

K-252a, a Potent Protein Kinase Inhibitor, Blocks Nerve Growth Factor–induced Neurite Outgrowth and Changes in the Phosphorylation of Proteins in PC12h Cells

Seiichi Hashimoto

Department of Biochemistry, Tohoku Dental University, Koriyama 963, Japan

Abstract. Nerve growth factor (NGF) promotes neuronal differentiation of PC12 pheochromocytoma cells. One of the most prominent and distinguishing features of neuronal differentiation is neurite outgrowth. The mechanism by which NGF causes the cells to elaborate neurites is unknown. This study shows that K-252a, a potent protein kinase inhibitor, blocks NGF-induced neurite outgrowth and the changes in protein phosphorylation elicited by NGF. In the experiment with intact cells phosphorylated with ^{32}P -orthophosphoric acid, an exposure of PC12h cells to NGF (50 ng/ml) caused an increase in the phosphorylation of tyrosine hydroxylase and a 35,000-D protein and a decrease in a 36,500-D protein. Pretreatment of PC12h cells with K-252a (100 nM) inhibited the effects of NGF on the phosphorylation of these three proteins. In the phosphorylation of cell-free extracts with [γ - ^{32}P] ATP, treatment of PC12h cells with NGF (50 ng/ml) caused a decrease in the phosphorylation of Nsp100. Pretreatment of the cells with K-252a (30 nM) almost completely blocked the NGF effect on the phosphorylation of Nsp100 elicited by subsequent treatment of the cells with NGF. Treatment of PC12h cells with NGF promoted outgrowth of neurites. The addition of

K-252a (100 nM) into the culture almost completely blocked the generation of neurites elicited by NGF. Earlier studies demonstrated that NGF-induced neurite outgrowth in PC12 cells involves at least two components: the first of these is transcription-dependent and the second is transcription-independent. To determine the component on which K-252a acts, experiments were carried out on NGF-induced priming or regeneration of neurites. When K-252a was present in the priming step, NGF induced only actinomycin D-sensitive neurites, showing that K-252a interferes with the transcription-dependent actions of NGF. When already primed cells were treated with NGF, actinomycin D-resistant neurites were formed and these were blocked by K-252a, showing that the inhibitor interferes with the transcription-independent actions of NGF as well. Although the exact mechanism of inhibition of NGF-promoted neurite formation by K-252a is unknown, the most probable explanation is that both transcription-dependent and -independent components are involved in at least one step of the activation of some specific protein kinase(s) that can be suppressed by K-252a.

NERVE growth factor (NGF)¹ is a polypeptide required for the survival and development of sympathetic and sensory nervous systems (9, 21, 29). The rat pheochromocytoma cell line PC12 has become the premier model for studying the mechanism of action of NGF. NGF promotes neuronal differentiation of PC12 cells. That is, PC12 cells respond to NGF over a period of days by their biochemical and morphological conversion from chromaffin-like cells to those with a sympathetic neuron-like phenotype that includes neurite outgrowth, electrical excitability, and cessation of cell growth (6, 10). These actions are initiated by the binding of NGF to its specific receptors on the surface of

PC12 cells. Although the exact mechanism of signal transduction after the interaction of NGF with its receptor is unknown, some kind of second messenger system must be involved (16).

The regulation of intracellular events by extracellular signals often involves reversible covalent modification of proteins. One of the most notable modifications is protein phosphorylation. Indeed, NGF regulates the intracellular phosphorylation of several proteins. These proteins include tyrosine hydroxylase (12), a 30,000-D nuclear protein (slow-migrating protein [SMP]; 25, 33), a 35,000-D insoluble protein (14), a 100,000-D nerve growth factor-sensitive protein (Nsp100; 7, 8), the ribosomal protein S6 (12, 23), and a 250,000-D cytoskeletal protein (20). With respect to these actions of NGF, it has also been reported that in PC12 cells NGF alters

1. *Abbreviations used in this paper:* dBcAMP, N,2'-dibutyryladenine 3',5'-cyclic monophosphate; NGF, nerve growth factor; Nsp, nerve growth factor-sensitive protein; SMP, slow-migrating protein.

the activity of the following kinases: Nsp100 kinase (30), SMP kinase (25), NGF-sensitive S6 kinase (22), kinase N (27), cAMP-dependent protein kinase (5, 12, 24), and Ca²⁺/phospholipid-dependent protein kinase (C kinase; 5, 13). It appears that a variety of biochemical and morphological changes accompanied by the NGF-induced neuronal differentiation is mediated at least in part by such modulation of protein phosphorylation.

It has been reported that K-252a, a microbial alkaloid isolated from the culture broth of *Nocardia* species, is a potent inhibitor of C kinase and cyclic nucleotide-dependent protein kinases. This compound inhibits these protein kinases competitively with respect to ATP (17). Therefore, it was expected that this kinase inhibitor would provide a useful tool for studies on changes in the phosphorylation of proteins and on other biological actions elicited by NGF. In this study, it is shown that K-252a blocks the NGF-mediated changes in the phosphorylation of proteins and neurite outgrowth.

Materials and Methods

Materials

NGF (2.5 s) was prepared by the procedure of Bocchini and Angeletti (1). K-252a was purchased from Kyowa Medex Co., Ltd. (Tokyo, Japan). N₂,2'-dibutyryladenine 3',5'-cyclic monophosphate (dBcAMP) was purchased from Sigma Chemical Co. (St. Louis, MO). Actinomycin D was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). ³²P-orthophosphoric acid and [γ -³²P] ATP were obtained from New England Nuclear (Boston, MA). SDS-PAGE molecular mass standards were from Bio-Rad Laboratories (Cambridge, MA).

Cell Culture

PC12 subclone h cells (15) were grown in DME supplemented with 7% FBS and 7% horse serum. They were kept in a humidified atmosphere containing 95% air and 5% CO₂ in 75-cm² tissue culture flasks or 35-mm tissue culture dishes at 37°C. The cells were subcultured once a week at a 1:6 or 1:8 split ratio, and the culture medium was changed once a week.

Phosphorylation in Intact Cells

The phosphorylation was accomplished by the incubation of PC12h cells with ³²P-orthophosphoric acid at a concentration of 100 μ Ci/ml for 2 h. NGF treatment was carried out by incubating the cells with NGF for the final 1 h of the 2-h labeling period. When used, K-252a was added to the culture 15 min before NGF addition. After the incubation, the medium was removed and cells were collected and washed with buffer A (composed of 0.32 M sucrose containing 3 mM CaCl₂, 1 mM MgCl₂, and 1 mM sodium phosphate, pH 6.5). Then the cells were suspended in 1 ml of lysis buffer composed of 0.24 M sucrose, 20 mM Na₂MoO₄, 5 mM ethylenediaminetetraacetate, and 10 mM sodium phosphate (pH 6.5), containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.2% Triton X-100, and left on ice for 15 min. The lysates were centrifuged at 1,500 g for 10 min to remove the crude nuclear fraction. The supernatants were then centrifuged at 100,000 g for 60 min to separate the soluble fraction from the insoluble fraction. The final supernatants and the insoluble pellets were, respectively, diluted with or dissolved in 1 ml of SDS sample buffer, then heated in a boiling water bath for 5 min.

Normalization of Protein-bound Radioactivity

The protein-bound counts of ³²P in each sample were measured by spotting portions of the sample 1.5 cm above the bottom edge of small strips (12 \times 1.5 cm) of a filter paper (Toyo No. 526) and chromatographing the strips in an ascending fashion for 10 min in 25% TCA. The strips were dried and the portions containing the origins were counted in Atomlight scintillation fluid (New England Nuclear, Boston, MA).

Phosphorylation in Cell-free Extracts

Culture medium was removed and PC12h cells were washed with, and then incubated in, DME without serum. The cells were treated with NGF for 3 h at a concentration of 50 ng/ml in the culture flasks. Pretreatment of PC12h cells with K-252a was carried out by the addition of K-252a at concentrations from 3 to 100 nM 1 h before NGF addition. At the end of the experiment, the medium was removed and the cells were collected and washed with buffer A. The cell pellets were taken up in 700 μ l to 1 ml of sonication buffer consisting of 100 mM Pipes, pH 6.9, 1 mM MgSO₄, 0.1 mM Na₂MoO₄, 10 mM EGTA, and 1 mM PMSF. The cell suspension was sonicated for 30 s (sonifier model No. 200; Branson Sonic Power Co., Danbury, CT) and centrifuged at 100,000 g for 60 min. The supernatant fractions were stored frozen at -80°C until assayed. The supernatants were diluted to an equal concentration of protein and the phosphorylation was carried out as described previously (8, 14). Protein determinations were done by the method of Bradford (2). The incubation mixture contained 50 mM 2-(N-morpholino)ethanesulfonic acid (MES; pH 6.2), 10 mM MgSO₄, 0.1 mM Na₂MoO₄, and 2-4 μ Ci of [γ -³²P]ATP in a final volume of 200 μ l. The incubations were carried out at 37°C for 10 min and terminated by the addition of 200 μ l of SDS sample buffer. After being boiled, the samples were subjected to SDS-PAGE.

PAGE

SDS-PAGE was performed at 150 V for 5-6 h using 10% or 7.5% acrylamide slab gel containing 0.1% SDS (19). Gels were stained overnight with Coomassie Blue staining solution (0.25% Coomassie Blue in 9% acetic acid and 45% ethanol) and destained initially with 42% ethanol and 7% acetic acid and finally with 7% acetic acid.

Autoradiography

The slab gels were dried in vacuo and exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY) at room temperature. After an appropriate period of time, the film was processed using standard procedures. Autoradiograms were scanned using a Shimadzu CS-900, and the areas under the relevant peaks were measured.

Results

Effect of Pretreatment with K-252a on the Action of NGF or of Dibutyryl cAMP in the Phosphorylation of Proteins in Intact PC12h Cells

The incubation of PC12h cells with ³²P-orthophosphoric acid led to phosphorylation of a number of proteins, including three with apparent molecular masses of 60,000, 36,500, and 35,000 D. Treatment of PC12h cells with NGF (50 ng/ml) in culture caused an increase in the incorporation of ³²P into the 60,000- and 35,000-D proteins, and a decrease in the 36,500-D protein. Of these, the 60,000-D protein has been identified as tyrosine hydroxylase (12, 14). Treatment of PC12h cells with K-252a produced no marked alterations in the phosphorylation of proteins except tyrosine hydroxylase. The phosphorylation of this enzyme was reduced by the treatment with K-252a at concentrations >300 nM in a dose-dependent manner (Figs. 1 A and 2 A, and Table I). Next, the effect of pretreatment with K-252a on NGF-mediated changes in the phosphorylation of proteins in PC12h cells was examined. Pretreatment of PC12h cells with K-252a inhibited the effects of NGF on the phosphorylation of these proteins. The inhibition was observed when the cells were treated with K-252a at concentrations >100 nM, and almost complete inhibition was obtained at 1 μ M (Fig. 1, A and B, and Table I). On the other hand, treatment of PC12h cells with dibutyryl cAMP (300 μ M) also caused an increase in

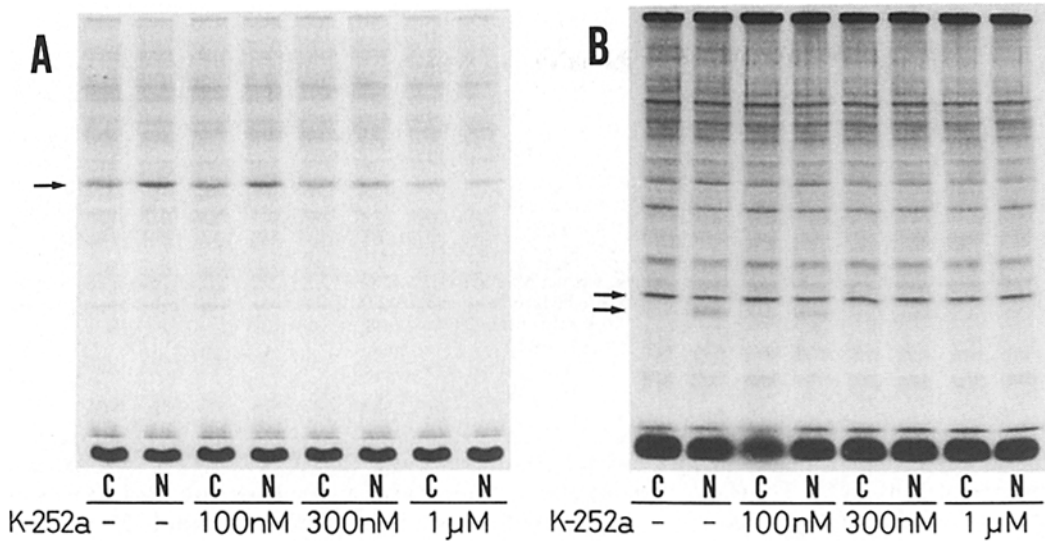


Figure 1. Effect of pretreatment with K-252a on the action of NGF in the phosphorylation of proteins in intact PC12h cells. PC12h cells were labeled with ^{32}P -orthophosphoric acid (100 $\mu\text{Ci}/\text{ml}$) in DME supplemented with 7% FBS and 7% horse serum for 120 min. **A** and **B** are autoradiograms from SDS-PAGE (10%) of respective 0.2% Triton X-100 detergent-soluble and -insoluble fractions from PC12h cells treated with control culture medium for the 120-min labeling period (C) or with NGF (50 ng/ml) for the last 60 min of the 120-min labeling period (N). As indicated, other similar cultures received various concentrations of K-252a (100 nM, 300 nM, or 1 μM) that was present during the last 75 min of the 120-min labeling period. In **A**, the arrow indicates the position of the 60,000-D protein. The relative densities of the 60,000-D protein band were as follows: (C) 100; (N) 140; (100 nM [C]) 104; (100 nM [N]) 125; (300 nM [C]) 88; (300 nM [N]) 98; (1 μM [C]) 70; (1 μM [N]) 62. In **B**, the upper and lower arrows indicate the position of 36,500- and 35,000-D proteins, respectively. The relative densities of the 36,500-D protein band were as follows: (C) 100; (N) 75; (100 nM [C]) 96; (100 nM [N]) 87; (300 nM [C]) 99; (300 nM [N]) 97; (1 μM [C]) 94; (1 μM [N]) 96. The relative densities of the 35,000-D protein band were as follows: (C) 100; (N) 320; (100 nM [C]) 100; (100 nM [N]) 220; (300 nM [C]) 80; (300 nM [N]) 120; (1 μM [C]) 80; (1 μM [N]) 80. (Values for the 35,000-D protein bands were rounded off since the density of the band for this protein from PC12h cells incubated in control culture medium was very low.)

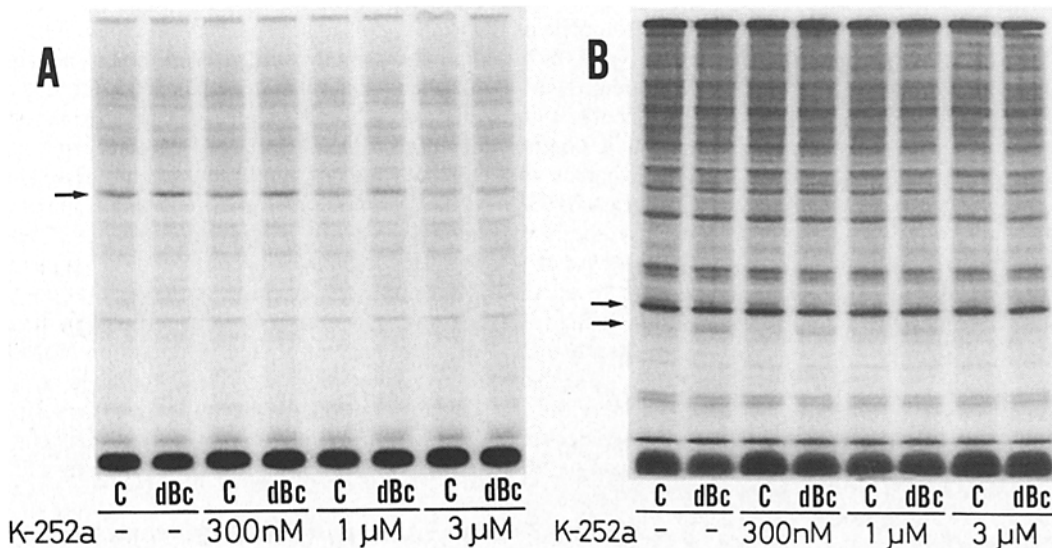


Figure 2. Effect of pretreatment with K-252a on the action of dibutyryl cAMP in the phosphorylation of proteins in intact PC12h cells. PC12h cells were labeled with ^{32}P -orthophosphoric acid in DME supplemented with 7% FBS and 7% horse serum for 120 min. **A** and **B** are autoradiograms from SDS-PAGE (10%) of respective 0.2% Triton X-100 detergent-soluble and -insoluble fractions from PC12h cells treated with control culture medium for the 120-min labeling period (C) or with dBcAMP (300 μM) for the last 60 min of the labeling period (dBc). As indicated, other similar cultures received various concentrations of K-252a (300 nM, 1 μM , or 3 μM) that were present during the last 75 min of the 120-min labeling period. In **A**, the arrow indicates the position of the 60,000-D protein. The relative densities of the 60,000-D protein band were as follows: (C) 100; (dBc) 113; (300 nM [C]) 87; (300 nM [dBc]) 102; (1 μM [C]) 69; (1 μM [dBc]) 81; (3 μM [C]) 49; (3 μM [dBc]) 54. In **B**, the upper and lower arrows indicate the position of 36,500- and 35,000-D proteins, respectively. The relative densities of the 36,500-D protein band were as follows: (C) 100; (dBc) 91; (300 nM [C]) 102; (300 nM [dBc]) 90; (1 μM [C]) 99; (1 μM [dBc]) 90; (3 μM [C]) 105; (3 μM [dBc]) 92. The relative densities of the 35,000-D protein band were as follows: (C) 100; (dBc) 210; (300 nM [C]) 100; (300 nM [dBc]) 180; (1 μM [C]) 100; (1 μM [dBc]) 130; (3 μM [C]) 90; (3 μM [dBc]) 100. (Values for these densities were rounded off as in the legend of Fig. 1.)

Table I. Phosphorylation of Proteins Extracted from PC12h Cells

Protein	Pretreatment with K-252a							
	None (n = 7)		100 nM (n = 3)		300 nM (n = 3)		1,000 nM (n = 4)	
	control	NGF	control	NGF	control	NGF	control	NGF
60,000-D	100	152 ± 29	102 ± 9	134 ± 16	83 ± 8	94 ± 3	75 ± 14	71 ± 10
36,500-D	100	77 ± 9	97 ± 5	86 ± 6	99 ± 7	95 ± 9	99 ± 8	98 ± 5
35,000-D	100	261 ± 44	103 ± 6	183 ± 32	93 ± 12	110 ± 10	90 ± 8	90 ± 14

PC12h cells were pretreated with various concentrations of K-252a, then treated with or without NGF at a concentration of 50 ng/ml. Relative density of 60,000-, 36,500-, and 35,000-D protein bands were measured by densitometry (see Materials and Methods) of autoradiograms, and are expressed as a percentage of untreated control values taken as 100%. The values represent the averages ± SD of 7, 4, or 3 determinations, as indicated.

the incorporation of ³²P into tyrosine hydroxylase and a protein that migrated at a position of an apparent molecular mass of 35,000 D on an SDS gel (Fig. 2, A and B). The effect of dibutyryl cAMP on the phosphorylation of the 36,500-D protein was not clear. Although the pretreatment of the cells with K-252a also inhibited the effects of dibutyryl cAMP on the phosphorylation of these proteins, much higher concentrations of the inhibitor were required (Fig. 2, A and B).

Effect of Pretreatment with K-252a on Subsequent Cell-free Phosphorylation of Soluble Proteins from Control PC12h Cells or Cells Treated with NGF

The incubation of soluble extracts from PC12h cells with [γ -³²P]ATP led to phosphorylation of a number of proteins, including one with an apparent molecular mass of 100,000 D,

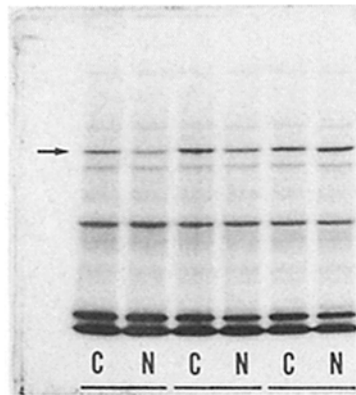
designated in earlier studies as Nsp100 (7, 8). In extracts from NGF-treated cells, phosphorylation of Nsp100 was markedly and selectively reduced, as described previously (14). In contrast, treatment of the cells with K-252a caused an increase in the phosphorylation of Nsp100 during the subsequent incubation of the extracts with [γ -³²P] ATP. Pretreatment of the cells with K-252a prevented the NGF effect on the phosphorylation of Nsp100 at concentrations >3–10 nM, and almost complete inhibition was obtained at 30 nM (Fig. 3).

Effect of K-252a on NGF-promoted Neurite Outgrowth

When PC12h cells were cultured with NGF (50 ng/ml), the generation of neurites proceeded with a lag time of >24 h. NGF-promoted neurite outgrowth was inhibited by coaddition of K-252a into the culture. The inhibition was clearly observed when the cells were cultured with NGF in the presence of K-252a at concentrations >30 nM and was almost complete at 100 nM (Fig. 4). Thus, the protein kinase inhibitor K-252a completely inhibited NGF-promoted neurite outgrowth. It has been demonstrated that NGF-promoted neurite outgrowth in PC12 cells involves at least two separable components: one of these requires RNA transcription, whereas the other is transcription independent (3). The transcription-dependent component may lead to the specific synthesis and accumulation of materials required for outgrowth of neurites. Accumulation of such materials appears to be necessary but not sufficient to promote generation of neurites (3). The transcription-independent component must be activated to promote generation and regeneration of neurites in the respective naive cells and NGF-pretreated cells. Therefore, the effect of K-252a on the respective transcription-dependent and -independent components in the mechanism of NGF-promoted neurite outgrowth was investigated.

Effect of K-252a on NGF-promoted Regeneration of Neurites from NGF-primed Cells

After the exposure of PC12h cells to NGF (50 ng/ml) for 8 d, the cells were triturated to shear off their neurites, then were subcultured with NGF. Most of cell clumps regenerated neurites within 24 h (Fig. 5 B). This regeneration of neurites was inhibited by the presence of K-252a in the culture medium in a dose-dependent manner, and almost complete inhibition was obtained at 100 nM (Fig. 5, D, E, and F). The measurement of neurite-bearing clumps was carried out by the counting of clumps with neuritic processes longer than twice the cell diameter. A minimum of 115 clumps per cul-



K-252a — — 3nM 30nM

Figure 3. Effect of pretreatment with K-252a on subsequent cell-free phosphorylation of soluble proteins from control PC12h cells or cells treated with NGF. Cells were treated with control culture medium (C) or NGF (N; 50 ng/ml) for 3 h. 1 h before the start of incubation, some of the cultures were given K-252a at a concentration of 3 or 30 nM. The cultures were then sonicated, and soluble extracts were obtained by centrifugation. The extracts were incubated with [γ -³²P]ATP for 10 min at 37°C. The reactions were terminated by addition of SDS sample buffer, and incubation mixtures were boiled and applied to 7.5% SDS polyacrylamide gels. The gels were stained, destained, dried, and then exposed to x-ray film for the appropriate time. The arrow indicates the position of Nsp100. The relative densities of the Nsp100 band were as follows: (C) 100; (N) 74; (3 nM [C]) 140; (3 nM [N]) 111; (30 nM [C]) 120; (30 nM [N]) 137.

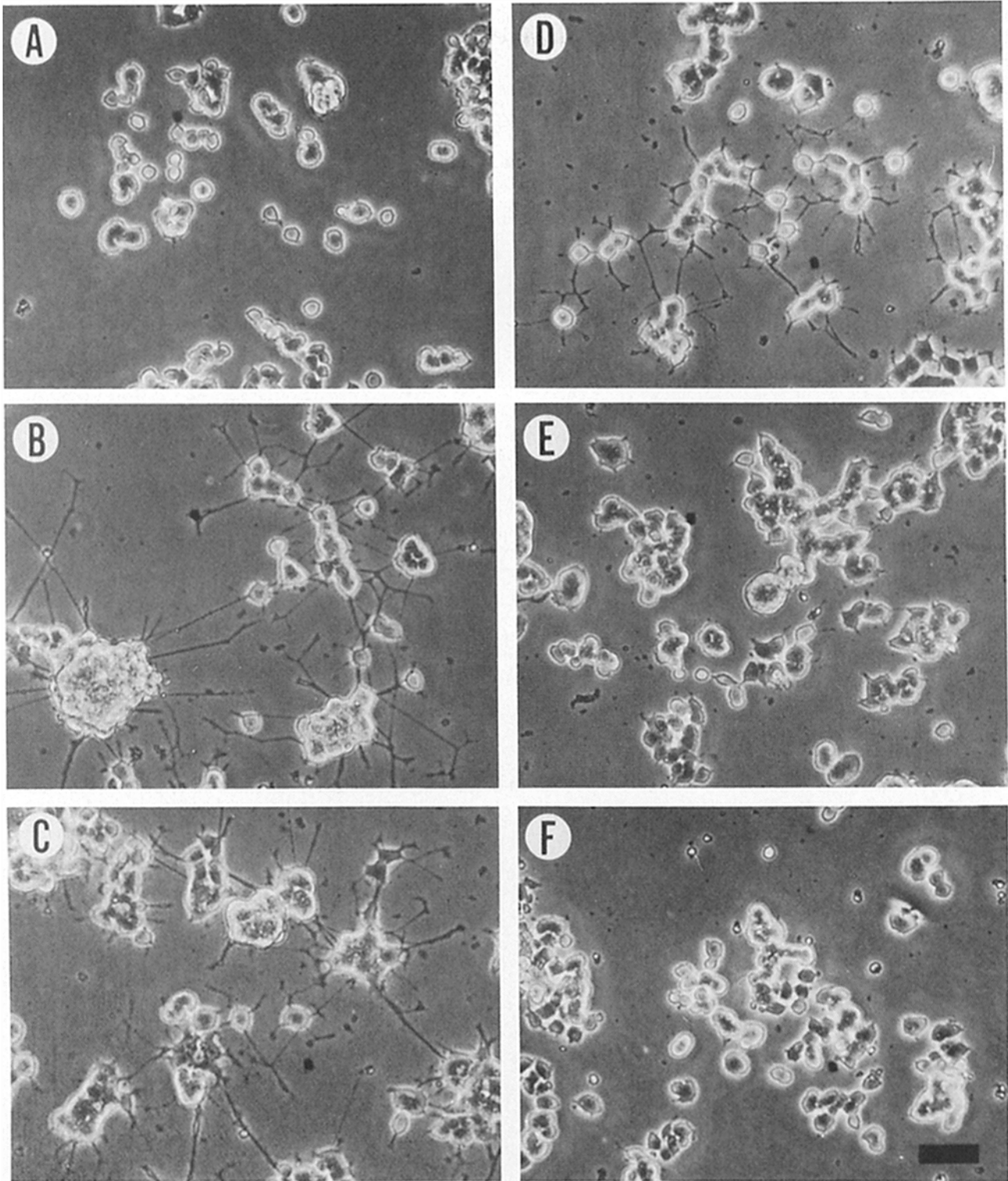


Figure 4. Effect of K-252a on NGF-promoted neurite outgrowth. PC12h cells were cultured for 8 d in control culture medium (*A*), in the presence of NGF (50 ng/ml; *B*), in the presence of NGF (50 ng/ml) and K-252a (10 nM; *C*), in the presence of NGF (50 ng/ml) and K-252a (30 nM; *D*), in the presence of NGF (50 ng/ml) and K-252a (100 nM; *E*), or in the presence of K-252a (100 nM; *F*). Bar, 50 μ m.

ture was examined. After 1 d in culture in the presence of NGF (50 ng/ml), 72.7% of the clumps exhibited long neurites, in contrast to only 9.3% short process-bearing clumps in untreated cultures. Coaddition of K-252a in NGF-treated

cultures reduced the percentage of neurite-bearing clumps from 72.7% to 53.8, 42.6, or 13.1% at a concentration of 10, 30, or 100 nM, respectively, and suppressed the elongation of neurite length with increasing doses (Fig. 5). The data in-

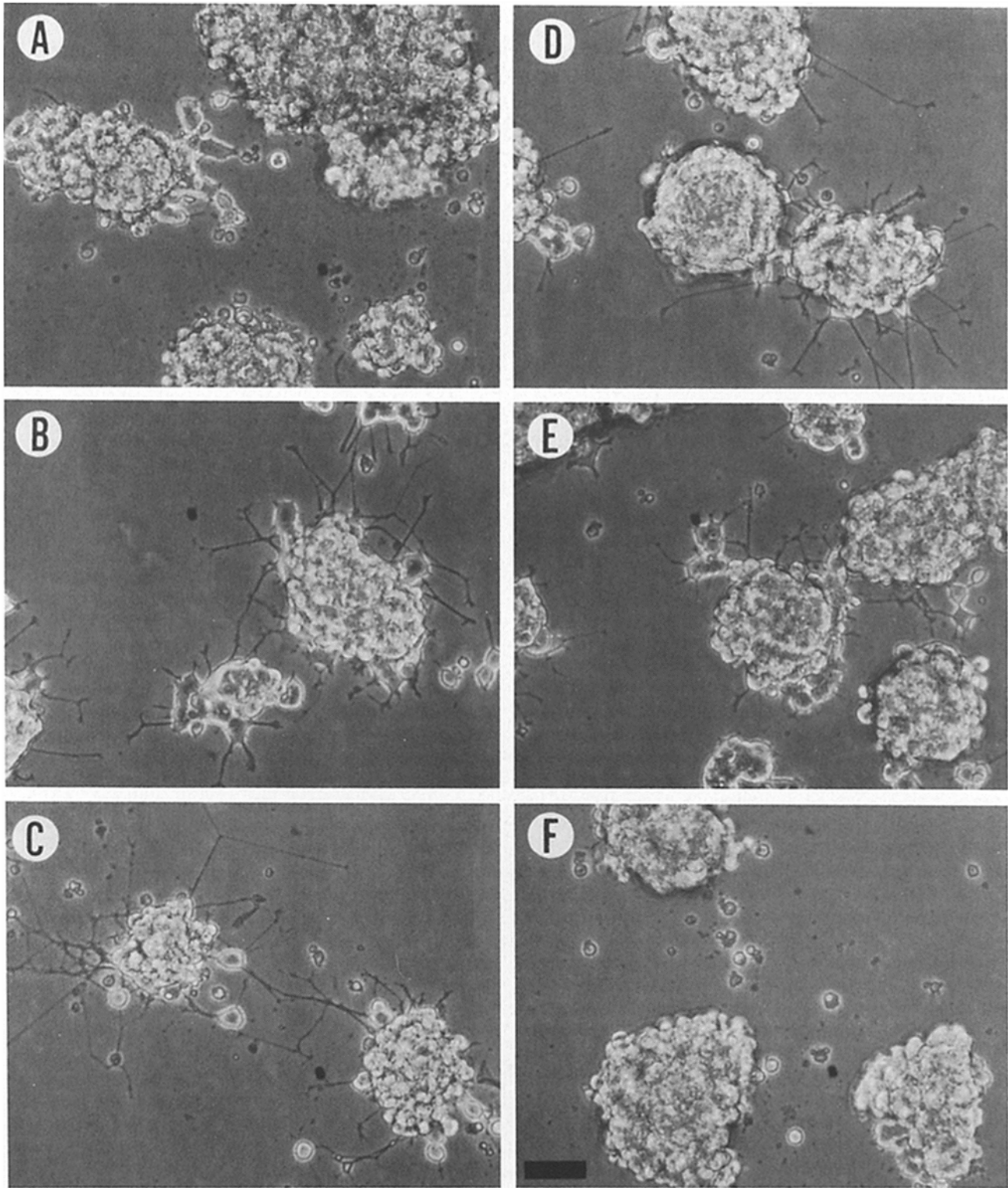


Figure 5. Effect of K-252a on NGF-promoted regeneration of neurites from NGF-primed cells. NGF-primed PC12h cells that had been preexposed to NGF (50 ng/ml) for 8 d were subcultured in control medium without NGF (*A*), with NGF (50 ng/ml; *B*), with NGF plus actinomycin D (1 nM; *C*), with NGF plus K-252a (10 nM; *D*), with NGF plus K-252a (30 nM; *E*), or with NGF plus K-252a (100 nM; *F*) for 24 h. Bar, 50 μ m.

indicated that K-252a suppresses NGF-induced neurites at concentrations higher than 10 nM. On the other hand, addition of actinomycin D to the culture did not affect the regeneration of neurites elicited by NGF (Fig. 5 *C*) as described in earlier studies (3, 11). These results indicate that

K-252a inhibits the transcription-independent component that is involved in the mechanism of promotion of neurite outgrowth elicited by NGF. Then what was investigated was whether the presence of K-252a in the PC12h culture would inhibit the priming elicited by NGF.

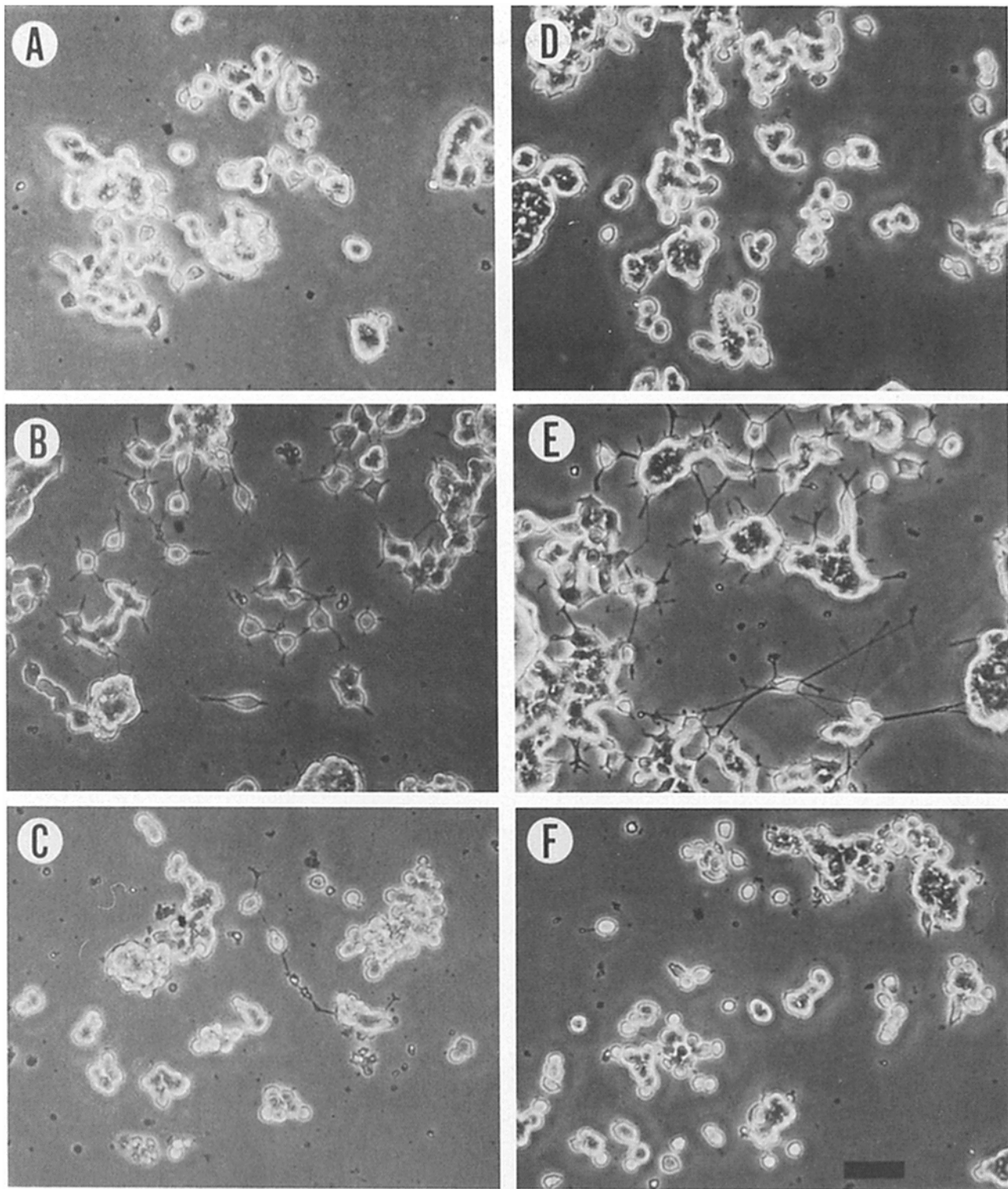


Figure 6. Effect of K-252a on NGF-promoted priming. PC12h cells were treated with NGF (50 ng/ml) plus K-252a (100 nM) for 8 d. Then the cells were washed three times with control culture medium (DME supplemented with 7% FBS and 7% horse serum) and cultured in control medium for 24 (A) or 48 (D) h, with NGF (50 ng/ml) for 24 (B) or 48 (E) h, or with NGF (50 ng/ml) plus actinomycin D (1 nM) for 24 (C) or 48 (F) h. Bar, 50 μ m.

Effect of K-252a on NGF-promoted Priming

After the treatment of PC12h cells with NGF (50 ng/ml) plus K-252a (100 nM) for 8 d, the cells were cultured in the presence or absence of NGF and/or actinomycin D. As shown in Fig. 6, NGF promoted short neurites within 24 h, and these

neurites extended with time (Fig. 6, B and E). However, coaddition of actinomycin D blocked NGF-dependent generation of neurites (Fig. 6, C and F). These data indicate that K-252a blocks a transcription-dependent component, since an inhibitor of RNA synthesis, actinomycin D, blocked NGF-

dependent neurite outgrowth in the cells that were preexposed to NGF in the presence of K-252a for 8 d.

Discussion

The data presented here show that K-252a prevented the effects of NGF on phosphorylation of proteins and neurite outgrowth in PC12h cells. It has been reported that K-252a is a potent inhibitor of C kinase and cyclic nucleotide-dependent protein kinases (17, 32). K-252a inhibits these protein kinases competitively with respect to ATP (17). Indeed, K-252a inhibited the effect of dibutyryl cAMP on phosphorylation of proteins in intact PC12h cells. This indicates that K-252a exactly acts as a kinase inhibitor in these cells. However, a much higher concentration of K-252a was required to inhibit the action of dibutyryl cAMP than to inhibit the action of NGF in these cells. Therefore, it seems unlikely that K-252a prevents NGF action via the inhibition of cAMP-dependent protein kinase. Some other protein kinases, including myosin light chain kinase and tyrosine kinase, are also inhibited by this agent (26; Dr. Matsuda [Kyowa Hakko Kogyo Co. Ltd., Tokyo Research Laboratories], unpublished data). The data presented here indicate that K-252a causes a fairly selective inhibition of NGF-sensitive kinase(s). Indeed, K-252a selectively prevented the effects of NGF on the phosphorylation of proteins including tyrosine hydroxylase, 36,500-D protein, 35,000-D protein, and Nsp100 without any remarkable alterations on the phosphorylation of other proteins in my experiments. Moreover, K-252a and NGF had an opposite effect on the phosphorylation of proteins in PC12h cells. On the other hand, our previous work has shown that wheat germ agglutinin and NGF also show opposite effects on the phosphorylation of proteins including tyrosine hydroxylase, 35,000-D protein, and Nsp100 and that this agglutinin also prevents the effect of NGF on the phosphorylation of proteins as did K-252a in this study. The inhibition is produced by the binding of this lectin to the sialic acid residues of the NGF receptor (14, 31), which in turn blocks the binding of NGF to its receptor and probably deprives the cell of a functional receptor.

The addition of K-252a to the cultures blocked both NGF-dependent regeneration of neurites by PC12h cells, which had been primed by long-term preexposure to NGF, and NGF-induced priming. It appears, therefore, that both transcription-independent and -dependent components, which must be activated by the binding of NGF to its receptors to promote neurite outgrowth, are suppressed by K-252a. These data suggest that both of these components must be involved in at least one phosphorylation step that can be suppressed by K-252a, if this agent acts only on protein kinases. The idea that two separable components required for NGF-induced neurite formation participate in the phosphorylation step is supported by the observation that methyltransferase inhibitors such as 5'-S-methyl adenosine and 5'-S-(2-methylpropyl)-adenosine, which block NGF-induced neurite formation by the blockade of both transcription-dependent and -independent components, also inhibit changes in the phosphorylation of proteins including tyrosine hydroxylase (28). Furthermore, it has also been reported that lithium ion inhibits NGF-induced neurite outgrowth without having any effect on NGF-promoted priming, suggesting that this ion may block a step in which the transcription-independent

component is involved and inhibits NGF-induced changes in the phosphorylation of microtubule-associated proteins (4).

At this point, the exact mechanism by which K-252a blocks the NGF action is unknown. Some other possibilities, for instance that K-252a affects some specific ATP-requiring enzyme in its activity, cannot be ruled out. To identify the exact site(s) on which K-252a acts, further studies are required.

After this manuscript was submitted, results similar to some of those reported here appeared elsewhere (18).

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