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Obligatory role of membrane events in the regulatory effect of metformin on the respiratory chain function

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Abstract

From recent findings about the indirect effect of metformin (MET) targeted on the respiratory chain complex I, we reconsidered this question and tried to determine the causality of any alteration at this enzymatic level using *Xenopus laevis* oocytes. Addition of MET (50 μM) reduced by 40% the rotenone-sensitive activity of complex I only in incubating intact oocytes but not in mitochondria isolated by differential centrifugation. The drug prior injected inside these cells had also no measurable effect. In spite of this and the weak binding of MET to the mitochondrial fraction, there was a fairly good correlation between the marked inhibitory action of MET on complex I and its progressive appearance within the oocyte cytoplasm. The intriguing observation that MET as a liposomal form was again able to exert its role when added directly to isolated mitochondria is in accordance with a membrane-mediated uptake and vesicular routing of MET. Furthermore, a temperature-dependent effect was clearly shown. At 4°, oocytes failed to take up efficiently MET and accordingly its subsequent action on respiration was therefore lost. Likewise, MET transport was hindered and inhibition of complex I totally disappeared when a structural analog, asymmetrical dimethylarginine (ADMA), was placed together with MET either at an identical concentration or in excess. These data strongly support the view that MET may recognise some specific membranous sites, likely belonging to effector systems, before penetrating the cell in a bound state *via* an obscure endocytotic event which still has to be identified.

Keywords: Metformin; *Xenopus laevis* oocyte; Mitochondrial function; Complex I; Complex IV; Subcellular fractionation

1. Introduction

The dimethylated compound MET (*N*, *N*-dimethylbiguanide) is an oral antihyperglycaemic agent widely used in the treatment of type 2 diabetes. This drug inhibits hepatic gluconeogenesis [1,2], and it also stimulates glucose transport and its utilisation by peripheral tissues sensitive to INS [3–5]. By taking advantage of the experimental model of *Xenopus laevis* oocyte, it was confirmed that MET potentiated the hormonal signalling by increasing the intrinsic tyrosine kinase activity of the insulin receptor [6,7]. This biochemical mechanism mainly leads to the activation of glycogen synthase in a concentration-dependent manner within the therapeutic range [8], this enzyme being considered as a major limiting factor in the causal defects of diabetes [9].

Many studies (recently reviewed in [10]) provide conclusive evidence that the primary site for MET action is confined at the cell membrane level. In the case of *Xenopus* oocytes, the best proof concerns the observation that a cortical oocyte preparation responds adequately to the presence of MET [7], which implies that the intracellular component is not required to produce its metabolic effect. On the other hand, when the drug was directly introduced in the cell interior by microinjection, the tyrosine kinase-utilising processes (e.g. egg maturation and glycogen synthase activation) were either delayed [11] or less efficient [8]. However, this data was not necessarily inconsistent with an action of MET within the subplasmalemmal area nearby the insulin receptor. Furthermore, the lack of correlation between the potentiating effect of MET on

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Abbreviations: MET, metformin; ADMA, asymmetrical dimethylarginine; MBS, modified Barth's solution; INS, insulin.

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INS-mediated glycogen synthase activity in *Xenopus* oocytes and the intracellular drug amounts strongly corroborates the emerging idea of a MET action on, or within, the plasma membrane [12], even though this result doesn't totally preclude that other functions could be altered by MET when internalised into the cytosol.

From basic investigations intended to probe the link between the physicochemical properties of biguanides in general and their biological activity, the capacity of some compounds to interfere with the oxidative phosphorylation machinery was claimed [13]. Nonetheless, these studies were principally conducted on isolated mitochondria with very high drug concentrations, and with biguanides of varying side-chain length. Besides, most data were extrapolated to all derivatives of this family thus including MET, whereas it was already observed that MET binding to mitochondrial membranes was very weak when compared with biguanides having a longer side-chain [14]. A reassessment of the MET pharmacodynamic features has largely questioned these old expectations and pointed to the absence of relationship between cell respiration and the antidiabetic potency of MET [15]. Ubl *et al*. [16] also noticed that MET *in vitro* does not modify the membrane potential of mitochondria. Other studies, showing that high doses of MET influenced the redox state of whole cells while energy metabolism was only slightly reduced [17,18], clearly illustrated that interference with this parameter was not likely to be explained by a direct effect of MET on the mitochondrial membrane. This hypothesis was further confirmed by a recent work reporting that MET (in doses ranging from 0.1 to 10 mM) suppressed cell respiration in intact hepatocytes *via* a selective inhibition of complex I located in the inner membrane of mitochondria [19]. The latter effect was temperature-dependent and also persisted after permeabilisation of hepatocytes using digitonin and when mitochondria were prepared from livers previously perfused with 10 mM MET. Despite the rapid onset of the process and the short time for reaching the maximal effect, there is no unequivocal proof that this MET action is attributable to its intracellular presence in a free state since no measurable effect was directly found on permeabilised cells or isolated organelles [19].

By using the *Xenopus* oocyte model, we previously observed that a prolonged incubation was necessary to detect radioactively labeled MET in a cytosolic compartment [12]. It is conceivable that this long-term requirement may be related to binding and/or transport characteristics of the drug across the lipid bilayer, assuming that MET needs to penetrate into the oocyte for acting on different physiological functions. Therefore, we decided here to revisit this issue by analysing the efficacy of MET on respiration in this cell type. For that purpose, the enzymatic activity of mitochondrial complex I was more particularly followed according to the mode of drug application or the contribution of certain factors influencing its physicochemical behaviour.

2. Materials and methods

2.1. Materials

Two batches of [^{14}C]MET (1891.1 and 4061 MBq/mmol) were kindly given by ISOTOPCHIM and unlabeled MET hydrochloride was a gift from LIPHA. [U-^{14}C] sucrose (486 mCi/mmol) was from Amersham Life Science. ADMA, cytochrome c, decylubiquinone, rotenone and all other reagent grade chemicals were purchased from Sigma Co, except for EDTA and NADH which were obtained from Merck and ICN Biomedicals, respectively. MBS buffer was composed of 10 mM HEPES (pH 7.6), 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$ and 0.41 mM $CaCl_2$. Formulation of the antidiabetic agent retained in liposomes (made from phospholipids with encapsulated MET and non-encapsulated MET in the ratio of 6:3:1; 0.12 mmol phosphatidylcholine, 0.06 mmol cholesterol and 0.02 mmol phosphatidylglycerol per 10 mL of buffer plus trehalose; liposome size 80 nm).

2.2. Preparative procedures and assay of mitochondrial complexes

After promptly removing ovaries from mature *Xenopus* females, the oocytes were manually dissociated from their connective tissue, sorted and defolliculated as previously described [8]. They were then thoroughly rinsed in MBS before their lysis in a buffered medium consisting of 10 mM Tris (pH 7.4), 1.5 mM EDTA, 0.5 mM EGTA, 255 mM sucrose, 2 mM $MgCl_2$, and 4 mM dithiothreitol (DTT). All subsequent manipulations were carried out at 0–4°. The oocytes were broken in a Dounce homogeniser using five hand-driven strokes of the loose-fitting pestle, and mitochondria were isolated by conventional differential centrifugation as follows. The homogenate was centrifuged twice for 10 min at 1000 *g* to eliminate cell debris, yolk proteins and nuclei. The cleared supernatant, whose samples were withdrawn and used for determination of enzyme activities described below, was transferred to clean centrifuge tubes and run at 11,000 *g* for 15 min. This step yielded the mitochondrial pellet which was carefully resuspended in the homogenisation buffer. The final suspension contained between 2 and 8 mg mitochondrial proteins per mL. This concentration range was measured with the Bio-Rad method, based on the Bradford dye-binding technique, using BSA as a standard. Considering that the protein content does not relate solely to mitochondria because of a moderate contamination of this fraction in fully-grown oocytes [20], the calculated specific activities must be underestimated.

Complex IV (cytochrome c oxidase) activity of the electron transfer chain was determined with the method of Cooperstein and Lazarow [21]. In brief, aliquots of supernatants or mitochondrial extracts properly diluted in

the presence of 0.04% (w/v) Triton X-100 were incubated at 22° with 17 μM reduced cytochrome c, 1 mM EDTA and 30 mM phosphate buffer (pH 7.4), in a total volume of 3 mL. The reaction was followed by recording the decrease in absorbance at 550 nm. One unit of marker enzyme corresponds to the amount which oxidises per min 90% of the reduced cytochrome c present in 100 mL of the incubation mixture.

For the assay of complex I (NADH-ubiquinone oxidoreductase), either 200 μL of supernatant or 40 μL of the mitochondrial fraction were added to 1 mL of lysis buffer containing 100 μM NADH and 1.5 mM KCN, but devoid of sucrose in order to break inner membrane by osmotic shock. The rate of NADH oxidation was monitored at 340 nm for 2 min in a UV spectrophotometer equipped with thermostatic control. An electron acceptor, decylubiquinone (80 μM), was then added and the stimulated rate of this process was taken as the complex I activity, using an extinction coefficient of 6.22/mM/cm. In order to assess the oxidation of NADH which was independent of this enzymatic activity, the same reaction was evaluated in the presence of rotenone (2.5 μM), a potent and specific inhibitor of complex I. One unit of this enzyme is the amount of complex I producing 1 μmol of oxidised NADH/min. Throughout the results, activities measured on low-speed supernatants were expressed in U per g of tissue, and those calculated from isolated mitochondria were expressed in nmol/min/mg mitochondrial protein.

2.3. Use of different approaches for the study of MET effect on oocyte complex I

The ability of MET to modulate the mitochondrial metabolism was examined under various conditions similar to those used by authors working on hepatocytes [19]. In one case, the oocytes were incubated for the required time periods into 2 mL of MBS alone or containing MET at either 50 μM or 10 mM. For the competition studies, this medium was supplemented with increasing concentrations of ADMA ranging from 500 nM to 2.5 mM accordingly. We moreover achieved incubations at 4° for analysing the temperature effect. Secondly, MET was injected through the use of microsyringes in the cytosol of cells at a final dose of 50 μM. This concentration was chosen with respect to intracellular levels of MET found with the radioactive method. These oocytes were thereafter placed in MBS for 3 hr, i.e. a time similar to that of incubation with MET added outside the cells. At the end of these multiple treatments, both intact and poked oocytes were disrupted and the activity of complex I was measured on low-speed extracts. In a third method, the drug was directly placed with mitochondria in an incubation bath at 25° for 20–30 min before assaying the enzyme activity. Lastly, we made use of MET enclosed into liposomes. This novel formulation for the molecule was tested on intact oocytes or isolated mitochondria, in experimental conditions identical to those applied for free MET.

2.4. Uptake of MET into oocytes and analysis of its cellular distribution

Batches of 2–8 mature *Xenopus* oocytes were incubated at ambient temperature or 4° in 250 μL of MBS containing separately 50 μM and 10 mM [^{14}C]-labeled MET or sucrose. At regular time intervals, the cells were quickly taken out of the medium, washed with cold MBS and lysed in an isolation buffer (10 mM HEPES, pH 7.9, 10 mM NaCl). The uptake of MET was mainly expressed as the percentage of radioactivity incorporated to whole cells (determined from aliquots of the initial lysate) vs. total external radioactivity. Competition studies were conducted at room temperature in the absence or presence of the structural analog ADMA at the required doses. To now examine the distribution of MET among the cellular components, two procedures were applied as in [12]. Either each oocyte was manually dissected in order to remove the bulk of cytoplasmic content from the cortex. As the oocyte cortex is formed by the plasma membrane and a cytoskeletal matrix in which subcellular organelles are embedded [22], it is likely that cytosol of cell periphery was not entirely extracted. In the second protocol, we achieved the fractionation of oocytes as earlier described, with some minor modifications. After the oocytes were treated with radioactive MET for 30 min and 3 hr, they were homogenised in the presence of 20 mM Tris (pH 7.2), 1 mM EGTA, and 250 mM sucrose. After differential centrifugation, four fractions were isolated: a nuclear fraction filled with yolk proteins and sedimented at 1000 *g* for 10 min (P1); a mitochondrial fraction at 11,000 *g* for 15 min (P2); a microsomal fraction at 100,000 *g* for 45 min (P3) and a cytosolic fraction being the final supernatant. In addition, we characterised the mitochondria-rich fraction biochemically by assaying the activity of cytochrome c oxidase as marker of these organelles.

2.5. Statistical analysis

Statistical significance of differences was determined by Student's *t*-test. A probability of 5% or less was accepted as significant. All data were presented as the means ± SEM ($N \geq 4$).

3. Results

3.1. Effect of MET on mitochondrial enzymatic complexes in intact cells

The high number of mitochondria stored in *Xenopus* oocytes [23], as well as the easy preparation of a

subcellular fraction enriched in these organelles are favourable elements for investigating in this cell type the interaction between a drug and cell respiration. In the first set of experiments, we tested the influence of MET on two mitochondrial complexes, complex IV and complex I. This study was achieved with respect to drug concentrations (50 μM and 10 mM) and incubation times of oocytes. As shown in Fig. 1, MET did not affect the activity of cytochrome c oxidase whatever the parameters applied. In addition, the enzyme activity was maintained after a sustained incubation of 16 hr, which proves the good viability of the oocytes in our experimental conditions. Conversely, MET induced a significant ($P < 0.01$) time-dependent inhibition of the complex I activity when used at both concentrations (Fig. 2a). However, the maximal effect (inhibition around 40–50%) was reached only after several hours of treatment in the physiological setting (50 μM), whereas a similar response was already recorded within 30 min with the non-therapeutical dose (10 mM). Although the enzyme measurements were performed on relatively crude extracts (low-speed supernatants from the first spin), the obtained values are certainly reliable in keeping with the expected results. Moreover, the extents of MET inhibitions were not significantly higher when examining the respective complex I specific activities which were assayed on mitochondria-rich fractions (Fig. 2b). This approach besides reveals rather small activities, as it is commonly the case in unenergised mitochondria, but the fraction showed a 60-fold enrichment as compared with whole-cell homogenate (data not shown). All these findings clearly indicate that the centrifugation scheme proposed is correct and calculation of enzyme activity on yolk-free fractions gives quite sufficient and valuable information.

To further confirm that complex I was the selective target of MET action, we studied the efficiency of rotenone using functionally isolated complex I from the above preparations (Fig. 2). Mitochondria catalysed electron transfer from NADH to oxidised quinone at a rate which was around 90% inhibited by rotenone, indicating that quinone reduction occurs at or near the physiological acceptor site. On the other hand, the inhibitory effect of this poison was only partial with supernatants, yet total enzyme activity in these extracts vs. isolated organelles was slightly increased. This suggests the presence of a quinone-mediated reductase activity with a rotenone-insensitive component but that this activity belongs probably to complex I itself [24]. Hence, the observation that mitochondrial chain activity was decreased in similar proportion by MET and a standard respiratory inhibitor such as rotenone is completely favourable to this site I-specific effect of MET.

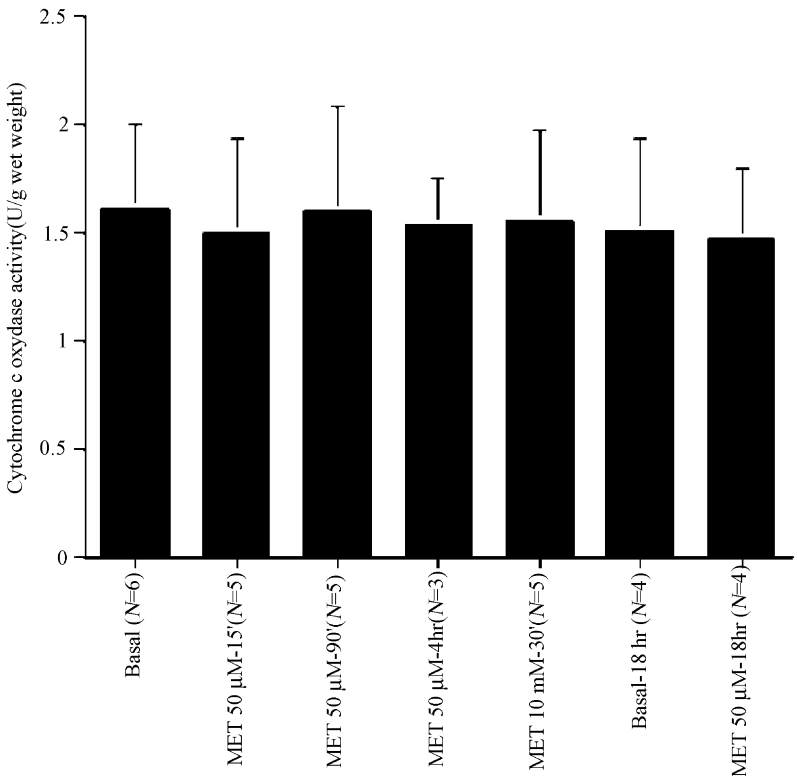

Fig. 1. Effect of MET on cytochrome c oxidase activity in intact oocytes. The oocytes were incubated in MBS alone (basal) or supplemented with MET at two concentrations for the indicated periods of time. After these incubations, each group of cells was homogenised, fractionated and the subsequent yolk-free extract was then used for measuring cytochrome c oxidase activity as described elsewhere. The data were expressed as the means ± SEM ($N = 3-6$). Measurements of complex IV specific activities performed on isolated mitochondria were resumed in Table 1.

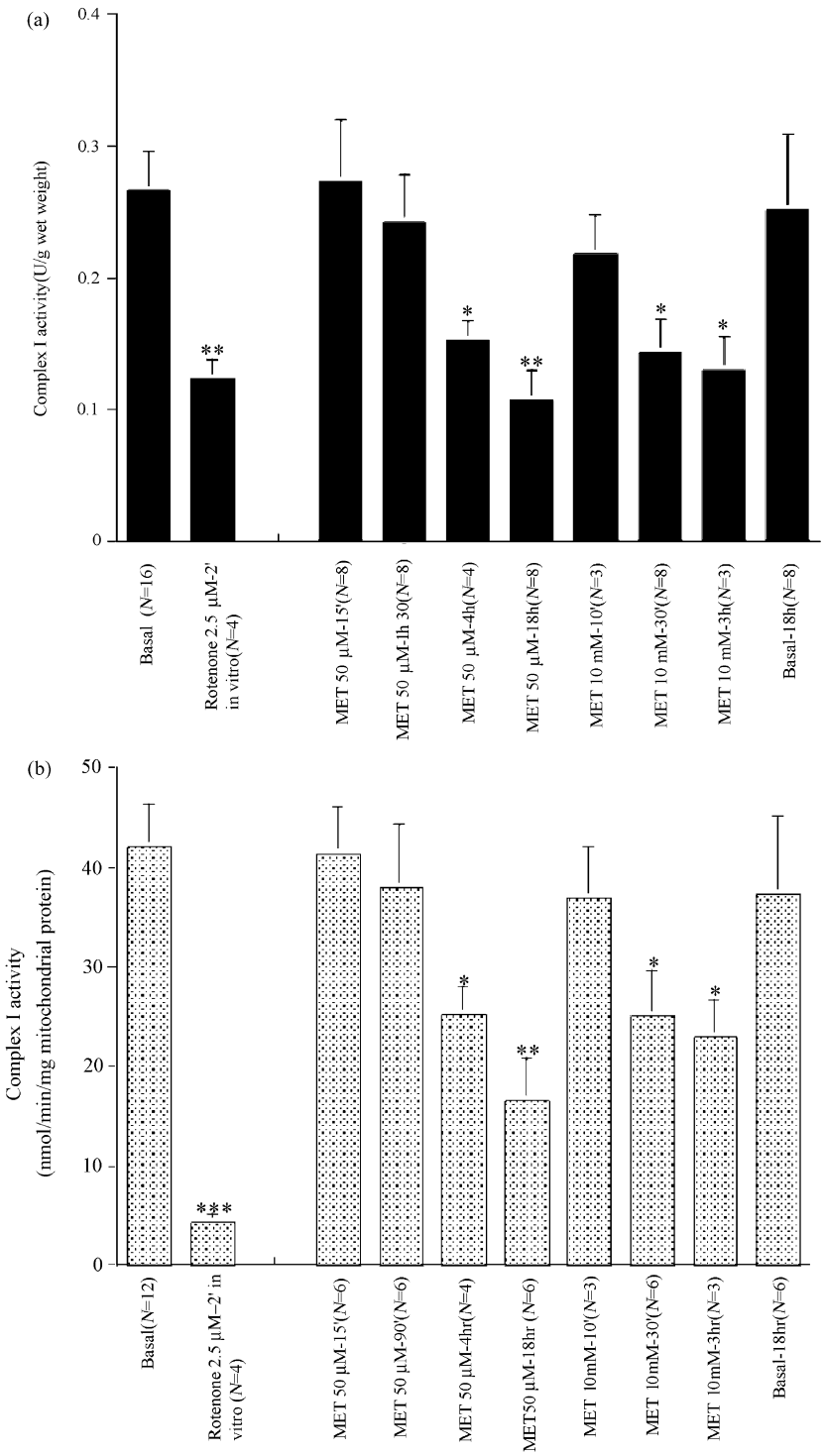

Fig. 2. Comparative effects of MET and rotenone on NADH-ubiquinone oxidoreductase activity. The oocytes were treated in the absence or presence of MET at two concentrations for the above-mentioned exposure times. After these treatments, groups of oocytes were processed as in Fig. 1 and complex I activity was assayed on yolk-free supernatants (a) and mitochondria-rich fractions (b). NADH oxidation by the rotenone-sensitive pathway was also tested on these both mitochondrial preparations as reported in Section 2. The values are presented as the means ± SEM ($N = 3-16$). MET effects were statistically different from basal conditions for $P < 0.01$ (indicated by *) and $P < 0.005$ (indicated by **). Rotenone action, when compared to control, was significant with $P < 0.005$ or $P < 0.001$ (indicated by ***).

3.2. Cellular location of MET and respiratory function

The major difference in the velocity of reaction when *Xenopus* oocytes are exposed to such extreme MET concentrations possibly reflects a peculiar relationship with the plasma membrane. In an attempt to ascertain this hypothetical link, we monitored the cellular uptake of radioactive drug at the above cited doses. Firstly, there is a very slow

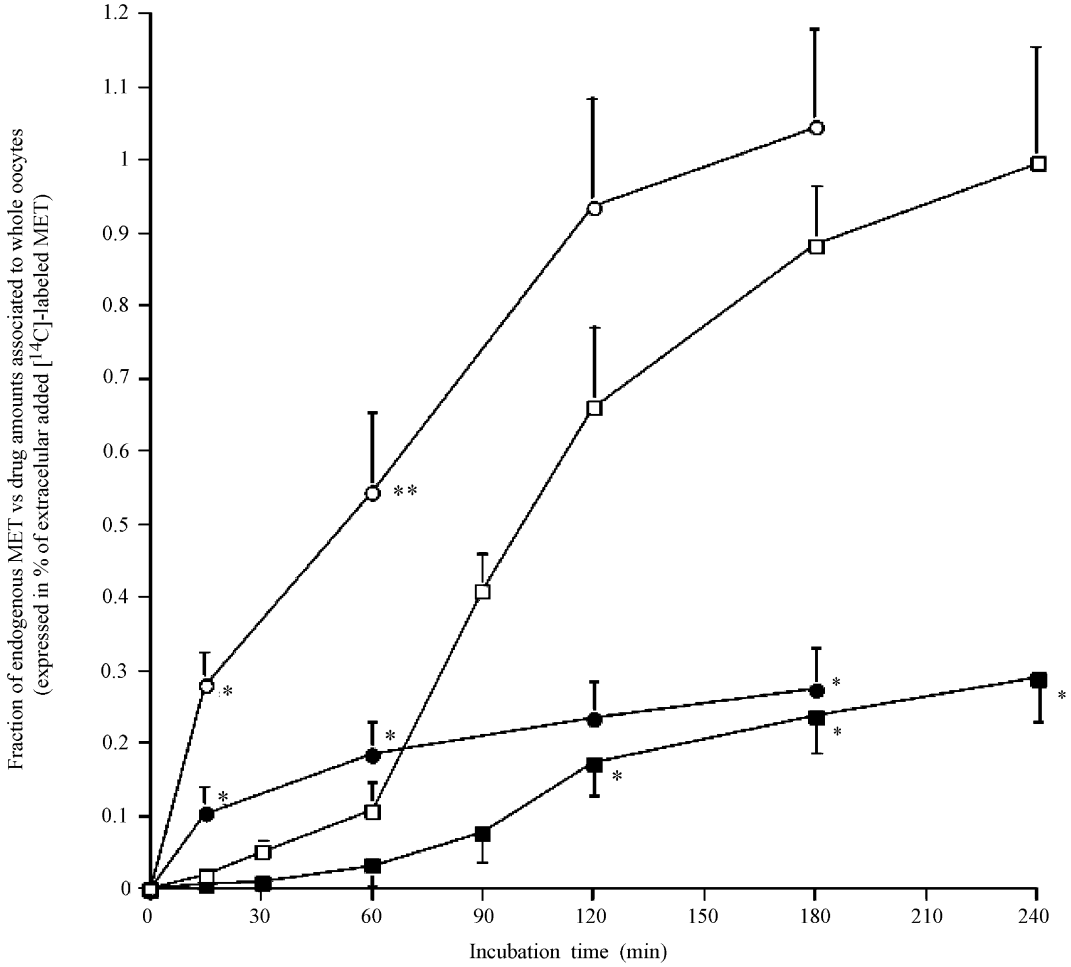

Fig. 3. Time-dependent variations in the amount of [^{14}C]MET taken up by *Xenopus* oocytes. The oocytes were exposed to the radioactive drug at two doses for up to 4 hr. After washing, they were handled as described in Section 2. The percentage of radioactivity associated to oocytes treated with 50 μM (□) and 10 mM (○) MET as well as the corresponding amount of MET present in a cytosolic compartment (■ and ●) were next determined. Results are means ± SEM of four experiments, each performed in duplicate. (*) denotes significance at $P < 0.05$; (**) denotes significance at $P < 0.01$.

permeation of 50 μM [^{14}C]MET across the lipid bilayer (Fig. 3). Less than 0.1% of extracellular radioactivity is associated to whole oocytes after 60 min. Yet, it is interesting to remind that the INS-potentiating effect of MET on glycogen synthase activity was fully elicited during this lag stage, characterised by a lack of detectable intracellular MET ([12], and present work). Afterwards, the oocytes begin to poorly accumulate the drug while a clear-cut appearance of drug within their cytosol becomes significantly ($P < 0.05$) effective after 2 or 3 hr. This duration corresponds precisely to the delay required for observing a marked effect of MET on complex I. At this moment, the amount of endogeneous MET taken up by the oocytes was equivalent to about 25% of the total radioactivity tethered to whole cells. This value, which was minimally changed if the incubation was extended to 16 hr [12], is possibly underevaluated because it doesn't take into account the presence of MET just beneath the membrane, in the cortical area (see Section 2). Nevertheless, these observations also showed that a substantial part of MET remained attached to (sub)membranous structures. On the other hand, by examining the subcellular distribution of MET at two relevant time points (30 min and 3 hr), trace amounts of radioactivity were detected in the mitochondrial fraction P2 (Table 1). This observation suggests that MET doesn't colocalise with mitochondria since its presence is not closely paralleled with the distribution pattern of cytochrome c oxidase used as enzyme marker. The labeling is mainly localised among the cytosol and the fraction P1 having plenty of components that can potentially bind MET in a non-specific fashion. Moreover, radioactivity in the latter augmented with time while it decreased in the former.

Interestingly, we recorded quantitatively similar results with the oocytes submitted to 10 mM radioactive MET, except that the rate of drug incorporation was faster than with the lowest dose during the first 60-min incubation (Fig. 3). This difference, supported by statistical analysis ($P < 0.01$), matches well the more rapid effect of MET on the mitochondrial metabolism at this higher concentration.

Table 1
Respective distribution of [^{14}C]-labeled MET and cytochrome c oxidase activity in oocytes and in subcellular fractions isolated by differential centrifugation

	Incorporation of MET[a]		Marker enzyme activity (U/g wet weight)[b]
	30 min	180 min	
Whole oocytes (=crude homogenate)	0.067	0.855	*1.82* (**6.5** U/g protein)
Subcellular fractions			
Fraction P1	9.8	28.5	*0.107* (**4.45** U/g protein)
Fraction P2	*<0.1*	3	*1.68* (**324** U/g protein)
Fraction P3	ND	0.4	ND
Cytosol (Final supernatant)	90.3	68	*0.06* (**1.9** U/g protein)
Total = 100%			
Recovery = 101.5%			

The cells were incubated with radioactive MET and oocyte fractions were obtained as described in Section 2.4. The values presented are the average means ($N = 4$); the errors bars are not mentioned for clarity of the table. ND, not determined. Values in bold are the specific activities from which the relative enrichment is calculated, whereas the recovery is evaluated from the sum of total activities (values in italics).

[a] Expressed in % of total externally added MET for whole oocytes, or in % of MET within the lysate for subcellular fractions.

[b] One unit of marker enzyme (cytochrome c oxidase) is defined in the Section 2.2.

3.3. Consequences of pretreatment with MET, either as a free form or encapsulated into liposomes, on complex I activity from isolated mitochondria or mitochondria in living cells

Although the percentage of MET bound to mitochondria is negligible, there is a striking parallelism between a cytosolic location of the drug and its pronounced inactivating effect on the complex I activity. To mimic this putative presence of MET inside the cells, two approaches were envisaged. Briefly, MET was added *in vitro* to a mitochondrial suspension or loaded into *Xenopus* oocytes by injection, this last technique being selected instead of the permeabilisation made for the cultured hepatocytes [19]. Unexpectedly, neither MET present together with mitochondria nor the drug introduced in the oocyte cytosol

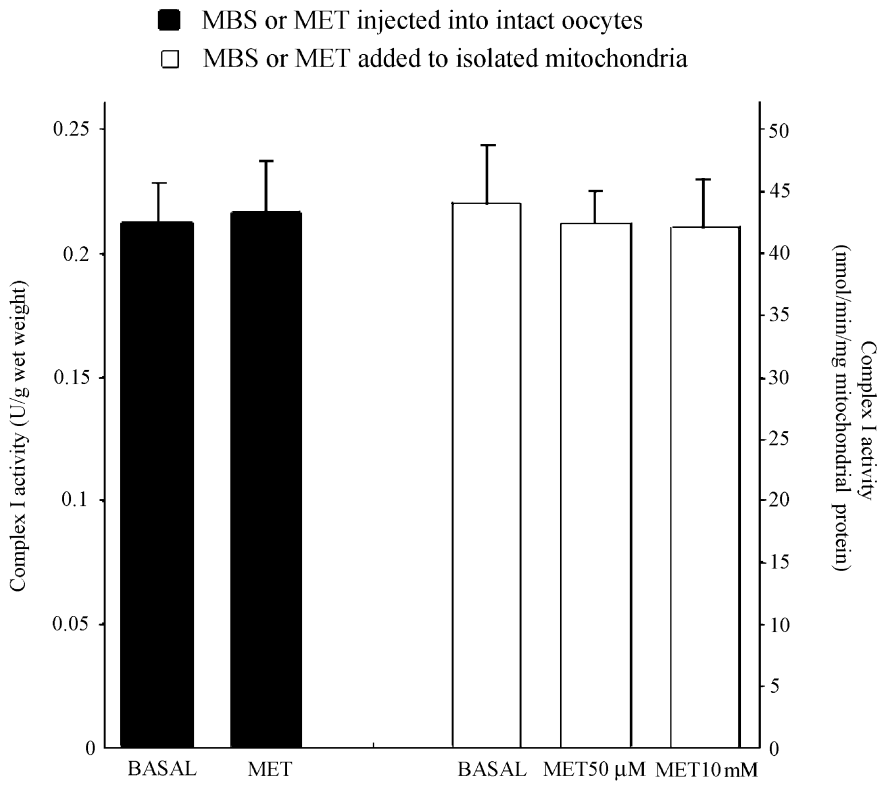

Fig. 4. Effect of MET on complex I activity following its microinjection inside the oocyte or its addition to isolated mitochondria. In the first case, oocytes were injected with MET (at a final concentration evaluated to 50 µM into each egg), or with an equivalent amount of MBS. Their incubation was then prolonged at room temperature for 3 hr before measuring the activity of complex I in the yolk-free fraction as previously. Results are the means ± SEM ($N = 4$). In the second case, the experiments performed at 25° were started after addition of oocyte mitochondria to a buffer containing or not MET at both concentrations (50 µM and 10 mM) during 20 min. The control specific activity of complex I, i.e. without preincubation stage (time 0 min) was 45.6 ± 4.2 nmol/min/mg mitochondrial protein. Results are the means ± SEM ($N = 5$).

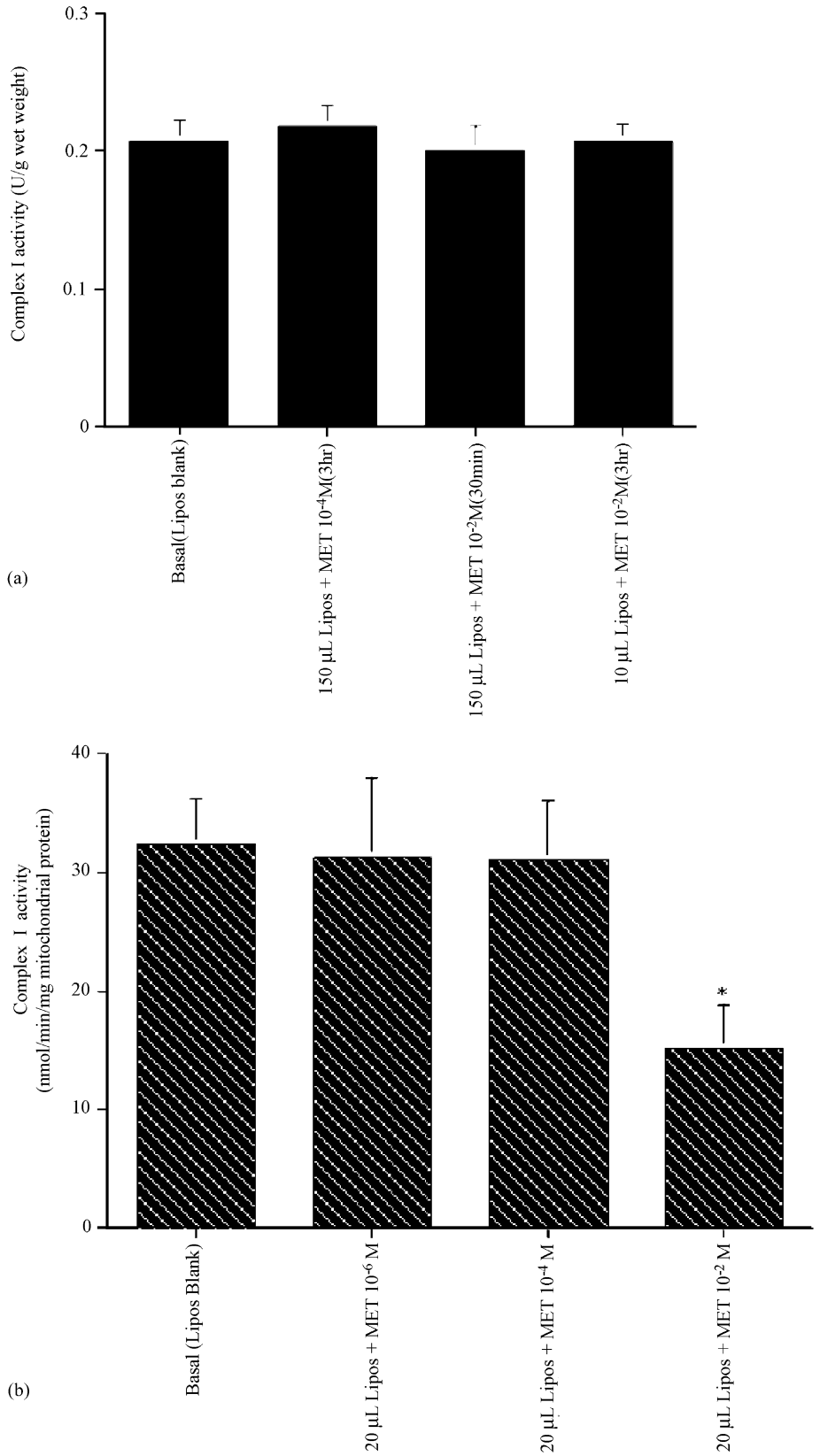

Fig. 5. Effect of liposome-encapsulated MET on complex I activity in intact oocytes or isolated mitochondria. Different amounts of liposomes (Lipos) enclosing MET were added either to the incubation bath of oocytes at room temperature for the times shown (a) or to isolated mitochondria incubating for 20 min at 25° (b). The intact oocytes underwent a prior treatment similar to that of Fig. 2, and the activity of complex I was assayed in both conditions as previously. Results are the means ± SEM ($N = 3$–5). (*) indicates effect of Lipos + 10^{-2} M MET significant with $P < 0.005$. Liposomes without the encapsulated drug were used as blank.

caused an inhibition of the complex I activity (Fig. 4). Isolated organelles were incubated in conditions compatible with the preservation of mitochondrial structural integrity since no significant loss of basal enzyme activity was recorded after a 20-min preincubation step at 25° (Fig. 4). Longer time intervals at higher temperatures (e.g. 37°) would logically not be suitable. With the help of microinjection, we placed in the cytosol of individual oocytes an amount of MET corresponding to the estimated level present inside the eggs when incubated in 50 μM or 10 mM MET. The lack of response was not unexpected since MET in similar conditions yielded a weaker and delayed effect on INS functions [8,11]. These findings obviously suggest that the drug trapped intracellularly does not act as a free form. Therefore, we thought of encapsulating MET into liposomes with the aim of facilitating its trafficking towards mitochondria. These vesicles, either containing MET at various dosages or deprived of this drug (=blank) were tested on intact oocytes or on isolated mitochondria. The liposomes applied in the cell medium were without effect regardless of their inner content (Fig. 5a). In contrast, liposomes enclosing 10^{-2} M MET caused a drastic inhibition of the enzyme ($P < 0.005$) when incubated with a mitochondrial fraction (Fig. 5b). The magnitude of this effect, comparable to that observed with externally added free MET, was not modified whatever the amount of vesicular MET added in relation to the quantity of mitochondrial proteins (data not shown).

3.4. Search for hypothetical mechanisms consistent with the oocyte membrane-mediated action of MET on mitochondrial complex I

Data referring to the effect of liposome encapsulated MET vs. free drug on mitochondria provide indirect support for an endocytosis-like event for its internalisation.

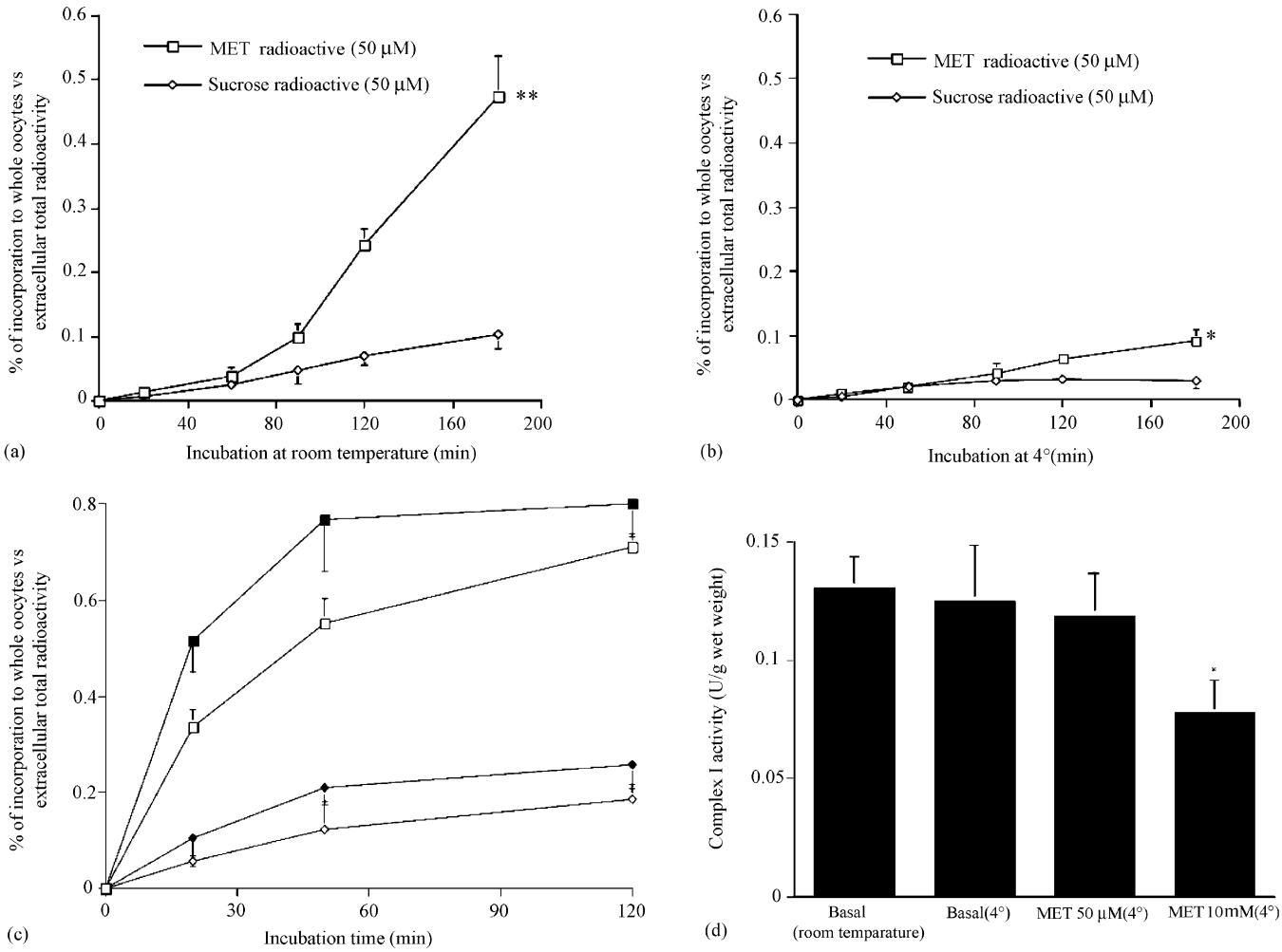

Fig. 6. Comparative study about the uptake of [^{14}C]MET and [^{14}C]sucrose by *Xenopus* oocytes. The oocytes were incubated for 3 hr at room temperature (panel A) or 4° (panel B) in the presence of 50 μM radioactive MET or sucrose. The panel C depicts the respective uptake of 10 mM drug (□)(■) or sugar (◇)(◆) in similar circumstances. After washing the cells, they were lysed and the percentage of incorporation to whole oocytes for both compounds was then calculated as described in Section 2. Values are means ± SEM of four experiments. (*) denotes significance at $P < 0.05$, (**) denotes significance at $P < 0.001$. The impact of low temperatures (4°) on complex I activity from untreated oocytes or treated with 50 μM and 10 mM MET for respectively 3 hr and 30 min is shown in (d). Here the effect of 10 mM MET significant with $P < 0.05$ is denoted by (*).

To gain information on this proposal, we compared the uptake of MET to that of sucrose in *Xenopus* oocytes incubated at ambient temperature or at 4°. The transport of sucrose, which raises almost linearly with an amplitude independent of the concentration used, i.e. 50 μM (Fig. 6a) and 10 mM (Fig. 6c), is in accordance with a classical fluid-phase endocytosis albeit the final amount of sugar accumulated by these cells remains low. No alteration in complex I activity was recorded with either dose (data not shown). Time course for MET uptake is, generally considered, not very different from the sucrose one (Fig. 6a). However, the percentage of MET incorporation is significantly ($P < 0.001$) more elevated than with sucrose at the end of exposure with the physiological dose. On the other hand, reducing the medium temperature to 4° dramatically diminished cell-associated radioactivity (Fig. 6b). The phenomenon was observed with both compounds but a significant ($P < 0.05$) difference remained between MET and sucrose after a 3-hr incubation. This decreased capacity of MET transport was accompanied by a concomitant loss of its action on complex I activity which returned to its baseline (Fig. 6d). Surprisingly, the uptake of sucrose or MET at high concentrations (10 mM) by oocytes in cold conditions was not attenuated (Fig. 6c) and, moreover, the

Table 2
Impact of ADMA on the cellular uptake of radioactive MET when present at 50 μM or 10 mM

Incubation time of oocytes	50 μM [^{14}C]MET (%)	+ADMA
30 min	0.052 ± 8.3 10^{-3}%	500 nM: 0.062 ± 0.015% 50 μM: 0.031 ± 0.016%[a] 2.5 mM: 9.4 10^{-3} ± 8.8 10^{-3}%[b]
3 hr	0.536 ± 0.026%	500 nM: 0.525 ± 0.043% 50 μM: 0.227 ± 0.045%[a] 2.5 mM: 0.109 ± 0.013%[b]
	10 mM [^{14}C]MET	+ 2.5 mM ADMA
30 min	0.26 ± 0.0408%	0.245 ± 0.021%
2 hr	0.637 ± 0.039%	0.562 ± 0.043%

Fully-grown oocytes were exposed at room temperature to radioactive MET alone or together with ADMA at the indicated concentrations. At relevant time intervals, the incubation was stopped by washing the oocytes in cold MBS. The cells were lysed and the uptake of MET was then estimated from their incorporated radioactivity in relation to total counts in the extracellular medium. The results are presented as the means ± SEM ($N = 4$).

[a] ADMA effect (at 50 μM) on MET transport was significant with $P < 0.01$.

[b] ADMA effect (at 2.5 mM) on MET transport was significant with $P < 0.005$.

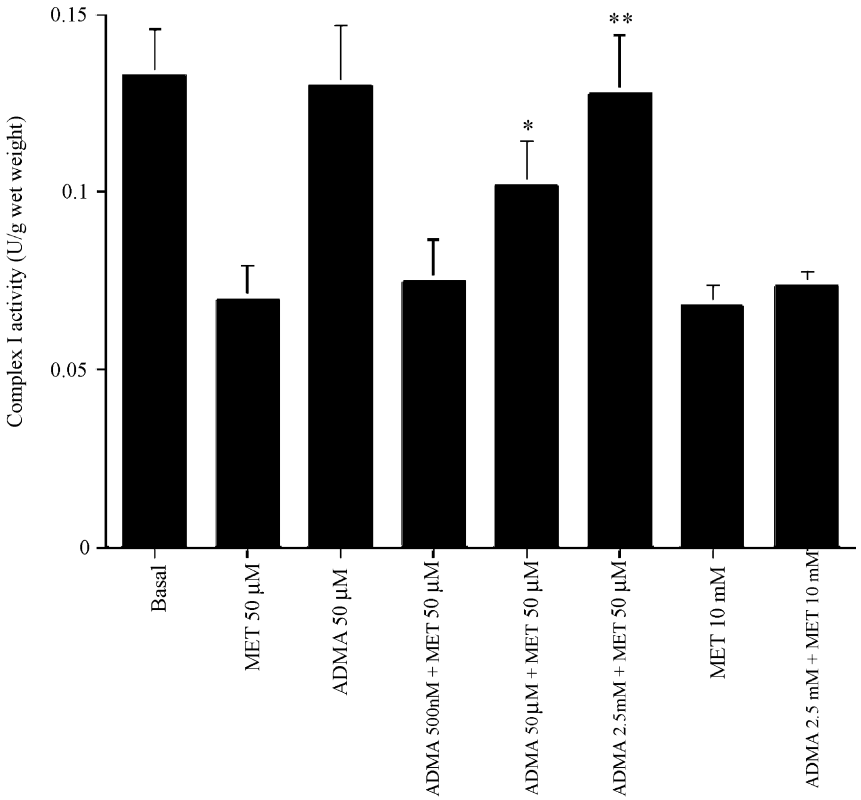

Fig. 7. Interference of ADMA with the effect of MET on complex I in *Xenopus* oocytes. The oocytes were incubated for 180 min in MBS alone, in MBS containing either both MET and ADMA separately at the same dose (50 μM) or MET with increasing concentrations of ADMA, and in MBS with 10 mM MET in the absence or presence of 2.5 mM ADMA. After these treatments, the oocytes were homogenised and the activity of complex I was assayed as described in Section 2. The data were expressed as the means ± SEM ($N = 3$–4). Competitive effects of 50 μM and 2.5 mM ADMA towards 50 μM MET action significant with (*)$P < 0.01$ and (**)$P < 0.005$, respectively.

inhibitory effect of MET on complex I was maintained even though it was significantly lower than in oocytes incubated at room temperature ($P < 0.05$ vs. $P < 0.01$).

The hydrophilicity and ionisation of MET suggest that its crossing of membranes is limited by a permeability barrier. The structural similarity between MET and ADMA, a methylated derivative of arginine which binds to the cationic amino acid transporters [25], led us to next investigate whether these molecules compete for the recognition of some specialised sites at the cell surface. Very importantly, an ADMA concentration either in equilibrium (50 μM) or in excess (2.5 mM) to the MET dose markedly prevents the uptake of [^{14}C]MET by *Xenopus* oocytes incubated for up to 3 hr (Table 2). This blockade in the process of drug internalisation likewise provoked a progressive loss of its inhibitory action on complex I activity (Fig. 7). It should be also noted that ADMA alone did not succeed in modulating the basal activity of this enzyme. On the other hand, both the uptake of MET at a supra-therapeutical dose (10 mM) and its corresponding effect on cell respiration were not counteracted by ADMA present at 2.5 mM (Table 2 and Fig. 7). This last result is probably indicative of abrupt change in the drug's pharmacological behaviour.

4. Discussion

While MET is the only biguanide in clinical use today, the accurate mechanisms of its cellular uptake and molecular action are still not confirmed since a variety of factors render the situation elusive. By taking advantage of the *Xenopus* oocyte model, we and others [6–8,12] demonstrated that MET, in a range of doses similar to those found in plasma of type 2 diabetic patients undergoing MET therapy [26], potentiated the INS-induced glycogenesis through an increase of insulin receptor tyrosine kinase activity. Furthermore, this enhancing effect of MET was fully achievable when MET was located inside or near the membrane [12]. Recently, MET was reported to actively inhibit cell respiration in hepatocytes, yet the drug was without effect on permeabilised cells or isolated mitochondria [19]. This data also argues in favour of a plasma membrane-mediated effect but the signalling leading to the cytosolic organelle is currently unknown. Given the possibility that a few molecules of MET getting into the oocyte cytoplasm could affect the energy metabolism and this, independently of the presence of INS, we presently tried to better clarify some key steps of this pathway putatively originating at the cell surface.

The preliminary objective was to determine how MET therapeutically modulated the mitochondrial function in intact *X. laevis* oocytes. Alterations of the respiratory chain at a rotenone-sensitive enzyme level without change of cytochrome c oxidase activity are consistent with recent data on hepatocytes showing that the inhibitory effect of MET is purely located on the complex I, not on the downstream oxidative phosphorylation machinery [19]. Additionally, the requirement of a long-term exposure of oocytes for attainment of optimal MET response (inhibition ranging from 40 to 50%) was also found by authors looking at the oxidation of glutamate and malate, the specific substrates of complex I, in hepatoma cells [27]. In fact, they observed a decrease in mitochondrial metabolism by 26 and 37% after a treatment with 100 μM MET lasting 24 or 60 hr, respectively. By next testing a concentration exceeding the therapeutic level (10 mM), the degree of enzyme activity inhibition was not greater than with 50 μM MET, but the effect settled in more rapidly. It is striking that the sustained action of MET on complex I is fairly well correlated with its gradual appearance in the cytoplasm of oocytes, although it took longer to be observed with 50 μM than with 10 mM (see Fig. 2). At this point, it is worthwhile to stress out that such high MET doses generated the progressive loss of both its stimulating action on glycogen synthase [8] and its mimetic effect on tyrosine kinase activity [28]. Therefore, the particular effect of MET on oocyte complex I in addition to the antagonistic events relating to the hormonal receptor would be partly explained by changes in cell membrane dynamics. As previously described [29], the preferential binding of MET to proteins at therapeutic dose vs. its linkage to phospholipids in the millimolar range may be responsible for these different responses. Accordingly, MET may require different lapse of time to reach an intracellular site at concentrations sufficient but yet below the threshold of the non-toxic inhibition of respiration. To check whether a complete internalisation of MET was needed for its action on mitochondrial respiration, the biguanide was microinjected in the oocyte interior or added to isolated mitochondria. In these conditions inhibition of complex I by MET was lost. This result is inconsistent with a recent report showing an inhibitory effect of MET in isolated organelles or submitochondrial particles from liver and heart [27]. However, the experimental conditions used (for instance, mitochondria incubated at 8°) as well as some contradictory comments evoked by authors (increase of double membrane permeability) severely limit the strength of their conclusions. Since cells (hepatocyte and oocyte) as functional entities with intact structure are requisite to observe an effect, we suggest that the MET-induced complex I inhibition is not the consequence of a direct interaction with the electron transport chain. The very weak or even irrelevant binding of MET to mitochondria, measured after subcellular fractionation of either oocytes incubated with MET (this study) or hepatocytes from rats orally treated with the radioactive drug [30], fully support this notion. By also taking into account the temperature-dependent nature of this phenomenon in hepatocytes, oxygen consumption decreasing by 50, 20 and 0% at 37, 25 and 15°, respectively, one may infer that the negative effect of MET on respiration is related to the physicochemical

state of the plasma membrane. The disappearance of the response in oocytes incubated at 4° could be equally connected with a modification of the physical properties of plasma membrane. It should be borne in mind that, as the *Xenopus* frog is a poikilothermic animal (its oocytes are kept best at lower temperatures than those used for hepatocytes), most experiments of this work were carried out at room temperature ($19 \pm 2°$), i.e. in situations for which the processes such as protein trafficking and kinetics may be altered. The membrane fluidity, which presumably plays an important role in the mode of MET action [31,32], should be differently affected in the oocyte and hepatocyte. Alternatively, this could explain the major differences between these cell types.

The binding of MET *in vivo* to a variable extent with many components (lipidic structures, proteins...), due to its single net positive charge at physiological pH, is a well-established fact [33]. Obviously, the ionisation of MET once freely internalised seems harmful for its effect on cell respiration as above described. Therefore, Leverve and his colleagues thought of encapsulating MET into liposomes and testing them on isolated rat liver mitochondria. In pilot experiments, MET engulfed in such vesicles at a final concentration of 10^{-4} or 10^{-2} M almost instantaneously blocked cell respiration while liposomes deprived of the molecule were without effect.[1] These artificial constructs were next tried out on oocyte mitochondria and, very interestingly, the inhibitory effect of MET on complex I activity was totally recovered, at least with the highest dosage. In contrast, no effect was found with the liposomes applied extracellularly. This difference must be attributed to the fact that lipid composition of liposomes was chosen for mimicking the mitochondrial membrane. Prior to its internalisation, MET thus appears to undergo a necessary process set up at the cell surface level, and enabling it to be routed efficiently towards its cellular target, namely the mitochondria. In HepG2 cells, Moats and Ramirez [34] have identified a similar plasma membrane-mediated uptake for estrogen which was translocated to the mitochondria where it inhibited the complex V of the respiratory chain. Furthermore, by making use of fluorescent markers, others described the fusion *in vitro* of liposomes with mitochondrial inner membranes [35] or their interaction with normal mitochondria when intravenously injected into mice [36]. Numerous reports highlighted the endocytotic capacity of *Xenopus* oocytes, involving various specific proteins in the regulation of vesicular traffic mediated by membrane receptors [37,38] or not [39]. These very promising data imply that the liposomes combined with those from experiments (transport and complex I activity) conducted at low temperature are strong elements clearly alluding to an endocytosis. Unfortunately, they don't provide conclusive evidence that MET builds up into the *Xenopus* oocyte by this way. Since a diffusion of MET through the membrane barrier can be certainly excluded on grounds of the charged nature of this drug and its pK_a value close to 12, an alternative explanation brought up by authors for its transmembrane shuttle is the operation of a saturable transporter. MET has many structural similarities with the amino acid arginine and its derivatives. In 3T3 cells, MET was actually found to be transported *via* a sodium dependent secondary active transport system, probably the carrier y^+, which is shared by cationic amino acids like arginine and lysine [40]. This interesting conclusion is nonetheless not entirely satisfactorily because of some controversial results. Firstly, conditions for measurement of MET penetration were not strictly the same as those for arginine uptake. Moreover, the value reached with the biguanide (up to 2000 nmol/mg protein) appears abnormally high considering the intrinsic capacity of the carrier. Finally, this result has never been corroborated afterwards on typical cell systems possessing significant amino acid transport activities. Hence, although their finding is partly favourable to the existence of a transporter for MET, no proof to that effect is nowadays available. The *Xenopus* oocyte, also equipped with endogenous transporter y^+ [41], is able to take up arginine at a rate which represents an accumulation level of approximately 20% into the cytosol [42]. This value is far above that encountered with MET since the maximum percentage of incorporation to whole cells is inferior to 1% over a period of several hours. In this context, data from distribution study with radioactive drug (Table 1) should be interpreted with caution. Under no circumstances does the predominant association of MET with the cytosol after only 30-min exposure mean that this drug uses the y^+ carrier for its actual transfer from the external medium to the cell interior. Rather, this is a misleading result primarily due to the methodology which is inappropriate for correctly dissecting such a process. Indeed, even if MET is thought to be firmly anchored within the lipid bilayer [43], recent washout studies in oocytes revealed that its ionic interactions with cell surfaces are weak [12]. These non-covalent binding properties may not be strong enough to fully withstand the gross steps (homogenisation and multiple spins) of fractionation protocol and a potential release of MET between subcellular fractions is consequently unavoidable. The extremely low amounts of MET incorporated to whole oocytes after 60 min also speak against any carrier-mediated action. Also in hepatocytes, there is practically no more penetration of MET *in vitro* than in oocytes [33]. *In vivo*, the situation appears somewhat different since MET will substantially bind with various substances, as outlined elsewhere, and thus lose its cationic charge before entering the liver. MET orally administered could be therefore internalised by hepatic cells in a bound form, thereby explaining its apparent accumulation in the cytosol vs. plasma levels [30]. This statement doesn't contradict the earlier proposed hypothesis of MET endocytosed by oocytes.

[1] Leverve X, unpublished data.

The prior observation that monomethylbiguanide was inactive on INS action [12], in spite of having the same charge as MET, emphasises that the structure-function relationship is critical for its biological activity. Structurally, MET exhibits a salient analogy with some compounds such as ADMA, known to interfere with arginine transport [44]. Both molecules bear the guanidine functional headgroup that is doubly methylated. This close resemblance further opens the possibility of addressing a plausible competition event. ADMA curtails dose-dependently the internalisation of MET and its subsequent action upon oocyte complex I activity. This result shows that ADMA succeeds in preventing the uptake mechanism either by competing with MET for relevant sites or by altering, for example due to steric constraints, other binding sites of MET to specific plasma membrane domains but unrelated to the transport function. This characteristic of MET may account for the main differences observed with sucrose (Fig. 6) and can be also responsible for the lag period preceding the physiological response. By virtue of its INS-like action, MET might preferably bind to the hormonal receptor behaving as a vehicle before the complex is internalised, although with a lesser effectiveness than in the presence of INS, and then routed towards mitochondria. It is worth the effort now of ascertaining the steps governing the biochemical machinery in the oocyte interior.

Lastly, it is useful to remind that the mitochondrion constitutes the primary source of reactive oxygen species which can also function acutely as regulators of aerobic metabolism, in addition to their deleterious effects [45]. In principle, this novel homeostatic control may be perturbed in the setting of certain disease states. More particularly, an overproduction of mitochondrial oxidants induced by hyperglycaemia was recently described to contribute to the main vascular abnormalities linked to diabetes, while a normalisation of the so produced superoxide level by metabolic pathways-specific inhibitors appeared to provide benefits against the progression of this illness [46]. An alteration in the respiratory chain may therefore be one potential strategy to reverse these damages. Since MET exerts remarkable vasculoprotective effects under pathological conditions [47,48], the inhibitory effect on oocyte complex I disclosed here fits perfectly into its therapeutical action from some of these diabetic complications.

Due to their large size and thus ease of handling, isolated *X. laevis* oocytes are elegant experimental tools for unravelling membrane phenomena. This work gives full of promise advances which considerably back up previous data obtained on a cell type as different as the hepatocyte. We clearly show that MET affects cell respiration in intact oocytes by selectively inhibiting the mitochondrial complex I. This effect relies on an indirect mechanism starting from the cell surface and involving the specific binding of MET to relevant domains of effector systems. On the other hand, the notion of drug partitioning within the plasma membrane itself could be also a crucial factor since at pharmacological dose (i.e. 10 mM) MET still exerts its action on respiration whereas its beneficial action on INS signalling is lost. Altogether, these data provide strong evidence that membrane changes largely represent a common parameter explaining MET intracellular effects complemented by those related to INS.

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