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INHIBITION OF CALMODULIN STIMULATION OF PHOSPHODIESTERASE AND Ca^{2+},Mg^{2+}-ATPase ACTIVITIES AND SHAPE CHANGE OF ERYTHROCYTE GHOSTS BY CHLOROQUINE

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Abstract—The effects of chloroquine on calmodulin (CaM)-related enzyme activities and the shape of human erythrocytes have been studied. It was found that the CaM activation of rat brain phosphodiesterase was abolished by the addition of chloroquine. CaM was included in the assay of phosphodiesterase activity at the concentration that gave half-maximal activation. The concentration of chloroquine that caused 50% inhibition of CaM stimulation of phosphodiesterase was 7×10^{-5} M. The type of inhibition was competitive with respect to CaM. The CaM-stimulated Ca^{2+}, Mg^{2+}-ATPase in erythrocyte membrane was also inhibited by chloroquine, the 50% inhibitory concentration of which was about 2×10^{-4} M. Its mode of action was also competitive with respect to CaM. The shapes of erythrocyte ghosts prepared by hypotonic hemolysis were examined in a solution consisting of 2 mM $MgCl_2$, 154 mM NaCl and 10 mM Tris–HCl (pH 7.4); they were discocytic in the presence of 2 mM ATP and in its absence. They were converted to the invaginated form by the addition of chloroquine in the concentration range of 1×10^{-4}–5×10^{-4} M. This concentration is similar to that which caused the inhibition of CaM activation of Ca^{2+},Mg^{2+}-ATPase.

Chloroquine has been widely used for the treatment of malaria, rheumatoid arthritis, and collagen vascular diseases [1]. However, its clinical use is restricted by the development of retinopathy [2] and myopathy [3] on prolonged drug administration. The mechanisms of the occurrence of these symptoms have not yet been established.

Chloroquine is one of the lysosomotropic drugs [4–8] and is known to cause large vacuoles and membranous bodies with a multilamellar appearance in macrophages [9] and rat liver [10]. Chloroquine inhibits various aspects of intralysosomal catabolism due to the rise in lysosomal pH [11–13]. It inhibits phospholipases A and C, which are responsible for catabolism of lysosomal phospholipid, and gives rise to fatty liver [4, 5, 8]. Some other inhibitory effects of the drug on protein synthesis [7] and ornithine decarboxylase [14] have also been reported. Studies with pure DNA from various sources demonstrated intercalation of chloroquine into DNA [15–17].

Calmodulin, a Ca^{2+}-regulatory protein, is known to activate brain phosphodiesterase [18, 19], brain adenylate cyclase [20, 21], and myosin light chain kinase [22, 23]. Furthermore, it has been reported to interact with several erythrocyte proteins including Ca^{2+},Mg^{2+}-ATPase [24, 25], spectrin [26] and other cytoplasmic surface proteins [27]. Some CaM inhibitors are known to work as cup-formers of erythrocytes [28]. However, the action of chloroquine against CaM and the effect on erythrocyte shape have not been examined in detail.

We have studied the effects of chloroquine on CaM activation of rat brain phosphodiesterase and Ca^{2+},Mg^{2+}-ATPase and on the shape of human erythrocyte ghosts.

MATERIALS AND METHODS

Materials. Chloroquine acetate was used for this experiment. Calmodulin (CaM), phosphodiesterase devoid of CaM from bovine brain, and 5′-nucleotidase of *Crotalus atrox* venom were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Preparation of erythrocyte ghosts for microscopic observation. Human erythrocytes obtained from citrate, phosphate, dextrose (CPD) treated blood (5–15 days old) were washed three times with 5 vol. of physiological saline and hemolyzed by 1:50 dilution within 10 mM Tris–HCl buffer (pH 7.4). The membranes were pelleted at 18,000 rpm for 20 min in a Sorvall SS-34 rotor, washed with 10 mM Tris–HCl buffer at 4°, and immediately used for assay of the shape change. These ghosts were suspended in 10 vol. of physiological saline (pH 7.4) containing 2 mM $MgCl_2$ or 2 mM Mg^{2+}-ATP [29].

Assay of cAMP phosphodiesterase. The assay mixture contained 40 mM Tris–HCl (pH 7.5), 2 mM

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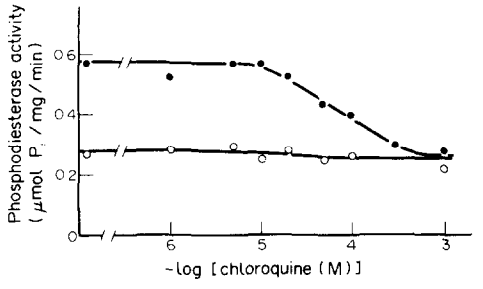

Fig. 1. Inhibition of brain phosphodiesterase activity by chloroquine. Phosphodiesterase activity was measured in the absence (○) and presence (●) of 0.1 μg of CaM with various concentrations of chloroquine. Each point is the mean of duplicate determinations. The other assay procedures are described in Materials and Methods.

cAMP, 5 mM $MgCl_2$, 4 mM imidazole, 1 mM 2-mercaptoethanol, 10 μM $CaCl_2$ and 1 μg of bovine brain phosphodiesterase with or without 0.1 μg of CaM in a volume of 0.5 ml. The amount of CaM giving half-maximal activity of phosphodiesterase was 0.1 μg. The mixture was incubated at 30° for 10 min. The reaction was terminated by boiling for 2 min. The mixture was brought to 30° and incubated with 20 μl of 5′-nucleotidase (25 units) for 10 min. The reaction was terminated by the addition of 100 μl of 40% trichloroacetic acid (TCA), and the precipitates were removed by centrifugation at 1000 *g* for 15 min. Inorganic phosphate in the supernatant fraction was determined by the method of Fiske and Subbarow [30]. To determine the effect of chloroquine on the enzyme activity, chloroquine was mixed with Ca^{2+} and CaM prior to the addition of the other constituents of the assay medium. After the enzyme reaction was stopped, chloroquine was removed by mixing with a suspension of Dowex 50W-X4 resin (H-form) followed by centrifugation.

Assay of Ca^{2+},Mg^{2+}*-ATPase.* Erythrocyte ghosts were prepared by the method described above for the observation of shape except that 1 mM EDTA was included in the hemolyzing medium. Ca^{2+},Mg^{2+}-ATPase was assayed in 0.5 ml of a solution containing 0.1 to 0.2 mg ghosts, 3 mM ATP, 5 mM $MgCl_2$, 140 mM NaCl, 14 mM KCl, 0.1 mM ouabain, 0.5 mM ethyleneglycolbis(amino-ethylether)tetra acetate (EGTA), 0.8 mM $CaCl_2$ and 30 mM imidazole–HCl (pH 7.4). Incubation was carried out at 37° for 45 min. For the determination of CaM-stimulated activity, various concentrations of CaM were added to the assay mixture. The reaction was stopped by the addition of 0.1 ml of 40% TCA, and the mixture was centrifuged at 1300 *g* for 10 min. The liberated P_i in the supernatant fraction was measured by the method of Fiske and Subbarow [30]. The effect of chloroquine was determined by the method described for the assay of phosphodiesterase.

Observation of shape of erythrocyte ghosts. Ghosts were examined with a dark-field light microscope (Zeiss Photo-microscope III) (×400) at room temperature

RESULTS

Inhibition of Ca^{2+},*CaM-stimulated bovine brain phosphodiesterase by chloroquine.* Ca^{2+},CaM-stimulated phosphodiesterase activity was inhibited by chloroquine (Fig. 1), though the basal activity, which is CaM-independent, was not affected. The concentration of chloroquine giving 50% inhibition of CaM-activated phosphodiesterase was 7×10^{-5} M. Phosphodiesterase activity was assayed in a two-enzyme coupling system, i.e. cAMP was hydrolyzed to 5′-AMP by phosphodiesterase first, and then the 5′-AMP was hydrolyzed further by 5′-nucleotidase to liberate inorganic phosphate. To examine the possibility that the 5′-nucleotidase was inhibited by chloroquine, we determined the hydrolysis of 5′-AMP in the absence or presence of 1×10^{-3} M chloroquine in the assay mixture. Chloroquine had no effect on the amount of 5′-AMP hydrolyzed (data not shown). The results provided evidence that the inhibitory action of chloroquine was on phosphodiesterase itself. To further establish the direct interaction between phosphodiesterase and chloroquine, we studied the effect of CaM concentration on the chloroquine inhibition of Ca^{2+},CaM-stimulated phosphodiesterase activity. The inhibition of the enzyme activity by chloroquine gradually recovered as the CaM concentration was increased (Fig. 2a). The Lineweaver–Burk plot showed clearly that the

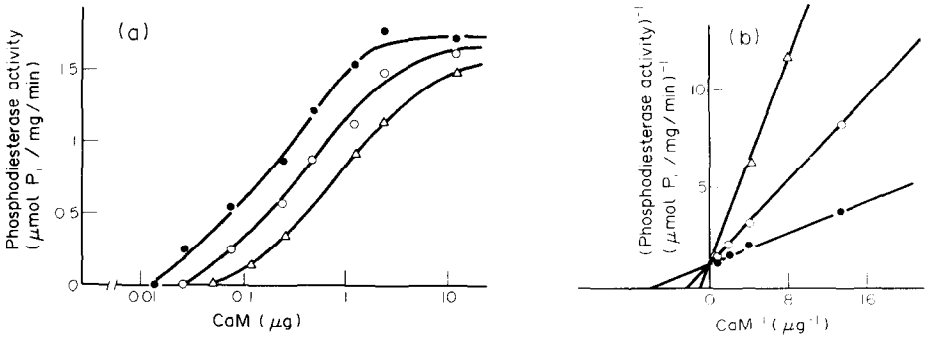

Fig. 2. (a) Effects of various concentrations of CaM on brain phosphodiesterase activity. The enzyme assay procedure is described in Materials and Methods. The activity was measured in the presence of 0 M (●), 5×10^{-5} M (○) and 1×10^{-4} M (△) chloroquine. (b) Kinetic analysis of the data shown in (a).

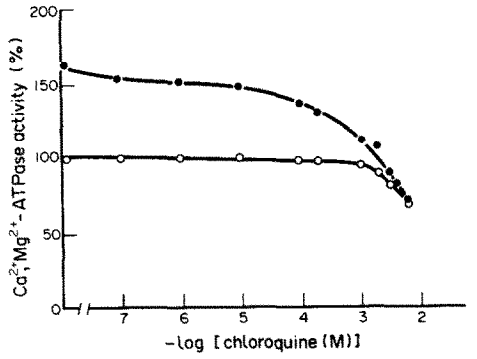

Fig. 3. Inhibition of Ca^{2+},Mg^{2+}-ATPase in erythrocyte membrane by chloroquine. Ca^{2+},Mg^{2+}-ATPase activity was measured in the absence (○) and presence (●) of CaM by adding various concentrations of chloroquine. Each point designates the mean of triplicate determinations. The other assay procedures were described in Materials and Methods.

inhibition was competitive (Fig. 2b). The V_{max} values for the Ca^{2+},CaM-stimulated phosphodiesterase activity in the absence and presence of 5×10^{-5}–1×10^{-4} M chloroquine were almost the same. The apparent K_m for CaM shifted to higher values with an increase in the chloroquine concentration. These results demonstrated that chloroquine was a competitive inhibitor with respect to CaM in phosphodiesterase activation.

Inhibition of Ca^{2+},Mg^{2+}*-ATPase in erythrocyte membrane by chloroquine.* The inhibition of CaM-stimulated Ca^{2+},Mg^{2+}-ATPase in erythrocyte membrane by chloroquine is shown in Fig. 3. The CaM-independent activity was not affected by chloroquine. On the other hand, CaM stimulation of Ca^{2+},Mg^{2+}-ATPase activity was inhibited by chloroquine, as shown in Fig. 4a. The inhibition was reversed by increasing the concentration of CaM when the level of chloroquine was below 3×10^{-4} M. However, CaM could not fully overcome the inhibition by chloroquine at concentrations higher than 1×10^{-3} M. As shown in the Lineweaver–Burk plot (Fig. 4b), chloroquine was a competitive inhibitor in relation to the activation by CaM of Ca^{2+},Mg^{2+}-ATPase in erythrocyte membrane at concentrations of less than 3×10^{-4} M.

Shape change of erythrocyte ghosts induced by chloroquine. When ghosts were prepared from CPD blood stored for less than 2 weeks by 1:50 dilution with 10 mM Tris–HCl buffer (pH 7.4), they were mostly spherocytes. When these spherocytic ghosts were incubated at 37° for 20 min in physiological saline containing 2 mM $MgCl_2$ or 2 mM Mg^{2+}-ATP, their shapes were changed to crenate [Fig. 5, (1)] or disc form [Fig. 5, (4)] respectively. When crenate ghosts in $MgCl_2$ were exposed to 0.1 and 0.5 mM chloroquine, they were invaginated to form a crenate-cup shape [Fig. 5, (2) and (3)]. Disc-type ghosts in 2 mM Mg^{2+}-ATP were transformed to typical cup-type ghosts by exposure to 0.1 and 0.5 mM chloroquine [Fig. 5, (5) and (6)].

DISCUSSION

The results show that chloroquine competitively inhibited CaM activation of brain phosphodiesterase and Ca^{2+},Mg^{2+}-ATPase in erythrocyte membrane. It is assumed that chloroquine directly binds to CaM or to a CaM-binding site on CaM-stimulated enzymes. A number of investigators have shown that the binding of calcium to CaM induces large conformational changes in the protein, which lead to the exposure of hydrophobic regions. Some CaM antagonists bind to the hydrophobic site of Ca^{2+}–CaM and thereby prevent the interaction of Ca^{2+}–CaM complex with target enzymes [31–35]. The hydrophobicity of drugs was considered to be important for interaction with CaM. Chloroquine is an amphipathic compound and is expected to be hydrophobic. Chloroquine was proved to be a CaM antagonist.

A morphological study clarified that chloroquine works as a cup-former in erythrocytes. The erythrocyte shape is related to the levels of intracellular ATP and calcium. Erythrocytes show the form of echinocytes or spherocytes when ATP is depleted, but they recover to discocytes upon restoration of the ATP level [36, 37]. They also become echinocytes when the internal calcium level is high [38]. Eryth-

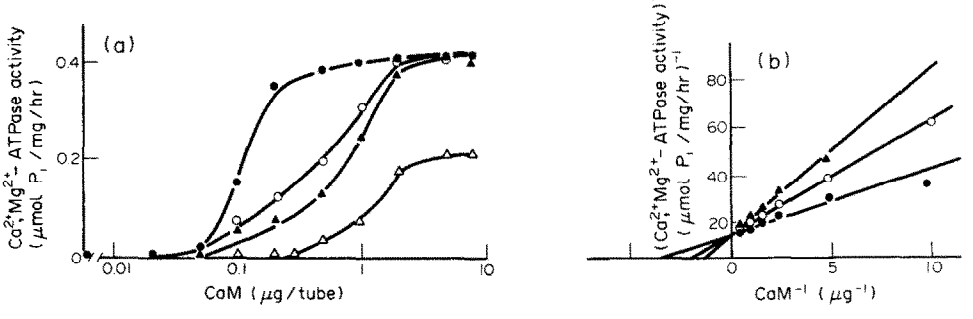

Fig. 4. (a) Effects of various concentrations of CaM on erythrocyte membrane Ca^{2+},Mg^{2+}-ATPase activity. The activity was measured in the presence of 0 M (●), 1×10^{-4} M (○), 3×10^{-4} M (▲) and 1×10^{-3} M (△) chloroquine. (b) Kinetic analysis of the data shown in (a) except for the curve with 1×10^{-3} M chloroquine.

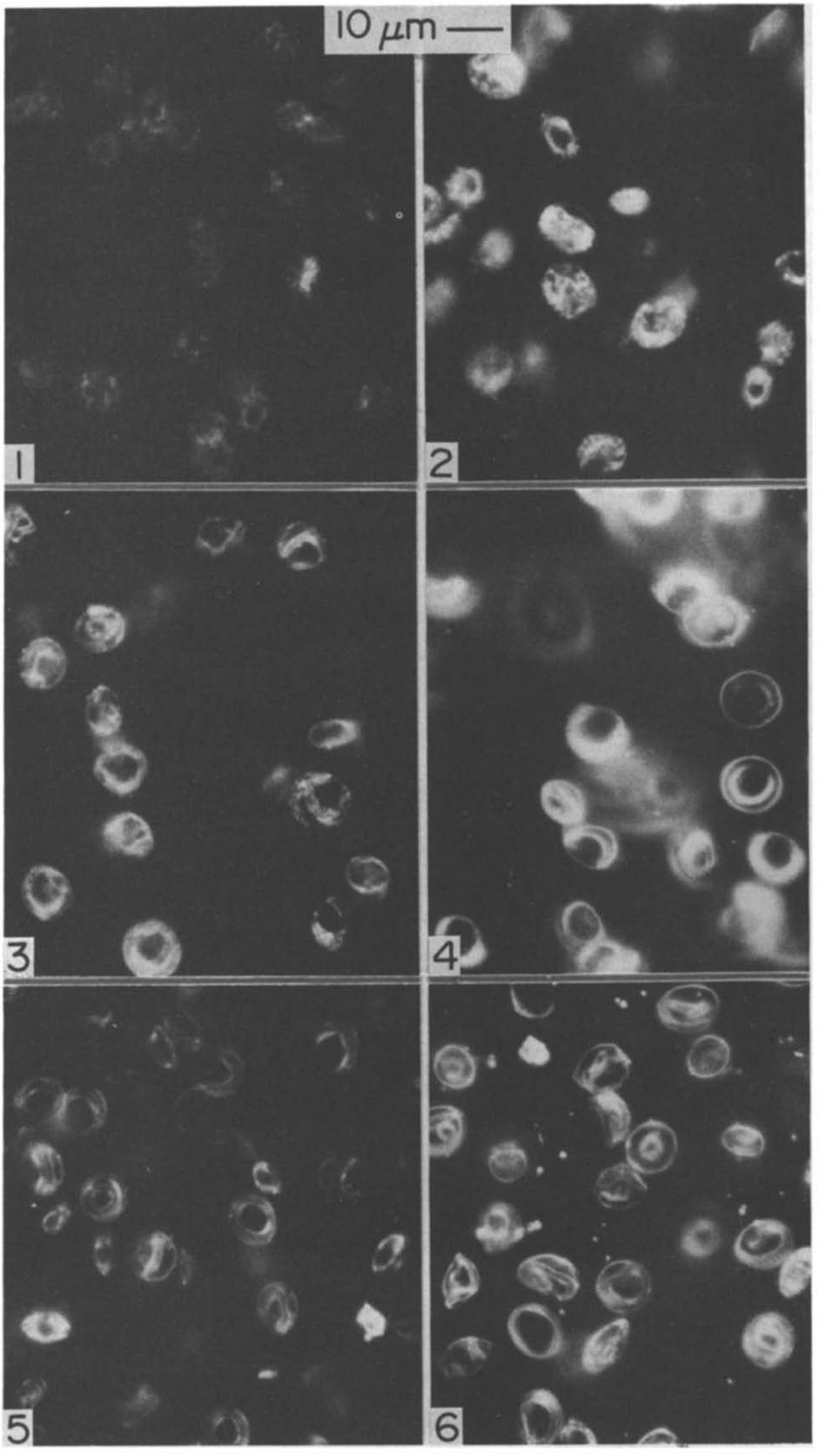

Fig. 5. Shape change of erythrocyte ghosts induced by chloroquine. Erythrocyte ghosts were suspended in 154 mM NaCl containing 2 mM $MgCl_2$ (1, 2 and 3) or 2 mM ATP and 2 mM $MgCl_2$ (4, 5 and 6). After incubation of the erythrocyte ghosts suspension at 37° for 20 min, chloroquine was added. Key: (1) and (4), control; (2) and (5), 0.1 mM chloroquine; (3) and (6), 0.5 mM chloroquine. The shape of ghosts was observed under a dark field light microscope. The bars show 10 μm.

rocyte cytoskeletal proteins such as spectrin, ankyrin, band 4.1, and actin play a major role in controlling the cell shape [29, 39]. Furthermore, CaM is known to interact with the Ca^{2+}-pump (Ca^{2+},Mg^{2+}-ATPase), spectrin and spectrin kinase in erythrocytes. CaM is required for the maintenance of normal erythrocyte morphology [28]. One explanation is that the binding of chloroquine to Ca^{2+}-CaM distorted the interaction of Ca^{2+}-CaM and CaM binding proteins in the erythrocyte membrane, resulting in shape change of the erythrocyte ghosts.

On the other hand, the bilayer coupling hypothesis [40, 41] cannot be excluded to explain the action of crenators and cup-formers of erythrocytes, such as chloroquine. Their activities are interpreted in terms of preferential partitioning of the compounds with cationic groups or anionic groups in the inner or outer lipid leaflets of the plasma membrane. This would expand one leaflet area relative to the other and result in shape change. Cup-forming of erythrocyte ghosts may depend on interaction with proteins in the cytoskeleton of the erythrocyte membrane or with the lipid bilayer or, possibly, both.

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