Recent Advances in Muscarinic Receptor Pharmacology & Therapeutics

April 4-5, 2008
San Diego Marriott Hotel & Marina
San Diego, CA

Organizers:
Richard Eglen
Nigel Birdsall
Christian Felder
Allison Fryer
Neil Nathanson

An ASPET Colloquium

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Day 1
Friday, April 4

7:45 am Registration/Continental breakfast

8:45 am Welcome remarks
Richard Eglen – *PerkinElmer Life & Analytical Sciences*

Session 1: Physical/Biophysical Studies: Chair: Nigel Birdsall

9:00 am Muscarinic receptor structure and function: Mutatis mutandis
Ed Hulme – *MRC National Institute for Medical Research, London*

9:45 am Muscarinic receptor dimers and clustering - single molecule studies on living cells
Nigel Birdsall – *MRC National Institute for Medical Research, London*

10:30 am Break

Session 2: Novel Aspects of Muscarinic Receptor Pharmacology: Chair: Chris Felder

10:45 am At long last - emerging selective muscarinic receptor pharmacology
Christian Felder – *Eli Lilly & Company, Indianapolis*

11:30 am Potential for allosteric activators of M_1 and M_4 muscarinic receptors in the treatment of schizophrenia
Carrie Jones – *Vanderbilt University*

12:15 pm Optimizing inhaled muscarinic receptor antagonist dissociation rates to enhance duration and subtype selectivity
Steven Charlton – *Novartis, Horsham, UK*

1:00 pm Lunch in Marriott Hall 3

2:00 pm Poster Session 1 in Marriott Hall 3

Session 3: Muscarinic Receptor Signaling and Phenotypes: Chair: Neil Nathanson

3:15 pm Localization and trafficking of muscarinic acetylcholine receptors
Neil Nathanson – *University of Washington*

4:00 pm Emerging data from novel muscarinic receptor mutant mouse models
Jurgen Wess – *NIDDK/NIH*

4.45 pm Break

5:00 pm Subtype-specific functions of mAChRs revealed by the use of knockout mice
Minoru Matsui – *Chiba Institute of Science, Japan*

5:45 pm Muscarinic modulation of striatal physiology in health and disease
James Surmeier – *Northwestern University*

6:30 pm Poster Session 2 in Marriott Hall 3

Recent Advances in Muscarinic Receptor Pharmacology and Therapeutics

Day 2, Saturday, April 5

7:00 am Continental breakfast

8:00 am Welcome remarks
Richard Eglen – *PerkinElmer Life & Analytical Sciences*

Session 4: Therapeutic Uses of Muscarinic Drugs: Chair: Allison Fryer

8:05 am Antimuscarinics and afferent signaling in the bladder
Karl-Erik Andersson – *Wake Forest University School of Medicine*

8:50 am Muscarinic receptor agonists: A novel treatment for schizophrenia
Anantha Shekhar – *Indiana University*

9:35 am Muscarinic antagonists and lung dysfunction
Allison Fryer – *Oregon Health & Science University*

10:20 am Break

Plenary Lecture

10:35 am Structure and dynamics of the human β_2 adrenergic receptor
Brian Kobilka – *Stanford University*

Selected Oral Presentations from Posters

11:20 am Use of acetylcholine nitrogen mustard to study allosteric interactions at the M1 muscarinic receptor
Katherine Figueroa – *University of California, Irvine*

11:35 am Differential effects on allosteric agonism and stimulus-trafficking by mutations in the orthosteric and allosteric pockets of M_2 muscarinic acetylcholine receptors
Karen J. Gregory – *Monash University*

11:50 am Kinetic Analysis Of The M3-Ach-Receptor Activation In Living Cells
Carsten Hoffmann – *Universität Würzburg*

12:05 pm Pharmacological characterisation of the M_3 muscarinic acetylcholine receptor in *Saccharomyces cerevisiae*
Greg Stewart – *Monash University*

12:20 pm Muscarinic Receptors on Eosinophils Inhibit PAF-induced Eosinophil Activation
Norah Verbout – *Oregon Health & Science University*

12:35 pm Lunch in Marriott Hall 3

1:30 pm Poster Session 3 in Marriott Hall 3

Session 5: Emerging Areas and Novel Concepts: Chair: Richard Eglen

2:30 pm The muscarinic receptors in keratinocytes: Signaling pathways and biologic effects
Sergei Grando – *University of California, Irvine*

3:15 pm Muscarinic receptors and apoptosis
Andrew Tobin – *University of Leicester, UK*

4:00 pm Break

Otto Loewi New Investigator Award & Lecture

4:15 pm Brain-specific deletion of M_3 muscarinic acetylcholine receptors causes pituitary hypoplasia and dwarfism in mice
Dinesh Gautam, *NIDDK, NIH*

5:00 pm Structure-function meets ligand-directed modeling: Towards novel muscarinic receptor chemotypes
Arthur Christopoulos – *Monash University, Australia*

5:30 pm **Presentation of the Ruth Levine Award Lecture**

5:40 pm Closing Remarks
Nigel Birdsall – *MRC National Institute for Medical Research, London*

Recent Advances in Muscarinic Receptor Pharmacology and Therapeutics
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Recent Advances in Muscarinic Receptor Pharmacology and Therapeutics

Speaker Abstracts

Muscarinic Receptor Structure and Function: Mutatis Mutandis[1]

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Systematic mutagenesis, in our hands alanine-scanning, has advanced our understanding of the structure-function relationships of M_1 muscarinic acetylcholine receptors(1). Like other GPCRs, M_1 mAChRs exhibit multiple conformational states with different affinities for and kinetics of activation of their cognate G proteins. Ligand binding alters the energetics of the transitions between these states, thereby promoting or inhibiting signalling. Alanine-substitution mutagenesis changes the relative contributions of amino acid side chains to the stability of the ground and activated states of the receptor and its complexes. The nature and magnitude of these perturbations determine the functional phenotypes of the mutant receptors. The development of a phenotypic classification scheme has allowed us to assign particular amino acid side chains to functional classes, and group them accordingly. Using homology models of the M_1 mAChR, we have developed a coherent view of how such clusters of residues may function in ligand anchoring, transduction of binding energy, global structural stabilization, and selective stabilization of the ground state or the activated state of the receptor. We have identified differences in ligand binding modes(2), and suggested inter and intra-molecular contacts that are weakened or broken, or formed or intensified during acetylcholine-induced activation(3). Many of the key interactions are reflected in the recently-determined high resolution structure of the β_2 adrenergic receptor(4). Recently, we have extended these methods to investigate how novel selective agonists bind to and activate the M_1 mAChR[2].

[1]Supported by the Medical Research Council, U.K.
[2] Supported by a GlaxoSmithKline Collaborative Research Agreement.

Reference List

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4. Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., Choi, H. J., Kuhn, P., Weis, W. I., Kobilka, B. K., and Stevens, R. C. (2007) *Science* **318,** 1258-1265

Muscarinic receptor dimers and clustering – single molecule studieson living cells

Nigel Birdsall
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There is much data (sometimes conflicting) regarding the existence, nature and composition of family A GPCR dimers, including muscarinic receptors, and whether they are constitutive dimers or higher oligomers. Using total internal reflectance fluorescence microscopy, single molecules of muscarinic acetylcholine receptors can be observed on the surface of living cells. Using automatic tracking and analytical algorithms, the behaviour of thousands of individual receptors on the surface of a single cell can be investigated with 20 msec time and 100 nm spatial resolution. It is possible to monitor any clustering of the receptors and their dimerisation status and to measure how they move on the cell surface.

References:

1. Zeng FY and Wess J. (1999) *J Biol Chem.* **274:** 19487-97. Identification and molecular characterization of M_3 muscarinic receptor dimers.
2. Chabre M and le Maire M (2005) *Biochemistry* **44:** 9395-9403. Monomeric G-protein-coupled receptor as a functional unit.
3. Goin JC and Nathanson NM (2006) *J Biol Chem.* **281**: 5416-25. Quantitative analysis of muscarinic acetylcholine receptor homo- and heterodimerization in live cells: regulation of receptor down-regulation by heterodimerization.
4. Whorton MR et al. (2007) *Proc Natl Acad Sci U S A.* **104**: 7682-7. A monomeric G protein-coupled receptor isolated in a high-density lipoprotein particle efficiently activates its G protein.
5. Ma AW et al. (2007) *Biochemistry.* **46**: 7907-27. Recovery of oligomers and cooperativity when monomers of the M_2 muscarinic cholinergic receptor are reconstituted into phospholipid vesicles.

At Long Last - Emerging Selective Muscarinic Receptor Pharmacology

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Neuroscience Division, Eli Lilly & Co., Indianapolis, IN 46236

Muscarinic receptors are widely distributed in the CNS and periphery and offer a rich family of targets for therapeutic development. However, the five muscarinic receptor subtypes share high sequence homology, particularly at their orthosteric ligand binding domains hampering discovery of selective ligands. Historical agonists and antagonists have shown promise in a variety of therapeutic indications, but their modest selectivity has resulted in significant unwanted side effects. Assigning physiologic functions to each receptor subtype has been challenging due to the lack of highly selective pharmacological tools. Significant progress has been made in understanding the role of each receptor subtype through the use of mice bred with constitutive or inducible genetic deletions of one or more of the muscarinic receptors. While these knockout studies have been very helpful in filling this knowledge gap, translation of muscarinic biology to human therapeutic applications requires the creation of selective and safe compounds.

Relatively recently, functionally selective orthosteric agonists have been created for the M1 receptor. These agents have been targeted primarily to CNS indications including cognitive deficits associated with neurological and neuropsychiatric diseases. None have yet provided clinical validation. An alternative approach targeting allosteric binding domains has generated receptor subtype selective modulators. Allosteric modulators may provide more physiologically relevant regulation of muscarinic receptor driven processes, thus avoiding side effects. Highly selective M1 and M4 positive allosteric modulators have been identified that have properties of positive cooperativity with acetylcholine binding as well as allosteric agonist activities. These agents are still currently being investigated in preclinical in vitro and in vivo studies. To date no negative allosteric modulators have been reported for muscarinic receptors. Yet to be determined are the relative levels of cooperativity or allosteric agonism or antagonism required to provide optimal in vivo activity. A better fundamental understanding of muscarinic receptor biology, coupled with novel pharmacological approaches should reinvigorate interest in this family of receptors for therapeutic development.

Relevant reviews:

1. Wess J. Eglen RM. Gautam D. Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. *Nature Reviews. Drug Discovery. 6(9):721-33, 2007 Sep.*

2. Langmead CJ. Christopoulos A. Allosteric agonists of 7TM receptors: expanding the pharmacological toolbox. *Trends in Pharmacological Sciences. 27(9):475-81, 2006 Sep.*

Potential for allosteric activators of M1 and M4 muscarinic receptors in the treatment of schizophrenia

C.K. Jones, A.E. Brady, J.K. Shirey, Z.J.M. Xiang, J. Marlow, A.S. Kane, T.M. Bridges, C.M. Niswender, A.L. Rodriguez, R. Williams, C.W. Lindsley, C.D. Weaver, P.J. Conn
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Schizophrenia is a severe, psychiatric disorder characterized by multiple symptom clusters, including positive and negative symptoms as well as cognitive impairments. While currently available antipsychotics provide efficacy for many of the positive symptoms, these therapies are largely ineffective in the treatment of the negative symptoms and cognitive impairments. Consequently, there is a tremendous need to develop novel therapeutic approaches with broader efficacy and fewer adverse effects than existing therapies. Previous clinical studies with muscarinic acetylcholine receptor (mAChR) agonists, such as M1/M4-preferring mAChR agonist xanomeline, indicate that selective activation of muscarinic receptors may reduce psychotic symptoms and behavioral disturbances in individuals suffering from various psychiatric and neurodegenerative disorders, including schizophrenia and Alzheimer's disease (AD). Unfortunately, the clinical effects of xanomeline, similar to acetylcholinesterase inhibitors, are also associated with adverse side-effects due to nonspecific activation of peripheral M2 and M3 mAChRs, including gastrointestinal distress, bradycardia, and salivation. Previous attempts to develop highly selective agonists of a single mAChR subtype have failed, likely due to high sequence conservation within the ACh (orthosteric) binding site of the five mAChR subtypes (M1-M5). An alternate approach to orthosteric muscarinic agonists is targeting allosteric sites to activate the receptor by actions at a site removed from the highly conserved ACh binding site. In recent years, we and others have successfully developed allosteric modulators of a number of GPCRs that provide unprecedented selectivity for the intended receptor and have behavioral effects in vivo that are very similar to those of orthosteric agonists. In this presentation, I will present recent findings for the *in vitro* and *in vivo* pharmacologic characterization of the selective M1 allosteric agonist TBPB and a novel series of M4 positive allosteric modulators. With the development of these novel subtype selective tools, we are beginning to understand the relative contributions of selective M1 or M4 activation to the antipsychotic effects observed with xanomeline, suggesting that subtype selective muscarinic agonists constitute an important novel treatment approach for schizophrenia.

Supported by grants from NIMH.

Key Words:
Schizophrenia, M1 allosteric agonist, M4 positive allosteric modulators

Optimizing inhaled muscarinic receptor antagonist dissociation rates to enhance duration and subtype selectivity

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Although optimization of pharmacokinetic parameters remains the most common approach to improve duration of drug action, there is growing evidence that the slow dissociation of a drug from its receptor can significantly enhance the extent of efficacy observed. Perhaps the best example of this phenomenon is the once-daily inhaled muscarinic receptor antagonist tiotropium, that was discovered retrospectively to derive a large part of its duration from a slow off-rate from the M_3 muscarinic receptor.

This talk will describe an approach to discover novel muscarinic antagonists with slow dissociation rates and demonstrate how in this class of compounds, off-rate from the receptor correlates well with in vivo duration of action. It will also touch on the concept of "kinetic selectivity" and discuss why we should perhaps begin to consider subtype selectivity in terms of "area under the curve" in addition to the degree of binding at C_{maz}.

Localization and Trafficking of Muscarinic Acetylcholine Receptors

Neil M. Nathanson
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The trafficking of neurotransmitter receptors to and from the cell surface can play an important role in the regulation of physiological responsiveness. Our laboratory has tested the hypothesis that muscarinic receptors possess sorting signals that direct specific receptor subtypes to specific regions of the cell surface. We have found that the M_2 and M_3 receptors are sorted to different domains on the surface of polarized epithelial cells and have identified regions of the receptors which are responsible for their respective localizations. Previous work has also suggested that the agonist-induced internalization of the M_2 receptor uses a distinct pathway from that utilized by other muscarinic subtypes. We have found that different subsets of small G-proteins regulate agonist-induced internalization of the M_2 and M_4 receptors.

Emerging Data From Novel Muscarinic Receptor Mutant Mouse Models

Jean-Marc Guettier, Dinesh Gautam, Jongrye Jeon, Iñigo Ruiz de Azúa, Marco Scarselli, Yinghong Cui, Jian Hua Li, and **Jürgen Wess**
Molecular Signaling Section, Lab. of Bioorganic Chemistry, National Institutes of Health, NIDDK, Bethesda, Maryland, USA

To better understand the physiological and pathophysiological roles of the individual muscarinic acetylcholine receptor (mAChR) subtypes, we used gene targeting technology to generate M_1-M_5 mAChR-deficient mice. During the past few years, we, with the help of many collaborators, have subjected these mutant animals to a series of physiological, pharmacological, behavioral, biochemical, and neurochemical tests. These studies have led to a wealth of novel information about the physiological functions of the M_1-M_5 mAChRs (reviewed in Wess *et al.*, *Nat. Rev. Drug Develop.* 6, 721-33, 2007). More recently, we started to employ Cre/loxP technology to generate mutant mouse lines that lack specific mAChR subtypes only in certain tissues or cell types. Phenotypic analysis of this new generation of mAChR mutant mice has led to novel information about the roles of the M_3 receptor subtype in beta-cell function and somatic growth. These studies should provide a rational basis for the development of novel muscarinic drugs.

Recently, based on a report by Armbruster *et al.* (*PNAS*, 104, 5163-8, 2007), we designed a series of mutant M_3 mAChRs which are unable to bind ACh but can couple to different classes of G proteins upon binding of the pharmacologically inert compound, clozapine-N-oxide (CNO). We started to generate transgenic mice that express these modified M_3 receptors in specific, metabolically relevant tissues and cell types. In these animals, administration of CNO allows temporal control of the activation of distinct G protein signaling pathways in a tissue-specific fashion. Studies with transgenic mice selectively expressing the various mutant M_3 receptors in pancreatic beta-cells have shed new light on the *in vivo* roles of distinct G protein signaling pathways in regulating beta-cell function. Since pancreatic beta-cells express a large number of GPCRs, these findings should be of considerable therapeutic relevance.

Subtype-specific functions of mAChRs revealed by the use of knockout mice

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In order to clarify functional roles of muscarinic receptor subtypes, we have established knockout mice lacking one or more of the subtype (s). This project started at the University of Tokyo about 10 years ago. The knockout mice were backcrossed to C57BL/6J or some other strains to establish congenic strains. The use of congenic strains should be beneficial in achieving well-controlled experiments. The overlapping expression pattern of multiple subtypes in the same tissues has hampered detailed analyses, which can be overcome by use of knockout mice laking multiple subtypes. Our mice has been useful in various research fields, and we have published dozens of important papers. Here, I will present previous, current and future aspects of this project.

Muscarinic modulation of striatal physiology in health and disease

D. James Surmeier
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Although the dysregulation of striatal muscarinic signaling has long been implicated in a variety of psychomotor disorders, the role played by these receptors in regulating cellular excitability and synaptic integration has been something of a mystery. The cholinergic innervation of the principal neurons of the striatum – medium spiny neurons – arises from giant, aspiny cholinergic interneurons. This innervation is very rich, making striatal levels of cholinesterase and choline acetyltransferase among the highest of any brain region. The postsynaptic effects of acetylcholine on medium spiny neurons are mediated primarily by M_1 muscarinic receptors localized largely to spiny dendritic regions that are synaptically coupled to cortical and thalamic projection neurons. Using a combination of patch clamp electrophysiology, two photon laser scanning microscopy and single cell molecular profiling in slices from BAC transgenic mice, our studies have revealed that M_1 receptor signaling cascades target several ion channels, including Kir2 and Kv4 K^+ channels, that shape not only local, short-term synaptic integration but also the linkage between somatic and dendritic compartments that enables global changes in activity and spiking to influence the induction of long-term changes in the strength of dendritic corticostriatal glutamatergic synapses. These neuromodulatory effects are tailored by variation in several factors, including ion channel architecture, resulting in cellular specificity of the responses to M_1 receptor activation. This cellular specificity plays an important role in the dendritic adaptations seen in Parkinson's disease models, as genetic deletion of M_1 receptors dramatically attenuates activity-dependent dendritic pruning of synaptic connectivity seen in specific neuronal populations.

This work was supported by grants from the Picower Foundation and NINDS (34696).

Antimuscarinics and afferent signaling in the bladder

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In the bladder, muscarinic receptors have been demonstrated not only on detrusor smooth muscle cells, but also on the urothelium and on other structures in the bladder wall (e.g., afferent nerves, interstitial cells).[1] It has been suggested that the urothelial/suburothelial muscarinic receptors may mediate mediate activation of afferent nerves.[2] Tolterodine given intravenously or intravesically to rats with and without pre-treatment with resiniferatoxin (to eliminate C fibers) increased bladder capacity in non-resiniferatoxin treated animals, but was less effective in those who had received the toxin.[3] The amplitude of contraction was not affected in any of the groups. In rats, antimuscarinics (oxybutynin, darifenacin, solifenacin) given systemically had inhibitory effects on both Aδ and C-fibres in the afferent part of the micturition reflex, as demonstrated by direct recording of afferent activity in the pelvic nerve.[4] Tolterodine also had a significant effect on afferent fibres, as studied by its actions on sensations evoked by intravesical electrical stimulation and during bladder filling in healthy female subjects. These findings are in line with the clinical observations that antimuscarinics at clinically recommended doses have little effect on voiding contractions and may act mainly during the bladder storage phase, increasing bladder capacity. During bladder filling there is normally no parasympathetic outflow from the spinal cord. However, there may be a basal release of acetylcholine (ACh) from nonneuronal (urothelial, lamina propria) as well as neuronal sources, as demonstrated in isolated human detrusor muscle. Thus, ACh, released during filling, may contribute to detrusor overactivity (DO) and the overactive bladder syndrome, by increasing bladder afferent activity.[2] This may be caused by a direct stimulatory effect on suburothelial afferents and interstitial cells, and by stimulation of contraction of detrusor muscle cells, which already have an increased myogenic activity in DO. In turn, enhanced myogenic contractions can generate enhanced afferent activity ("afferent noise"), contributing to urgency and/or initiation of the micturition reflex.[2] Thus, antimuscarinics may depress afferent signaling via blockade of muscarinic receptors on both detrusor smooth muscle cells and on non-muscle structures (afferent nerves, interstitial cells).

References:

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Muscarinic receptor agonists: A novel treatment for schizophrenia

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Significant unmet need exists in treating schizophrenia, especially for treatment of cognitive impairment, negative syndrome and loss of social function. A number of preclinical and limited human data suggest that agonists with selective affinity for acetylcholine muscarinic (M) receptors could provide a potentially new mechanism to treat schizophrenia.

Methods: We examined the efficacy of xanomeline, a relatively selectivite muscarinic type 1 and type 4 (M_1 and M_4) receptor agonist , on clinical outcomes in 20 subjects with schizophrenia in a pilot study, utilizing a double blind, placebo-controlled, 4-week treatment design. The outcome measures included Positive and Negative Symptom Scale for schizophrenia (PANSS); Brief Psychiatric Rating Scale (BPRS); Clinical Global Impression scale (CGI) and a test battery designed to measure cognitive function in schizophrenic patients

Results: The xanomeline treated group did significantly better than the placebo group on the total BPRS scores ($p<0.05$), and PANSS total scores ($p=0.039$). In the cognitive test battery, the xanomeline group showed improvements most robustly in measures of verbal learning and short term memory function.

Discussion: These results support further investigation of muscarinic agonists as one novel approach to treating schizophrenia is warranted. Additional aspects of developing cholinergic treatments for schizophrenia will be discussed.

Muscarinic Receptors and Lung Function in Asthma

Allison D Fryer
Oregon Health & Science University

Asthma is characterized by airway hyperreactivity, increased mucus, and inflammation. Acetylcholine from parasympathetic nerves causes bronchoconstriction and mucus secretion that are mediated by M3 muscarinic receptors. Release of acetylcholine is normally limited by prejunctional M2 muscarinic receptors on parasympathetic nerves. Dysfunction of these neuronal M2 receptors increases acetylcholine release and is a common feature of airway hyperreactivity induced by antigen challenge, viral infection, ozone exposure, or organophosphate pesticide exposure in animals, as well as in humans with severe asthma. Loss of M2 receptor function is linked to airway inflammation.

Selective loss of neuronal M2 receptor function results from blockade of receptors or down-regulation of receptor expression. Eosinophil major basic protein is an endogenous antagonist for M2 receptors, and plays a central role in allergic hyperreactivity by blocking M2 receptor function. Down-regulation of M2 receptor expression is important in virus induced airway hyperreactivity, and is likely to result from the effects of cytokines released in response to infection. For example, TNFalpha destabilizes M2 mRNA in parasympathetic nerves.

Regardless of the mechanism for neuronal M2 receptor loss, hyperreactivity mediated by the parasympathetic nerves should be blocked by anticholinergic drugs. However, early muscarinic antagonists failed to live up to expectations, particularly in the chronic treatment of asthma. We have recently shown that there are inhibitory muscarinic receptors on eosinophils (both guinea pig and human). As a result, anticholinergics increase eosinophil activation and degranulation that is induced by antigen challenge. Thus, anticholinergics, while acutely causing bronchodilation, at the same time potentiate eosinophil activation, possibly worsening the underlying airway disease. In conclusion, inflammation and inflammatory cells decrease M2 receptor expression and function, thereby increasing acetylcholine release and causing airway hyperreactivity. However, chronic use of anticholinergics may be contraindicated if the blockade of muscarinic receptors increases eosinophil activation in vivo.

Structure and Dynamics of the Human β_2 Adrenergic Receptor

Brian Kobilka
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G protein coupled receptors (GPCRs) are remarkably versatile signaling molecules. The β_2 adrenoceptor (β_2AR) is a prototypical Family A GPCR that mediates physiologic responses to adrenaline and noradrenaline. The function of the β_2AR can be modulated by a spectrum of synthetic ligands ranging from full agonists to inverse agonists. We have used crystallography to determine the three-dimensional structure of the β_2AR [1-3], and fluorescence spectroscopy [4] to map ligand-induced conformational changes and characterize the structure of β_2AR dimers. I will discuss what we these studies have taught us about the structural basis of β_2AR function.

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The Muscarinic Receptors in Keratinocytes:
Signaling pathways and biologic effects

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Acetylcholine is a ubiquitous molecule in life that in addition to neurotransmission plays important roles in various aspects of cell biology and homeostasis. The cholinergic control of keratinocytes can be mediated by synergistic, additive, and reciprocal effects triggered by activation of different subtypes of muscarinic acetylcholine receptors (mAChRs). Simultaneous stimulation of the mAChRs subtypes expressed on the cell surface may be required to synchronize and balance ionic and metabolic events, and a crosstalk between different mAChR subtypes may provide for fine tuning of the cholinergic signals. The diversity of signaling pathways mediating ACh effects downstream of mAChR explains the plethora of cholinergic effects in different types of non-neuronal cells and provides a basis for understanding biology of normal and malignant cells and identification of new targets for more effective therapies.

Muscarinic receptors and apoptosis

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G-protein coupled receptors (GPCRs) have proven to be excellent therapeutic targets for a host of diseases from heart failure to depression. The therapeutic potential of GPCRs has, however, been largely overlooked in diseases involving lesions in the programmed cell death or apoptotic pathways. Prominent among such diseases is cancer where tumour cells are able to resist apoptosis despite harbouring dramatic genomic re-arrangements. It has been known for some time that GPCRs are able to both promote and inhibit apoptosis in a manner that is dependent on the receptor subtype and cellular background. This ability can be attributed to the plethora of signaling pathways activated by these receptors many of which can interact with the apoptotic pathway. We have been interested in the notion that the signaling potential of GPCRs could be used to influence the apoptotic process initiated by chemotherapeutic agents. If this were the case then by pharmacologically targeting GPCRs expressed on the surface of tumour cells it may be possible to specifically augment the action chemotherapy.

We have been testing this notion in two ways. The first is by directly testing the principle that GPCR signalling can influence the apoptotic process in cancer cells. This we have done by focusing on the ability of the M_3-muscarinic receptor endogenously expressed on SH-SY5Y cells to inhibit apoptosis mediated by DNA-damaging chemotherapeutic agents. In this study we have found that the M_3-muscarinic receptor is able to act directly at the level of p53 in a manner previously unreported for a GPCR. The second approach we have taken is to define the GPCR expression profile in non-small cell lung carcinoma cells and determine if ligands specific to these receptors can regulate the activity of commonly used chemotherapeutic agents. We are currently at an early stage in this study and I plan to give an overview of progress to date.

Key words: Apoptosis, muscarinic, programmed cell death, p53, SH-SY5Y, phosphorylation.

Structure-function meets ligand-directed modeling:
Towards novel muscarinic receptor chemotypes

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Muscarinic acetylcholine receptors (mAChRs) are attractive drug targets [1]. However, subtype-selectivity remains a significant issue due to the high conservation of amino acids that comprise the orthosteric binding site. One approach to overcoming this problem is to target allosteric sites, which offer the potential for greater variability in binding affinity and pharmacological selectivity across the mAChRs [2]. Another method is to utilize structure-based approaches for discovering new chemotypes for these receptors. With the exception of bovine rhodopsin and the b_2 adrenergic receptor, however, atomic-level structural data for GPCRs is lacking, necessitating the use of homology modeling for generating high-quality structures of binding pockets. A limitation with the homology approach for ligand docking and virtual screening, however, is the common reliance on rigid-receptor/binding pocket models. An alternative approach is to incorporate ligand poses to explicitly shape and then optimize potential binding pockets. This "ligand-directed" modeling approach was recently used to successfully discover novel antagonist chemotypes for the human androgen receptor [3], and we have now applied the same method to the human M_2 mAChR. By using both rhodopsin and the b_2 receptor as templates, we have generated a large number of models of the M_2 mAChR based around multiple (different) binding poses of the antagonist, N-methylscopolamine (NMS), and subjected each of the NMS-directed binding pockets to a virtual screen of 1013 marketed drug structures deposited in the DrugBank. In both instances, but most strikingly when the b_2 receptor was used as a template, specific binding pockets were identified that were able to both enrich the highest scoring hits with known mAChR binders and to identify new chemotypes that were not previously appreciated for their antimuscarinic properties. By combining mutational data for orthosteric or allosteric sites on mAChRs with pocket-building driven by known binders for each of these sites, ligand-directed modeling offers a novel approach to discovering new and selective mAChR chemotypes via virtual ligand screening.

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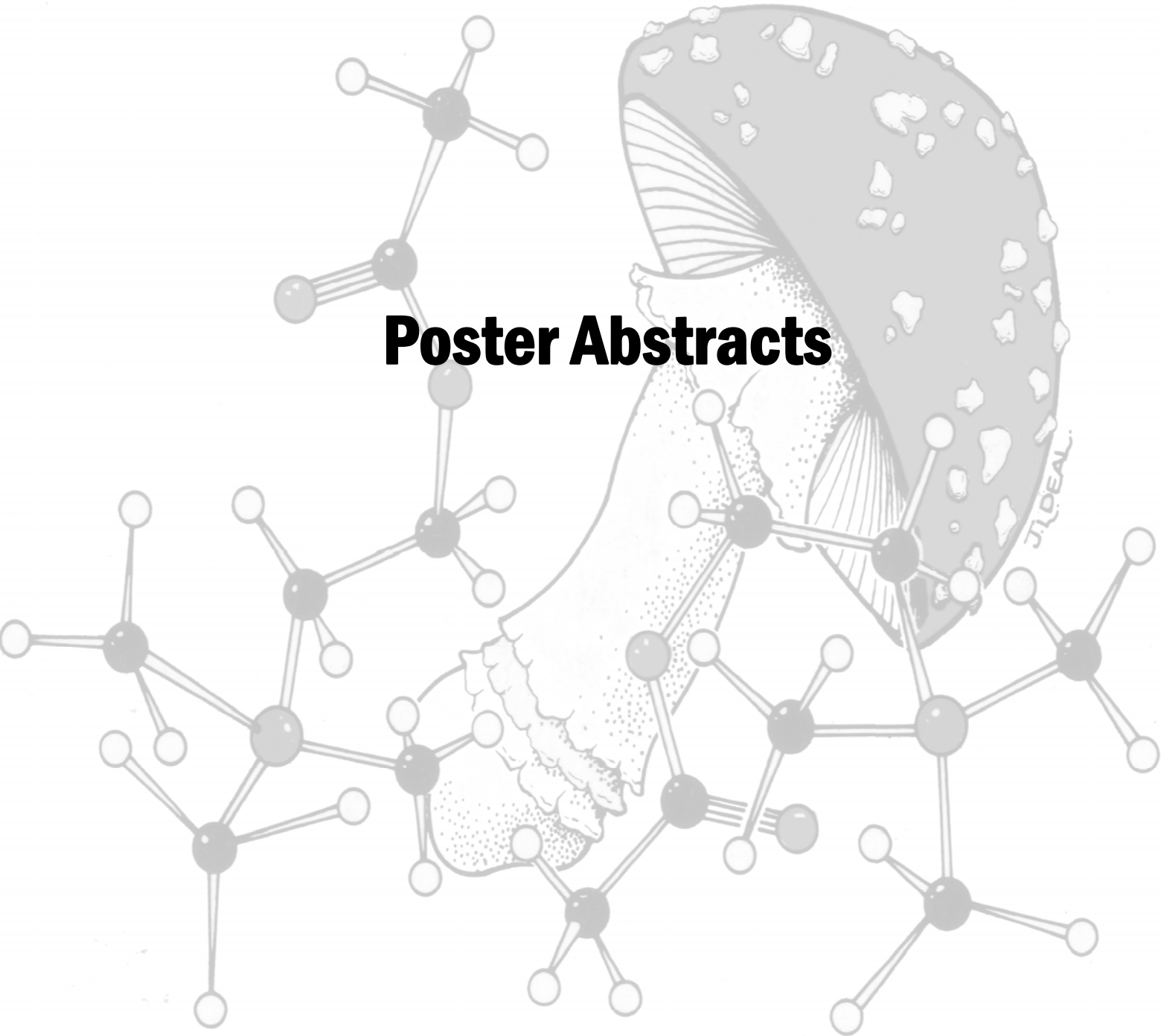
Recent Advances in Muscarinic Receptor Pharmacology and Therapeutics



Poster Abstracts

1

3d-Qsar Studies of 2,2-Diphenylpropionates to Aid Discovery of Novel Potent Muscarinic Antagonists

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Fifteen α-substituted 2,2-diphenylpropionate antimuscarinics were modeled using quantum chemical and pharmacophore generation to characterize antagonistic molecular mechanisms and design new therapeutics. Calculated molecular properties were compared with experimental data from three muscarinic receptor assays: [^{3}H]-NMS binding, α-amylase release, and ileum contraction (Gordon et al. 1989). Calculated total energies, bond distances, valence angles, and torsion angles, electronic properties including LUMO energy, reactivity indices, vibrational frequencies of ether & carbonyl moieties, and nitrogen atom proton affinity correlated with experimental data. *In silico* toxicity calculations correlated predicted rat oral LD_{50} values with binding and α-amylase, but not ileum assays. We developed a feature based 3D pharmacophore model using *in silico* CATALYST that contained a) a hydrogen bond acceptor site on the carbonyl oxygen atom and b) a ring aromatic attribute on one of the two aromatic rings. The model was used to search an in-house database, yielding some compounds equal in potency to atropine and, importantly, six not reported before as antimuscarinics. These results demonstrate that QSAR studies provide a molecular pharmacophore to aid in the discovery of novel potent muscarinic antagonists.

2

ACP-104 (*N*-desmethylclozapine) is a unique atypical antipsychotic agent with M_1 muscarinic activity

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ACP-104 (*N*-desmethylclozapine) is the primary circulating metabolite of clozapine. This compound has pharmacological features that suggest it will have potent antipsychotic activity (1-3). Importantly, ACP-104 is a muscarinic M_1 receptor agonist, a characteristic that is absent in all the other antipsychotic agents. M_1 agonist activity is responsible for pro-cognitive action in a variety of animal models of cognition, and these results suggest that ACP-104 has potential for improving cognitive function. Here, we show that ACP-104 activates M_1 muscarinic receptors in functional cell-based assays. *In vivo* studies show that ACP-104 activates MAP kinase in the rat prefrontal cortex. Moreover, ACP-104 displays pro-cognitive effects in recognition (NOR) and radial arm maze (RAM) models of cognitive functions. The pro-cognitive effects in RAM are reversed by pirenzepine. Taken together these findings suggest that ACP-104 pro-cognitive actions are M_1-mediated. Our studies show that ACP-104 has a unique antipsychotic profile and, because of its M_1 agonist activity, ACP-104 might also demonstrate a favorable outcome on cognitive measures and negative symptoms. ACADIA is presently developing ACP-104 as a novel treatment for schizophrenia.

3

Pharmacological characterization of AC-260584, a potent and selective M_1 muscarinic receptor agonist

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ACADIA has identified a number of allosteric muscarinic M_1 receptor agonists, including AC-42 and AC-260584 (Spalding, et al., 2002; Spalding, et al., 2006). We here report *in vitro* and *in vivo* pharmacology of this novel ligand, AC-260584. This molecule is a subtype-selective M_1 muscarinic receptor agonist, as multiple cell based assays demonstrate. Using extracellular signal-regulated kinase 1 and 2 phosphorylation in the hippocampus we show that AC-260584 activates M_1 receptors *in* vivo. Moreover, at 10 mg/kg SC this molecule improves novel object recognition (NOR) in mice, indicating pro-cognitive actions. Treatment with the M_1 receptor antagonist pirenzepine (0.03 mg/kg) 30 minutes prior to AC-260584 reversed this improvement. Compared to AC-42, AC-260584 has substantially greater efficacy, potency (20 nM in R-SAT and phosphotidyl inositol hydrolysis assays) and selectivity. Thus AC-260584 represents a major advancement in muscarinic receptor pharmacology.

4

Characterization of novel selective positive allosteric modulators (PAMs) of the M4 muscarinic acetylcholine receptor (mAChR)

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Recent clinical studies have revealed that the M_1/M_4 preferring mAChR agonist xanomeline induces a robust antipsychotic effect in schizophrenic patients, suggesting that mAChR agonists may be efficacious in reducing psychotic symptoms in patients suffering from a variety of neurodegenerative and psychiatric disorders. Nevertheless, a lack of highly selective compounds has made it impossible to conclusively verify whether one or both of these receptor subtypes is responsible for mediating these observed clinical effects. Previous compounds developed to selectively activate mAChR receptors have failed in clinical development due to a lack of true specificity and adverse effects associated with activation of peripheral M_2 and M_3 mAChR subtypes. Here we report characterization of a series of novel, selective M_4 PAMs which do not activate the receptor directly, but dramatically potentiate activation of the receptor by its natural agonist, acetylcholine. These novel M_4-selective agents can now be utilized as tools to ascertain whether selective potentiation of the M4 mAChR will mimic the *in vivo* effects of traditional orthosteric agonists and, more importantly, to rigorously test the hypothesis that activation of M_4 has effects in animal models predictive of antipsychotic efficacy in humans.

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5

Discovery and characterization of novel agonists, antagonists, and potentiators selective for the M1 muscarinic acetylcholine receptor

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Based on a large body of evidence, the muscarinic acetylcholine receptor subtype-1 (m1AChR/M1) is an attractive molecular target for numerous diseases and disorders of the CNS, including Alzheimer's disease (AD) and Schizophrenia. However, traditional muscarinic agonists carry severe side effects due to non-selective mAChR activation, particularly via peripheral M2 and M3. An absence of truly selective agents has also hindered advancement of basic research into the specific roles of the muscarinic receptors in the CNS. Here we report the discovery and initial characterization of novel compounds that allosterically activate the M1 receptor with high subtype-selectivity. We also report a highly potent M1 antagonist demonstrating unprecedented selectivity. Functional cell-based HTS and technology-enabled synthetic methods were employed to identify and optimize a number of novel chemical series. Functional Ca^{2+}-response and binding assays were used to further characterize the lead compounds. TBPB, an identified allosteric M1 agonist, demonstrated disease modification potential for AD by decreasing Aβ secretion in an in-vitro APP processing assay, and displayed in-vivo antipsychotic activity in rodent behavioral models. These novel compounds serve as useful tools for further pharmacological investigation of the muscarinic system, with TBPB in particular providing support for the therapeutic relevance of M1 activation in AD and Schizophrenia.

6

Characterization of the Intrinsic Efficacies of M1 Muscarinic Receptor Agonists

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Intrinsic efficacy is an important determinant of agonist actions. Intrinsic efficacy is often used to indicate the ability of an agonist to activate receptors, whereas efficacy usually refers to the ability of an agonist to induce responses in a given system. Thus in this context, intrinsic efficacy is an inherent property of a given agonist-receptor pair, whereas efficacy will be influenced by receptor reserve present among different systems. We sought to characterize the intrinsic efficacies of a series of M1 muscarinic receptor agonists that have been, or are currently in clinical development. We tested these compounds in cellular proliferation assays, GTP gamma S assays, and bioluminescence resonance energy transfer (BRET) assays, functional assays with very high, medium, and low receptor reserve, respectively, and in radioligand binding assays to determine receptor occupancy. Comparison of the potencies and efficacies in the various functional assays to the corresponding receptor occupancies revealed surprisingly large differences in intrinsic efficacy between M1 muscarinic receptor agonists. These results will be discussed in the context of what is known about the actions of these compounds *in vivo* and clinically.

7

Effect of ACP-104 (*N*-desmethylclozapine) on regulation of muscarinic M_1 receptors: comparison with allosteric and orthosteric ligands

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ACP-104 (*N*-desmethylclozapine) is currently under development by ACADIA Pharmaceuticals as an antipsychotic agent with potentially procognitive properties. ACP-104 has unique properties compared to other antipsychotic agents in that it is a muscarinic M_1 receptor partial agonist. We have previously compared ACP-104 to a family of allosteric agonists that bind and activate the M_1 muscarinic receptors (Spalding et al. Mol. Pharm. 70:1974-1983, 2006). Experiments demonstrated that M_1 receptors can be activated in distinct ways by ACP-104 compared to allosteric and orthosteric compounds. Here we present data comparing ACP-104 to allosteric and orthosteric muscarinic agonists in its ability to induce cellular trafficking of muscarinic M_1 receptors. Using different muscarinic radioligands, internalization of cell surface receptors and down-regulation of receptors were studied in HEK-293 and CHO cells, respectively. We demonstrate that ACP-104, similar to orthosteric muscarinic agonists, carbachol and oxotremorine-M induces receptor internalization and downregulation in a dose- and time-dependent manner. These results indicate that interaction of ACP-104 with the muscarinic M_1 receptors fully initiates receptor activation processes, including receptor trafficking. Moreover, we describe differences between allosteric and orthosteric compounds in their ability to induce internalization of cell surface receptors. These results indicate that allosteric and orthosteric muscarinic agonists behave differently in activating M_1 regulatory pathways.

8

Use of acetylcholine nitrogen mustard to study allosteric interactions at the M1 muscarinic receptor

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To determine the location of binding sites on the M_1 muscarinic receptor, we investigated the ability of ligands to prevent alkylation of the orthosteric binding site by a nitrogen mustard derivative of acetylcholine using radioligand binding methods in CHO-K_1 cells stably transfected with the M_1 receptor. The kinetics of the interaction of the aziridinium ion of acetylcholine mustard with the M_1 receptor was consistent with a two-step model in which the aziridinium ion first formed a reversible complex with the receptor (pK_D = 3.93) that converted to a covalent complex with a half time of approximately 6.3 min. Competitive binding experiments with [^{3}H]N-methylscopolamine and the aziridinium ion at 0°C yielded a similar estimate for the dissociation constant of the aziridinium ion (pK_I = 4.04). Modulatory effects of known competitive and allosteric muscarinic ligands on irreversible alkylation of the receptor were investigated. N-methylscopolamine caused a concentration-dependent slowing and ultimate cessation in the rate of alkylation, indicating competitive binding, whereas the inhibition by gallamine reached a plateau at high concentrations, consistent with an allosteric mechanism. Initial data using McN-A-343 shows concentration dependent slowing in the rate of alkylation therefore suggesting competition at the orthosteric site of the M1 receptor. WIN62,777 had no significant effect on the rate of M1 receptor alkylation by acetylcholine mustard. Our results illustrate a useful method for detecting allosteric interactions at the muscarinic receptor.

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9

Brain-specific deletion of M_3 muscarinic acetylcholine receptors causes pituitary hypoplasia and dwarfism in mice

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The M_3 muscarinic acetylcholine (ACh) receptor (M_3 mAChR) is widely expressed throughout the brain and in many peripheral organs. Whereas the peripheral M_3 mAChRs are known to mediate many of the parasympathetic actions of ACh, the physiological significance of neuronal M_3 mAChRs remains unclear. To address this question, we used Cre/loxP technology to generate mutant mice lacking M_3 mAChRs only in neurons and glial cells ('brain-M_3-KO mice'). Brain-M_3-KO mice did not show any significant metabolic alterations, including energy consumption, food intake, and glucose homeostasis. However, adult brain-M_3-KO mice exhibited a dwarf-like phenotype (~10% reduction in body length and ~20% decrease in body weight, as compared to control mice). Consistent with this phenotype, circulating IGF-1 and growth hormone (GH) levels were significantly decreased in brain-M_3-KO mice. Moreover, histological analysis revealed a marked hypoplasia of the anterior pituitary gland of brain-M_3-KO mice, associated with greatly reduced pituitary GH and prolactin levels. Additional studies suggested that the lack of hypothalamic M_3 mAChRs that normally regulate the activity of hypothalamic GHRH neurons contributes to this phenotype. These studies reveal a critical and unexpected role for neuronal M_3 mAChRs in anterior pituitary function and somatic growth.

10

Muscarinic and Anticholinesterase Effects of Betel Nut

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Betel nut is the most commonly used narcotic in the world after nicotine, ethanol and caffeine. The nut is chewed to impart different gastrointestinal (GI) and cardiovascular effects. This study was undertaken to evaluate some of its actions. A methanolic extract (Ac.Cr) of the nut was obtained while some commercially available pure compounds, known to be present in the nut, were tested. Tissue preparations: rabbit jejunum; guinea-pig ileum, gallbladder, atria; and rat aorta were used. In vitro assay for cholinesterase (ChE) inhibitory activity was performed. Effect on GI transit and laxative activity were monitored in mice. Effect on blood pressure (BP) was seen in vivo in rats while antiplatelet effect was recorded using human blood in vitro. Ac.Cr exhibited atropine sensitive, hexamethonium insensitive, spasmogenic effect in jejunum, ileum and gallbladder. Ac.Cr response was greater than that of acetylcholine (ACh) while also enhanced the effect of ACh. Ac.Cr inhibited acetyl- and butyryl-ChE. Ac.Cr enhanced GI transit and showed laxative effect in mice. Arecoline and arecaidine showed stimulant while diosgenin, tannic and gallic acid showed antiChE effects. Ac.Cr was 10 times more potent in its atropine sensitive relaxant effect in atria (with M_2 receptors) while showed atropine and L-NAME sensitive relaxation in rat aorta. In anaesthetized rats, Ac.Cr reduced BP, sensitive to atropine. Ac.Cr also showed antiplatelet activity. Arecoline showed cardiorelaxant while catechin: cardiorelaxant, vasodilator (via muscarinic and Ca^{2+} blockade) and antiplatelet effects. The study shows presence of multiple muscarinic constituents in betel nut, responsible for some of the actions of extract.

11

Differential effects on allosteric agonism and stimulus-trafficking by mutations in the orthosteric and allosteric pockets of M_2 muscarinic acetylcholine receptors

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Muscarinic acetylcholine receptors (mAChRs) contain at least one allosteric site that is topographically distinct from the acetylcholine (ACh)-binding orthosteric site. Although numerous studies have investigated the structural basis of allosteric modulation at these receptors, less is known about allosteric ligands that activate the receptor in their own right. We generated a number of M_2 mAChRs harboring different orthosteric-site mutations ($W^{99}A$, $Y^{104}A$, $Y^{403}A$), or allosteric-site mutations ($^{172}EDGE^{175}$-QNGQ, $Y^{177}A$), and investigated their impact on the binding and function (ERK phosphorylation and ^{35}S-GTPγS binding) of the putative allosteric agonists AC-42, 77-LH-28-1, McN-A-343 and *N*-desmethylclozapine (NDMC). As expected, orthosteric site mutations reduced the affinity and/or efficacy of the orthosteric agonists, ACh and pilocarpine, whereas the allosteric site mutations had little effect. Interestingly, ERK phosphorylation was severely attenuated for all agonists at the $Y^{104}A$ mutation, whereas ^{35}S-GTPγS binding was robust; indeed, NDMC and 77-LH-28-1 showed increased efficacy for this pathway, suggesting that Y^{104} is a key mediator of ligand-directed stimulus trafficking. $W^{99}A$ and $Y^{403}A$ caused significant increases in the affinity (100-300 fold and 3-10 fold respectively) and efficacy of AC-42 and 77-LH-28-1. NDMC showed increased efficacy, but not affinity, at $Y^{403}A$. McN-A-343 showed increased affinity and efficacy at $Y^{177}A$. Collectively, these findings can lead to a better understanding of the different modes of binding and activation of mAChRs.

12

Kinetic Analysis Of The M3-Ach-Receptor Activation In Living Cells

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The activation of G protein-coupled receptors (GPCRs) is the initial step in the signaling cascade which is triggered by binding of an agonist to a GPCR. Here we report the kinetics of the initial steps in the signaling cascade of a Gq-coupled receptor. The M_3-subtype of the muscarinic acetylcholine receptor (M_3-AChR) was employed as a model receptor to study Gq-signaling. Using a fluorescence resonance energy transfer (FRET) approach based on the FlAsH-tetracysteine tag technology in combination with CFP we generated a M_3-receptor construct that reports conformational changes during receptor activation by changes in the FRET-signal. Receptor activation occurred on a millisecond timescale and was induced by acetylcholine or carbachol with similar kinetics. However, receptor deactivation was significantly faster with carbachol than acetylcholine. Additional introduction of the mutation N514Y, which was previously shown to cause constitutive activity, allowed demonstration of inverse agonistic activity of atropine at the M_3-receptor construct. Furthermore, Receptor – G-Protein interaction was measured by FRET between a M_3-AChR-YFP construct and an N-terminally tagged CFP-γ_2 subunit of the heterotrimeric G-protein. The receptor – G-Protein coupling induced by carbachol was at a similar time scale as receptor activation. G-Protein activation was measured using YFP-tagged Gαq and an N-terminally tagged CFP-γ_2 subunit of the heterotrimeric G-protein. Activation of the Gq-Protein was significantly slower than receptor activation and undistinguishable for acetylcholine or carbachol.

13

Mapping of Contact Sites Between the M3 Muscarinic Acetylcholine Receptor and Gαq by Cross-linking Approaches

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How a G protein-coupled receptor (GPCR) interacts specifically with its cognate G protein(s) remains largely unknown. Many studies have identified distinct GPCR and G□ regions that are predicted to be critically involved in productive receptor/G protein coupling. However, little is known about the specific point-to-point interactions between GPCRs and their cognate G proteins. To address this question, we chose to study the M3 muscarinic acetylcholine receptor, a prototypic Gq-coupled receptor, as a model system. Cysteine (Cys) scanning mutagenesis was applied to introduce thiol groups for cross-linking into a mutant version of the M3 receptor that lacked most endogenous Cys residues. We also generated a mutant version of G□q lacking most native Cys residues as a template for Cys mutagenesis. The Cys-substituted mutant M3 receptors and G□q proteins were verified to remain functional by established biochemical techniques. Membranes were prepared from transfected COS-7 cells co-expressing various combinations of Cys-substituted M3 receptor and G□q constructs, followed by Cys-Cys cross-linking studies to screen for vicinal Cys pairs. Initial experiments have yielded interesting results regarding specific M3 receptor/G□q contact sites. Further mapping of the M3 receptor/G□q interface should provide detailed insights into the molecular basis of receptor-G protein interactions.

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14

Induced-fit docking of N-methylscopolamine and strychnine-like allosteric modulators to the M_2 muscarinic receptor homology model

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A model of the M_2 muscarinic receptor was build based on the coordinates of rhodopsine (1U19.pdb) using Prime module of Schrödinger Suite. First, N-methylscopolamine (NMS) was docked to the orthosteric binding site defined by Asp103, Tyr403, and Tyr430 using Induced Fit Docking (IFD) with an extraprecision final docking step. Subsequently strychnine and the strychnine-like allosteric modulators brucine, Wieland-Gumlich aldehyde (WGA) and propargyl-WGA were docked to the receptor-NMS complex at the allosteric binding site defined by residues 172-182 of the o2 loop and 410-422 of the o3 loop by IFD with o2 and o3 loops being treated as flexible and extraprecision final docking step. Stability of complexes was tested by simulation of 40 ns unconstrained molecular dynamics with implicit solvent (Macromodel) or explicit DPPC membrane/water/Na^+/Cl^- environment (gromacs). The estimated order of affinities for the best poses of each allosteric modulator agrees with experimental measurements. The model reveals substantial flexibility in the o2 and o3 loops of the M_2 receptor, their substantial change in conformation induced by binding of strychnine-like modulators, several equally valid poses with similar estimated affinities for each modulator, and propargyl-WGA oriented in a different way than WGA at the allosteric binding domain.

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Molecular and behavioral phenotypes caused by selective disruption of M_4 muscarinic acetylcholine receptors in D1 dopamine receptor-expressing cells

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The M_4 muscarinic acetylcholine (ACh) receptor (M_4 mAChR) is enriched in many regions of the forebrain including the striatum. Striatal M_4 mAChRs are preferentially found on a subset of striatal projection neurons that express D1 dopamine receptors and give rise to the so-called direct striato-nigral pathway. To study the functional roles of these striatal M_4 mAChRs, we used Cre/loxP technology to generate mutant mice that lack M_4 mAChRs only in D1 receptor-expressing neurons ('D1-M4-KO mice'). Adenylyl cyclase assays with striatal membranes showed that carbachol, a muscarinic agonist, was able to inhibit D1 receptor-mediated increases in striatal cAMP levels in control mice, but lacked this activity in tissues from D1-M4-KO mice. In behavioral studies, D1-M4-KO mice showed a significant increase in basal locomotor activity, as compared to control littermates. Furthermore, apomorphine, a non-selective dopamine receptor agonist, and SKF38393, a D1 receptor selective agonist, induced greater stimulatory locomotor responses in D1-M4-KO than in control mice. These studies provide novel insights into the functional roles of striatal M_4 mAChRs and their potential involvement in the pathophysiology of various CNS disorders including Parkinson's disease and schizophrenia.

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Development of muscarinic receptor agonists with functional selectivity for M_1, M_2 and M_4 receptor subtypes: Potential utility in Alzheimer's disease and schizophrenia

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Acetylcholine activates five muscarinic receptor subtypes, which mediate a variety of physiological responses throughout the central and peripheral nervous systems. Understanding the precise role for each muscarinic receptor subtype has been hampered by the lack of suitably selective agonists and antagonists. It is clear that muscarinic receptors are implicated in a variety of neurological disorders, including Alzheimer's disease and schizophrenia. Activation of muscarinic receptors might provide useful therapeutic approaches toward the treatment of memory and cognitive deficits, protection from neural degeneration and alleviation of psychiatric symptoms such as hallucinations and delusions associated with neurological disorders. Previous studies had identified CDD-0102 as a functionally selective M_1 agonist with potential utility in treating Alzheimer's disease. In contrast, CDD-0304 displays mixed agonist activity at M_1, M_2 and M_4 receptors, a profile suggesting potential utility in the treatment of schizophrenia. Recently, several new muscarinic agonists have been developed exhibiting improved functional selectivity. CDD-0316 displays muscarinic agonist activity at M_1 and M_4 receptors, while CDD-0317 is a selective M_2 agonist and CDD-0322 is a selective agonist at M_4 receptors. The five compounds provide an array of mixed and selective agonist activities that should be useful in helping to understand the role of relative contributions of M_1, M_2 and M_4 muscarinic receptor subtypes in neurological function. Pharmacological data (including receptor binding properties, biochemical activity and *in vivo* responses) will be presented and the therapeutic potential of selective muscarinic agonists will be discussed.

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Pharmacological characterization of M1 muscarinic acetylcholine receptors with a disabled orthosteric binding pocket

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M1 muscarinic acetylcholine receptor agonists improve cognition in various organisms including humans and are currently in development for the treatment of psychosis and cognitive dysfunction. Understanding drug-receptor interactions might help to improve selectivity and affinity of potential drug candidates. Three mutations have been described that severely impair interactions of orthosteric ligands with the human M1 receptor ($Trp^{3.28(101)}$Ala, $Tyr^{3.33(106)}$Ala and $Tyr^{6.51(381)}$Ala) while not disrupting, and in some cases potentiating the actions of other structural classes of M1 agonists. Here we combined the above mutations in order to create receptors that abolish function of ligands that bind to the orthosteric binding pocket while retaining or improving the activity for ligands that act via different contact points. We describe the pharmacological characteristics of the triple mutant and a double mutant ($Tyr^{3.33(106)}$Ala, $Tyr^{6.51(381)}$Ala) M1 receptor by presenting binding and functional data of a number of antagonists as well as agonists that are in preclinical and clinical development.

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A Single Conformation of the β_2 Adrenoceptor Suitable for Virtual Ligand Screening of Agonists

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The 2.4Å crystal structure of the β_2 Adrenoceptor (β_2AR) offers several advantages as a homology modeling template for Muscarinic and other biogenic amine receptors relative to the structure of bovine Rhodopsin (bRho). β_2AR exhibits higher sequence identity to the other biogenic amine receptors than bRho, and extracellular loop 2 of β_2AR is in a location more permissive to ligand binding within the transmembrane domain when compared with the occluded conformation seen in bRho. Here we evaluate the performance of the β_2AR structure, and several agonist-bound models derived from it, in virtual ligand screening. Importantly, β_2AR is crystallized with carazolol, a partial inverse agonist, and is thought to be in an antagonist bound conformation. In keeping with this, we found that virtual ligand screening with the new structure coordinates selectively retrieves known antagonists from a database of random GPCR ligands, and does not identify known agonist compounds. Subsequently, to generate hypothetical agonist bound conformations of β_2AR, rigid body movements of the transmembrane helices were generated and Monte Carlo sidechain refinement of the ligand binding pocket with the strong agonist isoproterenol was performed. These models were then evaluated for successful virtual screening of known agonists, and a single agonist-selective conformation was identified that retrieves partial as well as full agonists. This new conformation may reflect an early intermediate in the activation process. The use of this model as a template for generating agonist bound conformations of other receptors is discussed.

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Identification of a novel protein partner of the M_3 muscarinic acetylcholine receptor

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The M_3 muscarinic acetylcholine receptor is widely expressed throughout the central nervous system and is also found in many peripheral tissues and cell types. Several lines of work suggest that drugs acting on central or peripheral M_3 receptors might become clinically useful in a variety of pathophysiological conditions. Recent evidence suggests that the function of many G protein-coupled receptors (GPCRs) can be modulated by different classes of receptor-interacting proteins. The objective of the present study was to identify proteins that can interact with central M_3 muscarinic receptors. Recently, the split-ubiquitin yeast two-hybrid system has emerged as a novel approach to study membrane protein interactions. Using this system, we screened a human brain cDNA library using the full-length M_3 receptor as bait. The putative membrane protein, Tmem147, was identified 11 independent times in our screen. Tmem147 is predicted to span the membrane 6 or 7 times and has 99% amino acid conservation between human, mouse, rat and bovine species. We showed that Tmem147 heterodimerizes with the M_3 receptor in COS7 cells using biochemical approaches. Confocal imaging studies showed that this interaction can occur in the ER as Tmem147 is not expressed at the plasma membrane. We hypothesize that Tmem147 may act as a previously unidentified chaperone in the ER. Given the lack of ligands that can selectively activate or block M_3 receptors with high selectivity, the identification of novel protein partners may provide new insight into the regulation of M_3 muscarinic receptor function and provide possible new drug targets.

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An allosteric potentiator suggests a role for M_4 mAChR in modulation of excitatory hippocampal synaptic transmission

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Numerous animal and human studies suggest that muscarinic acetylcholine receptors (mAChRs), specifically M_1 and M_4, may be viable targets for the treatment of neurodegenerative disorders such as Alzheimer's disease (AD). We have used cheminformatic and medicinal chemistry to develop novel, highly selective M_4 allosteric potentiators. VU10010, the lead compound, potentiates the M_4 response to acetylcholine (ACh) 47-fold while having no activity at other mAChR subtypes. This compound binds to an allosteric site on the receptor and increases affinity for ACh and coupling to G proteins. Whole cell patch clamp recordings revealed that selective potentiation of M_4 with VU10010 increased carbachol-induced depression of transmission at excitatory but not inhibitory synapses in the hippocampus. The effect was not mimicked by an inactive analog of VU10010 and was absent in M_4 knockout mice. This provides compelling evidence for selective regulation of excitatory transmission in this important forebrain structure by M_4. We are now examining the effects of selective M_1 and M_4 activation on spike frequency adaptation and afterhyperpolarization currents in CA1 pyramidal cells to understand the involvement of these receptors in muscarinic regulation of neuronal excitability.

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Pharmacological characterisation of the M_3 muscarinic acetylcholine receptor in *Saccharomyces cerevisiae*

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Yeast strain *Saccharomyces cerevisiae* (*S.cerevisiae*) has been used as G protein-coupled receptor (GPCR) expression system for the purposes of ligand-screening and mutagenesis studies. However, yeast expression systems could also be used to assess pharmacological properties of ligands, such as pK_B values and G protein specific agonism or antagonism. Experiments were undertaken to investigate whether different pharmacological profiles can be detected in this system for ligands of the M_3 muscarinic acetylcholine receptor (M_3mAChR), and to what extent the findings correlated to a mammalian (NIH3T3) expression system. The rat $M_3\Delta$i3mAChR (with 3rd intracellular loop deletion (1)) was introduced into individual strains of *S. cerevisiae*, each expressing different chimeras from yeast and mammalian G_α subunits, followed by pharmacological characterisation . We tested rat $M_3\Delta$i3mAChR in yeast strains expressing chimeras of $G_{\alpha q}$, $G_{\alpha s}$, $G_{\alpha 12}$ and $G_{\alpha i1/2}$, which showed that in yeast, the rat $M_3\Delta$i3mAChR couples to $G_{\alpha q}$, $G_{\alpha 12}$ and $G_{\alpha i1/2}$ when stimulated with carbachol. The pA_2 values generated from analysis of the interaction between the M_3mAChR antagonist, atropine, and carbachol correlated with that observed in calcium flux assays in mammalian cells. Interestingly in the yeast assays, atropine appears to be an inverse agonist when coupled to $G_{\alpha q}$, but a weak partial agonist when coupled to $G_{\alpha 12}$. Preliminary studies in mammalian cells suggest that there is a correlation with results seen in the yeast assays.

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Acetylcholine mustard as a useful tool to study allosteric interactions at m_2 muscarinic receptor

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We explored the interaction of a nitrogen mustard derivative of acetylcholine with the human M_2 muscarinic receptor expressed in CHO cells using the muscarinic radioligand, [^{3}H]*N*-methylscopolamine ([^{3}H]NMS). Acetylcholine mustard caused a concentration-dependent, first order loss of [^{3}H]NMS binding at 37°C, with the half maximal rate constant occurring at 20.2 μM. We examined the effects of various ligands on the rate of alkylation of M_2 receptors by acetylcholine mustard. NMS and McN-A-343 competitively slowed the rate of alkylation, whereas the inhibition by gallamine reached a plateau at high concentrations, indicating an allosteric inhibition. In contrast, WIN51708 had no effect. We also measured the inhibition of [^{3}H]NMS binding by acetylcholine mustard at 0°C, conditions under which covalent binding couldn't be detected. In these experiments, the dissociation constants of NMS, gallamine, McN-A-343 and the aziridinium ion of acetylcholine mustard for their binding sites were 0.0001, 0.63, 18 and 12.3 μM, respectively. In contrast, the parent mustard and alcoholic hydrolysis product of acetylcholine mustard were without effect. Our results show that the aziridinium ion of acetylcholine mustard binds covalently to the orthosteric site of the M_2 receptor, whereas gallamine binds to an allosteric site. Measurement of the effects of ligands on the rate of inactivation of muscarinic receptors by a covalent orthosteric ligand is a powerful method for detecting allosterism.

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Differential Regulation of the M_1 Muscarinic Acetylcholine Receptor by Orthosteric and Allosteric Ligands

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AC-42 (4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl] piperidine hydrogen chloride) is a selective M_1 muscarinic receptor agonist with an allosteric mechanism of action. Here, we have investigated whether AC-42 and two orthosteric muscarinic agonists (oxotremorine-M (oxo-M) and pilocarpine) cause internalization and down-regulation of the M_1 muscarinic receptor. Oxo-M (EC_{50} 0.8 μM) caused the greatest increase in [^{35}S]-GTPγS-$G\alpha_{q/11}$ binding in membranes from Chinese hamster ovary cells stably expressing the M_1 muscarinic receptor, whereas AC-42 (EC_{50} 1.6 μM) and pilocarpine (EC_{50} 2.4 μM) stimulated similar responses that were approx. 30% of the oxo-M response. Using intact cell [^{3}H]-NMS and [^{3}H]-QNB binding assays, we next showed that after 24 h, oxo-M (100 μM) caused 68 ± 3% receptor internalization and 42 ± 3% receptor down-regulation. Over a similar time-course, pilocarpine (1 mM) caused 46 ± 7% internalization and 26 ± 8% down-regulation. In contrast, AC-42 did not cause significant receptor internalization or down-regulation. These data suggest that while AC-42 is able to activate selectively $G_{q/11}$-dependent signaling through the M_1 mACh receptor, it does not cause receptor internalization or down-regulation, at least over the time-course studied here.

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Modulation of cocaine discrimination by muscarinic ligands in mice

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Muscarinic receptors are modulators of dopaminergic function in various brain pathways including those thought to mediate cocaine's abuse-related effects. Some studies suggested a role for muscarinic receptors in mediating cocaine's effects, but the potential of muscarinic ligands as medications for cocaine dependence remains unclear. We tested the hypothesis that muscarinic agonists can blunt cocaine discrimination in mice. We also tested the hypothesis that muscarinic antagonists can exacerbate the discriminative stimulus of cocaine. Mice were trained to discriminate 10 mg/kg cocaine from saline in a two-lever operant procedure. After positive and negative controls were established, various muscarinic ligands were tested alone and in combination with cocaine. The non-selective muscarinic agonist oxotremorine and the putative M_1-selective agonist McN-A-343 dose-dependently blunted cocaine discrimination and shifted the cocaine dose-effect function to the right. The muscarinic antagonists scopolamine and methylscopolamine produced partial generalization to the discriminative stimulus of cocaine, and shifted the cocaine dose-effect function to the left in a manner consistent with synergistic interaction. The latter data confirm and extend an earlier report in which the antagonist atropine was evaluated. The new finding of this study is that muscarinic agonists can blunt cocaine discrimination, further supporting the implication of the muscarinic system in the abuse related effects of cocaine, and providing support for the investigation of muscarinic agonists as novel candidates for pharmacotherapy for cocaine dependence. Future studies are aimed at evaluating more selective muscarinic receptor ligands in this assay.

Muscarinic Receptors on Eosinophils Inhibit PAF-induced Eosinophil Activation

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In the lungs, eosinophils are clustered around parasympathetic nerves in asthmatic humans and animal models of asthma. Antigen challenge releases eosinophil major basic protein, an antagonist for M2 muscarinic receptors. Eosinophil mediated blockade of inhibitory M2 receptors on parasympathetic nerves increases acetylcholine release, which increases bronchoconstriction. Since eosinophils are associated with parasympathetic nerves, we tested whether they are reciprocally affected by acetylcholine. Eosinophils were isolated from guinea pig peritoneal lavage and human blood. RT-PCR and immunocytochemistry demonstrated that eosinophils express M3, M4 and M5, but not M1 or M2 muscarinic receptors. To test whether muscarinic receptors regulate eosinophil activation, pure eosinophils were treated with a nicotinic receptor antagonist hexamethonium (0.1uM), loaded with a calcium indicator (fluo-4; 5uM) and increases in intracellular calcium were measured via fluorescence microscopy. Eosinophil activation was measured as number of cells responding to an agonist within 1 minute. Platelet-activating factor (PAF) (0.01uM-10uM) increased the number of responding cells in a dose-dependent manner. Acetylcholine (1nM-10uM) alone did not increase intracellular calcium. However, the number of eosinophils activated by PAF (1uM) was dose-dependently decreased by acetylcholine (0.1-3uM; max response 49% of control at 3uM), an effect reversed by muscarinic blockade with atropine (1uM). Thus, eosinophils express 3 muscarinic receptors, one or more of which inhibit eosinophil activation in vitro. This inhibitory pathway may therefore be useful for treatment of asthma.

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