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EFFECT OF PROSTAGLANDINS ON PHOSPHORYLASE *b*

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Summary

Prostaglandins at mM concentration are able to induce a small activation to rabbit muscle glycogen phosphorylase *b* (1,4-α-D-glucan: orthophosphate α-D-glucosyltransferase, EC 2.4.1.1) in the absence of AMP. The extent of this activation depends on the nature of the molecular structure of prostaglandins. Saturated and unsaturated higher fatty acids were unable to substitute for prostaglandins. The main findings of our studies can be summarized as follows: (1) Prostaglandins inhibit the AMP-induced activation of phosphorylase *b*. (2) Modification of the AMP binding site with 2,3-butanedione could not inhibit the activity induced by prostaglandins. (3) Enzyme activation by prostaglandins is stimulated by spermine. (4) Phosphorylase *b* activation by prostaglandins and the observed stimulation of this activation by spermine were found to be temperature dependent. (5) Prostaglandins affect the quaternary structure of phosphorylase *b* inducing a partial enzyme tetramerization which is enhanced in the presence of spermine. The extent of this tetramerization is temperature dependent.

Introduction

It is known that rabbit skeletal muscle phosphorylase (1,4-α-D-glucan orthophosphate α-D-glucosyltransferase, EC 2.4.1.1) exists in two forms, an inactive form requiring AMP for activity which was named phosphorylase *b* and an active one in the absence of AMP which was named phosphorylase *a*. It is well established that the interconversion of these two forms of the enzyme arises from specific enzymic phosphorylation and dephosphorylation reactions [1]. Known physiological activators of phosphorylase *b* are the nucleotides AMP

and IMP [1]. High concentrations of substrate anions and several anions high on the Hofmeister series are also known to activate phosphorylase *b* in absence of AMP [2]. Furthermore, it has been reported that two polycationic molecules, spermine and a phosphopeptide derived from the NH_2-terminal region of phosphorylase *a* were able to induce an AMP-independent enzyme activity especially at low temperature [3]. Recently, activation of phosphorylase *b* in absence of AMP was achieved by several hydrophobic compounds [4—6]. The activation induced by hydrophobic solvents was attributed to a stabilization of the most active enzyme conformation [4]. It is also well known that the affinity of phosphorylase *b* for AMP may be enhanced by fluoride, divalent metal ions and polyamines [1], while phenothiazines [7], polycarboxylates [8], sulfate anions and sulfated polysaccharides [9] were also reported as stimulators of the AMP-dependent activity of the enzyme.

In the present study we have examined the interaction of phosphorylase *b* with prostaglandins. These compounds bear hydrophobic moieties and are known to affect a wide range of biological processes [10]. It was found that prostaglandins at mM concentration (non-physiological, [11]) enhanced the residual activity (AMP-independent activity) of phosphorylase *b* and that this activation was stimulated by spermine.

Materials and Methods

Phosphorylase *b* was isolated from rabbit skeletal muscle according to the method of Fischer and Krebs [12]. The enzyme was recrystallized four times, passed through a Sephadex G-25 column and then treated with acid washed Norit (1 mg/mg protein) to remove AMP [3] Oyster glycogen was purchased from British Drug Houses freed of AMP as described by Helmreich and Cori [13]. Glc-1-*P*, AMP and *t*-butanol were products of British Drug Houses, while spermine tetrahydrochloride was obtained from Serva. Crystalline prostaglandins were generously provided by the Upjohn Company (Kalamazoo, MI) through the kindness of Dr. A.D. Argoudelis. They were prepared for use by dissolving an appropriate amount of prostaglandin either in absolute ethanol or in the assay buffer, when it was possible, immediately before use and kept at 4°C until added to the incubation vessel. Saturated, unsaturated fatty acids, distearoyl phosphatidylcholine and Triton X-100 were products of Sigma. All other reagents were of the highest purity commercially obtainable.

Phosphorylase *b* was assayed in the direction of glycogen synthesis [14] and the reaction mixture had a final volume of 0.2 ml. P_i released in the reaction was measured by the method of Fiske and SubbaRow [15]. Activity of phosphorylase *b* was expressed in μmol product formed/min per mg enzyme. Enzyme concentration was measured spectrophotometrically using the extinction coefficient ($E^{1\%}_{1cm}$) at 280 nm of 13.2 [16]. Sedimentation velocity experiments were carried out using an MSE Centriscan 75 preparative and analytical ultracentrifuge with a 10-mm single-sector cell at a rotor speed of 60 000 rev./min. Sedimentation coefficients were obtained from direct measurements of the scanner traces, taken at 6-min intervals using a schlieren optical system, corrected for viscosity and density of the buffer to water at 20°C. The percentage of components with different sedimentation coefficients was estimated by

direct measurement of the area under the sedimentation peaks of the scanner traces (F_t) at the time t corrected for the radial dilution by multiplying the square of the distance of the peak (r_t^2) relative to the square of the distance of the meniscus (r_m^2) from the center of rotation, since the concentration, c, is proportional to F_t (r_t^2/r_m^2).

Results

Activation of phosphorylase b by prostaglandins in the absence of AMP

Prostaglandins increased the activity of phosphorylase *b* in the absence of AMP. Fig. 1 shows the activation of phosphorylase *b* in the absence of the nucleotide, as a function of the concentration of several prostaglandins. The residual activity of phosphorylase *b* in the presence of substrates but in the absence of AMP was taken as control value (100%). This activity amounted to 0.9% of that in the presence of substrates and AMP (1 mM). As can be seen, the residual activity of the enzyme was initially stimulated with increasing prostaglandin concentrations reaching a peak value which was dependent on the kind of prostaglandin used. Further increase of prostaglandin concentration negatively affects the activity of the enzyme. The second step of the activation curves, analogous to that observed with hydrophobic solvents [4], could not be detected for all prostaglandins used because the majority of them have very low solubility in aqueous solution and therefore their concentration in the assay mixture could not be increased. Fig. 1 also shows, that the molecular structure

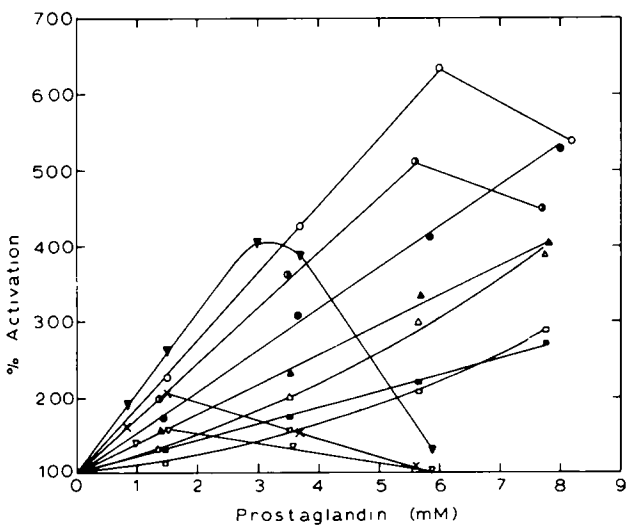

Fig 1 The effect of prostaglandins on the activity of phosphorylase *b* in the absence of AMP The enzyme (35 μg/ml) was assayed at 30°C in 1 mM EDTA/30 mM 2-mercaptoethanol/40 mM glycerol-2-*P* buffer, pH 6 8, with 32 mM Glc-1-*P*, 1% glycogen and various concentrations of prostaglandins. The prostaglandins were added in the assay system dissolved in ethanol and the final concentration of ethanol in the system was 5% (v/v). The activity induced by ethanol was deducted from each rate measurement The activity observed in the absence of prostaglandins and AMP (0.8 μmol P_i/mg per min) was taken as 100% activation Prostaglandins ○———○, B_1, ◑———◑, F_{2a}, ●———●, F_{1a}, ▲———▲, E_2, △———△, E_1, ▲———▲, A_1, □———□, F_{3a}, ■———■, E_3, x———x, A_2, ▽———▽, B_2

of prostaglandins greatly affects the activation pattern of phosphorylase *b*. It appears that in each type of prostaglandin used (prostaglandins A, B, E and F) the higher the degree of saturation the lower the prostaglandin concentration required to produce activation. Two exceptions were observed, prostaglandin E_2 is more effective than prostaglandin E_1 and prostaglandin F_{2a} is more effective than prostaglandin F_{1a}. The most efficient prostaglandin at the concentrations tested was B_1, which at 6 mM induced a 5% activation in respect to that produced by 1 mM AMP.

Possible contamination of prostaglandins by AMP was checked by TLC. 5 mg of each prostaglandin was dissolved in ethanol and chromatographed on silica gel plates (Merck 5721). The solvent system used was benzene/chloroform/*n*-butanol/ethanol (4 . 10 . 5 : 1, v/v) [17]. AMP chromatographed under the same conditions remained at the origin. The prostaglandins were visualised after chromatography by spraying with dodecamolybdophosphoric acid in ethanol (20%, w/v) and heating at 100°C until spots appeared. The spots of prostaglandins (not visualised) were extracted with ethanol, the extract was dried under nitrogen, dissolved in ethanol and tested for activity as in Fig. 1. Prostaglandins purified in this manner retained full capacity to activate phosphorylase *b*.

Since phosphorylase *b* activation by prostaglandins might be attributed to the hydrophobic moieties of these compounds, a number of saturated and unsaturated higher fatty acids, a phospholipid and a non-ionic detergent were tried as possible activators of the enzyme in order to obtain a measure of the specificity of prostaglandin action. The following substances were tested as in Fig. 1: lauric acid, palmitic acid, stearic acid, oleic acid, elaidic acid, linoleic acid, phosphatidylcholine (distearoyl), all at 2 mM concentration, and Triton X-100 at 0.05, 0.2 and 0.5%. Neither the fatty acids and phospholipid nor the non-ionic detergent served as activators of phosphorylase *b*. It was impossible to test higher concentrations of fatty acids or phospholipid because of their insolubility in the assay system. However, this concentration is sufficient if one takes into account the substantial activation induced by 2 mM prostaglandins.

Kinetic properties of phosphorylase b in the presence of prostaglandins

In order to examine the kinetic properties of phosphorylase *b* in the presence of prostaglandins, we have chosen prostaglandins F_{2a} and E_2, which exhibited the highest solubility in the assay system, to avoid possible interference of ethanol activation. Fig. 2 shows the Lineweaver-Burk plots of phosphorylase *b* reaction with respect to Glc-1-*P* (Fig. 2A) and glycogen (Fig. 2B) in the presence of 3 mM prostaglandin F_{2a}. The concentrations of Glc-1-*P* and glycogen required for half-maximal activity ($K_{0.5}$) are about 15-times higher than the K_m values for Glc-1-*P* and glycogen, respectively, measured in the presence of 1 mM AMP. Under the experimental consitions of Fig. 2, when 1 mM AMP was used instead of prostaglandin F_{2a}, phosphorylase *b* showed a K_m value for Glc-1-*P* of 6.8 mM and a K_m value for glycogen of 0.028%. The maximum velocity obtained with 3 mM prostaglandin F_{2a} was found to be 8% of that with 1 mM AMP. Furthermore, while under the experimental conditions used, at 1 mM AMP there was no homotropic cooperativity between Glc-1-*P* binding sites (Hill coefficient $n = 1$) [18,19], in the presence of 3 mM

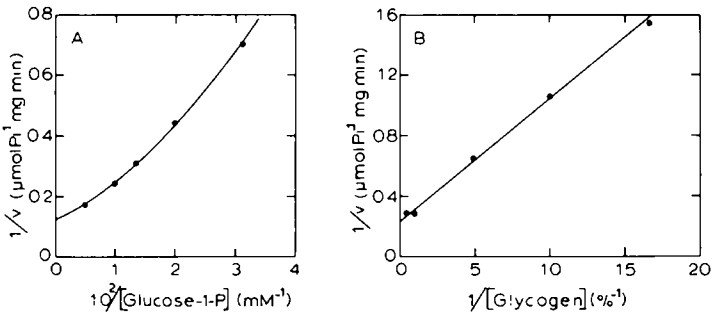

Fig 2. Lineweaver-Burk plots of phosphorylase *b* with respect to Glc-1-*P* (A) and glycogen (B) in the presence of prostaglandin F_{2a} Assay mixtures contained 1% or varying concentrations of glycogen, 80 mM or varying concentrations of Glc-1-*P*, 3 mM prostaglandin F_{2a} and 30 μg/ml phosphorylase *b* Reactions were carried out at 30° C and buffered as in Fig 1. $K_{0.5}$ for Glc-1-*P* was obtained by replotting the data of Fig 2A in the form of a Hill plot *n* = Hill coefficient (A) $K_{0.5}$ = 95 mM, *n* = 1.35, (B) $K_{0.5}$ = 0 42%

prostaglandin F_{2a} (Fig. 2A) a positive homotropic interaction was observed ($n = 1.35$). An analogous result has been obtained by Dreyfus et al. [4] when *t*-butanol was used as an activator.

The effect of prostaglandins on the kinetics of activation of phosphorylase *b* by AMP was further studied (Fig. 3), in comparison with *t*-butanol which has been reported to act synergistically with AMP on the enzyme activity [4]. In Fig. 3. Lineweaver-Burk plots are shown for AMP activation of phosphorylase *b* in the presence and absence of prostaglandin E_2 or *t*-butanol. In the absence of prostagalndin E_2 and *t*-butanol the enzyme shows the expected cooperative binding of AMP with a Hill coefficient $n = 1.5$. When 5% (v/v) of *t*-butanol is added, the cooperativity of AMP is decreased and the binding of the nucleotide to the enzyme is enhanced. In contrast, prostaglandin E_2 (5 mM) competitively inhibited AMP activation without affecting the cooperativity of the nucleotide binding. An analogous inhibitory effect was found in presence of prostaglandins B_1 (5 mM) or F_{2a} (3 mM).

The inhibition of AMP-induced activation by prostaglandin E_2 was further examined with respect to Glc-1-*P*. It was found that at 1 mM AMP, 1% glycogen and Glc-1-*P* concentrations between 2 and 32 mM, the prostaglandin is a competitive inhibitor with respect to Glc-1-*P*. The kinetic parameters of phosphorylase *b* relative to Glc-1-*P* in the absence or presence of 5 mM prostaglandin E_2 were *V*, 91 and 93 μmol P_i/mg per min, K_m (mean ± S.D. for three determinations), 4.2 ± 0.2 and 6.7 ± 0.3 mM, respectively. (The enzyme preparations used here and for Fig. 2 are different and the variation in the K_m value is not unusual [20].) This result may suggest that prostaglandin E_2 binds either at the nucleotide or the nucleoside site and inhibits phosphorylase *b* activity via allosteric competition with Glc-1-*P* [18,20,21].

In an effort to test whether the AMP site is the locus for prostaglandin action, we have examined the way by which the modification of phosphorylase *b* by 2,3-butanedione affected the activation by prostaglandins. We have treated phosphorylase *b* with 2,3-butanedione in the absence of AMP in order to modify the nucleotide binding site [22—24]. The effect of this modification on the catalytic activity of the enzyme as a function of time was studied. Enzyme

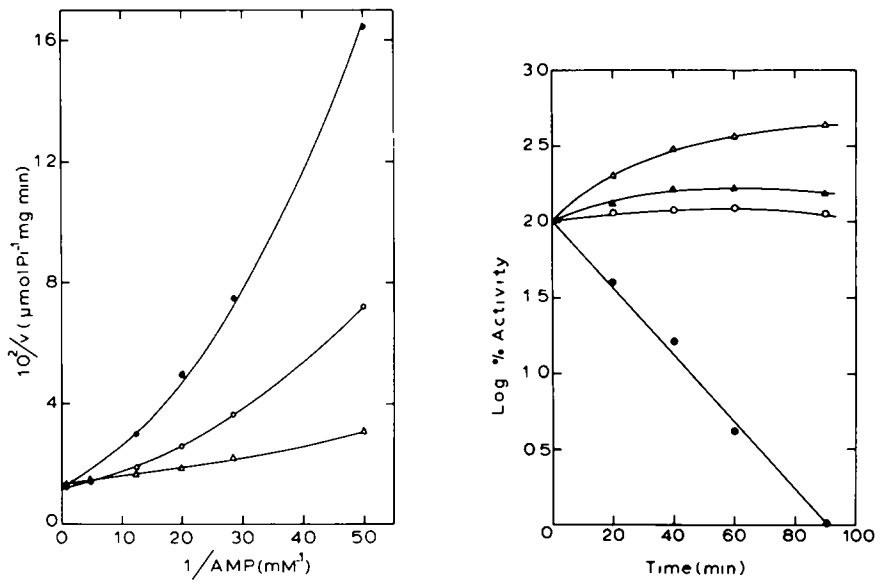

Fig. 3 Effect of prostaglandin E_2 and *t*-butanol on the kinetics of activation of phosphorylase *b* by AMP. The assay mixture contained in addition to AMP, 9 μg/ml enzyme, 32 mM Glc-1-*P* and 1% glycogen in the absence (○——○) (K_m = 54 μM, n = 1 5) or presence of 5 mM prostaglandin E_2 (●——●) (K_m = 110 μM, n = 1 55) 5% (v/v) *t*-butanol (△——△) (K_m = 25 μM, n = 1 2) Reactions were carried out at 30°C and buffered as in Fig 1 The activity induced by prostaglandin E_2 or *t*-butanol in the absence of AMP was deducted from each rate measurement K_m values for AMP and Hill coefficients (n) were obtained by replotting the data of Fig 3 in the form of a Hill plot

Fig 4. Kinetics of activity change after reaction of phosphorylase *b* with 2,3-butanedione. Activity of phosphorylase *b* was plotted as log% original activity as a function of incubation time in presence of 2,3-butanedione The enzyme (5 mg/ml) was reacted at 30°C with 10 mM 2,3-butanedione as in [22] After various reaction times, aliquots were diluted into assay buffer and tested for activity as in Fig. 1, in the presence of 1 mM AMP (●——●), 5 mM protaglandin E_2 (▲——▲), 5% *t*-butanol (○——○) or in absence of any activator (△——△) When AMP was present, the assay system contained 5 μg/ml phosphorylase *b* The percent activity in the presence of AMP, prostaglandin E_2 or *t*-butanol was calculated by deducting from each rate measurement the value of the residual activity observed at the corresponding reaction time.

activity was measured in presence of prostaglandin E_2 and was compared to that in presence of AMP, *t*-butanol or in the absence of any activator (residual activity). The following results were obtained (Fig. 4). 1. The residual activity of phosphorylase *b* was highly enhanced during modification (520%). 2. The above activation of phosphorylase *b* by 2,3-butanedione was found synergistic with the activation induced by prostaglandin E_2. 3. The activity induced by *t*-butanol was not substantially affected by the modification, in agreement with Dreyfus et al. [4]. 4. In contrast, as it was expected [4], the activity of the enzyme measured in the presence of AMP progressively disappeared.

Effect of spermine on the AMP-independent activity of phosphorylase b in the presence of prostaglandins

In order to obtain information about the role of the electrostatic interactions on the activation of phosphorylase *b* by prostaglandins, we studied the influence of spermine, an efficient polycationic stimulator of AMP activation, on the AMP-independent activity in the presence of prostaglandins. It has been

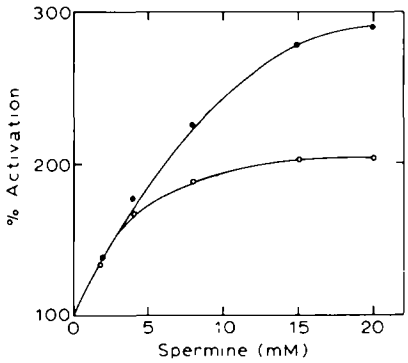

Fig 5. Effect of spermine on the activation of phosphorylase *b* by prostaglandins. The assay mixture contained 30 μg/ml enzyme, 80 mM Glc-1-*P*, 1% glycogen, various concentrations of spermine and 4 mM prostaglandin F_{2a} (●———●) or 5 mM prostaglandin B_1 (○———○) Reactions were carried out at 30°C and buffered as in Fig 1 Enzyme activity without spermine was taken as 100% Enzyme activity in the presence of spermine but in the absence of prostaglandin was deducted from each rate measurement Prostaglandin B_1 was dissolved in ethanol and the final concentration of the alcohol in the assay mixture was 5% (v/v) In this case, the enzyme activity in presence of spermine and ethanol was deducted from each rate measurement.

found that binding of spermine to phosphorylase *b* stimulated the activation of the enzyme by prostaglandins. Fig. 5 shows that spermine stimulated the activation of the enzyme induced by prostaglandins F_{2a} or B_1 and that the synergistic effect between spermine and prostaglandin F_{2a} was greater than that with B_1. It is interesting to note, that spermine under the previous experimental conditions is also able to stimulate the activation induced by *t*-butanol (5%, v/v) up to 200%

As has been shown previously [3], spermine activates phosphorylase *b* in the absence of AMP and this activation is temperature dependent. In order to test the effect of temperature on the activation of phosphorylase *b* by prostaglandins or hydrophobic solvents in the presence and absence of spermine, the

TABLE I

AMP-INDEPENDENT ACTIVITY OF PHOSPHORYLASE *b* IN THE PRESENCE OF PROSTAGLANDIN E_2 AND SPERMINE OR *t*-BUTANOL AND SPERMINE AT 10 OR 30°C

Reaction mixtures contained 32 mM Glc-1-*P*, 1% glycogen and buffer as in Fig 1 Other components were added as indicated When the reaction was carried out at 10°C the assay mixture contained 42 μg/ml phosphorylase *b*, while when the assay was carried out at 30°C the enzyme concentration was 5 μg/ml in the presence of AMP and 30 μg/ml in its absence Values in parentheses are activities expressed as a percentage of the activity in the presence of 1 mM AMP

Additions to reaction	μmol P_i/mg per min (%)	
	10°C	30°C
None	0 2 (2)	0 7 (1)
t-Butanol (5%, v/v)	3 6 (35)	9 5 (12)
Spermine (15 mM)[i]	1 4 (14)	3 1 (4)
t-Butanol (5%, v/v) + spermine (15 mM)	8 2 (80)	20 2 (25)
Prostaglandin E_2 (5 mM)	0 5 (5)	2 1 (2 5)
Prostaglandin E_2 (5 mM) + spermine (15 mM)	1 1 (11)	7 9 (10)
AMP (1 mM)	10 2 (100)	81 0 (100)

experiments of Table I were performed. As shown in this table, the percentage of activation of phosphorylase *b* by prostaglandin E_2 or *t*-butanol as compared to the activation of the enzyme by 1 mM AMP was significantly greater at 10 than at 30°C. A 2-fold increase was found for prostaglandin E_2 while the increase for *t*-butanol was 3-fold. In contrast to the previous findings in the absence of spermine, *t*-butanol and spermine together activated phosphorylase *b* synergistically both at 30 and at 10°C, while the activation of the enzyme by prostaglandin E_2 and spermine was found synergistic only at 30°C (Table I). It is worth emphasizing that the synergistic activation by spermine (15 mM) and *t*-butanol (5%, v/v) at 10°C was 80% of the activation induced by 1 mM AMP.

Effect of prostaglandins on the structure of phosphorylase b in the presence and absence of spermine

The effect of prostaglandin E_2 on the subunit association of phosphorylase *b* in the presence and absence of spermine, at 20 and 10°C, was examined ultracentrifugally, in order to see the relationship between the change in catalytic properties and the enzyme structure. At the protein concentration used in these experiments, phosphorylase *b* sediments as a dimer with a corresponding $s_{20,w}$ value of 8.5, while in the presence of AMP a second fast-sedimenting peak is formed with an $s_{20,w}$ of value of 13.3 corresponding to the tetramer [25]. As shown in Table II, phosphorylase *b* sedimented at 20 or 10°C as a single peak

TABLE II

TEMPERATURE-DEPENDENT EFFECT OF PROSTAGLANDIN E_2 AND *t*-BUTANOL ON THE SEDIMENTATION PROPERTIES OF PHOSPHORYLASE *b* IN THE PRESENCE AND ABSENCE OF SPERMINE

All ultracentrifugal sedimentation velocity measurements were carried out with 10 mg/ml enzyme in 1 mM EDTA/30 mM 2-mercaptoethanol/40 mM glycerol-2-*P* buffer, pH 6 8 Other components were added as indicated Temperature was maintained within ±0 5°C of the temperature indicated Other conditions were as described

Additions	°C	$s_{20,w}$	% of total
None	20	8 4	100
Spermine (20 mM)	20	8 0	96
		11 4	4
Prostaglandin E_2 (5 mM)	20	8 2	94
		11 5	6
Prostaglandin E_2 (5 mM) + spermine (20 mM)	20	8 0	62
		12 3	38
t-Butanol (5%, v/v)	20	8 4	100
t-Butanol (5%, v/v) + spermine (20 mM)	20	8 0	78
		11 4	22
None	10	8 2	100
Spermine (20 mM)	10	8 0	76
		12 2	24
Prostaglandin E_2 (5 mM)	10	8 3	76
		11 5	24
Prostaglandin E_2 (5 mM) + spermine (20 mM)	10	8 0	65
		12 8	35
t-Butanol (5%, v/v)	10	8 2	100
t-Butanol (5%, v/v) + spermine (20 mM)	10	8 1	46
		12 6	54

with an $s_{20,w}$ value of 8.4 and 8.2, respectively. Under our experimental conditions no tetramer formation has been observed at 10° C, while Kastenschmidt et al. [26] have reported an association of the dimer to an extent of 10% at 12.5° C. This difference is probably due to the lower enzyme and higher 2-mercaptoethanol concentrations used in our experiments. When the enzyme was treated with prostaglandin E_2 (5 mM) or spermine (20 mM), a fast-sedimenting peak corresponding to the tetramer appeared in addition to the peak of the dimer. The percentage of the tetramer was small at 20° C but is was substantially increased at 10° C. Carty et al. [3] did not observe tetramerization with spermine at 11° C, possibly because the concentrations of enzyme and polyamine used in their experiment were smaller. *t*-Butanol in contrast to prostaglandin E_2, could not promote tetramerization of phosphorylase *b* either at 20 or 10° C [4]. Furthermore, it was revealed that the tetramerization induced by spermine at 20 or 10° C was greatly increased in the presence of *t*-butanol. In contrast, when prostaglandin E_2 and spermine were added to the enzyme together, a synergistic action was observed only at 20° C.

Discussion

The data reported in the present study show that prostaglandins are able at mM concentration to increase the residual activity of phosphorylase *b* in the absence of AMP, and that this non-physiological activation [11] depends on the molecular structure of prostaglandins (Fig. 1). In this respect it has been reported that prostaglandin E_1 was able to stimulate phosphorylase in heart slices [27]. The fact that a number of saturated and unsaturated higher fatty acids, a phospholipid and a non-ionic detergent tested for activation were found to be unable to stimulate AMP-independent activity of phosphorylase *b*, suggests that the activation of the enzyme by prostaglandins is specific in nature. The observed difference between the action of prostaglandins and that of *t*-butanol on the catalytic and sedimentation properties of the enzyme (Fig. 3, Table 1, Table II) suggests a special type of interaction between phosphorylase *b* and prostaglandins.

As far as the site of action of prostaglandins is concerned nearly all experimental results suggest the nucleotide site as the locus of their binding· 1. Competition with AMP and increase in the Hill coefficient, similar to the effect of ATP which also binds there [18, 28, 29] (Fig. 3). 2. Competition with Glc-1-*P* ATP also competes with Glc-1-*P* [18] 3. Promotion of tetrameric state similar to AMP (Table II). 4. Stimulation of prostaglandin activation by spermine which is known to improve activation by poor nucleotide activators [1] (Fig. 5) The results of 2,3-butanedione treatment (Fig. 4), cannot rule out conclusively the possibility that the nucleotide site is the locus of action prostaglandins. Thus, the Arg 308/309 needed for AMP binding [30], may be hit first leaving other arginines or lysines in the area of the nucleotide pocket [29] available to bind the carboxyl group of the prostaglandin, while the cyclic moiety may interact with Tyr 75 to stabilize the active conformation as suggested by Fletterick and Madsen [30]. Taking into account our experimental results, partial binding of prostaglandins to the nucleoside effector site cannot be ruled out. An exclusive binding to this site should not be considered because compounds binding exclu-

sively to this site present only inhibitory action [20].

Since spermine is considered to be an allosteric effector [31], the enhancement of the action of prostaglandins on phosphorylase *b* by this polyamine arises presumably through a conformational change of the enzyme molecule. In addition interpreting the results of Tables I and II one can suggest, in agreement with Carty et al. [3], that at low temperatures a structure of the enzyme is favored, which can be influenced by prostaglandins, to permit association and induction of AMP-independent activity.

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