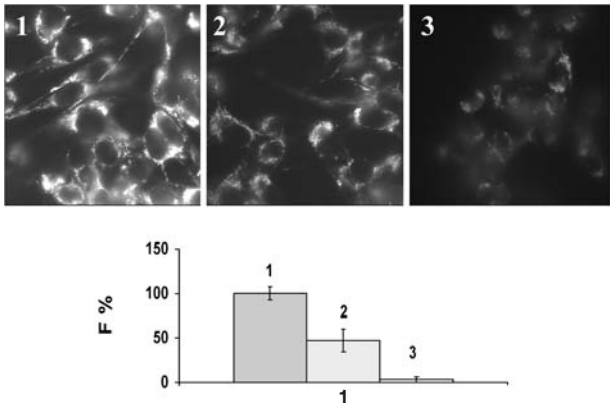

Figure 1. Fluorescence microscopy of glioma cells: mitochondria staining by rhodamine. Cells grown for 48 h on glass coverslips were incubated for 5 min at 37 °C in culture medium containing 0.5 µg/ml rhodamine 123, washed with PBS and mounted on a microscope slide for fluorescence observation equipped with a living chamber, obtained by removing a circular portion (about 1.5 cm) of silicon rubber (1.5 mm thick). Control cells (1), or cells treated before observation for 24 h with 20 nM morphine (2) or for 30 min with 0.5 mM N-methyl-D-aspartate (3).

The observation was extended to a larger number of cells (≥1×10^6 ml^{-1}), by fluorometrically monitoring in glioma cell suspensions the mitochondrial $\Delta\psi$ through the ability of mitochondria to accumulate the red-fluorescent JC_1 aggregates; the kinetics of the process were followed and analysed, and the spectra of the internalised probe were collected after reaching equilibrium, i.e. in about 20 min. The reaction was started by hyperpolarising mitochondria with a saturating amount of nigericin. As shown in figure 2, the fluorescence emission signal at 590 nm reached a maximum in about 30 min, thereafter decaying slowly (hours, not shown). The same experiment was carried out using cells pre-incubated for 24 h with desipramine and then treated for 24 h with morphine, in the presence of desipramine; as also shown in figure 2, after

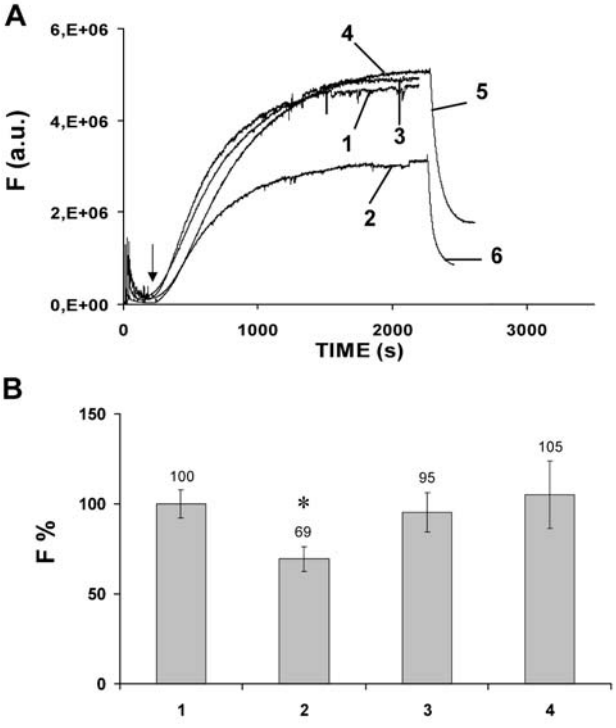

Figure 2. Effect of morphine on the time-dependent accumulation of JC_1 red aggregates into mitochondria of glioma cells. Cells 1×10^6 ml^{-1} were suspended in air-equilibrated medium and in the presence of 2 µM ouabain, and 0.4 µM JC_1; after signal stabilisation (about 150 s), 0.6 µM nigericin was added (arrow) and the fluorescence followed over time; desipramine-induced control cells (trace 1). Cells pre-treated with desipramine were incubated for 24 h with a saturating amount of morphine (20 nM) in the absence (trace 2) or presence of 200 nM naloxone (trace 3) or 1 mM L-NAME (trace 4). The addition of excess NO, as a 50 µM water solution, led to a rapid decay of fluorescence (trace 5), whereas the addition of valinomycin almost abolished the membrane potential (trace 6). Excitation wavelength at 575 nm, emission at 590 nm. Typical time courses (*A*) and maximal fluorescence expressed as percentage of fluorescence by taking the signal of control cells as 100% (*B*). T = 37 °C. The number of independent cell preparations assayed was n = 15 (morphine), n = 6 (other conditions). The statistical p value for morphine-treated cells was ≤ 0.01. For details see Materials and methods.

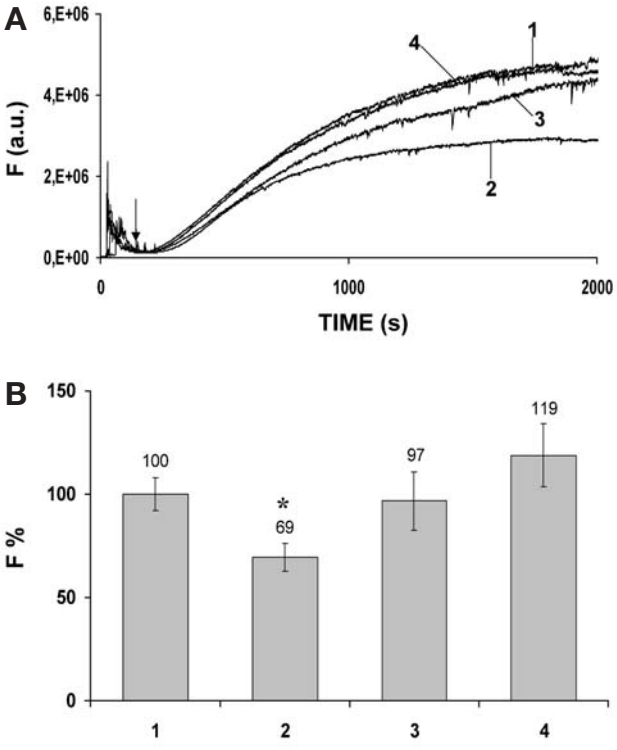

Figure 3. Effect of methadone and fentanyl on the time-dependent accumulation of JC_1 aggregates into mitochondria of glioma cells. The experiments, designed as in Figure 2, were carried out using cultured glioma cells (trace 1), cells incubated for 24 h with morphine (trace 2), 20 nM fentanyl (trace 3) or 20 nM methadone (trace 4). Traces and analysis of control and morphine-treated cells are reported for comparison with figure 2. T = 37 °C. The number of independent experiments was n = 15 (morphine), n = 3 (other conditions). For details see Materials and methods.

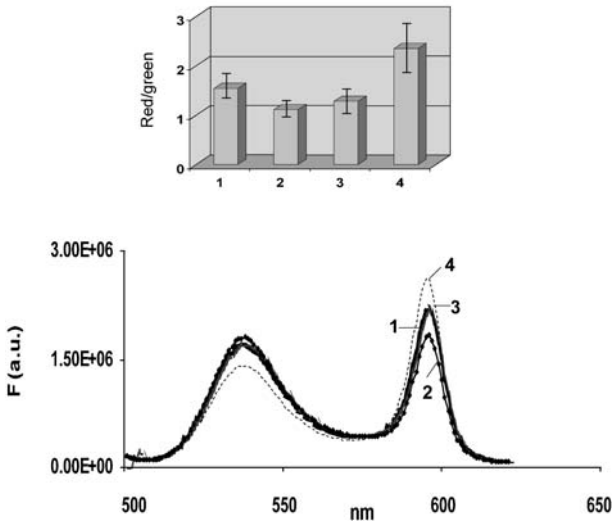

Figure 4. Fluorescence emission spectra of glioma cells after accumulation of the J-aggregates. Spectra were collected at the end of JC_1 red aggregate formation, under the experimental conditions described in figure 2. Spectra were accumulated and averaged after normalisation for the total number of cells suspended in the cuvette, and directly counted. Notice that an increase in fluorescence at 595 nm (red peak) corresponds to a decrease in fluorescence at 538 nm (green peak) as expected by the conversion of monomers into polymers. T = 37 °C.

morphine treatment, accumulation of the red aggregates occurred on a similar time scale but to a smaller extent, i.e. up to ~ 70% of untreated cells. The effect of morphine was prevented by naloxone, a commonly used antagonist of the morphine receptor, as well as by the NOS inhibitor L-NAME (see fig. 2). Naloxone alone had no effect on the kinetics and amplitude of the fluorescence signal which was comparable to that of untreated cells, whereas in the presence of L-NAME, some significant increase in the signal amplitude was often detected (up to ~20%).

The specificity of the effect induced by morphine was investigated by treating cells for 24 h with 20 nM fentanyl or 20 nM methadone. As shown in figure 3, cells treated with these drugs displayed the same ability to import JC_1 as control cells.

The addition of valinomycin, dissipating mitochondrial $\Delta\Psi$, caused a rapid and complete disappearance of the signal, confirming that the red fluorescence depends on the mitochondrial membrane potential (fig. 2). A similar behaviour was observed upon addition of excess (50 µM) NO gas directly in the cuvette and to the cell culture medium (fig. 2).

The time courses shown in figures 2 and 3 reflect the formation of JC_1 red aggregates; this was proved by collecting the fluorescence emission spectra of the cells at equilibrium, i.e. about 20–30 min after addition of nigericin (fig. 4). The spectra display the typical green (538 nm) and red (595 nm) band characteristic of JC_1 accumulated in the monomeric (low potential) and polymeric (high potential) state, respectively. As expected, the red/green peak ratio (595/538 nm) observed after morphine treatment was significantly lower (inset to fig. 4), indicating the existence of a smaller $\Delta\Psi$ than in control cells. Interestingly, treatment of glioma cells with excess morphine for a limited period of time (10 min) or even 24 h induced small changes (not shown) unless cells were pre-treated with desipramine for 24 h.

The production of NO by the cells accounting for the decrease in mitochondrial fluorescence was also directly monitored by measuring nitrite levels in the cell culture supernatant. As shown in figure 5, the Griess reaction revealed that glioma cells incubated with desipramine for 24 h and then for a further 24 h with morphine displayed an approximately 25% increase in nitrite level compared to control cells. Consistent with the mitochondrial fluorescence results, the nitrite increment was reversed by the NOS inhibitor L-NAME or by the antagonist of the morphine receptor, naloxone. In contrast, the nitrite concentration level of cells incubated with fentanyl or methadone for 24 h was similar to that of control cells (fig. 5).

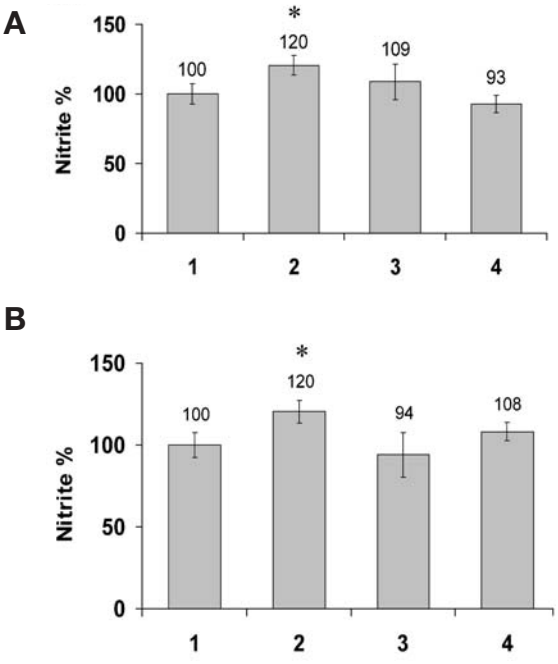

Figure 5. Accumulation of nitrite in the medium of cells treated with morphine and other effectors. Glioma cells were treated as for the experiments shown in figures 2 (*A*) and 3 (*B*), but were grown in modified Dulbecco's medium. At the end of treatment, the nitrite concentration was measured in the supernatant. The average concentration of nitrite in the supernatant of control glioma cells was $[NO_2^-] = 43 \pm 3$ µM mg^{-1} protein. Results are expressed as percentage of the nitrite concentration measured in control cells.

Discussion

We have shown that morphine treatment induces a substantial decrease in mitochondrial membrane potential in human glioma cells *via* an NO-dependent pathway. This novel effect is consistent with previous reports showing morphine-induced impairment of cell energy metabolism [28], and is assigned here to morphine-induced NO release, a reaction which has been intensively studied and characterised [19, 29, 30]. The reaction is inhibited by the NOS inhibitor L-NAME and is mediated by naloxone-sensitive receptors, whose expression was induced by 24 h incubation with desipramine. Sensitivity to desipramine strengthens the hypothesis that a µ-type receptor is involved, namely the μ_3-type according to some authors [31], without excluding the involvement of other binding sites [32].

In this report we show that mitochondria of glioma cells, exposed to morphine lose a significant fraction of the mitochondrial membrane potential. The effect is more clearly elicited if incubation with morphine occurs over a period of hours. According to previous reports [19, 29–32], binding of morphine activates NOS, particularly the constitutive cNOS. Consequently, NO is released in the (barely measurable) nanomolar range of concentration, and promptly diffuses through and within the cell membranes and compartments [33]. Under these conditions, mitochondrial CcOX is transiently inhibited [7, 8], and the mitochondrial membrane potential falls, although to a different extent and with different kinetics depending on the cell line under investigation [14, 34]. Consistently, glioma cells in the presence of morphine, when compared to untreated cells, display a decreased mitochondrial concentration of JC_1 red aggregates, whose formation requires high mitochondrial $\Delta\Psi$ regimes [22]. Thus, following a persistent (hours) morphine treatment, glioma cell mitochondria appear de-energized. The effect can be reversed by inhibiting morphine binding to the cell receptor with naloxone, or treating cells with NOS inhibitors such as L-NAME, which is to be expected if the effect on mitochondria is mediated by NO release. The same conclusion is supported by the finding that upon exposing glioma cells to morphine, the concentration of nitrite in the culture medium increases over the same period of time.

Interestingly, by simply inhibiting the NOSs with L-NAME, the observed mitochondrial fluorescence was often higher than in control cells. This finding is not surprising, as it had already been described in neuroblastoma cells [14]. It further suggests that, in cultured glioma cells also, there is a steady production of NO. This persistent nanomolar concentration of NO has been proposed to produce a partial inhibition of the respiratory chain at the level of CcOX [7, 35], and that inhibition would account for the higher, compared to the purified enzyme, apparent K_M of CcOX for O_2, when measured in situ, in cells and tissues [7, 8].

Although small (~10%), a mitochondrial fluorescence decrease is observed even at early incubation times with morphine (not shown), fully consistent with the reported release of NO that follows the activation of cNOS by the morphine-induced calcium entry [16]. A longer (chronic) treatment, on the other hand, is compatible with persistent NO production, and therefore a persistent depression in mitochondrial function and fluorescence, which becomes more clearly detectable. Whether on a longer time scale, in addition to the activation of the cNOS, the transcriptionally regulated inducible iNOS is also activated [36] needs to be unequivocally substantiated.

In experiments carried out with cardiomyocytes, McPherson et al. [37] proposed that on the time scale of minutes, morphine may open mitochondrial K_{ATP} channels, thereby inducing mitochondrial impairment. Interestingly, in these experiments, an overproduction of 2′,7′-dichlorofluorescein diacetate-sensitive radical species was demonstrated, also in agreement with previous reports [38]. Our results confirm that the NO radical is produced upon morphine treatment, accounting not only for the opening of mitochondrial K_{ATP} channels [39] but also for the inhibition of the mitochondrial respiratory

chain, with a decrease in mitochondrial membrane potential. Interestingly, the effect on the mitochondrial potential is specific for morphine and is not inducible by fentanyl or methadone. This finding might suggest that these opioids exert different effects on Ca^{2+} fluxes and thus, in our view, on NOS activation. Moreover, although possibly binding to the same μ-type receptors, their subtype [40, 41] and the chemistry involved [42] could be different.

According to several reports, an important side-effect of morphine treatment is cell pre-conditioning; i.e. morphine can be protective [43], an observation recently extended to purkinjie cells [44], and a potential clinical use of morphine to reduce ischemic brain injury is envisaged. An involvement of the mitochondrial K_{ATP} channels in the pre-conditioning events has been proposed. In addition, we would also draw attention to the recent observation that a significant but limited inhibition of the respiratory chain by NO can induce activation of glycolysis [34] and this process has also been suggested to be beneficial to neurons which are going to be exposed to ischemia [45]. In conclusion, our results suggest that not only the activation of the K_{ATP} channels but also the partial inhibition of the respiratory chain can contribute to the cell pre-conditioning induced by morphine. As recently hypothesised [31], and on the basis of our results, it will be of interest to verify whether endogenous morphine can play a bioenergetic role.

Acknowledgements. We thank A. Giuffrè (Institute of Molecular Biology and Pathology, CNR, Rome) for extended discussions on these issues. This work supported by Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) of Italy-PRIN 'Bioenergetica: aspetti genetici, biochimici e fisiopatologici', and by Fondo per gli Investimenti della Ricerca di Base (FIRB-RBAU01F2BJ) to P. S. and by Ministero della Salute (Progetti Speciali 2000–2002; RF 01.112) to E. A.

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