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Phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II and protein kinase C of sepiapterin reductase, the terminal enzyme in the biosynthetic pathway of tetrahydrobiopterin

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Abstract

Sepiapterin reductase, the terminal enzyme in the biosynthetic pathway of tetrahydrobiopterin, was stoichiometrically phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II and protein kinase C (Ca^{2+}/phospholipid-dependent protein kinase) in vitro. Maximal incorporation of phosphate into the enzyme subunit by these was 3.05 ± 0.05 (n = 4) and 0.74 ± 0.03 (n = 5) ^{32}P mol per mol enzyme subunit, respectively. The enzyme was not phosphorylated by cyclic nucleotide-dependent protein kinase of either the cAMP-dependent or cGMP-dependent type in this study. Dihydropteridine reductase, another enzyme working in direct supply of tetrahydrobiopterin, was also a good substrate for Ca^{2+}/calmodulin-dependent protein kinase II. Phosphorylation of sepiapterin reductase by these protein kinases modified the kinetic properties of the enzyme. It is likely that these multifunctional Ca^{2+}-activated protein kinases may play a role in the regulation of the physiological function of the BH4-generating enzymes in vivo, as was previously found in the case of BH4-requiring enzymes.

Key words: Tetrahydrobiopterin synthesis; Sepiapterin reductase; Phosphorylation; Ca^{2+}/calmodulin-dependent protein kinase II; Protein kinase C

1. Introduction

Tetrahydrobiopterin (BH4) is an essential H-donor cofactor for aromatic amino acid hydroxylases such as phenylalanine hydroxylase (PH), tyrosine hydroxylase (TH), and tryptophan hydroxylase (TpH), which enzymes are rate-limiting for the formation of tyrosine in the liver, catecholamines, and indoleamines in neural tissues, respectively [1] (Fig. 1). Recently, the requirement of the BH4 cofactor was also demonstrated for nitric oxide synthase (NOS) [2,3] for generating nitric oxide (NO), a messenger molecule involved in various processes in many tissues. It was previously reported that these BH4-requiring enzymes are partly regulated in their function by phosphorylation by various protein kinases. Mechanisms for the maintenance or rapid modulation of the BH4 level in the cell are also important for the regulation of the activities of the BH4-requiring enzymes to control the formation of catecholamines and indoleamines or NO in vivo.

We now report the phosphorylation of BH4-generating enzymes that are involved in the biosynthesis and regeneration of BH4 (Fig. 1). Sepiapterin reductase (SPR; EC 1.1.1.153), which is the terminal enzyme in the BH4 biosynthetic pathway [4] and regulated by diverse substances [5–8], was stoichiometrically phosphorylated by multifunctional Ca^{2+}-activated protein kinases in vitro. We also examined the phosphorylation of two other BH4-generating enzymes, dihydropteridine reductase (DHPR; EC 1.6.99.10) and dihydrofolate reductase (DHFR; EC 1.5.1.3.) (Fig. 1), and discuss the effect of the phosphorylation on the enzyme function in the light of generation and requirement of BH4.

2. Materials and methods

2.1. Chemicals

Some of the chemicals used and their sources were as follows: [γ-^{32}P]ATP (4000 Ci/mmol) from ICN, USA; ATP and cyclic AMP from Boehringer Mannheim, Germany; protein kinase C (PKC, rat brain), from Promega, USA; casein, from Merck, Germany; glycogen synthase fragment (sequence: 1–10), from Seikagaku Kogyo, Japan; molecular weight marker proteins, from Pharmacia-LKB, Sweden; sepiapterin, from Dr. Schircks Lab., Switzerland; 1,2-diolein (DL), from Serdary Research Lab., Canada; and phorbol 12,13-dibutyrate

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Abbreviations: BH4, tetrahydrobiopterin; SPR, sepiapterin reductase; DHPR, dihydropteridine reductase; DHFR, dihydrofolate reductase; CaM, calmodulin; CaM-KII, Ca^{2+}/calmodulin-dependent protein kinase II; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; EGTA, glycoletherdiamine tetraacetic acid; PDB, phorbol 12,13-dibutyrate; DL, 1,2-diolein; PS, phosphatidylserine.

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(PDB) and phosphatidylserine (PS), from Wako, Japan. Tubulin, MAP 1, MAP 2, and calmodulin (CaM) were a generous gift from Dr. M. Sano (Institute for Developmental Biology, Aichi, Japan). Purified DHPR (sheep liver) and DHFR (bovine liver), histones (type IIS, IIIS, IVS), protamine sulfate, myosin light chains from smooth muscle (chicken gizzard) and skeletal muscle (chicken), and all other chemicals were obtained from Sigma (USA).

2.2. Protein purification and peptide synthesis

SPR was purified to homogeneity from rat erythrocytes by PAGE and SDS-PAGE [6]. The catalytic subunit of cAMP-dependent protein kinase (PKA) was purified from bovine heart by the method of Reimann et al. [9]. cGMP-dependent protein kinase (PKG) was purified from silkmoth pupae as described by Takahashi [10]. Ca^{2+}/calmodulin-dependent protein kinase II (CaM-KII) was prepared from rat cerebral cortex according to the method of Yamaguchi and Fujisawa [11]. Activities of CaM-KII and PKC were determined with casein and histone type IIIS, respectively, used as substrates. Three peptide fragments (sequences: 152–159, 159–165, and 163–171) of the phosphorylation site domain of bovine MARCKS, of which the total amino acid sequence was determined by Stumpo et al. [12], were synthesized by use of an ABI peptide synthesizer (Model 430A) and purified by HPLC with a reverse-phase column (ODS-H-1251) (Senshu, Japan). The peptides were obtained from the column after 21 min by elution with the gradient solution of 0.1% trifluoroacetic acid (TFA) and 50% TFA/50% acetonitrile (flow rate, 1 ml/min) under detection at 210 nm.

2.3. Phosphorylation experiments

Phosphorylation reactions were conducted at 25°C for 1–10 min in a final volume of 100 μl. Purified enzymes (SPR, DHPR, and DHFR) and various other substrates of protein kinases were added at a concentration of 0.5 μM. The reaction mixture for CaM-KII contained 50 mM Tris-HCl buffer (pH 7.5), 0.5 μg CaM-KII, 0.5 μM CaM, 100 μM [γ-^{32}P]ATP (3000 cpm/pmol), 2 mM Mg acetate, and 0.15 mM $CaCl_2$. Phosphorylation by PKC was assayed in the reaction mixture containing 20 mM Tris-HCl buffer (pH 7.5), 25 ng PKC, 100 μM [γ-^{32}P]ATP (3000 cpm/pmol), 1.5 mM $CaCl_2$, 10 mM $MgCl_2$, and 2 μM PDB/600 μM PS/80 μM DL as activator. Phosphorylation by PKA [13] and PKG [10] (10 nM cAMP or cGMP, 200 units PKA or PKG) were performed in the presence of 0.1 mM [γ-^{32}P]ATP (3000 cpm/pmol) basically according to the references cited.

After incubation, the mixture was electrophoresed on 12.5% SDS-PAGE gels as described by Laemmli [14], and the gels were then autoradiographed. Proteins on the gel were stained with Coomassie brilliant blue R 250. Radioactive bands were cut out from the gel and subjected to liquid scintillation counting. Phosphorylation of peptide substrates was assayed according to the method of Glass et al. [15]. The phosphorylation reaction lacking substrate was performed as a control in the presence of Ca^{2+}. Rates of phosphorylation of the purified enzymes by protein kinases were compared with those of various other substrates by expressing them as a value relative to that of smooth muscle myosin light chains.

2.4. Enzyme assays

Activities of SPR [5] and DHPR [16] were determined photometrically (Hitachi Spectrophotometer U-3210) at 25°C as cited.

2.5. Protein determination

Protein was determined by the method of Lowry et al. [17]. Proteins on the gel were estimated densitometrically (with carbonic anhydrase, M_r 31 kDa as a standard) after the staining.

3. Results

SPR from rat erythrocytes consists of two homologous subunits of 28 kDa [6,18]. Subunits of sheep DHPR [19] and bovine DHFR [20] were 29 kDa and 24 kDa by SDS-PAGE, respectively, in this experiment. Purified enzymes and various other substrates were incubated with [γ-^{32}P]ATP in the phosphorylating systems of CaM-KII, PKC, PKA, and PKG. After SDS-PAGE, radioactivity was monitored by autoradiography (Fig. 2). In the CaM-KII system, a few bands were apparent around the position of 50–60 kDa corresponding to autophosphorylated subunits of CaM-KII (lanes 1–5, S,F and P). Obvious bands appeared in the lanes that contained typical substrates of CaM-KII (lanes 3,4) and PKC (lanes 2,3), while no band corresponding to substrate was found in lanes that contained poor substrates of CaM-KII (lanes 2, 5). In the CaM-KII and PKC systems, significant radioactive bands were also observed at the positions corresponding to the SPR subunit (Lane S); and in the CaM-KII system the DHPR subunit (lane P) was also phosphorylated. The radioactive band corresponding to SPR or DHPR subunit was not detected when CaM was omitted from the CaM-KII reaction, and the SPR band was missing when Ca^{2+} was omitted from, or 0.1 μM staurosporine was added to the PKC system (data not shown). These results indicate that SPR was phosphorylated by CaM-KII and PKC and that DHPR was phosphorylated by CaM-KII. DHFR was not phosphorylated by either CAM-KII (lane F) or PKC (lane F). None of the enzymes were phosphorylated by either PKA (lanes S,F,P) or PKG (lanes S,F,P) in this experiment.

Rates of phosphorylation of SPR and DHPR by CaM-KII and PKC were compared with those of other substrates of these kinases (Table 1). Initial rates of phos-

Table 1
Rate of phosphorylation of sepiapterin reductase and dihydropteridine reductase relative to that of smooth muscle myosin light chains by CaM-KII and PKC

Substrates	Relative initial rate of phosphorylation	
	Ca^{2+}/CaM-KII	PKC
Histone type II-S	0	0.30
III-S	0.95	11.8
IV-S	0	0.34
Protamine sulfate	3.11	12.8
Casein	0.93	0
Myosin light chains		
skeletal muscle	0	0
smooth muscle	1.00*	1.00*
MAP 1	0	2.60
MAP 2	16.6	3.50
Tubulin	1.00	0
Peptides		
glycogen synthase (1–10)	9.82	1.49
MARCKS (152–159)	1.91	1.14
(159–165)	1.00	4.33
(163–171)	0	0.37
Sepiapterin reductase	1.65	0.98
Dihydropteridine reductase	0.63	0
Dihydrofolate reductase	0	0

Phosphorylation of each substrate was determined at 25°C for 3 min as described in the text. Rates of phosphorylation of substrates are expressed as the value relative to that of smooth muscle myosin light chains (*).

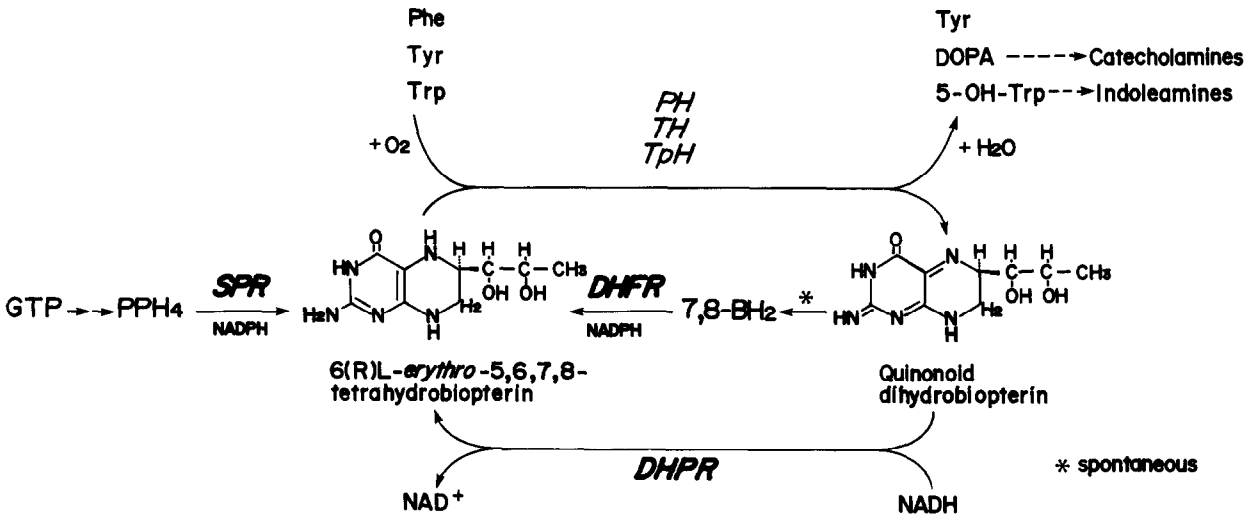

Fig. 1. Enzymes generating and requiring tetrahydrobiopterin. SPR, sepiapterin reductase; DHPR, dihydropteridine reductase; DHFR, dihydrofolate reductase; PH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; TpH, tryptophan hydroxylase; 7,8-BH_2, 7,8-dihydrobiopterin; PPH4, 6-pyruvoyl tetrahydropterin; 5-OH-Trp, 5-hydroxytryptophan.

phorylation of them relative to that of smooth muscle myosin light chains were determined. The results shown in Table 1 demonstrate that both SPR and DHPR were good substrates of CaM-KII, being comparable to casein and tubulin and that SPR, like a fragment of glycogen sythase (1–10), was also a good substrate of PKC. The

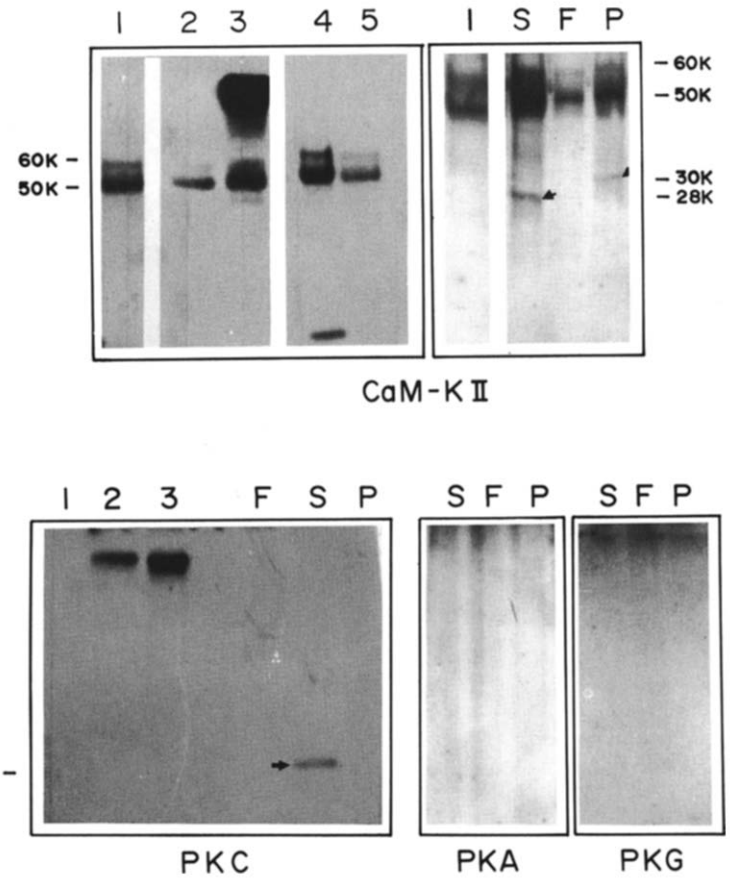

Fig. 2. Autoradiographs following SDS-PAGE of protein kinase-mediated phosphorylation reactions with purified enzymes of SPR, DHPR, and DHFR and other proteins used as substrates. Lanes S, F, and P contained SPR, DHFR, and DHPR, respectively. Lane 1 contained no substrate, and lanes 2–5 contained MAP 1, MAP 2, smooth muscle and skeletal muscle myosin light chains, respectively. Reactions were accomplished at 25°C for 3 min under the conditions described in section 2. In the CaM-KII experiment, the locations of subunits (50–60 kDa) of autophosphorylated CaM-KII are indicated in all lanes. Arrows indicate the purified enzymes phosphorylated.

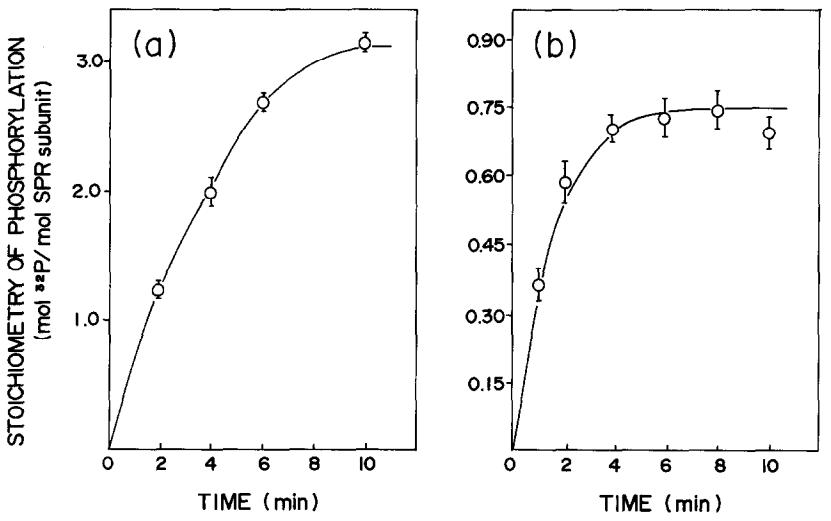

Fig. 3. Quantification of the time-dependent radioactive ^{32}P associated with SPR by phosphorylation of the enzyme by CaM-KII and PKC. SPR was phosphorylated as described in the text for the indicated times at 25°C. After SDS-PAGE, autoradiography was carried out, and stoichiometry of ^{32}P incorporation into SPR subunit was determined. Data are representative of experiments repeated 4 to 5 times (n = 4–5) with means and standard deviations. (a) time course for CaM-KII. (b) time course for PKC.

results also indicated that CaM-KII and PKC used in this study could phosphorylate two and all three fragment peptides, respectively, from the phosphorylation site domain in MARCKS (152–159, 159–165, 163–171) besides their substrates previously reported.

Fig. 3 shows substantial time-dependent incorporation of ^{32}P into SPR by CaM-KII (a) and PKC (b). Quantification of the radioactive ^{32}P associated with SPR indicated that the phosphorylation of SPR by CaM-KII reached its maximal level within 10 min. Half-maximal phosphorylation occurred at 3 min. The amount of phosphate incorporated into SPR after incubation for 10 min was 3.05 ± 0.05 (n = 4) mol of ^{32}P per mol of enzyme subunit. Phosphate incorporated into SPR by PKC at its maximal level was 0.74 ± 0.03 (n = 5) ^{32}P mol per mol subunit. These results indicate that monomer subunit of SPR could be maximally modified by the addition of 3 and 1 phosphate molecules by CaM-KII and PKC, respectively.

Effect of phosphorylation of SPR on the enzyme activity was examined by double reciprocal plotting of initial velocities. SPR was incubated with CaM-KII or PKC under the conditions in which SPR was maximally phosphorylated, and then the activity was measured. Results indicated that in vitro phosphorylation by either CaM-KII (Fig. 4) or PKC (Fig. 5) significantly modified the kinetical properties of SPR. Phosphorylation by CaM-KII decreased substrate inhibition at higher concentrations of pterin substrate (Fig. 4). On the other hand, phosphorylation by PKC changed the kinetic constants of K_m and V_{max}, which values increased about 2-fold over those of unphosphorylated SPR (Fig. 5). Phosphorylation of DHPR by CaM-KII, however, did not lead to any significant change in the activity when this enzyme was assayed with 1 μM concentration of either BH4 or 6-methy tetrahydropterin as the source of quinonoid dihydropterin substrate in the assay system of DHPR [16] (data not shown).

4. Discussion

The important finding of this study is that SPR and DHPR, both of which play an important role in the direct supply of BH4 for BH4-requiring enzymes, are phosphorylated by Ca^{2+}-activated kinases. The activity of PKC used in this experiment was Ca^{2+}-dependent. And thus it was Ca^{2+}/phospholipid-dependent protein kinase belonging to the conventional PKC group and requiring Ca^{2+} ion for activation [21]. Ca^{2+} play a central role as a second messenger with a regulatory involvement in many aspects of cellular signalling. A number of Ca^{2+}-activated protein kinases that may mediate Ca^{2+} action have been identified. Of these, only PKC and CaM-KII (and IV) are known to have a broad substrate specificity and the potential for regulation of numerous processes.

Our data establish that SPR belongs to that group of enzymes that are good substrates for CaM-KII and PKC (Ca^{2+}/phospholipid-dependent protein kinase), as evidenced by the following. (1) Phosphorylation of SPR was not enhanced by denaturation by urea (data not shown), indicating that native SPR contains specific sites capable of being phosphorylated by CaM-KII and PKC. (2) The rates of phosphorylation of SPR by CaM-KII and PKC were comparable to those of tubulin and casein (by CaM-KII), and MAP 1 and glycogen synthase fragment (1–10) (by PKC) under the same system of phosphorylation (Table 1). (3) SPR is stoichiometrically phosphorylated by CaM-KII and PKC (Fig. 3).

The primary structure of the SPR subunits of rats [18,22] and humans [23] as indicated by amino acid and cDNA sequences, contains three (S^{46}, S^{196}, S^{214}) and one (S^{213}) phosphorylation sites, respectively, for CaM-KII (R-X-X-S*/T*) [24]. Thus, the incorporation of each of the three molecules of ^{32}P into the SPR subunit by CaM-KII, as shown in this study, might be specified by each of these sequences. The primary structure of DHPR from sheep liver has not completely been analyzed; however, a partial structure of it shows high homology to that of DHPR of human origin [25], the latter of which has one sequence (S^{223}) for CaM-KII phosphorylation. DHFR from bovine liver [20] has one consensus sequence (T^{39}) for CaM-KII, but it was not phosphorylated by CaM-KII in this study. Rat SPR [18,22], sheep DHPR [19], and bovine DHFR [20] contain several consensus sequences for PKC [26]; however, only the phosphorylation of SPR by PKC was observed in this study.

The results of this study suggest that some portion of SPR and DHPR in cells is present in the phosphorylated form in vivo, and is isolated in the dephosphorylated form during the purification procedure.

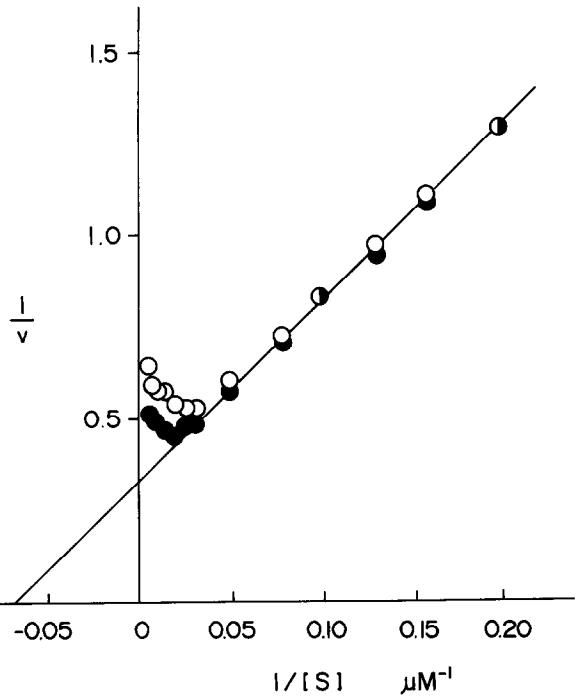

Fig. 4. Kinetical properties of SPR phosphorylated by CaM-KII. SPR (190 ng) was incubated in the phosphorylating system of CaM-KII containing 50 μM ATP instead of [γ-^{32}P]ATP in a final volume of 100 μl at 25°C. The reaction lacking Ca^{2+}/CaM was examined as a control for unphosphorylated SPR. After a 10-min incubation, 13 μl of a solution containing EGTA and NaF was added to the reaction mixture to give a final concentration of 9.8 mM for each. SPR activity in the resulted mixture was then assayed for 1 min at 25°C in the system containing 100 mM potassium phosphate buffer (pH 6.4), 100 μM NADPH, and various concentrations of sepiapterin in a final volume of 2 ml [5]. Activities are indicated in double reciprocal plots. Filled and open circles show phosphorylated and unphosphorylated SPR, respectively.

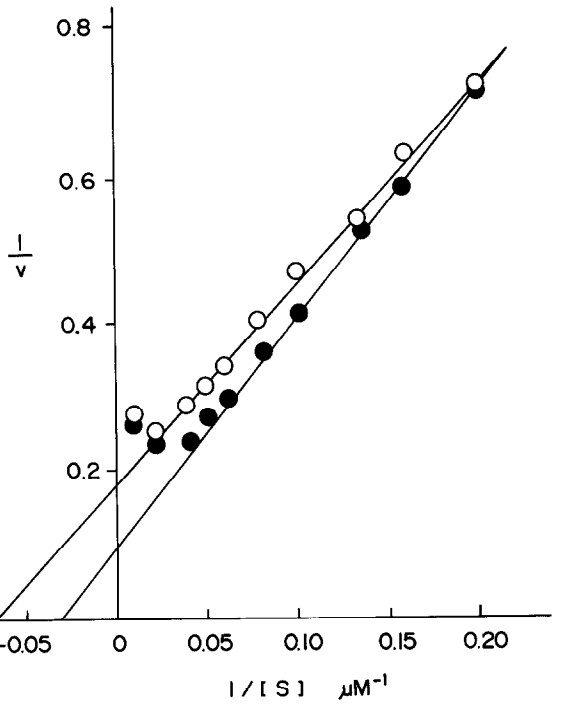

Fig. 5. Kinetical properties of SPR phosphorylated by PKC. SPR (310 ng) was incubated in the phosphorylating system of PKC containing 50 μM ATP instead of [γ-^{32}P]ATP in the presence of 50 μg bovine serum albumin (as stabilizer for SPR) in a final volume of 100 μl at 25°C. The reaction lacking PKC was assayed as a control for unphosphorylated SPR. And then SPR activity in the reaction mixture was assayed after the addition of EGTA and NaF as described in Fig. 4. Activities are indicated in double reciprocal plots. Filled and open circles show phosphorylated and unphosphorylated SPR, respectively.

It was previously demonstrated that BH4-requiring enzymes are regulated in their functions by phosphorylation with various protein kinases. Of interesting is that PH [27], TH [28], TpH [11], and NOS [29], all of which require BH4 for their activities (Fig. 1), are all good substrates of CaM-KII in vitro. Our present results are the first finding of phosphorylation by protein kinases of enzymes that are involved in BH4 formation. SPR was modified in its kinetical properties by phosphorylation in vitro by either CaM-KII or PKC in this study. We can thus conclude that the BH4 system including both BH4-requiring and -generating enzymes is totally regulated by Ca^{2+} ions in the cell through phosphorylation by multifunctional Ca^{2+}-activated protein kinases such as CaM-KII and PKC to control neurotransmitter synthesis.

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