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EPHAR Symposium

Molecular Pharmacology of G Protein Coupled Receptors and Signalling Partners

Organized by Turkish Pharmacological Society

June 06-07, 2011

Fulya Congress Center / Istanbul

Meeting Program and Abstracts Book

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This symposium is sponsored by EPHAR, with support from the British Pharmacological Society.
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Dear friends and colleagues,

I would like to thank you all for participating EPHAR Symposium organized by Turkish Pharmacological Society entitled "Molecular Pharmacology of G Protein Coupled Receptors and Signalling Partners" which is held in Fulya Congress Centre, Istanbul – TURKEY, in June 6-7 2011 on behalf of Turkish Pharmacological Society. It is a great honour and privilege for us to host you in Istanbul which a hundreds of centuries old city located on the Bosphorus strait, connecting Asia and Europe. I would also like to thank for Organizing Committee and especially to the symposium chair Ongun Onaran for all the efforts and the speakers who are really masters in their fields. My last thanks will be to EPHAR Executive Committee for giving us a change transforming our local event to an international landmark, and our sponsors that are listed in the cover page.

The most relevant speakers across Europe and Northern America participated. We also have 4 oral and 16 poster presentations. The details of the program and the abstracts are in the following pages. We are sure that this will be scientifically and socially memorable meeting.

Istanbul is a centuries old city located on the Bosphorus strait, connecting Asia and Europe. It was the capital of three empires; Roman, East Roman (Byzantine), and the Ottoman Empire. Historic areas of Istanbul were added to the UNESCO World Heritage List in 1985. The city is also known as "The City on Seven Hills" since the historic peninsula was built on seven hills, each bearing a historic mosque. Elected as the European Cultural Capital 2010, Istanbul is unique with its location, cultural and historical heritage, several palaces and monuments, museums, bazaars, blending with modern architecture, heavenly shopping centres, all sort of restaurants, pubs, clubs, and friendly wine houses.

Turkish Pharmacological Society will also host the 7th European Congress on Pharmacology, EPHAR2016 that be held in Istanbul at July 2016. The web page of the congress will be available soon. That year will all so be our societies' 50th anniversary and I am sure that we shall celebrate this important event together.

Öner SÜZER
President of Turkish Pharmacological Society

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Steve LANIER, *Medical University of South Carolina, USA*

Martin LOHSE, *University of Würzburg, Germany*

Graeme MILLIGAN, *University of Glasgow, UK*

Martine SMIT, *University of Amsterdam, The Netherlands*

Özlem UĞUR, *Ankara University, Turkey*

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Invited Speaker Texts

S-01

New Insights Into Mechanisms of Receptor-Mediated G Protein Activation

Heidi Hamm
Department of Pharmacology, Vanderbilt University, USA

GPCRs mediate the activation of multiple G protein pathways, and they do so by an exquisitely balanced interplay between the receptors, G proteins, regulators, and effectors. The formation of the R-G complex is the first step in receptor mediated signalling, which results in nucleotide exchange, alteration in Gα-βγ subunit affinity, and downstream effector activation. Since the structure of the complex has thus far eluded high-resolution structural determination, we have used a combination of electron paramagnetic resonance (EPR), fluorescence, and molecular modeling to reveal new movements of the Gα and Gβγ relative to each other in the high-affinity R-G complex. This iterative strategy has provided a refined model of the high-affinity R-G complex and the mechanism of receptor-mediated G protein activation based on relative changes in mobility and proximity in specifically labeled residues.

Activated GαGTP subunits signal to downstream effectors, and many of the conformational changes associated with activated Gα subunits alone and in complex with effectors have been determined from crystallographic studies, however these have were all obtained with unmyristoylated Gαi subunits. We have used a combination of hydrogen-deuterium exchange, fluorescence and crystallography to probe the environment of amino- and carboxyl terminal residues in myristoylated Gα proteins, and our results indicate a structural role for myristoylation of Gα proteins. The crystal structure of myristoylated Gαi.GDP.AlF4- shows the amino-terminal myristate group binding in the cleft between the α2 and α3 helices, where effectors are known to bind. There is a myristoylation-induced change of the carboxyl terminus, the site of interaction with activated receptor. The GTP-myristoyl switch which we have captured has implications for the spatial regulation of G protein signalling.

Keywords: GPCR, myristoilation, G-alpha subunit, structural mechanism of activation.

S-02

Molecular Mechanisms of Receptor Activation and Signalling

Martin J Lohse
University of Würzburg, Institute of Pharmacology and Toxicology, Germany

The mechanisms of ligand binding, activation and signalling by G-protein-coupled receptors (GPCRs) are being unraveled with a multitude of methods from various disciplines, which are beginning to be combined for a full picture of these key processes in cell regulation. We are using biochemical as well as fluorescence methods to study these processes in intact cells. Fluorescence methods are ideal to delineate the kinetics of the individual steps, the mobility of receptors and their interactions. I will present and discuss fluorescence resonance energy transfer (FRET) data to illustrate the kinetics of receptor activation and signalling to G-proteins, as well as the crosstalk between the subunits in dimeric GPCRs, such as opiate/alpha2-adrenergic and metabotropic glutamate receptor dimers. I will further present data on distinct signalling of receptors from specific subcellular localizations within cells: t-tubules for signalling of cardiac beta-adrenergic receptors, and intracellular signalling for TSH receptors. Finally, I will illustrate the power of single molecule analysis and examples of observations of individual receptors.

Keywords: G-protein-coupled receptors (GPCRs); fluorescence resonance energy transfer (FRET); adrenergic receptors; opiate receptors; glutamate receptors; receptor dimmers.

S-03

Insights into Receptor-G Protein Communication from Dynamic Structure Network Analysis

Francesca Fanelli
University of Modena and Reggio Emilia, Italy

The first event in scotopic vision is the absorption of a photon by the transmembrane G protein Coupled Receptor (GPCR) rhodopsin (1, 2), which causes the cis-trans isomerization of the retinal chromophore and the consequent formation of the active signalling state. The latter in turn catalyzes the exchange of bound GDP for GTP on an heterotrimeric intracellular G protein (transducin) (3). Protein Structure Network (PSN) analyses of Molecular Dynamics trajectories (4) served to infer the differences in the dark, photoactivated (Meta II, MII), and constitutively active apo-protein (opsin) states of rhodopsin, as well as the effects of opsin on the intrinsic dynamics of transducin. The latter study relied also on Essential Dynamics and Functional Mode analyses. The distribution of highly connected nodes in the network (i.e. hubs) reflects the existence of an intramolecular communication inside and between the two poles of the helix bundle. Most frequent hubs concern nodes that are either highly conserved or participate in the retinal binding site. The latter, including the chromophore, was suggested to participate in the stability core of the protein (5). Rhodopsin activation enhances the communication between retinal binding site and G protein binding regions, including the arginine of the E/DRY motif.
The molecular determinants of transducin activation by opsin seem to reside in the establishment of selected receptor-G protein contacts, which triggers inter-domain concerted motions in transducin. These motions correlate with solvent exposure of the nucleotide and result in changes in the structure network of the G protein. A potential nucleotide exit route could be also inferred.

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Keywords: GPCRs, Rhodopsin, transducin, molecular dynamics simulations, protein-protein docking, protein structure networks, structural communication.

S-04

Targeting G Protein-Coupled Receptor Folding and Their Export from Endoplasmic Reticulum for Drug Discovery

Michel Bouvier, Patricia René, Marc Palardy, Virginie Bernier, Jean Pierre Morello, Etienne Sauvageau, Fadi Hamdan, Yan Percherancier, Morad Oueslati, Driss Rochdi, Daniel Bichet
Institute for Research in Immunology and Cancer, Department of Biochemistry, Université de Montréal, Canada

Mutations in G protein-coupled receptors (GPCRs) genes cause various human diseases. In many instances, the mutations do not affect specific functional sites but rather cause conformational changes that are unnecessarily recognized by the endoplasmic reticulum (ER) quality control system as misfolded leading to their intracellular retention and degradation. Examples of such lost of function conformational diseases include nephrogenic diabetese insipidus (NDI) and early-onset severe obesity that result from mutations in the vasopressin receptor type 2 (V2R) and the melanocortin receptor type 4 (MC4R) respectively. For both V2R and MC4R, we found that cell-permeant ligands that bind to the receptors increase cell surface expression and rescue their function; an observation leading to the concept of pharmacological chaperones. By binding to the mutated receptors, pharmacological chaperones promote their proper folding, maturation and traficking. For NDI, a V2R selective pharmacological chaperone was found to significantly improve patients' condition in a small clinical trial establishing its therapeutic value. In order to establish an in-vivo proof of principle that the concept can be extended to early-onset severe obesity, we generated a knock-in mouse model substituting the mouse MC4R by a mutant MC4R known to cause obesity in humans. The mice expressing the mutant receptor developed hyperphagia, increased weight gain, increased in fat mass and linear growth similar to the human phenotype. The potential therapeutic value of MC4R pharmacological chaperones is currently being tested in this new model of genetic severe obesity. We also performed a bioluminescence resonance energy transfer (BRET)-based screen to identify proteins involved in the maturation of GPCRs. Cornichon homolog-4 was found to be involved in the ER export of many GPCRs. Whether such proteins may also represent targets for the treatment of conformational diseases remains to be established.

Keywords: pharmacological chaperones, endoplasmic reticulum, melanocortin receptor, vasopressin receptor, conformational diseases.

S-05

Viral Chemokine Receptors Activate Inflammatory and Oncogenic Signalling Networks

Martine J Smit
VU University Amsterdam, The Netherlands

Pathogenic herpesviruses alter cellular signalling after viral infection through expression of viral G protein coupled receptors (GPCRs). These viral GPCRs show highest homology to chemokine receptors, which are known to regulate the immune system but are also involved in the development of cancer. We have shown that several viral GPCRs, including the HCMV-encoded chemokine receptor US28, signal in a constitutive manner and hijack proliferative signalling pathways. We have recently shown that US28 stimulated the production and secretion of both vascular endothelial growth factor (VEGF) and the cytokine interleukin-6 (IL-6) in vitro and HCMV infected cells. US28 increased cell proliferation through activation of the IL-6-STAT3 axis. US28 expression promotes tumor formation in a xenograft model and was found to be expressed in tumor specimen of glioblastoma patients. By activating inflammatory and proliferative signalling pathways, viral GPCRs may effectively rewire cellular signalling networks and contribute to tumorigenesis. Insight into these mechanisms is crucial for the treatment of virus-associated pathologies.

Keywords: chemokine receptors, VEGF, IL-6, STAT3, oncogenic signalling networks.

S-06

Genetic Approaches to Study Opioid Receptors and Brain Function in vivo

Brigitte L Kieffer
IGBMC, CNRS/INSERM/ Université de Strasbourg, Illkirch, France

Opioid receptors are G protein coupled receptors (GPCRs) widely distributed throughout the nervous system, which control pain, stress responses and emotional, and addictive behaviors. To elucidate the role of each receptor in these responses, we have created mice lacking the mu, delta or kappa receptor gene. We have demonstrated that mu receptors mediate morphine analgesia and reward (Matthes 1996), as well as reinforcing properties of non-opioid drugs of abuse and natural rewards (Moles 2004, Contet 2004). We have demonstrated opposing roles of mu and delta receptors in the regulation of emotional responses (Filliol et al 2000) revealing delta receptors as a potential target for mood disorders, as well as in the control of motor impulsivity, thus opening a novel area of investigation (Olmstead PLoS ONE 2009). We have confirmed pain-relieving activity of delta receptors in chronic pain, and identified neurons where delta receptors attenuate chronic pain using conditional gene knockout (Gavériaux-Ruff 2011). Finally we have achieved functional imaging of delta receptors in vivo, using an eGFP knock-in strategy (Scherrer et al. 2006). This is the first example of a G protein coupled receptor directly visible in vivo. This unique tool led us to visualize real-time trafficking of the functional fluorescent receptor in live neurons, and demonstrate the physiological relevance of receptor internalization in vivo for analgesic efficacy (Pradhan 2009) and the development of distinct forms of tolerance (Pradhan 2010). Our findings have both fundamental and therapeutic implications in pain and addiction research, as well as for GPCR biology and disease.

Keywords: G protein coupled receptors, opioids, genes, pain, addiction, mouse mutagenesis, behavior and pharmacology.

S-07

Conditional Functional Selectivity of β2-Adrenergic Receptor Ligands

Özlem Uğur, Ongun Onaran, Ali Kaya, Gülnihal Özcan, Sezen Ballı
Ankara University, Ankara, Turkey

Activation of β2-aAdrenegic Receptor (β2-AR) leads to an increase in intracellular cAMP and activation of Extracellular Signal-Regulated Kinase (ERK). The receptor activates these pathways by interacting different signalling partners. We showed that the intrinsic activities of β2-AR ligands for stimulating cAMP production and ERK phosphorylation responses in HEK cells were not correlated. The lack of correlation resulted mainly from the discrepancy between the intrinsic activities of 2 groups of ligands for these two responses: The first group consisted of clenbuterol, cimaterol, procaterol and terbutalin which acted as full agonists for cAMP production, but displayed very weak or null effect on ERK phosphorylation. The second group, on the other hand, comprised adrenalin, noradrenalin and dopamine which displayed higher intrinsic activity for the ERK phosphorylation than for the cAMP response. Thus, both groups behaved as functionally selective ligands. The functional selectivity of the first group was observable only in adhered cells when confluency was around 100%. When cell-cell contact was minimized either by decreasing the density of the adhered cells or by bringing the cells into suspension, the first group of ligands gained the ability to stimulate ERK phosphorylation without a change in their effect on cAMP production. In contrast, selectivity of the second group was independent from the adherence state of the cells. Our results show that the inherent "bias" of ligands in coupling a GPCR to different transducers may not always be revealed as a functional selectivity when there is a "cross-talk" between the signalling pathways activated by the same receptor.

Keywords: Beta adrenergic receptor, cAMP, extracellular signal regulated kinase, functional selectivity, cell adherence.

S-08

Translating the Pharmacology of the Poorly Characterised G Protein Coupled Receptor GPR35

Graeme Milligan
University of Glasgow, College of Medical, Veterinary and Life Sciences, UK

GPR35 is a poorly characterised G protein-coupled receptor (GPCR) that has been suggested as a potential therapeutic target for the treatment of conditions including diabetes, hypertension and asthma. Although two endogenously produced ligands have been suggested as activators of GPR35, the relevance of these remains unclear and they are not suitable to assess the role of this receptor in vivo. Recently, a series of surrogate agonist ligands and the first antagonists of GPR35 have been identified and tentative first steps taken to define the mode of binding of the ligands. However, marked differences in the potency of agonists at species orthologues of GPR35 presents substantial challenges in translating pharmacology at the cloned human receptor into ex vivo and in vivo studies of the physiological function of this receptor in animal models. Currently identified agonists are unlikely to display high selectivity for GPR35, further complicating understanding of the function of GPR35. By contrast, comparisons of the potency of ligands at species orthologues of GPR35 have provided insight into the nature of the ligand binding pocket and may could result in the identification of more potent and selective ligands.

Keywords: diabetes, asthma, inflammation, cardiovascular disease, GPCR, species orthologues, drug screening.

S-09

New Insights into GPCR Pharmacology from the Use of Fluorescent Ligands in Single Living Cells

Stephen J Hill
School of Biomedical Sciences, University of Nottingham, Nottingham, UK

The talk will focus on the development of fluorescent ligand technology at Nottingham. Examples will be given of the development of fluorescent agonists and antagonists for the adenosine A1 and A3 receptors and the various technologies that can be used to visualise their binding in single living cells. The technique of fluorescence correlation spectroscopy will be introduced as a technique to monitor different ligand-receptor complexes in plasma membrane microdomains. Recent work will be presented illustrating how fluorescent ligands can be used to study the kinetics of ligand-binding at the single cell level. This work will also provide evidence for receptor allosterism and dimerisation in single living cells expressing the human adenosine A3 receptor. Finally, we will present recent data that provide the first insights into the study of allosteric interactions at the single molecule level in single living cells.

Keywords: GPCRs, adenosine, fluorescence, allosterism.

S-10

Activators of G-Protein Signalling: Function, Regulation and Pharmacological Implications

Stephen M Lanier
Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, USA

Activators of G-protein signalling define three primary groups of proteins that regulate the biology of G-protein systems and were discovered in a functional screen designed to identify receptor independent activators of G-protein signalling systems. The three groups of AGS proteins include guanine nucleotide exchange factors, guanine nucleotide dissociation inhibitors and proteins that interact with Gβγ. The discovery of AGS proteins and related entities led to the realization that Gα and Gβγ may exist complexed with alternative binding partners independent of the classical Gαβγ heterotrimer and that Gα and Gβγ[?]are processing signals within the cell distinct from their role as transducers for cell surface GPCRs, including protein trafficking, mitotic spindle dynamics and organelle function.

AGS proteins and related entities play unexpected and important functional roles in a number of systems and impact a number of signalling pathways that influence system adaptation, cell growth and differentiation. Rapidly accumulating data from disease tissue profiling and genome-based technologies suggest that selected AGS proteins play a role in specific diseases and are thus candidate signalling modules for therapeutic targeting. AGS1 (RasD1), which is growth inhibitory, is consistently down-regulated in various cancers. Renal AGS3 (GPSM1) is markedly elevated in polycystic kidney disease and in response to renal injury. In cell culture, AGS3 traffics into the aggresome pathway and it also increases autophagy. AGS2 (Tctex1), AGS3 and AGS5 (GPSM2, LGN) are involved in cell differentiation. AGS7 (TRIP13) was identified as a thyroid receptor interacting protein and AGS8 (FNDC1) promotes apoptosis of cardiac myocytes.

Group II AGS proteins are of particular interest with each member of this group containing one to four G-protein Regulatory (GPR) motifs that interact with Gαi/o-GDP and/or Gαt-GDP. Thus, Gαi-GDP can complex with a GPR motif free of Gβγ and this complex is an apparent target for non-receptor GEFs in a manner similar to the regulation of Gαβγ by cell-surface GPCRs. Recent data suggest that the Gαi-GPR signalling complex is also regulated by cell-surface GPCRs providing an unexpected opportunity for signal integration.

The unexpected mechanisms for regulation of G-protein signalling systems that are operative for AGS proteins and the expanded functional roles for this important signalling system may provide new opportunities for both diagnostics and pharmacological intervention.

Keywords: Activators of G-protein signalling, AGS, G protein mediated signalling, GPCR.

S-11

Signalling Networks at the Alpha1-Adrenergic Receptors

Susanna Cotecchia[1], *Giovanni Busco*[1], *Damiano Del Vescovo*[1], *Luca Cariolato*[2], *Dario Diviani*[2]
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[2]*Département de Pharmacologie et de Toxicologie, University of Lausanne, Switzerland*

The alpha1-adrenergic receptor (AR) subtypes (alpha1a, alpha1b and alpha1d) mediate several physiological effects of epinephrine and norepinephrine. Studies in recombinant systems and insight from genetically modified mice have highlighted their important involvement in cardiovascular homeostasis. However, our understanding of the physiological specificity of the alpha1-AR subtypes and its biochemical basis is still limited. Constitutive activity and receptor oligomerization might differentially regulate their function. Recently, we have observed important differences between the alpha1a and alpha1b receptor subtypes in their interaction with βarrestins. To further elucidate the biochemical mechanisms underlying the functional specificity of the alpha1-AR subtypes, we investigated their ability to activate, on one hand, Rho signalling and, on the other, the MAP kinase pathway. This lead to the identification of a novel pathway involving AKAP-Lbc, an A-kinase anchoring protein (AKAP) with an intrinsic Rho-specific guanine nucleotide exchange factor activity. AKAP-Lbc is critical for activating RhoA and transducing hypertrophic signals downstream alpha1-ARs. AKAP-Lbc can organize a signalling network including RhoA, Rho-Kinase and the IKK complex that is required for the activation of NF-kB transcription factor. This complex might play an important role in the cardiac hypertrophic response induced by the alpha1-ARs.

Keywords: Adrenergic receptors, signalling.

Oral Presentations

O-01

Agonist-Induced Desensitization of mu-Opioid Receptors Depends on Their Subcellular Localization

Janet D Lowe, Chris P Bailey
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In the central nervous system, numerous different G-protein coupled receptors (GPCRs) are located both on cell bodies and on nerve terminals of neurons where they play a key role in neurotransmitter release. Agonist-induced desensitization of GPCRs is a critical determinant of receptor function, however, the vast majority of GPCR desensitization studies have focussed on GPCRs located at cell bodies, rather than those located at nerve terminals. In this study, we examined agonist-induced desensitization of mu-opioid receptors (MOPrs) located both at neuronal cell bodies and nerve terminals and found profound differences in their desensitization profile depending on their subcellular localization. Using whole-cell patch-clamp electrophysiological methods in mouse brain slice, we assessed agonist-induced desensitization of cell body and nerve terminal MOPrs expressed in GABAergic interneurons within the ventral tegmental area (VTA), a brain region implicated in the rewarding properties of numerous drugs of abuse. In common with many other neuronal cell-types, agonist-induced MOPr desensitization is readily seen at VTA cell bodies, where the DAMGO-induced response declined by approximately 50% during a 10-minute application. In contrast, we have consistently failed to observe agonist-induced MOPr desensitization at MOPrs located at nerve terminals even in the absence of receptor reserve, by using a range of different agonists, or by exogenous activation of protein kinase C. Studies are ongoing to determine the mechanism underlying this effect, but these data suggest that MOPrs located at nerve terminals do not readily desensitize, or do so by different mechanisms to those located at cell bodies. Funded by MRC (UK).

Keywords: Mu-opioid receptor, desensitization, electrophysiology, ventral tegmental area.

O-02

Human 5-HT1D Receptor Utilizes Different Galpha Subtypes for Coupling to Different Cellular Responses

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[2]Ankara Univ. School of Medicine Dept. Pharmacology and Clinical Pharmacology. Ankara, Turkey

We used PTX resistant Galphai2 and GalphaoA-B proteins with a single amino-acid mutation (C351I or C352I) to investigate the coupling mechanisms of 5-HT1D receptor to different cellular responses such as; ERK 1-2 phosphorylation, adenylate-cyclase (AC) inhibition and intra-cellular [Ca2+] increase in LTK-8 cells. We transfected LTK-8 cells (a cell line that stably expresses human 5-HT 1D receptors) with one of the three pertusis toxin (PTX) resistant human Galphai or Galphao protein subtypes. In LTK-8 cells it is known that 5-HT application induces an inhibition of AC, phoshorylation of ERK 1-2 protein and an increase in [Ca2+]i, all of these three responses are fully sensitive to PTX treatment. After an overnight incubation with PTX, as expected, untransfected LTK-8 cells completely lost all the three 5-HT reposes (ERK phosphorylation, AC inhibition and [Ca2+]i increase). However after PTX treatment LTK-8 cells transfected by PTX resistant GalphaoA or GalphaoB proteins, while completely losing the AC inhibition and [Ca2+]i increase responses, retained a large portion of the ERK phosphorylation response. In contrast PTX resistant Galphai2 protein transfected cells retained, almost all of the three 5-HT responses. These results may suggest that while human 5-HT 1D receptor utilizes Galphai protein subtypes for coupling to a wide range of responses, it uses Galphao subtypes for coupling only to ERK phosphorylation.

Keywords: 5-HT1D receptor, Galphai, Galphao, signal trafficking.

O-03

Fragment Screening at the Adenosine A3 Receptor Using a Novel Fluorescence-Based Live Cell Binding Assay

Leigh A Stoddart[1], Andrea J Vernall[2], Stephen J Briddon[1], Barrie Kellam[2], Stephen J Hill[1]
[1]Institute of Cell Signalling, School of Biomedical science, Queen's Medical Centre, University of Nottingham, United Kingdom
[2]School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham, UK.

In recent years there has been a move to develop competitive binding assays GPCRs that use a fluorescent ligand rather than the traditional radiolabelled ligand. Using the adenosine-A3 receptor as a model system, a live cell competition binding assay utilising a fluorescently labelled A3-antagonist (CA200645) in conjunction with automated image capture and analysis was developed. The affinity values of known adenosine receptor agonists and antagonists were determined and were in close agreement with pKd values obtained using functional assays (R2=0.94).
Fragment based drug discovery sets out to identify relatively weakly binding low molecular mass molecules, enabling more effective exploration of the chemical space within a ligand binding pocket. Fragment screening is most often carried out using biophysical methods such as x-ray crystallography, NMR and SPR. However, due to the instability of GPCRs outside of their membrane environment, few fragment screens have been untaken using a GPCR target. We investigated whether our fluorescence-based competition binding assay could be used to identify weakly binding fragment-like molecules by deconstructing a known adenosine-A3 receptor antagonist into two lower molecular weight fragments and subsequently determining the affinity of a focused library of 40 related analogues. The binding affinities of these fragments were widely spread and generally increased with increasing molecular mass. Most importantly there were fragments which stood out from this trend, with affinity values in the low μM range.

These results indicate that fragment based drug discovery is possible at GPCRs in their native membrane environment using a fluorescence based live cell binding assay.

Keywords: Fluorescent ligand, fragment screening.

O-04

Epidermal Growth Factor Receptor Transactivation Contributes to Acute Effects of α1-Adrenoceptor Stimulation in Rat Aorta and A7r5 Cells

Nadir Ulu[1], Hakan Gurdal[2], Sjoerd W Landheer[3], Marry Duin[3], Hendrik Buikema[3], Robert H Henning[3]
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[2]Department of Medical Pharmacology, Faculty of Medicine, University of Ankara, Ankara, Turkey
[3]Department of Clinical Pharmacology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands

In cultured vascular smooth muscle cells (VSMCs), transactivation of epidermal growth factor receptor (EGFR) by α1-adrenoceptors (α1-ARs) has been implicated as a major pathway involved in catecholamine induced VSMC hypertrophy. Whereas induction of VSMC hypertrophy represents a long-term process, requiring several days of stimulation in cell culture, it is currently unknown whether the acute effects of α1-AR stimulation on VSMC also involve transactivation of EGFR. Therefore, we investigated the role of EGFR transactivation in the acute effects of α1-AR stimulation in rat aorta and A7r5 cells.
Thoracic aorta was isolated from 12-14 week old male Wistar rats. In vitro aortic contractile responses to cumulative doses of phenylephrine (PE) were characterized in the absence and presence of the EGFR kinase inhibitors. Involvement of signal transduction pathways was also investigated. Phosphorylation of EGFR and extracellular signal-regulated kinase1/2 (ERK1/2) was measured after short-term PE or EGF stimulation both in aorta segments and A7r5 cells.
EGFR inhibition either by AG1478 or DAPH concentration dependently attenuated PE-induced contractile responses both in endothelium-intact and -denuded aortic rings. Inhibition of phosphatidyl inositol 3-kinase (PI3K) prevented the effect of AG1478 on the responses to PE. PE induced phosphorylation of EGFR and it was partially blocked by AG1478. PE also increased phosphorylation of ERK1/2, time-dependently and was blocked by AG1478 and wortmannin.
Our data demonstrate that EGFR transactivation significantly contributes to acute effects of α1-AR stimulation via a PI3K and ERK1/2 dependent pathway in rat aorta and A7r5 cells.

Keywords: α1-adrenoceptor, epidermal growth factor receptor, extracellular signal-regulated kinase1/2, phenylephrine, phosphatidyl inositol 3-kinase, transactivation.

O-04

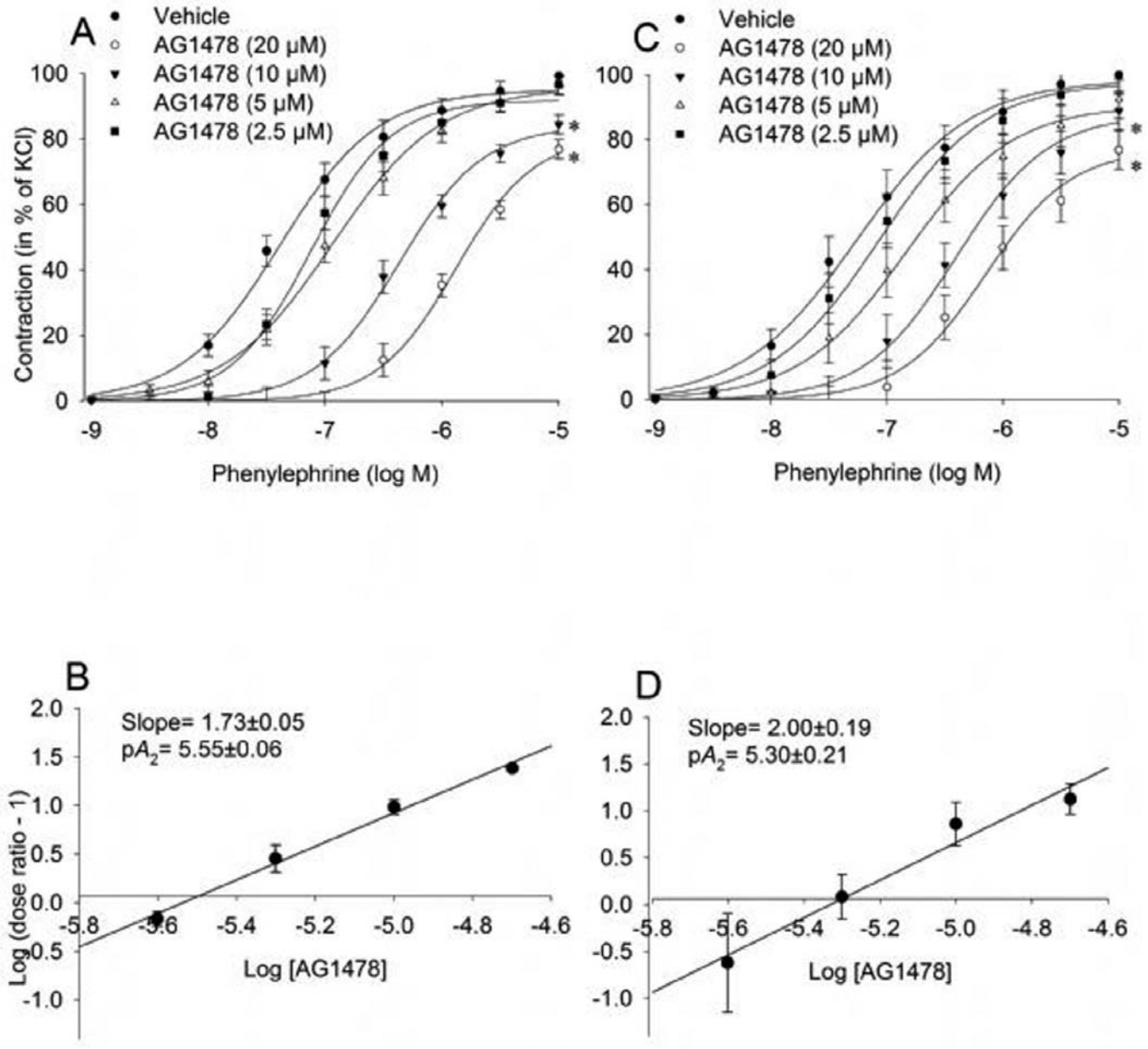

Figure 1. *Characterization of the inhibitory effect of the EGFR tyrosine kinase inhibitor on α1-AR mediated contraction. Characterization of the inhibitory effect of the EGFR tyrosine kinase inhibitor, AG1478, on α1-AR mediated contraction in rat thoracic aortic rings with intact (A, n=6) and denuded endothelium (C, n=6). Rings were pre-incubated with indicated concentrations of AG1478 (20 min) or vehicle (DMSO, 0.5% final concentration), prior to construction of cumulative concentration-response curves. Schild analysis was used to investigate the EGFR antagonism and calculated by plotting the log (dose ratio-1) against the log of the molar concentration of AG1478 (B, D). The slope was calculated both in endothelium-intact and -denuded rings and found to be significantly larger than unity. Data are expressed as mean±S.E.M. *P<0.05 versus vehicle curves (repeated measures ANOVA).*

Poster Presentations

P-01

Evaluation of Vitamin E in the Treatment of Erectile Dysfunction in Aged Rats

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The present study was designed to further elucidate the role of oxidative stress in erectile dysfunction associated with aging and to investigate the effect of 21-days treatment with vitamin E in this respect. Rats were divided into four groups: young (3-month-old, given peanut oil/day), aged rats (18-month-old, given peanut/day), aged rats given 80 IU of vitamin E/rat/day, aged rats given 5 mg/kg of sildenafil/day. Intracavernosal pressure/mean arterial pressure (ICP/MAP), nitric oxide production, TBARS, GSH levels and SOD activity in corpus cavernosum were measured. Significant decrease in ICP/MAP was observed in aged rats at both low and high frequency of stimulation. Significant increase in ICP/MAP was observed in aged rats treated with vitamin E over the range of 0.8 to 5 Hz but young control values were not restored. Percentage potentiation of ICP/MAP than aged group at 0.8 Hz was 326±41.3% and 897±72.2% for vitamin E and sildenafil respectively. Decreased levels of NO2/NO3 and SOD activity in the penile tissue observed with aging were elevated back to control by either vitamin E or sildenafil treatment. Penile concentration of TBARS was 20.86±0.83 for aged rats vs. 11.39±0.79 nmol/g tissue for young controls. Both Vitamin E and sildenafil reduced penile TBARS in aged rats. This study proves that antioxidant therapy with vitamin E ameliorates the age-associated erectile dysfunction. Sildenafil may exert some antioxidant properties which add to the advantages of its use on chronic basis. The effect of combinations of low-dose sildenafil and vitamin E on age-associated erectile dysfunction merits to be studied.

Keywords: Vitamin E, oxidative stress, sildenafil, aging, erectile dysfunction.

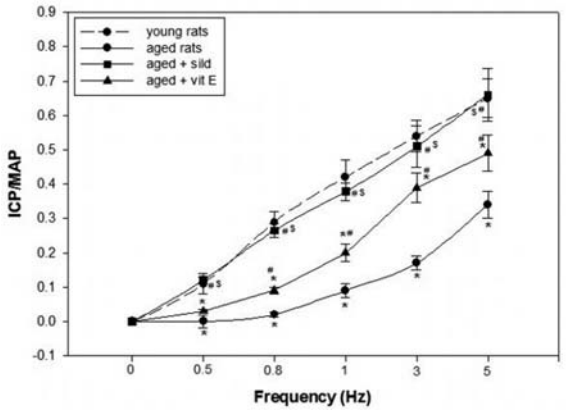

Figure 1. *Effect of sildenafil or vitamin E treatment on intracavernosal pressure/mean arterial pressure (ICP/MAP) in aged rats in comparison to young adult rats. Drugs were administered orally for 21-days. *denotes significant difference thanyoung adult group. # denotes significant difference thanaged group. $ denotes significant difference than vitamin E group. Significance was set at P < 0.05.*

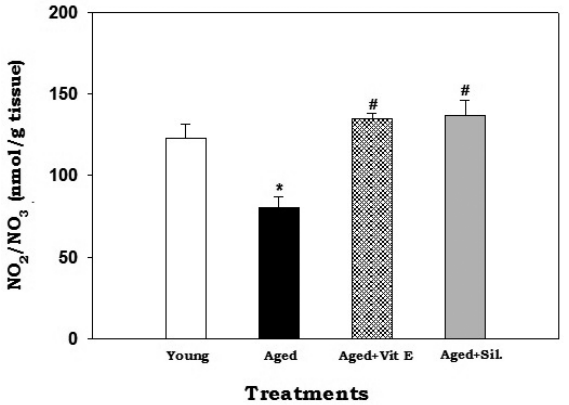

Figure 2. *Effect of vitamin E or sildenafil administration on age-induced changes in penile tissue NO2/NO3 levels. Drugs were administered orally for 21-days. *P < 0.05 compared with the young group. #P < 0.05 compared with the aged group.*

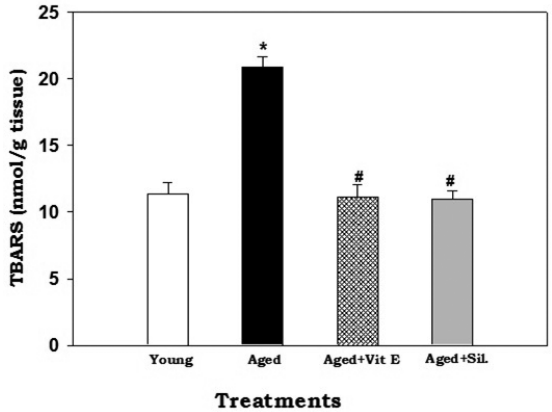

Figure 3. *Effect of vitamin E or sildenafil administration on age-induced changes in penile tissue TBARS. Drugs were administered orally for 21-days. *P < 0.05 compared with the young group. #P < 0.05 compared with the aged group.*

P-01

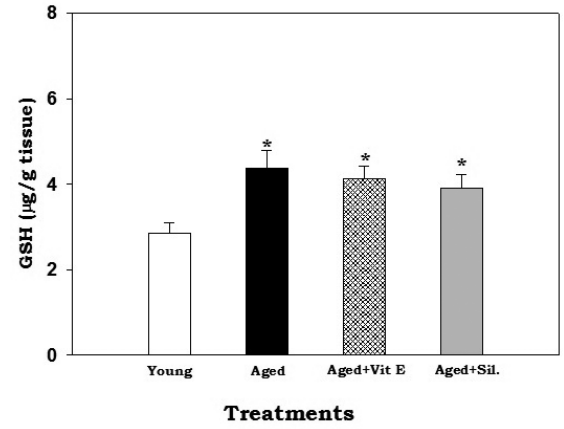

Figure 4. *Effect of vitamin E or sildenafil administration on age-induced changes in penile tissue GSH levels. Drugs were administered orally for 21-days. *P < 0.05 compared with the young group.*

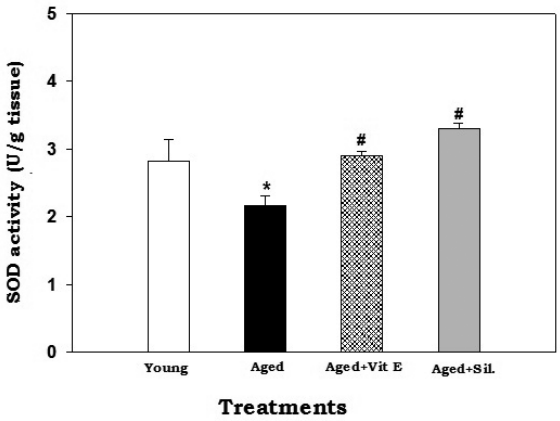

Figure 5. *Effect of vitamin E or sildenafil administration on age-induced changes in penile tissue SOD activity. Drugs were administered orally for 21-days. *P < 0.05 compared with the young group. #P < 0.05 compared with the aged group*

Table 1.

Group	Young	Aged
Brain MDA (nmol/g tissue)	37.31±2.41	39.27±0.99
Brain GSH (µg/g tissue)	74.12±3.75	67.83±3.83
Brain SOD (U/g tissue)	21.12±1.23	23.12±0.98
Brain NO2/NO3 (nmol/g tissue)	133.46±8.55	131.33±6.87
Plasma NO2/NO3 (nmol/ml)	72.84±3.97	76.40±6.84

Profile of brain biochemistry and plasma nitrite/nitrate level (Values are expressed as mean±SEM of 7 experiments)

P-02

Effect of a-Tocopherol or Simvastatin Treatment on Pro-Oxidant Antioxidant Balance in the Liver of Aged Rats

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The effect of α-tocopherol or simvastatin treatment on antioxidant defense in liver of old rats was investigated. Endogenous thiobarbituric acid reactive substances (TBARS) and total nitrite/nitrate (NO2/NO3) levels as well as non-enzymatic glutathione (GSH) and enzymatic antioxidants [glutathione-S-transferase (GST), superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) activities] were determined in livers of young (3 months), aged (22 months), α-tocopherol- or simvastatin-treated aged rats. Serum lipid profile and liver functions were also assessed in the 4 groups. Both α-tocopherol and simvastatin almost equally restored liver TBARS and CAT activity, serum aspartate aminotransferase (GOT), alanine aminotransferase (GPT) and alkaline phosphatase (ALP) back to control values. α-tocopherol, but not simvastatin, tended to restore GST and GPX activities in livers of aged rats. Simvastatin, on the other hand, counteracted the age-induced increases in serum cholesterol, TG, LDL, total hepatic NO2/NO3 level, and preserved a normal liver function during aging. Thus, either drug may be beneficial, in spite of a mechanistic difference in the antioxidant effect of both of them, in decreasing the age-induced liver injury.

Keywords: Liver dysfunction, Aging liver, Simvastatin,α-tocopherol, Antioxidant enzymes, Reactive oxygen species

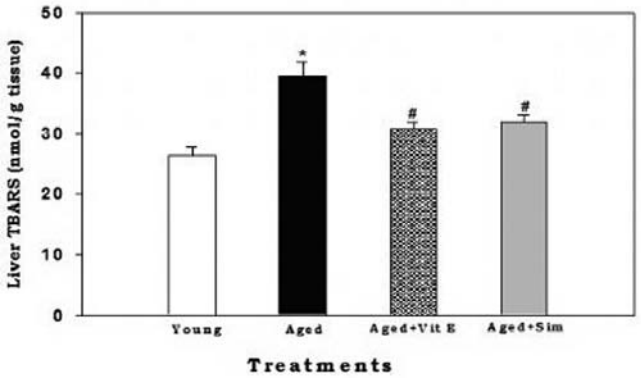

Figure 1. *Effect of α-tocopherol or simvastatin treatment on the age-induced changes in liver TBARS. Drugs were administered orally for 6-weeks. *P < 0.05 compared with the young group. #P < 0.05 compared with the aged group.*

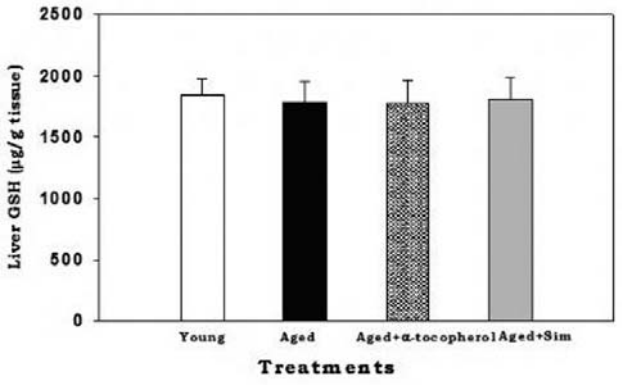

Figure 2. *Effect of α-tocopherol or simvastatin treatment on the age-induced changes in liver GSH content. Drugs were administered orally for 6-weeks. *P < 0.05 compared with the young group. #P < 0.05 compared with the aged group.*

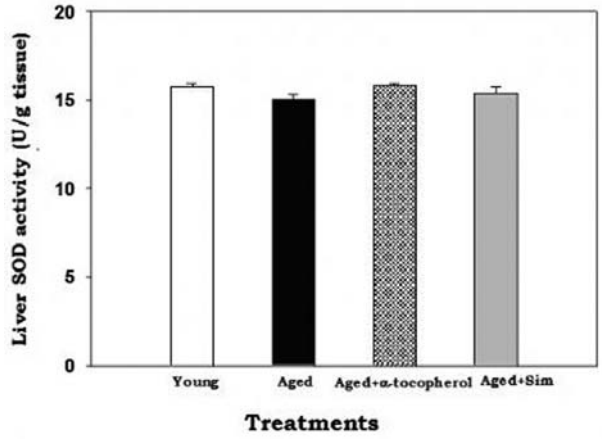

Figure 3. *Effect of α-tocopherol or simvastatin treatment on the age-induced changes in liver GST activity. Drugs were administered orally for 6-weeks. *P < 0.05 compared with the young group. #P < 0.05 compared with the aged group. +P < 0.05 compared with the aged+α-tocopherol group.*

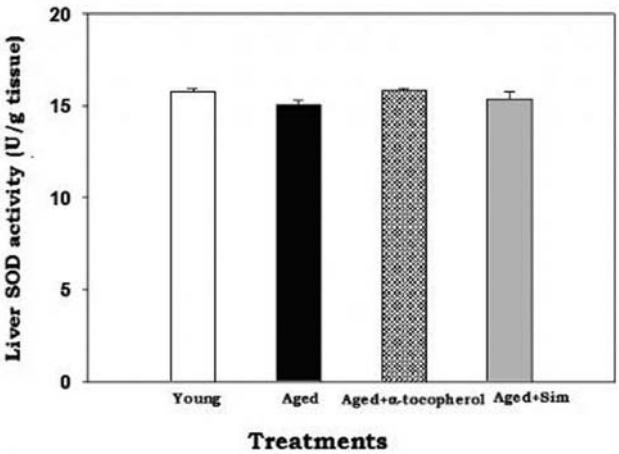

Figure 4. *Effect of α-tocopherol or simvastatin treatment on the age-induced changes in liver SOD activity. Drugs were administered orally for 6-weeks.*

P-02

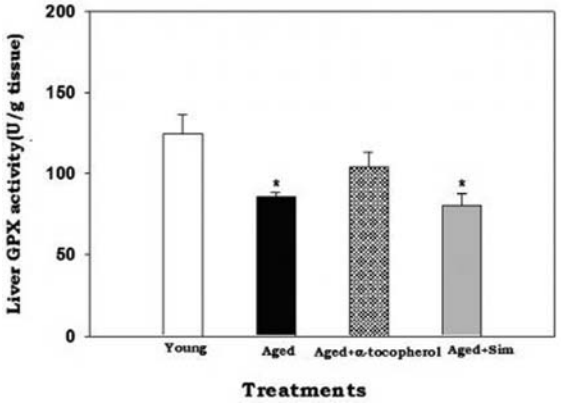

Figure 5. *Effect of α-tocopherol or simvastatin treatment on the age-induced changes in liver GPX activity. Drugs were administered orally for 6-weeks. *P < 0.05 compared with the young group.*

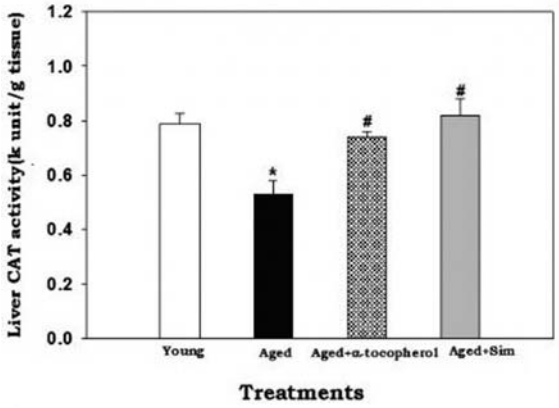

Figure 6. *Effect of α-tocopherol or simvastatin treatment on the age-induced changes in liver CAT activity. Drugs were administered orally for 6-weeks. *P < 0.05 compared with the young group. #P < 0.05 compared with the aged group.*

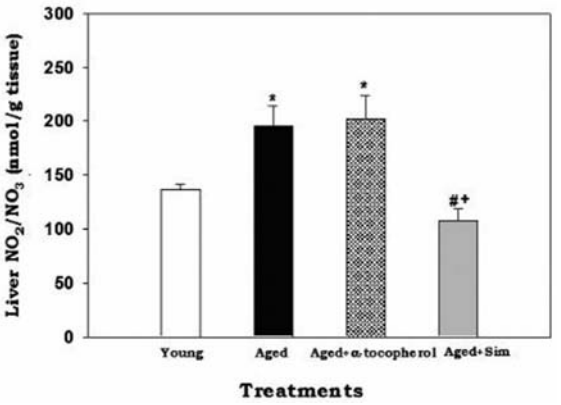

Figure 7. *Effect of α-tocopherol or simvastatin treatment on the age-induced changes in liver NO2/NO3 level. Drugs were administered orally for 6-weeks. *P < 0.05 compared with the young group. #P < 0.05 compared with the aged group. +P < 0.05 compared with the aged+α-tocopherol group.*

Table 1. *Effects of α-tocopherol or simvastatin treatment on the age-dependent changes in the serum levels of cholesterol, triglycerides (TG), low density lipoproteins (LDL) and high density lipoproteins (HDL).*

	Cholesterol (mg/dl)	TG (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
Young	29.28±1.96	17.62±1.62	18.27±1.92	5.02±0.12
Aged	52.86±4.24*	33.68±3.38*	34.02±2.65*	5.80±0.48
Aged+αf-tocopherol	49.52±3.39*	30.40±1.95*	21.21±2.04*	4.99±0.37
Aged+simvastatin	37.02±2.01#+	21.21±2.04#+	36.87±1.99#+	5.20±0.47

*Data are mean±SEM of 7 experiments. *P < 0.05 vs. the young group. #P < 0.05 vs. the aged group. +P < 0.05 vs. the aged+α-tocopherol group.*

Table 2. *Effects of α-tocopherol or simvastatin treatment on the age-dependent changes in the serum levels of aspartate aminotransferase (GOT), alanine aminotransferase (GPT), alkaline phosphatase (ALP), total bilirubin (Tbil), and α-glutamyltra*

	Young	Aged	Aged+α-tocopherol	Aged+simvastatin
GOT (IU/L)	120.16±8.47	192.78±9.78*	137.25±12.41#	128.70±10.88#
GPT(IU/L)	54.70±3.81	85.40±4.12*	60.60±3.07#	61.20±2.14#
ALP(IU/L)	145.30±5.70	187.98±9.31*	148.50±4.67#	150.41±8.77#
Tbil (mg/dl)	0.187±0.013	0.21±0.016	0.178±0.015	0.198±0.018
γ-GT(IU/L)	1.74±0.084	1.66±0.091	1.58±0.110	1.81±0.084

*Data are mean±SEM of 7 experiments. *P < 0.05 vs. the young group. #P < 0.05 vs. the aged group.*

P-03

Comparison of the Effects of Adrenaline Through β2-Adrenoceptors and Their Control By Phosphodiesterases PDE3 And PDE4 in 3 Regions of Juvenile Rabbit Hearts

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Barbuti et al. 2007 (J.Mol.Cell.Cardiol 42:71-78) proposed that stimulation of β2-adrenoceptors (β2AR) is the main mechanism, compared to β1-AR, by which heart rate is modulated through pacemaker channels. However, it is unknown whether β2-AR can mediate changes in cardiac contractility. We compared the (-)-adrenaline-evoked increases of contractile force in paced left atrial preparations and right ventricular papillary muscles and sinoatrial beating in right atria from juvenile rabbits (age 20-60 days) [exposed 90 min to phenoxybenzamine (5μM), β1-AR-selective antagonist CGP20712A (300nM) present]. Concentration-effect curves for (-)-adrenaline in the absence and presence of phosphodiesterase inhibitors revealed inconstant CGP20712A-resistant (β2-AR-mediated) components in right atria and papillary muscles but not left atria. Cilostamide (300nM) and rolipram (10μfM) were used to inhibit PDE3 and PDE4 respectively. Cilostamide and rolipram+cilostamide increased sinoatrial rate by 11±2 ($P<0.005$) and 34±5 ($P<0.002$) beats.min-1 respectively, but left ventricular force unaltered. The tachycardia to (-)-adrenaline was only 13±2% of the tachycardia to 200μM (-)-isoprenaline (ISO) in the absence of PDE inhibitors. The PDE inhibitors did not significantly change the –logEC50s of (-)-adrenaline but rolipram increased the chronotropic efficacy and rolipram+cilostamide increased the inotropic efficacy.

Conclusions: PDE3 and PDE4 suppress the β2-AR-mediated ventricular inotropic efficacy of (-)-adrenaline. PDE4 attenuates the (-)-adrenaline-evoked sinoatrial tachycardia mediated through β2AR.

Keywords: rabbit, heart, phosphodiesterases, (-)-adrenaline, β2-adrenoceptors.

Maximum responses to (-)-adrenaline mediated through β2-AR

	Control	Cilostamide	Rolipram	Rolipram+Cilostamide
Papillary Muscle				
Responders (n)	7/16	5/13	3/7	7/8
Δforce (%ISO)	9±4	14±2	21±6	42±13*
Sinoatrial node				
Responders (n)	7/9	7/8	7/8	6/7
Δbeats.min-1	18±5	24±6	76±12#	65±11#

**P<0.05, #P<0.002 vs control; n=number of rabbits*

P-04

Studying Ligand-Receptor Interactions at the Human β1-Adrenoceptor Using Two Fluorescent β-Adrenoceptor Ligands

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Recent evidence points to a second ligand binding site on the human β1-adrenoceptor, at which the purported antagonist CGP12177 exerts agonist activity. This low affinity "CGP12177" binding site is largely resistant to classical β-blockers such as propranolol. The use of fluorescent ligands will allow the investigation of receptor-ligand interactions at this binding site of the β1-adrenoceptor at the single cell level. In this study, we have characterised the binding of two fluorescent β-adrenoceptor ligands to the human β1-adrenoceptor: BODIPY-TMR-CGP12177 (BY-CGP) and BODIPY630/650-S-PEG8-propranolol, (BY-PROP). Functional responses were investigated in a CRE-SPAP gene reporter assay. Like CGP12177, BY-CGP stimulated an increase in CRE-mediated gene transcription. Both, propranolol and BY-PROP, acted as antagonists in this assay. Affinity values determined by Schild analysis showed reduced affinity of both fluorescent ligands for the β1-adrenoceptor compared to their respective parent compounds. However, both ligands allowed visualisation of receptor distribution, as confocal imaging showed clear concentration-dependent membrane labelling of CHO-β1-CRE-SPAP cells following incubation with BY-CGP or BY-PROP (3-100nM, 10min), which could be displaced by the β-adrenoceptor antagonist CGP20712 (100nM). Using these fluorescent ligands, affinity values of unlabelled compounds can also be obtained in a high-content-screening assay. Furthermore, kinetic parameters of the fluorescent ligands at the β1-adrenoceptor can be determined at the single cell level using confocal microscopy in conjunction with a novel perfusion system. As such, the two fluorescent β1-adrenoceptor ligands described here are suitable for imaging studies to investigate the kinetics of ligand-receptor interactions in single living cells.
This work was funded by the MRC.

Keywords: β1-adrenoceptor, fluorescent ligands, confocal microscopy.

P-05

Investigating the Interaction Between Arrestins and the Incretin Hormones

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Introduction: The incretin effect is mediated by two gut hormones; glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1. Both GIP and GLP-1 potentiate insulin secretion in a glucose dependent manner by binding to their respective receptors on the pancreatice beta-cell. The GIP and GLP-1 receptors are both Family B (Secretin) G protein-coupled receptors (GPCRs). In type 2 diabetes mellitus (T2DM) GIP loses its ability ability to potentiate insulin secretion so focus has shifted to the GLP-1 receptor (GLP-1R) as a target to treat T2DM. In this study the interaction between arrestin and both the GIP receptor (GIPR) and GLP-1R is investigated with aim to shed light on why type 2 diabetics remain responsive to GLP-1 but not GIP.

Methods: Arrestin recruitment to either GIPR or GLP-1R was measured using the PathHunter™ eXpress Kit (DiscoveRx Corporation Ltd., UK). Briefly, the kit detects the interaction of arrestin and activated receptor using enzyme fragment complementation.

Results: GLP-1 and GIP stimulated arrestin recruitment to their receptors with a pEC50 value of 8.2 (±0.14) and 8.1 (±0.27) respectively (means ± SEM, n=3). However the most striking finding was that GIPR displayed a significantly ($P<0.05$) higher basal binding to arrestin than GLP-1R (75% compared to 39%), when normalised to maximum arrestin binding.

Conclusion: GIPR displays significanlty higher basal binding to arrestin than GLP-1R when normalised to maximum arrestin binding. This may be due to GIPR's constitutive activity and may contribute to the loss of function of GIP in T2DM.

Keywords: Arrestin GLP-1R GIPR Incretin.

P-06

C-Src-Mediated Activation of G-Protein-Coupled Receptor Kinase 2 is Receptor-Specific

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G-protein-coupled receptor kinases (GRKs) catalyse the first step in the homologous desensitisation of G-protein-coupled receptors, namely phosphorylation of the agonist-activated receptor. There are seven GRKs, four of which (GRK2, GRK3, GRK5 and GRK6) show broad tissue distribution. The activities of GRKs may be regulated by other kinases and by interaction with other proteins. Here, we investigated the regulation of GRKs by the protein tyrosine kinase c-src. GRK activity was measured in transiently transfected HEK293 cells as FRET increase between YFP-labelled G-protein-coupled receptors (the beta(2)-adrenergic and the mu-opioid receptor) and CFP-labelled arrestins. Co-transfection of c-src decreased GRK2 steady state levels in HEK293 cells slightly but nevertheless accelerated arrestin recruitment to the beta(2)-adrenergic receptor two-fold. This effect was blocked by the c-src inhibitor, PP2, and required GRK2 residues Tyr86 and Tyr92. In contrast, co-transfection of c-src had no effect on GRK3- or GRK5-mediated arrestin recruitment to the beta(2)-adrenergic receptor. GRK6 transfection resulted in apparently constitutive beta(2)-adrenergic receptor phosphorylation under our conditions. Surprisingly, c-src had no effect on arrestin recruitment to the mu-opioid receptor, indicating receptor specificity of c-src-mediated GRK2 regulation. Mutation of Tyr350 in the beta(2)-adrenergic receptor to Phe abolished the effect of c-src on GRK2 activity. Our data show that the regulation of GRK2 by c-src is receptor-specific.

Keywords: Fluorescence resonance energy transfer, desensitisation.

P-07

Estrogen Modulator Effects of Resveratrol on Female Rats

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Resveratrol is known a phytoestrogen and has the ability to bind to estrogen receptors (ERs) and evoke biological effects that parallel those exerted by endogenous and synthetic estrogens. There are some discrepancies in the estrogenic activity of resveratrol. In the present study, the estrogenic effects of resveratrol were investigated in immature (21 days old) female rats. The activities of resveratrol were evaluated by using several parameters in the tissues which have estrogenic receptors. These parameters are the rates of uterine weight/body weight (UW/BW), uterine dry weight/uterine wet weight (UDW/UWW), the increase in ratio of body weight, and blood estradiol levels. The uteri were also evaluated histologically.
It was found that resveratrol increased the ratio of UW/BW as much as those of 17 alpha ethynyl estradiol (17αEE). The least significant activity was obtained with 10 mg/kg resveratrol (RES10). This activity was not decreased by tamoxifen but was significantly decreased by fulvestrant. In regard to the levels of estradiol, it was noticed that resveratrol decreased this parameter at 20 mg/kg dose. In conclusion, the dose of resveratrol may play an imortant role in its estrogenic activity. It can be thought that resveratrol may have an estrogenic modulatory activity.

Keywords: Estradiol, estrogen, fulvestrant, resveratrol selective estrogen receptor modulators (SERM), tamoxifen.

P-08

Antinociceptive Activity of Methanol Extract of Hyoscyamus Reticulatus L. in Mice

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Purpose: In the present study acute toxicity and possible antinociceptive effect of the metanolic extract of Hyoscyamus reticulatus L. was investigated in Swiss - albino mice of either sex.
Material-Methods: To assess Hyoscyamus reticulatus L. extract's acute toxicity it was given intraperitonelly to the mice (6 in each group) in dose 50, 100, 200, 400, 800, 1200 and 1600 mg/kg body weight. After probit analyze of mortality at 24, 48 and 72h after administration, 25, 50 and 100 mg/kg were determinate as safe doses for antinociceptive evaluation. Two models were used to study the effects of the extracts on nociception, acetic acid-induced writhing test and hot plate test in mice. Hyoscyamus reticulatus L. extract was administered intraperitoneally 30 minutes prior to pain induction.
Results: The metanolic extract in 50 mg/kg dose showed significant ($p<0.05$) analgesic activity comparable with diclophenac sodium, evidenced by increase in the reaction time by hot plate method and significant ($p<0.05$) reduction in acetic acid - induced writhings in mice in 100mg/kg dose with a maximum effect of 35.56 % reduction. These effects were compared with the control and standard drug, diclophenac sodium (50 mg/kg, p.o).
Conclusion: The results indicate that metanolic extract of Hyoscyamus reticulatus L. possesses a significant antinociceptive activity in both central and peripheral pain models in mice and therefore, it can be used as supplemental therapy in acute or chronic pain conditions.

Keywords: Antinociception, hot plate, Hyoscyamus reticulatus L., methanol extract, writhing test.

P-09

Ligand Efficacy and Ligand-Induced Conformational Changes in β-AR: A Stochastic Interpretation

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Ligand efficacy, defined as the ability of a ligand to change the "functional state" of receptor, is generally believed to be associated with ligand-induced conformational changes in the receptor. Such changes should eventually mediate an allosteric linkage between ligand binding site and distant functional domains of the receptor. Hence, one should be able to tell the structural basis of efficacy by comparing the structures of GPCRs in their agonist- or antagonist-bound forms. However, recent structural data surprisingly showed that agonist binding did not result in any significant conformational change in β adrenoceptors. Here, we therefore searched alternative mechanisms by which an observable efficacy can be generated without an observable ligand-induced conformational change in the receptor. We used a probabilistic model of allosteric linkage for the present analysis. The model is based on the fact that a protein molecule can assume a large number of conformational substates, and that ligand binding can modify the distribution of these substates. We found that 1) an allosteric linkage (as a measure of molecular efficacy) can indeed emerge without any ligand-induced change in the receptor on average; 2) in such cases, contribution of improbable substates of the receptor is the major determinant of the observed linkage; and 3) a considerable change in the receptor occurs only when the two allosteric partners are bound simultaneously (as observed in the case of β2-AR). We believe that the present approach may be of help to guide alternative experimental strategies to reveal molecular mechanisms of efficacy.

Keywords: Efficacy, allosteric linkage, conformational change, GPCR.

P-10

Functional Analysis of Some Designed Point-Mutations in Poorly Conserved Sites in Gi1α

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Based on a correlated mutation analysis in Gα family, and a cooperativity analysis in the native state ensemble of Gi1α, we chose to mutate 10 moderately or poorly conserved sites in Gi1α (6 in HD, 4 in RD) that are at least 5 Å away from the bound nucleotide, and possibly involved in the allosteric communication network of Gα. We evaluated a total of 21 mutations, along with the WT, in terms of their effects on basal or ALF-induced tryptophan fluorescence, basal GTPase activity, and the effect of DTT on these parameters in human Gi1α (expressed in e coli and purified by using a subtilisin-based affinity chromatography). We sought for non-trivial correlations in mutation effects, that could be considered as a signature of linkage among mutational perturbations. Indeed, we found a strong correlation between mutational effect on GTPase activity and sensitivity of mutational effect on ALF-induced Trp-fluorescence to DTT. Among these sites, V72 in the helical domain was particularly interesting, as it has been shown to be involved in AGS or RGS contact with Gα. Indeed, its mutations abolished the binding of purified AGS3-GPR to Gi1α, and thus the ability of AGS3-GPR to inhibit ALF-induced Giα activation at variable extent. Among all the mutations tested here, the V72D mutation had also the strongest effect on the basal GTPase activity of Gi1α. Therefore, we speculated that the site V72 may be critically involved in the allosteric mediation of AGS (or RGS) effect on Gα activation or GTPase activity.

Keywords: G protein alpha subunit, allosteric communication, GTPase, intrinsic tryptophan fluorescence, AGS3.

P-11

Prejunctional Facilitatory Effect of a Thiol-Alkylating Agent N-Ethylmaleimide on Neurogenic Contractions in Rat Prostate Smooth Muscle

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The effect of a non-specific thiol-alkylating agent N-ethylmaleimide was studied on neurogenic contractions induced by trains of electrical field stimulation (EFS) in prostate smooth muscle preparations from Wistar rats.
N-ethylmaleimide (100 μM) enhanced the EFS-evoked contractions without altering the basal tone. These effects were significantly suppresssed by an α1-adrenergic receptor antagonist (prazosin; 1 μM), a P2 purinergic antagonist (suramin; 100 μM), a specific P2X receptor antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS; 200 μM), an ATP analogue (alfa,beta-methylene ATP; 20 μM), or a calcium channel blocker (verapamil; 10 μM). This facilitating effect of N-ethylmaleimide did not occur following the administration of L-cysteine (1 mM) or glutathione (1 mM) which saturated N-ethylmaleimide with excess thiols. However, a thiol oxidant diamide failed to affect the contractions to EFS. An adrenergic neuron blocker (guanethidine) completely suppressed the responses to N-ethylmaleimide. On the other hand, an α2-adrenergic receptor blocker (yohimbine; 1-10 μM), a nitric oxide synthase inhibitor (N -nitro-L-arginine; 100 μM) or a cholinergic muscarinic receptor antagonist (atropine; 1 μM) did not significantly affect the facilitatory response of N-ethylmaleimide.
These findings suggest that N-ethylmaleimide has a prejunctional facilitatory action on the adrenergic nerves in rat prostate tissue to enhanced release of transmitters, noradrenaline and ATP. N-ethylmaleimide sensitive proteins involved in transmitter release mechanisms can play a role in this effect.

Keywords: Adrenergic nerves, N-ethylmaleimide, prostate smooth muscle, purinergic nerves.

P-12

The Subcellular Mechanism of Contractile Responses to Carbachol in the Bladder

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In this study we investigated the role of intracellular Ca2+ on contractile responses of cholinergic agonist carbachol (0.5 micromolar) in isolated detrusor muscle strips of the mouse urinary bladder. Ruthenium red (10-100 micromolar), an inhibitor of ryanodine receptors (intracellular Ca2+-channels), significantly inhibited the contractile responses of carbachol in a concentration-dependent manner. Similarly, Ca2+-free medium dramatically decreased these contractions. In addition, the combination of ruthenium red (10 micromolar) and Ca2+-free medium potentiated the inhibitory effect of Ca2+-free medium in isolated detrusor muscle strips of mice.
The exprimental results suggest that intracellular Ca2+ may have a role on contraction induced by carbachol in the detrusor muscle of the mouse urinary bladder.

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Keywords: Carbachol, intracellular Ca2+, mouse urinary bladder.

P-13

The Role of Nitrergic System on Contraction Induced by Caffeine

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In the present study, we evaluated the role of nitrergic system on contractile responses induced by caffeine (10 micromolar), intracellular Ca2+-releasing agent, in the detrusor muscle strips of the mouse urinary bladder. S-nitroso-L-cysteine (100 micromolar), the nitric oxide donor, significantly potentiated the caffeine-induced contraction. In contrast, ruthenium red (100 micromolar), an inhibitor of ryanodine receptors (intracellular Ca2+-channels), significantly inhibited the effect of S-nitroso-L-cysteine. In addition, pyrogallol (100 micromolar), a generator of reactive oxygen species, significantly decreased the contractile responses to caffeine in the related tissue. Similarly, Nω-nitro-L-arginine (100 micromolar), nitric oxide synthase inhibitor, caused a dramatic inhibition of the contractile responses. In addition, hemoglobin (100 micromolar), which binds nitric oxide, significantly decreased the contractions induced by caffeine in the detrusor muscle strips of the mouse urinary bladder. On the other hand, sodium nitroprusside (10 micromolar), the nitric oxide donor, increased the contractile response of caffeine but this increment was not statistically significant.
The exprimental results suggest that the nitrergic system may have a role on contraction induced by caffeine in the detrusor muscle of the mouse urinary bladder.

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Keywords: Caffeine, nitrergic system, urinary bladder.

P-14

In Vivo Detection of Heterodimerization of Two G-Protein Coupled Receptors: Dopamine D2R and Adenosine A2AR with FRET Method

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Recent studies demonstrate that there are several G-protein coupled receptors (GPCRs) that dimerize with other GPCRs and form heterodimers. Adenosine A2A-Dopamine D2 receptor interaction is one of the examples for GPCR heterodimerization. Both receptors bear very critical roles in physiological processes. Adenosine A2A receptor has functions in neurotransmission, cardiovascular system and immune response through its anti-inflammatory roles. On the other hand, dopamine receptors are the key point of dopaminergic system, which controls the regulation of memory, attention, food intake, endocrine regulation, psychomotor activity and positive reinforcement. Dysregulation in dopamine signalling could cause neurological disorders like Parkinson's disease and schizophrenia. Dopamine D2R and adenosine A2AR have been shown to interact in striatum and modulate dopaminergic activity. The purpose of the project is to investigate direct physical interaction between D2R and A2AR by tagging them with EGFP (enhanced green fluorescent protein) and mCherry (a fluorescent protein excited in red color range) to observe Förster Resonance Energy Transfer (FRET) between these receptors in N2a cell line using confocal microscopy.

Keywords: Dopamine, D2R, Adenosine, A2AR, Heterodimerization, GPCR, FRET.

P-15

Functional Expression of Potassium Channels in Cardiomyocytes Derived from Embryonic Stem Cells

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Royan B1-MHC-GFP stem cells are differentiating to cardiomyocytes (1). This developmental change is accompanied with a great deal in ion channel expression and functions (2). Mouse stem cell derived cardiomyoctes (ESCs) were prepared as described by Dr Baharvand (1). In this research we have investigated function expression of K current in ESCs using whole cell patch-clamp technique. The bath solution included 130mM NaCl and 1.5mM CaCl2. The intracellular pipette solution included 130mM KCl, 3mM ATP and 0.2mM EGTA unless otherwise stated. Drugs were applied via the bath solution.
When the holding potential (HP) was -60mV, a major outward current was elicited by square depolarizing pulses from -60mv to +50mV. This sustained outward K current was inhibited by tetraethylammonium (TEA 10mM), partially blocked by nifedipine (1μM) and attenuated by increasing concentration of EGTA (10 mM) in the pipette solution indicating activity a type of Ca2+ activated K channel in these cells.
In addition there was another kind of sustained outward K+ current which was resistance to TEA but was inhibited by 3,4-diaminopyridine (1mM), probably due to activation of delayed rectifier K+ channels. Undifferentiated stem cells had no voltage activated current. RT-PCR techniques confirmed expression of large conductance Ca2+ activated K channels and delayed rectifier K channels in these cells. Therefore, present study shows functional expression of two types K+ ionic currents in Royan B1 stem cell derived cardiomyocytes.
1- Baharvand H. et al., BBRC, 349 (2006) 1041-1049.
2- Boheler KR. et al., Circ. Res. 91 (2002) 189-201.

Keywords: Embryonic stem cells, Royan B1 line, Cardiomyocytes, K+ current, Tetraethylammonium, Patch-clamp, 3, 4-diaminopyridine, RT-PCR.

P-16

Dissecting the binding pocket of the imidazolylpyrimidine CXCR2 antagonists:

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The chemokine receptor CXCR2 is involved in different inflammatory diseases, like chronic obstructive pulmonary disease, psoriasis, rheumatoid arthritis and ulcerative colitis and therefore considered an attractive drug target. Different classes of small CXCR2 antagonists have been developed. Recently, we have pharmacologically characterized the distinct binding sites of low molecular-weight synthetic compounds at the chemokine receptor CXCR2 (de Kruijf et al., 2009 JPET 329:783-790). In these studies we further dissected the binding pocket of the imidazolylpyrimidine CXCR2 antagonists by making use of CXCR2 orthologs that show high homology to one another. We found that these orthologs show differential affinity for the imidazolylpyrimidine antagonist (compound I). Chimeras containing the human and baboon CXCR2 sequences were useful to localize the domains of the receptor that determine affinity of compound I for CXCR2. Furthermore, using site-directed mutagenesis and in silico modeling we propose two different binding modes of compound 1 at human CXCR2 in the trans-membrane region. These results open new possibilities in the structure-based design of allosteric modulators of CXCR2.

Keywords: GPCR, CXCR2, allosteric antagonist.

Notes