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Different Modes of Blockade by *p*-Phenylene-polymethylene Bis-ammonium Compounds of the Nicotinic Acetylcholine Receptor Channel in Skeletal Muscle Cells of Mice

HIROSHI NOJIMA, IKUKO KIMURA, MASASHI MUROI AND MASAYASU KIMURA

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan

Abstract—The structure-activity relationships of five newly synthesized *p*-phenylene-polymethylene bis-ammonium (PMBA: $C_6H_4[(CH_2)_nN^+R_3]_2$) compounds were investigated on the blockade of the nicotinic acetylcholine receptor (nAChR) channel. The cell-attached patch clamp configuration was used to measure single-channel currents in the endplate region of single flexor digitorum brevis muscle cells of adult mice. The bis-trimethylammonium compounds PMBA-1 (n=4, R=CH_3) and PMBA-23 (n=6, R=CH_3) produced channel opening above 0·3 μM and open channel blockade above 10 and 3 μM, respectively. The bis-triethylammonium compounds PMBA-43 (n=1, R=CH_2CH_3) and PMBA-24 (n=6, R=CH_2CH_3) showed no channel opening action, but PMBA-21 (n=4, R=CH_2CH_3) opened channels weakly at 3 and 10 μM. These bis-triethylammonium compounds exerted different blocking actions on acetylcholine-activated channel currents. Above 10 μM PMBA-43, like tetraethylammonium, blocked open channels by decreasing the mean open time by rapid partial closing of the channel during the open-phase. At 10 μM, PMBA-21 blocked open and closed channels by decreasing the opening frequency by means of an irregular sequence of short pulses. At 0·3 μM, PMBA-24 blocked closed or nonconducting channels by decreasing the opening frequency without producing changes in mean open time. These results indicate that by lengthening the distance between two nitrogen atoms in the bis-triethylammonium group of PMBA, open channel blockade changes to closed channel blockade. PMBA compounds were classified into three types of nAChR channel blockers: PMBA-43 as an open, PMBA-21 as an open and closed, and PMBA-24 as a closed or nonconducting channel blocker.

The nicotinic acetylcholine receptor (nAChR) is a membrane-associated glycoprotein assembly, forming the ion channel, the site of biological activity. The channel is linked functionally with recognition sites for acetylcholine, forming a regulator site (Changeux et al 1984; Changeux 1990). At the neuromuscular junction, the ion channel of the nAChR is blocked by various local anaesthetics (Neher & Steinbach 1978; Aracava et al 1984) and psychotropics (Carp et al 1983; Aguayo et al 1986; Albuquerque et al 1988). There are reports on the relation between chemical structure and nAChR channel blockade (Bakry et al 1982; Spivak et al 1982, 1983; Swanson et al 1991; Wood et al 1991) although the bis-ammonium series of compounds is open to examination.

In the present study, we investigated the effect of chemical structure on nAChR blockade, using newly synthesized *p*-phenylene-polymethylene bis-ammonium (PMBA) compounds (Fig. 1), which are not susceptible to enzymatic hydrolysis by cholinesterase. The simple chemical structures of these compounds make it possible to elucidate the essential steric distance between two nitrogen atoms in PMBA compounds. We also compared the nAChR blocking and depolarizing actions of PMBA compounds with those of tetraethylammonium (TEA), decamethonium, and succinylcholine.

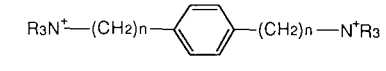

FIG. 1. Chemical structures of *p*-phenylene-polymethylene bis-ammonium (PMBA) compounds.

Correspondence: I. Kimura, Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan.

Materials and Methods

Adult male ddY mice, 30–38 g, were used.

Twitch tensions

Phrenic nerve-diaphragm muscle preparations were isolated and suspended in 5 mL Krebs-Henseleit solution (KHS) composition (mM): NaCl 118, KCl 5·4, $CaCl_2$ 2·5, $MgSO_4$ 0·57, $NaHPO_4$ 1·2, $NaHCO_3$ 12·8, glucose 11·1 (pH 7·3±0·1, 35–37°C) bubbled with 95% O_2–5% CO_2. The phrenic nerve and the muscle were stimulated alternately by means of bipolar platinum electrodes (0·2 Hz, 1 ms, supramaximal voltage of 0·4–0·5 and 1–2 V, respectively), and the twitch tensions were recorded isometrically under 1 g loading tension. Each drug was added cumulatively at 2 min intervals, and the 50% inhibitory concentration (IC50) was estimated.

Intracellular recordings

A conventional microelectrode technique was used. Glass microelectrodes (5–25 MΩ) filled with 3 M KCl were used to measure the resting membrane potential of muscle cells in modified KHS (mM): NaCl 137, KCl 5, $CaCl_2$ 2·5, $MgSO_4$ 1·2, $NaHCO_3$ 15, glucose 10, containing 0·3 μM tetrodotoxin. The depolarizing effects were examined by the local perfusion method (Katz & Miledi 1961). A microelectrode was inserted near an endplate region such that the rising times of

miniature endplate potentials were 1·0 ms or less. A glass pipette of about 0·5 mm in diameter was filled with the desired concentration of drug, and brought close to the surface of the recording cell. The drug was applied until stable depolarization was obtained.

Single channel recordings

The cell-attached patch clamp configuration was used for single channel recording (Hamill et al 1981). Single skeletal muscle cells were enzymatically isolated from the flexor digitorum brevis muscle. Details of the patch clamp experiment and enzyme treatment have already been described (Kimura et al 1991a). Experiments were performed 2 h after the enzyme treatment.

Currents through single channels at the endplate of each muscle cell were recorded in modified KHS (changing 5 to 2·5 mM KCl) at 24–26°C with a patch clamp system (Axopatch-1D, Axon Instruments, CA, USA). All patch currents, low-pass filtered at 2 kHz, were stored in a PCM data recorder (RP-880; NF electric Instruments, Japan). The sampling frequency was 56·88 kHz. Data were analysed using a histogram analyser (QC-111J, Nihon Kohden, Japan). Data replayed from a PCM data recorder were sampled at 100 μs per point and stored on a memory oscilloscope (VC-11, Nihon Kohden, Japan). The traces of the channel currents were drawn by a pen-oscillograph (RECTI-HORIZ-8K, NEC San-ei Instruments, Japan) from the stored data. The threshold used to detect transitions between opening and closing was set at a current amplitude of 2 pA. Only data of single-level channel opening were used. The mean open time was determined from an open time histogram at resting membrane potential (300–1700 events per patch), and opening frequency (the number of events per second) was determined simultaneously. For single channel recordings drugs were added to the pipette solution. The patch pipettes had a resistance of 15–20 MΩ when filled with modified KHS containing a PMBA compound. Data were recorded as soon as possible after seal formation and then analysed. At the end of these experiments, the cells were impaled with a patch pipette, and the intracellular membrane potentials were measured directly.

Drugs

The following drugs were purchased: acetylcholine chloride (Daiichi, Japan), succinylcholine chloride and tetraethylammonium (TEA) chloride (Nacalai Tesque, Japan), decamethonium dibromide (Wako Pure Chemical Industries, Japan), tetrodotoxin (Sankyo, Japan) and neostigmine methylsulphate (Sigma, St Louis, MO, USA). Hexamethyl-4, 4′-(1,4-phenylene)-dibutylammonium diiodide (PMBA-1), hexamethyl-6,6′-(1,4-phenylene)-dihexylammonium diiodide (PMBA-23), hexaethyl-1, 4-phenylene-dimethylammonium diiodide (PMBA-43), hexaethyl-4,4′-(1,4-phenylene)-dibutylammonium diiodide (PMBA-21) and hexaethyl-6,6′-(1,4-phenylene)-dihexylammonium dichloride (PMBA-24) were provided by Ome Research Laboratories, Tobishi Pharmaceuticals, Japan.

Results

Inhibitory effects of PMBA compounds on twitch tensions

All the bis-ammonium compounds used in this study blocked the nerve-evoked twitch response without affecting the contraction evoked by muscle stimulation in the isolated phrenic nerve-diaphragm muscle preparation of mouse. The IC50 values and their 95% confidence limits of these compounds were as follows (μM): PMBA-1; 22·8 (20·0–26·0), PMBA-23; 15·9 (13·4–19·0), PMBA-43; 638 (562–721), PMBA-21; 52·4 (45·7–60·0), PMBA-24; 4·3 (3·86–4·79), decamethonium; 101 (85·1–119), and succinylcholine; 25·2 (22·2–28·7). The blocking effects of these compounds were readily reversed on washout but were not reversed by 10 μM neostigmine, a cholinesterase inhibitor (Fig. 2), which fully reversed (+)-tubocurarine (6·5 μM)-induced inhibition of indirectly stimulated twitch tension (data not shown).

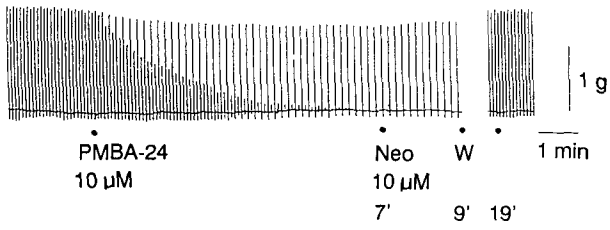

FIG. 2. Inhibitory actions of PMBA-24 (10 μM) on nerve-evoked twitch tension of isolated phrenic nerve-diaphragm muscle preparations from mice. Typical recordings of isometric twitch tensions evoked by alternating direct and indirect stimulations (supramaximal voltage, 0·2 Hz, 1 ms). Neo.: neostigmine, W: wash.

Depolarizing effects of PMBA compounds

The depolarizing effects of bis-trimethylammonium- (succinylcholine, PMBA-1 and PMBA-23) and bis-triethylammonium-derivatives (PMBA-43, PMBA-21 and PMBA-24) were examined at the endplate region of diaphragm muscles by the local perfusion method. Succinylcholine, PMBA-1 and PMBA-23 produced depolarization at concentrations above 1, 1, and 100 μM, respectively (Fig. 3A). The PMBA-23-induced depolarization declined much more rapidly than the succinylcholine-induced depolarization under continuous application of these agents (Fig. 3B). None of the bis-triethylammonium derivatives produced appreciable depolarization at concentrations of 10–1000 μM.

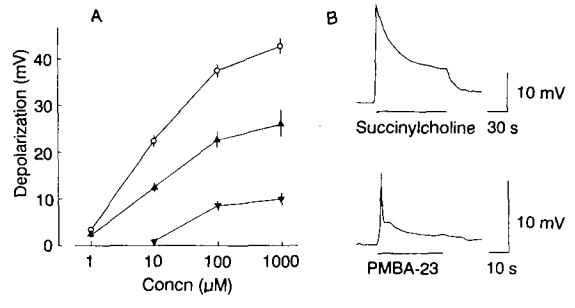

FIG. 3. Depolarizing effects of succinylcholine, PMBA-1 and PMBA-23 at the endplate region of diaphragm muscles. A. Depolarization-concentration curves for succinylcholine (○), PMBA-1 (▲) and PMBA-23 (▼). Each point represents the mean ± s.e. (n = 6–18). B. Typical recordings of 10 μM succinylcholine- and 100 μM PMBA-23-induced depolarization produced by local perfusion. The drug was applied continuously until stable depolarization was obtained. Horizontal bars represent periods of drug application. Note the fast decline in PMBA-23-induced depolarization.

Effects of bis-trimethylammonium- and bis-triethylammonium-derivatives on nAChR opening

The effects of trimethylammonium- (decamethonium, PMBA-1 and PMBA-23) and triethylammonium-derivatives (TEA, PMBA-43, PMBA-21 and PMBA-24) in a patch pipette on nAChR opening were examined at the endplate region of single muscle cells. Decamethonium and PMBA-1 at 1 μM caused individual channels to open at a steady rate. At 100 μM prolonged clusters of opening separated by long closed intervals were seen (Fig. 4). The mean open times of channel currents were similar (0·86–0·87 ms) for decamethonium, PMBA-1 and PMBA-23 at 1 μM, and decreased as the concentration was increased (Fig. 5A). Decamethonium, PMBA-1 and PMBA-23 increased the frequency of opening at concentrations higher than 0·3 μM, to maximum responses at 30 μM for decamethonium and PMBA-1, and 3 μM for PMBA-23 (Fig. 5B). TEA at 30–1000 μM, PMBA-43 at 3–100 μM and PMBA-24 at 0·1–100 μM were not able to activate the nAChR channel. PMBA-21 had an extremely weak effect on channel opening at 3 and 10 μM, and no effect at higher concentrations.

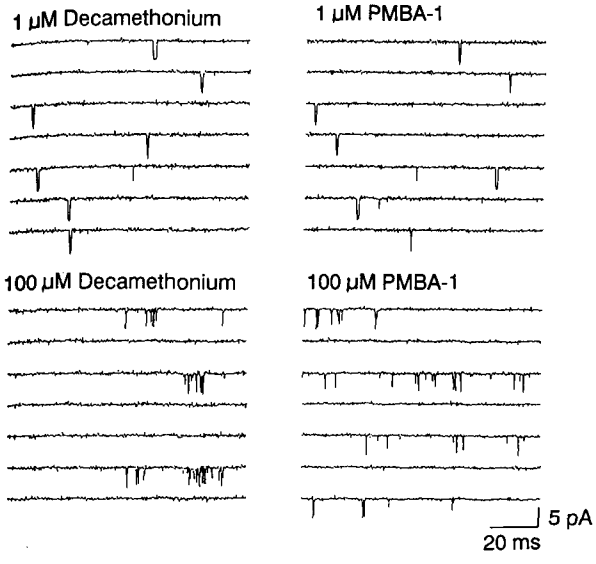

FIG. 4. Typical recordings of acetylcholine receptor-channel currents demonstrating agonism at 1 μM and channel blocking at 100 μM decamethonium and PMBA-1. Channel currents were recorded at resting membrane potentials (−72– −78 mV) several seconds after a pipette-membrane giga seal was established. Opening events are shown as downward deflections.

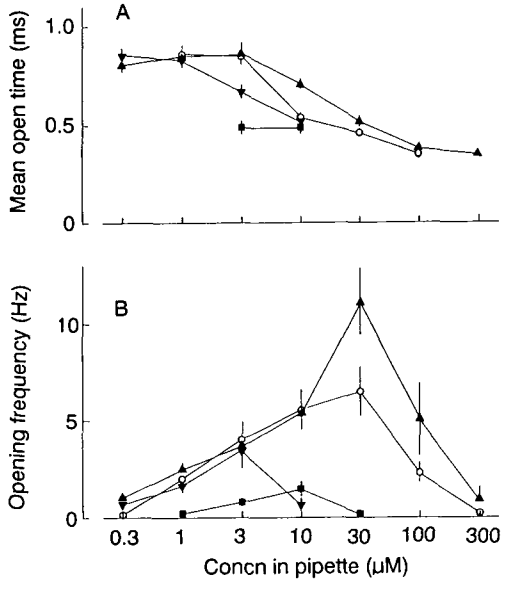

FIG. 5. Acetylcholine receptor-channel activity-concentration curves on the mean open time (A) and the opening frequency (B) for decamethonium (○), PMBA-1 (▲), PMBA-21 (■) and PMBA-23 (▼). Mean open time and opening frequency were estimated at resting membrane potentials (−72– −78 mV). Each point represents the mean ± s.e. (n = 5–10).

Blocking effect of bis-triethylammonium derivatives on acetylcholine-activated nAChR channel currents

The blocking effects of triethylammonium derivatives (TEA, PMBA-43, PMBA-21 and PMBA-24) were examined on acetylcholine (1 μM)-activated channel currents at resting membrane potential. When single channel currents were recorded with patch pipette solution containing 300 μM TEA plus 1 μM acetylcholine, TEA produced rapid partial closing of the channel during the open phase, termed flickering (Brett et al 1988; Fig. 6). This flickering by TEA was obvious at concentrations higher than 30 μM. TEA also shortened the mean open time, and at 300 μM increased the frequency of channel opening (Table 1). Single channel currents recorded with 30 μM PMBA-43 plus 1 μM acetylcholine in a patch pipette also produced flickering (Fig. 7). The flickering caused by PMBA-43 was noticeable at concentrations higher than 3 μM. This action of PMBA-43 shortened the mean open time and increased the frequency of opening, as in the case of TEA. PMBA-21 in amounts over 1 μM with 1 μM acetylcholine reduced the mean open time of acetylcholine-activated nAChR channel currents in a concentration-dependent manner. The blockade by PMBA-21 of open channels differed from those of TEA and PMBA-43: an irregular sequence of short pulses was observed instead of flickering. At 1 and 3 μM, PMBA-21 significantly reduced the mean open time without causing changes in opening frequency. The reduction of mean open time induced by PMBA-21 at 10 μM was accompanied by a decrease in the frequency of channel opening. PMBA-24 in amounts over 0·3 μM with 1 μM acetylcholine decreased the rate of opening of acetylcholine-activated channel currents in a concentration-dependent manner. The decreases in opening frequency induced by PMBA-24 at 0·3 and 1 μM were not accompanied by changes in mean open time.

Discussion

The structure-activity relationships of five newly synthesized PMBA compounds were investigated on the blockade of the nAChR channel. In the present study, we showed firstly that bis-trimethylammonium derivatives of PMBA compounds with n = 4 or 6 (Fig. 1) acted to open channels and block open channels of the nAChR complex, secondly, that the structural change from bis-trimethylammonium to bis-triethylammonium derivatives caused a large decrease in the potency of agonism and an increase in channel blocking potency, and thirdly that three bis-triethylammonium PMBA compounds with n = 1, 4 and 6 showed very different channel blocking actions.

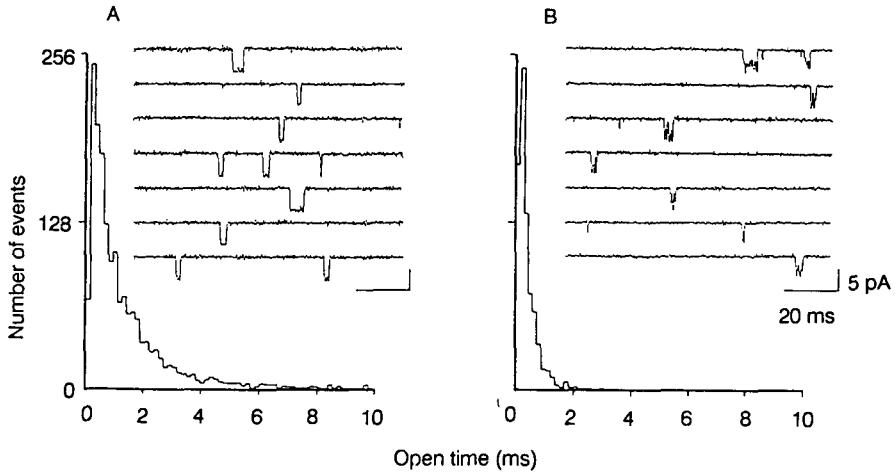

FIG. 6. Frequency distribution histograms for open time and typical recordings of acetylcholine (1 μM)-activated single channel currents at resting membrane potentials (−74 and −76 mV) in the absence (A) and presence (B) of 300 μM TEA. The mean open time in the absence of TEA was 1·29 ms from 1677 events. In the presence of TEA, the open time of each event was reduced because openings were interrupted by rapid closure. The mean open time was 0·52 ms from 775 events. The records are analysed quantitatively in Table 1.

The bis-trimethylammonium compound PMBA-1 was able to produce open channel blockade, in addition to opening and desensitization of the nAChR channel at elevated concentrations, as previously described for activation of the nAChR channel at desensitizing concentrations of nicotinic agonists (Sakmann et al 1980; Ogden & Colquhoun 1985; Marshall et al 1990). This condition was similar to that of decamethonium, where the concentrations that induced desensitization and open channel blockade were indistinguishable, unlike those of acetylcholine and succinylcholine, where the concentrations of the two differed (Eldefrawi et al 1982; Sine & Steinbach 1984; Ogden & Colquhoun 1985; Kimura et al 1991a). PMBA-23 had a powerful open channel blocking action, as indicated by the extremely fast decline in membrane depolarization it caused. These results suggest that, at the concentrations required to block neuromuscular transmission, bis-trimethylammonium compounds block open ion channels directly, and this mechanism contributes to neuromuscular blockade.

The bis-triethylammonium compounds PMBA-43 and PMBA-24 did not produce any channel opening action, although PMBA-21 showed a weak action characterized by extremely short pulses. These results support the suggestion that bis-triethylammonium compounds had a lower efficacy for channel opening than the corresponding bis-trimethylammonium compounds (Bovet 1951; Kitz et al 1969).

TEA produces a voltage-sensitive reduction in mean open time by blocking open channels (Adler et al 1979). In the present paper, we confirmed that acetylcholine-activated channel opening was interrupted by TEA. TEA may act only when the channel is open, because the product of mean open time and opening frequency was constant, regardless of the concentration of TEA. The channel-blocking property of the bis-triethylammonium derivative PMBA-43 was similar to that of TEA. PMBA-21 reduced the mean open time of acetylcholine-activated channel currents by producing short pulses, unlike the action of TEA or PMBA-43.

The reduction of mean open time results from steric hindrance of ion transport caused by direct interaction on the inside of the open channel (Lambert et al 1983). Depending on the affinity of the blocker for its site, two modes of open channel blockade have been reported (Neher & Steinbach 1978). Fast channel blockade is characteristic of a low-affinity blocker such as TEA and PMBA-43 at high concentrations: it manifests as a flickering that is interpreted as repeated binding and unbinding of the blocker at rates higher than the normal rate of channel closure. However, the flickering does not require direct ligand obstruction of the open channel. Ligands could bind to an allosteric site at a distance from the channel to explain the phenomenon (Brett et al 1988). On the other hand, slow channel blockade is

Table 1. Blocking effects of tetraethylammonium (TEA) and three *p*-phenylene-polymethylene bis-triethylammonium compounds (PMBA-43, PMBA-21 and PMBA-24) on acetylcholine-activated nAChR channel currents in single muscle cells of adult mice.

Drug in pipette	Mean open time (ms)		Opening frequency (Hz)	
1 μM Acetylcholine	1·20±0·04	(8)	4·62±0·71	(10)
+ 30 μM TEA	1·12±0·05	(6)	5·35±0·75	(8)
+100 μM	0·88±0·04**	(8)	7·19±1·40	(9)
+300 μM	0·49±0·02**	(7)	9·52±1·59**	(7)
1 μM Acetylcholine	1·22±0·03	(9)	5·27±0·74	(9)
+ 3 μM PMBA-43	1·13±0·05	(5)	6·79±0·75	(6)
+ 10 μM	0·81±0·03**	(7)	8·04±1·27	(8)
+ 30 μM	0·54±0·01**	(7)	8·60±1·08*	(8)
+100 μM	0·36±0·01**	(6)	7·20±0·76	(7)
1 μM Acetylcholine	1·23±0·04	(9)	5·53±0·81	(10)
+ 0·3 μM PMBA-21	1·14±0·06	(5)	5·16±0·98	(7)
+ 1 μM	0·93±0·06**	(5)	5·51±0·72	(8)
+ 3 μM	0·80±0·01**	(7)	3·63±0·58	(7)
+ 10 μM	0·60±0·01**	(7)	1·27±0·44**	(8)
+ 30 μM	N.D.		0·49±0·21**	(5)
1 μM Acetylcholine	1·24±0·04	(10)	5·00±0·69	(13)
+ 0·1 μM PMBA-24	1·23±0·04	(5)	3·58±0·63	(8)
+ 0·3 μM	1·20±0·06	(6)	1·57±0·18**	(9)
+ 1 μM	1·11±0·04	(5)	0·37±0·07**	(9)

Each value is the mean±s.e. for the number of cells in parentheses. Mean open time and opening frequency were estimated at a resting membrane potential. N.D.: not determined. Significant differences (*$P<0·05$, **$P<0·01$) from the control (1 μM acetylcholine) were determined using unpaired *t*-test.

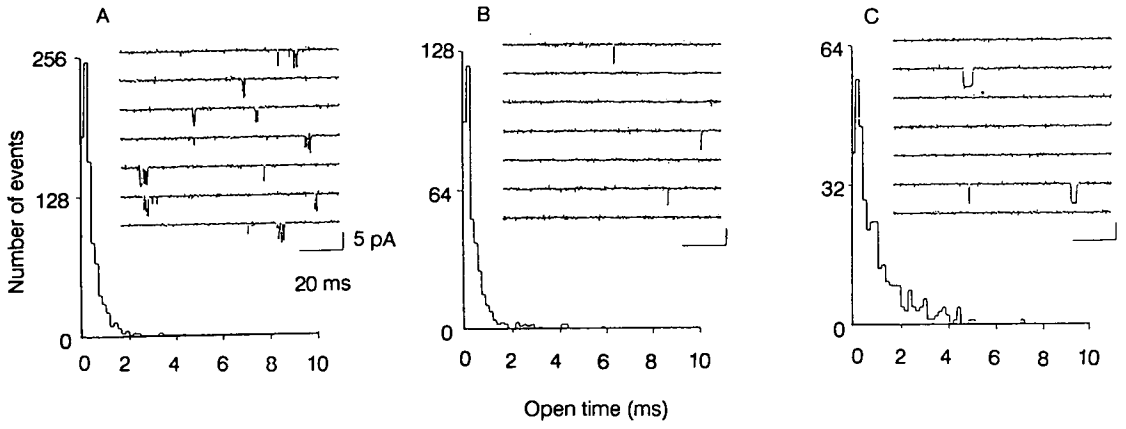

FIG. 7. Frequency distribution histograms for open time and typical recordings of acetylcholine (1 μM)-activated single channel currents at resting membrane potentials (−73, −75 and −76 mV) in the presence of 30 μM PMBA-43 (A), 10 μM PMBA-21 (B) or 1 μM PMBA-24 (C). Note that the distinct effects on channel blocking by the three PMBA compounds with different distances between two nitrogen atoms of the bis-triethylammonium group. The mean open times were 0·52 ms from 876 events in the presence of PMBA-43, 0·63 ms from 398 events for PMBA-21, and 1·16 ms from 359 events for PMBA-24.

characteristic of high-affinity blockers such as PMBA-21 and bupivacaine (Aracava et al 1984): it manifests as a short pulse, due to the reduction in mean open time, with dissociation of the blocker from the channel being too slow to produce flickering. The measured decrease in opening frequency induced by PMBA-21 may be seen as a consequence of the open channel block with a slow unblocking rate. Another possibility is that PMBA-21 may block closed or nonconducting channels in addition to open channels, because PMBA-21 is in the chemically intermediate positions between PMBA-43 (an open channel blocker) and PMBA-24 (a closed channel blocker, see below). Two effects of PMBA-21 on single channel activity, i.e. reductions in both mean open time and frequency of opening, indicate a phencyclidine-like channel blocking action, arising from blockade during both the open and closed states of the channel (Aguayo et al 1986; Kimura et al 1991b).

PMBA-24, which has two methylene groups more than PMBA-21 in the linking chains, blocked only closed or nonconducting channels, but not open channels. Similar findings have been reported for agents that accelerate the desensitization of nAChR such as chlorpromazine (Carp et al 1983; Heidmann et al 1983; Kimura et al 1991b) and meproadifen (Maleque et al 1982; Aracava & Albuquerque 1984). Action of PMBA-24 in reducing opening frequency may be due to closed channel block rather than receptor block, because the neuromuscular block was reversed readily on washout but not reversed by neostigmine. PMBA-24 may block channel openings reversibly by attacking a site different from the recognition site for acetylcholine.

In conclusion, lengthening the distance between two nitrogen atoms of bis-triethylammonium PMBA structures changed open channel blockade into closed channel blockade. PMBA compounds were classified into three types of nAChR channel blockers: PMBA-43 as an open, PMBA-21 as an open and closed, and PMBA-24 as a closed or nonconducting channel blocker. The availability of these three PMBA derivatives is useful for the identification of different sites at the ion channel of the nAChR complex which are known to bind a variety of noncompetitive blockers.

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