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The M₁ muscarinic receptor and its second messenger coupling in human neuroblastoma cells and transfected murine fibroblast cells

Mei, Lin, Ph.D.
The University of Arizona, 1989
THE M₃ MUSCARINIC RECEPTOR AND ITS SECOND MESSENGER COUPLING
IN HUMAN NEUROBLASTOMA CELLS AND TRANSFECTED
MURINE FIBROBLAST CELLS

By
Lin Mei

A Dissertation Submitted to the Faculty of the
COMMITTEE ON PHARMACOLOGY AND TOXICOLOGY (GRADUATE)
In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1989
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Lin Mei entitled The M1 Muscarinic Receptor and Its Second Messenger Coupling in the Human Neuroblastoma Cells and the Transfected Murine Fibroblast Cells and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director

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SIGNED: [Signature]
DEDICATION

This dissertation is dedicated to my wife, Wen Cheng, in appreciation for her love, understanding and encouragement, and my parents, my brother and my sister who gave me support. Knowing that they were behind me made this dissertation possible.
ACKNOWLEDGEMENTS

I thank my advisors Drs. Henry I. Yamamura and William R. Roeske who were willing to take a chance on a Chinese student, for their supervision, guidance and encouragement. For their time and input into my graduate training, thanks are given to my Graduate Committee members Drs. Thomas F. Burks, Thomas J. Lindell, and David L. Nelson. Sincere appreciation goes to the Dr. Thomas F. Burks for his support and interest in my scientific career. I appreciate the collaboration with Dr. Josephine Lal and her help. Thanks are also given to Drs. Raphael Gruener, Ryan Huxtable, Hugh Laird, Frank Porreca, and Glenn Sipes for their advice and suggestions. I express my gratitude to Dr. Jian-Xin Wang for showing me how to do my first radioligand binding assay and his assistance. Thanks are due to the post-doctoral fellows in the laboratory, old and new: Drs. Yutaka Fujiwara, Masaaki Ikeda, Richard Knapp, Mariangela Serra, Robert Speth and Mark Watson. I would like to thank Ms. Carol A. Haussler and Mr. Alfred C. Gallegos for cell culture assistance, Ms. Sue Waite for technical assistance and reading manuscripts, Ms. Pam Davis for secretarial help, and Ms. Pam Murray for help in graduate affairs.
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ABSTRACT

The data of this study indicate that pirenzepine (PZ)-high affinity muscarinic receptors (mAChRs) are coupled to the hydrolysis of inositol lipids and not to the adenylate cyclase system in human neuroblastoma SH-SY5Y cells. The maximal carbachol (CCh)-stimulated \(^{3}H\)IP\(_{1}\) accumulation in the SH-SY5Y cells was decreased in the presence of 1 \(\mu\)g/ml pertussis toxin, suggesting that a pertussis toxin sensitive G-protein may be involved in the coupling.

Several cell clones which express only M\(_{1}\) mAChR were generated by transfecting the murine fibroblast B82 cells with the cloned rat genomic M\(_{1}\) gene. The transfected B82 cells (cTB10) showed specific \(^{3}H\)(\(-\))QNB binding activity. The mAChRs in these cells are of the M\(_{1}\) type defined by their high affinity for PZ and low affinity for AF-DX 116 and coupled to hydrolysis of inositol lipids, possibly via a pertussis toxin sensitive G protein.

The relationship between the M\(_{1}\) mAChR density and the receptor-mediated hydrolysis of inositol lipids was studied in 7 clones. The M\(_{1}\) mAChR densities in these cells characterized by \(^{3}H\)(\(-\))MQNB binding ranged from 12 fmol/10\(^6\) cells in LK3-1 cells to 260 fmol/10\(^6\) cells in the LK3-8 cells. The Hill coefficients of the CCh/\(^{3}H\)(\(-\))MQNB competition curves were close to unity for
the LK3-1 cells and were less than one in the higher receptor clones. The percentage of the M₁ mAChRs which had high affinity for CCh decreased as the receptor density increased, suggesting the presence of endogenous factors in these cells which may be important for the agonist affinity state of the receptor. A significant correlation was observed between the density of the M₁ mAChR with high affinity for CCh and the maximum [³H]IP₁ accumulation in these cells. There is no significant difference among the CCh EC₅₀ values, its Kₐ values and the Kₖ values of the CCh/[³H](~)MQNB competition curves. These results suggest that the high affinity state for CCh may be the functional state of the M₁ mAChRs in these cells. Although a linear correlation between the total M₁ mAChR density and the maximum [³H]IP₁ accumulation was observed, there was evidence for the existence of spare M₁ receptors in the clones with high receptor densities but not in those with low receptor densities.
CHAPTER 1

INTRODUCTION

Origin of the receptor concept and acetylcholine receptors

The origin of receptors dates back to the late 19th century. John Newport Langley (1878) explained the mutual antagonism between atropine and pilocarpine by assuming that "there is a substance or substances in the nerve endings or gland cells with which both atropine and pilocarpine are capable of forming compounds." In 1905, he observed that nicotine caused a prolonged contraction of frog gastrocnemius muscles. Curare blocked this effect. "These two poisons are mutually antagonistic as regards stimulating effect on the muscles. Denervation of the muscles left essentially unaltered the contractile effects of nicotine but increased responsiveness to nicotine." He wrote, "I conclude that the poisons do not act directly on the contractile substance, but on other substances in the muscle which may be called receptive substances" (Langley, 1905). He generalized this concept by stating that not only "the majority of substances which are ordinarily supposed to act upon nerve-endings act upon the receptive substances of the cells", but also substances from the endocrinal glands "act on receptive substances, although in these cases the cells may be
unconnected with nerve fibers".

The term "receptor" was actually coined by Paul Ehrlich (1900). He presented his famous "side chain theory" first to explain the specific tissue staining by certain dyes, then to explain the interaction of bacterial toxins with the cells. A toxin has two groups "haptophore" as an anchorer, and "toxophile" as a poisoner. The cells have side chains to interact with the haptophore. Once the toxin is anchored to the cell through interaction of the haptophore with the side chain, the cell comes under the influence of the toxophore group (Ehrlich, 1900). Ehrlich referred to the side chains as "receptors". In 1907, he applied his side chain theory to drug actions. He stated: "some of the chemically defined substances are attached to the cell by atom groupings that are analogous to toxin-receptors; these atom groupings I will distinguish from the toxin-receptors by the name of 'chemoreceptors'" (for review, see Parascandola and Jasensky, 1974).

The phenomena that led Langley to coin the term "receptive substance" were actually involved with acetylcholine receptors in both cases. The term acetylcholine receptor was not accepted widely until the role of acetylcholine as a neurotransmitter was established by Otto Loewi (1922), who demonstrated that the so-called "vagusstoff" released into the perfusion fluid of a frog heart vagally stimulated, contained acetylcholine.

At the time, Langley did not notice the differences between the receptors for pilocarpine-atropine mutual antagonism and those
for nicotine-curare mutual antagonism. However, he did point out the receptive substance of cells, even of the same class, varies considerably (Langley, 1905). This difference is "mainly due to an inherent tendency to variation in the chemical nature of the cells." Some later years, Sir Henry Dale (1914) studied the actions of certain esters and ethers of choline and classified the pharmacological actions of acetylcholine into two different categories: "a 'muscarinic' action, paralysed by atropine, and a 'nicotine' action, paralysed by excess of nicotine". This simple classification was rapidly and fully endorsed by pharmacologists. Hereinafter, acetylcholine receptors are classified into two types: muscarinic acetylcholine receptors, muscarinic cholinergic receptors, or muscarinic receptors and nicotinic acetylcholine receptors, nicotinic cholinergic receptors, or nicotinic receptors. The nicotinic receptors are located at neuromuscular junctions, autonomic ganglions, or some areas in the central nervous system. They are activated by acetylcholine, nicotine, or carbachol and antagonized by d-tubocurare as observed in the mutual nicotine-curare mutual antagonism in frog striated muscle by Langley (1905).

Muscarinic receptors

Localization of muscarinic receptors

Autoradiographic studies showed that muscarinic receptors are widely distributed in the human central nervous system (Cortes
et al., 1987). The highest densities of muscarinic receptors were found in the striatum, olfactory tubercle and tuberal nuclei of the hypothalamus; intermediate densities were observed in the hippocampal formation, cerebral cortex and amygdala but low in the hypothalamus, globus pallidus and basal forebrain nuclei, and very low in the cerebellum and white matter tract. In the thalamus muscarinic receptors were heterogeneously distributed, with densities ranging from very low to high. In the brainstem, muscarinic receptors are associated with many nuclei including the nucleus facialis, the motor trigeminal nucleus, the nucleus solitarius, the parabrachial nuclei and some areas of the gray matter such as the periaqueductal gray matter and the ventral tegmental area (Cortes et al., 1984). Muscarinic receptors also distribute in certain areas of the spinal cord (Gillberg et al., 1984). Evidence has been shown that muscarinic receptors in the brain are associated with the synapses (Kuhar et al., 1981). Studies with dissociated cingulate cortical neurons found that they are present on the smooth surfaces of pyramidal and multipolar neurons (Vogt et al., 1987). Muscarinic receptors may locate on the presynaptic (Mash et al., 1985) and postsynaptic cholinergic synapses (Vogt et al., 1987). Muscarinic receptors in the brain also include those associated with the microvasculature (Estrada et al., 1983) and with the glia cells (Repke and Maderspach, 1982).

In the periphery, muscarinic receptors are located in the smooth muscle cells of the gastrointestinal tract (Ehlert et al.,
1980), the respiratory system (van Koppen et al., 1988), the blood vessels (Komori and Suzuki, 1987; O'Rourke and Vanhoutte, 1987), the urinary tract (Anderson et al., 1985), and the reproductive system (Doggrell, 1986); the heart (Fields et al., 1978); the exocrine glands (Culp and Marin, 1986; Batra, 1987; Martos et al., 1987) and the other tissues innervated by parasympathetic fibers (van Megen et al., 1988). The endothelium of the rabbit aorta also has muscarinic receptors (Stephenson and Summers, 1987).

In certain tissues such as the adrenal medulla which are innervated by sympathetic nerves also have muscarinic receptors (Yamanaka et al., 1986). The autonomic ganglia (Watson et al., 1986a) and the intracardiac neurons (Hassall et al., 1987) have muscarinic receptors. In addition, muscarinic receptors were observed in the nerve terminals of the adrenergic nerves (O'Rourke and Vanhoutte, 1987) and of the phrenic nerves (Wessler et al., 1987). Muscarinic receptors were also observed in the tissues or cells that have no innervation at all. These tissues or cells include erythrocytes (Aronstam et al., 1977) and lymphocytes (Adem et al., 1986; Maslinski et al., 1987).

Functions of muscarinic receptors

Stimulation of the peripheral muscarinic receptors generally causes contraction of the smooth muscles, secretion of the exocrine glands, bradycardia, and negative inotropic effects (Lindmar and Loffelholz, 1987). Muscarinic receptors in the brain are involved
in homeostatic regulation mechanisms such as thermoregulation, and feeding and drinking behaviors (Karczmar, 1976; Schiavone et al., 1987), hypothalamic control of pituitary hormone secretion (Lewis and Shute, 1978); motor behaviors (Lewis and Shute, 1978) and sensory mechanisms (Karczmar, 1976; Schiavone et al., 1987), hypothalamic control of pituitary hormone secretion (Lewis and Shute, 1978); motor behaviors (Lewis and Shute, 1978) and sensory mechanisms (Karczmar, 1976); and learning and memory (Brimblecombe, 1974; Fukuchi et al., 1987; Krnjevic, 1974). Administration of muscarinic agonists produced analgesia and potentiated the actions of narcotic and non-narcotic analgesics and had effects on arousal, attention and rapid eye movement sleep, affective disorders and depression, stress (Brimblecombe, 1974; Cortes et al., 1987; Lewis and Shute, 1978) and passive avoidance behaviors (Fukuchi et al., 1987).

Relationship of muscarinic receptors with G proteins

Muscarinic receptors are of the G protein-associated super-gene family including rhodopsin, adrenergic receptors, serotonin receptors, dopamine receptors, substance K receptors, angiotensin receptors and muscarinic receptors. G proteins are a group of membrane-associated guanyl nucleotide binding proteins which are heterotrimeric, with subunits designated α, β and γ. Interaction of the muscarinic receptors with the G proteins not only determines the binding characteristics of the receptors, but also,
and more importantly, transduces the signal accepted by the receptors to the subsequent macromolecules, usually the effectors. Muscarinic receptors in the lipid bilayer membrane catalyze the exchange of GDP for GTP binding to the $\alpha$ subunit of a specific G protein and dissociation of the $\beta\gamma$ subunits from the $\alpha$.GTP complex (Weiss et al., 1988). $\alpha$.GTP regulates the appropriate effectors such as the enzymes adenylate cyclase, or phosphatidylinositol-specific phosphodiesterase. Muscarinic receptors associated with the G proteins stay in the high affinity state for the agonist when the G proteins are bound with GDP. The binding of the G proteins with GTP to release GDP causes dissociation of the receptors when the latter have low affinity for the agonist (Gilman, 1987).

Evidence for the G protein involvement in the muscarinic receptor-second messenger coupling includes: decrease of muscarinic agonist (but not antagonist) binding affinity by guanyl nucleotides (Berrie et al., 1979; Rosenberger et al., 1979); dependence of muscarinic responses on GTP (Jakobs, 1979; Jakobs et al., 1979; Ollanas et al., 1983); imitation of muscarinic responses by agents which could directly stimulate G proteins, including GppNHp (Fleming et al., 1987), GTPyS (Cockcroft and Gomperts, 1985) or AF$^4_4$ (Paris and Pouyssegur, 1987; Jope, 1988) and sensitivity of muscarinic responses to specific bacterial toxins such as pertussis toxin or cholera toxin (Hughes et al., 1984; Helper et al., 1987). Reconstituted muscarinic receptors showed high affinity for the agonist when the receptors were associated with the G proteins (Haga
et al., 1986).

A history of muscarinic receptor heterogeneity studies

As early as 1951, Riker and Wescoe (1951) noted a selective muscarinic antagonistic effect of gallamine, a neuromuscular blocker. They reported that while it produced a potent blockade of the neuromuscular junction, gallamine had an atropine-like action which was confined to the cardiac vagus. It had no such action on sweating, salivary secretion, gut motility and the central actions of certain cholinesterase inhibitors (Riker and Wescoe, 1951). If muscarinic receptors were involved in the gallamine actions, these results should have indicated that different muscarinic receptor types exist among the different tissues. However, these results were virtually overlooked for almost 30 years. In 1961, Roszkowski (1961) reported a new muscarinic agonist, McN-A-343 [4-(m-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride], which had selective stimulating effects in sympathetic ganglia. Since atropine but not hexamethonium blocked its actions, it was believed to be a muscarinic agonist. It had no or little stimulating effect on the peripheral organs. This hint of nature was again missed. The results were explained only as evidence for the existence of muscarinic receptors in sympathetic ganglia.

Burgen and Spero (1968) studied the actions of acetylcholine and related parasympathetic drugs on the efflux of potassium and rubidium from smooth muscle of the guinea-pig intestine. These
drugs produced a very large increase in the efflux of potassium and rubidium from the longitudinal muscle with a much higher dose range than to produce contraction. If two distinct receptors were present they should display differences in their behavior to antagonists. Indeed, benzhexol, a muscarinic antagonist, was 8 times as active on efflux as on contraction. They concluded that 'the most satisfactory explanation of these results is that there are two types of the muscarinic receptor in the smooth muscle of the guinea-pig intestine'. In 1976, a study using weak antagonists indicated possible heterogeneity of muscarinic receptors in guinea pig ileum, mouse salivary gland, cat superior cervical ganglion and mouse CNS (Fisher et al., 1976). Barlow and colleagues (1976) presented a muscarinic antagonist, 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide), which had lower affinity for muscarinic receptors in guinea-pig ileum than for the receptors in the ileum. Muscarinic receptors were then classified into $m_1$ in the ileum and $m_2$ in the atria (Barlow et al., 1979).

Goyal and Rattan (1978) classified muscarinic receptors in the lower esophageal sphincter (LES) into two types. $M_1$ muscarinic receptors are those on the intramural autonomic ganglia while the muscarinic receptors on the effector organ LES are of the $M_2$ type. McN-A-343 exerts a preferential action on the $M_1$ muscarinic receptors and causes LES inhibition. Bethanechol, in contrast, is selective in stimulating $M_2$ muscarinic receptors and
mediates LES contraction.

During the time when pharmacologists were trying to classify muscarinic receptors by bioassays, \((-\text{quinuclidinyl benzilate})\) (QNB), a nonselective muscarinic antagonist with high affinity for muscarinic receptors was used in radioligand binding assays to identify muscarinic receptors (Yamamura and Snyder, 1974). The radioligand binding assays using \([^{3}\text{H}]\text{-QNB}\) avoids the biobarrier in the bioassays and makes it possible to study directly the interaction between the ligand and muscarinic receptors. This approach accelerated the studies of muscarinic receptor heterogeneity. Birdsall and colleagues (1978) noted the flattened agonist/\([^{3}\text{H}]\text{-antagonist}\) competition curves for muscarinic receptors in the rat brain. They identified three populations of muscarinic receptors in the rat brain based on their affinities for the agonists: a major component with low affinity (L), a smaller component with higher affinity (H) and a minor component with super high affinity (SH), none of which was distinguishable by the studied muscarinic antagonists available at that time (Hulme et al., 1978). Not until the introduction of pirenzepine, a drug used clinically in the treatment of gastric ulcer disease, did the heterogeneity of muscarinic receptors attract so much attention.

Hammer et al. (1980) observed that muscarinic receptors in the hippocampus and cerebral cortex had higher affinity for pirenzepine while those in the heart and smooth muscle had lower affinity. Three sites were compatible with \(K_d\) values of
pirenzepine which were 20 pM, 200 pM and 1000 pM, respectively. These findings were verified by using $^{3}$Hpirenzepine in the radioligand binding assay (Watson et al., 1982; Watson et al., 1986b). High densities of high affinity $^{3}$Hpirenzepine binding sites have been detected in homogenates of cerebral cortex, corpus striatum and hippocampus while low affinity $^{3}$Hpirenzepine binding sites predominate in homogenates of cerebellum and other areas of the CNS, and peripheral tissues such as the ileum and the heart.

**Different types of muscarinic receptors**

Radioligand binding properties of muscarinic receptor types

Muscarinic receptors are heterogeneous in the radioligand binding properties, the localizations, the second messenger couplings and the functions. The muscarinic receptors which have high affinity for pirenzepine were called M₁ muscarinic receptors while those which have low affinity for pirenzepine were called M₂ muscarinic receptors (Watson et al., 1986b). The dissociation constants of pirenzepine were about 10 nM for the M₁ muscarinic receptors and about 300 nM for the M₂ muscarinic receptors. Atropine has equal affinity for both types with a $K_d$ value of 0.3-1 nM (Watson et al., 1986d). The M₁ and M₂ types of muscarinic receptors had reverse affinities for AF-DX 116 (11-[[2-[(diethylamino)methyl]-1-piperidinyllacetyl]-5,11-dihydro-6H-pyrid[2,3-b][1,4]-benzodiazepine-6-one), a novel muscarinic
antagonist. The $K_d$ values of AF-DX 116 were about 500 nM for $M_1$ muscarinic receptors and about 40 nM for $M_2$ muscarinic receptors (Watson et al., 1986d; Wang et al., 1987c). Studies have shown that the heterogeneity of muscarinic receptors for pirenzepine binding in the rat forebrain and heart membranes disappeared after solubilization (Wang et al., 1987a). Also the thermodynamic properties of pirenzepine binding to the solubilized muscarinic receptors from forebrain and heart were similar (Mei et al., 1987). These studies suggest that the biomembrane microenvironment may play an important role in the heterogeneity of muscarinic receptors.

Interestingly, other types of muscarinic receptor may be characterized by having low affinity for both pirenzepine ($K_i$ of 430 nM) and AF-DX 116 ($K_i$ of 3700 nM) in the rat pancreas (Korc et al., 1987) or having high affinity for both pirenzepine ($K_i$ of 46 nM) and AF-DX 116 ($K_i$ of 34 nM) in the rabbit peripheral lung (Bloom et al., 1987). Himbacine could recognize a high affinity subtype of $M_2$ muscarinic receptors in the rat cerebral cortex labeled by $[^3H]AF-DX 116$ (Wang et al., 1988). Some muscarinic antagonists were reported selective for $M_3$ muscarinic receptors, including azaprophen (Witkin et al., 1987), 4-DAMP (Doods et al., 1987) and HHSiD (hexahydrosiladifenidol) (Akiba et al., 1988).

The heterogeneous muscarinic receptors could also be differentiated by agonist binding properties. Classical muscarinic agonists like carbachol and acetylcholine show significant selectivity for $M_2$ muscarinic receptors whereas pilocarpine and
McN-A-343 show significant selectivity for $M_1$ muscarinic receptors (Vickroy et al., 1984; Watson et al., 1986c). They may have different regulatory mechanisms. Guanyl nucleotides exert a specific regulatory function on the muscarinic receptors (Berrie et al., 1979; Rosenberger et al., 1979). The affinity of muscarinic receptors for the agonist could be decreased by the guanyl nucleotides. The agonist binding to $[^3H](\text{--})QNB$ labeled $M_2$ cardiac muscarinic receptors is more sensitive to GppNHp than $[^3H](\text{--})QNB$ labeled $M_1$ muscarinic receptors (Watson et al., 1986c). Divalent cations particularly magnesium ions have selective effects on muscarinic receptors. Magnesium ions were shown to enhance the affinity of $M_2$ muscarinic receptors for agonists but have little effect on muscarinic agonist binding to $M_1$ muscarinic receptors (Vickroy et al., 1983 and 1984). N-ethylmaleimide (NEM) reduced the labeling of $M_2$ muscarinic receptors by $[^3H]$oxotremorine-M by converting the high-affinity $M_2$ muscarinic receptors to a low affinity state, without affecting the affinity of the $M_1$ muscarinic receptors (Horvath et al., 1986).

Interestingly, treatment of the rat in vivo with methamphetamine had no effect of $[^3H]$pirenzepine binding in the brain but decreased the $M_2$ muscarinic receptors labeled by $[^3H]$N-methylscopolamine (McCabe et al., 1987). This decrease was accompanied by methamphetamine-induced decreases in tyrosine hydroxylase and tryptophan hydroxylase activities.
Distribution of different types of muscarinic receptors

The M₁ and M₂ muscarinic receptors are distributed unevenly in the brain. The autoradiogram using [³H]pirenzepine showed that [³H]pirenzepine binding was highest in the CA1 region of the hippocampus, in the molecular layer of the gyrus dentatus, in the corpus striatum and the superficial laminae of the parietal cortex and was lowest in the corpus callosum (Yamamura et al., 1983; Wamsley et al., 1984). In the spinal cord [³H](-)QNB labeled the dorsal horn with the substantia gelatinosa of the dorsal horn heavily labeled and the lateral aspect of the ventral horn whereas [³H]pirenzepine labeled only the substantia gelatinosa and to the rest of the dorsal horn and the ventral horn. [³H]Pirenzepine labeled sites have been observed in human stellate ganglia (Yamamura et al., 1984). The [³H](-)QNB binding sites in the cerebellum, nucleus tractus solitarius and facula nucleus were not labeled by [³H](-)QNB with pirenzepine as an inhibitor confirmed these results (Mash and Potter, 1986). In addition, these authors found M₁ muscarinic receptors in the olfactory tubercle, nucleus accumbens and amygdala. In agreement, an autoradiographic study using [³H]AF-DX 116 showed that the regions not labeled by [³H]pirenzepine could be identified by [³H]AF-DX 116 (Rogenold et al., 1987; Wang et al., 1989). Higher densities of the [³H]AF-DX 116 binding sites were found in the external plexiform layer of the olfactory bulb, septal nuclei, diagonal band of Broca, certain thalamic nuclei, habenula, superior colliculus,
interpeduncular nucleus, nucleus of the spinal tract of the trigeminal nerve, facial nucleus, nucleus of the solitary tract and hypoglossal nucleus. Lower densities were present in the striatum, some cortical areas, hypothalamic nuclei and cerebellum. Very low densities were seen in the globus pallidus, substantia nigra and locus coeruleus.

Second messenger systems of muscarinic receptor types

The activation of muscarinic receptors leads to stimulation of phosphoinositol-specific phosphodiesterase (Fisher and Agranoff, 1987), inhibition of adenylate cyclase (Murad et al., 1962) and guanylate cyclase (Hanley and Iversen, 1978); to activation of cAMP-specific phosphodiesterase (Meeker and Harden, 1982), to release of arachidonic acid (Conklin et al., 1988), to synthesis of prostaglandin (Reichman et al., 1987) or prostanoid (Busija et al., 1988), or to regulation of calcium dependent potassium channels (Baraban et al., 1985) or voltage-dependent calcium channels (Hescheler et al., 1987) (for review, see Nathanson, et al., 1987). The precise relationship between the different muscarinic receptor types and these second messenger systems has been the subject of a number of studies.

Pirenzepine was found to be more potent than AF-DX 116 in inhibiting the muscarinic receptor-mediated hydrolysis of inositol lipids (phosphatidylinositol hydrolysis, PI hydrolysis) in the rat cerebral cortex (Smith and Yamamura, 1985, 1987), mouse pituitary
tumor (AtT-20/D16-16) cells (Akiyama et al., 1986), rat retina
(Morol-Fetters et al., 1988), guinea pig colon (Noronha-Blob et al.,
1987) and human neuroblastoma SH-SY5Y cells (Serra et al., 1988),
suggesting that the M₁ muscarinic receptors are coupled to the
hydrolysis of inositol lipids. On the other hand, the muscarinic
receptors which are coupled to adenylate cyclase in a negative
manner may be classified into the M₂ type (Akiyama et al., 1986;
Gil and Wolfe, 1985). The differential couplings of muscarinic
receptor types could also be distinguished by muscarinic agonists.
Stimulation of muscarinic receptors elicited two distinct
biochemical responses in embryonic chick heart cells: inhibition of
cAMP formation and PI hydrolysis (Brown and Brown, 1984). Carbachol
and oxotremorine both inhibit cAMP formation whereas only carbachol
stimulates PI hydrolysis. The EC₅₀ values of carbachol in
inhibiting cAMP formation and stimulating PI hydrolysis were 0.2 μM
and 20 μM, respectively (Brown and Brown, 1984; Brown and Goldstein,
1986; Akiyama et al., 1986).

However, Fisher and Agranoff (1987) stated that both M₁
and M₂ muscarinic receptor types may be coupled to PI hydrolysis
in neural tissues since pirenzepine antagonized the PI response less
potently in the neostriatum, astrocytoma cell and brainstem. In
other studies, M₂ muscarinic receptors were found to be coupled to
PI hydrolysis in chick heart cells (Brown et al., 1985), sphincter
smooth muscle of rabbit iris (Akhtar et al., 1987), rat
pons-medulla, parotid gland and ileal smooth muscle (Lazareno et
al., 1985), and guinea pig bladder (Noronha-Blob et al., 1987). The precise correlation of the different types of the muscarinic receptors versus the second messenger systems was not clear because of the common presence of more than one type of the muscarinic receptors in most of the studied tissues or cell lines.

Muscarinic receptor-mediated inhibition of cAMP formation involved in a $G_i$ protein which is sensitive to pertussis toxin (Hoss and Messer, 1986). It is unknown, however, which G proteins act to stimulate the PI hydrolysis in the nervous system. No inhibitory effects of pertussis toxin on muscarinic receptor-mediated hydrolysis of inositol lipids were reported at that time. There was a hypothesis that the muscarinic receptors may be not coupled to hydrolysis of inositol lipids through a G protein which is sensitive to pertussis toxin (Masters et al., 1985).

Evidence from molecular biological studies

Several genes for both the $M_1$ and $M_2$ muscarinic receptors have been isolated from cDNA of genomic DNA libraries of porcine (Kubo et al., 1986a,b; Peralta et al., 1987a), rat (Bonner et al., 1987, Bonner et al., 1988; Braun et al., 1987; Gocayne et al., 1987; Lai et al., 1988; Stein et al., 1988), human (Bonner et al., 1987; Bonner et al., 1988; Peralta et al., 1987b) and mouse (Shapiro et al., 1988) sources. The amino acid sequences deduced from the 5 genes are different, suggesting that the muscarinic receptor types are different proteins although with homology in the
amino acid sequences. Studies on the hydropathic profile of the amino acid sequences encoded by these genes suggested that the secondary structures of these proteins may be similar to that of rhodopsin which have seven transmembrane domains with the N-terminal outside the membrane and the carboxy terminal inside the membrane.

It was found that one of these genes, designated m$_2$, encoded a muscarinic receptor with pharmacological characteristics of the M$_2$ type (Kubo et al., 1986b; Peralta et al., 1987a). However, four other genes, designated m$_1$, m$_3$, m$_4$ and m$_5$ expressed receptors which were reported to have relatively higher affinity for pirenzepine (Bonner et al., 1987; Bonner et al., 1988; Peralta et al., 1987b). Such apparent polymorphism of the M$_1$-like receptor, defined by high affinity for pirenzepine, remains to be clarified. Northern blot analyses and in situ hybridization studies have demonstrated that the mRNA of the m$_1$ gene from the porcine brain (Kubo et al., 1986a) and m$_1$, m$_3$ and m$_4$ genes from the rat brain (Bonner et al., 1987; Braun et al., 1987) are localized in the cerebral cortex and corpus striatum while the mRNA of the m$_2$ gene from the porcine heart (Kubo et al., 1986b; Peralta et al., 1987a) are expressed in the medulla-pons and atria.

In this dissertation, M$_1$, M$_2$ and M$_3$ are assigned for the pharmacologically distinguishable muscarinic receptor types, while m$_1$, m$_2$, m$_3$, m$_4$ and m$_5$ represent the muscarinic receptor genes that have so far been identified. The number of the amino acid residues of the proteins encoded by these genes were 460
in human (Peralta et al., 1987b; Allard et al., 1987 and Bonner et al., 1988), rat (Bonner et al., 1987) and porcine (Kubo et al., 1986a) for $m_1$; 466 in human (Bonner et al., 1987; Peralta et al., 1987b), rat (Gocayne et al., 1987) and porcine (Kubo et al., 1986b; Peralta et al., 1987a) for $m_2$; 590 in human (Peralta et al., 1987b) and porcine (Akiba et al., 1988) and 589 in rat (Bonner et al., 1987; Braun et al., 1987) for $m_3$; 479 in human (Bonner et al., 1987; Peralta et al., 1987b) and 478 in rat (Bonner et al., 1988) for $m_4$, and 532 in human (Bonner et al., 1988) and 531 in rat for $m_5$ (Bonner et al., 1988). The subscripts do not necessarily indicate a correlation with the pharmacological types.

The pirenzepine/[3H](-)QNB competition curves for the muscarinic receptors encoded by $m_3$ and $m_4$ genes (Bonner et al., 1987) and $m_1$ gene (Stein et al., 1988) were shallow and could be interpreted as multiple antagonist binding states for apparently a single type of transfected muscarinic receptor protein. AF-DX 116 inhibition of [3H](-)QNB binding to intact human embryonic kidney cells transfected with $m_1$, $m_2$ and $m_3$ genes could be fitted by a 2-site model (Peralta et al., 1987b). The pirenzepine rank order for $m_1$, $m_2$, $m_3$ and $m_4$ gene-encoded muscarinic receptors were $m_1 > m_4 > m_3 > m_2$ in COS-7 cells and $m_1 > m_3 > m_4 > m_2$ in human embryonic kidney cells. These discrepancies in the binding values of these expressed muscarinic receptor proteins may be attributed to differences in the assay systems employed, the use of membrane homogenates versus intact cells, or differences in the biology of
the host cells.

Using the probes corresponding to the N-terminal sequences of the muscarinic receptors, in situ hybridization studies indicated that the mRNA of the m₁, m₃ and m₄ genes were detected in the CA1-3 fields of Ammon's horn and polymoph layer of the dentate gyrus (Mel et al., submitted a). The grain densities of m₁ and m₃ probes were much higher than those of the m₄ probe. Interestingly, [³H]pirenzepine binding was high in the CA1 region of the hippocampus and in the molecular layer of the gyrus dentatus (Yamamura et al., 1983) and the m₁ probe also labeled these areas. As few muscarinic receptors were detected by autoradiography using [³H](-)QNB in the corpus callosum, the labeling by these four probes was faint in this area. These results confirmed that m₁, m₃ and m₄ mRNAs but not m₂ mRNA were detectable in the hippocampus and dentate gyrus either by using synthetic oligonucleotide probes (Buckley et al., 1988) or by using the m₃ antisense cRNA probe (Braun et al., 1987). The mRNA of the m₂ gene was detectable in the medial septum, diagonal band, olfactory bulb and pontine nuclei (Bckley et al., 1987), in agreement with the autoradiographic studies with [³H]AF-DX 116 (Rogenold et al., 1987; Wang et al., 1989).

Rationales of this study

The goals of this study include finding and characterizing a system which has muscarinic receptors to test the hypothesis that
the M₁ muscarinic receptor is coupled to the hydrolysis of inositol lipids. A human neuroblastoma cell line, SH-SY5Y, was chosen as a model. Previous studies in this laboratory showed that SH-SY5Y cells have muscarinic receptors and 70 percent of them are of the pirenzepine-high affinity type. The muscarinic receptor-mediated hydrolysis of inositol lipids and the effects of the muscarinic agonist carbachol on the cAMP formation in SH-SY5Y cells were investigated. Possible involvement of G proteins in the muscarinic receptor-mediated hydrolysis of inositol lipids was tested by characterizing the inhibitory effects of pertussis toxin.

When the gene for the M₁ muscarinic receptor was available, transfection of the gene into a murine fibroblast cell line, B82 was carried out in order to generate a system which has only one type of muscarinic receptor. Two lines of research have been done: characterization of M₁ muscarinic receptors in the transfected B82 cells and identification of the second-messenger system of the transfected M₁ muscarinic receptors in these cells to further test the above hypothesis. In addition, the relationship of the receptor density with the response in the transfected B82 cells was studied to test another hypothesis that the high affinity state of the M₁ muscarinic receptors for carbachol is the functionally coupled state.
CHAPTER 2

GENERAL EXPERIMENTAL METHODS

Cell Culture

SH-SY5Y Cells

Human neuroblastoma SH-SY5Y cells of 60-110 passages were grown in Costar 75 cm² tissue culture flasks (Costar, Cambridge, MA) in a mixture growth medium of RPMI 1640 (Irvine Scientific, Santa Ana, CA) with 10 percent fetal bovine serum (Irvine Scientific) supplemented with penicillin (100 u/ml) and streptomycin (100 µg/ml). The cells were incubated in an atmosphere of humidified air (95 percent) with CO₂ (5 percent) at 37°C. The cells were subcultured routinely with 0.54 mM EDTA solution. The cells were seeded into 2 cm² Costar culture wells (250,000 cells/well, 70-80 percent confluency) 24 hours before the experiments, and grown as a monolayer and used for PI hydrolysis and cAMP formation studies.

Murine fibroblast BB2 cells

The transfected murine fibroblast BB2 cells as well as cells transfected with the vector alone and non-transfected BB2 cells were maintained in a medium containing 45 percent Dulbecco's modified Eagle's medium, 45 percent Ham's F-12 medium and 5 percent fetal
bovine serum, 5 percent newborn calf serum, 100 u/ml penicillin, 100 
µg/ml streptomycin. Cells were grown in Costar 75 cm² tissue 
culture flasks and incubated in a humidified atmosphere of 95 
percent air and 5 percent CO₂. 24 hours before experiments, the 
cells were transferred to 2 cm² diameter wells at a concentration 
of 125,000-250,000 cells/well.

**Radioligand binding assay.**

[^H](-)QNB binding to the membranes

[^H](-)QNB binding to the membranes was performed as 
previously described by Yamamura and Snyder (1974) with slight 
modifications (Mei et al., 1987). Transfected cells cultured in 
monolayer in 150 mm culture plates were harvested by scraping cells 
with a rubber policeman in 6 ml/plate of 5 mM Tris buffer (pH 7.4) 
supplemented with 5 mM EDTA and 0.1 µM phenylmethylsulfonylfluoride 
(PMSF). The cell suspension was incubated on ice for 15 min, 
homogenized in a glass/glass Dounce homogenizer (20 strokes) and 
centrifuged at 1000 x g for 10 min at 4°C. The supernatant was 
centrifuged at 40,000 x g for 30 min at 4°C. The membrane pellet 
was resuspended in ice-cold 10 mM sodium-potassium phosphate 
(Na/K-P0₄) buffer, pH 7.4 (0.4 ml/flask) and stored at -70°C until 
use. Aliquots of cell membranes were incubated with [^H](-)QNB in 
the absence and presence of 1 µM atropine sulfate in a final volume 
of 2 ml in 10 mM Na/K-P0₄ buffer supplemented with 0.1 mM PMSF and 
1 mM MgCl₂, for 4 hr at 37°C. Binding reactions were determined
by rapid filtration of the suspension through Whatman GF/B filter strips with a Brandel cell harvester, followed by four rapid washes (approximately 2.5 ml each) with ice-cold 10 mM Na/K-PO₄ buffer (pH 7.4). After filtration the filters were placed in scintillation vials and 8 ml of scintillation cocktail was added to each of the vials. Radioactivity was counted in a liquid scintillation spectrophotometer (Searle Analytic 81) with 44 percent efficiency. For inhibition experiments of [³H](-)QNB binding by muscarinic antagonists, a final concentration of 200 - 250 pM [³H](-)QNB was incubated with 10 concentrations of the inhibitor and aliquots of cell membranes in a final volume of 2 ml, 2 hr at 37°C. For inhibition experiments of [³H](-)QNB binding by carbachol, 180-220 pM [³H](-)QNB was incubated with 10 concentrations of carbachol and aliquots of cell membranes, rat forebrain or heart membrane preparations (Mei et al., 1987), in modified Krebs-phosphate buffer (mM) (NaCl, 120; KCl, 4.8; MgSO₄, 1.2; CaCl₂, 1.3; NaH₂PO₄, 20.3; HCl, 3.2; and D-glucose, 10; pH 7.4), 3 hr at 25°C (Watson et al., 1986). In some of the carbachol/[³H](-)QNB inhibition experiments, a final concentration of 100 µM GppNHP (Pharmacia, Piscataway, NJ) was added. Specific binding was determined in parallel from the difference between [³H](-)QNB binding in the absence and in the presence of 1 µM atropine sulfate. Protein concentration of the membrane suspensions was measured by the method of Lowry et al. (1951).
[\textsuperscript{3}H](--)QNB and [\textsuperscript{3}H](--)MQNB binding to intact cells

[\textsuperscript{3}H](--)QNB binding and [\textsuperscript{3}H](--)MQNB binding to the intact B82 cells or the transfected B82 cells was performed as previously described for [\textsuperscript{3}H](--)QNB and [\textsuperscript{3}H](--)MQNB binding to human neuroblastoma SH-SY5Y cells (Serra et al., 1988). After aspirating the culture medium, the cells were incubated with [\textsuperscript{3}H](--)QNB or [\textsuperscript{3}H](--)MQNB in 1 ml of Iscove's modified Dulbecco's medium (IMDM) (Irvine Scientific) at 37°C for a duration as indicated. For ligand/[\textsuperscript{3}H](--)QNB competition or ligand/[\textsuperscript{3}H](--)MQNB competition assays, the cells were incubated with 10 concentrations of muscarinic antagonists and 200 pM [\textsuperscript{3}H](--)QNB or 300-400 pM [\textsuperscript{3}H](--)MQNB in 1 ml of IMDM, respectively. Specific binding was determined as the amount of binding inhibited by 1 μM atropine sulfate. The incubation was terminated by aspirating the medium and placing the cell culture trays on ice. The cells were rinsed once with 1 ml of ice-cold IMDM for 10 min at 4°C, and were then solubilized twice with 0.5 ml of 1 percent Triton X-100 and transferred to scintillation vials. Scintillation fluid was added and the samples were counted.

Phosphatidylinositol hydrolysis studies

A previously described method (Serra et al., 1986) modified from the technique of Berridge et al. (1982) was applied to measure the accumulation of [\textsuperscript{3}H]IP₁ after muscarinic stimulation in the presence of lithium chloride. SH-SY5Y cells were incubated with 0.2
µM myo-[2,3H]inositol (15 and 12.8 Ci/mmol, New England Nuclear, Boston, MA) in 0.5 ml IMDM after removal of the culture medium and allowed to equilibrate with 95 percent air-5 percent CO₂ at 37°C for 20-22 hours. After aspiration of the radioactive IMDM, 1 ml fresh IMDM was added to the cell culture and allowed to incubate for 10 min before starting the lithium chloride treatment. Then the cells were incubated with 10 mM lithium chloride for 10 min before the addition of various concentrations of carbachol to initiate the reaction. In the studies with muscarinic antagonists, various concentrations of atropine, pirenzepine or AF-DX 116 were added 5 min before carbachol. The reaction was terminated at the respective times as indicated by aspirating the medium and adding 0.31 ml methanol. The cells in methanol were scraped off from the wells with a Costar cell scraper (Costar, Cambridge, MA) and collected into chloroform-resistant tubes (American Scientific Products, McGaw Park, IL). Another 0.31 ml methanol was used to rinse the well and the solutions were collected. The cell suspensions were mixed with 0.62 ml of chloroform and 0.31 ml of double distilled water to obtain a two-phase separation. After a complete separation of the phases achieved by centrifugation at 1000 x g for 5 min, 0.7 ml aliquots of the upper aqueous phase were added to 2 ml distilled water and mixed by vortexing. The mixtures were transferred to mini-columns (Bio Rad Laboratories, Richmond, CA) consisting of 2 ml of 10 percent slurry of anion exchange resin in formate form (AG 1-X8, 100-200 mesh, Bio-Rad Laboratories). The resin was allowed to
set and then washed three times each with 5 ml of distilled water and 2 times each with 5 ml of 5 mM myo-inositol. The $[^{3}\text{H}]\text{IP}_1$, $[^{3}\text{H}]\text{inositol biphosphates (IP}_2)$ and $[^{3}\text{H}]\text{inositol triphosphates (IP}_3)$ were eluted by 2 ml 0.2 M ammonium formate/0.1 M formic acid, 0.4 M ammonium formate/0.1 M formic acid, and 1 M ammonium formate/0.1 M formic acid, respectively. Each form of the inositol phosphates eluted was separately collected in scintillation vials. Nine milliliters of AquaMix (ICN) were added and the radioactivity was counted.

**Cyclic AMP formation studies**

The cAMP formation studies were performed according to the method of Gilman (1970). The cells were rinsed with 1.0 ml of Hepes/phosphate buffer (in mM): NaCl (137), KCl (4.7), MgSO$_4$.7H$_2$O (1.0), CaCl$_2$ (1.3), MgCl$_2$.6H$_2$O (1.5), Na$_2$HPO$_4$ (3.24), KH$_2$PO$_4$ (0.76), HEPES (free acid, 12.5; sodium salt 12.5) and glucose (5.0) at pH 7.4 (37°C) and covered with 0.4 ml of 5 mM IBMX (Hepes/PO$_4$ buffer) 1 min before the addition of 100 μl of buffer or buffered drugs to initiate the reaction. The cells were then incubated at 37°C for 3 min. The reaction was terminated by aspirating the buffer and adding 150 μl of cold Tris/EDTA buffer (50 mM Trizma HCl/Trizma base containing 4 mM Na$_2$EDTA, pH 7.4 at 20°C) to each well on ice. The cells were dislodged from the wells with a Costar cell scraper and transferred to microfuge tubes, then heated in a boiling water bath for at least
5 min. A 50 μl-aliquot of the supernatant after centrifugation (Sorvall-Microspin) at 5600 x g for 2 min was analyzed for cAMP content using the competitive protein binding assay as follows. cAMP-dependent protein kinase (Sigma Chemical Co., St. Louis, MO) was prepared in a 50 mM Tris HCl-4 mM EDTA-0.1 percent bovine serum albumin buffer (60 μg protein kinase/ml, pH 7.5 at 25°C). A 50 μl-aliquot of either the supernatant from each sample or a standard (cAMP, 0.125 pM-64 pM) was incubated with 50 μl (0.9 pmol/50 μl) of [³H]cAMP (specific activity, 41 Ci/mmol, Amersham Corp.) and a 100 μl-aliquot of the protein kinase preparation on ice for 2 hours. Separation of the bound from the free [³H]cAMP was achieved by adding 100 μl of ice-cold activated charcoal (Norit Ultra C, American Norit Company Inc., Jacksonville, FL) in 50 mM Tris HCl/4 mM EDTA/2 percent bovine serum albumin to each tube. The samples were mixed vigorously and centrifuged immediately at 5600 x g for 45 sec. A 200 μl-aliquot of each supernatant was transferred to a scintillation vial and the radioactivity was counted in 4 ml of AquaMIX.

Data analyses

Data from kinetic experiments were analyzed using the first order equations. The pseudo-association rate constant (kₜₐₚ) was first estimated using a monoexponential equation:

$$B_t = B_e (1 - e^{-k_{obs}})$$

where $B_t$ and $B_e$ were specific [³H](-)QNB binding at time, t.
and at equilibrium, respectively. Dissociation rate constant 
\( k_{-1} \) was calculated using the equation:

\[
k_{-1} = 0.693/ t_{1/2}
\]

The association rate constant \( k_{+1} \) was then calculated by the
equation:

\[
k_{+1} = (k_{\text{obs}} - k_{-1})/[L]
\]

where \([L]\) was the concentration of free \[^3\text{H}(-)\text{QNB}].

All binding concentration-response parameters were
determined by logistic nonlinear least-squares analysis using a
computerized iterative procedure developed by Susan Yamamura for the
Apple II Computer. In the inhibition experiments the IC\(_{50}\) values
were corrected to \( K_i \) (Weiland and Molinoff, 1981) using the Cheng
and Prusoff equation (Cheng and Prusoff, 1973):

\[
K_i = IC_{50}/(1 + [L]/K_d)
\]

where \( L \) is the free labeled radioligand concentration and \( K_d \) is
the apparent dissociation constant of the labeled ligand.

The F values of comparing the 1-site versus 2-site fit were
calculated from the equation:

\[
F = \frac{SS_1 - SS_2}{df_1 - df_2}
\]

where \( SS_1 \) and \( SS_2 \) are residual sums of squares of errors with
corresponding degrees of freedom of \( df_1 \) and \( df_2 \) of the two
models, respectively.
Statistical differences were analyzed using the one way ANOVA for grouped data (Tallarida and Murray, 1987), Student's t-test for grouped data, and paired t-test. A value of $p<0.05$ was considered statistically significant.
CHAPTER 3

THE COUPLING OF MUSCARINIC RECEPTORS TO HYDROLYSIS OF INOSITOL LIPIDS IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS

The human neuroblastoma SH-SY5Y cells are of a neuroblastic subclone from SK-N-SH cells (Biedler et al., 1978). These cells can convert the radioactive precursors of dopamine, acetylcholine and GABA into their respective neurotransmitters. Recently, the muscarinic receptors in this cell line were characterized by using \(^{3}H\)pirenzepine in a radioligand binding assay (Serra et al., 1988). The majority of the muscarinic receptors in the SH-SY5Y cells have high affinity for pirenzepine. In this study, carbachol-induced hydrolysis of inositol lipids and cAMP formation in these cells were systematically characterized to test the hypothesis that the M₁ muscarinic receptors of this cell line were coupled only to the hydrolysis of inositol lipids. The results showed that the pirenzepine-high affinity receptors in the SH-SY5Y cells are associated with the hydrolysis of inositol lipids. Stimulation of muscarinic receptors in this cell line had neither a stimulatory nor an inhibitory effect on basal cAMP formation and did not inhibit agonist-induced stimulation of cAMP formation.

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Results

Carbachol-induced accumulation of $[^3H]IP_1$ in intact SH-SY5Y cells

The basal $[^3H]IP_1$ accumulation in the absence of carbachol was low in SH-SY5Y cells. The inositol phosphates formed in the cells treated with 10 mM LiCl to inhibit breakdown of inositol phosphates to free inositol were 25±1.4 cpm/10^5 cells for $[^3H]IP_1$, 14±0.92 cpm/10^5 cells for $[^3H]IP_2$ and 12±0.80 cpm/10^5 cells for $[^3H]IP_3$ 3 sec after starting incubation in IMDM and did not increase with increasing incubation time.

Carbachol stimulated phosphoinositol hydrolysis in the presence of 10 mM lithium chloride in SH-SY5Y cells. As shown in figure 1, carbachol (100 μM) induced the formation of the water-soluble inositol phosphates. Carbachol-stimulated hydrolysis of inositol lipids was rapid. Three seconds after the addition of carbachol, $[^3H]IP_3$ was the major product at 3 sec, 4.5 fold higher than $[^3H]IP_1$ and 3.0 fold higher than $[^3H]IP_2$. After a 30 sec incubation with carbachol, $[^3H]IP_1$ formation surpassed $[^3H]IP_3$ and $[^3H]IP_2$. Within the time course of 4 hours, $[^3H]IP_1$ formation was linear while the formation of $[^3H]IP_2$ and $[^3H]IP_3$ did not increase with increasing incubation time. In the following experiments, only $[^3H]IP_1$ was measured after incubation of the cells for 1 hr.

Carbachol concentration-dependently stimulated $[^3H]IP_1$.
Figure 1. Time course of [3H]inositol phosphate accumulation stimulated by carbachol. Data are shown as counts/min representing [3H]IP$_1$ ( ), [3H]IP$_2$ ( ) and [3H]IP$_3$ ( ) formed in intact SH-SY5Y cells in the presence of 100 µM carbachol minus those from control cells (basal), respectively. In A, the time scale is in seconds; in B, the time scale is in minutes. Each point represents the mean ± S.E.M of 3 experiments done in duplicate.
accumulation with an EC$_{50}$ value of 14 $\mu$M for $[^3$H]IP$_1$ formation (figure 2 and table 1). The maximal amount of $[^3$H]IP$_1$ accumulated in the presence of 10 mM LiCl was about 50-100 fold above the basal level. The dose-response curve had a slope factor of 0.98 generated from computer nonlinear least squares regression analyses.

Inhibition of carbachol-stimulated hydrolysis of inositol lipids by the muscarinic antagonists atropine, pirenzepine and AF-DX 116

Carbachol-stimulated hydrolysis of inositol lipids was blocked by the muscarinic antagonists atropine, pirenzepine and AF-DX 116. These three muscarinic antagonists were chosen in order to systematically study the possible types of muscarinic receptors involved in the carbachol-stimulated hydrolysis of inositol lipids, i.e., atropine, a classical nonselective muscarinic antagonist, pirenzepine, an M$_1$ selective antagonist and AF-DX 116, an M$_2$ selective antagonist. Neither of the three antagonists had any stimulatory or inhibitory effects on the basal levels of $[^3$H]IP$_1$ formation. Each antagonist reduced the accumulation of $[^3$H]IP$_1$ induced by carbachol. Figure 2 A, B and C shows the carbachol dose-response curves of the accumulation of $[^3$H]IP$_1$ in the absence and the presence of increasing concentrations of the antagonists. In the presence of increasing concentrations of the muscarinic antagonists, the carbachol dose-response curve was shifted to the right without a reduction of the maximal effect of
Table 1

Effects of muscarinic antagonists on carbachol-stimulated hydrolysis of inositol lipids in SH-SY5Y cells\(^a\).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EC(_{50}) ((\mu)M)(^b)</th>
<th>(E_{\text{max}}) (percent)(^c)</th>
<th>n(_h) (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbachol</td>
<td>14(7.1-27)</td>
<td>99±0.60</td>
<td>0.98±0.1</td>
</tr>
<tr>
<td>+Atropine ((\mu)M)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001</td>
<td>75(52-100)</td>
<td>100±7.5</td>
<td>0.83±0.1</td>
</tr>
<tr>
<td>0.01</td>
<td>440(180-700)</td>
<td>94±6.0</td>
<td>0.93±0.1</td>
</tr>
<tr>
<td>0.1</td>
<td>4000(1800-6400)</td>
<td>110±11</td>
<td>0.93±0.04</td>
</tr>
<tr>
<td>0.5</td>
<td>21000(13000-30000)</td>
<td>120±10</td>
<td>0.90±0.05</td>
</tr>
<tr>
<td>+Pirenzepine ((\mu)M)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>30(14-42)</td>
<td>100±4.9</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>0.03</td>
<td>55(35-70)</td>
<td>96±3.3</td>
<td>0.78±0.05</td>
</tr>
<tr>
<td>0.1</td>
<td>220(54-300)</td>
<td>100±7.1</td>
<td>0.62±0.05</td>
</tr>
<tr>
<td>0.3</td>
<td>480(150-740)</td>
<td>94±9.8</td>
<td>0.66±0.10</td>
</tr>
<tr>
<td>1.0</td>
<td>1600(940-4400)</td>
<td>92±7.3</td>
<td>0.49±0.03</td>
</tr>
<tr>
<td>3.0</td>
<td>5200(4200-6400)</td>
<td>110±2.8</td>
<td>0.59±0.33</td>
</tr>
<tr>
<td>10</td>
<td>16000(14000-26000)</td>
<td>120±9.0</td>
<td>0.66±0.05</td>
</tr>
<tr>
<td>30</td>
<td>20000(18000-23000)</td>
<td>96±4.9</td>
<td>0.87±0.08</td>
</tr>
<tr>
<td>100</td>
<td>140000(94000-180000)</td>
<td>97±2.5</td>
<td>0.61±0.10</td>
</tr>
<tr>
<td>+AF-DX 116 ((\mu)M)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>50(23-66)</td>
<td>99±4.0</td>
<td>0.82±0.11</td>
</tr>
<tr>
<td>10</td>
<td>450(200-890)</td>
<td>99±0.58</td>
<td>0.79±0.10</td>
</tr>
<tr>
<td>100</td>
<td>7000(2500-12000)</td>
<td>120±7.9</td>
<td>0.70±0.14</td>
</tr>
</tbody>
</table>

\(^a\)Data were analyzed by a computer program for logistic nonlinear least squares regression analysis. Carbachol-stimulated \([^{3}H]IP_{1}\) accumulation in the absence of the antagonist was first analyzed to generate an \(E_{\text{max}}\) value as 100 percent. \([^{3}H]IP_{1}\) accumulation in parallel studies with muscarinic antagonists was converted into the percentage over the \(E_{\text{max}}\) value.

\(^b\)Data are presented as geometric means with the variation range in the parentheses.

\(^c\)Data are presented as arithmetic means ± S.E.M.
Figure 2. Inhibition of carbachol-elicited $[^3H]IP_1$ accumulation in intact SH-SY5Y cells by muscarinic antagonists. $[^3H]IP_1$ accumulation was initiated by adding carbachol to the control cells or those 5 min-pretreated with different concentrations of atropine (in A), pirenzepine (in B) or AF-DX 116 (in C). The incubation was stopped 60 min later and $[^3H]IP_1$ formed was measured as described in chapter 2. Each point represents the mean percentage ± S.E.M. of the maximal stimulation above basal from 3 or more experiments. The maximal amount of $[^3H]IP_1$ accumulated that correspond to 100 percent response was 3700 ± 300 cpm/10^5 cells.
carbachol. The rightward shifts were dependent on the concentrations of the antagonists. Higher concentrations of the antagonists caused greater shifts to the right. The concentration response curves of carbachol in the presence of atropine, pirenzepine or AF-DX 116 were parallel to the original curve in the absence of the muscarinic antagonists. The tests of parallelism did not show any significant difference among the linearized concentration response curves. The EC\textsubscript{50} values, Hill coefficients and maximal efficacies of carbachol in the absence and in the presence of different concentrations of the antagonists are presented in table 1.

The Schild regression was applied to analyze the inhibition of the carbachol-stimulated hydrolysis of inositol lipids by the different concentrations of the three antagonists (figure 3). The pA\textsubscript{2} values obtained were 9.6, 8.1 and 6.3 for atropine, pirenzepine and AF-DX 116, respectively. The functional inhibition constants (K\textsubscript{i}) converted from pA\textsubscript{2} values were 0.24, 8.1 and 470 nM, respectively, which were in good agreement with the inhibition constants of these drugs from antagonist/[\textsuperscript{3}H]pirenzepine binding studies (table 2) (Serra et al, 1988). The slope of the Schild regression was 0.94 for atropine, 1.0 for pirenzepine and 1.2 for AF-DX 116.

Effects of phorbol esters on carbachol-induced [\textsuperscript{3}H]IP\textsubscript{1} accumulation
Figure 3. Schild regression analyses of the inhibition of carbachol-stimulated \(^{3}H\)IP\(_1\) accumulation by muscarinic antagonists. The original curves are from figure 2A for atropine (◼), figure 2B for pirenzepine (▲) and figure 2C for AF-DX 116(●). A' represents the EC\(_{50}\) value of carbachol in the absence of antagonist; A represents the EC\(_{50}\) values of carbachol in the presence of the respective antagonists at appropriate concentrations and B represents the concentrations of the antagonists in molar units. Linear regression analyses of \(\log_{10}(A/A' - 1)\) vs. \(-\log [B]\) generated values of 9.6 for atropine, 8.1 for pirenzepine and 6.3 for AF-DX 116 and the slopes of the Schild regression were 0.94, 1.0 and 1.2, respectively.
Table 2
Comparison of the inhibition constants from ligand/[3H]pirenzepine binding and those from [3H]IP\(_1\) accumulation studies

<table>
<thead>
<tr>
<th>Inhibition Constants (nM)</th>
<th>Atropine</th>
<th>Pirenzepine</th>
<th>AF-DX 116</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_1 ) (^a)</td>
<td>0.60</td>
<td>6.9</td>
<td>210</td>
</tr>
<tr>
<td>( K_1 ) (^b)</td>
<td>0.24</td>
<td>9.2</td>
<td>470</td>
</tr>
</tbody>
</table>

\(^a\) \( K_1 \) values were calculated from the IC\(_{50}\) values from ligand/[3H]pirenzepine studies with intact SH-SY5Y cells recently undertaken in our laboratory (Serra et al., 1988).

\(^b\) The \( K_1 \) values were the negative anti-log of pA\(_2\) values from fig 3.
The tumor promoter phorbol ester PMA (4β-phorbol, 12β-myristate, 13α-acetate) decreased carbachol-stimulated \([^{3}H]IP_{1}\) accumulation. In the presence of 10 μM PMA, the maximal effect of carbachol was reduced to 30 percent of that for carbachol alone. Taurine at the concentration of 10 mM did not reverse PMA's inhibitory effects (figure 4). The inactive phorbol ester 4α-phorbol 12β, 12α-didecanoate (αPDD) did not reduce the carbachol maximal effect. If the \([^{3}H]IP_{1}\) formed at 100 μM was taken as 100 percent, 3 nM PMA inhibited about 20 percent of the carbachol effects while 10 nM PMA inhibited about half of the carbachol effect. However, about 20 percent of the carbachol-induced \([^{3}H]IP_{1}\) accumulation was not blocked by 1 μM PMA. The half maximal inhibition concentration of PMA was 13 (7.3-41) nM (figure 5). A 30 min pretreatment of the cells with 10 μM 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) did not reverse PMA's inhibitory effects (data not shown).

**Forskolin-induced cAMP formation in SH-SY5Y cells**

The basal formation of cAMP was 5.3±1.9 fmol/10³ cells in the SH-SY5Y cells and 17±3.1 fmol/10³ cells in the medium. In order to determine the proper concentration of 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, for cAMP formation studies, 100 μM forskolin was added to stimulate cAMP formation in the presence of various concentrations of IBMX. As shown in figure 6A, IBMX enhanced the forskolin-elicited cAMP
Figure 4. Concentration-response curves of carbachol in inducing 
$[^3]H\text{IP}_1$ accumulation in intact SH-SY5Y cells in the 
absence and presence of 10 μM PMA. $[^3]H\text{IP}_1$
accumulation assay was done with the control cells (○) 
and those pretreated with 10 μM PMA (△) and rinsed with 1 
ml IMDM. In some experiments, PMA-treated cells were 
pretreated with 10 mM taurine (△) and taurine was present 
in the PMA treatment. The $E_{\text{max}}$ and $E_{50}$ values of 
carbachol were 100±1.8 and 17 (11-23) μM in the absence and 
30±5.4 and 85 (68-100) μM in the presence of 10 μM PMA, 
respectively. The $E_{\text{max}}$ and $E_{50}$ values of carbachol 
from taurine-preincubated PMA-treated cells were 27±3.5 and 
75 (69-81) μM, respectively.
Figure 5. Inhibition of carbachol-induced $[^3H]IP_1$ accumulation in intact SH-SY5Y cells by PMA. 100 μM carbachol was added to stimulate PI hydrolysis in control SH-SY5Y cells (100 percent of control) and in SH-SY5Y cells pretreated with various concentrations of PMA for 1 hr. The half maximal inhibition concentration of PMA was 13 (7.3-41) nM.
Figure 6. In A: The effects of IBMX on forskolin-elicited cAMP formation. 100 μM forskolin was added to stimulate cAMP formation in the presence of various concentrations of IBMX. The EC₅₀ value of IBMX was 0.68 mM. Data shown are from a representative experiment, repeated 3 times. In B: A representative concentration-response curve of forskolin in stimulating cAMP formation in intact SH-SYSY cells. Data are presented as percentages of the maximal stimulation above basal in the presence of 5 mM IBMX. The maximal cAMP formation stimulated by forskolin was 50-100 fold above the basal level of 3.0±0.15 fmol/10⁶ cells. The experiment was repeated three times with similar results.
formation. The enhancement of IBMX was saturable. The cAMP formed did not increase linearly with increasing IBMX concentrations after 1.2 mM and the amount of cAMP in the cells was increased from 75 fmol/10^3 cells at 0.05 mM IBMX to 300 fmol/10^3 cells at 2.5 mM which is close to the maximal effect of IBMX. Consequently, 5 mM IBMX was routinely used in the following experiments in order to inhibit phosphodiesterase activity. In the presence of 5 mM IBMX, the basal amounts of cAMP formed were 3.0±0.15 fmol/10^3 cells in the SH-SYSY cells and 28±6.7 fmol/10^3 cells in the medium.

Forskolin stimulated cAMP formation in a concentration-dependent manner with an EC_{50} value of 8.7 µM (figure 6B). At a concentration of 100 µM, forskolin's stimulatory effect became maximal with cAMP formation of about 50-100 fold above the basal level.

Effects of different pharmacological agents on cAMP formation in SH-SYSY cells

Since adeny1ate cyclase activity has been reported to be positively or negatively coupled to a variety of neurotransmitter receptors, we examined selected agonists for their possible coupling in SH-SYSY cells. As shown in figure 7, in addition to forskolin, sodium fluoride (NaF), adenosine and prostaglandin E_1 (PGE_1) significantly increased the amount of cAMP in the SH-SYSY cells. The increases were 22±9.8 fold above basal for 100 mM NaF, 6.3±2.5 for 1 mM adenosine and 5.0±0.71 for 100 µM PGE_1 in the presence of
Figure 7. Effects of different pharmacological agents on cAMP formation in intact SH-SY5Y cells in the presence of 5 mM IBMX. Data are presented as ratio of cAMP formation over basal from 3 or more experiments (means ± S.E.M.). The concentrations of the agents studied were 100 µM except those for adenosine 1 mM, isoproterenol (10µM) and NaF (100 mM). * p<0.05; ** p<0.01. Adenosine, AD; Dopamine, DA; Morphine, MORP; Histamine, HIS; Isoproterenol, ISO; Forskolin, FORS.
5 mM IBMX. A 40 percent increase in the medium cAMP was also observed at 10 μM PGE₁ in the absence of IBMX (data not shown). Morphine at a concentration of 100 μM significantly decreased the basal amount of cAMP in SH-SY5Y cells. The ratio of cAMP formed in the cells in the presence of 100 μM morphine sulfate over the basal formation was 0.25±0.1. In contrast, the muscarinic agonist carbachol (100 μM) showed neither inhibitory nor stimulatory effects on the basal amount of cAMP formed in the SH-SY5Y cells. In addition, dopamine (100 μM), histamine (100 μM) and isoproterenol (10 μM) did not significantly influence basal cAMP formation.

The stimulatory effects of adenosine and PGE₁ on cAMP formation in the SH-SY5Y cells were concentration-dependent. Figure 8 presents the concentration-response curves of these two drugs in stimulating cAMP formation. The maximal stimulation of cAMP formation by adenosine or PGE₁ were similar, with no significant difference between the E_max values (figure 8). However, PGE₁ was more potent than adenosine. In the presence of 5 mM IBMX the amount of cAMP in the medium were 2.5±0.18 fold above the basal at 1 μM PGE₁ but 0.90±0.26 fold at 10 μM PGE₁.

As shown in table 3, carbachol increased forskolin-stimulated cAMP formation in SH-SY5Y cells in the presence of 5 mM IBMX. However, this enhancement by carbachol was not blocked by either atropine (1 μM) or d-tubocurare (100 μM). Carbachol at a concentration of 100 μM did not reverse PGE₁'s or adenosine's stimulatory effect. In the absence of IBMX, carbachol
Figure 8. Concentration-response curves of PGE₁ (●) and adenosine (▲) in stimulating cAMP formation in intact SH-SY5Y cells. Each point represents a percentage mean ± S.E.M. of the maximal stimulation above the basal amounts of cAMP in the cells in the presence of 5 mM IBMX (n=3). The maximal cAMP formation stimulated by PGE₁ and adenosine were 17±5.0 fmol/10⁴ cell and 12±2.1 fmol/10⁴ cells, respectively. The EC₅₀ values for PGE₁ and adenosine were 4.7 μM and 54 μM, respectively.
Table 3
Effects of selected drugs on the cAMP formation in SH-SY5Y cells*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cAMP formedb</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGE₁, 100 μM</td>
<td>6.9±0.79</td>
<td></td>
</tr>
<tr>
<td>+ morphine, 100 μM</td>
<td>4.0±1.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>+ morphine, 100 μM + naltrexone 100 μM</td>
<td>6.2±2.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>+ carbachol, 100 μM</td>
<td>6.8±0.79</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Adenosine, 1 mM</td>
<td>3.8±0.24</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>+ carbachol, 100 μM</td>
<td>3.5±0.33</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Forskolin, 100 μM</td>
<td>49±7.5</td>
<td></td>
</tr>
<tr>
<td>+ carbachol, 100 μM</td>
<td>70±4.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>+ carbachol, 100 μM + atropine, 1 μM</td>
<td>76±20</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>+ carbachol, 100 μM + d-tubocurare, 100 μM</td>
<td>63±21</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*aCAMP formation studied were performed as described in chapter 2. Carbachol was added to the medium at the same time as the respective stimulant in experiments with carbachol.
bCAMP in the cells is expressed as the ratio over the basal level (means ± S.E.M.).
also had no stimulatory or inhibitory effects on the basal or PGE₁-stimulated cAMP formation in SH-SY5Y cells. The amounts of cAMP did not change significantly both in the cells and in the medium at 10 mM carbachol. In contrast, PGE₁-induced cAMP formation was reversed by 1 μM morphine. As shown in table 3, a 40 percent decrease in the amount of PGE₁-stimulated cAMP in the cells was observed by 100 μM morphine in the presence of 5 mM IBMX. A similar decrease in the amounts of PGE₁-stimulated cAMP in the medium was also observed. The inhibitory effect of 100 μM morphine was blocked by the opioid antagonist naltrexone at 100 μM.

Discussion

The findings of this study support the hypothesis that pirenzepine-high affinity muscarinic receptors are coupled to the hydrolysis of inositol lipids in the human neuroblastoma SH-SY5Y cell line. The muscarinic agonist carbachol stimulated the hydrolysis of inositol lipids as evidenced by carbachol-induced accumulation of [³H]IP₁. Muscarinic antagonists such as atropine, pirenzepine and AF-DX 116 blocked the carbachol-induced accumulation of [³H]IP₁, and pirenzepine was more effective than AF-DX 116. The functional inhibition constants of pirenzepine and AF-DX 116 were 9.2 nM and 470 nM, respectively. The rank order of potency of these three antagonists was atropine > pirenzepine > AF-DX 116. These data suggest that pirenzepine has higher affinity for muscarinic receptors in SH-SY5Y cells which are coupled to the
hydrolysis of inositol lipids.

$[^3H](-)QNB$ bound to the muscarinic receptors in the intact SH-SY5Y cells with a high affinity (Serra et al., 1988). The $B_{\text{max}}$ and $K_d$ values of $[^3H](-)QNB$ were 220 fmol/mg protein and 25 pM, respectively. Saturation experiments with $[^3H]$pirenzepine revealed a single class of binding sites with a total binding capacity of 160 fmol/mg protein and $K_d$ value of 13 nM. Accordingly, the majority of the total muscarinic receptors in the SH-SY5Y cells are of the $M_1$ type (Serra et al., 1988). Table 2 shows the inhibition constants of atropine, pirenzepine and AF-DX 116 from antagonist/$[^3H]$pirenzepine competition studies. The rank order of the affinity of these antagonists for $M_1$ muscarinic receptors in the SH-SY5Y cells is the same as revealed from the inhibition of carbachol-stimulated $[^3H]$IP$_1$ accumulation (table 2).

The breakdown of membrane inositol lipids produces two products with second messenger function: IP$_3$ and diacylglycerol (DAG) (Berridge et al., 1987). The primary function of IP$_3$ is to mobilize calcium from intracellular stores while DAG stimulates protein kinase C to mediate protein phosphorylation. We observed an increased $[^3H]$IP$_3$ formation at the early stage after muscarinic stimulation of SH-SY5Y cells (figure 1), followed by a steady accumulation of $[^3H]$IP$_1$ in the presence of 10 mM LiCl which inhibits inositol-1-monophosphatase responsible for IP$_1$ degradation (Berridge et al., 1982). The time course of $[^3H]$IP$_3$
and $[^{3}H]IP_{1}$ accumulation suggests a second messenger role of IP$_{3}$ in these cells. Heikkila et al. (1987) have shown that stimulation of muscarinic receptors in SH-SY5Y cells are linked to intracellular Ca$^{2+}$ mobilization. Carbachol-induced $[^{3}H]IP_{1}$ accumulation was linear within a time course of 4 hr in the presence of 10 mM LiCl. Similar time courses were reported from cultured rat cerebellar granule cells (Xu and Chuang, 1987), pig coronary artery slices (Sasaguri et al., 1986), human astrocytoma cells (Nakahata et al., 1987), and guinea pig ileal smooth muscle slices (Bielkiewicz-Vollrith et al., 1987).

PMA is a tumor-promoting phorbol ester that activates protein kinase C directly both in vitro and in vivo (Castagna et al., 1982). PMA potently inhibited carbachol-induced $[^{3}H]IP_{1}$ accumulation in SH-SY5Y cells with an average IC$_{50}$ value of 13 nM. This inhibitory effect of phorbol esters is specific since $aPDD$, an inactive form of phorbol ester, showed no inhibitory effects. Pretreatment of human neuroblastoma cells with 10 μM PMA resulted in a decrease in the maximal $[^{3}H]IP_{1}$ accumulation stimulated by carbachol, which was not reversed by 10 mM taurine, a membrane stabilizer which reduces cytoplasmic calcium concentration by increasing calcium binding to membrane lipids (Huxtable and Sebring, 1986). The effects of PMA on carbachol-induced $[^{3}H]IP_{1}$ accumulation may be due to its activation of protein kinase C in SH-SY5Y cells. Scott et al. (1985) reported that muscarinic agonists increased $^{45}$Ca$^{2+}$ influx only into the PMA-treated
SH-SY5Y cells while no response was observed with cells cultured in the absence of PMA. Furthermore, pretreatment of the SH-SY5Y cells with PMA produced a decrease in the affinity of carbachol for muscarinic receptors (Serra et al., 1986) and a decrease in the agonist sensitivity of the cells as measured by quin-2-fluorescence or $^{45}$Ca$^{2+}$ release from the cells (Heikkila et al., 1987).

If the $M_1$ muscarinic receptors are all coupled to the PI hydrolysis, we would expect that the muscarinic receptors in SH-SY5Y cells may not be coupled to the cAMP system. We tested a series of agonists which are known to stimulate cAMP formation: adenosine (van Calker et al., 1979) and PGE$_1$ (Sharma et al., 1975) as well as forskolin (Seaman and Daly, 1981). Treatment of SH-SY5Y cells with these three drugs resulted in an increase of cAMP formation (figure 6 and figure 8) while carbachol had neither stimulatory nor inhibitory effects on the basal cAMP formation. Stimulation of cAMP formation in SH-SY5Y cells by adenosine and PGE$_1$ suggests that these cells have receptors for these putative neurotransmitters and also have appropriate signal transducing systems such as G proteins. The adenosine receptors in these cells are most likely of the $A_2$ type since adenosine $A_1$ receptors are inversely coupled to the adenylate cyclase (van Calker et al., 1979). As shown in table 3 and figure 7, morphine not only decreased the basal cAMP formation but also inhibited the cAMP formation stimulated by PGE$_1$. The latter inhibitory effect of morphine was blocked by the opioid antagonist naltrexone. These results are in good agreement
with those by Kazmi and Mishra (1987) who found that PGE₁-stimulated cAMP formation was inhibited by opioid agonist Tyr-D-Ala-Gly-NMePhe-Gly-ol (DAGO). Carbachol had no inhibitory effect on adenosine or PGE₁-induced cAMP formation (table 3). Stimulation of muscarinic receptors in SK-N-SH cells was found to increase the basal and forskolin-stimulated cAMP level by 50 percent (Baumgold and Fishman, 1988). Interestingly, carbachol potentiated forskolin-stimulated cAMP formation in SH-SY5Y cells (table 3). Again, the mechanism of this effect is not clear as it was not blocked by 1 μM atropine or 100 μM d-tubocurare. These results indicated that interaction of muscarinic receptors in these cells with carbachol, however, had neither a stimulatory nor inhibitory effect on the basal cAMP formation and adenosine-, PGE₁- or forskolin-induced cAMP formation. These data suggest that muscarinic M₁ receptors are coupled to the hydrolysis of inositol lipids and not to the adenylate cyclase system in human neuroblastoma SH-SY5Y cells.
CHAPTER 4

EFFECTS OF PERTUSSIS TOXIN AND CHOLERA TOXIN ON CARBACHOL-STIMULATED HYDROLYSIS OF INOSITOL LIPIDS IN SH-SY5Y CELLS

The mechanism of receptor-mediated stimulation of the phosphatidylinositol-specific phosphodiesterase is not clear. A G protein is believed to exist and transduce the extracellular signal into the enzymatic activity. This has been demonstrated in non-neuronal systems, where PI turnover was blocked by pertussis toxin (Worley et al., 1987). At the present time, however, it is unknown which G proteins act to stimulate the PI system in the brain. Although pertussis toxin has been found to inhibit PI hydrolysis induced by \( \alpha_1 \)-adrenergic receptors in rat thyroid FRTL-5 cells (Corda and Kohn, 1986), \( \text{LTB}_4 \) in guinea pig alveolar macrophages (Holian, 1986) and thrombin in hamster fibroblast CC139 (Paris and Pouyssegur, 1986), no inhibitory effects of pertussis toxin on muscarinic receptor-mediated PI hydrolysis have been reported. Brown and colleagues have suggested that muscarinic receptors are not coupled to PI hydrolysis through a G protein which is sensitive to pertussis toxin (Masters et al., 1985).

We used the human neuroblastoma cell line to study the possible involvement of G protein in activating phosphodiesterase and found that the high affinity pirenzepine muscarinic
receptor-mediated PI hydrolysis in SH-SY5Y cells is sensitive to petussis toxin but not to cholera toxin.

Results

Treatment of the cells with 10 μg/ml of cholera toxin (Sigma, St. Louis, MO) or 10 μg/ml pertussis toxin (List Biological Laboratories, Inc., Campbell, CA) for 1 hr had no effects on either the carbachol-induced IP$_1$ accumulation, or the basal levels of IP$_1$ formation (figure 9). After a 22 hr-incubation with the cells, pertussis toxin decreased carbachol-induced IP$_1$ accumulation in a concentration dependent manner as shown in figure 10 and table 4 while it had neither stimulatory nor inhibitory effects on the basal [${}^3$H]IP$_1$ accumulation. Pertussis toxin changed the maximal [${}^3$H]IP$_1$ accumulation stimulated by carbachol from 51 fmol/10$^6$ cells in the absence to 42 fmol/10$^6$ cells (p<0.05) and 22 fmol/10$^6$ cells (p<0.01) in the presence of 1 μg/ml and 10 μg/ml pertussis toxin, respectively (figure 11). However, pertussis toxin did not change the carbachol EC$_{50}$ value in inducing IP$_1$ accumulation which were 18 μM in the absence vs. 18 and 22 μM in the presence of 1 μg/ml and 10 μg/ml pertussis toxin, respectively (p>0.05). Cholera toxin at a concentration of 1 μg/ml had neither stimulatory nor inhibitory effects even after 22 hr incubation. Both the EC$_{50}$ and the maximal stimulatory effects of carbachol in the presence of 1 μg/ml cholera toxin showed no significant change from those in its absence (table 4). Further
Figure 9. Effects of pertussis toxin (10 μg/ml) and cholera toxin (1.0 μg/ml) on the basal level of [3H]IP\(_1\) accumulation (inset) and on the carbachol (10 mM)-stimulated [3H]IP\(_1\) accumulation. The IP\(_1\) accumulation study was performed in parallel with control SH-SY5Y cells and with those pretreated with the respective toxins for 1 hr.
Figure 10. Effects of pertussis toxin (1 μg/ml, 10 μg/ml) and cholera toxin (1.0 μg/ml) on the basal level of [3H]IP₁ accumulation (inset) and on the carbachol (10 mM)-stimulated [3H]IP₁ accumulations. The IP₁ accumulation study was performed in parallel with control SH-SY5Y cells and with those pretreated with the respective toxins for 22 hr.
Figure 11. Concentration-response curves of carbachol in stimulating [3H]IP$_1$ accumulation in control SH-SYSY cells (○) and in the cells pretreated with pertussis toxin (△; 1 μg/ml; Δ, 10 μg/ml) or cholera toxin (●, 1 μg/ml). The IP$_1$ accumulation study was performed in parallel with control SH-SYSY cells and with those pretreated with the respective toxins for 22 hr.
Table 4

Effects of pertussis toxin and cholera toxin on carbachol stimulation of IP$_1$ accumulation in SH-SY5Y cells.$^a$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EC$_{50}$ (µM)$^b$</th>
<th>$E_{\text{max}}$ (fmol/10$^6$ cells)$^c$</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>carbachol alone</td>
<td>18 (8.2-43)</td>
<td>51±3.2</td>
<td>0.96±0.1</td>
</tr>
<tr>
<td>+ PT 1 µg/ml</td>
<td>18 (14-24)</td>
<td>42±1.2$^d$</td>
<td>0.90±0.2</td>
</tr>
<tr>
<td>+ PT 10 µg/ml</td>
<td>22 (18-26)</td>
<td>22±0.79$^e$</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>+ CT 1 µg/ml</td>
<td>15 (12-18)</td>
<td>47±3.6</td>
<td>1.1±0.4</td>
</tr>
</tbody>
</table>

$^a$P$_1$ accumulation studies were performed as described in the text with control cells or cells pretreated with the respective toxin for 22 hr.

$^b$Values are geometric means and the range of variation of three or more experiments analyzed by a nonlinear least squares regression program.

$^c$$E_{\text{max}}$ values and Hill coefficient ($n_H$) values are arithmetic means±S.E.M. of three or more experiments.

$^d$p<0.05

$^e$p<0.01
Table 5
Effects of pertussis toxins on the number of viable SH-SY5Y cells

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Number of viable cells (x 10^3)</th>
<th>p value b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>530±29</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>0.001</td>
<td>520±18</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>0.01</td>
<td>510±62</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>0.1</td>
<td>510±41</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>1</td>
<td>520±55</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>10</td>
<td>510±70</td>
<td>&gt;.05</td>
</tr>
</tbody>
</table>

aSH-SY5Y cells were incubated in IMDM supplemented without (as control) or with various concentrations of pertussis toxin for 22 hr. After aspirating the medium, 0.2 ml of 0.02 percent EDTA-PBS buffer was added to the culture. The cells were rinsed off and collected. Another 0.8 ml of IMDM was added to the culture to rinse the wells and the remaining cells were collected. One milliliter of trypan blue was added to give a final volume of 2 ml. The cells were counted by using a hemocytometer. The viable cells were considered as those who were trypan blue resistant.

bStudent's t test was used for statistical analysis of the control group vs pertussis toxin-treated group.
studies were done to determine whether pertussis toxin caused
detachment of the cells from culture wells or cell death. After
pertussis toxin treatment (10 μg/ml, 22 hr), the cells were washed
with 1 ml of IMDM and counted using a hemocytometer. The number of
the viable cells (trypan blue resistant cells) from the pertussis
toxin-treated group showed no significant change from that of the
control group (table 5).

Discussion

We employed a high concentration of pertussis toxin (1-10
μg/ml) for a long period (22 hr) of incubation and found that
muscarinic receptor-mediated PI hydrolysis in SH-SY5Y cells is
sensitive to pertussis toxin. In some of the previously reported
experiments studying muscarinic receptor-mediated PI hydrolysis, the
concentrations of pertussis toxin varied from 20 to 100 ng/ml
(Masters et al., 1985; Sasaguri et al., 1986), which were lower than
the concentrations (1 μg/ml) of pertussis toxin to inhibit LTB₄
(Holian, 1986) and thrombin (Paris and Pouyssegur, 1986) induced PI
hydrolysis. The incubation of rat thyroid FRTL-5 cells in 10⁻¹⁰ M
for four hours did not inhibit α₁-adrenergic receptor-mediated PI
hydrolysis but 20 hr-incubation at the same concentration did
inhibit (Corda and Kohn, 1986), suggesting appropriate incubation
time is also important. In addition, we offer two hypotheses for
our results. First, different muscarinic receptor subtypes are
involved in the initiation of PI hydrolysis in different systems.
The phosphatidylinositol hydrolysis coupling muscarinic receptors in SH-SY5Y cells have high affinity for pirenzepine (9.2 nM) and low affinity for AF-DX 116 (470 nM) (Serra et al., 1988). Muscarinic receptors in cultured cerebellar granule cells had a relatively low affinity for pirenzepine (120 nM) and muscarinic receptor-mediated PI hydrolysis in this system was not sensitive to pertussis toxin (Xu and Chuang, 1987) while the types of muscarinic receptors in human embryonic tumor cells (Flow 9000) have not been characterized (Lo and Hughes, 1987). It is possible that the muscarinic receptors of these two sources are different from those in SH-SY5Y cells. An alternative hypothesis is that different G proteins may be involved in the different systems. Pertussis toxin ADP-ribosylates several G proteins including $G_1 (\alpha_{41}\beta\gamma)$, transducin, $G_0 (\alpha_{39}\beta\gamma)$ and a new GTP-binding protein ($\alpha_{40}\beta\gamma$), purified from the porcine brain (Katada et al., 1987). In a number of tissues pertussis toxin has been shown to produce a blockade or an attenuation of the inhibition of adenylate cyclase activity by neurotransmitters and GTP, suggesting a pertussis toxin sensitive G protein is involved in the signal transducing from the receptors to the adenylate cyclase (Ui, 1984). Pertussis toxin has been found to inhibit the phosphodiesterase stimulated by $\alpha_1$-adrenergic agonists (Corda and Kohn, 1986), LTB$_4$ (Holian, 1986), angiotensin II (Pfeilschnitter and Bauer, 1986), chemotactic factors (Bradford and Rubin, 1985) and thrombin (Paris and Pouyssegur, 1986) in non-neuronal tissues or cells. Bradykinin-dependent hyperpolarization of NG 108-15 cells
was believed to result from PI hydrolysis via the stimulation of phosphodiesterase, which was also sensitive to pertussis toxin (Higashida et al., 1986). However, no inhibitory effect of pertussis toxin on muscarinic receptor-mediated PI hydrolysis has been previously reported. The sensitivity of muscarinic receptor-mediated PI hydrolysis to pertussis toxin in human neuroblastoma SH-SY5Y cells indicates that one of the pertussis toxin sensitive G proteins may be involved in the signal transducing cascade and that a pertussis toxin sensitive G protein may act to stimulate PI hydrolysis in the brain.
CHAPTER 5

CHARACTERIZATION OF M₁ MUSCARINIC RECEPTORS IN THE MEMBRANES OF THE TRANSFECTED MURINE FIBROBLAST B82 CELLS

Since most tissues or cell lines contain more than one type of muscarinic receptor, the studies of the precise relationship between the muscarinic receptor types and the signal transduction mechanism were often hampered. In order to derive a system which contains only one type of muscarinic receptor, a gene which was identified to be the m₁ from a rat genomic DNA library was introduced into an established murine fibroblast cell line, B82, by using the eukaryotic expression vector pMVS-neo (figure 12) (Lai et al., 1988). Such an application of gene cloning offers the advantage of an isolated, well characterized receptor gene product in a controlled environment. While parental B82 cells contain no muscarinic receptor ligand binding activity, the membranes of the transfected B82 cells exhibit a specific, saturable, high affinity binding of [³H](-)QNB.

Methods

The neomycin resistant clones isolated for the pharmacological characterization of the muscarinic receptors were treated with 1 μM dexamethasone 16-24 hr prior to cell harvest or
Figure 12. Construction of the M₁ muscarinic receptor gene into the eukaryotic expression vector pMSV-neo. A 2.2 kb TaqI-BamHI restriction fragment from c71 (linear map) containing the entire open reading frame (shaded box) was subcloned into pIBI 24 (IBI, New Haven, CT) and excised with endonucleases MluI and KpnI. This fragment was then inserted downstream from the mouse mammary tumor virus long terminal repeat (MMTV LTR) by directional cloning into the MluI/KpnI sites in the polylinker region (not shown). Neo<sup>r</sup>, neomycin resistance gene; Amp<sup>r</sup>, ampicillin resistance gene.
[3H]IP1 measurement to induce the expression of the gene.

Results

[3H](−)QNB binding to the membranes of the transfected B82 cells

[3H](−)QNB binding to membranes isolated from two transfected B82 clones, cTB10 and cTB9 from a single transfection experiment, had Kd and Bmax values of 3.7 (2.2-5.1) pM and 120±29 fmol/mg protein for cTB10 and 12 (5.3-22) pM and 53±9.2 fmol/mg protein for cTB9, respectively. For specific [3H](−)QNB binding, the saturation isotherms were best defined by an interaction of the radioligand with a single population of saturable high affinity binding sites (figure 13). The specific [3H](−)QNB binding was dependent on the concentration of the membranes. Non-specific binding increased linearly with increasing radioligand concentration and did not saturate over the radioligand concentrations used in the assays.

In the absence of dexamethasone treatment, the Bmax of [3H](−)QNB binding was reduced by an average of 50 percent. In contrast, the membranes prepared from non-transfected B82 cells in the presence or absence of dexamethasone showed no specific [3H](−)QNB binding. The counts per minute of [3H](−)QNB (200 pM) binding to the membranes of non-transfected B82 cells were 26±4.7 in the absence and 38±10 in the presence of 1 µM atropine sulfate (p>.05).

Inhibition of [3H](−)QNB binding to the membranes of the transfected B82 cells
Figure 13. [\(^3\text{H}\)](-)QNB binding to muscarinic receptors in the membranes of transfected B82 cells: cTB10 in A, cTB9 in B. Total binding is indicated by closed circles, nonspecific binding by open circles and specific binding by triangles. Points shown are from respective representative experiments which were repeated 3-4 times. The experiments shown were analyzed by a nonlinear least squares regression program. The \(K_d\) and \(B_{\text{max}}\) values were 5.1 pM and 170 fmol/mg protein for cTB10 and 5.3 pM and 64 fmol/mg protein for cTB9, respectively.
Figure 14. Inhibition of \(^{3}H\)(-)-QNB (250 pM) binding to transfected cell membranes by the muscarinic antagonists atropine (closed circles), pirenzepine (open circles) and AF-DX 116 (open triangles). Points shown are from representative experiments (n=3). In A, the IC\(_{50}\) values were 3.9 nM for atropine, 180 nM for pirenzepine and 7.5 \(\mu\)M for AF-DX 116; while in B the IC\(_{50}\) values were 7.3 nM for atropine, 83 nM for pirenzepine and 11 \(\mu\)M for AF-DX 116. The inhibition constant (\(K_I\)) values were converted from IC\(_{50}\) values by using the Cheng and Prusoff equation (1973). The \(K_d\) values of \(^{3}H\)(-)-QNB determined in parallel experiments were 5.1 pM for cTB10 and 12 pM for cTB9. In A, the \(K_I\) values were 75 pM for atropine, 4.4 nM for pirenzepine and 300 nM for AF-DX 116 while in B, the \(K_I\) values were 320 pM for atropine, 3.8 nM for PZ and 450 nM for AF-DX 116.
Muscarinic antagonists inhibited specific $[^3H](-)QNB$ binding (figure 14). The inhibition constants ($K_i$) for atropine, pirenzepine and AF-DX 116 were converted from the $IC_{50}$ values of the respective unlabeled ligand by using the Cheng and Prusoff equation (1973) (table 6). In cTB10 membranes, the $K_i$ values and the pseudo-Hill coefficients were $0.057 \ (0.039-0.075) \ \text{nM}$ and $0.91 \pm 0.09$ for atropine, $2.5 \ (1.8-4.4) \ \text{nM}$ and $1.1 \pm 0.22$ for pirenzepine, $180 \ (100-300) \ \text{nM}$ and $1.2 \pm 0.10$ for AF-DX 116; while in cTB9 membranes, the $K_i$ values and the pseudo-Hill coefficients were $0.20 \ (0.10-0.32) \ \text{nM}$ and $1.1 \pm 0.09$ for atropine, $7.3 \ (3.8-16) \ \text{nM}$ and $1.0 \pm 0.08$ for pirenzepine, and $560 \ (240-1600) \ \text{nM}$ and $0.84 \pm 0.22$ for AF-DX 116, respectively.

Effects of GppNHp on carbachol/$[^3H](-)QNB$ competition

In order to characterize the possible interactions of the transfected $M_1$ muscarinic receptors with the endogenous $G$ proteins in B82 cells, we carried out carbachol/$[^3H](-)QNB$ competition in cTB10 membranes in the absence or in the presence of $100 \ \mu \text{M}$ GppNHp. Rat forebrain and rat heart membranes were prepared as previously described (Mei et al., 1987), stored at $-70^\circ \text{C}$, and used as controls in parallel carbachol/$[^3H](-)QNB$ competition experiments. As shown in figure 15 and table 7, $100 \ \mu \text{M}$ GppNHp shifted the carbachol inhibition curves to the right in the cTB10 membranes. The $IC_{50}$ value increased from $190 \ \mu \text{M}$ to $1500 \ \mu \text{M}$ in the absence and in the presence of $100 \ \mu \text{M}$ GppNHp, respectively. It is of interest to note
Table 6
Comparison of the inhibition constants of muscarinic antagonists in cTB10 and cTB9 cells.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_i^{a,b}$ (nM)</th>
<th>$K_i^{a,c}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>0.057&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.20&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(0.039-0.075)</td>
<td>(0.10-0.32)</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>2.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(1.8-4.4)</td>
<td>(3.8-16)</td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>180&lt;sup&gt;d&lt;/sup&gt;</td>
<td>560&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(100-300)</td>
<td>(240-1600)</td>
</tr>
</tbody>
</table>

<sup>a</sup>The inhibition constants ($K_i$) were converted from the IC<sub>50</sub> values of the antagonist inhibiting [3H](−)QNB binding by using the Cheng and Prusoff equation (1973).

<sup>b</sup>These data were from cTB10 membranes.

<sup>c</sup>These data were from cTB9 membranes.

<sup>d</sup>The geometric mean of 3 experiments with the range of variation in the parentheses.
Figure 15. Inhibition of $[^3H](-)$QNB (180 pM) binding to transfected cell (cTB10) membranes (in A), rat forebrain membranes (in B) and rat heart membranes (in C) by carbachol in the absence or in the presence of 100 μM GppNHp. Points shown are from representative experiments which were repeated 3 times. The IC$_{50}$ values of carbachol were 240 μM in the absence and 1200 μM in the presence of 100 μM GppNHp in transfected cell membranes in A; 110 μM and 360 μM in rat forebrain membranes in B; and 5.4 μM and 91 μM in rat heart membranes in C, respectively.
Table 7

Effects of GppNHp on the inhibition by carbachol of \([^{3}H](-)QNB\) binding to membranes from transfected cells, rat forebrain or rat heart.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>+100 (\mu M) GppNHp</th>
<th>p value(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTB10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC(_{50}) ((\mu M))</td>
<td>190 (120-250)</td>
<td>1500 (1200-2000)</td>
<td>&lt; .01</td>
</tr>
<tr>
<td>(n_H)</td>
<td>1.0±0.32</td>
<td>1.5±0.33</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>Rat forebrain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC(_{50}) ((\mu M))</td>
<td>160 (100-360)</td>
<td>350 (230-540)</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>(n_H)</td>
<td>0.39±0.04</td>
<td>0.52±0.04</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>IC(_{50})(H) ((\mu M))</td>
<td>2.8 (1.0-7.3)</td>
<td>3.7 (1.6-8.3)</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>Percent(H)</td>
<td>22±2.5</td>
<td>16±1.4</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>IC(_{50})(L) ((\mu M))</td>
<td>600 (340-1500)</td>
<td>680 (410-1000)</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>Rat heart</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC(_{50}) ((\mu M))</td>
<td>9.2 (4.4-32)</td>
<td>90 (55-140)</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>(n_H)</td>
<td>0.53±0.09</td>
<td>0.68±0.07</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>IC(_{50})(H) ((\mu M))</td>
<td>1.8 (0.72-4.5)</td>
<td>9.9 (2.7-36)</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>Percent(H)</td>
<td>42±7.8</td>
<td>25±8.1</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>IC(_{50})(L) ((\mu M))</td>
<td>57 (29-130)</td>
<td>130 (78-340)</td>
<td>&lt; .05</td>
</tr>
</tbody>
</table>

\(^a\)Carbachol/[\(^{3}H\)](-)QNB competition studies were performed as described in Chapter 2. IC\(_{50}\) values are presented as geometric means with the variation ranges in the parentheses and pseudo-Hill coefficients (\(n_H\)) are presented as arithmetic means ± S.E.M. of 3 separate experiments. H = high affinity and L = low affinity binding sites determined from a 2-site model.

\(^b\)The Student's t-test was used for statistical analysis.
that the pseudo-Hill coefficients of the carbachol curves in cTB10 membranes were 1.0±0.32 in the absence and 1.5±0.33 in the presence of 100 μM GppNHp, while those in rat forebrain and rat heart membranes were less than one (table 7). The rightward shift of the carbachol inhibition curves in the presence of 100 μM GppNHp was greatest in the rat heart membranes as previously described (Watson et al., 1986).

Discussion

While membranes of non-transfected B82 cells display no \[^{3}\text{H}](-)QNB binding, those of transfected B82 cells exhibit a specific, saturable, high affinity binding of \[^{3}\text{H}](-)QNB (figure 13). The density of the expressed muscarinic receptors in cTB9 cells is about 44 percent of that in cTB10 cells. Competitive inhibition of \[^{3}\text{H}](-)QNB binding by atropine, pirenzepine and AF-DX 116 shows that this receptor has high affinity for pirenzepine and low affinity for AF-DX 116, closely approximating the pharmacological characteristics of the M₁ type of the muscarinic receptor in rat brain (Watson et al., 1986c). The unity of the pseudo-Hill coefficients for these selective antagonists suggest that only one binding site, identified by \[^{3}\text{H}](-)QNB, was produced by the transfected system. Similar binding properties have also been reported for the cloned porcine (Fukuda et al., 1987) and rat (Brann et al., 1987) M₁ muscarinic receptors.

The inhibition constant converted from the IC\(^{50}\) value was
13 (9.8-17) μM. The non-hydrolyzable guanine nucleotide (GppNHp) (100 μM) caused a significant rightward shift of the carbachol inhibition curve of [3H](-)QNB binding to the muscarinic receptors in the cTB10 membranes under the conditions which optimize G protein modulation (Watson et al., 1986c). The IC_{50} value of carbachol was increased from 190 μM in the absence to 1500 μM (p<.01) in the presence of 100 μM GppNHp. The rightward shift of the carbachol inhibition of [3H](-)QNB binding in the presence of 100 μM GppNHp to rat forebrain and heart membranes was about 2.2 and 8.7 fold of the control, which is in good agreement with the previously published data from our laboratory (Watson et al., 1986c). These data suggest that the transfected M₁ muscarinic receptors may be coupled to an endogenous G protein in the transfected B82 cells.

The expressed rat M₁ receptor in the A9L cells had a much higher affinity for carbachol (IC_{50} of 0.52 μM) (Brann et al., 1987) than that in the transfected B82 cells (IC_{50} of 190 μM). Addition of 300 μM GTP induced a four fold decrease in the affinity of carbachol in the A9L cells, compared to an eight fold decrease in the affinity observed for the M₁ receptor in the transfected B82 cells in the presence of 100 μM GppNHp. The pseudo-Hill coefficients (0.4 with no GTP and 0.7 with 300 μM GTP) of carbachol/[3H](-)QNB competition curves in the A9L cells suggested multiple affinity states for carbachol. In contrast, the pseudo-Hill coefficient of carbachol/[3H](-)QNB inhibition curves were 1.1±0.32 in the absence and 1.5 ±0.33 in the presence of 100 μM
GppNHp. Besides differences in the experimental conditions employed in the two studies, differences in the microenvironment of the transfected receptors in the two cell lines may exist.
CHARACTERIZATION OF M₁ MUSCARINIC RECEPTORS IN THE INTACT TRANSFECTED B82 CELLS AND THEIR SECOND MESSENGER COUPLING

This study characterized the [³H](-)QNB binding to the muscarinic receptors in the intact cTB10 cells, a cell clone which had higher receptor density. The carbachol-stimulated [³H]IP₁ accumulation in these cells was studied by applying the Schild analysis of the inhibitory effects of various concentrations of muscarinic antagonist. The effects of pertussis toxin and phorbol ester on carbachol-stimulated [³H]IP₁ accumulation were studied. The effects of muscarinic agents on the basal and the PGE₁-stimulated cAMP formation in the cTB10 cells were also evaluated.

Results

Kinetic studies.

At 37°C, specific [³H](-)QNB (50 pM-60 pM) binding to the intact cTB10 cells was half maximal at 30±3.6 min and reached equilibrium at 90 min (figure 16). The specific binding after equilibrium did not change for at least 150 min. Nonspecific [³H](-)QNB binding was not time-dependent and remained unchanged during the 4 hr time course examined. The association rate constant
Figure 16. A representative experiment of association of specific [3H](-)-QNB (50 pM) binding to the intact cTB10 cells. Incubation was performed at 37°C during a time span from 5 min to 4 hr. Inset, dissociation was initiated by adding atropine sulfate into the binding reaction buffer (final concentration of 1 μM) which had been preincubated for 90 min. The data are plotted as the natural logarithm of the quotient of the amount (B) of [3H](-)-QNB bound at each time divided by the initial amount (B₀) of radioligand bound. The k⁺ and k⁻ values of this experiment were 1.3 x 10⁸ M⁻¹ min⁻¹ and 4.1 x 10⁻³ min⁻¹. The kinetic Kᵅ value was 31 pM.
was $1.4 \times 10^8 (1.1 \times 10^8 - 1.4 \times 10^8) \text{ min}^{-1} \text{ M}^{-1}$. Atropine sulfate at a final concentration of 1 μM was added to the binding reaction medium to initiate the dissociation of specifically bound $[^3H](-)\text{QNB}$ from the intact cells which had been preincubated with $[^3H](-)\text{QNB}$ for 90 min (figure 16). The half time of dissociation was 130±16 min. The dissociation rate constant was calculated as $5.4 \times 10^{-3} (4.1 \times 10^{-3} - 7.4 \times 10^{-3}) \text{ min}^{-1}$. The kinetic $K_d$ of $[^3H](-)\text{QNB}$ binding to the intact cTB10 cells was 39 (29 - 53) pM. In order for all concentrations of $[^3H](-)\text{QNB}$ to achieve steady-state, $[^3H](-)\text{QNB}$ saturation binding studies were carried out for 4 hr and ligand/$[^3H](-)\text{QNB}$ competition studies for 3 hr at 37°C.

Saturation studies.

$[^3H](-)\text{QNB}$ bound to the intact cTB10 cells in a saturable manner. As shown in figure 17, the specific binding increased from 6.3±1.2 fmol/10^6 cells to 15±2.8 fmol/10^6 cells as the free $[^3H](-)\text{QNB}$ concentration was increased from 5.8 pM to 200 pM. The total $[^3H](-)\text{QNB}$ bound was less than 10 percent of $[^3H](-)\text{QNB}$ added at the lowest concentration and about 3 percent of $[^3H](-)\text{QNB}$ added at the highest concentration. Nonspecific binding was less than 10 percent of total binding at the concentration of free $[^3H](-)\text{QNB}$ below the $K_d$ and about 25 percent of the total binding at the maximal concentrations (figure 17). The $K_d$ value of $[^3H](-)\text{QNB}$ binding to the intact cTB10 cells was 39 (29 - 53) pM.
Figure 17. [3H](-)QNB binding to the intact cTB10 cells. Points are shown as arithmetic means ± S.E.M. of 3 separate experiments. The curves were generated by a nonlinear least-squares regression program. The $K_d$ and $B_{max}$ values of the pooled data were 9.2 pM and 17 fmol/10^6 cells, respectively.
cells was 12 (9.5-16) pM with the $B_{\text{max}}$ value of 17±0.74 fmol/10^6 cells. In contrast, the B82 cells and those transfected with the vector (pMSV-neo) did not show any observable specific $[^3\text{H}](\text{-})$QNB binding under the same assay conditions. $[^3\text{H}](\text{-})$QNB binding to the B82 cells was 480±68 cpm/10^6 cells in the absence and 570±61 cpm/10^6 cells in the presence of 1 μM atropine sulfate (p>0.05).

Inhibition of $[^3\text{H}](\text{-})$QNB binding to intact cTB10 cells.

The muscarinic antagonists atropine sulfate, pirenzepine and AF-DX 116 inhibited specific $[^3\text{H}](\text{-})$QNB (200 pM) binding to intact cTB10 cells (figure 18). The IC_{50} values were 5.5 (3.9-6.8) nM for atropine, 630 (570-690) nM for pirenzepine and 26 (18-45) μM for AF-DX 116. The inhibition curves of these three ligands of $[^3\text{H}](\text{-})$QNB binding were best fitted by a one-site nonlinear least-squares regression model. The pseudo-Hill coefficients were 1.1±0.09 for atropine, 0.90±0.04 for pirenzepine and 0.95±0.08 for AF-DX 116. By using the Cheng and Prusoff (1973) equation, we converted the IC_{50} values into the inhibition constant ($K_\text{i}$) values as summarized in table 8. Pirenzepine had a higher affinity for the muscarinic receptors expressed in the cTB10 cells than AF-DX 116 since the $K_\text{i}$ values were 33 (28-37) nM and 1.4 (0.75-2.9) μM, respectively. Muscarinic antagonists HHSiD and himbacine at 10 μM, and muscarinic agonists oxotremorine, pilocarpine and McN-A-343 at 1 mM totally inhibited specific $[^3\text{H}](\text{-})$QNB binding. Carbachol at 1 mM inhibited specific $[^3\text{H}](\text{-})$QNB binding by 12±2.5 percent.
Figure 18. Inhibition of specific $[^{3}H](-)$QNB binding to the intact cTB10 cells by muscarinic antagonists atropine, pirenzepine and AF-DX 116. Points are shown as arithmetic means ± S.E.M. of 3 separate experiments. The curves were generated by a nonlinear least-squares regression program. The IC$_{50}$ values were 5.5 (3.9-6.8) nM for atropine, 630 (570-690) nM for pirenzepine and 26 (18-45) µM for AF-DX 116. GABA and norepinephrine at 1 mM, hemicholinium-3, hexamethonium and d-tubocurare at 10 µM had no inhibitory effect on $[^{3}H](-)$QNB binding to the intact cTB10 cells. Serotonin at 1 mM and propranolol at 10 µM decreased $[^{3}H](-)$QNB binding by 30 percent and 33 percent, respectively.
Table 8

Inhibition constants of atropine, pirenzepine and AF-DX 116 from ligand/[3H](-)QNB competition studies and from PI hydrolysis studies in the cTB10 cells

<table>
<thead>
<tr>
<th>Slope</th>
<th>( \text{Ki}^\text{b} ) (nM)</th>
<th>( \text{nH}^\text{c} )</th>
<th>( \text{Ki}^\text{d} ) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>0.29 (0.25-0.36)</td>
<td>1.1±0.09</td>
<td>0.40 1.1</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>33 (28-37)</td>
<td>0.90±0.04</td>
<td>9.7 1.1</td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>1400 (750-2900)</td>
<td>0.95±0.08</td>
<td>390 1.2</td>
</tr>
</tbody>
</table>

\( ^a \)Ligand [3H](-)QNB competition studies and inositol lipid hydrolysis studies were performed as described in Chapter 2.

\( ^b \)Inhibition constants (Ki) from ligand/[3H](-)QNB competition studies were converted from the IC_{50} values by using the Cheng and Prusoff (1973) equation. Data are presented as geometric means with ranges in the parentheses (n=3).

\( ^c \)Pseudo-Hill coefficients (nH) from ligand/[3H](-)QNB competition studies were generated by a one-site non-linear least-squares regression analysis. Data are presented as arithmetic means ± S.E.M.

\( ^d \)Functional inhibition constants (Ki) were converted from the pA2 values. The slopes were from the Schild plots, insets of figure 19.
Carbachol-stimulated $[^3$H]$IP_1$ accumulation in the cTB10 cells

The basal $[^3$H]$IP_1$ accumulation was 4100±120 cpm/10$^5$ cells in the non-transfected B82 cells and 1600±50 cpm/10$^5$ cells in the cTB10 cells (p<0.01). Carbachol had no stimulatory or inhibitory effect on the $[^3$H]$IP_1$ accumulation in non-transfected B82 cells. $[^3$H]$IP_1$ formed in the presence of 0.10 mM carbachol was about 98±7.7 percent of the basal level (p>0.05). In contrast, carbachol at the concentration of 1 μM significantly increased $[^3$H]$IP_1$ formation in the cTB10 cells. Preliminary studies of the time course of carbachol-stimulated $[^3$H]$IP_1$ accumulation showed that the amount of $[^3$H]$IP_1$ stimulated by 0.1 mM carbachol was linear during the first 2 hr period of the study. The stimulatory effect of carbachol on $[^3$H]$IP_1$ formation in the cTB10 cells is concentration-dependent as shown in figure 19. The maximal effect, induced by 1 mM carbachol, was 3-5 fold above the basal level. The EC$_{50}$ value of carbachol in inducing $[^3$H]$IP_1$ formation in the transfected cells was 18 (8.9-43) μM (n=14). The concentration-response curve of carbachol was steep. The pseudo-Hill coefficient of 1.2±0.1 was generated by nonlinear least-squares regression analysis.

Inhibition of carbachol-induced $[^3$H]$IP_1$ accumulation in cTB10 cells by muscarinic antagonists.

The muscarinic antagonists atropine, pirenzepine and AF-DX 116 all attenuated $[^3$H]$IP_1$ formation in the cTB10 cells.
Figure 19. Concentration-response curves of carbachol in stimulating [3H]IP$_1$ accumulation in the cTB10 cells in the absence and in the presence of different concentrations of muscarinic antagonists: atropine (in A), pirenzepine (in B) and AF-DX 116 (in C). The inset of each panel shows the Schild plot of the inhibition data in that panel. Points are shown as arithmetic means of 3 separate experiments with the average S.E.M. values of 10 less than 15 percent of the value indicated by the corresponding point. The EC$_{50}$ values and E$_{max}$ of carbachol in the absence and in the presence of muscarinic antagonists are summarized in table 9.
stimulated by carbachol. None of these drugs had any stimulatory or inhibitory effect on the basal levels of $[^3H]IP_1$ formation. The functional inhibition constant of each of these drugs on the concentration-response curve of carbachol in stimulating $[^3H]IP_1$ formation in the cTB10 cells was obtained by applying the Schild analysis (1949).

Increasing concentrations of the muscarinic antagonists shifted the carbachol concentration response curve to the right without a reduction of the maximal effect of carbachol (figure 19). The EC$_{50}$ values and maximal effects of carbachol in the absence and in the presence of different concentrations of antagonists are presented in table 9. The insets of figure 19 show the Schild plots of the inhibition of carbachol-stimulated $[^3H]IP_1$ accumulation in the cTB10 cells by muscarinic antagonists. The pA$_2$ values obtained were 9.4, 8.0 and 6.4 for atropine, pirenzepine and AF-116, respectively. The functional inhibition constants converted from the pA$_2$ values and the slopes of the Schild plots for the muscarinic antagonists are shown in table 8.

Effects of pertussis toxin and PMA on carbachol-stimulated $[^3H]IP_1$ accumulation in the cTB10 cells.

Pretreatment of the cTB10 cells with pertussis toxin for 22 hr inhibited carbachol-stimulated $[^3H]IP_1$ accumulation. At the lowest concentration of pertussis toxin (1 ng/ml), carbachol-induced $[^3H]IP_1$ formation by 18 percent (figure 20). This inhibitory
Table 9
Effects of muscarinic antagonists on carbachol-induced hydrolysis of inositol lipids in the cTS10 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EC50(µM)b</th>
<th>Emax(percent)c</th>
<th>nH c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbachol</td>
<td>18(8.9-43)</td>
<td>99±1.2</td>
<td>1.2±0.10</td>
</tr>
<tr>
<td>+Atropine(µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.003</td>
<td>220(160-280)</td>
<td>92±2.7</td>
<td>1.1±0.26</td>
</tr>
<tr>
<td>0.01</td>
<td>420(120-990)</td>
<td>88±14</td>
<td>1.5±0.08</td>
</tr>
<tr>
<td>0.03</td>
<td>2400(1600-4500)</td>
<td>96±5.3</td>
<td>0.95±0.21</td>
</tr>
<tr>
<td>0.10</td>
<td>8100(5200-11000)</td>
<td>100±2.5</td>
<td>0.88±0.04</td>
</tr>
<tr>
<td>+Pirenzepine(µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>38(28-58)</td>
<td>100±7.3</td>
<td>1.0±0.24</td>
</tr>
<tr>
<td>0.03</td>
<td>91(26-180)</td>
<td>92±4.8</td>
<td>1.2±0.28</td>
</tr>
<tr>
<td>0.10</td>
<td>410(100-670)</td>
<td>100±3.8</td>
<td>0.59±0.01</td>
</tr>
<tr>
<td>0.30</td>
<td>1900(890-3600)</td>
<td>100±1.8</td>
<td>0.68±0.15</td>
</tr>
<tr>
<td>+AF-DX 116(µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>60(33-150)</td>
<td>99±7.8</td>
<td>1.1±0.21</td>
</tr>
<tr>
<td>3.0</td>
<td>310(220-400)</td>
<td>94±7.0</td>
<td>0.69±0.07</td>
</tr>
<tr>
<td>10</td>
<td>730(630-871)</td>
<td>95±4.9</td>
<td>0.77±0.02</td>
</tr>
<tr>
<td>30</td>
<td>2900(1900-4400)</td>
<td>110±15</td>
<td>0.76±0.14</td>
</tr>
</tbody>
</table>

aData were analyzed by a computer program for logistic nonlinear least-squares regression analysis. Carbachol-stimulated [3H]IP1 accumulation in the absence of the antagonists was first analyzed to generate an Emax value as 100 percent. [3H]IP1 accumulation in parallel studies with muscarinic antagonists was converted into the percentage of the Emax value. The basal [3H]IP1 accumulation in the cTS10 cells in the presence of 10 mM LiCl was 1600 ± 53 cpm/10^5 cells and the maximal [3H]IP1 accumulation by carbachol was 5500 ± 50 cpm/10^5 cells. Cholecystokinin (1 µM), bradykinin (10 µM), platelet activating factor (1 µM), histamine (1 mM), serotonin (1 mM) and norepinephrine (1 mM) did not change the basal [3H]IP1 accumulation in the B82 or the cTS10 cells.

bData are presented as geometric means with variation range in parentheses (n=3 except for carbachol control group, n=14).

cData are presented as arithmetic means ± S.E.M.
Figure 20. Effects of pertussis toxin on carbachol-stimulated $[^3H]IP_1$ accumulation in the cT810 cells. The amount of $[^3H]IP_1$ accumulated at 0.1 mM carbachol in the control cells was taken as 100 percent, while the amount of $[^3H]IP_1$ accumulated in the pertussis toxin-pretreated (22 hr) cells at 0.1 mM carbachol was expressed as a percentage of that in the control cells. Data are shown as arithmetic means ± S.E.M. (n = 3).
effect of pertussis toxin was concentration-dependent. In the cells pretreated with 10 μg/ml of pertussis toxin, the $[^3\text{H}]$IP$_1$ formation was only 6.5 percent of the control group.

Pretreatment of the cTB10 cells with the phorbol ester, PMA, also decreased the $[^3\text{H}]$IP$_1$ formation stimulated by 0.1 mM carbachol. The carbachol-stimulated $[^3\text{H}]$IP$_1$ formation in the cells pretreated with 0.3 mM PMA for 1 hr was 83 percent of the control. As shown in figure 21, increasing concentrations of PMA produced a greater inhibition of carbachol-stimulated $[^3\text{H}]$IP$_1$ accumulation in the cTB10 cells. The inhibitory effect of PMA was maximal at 10 nM when $[^3\text{H}]$IP$_1$ formation was 39±1.8 percent of the control. A further increase in PMA concentration did not enhance the inhibition. The half-maximal inhibitory concentration (IC$_{50}$) of PMA was 0.81 (0.32-1.8) nM.

Cyclic AMP formation in the non-transfected B82 and cTB10 cells

The basal level of cAMP in the non-transfected B82 cells and the cTB10 cells were 2.6±0.54 pmole/10$^6$ cells and 3.3±0.58 pmole/10$^6$ cells, respectively (p>0.05). In the non-transfected B82 cells, PGE$_1$ has been shown to stimulate cAMP formation (Fraser et al., 1987). In agreement with this, we observed a 28-fold increase in cAMP formation in the B82 cells at 0.1 mM PGE$_1$.

Figure 22 illustrates the effects of a variety of agents on the cAMP formation in the non-transfected cells as well as cTB10 cells. Adenosine at 1 mM increased cAMP formation by 2.6±0.62 fold of the
Figure 21. Effect of PMA on carbachol-stimulated [\[^3\text{H}\]IP\(_1\)] accumulation in the cTB10 cells. The amount of [\[^3\text{H}\]IP\(_1\)] accumulated at 0.1 mM carbachol in the control cells was taken as 100 percent, while the amount of [\[^3\text{H}\]IP\(_1\)] accumulated in the PMA-pretreated (1 hr) cells at 0.1 mM carbachol was expressed as a percentage of that in the control cells. Data are shown as arithmetic means ± S.E.M. (n = 3).
Figure 22. Effects of different drugs on cAMP formation in the B82 (A) and the cTB10 (B) cells. Data are presented as means ± S.E.M. as ratio of cAMP formed over the basal (n = 3). The concentrations of the drugs are 0.1 mM for carbachol (CCh), forskolin (FOS) and PGE₁ except those for adenosine (AD) at 1 mM and atropine (ATROP) at 1 μM.
basal in the non-transfected cells, forskolin at 0.1 mM by 14±4.1 fold, and PGE₁ at 0.1 mM by 28±7.2 fold (figure 22A). There was no detectable change in the cAMP content in the reaction medium at 0.1 mM PGE₁. The basal level of cAMP in the cell medium was 0.79±0.10 pmol/10⁶ cells while the cAMP in the medium of cells stimulated by 0.1 mM PGE₁ was 100±0.02 percent of the basal value. As shown in figure 22, carbachol, at 0.1 mM, had no effect on the basal, the forskolin-induced, or PGE₁-induced cAMP formation in the B82 cells or in the cTB10 cells.

The concentration-response curves of PGE₁ in stimulating cAMP formation in the cTB10 cells in the absence and the presence of 10 mM carbachol with or without 1 μM atropine sulfate were shown in figure 23. At 10 nM, PGE₁ significantly increased cAMP formation with the amount of cAMP being 3.5±0.36 pmol/10⁶ cells (that stimulated by PGE₁ minus the basal). Table 10 summarizes the EC₅₀ values and the E₉₀ values of PGE₁. Carbachol at 10 mM had no effect on the PGE₁-stimulation of cAMP formation.

Discussion

The muscarinic receptors in the intact cTB10 cells have also been characterized by radioligand binding assays. The Kᵋ value (12 pM) for [³H](-)QNB from the saturation experiments is close to that obtained from the kinetic experiments (39 pM). The specific [³H](-)QNB binding to the intact cTB10 cells was displaceable by muscarinic antagonists pirenzepine and AF-DX 116. The Kᵋ values
Figure 23. Concentration-response curves of PGE1 in stimulating cAMP formation in the cTB10 cells in the absence (○) or in the presence of 10 mM carbachol (□) or 10 mM carbachol and 1 µM atropine (△). Each point represents a mean ± S.E.M. of the values from 3 or more experiments. Table 3 details the values of EC50, Emax and nH.
Table 10

Effects of carbachol on PGE₁-induced cAMP formation in the cTB10 cells.

<table>
<thead>
<tr>
<th></th>
<th>EC₅₀ (nM)ᵇ</th>
<th>Eₘₐₓ (pmol/10⁶ cell)ᶜ</th>
<th>nₜ c</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGE₁</td>
<td>180 (73-350)</td>
<td>57±14</td>
<td>0.64±0.07</td>
</tr>
<tr>
<td>+carbachol, 10 mM</td>
<td>150 (95-260)</td>
<td>59±8.4</td>
<td>0.87±0.06</td>
</tr>
<tr>
<td>+carbachol, 10 mM +atropine, 1 μM</td>
<td>320 (230-440)</td>
<td>67±17</td>
<td>0.71±0.09</td>
</tr>
</tbody>
</table>

ᵇData were analyzed by a computer program for logistic nonlinear least-squares regression analysis.
ᶜData are presented as geometric means with variation range in parentheses (n = 4).
ᶜᶜData are presented as arithmetic means ± S.E.M.
were 33 nM for pirenzepine and 1.4 \mu M for AF-DX 116. These values are similar to those defined as the M₁ muscarinic receptor in the brain by \(^{3}H\)pirenzepine binding, 11 nM for pirenzepine and 0.53 \mu M for AF-DX 116 (Watson et al., 1986b) and in the SH-SY5Y cells, 6.9 nM for pirenzepine and 0.21 \mu M for AF-DX 116 (Serra et al., 1988). In COS-7 cells transfected with the m₁ gene, the \(K_i\) value of pirenzepine which we converted from the published IC₅₀ value (Bonner et al., 1987) by the Cheng and Prusoff (1973) equation was 8.5 nM, in agreement with the \(K_i\) value of pirenzepine for the transfected m₁ muscarinic receptor in our system.

Inhibition of the specific \(^{3}H\)(-)-QNB binding to the intact cTBI0 cells was observed by the muscarinic agonists carbachol, McN-A-343, pilocarpine or oxotremorine. The full agonist carbachol at 1 mM inhibited 12 percent of the specific \(^{3}H\)(-)-QNB binding while the other three muscarinic agonists at 1 mM totally inhibited specific \(^{3}H\)(-)-QNB binding. Since carbachol stimulates the hydrolysis of inositol lipids in the cTBI0 cells with an EC₅₀ value of 18 \mu M, such a disparity between carbachol's high efficacy and potency in the functional system, yet inability to inhibit \(^{3}H\)(-)-QNB binding may be due to a number of factors. There is a difference between the time courses of the binding studies and the biofunctional studies. \(^{3}H\)IP₁ in the cTBI0 cells was accumulated in the presence of 10 mM LiCl linearly for 1 hr after starting the incubation of the cells with carbachol. In contrast, the carbachol/\(^{3}H\)(-)-QNB competition binding assay was performed
for 3 hr incubation in order to achieve equilibrium, during which time internalization or desensitization may have occurred. In the homogenates of the cTB10 cells where such regulation cannot take place, carbachol at 1 mM inhibited 75 percent of the specific \[^3\text{H}(-)\text{QNB}\] binding (Lai et al., 1988). In Chapter 7, carbachol at 1 mM inhibited 90 percent of the specific binding of \[^3\text{H}(-)\text{methyl-QNB} ([^3\text{H}(-)\text{MQNB})\], a quaternary non-selective ligand, to the muscarinic receptors in the intact cells. Alternatively, carbachol may have limited access to some of the muscarinic receptor sites in intact cells which are labeled by the lipophilic \[^3\text{H}(-)\text{QNB}\]. The muscarinic receptors in intact cells, upon prolonged incubation, may be in an uncoupled state which has very low affinity for carbachol and/or other ligands.

Stimulation of the muscarinic receptors in the cTB10 cells mediated the hydrolysis of inositol lipids. The muscarinic antagonists atropine, pirenzepine and AF-DX 116 were competitive in inhibiting carbachol-stimulated \[^3\text{H}\text{IP}_1\] accumulation in the cTB10 cells. First, each of the muscarinic antagonists caused a rightward shift of the carbachol concentration response curve without a reduction in the maximal effect of carbachol. Secondly, the rightward shifted curves were parallel to the original concentration response curve in the absence of antagonists. No significant differences of the linearized carbachol concentration response curves (probit analysis) were observed from the tests for parallelism (Tallarida and Murray, 1987). Finally, the slopes of
Schild linear regression of the inhibitions were close to one. A Schild regression slope of unity implies a one-to-one relationship between the antagonist and the receptor with no substantial cooperative effects (Kenakin, 1984). The Schild regression analysis used in this study to evaluate the inhibition of carbachol-induced $[^3H]IP_1$ accumulation by a series of concentrations of muscarinic antagonists confirms that pirenzepine had a higher affinity than AF-DX 116 for the muscarinic receptors which mediated $[^3H]IP_1$ accumulations in the cTB10 cells. The functional $K_i$ values of pirenzepine and AF-DX 116 were 9.7 nM and 390 nM, respectively. We found that the majority of muscarinic receptors in human neuroblastoma SH-SY5Y cells are of the $M_1$ type and stimulation of them mediates the hydrolysis of inositol lipids (Serra et al., 1988; Mei et al., submitted b). The functional $K_i$ values of pirenzepine and AF-DX 116 in SH-SY5Y cells, 9.2 nM and 470 nM, respectively, are very similar to those obtained in the cTB10 cells. These data suggest that the transfected $m_1$ gene encodes an $M_1$ type muscarinic receptor which is functionally coupled to the hydrolysis of inositol lipids in the cTB10 cells.

Hydrolysis of inositol lipids is catalyzed by phosphoinositide-specific phosphodiesterase (Hawthorne, 1983). The mechanism of the $M_1$ muscarinic receptor-mediated stimulation of this enzyme is not clear. The following observations suggest that a pertussis toxin-sensitive G-protein may be involved in the coupling of the stimulated muscarinic receptor to the
phosphodiesterase. Carbachol binding to the M$_1$ muscarinic receptors in the membranes of the cTB10 cells was shifted from a high-affinity state to a low-affinity state by 100 µM GppNHp, a non-hydrolyzable guanyl nucleotide (Lai et al., 1988). Pertussis toxin was found to inhibit carbachol-stimulated $[^3$H]$IIP_1$ accumulation in the human neuroblastoma SH-SY5Y cells (Mei et al., 1988) and in CHO cells transfected with the procine m$_2$ gene (Ashkenzai et al., 1987). In this study we found that carbachol-induced $[^3$H]$IIP_1$ accumulation in the cTB10 cells is also sensitive to pertussis toxin (figure 20). Pertussis toxin at the low concentration of 1 ng/ml inhibited carbachol-stimulated $[^3$H]$IIP_1$ accumulation by 18 percent.

PMA inhibits the muscarinic receptor-mediated hydrolysis of inositol lipids in the human neuroblastoma SH-SY5Y cells (Serra et al., 1986), rat cerebellar granule cells (Xu and Chuang, 1987), human 1321N1 astrocytoma cells (Orehana et al., 1985) and human embryonic pituitary cell line Flow 9000 (Lo and Hughes, 1987). In our transfected cell system, we found that the pretreatment of the cTB10 cells with 100 nM PMA for 1 hr did not significantly change the $B_{max}$ and the $K_d$ values of $[^3$H](−)QNB binding to the cTB10 cells (data not shown) but inhibited 50 percent of carbachol-stimulated $[^3$H]$IIP_1$ accumulation. Consistent with this finding is that there is no difference in the $B_{max}$ and $K_d$ values of the receptor in human 1321N1 astrocytoma cells for $[^3$H]N-methyl scopolamine in control cells versus cells treated with 10 µM PMA.
(Orehana et al., 1985). Furthermore, pretreatment of SH-SY5Y cells with phorbol esters was found to slightly increase the EC$_{50}$ values of carbachol in stimulating $[^3$H$]$IP$_3$ accumulation, but it dramatically decreased the maximal effect of carbachol (Mei et al., submitted b). These results suggest that the muscarinic receptor is probably not the primary site of PMA activity. The G protein that couples the receptor to the phosphodiesterase may be a target of the protein kinase C stimulated by PMA. Protein kinase C can phosphorylate $G_{1\alpha}$ in vitro and this reaction is suppressed by $G_{By}$ (Gilman, 1987). Alternatively, the phosphoinositide-specific phosphodiesterase may be modified by protein kinase C-catalyzed phosphorylation. Interestingly, there was a plateau of PMA inhibition of carbachol-stimulated $[^3$H$]$IP$_3$ accumulation in the cTB10 cells (figure 21) suggesting that the signal transducing cascade flows via some pathway which is not sensitive to phosphorylations by protein kinase C, i.e., that the G proteins or the phosphodiesterases involved are heterogeneous.

PGE$_1$ stimulated cAMP formation in both the B82 cells and the cTB10 cells in the presence of 5 mM IBMX, demonstrating the presence of an endogenous adenylate cyclase. Carbachol at 10 mM had no stimulatory or inhibitory effect on either the basal or PGE$_1$-stimulated cAMP formation in the presence of 5 mM IBMX (figure 23). In the absence of IBMX, preliminary data show that carbachol also has no effect on the basal or PGE$_1$-stimulated cAMP in the cTB10 cells suggesting that carbachol does not interact with
the phosphodiesterase system. Thus, there is no evidence that the M₁ muscarinic receptor is coupled in an inhibitory manner to the adenylate cyclase in the cTB10 cells. The possibility remains that a G protein required for such a coupling is not optimal in this system. We also failed to observe any stimulatory or inhibitory effect of a muscarinic agonist on cAMP formation in human neuroblastoma SH-SYSY cells (Mei et al., submitted b). In contrast, an M₁ muscarinic receptor in the transfected RAT-1 cells was found to be inversely coupled to adenylate cyclase (Stein et al., 1988). In addition, in the human kidney cells transfected with the human m₁ gene, carbachol potentiated forskolin-stimulated cAMP formation (Peralta et al., 1988). Our finding shows that the forskolin-stimulated cAMP formation was slightly elevated in the presence of carbachol in the cTB10 cells, however, this difference was not statistically significant. These disparities emphasize the importance of the choice of the host cell line.

This study shows that the stable expression of the rat m₁ muscarinic receptor gene in the murine fibroblast B82 cells (cTB10) gives rise to a homogeneous population of the muscarinic receptors which exhibit characteristics similar to those defined as the M₁ type. These receptors are coupled to the hydrolysis of inositol lipids, possibly through a pertussis toxin sensitive G protein, but they do not appear to interact with the PGE₁-stimulated cAMP formation system in the cTB10 cells. Stable expression of the putative M₁ muscarinic receptor has been achieved by transfecting
the gene into mouse fibroblast A9 L cells (Brann et al., 1987) and of the M2 muscarinic receptor into Chinese hamster ovary (CHO) cells (Peralta et al., 1987a; Ashkenazi et al., 1987). These studies, together with ours, demonstrate that the continuous expression of one type of muscarinic receptor encoded by the transfected gene provides an unlimited source of receptor for characterizing its binding properties and its signal transduction mechanisms.
CHAPTER 7

THE RELATIONSHIP BETWEEN AGONIST STATES OF THE M₁ MUSCARINIC RECEPTOR AND THE HYDROLYSIS OF INOSITOL LIPIDS IN TRANSFECTED MURINE FIBROBLAST CELLS EXPRESSING DIFFERENT RECEPTOR DENSITIES

Since the origin of the receptor concept, the drug-receptor interaction has been characterized extensively using drugs as the variable. With the advent of recombinant DNA techniques, we can now alter directly the receptor portion of the drug-receptor interaction. For example, a receptor can be structurally modified directly by site-directed mutagenesis (O'Dowd et al., 1988) or chimeric constructions (Kobilka et al., 1988). A receptor can also be manipulated at the level of its expression in a particular system. This strategy tests the effect of concentration changes of the receptor on drug-receptor interaction and receptor-second messenger coupling.

In this study, we generated a series of 7 clones of murine fibroblast cells which express various amounts of M₁ muscarinic receptors. Radioligand binding assays were performed in intact cells with [³H](-)MQNB. A correlation of the [³H](-)MQNB binding capacity with the maximal muscarinic receptor-mediated response was assessed. The results indicate that the carbachol high affinity state of the M₁ muscarinic receptors in the transfected cells may be the functionally coupled state. The carbachol
concentration-occupancy relationship was compared with its concentration-response relationship, and the data suggest the presence of spare M₁ muscarinic receptors in the clones with high receptor densities but not in those with low receptor densities.

Methods

Cell Culture

The m₁ muscarinic receptor gene under the constitutive control of the human β-actin promoter in the vector pHBAPr-1-neo vector (figure 24) (Gunning et al., 1987) was transfected into the murine fibroblast (B82) cells (Lai et al., 1988; Lai et al., in preparation). Transfected cells were cloned and screened for muscarinic receptor binding activity. A series of 7 clones which exhibited various [³H](-)MQNB binding capacities from a single transfection experiment were maintained as described previously (Chapter 2).

Radioligand binding assays

[³H](-)MQNB binding to the intact cells was performed as previously described (Chapter 2). In LK3-1 cells, the specific binding and non-specific binding were 38±5.2 cpm/well and 15±2.0 cpm/well, respectively with the total counts of 2500±130 cpm/well at 38 pM [³H](-)MQNB; 190±14 cpm/well and 330±25 cpm/well, respectively with the total counts of 63000±1800 cpm/well at 960 pM [³H](-)MQNB. In LK3-8 cells, the specific and non-specific
binding were 460±110 cpm/well and 30±15 cpm/well, respectively with the total counts of 2300±120 cpm/well at 35 pM \(^{3}H\)(-)MQNB; 2800±890 cpm/well and 320±21 cpm/well with the total counts of 54000±5100 cpm/well at 820 pM \(^{3}H\)(-)MQNB.

**Results**

\(^{3}H\)(-)MQNB binding to intact cells

There was no specific \(^{3}H\)(-)MQNB binding to intact non-transfected B82 cells or cells transfected with the pHBAPr-1-neo vector alone (LK4-V). \(^{3}H\)(-)MQNB binding (900 pM) to the former was 1500±36 cpm/10⁶ cells in the absence and 1500±92 cpm/10⁶ cells in the presence of 1 μM atropine sulfate (p>0.05, paired t-test) while \(^{3}H\)(-)MQNB binding (900 pM) to the latter was 1200±49 cpm/10⁶ cells in the absence and 1200±39 cpm/10⁶ cells in the presence of 1 μM atropine sulfate (p>0.05, paired t-test).

\(^{3}H\)(-)MQNB binding to the 7 clones transfected with the m₁ muscarinic receptor gene was saturable, with the B\(\text{max}\) values ranging from 12 to 260 fmol/10⁶ cells (62 to 1300 fmol/mg protein) (figure 25 and table 11). The rank order based on the receptor density of these clones was LK3-8=LK3-3>LK7-2>LK3-7>LK7-6>LK3-4>LK3-1 (paired t-test). Although the receptor densities of these clones varied, the dissociation constants of \(^{3}H\)(-)MQNB for the muscarinic receptors of the different clones were similar (table 11). \(^{3}H\)(-)MQNB labeled a homogeneous population of muscarinic receptors since the Hill coefficients of its binding to the
Figure 24. Construction of the m₁ muscarinic receptor gene into the eukaryotic expression vector pHβAPr-1-neo. The 1.4 kb open reading frame (o.r.f.) and 0.15 kb of the 3'-untranslated region of the m₁ gene was excised from a 2.2 TaqI/BamHI restriction fragment (linear map) with HpaII. The 1.4 kb fragment was subcloned into pIBI24 and excised with HindIII (5') and BamHI (3'). This fragment was inserted into the unique HindIII and BamHI sites downstream from the human β-actin promoter. 5'-UT, 5'-untranslated region of β-actin gene; IVS1, intervening sequence 1 of β-actin gene; AMPr, ampicillin resistance gene; Neo r, neomycin resistance gene; Ori, origin of replication.
Figure 25. Saturation isotherms of the specific [3H](–)MQNB binding to the series of clones of the transfected B82 cells: LK3-1 (●), LK3-4 (○), LK7-6 (□), LK3-7 (△), LK7-2 (▲), LK3-3 (▽) and LK3-8 (▽). The data points represent the means of 3 independent experiments. The standard errors of the means which were less than 10 percent of the values indicated were omitted from the figure. The lines are the best fit from the computer-generated nonlinear least-squares regression analyses of a three parameter logistic equation. [3H](–)MQNB was incubated with the intact cells in 1 ml of IMDM at 37°C for 3 hr (see Methods). For the curves shown, the Kd values (Hill coefficients) and the B_{max} values are 100 pM (1.0) and 12 fmol/10^6 cells in LK3-1, 110 pM (0.96) and 18 fmol/10^6 cells in LK3-4, 100 pM (1.0) and 30 fmol/10^6 cells in LK7-6, 140 pM (1.1) and 95 fmol/10^6 cells in LK3-7, 140 pm (1.0) and 140 fmol/10^6 cells in LK7-2, 160 pM (1.00) and 240 fmol/10^6 cells in LK3-3, and 220 pM (0.98) and 230 fmol/10^6 cells in LK3-8. The K_d values, the B_{max} values and the Hill coefficients of the individual experiments are shown in table 11.
Table 11
The binding parameters of the H1 muscarinic receptors in a series of transfected B82 cells.

<table>
<thead>
<tr>
<th></th>
<th><a href="-">3H</a>MQNB binding</th>
<th>carbachol/<a href="-">3H</a>MQNB binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bmax a (fmol/10^6 cells)</td>
<td>Kd b (µM)</td>
</tr>
<tr>
<td>LK3-1</td>
<td>12±0.98</td>
<td>100</td>
</tr>
<tr>
<td>LK3-4</td>
<td>18±1.5</td>
<td>110</td>
</tr>
<tr>
<td>LK7-6</td>
<td>97</td>
<td>(67-120)</td>
</tr>
<tr>
<td>LK3-7</td>
<td>96±15</td>
<td>(120-180)</td>
</tr>
<tr>
<td>LK7-2</td>
<td>150</td>
<td>(140-160)</td>
</tr>
<tr>
<td>LK3-3</td>
<td>160</td>
<td>(140-180)</td>
</tr>
<tr>
<td>LK3-8</td>
<td>190</td>
<td>(91-310)</td>
</tr>
</tbody>
</table>

a p<0.001, one-way ANOVA. Differences between the clones are presented in the results.
b p>0.05, one-way ANOVA. All the Hill coefficients are not significantly different from unity.
c p>0.05, one-way ANOVA. The Hill coefficient of LK3-1 is not significantly different from unity while the others are significantly different from unity.
d The Kd value was corrected from the IC50 value using the Cheng and Prusoff (1973) equation. The IC50 values were 34 (17-47) µM in LK3-1, 39 (35-59) µM in LK3-4, 34 (25-42) µM in LK7-6, 46 (36-52) µM in LK3-7, 68 (53-77) µM in LK7-2, 55 (38-64) µM in LK3-3 and 45 (37-52) µM in LK3-8 (p>0.05, one-way ANOVA).
e p<0.05, one-way ANOVA. The Hill coefficient of LK3-1 is not significantly different from unity while the others are significantly different from unity.
f p>0.05, one-way ANOVA. The Kd values were corrected from the IC50 values using the Cheng and Prusoff (1973) equation. The corresponding IC50 values were 7.5 (4.9-11) µM in LK3-1, 17 (8.0-32) µM in LK7-6, 19 (10-30) µM in LK3-7, 24 (15-46) µM in LK7-2, 15 (4.0-45) µM in LK3-3 and 6.6 (2.4-12) µM in LK3-8 (p>0.05, one-way ANOVA).
g p<0.05, one-way ANOVA of the group including the percentage values of LK3-1 cells (96.4±0.4).
h p>0.05, one-way ANOVA. The Kd values were corrected from the IC50 values using the Cheng and Prusoff (1973) equation. The corresponding IC50 values were 200 (110-430) µM in LK3-4, 150 (68-430) µM in LK7-6, 130 (97-160) µM in LK3-7, 180 (130-360) µM in LK7-2, 140 (94-200) µM in LK3-3 and 150 (131-170) µM in LK3-8 (p>0.05, one-way ANOVA).
intact cells were close to one (table 11). The non-specific binding of \(^3\text{H}\)[(-)MQNB defined by 1 \(\mu\)M atropine sulfate was similar among these clones. To ensure that the muscarinic receptors in the newly transfected cells had the expected M\(_1\) muscarinic receptor pharmacology, we characterized the muscarinic antagonist inhibition of \(^3\text{H}\)[(-)MQNB binding to one of the clones, LK3-8, which had the highest receptor density. Pirenzepine had high affinity (\(K_i\) of 8.7 nM with a range of 5.1-15 nM) while AF-DX 116 had low affinity (\(K_i\) of 740 nM with a range of 720-770 nM) for the muscarinic receptors. The \(K_i\) value of atropine sulfate was 0.29 nM with a range of 0.23-0.39 nM.

Carbachol inhibition of \(^3\text{H}\)[(-)MQNB binding to the muscarinic receptors in the intact cells

The muscarinic agonist carbachol inhibited specific \(^3\text{H}\)[(-)MQNB binding to the muscarinic receptors in the intact cells. The IC\(_{50}\) values of carbachol in inhibiting \(^3\text{H}\)[(-)MQNB binding to the muscarinic receptors in the intact cells were in a range of 34 \(\mu\)M - 68 \(\mu\)M (p>0.05, one-way ANOVA) and are summarized in the footnote to table 11. The Hill coefficients of carbachol/\(^3\text{H}\)[(-)MQNB binding are shown in table 11. The Hill coefficient values in the LK3-1 cells (0.95 ± 0.10) are not significantly different from unity. However, a better fit by a 2-site model versus the 1-site model was observed in carbachol/\(^3\text{H}\)[(-)MQNB competition curves in the other 6 clones.
The IC_{50} value from each of the 7 clones was corrected into either the K_I, K_H or K_L value using the corresponding K_d value by the Cheng and Prusoff equation (1973) depending on the best fit and is shown in Table 11.

The K_H values of carbachol for the muscarinic receptors in these clones ranged from 1.9 μM to 7.1 μM and did not differ significantly from each other (p>0.05, one-way ANOVA). However, a significant variation was estimated from the Hill coefficients of carbachol/[^3H](-)MQNB competition curves (p<0.05, one-way ANOVA) and of the percentage of the population of the carbachol high affinity state (p<0.05, one-way ANOVA). As the receptor density increased from 12 fmol/10^6 cells in the LK3-1 cells to 260 fmol/10^6 cells in the LK3-8 cells, the Hill coefficient decreased from 0.95±0.10 to 0.69±0.05 (p<0.05, paired t test) while the population of the high affinity state for carbachol decreased from 96±4.0 percent to 42±5.9 percent (p<0.05, paired t-test).

Carbachol stimulation of the hydrolysis of inositol lipids

The non-transfected B82 cells and the LK4-V cells had basal [^3H]IP_1 accumulation of 12000±600 cpm/10^6 cells and 11000±1300 cpm/10^6 cells, respectively. The basal [^3H]IP_1 accumulation in the 7 clones ranged between 9600 cpm/10^6 cells and 13000 cpm/10^6 cells (Table 12) (p>0.05, one-way ANOVA). The muscarinic agonist carbachol stimulated [^3H]IP_1 accumulation in these 7 clones in the presence of 10 mM LiCl (Figure 26) but not in the
Table 12

Carbachol stimulation of [3H]IP$_1$ accumulation in the series of transfected cells

<table>
<thead>
<tr>
<th></th>
<th>Basal [3H]IP$_1$ Accumulation$^a$ (cpm/10^6 cells)</th>
<th>Carbachol-stimulated [3H]IP$_1$ Accumulation$^c$ (cpm/10^6 cells)</th>
<th>EC$_{50}^b$ (µM)</th>
<th>E$_{max}^c$ (cpm/10^6 cells)</th>
<th>n$_H^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LK3-1</td>
<td>12000±1300</td>
<td></td>
<td>13</td>
<td>10000±2700</td>
<td>1.2±0.30</td>
</tr>
<tr>
<td></td>
<td>(9.3-19)</td>
<td></td>
<td></td>
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<tr>
<td>LK3-4</td>
<td>11000±1000</td>
<td></td>
<td>6.7</td>
<td>7300±2600</td>
<td>1.4±0.23</td>
</tr>
<tr>
<td></td>
<td>(1.6-23)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>LK7-6</td>
<td>13000±610</td>
<td></td>
<td>26</td>
<td>23000±1100</td>
<td>0.95±0.04</td>
</tr>
<tr>
<td></td>
<td>(21-42)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LK3-7</td>
<td>9600±1500</td>
<td></td>
<td>13</td>
<td>44000±9800</td>
<td>1.1±0.20</td>
</tr>
<tr>
<td></td>
<td>(9.4-19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LK7-2</td>
<td>12000±1500</td>
<td></td>
<td>14</td>
<td>55000±5300</td>
<td>1.1±0.08</td>
</tr>
<tr>
<td></td>
<td>(9.5-27)</td>
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</tr>
<tr>
<td>LK3-3</td>
<td>10000±880</td>
<td></td>
<td>4.0</td>
<td>86000±1100</td>
<td>1.1±0.11</td>
</tr>
<tr>
<td></td>
<td>(1.5-11)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>LK3-8</td>
<td>13000±1600</td>
<td></td>
<td>3.5</td>
<td>78000±8300</td>
<td>1.1±0.16</td>
</tr>
<tr>
<td></td>
<td>(3.1-3.9)</td>
<td></td>
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</table>

$^a$ p>0.05, one-way ANOVA.

$^b$ p<0.01, one-way ANOVA.

$^c$ [3H]IP$_1$ accumulation was calculated as the difference between the carbachol-stimulated and the basal [3H]IP$_1$ accumulation. p<0.01, one-way ANOVA. Differences between the clones are presented in the results.
Carbachol stimulation of the [³H]IP₁ accumulation in the series of the transfected B82 cells: LK3-1(●), LK3-4(○), LK7-6(□), LK3-7(△), LK7-2(▲), LK3-3(▼) and LK3-8(▼). The data points represent the means of 3-4 independent experiments. The standard errors of the means which were less than 15 percent of the values indicated were omitted from the figure. The lines are the best fit from the computer-generated nonlinear least-squares regression analyses of a three parameter logistic equation. For these curves, the EC₅₀ values (Hill coefficients) and the E₅₀ values are 19 μM (0.93) and 9600 cpm/10⁶ cells in LK3-1, 6.2 μM (1.3) and 7300 cpm/10⁶ cells in LK3-4, 26 μM (0.91) and 23000 cpm/10⁶ cells in LK7-6, 14 μM (0.94) and 44000 cpm/10⁶ cells in LK3-7, 15 μM (1.2) and 55000 cpm/10⁶ cells in LK7-2, 4.9 μM (1.2) and 87000 cpm/10⁶ cells in LK3-3 and 3.5 μM (1.2) and 78000 cpm/10⁶ cells in LK3-8. The maximal effects, the EC₅₀ values and the Hill coefficients as well as the basal [³H]IP₁ accumulation of the individual experiments are shown in table 12.
non-transfected B82 cells (\[^3\text{H}\]IPI\(_1\) accumulation of 11000±1000 cpm/10\(^6\) cells at 10 mM carbachol) or the LK4-V cells (\[^3\text{H}\]IPI\(_1\) accumulation of 11000±2000 cpm/10\(^6\) cells at 10 mM carbachol). The EC\(_{50}\) values of carbachol ranged from 3.5 \(\mu\)M to 26 \(\mu\)M and are shown in Table 12. The functional dissociation constants (\(K_A\)) were calculated by using the Furchgott and Bursztyn (1967) method with the concentration-response curve in the LK3-8 cells as the control. The \(K_A\) values were 7.4 \(\mu\)M in the LK3-1 cells, 5.4 \(\mu\)M in the LK3-4 cells, 1.9 \(\mu\)M in the LK7-6 cells, 2.8 \(\mu\)M in the LK3-7 cells, 7.0 \(\mu\)M in the LK7-2 cells and 5.4 \(\mu\)M in the LK3-3 cells. With the LK3-8 cells as 100 percent, the calculated fraction of the receptors (\(q\)) was 0.08 in the LK3-1 cells, 0.09 in the LK3-4 cells, 0.35 in the LK7-6 cells, 0.55 in the LK3-7 cells, 0.42 in the LK7-2 cells and 0.88 in the LK3-3 cells. The averaged functional dissociation constant (\(K_A\)) of carbachol was 4.5 (1.9-7.4) \(\mu\)M. The calculated fraction of the receptors (\(q\)) in each of the clones agrees well with that from the \[^3\text{H}\](-)MQNB binding experiments. The maximal amount of \[^3\text{H}\]IPI\(_1\) accumulation (\(E_{\text{max}}\)) calculated from the corresponding dose-response curves was dependent on the muscarinic receptor density of the clone. The rank order based on the \(E_{\text{max}}\) values of these clones was LK3-B=LK3-3>LK7-2=LK7-6>LK3-4=LK3-1 (paired t-test). The correlation coefficient of the linear regression analyses of the average \(E_{\text{max}}\) values versus the average \(B_{\text{max}}\) values of \[^3\text{H}\](-)MQNB binding for each of the 7 clones was 0.98 (p<0.001) (figure 27). Furthermore, a significant correlation
Figure 27. Correlation of the carbachol-stimulated maximal 
$[^3H]IP_3$ accumulation ($E_{\text{max}}$) versus the maximal 
$[^3H](-)MQNB$ binding capacities ($B_{\text{max}}$) in different 
clones of the transfected B82 cells. The linear 
regression equation ($Y = mX + B$) is $E_{\text{max}} = 310B_{\text{max}} + 7100$ with the correlation coefficient of 0.98 ($p<0.001$).
Figure 28. Correlation of the carbachol-stimulated maximal \([^3H]IP_1\) accumulation \((E_{\text{max}})\) versus the high (panel A, \(B_{\text{max}} H\)) and low (panel B, \(B_{\text{max}} L\)) affinity state of the \(M_1\) muscarinic receptors for carbachol in the transfected B82 cells. The linear regression equations are \(E_{\text{max}} = 770 \times B_{\text{max}} H + 2200\) in A and \(E_{\text{max}} = 500 \times B_{\text{max}} L + 11000\) in B with the correlation coefficients of 0.98 \((p<0.001)\) and 0.96 \((p<0.001)\), respectively.
was observed from the linear regression analyses of the PI responses versus the densities of the high affinity states of muscarinic receptor for carbachol (figure 28A). A linear regression was also computed between the PI responses and the densities of the low affinity states of muscarinic receptors for carbachol (figure 28B). No significant correlation existed between the receptor density and the basal $[^3H]IP_1$ accumulation in the transfected B82 cells ($r=-0.098, p>0.4$). There was a weak negative linear correlation between the $B_{max}$ values and the $EC_{50}$ values of carbachol in stimulating $[^3H]IP_1$ accumulation ($r=-0.63, p<0.05$).

Comparison of carbachol stimulation of $[^3H]IP_1$ accumulation versus its occupancy of muscarinic receptors in the transfected cells

Figure 29 presents the concentration-response curves and concentration-occupancy curves of carbachol in the cells of clones LK3-1, LK3-7 and LK3-8. In the LK3-1 cells with a muscarinic receptor density of 12 fmol/10$^6$ cells, the functional concentration-response curve and the concentration-occupancy curve were superimposable. However, as the receptor density increased to 96 fmol/10$^6$ cells in the LK3-7 cells, the two curves separated from each other. The carbachol concentration-response curve was shifted to the left in the clones which expressed higher receptor densities. As shown in figure 29, in order for carbachol to initiate a half maximal effect, it needs to occupy about 50 percent
Figure 29. The concentration-response curves for carbachol compared with concentration-occupancy curves in 3 clones. The data of the concentration-response curves are reanalyzed from figure 26 using the estimated E_{\text{max}} values as 100 percent. The data of the concentration-occupancy curves are calculated from the carbachol/[^{3}H](-)MQNB competition experiments (see Methods). Each point represents the mean of 3 independent experiments with the SEM less than 15 percent. The EC_{50} values and Hill coefficients are presented in table 11. The IC_{50} values are shown in the legend to table 12.
of the muscarinic receptors in the LK3-1 cells, about 25 percent of the receptors in the LK3-7 cells, and 12 percent of the receptors in the LK3-8 cells (25 percent of the receptors in the LK3-4 cells, 42 percent of the receptors in the LK7-6 cells, 22 percent of the receptors in the LK7-2 cells and 14 percent of the receptors in the LK3-3 cells, data not shown). Significant differences were estimated in the pairs of the $EC_{50}$ values and the $IC_{50}$ values in the cells of the clones LK3-4, LK3-7, LK7-2, LK3-3 and LK3-8 but not in the cells of the clones LK3-1 and LK7-6 (student's $t$-test).

**Discussion**

The major findings of this study are: 1) A significant correlation was observed between the $M_1$ muscarinic receptor density and the maximum response induced by the muscarinic agonist carbachol in the 7 transfected clones of the murine fibroblast B82 cells with different receptor densities; 2) The $M_1$ muscarinic receptors in LK3-1 cells which expressed a low receptor density were in the high affinity state for carbachol whereas those in the clones which expressed higher receptor densities have both high and low affinity states for carbachol; 3) The high affinity state of the $M_1$ muscarinic receptors for carbachol may be the functionally coupled state; 4) Spare receptors were not present in the clones with lower receptor densities but were manifested in clones with higher receptor densities.

The classical receptor occupancy theory proposed by Clark
states that the magnitude of the pharmacological effect of a
drug is related to the number of receptors occupied by the drug
using the dose-response curve. This theory has been examined using
the drug as the only variable of the drug-receptor interaction. We
used an in vitro gene expression system to study the M₁ receptor
pharmacology by manipulating the expression of the receptor.
Transfection of murine fibroblast B82 cells with the m₁ gene
yielded several clones which expressed different densities of
muscarinic receptors (Lai et al., 1988). The muscarinic receptors
expressed in these transfected B82 cells were of the M₁ type since
they exhibited high affinity for pirenzepine and low affinity for
AF-DX 116. No difference in the basal [³H]IP₁ accumulation in
the B82 cells, LK4-V cells and several clones of the transfected
cells suggests that the transfection of the original B82 cells with
the m₁ muscarinic receptor gene did not interfere with the PI
turnover in the B82 cells. No specific [³H](-)MQNB binding was
detected in the B82 and LK4-V cells. Consequently, we observed that
carbachol had no effects on the basal [³H]IP₁ accumulation in
these cells. The significant correlation of the Bₘ₉ values of
[³H](-)MQNB binding and the Eₘ₉ values of carbachol-stimulated
[³H]IP₁ accumulation indicates that the carbachol-initiated
response is not only dependent on the carbachol concentration as
shown from the concentration-response curves (figure 26) but also
dependent upon the M₁ receptor density in these clones (figure
27). This agrees with a recent report that increases in the PI
response in CHO cells were \( M_1 \) receptor density-dependent (Ashkenazi et al., 1989). A proportionality between the cloned muscarinic receptor density and the mediated response was also observed for the \( M_2 \) muscarinic receptor in Chinese hamster ovary cells (Ashkenazi et al., 1987) and for the \( m_3 \) gene-encoded receptor in human kidney cells (Peralta et al., 1988).

Muscarinic receptors are of the G protein-associated super-gene family (for review, see Barnard, 1988). Muscarinic receptors in the lipid bilayer membrane are associated with the G proteins and stay in the high affinity state for the agonist when the G proteins are bound with GDP (figure 31). The binding of the G proteins with GTP to release GDP causes dissociation of the receptors (Gilman, 1987). Haga et al. (1986) reported that the proportion of the agonist high affinity state increased with increasing concentrations of the G proteins in a reconstitution system. In the present study, all the transfected clones originated from the same parental B82 line; therefore, the concentration and the type of the G proteins should be similar in these clones. If the concentration of G proteins were a limiting factor in their association with a large amount of receptors in the clones expressing high receptor densities, we would observe a larger ratio of the high/low carbachol affinity state in the clones with low receptor densities and a smaller ratio in those with high receptor densities. We found that in the LK3-1 cells which have the lowest receptor density, all of the \( M_1 \) muscarinic receptors were in the
high affinity state. For the clones which contain progressively higher receptor densities, a decreasing percentage of the high affinity state for carbachol was observed (table 11). Our previous study has already demonstrated that the $M_1$ muscarinic receptors in the cTB10 cells which had a $B_{\text{max}}$ value of $17 \text{ fmol/10}^6 \text{ cells}$ exhibited a single population of carbachol affinity state with the $K_i$ value of $13 \mu\text{M}$ (Lai et al., 1988; Mei et al., 1989). A nonhydrolyzable GTP analogue GppNHp shifted the high affinity state of the muscarinic receptors for carbachol in the cTB10 cell membranes to a low affinity state (Lai et al., 1988). These results indicate that the amount of G proteins available for the association with receptors may influence the agonist affinity state of the receptors in this system. If the G protein were the only determinant of the high affinity state for the agonist, we would expect that the amount of receptors which had high affinity for carbachol would be saturable when the amount of G proteins becomes limiting. However, a population of reconstituted muscarinic receptors showed high affinity for the agonist in the absence of G proteins (Haga et al., 1986). In agreement with Haga et al. (1986), the amount of the $M_1$ muscarinic receptors in the high affinity state for carbachol was not saturable in our system (figure 30).

Investigators have shown that high and low affinity states of muscarinic receptors for the agonist can be functionally distinguished. A good correlation between the $EC_{50}$ values of tertiary amines for stimulation of contractions of guinea pig ileum
Figure 30. Relationships of the carbachol high affinity receptor density and the functional receptor density to the $B_{\text{max}}$ values of $[^3H](-)\text{MQNB}$ binding in the transfected B82 cells. The carbachol high affinity receptor density was calculated by using the $B_{\text{max}}$ values x percentage of the high affinity population (table II). The functional receptor density was calculated by using the $B_{\text{max}}$ values x corresponding occupancy percentage at EC$_{50}$. 

Figure 31. Interaction between muscarinic receptor and G protein.

- **CTX**: cholera toxin
- **G**: G protein
- **H**: hormone or neurotransmitter
- **PTX**: pertussis toxin
- **R**: low affinity state of receptor
- **R^***: high affinity state of receptor
and their high-affinity dissociation constant determined from agonist/[\[^{3}H\](-)QNB binding experiments on the rat brainstem was observed (Ehlert and Jenden, 1984). Comparison of the concentration-response curves of carbachol (EC\textsubscript{50} of 3.4 \(\mu\)M) with the binding studies indicated that Ca\textsuperscript{2+} mobilization was triggered via the high-affinity state (17 \(\mu\)M for carbachol) of muscarinic receptors in chick embryo cells (Oettling et al., 1985). The high affinity state of the M\textsubscript{1} muscarinic receptors for carbachol in the transfected B82 cells may be the functionally coupled state for several reasons. First, EC\textsubscript{50} values are similar to the K\textsubscript{i} value of carbachol in LK3-1 cells and the K\textsubscript{H} values of the rest of the clones (\(p>0.05\), one-way ANOVA) but significantly different from the K\textsubscript{L} values (\(p<0.001\), one-way ANOVA). Secondly, the K\textsubscript{A} values calculated by using the Furchgott method are similar to the K\textsubscript{H} values. Thirdly, the E\textsubscript{max} of carbachol-stimulated [\[^{3}H\]IP\textsubscript{1}] accumulation correlated well with the density of the high affinity state for carbachol (figure 28A). The intercept on the Y-axis is not significantly different from zero (\(p>0.05\)). Although a significant correlation between the E\textsubscript{max} and the density of low affinity state for carbachol was also observed (figure 28B), we think that the latter regression analysis is misleading. The data points are scattered away from the regression line with a larger sum of the squares of errors and the regression line does not cross the Y-axis at the origin. The intercept on the Y-axis is significantly different from zero (\(p<0.001\)) which is not expected theoretically.
since at a zero receptor density no response should be observed.

In other systems the low affinity state (carbachol $K_L$ of 0.5 μM) of muscarinic receptors for the agonist was found to be coupled to the functional responses (Halvorsen and Nathanson, 1981). The low affinity state (carbachol $K_L$ of 50 μM) of muscarinic receptors in murine fibroblast NIE-115 cells was involved in cGMP formation while the high affinity state (carbachol $K_H$ of 0.1 μM) was coupled to the inhibition of prostaglandin E₁-stimulated cAMP formation (McKinney and Richelson, 1984).

Recently McKinney et al. (1989) reported that the cortical M₁ receptor-mediated PI metabolism is elicited by carbachol through a low affinity agonist-receptor complex ($K_d$ of 90 μM) whereas the cortical and striatal M₂ receptor-mediated inhibition of cAMP formation is mediated by a high affinity agonist-receptor complex ($K_d$ of 2.9 and 8.5 μM).

If the muscarinic receptor functions through G proteins and the transducing machinery is completed by dissociations of the G proteins from th receptors (Gilman, 1987), the agonist high affinity state may be the functional one since the receptors associated G proteins have high affinity for the agonist. However, the possibility should not be eliminated that the receptors may not stay associated with G proteins in the absence of the agonist in some systems. In the latter case, the receptors have low affinity for the agonist. This may be why the agonist low affinity state of muscarinic receptor was found to be the functionally coupled state.
Such discrepancies may be due to the different systems studied which may have different types of and/or different concentrations of the G proteins (Kenakin and Morgan, 1989).

The existence of spare receptors has been demonstrated for histamine in the guinea pig ileum (Furchgott, 1955; Nickerson, 1956), for epinephrine in rabbit aorta, and for carbachol in rabbit stomach, aorta and guinea pig atrium (Furchgott, 1955) by observing that an irreversible blocking agent would shift the concentration-response curve of agonist to the right in a parallel fashion before it decreased the maximum effect in some tissues. This approach is commonly used to study muscarinic spare receptors (Grandordy et al., 1986; Ringer et al., 1987; Fisher and Snider, 1987; Keen and Nahorski, 1988) with the muscarinic receptor densities varying from 275-1500 fmol/mg protein. Mahan and Insel (1986) used another approach to explore the relationship between the receptor number and response in intact S49 lymphoma cells. They synchronized the population of S49 cells. The receptor number increased in proportion to cell size and transition through the cell cycle from G1 to mitosis. Spare ß-adrenergic receptors were observed in the synchronized S49 cells.

This study employed a eukaryotic in vitro expression system to generate stable cell lines which expressed different densities of M1 muscarinic receptors to study the spare receptor hypothesis. When the occupancy curves of carbachol from the carbachol/[3H](-)MQNB competition studies were compared with the
concentration-response curves of the same agonist (figure 29), we found evidence for spare receptors. Linear regression of the ratios of the IC$_{50}$ values over the EC$_{50}$ values versus the receptor density of each of the different clones gave a significant correlation for the two parameters (p<0.001). The one-to-one correspondence between the receptor occupancy and the response occurs when the receptor density is 19 fmol/10$^6$ cells (95 fmol/mg protein). In addition, a comparison of the PI response with the fractional receptor occupancy (q= [A]/(K$_A$ + [A]), Furchgott and Bursztyn, 1967) also revealed that spare receptors exist in the clones with high receptor densities but not in those with low receptor densities (data not shown). The similarity between the carbachol EC$_{50}$ values, the K$_A$ dissociation constants and the K$_H$ values, and the excellent correlation between the E$_{max}$ and the high affinity state (figure 28A) imply that the functional receptors may be a portion of the receptors which have high affinity for the agonist.

I noticed that the functional receptor population needed for the carbachol half maximum effect increased from 5.3 fmol/10$^6$ cells in the LK3-1 cells to 30 fmol/10$^6$ cells in the LK7-2 cells with no further increases in the LK3-3 cells (33 fmol/10$^6$ cells) and in the LK3-8 cells (32 fmol/10$^6$ cells) (figure 30). This result suggests that there may be an endogenous factor which limits the formation of the functional receptors. In contrast, we found a linear increase in the receptor-mediated PI response in these cells,
suggesting that this factor does not limit the functional response of the receptors although it limits the functional receptor complex formation. In other words, more than one receptor molecules can react with a single molecule of this endogenous factor. This endogenous factor is very likely to be the G proteins (or the subunits).

The relationship of the total receptor concentration versus the $E_{\text{max}}$ was also analyzed using a computerized nonlinear least-square regression program as an alternative approach to the study of spare receptors. Interestingly, a saturating tendency for the relationship in figure 27 was observed although we obtain a significant linear correlation for the plotted data. Fitting the data to a 3 parameter logistic equation, we found the maximal $[^3\text{H}]\text{IP}_1$ accumulation at the saturating receptor density estimated to be 190000 cpm/$10^6$ cells. This curve had a half maximal value of 360 fmol/$10^6$ cells and a Hill coefficient of 0.91. The probit linearized plot of the percentage of the carbachol-stimulated $[^3\text{H}]\text{IP}_1$ accumulation versus the logarithmic receptor concentration gave a receptor density of 13000 fmol/$10^6$ cells (65000 fmol/mg protein) at the 99 percent of the saturated $[^3\text{H}]\text{IP}_1$ accumulation. In other words, the theoretical $[^3\text{H}]\text{IP}_1$ accumulation response could be increased with increasing receptor density in the transfected B82 cells until the receptor density reached 13000 fmol/$10^6$ cells. Then, further increases in the carbachol stimulated $[^3\text{H}]\text{IP}_1$ accumulation would not be
observed. Therefore, such an analysis predicted a receptor density of 13000 fmol/10^6 cells as the starting point for spare receptors. In contrast, we observed that spare receptors exist in the cells with receptor densities higher than 19 fmol/10^6 cells (nearly 680 fold lower). There has been no report of such a high density of muscarinic receptor (as 65000 fmol/mg protein) in any tissue examined (Venter et al., 1988). Accordingly, the relationship between the PI response and the total receptor densities in the transfected cells must be complex (figure 27) in view of the existence of high and low affinity states for agonists and the presence of spare receptors. As shown in figure 29, such a simplified correlation between the responses and the total receptor densities is not a sufficient method for demonstrating spare receptors.

In summary, the results obtained in this study show a significant correlation between the densities of the high affinity state of M₁ muscarinic receptors for carbachol and the PI hydrolysis response in 7 clones of the transfected B82 cells. The high affinity state of the M₁ muscarinic receptors for carbachol in this system may be functionally coupled to the PI response. Spare M₁ muscarinic receptors were observed in the clones with high receptor densities but not in those with low receptor densities. Transfection of cloned receptor genes to obtain cells with different densities of receptors should prove to be a good model for studying mechanisms of receptor-signal transduction.
including the interactions of the receptor with G proteins (Mei et al., submitted c).
CHAPTER 8

CONCLUSIONS

We have characterized the possible second messenger systems coupled to the muscarinic receptors in the human neuroblastoma SH-SY5Y cells (chapter 3). Stimulation of muscarinic receptors in the SH-SY5Y cells mediated hydrolysis of inositol lipids. The muscarinic receptors which were coupled to the hydrolysis of inositol lipids in the SH-SY5Y cells had high affinity for pirenzepine and low affinity for AF-DX 116, which supports the hypothesis that M₁ muscarinic receptors are coupled to the hydrolysis of inositol lipids. Pertussis toxin but not cholera toxin inhibited carbachol-induced [³H]IP₃ accumulation in the SH-SY5Y cells (chapter 4). A pertussis toxin-sensitive G protein may be involved in the muscarinic receptor-PI specific phosphodiesterase coupling in the SH-SY5Y cells. Stimulation of muscarinic receptors in this cell line had no effect on the cAMP formation.

The gene which encodes M₁ muscarinic receptor was transfected into a murine fibroblast cell line, B82, in order to generate a system which has only one type of muscarinic receptor. While the parental non-transfected B82 cells had no muscarinic receptors, the transfected B82 cells exhibited specific [³H](--)-QNB
The muscarinic receptors in the transfected B82 cells had high affinity for pirenzepine and low affinity for AF-DX 116. Stimulation of the muscarinic receptors produced hydrolysis of inositol lipids but did not change the basal or PGE₁-stimulated cAMP formation in the transfected B82 cells. The following results suggest that the G protein may play an important role in the muscarinic receptor-effector coupling. First, GppNHp shifted the M₁ muscarinic receptor in the membranes of the transfected B82 cells from the high affinity state to the low affinity state for carbachol (chapter 5). Secondly, the carbachol-induced [³H]IP₁ accumulation in the transfected B82 cells was sensitive to pertussis toxin (chapter 6).

In chapter 7, we have studied the relationship of the density of the transfected M₁ muscarinic receptor versus the PI response. The results suggest that the high affinity state of the muscarinic receptors for carbachol may be the functionally coupled state. By varying the receptor density in the transfected cells, we were able to show that there was an endogenous factor in the transfected murine fibroblast B82 cells that influences the formation of functional receptors. This factor may be G proteins or one of the G proteins. Correlation of the total M₁ muscarinic receptor densities versus the PI response is not a sufficient method for demonstrating spare receptors in the transfected B82 cells. Transfection of a cloned receptor gene to obtain a cell line which express a single type of muscarinic receptor will shed light on the
study of the relationship of the muscarinic receptor types versus the biofunctional responses. The transfected cells with different densities of receptors should prove to be a good model for studying mechanisms of receptor-second messenger coupling, especially, the interactions of the receptor with the G proteins and/or the effectors.
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