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Potential Role of Ca^{++} on the Differentiation of Erythroid Progenitor Cells

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In order to gain insight into the mechanisms by which erythropoietin promotes erythropoiesis, effects of various inhibitors on the erythropoietin-promoted differentiation of erythroid progenitor cells and on the erythropoietin-promoted Ca^{++} uptake in the progenitor cells were determined, and the relationship between the inhibitory activity of each inhibitor toward the differentiation and Ca^{++} uptake were examined. The inhibitors used were a tyrosine kinase inhibitor (genistein), a Ca^{++}-channel blocker (verapamil), a Ca^{++} chelator (EDTA) and a protein kinase C inhibitor (staurosporine). All of these agents inhibited both the erythropoietin-mediated differentiation of the erythroid progenitor cells, as determined by the incorporation of ^{59}Fe into heme, and Ca^{++} uptake in a concentration dependent manner. In the cases of verapamil and EDTA, the half-miximal inhibitory concentration (IC_{50}) values for differentiation of the progenitor cells may be the consequence of the inhibition of the Ca^{++} uptake by the inhibitior. On the other hand, in the cases of genistein and staurosporine, the IC_{50} values for inhibition of differentiation were significantly different from that for inhibition of Ca^{++} uptake. These results suggest that the mechanism of inhibition of differentiation by these two inhibitors is complex. However, taken all together, the above results support the proposition that Ca^{++} uptake may paly a role in the erythropoietin-mediated differentiation of erythoid progenitor cells.

Key words : Erythropoietin, Ca^{++} uptake, Erythroid progeitor cells, Genistein, Verapamil, H7, Staurosporine, Erythropoiesis

INTRODUCTION

Despite wide use of erythropoietin in the treatment of patients with anemia, little is known about the mechanisms of erythropoietin-mediated erythropoiesis. Progress in the understanding of the mechanism of erythropoiesis has been hampered by the difficulty in obtaining an adequate amount of a homogeneous population of erythroid progenitor cells. It has been established a procedure for the preparation of a large amount of homogenous popluation of erythroid cells from the spleens of mice infected with an anemia-inducing stain of Friend virus (Sawyer et al., 1987). These erythroid progenitor cells are erythropoietin-responsive and thus are a useful model system for studing erythropoietin-mediated differentiation of erythroid cells (Koury et al., 1984; Koury et al., 1988).

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It has been shown that Ca^{++} plays a role in erythropoiesis (Misiti et al., 1979) and that erythropoietin stimulates Ca^{++} uptake in the erythroid progenitor cells (Sawyer et al., 1984; Miller et al., 1988). The erythropoietin-stimulated Ca^{++} uptake by the erythroid progenitor cells may play an important role in the differentiation of these cells (Sawyer et al., 1984). However, the direct relationship between the erythropoietin-stimulated Ca^{++} uptake and the differentiation of erythroid progenitor cells is not established. Previously, it has been shown that erythropoietin plays a role in the maintenance of cellular energy levels during differentiation of erythroid progenitor cells (Kim et al., 1989; Kim et al., 1991). Recently, partially Purified erythropoietin receptors were used along with the antiphosphotyrosine antibody to demonstrate that the erythropoietin-induced phosphorylation of the erythropoietin receptor occurs. As an initial step for the studies on the function of tyrosine kinase action of erythropoietin receptors, we investigated the effects

of a tyrosine kinase inhibitor, genistein, on the erythropoietin-mediated differentiation of erythroid progenitor cells and Ca^{++} uptake in the progenitor cells.

In gaining insight into biological roles for protein kinase C, the use of activators (e.g.,phorbol esters) and inhibitors (e.g., staurosporine and H7) of the enzyme have been beneficial. Phorbol esters have been implicated to plays a role in erythropoiesis (Miao et al., 1978). Recently, we found that staurosporine inhibits erythropoietin-mediated differentiation of erythoid progenitor cells (submitted for publication). In the study, the effect of staurosporine on Ca^{++} uptake was assesed to determine the relationship between the inhibition erythropoietin-mediated differentiation and inhibition of Ca^{++} uptake by staurosporine.

In order to find out whether Ca^{++} uptake plays a role in the erythropoietin-mediated differentiation of erythoid progenitor cells, in addition to genistein and stausporine, the effects of verapamil and EDTA on the erythropoietin-mediated differentiation of the progenitor cells and the Ca^{++} uptake were also examined.

In this communication we report the suppression of erythropoietin-mediated differentiation of erythoid progenitor cells and inhibition of Ca^{++} uptake in the progenitor cells by genistein, verapamil, EDTA and staurosporine.

MATERIALS AND METHODS

Materials

Erythropoietin was a generous gift from the Genetics Institute (Cambridge, MA). Drabkin's reagant, Iscove's modified Dulbecco's medium, bovine serum albumin, cyclohexanone, penicillin G, streptomycin sulfate, α-monothioglycerol, calcium ionophore A23187, verapamil and staurosporine were purchased from Sigma Chemical Company (St. Louis, MO). Genistein was obtaind from ICN Biochemicals, Inc. (Irvine, CA), human transferrin from CalBiochem (San Diego, CA) and fetal bovine serum was from Gibco Laboratories (Grand Island, NY). $^{59}FeCl_3$ (16 mCi/mg) and $^{45}CaCl_2$ (23.7 mCi/mg) were purchased from duPont-New England Nuclear (Boston, MA).

Effects of various agents on the erythropoietin-mediated in vitro differentiation of erythroid progenitor cells

Erythroid progenitor cells were prepared from spleens of anemia-inducing stain of Friend virus infected mice (Sawyer et al., 1987). The effects of various agents on the erythropoietin-mediated in vitro differentiation of erythroid progenitor cells were determined (Koury et al., 1984; Koury et al., 1988). In this procedure, the extent of the differentiation was determined by the amount of ^{59}Fe incorporation into heme. The viability of the gravity-seperated erythroid progenitor cells was checked with 0.4% Trypan blue. The cells ($2x10^6$ cells) were suspended in 1 ml of Iscove's modified Dulbecco's medium containing 0.2 U/ml of erythropoietin, 30% fetal bovine serum, 1% bovine serum albumin, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.1 mM -thioglycerol, and then cultured in 16 mm diameter plastic wells at 37°C in humidified air plus 4% CO_2. At the begining of cell culture 0.2 U/ml of erythropoietin was added, and then 12 hours before the determination of ^{59}Fe incorperation into heme, 50 μl of human trasferrin (2.4 mg/ml Iscove's modified Dulbecco's medium), 10 mCi of $^{59}FeCl_3$, and varying amounts of an inhibitor in 10 μl of dimethylsulfoxide were added to 1 ml aliquots of the cell culture. After 12 hr culture, the cells were treated with 0.4% Trypan blue and viable cells were counted. The cells were collected, washed, then lysed with Drabkin's solution. To the lysates, HCl was added to give the final concentration of 0.1 N, and then the ^{59}Fe incorporated into heme was extracted with 2 ml of cyclohexanone. The amount of cyclohexanone-extracted ^{59}Fe was determined in a gamma counter.

Effects of various inhibitors on the erythropoietin-promoted $^{45}Ca^{++}$ uptake in the erythroid progenitor cells

The incubation medium used was Iscove's modified Dulbecco's medium containig 1% bovine serum albumin and 0.2 U/ml of erythropoietin. The erythroid progenitor cells ($1x10^7$ cells) were suspended in 200 μl of the incubation medium (control samples) and preincubated for 30 min at 37°C. The inhibitory samples were suspended in 100 μl of the incubation medium and preincubated for 30 mm at 37°C. After preincubation, the samples were cooled on ice and 100 μl of chilled incubation medium containing $^{45}Ca^{++}$ (2.5 μCi) was added. The samples were incubated for 10 min at 37°C, then cooled on ice and diluted with 1 ml of cold incubation medium. The samples were vortexed and centrifuged at 800×g for 5 min at 4°C. The supernatants were carefully removed by aspiration and the cell pellets were lysed by incubation at 37°C overnight with 0.6 ml of 0.2M NaOH. The lysed cell cultures were neutralized with 60 μl of 2M HCl and radioactivity was determined in a scintillation counter.

RESULTS

Effects of genistein, verapamil and EDTA on the

erythropoietin-mediated differentiation of erythroid progenitor cells

In order to determined whether inhibition of tyrosine kinase suppresses the erythropoietin-mediated differentiation of erythroid progenitor cells, the effect of genistein on ^{59}Fe incorperation into heme during the culture of the progenitor cells was assayed. During the culture with erythropoietin, the cell death was less than 5%. However, in the absence of erythropoietin, virtually no ^{59}Fe incorporation into heme was observed and 95% of the cells were dead. A typical result of four seperate experiments for the inhibition of ^{59}Fe incorporation by genistein is shown in Fig. 1A. The ^{59}Fe incorporation into heme reached a peak at 24 hours of culture, then the ^{59}Fe incorporation declined sharply after 36 to 48 hours. The data for the 24 hour culture period are replotted in terms of % ^{59}Fe incorporation versus genistein concentration (Fig. 1B). Fig. 1B shows that the inhibition of ^{59}Fe incorporation is dependent on the concentration of genistein and the half-maximal inhibition concentration of genistein is approximately 122 μM.

The effect of the Ca^{++}-channel blocker, verapamil, on the inhibition of ^{59}Fe incorporation into heme during culture of erythroid progenitor cells in the presence of erythropoietin was assesed by the same procedure as above. A typical result of three seperate experiments is shown in Fig. 2A. The pattern of inhibition of ^{59}Fe incorporation into heme by verapamil was similar to that for genistein. In Fig. 2B, the data for the 24 hour culture period are replotted in terms of % ^{59}Fe incorporation versus verapamil concentration. Fig. 2B shows that the inhibition of ^{59}Fe incorporation into heme is dependent on the concentration of verapamil and the half-maximal inhibition concentration of verapamil is approximately 7.3 μM The effects of various concentrations of EDTA on the erythropoietinmediated ^{59}Fe incorp_ oration into heme were assayed and a typical result of three separate experiments is shown in Fig.3A. In Fig.3B, the data for the 24 hour culture period are replotted in terms of ^{59}Fe incorporation versus EDTA concentration. Fig.3B shows that the inhibition of ^{59}Fe incorporation into heme is dependent on the concentration of EDTA and the IC_{50} of EDTA is approximately 375 μM.

Effect of genistein, verapamil, EDTA and staurosporine on the Ca^{++} uptake in the erythroid progenitor cells

The results of the effects of genistein, verapamil,

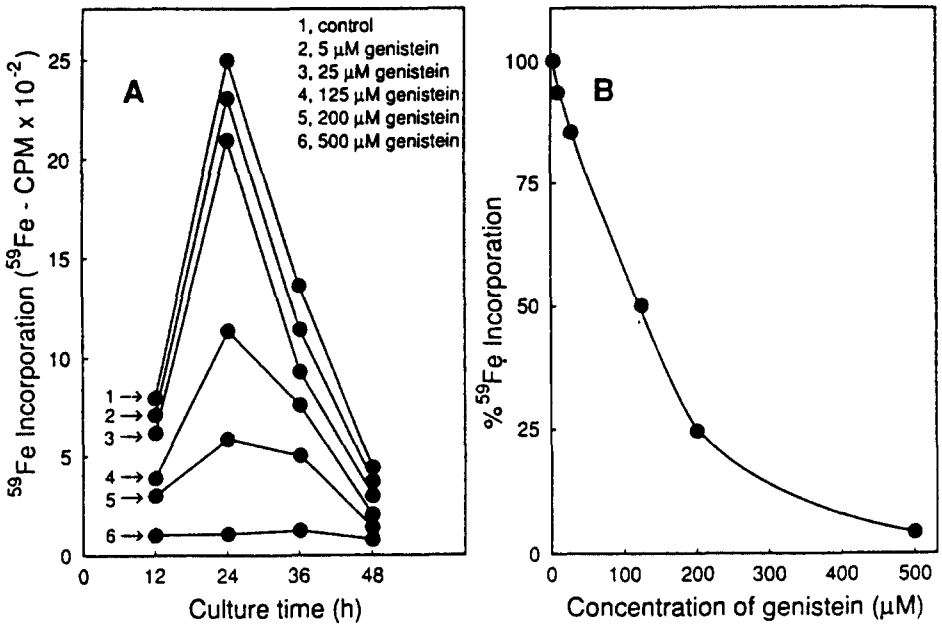

Fig. 1. Effect of genistein on the incorporation of ^{59}Fe into heme during culture of erythroid progenitor cells in the presence of erythropoietin. (A) Erythropoietin (0.2U/ml) was added to gravity purified erythroid progenitor cells ($2x10^6$ cells/ml) and the cells were cultured. twelve hours before the determination of ^{59}Fe incorporation into heme, 10 μCi of $59FeCl_3$ and varying amounts of genistein were added each cell well. At 12 hour intervals, the viability of the cells was examined by Trypan blue dye exclusion method, and the amounts of ^{59}Fe incorporation into heme were determined as described in " Methods". (B) The ^{59}Fe incorporation data for the 24 hour period in (A) are replotted in terms of % ^{59}Fe incorporation versus genistein concentration. A representative result of four separate experiments is shown. Each point represents the mean of triplicate cultures. Standard deviations were all less than 5% of the mean values.

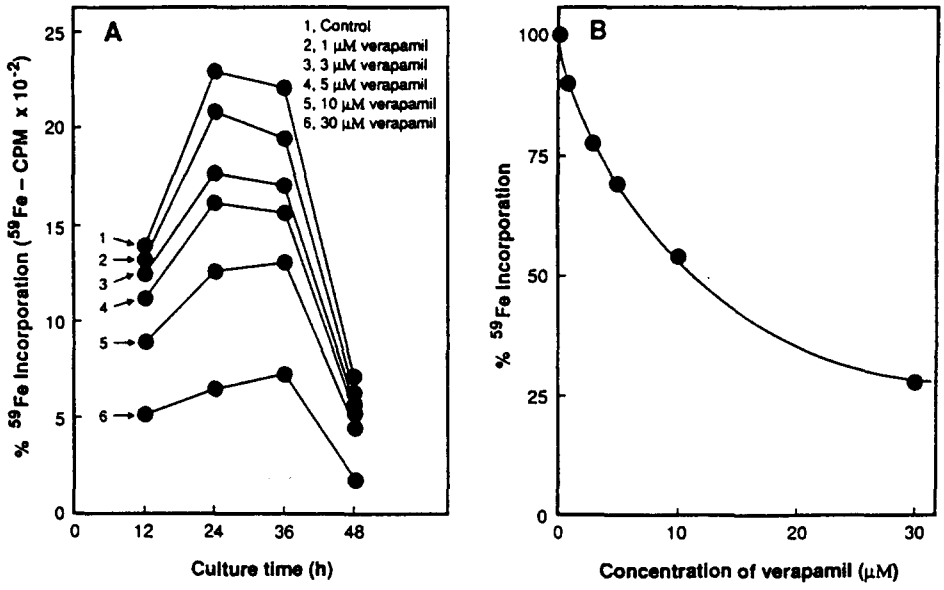

Fig. 2. Effect of verapamil on the incorporation of ^{59}Fe into heme during culture of erythroid progenitor cells in the presence of erythropoietin. The experimental procedure was the same as for Fig.1. (A) Effects of various concentrations of verapamil on the ^{59}Fe incorporation into heme. (B) The data for the 24 hour culture period in (A) are replotted in terms of % ^{59}Fe incorporation versus verapamil concentration. A typical result of three separate experiments is shown. Each point represents the mean of triplicate samples. Standard deviations were all less than 5% of the mean values.

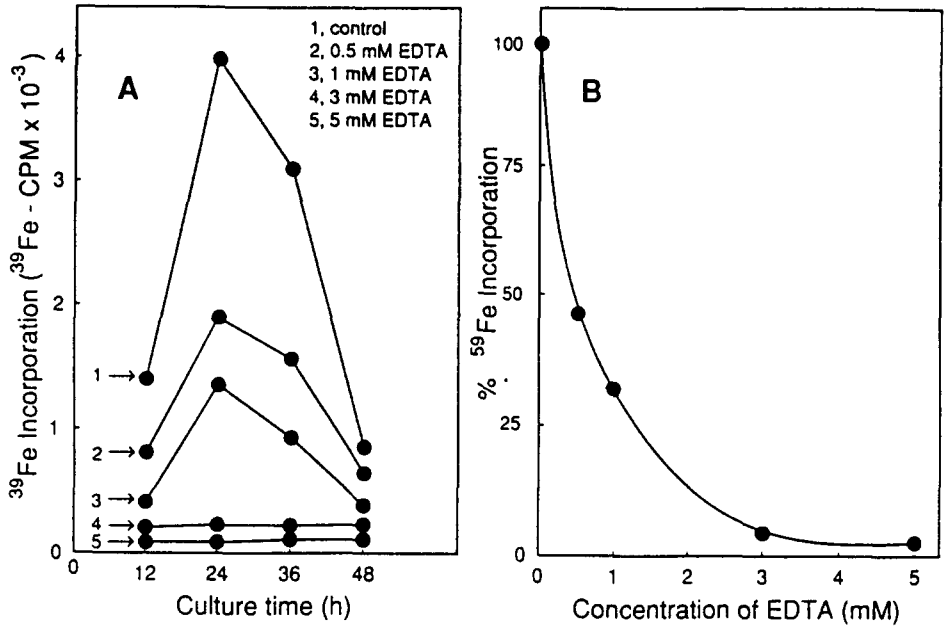

Fig. 3. Effect of EDTA on the ^{59}Fe incorporation into heme during culture of erythroid progenitor cells in the presence of erythropoietin. The experimental procedure was the same as for Fig.1. (A) Effects of various concentrations of EDTA on the ^{59}Fe incorporation into heme. (B) The data for the 24 hour culture period in (A) are replotted in terms of % ^{59}Fe incorporation versus EDTA concentration. A typical result of three separate experiments is shown. Each point represents the mean of triplicate samples. Standard deviations were all less than 5% of the mean values.

EDTA and staurosporine on Ca^{++} uptake by erythroid progenitor cells are shown in Fig. 4. In Fig. 4, each panel show a typical result of three separates and each point represents an average of triplicate samples. Fig.4 shows that all the agents tested inhibited Ca^{++} uptake in a concentration dependent manner, and the half-maximal inhibition concentrations of genistein, verapamil, EDTA and staurosporine are 20 μM,

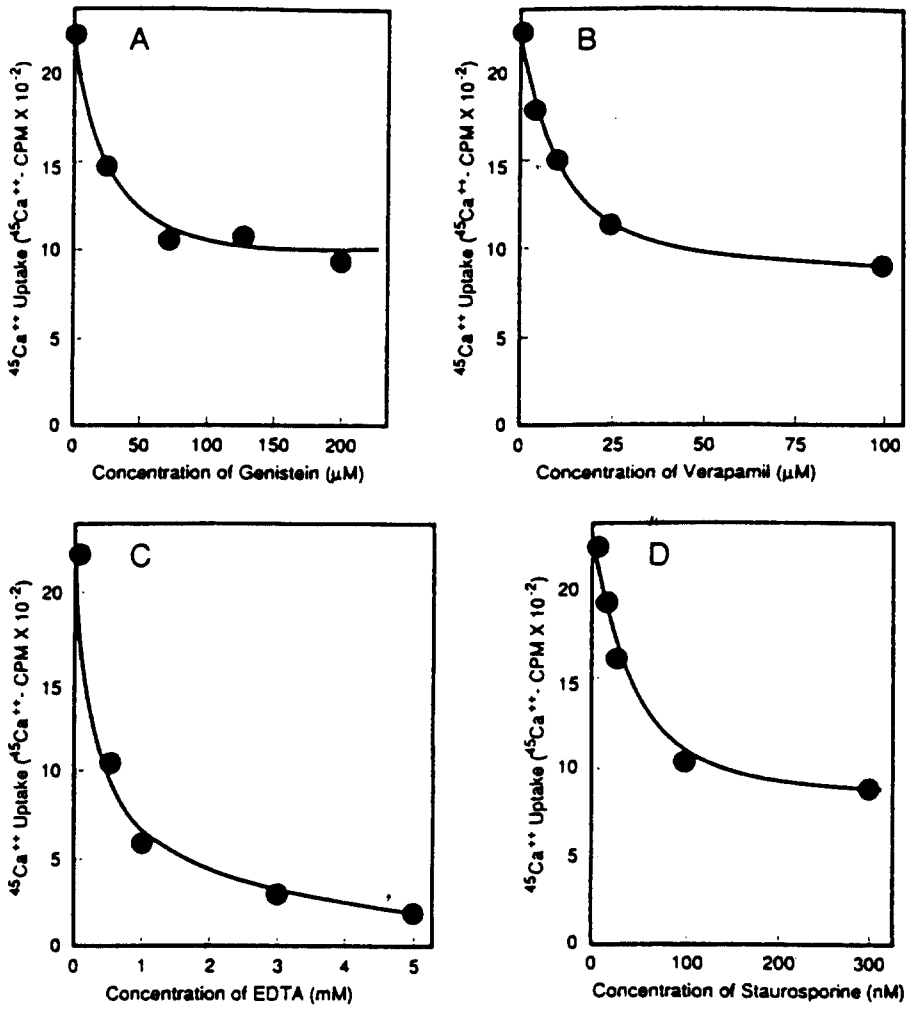

Fig. 4. Effect of genistein, verapamil, EDTA and staurosporine on the erythropoietin-promoted $^{45}Ca^{++}$ uptake in the erythroid progenitor cells. (A) Effect of genistein on the $^{45}Ca^{++}$ uptake. (B) Effect of verapamil on the $^{45}Ca^{++}$ uptake. (C) Effect of EDTA on the $^{45}Ca^{++}$ uptake. (C) Effect of staurosporine on the $^{45}Ca^{++}$ uptake. Erythroid progenitor cells ($1x10^7$ cells) were suspended in 200 μl of the incubation medium (Iscove's modified Dulbecco's medium containig 1% bovine serum albumin and 0.2U/ml of erythropoietin). To the samples, 100 μl of varing concentrations of an inhibitor was added and preincubated for 30 min at 37°C, then the samples were cooled on ice. To the cooled samples, 100 μl of $^{45}Ca^{++}$ (2.5 μCi) was added and incubated for 10 min at 37°C. The samples were cooled on ice and 1ml of cold incubation medium was added, then centrifuged for 5 min at 800 x g at 4°C. The cell pellets were lysed with 0.6ml of 0.2M NaOH, the lysed samples were neutralized with 2M HCl, and radioactivity was determined in a scintillation counter. For details see "Method". Each panel represents a typical result of three seperate experiments and each point represents the mean of triplicate samples. Standard deviations were all less than 5% of the mean values.

8.1 μM, 491 μM and 0.024 μM, respectively.

Relationship between the inhibition of Ca^{++} uptake and erythropoietin-mediated ^{59}Fe incorporation into heme in the erythroid progenitor cells

In order to examine the relationship between the inhibition of the erythropoietin-mediated differentiation of erythroid progenitor cells and inhibition of Ca^{++} uptake in the progenitor cells by various inhibitors, the values of IC_{50} of the inhibitors for the erythropoietin-mediated differentiation of the progenitor cells and for the Ca^{++} uptake in the progenitor cells are summarized in Table I. Table I shows that the order of potency of the inhibitors for the erythropoietin-mediated differentiation of the progenitor cells and for the Ca^{++} uptake in the progenitor cells is same. Table I also shows that in the case of verapamil and EDTA, the IC_{50} values for the differentiation of Ca^{++} uptake are nearly the same. However in the case of genistein and staurosporine, the IC_{50} values for the erythropoietin-mediated differentiation of the progenitor cells are signific-

Table I. Half maximal inhibition concentrations of various agents for the erythropoietin-mediated differentiation of erythroid progenitor cells and for the Ca^{++} uptake in the erythroid progenitor cells

Agents	IC_{50} (μM) Differentiation of progenitor cells	IC_{50} (μM) Ca^{++} uptake
Genistein	122.0±5.0	20.0±0.8
Verapamil	7.3±0.2	8.1±0.3
EDTA	375.0±11.0	491.0±15.0
Staurosporine	0.004±0.002	0.024±0.008

*Data were expressed as a mean±S.E. of the three different experiments.

antly different from the IC_{50} values for the Ca^{++} uptake.

DISCUSSION

Erythropoietin promotes erythropoiesis through erythropoietin receptors. Two isoforms of erythropoietin receptor with molecular masses of 85 Kd and 105 Kd were identified from erythropoietin-responsive erythroid progenitor cells (Hosoi *et al.*, 1991), and it was shown that the two proteins are structurally similar (Sawyer, 1989). An erythropoietin receptor cDNA was isolated from erythropoietin-nonresponsive murine leukemia cells (D'Andrea *et al.*, 1989). The cDNA is supposed to encode a 507 amino acid peptide with no tyrosine kinase domain. However, when cDNA was expressed in COS cells, for unknown reasons, two erythropoiein binding polypeptides with molecular masses of 66 Kd and 105 Kd were found. Contrary to the report by D'Andrea et al. (1989) that erythropoietin receptor cDNA sequnce does not contain tyrosine kinase domain, Im et al. (1990) showed erythropoietin promoted autophosphorylation of erythropoietin-responsive 105 Kd erythropoietin receptors by isolation of phosphorylated erythropoietin receptors with the use of antityrosine antibody. This result suggested the possibility that tyrosine kinase inhibitor may inhibit erythropoietin-mediated differentiation of erythroid progenitor cells. Our present results clearly show that genistien inhibits both the erythropoietin-mediated differentiation of erythroid progenitor cells and Ca^{++} uptake (Fig. 1 and 4). Recently, it was reported that the terminal erythroid differentiation of erythropoietin-nonresponsive murine leukemia cells was induced by genistein (Watanabe *et al.*, 1989). The reason for the contradicting results may be due to the differences in the mechanisms for differentiation of erythropoietin-responsive cells and erythropoietin-nonresponsive cells.

Although protein kinase C has long been implicated to play a role in the regulation of erythropoiesis, phorbol 12-myristate 13-acetate action on the differentiation of erythroid cells is controversial. Phorbol 12-myristate 13-acetate exerts effects on various proerythroid cells (Miao *et al.*, 1978). While it was shown that phorbol 12-myristate 13-acetate increases the number and size of burst forming unit-erythroid (Fibach *et al.*, 1980). And also it was reported that an inhibition of brust formation by bone marrow-derived early erythoid progenitor cells was observed (Sieber *et al.*, 1981). On the other hand, it was reported that phorbol 12-myristate 13-acetate has no effect on the differentiation of erythroid cells (Sawyer *et al.*, 1990). Recently we found that protein kinase C inhibitors, staurosporine and H7, inhibit erythropoietin-mediated differentiation of erythroid progenitor cells in a concentration dependent manner (submitted for publication). However, the effect of protein kinase C inhibitors on the erythropoietin-promoted Ca^{++} uptake in the erythroid progenitor cells has not been reported.

Calcium is not only involved in the commitment of eythroid cells to differentiation (Bridges *et al.*, 1981; Levenson *et al.*, 1980) but also plays an important role in the erythropoietin-mediated erythropoiesis (Misiti *et al.*, 1979). Although it was reported that erythropoietin has no effect on the intracellular Ca^{++} concentration of erythroid cells (Imagawa *et al.*, 1989; Linch *et al.*, 1987; Thomson *et al.*, 1988), several laboratories have demonstrated that erythropoietin promotes Ca^{++} uptake in the erythropoietin-responsive erythroid cells (Sawyer *et al*, 1984; Miller *et al.*, 1988; Miller *et al.*, 1989; Yelamarty *et al.*, 1990; Mladenovic *et al.*, 1988; Worthington *et al.*, 1989). However, up to now, inhibition of erythropoietin-promoted Ca^{++} uptake by various agents, and the relationship between the Ca^{++} uptake and the erythropoietin-mediated differentiation of erythroid progenitor cells have not been reported. Therefore, based on the above observations, the present studies were undertaken to determine the relationship of the Ca^{++} uptake and the erythropoietin-mediated differentiation of erythroid progenitor cells.

Our results show that genistein, verapamli, EDTA, and staurosporine inhibit both the erythropoietin-mediated differentiation of erythroid progenitor cells and Ca^{++} uptake (Fig. 1-4 and Table I). These results suggest the possibility that tyrosine kinase and protein kinase C may play roles in the erythropoietin-mediated differentiation of erythroid progenitor cells and Ca^{++} uptake, and the possibility that there may be a relationship between the erythropoietin-mediated differentiation of erythroid progenitor cells and Ca^{++} uptake. The observation that IC_{50} values of verapamil and EDTA for both the erythropoietin-mediated dif-

ferentiation of erythroid progenitor cells and the Ca^{++} uptake are essentially the same (Table I), suggests the possibility that the inhibition of the erythropoietin-mediated differentiation of erythroid progenitor cells by verapamil and EDTA may be the consequence of inhibition of the Ca^{++} uptake. On the other hand, the IC_{50} of genistein for the erythropoietin-mediated differentiation is about 6-fold greater than that for the Ca^{++} uptake (Table I). In contrast, the IC_{50} of staurosporine for the erythropoietin-mediated differentiation is about one sixth of that for the Ca^{++} uptake (Table I). These indicate that, in the case of genistein and staurosporine, the relationship of the inhibition of Ca^{++} uptake to the erythropoietin-mediated differentiation of erythroid progenitor cells are complex, and the delineation of the relationship needs further study.

Earlier, it has been shown that 10nM calcium ionophore A23187 increased the erythropoietin-induced erythroid colony formation by 24% (Misiti et al., 1979). Recently, the enhancement of the erythropoietin-mediated differentiation of erythroid progenitor cells by A 23187 was reported (Sawyer, 1990). We have investigated the effects of A23187 on the erythropoietin-mediated differentiation of erythroid progenitor cells and obtained a result similar to that reported by Sawyer (1980). In the presence of varing concentrations of erythropoietin (0.1 U/ml to 0.5U/ml), low concentrations of A23187 (1nM to 10nM) stimulated differentiation of the progenitor cells up to 30%, but further increase in the concentration of A23187 did not further stimulate the differentiation (data not shown). These observation suggest that the stimulation of the Ca^{++} uptake to a certain optimum intracellular Ca^{++} concentration promotes the erythropoietin-mediated erythrpoiesis. However, further stimulation of the Ca^{++} uptake beyond the optimum intracellular Ca^{++} concentration does not promote the erythropoietin-mediated erythrpoiesis any more.

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