175P INHIBITORS OF PROTEIN KINASE C: THEIR BIOLOGY AND PHARMACOLOGY

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The structural lead provided by staurosporine has been used as the basis for the design of a series of substituted bis-indolylmaleimides with much improved selectivity for protein kinase C (PKC) over that shown by staurosporine.

The advent of these inhibitors has led to a reappraisal of the wide ranging role that PKC was thought to play in signal transduction processes. A number of lines of evidence suggest that PKC plays an important role in antigen-driven T-cell proliferation, and potent bisindolylmaleimide PKC inhibitors such as Ro31-8425 are potent inhibitors of this process. Furthermore, orally bioavailable bisindolylmaleimide PKC inhibitors such as Ro32-0432 block phorbol ester-induced inflammation in rodents and selectively inhibit T cell-mediated inflammatory responses in animal models of arthritis and encephalomyelitis.

176P G-PROTEIN-COUPLED RECEPTOR KINASES AS POSSIBLE DRUG TARGETS

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G protein-coupled receptors (GPCRs) are common drug targets, with a high percentage of marketed drugs being targeted against them.

The underlying reasons are manifold. They are numerous and mediate a wide spectrum of cellular functions ranging from neurotransmission, olfaction and light detection to endocrine and immune functions. Being located on the cell surface, GPCRs are easily accessible by specific agonist or antagonist drugs which trigger or inhibit various GPCR-mediated functions to relieve the body of disease states. Most GPCRs are strictly regulated through the process of desensitization, which overrides receptor activation by its agonists. This process contributes to the homeostatic regulation of cellular functions, and enables the cell to respond efficiently to the ever-changing extracellular milieu.

In the arena of therapeutics, receptor desensitization can have an impact on the efficacy of receptor-active drugs by limiting the activation of receptors. Furthermore, abnormalities in desensitization may also lead to disease states. The G protein-coupled receptor kinases (GRKs) are therefore of great interest since they are dedicated to carrying out this process of desensitization.

GRKs are serine/threonine kinases, with six subtypes being identified so far, named GRK1 to GRK6. Though not all subtypes have been studied in detail, the general consensus is that they serve to phosphorylate agonist-bound GPCRs within seconds or minutes of receptor occupation. The receptors thus phosphorylated have increased affinity for arrestin proteins, the binding of which uncouples the GPCR from Gprotein, rendering it inactive (desensitized).

Not surprisingly, the functional state of GRKs, and consequently the efficiency of the desensitization process, is under active regulation through various intracellular mechanisms. Increases in GRK expression/activity cause enhanced efficiency of receptor desensitization, hence reduced receptor activation. Inappropriate regulation of GRKs may lead to pathological states, as exemplified by the association of increased cardiac GRK2 expression with heart failure.

Such findings have kindled the interest in GRKs as possible drug targets, and will form the basis of this presentation.

177P INHIBITION OF THE MITOGEN-ACTIVATED PROTEIN KINASE PATHWAYS

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Mitogen-activated protein kinases (MAPK) are activated in response to a variety of extracellular signals by dual phosphorylation on threonine and tyrosine residues. Recently, it has become apparent that, as in yeast, mammalian cells contain multiple MAPK cascades, and three pathways have been identified: ERK1/ERK2, SAPK/JNK and p38.

It is becoming increasingly apparent that regulation of these distinct signalling cascades is central to the control of important cellular processes, suggesting that pharmacological interference could be beneficial in certain disease states.

This is illustrated by the bicyclic and triaryl-imidazoles which represent a novel class of compounds (CSAID™) that regulate the biosynthesis of inflammatory cytokines, primarily at the level of protein translation. These compounds, which are potent inhibitors of the protein kinase p38, have been shown to be effective in many animal models of joint and bone disease.

178P REGULATION OF PHOSPHOLIPASE A_2 AND PHOSPHOLIPASE C_γ BY PHOSPHORYLATION

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A large number of intracellular events are regulated by protein kinase cascades leading to phosphorylation of a target protein which plays a fundamental role in the final response.

The consequences of phosphorylation can be manifold, such as activation in the case of enzymes or translocation to the membrane or cytoskeleton etc. A remarkable degree of redundancy appears to exist for many protein kinase cascades as a consequence of expression of multiple protein isoforms within a cell or through 'cross-talk' with other signalling pathways etc. The experimental investigator is therefore faced with a considerable challenge in identifying the series of events which underlie a response regulated by phosphorylation, especially as this can vary widely between cells. On the other hand, the existence of this 'redundancy' may offer an opportunity for selective therapeutic intervention with only limited side effects.

My talk illustrates aspects of this through our work on the regulation of phospholipase C (PLC) and cytosolic phospholipase A_2 (PLA_2) in blood platelets.

The majority of platelet agonists induce activation through the generation of the PLC-derived messengers inositol 1,4,5-trisphosphate and protein kinase C. We have shown that collagen fibres activate this pathway through a mechanism that is distinct from that used by other stimuli, namely via tyrosine phosphorylation of the PLCγ2 isoform. The use of selective inhibitors such as the src family kinase inhibitor PP1 (CP 118,556) or studies on platelets from knockout mice have shown that PLCγ2 is regulated by collagen through a kinase cascade involving members of the src and syk family of tyrosine kinases. In contrast, platelet activation by G protein-coupled receptor agonists is not altered under these conditions despite activation of the same kinases.

$cPLA_2$ is widely recognised as the major enzyme underlying receptor release of arachidonic acid leading to formation of cyclooxygenase and lipoxygenase products. $cPLA_2$ is regulated through phosphorylation on serine residues and by Ca^{2+}. Although elevation of intracellular Ca^{2+} is essential for the action of $cPLA_2$, its intrinsic activity can be increased by phosphorylation on serine 505 which falls within a consensus site for MAPkinase. The use of selective inhibitors of the p42/44 MAPkinase (PD98059) and p38 MAPkinase (SB203580) pathways has shown that both of these cascades give rise to phosphorylation of $cPLA_2$, but that their importance is agonist-dependent. Moreover, phosphorylation is only important at concentrations where intracellular Ca^{2+} is sub-maximal.

The existence of multiple pathways capable of regulating a given response gives rise to the possibility that kinases within these cascades represent targets for pharmaceutical intervention without necessarily giving rise to widespread side effects.

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179P STRUCTURAL STUDIES OF PROTEIN PHOSPHATASES

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Protein phosphatases are signal transduction enzymes that dephosphorylate proteins on their serine, threonine and tyrosine residues. From a structural viewpoint, these enzymes are classified into three families; the PPP and PPM families dephosphorylate serine and threonine residues, whereas the PTPs dephosphorylate phosphotyrosine-containing proteins and peptides. The PTPs also include a sub-family known as the dual specificity phosphatases which dephosphorylate all three phospho-amino acids.

The modification of proteins by protein phosphorylation is the major mechanism for the regulation of intracellular activities by extracellular signals that include hormones, mitogens and nerve impulses. Protein phosphorylation regulates metabolism, gene transcription, the cell cycle, differentiation and transport processes. Protein phosphatases, together with the protein kinases regulate the overall levels of intracellular protein phosphorylation. The genes encoding these enzymes account for ~5% of the genes within the eukaryotic genome and it has been estimated that 30% of intracellular proteins are phosphorylated.

The studies in my laboratory have been aimed at understanding the mechanism of catalysis, substrate specificity and regulation of these enzymes. In my presentation, I shall compare and contrast the representative members of each of the protein phosphatase families, namely PTP1B, a protein tyrosine phosphatase, PP1 and PP2C, two serine/threonine specific protein phosphatases of the PPP and PPM family, respectively and finally KAP (kinase associated phosphatase) that functions during the cell cycle to dephosphorylate Thr 160 of cyclin dependent kinase 2.

These studies have revealed the basis for the substrate specificity of PTP1B for phosphotyro-sine-containing peptides and proteins and the nature of an induced conformational change within the protein that accompanies substrate binding. The catalytic site of KAP is similar to that of PTP1B, however differences in the structure of the two enzymes account for the ability of KAP to recognise phosphothreonine residues. Both PP1 and PP2C belong to two different gene families sharing no sequence similarities, however the deduced 3-dimensional structures of these proteins reveal similarities in their overall protein architecture and mechanism of catalysis. Both enzymes are metalloenzymes which bind two divalent metal ions at their catalytic sites within a binuclear centre that acts to coordinate the substrate phosphate group and activates a water molecule for nucleophilic attack onto the substrate.

Finally, I shall discuss the mechanism by which PP1 is regulated via targetting and regulatory subunits and the implications of knowledge of protein phosphatase structures for rational drug design.

180P NEW LIGANDS FOR STUDYING IMIDAZOLINE RECEPTORS

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The development of selective ligands for imidazoline receptors is vital to the future understanding of these novel sites. Although they were originally thought of as a single entity, it is now clear that they are heterogeneous and can be divided into I_1, I_2 and an atypical or I_3 site. Agmatine has been reported as the putative endogenous ligand.

I_1 receptors are found in the brainstem, where their activation lowers blood pressure. It has been proposed that the antihypertensive actions of clonidine are mediated by I_1 receptors whilst the unwanted side effects such as sedation are mediated by α_2-adrenoceptors. I_1 sites have been labelled by a number of non-selective ligands, notably [^{3}H]clonidine, [^{3}H]*p*-aminoclonidine and [^{125}I]*p*-aminoclonidine (Table 1). The use of these has also revealed the existence of peripheral sites, notably in kidney, where I_1 receptor activation leads to increased osmolar clearance (Smyth *et al.*, 1995). There are at least two I_1 receptor selective ligands, moxonidine and rilmenidine (Table 1), both of which are antihypertensive in patients and are reported as less sedative than clonidine (see Reid, this symposium).

The functions of the I_2 receptor are less clear, due in part to the previous non-availability of selective ligands. Until recently, the α_2-adrenoceptor antagonist idazoxan was the only radioligand used routinely to label I_2 receptors. Fortunately, there are now several selective ligands (Table 1) including [^{3}H]2BFI (2-(2-benzofuranyl)-2-imidazoline), [^{3}H]RS-45041-190 (4-chloro-2-(imidazolin-2yl)isoindolene) and a photoaffinity ligand, 2-(3-azido-4-^{125}I-iodophenoxy)methyl-imidazoline. Our own studies have focused on 2BFI and BU224, (2-(4,5-dihydroimidaz-2-yl)-quinoline) for both *in vitro* and *in vivo* experiments. [^{3}H]2BFI has proved to be an excellent ligand for receptor binding and autoradiography in a range of species. *In vivo* brain dialysis reveals that both BU224 and 2BFI dose-dependently increase extracellular noradrenaline in rat frontal cortex and hippocampus. Behavioural studies reveal 2BFI prolongs swim time in the Porsolt model of depression and like RS-45041-190 and LSL60101 (2-(2-benzofuranyl)-2-imidazole), induces acute hyperphagia in rats (Nutt *et al.*, 1995; Brown *et al.*, 1995; Menargues *et al.*, 1994).

The atypical or I_3 receptor is that site associated with the pancreatic β-cell where some compounds with an imidazoline moeity can act as insulin secretagogues. One such drug is the α_2-adrenoceptor antagonist efaroxan, where the (-) enantiomer shows greater potency on insulin release than the (+) form (see Morgan *et al.*, this symposium).

Although the mechanisms behind some of the above effects are unresolved at present, what is clear is that the introduction of selective ligands is a landmark in the future study and understanding of these novel receptors. These new tools, both labelled and unlabelled, should help researchers to determine the exact structure, location, function and therapeutic potential of imidazoline receptors.

Table 1 A selection of ligands used for imidazoline receptor studies

Ligand	*Subtype*	*Comment*
[^{3}H]*p*-aminoclonidine	I_1	non-selective
[^{3}H]Moxonidine	I_1	selective, antihypertensive
[^{3}H]Rilmenidine	I_1	selective, antihypertensive
[^{3}H]Idazoxan	I_2	also α_2-adrenoceptor antagonist
[^{3}H]2BFI	I_2	highly selective
[^{3}H]RS-45041-190	I_2	highly selective
Efaroxan	I_3	also α_2-adrenoceptor antagonist, also I_1

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