J. Pest Cont. & Environ. Sci. 6 (1): 35-48 (1998)

PROTEIN KINASES: ACTIVITY AND INHIBITION BY INSECTICIDES AND NATURAL PRODUCTS

By

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ABSTRACT

Calcium-phospholipid-dependent protein kinase (PKC), polypeptide-dependent protein kinase (PKP) and epidermal growth factor-receptor (EGF-R) as a tyrosine kinase were prepared from rat brain, yeast, and A431 human epidermoid carcinoma cells, respectively. Their Phosphorylation activities and inhibition assays were carried out radiographically by two independent methods, filter paper assay and autoradiogram of polyacrylamide gel electrophoresis. The autoradiogram of the A431 tyrosine kinase activity showed its ability to phosphorylate itself of approximately 170 KD. Mammalian hormone epidermal growth factor (EGF) showed that A431 tyrosine kinase at the same band (170 KD) was highly stimulated by EGF. On the contrary, the autophosphorylation of this tyrosine kinase was inhibited by over 50% at 10 μM gossypol concentration, where as in the presence of EGF, the inhibition was only 32% at the same concentration of gossypol. Moreover, several chlorinated hydrocarbon compounds, DDT, endrin, dieldrin, aldrin and chlordane and deltamethrin from synthetic pyrethroids, all showed inhibitory effects of the activity of PKP. Staurosporine (microbial chemical product), aldrin, DDT, and deltamethrin showed strong inhibitory effects of the activity of PKC. The results were discussed in view of mammalian toxicity of these compounds and their selectivity as insecticides.

INTRODUCTION

Protein kinases play fundamental role in protein phosphorylation in almost all types of cells including mammalin, yeast, insects and plants. They catalyze the transfer of the terminal phosphate group of ATP to the hydroxyl group of serine, therionine, or tyrosine residues of substrate proteins (Nestler and Greengard, 1984). These phosphorylation and dephosphorylation reactions have been recognized as regulatory mechanisms of metabolism, membrane function and strucural as well as contractile protein (Abdel-Ghany *et al.*, 1989). Some protein kinases are known to play a role in the regulation of normal cell metabolism such glycogen synthesis, while a number of protein kinase activities were shown to be associated with growth factor receptors (Nestler and Greengard, 1984). Yet, the effect of natural and synthetic insecticides on the activity of protein kinases has not been well understood.

In view of the effect of pesticides on the fundamental enzymes involved in signal transduction pathway, a little information is known of such effect and its correlation with diseases and insect as well as human toxicity. In the recent years, there are many human diseases widely occurring such as kidney, liver, and skin cancer diseases. Although, pesticide application has been used in pest control several years ago, still the effect of pesticide residues in the environment and foodstuff and its relation with human diseases has not been fully understood. However, eukaryotic protein kinases and phosphatases are known to be inhibited by a variety of both natural and synthetic compounds, including medicines (immuno supperssants, tumor suppressants and anti-inflammatory agents), potions (a purported aphrodisiac that doubles as a wart remover) and poisons (diarrhetic toxins, liver toxins, tumor promotrs, insect defense chemical and herbicides (Mackintosh and Mackintosh 1994).Gossypol which is considered the most commoh naturally occurring terpenoid, has been recognized as an insect repelling component of cotton plants (Abou-Donia 1976; Sherby 1979 and El-Sebae *et al.*, 1981) ,however, the way it exerts this effect on insects is not well known.On the other hand, several insecticidal compounds of the chlorinated hydrocarbon group as well as the synthetic pyrethroid,deltamethrin, often show diverse effects on several biological systems. Yet, there has been no general agreement about their mode of action.

In spite of the vital role of the above group of enzymes in the cellular metabolism, little has been done in regard to their role in host plant resistance or in the mode of action of chemical insecticides. In the present investigation we tried to shed light on the possible relationship between the physiological function of such group of enzymes and the role of certain natural compounds as an insect-resistance conferring component of plant crops. Furthermore, the effect of some chemical insecticides on the acitivity of these enzymes has been evaluated in terms of their mode of action. Accordingly, we evaluated the effect of staurosporine as a fungally derived indole carbazole compound, and gossypol as a natural inhibitor, deltamethrin from the synthetic pyrethroids and DDT, endrin, dieldrin, aldrin and chlordane from chlorinated hydrocarbon compounds on the activity of the above mentioned protein kinases as a probable target for insecticides.

MATERIALS AND METHOD

[γ-^{32}P]ATP was obtained from Amersham, gossypol, Whatman 3MM, polyacrylamide, Bis-acrylamide, $CaCl_2$ were from Sigma, staurosporine from GIBICO, *p,p'*-(dichloro diphenyl) trichloroethane (DDT), endrin, dieldrin, aldrin, chlordane, and deltamethrin were from EPA (Triangle Parke USA), glycerol, Hepes, EDTA, TritonX-100 were obtained from Boehringer Mannheim, thioglycerol from Evans Chemitics (Waterloo, N.Y), Epidermal growth factor was a generous gift from Dr.M. Abdel-Ghany (Cornell University, Ithaca, NY), X-ray films were from Kodak. A-431 human epidermoid Carcinoma cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum.

Isolation of EGF-receptor :

Membranes (200mg) from A431 Carcenoma cells were prepared as described by (Braun *et al.*, 1986). These membranes were solubilized at 0°C for 30 min with solubilization buffer (20 mM Tris-HCl, pH 7.2, 1mM EDTA, 10% glycerol and 0.5% TritonX-100). After centrifugation at 150,000 xg for 30 min, the supernatant was used as a source of EGF-receptor.

Assay of EGF-receptor kinase activity :

The assay mixture contained, in final volume of 50 µl, 20 mM Na Hepes pH 7.4, 5mM $MgCl_2$, 8 µg EGF-receptor, 10 µM [γ^{32}P]ATP (5000 cpm/pmol) and with or without 10 ng of animal hormone, EGF. Gossypol was added to the assay mixture as an alcoholic solutions. The enzyme reaction was started by adding the radioactive ATP. After 10 min at room temperature, 20 µl were placed on Whatman 3MM filter paper and the rest of reaction mixture was loaded on 10 % SDS polyacrylamide gel and analyzed as described by Abdel-Ghany *et al.*(1987).

Isolation and purification of polypeptide-dependent protein kinase (PKP) :

Membranes (500 mg) from Baker's yeast were prepared as described by Yanagita *et al.*(1987). These membranes were solubilized at 4°C for 20 min with solubilization buffer (20 mM Hepes pH 7.4, 10 mM thioglycerol, 10% glycerol and 0.5% TritonX-100). After centrifugation at 120,000xg, the resulting supernatant was use in the purification steps and the activity of the PKP was performed as dscribed by Yanagita *et al.* (1987).

Isolation of Ca^{2+}-phospholipid-dependent protein kinase (PKC) :

This enzyme was isolated and purified from fresh rat brain as described by Wodgett and Hunter (1987).

Assay of PKC activity :

In a final volume of 50µl the assay contained, 50µl/ml freshly sonicated phosphatidyl serine, 5 µg diolein/ml, with or without 5 µg histone, 10 µM[γ-^{32}P]ATP (5000 cpm/pmol) and 20 ng of PKC in 20 mM Hepes pH 7.4, 10 mM $MgCl_2$, and 0.5 mM $CaCl_2$. The PKC reaction mixture was started by adding radioactive ATP. After 10 min at room temperature, 20 µl were placed on Whatman 3MM filter paper and analyzed as described by Woodgett and Hunter, 1987. The remaining samples were separated after being heated for 5 min at 90°C by SDS-PAGE (12% polyacrylamide gel according to the methods of laemmli,1970), the gel was dried and autoradiography was performed according to Abdel-Ghany *et al.* (1987).

RESULTS AND DISCUSSION

1. Effect of natural inhibitors on the activity of protein kinases:

a) Effect of gossypol and animal hormone, EGF on EGF-receptor tyrosine kinase activity:

The Ethe ability to phosphorylate itself in absence of additional substrate (so-called autophosphorylation) as shown in Fig. 1 (lane 1).

When animal hormone, EGF, was added at concentration of 5ng to the EGF-receptor, the autophosphorylation of endogenous proteins of the EGF-receptor was highly stimulated (lane 2). Our results are consistent with that reported by Ushiro and Cohen (1980), who found that the EGF-receptor protein itself possesses protein kinase activity with specificity for tyrosine residues in substrate proteins. In addition, membranes prepared from A-431 cells capable to phosphorylate specific endogenous membrane proteins as well as exogenously added histone.

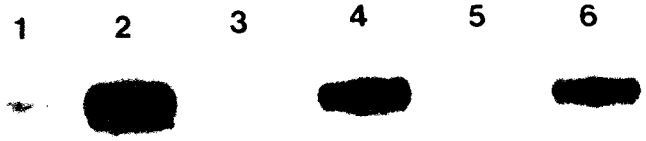

Fig.(1): Autoradiogram showing the autophosphorylation of EGF-receptor in absence (odd lanes) and presence (even lanes) of epidermal growth factor, with and without different concentration of gossypol; lanes 1 and 2 (control); 3 and 4 with 20μM; lanes 5 and 6 with 40μM gossypol.

Cotton seed natural inhibitor, gossypol have showed inhibitory effect of EGF-receptor phosphorylation occurred either in presence or absence of animal hormone, EGF (see, lane 3,4,5 and 6). Filter paper assay was performed to measure the percent of inhibition of EGF-receptor by gossypol. Tyrosine kinase activity of EGF-receptor was inhibited by 32% at 40μM concentration of gossypol.This data are in agreement with our previous data reported by Abo-El-Saad (1992), in which we found that tyrosine kinase isolated from pig brain was significantly inhibited by similar concentration of gossypol.

The present data show that the EGF-receptor tyrosine kinase activity was significantly inhibited by gossypol. Decreasing in such activity might be correlated to certain diseases such as diabetes which have been reported by Levitzki and Gazit (1995), who showed that the decrease in the activity of tyrosine kinase of insulin receptor is the cause of various types of diabetes. Moreover, the gossypol content in cotton plants is playing an important role in conferring resistance to cotton plants against insect attack, suggesting that protein kinases from insect could be inhibited by gossypol content during insect feeding. This might explain why do insects are attacking free gossypol cotton plants rather than the ones having gossypol.

b) Effect of microbial product, staurosporine on the PKC activity :

Microbial agent as a fungally derived indole carbazole compound, staurosporine have been tested on the activity of PKC. Nanomolar range of staurosporine was found to have strong inhibitory effect on the activity of this enzyme. Enzymatic activity of PKC was dramatically inhibited by concentrations of staurosporine ranged in 10 to100 nM as a fingerprint of phosphorylated bands when the dried gel was exposed to X-ray film as shown in Figs. (2 and 3). Analysis of the enzyme activity was also performed by using the filter paper assay to calculate the percent inhibition of the enzyme activity. This analysis was indicated that the PKC activity was strongly inhibited by 85% at 0.1μM staurosporine concentration.

Recently, it has been reported by Tamaoki (1991) that staurosporine have the ability to inhibit several protein kinases. Sugesting, that limited selectivity of staurosporine for different protein kinases due to its interaction with the essential region of the catalytic domain of protein kinases that share a homologous region.

Since fungi are capable to produce large number of toxins such as staurosporine which is produced by *Streptomyces* spp, recently it has been widely used as a commercial bioagent. Recently, the EPA have approved use of a product made with a whitefly-killing fungus. Thus, it possibly that the toxicity of such agent for insects due to their contents of staurosporine which may inhibit the activity of insect protein kinases.

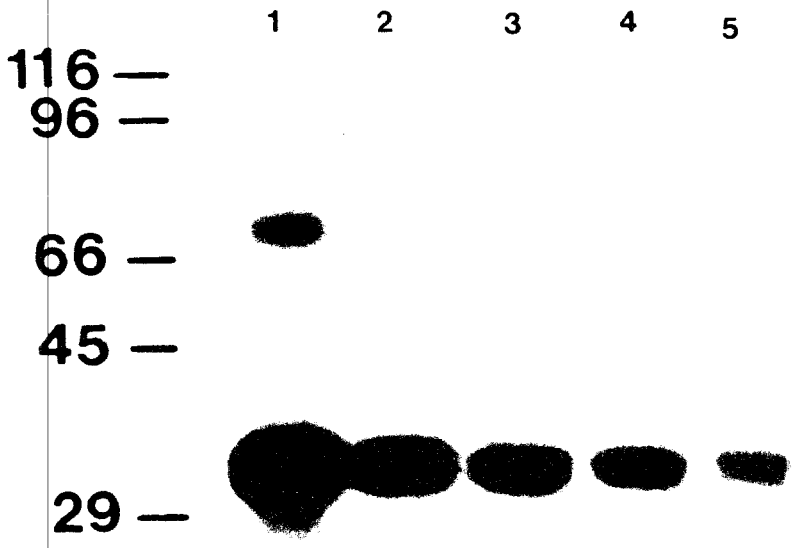

Fig.(2): Autoradiogram showing the phosphorylation of histone 1 by protein kinase-C (PKC) and inhibition by different concentrations of staurosporine; lane 1 (control); lane 2, 3, 4 and 5 with 10, 30, 50 and 100nM gossypol.

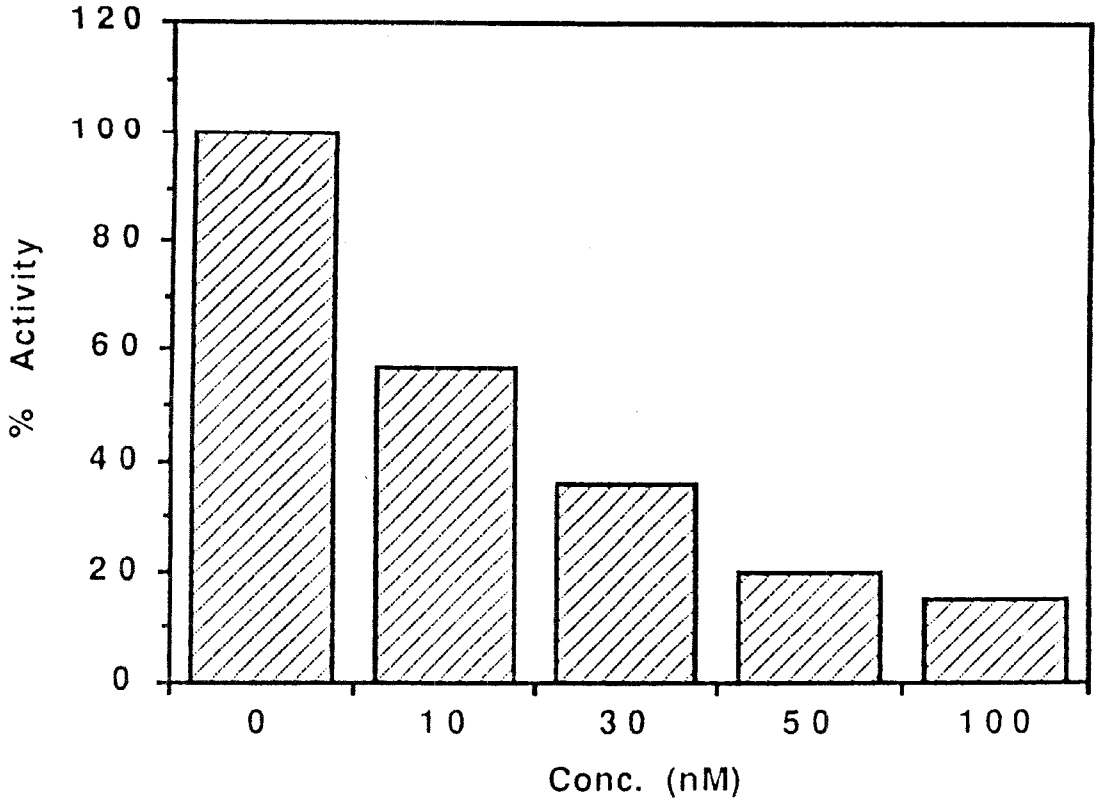

Fig.(3): Inhibitory effect of staurosporine of histone 1 by PKC in using filter paper assay as described under methods.

2. Effect of synthetic insecticdes on the activity of protein kinases :

a) Effect of certain chlorinated hydrocarbons and deltamethrin on the activity of PKP :

The activity of polypeptide-dependent protein kinase (PKP) was affected by several insecticides of chlorinated hydrocabons, α-chlordane, aldrin, dieldrin and DDT, as well as pyrethroid insecticide, deltamethrin. It can be seen from Table (1) that DDT at 10μM concentration slightly inhibits the activity of PKP,whereas, the activity of this enzyme at the same concentration of deltamethrin was inhibited by 21%. However, aldrin, chlordane, endrin and dieldrin at 10 μM concentration gave higher inhibition of the enzyme activity by 60%, 55%, 36% and 27% respectively.

Table (1): Effects of certain chlorinated hydrocarbon compounds and deltamethrin on the activity of polypeptide-dependent protein kinase (PKP).

Additions	Activity (as % of control)
50 ng PKP	100.0
+ 10 μM α-chlordane	45.4
+ 10 μM β-chlordane	59.4
+ 10 μM aldrin	40.3
+ 10 μM dieldrin	73.0
+ 10 μM endrin	64.7
+ 10 μM DDT	95.2
+ 10 μM deltamethrin	79.4

The data did not match with that known of the toxicity of these compounds in the order of endrin>dieldrin≥aldrin in their toxicity to mammals. This difference might be due to two reasons 1) PKP enzyme was prepared from yeast, not from mammals and 2) that the present work was done *in vitro*. It seems likely that the protein kinases are not the direct target for DDT and other chlorinated hydrocarbons which is consistent with recently reported by Younis *et al.* (1997), who showed that DDT as low as of 2 μ M of DDT selectively inhibits the F_oF_1-ATPase complex isolated from insects, however, they found that the enzyme from mammalian source is not inhibited by, DDT.

Recently, it has been reported by (Matesic *et al.*, 1994) that chlorinated hydrocarbon compounds such as dieldrin inhibit phosphorylation of gap-junction (membrane channels that permit the transfer of ions and small molecules between contiguous cells). In our study, the data showed that PKP can be inhibited by various chlorinated hydrocarbon compounds which are high persistent in adipose tissues, suggesting that protein kinases might be involved in the toxicity of these compounds.

b) Effect of DDT, aldrin and deltamethrin on the activity of PKC :

The two compounds (aldrin, DDT) which have lowest inhibitory effect on the above enzyme, PKP and deltamethrin which have the highest inhibitory effect on the same enzyme were chosen for testing on PKC

activity. These compounds at different concentrations of 10, 50 and 100 μ M were examined. Fig. (4) shows that aldrin at 50 μM slightly inhibits the activity of PKC, whereas at 100 μM gave 53 % inhibition of the enzyme activity. DDT at 10 μM concentration had no inhibitory effect on PKC activity, whereas at 50 μM and 100 μM, the inhibition of the enzyme activity was 37% and 49% respectively. Furthermore, deltamethrin, at the same concentrations of 10, 50, 100 μM inhibits the enzyme activity by 25%, 42%, and 55% respectively. These data are in agreement with that reported by (Enan and Matsumura 1993; El-Sebae *et al.*,1993; leng and Xiao 1995) who found that protein phosphorylation from rat, housefly brain and human brain tau (microtubule associated protein) were inhibited by deltamethrin and Al^{3+} as well as the level of depolarization-induced protein phosphorylation was increased by deltamethrin.

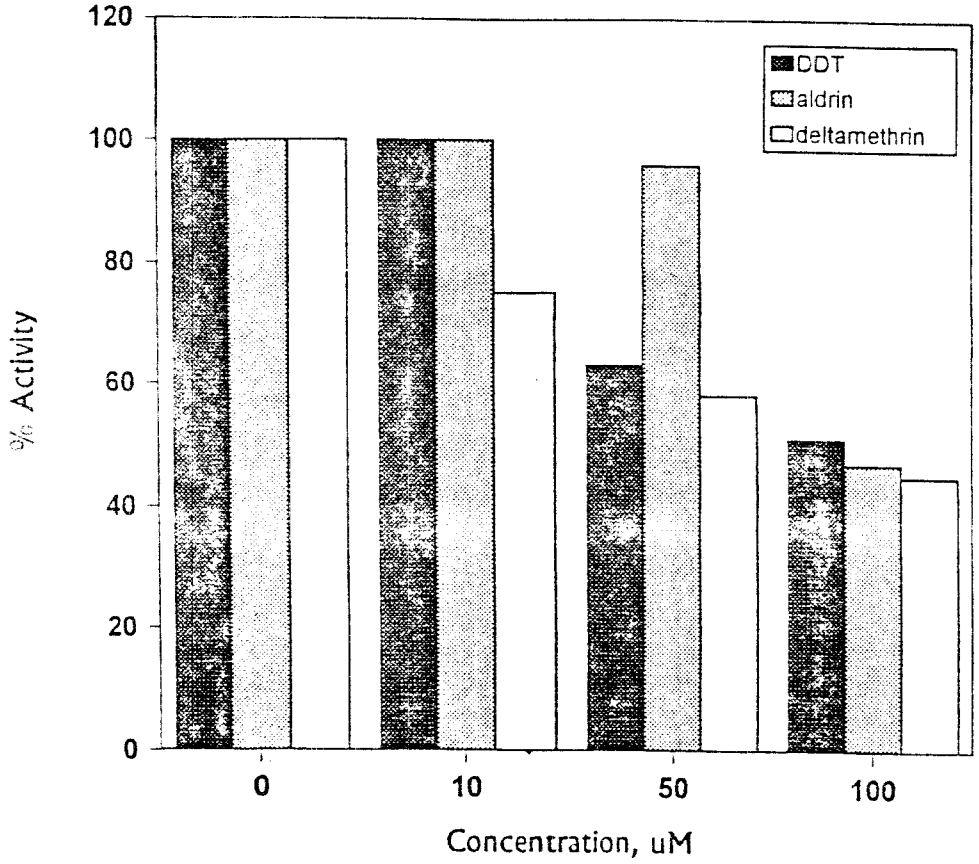

Fig (4). Effect of DDT, aldrin and Deltametrin on the activity of protein kinase

Among natural inhibitors, gossypol which is known as polyphenolic compound and staurosporine as a fungally derived indole carbazole compound are tested in on the activity of EGF-R and PKC respectively. The present data show that both enzymes were strongly inhibited by the above compounds. Decreasing in such activity might be correlated to certain diseases such as diabetes which have been reported and Gazit(1995), who showed the decreasing in the activity of tyrosine kinase of insulin receptor is the cause of various types of diabetes. Moreover, the gossypol content in cotton plants playing a important role in conferring cotton plants resistant against insect attack, suggesting that protein kinases from insect could be inhibited by gossypol content during insect feeding. This might explain why do insects attacking cotton plants having no gossypol rather than ones having gossypol.

Because of high presistance of chlorynated hydrocarbons and their accumulation in animal tissues, it can caused serious toxicities. The biochemical data of this group of insecticides presented in the current investigation on protein kinases can be a part of the basis for such toxicity, however, many more data are needed to correlate such *in vitro* findings to the toxicity occurring *in vivo*.

ACKNOWLEDGMENT

I like to acknowledge Prof. M. Abdel-Ghany, for all the facilities provided to finish this work at Cornell University, Ithaca, N.Y. and Prof. Hassan M. Younis for critical comments and discussions of the manuscript.

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الملخص العربى

الإنزيمات الناقلة : نشاطها وتثبيطها بالمبيدات والنواتج الطبيعية

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اجريت الدراسة على ثلاثة انواع من الإنزيمات الناقلة وهى البروتين كينيز ـ سى (PKC) والبروتين كينيز بى (PKP) ومستقبل عامل النمو EGF-R المحتوى على الإنزيم الناقل من النوع التيروسينى وهذه الإنزيمات تم عزلها من مخ الفئران والخميرة ومن الخلايا السرطانيه من النوع A431 على الترتيب. فسفرة هذه الإنزيمات و تثبيطها تم بإستخدام طريقتين هما تقدير الفسفرة على ورق الترشيح والهجره الكهربائية للبروتينات المفسفرة على البولى أكريلاميد جيل. ولقد أوضحت الدراسة أن الإنزيم الناقل التيروسينى له القدرة على فسفرة نفسه عند الوزن الجزيئى ١٧٠ كيلو دالتون وأن هذه الفسفرةمن تضاعفت عدة مرات فى وجود الهرمون الـ EGF. على النقيض فإن هذا الإنزيم وجد أنه عند إضافة ١٠ ميكرومولر من الجوسيبول أنه يُثبط بأكثر من ٥٠ ٪ فى حالة عدم إضافة هذا الهرمون بينما عند إضافته يُثبط بمقدار ٣٢ ٪ بالإضافة إلى ذلك تم دراسة سلسلة من المركبات الهيدروكلورونية المكلوره مثل د.دت، اندرين، دايلدرين، الدرين، الكوردان وكذلك أحد مركبات البيروثيرويدات مثل الدلتامثيرين على البروتين كينيز ـ بى ووجد أن هذه المركبات مثبطه لهذاالنوع الإنزيمى. كما أختبر الـ د.د.ت ،الألدرين ،الدلتا ميثرين وبعض المركبات الميكروبيه الطبيعية مثل الـ Staurosporine على البروتين كينيز ـ سى ووجد أن هذه المركبات لها تأثير تثبيطى واضح على هذا لنوع الانزيمات.