A PAIR OF NOVEL ORPHAN RECEPTORS ARE EXPRESSED IN THE RECTAL GLAND OF THE SPINY DOGFISH, SQUALUS ACANTHIAS.

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The rectal salt gland of the spiny dogfish is a hollow tubular epithelial gland situated on the posterior abdominal wall. Analogous to the mammalian kidney ascending loop of Henlé, it has become established as a powerful physiological model system for the study of salt transport mechanisms. Adenosine analogues have been shown to be potent stimulators of ion transport when perfused into isolated glands. The target receptor(s) however have not been identified in the dogfish. Recent molecular biological techniques using the powerful polymerase chain reaction (PCR) and redundant oligonucleotide primers have identified cDNAs encoding both stimulatory and inhibitory adenosine receptors from the dog thyroid gland. The sequences of these cDNAs when translated into amino acid residues are members of the protein superfamily of G-coupled receptors. Prior to their expression and performance of binding studies the natural ligands for these receptors are unknown, and are therefore termed "orphan". Characteristically they possess a similar tertiary structure of seven hydrophobic transmembrane (7TM) spanning regions. We predicted that the adenosine receptors within the rectal gland will have a similar structure, and will provide an ideal system for subsequent detailed molecular physiological studies. In addition, this approach has the potential to identify all expressed cDNAs encoding G-coupled receptors within the rectal gland. For example, the human vasoactive inhibitory peptide (VIP) receptor has been cloned and shown to be a 7TM G-coupled receptor. VIP perfused into the rectal gland has been shown to be active in stimulating water and ion secretion, and is probably active via an homologous receptor.

Immediately following collection a single rectal gland was flash frozen by dropping into liquid nitrogen. This was in order to inhibit the activity of endogenous ribonucleases. Poly-A^+ messenger RNA (mRNA) was prepared (Fast-Track™, InVitrogen San Diego CA), and first strand cDNA reverse transcribed to serve as template for amplification (RT-PCR kit, InVitrogen San Diego CA). A programmable thermocycler (Techne PHC-2™, Princeton NJ) was used to amplify regions of G-coupled receptors using a pair of degenerate oligonucleotide primers designed to hybridise to segments of the third and sixth transmembrane domains. An amplification profile for 35 cycles was: dissociation at 95°C for 0.5 min, annealing at 40°C for 0.5 min, and annealing at 60°C for 1 min. A slow ramp rate was programmed between annealing and extension to maximise stability of the redundant primer extension products. Taq polymerase enzyme was from Cetus, Norwalk CT. The reaction products ranging in size from 200bp to 1Kb were separated in a 1% low melting agarose gel, and purified onto glassmilk (USBioclean™, USB Cleveland OH). Individual products were TA-cloned (Marchuk et al, Nucl. Acids Res. 19, 1154,1991; TA-cloning kit, InVitrogen, San Diego CA) and recombinants screened utilising blue-white selection (Ullmann et al, J. Mol. Biol. 24, 339-343, 1967). Double-stranded plasmid DNA sequencing use Sequenase 2.0™ enzyme (USB, Cleveland OH).

Sequence manipulation utilised the DNAStar computer program (Lasergene™) running on an Apple Macintosh IIcx microcomputer. A single open-reading frame continued from the translated 3'end of the IIITM primer for two clones of about 450bp. Alignment with each other showed a 50% amino acid absolute identity. Homology was greatest at the predicted IV and V transmembrane regions. However, database comparison with Genbank revealed only weak homology to the rat substance K receptor sequence, again at predicted IVTM and VTM predicted segments. There is no significant homology with the published sequences of

dog adenosine stimulatory (RCD8) or inhibitory (RCD7) receptors (Libert et al, Science 244, 569-572, 1989; figure 1).

Figure1: Amino acid alignment of shark rectal gland orphan receptors (SOR1, SOR2), rat substance K receptor (RATSUBK), dog adenosine receptors (RCD8, RCD7), rat testis adenosine receptor (TGPCR1), rat muscarinic M1 receptor (RATM1), rat serotonin HT1 receptor (RAT5HT1), and human vasoactive inhibitory peptide receptor (HUMVIP). Asterisks (*) denote highly conserved residues between receptors. The amino acids of the fourth transmembrane domain (TM IV) are underlined.

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SOR1      VVVAYPIRQRIRPRSCAYIVAF--IWLVSIGVS-MPSSLHT
              *                *        *
SOR2      VVVAYPIRQRITLSCCGLIMG--AIWVLSMALAPQPPSTSC
              *                *        *
RATSUBK   MAIVHPFQPRLSAPSTKA-IIAGI-WLVALALAS-PQC-F-YS
              *                *        *
RCD8      IAIRIPLRYNGLVTGTRAKGIIAVCWVLSFAIGLTPM--LGWN
              *                *        *
RCD7      LRVKIPLRYKTVVTPRRAAVAIAGCWILSFVVGLTPL--FGWN
              *                *        *
TGPCR1    LRVKLTVRYRTVTTQRRIWLFLGLCWLVSFLVGLTPM--FGWN
                               *        *
RATM1     FSVTRPLSYRAKRTPRRAALMIGLAWLVSFVLWA-PAILF-WQ
              *                *        *
RAT5HT1   VAIRNPIEHSRFNSRTKAIMKIAIVWAISIGVSV-PIPVIGLR
              *                *        *
HUMVIP    LSITYFTNTPSSRKKMVRRVVCILVWLLAFCVSL-PDTYYLKT
                                          TM IV
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The pair of putative orphan 7TM receptor encoding cDNAs isolated from the dogfish rectal gland were used as probes to screen a rectal gland cDNA library. No positives were detected either on screening this library or another available rectal gland library provided by Dr. E. Benz. This may reflect a very low abundance message, undetectable by conventional cDNA library screening. We are proceeding to construct a shark total genomic DNA library, and plan to screen this with the orphan receptors.

It remains an open question as to whether the sequences of the two orphan receptors are those coding for dogfish stimulatory and inhibitory receptors. Even though the sequences are markedly different from the adenosine receptor of "higher" species, we have previously shown that the dogfish atrial prepronatriuretic factor (prepro-CNP) exhibits poor homology with the equivalent cDNA sequences of all other species. An alternative is that these new orphan receptors may be the shark VIP or substance K receptors for example. These questions will be resolved following isolation and expression of the full-length cDNA sequences in conjunction with ligand-binding studies, i.e. exactly the route taken to identify canine RCD7 and RCD8 as adenosine receptors.

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