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ABSTRACT

α_1-Adrenoceptor has been discovered to exist in many human tissues and mediates important physiological functions. The purpose of this study was to detect the expression, distribution, and function of α_1-adrenoceptor subtypes in human submandibular glands. α_{1A}- and α_{1B}-Adrenoceptor mRNAs were identified by reverse-transcription/polymerase chain-reaction (RT-PCR), and their proteins were detected by Western blotting. No expression of the α_{1D}-adrenoceptor mRNA and protein was found. By *in situ* hybridization and immunohistochemistry, α_{1A}- and α_{1B}-adrenoceptor mRNAs and proteins were shown to be widespread in both ductal and acinar cells. By confocal microscopy, phenylephrine (stimulating both α_{1A}- and α_{1B}-adrenoceptors) or A61603 (α_{1A}-selective agonist) induced an increase in intracellular calcium by 2.33 ± 0.18-fold and 1.81 ± 0.43-fold, respectively, while 5-methylurapidil (α_{1A}-selective antagonist) partly blocked calcium mobility stimulated by phenylephrine. The results indicated that functional α_{1A}- and α_{1B}-adrenoceptors were expressed in human submandibular glands, and might contribute to the regulation of saliva synthesis and secretion.

KEY WORDS: α_1-adrenoceptor, receptor subtypes, neurotransmitter, submandibular gland, tissue distribution.

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INTRODUCTION

α_1-Adrenoceptor plays important roles in mammalian physiology, such as function regulation, energy metabolism, and stress response, by interacting with catecholamine (for review, see Docherty, 1998). α_1-Adrenoceptor has been characterized according to its different affinities for the competitive antagonist WB4101 and prazosin by ligand-binding (Morrow and Creese, 1986), and has been distinguished by the selective alkylating agent chloroethylclonidine in inactivating the α_{1B}, but not α_{1A}, subtype (Han *et al.*, 1987). Until now, three distinct α_1-adrenoceptor subtypes (α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor) have been identified through molecular cloning techniques and pharmacological assays (for review, see Gregory *et al.*, 2000). The cDNAs encoding human α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptors have also been cloned (Schwinn *et al.*, 1990; Hirasawa *et al.*, 1993; Weinberg *et al.*, 1994; Esbenshade *et al.*, 1995), and different α_1-adrenoceptor subtypes have been localized in several human organs, such as the heart, liver, kidney, spleen, and prostate (Price *et al.*, 1994; for review, see Ruffolo *et al.*, 1994).

It has been suggested that the secretory function of mammalian salivary glands is primarily regulated through the complicated action of distinct receptors such as α-adrenoceptors, β-adrenoceptors, muscarinic-cholinergic receptors, and peptidergic receptors (for review, see Baum, 1993; Ekström *et al.*, 1993; Baum and Wellner, 1999). The α-adrenoceptor has been found to regulate the secretion of fluid and electrolytes and may have an effect on the secretion of protein in saliva (Klein, 2002). Secretory responses evoked by sympathetic neurotransmitters could be mediated through the activation of the α_1-adrenoceptor in both innervated and denervated rat submandibular glands (Bylund *et al.*, 1982; Elverdin *et al.*, 1984), and the stimulating effect of the α_1-adrenoceptor on rat parotid acinar cells was confirmed *in vitro* by an increase in potassium release (Ito *et al.*, 1982). The presence of α_{1A}- and α_{1B}-adrenoceptor mRNA has been confirmed in rat submandibular glands (Faure *et al.*, 1994; Rokosh *et al.*, 1994; Nishiura *et al.*, 2001). α_{1A}- and α_{1B}-Adrenoceptor proteins have been identified in rat submandibular glands and an acinar cell line (SMG-C10) (Bockman *et al.*, 2004).

Until now, however, limited information exists regarding the α_1-adrenoceptor subtypes in human submandibular glands, and their physiological and pathological significance remains to be determined. In this report, we have detected the expression, distribution, and function of α_1-adrenoceptor subtypes in human submandibular glands and have attempted to explore the possible regulatory function of α_1-adrenoceptor subtypes in saliva secretion.

MATERIALS & METHODS

Materials

Fresh submandibular gland tissues were obtained from ten patients (mean age,

Table. Primers for the α_1-adrenoceptor Subtype mRNA

	Upper Primer	Lower Primer	Fragment Size	Temp.
α_{1A}-adrenoceptor	5'-CCA TCT CCC TGG TCA TAT CC-3'	5'-CCA TGT CCT TGT GTT GCC-3'	651 bp	57.6°C
α_{1B}-adrenoceptor	5'-CCT TCC GAG CCC AAT CAT-3'	5'-CCT TGG CCT TGG TAC TGC TA-3'	841 bp	59.2°C
α_{1D}-adrenoceptor	5'-CGA ACC CCC AGG CAC GCC CGA GA-3'	5'-TTA CCC CCA AGC CCA GCA CAC TC-3'	538 bp	64.0°C
β-actin	5'-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG GCG-3'	5'-CGC CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3'	843 bp	60.0°C

61.6 yrs [52-71]; three females, seven males) receiving functional neck dissection for primary oral squamous cell carcinoma with negative cervical lymph node metastasis. In this study, human samples were used with the patients' informed consent and the local ethics committee's approval. The obtained submandibular gland tissue was histologically normal, by hematoxylin and eosin staining, and no clinical or pathological metastasis and inflammatory cells were detected.

Reverse-transcription/Polymerase Chain-reaction (RT-PCR)

Total RNA was isolated by the Trizol method, and each sample was quantitated spectrophotometrically at OD260/280. RT-PCR was carried out according to the manufacturer's protocol (Gibco-BRL, Gaithersburg, MD, USA), with 1.0 μg of total RNA. The α_1-adrenoceptor subtype-specific primers (Table) were designed and synthesized (AoKe, Beijing, China). Amplification of β-actin was performed for internal standardization. cDNAs from human submandibular gland tissues without reverse-transcriptase treatment and human α_1-adrenoceptor subtype full-length cDNAs were amplified as negative and positive controls, respectively. The band densities were analyzed semi-quantitatively by image analysis after gel electrophoresis (LEICA550IW).

Probes for *in situ* Hybridization

The human α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor probes consisted of 440-bp, 528-bp, and 525-bp *Eco*RI-*Bam*HI fragments corresponding to nucleotides 962 to 1401 of cloned human α_{1A}-adrenoceptor cDNA (GenBank L31774), 1028 to 1554 of cloned human α_{1B}-adrenoceptor cDNA (GenBank L31773), and 1192 to 1716 of cloned human α_{1D}-adrenoceptor cDNA (GenBank L31772), respectively. They were linked to the pGEM-7Z vector, which was confirmed by restriction nuclease digestion and identification. Antisense and sense single-stranded digoxigenin-labeled RNA probes were generated from linearized α_1-adrenoceptor subtype cDNA constructs by RNA polymerase T7 and SP6 by *in vitro* reverse-transcription as described in the Promega protocols.

In situ Hybridization

Small tissue blocks were quickly excised and fixed in freshly prepared 4% (w/v) paraformaldehyde in PBS, pH 7.4, at 4°C. After being dehydrated with a series of ascending grades of ethanol, tissues were embedded in paraffin wax, sectioned, and mounted. The sections were deparaffinized, rehydrated, digested with proteinase K (2 μg/mL in PBS), and fixed according to established procedures (Yang *et al.*, 1999). Subsequently, the sections were hybridized with the specific digoxigenin-labeled probes and incubated at 42°C for 16 hrs. After hybridization, the sections were blocked by treatment with horse serum and incubated with anti-digoxigenin-alkaline phosphatase, prior to color development with nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate in the dark.

Immunohistochemistry

Frozen tissue specimens 5 μm thick were prepared. The cryostat sections were fixed in acetone:chloroform (1:1) and immunostained with the primary polyclonal antibodies diluted 1:100 in 2% BSA/PBS for α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptors (Santa Cruz, Santa Cruz, CA, USA), according to routine immunohistochemistry protocols (Cohen *et al.*, 1995).

Western Blotting

Tissues were minced in lysis buffer (pH 7.4) containing 20 mM Tris, 0.5 mM EDTA, 25 μg/mL aprotinin, 25 μg/mL leupeptin, 20 μg/mL pepstatin A, and 174.2 μg/mL phenylmethylsulfonyl fluoride, and homogenized at 4°C. After centrifugation, the membrane pellet was solubilized in the above buffer containing 0.5% sodium deoxycholate, 1.5% NP-40, and 0.1% SDS. The protein concentration was determined by the method of Lowry *et al.* (1951).

Aliquots (50 μg) of the soluble proteins were denatured in the loading buffer and resolved on 10% SDS-PAGE gels. The proteins were then electroblotted onto PVDF membrane at 100 V for 90 min after the gels were run. The membranes were probed overnight with goat anti-α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor antibodies (diluted 1:100 in blocking buffer) at 4°C, and then incubated with horseradish-peroxidase-conjugated anti-goat IgG antibody. The immunoreactive bands were visualized by chemiluminescence detection (Amersham, Oakville, ON, Canada).

Submandibular Gland Cell Culture

Acinar cells from human submandibular glands were freshly isolated, as described previously (Horn *et al.*, 1988), in Hanks' balanced salt solution buffered with HEPES to pH 7.5 (containing 0.05% BSA, 100 U/mL collagenase, 0.2 mg/mL hyaluronidase). The resulting cell suspension was centrifuged at 400 *g* for 5 min and gently re-suspended in DMEM medium, then plated on a 35-mm Petri dish coated with 50 μg/mL poly-D-lysine and cultured in an atmosphere of 5%CO_2 in air at 37°C for 12 hrs.

Intracellular Calcium Imaging

The cultured cells were washed with Hanks' balanced salt solution, then loaded with 10 μM fluo-3-AM at 37°C for 30 min. The cells were washed and stimulated with phenylephrine (1 μM) or A61603 (100 μM; Sigma, St. Louis, MO, USA). For some experiments, 5-methylurapidil (0.1 μM) was added 30 min before the treatment with phenylephrine. The addition of KCl (50 mM) was used as the positive control. Calcium fluorescence images were examined by confocal microscopy and quantified as described previously (Premkumar and Ahern, 2000).

Statistical Analyses

Data were analyzed by unpaired Student's *t* test between groups. A probability of less than 0.05 was assumed to be significant. Data were presented as means ± SEM.

RESULTS

Reverse-transcription/ Polymerase Chain-reaction

To detect α_1-adrenoceptor subtypes in human submandibular gland, we designed the specific primers according to their sequences. The specificity of the primers was confirmed by DNA sequencing of the corresponding gene products and by amplification of human α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor full-length cDNAs (Figs. 1A, 1B, 1C) as positive controls. The amplification efficiencies of the target cDNAs and β-actin were detected at various cycle numbers from 30 to 39 (data not shown). α_{1A}- and α_{1B}-Adrenoceptor mRNAs were identified in all specimens tested (n = 10; Figs. 1D, 1E, 1F). The relative intensities of transcripts for α_{1A}- and α_{1B}-adrenoceptor mRNA in the submandibular gland samples were compared, with normalization to the house-keeping gene β-actin (Fig. 1G). α_{1A}-Adrenoceptor mRNA was more abundantly distributed than α_{1B}-adrenoceptor mRNA in human submandibular glands. No expression of α_{1D}-adrenoceptor mRNA was identified.

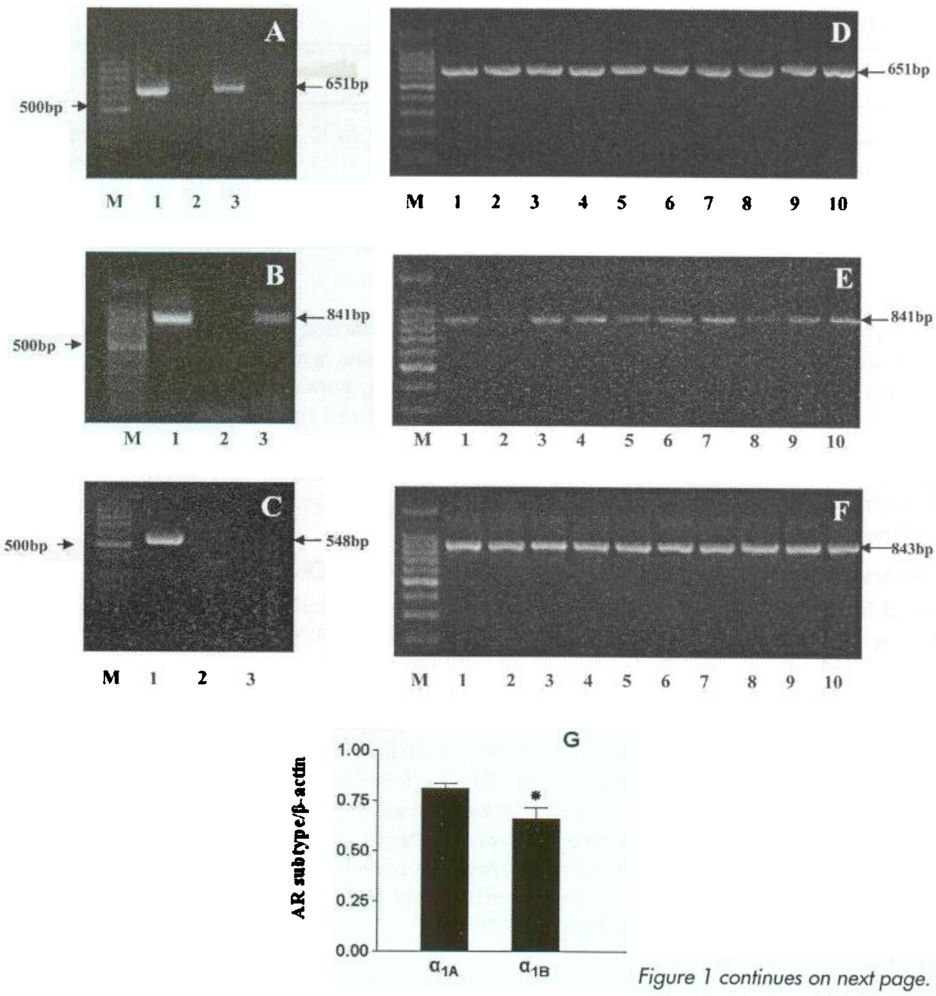


Figure 1 continues on next page.

Figure 1. The gene imaging and protein expression of α_1-adrenoceptor subtypes in human submandibular glands. RT-PCR products from human submandibular glands were eletrophoresed on 2% agarose gels and visualized with ethidium bromide. M, Marker (100-bp DNA ladder; the lightest band is 500 bp). **(A,B,C)** α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor gene expression, respectively. Lane 1 in A, B, and C indicates RT-PCR product amplified from human α_1-adrenoceptor subtype full-length cDNA, as a positive control. Lane 2 in A, B, and C indicates RT-PCR product without using reverse-transcriptase, as a negative control. Lane 3 in A, B, and C indicates RT-PCR product amplified from human submandibular gland. **(D,E,F)** RT-PCR products of α_{1A}-, α_{1B}-adrenoceptor and β-actin amplified from individuals. **(G)** Comparison of the relative amounts of transcripts for α_{1A}-adrenoceptor subtype mRNA with α_{1B}-adrenoceptor subtype mRNA. Data were expressed as mean ± SEM (n = 10, *P* < 0.05). **(H,I)** α_{1A}- and α_{1B}-adrenoceptor gene expression by *in situ* hybridization (paraffin section), respectively. Intracytoplasmic hybridization signal was observed in acinar and ductal cells. **(J,K)** α_{1A}- and α_{1B}-adrenoceptor protein expression by immunohistochemistry (frozen section), respectively. Intracytoplasmic and membrane staining was seen in acinar and ductal cells. (Arrow a indicates acinar cells, arrow d indicates ductal cells.) **(L,M)** α_{1A}- and α_{1B}-adrenoceptor protein expression by Western blotting. Intensively immunoreactive bands of 55 kDa and 60 kDa, corresponding to the α_{1A}- and α_{1B}-adrenoceptors, were detected. 'Cont' indicates the positive control, with HEK293 cells transfected with the cDNAs encoding α_1-adrenoceptor subtypes. Lanes 1, 2, and 3 in L and M, respectively, indicate the expression of α_{1A}- and α_{1B}-adrenoceptors in human submandibular glands from three persons.

In situ Hybridization

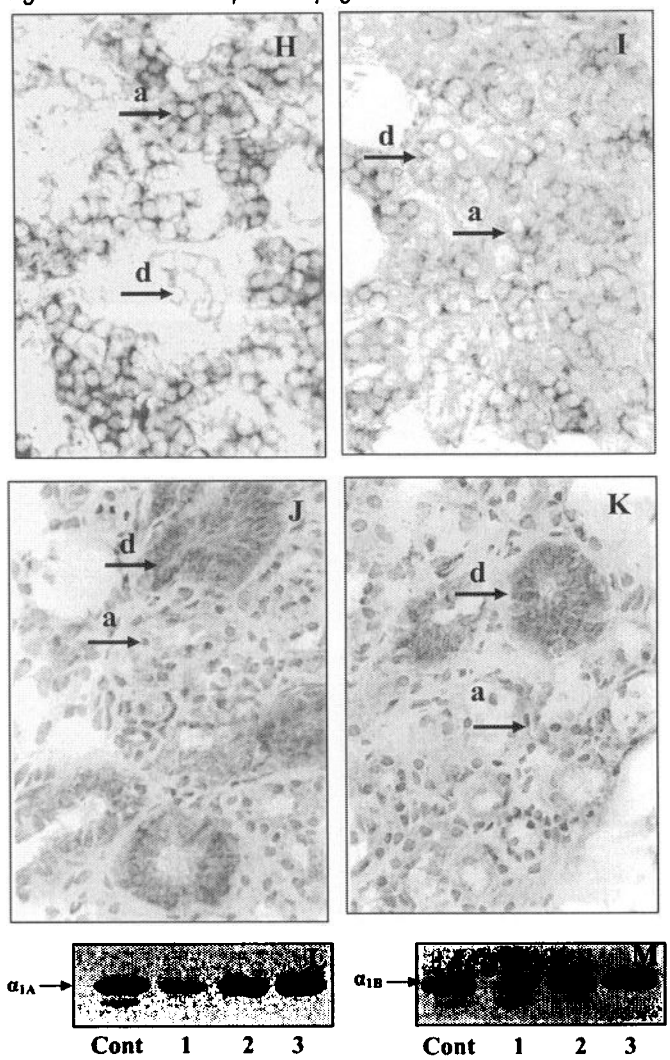
The specific probes corresponded to nucleotides that encoded mRNA from the end of the third cytoplasmic loop region of α_1-adrenoceptor subtypes, showed no homology, and were distinct from the transmembrane regions. The cDNA sequencing results confirmed the specificity of each probe (data not shown).

High expression levels of alpha-1 adrenoceptor were observed in the acinar cells of the submandibular glands. The positive hybridization signal was localized mainly in acinar cell cytoplasm (Fig. 1H). Much lower levels of labeling were seen in the striated, intercalated, and excretory ductal cells. The expression of α_{1B}-adrenoceptor mRNA had a similar distribution in submandibular glands (Fig. 1I), was expressed intensely in acinar cells, and at lower intensities in ductal cells. No expression of α_{1D}-adrenoceptor mRNA was detected.

Immunohistochemistry

In the frozen sections, the submandibular glands consisted mainly of serous acini and all types of ducts; a few mucous acini and mixed acini were also observed. All specimens (n = 10) showed strong intracytoplasmic and membrane immunoreactivity with specific α_1-adrenoceptor subtype antibodies. The immunoreactivity of α_{1A}- and α_{1B}-adrenoceptor was most intense in striated, intercalated, and excretory ductal cells (Figs. 1J, 1K). The staining intensity of ductal cells was distinctly stronger than that of acinar cells. The positive staining of α_{1D}-adrenoceptor was barely detectable in both acinar and ductal cells.

Figure 1 continued from previous page.



Western Blotting

The cDNAs encoding the α_1-adrenoceptor subtypes were transfected in HEK293 cells, and the cell lysates were immunoblotted as a test of the specificity of the antibodies. We evaluated quantification range by controlling the protein dependency of the immunoreaction, and it was confirmed that the reaction was linear (data not shown). Distinct immunoreactive bands of 55 kDa and 60 kDa, corresponding to the α_{1A}- and α_{1B}-adrenoceptor, respectively, were detected by receptor-specific antisera in human submandibular glands (n = 3; Figs. 1L, 1M).

Intracellular Calcium Imaging

To determine the function of α_1-adrenoceptor subtypes, we performed quantitative analysis of intracellular calcium ($[Ca^{2+}]_i$) in single cells. A small peak of fluorescence intensity was observed at about 400 sec, and the maximum value was reached at about 1500 sec after agonist treatment. Either phenylephrine (Figs. 2A, 2B) or A61603 (Figs. 2C, 2D) induced a significant increase in $[Ca^{2+}]_i$ by 2.33 ± 0.18-fold and 1.81 ± 0.43-fold (n = 10 cells from three different submandibular glands), respectively. The addition of KCl significantly increased $[Ca^{2+}]_i$ (Fig. 2H). The phenylephrine-induced $[Ca^{2+}]_i$ was markedly attenuated by 65% with the pre-treatment of 5-methylurapidil (Figs. 2E, 2F).

DISCUSSION

In this study, we report for the first time the expression, distribution, and function of α_1-adrenoceptor subtypes in human submandibular glands. The results showed that both α_{1A}- and α_{1B}-adrenoceptor mRNAs and proteins were widespread in ductal and acinar cells, and no α_{1D}-adrenoceptor expression was found. α_{1A}- and α_{1B}-Adrenoceptors might be involved in the regulation of submandibular gland function.

α_1-Adrenoceptor subtypes have been characterized in many mammalian tissues, but little is known about the human submandibular gland because of the limitation of available normal human tissues. It is noteworthy that most of our experiments were done with submandibular gland tissues from patients undergoing functional neck dissection for oral carcinoma at the stage of clinically negative lymph node metastasis. Because glands excised for chronic sialadenitis, submandibular gland calculus, or tumors generally have pathological changes, the specimens in our experiment appear to be the only possible source of normal human submandibular gland tissue for research purposes.

Species heterogeneity exists in the distribution of α_1-adrenoceptor subtypes in submandibular glands. In the rat, the expression of α_{1A}- and α_{1B}-adrenoceptor mRNAs has been detected, and the mRNA expression level of the α_{1A}-adrenoceptor was higher than that of the α_{1B}-adrenoceptor (Rokosh *et al.*, 1994; Nishiura *et al.*, 2001). α_{1D}-Adrenoceptor mRNA was not detected in previous studies, but has recently been identified by RT-PCR (Bockman *et al.*, 2004). In the human submandibular gland, we found that the expression of α_{1A}-adrenoceptor mRNA was relatively higher than that of the α_{1B}-adrenoceptor by semi-quantitative RT-PCR, and no expression of α_{1D}-adrenoceptor mRNA was identified. The expression of α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor proteins has been detected in the rabbit (Piao *et al.*, 2000). α_{1A}- and α_{1B}-Adrenoceptors, but not α_{1D}-adrenoceptors, have been found in rat submandibular glands (Bockman *et al.*, 2004). Using specific α_1-adrenoceptor subtype antibodies, we detected the presence of α_{1A}- and α_{1B}-adrenoceptors in human submanibular glands. These results indicated the similar pattern of expression of α_1-adrenoceptor subtypes between rat and human submandibular glands.

There has been no report about the distribution of α_1-adrenoceptor subtype mRNA and protein in human submandibular glands. In our study, α_{1A}- and α_{1B}-adrenoceptor mRNA was localized in human submandibular glands by *in situ* hybridization. The expression patterns for α_{1A}- and α_{1B}-adrenoceptor mRNAs had distinct cellular profiles, with acinar cells showing a very intense hybridization signal, while duct cells showed a very low level of gene expression. The distribution of α_{1A}- and α_{1B}-adrenoceptor proteins in human submandibular glands was not in accord with that of their mRNA, since the immunoreactivity for α_{1A}- and α_{1B}-adrenoceptors was located mainly in duct cells and partly in acinar cells, as determined by immunohistochemistry.

It is known that the expression of mRNA may not always parallel that of the protein. Not all adrenoceptor mRNAs expressed strictly reflect functional receptors; similar phenomena can be observed in other tissues. For example, in the rabbit thalamus, the fact that α_{1B} binding sites were not detected, despite the expression of α_{1B}-adrenoceptor mRNA, is significant (Piao *et al.*, 2000). The expression variance between mRNA and corresponding protein may result from translational efficiency, half-life, or instability of the mRNA (Nishiura *et al.*, 2001). The expression of α_{1A}- and α_{1B}-adrenoceptor mRNAs indicated their transcription in human submandibular glands; however, the synthesis of the adrenoceptor proteins may be influenced at the translational or post-translational level.

It has been demonstrated that the formation of plasma-like initial salivary secretion takes place in the acini. Through the duct system of salivary glands, the composition of this initial salivary secretion is modified, due to re-absorption of sodium and chloride, and the secretion of potassium and bicarbonate (for review, see Baum, 1993). In the process of salivary secretion, the α_1-adrenoceptor may have an important effect on salivary gland duct cells in re-absorbing fluid and electrolytes (Klein, 2002). The distribution of functional α_{1A}- and α_{1B}-adrenoceptors has been evaluated in SMG-C10, an acinar cell line cloned from rat submandibular glands, according to the different affinity values for α_1-adrenoceptor subtype selective antagonists (Bockman *et al.*, 2004).

It has been reported that activation of the α_1-adrenoceptor could induce an increase in $[Ca^{2+}]_i$, which mediates electrolyte mobility and fluid secretion (Martinez and Reed, 1988; Quissell *et al.*, 1992). We evaluated the function of α_{1A}- or α_{1B}-adrenoceptors in human submandibular gland cells using the intracellular calcium imaging indicator, fluo 3-AM. Phenylephrine, which stimulates both α_{1A}- and α_{1B}-adrenoceptors, induced a significant increase in $[Ca^{2+}]_i$, while A61603, an α_{1A}-adrenoceptor seletive agonist, also induced an increase in $[Ca^{2+}]_i$, but the extent of $[Ca^{2+}]_i$ increase was lower than that induced by phenylephrine. In addition, 5-methylurapidil, the selective α_{1A}-adrenoceptor antagonist, showed distinct but incomplete inhibition of the increase in $[Ca^{2+}]_i$ induced by phenylephrine. The results indicated that the α_{1A}-adrenoceptor subtype could contribute to the regulation of saliva synthesis and secretion. Since the selective α_{1B}-adrenoceptor antagonist at present is controversial (Zhong and Minneman, 1999), the function of α_{1B}-adrenoceptor in human submandibular glands needs to be further investigated.

In conclusion, we have presented here the first evidence of the expression of functional α_{1A}- and α_{1B}-adrenoceptors in human submandibular glands, which may be significant for our understanding of the involvement of human α_1-adrenoceptor subtypes in salivary synthesis and secretion.

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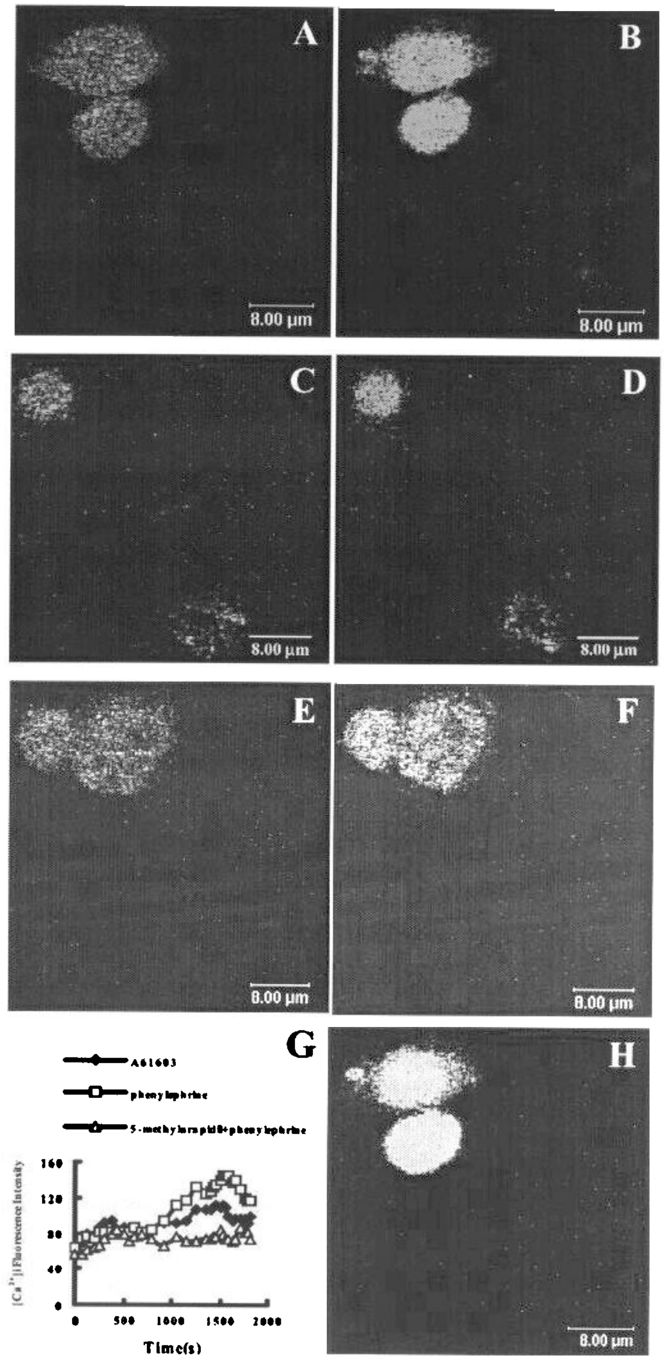

Figure 2. Effects of α_1-adrenoceptor subtype selective agonists and antagonist on $[Ca^{2+}]_i$ in human submandibular gland cells. **(A,C,E)** Fluorescence image of fluo-3AM-loaded cells before treatment with phenylephrine, A61603, or 5-methylurapidil. No change in $[Ca^{2+}]_i$ fluorescence intensity (FI) was observed after pre-treatment with 5-methylurapidil (E). There were no marked differences in FI among A, C, and E. The maximum of FI was observed at about 1500 sec after agonist treatment. **(B,D)** A significant increase in $[Ca^{2+}]_i$ FI at the individual cell level after treatment with phenylephrine (1 μM) or A61603 (100 μM), respectively. **(F)** A mild increase in $[Ca^{2+}]_i$ FI at the individual cell level with 1 μM of phenylephrine after pre-treatment with 5-methylurapidil (0.1 μM). **(G)** The dynamics of FI at the individual cell level treated with phenylephrine, A61603, or 5-methylurapidil plus phenylephrine in the human submandibular gland cells. **(H)** Fluorescence image of fluo-3AM-loaded cells with KCl stimulation (50 mM).

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