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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181P MOLECULAR ASPECTS AND PROPERTIES OF IMIDAZOLINE RECEPTORS

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Imidazoline binding sites (IBS) are subclassified in two major subtypes based on their high (I_1) or low (I_2) affinity for clonidine. In addition to the different pharmacological profiles, I_1 and I_2 binding sites display differences in their subcellular and tissue distribution. I_1BS are localised in the plasma membrane and are found predominantly in the central nervous system and in platelets. I_2BS are mainly located in mitochondria and are widely distributed in the brain and in the periphery. Such differences strongly suggest that I_1 and I_2 binding sites represent distinct proteins with specific functional properties.

Using biochemical and molecular biology approaches, we recently demonstrated that at least one type of I_2BS is located on the A and B isoforms of monoamine oxidase (MAO), two mitochondrial membrane enzymes catalysing the oxidative deamination of neurotransmitter (noradrenaline, dopamine and serotonin) and exogenous amines.

The identity of I_2 sites and MAO is indicated by:

1) the apparent molecular weights of I_2 site correspond to those expected for MAO-A and MAO-B;

2) partial amino acid sequencing of purified I_2 sites indicates homology to MAO;

3) expression of MAO in yeast results in the generation of I_2 sites;

4) immunoprecipitation of photoaffinity labelled I_2 sites in human placenta and liver with monoclonal antibodies directed against MAO-A and MAO-B.

Additional pharmacological and biochemical studies indicate that the imidazoline binding domain on MAO is distinct from the catalytic site, the FAD prosthetic group or the binding domain of classical MAO inhibitors. Another major point concerning MAO and I_2 sites is that there is a stoichiometric discrepancy in the relative detection of the two entities. This finding is particularly interesting as it suggests that I_2BS may represent the first target for the identification and the pharmacological control of a specific subpopulation of the enzyme.

Several studies also suggest the existence of additional I_2BS in bovine and rat tissues. The apparent molecular weight of these proteins (70, 45, and 29-30 kDa) does not correspond to that of MAO suggesting the existence of additional I_2BS that could be distinct from MAOs.

The structural properties of I_1BS are still unknown. Some radioligand and functional studies supplied indirect evidence suggesting that I_1BS could belong to the family of G-protein coupled receptors. Other studies did not support this hypothesis and recently the possible association of I_1BS with the intrinsic ion channel of the nicotinic acetylcholine receptor has been reported. These discrepancies between the different studies may be related to the experimental conditions or may reflect structural and functional heterogeneity of I_1BS.

In conclusion, I_1 and I_2 binding sites represent a heterogeneous family of membrane proteins. Their molecular characterisation will be a critical step toward the full understanding of their functional relevance.

182P IMIDAZOLINE RECEPTORS IN HUMAN BRAIN

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Pharmacological criteria have defined two main types of imidazoline receptors (IR): the clonidine-preferring receptor (I_1-type) labelled by [^{3}H]-clonidine and its derivatives, and the idazoxan-preferring receptor (I_2-type) labelled by [^{3}H]-idazoxan and [^{3}H]-2-BFI. I_2-IR have been further subclassified into the I_{2A}-subtype (high affinity for the guanidide amiloride) and the I_{2B} -subtype (low affinity for amiloride). Functional studies have revealed additional IR which cannot be grouped into any of the previous types (i.e., non I_1/I_2-type; e.g. those regulating the release of noradrenaline in peripheral tissues of the rabbit and insulin secretion from rat and human islets of Langerhans).

At present, only I_1- and I_2-IR have been characterized in the human brain. The I_2-IR appears to belong to the I_{2B}-subtype. IR are expressed in most regions of the human brain, although the I_1-IR shows a more limited distribution. Autoradiographical studies with [^{3}H]-clonidine showed the highest densities of I_1-IR in the basal ganglia (putamen, nucleus caudatus), hippocampus, amygdala, substantia nigra and brainstem. Autoradiography with [^{3}H]-idazoxan revealed higher densities of I_2-IR in cortex, hippocampus, basal ganglia and brainstem. I_1-IR are plasma membrane G protein-coupled receptors and appear to regulate arterial pressure by inhibiting adrenergic nerve activity in the brainstem. I_2-IR are mainly localized to mitochondria (e.g. in glial cells) and show a relation with the enzyme monoamine oxidase (MAO). Brain I_2-IR appear to regulate the expression of the glial fibrillary acidic protein and to be up-regulated by the process of ageing (parallel increases with MAO-B). At present, however, the nature, signal transduction pathways and physiological functions of brain IR remain largely unknown (García-Sevilla *et al.*, 1995a).

Several immunoreactive IR proteins have been identified in the human brain which has confirmed the heterogeneity of IR and the association of some of these proteins with specific IR types. Thus, various brain regions (frontal cortex, hippocampus, hypothalamus, nucleus caudatus and brainstem) express a double band of 29-30 kDa and a less intense band of 45 kDa (Escribá *et al.*, 1994). This brain 45-43 kDa IR protein has been suggested to represent the binding entity of I_1-IR (Bennai *et al.*, 1996). The 29/30-kDa IR protein has been associated with I_{2B}-IR in the human brain during the process of ageing (parallel increases) (García-Sevilla *et al.*, 1995b). Although I_2-sites identified with [^{3}H]-idazoxan have been suggested to be previously unknown regulatory sites located on MAO (Parini *et al.*, 1996), certain IR proteins (e.g. 29-30 kDa and 45-kDa) represent molecular species that are distinct from MAO (molecular mass of 59-63 kDa).

Various pathological states (psychiatric disorders, neurodegenerative diseases, opioid addiction) have been associated with disturbances of brain IR. In depression the 45 kDa IR protein (putative I_1-IR) has been shown to be up-regulated in brains of suicide victims (51%) and in platelets of depressed patients (40%) (García-Sevilla *et al.*, 1996). Similarly, the density of platelet I_1-IR ([^{125}I]-p-iodoclonidine binding) was also shown to be increased in depressed patients (135%) (Piletz *et al.*, 1996). In contrast, the 29-30 kDa IR protein (putative I_{2B}-IR) was found to be down-regulated (19%) in brains of suicides in parallel with a reduction (40%) in the density of I_{2B}-IR (García-Sevilla *et al.*, 1996). The density of I_{2B}-IR has been shown to be increased in Alzheimer's disease (63% in frontal cortex) (Ruiz *et al.*, 1993) and decreased in Huntington's disease (56% in putamen) (Reynolds *et al.*, 1996). In contrast, brain I_{2B}-IR (putamen and frontal cortex) appear not to be altered in the chronic phase of Parkinson's disease (Gargalidis-Moudanos *et al.*, 1997). The density of I_{2B}-IR and the abundance of the related 29-30 kDa IR protein were found to be decreased (39% and 28%) in postmortem brains of heroin addicts (Sastre *et al.*, 1996). The possible functional relevance of these findings in the context of the pathogenesis of these disorders remains to be elucidated.

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183P IMIDAZOLINE-I_2BINDING SITES IN ADIPOSE TISSUE: RELATIONSHIP WITH AMINE OXIDASE ACTIVITY AND GLUCOSE METABOLISM

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White adipocytes of various mammal species, including man, contain a large population of imidazoline-I_2 binding sites exhibiting higher affinity for idazoxan than for clonidine. As some interactions between I_2-site ligands and monoamine oxidase (MAO) have been previously described in rat liver (Carpéné *et al.*, 1995), the present work focused on the interactions between amine oxidases and I_2-sites in rat adipocytes. Binding studies and assays of tyramine oxidation were conducted as previously reported (Carpéné *et al.*, 1995) on crude membrane preparations obtained from white fat cells. Glucose transport was determined on intact rat adipocytes by the [^{3}H]-2-deoxyglucose uptake technique and lipogenesis was measured by the incorporation of [^{3}H]-glucose into cellular lipids.

The presence of I_2-sites was reassessed using [^{3}H]-benzo-furanyl imidazoline (BFI), a selective I_2-radioligand. Binding characteristics on rat fat cell membranes were: B_{max} = 428±87 fmol mg^{-1} protein and Kd = 9.1±1.0 nM (n=5), in accordance with previous data obtained with the classic I_2-ligand [^{3}H]-idazoxan. Amiloride was a weak competitor of [^{3}H]-BFI binding, which is consistent with the definition of I_{2B}-sites in this model. In rat fat cell membranes, MAO inhibitors (pargyline, clorgyline and selegiline) displaced [^{3}H]-BFI and [^{3}H]-idazoxan from their binding sites with a lower potency than the I_2-ligands (idazoxan, BFI, cirazoline, naphazoline). In the same preprations, tyramine oxidation was characterized by a Km of 85± 18 µM and a Vm of 12±1 nmol/mg prot/min and was dose-dependently inhibited by both MAO inhibitors and I_2-ligands. However, half of the tyramine oxidation was resistant to the compounds tested. Complete inhibition was only reached in the presence of semicarbazide, suggesting the presence of a semicarbazide-sensitive amine oxidase (SSAO).

Similar characteristics of tyramine oxidation were found in intact adipocytes reflecting the action of at least three distinct enzymes: MAO-A, MAO-B and SSAO. As MAO and SSAO generate hydrogen peroxide during substrate oxidation, we tested whether the endogenous production of this insulin-mimicking agent by tyramine catabolism could stimulate glucose metabolism in intact fat cells. No activation of 2-deoxyglucose transport was found when rat adipocytes were incubated in the presence of increasing concentrations of tyramine, phenylephrine or 5-HT. The addition of 0.1 mM vanadate to the incubation medium was inefficient by itself, but potentiated the action of tyramine on glucose transport: vanadate plus tyramine (1 mM) stimulated basal transport fourfold, an activation equivalent to 54±6% of the maximal insulin stimulation (n=22). The effect of tyramine plus vanadate was inhibited in a dose-dependent manner by SSAO or MAO inhibitors and I_2-ligands. However, total blockade was reached with the former, while only partial inhibition was obtained with the latter. The fact that 1 mM tyramine totally inhibited [^{3}H]-idazoxan binding on I_2-sites can partly explain this difference in the blocking capacity between MAO inhibitors and I_2-ligands. Agmatine, a putative endogenous imidazoline ligand, was neither oxidized by adipocyte amine oxidases nor able to stimulate glucose transport. Tyramine plus vanadate not only synergistically stimulated glucose transport but also glucose incorporation into cellular lipids. This lipogenic effect was blocked by catalase, thus arguing for the crucial role played by the hydrogen peroxide formed during the oxidative deamination of tyramine.

The combination of exogenous 1 mM hydrogen peroxide with 0.1 mM vanadate stimulated glucose transport and metabolism in a catalase-sensitive and MAO-independent manner. We thus hypothesize that, in intact adipocytes, the hydrogen peroxide endogenously generated during amine oxidation interacts with vanadate to form peroxovanadate and thus confer to tyramine its insulin-mimicking effects.

These observations demonstrate for the first time that the I_2-sites present in white adipocytes are associated with a high level of amine oxidase activity which can be involved in glucose metabolism. Whether the amine oxidase-dependent activation of hexose transport is physiologically relevant or can be regulated by imidazoline compounds remains to be defined.

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184P IMIDAZOLINE RECEPTORS IN THE ENDOCRINE PANCREAS: POSSIBLE THERAPEUTIC TARGETS?

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It is now well established that a range of compounds bearing an imidazoline (or closely related) moiety are able to stimulate insulin secretion from pancreatic β-cells. This effect occurs both *in vivo* and *in vitro* and appears to be mediated by a receptor mechanism since the response shows stereospecificity and agonist specific regulation and is sensitive to blockade by imidazoline receptor antagonists. The pharmacology of the islet imidazoline receptor is different from that exhibited by other members of the family (which are now defined as I_1 or I_2 receptors) suggesting that the molecule may represent a convenient selective target for a new generation of anti-hyperglycaemic agents.

In pancreatic β-cells, activation of the imidazoline receptor results in stimulation of insulin secretion in a glucose-dependent manner and this is accompanied by a marked decrease in the potassium permeability of the plasma membrane. The change in potassium permeability derives from inhibition of ATP-sensitive potassium channels, a response which is measureable in isolated patches of membrane under patch-clamp conditions. The relationship between the ion-conducting pore of the channel and imidazoline receptor has not been defined but the binding site cannot be located within the channel pore since the two components can be regulated independently.

Sulphonylureas are well characterised regulators of islet cell potassium channel activity and, like imidazoline secretagogues, they stimulate insulin secretion by causing channel closure. There is no interaction between sulphonylureas and imidazolines at the level of receptor binding but increasing evidence indicates that control of potassium channel activity by these two types of drug is subject to mutual interaction. Thus, some imidazoline antagonists can block the insulin secretory effects of sulphonylureas and synergistic interactions between certain imidazolines and sulphonylureas with respect to stimulation of insulin secretion, have been observed.

This lecture will focus on the current status of the islet imidazoline receptor as a therapeutic target and will draw attention to the unique pharmacological characteristics of this site. The interactions between sulphonylureas and imidazoline insulin secretagogues will also be reviewed.

185P IMIDAZOLINE RECEPTORS IN HYPERTENSION

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Drugs with an action on the central nervous system have been employed for over 40 years in the treatment of hypertension. Reserpine in the 1950s, methyldopa from the 1960s and clonidine in the 1970s were introduced into clinical practice. While their efficacy is not disputed, the frequency of symptom side effects has limited their use in recent years.

The availability of better tolerated drugs active at extracerebral sites has resulted in a marked reduction in the use of centrally acting drugs. The most common side effects were sedation (all central drugs) depression (reserpine and methyldopa) and dry mouth and withdrawal reactions (clonidine). All three classes were believed to act by modifying catecholamine function in the brain and periphery: in the case of methyldopa by formation of a 'false' transmitter, alpha methylnoradrenaline, and in the case of clonidine as an agonist at 'atypical' α_2 receptors in the brain stem. These receptors were considered to be the site and mechanisms not only of the blood pressure fall but also the central side effects, including sedation.

In recent years evidence has accumulated of a new class of receptors which preferentially bind imidazoline drugs and lead to hypotension but not sedation. These imidazoline receptors and putative endogenous ligands have provided the rationale for a new group of drugs which are imidazoline-preferring compounds. Rilmenidine and moxonidine are examples of this group and have been introduced into clinical practice as antihypertensive drugs. These agents have less affinity for α_2 adrenoceptors than clonidine and are relatively selective ligands at the imidazoline I_1 receptor which is linked to inhibition of sympathetic outflow in the rostralventrolateral medulla of the brain stem. These I_1 receptors have also been identified in renal tubules, platelets and adrenal medulla.

Clinical trials with rilmenidine and moxonidine confirm comparable efficacy compared to other established classes of antihypertensive drugs. Side effects, particularly dry mouth and sedation, are less common than with clonidine in controlled trials. Reversal of antihypertensive actions is gradual over 3-4 days, unlike clonidine, and withdrawal reactions have not been reported.

The precise role of imidazoline-preferring agents in hypertension remains to be established. They offer an alternative strategy in patients with side effects on other classes of drugs; they can be useful in a wide range of patients with few absolute or relative contraindications and can be used safely and successfully in combination with most other drugs in patients requiring more than one drug. There is potential for the further improvement of the selectivity of this class of drug and there are possible additional indications, including heart failure patients.

186P THE PHARMACOLOGICAL BASIS OF DIABETES THERAPY: AN OVERVIEW

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Non-insulin dependent diabetes mellitus (NIDDM) has been described by the British Diabetic Association as the hidden epidemic, affecting an increasing proportion (over 5%) of the adult population of the UK and accounting for 10% of the National Health Service's total budget. In addition to dietary control, the principal aims of drug treatment are to facilitate the disposal of blood glucose following ingestion of carbohydrate and to blunt the rise of plasma free fatty acids which ensues from lipolysis. In the insulin-resistant NIDDM patient these beneficial effects can be achieved by restoring insulin sensitivity.

Very many natural products have been examined for their potential hypoglycaemic activity and no less than 343 medicinal plants identified (Rahman & Zaman, 1989). However, with the exception of the sulphonylureas, whose hypoglycaemic properties were discovered somewhat serendipitously in the early 1950s, advances in the pharmacological treatment of diabetes have been notably lacking. The first biguanide (Synthalin B) appeared in 1926 and eventually led to the development of dimethylbiguanide (Metformin) in 1957. The stimulation of insulin release by activation of the K^+-ATP channel-linked sulphonylurea receptor and the potentiation of glucose transport (by the biguanides for example) have remained the principal targets for drug action. Thiazolidinediones such as troglitazone initially proved successful in this last respect, but the early compounds were associated with unacceptable toxicity. In addition, it now appears that a novel imidazoline binding site on pancreatic β cells may be activated to provoke insulin release (Chan *et al.*, 1994). The association of NIDDM with obesity has also directed interest to the anti-diabetic potential of anti-obesity drugs, and a large number of appetite suppressants are used clinically.

More recently, the discovery of new hormones such as the *obese* gene product leptin, and new roles for established hormones, such as CCKA and GLP-1, has yielded a number of novel potential mechanisms for regulating satiety and energy balance which are now rapidly being exploited. At the same time, technical advances such as time-lapse imaging of intracellular events *in vivo* by confocal microscopy (Oatey *et al.*, this meeting), are making it feasible to observe functional responses to drugs and hormones at the subcellular level. Probably at no time since the initial discovery of the role of insulin in juvenile diabetes has there been such an opportunity to develop novel treatments for this hidden epidemic.

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