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The Antidiabetic Drug Metformin Elevates Receptor Tyrosine Kinase Activity and Inositol 1,4,5-Trisphosphate Mass in *Xenopus* Oocytes*

BRADLEY J. STITH, MARC L. GOALSTONE, RONALD ESPINOZA, CORI MOSSEL, DAWN ROBERTS, AND NICHOLAS WIERNSPERGER

University of Colorado at Denver (B.J.S., M.L.G., R.E., C.M., D.R.), Biology 171, PO Box 173364, Denver Colorado 80217; and Lyonnaise Industrielle Pharmacentique (LIPHA) Laboratories (N.W.), F-69379 Lyon Cedex 08, France

ABSTRACT

Although metformin is an important antidiabetic, its mechanism of action is still unknown. To study its mechanism, we examined metformin stimulation of insulin action on the *Xenopus* oocyte. Similar to therapeutic concentrations, maximal stimulation of insulin-induced meiotic cell division was achieved at about 1–10 μg/ml (or 7.7–77 μM) metformin. An equivalent concentration of metformin was required to elevate receptor tyrosine kinase activity (in whole cells or a membrane-cortex preparation) and, through this tyrosine kinase activation, inositol 1,4,5-trisphosphate (IP_3) production. With whole cells, the preincubation time for metformin stimulation of insulin action (~1 h) was equivalent to the time required for metformin to maximize tyrosine phosphorylation and raise IP_3 levels. With the membrane-cortex preparation, metformin was active within minutes; thus, metformin may act at an intracellular site. Since metformin can increase IP_3 mass, we prevented elevation of calcium by prior microinjection of a calcium chelator or heparin (a drug that inhibits IP_3 binding to the IP_3 receptor). Both the chelator and heparin blocked metformin stimulation of insulin action on whole cells. Since microinjection of IP_3 also stimulates insulin action, metformin may stimulate insulin action by elevation of intracellular calcium in addition to activation of the receptor tyrosine kinase. (*Endocrinology* **137:** 2990–2999, 1996)

METFORMIN (*N,N'*-dimethylbiguanide) is an oral hypoglycemic drug used in the treatment of diabetes (1), and this drug has been recently introduced in the United States (2). Metformin increases insulin-stimulated glucose uptake (3); however, its effect on the insulin receptor kinase is controversial [two groups (3, 4) found no effect; another report (5) suggested that metformin can stimulate the receptor kinase]. The initial steps by which the drug stimulates insulin action have not been described.

We and others have used the *Xenopus* oocyte as a model system for the study of both insulin and metformin action. As in other responsive cells, the addition of insulin to oocytes can stimulate glucose uptake (6–8), glycogen synthesis (9), and the mitogen-activated protein kinase pathway and increased intracellular pH (10, 11), type III cAMP phosphodiesterase (12), receptor-mediated endocytosis (13), protein synthesis (14), phosphorylation of annexin (15), and phospholipid turnover (16). In addition, at about 5–7 h after hormone addition, insulin induces germinal vesicle breakdown (GVBD). GVBD represents continuation of meiotic prophase or maturation of the oocyte (for review, see Ref. 10). Various laboratories have used the induction of GVBD or meiosis as a measure of insulin action since the process is rapid and easily followed in a large number of cells. For example, microinjection of the insulin receptor substrate (IRS-1) accelerates GVBD (17, 18) whereas a tyrosine phosphatase inhibits insulin induction of GVBD and receptor tyrosine phosphorylation (19).

The *Xenopus* oocyte has also been used as a model system for metformin. Without changing insulin binding, metformin stimulates the ability of insulin to induce GVBD and potentiated insulin-induced phosphorylation of annexins (lipocortins) (15).

In many studies, it was suggested that insulin acts through the insulin-like growth factor 1 (IGF-1) receptor since high concentrations (EC_{50} ≈50 nM) of insulin were required. For maximal induction of GVBD, ribosomal S6 phosphorylation, elevation of intracellular pH, or phospholipase C stimulation, approximately 100 nM insulin was required (10, 16, 20). Surprisingly, four laboratories have suggested that the insulin receptor is present in the oocyte (20–23). Maller and Koontz (20) found 40 million high affinity [dissociation constant (K_d) ≈ 1.4 nM] insulin-binding sites per oocyte, Hainaut *et al.* (21) estimated 70 million high affinity (K_d ≈ 0.2 nM) sites, and Diss and Greenstein (23) found 97 million sites (K_d ≈ 0.89 nM). Scavo *et al.* (22) found more IGF-1 receptors than insulin receptors, and Hainaut *et al.* (21) estimated 120 million IGF-1 sites with a K_d of 0.8 nM for IGF-1. Collagenase treatment for isolation of oocytes [it reduces functional acetylcholine muscarinic receptors and may reduce insulin receptor number (21)], presence of insulin receptor-IGF-1 receptor hybrids, different hormonal levels of the original animal (at different times of the year, some frogs produce oocytes with reduced responsiveness; some researchers use PMSG to prime and increase the responsiveness of oocytes to insulin) could explain differences in insulin receptor-bind-

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Address all correspondence and requests for reprints to: Bradley J. Stith, University of Colorado at Denver, Biology 171, P.O. Box 173364, Denver, Colorado 80217-3364. E-mail: bstith@castle.cudenver.edu.
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ing studies. The apparent difference between the conclusions of the binding data and the dose-response relationship may be explained if the insulin receptor is present but uncoupled or inactive.

Due to the requirement for low insulin concentrations and the efficacy of an insulin receptor antibody, we suggest that a plasma membrane-cortex preparation from the *Xenopus* oocyte has an active insulin receptor. We suggest that metformin acts at an intracellular site to activate a receptor tyrosine kinase and, through this kinase, stimulates phospholipase C [elevating inositol 1,4,5-trisphosphate (IP_3), and intracellular calcium ($[Ca^{++}]_i$)]. The drug mimicked some (receptor kinase and phospholipase C activation) but not all (large DAG production, induction of GVBD) of the effects of insulin on *Xenopus* oocytes.

Materials and Methods

Induction of meiotic cell division in Xenopus oocytes

Xenopus females were obtained from Xenopus One (Ann Arbor, MI) and maintained on a diet of cubed beef heart (animal care procedures have been approved by the University Institutional Animal Care and Use Committee). Approximately 1–2 weeks before use, female frogs were primed with injection of 35 IU PMSG (Sigma, St. Louis, MO). This procedure did not cause spontaneous maturation but reduced the time required for maturation and increased the synchrony of GVBD (14). Oocytes were obtained by manual dissection of ovarian fragments obtained after surgery or after guillotining (anesthesia: 0.12% MS-222).

To follow insulin-induced GVBD, groups of 15–20 oocytes were placed into multiwell dishes with 3 ml modified 0-R2 (83 mM NaCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.9). The concentration of the insulin (porcine, Lilly, Indianapolis, IN) stock (154 μM) was determined spectrophotometrically, kept in capped glass test tubes, and used at a final concentration of 1 μM. One of the early prophase events of insulin-induced meiosis is that the nucleus moves to the animal pole surface and pushes pigment granules to the side. The resulting white spot can be found with a dissecting scope (15×). A second measure of meiosis was used; the breakdown of the nuclear envelope (or GVBD) was confirmed (in each experiment, cells were fixed in 5% trichloroacetic acid (TCA) and dissected to determine whether nuclear envelope breakdown occurred). Spontaneous induction of meiosis in the absence of insulin was zero.

Thus, insulin action is recorded by the number of oocytes that enter meiosis and the time of their entry. The number of oocytes entering meiosis is reported as the percentage of cells that have undergone GVBD (see ordinates that show percent GVBD). Metformin (LIPHA labs, Lyon, France) was added to the external medium at various concentrations before or after insulin addition to examine its ability to stimulate insulin action. In some experiments, a more effective metformin derivative, methylglyoxyl bis(guanylhydrazone)dihydrochloride hydrate (MGBG)(Aldrich, Milwaukee, WI) was added to oocytes.

Measurement of IP_3

An IP_3 assay kit (a membrane preparation from calf cerebellum containing labeled IP_3 bound to the IP_3 receptor; New England Nuclear, Boston, MA) has been used to follow this second messenger after insulin addition to *Xenopus* oocytes (16). Although this procedure has been described more completely, we note that groups of 15 oocytes were homogenized in TCA, acid was extracted with 3:1 freon-trioctylamine, and a portion of the extract was incubated with the $[^3H]IP_3$ and IP_3 receptor mix. The IP_3 in the oocyte extract displaced the $[^3H]IP_3$ from the receptor over a 1-h incubation period. The remaining radioactivity in the pellet was quantified by liquid scintillation. The decrease in counts associated with the IP_3 receptor was converted to the amount of IP_3 in the sample (femtomoles of IP_3 per oocyte) with a standard line. For maximum accuracy, four standards were run with each experiment. Nonspecific counts (typically, 6% or less of the experimental values) were obtained with the use of inositol hexakisphosphate.

DAG measurement

The procedure used to measure DAG mass in *Xenopus* oocytes (24) involved homogenization of groups of 10 oocytes in 1:2 chloroform-methanol. After the monophase was broken, the organic phase DAG was converted to [^{32}P]phosphatidic acid with DAG kinase (Lipidex, Westfield, NJ). After separation by TLC, the amount of radioactivity in the phosphatidic acid can be used to determine the mass of DAG in the original sample. Standards (Avanti Polar Lipids, Birmingham, AL) were used with each experiment.

Phosphatidylcholine (PC) hydrolysis by phospholipase D or phospholipase C

These two phospholipase activities were assayed by recording the mass of choline (produced by PC-specific phospholipase D) or phosphocholine (produced by PC-specific phospholipase C)(25). Briefly, oocytes were homogenized in 2:1 chloroform-methanol (vol/vol) with 20 mM formic acid. After phase was broken, the aqueous layer was evaporated with nitrogen and reconstituted with [^{32}P-γ]ATP and choline kinase. After an incubation period, the phosphorylated choline was eluted from Dowex 1-X8 (200–400 mesh, formate form, Bio-Rad Labs, Hercules, CA) and the counts per min in the eluate was equivalent to the mass of choline present in the original sample. For phosphocholine, half of the original homogenate was incubated with alkaline phosphatase (to produce choline) before choline analysis. The difference between the amount of choline found in samples treated with the alkaline phosphatase and the amount of choline in the untreated group represented the amount of phosphocholine in the original homogenate. Standards were run with each experiment (resulting in regression coefficients of 0.96–0.98), and the assay was linear with the number of cells (over the range of 20–150 cells).

As opposed to label turnover, the DAG, IP_3, choline, and phosphocholine mass assays are not dependent upon labeling to near equilibrium and, in the case of DAG and IP_3, they record only the active isomer.

1,2-bis(2-aminophenoxy)ethane N,N,N′,N′-tetraacetic acid (BAPTA) and heparin experiments

A 1.56:1 BAPTA:Ca stock solution (that buffers calcium to 345 nM) was made following our published method (26). Eight nanoliters were injected into each oocyte [intracellular free volume of 450 nl (14)] for a final intracellular BAPTA concentration of 0.65 mM. Twenty minutes after BAPTA injection (to allow for BAPTA diffusion from the injection site), 10 μg/ml (77 μM) metformin was added. The cells were incubated for 1.5 h after which 1 μM insulin was added. The ability of metformin to stimulate insulin action (induction of meiosis) was determined with these BAPTA-injected oocytes. Control cells were poked with a micropipette (to mimic BAPTA injection) and incubated for 1.5 h, and then insulin was added.

The ability of 10 μg/ml metformin to stimulate insulin action was also measured after a heparin stock solution (0.6 mg/ml; 8–16 nl) was injected to an estimated intracellular concentration of 10.7–21.4 μg/ml. Heparin blocks IP_3 action in the *Xenopus* egg (26).

Measurement of tyrosine kinase activity in Xenopus oocytes using Western blotting

At various times after metformin addition, groups of 35 oocytes were homogenized in 275 μl 12 mM Tris-HCl (pH 7.5), 40 μM phenylmethylsulfonylfluoride, and 1.6 mM EGTA (Sigma Chemical Co). To extract yolk proteins, 600 μl 1,1,2-trichlorotrifluoroethane, 230 μl 15 mM Tris, and 40 μl phenylmethylsulfonylfluoride were added to the homogenate. After centrifugation (10 min, 4 C, 16,000 × g), the supernatent was run on an 8.5% polyacrylamide gel and proteins were separated by electrophoresis. An equivalent amount of protein (as measured by Bradford or bicinchoninic acid-$CaSO_4$ assays) was added to each lane in the gel. Proteins were transferred to polyvinylidene difluoride membranes

(Sigma) and antiphosphotyrosine antibody linked to alkaline phosphatase (RC20 from Transduction Labs, Lexington, KY) was incubated with the blot. Color development was achieved by addition of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma). Band intensity was quantified by digitalizing the blot with a Scanjet IIcx (Hewlett-Packard, Minneapolis, MN), and the pixel intensity was plotted as a function of horizontal distance across the gel. Next, the area under the pixel intensity line was determined, and this area was plotted on the Y axis of Figs. 3 and 4 as the band intensity.

A 178-kDa band was identified as IRS-1 since it was detected by the antiphosphotyrosine antibody in homogenates of cells that had been treated with insulin. In addition, this band was equivalent to an expected molecular weight, and anti-IRS-1 identified a band at the same location. A band (sometimes a doublet) at 94 kDa was identified as the β-subunit of the IGF-1/insulin receptor due to its size and detection by antiphosphotyrosine antibody after insulin addition to whole cells.

Xenopus oocyte membrane-cortex preparation

A plasma membrane-cortex preparation was made from primed *Xenopus* oocytes in the manner described by Sadler and Maller (27). Briefly, oocytes were torn open in ice-cold buffer (10 mM NaCl, 10 mM HEPES, pH 7.9), and the membrane and associated cortex were washed of cytoplasm and nuclear material. Each group of 15 membrane-cortices were kept in 100 μl buffer at 15 C, and metformin or insulin was added for 15 min. Since there were four to six groups per treatment, each treatment represented the response of 60–120 membrane-cortices per experiment.

To measure IGF-1/insulin receptor kinase activity in this membrane-cortex preparation, 1 μM insulin or 10 μg/ml metformin, 250 μM [^{32}P]ATP (1776 cpm/pmol), and a substrate of the receptor tyrosine kinase, RR-SRC (0.5–2.5 mM)(Biomol, Plymouth Meeting, PA) were added to 30 membrane-cortices. After 15–20 min, membrane-cortices were homogenized in 10% TCA and 0.1% Triton X-100. Labeled substrate was separated from ATP by use of phosphocellulose microfuge tubes (Pierce, Rockford, IL) and quantified by liquid scintillation counting.

To measure IP_3 production in these membrane-cortices, 100–200 μl of 25% TCA were added at 15 min after hormone addition. The preparation was homogenized and analyzed for IP_3 mass (16).

In some experiments with membrane-cortices, calcium was buffered to low (45 nM), moderate (970 nM), or high (97 μM) levels through the use of EGTA (for methodology, see Ref. 26). Tyrphostin B46 (an inhibitor of tyrosine kinases) was added 10 min before insulin while an inactive form (tyrphostin A1) was added to control groups (both tyrphostins were from Calbiochem-Novabiochem, San Diego, CA). An affinity-purified antibody to the C terminus of the β-subunit of the human insulin receptor (Santa Cruz Biotechnology, Santa Cruz, CA) was added to cortices for 30 min before tyrosine kinase substrate and ATP were added.

Data analysis

Unless noted, a two-tailed Student's *t* test was used for tests of significance, and standard error bars are shown in figures and reported in the text.

Results

First, we examined whether metformin could induce meiotic cell division (GVBD) by itself. Over seven experiments, we could not detect any induction by 0.001–200 μg/ml metformin. However, metformin stimulated insulin action on *Xenopus* oocytes (Fig. 1). Note that metformin stimulated insulin action in two ways; the drug stimulated the rate at which insulin induced oocytes to undergo meiotic cell division, and it stimulated the number of cells that respond to insulin (by the final percentage of GVBD; see also Fig. 2A). There was variability among oocytes from different frogs; cells from other animals were less responsive to 1 μg/ml and required 10 μg/ml metformin for maximal stimulation. Maximal stimulation of insulin action occurred at metformin concentrations in the range of 1–10 μg/ml (~7.7–77 μM)(higher concentrations were not more effective). This concentration is similar to that found to be therapeutic in diabetic humans (1).

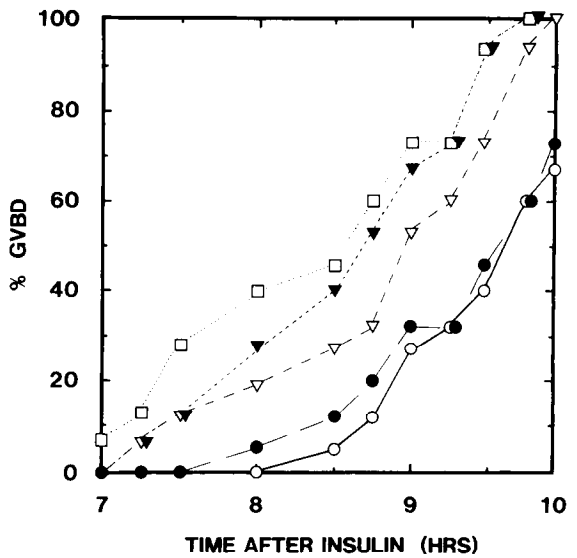

FIG. 1. Dose-response relationship of metformin stimulation of insulin action. The percentage of oocytes entering meiosis (% GVBD) is a measure of insulin action. Metformin was preincubated with oocytes for 0.5 h before insulin (1 μM) was added. The groups shown are: insulin alone (*open circle*), insulin and 0.001 μg/ml metformin (*closed circle*), insulin and 0.01 μg/ml metformin (*open triangle*), insulin and 0.1 μg/ml metformin (*closed triangle*), and insulin and 1 μg/ml metformin (*open square*). Similar results were obtained in four other experiments.

We then wanted to optimize the time of metformin addition relative to the time of insulin addition. We noted that a similar level of stimulation was obtained when metformin was added from about 0.5–3 h before insulin (Fig. 2). However, when metformin was added 5 min before insulin or up to hours after insulin addition, metformin did not stimulate insulin action (Fig. 2B).

In whole cells, metformin stimulates IGF-1/insulin receptor kinase

In a Western blot using an antiphosphotyrosine antibody, higher concentrations (1–10 μg/ml) of metformin increased phosphotyrosine in two proteins at 178 and 94 kDa (Fig. 3). Due to other evidence (see *Materials and Methods*), the band corresponding to a higher molecular weight protein hormone was probably IRS-1 and the other, the β-subunit of the IGF-1 or insulin receptor. Maximal receptor tyrosine phosphorylation required about 1 h (Fig. 4). Note that the dose and the preincubation time required for metformin stimulation action were similar whether one examines metformin stimulation of insulin action (*i.e.* GVBD) or metformin stimulation of the receptor kinase. The presence of a β-subunit doublet was often noted by ourselves and three other groups (28–30); a doublet could reflect IGF-1 receptor-insulin receptor hybrids (31).

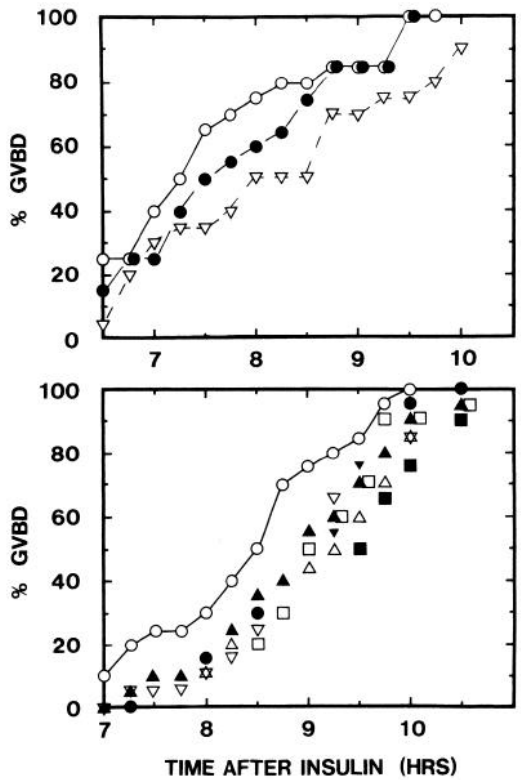

FIG. 2. Metformin stimulation of insulin action. Similar results were obtained with four other experiments. A, The *top figure* shows that insulin action (the rate of induction of meiosis) was accelerated when 1 μg/ml metformin was added 1 h (*closed circle*) or 3 h (*open circle*) before insulin. These groups were compared with the group that received insulin only (*open triangle*). B, In the *bottom* figure (cells were from a different frog), addition of metformin 0.5 h before insulin also stimulated (*open circle*). However, if metformin was added 5 min earlier than insulin (*closed circle*), 30 min after insulin (*open triangle*), 1 h after insulin (*closed triangle*), 2 h after insulin (*open square*), 3 h after insulin (*closed square*), or 4 h after insulin (*open triangle*), insulin action was not stimulated when compared with the insulin alone group (*closed triangle*). Although some points were omitted for clarity, all groups showed 90–100% GVBD by 10.5 h.

Metformin Increases IP_3 mass

Since we have found that insulin can stimulate IP_3 production in *Xenopus* oocytes (16), we wanted to determine whether metformin would enhance insulin's ability to produce IP_3. In three experiments, insulin (1 μM) raised IP_3 mass from control levels (59 ± 38 fmol/oocyte; n = 12) to 165 ± 72 fmol/oocyte (n = 11) ($P < 0.0001$) by 15 min. However, there was no further increase in IP_3 mass when a combination of metformin and insulin was used (10 μg/ml metformin was preincubated for 1 h; then 15 min after insulin addition, samples were analyzed: 130 ± 41 fmol/oocyte; n = 10; compare with above). A longer incubation time in insulin also did not result in a metformin stimulation of insulin's ability to raise IP_3 mass (after a 3-h incubation in insulin alone: 148 ± 15 pmol/oocyte; after a 1-h preincubation in metformin followed by a 3 h incubation in insulin: 140 ± 12 pmol/oocyte). Thus, there is no support for the idea that metformin acts through a stimulation of insulin's ability to produce IP_3. The fact that metformin did not elevate IP_3 mass above insulin-

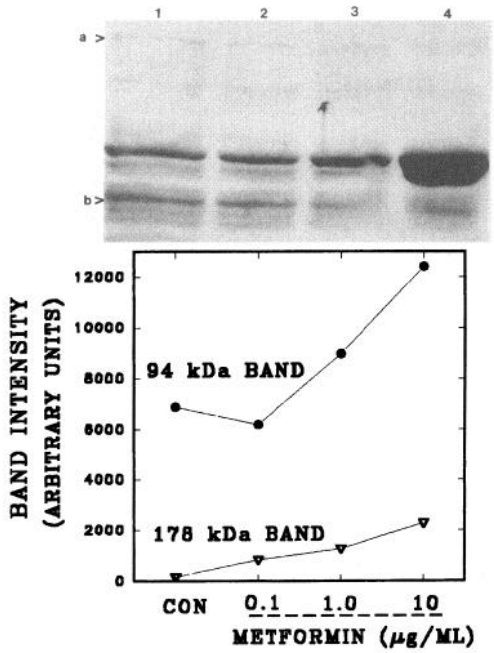

FIG. 3. Metformin action on the receptor tyrosine kinase activity in whole oocytes. A, After 1.5 h in 10 μg/ml (lane 1), 1 μg/ml (lane 2), 0.1 μg/ml metformin (lane 3), or no metformin (lane 4), oocytes were homogenized and equivalent amounts of protein were electrophoresed. Tyrosine phosphorylation on proteins was quantified by Western blotting. In the blot, a> represents the 178-kDa IRS-1 protein and b> represents the 94-kDa β-subunit of the IGF-1/insulin receptor. The large band above b are yolk proteins. B, The intensity of the 94- and 178-kDa bands were quantified (see *Materials and Methods*) and plotted for the control group (CON) and various metformin concentrations.

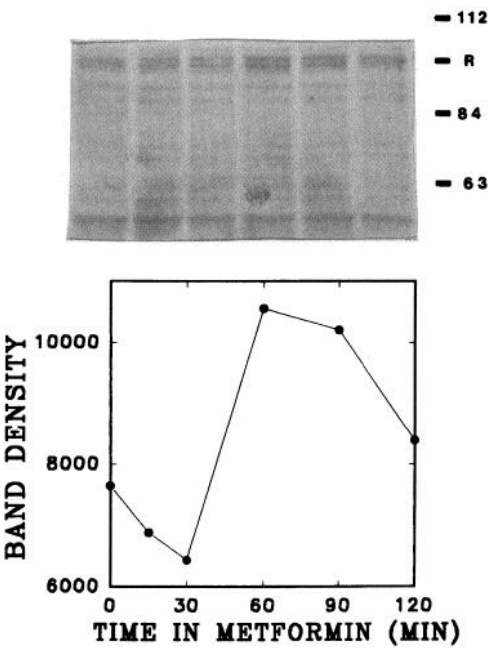

FIG. 4. Incubation time for metformin stimulation of tyrosine kinase activity. With antiphosphotyrosine antibodies and Western blotting, tyrosine kinase activity was recorded by band density. In the *upper* part, R represents the receptor tyrosine kinase, and the pixel density of this doublet is graphed *below*.

stimulated levels may mean that metformin acts upon the same pathway as insulin.

The experiments noted above included a metformin alone group, and this group showed elevated IP_3. We pursued these preliminary results and found that metformin was able to mimic insulin. Concentrations of metformin that stimulate insulin action also raise IP_3 mass (Fig. 5). We then measured the time course of IP_3 elevation by metformin (Fig. 6). Maximal IP_3 production also occurs at about 1 h after metformin addition to oocytes.

Blocking IP_3 action with two methods blocks metformin stimulation of insulin action

To examine the importance of the IP_3 increase in metformin stimulation of insulin action, IP_3 action was blocked with two different methods. First, we microinjected a calcium chelator (BAPTA) that would block metformin elevation of $[Ca^{++}]_i$ but still allow insulin to act upon the oocytes. Many laboratories have microinjected BAPTA into *Xenopus* cells to prevent $[Ca^{++}]_i$ increases (see Ref. 26). Second, we injected heparin; this competitive inhibitor of IP_3 binding to the IP_3 receptor also has been used extensively in *Xenopus* cells (26).

In four experiments in which BAPTA was preinjected into the oocyte, metformin was unable to stimulate insulin action. The time for half the control cells to enter meiosis was 5.1 ± 1.5 h after insulin addition (range of 4–7 h). As evaluated by a paired, two-tailed Student's *t* test, this time was not significantly different from the time required for metformin-treated, BAPTA-injected cells (4.9 ± 1.3 h). In five other experiments, the time course was not followed but metformin did not increase the final percentage of oocytes that entered meiosis after insulin addition.

Metformin was also not able to stimulate insulin action in oocytes preinjected with heparin. Half the control oocytes entered meiosis at 4.0 ± 1.6 h after insulin addition, whereas heparin-injected oocytes that had been treated with metformin entered at 4.1 ± 1.7 h (six experiments).

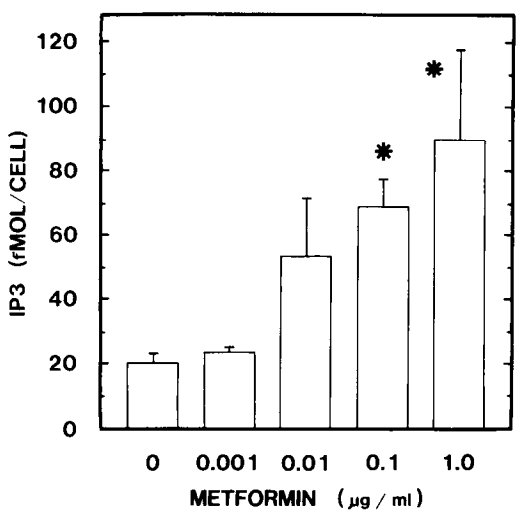

FIG. 5. Metformin action on phospholipase C. Metformin was incubated with three to five groups (each group had 15 oocytes) for 30 min before cells were analyzed for IP_3 mass. *Asterisks* denote significance at $P < 0.05$.

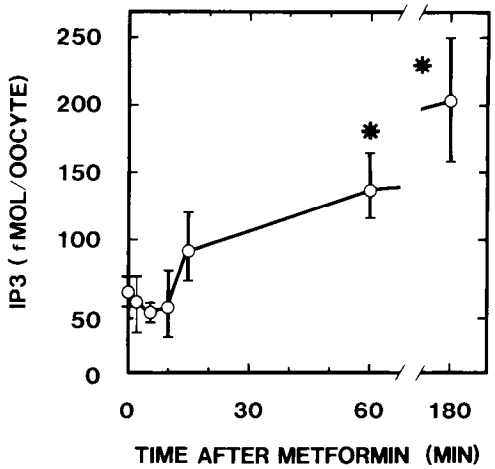

FIG. 6. Preincubation period required for metformin stimulation of phospholipase C. Metformin (1 μg/ml) was added to oocytes (15 per group, three to five groups per time point), which were homogenized at the times noted, and analyzed by an IP_3 receptor binding assay. *Asterisks* denote significance at $P < 0.02$.

Since the calcium chelator or heparin blocked metformin stimulation of insulin action, metformin may act (at least in part) through IP_3 production.

Metformin does not increase DAG, choline, or phosphocholine mass

Addition of insulin to the oocyte causes a large increase in DAG levels (24) that is probably due to the breakdown of PC rather than phosphatidyl 4,5-bisphosphate (PIP_2). We wanted to determine whether metformin could also stimulate a large production of DAG from non-PIP_2 sources. However, neither long (2–3 h; 115 ± 43 pmol/oocyte) nor short (5–30 min; 103 ± 36 pmol/oocyte) incubation times in 1 μg/ml metformin produced any measurable change in DAG mass (control DAG level was 113 ± 45 pmol/oocyte) (three experiments; within each experiment, each treatment group had five groups of 10 oocytes). A higher metformin concentration (10 μg/ml) and a longer incubation time (3 h) also did not change DAG levels (metformin-treated groups were 99 ± 13% of control; 11 determinations in two experiments). Thus, metformin does not share the ability of insulin to stimulate the production of large amounts of DAG. Since metformin does increase IP_3, one would expect that an equivalent amount of DAG would also be produced from PIP_2; this increase would represent an increase from a basal DAG level of 110 pmol to 110.1 pmol and was not measurable.

As another method to examine whether metformin could stimulate a large production of DAG, we measured whether metformin could stimulate PC-specific phospholipases. Activation of PC-specific phospholipase C would increase DAG and phosphocholine mass whereas activation of PC-specific phospholipase D would increase phosphatidic acid (which can be dephosphorylated to DAG) and choline.

However, metformin was unable to increase either choline or phosphocholine. One and one half hours after metformin (10 μg/ml) addition, oocytes were homogenized and analyzed by choline or phosphocholine mass. Control levels (choline: 25 ± 16 pmol/oocyte; phosphocholine: 8294 ± 7415

pmol/oocyte) were equivalent (by paired *t* test since there was much variation in basal levels between experiments) to metformin-treated groups (choline: 29 ± 27 pmol/oocyte; phosphocholine: 12,420 ± 11,790 (n = 8, and each group consisted of 50 oocytes). These results are in agreement with the DAG assay which, within its limits of sensitivity, found no metformin-induced increase in DAG. These results also suggest that the IP_3 path, rather than the DAG [and protein kinase C (PKC)] path, is more important in metformin action.

In a membrane-cortex preparation, metformin stimulates receptor tyrosine kinase activity

To examine early events in insulin action, we made use of an unusual property of the oocyte. After opening the cell with the tips of forceps, the plasma membrane and cortex can be isolated by washing away the cytoplasm and nucleus. This model system allows an examination of early cell signaling events in the absence of 99+% of cell protein. This plasma membrane-cortex (PMC) preparation was responsive to metformin or insulin; that is, when added to the PMC preparation, either drug or hormone stimulated tyrosine kinase activity and increased IP_3.

After determining the optimal time of incubation (~15–20 min), basal incorporation of $^{32}PO_4$ into tyrosine kinase substrate amounted to 1739 ± 1481 cpm. Metformin increased incorporation to 3393 ± 1747 cpm, and 500 μM tyrphostin B46 pretreatment (10 min) reduced ($P < 0.005$; paired *t* test) this to 1931 ± 1909 cpm (three experiments). Note that the inactive form of tyrphostin (A1) was added to controls, that the tyrosine kinase substrate RR-SRC does not have a serine or threonine, and absence of this peptide resulted in no measurable increase in phosphorylation after insulin or metformin addition. In terms of a dose-response relationship, 0.01 and 0.1 μg/ml metformin were unable to significantly increase tyrosine kinase activity but 1 μg/ml metformin raised activity 3.9-fold (one experiment). Higher concentrations of metformin greater than 10 μg/ml (200 μg/ml) were not more effective than 1–10 μg/ml.

Due to the variability of basal incorporation, the data from these and other experiments were summarized by the fold increase in tyrosine phosphorylation of substrate over control levels (Fig. 7). Insulin (1 μM) stimulated phosphorylation about 7-fold over control levels, whereas metformin was able to stimulate activity only about 3-fold. Elevating calcium to high levels (97 μM) or addition of more effective metformin derivative, MGBG, was able to stimulate tyrosine kinase activity to a level approximately equal to that stimulated by insulin. In a separate experiment, raising calcium to a more physiological level was also able to stimulate kinase activity (in the control group where calcium was buffered to 45 nM: 414 cpm were incorporated into RR-SRC; after calcium was raised to 970 nM: 2553 cpm).

Membrane-cortices were treated with insulin or metformin and homogenized, and proteins were separated on a 8% polyacrylamide gel. After autoradiography, metformin or insulin increased ^{32}P-phosphorylation of a 94 kDa band (data not shown). This result and the fact that tyrosine phosphorylation by insulin and metformin were not additive (control: 49 ± 19 cpm; insulin: 258 ± 174; insulin + metformin: 216 ± 42; two experiments) suggest that metformin acts directly upon the IGF-1/insulin tyrosine kinase receptor.

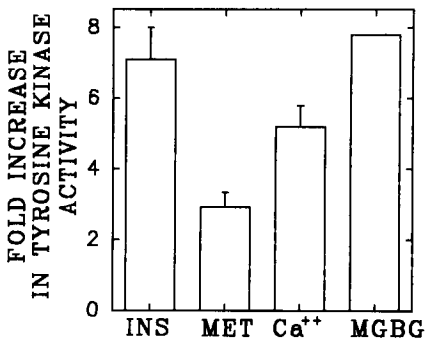

FIG. 7. Metformin and insulin action on tyrosine kinase activity in a membrane-cortex preparation from *Xenopus* oocytes. One micromolar insulin (INS; n = 13), 10 μg/ml metformin (MET; n = 7), an EGTA-$CaCl_2$ mixture that elevates and buffers calcium to 97 μM (Ca^{++}; n = 3), or a more effective metformin derivative (MGBG; n = 1; 2 mM) was added with tyrosine kinase substrate (1 mM RR-SRC) and 15 membrane-cortices. For the EGTA experiments, an EGTA solution that buffers calcium to 45 nM was added to control groups (this did not alter control values). After 15 min, the membrane-cortices were homogenized and added to phosphocellulose membranes. The counts per min incorporated into substrate was determined by liquid scintillation counting and expressed relative to basal levels.

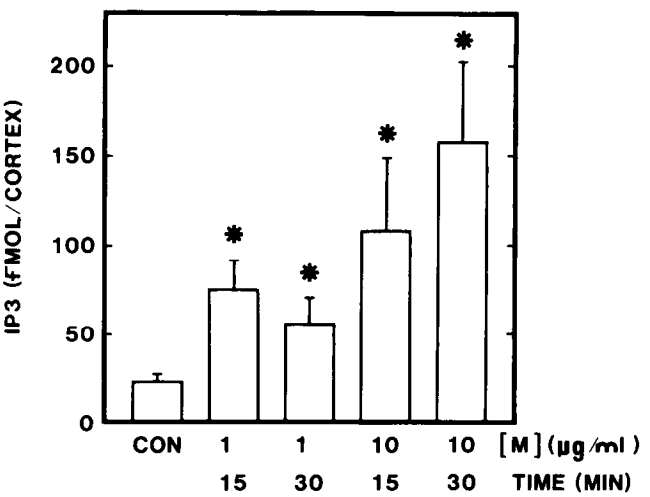

FIG. 8. The effect of metformin on IP_3 production. Two different concentrations (1 or 10 μg/ml) were used with two different incubation periods (15–30 min). After the incubation, membrane-cortices were homogenized and analyzed for IP_3 mass. *Asterisks* denote a significant ($P < 0.05$) difference as compared with control values.

In membrane-cortices, metformin also stimulates IP_3 production and tyrphostin inhibits this stimulation

We also used this PMC preparation to examine metformin's stimulation of another membrane event, that of IP_3 production. In these membrane-cortices (as with the prior work with whole cells), 1–10 μg/ml metformin was required to stimulate IP_3 production (Fig. 8). Note also that the time required for maximal production was similar to that required for maximal tyrosine phosphorylation (only ~15 min); this was much faster than metformin's action on whole cells

(~1 h). Since the increase in IP_3 was approximately the same as when metformin was added to the whole cell or the membrane-cortex preparation (compare Figs. 6 and 8), metformin probably stimulates IP_3 production in the plasma membrane-cortex area of the whole cell (not deep within the cell).

Using this PMC preparation, addition of a tyrosine kinase inhibitor decreased both insulin (Stith, B. J., K. Woronoff, and R. Espinoza, manuscript in preparation) and metformin stimulation of IP_3 production. In six experiments, 500 μM tyrphostin B46 was added 10 min before 10 μg/ml metformin, and, after another 15 min, the membrane-cortices were homogenized and analyzed for IP_3 mass. The combination of tyrphostin A1 (ineffective derivative) and metformin increased IP_3 mass from a basal level of 47 ± 15 to 221.0 ± 44 fmol/cortex ($P < 0.01$). When tyrphostin B46 was added before metformin, IP_3 production was reduced to 99 ± 23 fmol/cortex. In addition, 200 μM GTP-τ-S, which activates G proteins, stimulated IP_3 production in the membrane-cortex preparation and was not inhibited by tyrphostin B46 (control: 48.3 ± 19 fmol/cortex; tyrphostin A1 and GTP-τ-S: 161.4 ± 27.3; tyrphostin B46 and GTP-τ-S: 201.9 ± 22.2). These results suggest that both insulin and metformin stimulate IP_3 production through a tyrosine kinase (not through a G protein).

Metformin affects both insulin and IGF-1 action (32), and there is evidence for an IGF-1 receptor in the intact *Xenopus* oocyte (see Introduction). We wanted to examine whether a functional insulin receptor was present in the PMC preparation.

Although the oocyte has a high affinity ($K_d \approx 0.2$–1 nM) site for insulin that is typical for the insulin receptor, the actions of insulin in the intact oocyte are presumed to be through the IGF-1 receptor (insulin binds this receptor with a K_d of ~50–200 nM). To detect the presence of an active insulin receptor in the PMC, we examined the dose-response for insulin stimulation of tyrosine kinase and phospholipase C activity. Since maximal stimulation of tyrosine kinase or phospholipase C activity was found at very low concentrations of insulin (0.1 and 1 nM; Fig. 9), there is evidence that the PMC contains an active insulin receptor. There was a unexpected decline in kinase activity at 100 nM insulin, followed by an increase. The second rise in tyrosine kinase activity may reflect insulin binding to the IGF-1 receptor, and this binding may be related to a decline in insulin receptor activity at 100 nM insulin. Although tyrosine kinase activity is maximal at 0.1 nM insulin, note that this concentration does not produce maximal phospholipase C activation. This difference could reflect differences between cells from different animals. Since maximal responses were obtained by 1 nM insulin, this suggests that the insulin receptor (or at least a high affinity binding site similar to the insulin receptor) is present and active.

Further evidence for the presence of an insulin receptor is that an affinity-purified antibody to the C terminus (amino acids 1365–1382) of the β-subunit of the insulin receptor (final concentration, 83 μg/ml) was able to stimulate tyrosine kinase activity 5.0 ± 0.8-fold over controls (n = 9). Boiling (60 min) of the antibody before use inhibits its ability to stimulate the tyrosine kinase by 65 ± 6% (n = 4 for boiled and n = 4 for native antibody). By Western blots and due to the different amino acid sequence of the C termini of the two receptors (this is the site of negligible sequence homology between the two receptors), this antibody does not bind the IGF-1 receptor (Santa Cruz technical service, Santa Cruz, CA). IGF-1 or IGF-1 receptor antibody (to the C terminus) also stimulated tyrosine kinase activity (Stith, B. J., K. Woronoff, and R. Espinoza, manuscript in preparation). These data suggest that the insulin receptor is present but uncoupled in the intact oocyte, but this inhibition is lost when the cytoplasm is removed and the PMC preparation is obtained.

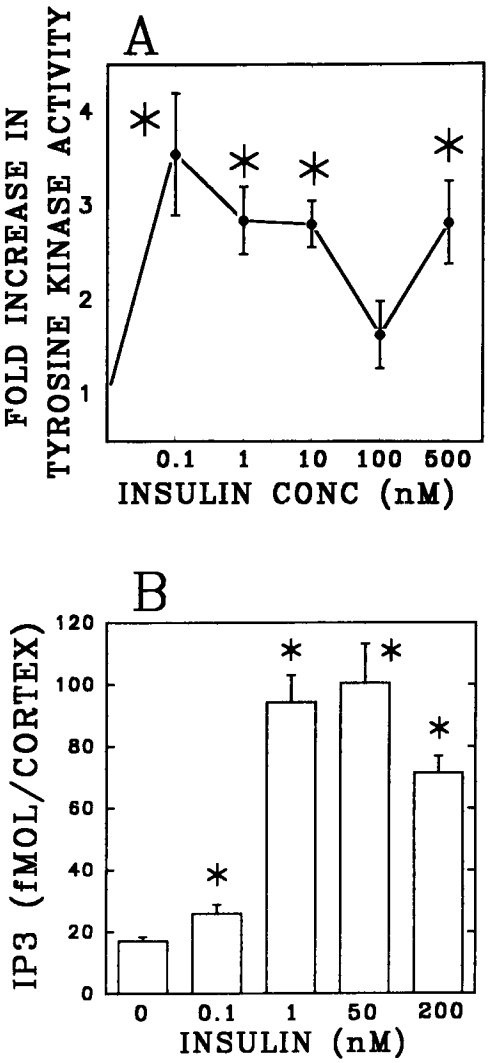

FIG. 9. The effect of low concentrations of insulin on tyrosine kinase and phospholipase C activity in the membrane-cortex preparation. Insulin and 0.1% fatty acid-free BSA were added to the membrane-cortex for 15 min and $^{32}PO_4$ incorporation into the tyrosine kinase substrate (A) and IP_3 production (B) were measured as described earlier. *Asterisks* denote significance at $P < 0.03$.

Discussion

Our experiments suggest six ideas about the mechanism of action of metformin. First, metformin must be internalized to act. The time required for metformin stimulation of insulin action in whole cells (induction of GVBD, tyrosine phosphorylation, IP_3 production) was about 1 h; this was much

slower than the equivalent responses measured after metformin addition to the membrane-cortex preparation. Entry of the drug into the cell (requiring an estimated 1 h) would account for this discrepancy. Khan *et al.* (33) found that metformin enters *Xenopus* oocytes, but that metformin linked to Sepharose beads did not enter or stimulate insulin action. Matthaei *et al.* (3) and Hundal *et al.* (34) found that a preincubation period of hours was required for metformin stimulation of insulin action on glucose transport in rat adipocytes and cultured L6 myotubes. One reason that Fischer *et al.* (35) suggested that insulin and metformin do not act upon the same signaling path was that insulin required minutes to stimulate glucose uptake into rat cardiomyocytes whereas relatively weak stimulation by metformin occurred only after a 90-min lag period. Since they demonstrated that this lag period was not due to protein synthesis, and in light of our data, we suggest that the lag period is required for entry of metformin into the cell.

Second, since metformin did not stimulate insulin action if added 5 min before insulin and since metformin requires about 1 h to build up within the cell, there is evidence that metformin stimulates an event that occurs within 1 h of insulin addition. We found that metformin activated two early membrane events: elevation of IGF-1/insulin receptor kinase activity and phospholipase C activation (*i.e.* elevation of IP_3 mass). Our evidence that metformin stimulates both insulin (in the PMC preparation) and IGF-1 receptors (based on whole oocyte results) may be reflected in mammalian tissue [metformin stimulates the action of insulin and nanomolar concentrations of IGF-1 on rat hepatocytes (33)].

There has been a controversy over whether the receptor is affected by metformin. Rossetti *et al.* (5) found that metformin increased rat liver wheat germ agglutinin-enriched insulin receptor tyrosine kinase activity. Matthaei *et al.* (3) and Jacobs *et al.* (4) found that metformin did not stimulate the receptor tyrosine kinase in rat adipocytes. The inability of some others to record stimulation of the receptor tyrosine kinase by metformin may be due to tissue differences or the inability to record small increases in the density of autoradiogram bands. Our system does not involve removal of the receptor from the membrane with detergent and is sufficiently sensitive to discriminate between weak metformin stimulation and basal levels of tyrosine phosphorylation.

Related to the weak efficacy of metformin, metformin (as opposed to insulin) may not induce GVBD since it is not a full mimetic of insulin and because the drug is a relatively poor activator of the receptor tyrosine kinase. The more effective metformin derivative MGBG was able to induce GVBD when added to oocytes (Ref. 33 and our unpublished results) and increased tyrosine kinase activity to high levels. However, elevated calcium also induced high levels of phosphorylation, yet elevated $[Ca^{++}]_i$ does not induce GVBD (16).

Third, metformin mimics insulin. That is, the drug can act independently of insulin to stimulate receptor kinase activity and IP_3 production. Although some have found no effect of metformin alone [*e.g.* rat adipocytes (3)], addition of metformin has been found to mimic insulin action in cultured human skeletal muscle (34, 36), human erythrocytes (37), rat cardiomyocytes (35), rat adipocytes (38, 39), and mouse soleus muscle (40).

Fourth, metformin acts through a tyrosine kinase to activate phospholipase C and produce IP_3. Since only one isozyme has been found to be regulated by tyrosine phosphorylation, metformin may stimulate the γ-isoform of phospholipase C.

Fifth, metformin's ability to stimulate insulin action may depend upon elevation of IP_3 and $[Ca^{++}]_i$. Since metformin induced a 3-fold increase in IP_3 levels, the drug should also elevate $[Ca^{++}]_i$ (see Ref. 26 for a discussion of *in vivo* IP_3 dose-response values in *Xenopus* cells). We have also shown (Fig. 7) that elevating calcium was able to stimulate a tyrosine kinase in the membrane-cortex preparation. There is weak support for the importance of elevated $[Ca^{++}]_i$ in metformin action through correlation (with whole cells, the concentration and preincubation time of metformin to stimulate insulin action were similar to those required to stimulate IP_3 formation). More importantly, a calcium chelator (BAPTA) or an IP_3 receptor blocker (heparin) were able to inhibit metformin stimulation of insulin action on the oocyte.

Many reports have found that elevated $[Ca^{++}]_i$ stimulates the insulin receptor (41–46). In addition, microinjection of IP_3 into the *Xenopus* oocyte stimulates insulin induction of GVBD (47). Finally, two groups have shown that extracellular calcium was needed for metformin stimulation of insulin action in cultured L6 myotubes (34, 48). The requirement for extracellular calcium may be related to a metformin-induced increase in IP_3 and $[Ca^{++}]_i$. Further study is needed to conclude that elevated $[Ca^{++}]_i$ plays a role in metformin action in human adipocytes or muscle.

Finally, there is evidence against the idea that metformin acts through the other pathway of polyphosphatidyl turnover; *i.e.* production of DAG and subsequent stimulation of PKC. Metformin was unable to mimic insulin in that the drug did not significantly increase DAG. Even if metformin was able to induce a small DAG increase, PKC is believed to inhibit the insulin receptor. With the use of different activators and inhibitors, we found that PKC inhibits initial insulin events in the oocyte (49). Thus, it is not likely that metformin acts through PKC activation.

In summary, we have used whole *Xenopus* oocytes or a plasma membrane-cortical preparation from these cells as a model system for metformin action. We present two lines of evidence that the insulin receptor is present and active in the plasma membrane-cortical preparation. Future work will examine the possibility that a soluble inhibitor blocks the insulin receptor in whole oocytes and that this inhibition is lost when the plasma membrane-cortex is isolated. There is additional data suggesting that the IGF-1 receptor is present and active in both the oocyte and membrane-cortex preparation. Although metformin stimulates the action of both insulin and IGF-1 in rat adipocytes, further work is needed to conclude that the results found with the *Xenopus* oocyte are relevant to the effects of metformin in the diabetic patient.

We demonstrate that metformin alone mimics insulin's ability to increase receptor tyrosine kinase activity and stimulate an enzyme (phospholipase C). Other data indicate that metformin stimulates an early step in the insulin path. Since whole cells respond slowly to metformin, yet isolated membrane preparations respond within minutes, there is support for the idea that metformin must enter the cell and act at an

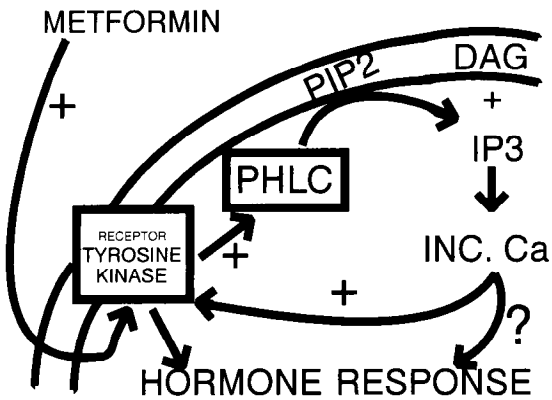

FIG. 10. Summary of model for mechanism of the antidiabetic drug metformin. After entry into the cell, metformin would activate the IGF-1/insulin receptor tyrosine kinase that, in turn, would activate the γ-isozyme of phospholipase C (PHLC) to produce IP_3. IP_3 would release calcium and initiate a positive feedback loop that would stimulate the receptor tyrosine kinase activity.

intracellular site. Through stimulation of phospholipase C and elevation of $[Ca^{++}]_i$, a positive feedback loop may also play a role in metformin action (Fig. 10).

Acknowledgments

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