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KT5926, a Potent and Selective Inhibitor of Myosin Light Chain Kinase

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SUMMARY

KT5926, (8*R**,9*S**,11*S**)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-14-*n*-propoxy-2,3,9,10-tetrahydro-8,11-epoxy,1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[*a*,*g*]cylcoocta[*cde*] trinden-1-one, was found to be a potent and selective inhibitor of myosin light chain kinase. The compound inhibited both Ca^{2+}/calmodulin-dependent and -independent smooth muscle myosin light chain kinases to a similar extent. The inhibition was not affected by the concentration of calmodulin. Kinetic analyses showed that the mode of inhibition was of the competitive type with respect to ATP (K_i, 18 nM) and of the noncompetitive type with respect to myosin light chain (K_i, 12 nM). These results indicated that KT5926 directly interacted with the enzyme at the catalytic site. KT5926 also inhibited other protein kinases, but with relatively high K_i values; the values for protein kinase C, cAMP-dependent protein kinase, and cGMP-dependent protein kinase were 723, 1200, and 158 nM, respectively. Ca^{2+}-ATPase, Na^+/K^+-ATPase, hexokinase, and 5′-nucleotidase were not inhibited by KT5926 at less than 10 μM. The effect of KT5926 on serotonin secretion and protein phosphorylation induced by platelet-activating factor or phorbol ester was examined in rabbit platelets. KT5926 inhibited the phosphorylation of a 20-kDa protein but had no effect on the phosphorylation of a 40-kDa protein, thereby indicating that the compound exerts its selective inhibition of myosin light chain kinase in intact cells. The compound inhibited serotonin secretion induced by platelet-activating factor, but its potency was significantly less than that of K-252a, (8*R**,9*S**,11*S**)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[*a*,*g*]cycloocta[*cde*]trinden-1-one, which inhibited the phosphorylation of both the 20-kDa protein and the 40-kDa protein. Phorbol ester-induced secretion was not suppressed by KT5926. These results provide the evidence that both the 20-kDa protein phosphorylation by myosin light chain kinase and the 40-kDa protein phosphorylation by protein kinase C substantially contribute to the secretion response in platelets.

Myosin light chain kinase appears to play a crucial role in the contraction of smooth muscle and the activation of non-muscle cells (for reviews, see Refs. 1 and 2). The kinase catalyzed the transfer of the γ-phosphate from ATP to the 20-kDa myosin light chain, and its activity completely depends on Ca^{2+}/calmodulin. The light chain phosphorylation is obligatory for the activation of actomyosin ATPase, a prerequisite for tension development, in both smooth muscle and non-muscle cells. Myosin light chain kinase has been isolated from many sources including smooth muscle, skeletal muscle, cardiac muscle, platelets, pancreas, and brain.

In platelets, physiological stimuli such as thrombin, collagen, and platelet-activating factor induce prominent increases in the phosphorylation of two proteins, with apparent molecular weights of 20,000 and 40,000. The 20-kDa protein has been identified as a regulatory light chain of platelet myosin and the phosphorylation is catalyzed by myosin light chain kinase (3). Although the function of the 40-kDa protein is unknown, protein kinase C is responsible for its phosphorylation (4). Activation of these two protein kinases in platelets occurs when physiological stimuli induce a rapid breakdown of inositol phospholipid to elevate cytosolic free calcium and to produce diacylglycerol. These two pathways, that is, free calcium elevation followed by myosin light chain kinase activation and diacylglycerol production followed by protein kinase C activation, are hypothesized to function synergistically for full activation of dense granule secretion (4–6). The hypothesis was based on the synergistic action of Ca^{2+} ionophores and phorbol esters, the direct activators of the respective pathways, in platelet activation. Another way to provide support for the hypothesis is utilization of specific inhibitors.

In previous papers (7, 8), we reported that K-252a, a novel metabolite isolated from *Nocardiopsis* sp., was a potent inhibitor of cyclic nucleotide-dependent protein kinases, protein kinase C, and myosin light chain kinase, with K_i values of ~20 nM. This compound seriously affected not only functions of various cells and tissues, such as platelets (9, 10), mast cells (11), neutrophils (11, 12), basophils (13). pheochlomocytoma PC12 cells (14), chick embryo dorsal root ganglion cells (15), and smooth muscle strips (16), but also responses of whole

ABBREVIATIONS: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

animals (17). In platelets, K-252a inhibited the phosphorylation of proteins and secretion of serotonin, and the cause-effect relationships between protein phosphorylation and serotonin secretion under several conditions were investigated by using this compound (9, 10).

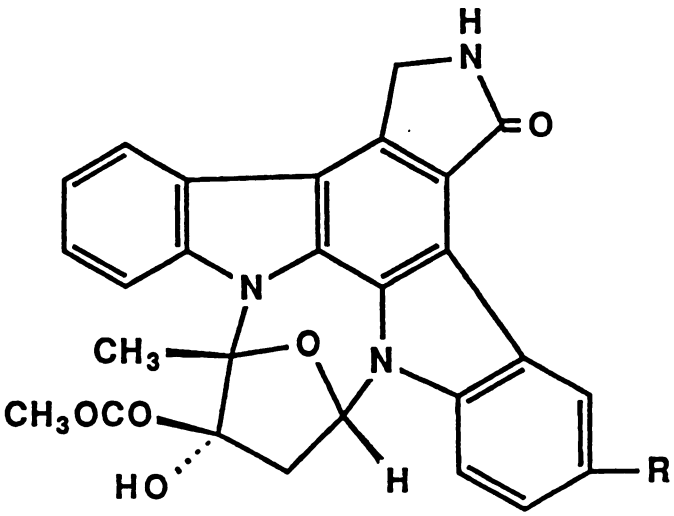
Although K-252a was shown to be a good tool to study the functions of a variety of protein kinases, the compound has intrinsic limitations in distinguishing the contribution of each protein kinase concerned, because it inhibits various protein kinases in a similar concentration range. Therefore, attempts were made to search for selective inhibitors among other K-252-related metabolites (18) and chemically synthesized derivatives. As a result, we have found that a new compound, KT5926, the 14-*n*-propoxy derivative of K-252a, is a potent and selective inhibitor of myosin light chain kinase. Utilizing KT5926 together with K-252a, we could study the specific function of myosin light chain kinase in various cells. In this paper, we report the biochemical properties, inhibition mechanism, and effects on platelet activation of KT5926. From these experiments, we provide a new line of evidence that both 20-kDa protein phosphorylation and 40-kDa protein phosphorylation are involved in serotonin secretion in rabbit platelets induced by platelet-activating factor.

Experimental Procedures

Reagents and Materials

Trypsin (type XIII, from bovine pancreas), trypsin inhibitor (type II-S, from soybean), histone H1 (type III-S, from calf thymus), histone IIA (type II-AS, from calf thymus), cAMP, cGMP, platelet-activating factor, Na^+/K^+-ATPase, and 5′-nucleotidase were purchased from Sigma Chemical Co.; hexokinase (from *Saccharomyces* sp.) and glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*) were from Toyobo Co., Ltd. (Japan); phosphatidylserine was from Serdary Research Laboratories; diolein and trifluoperazine were from Nacalai tesque Inc. (Japan); [γ-^{32}P]ATP was from Amersham Corp.; $^{32}P_i$ was from New England Nuclear. K-252a was isolated from the culture broth of *Nocardiopsis* sp. K-252, as described previously (19). KT5926 was prepared from K-252a as follows. Briefly, K-252a was acetylated with acetic anhydride and dimethylaminopyridine at the −NH and −OH positions, the acetate obtained was acetylated by a Friedel-Crafts reaction and oxidized by a Baeyer-Villiger reaction at the 14-position, the acetyl groups were removed by treatment with sodium methoxide, and the 14-hydroxy group introduced as above was *n*-propylated with sodium hydride and *n*-propyl iodide. The precise methods of the synthesis will be described elsewhere. The following spectroscopic data established the identity of the product as KT5926: mass spectrum, *m/z* 526 (M^+); NMR, δ (ppm in [^{2}H]dimethylsulfoxide) 1.07 (3 H, t, J = 8 Hz, C*H*$_3$), 1.72–2.24 (3 H, m, C*H*$_2$, *H*10), 2.16 (3 H, s, CC*H*$_3$), 2.90-3.40 (1H, m, *H*10), 3.94 (3 H, s, COOC*H*$_3$), 4.08 (2 H, t, *J* = 7 Hz, OC*H*$_2$), 5.04 (2 H, broad s, C*H*$_2$NHCO), 6.34 (1 H, s, O*H*), 7.00–7.24 (2 H, m, *H*11, aromatic), 7.32–7.60 (2 H, m, aromatic), 7.76–8.16 (3 H, m, aromatic), 8.60 (1 H, s, N*H*), 8.87 (1 H, d, *J* = 2 Hz, *H*15). The purity of KT5926 was estimated to be 99.2% by high pressure liquid chromatographic analysis (column, Nucleosil C_{18} 4.6 × 250 mm; solvent, 50% acetonitrile containing 0.1% trifluoroacetic acid; flow rate, 1.5 ml/min; temperature, 40°; detection, absorbance at 220 nm). The structures of K-252a and KT5926 are shown in Fig. 1. Other reagents were of analytical grade.

Myosin light chain kinase and mixed light chains from chicken gizzard and calmodulin from bovine brain were prepared as described previously (8). The partially purified protein kinase C (third Sephadex G-150 step) from rat brain, the partially purified holoenzyme of cAMP-dependent protein kinase type I (second DE-52 step) and its purified



K-252a R : H
KT5926 R : $OCH_2CH_2CH_3$

Fig. 1. Structures of K-252a and KT5926.

catalytic subunit from rabbit skeletal muscle, and cGMP-dependent protein kinase from pig lung were obtained as described (7). Calmodulin-dependent cyclic nucleotide phosphodiesterase was partially purified from bovine brain cortex as described (19). Ca^{2+}-ATPase was prepared from rabbit red blood cells as described by Ozaki *et al.* (20).

Trypsin treatment of myosin light chain kinase was carried out by the method of Ikebe *et al.* (21). Briefly, myosin light chain kinase (0.28 mg/ml) was incubated in a reaction mixture (0.1 ml) containing 25 mM Tris·HCl (pH 7.5), 1 mM EGTA, 400 mM KCl, and 69 μg/ml trypsin. After digestion for 20 min at 25°, the reaction was terminated by addition of 172 μl of 0.1 mg/ml trypsin inhibitor.

Enzyme Assay

Myosin light chain kinase. Myosin light chain kinase activity was assayed as described (8). The reaction mixture contained, in a final volume of 0.25 ml, 25 mM Tris·HCl (pH 7.5), 0.5 mg/ml bovine serum albumin, 4 mM $MgCl_2$, 0.5 mM $CaCl_2$, 8.4 nM calmodulin, 108 μg/ml mixed light chains, 5 μM [γ-^{32}P]ATP (100–900 cpm/pmol), and 0.11 μg/ml myosin light chain kinase. After a 3-min incubation, the reaction was started by addition of ATP and was performed for 5 min at 25°. In order to terminate the reaction, 0.5 volume of 20% trichloroacetic acid was added to the reaction mixture. The acid-precipitable materials were collected on a nitrocellulose membrane filter (Toyo Roshi Co., Ltd., Japan) and washed with four 1-ml aliquots of 5% trichloroacetic acid. The radioactivity on the filter was counted in a toluene scintillator fluid, using a Packard Tri-Carb liquid scintillation spectrometer (model 4530).

Protein kinase C. Protein kinase C activity was assayed as described (22). The reaction mixture contained, in a final volume of 0.25 ml, 20 mM Tris·HCl (pH 7.5), 200 μg/ml histone H1, 10 mM $Mg(CH_3COO)_2$, 2.5 mM EGTA, 2.5 mM $CaCl_2$, 80 μg/ml phosphatidylserine, 3.2 μg/ml diolein, 5 μM [γ-^{32}P]ATP (100–900 cpm/pmol), and 3.2 μg/ml enzyme. After a 3-min preincubation at 30°, reaction was performed for 3 min at 30° and terminated by addition of 1 ml of 25% trichloroacetic acid. The radioactivity incorporated into the acid-precipitated material was counted as described above.

cAMP dependent protein kinase. The activity of the holoenzyme of cAMP-dependent protein kinase was assayed under the conditions described (23), in a reaction mixture of final volume of 0.25 ml containing 40 mM phosphate buffer (pH 7.0), 200 μg/ml histone H1, 10

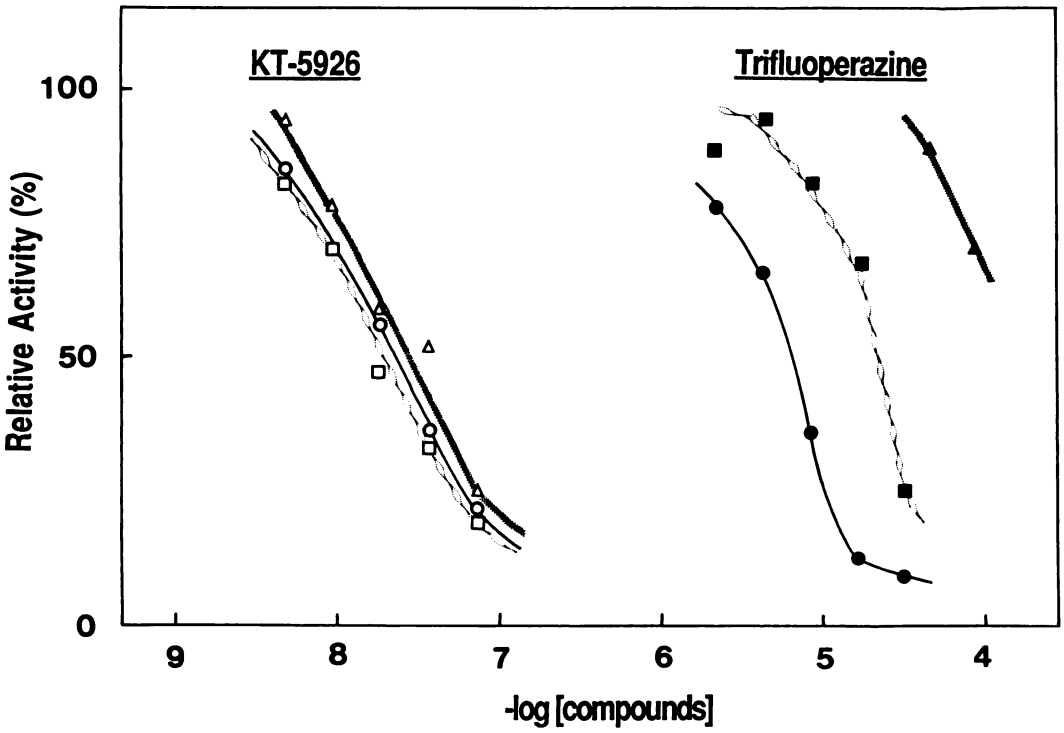


Fig. 2. Effects of KT5926 and trifluoperazine on chicken gizzard myosin light chain kinase. Myosin light chain was phosphorylated in the presence of KT5926 (*open symbols*) or trifluoperazine (*closed symbols*) by myosin light chain kinase (*circles* and *squares*) or by the trypsin-treated enzyme (*triangles*). Calmodulin concentration was 1.2 (*circles*) or 120 nM (*squares*). All other conditions were described in Experimental Procedures.

mM $Mg(CH_3COO)_2$, 3.2 μM cAMP, 5 μM [γ-^{32}P]ATP (100–900 cpm/pmol), 2 mM theophylline, and 538 μg/ml enzyme. Reaction was performed and the radioactivity incorporated into the acid-precipitable material was measured by the same method as that for protein kinase C.

cGMP-dependent protein kinase. The activity of cGMP-dependent protein kinase was assayed as described (24). The reaction mixture contained, in a final volume of 0.25 ml, 20 mM Tris·HCl (pH 7.5), 100 μg/ml histone IIA, 100 mM $Mg(CH_3COO)_2$, 0.1 μM cGMP, 10 μM [γ-^{32}P]ATP (100–900 cpm/pmol), and 10 μg/ml enzyme. Reaction was performed and the radioactivity incorporated into the acid-precipitable material was measured by the same method as that for protein kinase C.

Calmodulin-dependent cyclic nucleotide phosphodiesterase. The activity of calmodulin-dependent cyclic nucleotide phosphodiesterase was assayed as described previously (7); the reaction mixture contained, in a final volume of 0.5 ml, 80 mM imidazole·HCl (pH 6.9), 3 mM $MgSO_4$, 0.3 mM dithiothreitol, 100 mM NaCl, 1.2 mM cAMP, 50 μM $CaCl_2$, 60 nM calmodulin, and 100 μg/ml enzyme. After incubation for 30 min at 30°, the reaction was stopped by boiling for 5 min. Then, 6 μmol of $MnCl_2$ and a sufficient amount of 5′-nucleotidase was added, and the mixture was incubated for another 30 min at 30°. The reaction was terminated by the addition of 3 ml of perchloric acid, and the liberated inorganic phosphate was measured by the method of Ames (25).

Ca^{2+}-ATPase. The Ca^{2+}-ATPase assay was performed at 25° in a reaction mixture of a final volume of 1 ml containing 0.5 mg/ml erythrocyte membrane, 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 20 mM Tris-maleate (pH 6.8), 0. 1mM ouabain, 60 nM calmodulin, 20 mM EGTA, and 18.2 mM $CaCl_2$, as described (20). The reaction was started by the addition of 2 mM ATP and terminated by the addition of 5% trichloroacetic acid. The amount of inorganic phosphate liberated during a 30-min incubation was determined by the method of Martin and Doty (26).

Na^+/K^+-ATPase. Na^+/K^+-ATPase was assayed at 37° in a reaction mixture of a final volume of 1 ml containing 50 mM Tris·HCl (pH 7.6), 100 mM NaCl, 15 mM KCl, 5 mM $MgSO_4$, 2 mM EDTA, 25 μg/ml enzyme, and 2 mM ATP, as described (27). The reaction was started by the addition of ATP and terminated by the addition of 1 ml of 20% trichloroacetic acid. Inorganic phosphate liberated after 20 min of incubation was determined (26).

Hexokinase. Hexokinase was assayed by the method described (28); the rate of phosphorylation of glucose was measured, in a reaction mixture of a 9 final volume of 1 ml containing 50 mM Tris·HCl (pH 8.0), 111 mM glucose, 550 μM ATP, 226 μM NAD^+, 1.6 μg/ml glucose-6-phosphate dehydrogenase, and 0.10 μg/ml hexokinase at 30°, by observing the change in absorbance at 340 nm.

5′-Nucleotidase. 5′-Nucleotidase activity was assayed in a reaction mixture of a final volume of 0.75 ml containing 80 mM imidazole·HCl (pH 6.9), 3 mM $MgSO_4$, 0.3 mM dithiothreitol, 60 mM $MnCl_2$, 375 μM 5′-AMP, and 0.57 μg/ml enzyme. After incubation for 30 min at 30°, the reaction was terminated by the addition of 3 ml of 10% perchloric acid, and liberated inorganic phosphate was determined by the method of Ames (25).

Protein Phosphorylation and Serotonin Release in Platelets

Protein phosphorylation and serotonin secretion in platelets were measured as described previously (10). Blood anticoagulated with acid-citrate-dextrose was obtained from male rabbits and was centrifuged for the separation of platelet-rich plasma. The platelet-rich plasma was incubated with 0.1 mCi/ml $^{32}P_i$ for 90 min at 22°. The platelets were pelleted from the platelet-rich plasma by centrifugation at 600 × *g* for 10 min, washed twice, and suspended to 2 × 10^9 cells/ml in Tyrode's solution containing 138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 2.0 mM $MgCl_2$, and 5.5 mM glucose. After stabilization for 30 min, 1 mM $CaCl_2$ was added to the suspension. A suspension (1 × 10^9 cells/ml) of ^{32}P-labeled platelets was preincubated for 3 min at 37° with the indicated concentration of inhibitors, and then 10^{-9} M platelet-activating factor was added. After incubation for 30 sec, the phosphorylation reaction was terminated by addition of a solution containing final concentrations of 1.1% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, and 50 mM EDTA. The mixture was boiled for 2 min at 100° and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described by Laemmli (29). The gel was stained with Coomassie brilliant blue, dried, and exposed to Fuji X-ray film. The film was scanned at 430 nm by a Shimadzu dual wave length chromatogram scanner (model CS-930), to determine the relative intensity of each band.

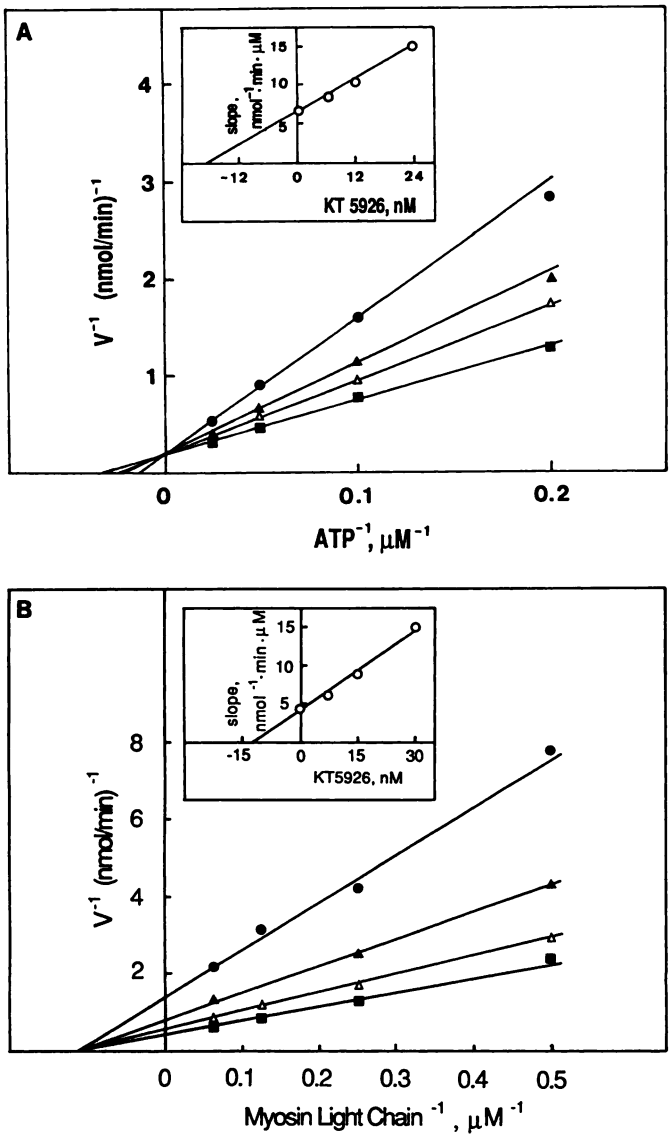


Fig. 3. Kinetic analyses of inhibition of chicken gizzard myosin light chain kinase by KT5926. The enzyme activity was assayed with ATP or myosin light chain as a variable substrate, as described in Experimental Procedures. A, Reciprocal velocity versus 1/[ATP] at 108 μg/ml mixed light chains. B, Reciprocal velocity versus 1/[myosin light chain] at 5 μM [γ-^{32}P]ATP. *Insets*, the secondary replots of the slopes of the lines as a function of KT5926 concentration.

TABLE 1

K_i values of K-252a and KT5926 for ATP with various protein kinases

The activities of protein kinases were assayed in the presence and absence of inhibitors, as described in Experimental Procedures. For determination of K_i values, 1.25, 2.5, 5.0, 10, 20, and 40 μM [γ-^{32}P]ATP was used as variable substrate, at a fixed concentration of phosphoryl-accepting substrates. The K_i values were calculated from the replots of double-reciprocal plots, as described in Fig. 3. K_i values are means from triplicate experiments and the data are representative of three such experiments made with different enzyme preparations.

Enzyme	K_i	
	K-252a	KT5926
	nM	
Myosin light chain kinase	20	18
Protein kinase C	25	723
cAMP-dependent protein kinase	18	1200
cGMP-dependent protein kinase	20	158

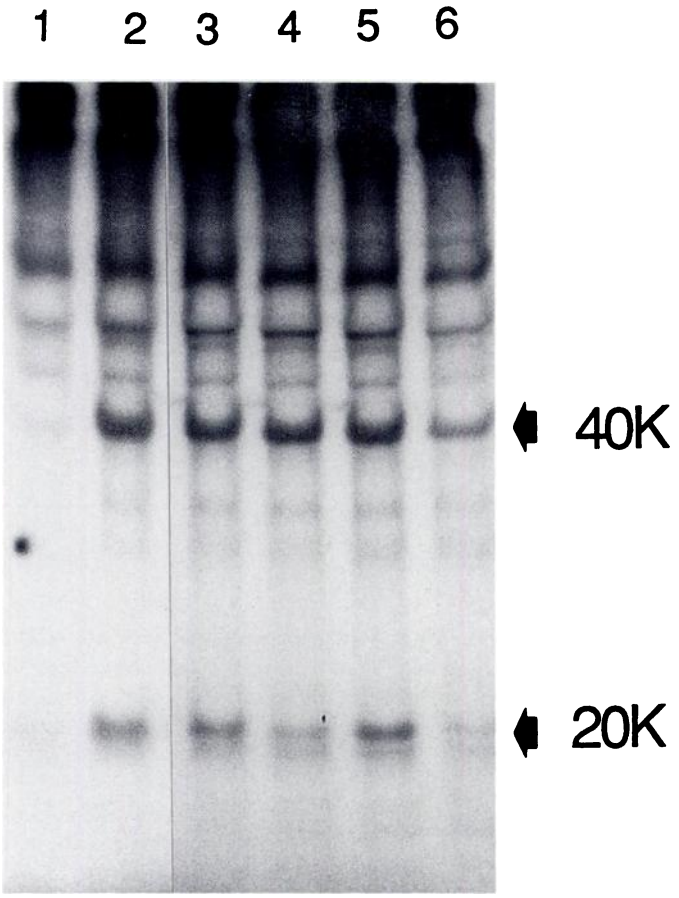


Fig. 4. Autoradiogram of phosphoproteins in platelet-activating factor-stimulated platelets. After preincubation with vehicle (dimethyl sulfoxide, *lanes 1* and *2*), 3 μM KT5926 (*lane 3*), 10 μM KT5926 (*lane 4*), 3 μM K-252a (*lane 5*), or 10 μM K-252a (*lane 6*), $^{32}P_i$-labeled platelets were stimulated by 0 M (*lane 1*) or 10^{-9} M platelet-activating factor (*lanes 2–6*). Phosphoproteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography, as described in Experimental Procedures.

[2-^{14}C]Serotonin-labeled platelets were prepared by the same method as described above, except that [2-^{14}C]serotonin (0.2 μCi) instead of $^{32}P_i$ was incubated with 10 ml of platelet-rich plasma for 60 min at 37°. After preincubation of a suspension (5×10^8 cells/ml) of radiolabeled platelets with inhibitors, platelets were treated with either 10^{-10} M platelet-activating factor for 3 min or 10^{-7} M TPA for 5 min. Serotonin secretion was terminated by the addition of ice-cold solution containing 0.1 mM formaldehyde and 5 mM EGTA. The mixture was centrifuged for 3 min at 1000 × *g*, and the radioactivity in the supernatant was counted with a liquid scintillation counter.

Protein Concentrations

Protein concentration of myosin light chain kinase and calmodulin were determined by UV absorption, with $E^{1\%}_{278\ nm}$ = 11.40 (30) and $E^{1\%}_{277\ nm}$ = 2.00 (31), respectively. Other protein concentrations were determined by the method of Bradford (32), using bovine serum albumin as a standard. Molarity of the 20-kDa light chain in mixed light chains was determined by measuring the radioactivity incorporated after complete phosphorylation.

Results

Inhibition of myosin light chain kinase by KT5926. KT5926 potently inhibited the activity of chicken gizzard myosin light chain (Fig. 2). The IC_{50} was 14 nM under the condi-

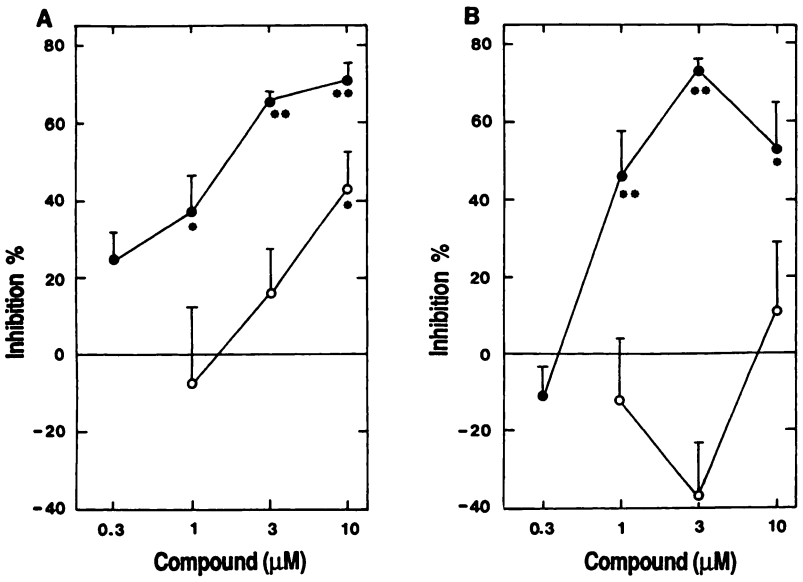


Fig. 5. Effects of KT5926 and K-252a on serotonin secretion in rabbit platelets. [2-^{14}C]Serotonin-labeled platelets were preincubated with the indicated concentrations of KT5926 (○) or K-252a (●) and stimulated by 10^{-10} M platelet-activating factor (A) or 10^{-7} M TPA (B). [2-^{14}C]Serotonin secretion was determined as described in Experimental Procedures. *Points* and *bars*, mean values and standard errors, respectively, $^{*}P < 0.05$, $^{**}p < 0.01$.

tions described in Experimental Procedures. The inhibitory potency of the compound is very much higher than that of trifluoperazine, a well known calmodulin inhibitor. As shown in Fig. 2, the inhibition curve of trifluoperazine was shifted rightward by calmodulin added at a 100-fold higher concentration. Under the same conditions, the inhibition curve of KT5926 was not affected at all. The catalytic domain generated by tryptic digestion of myosin light chain kinase was inhibited by KT5926 with a concentration dependency similar to that of the native enzyme (Fig. 2). The activity of this catalytic domain was independent of Ca^{2+}/calmodulin and was not inhibited by trifluoperazine, even at 30 μM (Fig. 2). These data indicated that the compound acted on the catalytic domain of myosin light chain kinase but not on calmodulin or on the regulatory domain of the enzyme.

Inhibition mechanism of myosin light chain kinase by KT5926. We next tried to clarify the precise inhibition mechanism of KT5926 by kinetic analyses. The IC_{50} value was remarkably affected by ATP concentration; the values were 14, 22, 29, 52, 72, and 390 nM in the presence of 5, 10, 20, 40, 200, and 1600 μM ATP, respectively. Double-reciprocal plot of ATP concentration versus reaction rate showed that the inhibition was competitive with respect to ATP (Fig. 3A). The K_i value estimated from the secondary replot was 18 nM (Fig. 3A, *inset*). Alternatively, KT5926 inhibited the enzyme noncompetitively with respect to the myosin light chain (Fig. 3B). The K_i value for the myosin light chain was 12 nM.

Selectivity of KT5926. The K_i value of KT5926 for myosin light chain kinase was comparable to that of K-252a. However, unlike K-252a, which inhibited various protein kinases to a similar degree, KT5926 had significantly decreased inhibitory potencies for other protein kinases. The inhibition mode of the compound for other protein kinases was also competitive with respect to ATP (data not shown). The K_i values for cAMP-dependent protein kinase, cGMP-dependent protein kinase, and protein kinase C were 1200, 158, and 723 nM, respectively (Table 1).

K-252a has been shown to inhibit the calmodulin-dependent activity of cyclic nucleotide phosphodiesterase, with an IC_{50} value of 2.9 μM,. and not inhibit the independent activity (19). The inhibition was antagonized by calmodulin (33). In contrast, KT5926 had no effect on the calmodulin-dependent activity, even at 10 μM. Furthermore, Na^+/K^+-ATPase, hexokinase, 5′-nucleotidase, and calmodulin-dependent Ca^{2+}-ATPase were not inhibited by the compound at less than 10 μM. These results suggest that KT5926 is a selective inhibitor of myosin light chain kinase, with no calmodulin-inhibiting activity.

Inhibition of protein phosphorylation in intact platelets. In order to examine whether KT5926 exerts its effect in intact cell systems, the effect of the compound on protein phosphorylation in rabbit platelets was investigated. As shown in Fig. 4, the phosphorylation of the 20- and 40-kDa proteins is markedly increased in platelets stimulated by platelet-activating factor in the presence of extracellular Ca^{2+} (Fig. 4, *lane 2*). K-252a inhibited the phosphorylation of both proteins significantly, although the inhibition of 40-kDa protein phosphorylation was much weaker than that of 20-kDa protein phosphorylation (Fig. 4, *lanes 5* and *6*). From the experiments of densitometric scanning of the gel, the inhibition of the phosphorylation by 3 and 10 μM K-252a was estimated to be 18 and 87%, respectively, for the 20-kDa protein, and 7 and 37%, respectively, for the 40-kDa protein. KT5926 inhibited the 20-kDa protein phosphorylation with potency comparable to that of K-252a; the percentage of inhibition at 3 and 10 μM was 19 and 64%, respectively. However, in contrast to K-252a, KT5926 showed no inhibition toward 40-kDa protein phosphorylation up to 10 μM (Fig. 4, *lanes 3* and *4*). Those findings indicated that KT5926 showed selectivity for myosin light chain kinase in intact platelets.

Inhibition of serotonin secretion in platelets. The effect of KT5926 on secretion response in platelets was examined under two different conditions, 1) TPA-stimulated serotonin secretion, where the 40-kDa protein phosphorylation was markedly elevated and no increase in the 20-kDa protein phosphorylation was observed (9), and 2) platelet-activating factor-induced serotonin secretion in Ca^{2+}-containing medium, where both 20- and 40-kDa proteins were prominently phosphorylated (Fig. 4). K-252a at 1–10 μM inhibited platelet-activating factor-induced serotonin secretion in a dose-dependent manner (Fig. 5A). The inhibition by K-252a at 1, 3, and 10 μM was 38, 66,

and 71%, respectively. KT5926 also inhibited the secretion response, but its potency was significantly lower than that of K-252a at any concentration examined. As shown in Fig. 5A, KT5926 at 1 μM showed no effects on the secretion response, and at 3 and 10 μM inhibited the response by 16 and 43%, respectively (Fig. 5A). The inhibition occurred within the concentration range for inhibition of the 20-kDa protein phosphorylation with no effect on the 40-kDa protein phosphorylation (Fig. 4). TPA-induced serotonin secretion was inhibited by K-252a at 1–10 μM (Fig. 5B), whereas KT5926 at 1–10 μM had no effect on this secretion response (Fig. 5B).

Discussion

KT5926 appears to inhibit myosin light chain kinase by interacting with the ATP-binding site in the catalytic domain. The following evidence supported this inhibition mechanism. 1) Inhibition by KT5926 was not recovered by higher concentrations of calmodulin (Fig. 2), 2) the compound inhibited the catalytic domain obtained by partial tryptic digestion of the enzyme with the same potency as the native enzyme (Fig. 2), and 3) the mode of inhibition was competitive with respect to ATP and noncompetitive to the myosin light chain (Fig. 3). KT5926 shares this inhibition mechanism with the lead compound, K-252a (8). Although the 14-*n*-propoxylation of K-252a caused no change in inhibitory potency for myosin light chain kinase, it markedly reduced the potencies not only for other protein kinases (Table 1) but also for calmodulin. Various compounds that inhibit myosin light chain kinase by antagonizing calmodulin have been reported (for review, see Ref. 34), but few compounds are known to inhibit the kinase by acting on the enzyme itself and, in addition, selectively. Hidaka and his co-workers (35) synthesized 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine hydrochloride (ML-9), which selectively inhibited myosin light chain kinase by competing with ATP (K_i, 3.8 μM). KT5926 shares the mode of inhibition with ML-9 but its affinity for myosin light chain kinase is much higher (K_i, 18 nM).

The selectivity of KT5926 for myosin light chain kinase was demonstrated also in intact platelets; the compound inhibited the phosphorylation of the 20-kDa protein but not the 40-kDa protein in rabbit platelets stimulated by platelet-activating factor (Fig. 3). In recent years, Naka *et al.* (36, 37) have reported that, in platelets stimulated by phorbol ester or thrombin, the 20-kDa light chain was phosphorylated also by protein kinase C at a site different from that phosphorylated by myosin light chain kinase. At least under our experimental conditions, the inhibition of 20-kDa protein phosphorylation by KT5926 may be due to the inhibition of myosin light chain kinase, because KT5926 had no effect on the 40-kDa protein phosphorylation and, therefore, on other protein phosphorylation mediated by protein kinase C.

The concentration of KT5926 required for effective inhibition in intact cells was very much higher than the K_i value for chicken smooth muscle myosin light chain kinase measured *in vitro*. In rabbit platelets, the compound inhibited the 20-kDa protein phosphorylation and serotonin secretion at 3–10 μM (Figs. 2 and 3) and, in rabbit aorta, it inhibited the contraction evoked by KCl or noradrenalin at 0.3–3 μM.[1] Although a permeability barrier or difference in enzyme species should be taken into consideration, the main cause is that a high intracellular content (~10^{-3} M) of ATP reversed the inhibition of KT5926, e.g., at 1.6 mM ATP, its IC_{50} value for the chicken gizzard enzyme *in vitro* increased at 0.39 μM. An additional factor is the relatively high concentration of myosin light chain kinase in platelets (micromolar), compared with the concentration used *in vitro* (nanomolar).

In previous papers (9, 10), we reported the correlation of protein phosphorylation with serotonin secretion in platelets, using K-252a under various experimental conditions. TPA (9) or platelet-activating factor in Ca^{2+}-free medium (10) induced an increase of only the 40-kDa protein phosphorylation, to release small amounts of serotonin. K-252a concomitantly inhibited the 40-kDa protein phosphorylation and serotonin secretion, suggesting that the phosphorylation is a prerequisite for serotonin secretion. The serotonin secretion induced by TPA was not inhibited by KT5926, probably because of the lack in inhibitory effects of KT5926 on the 40-kDa protein phosphorylation. When platelets were stimulated by platelet-activating factor in Ca^{2+}-containing medium, a prominent increase in the phosphorylation of both the 20-kDa protein and the 40-kDa protein was accompanied by secretion of large amounts of serotonin (10). Under such conditions, K-252a inhibited the 20-kDa protein phosphorylation preferentially, and this inhibition was closely correlated with the inhibition of serotonin release (Figs. 4 and 5A) (10). However, the 40-kDa protein phosphorylation was inhibited by K-252a only weakly. Based on these results, we proposed in the previous paper that K-252a inhibited the serotonin release mainly due to the inhibition of the 20-kDa protein phosphorylation. Thus, the real function of the 40-kDa protein phosphorylation in dense granule secretion remained unclear. In this paper, we have found that KT5926 at 3 and 10 μM inhibited both serotonin secretion and the 20-kDa protein phosphorylation, but not the 40-kDa protein phosphorylation, when platelets were stimulated by platelet-activating factors in the presence of extracellular Ca^{2+} (Figs. 4 and 5). These data confirmed a crucial role of the 20-kDa protein phosphorylation in the secretion response. In addition, the results provide evidence on the function of the 40-kDa protein phosphorylation in serotonin secretion. KT5926 inhibited the 20-kDa protein phosphorylation with potency comparable to that of K-252a (Fig. 4), but its potency to inhibit serotonin release was significantly lower than that of K-252a at any concentration examined. The low efficacy of KT5926 in the suppression of the secretion response is apparently brought about by the lack of inhibition of the 40-kDa protein phosphorylation (Fig. 4). These findings indicate that the 40-kDa protein phosphorylation substantially contributes to the secretion response. Furthermore, the results may support the hypothesis (4) of synergistic action of the 20-kDa protein and 40-kDa protein phosphorylation in serotonin secretion.

In conclusion, we have found KT5926 to be a potent and selective inhibitor of myosin light chain kinase. The compound should be an excellent tool to study specific function of myosin light chain kinase and provide further insight into the function of the enzyme.

[1] Unpublished data.

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