Modifications of Single Acetylcholine-activated Channels in BC3H-1 Cells

Effects of Trimethyloxonium and pH

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ABSTRACT We have examined the effects of chemical modification with trimethyloxonium (TMO) and changes in external pH on the properties of acetylcholine (ACh)-activated channels in BC3H-1 cells, a clonal muscle cell line. TMO reacts covalently and specifically with carboxylic acid moieties in proteins to convert them to neutral methyl esters. In BC3H-1 cells TMO modification reduces the whole-cell response to ACh measured at negative membrane potentials by ~60%. GΩ seal patch-clamp recordings of single ACh channel currents showed that the reduction in ACh sensitivity is due to alterations in both the current-carrying and the kinetic properties of the channels. Under all our experimental conditions, i.e., in external solutions of normal or low ionic strength, with or without external divalent cations, and at external pHs between 5.5 and 8.1, TMO treatment reduced ACh single-channel conductance to 70-90% of normal. The effects of TMO on channel kinetics were dependent on the ionic conditions. In normal ionic strength solutions containing both calcium and magnesium ions TMO modification reduced the channel average open time by ~25%. A similar reduction in open time was seen in calcium-free solution, but was not present when both calcium and magnesium ions were absent from the external solution. Lowering the ionic strength of the solution increased the mean open time in normal channels by about threefold, but did not affect the kinetics of modified channels. In low ionic strength solutions normal ACh channel open times were maximal at ~pH 6.7 and decreased by three- to fourfold at both acid and alkaline pH. TMO modification removed the pH dependence of channel kinetics, and average open times were short at all pHs between 5.5 and 8.1. We suggest that TMO modifies normally titratable groups on the external surface of ACh channels that help to determine both the gating and permeability properties of ACh channels.
INTRODUCTION

The nicotinic acetylcholine (ACh)-activated channel has been extensively studied for a number of years using biochemical, biophysical, and molecular genetic techniques, and is currently the best-characterized of the physiologically important ion channels. Although the primary structure of the channel has been known for some years now (Noda et al., 1982, 1983a, 1983b; Claudio et al., 1983; Devillers-Thiery et al., 1983), we do not know enough about the rules governing tertiary protein structure in membranes to be able to predict the relations between chemical and structural features of the channel with confidence (Guy and Hucho, 1987). Further, we are only beginning to learn how structural features of the channel determine the permeability properties of the pore.

Both structural (Toyoshima and Unwin, 1988) and biophysical (Neher and Steinbach, 1978; Adams et al., 1980; Dwyer et al., 1980; Horn et al., 1980) measurements indicate that the ACh channel has a large "vestibule" forming the outer region of the aqueous pore. Several lines of evidence indicate that negatively charged or polarized chemical groups are associated with this region of the molecule. ACh channels are highly glycosylated, and contain a large number of negatively charged sugar residues on their outer surface. ACh channels are perfectly selective for cations over anions (Adams et al., 1980), indicating that there must be some degree of negativity (although not necessarily a net negative charge) encountered by ions traversing the channel. Selectivity measurements show concentration-dependent permeability ratios in mixtures of monovalent and divalent cations that are consistent with a low density negative surface charge at the exterior face of the channel (Lewis, 1979; Lewis and Stevens, 1979; Adams et al., 1980). Many complicated features of ion permeation through ACh channels such as the anomalously high conductance of the channel in low concentrations of cations and the nonlinear, inwardly rectifying current-voltage relations seen in the absence of divalent cations can be relatively simply accounted for by the presence of negative charge influencing the entrance of ions into the channel (Dani and Eisenman, 1987). Finally, studies examining the effects of pH on ACh receptor channels indicate that titrating groups with acidic $pK_a$ values can reduce channel conductance (Landau et al., 1981; Huang et al., 1978).

Carboxylic acid moieties, either contained in aspartic or glutamic acid residues of the protein, or associated with the glycosylation of the receptor are likely sources of some of the negative charge of the ACh channel. The $pK_a$'s determined from hydrogen ion block experiments in ACh channels are consistent with the titration of a carboxylic acid group. There is evidence from chemical modifications of ACh receptor channels that carboxylic acids, either forming part of the channel protein or associated with other membrane components, can affect the properties of ACh channels. Carboxyl group specific reagents reduce the acetylcholine sensitivity of muscle (Edwards et al., 1970; Stuesse and Katz, 1973; Nachshen and Landau, 1977), decrease the agonist-induced flux from vesicles containing ACh receptors (Chao et al., 1975), and reduce the single-channel conductance and kinetics of muscle ACh receptor channels measured using noise analysis (Adams, 1983). Recently, results of more direct molecular biological experiments have shown that replacement of glutamate or aspartate residues by neutral or positively charged amino acids decreases the single-channel conductance (Imoto et al., 1986, 1988). These results
all support the idea that carboxylic acids contribute functionally important negativity to ACh channels.

Modification of ion channel proteins with trimethyloxonium (TMO) has been used to study the functional roles of carboxylic acid groups in both voltage-gated and ligand-gated channels. TMO covalently modifies ionizable carboxyl groups to neutral methyl esters, and in proteins in aqueous solution the reaction seems to be specific for carboxyl groups (Parsons et al., 1969; Reed and Raftery, 1976). TMO modification reduces the single-channel conductance of voltage-gated sodium channels (Sigworth and Spalding, 1980; Worley et al., 1986) and calcium-activated potassium channels (MacKinnon and Miller, 1989a, b). Results from fluctuation analysis of frog muscle endplate currents suggested that TMO modification has similar effects on ACh channel permeability properties, decreasing the apparent single-channel conductance by 50% (Adams, 1983).

We began the experiments presented here to characterize the effects of TMO modification on the permeation process in ACh channels at the single-channel level. We used GΩ seal patch recording methods to examine the effects of TMO modification on the behavior of ACh channels in cells from a clonal muscle cell line, BC3H-1. To our surprise, we found that TMO modification has only small effects on channel conductance properties in our cells, but substantially alters channel kinetics. To further characterize the groups reacting with TMO we also examined the effects of changes in external pH on normal and modified channels. Preliminary accounts of these results have been presented in abstract form (Barchfeld and Pappone, 1988; Pappone and Barchfeld, 1989).

METHODS

Cell Cultures

BC3H-1 cells (#CRL 1443; American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (10% DMEM; Gibco, Grand Island, NY) or 10% fetal calf serum/horse serum (Sigma Chemical Co., St. Louis, MO) in a humidified atmosphere of 5% CO₂, 95% air, at 37°C. Cells were cultured and prepared for experiments as previously described (Patrick et al., 1977). Briefly, stock cultures were grown in 60-mm culture dishes and subcultured every 3 or 5 d. Subculturing consisted of removing the growth media, rinsing with Hank's balanced salt solution (HBSS; Sigma Chemical Co.), applying 0.025% pancreatin (Gibco) in HBSS for 3–4 min, gently loosening the cells by scraping with a nylon policeman, then adding 10% DMEM. The cell suspension was centrifuged at 1,800 rpm for 5 min, the supernatant was removed, and fresh 10% DMEM was added. The pellet was gently triturated to resuspend the cells. For stock cultures, the cell suspension was plated onto culture dishes at a cell density of ~0.5 x 10⁶ cells/ml. Cells to be used in experiments were similarly plated on collagen-coated (rat tail Type I; Sigma Chemical Co.) glass coverslips in 35-mm culture dishes. 1 d after plating the serum concentration was reduced to 0.5% to induce expression of nicotinic ACh receptors (Olsen et al., 1983). Cells were used for experiments 8–18 d after the switch to low serum media.

Solutions

For whole-cell experiments the bath solution contained 140 mM CaCl₂, 5.4 mM NaCl, 1.8 mM CaCl₂, 1.7 mM MgCl₂, 9.5 mM Na-HEPES, adjusted to pH 7.4 with NaOH. The pipette
solutions contained 140 mM KCl or CsCl, 11 mM K$_2$-EGTA, 1 mM CaCl$_2$, 10 mM K-HEPES, adjusted to pH 7.2 with KOH.

Single-channel currents were recorded in the cell-attached patch-clamp configuration using pipette solutions having both normal and low ionic strength. Cesium was the major external cation in the pipette in all the single-channel recordings to help eliminate possible contributions to the current record from other channel types. For these recordings the cells were superfused with the above bath solution, but with potassium replacing cesium to set the resting membrane potential near 0 mV. The normal ionic strength solution was the same as the cesium bath solution above. The 0 Ca solution contained 140 mM CsCl, 11 mM Cs-EGTA, 1 mM MgCl$_2$, and 10 mM Cs-HEPES. The 0 Ca, 0 Mg solution was the same except that the MgCl$_2$ was omitted. Low ionic strength solutions contained 10 mM CsCl, 5 mM Cs-HEPES, and 250 mM sucrose and either 0 Ca and 1 mM Cs-EGTA or 1.8 mM Ca. Low ionic strength solutions contained no added magnesium. The pH of the solutions was normally 7.4. For experiments examining the effects of pH, the pH of the low ionic strength solution was adjusted with HCl or NaOH. The ACh concentration present in the pipette was 5 μM in all the single-channel experiments reported here. ACh was stored as a frozen concentrate. Before each experiment, the ACh stock was thawed and diluted into the bath or pipette solution.

**TMO Modification**

The external surface of BC3H-1 cells was modified using TMO tetrafluoroborate (Alfa Products, Danvers, MA) as previously described by Spalding (1980). TMO crystals sufficient to give a final concentration of 50 mM were added to an iced solution consisting of 80 mM Na-MOPS, 2 mM CaCl$_2$, and 60 mM NaCl, pH 8. Immediately after the addition of TMO the reaction solution was rapidly washed onto the cells. The cells were exposed to the reaction solution for 2 min. They were then washed extensively with bath solution. Because TMO is rapidly hydrolyzed the concentration of TMO reaching the cell membrane is not known. The cells were in bath solutions at least 5 min before electrical recording began.

**Voltage-Clamp Measurements**

Patch electrodes were pulled from Corning 7052 glass (Garner Glass, Claremont, CA) with a two step vertical puller (Narishige, Tokyo, Japan), coated with Sylgard (Dow Corning Corp., Midland, MI), and heat polished to an electrode resistance of 2–6 MΩ. The electrode was pressed against the cell surface and suction applied to form a high resistance seal of 10–80 GΩ. Seal resistances were generally higher and more stable in acid solutions than in neutral or alkaline solutions.

Patch-clamp currents were measured using a List L/M EPC 7 patch-clamp amplifier. The current signal was filtered with an 8-pole lowpass Bessel filter (Frequency Devices, Inc., Haverhill, MA) and recorded through a Cheshire Data Interface (INDEC, Sunnyvale, CA) run by an LSI 11/73 PDP computer (Digital Equipment Corp., Marlboro, MA). Membrane current recordings were stored directly on the disk of the computer or recorded on videotape for later analysis using a modified digital audio processor with 16-bit resolution (Bezanilla, 1985; Unitrade, Philadelphia, PA) and a video cassette recorder. All experiments were done at ambient temperature (22–25°C).

**Data Analysis**

Channel conductances and reversal potentials were measured from inward cesium currents and analyzed using one of three methods. (a) Steady-state records of channel events at different membrane holding potentials were used to generate amplitude histograms at each potential, the peaks of which were fitted by eye to determine the single-channel current
amplitude and baseline current amplitude. The single-channel current amplitudes at different membrane potentials between -200 and -20 mV were least-squares fitted by a line, the slope of which gave the single-channel conductance, \( \gamma \), and the intercept of which estimated the reversal potential, \( E_r \). (b) Multiple measurements by eye of single-channel current amplitudes occurring during steps in membrane potential were averaged to determine the mean current amplitude. \( \gamma \) and \( E_r \) were determined from these data as above. (c) Currents measured during ramps of applied potential were sorted into open and closed events, averaged independently, and the closed average was subtracted from the open average. The resultant open-channel current-voltage relation was fitted with a line between -200 mV and \( E_r \) and \( \gamma \) and \( E_r \) determined as above. Methods a and b were used to analyze currents recorded in normal ionic strength solutions and methods a and c were used for experiments in low ionic strength solutions. There was no significant difference in the values of \( \gamma \) obtained using the different methods.

Channel average open times were determined from 2-20-min recordings of channel openings measured during steady-state exposure to 5 \( \mu \)M ACh. The recordings were filtered at 1.6 KHz and sampled at 125 \( \mu \)s/point. The raw current records were converted to idealized records using a semiautomated process in which events that crossed half the mean single-channel current amplitude were confirmed or rejected by the operator. The minimum channel open time or closed time that could be resolved was 113 \( \mu \)s in neutral or alkaline pH solutions. The smaller amplitude of the channel events at low pH reduced the temporal resolution possible under these conditions. The idealized records were used to construct open-time histograms. In the results the average open time refers to the arithmetic mean duration of channel opening events detected by the 50% amplitude criterion. Mean open times were determined by fitting one or two exponential functions to these data using a least-squares method. A minimum of 150 events was used to generate the histograms, and more typically they contained 300-500 events. Averages are reported \( \pm \) SEM throughout.

**RESULTS**

**Effects of TMO Modification on Macroscopic Currents**

We examined the effects of TMO modification on the currents recorded from BC3H-1 cells in the whole-cell configuration to determine whether TMO modification reduced ACh-induced currents as had been reported in frog muscle (Adams, 1983), and to test for the presence of other currents in modified or unmodified cell membranes which might interfere with our measurements of ACh channel properties. BC3H-1 cells are large; cell resistances measured in whole-cell voltage clamp were 0.1-0.3 G\( \Omega \) and cell capacitances ranged from 40 to 90 pF. Because of their large size, the cells were not well voltage clamped, hence these results can only be used for a qualitative assessment of cell membrane properties.

Fig. 1 shows the whole-cell response of a BC3H-1 cell to ACh with cesium as the major cation in both the extracellular and intracellular solutions. In A, 1 \( \mu \)M ACh causes a threefold increase in the cell conductance in the steady-state before exposure to TMO. As shown in B, subsequent treatment of the cell membrane with TMO dramatically decreased the amount of cell conductance induced by ACh and slightly increased the resting conductance. In the cell of Fig. 1, 1 \( \mu \)M ACh caused only 32% as much of a conductance increase as it had in the control application of ACh to the same cell before modification. TMO modification consistently reduced the response of the cells to ACh, but the extent of this reduction was variable, and
ACh responses in modified cells ranged from 32–61% of control values, with an average response of 38% ± 8% (n = 4) of control. The variability is probably due at least in part to the difficulty in exposing the cells to a consistent dose of TMO because of its extreme lability in aqueous solution. There was no significant change in the reversal potential, $E_r$, after TMO modification, nor were there substantial changes in the shape of the current-voltage relation. With cesium as the major cation outside and inside the cell, $E_r$ averaged 1.7 mV ($±2.3$, $n = 3$) in controls and 0.8 mV ($±2.0$, $n = 3$) in TMO-modified cells. With cesium outside the cell and potassium inside, the values of $E_r$ were 0.2 mV ($±1.8$, $n = 5$) in controls and $-1.7$ mV ($±1.7$, $n = 3$) after TMO treatment. The resting conductance increased after TMO modification, e.g., by 30% in the cell shown in Fig. 1 (average 27% in four whole-cell experiments). The reversal potential of the leak conductance was not changed by TMO and the effects on cell resting conductances were not examined further. Thus, our results from whole-cell measurements in BC3H-1 cells confirm the finding in frog endplate that TMO substantially reduces the response to ACh.

In our initial experiments, the only voltage-dependent currents that we observed in BC3H-1 cells were delayed-rectifier type potassium currents, as shown in Fig. 2. The potassium currents activated at membrane potentials positive to $-30$ mV in both control and TMO-modified cells. These currents could be largely eliminated by replacing internal potassium with cesium, as shown in Fig. 2 B. No other voltage-dependent currents were apparent in these records, even with the large potassium currents blocked. TMO modification had little effect on the magnitude or voltage dependence of the potassium currents in BC3H-1 cells (not shown) and they remained largely blocked by cesium, as shown in Fig. 2 B. No other voltage-dependent currents were induced by modification of the membrane with TMO. Thus, measurements made at negative membrane potentials in the presence of

![Figure 1](image-url)
extracellular cesium should be uncontaminated by currents through voltage-gated channels. We therefore confined our measurements of single ACh channel properties to these conditions. In some later experiments voltage-gated sodium currents were present in the cells as well, as has been reported previously for BC3H-1 cells grown under similar conditions (Caffrey et al., 1987). The cells used in the later experiments were grown in a culture medium containing a mixture of fetal calf and horse serums rather than the fetal calf serum alone as was used in the earlier experiments and those of Caffrey et al. (1987). It is possible that differences in serum composition may be responsible for the different channel expression seen in these experiments.

**Figure 2.** Voltage-activated currents in BC3H-1 cells. Currents were recorded from cells voltage clamped in the whole-cell configuration during 200-ms depolarizing voltage-clamp pulses from the holding potential of −80 mV. Linear currents were subtracted from the records using the P/4 procedure (Armstrong and Bezanilla, 1974). (A) Currents in an unmodified cell with K⁺ as the major internal cation and Cs⁺ as the major external cation. Shown are superimposed current records from pulses to potentials between −50 and +30 mV in 10 mV increments. (B) Voltage-clamp currents recorded from a cell before (upper) and after (lower) exposure of the cell to TMO with Cs⁺ as the major extracellular and intracellular cation. Each set of records shows superimposed records measured during depolarizations to potentials between −50 and +30 mV in increments of 20 mV.

**Channel Permeability Properties**

**Effects of TMO modification.** Treatment with TMO substantially reduces the single-channel conductance, γ, of voltage-gated sodium channels (Sigworth and Spalding, 1980; Worley et al., 1986) and calcium-activated potassium channels (MacKinnon and Miller, 1989a, b). We expected that the substantial reduction we saw in ACh-induced currents in the whole-cell experiments on BC3H-1 cells was similarly due to a change in channel permeability properties, as suggested by the results of fluctuation analysis in frog muscle endplates (Adams, 1983). However, as shown in Fig. 3, single-channel current amplitudes were only slightly lower in modified than in unmodified membranes. Neither the single-channel records of Fig.
3 B, nor the amplitude histogram constructed from the currents measured during epochs containing 275 channel openings shown in Fig. 3 D show any evidence of smaller conductance channel events in addition to the major, near normal amplitude events in TMO-modified membranes. All the channel events following TMO modification were of similar amplitude, that is slightly reduced relative to control values. There was never any evidence of a "hump" on the amplitude histogram corresponding to the current amplitude expected from normal conductance channels. We know that the channels of Fig. 3 D had been modified by TMO because their kinetics were altered (see below). If TMO modification substantially reduces γ of some ACh channels in BC3H-1 cells, it must be to a level well below the 50% normal amplitude suggested by the noise experiments in frog muscle.

The decrease in single-channel currents after TMO modification results from a small but consistent reduction in single-channel conductance as shown in Fig. 4 and

![Figure 3](image)

**Figure 3.** (A) Single-channel records from cell-attached patches recorded before (Control, left) and after (TMO, right) modification of the cell membrane with TMO. The recordings are from two different cells, measured at a holding potential of −100 mV. The extracellular solution in the pipette contained 140 mM Cs⁺, 1.8 mM Ca²⁺, and 5 μM ACh. Records were filtered at 1,600 Hz and sampled at 125 μs/point. Inward currents are shown as downward deflections. (B) Amplitude histograms generated from single-channel recordings from an unmodified cell and a TMO-modified cell made under the same conditions as in A. The baseline current level is considered to be zero current. Current amplitudes of the histogram peaks are −5.3 pA in the control and −4.6 pA in the TMO-modified patch.

Table I. TMO modification reduced γ in solutions of normal and low ionic strength, in the presence and absence of divalent cations, and at all pHs between 8.1 and 6 (see Fig. 5 below). These 7–28% reductions in ACh channel conductance are insufficient to explain the ~60% reduction in ACh-induced currents seen in the whole-cell experiments.

While TMO treatment reduced ACh channel conductance under a number of experimental conditions, TMO seems to have little other effect on other channel permeability properties. Fig. 4 shows single-channel current-voltage relations for normal and modified channels under conditions of differing ionic strength, divalent cation concentration, and pH. TMO modification did not alter the shape of the current-voltage relations nor did it affect the reversal potential for the currents in any of these solutions. The average reversal potential changed <5 mV after TMO modification under any of our ionic conditions, indicating that channel selectivity
was not significantly affected by the treatment. The single-channel conductances of both control and TMO-modified channels were reduced to a similar extent by changes in permeant ion concentration as shown in Table I. Decreasing the external cesium concentration from 140 to 10 mM decreased $\gamma$ by 40% in unmodified channels and by 38% in modified channels in the absence of divalent cations. ACh channel conductances in normal and TMO-modified channels also responded similarly to changes in divalent cation concentrations. As has been previously reported for ACh channels from other sources (Lewis, 1979; Bregestovski et al., 1979; Magleby and Weinstock, 1980; Dani and Eisenman, 1987; Mathie et al., 1987), increasing the external calcium concentration decreases $\gamma$ in BC3H-1 cells. In unmodified patches, increasing the calcium concentration from 0 to 1.8 mM reduced the single-channel conductance by 27% in normal ionic strength solution. Calcium had a more marked effect in solutions of low ionic strength. Unmodified channel conductance decreased by 64% in 1.8 mM calcium compared with 0 calcium

FIGURE 4. Effects of TMO modification on single-channel conductances measured under conditions of differing ionic strength, divalent cation concentration, and pH. Each condition represents data from a different patch. (A and B) single-channel current-voltage relations with normal ionic strength solution in the pipette containing 140 mM $\text{Cs}^+$ with (A) and without (B) 1.8 mM $\text{Ca}^{2+}$. Solutions in both A and B contained $\text{Mg}^{2+}$. Currents at the different membrane potentials were measured during 200-ms steps in membrane potential. Open symbols are from unmodified patches and filled symbols are from TMO-modified membranes. Where only one symbol is shown the two measurements superimpose. The lines are from a least-squares fit of the data from unmodified membranes and have slopes of $\gamma = 48$ pS in A and $\gamma = 61$ pS in B. (C and D) Single-channel current-voltage relations in low ionic strength solutions in pH 7.2 (C) and pH 6.7 (D) external solutions in unmodified (solid lines) and TMO-modified (points) patches. Currents are averages made from open-channel currents occurring during ramps of applied voltage between -200 and -20 mV. (C) Control $\gamma = 43$ pS, TMO $\gamma = 41$ pS. (D) Control $\gamma = 39$ pS, TMO $\gamma = 36$ pS. The average baseline conductance has been subtracted from the records. The solutions contained 10 mM $\text{Cs}^+$, 0 $\text{Ca}^{2+}$, and 0 $\text{Mg}^{2+}$. 


when the external solution contained only 10 mM cesium. TMO modification did not alter these effects of calcium. 1.8 mM calcium caused a 20% decrease in \( \gamma \) in normal ionic strength solution and a 70% decrease in the low ionic strength solution in TMO-treated patches. Removing magnesium as well as calcium from the pipette solution also increased channel conductance to a similar degree in normal and modified channels. The similarities in the effects of changing divalent and permeant ion concentrations on channel conductance before and after TMO modification indicate that TMO reduces channel conductance through some mechanism other than a neutralization of negative surface charges.

**Effects of pH.** To test whether TMO reduces \( \gamma \) through the modification of a titratable group or groups associated with the channel, we assessed the effects of changing external pH on the single-channel conductance. As shown in Fig. 5, increasing pH from 7.4 to 8.1 had little effect on \( \gamma \) measured in normal channels. Decreasing pH affected \( \gamma \) more substantially. Lowering the pH from 7.4 resulted in a gradual reduction of \( \gamma \) to ~50% of its value in neutral solution at pH 5.5. Assuming that the conductance would be completely blocked at sufficiently acidic pH, this result would suggest that a group with a pK\(_a\) of ~5.5 is necessary for ion permeation. However, the relation between \( \gamma \) and pH is less steep than would be expected for titration of a single group, indicating that multiple groups are involved in the pH effects on permeation. Unfortunately, we were not able to measure channel activity reliably at more acidic pHs because of the small size, short open-time duration, and rapid disappearance of channel activity under these conditions (see below). TMO-modified channels respond similarly to increases in the external hydrogen ion concentration as shown in Fig. 5. \( \gamma \) in modified patches is ~20% less than in untreated membranes at all pHs between 8.1 and 5.5, indicating that the modified channels are equally sensitive to protonation in this range. In addition, these results suggest that if TMO treatment is affecting channel permeation proper-

### Table 1

| Conditions      | Control | TMO modified | Ratio |
|-----------------|---------|--------------|-------|
| \( \gamma \)    | \( \gamma_{\text{TMO}} \) | \( \gamma_{\text{TMO}}/\gamma \) |
| \( pS \)        | \( pS \) |
| 140 Cs, 1.8 Ca, 1.0 Mg | 47.7 ± 0.9 | 44.2 ± 1.1 | 0.93* |
| 140 Cs, 0 Ca, 1.0 Mg | 65.5 ± 2.6 | 55.4 ± 0.3 | 0.85* |
| 140 Cs, 0 Ca, 0 Mg | 73.6 ± 1.3 | 58.9 ± 1.9 | 0.80* |
| 10 Cs, 1.8 Ca, 0 Mg | 19.2 ± 1.0 | 13.9 ± 0.2 | 0.72* |
| 10 Cs, 0 Ca, 0 Mg | 44.4 ± 2.0 | 36.3 ± 2.5 | 0.82* |

Single-channel conductance, \( \gamma \), is the slope conductance determined by a linear fit to inward single-channel currents. Currents were either measured at three to six membrane potentials between -120 and -20 mV or determined from currents measured during ramp changes in membrane potential between -200 and 0 mV. Concentrations of the Cs\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\) chloride salts in the pipette solution are given in millimolar. The pH of the pipette solution was 7.4. Mean values are given ±SEM. \( N \) is the number of patches for which each conductance measurement was made. The asterisk indicates measurements in which the average conductance of the TMO-modified channels differed significantly from the control conductance (\( P < 0.05 \)) using a two-tailed Student's \( t \) test.
ties by neutralizing a negatively charged titratable group, the group must have a pKₐ more acidic than 5.5.

**Single-Channel Kinetics**

**Effects of TMO modification.** The decreased whole-cell response to ACh after TMO exposure is due in large part to changes in channel gating properties caused by the modification. In the presence of divalent cations, TMO treatment resulted in channels with shorter open times. This can be seen by comparing the single-channel current records of Fig. 3, A and B, in which more brief openings are evident in the modified channels and the longer openings are interrupted by brief closing events much more frequently than in the controls.

We assessed the effects of TMO on channel kinetics quantitatively by examining the distribution of open times for ACh channels before and after TMO modification.

**Fig. 5.** Single-channel conductance, γ, as a function of external pH in normal (circles) and TMO-modified (triangles) ACh channels. γ was determined from a linear fit of inward single-channel currents measured during ramps of applied membrane potential from Eᵣ to −200 mV. All measurements were made with 10 mM Ca²⁺, 0 Ca²⁺, and 0 Mg²⁺ in the pipette solution. Each point represents the average of measurements from 6–11 patches. Error bars are shown when they are larger than the symbol.

Fig. 6 shows that in normal ionic strength solutions, both in the presence of calcium and magnesium (A, B) and in the presence of magnesium but no calcium (C, D), channel open times are shorter in the modified membranes. In the examples of Fig. 6, TMO treatment reduced the average channel open time to 80% its control value in the presence of calcium and magnesium, and to 65% of control with magnesium as the only divalent cation present. The histograms of Fig. 6 are from our longest recordings, and so have the greatest numbers of events.

The effects of TMO modification were sensitive to both the permeant ion concentration and to the presence of divalent cations. As shown in Table II, average open times averaged 23–39% shorter than normal in modified channels in the presence of divalent cations, either calcium and magnesium or magnesium alone, but open times were 33% longer in the modified channels in the absence of all divalent cations. The difference between the normal and modified channels is due to an ~50% decrease in channel open times caused by removing magnesium in the normal
channels that is absent in the TMO-modified channels. In addition to their relative insensitivity to external magnesium ions, the kinetics of TMO-modified channels were also less sensitive to changes in permeant ion concentration. Decreasing the cesium concentration from 140 to 10 mM caused a threefold increase in the average open time of normal ACh channels, but had no effect on the modified channels.

The decrease in channel open time produced by TMO was present and of similar magnitude at all membrane potentials between -20 and -120 mV. Channel open times were weakly voltage dependent in both control and modified channels, with open times being longer at more hyperpolarized membrane potentials. Both modified and unmodified channels showed a similar voltage dependence. The changes in

kinetics seen with TMO modification would be consistent with either a shift in the voltage dependence of channel closing rates by ~30 mV towards more negative membrane potentials in the modified channels, or with an overall reduction in open time at all membrane potentials by the modification. The open-time histograms from measurements in the presence of divalent cations could be fitted reasonably well with the sum of two exponential functions, indicating that there are at least two open states of the ACh channels in BC3H-1 cells under these conditions as has been reported previously (Sine and Steinbach, 1984). When the control data in calcium- and magnesium-containing solutions were analyzed in this way about two-thirds of the openings were of long duration, with $\tau = 6.7 \pm 1.0$ ms ($n = 5$) and about

![Open-time histograms measured under different conditions. Steady-state current records measured at -100 mV were idealized and a mean open time was determined as described in the Methods. (A) Unmodified, 140 mM Cs\(^+\), 1.8 mM Ca\(^{2+}\), and 1.0 Mg\(^{2+}\) in the pipette, $\tau = 6.9$ ms. 552 events. (B) TMO-modified, 140 mM Cs\(^+\), 1.8 mM Ca\(^{2+}\), and 1.0 Mg\(^{2+}\) in the pipette. $\tau = 5.6$ ms. 436 events. (C) Unmodified, 140 Cs\(^+\), 0 Ca\(^{2+}\), and 1.0 Mg\(^{2+}\) in the pipette. $\tau = 2.8$ ms. 806 events. (D) TMO-modified, 140 Cs\(^+\), and 1.0 Mg\(^{2+}\) in the pipette. $\tau = 1.8$ ms. 315 events.](image)
one-third of the openings had a shorter time constant, $\tau = 1.6 \pm 0.2$ ms ($n = 5$). TMO modification decreased the duration and increased the proportion of short openings. The fast time constant was reduced by half, to $0.8 \pm 0.1$ ms ($n = 6$), and the proportion of short openings increased to ~70% of the total. This state probably corresponds to the intermediate duration open state described by Sine and Steinbach (1984, 1986), although a direct comparison is not possible because their experiments were done at low temperature. There was no significant change in the average duration of the longer openings after TMO modification.

**Effects of pH.** We examined the pH dependence of TMO's effects in low ionic strength, divalent cation-free solutions. TMO modification had dramatic effects on channel kinetics measured at pH 6.7 as shown in Fig. 7. Channel openings in control membranes were as long as 50–150 ms in pH 6.7 solution, as seen in Fig. 7A, but in modified membranes they were of short duration. This effect is shown quantitatively in the open-time histograms of Fig. 7, C and D, which show a fivefold difference in the average open time between the modified and unmodified channels at pH 6.7.

### TABLE II

| Conditions | Control $\tau_C$ (ms) | Control $N$ | TMO modified $\tau_{TMO}$ (ms) | TMO modified $N$ | Ratio $\tau_{TMO}/\tau_C$ |
|------------|-----------------------|-------------|----------------------------------|------------------|--------------------------|
| 140 Cs, 1.8 Ca, 1.0 Mg | 6.6 ± 0.8 | 5 | 5.1 ± 0.6 | 6 | 0.77* |
| 140 Cs, 0 Ca, 1.0 Mg | 3.1 ± 0.3 | 4 | 1.9 ± 0.1 | 2 | 0.61* |
| 140 Cs, 0 Ca, 0 Mg | 1.5 ± 0.1 | 13 | 2.0 ± 0.2 | 7 | 1.33* |
| 10 Cs, 0 Ca, 0 Mg | 4.5 ± 0.6 | 6 | 1.6 ± 0.4 | 5 | 0.36* |

Mean open time, $\tau$, is the simple average of the measured single-channel open times from current records measured at -80 or -100 mV as described in the Methods. Each record contained from 150 to 1,400 events. Values are means ± SEM. $N$ is the number of patches from which measurements were made under each condition. The concentration of ACh in the pipette was 5 $\mu$M and the pH was 7.4. The asterisk indicates measurements in which the average open time of the TMO-modified channels differed significantly from the control ($P < 0.05$) using a two-tailed Student’s $t$ test.

The differences in channel kinetics between normal and modified membranes were most pronounced near neutral pH, as can be seen in Fig. 8, which compares average open times measured at pHs between 8.1 and 5.5 in normal and treated patches. In normal membranes decreasing the pH from 8.1 to 6.7 causes an approximately fourfold increase in channel open time. Further acidification reduces the channel open time to near the values measured at alkaline pH. This bimodal effect of pH in normal ACh channels suggests that at least two distinct titratable or interacting groups are involved in determining open-channel stability. The influence of both alkalinization and acidification is absent in the kinetics of the TMO-modified channels. Average open times in modified channels are insensitive to changes in external pH in the range between pH 8.1 and 5.5. Two-way analysis of variance of the data in Fig. 8 indicates that the mean open time is independent of pH in the modified channels over this range. The lack of effect of pH changes in treated channels suggests that the TMO reaction modifies the effects of chemical groups
involved in channel gating having both acidic and alkaline pKₐ’s. Modification prevents protonation or deprotonation of these groups, and thus removes the sensitivity of channel kinetics to pH. Attempts to measure channel kinetics in more acidic solutions failed because in both modified and unmodified channels decreasing pH drastically reduced the steady-state level of channel activation. At pH 6.5, channel open events occurred on average only every ~4 s in normal patches and every ~12 s in TMO-modified patches in the steady state. Events were even less

FIGURE 7. Effects of pH on channel kinetics. (A and B) Single-channel current records in pH 6.7 external solution in an unmodified (A) and a TMO-modified (B) patch measured at a membrane potential of -100 mV. Opening events are downward deflections. Current records were filtered at 1,600 Hz and sampled at 125 µs/point. Records are not continuous. (C and D) Open-time histograms from channel openings measured in an unmodified (C) and a TMO-modified (D) patch at -100 mV in pH 6.7 external solution. The simple average open times of the distributions are 7.7 ms in C and 1.5 ms in D.

FIGURE 8. Average open time as a function of external pH. Simple average open time was measured at -100 mV from ACh channels in normal (circles) and TMO-modified (triangles) cells. The external solution contained 10 mM Cs⁺, 0 Ca²⁺, and 0 Mg²⁺. Each point is the mean of the open times determined from three to six different patches. Error bars are shown when they are larger than the symbol.
frequent at more acidic pHs. Thus, the open times reported here at pHs <6.7 are not steady-state measurements, but rather were made in the first 1–2 min after GΩ seal formation, before desensitization reached its essentially complete steady-state level.

**DISCUSSION**

Our results show that both conductance and kinetic properties of ACh channels can be modified by reaction with TMO and by changes in external pH. Chemical modification of ACh channels by TMO leads to a substantial reduction in the response of BC5H-1 cells to ACh in macroscopic, whole-cell recordings. Examination of currents in single ACh channels showed that the decreased response to ACh is due to changes induced by TMO in both the permeation properties and kinetic responses of the channels. Single-channel conductance measured in normal permeant and divalent cation concentrations in TMO-treated membranes were ~90% of normal. Channel kinetics were more substantially altered by TMO, with channel average open times reduced to ~80% of normal under these conditions. The simple average open time used in our kinetic analysis underestimates the decrease in true mean channel open time produced by TMO because there are probably a greater proportion of missed short-duration events in the TMO-modified membranes than in the controls. Thus, when this bias is taken into account, the observed changes in channel kinetic and permeability properties are probably sufficient to explain the 60% reduction in ACh response seen in our macroscopic measurements. Increasing the external hydrogen ion concentration in low ionic strength solution decreases the single-channel conductance for inward currents, and has a bimodal effect on channel open time. Channel open time was lengthened in moderately acidified solution, but shortened at more acidic pH. Decreases in ACh sensitivity in other preparations at acidic pH have been reported using macroscopic methods (Huang et al., 1978; Landau et al., 1981). Our results indicate that the decrease in ACh response in these experiments may be due to decreases in both γ and τ at acidic pH, as suggested previously from the results of fluctuation analysis (Landau et al., 1981). TMO modification alters the response of the channels to pH changes, indicating that the reactive chemical groups involved are normally titratable in the pH range between 5.5 and 8.1.

**Permeation Properties**

Single-channel conductance, γ, was reduced by TMO by 10–30% under all our experimental conditions, i.e., at normal and reduced ionic strength, in the presence and absence of divalent cations, and at pHs from 5.5 to 8.1. In both normal and modified channels increasing the external hydrogen ion concentration reduced the single-channel conductance with an apparent pKₐ for channel block of ~5.5. The decrease in γ produced by TMO was present and of similar magnitude over the entire pH range between 8.1 and 5.5, suggesting that if neutralization of a charged group was responsible for TMO's conductance effects, the modification affected a group or groups having a more acidic pKₐ than 5.5.

Our finding that TMO reduced γ by only ~10% under normal ionic conditions
differs from that of Adams (1983) who concluded from fluctuation analysis of the ACh response in frog endplate that TMO modification reduced the single-channel conductance of ACh channels by 50%. While the difference between his results and ours could be due to a species difference, it is equally likely that the discrepancy is due to the dissimilar techniques used in the two studies. Noise analysis gives lower single-channel conductances for ACh channels activated by some weak agonists compared with γ measured with ACh as the agonist, although single-channel recordings under the same conditions show no difference in single-channel current amplitudes (Gardner et al., 1984). Discrepancies in conductances and time constants measured using the two techniques in the same preparation have also been observed for glutamate-activated channels (Ascher et al., 1988; Ascher and Nowak, 1988). Since recording of single-channel current events is a more direct measure of channel properties, we believe our results more reliably reflect the effects of TMO than does the fluctuation analysis. The difference may be due to significant contributions to noise measurements from attenuated brief channel openings, which increase in frequency and decrease in duration after TMO modification. Since we only used channel openings that were long enough to be fully resolved by our recording system for our conductance measurements, they are not prone to this error.

The small effect TMO has on ACh channel conductance in our cells also differs from the results of TMO modification in other cation-selective channels. In both voltage-gated sodium channels (Sigworth and Spalding, 1980; Worley et al., 1986) and calcium-activated potassium channels (MacKinnon and Miller, 1989a, b), TMO treatment reduced the single-channel conductance by 30–70%, although in the potassium channels a substantial effect was only seen at low ionic strength. In sodium channels the decrease in γ, the shift in pKₐ for hydrogen ion block of the channel, and the reduction in sensitivity to block by external divalent cations by TMO are consistent with a reduction in the negative surface potential at the outer face of the channel by the modification. Similarly, the concentration dependence of TMO's effects on conductance in potassium channels, its effects on channel block by a cationic toxin, and changes in voltage-dependent gating after TMO modification are indicative of an altered surface potential. In contrast, we find that TMO reduces γ without reducing the apparent pKₐ for hydrogen ion block of affecting the sensitivity of the conductance to divalent cations, and that the reduction in conductance is similar whether measured in solutions of normal or low ionic strength. These findings indicate that the reduction in ACh channel conductance produced by TMO is not the result of a reduction in the negative surface charge at the channel mouth that normally concentrates cations there, but must be due to some other change in channel structure.

Recent results of molecular biological experiments in Torpedo ACh channels expressed in Xenopus oocytes indicate that negative charges from glutamate and aspartate residues influence the permeability properties of ACh channels (Imoto et al., 1986, 1988). In these experiments replacing negatively charged carboxylic acids with neutral or positively charged amino acids on any of the subunits reduced γ, with the effect being greater the more positive the change in charge. If TMO is modifying homologous charged groups in BC3H-1 cells (LaPolla et al., 1984; White et al., 1985), the decrease in γ seen after TMO treatment could be explained by the
neutralization of two to three negatively charged groups on the outer face of the channel, which reduced $\gamma$ to 75-90% of control values in the experiments on *Torpedo* channels. Our results are not consistent with modifications of the hypothesized "intermediate ring" of negative charge postulated to be within the pore of the channel, where even a single change in charge resulted in much more substantial changes in single-channel conductance than we see, nor with changes in the internal negative charge ring, where changes affected outward, but not inward currents through the channels. Our titration curve for channel conductance is less steep than would be predicted for a single class of titratable groups indicating that more than one acidic moiety is affecting channel conductance properties. In addition, $\gamma$ is affected by pH changes in a more alkaline range than would normally be expected for glutamic and aspartic acid residues. Both the shallowness and the alkaline shift of our titration curves relative to that expected for a carboxylic acid can be explained by the proposed model for *Torpedo* channels, since the hypothesized ring carboxyl groups would be electrically close enough in our low ionic strength solutions to be influenced by neighboring negatively charged ring groups (Edsall and Wyman, 1958).

**Kinetic Properties**

The open time of normal ACh channels in BC3H-1 cells was very sensitive to changes in external pH, while this effect was absent in TMO-modified channels. In normal channels the average mean open time was maximal at pH 6.7 and was as much as four times shorter both at more alkaline and acidic pHs. Channel open times were insensitive to changes in external pH in the modified channels. In contrast to the consistency of TMO's effects on conductance under different external ionic conditions, the effects of TMO on channel open time varied depending on the external milieu. TMO dramatically shortened channel open time at near neutral pH in low ionic strength, divalent cation-free solutions, but lengthened open times measured in neutral, divalent cation-free solution containing 140 mM cesium.

In normal BC3H-1 ACh channels our results show that mean open time is affected by the permeant ion concentration, the concentration of divalent cations, and the external pH. Reducing the external cesium concentration from 140 to 10 mM resulted in a 50% increase in the average open time. Removing calcium from the external solution caused a 50% reduction in open time and removing magnesium as well caused a further 50% decrease in open time. Increasing calcium concentrations above the normal level has been reported to decrease (Bregestovski et al., 1979), increase (Ascher et al., 1978), or have no effect (Magleby and Weinstock, 1980) on ACh channel mean open times in other preparations measured using fluctuation analysis. In low ionic strength, divalent cation-free solutions, normal ACh channels had a maximum average channel open time at pH 6.7, with open times that were three- to fourfold shorter at more acidic and alkaline pH. This bimodal pH dependence of ACh channel open time is similar to that reported from fluctuation analysis in frog endplates (Landau et al., 1981).

The kinetics of TMO-modified channels were less sensitive to changes in the external solution than were the normal channels. They showed a sensitivity to calcium that was similar to the unmodified channels, but neither removing external
magnesium nor reducing the cesium concentration of the extracellular solution affected the channel open time of TMO-modified channels. In addition, the channel open time was insensitive to changes in the external hydrogen ion concentration in the modified channels. These results suggest that the TMO-reactive site is normally involved in the channel kinetic responses to both mono- and divalent cations, and that modification by TMO prevents these interactions.

Which Groups Are Modified by TMO?

The most likely site of the groups reacting with TMO is on the external face of the channel, since the reagent was applied externally and its positive charge would prevent permeation through the cell membrane. ACh channels are measurably permeant to organic cations with sizes similar to TMO (Dwyer et al., 1980), so it is possible that TMO could traverse the channel to react with sites in the channel pore. However, since the modification was done in the absence of agonist, it is unlikely that TMO could reach the intracellular surface of the channel. The increase in the proportion of brief openings seen after TMO modification is somewhat similar to what is seen when early steps in the normal glycosylation pathways involved in receptor synthesis are blocked, altering the carbohydrate content of the receptors (Covarrubias et al., 1989). However, it seems unlikely that TMO acts by modifying carboxyl groups associated with the carbohydrate portion of the ACh channel since more selective block of carbohydrate trimming steps necessary for the addition of carboxyl-containing sialic acid residues did not affect channel kinetics (Covarrubias et al., 1989).

The chemical identity of the reacted group or groups on ACh channels modified by TMO cannot be determined with certainty from our experiments. The reaction of TMO seems to be specific for carboxylic acid moieties of proteins under aqueous conditions like those used in these experiments (Paterson and Knowles, 1972; Reed and Raftery, 1976), and the macroscopic effects of TMO modification of ACh channels are similar to those of modification with water-soluble carbodiimides (Edwards et al., 1970; Nachshen and Landau, 1977) or N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (Stuesse and Katz, 1973). Carboxyl groups are the only known common reaction sites for these reagents. Our results are also consistent with the effects on ion flux seen in studies in which the ACh channel was enzymatically carboxymethylated (Yee and McNamee, 1985). However, we cannot rule out the possibility that TMO is acting at some site other than a carboxyl group or groups. The related reagent triethylxonium will react with the imidazole ring of histidine and the sulfhydryl group of cysteine in free amino acid form (Hamada and Yonemitsu, 1971), although these reactions have not been reported in proteins treated with the reagent. The effects of pH on open time in normal channels indicate that groups with both acidic and alkaline pKₐ's normally influence channel kinetics. The lack of effect of both increases and decreases in hydrogen ion concentration in the modified channels could indicate that both kinds of groups are modified by TMO, i.e., that both histidine and cysteine residues react with the reagent. Sulfhydryl group reagents have been shown to affect ACh channel functions in ways that would be consistent with the effects of TMO in measurements using both electrophysiolog-
ical (Landau and Ben-Haim, 1974; Ben-Haim et al., 1975; Terrar, 1978; Steinacker, 1979; Adams, 1983; Pradier et al., 1989) and flux (Yee et al., 1986) methods.

A second possibility, more in keeping with the known specificity of TMO for carboxyl groups in other proteins, would be that TMO reacts with a carboxylic acid moiety that is normally involved in salt bridge formation with a protonated, more alkaline residue, such as the imidazole of histidine. Formation of the salt bridge could be involved in stabilizing the open conformation of the channel at pHs where the carboxyl group was charged and the alkaline group was protonated. TMO modification of the carboxyl group would prevent formation of the salt bridge and so prevent the pH-dependent stabilization. While this hypothesis requires that the modified carboxyl group is protonated at relatively alkaline pHs in our experiments, this is plausible given the low ionic strength of the solutions we used for the pH measurements and the proposed proximity of the alkaline group. A similar proposal has been put forth to explain the pH-dependent stabilization of a conductance state of voltage-gated calcium channels (Prod’hom et al., 1989), and it is possible that this may be a general mechanism by which channels stabilize transitory open conformations.

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