

A negative charge in the M2 transmembrane segment of the neuronal $\alpha 7$ acetylcholine receptor increases permeability to divalent cations

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Threonine-244 (T244) in the putative channel-forming M2 segment of the neuronal $\alpha 7$ acetylcholine receptor (AChR), a residue proposed to form part of the selectivity filter, was mutated to aspartic acid to examine the influence of a negative charge on AChR ion permeation properties. Wild type (AChR $\alpha 7$ wt) and mutant (AChR $\alpha 7$ D244) acetylcholine receptors expressed in *Xenopus* oocytes give rise to acetylcholine (ACh)-activated, α -bungarotoxin-sensitive, cation-selective ionic currents. AChR $\alpha 7$ D244 exhibited larger currents than AChR $\alpha 7$ wt that, in addition, activated at lower ACh concentrations. The relative ionic permeability (P_X/P_{Na}) of AChR $\alpha 7$ wt to K^+ was $P_K/P_{Na} = 1.2$, and to Ba^{2+} , $P'_{Ba}/P_{Na} = 1.4$. In contrast, AChR $\alpha 7$ D244 was less selective in discriminating between K^+ and Na^+ , $P_K/P_{Na} = 0.95$, but exhibited a remarkable increase in permeability to Ba^{2+} , $P'_{Ba}/P_{Na} = 3.7$. Furthermore, only mutant receptors were permeable to Mg^{2+} . Hence, a ring of negatively charged residues in the putative pore-forming segment of the receptor increases the permeability to divalent cations. Our results substantiate the notion that T244, or its equivalent, in the M2 transmembrane segment of cholinergic receptor channels is a key structural determinant of the selectivity filter.

Channel protein; Ionic selectivity; Ionic pore; α -Helix; Signal transduction

1. INTRODUCTION

Ligand-activated ion channels play a fundamental role in signal transduction processes in the nervous system [1]. On the basis of amino acid sequence and hydrophobicity plots [1–5], members of the superfamily of ligand-gated channels display the occurrence of three major structural domains: (i) a large extracellular N-terminal domain, presumably containing the ligand binding sites; (ii) a large intracellular domain with multiple consensus sequences for protein kinases; and (iii) four putative transmembrane segments, M1, M2, M3, and M4, with predicted α -helical secondary structure. However, the structure of the acetylcholine receptor (AChR) from *Torpedo* at 9 Å resolution indicates that the pore is formed by a bundle of five α -helices, one contributed by each subunit, perhaps surrounded by a rim of β -sheets [6].

Several lines of evidence point to M2 as the transmembrane segment implicated in forming the pore of the ligand-gated ion channels: (i) M2 is amphipathic

and highly conserved among all members of this superfamily [4,6–8]; (ii) in cation-selective channels M2 contains negatively charged residues at the N- and C-termini, whereas in anion selective channels it has positively charged residues [6–8]; (iii) mutations of residues in M2, presumed to face the aqueous pore, modulate ion permeation properties of the receptor channel [2,9–16]; (iv) photolabeling experiments using open channel blockers identified specific amino acid residues of M2 as part of the high affinity binding site [2]; (v) synthetic monomeric peptides that mimic the amino acid sequence of M2 from *Torpedo californica* AChR δ subunit and from the Glycine receptor α subunit form, when reconstituted in lipid bilayers, discrete cation and anion selective ion channels, respectively [17–19]; (vi) NMR studies show that the M2 peptides from the AChR δ subunit [20] and the Glycine receptor α subunit [21] are indeed α -helical in lipid bilayers and are oriented perpendicular to the membrane plane [20,21]; (vii) a synthetic channel protein consisting of five identical M2 segments designed to mimic the predicted five helix bundle motif of the *Torpedo* AChR pore, forms cation-selective channels that match the single channel conductance of the authentic receptor [22].

Here, we address the question of how a negatively charged residue in M2, at a position thought to be exposed to the luminal face of the pore, modulates the ion permeation properties of ligand-gated ion channels. We use the neuronal $\alpha 7$ AChR as a prototype member of this superfamily of ion channels. The rationale for selecting this receptor is its structural simplicity

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Abbreviations: AChR $\alpha 7$ wt, wild type $\alpha 7$ acetylcholine receptor; AChR $\alpha 7$ D244, site-specific mutant of AChR $\alpha 7$; P_X/P_{Na} , relative permeability of ion X with respect to Na^+ ; ACh, acetylcholine; V_r , reversal potential; I , ionic current; NMG, *N*-methyl-D-glucamine; GHK, Goldman-Hodgkin-Katz relative permeability equation, the single-letter amino acid code is used.

[14,23,24], namely the $\alpha 7$ receptor is a homo-oligomer, presumably with a pentameric stoichiometry. Specifically, we have investigated the functional consequences of mutating the polar residue T244 in M2 to aspartic acid. Our results show that negative charges lining the aqueous pore markedly increase the AChR channel permeability to divalent cations.

2. MATERIALS AND METHODS

2.1. Site-directed mutagenesis

A cDNA encoding a neuronal $\alpha 7$ acetylcholine receptor from chicken brain, pCH2934-SP6 [25], was kindly provided by Dr. Ralf Schoepfer. Mutagenesis was performed using an oligonucleotide-directed in vitro mutagenesis kit (Bio-Rad). Missense mutations were introduced using *du^r* and *ung^r* selection [26]. T244 was mutated to aspartic acid (D244). Mutations were confirmed by DNA sequencing [27] using a sequenase kit (United States Biochemicals).

2.2. cRNA preparation and microinjection

cRNA transcripts were prepared from linearized cDNA using the SP6 RNA promoter/polymerase system [28]. Oocyte microinjection was as described [29,30]. Briefly, oocytes were removed from anesthetized adult female *Xenopus laevis* (Nasco, Fort Atkinson, WI) and dissociated with 2 mg/ml of collagenase (Type 1A, Sigma Chemical Co.) in Ca^{2+} -free Barth's medium [in mM: 88 NaCl, 1 KCl, 2.4 NaHCO_3 , 0.82 MgSO_4 , 5 *N*-tris[hydroxymethyl]-2-aminoethanesulfonic acid (TES), pH 7.4]. Isolated follicle-free stage V and VI oocytes were injected with 50 nl cRNA dissolved in diethylpyrocarbonate-treated water at 0.04 mg/ml, and maintained at 19°C in Barth's medium [Ca^{2+} -free Barth's supplemented with 0.4 mM CaCl_2 and 0.33 mM $\text{Ca}(\text{NO}_3)_2$].

2.3. Electrophysiological measurements

Oocytes were transferred (2–6 days) to a recording chamber ($V=0.2$ ml) and were continuously perfused (2–4 ml/min) with Ba^{2+} -Ringer's solution [in mM: 115 NaCl, 2.8 KCl, 1.8 BaCl_2 , 5 TES, pH 7.4], in the absence or presence of ACh supplemented with 2 μM atropine. For permeability studies the following Ringer's solutions were used: 115Na/2Ba (in mM): 115 NaCl, 1.8 BaCl_2 ; 90Na/2Ba: 90 NaCl, 25 *N*-methyl-D-glucamine (NMG), 1.8 BaCl_2 ; 50Na/2Ba: 50 NaCl, 65 NMG, 1.8 BaCl_2 ; 5Na/2Ba: 5 NaCl, 110 NMG, 1.8 BaCl_2 ; 0Na/2Ba: 115 NMG, 1.8 BaCl_2 ; 0Na/5Ba: 110 NMG, 5 BaCl_2 ; 0Na/10Ba: 100 NMG, 10 BaCl_2 ; 0Na/20Ba: 80 NMG, 18 BaCl_2 ; 0Na/2Mg: 115 NMG, 2 MgCl_2 ; 0Na/20Mg: 80 NMG, 20 MgCl_2 ; all supplemented with 2.8 KCl and 5 TES, pH 7.4.

Whole cell currents were recorded under voltage clamp using two-microelectrodes (Turbo TEC 01C, NPI Electronics, Tamm, Germany) [29–31]. Records were digitized at 0.5–2 kHz and filtered 0.1–1 kHz using an Axon TI-1 interface (Axon Instruments, Burlingame, CA) connected to a 386-based computer (Everex, Fremont, CA). Axotape, pClamp 5.5 and SigmaPlot 4.1 program packages were utilized for data acquisition and analysis. Oocyte experiments were performed at $22 \pm 2^\circ\text{C}$. All processed data are given as mean \pm S.D. with $n \geq 4$.

3. RESULTS AND DISCUSSION

3.1. AChR $\alpha 7$ D244 expresses functional receptors in oocytes

Injection of AChR $\alpha 7$ wt or AChR $\alpha 7$ D244 cRNA into oocytes evoked ACh-activated responses, as illustrated in Fig. 1A and B. Perfusion of oocytes expressing the homomeric AChR $\alpha 7$ wt with 0.1 μM or 1 μM ACh, at

holding potential $V_h = -80$ mV, did not elicit a detectable current; application of 10 μM ACh, however, produced a small inward current that activated and desensitized in presence of agonist (Fig. 1A). AChR $\alpha 7$ D244 exhibited larger ionic currents than those observed with wild type receptors (Fig. 1B). Whereas perfusion with 0.1 μM ACh did not produce a measurable current, ACh-activated inward currents were observed at 1 μM and 10 μM . The peak current exhibited by AChR $\alpha 7$ D244 receptors was ~ 200 -fold larger than that detected with AChR $\alpha 7$ wt (Fig. 1C, inset). Channel activity of both AChR $\alpha 7$ wt and AChR $\alpha 7$ D244 was blocked ($\geq 98\%$) after 5 min preincubation with 10 nM α -bungarotoxin ($n = 4$; data not shown).

The mutation in M2 also altered the ACh sensitivity of the receptor, as illustrated by the dose-response curves for ACh (Fig. 1C). The ACh concentration at which the current amplitudes were 50% of the maximal response (K_c , efficacy constant) were 250 ± 25 μM ($n = 8$) and 7.5 ± 0.5 μM ($n = 5$) with Hill coefficients of 1.2 ± 0.3 and 1.4 ± 0.5 for AChR $\alpha 7$ wt and AChR $\alpha 7$ D244, respectively. Together, these results show that introduction of a negatively charged residue in the putative channel domain of the receptor alters its sensitivity to the ligand, by increasing both the ligand potency (200-fold larger currents) and its efficacy (35-fold lower K_c).

3.2. AChR $\alpha 7$ D244 is permeable to Na^+ , Ba^{2+} and Mg^{2+}

The ionic permeability of wild type and mutant receptors to monovalent and divalent cations was investigated. To minimize the contribution of the endogenous Ca^{2+} -activated, -voltage dependent chloride channel to the measured currents [32,33], Ba^{2+} and Mg^{2+} , which poorly activate [32] this channel, were used. Niflumic and flufenamic acids, blockers of this endogenous channel [34], were not used because they precipitated in presence of Ba^{2+} and Mg^{2+} . To discern the currents carried by divalent cations, the experiments were performed in Na^+ -free Ringer's solution.

ACh activated inward currents in both AChR $\alpha 7$ wt (Fig. 2, top panels) and AChR $\alpha 7$ D244 (Fig. 2, bottom panels) at two different extracellular $[\text{Ba}^{2+}]$: 1.8 mM (0Na/2Ba) and 18 mM (0Na/20Ba). Wild type receptors exhibited a small inward current in 0Na/2Ba Ringer's solution, that augmented with increasing the extracellular $[\text{Ba}^{2+}]$ (0Na/20Ba Ringer's medium). A similar effect was observed when the external $[\text{Na}^+]$ was increased to 115 mM (115Na/2Ba Ringer's solution, Fig 1A.). Under the same conditions, AChR $\alpha 7$ D244 displayed much larger ACh-evoked ionic currents (Fig. 2, bottom). Together, these data indicate that both wild type and mutant receptors are permeable to cations.

A more striking result was obtained with Mg^{2+} . At extracellular $[\text{Mg}^{2+}]$ of 2 mM (Fig. 2, 0Na/2Mg) or 20 mM (Fig. 2, 0Na/20Mg), AChR $\alpha 7$ wt did not produce detectable ACh-evoked ionic currents. This lack of

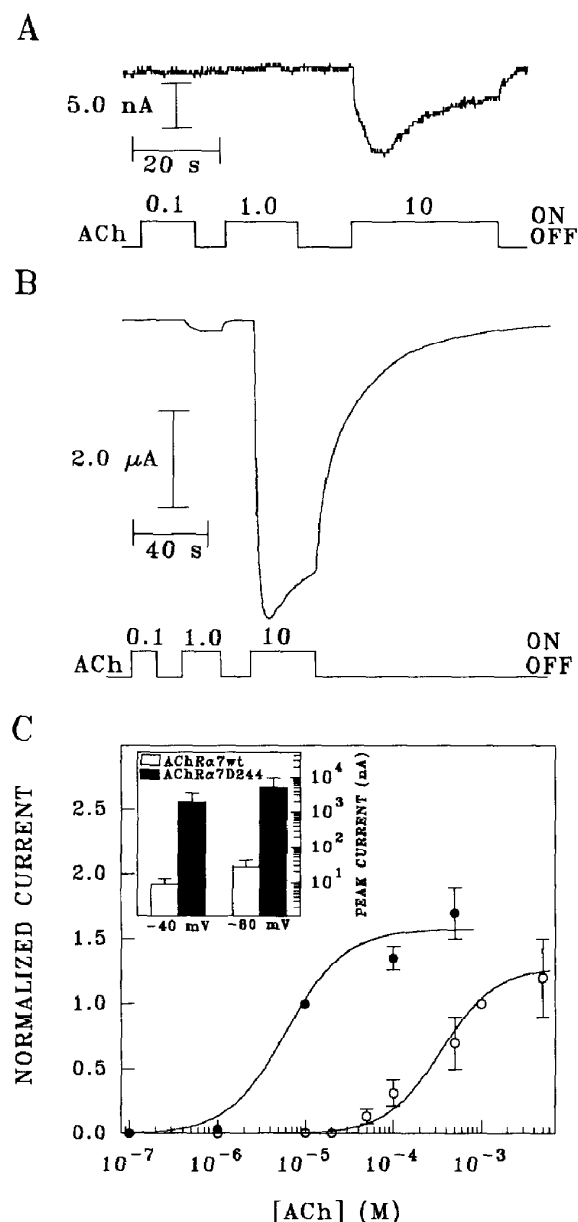


Fig. 1. AChR α 7wt and AChR α 7D244 express ACh-activated currents in *Xenopus* oocytes with distinctive functional properties. Current responses elicited by ACh (μ M) at $V_h = -80$ mV from oocytes expressing wild type (A) and mutant (B) receptors. Traces were filtered at 300 Hz. A downward deflection indicates inward current flow. ON and OFF denote initiation and cessation of ACh pulse. (C) Dose-response curves. ACh-elicited peak currents were measured at $V_h = -40$ mV, and normalized to that evoked by 1 mM ACh and 10 μ M ACh for wild type and mutant receptors, respectively. Each point represents the mean \pm S.D. with $n \geq 4$. Solid lines depict theoretical fits to a Michaelis-Menten binding isotherm given by $I/I_{\max} = K_e[\text{agonist}]^h / (1 + [\text{agonist}]^h)$, where I is the peak current, h the Hill coefficient, K_e the efficacy constant for the agonist and I_{\max} the maximal current. Inset. Peak currents elicited by 10 μ M ACh. Values are given as mean \pm S.D. with $n \geq 10$.

channel activity was independent of the applied voltage or the agonist concentration, indicating that wild type receptors are not permeable to Mg^{2+} . Furthermore, no

ACh-activated outward current, reflecting the efflux of K^+ , was observed at the voltages tested ($-80 \leq V \leq +20$ mV), suggesting that Mg^{2+} may block the channel. Conversely, mutant receptors did exhibit ACh-activated currents in presence of extracellular Mg^{2+} and, as for Ba^{2+} , currents increased with extracellular $[\text{Mg}^{2+}]$ as expected for a permeant ion.

The permeability of both receptors to monovalent and divalent cations was determined from reversal potential measurements (V_r) (Fig. 3). In normal Ringer's solution (115Na/2Ba), wild type receptors exhibited $I-V$ curves with a conspicuous inward rectification (Fig. 3A), and a $V_r = -4.3 \pm 3.2$ mV ($n = 10$). Removal of Na^+ from the external medium, shifted the V_r closer to the resting potential for K^+ , $V_r = -54 \pm 4$ mV, ($n = 7$). This effect was reversed by increasing the external $[\text{Ba}^{2+}]$ (Fig. 3A); in 0Na/20Ba Ringer's solution, $V_r = -1.0 \pm 4$ mV ($n = 13$).

For mutant receptors, the V_r measured in normal Ringer's medium was shifted towards more positive potentials, $+5.1 \pm 3.8$ mV ($n = 13$) (Fig. 3B). In addition, incorporation of a negative charge in the channel domain removed the strong inward rectification shown by AChR α 7wt (Fig. 3A). In the absence of extracellular Na^+ , $V_r = -32 \pm 3$ mV ($n = 9$). As for wild type channels, an increment of the external $[\text{Ba}^{2+}]$ shifted V_r to $+17 \pm 5$ mV ($n = 8$) (Fig. 3B). In all conditions explored, AChR α 7D244 reversed at more positive potentials than those displayed by AChR α 7wt (Fig. 3C), suggesting a higher permeability of the mutant channel to Na^+ and Ba^{2+} .

The V_r of the mutant receptor was also modulated by the external $[\text{Mg}^{2+}]$ (Fig. 3C). In 0Na/2Mg Ringer's solution $V_r = -57 \pm 6$ mV ($n = 9$) and shifted to -25 ± 4 mV ($n = 10$) when the external buffer was 0Na/20Mg. Note that for wild type receptors no ionic current was detected in Mg^{2+} -Ringer's solutions.

Collectively, these data demonstrate that both wild type and mutant receptors are permeable to monovalent and divalent cations. Furthermore, AChR α 7D244 displays a higher permeability to divalent cations than AChR α 7wt. In addition, the AChR α 7D244 exhibits a higher permeability to Ba^{2+} than to Na^+ and Mg^{2+} , suggesting a correlation between dehydration energy of the cation and permeation through the channel [1].

3.3. AChR α 7D244 has a higher relative permeability to divalent over monovalent cations

To rigorously assess the increment in divalent cation permeability consequent to the mutation, we measured reversal potentials at different extracellular concentrations of Na^+ and Ba^{2+} (Fig. 4). For both cations, V_r increases linearly with the logarithm of the external cation concentration (Fig. 4, insets), as predicted by the Goldman-Hodgkin-Katz (GHK) equation for a cation-selective channel [1]. To include both monovalent and divalent cations, the GHK equation is [35, 36]:

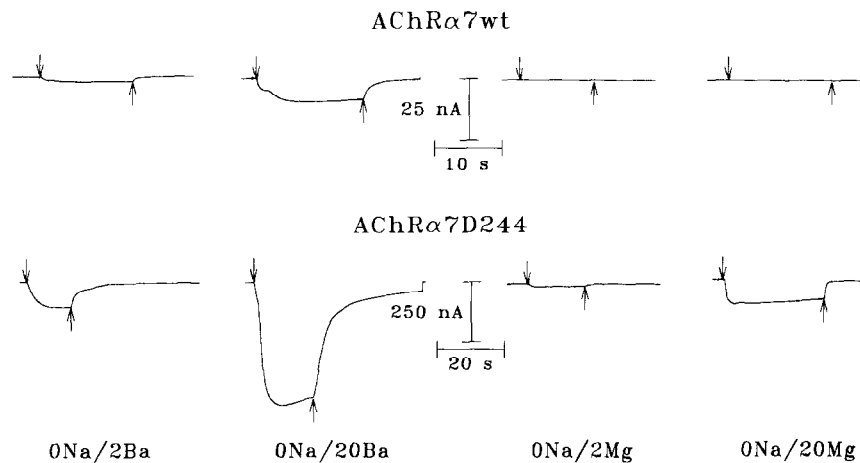


Fig. 2. AChR α 7D244 is permeable to Ba^{2+} and Mg^{2+} . ACh-evoked inward currents recorded in 0Na/2Ba, 0Na/20Ba, 0Na/2Mg and 0Na/20Mg Ringer's solutions from AChR α 7wt (top) and AChR α 7D244 (bottom) expressed in oocytes. ACh was 20 μM and $V_h = -80$ mV. \downarrow and \uparrow denote initiation and cessation of agonist pulse, respectively. Recordings were filtered at 100 Hz. Composition of Ringer's solutions as detailed in section 2.

$$V_r = \frac{RT}{F} \ln \frac{[\text{Na}]_o + \frac{P_K}{P_{\text{Na}}} [\text{K}]_o + 4 \frac{P'_{\text{Ba}}}{P_{\text{Na}}} [\text{Ba}]_o}{[\text{Na}]_i + \frac{P_K}{P_{\text{Na}}} [\text{K}]_i + 4 \frac{P'_{\text{Ba}}}{P_{\text{Na}}} [\text{Ba}]_i \exp(FV_r/RT)}$$

where $P'_{\text{Ba}} = \frac{P_{\text{Ba}}}{1 + \exp(FV_r/RT)}$, P_K/P_{Na} and $P'_{\text{Ba}}/P_{\text{Na}}$ represent the relative permeabilities of K^+ and Ba^{2+} , $[\text{X}]_o$ and $[\text{X}]_i$ are the extracellular and intracellular concentrations of the permeant ion (Na^+ , K^+ , and Ba^{2+}), and RT/F is 25.3 mV at 20°C [1]. Hence, the relative ionic permeabilities, P_K/P_{Na} and $P'_{\text{Ba}}/P_{\text{Na}}$, are readily obtained from measurements of V_r at different external ion concentrations. We assume that $[\text{Na}^+]_i = 10$ mM, $[\text{K}^+]_i = 120$ mM, $[\text{Ba}^{2+}]_i = 0$, and consider negligible the anionic contribution to the GHK equation. The experimental data depicted in Fig. 4A are readily fitted to the GHK equation using a non-linear least-squares regression algorithm [37] and substituting $[\text{K}^+]_o = 2.8$ mM and $[\text{Ba}^{2+}]_o = 1.8$ mM. The relative permeability values obtained for wild type AChRs were $P_K/P_{\text{Na}} = 1.2 \pm 0.1$ (best fitted value \pm error fit) and $P'_{\text{Ba}}/P_{\text{Na}} = 1.4 \pm 0.2$. Corresponding values for mutant receptors were $P_K/P_{\text{Na}} = 0.95 \pm 0.1$ and $P'_{\text{Ba}}/P_{\text{Na}} = 3.7 \pm 0.1$. A similar result was obtained when the data shown in Fig. 4B were fitted to the GHK equation, using $[\text{Na}^+]_o = 0$. Notice, however, that at high $[\text{Ba}^{2+}]_o$ a deviation from the theoretical prediction occurs, indicating that the constant field approximation of the GHK model can not account for the experimental data. More complex models, which consider specific interactions of the permeant ions with residues lining the lumen of the pore, should be taken into account to explain this deviation [1,36].

Here, we report that a ring of acidic residues lining the proposed aqueous pathway of the neuronal $\alpha 7$ re-

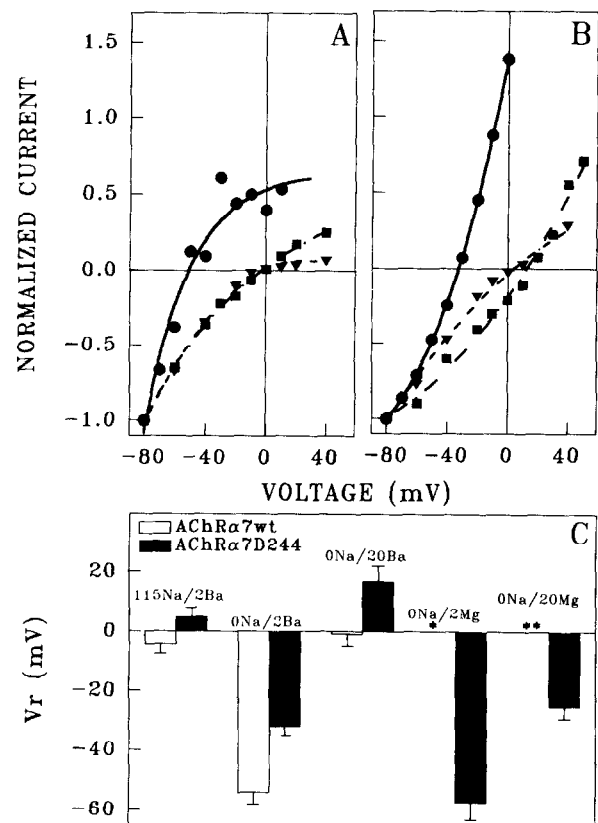


Fig. 3. AChR α 7D244 has a higher relative permeability to divalent cations than AChR α 7wt. I - V curves for wild type (A) and mutant (B) receptors. (\circ -) 0Na/2Ba, (\bullet -) 115Na/2Ba and (\blacksquare -) 0Na/20Ba Ringer's solutions. Currents were elicited by 100 μM ACh, and normalized to that evoked at $V_h = -80$ mV. Each data point has an experimental error of $\leq 20\%$ ($n \geq 8$). (C) V_r obtained from I - V curves. (*) and (**) indicate that no ACh responses were detected in 0Na/2Mg or 0Na/20Mg Ringer's medium. Values are given as mean \pm S.D. with $n \geq 8$.

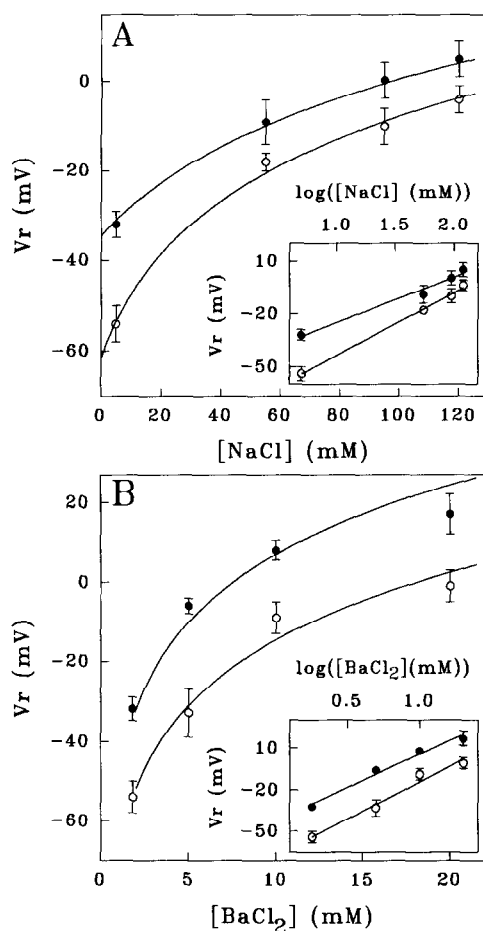


Fig. 4. AChR α 7D244 exhibits a higher relative permeability to divalent over monovalent cations. V_r was obtained from I - V curves (Fig. 3). External ionic concentrations are detailed in section 2. (—○—) AChR α 7wt, (—●—) AChR α 7D244. Values are mean \pm S.D. with $4 \leq n \leq 13$. Solid lines depict theoretical fits to the GHK equation (see section 3).

ceptor modulates its ionic selectivity, primarily increasing the relative permeability to divalent over monovalent cations. It is intriguing, however, that the incorporation of a single negatively charged residue in the putative channel-forming M2 segment drastically alters both its ion permeability and its sensitivity to ACh. It might be argued that the observables are consequence of a major conformational alteration of the protein to accommodate the negative charge, rather than a specific effect of introducing an acidic residue to the pore lining [12,13,23], or that the expression of mutant receptors in oocytes is more efficient than that of wild type. However, the mutant receptor exhibits the full complement of properties characteristics of the wild type receptors suggesting that a drastic folding change on the mutant protein is unlikely [21,22]. Since the mutation is in a domain distant from the ligand binding site [2,6], the alterations on ACh sensitivity may reflect a perturbation in the energetics of channel opening rather than

a change in ligand affinity [38]. The larger ACh responses of mutant receptors may be accounted for by an increase in the single channel conductance or in channel open probability or in both. Considering that the aqueous pore is formed by a pentameric arrangement of M2 segments [6], computer models show that a ring of negatively charged residues enlarges the pore area at the mutated position (data not shown). Accordingly, an increment of the single channel conductance would be anticipated. Preliminary single channel recordings indeed show that AChR α 7D244 exhibits a single channel conductance of ≈ 90 pS (Patten, C., Ferrer-Montiel, A.V. and Montal, M. unpublished observations), contrasted with the 45 pS conductance characteristic of AChR α 7wt [23,24].

In conclusion, our data support the notion that T244, or its equivalent, in the M2 transmembrane segment of cholinergic receptor channels is a fundamental structural determinant of the selectivity filter. The drastic increase in sensitivity of the mutant receptor to ACh implies a concerted interplay between two distant domains, namely the extracellular domain containing the agonist binding sites and the pore-forming domain.

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