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STIMULATORY EFFECT OF CALMODULIN ANTAGONISTS ON PHOSPHOLIPID BASE-EXCHANGE REACTIONS IN RABBIT PLATELET MEMBRANES

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Abstract—The properties of Ca^{2+}-dependent incorporation of [^{3}H]serine, [^{3}H]ethanolamine and [^{3}H]choline into the corresponding phospholipids mediated by base-exchange enzymes in rabbit platelet membranes were studied in the presence or absence of the calmodulin antagonists chlorpromazine, trifluoperazine and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), all of which markedly activate three base-exchange reactions. The base-exchange activities were dependent on Ca^{2+} both in the presence and absence of the drugs. Other metal ions tested did not stimulate the base-exchange reactions, even in the presence of the drugs. Apparent K_m values for serine, ethanolamine and choline were not affected significantly by the concentration of Ca^{2+}, with or without the drugs. [^{3}H]Serine incorporation into phospholipid was competitively inhibited by ethanolamine and choline, [^{3}H]choline incorporation was competitively inhibited by serine and ethanolamine, whereas [^{3}H]ethanolamine incorporation was competitively inhibited by serine and noncompetitively by choline. These competitive and noncompetitive relations between each base were also not affected by the drugs. The amount of $^{45}Ca^{2+}$ binding to platelet membranes was decreased by the drugs dose dependently. A weaker calmodulin antagonist, *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5), only slightly stimulated the base-exchange reactions, but did clearly inhibit $^{45}Ca^{2+}$ binding to the membranes, in the same manner as that of the other calmodulin antagonists used. The concentration of chlorpromazine, trifluoperazine, W-7 and W-5, required to produce half-maximal inhibition of Ca^{2+} binding, was approximately 30 μM. These results suggest that the calmodulin antagonists used activate the base-exchange reactions only in the presence of Ca^{2+} without changing the affinity of each free base to base-exchange enzymes. The activation of the base-exchange reactions was not due to the increase in free Ca^{2+} caused by the drug-induced inhibition of Ca^{2+} binding to platelet membranes.

Enzymatic properties of base-exchange reactions have been studied in membrane fractions of rat liver [1–3], rat brain [4–6], hamster lung [7], human leukocytes [8], rabbit retina [9] and rat heart [10]. These reactions essentially require Ca^{2+} as a cofactor. Recently, we reported some enzymatic properties of base-exchange reactions in rabbit platelet membranes [11]: not only the base-exchange activities but also optimum pH for the reactions are clearly dependent on the concentration of Ca^{2+}, as is the case in the liver [2] and brain [5]. In addition, calmodulin antagonists such as chlorpromazine, trifluoperazine and W-7, which inhibit platelet aggregation and secretion induced by various agonists [12–16], markedly stimulate Ca^{2+}-dependent incorporation of not only serine but also ethanolamine and choline into the corresponding phospholipids in isolated rabbit platelet membranes. However, we found that the drug treatment of intact platelets causes a marked decrease in the contents of endogenous free serine, whereas the contents of free ethanolamine and choline are increased dose dependently. Furthermore, the drugs stimulate only the synthesis of phosphatidylserine [17]. These results strongly suggest that, in intact platelets, the drugs stimulate only the serine-phospholipid base-exchange reaction.

Therefore, to elucidate the regulatory mechanisms of the base-exchange reactions, we analyzed the kinetics of these reactions, in the presence or absence of calmodulin antagonists.

MATERIALS AND METHODS

Materials. Chlorpromazine and trifluoperazine were purchased from the Yoshitomi Pharmaceutical Co. (Japan). *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7‡) and *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5) were donated by Professor H. Hidaka, Mie University. L-[3-^{3}H]Serine (29 Ci/mmole), [1 - ^{3}H]ethan - 1 - ol - 2 - amine hydrochloride (30 Ci/mmole) and [^{3}H-*methyl*]choline chloride (60 Ci/mmole) were obtained from The Radiochemical Centre (Amersham, England). $^{45}CaCl_2$ (1.1 Ci/mmole) was obtained from New England Nuclear (Boston, MA), and phospholipid standards were from GIBCO. All other chemicals were of analytical grade.

Assay of base-exchange enzymes. Rabbit platelet membranes were prepared as described previously [18], and membranes were suspended in 0.25 M sucrose. The standard assay mixture (0.5 ml) con-

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‡ Abbreviations: W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; and HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

tained 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.5), 25 mM sucrose, 0.35 mg of membrane protein and 0.1 mM [^{3}H]serine (2 μCi) or [^{3}H]ethanolamine (2 μCi) or [^{3}H]choline (4 μCi). The reaction was carried out at 37° for 10 min and terminated with 0.3 ml of 0.5 N HCl and 3 ml of chloroform–methanol (1:2, v/v). Radioactive lipid was extracted as described by Bligh and Dyer [19]. The reaction products were quantified and identified as described previously [11].

Equilibrium dialysis. The membrane protein (0.5 mg, 0.3 ml) was dialyzed against 10 ml of HEPES buffer (50 mM, pH 7.5) containing $^{45}CaCl_2$ (0.1 mM, 5 μCi), with or without various drugs for 12 hr at 4° in the dark. After dialysis, the concentration of membrane protein in the inner solution was determined by the method of Lowry *et al.* [20], using bovine serum albumin as the standard, and the radioactivity in 0.1 ml of the inner and outer solutions was measured in 10 ml of scintillation mixture, using a Packard Tri-Carb scintillation counter.

RESULTS

Metal requirement. Among the various divalent metal ions tested, only Ca^{2+} stimulated base-exchange reactions in the presence or absence of various calmodulin antagonists—chlorpromazine, trifluoperazine and W-7; the concentrations of the drugs required for maximal stimulation are about 75 μM [11]. Other metal ions, Mg^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, CO^{2-}, Zn^{2+}, Hg^{2+}, Sn^{2+}, Sr^{2+}, Fe^{2+}, Fe^{3+}, and La^{2+}, at concentrations of 50 and 100 μM and 1 mM, did not stimulate the base-exchange reactions, even in the presence of the calmodulin antagonists (75 μM) (data not shown).

Kinetics of the base-exchange reactions. As shown in Fig. 1, apparent K_m values for serine, ethanolamine and choline were similar in the presence of 0.1 and 3 mM Ca^{2+}, with or without 75 μM chlorpromazine. Trifluoperazine and W-7 had almost the same effects on the three base-exchange reactions. Table 1 shows the apparent K_m and V_{max} values under various assay conditions. As shown in Fig. 2, panels A, B, E and F, the incorporation of [^{3}H]serine and that of [^{3}H]choline were competitively inhibited by ethanolamine or choline, and serine or ethanolamine, respectively. In contrast, [^{3}H]ethanolamine incorporation was inhibited competitively by serine, but noncompetitively by choline (Fig. 2, panels C and D). The respective K_i values of serine in the formation of phosphatidylethanolamine and phosphatidylcholine were 80 and 140 μM, those of ethanolamine in the formation of phosphatidylserine and phosphatidylcholine were 220 and 450 μM, and those of choline in the formation of phosphatidylserine and phosphatidylethanolamine were 0.78 and 12.5 mM. Inhibitory properties of each base on the exchange reactions and the K_i values were essentially the same as those obtained under the assay condition with 3 mM Ca^{2+} alone.

Each base-exchange activity was dependent on the concentration of the substrate and was inhibited by the other bases, as described above. In the cell, however, these three bases are considered to exist as a mixture, the contents of free serine, ethanolamine and choline being 4.8 ± 0.12, 1.8 ± 0.05 and 1.5 ± 0.06 nmoles/10^9 cells [17]. The volume of one rabbit platelet was determined to be about 4.5 × μm^3 using a platelet analyzer Baker 810. Therefore, the concentrations of free serine, ethanolamine and choline were calculated to be approximately 1.1, 0.40 and 0.33 mM, respectively, on the assumption that the free bases are evenly distributed in the platelet. As shown in Table 2, in the presence of the three bases together at the concentrations described above, [^{3}H]serine incorporation into phospholipid was only slightly inhibited by ethanolamine and choline, as compared with the activity in the presence of 1.1 mM serine alone. In contrast, [^{3}H]ethanolamine incorporation was inhibited by 60–70% and

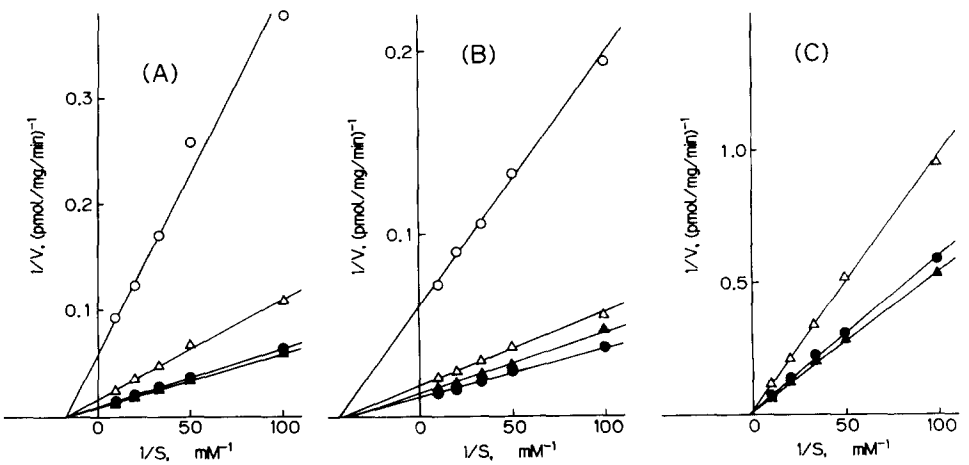

Fig. 1. Effects of various concentrations of serine, ethanolamine and choline on base-exchange reactions. Platelet membrane protein (0.35 mg) was incubated for 10 min as described in Materials and Methods in the presence of various concentrations of serine (A), ethanolamine (B) or choline (C) at 0.1 or 3 mM Ca^{2+} with or without chlorpromazine as indicated. Key: (○) 0.1 mM Ca^{2+}; (●) 0.1 mM Ca^{2+} with 75 μM chlorpromazine; (△) 3 mM Ca^{2+}; and (▲) 3 mM Ca^{2+} with 75 μM chlorpromazine.

Table 1. Apparent K_m and V_{max} values for serine, ethanolamine and choline in various assay conditions

Calmodulin antagonist	Ca^{2+} concn (mM)	Serine K_m (μM)	Serine V_{max} (pmoles/mg/min)	Ethanolamine K_m (μM)	Ethanolamine V_{max} (pmoles/mg/min)	Choline K_m (μM)	Choline V_{max} (pmoles/mg/min)
None	0.1	58	17	23	17	360	15
CPZ	0.1	67	126	27	95	400	73
TFP	0.1	65	126	25	95	380	80
W-7	0.1	63	136	25	86	370	75
None	3	65	64	24	59	400	58
CPZ	3	65	141	29	83	360	80

The medium (0.5 ml) for base-exchange reactions contained 50 mM HEPES buffer (pH 7.5), 0.34 mg of membrane protein, 0.1 or 3 mM $CaCl_2$, with or without 75 μM calmodulin antagonists, and various concentrations of labeled substrates as described in Fig. 1. Reactions were carried out for 10 min at 37°. Abbreviations: CPZ, chlorpromazine; and TFP, trifluoperazine.

[^{3}H]choline incorporation was inhibited markedly in the presence of the other bases. These findings suggest that only the serine base-exchange activity occurs under normal circumstances.

Effects of calmodulin antagonists on $^{45}Ca^{2+}$ binding (and/or uptake) to platelet membranes. The $^{45}Ca^{2+}$ binding (and/or uptake) to platelet membranes was studied using an equilibrium dialysis method. The binding (and/or uptake) activity was increased with the increase in the concentration of Ca^{2+} (Fig. 3). As shown in Fig. 4, $^{45}Ca^{2+}$ binding was dose-dependently inhibited by chlorpromazine, trifluoperazine,

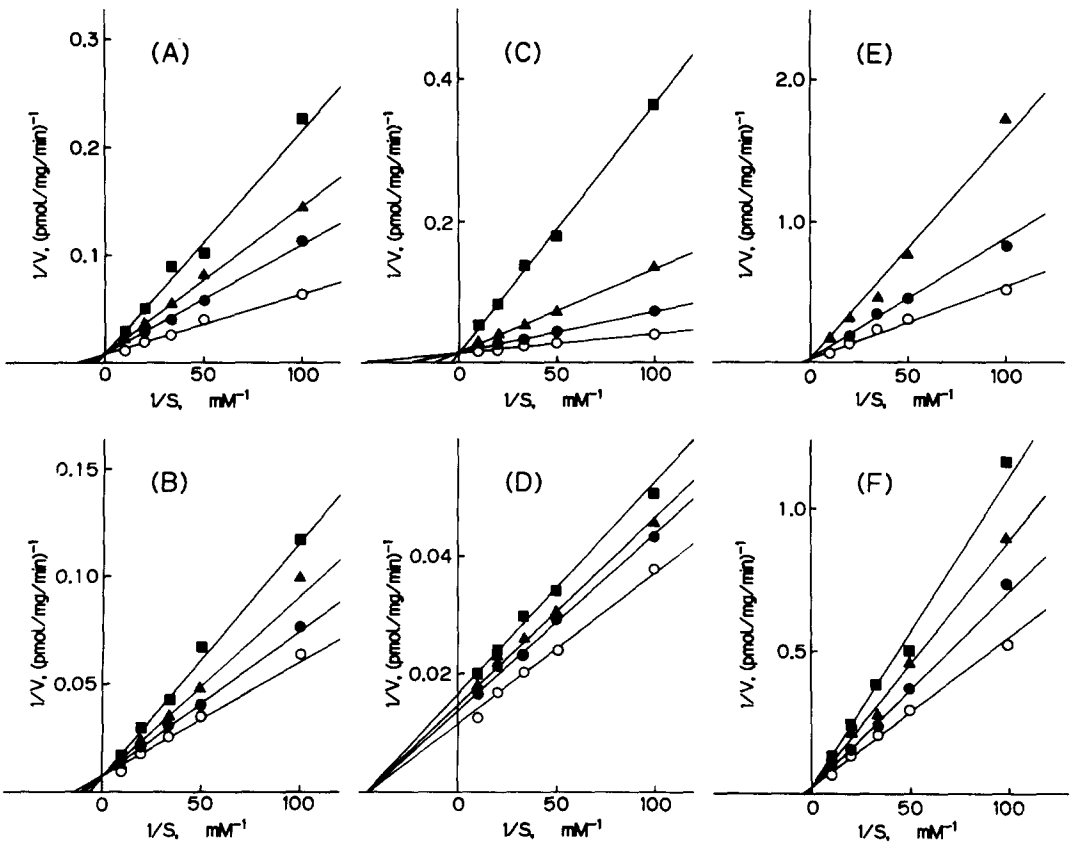

Fig. 2. Inhibition of serine, ethanolamine and choline on base-exchange reactions. Reactions were performed as described in Fig. 1 in the presence of 0.1 mM $CaCl_2$ and 75 μM chlorpromazine with various concentrations of [^{3}H]serine (A, B), [^{3}H]ethanolamine (C, D) or [^{3}H]choline (E, F) and with unlabeled serine, ethanolamine and choline. A and F: ethanolamine, 0 mM (○), 0.1 mM (●), 0.3 mM (▲), 1.0 mM (■). B: choline, 0 mM (○), 0.1 mM (●), 0.3 mM (▲), and 1.0 mM (■). D: choline, 0 mM (○), 1.0 mM (●), 3.0 mM (▲), and 10 mM (■). C and E; serine, 0 mM (○), 0.1 mM (●), 0.3 mM (▲), and 1.0 mM (■).

Table 2. Effects of mixed serine, ethanolamine and choline on base-exchange reactions

	Labeled base incorporation into lipid (pmoles/10 min/mg protein)			
	0.1 mM Ca^{2+}		3 mM Ca^{2+}	
	None	CPZ	None	CPZ
Mix A	114	1160	590	1300
Mix B	110	980	490	1200
Mix C	104	900	580	810
Mix D	29	280	190	260
Mix E	27	330	280	440
Mix F	6	39	18	51

The reaction mixture (0.5 ml) contained 50 mM HEPES buffer (pH 7.5), 0.4 mg membrane protein, 0.1 or 3 mM $CaCl_2$ with or without chlorpromazine (CPZ, 75 μM) and various labeled substrates (Mix A, B, C, D, E and F). The data are representative of results obtained in two determinations. Mix A: [^{3}H]serine (1.1 mM); Mix B: [^{3}H]serine (1.1 mM), unlabeled ethanolamine (0.4 mM) and choline (0.33 mM); Mix C: [^{3}H]ethanolamine (0.4 mM); Mix D: [^{3}H]ethanolamine (0.4 mM), unlabeled serine (1.1 mM) and choline (0.33 mM); Mix E: [^{3}H]choline (0.33 mM); and Mix F: [^{3}H]choline (0.33 mM), unlabeled serine (1.1 mM) and ethanolamine (0.4 mM).

W-7, and the weaker calmodulin antagonist, W-5. The concentration required to produce half-maximal inhibition was about 30 μM for all four drugs. On the other hand, in the presence of 0.1 mM Ca^{2+}, chlorpromazine, trifluoperazine and W-7 activated the three base-exchange activities dose dependently (serine and choline base-exchange reactions are not shown). Although W-5 also inhibited Ca^{2+} binding (and/or uptake) to the membranes, this drug had only a slight activation of the base-exchange reactions.

DISCUSSION

We previously demonstrated in rabbit platelet membranes that some calmodulin antagonists stimulate the incorporation of serine, ethanolamine and choline into phospholipids, in the presence but not in the absence of Ca^{2+} [11]. The present study further confirmed that, among the various metals tested, only Ca^{2+} was active in the base-exchange reactions, even in the presence of the drugs. With low concentrations of Ca^{2+}, the calmodulin antagonists stimulated the reactions without changing apparent K_m values for the bases (Fig. 1 and Table 1), and shift the optimal pH from a higher to a lower range [11], in a manner similar to that induced by Ca^{2+}. These results suggest that the high concentration of Ca^{2+} increases the enzyme activities in the same manner as the drugs. Amphiphilic compounds such as chlorpromazine interact with phospholipids [21] and inhibit the binding of Ca^{2+} to phospholipid vesicle [22]. Therefore, we examined the effects of the calmodulin antagonists on Ca^{2+} binding to the platelet membranes and attempted to determine whether or not the effects of the drugs on the Ca^{2+} binding correlated with the stimulation of the reactions induced by the drugs. Chlorpromazine, trifluoperazine and W-7 inhibited $^{45}Ca^{2+}$ binding (and/or uptake) and activated the base-exchange reactions, dose dependently (Fig. 4). The inhibitory and stimulatory effects of the drugs seem to be closely correlated. However, the weaker calmodulin antagonist, W-5, which stimulated the base-exchange reac-

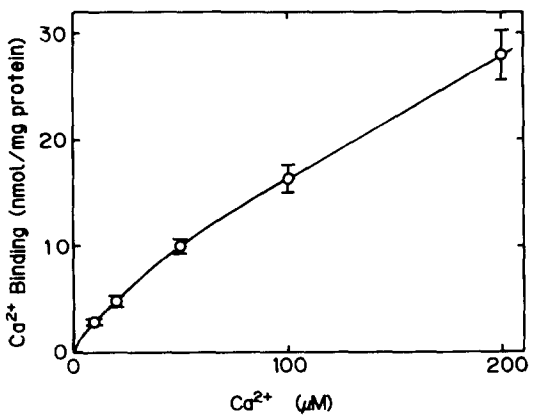

Fig. 3. Ca^{2+} binding to platelet membranes. Platelet membrane protein (0.5 mg) was dialyzed as described in the section on "Equilibrium dialysis" in the presence of various concentrations of $^{45}Ca^{2+}$ (5 μCi). Each experimental point is the mean of triplicate determinations with standard errors.

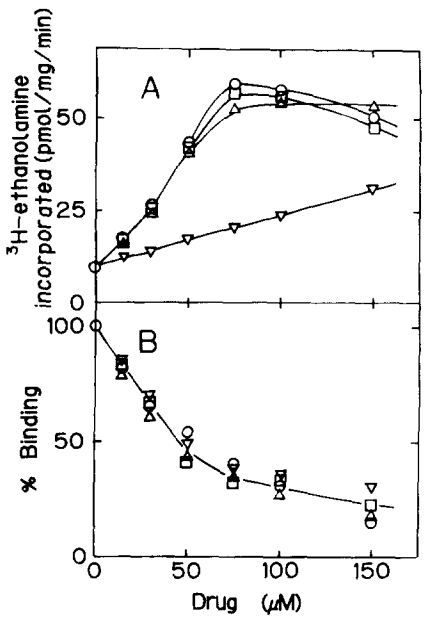

Fig. 4. Effects of various calmodulin antagonists on the base-exchange activity and Ca^{2+} binding to platelet membranes. (A) Platelet membrane protein (0.35 mg) was incubated with 2 μCi of [^{3}H]ethanolamine (0.1 mM) in the presence of 0.1 mM Ca^{2+} with the indicated concentrations of calmodulin antagonists. (B) Platelet membrane protein (0.5 mg) was dialyzed as described in Fig. 3, in the presence of 5 μCi of $^{45}Ca^{2+}$ (0.1 mM) with indicated concentrations of the drugs. Each experimental point is the mean of triplicate determinations with standard errors of less than 10%. Key: chlorpromazine (○), trifluoperazine (□), W-7 (△), and W-5 (▽).

tions only slightly, also inhibited $^{45}Ca^{2+}$ binding (and/or uptake) in the same manner as the other calmodulin antagonists. These results suggest that the stimulation of base-exchange activities by the antagonists is not simply due to large amounts of free Ca^{2+} being available for the base-exchange enzymes. The drug-induced inhibition of $^{45}Ca^{2+}$ binding and the stimulation of base-exchange reactions seem to be different phenomena. Although exogenously added calmodulin inhibits only choline incorporation into phospholipid [11], the Ca^{2+}–calmodulin complex may inhibit the Ca^{2+}-induced base-exchange reactions, since platelet membranes contain considerable amounts of endogenous calmodulin [23]. Further experiments using solubilized and purified base-exchange enzymes [24, 25], free from calmodulin and endogenous phospholipids, are required to elucidate whether the drugs interact directly with calmodulin, phospholipids or the base-exchange enzymes.

The apparent K_m values for serine, ethanolamine and choline, did not vary significantly with the Ca^{2+} concentrations, being about 65, 25 and 400 μM, respectively, with or without a calmodulin antagonist. These values were similar to those obtained using rat liver [2], rat brain [5] or canine heart [26]. The differences might be due to the various assay conditions used, since the apparent K_m value has been reported to vary with the Ca^{2+} concentration, buffer system and pH used [2, 5].

The incorporation of serine into phospholipids was inhibited competitively by ethanolamine and choline, and choline incorporation was inhibited competitively by serine and ethanolamine. These findings are in a good agreement with the results of the base-exchange reactions in rat liver [2] and rat brain [4]. On the other hand, ethanolamine incorporation was inhibited competitively by serine and noncompetitively by choline in platelet membranes. This relationship between each base in ethanolamine phospholipid base-exchange reaction in platelet membranes was quite different from that in rat liver [2]. In rat liver, ethanolamine incorporation is inhibited uncompetitively by serine and noncompetitively by choline. The reason for these discrepancies is not apparent, but they may be due to the different tissue, species or assay conditions (pH, buffer systems or Ca^{2+} concentrations) used.

Although it is generally difficult to determine the exact concentration of the various compounds in platelets, the intracellular concentrations of free serine, ethanolamine and choline were calculated to be 1.1, 0.4 and 0.33 mM respectively. Under the assay conditions containing a mixture of 1.1 mM serine, 0.4 mM ethanolamine and 0.33 mM choline, the rate of serine incorporation into phospholipid was the highest among the three base-exchange reactions, at either 0.1 or 3 mM of Ca^{2+}, with or without the calmodulin antagonists. Whether or not the three free bases and three base-exchange enzymes are distributed evenly in platelets remains unknown. Our results suggest that serine incorporation is the most active among the three base-exchange reactions under the physiological concentrations of the free bases in rabbit platelets.

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