Vol. 142, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1987 Pages 53-62

COMPARISON OF THE ROLES OF CALMODULIN AND PROTEIN KINASE C IN ACTIVATION OF THE HUMAN NEUTROPHIL RESPIRATORY BURST

Clifford D. Wright* and Michael D. Hoffman

Pharmacology Department,
Warner-Lambert/Parke-Davis Pharmaceutical Research,
Ann Arbor, Michigan 48105

Received August 29, 1986

SUMMARY: The roles of calmodulin and protein kinase C in the activation of the human neutrophil respiratory burst were characterized pharmacologically. The protein kinase C inhibitors 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) and N-(2-aminoethyl)-5-isoquinolinesulfonamide (H-9) did not inhibit superoxide anion generation by neutrophils stimulated for 30 minutes with N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) or 4 beta-phorbol 12 beta-myristate 13 alpha-acetate (PMA). However, H-7 did depress superoxide production during the first 5 minutes following stimulation. In contrast, the specific calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and the dual calmodulin antagonist/protein kinase C inhibitor trifluoperazine (TFP) were potent inhibitors of the response throughout the 30 minute incubation. Stimulation of neutrophils with submaximal doses of FMLP or PMA failed to promote inhibition of the respiratory burst by H-7 or H-9, but did stimulate a respiratory response which was not inhibited by TFP or W-7. These results suggest that while protein kinase C may play a role in the initiation of the respiratory burst response, propagation of the response is dependent on calmodulin-dependent processes. The inability of TFP and W-7 to inhibit superoxide anion generation in response to submaximal stimulatory doses of FMLP or PMA suggests that calmodulin-independent processes may also be involved in activation of the respiratory burst.

Receptor-ligand interactions stimulate phospholipase C to hydrolyze phosphatidylinositol 4,5-bisphosphate to produce the second messenger molecules inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DG) (1). IP_3 mobilizes intracellular calcium from the endoplasmic reticulum to promote the activation of calcium/calmodulin-dependent protein kinase(s) while DG promotes the activation of protein kinase C. Protein phosphorylation catalyzed by these kinases is essential for cell activation.

In the past, characterization of the roles of calmodulin and protein kinase C in cell activation has relied on the use of the phenothiazine

0006-291X/87 $1.50

antipsychotic drugs such as trifluoperazine (TFP). However, biochemical characterizations using such compounds are limited by the ability of such compounds to both antagonize calmodulin and inhibit protein kinase C (12,18). The recent development of specific calmodulin antagonists [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide] (W-7) (15) and protein kinase C inhibitors [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) and N-(2-aminoethyl)-5-isoquinolinesulfonamide (H-9)] (7) has provided tools to examine the participation of the IP_3- and DG-dependent second messenger pathways in cell activation.

We have recently reported that the isoquinolinesulfonamide protein kinase C inhibitors, H-7 and H-9, fail to inhibit human neutrophil respiratory and secretory responses to either soluble or particulate stimuli. In contrast, the naphthalenesulfonamide calmodulin antagonist, W-7, was a potent inhibitor of these responses, suggesting the importance of the IP_3-dependent second messenger pathway in neutrophil activation (19). Sha'afi, et.al.(14) have recently confirmed the inability of H-7 to inhibit neutrophil secretory responses and have suggested that protein kinase C may play a negative modulatory role in neutrophil regulation. In contrast, H-7 and H-9 have been shown to inhibit phorbol ester-stimulated histamine release from human basophils (16).

The purpose of this study is to further examine the roles of calmodulin and protein kinase C in neutrophil activation by comparing the effects of H-7, H-9, TFP, and W-7 on the respiratory burst of human neutrophils. The effects of these compounds on the time course of superoxide anion generation and stimulatory dose response to FMLP and PMA are examined.

MATERIALS AND METHODS

Isolation of human neutrophils. Venous blood was drawn from healthy human volunteers into heparin (10 U/ml), and the neutrophils were isolated by the method of Ferrante and Thong (5). The cell preparations consisted of greater than 98 percent neutrophils. The cell suspension medium used throughout this study was Dulbecco's phosphate buffered saline (PBS) (GIBCO Laboratories, Grand Island, NY).

Human neutrophil respiratory burst activity. The respiratory burst of human neutrophils was measured as superoxide anion-mediated reduction of horse

heart cytochrome C (Sigma Chemical Co., St. Louis, MO), as previously described by DeChatelet, et al. (4). Superoxide generation was stimulated by addition of soluble stimuli to a 1.0 ml reaction mixture containing 1 x 10^6 neutrophils and 2.6 x 10^{-5} M cytochrome C. The soluble stimuli 4 beta-phorbol 12 beta-myristate 13 alpha-acetate (PMA) (Sigma) and N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (Behring Diagnostics, La Jolla, CA) were used to activate the respiratory burst. Following incubation at 37°C for the appropriate time period, the respiratory burst was stopped by placing the reaction tubes in ice water. Cells were removed by centrifugation and the reduction of cytochrome C was measured as the increase of the absorbance of the supernate at 550 nm. The effect of the test compounds on acellular generation of superoxide was determined using xanthine oxidase as previously described (10).

H-7 and H-9 were provided by Gödecke AG (Freiburg, Federal Republic of Germany). Trifluoperazine (TFP) was obtained from Behring. N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) was obtained from Sigma.

RESULTS

Inhibition of the human neutrophil respiratory burst.

Figure 1 compares the effects of the calmodulin antagonists and protein kinase C inhibitors on the generation of superoxide anion by human neutrophils

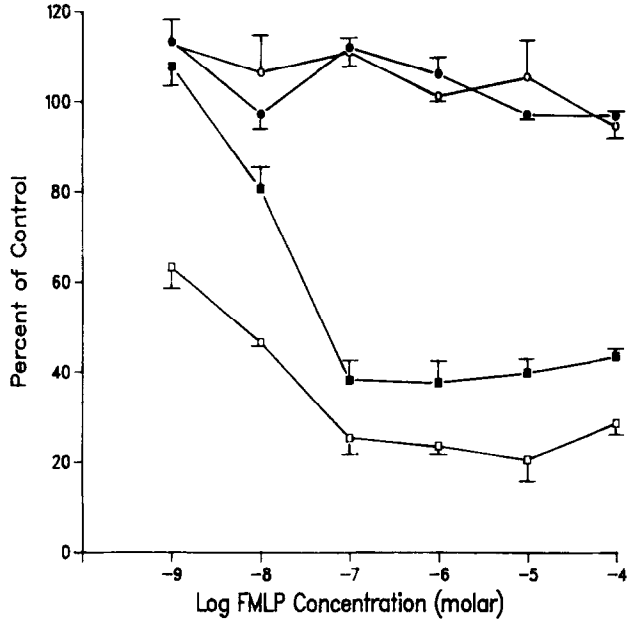

Figure 1. Effects of selected calmodulin antagonists and protein kinase C inhibitors on superoxide anion generation by FMLP-stimulated human neutrophils. Neutrophils ($1x10^6$) were stimulated with varying concentrations of FMLP for 30 minutes at 37°C. H-7 (-•-), H-9 (-o-), TFP (-▪-), and W-7 (-□-) were tested at concentrations of $1x10^{-4}$ molar. The effects of the compounds are presented as the percent of control superoxide anion generation (mean ± s.e.m. for a minimum of 2 experiments, each run in duplicate).

in response to varying doses of FMLP. An FMLP concentration range from $1x10^{-9}$ and $1x10^{-4}$M stimulated the generation of 4.9 to 15.9 nanomoles of superoxide by $1x10^{6}$ neutrophils during a 30-minute incubation at 37°C. Superoxide generation over this FMLP dose range was not inhibited by 100 micromolar H-7 or H-9. In contrast, both TFP and W-7 inhibited superoxide anion generation from $1x10^{-7}$ to $1x10^{-4}$ molar, with 60.4 ± 1.3 and 75.6 ± 1.7 mean percent inhibition, respectively. The inhibitory activity of TFP and W-7 was reduced when the neutrophils were stimulated with FMLP doses of $1x10^{-8}$ and $1x10^{-9}$ molar. At $1x10^{-9}$ M, FMLP-stimulated superoxide generation was not inhibited by TFP, while inhibition of the response by W-7 was reduced to 36.8 percent. Lack of inhibition by TFP and W-7 of xanthine oxidase-catalyzed superoxide production indicates that the compounds do not act as

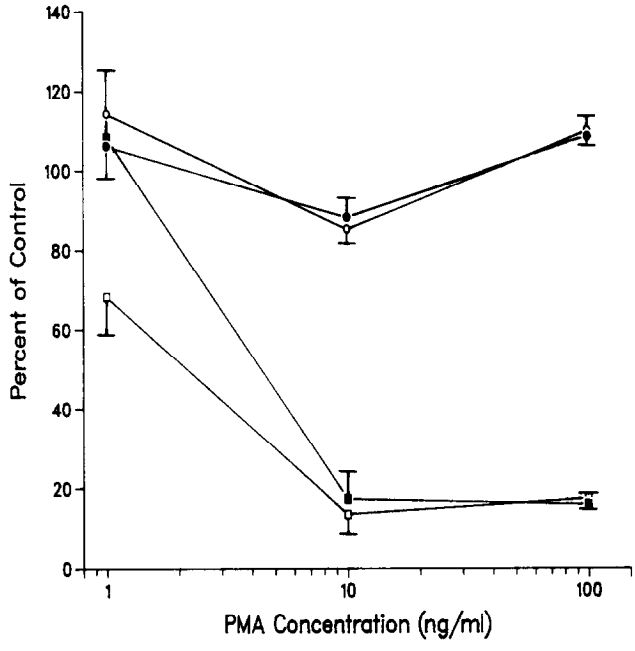

Figure 2. Effects of selected calmodulin antagonists and protein kinase C inhibitors on superoxide anion generation by PMA-stimulated human neutrophils. Neutrophils ($1x10^{6}$) were stimulated with varying concentrations of PMA for 30 minutes at 37°C. H-7 (-•-), H-9 (-o-), TFP (-▪-), and W-7 (-□-) were tested at concentrations of $1x10^{-4}$ molar. The effects of the compounds are presented as the percent of control superoxide anion generation (mean ± s.e.m. for a minimum of 2 experiments, each run in duplicate).

oxygen radical scavengers. In addition, W-5 (an inactive analogue of W-7) had no effect on superoxide generation (19).

Figure 2 compares the effects of the inhibitors on the generation of superoxide anion by human neutrophils in response to varying doses of PMA. PMA from 1 to 100 ng/ml stimulated the generation of 4.1 to 25.6 nanomoles of superoxide by $1x10^6$ neutrophils during a 30 minute incubation at 37°C. As with FMLP, H-7 and H-9 failed to inhibit PMA-stimulated superoxide anion generation over the dose range tested. Both TFP and W-7 inhibited the response to PMA at 10 and 100 ng/ml, with 83.3 ± 0.7 and 84.5 ± 2.0 mean percent

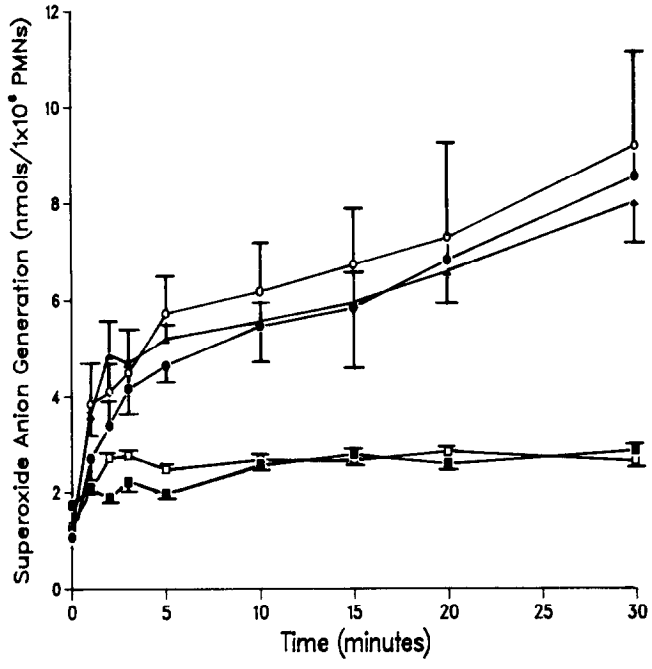

Figure 3. Effects of selected calmodulin antagonists and protein kinase C inhibitors on the time course of superoxide anion generation by FMLP-stimulated human neutrophils. Neutrophils ($1x10^6$) were incubated with $1x10^{-6}$ molar FMLP at 37°C for varying times. (H-7 (-●-), H-9 (-o-), TFP (-■-), and W-7 (-□-) were tested at concentrations of $1x10^{-4}$ molar and compared with control release (-▲-). Superoxide anion generation is quantitated as nanomoles of superoxide produced per $1x10^6$ neutrophils (mean ± s.e.m. for a minimum of 2 experiments, each run in duplicate).

Table 1. Time-Dependent Inhibition of Superoxide Anion Generation by Calmodulin Antagonists and Protein Kinase C Inhibitors

	FMLP[a]		PMA[b]	
	2 min	10 min	2 min	10 min
Control	4.87 ± 1.34[c]	5.56 ± 1.31	6.88 ± 0.74	23.30 ± 1.49
H-7[d]	3.39 ± 1.36	5.46 ± 1.48	3.25 ± 1.06*	24.70 ± 1.76
H-9[e]	4.10 ± 1.83	6.17 ± 1.96	7.90 ± 0.83	25.91 ± 0.14
TFP[f]	1.88 ± 0.02[+]	2.57 ± 0.10[+]	2.52 ± 0.03*	2.87 ± 1.12*
W-7[g]	2.70 ± 0.08[+]	2.67 ± 0.09[+]	1.87 ± 0.06*	2.23 ± 0.12*

*$p < 0.05$

[+]$p < 0.1$

[a]N-Formyl-L-methionyl-L-leucyl-L-phenylalanine, $1x10^{-6}$M.

[b]4 beta-phorbol 12 beta-myristate 13 alpha-acetate, 100 ng/ml.

[c]Nanomoles $\cdot O_2^-/1x10^6$ human neutrophils, mean ± SEM.

[d]1-(5-isoquinolinylsulfonyl)-2-methylpiperazine, $1x10^{-4}$M.

[e]N-(2-aminoethyl)-5-isoquinolinesulfonamide, $1x10^{-4}$M.

[f]Trifluoperazine, $1x10^{-4}$M.

[g]N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, $1x10^{-4}$M.

inhibition. However, the respiratory response stimulated by 1 ng/ml PMA was not inhibited by TFP, while inhibition by W-7 was reduced to 31.7 percent. Decreasing inhibition of superoxide generation by TFP and W-7 was also observed upon stimulation of neutrophils with decreasing concentrations of calcium ionophore A23187.

Inhibition of the time course of superoxide anion generation by human neutrophils.

Figure 3 compares the effects of the calmodulin antagonists and protein kinase C inhibitors on the time course of superoxide anion generation by human neutrophils stimulated with $1x10^{-6}$ molar FMLP. The total response determined 30 minutes after stimulation was not inhibited by $1x10^{-4}$ molar H-7 or H-9. However, H-7 did inhibit superoxide production during the first 5 minutes of activation (30.5 percent inhibition, two minutes after stimulation) (Table 1).

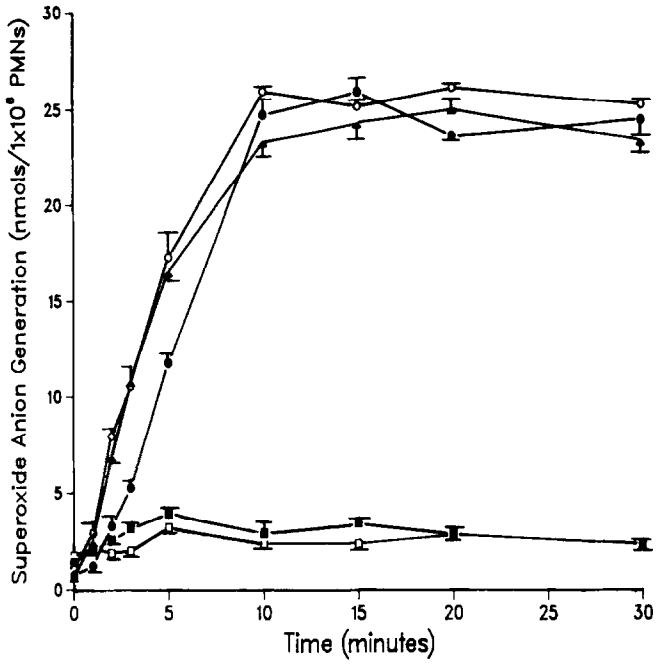

Figure 4. Effects of selected calmodulin antagonists and protein kinase C inhibitors on the time course of superoxide anion generation by PMA-stimulated human neutrophils. Neutrophils ($1x10^6$) were incubated with 100 ng/ml PMA at 37°C for varying times. H-7 (-•-), H-9 (-o-), TFP (-▪-), and W-7 (-□-) were tested at concentrations of $1x10^{-4}$ molar and compared with control release (-▲-). Superoxide anion generation is quantitated as nanomoles of superoxide produced per $1x10^6$ neutrophils (mean ± s.e.m. for a minimum of 2 experiments, each run in duplicate).

At similar doses, TFP and W-7 were potent inhibitors of superoxide anion generation, exhibiting 64.3 and 67.1 percent inhibition, respectively, after a 30 minute incubation.

Figure 4 compares the effects of the inhibitors on the time course of superoxide anion generation by human neutrophils stimulated with 100 ng/ml PMA. As with FMLP stimulation, $1x10^{-4}$ molar H-7 and H-9 failed to inhibit superoxide generation over the 30 minute time course. However, H-7 did inhibit the early generation of superoxide by 52.8 percent two minutes after stimulation. At similar doses, TFP and W-7 inhibited superoxide generation by 90.3 and 89.8 percent, respectively, after a 30 minute incubation.

DISCUSSION

Protein phosphorylation initiated by the second messengers inositol 1,4,5 trisphosphate and diacylglycerol is essential for cell activation. These second messengers have been shown to act synergistically to stimulate oxygen radical generation by human neutrophils by the use of combinations of suboptimal concentrations of calcium ionophore A23187 and PMA to increase intracellular calcium concentrations and activate protein kinase C, respectively (3,13).

Our data suggest that inhibition of protein kinase C alone is not sufficient to inhibit neutrophil activation. While generation of oxygen radicals is not inhibited over a 30 minute incubation period by the specific protein kinase C inhibitors, H-7 does reduce superoxide production during the first five minutes following stimulation. In contrast, calmodulin antagonists inhibit the entire respiratory response. These results suggest that protein kinase C may play an essential role in the generation of priming signals for activation of the NADPH oxidase while calmodulin-dependent processes are required for maintenance of oxidase activity. Incomplete inhibition of protein kinase C could allow sufficient priming of the cells to allow activation of the respiratory burst. The requirement of both priming and activation signals for regulation of NADPH oxidase has been proposed by McPhail, et.al. (11). These results support the hypothesis of Hirasawa and Nishizuka (8) that protein kinase C is required for priming cells to be fully responsive following the elevation of the intracellular calcium concentration. The critical role of calmodulin in the generation of oxygen radicals by human neutrophils is evidenced by the potent inhibition of FMLP or PMA-stimulated superoxide generation by the calmodulin antagonists. The ability of W-7 to inhibit cellular responses to PMA suggests that calmodulin-dependent processes involved in the respiratory burst occur subsequent to the protein kinase C-specific stimulation by phorbol ester. These results suggest that the processes required for maintenance of the neutrophil respiratory burst are more sensitive to inhibition than is the priming process.

Since H-7 and H-9 were originally demonstrated to inhibit protein kinase C isolated from rabbit brain, it can be argued that H-7 and H-9 failed to block human neutrophil activation because of an inability to inhibit neutrophil protein kinase C. However, we have found H-7 and H-9 to have Ki values of 9.3 and 3.0 micromolar, respectively, for human neutrophil protein kinase C as determined by the procedure of Kuo, et. al. (9). Rabbit brain protein kinase C has been reported to be inhibited by the isoquinolinesulfonamides with Ki values of 6.0 and 18.0 micromolar, respectively (7).

The role of protein kinase C in the neutrophil respiratory burst has been demonstrated by Cox, et.al. (2). Using a reconstituted cell-free system, NADPH oxidase was activated by protein kinase C in the presence of substrate for both NADPH oxidase and protein kinase C. However, the availability of substrate for NADPH oxidase in activated cells is dependent on calmodulin-dependent NAD kinase (17). This enzyme is responsible for elevating cellular levels of NADP, which can be reduced to form NADPH, the substrate for NADPH oxidase-dependent superoxide anion generation. Thus, a calmodulin antagonist could inhibit superoxide production by limiting the available substrate for NADPH oxidase.

In addition to the calmodulin- and protein kinase C-dependent processes involved in the neutrophil respiratory burst, another enzyme or cofactor may also be critical. The requirement of a third functional activation pathway is suggested by the inability of the calmodulin antagonists and protein kinase C inhibitors to block the respiratory burst in response to suboptimal concentrations of either FMLP or PMA. The involvement of such an additional component may account for the inability of TFP or W-7 to completely inhibit superoxide anion generation.

Gerard, et. al. (6) have recently described 1-(5-isoquinolinesulfonyl)-piperazine (C-I) as a potent inhibitor of protein kinase C. Unlike H-7, this demethylated analogue of H-7 also inhibits superoxide release from PMA-stimulated human neutrophils. However, such a structural modification may have provided C-I with additional biochemical properties which are responsible for

its ability to block neutrophil activation. Additional studies are required to compare the differences in the activities of H-7 and C-I.

Further research will be required to determine the roles of calmodulin and protein kinase C in the stimulus-response coupling of other cell types. Such studies may provide information concerning variations in cellular responses to different stimuli.

REFERENCES

1. Berridge, M.J. (1984) Biochem. J. 220, 345-360.
2. Cox, J.A., Jeng, A.Y., Sharkey, N.A., Blumberg, P.M., and Tauber, A.I. (1985) J. Clin. Invest. 76, 1932-1938.
3. Dale, M.M., and Penfield, A. (1984) FEBS Letters 175, 170-172.
4. DeChatelet, L.R., Shirley, P.S., and Johnson, R.B. (1976) Blood 47, 545-554.
5. Ferrante, A., and Thong, Y.H. (1978) J. Immunol. Methods 24, 389-393.
6. Gerard, C., McPhail, L.C., Marfat, A., Stimler-Gerard, N.P., Bass, D.A., and McCall, C.E. (1986) J. Clin. Invest. 77, 61-65.
7. Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. (1984) Biochemistry 23, 5036-5041.
8. Hirasawa, K., and Nishizuka, Y. (1985) Ann. Rev. Pharmacol. Toxicol. 25, 147-170.
9. Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I. Brackett, N.L., Katoh, N., Shoji, M., and Wrenn, R.W. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 7039-7043.
10. McCord, J.M., and Fridovich, I. (1968) J. Biol. Chem. 243, 5753-5760.
11. McPhail, L.C., Clayton, C.C., and Snyderman, R. (1984) J. Biol. Chem. 259, 5768-5775.
12. Prozialeck, W.C., and Weiss, B. (1982) J. Pharmacol. Exp. Ther. 222, 509-516.
13. Robinson, J.M., Bawdey, J.A., Karnovsky, M.L., and Karnovsky, M.J. (1984) Biochem. Biophys. Res. Commun. 122, 734-739.
14. Sha'afi, R.I., Molski, T.F.P., Huang, C.-K., and Naccache, P.H. (1986) Biochem. Biophys. Res. Commun. 137, 50-60.
15. Tanaka, T., Ohmura, T., Yamakado, T., and Hidaka, H. (1982) Mol. Pharmacol. 22, 408-412.
16. Thueson, D.O., Kennedy, J.A., and Conroy, M.C. (1986) Fed. Proc. 45, 386.
17. Williams, M.B., and Jones, H.P. (1985) Arch. Biochem. Biophys. 237, 80-87.
18. Wise, B.C., and Kuo, J.F. (1983) Biochem. Pharmacol. 32, 1259-1265.
19. Wright, C.D., and Hoffman, M.D. (1986) Biochem. Biophys. Res. Commun. 135, 749-755.