Mechanism of activation of ERK and H-K-ATPase by isoproterenol in rat cortical collecting duct

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Mechanism of activation of ERK and H-K-ATPase by isoproterenol in rat cortical collecting duct. Am J Physiol Renal Physiol 284: F948–F954, 2003; 10.1152/ajprenal.00394.2002.—Isoproterenol stimulates H-K-ATPase activity in rat cortical collecting duct β-intercalated cells through a PKA-dependent pathway. This study aimed at determining the signaling pathway underlying this effect. H-K-ATPase activity was determined in microdissected collecting ducts preincubated with or without specific inhibitors or antibodies against intracellular signaling proteins. Transient cell membrane permeabilization with streptolysin-O allowed intracellular access to antibodies. Isoproterenol increased phosphorylation of ERK in a PKA-dependent manner, and inhibition of the ERK phosphorylation prevented the stimulation of H-K-ATPase. Antibodies against the monomeric G protein Ras or the kinase Raf-1 curtailed the stimulation of H-K-ATPase by isoproterenol, whereas antibodies against the related proteins Rap-1 and B-Raf had no effect. Pertussis toxin and inhibition of tyrosine kinases with genistein also curtailed isoproterenol-induced stimulation of H-K-ATPase. It is proposed that activation of PKA by isoproterenol induces the phosphorylation of β-adrenergic receptors and the switch from Gs to Gi coupling. In turn, β3-subunits released from Gα resulted in Gβγ coupling. H-K-ATPases are P-type ATPases that exchange intracellular proton against extracellular potassium at the expense of ATP hydrolysis (10). Gastric H-K-ATPase was the first H-K-ATPase to be discovered and remains the archetype of this family of ATPases. It is a ouabain-insensitive, omeprazole- and Sch-28080-sensitive ATPase located in the apical membrane of gastric parietal cells where it energizes HCl secretion (9). Subsequently, other H-K-ATPases were characterized in the distal colon and in the kidney collecting duct (13, 28). Based on the finding that Sch-28080-sensitive ATPase activity is increased in the collecting duct of potassium-deprived rats compared with normal ones, it was initially thought that renal H-K-ATPase was mainly involved in potassium transport (19). Later, functional studies demonstrated that collecting duct H-K-ATPase also participates in the regulation of acid-base balance (10). However, regulation of potassium and acid-base balances in the collecting duct may be accounted for by different H-K-ATPases. As a matter of fact, collecting ducts from normal and potassium-deprived rats express at least two distinct Sch-28080-sensitive ATPases that are insensitive and sensitive to ouabain, respectively (4).

Rat cortical collecting ducts (CCDs) consist of at least three cell types characterized by distinct morphological and functional features. Principal cells are involved in sodium, potassium, and water transport, whereas α- and β-intercalated cells are the site of proton and bicarbonate secretion, respectively. Functionally, the three cell types of the rat CCD also differ by the presence of specific G protein-coupled hormone receptors activating adenylyl cyclase: principal cells express vasopressin V2 receptors, whereas α- and β-intercalated cells express calcitonin and β-adrenergic receptors, respectively. This hormone selectivity was used to localize functionally H-K-ATPases in the different CCD cell types: Sch-28080-sensitive ATPase is stimulated by calcitonin and isoproterenol in normal rats, demonstrating that it is expressed in α- and β-intercalated cells, whereas in potassium-deprived rats it is activated by vasopresin and therefore originates from principal cells (15). These findings further support the hypothesis that H-K-ATPase-mediated regulation of acid-base balance and potassium balance in collecting duct might be accounted for by two distinct forms of H-K-ATPase originating from different cell types. They also demonstrate that besides long-term regulation of expression, H-K-ATPase activity is modulated in the short term by posttranscriptional mechanisms. The stimulation of H-K-ATPase by calcitonin and isoproterenol observed in CCDs of normal rats, along with that of H-ATPase previously reported (25), likely participates to the short-term regulation of proton transport by these two hormones (25, 26).
The signaling pathways underlying calcitonin- and isoproterenol-induced activation of H-K-ATPase are different. Although stimulation of H-K-ATPase by either hormone is mediated by cAMP (15), the action of isoproterenol relies on the activation of PKA, whereas that of calcitonin is independent of PKA. The PKA-independent mechanism of activation of H-K-ATPase by calcitonin in α-intercalated cells has been characterized: the calcitonin-induced increase in cAMP activates the cAMP-activated guanine-nucleotide exchange factor Epac I, which in turn activates a cascade that includes the monomeric G protein Rap-1, the B-Raf kinase, the MAP kinase kinase MEK, and the extracellular signal-regulated kinases ERK1/2 (16). Activation of ERK1/2 leads to the stimulation of H-K-ATPase activity through a nontranscriptional mechanism. In contrast, the PKA-dependent signaling mechanism of isoproterenol in β-intercalated cells has not been identified as yet and was therefore investigated in the present study. Because PKA-dependent activation of ERK has been reported in the literature (5, 11, 32), we determined the potential involvement of ERK in isoproterenol-induced activation of H-K-ATPase and the cascade leading to ERK phosphorylation.

MATERIALS AND METHODS

Animal preparation and tubule microdissection. Experiments were carried out in male Sprague-Dawley rats anesthetized with pentobarbital sodium (50 mg/kg body wt). CCDs were dissected at 4°C from collagenase-treated kidneys as described previously (12). After microdissection, they were photographed to determine their length, which served for normalization of the results. Unless indicated otherwise, microdissection was carried out in a solution containing (in mM) 120 NaCl, 5 KCl, 1 MgSO4, 4 NaHCO3, 0.2 NaH2PO4, 0.15 Na2HPO4, 5 glucose, 0.5 CaCl2, 0.08 dextran, 2 lactate, 20 HEPES, and 4 essential and nonessential amino acids, as well as 0.03 vitamins and 1 mg/ml BSA. The pH was adjusted to 7.4 and osmotic pressure to 400 mosmol/kgH2O, with mannitol up to 400 mosmol/kgH2O, at pH 7.4. After transfer within 0.5 μl of rinsing solution into a 96-well flat-bottom plastic microplate, samples were permeabilized by adding 2 μl of hypotonic solution (10 mM Na+-free Tris-HCl, pH 7.4, with or without 10−4 M Sch-28080) to each sample and freezing on dry-ice. After thawing and addition of 10 μl of assay medium (see composition below), the microplate was incubated at 37°C for 15 min. Incubation was stopped by cooling and adding 300 μl of an ice-cold suspension of 15% (w/v) activated charcoal. After centrifugation, 50-μl aliquots of each supernatant were transferred to a 96-well sample microplate for Cerenkov counting (Trilux microbeta 1450, Wallac, Finland).

H-K-ATPase activity assay. H-K-ATPase activity was determined with the radiolabeled microassay previously described (4) and adapted for microplate assay. Briefly, to avoid contamination with Na-K-ATPase activity, CCDs were rinsed in a cold Na+-free solution containing (in mM) 0.8 MgSO4, 1 MgCl2, 0.5 CaCl2, 100 Tris-HCl, 1 mg/ml BSA, and mannitol up to 400 mosmol/kgH2O, at pH 7.4. After transfer within 0.5 μl of rinsing solution into a 96-well flat-bottom plastic microplate, samples were permeabilized by adding 2 μl of hypotonic solution (10 mM Na+-free Tris-HCl, pH 7.4, with or without 10−4 M Sch-28080) to each sample and freezing on dry-ice. After thawing and addition of 10 μl of assay medium (see composition below), the microplate was incubated at 37°C for 15 min. Incubation was stopped by cooling and adding 300 μl of an ice-cold suspension of 15% (w/v) activated charcoal. After centrifugation, 50-μl aliquots of each supernatant were transferred to a 96-well sample microplate for Cerenkov counting (Trilux microbeta 1450, Wallac, Finland).

H-K-ATPase activity was distinguished from other ATP-hydrolyzing activities on the basis of its sensitivity to Sch-28080, a specific inhibitor of renal H-K-ATPase activities (4). Thus, for each experimental condition, eight samples were divided into two groups, one for measuring total ATPase activity and the other for measuring ATPase activity in the presence of Sch-28080. H-K-ATPase activity was calculated as the difference between these two mean ATPase activities.

The ATPase assay medium contained (in mM) 25 Tris-HCl, 10 MgCl2, 1 EGTA, 2.5 KCl, 50 Tris-ATP, and 200 nCi of [γ-32P]ATP with or without 300 μM Sch-28080.

Western blot analysis of phospho-ERKs. Pools of 30 CCDs dissected in medium supplemented with leupeptin (25 μg/ml) and aprotinin (25 μg/ml) were preincubated at 37°C for 1 h with or without H-89 and then at 37°C for 10 min with or without isoproterenol. Samples were then transferred with 0.5 μl of incubation medium into 14.5 μl of lysis buffer containing (in mM) 100 NaCl, 1.5 MgCl2, 2 sodium pyrophosphate, 2.5 glycerophosphate, 30 NaF, 1 EGTA, 20 HEPES, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml AEBSF, and 10 μg/ml antipain at pH 7.4, vortexed, and kept on ice for 1 h. Cell lysate was centrifuged at 15,000 g for 10 min, and the supernatant was removed and stored at −80°C until use.

Supernatants were resuspended (vol/vol) in 2× Laemmli, heated at 100°C for 5 min, and analyzed by SDS-PAGE. After electrophoresis on 10% polyacrylamide gels, proteins were electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) and incubated for 1 h with anti-
phospho-ERK1/2 antibody (dilution 1/2,000) in PBS with 0.1% Tween (vol/vol) and with 5% (wt/vol) nonfat dry milk. After washing in TBS-Tween, membranes were incubated for 45 min with an anti-rabbit IgG antibody (dilution 1/5,000) coupled to horseradish peroxidase (Transduction Laboratories, Lexington, KY) in TBS-Tween. The antigen-antibody complexes were detected by chemiluminescence with the Super Signal Substrate method (Pierce) according to the manufacturer’s instructions.

In each experiment, the amounts of proteins loaded onto each lane of the electrophoresis gel corresponded to the same initial length of isolated CCDs (±5%). The chemiluminescence of each lane was quantified by densitometry (arbitrary units) and expressed in each experiment as percentage of the control lane (no isoproterenol treatment). Results are expressed as means ± SE from several animals.

Measurement of inositol phosphate production. Assays were performed by using the technique developed for proximal tubules (20) with slight modifications (6). Briefly, CCDs were microdissected in the dissection solution supplemented with ibuprofen (10 μM) and adenosine deaminase (0.5 U/ml). Samples were radiolabeled in 50 μl of this medium containing 50 μCi myo-[3H]inositol (1 mCi/ml, Amersham Pharma Biotech, Orsay, France) and 2 mM CaCl2 for 2 h at 30°C. Thereafter, tubules were successively rinsed five times in 1 ml of incubation solution (similar to dissection solution except that CaCl2 was 0.5 mM), and pools of CCDs (4- to 7-mm length) were incubated in the presence or absence of isoproterenol at 37°C for 15 min in the incubation solution supplemented with 20 mM LiCl. The reaction was stopped, and the phosphoinositides, free inositol, glycerophospho inositol, and inositol phosphates (IPs) were separated by Dowex chromatography, and their associated radioactivity was counted. Production of IPs was expressed as the percentage of the total radioactivity incorporated in tubules. For each condition, production of IPs was determined in quintuplicate and expressed as the mean value.

Statistics. Results are given as means ± SE from different animals. Data were compared according to either paired or unpaired Student’s t-test, as appropriate, or, when more than two groups were compared, according to ANOVA with Fisher’s protected least significant difference test.

RESULTS

Role of ERKs in isoproterenol-induced stimulation of H-K-ATPase. Because activation of the ERK pathway mediates the stimulation of H-K-ATPase by calcitonin in α-intercalated cells of the rat CCD (16), we evaluated whether 1) isoproterenol activates ERK1/2 in CCDs and 2) activation of ERK1/2 mediates the stimulation of H-K-ATPase.

Western blot analysis using an antibody against phospho-ERKs p42 and p44 demonstrated that isoproterenol increased the phosphorylation of ERK1/2 by >50% and that this effect was abolished by the PKA inhibitor H-89 (Fig. 1A).

This finding is compatible with the involvement of ERK activation in the stimulation of H-K-ATPase not only by calcitonin but also by isoproterenol. Indeed, the preincubation with 5 μM U-0126, an inhibitor of the ERK kinase MEK (8), abolished the stimulation of H-K-ATPase induced by isoproterenol (Fig. 1B).

Activation pathway of MEK by isoproterenol. MEK may be activated through phosphorylation by a kinase of the Raf family, Raf-1 or B-Raf. Raf kinases are themselves activated by monomeric G proteins of the Ras family. We therefore evaluated the possible role of Raf kinases and Ras G protein in isoproterenol-induced stimulation of H-K-ATPase.

The pretreatment of CCDs with a monoclonal antibody that inhibits the activity of Ras curtailed the stimulatory effect of isoproterenol (Fig. 2A). In contrast, pretreatment with a polyclonal antibody directed against the related G protein B-Raf, previously shown to inhibit calcitonin-induced stimulation of H-K-ATPase (16), did not alter the effect of isoproterenol on H-K-ATPase (Fig. 2B, left). Similarly, the pretreat-

![Fig. 1. Activation of ERK mediates isoproterenol action. A: isoproterenol (Iso)-induced phosphorylation of ERK1/2: effect of H-89. After preincubation at 30°C for 1 h in the absence (control; C) or presence of 1 μM H-89 (H89), rat cortical collecting ducts (CCDs) were incubated at 37°C for 45 min in the absence or presence of isoproterenol (shaded bars). Thereafter, samples were solubilized in Triton X-100, and phospho-ERK1/2 (p42 and p44) was revealed by Western blotting using a specific anti-phospho-ERK antibody. Top: representative gels from the same animal. In each experiment, the densitometry of each lane (p42 and p44) was expressed as the percentage of the control value (absence of H-89 and of isoproterenol). Bottom: values are means ± SE of results from 4 animals. Statistical comparison between groups was performed by ANOVA with Fisher’s protected least significant difference (PLSD) test. *P < 0.05 vs. control values. B: role of ERK1/2 on hormone-induced stimulation of H-K-ATPase. After preincubation at 30°C for 45 min without (control) or with 5 μM U-0126, CCDs were incubated at 37°C for 10 min without (filled bars) or with 1 μM isoproterenol (shaded bars). Thereafter, H-K-ATPase activity was determined. N, no. of experiments. Statistical comparison between groups was performed by ANOVA with Fisher’s PLSD test. **P < 0.001 vs. control values (no isoproterenol).](http://ajprenal.physiology.org/Downloaded/from)
ment with a monoclonal antibody mapping a COOH-terminal epitope of Raf-1 abolished the stimulatory effect of isoproterenol on H-K-ATPase (Fig. 2A, right), whereas an antibody against B-Raf, which inhibits calcitonin-induced activation of H-K-ATPase (16), had no effect (Fig. 2B, right). The specificity of action of the Ras and Raf-1 antibodies toward their cognate protein is assessed by the fact that these two antibodies had no effect on the related proteins Rap1 and B-Raf (16). Together, these findings suggest that isoproterenol-induced activation of ERK1/2 and H-K-ATPase is mediated by the Ras/Raf-1/MEK1/2 cascade.

Because Ras can be activated by PLC, we investigated whether isoproterenol might stimulate PLC. Results in Fig. 3 indicate that in CCDs, isoproterenol did not increase the production of IPs, whereas vasopressin and prostaglandin E2 used as positive controls were efficient.

**Evidence for G$_s$-to-G$_i$ switch of the β-adrenergic receptor.** Results reported above indicate that stimulation of H-K-ATPase by isoproterenol is mediated by activation of PKA and MEK but is independent of B-Raf, a classic effector of PKA. Several reports have shown that PKA-mediated phosphorylation of the β-adrenergic receptor can induce a switch from G$_s$ to G$_i$ coupling (5, 32). Such a switch may lead to the release of βγ-subunits from G$_i$, the stimulation of Ras by non-receptor tyrosine kinases such as Src, and the activation of Raf-1 and MEK (5, 11). Thus we evaluated whether this pathway might account for isoproterenol-induced stimulation of H-K-ATPase in CCD β-intercalated cells. For this purpose, we determined the role of G$_i$ and tyrosine kinase activity on the stimulation of H-K-ATPase by isoproterenol.

Preincubation with 0.5 μg/ml of the G$_i$ inhibitor pertussis toxin (Fig. 4A) or with 100 μM of tyrosine kinase inhibitor genistein (Fig. 4B) did not modify basal H-K-ATPase activity but totally blocked its stimulation by isoproterenol.

**Fig. 2. Role of Ras and Raf-1 in isoproterenol-induced stimulation of H-K-ATPase.** Rat CCDs were preincubated at 4°C for 90 min in the absence (control) or presence of antibodies (dilution 1:100) directed against Ras (A, left), Raf-1 (A, right), Rap-1 (B, left), or B-Raf (B, right) after streptolysin-O permeabilization. Thereafter, samples were incubated at 37°C for 10 min without hormone (filled bars) or 1 μM isoproterenol (shaded bars) before measurement of H-K-ATPase activity. N, no. of experiments. Values are means ± SE. Statistical comparison between groups was performed by ANOVA with Fisher’s PLSD test. *P < 0.05, **P < 0.005, and ***P < 0.001 vs. control groups (no isoproterenol).

**Fig. 3. Isoproterenol does not stimulate phospholipase C.** After rat CCDs were loaded with [myo-3H]inositol, they were preincubated at 37°C for 15 min without (basal) or with 1 μM isoproterenol, 1 μM PGE$_2$, or 100 nM AVP. Thereafter, inositol phosphates (IPs) were separated and their radioactivity was determined. Values are means ± SE of results from 4 animals and are expressed as the percentage of the total radioactivity incorporated in CCDs. Statistical comparison between groups was performed by ANOVA with Fisher’s PLSD test. *P < 0.01 and **P < 0.001 vs. basal.

**Fig. 4. Role of G$_i$ and tyrosine kinases on isoproterenol-induced stimulation of H-K-ATPase.** Rat CCDs were preincubated at 30°C for 120 min without inhibitor (control) or with 0.5 μg/ml pertussis toxin (PTX; A) or 100 μM genistein (B). Thereafter, samples were incubated at 37°C for 10 min without hormone (filled bars) or with 1 μM isoproterenol (shaded bars) before measurement of H-K-ATPase activity. N, no. of experiments. Values are means ± SE. Statistical comparison between groups was performed by ANOVA with Fisher’s PLSD test. **P < 0.005 vs. control groups (no isoproterenol).
DISCUSSION

In the present study, we determined the signaling pathways responsible for the stimulation of H-K-ATPase by isoproterenol in CCD β-intercalated cells (Fig. 5). Although the signalization of the effect of isoproterenol on H-K-ATPase in β-intercalated cells is different from that of calcitonin in α-intercalated cells, results indicate that the initial and terminal steps of H-K-ATPase stimulation are similar in the two cell types: the stimulation is initiated by the production of cAMP and finally results from the activation of ERKs. However, the coupling between cAMP production and activation of ERK is distinct in the two cell types.

As previously shown, ERK activation in α-intercalated cells is a PKA-independent process involving the cAMP-activated guanine nucleotide exchange factor Epac I, the monomeric G protein Rap-1 and the B-Raf kinase (16). In contrast, in β-intercalated cells, it is dependent on PKA (16) and is not related to the activation of Rap-1 and B-Raf (Fig. 2B, left and right), as previously reported in response to β-adrenergic agonists in HEK-293 cells (23). Instead, activation of ERK in β-intercalated cells relies on the stimulation of the classic Ras/Raf-1 pathway (Fig. 2A, left and right). Data from the literature indicate that activation of the monomeric G protein Ras is controlled by the guanine nucleotide exchange factor Sos. Activation of Sos may result either from activation of membrane receptor tyrosine kinases and the recruitment of Grb2 and Sos to the membrane or from a cascade of non-receptor tyrosine kinases (in particular, Pyk2 and Src) triggered by release of calcium in response to PLC activation by protein Gα-coupled receptors. Although activation of the Ras/Raf-1 pathway by isoproterenol in CCDs seemed dependent on tyrosine kinase activity, because it was abolished by genistein (Fig. 4B), it is unlikely to result from the two classic modes of activation of Ras because 1) isoproterenol binds to a G protein-coupled receptor and not to tyrosine kinase receptors; 2) isoproterenol does not activate phospholipase C in β-intercalated cells (Fig. 3); and 3) isoproterenol's effects on ERKs and H-K-ATPase rely on the activation of PKA (16).

An alternate mode of activation of Ras by β2-adrenergic receptors has been observed in HEK-293 cells (5) and in cardiomyocytes (32). This PKA-dependent pathway involves direct activation of the non-receptor tyrosine kinase Src, and hence of Sos and Ras, by the βγ-subunits of pertussis toxin-sensitive G proteins. Activation of this pathway requires phosphorylation of the βγ-adrenergic receptor by PKA, because it is blocked by H-89 or in the presence of a mutant receptor lacking the PKA phosphorylation site. It has been proposed that PKA phosphorylation of the receptor, known to induce its heterologous desensitization, also allows the receptor to switch its coupling from Gs to Gi, thereby triggering the release of βγ-subunits. That inhibition of PKA (16), Gi (Fig. 4A), and of tyrosine kinase activity (Fig. 4B) blocked the stimulation of H-K-ATPase by isoproterenol is consistent with the involvement of this signaling pathway in isoproterenol-induced stimulation of H-K-ATPase in β-intercalated cells of rat CCD. It is worth mentioning that PKA has been reported to inhibit the Ras/Raf-1-dependent activation of ERKs through either inhibition of Ras binding (31) or direct inhibition of Raf-1 kinase activity (18, 22). The absence of such an inhibition in β-intercalated cells may result from the different intracellular compartmentalization of PKA on one hand and of Ras/Raf-1 on the other, or from a difference in the time course of activation of PKA and Raf-1. According to the above-mentioned hypothesis, the Gα-to-Gi switch that promotes the activation of Ras and Raf-1 is accompanied by the desensitization of the isoproterenol receptor and therefore also promotes the deactivation of the cAMP-PKA pathway. Thus it is likely that Raf-1 activation may occur at a time when PKA is no longer activated.

Although a causal relationship between activation of ERK and stimulation of H-K-ATPase can be established, on the basis of the inhibition of isoproterenol-induced stimulation of H-K-ATPase by U-0126 (Fig.

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Fig. 5. Schematic pathway underlying the stimulation of H-K-ATPase activity by isoproterenol in β-intercalated cells of CCDs. AC, adenylyl cyclase; R, receptor.
Gs-to-Gi switch in receptor signalization in native kidney cells because it activates transcription factors through activation of transcription complexes, which translocate into the nucleus, where it controls the expression of specific genes through activation of transcription factors. However, the time course of isoproterenol-induced activation of H-K-ATPase is not compatible with de novo protein synthesis but suggests that phosphorylated ERKs may activate preexisting ATPase units. Several observations support the notion that isoproterenol may stimulate H-K-ATPase activity through exocytotic insertion into the plasma membrane of intracellular H-K-ATPase units: 1) in CCDs, H-K-ATPase colocalizes with H-ATPase, 2) another proton pump that is controlled through exocytosis/endoctyosis in CCDs (24); 3) ERKs control exocytosis in several cell types (14, 21, 29).

In conclusion, the present study provides original data regarding several aspects of protein Gs-coupled receptor signalization in native kidney cells because it characterizes H-K-ATPase as a cytosolic effector of ERK signalization and provides a new example of the Gs-to-Gi switch in β-adrenergic signalization in native cells.

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5. Daaka Y, Luttrell LM, and Lefkowitz RJ. Switching of the mechanism of ERK action has not been investigated. Phosphorylation of ERK is known to trigger its translocation into the nucleus, where it controls the expression of specific genes through activation of transcription factors. However, the time course of isoproterenol-induced activation of H-K-ATPase is not compatible with de novo protein synthesis but suggests that phosphorylated ERKs may activate preexisting ATPase units. Several observations support the notion that isoproterenol may stimulate H-K-ATPase activity through exocytotic insertion into the plasma membrane of intracellular H-K-ATPase units: 1) in CCDs, H-K-ATPase colocalizes with H-ATPase, 2) another proton pump that is controlled through exocytosis/endoctyosis in CCDs (24); 3) ERKs control exocytosis in several cell types (14, 21, 29).

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