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# Effects of pH on Acetylcholine Receptor Function

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**Summary.** We have examined the effects of changing extracellular pH on the function of nicotinic acetylcholine receptors from *Torpedo californica* using ion flux and electrophysiological methods. Agonist-induced cation efflux from vesicles containing purified, reconstituted receptors showed a monotonic dependence on external hydrogen ion concentration with maximal fluxes at alkaline pH and no agonist-induced efflux at pH's less than ~5. A similar pH dependence was measured for the peak agonist-activated membrane currents measured in microelectrode voltage-clamped *Xenopus* oocytes induced to express *Torpedo* receptor through mRNA injection. Half-maximal inhibition occurred at a similar pH in both systems, in the range of pH 6.5–7.0. Single-channel currents from *Torpedo* ACh receptors measured in patch-clamp recordings were also reduced in amplitude at acid pH with an apparent $pK_a$ for block of <5. Measurements of channel kinetics had a more complicated dependence on pH. The mean channel open time determined from patch-clamp measurements was maximal at neutral pH and decreased at both acid and alkaline pH's. Thus, both channel permeability properties and channel gating properties are affected by the extracellular pH.

**Key Words** nicotinic acetylcholine receptors · pH · *Torpedo californica* · oocyte expression system · reconstitution

## Introduction

The nicotinic acetylcholine receptor (AChR) has been the subject of extensive biochemical and biophysical analysis and is currently the best-characterized of the physiologically important ion channel proteins. Structural studies indicate that the pathway traversed by permeant ions consists of a large extracellular vestibule which connects to a narrow intramembranous pore region leading to the cell interior (e.g., Toyoshima & Unwin, 1988). This structure contributes to the AChRs high ion transport rate, with the wide mouth effectively collecting and funneling cations to a short, narrow, sieving region near the cytoplasmic face of the channel. The narrower intramembranous region of the pore is presumed to contain the "selectivity filter" where the passage of ions greater than ~7 Å in diameter is halted (Dwyer, Adams & Hille, 1980). Streaming potential measurements indicate that this most narrow region of the channel is probably only a few water molecules long (Dani, 1989), suggesting its design has evolved to minimally slow permeant ions during the selection process.

Structural and electrophysiological studies show that both the vestibule and pore region of the channel contain functionally important negatively charged groups. A number of electrophysiological experiments indicate that the vestibular region contains a low density of negative charge that can act to concentrate cations at the channel mouth (Lewis, 1979; Lewis & Stevens, 1979; Adams, Dwyer & Hille, 1980; Dani & Eisenman, 1987). Negative groups contributing to the selectivity filter are expected to contribute to the perfect cation selectivity of the channel (Adams et al., 1980; Dwyer et al., 1980). More recently, site-directed mutagenesis studies on *Torpedo* AChRs have suggested that concentric rings of negatively charged carboxylic acid moieties in both the vestibule and pore regions of the channel form the structural bases for these electrophysiological results (Imoto et al., 1986, 1988).

Previous studies on frog, mammalian, and chick AChRs have shown that extracellular pH changes affect the flux of ions through these channels via alterations in both conductance and kinetic properties of the channels (Huang, Catterall & Ehrenstein, 1978; Landau et al., 1981; Pappone & Barchfeld, 1990) without affecting agonist binding (Huang et al., 1978). Reducing extracellular pH decreases both the single-channel conductance and the mean open time of AChRs in these preparations, suggesting that normally negatively charged groups are titrated by increasing hydrogen ion concentration. The effects of external pH on the properties of *Torpedo* AChRs have not been systematically studied. In this paper we show that *Torpedo* AChRs survive exposure to a wide range of external pH's, making it possible to

use changes in external pH to explore the role of charged groups in determining *Torpedo* AChR function. Using flux assays in reconstituted receptors and macroscopic and microscopic voltage-clamp techniques on AChRs expressed in oocytes, we find that changes in external pH have similar effects on the ion flux, the single-channel conductance, and the mean open time of *Torpedo* AChRs as has been seen in receptors from other species. A preliminary report of this work has appeared in abstract form (Palma et al., 1990).

## Materials and Methods

### AChR Preparations

#### *Acetylcholine Receptor Purification and Reconstitution*

A crude membrane preparation partially enriched in AChR was prepared as described (McNamee, Jones & Fong, 1986) using frozen *Torpedo californica* electroplax obtained from Dr. H. Wang, University of California, Santa Cruz, CA. AChR was purified from AChR-rich membranes by affinity chromatography (Fong & McNamee, 1986) with a modification of the affinity column preparation to take advantage of significant cost savings and product reliability made possible by using Affi-Gel 10 instead of Affi-Gel 401 (Bhushan & McNamee, 1990). Briefly, Affi-Gel 10 (Bio-Rad, Richmond, CA) was suspended in 50 ml of 0.054 M cystamine hydrochloride in 20 mM MOPS at pH 7.4 for 1 hr in a 3 × 20 cm Bio-Rad Econo-column. The gel was washed with water and resuspended in 50 ml of 0.1 M DTT in 0.1 M MOPS at pH 8.0 for 30 min. The suspension was then allowed to pack and washed with water to remove DTT. The gel was then suspended in 0.1 M MOPS at pH 7.4 and 500 mg of bromoacetylcholine bromide was added with vigorous mixing and incubated for 30 min. The column was washed with 100 ml of water, and residual sulfhydryl residues were alkylated by adding 50 mg of iodoacetamide. The column was washed with 0.1 M sodium acetate at pH 4 for storage at 4°C and washed with buffer A (100 mM NaCl, 10 mM MOPS, 0.1 mM EDTA, 0.02% $NaN_3$ at pH 7.4) before use. All the following purification steps were carried out at 0 to 4°C.

Crude membranes were diluted with buffer A to a protein concentration of 2 mg/ml and solubilized by adding sodium cholate, with gentle stirring, to a final concentration of 1.2% (wt/vol) cholate. This mixture was centrifuged in a Beckman Type 35 rotor at 35,000 rpm for 60 min (95,000 × *g*). The supernatant was filtered through four layers of cheesecloth and applied to the affinity column. The column was washed with three-column bed volumes of a solution containing 2 mg/ml of dioleoylphosphatidylcholine (Avanti Polar Lipids, Birmingham, AL) and 1% sodium cholate in buffer A. AChR was then eluted by applying 100 ml of final wash containing 10 mM carbamylcholine (Carb) as eluent in the same buffer used for the washing steps. Protein concentration was determined by $A_{280}$ (protein mg/ml = $A_{280}$ × 0.6), and fractions were pooled to achieve a protein concentration of 1 mg/ml and dialyzed for 48 hr at 4°C against 4 liters of buffer A with three buffer changes.

For reconstitution, two volumes of the purified receptor at 1.5 mg/ml protein was mixed with one volume of a solution containing 6% sodium cholate and 60 mg/ml asolectin (Applied Concentrates, Woodside, NY) in buffer A. The mixture was dialyzed for 48 hr against four changes of buffer A (4 liters each) to give a final reconstituted membrane sample containing 1 mg/ml AChR and 20 mg/ml asolectin.

### *pH Modifications*

In most experiments, the pH was altered by exchanging buffer A with a universal buffer solution that was adjusted to pH values ranging from 4–9. The buffer consisted of (in mM): 10 MOPS, 10 acetic acid, 100 NaCl, 0.1 EDTA, 0.02% $NaN_3$. The buffer exchange was rapidly achieved using 2 ml BioLab QS4 columns packed with Sephadex G-25 (medium), equilibrated with the desired buffer, and partially dehydrated by 1-min centrifugation using a table-top centrifuge. Aliquots of the reconstituted membranes (200–500 μl) were then applied to the columns and centrifuged under the same conditions. The "spin column" method resulted in nearly complete recovery of membranes with minimal dilution. Return of the pH to the original value was achieved by reapplication of the sample to a new column equilibrated with the control pH (7.4).

### *AChRs Expressed in Oocytes*

*Torpedo californica* mRNA was prepared and injected into *Xenopus laevis* oocytes as described in Pradier, Yee and McNamee (1989). Briefly, the cDNA encoding for each of the individual subunits was subcloned into separate plasmids downstream from the specific *Samonella* phage SP6 promoter. The plasmids were linearized with the appropriate restriction enzyme (*SmaI* for α, γ, δ and *SacII* for β) to serve as a template for in-vitro transcription. Transcripts for the different subunits were dissolved in water to yield an $\alpha:\beta:\gamma:\delta$ mRNA weight ratio of 2 : 1 : 1 : 1 at a total final concentration of 200–400 ng/μl. Ovarian lobes were surgically removed from anesthetized *X. laevis* females and were dissected into clumps of 20–50 oocytes in ~5 volumes of Modified Barth's solution (MB; 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_2$, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 10 mM HEPES-NaOH, pH 7.6) containing 2 mg/ml collagenase (type 1A, Sigma). The oocytes were incubated in this solution with gentle shaking at room temperature for 1–2 hr to remove the follicle cells. After extensive washing of the oocytes with MB, the oocytes were transferred to MB supplemented with 300 μg/ml pyruvate, 100 μg/ml gentamycin, 10 μg/ml penicillin/streptomycin, 90 μg/ml theophylline, and 200 μg/ml BSA (fraction V, Miles Scientific). Any remaining follicle cells were removed manually with forceps. Healthy stage V and VI oocytes were selected for microinjection following 2–3 hr incubation at room temperature in supplemented MB. Injections were performed using a 10-μl micropipette (Drummond) and glass injection needles with a tip diameter of 20–30 μm. Approximately 50 nl (10–20 ng) of the mRNA transcript mix was injected into the equatorial region of an oocyte. Oocytes were incubated at 18–19°C in supplemented MB, and the media changed daily. The oocytes were used for voltage-clamp recordings after a minimum of 2–3 days incubation and were patch clamped after a minimum of five days.

## Assays of AChR Function

### *Ion Flux and Ligand Binding Assays*

The ion permeability response of the reconstituted membranes was measured essentially as described by McNamee et al. (1986). Briefly, 50-$\mu$l aliquots of membrane were mixed with 15 $\mu$l of buffer containing $^{86}Rb^+$ (2 mCi/ml) with or without 5 mM Carb. The buffers were at the same pH as the membrane samples. After 30 sec, a 50-$\mu$l aliquot was applied to a 3-ml Isolab QSY column packed with Dowex 50W-X8 cation exchange resin. The column was washed with 3 ml of 170 mM sucrose, and the entire eluate containing the membranes and trapped rubidium was counted using a liquid scintillation counter. Chernekov counting was used thus avoiding the need to add scintillation cocktail. The ion flux response was measured as the difference in cpm between the plus and minus Carb samples normalized to the total internal volume of the vesicles measured by allowing influx to continue for 48 hr prior to ion exchange chromatography.

Total receptor concentration was measured using the equilibrium $\alpha$-bungarotoxin binding assay as described by Jones, Earnest and McNamee (1987). Briefly, AChR-containing samples that had been equilibrated with different buffer solutions were diluted with buffer containing 10 mM NaCl, 10 mM MOPS and 0.2% Triton X-100 (pH 7.4) to give 200 $\mu$l of AChR at a concentration of approximately 20–50 nM. Fifty microliter aliquots of receptor were incubated with 50 $\mu$l of 150 nM $^{125}$I-$\alpha$-bungarotoxin (Amersham; 80,000 dpm/pmol) in the same buffer. The mixture was incubated for 30 min at room temperature and then diluted 100-fold with buffer and filtered under low vacuum through two DE52 filters and washed twice with buffer. The filters were counted on a Packard Gamma Counter. Controls included samples preincubated with a large excess of unlabeled toxin.

### *Microelectrode Voltage Clamp*

The whole-cell responses of *Xenopus* oocytes expressing *Torpedo* AChRs were measured 2 to 3 days following injection of AChR mRNA using an Axoclamp 2A two-electrode voltage-clamp (Axon Instruments, Foster City, CA). The voltage and current electrodes were filled with 3 M KCl and had tip resistances of 0.5–5 M$\Omega$. The recording chamber had a volume of ~1 ml and was continuously perfused at a rate of 25–30 ml/min with a standard bath solution (MOR2) consisting of (in mM): 82 NaCl, 2.5 KCl, 1 $Na_2HPO_4$, 5 $MgCl_2$, 0.2 $CaCl_2$, and 5 buffer. The buffers used were MES (pH 5.5–6.5), HEPES (pH 7.0–8.5), and CHES (pH 9.0). Agonist solutions contained in addition 1 $\mu$M ACh and 0.5 $\mu$M atropine. The holding potential in all experiments was −80 mV. For the pH studies, a single oocyte was preincubated in MOR2 at the pH to be tested for 5 min, then switched to agonist solution at the same pH. The bath solution was then exchanged for pH 7.4 MOR2 for 30 min before the next application of ACh. ACh responses at test pH's were bracketed by responses measured at pH 7.4. Current responses were recorded on a pen recorder.

### *Patch Voltage Clamp*

Single-channel currents from *Torpedo* AChRs expressed in *Xenopus* oocytes were recorded from excised outside-out patches as described by Methfessel et al. (1986). Patch pipettes were made from Corning 7502 glass and typically displayed open pipette resistances of 4–5 M$\Omega$. The pipette (internal) solution was composed of (in mM): 80 KF, 20 KCl, 10 K-EGTA, 10 HEPES, pH 7.2 (at room temperature). The bath solution for seal formation consisted of (in mM): 100 KCl, 1 $MgCl_2$, 10 HEPES, Ph 7.2 (at room temperature). Following gigohm seal formation (>20 G$\Omega$), the membrane underneath the patch pipette was ruptured by the application of a pulse of suction or voltage, and then the pipette was backed away from the oocyte to form the outside-out patch. The chamber was cooled to 8 ± 2°C and the solution in the bath was exchanged for the recording solution which consisted of 1 $\mu$M ACh, 100 mM KCl and 10 mM buffer. The buffers used were the same as for the microelectrode voltage-clamp experiments. pH was measured at 8°C and adjusted to the desired value with KOH. Single-channel currents were measured with either a Yale MkV or Dagan 3900 patch-clamp amplifier interfaced with a Cheshire Data Interface to a DEC LSI 11/73 computer system (INDEC Systems, Sunnyvale, CA). Currents were recorded directly onto the computer system or recorded on video tape for later analysis using a modified digital audio processor with 16-bit resolution (Bezanilla, 1985; Unitrade, Philadelphia, PA) and a video cassette recorder. Current data was filtered at 2 kHz and sampled with a 100-$\mu$sec interval. Single-channel conductances are slope conductances derived from either (*i*) open-channel currents measured during the application of voltage ramps from −100 to +100 mV or (*ii*) the construction of an *I-V* curve by measuring the current amplitudes at various holding potentials. Open times were calculated from steady-state recordings taken at a potential of −80 mV idealized using the half-amplitude criterion and analyzed with computer programs kindly provided by T. Hoshi and W. Zagotta.

## Results

### AChRs Reconstituted in Lipid Vesicles

Cation flux through AChRs reconstituted into vesicles decreases at acid pH. Fig. 1 shows normalized $^{86}Rb^+$ fluxes measured in response to activation of receptors with 1 mM carbamylcholine at different extracellular pH's. Increasing the extracellular pH from the normal value of pH 7.4 had only small effects on the cation flux, while decreasing pH dramatically reduced the $Rb^+$ influx. At pH's of 5.0 and below AChR activity is almost completely absent. The curve in Fig. 1 is drawn assuming a single site for hydrogen ion block of AChR activity with a $pK_a$ for block of 6.5. Given the scatter in the data the curve fits reasonably well, suggesting that an acidic group or groups with $pK_a$'s in the range between 6.0 and 6.5 are involved in channel function. Since these experiments measured the ion flux during a 30-sec exposure to agonist the values are a function of channel conductance and gating properties, both of which are affected by external pH (*see below*). Therefore, the fact that the data is not fitted perfectly by a single-site titration curve is not surprising.

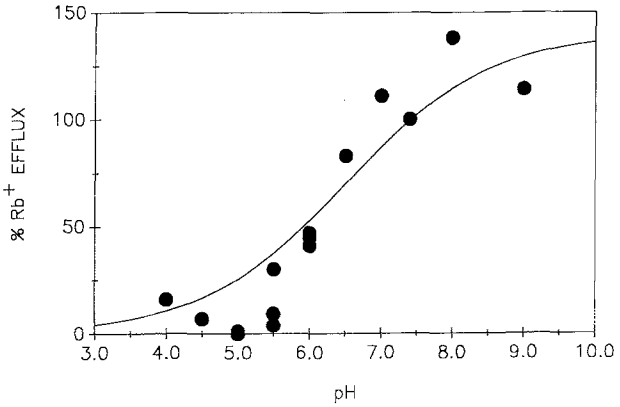


**Fig. 1.** Effects of extracellular pH on the carbamylcholine-induced $^{86}Rb^+$ efflux from lipid vesicles containing purified *Torpedo* AChRs. Agonist-induced $Rb^+$ efflux was measured as the difference between the efflux during a 30-sec incubation in the presence or absence of 1 mM carbamylcholine. Activated efflux is plotted relative to that measured at pH 7.4 in the same vesicle preparation. Each point represents the measurement from a single experiment. The curve is the relation expected if efflux were blocked by titration of a single group with a $pK_a$ of 6.5 and a maximum flux at alkaline pH that is 140% that at pH 7.4

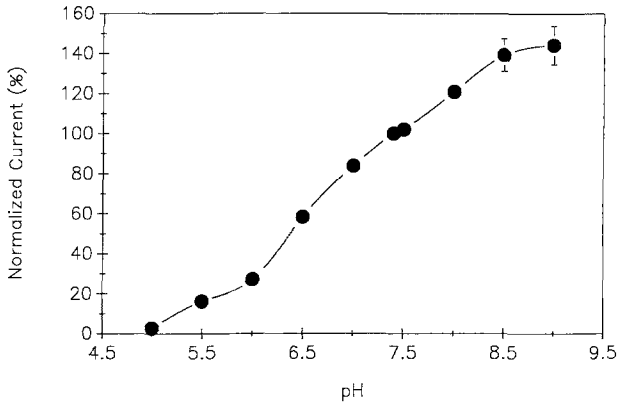


**Fig. 2.** Effects of extracellular pH on the acetylcholine-induced currents in voltage-clamped oocytes expressing *Torpedo* AChRs. Shown is the peak current measured during the exposure of oocytes to 1 μM acetylcholine. The cells were voltage clamped to a holding potential of −80 mV. The current responses at different pH's were normalized to the value measured at pH 7.4 in the same oocyte. Each point represents the average of 10 measurements on 10 different oocytes. error bars are the SEM

The effects of moderately low pH on ligand-induced flux are reversible. Prior exposure of receptors, either in solution or reconstituted into vesicles, to pH 6.0 buffer for 1–1.5 hr had no effect on the flux subsequently measured at pH 7.4, compared to controls maintained at pH 7.4 for the entire period. The effects of exposure to lower pH solution were less readily reversed, and 1-hr incubation of AChRs at pH 7.4 restored only ~½ the flux activity in vesicles exposed to pH 5.5 solution for 1 hr. In contrast, α-bungarotoxin binding activity was unaffected by 1-hr preincubation at pH's as low as 5.0.

## Receptors Expressed in Oocytes

Changes in external pH affected the peak amplitude of the currents produced by *Torpedo* ACh receptors expressed in *Xenopus* oocytes. Oocytes expressing receptor were voltage clamped to a holding potential of −80 mV. Rapid (<3 sec) application of 1 μM ACh produced large inward currents ranging from 200–1000 nA in amplitude. The current reached a peak amplitude within a few seconds and decayed with an approximately exponential time course to near zero. Figure 2 shows that acidification reduced and alkalinization increased the peak amplitude of the AChR current relative to that measured at pH 7.4. The peak current had a maximal value at pH's between 8.5 and 9.0 that was 44% greater than at normal pH. Increasing hydrogen ion concentration reduced the amplitude of the current, which was absent at pH's ≤ 5.0. The currents were half their maximal value at a pH of 7.0. The relationship between pH and peak AChR current was less steep than that seen in the flux experiments, and was also less steep than would be predicted for the titration of a single group with an acidic $pK_a$. Like the flux measurements however, the peak current measurement can reflect both conductance and kinetic properties of the channel and so could show complex pH dependence even if a single titratable group were involved.

## Single-Channel Recordings

Patch-clamp recordings of single AChR channel currents showed that both the single-channel conductance and the channel mean open time were sensitive to changes in extracellular pH. Figure 3 shows single-channel conductances measured from the open-channel current-voltage relation between −100 and +100 mV as a function of pH. The single-channel current-voltage relations were ohmic at all pH's. Channel conductance is relatively insensitive to pH changes between 9.5 and 6.5, but begins to decline at higher hydrogen ion concentrations. It was not possible to determine channel conductances at pH's more acid than 5.5 because of the infrequency of the opening events and their short duration (*see below*).

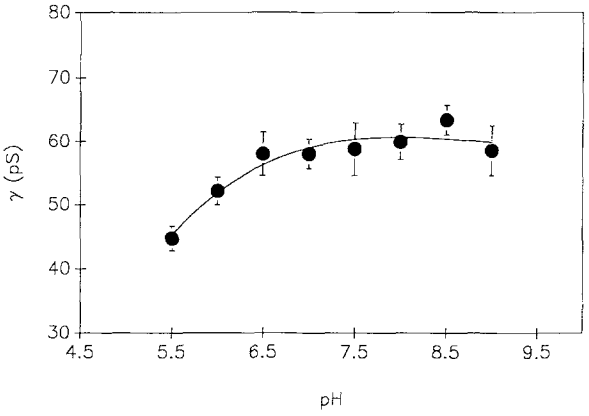


**Fig. 3.** Single-channel conductance as a function of pH. Single-channel conductance, $\gamma$, was determined from the slope of open single-channel current-voltage relations between −100 and +100 mV. Each point is the average of 3–4 measurements. Error bars represent the SEM. The line is a polynomial curve best fitted to the data

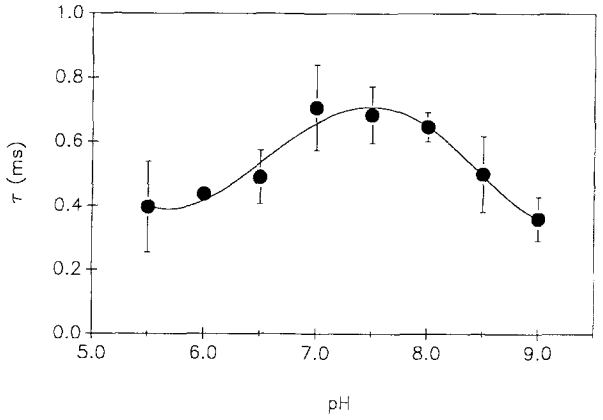


**Fig. 4.** Channel open times as a function of pH. Mean channel open time, $\tau$, was calculated from steady-state recordings made and a holding potential of −80 mV. Open-time histograms containing 88–287 events were fitted with a single exponential function to determing $\tau$. Each point is the average of 3–5 measurements made on different patches. Error bars represent the SEM. The line is a polynomial function best fitted to the data points

The effects are consistent with the titration of a single group with a $pK_a$ acid to 5.5.

In contrast, the channel mean open time had a bell-shaped dependence on pH as shown in Figure 4. The figure shows mean open times determined by fitting a single exponential function with time constant $\tau$ to the open-time histogram for events measured at a holding potential of −80 mV. Both increasing and decreasing pH from pH 7 caused a reduction in channel mean open time, indicating that channel open time is influenced by at least two different titratable groups, one acidic and one basic.

## Discussion

In this report we describe the effects of changing extracellular pH on the function of *Torpedo* acetylcholine receptors assessed using both ion flux and electrophysiological methods. Agonist-induced cation efflux from vesicles and peak agonist-induced membrane currents from oocytes expressing receptor both were maximal at alkaline pH's and decreased monotonically as the hydrogen ion concentration increased. Half-maximal inhibition occurred at a similar pH in both systems, in the range of pH 6.5–7.0. The microscopic measurement of single-channel conductance also showed a simple decrease at acid pH, although the apparent $pK_a$ for the effect is less than 5.0. In contrast, measurements of single-channel kinetics had a more complicated dependence on pH. The mean channel open time was maximal at neutral pH and decreased at acid and alkaline pH's. In addition, channel opening probability must be increased at alkaline pH, since whole-cell currents increased in amplitude between pH 8 and 9, while channel open time decreased dramatically in this range. Thus, both channel permeability properties and channel gating properties are affected by the extracellular pH, although apparently by different titratable groups on the channel.

This study represents the first examination of pH effects on *Torpedo* AChR function and the first time that AChR function has been assessed using these three methodologies on AChRs from the same source. We find that changes in extracellular pH have consistent effects on AChR function whether evaluated using flux or electrophysiological methods and whether the electrophysiological recordings are from many channels or single channels. These effects of external pH on *Torpedo* AChRs are also consistent with those described previously for AChRs from other sources. Agonist-induced $Na^+$ influx into cultured chick muscle cells is blocked at acid pH, with a pH dependence that is well fit by a titration curve for a single group with an apparent $pK_a$ of 4.8 (Huang et al., 1978). The single-channel conductance measured in frog muscle using fluctuation analysis decreases at acid pH (Landau et al., 1981), and single-channel conductance measured directly from mouse AchRs using patch-clamp techniques shows a similar monotonic pH dependency (Pappone & Barchfeld, 1990). Finally, the bell-shaped dependence of channel open times on pH described in the present experiments is similar to

those seen for the rate of decline of endplate currents in frog muscle and the mean channel open times measured in frog or mouse muscle. The similarities seen in the functional effects of pH on different AChRs indicates that the groups being titrated are conserved in receptors from quite a wide range of sources.

The pH-sensitive groups affecting channel conductance properties are most likely carboxylic acids associated with either glutamic or aspartic acid residues. It has been suggested on the basis of site-directed mutagenesis studies on *Torpedo* receptors that there are two externally facing rings of carboxylic acids lining the vestibular region of the permeation pathway that are important in determining the conductance properties of the channel (Imoto et al., 1986, 1988). The titration of single-channel conductance with a $pK_a$ less than 5 is consistent with this hypothesis.

The bell-shaped dependence of channel kinetics on pH suggests that at least two distinct chemical species participate in determining channel open times, one with an acid $pK_a$ and one with an alkaline $pK_a$. From the limited data of Fig. 4 the apparent $pK_a$ values of 6.6 and 8.5 can be estimated. The apparent values for the $pK_a$s in the present experiments would suggest the involvement of imidazole and sulfhydryl groups, as would the apparent $pK_a$'s measured for mouse receptors (Pappone & Barchfeld, 1990). In frog muscle, the open times measured for AChRs from the American frog, *Rana pipiens*, would be consistent with the titration of a carboxylic acid and an amino group, while the relationship for receptors from the European frog, *Rana ridibunda*, would be more consistent with the titration of a carboxyl group and an imidazole (Landau et al., 1981). A possible explanation of these findings would be that the protonation/deprotonation of carboxyl, imidazole, and sulfhydryl groups could all exert similar affects on channel gating properties in different receptor species. However, such an explanation implies opposite effects of imidazole group titration in *Rana pipiens* and *Torpedo* or mouse receptors. Alkalinization reduces channel open time in *Rana*, whereas alkalinization in the same pH range increases channel open time in *Torpedo* and mouse receptors.

An additional difficulty with a simple interpretation of the pH results comes from chemical modification experiments in which the pH dependence of channel open time of mouse AChRs is completely abolished by modification of the receptors with a carboxyl-group specific reagent (Pappone & Barchfeld, 1990). It was suggested that the chemical modification results could be explained by the formation of a salt bridge between a negatively charged carboxylic acid and a positively charged imidazole in the course of channel opening. Salt bridge formation could stabilize the open state of the channel only when both groups were charged, leading to the bell-shaped dependence of channel open time on pH. A similar explanation could hold true for receptors from other sources as well if there are significant differences in the local environment of the tritratable groups that shift their pH dependence. Positive identification of the groups involved will require studies using site-directed mutagenesis of the receptor. Once the moieties involved are identified, further study of their pH dependence could prove useful in making inferences about the electrostatic environment of the titratable groups.

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