Urol Res (2001) 29:325–329
DOI 10.1007/s002400100207

ORIGINAL PAPER

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G-protein coupled receptor kinase 2 and 3 expression in human detrusor cultured smooth muscle cells

Received: 7 February 2001 / Accepted: 4 July 2001 / Published online: 28 August 2001

Abstract The aim of this study was to investigate the expression of G-protein coupled receptor kinases (GRKs) mRNA by RT-PCR and GRKs protein by immunohistochemistry in human detrusor cultured smooth muscle. Primary cultures of human detrusor smooth muscle cells were established using the explant method from three normal bladders. The expression of each GRK, β-adrenergic receptor and muscarinic acethylcholine receptor (mAchR) mRNA was examined by reverse transcription polymerase chain reaction (RT-PCR). Immunohistochemical staining was also performed using primary antibodies for GRKs. The GRK2 and GRK3 transcripts were detected by RT-PCR. The m2, m3 and m5 mAchR and β1, β2 and β3 adrenergic receptor subtypes mRNA were also detected. Using immunohistochemistry, both GRK2 and GRK3 were found to be expressed in detrusor smooth muscle cells. These results demonstrated the existence of GRK2 and GRK3 and the co-expression of m2, m3 and m5 mAchR and β1, β2 and β3 adrenergic receptor subtypes in detrusor smooth muscle cells. The possibility exists that these kinases play a role in the desensitization mechanism of mAchR and β adrenergic receptors.

Keywords G-protein coupled receptor kinase · Muscarinic acethylcholine receptor · β adrenergic receptor · Human detrusor cultured smooth muscle cells

This work was supported by the Ministry of Education, Science and Culture of Japan Grants in Aid for Scientific Research 12770856 (K.O.)

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Introduction

Receptor desensitization is a readily reversible process whereby membrane receptors become refractory in their actions. Desensitization can occur within seconds and involves phosphorylation of the receptor and its associated G proteins which results in the uncoupling of the receptor from its G protein and effector [21]. G protein-coupled receptor kinases (GRKs) are a recently identified family of enzymes that phosphorylate seven-trans-membrane receptors. GRKs phosphorylate G-protein coupled receptors(GPRs) in an agonist-dependent manner and receptor phosphorylation is a crucial step in the process of desensitization. Six different mammalian GRKs have been cloned and characterized. Although GRK1 (rhodopsin kinase) and GRK4 are expressed exclusively in retinal photoreceptor cells and testis respectively, β-adrenergic receptor kinase 1 (GRK2) and β-adrenergic receptor kinase 2 (GRK3), as well as GRK5 and GRK6, are widely distributed throughout the body. GRKs mediate rapid activation-dependent loss of receptor responsiveness through their unique ability to recognize and phosphorylate their receptor substrate [28]. GRKs have been shown to specifically phosphorylate the activated form of multiple GPRs in vitro, including the β-adrenergic receptors [2], muscarinic acetylcholine receptors (mAchRs) [17], α2-adrenergic receptors [3], substance P receptors [18] and endothelin receptors [10].

Human detrusor smooth muscle contraction is controlled by the autonomic nervous system via the cholinergic innervation of muscarinic receptors [15]. Both functional and receptor binding studies have demonstrated the existence of β-adrenergic receptors in the detrusor muscle of several mammals, including humans [22, 25 26]. We have already demonstrated the existence of both ETA and ETB endothelin receptor mRNA in human detrusor cultured smooth muscle cells by reverse transcription polymerase chain reaction (RT-PCR) and in situ hybridization [27].

In this article, we report the mRNA expression of GRKs, each β-adrenergic receptor and mAchR

subtype by RT-PCR in primary cultures of human detrusor smooth muscle cells. The expression of GRKs protein is also demonstrated by immunohistochemistry.

Materials and methods

Establishment of primary cultures of human detrusor smooth muscle cells

Detrusor smooth muscle cell culture was established by the explant method as previously reported [27]. In brief, cold cup pinch biopsies of detrusor muscle were obtained from three patients undergoing cystoscopy. These biopsies were denuded of the accompanying mucosa and cut into approximately 1 mm^3 pieces which were transferred onto tissue culture plastic dishes and covered with tissue culture medium RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The flask was placed in a humidified cell culture incubator in an atmosphere of 5% CO2 and 95% air at 37°C to allow outgrowth of the detrusor smooth muscle cells. After 10 days, the tissue explants were removed, and the media was replaced with freshly supplemented RPMI 1640 medium. Immunohistochemistry was used to confirm the identity of the muscle cells by staining specifically for human smooth muscle α-actin. All experiments replicated twice.

Reverse transcription polymerase chain reaction

Total RNA was extracted from human detrusor smooth muscle cells using the acid guanidinium phenol chloroform method [6]. Total RNA was reverse-transcribed into cDNA using a DNA amplification reagent kit (GIBCO BRL, USA) for use in the PCR. The cDNA was synthesized from 5 μg of total RNA and 1/20 of the first strand cDNA solution was then used for PCR with primers designed to amplify fragments of each GRK cDNA sequence. Expression of the β-adrenoceptors and mAchRs mRNA were also examined by RT-PCR. The PCR primer sequences for each GRK, β-adrenoceptor and mAchR are shown in Table 1. PCR was performed in a 50 μl reaction volume containing buffer (50 mM/l KCl; 10 mM/l Tris-HCl, pH 8.4; 1.5 mM/l MgCl2), 160 mM/l of each dNTP, 0.8 mM/l of each oligonucleotide primer, and 2.5 U of Taq DNA polymerase (Roche Diagnostics, USA). A 500-bp fragment of the human β-actin gene was amplified as a positive control. To rule out the possibility of amplifying genomic DNA, in some experiments PCR was performed without prior reverse transcription of the RNA. PCR products were size fractionated by 3% agarose gel electrophoresis. DNA bands were visualized with an ultraviolet transilluminator (Spectoline, Funakoshi, Tokyo, Japan). Each PCR products were sequenced using an autosequencer.

Immunohistochemistry

Primary culture cells were grown on Lab-Tek chamber glass slides and fixed for10 min in cold acetone. Expression of GRK2 and GRK3 were confirmed by immunohistochemistry, using the following antibodies: C-15, a polyclonal anti-GRK2 antibody and C-14, a polyclonal anti-GRK3 antibody (Santa Cruz Biotechnology, Calif.). Staining was performed according to a streptavidin-biotin-peroxidase protocol. All slides were viewed using an Olympus microscope (Olympus, Tokyo, Japan).

Results

Reverse transcription polymerase chain reaction

Total RNA from primary cultures of human detrusor smooth muscle cells was used to construct cDNA. PCR products from three detrusor smooth muscle cell cDNAs revealed positive amplification of both GRK2 and GRK3 genes. The RT-PCR results for GRK2 and GRK3 from the three cultures are shown in Fig. 1. The m2, m3 and m5 mAchR and β1, β2 and β3 adrenoceptor subtypes mRNA were also detected in all three cultures. No differences were seen in expression pattern between three different cultures. Representative results from a single culture are shown in Fig. 2, 3. PCR products without prior reverse transcription of the RNA, or without prior mRNA, did not reveal positive bands. All PCR products were sequenced using an autosequencer to confirm their identity.

Morphology and immunohistochemistry

The detrusor smooth muscle cells were spindle shaped and grew in a whorl like pattern when grown on cover-

Table 1 Specific primers for RT-PCR

	Primer 5′–3′	Product (bp)
GRK2	CAC ACG GTT TTG CCA GTG GA CCA GGA TGA AGC TGA GCT TG	302
GRK3	GTC ATC TCT GAA CGC TGG CA GGC CTC CTT GAA GGT TTC GA	429
m1	AAA TAC AGT CAA GAG GCC GAC TAA G CTT GTC CCA GCG GCA AAG CAG C	349
m2	CTA AGC AAA CAT GCA TCA GAA TTG G AAG GTG CAC AAA AGG TGT TAA TGA G	288
m3	ACC CAG CTC CGA GCA GAT GGA C CGG CTG ACT CTA GCT GGA TGG G	341
m4	CAG CCA TTG AGA TTG TGC CTG CC GGT GGC GTT GCA CAG AGC ATA G	314
m5	TCA GAA ATG TGT GGC CTA TAA GTT C TGA CTG GGA CAC ACT TGT CAC AG	304
β1	TCG TGT GCA CCG TGT GGG CC AGG AAA CGG CGC TCG CAG CTG TCG	261
β2	GCC TGC TGA CCA AGA ATA AGG CC CCC ATC CTG CTC CAC CT	327
β3	GCT CCG TGG CCT CAC GAG AA CCC AAC GGC CAG TGG CCA GTC AGC G	312

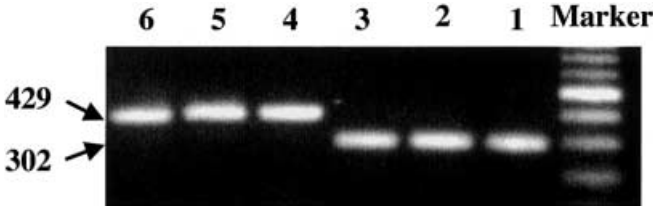

Fig. 1 Detection of GRK2 and GRK3 mRNA in primary culture of human detrusor smooth muscle cells by reverse transcription polymerase chain reaction (RT-PCR). Three different cell cultures were pooled and used to construct cDNA. *Lanes 1*, *2*, *3*: GRK2 RT-PCR, expected reaction product size: 302 bp. Each lane is a PCR product from the three different cultures. *Lanes 4*, *5*, *6*: GRK3 RT-PCR, expected reaction product size: 429 bp. *Lanes 1* and *4*, *lanes 2* and *5*, *lanes 3* and *6* were PCR products from same culture cDNA, respectively

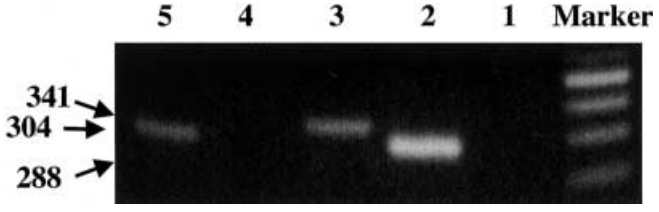

Fig. 2 Detection of m1–m5 mAchR subtype mRNA in primary culture of human detrusor smooth muscle cells by reverse transcription polymerase chain reaction (RT-PCR). Positive amplification of m2, m3 and m5 mAchR subtype genes were detected. *Lane1*: m1, *lane2*: m2, *lane3*: m3, *lane4*: m4, *lane5*: m5. Expected band size of m2, m3 and m5 are 288, 341 and 304 bp, respectively. Representative results from a single culture were shown

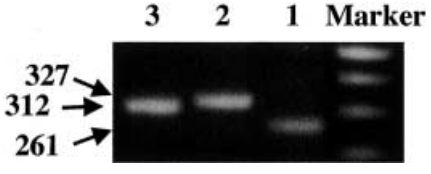

Fig. 3 Detection of β-1, β-2 and β-3 adrenergic receptor subtype mRNA in primary culture of human detrusor smooth muscle cells by reverse transcription polymerase chain reaction (RT-PCR). Positive amplification of β-1, β-2 and β-3 adrenergic receptor subtype genes were detected. *Lane 1*: β-1, *lane 2*: β-2, *lane 3*: β-3. Expected band sizes of β-1, β-2 and β-3 are 261, 327 and 312 bp, respectively. Representative results from a single culture were shown

slips in the confluent state. However, cells tended to be more stellate in shape with visible phase dense stress fibers in the nonconfluent state. By immunohistochemistry, GRK2 and GRK3 were expressed in detrusor smooth muscle cells. Representative results are shown in Fig. 4.

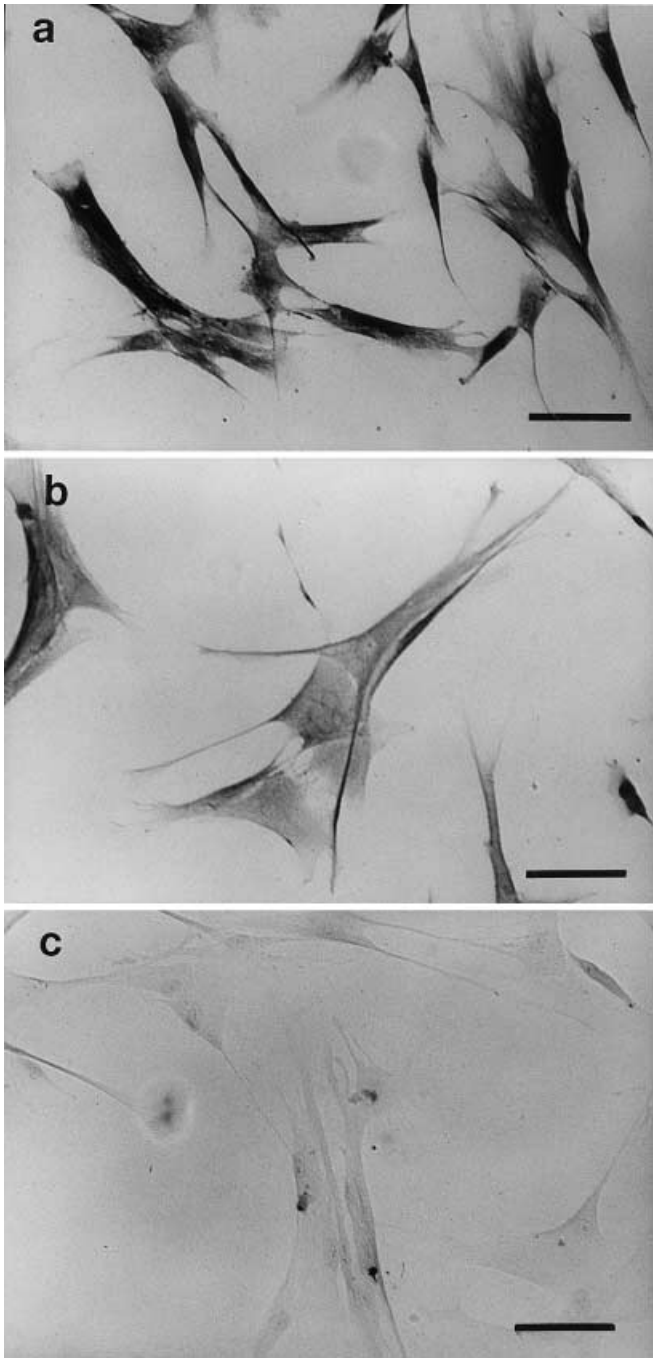

Fig. 4a–c Immunohistochemistry using GRK2 and GRK3 specific antibodies in primary culture of human detrusor smooth muscle cells. Both GRK2 (**a**) and GRK3 (**b**) were expressed in human detrusor smooth muscle cells. The staining procedure was controlled by an unspecific antibody (**c**). Representative results of immunohistochemistry in three patients. *Scale bar* = 200 μm

Discussion

In the present paper we have shown the expression of GRK2 and GRK3 mRNA and protein in human detrusor smooth muscle cells. We also detected the m2, m3 and m5 mAchR and β1, β2 and β3 adrenergic receptor subtype mRNAs which are key autonomic receptors in bladder function.

Muscarinic receptors mediate the main part of bladder contraction in humans [1]. Histochemical studies have demonstrated a rich plexus of intramural acetylcholinesterase-positive nerves [9]. Several investigators

have demonstrated that the pharmacologically defined M3 receptor mediates bladder contraction [8]. The human detrusor smooth muscle cells in primary culture express M3 muscarinic receptors which are linked to phosphoinositide hydrolysis [13]. Molecular cloning studies have isolated the genes m1, m2, m3 and m4 encoding M1, M2, M3 and M4 muscarinic receptor subytpes, with one additional distinct subtype, the m5 gene [5, 7, 16, 24, 29]. It has been suggested that M2 and M4 muscarinic receptors are linked to the Gi/Go family of G proteins and coupled to inhibition of adenylate cyclase, whereas M1, M3 and M5 receptors are linked to Gq family G proteins and coupled to the activation of phospholipase C [4]. Muscarinic receptors as well as other GPRs are known to undergo desensitization following exposure to agonists [14]. In human detrusor cultured smooth muscle cells, carbachol induced a concentration-dependent increase in phosphoinositide turnover, the response being rapid and evident after only a 30 s exposure to the agonist. Preincubation of the cells with carbachol produced a concentration-dependent decrease in the inositol phosphate response to a second challenge. Preexposure to carbachol for only 5 min reduced the second inositol phosphate response to 49% [23]. These experiments suggest that the receptor desensitization mechanism is rapid and that the level of the receptor itself plays a major role.

Both functional and receptor binding studies have demonstrated the presence of β-adrenergic receptors in the human detrusor smooth muscle. The results of receptor-binding studies suggest that β-adrenergic receptors of the human detrusors are primarily of the $\beta 2$ subtype [20]. However, we detected the existence of the $\beta 3$ adrenergic receptor subtype in detrusor smooth muscle cells. We also demonstrated the participation of $\beta 3$ adrenergic receptor in the relaxant effect on the carbacol-induced muscle contraction using the isometric contraction technique [30]. Igawa and co-workers also showed the existence of all three β-adrenergic receptors in human detrusor muscle cells and suggested that adrenergic stimulation caused the human detrusor relaxation mainly by stimulating $\beta 3$ adrenergic receptors [15].

Three different processes of desensitization are known: uncoupling of receptors from G proteins, sequestration or internalization of receptors from the cell surface and decrease in the numbers of receptors [19]. Kinases that phosphorylate GPRs in an agonist-dependent manner are known as GRKs. Presently, six members of the GRKs family have been identified(GRK1–6). GRKs stimulate short-term desensitization(< 5 min) by phosphorylating GPRs, and also participate in receptor sequestration, which may relate to intermediate-term desensitization(30–60 min). The specificity of the GRKs for particular receptors remains incompletely understood. However, several investigators have shown that muscalinic acetylcholine receptor(mAchR) M1, M2, M3 and β-adrenergic receptors might be phosphorylated by GRK2 and GRK3 [11, 12].

In summary, we established primary cultures of human detrusor smooth muscle cells and demonstrated the existence of GRK2 and GRK3 mRNA and protein. We also detected the co-expression of m2, m3 and m5 mAchR and $\beta 1$, $\beta 2$ and $\beta 3$ adrenergic receptor subtypes by RT-PCR. Although further experiments are needed before any conclusion can be drawn on the functional role of GRKs in detrusor smooth muscle cells, there is a possibility that these kinases correlate to the desensitization mechanism of mAchR and β adrenergic receptors. To our knowledge, this is the first report which has demonstrated the existence of GRKs in human detrusor smooth muscle cells.

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