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Cellular and Molecular Mechanisms Involved in Insulin's Potentiation of Glycogen Synthase Activity by Metformin

Dominique Detaille,† Nicolas Wiernsperger‡ and Pierre Devos**

*Laboratory of Comparative Biochemistry and Physiology, Facultés Universitaires Notre-Dame de la Paix, FUNDP, B-5000 Namur, Belgium; and ‡Lyonnaise Industrielle Pharmaceutique, LIPHA s.a., F-69379 Lyon, France

ABSTRACT. By taking advantage of the *Xenopus* oocyte model, we recently confirmed the *in vitro* enhancing effect of metformin (MET) on glycogen synthase (GS) activity when induced by insulin (INS). We now investigated some mechanistic aspects of its modulatory role upon the hormonal regulation of this rate-limiting enzyme. The action of 20 μM MET (~3.3 μg/mL) was measurable at early steps in the intracellular metabolic pathway: the amount of adenosine 3′,5′-cyclic monophosphate (cAMP) was markedly decreased in the presence of the biguanide plus 50 nM INS (to about 60% of control vs 25% with INS alone). The injection of tyrphostin B46, a potent inhibitor of insulin receptor (IR)-associated tyrosine kinase activity, led to a drastic reduction in MET-stimulated GS activity in the presence of INS. MET failed to increase the activity of type 2 protein phosphatases whether INS was present or not. However, a specific inhibitor of type 1 phosphatases, when microinjected, blocked both the hormonal effect on GS and its potentiation by MET. The salient feature of this study was that there was almost no accumulation of radiolabeled MET in oocytes: less than 0.1% was found in the cytosol of cells which had been exposed to MET at a therapeutic dose (10 μM) for up to 16 hr. Moreover, a lack of detectable intracellular MET after a 60-min incubation nevertheless correlated with its sustained action on INS-regulated GS activity. From these results, it could be inferred that the major site of MET action may reside within some membrane components of a signaling complex most likely linked to the IR, but in any case located upstream of the branching of reactions which tightly control GS activity. BIOCHEM PHARMACOL **58**;9: 1475–1486, 1999. © 1999 Elsevier Science Inc.

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It is now well documented that GS§ activity from various responsive cell types is under the tight control of phosphorylation–dephosphorylation events which are rapidly triggered after hormonal induction (recently reviewed in Ref. [1]). Studies performed on muscle cells and adipose tissue established that INS promotes the dephosphorylation and activation of GS by inhibiting glycogen synthase kinase-3 via the protein kinase B pathway [2]. This step may be assisted by a concurrent stimulation of the relevant phosphatase lying upstream of GS [3]. Likewise, data from our laboratory indicated that the activation of oocyte GS in response to INS probably proceeded through similar mechanisms which should also operate in concert, namely a stimulation of some protein phophatases coupled with an inhibition of certain protein kinases [4]. Furthermore, the specific tyrosine kinase activity of the IR is known to play a pivotal role in the control of the intracellular cascade leading to glycogen synthesis [5]. Missing links remain, however, between tyrosine phosphorylation events at the level of transmembrane receptor and downstream signaling elements implicated in this metabolic route.

Insulin resistance in type 2 diabetes, albeit still poorly understood in biochemical terms, is probably connected with or could be the result of reduced signal transduction of INS. The biguanide drug MET (*N*,*N*-dimethylbiguanide; commercial name: glucophage), widely used in the treatment of this disease, has been shown in many studies to improve INS action [6–8]. With the help of the *Xenopus laevis* oocyte model that has proved valuable over the past few years, we recently highlighted the biological relevance of GS activity in the potentiating action of MET. Apart from its well-known effect on INS-stimulated glucose uptake, MET either added to the incubation medium or, when directly microinjected into oocytes, was found to increase the activity of GS in the presence of INS [9]. This

† Corresponding author: Dr. Dominique Detaille, Laboratory of Comparative Biochemistry and Physiology, Facultés Universitaires Notre-Dame de la Paix, rue de Bruxelles, 61, B-5000 Namur, Belgium. Tel. (32) (81) 72 42 87; FAX (32) (81) 72 44 20; E-mail: dominique.detaille@fundp.ac.be

§ *Abbreviations:* MET, metformin; GS, glycogen synthase; INS, insulin; cAMP, adenosine 3′,5′-cyclic monophosphate; IR, insulin receptor; PP-1, type 1 protein phosphatase; PP-2A, type 2A protein phosphatase; MBS, modified Barth's solution; PMC, plasma membrane-cortex complex; MMB, momomethylbiguanide; and OA, okadaic acid.

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interesting finding clearly suggested that the biguanide, although inactive in the absence of the hormone, modulated some upstream steps in the signaling pathways set up by INS. Information on a possible mode of MET action upon this complex network of pathways is relatively scarce and, moreover, the high number of potential candidates which could be affected by the biguanide complicate the analysis. For a better understanding of the potential effects of MET, it was therefore critical to know whether its specific action took place at the cell surface near the receptor or at post-receptor sites. Some *in vivo* and *in vitro* studies demonstrating the ability of the drug to increase tyrosine kinase activity initiated by INS, without affecting hormone binding, pointed towards MET action at a post-binding location [10–12]. This effect of MET was also found in the oocyte [13, 14]. The biguanide, characterized as a relatively poor activator of the receptor tyrosine kinase by itself, can also work independently of INS to stimulate phospholipase C and inositol 1,4,5-triphosphate production [14]. Finally, MET was found to modulate the activity of specific protein tyrosine phosphatases towards IR in skeletal muscle fractions from insulin-resistant animal models [15].

Taking into account these admitted potentiating and/or mimetic effects of MET, we decided to explore in the current study the importance of its membrane versus cytosolic action(s), more precisely its mechanism on GS regulation which, when defective, is thought to largely underlie the resistance to the biological action of INS. We also attempted to delineate the signaling chain whereby physiological doses of MET enhanced glycogen synthesis by identifying some target steps which might be responsible for significant metabolic changes.

MATERIALS AND METHODS

Materials

[^{14}C]MET (1891.1 MBq/mmol) was kindly provided by Prof. J. Rapin (University of Dijon, France). Unlabeled MET hydrochloride (dimethylbiguanide) and MMB were gifts from LIPHA Labs. Tyrphostin B46, inhibitor-2 (>500,000 U/mg), and a water-soluble form of OA were supplied by Calbiochem-Novabiochem. Uridine diphospho-D-[U-^{14}C] glucose was from Amersham Life Science and liquid scintillant Aqualuma, from Lumac LSC. Porcine INS, collagenase type IA (436 U/mg), and all other reagent grade chemicals were purchased from the Sigma Chemical Co., except for EDTA, 2-mercaptoethanol, and DMSO, which were obtained from Merck. MBS buffer consisted 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 $CaCl_2$.

Oocyte Incubations with Radioactive Metformin

After isolation and selection of *Xenopus oocytes* according to the method previously described [9], several groups of 2 to 10 defolliculated mature eggs (stage V and VI) were incubated at room temperature in 250 μL of MBS containing [^{14}C]-labeled MET at 10 μM (final concentration). At relevant time intervals, the uptake of this substance was stopped by gently washing the oocytes three times in 10 mL of ice-cold fresh MBS. They were then pooled and resuspended in an isolation buffer (10 mM NaCl, 10 mM HEPES, pH 7.9). Two procedures were adapted in order to analyze the accumulation of radioactive MET and its distribution throughout the cell. The first method was a manual dissection of each oocyte in the manner described by Sadler and Maller [16]. Briefly, after a small slit in the animal hemisphere, the oocytes were torn open with the tips of forceps to extract the bulk of the cellular content. The complex formed by the plasma membrane and the cortex, termed PMC, was directly counted. Nuclear material and cytoplasm were collected on filter paper and transferred to a scintillation vial for counting. In a second protocol, we applied a proven technique of density gradient ultracentrifugation for separating out a membrane fraction [17]. About 80 denuded oocytes were brought to 5 mL of a cold homogenization buffer consisting of 20 mM HEPES (pH 7.2), 100 mM K^+ glutamate, 10 mM EGTA, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 μg/mL soybean trypsin inhibitor. A Dounce homogenizer was used to grind the cells with 10–15 strokes of the loose-fitting pestle (pestle B). The homogenate was appropriately diluted, layered onto a 0.5 volume sucrose cushion (1.5 M sucrose in the same buffer), and centrifuged at 4° in an SW 27.1 rotor at 16,000 *g* for 15 min. Fractions were then collected from the top in three distinct bands (the interface being the membrane fraction) plus the residual pellet containing yolk proteins, pigment granules and, probably, the subcortical matrix. The interface was removed, rinsed, and thereafter centrifuged in a microfuge at 16,000 *g* for 10 min to sediment total plasma membranes. The percentage of MET bound to this membrane-rich extract was then measured after resuspension in the above buffer. Radioactivity was also checked in the other fractions. The main results are expressed in relation to the total radioactivity determined from aliquots of the initial lysate; this latter value corresponds to MET associated with whole oocytes irrespective of its cellular localization. In addition, we defined the plasma membrane fraction biochemically by assaying the activity of a specific marker enzyme, alkaline phosphodiesterase, as previously described [18].

Microinjection of Tyrphostin B46

The contribution of tyrosine kinase activity to the enhancing effect of MET on hormonal action was investigated by measuring GS activity after treatment with tyrphostin B46, a potent inhibitor of this IR-associated intrinsic enzyme activity. A diluted solution of tyrphostin B46 (prepared from a 15-mM stock solution in DMSO) was preinjected

into each mature oocyte in order to reach an intracellular final concentration ranging from 50 to 500 μM. We choose this dose range because others have found that a 300-μM concentration of another tyrphostin, with a selectivity similar to that of tyrphostin B46, blocked the hormone-induced oocyte maturation through inhibition of β-subunit phosphorylation [19]. Control oocytes were injected with an equivalent quantity of tyrphostin A1 used as inactive inhibitor. About 20 min after injection of tyrphostin B46 (or of its inactive form), the cells were transferred into 2 mL MBS containing INS alone or INS combined with MET. At the end of a 60-min incubation, the oocytes were sonicated in a cold buffer containing 50 mM Tris–HCl (pH 7.8), 5 mM EDTA, and 250 mM sucrose. After centrifugation at 16,000 *g* for 15 min, GS activity was assayed in the supernatant, either in the presence of 7 mM glucose-6-phosphate (total form) or in its absence (active form, also called synthase *a*) as reported elsewhere [20].

Use of a Biguanide Derivative Less Active than Metformin

The ability of MMB to stimulate INS-induced oocyte GS activity was examined under two approaches similar to those previously used for MET. The first method entailed the addition of the drug (20 μM), alone or with the hormone, to the external glucose-free medium. In the second method, MMB was injected into the oocyte cytoplasm at a final concentration of 20 nM, and the oocytes were then incubated for 60 min in the presence of INS. At the end of the treatment, they were homogenized and the activity of synthase *a* was assayed on low-speed extracts.

Measurement of cAMP

A commercial cAMP radioimmunoassay kit developed by Dupont-New England Nuclear was used to follow this second messenger after MET addition to *Xenopus* oocytes. Briefly, once the cells were incubated in MBS containing the required additions, they were treated or quickly frozen in liquid nitrogen and stored at −20° prior to perchloric acid extraction. After centrifugation (3500 *g* for 5 min), the supernatant was buffered with K_2CO_3 before undergoing a second spin (4800 *g* for 10 min). The final extract, correctly diluted with assay buffer, was incubated overnight at 4° with the anti-cAMP antibody in the presence of [^{125}I]-iodinated ligand. Separation of bound and free antigen was achieved by the addition of a precipitation enhancer followed by centrifugation. The supernatant was discarded and the radioactivity of the pellet was quantified by liquid scintillation. Total cAMP cellular content for all the samples was determined by interpolation from a standard curve which was made for each experiment. It should be noted that this assay is accurate over a wide range of values and has a high degree of specificity. The extraction efficiency (or recovery) was determined by adding *ca*. 4000 cpm of [^{3}H]cAMP marker to each oocyte extract.

Measurement of Protein Phosphatase Activity

To examine whether serine/threonine phosphatases are implicated in the mechanism of MET action, we used the non-radioactive assay system from Promega, well designed for specific detection of these enzyme activities in crude tissue extracts. Nevertheless, it is useful to mention here that the kit allows one to measure the activity of PP-2 but not PP-1, since the supplied phosphopeptide is a poor substrate for these type 1 enzymes. The first step of this assay was the sample preparation. Once the oocytes were treated with MET alone (20 μM), INS alone at two concentrations (80 nM and 10 μM), or with INS (80 nM) combined with MET, the cells were broken up by sonication in a cold storage buffer containing 25 mM Tris–HCl (pH 7.5), 2 mM EDTA, 0.01% (w/v) 2-mercaptoethanol, 0.1 mM PMSF, 1 mM benzamidine, and 27 milli-trypsin inhibitory units/mL aprotinin. After centrifugation at 100,000 *g* for 45 min, the clear supernatant was loaded onto a Sephadex G-25 spin column (equilibrated with the same storage buffer) to remove free phosphate prior to the phosphatase activity determination. Assay conditions and procedures were followed according to the recommendations of the manufacturer. In brief, 5 μL of enzyme sample was incubated with 100 μM phosphopeptide substrate for 30 min at 30° in 50 μL (final volume) of reaction buffer which consisted of 25 mM imidazole (pH 7.2), 0.2 mM EGTA, 0.02% (w/v) 2-mercaptoethanol, and 0.1 mg/mL BSA. The reaction was terminated by adding an equal volume of the molybdate dye/additive mixture to all wells, including controls and standards. The use of a 10-μL oocyte extract for a 50-μL reaction allowed an accurate response as well (data not shown). This system determines the amount of free phosphate generated from the substrate by measuring the absorbance of a molybdate/malachite green/phosphate complex at 660 nm with a plate reader. The linear range for the detection of phosphate released is 50 to 4000 pmol of phosphate. For calculation of specific activities, we measured the protein concentration in the phosphate-deprived samples. We evaluated only the activity of PP-2A (removing phosphate from the phosphopeptide in a time-dependent manner), since there exist few-if any-published works demonstrating some implication of the other forms (PP-2B and PP-2C) in INS metabolic signaling.

The experimental set was completed by additionally measuring protein phosphatase activity as a function of OA concentration, since this compound is a potent and selective inhibitor of both classes of phosphatases [21]. OA was used *in vitro* by supplementing the above reaction solution with different doses of inhibitor in the nanomolar range. Another approach was to microinject *Xenopus* oocytes with OA at a higher dose (final concentration of 5 μM), before incubating the cells in the presence of test molecules (MET

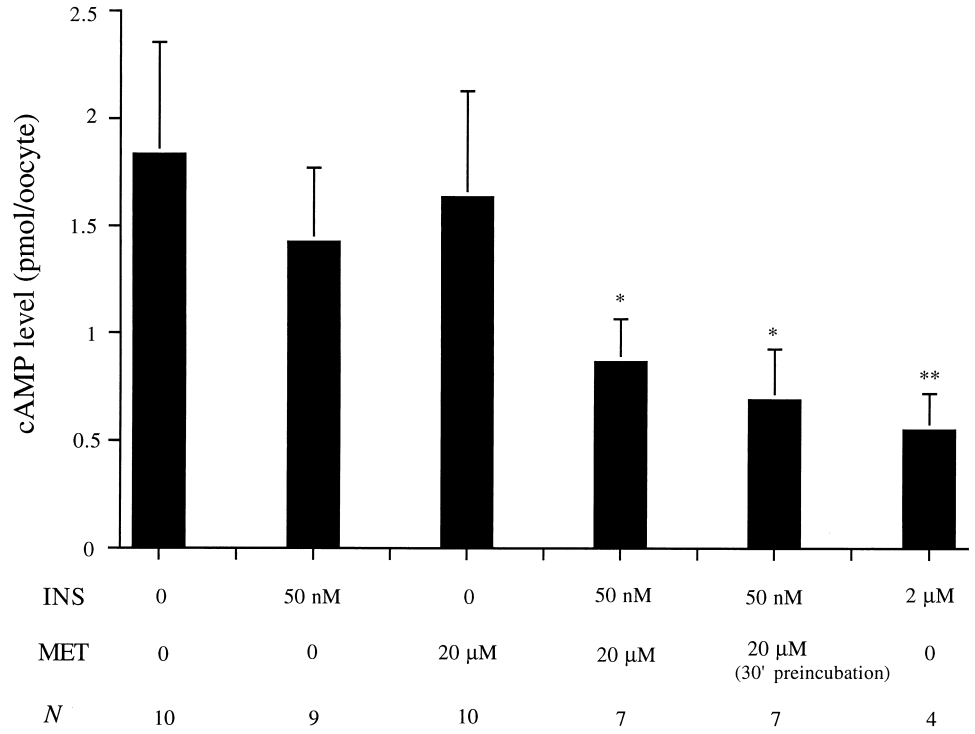

FIG. 1. Effect of MET on the cAMP content of mature oocytes treated or not with INS. The oocytes were first incubated for 60 to 90 min in the presence of test molecules at the above-mentioned concentrations. Afterwards, the level of cAMP was assayed on each sample by using a radioimmunoassay kit. The values are presented as the means ± SEM (N = 4 to 10). **INS effect significant with $P < 0.0005$. *MET plus INS action significant with $P < 0.001$.

and/or INS) and examining phosphatases in the cell-free extracts.

Other Metabolic Studies with OA and Inhibitor-2

The putative role played by PP-1 in the combined action of MET and INS was indirectly investigated by measuring GS activity after treatment with inhibitor-2, known to specifically inhibit the catalytic subunit of PP-1 [22]. For this purpose, inhibitor-2 was introduced into whole cells at a final concentration of 2 μM. About 30 min after this microinjection step, the oocytes were placed in 2 mL of MBS containing INS alone or INS combined with MET for 80 min. At the end of this incubation, oocytes were homogenized, the lysate was centrifuged, and synthase *a* activity was assayed on extracts. We reproduced the same kind of experiments by replacing inhibitor-2 by OA, except that OA was added to the assay medium.

As regards the studies on phosphatases, it is also noteworthy that the concentrations and preincubation periods used for inhibitor-2 and OA under microinjection conditions as well as OA doses applied to extracts are known to totally inhibit the corresponding oocyte phosphatase activities controlling several major steps in the cell cycle [23, 24].

Statistical Analysis

Statistical significance of differences was tested by using a Student's *t*-test (unpaired observations), and all data were presented as the means ± SEM ($N \geq 3$).

RESULTS

Metformin Action and the Intracellular Signaling Pathway

Considering that GS is under tight regulation by INS, we addressed the question of whether MET *in vitro* was able to alter the functioning of intracellular modulatory events in *Xenopus* oocytes. In the first set of experiments, we investigated the effect of MET alone or in conjunction with INS on the cAMP level. Neither substance, when used separately at a low concentration, caused a significant modification of the second messenger basal amount which averaged 1.7 pmol per oocyte (Fig. 1). When oocytes were incubated with 20 μM MET plus 50 nM INS, or were preincubated in the presence of the biguanide before hormone addition, we recorded, in both cases, a significant drop ($P < 0.001$) in the intraoocyte content of cAMP, which was similar to the value observed after INS treatment at a high concentration (2 μM).

A second approach was to elucidate the possible involvement of serine/threonine phosphatases in the mode of MET

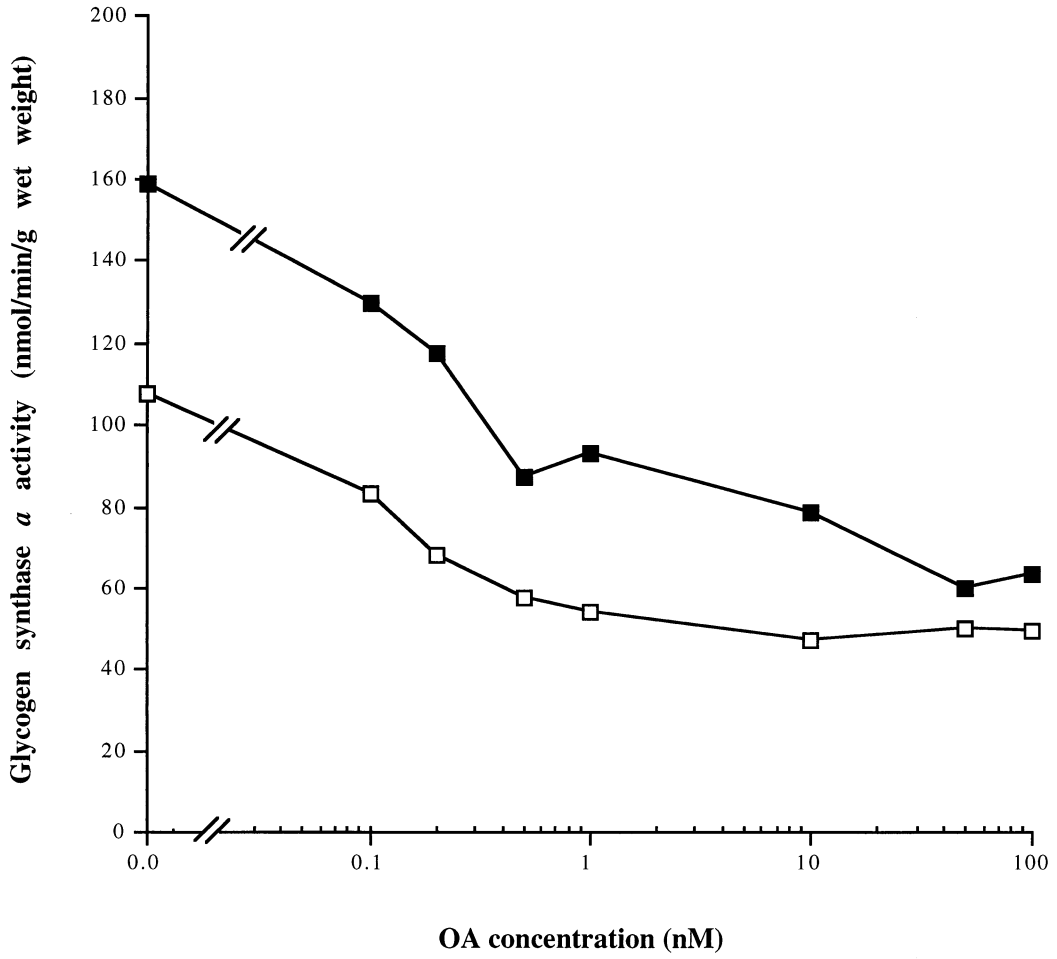

FIG. 2. Dose–response curves for the inhibitory effect of OA on GS activity induced by INS alone or by INS combined with MET. The oocytes were incubated in the presence of 50 nM INS alone (—□—) or in association with 20 μM MET (—■—) for 90 min. After this treatment, each group of cells was homogenized and an *in vitro* preparation of GS was subsequently obtained following a low-speed centrifugation as described in Methods. GS from the supernatant was then assayed after a 10-min incubation at 30° with increasing concentrations of OA. Each point represents the mean value of two separate experiments performed in duplicate. The basal activity of synthase *a* in untreated oocytes was 53.6 ± 5.1 nmol/min/g wet weight, whereas the total activity of GS was 240.1 ± 15.9 nmol/min/g wet weight.

action on glycogen metabolism. The use of OA as a selective inhibitor of these enzymes should allow us to assess the roles of PP-2 and PP-1, which are sensitive to low and high doses of OA, respectively. To begin with, we tested the inhibitory effect of OA on the oocyte GS (from a low-speed extract) that was previously activated by INS alone or INS combined with MET. Figure 2 shows that OA dose dependently suppressed the stimulation of GS by INS; the maximal inactivation was achieved at approximately 1 nM OA. We also found that the additional increase in INS-induced GS activity specifically attributable to MET action was only partly thwarted by OA, and at higher doses of this inhibitor (to which PP-1 is more sensitive). Moreover, the baseline activity of GS was not restored as compared with the former case. Direct measurement of phosphatase showed that INS increased the activity of PP-2 in a dose-dependent manner, yet MET was without effect whether the hormone was present or not (Fig. 3). Furthermore, microinjected OA (5 μM/oocyte) erased this INS action. A similar inhibition level was observed when OA was used *in vitro* at a final concentration of 50 nM. It is interesting to note that the basal activity of PP-2 was less affected in the presence of 1 nM OA than with 50 nM. On the other hand, results from studies carried out with inhibitor-2, the powerful specific inhibitor of PP-1, indicated that this preinjected agent almost completely decreased GS activity stimulated by INS together with MET (Fig. 4). The activity of GS therefore returns to a highly phosphorylated inactive form which corresponds to its status under basal conditions.

Assessment of the Role of the Insulin Receptor Protein Tyrosine Kinase in Mono- and Dimethylbiguanide Effects

Studies intended to test the possible link of MET to the INS-regulatable tyrosine kinase activity were achieved by

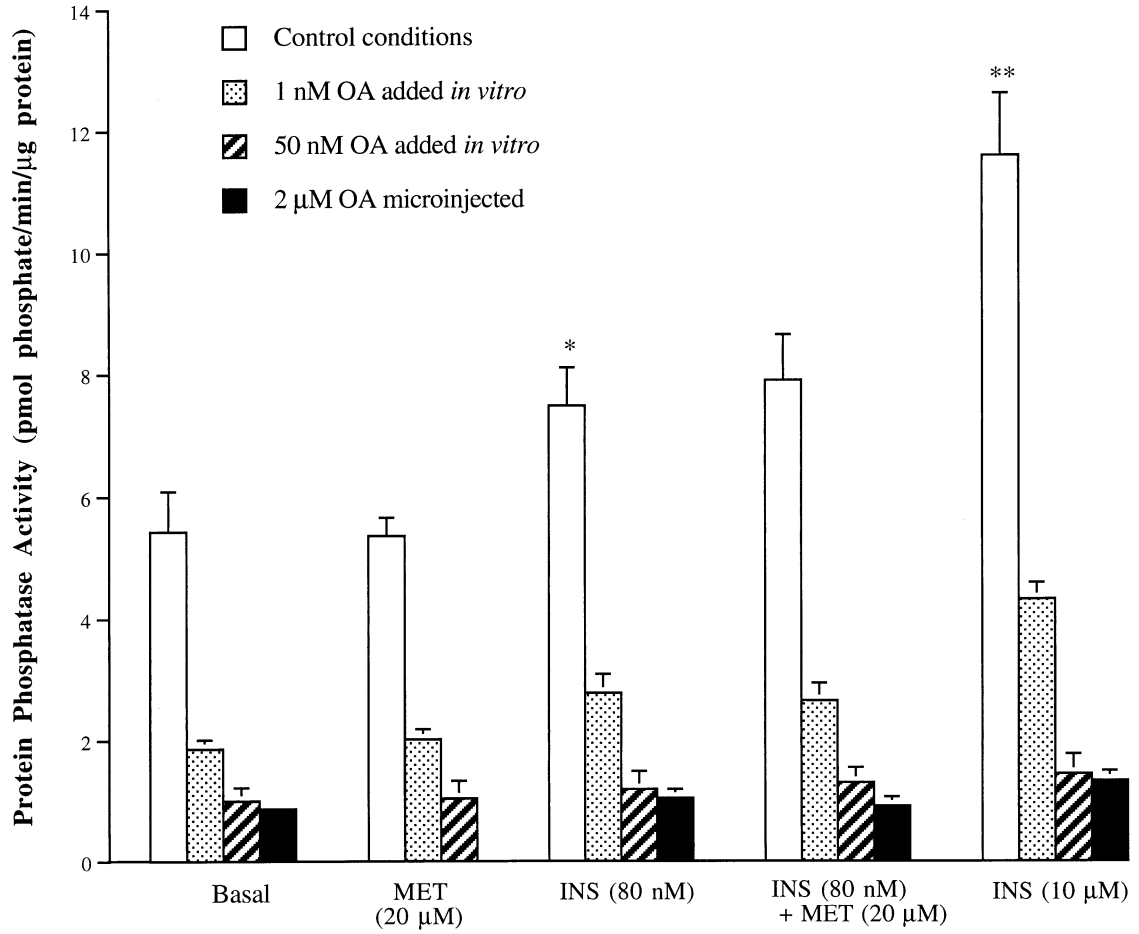

FIG. 3. Determination of PP-2 activity as a function of OA concentration after INS and/or MET action. Oocytes were incubated for 80 min either with MET or INS alone, or with both substances together at the above-indicated concentrations. After disruption of the cells and lysate processing, PP-2 activity from a clear supernatant was then assayed in the presence of OA (1 and 50 nM) as described in Methods. On the other hand, the oocytes were preinjected with OA at a final concentration of 2 μM. They were then incubated and treated before the activity of PP-2 was measured as before. The data were expressed as the means ± SEM (N = 5). *INS (80 nM) effect significant with $P < 0.01$. **INS (10 μM) effect significant with $P < 0.005$.

determining the influence of a potent inhibitor of receptor-associated tyrosine kinase(s) on GS activity. When tyrphostin B46 was first microinjected into oocytes at a final concentration of 200 μM, before incubation in the presence of the drug together with INS, the activity of synthase *a* was significantly reduced ($P < 0.005$; Table 1). The combination of tyrphostin A1 (an ineffective derivative) and MET led to an enhanced enzyme activity comparable to that observed in its absence. In terms of a dose–response relationship, we observed that concentrations of tyrphostin B46 ranging from 10 to 50 μM were able to block INS action alone, but failed to inhibit the activatory effect of MET; higher doses (≥500 μM) were required for a maximal inhibition of GS activity (data not shown). In another set of experiments, we investigated the metabolic action of MMB, a derivative of MET which appears to exert, *in vivo*, a very weak antihyperglycaemic effect.* Its potential efficacy on INS-mediated GS stimulation was tested under conditions where the drug was either simply added to the incubation medium of oocytes or microinjected into these cells. In contrast to what was observed for MET [9], there was no amplification of the metabolic response whatever the mode of MMB application to oocytes (Table 2). Moreover, once preinjected, the substance even exerted an inhibitory effect on the enzyme activity. As suggested by Stith *et al.* [25], who made use of the intracellular portion of the human IR as experimental tool, the different behavior (inhibition vs activation) of these two structurally very similar derivatives belonging to the biguanide family on insulin signaling was consistent with their divergent action at the level of the IR tyrosine kinase.

Cellular Location and Biological Effect of Metformin

The goal of the following series of experiments was to verify whether an accumulation of MET in the whole oocyte was necessary to elicit its metabolic action on GS activity.

* Wiernsperger N, unpublished observations.

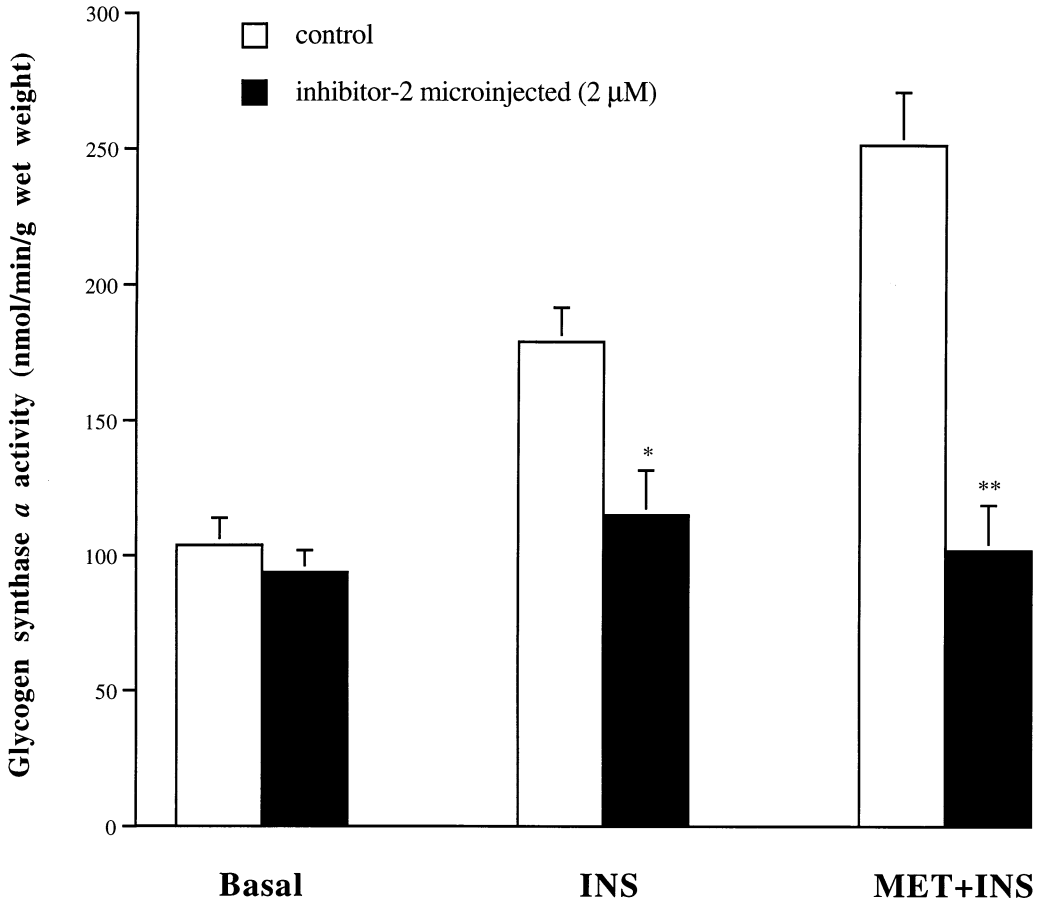

FIG. 4. Effect of inhibitor-2 on basal GS activity or on GS activity stimulated by INS in the presence or absence of MET. Oocytes were first microinjected with inhibitor-2 at an internal concentration of 2 μM (solid bars) or with an equivalent amount of MBS buffer (open bars). They were then incubated for a further 60 min in the presence of 80 nM INS together with 20 μM MET, before GS activity was assayed in extracts as reported in Methods. The values are presented as the means ± SEM (N = 4). *Inhibitor-2 effect on INS action significant with $P < 0.01$. **Inhibitor-2 effect on INS plus MET action significant with $P < 0.001$. The total activity of GS in this set of experiments is 273.2 ± 14.3 nmol/min/g wet weight.

Once the cells were incubated in the presence of 10 μM radioactive MET for different periods of time and thereafter washed, two oocyte fractions were differently prepared as described earlier (see Methods). Results with the PMC preparation clearly show that, after 1 hr of incubation, [^{14}C]MET was almost exclusively detected in the membranous compartment (Fig. 5). The process of drug "internalization" occurred only at the end of this lag stage. The percentage of endogenous MET taken up by oocytes, after 3 hr, was equivalent to about 0.1% of the total MET present in the external medium; it represents an actual amount of 2 pmol MET that entered the 0.5-μL inner water space of the oocyte. This value was only minimally altered if the incubation was extended to 16 hr. By contrast, the percentage of MET bound to the PMC complex increased in the course of incubation, suggesting that this molecule remains predominantly attached to submembranous structures just beneath the oocyte periphery. A very clear-cut difference between the fractions (middle vs lower curves in Fig. 5) is largely supported by statistical analysis. In addition, we recorded similar results when the oocytes were treated with a higher dose of MET (10 mM), except that the radioactivity associated with PMC after an incubation of 15–30 min was more elevated than with 10 μM MET (data not shown). To subsequently identify a possible correlation between the biological function of MET, when used at a therapeutic concentration (10 μM) as effective as the dose previously applied (20 μM), and its cellular location (membrane vs cell interior), we monitored its particular action on INS-induced GS activity as a function of preincubation time with this biguanide. The inset of Fig. 5 depicts pooled data from experiments performed with different non-radioactive MET pre-exposure periods which did not exceed 60 min, i.e. a time where the drug had not penetrated into the oocyte cytoplasm. Indeed, a significant increase in INS-induced GS activity was recorded when oocytes were pretreated with MET for 15 ($P < 0.01$) or 45 min ($P < 0.005$) before these cells were washed and transferred into fresh MBS containing 80 nM INS alone. We also observed an amplified biological response when the

TABLE 1. Inhibitory effect of tyrphostin B46 on INS-regulated GS activity, in the presence or absence of MET

	GS activity (nmol/min/g wet weight)
Control	73.1 ± 9.4
INS (50 nM)	138.6 ± 14.1
MET (20 μM)/INS (50 nM)	195.4 ± 22.2
Tyrphostin A1 (200 μM) + INS (50 nM)	140.4 ± 8.2
Tyrphostin A1 (200 μM) + MET (20 μM)/INS (50 nM)	189.1 ± 15.5
Tyrphostin B46 (200 μM) + INS (50 nM)	88.5 ± 8.1*
Tyrphostin B46 (200 μM + MET (20 μM)/INS (50 nM)	102.1 ± 19.3†

Tyrphostin B46 was first microinjected into mature oocytes at a final concentration of 200 μM before their incubation for 1 hr in the presence of INS alone or in association with MET. GS activity was then assayed as described in Methods. Tyrphostin A1, injected at an equivalent dose, was used as negative control. The results are presented as the means ± SEM (N = 5).

*Inactivatory effect of tyrphostin B46 on INS action was significant with $P < 0.001$ compared to INS alone.

†Inactivatory effect of tyrphostin B46 on MET action was significant with $P < 0.005$ compared to INS and MET together.

compounds were added simultaneously and exerted their action together for no more than 60 min. Conversely, MET used at its highest concentration (10 mM) failed to elevate the activity of GS initially stimulated by INS (147.6 ± 9.8 vs 167.2 ± 16.8 nmol/min/g wet weight, N = 4; NS, not significant).

The other study, based on a membrane-rich extract (prepared by lyse of oocytes followed by centrifugation on a sucrose gradient), gives apparently contradictory results (Table 3). To ascertain that oocyte membranes were correctly isolated, we determined specific alkaline phosphodiesterase activities in the various subcellular fractions. The fraction containing only the plasma membrane (pellet from fraction F2 spun at 16,000 *g*) showed an enrichment of at least 125-fold for this marker enzyme, as compared with whole-cell homogenate. Yet, no or a negligible trace of radioactivity was detected in this pellet following a period of 30- and 120-min incubation with MET. The labeling was distributed between the fraction F1 (corresponding to the cytosol) and the yolk protein-filled residual pellet (F4), but also having some components (numerous pigment and cortical granules) that can bind non-specifically to many types of molecules, probably including MET.

TABLE 2. Effect of MMB, the monomethylated analog of MET, on INS-regulated GS activity

	GS activity (nmol/min/g wet weight)
Control	64.2 ± 16.9
INS (50 nM)	115.6 ± 13.5
MMB (20 μM, incubated)	70.5 ± 11.7
MMB (20 μM)/INS (50 nM) (incubated)	96.1 ± 11.8
MMB preincubated (20 μM) + INS (50 nM)	88.4 ± 14.4*
MMB preinjected (20 nM)	58.1 ± 5.8
MMB preinjected (20 nM) + INS (50 nM)	78.1 ± 8.2†

Fully grown oocytes were either preincubated in the presence or absence of MMB for 45 min before INS addition, or their incubation was performed with the two molecules added at the same time. The action of this MET derivative was also tested following its microinjection into the oocyte cytoplasm at a final concentration of 20 nM. After each treatment, the activity of GS was measured as described in Methods. The results are presented as the means ± SEM (N = 4 or 6).

*MMB effect (if preincubated) was significant with $P < 0.05$ compared to INS action.

†MMB effect (if injected) was significant with $P < 0.01$ compared to INS action.

DISCUSSION

Recently, we and others [9, 13, 14, 26] pointed out the pertinence of the *Xenopus laevis* oocyte as an experimental model to better characterize the potentiating role of MET on INS action. The synergistic action of both compounds on glycogen metabolism was illustrated by an increased stimulation of GS activity, the key enzyme which catalyzes a rate-limiting step for glycogen deposition. Since a predominant impairment in non-insulin-dependent diabetic subjects is precisely a substantial decrease in glucose storage [27], it becomes crucial to clarify some molecular events whereby MET could alleviate insulin resistance and exert its antihyperglycaemic role. The main purpose of this work was, therefore, to identify along the signaling route, starting from the transmembrane IR down to the regulatory system directly linked to GS activity, some important target sites for MET action.

To examine the fundamental issue of whether MET acted intra- or extracellularly, we made use of an unusual property of the oocyte (isolation of the plasma membrane with the contiguous underlying layer, the cortex). Because biguanides are weak bases with extremely high pKa values, they exist in a cationic form at physiological pH, which should severely limit their ability to cross biological membranes. As expected, we found that oocytes incubated with [^{14}C]-labeled 10 μM MET began to weakly accumulate the biguanide only after a 60-min lag period; at this moment less than 0.1% was found in the cytoplasm of these cells. In contrast to authors [14] who suggested that this delay was mainly required for the entry of MET into the cell, allowing its subsequent action at an intracellular site, we propose another explanation. In the light of our data concerning the metabolic study which was conducted in parallel with the measurement of MET uptake (see Fig. 5 + inset), there is evidence that the potentiating effect of MET (10 μM) on INS action is triggered before it reaches the cytosol. We actually observed that MET stimulated the active form of GS within 1 hr of hormonal action or 15 to 45 min before INS addition, i.e. a time period where the majority of MET resides inside the PMC complex. It also appeared that the withdrawal of free MET surplus by rinsing the pre-exposed cells before adding INS was not harmful to its enhancing action. While bearing these data in mind, the lack of MET

Preincubation	Incubation (60 min)	GS activity (nmol/min/g wet weight)
10 μM MET (15 min)	INS (80nM)	240.4 ± 16.9 ($N = 4$, $P < 0.01$)
10 μM MET (45 min)	INS (80 nM)	267.4 ± 13.2 ($N = 4$, $P < 0.005$)
10 μM MET (3 hr)	INS (80nM)	231.6 ± 10.7 ($N = 4$, $P < 0.01$)
—	MET + INS	227.1 ± 21.4 ($N = 5$, $P < 0.01$)
—	INS (80 nM)	167.2 ± 16.8 ($N = 5$)
	Basal	90.1 ± 17.5 ($N = 5$)

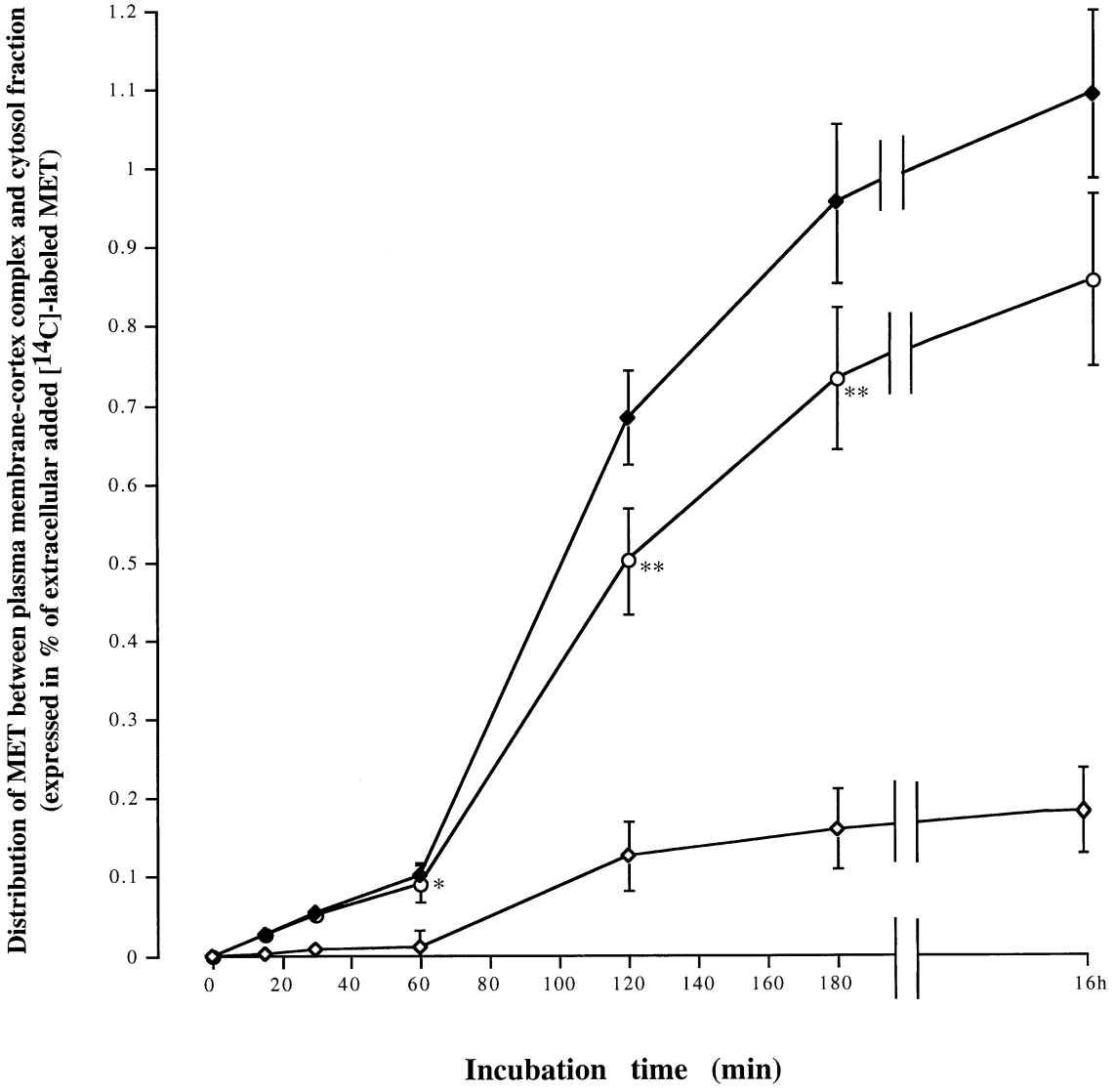

FIG. 5. Time-dependent changes in the amount of [^{14}C]MET taken up by intact oocytes. The oocytes were incubated in the presence of 10 μM radioactive MET for the indicated periods of time. After washing, they were processed as described in Methods. The total radioactivity associated with whole oocytes (—◆—) as well as the estimated amount of MET, either bound to a membrane–cortex complex (—○—) or present in the cytosol (—◇—), were then determined. Values are means ± SEM of 3 experiments, each performed in duplicate (asterisk denotes significance at $P < 0.05$; double asterisk denotes significance at $P < 0.01$). The inset shows the measurements of glycogen synthase *a* activity either after preincubation of oocytes with unlabeled MET (10 μM) for short (15 and 45 min) or long (3 hr) periods of time followed by their incubation in the presence of 80 nM INS, or after their incubation in the presence of drug together with INS for only 60 min.

TABLE 3. Respective distribution of [^{14}C]-labeled MET and alkaline phosphodiesterase activity in whole cells and in fractions separated by continuous sucrose gradient

	Incorporation of MET* after: 30 min	120 min	Marker enzyme activity (U/g wet weight)†
Whole oocytes (= crude homogenate)	**0.063%**	**0.44%**	**1.96** (**5.2** mU/mg protein)
	Subcellularfractions (Total = 100%)		Recovery† = 99.7%
Fraction I (F1)	67.4%	53%	0.44 (**9.54** mU/mg protein)
Fraction II, slit into:	10.5%	11.7%	
-Supernatant (SN)	10.6%	11.4%	ND
-Final pellet (membrane-rich fraction)	ND	0.26%	0.677 (**651** mU/mg protein)
Fraction III (F3)	>0.5%	1.3%	0.002 (0.25 mU/mg protein)
Residual pellet (F4)	20%	31%	0.835 (**3.15** mU/mg protein)

The cells were incubated with radioactive MET and oocyte fractions were prepared as described in Methods. The values presented are the average means (N = 3); the error bars are not indicated in the table for the sake of clarity. ND, not determined. Values in bold are the specific activities from which the relative enrichment is calculated, whereas the recovery is evaluated from total activities (underlined values).

*Expressed in % of total external MET for whole oocytes, or in % of MET within the homogenate for subcellular fractions.

†One unit of marker enzyme equals μmol *p*-nitrophenol formed/min.

remaining attached to a membrane-rich pellet seems, at first glance, rather surprising (see Table 3). However, the experimental conditions required for this preparation, e.g. oocytes triturated by homogenization in a MET-free medium and resuspension of the pellet after each spin, make this protocol inappropriate for correctly examining such a process. Additionally, as MET does not bind covalently to the membranes [28], it is easily released by multiple washing so that we logically encountered great difficulty in measuring high levels of MET incorporation into intact oocytes.

Even if we do not rule out the possibility that MET gets into the deep cytosol, it is unlikely that these few transported biguanide molecules exert some effect in close relation with GS activation. Indeed, there was not any amplification of the INS action after 2 or 3 hr of treatment with MET (see the inset of Fig. 5, line 3). Furthermore, we previously demonstrated that MET was without effect on hormonal stimulation of GS when it was added to the external medium 15 min after INS [9]. Our results are somewhat different from those found by others who analyzed the same phenomenon in oocytes or in other cell types [26, 29]. These authors state that MET does build up within the cell, possibly using an amino acid transport system. However, measurements for MET penetration were achieved only after 44 hr of incubation in the MBS medium. This duration is entirely inconsistent with the drug acting at an early step in the mechanism of INS action. Moreover, their values (up to 120 nmol MET entering the oocyte) correspond to a concentration range which seems to us abnormally high in keeping with the intracellular volume of an average oocyte. Altogether, we can not unequivocally preclude one cytoplasmic site of MET action but, for the biological effects currently investigated, MET does not need to reach cytosol. Hence, we believe that the prime site for MET action is located within the plasma membrane or near the cortical area of the whole cell. Numerous studies [30–32] showing that MET at therapeutic concentrations increases or restores membrane fluidity support this hypothesis.

The close similarity in terms of cationic charge between MET (also called dimethylbiguanide) and one of its derivatives, namely the MMB, led us to compare their respective efficacy. Although both compounds share the same structural characteristics, i.e. a guanidine moiety with an NH_2-terminal end, only MET allowed for a positive biological response. We also observed that the tyrosine kinase inhibitor, tyrphostin B46, prevented GS stimulation by MET. In the light of these results, it could be argued that the major target of MET in enhancing INS-mediated GS activity is the IR β-subunit. The very striking similarity between the bell-shaped curves for MET action on GS activity [9] and for MET activation of tyrosine kinase activity [25] corroborates this conclusion. The lack of stimulation by 10 mM MET on INS-mediated GS activity correlates with the fact that high concentrations of MET (50–200 μg/mL) inhibit tyrosine kinase activity in the *Xenopus* oocyte membrane [14, 25]. Although the tyrosine kinase domain of the IR is located intracellularly, it is unlikely that MET exerts its full stimulatory action on this enzyme in a direct way. The fact that preinjected MET is also active, but to a lesser extent, does not at all mean that this molecule acts intracellularly. Our previous results showing that the level of MET action on INS-stimulated GS activity was lower in microinjected versus incubated oocytes [9] partially support this view. According to the

discussion above, it seems more accurate to assume that MET, at least to some degree, needs to insert into the PMC compartment (not deep within the cell) to enhance INS action at an early event of the cascade-regulating GS activity.

Another important task of this work was to determine which conditions are required for GS stimulation, notably by identifying some intracellular downstream processes likely to be indirectly affected by MET in the presence of INS. Our data revealed that MET combined with 50 nM INS markedly reduced (to about 60% of basal) the content of cAMP in oocytes. A similar decrease in the cyclic nucleotide content also occurred in oocytes treated with INS alone at a higher concentration (2 μM). Such a drop in the cAMP pool, consistently observed by others using the same cell type [33, 34], would here reflect an incremental effect of INS plus MET on certain membrane-bound enzymes. For example, adenylate cyclase should be a target, since Gawler *et al.* [35] found that MET restored the ability of INS to inhibit this enzyme activity under diabetic conditions. At this point, it is also worthwhile to mention previous results from our laboratory showing that INS (2 μM) causes a rapid decrease in the phosphorylation level of proteins associated with a glycogen-rich pellet from *Xenopus* oocytes [4]. Taken together with the demonstration that MET exerts its action by reducing INS requirements for GS stimulation (20 μM MET combined with 50 nM INS gives the same response as INS alone when tested at a concentration 10- to 20-fold greater [9]), it is tempting to put forward that MET may enhance the inhibition of protein kinase A, leading in turn to an increased degree of GS dephosphorylation and hence to its higher activation. Besides protein kinases, protein phosphatases are known to fulfill an important role in INS signaling [36, 37]. Our preliminary results showed that a complete inhibition of GS activity by nanomolar concentrations of OA (0.5–5.0 nM) is consistent with a possible involvement of PP-2 (sensitive to these low doses) in INS action. By contrast, the additional increase in GS activity following MET treatment was only moderately reduced in the presence of similar, or even higher, amounts of this inhibitor agent (see Fig. 2). This kind of interpretation must certainly be made with caution in view of the phosphatase activities observed, at least for PP-2. By using OA and a direct assay method for these enzymes, we demonstrated that the basal activity of PP-2 should play a role only in the action of INS. It has recently been shown that the IC_{50} for purified oocyte PP-2A is 1 nM, while 1 μM OA is required to inhibit 50% of PP2 activity in a crude oocyte extract, and this because oocytes contain high levels of phosphatases [38]. Our data are in agreement with this observation and support the notion that OA acts *in vivo* by a selective inhibition of PP-2 under microinjection conditions. Nonetheless, as GS activity may be increased up to 5-fold by 10 μM INS, i.e. a dose ensuring the maximal hormonal effect [9], whereas the activation of PP-2 is only doubled (both over control levels), this means that PP-2 are probably not sufficient to explain the overall action of INS. Concerning the effect of MET, we provide conclusive evidence that this biguanide does not act through a stimulation of PP-2 activity, whether INS is absent or not. Results with microinjected inhibitor-2, specifically inhibiting PP-1 activity, suggest that more than one class of phosphatases are partially involved in the whole process, since a total conversion of GS stimulated by INS, alone or with MET, into its inactive form was actually recorded. However, further work is needed to conclude whether direct activation of PP-1 is fully responsible for MET or INS action on glycogen metabolism. Taken together, our results tend at best to demonstrate that MET in the presence of INS enhances some pathways of the metabolic regulation by affecting the cycle of phosphorylation/dephosphorylation, at a step still remaining unclear.

In conclusion, we believe that the oocyte of *Xenopus laevis* is a reliable cellular model for investigating a drug mechanism of action, as demonstrated in the case of MET. The improvement of INS action by MET, which characterizes its clinical efficacy, is easily seen in such cells when considering various metabolic effects of the hormone [9, 13, 14]. Interestingly, we provide strong evidence that MET, at therapeutically relevant concentrations, does not primarily act at an intracellular site, but inside a restricted area of the cell surface from which early events of INS signaling, such as the stimulation of the IR-associated tyrosine kinase activity, should be increased.

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