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ACTIVATION OF GLYCOGENOLYSIS BY METHOTREXATE

INFLUENCE OF CALCIUM AND INHIBITORS OF HORMONE ACTION

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Abstract—The influence of Ca^{2+} and the possible action of hormone blockers on the activation of glycogenolysis by methotrexate were investigated. Methotrexate was inactive on glycogenolysis and oxygen uptake when the liver, depleted of intracellular Ca^{2+}, was perfused with Ca^{2+}-free medium. The action of methotrexate in calcium-depleted hepatocytes could be restored by the addition of extracellular Ca^{2+}. When Ca^{2+} was absent in the extracellular medium, but the intracellular stores were not depleted, methotrexate produced transient and progressively attenuated increases in glycogenolysis and oxygen uptake. Like many agonists, methotrexate produced transient increases in Ca^{2+} efflux. The action of methotrexate was not blocked by the antagonists of norepinephrine, phenylephrine, isoproterenol, vasopressin and angiotensin II. It was concluded that methotrexate acts through a Ca^{2+}-dependent mechanism, which is similar to that of the Ca^{2+}-dependent agonists. This action, however, seems not to be receptor mediated.

Methotrexate is well known for its anti-tumor action [1], but the compound also exerts metabolic effects. It has been demonstrated, for example, that the compound inhibits oxygen uptake in isolated mitochondria as well as in tumor cells [2–5]. Possibly, this is a consequence of several effects, especially inhibition of pyridine-nucleotide linked dehydrogenases. Further, the drug inhibits transplasma-membrane redox activity and ferricyanide-induced proton extrusion in HeLa cells [6]. In the liver, there is another effect which seems to be independent of the action on dehydrogenases: methotrexate increases hepatic glycogenolysis, as demonstrated in the isolated perfused rat liver [7]. At a 0.22 mM concentration of methotrexate in the perfusate, glucose release by the perfused liver was doubled [7], whereas significant effects on mitochondrial energy metabolism occurred only at substantially higher concentrations. The effect on glycogenolysis was accompanied by relatively small increases in oxygen uptake and by slight, frequently transient, increases in lactate and pyruvate release. These effects of methotrexate were not observed with folic acid, a biologically important analogue [7].

The purpose of the present work was to investigate the influence of Ca^{2+} and of inhibitors of hormone action on the activation of hepatic glycogenolysis by methotrexate in the hemoglobin-free perfused liver. The possible involvement of Ca^{2+} and/or hormone receptors was suggested by some experimental findings. First, activation of glycogenolysis by methotrexate seems not to be a consequence of an inhibition of mitochondrial energy metabolism, because in the intact liver cells this effect could not be detected [7]. Second, methotrexate increases Ca^{2+} efflux from isolated mitochondria [8]. If one considers that several agonists, including the α-adrenergic hormones, vasopressin and angiotensin II [9], increase glycogenolysis through Ca^{2+}-mediated mechanisms, it seems reasonable to expect participation of that ion in the action mechanism of methotrexate. The experiments should also help to clarify whether the action of methotrexate is mediated by some of the already identified hormone receptors.

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MATERIALS AND METHODS

Materials. The perfusion apparatus was built in the workshops of the University of Maringá. Hormones, hormone antagonists, and all enzymes and coenzymes used in the metabolite assays were products of the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium methotrexate was purchased from Lederle Parenterals (Carolina, Puerto Rico). The reagent grade chemicals were from Merck (Darmstadt, FRG), Carlo Erba (Sao Paulo, Brazil) and Reagen (Rio de Janeiro, Brazil). [^{45}Ca]$CaCl_2$ (NEZ-013) was purchased from the Du Pont Co. (Boston, MA, U.S.A.).

Liver perfusion. Male albino rats (Wistar strain; 200–250 g) received a standard laboratory diet (Purina) and water *ad lib.* prior to the surgical removal of the liver under pentobarbital anesthesia

(50 mg/kg). The perfusion technique described Scholz *et al.* [10] was used. The perfusion fluid was Krebs/Henseleit–bicarbonate buffer, pH 7.4, saturated with an oxygen/carbon dioxide mixture (95/5%) and containing fatty acid free bovine serum albumin (25 mg/100 mL). The fluid was pumped through a temperature-regulated (37°) membrane oxygenator prior to entering the liver via a cannula inserted in the portal vein. Samples of the effluent perfusion fluid were collected in 15- to 120-sec intervals and analyzed for glucose, lactate and pyruvate.

Analytical. Glucose, lactate and pyruvate were measured by standard enzymatic techniques [11]. Interference by methotrexate and metabolites at 340 nm was excluded by running a blank for each sample. The oxygen concentration in the venous perfusate was monitored continuously, employing a teflon-shielded platinum electrode, and the rates of uptake were calculated from the portal-venous concentration differences and the flow rate. The rates of metabolite release (glucose, lactate and pyruvate) were calculated from the venous concentrations and the flow rate. All metabolic rates were referred to the wet weight of the liver.

Radioactivity of $^{45}Ca^{2+}$ was measured by liquid scintillation spectroscopy. The following scintillation solution was used: toluene/ethanol (2/1) containing 5 g/L 2,5-diphenyloxazole (PPO)* and 0.15 g/L 2,2′-*p*-phenylene-bis(5-phenyloxazole) (POPOP).

RESULTS

Influence of extracellular calcium on the action of methotrexate. The first experiments were performed to verify if Ca^{2+} is involved in the activation of glycogenolysis by methotrexate. Panels A and B of Fig. 1 show the mean results of experiments in which the perfusion fluid was switched from "low-Ca^{2+}" to "high-Ca^{2+}" medium, either in the absence (Fig. 1A) or in the presence of 0.4 mM methotrexate (Fig. 1B). The low-Ca^{2+} medium contained only the traces of Ca^{2+} which generally contaminate distilled water. This medium also contained 0.1 mM EDTA in order to keep the concentration of free Ca^{2+} at a mimimum. The sudden introduction of 2.5 mM Ca^{2+}, as $CaCl_2$, in the absence of methotrexate produced a small and transient decrease in oxygen consumption, and a small and transient increase in glucose release. Lactate and pyruvate release were shifted to slightly higher steady states. This picture changed when methotrexate was present. As shown in Fig. 1B, the infusion of methotrexate during low-Ca^{2+} perfusion produced only a small and transient increase in glucose and lactate release. Oxygen uptake was not affected. As shown by Oliveira *et al.* [7], 0.4 mM methotrexate produces nearly 150% activation of glucose release when liver perfusion is carried out with high-Ca^{2+} medium. This is indeed what happened when Ca^{2+} was introduced suddenly into the perfusate: glucose release was more than doubled

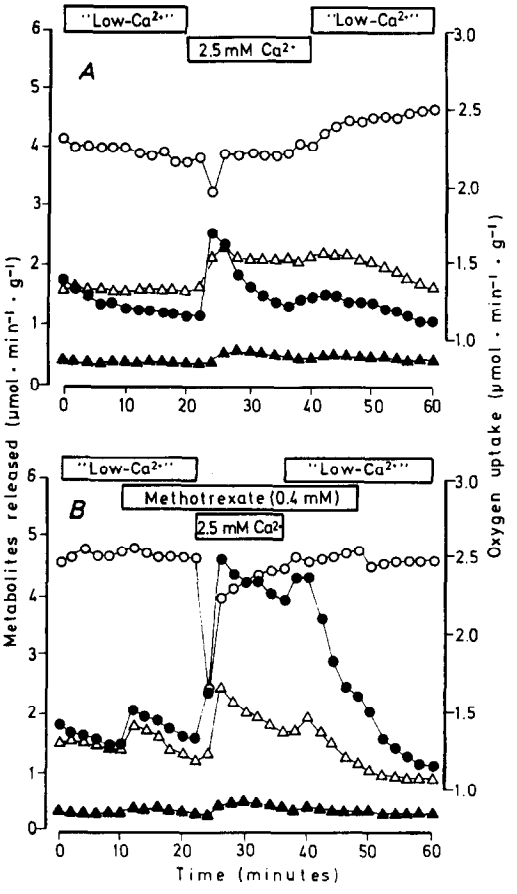

Fig. 1. Influence of Ca^{2+} on glycogen catabolism in the absence (A) and presence (B) of methotrexate. Livers from fed rats were perfused with Krebs/Henseleit–bicarbonate buffer (pH 7.4) containing 1.0 mM EDTA and without Ca^{2+} ("low-Ca^{2+}"), as indicated in the horizontal bars. In panel A, 2.5 mM Ca^{2+} was infused at about 20–40 min in the absence of methotrexate. In panel B, 0.4 mM methotrexate was infused at 10–48 min and 2.5 mM Ca^{2+} at 22–36 min. EDTA was excluded from the perfusion medium when Ca^{2+} was infused. Samples were taken for the measurement of glucose (●—●), lactate (△—△) and pyruvate (▲—▲). Oxygen was measured polarographically (○—○). Each data point is the mean of three experiments with identical protocol.

and remained elevated as long as the Ca^{2+} concentration in the medium was high. This is similar to what occurred when norepinephrine was used in place of methotrexate, but differed from the action of glucagon on glucose release, which was not further increased upon Ca^{2+} infusion (not shown). The Ca^{2+}-induced transient increase in oxygen consumption, which also occurred in the absence of methotrexate, was much more pronounced in its presence. The same may be said about the transient increase in lactate production. When 2.5 mM Ca^{2+} was replaced by a low-Ca^{2+} medium, glucose release declined even before the infusion of methotrexate was stopped.

* Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 2,2′-*p*-phenylene-bis(5-phenyloxazole)]; A_{angio}, [Sar^1,Ile^B]-angiotensin II; and A_{vasopr}, [β-mercapto-β,β-cyclopentamethylenepropionyl^1,*O*-Et-Tyr^2,Val^4,Arg^B]-vasopressin.

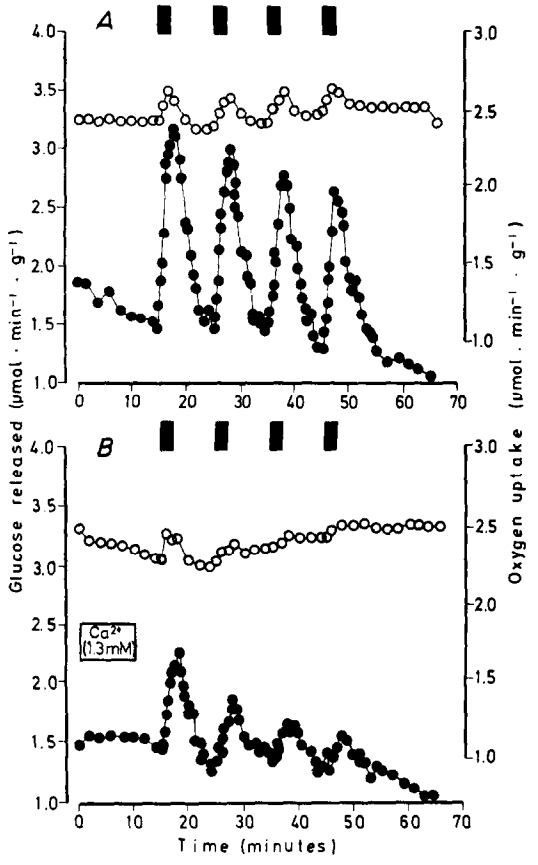

Fig. 2. Effects of methotrexate pulses on glucose release and oxygen uptake in livers perfused with "high-calcium" (A) and "low calcium" (B) perfusion media after loading the cells with calcium for 10 min. Livers from fed rats were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) as described in Materials and Methods. In panel A, the calcium chloride concentration was 1.3 mM ("high-calcium"). In panel B, Ca^{2+} was omitted from the perfusion fluid ("low-calcium"), except for a short period between 0 and 10 min during which the calcium chloride concentration was 1.3 mM. The black bars indicate the short 2-min pulses of 0.8 mM methotrexate. Samples were taken for the measurement of glucose (●—●). Oxygen uptake (○—○) was followed polarographically. Each data point is the mean of three experiments with identical protocol.

Influence of endogenous calcium. The involvement of Ca^{2+} in the activation of glycogenolysis by methotrexate is demonstrated further in the experiments of Fig. 2. In Fig. 2A, livers from fed rats were perfused from the beginning with a medium containing 1.3 mM Ca^{2+}, the same concentration used by Reinhart *et al.* [12] in their experiments with α-adrenergic agonists. Successive pulses of 0.8 mM methotrexate (2 min) produced large increases in glucose release and small increases in oxygen consumption. The successive activations of glycogenolysis were somewhat attenuated, but this can be attributed to a decrease in glycogen content, as suggested by the declining base line. When the livers were perfused with low-Ca^{2+} medium, as in the experiments of Fig. 2B, the effects of methotrexate were diminished and modified. In the experiments of Fig. 2B, Ca^{2+} was not present in the perfusion fluid during the pre-perfusion period, which is the time between the surgical removal of the liver and the stabilization of oxygen consumption (around 25 min). However, Ca^{2+} was infused during 10 min (1.3 mM), just after the onset of sample collection (between 0 and 10 min, as indicated by the horizontal bar in Fig. 2B). This allows intracellular reaccumulation of Ca^{2+} [12]. Under these conditions, the first infusion of methotrexate (0.8 mM) was far less effective on glucose release compared to the condition of high-Ca^{2+} perfusion (Fig. 2A). Furthermore, with the successive infusions the effects were increasingly reduced. These results suggest that intracellularly accumulated Ca^{2+} is also involved in the action mechanism of methotrexate, but exogenous Ca^{2+} is required in order to sustain continuous activation of glycogenolysis. The results in Fig. 2 are similar to those obtained by Reinhart *et al.* [12] with α-agonists, except for the fact that the oxygen consumption activation produced by methotrexate was less pronounced than that caused by phenylephrine [12].

Effect of methotrexate on $^{45}Ca^{2+}$ efflux. Hormones which increase glycogenolysis by Ca^{2+}-dependent mechanisms also promote transient net Ca^{2+} efflux from the liver cells [12, 13]. To determine if the same result occurs with methotrexate, experiments were performed in which the intracellular calcium pools of the liver cells were labeled with $^{45}Ca^{2+}$. This was accomplished by recirculating 100 mL of perfusion fluid containing 2.5 mM $CaCl_2$ and 0.5 μCi/mL $^{45}Ca^{2+}$ during 40 min. After this time, perfusion was continued with a Ca^{2+}-free medium, supplemented with 1.0 mM EDTA, in the non-recirculating mode. Samples of the effluent perfusate were collected and assayed for radioactivity. The results of typical experiments are shown in Fig. 3. Short infusions of 0.8 mM methotrexate produced increases in the rate of $^{45}Ca^{2+}$ efflux; the response to a second infusion was negligible. The effects of methotrexate on $^{45}Ca^{2+}$ efflux were comparable to those of norepinephrine, as revealed by the experiment shown in Fig. 3B. Similar increases in $^{45}Ca^{2+}$ efflux have been observed upon the infusion of several hormones [13, 14].

Influence of hormone antagonists. The results described thus far resemble the action of agonists operating by Ca^{2+}-dependent mechanisms [12, 14, 15]. Since the action of agonists is receptor-mediated, the possibility exists that methotrexate also operates as a glycogenolytic agonist. If it operates through one of the already described receptors, however, its action should be sensitive to one of the corresponding antagonists. To investigate this, experiments were performed with a series of antagonists. The results are shown in Figs. 4 and 5. Prazosin, an α_1-adrenergic antagonist, completely abolished the action of norepinephrine on glucose release, oxygen uptake and lactate production (Fig. 4A). However, it had no significant effect on the alterations caused by methotrexate (Fig. 4B). Experiments similar to those shown in Fig. 4 were

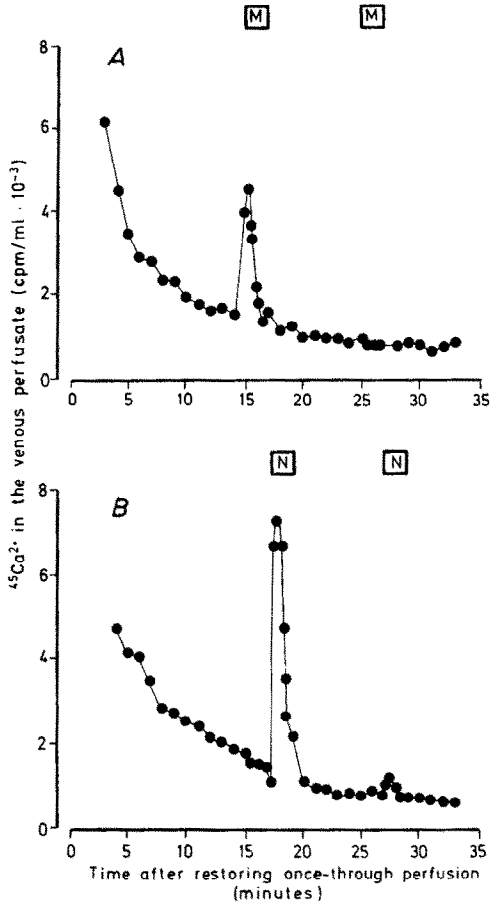

Fig. 3. Effect of methotrexate (A) and norepinephrine (B) on $^{45}Ca^{2+}$ net efflux from the perfused liver. Livers from fed rats were perfused initially in an open system with Krebs/Henseleit–bicarbonate buffer (pH 7.4), containing 2.5 mM $CaCl_2$, as described in Materials and Methods. At approximately 20 min after the surgical procedure, [^{45}Ca]-$CaCl_2$ was added to 100 mL of the perfusion fluid (0.25 μCi/mL) and the system was switched to a recirculating one. Recirculation was continued for 40 min. After this time, the perfusion fluid was switched to Ca^{2+}-free Krebs/Henseleit–bicarbonate buffer and perfusion was continued in the open mode. Samples (500 μL) for the measurement of radioactivity were collected at 15- to 60-sec intervals. Methotrexate (0.8 mM) and norepinephrine (0.5 μM) infusions were performed at the times indicated by the bars labeled with "M" and "N", respectively. The radioactivity in 500-μL samples was plotted against the time after restoration of the non-recirculating perfusion.

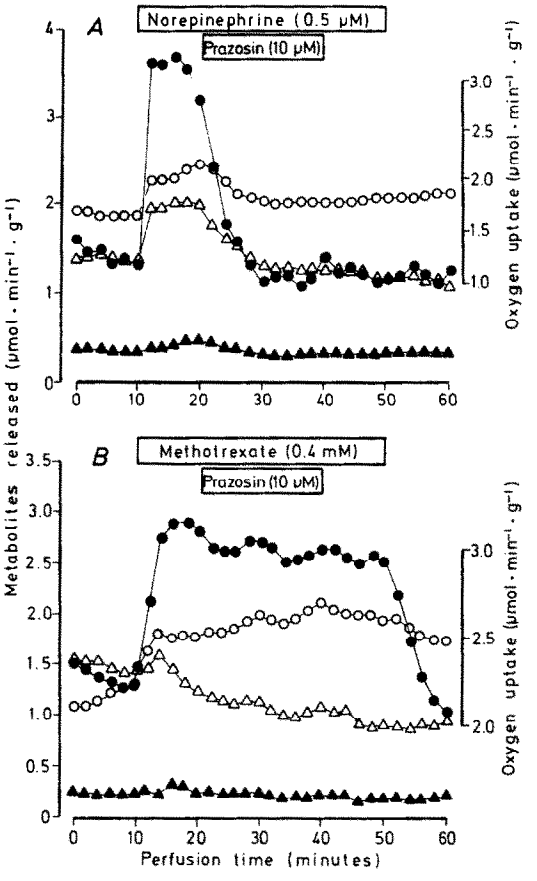

Fig. 4. Action of prazosin on the metabolic effects of norepinephrine (A) and methotrexate (B). Livers from fed rats were perfused with Krebs/Henseleit–bicarbonate buffer (pH 7.4), as described in Materials and Methods. In panel A, 0.5 μM norepinephrine and 10 μM prazosin were infused at the times indicated by the horizontal bars; in panel B, 10 μM prazosin and 0.4 mM methotrexate were infused at the times also indicated by the horizontal bars. Samples were taken for the measurement of glucose (●—●), lactate (△—△) and pyruvate (▲—▲). Oxygen was measured polarographically (○—○). Each data point is the mean of three experiments with identical protocol.

performed with other agonists and the corresponding antagonists. The results are summarized in Fig. 5, which shows the increases in glucose release in the presence of the agonists, methotrexate and the effects of the corresponding antagonists. The concentrations of the antagonists of phenylephrine, isoproterenol, angiotensin II and vasopressin used in the experiments were highly effective, as can be judged from their inhibitory effects. However, they were inactive on the increased rate of glucose release caused by methotrexate. The oxygen uptake increase caused by methotrexate was also insensitive to the antagonists (not shown). Consequently, it is unlikely that methotrexate operated through any of the hormone receptors investigated in this work.

DISCUSSION

The results of this work allow the conclusion that methotrexate increases glycogenolysis by a mechanism in which Ca^{2+} is involved. Several agonists are believed to operate by Ca^{2+}-dependent mechanisms, and these mechanisms present several characteristics which were also observed in this work for methotrexate: (1) methotrexate and several agonists are inactive on glycogenolysis when the liver, depleted of intracellular Ca^{2+}, is being perfused

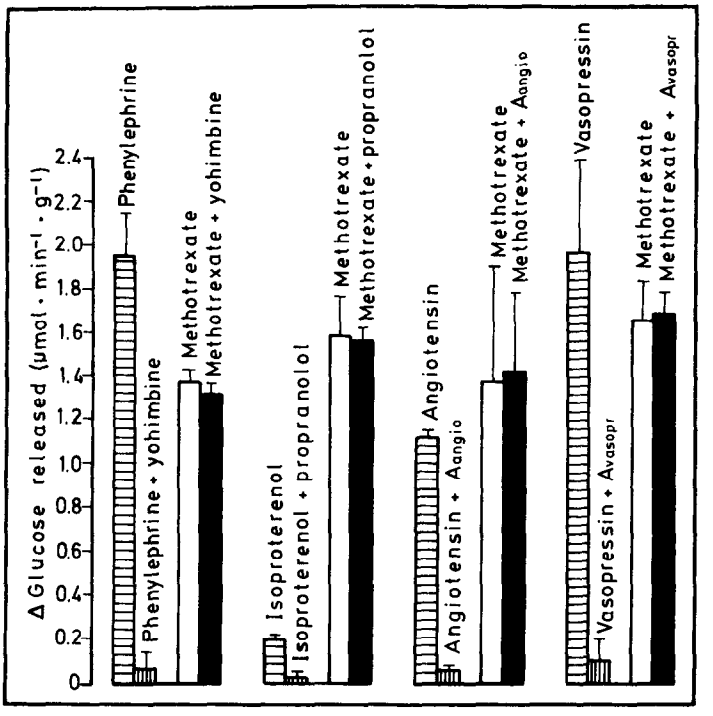

Fig. 5. Effects of various antagonists on the activation of glucose release by the corresponding hormone and by methotrexate. The experimental protocol was similar to that described in the legend of Fig. 4 with minor variations. The concentrations of methotrexate were between 0.35 and 0.45 mM. The concentrations of phenylephrine, isoproterenol, angiotensin II and vasopressin were 2.0, 20.0, 0.01 and 0.01 μM, respectively. The concentrations of yohimbine, propranolol, [Sar^1,Ile^8]-angiotensin II (A_{angio}) and [β-mercapto-β,β-cyclopentamethylenepropionyl^1,*O*-Et-Tyr^2,Val^4,Arg^8]-vasopressin (A_{vasopr}) were 100.0, 50.0, 0.1, and 0.1 μM, respectively. The bars represent the mean (±SEM) increases (N = 3) in glucose release observed under each of the specific conditions.

with Ca^{2+}-free medium [16]; (2) the action of methotrexate and of Ca^{2+}-dependent agonists in the Ca^{2+}-depleted hepatocytes can be restored by the addition of extracellular Ca^{2+} [15]; (3) when Ca^{2+} is absent from the extracellular medium, but the intracellular stores are not depleted, both methotrexate and Ca^{2+} dependent agonists produce transient and progressively attenuated increases in glycogenolysis [12]; (4) like several agonists, methotrexate mobilizes the intracellular Ca^{2+} stores and, therefore, increases efflux [9]; (5) like most Ca^{2+}-dependent agonists, methotrexate increases oxygen uptake and has a small, frequently transitory, stimulatory effect on lactate and pyruvate production (glycolysis) [9, 17].

In spite of the similarities listed above, the action of methotrexate was not inhibited by the specific blockers of norepinephrine (prazosin, an α_1-adrenergic blocker [17]) phenylephrine (yohimbine, an α_1- and α_2-adrenergic blocker [17]), angiotensin II [18, 19] and vasopressin [20], nor was it blocked by the antagonist of isoproterenol, a β-adrenergic blocker [21]). These observations exclude the α- and β-adrenergic receptors as a site of action for methotrexate. The possibility that the drug acts through the glucagon receptor is also unlikely, because glucagon characteristically inhibits glycolysis [22, 23], whereas methotrexate, depending on the concentration and the conditions, is either without effect or produces activation [7]. Moreover, as already mentioned in the Results, the effect of glucagon on glucose release occurred even in the absence of Ca^{2+} and was not increased further upon Ca^{2+} infusion, whereas the opposite occurred with methotrexate. Taken together, all these observations are strong arguments against the participation of cyclic AMP in the mechanism of action of methotrexate. The effects of the drug are also different from those compounds acting through purinergic receptors, i.e. adenosine diphosphate and analogues. The glucose release activations produced by the latter are rather transitory [24], but methotrexate generated stable increases in the presence of Ca^{2+}. Thus, it is unlikely that the mechanism of action of methotrexate involves the purinergic receptors, inasmuch as the chemical structure of the drug shows little resemblance to that of adenosine diphosphate and analogues.

If the action of methotrexate is not mediated by any of the already described receptors, two possibilities remain: it could be that methotrexate acts through an up to now unknown receptor or that it acts intracellularly, with or without participation of secondary messengers, at one or various steps of

the complex sequence of events by which Ca^{2+} and other factors increase the activity of glycogen phosphorylase [25–27]. There is no doubt that the compound permeates the cell membrane, because it exerts its therapeutic effect inside the cells [1]. Moreover, in the liver cells, the drug is metabolized and one cannot exclude the possibility that some of its metabolites could be responsible for the activation of glycogenolysis.

The hypothesis that methotrexate acts intracellularly, without participation of receptors and secondary messengers, has some experimental support. It has been shown that the drug simultaneously decreases influx and increases efflux [8] of Ca^{2+} in isolated rat liver mitochondria. The latter observation is of particular importance because it reveals that methotrexate by itself can affect Ca^{2+} transport. It is indeed possible that the transient increases in Ca^{2+} efflux observed in this work, which must result from the mobilization of intracellular Ca^{2+} stores, are a consequence of that phenomenon [8]. The mobilization of intracellular Ca^{2+} pools, however, can be solely responsible for the initial phase of glycogenolysis activation. In the subsequent phases, as can be deduced from the experiments presented in this work, extracellular Ca^{2+} certainly participates. The mechanism by which methotrexate promotes the participation of exogenous Ca^{2+}, however, cannot be inferred from the data presented in this work. This is an aspect which remains obscure even for the intensively studied α-adrenergic hormones [28]. If methotrexate acts directly, without participation of secondary messengers or derived metabolites, it will become useful as a tool in the study of the action mechanism of Ca^{2+}-dependent agonists.

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