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ACTIVATION OF GLYCOGEN SYNTHASE IN LEUKOCYTES IS INHIBITED BY THE INTRACELLULAR Ca-ANTAGONIST TMB-8.

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SUMMARY: Activation of glycogen synthase in human polymorphonuclear leukocytes by addition of glucose or glucosamine to the incubation buffer is prevented, and already activated glycogen synthase is inactivated, by addition of 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate, which prevents mobilization of Ca^{2+} from membrane stores. The results suggest that glycogen synthase phosphatase or a modulator of the enzyme is dependent on Ca^{2+}.

With the glycogen-protein complex of human polymorphonuclear leukocytes, it was originally observed that phosphorylase phosphatase and synthase phosphate activity separated by chromatography on concanavalin A-Sepharose (1). Recently (H. Juhl, N. Nahas and V. Esmann, unpublished observations), both these enzyme activities were found associated with the microsomal fraction and upon solubilization separated on Sephadex G-200 with apparent molecular weight of 380 000 (synthase phosphatase activity) and 250 000 (phosphorylase phosphatase activity). Ethanol treatment released from both enzyme activities a catalytically active M_r 30 000 fragment and increased the phosphorylase phosphatase activity four-fold, while the synthase phosphatase activity remained stable. The holoenzymes as well as the M_r 30 000 fragments showed different temperature sensitivities.

Abbreviation: TMB-8, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate.

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Involvement of Ca^{2+} in the regulation of the phosphorylation state of glycogen synthase was suggested in experiments with the glycogen-protein complex, when the rate of dephosphorylation of phosphorylated synthase was found increased by both Mn^{2+} and Ca^{2+} (1). In the present experiments the role of intracellular Ca^{2+} was investigated using TMB-8, which acts by preventing mobilisation of Ca^{2+} from membrane stores ((2) and unpublished observations). TMB-8 was also found to inhibit adenylate cyclase activity and prevent activation of unphosphorylated phosphorylase kinase in leukocytes.

EXPERIMENTAL PROCEDURE

TMB-8 was synthesized by Clauson-Kås Company, Copenhagen and $^{54}Mn^{2+}$ was from the Radiochemical Centre, Amersham. All other chemicals were purchased as in (3).

Human polymorphonuclear leukocytes were prepared (3) and suspended at 4 x 10^7 cells/ml in buffer: 138 mM NaCl/ 2.7 mM KCl/8.1 mM $NaHPO_4$/1.5 mM KH_2PO_4/0.6 mM $CaCl_2$/1.0 mM $MgCl_2$/10 mg/ml human serum albumin, pH 7.4. The suspension was incubated at 37°C and a preincubation period of 60 min was used to reduce glycogen concentration and allow synthase dephosphorylation to occur. The experimental period was started with the addition of 1 mM glucose or 1 mM glucosamine. TMB-8 was added two min before start of the experiments.

For assay of glycogen synthase and phosphorylase 400 µl samples were added to 100 µl ice-cold 250 mM Tris (pH 7.4), 50 mM EDTA/25 mM EGTA/150 mM NaF/5 mM dithiotreitol, sonicated, centrifuged, and assayed as in (3). The samples for determination of cyclic AMP were taken, assayed, and expressed as in (3). Damage to cells were routinely assayed by determination of lactate dehydrogenase leakage and never exceeded 5%. Glycogen synthase activity is expressed as RI (ratio of independence), which is the ratio of enzyme activity assayed without and with 6.7 mM glucose-6-P. The relation of RI to the phosphorylation state of synthase has been discussed in (4). For Mn-efflux studies leukocytes, 5 x 10^6/ ml, were incubated in Hank's balanced salt solution for one hour in the presence of $^{54}Mn^{2+}$ (0.01 mCi/ml, specific activity 100 µCi/µg Mn). The cells were washed three times and Mn^{2+}-efflux was measured by a silicone-oil method.

RESULTS AND DISCUSSION

In confirmation of earlier results (5), addition of glucose to leukocytes with reduced glycogen content rapidly inactivated phosphorylase and caused a transient acti-

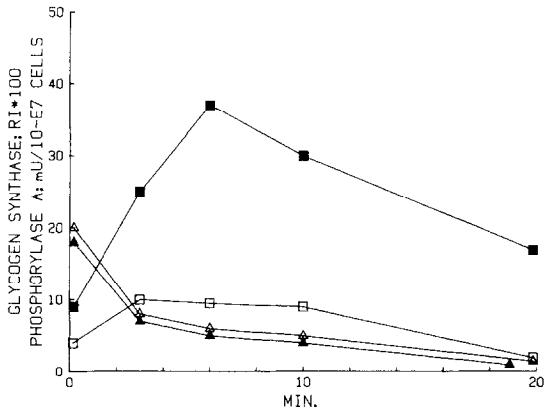

Fig. 1. Leukocytes (4 . 10^7/ml) were incubated for one hour without glucose. At zero time 1 mM glucose was added. TMB-8 (0.7 mM) was added at -2 min. RI of glycogen synthase without (■), and with (□) TMB-8. Phosphorylase a activity without (▲) and with (△) TMB-8. (The shown experiment is representative for 4 separate expt.).

vation of glycogen synthase with maximum RI at 6 min (Fig. 1). Also, as observed previously, glucosamine caused a sustained activation of synthase lasting 30 min and a slight inactivation of phosphorylase (Fig. 2). TMB-8 prevented activation of synthase whether mediated by addition of glucose or glucosamine, while the effect on phosphorylase was different in the two cases. TMB-8 did not affect glucose inactivation of phosphorylase (Fig. 1), but surprisingly caused a slow phosphorylase activation in glucosamine stimulated cells (Fig. 2). When glycogen synthase was activated by addition of glucosamine the further addition of TMB-8 causes an inactivation of synthase (Fig. 3), presumably from the activity of cyclic AMP independent synthase kinase. As in Fig. 2, TMB-8 elicits a slow increase in phosphorylase a activity. Addition of glucose now allows phosphorylase phosphatase activity to manifest itself, while synthase phosphatase remains inactive. Control experiments showed that when TMB-8 alone was added to one hour starved cells, phosphorylase a remained unchanged, while RI of synthase decreased

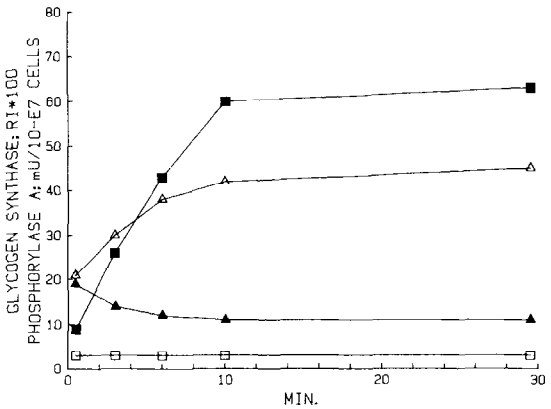

Fig. 2. Experimental conditions and symbols as in Fig. 1, except that 1 mM glucosamine was added at zero time.

from 0.10 to 0.05 (not shown). Cyclic AMP concentration was constant and at basal levels (1 pMol/10^6 cells) in all types of experiments (not shown).

The glucose-mediated inactivation of phosphorylase (Fig. 1 and 3) is thought mediated by allosteric modification of phosphorylase a (6), making the enzyme a better substrate for the phosphorylase phosphatase, and it is not surprisingly that TMB-8 does not influence the course of events. However, the slow increase in phosphorylase a activity

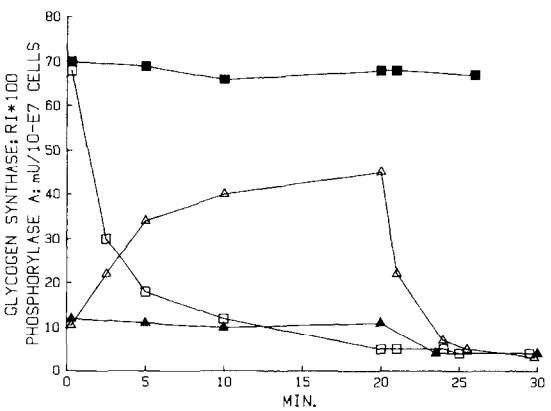

Fig. 3. Leukocytes (4 . 10^7/ml) were preincubated for one hour without glucose and then for 30 min with 1 mM glucosamine. At zero time 0.7 mM TMB-8 was added and at 20 min 1 mM glucose was added to all incubations. RI of glycogen synthase without (■) and with (□) TMB-8. Phosphorylase a without (▲) and with (△) TMB-8. (The shown experiment is representative for 5 separate expt.).

caused by TMB-8 in glucosamine stimulated cells (Fig. 2 and 3), which may vary somewhat in extent, but has been repeatedly observed, is unexplained. Thus, activation of phosphorylase kinase by phosphorylation is ruled out, as the concentration of cyclic AMP did not increase. Also, activation of unphosphorylated phosporylase kinase by Ca^{2+} occurs within one min of stimulating Ca^{2+} release and is completely prevented by TMB-8 (unpublished observations). Finally, phosphorylase phosphatase is not inhibited, but remains potentially active under these experimental circumstances (Fig. 3).

The glucose mediated activation of glycogen synthase is not caused by glucose, but by the allosteric activator glucose-6-P (1) and may be prolonged by using glucosamine, as glucosamine-6-P is not metabolized. The phosphorylation state of glycogen synthase is the resultant of the action of kinases and phosphatase(s). The effect of TMB-8 might be thought caused by an effect on the activity of the kinases. Four kinases are known to phosphorylate glycogen synthase I (7), the cyclic AMP dependent protein kinase, the cyclic AMP independent synthase kinase, the cyclic AMP independent phosvitin kinase, and (muscle) phosphorylase kinase. Neither of these enzyme activities are modified by Ca^{2+}, except unphosphorylated phosphorylase kinase, which is activated by Ca^{2+}. A Ca-calmodulin dependent synthase kinase has been found present in liver (8), but it is not known whether a similar enzyme is present in leukocytes. However, in neither of these cases will a decreased cytosolic Ca^{2+} concentration lead to an increased kinase activity. An increased activity of the cyclic AMP dependent protein kinase and of phosphorylated phosphorylase kinase is ruled out as the concentration

of cyclic AMP was not increased and phosphorylase not activated. What remains is the possibility of an effect of TMB-8 on a hitherto unknown Ca-inhibited protein kinase or on the activity of glycogen synthase phosphatase. The presence of a Ca-inhibited protein kinase in leukocytes cannot be ruled out, but the increased rate of activation of glycogen synthase by Ca observed using the glycogen-protein complex occurred in the abscence of any protein kinase activity using Mg-ATP as the other substrate (1). The effect of TMB-8 on glycogen synthase phosphatase activity should then be an effect of inactivation, implying that synthase phosphatase or a modulator of the enzyme is dependent on Ca^{2+}. Neither of the known phosphoprotein phosphatases of glycogen metabolism have been reported activated by Ca^{2+} (9, 10, 11), but activation by Mn^{2+} and Mg^{2+} is well known. It is unlikely that TMB-8 acts by inhibiting Mg-dependent processes as Mg-ATP dependent phosphorylations proceed normally in the presence of TMB-8. To exclude the possibility of an effect on the availability of Mn^{2+} from a hypothetical store, Mn^{2+}-efflux was studied after labelling the cells with ^{54}Mn. No efflux of Mn^{2+} occurred neither in the abscence nor the presence of TMB-8, signifying that no stores of Mn^{2+} have been labelled (data not shown). Thus, the inhibitory effect of TMB-8 on synthase phosphatase activity cannot be assigned to a possible inhibition of mobilisation of Mn^{2+} from a hypothetical store.

In conclusion this preliminary evidence has shown a functional difference between glycogen synthase phosphatase and phosphorylase phosphatase in the intact cell which may implicate a structural difference, synthase phosphatase activity being dependent on Ca, while phosphorylase phosphatase activity

is not. While these activities reside in the same protein in rabbit muscle (12, 13), separation of synthase phosphatase and phosphorylase phosphatase has also been observed in liver (11, 14, 15).

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