Effects of AF64A on the mRNA Levels of Muscarinic Receptor Subtypes in the Rat Iris Sphincter

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Abstract

The reduction of parasympathetic nerve activity by the treatment with ethylcholine mustard aziridinium ion (AF64A) in vivo induced both specific and non-specific supersensitivity in the rat iris sphincter (Tanaka et al., 1999). Changes in the expression of muscarinic receptor subtypes, which could be a cause of specific supersensitivity induced by the treatment with AF64A, were examined using competitive PCR techniques. Muscarinic receptor population is composed of m2, m3, and m4 subtypes in the rat iris (Furuta et al., 1998). Interestingly, m4 mRNA was much more abundantly expressed than m2 and m3 in the rat iris sphincter. The treatment with AF64A significantly increased the mRNA levels of m2 and m3 subtypes to 370 and 330% of the control but not that of m4 (approximately 90% of the control). In addition, the total protein contents were increased to approximately 125% of the control. The up-regulation of the mRNA levels of m2 and m3 subtypes by the treatment with AF64A was significant when they were compensated for the increase in total protein contents. The down-regulation of m4 mRNA expression was not significant even after being corrected for the protein content. These results suggest that the up-regulation of the mRNA levels of m2 and m3 subtypes may be, at least in part, responsible for the supersensitivity to muscarinic agonists after the treatment with AF64A in vivo.

Key words: rat iris sphincter, AF64A, supersensitivity, muscarinic receptor subtypes, mRNA expression level, competitive PCR

Introduction

Muscarinic acetylcholine receptors are widely distributed in various types of smooth muscles including the iris sphincter (Eglen et al., 1996 and 1997). Four subtypes (M1–M4) on the basis of the pharmacological profiles and five subtypes (m1–m5) on the basis of molecular analysis have been identified (Caulfield, 1993). Pharmacologically, M1–M4 receptor subtypes correlate with m1–m4 gene products, respectively. Among them, m1, m3, and m5 subtypes preferentially couple to G proteins of the Gq/11 class to mediate a stimulation of phos-
phosphoinositide hydrolysis, whereas m2 and m4 subtypes are selectively linked to G proteins of the G_{i/o} family to mediate an inhibition of adenylate cyclase activity (Caulfield, 1993; Offermanns et al., 1994).

In smooth muscles, muscarinic receptor population is composed of mainly m2 and m3 subtypes (Ehlert et al., 1997; Eglen et al., 1996), whereas the most abundant subtype is m3 subtype in the bovine and human iris sphincters (Honkanen et al., 1990; Gil et al., 1997). Our previous studies suggested that the functional receptors that mediate contractile responses to muscarinic agonists in the rat iris sphincter muscle belong to the M3 subtype on the basis of a study with selective muscarinic antagonists (Masuda et al., 1995) and that mRNAs encoding m2, m3, and m4 subtypes are predominantly expressed in the rat iris (Furuta et al., 1998). The relative mRNA levels of these subtypes are, however, not determined in the rat iris sphincter.

The rat iris sphincter muscle is innervated by parasympathetic nerves (Csillik and Koelle, 1965; Huhtala et al., 1976; Narita and Watanabe, 1981). It has been shown by our group that the surgical parasympathetic denervation by ciliary ganglionectomy induces both specific and non-specific supersensitivities (Hasegawa et al., 1987 and 1988; Banno et al., 1987; Hashimoto et al., 1993). Ethylcholine mustard aziridinium ion (AF64A) causes selective degeneration of cholinergic neurons in the central nervous system (Fisher and Hanin, 1980). The administration of AF64A to cerebral ventricle elicits the following phenomena: 1) several biochemical markers such as choline acetyltransferase activity and high affinity choline uptake are reduced (Walsh et al., 1984), 2) the density of muscarinic recognition sites in brain remains virtually unchanged (Vickroy et al., 1985), and 3) the activation of phosphoinositide turnover or the inhibition of cyclic AMP accumulation elicited by muscarinic receptor agonists is facilitated (Eva et al., 1987). Our preceding study (Tanaka et al., 1999) showed that the intracocular injection of AF64A induces both specific and non-specific supersensitivities which are the same as the results of parasympathetic denervation by ciliary ganglionectomy in the rat iris sphincter (Hasegawa et al., 1987). In the rat urinary bladder, denervation induces both hypertrophy and a supersensitivity to muscarinic agonists (Gunasena et al., 1995) and an increase in M3 subtype density with no change in M3 subtype density (Braverman et al., 1998).

The present study was undertaken to 1) determine the relative mRNA levels of the m2, m3, and m4 subtypes and 2) investigate the effects of the treatment with AF64A in vivo on the mRNA levels of muscarinic receptor subtypes in the rat iris sphincter by competitive PCR.

Methods

Preparation of RNA standards

To prepare the RNA standards for m2, m3, and m4 subtypes, deletion mutagenesis of these subtypes was performed by restriction enzyme digestion. The PCR fragments amplified with brain cDNA and specific primers (m2-1, m3-1, and m4-1; see Table) were ligated into pBlue-script II SK(+) (Stratagene). Following this, insert DNAs were double-digested with Spe I/ Sty I for m2, Hind III/Pst I for m3, and Pst I/Sty I for m4, respectively. Digested sites were blunted by T4 DNA polymerase and then were self-ligated. Resulting mutagenesis was confirmed by the sequencing on a DSQ-1000L DNA sequencer (Shimadzu). RNA standards

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were transcribed using MEGAscript (Ambion) and RNA concentrations were determined by the measurements of O.D.\textsubscript{260}.

\textit{Pretreatment with AF64A in vivo}

For application of AF64A (RBI) \textit{in vivo}, 8–9 week-old male Wistar rats were anesthetized with an i.p. injection of thiopental sodium (25 mg/kg) and amobarbital sodium (50 mg/kg). As described by Tanaka \etal{} (1999), AF64A was applied into the anterior chamber of the eye by slow injection of 10 mM AF64A in 5 μl saline (50 nmol) using sterilized Hamilton syringe and 26 gage injection needle. On the third day after the first injection, the another addition of 50 nmol of AF64A was performed to same eyes. On the fourth day after the second injection, eyes were dissected and irides were used for the experiments. The contralateral eyes were used for control experiments. In the preliminary experiments, the treatment with 50 nmol AF64A twice resulted in both specific increase in sensitivity to muscarinic agonist, acetylcholine, by the shift of pD\textsubscript{2} from 5.0 to 5.6 and the increase in the maximum response by 53\% at the forth day after the second injection (n=10). These changes were comparable to and slightly smaller than those in the preceding study, respectively (Tanaka \etal{}, 1999). As shown in the preceding study, the injection of 100 nmol AF64A occasionally gave irreversible damages to eyes, we preferred to 50 nmol injection, which did not induced apparent damages in over 20 eyes used in the preliminary experiments.

\textit{Competitive PCR}

The rat iris sphincter with or without AF64A treatment was homogenized by 1 ml of conventional acid guanidium thiocyanate solution, and four-fold serial dilution of RNA standard was set up for competitive PCR. 250 μl of homogenate (1/4 portion of an iris sphincter) and 25 μl of adequate concentration of RNA standard were transferred into clean and dry test tube, and then total RNA and RNA standard were co-extracted by conventional acid guanidium thiocyanate–phenol methods. According to Gibco BRL’s protocol, reverse transcription (RT) was performed using the each subtype–specific antisense primer (m2-1, m3-1, and m4-1; see Table) as reverse primer. Resulting RT products were amplified using GeneAmp 2400 (Perkin Elmer). The following thermal cycler program was used for the first PCR amplification: a 0.5 min denaturation step at 94°C, a 0.5 min annealing step at 55°C, and 0.5 min extension at 72°C for 10 cycles. The reaction mixtures were five-fold diluted with distilled

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|l|}
\hline
sense primer & antisense primer & predicted product size (bp) & GenBank accession number \\
\hline
m2-1 & 5'-GCCTGGAACACAACAAGATCC-3' & 5'-GTGGAGGCTTCTTTTTGCGAG-3' & J00205 \\
m2-2 & 5'-TGTTTTCAAGGGAGGAG-3' & 5'-CGTTCTGTCTTTCTATCCC-3' & 259 \\
m3-1 & 5'-AGTGCCGTCGCTCCTTT-3' & 5'-CTCTAACTGGATGGAAAGC-3' & M16407 \\
m3-2 & 5'-ACACGCAGCGAGCACTTG-3' & 5'-CATGCTGTCTGCGGCCTGA-3' & 251 \\
m4-1 & 5'-AGGAGAAGAAGGCCA-3' & 5'-AACCTTCGCGCCACA-3' & M16409 \\
m4-2 & 5'-AGGAGAAGAAGGCCA-3' & 5'-GCCGTACACATTCATGCCC-3' & 329 \\
GAPDH & 5'-GCCAAAAGGGTTCATCATCCTGCC-3' & 5'-ACATGGGCTAGGAACACCGA-3' & 373, X02231 \\
\hline
\end{tabular}
\end{table}
water and reamplified using the other set of primers (m2–2, m3–2, and m4–2) as shown in Table for 22–26 cycles.

Data analysis
Amplified products were separated on 2% agarose gel electrophoresis and visualized with ethidium bromide. Resultant gels were imaged by a FluorImager 595 (Molecular Dynamics) and the digitized signals were quantified with ImageQuaNT software (version 4.2a, Molecular Dynamics). The log of the ratio of the ‘target’ band intensity to ‘standard’ one was then plotted against the log of ‘standard’ molecules added to the PCR reaction (see Results). The lines were drawn from a linear regression analysis of the data points excluding the values, which fell out the linear portion of the curve. The amount of target mRNA was calculated by determination of the x–intercept for the point on the curve where the ratio of ‘target’ to ‘standard’ equals one.

Measurement of the total protein contents
The rat iris sphincter treated with or without AF64A was frozen in acetone/5% (w/v) trichloroacetic acid on dry ice for 30 min. Then the tissue was homogenized with 1 ml of PBS buffer containing 137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl and 1.47 mM KH₂PO₄. 500 μl of homogenate was centrifuged (4°C, 15,000 rpm, 20 min) and supernatant fraction was discarded. Pellet was dissolved by 500 μl of 1 N NaOH at room temperature. After this procedure, the sample was added to 500 μl of distilled water and was centrifuged (4°C, 3,000 rpm, 2 min). Supernatant fraction was measured with the Bio–Rad DC Protein Assay Kit II (Bio–Rad) according to Bio–Rad's protocol.

Statistics
Pooled data were expressed as means±SE. Statistical significance was tested according to paired t–test for the comparison between two groups, and p level (p<0.05 or 0.01) was represented by * or **, respectively.

Results
Comparison of the mRNA levels of muscarinic receptor subtypes in the iris sphincter by competitive PCR and effects of AF64A treatment
Using conventional RT–PCR methods, we have suggested that m2, m3, and m4 but not m1 and m5 subtype mRNAs were abundantly expressed in the rat iris (Furuta et al., 1998). The present study focused on the quantitative analysis of the mRNA expression levels of m2, m3, and m4 subtypes and their changes by the treatment with AF64A in vivo by micro–injection into the anterior chamber of the eye. Competitive PCR (cPCR) (Gilliland et al., 1990; Jung et al., 1998) was performed for the quantification of mRNA levels of muscarinic receptor subtypes.

Figure 1A showed competitor titration experiments with RNA standards and total RNAs from the iris sphincters treated with AF64A (right panels, AF64A) and no–treated (left panels, control). Total RNAs were co–extracted with four–fold serial dilutions of RNA standards prepared as described in Methods, whose ranges were from 6.3×10⁷ to 2.4×10⁵ molecules (left
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Fig. 1. A: Competitor titration experiments with RNA standards for m2, m3, and m4 subtypes in the iris sphincter treated with AF64A (right panels, AF64A) or non-treated (left panels, control). The numbers of standard RNA in each reaction were 62.5, 15.6, 3.91, 0.98, 0.24 × 10⁶ molecules (left to right) in both m2 and m3 subtypes, whereas 62.5, 15.6, 3.91, 0.98, 0.24 × 10⁷ molecules (left to right) in m4 subtype. B: The mRNA levels of m2, m3, and m4 subtypes were calculated and expressed as the mRNA molecules per an iris sphincter. Open and closed columns indicate the summarized data obtained in the control (control) and AF64A-treated (AF64A) iris sphincters, respectively. Data are averages of six independent experiments (± SE). * and ** indicate statistical significance versus control at \( p < 0.05 \) and \( p < 0.01 \), respectively.

to right; m2 and m3) and from \( 6.3 \times 10^6 \) to \( 2.4 \times 10^6 \) molecules (m4). The amplified products derived from mRNAs from the iris sphincter were detected as ‘target’ and to those derived from RNA standards as ‘standard’ (Fig. 1A). The specificity of each amplified product was established from the size of the fragment stained with ethidium bromide and confirmed by DNA sequencing analyses. The mRNA levels of muscarinic receptor subtypes were expressed as molecules per one sphincter. The summarizing data were shown in Figure 1B.

The basal mRNA levels of m2, m3, and m4 subtypes were \( 2.06 \pm 0.40 \times 10^7 \), \( 1.24 \pm 0.10 \times 10^7 \), and \( 2.96 \pm 0.43 \times 10^6 \) molecules, respectively (\( n = 6 \) for each) (Fig. 1B). The mRNA levels expressed as percentage of total muscarinic receptors were 6, 4, and 90% for m2, m3, and m4 subtypes, respectively. These results suggested that m4 subtype mRNA was expressed predominantly, whereas m2 and m3 mRNAs were expressed at much lower levels in the rat iris sphincter. This is the first finding that m4 subtype mRNA is expressed at such high level in a smooth muscle.

The treatment of the rat iris sphincter with AF64A \textit{in vivo} resulted in significant increase
in the mRNA levels of m2 and m3 subtypes: \(7.50 \pm 1.31 \times 10^7\) and \(4.10 \pm 1.00 \times 10^7\) molecules for m2 and m3 subtypes, respectively \((n=6\text{ for each, } p<0.01 \text{ (m2) and } p<0.05 \text{ (m3) vs. respective controls}), \) whereas there was no significant change in that of m4 subtype: \(2.77 \pm 0.40 \times 10^9\) molecules \((n=6)\) (Fig. 1B). In the iris sphincters treated with AF64A, the mRNA levels expressed as percentage of total muscarinic receptors were 19, 10.5, and 70.5\% for m2, m3, and m4 subtypes, respectively. The mRNA levels of m2 and m3 subtypes were increased to \(402 \pm 80\) and \(330 \pm 69\%\) of respective controls, whereas that of m4 subtype were not changed \((94 \pm 13\%\) of control).

To compensate for the changes in the total mRNA levels, we also quantified the mRNA level of glyceroldehyde-3-phosphate dehydrogenase (GAPDH), which is in general used as an internal standard. The mRNA level of GAPDH in the iris sphincter treated with AF64A was significantly increased to approximately 550\% of that in control \((\text{control}=3.91 \pm 0.69 \times 10^7),\) \(\text{AF64A}=21.3 \pm 1.98 \times 10^7\) molecules, \(n=6\) for each \) (data not shown). These suggested that the treatment of the iris sphincter with AF64A induced the changes in the mRNA levels of not only muscarinic receptor subtypes but also GAPDH. Therefore, the compensation of the mRNA levels of muscarinic receptor subtypes for that of GAPDH were not performed in the present study.

*Quantification of the total protein contents in the iris sphincter treated with or without AF64A*

As described above, the mRNA levels of muscarinic receptor subtypes in the iris sphincter treated with or without AF64A could not be compensated for the mRNA level of GAPDH. The total protein contents were therefore determined in both the control and AF64A–treated iris sphincters using the Bio-Rad DC Protein Assay Kit II (Bio-Rad). The total protein contents were \(48.3 \pm 5.01\) and \(59.8 \pm 4.77\mu g\) (per a sphincter) in the control and AF64A–treated iris sphincters, respectively \((n=6\text{ for each})\) (data not shown). The total protein contents in AF64A–treated sphincters were significantly increased \((126 \pm 10\%\) of control). When the mRNA levels of m2 and m3 subtypes were compensated for the total protein contents, they

![Fig. 2. Compensation of the mACHR mRNA levels for the changes in total protein contents. The mRNA levels of m2, m3, and m4 subtypes were calculated and expressed as the mRNA molecules per 1\mu g of protein contents. Open and closed columns indicate the data obtained in the control (control) and AF64A–treated (AF64A) iris sphincters, respectively. Data are averages of six independent experiments (±SE). * and ** indicate statistical significance versus control at \(p<0.05\) and \(p<0.01\), respectively.](image-url)
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were still significantly higher in the AF64A treated iris sphincter than in the control (m2: control = 4.26 ± 0.84 (× 10^6), AF64A = 12.74 ± 2.18 (× 10^6) molecules/μg protein (n=6, p < 0.01); m3: control = 2.56 ± 0.21 (× 10^6), AF64A = 6.89 ± 1.68 (× 10^6) molecules/μg protein (n=6, p < 0.05)) (Fig. 2). Consequently, in the iris sphincter treated with AF64A, the mRNA levels of m2, m3, and m4 subtypes were increased or decreased to 325 ± 65, 266 ± 56, and 80 ± 11% of respective controls.

Discussion

We have previously reported that the molecular components of muscarinic receptor subtypes expressed in the rat iris are m2, m3, and m4 (Furuta et al., 1998). In general terms, most smooth muscles express both m2 and m3 subtypes with the majority of m2, and the contractile response is mediated by mainly M3 subtype (Eglen, 1997). It has been reported that the predominant muscarinic receptor in the human iris sphincter is m3 subtype (Gil et al., 1997). Surprisingly, in the rat sphincter, the predominant subtype is m4 (90%), and m2 and m3 subtypes were expressed at much lower levels (6 and 4%, respectively). As far as we know, this is the first report which shows the predominant expression of m4 mRNA in a smooth muscle tissue. Since the functions of m4 subtype remain unclear (Eglen, 1997), this finding may provide a novel preparation which is suitable to investigate the presynaptic and/or postsynaptic m4 signaling and its function in a smooth muscle tissue.

The significant up-regulation of the mRNA levels of m2 and m3 subtypes was observed after the treatment with AF64A. Although the total protein contents were also increased by 25%, up-regulation of m2 and m3 mRNA levels was still significant after corrected for the increase in the protein contents. Thus, one possible explanation for the supersensitivity to muscarinic agonists by the treatment with AF64A in the iris sphincter is the increase in the mRNA levels of m2 and m3 subtypes. Interestingly, Braverman et al., (1998) have shown the similar increase in the density of M2 subtype with no change in the density of M3 subtype in denervated urinary bladder. Recently, it has been suggested that M2 subtype is involved in contractile responses in several types of smooth muscles and that an interaction between M2 and M3 subtypes can result in a pertussis toxin-sensitive (PTX), M3-like response with a M3 antagonistic profile (Ehlert et al., 1999). An alternative possibility is that activation of M3 receptor subtype induces a sustained depolarization by the suppression of potassium conductance in smooth muscles as shown by Kotlikoff et al. (1999). However, in the rat iris sphincter, PTX-insensitive M3 subtype is involved in the contractile responses (Masuda et al., 1995) and muscarinic receptor activation does not elicit any active changes in the membrane potential (Imaizumi et al., 1984; Banno et al., 1985).

The major muscarinic receptor expressed in the iris sphincter was m4 subtype mRNA (approximately 90%). Significant reduction has been observed in M4 subtype of the denate gyrus of Alzheimer patients compared to controls, suggesting that M4 subtype may be related to the dysfunction of the central or peripheral cholinergic systems (Adem and Karlsson, 1997). Of interest is that mRNA level of m4 subtype was slightly decreased after the treatment with AF64A, and consequently, the total mRNA levels of m4 subtype compensated for the total
protein contents was rather tend to decrease (approximately 80% of the control). Hasegawa et al. (1987) have shown that the muscarinic receptor density resulting from the binding assay using 3H-QNB tends to decrease in the rat iris sphincter denervated by ciliary ganglionectomy in comparison with normal sphincter. That finding may reflect the decrease in m4 receptor protein. Recent studies have suggested that autoinhibition of acetylcholine release is mediated via M₄ subtype in several smooth muscles (duodenum, urinary bladder, vas deferens, and trachea) (Kilbinger et al., 1995; D'Agostino et al., 1997; Akbulut et al., 1999). Functions of m4 and their changes after denervation or AF64A treatment remain to be determined.

In addition to specific supersensitivity to muscarinic agonists, our previous work showed the increase in maximum contractile response to 80 mM K⁺ in the iris sphincter treated with AF64A (Tanaka et al., 1999). In the present study, the total protein contents were increased in AF64A-treated iris sphincter by 25%. In addition to the increase in the maximum response, the sensitization of depolarized sphincter to calcium was clearly demonstrated in the preceding study. Non-specific supersensitivity may therefore be profoundly related to changes in various contractile protein profiles, which result in both the increase in the maximum response and Ca²⁺ sensitivity of contractile system. Another surprising finding in this study was the marked increase in the expression level of GAPDH, which is usually taken as the internal standard of stable expression. Similar up-regulation of GAPDH mRNA level has been reported by Yan et al. (1999) and Ishitani et al. (1996). Although the meaning of this phenomenon is not clear, it may suggest that there might be more genes whose transcription is markedly enhanced by AF64A treatment but not detected in this study.

In conclusion, in addition to mRNAs of m2 and m3 muscarinic receptor subtypes, m4 mRNA was abundantly expressed in the rat iris sphincter. The treatment with AF64A up-regulated the mRNA expression levels of m2 and m3 subtypes but not that of m4. The increased sensitivity to ACh may be due to the increase in the mRNA levels of m2 and m3 subtypes. The molecular mechanisms involving in non-specific supersensitivity, however, remain to be determined.

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References


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