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Peripheral-type benzodiazepine receptor ligands: mitochondrial permeability transition induction in rat cardiac tissue

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Abstract

Strong evidence is emerging that mitochondrial permeability transition (MPT) may be important in certain physiological conditions and, above all, in the processes of cell damage and death. Reversible MPT, triggered by inducing agents in the presence of calcium ions, has resulted in the opening of a dynamic multiprotein complex formed in the inner mitochondrial membrane and has caused large-amplitude mitochondrial swelling. In the present work, the exposure of de-energized rat cardiac mitochondria to peripheral benzodiazepine receptor (PBR) ligands (1-(2-chlorophenyl-*N*-methyl-1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195), 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (Ro5-4864), and diazepam) produced a dose-dependent and cyclosporin A (CSP)-sensitive loss of absorbance, which was indicative of mitochondrial swelling. By contrast, the addition of a high-affinity central benzodiazepine receptor ligand (clonazepam) was ineffective, even at the highest concentration tested. The ultrastructural changes associated with swelling were similar in mitochondria exposed either to PK 11195 or to calcium. Supporting the apoptotic role of PK 11195-induced swelling, supernatants from mitochondria that had undergone permeability transition caused apoptotic changes in isolated cardiac nuclei. In addition, ultrastructural abnormalities were observed in rat cardiac tissue following *in vivo* PK 11195 administration, with these abnormalities being prevented by CSP co-administration. These data indicate that PBR ligands induce mitochondrial permeability transition and ultrastructural alterations in isolated cardiac mitochondria as well as in myocardiocytes, suggesting a novel strategy for studying the implication of PBR ligands as apoptosis inducers, through a probable effect on the MPT pore.

Keywords: Permeability transition; Benzodiazepine receptor ligands; Cardiac mitochondria; Electron microscopy; Apoptosis; PK 11195; *In vivo* administration

1. Introduction

One of the bases of the chemiosmotic model of energy transduction is the impermeability of the inner mitochondrial membrane to solutes not endowed with specific transport systems [1]. However, isolated mitochondria are known to undergo a reversible permeability transition in the presence of an inducing agent and matrix calcium ions, a reaction that results in the opening of a pore formed at the contact site between the inner and outer mitochondrial membranes [2]. Although the exact molecular composition of this pore has not been definitively established, proteins such as hexokinase, PBR, porin, creatine kinase, adenine nucleotide carrier (ANC), and cyclophilin D have been suggested to be implicated in MPT pore formation and/or regulation [1,3–8]. The MPT pore, also called the megachannel or multiple conductance channel, functions as a Ca^{2+}-, voltage-, pH-, and redox-gated channel with several levels of conductance, and little, if any, ion selectivity [1,9–11]. The opening of the MPT pore, which is inhibited specifically by the drug cyclosporin A, can cause the dissipation of inner mitochondrial transmembrane potential ($\Delta\Psi m$) and colloid osmotic swelling of the mitochondrial matrix, disrupting mitochondrial structure and leading to the release of proapoptotic intermembrane proteins from the mitochondrion when the MPT pore opening is both extensive and prolonged [12–15]. On the contrary, when the opening of the MPT pore is transient, a rapid resealing allows a total recovery of mito-

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Abbreviations: MPT, mitochondrial permeability transition; PBR, peripheral benzodiazepine receptor; TEM, transmission electron microscopy; and CSP, cyclosporin A.

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chondrial function: during cardiac ischemia, it has been recognized that pore opening does not occur, whereas during reperfusion a significant opening does occur, and recovery of the heart depends on subsequent pore closure [16].

As a result, several papers have suggested that MPT may act as a "central executioner" of cells subjected to a range of insults (such as oxidative stress, growth factor removal, or exposure to cytokines), determining not only whether cells live or die, but also whether death occurs by apoptosis or necrosis [17,18].

Recently, many works have reported that ligands to PBR, a presumed constituent of the pore, show an antiproliferative effect in normal and transformed cells, and several studies are now in progress to evaluate their activity as antitumor drugs [19–24]. Interestingly, a change in PBR density has been demonstrated in several neoplastic tissues, and accumulating data indicate a possible role for PBR in the adaptation of the organism to stress and brain damage (for a review, see [25]). It has often been suggested that PBR is involved in the regulation of cholesterol transport from the cytoplasm to the mitochondrial matrix [26–29]; furthermore, it has been demonstrated that PBR ligands control the immune function [25,26,30] and cell proliferation [25,31–34]. In particular, PK 11195, a specific PBR ligand, facilitates mitochondrial transmembrane potential disruption and subsequent apoptosis, induced by a number of different agents including agonists of the glucocorticoid receptor, chemotherapeutic agents (etoposide, doxorubicin), and γ-irradiation, in a variety of cell types [35]. A potentiation of the cytotoxicity of several agents by PBR ligands has also been demonstrated on mouse fibroblasts [36] and rat hepatocytes [37], and this has been related to the induction of MPT, as assessed by the loss of rhodamine fluorescence in fibroblasts [36] and by light-scattering changes in isolated liver mitochondria [37].

Based on these considerations, in the present work we first tested the ability of selective and non-selective PBR ligands to induce rat isolated heart mitochondrial swelling in a de-energized medium using the electron transport inhibitors rotenone and antimycin A. Secondly, nuclear apoptotic changes were investigated by the use of a "cell-free system" assay, mixing together mitochondrial extracts after MPT induction and purified nuclei. The "cell-free system" was designed to reproduce the essential steps of the apoptotic process *in vitro* by mixing together cell extracts and purified organelles [12,13,38,39]. Finally, in order to reveal possible submicroscopic damage, we investigated both isolated mitochondria exposed to PK 11195 and myocardial tissue after *in vivo* PK 11195 administration.

2. Materials and methods

2.1. Materials

1-(2-Chlorophenyl-*N*-methyl-1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195), Diazepam, Clonazepam, 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (Ro5-4864), soybean trypsin inhibitor, bacitracine, and benzamidine were obtained from the Sigma Chemical Co. Rotenone, antimycin A, and cyclosporin A were obtained from Calbiochem. All other compounds were purchased from chemical sources.

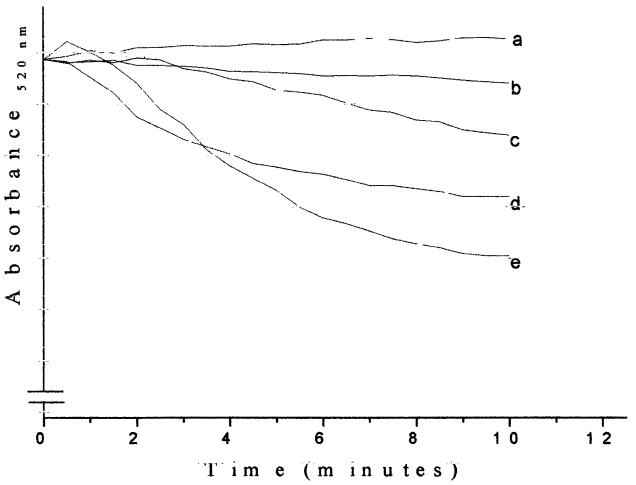

Fig. 1. Effects of calcium on MPT. Mitochondria (~1 mg protein/mL) were incubated in the presence of calcium, and the absorbance at 520 nm was measured for 10 min, as described in Materials and Methods. MPT was induced at time zero by the addition of calcium. Trace a: 250 μM Ca^{2+} plus 5 μM CSP; trace b: 25 μM Ca^{2+}; trace c: 100 μM Ca^{2+}; trace d: 250 μM Ca^{2+}; trace e: 500 μM Ca^{2+}. All traces were redrawn to start at exactly the same point, so absolute absorbance values are not given. Adjustments were less than 3%. All traces represent typical experiments repeated on at least four separate mitochondrial preparations with similar results.

2.2. Cardiac mitochondria preparation

Mitochondria were prepared from hearts of male Wistar rats (200–250 g body wt) essentially as previously described [40], with minor modifications. Briefly, after rapid removal and dissection, the heart was gently homogenized in 20 volumes (w/v) of ice-cold 5 mM Tris–HCl buffer, pH 7.4, containing 0.32 M sucrose, 1 mM EDTA, and protease inhibitors (0.2 mg/mL of soybean trypsin inhibitor, 0.2 mg/mL of bacitracine, and 0.16 mg/mL of benzamidine) (T_1 buffer), using an Ultra-Turrax homogenizer. The homogenate was centrifuged at 700 $\times$ *g* for 10 min and the pellet was suspended in the same initial volume of 50 mM Tris–HCl buffer, pH 7.4, containing 0.32 M sucrose and protease inhibitor (T_2 buffer) and centrifuged at the same speed three times. The resulting supernatants were recovered and centrifuged at 6500 $\times$ *g* for 20 min and the resulting pellet, suspended in the T_2 buffer and centrifuged twice at 10,000 $\times$ *g* for 10 min, was designated as the "mitochondrial fraction." All operations were carried out at 4°. Protein concentration was determined by Lowry's method modified by Peterson [41], using BSA as the standard.

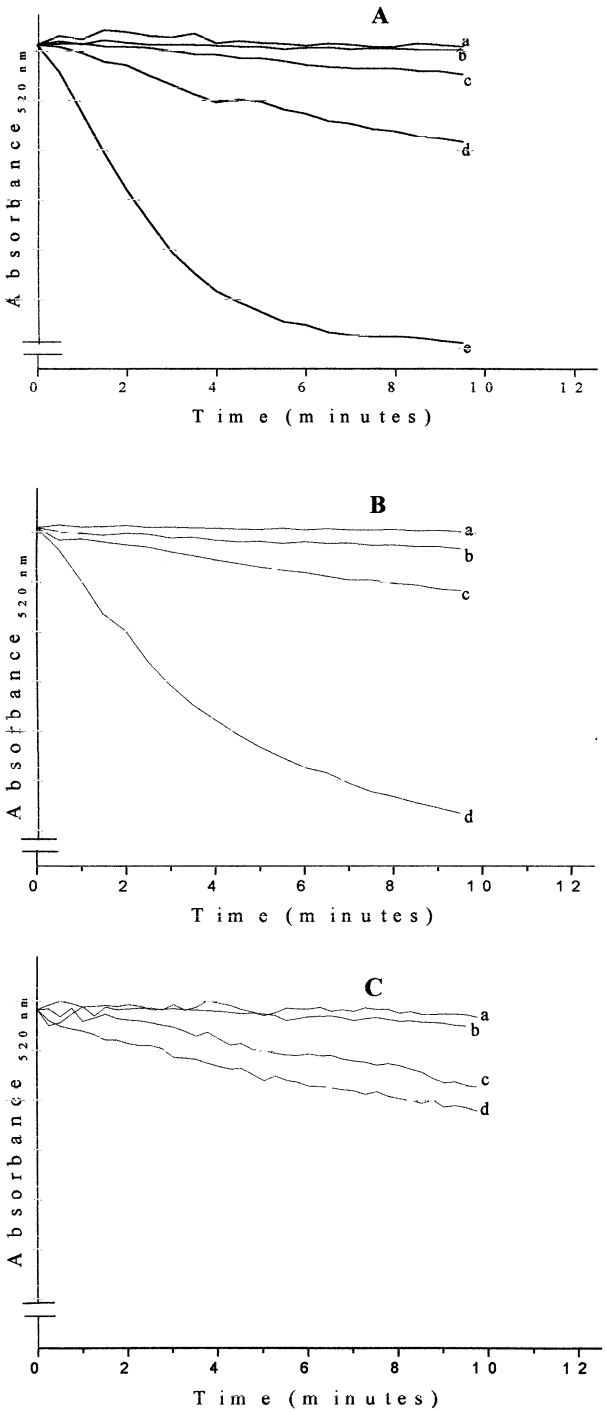

Fig. 2. Effects of PBR ligands on MPT. Mitochondria (~1 mg protein/mL) were incubated in the presence of PBR ligands, and the absorbance at 520 nm was measured for 10 min, as described in Materials and Methods. MPT was induced at time zero by addition of the test ligand. (A) 20, 40, 100, or 250 μM PK 11195 (traces b, c, d, and e, respectively) or 40 μM PK 11195 plus 5 μM CSP (trace a) was added. (B) 40, 100, or 250 μM Ro5-4864 (traces b, c, and d, respectively) or 40 μM Ro5-4864 plus 5 μM CSP (trace a) was added. (C) 40, 100, or 250 μM diazepam (traces b, c, and d, respectively) or 100 μM diazepam plus 5 μM CSP (trace a) was added. All traces were redrawn to start at exactly the same point, so absolute absorbance values are not given. Adjustments were less than 3%. All traces represent typical experiments repeated on at least four separate mitochondrial preparations with similar results.

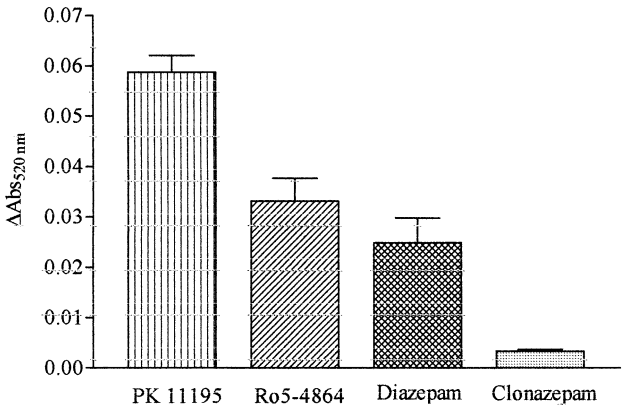

Fig. 3. Effects of benzodiazepine receptor ligands on MPT. Swelling of de-energized mitochondria was initiated by the addition of 100 μM benzodiazepine receptor ligands to the sample cuvette and monitored for 10 min, as described in Materials and Methods. Results are expressed as the means ± SEM of four separate experiments analyzed using the GraphPad InStat program.

2.3. De-energized cardiac mitochondria swelling measurement

Induction of MPT was monitored by following the absorbance decrease associated with mitochondrial swelling [1,42,43]. The procedure was essentially as described by Halestrap [44]: fresh heart mitochondria were added to 10–15 mL of a buffer containing 150 mM sucrose, 10 mM Mops (3-[*N*-morpholino]-propanesulfonic acid), 5 mM Tris, and electron transport inhibitors (0.5 μg/mL of rotenone and 0.5 μg/mL of antimycin A) at pH 7.2 ("swelling buffer"). After mixing, 1.0 mL portions of the suspension (final concentration approximately 1 mg protein/mL) were added to both sample and reference cuvettes of a split-beam spectrophotometer. Swelling was initiated by addition of calcium or benzodiazepine receptor ligands to the sample cuvette, and the absorbance at 520 nm (Abs_{520nm}) was monitored with an on-line computer at 30° for 10 min. In some samples, CSP (5 μM) was added in the presence of the test ligands. The ethanol concentration in the incubation medium was maintained below 0.5% (v/v).

2.4. Electron microscopy

2.4.1. Isolated cardiac mitochondria

Control and Ca^{2+}- or PK 11195-treated mitochondria were fixed by mixing equal amounts (v/v) of the mitochondrial suspensions with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After a primary fixation of approximately 5 min, the mitochondria were pelleted by centrifugation and subsequently fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 hr at 4°. After rinsing in cacodylate buffer, mitochondrial pellets were postfixed in 1% cacodylate-buffered osmium tetroxide for 2 hr at room temperature, then dehydrated in a graded series of ethanol,

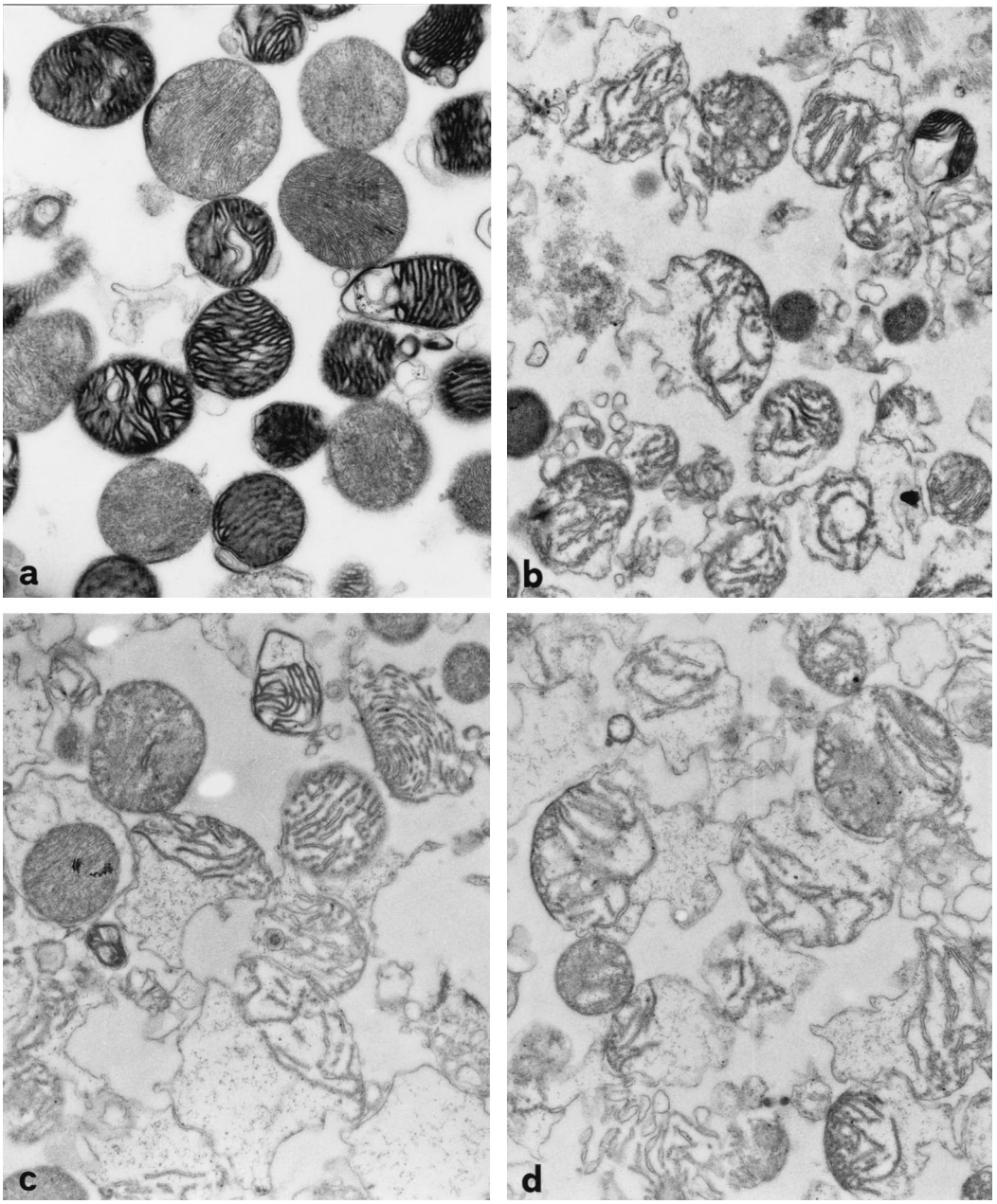

Fig. 4. Electron micrographs of de-energized cardiac mitochondria. (a) Mitochondria not exposed to any agent (control specimens), × 15,000; (b) mitochondria exposed to 250 μM Ca^{2+}, × 11,500; (c) mitochondria exposed to 40 μM PK 11195, × 13,000; and (d) mitochondria exposed to 100 μM PK 11195, × 13,000.

briefly transferred to propylene oxide, and embedded in Epon-Araldite. Ultrathin sections (60–80 nm thick) were cut with a diamond knife, placed on Formvar carbon-coated copper grids (200 mesh), stained with uranyl acetate and lead citrate, and observed with a Jeol 100 SX transmission electron microscope.

2.4.2. Cell-free system of apoptosis

For the *in vitro* apoptosis assay, rat cardiac nuclei were prepared essentially as reported by Jackowski [45], and suspended (final concentration 20 mg protein/mL) in a buffer containing 250 mM sucrose, 1 mM dithiothreitol, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 μM phenylmethylsulphonyl fluoride, and 10 mM Mops (3-[*N*-morpholino]-propanesulfonic acid), 5 mM Tris, pH 7.4. Cardiac mitochondrial swelling was induced by 100 μM PK 11195. After 10 min, the PK 11195-exposed and non-exposed mitochondria were pelleted by centrifugation (13,000 × *g* for 5 min). The corresponding supernatants were added to 1 mM ATP, 1 mM $MgCl_2$, and 25 μL nuclei and incubated for 2 hr at 37°. Finally, the nuclear suspensions were centrifuged at 1000 × *g* for 10 min, and the pellets were processed for TEM analysis, as previously described for the mitochondrial pellets.

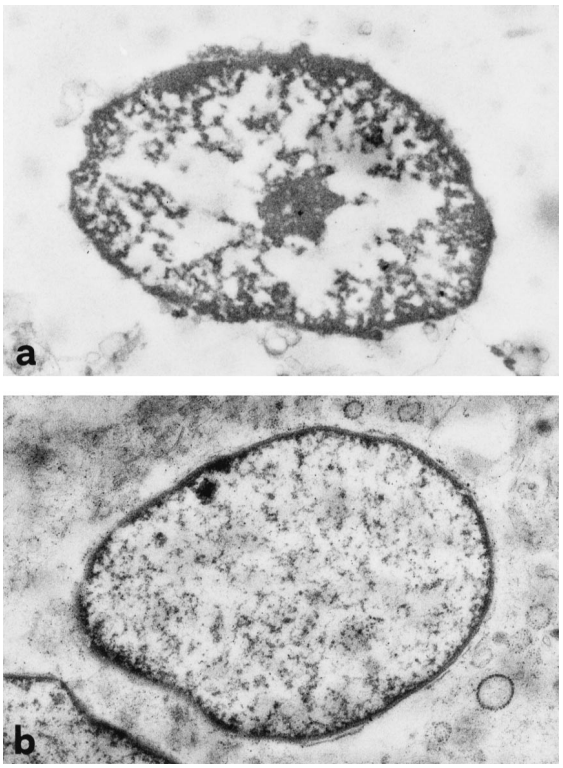

Fig. 5. Electron micrographs. (a) Nucleus exposed to supernatant from PK 11195-treated mitochondria. Note the condensation and margination of chromatin typical of early stages of apoptosis, × 16,000. (b) Nucleus exposed to supernatant from untreated mitochondria. The chromatin shows the characteristic diffuse pattern, × 15,000.

2.4.3. Myocardiac tissue after in vivo *PK11195 administration*

In *in vivo* experiments, three groups of animals were injected intraperitoneally (1.0 mL) with PK 11195 (5 mg/kg), PK 11195 (5 mg/kg) plus CSP (10 mg/kg), and CSP (10 mg/kg), respectively, suspended in 20% (w/v) dilute Emulphor in saline solution (dilute Emulphor is Emulphor diluted 1:1, v/v, with ethanol). In parallel, one group of rats (control) received an i.p. administration of the vehicle (Emulphor saline solution). After 30 min, control and treated animals were killed by cervical dislocation and thoracotomized, and the hearts, still beating, were cut into small pieces (1 mm^3) and immediately fixed in glutaraldehyde 3% in 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide in the same buffer for 2 hr at room temperature. Later steps were performed as described above for TEM observation. The care and handling of the animals was in accordance with the provisions of the European Community (EC) Council Directive 86-609 recognized and adopted by the Italian Government.

3. Results

3.1. De-energized cardiac mitochondrial swelling

A greater decrease in light-scattering (ΔAbs_{520nm}) was observed when cardiac mitochondria were exposed to increasing calcium concentrations, and the calcium effect was totally inhibited by 5 μM cyclosporin A (Fig. 1). Subsequently, we assessed the ability of benzodiazepine receptor ligands to induce swelling in de-energized rat cardiac mitochondria. PK 11195 and Ro5-4864 induced MPT in a dose-dependent manner (Fig. 2, A and B): the exposure of mitochondria to less than 40 μM of either PK 11195 or Ro5-4864 had no detectable effect, whereas exposure to 40, 100, or 250 μM PBR ligands induced a loss of absorbance. The decrease in absorbance induced by either PK 11195 or Ro5-4864 was prevented by cyclosporin A, the specific inhibitor of MPT pore opening. Fig. 2C shows that the benzodiazepine agonist, diazepam, similarly induced swelling of de-energized mitochondria, although a dose-dependent decrease in absorbance was obtained on addition of 100 or 250 μM diazepam to mitochondria, while the 40 μM concentration showed only a slight effect. Again, cyclosporin A completely prevented the swelling induced by 100 μM diazepam.

Mitochondrial light-scattering changes induced by a single concentration (100 μM) of the test compounds are illustrated in Fig. 3. Selective and non-selective PBR ligands induced MPT; by contrast, no swelling was found in the presence of the central-type benzodiazepine receptor ligand, clonazepam, even at 250 μM, the highest concentration tested (data not shown).

3.2. Ultrastructural observation of calcium- or PK 11195-exposed mitochondria

In order to visualize ultrastructural alterations in isolated rat cardiac mitochondria after exposure to calcium or PK 11195, the mitochondrial samples were observed with a transmission electron microscope. Control specimens showed well-preserved mitochondria with an electron-dense matrix and well-arranged cristae (Fig. 4a). In the specimens treated with either Ca^{2+} (Fig. 4b) or PK 11195 (Fig. 4, c or d), the majority of mitochondria were grossly damaged. In particular, they showed swelling, hypertrophy, cristolysis, and matrix dilution (Fig. 4, b–d vs Fig. 4a). In the presence of 40 μM PK 11195 (Fig. 4c), some mitochondria appeared to have completed, or partially completed, the morphological changes associated with becoming permeable and swelling, while others still displayed the characteristic ultrastructure of the intact organelle. In the presence of 100 μM PK 11195 (Fig. 4d), almost all mitochondria showed hypertrophy and severe disturbance in cristae arrangement.

3.3. Ultrastructural observation of cardiac nuclei

To determine whether apoptogenic proteins were released from cardiac mitochondria during PK 11195-induced permeability transition, supernatant from mitochondria which had undergone MPT was incubated with isolated rat cardiac nuclei. The addition of supernatant from treated mitochondria to myocardiocyte nuclei led to a morpholog-

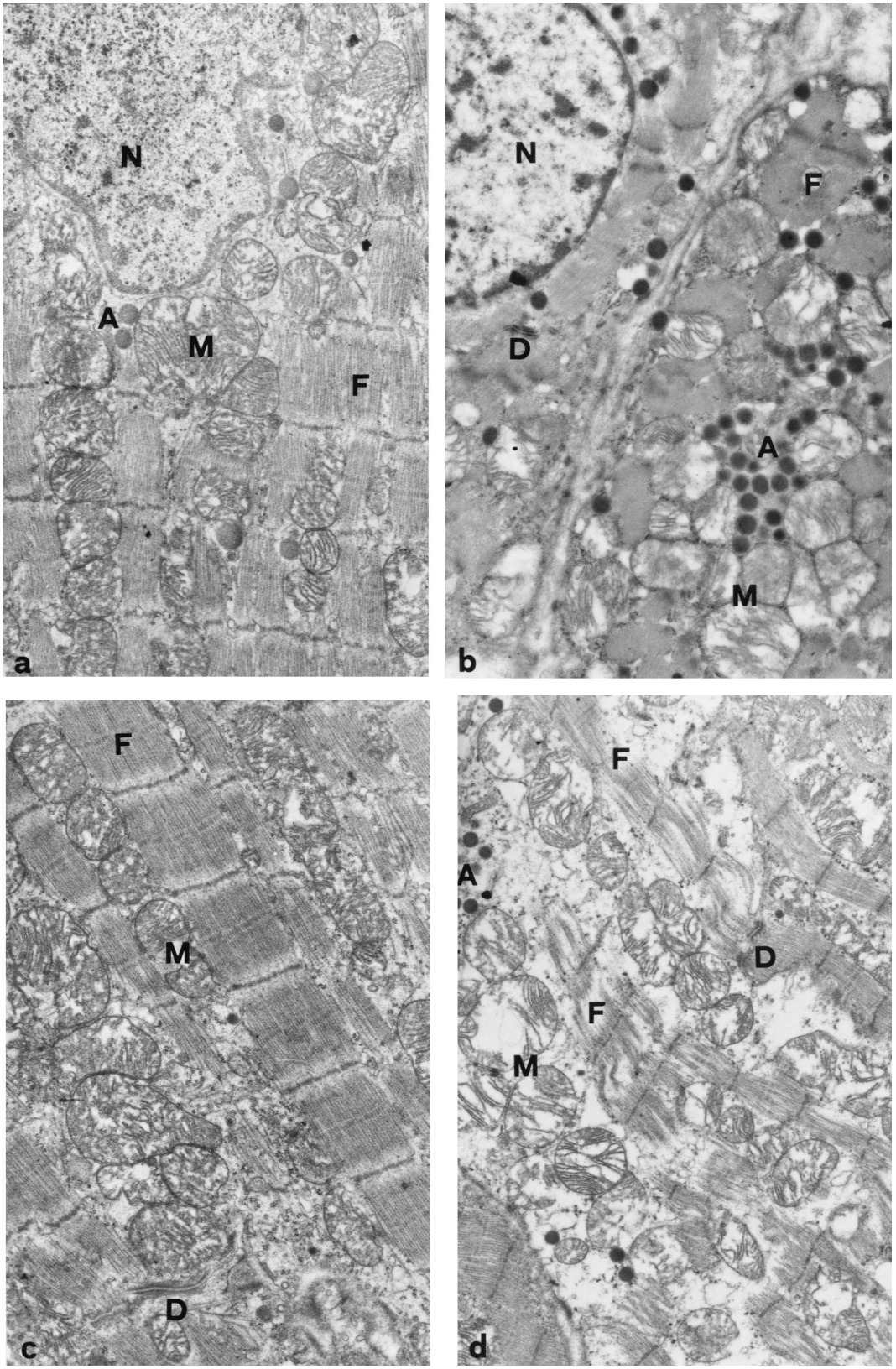

Fig. 6. Electron micrographs of rat myocardium. (a) Longitudinal section of atrial cardiomyocyte from control rat, × 12,000. (b) Section of atrium from rat treated with PK 11195. Some clusters of mitochondria (M) show an increased volume, matrix dilution, and cristolysis, × 10,000. (c) Longitudinal section of atrial cardiomyocyte from control rat. Well-preserved mitochondria (M) are oriented among myofibrils (F) exhibiting a normal arrangement, × 12,000. (d) Section of atrium from rat treated with PK 11195. Altered mitochondria (M) and disarranged myofibrils (F) are visible, × 8,800. Abbreviations used in the figure: (A) atrial granules; (D) intercalated discs; (F) myofibrils; (M) mitochondria; and (N) nucleus.

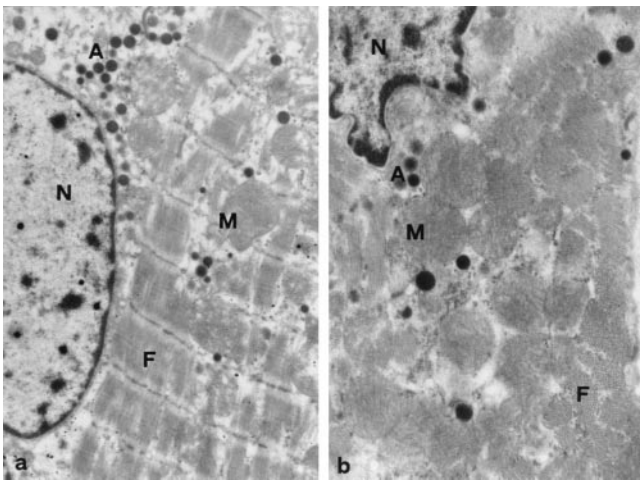

Fig. 7. Electron micrographs of rat myocardium. (a) Longitudinal section of atrial cardiomyocyte from rat treated with PK 11195 plus cyclosporin A. Intact mitochondria (M) and myofibrils (F) with a normal arrangement are visible, × 9,200. (b) Section of atrium from rat treated with cyclosporin A. Well-preserved mitochondria (M) and myofibrils (F) are visible as in (a), × 14,000. Abbreviations used in the figure: (A) atrial granules, (F) myofibrils, (M) mitochondria, and (N) nucleus.

ical pattern of early apoptosis. Fig. 5a shows an apoptotic nucleus with condensation of chromatin into dense masses, mainly adjacent to the inner nuclear envelope. In contrast, nuclei incubated with supernatant from untreated mitochondria revealed no apoptotic features (Fig. 5b). In addition, isolated nuclei directly exposed to PK 11195 did not reveal any morphological alteration (data not shown).

3.4. Ultrastructural observation of myocardiac tissue after in vivo *PK 11195 administration*

In order to investigate whether *in vivo* PK 11195 administration was able to induce ultrastructural alterations in rat cardiac tissue, one group of animals was injected intraperitoneally with the selective PBR ligand, PK 11195 (5 mg/kg), a second group with PK 11195 (5 mg/kg) plus CSP (10 mg/kg), a third group with CSP (10 mg/kg), and a fourth group (control group) received only the vehicle. Atrial cardiomyocytes from control specimens displayed a normal subcellular structure, showing well-preserved intermyofibrillar mitochondria and scattered electron-dense atrial granules (Fig. 6, a and c). By contrast, cardiomyocytes from PK 11195-treated animals exhibited altered mitochondria with different degrees of swelling in mitochondrial chambers. In addition, mitochondria showed disruption of the crests and loss of matrix substance (Fig. 6, b and d). A marked heterogeneity of myofibril patterns was observed. A few areas showed a normal structure, while in others, disarranged myofibrils as well as rupture of the myofilaments were visible (Fig. 6d). Atrial granules and intercalated discs appeared to have a normal structure (Fig. 6, b and d). Atrial cardiomyocytes from PK 11195 plus CSP-treated animals (Fig. 7a) exhibited intact mitochondria and well-preserved myofibrils and atrial granules, as in cardiomyocytes from CSP-treated rats (Fig. 7b).

Ventricular cardiomyocytes from control rats displayed a normal subcellular architecture as in standard descriptions (Fig. 8a). On the contrary, pictures from PK 11195-treated rats showed mitochondria with considerable alterations, consisting of an increased volume (hypertrophy), dilution of the matrix, and cristolysis (Fig. 8b). In some cases, lacunar areas corresponded to the disappearance of mitochondrial crests and the enlargement of the space between inner and outer membranes (Fig. 8b, inset). Disarrangement in myofibrils was sometimes observed, but no alteration of intercalated discs was found. Ventricular cardiomyocytes from PK 11195 plus CSP-treated rats (Fig. 8c) showed well-preserved mitochondria and myofibrils, as in ventricular sections from CSP-treated animals (Fig. 8d).

4. Discussion

PBR ligands induce mitochondrial permeability transition in heart mitochondria in a concentration-dependent and cyclosporin A-sensitive manner, as confirmed by light-scattering changes (ΔAbs_{520nm}) and electron microscope visualization. PK 11195-treated mitochondria showed complete or partially complete round structures, consistent with their becoming permeable, and swollen mitochondria, in contrast with well-preserved control organelles, showing an electron-dense matrix and well-arranged cristae. The "cell-free system" assay provided evidence of apoptotic changes in isolated cardiac nuclei after their incubation with supernatants from swollen mitochondria.

The selective and non-selective peripheral-type receptor ligands, PK 11195, Ro5-4864, and diazepam, promote MPT; on the other hand, the central-type receptor-selective ligand, clonazepam, was found to be ineffective, even at the highest concentration tested. MPT can be commonly observed after calcium accumulation in the presence of a surprisingly wide variety of inducing agents, ranging from Me^{2+} ions to local anaesthetics, scavengers of oxygen radicals, and many others, with no obvious functional or structural features in common [2].

In our hands, a cyclosporin A-sensitive induction of MPT was demonstrated following exposure of rat cardiac mitochondria to an increasing calcium concentration, in agreement with the data reported by Halestrap [44]. Typically, after a lag phase during which membrane permeability remains normal, Ca^{2+} uptake is followed by a spontaneous increase in permeability. This seems to be due to the opening of a non-selective channel, specifically inhibited by cyclosporin A [44,46–48], with a minimum diameter of 2.8 nm, allowing equilibration of solutes with molecular masses up to 1200 Da [49]. Several authors have suggested that the MPT pore coincides with the mitochondrial megachannel (MMC) [42,50–52], identified by patch-clamp studies [53,

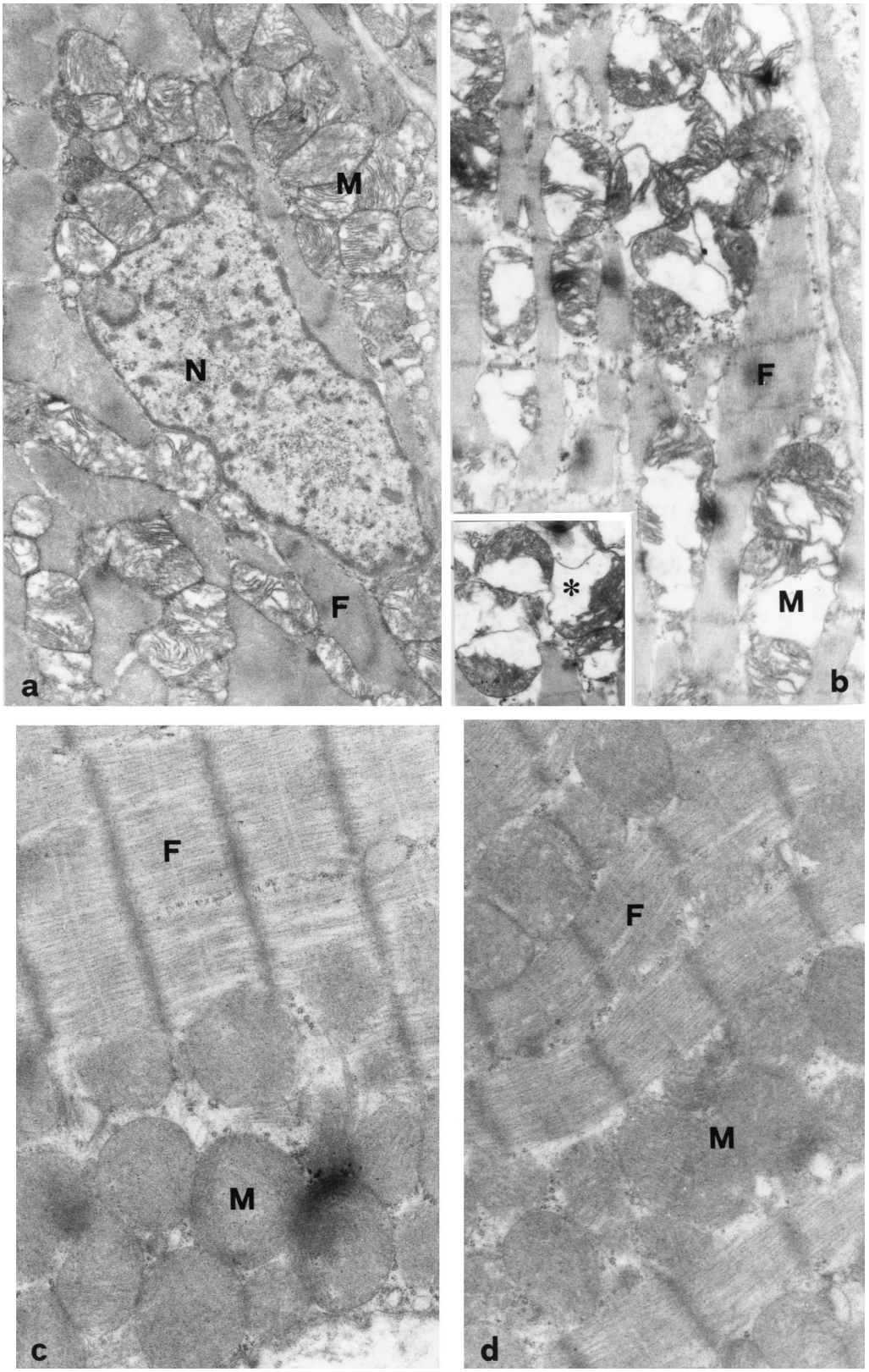

Fig. 8. Electron micrographs of rat myocardium. (a) Section of ventricular cardiomyocyte from control rat, × 8,800. (b) Section of ventricular cardiomyocyte from rat treated with PK 11195. The mitochondria (M) show significant alterations consisting of cristolysis and large areas of matrix dilutions, × 13,200. In the inset, note the considerable widening (*) between the outer and inner mitochondrial membranes, × 8,800. (c) Longitudinal section of ventricular cardiomyocyte from rat treated with PK 11195 plus cyclosporin A. Myofibrils (F) with a normal arrangement and well-preserved mitochondria are visible, × 16,800. (d) Longitudinal section of ventricular cardiomyocyte from rat treated with cyclosporin A. Normal myofibrils (F) and mitochondria (M) are visible as in (c), × 16,000. Abbreviations used in the figure: (F) myofibrils, (M) mitochondria, and (N) nucleus.

54]. Multiple regulatory steps are involved in the modulation of the pore activity, although all known inducers require matrix calcium accumulation [2], with the exception of the bifunctional SH group reagent, phenylarsine oxide [55,56]. The PBR ligands induced MPT without any calcium addition, probably either by facilitating endogenous calcium binding to its own sites and/or by acting directly on the proteinaceous pore. This hypothesis is in agreement with the suggested molecular pore structure, consisting of two copies of the 18-kDa peptide (PK 11195-binding site) associated with dimer porin molecules and with two molecules of the adenine nucleotide carrier [1]. In addition, Kinnally *et al*. [5] have reported that the benzodiazepine Ro5-4864 inhibits MMC activity, while clonazepam and Ro15-1788, which bind specifically to the central-type receptor, are ineffective.

Our data may facilitate the understanding of some PBR ligand effects shown in other cell models: indeed, in recent works, PK 11195 has been shown to facilitate the MPT pore opening induced by the tumor necrosis factor in L929 cells [36]. Furthermore, protoporphyrin IX, an endogenous substance with a high affinity for PBR, has been shown to induce swelling in isolated liver mitochondria and to potentiate killing of hepatocytes by rotenone [37]. It has been suggested that the opening of the mitochondrial pore may explain how PK 11195 facilitates apoptosis triggered by a variety of different agents, as reported by Hirsch and coworkers in several cell lines [35]. Indeed, apoptosis research has recently undergone a change from a model in which the nucleus determined the apoptotic process to one in which mitochondria constitute the center of death control [57]. Using a "cell-free system" assay, it has been demonstrated that mitochondria from healthy tissue release apoptogenic factors on addition of inducers of MPT, such as Ca^{2+}, pro-oxidants, caspases, and perhaps protein kinases. These proteins, the so-called apoptosis-inducing factors, then cause apoptotic changes in normal purified nuclei [12,15, 58]. Supporting an apoptotic role of PK 11195-induced permeability transition in isolated mitochondria, supernatants from swollen mitochondria caused changes in isolated cardiac nuclei. TEM analysis of treated nuclei revealed condensation and margination of chromatin, as is typical of the early stage of apoptosis, while no apoptotic features were found in nuclei exposed to supernatants from untreated mitochondria or to PK 11195 solution.

Our findings revealed mitochondrial swelling and myofibril disarrangement in atrial and ventricular cardiomyocytes of rats after *in vivo* PK 11195 administration. The damage found was probably due to the accumulation of the non-metabolized compound in the heart, as suggested by previous reports on the *in vivo* evaluation of radiolabeled PK 11195 distribution and metabolism [59,60]. However, no apoptotic changes were found in cardiomyocyte nuclei. Simultaneous PK 11195 plus CSP *in vivo* administration protected cardiac tissue; no ultrastructural damage was observed in either atrial or ventricular cardiomyocytes, suggesting a resilience of the cardiac tissue as a result of CSP-induced MPT pore closure, as reported in previous papers [1,16,43,44]. The ultrastructural alterations induced by PK 11195 and prevented by CSP in *in vivo* functional mitochondria confirmed that the effects observed in isolated de-energized mitochondrial fractions were not related to experimental variables.

In conclusion, our present data demonstrate that PBR ligands trigger MPT in a dose-dependent and CSP-sensitive manner, suggesting a novel strategy to investigate the molecular mechanism of MPT induction. Furthermore, supernatants from PK 11195-exposed mitochondria induce nuclear apoptotic changes. These findings encourage further studies on the critical role of MPT in the apoptotic pathway.

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