# Catecholamine Regulation of Human Erythrocyte Membrane Protein Kinase

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ABSTRACT The effect of catecholamines on membrane-associated protein kinase in the mature human erythrocyte was investigated. Protein kinase activity was assayed after isolation of membranes from intact erythrocytes incubated with and without catecholamines. Activation of the enzyme is expressed as the ratio of the extent of phosphorylation of exogenous protein substrate in the absence to that in the presence of 2.5  $\mu$ M cyclic AMP (cAMP). The potent  $\beta$ -adrenergic agonist, (-)isoproterenol (2  $\mu$ M), (-)epinephrine (10  $\mu$ M) and (-)norepinephrine (10  $\mu$ M) stimulated the cAMP-dependent protein kinase in membranes,  $38\pm7\%$ ,  $31\pm6\%$ , and  $30\pm6\%$ , respectively. Maximal stimulation of membrane protein kinase by  $10 \,\mu M$  (-)epinephrine was obtained  $\approx 30$  min after initiation of the incubation of erythrocytes with the hormone. The concentrations of (-)catecholamines that gave half-maximal stimulation of the membrane protein kinase were 0.17  $\mu$ M for isoproterenol, 0.35  $\mu$ M for epinephrine, and 0.63  $\mu$ M for norepinephrine. The membrane protein kinase response to  $\beta$ -adrenergic agonists was found to be stereospecific. The stimulation of membrane protein kinase by  $10 \,\mu M$  (–)epinephrine was inhibited by the  $\beta$ adrenergic antagonist, (-) propranolol with EC<sub>50</sub> = 0.60  $\mu$ M, and the inhibition of agonist stimulation of the cAMP-dependent protein kinase by propranolol was stereospecific. These studies suggest that a functional  $\beta$ adrenergic receptor exists in the mature human erythrocyte.

## INTRODUCTION

Recently, with improved methodology, hormonesensitive adenylate cyclase has been detected in erythrocytes of several mammalian species, i.e., rat (1-3), mouse (1), and rabbit (4). To our knowledge only two groups have reported the presence of catecholamineresponsive adenylate cyclase in human erythrocytes (5, 6). However, because of low activity as well as the small degree of stimulation of adenylate cyclase by catecholamines (5, 6), it has not been clear whether this enzyme plays a functional role in the mature erythrocyte or is merely a vestigial function retained during erythroid differentiation. However, in vitro studies indicate that mature human erythrocytes possess a functional  $\beta$ -adrenergic receptor. (–)Isoproterenol and (-)epinephrine increased the degree of hypotonic hemolysis and decreased the deformability of human erythrocytes (3, 7). The catecholamines also decreased the size of a rapidly exchangeable calcium pool (3). Epinephrine decreased the fatty acid-chain flexibility in human erythrocytes (8). Further, those catecholamine effects on human erythrocytes had the characteristics of a  $\beta$ -adrenergic effect (7, 8). The effects of catecholamines on human erythrocyte physiology may possibly be a result of the elevation of the intracellular cyclic AMP (cAMP)<sup>1</sup> concentration via stimulation of adenylate cyclase because the observed effects could be mimicked by cAMP(7, 8).

Recent observations indicate that phosphorylation and dephosphorylation of membrane proteins play a central role in affecting cell membrane functions, and possibly control of cell deformability and shape in human erythrocytes (9, 10). It has been demonstrated that human erythrocyte membrane phosphorylation by ATP is mainly mediated by cAMP-dependent and cAMP-independent protein kinase (11–14).

We have observed the stimulation of human erythrocyte membrane protein kinase by catecholamines (15). This study was directed to examine the characteristics of catecholamine stimulation of human erythrocyte membrane protein kinase.

#### METHODS

Materials. cAMP and ATP, disodium were obtained from Schwarz/Mann, Orangeburg, N. Y. (-)- and (+)propranolol hydrochloride were generous gifts from Dr. D. J. Marshall, Ayerst Research Laboratories, Montreal, Canada. The (+)isomers of epinephrine bitartrate and norepinephrine bitartrate

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<sup>&</sup>lt;sup>1</sup>Abbreviation used in this paper: cAMP, cyclic AMP.

were generous gifts of Dr. F. C. Nachod, Sterling-Winthrop Research Institute, Rensselaer, N. Y. The (-)isomers of isoproterenol bitartrate, epinephrine bitartrate, norepinephrine bitartrate, as well as (±)normetanephrine hydrochloride, (±)3,4-dihydroxymandelic acid, (±)phenylalanine, (-)3,4dihydroxyphenylalanine, (-)tyrosine, adrenochrome, and protamine sulfate were purchased from Sigma Chemical Co., St. Louis, Mo. Phentolamine hydrochloride was kindly supplied by Dr. C. A. Brownley, CIBA Pharmaceutical Co., Summit, N. J. Hank's balanced salt solutions were obtained from Grand Island Biological Co., Grand Island, N. Y. Lymphoprep was obtained from Accurate Chemical & Scientific Corp., Hicksville, N. Y. [ $\gamma$ -<sup>32</sup>P]ATP (20-25 Ci/mmol) was purchased from International Chemical & Nuclear Corp., Burbank, Calif.

Preparation of erythrocytes and erythrocyte membranes. Blood was collected directly into heparinized vacutainer tubes from young healthy male blood donors between the ages of 19 and 31 yr. The blood was centrifuged at 4°C and washed five times with 10 mM Tris buffer which contained 150 mM NaCl (pH 7.8). Each erythrocyte preparation was monitored with a cell count of erythrocytes, reticulocytes, and leukocytes including a leukocyte differential count. The residual leukocyte count in the erythrocyte preparations was 0.013±0.002% with 0.066±0.021% reticulocytes. Membranes were prepared from washed erythrocytes essentially by a modification (16) of the method of Dodge et al. (17). Hemolysis was initiated by thoroughly mixing 2.0 ml of washed erythrocyte suspension (50% hematocrit) with 20 ml of 10 mM Tris buffer (pH 7.8) for 10 min at 0°C. The membranes were sedimented at 29,000 g for 30 min (noted as first washed membranes). Membranes obtained by subsequent washes are noted in succession as second washed membranes (pink colored), third washed membranes (slightly pink colored) and fourth washed membranes (colorless). The membranes used in these studies are described in Results.

Leukocyte preparations. Human mononuclear leukocytes and polymorphonuclear leukocytes were separated from heparinized blood by the method of Böyum (18).

Protein kinase assay. Protein kinase activity was determined by measuring the amount of <sup>32</sup>P incorporated into exogenous protein substrate (protamine). The reaction mixture (0.3 ml) contained 40 mM of potassium phosphate buffer (pH 7.0), 10 mM MgSO<sub>4</sub>,  $3.5 \mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (1,500–2,200 cpm/pmol), 300  $\mu$ g of protamine sulfate, and 75–105  $\mu$ g of membrane proteins. When cAMP was present in the reaction mixture, its concentration was  $2.5 \mu M$ . Incubations were performed at 0°C for 90 min. The reaction was terminated by adding 100  $\mu$ l of reaction mixture to 2.5 ml of ice-cold, 11% TCA together with 20  $\mu$ l of 2.5% bovine serum albumin solution. The amount of <sup>32</sup>P transferred to protamine and membranes was determined by the method of Erlichman et al. (19) except that 5% TCA was replaced by 10% TCA. The amount of <sup>32</sup>P transferred to membranes was determined in parallel assays in which protamines were omitted, and all data on phosphorylation of exogenous protein substrates were corrected for endogenous membrane phosphorylation unless otherwise noted. As blanks, boiled membranes were used in the assays. The assay error in duplicate was within 5%. In this study, protein kinase activity is expressed as the amount of transferred phosphate from ATP to exogenous protein substrate in 90 min at 0°C. In other cases, the kinase activity is expressed as the protein kinase activity ratio, i.e., the ratio of kinase activity in the absence to that in the presence of added cAMP (20).

Determination of catecholamine stimulation of membrane protein kinase. In the experiments related to catecholamine stimulation of human erythrocyte membrane protein kinase, initially, isolated intact erythrocytes (38-42% hematocrit)

were incubated with catecholamines. Well-washed human erythrocytes were additionally washed two times with the incubation buffer (Hank's balanced salt solution without Ca or Mg) and mixed with a small portion of the incubation buffer which contained catecholamines or acids used to form salts with catecholamines (control) at 0°C. The incubation medium was transferred into tubes placed in a 30°C shaking water bath (140 cycle/min). This was defined as zero time in this study. The incubation time was 30-45 min unless otherwise noted. At the end of the incubation, the incubation medium was diluted six times with the ice-cold incubation buffer and immediately centrifuged at 3,020 g for 5 min at 4°C. Erythrocyte pellets were resuspended in 10 mM Tris buffer containing 150 mM NaCl (pH 7.8) and washed once by the buffer, and then erythrocyte membranes were prepared. The protein kinase activity associated with isolated membranes was determined after being frozen and thawed. The membrane proteins in each assay tube were carefully adjusted to the same concentration.

Chemical analysis. Protein was determined by the method of Lowry et al. (21). Hemoglobin associated with membranes was measured with the cyanmethemoglobin method (22). The membrane hemoglobin was subtracted from the total protein to give the nonhemoglobin protein (membrane protein) in the case of the first, second, and third washed membranes. Nonhemoglobin protein in membranes was also determined by the method of Bodemann and Passow (23).

Statistical methods. All the results are expressed as mean percentage of stimulation  $\pm$  SEM unless otherwise noted. Statistical significance was determined by student's t test based on paired data (24). Statistical significance was assigned if P < 0.05.

#### RESULTS

The effect of incubation time on phosphorylation of protamine by human erythrocyte membrane protein kinase. The protamine phosphorylation by membrane protein kinase at 0°C in the presence and absence of cAMP was almost linear up to 120 min with concentrations of 0.25-0.35 mg/ml of membrane proteins (Fig. 1). The protein kinase activity ratio determined at 0°C increased with incubation time up to 80 min and reached a constant value between 90 and 100 min of incubation (Fig. 1).

Effect of membrane protein concentration on phosphorylation of protamine. The incorporation of [<sup>32</sup>P]phosphate into protamine in the presence or absence of cAMP was directly proportional to the concentration of human erythrocyte membrane protein up to 0.37 mg/ml of the incubation medium at 0°C for 90 min of incubation (Fig. 2). Furthermore, the protein kinase activity ratio remained almost constant between 0.1 and 0.37 mg/ml of the incubation medium (Fig. 2).

Kinetics of stimulation of membrane protein kinase. The effect of incubation time at 30°C on 10  $\mu$ M (-)epinephrine stimulation of membrane protein kinase in human erythrocytes is illustrated in Fig. 3. Stimulation of the membrane protein kinase by (-)epinephrine (10  $\mu$ M) was initiated 10 min after addition of the hormone, the earliest time measured. (-)Epinephrine stimulation of the membrane protein kinase reached



FIGURE 1 Effect of incubation time at 0°C on protein kinase activity and the protein kinase activity ratio in human erythrocyte membranes (0.3 mg/ml of reaction medium). The concentration of cAMP was 2.5  $\mu$ M when introduced into the reaction medium.

a maximum at  $\cong 30 \text{ min } (P < 0.001)$  and decreased gradually during the subsequent 90 min of incubation. Significant levels of stimulation (33-41%) of the membrane protein kinase were observed between 30 and 60 min of incubation with (-)epinephrine.



FIGURE 2 Effect of membrane protein concentration on the protein kinase activity and the protein kinase activity ratio in human erythrocyte membranes. Incubation of membranes with  $[\gamma^{-32}P]$ ATP was conducted for 90 min at 0°C.

Effect of catecholamine concentration on stimulation of erythrocyte membrane protein kinase. Fig. 4 shows the levels of the membrane protein kinase activity ratio in response to increasing concentrations of  $\beta$ -adrenergic agonists. Assays were made with membranes isolated from erythrocytes exposed (30 min at 30°C) to various concentrations of the catecholamines.

The protein kinase activity ratio increased in proportion to the concentration and reached a maximum at 1-10  $\mu$ M (-)catecholamines (Fig. 4). For example, the protein kinase activity ratio of membrane protein kinase exposed to 10  $\mu$ M (-)epinephrine was 0.173, 38% above the control value (0.125). The concentrations of (-) $\beta$ -adrenergic agonists required to stimulate membrane protein kinase half-maximally ( $K_m$ ), were graphically obtained to be about 0.17  $\mu$ M for (-)isoproterenol, 0.35  $\mu$ M for (-)epinephrine, and 0.63  $\mu$ M for (-)norepinephrine. The effect of catecholamine on membrane protein kinase was stereospecific as shown in Fig. 4. The (+)isomer of one of the catecholamines, (+)epinephrine, was markedly less potent than (-)epinephrine (Fig. 4).

Statistical analysis of catecholamine stimulation of erythrocyte membrane protein kinase indicated significant stimulation as shown in Fig. 5. After incubation with  $\beta$ -adrenergic agonists, the membrane protein kinase activity ratio increased  $38\pm7\%$  (n = 10, P < 0.002),  $31\pm6\%$  (n = 13, P < 0.001), and  $30\pm6\%$  (n = 6, P < 0.005), by 2  $\mu$ M (-)isoproterenol, 10  $\mu$ M (-)epinephrine, and 10  $\mu$ M (-)norepinephrine, respectively.

Characteristics of catecholamine stimulation of membrane protein kinase in human erythrocytes.



FIGURE 3 Effect of (-)epinephrine on human erythrocyte membrane protein kinase as a function of time. Isolated erythrocytes (40% hematocrit) were incubated in Hank's balanced salt solution at 30°C for the time indicated in the absence (control) or presence of 10  $\mu$ M (-)epinephrine. Protein kinase activities associated with membranes (second washed membranes) were determined according to the method given in Methods. Values are the means±SEM of replicate determinations from four experiments. Significant effects of (-)epinephrine on membrane protein kinase are depicted by \*, P < 0.05 and \*\*, P < 0.001.

 $\beta$ -adrenergic agonist stimulation of membrane protein kinase in human erythrocytes was progressively inhibited with increasing concentrations of the  $\beta$ -adrenergic blocker, (-)propranolol (Fig. 6). The concentration of (-)propranolol required to produce half-maximal inhibition of 10  $\mu$ M (-)epinephrine stimulation of the membrane protein kinase was 0.6  $\mu$ M (Fig. 6).

The concentration of (-)propranolol required to inhibit 100% of the effect of 10  $\mu$ M (-)epinephrine on membrane protein kinase was 30  $\mu$ M (Fig. 6).

The dissociation constant of the (-)propranolol effect on epinephrine stimulation of membrane protein kinase was calculated to be 20 nM with the equation (25)

$$K_d = \frac{\mathrm{EC}_{50}}{1 + \mathrm{S}/\mathrm{K}_\mathrm{m}}$$

where S is the concentration of (-)epinephrine used to stimulate membrane protein kinase,  $K_m$  is the constant for half-maximal (-)epinephrine stimulation, and EC<sub>50</sub> is the concentration of (-)propranolol required to produce half-maximal inhibition of (-)epinephrine stimulation of the membrane protein kinase.

In contradistinction, the inhibition of 10  $\mu$ M (-)epinephrine stimulation of membrane protein kinase activity by the (+)isomer of propranolol was much less. The concentration of (+)propranolol required to produce half-maximal inhibition of 10  $\mu$ M (-)epinephrine stimulation of the membrane protein kinase was 16  $\mu$ M. Thus, the inhibition of  $\beta$ -adrenergic agonist stimulation of erythrocyte membrane protein kinase by propranolol was shown to be stereospecific. Although the dose-response curves for the effect of (-)propranolol on the (-)isoproterenol and (-)norepinephrine stimulation of membrane protein kinase were not



FIGURE 4 Effect of catecholamine concentration on membrane protein kinase in human erythrocytes. Erythrocytes (42% hematocrit) were incubated for 30 min at 30°C with Hank's balanced salt solution containing the indicated concentration of catecholamines. Preparation of membranes (second washed membranes) for protein kinase, is described under Methods. Each point represents the mean±SEM of values of replicate determinations from three experiments.



FIGURE 5 Effect of catecholamines on the membrane protein kinase in human erythrocytes. Isolated erythrocytes (38-42%)hematocrit) were incubated in Hank's balanced salt solution for 40 min at 30°C in the absence (control) or presence of catecholamines. Membrane protein kinase activities were determined as described in Methods. Third washed membranes were used. Values are the mean±SEM, and *n* is the number of experiments. Significant effects of catecholamines on membrane protein kinase compared with solvent controls are indicated as \*, P < 0.005; \*\*, P < 0.002; and \*\*\*, P < 0.001.

determined, complete inhibition of  $2 \mu M$  (-)isoproterenol and 10  $\mu M$  (-)norepinephrine stimulation of membrane protein kinase by 50  $\mu M$  (-)propranolol was found (data not shown). The  $\alpha$ -adrenergic blocker, phentolamine, at concentrations up to 0.1 mM failed to inhibit significantly the (-)epinephrine stimulation of membrane protein kinase (data not shown).

In addition, the effect of catecholamine precursors  $[(\pm)phenylalanine, (-)tyrosine, and (-)3,4-dihydroxy-$ 



FIGURE 6 The effect of various concentrations of propranolol on (-)epinephrine stimulation of protein kinase activity in human erythrocyte membranes. In this assay, 39-41% hematocrit and 35 min at 30°C were employed as the incubation conditions. Second washed membranes were used for the membrane protein kinase assay. For details see Methods.

phenylanine] and metabolites  $[(\pm)3,4$ -dihydroxymandelic acid,  $(\pm)$  normetanephrine, and adrenochrome)] on the membrane protein kinase was tested. No significant change of the membrane protein kinase was observed after incubation of erythrocytes with these compounds (data not shown).

### DISCUSSION

Although there have been several investigations of the effects of hormones on cAMP concentration and adenylate cyclase activity in human erythrocytes (1, 3, 5, 6), no detailed studies have been reported on the hormonal regulation of human erythrocyte protein kinase. We have found that catecholamines activated the protein kinase associated with human erythrocyte membranes. All of the  $\beta$ -adrenergic agonists [(-)isoproterenol, (-)epinephrine, and (-)norepinephrine] tested in this study, stimulated membrane protein kinase significantly (Figs. 4 and 5). Determinations of the protein kinase activity ratio of human erythrocyte membranes were performed under conditions where the activity ratio was not influenced by slight differences in membrane protein content and incubation times (Figs. 1 and 2). Therefore, the observed increase in the membrane protein kinase activity ratio after exposure to catecholamines reflected a real increase in the active state of protein kinase associated with membranes rather than an artifact such as membrane sampling.

The protein kinase activity and the activity ratio were very sensitive to Mg<sup>2+</sup> concentration, exogenous protein substrates, concentration of  $[\gamma^{-32}P]ATP$ , temperature, and freshness of membranes (data not shown).<sup>2</sup> This may account for the large differences in the cAMP dependency of membrane protein kinase in human erythrocytes reported by several investigators (26-28). Therefore, the assay conditions have been rigorously the same throughout this entire study. Notwithstanding, there is still some variation in the basal protein kinase activity ratio. When the basal protein kinase activity was higher, we noted a smaller catecholamine effect on the membrane protein kinase. This may account for differences in absolute maximal stimulation of the membrane cAMP-dependent protein kinase by catecholamines in several assays. It is possible that the concentrations of catecholamines or other factors in human blood may modify the membrane protein kinase in erythrocytes to varying extents.

The range of percentage of stimulation of the membrane cAMP-dependent protein kinase by 10  $\mu$ M (-)epinephrine as well as 2  $\mu$ M (-)isoproterenol was 20-80%. When we observed 80% maximal stimulation (1.8-fold increase in the protein kinase activity ratio) of the membrane protein kinase by  $10 \mu M$  (-)epinephrine, a significant increase in the protein kinase activity was observed with exposure to 50 nM (-)epinephrine (data not shown).<sup>2</sup>

Great care was necessary in the preparation of membranes, after incubation of erythrocytes with catecholamines. Hemolysis of the erythrocytes at 0°C resulted in less variation in the values of catecholamine stimulation of membrane protein kinase activity as well as basal protein kinase activity than hemolysis at room temperature. It may be that there is increased membrane heterogeneity with resealing at temperatures above 0°C (23). Catecholamine stimulation of membrane protein kinase was observed in first, second, third, and fourth washed membranes with carefully controlled washing. However, after the fourth washing, there was diminished catecholamine stimulation of membrane protein kinase with resultant greater variance in the data.

The small amount of leukocyte  $(0.013\pm0.002\%)$ and reticulocyte  $(0.066\pm0.021\%)$  contamination in our human erythrocyte preparations cannot account for the stimulation of membrane protein kinase by catecholamines. In our studies, up to 0.05% (highest contamination tested) of mononuclear leukocytes and polymorphonuclear leukocytes in human erythrocyte preparations did not produce any significant changes in the extent of catecholamine stimulation of the membrane protein kinase as well as the basal protein kinase activity. Moreover, with various human erythrocyte preparations, up to 0.5% reticulocytes and 1.0% platelets did not produce any significant changes in the catecholamine stimulation of membrane protein kinase or basal protein kinase activity.

We were not able to detect any significant difference in endogenous phosphorylation of membrane proteins between membranes obtained from erythrocytes exposed to catecholamines and control with the protein kinase assay employed here. However, by employing different assay conditions, we have observed stimulation of membrane phosphorylation by catecholamines (29). Preliminary experiments suggest that incubation of isolated membranes (leaky ghosts) with catecholamines cause very little activation of membrane protein kinase.

Because we have observed much greater stimulation of membrane protein kinase by incubating intact erythrocytes with catecholamines, we have conducted our study with intact erythrocytes.

The human erythrocyte membrane protein kinase response to catecholamines appeared to be of the  $\beta$ -adrenergic type as indicated by the following observations. (a) The order of potency of  $\beta$ -adrenergic agonists to stimulate membrane protein kinase was (-)isoproterenol > (-)epinephrine > (-)norepinephrine (Fig. 4), although the difference of the potency

<sup>&</sup>lt;sup>2</sup> Results documenting these phenomena will be provided upon request.

between (-)isoproterenol and (-)epinephrine was smaller than that observed in various other tissues with respect to activation of adenylate cyclase (30). (b) The effect of (-)epinephrine was stereospecific, the (-)-form of epinephrine being much more effective than (+)epinephrine in stimulating erythrocyte membrane protein kinase (Fig. 4). (c) The catecholamine stimulation of membrane protein kinase was stereospecifically blocked by  $\beta$ -adrenergic antagonists (Fig. 6). (d) The  $\alpha$ -adrenergic blocker did not block the effect of catecholamines on the membrane protein kinase.

The mechanism whereby membrane cAMP-dependent protein kinase is stimulated by catecholamines is not clear at present except that it appears to be of the  $\beta$ -adrenergic type.  $\beta$ -adrenergic actions in various tissues including erythrocytes have been considered to be mediated by increased intracellular cAMP concentrations by activation of adenylate cyclase (31). Thus, it is likely that catecholamine stimulation of membrane cAMP-dependent protein kinase will most likely be through activation of adenylate cyclase and increased intracellular cAMP concentration. This change may be too small to measure accurately with currently available methods.

In many cell systems, it has been reported that the time-course of the catecholamine effects on  $\beta$ -adrenergic receptors, particularly with regard to adenylate cyclase stimulation, reach a maximum within 5–10 min and decrease rapidly (32-34). However, in erythrocytes, the response of  $\beta$ -adrenergic receptors appears to be a much slower process. For example, the effect of isoproterenol on cellular cAMP accumulation in turkey erythrocytes did not reach a maximum even after 90 min of incubation (35). Furthermore, the effect of isoproterenol on sodium influx reached a maximum at approximately 30 min, and after 30 min there was a steady decrease in the effect of isoproterenol so that by 2 h sodium influx in cells exposed to isoproterenol was only slightly greater than for cells incubated for the same period of time without isoproterenol (35). Thus, the kinetics of the effect of isoproterenol on sodium influx in turkey erythrocytes appears to be quite similar to that of the effect of epinephrine on membrane protein kinase in human erythrocytes.

It may be possible that in the human erythrocyte, a  $\beta$ -adrenergic action may be unrelated to the adenylate cyclase system (3). There are reports that indicate that certain hormones stimulate protein kinase directly, i.e., independent of adenylate cyclase activation (36). An increase in membrane protein kinase activity by catecholamines may be the consequence of an increased association of the catalytic subunits of protein kinase from the cytosol to membranes (20, 37).

Studies are in progress in this laboratory to clarify the mechanism of catecholamine stimulation of membrane protein kinase in human erythrocytes.

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