Cationic uncouplers of oxidative phosphorylation are inducers of mitochondrial permeability transition

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Abstract To determine whether cationic uncouplers of oxidative phosphorylation induce permeability transition in mitochondria, the effects of the divalent cationic sulfhydryl cross-linker copper-*o*-phenanthroline ($Cu(OP)_2$) and the cyanine dye tri-S-C_4(5) on rat liver mitochondria were examined. Like Ca^{2+}, they accelerated mitochondrial respiration with succinate and induced mitochondrial swelling when inorganic phosphate (Pi) was present in the incubation medium. The acceleration of respiration and swelling were inhibited by the SH-reagent *N*-ethylmaleimide, and by the specific permeability transition inhibitor cyclosporin A (CsA). In addition, these cations, like Ca^{2+}, induced release of ADP entrapped in the mitochondrial matrix space, and the morphological change of mitochondria induced by these cations was essentially the same as that induced by Ca^{2+}. It is concluded that the uncoupling actions of $Cu(OP)_2$ and tri-S-C_4(5) are due to induction of permeability transition in the inner mitochondrial membrane.

Key words: Uncoupler; Permeability transition; Ca^{2+}; Oxidative phosphorylation; Liver mitochondrion; Rat

1. Introduction

Uncouplers of oxidative phosphorylation inhibit ATP synthesis in mitochondria by dissipating the H^+-electrochemical gradient across the inner membrane, which is a driving force for ATP synthesis. Potent uncouplers, mostly with effective concentration ranges of 10–100 nM, are hydrophobic weak acids, and they act as protonophores making the H^+-impermeable mitochondrial membrane permeable to H^+ (for reviews, see [1–3]). Besides protonophoric weakly acidic uncouplers, divalent organic cations, such as cyanine dyes [4,5], copper-*o*-phenanthroline ($Cu(OP)_2$) [6] and crystal violet [7], exhibit uncoupling activity in mitochondria. Their actions are sometimes thought to be due to dissipation of the H^+-electrochemical potential by their transfer into the matrix space across the inner mitochondrial membrane according to the inside-negative membrane potential [8,9], although no proof for this action mechanism has been reported. The action of a cationic uncoupler is in some respects different from that of a weakly acidic uncoupler, such as in its requirement for Pi, its abolishment by the SH-reagent *N*-ethylmaleimide (NEM) and its induction of mitochondrial swelling [4,6].

These features of its action in mitochondria are very similar to those of metal cations, such as Ca^{2+} and Cd^{2+} [10,11]. Accumulation of Ca^{2+} in the mitochondria induces permeability transition in cooperation with Pi (for reviews, see [12–14]), and this results in the 'uncoupling' of mitochondria, i.e. acceleration of respiration, swelling and activation of ATPase, as in the action of a cationic uncoupler [4–7]. NEM at low concentration also inhibits 'Ca^{2+} uncoupling'. Therefore, it is possible that uncoupling by organic cations is due to the induction of permeability transition.

In this study, we examined the effects of the cyanine dye tri-S-C_4(5) and sulfhydryl cross-linker $Cu(OP)_2$ on rat liver mitochondria under various conditions. In addition, as cyclosporin A (CsA) is known to be a very effective inhibitor of permeability transition by inhibiting induction and causing closure of permeability transition by Ca^{2+} [15], we examined its effect on the action of cationic uncouplers. The results show that cationic uncouplers exert their activities by inducing permeability transition in the inner mitochondrial membrane.

2. Materials and methods

2.1. Materials

CsA was a gift from Novartis Pharma Co. (Tokyo), and tri-S-C_4(5) from Nippon Kankohshikiso Research Laboratory (Okayama). [2,8-^{3}H]ADP (code number NET-241) was purchased from DuPont-New England Nuclear (Wilmington, DE). $Cu(OP)_2$ was prepared as described previously [6].

2.2. Isolation of mitochondria

Mitochondria were isolated from the liver of adult male Wistar rats as described previously [16] in medium consisting of 250 mM sucrose, 1 mM EDTA and 2 mM Tris-HCl buffer, pH 7.4. Mitochondrial preparations were washed three times with the same medium, but without EDTA in the last wash. Mitochondrial protein was determined by the biuret method.

2.3. Measurement of mitochondrial respiration

Mitochondrial respiration was measured in medium consisting of 200 mM sucrose and 10 mM potassium phosphate buffer (pH 7.4). Respiration of mitochondria (0.7 mg protein/ml) in a total volume of 2.2 ml was started by adding 10 mM succinate plus rotenone (0.6 μg/ml). Time-dependent oxygen consumption was monitored with a Clark oxygen electrode (Yellow Spring, YSI 5331) at 25°C.

2.4. Measurement of swelling

The swelling of mitochondria was monitored as the decrease in the absorbance at 540 nm in studies with Ca^{2+} and $Cu(OP)_2$, and at 700 nm in studies with tri-S-C_4(5) in a spectrophotometer, Shimadzu UV-3000. Time-dependent change in the absorbance of the mitochondrial

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Abbreviations: Tri-S-C_4(5), 2,2′-[3-[2-(3-butyl-4-methyl-2-thiazolin-2-ylidene) ethylidene] propenylene]-bis[3-butyl-4-methyl thiazolinium iodide]; Tri-S-C_7(5), 2,2′-[3-[2-(3-heptyl-4-methyl-2-thiazolin-2-ylidene)ethylidene]propenylene]-bis[3-heptyl-4-methyl thiazolinium iodide]; $Cu(OP)_2$, complex of copper with *o*-phenanthroline; CsA, cyclosporin A; NEM, *N*-ethylmaleimide; TEM, transmission electron microscopy; Pi, inorganic phosphate

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suspension was monitored under identical conditions to those for measurements of respiration.

2.5. Measurement of release of matrix ADP into the medium

For exchange of inner mitochondrial ADP with [^{3}H]ADP, 12.5 μl of [^{3}H]ADP (specific radioactivity 807 GBq/mmol, 37 MBq/ml) undiluted by cold ADP was added mitochondrial suspension (35 mg/ml) in 1.0 ml of 250 mM sucrose containing 2 mM Tris-HCl buffer, pH 7.4. The suspension was stood on ice for 40 min, and then untrapped [^{3}H]ADP was removed by three washes with centrifugation. For measurement of the permeability of the inner mitochondrial membrane, this preparation was added at a final protein concentration of 0.7 mg/ml to 1.0 ml of the same medium as used for measurement of respiration. Then, test compounds were added, the reaction mixture was incubated at 25°C for 7 min, and the radioactivity of [^{3}H]ADP remaining in the mitochondria was determined in an Aloka liquid scintillation counter LSC-700. For examination of the effect of CsA, the mitochondria were preincubated with 600 nM CsA for 3 min, and then incubated with cations.

2.6. Transmission electron microscopy (TEM)

TEM analysis of mitochondria treated with various cations was carried out essentially as described previously [17]. Briefly, mitochondria were precipitated, fixed with 2.5% glutaraldehyde and then post-fixed in 2% OsO_4. After dehydration, the mitochondria were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by TEM with a Hitachi H-800MT electron microscope.

3. Results

3.1. Effects of cationic uncouplers on the respiration of mitochondria

We have reported that divalent hydrophobic cationic cyanine dyes such as tri-S-C_4(5) and tri-S-C_7(5), and the sulfhydryl cross-linker $Cu(OP)_2$ induce uncoupling of oxidative phosphorylation in rat liver mitochondria [4–6]. As shown in Fig. 1, 8 μM $Cu(OP)_2$ and 40 μM tri-S-C_4(5) accelerated mitochondrial respiration with succinate (plus rotenone) as a respiratory substrate. Unlike protonophoric uncouplers, they induced progressive acceleration of respiration with time after their addition.

The initial regulated respiration with succinate (37 nmol/mg protein/min) was finally increased to 98 nmol/mg protein/min with 8 μM $Cu(OP)_2$ and 111 nmol/mg protein/min with 40 μM tri-S-C_4(5) (Fig. 1). Respiration increased with increase in their concentrations in a sigmoidal manner [4,6], the concentrations required for 50% acceleration being 9.5 μM with

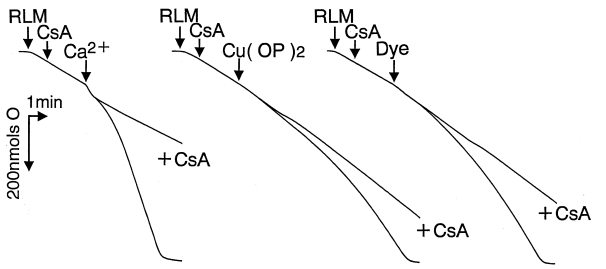

Fig. 1. Effects of Ca^{2+}, $Cu(OP)_2$ and tri-S-C_4(5) on respiration of rat liver mitochondria. Rat liver mitochondria (0.7 mg/ml) were suspended in 2.2 ml of medium consisting of 200 mM sucrose and 10 mM potassium phosphate buffer (pH 7.4) in a total volume of 2.2 ml. Respiration of rat liver mitochondria (RLM) was induced with 10 mM succinate plus rotenone (0.6 μg/ml) at 25°C, and the effects of 100 μM Ca^{2+}, 8 μM $Cu(OP)_2$ and 40 μM tri-S-C_4(5) (Dye) on the respiration were examined. The effect of prior addition of 600 nM CsA on the acceleration of respiration induced by these cations was also examined.

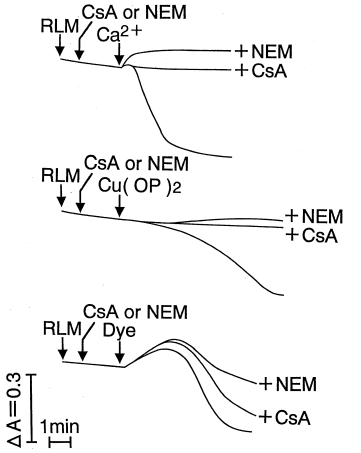

Fig. 2. Induction of mitochondrial swelling by Ca^{2+}, $Cu(OP)_2$ and tri-S-C_4(5). Change in the optical absorbance at 540 nm was recorded under the conditions described in the legend to Fig. 1. The effect of tri-S-C_4(5) was monitored at 700 nm due to its considerable absorption at 540 nm. The final concentrations of cations added to the suspension of rat liver mitochondria (RLM) were 100 μM Ca^{2+}, 8 μM $Cu(OP)_2$ and 60 μM tri-S-C_4(5) (Dye). The effects of 60 μM NEM and 600 nM CsA on the swelling were also examined.

$Cu(OP)_2$ and 46 μM with tri-S-C_4(5). These stimulations of respiration required Pi, and were inhibited by the sulfhydryl reagent NEM (data not shown), as reported previously [4,6]. As with Ca^{2+}, the specific inhibitor of permeability transition CsA inhibited their acceleration of respiration (Fig. 1).

Like Ca^{2+}, $Cu(OP)_2$ and tri-S-C_4(5) also induced swelling, measured as decrease in optical absorbance at 540 nm and 700 nm, respectively, and 60 μM NEM or 600 nM CsA inhibited their induction of swelling (Fig. 2). Transit shrinkage of mi-

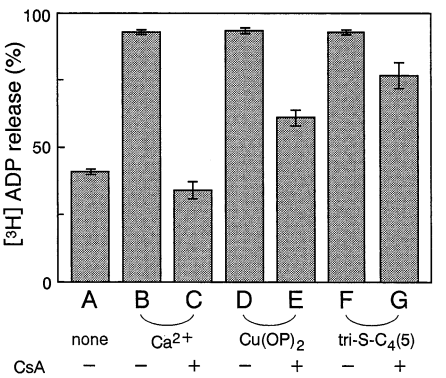

Fig. 3. Release of entrapped [^{3}H]ADP from rat liver mitochondria by various cations. [^{3}H]ADP was loaded into rat liver mitochondria as described in Section 2. The mitochondria (0.7 mg/ml) were suspended in medium consisting of 200 mM sucrose and 10 mM potassium phosphate buffer (pH 7.4), and energized with 10 mM succinate plus rotenone (0.6 μg/ml). Then they were incubated with 100 μM Ca^{2+} (B), 8 μM $Cu(OP)_2$ (D) and 40 μM tri-S-C_4(5) (F) for 7 min at 25°C, and promptly centrifuged, and their remaining radioactivity of [^{3}H]ADP was determined. For determination of the effect of CsA, mitochondria were incubated with 600 nM CsA for 3 min before incubation with the cations. Results on the effect of CsA are shown in columns C (with Ca^{2+}), E (with $Cu(OP)_2$) and G (with tri-S-C_4(5)). The result with non-energized mitochondria without addition of succinate (A) is also shown. The total radioactivity of [^{3}H]ADP incorporated into mitochondria was taken as 100%. Means (±S.D.) of three separate runs are shown.

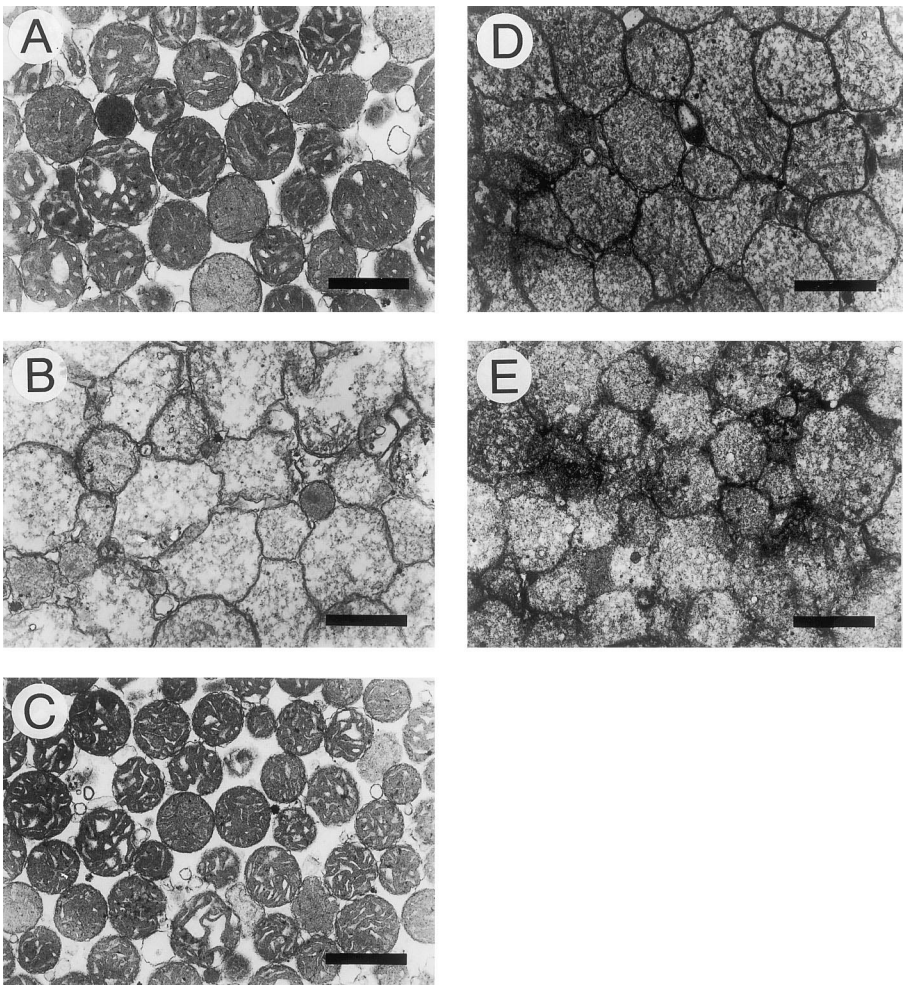

Fig. 4. Transmission electron microscopic observation of rat liver mitochondria treated with various cations. Rat liver mitochondria energized with 10 mM succinate plus rotenone (0.6 μg/ml) were incubated with Ca^{2+} (with or without CsA), tri-S-C_4(5) and $Cu(OP)_2$ for 7 min at 25°C, as described in the legend to Fig. 3. Then, mitochondrial samples for TEM analysis were prepared as described in Section 2. The appearances by TEM of energized mitochondria without further treatment (A), treated with 100 μM Ca^{2+} (B), treated with 600 nM CsA before treatment with 100 μM Ca^{2+} (C), treated with 60 μM $Cu(OP)_2$ (D) and treated with 60 μM tri-S-C_4(5) (E) are shown. Bars indicate 1.0 μm.

tochondria, as reflected by an increase in the absorbance, was observed in all cases; most with tri-S-C_4(5), least with $Cu(OP)_2$, and intermediately with Ca^{2+}. These results suggest that $Cu(OP)_2$ and tri-S-C_4(5) induce permeability transition of mitochondria.

3.2. *Release of ADP entrapped in the matrix space of mitochondria*

Induction of permeability transition in the inner mitochondrial membrane has usually been examined as a decrease in optical density associated with swelling of mitochondria. However, in this way it is difficult to distinguish the swelling associated with permeability transition from that without permeability transition (simple swelling), such as swelling caused by valinomycin in the presence of K^+. Therefore, we measured the induction of permeability transition by cationic uncouplers directly as release of ADP from the matrix space. For this, after replacement of the endogenous ADP in the matrix space by [^{3}H]ADP (see Section 2), the mitochondria were incubated with Ca^{2+}, $Cu(OP)_2$ and tri-S-C_4(5) for 7 min at 25°C under various conditions, and then release of the incorporated [^{3}H]ADP was determined by measuring the radioactivity of mitochondria precipitated by centrifugation. From the effects of the ADP/ATP carrier inhibitors carboxyatractyloside and bongkrekic acid, we confirmed that the entrapped [^{3}H]ADP was not released via the ADP/ATP carrier in the absence of exchangeable ADP in the incubation medium. Therefore, release of the entrapped [^{3}H]ADP from mitochondria should be a measure of induction of permeability transition.

As shown in Fig. 3, massive [^{3}H]ADP release was observed on incubation with 100 μM Ca^{2+}, 8 μM $Cu(OP)_2$ and 40 μM tri-S-C_4(5), showing that, like Ca^{2+}, the cationic uncouplers induced permeability transition. As 600 nM CsA inhibited the release of [^{3}H]ADP induced by $Cu(OP)_2$ or tri-S-C_4(5), the effects of these compounds were CsA-sensitive. However, the effect of CsA on the permeability transition by the cationic uncouplers was partial, unlike its complete inhibition of permeability transition induced by Ca^{2+}, suggesting that the effects of cationic uncouplers were stronger than that of Ca^{2+}. In fact, CsA did not completely inhibit the accelerated respiration and swelling of mitochondria caused by higher concentrations of $Cu(OP)_2$ and tri-S-C_4(5) (data not shown). Although CsA prevented the acceleration of respiration and induction of swelling by $Cu(OP)_2$ and tri-S-C_4(5) almost completely (Figs. 1 and 2), it did not completely inhibit the release

of entrapped [^{3}H]ADP induced by these cations. This could be because we determined total [^{3}H]ADP release on incubation with these cations for 7 min, and so detected the induction of slight permeability transition by measuring release of the entrapped [^{3}H]ADP in this way. Therefore, $Cu(OP)_2$ and tri-S-C_4(5) were concluded to be inducers of permeability transition. In addition, as with Ca^{2+} [18], we observed that $Cu(OP)_2$ and tri-S-C_4(5) increased the sucrose space of mitochondria due to increase in permeability to sucrose associated with permeability transition: the original sucrose space of 2.0 μl/mg protein was increased to 2.5–3.0 μl/mg protein on treatment of mitochondria with $Cu(OP)_2$ and tri-S-C_4(5), as observed with Ca^{2+} [18]. However, it is difficult to distinguish whether this increase was due to formation of permeability transition or the swelling of the matrix space, as in the measurement of optical absorbance. Therefore, measurement of release of the entrapped [^{3}H]ADP is a good measure of formation of permeability transition. Details of this method will be published elsewhere.

3.3. Electron microscopic analysis of mitochondrial configuration

When permeability transition is induced by Ca^{2+}, the structure of the mitochondrial inner membrane is drastically disrupted [19–21]. As shown in Fig. 4, disruption of the inner mitochondrial membrane was observed on treatment of mitochondria with 100 μM Ca^{2+}, and mitochondria remained intact on treatment with 600 nM CsA. Similarly, 60 μM $Cu(OP)_2$ and 60 μM tri-S-C_4(5) caused disruption of the inner mitochondrial membrane (Fig. 4), and the disruption was significantly inhibited by CsA (data not shown). These results showed that the mitochondrial inner membrane was disrupted by treatment of mitochondria with $Cu(OP)_2$ and tri-S-C_4(5), and thus, that these cations actually induce permeability transition.

4. Discussion

Uncoupling of mitochondrial oxidative phosphorylation by the organic divalent cationic cyanine dye tri-S-C_4(5) and the sulfhydryl cross-linker $Cu(OP)_2$ is different from that by protonophoric weakly acidic uncouplers in showing requirement for Pi and progressive acceleration of respiration, being abolished by the SH-reagent NEM and inducing swelling of mitochondria [4,6]. Some of these features are shown in Figs. 1 and 2. Although the effects of cationic uncouplers have been characterized in detail [4–7], the mechanism by which they induce uncoupling is not understood. As the effects of cationic uncouplers are very similar to those of Ca^{2+} 'uncoupling' due to induction of permeability transition, we examined whether cationic uncouplers induce permeability transition.

As low concentrations of tri-S-C_4(5) and $Cu(OP)_2$ released much of the [^{3}H]ADP entrapped in mitochondria, and this effect was prevented by CsA, and they also disrupted the inner mitochondrial membrane, their uncoupling action, like that of Ca^{2+}, was concluded to be due to induction of permeability transition. Namely, $Cu(OP)_2$ and tri-S-C_4(5) are inducers of permeability transition. These results are consistent with our previous observation that tri-S-C_4(5) induces a leakage type pathway only in the presence of Pi in phospholipid bilayer membranes [5]. It is noteworthy that the cyanine dye, in association with Pi, induces alteration of phospholipid bilayer organization, even though proteins are not present in the membrane.

However, their actions did not seem to be completely the same as that of Ca^{2+}. Ca^{2+} induces permeability transition on its accumulation in mitochondria after its transfer, and the resultant induced permeability transition is reversible and is completely inhibited by CsA. In contrast, the effect of CsA on the actions of cationic uncouplers was only partial, suggesting that their effects were greater than that of Ca^{2+}, especially that of tri-S-C_4(5), and that their induced permeability transition was irreversible. The mechanism of induction of permeability transition by $Cu(OP)_2$ and tri-S-C_4(5) may be different from that by Ca^{2+}. Possibly, cationic uncouplers induce permeability transition by interaction with the mitochondrial membrane. If this is so, the mechanism by which low concentrations of cationic uncouplers change the membrane organization is of importance for full understanding of the mechanism of induction of permeability transition.

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References

[1] McLaughlin, S.G. and Dilger, J.P. (1980) Physiol. Rev. 60, 825–863.
[2] Terada, H. (1981) Biochim. Biophys. Acta 639, 225–242.
[3] Terada, H. (1990) Environ. Health Perspect. 87, 213–218.
[4] Terada, H., Nagamune, H., Morikawa, N. and Ikuno, M. (1985) Biochim. Biophys. Acta 807, 168–176.
[5] Takeguchi, N., Saitoh, T., Morii, M., Yoshikawa, K. and Terada, H. (1985) J. Biol. Chem. 260, 9158–9161.
[6] Shinohara, Y. and Terada, H. (1987) Biochim. Biophys. Acta 890, 387–391.
[7] Moreno, S.N., Gadelha, F.R. and Docampo, R. (1988) J. Biol. Chem. 263, 12493–12499.
[8] Bakeeva, L.E., Grinius, L.L., Jasaitis, A.A., Kuliene, V.V., Levitsky, D.O., Liberman, E.A., Severina, I.I. and Skulachev, V.P. (1970) Biochim. Biophys. Acta 216, 13–21.
[9] Liberman, E.A. and Skulachev, V.P. (1970) Biochim. Biophys. Acta 216, 30–42.
[10] Nicholls, D. and Akerman, K. (1982) Biochim. Biophys. Acta 683, 57–88.
[11] Koike, H., Shinohara, Y. and Terada, H. (1991) Biochim. Biophys. Acta 1060, 75–81.
[12] Gunter, T.E. and Pfeiffer, D.R. (1990) Am. J. Physiol. 258, C755–C786.
[13] Bernardi, P., Broekemeier, K.M. and Pfeiffer, D.R. (1994) J. Bioenerg. Biomembr. 26, 509–517.
[14] Zoratti, M. and Szabo, I. (1995) Biochim. Biophys. Acta 1241, 139–176.
[15] Broekemeier, K.M., Dempsey, M.E. and Pfeiffer, D.R. (1989) J. Biol. Chem. 264, 7826–7830.
[16] Myers, D.K. and Slater, E.C. (1957) Biochem. J. 67, 558–572.
[17] Shinohara, Y., Sagawa, I., Ichihara, J., Yamamoto, K., Terao, K. and Terada, H. (1997) Biochim. Biophys. Acta 1319, 319–330.
[18] Crompton, M., Ellinger, H. and Costi, A. (1988) Biochem. J. 255, 357–360.
[19] Beatrice, M.C., Stiers, D.L. and Pfeiffer, D.R. (1982) J. Biol. Chem. 257, 7161–7171.
[20] Petronilli, V., Cola, C., Massari, S., Colonna, R. and Bernardi, P. (1993) J. Biol. Chem. 268, 21939–21945.
[21] Jung, D.W., Bradshaw, P.C. and Pfeiffer, D.R. (1997) J. Biol. Chem. 272, 21104–21112.