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Mutation screening of the muscarinic M_2 and M_3 receptor genes in normal and asthmatic subjects

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1 Muscarinic receptors are important in the development of airway hyperresponsiveness, and dysfunction of these receptors has been suggested to be present in asthma.

2 The human muscarinic M_2 and M_3 receptor genes were screened for polymorphic variation using single-stranded conformation polymorphism (SSCP) analysis, complemented by direct fluorescent sequencing. Forty-six random DNA samples and 46 respiratory physician diagnosed asthmatic samples were used as a template for analysis.

3 Within the muscarinic M_2 receptor gene, we identified two degenerate single base substitutions (1197T→C, Thr→Thr and 976A→C, Arg→Arg) in one random and one asthmatic sample respectively. Analysis of the 3′ UTR region revealed an additional 'A' at bp 1793 (c.f. ATG). This was present in all of 49 samples analysed by sequencing or BsmI digest, suggesting that the published sequence (GenBank Accession No. M16404) is incorrect. A common 3′ UTR polymorphism (T→A) was found at bp 1696 (c.f. ATG) (allelic frequency = 65%, $n = 60$), but this does not alter transcription factor recognition sites.

4 We were unable to identify any polymorphic variation within the muscarinic M_3 coding region or the flanking regions investigated, using the methods described.

5 The coding regions for the human muscarinic M_2 and M_3 receptor genes are both highly conserved. These data suggest that polymorphic variation within these coding sequences is unlikely to account for inter-individual variability in response to methacholine or anticholinergic therapy. The potential functional significance of the muscarinic M_2 receptor 3′ UTR polymorphism (bp 1696) remains to be determined.

British Journal of Pharmacology (2001) **133,** 43–48

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Abbreviations: DAG, diacylglycerol; EPO, eosinophil peroxidase; IL-1β, interleukin 1-beta; IP_3, inositol triphosphate; MBP, major basic protein; PCR, polymerase chain reaction; PIP_2, phosphatidylinositol-4,5-biphosphate; PKC, protein kinase C; PLC, phospholipase C; RFLP, restriction fragment length polymorphism; SSCP, single-stranded conformation polymorphism; TNFα, tumour necrosis factor-alpha; UTR, untranslated region

Introduction

Muscarinic receptors are members of the family of G-protein coupled seven trans-membrane spanning proteins. They are responsible for mediating effects of cholinergic neurotransmission, *via* signal transduction mechanisms which are activated following binding of acetylcholine to receptor. Five receptor subtypes have been identified to date (M_1 to M_5), of which only the first three are found in human lung (Barnes, 1992). In the airways, M_1 receptors are mainly localized to parasympathetic ganglia, and their stimulation acts to facilitate cholinergic neurotransmission (Mak & Barnes, 1989). Muscarinic M_2 receptors are found both prejunctionally on postganglionic cholinergic nerve terminals, as well as postjunctionally on effector organs, mainly airway smooth muscle. Prejunctional M_2 receptors modulate acetylcholine release *via* a negative feedback mechanism, and thus function as autoreceptors (Minette & Barnes, 1988). Acute stimulation of postjunctional M_2 receptors acts to inhibit adenylate cyclase activation *via* coupling to an inhibitory G-protein G_i, and they therefore decrease the degree of cyclic AMP-induced airway smooth muscle relaxation, whereas chronic stimulation results in sensitization of adenylate cyclase (Billington *et al*., 1999). Muscarinic M_3 receptors are present on human airway smooth muscle and sub-mucosal glands (Mak & Barnes, 1990). They activate phospholipase C (PLC) *via* coupling to $G_{q/11}$. This results in the production of inositol triphosphate (IP_3) (Chilvers *et al*., 1990) and diacylglycerol (DAG) from phosphatidylinositol-4,5-biphosphate (PIP_2) (Baron *et al*., 1984). DAG activates protein kinase C (PKC) (Nishizuka, 1986) resulting in the phosphorylation of many key intracellular proteins, including G_s, adenylate cyclase and the β_2-adrenoceptor. IP_3 acts to release Ca^{2+} ions from the endoplasmic reticulum, *via* IP_3-sensitive Ca^{2+} channels. M_3 receptor activation, therefore both promotes Ca^{2+}-induced smooth muscle contraction and inhibits sympathetically-induced relaxation.

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The genes for the human M_2 and M_3 receptors were both initially cloned and characterized in 1987 by two groups working independently (Bonner *et al*., 1987; Peralta *et al*., 1987). The human M_2 receptor gene is 1.4 kb long and is located on chromosome 7q35-qter. It codes for a 466 amino acid protein. The M_3 gene (1.77 kb) is localized to chromosomal region 1q43-44 and codes for a 590 amino acid protein. Both genes are intronless. There has been some confusion in the early scientific literature regarding muscarinic receptor nomenclature, following the reversal of the M_3 and M_4 subtype names by the two groups which originally cloned the receptors. The International Union of Pharmacology (IUPHAR) subcommittee on muscarinic acetylcholine receptors has however officially adopted the nomenclature originally used by Bonner *et al*. (1987), and this nomenclature has been used throughout this paper.

Published studies have suggested that a degree of M_2 receptor dysfunction may be present in asthmatic patients. This would be predicted to have two functional consequences. First, muscarinic M_2 receptor dysfunction could lead to excessive acetylcholine release, resulting in increased activation of postjunctional muscarinic M_3 receptors on airway smooth muscle and submucosal glands. Evidence for this comes from studies using pilocarpine, an M_2 receptor agonist, which exerts an inhibitory effect on SO_2-mediated bronchoconstriction in healthy individuals, but fails to do so in asthmatics (Minette *et al*., 1989). Secondly, dysfunctional postjunctional muscarinic M_2 receptors on airway smooth muscle would result in a reduced ability to inhibit relaxant mechanisms because of reduced inhibition of adenylate cyclase. Dysfunctionality of muscarinic receptors, mostly due to allosteric modulation of the receptor, has been already shown to occur in the presence of inflammatory proteins normally present in asthmatic airways, such as major basic protein (MBP) and eosinophil peroxidase (EPO) (Jacoby *et al*., 1993; Costello *et al*., 1998). Exposure to viral neuraminidase (e.g. from parainfluenza virus type 1 infections) has also been shown to exert such an influence (Fryer *et al*., 1990; Fryer & Jacoby, 1993). Tumour necrosis factor-alpha (TNFα) and Interleukin 1-beta (IL-1β) have been reported to act in synergy to downregulate M_2-receptor mRNA and consequently expressed protein (Barnes *et al*., 1997).

Whilst the above mechanisms might account for the effects of inflammation on muscarinic receptor functioning, they would not explain the inter-individual variation in responsiveness which has been reported (Ihre & Larsson, 1990). We therefore postulated that any potential polymorphic variation within the M_2 and M_3 genes might contribute towards altered cholinergic-mediated symptomatology in asthma, as well as help in understanding the wide inter-patient variation observed in the clinical respose to anticholinergic therapy. We therefore undertook the screening of the entire coding regions of the muscarinic M_2 (GenBank Accession No. M16404) and M_3 (GenBank Accession No. U29589) receptors for polymorphisms by an approach which involved single-stranded conformation polymorphism (SSCP) analysis supported by direct fluorescent sequencing.

Methods

Selection of subjects

Maltese asthmatic patients Whole blood (4 ml) was collected from patients ($n=46$, male = 25, female = 21) attending the Asthma Outpatients Clinic at St Luke's Hospital, Guardamangia, Malta. All patients were physician diagnosed asthmatics, and all showed a positive skin-test response to at least one common allergen. The mean age was 48.8 years (range: 15–81 years).

Maltese random population Random samples from cord blood were obtained from an anonymous DNA bank of Maltese individuals, held by the Laboratory of Molecular Genetics, Department of Pathology on Campus, University of Malta. Whilst no phenotype information is available on these subjects, the prevalence of physician-diagnosed childhood asthma in Malta is 7.5% (ISAAC, 1998) and hence the majority of these subjects would be expected to be non-asthmatic.

Approval by the Ethics Committee of the Faculty of Medicine and Surgery, University of Malta, and informed patient consent were obtained prior to sampling.

DNA extraction and PCR

DNA was extracted from whole blood using the method of salting out (Miller *et al*., 1988). Suitable oligonucleotide primers were designed in order to generate adjacent overlapping PCR products from each of the M_2 and M_3 coding regions (Figure 1, Table 1). Parts of the 5′ and 3′ untranslated regions (UTR) were also included (M_2: -174 to $+459$, M_3: -128 to $+53$). The degree of overlap between adjacent fragments was 46 bp or more, and the maximum fragment length was 298 bp.

PCR reactions (25 μl) were prepared, using the following final concentrations per tube: forward primer: 0.5 μM; reverse primer: 0.5 μM; dATP, dTTP, dGTP, dCTP: 0.2 mM of each; genomic DNA: approximately 0.2 μg, Supertaq^R DNA polymerase: 0.5 U per tube, in a buffered solution containing (mM) Tris-HCl 10, $MgCl_2$ 1.5, KCl 50 and 10^{-4}% w/v Triton X-100. PCR reaction mixes were overlaid with one drop of mineral oil in order to minimize losses by evaporation. All PCR reagents were purchased from Kramel Biotech, Northumberland, UK. Oligonucleotide primers were purchased from Oswel, Southampton, UK and Laboratori Genenco, Firenze, Italy. The cycling reactions were carried out in a Techne^R Cyclogene or Techne^R GeneE thermal cycler at the following conditions: hotstart: 96°C for 5 min, denaturation: 94°C for 1 min, annealing: x°C for 1 min, extension: 72°C for 1 min, for 30 cycles, followed by a final extension at 72°C for 10 min. Values for the annealing temperatures x are given in Table 1.

Mutation screening

Single-stranded conformation polymorphism (SSCP) analysis was used initially to screen each fragment for potential mutations. The method is based on the sequence-dependent migration of short lengths of single-stranded DNA in a non-denaturing polyacrylamide gel, and when carried out twice

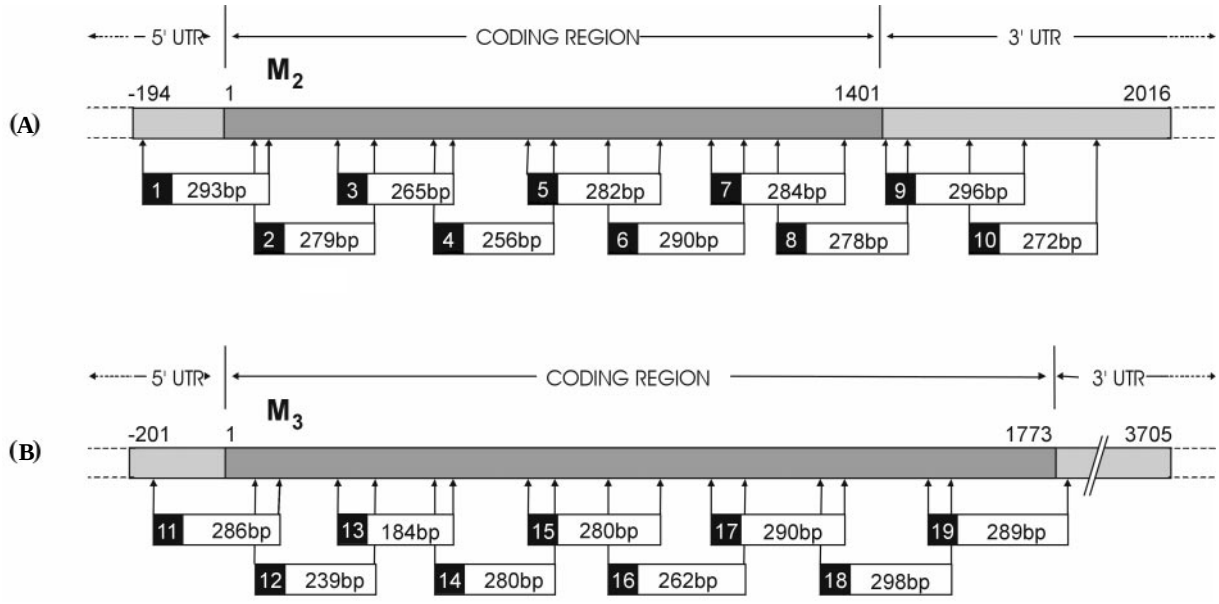

Figure 1 Position and size of PCR fragments used to screen the (A) muscarinic M_2 and (B) muscarinic M_3 receptor genes for polymorphic variation. The fragment numbering is included for reference.

Table 1 Data for the oligonucleotide primers used to PCR fragments from the muscarinic M_2 receptor (sets 1–10) and the muscarinic M_3 receptor (sets 11–19)

PCR primer	*Sequence*	*Fragment length* (bp)	*Overlap with previous fragment* (bp)	*Annealing temperature used during PCR* (°C)
1F	5′- GAC ACA GTA ATC ATG CAG GGG AAG G -3′	293	–	59
1R	5′- CCG ATA ATG GTC ACC AAA CTG AGG G -3′			
2F	5′- AAG TGG TGT TTA TTG TCC TGG TGG -3′	279	55	59
2R	5′- GCA GAT TCA TAA CTG AGG CAT TGC -3′			
3F	5′- CCC TCT ACA CTG TGA TTG GT -3′	265	102	56
3R	5′- CTC ACC CCT ACA ATG AAC TG -3′			
4F	5′- CCT CTC TTT CAT CCT CTG GGC -3′	256	60	60
4R	5′- ACT TGG AGA AAC GGG GTC TTG -3′			
5F	5′- GGA TAA AGA AGG ACA AGA AGG AGC -3′	282	56	60
5R	5′- TGG AAA CTG TGT TTT CAT CCT GGG -3′			
6F	5′- ACT GTG TTC AGG GAG AGG AGA AGG -3′	290	111	57
6R	5′- AGT CAT CTT CAC AAT CTT GCG GGC -3′			
7F	5′- AGT GGG GTC TTC AGG TCA GAA TGG -3′	284	70	57
7R	5′- CAT AGC AGG CAG GGT TGA TAG TGC -3′			
8F	5′- GTT GGC TTT CAT CAT CAC TTG GGC -3′	278	143	59
8R	5′- CCT TTT ATT CTT CTC AAG CTC CCC -3′			
9F	5′- TGA AAA AGA TAG AAG GTG GG -3′	296	47	50
9R	5′- AAT TGT AGA ATC AGA AGC CC -3′			
10F	5′- AAA CTG TCA GTA TTA GGA GC -3′	272	117	50
10R	5′- TAT GTC CCC ATT GTA TAG TG -3′			
11F	5′- TTC AGC ACC CTG TAA TAG GC -3′	286	–	56
11R	5′- TCT GGA GAG GAG AAA TTG CC -3′			
12F	5′- GGC AGC TAC AAT GTT TCT CG -3′	239	47	56
12R	5′- ATA ATC AGA TCG GCA CAG GC -3′			
13F	5′- AAG ACG GTC AAC AAC TAC TTC C -3′	184	53	54
13R	5′- CCA GAA GAT TCA TAA CAG AGG C -3′			
14F	5′- ATG GGC CTT AGG GAA CTT GG -3′	280	83	56
14R	5′- GAG GAA CTG AAT GAA GCA CTC -3′			
15F	5′- GTT GGA AAG AGA ACT GTG CC -3′	280	48	56
15R	5′- TGG AGC GTT TCA TGC TTT GC -3′			
16F	5′- ACG GGC AGT TCT CGA AGC T -3′	262	61	56
16R	5′- GCT TGA GCA CGA TGG AGT AG -3′			
17F	5′- AGG ACA TTG GCT CCG AGA CG -3′	290	48	56
17R	5′- CGT GCT CTT ACC CAC TGA GG -3′			
18F	5′- CAC AGC TAA GAC TTC TGA CG -3′	298	46	56
18R	5′- GAT GTA GCA CAG CCA GTA GC -3′			
19F	5′- TGA ACA CCT TTT GTG ACA GC -3′	289	68	56
19R	5′- AGG TCT GTG GGT TGA TGT GT -3′			

per sample, using different temperatures, screening sensitivities are reported to be higher than 90% especially when the DNA fragments do not exceed 300 bp in length (Vidal-Puig & Moller, 1994; Jordanova *et al*., 1997; Hayashi & Yandell, 1993).

PCR products (4 μl) were denatured by heating with 4 μl denaturing solution (0.04% bromophenol blue and 0.05% xylene cyanol in 99% formamide) at 96°C for 6 min, and immediately transferred to ice. The denatured samples were loaded onto a precast Pharmacia^R GeneGel Excel 12.5/24 polyacrylamide gel (5 μl per well), and electrophoresis was carried out on a peltier-cooled Pharmacia^R GenePhor electrophoresis unit for 3.5 h at 600 V. SSCP analysis was carried out twice per sample, using temperatures of 5 and 15°C respectively.

The gels were subsequently silver-stained according to conventional methods (Gustav *et al*., 1997), and scanned using a Biorad^R Fluor-S MultiImager.

Automated direct fluorescent sequencing of PCR products was used to confirm the presence of polymorphisms where suggested by SSCP.

In order to identify the presence of any potential common polymorphisms which were not resolved by SSCP, direct automated sequencing of the coding regions of both the M_2 and M_3 genes was carried out on a second set of 10 Maltese patient DNA samples. The sequencing strategy ensured that sequence data obtained for successive parts of the genes, overlapped by at least 100 bp. This was done in order to avoid internal sequence gaps due to the difficulty normally encountered in obtaining readable sequence for the terminal segments of a DNA fragment.

Results

Screening of the muscarinic M_2 receptor gene

Four novel polymorphic variants were identified within the muscarinic M_2 receptor gene and its flanking regions (Table 2). These were: (i) A single base substitution (T→C) within the coding region at nucleotide 1197 (c.f. ATG). This was only observed in one sample from the random population, and it was observed as a mobility shift on the SSCP gel (Figure 2). The substitution was degenerate (ACT→ACC, Thr→Thr) i.e. it did not alter the amino acid sequence at the receptor. Restriction mapping revealed that this polymorphism introduced restriction sites for the following enzymes: *Hph*I, *Scr*FI, *Bst*NI, *Bsa*JI, *Psp*GI and *Bss*KI. (ii) A single base substitution (T→A) in the 3′ UTR at position 1696 (*c.f.* ATG) (Figure 2). This was also observable by SSCP. The polymorphism did not modify the restriction map of the fragment nor did it alter any transcription factor consensus sequence. Its allelic frequency was determined by direct sequencing to be 63% in Maltese asthmatics ($n=40$) and 68% in the random population ($n=20$). (iii) A coding region A→C substitution at position 976 (*c.f.* ATG). This was identified in one patient sample from the directly sequenced group of 10 patients. The substitution is degenerate (AGA→CGA, Arg→Arg) and introduces new restriction sites for *Bst*FSI and *Fok*I. (iv) An additional 'A' in the 3′ UTR at position 1793 (*c.f.* ATG) which was present in each of 10 sequenced patient samples. This insertion results in the deletion of a *Bsm*I recognition site. Sequencing of a further 10 samples (random samples $n=5$, patient samples $n=5$) and restriction fragment length polymorphism (RFLP) analysis of an additional 29 samples (random samples, $n=14$, patient samples, $n=15$) suggested the presence of this insertion in all samples. This insertion introduces a previously unrecognised C-Rel/NF-κB consensus sequence into the 3′ UTR.

A search for potential splice site sequences throughout the whole M_2 coding region and flanking sequences, was carried out using the potential splice site finder facility available as part of the Baylor College of Medicine Gene Finder available online at http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html. None of the polymorphisms listed above was found to potentially contribute to altered splicing patterns.

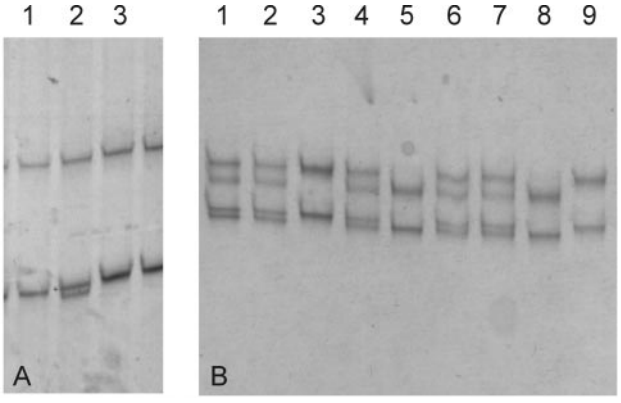

Figure 2 SSCP gels showing muscarinic M_2 receptor fragment No. 7 (A) and fragment number 10 (B). (A) Lanes 1,3: Wild type fragments; Lane 2: 1197T→C heterozygous substitution. (B) Lanes 3,9: Wild type fragments; Lanes 5,8: 1696T→A homozygous substitution; Lanes 1,2,4,6,7: 1696T→A heterozygous substitution.

Table 2 Polymorphisms identified in the muscarinic M_2 and M_3 receptors

Polymorphism	*Location*	*Comments*
Muscarinic M_2 receptor		
1197T→C	Coding region	Degenerate (ACT→ACC, Thr→Thr)
1696(T→A)	3′ UTR	Common polymorphism
976(A→C)	Coding region	Degenerate (AGA→CGA, Arg→Arg)
1793A	3′ UTR	Error in published sequence
Muscarinic M_3 receptor		
None		

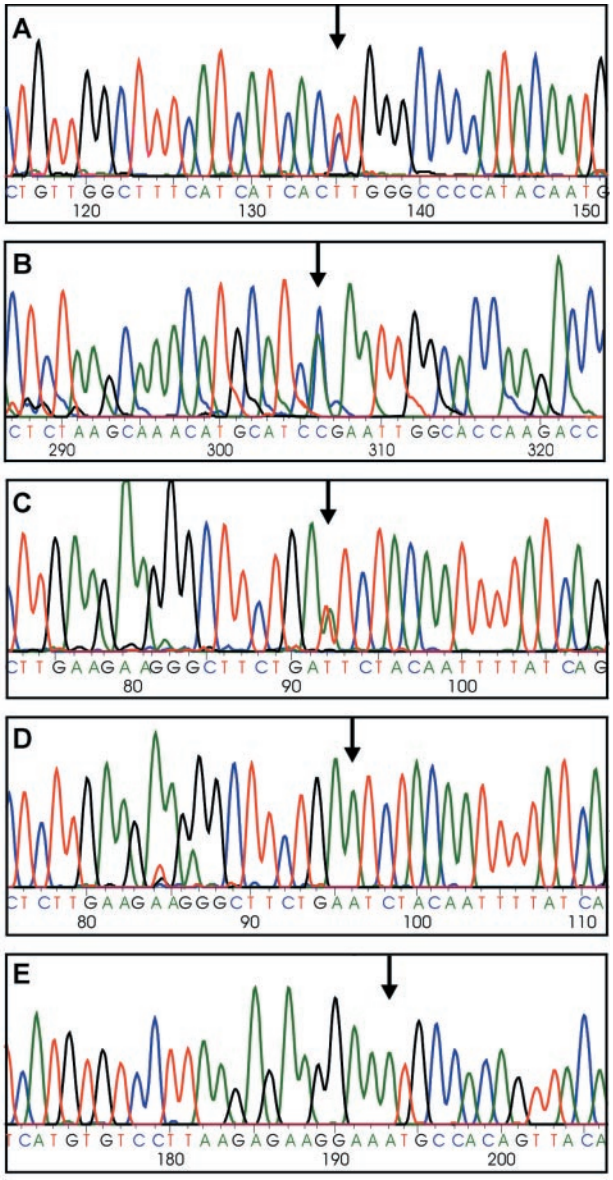

Figure 3 DNA sequencing electropherograms showing the (A) 1197T→C heterozygous substitution, (B) 976A→C heterozygous substitution, (C) 1696T→A heterozygous substitution, (D) 1696T→A homozygous substitution and (E) 1793A homozygous insertion.

Screening of the muscarinic M_3 receptor gene

We were unable to identify any polymorphic variation within the muscarinic M_3 coding region or the flanking regions investigated, using the methods described. Further to the complete sequencing of 10 extra patient DNA samples mentioned earlier, we also sequenced 30 randomly chosen fragments from the 46 asthmatic and 46 random DNA samples which had already been analysed by SSCP. This was carried out in order to intensify the search for any polymorphisms which were SSCP-silent. All sequencing data obtained, was however in perfect agreement with the published wild type sequence.

Discussion

We have identified four novel polymorphic variants of the muscarinic M_2 receptor gene. Two of these variants are degenerate single base substitutions in the coding region which do not appear to alter splicing patterns. A third variant (1696T→A) is a common polymorphism found in the 3′ UTR about 300 bp away from the stop codon, which however does not alter transcription factor consensus sequences. Costello *et al*. (2000) have found no relationship between this polymorphism and the change in airway resistance following vagally-induced bronchoconstriction. The fourth variant (1793A) was identified in every sample ($n=49$) which has either been directly sequenced or analysed by RFLP. We therefore suggest that the published muscarinic M_2 receptor gene sequence (GenBank Accession No. M16404) contains a missing 'A' in the 3′ UTR at position 1793 (*c.f.* ATG). The introduction of a previously unrecognized C-Rel/NFκB consensus sequence by this 'insertion' updates the transcription factor map downstream of the gene. Regulation of M_2 receptor expression at a transcriptional level is however not yet clearly understood and the significance of this recognition sequence remains to be determined.

In contrast to the M_2 receptor, we were unable to find any polymorphic variation within the M_3 receptor gene. Although we may have missed polymorphisms using SSCP alone, an intensified search using a direct sequencing approach indicates that any polymorphisms within this gene must be rare in the European population. This is unlikely to be due to isolation of the Maltese population studied, because other genetic studies in this population, such as haplotype analysis of neutral β-thalassaemia polymorphisms have shown trends which are similar to other Mediterranean based populations (Pulis, 2000). Given that direct sequencing of the entire coding region of the M_3 gene was performed for 10 individuals (i.e. 20 alleles), it is unlikely that any polymorphism exists with a frequency greater than 5% in this population. Goldman's group has however recently described five single nucleotide polymorphisms in the human muscarinic M_3 receptor gene in the USA (Rho *et al*., 2000).

The polymorphism picture presented by muscarinic M_2 and M_3 receptor genes contrasts markedly with the β_2-adrenoceptor which exhibits a much higher rate of polymorphic variation. Nine single base substitutions have to date been identified in the coding region. Whilst five of these are degenerate, and are unlikely to be functionally significant, three of the other four (all of which result in single amino acid substitutions) have demonstrable *in vivo* functional effects (Reihsaus *et al*., 1993; Hall *et al*., 1995; Hall, 1996). In addition, at least eight other single nucleotide polymorphisms exist in a 1.5 kilobase upstream region containing the β_2-adrenoceptor gene promoter (Scott *et al*., 1999).

We conclude that the coding regions of the human muscarinic M_2 and M_3 receptor genes are highly conserved in the population studied. Polymorphic variation within these regions is unlikely to contribute to muscarinic receptor dysfunction in asthma or to the observed inter-patient variation in clinical response to anticholinergic treatment. The potential significance of the M_2 3′ UTR polymorphism at position 1696 remains to be determined.

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