

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

AD-A244 419



is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this reporting burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Avenue, S.W., Washington, D.C. 20540-6047, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

REPORT DATE
June 21, 19913. REPORT TYPE AND DATES COVERED
Final, May 1988 to June 1991Regulation of Brain Muscarinic Receptors by
Protein Kinase C

5. FUNDING NUMBERS

DAAL03-88-K-0078

6. AUTHOR(S)

Esam E. El-Fakahany, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Dept. of Pharmacology and Toxicology, School of Pharmacy,
University of Maryland at Baltimore.
20 N. Pine Street
Baltimore, Maryland 21201

PERFORMING ORGANIZATION
REPORT NUMBER

JAN 08 1992

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U. S. Army Research Office
P. O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING/MONITORING
AGENCY REPORT NUMBER

ARO 25468.1-25

11. SUPPLEMENTARY NOTES

The view, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

We investigated the pharmacological classification of the subtypes of muscarinic receptors which are coupled to increased hydrolysis of phosphoinositides in rat cerebral cortex. Our results indicated that both M1 and M3 receptors mediate such a response. This response to muscarinic receptor stimulation is partially blocked by tetrodotoxin or by protein kinase C activators. The component of the response that is sensitive to blockade by such agents does not correspond to a certain receptor subtype. We investigated the role of protein kinase C in desensitization of muscarinic receptor function in a neuronal clone. While this kinase was clearly involved in the effects of phorbol esters, it did not play a role in agonist-induced receptor desensitization. We also studied some of the molecular events which accompany muscarinic receptor desensitization and down-regulation. These studies indicated that prolonged in vivo treatment of rats with an irreversible acetylcholinesterase decreased the concentration of the mRNA encoding the M2 muscarinic receptor.

14. SUBJECT TERMS

Muscarinic receptors, brain, neurons, protein kinase C,
Signal transduction, cyclic GMP, phosphoinositides.

15. NUMBER OF PAGES

8

16. PRICE CODE

17. SECURITY CLASSIFICATION
OF REPORT

UNCLASSIFIED

18. SECURITY CLASSIFICATION
OF THIS PAGE

UNCLASSIFIED

19. SECURITY CLASSIFICATION
OF ABSTRACT

UNCLASSIFIED

20. LIMITATION OF ABSTRACT

UL

92-00551

**Regulation of Brain Muscarinic Receptors
by Protein Kinase C**

FINAL REPORT

Esam E. El-Fakahany, Ph.D.

June 20, 1991

U.S. Army Research Office

Proposal 25468-LS

(Research Agreement Number DAAL03-88-K-0078)

University of Maryland at Baltimore

Approved for Public Release

Distribution Unlimited

Accession For	
NTIS CRA&I	☒
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution/	
Availability Code	
Dist	Availability Code Special
A-1	

The views, opinions, and/or findings contained in this report are those of the author and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

Statement of the Problem Studied:

The main purpose of the work was to elucidate the characteristics and the mechanisms of regulation of the signal transduction mechanisms coupled to neuronal muscarinic receptors, with emphasis on the role of protein kinase C.

Summary of Research Findings:

We have investigated the similarities in the mechanisms underlying the changes in muscarinic acetylcholine receptor sensitivity in neuronal tissue upon their exposure to protein kinase C-activating phorbol esters or to high concentrations of muscarinic agonists. Neuronal mouse neuroblastoma cells maintained in culture (clone N1E-115) were used as a model for these studies since they provide an excellent model to study regulation of the function of muscarinic receptors in the central nervous system.

Preincubation of cells with either phorbol esters or carbachol resulted in a significant loss of the ability of a subsequent addition of carbachol to stimulate cyclic GMP formation or phosphoinositide hydrolysis. However, it appears that different mechanisms mediate the effects of these two groups of agents on muscarinic receptor sensitivity. Thus, while the effects of phorbol esters are mediated by activation of protein kinase C, those of muscarinic agonists are independent of such a mechanism. Evidence for such divergent mechanisms is summarized as follows:

- a. Desensitization induced by phorbol esters was heterologous while that induced by receptor agonists was homologous.
- b. Only receptor desensitization induced by receptor agonists was accompanied by a significant decrease in the density of cell surface muscarinic receptors.
- c. Effects of phorbol esters were reversed by protein kinase C inhibitors while those of agonists were not affected.
- d. Depletion of cellular protein kinase C by prolonged incubation with phorbol esters significantly diminished the effects of these agents on muscarinic receptor sensitivity. However, this procedure had no effect on receptor desensitization induced by muscarinic agonists.

We have also studied the effects of phorbol esters and tetrodotoxin on muscarinic receptor-mediated phosphoinositide (PI) hydrolysis in rat cerebral cortex. Dissociated cerebral cortex cells were labeled with [³H]inositol and labeled inositol phosphates were isolated by a chromatographic procedure. Muscarinic agonists increased PI hydrolysis by 3-4 fold in this

preparation. Phorbol esters caused only a partial (40-50%) inhibition of muscarinic receptor-mediated PI hydrolysis. Similar results were obtained using tetrodotoxin, a voltage-dependent sodium channel blocker. Neither of these two agents affected binding of ligands to muscarinic receptors. The partial inhibition of the PI response was not attributed to a selective blockade of the response mediated by a specific muscarinic receptor subtype.

In addition, we have investigated whether phosphoinositide (PI) hydrolysis in rat cerebral cortex is mediated through the activation of one or more muscarinic receptor subtypes. PI hydrolysis was measured in dissociated brain cell aggregates by a precursor labeling technique. Muscarinic agonist-induced activation of PI hydrolysis was antagonized in a concentration-dependent manner by several muscarinic receptor antagonists, including atropine, pirenzepine, telenzepine, AF-DX 116, 4-DAMP and HHSid. These inhibition curves were monophasic, except in the case of the m1 selective antagonists pirenzepine and telenzepine. In addition, Schild plots of pirenzepine were curvilinear. However, these curves were linearized after the selective alkylation of pirenzepine low affinity binding sites. These results suggest that at least two muscarinic receptor subtypes are coupled to activation of PI hydrolysis in rat cerebral cortex. The most likely candidates are the M1 and M3 receptor subtypes.

We have previously reported that the increase in guanylate cyclase activity by muscarinic receptor agonists in a neuronal cell line is closely related to the increase in phosphoinositide hydrolysis and mobilization of intracellular calcium ions. Based on these findings we have performed experiments which demonstrated that the role of this increased intracellular calcium is to trigger the formation of an intermediate derived from L-arginine, probably similar to endothelium-derived relaxing factor (EDRF) which would activate guanylate cyclase.

We continued investigating the mechanisms underlying agonist-induced regulation of muscarinic cholinergic receptors using Chinese hamster ovary cells stably transfected by the m1 muscarinic receptor gene. The m1 receptor sequence contains conserved aspartic acid residues in transmembrane regions II and III which are thought to contribute to the binding of the positively charged neurotransmitter acetylcholine. Interestingly, we discovered that mutation of the aspartic acid residue at position 71 to asparagine abolished agonist-induced down-regulation of receptor concentration.

Relationship between in vivo down-regulation of cardiac muscarinic receptors and changes in their encoding mRNA was investigated. Rats were treated either once or for ten days with the irreversible inhibitor of acetylcholinesterase, DFP, followed by measurements of cardiac acetylcholinesterase, in addition to the density and affinity of muscarinic receptors. Messenger RNA was

quantitated using the sensitive method of DNA-excess solution hybridization. Our data indicate that while short-term treatment resulted in a marked decrease in the density of cardiac muscarinic receptors, there was no accompanying significant change in the concentration of their mRNA. In contrast, long-term inhibition of acetylcholinesterase significantly decreased the concentration of mRNA coding for these receptors. These results are indicative of multiple mechanisms of down-regulation of cardiac muscarinic receptors, some of which might involve alterations at the transcriptional level.

List of Publications:

1. Lai, W.S. and E.E. El-Fakahany: Phorbol Ester-Induced Inhibition of Cyclic GMP Formation by Muscarinic Receptors in Murine Neuroblastoma Cells. *Journal of Pharmacology and Experimental Therapeutics*, 241, 366-373, 1987.
2. Lee, J.-H. and E.E. El-Fakahany: Agonist-induced Desensitization of Muscarinic Acetylcholine Receptor in Rat Brain. *Archives of Pharmacological Research*, 10, 212-218, 1987.
3. Ramkumar, V. and E.E. El-Fakahany: Morphine Treatment Increases [3H]Nimodipine Binding Sites in Rat Brain; Attenuation by Nimodipine and Other Calcium Channel Antagonists of Naloxone-Precipitated Withdrawal in Morphine-Dependent Animals. *Annals of the New York Academy of Sciences*, 522, 207-209, 1988.
4. Lai, W.S. and E.E. El-Fakahany: Regulation of [3H]Phorbol-12,13-Dibutyrate Binding Sites in Mouse Neuroblastoma Cells: Simultaneous Down-Regulation by Phorbol Esters and Desensitization of Their Inhibition of Muscarinic Receptor Function. *Journal of Pharmacology and Experimental Therapeutics*, 244, 41-50, 1988.
5. Ramkumar, V. and E.E. El-Fakahany: Prolonged Morphine Treatment Increases Rat Brain Dihydropyridine Binding Sites; Possible Involvement in Development of Morphine Dependence. *European Journal of Pharmacology*, 146, 73-83, 1988.
6. Mattia, A., A.P. Leccese, K.L. Marquis, E.E. El-Fakahany and J.E. Moreton: Electroencephalographic (EEG), Behavioral, and Receptor Binding Correlates of Phencyclidinoids in the Rat. *Journal of Pharmacology and Experimental Therapeutics*, 246, 797-802, 1988.

7. Lee, N.H. and E.E. El-Fakahany: Influence of Ligand Choice on the Apparent Binding Profile of Gallamine to Cardiac Muscarinic Receptors; Identification of Three Main Types of Gallamine-Muscarinic Receptor Interactions. *Journal of Pharmacology and Experimental Therapeutics*, 246, 829-838, 1988.
8. Cioffi, C.L. and E.E. El-Fakahany: Lack of Alterations of Muscarinic Receptor Subtypes and Phosphoinositide Hydrolysis upon Acute DFP Treatment. *European Journal of Pharmacology*, 156, 35-45, 1988.
9. McKinney, M., N.H. Lee, D.J. Anderson, L. Vella-Rountree and E.E. El-Fakahany: Nonselectivity of Amitriptyline for Subtypes of Muscarinic Receptors Demonstrated in Binding and Functional Studies. *European Journal of Pharmacology*, 157, 51-60, 1988.
10. El-Fakahany, E.E., W. Surichamorn, C.L. Amrhein, S. Stenstrom, C.L. Cioffi, E. Richelson and M. McKinney: Pseudo-Noncompetitive Antagonism of Muscarinic Receptor-Mediated Cyclic GMP Formation and Phosphoinositide Hydrolysis by Pirenzepine. *Journal of Pharmacology and Experimental Therapeutics*, 247, 934-940, 1988.
11. Surichamorn, W., O.N. Kim, N.H. Lee, W.S. Lai and E.E. El-Fakahany: Effects of Aging on the Interaction of Quinuclidinyl Benzilate, N-Methylscopolamine, Pirenzepine and Gallamine with Brain Muscarinic Receptors. *Neurochemical Research*, 13, 1183-1191, 1988.
12. Cioffi, C.L. and E.E. El-Fakahany: Differential Sensitivity of Phosphoinositide and Cyclic GMP Responses to Short-Term Regulation by a Muscarinic Agonist in Mouse Neuroblastoma Cells: Correlation with Down-Regulation of Cell Surface Receptors. *Biochemical Pharmacology*, 38, 1827-1834, 1989.
13. Surichamorn, W., C.L. Amrhein, C. Forray and E.E. El-Fakahany: Inhibition of Cyclic AMP Formation in N1E-115 Neuroblastoma Cells is Mediated by a Noncardiac M2 Muscarinic Receptor Subtype. *Brain Research*, 493, 320-325, 1989.
14. Lee, N.H. and E.E. El-Fakahany: Mixed Competitive and Allosteric Antagonism by Gallamine of Muscarinic Receptor-Mediated Second Messenger Responses in N1E-115 Neuroblastoma Cells. *Journal of Neurochemistry*, 53, 1300-1308, 1989.

15. McKinney, M., D. Anderson, C. Forray and E.E. El-Fakahany: Characterization of the Striatal M2 Muscarinic Receptor Mediating Inhibition of Cyclic AMP Using Selective Antagonists: A Comparison with the Brainstem M2 Receptor. *Journal of Pharmacology and Experimental Therapeutics*. 250, 565-572, 1989.
16. Lai, W.S. and E.E. El-Fakahany: The Diacylglycerol Kinase Inhibitor R59022 antagonizes Muscarinic Receptor-Mediated Cyclic GMP Formation and Binding of [³H]N-Methylscopolamine. *Biochemical Pharmacology*, 39, 221-222, 1990.
17. Lee, N.H., C. Forray and E.E. El-Fakahany: Methoctramine, a Cardiosensitive Muscarinic Antagonist, Stimulates Phosphoinositide Hydrolysis in Rat Cerebral Cortex. *European Journal of Pharmacology*. 167, 295-298, 1989.
18. Surichamorn, W., E.A.M. Abdallah and E.E. El-Fakahany: Aging does not Alter Brain Muscarinic Receptor-Mediated Phosphoinositide Hydrolysis and its Inhibition by Phorbol Esters, Tetrodotoxin and Receptor Desensitization. *Journal of Pharmacology and Experimental Therapeutics*. 251, 543-549, 1989
19. Lee, N.H., A.D. Fryer, C. Forray and E.E. El-Fakahany: Different Mechanisms of Antagonism by Methoctramine of Two Neuronal Muscarinic Receptor-Mediated Second Messenger Responses. *Journal of Pharmacology and Experimental Therapeutics*. 251, 992-999, 1989.
20. Fryer, A.D. and E.E. El-Fakahany: An Endogenous Factor Induces Heterogeneity of Binding Sites of Selective Muscarinic Receptor Antagonists in Rat Heart. *Membrane Biochemistry*. 8, 127-132, 1989.
21. Lai, W.S., T.B. Rogers and E.E. El-Fakahany: Protein Kinase C is Involved in Desensitization of Muscarinic Receptors Induced by Phorbol Esters but not by Receptor Agonists. *The Biochemical Journal*, 267, 23-29, 1990.
22. Surichamorn, W., C. Forray and E.E. El-Fakahany: The Role of Intracellular Ca²⁺ in Muscarinic and Histamine Receptor-Mediated Activation of Guanylate Cyclase in Cultured Mouse Neuroblastoma Cells (Clone N1E-115). Assessment of the Arachidonic Acid Release Hypothesis. *Molecular Pharmacology*. 37, 860-869, 1990.
23. Abdallah, E.A.M., C. Forray and E.E. El-Fakahany: Relationship between the Partial Inhibition of Muscarinic Receptor-Mediated Phosphoinositide Hydrolysis by Phorbol Esters and Tetrodotoxin in Rat Cerebral Cortex. *Molecular Brain*

Research. 8, 1-7, 1990.

24. Lee, N.H. and E.E. El-Fakahany: The Allosteric Binding Profile of Himbacine; a Comparison with Other Cardiosensitive Antagonists. *European Journal of Pharmacology*. 179, 225-229, 1990.
25. Fryer, A.D., E.E. El-Fakahany and D.B. Jacoby: Parainfluenza Virus Type 1 Reduces the Affinity of Agonists for Muscarinic Receptors in Guinea-Pig Lung and Heart. *European Journal of Pharmacology*, 181, 51-58, 1990.
26. Forray, C. and E.E. El-Fakahany: On the Nature of Multiple Muscarinic Receptor Subtypes that Activate Phosphoinositide Metabolism in Rat Cerebral Cortex. *Molecular Pharmacology*. 37, 893-902, 1990.
27. Fryer, A.D. and E.E. El-Fakahany: Identification of Three Muscarinic Receptor Subtypes in Rat Lung Using Binding Studies with Selective Antagonists. *Life Sciences*. 47, 611-618, 1990.
28. Silverman, H.J., N.H. Lee and E.E. El-Fakahany: Regulation of Beta-Adrenergic Receptors in Canine Endotoxic Shock; Role of Catecholamines. *Circulatory Shock*, 32, 293-306, 1990.
29. Hu, J. and E.E. El-Fakahany: Selectivity of McN-A-343 in Stimulating Phosphoinositide Hydrolysis Mediated by M1 Muscarinic Receptors. *Molecular Pharmacology*. 38, 895-903, 1990.
30. Abdallah, E.A.M., W.S. Pou and E.E. El-Fakahany: Aging Does not Alter Muscarinic Receptor-Mediated Inhibition of Cyclic AMP Formation in the Striatum and Hippocampus. *Brain Research*. 534, 234-236, 1990.
31. Pou, W.S., S. Pou, G.M. Rosen and E.E. El-Fakahany: EDRF Release is a Common Pathway for the Activation of Guanylate Cyclase by Receptor Agonists and Calcium Ionophores. *European Journal of Pharmacology*. 182, 393-394, 1990.
32. Wang, S.Z., J. Hu, R.M. Long, W.S. Pou, C. Forray and E.E. El-Fakahany: Agonist-Induced Down-Regulation of m1 Muscarinic Receptors and Reduction of their mRNA Level in a Transfected Cell Line. *FEBS Letters*. 276, 185-188, 1990.
33. Pou, S., W.S. Pou, G.M. Rosen and E.E. El-Fakahany: N-Hydroxylamine is not an Intermediate in the Conversion of L-Arginine to an Activator of Soluble Guanylate Cyclase in Neuroblastoma N1E-115 Cells. *The Biochemical Journal*.

273, 547-552, 1991.

34. Arroyo, C.M., C. Forray, E.E. El-Fakahany and G.M. Rosen: Receptor-Mediated Generation of an EDRF-Like Intermediate in a Neuronal Clone Detected by Spin Trapping Techniques. *Biochemical and Biophysical Research Communications*. 170, 1177-1183, 1990.
35. Lee, N.H. and E.E. El-Fakahany: Allosteric Interactions at the m1, m2 and m3 Muscarinic Receptor Subtypes. *Journal of Pharmacology and Experimental Therapeutics*. 256, 468-479, 1991.
36. Jett, D.A., E.A.M. Abdallah, E.E. El-Fakahany, M.E. Eldefrawi and A.T. Eldefrawi: The High Affinity Activation by Paraoxon of a Muscarinic Receptor Subtype in Rat Brain Striatum. *Pesticide Biochemistry and Physiology*. 39, 149-157, 1991.
37. Hu, J., S.Z. Wang and E.E. El-Fakahany: Effects of Agonist Efficacy on Desensitization of Phosphoinositide Hydrolysis Mediated by m1 and m3 Muscarinic Receptors Expressed in CHO Cells. *Journal of Pharmacology and Experimental Therapeutics*, in press.
38. Brezenoff, H.E., E.E. El-Fakahany and Y.F. Xiao: The Effect of 4-DAMP Mustard on Blood Pressure and Muscarinic Binding in Spontaneously Hypertensive Rats. *Annals of Neuroscience*. 1, 24-33, 1990.
39. Fernando, J.C.R., E.A.M. Abdallah, M. Evinger, C. Forray and E.E. El-Fakahany: The Presence of an M4 Subtype Muscarinic Receptor in the Bovine Adrenal Medulla Revealed by mRNA and Receptor Binding Analyses. *European Journal of Pharmacology (Molecular Section)*, in press.
40. Zhu, S.Z., S.Z. Wang, E.A.M. Abdallah and E.E. El-Fakahany: In Vivo Regulation of Cardiac Muscarinic Receptor mRNA Measured by DNA-Excess Solution Hybridization. *Life Sciences*. 48, 2579-2584, 1991.

List of participating scientific personnel:

Jingru Hu, Ph.D.
Shi-Zhen Tao

Report of inventions:

No inventions.