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PHARMACOLOGY OF PROTEIN KINASE INHIBITORS

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INTRODUCTION

A complete understanding of the organization and functioning of the second messenger system requires the expertise and cooperation of several different scientific disciplines, such as molecular pharmacology, genetic manipulation, biochemistry, and cell biology. The advent of a new class of effective pharmacological agents is always an event of considerable interest, in particular when this class consists of new types of antagonists that act by specifically blocking one or more of the steps in intracellular signaling systems (1). Although various aspects of protein-phosphorylation systems have been investigated, uncertainties concerning the complex cellular responses in the second messenger system remain (2–4). Improved and sophisticated methods must be designed to estimate changes in the activities of cellular response elements after extracellular stimuli. While our comprehension of the biochemistry and molecular biology of protein kinases has progressed, the function of these enzymes in intact cells has been much more difficult to understand. For this reason researchers studying second messenger systems have long sought the development of specific and effective protein kinase inhibitors that would permit the definitive determination of the physiological role of the protein kinases (5). Protein kinase inhibitors can be used to determine the physiological significance of the protein phosphorylation systems in various types of cells. To elucidate the physiological function of each

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protein kinase, pharmacological probes should meet the following criteria (*a*) direct binding to the protein kinase, (*b*) strict specificity for a protein kinase, and (*c*) cell-membrane permeability. Such compounds, if available would promote understanding of the role of second messengers and second messenger-related protein kinases in cellular responses. Similarly, specific stimulators of protein kinases would help our understanding of the physiological significance of the protein kinases. The focus of this review is on synthetic inhibitors, especially those that can be used to study intact cells.

MYOSIN LIGHT CHAIN KINASE INHIBITOR, ML-9

In 1977, the calmodulin antagonist, naphthalenesulfonamide, W-7 [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide], which was synthesized in our laboratory, was first introduced at the USA-Japan Joint Congress in Hawaii. W-7 relaxes vascular smooth muscle and inhibits actomyosin superprecipitation, thereby indicating a role for calmodulin in smooth muscle contraction (6). This hypothesis was supported by two types of experiments, reported by Dabrowska et al (7) and Hidaka et al (8). We proposed that calmodulin antagonists be defined as compounds that directly bind to calmodulin in a Ca^{2+}-dependent manner, and selectively inhibit the Ca^{2+}/calmodulin-dependent enzymes (9). While a new calmodulin inhibitor was being synthesized from the derivatives of W-7, we discovered that shorter alkyl chain derivatives of naphthalenesulfonamide markedly inhibited several protein kinases such as cAMP-dependent protein kinase, cGMP-dependent protein kinase, and myosin light chain kinase. A shorter alkyl chain derivative of W-7, N-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide (A-3), directly inhibited these protein kinases without inhibiting calmodulin (10, 11). One derivative, ML-9 [1-(5-chloronaphthalene-1-sulfonyl)-1-H-hexahydro-1,4-diazepine], proved to be a specific inhibitor of myosin light chain kinase without inhibiting calmodulin (12). Double-reciprocal plot analysis indicated that ML-9 inhibited myosin light chain kinase activity competitively with respect to ATP but not to calmodulin or myosin light chain. The Ki value of ML-9 against myosin light chain kinase was 3.8 μM (Table 1, Figure 1). According to these data, ML-9 appears to interact with myosin light chain kinase at the binding site of ATP, i.e. a catalytic site. ML-9 was then used to clarify the roles of myosin light chain kinase. ML-9 was useful for studying the physiological role of myosin light chain kinase-dependent phosphorylation in various types of tissue. ML-9 relaxed vascular strips contracted by high K^+. The relaxation induced by the compound was not affected by treatment with adrenergic and cholinergic blocking agents. Thus, ML-9-induced relaxation is not due to a block of membrane-receptor associated mechanisms. Moreover, ML-9 inhibited the Ca^{2+}-induced contraction in

Table 1 Inhibition constants (K_i) of H-series inhibitor for various protein kinases

	A-kinase[a]	G-kinase[b]	CaM KII[c]	MLCK[d]	C-kinase[e]	CKI[f]	CKII[g]
Inhibitor							
A-3	4.3	3.8	—	7	47	80	5.1
ML-9	32	—	—	4	54	—	
H-7	3	5.8	—	97	6	100	780
H-9	1.9	0.9	60	70	18	110	>300
H-8	1.2	0.5		68	15	133	950
H-88	0.4	0.8	70	50	80	60	100
H-89	0.05	0.5	30	30	30	40	140
H-85	>100	>100	47	28	>100	50	>100
KN-62	>100	—	0.9	>100	>100	>100	—
CKI-6	>1000	—	840	—	>1000	50	>300
CKI-7	550		195	—	>1000	9.5	90
CKI-8	80	260		25	>100		

[a] cAMP-dependent protein kinase; [b] cGMP-dependent protein kinase; [c] Ca^{2+}/calmodulin kinase II; [d] myosin light chain kinase; [e] protein kinase C; [f] casein kinase I; [g] casein kinase II.

chemically skinned vascular smooth muscle, suggesting that ML-9 is not a Ca^{2+}-channel blocker. Increasing the concentration of ML-9 selectively inhibited the Ca^{2+}-dependent phosphorylation of human platelet myosin light chain. ML-9 also inhibited human platelet aggregation and serotonin secretion induced by collagen and thrombin. These results suggest that myosin light chain kinase is responsible for regulating platelet function among the many calmodulin-dependent pathways in platelets (1).

PROTEIN KINASE C INHIBITOR, H-7

Many studies have been conducted on protein kinase C. In addition, there have been more studies on protein kinase C inhibitors than on the inhibitors of any of the other protein kinases. Since protein kinase C is present in almost all eukaryotic cells and plays a role in many physiological and pathological processes, there is strong interest in developing and using inhibitors for protein kinase C.

At higher concentrations, W-7 inhibits protein kinase C in a manner competitive with phospholipids (11). When the naphthalene structure of the naphthalenesulfonamides was replaced by an isoquinoline ring, the derivatives directly suppressed protein kinase activities, and were no longer calmodulin- or phospholipid-interacting agents. Among the isoquinoline derivatives, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine, H-7 is a relatively specific inhibitor of protein kinase C (13). The K_i value of H-7 against protein kinase C is 6.0 μM (Table 1, Figure 1). Double reciprocal plot analyses indicated that inhibition of protein kinase C by H-7 was competitive to ATP.

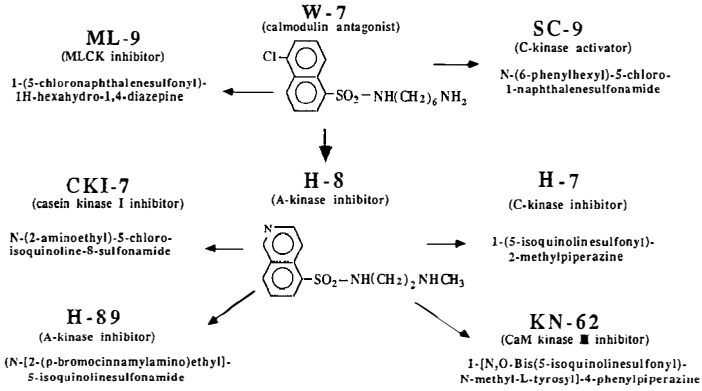

Figure 1 Chemical structures of H-series inhibitors

Thus, H-7 inhibits protein kinase C directly, and not through interaction with phospholipids or Ca^{2+}. Since H-7 is a relatively potent inhibitor of protein kinase C, it has been widely used to evaluate the role of this kinase in a variety of cell types (14–29). H-7 has significant effects on various functions caused by inhibition of protein kinase C-induced phosphorylation (Table 2). SC-9 [N-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide], a derivative of W-7, is an activator of protein kinase C (30). SC-9 and phosphatidylserine have similar effects on protein kinase activity and are both Ca^{2+}-dependent activators. In addition, TPA reduces the requirement for Ca^{2+} of the SC-9-activated protein kinase C in a similar manner to that of phosphatidylserine-activated enzyme. SC-9, a pharmacological activator of protein kinase C, should aid in elucidating the role of protein kinase C in intact cells. For example, exposure of Swiss 3T3 cells to SC-9 increased hexose uptake, which also occurred when the cells were treated with TPA (31).

As mentioned above, isoquinolinesulfonamides inhibited several protein kinases, but to different extents. Among the derivatives, H-9 [N-(2-aminoethyl)-5-isoquinolinesulfonamide] possesses a primary amino group and can be used as a ligand for affinity chromotography. Immobilized H-9 was prepared by direct coupling to agarose, using the cyanogen bromide activation method. The Ki value of H-9 against protein kinase C is 18 μM (13) (Table 1, Figure 1). Large scale purification to homogeneity was achieved for protein kinase C by the use of the H-9 affinity column (32).

CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASE INHIBITORS, H-8, H-88, H-89

By the time cAMP-dependent protein kinase was discovered, Sutherland and other researchers had established that cAMP was a second messenger for

Table 2 Effects of protein kinase C inhibitor

Inhibitor	Action	Cell type	Reference
H-7, W-7	Superoxide production	Human histocytic leukemia cell (U937)	75
H-7	Antigen and IL-2-induced proliferation	Murine T-cell	76
H-7	Oxidative burst and degranulation	Human neutrophil	77
H-7	Glucocorticoid-dependent enzyme induction and glucocorticoid receptor translocation	Rat hepatocyte	78
H-7	Chemotaxis	Human neutrophil	79
H-7	Superoxide production and secretion	Rabbit peritoneal neutrophil	80
H-7	β-2 Adrenergic receptor activation and desensitization	Human lymphocyte	81
H-7	Cell locomotion	Human polymorphonuclear neutrophil	82
H-7, W-7	Atrial natriuretic peptide release	Rat atrium	83
H-7	Hormonal receptor-adenylate cyclase complex modulation	Human lymphocyte	84
H-7, polymyxin B, staurosporine	Platelet aggregation	Human platelet	85
H-7, HA1004	Calcitriol-induced differentiation	HL-60 cell	86
H-7, W-7, HA1004	Morphological change	Rat astrocyte	87
H-7	Neurite outgrowth	Mouse neuro-blastoma & cerebellar cells	88
H-7	Long-term potentiation	Rat Cal pyramidal cell	89
H-7	Cell polarity and locomotion	Walker-256 cell	90
H-7	Alteration of actin-cytoskeleton	Swiss 3T3 cell	91
H-7	Intracellular actin level	HL-60 cell	92
H-7, W-7	Pertussis toxin-induced IL-1 production	Human monocyte	93
H-7	Cell lysis and proliferation	Human lymphocyte	94
H-7	Natural-killer activity	Human granular lymphocyte	95
H-7	Tumor promotion	Epidermal JB cell	96
H-7	Stress, crossbridge phosphorylation, muscle shortening, and inositol phosphate production	Rabbit artery	97
H-7, Sphingosine	Cellular proliferation	Swiss 3T3 cell	98

many extracellular stimuli (33). The enzyme is characterized by having a broad tissue distribution as well as an involvement in diverse responses.

We found that derivatives of W-7 exhibit a relatively selective inhibition toward cAMP- and cGMP-dependent protein kinases and that H-8, N-[2-(methylamino)ethyl]-5-isoquinlinesulfonamide, was a potent inhibitor of cyclic nucleotide-dependent protein kinases (13; Table 1, Figure 1). The

inhibition was of the competitive type with respect to ATP. We examined the interaction of H-8 with cAMP-dependent protein kinase using several affinity-labeling reagents of the ATP-binding sites. We found that H-8 specifically binds to the ATP binding site of the catalytic subunit with a binding ratio of 1:1. H-8 has unique characters that differ in the following respects from the ATP analogues reported Flockhart et al (34): (*a*) H-8, in the presence of other protein kinases, specifically inhibits cyclic nucleotide-dependent protein kinase; (*b*) the binding constant of H-8 to the enzyme is much lower than that of ATP; (*c*) the binding of H-8 to the enzyme is independent of Mg^{2+}; (*d*) the binding site of H-8 of the enzyme differs slightly from that of ATP (35).

A more specific inhibitor of cAMP-dependent protein kinase is now available. A newly synthesized isoquinolinesulfonamide, H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) much more markedly inhibits cAMP-dependent protein kinase than H-8 (36).

The effects of these newly synthesized isoquinoline-sulfonamides on cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C, myosin light chain kinase, Ca^{2+}/calmodulin kinase II, and casein kinase I and II have been investigated. H-88, N-(2-cinnamylaminoethyl)-5-isoquinolinesulfonamide, had a potent inhibitory effect on both cAMP- and cGMP-dependent protein kinases (Table 1). H-89, a brominated derivative of H-88, was the most selective and potent inhibitor of cAMP-dependent protein kinase among the isoquinolinesulfonamide derivatives tested. Its K_i value for cAMP-dependent protein kinase was 0.05 μM while the K_i value for cGMP-dependent protein kinase was 10 times higher, suggesting that the inhibitor was specific for cAMP-dependent protein kinase (Table 1). H-89 was shown to be a much weaker inhibitor of other kinases such as protein kinase C, myosin light chain kinase, Ca^{2+}/calmodulin kinase II, and casein kinase I and II. The structures of these inhibitors are shown in Figure 1. To elucidate the mechanisms involved in the inhibition of cAMP-dependent protein kinase activity, both H-88 and H-89 were tested for their ability to compete with ATP binding to this enzyme. Figure 2 shows double-reciprocal plots of the data obtained with H-88 and H-89. H-88 and H-89 inhibited cAMP-dependent protein kinase activity competitively with ATP. To investigate whether H-89 could serve as a pharmacological probe for examining intact cells, we studied the effect of this inhibitor on forskolin- and NGF-induced phosphorylation in PC12D cells. In the absence of forskolin, ^{32}P-radioactive phosphorylated proteins were not detected in PC12D cells. Pretreatment of the cells with H-89, 1 h before the addition of forskolin, markedly inhibited forskolin-induced protein phosphorylation in a dose-dependent fashion. These inhibitory effects were observed even 8 h after the addition of forskolin. The inhibition of nerve growth factor (NGF)-induced protein phosphorylation was not observed in the PC12D cells pretreated with H-89. These results suggest

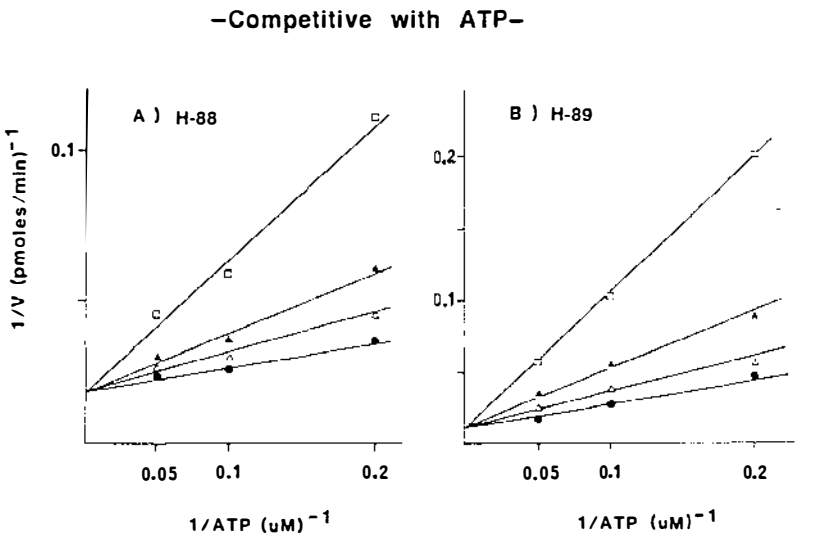

Figure 2 Kinetic analysis of inhibition of cyclic AMP-dependent protein kinase by N-88 and H-89

that H-89 is a useful probe with respect to the selective inhibition of cAMP-dependent protein kinase in intact cells.

The effects of H-89 on forskolin-induced cAMP accumulation in PC12D cells were also examined. The addition of H-89 along with forskolin did not affect forskolin-induced increase in the cAMP levels in PC12D cells. When examining the effect of H-89 on adenylate cyclase and cyclic nucleotide phosphodiesterases, this compound did not inhibit these enzymes at concentrations up to 100 μM. Thus, H-89 may act directly on cyclic AMP-dependent protein kinase in the intact PC12D cells.

To elucidate the role of cAMP-dependent protein kinase on the differentiation of PC12D cells, the effect of H-89 on dibutyryl cAMP-, forskolin-, and NGF-induced neurite outgrowth was investigated. Neurite outgrowth of PC12D cells was maximal at 16 h after the addition of forskolin, and the outgrowth was maintained for 48 h. When the cells were pretreated with H-89 for 30 min before the addition of forskolin, H-89 significantly inhibited the neurite outgrowth from 8 to 48 h after the addition of forskolin. In addition, H-89 inhibited dibutyryl cAMP-induced neurite outgrowth in a dose-dependent manner. Next, the effects of H-89 on NGF-induced neurite outgrowth were examined. Exposure of NGF stimulated neurite outgrowth in a time-dependent manner, with the maximal effect seen at 24 h. Pretreatment with H-89 for 30 min before the addition of NGF exerted to inhibitory action

on the neurite outgrowth of PC12D cells. Thus, cAMP-dependent protein kinase appears to act as a mediator of forskolin- or dibutyryl-cAMP-induced neurite outgrowth, but not of NGF-induced neurite outgrowth. When the cells were treated with ML-9, H-7, CKI-7 (a selective casein kinase I inhibitor, see below), and KN-62 (a specific Ca^{2+}/calmodulin kinase II inhibitor, see below), none inhibited a forskolin-induced neurite outgrowth of PC12D cells. H-85, N-[2-(N-formyl-p-chlorocinnamylamino)ethyl]-5-isoquinolinesulfonamide, was chosen as a control agent for H-88 and H-89 (Table 1). The IC_{50} values of H-85 for various protein kinases were as follows: >100 μM for cAMP- and cGMP-dependent protein kinases; >100 μM for protein kinase C; 28 μM for myosin light chain kinase; 47 μM for Ca^{2+}/calmodulin kinase II; 50 μM for casein kinase I; and >100 μM for casein kinase II. H-85 was not selective in inhibiting cAMP-dependent protein kinase but did produce a similar inhibition on other kinases; hence this compound can be used as a negative control for H-89. Thirty μM of H-85 did not inhibit the forskolin-induced neurite outgrowth of PC12D cells. There is some discrepancy regarding the effective dose of H-89 in in vitro and in vivo systems. This discrepancy is presumably related to the permeability of H-89 in cell membranes and the high concentration of ATP in the initial phase of the experiment, as observed in the case of H-8 (37). H-89 is a derivative of H-8, a specific inhibitor of cyclic nucleotide-dependent protein kinase. H-89 is 30 times more potent H-8 in inhibiting cAMP-dependent protein kinase activity, whereas it is 10 times less potent in inhibiting cGMP dependent protein kinase activity. These newly synthesized inhibitors H-88 and H-89 should serve as useful probes for clarifying the physiological roles of cAMP-dependent protein kinase (6).

Ca^{2+}/CALMODULIN KINASE II INHIBITOR, KN-62

Ca^{2+}/calmodulin-dependent protein kinases constitute an important group of enzymes involved in many aspects of calcium signaling (38, 39). One of the Ca^{2+}-calmodulin-dependent enzymes, Ca^{2+}/calmodulin kinase II, is the most abundant in the central nervous system and has emerged as a multifunctional, neuronal Ca^{2+}-operated switch. For example, phosphorylation of tyrosine hydroxylase and tryptophan monoxygenase (40, 41) by the protein kinase may alter neurotransmitter synthesis, whereas neurotransmitter release may be facilitated by phosphorylation of synapsin I (42). Although the enzymology of the protein kinase has been well studied, the physiological role of the enzyme remains unclear. To clarify the physiological function of Ca^{2+}/calmodulin kinase II, we developed specific Ca^{2+}/calmodulin kinase II inhibitors and eventually synthesized KN-62 [1-[N,O-bis(1,5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenyl-piperazine] (43).

Figure 2 shows the effect of KN-62 on the activities of Ca^{2+}/calmodulin kinase II, cAMP-dependent protein kinase, protein kinase C, and myosin light chain kinase. While over 80% of Ca^{2+}/calmodulin kinase II activity was inhibited by adding 10^{-6}M KN-62, the activities of the other kinases were only slightly affected in the presence of even higher concentrations of KN-62 (Table 1). Thus, this newly synthesized compound seems to be a potent and selective inhibitor of Ca^{2+}/calmodulin kinase II. To determine the mechanisms involved in the inhibition of Ca^{2+}/calmodulin kinase II activity, KN-62 was tested for its ability to compete with calmodulin or ATP for binding to the enzyme. Double-reciprocal plots revealed that inhibition of Ca^{2+}/calmodulin kinase II by KN-62 was competitive with calmodulin and noncompetitive with respect to ATP. Since autophosphorylated Ca^{2+}/calmodulin-kinase II is no longer calmodulin-dependent, the effects of KN-62 on the activity of autophosphorylated Ca^{2+}/calmodulin kinase II were also examined. An approximately 75% decrease in the exogenous substrate phosphorylation of Ca^{2+}/calmodulin kinase II occurred when KN-62 was added before autophosphorylation, whereas the Ca^{2+}/calmodulin kinase II activity seen after the addition of KN-62 after autophosphorylation was essentially unchanged (Figure 3). KN-62 had no effect on the calmodulin-independent activity of the enzyme.

To demonstrate that KN-62 binds directly to Ca^{2+}/calmodulin kinase II, the

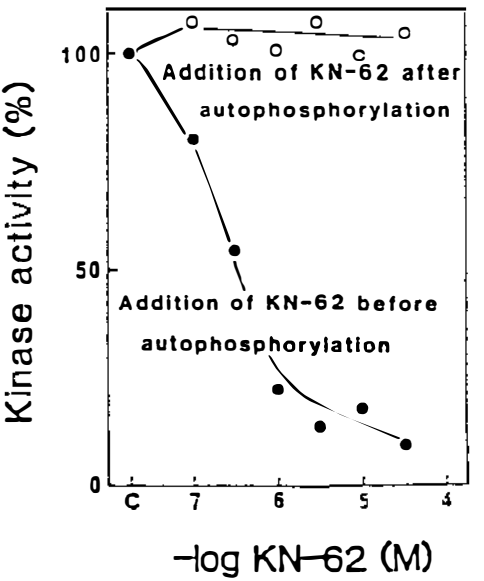

Figure 3 Effect of KN-62 on the autophosphorylated Ca^{2+}/CaM-dependent protein kinase II

mixture of the enzyme and calmodulin in the presence of EGTA was applied to a KN-62-coupled Sepharose 4B column. A pass-through fraction and a bound fraction were analyzed by sodium dodecyl sulfate gel electrophoresis (SDS/PAGE). Calmodulin was eluted in the through fractions but Ca^{2+}/calmodulin kinase II did not appear in these fractions. The enzyme was eluted by boiling in SDS/PAGE sample buffer from the affinity matrix, suggesting that KN-62 binds directly to Ca^{2+}/calmodulin kinase II and is not a calmodulin antagonist (Figure 4).

The physiological role of Ca^{2+}/calmodulin kinase II was postulated to prolong the effects triggered by transient Ca^{2+} signals and to play a role in the long-term modulation of synaptic transmission (44–46). Treatment of PC12 cells with extracellular signals such as nerve growth factor was found to regulate the phosphorylation of several intracellular substrate proteins (47–49). The PC12 cell therefore is an appropriate system for observing events related to Ca^{2+}/calmodulin kinase II. PC12D cells were labeled with ^{32}P-phosphate and stimulated by A-23187 in the presence and absence of KN-62.

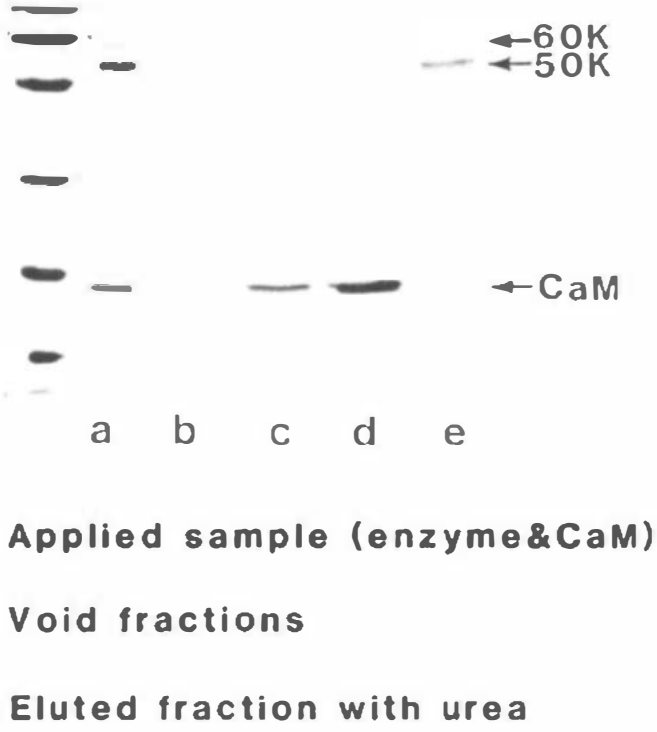

Figure 4 KN-62 affinity chromotography. Direct binding of KN-62 to Ca^{2+}/CaM kinase II

Phosphoproteins immunoprecipitated with an anti-Ca^{2+}/calmodulin kinase II antibody were resolved by SDS/PAGE and visualized by autoradiography. Autophosphorylation of the immunoprecipitated Ca^{2+}/calmodulin kinase II (53 kd) induced by the ionophore was inhibited by the treatment of KN-62 in a dose-dependent manner. These results suggested that KN-62 is cell permeable and blocks Ca^{2+}/calmodulin kinase II activity in PC12D cells (Figure 5). Several studies with KN-62 are currently underway and are clarifying the physiological significance of Ca^{2+}/calmodulin kinase II. For example, the possible involvement of Ca^{2+}/calmodulin kinase II in the long-term potentiation in CA1 cells was examined. When a hippocampal slice was treated with KN-62, long-term potentiation was completely suppressed. Cholinergic stimulation of gastric parietal cell secretion is mediated by Ca^{2+}. To define the role of Ca^{2+}/calmodulin kinase II in parietal cell secretion, the effects of

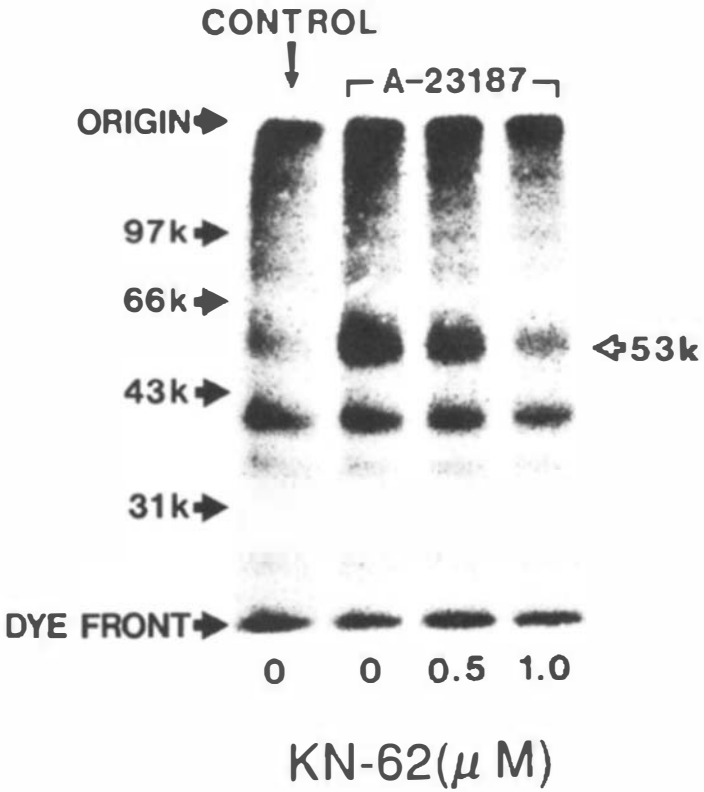

Figure 5 Effect of KN-62 on A-23187-induced phosphorylation of Ca^{2+}/CaM kinase II in PC12 D cells

Table 3 Application of KN-62 to in vivo assay systems

	Phenomena that might be modulated by molecular mechanisms, including CaMKII, through the effect of KN-62	Reference
1.	Catecholamine metabolism in PC12h cells[a]	99
2.	Long-term potentiation formation in CA1 region in rat hippocampal slices[b]	100
3.	Long-term desensitization formation in rat visual cortex[c]	101
4.	Chloride current caused by neurotransmitters in rat brain mRNA-injected *Xenopus* oocytes[d]	102
5.	Aminopyrine uptake into rabbit parietal cells[e]	103

Results obtained in collaboration with: [a] Department of Biochemistry, Nagoya University School of Medicine; [b] Department of Biology, Kyushu University; [c] Department of Neurophysiology, Osaka University School of Medicine; [d] Department of Pharmacology, Hokkaido University; [e] Department of Surgery, Yale University School of Medicine.

KN-62 on acid secretion in isolated parietal cells were determined. Pretreatment with KN-62 inhibited ^{14}C-aminopyrine uptake (which is correlated to acid secretion) over a concentration range of 3–60 μM. Other examples are listed in Table 3.

CASEIN KINASE I INHIBITOR, CKI-6, CKI-7, CKI-8

Casein kinase I has been purified from many tissues, including calf thymus (50), rabbit reticulocytes (51), liver (52), and skeletal muscle (53). The widespread distribution of the enzyme suggests it is important in cellular function, although exactly how remains to be clarified. H-9 has a weak inhibitory effect on casein kinase I and II. When the 5-aminoethylsulfonamide chain of H-9 was moved to position 8 on the aromatic ring, the derivative, CKI-6 [N-(2-aminoethyl)-isoquinoline 8-sulfonamide], produced a more potent inhibition of casein kinase activity than did H-9 (54).

CKI-7 [N-(2-aminoethyl)-5-chloroisoquinoline 8-sulfonamide, the chlorinated derivative of CKI-6], potently inhibited casein kinase I with an IC_{50} value of 9.5 μM for casein kinase I and 90 μM for casein kinase II (Table 1). CKI-7 at a concentration of up to 1000 μM only weakly inhibited protein kinase C. IC_{50} values of CKI-7 were 550 μM for cAMP-dependent protein kinase and 195 μM for Ca^{2+}/calmodulin kinase II activity. Double reciprocal analyses showed that CKI-7 inhibited casein kinase I competitively with respect to ATP. CKI compounds were also used as affinity ligands to purify casein kinase I. Two kinds of affinity columns were used incorporating CKI-7 or CKI-8 [1-(5-chloro-8-isoquinolinesulfonyl)-piperazine], as affinity ligands. To compare the properties of the two affinity ligands, partially purified casein kinase I and II were applied to these affinity columns. The CKI-7 affinity column absorbed casein kinase I only and casein kinase II did

not bind to it. The CKI-8 affinity column, by contrast, absorbed both casein kinase I and II. These results suggest that CKI-7 has a specific affinity for casein kinase I. CKI-8 does not have a specific affinity for casein kinase I and demonstrates an inhibition for casein kinase I that is approximately 10 times weaker than CKI-7. However, the recovery of casein kinase I from the CKI-7 column is lower than the CKI-8 affinity column, suggesting that casein kinase I was bound tightly to the column. The CKI-8 affinity column was thus chosen as the final step in purifying casein kinase I. Casein kinase I eluted from the affinity column at an L-arginine concentration between 0.53 and 0.8 M. Eighteenfold purification was achieved by this step alone. Although several casein kinase II inhibitors have been used to investigate the physiological role of the enzyme, a selective casein kinase I inhibitor has not been available. CKI-7 and CKI-8 should prove useful in determining the physiological role and distribution of casein kinase I in different tissues (54).

OTHER PROTEIN KINASE INHIBITORS

Another approach to developing inhibitors of protein kinases has been to screen natural products. An important group are those protein kinase inhibitors that contain the indole carbozole chromophore. The first compound in this group was staurosporine (Table 4), which is a very effective inhibitor of protein kinase C. The K_i of staurosporine value for protein kinase C is 1–3 nM, the lowest K_i for any protein kinase inhibitor thus far discovered (55–57). However, staurosporine inhibits several other protein kinases as well, including cAMP-dependent protein kinase (58), phosphorylase kinase (59), S6 kinase (59), $pp60^{v\text{-}src}$ (58), EGF receptor/kinase (59), and IGF receptor/kinase (59). The binding of staurosporine to a variety of protein kinases with a greater affinity than that of ATP suggests that the drug can recognize ATP-binding sites of these enzymes. Another indole carbozole isolated from microbial organisms is K265a (60, 61). The chemical structure of K252a is very similar to that of staurosporine. Like staurosporine, K252a has a broad specificity for inhibiting protein kinases, but seems to be less potent. Other indole carbozoles are listed in Table 5.

Calphostin C and related compounds have been isolated from a soil fungus (62–64). These compounds have a complex multiring quinone structure and are named calphostins. Calphostin C (1 μM) completely inhibited the binding of ^{3}H-phorbol dibutyrate (50 nM) to protein kinase C. Calphostin C inhibited the alpha, beta, and gamma isozymes of protein kinase C although at 50 μM it had no effect on the activity of either cAMP-dependent protein kinase or $pp60^{v\text{-}src}$.

Sphingosine is an 18-carbon chain lipid base and inhibits protein kinase C competitively with respect to diacylglycerol, Ca^{2+}, and phosphatidyl serine

Table 4 Chemical structure and inhibition constant of staurosporine

Protein kinase	$IC_{50}(\mu M)$
Protein kinase C	0.0027
cAMP-dependent protein kinase	0.0082
$pp60^{v\text{-}src}$	0.0064
EGF receptor/kinase	0.63

(65). Sphingosine is thought to be a natural negative regulator of protein kinase C in intact cells. However, sphingosine has other effects in addition to the inhibition of protein kinase C, e.g. as an antagonist of calmodulin (66). Sphingosine has been shown to activate EGF receptor/kinase (67, 68), to inhibit $pp60^{v\text{-}src}$ activity (69), and to affect other biological processes independently of its ability to inhibit protein kinase C. Sphingosine is thus not suitable for studies of protein kinase in intact cells.

As with the serine/threonine protein kinases, natural products have been an important source of tyrosine protein kinase inhibitors. It was previously observed that quercetin, a flavonoid compound, could inhibit tyrosine protein kinase activities as well as serine/threonine protein kinases (70, 71). In 1986, Ogawara et al isolated genistein, an isoflavonoid, from a strain of pseudomonas as a EGF receptor/kinase inhibitor (72). Both genistein and quercetin inhibited EGF receptor/kinase and $pp60^{v\text{-}src}$ with micromolar Ki's. Kinetic analyses showed that both compounds inhibited EGF receptor/kinase competitively with respect to ATP (73). However, quercetin can also inhibit protein kinase C and phosphorylase kinase, whereas genistein had little or no effect on these enzymes. Genistein could not inhibit a tyrosine protein kinase purified from thymus and thus the compound is not effective against all tyrosine protein kinases. Most studies with genistein has been with intact cells such as T cells, keratinocytes, and platelets (74). Another inhibitor of tyrosine protein kinase was isolated from culture medium of a strain of streptomyces (75). This compound is a tertiary amine substituted with three phenyl groups

Table 5 Structure and inhibition constant of K-252 compounds

Compound	Structure				K_i, nM			
	R_1	R_2	R_3	R_4	C-kinase[a]	A-Kinase[b]	G-kinase[c]	MLCK[d]
K-252a	H	CH_3	H	H	25	16	15	20
K-252b	H	H	H	H	20	90	100	147
KT5720	H	nHex	H	H	>2000	56	>2000	>2000
KT5823	CH_3	CH_3	CH_3	H	4000	>10000	234	>10000
KT5926	H	CH_3	H	OnPro	723	1200	158	18

[a] Protein kinase C; [b] cAMP-dependent protein kinase; [c] cGMP-dependent protein kinase; [d] myosin light chain kinase

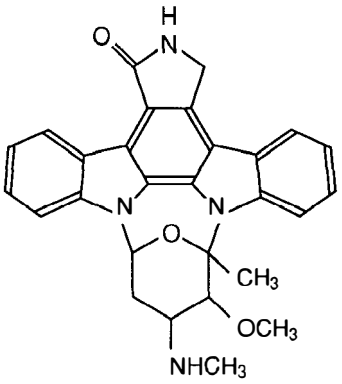

Figure 6 Chemical structure of staurosporine

and is named lavendustin A. The compound inhibited EGF receptor/kinase activity with a K_i value of 12 nM and was competitive with respect to ATP. Lavendustin A had no effect on protein kinase C and weak inhibitory effect on cAMP-dependent protein kinase.

In 1989, Umezawa et al reported an EGF receptor/kinase inhibitor isolated from a strain of streptomyces (76). The compound was named erbstatin. Erbstatin inhibited EGF receptor/kinase at micromolar concentrations but had almost no effect on cAMP-dependent protein kinase. This group also reported that the two hydroxyl groups on the ring of erbstatin were necessary for inhibitory activity (77, 78). Erbstatin is a competitive inhibitor of EGF receptor/kinase with respect to peptide substrate (79). The compound is relatively small and uncharged and thus can penetrate intact cells.

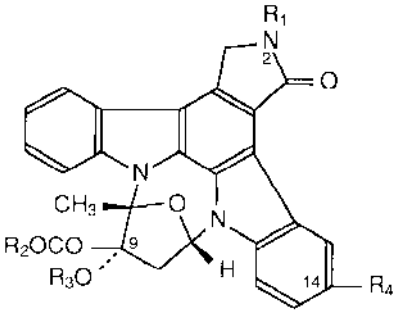

Figure 7 Chemical structure of K-252

CONCLUSION

There is now convincing evidence that protein phosphorylation is a common cellular mechanism in biological regulation. The large number of cellular mechanisms involving protein phosphorylation have been demonstrated; however, the interrelationship among each protein phosphorylation (i.e. each protein kinase) is complex and many uncertainties concerning the complex cellular responses in these systems remain. To clarify the physiological role and molecular mechanisms of the protein phosphorylation systems, selective inhibitors will be useful as pharmacological probes (80).

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