456P P2X RECEPTORS: A THIRD FAMILY OF LIGAND-GATED ION CHANNELS

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Adenosine 5'-triphosphate and/or related nucleotides act at bothionotropic (P2X) and metabotropic (P2Y) receptors. P2X receptor subunits (P2X1–P2X7) are encoded by distinct genes, having ten to twelve exons. They form ligand-gatedcation channels, either as homo-multimers or heteromultimers. P2X subunits are expressed by some smooth muscle and ventricular myocytes; in certain arterioles, ATP is the main sympathetic neurotransmitter. P2X4 subunits are expressed by several exocrine glands; together with P2X6 subunits, they are also widespread in the central nervous system. All the subunits are found in primary afferent neurons, and P2X 3 subunits participate in channels expressed by nociceptive sensory neurons.

P2X7 subunits are expressed by macrophages and brain microglia; they are unique in that the application of the agonist not only opens a cation channel (about 0.8 nm) but also leads to the development of a large pore (about 4 nm). P2X receptor subunits have intracellular N- and C-termini, with most of the protein forming a glycoslyated extracellular loop; cysteine scanning mutagenesis indicates that the second of the two transmembrane domains of each subunit contributes to the ion permeation pathway. This structure is thus fundamentally different from that of ligand-gated ion channels within the nicotinic acetylcholine or glutamate super-families; it resembles in general terms the topology of the superfamily of epithelial sodium channels/C. *elegans* degenerins, which now includes some ligand-gated members (protons/ FMRFamide).

457P THE MOLECULAR BIOLOGY OF ENDOTHELIN-CONVERTING ENZYME

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Three related genes encode the endothelin precursors or preproendothelins. Processing of these 200 amino-acid polypeptides occurs in two stages. Dibasic amino-acid endopeptidase and carboxypeptidase activities first release the inactive big endothelins (40 residues). Big endothelins are then further cleaved at Trp-Val (Trp-Iso for big ET-3) bond to generate endothelins.

Very early it was suggested that this second step of the endothelin synthesis was mainly achieved by a specific "endothelin-converting enzyme" (ECE). This enzyme was termed ECE-1. Cloning also revealed the existence of ECE-2, a closely related enzyme sharing the same ability to convert big endothelins into endothelins. However, the acidic pH optimum and the low expression levels of ECE-2 favor the hypothesis that ECE-1 is the main enzyme involved in the endothelin conversion.

The gene encoding ECE-1 has been characterized: it encompasses at least 70kB and 20 exons, and is localised on human chromosome 1 (1p36). The organization of its 5'regions is extremely complex due to the presence of 3 alternate promoters. A consequence of this complexity is the existence of three ECE-1 isoenzymes which display divergent N-terminal extremities. When expressed in CHO cells, these three ECE-1 isoforms process big endothelins with similar efficiencies but display different intracellular localizarions.

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458P STRUCTURE AND LOCALISATION OF ENDOTHELIN-CONVERTING ENZYME

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The endothelin (ET) peptides are synthesized through the consecutive actions of a furin-like enzyme and endothelin-converting enzyme (ECE) on proendothelin. ECE is a membrane-bound zinc metalloprotease similar to neutral endopeptidase-24.11 (NEP; neprilysin), and the KELL and PEX proteins (Turner & Tanzawa, 1997).

The membrane topology, quaternary structure and functionally important residues in ECE-1 have been compared with those in NEP. There have been differing reports on the subcellular location of ECE-1. Here, we have now used immunogold staining and ultrathin cryosections of pig and rat lung to co-localise ECE-1 both with big ET-1 on intracellular vesicles of 50-100 nm in diameter and with angiotensin converting enzyme on the luminal surface of endothelial cells. The presence of a proportion of ECE-1 on the plasma membrane has been confirmed by cell-surface biotinylation of endothelial cells.

Following chloroquine treatment of endothelial cells, ECE was shown to redistribute and be highly localised to an intracellular compartment, probably endosomal, a phenomenon also observed with both furin and the trans-Golgi network marker, TGN38. Thus ECE-1 appears to be able to cycle between the cell-surface and an intracellular location consistent with sorting motifs present in its N-terminal domain. Finally, an exclusive role for ECE in production of endothelin must be reconsidered in the light of the recent identification of a novel physiological substrate for ECE.

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459P BIOCHEMISTRY OF ENDOTHELIN-CONVERTING ENZYME

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Endothelin-l (ET-1) is synthesised in a number of cell types including endothelial, epithelial and smooth muscle cells. Initial biosynthesis occurs as a protein precursor, preproendothelin-l (preproET-1). This is processed intracellularly to the inactive intermediate big ET-1 which is hydrolysed by endothelin converting enzyme (ECE) to generate ET-1. The precise identity of the physiologically relevant ECE has yet to be confirmed. Although ET-1 is synthesised in the constitutive secretory pathway, many characteristics of the selective processing of proET-1 are comparable to classical peptide hormones such as insulin or ACTH. Thus, for ET-1 biosynthesis, pertinent features should be: co-ordinated expression of preproET-1 with ECE, packaging of ECE with proET-1 in the *trans* Golgi apparatus followed by processing to ET-1 during vesicle transport, and finally co-release of ECE with ET-1. To identify a physiologically relevant ECE we have investigated the relationship between the biosynthesis of ECE and ET-1.

Firstly, because of its sensitivity to phosphoramidon one candidate ECE is the metalloprotease referred to as endothelin-converting enzyme- I (ECE-1) (Shimada *et al.*, 1994; Xu *et al.*, 1994). Therefore, we have used RT-PCR to assess whether ET-1 mRNA in endothelial cells is regulated in parallel with mRNA for the ECE-1 isoforms (ECE-1a/1b). ET-1 synthesis was stimulated with TNFα or TGFβ, and inhibited with 2 chloroadenosine (CADO) or staurosporine. RT-PCR estimates for changes in proET-I expression correlated well with changes in ET-1 release. In contrast, measurements of the ECE-1 isoforms showed no correlation with proET-1, indicating that expression of ECE-1 is not co-ordinated with ET-1 synthesis. Secondly, immunoelectron microscopy has shown that enzymatic processing of big ET-1 occurs intracellularly in constitutive secretory vesicles (Harrison et *al.*. 1995).

To evaluate the role for a specific ECE in ET-1 biosynthesis, secretion of ET-1 and ECE activity was studied using endothelial cells. In three separate experiments, secretion of a phosphoramidon-sensitive ECE activity was closely correlated with ET-1. Hence, at the level of gene expression, the identified ECE is probably regulated in parallel with ET-1. Further characterisation of this activity is likely to yield the physiologically relevant ECE.

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460P PHARMACOLOGY OF ENDOTHELIN RECEPTORS

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Endothelin-1 (ET-1) is the most powerful endogenous constrictor of human blood vessels with an unusually long-lasting action and contributes to the maintenance of normal vascular tone (Haynes and Webb, 1994). Overproduction of this peptide may disturb the complex balance between constricting and dilating factors in the human vasculature, contributing to the vasoconstriction associated with cardiovascular disease. Two receptor sub-types, ET_A and have been cloned in humans. Our aim has been to characterise these sub-types and determine their localisation in the human cardiovascular system and to measure changes in receptor expression associated with disease. Whilst we have shown mRNA encoding both subtypes, present in the smooth muscle layer (or media} of human vessels, radioligand binding as show that the ET sub-type predominates (>85%) in both conduit and smaller resist; vessels from the human brain, heart, kidney and lung (Davenport *et al.,* 1994,1995a,b) Electron microscope autoradiography confirmed the ultra-structural localisation of receptors to the plasma membrane of smooth muscle cells of coronary arteries (Russell *et al.,* 1997}.

In patients with coronary artery disease, ET levels were significantly increased in vessels with atherosclerotic lesions. This may contribute to the initiation of atherosclerosis, vasospasm or the events leading to plaque rupture. We have synthesised a new low molecular weight non-peptide radioligand, [^{125}I]-PD164333, an analogue of the orally active butenolide antagonists of the ET_A receptor. Using this ligand, we have shown that ET_A receptors predominate in the media of atherosclerotic vessels but surprisingly, the proliferating smooth muscle cells within the intimal thickening in advanced plaques did not express either sub-type. However, ET_B receptors were localised to endothelial cells of neovascularization and macrophages (Bacon *et al,* 1996). Similarly, in occluded saphenous vein grafts, ET_A receptors were localized to the media with little or no binding to the proliferating cells. At present it is unclear whether progression of smooth muscle cells through successive cells divisions is accompanied by down regulation of ETA receptors or whether absence reflects the undifferentiated nature of these cells.

In agreement with ligand binding, functional studies using isolated vessels show that the ET-1-induced vasoconstriction is mainly mediated via the ET sub-type in both large conduit arteries (Maguire and Davenport, 1994) and vessels likely to contribute to vascular resistance. Recently developed orally active antagonists such as PD156707 that are highly, selective for the ETA receptor sub-type (Maguire *et al.,* 1997) block the constrictor action ET-1 *in vitro.* ET-1 has been implicated as a cause of vasospasm but importantly these antagonists are able to fully reverse an established constrictor response to this peptide in normal coronary arteries as well as those containing advanced atherosclerotic lesions, indicating no functional evidence for an increase in ET_B receptors with disease. Selective blockade of the vascular ET_A receptors may therefore produce beneficial vasodilatation represent a new therapeutic strategy in cardiovascular disease.

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461P CLINICAL PHARMACOLOGY OF ENDOTHELIN

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The discovery in 1988 of endothelin-1 (ET-1), a novel endothelium-derived 21 amino acid peptide (Yanagisawa *et al.,* 1988) and currently the most potent vasoconstrictor and pressor agent known, generated tremendous interest among the cardiovascular research community. These actions, together with its actions as a co-mitogen, suggested a potential pathological role for ET-1 in conditions associated with sustained vasoconstriction or with vasospasm.

Clinical pharmacology studies with ET-1 began as soon as the peptide could be synthesized. The first studies confirmed that ET-1 is a potent and sustained vasoconstrictor, venoconstrictor and pressor agent in humans; the sustained pressor effect mediated predominantly through an increase in peripheral resistance and associated with a reduction of cardiac output. In the forearm circulation, sustained arterial administration of ET-1 and the ETB selective agonist, sarafotoxin S6c (S6c), cause vasoconstriction though the latter to a lesser extent – suggesting that both ETA and ETB receptors in vascular smooth muscle may contribute to vasoconstriction. Brief high dose ET-1 and S6c cause transient initial vasodilatation, probably via the endothelial ETB receptor and in the resistance vessels mainly through generation of nitric oxide (NO).

Clinical studies with ET antagonists are likely to be more informative about the functional role of ET and these followed rapidly on the development of such drugs. The first endothelin 'antagonist' described was the ECE inhibitor, phosphoramidon, which abolishes forearm vasoconstriction to big endothelin-1 and produces a slowly progressive vasodilatation when given alone, consistent with a role for ET-1 in maintenance of basal vascular tone. Confirmation that endogenous ET-1 generation contributes to the maintenance of basal tone came from studies with the ETA receptor antagonist, BQ-123, and the combined ETA/B antagonist TAK-044, given via the brachial artery. Both agents caused progressive vasodilatation. Together, the L-arginine/nitric oxide system, the sympathetic nervous system and the ET system are currently the only mediator systems known to maintain basal vascular tone.

The greater effect of BQ-123 than a substantial local dose of TAK-044 would be consistent with a mainly dilator role for the ETB receptor. Further and more direct support for this view comes from recent arterial experiments with an ETB selective inhibitor, BQ-788. This agent causes progressive forearm vasoconstriction in healthy subjects (Love *et al.,* 1996a), consistent with greater functional importance of the dilator than the constrictor ETB receptor under physiological circumstances. Recent studies also suggest that a proportion of the dilator response to BQ-123 is mediated by NO acting through the unopposed endothelial ETB receptor. Interestingly, vasoconstriction appears to be mediated solely by ETA receptors in the human skin microcirculation, again on the basis of studies with antagonists (Wenzel *et al.,* 1994).

Systemic placebo-controlled studies with TAK-044 (10-1000 mg over 15 min i.v.) in healthy subjects showed TAK-044 was generally well tolerated. TAK-044 1000 mg reduced systolic BP by ~4%, diastolic BP by ~18%, and peripheral resistance by ~26% over a 24 h period, suggesting that a major part of its effect is mediated in the resistance vessels and confirms that endogenous ET-1 plays an important role in regulating arterial pressure; since confirmed in other studies. The major long lasting effecton systemic vascular resistance is remarkable given the short circulating half life of this peptide. TAK-044 also abolished vasoconstriction to ET-1 infused via the brachial artery for up to 3 hours but effectswaned thereafter. Such studies can usefully assess the efficacy and duration of action of ET receptor antagonists.

Important unresolved issues remain the province of clinical pharmacology studies. These include: investigation of the role of ET-1, and the effectsof its antagonists, on the function of other organs, such as the heart, kidney and gut, and on autonomic function; an understanding of the factors that up- or down-regulate the ET system in humans; the choice of therapeutic target in 'proof of concept' studies; and the further investigation of whether ETA, ETA/B or ECE antagonism is likely to be of most benefit in cardiovascular disease. This remains a very exciting area of clinical research which is likely in the near future to yield new agents for therapeutic use in cardiovascular disease, and perhaps other important areas.

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462P ENDOTHELIN ANTAGONISTS IN PULMONARY HYPERTENSION: CLINICAL AND EXPERIMENTAL STUDIES

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Pulmonary artery hypertension (PAH) results from constriction and remodeling of pulmonary vessels. Several observations now suggest that endothelins play an important role in these processes. Evidence for increased ET-1 production has been provided in humans and animals with PAH. Plasma concentrations of ET-1 and gene transcript levels for ET-1 and the ETA and ETB receptors in the lung have been reported in experimental models of PAH. Hypoxia stimulates ET-1 gene expression in cultured endothelial cells as well as in the lung from rats exposed to chronic hypoxia and developing PAH. Increased levels of endothelin-l like immunoreactivity and preproendothelin mRNA have been observed in vascular endothelium from lung tissue obtained from patients with both primary and secondary PAH. The greatest degree of immunostaining is found in the vessels which are the most severely affected by the morphologic abnormalities of PAH. Important changes in vasoreactivity to ET-1 have also been found in experimental models of PAH. Exposure of rats to chronic hypoxia leads to a loss of the vasodilator and enhancement of the vasoconstrictor effects of ET-1. The increased pulmonary vasoconstrictor effect of ET-1 in chronic hypoxic pulmonary hypertension seems to be mediated by both ETA and ETB receptors.

Reports of the beneficial effect of chronic treatment with specific ET-receptor antagonists in experimental pulmonary hypertension gives further support to the role of ET-1 in the initiation or progression of the lesions of pulmonary vessels. Chronic treatment with the ETA receptor antagonist, BQ- 173 or the mixed ETA and ETB receptors antagonist, bosentan, attenuates the development of hypoxic pulmonary hypertension. Treatment with bosentan also attenuates the pulmonary vascular remodeling in response to chronic hypoxia. decreasing the wall thickness of distal pulmonary arteries as well as the muscularization of arteries at both the alveolar duct and alveolar wall levels. Furthermore. when BQ-123 or bosentan treatment is started after 2 weeks of hypoxia and continued for 4 weeks. there is a significant reversal of pulmonary hypertension. right heart hypertrophy and pulmonary vascular remodeling despite continuing hypoxic exposure. Beneficial effect of BQ-123 has also been shown in neonatal pulmonary hypertension due to ligation of the ductus arteriosus in fetal lambs. These findings support the hypothesis that endogenous ET-1 contributes to the pulmonary vascular remodelling of PAH and failure of pulmonary circulation to adapt at birth.

463P ENDOTHELIN AND EXPERIMENTAL MODELS OF HEART FAILURE

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Endothelin (ET-1) is a potent vasoconstrictor peptide, which also exerts positive chronotropic and inotropic effects, and may stimulate vascular and cardiac hypertrophy. Production of ET-1 is increased in congestive heart failure (CHF). Indeed, plasma levels are correlated with the severity of cardiac dysfunction and are a major prognostic factor of mortality in human CHF. CHF also upregulates vascular ET receptors. Given the pharmacological profile of ET-1, this peptide may play a deleterious role in CHF.

The role of ET-1 in CHF was recently demonstrated experimentally using ET antagonists (either ETX or mixed ET_A-ET_B antagonists). In various experimental models of CHF, acute administration of ET antagonists decreased blood pressure and exerted favourable haemodynamic effects (Teerlink *et al.*, 1996; Sakai *et al.*, 1996a; Shimoyama *et al.*, 1996; Spinale *et al.*, 1997). In addition, in a recent study in rats, the peptide ET_A antagonist BQ-123 improved short term (2 months) survival, and reduced left ventricular hypertrophy and dilatation (Sakai *et al.*, 1996b). Moreover, in rats with CHF, we recently demonstrated that long term (9 months) treatment with the non peptide, ET_A-ET_B antagonist bosentan increased survival (survival after 9 months of treatment: untreated 47%, bosentan 65%, $p<0.01$). This effect on survival was similar to that induced by ACE inhibitors. The marked increase in survival after bosentan was associated with decreases in arterial pressure, heart rate, central venous pressure and left ventricular and diastolic pressure, as well as plasma catecholamines. The ET antagonist also reduced left ventricular dilatation and cardiac fibrosis, and increased contractility and cardiac output, assessed by echocardiography (Mulder *et al.*, 1997).

These experimental data suggest that chronic treatment with ET antagonists might be beneficial in human CHF and might increase long term survival in this pathology

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