Multiple Activities on Phosphorylase Kinase. 1. Characterization of Three Partial Activities by Their Response to Calcium Ion, Magnesium Ion, pH, and Ammonium Chloride and Effect of Activation by Phosphorylation and Proteolysis†

Manfred W. Kilimann and Ludwig M. G. Heilmeyer, Jr.*

ABSTRACT: Three separate enzymatic activities of phosphorylase kinase toward phosphorylase *b* are differentiated according to their dependence upon Ca^{2+}, Mg^{2+}, NH_4Cl, and pH. A Ca^{2+}-independent activity, A_0, contributes a small portion (20–30 milliunits/mg) of the enzyme's total activity under optimal conditions; it is only revealed at Ca^{2+} concentrations below 10 nM or at pH 6.0 where the other activities are not expressed. A high-affinity, Ca^{2+}-dependent activity, A_1, which requires free Mg^{2+} (K_m approximately 0.25 mM at pH 6.8) contributes most of the total enzymatic activity at pH 6.8 [$K'_m(Ca)$ = 1.4 μM; V_{max} = 80–160 milliunits/mg in 10 mM Mg^{2+}]. High Mg^{2+} inhibits (K_i = 3.5 mM) due to displacement of the activating Ca^{2+}. At pH 6.8, a third low-affinity Ca^{2+}-dependent activity, A_2, is induced above 20 mM Mg^{2+}. Increases of pH up to 9.0 enhance both the affinity of A_2 for Mg^{2+} and 10–25-fold its V_{max}. Half-maximal stimulation by Ca^{2+} occurs at 10–70 μM (depending on pH and Mg^{2+} concentration); calcium stimulation appears to be cooperative; its affinity is enhanced by Mg^{2+}. All three activities are inhibited at high (millimolar) concentrations of Ca^{2+}; this inhibition is antagonized competitively by Mg^{2+}. Judged from the Ca^{2+} and Mg^{2+} affinities, the inhibitions of A_0 and A_1 on the one hand and of A_2 on the other hand occur through different mechanisms: NH_4Cl (150 mM) lowers the maximum velocity of A_1 and A_2 but not of A_0. It does so by enhancing the K'_m and K'_i values for Ca^{2+} stimulation and inhibition of A_2 but has the opposite effect on both parameters for A_1. Phosphorylation predominantly in the β or in the α and β subunits catalyzed by the cAMP-dependent protein kinase selectively enhances the V_{max} value for A_2. The $K'_m(Ca)$ of 7–10 μM is approximately identical with that determined at pH 9.0 for the nonphosphorylated enzyme. Self-phosphorylation of phosphorylase kinase leads to the stimulation of both A_1 and A_2. Again, this is predominantly due to an increase in V_{max} rather than to a change in $K'_m(Ca)$. Proteolysis by trypsin stimulates the V_{max} values of all three activities; it also increases the Ca^{2+} affinity of A_1. It is proposed that the activation of glycogen breakdown during muscle contraction is principally due to the A_1 activity, whereas the hormonal activation occurs through the activities A_2 or A_0 or both.

Phosphorylase kinase, a key enzyme of glycogen metabolism, is a large oligomeric enzyme of the subunit composition $(\alpha\beta\gamma\delta)_4$ which is stimulated by micromolar Ca^{2+} [for reviews, see Fischer et al. (1971), Krebs & Beavo (1979), and Cohen (1980)]. The δ subunit is very similar to calmodulin (Cohen et al., 1978; Grand et al., 1981) and probably represents the only Ca^{2+} binding subunit of this enzyme (Kohse & Heilmeyer, 1981).

Ca^{2+} binding to phosphorylase kinase (Kilimann & Heilmeyer, 1977) as well as to isolated calmodulin (Kohse & Heilmeyer, 1981) occurs in two steps. Two sites, N_{1a}, on calmodulin, termed Ca^{2+}/Mg^{2+} sites, bind these two metal ions competitively; additionally, two Ca^{2+}-specific sites, N_2, are inducible by Mg^{2+} in the holoenzyme, whereas Mg^{2+} is not required for Ca^{2+} saturation of these sites in isolated calmodulin.[1]

Biphasic Ca^{2+} binding as well as Ca^{2+} activation of purified phosphorylase kinase was observed earlier (Brostrom et al., 1971). However, the effects of Mg^{2+} and ionic strength on Ca^{2+} binding were not known. Kinetic studies of partially purified phosphorylase kinase showed a pronounced lower Ca^{2+} affinity than that of the purified enzyme (Heilmeyer et al., 1970; Cohen, 1980). Ca^{2+}-stimulated phosphorylase kinase can further be activated by phosphorylation which can be catalyzed either by cAMP-dependent protein kinase (EC 2.7.1.37) or by autocatalysis (Walsh et al., 1971). A third efficient mode of activation occurs by limited proteolysis (Krebs et al., 1964). In earlier work on the purified enzyme, phosphorylated by the cAMP-dependent protein kinase, an increased Ca^{2+} sensitivity was found (Brostrom et al., 1971), whereas other authors did not observe such an effect (Cohen et al., 1976). With partially purified phosphorylase kinase, again an increased Ca^{2+} sensitivity upon this kind of phosphorylation was described (Cohen, 1980). Limited proteolysis results in a 10-fold increase in Ca^{2+} sensitivity (Brostrom et al., 1971; Cohen, 1980). The Ca^{2+} dependence of the enzyme phosphorylated autocatalytically has not yet been studied.

The Ca^{2+}/Mg^{2+} sites, N_{1a}, as well as the Ca^{2+}-specific sites, N_2, can potentially become saturated with Ca^{2+} during contraction (Kilimann & Heilmeyer, 1977; Heilmeyer et al., 1980a,b). Therefore, the question arises as to which of these two Ca^{2+} binding sites is responsible for activation of phosphorylase kinase during either contraction or hormonal stimulation of glycogenolysis. More importantly, does Ca^{2+} binding in two steps imply that more than one Ca^{2+} controlled activity exist on this enzyme?

The present publication will describe the Ca^{2+} activation of freshly isolated phosphorylase kinase as well as that of the

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[1] So that the correlation between binding sites and activities could be expressed more clearly, the nomenclature used in previous publications (Kilimann & Heilmeyer, 1977; Heilmeyer et al., 1980a) has been revised as follows: $n_{1a} = N_{1a}$, $n_{1b} = N_{1b}$, $n_4 = N_2$, $n_2 = N_3$, and $n_3 = N_4$ and $a_0 = A_0$, $a_1 = A_1$, and $a_4 = A_2$.

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phosphorylated or partial proteolytically digested enzyme.

Experimental Procedures

For determination of phosphorylase kinase activity in the manual assay, equal volumes of phosphorylase *b* (15 mg/mL in 1.5 mM sodium glycerol 2-phosphate and 0.75 mM dithioerythritol), of 18 mM ATP containing between 18 and 189 mM $MgCl_2$, adjusted to 3 times the total desired Mg^{2+} concentration, and of the enzyme sample were combined. Unless otherwise stated, the enzyme diluent buffer (1.4 mM sodium glycerol 2-phosphate, 0.75 mM dithioerythritol, and in some experiments 3 mM EGTA[2] to establish low free Ca^{2+} concentrations) was also used to adjust the ionic strength with NH_4Cl corresponding to the required $MgCl_2$ and $CaCl_2$ concentrations. In each reagent contaminant, Ca^{2+} was determined as described below and taken into account for calculation of total and free Ca^{2+}. All solutions, adjusted to the pH values required in the assay, were mixed within 30 s; the reaction was started with the enzyme. Aliquots (four to six) were taken between time 0 and 9–14 min, depending on the rate, and immediately diluted in ice-cold 100 mM NaF, 100 mM sodium maleate, 40 mM mercaptoethanol, 1 mg/mL bovine serum albumin, and 3 mM EDTA, pH 6.5. This dilution was assayed for phosphorylase *a* activity within 30 min on an autoanalyzer.

The convertibility of phosphorylase *b*, unlike its specific activity, was found to differ (±30%) between preparations and to decrease considerably during storage (−50% after 10 days being kept crystalline at 0 °C in 10 mM Tris-HCl, 1 mM AMP, 10 mM $MgCl_2$, and 30 mM mercaptoethanol, pH 7.0). Therefore, a quantitative comparison of absolute activities is maintained only for measurements that were performed simultaneously, usually meant for measurements illustrated in any one figure. Phosphorylase kinase concentrations in the assays were between 0.4 and 7.5 μg/mL; in this range, the concentration dependence of activity was linear.

The pH value of the assay mixture was identical with that of the reagent solutions over the whole Ca^{2+} and Mg^{2+} concentration range. The amount of phosphorylase *a* formed increased linearly between 1 and 14 min. No activation of phosphorylase occurred in control assays that did not contain phosphorylase kinase. One unit of kinase activity corresponds to 1 μmol of P_i transferred to phosphorylase *b* subunit (M_r 100000) per min, the specific activity of phosphorylase *a* taken as 50 units/mg. NH_4Cl was chosen instead of KCl because the latter substance quenches the detection of calcium by atomic absorption in the graphite furnace. The absolute enzymatic activities obtained in this test were lower than those obtained in the conventional test. The activity at elevated pH was depressed more than at neutrality. This activity depression is mainly due to the low glycerol 2-phosphate concentration which enhances phosphorylase kinase activity as well as the rate of autophosphorylation (Krebs et al., 1964; our unpublished data). However, the low concentration was chosen to be able to compare the kinetics to the Ca^{2+} binding data previously determined under the same conditions.

Phosphorylation of phosphorylase kinase predominantly in the β subunit was carried out according to Cohen (1973) by incubating the enzyme (0.24 mg/mL) in 8 mM sodium glycerol 2-phosphate, 4 mM EGTA, 0.3 mM EDTA, 32 mM NaCl, and 1.6 mM $MgCl_2$, pH 7.0, at 30 °C with 0.3 μg/mL cAMP-dependent protein kinase [(catalytic subunit; purified according to Beavo et al. (1974)] and 0.2 mM [γ-^{32}P]ATP. The reaction was stopped by a 10-fold dilution after 90 s in ice-cold 100 mM NH_4Cl, 1.5 mM sodium glycerol 2-phosphate, and 0.75 mM dithioerythritol, pH 6.8.

For autocatalytic phosphorylation, 150 μL of phosphorylase kinase (0.8 mg/mL in 1.5 mM sodium glycerol 2-phosphate and 0.75 mM dithioerythritol, pH 6.8) was incubated with 50 μL of 18.6 mM ATP and 48.6 mM $MgCl_2$, pH 9.0, and 25 μL of 11.2 mM [γ-^{32}P]ATP at 30° C. The incubation was stopped after 90 min by a 200-fold dilution. Aliquots were taken shortly before and afterward for determination of total phosphate incorporation and its distribution between subunits. Phosphorylase kinase was partially degraded with trypsin by incubating 15 μg in 1.5 mM sodium glycerol 2-phosphate and 0.75 mM dithioerythritol, pH 6.8, with 3.8 ng of bovine pancrease trypsin at 30 °C. So that the following activity assay with Ca^{2+} was not contaminated, proteolysis was stopped after 35 min by 250-fold dilution only. At this dilution the effect of trypsin on the phosphorylase *a* assay is negligible.

Concentrations of the various ion and complex species in the presence of ATP and/or EGTA were calculated on a digital computer from the total concentrations and complex species in the presence of ATP and/or EGTA (Sillen & Martell, 1964, 1971) by using a Fortran program modified from that of Perrin & Sayce (1967). The logarithmic association constants employed were as follows: Ca^{2+} to ATP^{4-}, 3.97; Mg^{2+} to ATP^{4-}, 4.22; H^+ to ATP^{4-}, 6.53; K^+ (instead of NH_4^+) to ATP^{4-}, 0.99; Ca^{2+} to $ATPH^{3-}$, 2.13; Mg^{2+} to $ATPH^{3-}$, 2.24; H^+ to $ATPH^{3-}$, 4.06; K^+ to $ATPH^{3-}$, 0.25; Ca^{2+} to $EGTA^{4-}$, 11.00; Mg^{2+} to $EGTA^{4-}$, 5.30; H^+ to $EGTA^{4-}$, 9.46; Ca^{2+} to $EGTAH^{3-}$, 5.33; Mg^{2+} to $EGTAH^{3-}$, 3.37; H^+ to $EGTAH^{3-}$, 8.85; H^+ to $EGTAH_2^{2-}$, 2.68; H^+ to $EGTAH_3^-$, 2.00.

Metal ion concentrations given in this paper always refer to the free metal ions unless stated otherwise. Nonlinear least-squares fits of kinetic and binding data to the Michaelis–Menten function $v = V[X]/(K_m + [X])$ were performed with a modified version of the Fortran program HYPER of Cleland (1967). Least-squares fits to the function $v = \sum_i [V_i[X]/(K_{m,i} + [X])]$ were performed by a modified form of a Fortran program FIT kindly supplied by Dr. J. Gergely, Boston.

Nonlinear regression of maximum curves, especially for the activity A_2 as a function of the Ca^{2+} concentration, was carried out with the program FIT by assuming sequential binding of Ca^{2+} to two sites, or groups of sites, of which the one of higher affinity was activating and the one of lower affinity inactivating. They were supposed to be noninteractive, and of the possible complexes E, E–Ca_{act}, E–Ca_{inact}, and Ca_{act}–E–Ca_{inact}, only the complex E–Ca_{act} was assumed to be active. The partial activities were taken to be additive.

Calcium binding to phosphorylase kinase or phosphorylase *b* as well as calcium determinations was carried out as described previously (Kilimann & Heilmeyer, 1977). Protein was determined according to Lowry et al. (1951) on an autoanalyzer. Phosphorylase *b* and *a* activities were assayed on an autoanalyzer according to Haschke & Heilmeyer (1972). Phosphorylase kinase activity was, besides the manual test, also determined on an autoanalyzer according to Jennissen & Heilmeyer (1974).

Materials. Solutions, prepared from "suprapur" reagents (Merck when obtainable; otherwise analytical grade) and quartz doubly distilled water (ca. 20 nM Ca^{2+}), were not allowed to come into contact with glass; only new plastic bottles, test tubes, and pipet tips rinsed with quartz doubly

[2] Abbreviation: EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

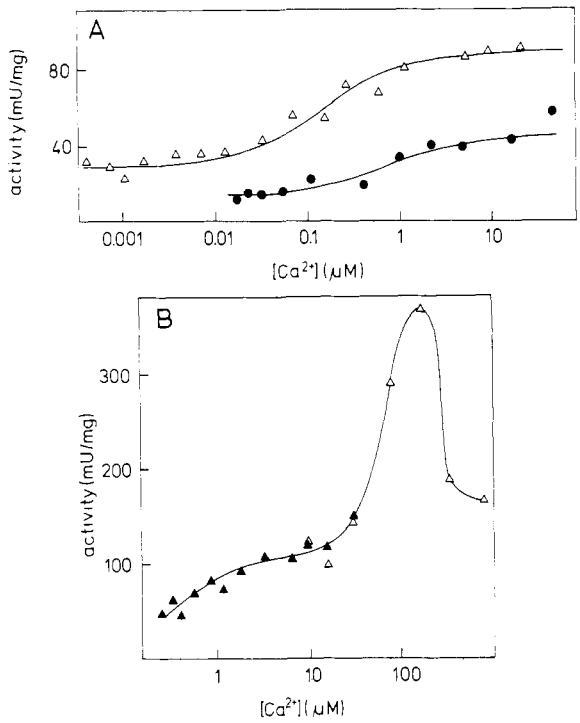

FIGURE 1: Three partial activities, A_0, A_1, and A_2. Phosphorylase kinase assays were carried out as described under Experimental Procedures under the following conditions: (A) (△) 10.7 mM Mg^{2+}, pH 6.8; (●) 2.1 mM Mg^{2+}, pH 6.0; both at 1 mM EGTA, 24 °C. (B) 57.4 mM Mg^{2+}, pH 6.8; 30 °C. Open and closed symbols represent two sets of experiments.

distilled water were used throughout. Phosphorylase *b* was purified according to Fischer & Krebs (1958) and freed from Ca^{2+} and AMP by filtration over Sephadex G-50 superfine preequilibrated in 1.6 mM sodium glycerol 2-phosphate and 0.75 mM dithioerythritol, pH 6.8 [a 16 mg/mL solution ($A_{260/280}$ ca. 0.55) contained about 0.3 μM Ca^{2+}]. Alternatively, filtration over charcoal was applied for the removal of AMP (Fischer & Krebs, 1958).

A solution of commercially available reagent-grade sodium ATP (Boehringer, Mannheim) contained about 3 μM Ca^{2+}/mM. It was bound to a column of DEAE-Sephadex A-25 (Pharmacia). Na^+ together with about half of the Ca^{2+} was eluted with water; 45 mM $MgCl_2$ then eluted the rest of the Ca^{2+}. Following extensive water washes, ATP was eluted with freshly prepared 0.6 M triethylammonium hydrogen carbonate. The eluate was evaporated under vacuum, and the residue was redissolved in 2 mL methanol and reevaporated 2 times. A solution, 18 mM in ATP, contained 100 nM Ca^{2+} and 5.7 mM Mg^{2+} (yield ca. 90% according to A_{260}). Enzymatic determination of ADP and ATP showed no decomposition of the triphosphate.

Phosphorylase kinase was purified according to Cohen (1973) and following the modification of Jennissen & Heilmeyer (1975). Its specific activity was ~8 units/mg at pH 8.2 and 100 μM Ca^{2+} (automatic test; the pH 6.8/8.2 ratio was 0.05). Homogeneity and subunit composition were checked by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Cohen, 1973). Subunit stoichiometry, determined for seven enzyme preparations from Coomassie blue staining densitometry, was found to be as follows: α, 1.03 ± 0.13; β, 1.00; γ, 1.53 ± 0.19; δ, 1.07 ± 0.13 (normalized to β). The enzyme was stored at −20 °C in 50 mM sodium glycerol 2-phosphate, 200 mM NaCl, 2 mM EDTA and 1 mM dithioerythritol, pH 7.0. This solution contained about 30 μM Ca^{2+}.

Table I: Dependence of A_0 from $[Mg^{2+}]$, $[H^+]$, and Salt Concentration [a]

pH	$[Mg^{2+}]$ (mM)	V (milliunits/mg)
6.0	2.1	13.9
	56.8	19.5
6.8	10.7	29.7
	10.7	31.0 [b]
	56.2	30.2

[a] The experiments were carried out in analogy to those of Figure 1A. V values were calculated by the program FIT as described under Experimental Procedures by taking into account the contributions of A_1 and A_2 to total activities. [b] Without NH_4Cl.

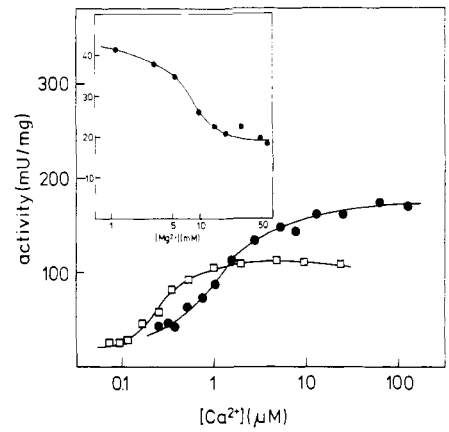

FIGURE 2: Competitive inhibition of A_1 by Mg^{2+}. Phosphorylase kinase assays were carried out as described under Experimental Procedures. (□) 1.14 mM Mg^{2+}; (●) 10.2 mM Mg^{2+}; both pH 6.8, 30 °C. Inset: 0.43 μM Ca^{2+}, pH 6.8; 25 °C.

Results

Three partial activities, termed A_0, A_1, and A_2, are exhibited by phosphorylase kinase at variable free Ca^{2+} and Mg^{2+} concentrations. Figure 1A demonstrates that below 10 nM free Ca^{2+} a constant residual activity, A_0, is observed. The high-affinity Ca^{2+}-dependent activity, A_1, superimposes itself on this basal activity, A_0, as the free Ca^{2+} rises to 1 μM, increasing the total activity ca. 3-fold (Figure 1A). The low-affinity Ca^{2+}-dependent activity, A_2, requires the induction by Mg^{2+} (>20 mM at pH 6.8). It forms a characteristic maximum around 100 μM Ca^{2+} (Figure 1B).

Thus, their different Ca^{2+} dependence is the main criterion by which the three partial activities are defined. The following will show that they differ in their response to Mg^{2+}, pH, and salt concentration as well as to phosphorylation and limited proteolysis.

A_0 is stimulated by 50% between pH 6.0 and pH 6.8. It is enhanced by Mg^{2+}, whereas 150 mM NH_4Cl has no effect (Table I). Characteristically the Ca^{2+} affinity of A_1 decreases when Mg^{2+} is increased in the assay (Figure 2). Likewise, an inhibition is observed when Mg^{2+} is increased at a constant $[Ca^{2+}]$ close to the K_m(Ca) (Figure 2 inset). In the latter experiment the total activity does not approach zero but reaches a final residual level above 20 mM Mg^{2+} representing A_0. K_m(Ca) and K_i(Mg) values can be calculated for A_1 (Table II) in good agreement from both experiments.

Besides this competitive displacement of the A_1 activation curve to higher Ca^{2+} concentrations, Mg^{2+} enhances the V_{max} of A_1 (Figure 2; Table II). Competitive inhibition by Ca^{2+} for this Mg^{2+} stimulation is observed by varying Mg^{2+} concentration at constant $[Ca^{2+}]$ (not shown). A true K_m(Mg) value of 0.25 mM and a K_i(Ca) of 0.035 mM can be calculated. Above 10 mM, Mg^{2+} is inhibitory; between ca. 10 and

Table II: Ca^{2+} and Mg^{2+} Dependence of A_1 [a]

constant metal ion	concn (mM)	V (milliunits/mg)	K'_m(Ca) (μM)	K'_i(Mg) (mM)	K_m(Ca) (μM)	K_i(Mg) (mM)
Mg^{2+}	1.14	128 ± 15	0.43 ± 0.12			
	10.2	156 ± 3	1.23 ± 0.10		0.33 ± 0.10	3.7 ± 1.5
Ca^{2+}	0.43×10^{-3}	29.4		7.5		3.2 [b]

[a] The experimental data are from Figures 1B and 2. The parameters of Ca^{2+} activation (Figure 1B) were computer fitted; the data from Figure 2 were fitted graphically by Eadie-Scatchard plots. The contribution of A_0 to total activity was taken as 13.9 (1.14 mM Mg^{2+}; Table I) and 20 (10.2 and 57.4 mM Mg^{2+}; fitted with the program FIT from Figure 1B) milliunits/mg. In Figure 2, A_0 was determined as 18 milliunits/mg. [b] Calculated from K'_i(Mg) by using the K_m(Ca) = 0.33 μM.

Table III: Effect of Salt Concentration on Ca^{2+} Stimulation and Inhibition of A_1 and A_2 [a]

act.	ionic strength (mM)	V (milliunits/mg)	K'_m(Ca) (μM)	K'_i(Ca) (mM)
A_1	200	56	0.11	
	50	69	0.99	
A_2	200	2050	31.7	0.43
	30	2510	10.4	0.13

[a] A_1 was determined at 10.7 mM Mg^{2+}, pH 6.8, and 1 mM EGTA; A_2 was determined at 2.8 mM Mg^{2+}, pH 9.0; all were at 24 °C.

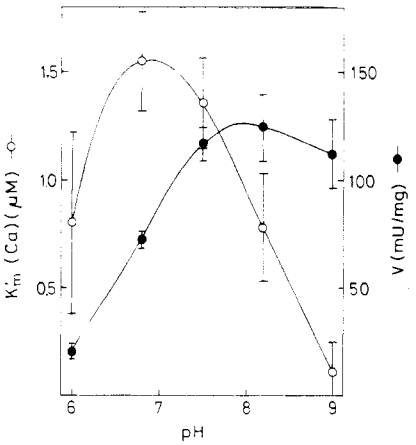

FIGURE 3: pH Dependence of the kinetic parameters of A_1. Phosphorylase kinase activity was determined as a function of $[Ca^{2+}]$ at 10.2 mM Mg^{2+}, 26 °C, as described under Experimental Procedures. K'_m(Ca) and V values were calculated as described under Experimental Procedures with the program HYPER. The contribution of A_0 to total activity was determined as 13.0 milliunits/mg at pH 6.0.

60 mM Mg^{2+}, the V_{max} of A_1 decreases from 156 ± 3 to 103 ± 5 milliunits/mg (cf. Figures 1B and 2).

The Ca^{2+} affinity of A_1 decreases 9-fold upon omission of NH_4Cl. Its V_{max} increases by ~20% (Table III) or up to ~2.5-fold if the free Mg^{2+} concentration is lowered to 1.2 mM (not shown). Increase of pH from 6.0 to 9.0 enhances the V_{max} of A_1 6-fold. It is more than half-maximal at pH 6.8 and remains virtually constant between pH 7.5 and pH 9.0 (Figure 3). In contrast, the apparent K'_m(Ca) reaches a maximum around pH 6.8 and decreases ca. 15-fold with increased pH with a maximal value observed around pH 8.3 (Figure 3).

Millimolar Ca^{2+} inhibits completely both activities, A_0 and A_1. The curves can be fitted best by assuming only one apparent inhibitor constant K'_i(Ca) for both partial activities (Figure 4). This Ca^{2+} inhibition is antagonized competitively by Mg^{2+} (Table IV). Omission of NH_4Cl from the assay enhances the K'_i(Ca) from 0.50 (Table IV) to 1.40 mM (not shown).

A_2 appears as a bell-shaped curve as a function of Ca^{2+} concentration (cf. Figure 1B); it is stimulated considerably by an increase of pH from 6.8 to 9.0 but maintains its maximum

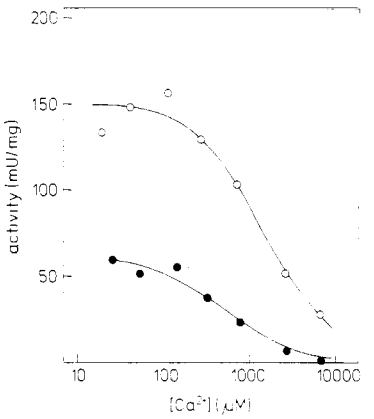

FIGURE 4: Concomitant inhibition of A_0 and A_1 by millimolar Ca^{2+}. Phosphorylase kinase was assayed as described under Experimental Procedures under the following conditions: (●) 1.2 mM Mg^{2+}; 180 mM NH_4Cl [V = 63 milliunits/mg; K'_i(Ca) = 0.50 mM]; (○) 1.0 mM Mg^{2+}, without added NH_4Cl [V = 155 milliunits/mg; K'_i(Ca) = 1.40 mM]; both at pH 6.8, 30 °C. The curves were fitted with the program FIT.

Table IV: Concomitant Inhibition of A_0 and A_1 by Millimolar Ca^{2+}: Competitive Effect of Mg^{2+} [a]

$[Mg^{2+}]$ (mM)	V (milliunits/mg)	K'_i(Ca) (mM)	K_m(Mg) (mM)	K_i(Ca) (mM)
1.2	63	0.50	0.29	0.10
59.4	170	20	0.29	0.10

[a] Ca^{2+} dependence of activity was determined at pH 6.8, 30 °C; K_m and K_i values were calculated by assuming competition between Mg^{2+} and Ca^{2+}.

Table V: Dependence of A_2 from $[Mg^{2+}]$ and $[H^+]$ [a]

$[Mg^{2+}]$ (mM)	pH	V (milliunits/mg)	K'_m(Ca) (μM)	K'_i(Ca) (mM)	K_m(Mg) (mM)	K_i(Ca) (mM)
10.2	6.8	0	–	–		
	7.5	95	40	1.05		
	8.2	1310	85	0.55	3.15	0.13
	9.0	1960	33	0.44	2.75	0.09
57.4	6.8	80	34	0.28		
	7.5	280	76	0.96		
	8.2	520	16	2.50	3.15	0.13
	9.0	640	11.2	2.01	2.75	0.09

[a] The least-squares fits for calculation of A_2 parameters take account of the contribution of A_0 and A_1 to total activity determined simultaneously under appropriate conditions (pH 6.0 and 6.8). K_m(Mg) and K_i(Ca) values were calculated by assuming competitive antagonism of Mg^{2+} to Ca^{2+} inhibition. At 10.2 mM Mg^{2+}, pH 6.8, activity A_2 is not expressed (–). Assay temperature was 26 °C.

at ca. 100 μM Ca^{2+} (Figure 5). This pH effect is essentially due to an increase in V_{max}; the values for K'_m(Ca) are less markedly influenced and seem to go through a maximum (Table V).

The ascending branches of some of the experimental curves in Figure 5 are steeper than those expected from the model

Table VI: Mg^{2+} Dependence of A_2 at pH 9.0[a]

[Mg^{2+}] (mM)	V (milliunits/mg)	K'_m(Ca) (μM)	K'_i(Ca) (mM)	K_m(Mg) (mM)	K_i(Ca) (mM)
2.8	2050	31.7	0.43	6.2	0.30
7.4	1460	24.4	1.49		
20.0	1850	26.8	0.77		
56.5	1270	7.9	3.00	6.2	0.30

[a] The K_m and K_i values of the last two columns were calculated from the K'_i values at 2.8 and 56.5 mM Mg^{2+} by assuming that Mg^{2+} antagonizes competitively the inhibition by Ca^{2+}. Assay temperature was 24 °C.

of successive Ca^{2+} binding to an activating and an inhibiting site (Experimental Procedures) which may indicate cooperativity of the Ca^{2+}-activating mechanism.

In 57 mM Mg^{2+}, the increase in A_2 activity as a function of pH takes off earlier (pH 6.8) than at lower Mg^{2+} concentration and is half-terminated at about pH 7.7. In contrast, at 10 mM Mg^{2+}, it only increased markedly at approximately this pH, and it reaches higher levels of activities (Table V). An increase from 10 to 57 mM Mg^{2+} stimulates A_2 at pH 7.5, whereas it inhibits it above pH 8.2 (Table V). Table VI shows that at pH 9.0 this inhibitory effect commences already above 2.8 mM Mg^{2+} which is similar to the inhibition of A_1 observed at high Mg^{2+} concentrations (see above). Contrary to the behavior of A_1, the K'_m(Ca) of A_2 activation does not increase with increasing Mg^{2+} concentrations but rather decreases (Table VI). Ca^{2+} above 200 μM inhibits A_2, and this inhibition is antagonized by Mg^{2+}. K_m(Mg) and K_i(Ca) values can be calculated for this latter effect (Tables V and VI).

Omission of NH_4Cl increases by ca. 1.25-fold the peak of activity (Table III) and shifts the A_2 maximum toward a lower Ca^{2+} concentration (~20 μM) (not shown); i.e., the affinity of both the activating and inactivation sites for Ca^{2+} are increased (Table III). Ca^{2+} activation of A_1 and A_2 as well as Ca^{2+} inhibition of all three partial activities are fully reversible as proven by dilution experiments or by sequestering the excess of Ca^{2+} with EGTA (data not shown). Phosphorylase b binds, probably unspecifically, low amounts of Ca^{2+} only at high pH and at very high free Ca^{2+} concentrations (maximally 0.28 mol/100 000 g of protein at 190 μM Ca^{2+}, pH 9.0).

Phosphorylase kinase phosphorylated predominantly in the β subunit by the cAMP-dependent protein kinase under conditions where autophosphorylation contributed less than 1% to the total phosphate uptake exhibits a selective increase in A_2 activity as compared to that of a nonphosphorylated control (Figure 6). A_1 seems not to be detectably influenced and A_0 only very slightly. The nonphosphorylated enzyme displays

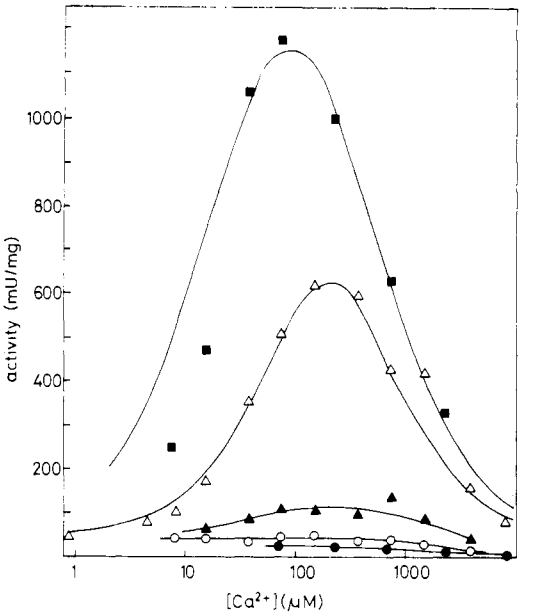

FIGURE 5: pH dependence of A_2. Phosphorylase kinase activity was assayed at (●) pH 6.0, (○) pH 6.8, (▲) pH 7.5, (△) pH 8.2, and (□) pH 9.0 at 10.2 mM Mg^{2+}, 26 °C, as described under Experimental Procedures. The curves are fitted with the program HYPER by assuming noncooperativity; the deviation between this fit and the data at pH 8.2 and pH 9.0 is commented upon in the text.

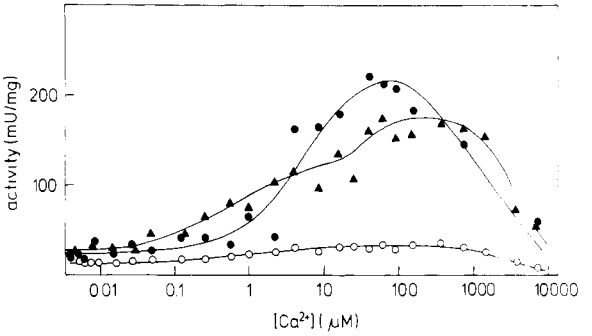

FIGURE 6: Ca^{2+} dependence of phosphorylase kinase activated by cAMP-dependent protein kinase and by autophosphorylation. The preparation of the enzymes is described under Experimental Procedures. Activity was determined at 9.8 mM Mg^{2+} and 1 mM EGTA, pH 6.5, 30 °C. (○) Nonmodified enzyme; (●) enzyme phosphorylated by cAMP-dependent protein kinase (0.08 mol of phosphate/mol of α and 0.44 mol/mol of β); (▲) autophosphorylated enzyme (0.82 mol of phosphate/mol of α and 0.24 mol/mol of β). The curves are computer fitted by the program FIT.

only the A_0 and A_1 activities under these conditions. The K'_m(Ca) value for A_2 for this phosphorylated form of the

Table VII: Kinetic Parameters of Covalently Modified and Nonmodified Phosphorylase Kinase[a]

enzyme modification	V (milliunits/mg)			K'_m(Ca) (μM)	
	A_0	A_1	A_2	A_1	A_2
nonmodified, pH 6.5	13.6	18.7	0	1.19	–
nonmodified, pH 9.0	(80)[b]		1710	0.1[b]	12.9
predominantly phosphorylated in β, pH 6.5 (0.08 mol/mol of α; 0.44 mol/mol of β)	26	19[c]	210	1.2[c]	6.9
phosphorylated in α and β, pH 6.5 (0.87 mol/mol of α; 0.54 mol/mol of β)	31	19[c]	390	1.2[c]	10.3
autophosphorylated, pH 6.5					
$\alpha = 0.82$; $\beta = 0.24$ mol/mol	28	85	110	0.45	87
$\alpha = 2.84$; $\beta = 1.35$ mol/mol	50	230	440	1.4	39
proteolyzed, pH 6.5	370	610	2510	0.032	95

[a] Assay conditions: 9.8 mM Mg^{2+}; 1 mM EGTA; 30 °C. Parameters were computer fitted. The K'_i(Ca) values (in mM) obtained by the least-squares fits are (top to bottom) as follows: 3.8 (A_1), 3.8 (A_1), and 1.7 (A_2); 3.8 (A_1) and 0.79 (A_2); 3.8 ($A_1 + A_2$); 2.65 ($A_1 + A_2$); 0.72 ($A_1 + A_2$); 1.05 ($A_1 + A_2$). [b] Taken as average values from Tables I and III. For the value in parentheses, experimental data do not allow discrimination between two particular activities. [c] Assumed as approximately identical with the values determined for the nonmodified enzyme at pH 6.5.

Table VIII: Synopsis of Ca^{2+} Binding and Ca^{2+} Activation Properties of Phosphorylase Kinase [a]

Ca^{2+} binding				Ca^{2+} activation			
buffer conditions	site	mol of Ca^{2+}/ 1.28×10^6 g of protein	K'_D (μM)	buffer conditions	act.	V (milli-units/mg)	K'_m (μM)
10 mM Mg^{2+}, pH 6.8	N_{1a}	6.8	0.33	10.2 mM Mg^{2+}, pH 6.8	A_1	120	1.5
		6.5	0.37			160	1.2
		6.8 [b]	0.61 [b]			170 [b]	0.6 [b]
	N_2	4.0	2.9	10.2 mM Mg^{2+}, pH 7.5–9.0	A_2	100–2000	13–85
		8.4	4.6				
		>10 [b]	>25 [b]	57.4 mM Mg^{2+}, pH 6.8	A_2	80	34
						– [b]	(>>170) [b]

[a] Kinetic data are from this paper. The first value of each set of binding parameters is taken from Kilimann & Heilmeyer (1977); the other data were measured under the same conditions with different enzyme preparations. [b] The last preparation had been subject to partial endogenous proteolysis (0.58 mol of α/mol of $\beta\gamma\delta$ subunits).

enzyme is approximately identical with that of the nonmodified kinase at pH 9.0 (Table VII). Additional phosphate incorporation in both the α and β subunits by cAMP-dependent protein kinase further increases the V_{max} of A_2 (Table VII). By contrast, self-phosphorylated phosphorylase kinase shows an increase of both A_2 and A_1 and even a minor stimulation of A_0. Table VII shows that during the course of self-phosphorylation, in which the α subunit is phosphorylated faster than β, A_1 increases earlier than A_2 though A_2 reaches a higher level. No signs of endogenous proteolysis were detected by gel electrophoresis in any of the phosphorylated kinase species described above.

In trypsin-activated phosphorylase kinase, the α and β subunits are degraded. A peptide of M_r 82000 is generated, presumably identical with the component "Y" described by Cohen (1973). All three activities are strongly stimulated, predominantly A_2 (Figure 7). The K'_m(Ca) of A_1 is decreased by more than 1 order of magnitude (Table VII).

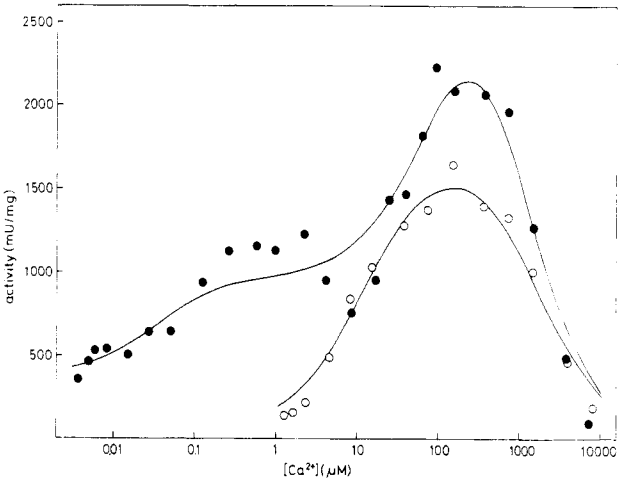

FIGURE 7: Ca^{2+} dependence of the activity of phosphorylase kinase activated by trypsin. The method for proteolysis is given under Experimental Procedures. Activity was determined at 9.8 mM Mg^{2+} and 1 mM EGTA, 30 °C. (●) Tryptically activated enzyme, pH 6.5; (○) nonmodified enzyme, pH 9.0. The curves are computer fitted by the program FIT.

Discussion

The phosphorylase kinase concentration in the activity assay is ca. $^1/_{1000}$ of that in the corresponding Ca^{2+} binding measurement. Dextran was present only in the Ca^{2+} binding assay; phosphorylase kinase is known to bind to polysaccharide (Krebs et al., 1964; Kilimann & Heilmeyer, 1977), whereas ATP together with a protein substrate that can be phosphorylated are present in the kinetic experiments. Due to these variations, a correlation between Ca^{2+} activation and binding (see Table VIII) has to rely not only on affinity and activation constants but also on specific qualitative properties of both.

The A_0 activity seems to be Ca^{2+} independent. If it were Ca^{2+} dependent at lower Ca^{2+} concentrations than those covered in the reported experiments, the kinetic data would yield the extremely low value of 40 pM as an upper limit for a possible K_m(Ca) (conditions: 10 mM Mg^{2+}, 150 mM NH_4Cl, pH 6.8). The binding curve shows no sign of heterogeneity of the eight N_{1a} binding sites and extrapolates toward zero (Kilimann & Heilmeyer, 1977). Therefore, it seems highly improbable that there exists a Ca^{2+}-dependent activity or Ca^{2+} binding sites of higher affinity than A_1 and N_{1a}, respectively. Phosphorylase kinase is known to possess substantial Ca^{2+}-independent activity in the range of 0.2–100 nM Ca^{2+} toward troponin I (Huang et al., 1974; Moir et al., 1974; Sperling et al., 1979) and in the course of self-phosphorylation (Kilimann & Heilmeyer, 1982). Also, phosphorylase kinases from blowfly muscle (Sacktor et al., 1974), rabbit liver (Sakai et al., 1979), and silkworm fat body (Ashida & Wyatt, 1979) were shown to possess a pronounced Ca^{2+}-independent activity component.

The K'_m(Ca) of A_1 and the K_D(Ca) of N_{1a} differ only by a factor of 3.5 at 10 mM Mg^{2+}, pH 6.8 (Table VIII). Characteristically, A_1 and N_{1a} both exhibit Mg^{2+} competition. It is therefore concluded that binding of Ca^{2+} to the N_{1a} sites results in stimulation of A_1. Likewise, the Ca^{2+} affinity of A_2 is 3–10-fold lower than that of the N_2 sites. These N_2 binding sites are induced by 10 mM Mg^{2+} (Kilimann & Heilmeyer, 1977); similarly, A_2 requires Mg^{2+} above 20 mM to appear at pH 6.8 (Figure 1B). By employment of a partially proteolyzed enzyme, the N_2 binding sites cannot be observed between 2 and 10 μM Ca^{2+} (Table VIII); likewise 57 mM Mg^{2+} is unable to induce the A_2 activity at pH 6.8 up to 170 μM Ca^{2+} in this particular enzyme (Table VIII). Activity and binding capacity of A_2 and N_2, respectively, approach their maximum level within a narrower concentration range than would be expected from a hyperbolic binding curve (Kilimann & Heilmeyer, 1977) or a Michaelis–Menton function (Figure 5); i.e., they both show apparent positive cooperativity. These qualitative similarities suggest that Ca^{2+} saturation of the N_2 sites leads to the expression of A_2. This A_2 activity must be responsible for the activation seen at high pH and commonly used to characterize phosphorylase kinase when the pH 6.8/8.2 activity ratio is measured (Krebs et al., 1964). Since A_2 is fully stimulated by 2.8 mM Mg^{2+} at pH 9.0 (Table VI) whereas at pH 6.8 it requires Mg^{2+} > 20 mM (Figure 1; Table VI) to be induced, the stimulatory effect of pH can be probably attributed to an increase in affinity of the Mg^{2+} binding sites that induce A_2 [cf. Heilmeyer et al. (1981)]. In contrast to the triphasic increase of phosphorylase kinase activity shown here, a biphasic increase with Ca^{2+} was observed earlier

(Brostrom et al., 1971). Those measurements were performed in 60 mM Mg^{2+} and without additional salt, conditions under which the K'_m(Ca) of A_1 is enhanced and that of A_2 is lowered. This results in a confluence of the two activities A_1 and A_2 which might have prevented the detection of the three partial activities described here.

The reversibility of all the described Ca^{2+} effects rules out that they are mediated by autophosphorylation, denaturation, or proteolysis. The Ca^{2+} binding measurements on phosphorylase *b* exclude that the activation of A_1 and A_2 is mediated by Ca^{2+} binding to this substrate whereas the inhibition of A_0, A_1, and A_2 by millimolar Ca^{2+} still could occur by such a mechanism. However, a similar Ca^{2+} inhibition can be observed in the autophosphorylation of phosphorylase kinase and in the phosphorylation of troponin T which also rules out this latter possibility (Kilimann & Heilmeyer, 1982). No significant shifts in the concentrations of $MgATPH^-$, ATP^{4-}, $ATPH^{3-}$, NH_4ATP^{3-}, $MgATP^{2-}$, and $CaATP^{2-}$ accompanies the rise in Ca^{2+} concentration observed at constant pH within the range in which A_1 and A_2 are stimulated (calculated as described under Experimental Procedures).[3] The inhibition of A_0, A_1, and A_2 above 200 μM Ca^{2+} is accompanied by a moderate decrease of [$MgATP^{2-}$], and a rise of [$CaATP^{2-}$] proportional to the rise of [Ca^{2+}]. As [Ca^{2+}] and [$CaATP^{2-}$] are coupled strictly by the law of mass action, inhibition by millimolar Ca^{2+} due to competition of $CaATP^{2-}$ with $MgATP^{2-}$ cannot be completely ruled out (Krebs et al., 1959). However, an analogous Ca^{2+} inhibition of liver phosphorylase kinase was observed by Chrisman et al. (1979), but only above 1 μM. This would demand a K_i for $CaATP^{2-}$ more than 3 magnitudes lower than that for $MgATP^{2-}$. Therefore, a direct competition between Ca^{2+} and Mg^{2+}, but not $CaATP^{2-}$ with the substrate $MgATP^{2-}$, seems more likely.

The strong enhancement of V_{max} for A_2 upon phosphorylation by cAMP-dependent protein kinase mimics the effect of alkaline pH on nonmodified phosphorylase kinase: both are known to lower the K'_m for phosphorylase *b* (Krebs et al., 1964; DeLange et al., 1968). Both effects could be explained if A_2 had a higher affinity for phosphorylase *b* than A_0 and A_1.

The increase in V_{max} for A_1 following proteolysis or autophosphorylation greatly exceeds that provided by high pH. This may explain the earlier observations that a higher pH 6.8/8.2 activity ratio is attained by extensive autophosphorylation or proteolysis than by the cAMP-dependent phosphorylation (Wang et al., 1976; Cohen, 1973). The latter increases the A_2 activity only, whereas the first two modifications stimulate the activity of A_1 (or A_0 and A_1), far above the level reached by pH enhancement.

The three partial activities probably do not represent different conformers of the enzyme because the enhancement of a particular partial activity by covalent modification or increase in pH never coincides with the suppression of another. The activities A_0 and A_1 could represent modified forms of the enzyme. However, the methods of covalent modification applied here always activate A_2 to a far greater extent than A_0 or A_1, whereas nonactivated phosphorylase kinase expresses both activities in the absence of A_2 (Figure 6).

Finally, dissociation of the Ca^{2+} binding δ subunit, e.g., by proteolysis, would abolish its inhibitory action. This would render the activities A_1 or A_2 Ca^{2+} independent and yield an A_0-like activity with a modified response to Mg^{2+}, NH_4Cl, and pH (see above) and a wider protein substrate specificity (Kilimann & Heilmeyer, 1982). Even though A_0 was not found to be concentration dependent (it can be observed even at the high enzyme concentrations at which autophosphorylation is carried out) and its stimulation by trypsin coincides with an even more prominent stimulation of A_2, this cannot be excluded.

Phosphorylase *a* formation upon electrical stimulation (Posner et al., 1965; Drummond et al., 1969; Stull & Mayer, 1971) can be attributed to the stimulation of the A_1 activity, the only one of the two Ca^{2+}-dependent activities, observed at physiological pH and Mg^{2+} concentrations. Its Ca^{2+} sensitivity (taking into account the competitive influence of Mg^{2+}) is in the concentration range in which muscle contraction is regulated (Hasselbach, 1976). The free sarcoplasmic Mg^{2+} concentration is not known. However, 10 mM can be considered an upper limit, since it represents the total magnesium content of skeletal muscle (Walser, 1967). At pH 6.8 this concentration may not be sufficient to induce the A_2 activity of the nonmodified enzyme in intact muscle cells. Adrenalin leads to the phosphorylation of phosphorylase kinase by cAMP-dependent protein kinase (Yeaman & Cohen, 1975). Probably as a result of an increase in the affinity of the inducing sites for Mg^{2+} (Heilmeyer et al., 1980a,b), the A_2 activity can now appear at physiological pH. One might assume that it mediates the hormonal signal. Administration of adrenalin to resting muscle where free Ca^{2+} is ca. 50 nM (Hasselbach, 1976) can lead to an almost complete conversion of phosphorylase to the *a* form (Drummond et al., 1969). For A_2 to be responsible for this *b* to *a* conversion, its K'_m(Ca) would have to be at least 1 order of magnitude lower under physiological conditions than that observed here. This would be required to maintain a catalytic activity at 50 nM Ca^{2+} that is at least as high as that of A_1 during contraction. Alternatively, if the Ca^{2+} affinity of A_2 under in vivo conditions did not differ significantly from that found in vitro, the *b* to *a* conversion in resting muscle upon adrenalin stimulation would rely solely on A_0 [compare Heilmeyer et al. (1980a)]. In that case an increase in the level of phosphorylase *a* could only result from an inhibition of phosphorylase phosphatase which could occur through a cAMP-dependent phosphorylation of inhibitor 1 (Huang & Glinsmann, 1976, 1979; Foulkes & Cohen, 1979). Stimulation of the *b* to *a* conversion by adrenalin in contracting muscle particularly during fatigue (Danforth & Helmreich, 1964) could additionally involve A_2. In any case, nervous and hormonal stimulations of glycogenolysis would then follow separate pathways at the level of phosphorylase kinase, just as they are expressed by the two second messengers, Ca^{2+} and cAMP, higher up in the regulatory "cascade".

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[3] Data not shown but will be furnished to the interested reader by writing directly to the author.

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